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# Mitigating thermal mechanical damage potential during two-photon dermal imaging

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**Abstract.** Two-photon excitation fluorescence microscopy allows in vivo high-resolution imaging of human skin structure and biochemistry with a penetration depth over 100  $\mu\text{m}$ . The major damage mechanism during two-photon skin imaging is associated with the formation of cavitation at the epidermal-dermal junction, which results in thermal mechanical damage of the tissue. In this report, we verify that this damage mechanism is of thermal origin and is associated with one-photon absorption of infrared excitation light by melanin granules present in the epidermal-dermal junction. The thermal mechanical damage threshold for selected Caucasian skin specimens from a skin bank as a function of laser pulse energy and repetition rate has been determined. The experimentally established thermal mechanical damage threshold is consistent with a simple heat diffusion model for skin under femtosecond pulse laser illumination. Minimizing thermal mechanical damage is vital for the potential use of two-photon imaging in noninvasive optical biopsy of human skin in vivo. We describe a technique to mitigate specimen thermal mechanical damage based on the use of a laser pulse picker that reduces the laser repetition rate by selecting a fraction of pulses from a laser pulse train. Since the laser pulse picker decreases laser average power while maintaining laser pulse peak power, thermal mechanical damage can be minimized while two-photon fluorescence excitation efficiency is maximized.

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Keywords: two-photon microscopy; cavitation; human skin; pulse picker; thermal mechanical damage.

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## 1 Introduction

In vivo optical imaging of human skin is a major technological challenge. While optical low-coherence reflectometry and confocal microscopy are useful to image the epidermal and dermal skin morphologies,<sup>1–5</sup> it is the application of multiphoton excitation microscopy that provides both structural and spectroscopic characterization of skin epithelial and dermal components.<sup>6,7</sup> Comparisons of confocal microscopy and multiphoton excitation microscopy on the same specimens of human skin in vivo have been previously published.<sup>8</sup> Applications of multiphoton microscopy in dermatology include noninvasive optical biopsy of skin lesions, the study of wound healing processes, and transdermal drug delivery.<sup>9,10</sup>

Multiphoton excitation microscopy is a nonlinear optical imaging technique that excites specimen fluorescence using infrared radiation.<sup>11</sup> The applications of multiphoton microscopy have been reviewed in a number of books<sup>12–14</sup> and journal special issues.<sup>15</sup> This technique has two major advantages over confocal microscopy for the imaging of thick, highly scattering specimens: (1) near-infrared light has a deeper tissue penetration depth than ultraviolet light and therefore can

image deeper structures, (2) the localization of the excitation volume and the use of infrared, instead of ultraviolet radiation, results in less photodamage. Since reducing photodamage is a key reason to use two-photon microscopy for human dermal imaging, an understanding of the skin photodamage mechanisms in two-photon excitation microscopy is critical for its proper application.

There are three major photodamage mechanisms: (1) Photodamage can be caused by two-photon excitation of intracellular chromophores. In cellular systems, photodamage caused by two-photon excitation is similar to that of ultraviolet irradiation.<sup>16,17</sup> Intracellular coenzymes and porphyrins act as photosensitizers in photo-oxidative processes. Photoactivation of these chromophores results in the formation of reactive oxygen species which trigger the subsequent biochemical damage cascade in cells. These processes can also result in genetic alterations and damage. (2) Photodamage can also be caused by other mechanisms, such as dielectric breakdown, related to the high electromagnetic fields of the femtosecond laser pulses.<sup>16</sup> (3) Another source of photodamage can also be single-photon absorption of the infrared radiation. This damage mechanism is of thermal origin.<sup>18–20</sup>

Multiphoton microscopes often use femtosecond mode-locked pulse lasers, such as titanium-sapphire lasers. These

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infrared lasers deliver a train of 100- to 400-fs pulses at a repetition rate of 80 MHz with an average power up to 2 W. Since the average power required for two-photon excitation microscopy ranges from 1 to 100 mW, which is about one to two orders of magnitude higher than that of conventional one-photon confocal microscopy systems where fluorophore excitation saturation occurs at tens of microwatts, the potential of thermal mechanical damage should be considered.

