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

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Chapter 5

Estimating the risk of squamous cell cancer induction in skin following nonlinear optical imaging

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From

Abstract

High power femtosecond (fs) laser pulses used for *in vivo* nonlinear optical imaging (NLOI) can form cyclobutane pyrimidine dimers (CPD) in DNA, which may lead to carcinogenesis via subsequent mutations. Since UV radiation from routine sun exposure is the primary source of CPD lesions, we evaluated the risk of CPD-related cutaneous squamous cell carcinoma due to NLOI relative to that from sun exposure. We developed a unique cancer risk model expanding previously published estimation of risk from exposure to continuous wave (CW) laser. This new model showed that the increase in CPD-related squamous cell carcinoma in skin from NLOI is negligible above that due to regular sun exposure.
5.1 | Introduction

Nonlinear optical imaging (NLOI) based on processes like multiphoton excitation (MPE) and second harmonic generation (SHG), is gaining importance in the field of biomedical imaging, like visualising collagen, elastin and cellular metabolic activity [1-4]. Since NLOI relies on MPE, it is advantageous in that it can use low energy near infrared (NIR) wavelengths to excite absorption bands of bio-molecules, whereas conventional imaging modalities can only do so using short wavelength photons like ultraviolet (UV) and visible light that are highly scattered in tissue. NLOI using NIR photons results in tissue imaging at higher depths and lower photobleaching. Since this process is nonlinearly dependent on the excitation intensity, excitation is limited to the focal area, which excludes out-of-focus excitation signals [1-4].

Despite such advantages, the nonlinear processes needed for NLOI require an extremely high concentration of NIR photons to excite biological fluorophores. With conventional lasers, this could lead to overheating and tissue destruction. However, the high photon fluxes can be conveniently provided by femtosecond (fs) pulsed lasers. These lasers can supply high irradiance in the range of GWcm\(^{-2}\) for transient durations as short as 10\(^{-9}\) to 10\(^{-15}\) seconds within the focal volume. Even so, during an ultra-short pulse duration, high peak irradiance delivered may still damage the irradiated cells by eliciting a variety of undesired biological responses [4-10].

However, the mentioned harmful cellular effects are restricted to the irradiated tissue and its immediate surroundings and do not have long term effects. On the other hand, studies have shown that MPE processes can form DNA UV photoproducts such as cyclobutane pyrimidine dimers (CPDs) [11]. If left unrepaired by the cellular DNA repair mechanisms [12,13], these lesions may eventually lead to carcinogenesis.

CPDs are generally formed by the absorption of single UV photons in the wavelength region of 250 to 400 nm. The corresponding two- or three-photon absorption for NLOI wavelengths (750 – 800 nm) [14] would lie in the UV range, as two- or three-photon processes involved lead to effective absorption of one half and one third of the imaging NIR wavelength respectively. Thus it should then be a major concern that NLOI may trigger cancer by forming CPDs from NIR wavelengths via MPE processes. It is therefore essential to evaluate the safety aspects of NLOI biopsies, with an emphasis on DNA lesions, such as CPDs, that could have long term effects. However, it must be noted that CPDs are also produced routinely in human skin by sunlight as well. The UV component of sunlight is mainly responsible for this [15,16]. An individual is therefore already at a certain risk for skin carcinogenesis, especially cutaneous squamous cell carcinoma (cSCC) due to chronic exposure to sunlight [17,18]. Thus rather than evaluating an absolute carcinogenic risk due to NLOI, it is more sensible to estimate the relative carcinogenic risk of cSCC from NLOI above the risk due to regular sunlight exposure.

In order to estimate the relative carcinogenic risk due to NLOI, we adopted the risk analysis model developed by Sterenborg \textit{et al.} [19]. Sterenborg \textit{et al.}'s model was intended to estimate the additional risk of skin cancer due to occupational exposure to continuous wave (CW) UV
lasers in a worker, above that routine exposure to sunlight UV. While Sterenborg et al’s model dealt with CW UV, in our study we wanted to investigate the risk for cSCC associated with ultra-short pulsed NIR light used in NLOI. In order to modify this model for our study, we therefore needed to know how effective NIR femtosecond laser pulses are at inducing carcinogenic DNA lesions, relative to that of regular CW UV sources. This was assessed by comparing the levels of DNA mutations (CPDs) induced in Chinese Hamster Ovary (CHO) cells in vitro, by pulsed NIR from NLOI with those induced by regular CW UV.

This study thus aimed at providing an estimate of the relative risk for CPD-based SCC arising in skin from NLOI, by utilising an established risk model from literature. The main motive of this study was to see if an annual NLOI biopsy increases the carcinogenesis risk significantly above the existing risk from sunlight exposure. The derived model is unique and the first of its kind because it assesses the risk of CPD related carcinogenesis due to both two- and three-photon effects. In addition, the role of excitation wavelength and laser power on the risk was studied. Furthermore, the model has been designed to present the reader with the carcinogenic risk attributable to NLOI biopsies for both realistic as well as worst-case scenarios.

5.2 | Materials and methods

5.2.1 | Cell culture
CHO cells were cultured in flasks containing Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Breda, the Netherlands) supplemented with 7.5% fetal calf serum (Invitrogen, Breda, The Netherlands), 2mM 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. These Petri dishes had a built-in glass cover slip of 0.16 to 0.19 mm thickness and 20 mm diameter at their centre for optimal laser irradiation (MatTek, Massachusetts, U.S.A.). These cover glass slips were demarcated into 16 squares, using water resistant ink. The positions of these squares were noted to identify the spot of irradiation after immuno-fluorescent staining. The cells were incubated for another day under the above conditions to obtain a confluent monolayer of cells.

5.2.2 | Fs-laser and UV irradiation
The laser beam used in this study was from a compact mode-locked Ti:Sapphire laser (Chameleon model, Coherent, California, U.S.A.) and coupled to an inverted confocal laser scanning microscope (C1, Nikon, Tokyo, Japan) equipped with a water immersion objective (Plan Neofluar 40X, N.A. 0.8, water). The laser was operated at 80-MHz pulse repetition frequency and produced 175 fs output pulses. A half-waveplate (Thorlabs, New Jersey, U.S.A.) and a polarising cube were used to attenuate the laser power. Pulse width of the excitation was measured at the focal plane with an autocorrelator (Carpe, APE GmbH, Berlin, Germany).
Monolayers of CHO cells were scanned using the fs laser with a beam spot diameter of 400 nm diameter and pixel dwell time of 30 μs, over a region of 160 × 160 μm at a pixel resolution of 512 × 512. Each CHO cell monolayer (≈ 5 – 6 μm thickness) received 5 such horizontal scans in a z-stack at depth intervals of 1 μm to ensure complete irradiation of cell nuclei. The cell irradiation was performed in select squares of the 16 squares marked in the Petri dish cover slip. The positions of these irradiated regions were observed with respect to the marked squares and noted in a log book. The logbook thus served as a reference to identify zones of irradiated cells and unexposed cells during immunofluorescent analysis.

Cell irradiation was performed at varying laser powers (7 – 20 mW, peak irradiance: 0.3 – 1 TW cm⁻², pulse energy fluence: 175 – 500 J m⁻²), for three different wavelengths – 711 nm, 750 nm and 780 nm. Non-irradiated cells served as an internal negative control for visual comparison. For UV irradiation, the entire surface of the Petri dishes was illuminated under 254 nm (UVC) and 365 nm (UVA) in a UV cabinet (Chromato Vue CC-20, California, U.S.A.). The irradiance of the UV radiation delivered to the cells was measured with a power meter (Gigahertz Optik, Alphen aan den Rijn, the Netherlands). The UV irradiation was performed at increasing doses of 30 J m⁻² – 1035 J m⁻² for UVC and 100 KJ m⁻² – 500 KJ m⁻² for UVA. This was essential to assess if CPD levels were related linearly or otherwise, to the varying UV doses.

