

Risk estimation of skin damage due to ultrashort pulsed, focused near-infrared laser irradiation at 800 nm

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1 Introduction

A few decades ago, ultrashort pulsed laser sources, e.g., femtosecond-laser (fs-laser) systems, became available.^{1,2} But it was only recently that they found interest in biomedical research.³ They possess potentials for a multitude of new methods and techniques in different fields of biomedical research, like controlled cell damage in the micrometer range,⁴ nanosurgery,^{5,6} multiphoton laser scanning microscopy (MPLSM),^{2,4} DNA-protein cross-linking,^{6,7} and optical coherence tomography (OCT).⁸ So far, these techniques were applied in routine use *in vivo* only for laser surgery of the cornea, although some other *in vivo* applications seem to be possible and desirable.⁹

One reason for the delayed development of *in vivo* equip-

Abstract. New imaging techniques using near-infrared (NIR) femtosecond lasers (fs-lasers) in multiphoton laser scanning microscopy (MPLSM) have great potential for *in vivo* applications, particularly in human skin. However, little is known about possible risks. In order to evaluate the risk, a "biological dosimeter" was used. We irradiated fresh human skin samples with both an fs-laser and a solar simulator UV source (SSU). DNA damage introduced in the epidermis was evaluated using fluorescent antibodies against cyclobutane-pyrimidindimers (CPDs) in combination with immunofluorescence image analysis. Four fs-irradiation regimes (at 800-nm wavelength) were evaluated differing in laser power and step width of horizontal scans. Fs-irradiation did not give CPDs at 15-mW or 30-mW irradiation power using 10 horizontal scans every 5 microns. CPDs could be seen at 60-mW laser power and 5- μ m step size and at 35-mW using 1-micron step width. Quantitative comparison of SSU-induced CPDs showed that the 60-mW laser irradiation regime is comparable to UV-irradiation, giving 0.6 minimal erythral dose (MED). The 1-micron irradiation regime was comparable to 0.45 MED. Under these experimental conditions, the risk of DNA damage due to fs-laser irradiation on skin is in the range of natural UV-exposure. © 2008 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2960016]

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ment and studies could be related to the fact that little is known about the risk of this type of laser application *in vivo*, although cell damage has been shown.¹⁰ Damage thresholds for *in vitro* applications can be found.^{11,12} For example, the laser power leading to cell damage in microscopy applications was found¹² to start with irradiances as low as 2 mW. The threshold power depends on pulse duration, mean power, and peak power.¹¹

Using ultrashort pulsed, focused laser irradiation in the near-infrared (NIR) wavelength range, it is possible to reach deeper skin layers (e.g., the *stratum germinativum*) compared to UV.¹³ Due to the nonlinear effects of ultrashort focused laser irradiation, biologically relevant skin chromophores are excited comparable to one-photon excitations in the UV range. If the irradiance is high enough, two or more photons can be absorbed simultaneously. Two-photon absorption

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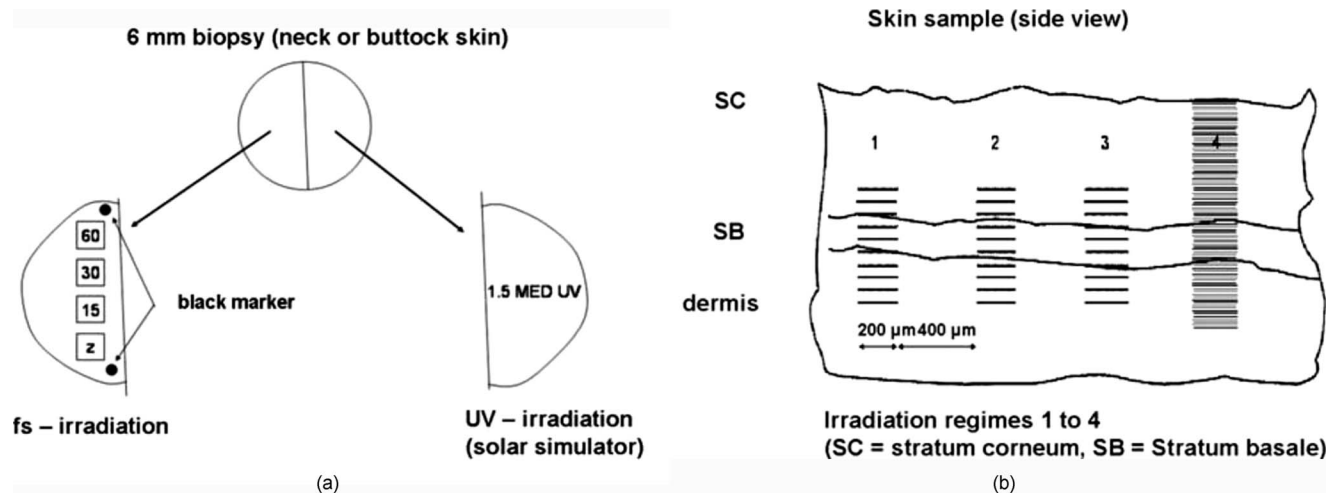


Fig. 1 Sample preparation and irradiation procedure. (a) Biopsy is divided and irradiated using fs-irradiation (left) or UV-irradiation (right). (b) Fs-irradiation regime 1 to 3 = 10 horizontal scans around the basal membrane; fs-irradiation regime 4 = 150 horizontal scans from the skin surface to the dermis.

