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Investigation of *in-vivo* skin autofluorescence lifetimes under long-term cw optical excitation

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Abstract. **The main results obtained during the last five years in the field of laser-excited *in-vivo* human skin photobleaching effects are presented. The main achievements and results obtained, as well as methods and experimental devices are briefly described. In addition, the impact of long-term 405-nm cw low-power laser excitation on the skin autofluorescence lifetime is experimentally investigated.**

Keywords: *skin autofluorescence, photobleaching, lifetime spectroscopy.*

1. Introduction

A decrease in the fluorescence intensity as a result of long-term optical excitation is known as photobleaching. Laser-excited tissue autofluorescence photobleaching (AFPB) has been studied extensively over the last few decades. Most of the authors observed that a decrease in the skin autofluorescence (AF) intensity can be well described empirically by a double exponential function [1–6]. Under cw excitation, the autofluorescence intensity I mainly decreases during the first 10–15 s, followed by a slow-down. In this case, there is a residual intensity, which asymptotically strives to a constant level A:

$$I(t) = a\exp(-t/\tau_1) + b\exp(-t/\tau_2) + A. \quad (1)$$

Here, τ_1 and τ_2 characterise the fast and slow phase of AFPB, respectively; a, b and A are the constants; and t is the time. Our previous research has shown that the photobleaching effect was evident in all skin types and in some skin pathologies under UV/VIS laser excitation. The photobleaching effect can be approximated with a double exponential function for all skin types and most skin pathologies [7, 8]. The authors of [8, 9] demonstrated that the temporal analysis of AF photobleaching indices for different areas of the skin may vary, and the bleaching rates distribution over the skin surface is uneven. A subsequent study of laser-excited skin photobleaching has demonstrated that AF intensity recovery (Fig. 1) after 2-min cw excitation is a long-term process. The measurements of the recovery kinetics show that even after 125 hours of relaxation, the AF intensity of the skin recovered to only 80% of its initial value. And the consequence of the long-term recovery of intensity, the skin 'photo-memory' effect, has been experimentally demonstrated: low-power laser irradiation (below the standard skin safety limits) leaves traces in skin for several days [10, 11].

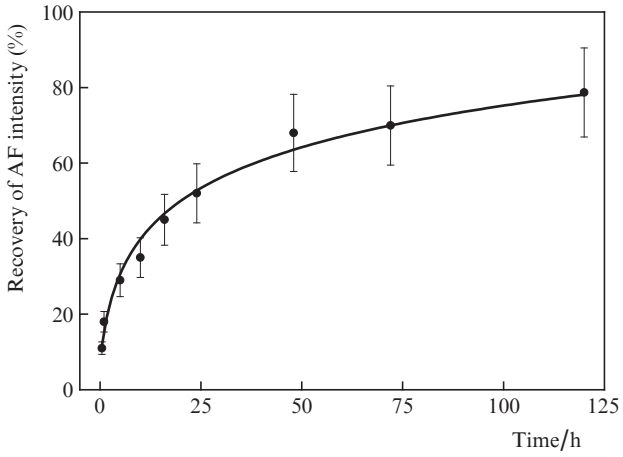

Figure 1. Kinetics of skin AF recovery after 2-min cw excitation by a 532-nm laser with a power density of 85 mW cm^{-2} [10].

Although the cause that gives rise to the photobleaching phenomenon remains unclear, it has become the focus of attention for its potential as a clinical diagnostic tool. In our previous clinical research we have focused on AFPB analysis of different pigmented, vascular and malignant skin pathologies. The analysis of dermatological pathologies showed that the most sensitive from the point of view of diagnosis are the parameters τ_1 and A. The pronounced difference in the A values (Fig. 2) for skin basal cell carcinomas appears to show the most promise for clinical implementation [12, 13].

In studying experimentally the AFPB of human skin we have found that under long-term cw irradiation the AF spectra as well as the diffuse reflectance spectra (Fig. 3) exhibit re-absorption peaks, which correspond to the absorption bands of oxy-haemoglobin. Thus, the appearance of the absorption peaks in the green (540 nm) and yellow (580 nm) regions of the visible spectrum during the experiment is an indication of a certain role of oxy-haemoglobin in the photobleaching process [14].

