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

Thomas, G.

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

Giju Thomas (2015)

INVITATION
To the public defense of the PhD thesis ‘Nonlinear optical imaging as a diagnostic tool for cutaneous squamous cell carcinoma’
on Wednesday, January 14th 2015 at 13.00h in the auditorium of Aula - Gude Lutherse Kerk Singel 411, 1012 XA Amsterdam, The Netherlands

The reception afterwards will take place in the Tetterode Bibliotheek (Library) next to the auditorium

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

Giju Thomas
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Cover Illustration: Giju Thomas
The central image shows a transverse nonlinear optical scan taken from a skin tumour of 1 mm diameter in a hairless mouse. Laser beam background obtained freely from the online public domain www.wallpapers-xs.blogspot.com.

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

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D. C. van den Boom
ten overstaan van een door het College voor Promoties ingestelde commissie, in het openbaar te verdedigen in de Aula der Universiteit
op woensdag 14 januari 2015, te 13.00 uur

door

Giju Thomas

geboren te Fujairah, Verenigde Arabische Emiraten
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               Prof. dr. M.A.M.J. van Zandvoort
               Prof. dr. J. van Rheenen

Faculteit der Geneeskunde
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Abbreviations used in this thesis

AK  actinic keratoses
AUC  area under curve
BSA  bovine serum albumin
cBCC  cutaneous basal cell carcinomas
cMM  cutaneous malignant melanomas
cSCC  cutaneous squamous cell carcinomas
CCD  charge coupled device
CHO  Chinese Hamster Ovary
CPDs  cyclobutane pyrimidine dimers
CSLM  confocal scanning laser microscopy
CT  computerised tomography
CW  continuous wave
DAB  3, 3’-diaminobenzidine
DAPI  4’, 6-diamidino-2-phenylindole
DMBA  7, 12-dimethylbenz(a)anthracene
DNA  deoxyribonucleic acid
DRS  diffuse reflectance spectroscopy
FAD  flavin adenine dinucleotide
EMCCD  electron multiplying charge coupled device
FPs  family practitioners
Fs  femtoscond
GPs  general practitioners
GW  gigawatt
H&E  haematoxylin and eosin
HFUS  high frequency ultrasound
HPV  human papilloma virus
LIFS  laser-induced fluorescence spectroscopy
MAF  multi-photon autofluorescence
MOPS  3-(N-morpholino) propanesulfonic acid
MPE  multi-photon excitation
MRI  magnetic resonance imaging
mW  milliwatt
NADH  nicotinamide adenine dinucleotide
NIR  near infrared
NLOI  nonlinear optical imaging
NLSI  nonlinear spectral imaging
nm  nanometre
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>NMSC</td>
<td>non-melanoma skin cancer</td>
</tr>
<tr>
<td>OCT</td>
<td>optical coherence tomography</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>phosphate buffered saline with 0.1% Tween.</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PHCCs</td>
<td>primary health care clinicians</td>
</tr>
<tr>
<td>PMT</td>
<td>photomultiplier tube</td>
</tr>
<tr>
<td>RGB</td>
<td>red, green and blue</td>
</tr>
<tr>
<td>SHG</td>
<td>second harmonic generation</td>
</tr>
<tr>
<td>THG</td>
<td>third harmonic generation</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TPEF</td>
<td>two-photon excitation fluorescence</td>
</tr>
<tr>
<td>TW</td>
<td>terawatt</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
</tbody>
</table>
‘And God said, “Let there be light,” and there was light.

God saw that the light was good,

and He separated the light from the darkness.’

Genesis 1: 3 – 4, The Holy Bible

For my Mama and Papa

For Christ, my Saviour and Redeemer
1 | The skin

The skin is a complex multifunctional organ that constitutes the soft outer covering of the body. It is the largest organ in the human body, having a surface area of about 1.5 m² – 2 m² and constituting 12% – 15% of total body weight [1].

1.1 | Function of skin

The skin along with appendages such as hairs and nails form the integumentary system that serves as a protective layer for the body. As skin is located at the interface between the organism and the environment, it has various vital functions such as: (i) thermoregulation, (ii) minimising excessive water loss, (iii) providing a water-proof protective barrier to deeper organs, (iv) protection against environmental hazards as UV radiation, chemical hazards, physical injury or pathogens, (v) waste excretion, (vi) source of sensation in response to external stimuli such as pain, pressure and temperature (vii) synthesis of vitamin D from sunlight and (viii) protection of vitamin B folates.

1.2 | Structure of skin

As seen in Figure 1, the skin is a multilayered organ that can be broadly divided into three distinct layers: (a) the epidermis, (b) the dermis and (c) the hypodermis.

![Figure 1 | The skin is composed of three distinct layers: (a) the epidermis composed of mainly keratinocytes, (b) the dermis composed of dense connective tissue that also contains hair follicles, blood vessels, sweat and sebaceous glands, and (c) the hypodermis that consists mainly of loose connective and fatty tissues. (Reproduced from 'Layers of the Skin' that is an online textbook, with permission from OpenStax College [2]).]
1.2.1 | The epidermis

The epidermis serves as the first major barrier of the body against the environment. It receives oxygen almost exclusively by diffusion from the surrounding air [3], while it obtains its nutrients from the deeper lying dermis that is well supplied by blood vessels. The epidermis is organised in definite layers that is composed mainly of keratinocytes. Other components of the epidermis are melanocytes (pigment cells), Merkel cells (touch sensation), Langerhans cells (antigen-presenting cells) and T lymphocytes (immune defence).

The epidermis is composed of four or five layers of epithelial cells, depending on its location in the body. Most of the skin can be classified as thin skin and this type of skin has four layers of cells. From superficial to deep, these layers are the stratum corneum, stratum granulosum, stratum spinosum and stratum basale [4]. On the other hand, thick skin that is found only on the palms of the hands and the soles of the feet has an additional fifth layer called the stratum lucidum [2]. This layer is typically located between the stratum corneum and the stratum granulosum.

Figure 2 | The various layers and constituents of epidermis. The layers are composed of stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale. The epidermal constituents are mainly keratinocytes along with other components such as melanocytes and Merkel cells. (Reproduced from ‘Layers of the Skin’ that is an online textbook, with permission from OpenStax College [2]).
As depicted in Figure 2, stratum corneum is the most superficial layer of the epidermis. It is a cornified layer composed of polyhedral, enucleated and flattened dead cells called corneocytes and keratin. The stratum lucidum, if present as in thick skin, is seen as a thin translucent layer just below the stratum corneum. Stratum lucidum is composed of dead and flattened keratinocytes that are densely packed with eleiden, a clear protein rich in lipids, derived from keratohyalin [2]. The subsequent layer is named stratum granulosum, after the granules that are present in the cytoplasm of keratinocytes within this layer. This layer provides the first barrier against environmental threats. The next layer, stratum spinosum, is 3 to 4 cell layers thick and contains differentiated keratinocytes. The deepest layer of the epidermis is the stratum basale that is located on the basement membrane which separates the epidermis from the dermis. The stratum basale is the germinative layer that is composed of proliferative keratinocytes, which produce daughter keratinocytes that move up into the overlying layers. As these cells migrate upwards from the basal layer, they stop dividing and eventually undergo terminal differentiation. They move outwards to the stratum corneum where they are eventually shed during epidermal turnover. In addition to the proliferative keratinocytes, the stratum basale is also composed of non-proliferating keratinocytes, melanocytes (responsible for melanin production) and Merkel cells (closely associated with deeper cutaneous nerves).

1.2.2 | The dermis
Dermis is the intermediary layer between the epidermis and hypodermis. The dermis along with the epidermis constitutes the cutis. Dermis consists of mainly connective tissue and is composed of collagen, elastin and extracellular matrix [4]. The dermis is divided into two layers – the superficial region adjacent to epidermis is called papillary dermis, while the deeper thick region is called reticular dermis. The dermis is rich in mechanoreceptors, hair follicles, sweat and sebaceous glands, blood and lymphatic vessels. The blood vessels provide nourishment and remove waste from the dermal and epidermal cells, in addition to aiding in thermoregulation.

1.2.3 | The hypodermis
Hypodermis or subcutaneous tissue is the layer that lies immediately below the dermis of vertebrate skin. It consists of mainly loose connective tissue and fat lobules. Compared to the dermis, the hypodermis contains larger blood vessels and nerves. The cells present in hypodermis are mainly adipocytes, fibroblasts and macrophages.

1.3 | Skin cancer

Skin cancer is the most common form of cancer globally among the Caucasian population and the incidence of skin cancer is on the rise since the last few decades [5-10]. Skin cancer arises due to development of cancerous cells in any of the three layers of the skin and eventually
invades or spreads to other regions of the body. Skin cancer arises mainly from the epidermis and can be categorised into three major groups: (a) cutaneous basal cell carcinoma (cBCC), (b) cutaneous squamous cell carcinoma (cSCC) and (c) cutaneous malignant melanoma (cMM). The less common skin cancers arise from the dermis and include – Merkel cell carcinoma (arising from Merkel cell receptors), sebaceous carcinoma (arising from sebaceous glands), spindle cell sarcoma (arising from dermal connective tissue) and dermatofibrosarcoma protuberans (rare sarcoma from dermal layer). These less common skin cancers along with cBCC and cSCC are categorised together under non-melanoma skin cancers (NMSC).

The aetiology of skin cancer can be attributed to ultraviolet (UV) radiation exposure in more than 90% of the cases [11]. The recent rise in incidence of skin cancer is related to increased exposure to UV radiation, which can be caused by: (a) ongoing depletion of the ozone layer that normally filters out the solar UV radiation [6], (b) frequent use of tanning beds that is a major indoor source of UV radiation [6,12], (c) increased sun-seeking behaviour amongst the Caucasian population [13-16] and (d) a global increase in the size of the ageing population who are at a higher risk for skin cancer [17]. Certain studies show that increased episodes of sunburn or acute exposure to UV particularly during childhood was associated with a higher risk for cBCC or cMM [18-20]. On the other hand, the total or cumulative exposure to UV radiation plays a significant role in the risk of developing cSCC, irrespective of the age at exposure. Other pre-disposing factors that may contribute to the aetiopathogenesis of skin cancer are geographic location, skin phototype, genetic background (such as xeroderma pigmentosum), occupation, presence of pre-existing moles or naevi, previous history of skin malignancies, presence of chronic non-healing wounds, exposure to chemical carcinogens, infection by viral pathogens such as human papilloma virus (HPV), organ transplantation and immunocompromised status [21-27].

Of all the major types of skin cancer, cMM are the most aggressive and has the highest mortality at almost 75% of the deaths resulting from skin cancer [28]. However cMM is rarer compared to NMSC which is about 20 times more common worldwide.

1.3.1 | Non-melanoma skin cancer (NMSC)

NMSC is the most common type of skin cancer with a global incidence of 2 – 3 million people per year, compared to the global incidence of cMM at just 132,000 people per year [20]. Although studies show that the mortality of NMSC is quite low at just ~ 0.69 deaths/10,000 NMSC cases per year [29], the associated morbidity arising from NMSC can be debilitating for the patient that results in an increased burden to the healthcare system [30,31]. 75% of the NMSC cases are diagnosed as cBCC, while 20% can be attributed to cSCC and the remaining 5% to the rarer non-melanoma skin cancers. Among NMSC diagnosed cases, cBCC rarely metastasizes and is easily treatable. On the other hand, cSCC present as rapidly growing tumours that spreads more rapidly and aggressively to adjacent tissues than cBCC. The mortality rate and frequency of metastasis was also found to be higher for cSCC when compared to cBCC [32].
1.3.2 | Cutaneous squamous cell carcinoma (cSCC)

The incidence of cSCC ranges from 0.03 – 3.5 cases/10,000 people per year on a global basis [33]. The incidence of cSCC is the highest in Australia, where this high incidence can be attributed to a predominant Caucasian-skinned population residing in a region that receives extensive sun exposure [34]. Lesions diagnosed as cSCC normally present on the sun exposed regions of the body such as face, ears, lips, neck, hands or arm. The lesions that develop on ears and lips metastasise more rapidly by spreading to lymph nodes [33]. Among the cSCC cases, the recurrence rate and the metastasis rate five years after surgery could be as high as 8% and 5% respectively [35-38]. Due to the associated rapid growth and invasion of cSCC lesions, timely diagnosis of cSCC plays a crucial role in better patient prognosis and effective therapy.

1.4 | Current mode of diagnostics for cSCC

Detection of cSCC usually occurs during clinical examination. The clinical signs for cSCC are highly variable. The typical presentation of cSCCs is that of an ulcerated lesion with hard and raised edges. However cSCC can also clinically present as a hard plaque or papule that are firm, skin-coloured or pink. These papules could have a surface that is smooth or hyperkeratotic. Patients may provide history of the lesions being itchy or painful non-healing wounds that persistently bleeds upon contact [35]. However at this stage of cSCC, treatment would essentially involve surgical excision of the lesion along with a free margin of healthy tissue or Mohs surgery [39]. Although these surgeries are highly effective, additional radiation therapy may be required depending upon the associated risks, size and extent of cSCC lesion. In effect, following efficient excision of cSCC lesions, the patient may still be left with residual disfiguring cosmetic scars. In addition, possible recurrence or metastasis associated with cSCC following surgery may add onto the patient's morbidity.

Therefore the current focus of skin cancer diagnostics should be to detect cSCC in its earlier or precursor stages for effective and yet minimally invasive treatment. The two most common precursors to cSCC are actinic keratoses (AK) and Bowen's disease. AK typically present as scaly lesions, typically 2 to 6 mm in diameter that may be the same colour as the skin [35]. Patients with Bowen's disease on the other hand, present with sharply demarcated, erythematous, velvety, or scaly plaques on sun-exposed areas. The typical clinical manifestations of cSCC, AK and Bowen's disease are shown in Figure 3.

The responsibility of early detection of cSCC or its precursors presently lies with the primary health care clinicians (PHCCs), family practitioners (FPs) or general practitioners (GPs) who form the first line of patient healthcare. However due to high variability in the clinical features for cSCC and its precursors, diagnosis made purely by clinical examination can be erroneous. Certain studies have reported that FPs diagnosed cSCC or its precursors with poor sensitivity or specificity. In the study by Morrison et al., FPs were able to clinically identify only 22% of cSCC (2 out of 9) and 0% of AK (0 out of 18) cases that were later diagnosed histologically [40].
In another study by Whited et al., the sensitivity of clinically detecting skin malignancy by PHCCs was just 38%. Of 109 lesions in 61 patients, the primary care clinicians misdiagnosed 37 malignant NMSCs as benign (false negative ~ 34%) and 27 benign skin conditions as malignant (false positive ~ 25%) [41]. Westbrook et al. found that of the 70 cases referred as cSCC by GPs, only 13 were confirmed to be cSCC histologically (sensitivity ~ 19%) [42]. Low sensitivity and specificity in detection of cSCC by PHCCs, FPs or GPs lead to (a) missing out diagnosis of premalignant or malignant stages of cSCC and (b) unnecessary referrals of benign skin conditions to the dermatologists or the plastic surgeons.

Figure 3 | Clinical manifestation of cSCC (left) and its precursor lesions – actinic keratosis (centre) and Bowen's disease (right). cSCC appears as a firm hyperkeratotic papule located on the right ear (as shown in left figure) which is a high risk region for metastasis via lymph nodes. The centre figure shows several reddish scaly patches on the scalp denoting multiple sites of actinic keratosis with a single central reddish lesion having a thick overlying black scale, suggestive of transformation of actinic keratosis to invasive cSCC at that site. The extreme right figure displays sharply demarcated erythematous lesion near the sun exposed temple indicative of Bowen's disease. (Reproduced with permission from ‘Cutaneous squamous cell carcinoma by Alam et al. (2001)’ [35], Copyright Massachusetts Medical Society).

In contrast, the dermatologists and the plastic surgeons are equipped with a more specialised clinical training in identifying cancerous skin lesions, compared to the PHCCs, FPs or GPs. Therefore in contrast to PHCCs, FPs and GPs, the dermatologists and plastic surgeons record a higher sensitivity and specificity in cSCC diagnosis clinically, as revealed in various studies [40-43]. However, the dermatologists and plastic surgeons also find it more complicated to rightly identify cSCC or its precursors, when compared to cBCC and cMM [43]. In the study by Cooper and Wojnarowska, while the dermatologists demonstrated a high sensitivity of 90.5 for cSCC, the specificity was only moderate at 75.3% [44]. In contrast, only 60% of the malignant lesions were correctly identified by plastic surgeons in the study conducted by Hallock and Lutz, at a moderate sensitivity of 73% [45]. The sensitivity of clinically diagnosing cSCC by
plastic surgeons was even lower at 56% in the study conducted by Ek et al. [46]. However, the dermatologist and plastic surgeons always have the option to perform invasive punch or excision biopsies of these skin lesions to confirm the diagnosis histologically. This is because the current gold standard for dermatopathologic diagnosis is evaluating the conventional haematoxylin and eosin (H&E) stained skin section obtained by biopsy.

