*Armenian Journal of Physics, 2016, vol. 9, issue 2, pp. 183-187*

# Second Harmonic Diagnostics for Real-Time Monitoring of Biological Macromolecules Structural Changes

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Received 15 January 2016

**Abstract.** Peculiarities of second harmonic generation (SHG) have been studied in the samples of native tissue with different ordering at biotissue ablation and welding laser procedures. The dependence of magnitude of a second harmonic signal in samples of native tissue with different types of structural organization has been determined. Revealed polarization dependence of SHG on collagen macromolecules organization in tissue during photoheating demonstrates that SHG technique could be a powerful tool in early detection of diseases accompanying with tissue disordering.

**Keywords**: biotissue, laser ablation, laser welding, second harmonic diagnostics

## Introduction

As was shown in the latest research reports, new unique possibilities for researchers and practitioners in the field of biomedical optics can give nonlinear optical methods. As we known, in 1986 the Freund and coworkers [1] were first who demonstrated the applicability of SHG technique for soft biotissue by using a transmission type of SHG microscopy. In that work the rare type of biotissue such as relatively optically transparent wet rattail tendon tissue was chosen as biosample where effective SHG can be easy detected. In 1993 Altshuler et al. [2] demonstrated the nonlinear-optical phenomenon of frequency conversion in hard tooth tissue (enamel, dentin). This kind of hard tissue contains the high amount of non-centrosymmetrical nonorganic microcrystals of hydroxyapatite and can effectively generate optical second harmonic. Requirement of good optical transparency is a strong limitation factor for the further development of biomedical applications of SHG technique for optical analysis of biotissue, since in variety types of biological tissue the strong scattering of laser irradiation takes place. This leads to attenuation of probing laser beam intensity and consequently to sharply decrease of altitude of nonlinear response.

However, in our previous works [3-4] we demonstrated that during picosecond Nd:YAG irradiation the effective SHG occurs for various types of nontransparent biological tissues particularly in the samples of muscle, fat, skin tissues that represent the strong turbid media for light transport. Yici Guo et al. [5] reported similar results at picoseconds, as well as for femtosecond laser irradiation. Later, several groups have demonstrated second harmonic tomography techniques for tissue optical analyses [6-8]. Particularly, Yici Guo et al. [6] demonstrated possibility to evaluate the subsurface layer structure of mucosal tissues by using femtosecond laser. 2-D images of hamster cheek pouch mucosa were obtained by scanning the second harmonic signal at various lateral and axial positions.

In this paper, a laser ablation and welding of biotissue have been studied simultaneously in the biosamples with tissue different structure by SHG technique under the picosecond laser irradiation. The polarization properties of second SHG have been investigated in ordered tissue samples comparing with other two-order nonlinear optical phenomena such as two-photon fluorescence (TPF).

**Experimental results and discussion**

For the study of nonlinear response from biotissues, the pulsed passive mode-looked picosecond YAG:Nd laser was used. Laser output with energy 20 mJ was linearly polarized and represented the train of picosecond pulses (single pulse duration $\tau_{imp}$ =33ps). The nonlinear optical response was registered by monochromator (MDR-23) -PMT- Signal Amplifier- PC system. The presence of optical anisotropic birefringence structures stipulates the SHG phenomena in biotissue. One of such structure is microfibril, which contains the biological macromolecules. In animal and human organisms, microfibrils form arbitrary large size fibrils. Such structure represents the optically anisotropic system with optical properties similar to the molecular organic nonlinear crystal. Among other biofibrils the collagen fibrils produce the strongest SHG because of their largest sizes (7x40 microns) that are in order of coherence length of harmonic generation nonlinear optical process.

The continuous wave Nd:YAG laser with output beam diameter ≈ 2mm and power up to 4W was used as photoheating source of radiation. As the result of laser heating of tendon and fascia tissue samples the amplitude of SH nonlinear signal decreased more than 6 times in the surface area in the neighborhood of irradiation spot. Fig.1. shows the 2D map of SHG signal in tendon (a) and fascia (b) samples. Note here that in these types of biotissue the collagen molecules are dense packaged in the microfibrils, which form specific types of tissue. In the case of tendon tissue, the collagen fibrils have the parallel orientation concerning each other. In case of fascia tissue the parallel oriented collagen fibrils form tissue layers (lamellar organization), where orientation of fibril is different. As the result of laser heating, the collagen molecules significantly lose their ability to produce SH signal. This result corresponds to our previous data [4], where tissue sample was heated in water containing thermo-regulated box. SHG signal decreased sharply in temperature interval of 59-64$^0$C, which corresponded to spiral - coil transitions of collagen.