(2PA) and three-photon absorption (3PA) allow excitation of higher energy levels in tissue chromophores. For example, 800 nm reaches energy states that are usually accessible only by 400-nm (2PA) or 267-nm (3PA) irradiation. Tissue chromophores like flavins, porphyrines, and/or lipoproteins can be excited by two photons. Simultaneous absorption of three 800-nm photons can excite DNA, NADH, collagen, proteins, and other tissue molecules.

Because of the low pulse energy in the nanojoule range and the low average power (milliwatt) of fs-laser irradiation used in biomedical research, especially in MPLSM, all effects on tissue are connected with nonlinear effects, which occur due to the high peak irradiance, which reaches several hundred GW/cm^2 . Photochemical damaging induced by linear absorption, which can also occur at low irradiances, is not expected to play a role in the wavelength range between 700 nm and 1300 nm, which is usually used with fs-lasers. With the exception only of melanin, the endogenous chromophores do not absorb in this wavelength range.^{14–17} Therefore, in tissue with low melanin content, the risk of damage due to one-photon absorption (1PA) is expected to be negligibly low. Only biostimulation effects of low-irradiance NIR radiation (below $300 \text{ mW}/\text{cm}^2$) were described.^{18–22}

Therefore, only two mechanisms are expected to be relevant for tissue damage, photochemical effects caused by multiphoton absorption and optical breakdown. It is well known that blue and UV light irradiation is able to damage DNA and/or other biomolecules, which absorb in this wavelength range. In skin, photochemical damage results in immediate tissue response (e.g., sunburn) and long-term effects like photoaging or even skin cancer.^{3,14}

Little is known about the action of fs-lasers in skin. Methods based on “biological dosimetry” are the only option to observe biological effects. Some dosimetric methods use the sensitive and quantitative assay of cyclobutane-pyrimidine-dimers (CPDs) in DNA visualized by immunofluorescence detection.²³ Cell aberrations may result if induced CPDs are not repaired or are misrepaired by the polymerase repair system.^{24,25} The fluorescence intensity of the

fluorescent-labeled CPD antibodies can be used as a measure for laser-induced CPD formation. We compared CPDs induced by 1.5 minimal erythema dose (MED) of solar UV.

2 Materials and Methods

2.1 Skin Samples

Skin samples were obtained from healthy volunteers (phototypes II to III) after written consent. Each of eight volunteers gave two skin biopsies, 6 mm in diameter, one from the neck and one from the buttock. Samples from the neck were included in this study in order to be able to distinguish effects of extended UV irradiation due to sun exposure from fs-NIR-irradiated skin. After excision, the skin samples were cut in half.

2.2 Irradiation

Before skin sample excision, the sensitivity of the volunteers to UV-irradiation was quantified. One minimal erythema dose (MED) was evaluated by visual assessment of six spots irradiated using increasing radiation power of the UV source. The erythema was defined according to COLIPA (The European Cosmetic Toiletry and Perfumery Association) as the first perceptible, clearly defined, unambiguous reddening of the skin 24 h after irradiation. UV- and fs-irradiations were performed on freshly excised samples (maximum 1 h after excision; see Fig. 1).

For the UV-irradiation, the whole surface of one half of the sample was irradiated using a solar simulator (SU 5000, mut GmbH, Hamburg, Germany) at a UV dose according to 1.5 MED. The UV-irradiation took about 15 min. After the UV-irradiation, the samples were frozen in liquid nitrogen for further use. A spectrum of the solar simulator emission is shown in Fig. 2.

