Characterization of collagen in non-small cell lung carcinoma with second harmonic polarization microscopy

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Abstract: Polarization second harmonic microscopy was used for collagen imaging in human non-small cell lung carcinoma and normal lung tissues ex vivo and revealed significant differences in the nonlinear susceptibility component ratio, demonstrating potential use in cancer diagnosis.

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1. Introduction

Lung cancer is the second most commonly diagnosed cancer in the developed world, with non-small cell lung carcinoma (NSCLC) constituting 85-90% of all lung cancer cases [1]. Diagnosis is a vital step in cancer management and typically involves histological imaging of biopsied or resected tissue sections. Structural alteration of the extracellular matrix (ECM)
during tumor initiation and progression has been shown to occur for several epithelial carcinomas [2, 3]. Probing these alterations can lead to development of new biomarkers for cancer diagnosis. Collagen is the major constituent of the ECM. It can be readily visualized with second harmonic generation (SHG) microscopy in histology sections and its organization can be probed with SHG polarization measurement [4–6]. We recently showed that structural details of collagen organization in the tissue can be studied with polarization-in, polarization-out (PIPO) SHG microscopy, where for each orientation of incoming laser polarization a set of outgoing SHG polarizations is measured [7]. The PIPO analysis can reveal the second-order susceptibility component ratio in each pixel of the image, which reflects the hierarchical organization of collagen in the tissue [6]. The values of susceptibility tensor components is influenced by several factors, including the amino acid composition and sequence of the collagen triple helix, organization of the triple helices in the collagen fibrils, arrangement of these fibrils in the fibers and finally fiber orientation with respect to the tissue section plane [6]. In addition, PIPO analysis renders an average fiber orientation in each pixel of the image, and provides information on the orientation distribution of collagen in the tissue. Hence, polarization SHG is a promising technique to detect collagen alterations in the ECM during cancer progression. PIPO SHG microscopy is a label free imaging technique, therefore it enables pathologists to perform a live biopsy, for example, in the endoscopic setting, or provides a quick histopathology investigation possibility that does not require staining. Furthermore, having additional imaging tool can be beneficial for cases that are difficult to diagnose.

In this paper, we demonstrate that PIPO SHG microscopy can detect significant differences in collagen organization between cancer and normal lung tissue. The susceptibility component ratio extracted from the PIPO measurements for each image pixel can be presented as a color-coded map that reveals areas of altered collagen. Therefore, the second-order susceptibility component ratio can be utilized as additional information beyond the visualized distribution of collagen density in the tissue.

2. Materials and methods

Histology sample preparation

Samples of normal human lung and primary NSCLC tissues from 4 patients were obtained with informed consent and institutional approval from University Health Network, Toronto, Canada. These were handled as per standard clinical histology protocols. 5 µm thick sections were cut from formalin-fixed tissues and mounted on glass slides for the SHG PIPO imaging, as described below. Adjacent sections were stained by hematoxylin & eosin (H&E) for histopathologic analysis. For each patient, slides of one tumor and one normal lung section were investigated. In each slide at least 5 regions of interest, as identified by a lung pathologist (S.S. and/or M.T.), were scanned. A total of 36 tumor and 26 non-tumor regions were imaged in the four patients to determine quantitative differences between tumor and non-tumor tissue.

SHG PIPO microscope setup

A custom-built nonlinear laser scanning microscope was used for the SHG PIPO imaging, as described elsewhere [7]. A custom diode-pumped Yb-ion-doped potassium gadolinium tungstate (Yb:KGW) crystal based oscillator operating at 1028 nm and generating ~430 fs pulses at 14.3 MHz repetition rate was used as the excitation source [8]. The nonlinear microscope contained a linear polarizer (Laser Components Inc.) and a half-wave plate (Comar Optics) placed before the excitation objective (20x, 0.75 NA air objective, Carl Zeiss). For SHG polarization analysis, the signal was collected in the forward direction with a home-built objective and passed through a linear polarizer (Laser Components Inc.) placed in front of the SHG detector (Hamamatsu model H7421-40). A BG 39 filter and a 510-520
nm band-pass interference filter were used to separate SHG from the laser radiation. The half-wave plate was rotated to 10 different angles in equal increments from 0 to $\pi/2$ radians, while the analyzer was rotated to 10 different evenly-spaced excitation angles for each polarizer angle.

**Polarization image analysis**

Polarization-in, polarization-out SHG imaging was performed to characterize the structural properties of collagen. The experimental geometry is presented in Fig. 1. The sample section is located in the XZ-plane of the Cartesian laboratory coordinate system and the probing laser beam propagates along the Y-direction. The average orientation of the collagen fibers in a pixel is defined by modified spherical angles $\delta$ and $\alpha$ as indicated in the Fig. 1.

