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Photobleaching effects on *in vivo* skin autofluorescence lifetime

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Abstract. The autofluorescence lifetime of healthy human skin was measured using excitation provided by a picosecond diode laser operating at a wavelength of 405 nm and with fluorescence emission collected at 475 and 560 nm. In addition, spectral and temporal responses of healthy human skin and intradermal nevus in the spectral range 460 to 610 nm were studied before and after photobleaching. A decrease in the autofluorescences lifetimes changes was observed after photobleaching of human skin. A three-exponential model was used to fit the signals, and under this model, the most significant photoinduced changes were observed for the slowest lifetime component in healthy skin at the spectral range 520 to 610 nm and intradermal nevus at the spectral range 460 to 610 nm. [DOI: 10.1117/1.JBO.20.5.051031]

Keywords: lifetime; skin autofluorescence; autofluorescences photobleaching.

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1 Introduction

The intensity of the photobleaching of skin autofluorescence decreases during temporally stable irradiation,[1] which indicates photoinduced structural changes in the skin. The mechanism of this effect has not been explained in detail so far, and only a few hypotheses have been examined experimentally. One of these hypotheses suggests that the mechanism involves the irreversible destruction of a fluorophore in its excited state due to its interactions with molecular oxygen or other surrounding molecules; the chemically modified fluorophore then returns to the ground state as a new molecule that no longer absorbs light at the excitation wavelength.[2] Skin autofluorescence photobleaching was previously investigated for healthy and pathological skin under 405-, 473-, and 532-nm laser excitation.[3,4] The specific distribution of photobleaching parameters in healthy and pathological skin had been examined earlier.[5] Our previous studies showed that the restoration of autofluorescence intensity after photobleaching is a long-term process and lasts for hours.[4] The influence of continuous wave (cw) laser irradiation on skin diffuse reflectance in the spectral range 500 to 600 nm was also demonstrated;[6] this study confirmed that the low-power cw laser irradiation increased the skin's oxy-hemoglobin content.

In this study, a 405-nm picosecond laser was used for fluorescence lifetime measurements, and a cw 405-nm laser initiated the photobleaching. Radiation with wavelength 405 nm is absorbed by a number of fluorophores: keratin (absorption maximum at 380 to 400 nm and 450 to 470 nm, emission maximum at 500 to 550 nm), reduced nicotinamide adenine dinucleotide (absorption maxima at 290 nm and 350 to 370 nm, emission maximum at 440 and 460 nm), flavin adenine dinucleotide (FAD) (absorption maximum at 420 to 450 nm, emission maximum at 520 to 550 nm), phospholipids (absorption maximum at 430 nm, emission maximum at 500 and 540 nm), and lipofuscin (absorption maximum at 340 to 395 nm, emission maximum at 430 to 460 nm and 540 nm).[7,8]

The influence of photobleaching on autofluorescence lifetimes of healthy skin and intradermal nevus is studied in this work. Autofluorescence decay curves were approximated by three exponential decay functions, and spectral responses of fluorescence lifetimes before and after photobleaching were recorded and displayed on a plot. The three-exponential model was chosen because it was more informative[9,10] and achieved a better fit. This model shows that the fluorophores with emission in the spectral range from 520 to 610 nm are more sensitive to photobleaching. Time-resolved emission spectra were plotted as time-resolved area-normalized emission spectra (TRANES).[11] Changes in lifetimes and peak shifts in TRANES can be related to photoinduced changes of specific skin fluorophore or chromophore concentrations.

2 Methods and Materials

The single-spot measurement setup scheme is shown in Fig. 1, and it comprised a laser controller and a picosecond/cw laser (PicoQuant: 405 nm, pulse half-width 59 ps, repetition rate 20 MHz, mod. LDH-D-C-405) with 200-μm silica core optical fiber output via the SMA-connector. The excitation fiber represented one leg of a Y-shaped optical fiber bundle that contained an additional six fibers to deliver the fluorescent light via a monochromator to the photon counting detector (Becker&Hickl, PMC-100-4). The laser controller and photon counting detector were connected to a PC with a data processing card (Becker&Hickl, TCSPC, mod. SPC-150). The fiber-optic probe was tightly fixed such that the distance between the skin surface and tip of the Y-shaped fiber bundle was 3 mm. The diameter of the irradiated skin spot was ~3 mm. The same probe was used for lifetime measurements under pulsed excitation as well as for inducing photobleaching by cw laser irradiation.