Previous studies of cellular damage have concluded that thermal mechanical damage is not an important process in most cases during multiphoton imaging.<sup>21</sup> However, the presence of highly absorbing melanin in human skin is a unique case where thermal mechanical damage can occur. In this report, we verified that the primary damage process in Caucasian ex vivo human skin during two-photon imaging is of thermal mechanical origin. We also measured the power threshold to initiate this damage process. We demonstrated that the thermal mechanical damage threshold can be predicted based on a simple heat diffusion model provided that the tissue infrared absorption coefficient ( $\mu_a$ ) is known. Furthermore, we used a laser pulse picker in order to reduce the thermal mechanical damage during two-photon imaging.

## 2 Materials and Methods

### 2.1 Sample Preparation

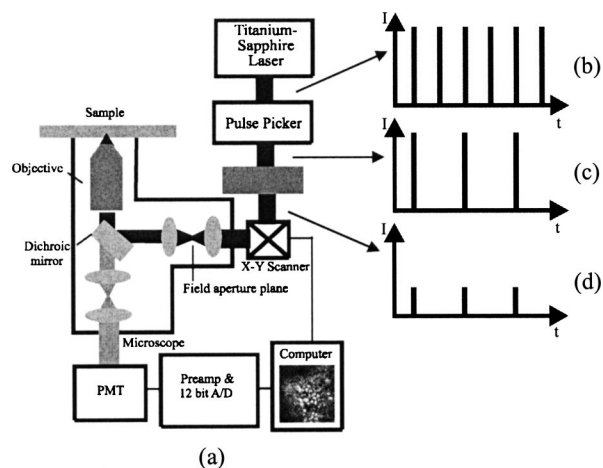
Frozen human cadaver skin was obtained from the expired stock of a skin bank. The specimen was defrosted and stored at 4°C before use. Three specimens, 1 cm by 1 cm, from the skin of three Caucasian subjects were studied. Each sample of skin was prepared by placing it in a hanging drop microscope slide containing a piece of damp sponge. The specimen was sandwiched between the coverslip and the damp sponge in order to maintain its moisture. All studies are performed in room temperature.

Calibration grade fluorescein (Molecular Probes, Eugene, OR) was dissolved in pH 8.0 HEPES buffer at a concentration of approximately 100  $\mu$ M. The fluorescein solution is sealed in a hanging drop slide with a standard microscope coverslip.

### 2.2 Two-photon Microscopy

The basic two-photon microscope design [Fig. 1(a)] has been described previously.<sup>6</sup> The major modification in this study is the addition of a combination of a laser pulse picker and a Glan-Thomson polarizer to control the excitation laser pulse train profile generated from a Titanium-Sapphire laser [Fig. 1(b)].

A laser pulse picker based on an acousto-optical modulator allows the control of the laser pulse train repetition rate (Pulse Picker 9200, Coherent Inc., Santa Clara, CA). In the ideal situation, the pulse picker only reduces the pulse train repetition rate but not the instantaneous power of the pulses [Fig. 1(c)]. It should be noted that the excitation efficiency is often decreased due to pulse dispersion as the light is diffracted by the tellurium oxide (TeO<sub>2</sub>) crystal with group velocity dispersion (GVD) coefficient 10,000 fs<sup>2</sup>. This dispersive effect can be compensated by temporal pulse shaping.<sup>22</sup> The use of an acousto-optical modulator has two additional effects that can further reduce laser peak power. First, due to the limited efficiency of the acousto-optical crystal, the typical deflection efficiency of the emitted beam is about 50% to 60%, which



**Fig. 1** (a) Schematics of a two-photon excitation microscope. The pulse profile of the excitation light is controlled using a combination of a laser pulse picker and an attenuator. (b) Pulse profile from the titanium-sapphire laser. (c) Pulse profile after the laser pulse picker. (d) Pulse profile after the attenuator.

causes an irreversible reduction of instantaneous laser power by a factor of two. The acousto-optical modulator may also introduce spherical aberration, which affects beam focusing. Investigations into compensating for aberration effects based adaptive optics and other methods are under way.<sup>22,23</sup>