![Fig. 1. Orientation of collagen fibers in the optical setup. XYZ is the laboratory coordinate system, and xyz is the fiber coordinate system. The red fiber indicates the weighted average of all fibers in the cone. The average-fiber orientation is defined by modified spherical coordinate angles $\alpha$ and $\delta$ in the laboratory frame. The incoming beam is shown by $k_\omega$ and the outgoing SHG radiation is determined by $2k_\omega$. Variables $\theta$ and $\varphi$ are the polarizer and analyzer angles with respect to the Z-axis, respectively.](image)

The ratio of second-order susceptibility tensor components, $\chi^{(2)}_{ZZZ}/\chi^{(2)}_{ZZX}$, for the sample voxel that contains cylindrical fibers of collagen of susceptibility component ratio, $\chi^{(2)}_{zz}/\chi^{(2)}_{xx}$, and oriented arbitrarily in space at angles $\alpha$ and $\delta$, is given by:

$$\chi^{(2)}_{ZZZ}/\chi^{(2)}_{ZZX} = \frac{\left(\frac{\chi^{(2)}_{zz}}{\chi^{(2)}_{xx}} - 3\right) \cos^2 \alpha \cos^2 \delta + 3}{\left(\frac{\chi^{(2)}_{zz}}{\chi^{(2)}_{xx}} - 3\right) \cos^2 \alpha \sin^2 \delta + 1}$$

(1)

The images at different excitation polarization $\theta$ and analyzer $\varphi$ angles (defined in Fig. 1) were assembled and polarization intensity analysis for each pixel of the image was performed applying the following Eq. (2), where birefringence was neglected because of the small (5 µm) sample thickness and also assuming Kleinman symmetry [6]:

$$I_{zz} \propto \sin (\varphi - \delta) \left(\frac{\chi^{(2)}_{zz}}{\chi^{(2)}_{xx}}\sin (\theta - \delta) + \sin 2 (\theta - \delta) + \frac{\chi^{(2)}_{zz}}{\chi^{(2)}_{xx}} \cos^2 (\theta - \delta)\right)$$

$$+ \cos (\varphi - \delta) \left(\sin^2 (\theta - \delta) + \frac{\chi^{(2)}_{zz}}{\chi^{(2)}_{xx}}\sin 2 (\theta - \delta) + \frac{\chi^{(2)}_{zz}}{\chi^{(2)}_{xx}} \cos^2 (\theta - \delta)\right)$$

(2)
\( \chi'_{Jk} \) is the measured susceptibility tensor component in the laboratory frame. Using a custom Levenberg-Marquardt fitting algorithm, the intensity variation of every pixel of the image as a function of \( \theta \) and \( \phi \) was fitted with Eq. (2), and best-fit parameter values for each pixel having goodness-of-fit \( R^2 \geq 0.80 \) are presented in the images. The susceptibility ratio calculated from each pixel of the imaged area in both tumor and non-tumor samples were then plotted as an occurrence histogram.

3. Results

H&E stained histopathology sections of human lung tissue with non-small cell lung carcinoma were investigated with SHG polarization microscopy, and representative NSCLC and normal lung images are shown in Fig. 2. The SHG images in Figs. 2(b) and 2(f) reveal collagen fibers in the tissue.

![SHG images of non-tumor and tumor areas](image)

The collagen fiber distribution morphology in the tissue and SHG intensity of the fibers show large variation between different tissue regions. The SHG intensity depends on the number density of collagen molecules in the focal volume, therefore large area scanning is required for morphological distinction between the tumor and non-tumor regions using SHG microscopy. On the other hand, nonlinear susceptibility component ratio can characterize the ultrastructure of individual collagen fibers independently of the concentration of the collagen molecules in the focal volume, and therefore provides diagnostic possibilities based on the properties of the fibers rather than collagen distribution morphology in the tissue.

By using SHG PIPO microscopy, the susceptibility tensor components ratio, \( \frac{\chi^{(2)}_{ZZZ}}{\chi^{(2)}_{ZXX}} \), for each pixel of the image can be obtained and is shown in Figs. 2(c) and 2(g). Only pixels with good fits \( (R^2 \geq 0.80) \) using Eq. (2) are shown and other pixels are
presented in black. The $\chi_{zz}\chi_{xx}$ values are represented with a color palette from blue to red corresponding to increasing ratio values. Comparison of Figs. 2(c) and 2(g) reveals marked differences between non-tumor and tumor tissues; the tumor has more orange and red pixels, while the non-tumor image is dominated by yellow and some blue pixels demonstrating that the collagen fiber ultrastructure is affected in the tumor tissue. Figures 2(d) and 2(h) show the corresponding histograms of occurrence of the susceptibility ratio, revealing a single-peak distribution with a shoulder towards the higher susceptibility ratios, indicating that two populations of collagen are present in the image. The modified Eq. (1) (for $\delta = 0$) was used to fit the occurrence histograms:

$$
\frac{\chi_{zz}}{\chi_{xx}} = A_1 \left[ f_1 \left( \frac{\Delta \chi_{zz}}{\chi_{zz}} \right) \cos^2 (\alpha) + 3 \right] + A_2 \left[ f_2 \left( \frac{\Delta \chi_{zz}}{\chi_{zz}} \right) \cos^2 (\alpha) + 3 \right] \quad (3)
$$