The other half of the skin sample was irradiated using an ultrashort pulsed, focused laser beam of a Mai-Tai laser (Spectra Physics, Darmstadt, Germany) at a wavelength of 800 nm, using a repetition rate of 80 MHz and a pulse width at sample layer of about 150 fs. For scanning and focusing

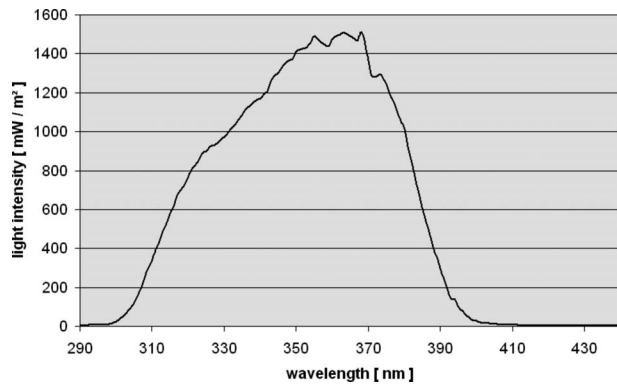


Fig. 2 Emission spectrum of the solar simulator SU 5000.

purposes, a MPLSM (DermaInspect, Jenlab, Jena, Germany) equipped with a Zeiss Plan/Neofluar 40 \times NA 1.3 objective lens (oil immersion) was used. The DermaInspect device contains a laser emitting NIR in the range between 750 and 850 nm according to the specification by the manufacturer. The focus of the laser beam was scanned at various depths within the epidermis and papillary dermis of the excised half of the skin sample. Each horizontal scan was 200 \times 200 microns wide and took 32 s. Automatic horizontal scanning (parallel to the skin surface) using a fixed step width in the depth of the microscope focus was performed. Four laser irradiation regimes were chosen (Fig. 1):

- 10 horizontal scans at 15 mW laser power (96 J/cm² peak fluence, 0.64 TW/cm² peak irradiance), 5 microns in depth apart from each other;
- 10 horizontal scans at 30 mW laser power (191 J/cm² peak fluence, 1.28 TW/cm² peak irradiance), 5 microns in depth apart from each other;
- 10 horizontal scans at 60 mW laser power (383 J/cm² peak fluence, 2.55 TW/cm² peak irradiance), 5 microns in depth apart from each other;
- 150 horizontal scans at linear increasing laser power with depth (2 mW to ca. 35 mW, 13 to 223 J/cm² peak fluence, 0.085 to 1.5 TW/cm² peak irradiance), 1 micron in depth apart from each other. Power was increased linearly, since image quality was found to be best when linearly increasing the power with depth.

Peak fluence (or peak radiant fluence) is the time integral of the spherical peak irradiance; it combines the two main parameters influencing dosimetry and hence photochemical effects, in our case, the irradiance and the time. The wavelength and the irradiation time were kept constant during experiments in order to investigate the effects of one parameter, the peak irradiance.

The first three regimes were applied around the basal membrane in order to make sure that the most sensitive cells for risk evaluation, the basal layer cells, will receive the laser light. Three different laser powers (15, 30, and 60 mW) were applied, because with tissue imaging, these laser powers had been shown to cover the possible working range when imaging horizontal scans at basal membrane depth. Laser power was measured using a power meter (Ophir Laserstar) with a coverglass and immersion oil in front of the detector. In this depth, at 15 mW, imaging starts to be possible. At 30 mW,

images can be taken with good contrast. At 60 mW, first damaging occurs during scanning (fluorescent scars, as described elsewhere⁴). The fourth regime covered the whole depth from the surface to the papillary dermis using linearly increasing laser power (Fig. 1). The most suitable laser power to achieve best imaging results was used in order to simulate tissue imaging as one possible *in vivo* application.

The four irradiation regimes were applied side by side on the second half of the original skin sample, producing irradiated areas of about 200 \times 200 μ m. Between irradiated areas, a space of 400- μ m unirradiated tissue was left in order to avoid cross talk between irradiated areas due to light scattering in the skin. At each side of the row of the four irradiated areas, the skin was marked with a black spot in order to retrieve the tiny irradiated areas for the subsequent CPD analysis. The whole fs-irradiation took about 15 to 20 min; samples were frozen in liquid nitrogen at -196 $^{\circ}$ C after fs-irradiation for further analysis.