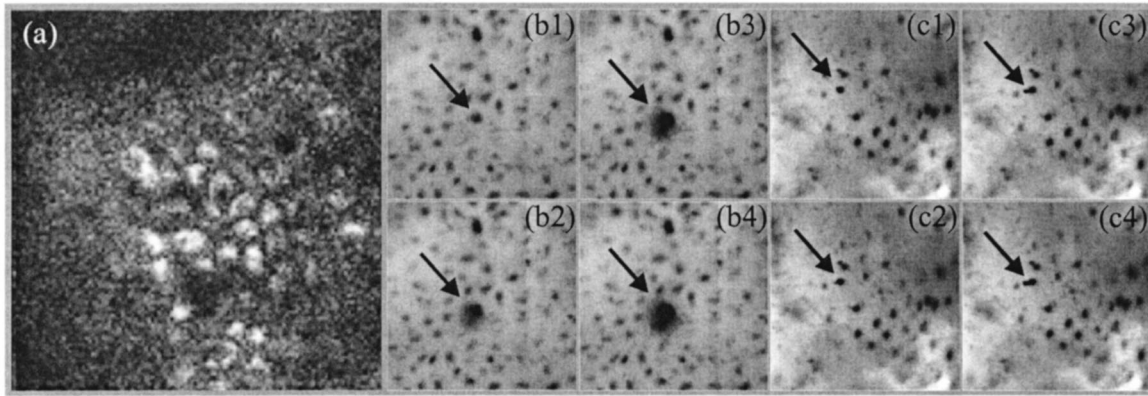
The laser pulse energy can be further controlled by an attenuator, such as a polarizer. For a polarizer, the attenuation factor depends on the square of the cosine of the relative angle between the polarization orientations of the laser beam and the polarizer. The laser's peak and average power are both decreased by this method while the laser repetition rate is unchanged [Fig. 1(d)].

### 2.3 Two-photon Microscopic Imaging

Two-photon imaging of the ex vivo human skin specimen was performed as previously described.<sup>6</sup> Two-photon imaging is performed at 780-nm excitation wavelength using 10-mW average power using a Zeiss 40 $\times$  Fluar objective (1.2 NA). Pixel residence time is 0.1 ms. Melanin capped basal cells at the epidermal-dermal junction are visualized based on their autofluorescence.

### 2.4 Measurement of Skin Thermal Mechanical Damage

Skin thermal mechanical damage measurement was performed in a standard two-photon microscope. The laser pulse train profile from the titanium-sapphire laser was regulated by the laser pulse picker and a polarizer. Thermal mechanical damage probability was measured for laser pulse energy in the range from 0.05 to 1.8 nJ, and the laser repetition rate ranged from 1 kHz to 10 MHz. The specimen and the photodamage process were visualized under dark field condition using white light illumination. A high-sensitivity video rate CCD camera was used to record the time course of photodamage (PlutoCCD, PixelVision, Tigard, OR). The *x-y* scanning mechanism of the microscope was disabled so that the excitation beam can be manually placed at a chosen melanin cap at the epidermal-dermal junction. As a function of laser average



**Fig. 2** (a) Two-photon imaging of ex vivo human skin epidermal-dermal junction based on autofluorescence from NAD(P)H and melanin associated chemical species. (b) Cavitation process observed in epidermal-dermal layer imaged by white-light wide-field imaging. The laser pulse energy is 1.4 nJ operating at a 10-MHz repetition rate. The time separations between frames are 15 sec. (c) Cavitation process observed in epidermal-dermal layer imaged by white-light wide-field imaging. The laser pulse energy is 1.4 nJ operating at a 1-kHz repetition rate. The arrows in parts (b) and (c) point to the melanin caps at which the laser was focused. The time separations between frames are 15 sec.

power and repetition rate, we evaluated the presence of photodamage based on the observation of morphological changes. Thermal mechanical damage was considered to occur at a given average power and repetition rate if we observed a morphological transition characterized by cavitation: an explosive evaporation of melanin containing skin tissue components.<sup>24</sup> The lack of thermal mechanical damage corresponds to cases in which no morphological changes were observed after 120 sec of laser beam illumination. For a given combination of average power and repetition rate, nine experiments were performed at independent skin sites. Illumination sites were always chosen to be the center of melanin caps by visual inspection. Three skin specimens from different subjects were used and three sites were chosen from each specimen.