However, this route of diagnosis involving invasive biopsies has its own associated disadvantages. The dermatologists/plastic surgeons are sometimes left in a dilemma to decide if a skin lesion needs a biopsy or not. They have to rely entirely on their clinical experience to make this decision. Decisions to perform biopsy based purely on clinical diagnosis can lead to either a) the dermatologists/plastic surgeons skip obtaining a biopsy of a premalignant or malignant skin lesion or b) they may perform an unnecessary biopsy on a benign lesion. The former would result in worsened patient morbidity/mortality, while the latter results in unnecessary cosmetic scarring for the patient and needless workload for the dermatopathologists. Moreover, the diagnosis following a biopsy is never immediate and could often be delayed depending on the backlog of the available dermatopathologists. This can result in delay in follow-up and timely therapy for the patient. In addition, the plastic surgeons are always dependant for the pathologist’s report to decide if a conservative excision or radical excision is needed for the suspect skin lesions. Delay in the arrival of the pathology report results in the postponement of this vital therapeutic decision by the plastic surgeons. Therefore there is a dire need for newer non-invasive diagnostic modalities that will serve the following functions:

(a) To guide the PHCCs, FPs or GPs in deciding which candidate needs an urgent referral to the dermatologist/plastic surgeon. In addition, it should be useful for them to perform surveillance and screening for precancerous changes in asymptomatic individuals who are at risk.

(b) To assist the dermatologist/plastic surgeon determine if a suspect skin lesion requires an invasive biopsy or not.

(c) To aid the plastic surgeon in real-time during surgery to determine if a skin lesion needs conservative excision or radical excision.

1.5 Newer non-invasive diagnostic modalities for cSCC detection

Although H&E stained sections obtained from skin biopsies are considered the ‘gold’ standard for dermatopathologic diagnosis, the procedures involved to obtain it can be time-consuming, expensive and invasive for the patient. Therefore non-invasive diagnostic modalities have emerged that could be useful for the clinicians and dermatologists at the primary and specialised line of health care respectively. The non-invasive modalities that are already available at clinics at present include total body photography, dermatoscopes, high frequency ultrasound (HFUS) and Doppler sonography, computerised tomography...
(CT), positron emission tomography (PET) and magnetic resonance imaging (MRI). Newer non-invasive diagnostic modalities are currently still being investigated for their efficacy in skin cancer detection with in vivo animal models, in vivo human skin or ex vivo human skin biopsies. One such technique that is being utilised involves tape-stripping the skin lesions and analysing the mRNA of the epithelial cells that adhere to the tape. Other modalities include tetrahertz imaging, electrical bio-impedance analysis, photodynamic diagnosis, optical coherence tomography (OCT), optical spectroscopy based on laser-induced fluorescence spectroscopy (LIFS) or diffuse reflectance spectroscopy (DRS), and confocal scanning laser microscopy (CSLM). An overview of all the mentioned modalities has been provided in Table 1a and 1b, which describes the principle involved, advantages and disadvantages associated with each diagnostic modality.

1.6 | Nonlinear optical imaging for cSCC diagnosis

Of the mentioned diagnostic non-invasive modalities, the optimum image resolution to visualise essential cellular details that may be useful for a pathologist can be provided non-invasively by only CSLM. However CSLM has certain disadvantages such as a) higher risk of photobleaching and photodamage in samples and b) limited depth penetration while imaging as already mentioned in Table 1. Moreover CSLM also relies heavily on exogenous labels to enhance contrast for cellular details.

A newer non-invasive imaging modality that has shown promise for cancer diagnosis is nonlinear optical imaging (NLOI). NLOI can generate real-time images with fine cellular details having a comparable resolution to CSLM, without having to rely on exogenous labels. Since NLOI is based on non-linear interaction of photons with the imaged tissue, fluorescent emission occurs only in the focal plane resulting in reduced photobleaching and photodamage compared to CSLM [53]. Furthermore, use of longer imaging wavelengths in the near infrared range by NLOI enables deeper tissue imaging when compared to CSLM [53]. Due to these merits, NLOI is rapidly emerging as a viable non-invasive diagnostic tool for cancer detection [54-57]. The potential and advances of in vivo NLOI as a diagnostic tool for cancer is covered comprehensively in Chapter 2.
Table 1a | Non-invasive diagnostic modalities for skin cancer presently available in clinics [43,47,48]

<table>
<thead>
<tr>
<th>Non-invasive diagnostic modalities</th>
<th>Principle involved</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Total body photography            | Based on clinical acquisition of digital photographs from head to toe of entire skin surface. | - Useful for surveillance and detection of new lesions in cMM.  
- Useful for long distance consultation with specialists from primary health care centres. | - Only macroscopic visualisation.  
- Not very useful for NMSC.  
- Poor patient compliance due to requirement of regular follow-up.  
- Privacy issues regarding the storage and use of photographs. |
| Dermatoscopy                      | Based on examination by a magnifier (10X) and illumination of the lesion by a non-polarised/polarised light source. | - Simple handheld device.  
- Inexpensive.  
- Increased sensitivity for cMM and pigmented cBCC. | - Sensitivity and specificity vary between different users based on experience.  
- Not useful for cSCC.  
- Increased examination time.  
- No useful cellular details for pathologists. |
| HFUS and Doppler Sonography.      | Based on ultrasound pulses sent to tissue using a **transducer**. The sounds reflect off the tissue and the resultant echoes are converted to images. | - Useful for depth determination of lesions and imaging skin layers.  
- HFUS provides resolution of 20 microns. | - Poor sensitivity to lesions localised to epidermis/thin lesions.  
- Cannot differentiate between benign and malignant lesions.  
- No useful cellular details for pathologists. |
| CT                                | Based on X-ray imaging by tomography or 'sections' of whole body. | - Useful in assessing invasiveness and distant metastasis through lymph nodes and perineural route.  
- Useful for follow-up assessment for recurrence or metastasis following surgical/radiation/chemotherapy treatment. | - Not useful for early detection or screening.  
- No useful cellular details for pathologists.  
- Risk of ionising radiation from CT. |
## Non-invasive diagnostic modalities

<table>
<thead>
<tr>
<th>Modalities</th>
<th>Principle involved</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET</td>
<td>Imaging based on detecting pairs of gamma ray emissions due to collision between positrons emitted from a tracer and electrons in tissue.</td>
<td>– Useful in monitoring the metabolic activity of the tumour/skin lesion.</td>
<td>– Not useful for early detection or screening. – No useful cellular details for pathologists. – Exogenous positron emitting radioactive tracer required for PET.</td>
</tr>
<tr>
<td>MRI</td>
<td>Imaging based on the switch in magnetic alignments of protons in different tissues in response to turning a magnetic field on and off.</td>
<td>Similar to CT</td>
<td>Acquisition period ~ 30 minutes. – Not useful for early detection or screening. – No useful cellular details for pathologists.</td>
</tr>
</tbody>
</table>
Table 1b | Non-invasive diagnostic modalities for skin cancer presently under investigation [43, 47-52].

<table>
<thead>
<tr>
<th>Non-invasive diagnostic modalities</th>
<th>Principle involved</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Tape-strip mRNA analysis          | Diagnosis based on mRNA analysed from epithelial cells adhering to the tape after stripping it off from lesions. | – Sensitive for early melanomas.  
– Maybe able to detect genomic changes before morphologic changes. | – Only few studies to confirm specificity and sensitivity of diagnosis.  
– No studies on NMSC. |
| Tetrahertz Imaging                | Imaging based on using electromagnetic radiation pulses with frequencies ranging from 0.1 – 10 THz, as water has strong absorption in this range and thus tissue water content serves as contrast. | – Able to distinguish between cBCC and normal skin. | – Limited studies.  
– All studies on cBCC.  
– Thermal effects (increase in temperature)  
– Limited depth penetration for imaging.  
– No useful cellular details for pathologists. |
| Electrical bio-impedance analysis | Detection based on the fact that electric impedance spectrum is unique for each biological tissue, thus serving as the source of distinguishing between different tissue types. | – Able to detect cMM, cBCC and cSCC from normal skin. | – Since the modality relies on electric impedance spectra, no visual/cellular details are available for analysis.  
– High intra-group variance, leading to inability to differentiate one cancer type from another. |
| Photodynamic diagnosis           | Based on selective accumulation of exogenous/endogenous photosensitisers in tumours compared to normal tissues. These photosensitisers elicit characteristic fluorescence emission when exposed to lights of specific wavelengths or energy dose. | – Application of photosensitisers like aminolevulinic acid (ALA) has been found useful in detecting cBCC and cSCC.  
– Useful in detecting recurrence or local spread of the lesions in skin cancer. | – Requires an exogenous photosensitiser to provide contrast.  
– Highly sensitive to the pharmacokinetics and tissue distribution of the photosensitiser. |
<table>
<thead>
<tr>
<th>Non-invasive diagnostic modalities</th>
<th>Principle involved</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| **OCT**                           | Imaging is based on sensing near infrared light reflected back from tissue and applying interferometric methods to generate images. These images depend on scattering, refractive index and birefringence properties that are unique to each tissue types. | - Image resolution at ~ 10 microns that is better than most optical imaging techniques.  
- Deeper imaging as high as 500 mm.  
- Can visualise tissue architectural difference between cancerous and normal skin. | - Cannot differentiate between cBCC and AK.  
- Images show variation from person to person and on different sites of the same person.  
- Resolution is not optimal for subcellular details needed for pathology diagnosis. |
| **Optical spectroscopy based on LIFS/DRS** | Diagnosis based on the unique fluorescence emission spectrum or reflectance spectrum obtained from tissue upon illumination with a UV/visible/near infrared light. | - Cost effective.  
- Quick acquisition of data from skin region.  
- Good sensitivity in distinguishing NMSC from normal skin. | - Spectral based analysis with no visual details for pathologic diagnosis.  
- Complex algorithm implementation to account for all sources of absorption and scattering in tissue.  
- Highly sensitive to fluctuations in probe pressure and bending of optical detection fibre leading to high inter-user variation. |
| **Confocal Scanning Laser Microscopy** | Imaging is done by focussing a laser beam onto a specific region and then detecting the light reflected from the focal point through a confocal pinhole filter, which removes light reflected outside the focal plane. The detected light generates an electrical signal that is converted into an image. | - Optical imaging method that provides the best image resolution at < 1 micron.  
- Subcellular details are visualised clearly with precision closest to conventional histology slides.  
- High sensitivity in detection of cMM and cBCC. | - Tissue depth penetration not more than 100 mm.  
- Associated photobleaching and photodamage in the sample during prolonged imaging.  
- Costlier than other techniques.  
- Poorer intra-nuclear details compared to histology. |
1.7 | Objectives of the thesis

The main objective of the thesis is to explore the potential of label-free in vivo NLOI as a non-invasive imaging modality in the diagnosis of cSCC and how this application can be extrapolated for cancer diagnostics in other organs as well. The focus of the thesis can be broadly categorised into three segments:

(a) To assess and optimise the biosafety issues and possible laser radiation associated risk caused by in vivo NLOI to ensure minimal hazards to the patient upon clinical translation (Chapter 4 and 5).

(b) To develop a skin cancer model in a SKH1-hr strain mice for assessment by in vivo NLOI. Use of this specific murine cancer model for in vivo imaging ensures a better translation for human subjects as this particular strain of mice are immunocompetent and thus ensures a more realistic carcinogenesis process as compared to carcinogenesis in immunocompromised nude mice. In addition, these murine models have hair density comparable to humans that is advantageous for NLOI while imaging skin, as there is minimal interference from hair during the procedure unlike as in wild strain mice (Chapter 6).

(c) To identify diagnostic optical signatures during different stage of skin carcinogenesis using non-invasive in vivo NLOI in the developed mice skin cancer model (Chapter 7 and 8).

The various studies described in this thesis can be outlined as follows:

Chapter 2 gives a comprehensive review about the advances made by NLOI in the field of translational cancer research. Initially it covers the physics and principles that is involved during in vivo NLOI. This is followed by a brief overview on endogenous optical fluorescence that has enabled to use NLOI in a label-free manner for optical diagnostics. Subsequently, the sensitivity and specificity of NLOI based diagnosis is compared against conventional histopathology. Advances in NLOI as a tool to monitor spectroscopic, metabolic and collagen related changes during carcinogenesis are elaborated in detail in the latter segments. The chapter ends discussing about the present challenges involved for the clinical translation of in vivo NLOI and potential areas of future research involving label-free NLOI.

Chapter 3 gives a brief description of the NLOI setup used for in vivo studies described in this thesis.

In Chapter 4, the risk of DNA damage in the form of mutations such as cyclobutane pyrimidine dimers (CPDs) caused by irradiation from NLOI was investigated. The role of laser power, imaging wavelength, pulse duration and scan speed in determining levels of DNA damage was evaluated in this chapter.

Using data from Chapter 4, a unique cancer risk model based on epidemiological data was developed to evaluate the additional risk of cSCC in humans that are potentially exposed to irradiation from NLOI when compared to natural solar UV exposure in Chapter 5. This
model investigated how various NLOI parameters, optical biopsy frequency, age of individual and body surface area exposed to irradiation from NLOI could influence risk of cSCC in that individual's lifetime.

Chapter 6 describes how a cSCC murine model was developed for the purpose of further in vivo NLOI investigations. This cancer model was established in hairless, immunocompetent mice strains for the first time using chronic weekly exposure to a chemical carcinogen 7, 12 dimethylbenz(a)anthracene (DMBA). This study also involved observing the course of histopathological and immunohistochemical changes in different stages of DMBA-induced carcinogenesis in murine skin.

In chapter 7, the cSCC murine model developed as described in chapter 6 was assessed using in vivo NLOI to study the various optical microscopic changes in various stages of skin carcinogenesis. A diagnostic index based on TPEF and SHG was formulated and was used for comparison accordingly.

Chapter 8 reports on the spectroscopic changes observed using in vivo NLOI in the developed murine cSCC models. In addition, the mean epidermal autofluorescence intensity, mean SHG intensity and the metabolic status were assessed and compared for the different stages of DMBA induced carcinogenesis observed in murine skin.

Chapter 9 provides an overview of the results and subsequent conclusions of the studies described in this thesis. Additionally, a brief outlook on the future use of in vivo NLOI for cancer diagnostics in skin and other organs are covered.
References

Chapter 2

Advances and challenges in label-free nonlinear optical imaging using two-photon excitation fluorescence and second harmonic generation for cancer research

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Abstract

Nonlinear optical imaging (NLOI) has emerged to be a promising tool for bio-medical imaging in recent times. Among the various applications of nonlinear optical imaging, its utility is the most significant in the field of preclinical and clinical cancer research. This review begins by briefly covering the core principles involved in NLOI, such as two-photon excitation fluorescence (TPEF) and second harmonic generation (SHG). Subsequently, there is a short description on the various cellular components that contribute to endogenous optical fluorescence. Later on the review deals with its main theme – the challenges faced during label-free NLOI in translational cancer research. While this review addresses the accomplishment of various label-free NLOI based studies in cancer diagnostics, it also touches upon the limitations of the mentioned studies. In addition, areas in cancer research that need to be further investigated by label-free NLOI are discussed in a latter segment. The review eventually concludes on the note that label-free NLOI has and will continue to contribute richly in translational cancer research, to eventually provide a very reliable, yet minimally invasive cancer diagnostic tool for the patient.
2.1 | Introduction

Nonlinear optical imaging (NLOI), based on the nonlinear excitation of fluorophores has taken rapid strides in the field of biomedical imaging since the last two decades. Technological innovations and research have steadily progressed in trying to develop NLOI as a diagnostic tool for the patient in a clinical environment [1-7]. Simultaneously NLOI based research has also been able to provide fundamental researchers with new perspectives in cancer research, especially with regard to studying pathogenesis of cancer and drug development for cancer therapy [8-14]. Numerous NLOI studies have given deep insights about cancer progression and the steps involved in angiogenesis and metastasis [8,9,15-26]. These studies however have been performed by administering exogenous fluorophores that enhanced contrast. Nonetheless NLOI can also be performed relying solely on endogenous fluorescence provided by the biological sample itself [27-32]. This review will thus cover cancer research studies that rely on label-free NLOI using only endogenous optical fluorescence.