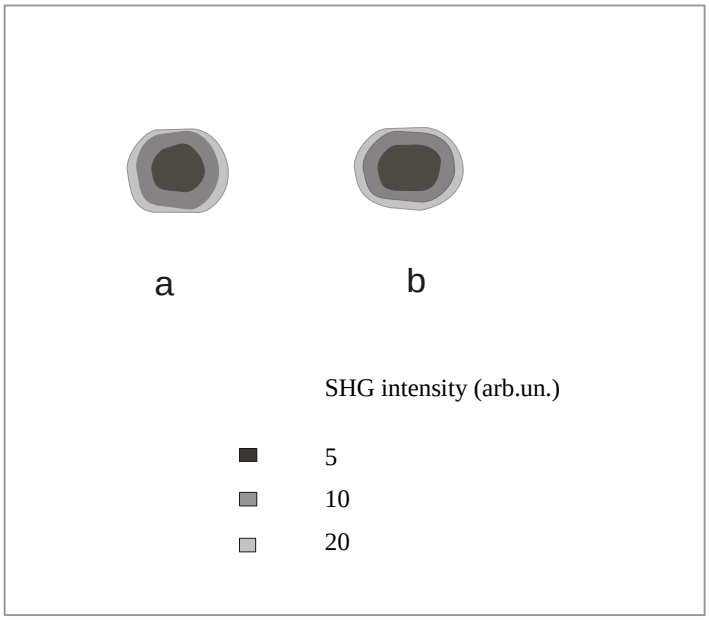


**Fig. 1**. Two-dimensional map of SHG signal after laser heating of fascia (a) and tendon (b) tissue samples.

As we can see on Fig. 1 the decreasing of SHG signal in the laser heated area surface is different in samples of fascia and tendon tissues. On the surface of fascia, the laser irradiation induces denaturation of collagen molecules, which occurs in the symmetrical geometry and is independent from direction of the scanning probe beam. On the surface of tendon, we have a picture with the

elliptical geometry where the large axis is parallel to the fiber orientation. This result can be explained by assuming that the heat transfer in tendon and fascia is different due to different tissue organization. In case of lamellar type of tissue organization each layer have preferable orientation where heat transfers more effectively, however, the impact of the several layers give symmetrical distribution of SHG response. In case of the strong oriented tendon tissue where fibers are oriented in only one direction, the distribution of SHG response from the sample is elliptical.

Polarization dependence of SHG has been studied, comparing with another nonlinear process - two-photon fluorescence (TPF). For excitation of TPF with signal level in order of SHG, the samples of strong ordered tendon tissue were colored by the organic dye with the high fluorescence quantum yield. The tissue samples were held for 5 min in physiological saline with Rhodamine 6G at concentration of $10^{-4}$ M. Then, the samples were washed again with pure physiological saline for 5 min. In the case of nanosecond irradiation, when irradiation intensity is decreased in about three orders, and the conditions of registration scheme and energy density remained the same, SHG was not observed. Both TPF and SHG nonlinear optical phenomena were observed simultaneously in the samples of tendon tissue with the thickness of 0.5 mm. The samples were irradiated by linearly polarized beam in the direction, which was perpendicular to biofibers. Using of the picosecond train allowed to easily register the signals of two-photon fluorescence and second harmonic generation in distinguish from the case of single picosecond pulse, when additional optical amplifier stage is required.

Second harmonic generation and two-photon fluorescence phenomena exhibited the different polarization dependence. Magnitude of SHG signal was about 2.5 times larger in case when polarization of laser beam was parallel to collagen fibers (Fig. 2a) than in the perpendicular case (Fig. 2b). Since TPF and SHG processes occur at the same time and both depend on intensity by square-law, the changing of irradiation intensity due to scattering would have the same impact on both those processes. However, in contrast to SHG the TPF signal did not show any dependence on the polarization of the incident beam. Note here, that SHG signal was produced under the linear polarized irradiation and was also polarized. For 0.5mm thickness tendon samples the polarization of SHG was: $P(2\omega) = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp}) = 0.5$.

Because the TPF signal was not polarized, the difference in polarization properties of SHG and TPF can be explained by assuming that both nonlinear signals are produced mainly in thin subsurface layer of sample. Obviously, in this case the polarization of incident beam will have a weak influence on TPF.

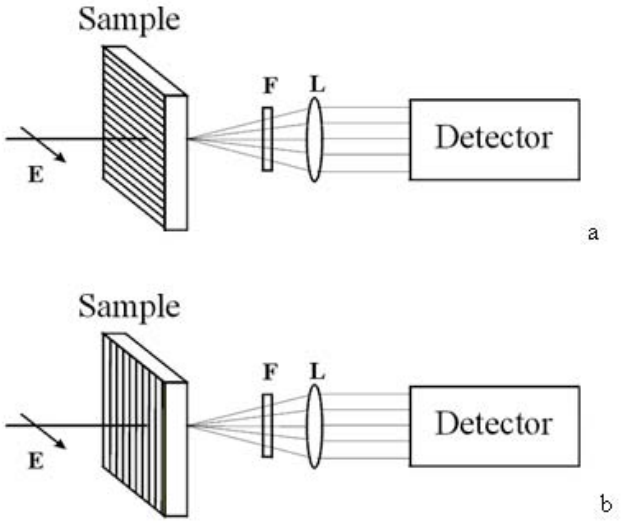


**Fig. 2**. Two cases of irradiation of samples: (a) polarization of laser beam was parallel to collagen fibers; (b) polarization of laser beam was perpendicular to collagen fibers.