5.2.3 | Immunofluorescence Assay for CPD

After irradiation, cells were fixed in the Petri dish for 10 minutes with 4% formalin (Sigma Aldrich, Zwijndrecht, the Netherlands) in phosphate buffered saline (PBS). After fixation, the samples were washed twice with 2 ml of PBS and incubated with 0.5% Triton X-100 in PBS for 5 minutes on ice to permeabilise the cell membranes for antibody penetration. The samples were then treated with 2N HCl to denature cellular DNA, and kept at room temperature for 30 minutes. Then the samples were incubated with 5% albumin (BSA) in PBS for 30 minutes at room temperature to prevent non-specific antibody binding. This step was followed by sample incubation with a monoclonal antibody specific for CPD (Cosmobio, Tokyo, Japan) diluted 1:500 in 5% BSA in PBS for one hour at room temperature. The cells were then incubated with goat derived anti-mouse IgG conjugated with AlexaFluor-594 (Invitrogen, Breda, the Netherlands) diluted 1:100 in 5% BSA in PBS for 30 minutes. The cells were finally treated with 0.05 μg/ml DAPI in PBS for nuclear staining. Every step was followed by washing the cells five times with 2 ml PBS. The samples were dried and mounted with Vectashield anti-fade medium, next the Petri dish was closed and sealed with paraffin tape. The CPD immunofluorescence in the irradiated cells was visualised using a Nikon PCM-200 camera coupled to a fluorescence microscope (Nikon, Tokyo, Japan).

Immunofluorescent 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. The fluorescence quantification process was done as follows: At first, the fluorescence intensity levels seen in non-irradiated cells was measured and denoted as \( F_{\text{unexp}} \). This is primarily due to diffuse non-specific immuno-staining. Fluorescent levels in irradiated cells (\( F_{\text{exp}} \)) were obtained after subtracting \( F_{\text{unexp}} \) from the original intensity levels. The CPD immuno-fluorescence intensity was averaged over at least 20 – 25 CHO cell nuclei per image, for both irradiated and non irradiated cells. The CPD levels quantified correspond to lesions produced from 5 horizontal scans in a z-stack per nucleus. Previously it had been demonstrated that CPD levels increase linearly with pixel dwell time [20], and hence CPD levels increased proportionally with the number of scans. For simplicity it was assumed that DNA is homogenously distributed in the nuclei and CPD levels were accordingly calculated for a single scan. CPD levels per scan were obtained by dividing the total amount of CPDs generated per nucleus by the number of scans received by a nucleus. This then served as an input parameter in the derived risk model.

5.2.4 | Risk Analysis Model and Calculations

5.2.4.1 | Existing Risk Model for continuous wave UV

Sterenborg et al. [19] had estimated the relative risk of skin carcinogenesis due to long term occupational exposure to UV laser emissions as compared to daily sunlight exposure using the following risk model

\[
RR(t) = \left( 1 + \frac{CD_c(t)}{CD_s(t)} \right)^{cp} 
\]

(1)

Here, \( RR(t) \) is the relative risk for carcinogenesis in an individual at a particular age ‘t’, while \( CD_c(t) \) is the cumulative dose of CW UV laser radiation received by the individual up to that age ‘t’. \( CD_s(t) \) is the cumulative dose of regular sunlight exposure radiation received by the individual until age ‘t’, while \( cp \) is a biologic amplification factor valued at 2.3 ± 0.5 [21] for SCC. Thus \( CD_s(t) \) is calculated as follows:

\[
CD_s(t) = \text{Solar(annual)} \times t
\]

(2)

Here Solar (annual) is the annual dose of sunlight radiation received by an indoor worker in Jm\(^{-2}\).

5.2.4.2 | Derivation of model for pulsed NIR in NLOI

In our study, instead of CW UV laser radiation, \( CD_c(t) \) now denotes the cumulative radiation received due to pulsed NIR wavelengths from NLOI biopsies in an individual’s lifetime. So, \( CD_c(t) \) would now be calculated as in Equation 3 below:

\[
CD_c(t) = Q \times k \times N \times \left( t - t_0 \right)
\]

(3)
Here \( Q \) is the effective carcinogenic radiation dose delivered in one fs-laser scan, \( k \) indicates the number of scans per biopsy site, \( N \) denotes the number of biopsies per year and \( t_0 \) equals the patient’s age at the first biopsy. Thus Equation 1 becomes:

\[
RR\,(t) = \left( 1 + \frac{Q \times k \times N \times (t - t_0)}{Solar(annual) \times t} \right)^{cp}
\]

Previously, the effective dose \( Q \) that causes carcinogenesis for continuous broadband wave radiation such as UV was calculated as:

\[
Q = \int S(\lambda)\alpha(\lambda)\,d\lambda
\]

where, \( S(\lambda) \) is the spectral energy distribution of the incident CW radiation and \( \alpha(\lambda) \) is the action spectrum for skin carcinogenesis as defined by Sterenborg \textit{et al.} [19].

Sterenborg \textit{et al.’s} model dealt with carcinogenic risk arising from broad spectrum CW radiation. However in our study, we calculated \( Q \) for pulsed exposure by fs lasers at a fixed excitation wavelength. Therefore, when \( Q \) was calculated, certain points were considered specifically for linking the available epidemiological skin cancer data caused by CW sunlight exposure to the potential cancer risk from pulsed lasers. First, \( S(\lambda) \) is measured at the single imaging NIR wavelength thus negating the need for integration as in the case of a broadband continuous UV source from Sterenborg \textit{et al.’s} model. The action spectrum used for calculating \( Q \) must be obtained for the wavelength that measures half of the imaging wavelength \( \lambda \) to account for effective wavelength absorption involved in a 2-photon process. Similarly, the action spectrum of the wavelength that measures \( 1/3^{rd} \) of \( \lambda \) must be used for a 3-photon process. Thus for \( S(750 \text{ nm}) \), theoretically the value of \( \alpha(375 \text{ nm}) \) and \( \alpha(250 \text{ nm}) \) should be used. However, for the \textit{in vitro} experiment a filtered mercury UV lamp that emitted either only 254 nm or 365 nm was used. But the difference between \( \alpha(250 \text{ nm}) \) and \( \alpha(254 \text{ nm}) \) was negligible, and the same was the case for \( \alpha(375 \text{ nm}) \) and \( \alpha(365 \text{ nm}) \). Thus the effective action spectrum for an MPE process will be denoted as \( \alpha(\lambda/n) \), where \( n = 2 \) or 3 depending on the MPE process.

Second, it has been shown by Denk \textit{et al.} [1] that MPE events are nonlinearly related to the excitation irradiance \( I \) and linearly related to the time \( T \) it is delivered. Thus Equation 5 can be rewritten as below, using the following parameters for intensity delivered from a pulsed wave laser:

\[
Q = I^{\text{fs vscw}}(\lambda) \cdot T \cdot \alpha(\lambda/n) \cdot C_{\text{fs vs cw}\,\lambda/n}
\]

Here, \( S(\lambda) \) is replaced by \( I(\lambda) \) and \( T \), where \( I(\lambda) \) is the irradiance delivered to tissue by the pulsed laser at wavelength \( \lambda \). \( T \) is the duration for which the intensity is delivered. Meanwhile \( C_{\text{fs vs cw}\,\lambda/n} \) is the factor that accounts for the CPD production efficiency of a pulsed radiation of
wavelength $\lambda$ as compared to CW radiation of wavelength $\lambda/2$ or $\lambda/3$; $n$ is 2 or 3 depending on the type of MPE process.

It has been shown in the work of Nadiarnykh et al. [20] that at wavelengths below 780 nm, CPDs produced by pulsed laser irradiation result from a combination of two- and three-photon process, where their relative contributions depend on the user-chosen imaging parameters.. At a fixed wavelength and pulse duration, the relative share of two- and three-photon process in CPD production depends on the irradiance ($I$) delivered to the cell. Equation (6) is thus modified as:

$$Q = \left[ I^2(\lambda) \cdot T \cdot \alpha(\lambda/2) \cdot C_{fs,\lambda/2} \cdot \beta_{2p} \right] + \left[ I^3(\lambda) \cdot T \cdot \alpha(\lambda/3) \cdot C_{fs,\lambda/3} \cdot \beta_{3p} \right]$$

(7)

Here $\beta_{2p}$ is the fraction of CPDs produced by two photon processes, while $\beta_{3p}$ is the same for three photon processes. The values for $\beta$ were calculated from the results by Nadiarnykh et al. [20].