The key aim of this review is to evaluate the advances made and challenges faced in label-free NLOI, as researchers attempt to use this technology for cancer diagnosis in a clinical scenario. The authors have tried to highlight the accomplishments and assess the limitations of the reviewed studies. This review eventually raises certain pertinent questions that haven't been investigated by researchers till date, which gives scope for further studies.

2.1.1 | Physics of Nonlinear Optical Imaging
2.1.1.1 | Two-photon Excitation Fluorescence (TPEF)

In TPEF, two near infrared (NIR) photons are absorbed simultaneously, where each photon provides half of the energy, which is normally required to excite the fluorophore into a higher electronic state as seen in Figure 1. Therefore, emission of fluorophores in wavelengths that fall in visible light or UV region can be induced with low-energy NIR photons. The NIR excitation spectra is unique as the linear absorption and scattering coefficients contributed by cells and tissues is low in this wavelength range, which leads to a high light penetration depth. In addition, the two-photon absorption occurs only in the plane of focus, minimising background scatter from regions outside focus. Though Maria Göppert-Meyer had formulated the theory of TPEF in 1931 [33], it was only observed three decades later by Kaiser et al.[34] and Abella et al. [35]. Eventually it was put into application by Denk, Strickler and Webb who built the first TPEF microscope and observed intracellular fluorescent probes [36]. TPEF, however, is achieved only at very high photon concentration in space and time, requiring extremely high NIR laser intensities [34]. However the development of ultra-short pulsed lasers [37] can now provide transient intensities of GW cm\(^2\) in a pulsed form, with the pulse duration ranging in the femtoseconds and at a high pulse frequency of 80 – 90 MHz. As a result TPEF signals can be generated effectively at average laser powers lower than 5 mW [38] incident on the sample.
2.1.1.2 | Second Harmonic Generation (SHG)

SHG is a nonlinear optical process where two photons combine together to produce a new photon with twice the energy or half the wavelength emission of the incident photons as shown in Figure 1. Unlike in TPEF, there is no non-radiative energy loss involved in SHG. SHG was first demonstrated in 1961 by Peter Franken, A. E. Hill, C. W. Peters, and G. Weinreich [39] and a year later N. Bloembergen and P. S. Pershan described the formulation of SHG [40]. In order to obtain SHG, an intense laser beam from ultra short pulsed NIR laser should pass through materials with a specific molecular orientation. These materials are generally composed of non-centrosymmetric (no inversion symmetry) molecular structures. Certain biological materials such as collagen, microtubules (tubulin), and muscle myosin are highly polarisable, as these materials are assembled from fairly ordered, large non-centrosymmetric structures. Therefore the secondary, tertiary and quaternary structure of proteins that involve specific folding of the proteins into its unique 3-dimensional conformation play a major role in determining the polarisability of biological materials and its ability to produce SHG. Alteration of biological materials at the secondary, tertiary or quaternary structural levels in a diseased state will therefore affect the level of SHG obtained from the imaged tissue, making it a useful optical property for diagnostic purposes by NLOI.

Figure 1 | Jablonski energy diagram showing the process involved for two photon excited fluorescence (TPEF) and second harmonic generation (SHG). TPEF requires the existence of an actual excited state, while SHG does not.
2.1.3 | Comparison of NLOI over other Linear Imaging methods

NLOI provides distinct advantages over other linear imaging methods as:

(i) Linear imaging techniques can only use UV (200 – 400 nm) or visible wavelengths (400 – 700 nm) for imaging and these wavelengths have a very poor tissue depth penetration. This is due to increased scattering and absorption of shorter wavelengths in biological samples. By contrast, NLOI uses near infra red (NIR) light which allows deeper tissue imaging up to a depth of about 1 mm as depicted in Figure 2 [41,42].

(ii) UV and visible wavelengths used for linear imaging technique leads to increased photodamage and photobleaching in tissue sample, which is minimal for NLOI techniques which rely on NIR wavelengths [36,43].

(iii) In confocal microscopy, the out-of-focus molecules are also excited in addition to the focal plane. The pinhole aperture seen in confocal microscopy filters the out-of-focus fluorescence signals. However, the detection level of in-focus fluorescence signals is decreased because of the pin-hole. NLOI on the other hand does not require a pin-hole aperture, as the fluorescence arises only from the point of focus where TPEF takes place as depicted in Figure 2 [44].

(iv) In conventional linear fluorescence microscopy, the in-focus structures are washed out by the fluorescence signals from outside plane of focus. Thus linear imaging techniques often require labelling with exogenous fluorophores or fluorescent proteins for improved detection. Since in NLOI there is virtually no fluorescence outside the focus, the endogenous fluorescence in focal plane is well detected without being washed out, nullifying the need for an exogenous label. Therefore NLOI can provide subcellular resolution without relying on an exogenous label, making it advantageous over confocal microscopy and other biomedical imaging techniques.

(v) In addition to TPEF, NLOI can also rely on SHG to provide additional cellular details as compared to classic fluorescence and confocal microscopy.

(vi) NLOI setups generally comprise of scanners in x-, y- and z- direction as indicated in Figure 3, enabling three dimensional localisation of tissue fluorescence and study of tissue morphology changes in subcellular range that cannot easily be visualised by other imaging modalities [45,46].
Figure 2 | Comparison of single photon excitation fluorescence in confocal microscopy and two photon excitation fluorescence in nonlinear optical imaging.

2.2 | Endogenous Optical Fluorescence

Biological tissues contain many sub-cellular components that are fluorescent and this property allows us to induce fluorescence without any addition of exogenous fluorophores. These fluorescent components are composed of a) vitamins or vitamin derivatives as retinol, cholecalciferol, riboflavin or pyridoxine that emit in the visible light range or b) aromatic amino acids tyrosine, phenylalanine and tryptophan that emit in the UV range [47-50]. There are predominantly two sources of endogenous fluorescence in tissue: i) Intracellular and ii) Extracellular. Intracellular fluorophores can vary depending on the cell or tissue type and normally include nicotinamide adenine dinucleotide (NADH) and its phosphate derivative (NADPH), flavins as flavin adenine dinucleotide (FAD), retinol, tryptophan, serotonin, melatonin, melanin, porphyrins and lipofuchsin. Extracellular fluorophores on the other hand, comprise of collagen and elastin. Meanwhile fluorophores like keratin and phospholipids are present both intracellularly and extracellularly [47-50]. An overview of endogenous fluorophores, along with their excitation and emission maxima is shown in Table 1.
Table 1 | Representative Endogenous Fluorophores Responsible for Optical Contrast.

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Excitation</th>
<th>Emission</th>
<th>Source</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD(P)H free</td>
<td>300 – 380 nm</td>
<td>450 – 500 nm</td>
<td>Cytosol, Mitochondria</td>
<td>[48,50]</td>
</tr>
<tr>
<td>Bound NAD(P)H</td>
<td>300 – 380 nm</td>
<td>450 – 500 nm</td>
<td>Cytosol, Mitochondria</td>
<td>[48,50]</td>
</tr>
<tr>
<td>FAD</td>
<td>420 – 500 nm</td>
<td>520 – 570 nm</td>
<td>Mitochondria</td>
<td>[48,50]</td>
</tr>
<tr>
<td>Bound FAD</td>
<td>420 – 500 nm</td>
<td>520 – 570 nm (weak)</td>
<td>Mitochondria</td>
<td>[50]</td>
</tr>
<tr>
<td>Retinol</td>
<td>325 – 350 nm</td>
<td>490 – 500 nm</td>
<td>Cytosol</td>
<td>[48,50]</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>540 nm</td>
<td></td>
<td>Cellular and organelle membranes</td>
<td>[48,50]</td>
</tr>
<tr>
<td>Collagen</td>
<td>280 – 350 nm</td>
<td>370 – 440 nm</td>
<td>Fibrous tissue – dermis of skin, tendon, ligament</td>
<td>[48,50]</td>
</tr>
<tr>
<td>Elastin</td>
<td>300 – 370 nm</td>
<td>420 – 460 nm</td>
<td>Elastic tissue – Skin, Blood vessels, Lungs, Bladder, Stomach</td>
<td>[48,50]</td>
</tr>
<tr>
<td>Keratin</td>
<td>450 – 500 nm</td>
<td></td>
<td>Skin, Nail, Hair</td>
<td>[51]</td>
</tr>
<tr>
<td>Melanin</td>
<td>300 – 800 nm</td>
<td>520, 575 nm</td>
<td>Skin, Hair, Iris</td>
<td>[50]</td>
</tr>
<tr>
<td>Porphyrin</td>
<td>400 – 450 nm</td>
<td>635, 710 nm</td>
<td>Red Blood Cells</td>
<td>[48]</td>
</tr>
<tr>
<td>Lipofuscin</td>
<td>340 – 395 nm</td>
<td>540, 430 – 460 nm</td>
<td>Brain, Retina, Liver and Age spots in skin</td>
<td>[48,50]</td>
</tr>
<tr>
<td>Pheophorbide-a</td>
<td>400 – 410 nm</td>
<td>674 nm</td>
<td>Mice skin (from chlorophyll diet)</td>
<td>[52]</td>
</tr>
</tbody>
</table>

2.3 | Advances and challenges in label-free NLOI for in vivo cancer diagnostics

2.3.1 | Bio-safety evaluation

Although label-free NLOI can be performed optimally over the sample at average power lower than 5 mW [38], the potential negative effects of ultra-short NIR laser pulses cannot be ignored. Due to high peak power albeit for pulse duration of femtoseconds, NLOI can induce generation of damaging reactive oxygen species and localized heat effects, leading to loss of cell viability and cell death [43,53-57]. These thermo-chemical effects are however confined spatially just to the imaged zones, making it no less harmful than an invasive tissue biopsy. On the other hand, studies have shown that NLOI based multi-photon processes can form various mutations such as UV photoproducts, double strand breaks, single strand breaks and
oxidative lesions in the DNA [58-60]. If not repaired, these lesions can later lead to defective cell function and even carcinogenesis. It is these types of undesired effects that need to be thoroughly investigated prior to a full scale translation of NLOI devices to the clinic.

Various studies have been performed to investigate the biosafety aspects of label-free NLOI. The notable one among them are the studies by Fischer et al. [61,62], where the risk from an NLOI biopsy was compared with natural UV exposure and the increased risk for skin cancer was found to be just \(0.045\% - 0.06\%\). In another study, Thomas et al. [63] used an epidemiological model to show that the additional risk for squamous cell carcinoma of skin in an individual’s lifetime was much lower for 40 optical biopsies at 20 mW, as compared to 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 considerably. While these studies were restricted to risk of skin cancer, Dela Cruz et al. performed a detailed study on the laser power required to image various internal organs and the associated mutation risks [64]. The study showed that colon which is highly auto fluorescent required a laser power lower than 20 mW to generate an image. On the other hand, it took a power as high as 120 mW to collect an image from the bladder, which has lower intrinsic fluorescence. The study later showed that higher laser power for imaging caused increase in mutation frequency in imaged V79 cells. However, mutation levels are not reliable indicators of the actual cancer risk in the imaged tissue, as factors like cell turnover rate and innate cellular mutation repair mechanisms are overlooked. While Thomas et al. [63] assessed the risk for skin cancer from an epidemiological model that exists for radiation induced skin cancer, a similar epidemiological model does not exist for organs other than skin. It is also not known as to how the susceptibility to DNA damage and tissue reparative capability would vary from organ to organ. This implies the need for an NLOI imaging regime that can minimise carcinogenic risk and have doses customised for individual organs. Thus more investigations for bio-safety aspects of label-free NLOI are essential, in parallel with translational cancer research.

2.3.2 | Reliability of label-free NLOI diagnostic parameters
2.3.2.1 | Label-free NLOI versus conventional histopathology
Various studies have utilised label-free NLOI on unstained tissue sections to observe the morphological changes that arise in diseased or cancerous tissues. Often these studies have utilised TPEF and SHG to generate images, comparable to standard haematoxylin-eosin (HE) stained slides typically used by pathologists. Rogart et al. [65] demonstrated excellent comparability between label-free NLOI and HE staining in differentiating between various normal and neoplastic tissues of the gastro-intestinal tract. Later on, Yan et al. was able to provide cytostructural details that could identify normal and gastric cancer samples [66]. Similarly good correlation was demonstrated between ex vivo label-free NLOI and HE stained slides in various other organs such as oral cavity [14], lungs [67-69], breast [70], ovaries [29,71, 72], prostate [10], colon [73,74], rectum (as shown in Figure 3) [75] and bladder [76-78].
Figure 3 | Comparison of label-free NLOI and HE stained images of a normal rectal mucosa (A and B) and a cancerous one (C and D) A – NLOI of fresh, unfixed, and unstained normal rectal mucosal section showing regular tissue architecture with central, round crypt openings, and glands lined by epithelial and goblet cells. The green signal seen in A and C arise from TPEF in rectal epithelial and goblet cells. SHG signals (red circles) could be seen around the glands. B – The corresponding HE image illustrates a similar arrangement of rectal mucosa. C – NLOI of fresh, unfixed, and unstained section of cancerous rectal mucosa showing irregular tubular structures, gland epithelium proliferation and reduced stroma. SHG signals were significantly decreased in the cancerous area. D – The corresponding HE image shows similar features that correspond to the NLOI image C [75].

While the mentioned studies were based on qualitative diagnosis, certain studies have been able to distinguish normal tissue from various grades of cancer by simply quantifying tissue specific morphologic variables in the optical image itself. Skala et al. [79] measured key features in the hamster cheek such as nuclear density ratio, keratin and epidermal thickness and found it to be increased in dysplastic, carcinoma in situ and squamous cell carcinoma lesions as compared to normal tissue. Zhuo et al. [80] quantified the goblet cell population in the obtained images and found a significant drop of goblet cells in colonic adenoma as compared to normal tissue. They also observed the diameter of colonic crypts in basement membrane to vary among normal, precancerous and cancerous stages in a later study [81]. Meanwhile Chen et al. [82] observed increased size, variability and orientation angle of gastric glands in gastric cancer samples from in ex vivo biopsies.

Although various studies have shown the potential of label-free NLOI as a substitute to conventional histopathological diagnosis, there are still associated limitations. Among
all microscopic information that can be provided by label-free NLOI, nuclear details are the most limited [70,83]. Nuclear detail such as nuclear hyperchromaticity is essential for the pathologist to identify the grade of dysplasia. This eventually determines the prognosis and course of treatment for the patient. Secondly, majority of label-free NLOI studies that showed images with structural details comparable to HE stained slides, were \textit{ex vivo} studies performed on tissue biopsies. The main challenge would be to achieve similar images in real time \textit{in vivo} without being invasive to the patient. While \textit{in vivo} label-free NLOI studies have been performed mainly in animals and limitedly in humans, these images appear to have lower resolution and lesser cellular details than its \textit{ex vivo} counterparts. This could be due to accompanying motion artefacts and increased tissue scattering from thicker biological sample present during \textit{in vivo} imaging. These limitations may however be addressed by future technological advancements like (a) spatial overlap modulation NLOI [84] to reduce background noise, (b) use of advanced motion compensations and adaptive optics during optical imaging [85] to reduce motion artefacts from the specimen or the imaging setup itself, (c) use of lasers with longer wavelengths > 1000 nm [86] to enable deeper imaging and (d) incorporation of photon counting detection to improve signal-to-noise ratio [87]. These advancements may enable label-free NLOI to be a real time non invasive diagnostic tool in the clinic.

2.3.2.2 | TPEF spectral analysis

During neoplastic transformation, production of proteins, amino acids, lipids and other cytoskeletal structures is often up- or down regulated, resulting in a modified biochemical composition of the involved cells or tissue. This is often reflected in the expression levels of the intracellular and extracellular endogenous fluorophores as well, resulting in altered spectral properties of the affected tissue.

One of the approaches utilised for this type of analysis is tissue spectral characterisation. The method involves obtaining the autofluorescence emission spectra of the neoplastic tissue in organ by label-free NLOI. The spectral characteristics like spectral shape, spectral symmetry and emission peak is then compared with the autofluorescence emission spectra obtained from a normal region in the same organ. TPEF based spectral characterisation has been useful in distinguishing between various skin malignancies. In the work of Giorgi et al. [88] multi-spectral imaging could discriminate BCC from normal skin. Later Leupold et al. [89] was able to spectrally characterise and distinguish clearly between malignant melanoma, common nevi, oculocutaneous albinism and vitiligo.

