

Optical indication of skin collagen denaturation during laser heating

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Optical diagnostics is the most effective method for non-invasive monitoring of tissue condition during non-ablative infrared laser exposure. In this work, laser radiation with a wavelength of 1560 nm and a power of 1–3 W was used to create microscopic zones of thermal damage in rabbit skin. The dynamics of the intensity of diffusely reflected and diffusely transmitted laser radiation from the sample were recorded using an integrating sphere. After laser exposure, the completeness of collagen denaturation in the irradiated area was assessed by the resistance of the tissue to the proteolytic enzyme treatment. A comparison of the optical characteristics dynamics of the sample and the degree of collagen denaturation allowed us to establish a correlation between the onset of collagen denaturation and changes in optical parameters: by reducing diffuse light transmission and increasing diffuse reflection. The time of the output of the diffuse transmission signal to a stationary level clearly correlates with the complete denaturation of collagen throughout the entire depth of the skin sample. Monitoring the dynamics of light transmission and reflection makes it possible to quickly and effectively adjust the geometric and energy parameters of laser radiation, which ensures the achievement of targeted local coagulation with minimal risk of overheating of surrounding tissues

Keywords: IR laser irradiation, skin, diffuse transmission, collagen denaturation.

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Introduction

IR laser radiation is an important medical tool for local hyperthermia of connective tissues followed by its target modification. Thus, fractional photothermolysis has become very popular and involves creation of multiple microscopic thermal tissue injury zones, initiating regenerative processes both in the affected area and adjacent tissues [1–3]. Such approach can cover not only cosmetic procedures, but also laser treatment of various pathological conditions including fibrotic changes. It should be kept in mind that the employed laser exposure parameters, on the one hand, shall ensure the target outcome and, on the other hand, shall minimize overheating of tissues surrounding the affected zone [1].

Collagen denaturation is a marker of occurring thermal injury zone. Therefore, tissue monitoring during laser exposure at the initial stage can be reduced to collagen denaturation control in the irradiation region. The coagulated zone volume can be estimated via an analysis of a non-steady temperature field formed during irradiation, which is, in turn, calculated on the basis of a light field taking into account the known absorption coefficient μ_a , scattering coefficient μ_s and anisotropy factor g [2,4]. These optical characteristics are predominantly personalized for each subject, however, they can be successfully evaluated via a combination of mathematical simulation and experimental light absorption and scattering data [5–9]. Nevertheless,

accurate calculation of the degraded tissue volume encounters two substantial challenges. First, μ_s and g demonstrate a temperature dependence, establishment of which requires that the experimental technique be significantly sophisticated [7,8]. Second, collagen denaturation in tissue during laser heating depends on the irradiation parameters and can be initiated at temperatures other than those established during traditional heating [10,11]. Therefore, preliminary prediction of laser exposure outcome on the basis of estimates becomes too approximate, which is an unacceptable restriction taking into account the potential use of this method in medicine. Thus, histological evaluation is a standard for evaluating thermal tissue injury. Effective modern techniques for monitoring changes in tissues include the optical coherence tomography. In particular, for skin this technique provides noninvasive monitoring of all layers up to 1 mm in depth [12,13]. However, both techniques cannot be used for detecting tissue condition directly during irradiation. Thus, optical diagnostics techniques based on light absorption and scattering are still the most convenient tool for noninvasive tissue monitoring during laser exposure. Thus, it is shown in [7–9] that μ_s and g change significantly after thermal treatment of tissues, which can affect the form of transmission and reflection of radiation that initiates morphological changes in tissues.

The purpose of this study is to identify skin modification during non-ablative laser exposure by comparing the behavior of diffusely reflected and diffusely transmitted laser radiation with a degree of collagen denaturation.

