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

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In vivo nonlinear optical imaging to monitor early microscopic changes in a murine cutaneous squamous cell carcinoma model

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Abstract

Early detection of cutaneous squamous cell carcinoma (cSCC) can enable timely therapeutic and preventive interventions for patients. In this study, in vivo nonlinear optical imaging (NLOI) based on two-photon excitation fluorescence (TPEF) and second harmonic generation (SHG), was used to non-invasively detect microscopic changes occurring in murine skin treated topically with 7,12-dimethylbenz(a)anthracene (DMBA). The optical microscopic findings and the measured TPEF-SHG index show that NLOI was able to clearly detect early cytostructural changes in DMBA treated skin that appeared clinically normal. This suggests that in vivo NLOI could be a non-invasive tool to monitor early signs of cSCC.
7.1 | Introduction

Cutaneous squamous cell carcinoma (cSCC) is the second most common skin cancer occurring in the Caucasian population worldwide after basal cell carcinoma and the global incidence of cSCC has been increasing lately [1-4]. This increase could be attributed due to a) increased ultraviolet (UV) exposure from the sun or tanning beds and b) improved awareness and reporting of incidences [5,6]. The main areas affected by cSCC are the heavily sun exposed regions such as the face, the scalp and neck. Although the mortality is quite low, undiagnosed cSCC can later cause local invasion of adjacent tissue and visible disfigurement [7]. This leads to significant morbidity in cSCC affected population resulting in an increased burden to health care.

Currently diagnosis of cSCC or precursor lesions occurs during routine clinical evaluation, often followed by biopsies taken for standard histopathology. This approach however has the following disadvantages: a) invasive biopsies performed on benign skin lesions do not benefit the patient and provide unnecessary workload for the pathologist and b) due to dependency on the pathologist's report, there is associated delay in diagnosis and potential loss of patient follow-up. This necessitates the need of non-invasive imaging modalities that can be sensitive to early skin cancer changes and provide rapid diagnosis. Since the last two decades, the use of dermatoscopes has improved clinical diagnosis of cSCC [8]. However the dermatoscope fails to visualise the cellular details typically utilised by pathologists to differentiate between benign and malignant lesions. Newer non-invasive modalities for cancer diagnostics have emerged now such as high-frequency ultrasound (HFUS), optical coherence tomography (OCT), confocal microscopy and non-linear optical imaging (NLOI). Of these diagnostic modalities, the optimum image resolution to visualise essential cellular details that may be useful for a pathologist can be provided non-invasively by confocal microscopy and NLOI. However confocal microscopy has certain disadvantages such as a) higher risk of photobleaching and photodamage in samples and b) limited depth penetration while imaging.

On the other hand, NLOI generates subcellular images based on nonlinear interaction of two or more photons with molecules present in the tissue resulting in optical phenomena like a) Two-Photon Excitation Fluorescence (TPEF) arising from endogenous fluorophores present in the tissue, without the need of exogenous dyes or stains [9,10] and b) Second Harmonic Generation (SHG) produced as a result of back scattering of light originating uniquely from collagen [11]. Due to the nonlinear mechanisms involved, fluorescent emission occurs only in the focal volume resulting in 3-D optical tissue sectioning capability with reduced photobleaching and photodamage [12]. In addition, use of longer imaging wavelengths in the near infrared range by NLOI enables deeper sample imaging when compared to confocal microscopy. Due to these merits, NLOI is rapidly emerging as a viable tool for cancer detection in various organs [13-18].

This study aimed to investigate whether NLOI is able to visualise cytostuctural changes in various stages of carcinogenesis in vivo, in hairless mice skin. It was unique as it also evaluated
Chapter 7

7.2 | Materials and Methods

7.2.1 | Animal cancer model
The Animal Research Committee of the Erasmus University, Rotterdam approved the use of 48 six week old female albino hairless mice (SKH1-hr, Charles River, Someren, the Netherlands) in this study. Female mice were chosen for this study, as they were less aggressive than their male counterparts and were thus easier to handle during the cancer induction protocol and the in vivo NLOI procedure. The mice were randomly divided into three groups of 16 each. The first control group received no treatment. The second control group was treated topically with acetone (Sigma Aldrich Chemie, Zwijndrecht, the Netherlands) on skin once a week for 20 weeks. The test group received a similar regimen using 0.15 mg 7, 12-dimethylbenz[a]anthracene (DMBA) (Sigma Aldrich Chemie, Zwijndrecht, the Netherlands) dissolved per ml of acetone [21]. The mice were clinically assessed every week for skin changes and tumour formation since its first DMBA application for a period of 27 weeks. The mice were monitored for 7 more weeks following 20 weekly DMBA applications, as the incidence of malignant neoplasms increased after the 20th week of DMBA application in murine skin cancer models [21,22]. This step was therefore essential as the study aimed to perform NLOI on the complete spectrum of skin carcinogenesis, including the malignant stage of cSCC.