Although the mechanism of skin autofluorescence photobleaching has not been established in detail so far, we can assume that long-term cw laser irradiation most probably causes the photochemical process that leads to degradation of endogenous fluorophores. In turn, the degradation of skin fluorophores should affect the resulting skin autofluorescence lifetimes. The skin fluorophores that emit fluorescence under violet-blue excitation are NADH, flavins, porphyrins, localised

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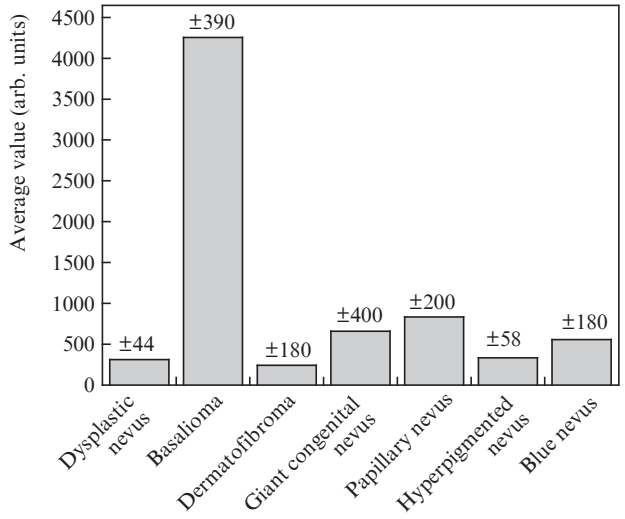

Figure 2. Average values of the photobleaching parameter A for different skin pathologies [12].

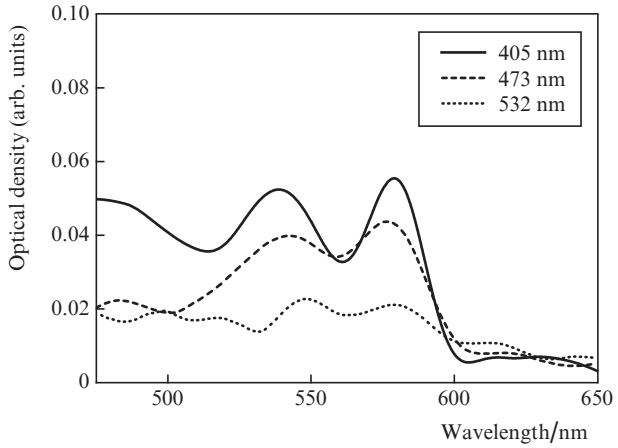

Figure 3. Typical oxy-haemoglobin absorption peaks in post-irradiated diffuse reflectance spectra of skin [14].

in the epidermis keratin as well as dermal elastin and collagen [5]. The radiative lifetime characteristics of each fluorophore are unique and can provide specific information for tissue identification. The fluorescence lifetimes for these skin fluorophores fall within range from 0.2 to 15 ns. However, in tissue experiments there are a number of de-excitation processes that decrease the characteristic lifetime. In addition, tissue fluorescence lifetime values are dependent on many environmental factors, such as the experimental setup, fluorophore localisation, skin pH balance, viscosity, temperature, etc. [15–17]. Taking into account the aforementioned circumstances, the selective analysis and separation of individual fluorophores underlying multi-exponential decays of *in-vivo* skin autofluorescence is still a challenging problem.

The goal of the experiment described in this paper is to investigate *in-vivo* skin AF lifetimes during long-term cw low-power laser excitation.

2. Experimental

The main focus of this study was to investigate *in-vivo* skin AF lifetimes under long-term optical excitation by a low-power laser at 405 nm. For this purpose an experimental setup to carry out parallel point measurements of skin AF lifetimes and photobleaching rates was assembled (Fig. 4). The setup comprises a picosecond/cw laser (PicoQuant LDH-D-C-405: pulse half-width 59 ps, 405nm), a laser controller, a monochromator, a photon counting detector with a temporal resolution of 180 ps (Becker&Hickl PMC-100-4), a data processing system for 'time-correlated single photon counting' with time resolution 6.6 ps (TCSPC, model SPC-150, Becker&Hickl GmbH), a fibre-optic probe, a CCD spectrometer (AvaSpec 2048) and a computer. The surface of healthy skin was excited via optical fibre by a pulsed laser for the lifetime measurements or by a cw laser for photobleaching measurements.

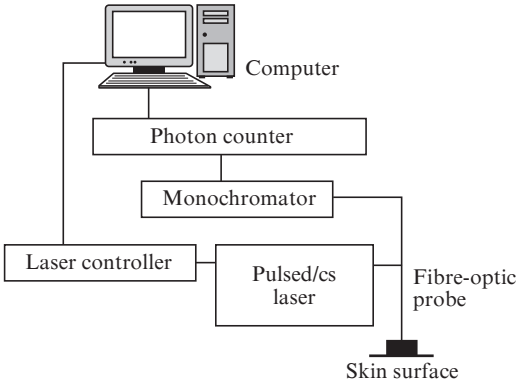

Figure 4. Experimental setup for parallel measurements of skin AF lifetime and photobleaching rate.