Besides skin, TPEF based spectral characterisation has also been studied in oral cavity cancer models in hamster. In an \textit{in vivo} study conducted by Edward et al. [90] they observed the normal oral cavity in the hamster to have symmetrically shaped autofluorescence spectra with an emission peak at 515 nm. On the other hand, they observed asymmetric shaped autofluorescence spectra in the dysplastic regions induced by chemical carcinogenesis in hamster oral cavity. They also noted that the emission peak was now shifted to approximately 480 nm as clearly shown in Figure 4. This spectral shift towards ‘blue’ side was observed in the
stratum corneum, epithelial layers and to a lesser extent in the stroma of dysplastic regions in hamster oral cavities. In another study, Palero et al. [91] too observed spectral changes in hairless mouse skin in vivo following UV irradiation. Their results depict a similar blue shift in the emission peak of UV irradiated mice skin (~450 nm) in the stratum spinosum layer, as compared to the emission peak of the corresponding layer in non-irradiated skin (~475 nm). However it should be duly noted that since the biochemical composition vary from organ to organ, the nature of emission spectral change during carcinogenesis would be unique for that specific organ. Therefore further research should be invested in using label-free NLOI to identify the characteristic spectral changes during carcinogenesis in other organs as well that have high incidence of malignancies such as colon, breast and ovaries.

Another method of spectral analysis utilised in label-free NLOI studies involves unmixing the tissue emission spectra to obtain the relative contribution or quantity of individual fluorophores in the tissue section. This gives a clearer picture on the biochemical changes happening in tissue during carcinogenesis. Traditionally, spectral unmixing involves measuring fluorescence emission using various channels, each covering a region of the emission spectrum. Before imaging, a calibration is performed with a solution containing multiple fluorophores of interest in known concentrations. The contribution of each fluorophore species in solution to each emission channel is then measured. Thereby a sample containing a mixture of fluorophore species of interest can then be quantitatively unmixed using this calibration information. This method is known as linear unmixing [92]. In contrast to linear unmixing which can be used only for samples containing known fluorophores, other unmixing techniques are available to quantitatively unmix a sample composed of unknown fluorophores. These methods include parallel factor analysis (PARAFAC) [93], principle component analysis (PCA) [94], non negative matrix factorisation (NMF) [95] and spectral phasor analysis (SPA) [96]. These methods use various unique algorithms to unmix multiple fluorophores in spectrally resolved microscopy image. Most label-free NLOI in cancer research have utilised linear unmixing to quantify known intracellular fluorophores such as NADH and FAD to obtain information on cellular metabolism during carcinogenesis. This aspect of spectral unmixing is covered in more detail in the next section. Further studies should be done in cancer models by using the other unmixing techniques such as SPA, PARAFAC, NMF and PCA to obtain additional spectral details from unknown tissue fluorophores. The new information may throw more light on the biochemical changes during carcinogenesis.
Figure 4 | *In vivo* auto fluorescent emission spectra of stratum corneum in normal oral cavity as compared to moderate and severe dysplasia of the oral cancer model in hamster (Excitation wavelength = 780 nm). The emission peak of normal region in oral cavity lies at 515 nm, while the emission peak of the dysplastic region is ‘blue-shifted’ to 480 nm [90].

2.3.2.3 | Metabolic assessment of normal versus cancer cells
During carcinogenesis, cells divide and grow uncontrollably often resulting in increased substrate consumption and energy expenditure. Due to this increased cellular metabolism, it is hypothesised that the cancerous cells shift from oxidative phosphorylation to the glycolytic pathway either in absence of oxygen (called diminished Pasteur effect) or in presence of oxygen (called aerobic glycolysis or Warburg effect) [97-99]. As this transition occurs, oxidative phosphorylation is relatively down-regulated or diminished resulting in altered equilibrium between NADH and FAD. This altered metabolic profile in cancer cells may be assessed by monitoring the fluorescent forms of reduced NADH and oxidised FAD using label-free NLOI.

An indicator of cellular metabolic activity often studied by researchers is the redox ratio (reduction oxidation ratio) which is obtained by quantifying fluorescent intensities from NADH and FAD and expressing it as a ratio. Since its formulation [100,101], the redox ratio has been researched on widely in many preclinical animal and human cancer models. The redox ratio equation is often expressed as [FAD+] / [NADH] or another derivative format of the same. The most notable of the studies investigating redox ratio was performed by Skala *et al.* [102-104] in an oral cancer model of hamster cheek *in vivo*. They formulated the ratio as FAD/FAD+NADH and observed it to decrease in precancerous stages.

Majority of the *ex vivo* studies that investigated the redox ratio observed a decrease in the redox ratio [76,105,106] which was is in agreement with the findings of Skala *et al.* Other *ex vivo* studies done by Zhuo *et al.* (as depicted in Figure 5) [107] and Shah *et al.* [108] found redox ratio calculated inversely as NADH/FAD to be increased in cancerous colonic biopsies and malignant cell lines respectively. *In vivo* studies conducted by Walsh *et al.* in mouse xenograft models for breast cancer [109] and Edward *et al.* in oral cancer models in hamsters [90] also showed similar findings. However a rare exception would be the work of Kirkpatrick *et al.* [29] where the redox ratio (equated as FAD/FAD+NAD(P)H) was found to be increased in high-risk
normal tissue and even higher in cancerous ovarian biopsies, as compared to low-risk normal tissues. The authors reasoned that the decreased redox ratio in normal-low risk tissue indicated a normal functioning surface epithelium that still relied on mitochondrial aerobic metabolism. On the other hand, increased redox ratio measured in high-risk normal and cancerous ovarian tissues may have been due to substrate (glucose) limitation to the tissue leading to reduced intracellular NAD(P)H levels.

Among recent studies, the redox ratio has also been used for monitoring metabolic response to targeted cancer therapies. Walsh et al. [109] monitored the effect of Trastuzumab, an HER2 inhibitor, on the metabolic profile of Trastuzumab responsive and resistant breast cancer xenografts in mice in vivo. A drop in the redox ratio was clearly observed in the Trastuzumab responsive xenograft suggesting a restoration of normal oxidative phosphorylation from glycolysis and hence a positive therapeutic response.

When investigating the role of redox ratios, it must be borne in mind that the molecular mechanisms involved in carcinogenesis are dynamic and would fluctuate according to tumour microenvironment changes. Gatenby et al. [110], clearly explains that the cells involved in malignant transformation are all in different physiological states such as hypoxic, glycolytic, acidotic and motile. The redox ratio would vary for these different stages, thus making redox ratio extremely spatially dependant. It is highly probable that the necrotic region of the tumour would possess a different redox ratio compared to its proliferative zone. So it would be erroneous to generalise the ratio for the entire tumour volume, while only interpreting data
from the surface of the lesion. Certain *in vivo* and *ex vivo* label-free NLOI studies have observed the redox ratio to decrease with depth [103,105] as the more metabolically active proliferative cells are located deeper. However no concrete studies have been performed on the regional or spatial variation of redox ratios within a tumour and normal tissue as such.

### 2.3.2.4 SHG based quantitative and morphometric analysis of collagen

It has been well established that changes in extracellular collagen play a key role in facilitation of tumour progression, invasion, angiogenesis and metastasis [111-115]. Bearing this in mind, many preclinical clinical studies have utilised SHG from collagen as a diagnostic marker in label-free NLOI. Provenzano *et al.* [116] observed that high collagen density promoted tumourigenesis, invasion and metastasis in a transgenic mice breast cancer model. In stark contrast to the mentioned study, Xiong *et al.* [117] found that collagen appeared broadened, integrated and well distributed in normal skin, while it was fragmented, reduced and disordered in cutaneous squamous cell carcinoma (cSCC) and basal cell carcinoma (cBCC). Loss of collagen associated SHG was also observed in neoplasia of oropharynx, [118], lungs [119], rectum [75], oesophagus [120], while SHG was observed to be absent in breast carcinoma [70]. This disparity could arise due to variation in species, related organ or the tumour microenvironment involved for that specific cancer subtype.

In another group of studies, researchers have expressed the SHG relative to endogenous autofluorescence as a quantitative ratio. Zhuo *et al.* [121] quantified the ratio as SHG to TPEF in oesophagus and found it to be considerably decreased in neoplastic stroma. According to the study, the results seemed to imply that the SHG from intact collagen accounted for most of the signal in the normal stroma, while collagen structures in the neoplastic tissue are disrupted leading to diminished SHG signal. In the neoplastic condition, increased elastin enhanced the TPEF signals, causing the SHG-TPEF ratio to decrease further. Later Wang *et al.* [13] used a parameter called as multi-photon autofluorescence (MAF) to SHG index (MAFSI) and defined it as MAF-SHG/MAF+SHG. MAFSI was applied in human lung biopsies and found to be higher for lung adenocarcinoma and squamous cell carcinoma as compared to normal.

Researchers have recently discovered that collagen alignment and organisation also play a prominent role in cancer progression. It was seen in the work of Nadiarnykh *et al.* [122] and Kirkpatrick *et al.* [29] that malignant tissue was found to have more ordered and regularly packed collagen as compared to normal tissue. Newer studies have focussed on definitive parameters like collagen alignment angle and inter collagen fibre separation to assess the level of order in stromal collagen organisation. In a very interesting study, Conklin *et al.* [123] observed that increase in the number of collagen fibres aligned perpendicularly to the tumour boundary was associated with decreased survival in the patient. Similar findings were also observed in human ovarian biopsies by Adurs *et al.* [124], where the majority of collagen stromal fibres in malignant tissue were aligned perpendicular to the epithelium and had a collagen alignment angle of 90°. However in normal ovarian biopsies, the fibres lie in parallel to epithelium with a collagen alignment angle of 0°. Meanwhile Zheng *et al.* [125] found the
inter-collagen fibrillar distance to be decreased in breast adenocarcinoma, implying more densely packed collagen in breast cancer tissue as compared to normal. This is in agreement with the findings of Provenzano et al. [116] mentioned earlier.

While all these studies firmly establish collagen based SHG as a prime cancer signature, its potential as a biomarker for premalignant changes is yet to be fully realised. Certain studies have already indicated that alteration in collagen matrix starts at a much earlier stage of carcinogenesis [126-129]. Future SHG related research should thus investigate as to when do the earliest alterations in SHG signal take place. Collagen based SHG could then serve as an informative descriptor that identifies early carcinogenesis even before cellular changes are visible in standard histopathology.

2.3.3 | Translating label-free NLOI research to the clinic

The majority of the studies that used label-free NLOI in translational cancer research have employed in vitro or ex vivo cancer models. Samples used commonly for in vitro or ex vivo studies are obtained either from cell lines, tissue biopsies from experimental rodents or human patients. The organs often used as source for these samples include the skin, breast, the gastrointestinal tract, ovaries lungs, pancreas, liver, bladder and prostate. The in vitro cell lines are often imaged in the cell culture media. On the other hand, ex vivo tissue samples after biopsy are often kept cold on ice to prevent tissue deterioration till the time of NLOI and imaged freshly, being kept moist with phosphate buffered saline throughout the imaging procedure. However, in other studies that compared metabolic activity between normal and neoplastic samples, the tissues were maintained in a buffer media at 37°C to preserve and maintain the metabolic activity of the ex vivo tissue sample. In fact, Kirkpatrick et al. [29] constructed an oxygenated tissue perfusion chamber to keep ovarian tissue viable throughout the imaging procedure.

Although label-free NLOI is relatively simpler to perform on in vitro and ex vivo models, it still remains unclear to what extent these results relate to what happens in the intact organism. It is likely that the cellular microenvironment would differ in an ex vivo and in vivo models, owing to the role of the external environment around it. It remains to be seen if all label-free NLOI findings obtained from in vitro and ex vivo cancer models can be confirmed in cancer models in vivo with comparable sensitivity and specificity.

In vivo studies have been used to investigate cancer related changes in oral cavity of hamster [103,104], skin [12], breast [109] and ovaries in rodents [130]. As mentioned before, the hamster model has been demonstrably useful to study the difference in cellular metabolism between normal, precancerous and cancerous oral cavity lesions. Besides this, Wang et al. [12] had utilised the window chamber mice model to study development of skin cancer using label-free NLOI in hairless mice. However, in vivo label-free NLOI cancer studies that have been performed till date have been much fewer than ex vivo studies.

In vivo imaging trials in human subjects using label-free NLOI is in an even more nascent stage, as very few multiphoton setups have been able to translate successfully to the clinical
domain. Among the few in vivo human studies, Dimitrow et al. [131] demonstrated detection of melanoma with a sensitivity of 85.45%, while Koehler et al. [132] in another study was able to distinguish between actinic keratosis, pemphigus vulgaris, stukkeratosis and skin scars in human subjects. However at present, by far in vivo human label free NLOI cancer research studies is limited to only skin. On a clinical scale, commercial devices such as DermalInspect and MPTFlex from JenLabs, Germany [3,133] or similarly built devices are currently available for dermato-pathological diagnostics.

However, label-free NLOI holds tremendous value for cancer diagnostics in various other organs as well as demonstrated in the ex vivo studies. In order to extend its applications, miniaturisation of the working setup and development of a fibre based optic probe is being tried [6,134,135], which would allow the clinician to access deep seated organs through the endoscopic or laparoscopic routes. Due to technological advancements in the forms of photonic crystal fibres, miniaturised imaging lenses (gradient index (GRIN) lenses) and scanning systems, fibre based NLOI devices have been currently developed [5,136-138]. While fibre based label-free NLOI has been demonstrated in live tissue [65,138], no such study has been performed yet with regard to cancer research. Nonetheless with further advancements in the flexible fibre prototype for NLOI micro endoscopy, in vivo human trials for cancer research in organs like oesophagus, stomach, colon, breast and ovaries may happen in the times ahead.

2.4 | Future of label-free NLOI based cancer research

Due to technological advancements and scientific innovations, label-free NLOI has taken giant strides towards becoming a valuable imaging modality in cancer diagnosis. However the efficiency of such a label-free NLOI setup in a clinical scenario is yet to be established. A setup like DermalInspect at present offers an in vivo sensitivity of 75% and specificity of 80% in detecting melanoma, as compared to an ex vivo sensitivity of 93% and specificity of 74% [131]. It is yet to be seen if the diagnostic sensitivity can be improved and how the clinical NLOI setup fares for in vivo detection of malignancies in other organs. In case there is a further need of improving the detection threshold of the setup, it would be feasible to combine label-free NLOI with other imaging modalities like optical coherence tomography [139-141] and coherent anti-Stokes Raman’s spectroscopy [3,133,142-144]. Multimodal imaging that is achieved by combination with one of these modalities has the advantage of providing additional information that could not be provided by label-free NLOI. Often anatomical, physiological as well as biochemical details can be extracted when these imaging modalities are combined, increasing the sensitivity of the device in detecting cancer.
This review had mainly focused on studies that were based on TPEF and SHG. But the avenue of three photon based MPEF (or 3PEF) [145,146] and third Harmonic Generation [147-150] (THG) is yet to be fully explored. The biggest advantage here is that 3PEF based label-free NLOI use longer NIR wavelengths than those employed by TPEF based NLOI. These NIR wavelengths penetrates the tissues even deeper and thus enable label-free NLOI at greater tissue depths than TPEF based NLOI. In the work of Horton et al. [145] in vivo three-photon microscopy was performed on an intact mice brain and subcortical structures could be visualised at depths greater than 1mm. Meanwhile THG, like SHG, occurs when three photons are fused to create a single photon at three times the frequency or one third the wavelength. THG had been employed by Tsai et al. [147] and they were able to differentiate between pigmented basal cell carcinoma, seborrhoeic keratosis and melanocytic nevi. It is highly probable that 3PEF and THG will have a significant role to play in the future of NLOI based cancer research, as the required technology is currently available.