Strong polarization dependence of SHG can be explained by different action of the light scattering on non-polarized TPF and polarized SHG signals during propagation in the ordered tissue. To reveal the dependence of the observed phenomenon on scattering of polarized beam, the attenuation of the control linear polarized beam with weak intensity after the passing through the same sample and same experimental scheme was measured. Our measurements shown, that the intensity of the passed beam was 4.9 times larger (low scattering) at parallel polarization than at perpendicular (strong scattering).

Thus, we can conclude, that scattering of polarized beam is the main mechanism explaining the obtained experimental results. We also studied the changes of SHG signal magnitude during laser ablation and welding of skin tissue. The welding and ablation were performed on samples of chicken skin, which were irradiated by CW laser and then were tested by probing picosecond beam. The SHG signal is detected during scanning of sample in plane perpendicular to the sample`s surface. The altitude of SHG signal was independent from polarization of the probing laser beam. Fig.3. shows the altitude of SHG signal intensity at welded and ablated spot and its surrounding. The SHG signal is detected at scanning of the probing beam across the irradiated area of the sample. The experimental points were obtained by averaging 60 pulses.

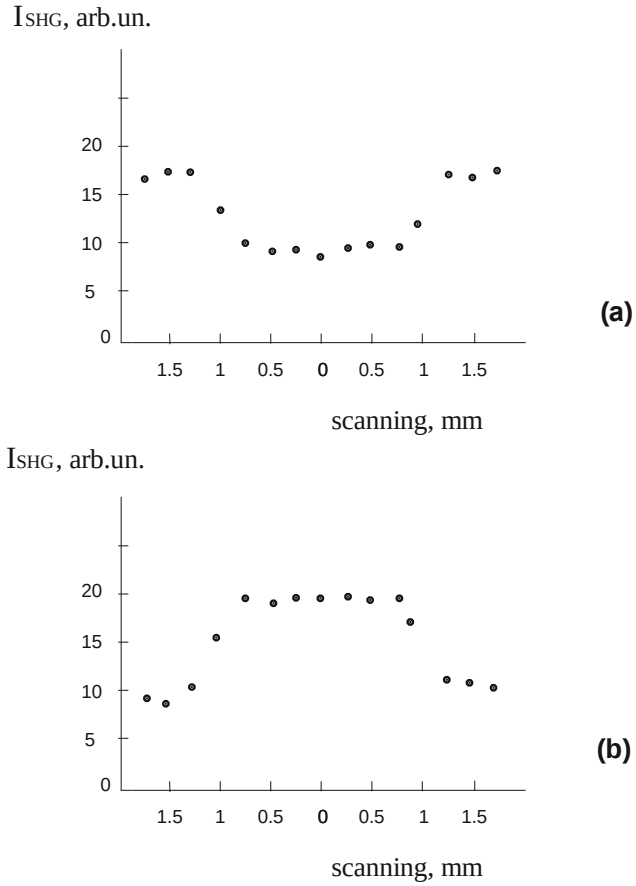


**Fig.3**. SHG intensity during scanning of the probing beam across the welded (a) and ablated (b) area of the skin sample.

The amplitude of the SHG signal was independent from polarization of the probing beam. As a result of laser heating during welding of tissue sample the SHG nonlinear signal decreased on factor 1.7 in the area of skin surface exposed to laser irradiation (Fig. 3a). Fig.3b. shows the change of the SHG signal intensity from the ablated spot. Measurements were performed on several ablated spots of the sample. As can be seen in Fig. 3b, the intensity of the SHG signal grows in the area of the

skin surface exposed to laser ablation. It is worth to mention that the layer of the skin tissue containing mainly collagen macromolecules provides a major impact on the nonlinear response. This layer with elastic netlike structure of collagen fibers is located deep in the skin. The intensity of the probing beam is attenuated significantly by reaching this inner layer due to strong scattering after passing through the surface layer (epidermis). Attenuation of irradiation can significantly influence on the SHG phenomenon because the intensity of SHG signal depends on the intensity of the probing beam in square-law. The removal of the superficial layer during ablation leads to the increase in intensity of the probing beam in collagen layer due to a decrease in the thickness available for scattering and absorbing media located before collagen layer.

Thus, in the ablated area of the skin where the superficial layer is removed but the inner collagen layer is not damaged, the intensity of the SHG signal increases. The altitude of SH signal increases approximately by two times after laser ablation of skin sample.

In conclusion, we have demonstrated the potential of the SHG technique to monitor structural changes of biotissue during laser ablation and welding surgical procedures. Obtained results show, that the SHG nonlinear phenomenon gives a new possibility to monitor the photothermal tissue damages connected with photothermal changes of collagen molecules during laser heating process in real time. It is especially important that this technique allows sensing the early changes in biotissue at temperature level about $64^0$C and before reaching a temperature values, when significant and irreversible photodamages in biotissue will be produced. Revealed polarization dependence of SHG on type of collagen macromolecules organization in tissue demonstrates that SHG technique could also be a powerful tool in early detection of diseases accompanying with tissue disordering.

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