7.2.2 | In vivo NLOI protocol for the murine cSCC model
The NLOI setup used for this study was conducted on a homebuilt imaging system that has been described previously as in Chapter 3 [23, 24]. Two mice from each group were taken for assessment by in vivo NLOI at weeks 4, 7, 11, 14, 17, 20, 23 and 27 after the first DMBA application as shown in Figure 1. Prior to imaging, the mice were anaesthetised using intra-peritoneal injection of a combination of ketamine (75 mg/kg of mouse body weight) and medetomidine (1 mg/kg of mouse body weight) in normal physiological saline solution (Janssen Pharmaceutica, Tilburg, the Netherlands). The skin region of interest to be imaged was pre-marked with black ink for spot identification later. At an 80 MHz pulse repetition frequency for laser, NLOI was performed with an infinity corrected water-immersion objective (40x, Numerical Aperture = 0.8, Nikon, Japan). For imaging, excitation light source of wavelength 765 nm was used and pixel dwell time was fixed at 128 μs. The laser power incident on mice skin surface ranged from 17 – 30 mW for clinically normal skin and higher laser power was used
with increasing tissue depth. While imaging visible tumours, the laser power was increased further to 35 – 45 mW due to increased tissue scattering in the lesions. The imaging regimen for one region involved first performing an axial x-z scan (100 μm × 60 μm). This was followed by a series of transverse x-y scans (100 μm × 100 μm), at the rate of one scan per 3 μm interval extending down to a depth of 60 μm. In vivo NLOI was performed on clinically normal skin or visible tumours occurring near the thigh region of the mice for two reasons: a) respiratory motion artefacts were the least at this site and b) to minimise variation in murine skin from different anatomical regions. Following the imaging procedure, each mouse was euthanised by cervical dislocation. Skin punch biopsies were obtained from the pre-marked region, fixed in 10% formalin and sent for histopathological evaluation by a certified veterinarian pathologist. All experiments were performed in compliance with the relevant laws and institutional guidelines in accordance with the ethical standards of the Declaration of Helsinki.

Figure 1 | A schematic diagram describing the DMBA carcinogenesis protocol and the in vivo NLOI imaging methodology used during this study (DMBA – 7, 12-dimethylbenz(a)anthracene, NLOI – nonlinear optical imaging).

7.2.3 | Data analysis of NLOI experimental data
Due to considerable loss of signal intensity, scans deeper than 48 μm were excluded from data analysis. The recorded image data were corrected for background noise to eliminate background variation with time and converted to RGB (red-green-blue) images as described by Bader et al. [24] and these images were used to analyse the microscopic features. The acquisition and unmixing of spectral information from the image data file was done on the ImageJ software (available at http://imagej.nih.gov/ij/) using a home built ImageJ plug-in that is based on the phasor approach described by Fereidouni et al. [25]. Spectral unmixing yielded the fractional intensities of SHG from collagen and TPEF from epidermal fluorophores, which included keratin, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD). The reference spectral values used for unmixing were obtained from ex vivo rat tail
tendon collagen, 8.1 g/ml aqueous solution of human epidermal keratin, 2 mg/ml FAD in phosphate buffered saline (PBS) solution and 2.5 mM NADH in 1 ml of MOPS buffer solution – pH 7.4 (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands). For each scan, the fractional intensities of SHG (collagen) and TPEF (epidermal cells) were applied to calculate the TPEF – SHG index as shown in Equation 1 below:

\[
\text{TPEF–SHG Index} = \frac{\text{TPEF}}{\text{TPEF} + \text{SHG}}
\]  

Based on the histopathological classification, the index values were averaged for each category and then compared between the different groups. The variation of TPEF-SHG index with skin depth was also evaluated for the different groups. Statistical analysis was performed using two tailed sample-t-tests (unequal variance) and considered significant with a p-value ≤ 0.05.