Materials and methods

Skin of $350 \pm 50 \mu\text{m}$ in thickness in the inner upper area of a rabbit's ear auricle served as a subject of study [14]. The LSK 1.56 1560 nm surgical laser (IRE Polyus, Russia) was used for radiation. Laser radiation power (P) was varied from 1 to 3 W and controlled by the UP12-H power meter (Gentec Electro-Optics, Canada). Exposure time τ_f in the main series of experiment was 50 ms. For a power of 2 W and higher, the second series of experiment was performed with τ_f decreased to 30 ms. Contact exposure was used with radiation delivered to samples using 600 μm optical quartz fiber with a numerical aperture of 0.22. A separate experiment on a sample thermally treated on a water bath at 90°C for 1 min was carried out additionally (for comparison).

Diffuse transmission and diffuse reflection of the tissue were measured using an integrating sphere [4] with two holes. In the first case the sample was placed in front of the sphere inlet, in the second case the sample was placed outside the sphere near the outlet. In both cases, the optical fiber end was placed in immediate proximity to the sample surface. A signal associated with the diffuse transmission (T_d) and diffuse reflection (R_d) of the laser radiation was measured by the InGaAs/InP photodiode with a spectral range of 1.1–1.62 μm and time resolution of 100 μs . Photodiode was placed on the inner surface of the sphere and shielded from the sample by a damper. Typical times of change in T_d and R_d (τ_T and τ_R , respectively) during sample exposure were determined after processing the primary photodiode signal in OriginPro using the *Smooth* procedure for 20 points via the *Adjacent-Averaging* method followed by differentiation of the smoothed curve and repeating the *Smooth* procedure on the already differentiated curve for 20 points via the *FFT filter* method.

Denaturation completeness throughout the sample depth was determined from the trypsin resistance of intact collagen. Trypsinization was performed in a solution with an enzyme concentration of 1.5 mg/ml with addition of sodium azide at 37°C for 24 h. Holes in the tissue (perforation) were determined using the Digital Industrial Microscope GS-23. For each power, 6 to 9 applications were performed.

A separate experiment was carried out to determine the temperature field behavior on the front and back surfaces of samples with laser heating. The FLIR 655sc thermal imaging camera with an additional FOL25 lens (FLIR Systems Inc., USA) with a maximum frame frequency of 200 Hz and spatial resolution of $\sim 10 \text{ px/mm}$ was used for measurements. FLIR ReserchIRMax software was used to extract the information about the temperature behavior in the center of the laser spot (maximum sample surface temperature).

At least five measurements of radiation transmission and reflection were carried out for each power. Difference between the mean τ_T and τ_R of independent samples was evaluated using the two-sample unpaired Student t -test. An *a priori* significance level was set to $p = 0.05$.

Temperatures in the center of the laser spot for the typical times τ_T and τ_R were calculated by averaging over 3–5 temperature field measurements for each power.

Results

Figure 1 shows typical time curves of T_d reflecting the change of diffuse transmission of laser radiation through the sample. Time dependences of T_d demonstrated three distinct types of behavior caused by different P . Type I curve features a stable level of signal T during exposure or a change of the signal with a minor trend (Figure 1, curve 1). Type I dynamics was observed at relatively low P not greater than 1.7 W. As P increased to 1.7 W, significant reduction of T was detected after a stable period. These dependences are classified as Type II (Figure 1, curve 2). At powers of 2.2 W and higher, signal T achieved the plateau after the initial drop (Type III dependence, Figure 1, curve 3).

Start times of signal T_d (τ_T) drop for Type II and III dependences are shown in the table. Note that τ_T decreases as P grows.

For powers of 2.5 W and 3 W, at which time dependences of signal T_d corresponding to Type III were recorded, additional tests were performed. In the first experiment, when the same tissue region was repeatedly exposed to laser radiation, signal T_d maintained at the level achieved after the plateau in the first pulse (Figure 2, curve 2). In the second experiment, a thermally treated material sample (90°C, 1 min) was exposed to radiation. In this case, signal T_d turned out to be much lower than in the intact material and remained unchanged throughout the laser exposure period (Figure 2, curve 3).