### 3 Results and Discussion

#### 3.1 Visualization of Skin Morphological Damage Caused by a Femtosecond Pulse Laser

Two-photon imaging of skin structures based on autofluorescence allow the clear identification of epidermal and dermal strata including stratum corneum, stratum spinosum, epidermal-dermal junction, and the dermal layer. A challenge in two-photon imaging of human skin is to avoid tissue photodamage. Photodamage of skin in two-photon imaging often occurs at the epidermal-dermal junction where the basal cells can be identified by their unique morphology and the presence of melanin caps. Two-photon excited autofluorescence from this layer can be attributed to signals from reduced pyridine nucleotides [NAD(P)H] and melanin (or melanin associated fluorophores) [Fig. 2(a)]. Two-photon photodamage at the basal layer is associated with clear morphological changes accompanied by cavitation (explosive evaporation) at the location of the laser beam [Figs. 2(b) and 2(c)].

#### 3.2 Validation of the Thermal Origin of Skin Damage during Two-Photon Imaging

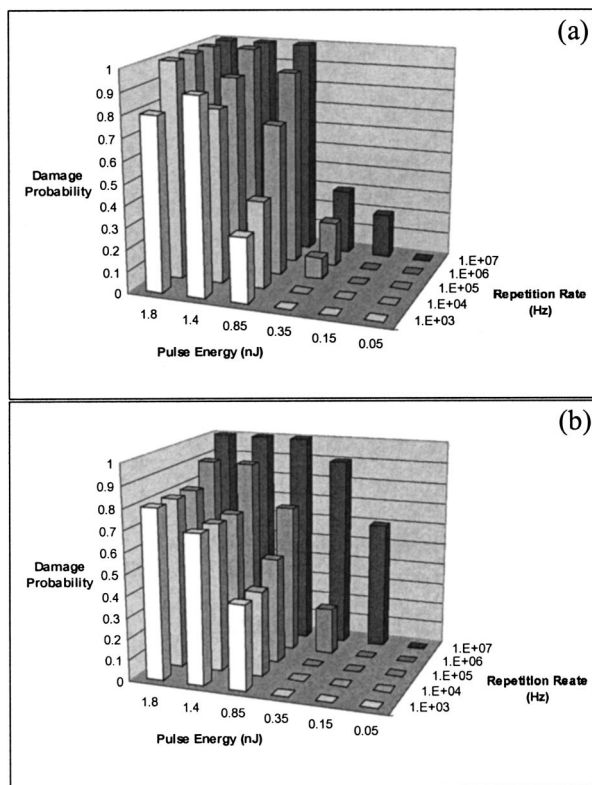
Thermal mechanical damage mechanism is consistent with the formation of local cavitation.<sup>25–28</sup> Under typical two-photon microscopic imaging conditions, previous studies

show that thermal mechanical damage due to either one- or two-photon absorption processes is negligible for typical physiological specimens.<sup>21,29</sup> However, skin and a few other pigment containing tissues, such as the retina, are exceptions. In these tissues with a high concentration of pigments, strong one-photon infrared absorption may result in a significant local temperature increase, cavitation, and resulting morphological damage.

Since skin damage during two-photon imaging may have a thermal origin, it is important to understand heat deposition mechanisms in tissues. Consider a typical two-photon microscope that uses an infrared laser source that generates an 80-MHz laser pulse train containing 100-fs duration pulses. The temperature rise in a specimen due to the absorption of this pulse train can be approximated as two independent processes.<sup>21,29–31</sup> First, since individual pulses are femtoseconds in duration, which is significantly shorter than thermal relaxation time in liquid, which is on the order of 70 ns in water, the heating due to each pulse can be considered instantaneous. The specimen temperature change resulting from the absorption of a single laser pulse will exhibit a fast rise time characterized by the laser pulse width and a fall time characterized by the thermal diffusion time of the water. Second, when the pulse repetition rate is in the same time scale as the thermal relaxation of water, there will be thermal buildup as multiple pulses are incident upon the same location in the sample. For pulse separation time shorter than the thermal relaxation time, the temperature profile of the sample can be approximated as a slow temperature rise plus a fast, periodic (at the laser pulse repetition rate) temperature fluctuation due to the absorption of individual laser pulses. The maximum temperature rise  $T_{\max}$  is a sum of the maximum temperature rise due to cumulative effect  $T_{\max}^{\text{Cumulative}}$  and individual pulses  $T_{\max}^{\text{Pulse}}$ :

$$T_{\max} = T_{\max}^{\text{Cumulative}} + T_{\max}^{\text{Pulse}} = \left[ \frac{\mu_a E f_p}{4 \pi k_t} \ln \left( 1 + \frac{2t_{\text{res}}}{\tau_C} \right) \right] + \left[ \frac{\mu_a E}{2 \pi k_t \tau_C} \right], \quad (1)$$