It should be noted that $I(\lambda)$ can be expressed as a product of the following parameters-pulse energy fluence $\Phi$ at the focus, pulse repetition rate $f$ of the laser and pulse duration $\tau$. Meanwhile, $T$ can be denoted in terms of the pixel dwell time $s$. These parameters are user – defined for optimum image quality and resolution. Thus Equation 7 becomes as given below:

$$Q = \left[ \left( \frac{\phi^2(\lambda) \cdot f \cdot s}{\tau} \right) \cdot \alpha(\lambda/2) \cdot C_{fs,\lambda/2} \cdot \beta_{2p} \right] + \left[ \left( \frac{\phi^3(\lambda) \cdot f \cdot s}{\tau^2} \right) \cdot \alpha(\lambda/3) \cdot C_{fs,\lambda/3} \cdot \beta_{3p} \right]$$

(8)

This equation provides a comprehensive description of the parameters that determine the relative risk for skin carcinogenesis from a NIR fs-pulsed laser.

5.2.4.3 | Determining $C_{fs,\lambda/n}$

$C_{fs,\lambda/n}$ is a factor that is crucial to this risk model. It links the damage generated by the femto-second pulses to an equivalent CW UV dose. Determining it involved two steps. Firstly, we evaluated the CPD levels produced per unit irradiance for CW UV as well as fs laser in CHO cells This is necessary in order to actually compare the efficiency of CPD production from a pulsed laser scan as against CW UV irradiation. However, connecting CPD levels generated by fs pulsed lasers to those produced from CW UV is complex. As CPDs produced from fs pulsed laser are a result of MPE, the CPD levels can be affected by various parameters like pulse energy fluence $\Phi$, pulse duration $\tau$, scan time $s$ and fraction of CPDs $\beta$, produced by two- or three-photon, as seen in the work of Nadiarnykh et al.[20]. Meanwhile, CPDs levels produced by CW UV source depends just linearly on the energy dose delivered, since these lesions arise from single photon events.

Secondly, the irradiated tissue volumes by CW UV radiation and NIR fs laser would be different. The UV light is distributed evenly across the exposed tissue area and is strongly attenuated by the tissue. In contrast, the pulsed laser light is concentrated in a small spot in
the focal volume and attenuation is low. In addition, the skin carcinogenesis action spectrum $\alpha(\lambda)$ has been defined at the skin surface by Sterenborg et al. [19], and not at a deeper level in tissue where the fs laser would be focussed. Only 0.03% of surface incident UVC (254 nm) and 19% of surface incident UVA (365 nm) reaches the basal layer (at 70 μm depth) of human skin [22]. On the other hand, as there is negligible absorption for the NIR wavelengths in skin, fs laser light is assumed to reach the skin basal layer at 100% of the incident intensity. The role of scattering of NIR wavelengths is not included for simplicity: for calculation, we use the intensity of excitation delivered to the focal volume. Thus the volume difference of affected DNA between continuous wave and pulsed wave radiation in tissue requires adjustment of the model.

Based on the data from the study of Bruls et al. [22], the attenuation distance $D_{\lambda/n}$ was calculated to be as 9 μm for UVC (254 nm) and 40 μm for UVA (365 nm) in human skin. Attenuation distance $D_{\lambda/n}$ for CW radiation in skin is the distance (skin depth) at which the irradiance drops to 37% of the original incident value at skin surface. Thus $D_{\lambda/n}$ is an important component for determining $s_{\lambda/n}$, which is the tissue volume fraction affected by CW UV. Due to negligible absorption, there is no attenuation component in determination of $\sigma_{\lambda/n}$ which is the tissue volume fraction affected by the fs laser light. Calculation for this volume difference, given by the ratio $\sigma_{fs} / \sigma_{\lambda/n}$, is derived in detail in the supplement section. The ratio $\sigma_{fs} / \sigma_{\lambda/n}$ thus compensates for two factors: (i) the carcinogenesis action spectrum $\alpha(\lambda)$ which was originally defined for CW UV at skin surface can now be applied for deeper layers due to the attenuation distance $D_{\lambda/n}$ component and (ii) it also accounts for the difference in tissue volume affected by CPD induced by CW UV and fs laser irradiation.

$C_{fs vs cw \lambda/n}$ is described by:

$$C_{fs vs cw \lambda/n} = \frac{\frac{CPD_{fs,\lambda} \times \beta_{n,exp}}{\phi_{exp} \cdot f_{exp} \cdot s_{exp} \cdot \tau_{exp}^{-1}}}{\frac{CPD_{cw(\lambda/n)}}{Dose_{cw(\lambda/n)}}} \times \frac{\sigma_{fs}}{\sigma_{\lambda/n}}$$

(9)

where $CPD_{fs,\lambda}$ denotes CPD levels produced by a single scan from a fs laser, while $CPD_{cw \lambda/n}$ denotes the CPD levels from UV irradiation of a CHO cells monolayer in vitro. CPD levels were measured in terms of fluorescent intensity averaged per pixel from the CPD immuno-fluorescence assay images. For fs laser, CPD levels were normalised to the in vitro experimental parameters: the pulse fluence at the focus $\Phi_{exp}$, the laser pulse repetition rate $f_{exp}$, the pulse duration $\tau_{exp}$ and the pixel dwell time $s_{exp}$. $\beta_{n,exp}$ indicates the fraction of CPD generated from 2 or 3 photon process at the experimental pulse fluence $\Phi_{exp}$ where $n = 2$ or 3. CPDs arising from CW UV irradiation were simply normalised to $Dose_{cw \lambda/n}$, i.e., the experimental CW UV dose
delivered to CHO cells in Jm². $\sigma_{fs}$ and $\sigma_{\lambda/n}$ represent the tissue volumes affected by the fs laser and CW UV respectively.

5.2.4.4 | Calculation of $\sigma_{fs}$ and $\sigma_{\lambda/n}$

When using a NIR ultra-short or fs pulsed laser to scan through a tissue to a depth D, the deeper scans receive lower irradiance level. This is because the NIR radiations commonly used for NLOI undergo attenuation in tissue, mainly due to optical scattering. To counter this efficiency loss with depth, the laser power is commonly increased with increasing tissue or skin depth to get images of optimal quality. For this model, it is assumed that laser power is increased with increasing imaging depth.

Figure 1 | (a) Irradiance at varying tissue depth for fs-pulsed scans. Note that since laser power is increased for greater depth, the irradiance received by each skin or tissue layer from successive fs pulsed scans stays constant, irrespective of skin depth D. (b) A diagrammatic representation of DNA damage distribution from fs pulsed laser scans in a tissue volume cross section of radius $\omega_0$ and skin depth D. The region shaded in yellow represents the tissue volume section that has received a pulsed laser scan and has resultant CPD formation. The region shaded in orange represent the region outside the scan that is affected by CPD formation as well, at a distance of z (axial coordinate) from the focal plane.

In this model, each scan receives the same peak irradiance, irrespective of imaging depth as seen in Figure 1a. Therefore factors like absorption, scattering and loss of focus for fs pulsed NIR radiation with depth will not be considered for measuring $\sigma_{fs}$ (tissue volume section affected by CPD products due to fs laser irradiation). So with peak irradiance per scan being kept constant, we determine the irradiance variation within a single pulsed laser scan (Figure 1b) using the following Gaussian beam equation:
\[ I_{fs-laser}^n(r, z) = \frac{I_{0\, fs-laser}^n}{(\pi\omega^2(z))^n} \cdot \exp\left(-\frac{2r^2 \cdot n}{\omega^2(z)}\right) \]  

Here \( z \) = axial coordinate, \( r \) = radial coordinate, \( \omega(z) \) = beam spot radius at \( z \) and is calculated as:

\[ \omega^2(z) = \omega_0^2 \left(1 + \left(\frac{\lambda \cdot z}{\pi\omega_0^2}\right)^2\right) \]

By integrating Equation 10 from \( z = -3 \, \mu m \) to \( +3 \, \mu m \), the volume affected by 2- and 3-photon processes for a 6 \( \mu m \) thick CHO cell monolayer is obtained. This accounts for the CPD production in a volume up to a distance of 3 \( \mu m \) above and below the actual irradiated focal plane of a single scan. Next, we estimated the probable volume that would be affected with DNA damage if a scan was performed in skin tissue (for \( z = -20 \, \mu m \) – \( +20 \, \mu m \)). This step was essential to know if the volume affected by CPD formation from a scan is larger in skin tissue as compared to a CHO monolayer. The difference between the tissue volume affected by 2- and 3-photon processes from a single fs laser scan in skin and CHO cell monolayer was minimal (ratio \( \approx 1.02 \) for 2-photon and 1.00 for 3-photon). The variation in volume sections affected by the different NIR wavelengths was negligible. Hence an averaged value of \( \sigma_{fs} \) is assigned for the 3 NIR wavelengths, as shown in Table 1.