2.3 Cyclobutane-Pyrimidin-Dimer Antibodies and Staining Protocol

Sections (7 μ m thick) were cut from frozen tissue using a cryostat (Microm, Walldorf, Germany) and were sequentially fixed after slide preparation for 5 min in methanol and acetone at -20 $^{\circ}$ C. After fixation, the slides were stored at -20 $^{\circ}$ C. For CPD staining, the samples were rehydrated in TKT 100 (50 mM Tris pH 7.2; 1 M KCl, 0.3% Triton X-100) for 60 min at room temperature, washed once in phosphate buffered saline (PBS) for 5 min and two times for 5 min in 2 \times SSC (300 mM NaCl, 30 mM Na citrate). The sections were denatured in 0.1 N NaOH/70% EtOH for 3 min; dehydrated for 1 min each in 70%, 90%, and 100% EtOH; air dried; and incubated with Proteinase K (10 μ g per ml; Sigma, Taufkirchen, Germany) at 37 $^{\circ}$ C for 10 min. After three washes for 5 min each in PBS, they were incubated with 5% goat serum (Dako, Hamburg, Germany) for 30 min at room temperature, rewashed three times for 5 min in PBS, and incubated overnight at 4 $^{\circ}$ C with monoclonal antibody specific for CPD (Kamiya Biochemical, Seattle, Washington) diluted 1:1000 in PBS. Sections were then washed three times for 5 min each in PBS and incubated with goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC; Dianova, Hamburg, Germany) diluted 1:100 in PBS. After three additional 5-min washes in PBS, slides were mounted with "antifade" (2.3% DABCO; Sigma, Taufkirchen, Germany) in 90% glycerol in 20 mM Tris, pH 8.0 and covered. Sections were visualized using a CCD camera (Kappa, Gleichen, Germany) coupled to a fluorescence microscope (Leica DM, Leica, Wetzlar, Germany). Fluorescence intensity was quantified using a digital imaging system (OPTIMAS, Bothell, Washington). Under the premise that the images of the different samples were taken under the same camera conditions and a careful handling of the probes to minimize the influence of photobleaching, the fluorescence intensity is a reliable measure for the amount of induced CPDs (e.g., Refs. 26 and 27). Comparing the data of laser-induced CPDs with those induced by 1.5 MED solar UV radiation (UVR), the method can be used to establish the possibility for estimation of skin cancer risks according to current models.

3 Results

First, an astonishingly high amount of CPD-related fluorescence could be found in unirradiated neck tissue, indicating a recent UV exposure of volunteers. An example using green fluorescent CPD antibodies is shown in Fig. 3(a). For comparison, a CPD-stained buttock tissue slice is shown in Fig. 3(b). Apart from the nuclei, the images exhibit a low overall fluorescence in the tissue due to a slight unspecific binding of the antibody. While in the neck skin [Fig. 3(a)] almost all cell nuclei show green fluorescence, the nuclei of buttock tissue are black [Fig. 3(b)]. Hence, UV-induced CPDs could already be detected in unirradiated neck tissue (due to recent solar UV exposure), while the buttock skin cells show no damage.

Due to the high amount of CPDs found in native neck tissue (not irradiated during the experiment protocol), a comparison between unirradiated and irradiated neck tissue in order to quantify changes due to fs-laser irradiation was not possible (data not shown). In order to compare the effect of the different irradiation regimes (unirradiated, UV-irradiated, and fs-laser irradiated) only buttock tissue was evaluated. Examples are shown in Fig. 4.

In the unirradiated buttock samples of all participating volunteers, no CPD fluorescence could be detected [Fig. 4(a)]. In contrast, many nuclei of the solar simulator irradiated buttock tissues (UV dose equivalent to 1.5 MED) showed CPD fluorescence with high fluorescence intensity in all volunteers [Fig. 4(b)]. In the fs-irradiated buttock tissue of all volunteers, spatially confined areas of CPD fluorescence could be found [Figs. 4(c) and 4(d)]. Horizontal dimensions of these areas were about 150 μm , which is about the field of view of the *in vitro* multiphoton microscope setup used. With the known location of the irradiation regimes due to the marking used on the skin, it seems safe to assume that this fluorescence originates from the CPDs produced using 60-mW fs-irradiation. Additionally, in three buttock samples, a second area with CPD fluorescence could be found [Fig. 4(d)]. CPD fluorescence in this area was lower and restricted in the horizontal dimensions to a small area. (Only four nuclei show fluorescence signal.) This fluorescence is assumed to be due to the femtosecond irradiation regime applied at varying power from 2 to 35 mW every 1 μm in depth.

A quantitative analysis of the CPD fluorescence found in UV-irradiated and fs-irradiated buttock skin for all eight skin samples is given in Fig. 5. In order to quantify the induced DNA damage, the fluorescence intensity of up to 40 nuclei per slice was measured [arbitrary units] and average values for CPDs per nucleus were calculated.

From an area of $200 \times 200 \mu\text{m}^2$, only within an area of $160 \times 80 \mu\text{m}^2$ for Fig. 3(c) and $80 \times 40 \mu\text{m}^2$ for Fig. 3(d) were CPD-labeled nuclei found, whereas after 1.5 MED irradiation, the complete viable epidermis was highlighted.

The amount of CPD-related fluorescence per nucleus found after UV-irradiation varied highly between different individuals, both for solar UV and for fs-irradiation. However, for fs-irradiation, the variation was smaller. In Table 1, the average for all eight volunteers is given, together with its standard deviation, which indicates the described variations.

According to the mean values shown in Table 1, the second-highest CPD-related fluorescence was found in neck tissue irradiated with the fs-laser. As described, CPDs were

Table 1 Average and standard deviation of the relative amount of CPDs per nucleus for UV irradiation (1.5 MED) on buttock skin and fs-irradiation on buttock and neck skin.