7.3 | Results

7.3.1 | Histopathological findings of DMBA induced skin carcinogenesis

Based on the initial clinical findings and subsequent histopathological evaluations, the samples were categorised as follows: (i) Normal skin: No microscopic changes related to neoplasia or clinically visible skin changes, (ii) Skin with preclinical changes: Positive microscopic changes of epidermal hyperplasia (acanthosis) with no clinically visible skin changes , (iii) Early clinical lesions: Positive microscopic changes of preneoplasia or neoplasia with presence of clinically visible lesion lesser than 2 mm diameter and (iv) Late or advanced clinical lesions: Positive microscopic changes of neoplasia with presence of clinically visible lesion greater than 2 mm diameter.

Upon clinical examination, the untreated and acetone treated mice remained asymptomatic throughout the experiment, while the DMBA treated mice exhibited the first visible lesion at around 11 weeks after the first DMBA application. Upon histopathological evaluation, normal skin regions were detected from all three groups of mice and categorised together. However murine skin with preclinical changes, early and late clinical lesions were exclusively found in DMBA treated mice group. Skin samples with preclinical changes were identified histopathologically as mild to moderate epidermal hyperplasia (acanthosis) with no atypia (dysplasia). Within the early and late clinical lesions, the lesions had been diagnosed as: (i) squamous cell papilloma (with or without invasion) – 35%, (ii) exophytic and endophytic acanthoma (with or without invasion) – 29%, (iii) keratoacanthoma (with or without invasion) – 20%, (iv) squamous cell carcinoma in-situ (Bowenoid lesion) – 2% and (v) squamous cell carcinoma (well or moderately differentiated) – 14%. The incidence of malignant lesions diagnosed was increased after 20 weeks of DMBA application. Additionally, 14 out of 16 DMBA treated mice exhibited clinical skin lesions that underwent progression to the malignant stage of skin carcinogenesis after 20 weeks of DMBA application.
7.3.2 | Comparison of axial x-z NLOI scans with standard histopathology

The axial x-z scan shown in Figure 2-A2 from normal skin revealed the typical layered architecture of murine skin that is comparable with the corresponding haematoxylin-eosin (H&E) stained section as shown in Figure 2-A3. At the layer of stratum corneum, TPEF from keratin and lipids from dead corneocytes (coloured in green) was visible. This autofluorescence results from the combination of keratin emission peak at 480 nm and lipid emission peak at 540 nm. The underlying stratum spinosum and basale were characterised by TPEF from intracellular NADH that has an emission peak of 460 nm that is shown as autofluorescence (coloured in blue) in epidermal cells. The remaining portion of skin was constituted by the dermis, which was clearly visualised by SHG of dermal collagen (coloured violet). The SHG occurred at an emission wavelength of 382 nm that was approximately half of the imaging wavelength (765 nm) used in this study.

Figure 2 | Clinical photograph of pre-marked region in black ink, the axial x-z nonlinear optical imaging (NLOI) scan and haematoxylin and eosin (H&E) stained histopathology section of murine skin for normal skin (A1-A3), skin with preclinical changes (B1-B3), early clinical lesion (C1-C3) and late clinical lesion (D1-D3) respectively. Axial NLOI scans could show 3 distinct layers in skin – a) stratum corneum with TPEF from keratin and lipids, b) stratum spinosum and basale from TPEF of intracellular NADH and c) dermis from SHG of collagen. The NLOI image has been colour coded to correspond to the wavelengths shown in emission spectra below the NLOI images. H&E stained sections show normal murine skin in A3, epidermal hyperplasia (acanthosis) in B3, severe epidermal hyperplasia with atypia (part of an acanthoma) in C3 and keratinous region in cutaneous squamous cell carcinoma in D3. H&E stained images obtained from biopsy of the imaged spot do not correspond to the exact position of the imaged spot, but is representative of tissue architecture in the imaged area and adjacent skin regions (Pixels scanned per NLOI image: 224 × 224 pixels, Average laser power at focal plane – 17 – 20 mW for normal skin, 25 – 30 mW for skin with preclinical changes and 35 – 45 mW for clinical lesions).
Skin regions with preclinical changes appeared clinically normal as shown in Figure 2-B1. However upon histopathologic evaluation, these regions showed an evident increase in epidermal thickness (Figure 2-B3). This microscopic change was clearly demonstrated in the NLOI x-z scan as seen in Figure 2-B2. In contrast SHG from dermal collagen was visibly diminished as compared to normal skin tissue.