<table>
<thead>
<tr>
<th>NIR wavelength</th>
<th>( \sigma_{fs} ) (m(^3)) from 1 pulsed laser scan in CHO cell monolayer ((z = -3 , \mu m , \text{to} , +3 , \mu m))</th>
<th>( \sigma_{fs} ) (m(^3)) from 1 pulsed laser scan in skin tissue ((z = -20 , \mu m , \text{to} , +20 , \mu m))</th>
</tr>
</thead>
<tbody>
<tr>
<td>711 nm</td>
<td>4.06 x 10(^{-13}) 2.26 x 10(^{-13}) 4.14 x 10(^{-13}) 2.26 x 10(^{-13})</td>
<td>4.14 x 10(^{-13}) 2.26 x 10(^{-13})</td>
</tr>
</tbody>
</table>

For a collimated CW of UV radiation, while propagating in tissue or skin of depth \( D \), the irradiance \( I \) decreases roughly exponentially (Figure 2a and 2b). This exponential decrease depends on the attenuation distance \( D_{\lambda_n} \) of the CW UV wavelength. The attenuation distance...
is the distance at which the irradiance drops to 1/e, or 37%, of the initial value due to intrinsic tissue properties such as absorption and scattering.

So, if \( I_{0UV} \) refers to the incident UV irradiance before attenuation in tissue, \( D_{\lambda/n} \) denotes attenuation distance of UV radiation \( \lambda/n \) used, \( D \) stands for skin depth and \( n \) indicates the number of photons involved in process, then the irradiance at depth ‘\( D \)’ is denoted as:

\[
I_{UV}(D) = I_{0UV} e^{-\frac{D}{D_{\lambda/n}}}
\] (12)

Therefore, DNA damage from UV across the full depth of the tissue sample would be proportional to an integral of the UV dose absorbed in each layer. This can be obtained by integrating Equation (12) up to infinity.

\[
\text{Damage}_{cwUV} \propto \int_0^\infty I_{UV}(D) dD = I_{0UV} \int_0^\infty e^{-\frac{D}{D_{\lambda/n}}} dD = I_{0UV} D_{\lambda/n}
\] (13)

The tissue volume at risk of DNA damage from a CW UV was measured for an equivalent radius of \( \omega_0 \) similar to the radius of tissue volume section affected from an fs pulsed laser scan. Thus the tissue volume in which probable DNA damage (CPD production) occur per unit irradiance (\( I_{0UV} = 1 \text{ Wm}^{-2} \)) can be measured as:

\[
\sigma_{cwUV} \approx \pi \omega_0^2 \times D_{\lambda/n}
\] (14)
The values calculated for tissue volume affected by CPD from CW UV is shown in Table 2.

Table 2 | Tissue volume sections ($\sigma_{cw\ UV}$) in which CPD production would occur from CW UV. Beam spot $\omega$ radius = 398 + 20 nm. Attenuation distance measured from the results of Bruls et al. [22].

<table>
<thead>
<tr>
<th>CW UV wavelength</th>
<th>Attenuation Distance (m) of CW UV in human skin</th>
<th>$\sigma_{cw\ UV}$ (m$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>254 nm</td>
<td>9 x 10$^{-6}$</td>
<td>4.91 x 10$^{-18}$</td>
</tr>
<tr>
<td>365 nm</td>
<td>40 x 10$^{-6}$</td>
<td>2.15 x 10$^{-17}$</td>
</tr>
</tbody>
</table>

**2.4.5 | Role of the Area factor $\varepsilon$ in the risk model**

The role of the skin area exposed to the fs laser during a biopsy procedure, as compared to the skin area that is routinely exposed to sunlight, should also be considered in this risk model. This is denoted by the Area factor $\varepsilon$:

$$\varepsilon = Area\ factor = \frac{Area_{(fs\ biopsy + Sun)} \times b}{Area_{(Sun\ only)}}$$  \hspace{1cm} (15)

Here, $Area_{(fs\ biopsy + Sun)}$ represents the area that receives radiation from one laser scan in addition to UV radiation from sun, while $Area_{(Sun\ only)}$ denotes the area that receives radiation only from the sun. And $b$ indicates the number of biopsy sites during a routine diagnostic procedure. An average adult male of body surface area of 1.9 m$^2$, wearing full sleeves shirts, long pants and no hat would have 11% of his surface area exposed to sunlight (4% for hands + 7% for face and neck) [23]. So the solar dose is normally distributed over an average area of $\approx 0.2$ m$^2$. In contrast, the non linear biopsy dose would be typically concentrated over an area of $\approx 2.6 \times 10^{-6}$ m$^2$.

We note that Sterenborg et al.’s [19] work shows the radiant energy when delivered to a smaller surface area increases the risk more strongly. As a high fluence from fs laser is distributed over a small area that also additionally receives solar radiation, there are increased chances of tumour formation in these irradiated tissue areas. This is called the Area factor effect. Putting the Area factor denoted by $\varepsilon$ in Equation 1, it can be rewritten as follows:

$$RR''(t) = 1 - \varepsilon + \varepsilon \left(1 + \frac{CDc(t)}{CDs(t)}\right)^{cp}$$  \hspace{1cm} (16)

Since $\varepsilon$ is in the order of $< 10^{-7}$, much less than 1, Equation 11 can be re-written as:

$$RR''(t) = 1 + \varepsilon \left(1 + \frac{Q \times k \times N \times (t - t_0)}{Solar(annual) \times t}\right)^{cp}$$  \hspace{1cm} (17)
So, from Equation 1 and 4, the relative risk model for skin cancer from NLOI biopsies can finally be written as:

\[
RR''(t) = 1 + e^{\left(1 + \frac{Q \times k \times N \times (t - t_0)}{Solar\text{annual} \times t}\right)^{cp}}
\]

(18)

Here Q can be determined from Equation 8 to Equation 14

5.2.4.6 | Risk calculations

The relative risk of a person receiving NLOI biopsies was evaluated over different scenarios such as (a) after a single NLOI biopsy at the age of 30 years, (b) after 10 annual NLOI biopsies at the age of 40, (c) after a single NLOI biopsy in a lifetime of 80 years and (d) after 40 NLOI biopsies in a lifetime of 80 years.

For each scenario, the risk was evaluated for two pulse energy fluences – 250 and 500 Jm\(^{-2}\). High contrast NLOI images were obtained in our setup using a pulse energy fluence of 250 Jm\(^{-2}\), whereas cell detachment was noticed above 500 Jm\(^{-2}\). Risk evaluations were carried out at three NIR wavelengths – 711 nm, 750 nm and 780 nm, realistic wavelengths in NLOI. The risk evaluation was also calculated for differing biopsy regimes by varying the number of biopsy sites ‘b’ and the number of scans per biopsy sites ‘k’. The parameters \(\Phi_{exp}, f_{exp}, \tau_{exp}\) and \(s_{exp}\) correspond to our experimental laser setup.

The yearly dose of sunlight (S) measured using UV dosimeter at the wrists of Danish indoor workers on an annual basis was found to be 13.2 SED/year. Taking into account the additional UV radiation incident on the face, Thieden et al. [24] estimated the annual UV dose received by the indoor worker to be 26.4 SED, i.e. 26,400 Jm\(^{-2}\). This value served as the CDs(t) in our risk calculation.

5.3 | Results

5.3.1 | Immuno-fluorescence Assay for CPD

In Figure 3a, it can be seen that CPD fluorescence intensity exhibited linear dependence on UVA dose. It should be noted that on extrapolation, the graphical linear fit indicates no CPD fluorescence at UVA dose of 63 KJm\(^{-2}\). On the other hand as seen in Figure 3b, CPD production for UVC stayed linear till 250 Jm\(^{-2}\) and partial saturation is evident above this energy dose.