First, the measurements were taken from 10 different spots of healthy skin on the inner part of the forearm and from 10 different spots of the back of the hand of 10 volunteers ranging in age from 24 to 48 years and having skin types II and III according to

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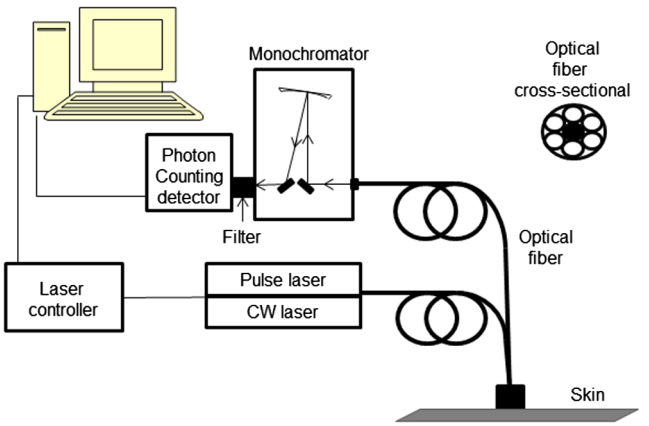

Fig. 1 Experimental setup for skin autofluorescence lifetime measurements.

the Fitzpatrick classification.[12] Data were collected at 475- and 560-nm emission wavelengths using a monochromator and interference filters.

Next, measurements of autofluorescence lifetimes of healthy *in vivo* skin and intradermal nevus before optical provocation (photobleaching) and immediately afterward were taken. Autofluorescence lifetimes were determined within the spectral range 460 to 610 nm with a step size of 10 nm via the monochromator (Fig. 2). Then, the laser was switched to cw mode and the same area was irradiated for 3 min at a laser power density of 40 mW/cm^2, well below the skin laser safety limit [200 mW/cm^2, exposure time up to 10^3 s (Ref. 13)]. Immediately after optical provocation, the laser was again switched to the pulsed mode and autofluorescence lifetime data were collected (which took ~10 s). The same procedure was applied during measurements of the intradermal nevus. The measurements were repeated three times for healthy skin on the inner part of the forearm of three volunteers and three times for the intradermal nevus of one volunteer. The safety and well-being of the human subjects involved in all clinical measurements were assured through the supervision of the local ethics committee that authorized the studies.

The obtained results were automatically processed using the Becker&Hickl SPCImage program. The results were approximated by a three-exponential decay function:

$$I = a_1 e^{-T/\tau_1} + a_2 e^{-T/\tau_2} + a_3 e^{-T/\tau_3}, \quad (1)$$

where I is the decaying intensity after pulsed laser excitation, T is the time, τ_i is the lifetime of the i'th component, and a_i is its amplitude ($i = 1$, 2, or 3).[9,10]

3 Results

The obtained average values of τ_1, τ_2, τ_3, and X_r^2 with standard deviation (SD) for healthy skin are presented in Table 1. It summarizes the results of 200 autofluorescence lifetime measurements: for each of 10 volunteers, 10 different spots of healthy skin on the inner part of the forearm and 10 different spots of the back of the hand. In all cases, the healthy skin autofluorescence lifetimes for all volunteers were similar (within a range of ±20%), which is confirmed by relatively low deviations from the average values. The three-exponential fitting resulted in the relatively low amplitude of the third component: $\tau_3 = 7.6 \pm 1.1$ ns, $a_3 = 6.4 \pm 0.4\%$ at 475 nm and $\tau_3 = 9.1 \pm 1.0$ ns, $a_3 = 7.8 \pm 2.7\%$ at 560 nm.

The spectral dependencies of the time-resolved emission intensity before and after photobleaching of healthy skin and the intradermal nevus are presented in Figs. 2 and 3, plotted

