Nonlinear optical imaging as a diagnostic tool for cutaneous squamous cell carcinoma

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Carcinogenic damage induced to deoxyribonucleic acid by femtosecond laser pulses via combination of two- and three-photon absorption during nonlinear optical imaging

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From
Abstract

Nonlinear optical imaging (NLOI) applied in vivo are increasingly promising for clinical diagnostics and the monitoring of cancer and other disorders, as they can probe tissue with high diffraction-limited resolution at near infrared (NIR) wavelengths. However, high peak intensity of femtosecond laser pulses required for two-photon excitation fluorescence (TPEF) can cause formation of cyclobutane pyrimidine dimers (CPDs) in cellular deoxyribonucleic acid (DNA) similar to damage from exposure to solar ultraviolet (UV) light. Inaccurate repair of subsequent mutations increases the risk of carcinogenesis. This study investigated CPD damage that results in Chinese hamster ovary cells in vitro from imaging them using NLOI based on TPEF. The CPD levels were quantified by immunofluorescent staining. We further evaluated the extent of CPD damage with respect to varied wavelength, pulse width at focal plane, and pixel dwell time as compared with more pronounced damage from UV sources. While CPD damage has been expected to result from three-photon absorption, the results revealed that CPDs were induced by competing two and three-photon absorption processes, where the former accesses UVA absorption band. This finding was independently confirmed by nonlinear dependencies of damage on laser power, wavelength, and pulse width.
4.1 | Introduction

In recent years, nonlinear optical imaging (NLOI) modalities based on near infrared (NIR, 700 to 1000 nm) femtosecond (fs) laser sources have been increasingly utilised in biomedical research and clinical applications. In vivo tissue imaging is especially promising for investigation, diagnostics, and monitoring of cancers and other disorders. Multiphoton laser scanning microscopy (multiphoton excited fluorescence, second and third harmonic generation) can probe tissue structure at high diffraction-limited resolution (0.3 μm), with 100- to 300-μm penetration depth, intrinsic three-dimensionality (3-D), and no out-of-focus volume photobleaching or photodamage, since there is no one-photon absorber except melanin at NIR wavelengths.

Cells and tissues contain a host of endogenous fluorophores that can be excited using multiphoton imaging techniques. Contrast in tissue arises from two photon excitation fluorescence (TPEF) of nicotinamide adenine dinucleotide (NADH, absorption maximum at 340 nm) [1], flavin adenine nucleotide (FAD, 440 nm) [1], melanin (broad-band absorption through all visible wavelengths) [2], collagen (Type I, 375 nm) [3] and various lipoproteins, while noncentrosymmetric protein arrays (collagen, myosin and tubulin) produce a second harmonic generation signal (SHG) [4] and interfaces can be visualised with third harmonic generation [5,6]. All these distinct endogenous sources can be excited simultaneously and discriminated in a spectral imaging system [7-9]. NLOI based on label-free autofluorescence has been demonstrated in vivo not only in skin [10] but also in highly dynamic tissues such as lymphoid tissue [11] and intestine [12]. Moreover, in vivo monitoring of protein-bound and free NADH has been achieved recently with NLOI [13].

Thus NLOI allows for probing and understanding of cells and tissues in their natural unperturbed state. The ability to generate images using intrinsic sources opens new possibilities for development of NLOI in medical applications in hospital settings, where label-based imaging must be avoided. To this date, NLOI has been shown to successfully visualise and distinguish with statistical significance between control and diseased states in various tissue disorders [14-22] as well as detect and monitor changes in free and bound NAD(P)H in cancer versus normal cells [23] and during apoptosis [13].

Apart from technical challenges for in vivo microscopy, one of the reasons for a delay in routine NLOI in clinical applications is insufficient experimental data on possible risks involved, with carcinogenic cell damage being the most serious concern [24,25]. For a successful transition from laboratory bench to clinics, biological safety must be investigated thoroughly. Since all multiphoton processes are based on simultaneous nonresonant absorption or resonant up-conversion of two or three excitation photons in the sub-femtolitre focal volume of a high NA objective, they require fs laser pulses with high peak intensity in the range of several hundreds of GW/cm². This level of photon density is high enough to cause destructive nonlinear photochemical effects in live cells, with acute damage ranging from irreversible impairment of cell division [26], formation of destructive oxygen radicals and singlet oxygen...
to plasma formation and morphological destruction, where the latter require an order of magnitude higher intensity [28]. In fact, thermal damage resulting from multiphoton water absorption has been estimated to be insignificant throughout the NIR imaging range on the order of 1 mK for typical excitation power of 5 – 30 mW [29].