The UVC:UVA ratio obtained in the UV dose calibration experiments were compared with the ratio found to be \(10^{0.45}:1\) in the study of Matsunaga et al. [25]. A consistent UVC: UVA ratio of \(10^{4.1}:1\) was obtained for UVC dose up to 250 Jm\(^{-2}\) and the ratio dropped to \(10^{2.25}:1\) at 1035 Jm\(^{-2}\). Therefore CPD fluorescence intensity values for UVC were considered only up to 250 Jm\(^{-2}\), for calibration of continuous UV against fs laser in the risk model, as the UVC:UVA ratio was in agreement with the mentioned study of Matsunaga et al. only till that particular dose.
Estimating the risk of squamous cell carcinoma induction in skin following NLOI

Figure 3 | (a) Levels of CPDs vs irradiance (Jm⁻²) of (a) UVA – 365 nm and (b) UVC – 254 nm based on fluorescent intensity from CPD immuno-fluorescence assay.

In Figure 4, difference in CPD immunofluorescence staining intensity for UVA, UVC and fs laser irradiated CHO cells can be observed visually. The CPD levels per unit irradiance, quantified based on CPD immunofluorescence intensity averaged per pixel over 20 – 25 CHO cell nuclei, has been plotted on a logarithmic scale as shown in Figure 5. It can be seen that the CPD levels per unit irradiance in UVC irradiated cells were about 10⁴ folds higher than those in cells exposed to UVA. The CPD levels per unit irradiance measured in fs laser irradiated cells were about 10 – 30 folds lower than that of UVC irradiated cells. Among the different NIR wavelengths, the CPD levels produced were the highest at 711 nm, as compared to 750 and 780 nm. It should be noted, however, that CPDs were not detectable below a pulse energy fluence of 175 Jm⁻² (peak irradiance: 0.35 TWcm⁻², average power at cell monolayer ≈ 7mW) in our experimental setup.

Figure 4 | Immuno-fluorescence images of CPD formation at 40X magnification in the nuclear DNA of CHO cells that were (a) unexposed, (b) exposed to UVA (365 nm) at 300 KJm⁻², (c) exposed to UVC (254 nm) at 250 Jm⁻² or exposed to 5 scans of femto-second laser irradiation by wavelengths (d) 711 nm, (e) 750 nm and (f) 780 nm at pulse energy fluence 500 Jm⁻².
5.3.2 Relative Risk Calculations for Nonlinear Optical Biopsy

The determined values for the CPD production efficiency factor \( (C_{\text{fs vs cw} \lambda/n}) \), tissue volume section affected by UV and pulsed laser irradiation \( (\sigma_{\text{cw UV}} \text{ and } \sigma_{\text{fs}}) \) and fraction of two- and three-photon \( (\beta-2p, \beta-3p) \) are listed in table 3.1, 3.2 and 3.3. Action spectrum for carcinogenesis \( (\alpha(\lambda)) \) was acquired from Sterenborg et al. [19] \( (\alpha(365 \text{ nm}) = 0.00029, \alpha(254 \text{ nm}) = 0.156) \).

Calculating \( \sigma_{\text{cw UV}} \) required determining attenuation distance \( D_{\lambda/n} \), for continuous UV of 254 and 365 nm in skin. This was obtained from the results of Bruls et al. [22]. \( (D_{254 \text{ nm}} \approx 9 \times 10^{-6} \text{ m}, D_{365 \text{ nm}} \approx 40 \times 10^{-6} \text{ m}) \).

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>( C_{\text{fs vs 365nm}} \text{ (Wm}^{-2}) )</th>
<th>( C_{\text{fs vs 254nm}} \text{ (W}^2\text{m}^{-4}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>711 nm</td>
<td>( 2.41 \pm 0.26 \times 10^{-13} )</td>
<td>( 1.39 \pm 0.15 \times 10^{-31} )</td>
</tr>
<tr>
<td>750 nm</td>
<td>( 0.35 \pm 0.05 \times 10^{-13} )</td>
<td>( 1.16 \pm 0.17 \times 10^{-31} )</td>
</tr>
<tr>
<td>780 nm</td>
<td>( 0.00 \pm 0.00 \times 10^{-13} )</td>
<td>( 1.06 \pm 0.10 \times 10^{-31} )</td>
</tr>
</tbody>
</table>
Table 3.2 | Tissue volume sections (σcw UV and σfs) in which CPD production would occur from CW UV and pulsed NIR irradiation. Beam spot diameter = 398 ± 20 nm. An averaged value of σfs is assigned for the three NIR wavelengths, due to negligible difference between them.

<table>
<thead>
<tr>
<th>CW UV wavelength</th>
<th>σcw UV (m³)</th>
<th>Pulsed NIR wavelength</th>
<th>σfs (m³) from 1 pulsed laser scan</th>
<th>Section affected by two-photon events</th>
<th>Section affected by three-photon events</th>
</tr>
</thead>
<tbody>
<tr>
<td>254 nm</td>
<td>4.91 × 10⁻¹⁸</td>
<td>711 nm</td>
<td>750 nm</td>
<td>780 nm</td>
<td>4.14 × 10⁻¹³</td>
</tr>
<tr>
<td>365 nm</td>
<td>2.15 × 10⁻¹⁷</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3 | Fraction of CPDs produced by two-photon processes (β2p) and three-photon processes (β3p) for the wavelengths 711 nm, 750 nm and 780 nm at varying peak intensity. Values were determined based on the graphical fits obtained from the results of Nadiarnykh et al. [20].

<table>
<thead>
<tr>
<th>Peak Irradiance TWcm⁻²</th>
<th>711 nm</th>
<th>750 nm</th>
<th>780 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β2p</td>
<td>β3p</td>
<td>β2p</td>
</tr>
<tr>
<td>0.1</td>
<td>0.65</td>
<td>0.35</td>
<td>0.26</td>
</tr>
<tr>
<td>0.2</td>
<td>0.48</td>
<td>0.52</td>
<td>0.15</td>
</tr>
<tr>
<td>0.3</td>
<td>0.38</td>
<td>0.62</td>
<td>0.10</td>
</tr>
<tr>
<td>0.4</td>
<td>0.32</td>
<td>0.68</td>
<td>0.07</td>
</tr>
<tr>
<td>0.5</td>
<td>0.27</td>
<td>0.73</td>
<td>0.06</td>
</tr>
<tr>
<td>0.6</td>
<td>0.23</td>
<td>0.77</td>
<td>0.05</td>
</tr>
<tr>
<td>0.7</td>
<td>0.21</td>
<td>0.79</td>
<td>0.05</td>
</tr>
<tr>
<td>0.8</td>
<td>0.19</td>
<td>0.81</td>
<td>0.04</td>
</tr>
<tr>
<td>0.9</td>
<td>0.17</td>
<td>0.83</td>
<td>0.04</td>
</tr>
<tr>
<td>1.0</td>
<td>0.15</td>
<td>0.85</td>
<td>0.03</td>
</tr>
</tbody>
</table>

5.3.2.1 | Risk variation with NIR wavelength and laser power
As seen in Table 4.1, the relative risk for carcinogenesis slightly drops with longer NIR imaging wavelengths. However, the drop is not significant. The relative risk from a single biopsy at a pulse energy fluence of 250 Jm⁻² was estimated to be the highest for 711 nm at 1.0000061 for cSCC. This means that the NLOI biopsy would increase the existing skin carcinogenic risk of an individual from sun, by 0.00061% for cSCC. When the wavelength is increased, the risk for cSCC falls to 1.0000035 for 750 nm and 1.0000034 for 780 nm. Increasing the laser power for imaging, increases the energy dose delivered to the tissue or cell monolayer. This raises the risk noticeably. As shown in table 4.1, NLOI at a pulse energy fluence of 250 Jm⁻² (average power measured at the cell monolayer ≈ 10 mW, peak irradiance of 0.5 TWcm⁻²) and wavelength of 750 nm, gives a relative risk of 1.0000035 for cSCC in a 30 year old patient. The risk increases
17 folds to 1.000061 when pulse energy fluence was doubled to 500 Jm\(^{-2}\) (average power measured at cell monolayer ≈ 20 mW, peak irradiance of 1 TWcm\(^{-2}\)).