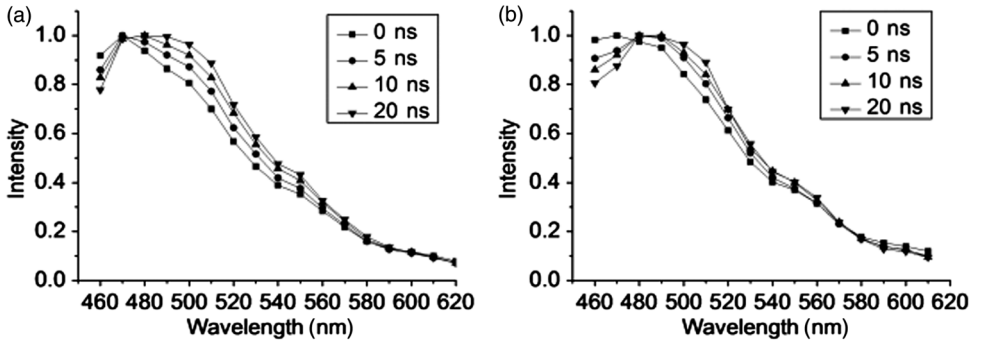

Fig. 2 Time-resolved area-normalized emission spectra (TRANES) of healthy skin before (a) and after (b) photobleaching.

Table 1 Averaged autofluorescence lifetime components based on *in vivo* measurements without photobleaching from the skin of 10 volunteers of second and third phototypes (SD—standard deviation).

	Triple-exponential decay						
	$\tau_1 \pm$ SD (ns)	$a_1 \pm$ SD (%)	$\tau_2 \pm$ SD (ns)	$a_2 \pm$ SD (%)	$\tau_3 \pm$ SD (ns)	$a_3 \pm$ SD (%)	$X_r^2 \pm$ SD
475 nm	1.2 ± 0.2	74.1 ± 1.1	4.4 ± 0.6	19.5 ± 0.8	7.6 ± 1.1	6.4 ± 0.4	1.3 ± 0.3
560 nm	1.1 ± 0.1	65.4 ± 4.1	4.3 ± 0.5	26.8 ± 1.6	9.1 ± 1.0	7.8 ± 2.7	1.1 ± 0.1

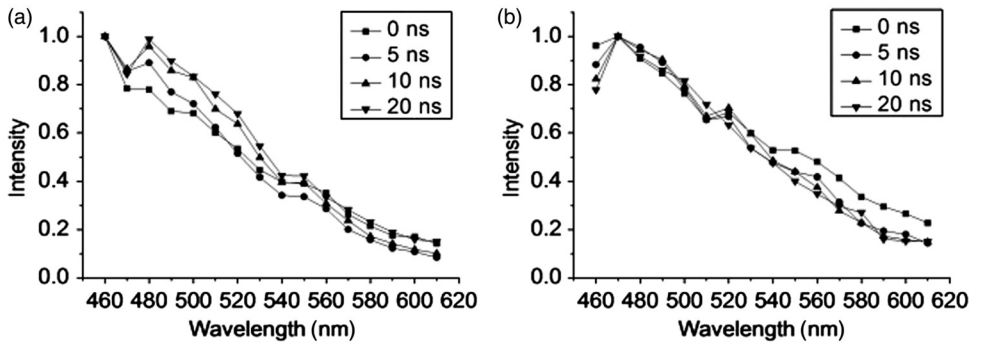

Fig. 3 TRANES of intradermal nevus before (a) and after (b) photobleaching.