However, the biggest safety concern has always been the direct DNA damage from simultaneous absorption of three photons, as strong absorption bands of nucleic acids lie below 290 nm, peaking at 257 nm. The DNA lesions induced are similar to those from natural UV exposure resulting in formation of cyclobutane pyrimidine dimers (CPDs), 6-4 photoproducts, single- and double-strand breaks, and numerous modified bases [25,30-32]. Among all these lesions, CPDs are primarily responsible for carcinogenesis [33] resulting from a series of mutations in cells with unrepaired damage [34]. The genotoxic cellular effects of the shorter UV wavelengths (UVC band, 200 to 290 nm, and UVB band, 290 to 320 nm) have been long known and thoroughly investigated [30,35-37]. However, the DNA damaging effects of longer wavelengths (UVA band, 320 to 400 nm) causing strong mutagenic properties have been recognized only recently [30,38-40]. There is an ongoing debate regarding the direct and indirect pathways leading from UVA absorption to CPDs. Several studies have claimed that UVA absorption produces reactive chemical species that can damage DNA [14,38-42]. However, two recent studies strongly suggested that CPDs are produced by UVA via direct photochemical mechanism, without mediation of a cellular photosensitiser or intermediate reactive species [43,44]. Moreover, Mouret et al. showed evidence that UVA-induced CPDs are less repairable [38]. In their experiment, 48 hours after exposure to UVA radiation the level of unrepaired CPDs was unexpectedly higher than after UVB induced damage. There are no indications that different CPDs are produced by absorption of UVA and UVC photons. However, the possible explanations for persistence of UVA induced CPDs include different in-cell cycle arrest after irradiation, degradation of DNA repair protein by the UVA induced reactive oxygen species [37] as well as alteration of cell behaviour through changes in cell signalling pathways by UVA radiation [45].

Theoretically, in the spectral range typically used for NLOI of endogenous fluorophores both UVC and UVA absorption bands can be excited by fs laser pulses via three- and two-photon absorption, respectively. Previously, only third-order process and UVC-like damage has been considered [25]. Therefore there is a need to investigate the mechanism of CPD damage by NIR laser pulses. Specifically it is important to understand if CPD damage results from concurrent three- and two-photon absorption, and if it does, what their contributions are.
4.2 | Materials and Methods

4.2.1 | Cell Culture

Chinese hamster ovary (CHO) cells were cultured in flasks containing Dulbecco’s modified Eagle’s medium (Invitrogen, Breda, The Netherlands) containing 7.5% fetal calf serum (Invitrogen, Breda, The Netherlands), 2 mM L-glutamine (Invitrogen, Breda, The Netherlands), and 2% antibiotic mix (Invitrogen, Breda, The Netherlands) at pH 7.4. Cultures were maintained at 37°C in an incubator aerated with 5% CO₂.

For experiments, cells were trypsinised and 3 ml of the cell suspension was transferred into sterile Petri dishes of 35 mm diameter with an inbuilt glass cover slip of 0.16 to 0.19 mm thickness and 20 mm diameter (MatTek, Massachusetts, U.S.A.), located in the Petri dish centre for optimal laser irradiation/imaging. Grids were drawn on the Petri dish bottom in order to image known groups of cells with varying laser parameters. To obtain a confluent monolayer of cells the cell chambers were additionally incubated for another day under the above conditions.

4.2.2 | Laser irradiation

The cells were imaged with a laser scanning unit (C1, Nikon, Japan) mounted on an inverted microscope (TE2000, Nikon, Tokyo, Japan) coupled to a mode-locked titanium sapphire fs laser (Chameleon model, Coherent, California, U.S.A.) with 80-MHz pulse repetition rate, and 130-fs pulse width. Excitation intensity was controlled with a combination of a half-waveplate and a polarising cube (Thorlabs, New Jersey, U.S.A.). Pulse width was adjusted with a group velocity dispersion compensator consisting of a pair of gratings (Thorlabs, New Jersey, U.S.A.) and measured at the focal plane with an autocorellator (APE Gmbh, Berlin, Germany). All experiments were performed over a range of excitation wavelengths between 695 and 810 nm with varied intensity and pulse width using a water immersion 40 × 0.8 N.A. objective (Nikon, Tokyo, Japan) with autofluorescence signal acquired in a nondescanned configuration.