5.3.2.2 | Risk variation with number of biopsies, biopsy regimes and age of the individual

Another factor contributing to the relative risk is the frequency of biopsies performed upon an individual. Table 4.1 shows that the relative risk for cSCC increases from a negligible 1.0000026 for a single biopsy performed at 711 nm and energy fluence 500 Jm\(^{-2}\) in a lifetime of 80 years to 1.035 if 40 NLOI biopsies were performed in the same lifetime.

For a NLOI biopsy regime of 200 scans, if the protocol involved performing 20 scans/site and the number of biopsy sites 'b' = 10, then area factor ε was calculated to be 1.3 × 10\(^{-6}\). In such a scenario, if the protocol was followed at 711 nm and pulse energy fluence 500 Jm\(^{-2}\), the risk for cSCC stands at 1.000097. As seen in Table 4.2, if number of biopsy sites 'b' was increased to 20 and 40 sites, the relative risk increased to 1.00019 and 1.00039 respectively. Choosing more scans per site 'k' lead to higher relative risk for cSCC. The risk went up from 1.000027 for k = 10 to 1.000097 for k = 20 and even further to 1.0004 for k = 40. On the other hand, if total number of scans in regime was kept constant at 200 scans, then the relative risk for cSCC changes accordingly. For 20 biopsy sites and 10 scans per site, the relative risk for cSCC decreases to 1.000055. The risk drops even further to 1.000039 for a biopsy regime of 40 biopsy sites and 5 scans per site. Therefore the number of scans per site appears to have an even more significant role in affecting the risk than the actual number of biopsy sites in this model.

The relative risk of NLOI biopsy is markedly lower at an older age. As seen from Table 4.1, the relative risk of cSCC from a single biopsy performed at the age of 80 years is about 2 – 5 times lower than the same if the biopsy was performed at the age of 30 years.

5.3.2.3 | Relative cancer risk from sun-bathing and outdoor profession

Spending one afternoon on a beach in northern Europe increases the cumulative solar radiation received by about 460 Jm\(^{-2}\), i.e. 4.6 Standard Erythema Dose (SED) [1 SED = 100 Jm\(^{-2}\)], while it increases by 250 Jm\(^{-2}\) (2.5 SED) when spent outdoor elsewhere [24]. If an individual was to spend one such afternoon every year in his or her lifetime of 80 years, the cumulative dosage would correspond to 80 afternoons leading to a relative risk for cSCC at 1.04 for afternoons spent at beaches and 1.02 for afternoons spent outdoors elsewhere. If an individual spends 3 such afternoons a year on average, the cumulative solar radiation received increases for 240 afternoons in lifetime and hence corresponds to a relative risk of cSCC at 1.12 and 1.07. The relative cancer risks for cSCC in an outdoor worker were found to vary in different studies. In the study of Vishvakarman et al. performed in Australia [26], post mail delivery personnel had a relative risk for cSCC as high as 5.5, while physical education teachers had a relative risk of 2.3. Another study conducted by Radespiel-Tröger et al. [27] in Germany, found the relative risk for cSCC to range from 2.5 – 3.6 for an outdoor worker, as compared to an indoor worker.
Table 4.1 | Relative Risk of carcinogenesis from NLOI biopsies compared to UV radiation from sunlight for cutaneous squamous cell carcinoma (cSCC) for a biopsy regime of 20 scans per site at 10 sites. [* - Uncertainty range for risk values: ± 0.028 to ± 0.0000029. The mentioned limits cover the uncertainty range for all risk values in the table].

<table>
<thead>
<tr>
<th>NLOI Biopsy Frequency</th>
<th>Imaging NIR Wavelength</th>
<th>711 nm</th>
<th>750 nm</th>
<th>780 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pulse Energy (in Jm²)</td>
<td>250</td>
<td>500</td>
<td>250</td>
</tr>
<tr>
<td>After 1 Biopsy at age of 30 years</td>
<td>1.0000061</td>
<td>1.000097</td>
<td>1.000035</td>
<td>1.000061</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.000029</td>
<td></td>
<td></td>
</tr>
<tr>
<td>After 10 Biopsies at age of 40 years</td>
<td>1.00016</td>
<td>1.0072</td>
<td>1.000053</td>
<td>1.0042</td>
</tr>
<tr>
<td>After 1 Biopsy in Lifetime of 80 years</td>
<td>1.0000027</td>
<td>1.000017</td>
<td>1.000020</td>
<td>1.000012</td>
</tr>
<tr>
<td>After 40 Biopsies in Lifetime of 80 years</td>
<td>1.00069</td>
<td>1.035</td>
<td>1.00021</td>
<td>1.019</td>
</tr>
</tbody>
</table>

Relative Risk for cSCC at 80 summer afternoons in lifetime of 80 years (1 afternoon/year): 1.041 (at beach) or 1.021 (elsewhere)

Relative Risk for cSCC at 240 summer afternoons in lifetime of 80 years (3 afternoons/year): 1.12 (at beach) or 1.07 (elsewhere)

Relative Risk for cSCC for an outdoor worker: 2.3 – 5.5 (depending on profession and latitude of country)
Table 4.2 | Relative Risk of carcinogenesis from NLOI biopsies for different biopsy regimes at wavelength of 750 nm and energy fluence of 500 Jm\(^{-2}\).

<table>
<thead>
<tr>
<th>Number of scans per site 'k'</th>
<th>Number of biopsy sites 'b'</th>
<th>Total number of scans = 200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of biopsy sites = 10 (constant)</td>
<td>Number of scans per site 'k' = 20 (constant)</td>
<td></td>
</tr>
<tr>
<td>k = 10</td>
<td>b = 10</td>
<td>k = 20 scans/site</td>
</tr>
<tr>
<td>1.000027</td>
<td>1.000097</td>
<td>Total scans = 200</td>
</tr>
<tr>
<td>k = 20</td>
<td>b = 20</td>
<td>k = 10 scans/site</td>
</tr>
<tr>
<td>1.000097</td>
<td>1.00019</td>
<td>Total scans = 200</td>
</tr>
<tr>
<td>k = 40</td>
<td>b = 40</td>
<td>k = 5 scans/site</td>
</tr>
<tr>
<td>1.0004</td>
<td>1.00039</td>
<td>Total scans = 200</td>
</tr>
</tbody>
</table>

5.4 | Discussion

The newly derived risk model in this study indicates that the increase in CPD-induced cSCC risk from NLOI biopsy is insignificant compared to the existing risk from sunlight. The findings of this study are comparable with the results of Fischer et al. [28,29] who performed laser irradiation damage studies on freshly excised human skin. While the risk studies in Fischer’s work were based on comparisons between DNA damage from a fs laser and from a solar simulator, the present study was based on estimating the relative risk of carcinogenesis by uniquely incorporating in vitro data into an established carcinogenic risk model for humans. In addition, it was seen that the risk significantly increases by increasing the NLOI laser power. Other factors, like imaging wavelength, number of biopsies and biopsy regimes were also found to affect the risk in varying degrees.

5.4.1 | Immunofluorescence Assay for CPD

It was observed that CPD fluorescence intensity exhibited linear dependence on UVA dose. The finding that on extrapolation there is no CPD fluorescence at or below the UVA dose of 70 KJm\(^{-2}\), might be an indicator of the sensitivity of the assay. This may mean that the CPDs produced, if any, do not provide a detectable fluorescent signal below this energy dose. On the other hand, when observing the CPD level relation with variation UVC irradiance, partial saturation is evident above the energy dose of 250 Jm\(^{-2}\). A consistent UVC: UVA ratio of 10\(^{4.1}\):1 was obtained for UVC dose up to 250 Jm\(^{-2}\), which was in agreement with the study of Matsunaga et al. [25]. The ratio decreases with UVC irradiance above 250 Jm\(^{-2}\) and drops to 10\(^{1.25}\):1 at 1035 Jm\(^{-2}\). This might be due to saturation of fluorescence signals at the higher UVC doses, due to extremely high amounts of CPDs being formed. Therefore CPD fluorescence
Estimating the risk of squamous cell carcinoma induction in skin following NLOI intensity values for UVC was considered only up to 250 Jm\(^{-2}\), for calibration of CW UV against fs laser in this risk model.