Finally, the importance of using label-free NLOI to monitor cancer related changes right from the earliest time-point cannot be stressed enough. In most studies that utilised label-free NLOI to differentiate cancer from healthy samples, the event of neoplastic transformation had already occurred. Only few studies [73,130] have tried to temporally monitor early tissue changes right from the start of cancer induction. The ability of NLOI to detect changes in this stage would make it a very useful screening tool, as a timely intervention in the early stages can reverse the carcinogen induced changes. Most researchers often focus on label-free NLOI as a clinical diagnostic tool, that its potential as a cancer risk screening tool is often overlooked. Once advances are made in these potential areas of research, label-free NLOI can be successfully established as an invaluable tool for the fundamental researchers to understand cancer mechanisms as well as for the clinicians to non-invasively diagnose cancer and provide timely cancer therapy to the patient.
References

27. Y. Wu and J. Y. Qu, Optics Letters 30(22), 3045-3047 (2005).
Advances and challenges in label-free NLOI using TPEF and SHG for cancer research

Advances and challenges in label-free NLOI using TPEF and SHG for cancer research

Chapter 3

Systems Overview
3.1 | Introduction

The combination of optical tissue imaging and fluorescence spectroscopy gave rise to a novel modality of imaging known as spectral imaging. Also known as multi-spectral imaging or hyperspectral imaging, this technique acquires images and then allows fluorescent compounds to be distinctly discriminated, despite the highly overlapping fluorescence emission. As a result, spectral imaging has now become a powerful tool for advanced fluorescence imaging modalities [1,2]. Spectral imaging can distinguish between various fluorophores based on the differences in its fluorescence decay kinetics or emission spectra. Spectral imaging based on fluorescence decay kinetics, generally relies on lifetime imaging methods that utilise time-correlated single photon counting [3] or time-gating [4]. On the other hand, spectral imaging based on differences in emission spectra can be performed using a set of emission filters [5, 6] or dispersive optics and a photomultiplier tube (PMT) array [7] or charge coupled device (CCD) camera [8]. Compared to lifetime imaging methods, spectral imaging based on emission spectra is advantageous in being able to offer detailed spectroscopic analysis in high resolution images, as shown in earlier in vivo studies [9,10]. Spectral and morphological information can be obtained simultaneously in real time by ‘real colour representation of the spectral images in the red-green-blue (RGB) format [9,10]. In addition, individual endogenous fluorophores can be identified in the imaged tissue based on their distinct spectral signature [9,10].

At present, spectral imaging has been successfully incorporated for commercially available confocal fluorescence microscopes [11-15]. On the other hand, newer studies have now demonstrated the merits of utilising spectral imaging in combination with nonlinear optical microscopy [9,10,16-21]. As mentioned earlier in Chapter 1 and 2, compared to confocal fluorescence microscopy, nonlinear optical microscopy benefits from: (a) reduced photobleaching, (b) deeper image penetration depth and (c) increased fluorescence collection efficiency. Therefore nonlinear spectral imaging would be more sensitive and efficient when performed in vivo on a thicker biological specimen, when compared to spectral imaging with confocal fluorescence microscopy that is limited to mainly optically thinner ex vivo tissue sections.

The ability of our particular setup to provide high quality spectral images and reveal emission spectral difference in normal murine and human skin layers in vivo has already been demonstrated in the work of Palero et al. and Bader et al. [9,10,17]. In this chapter, a brief description of the setup is provided along with the calibration techniques adopted prior to the imaging experiments described in the later chapters of this thesis.
3.2 | Description of the setup

The setup was based on a home-built inverted microscope and consisted of a mode-locked titanium: sapphire (Ti: Sa) laser (Tsunami, Spectra-Physics, Sunnyvale, California, U.S.A), pumped by a neodymium yttrium vanadate (Nd: YVO₄) laser (Millennia, Spectra-Physics, Sunnyvale, California, U.S.A). The laser can generate pulses with duration ranging from 70 to 100 femtoseconds, at a repetition rate of 80 – 82 MHz. The excitation wavelength of the laser can be tuned from 700 nm to 1000 nm with an output average power of 1 W.

The laser beam was attenuated by a long-pass filter glass (Model 5254, New Focus, California, U.S.A). After passing through the filter, the laser excitation beam was directed to a galvanometer scanning mirror (040EF, LSK, Stallikon, Switzerland) that moves the focus in the x-y plane. A temperature controlled z-piezo translation stage (Mad City Labs, Madison, WI, USA) was also incorporated to scan the sample in z plane. The scanned laser beam was then focussed by infinity corrected water-immersion objective (40 X, NA = 0.8, Nikon, Japan) onto the sample. The same objective lens collected the resultant emission beam from the sample and directed it back to the scanning mirror that reflected it towards a dichroic mirror (Laseroptik, Garbsen, Germany).

The emission beam then passed through the dichroic mirror and a multi-photon emission filter (FF01-680/SP-25, Semrock, Rochester, New York, U.S.A). A fused silica equilateral prism (Linos, Gottingen, Germany) dispersed the filtered emission beam and focused it on an electron multiplying charge coupled device (EMCCD) camera (Cascade 128þ EMCCD, Photometrics, Tucson, AZ, U.S.A). The EMCCD was equipped with on-chip multiplication gain, where the signal was amplified before the readout electronics. Therefore, this camera was very suitable for detecting low light levels. A schematic diagram of the nonlinear optical spectral imaging system is shown in Figure 1.

Spectral acquisition and processing was performed by software written in V++ (Digital Optics, Auckland, New Zealand). The raw data file was processed in V++ by a script written in the built-in programming language called Vpascal. This script executed the following functions: (a) averaging and subtraction of background spectra from the fluorescence emission spectrum, (b) applying a threshold, (c) creating 8-bit auto-scaled intensity images by summation over the whole emission spectrum and (d) applying 8-bit RGB colouring. The spectral images were then converted to real colour RGB images by multiplying the emission spectrum by the spectra for the red, green and blue sensitivities of the human eye (flat-field correction is included). The sums of these multiplied spectra gave the R, G, and B values, respectively. The R/G/B values were then scaled from 0 to 1 (the latter being the largest of R, G, and B) and multiplied by the intensity (8-bit) of the pixel.
3.3 | Calibration of the setup

Wavelength calibration was accomplished by using standard red, green and blue RazorEdge filters (Semrock, Rochester, New York, U.S.A) and inserting a multiband band-pass filter (Semrock FF01-390/482/563/640-25) in the emission path of the microscope. The multiband pass filter has steep edges (~ 1 nm) and by dividing spectral recordings with and without the filter, positions of the edges are obtained. Calibration was finalised by fitting the pixel positions with a 2nd order polynomial. The wavelength was additionally monitored with an accuracy of up to 1 nm by measuring the SHG peak position from collagen in an \textit{ex vivo} sample of pig skin.

The correction for the wavelength dependent sensitivity of the system (flat-field) was composed of the spectral response of the camera multiplied by the transmission curves of the optical components in the emission path (e.g., dichroic and filter) and a correction for the wavelength dependent dispersion of the prism. Two-photon spectra from solutions of Lucifer Yellow (Life Technologies Europe, Bleiswijk, the Netherlands) and Coumarin-120 (Sigma Aldrich Chemie, Zwijndrecht, the Netherlands) were used to do the flat-field calibration of the nonlinear optical spectroscopy setup. The emission maxima for Lucifer Yellow and Coumarin-120 were recorded to be at 545 nm and 440 nm respectively.
3.4 | Summary

The nonlinear spectral imaging system used for the experiments described in this thesis was capable of the following:

(a) Produce spectral images with optimal spectral sensitivity in the emission wavelength range of 350 to 600 nm.
(b) Provide spectral resolution of ~ 5 nm.
(c) Acquire spectral images at 6.5 seconds and 2.25 minutes for an individual scan and a stack of 20 scans respectively. The use of an EMCCD based spectrograph that can acquire 8000 spectra per second enabled the spectral imaging to be fast and robust. As a result, motion artefacts were minimised because of reduced acquisition time.
(d) Simultaneous acquisition and correction for the background noise. The described software accurately corrected for stray light and temporal variation from the background.
(e) The emission was detected with high quantum efficiency by the EMCCD camera, due to the on chip multiplication gain. As a result, sufficient signal for RGB visualisation and detailed spectral analyses could be performed with an excitation laser power ranging from 5 – 20 mW on normal skin tissue.

Due to the aforementioned aspects of the described setup, it was possible to produce high quality spectral images that can be used to distinguish between normal and cancerous mice skin based on morphological and spectroscopic changes. Those findings have been explained in detail in chapters 7 and 8 of this thesis.
References

Chapter 4

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

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From

Abstract

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

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

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

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

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

However, the biggest safety concern has always been the direct DNA damage from simultaneous absorption of three photons, as strong absorption bands of nucleic acids lie below 290 nm, peaking at 257 nm. The DNA lesions induced are similar to those from natural UV exposure resulting in formation of cyclobutane pyrimidine dimers (CPDs), 6-4 photoproducts, single- and double-strand breaks, and numerous modified bases.

Among all these lesions, CPDs are primarily responsible for carcinogenesis resulting from a series of mutations in cells with unrepaired damage. The genotoxic cellular effects of the shorter UV wavelengths (UVC band, 200 to 290 nm, and UVB band, 290 to 320 nm) have been long known and thoroughly investigated. However, the DNA damaging effects of longer wavelengths (UVA band, 320 to 400 nm) causing strong mutagenic properties have been recognized only recently. There is an ongoing debate regarding the direct and indirect pathways leading from UVA absorption to CPDs. Several studies have claimed that UVA absorption produces reactive chemical species that can damage DNA. However, two recent studies strongly suggested that CPDs are produced by UVA via direct photochemical mechanism, without mediation of a cellular photosensitiser or intermediate reactive species. Moreover, showed evidence that UVA-induced CPDs are less repairable. In their experiment, 48 hours after exposure to UVA radiation the level of unrepaired CPDs was unexpectedly higher than after UVB induced damage. There are no indications that different CPDs are produced by absorption of UVA and UVC photons. However, the possible explanations for persistence of UVA induced CPDs include different in-cell cycle arrest after irradiation, degradation of DNA repair protein by the UVA induced reactive oxygen species as well as alteration of cell behaviour through changes in cell signalling pathways by UVA radiation.

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

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

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

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

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

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

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

4.3 | Results and Discussion

A typical image of TPEF from CHO cells is shown in Figure 1(a) along with the corresponding one photon immuno-fluorescence from CPDs in the nuclei of fixed cells as seen in Figure 1(b). Very minimal cell migration was noted between the start of irradiation and fixation with formalin. A clear border of region with CPD damage corresponds to the edge of the x-y scan; the cells in the area unexposed to laser exhibit a very low level of CPD immuno-fluorescence. The background level of CPDs in the cells can be attributed to CPDs formed by exposure to stray white light during culturing and especially during the experiment. Non-specificity of the CPD staining was ruled out by a separate negative control experiment. As expected, CPD damage from imaging with fs laser was evenly distributed within cell nuclei as equal doses of radiation were delivered to every pixel. Any inhomogeneity in the damage pattern corresponded to local concentration of available DNA. On the contrary a seen in Figure 1(c), CPDs induced by UVC lamp (positive control) showed a doughnut-shape distribution with stronger damage at the nuclei periphery. This finding could be explained by strong absorption of 254-nm photons by DNA. It has been shown that in some laser-damaged cells, DNA lesions
Carcinogenic damage induced to DNA by fs laser pulses via 2- & 3-photon absorption during NLOI

...tend to migrate toward the periphery of the nuclei, which might explain the doughnut shape distribution of CPDs [32]. Therefore the possibility that uniformly damaged nuclei might carry different consequences for cell viability, repair, and carcinogenic mutations requires further investigation.

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

4.3.1 | Intensity versus Damage

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

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

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

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

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

For n-photon absorption process, the number of photons absorbed per molecule (nₐ) is given by Equation 1 [46].
Carcinogenic damage induced to DNA by fs laser pulses via 2- & 3-photon absorption during NLOI

(1) \[ n_\sigma \approx \frac{P^n_0 \sigma_{n-ph}}{f_{rep}^{(n-1)} \tau^{(n-1)}} \left( \frac{NA^2}{2hc\lambda} \right)^n \]

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

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

![Figure 3](image-url)  

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

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

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

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

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

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

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

Figure 4 | CPD immunofluorescence signal dependence on peak intensity of fs laser. The fits to Eq. (2) \( \text{CPD} \sim A_0 + A_2I^2 + A_3I^3 \) were used to decouple the relative contributions of two- and three-photon absorption to CPD damage. Pixel dwell time: 30 μs, pulsewidth at the focal plane: 164 fs.

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

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

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

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

![Figure 5](image)

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

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

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

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

![Figure 6](image.png)

Figure 6 | Effect of pixel dwell time on induction of CPD lesions.

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

4.4 | Conclusion

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


Chapter 5

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

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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 Jm⁻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 Jm⁻² – 1035 Jm⁻² for UVC and 100 KJm⁻² – 500 KJm⁻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 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:

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

(2)

Here $Solar(\text{annual})$ 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:

$$CD_c(t) = Q \times k \times N \times (t - t_0)$$

(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:

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

(4)

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

$$Q = \int S(\lambda) \alpha(\lambda) d\lambda$$

(5)

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 et al. [19].

Sterenborg 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 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 $1/3$ of $\lambda$ must be used for a 3-photon process. Thus for $S(750 \text{ nm})$, theoretically the value of $a(375 \text{ nm})$ and $a(250 \text{ nm})$ should be used. However, for the in vitro experiment a filtered mercury UV lamp that emitted either only 254 nm or 365 nm was used. But the difference between $a(250 \text{ nm})$ and $a(254 \text{ nm})$ was negligible, and the same was the case for $a(375 \text{ nm})$ and $a(365 \text{ nm})$. Thus the effective action spectrum for an MPE process will be denoted as $a(\lambda/n)$, where $n = 2$ or $3$ depending on the MPE process.

Second, it has been shown by Denk 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:

$$Q = I^n(\lambda) \cdot T \cdot \alpha(\lambda/n) \cdot C_{fs vs. cw \lambda/n}$$

(6)

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 $C_{fs vs. cw \lambda/n}$ 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:

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

(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:

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

(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 $C_{\text{fs} \text{vs} \text{cw} \lambda/n}$

$C_{\text{fs} \text{vs} \text{cw} \lambda/n}$ 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 \( \mu \text{m} \) 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 \( \mu \text{m} \) for UVC (254 nm) and 40 \( \mu \text{m} \) 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.

\[
C_{fs vs cw \lambda/n} \text{ is described by:}
\]

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

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 \( Dose_{exp \text{UV}} \), i.e., the experimental CW UV dose.
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.

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:
\[ I_{fs-laser}^{n} (r, z) = \frac{I_{0 \, fs-laser}^{n}}{(\pi \omega^2(z))^n} \cdot \exp \left( -\frac{2r^2 \cdot n}{\omega^2(z)} \right) \]  \hspace{1cm} (10)

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

\[ \omega^2(z) = \omega_0^2 \left( 1 + \left( \frac{\lambda \cdot z}{\pi \omega_0^2} \right)^2 \right) \]  \hspace{1cm} (11)

Here, \( \omega_0 = \) beam spot radius at focus.

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 \( \sigma_n \) is assigned for the 3 NIR wavelengths, as shown in Table 1.

Table 1 | Tissue volume sections \((\sigma_n)\) in which CPD production would occur from one pulsed laser scan. Beam spot \( \omega_0 \) radius = 398 ± 20 nm (2p – 2 photon, 3p – 3 photon, \( z \) – distance from scanned layer in \( z \)-axis).

<table>
<thead>
<tr>
<th>Pulsed NIR wavelength</th>
<th>( \sigma_n ) ((m^3)) from 1 pulsed laser scan in CHO cell monolayer ((z = -3 \mu m \ to \ +3 \mu m))</th>
<th>( \sigma_n ) ((m^3)) from 1 pulsed laser scan in skin tissue ((z = -20 \mu m \ to \ +20 \mu m))</th>
</tr>
</thead>
<tbody>
<tr>
<td>711 nm</td>
<td>4.06 ( \times ) 10(^{-13} )</td>
<td>4.14 ( \times ) 10(^{-13} )</td>
</tr>
<tr>
<td>750 nm</td>
<td>2.26 ( \times ) 10(^{-13} )</td>
<td>2.26 ( \times ) 10(^{-13} )</td>
</tr>
<tr>
<td>780 nm</td>
<td></td>
<td></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:

$$I_{UV}(D) = I_{0UV} e^{-D/D_{\lambda/n}}$$

(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.

$$Damage_{cwUV} \propto \int_0^\infty I_{UV}(D) dD = I_{0UV} \int_0^\infty e^{-D/D_{\lambda/n}} dD = I_{0UV} D_{\lambda/n}$$

(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)

Figure 2 | (a) Irradiance variation with skin depth $D$ for a CW UV radiation. $D_{254\text{nm}}$ and $D_{365\text{nm}}$ stand for the attenuation distances for the corresponding CW UV wavelengths in skin (b) Area shaded in orange represents the region of potential DNA damage distribution from CW UV in a tissue volume cross section of radius $\omega_0$ and depth $D$, where $D_{\lambda/n}$ is the attenuation distance of CW UV $\lambda/n$. Note that the tissue cross section for CW UV is also considered as $\omega_0$ as tissue volume affected by CW UV irradiation needs to be compared to that by fs pulsed laser irradiation for an equal area cross section of tissue.
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 $\omega_0$ 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</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 $\varepsilon$:

$$\varepsilon = \textit{Area factor} = \frac{\text{Area}_{(\text{fs biopsy + Sun})} \times b}{\text{Area}_{(\text{Sun only})}}$$ (15)

Here, Area$_{(\text{fs biopsy + Sun})}$ represents the area that receives radiation from one laser scan in addition to UV radiation from sun, while Area$_{(\text{Sun only})}$ 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 $\approx 2.6 \times 10^{-8}$ 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:

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

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

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

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

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 \( \Phi_{\text{exp}}, f_{\text{exp}}, \tau_{\text{exp}} \) and \( s_{\text{exp}} \) 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 \( 10^{4.045:1} \) 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.
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 $10^4$ 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 5 | CPDs produced per unit irradiance (Jm⁻²) from UVA, UVC and one scan of femtosecond (fs) laser irradiation for different NIR wavelengths, based on fluorescent intensity from CPD immunofluorescence assay.