In Figure 2-C3, the H&E stained section of the early clinical lesions (preneoplastic or neoplastic) showed an even greater degree of epidermal proliferation as compared to the skin with preclinical changes. In the corresponding x-z NLOI image, TPEF from epidermal layer constituted the majority of the fluorescence as shown in Figure 2-C2. From this stage of skin carcinogenesis onward, the SHG signal from collagen was not visualised anymore. On the other hand, the late or advanced clinical lesions with diameter over 2 mm were heavily keratinised. In Figure 2-D2, the only visible signal was due to TPEF arising from keratin and dead corneocytes. This is validated by the corresponding H&E stained section in Figure 2-D3 which reveals an abnormal quantity of keratin at the tumour surface.

It should however be duly noted that the precise skin spot evaluated by in vivo axial NLOI (100 μm × 60 μm) could possibly be missed during histopathology slide preparation. Therefore the corresponding H&E stained image may not depict the exact region scanned by in vivo NLOI, but would be representative of murine skin that lie in close range to the imaged spot.

7.3.3 | Microscopic changes in transverse NLOI scans with depth during different stages of skin carcinogenesis

In normal skin, a transverse x-y scan at a depth of 3 μm from surface (shown in Figure 3-A1) revealed the characteristic polygonal shaped corneocytes present in stratum corneum. Further below at 6 μm, TPEF from the spinous cells in stratum spinosum was observed as seen in Figure 3-A2. The cells began to decrease in its size as the scans approached the stratum basale located at about a depth of 12 μm as shown in Figure 3-A3. Below this depth, SHG from dermal collagen predominated for the subsequent transverse x-y scans. Meanwhile in skin with preclinical changes as seen in Figure 3-B1 to B4, TPEF from epidermal cells persisted down to depth of 24 μm, which is twice as deep compared to normal mice skin. Another notable finding was that no change in cytonuclear morphology was observed for skin with preclinical changes. This was validated histopathologically as these skin regions showed no atypia and only displayed epidermal hyperplasia (acanthosis). In addition, SHG from dermal collagen could be visualised only from x-y scans performed deeper than 24 μm as shown in Figure 3-B4 and B5.

In early clinical lesions, the scans revealed a more disorganised stratum corneum as deep as 6 μm (Figure 3-C2), which points to thickening of stratum corneum in these lesions. The subsequent scans demonstrated that presence of TPEF from epidermal cells down to a depth of 48 μm. One key observation in the scans of early clinical lesions was the presence of transformed cytonuclear morphology. This was characterised by presence of increased number of cells with a) irregular and variable cell size, b) cells with enlarged nuclei and c)
actively dividing mitotic cells. These features could be clearly seen from a depth of 12 μm to 48 μm as shown in Figure 3-C3 to C5. This was confirmed histopathologically, as features of abundant mitotic figures and features of atypia were observed in the corresponding H&E stained sections. Another notable feature in these lesions was that dermal collagen SHG could not be detected even from the deepest x-y scan.

Figure 3 | Transverse x-y nonlinear optical imaging (NLOI) scans performed at depths of 3 μm, 6 μm, 12 μm, 24 μm and 48 μm from murine skin surface for normal skin (A1-A5), skin with preclinical changes (B1-B5), early clinical lesions (C1-C5) and late clinical lesions (D1-D5). Cells with enlarged nuclei (marked in dotted white circle) and mitotic cells (marked in continuous yellow circle and arrow) were abundant in the early clinical lesions. (HF-hair follicle, Scale-100 μm × 100 μm, Pixels scanned per NLOI image: 224 × 224 pixels, Average laser power at focal plane – 17 – 20 mW for normal skin, 25 – 30 mW for skin with preclinical changes and 35 – 45 mW for clinical skin lesions).
In late or advanced clinical skin lesions, scans revealed layers of disorganised corneocytes with TPEF from keratin (Figure 3-D1 to D5). Unlike early clinical lesions, the x-y scans performed here could not provide any additional information on cellular details due to the extreme degree of keratinisation. SHG from dermal collagen was notably absent in the large neoplastic lesions as well. It must also be noted that unlike the axial x-z scans, the transverse x-y scans was clearly able to provide finer cytonuclear details for all samples.