Confluent monolayers of CHO cells were exposed to NIR irradiation doses under varying imaging conditions. The acquired xyz optical stacks contained between 10 and 12 images of 512 × 512 pixel (170 × 170 um) size with axial intervals of 1 mm. The cells in the non-irradiated regions served as an internal negative control. UV irradiation was performed in a UV cabinet (Chromato Vue CC-20, California, U.S.A.) over the whole surface of the Petri dishes at two individual wavelengths: 254 nm corresponding to UVC band, and 365 nm corresponding to UVA band.

4.2.3 | Immuno-fluorescence assay for CPDs

After the NIR irradiation the cells were fixed for 10 min with 4% formalin (Sigma Aldrich, Zwijndrecht, the Netherlands) in phosphate buffered saline (PBS). Washing the cells with 2 ml PBS five times followed this step and every subsequent one. Fixed cells were incubated for 5 min on ice with 0.5% Triton X-100 in PBS in order to permeabilise the cell membranes
for antibody penetration. The cellular DNA was then denatured by treating the samples with 2N HCl at room temperature for 30 min. Primary monoclonal antibodies specific for CPDs (Cosmobio, Tokyo, Japan) were diluted 1:500 in 5% bovine serum albumin in PBS and incubated with the cells for 1 h at room temperature. For the secondary antibodies, we used goat anti-mouse IgG conjugated with AlexaFluor-594 (Invitrogen, Breda, The Netherlands) diluted 1:100 in 5% bovine serum albumin in PBS, where incubation was performed for 30 min at room temperature. Finally, the cells were stained with 0.05 μg/ml DAPI in PBS to facilitate visual localisation of nuclei during the analysis. The samples were dried and mounted with Vectashield antifade medium, and the Petri dishes were sealed with paraffin tape.

The one-photon CPD immuno-fluorescence from the stained cells was recorded using an EMCCD camera (Cascade model, Photometrics, Arizona, U.S.A.) coupled to a fluorescence microscope (TE2000, Nikon, Tokyo, Japan) with a 20 × 0.75 NA PlanApo air objective (Nikon, Tokyo, Japan). Since formalin fixation is known to flatten cells, acquisition of a single optical section from the axial centre of cells is a sufficient indicator of the total fluorescence. Signal intensity was quantified using ImageJ software (http://rsbweb.nih.gov/ij/), where pixel intensity values were obtained from individual nuclei, and then averaged between all the cells irradiated under the same conditions. All samples were imaged using the same acquisition parameters with minimal and similar levels of photobleaching. Therefore the quantified fluorescence intensity serves as a valid metric for the amount of induced CPD lesions. Every set of simultaneously processed samples had one UVC-irradiated sample that served as positive control and provided a normalisation factor for comparison between different sets to account for unavoidable fluctuations in staining efficiency and inhomogeneity.

4.3 | Results and Discussion

A typical image of TPEF from CHO cells is shown in Figure 1(a) along with the corresponding one photon immuno-fluorescence from CPDs in the nuclei of fixed cells as seen in Figure 1(b). Very minimal cell migration was noted between the start of irradiation and fixation with formalin. A clear border of region with CPD damage corresponds to the edge of the x-y scan; the cells in the area unexposed to laser exhibit a very low level of CPD immuno-fluorescence. The background level of CPDs in the cells can be attributed to CPDs formed by exposure to stray white light during culturing and especially during the experiment. Non-specificity of the CPD staining was ruled out by a separate negative control experiment. As expected, CPD damage from imaging with fs laser was evenly distributed within cell nuclei as equal doses of radiation were delivered to every pixel. Any inhomogeneity in the damage pattern corresponded to local concentration of available DNA. On the contrary, a seen in Figure 1(c), CPDs induced by UVC lamp (positive control) showed a doughnut-shape distribution with stronger damage at the nuclei periphery. This finding could be explained by strong absorption of 254-nm photons by DNA. It has been shown that in some laser-damaged cells, DNA lesions
tend to migrate toward the periphery of the nuclei, which might explain the doughnut shape distribution of CPDs [32]. Therefore the possibility that uniformly damaged nuclei might carry different consequences for cell viability, repair, and carcinogenic mutations requires further investigation.

Figure 1 | Experimental recording of CPD damage: (a) TPEF from cells imaged at 730 nm; (b) corresponding immunofluorescence image of cell nuclei with CPD damage (note on the left side the distinct border of the laser scanned with residual level of fluorescence signal); (c) CPD immunofluorescence from cell nuclei irradiated with UVC light (note the mostly peripheral distribution of CPD lesions).