5.3.2 | Relative Risk Calculations for Nonlinear Optical Biopsy

The determined values for the CPD production efficiency factor ($C_{fs \text{ vs } cw \lambda/n}$), tissue volume section affected by UV and pulsed laser irradiation ($\sigma_{cw\text{ UV}}$ and $\sigma_{fs}$) 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 nm) = 0.00029, $\alpha$(254 nm) = 0.156)

Calculating $\sigma_{cw\text{ UV}}$ 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 3.1 | CPD production efficiency factors ($C_{fs \text{ vs } cw \lambda/n}$) for different NIR wavelengths.

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>$C_{fs \text{ vs } 365\text{ nm}}$ (Wm⁻²)</th>
<th>$C_{fs \text{ vs } 254\text{ nm}}$ (Wm⁻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 (σ_{cw UV} and σ_{fs}) in which CPD production would occur from CW UV and pulsed NIR irradiation. Beam spot diameter = 398 ± 20 nm. An averaged value of σ_{fs} is assigned for the three NIR wavelengths, due to negligible difference between them.

<table>
<thead>
<tr>
<th>CW UV wavelength</th>
<th>σ_{cw UV} (m³)</th>
<th>Pulsed NIR wavelength</th>
<th>σ_{fs} (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>4.14 × 10⁻¹³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>365 nm</td>
<td>2.15 × 10⁻¹⁷</td>
<td>750 nm</td>
<td>2.26 × 10⁻¹³</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3 | Fraction of CPDs produced by two-photon processes (β_{2p}) and three-photon processes (β_{3p}) 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>β_{2p}</td>
<td>β_{3p}</td>
<td>β_{2p}</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 $\approx$ 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 $\varepsilon$ was calculated to be $1.3 \times 10^{-6}$. 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>Pulse Energy Fluence (in Jm⁻²)</th>
<th>Pulse Energy Fluence (in Jm⁻²)</th>
<th>Pulse Energy Fluence (in Jm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>711 nm</td>
<td>750 nm</td>
<td>780 nm</td>
<td></td>
</tr>
<tr>
<td>After 1 Biopsy at age of 30 years</td>
<td>1.0000061</td>
<td>1.000097</td>
<td>1.0000035</td>
<td>1.000061</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>500</td>
<td>250</td>
<td>500</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>1.0000020</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>1.0021</td>
<td>1.019</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>500</td>
<td>250</td>
<td>500</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></td>
<td></td>
</tr>
<tr>
<td>k = 10</td>
<td>b = 10</td>
<td>1.000027</td>
</tr>
<tr>
<td>k = 20</td>
<td>b = 20</td>
<td>1.000097</td>
</tr>
<tr>
<td>k = 40</td>
<td>b = 40</td>
<td>1.00039</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>b = 10 sites</th>
<th>b = 20 sites</th>
<th>b = 40 sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>k = 20 scans/site</td>
<td>k = 10 scans/site</td>
<td>k = 5 scans/site</td>
</tr>
<tr>
<td>Total scans = 200</td>
<td></td>
<td>Total scans = 200</td>
</tr>
<tr>
<td>1.000097</td>
<td>1.000055</td>
<td>1.000039</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
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 (\(\varepsilon\)) was not taken into account for their risk calculation. The role of area factor (\(\varepsilon\)) 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 ≈ 7mW), 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 \(\approx 20\text{mW}\)) 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

Investigation of 7, 12-dimethylbenz(a) anthracene as a complete carcinogen in development of cutaneous squamous cell carcinomas by chronic exposure in immunocompetent hairless mice

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Abstract

Earlier studies have already established that the chemical carcinogen 7, 12-dimethylbenz(a)anthracene (DMBA) can initiate cutaneous squamous cell carcinoma (cSCC) formation via hair follicle stem cells in murine models. However, tumour development requires additional topical application of a promoter agent. In this study, it was investigated if DMBA can function as a complete carcinogen to produce cSCCs in hairless SKH1-hr mice that have abnormal hair follicles. The DMBA application regimen was optimised to improve the yield of malignant cSCCs over benign skin tumours in SKH1-hr mice. The results showed that DMBA at 30 μg in 100 μl once a week and 15 mg in 100 ml twice a week produced skin tumours after just 7 – 8 weeks. However, mice exposed to these two regimes could be studied only until week 14 as it resulted in a heavy tumour yield leading to early euthanasia, while the all tumours were found to be benign. It was seen that lowering DMBA dose to 15 μg in 100 ml just once a week produced tumours at a slower rate but allowed the mice to be studied further till week 27. Despite the slower tumour induction rate, this low dose DMBA regime yielded a high percentage of malignant tumours (59%) after just 23 weekly applications. In addition, the histomorphological changes and expression of markers for Ras activation (p-Erk1/2), basal or undifferentiated keratinocytes (keratin 14), proliferation (Ki67), tumour suppression (p53) and apoptosis (cleaved caspase 3) were assessed at different stages of skin carcinogenesis. Keratin 14 and Ki67 showed an increased expression that started in early keratinocyte proliferation stages and continued to increase with further tumour progression. In contrast, p53 and cleaved caspase 3 were detected only towards the late malignant stages of skin carcinogenesis. Interestingly p-Erk1/2 showed a marked shift from an expression in terminally differentiated keratinocytes in normal mice skin to proliferating compartments in cSCCs. This hairless and immunocompetent mice model of cSCC induction using DMBA can be reliably replicated and be further developed into a highly valuable model for biomedical research on skin cancer diagnosis and therapy.
6.1 | Introduction

In the past few decades, the global incidence of cSCC has been on the rise [1,2] due to a) a longer life expectancy leading to an ever increasing geriatric population who are particularly at a higher risk for cSCC [3], and most likely for a major remaining part due to b) increased ultraviolet (UV) exposure from the sun and tanning beds [4-7], and c) improved awareness, registration and reporting of incidences [2]. The progression of cSCCs to terminal or morbid stages occurs – at least in part – due to genetic instability and a cumulative build up of irreversible genetic mutations in skin. Emulating the tumour progression in animal models provides insight into the process and the possibility of preventive or therapeutic interventions.

Various murine strains have been utilised for the development of skin cancer models. Notable murine strains that have been investigated for this purpose include SENCAR, CD-1, C57BL/6, BALB/c and DBA/2 mice [8-10]. The introduction of hairless strains of mice in the 1960s-70s has proved highly advantageous to skin cancer research [11,12]. This is mainly because hair depilation, which is time consuming and can cause inflammation in skin, is rendered unnecessary for hairless mice. As a result the carcinogen and potential chemopreventive/chemotherapeutic compounds can be applied topically to hairless skin with ease. Moreover, tumours can be easily identified and observed from their earliest stages, in contrast to the conventional hairy murine models. In addition, the varying effects of hair cycle on skin carcinogenesis are minimised.

Among the various hairless murine strains commercially available, outbred albino SKH1-hr mice are the most widely used in dermatologic research [11]. The popularity of the SKH1-hr mice for experimental carcinogenesis is predominantly because of the following factors: (a) UV-induced tumours formed in these mice resemble cSCCs in man at the morphologic and molecular levels, (b) the strain is nonpigmented and therefore enables easy visualisation of cutaneous response to carcinogens and (c) the SKH1-hr mice are thymic and immunocompetent which makes the onset and progression of skin cancer in these models more comparable to humans in contrast to immunocompromised hairless murine strains such as SHO, SHC and SHrN.

The use of the various aforementioned murine strains in skin cancer models has clearly confirmed the sequential, multistage nature of skin carcinogenesis, which conceptually involves the following stages:

(i) **Initiation**: This is the first stage of carcinogenesis during which the normal keratinocytes undergoes the first somatic mutation and by which the cell is conditioned for further tumorigenesis. Murine skin carcinogenesis initiation is generally accomplished by a single exposure to a subcarcinogenic dose of the initiator agent (mutagen and carcinogen). This step is irreversible and occurs within 2 weeks of exposure to the initiator agent.

(ii) **Promotion**: The next stage of skin carcinogenesis involves repeated and chronic exposure to a “promoter”, a non-mutagenic and hyperproliferation inducing agent. In contrast to the initiation stage, the promotion step is lengthier and reversible. The promotion stage lasts 10 – 40 weeks.
(iii) **Malignant progression:** The promotion stage is sometimes followed by malignant transformation manifested by the invasive nature of the tumour and commonly linked to genetic instability and chromosomal aberrations. This stage can be enhanced by an additional post-promotion application of a mutagen (initiator) [13].

The most commonly used initiator agents are 7, 12-dimethylbenz(a)anthracene (DMBA), benzo(a)pyrene, N-methyl-N-nitrosourea (MNU), bis(chloromethyl)ether, cisplatinum, b-propiolactone and UV irradiation while the promotion stage of carcinogenesis can be achieved with 12-O-tetradecanoylphorbol-13-acetate (TPA), chrysurbin, benzoyl peroxide, UV irradiation or just skin wounding [8].

SKH1-hr mice skin cancer models generally use UV as a complete carcinogen, assumed to include both initiating and promoting activities. In SKH1-hr mice, complete carcinogenesis has so far been attained only using UV irradiation [14]. Earlier studies have shown that at subacute daily exposures, first tumours (1 mm diameter) are induced in 7 – 8 weeks, and 50% of the animals are tumour-bearing in 11 weeks [15]. These small tumours were benign actinic keratoses – precursor lesions of cSCCs. At 19 weeks 50% of the animals had tumours with diameters > 4 mm, and those tumours were mostly malignant cSCCs.

The two stage chemical carcinogenesis protocol using DMBA as the initiator and TPA as the promoter produces skin tumours with comparable latency times. The DMBA-TPA protocol in SENCAR strain mice produced skin tumours as early as 6 weeks with malignant transformation occurring at 18 weeks [16]. However it must be noted that efficiency and speed of DMBA-TPA carcinogenesis protocol again relies highly on the murine strain [8]. Two stage chemical carcinogenesis has not been utilised for the SKH1-hr mice; although Nijhof et al. [17] had used UV and TPA as initiator and promoter respectively, in SKH1-hr to investigate whether p53-mutant clones could thus be induced in the skin. Van Schanke et al. used neonatal DMBA initiation followed by chronic UV exposure as promotion on SKH2-hr pigmented cross bred hairless mice to raise melanomas, but it resulted mostly in papillomas and cSCCs [18].

One limitation of the two stage DMBA-TPA protocol is that it tends to initially produce more benign papilloma and hyperplastic lesions (possibly actinic keratosis-like lesions when evaluated by human pathologists) that regress upon discontinuation of TPA. On the other hand, certain studies have shown that DMBA can function as a complete carcinogen [19-24]. Since the carcinogenic action of DMBA is mainly exerted by mutation induction in the quiescent stem cells [8,25,26] present in hair follicle bulge region, it is not clear whether skin carcinogenesis with only DMBA would be successful in SKH1-hr mice that possess abnormal hair follicles.

In this study, we have investigated if a cSCC model can be successfully established in SKH1-hr mice by using DMBA as a complete carcinogen. This study first tried to identify the susceptibility of SKH1-hr mice to complete carcinogenesis with DMBA, by utilising different DMBA dosing regimes in a pilot study. The DMBA ‘dose of choice’ was then adopted for a main study wherein the course of skin tumour formation and malignant transformation was studied.
This main study was therefore used to histopathologically observe the various types of skin lesions induced by DMBA over time in SKH1-hr mice. Additionally in order to understand the various molecular changes involved in complete carcinogenesis with DMBA in SKH1-hr mice, expression of various nuclear and cytoplasmic markers such as phosphorylated Erk1/2 (p-Erk1/2), keratin 14, Ki67, p53 and cleaved caspase 3 were investigated.

6.2 | Materials and Methods

6.2.1 | Pilot Study
This experimental animal protocol was discussed and approved by the Animal Research Committee of the Erasmus University, Rotterdam. The pilot study was essential to determine the susceptibility of SKH1-hr mice to carcinogenesis using only DMBA. Moreover it helped in determining the time-frame for onset of tumour formation and the optimal DMBA dosage regimen for the main study. In the pilot study, 25 female albino SKH1-hr mice aged 6 – 8 weeks (Charles River Laboratories, Someren, the Netherlands) were housed under standard housing conditions and fed ad libitum. The mice were randomly and equally distributed over five groups. In the first group, the mice were left untreated, while the mice were treated topically with acetone once a week in the second group (Sigma Aldrich Chemie, Zwijndrecht, the Netherlands). In the third and fourth group, mice were treated similarly with a weekly topical application of 0.15 mg and 0.3 mg of DMBA (Sigma Aldrich Chemie, Zwijndrecht, the Netherlands) dissolved per ml of acetone. In the fifth group, the mice were exposed to biweekly topical application of 0.15 mg DMBA dissolved per ml of acetone. In all cases, the topical application measured 100 µl per mice. The grouping of the mice is shown in Table 1.

The mice were clinically examined every week post the first topical DMBA application, in terms of skin changes, time of first tumour appearance, number of tumours and tumour size. All tumours with diameter greater than 1 mm were included for the total tumour count per mice group at each time point. Increased tumour load (total tumour volume exceeding 2 cm³ per mouse), weight loss of more than 20% from the starting weight, signs of respiratory or circulatory distress was considered as criteria for euthanasia. Euthanasia was performed by cervical dislocation of mice that were anaesthetised by isofluorane inhalation. After euthanasia, dorsal skin or skin tumour biopsies were fixed in standard 10% buffered formalin solution and evaluated histopathologically. The optimal carcinogen dose for the main study was primarily finalised based on the dosing regime efficiency to produce malignant skin lesions (final stage of carcinogenesis) in the mice, without resulting in a heavy benign papilloma load. A heavy benign papilloma load leads to premature euthanasia of the mouse for ethical reasons, even if the mouse has not met the malignant transformation end point. The tumour rate formation between the different groups were compared using 2-tailed student t-test for unequal variance, with p-value < 0.05 being considered significant.
Table 1 | Various DMBA dosing regimes used for the pilot study.

<table>
<thead>
<tr>
<th>Categories</th>
<th>Reagent and Concentration</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1, n = 5 mice</td>
<td>Untreated (control)</td>
<td>-</td>
</tr>
<tr>
<td>Group 2, n = 5 mice</td>
<td>Acetone treated (control) at 100 µl/mice</td>
<td>Once a week</td>
</tr>
<tr>
<td>Group 3, n = 5 mice</td>
<td>15 mg DMBA per mice (0.15 mg DMBA/ml acetone at 100 µl/mice)</td>
<td>Once a week</td>
</tr>
<tr>
<td>Group 4, n = 5 mice</td>
<td>30 mg DMBA per mice (0.30 mg DMBA/ml acetone at 100 µl/mice)</td>
<td>Once a week</td>
</tr>
<tr>
<td>Group 5, n = 5 mice</td>
<td>15 mg DMBA per mice (0.15 mg DMBA/ml acetone at 100 µl/mice)</td>
<td>Twice a week</td>
</tr>
</tbody>
</table>

6.2.2 | Main study
After determining the optimal DMBA dosing regime, 64 SKH1-hr hairless female mice aged 6 – 8 weeks were utilised for the main study. The mice were randomly divided into three groups: Group 1 (untreated), Group 2 (acetone treated) and Group 3 (treated with DMBA dosage determined from the pilot study). After 4, 7, 11, 14, 17, 20, 23 and 27 weeks from the first topical application, 2 untreated, 2 acetone treated and 4 DMBA treated mice were sacrificed at each time point. Following euthanasia, the total number of tumours formed in DMBA treated mice at each time point was counted, based on the tumour diameter as (i) > 1 mm and < 2 mm, (ii) > 2 mm and < 4 mm and (iii) > 4 mm. These values were then plotted in terms of number of weekly DMBA application. As in the pilot study, the dorsal skin or skin tumour biopsies were fixed in standard 10% buffered formalin solution, processed and sent for histopathologic evaluation by a certified veterinarian pathologist.