7.3.4 | Analysis of TPEF-SHG index and its variation with skin depth

The separation of the TPEF and SHG components of the in vivo NLOI images were performed by using the spectral phasor based approach [25]. In the spectral phasor approach, a sinusoidal wave such as the tissue emission spectrum is converted into a vectorial representation of the same. With this method, the Fourier transformation of the spectrum is calculated and the resulting complex number is plotted on a 2-dimensional plot. By this process, emission spectrum from each pixel in the image is transformed into a unique position on this 2D phasor plot depending on its emission peak or spectral width. By selecting specific regions on this 2D phasor plot that corresponds to a particular emission wavelength or a range of wavelengths, the acquired NLOI image can be unmixed accordingly. The details of the spectral phasor unmixing approach are displayed in Figure 4.

TPEF-SHG index calculated from the unmixed spectral components revealed the normal skin has the lowest index value compared to the rest of the samples. The TPEF-SHG index values rose considerably for the skin with preclinical epidermal hyperplasia. This increase was about three fold when compared to the index values of normal skin. On the other hand, skin regions with preclinical changes had the largest standard deviation when compared to the other groups. The index attained its highest value in the early and late clinical skin lesions. However, there was no observable difference between the TPEF-SHG index values for the early or late clinical skin lesions. The TPEF-SHG variation for the different groups categorised by pathology grading is depicted in Figure 5.
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Figure 4 | The spectral phasor plot displaying the two-photon excitation fluorescence (TPEF) and second harmonic generation (SHG) components, the composite RGB nonlinear optical imaging (NLOI) scan, the unmixed TPEF and SHG intensity images for normal skin (A1-A4), skin with preclinical changes (B1-B4), early clinical skin lesion (C1-C4) and late clinical skin lesion (D1-D4) respectively. When compared to normal skin as seen in A1, the spectral phasor image shows a clear decrease in the contribution of SHG component in B1 for skin with preclinical changes and almost no contribution to SHG component for early and late clinical lesions as seen in C1 and D1. This is clearly reflected in the unmixed SHG intensity images shown from A4 to D4.

Figure 5 | A comparative bar diagram showing the variation of TPEF-SHG index values for mice skin with differing pathology diagnosis. (#: p-value ≤ 0.05 between normal skin and skin with preclinical changes, ^: p-value ≤ 0.05 between skin with preclinical changes and clinical skin lesions, *: p-value ≤ 0.05 between normal skin and early clinical skin lesion, **: p-value ≤ 0.05 between normal skin and late clinical skin lesion).
The TPEF-SHG index values were also measured with increasing depth for normal skin, skin with preclinical changes, early and late clinical skin lesions. It can be seen in Figure 6, that the TPEF-SHG index values dropped sharply and was constant for normal skin from a skin depth of 12 μm onwards. The index values reached its lowest value of around 0.2 for this group at about 18 μm and remained steady thereafter around that range. In stark contrast, the drop in index values for murine skin regions with preclinical changes varied highly from sample to sample. The depth at which the index value started to decrease ranged from 12 μm to 36 μm. Despite the high variability, the index values for skin with preclinical changes stayed consistently above that of normal skin till a skin depth of 45 μm, following which the TPEF-SHG index dropped to its lowest value. In early and late clinical skin lesions, the TPEF-SHG index showed minimal fluctuation from its starting value throughout the imaged skin depth. The values stayed consistently around 0.9 even at depth of 48 μm. No difference was observed between the index values of early and late clinical lesions. From Figure 6, it can also be seen that the TPEF-SHG index values could ideally be used to distinguish normal skin, skin with preclinical changes and early clinical skin lesions at a depth of 18 – 45 μm.

Figure 6 | Graph showing the variation of TPEF-SHG index with increasing murine skin depth for normal skin, skin with preclinical changes (denoted as Preclinical), early clinical skin lesions and late clinical skin lesions. The TPEF-SHG index for skin regions with preclinical changes were plotted individually, due to high variability between the index trend lines in this group.
7.4 | Discussion