The obtained results show that laser exposure with a power greater than 2.5 W and thermal treatment induce irreversible changes in skin tissue. However, note that

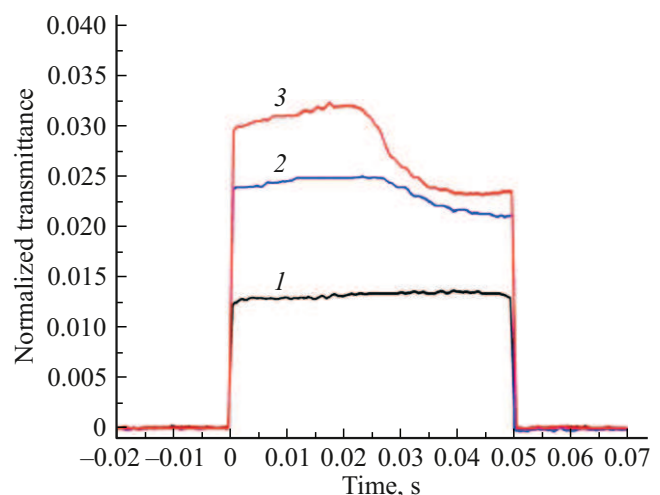


Figure 1. Behavior patterns of T_d at different exposure powers: type I (curve 1, $P = 1 \text{ W}$), type II (curve 2, $P = 2 \text{ W}$) and type III (curve 3, $P = 2.5 \text{ W}$).

Characteristics of radiation transmission and reflection behavior (see the text for designations) and corresponding maximum surface temperatures at certain τ

P, W	1	1.5	1.7	2	2.2	2.5	2.7	3
$\tau_T \pm SD, ms^{*,***}$	n/a	n/a	38 ± 2	26 ± 3	22 ± 2	20 ± 2	18 ± 1	15 ± 1
$T_B(\tau_T) \pm SD, ^\circ C^{**}$	—	n/a	42 ± 2	44 ± 1	42 ± 1	44 ± 1	44 ± 1	39 ± 1
$T_F(\tau_T) \pm SD, ^\circ C$	—	n/a	55 ± 1	55 ± 1	56 ± 5	56 ± 1	55 ± 5	56 ± 1
$\tau_R \pm SD, ms^{*,***}$	n/a	40 ± 2	31 ± 2	22 ± 2	19 ± 2	15.5 ± 1.0	13 ± 1	11 ± 1
$T_F \pm SD(\tau_R)^{**}$	—	53 ± 2	52 ± 2	51 ± 2	51 ± 1	50 ± 2	50 ± 2	48 ± 2

* Mean values of τ were statistically significantly different for different P .

** Mean values of T were statistically significantly different for different P , except for the data for $P = 3 W$.

*** Mean values of τ_R and τ_T were statistically significantly different for one power, except for 2.2 W.

„—“ — no measurements were performed;

„no“ — there is no effect.

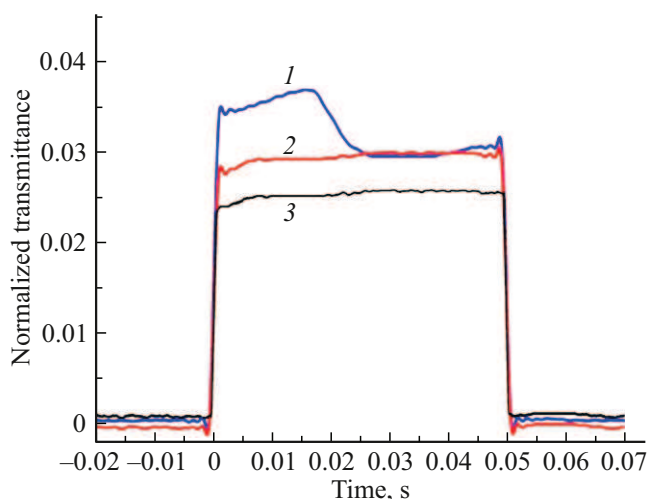


Figure 2. Signal T_d behavior during initial laser exposure $P = 3 W$ (curve 1), repeated exposure of the same location (curve 2) and thermal treatment of the intact material (curve 3).

thermal treatment caused a 1.5–1.7 times increase in the test skin sample thickness and a decrease in the sample surface area.