Treatment	Average fluorescence intensity/nucleus [a.u.]	SD fluorescence intensity/nucleus [a.u.]
1.5 MED-UV	58.4	19.1
Fs (neck)	27.9	7.3
Fs (buttock)	24.8	8.0
Fs (buttock 2)	18.1	2.1

found in unirradiated areas of the neck tissue as well, probably due to recent solar exposure of the volunteer [see Fig. 4(a)]. The areas where fs-irradiation was applied could not be distinguished from the surrounding unirradiated tissue. Hence, the numbers given in Fig. 4 and Table 1 for fs-irradiated neck tissue may be mainly ascribed to CPDs found in native tissue. The fs-irradiation has little or no additional effects in this case. Neck tissue values are given here only for comparison in order to be able to classify sun-exposed tissue in terms of CPD induction.

Buttock skin irradiated at 15-mW and 30-mW fs-laser light using 10 horizontal scans around the basal membrane did not show any CPDs. At 60-mW fs-irradiation, 42% of the CPDs observed in 1.5 MED UV-irradiated buttock skin were found (Table 1). Thus, the amount of CPDs induced by 60-mW fs-irradiation is equivalent to a dose of about 0.6 MED for that small irradiated area.

Irradiation regime four (2 to 35 mW) resulted in 30% CPDs compared to the 1.5 MED UV irradiation. Therefore, this irradiation regime gave 0.45 MED. The fs-irradiated area was much smaller than the UV-irradiated area, resulting in much smaller amounts of fs-irradiated cells.

4 Discussion

4.1 UV- and Fs-NIR-Irradiation Effects in Human Skin

In the unirradiated areas of fs-exposed samples and untreated buttock samples, no CPD-related fluorescence could be observed [Figs. 4(a) and 4(c)]. In contrast, unirradiated areas of fs-exposed neck samples showed already a high amount of CPDs, corresponding to 48% of CPDs induced by a single 1.5 MED solar-simulated UV-radiation (UVR). Since the neck is a body site that is naturally heavily sun exposed, it is assumed that the detected CPDs were induced by solar irradiation. Although CPDs can be repaired by epidermal cells very efficiently,^{28–32} it has been observed that even 10 days after irradiation, about 2% of epidermal cells still contain CPDs.³³ In all the investigated neck samples here, heavily damaged cells in the basal layer of the epidermis can be observed (Fig. 3(a)). These CPD-retaining basal cells (CRBCs) occur in human skin depending on the amount of sun exposure. Some evidence exists that they may represent epidermal stem cells,³⁴ which are thought to be the most important targets for cutaneous cell aberrations, possibly leading to skin carcinogenesis.³⁵

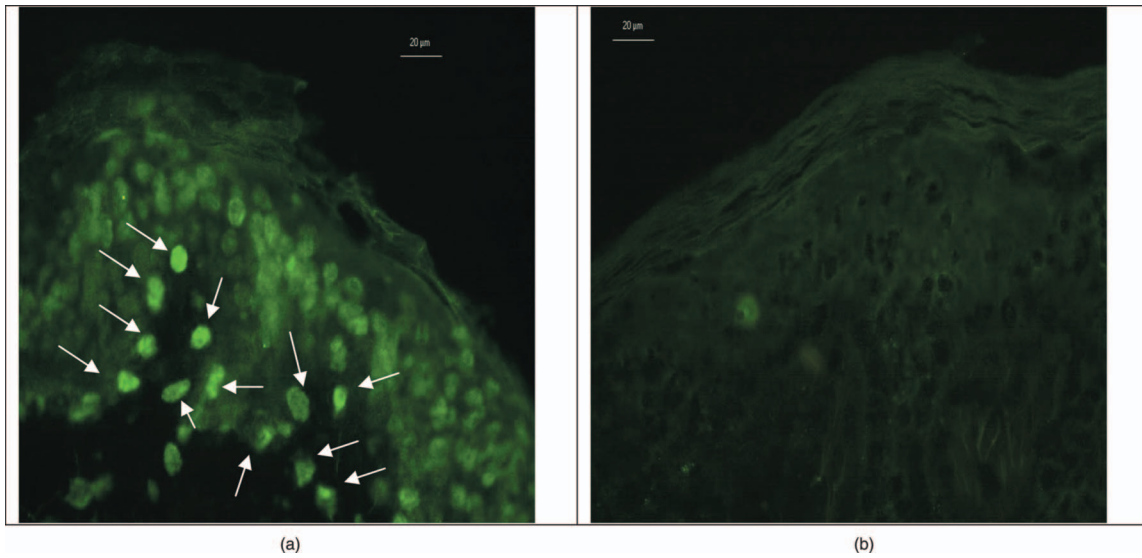


Fig. 3 Example of an unirradiated neck tissue slice (a) versus unirradiated buttock tissue (b). Both slices were stained against CPDs. Note the natural amount of CPD in the sun-exposed body area and heavily stained nuclei in the basal membrane (white arrows).