**Fig. 3** (a) Measured thermal mechanical damage probability in ex vivo human skin as a function of laser pulse energy and repetition rate. (b) With the assumption that cavitation occurs when local tissue temperature increases above  $110^{\circ}\text{C}$ , least square fitting of the experimental data to the tissue heating theory indicates that the most probable tissue absorption coefficient ranges between  $4.0 \times 10^3$  to  $5.4 \times 10^4 \text{ m}^{-1}$ . The skin thermal mechanical damage probability distribution resulted for this range of tissue absorption coefficients is plotted.

where  $E$  is the energy of each pulse,  $f_p$  is the laser repetition rate,  $k_t$  is tissue thermal conductivity with a typical value of  $0.6 \text{ WK}^{-1} \text{ m}^{-1}$ ,  $\tau_c$  is the thermal time constant, which is about  $70 \text{ ns}$  in water, and  $t_{\text{res}}$  is the residence time of the laser at a specific position in the specimen, which ranges from  $10 \mu\text{s}$  to  $10 \text{ ms}$  in a typical imaging situation. Two-photon imaging parameters that can be adjusted experimentally are laser pulse energy  $E$  and the laser repetition rate  $f_p$ .

The probability of observing this type of morphological damage at the skin epidermal-dermal junction in the Caucasian skin specimens as a function of laser pulse energy  $E$  and the laser repetition rate  $f_p$  has been quantified [Fig. 3(a)]. The laser pulse energy was varied from  $0.5$  to  $18 \text{ nJ}$ . By least square fitting of the measured damage probability [Fig. 3(a)] to the heat deposition model [Eq. (1)], one can theoretically estimate the range of the tissue optical absorption coefficient using an additional assumption that the occurrence of cavitation corresponds to local tissue temperature rise above  $110^{\circ}\text{C}$ , based on previous study results.<sup>24</sup> Least square fit indicates that the most probable tissue absorption coefficient ranges between  $4.0 \times 10^3$  to  $5.4 \times 10^4 \text{ m}^{-1}$ . This range of tissue absorption coefficients is consistent with previous studies of the melanin absorption coefficient in skin ranging from  $7.8 \times 10^3$  to  $1.3 \times 10^5 \text{ m}^{-1}$ .<sup>24</sup>

The theoretical photodamage probability distribution con-

sistent with this range of absorption coefficients based on the heat deposition model is presented in Fig. 3(b) and is consistent with experimental observation. Specifically, morphological damage is observed for laser pulse energy above a threshold of  $1.0$  to  $1.2 \text{ nJ}$ . Above this threshold, damage is uniformly observed independent of laser repetition rates. This observation is consistent with the mechanism that thermal mechanical damage is dominated by the single pulse heating. For pulses with sufficient energy, local heating above  $110^{\circ}\text{C}$  can result even in the absence of cumulative heating effect. For pulse energy below the threshold level, damage probability is observed to increase with increasing laser repetition rate consistent with the model that cumulative heating process is important at lower pulse energy and higher repetition rate.

### 3.3 Using Laser Pulse Picker to Mitigate Thermal Mechanical Damage during Two-photon Skin Imaging

From the heat deposition model, it is clear that the energy of an individual laser pulse must be sufficiently low to avoid cavitation. However, even if a single laser pulse does not deposit sufficient energy to induce thermal mechanical damage, tissue heating due to cumulative effects can still be important. The relative heating contribution from pulse and cumulative heating can be estimated as:

$$\frac{T_{\text{max}}^{\text{Cumulative}}}{T_{\text{max}}^{\text{Pulse}}} = \frac{f_p \tau_c}{2} \ln \left( 1 + \frac{2t_{\text{res}}}{\tau_c} \right). \quad (2)$$