Visual analysis of trypsinized samples has identified the following patterns:

- In samples exposed to laser with Type I temperature behavior (powers 1 W and 1.5 W), there were no surface changes.

- In samples with Type II temperature behavior (powers 1.7 W and 2.0 W), thinning of the exposed zone was observed (Figure 3, a).

- In samples with Type III temperature behavior (2.2–3 W), tissue perforation occurred in the affected zone (Figure 3, b).

Additional experiments with trypsinization of samples irradiated during 30 ms has shown that perforation on the affected zone occurred only when powers of 2.7 W and 3 W were used. At 2.5 W, the affected area grew thinner as shown in the microphotograph in Figure 3, a.

Typical time dependences of diffuse reflection signal are shown in Figure 4.

At $P = 1 W$, signal R_d remains almost constant throughout the laser exposure time (curve 1). At $P = 1.5 W$, signal R_d starts growing by the end of laser exposure (curve 2). As P increases to 1.7 W and higher, signal R_d stops increasing and remains almost unchanged (curve 3). Signal growth start times of R_d (τ_R) are listed in the table. Note a decrease in τ_R with growth of P and statistically significant differences in τ_R and τ_T for the same radiation power P .

On the basis of the measured maximum sample surface temperature behavior, back surface temperature (T_B) and front surface temperature (T_F) corresponding to τ_T were chosen. T_B and T_F reach $\sim 44^\circ C$ and $\sim 55^\circ C$, respectively, and don't depend on P . For τ_R , only the front surface temperature was measured. Note that T_F , at which the diffuse reflection signal starts changing, is $\sim 51^\circ C$ and is almost the same at different powers (table).

Discussion of results

Intensity drop of the radiation transmitted through the tissue starting from τ_T is associated with the start of tissue modification induced by laser exposure. While the effect is observed only when some threshold power P_{cr} is exceeded, and the higher the laser power, the earlier the change starts (τ_T decreases, table). At sufficiently high powers ($P \geq 2.2 W$), signal T_d decreases to the lower limit and achieves the plateau. In these cases, tissue transformation completely stops throughout the depth in the exposed zone during the laser exposure time. During repeated exposure of this region, no changes are observed in T_d (Figure 2, curve 2). Thus, tissue modification is irreversible. Comparison of temperature and T_d behavior shows that the same maximum temperatures on the front and back surfaces of the sample correspond to τ_T : $T_F \sim 55^\circ C$ and $T_B \sim 44^\circ C$. This indicates that it is laser-induced heating that causes irreversible changes in the tissue structure. This is proved by the transmission analysis of samples