where Gaussian distribution functions $f (\chi_{zz}/\chi_{xx})$ and von Mises distribution $g(\alpha)$ are assumed for the variability of the susceptibility component ratio and the tilt angle variations across the image, respectively. The indices 1 and 2 indicate first and second population of the collagen fibers in the image, respectively. The fitting parameters are mean value of fibrilar susceptibility ratio $\chi_{zz}/\chi_{xx}$, the width of fibrilar susceptibility ratio distribution, the mean value of $\alpha$ and the width of its distribution. Also for each population a relative amplitude value is considered, $A_1$ and $A_2$, which shows the relative amounts of each of the two populations present in the image. Fitting of the distributions revealed that the mean tilt angle $\alpha$ value fluctuates around 0, and the shape factor of von Mises distribution can be maximized to constrain the tilt angel distribution to a narrow range. This indicates that visualized collagen fibers appear oriented close to the image plane.

Fits of occurrence histograms as well as the two populations of the susceptibility component ratio distributions are included together with the ratio occurrence histograms in Figs. 2(d) and 2(h). The presented results in Fig. 2 are typical for the 62 scanned regions investigated from 4 patients in this study. Table 1 presents the fitting parameters of the susceptibility ratio histograms. The lower susceptibility component ratio population has higher relative occurrence than the higher ratio population.

| Fitting parameter | Amplitude (%) | Susceptibility ratio | FWHM** of ratio |
|-------------------|---------------|----------------------|-----------------|
| Non-Tumor         | 58 ± 2***     | 42 ± 2               | 1.94 ± 0.02     |
| Tumor             | 65 ± 2        | 35 ± 2               | 2.12 ± 0.01     |
|                    |               |                      | 0.45 ± 0.01     |
|                    |               |                      | 0.87 ± 0.02     |
| p-value           | 0.02          | 0.02                 | 0.0001          |
|                   | 0.05          | 0.4                  | 0.1             |

* Data is based on 36 tumor and 26 normal lung regions scanned in 4 patients with matched normal and NSCLC tissue samples.
** Full width at half-maximum of the susceptibility ratio distribution.
***All errors are standard error of the mean values

When compared the relative amplitudes for normal and tumor tissue, slightly more of the smaller ratio population is present in the tumor tissue. However, this does not prevent the whole susceptibility component ratio occurrence histogram to shift to the higher ratios for the tumor tissue.

To validate the distinction between mean values of fitting parameters in tumor and non-tumor tissue the two-tail t-tests were performed and p-values are included in Table 1. Comparing four different patients, there would be four replicates of treatments, which make the degree of freedom 6. The susceptibility ratio value of the first and the second population
in tumor and non-tumor are highly significantly different, and significantly different, respectively. Furthermore, relative amplitude of both populations are significantly different, while the widths of the ratio distributions is not significantly different between tumor and non-tumor tissue. The statistical analysis shows that tumor and non-tumor tissue can be robustly distinguished base on the susceptibility ratio.

4. Discussion and conclusions

The susceptibility component ratio of either population in tumor and non-tumor are significantly different. The measured susceptibility ratio in a pixel can be related to the structure of collagen using hierarchical organization described by Tuer et al. [6, 7]. Differences in susceptibility ratio can originate from the three structural hierarchy levels in collagen and this appears to be affected in tumor tissue. Thus, differences in the amino acid composition of collagen can alter the susceptibility ratio of triple helices [9]. In turn, the triple helices can assemble parallel or they can coil into fibrils with a specific helical pitch, which also influences the susceptibility component ratio [10]. The same argument holds for assembly of the fibrils into collagen fibers with different coiling pitch angle. The fibers can also be arranged with a specific angular spread within a single image pixel. Thus, a larger spread of the fibers from the dominant direction yields a higher effective susceptibility component ratio. From the higher susceptibility ratios of tumor in Table 1 we can speculate that collagen is less compact and has larger disorder in the tumor tissue. This is independent of the overall collagen content and distribution that is revealed in standard SHG microscopy. Hence, SHG polarization imaging provides complementary information for visualizing cancerous areas in pathology diagnosis. The susceptibility component ratio can be used as a new marker for analyzing the histological sections in cancer diagnosis/prognosis. Pathologists, for example, could use the color-coded ratio images Figs. 2(c) and 2(g) to locate tumor areas in the digital image of a histology section. In addition, by analyzing the frequency histograms of the susceptibility ratios in the images the structural organization parameters of collagen can be determined independent of the total collagen content, and could serve to characterize the tissue structure in a quantitative and non-subjective manner. These observations of altered collagen structural hierarchy will be extended to study underlying biological mechanisms using biochemical analysis in combination with laser capture microdissection technique [11] and AFM imaging. Future investigations will include the assessment of whether or not these changes are specific to lung cancer or represent a more universal phenomenon in tumor initiation and progression.

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