Among other studies that assessed the carcinogenic risk from pulsed lasers, the work of Fischer et al.'s was the most comparable study. In their study, 10 horizontal scans were performed per imaging site at 15 and 30 mW laser power at a wavelength of 750 nm [28]. While their study reported no CPD formation in irradiated human buttock skin at these doses, CPDs were detected when they performed 10 horizontal scans at 5 micron steps using a laser power of 60mW and 150 horizontal scans at 1 micron steps increasing the laser power linearly with depth from 2 to 35 mW. In our study, the samples received 5 horizontal scans in z-stack per CHO monolayer at peak irradiances 0.5 and 1 TWcm\(^{-2}\). The average power delivered at the cell monolayer interface was measured to be about 10 and 20mW respectively. We observed notable CPD formation at both these energy fluences, despite the average power being lower than the laser power of 15 mW and 30 mW used by Fischer et al. This disparity in DNA damage observed can however be attributed to the different biological samples used. Our CPD immunofluorescence data were obtained from in vitro irradiated CHO cells, while Fischer et al.'s experiments were performed on excised human skin. In Fischer et al.'s study, CPDs could be produced only at higher energy fluence, because cells located in stratum basale of skin receive a lower dose of laser radiation, compared to what was actually measured at the sample surface, in their experiment. This loss of laser power could be due to scattering which is the main source of attenuation of NIR excitation in stratum corneum and suprabasal skin layers. This has a strong effect on the level of CPDs produced in deeper skin layers, since the process is sub-third order dependant (exponent of 2.5 – 3), from the study by Nadiarnykh et al. [20]. If irradiance reaching the deeper skin layers decreases by a factor of 2 due to scattering, the CPD levels would reduce by a factor of 5 – 6. Since the CHO cells are directly exposed to radiation, there is no loss of irradiance reaching cell layer from scattering. This fact is however useful to extrapolate our risk model from cells to tissue, as the peak irradiance measured at the CHO cell monolayer would be equivalent to the final peak irradiance received at stratum basale in skin, after events like scattering in the superficial layers.

Another notable difference seen in Fischer et al.'s study is that the risk for DNA damage was estimated by comparing CPD levels obtained from fs-laser samples and control samples exposed to a solar simulator (dose 1.5 MED). The control samples in our studies were irradiated by UVC (254 nm) and UVA (365 nm) lamps. It must be noted that results obtained from our in vitro experiments only compare the efficiency of CPD formation between a pulsed and continuous radiation source. The experimental data by itself is not indicative of the carcinogenic risk and only serves to supplement the derived mathematical model which measures the relative risk.

5.4.2 | Role of sunlight UV and multi-photon processes in the risk model

As researchers are now developing NLOI modalities for clinical applications, it is essential that the safety aspects be examined, especially considering the potential long-term effects of NLOI. Trying to prevent CPD formation by reducing the peak irradiance in the focal plane is not
practically feasible since CPDs have been detected for peak irradiances as low as 250 GWcm\(^{-2}\) [30]. Since the average laser power required for optimal NLOI is typically 3-10 mW, the peak irradiance at the focus is bound to be as high as 0.1 – 1 TWcm\(^{-2}\). Therefore CPD formation is inevitable in any realistic NLOI condition.

Despite this risk, it must be noted that CPD formation can also occur in individuals exposed to UV sources on a regular basis. Therefore it would be more relevant to evaluate the risk of cancer induction from NLOI relative to UV sources. These UV sources are diverse in the clinical field, ranging from the 193 nm ArF excimer laser used for corneal shaping to the 308 nm XeCl excimer laser for coronary angioplasty [19] and the various UV sources used for phototherapy. However, the largest source of UV exposure of the skin is the sun. Sunlight UV can therefore serve as the reference point for NLOI with regard to cancer risk studies in skin, since sunlight UV is already known to cause cSCC via CPDs [16,18,31].

Since NLOI primarily deals with wavelengths in the range from 700 – 800 nm, it is essential to know how CPDs produced from MPE processes with pulsed NIR wavelength light compare with UV action spectra for CPDs. It was previously known that the UVC (230 – 280 nm) and UVB components (280 – 320 nm) of sunlight were mainly responsible for CPD formation and eventual carcinogenesis [15,16]. So a three-photon process from the laser would result in absorption between 233 – 267 nm, lying in UVC region, resulting in CPD production. However, the role of UVA component (320 – 400nm) in forming CPDs has only been recently confirmed [32,33]. Therefore, two-photon processes, with absorption between 350 – 400 nm, can also lead to CPD formation. Though the incidence of CPD formation drops drastically above wavelengths of 290 – 300 nm, as seen in the work of Freeman et al. [34], notable CPD formation is still present up to wavelengths of 370 nm. In the work of Nadiarnykh et al. [20], it has been demonstrated that CPDs produced from NIR irradiation are due to a combination of two- and three-photon processes, rather than isolated events. The findings of that study necessitated the investigation of the risk for CPDs produced by two- and three-photon processes in our analysis.

5.4.3 | Estimating carcinogenic risk increase from nonlinear biopsy relative to sunlight

The relative risk for cSCC from a NLOI biopsy performed at a pulse energy fluence of 250 Jm\(^{-2}\) (peak irradiance of 0.5 TWcm\(^{-2}\)) and wavelength of 711 nm – is almost negligible at 1.0000061. The result implies that NLOI biopsy if performed at the age of 30 years, would increase the existing risk of cSCC from sunlight at the age of 30 years by just 0.00061%. For the two comparable imaging regimes adopted by Fischer et al. [28], the additional risk increase for skin cancer from a lifetime biopsy was found to be 0.045 – 0.06% for a wavelength of 750 nm. Our risk values were lower than those calculated by Fischer because the area factor (ε) was not taken into account for their risk calculation. The role of area factor (ε) was, however, mentioned in their study.

The different laser parameters used for imaging were found to have a significant role in this risk model. When pulse energy fluence was increased from 250 Jm\(^{-2}\) to 500 Jm\(^{-2}\) (peak irradiance
from 0.5 to 1 TWcm\(^2\)), the relative risk grew 15 – 20 fold. In addition, it should be pointed out that good quality NLOI biopsy images could be routinely obtained in our experimental setup at a pulse energy fluence of just 175 Jm\(^2\) (peak irradiance: 0.35 TWcm\(^2\), average power at cell monolayer \(\approx 7\) mW), which did not produce any detectable CPDs. However this could be due to the sensitivity of the immunofluorescent assay used, which might not be able to detect CPDs produced at lower energy fluences. On the other hand, the NIR excitation wavelength also influences cSCC risk. The risk was found to be reduced at higher wavelengths. These findings therefore imply the need for judicious monitoring of the functional laser parameters to at least minimise the production of CPDs and other similar lesions without affecting the quality of NLOI biopsy images.

The frequency of biopsies and type of NLOI biopsy protocol also affects the relative risk for skin cancer. It is understandable that an increase in frequency of biopsies in a year or number of annual NLOI biopsies performed in an individual’s lifetime increases the relative risk for skin carcinogenesis. While the risk from a single NLOI biopsy is negligible, follow up NLOI biopsies in the same individual should be performed after weighing the diagnostic benefit from the biopsy. This is essential to avoid unnecessary NLOI biopsies. Alternatively, increasing the depth distance between successive horizontal scans at a biopsy site could spare the cells in the inter-scan gap from pulsed irradiation and thus CPD production. This might decrease the cancer risk in the tissue volume that has been biopsied.

The biopsy regime also has an important role, where the number of biopsy sites and scans per biopsy site are key factors. The risk for cSCC increases linearly with an increase in the number of biopsy sites. In addition, increasing the number of scans per site could elevate the risk significantly. When the risk calculations considered more biopsy sites and fewer scans per biopsy site to obtain a constant scan total, the relative risk for cSCC dropped significantly. This shows that the risk model is affected even more by the number of scans per site than the number of biopsy sites. The likely explanation could be that the number of scans per site, which is an indicator of energy dose delivered, has a nonlinear effect on the risk. On the other hand the number of biopsy sites, which formulates the area factor \(\varepsilon\) has only a linear effect on the risk. These findings indicate that NLOI biopsies should be performed with a sensible, well planned protocol.