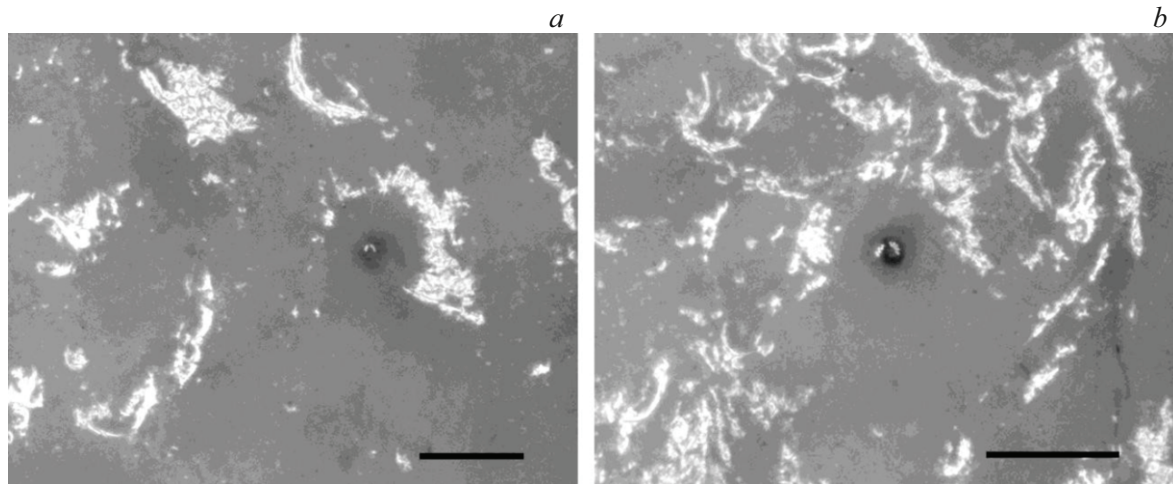


Figure 3. Microphotographs of the exposed sample after trypsinization: (a) thinning of the exposed zone, $P = 2$ W, (b) tissue perforation, $P = 2.2$ W. Scale bar length is $500\ \mu\text{m}$.

subjected to hydrothermal treatment at 90°C . Such samples demonstrate constant transmission when exposed to laser radiation from 2.5 W to 4 W (Figure 2, curve 3).

Thermal action on skin starting from temperatures higher than 65°C is known to result in collagen denaturation [10]. Collagen denaturation in a local laser exposure region was determined by the trypsinization method. An enzyme known as trypsin accelerates proteolysis of all proteins manifold, except for intact collagen macromolecules [15]. A sample subjected to hydrothermal treatment is completely dissolved under the trypsin action. In case of denaturation, perforation occurs in the laser exposure region throughout the sample depth after treatment with trypsin solution. In this study, perforation in the exposed region of trypsinized samples was clearly observed in samples subjected to irradiation with 2.2 W and higher (Figure 3, b). In these conditions, denaturation occurs throughout the sample depth and signal T_d achieves the plateau and remains unchanged during further laser exposure. The obtained data show that decreased radiation transmission of tissue is caused by the thermostimulated collagen denaturation.

At $P = 2$ W, after $\tau_T = 26$ ms signal T_d steadily drops until the end of exposure without having achieved the steady-state value (Figure 1). Microscopic analysis only detects tissue thinning in the exposure zone (Figure 3, a), indicating a partial denaturation throughout the sample depth. When power decreases to 1.7 W, the initial drop of signal T_d has a delay of $\tau_T \sim 40$ ms. However, the next 10 ms of nonuniform volumetric heating appear to be insufficient to achieve the degree of denaturation required for visualization of morphological changes. Additional experiment with reduced exposure time to 30 ms proves a hypothesis of dependence between denaturation completeness and laser exposure time. In particular, stabilization of signal T_d is achieved by 30 ms at $P \geq 2.7$ W. In the same conditions, the samples after trypsinization show distinct perforation in exposure zones. At 2.5 W, signal T_d starts