4.3.1 | Intensity versus Damage
Experimentally measured dependence of CPD damage on peak intensity for three different wavelengths at fixed pulse width of 175 fs (measured at the sample) was plotted as shown in Figure 2. An apparent minimal peak intensity is observable at around 0.35 TW/cm² that produces detectable damage just above the background level. However, this was indicative of the overall staining method sensitivity at lower levels of CPDs rather than a threshold behaviour. Nevertheless, these laser intensities were lower than required for tissue imaging.
(0.5 to 0.8 TW/cm²), and the damage over this practical range of intensities was investigated here. As evident from the data, the minimal peak intensity that produced detectable damage decreased, while the level of CPD damage increased for shorter wavelengths.

![Logarithmic plot of CPD immunofluorescence signal dependence on peak intensity of laser.](image)

Figure 2 | Logarithmic plot of CPD immunofluorescence signal dependence on peak intensity of laser. The slopes of the linear fits are 3.40 ± 0.33, 2.94 ± 0.18, and 2.81 ± 0.16 for 780, 750, and 711 nm respectively. Scan speed: 30 μs, pulse width at the focal plane: 164 fs.

Any third-order process would be governed by cubic power dependence. While accounting for the background level (parameter A₀, Table 1) of CPD immunofluorescence from non-irradiated cells, linear fits of the experimental data on the log – log scale yielded slopes of 3.40 ± 0.33, 2.94 ± 0.18, and 2.81 ± 0.16 for 780, 750, and 711 nm, respectively. Clearly, these results show within the experimental error a three-photon nature of CPD damage at longer wavelengths. However, the decline in slope value of power dependence with shorter wavelengths indicates a probable sub-third-order behaviour at 711 nm. In fact, if shorter wavelengths gave rise to two-photon absorption corresponding to UVA absorption band, the total CPD damage could have resulted from a superposition of the second and the third-order events.

In order to independently verify the finding that CPD lesions may be induced by simultaneous combination of two- and three- photon absorption below 780 nm, the dependence of CPD damage on pulse width of excitation laser at the wavelength of 750 nm and constant intensity was investigated.

For n-photon absorption process, the number of photons absorbed per molecule (n_a) is given by Equation 1 [46].
\[ n_{\text{a}} \approx \frac{P^n \sigma_{n-\text{ph}}}{f_{\text{rep}}^{(n-1)} \tau^{(n-1)}} \left( \frac{N A^2}{2 h c \lambda} \right)^n \]  

Here \( n \) equals 2 and 3 for two- and three-photon absorption, respectively, \( P \) is the average excitation power, \( \sigma_{n-\text{ph}} \) is the multiphoton absorption cross-section (in context of this discussion corresponds to CPD-inducing transitions), \( f_{\text{rep}} \) is laser repetition rate, \( \tau \) is the excitation pulse width, \( \lambda \) is the excitation wavelength, \( c \) is the speed of light, \( h \) is Planck’s constant. It can be noted here that two-photon absorption is inversely proportional to the excitation pulse width, while three-photon absorption scales down proportionally to the square of the pulse width. However, for coexisting multiphoton processes one would probably expect an intermediate coupled behaviour.

The pulse widths used for imaging were normally above 100 fs, as shorter pulses get severely broadened by the microscope optics through group velocity dispersion. In this study, CPD damage was recorded at 750 nm with constant intensity of 14 mW while varying pulse width at the focal plane from 164 to 425 fs with a grating pair, and the results were plotted as shown in Figure 3. While the intensity of 14 mW was on the high end of the levels expected for cell imaging, it provided the necessary dynamic range for excitation with 425 fs pulse width. As described above, several spots in the same cell dish were imaged with different pulse widths in order to have the same staining conditions within the data series.

Figure 3 | Pulse width dependence of CPD damage recorded at 750 nm with 14 mW average intensity and 30 μs pixel dwell time.

Using, Eq. (1), the fit of the experimental data yielded the exponential value \((n-1)\) of 1.76 ± 0.18 confirmed the supposition that damage may arise from a mixture of two- and three-photon.
absorption. This dependence suggests that at longer pulse widths, contribution from two-photon absorption increases while reduced photon density makes three-photon absorption less efficient.