An individual’s age at the time of biopsy can also affect this risk model calculation. The relative risk for cSCC, when assessed for the present age of the individual is found to be higher, if the first biopsy is done at a younger age, e.g. 30 years, as compared to an older age e.g. 80 years. This simply means that the increase in the existing risk of cSCC for a 30 year old from NLOI biopsy is higher as compared to the existing risk for an 80 year old. This however does not mean that the absolute risk from NLOI biopsy is lower at an older age. It only implies that additional risk from NLOI biopsies at an older age is insignificant compared to the existing carcinogenic risk an individual would have acquired from sunlight itself every passing year. Therefore, a marginal additional risk makes NLOI biopsy a relatively safe procedure for monitoring skin lesions in older patients.
In a worst case scenario, if 40 NLOI biopsies at a wavelength of 711 nm and pulse energy fluence as high as 500 Jm\(^{-2}\) (peak intensity: 1 TWcm\(^{-2}\), average power at cell monolayer ≈ 20mW) were performed in a lifetime of 80 years, the relative risk for an indoor worker would stand at a notable 1.035 for cSCC. However, to get a better perspective on the severity of cSCC risk from NLOI biopsy in a worst case scenario, it is important to assess the cSCC risk from sun exposure itself.

Thieden et al. [24] showed that risk increasing activities like sunbathing or exposing the shoulder or upper body to sun during the summer, results in an adult receiving a median UV radiation dose of 4.6 SED per day in a northern European beach and 2.5 SED outside the beach. Such behaviour corresponds to indoor workers receiving an additional dose of 460 Jm\(^{-2}\) at the beach and 250 Jm\(^{-2}\) outdoor elsewhere on a yearly basis. According to this risk model, if an indoor worker spent one such afternoon every year in a lifetime of 80 years, i.e. 80 afternoons, the lifetime relative risk for cSCC would be 1.04 for afternoons spent at beaches and 1.02 for afternoons at outdoors elsewhere. An indoor worker who on average spends three such afternoons outdoor every year, i.e. 240 afternoons in a lifetime of 80 years, would thus have a higher risk for cSCC at 1.12 for afternoons spent at beaches and 1.07 for afternoons at outdoors elsewhere. So sunbathing at a beach for one day every year has a higher lifetime relative risk for cSCC than having 40 NLOI biopsies at a peak energy fluence of 500 Jm\(^{-2}\) in lifetime. Outdoor workers on the other hand, have an even higher relative risk for cSCC ranging from 2.3 – 5.5, depending on outdoor profession and latitude of country [26,27]. Thus the risks for cSCC from such circumstances are much higher than that from NLOI.

5.4.4 | Absolute Risk from NLOI Biopsies

As the incidence of cSCC is negligible at the younger age of 30, no data was found with regard to incidence and absolute risk of cSCC in that age group. In the study of Hollestein et al. conducted in the Netherlands [35], the absolute risks for cSCC were found to be 520 per 100,000 in males and 231 per 100,000 in females, at or above the age of 80 years. So the absolute risk for cSCC from a single NLOI biopsy at 711 nm and pulse energy fluence of 500 Jm\(^{-2}\), at the age of 80 years was calculated to be 520.0089 for males and 231.0040 for females. This means that the absolute risk for cSCC from a single NLOI biopsy increases by just .0089 and .0040 per 100,000 for males and females in that age group respectively. The absolute risk for cSCC increases from 520 to 538.2 per 100,000 for males and from 231 to 239 per 100,000 for females, if 40 NLOI biopsies are performed in the individual’s lifetime.

It must also be noted that the present model calculated the risk for cSCC from NLOI in regularly sun-exposed skin areas and not in unexposed skin areas like buttocks or trunk. However it should be noted that the body areas not being exposed to the sun earlier, also receive UV radiation with the current popularity of indoor tanning and sunbathing [24,36]. This is evident from the study of Hollestein et al. [35] that cSCC can also occur in the trunk and have an absolute risk of 2 per 100,000. But this is still low as compared to 11.9 per 100,000 for face. However, if the present relative risk model were to be applied for an area not regularly
exposed to the sun like trunk, the relative risk of cSCC from NLOI would increase considerably higher in such a skin region, due to a lower cumulative UV received. But since the occurrence or absolute risk of cSCC in such a skin region is negligible, the absolute risk from NLOI biopsy would be quite low, despite the elevated relative risk.

5.4.5 | Limitations of the model
The unique model derived in this study aims to provide a reliable estimate of the potential skin cancer risk from NLOI relative to UV sources. The following limitations of this model however require further investigation.

The risk analysis has been restricted to cSCC in this study, as the etiology of cutaneous basal cell carcinoma (cBCC) and cutaneous malignant melanoma (cMM) is more complex. The review of English et al. [17] and the study of Fartasch et al. [37] indicate that there is a positive correlation between sun-exposure and incidence of cBCC. However, the study points out that the degree of correlation is lower than for cSCC. The past history of sunburns and the degree of sun exposure in early life has an additional role in the etiology of cBCC [38]. On the other hand, there is no quantitative model for the relationship between melanomas and sun exposure in which sunburns are taken into account. Therefore the risk model presented in this study, which is based on cumulative sun exposure in lifetime, would not be an apt predictor for cBCC and cMM risk estimation. Nonetheless, it is important to investigate the risk of cBCC and cMM from NLOI biopsy.

Secondly, it must be borne in mind that these risks apply mainly to individuals with the Caucasian skin phototype, i.e. Fitzpatrick Skin Type I-III, who are already at a higher carcinogenic risk from sunlight UV. This might not be the case for Type IV-VI individuals who are relatively protected from sunlight by the presence of melanin. The relative risk from NLOI skin biopsy might thus be higher for these individuals as their risk to exposure to sunlight is smaller. In addition due to the light absorption by melanin, NLOI may require higher laser powers in these individuals.

It must also be noted that we cannot fully overlook the role of DNA lesions other than CPDs such as oxidative lesions (8-oxo-guanine), DNA single strand breaks, and DNA double strand breaks in causing mutations. Since these lesions are also formed in the UVA range, mainly at 360-400 nm[15], more insight is also needed for the carcinogenesis risk from these lesions as well, especially in case of two-photon processes from imaging wavelengths between 720 – 800 nm. In addition, three-photon processes for wavelengths above 800 nm at high peak intensity can produce CPDs as well, as these MPE processes would be equivalent for UVB irradiation lying between 280 – 320 nm. Thus the potential long term effects of deeper penetrating wavelength above 800 nm should be considered.

The possibility of a four-photon process must also be investigated. In the present wavelength range of 700 – 800 nm, effective four-photon absorption would lie between 175 – 200 nm, where CPDs could be formed according to the study of Hieda et al. [39]. However extremely high photon flux would be needed to necessitate a four-photon process that could induce CPDs in DNA and the cancer induction risk has to be considered in such a scenario.
The current risk model is applicable only to skin since this is the only organ for which there exist established epidemiological data. Since in vivo NLOI has immense potential for visceral organs, a different model must be developed to evaluate the relative carcinogenesis risk in these organs. These internal tissues are not usually exposed to UV and there is no epidemiological data on UV induced cancer in these tissues. In fact these organs might be more susceptible to fs-laser or UV like damage due to the lack of any protection mechanisms, unlike skin. Nonetheless, the human cell should be able to efficiently repair CPDs induced by fs laser or UV by nuclear excision repair [31], irrespective of tissue origin. However, it would be interesting to investigate if there is a difference in the reparative capability of fs laser and UV induced DNA damage in both melanin protected skin and unguarded visceral tissue.

5.5 | Conclusion

This new risk model demonstrates that the additional risk of cSCC arising from CPDs in skin due to NLOI is negligible above that from regular exposure to UV radiation in sunlight. The relative risk from 40 or more NLOI biopsies over is notably higher, but this increase is still lower than the risk arising from sunbathing or having an outdoor profession. However, the risk could become significant if the NLOI biopsies are performed without discretion, i.e. by using unnecessarily high energy fluence for imaging, performing too many scans over the same tissue site or carrying out excessive NLOI biopsies. Thus it is necessary to delineate an efficient protocol for NLOI biopsy in the clinic to ensure its efficacy as a diagnostic tool and also minimise possible long-term effects as mentioned in this study. This customisable model is also likely to be useful to clinicians and microscopists who want to arrive at a reasonable estimate of CPD-linked cSCC risk arising from the NLOI regime adopted in their own field of expertise.
References