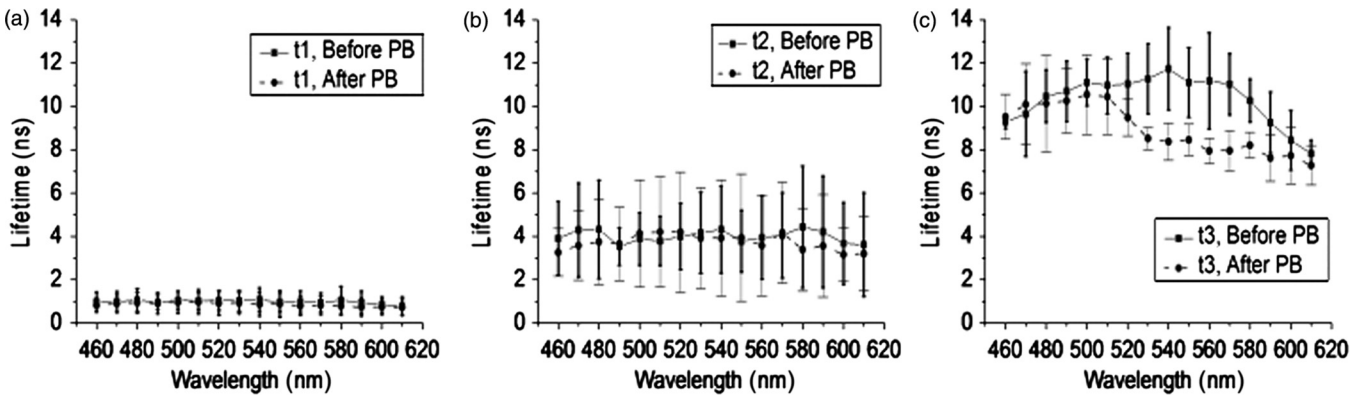

Fig. 4 Autofluorescence lifetimes of healthy skin before and after photobleaching. The parameters t1 (a), t2 (b), and t3 (c) are the averaged fluorescence lifetime components with error bars indicating the standard deviation (SD).

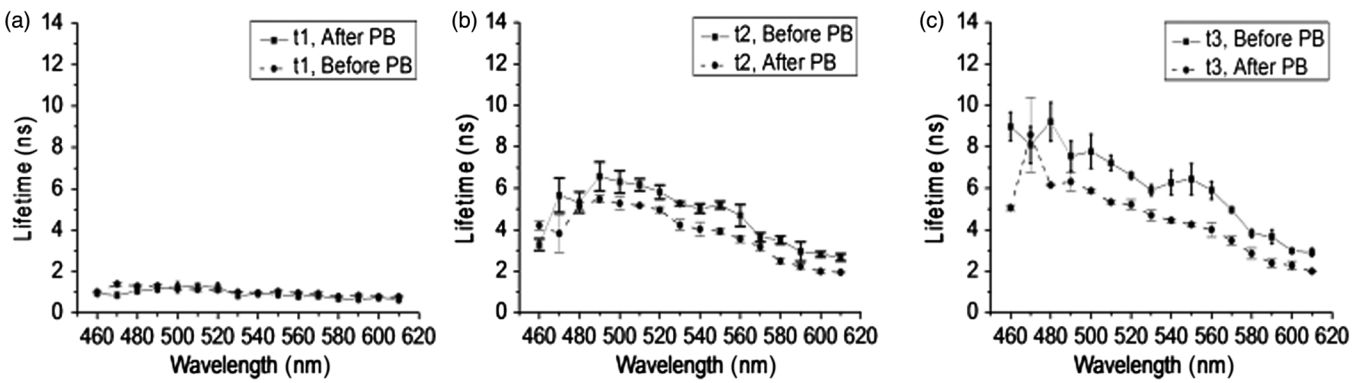

Fig. 5 Autofluorescence lifetimes of intradermal nevus before and after photobleaching. The parameters t1 (a), t2 (b), and t3 (c) are the averaged fluorescence lifetime components with error bars indicating the SD.

as TRANES; Figs. 4 and 5 show the spectral changes of the three lifetime components as a result of photobleaching. The typical numbers were: ~300,000 photons at the maximum and 19,500 photons at the minimum of healthy skin spectra before photobleaching, and 82,000 photons at the maximum and 11,000 at the minimum after photobleaching. In the cases of the intradermal nevus, about 50,000 photons were recorded at the maximum and 7000 photons at the minimum before photobleaching, and 30,000 photons at the maximum and 5000 at the minimum after photobleaching.

The third lifetime component appeared to be spectrally sensitive to photobleaching; it notably decreased in the spectral range 520 to 610 nm. In the case of the intradermal nevus, the third lifetime component decreased over the whole spectral range. The photobleaching-caused spectral changes were observed in the area-normalized emission spectra as well (Figs. 2 and 3). Figure 6 illustrates the spectral variations of the amplitude values.

4 Discussion

The reported results demonstrate that the three-exponential fitting of the obtained skin autofluorescence decay distribution is informative, and the third component with a relatively high lifetime and a low amplitude is spectrally sensitive. This decay component was observed by several authors under violet excitation.[9,10]

