Structural characterization of collagen using polarization-resolved second harmonic generation (SHG) microscopy

N Mazumder$^{1,*}$, Sindhoora K M$^1$ and F-J Kao$^2$

$^1$Department of Biophysics, Manipal School of Life Sciences, Manipal Academy of Higher Education, Manipal, Karnataka, India 576104
$^2$Institute of Biophotonics, National Yang-Ming University, 11221 Taipei, Taiwan

*E-mail: nirmal.mazumder@manipal.edu

Abstract. Second harmonic generation (SHG) microscopy is an effective analytical tool for a detailed investigation of the microscopic structure of non-centrosymmetric molecules. We developed a four-channel photon-counting-based Stokes polarimeter integrated to the SHG microscope for spatial characterization of polarization effects in the SH signal. We implemented Stokes-vector-based polarization-resolved SHG imaging to perform quantitative polarimetry, with a view to applications in biomedicine, which can measure the full polarization state of the SH light. In this work, we describe the application of a Stokes-vector-based four-channel photon counting SHG microscope to determine the molecular interpretation of the SH light from collagen in normal, scar, and keloid tissue.

1. Introduction
The second harmonic generation (SHG) is a second-order non-linear coherent optical process that has been widely used for imaging non-centrosymmetric molecular structures, partly due to the lack of photo-bleaching [1, 2]. SHG provides a unique contrast mechanism on a wide range of materials, as well as the capacity to image with a higher spatial resolution and at sub-millimeter depths [1, 2]. Recently, polarization-resolved SHG microscopy was used to investigate the relative molecular orientation and disorder in the structure of human tissues, such as the dermis [3], cornea [4,5], and myosin of the skeletal muscle [6]. However, the technique based on Jones methodology is applicable to only perfectly polarized light beams [7, 8] and hence is unsuitable for many related applications. In 2012, for the first time, Mazumder et al. reported the measurements and characterization of polarization properties of second harmonic (SH) signals using a four-channel photon-counting-based Stokes polarimeter, and critical polarization parameters were obtained concurrently without repeated image acquisition. The critical polarization parameters, including the degree of polarization (DOP), the degree of linear polarization (DOLP), and the degree of circular polarization (DOCP), were extracted from the reconstructed Stokes-vector-based SH images in a pixel-by-pixel manner [9, 10]. We wish to investigate the morphological alterations of the wound tissue progression, an important global health problem [11].

The dynamics of metabolism in the wound are not well understood at the cellular level [12, 13]. To understand the collagen structural information in the extracellular matrix in wounded tissue, SHG microscopy was used. Simultaneous collection of Stokes vector SHG images can provide information
on morphological alterations of fibrillar collagen and its density during tissue regeneration could depict the fate of wound healing with therapeutic outcomes. The identification of differences in collagen structure during the progression of wound healing could be a decisive factor in determining the influence of therapeutic interventions [12]. In the case of burn wounds, metalloproteinase expression regulated early during tissue remodeling can lead to altered collagen deposition [13]. The SH signal generation is strongly dependent on the angle between the input state of laser polarization and collagen alignment [9], and thus the loss of collagen orientation can be determined through the degree of polarization (DOP).

The birefringence and chiral behavior of collagen fiber can be differentiated by the degree of linear polarization (DOLP) and the degree of circular polarization (DOCP) [9, 10]. Hence, SHG microscopy integrated with Stokes polarimeter can be a useful tool to study scars tissue. We measured the Stokes vectors and various polarization properties of the SHG signal of collagen in normal, scar, and keloid tissue.

2. Materials and methods

The experimental design of Stokes-vector-based SHG microscopy is described in detail in [9, 10]. A Stokes polarimeter measures the four Stokes vectors of SHG light using four detectors simultaneously and reconstructs the 2D images. The 800-nm output wavelength from a femtosecond Ti:Sapphire laser is used as the excitation source with pulse duration of ~100 fs, repetition rate ~76 MHz, and average power ~550 mW. The system consists of a polarization state generator (PSG) placed in the excitation arm, a sample, and a polarization state analyzer (PSA) after the sample. We have incorporated them into an Olympus IX71 microscope [see figure 1, ref. 9] for implementation. The SHG signal from the tissue sample is passed through the PSA and imaged by four photo-multiplier tubes (PMTs) which are integrated into time-correlated single-photon counting electronics (TCSPC, PicoHarp300, PicoQuant GmbH, Germany). The SHG Stokes vector \( S_{\text{out}} \) is measured from the four intensity images, \( I \), using the equation: \( S_{\text{out}} = (A_{4\times4})^{-1} \cdot I \). The 2D Stokes vector and various polarization parameters images are reconstructed using the MATLAB (MathWorks, R2009b, Natick, MA) program. In this experiment, human skin tissue of different stages of scar formation was used for SHG microscopy. The SH signals were measured from histological tissue sections of thickness of 5 μm (eosin and hematoxylin stained, fixed and placed on a glass slide which is covered by coverslip). We collected five images from each tissue section of normal, scar, and keloid. We used 400/20-nm bandpass filter in combination with a short-pass filter (Brightline 680 SP, Semrock) for SHG signal collection in forward direction. This helps to block the auto-fluorescence and fluorescence signals from eosin and hematoxylin.