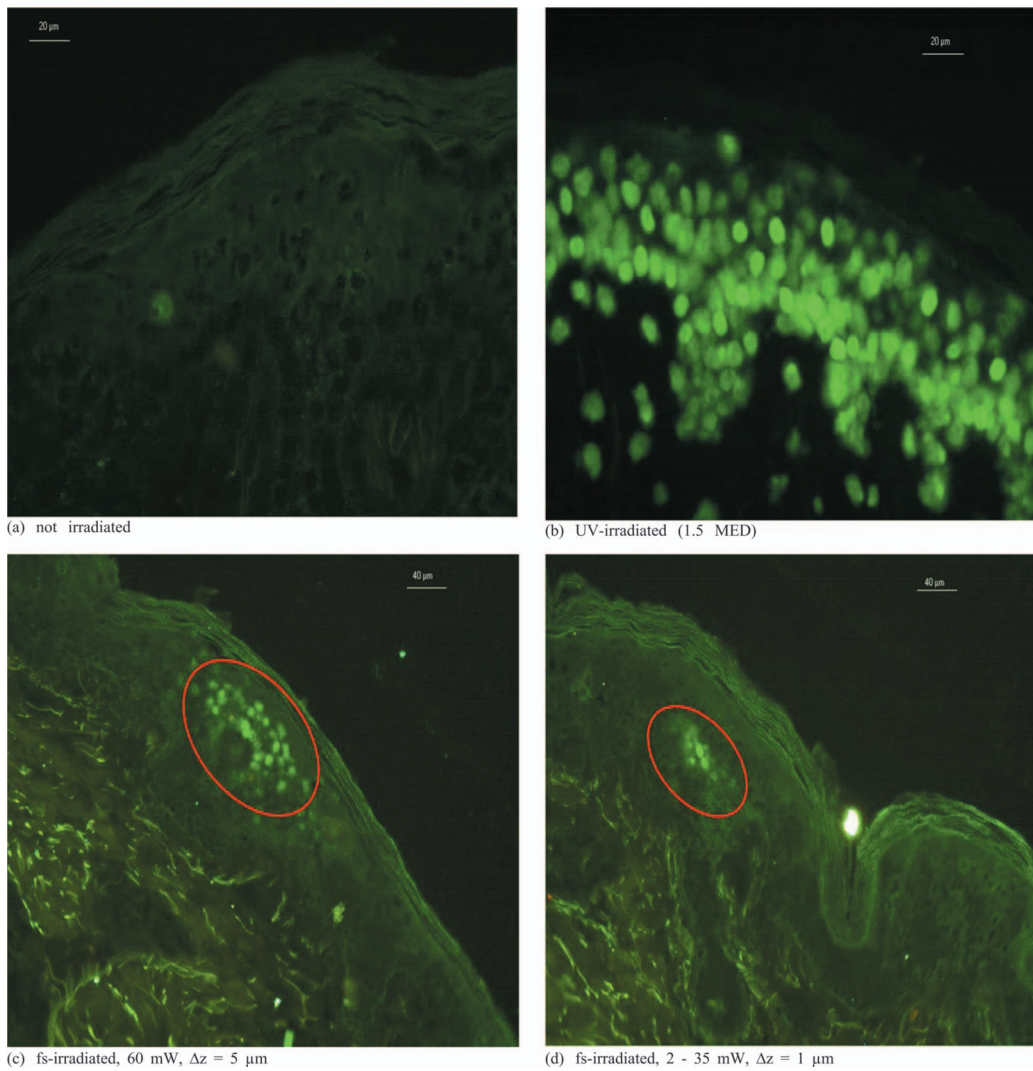


Fig. 4 Examples of (a) not irradiated, (b) UV-irradiated (1.5 MED), and (c) and (d) fs-irradiated buttock tissue slices. In image (c), one area of CPD fluorescence due to fs-irradiation was found (red ellipse); image (d) shows the second area (red ellipses), found in three samples.