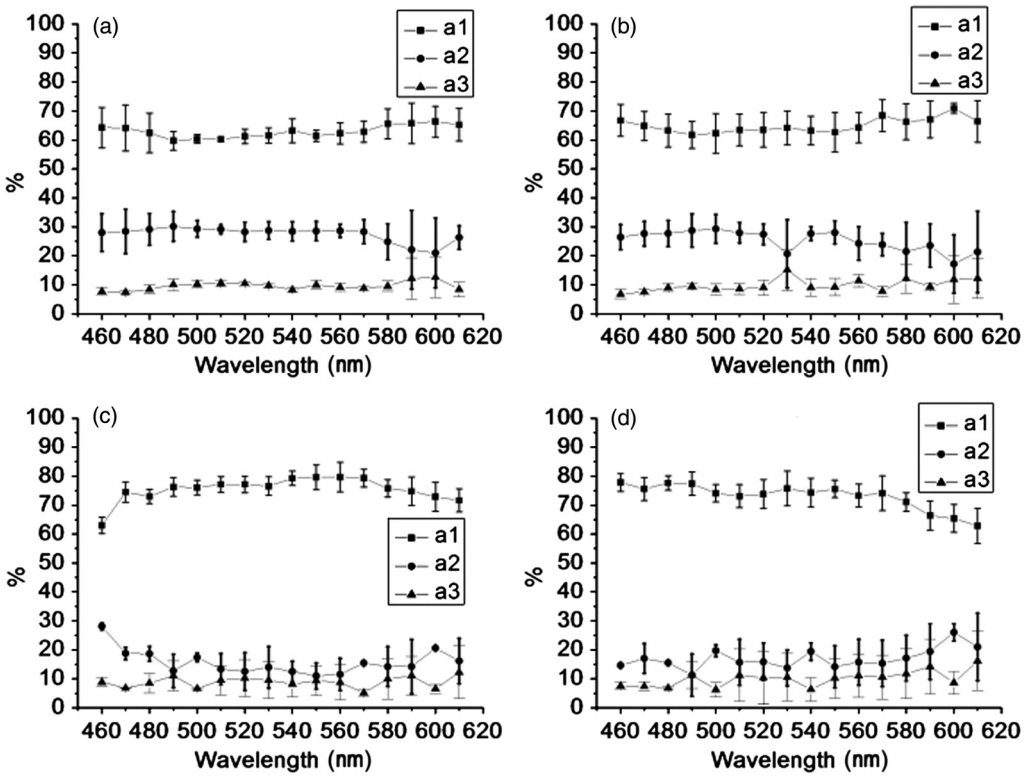

Fig. 6 Autofluorescence lifetime amplitudes of healthy skin and intradermal nevus before and after photobleaching. The parameters a1, a2, and a3 are the averaged fluorescence lifetime amplitude components with error bars indicating the SD. (a) Healthy skin before photobleaching; (b) healthy skin after photobleaching; (c) intradermal nevus before photobleaching; and (d) intradermal nevus after photobleaching.

Under normal conditions, the lifetime components for different subjects were similar (within ±20%, see Table 1). The results were obtained from 10 volunteers with second and third skin phototypes without extraillumination to induce photobleaching.

However, cw low-power laser irradiation caused notable changes in the lifetime components (especially τ_3) as well as in their spectral distributions (Figs. 4 and 5). The results of the experiment show a notable decrease of the third lifetime component in the spectral range 520 to 610 nm after photobleaching. TRANES show the emission intensity changes in the same spectral range; consequently, the decay becomes faster. The shift of the emission maxima in TRANES might be the result of changes in the composition and/or concentration of fluorophores (Figs. 2 and 3). In all cases, the corresponding amplitudes were unchanged.

Skin contains a number of different fluorophores, so it is still difficult to determine exactly which fluorophore(s) are most involved in the photobleaching phenomenon. The main fluorophores emitting in this spectral range are FAD, flavins, lipofuscin, and phospholipids. On the other hand, the 405-nm cw irradiation increases the oxy-hemoglobin content in the skin (perhaps as the result of photoinflammation),[6] which results in increased absorption and decreased lifetime. This phenomenon might be the major factor in our experimental conditions.

5 Conclusions

To the authors' knowledge, this investigation was the first attempt to study how external cw irradiation affects skin autofluorescence lifetimes. The observed variations in spectral responses of the three fluorescence lifetime components after photobleaching of healthy skin and the intradermal nevus may lead to a better understanding of the photobleaching mechanism. The results show the influence of photobleaching on FAD, flavins, and/or lipid fluorophores. The results point to changes in the composition and/or concentration of fluorophores as a result of photobleaching. Also, different lifetime spectral responses of healthy skin and the intradermal nevus were observed. The different responses of healthy and pigmented skin may provide additional information for skin diagnostics. More detailed clinical studies involving a larger number of volunteers of different ages, skin phototypes, and pathological structures have to be performed in the future.

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