3. Results and discussion

Stokes parameters of the SH signal from normal, scar, and keloid tissue were measured to investigate the alignment and orientation of collagen fibers. Collagen fibers are the main component that changes in different types of scar tissue, including its molecular architecture. They can be visualized under optical microscopy when stained by a dye, e.g., hematoxylin and eosin. The SHG microscopy improves the image contrast due to the birefringence nature of collagen, and also the quadratic dependence on the SHG active molecules. Previously, it was reported that SHG microscopy can quantify the collagen in human scar tissue [8]. Figure 1 shows the reconstructed 2D Stokes vector SHG images of normal, scar, and keloid tissue with a horizontally polarized input laser beam. Figure 2 shows the variation of the polarization properties of the SHG signal in terms of the DOP, DOLP, DOCP, and anisotropy, \( r \). The polarization properties are derived from the four Stokes parameters.

Scar in the skin results from wound healing and is a natural process. Scar tissue replaces the normal skin and heals it through the production of collagen fibers; they are aligned in a single direction. Overproduction of collagen fiber occurs in the case of keloid and causes abnormal wound healing. In this stage, the amount of collagen fibers is larger, thicker, and randomly oriented as compared to normal scar. Figure 1 shows the Stokes vector SHG images of scars tissue and depicts the high second-order nonlinearity due to the densely-packed collagen fibers. The polarization states of the SHG signals are identified by the Stokes parameters and showed variance in the different types of scar tissues. The
SHG signal is more polarized in the case of normal than the other types of tissue samples, which is due to the molecular arrangement of the collagen fiber.

![Experimental reconstructed 2D Stokes vector images of SH light from normal, scar, and keloid tissue with a horizontally polarized input laser beam](image)

**Figure 1.** Experimental reconstructed 2D Stokes vector images of SH light from normal, scar, and keloid tissue with a horizontally polarized input laser beam. The color scale shows the values of each parameter.

Various polarization parameters are analyzed in each pixel of the image from the Stokes vector and 2D images are reconstructed (as shown in figure 2). The polarization values are higher for normal than scar tissues. For a perfectly polarized light, the DOLP values are equal to 1, whereas they are 0 for depolarized light. We observed that the DOLP values in the case of a keloid scar are close to 0.7 and 0.9 for normal tissue. The DOCP of SH light is attributed to the signal depolarization and birefringence of collagen. Further, the molecular arrangement of collagen fiber is measured by the anisotropy $r$ and ranges from -0.5 to 1. The anisotropy image indicates that the average value of $r$ is 0.9 for normal tissue and 0.6 for keloid. Higher anisotropy values suggest that collagen fibers in a normal tissue is more organized and aligned with respect to keloid.
4. Conclusion
We discuss the application of a Stokes polarimeter integrated with SHG microscopy to fully characterize the polarization states and various polarization parameters of the SH signal. The Stokes vector and polarization parameters of the SH signal are measured in each pixel of the 2D images. The polarization parameters, mainly DOP and anisotropy, can distinguish between the molecular arrangements in scar tissue with a high spatial resolution. The DOP is higher in the case of a normal than a keloid tissue. This suggests that the SHG signal from collagen fiber is highly dependent on the molecular arrangement in the tissue. Therefore, the DOP and anisotropy provide a higher sensitivity for collagen fiber and may provide good diagnostic accuracy. The technique has a potential for applications in biomedicine, where the structure or orientation of the collagen molecules are key factors in the e.g., the progress of wound healing.

Acknowledgments
We thank the Department of Science and Technology (Project No.: DST/INT//BLG/P-03/2019), Government of India for the funding. We thank Manipal School of Life Sciences, Manipal Academy of Higher Education, Karnataka, Manipal, India for providing the infrastructure during the preparation of the manuscript. The authors also thank the National Science Council, Taiwan (NSC99-2627-M-010-002, NSC98-2627-M-010-006, NSC97-2112-M-010-002-MY3, and NSC98-2112-M-010-001-MY3).

Compliance with ethical standards
We thank Dr. Wei-Wen Wu of Taipei City Hospital, Division of Plastic & Reconstructive Surgery, Department of Surgery, Heping Fuyou Branch, Taipei, Taiwan, for providing the tissue samples studied.

Conflict of interests
The authors declare no conflict of interest.

References
[1] Su P J et al. 2009 Opt. Express 17 11161-11171
[2] Han M, Giese G and Bille J F 2005 *Opt. Express* **13** 5791–5797
[3] Mainil-Varlet P *et al.* 2003 *J. Bone Joint. Surg. Am.* **85-A** Suppl 2 45-57
[4] Mansfield J C, Mandalia V, Toms A, Winlove C P and Brasselet S 2019 *J. R. Soc. Interface* **16** 20180611
[5] Strupler M, Pena A M, Hernest M, Tharaux P L, Martin J L, Beaurepaire E and Schanne-Klein M C 2007 *Opt. Express* **15** 4054–4065
[6] Sun Y *et al.* 2006 *Biophys J.* **91** 2620–2625
[7] Provenzano P P, Eliceiri K W, Campbell J M, Inman D R, White J G and Keely P J 2006 *BMC Med.** 4** 1–5
[8] Cicchi R, Sestini S, De Giorgi V, Massi D, Lotti T and Pavone F S 2008 *J. Biophoton.* **1** 62–73
[9] Mazumder N *et al.* 2012 *Opt. Express* **20** 14090–14099
[10] Mazumder N, Hu C W, Qiu J, Foreman M R, Romero C M, Török P and Kao F J 2014 *Methods* **66** 237–245
[11] Deka G, Wu W and Kao J F 2012 *J. Biomed. Optics.* **18** 061222
[12] Jake D J, Hallie E R, Woessner A E and Quinn P K 2018 *Communications Biology* **1** 19
[13] Prabhu V Rao S B, Fernandes E M, Rao A C, Prasad K and Mahato K K 2014 Objective assessment of endogenous collagen in vivo during tissue repair by laser induced fluorescence. *PLoS One.* **29** (5) e98609