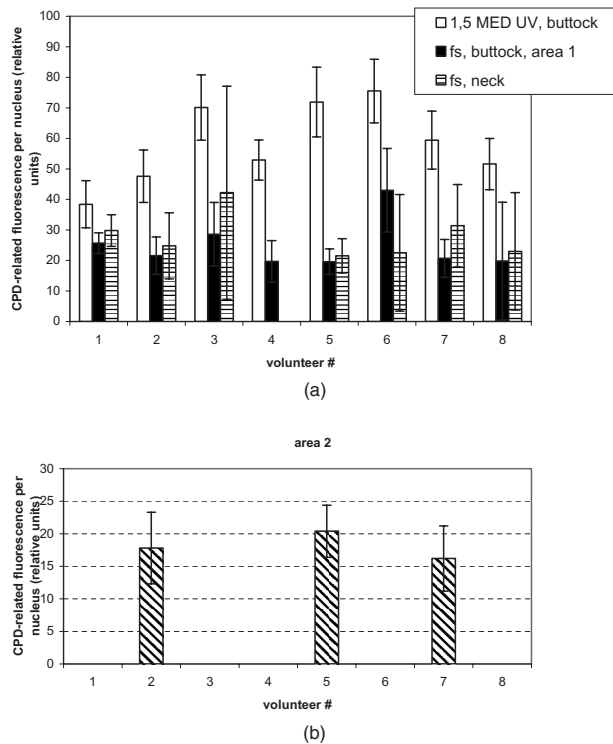


Fig. 5 Quantitative analysis of CPDs found in the skin sample of each volunteer after various irradiation regimes, measured as CPD-related fluorescence. (a) □=1.5 MED UV-irradiation of buttock skin; ■=fs-irradiation of buttock skin (area 1); and ▨=fs-irradiation on neck skin for comparison. (b) Area 2 found for fs-irradiation (buttock skin).

In the fs-irradiated buttock samples of all volunteers, a region with CPD-related fluorescence of cell nuclei was observed that can be attributed to the 60-mW fs-NIR-irradiation scheme [see Fig. 5(a), fs-buttock, area 1]. This region has a maximum area of $2 \times 10^{-3} \text{ mm}^2$ [Fig. 4(c)]. Additionally, a second region could be found in three out of eight volunteers [see Fig. 5(b), fs-buttock, area 2]. This region can be attributed to the area irradiated by the scheme using the z-scan with increasing laser power (regime 4). These second CPD-containing regions are small, representing $2.5 \times 10^{-4} \text{ mm}^2$ [Fig. 4(d)], respectively. Obviously, after 60-mW fs-irradiation with a step width of $5 \mu\text{m}$ or after 150 horizontal scans with linearly increasing laser power (2 mW to ca. 35 mW) and a step width of 1 micron, the number of two- and three-photon excitations is sufficient to induce CPDs in deeper cell layers. Taking into account that the microscope focus has a z dimension of about $1.5 \mu\text{m}$, a double exposure occurs in every $1\text{-}\mu\text{m}$ scan-step of two consecutive scans and enhances the formation of CPDs locally. On the other hand, due to skin scattering, deeper tissue layers will receive less power. Sun-simulator-exposed skin, on the other hand, shows significant CPD formation in all viable cell layers of the epidermis and dermis, and in the basal layer not limited to a restricted area.

4.2 Assessment of the Biological Effectiveness of UV Damage by Fs-NIR-Laser Irradiation

For a better assessment of the damage induced by fs-NIR-laser irradiation, one has to compare the amount of CPD for-

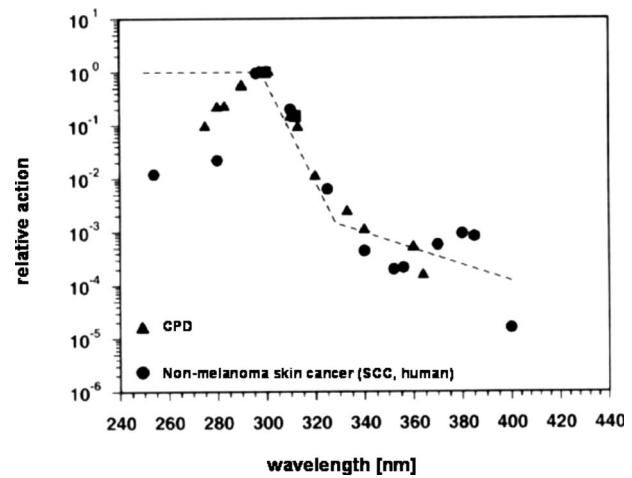


Fig. 6 Action spectrum for the development of CPDs (\blacktriangle) in the epidermis compared to the action spectrum for skin cancer induction (\bullet). For comparison, the erythema action spectrum of the Commission Internationale de L'Eclairage (International Commission on Illumination, CIE) is included (dotted line). Modified from Ref. 24; additional references can be found there.

mation with the natural amount of DNA damage after sun exposure and evaluate the amount of remaining CPD based on data from the literature. The outermost risk of UV irradiation can lead to three types of skin cancer, basal cell carcinomas (BCC), squamous cell carcinomas (SCC), and malignant melanoma (MM).^{36,37} The first two types have a relatively low lethality. On the other hand, late malignant melanoma stages lead to the death of the patient in a number of cases, if the melanoma is not removed in time. The connection between UV irradiation and the appearance of BCC or SCC is well proven epidemiologically. With MM, the connection is likely, but not equally well understood. Using animal investigations for nonmelanoma tumors, an action spectrum has been defined by de Gruijl and van der Leun.⁴¹

In the UVB and UVA range, this action spectrum follows the erythema action spectrum of the Commission Internationale de L'Eclairage (International Commission on Illumination, CIE; see dotted line, Fig. 6). Due to the absorption of shorter wavelengths in the skin the radiation is less effective in the UVC range, reaching a maximum at a wavelength of 290 to 310 nm, as these data include the protecting effect of the upper skin layers in the estimation of damage induction in deeper epidermis layers (e.g., basal cells).