7.4.1 | In vivo NLOI for monitoring preclinical stages of skin cancer

The use of TPEF and SHG based NLOI as a useful tool in skin cancer diagnosis has been investigated by various researchers \[26-28\]. Nonetheless the potential of \textit{in vivo} NLOI in evaluating cytostuctural changes during the early stages of cancer is yet to be fully explored. Certain NLOI studies have attempted to monitor early changes in the gastro-intestinal tract \[29\] and ovaries \[30\], while similar investigations in skin cancer have not been adequate. Earlier Wang \textit{et al.} \[31\] had used intra-vital microscopy to conduct an experiment comparable to this present study in a complex window chamber model. In their work, the study was followed till 7 weeks of DMBA applications following which they histopathologically observed malignant squamous cell carcinoma of the skin. However in their study, only multi-photon images performed in normal skin and carcinogen treated skin (with epidermal hyperplasia) were demonstrated. In this study, an attempt was made to provide NLOI images in all stages of carcinogenesis – preclinical (hyperplasia), early clinical (preneoplasia/neoplasia) and late clinical (neoplasia) stages. Furthermore, this study attempted to evaluate preclinical cancer changes in mice skin using axial x-z scans. These axial x-z scans are more analogous to standard H&E slides and would be easier to relate to for the pathologist. In addition, while Wang \textit{et al.} used the complex window chamber murine skin model for their investigation, a simpler non-invasive approach with minimal interference to the imaged skin was used in the present study.

When compared to normal skin, \textit{in vivo} NLOI performed on the skin regions with preclinical changes showed a notable increase in the epidermal thickness. These changes in epidermis are typically due to a) pronounced keratin deposition and elevated corneocyte turn-over that lead to thickened stratum corneum (hyperkeratosis) and b) epidermal proliferation which is characterised by the concurrent increase in the extent of NADH associated fluorescence in deeper epidermal layers in skin with hyperplasia (acanthosis). The significant aspect of these findings was that NLOI could detect these microscopic changes, even as such skin regions appeared clinically normal. This shows that \textit{in vivo} NLOI has immense potential as a non-invasive tool to visualise preclinical changes in skin and other organs prior to visible tumourigenesis. This is particularly important as preclinical skin changes such as epidermal hyperplasia (acanthosis) can be reversed with the necessary preventive or therapeutic measures. For the clinician, this property of NLOI could be used to monitor skin changes in patients at high risk or follow through for recurrence in patients with previous history of skin malignancies. Additionally while precisely excising around the margins of malignant skin lesions during Mohs surgery \[32\], surgeons can utilise \textit{in vivo} NLOI to resect high risk precancerous skin that appears clinically normal to minimise recurrence.

On the other hand, NLOI x-z scans of the more advanced stages of carcinogenesis, i.e. early clinical lesions continued to show extreme epidermal proliferation. The corresponding x-y scans was able to clearly show an increase in number of cells with enlarged nuclei and actively dividing cells throughout the scans. Taken together, this suggests of altered nuclear-
cytoplasmic ratio and increased mitotic activity, which are features characteristically seen in epithelial dysplasias (atypia) and carcinomas. Therefore clinicians could use *in vivo* NLOI to monitor epithelial dysplasias and detect development of carcinoma or neoplasia non-invasively in real-time.

### 7.4.2 TPEF-SHG index as an indicator for epidermal proliferation

Intrinsic TPEF to SHG ratio has served as a quantitative biomarker for carcinogenesis in different organs [19,20,33]. During cancer progression, epidermal proliferation enhances intrinsic TPEF. On the other hand, collagen can be degraded or damaged as a result of epidermal infiltration and invasion, which results in reduced SHG [34]. Depending on the formulation of TPEF and SHG, this ratio or index can fluctuate sensitively during cancer progression.

In this study, it was investigated to see if the TPEF-SHG index, expressed as TPEF/ (TPEF + SHG), was a reliable indicator for skin cancer progression. The results revealed that the TPEF-SHG index rose considerably in the early preclinical stage and then continued to rise for the clinical stage of skin carcinogenesis. This rise is possible due to two reasons: (i) Increased TPEF due to hyper-proliferative epidermis and (ii) Dermal collagen being displaced deeper due to a thicker epidermis, thus resulting in lower SHG detection by NLOI. This may also be the reason for the high index values for the clinical skin lesions. Because of reduced signal-to-noise ratio with increasing depth, the efficiency of the data analysis software to spectrally unmix and detect SHG was considerably decreased beyond 48 μm. Thus it was unlikely for the described setup to detect SHG from dermal collagen that was located very deeply in neoplastic lesions. Due to very low SHG detection for both early and late clinical lesions, the index values stayed as high as 0.9 with no observable difference between the two. It would be interesting to re-evaluate the trend in TPEF-SHG index from early non-keratinised skin lesions to the advanced larger keratinised skin lesions using a NLOI setup that could achieve higher signal-to-noise at greater depths during imaging.