The histopathologic diagnosis given by the pathologist was eventually converted into a numerical score in a semi-quantitative manner as shown in Table 2. The scores obtained from untreated, acetone and DMBA treated mice were averaged for each selected time point (weeks of DMBA or acetone application). The averaged pathology scores were then plotted versus weeks of treatment received by the group.
Table 2 | Quantitative histopathology scoring system.

<table>
<thead>
<tr>
<th>Histopathological feature</th>
<th>Scoring system</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acanthosis (Hyperplasia)</td>
<td>None – 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mild – 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moderate – 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severe – 3</td>
<td></td>
</tr>
<tr>
<td>Hyperkeratosis</td>
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</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>Moderate – 2</td>
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</tr>
<tr>
<td></td>
<td>Severe – 3</td>
<td></td>
</tr>
<tr>
<td>Parakeratosis</td>
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<tr>
<td></td>
<td>Preneoplastic – 15</td>
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<tr>
<td></td>
<td>Neoplastic – 30</td>
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<tr>
<td>Malignant transformation</td>
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<td></td>
<td>Progression to invasion – 15</td>
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<tr>
<td></td>
<td>Complete malignant transformation – 30</td>
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</tr>
<tr>
<td>Differentiation in tumour</td>
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<tr>
<td></td>
<td>Moderately differentiated – 5</td>
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<td></td>
<td>Poorly Differentiated – 10</td>
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<tr>
<td>Cellular Atypia (Dysplasia)</td>
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<td></td>
<td>Moderate – 3</td>
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<td>Mitotic Activity</td>
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</tr>
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<td></td>
<td>Severe – 3</td>
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<tr>
<td>Invasion</td>
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<td>Distal invasion – 10</td>
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<td>Inflammation</td>
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<td></td>
<td>Severe – 3</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>Maximum – 100</td>
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6.2.3 | Comparison of mean epidermal thickness of clinically normal control and DMBA treated skin

H&E slides were digitally scanned using the Nanozoomer 2.0 HT (Hamamatsu Photonics, Hamamatsu, Japan). Epidermal thickness was measured from the digital scans obtained from H&E slides of clinically normal skin taken from untreated, acetone treated and DMBA treated mice using the NDP 2.0 software. The mean epidermal thickness was then compared between each group. The changes in mean epidermal thickness for untreated, acetone treated and DMBA treated mice was also studies with respect to weeks of DMBA application.

6.2.4 | Immunohistochemistry for p-Erk 1/2, keratin 14, Ki67, p53 and cleaved caspase 3

5 µm thick paraffin slices were obtained on standard slides from the formalin fixed and processed murine skin biopsies. The slides were de-paraffinised by standard protocol and then subject to heat induced antigen retrieval in 10 mM Trisodium citrate solution pH 6.0 (Sigma Aldrich, Zwijndrecht, the Netherlands) for 20 minutes at 95ºC. The slides were then allowed to cool for another 20 minutes. After triple washing with 1X phosphate buffered solution containing 0.1% Tween (PBS-T), the slides were incubated with a endogenous peroxidase blocking solution composed of 3% hydrogen peroxide in 1X PBS-T. Following this the tissue sections were incubated with a solution of 5% bovine serum albumin (BSA) in 1X PBS-T for 30 minutes to minimise non-specific antibody binding.

The tissue sections were later incubated with primary antibodies for the respective antigens/markers for 1 hour. The negative control slides were plainly incubated with 1X PBS-T. The primary antibodies used were polyclonal rabbit anti-mouse antibodies for phosphorylated Erk 1/2 (Cell Signaling, Leiden, the Netherlands) at 1:100 dilution, keratin 14 (Thermo Scientific, Dreieich, Germany) at 1:400 dilution, Ki67 (Monosan, Uden, the Netherlands) at 1:200 dilution, CM5 for wild and mutant p53 (Monosan, Uden, the Netherlands) at 1:200 dilution and cleaved caspase 3 (Cell Signaling, Leiden, the Netherlands) at 1:50 dilution. Tissue sections were subsequently incubated first with the secondary goat anti-rabbit biotinylated antibody at 1:200 dilution (DakoCytomation, Heverlee, Belgium) and then Streptavidin conjugated with horseradish peroxidase at 1:300 dilution (ITK Diagnostics, Uithoorn, the Netherlands) for 30 minutes at each step. These steps were preceded and succeeded by triple washings in 1X PBS-T. The primary antibodies, biotinylated secondary antibodies and streptavidin conjugated with HRP were all diluted in 1X PBS-T. The slides were finally incubated in 2% solution of 3, 3’ diaminobenzidine (DAB) (Sigma Aldich Chemie, Zwijndrecht, the Netherlands) in 1X PBS containing 0.01% hydrogen peroxide for 5 minutes. The DAB colouring reaction was stopped by immersing the slides in deionised water, following which the tissue sections were counterstained in haematoxylin. The slides were then dehydrated successively with standard protocol and coverslipped using Permound mounting medium. While all 5 markers were assessed in a qualitative manner, quantitative scoring was additionally performed for Ki67 by counting the total number of Ki67 positive keratinocytes per mm of interfollicular epidermis.
This was performed only on clinically normal skin from control mice and DMBA treated mice to evaluate if DMBA applications contributed to early proliferation related changes in interfollicular murine epidermis.

6.3 | Results

6.3.1 | Pilot Study
The mice from the control groups 1 and 2 (untreated and acetone treated) did not show any skin changes or tumour formation throughout the pilot study. The first DMBA-induced tumour with diameter > 1 mm was visible at week 7 for mice from group 5 (15 μg DMBA/mice biweekly). This was succeeded by appearance of similar sized tumours in mice from group 4 (30 μg DMBA/mice weekly) at week 8. All mice from groups 4 and 5 were tumour positive by week 10. Mice from group 3 (15 μg DMBA/mice weekly) were the last to develop skin tumours at week 10 and all mice from this group contracted tumours at week 14. Till week 14, the rate of tumour formation for group 5 was significantly faster than group 3 with a p-value = 0.03, while group 4 was more rapid than group 3 as well but not significantly (p-value = 0.07). There was no significant difference observed in the rate of tumour formation between group 4 and 5 (p-value = 0.29) till week 14.

Figure 1 | Graph showing the variation of total tumour count in different groups at each time point (* and ** denotes euthanasia for mice from group 4 and 5 due to increased tumour load for mouse – maximal total tumour volume permitted per mouse was 2 cm³); for total tumour count in each group, all tumours with diameters > 1 mm were considered.

As seen from the graph in Figure 1, the tumour yield increased quite considerably for mice from Group 4 and 5 and met euthanasia criteria by week 14. Due to euthanasia performed because
of heavy tumour yield, the mice from these groups could not be followed beyond week 14. The tumour formed in these mice had diameters ranging from 1 mm to 4 mm. Tumours biopsied at this stage were found to be mainly benign papillomas. No malignant lesions were observed at this time point. Therefore the DMBA dosage used for group 4 and 5 was considered unsuitable for the main study, as it produced high amount of benign skin tumours too quickly. In contrast, mice from Group 3 could be followed up to week 23 following which they were euthanised. Biopsies obtained from tumours taken at that time point comprised of both benign papillomas and malignant lesions as cSCC. It should be noted that this particular DMBA dose regime allowed the murine model to be followed through all skin carcinogenesis stages up to the malignant transformation endpoint, without resulting in a heavy tumour load for the mice. Therefore weekly topical application of 15 μg DMBA per mouse was selected as the standard carcinogenesis protocol for the main study.

6.3.2 | Main Study
In the main study, mice were subjected to the protocol of weekly topical application of 15 μg DMBA per mouse as optimised in the pilot study. The first visible tumours (> 1 mm diameter) were detected during inspection at 11th week of DMBA application. The total number of tumours counted at each time point for DMBA treated mice increased with subsequent weeks of DMBA application as seen in Figure 2. Tumours that appeared initially were mainly > 1 mm and < 2 mm in diameter. These smaller tumours predominated from week 11 to week 17 of DMBA applications. From week 20 onwards, slightly bigger tumours with diameter > 2mm and < 4 mm formed majority of the tumours and this trend was observed till the last time point of the study, i.e. week 27. Large tumours with diameter > 4mm first appeared at week 17 and showed a gradual upward trend with subsequent weeks of DMBA application.

![Figure 2](image_url) | Total tumour count according to tumour diameters in DMBA treated mice at each time point of DMBA application.
As the mice were euthanised at regular time points, it was possible to histopathologically evaluate the tumours at those corresponding time points. All counted tumours were biopsied at weeks 11, 14 and 17. Due to high tumour yield from week 20 to week 27, not all tumours counted in mice could be biopsied and diagnosed. From all tumours with similar size and appearance, only two or three tumours were eventually biopsied. Therefore percentage of tumours biopsied and diagnosed was 36% at 20 weeks, 37% at 23 weeks and 14% at 27 weeks. All tumour biopsies taken at 11 weeks of DMBA application were benign or non-invasive in nature and consisted mainly of papillomas and acanthomas that had no atypia. At 14 weeks, benign tumours constituted 75% of the lesions, while the remaining 25% tumours were categorised premalignant. The former included keratoacanthomas and papillomas with no atypia, while the latter included Bowenoid lesions (squamous cell carcinoma-in situ or SCCIS) and papillomas with atypia. However no invasive or malignant tumours were observed during this time period. Malignant transformation of skin tumours was first observed only after 17 weeks of DMBA application. These included keratoacanthomas, acanthomas and papillomas progressing to dermal invasion or cSCCs. The percentage of malignant tumours diagnosed increased with subsequent weeks of DMBA application as seen in Figure 3.

![Figure 3](image)

Figure 3 | Percentage of skin tumour biopsies diagnosed benign, premalignant and malignant at each time point of DMBA application. It should be noted that at week 27 all the tumours biopsied were malignant, but only 17% of the counted tumours were biopsied eventually due to the heavy tumour yield at that time point.

The histopathological evaluation of clinically normal skin biopsies from the control groups (untreated and acetone treated mice) showed the typical architecture of murine skin tissue as seen in Figure 4a. However the skin in control SKH1-hr hairless mice also showed characteristic histological features associated with the strain such as – (a) presence of multiple dermal cysts
lined by keratinised epithelium, (b) presence of utriculus which is an ampuliform structure connected to the skin surface and is lined by hyperkeratotic epithelium, (c) absence of melanin pigments, (d) sebaceous gland hyperplasia and (e) dermal inflammation characterised by infiltration of mast cells. Clinically normal skin obtained from DMBA treated mice either showed (i) no changes when compared to control murine skin samples or (ii) they exhibited mild to moderate epidermal hyperplasia (acanthosis) as seen in Figure 4b. These features of epidermal hyperplasia could be observed quite early in DMBA treated mice after just 4 weeks of DMBA application. In addition, the epidermal hyperplasia observed in DMBA-treated skin was more focal in nature and not diffuse. However epidermal hyperplasia or acanthosis seen in this stage was not associated with atypia or dysplastic features.

In the period from 12 – 17 weeks of DMBA application, the tumours that were biopsied from DMBA treated mice included – papillomatous hyperplasia (Figure 4c), acanthomas (Figure 4d), papillomas (Figure 4e) and keratoacanthomas (Figure 4g). From week 17 onwards, histopathological evaluations revealed the presence of premalignant Bowenoid lesions (squamous cell carcinoma-in situ or SCCIS) (Figure 4f) or full blown malignant cSCC (Figure 4i). This time period was also increasingly associated with pre-existing benign lesions such as papillomas, acanthomas or keratoacanthomas turning invasive (Figure 4h).

Figure 4 | H&E images of skin biopsies obtained from untreated, acetone treated and DMBA treated mice. The histopathological diagnosis were given as : (a) normal mice skin (in SKH1-hr mice), (b) mice skin with moderate hyperplasia (acanthosis), (c) papillomatous hyperplasia, (d) exophytic acanthoma, (e) papilloma, (f) Bowenoid lesion (squamous cell carcinoma in situ), (g) keratoacanthoma, (h) keratoacanthoma with progression to invasive carcinoma and (i) moderately differentiated cSCC. Mice from all three groups showed normal skin as in (a), while features as seen from (b) to (i) was seen only in DMBA treated mice.
When the pathologist’s diagnosis was converted to a semiquantitative score with a maximal value of 100, the control murine skin score averaged at 2.75 ± 1.17 for untreated skin and 2.65 ± 1.02 for acetone treated skin. On the other hand DMBA induced benign tumours scored higher at about 36.90 ± 2.70, while invasive or malignant lesions scored the highest at approximately 75.82 ± 3.70. The histopathological score obtained from the diagnosis of the biopsies when plotted as a function of time (weeks of DMBA application) also showed differences in the trend lines for DMBA treated mice when compared to the controls. The scores for biopsies from untreated and acetone treated mice stayed consistently between 1.5 – 3.75 at all time points. The scores for biopsies from DMBA treated mice were similar to the control mice up to week 7, following which it demonstrated an upward trend from week 11 onwards as seen in Figure 5. On the other hand, the standard deviation of the scores was quite high, especially at week 17, 20 and 23 of DMBA applications. This could arise due to the different types of lesions (benign and malignant) being diagnosed at each time point, resulting in a high variability of the score per time point. This variability decreases for the 27th week of DMBA application as all the lesions were diagnosed to be malignant at this time point.

![Figure 5](image.png)

**Figure 5 | Variation of histopathology score in time (weeks of topical application) for untreated, acetone treated and DMBA treated mice. The y-bars denote the standard deviation of the mean value.**

### 6.3.3 Mean epidermal thickness in control and DMBA treated mice skin

The mean epidermal thickness was assessed in terms of weekly topical applications received by mice as shown in Figure 6 (a-f). Mean epidermal thickness in control mice did not differ significantly from weeks 4 to weeks 27. In contrast to the control mice, DMBA treated clinically normal murine skin showed a gradual increase in epidermal thickness with successive weekly DMBA applications as seen in Figure 7. It is notable that the mean epidermal thickness in DMBA treated mice skin was significantly higher by about 40% compared to control mice after just 4 weekly DMBA applications. The increase was even higher at 92% and 135% after 11 weeks and 23 weekly DMBA applications respectively.
Figure 6 | Epidermis in clinically normal skin of SKH1-hr mice after 4, 11 and 23 weeks of treatment with acetone (a, c, e) and DMBA (b, d, f).

Figure 7 | The variation of mean epidermal thickness in time (weeks of topical application) for clinically normal untreated, acetone treated and DMBA treated mice skin. Y-bars in the graphs denote the standard deviation from the mean value.
6.3.4 | Immunohistochemistry in control and DMBA treated murine skin

6.3.4.1 | Markers for H-ras activation (p-Erk1/2)

The immunohistochemical staining for p-Erk1/2 showed a unique pattern in clinically normal murine skin obtained from control mice. As seen in Figure 8, the staining pattern for p-Erk 1/2 was particularly strong in the suprabasal and infundibular regions of the present hair follicles. In the interfollicular epidermis, p-Erk1/2 stained strongly all the flattened keratinocytes in the terminally differentiated layer underneath stratum corneum (i.e. stratum granulosum). The pattern was similar to DMBA treated skin with hyperplasia, with occasional positive staining of keratinocytes in suprabasal and basal layers of skin as well. In the DMBA induced lesions, p-Erk1/2 staining had an irregular pattern, localised preferentially in differentiated keratinocytes when compared to proliferating keratinocytes. This staining pattern was consistent for papillomas, keratoacanthomas, acanthomas and SCCIS. On the other hand, p-Erk 1/2 was preferentially strong in the proliferating zones in tumours undergoing malignant transformation (as shown for acanthoma with dermal invasion in Figure 8) and cSCC.

6.3.4.2 | Marker for basal keratinocyte or undifferentiated keratinocytes (keratin 14)

Keratin 14 was strongly positive in the cytoplasm of proliferative cells in (a) the hair follicle and (b) the stratum basale layer in normal interfollicular epidermis skin of control mice as seen in Figure 8. However staining became weaker in the suprabasal layer and was absent in the outer layer with flattened keratinocytes (stratum granulosum). In contrast to normal mice skin, skin with hyperplasia from DMBA treated mice showed moderate to strong keratin 14 positive staining in all layers of the epidermis as seen in the representative image in Figure 8. Keratin 14 staining was also positive in all the DMBA induced tumours, irrespective of whether the lesion was benign, turning invasive or malignant. The staining intensity of keratin 14 in the proliferating regions of the tumour was also found to be stronger compared to the staining intensity observed in the differentiated zones of the tumour.