Since the primary laser irradiation of the MPLSM used has a typical wavelength range of 750 to 850 nm, acute direct DNA damage by NIR radiation can be excluded. Hence, a real consideration of DNA damage has to take into account excitation of energy states usually accessible only by UV absorption but now generated by multiphoton excitation processes in viable skin layers. Further, the natural DNA repair efficiency after a single exposure by the fs-NIR-laser has been estimated.

One has to bear in mind, however, that the action spectra for BCC and SCC, as shown in Fig. 6, could be different in the fs-irradiation situation, because they are related to the effect of irradiation dose at the skin surface. For the mutation induction in mammalian cells not protected by cornified skin

layers, an action spectrum for cells in culture was published previously.³⁸ This can also be applied mainly to deeper skin layers (e.g., basal layer) because they resemble a kind of 3-D cell culture domain.

In comparison to the BCC/SCC action spectrum, a much higher effectiveness can be observed at 250 to 280 nm in nonprotected cells. This is important, since in addition to the main 425 to 325 nm, two-photon excitation, there could be a nonnegligible amount of short-wavelength UV effects around 267 nm being produced in deeper skin layers due to three-photon excitation produced by ultrashort focused laser irradiation.

The fs-NIR-irradiation experiments have proven that the energy density is high enough to lead to CPD formation, but whether these CPDs were induced by a two- or three-photon process remains open at the moment.

At a minimum, the measurement of the amount of CPDs offers a possibility of a very rough quantitative risk estimation at the level of DNA damage. The parallel exposure to 1.5 MED simulated sun radiation can serve as a “biological reference” leading to an exposure of 0.6 and 0.45 MED by fs-NIR-irradiation regimes 3 and 4, respectively. Hence, it is not necessary to measure the local photon dose in deeper skin layers. For BCC and SCC, a risk estimation was performed.³⁹ With a yearly UV dose of 100 MED over 30 years at ages from 15 to 45, the risk increases until age 75 by a factor 4. Assuming a linear dose relationship—as is usual in this kind of considerations³⁹—in a rough estimation, one can conclude that one additional exposure by fs-NIR-irradiation regime 3 or 4 increases the risk locally by 0.6% or 0.45%, respectively.

These estimations do not include the fact that only very small, defined areas are irradiated (e.g., in our case, $200 \times 200 \mu\text{m} = 0.04 \text{ mm}^2$) by MPLSM investigations compared to a sun exposure of total body parts or the whole body. For example, if the face is sun exposed, its area of about $10 \times 10 \text{ cm} (=10^4 \text{ mm}^2)$ is by a factor 250,000 larger than that exposed during fs-NIR-irradiation.

One has to keep in mind that the risk estimation is based on standard values for a Caucasian skin with phototype II or III and on limited number of *ex vivo* experiments with a limited number of possible irradiation parameters. Subjects with phototype I are more sensitive to cancer risk due to UV-irradiation and may be more sensitive to fs-irradiation as well. Since the tissue damage is caused by a nonlinear effect, changing laser parameters may result in more severe tissue damage (see Ref. 40).

If the mechanisms of DNA damage differ from that of UV-irradiation, especially when three or more photons are involved that excite to energy states not be reached by UV-irradiation due to the protection of the upper layer of the skin, these experiments may underestimate the cancer risk. In addition, a specially targeted irradiating of cells that are especially important for tumorigenesis (e.g., CRBCs) can increase the tumor risk over a linear extrapolation of the dose or irradiated area.

For future work, it will be interesting to see which role the DNA repair process may play after fs-NIR-irradiation and whether CRBCs can be induced or remain. Furthermore, it is important to investigate alternative UV-induced DNA lesions like 8-oxo-guanine or, even more important, DNA double-

strand breaks, which recently have been shown to be induced by UVA-radiation. Nevertheless, the large amount of CPDs produced in the neck area after natural sun exposure is usually repaired by the DNA polymerase repair system in a very efficient way.

Taking all the findings and considerations together, at our irradiation parameters, the risk increase by an fs-NIR-irradiation of a defined small skin area can be assumed to be reduced to negligible values. Even several fs-NIR-irradiation sessions delivered at different 0.04-mm^2 areas will not increase the risk of remaining DNA damage in a measurable way.

Acknowledgments

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