While analysing TPEF-SHG index variation in depth, it was seen that all normal skin regions displayed a uniform drop in the index value from 12 μm onwards. However for early and late clinical skin lesions, the TPEF-SHG index stayed constantly around 0.9 with no drop. As mentioned earlier, this was due to the inability to detect SHG from deeper dermis in these lesions giving minimal SHG and thus elevated index values at all scanning depths for these lesions. On the other hand in skin with preclinical changes, the depth at which the index values started to drop varied from sample to sample. This indicates a high variability for this group as seen in Figure 6. This could be due to higher variation of epidermal thickness or epidermal hyperplasia in this particular group when compared to the normal group. From this analysis, it was seen that there was a clear distinction between these group of skin samples in a diagnostic window that extended between a skin depth of 18 μm and 45 μm. Applying the TPEF-SHG index in this depth range during *in vivo* NLOI could be useful in distinguishing between normal skin, skin with preclinical changes and early clinical changes more clearly. However, this diagnostic window may occur at a different depth range in human skin...
7.4.3 | Present limitations and future implications

The major challenge encountered in this study was depth limitation with a significant loss of signal intensity at depths below 48 μm upon using the described NLOI setup. Therefore it was only possible to image the superficial layers of early and smaller clinical lesions that had an epidermal thickness of around 200 μm. The advanced and larger lesions were even more difficult to image, due to the high degree of keratinisation. This is probably due to increased scattering of signal by the highly keratinised layers of dead cells. Corresponding histology showed that the larger clinical lesions were diagnosed to be mainly keratoacanthomas and squamous cell carcinomas, which were generally associated with extreme keratinisation. Due to the mentioned depth limitation of NLOI, no microscopic changes could be visualised from the proliferative core of these advanced lesions. Evaluation of this proliferative cellular core at the epidermo-dermal junction is needed by the pathologist to establish the invasiveness of a lesion. Since this zone was located at a depth beyond the imaging limit of the described NLOI setup, it was not possible to determine if a lesion was invasive or non-invasive in this study. On the other hand, the inability of in vivo NLOI to non-invasively image advanced or late clinical lesions would not improve or worsen the outcome of the disease. As in such an advanced state, surgical resection of the lesion is inevitable for either histopathological diagnosis or improved patient’s quality of life. In addition, the inability to image deeper is limited only to this particular study as in vivo NLOI has been used successfully in other studies to image as deep as 135 – 320 μm in human skin [27,35]. In those studies, NLOI could be performed at a much greater depth because of the following reasons: a) the numerical aperture (NA) of the objective used by Koenig et al. was 1.3, when compared to 0.8 used in the current study. Objectives with higher NA are more efficient in collecting emission signal and thus can influence the maximum achievable imaging depth, b) Koenig et al. used an oil immersion objective that have a lower refractive index mismatch induced spherical aberrations and hence can collect more autofluorescence signals at increasing depths, compared to the water-immersion objective that was used in this study [36] and c) in the study by Paoli et al., the maximum laser power used at the focal plane for imaging was about 5 times higher than the power employed in this study.

Another prominent aspect that needs to be investigated with regard to clinical feasibility of NLOI is its biosafety aspect and associated DNA damage. Nadiarnykh et al. showed that multiphoton imaging could cause DNA mutations in the form of cyclobutane pyrimidine dimers (CPDs) via a combination of two- and three-photon absorption [37]. The associated carcinogenic risks from such events have been evaluated in the studies by Fischer et al. [38] and Thomas et al. [39]. In the study conducted by Fischer et al., the risk from a typical NLOI biopsy where the average laser power being increased from 2 – 35 mW as NLOI was performed from skin surface to a depth of 150 μm was compared with natural UV exposure. The increase in risk for skin cancer from such a biopsy was found to be just .045% for a lifetime biopsy and 1.35% for an annual biopsy. However this result did not consider the role of skin area being exposed to NLOI or UV radiation. On the other hand, Thomas et al. used a risk model
that also accounted for the small skin area imaged by NLOI compared to the much larger skin area that is exposed to solar UV. The results of that study showed that the additional risk for squamous cell carcinoma of skin in an individual’s lifetime from 40 optical biopsies at 20 mW was much lower than spending one afternoon exposed to solar UV every year. The same study also demonstrated that longer NIR imaging wavelengths, reduced peak powers and quicker scan times reduced the carcinogenic risk even more. However, these cancer risk studies had focussed on solely CPD forms of DNA mutations. Further evaluation of carcinogenic risk associated with other types of DNA mutations such as double strand DNA breaks and oxidative DNA lesions is required.