A rapid decrease of induced CPD damage was evident between 164 and 200 fs, while this trend slowed down as excitation pulse was broadened further. Earlier, König et al. reported the evidence of a strong effect of excitation pulse width at 780 nm on CPD damage [47]. However, the damage was shown as purely two-photon following the \( (P^2/\tau) \) relation. The discrepancy between the findings of this study and the study by König et al. could be due to the different methods of damage assessment. While the data from this study accounted for total induced CPD damage based on immediate immuno-fluorescent assay, Konig et al. estimated residual damage by monitoring cell-cloning efficiency for 48 hours after the exposure to laser. The latter approach obviously includes not only the direct damage, but also the subsequent cellular response (repair and apoptosis). Therefore it cannot be indicative of the actual order of absorption and the physics involved. On the other hand, if the two findings were considered together – (a) second- and third-order dependence of direct CPD damage and (b) second order dependence of cloning efficiency on laser intensity at 780 nm, one can speculate that stronger initial damage has sublinear effect on cell viability.

The pulse width dependence investigated here has implications for \textit{in vivo} imaging with ultra-short pulses, such as two-photon microscopy with pulse widths as low as 12 fs [48]. On one hand, decreasing the pulse width translates into linear increase of TPEF intensity. At the same time, as demonstrated here, the CPD damage increases as \( \tau^{1.76} \) (on average), and proportionally to the subcubic peak intensity under the typically used imaging conditions. For much shorter pulses, the damage will be caused by almost 100% third-order absorption overshadowing the gain in fluorescence efficiency. The increased damage can only be offset by the corresponding decrease in the average excitation intensity in order to maintain the same peak intensity of the pulses. However, this damage cannot be compensated for without loss of intensity and, consequently, image quality.

Having demonstrated the superposition of competing two- and three-photon processes, their relative contributions were decoupled by refitting the data from Figure 2 with Equation 2 given below:

\[
CPD \sim A_0 + A_2 I^2 + A_3 I^3
\]  

(2)

Here \( A_0 \) was the background CPD level in non-exposed cells, \( I \) was the damaging intensity, while \( A_2 \) and \( A_3 \) were the fitting parameters. The results were plotted in Figure 4 and listed in Table 1.
Carcinogenic damage induced to DNA by fs laser pulses via 2- & 3-photon absorption during NLOI

Figure 4 | CPD immunofluorescence signal dependence on peak intensity of fs laser. The fits to Eq. (2) \( \text{CPD} \sim A_0 + A_2 I^2 + A_3 I^3 \) were used to decouple the relative contributions of two- and three-photon absorption to CPD damage. Pixel dwell time: 30 μs, pulsewidth at the focal plane: 164 fs.

Table 1 | Results of fitting the experimental data of CPD damage dependence on peak intensity.

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>A0</th>
<th>A2</th>
<th>A3</th>
<th>R-Squared value</th>
</tr>
</thead>
<tbody>
<tr>
<td>711 nm</td>
<td>63</td>
<td>886 ± 655</td>
<td>4772 ± 927</td>
<td>0.9717</td>
</tr>
<tr>
<td>750 nm</td>
<td>40</td>
<td>126 ± 433</td>
<td>3663 ± 624</td>
<td>0.9769</td>
</tr>
<tr>
<td>780 nm</td>
<td>130</td>
<td>0 ± 470</td>
<td>1599 ± 589</td>
<td>0.9422</td>
</tr>
</tbody>
</table>

The R-squared values were not significantly different from those of the earlier fits plotted in Figure 2, and fall between 0.942 and 0.977. However, this simple model now shows relative contributions of two- and three-photon absorption (UVA- and UVC like CPD damage, respectively), where the latter dominated at all wavelengths investigated, even at 711 nm. Moreover, the fits estimated how this distribution changed with increasing intensity, eventually becoming almost exclusively third order. The data showed that at 711 nm, the share of two-photon process fell from 38% to 16% as peak intensity increased from 0.3 to 1 TW/cm². A weak but non-negligible contribution of second order absorption was observed at 750 nm (decreased from 10% to 3% over the same intensity range), while only three-photon absorption was observed at 780 nm. In fact, the 780 nm data series exhibits better conversion when fitted with only third-order term.
4.3.2 | Wavelength Dependence