In order to avoid artefacts caused by the movements of the body, the arm was fixed during the measurements in a special position-stabilised holder. Autofluorescence was excited and measured on healthy, previously non-irradiated skin (at a distance of 6 mm from its surface) in the inner part of the forearm. Autofluorescence decay was measured on the same portions of skin prior to irradiation and immediately after 6-min cw excitation with the laser power density of 20 mW cm^{-2}. In all cases the time required for the collection of the AF lifetime data was 10 s at 480±10 nm. During 6 minutes of continuous cw excitation an AF intensity decrease was recorded by a spectrometer in the 450–800 nm spectral range. The autofluorescence intensity of skin was investigated using SPCM software (Becker&Hickl) by collecting photon counts during fixed time intervals. For the analysis of AF lifetimes, a multi-exponential fluorescence decay distribution model was applied:

$$f(t) = \sum_{i=0}^{n} a_i \exp(-t/\tau_i) + c, \tag{2}$$

where $f(t)$ is the AF intensity at time t after the excitation pulse, n is number of decaying species in the exponential sum and c is a background level of light in each particular case. The applied multi-exponential fitting model allowed us to obtain characteristic lifetimes of the exponential components, τ_i, and their amplitudes, a_i.

The program uses a deconvolution technique in conjunction with the measured instrumental response function to obtain an autofluorescence decay curve corrected for the instrument

Table 1. Averaged values and standard deviations (in brackets) of autofluorescence lifetime components, τ_i, and their relative amplitudes a_i.

Instant of time	Two-exponential fitting				Three-exponential fitting					
	a_1 (%)	τ_1/ns	a_2 (%)	τ_2/ns	a_1 (%)	τ_1/ns	a_2 (%)	τ_2/ns	a_3 (%)	τ_3/ns
Before photobleaching	72.48 (2.42)	1.63 (0.17)	27.52 (2.42)	7.15 (1.00)	69.26 (3.40)	1.52 (0.13)	22.12 (1.33)	6.02 (1.03)	8.6 (2.47)	8.10 (1.68)
After 3 min of photobleaching	71.66 (2.56)	1.29 (0.15)	28.34 (2.56)	6.22 (1.07)	72.14 (2.53)	1.31 (0.13)	20.94 (3.05)	6.25 (0.74)	6.94 (0.66)	6.81 (2.30)
After 6 min of photobleaching	70.9 (3.15)	1.13 (0.13)	29.1 (3.15)	5.37 (0.88)	71.32 (2.9)	1.13 (0.11)	21.22 (3.57)	4.95 (0.62)	7.46 (0.81)	5.97 (1.03)

response. Thus, the setup allows measurements of lifetime durations up to 0.5 ns. The measurements were collected from 3 different spots of healthy skin from the inner part of the forearm. For further analysis, the obtained AF decay time distributions were approximated with a two- and three exponential decay model. In total, ten volunteers with different skin photo types were involved in the study.

3. Results

Table 1 represents the averaged values of AF lifetimes and their relative amplitudes obtained from 10 different volunteers. Autofluorescence decay distributions were collected at three different time moments, before photobleaching, after 3 min of photobleaching and after 6 min of photobleaching. In all cases the healthy skin AF lifetimes and their contribution for all volunteers were virtually equal. The standard deviations (SD) show relatively low deviations from the average values.

The AF decay distribution of previously non-irradiated skin can be characterised by the parameters $\tau_{1,2}$ and $a_{1,2}$ or $\tau_{1,2,3}$ and $a_{1,2,3}$ (depending on the fitting model). In fitting one and the same AF decay distribution by two and three-exponential functions we obtain the same values of the parameters τ_1 and τ_2 within the standard deviation. However, with respect to three-exponential fitting an additional decay component with a relatively high lifetime $\tau_3 \sim 8.1$ ns and a low amplitude $a_3 \sim 8.6\%$ can be distinguished from the resulting decay distribution.