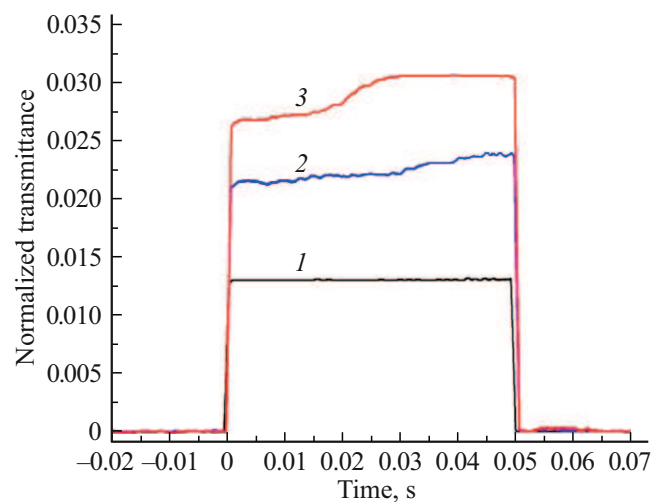


Figure 4. Behavior time patterns of R_d at different exposure powers: curve 1, $P = 1$ W, curve 2, $P = 2$ W, curve 3, $P = 2.5$ W.

declining from $\tau_T \sim 20$ ms, however, the remaining 10 ms of laser exposure are not sufficient for collagen denaturation throughout the sample depth. This case is similar to the exposure outcome with 2 W and 50 ms. All data together indicate a correlation between signal T_d behavior and collagen denaturation process in the exposed volume.

On the molecular level, collagen denaturation is described by transition of triple-stranded elongated collagen macromolecules into a random coil conformation. This results in the loss of quasi-crystalline uniaxial structure of fibrils and fibers or in amorphization [16]. At the macroscopic level, collagen-containing material thickness increases and dimensions decrease [17]. This effect is known as tissue shrinkage. Increase in the tissue thickness in the denaturation region can be interpreted as an increase in the optical thickness and can explain the decrease in transmission of

the initiating radiation. On the other hand, the effect of a significant change in absorption and scattering coefficients, and anisotropy factor on the integral transmission due to collagen denaturation cannot be excluded. Such change of optical properties was reported in [9]. Though the cause of drop of signal T_d cannot be exactly identified, this change can be definitely associated with the collagen denaturation process.

The pattern of signal R_d behavior critically depends on the radiation power. However, there are differences in the form of time dependences of signals associated with transmission and reflection of initiating radiation (table):

— signal R_d after τ_R starts increasing at a threshold power of 1.5 W (which is lower than 1.7 for T_d);

— τ_R is a little shorter than τ_T and T_F at τ_R is lower than T_F at τ_T .

Signal R_d is to a large extent associated with the sample surface condition and its change primarily reflects a change of the surface and near-surface layer. At the same time, for signal T_d to decrease, significant changes shall occur in tissue. This process requires some time necessary for heating to the depth of the sample. This can explain a minor shift of τ_R and power at which signal stationarity starts changing downwards compared with T_d . Nevertheless, there is correlation between the behavior of T_d and R_d and laser-induced irreversible collagen denaturation in tissue.

Conclusion

Optical methods have once again demonstrated the efficiency of real-time tissue monitoring under laser exposure. Such optical properties of skin sample as absorption coefficient μ_a , scattering coefficient μ_s and scattering anisotropy factor g haven't been determined, however, identification of changes in integral characteristics (diffuse transmission and reflection) has appeared to be sufficient for indicating the start and stop of morphological changes in skin matrix *in vitro*. Avoiding labor-consuming and long-term histological or physical and chemical analyses often complicated by a limited affected region, it becomes possible to correct geometrical and energy parameters of radiation quickly and effectively. This provides the achievement of target local coagulation, minimizing the risk of overheating of surrounding tissues.

The second important finding is the expansion of tissue monitoring capabilities under laser exposure *in vivo* via reflected light detection. This approach will allow proceeding to creating a diagnostics system for tissue monitoring during laser heating.

It should be noted that diagnostics based on integral absorption in all skin layers cannot be used to refer the detected signal to a change in each individual layer. As for the scattering signal, conclusions regarding any volumetric changes in tissue cannot be made either without evaluating the predominant probing depth. Development of the described approach requires further investigations combining

it with computational and experimental methods that allow analysis of changes in all layers of a test object [12,13].

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Declaration of competing interest

The authors declared that they had not any conflict of interests.

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