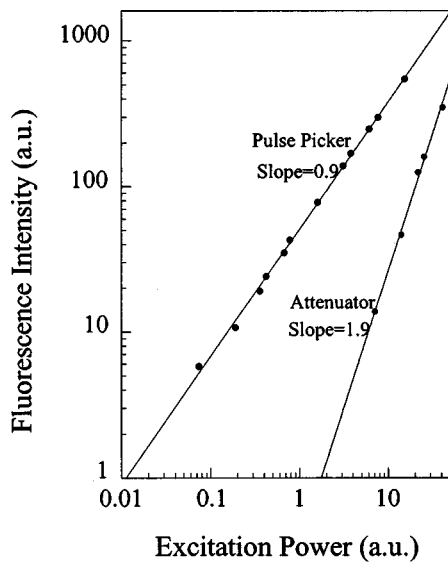
For typical experimental parameters with  $f_p = 80 \text{ MHz}$ ,  $\tau_c = 70 \text{ ns}$ , and  $t_{\text{res}} = 10$  to  $100 \mu\text{s}$ , this ratio is between  $15$  to  $25$ . Under the typical operation conditions of a two-photon microscope, the slow temperature rise resulting from the cumulative effect of the multiple pulses is about an order of magnitude higher than the effect of a single pulse.

In the regime where energy of individual pulses is insufficient to produce thermal mechanical damage, the effect of cumulative heating can be minimized by: (1) a further reduction of the pulse instantaneous power or (2) a decrease of laser repetition rate.

Given that either reducing laser pulse energy or laser repetition can reduce the cumulative heating, the choice of thermal mechanical damage management should be dictated by the method that best maintains two-photon excitation efficiency. Reducing the laser pulse repetition rate is the preferred method. The number of photon pairs absorbed per second via two-photon excitation is  $N_a$ :

$$N_a \propto \frac{E^2 f_p \delta}{\tau_p}, \quad (3)$$

where  $\tau_p$  is the pulse width and  $\delta$  is the two-photon cross section. Note that the two-photon excitation probability fluorescence intensity is a linear function of pulse repetition rate, and a quadratic function of laser pulse energy (Fig. 4). Therefore, a reduction of the pulse repetition rate will reduce correspondingly the fluorescence signal linearly. However, if we choose to reduce the laser pulse energy, a significantly greater reduction in fluorescence signal will result. Therefore, in the case where thermal mechanical damage is limited by heat



**Fig. 4** Two-photon fluorescence intensity as a function of excitation power for a 10  $\mu\text{M}$  fluorescein solution. When the excitation power is controlled by the division rate of an acousto-optical pulse picker, a linear dependence of the two-photon fluorescence intensity as a function of excitation power is expected. When the excitation power is controlled by a cross polarizer, a quadratic dependence is expected. These power dependences are clearly observed.

accumulation, power reduction by reducing repetition rate is preferable and can be accomplished by using a laser pulse picker. The heating contribution from cumulative heating is approximately 20 times higher than heating by individual pulses. In terms of thermal mechanical damage, reducing the pulse repetition rate significantly beyond a factor of 20 using a laser pulse picker does not afford significant additional advantage, because the single pulse heating effect will become the dominant factor. When the thermal mechanical damage from a single pulse is significant, this effect cannot be mitigated by a pulse picker. Instead, the laser pulse energy must be reduced.

We have demonstrated that a major photodamage mechanism in two-photon skin imaging is of thermal origin. This damage process is characterized by cavitation driven by local temperature rise as melanin absorbs infrared radiation via a one-photon process. We further demonstrate that the heating process can be separated into two parts corresponding to instantaneous heating from absorbing the femtosecond laser pulses and cumulative heating when energy from individual pulses is accumulated in the sample region faster than can be dissipated by conductive heat transfer. This model allows prediction of the thermal mechanical damage threshold as a function of the skin infrared absorption coefficient. This model further allows the proper choice of laser power thresholds for two-photon imaging of skin with different photogrades based on their infrared absorption coefficients. In addition, we demonstrated that the cumulative heating problem can be mitigated by the use of a laser pulse picker to reduce the pulse repetition rate, which allows sufficient time for heat dissipation between pulses.

While this experimental investigation analyzed the thermal mechanical origin of the laser damage, we have not analyzed

the possible biochemical and genetic damage that could also have occurred. In fact, biochemical and genetic damage is likely to occur at a lower power threshold than thermal mechanical type processes. A further caveat of our study is that it was performed on ex vivo human skin. This is due to ethical and legal considerations. In vivo human skin has a vascular supply that may contribute to heat dissipation and may increase the damage threshold. However, the epidermis in both ex vivo and in vivo human skin are both avascular. The vascular structure is situated below the dermal-epidermal junction and its effect on epidermal heat dissipation is likely to be minimal.

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