Figure 5 demonstrates *in-vivo* skin AF lifetime decay distributions before and after 6 min of cw excitation. As shown, AF lifetimes of skin after 6 min pre-irradiation have changed. Respectively, a significant decrease in τ_1 and τ_2 values is observed. The value of τ_1 decreased from 1.52±0.13 to 1.13±0.11 ns, τ_2 decreased from 6.02±1.0 ns to 4.95±0.62 ns and τ_3 – from 8.1±1.68 ns to 5.97±1.03 ns. At the same time, the amplitudes of the decay components demonstrate relatively insignificant changes. We have found that the changes in the AF decay distribution are a consequence of uneven bleaching of tissue fluorophores during long-term irradiation. The photon count at different instants of time after excitation shows a nonuniform decrease in the number of AF photons. Thus, the number of collected photons in the time range from 3 ns to 4 ns decreased approximately by 45%, in the range from 5 to 6 ns by 55% and photons collected in range from 8 ns to 9 ns decreased by 60%. Also after 6 min of cw irradiation, the quantile χ^2_r decreased from 1.4±0.2 to 1.1±0.1. At the same time we have found that the lifetime indices τ_2 and τ_3 become equal within the standard deviation after 6 min of optical excitation.

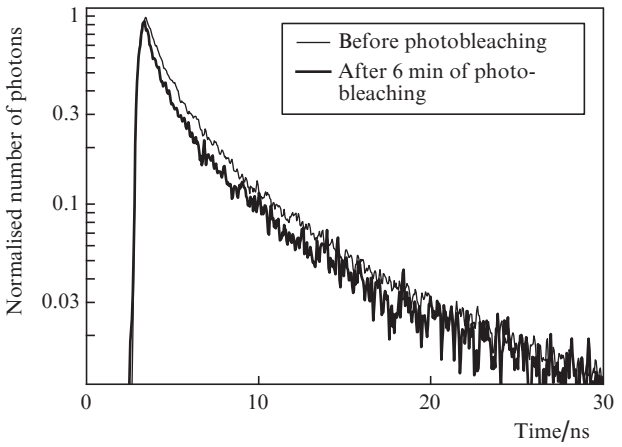

Figure 5. Skin AF lifetime decay curves before and immediately after 6 min of 405-nm cw laser irradiation.

4. Discussion

The reported results demonstrate that three-exponential fitting of the obtained skin autofluorescnce decay distribution is more informative and reveals a third component with a relatively high lifetime and a low amplitude. This decay component was observed by several authors under violet excitation, and probably is associated with collagen cross links formed by advanced glycation end-products [18, 19]. At the same time, the components, τ_1 and τ_2, represent the averaged lifetimes of all emitting fluorophores (under 405-nm excitation), such as, collagen, elastin, keratin, NADH and flavines. The lifetimes of these fluorophores are characterised by short and long components. Moreover, the short components of these fluorophores lies in the picosecond range [15]. Thus, the precise estimation of the short AF decay component is impeded by system response time and serious contribution of laser excitation (scattering and reflection) [20, 21]. However, a significant decrease in the number of collected photons in the entire nanosecond time scale range during the bleaching process indicates a noticeable contribution of tissue fluorescence to the resulting decay distribution.

The main result of this work is an experimental demonstration of a decrease in the skin AF lifetime during the photobleaching process. The lifetime decrease indicates that the skin fluorophore content changes during the photobleaching process. This decrease is most probably caused by the equalisation of the fluorophore content of skin during this process. In addition, after photobleaching the tendency to equalisation of lifetime components $\tau_{2,3}$ suggests that the third component of the exponential expansion disappears, which may indicate that a certain fluorophore or group of fluorophores that had given rise to the parameter τ_3 has become extinct or changed concentration. Taking into account the long-term recovery of the AF intensity after photobleaching [10], a decrease in the

lifetimes is probably caused by a nonuniform degradation or photodamage of skin fluorophores after long-term optical excitation. We also should mention the fact that during the 7th or 8th minute of 15-min excitation the number of photons collected began to increase after a steady decrease, leading to a significant increase in the third decay component (τ_3) from 5.67 ns to 15.0 ns. An increase in the AF intensity and lifetimes indicates either heterogenous degradation of skin fluorophores or formation of their new compositions. Undoubtedly, this phenomenon requires additional study to determine the exact mechanism of photobleaching and its influence on the skin physiology.

5. Conclusions

This paper reviews the main achievements of cw laser-excited skin autofluorescence photobleaching and its potential applications in clinical diagnostics. In the experimental part of work we have demonstrated uneven bleaching of skin fluorophores under long-term optical excitation, which leads to a change in the autofluorescence decay distribution. This effect of uneven bleaching of autofluorescence requires additional studies to identify the fluorophores responsible for the change in the lifetime distribution. In addition, studies should involve more volunteers of different age with all skin photo types, and also include a variety of pathological structures.

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