A notable disadvantage of the axial x-z scans was the poor visualisation of finer details such as cellular cohesiveness and cytonuclear morphology, although a change in the skin layer organisation could be clearly detected. Due to lack of cytonuclear details in the x-z scan, these lesions could not be correlated with its specific histopathological diagnosis. One possible solution to this problem could be to perform a quick x-z scan to identify the suspect region of interest in skin. Following this, finer tissue details could be obtained by performing a series of transverse x-y scans from the region of interest to identify the cytonuclear changes. Therefore the image acquisition time would also play a decisive role for the successful translation of in vivo NLOI to the clinic. An axial x-z scan or a transverse x-y scan in this study took 6.5 seconds (performed over 224 x 224 pixels at 128 μs per pixel). If a routine NLOI biopsy was performed to study finer cytonuclear details as mentioned earlier, it would consist of a stack of 20 transverse x-y scans performed in the x-z direction up to a specific depth at regular intervals. In such a scenario, the process would then take about 2 – 2.5 minutes which is clinically feasible for the patient. However the problems that can arise from imaging time periods this long are mainly motion artefacts. On the other hand, studies are currently attempting to utilise adaptive optics to compensate for motion artefacts during optical imaging [40]

It should also be noted though that by solely using in vivo NLOI, it is extremely difficult to arrive at a specific histopathological diagnosis for a skin lesion or to differentiate one cancer subtype from the other. This is essentially because in vivo NLOI provides very poor intranuclear details at present [18,41]. Nuclear details such as nuclear hyperchromaticity are often needed by the pathologist to determine the dysplasia grade. With the present limitations, the current scope of in vivo NLOI would be to determine the requirement of an invasive biopsy on a suspect region, rather than to provide an accurate pathology diagnosis. However in the near future, it is possible that technological advancements could deliver (a) improved deeper tissue imaging [42,43], (b) compact multi-photon microendoscopy [44,45], (c) reduced background noise by using spatial overlap modulation NLOI [46], (d) correction of motion artefacts by use of advanced motion compensations during optical imaging [40] and (e)improved signal-to-noise ratio by incorporation of photon counting detection [47]. These advancements will substantially improve the quality of NLOI images to provide non-invasive histopathologic diagnosis with the required sensitivity and specificity in the future.
On the other hand, NLOI setups are quite complex and expensive at present for diagnostic or surveillance purposes. There is a dire need currently for practical and cheaper skin cancer surveillance modalities. One of the solutions would be to design inexpensive non-imaging diagnostic modalities like a depth-based epidermal or dermal autofluorescence detecting device that can thus indirectly measure the degree of epidermal proliferation. Nonetheless, the cost-effectiveness of such a modality is obtained by foregoing the useful image acquisition capability of NLOI. However, it should also be borne in mind that most NLOI devices are expensive at present only because these devices are either home built or used solely for academic or research purposes. There are attempts to make NLOI more cost-effective as demonstrated in the study by Kieu et al. [48] where low cost compact Er\textsuperscript{3+}-doped femtosecond fibre laser mode-locked by a single-walled carbon nanotube (CNT) was used successfully to perform NLOI on biological samples. Cheaper and simpler prototypes of NLOI would eventually begin to be manufactured by commercial industries depending on the successful translation of NLOI into clinics. Once it has been made cost-effective, in vivo NLOI would indeed be a suitable non-invasive alternative to conventional histopathology.

### 7.5 Conclusion

The findings of this study suggest that in vivo NLOI could clearly detect early microscopic changes in clinically normal skin undergoing latent carcinogenesis for cSCC. The results also show that axial x-z NLOI scans could clearly differentiate between the various layers in skin and detect abnormal proliferation in any of these layers. Complimentary transverse x-y scans were able to provide cytonuclear details that could be useful to determine the extent of epithelial atypia. Furthermore, it was seen that the TPEF-SHG index was a reliable indicator of epidermal proliferation and was useful to distinguish skin with early preclinical changes from normal skin regions. To summarise, in vivo NLOI has immense potential as a diagnostic modality to monitor early cancerous changes in skin. Thus it can be used by the clinicians to screen for skin changes in patients who are at high risk to provide the needed timely intervention.
References