The relative contributions of two- and three-photon absorption were further investigated by assessing the spectral response of CPD damage. A strong dependence of induced CPD damage on excitation wavelength was found, as can be seen in Figure 5. For this experiment, only the wavelength was varied, while intensity and pulse width were maintained constant. The damage level was found to be low for wavelengths longer than 780 nm, where the absorption is purely three-photon according to the intensity dependence discussed above. In contrast, a moderate increase in damage with shorter wavelengths was observed for the 780 to 750 nm range, followed by a dramatic threefold rise of CDP formation over 750 to 710 nm range. The factors that must be considered for the explanation of this trend act oppositely. On one hand, one-photon DNA absorption is considerably stronger at UVC range (peaking between 255 and 260 nm) than at UVA. This explains the eightfold higher CPD damage produced by UVC lamp as compared with that from UVA lamp in the positive control experiment performed in this study. On the other hand, compared with three-photon absorption, two-photon process has at least an order of magnitude higher probability and occurs over a larger focal volume. It is possible that at longer wavelengths, the absorption bands of endogenous cellular fluorophores were excited primarily. However, when the excitation wavelength becomes shorter (< 750 nm) to access the low UVA band, the two-photon absorption could become an important and efficient mechanism of CPD damage, although with a lower efficiency than the three-photon effect.

Figure 5 | Spectral dependence of CPD damage production. Pixel dwell time: 30 μs, pulse width at the focal plane: 164 fs.

It can also be seen that there is excessive CPD formation at 695 nm, where the data has the largest error bars. In fact, cells were found to detach quickly following exposure at 695 nm and prior to formaldehyde fixation. The cell loss was so high that measurement of excitation power
dependence at this wavelength was not feasible, as it required successive irradiation of several spots with 695-nm wavelength. Here it can be assumed that the laser easily excited UVA and UVC absorption bands with high efficiency. It can be hypothesised that the cumulative absorption at 695 nm was so strong that cell necrosis and/or apoptosis mechanisms were triggered immediately leading to cell detachment. Fortunately, for \textit{in vivo} imaging, all endogenous fluorophores can still be excited above 750 nm, so the extreme levels of CPD damage observed at shorter wavelengths does not present additional practical concerns.

The results therefore suggests that imaging with wavelengths above 1000 nm would dramatically, if not completely, reduce the CPD carcinogenic risk since only the long-wavelength edge of UVA absorption band might be excited with three-photon absorption.

\textbf{4.3.3 | Effect of pixel dwell time}

A linear dependence of CPD damage on pixel dwell time was observed in this study experiments, as constant peak intensity was maintained at 750 nm (Figure 6). The number of multiphoton absorption events in DNA was therefore directly proportional to the pixel dwell time. There was an apparent saturation of CPD formation at exposure time of 40 μs. This could be due to complete DNA dimerisation and/or acute effects that cause cells detachment and loss prior to fixation and staining.

![Figure 6](image)

\textit{Figure 6 | Effect of pixel dwell time on induction of CPD lesions.}

\textbf{4.3.4 | Considerations for Tissue Imaging}

The main finding of this study was that the CPD damage was induced by a combination of two- and three-photon absorption processes, where the relative contributions were dependent on imaging parameters. The conclusions were made based on DNA absorption properties within
one-pixel resolution. When translating these findings into the highly scattering environment of biological tissues, attenuation of excitation as well as defocussing must be considered. The attenuation will reduce average excitation intensity, while defocussing will further decrease the peak excitation intensity at the focal volume. Additionally, the relative volumes that are subject to significant two and three-photon fluorescence (based on $1/e^2$ intensity profile) will change. However, as can be estimated [49] with focused Gaussian beam approximation, the volume changes alone does not significantly alter the total CPD production over 200 μm depth in human skin. Finally, it can be expected that the scattering effectively affects these imaging parameters resulting in the corresponding combination of two- and three-photon CPD damage within the exposed focal volume.

4.4 | Conclusion

This paper demonstrated for the first time that the CPD damage of the cellular DNA induced during imaging with fs NIR laser could result from concurrent two- and three-photon absorption. Effectively, these processes correspond to damage caused by UVA and UVC irradiation, respectively. The third-order absorption was found to be the dominant mechanism, where its contribution increased with longer NIR wavelength and higher peak intensity, while it decreased with pulse width. At all wavelengths required for practical in vivo tissue imaging the third-order absorption (UVC-like damage) was found to be responsible for more than 85% of the CPDs induced. However, as discussed earlier, the mechanisms and implications of the CPD damage caused by absorption in the UVA band are still debated in the literature, as they might be different from those at the UVC band and might include a combination of direct absorption by DNA and damage by intermediate reactive species. Therefore, for a thorough evaluation of any risks involved during in vivo NLOI using TPEF, both mechanisms of CPD formation must be considered separately with decoupled relative contributions and according to their carcinogenic potential. Finally, post-exposure cellular responses (repair and apoptosis) to CPD lesions with respect to different proportion of UVA- and UVC- like damage warrant further investigation, preferably in vivo, where various cell types must also be studied.
References