6.3.4.3 | Proliferative marker (Ki67)

As seen in the corresponding image in Figure 8, Ki67 staining was nuclear in nature in comparison to the cytoplasmic staining of keratin 14. Similar to keratin 14 staining, Ki67 staining was positive in the stratum basale layer of normal murine epidermis and highly increased in the proliferating regions in benign, premalignant and malignant DMBA induced tumours.
Figure 8 | (From top to bottom) H&E stain and the immunohistochemical staining of neighbouring skin sections for p-Erk1/2, keratin 14, Ki67, p53 and cleaved caspase 3. (From left to right) Normal mice skin, skin with hyperplasia, benign papilloma, keratoacanthoma, squamous cell carcinoma in-situ, endophytic acanthoma turning invasive and cutaneous squamous cell carcinoma. Scale denoted by black line corresponds to 200 µm.
Although Ki67 stain was present in the basal layer of epidermis, it can be seen from Figure 9a and 9b that not all basal keratinocytes were Ki67+ in untreated and acetone treated normal murine skin. On the other hand, more basal keratinocytes were Ki67+ in the epidermis of DMBA treated normal skin as shown in Figure 9c, even though there was no visible epidermal thickening or proliferation. In DMBA treated murine skin with hyperplasia, Ki67+ keratinocytes were even more frequent and expanded into the suprabasal layer as well as shown in Figure 9d.

Figure 9 | Immunohistochemical staining for Ki67 at 11 weeks after initiation of the study in (a) untreated normal skin, (b) acetone treated normal, (c) DMBA treated normal skin and (d) DMBA treated skin with hyperplasia.

Figure 10 | Comparison of number of Ki67+ cells per mm of mice epidermis in untreated normal, acetone treated normal, DMBA treated normal skin and DMBA treated skin with hyperplasia. The y-bars in the graphs denote the standard deviation from the mean value. (*) and (**) denote a p-value < 0.01 with respect to the control murine skin.
Upon quantifying the number of Ki67+ cells per mm of interfollicular epidermis of mice skin, it was found that DMBA treated normal mice epidermis had about twice as many Ki67+ cells than untreated and acetone treated normal epidermis as seen in the graph shown in Figure 10. The increase in Ki67+ keratinocytes per mm of interfollicular epidermis was even greater for DMBA treated skin with hyperplasia and was four times higher compared to normal skin from the control mice.

6.3.4.4 | Markers for tumour suppression (p53) and apoptosis (cleaved caspase 3)
Upon analysing for tumour suppressor markers such as p53, occasional p53+ stained cells could be observed in control mice skin, DMBA treated skin with hyperplasia and benign skin tumours like papillomas and keratoacanthomas. The number of p53+ stained cells was notably higher in benign lesions that were turning invasive as seen in Figure 8. The strongest p53 staining was observed in the proliferative zones of cSCC. On the other hand, the staining for apoptotic marker such as cleaved caspase 3 was positive only for cSCC biopsies in a highly localised manner and absent in all other samples.

6.4 | Discussion
6.4.1 | Susceptibility of SKH1-hr mice to complete carcinogenesis by DMBA
The outbred albino SKH1-hr mice are the most widely used for translational cancer research, among all commercially available strains. These mice are highly susceptible to photocarcinogenesis, probably due to the corrupted hairless gene (‘hr’) itself. This was demonstrated in C3H, another hairless mice strain by Davies et al. [27], where hairless mice were shown to be more vulnerable to photocarcinogenesis than haired mice of the same strain. However multistage chemical carcinogenesis is not commonly employed for hairless mice, probably due to the fact that DMBA functions mainly as an initiator by inducing irreversible mutations in the stem cells of active hair follicles [8,28] which are sparse or abnormal in hairless mice. However certain studies have demonstrated that hairless mice can still be susceptible to chemical carcinogenesis [29-31]. However, it has been thus far not known if SKH1-hr mice are susceptible to carcinogenesis by using DMBA as a complete carcinogen, i.e. with assumed initiator and promoter activities. While carcinogenesis by DMBA only has been shown earlier in various other mice strains [19-23,32], this study aimed to demonstrate it in SKH1-hr mice for the first time, and optimise the dose regimen for the yield of malignant cSCCs.

The pilot study data clearly shows that the hairless SKH1-hr mice were highly prone to skin carcinogenesis with just DMBA applications in all three test groups. It maybe that despite the sparsity of active hair follicles or presence of abnormal hair follicles in SKH1-hr mice, DMBA may still be able to initiate carcinogenesis from the interfollicular epidermis to a lesser extent [8] or rudimentary pilosebaceous appendages [31]. Another notable finding was that increased frequency of DMBA applications produced skin tumours faster (p-value of
significance = 0.03) in SKH1-hr mice. Although increasing the DMBA dose concentration per application increased the rate of tumour formation, the difference was not as significant (p = 0.07) when compared to increasing the frequency of DMBA application. This finding implies that the frequency of DMBA application has more influence than the concentration of DMBA in terms of tumour formation rate. This finding was in agreement with the finding of Iversen et al. [23], where 50 doses of 1 μg DMBA application resulted in 100% of mice developing skin tumours in comparison with 1 dose of 51.2 μg DMBA application that produced skin tumours in only 40% of the mice.

However, the interplay between frequency of carcinogen application and the carcinogen concentration per application needs to be tailored for an optimal murine cancer model. In this study, it was found that although higher dosing frequency of DMBA or higher DMBA concentration per application produced tumours faster, all the produced tumours were benign in nature. The resultant heavy tumour load led to premature euthanasia to prevent animal discomfort and hence did not allow the mice to be studied towards further stages of malignant transformation. Therefore a more suitable route to achieve a complete carcinogenesis model in SKH1-hr mice would be to adopt weekly DMBA applications at a lower concentration range such as 10 μg – 15 μg DMBA per mouse. However, the same protocol would need to be modified for another hairless mice strain depending upon its sensitivity to complete carcinogenesis by DMBA.

On the other hand, it should be noted that skin in SKH1-hr mice is also characterised by dermal inflammation [11] and increased risk for follicular cystitis. Therefore it is not known if this pre-existing dermal inflammation could contribute to the susceptibility of SKH1-hr mice towards experimental carcinogens when compared to other mice strains. However this hypothesis needs to be validated with further investigations.

6.4.2 | Higher carcinoma yield with DMBA only carcinogenesis in SKH1-hr mice skin.

The early visible tumours measuring between 1 mm – 2 mm diameter that formed after 11 weeks of DMBA applications consisted mainly of papillomas and acanthomas. This was in agreement with earlier studies that used UV and DMBA-TPA carcinogenesis in different murine strains [8,9,14,33]. Tumours with diameter between 2 mm and 4 mm were seen from week 14, but predominated from week 20 onwards. These tumours were either benign like keratoacanthomas or premalignant like SCCIS and papillomas with atypia. Larger diameter > 4 mm comprised of either benign lesions such as keratoacanthoma or malignant ones such as acanthomas progressing to invasion or cSCCs. The malignant tumours were first observed after 17 weekly DMBA applications. In addition, 59% of the biopsied tumours were diagnosed malignant after 23 weekly DMBA applications. However it should be noted that although keratoacanthomas regularly progresses to cSCC in murine models, these lesions rarely turn malignant in humans. Therefore cancer model outcomes which involve keratoacanthomas
turning invasive in mice should not ideally be extrapolated for human skin carcinogenesis, as done in the case of benign papillomas turning malignant [11].

The results in this study show that tumour formation with weekly DMBA application in SKH1-hr mice was slower than that with UV carcinogenesis as shown in earlier studies [15]. However, this hairless immunocompetent skin cancer model relied only on low dose weekly DMBA applications and succeeded in producing a high malignant carcinoma yield (50% at 20 weeks and 59% at 23 weeks) that is comparable to UV carcinogenesis [15,34] and clearly higher than that produced by multistage chemical carcinogenesis using DMBA-TPA [35]. This finding may be explained by the study of Iversen et al. [19] where it was seen that after DMBA initiation, mice developed more carcinomas upon frequent application with 10 nmol of DMBA when compared to those that received 10 nmol of TPA. The underlying mechanism hypothesised is that DMBA always remains an initiator at low dose levels, but functions as a complete carcinogen at higher doses [36]. However it is still not clear if repeated applications of DMBA actually serve the role of a classic tumour promoter. Since the changes of a promoter are reversible, discontinuation of the promoter should result in regression of the tumours, as in the case of TPA and other promoters [8,11]. Therefore, in order to validate the role of DMBA as a promoter, future studies should investigate if skin tumours would regress upon cessation of DMBA applications. On the other hand, DMBA is known to cause irreversible mutations and it is highly probable that repeated or chronic DMBA application functions more like successive tumour initiation steps with cumulative irreparable mutations over time. In that sense, carcinogenesis arising from multiple DMBA application could be synergistic and may resemble skin carcinogenesis arising from repeated UV exposures. However the signature mutations induced by UV and DMBA are different, as the former involves mutations of the p53 gene via cyclobutane pyrimidine dimers [37] and the latter causes mutational activation of the H-ras gene [38]. Therefore further experiments are required to study the similarity or dissimilarity of complete carcinogenesis by UV and DMBA in parallel for the hairless SKH1-hr mice.

6.4.3 | DMBA induces epidermal hyperplasia in SKH1-hr mice skin

Earlier studies have reported epidermal hyperplasia induced by DMBA in oral cavity of hamsters [39] and rat mammary tissues [40,41]. In conventional mice skin cancer models, the role of DMBA has been limited to that of an initiator, while epidermal hyperplasia was typically induced by the promoters such as TPA [8,16] or UV [42]. The possibility of DMBA solely being able to induce epidermal hyperplasia in hairless mice skin still remains largely unexplored. One of the other important histomorphological findings in this study conducted on SKH1-hr mice was that repeated application of DMBA itself was capable of inducing epidermal hyperplasia. DMBA induced hyperplasia was observable in SKH1-hr mice skin after just 4 weeks of DMBA applications and was further elevated after subsequent weekly applications.

Another notable feature observed in this study was that DMBA induced hyperplasia in mice skin was focal in nature and did not resemble the diffuse epidermal hyperplasia associated with
UV irradiation in SKH1-hr mice skin [43]. While this finding has not been reported earlier for SKH1-hr mice, similar hyperplastic foci in interfollicular epidermis of skin from SENCAR strain mice subject to DMBA-TPA carcinogenesis protocol has been reported by Binder et al. [44]. That study suggested that these hyperplastic foci were clusters of initiated keratinocytes that potentially served as precursors for papilloma generation. This distinction in the epidermal hyperplasia pattern induced by chemical carcinogenesis and UV irradiation, again suggests that initiation and proliferation of keratinocytes occur in a different manner for the two types of mice skin cancer models.

6.4.4 | Role of p-Erk 1/2 and keratin 14 in differentiation and proliferation associated with DMBA induced skin carcinogenesis

Extracellularly regulated kinases or Erks belongs to the family of mitogen activated protein kinases (MAPK) that is very crucial for major cytoplasmic signal transduction pathways. Erks have been known to play a pivotal role in differentiation, proliferation, cell migration, UV responses, integrin- and growth factor-mediated signaling, and responses to mechanical stretching in the epidermis [45,46]. The Erk pathway is eventually responsible for promoting epidermal differentiation, which is achieved by (a) promoting production of the RNA splicing machinery and nuclear envelope components and (b) suppressing steroid synthesis and mitochondrial energy production [46].

Several studies show that continual phosphorylation of Erk1/2 (p-Erk1/2) resulted in keratinocyte proliferation. In contrast, the study by Seo et al. has shown that murine epidermal keratinocyte differentiation occurred through protein kinase C activation with p-Erk1/2 [41]. It may then be assumed that p-Erk1/2 can drive mice keratinocytes towards differentiation in the absence of a carcinogenic stimulus. This may explain the expression p-Erk1/2 predominantly in the terminally differentiated corneocytes underneath stratum corneum of untreated and acetone treated mice skin. The positive expression of p-Erk1/2 in the suprabulbar and infundibular region near the hair follicle on the other hand maybe connected to its role associated with proliferation, differentiation and migration of keratinocytes. Since p-Erk1/2 is highly interconnected to pathways involved in proliferation, differentiation, migration and inflammation, it could explain the irregular distribution of p-Erk1/2 positive cells in DMBA induced tumours in SKH1-hr mice. In this study, it was seen that p-Erk1/2 expression were preferentially localised to the differentiated keratinocytes in benign lesions such as papillomas, acanthomas and keratoacanthomas, while it was highly positive in the region of actively dividing keratinocytes in cSCC or benign lesions that were turning invasive. This implies that p-Erk 1/2 is more of a pro-differentiation agent in early benign skin neoplasms. It could be possible that role of p-Erk1/2 may get altered towards anti-differentiation and hyperproliferation of keratinocytes upon malignant conversion of these benign tumours.

Keratin 14 belongs to the type 1 keratin family of intermediate filament proteins. Along with keratin 5, keratin 14 is strongly expressed in basal keratinocytes in skin. As the keratinocytes differentiate and move upwards towards the suprabasal layers, keratin 14 expression is down
regulated [47]. This is consistent with the results of this study where keratin 14 staining in the control mice skin was strong in the stratum basale and got progressively weaker for the suprabasal and stratum corneum layers. In contrast to the control normal skin, DMBA treated skin with hyperplasia showed positive keratin 14 staining extending to the suprabasal layers as well. Similar findings have also been reported in DMBA induced hamster cheek pouch carcinogenesis [48] and human hyperplastic epidermis [49]. In the DMBA induced tumours, irrespective of whether it was benign or malignant, keratin 14 stain was strong in the actively dividing zones and weak in the inactive differentiated zone. This again confirms the role of keratin 14 being an indicator of proliferation during different stages of skin carcinogenesis.

6.4.5 | Role of proliferation marker Ki67 in DMBA induced skin carcinogenesis

It is known that Ki67 is expressed broadly in late G1 (growth 1), S (DNA synthesis), G2 (growth 2) and M (mitotic) stage of the cell cycle [50-52], i.e. it labels cycling cells actively contributing to cell proliferation. It was observed that Ki67 displayed nuclear staining only in a fraction of keratin 14+ cells in the control mice skin. It could be because Ki67- basal keratinocytes cells are probably in the resting phase or G0 phase of the cell cycle [50,53]. In this study, a marked increase in the number of Ki67+ basal keratinocytes in the interfollicular epidermis of DMBA treated skin was observed when compared to the control samples. Moreover, these observations were made on DMBA treated skin which still appeared normal histologically with no features of hyperplasia. This clearly suggests that DMBA application caused ‘resting’ basal keratinocytes to proceed for further cell division. This may be a possible explanation on how repeated DMBA applications could trigger epidermal proliferation resulting in the hyperplastic or papillomatous stage of carcinogenesis. An earlier study conducted by Olsen et al. observed early changes in cell division kinetics after low dose applications of DMBA on hairless mice skin [32]. However these changes were transient and not necessarily related to carcinogenicity, as the study was followed for only up to 3 days.

6.4.6 | Role of tumour suppressor p53 and apoptotic marker cleaved caspase 3 in DMBA induced skin carcinogenesis

In the control samples, skin with hyperplasia and benign skin tumours, p53+ve cells were occasionally seen. It is likely that these cells may be expressing wild p53 which was detected by the CM-5 polyclonal antibody employed in this study, as CM-5 detects both wild and mutant tumour suppressor p53. On the other hand, there was a notable increase in p53+ cells in benign tumours that were turning malignant and cSCC. It is possible that the p53 expressed in these tumours may belong to the mutant variant of p53. However, to specifically identify incidence of mutant p53 positive cells, the PAb240 monoclonal antibody should be employed; or the presence of p53 mutation can be directly determined by sequence analysis. Cleaved caspase 3 on the other hand, showed positive staining only in the stage of cSCC.

The absent expression of tumour suppressor p53 and apoptotic marker cleaved caspase 3 during the early stages of DMBA induced skin carcinogenesis in this study could be because
the early keratinocyte responses involving p53 and cleaved caspase 3 may have been missed. In a very interesting study performed by Berg et al. on SKH1-hr mice, it was shown that the levels of p53+ve cells peaked as early as 24 hours and then reduced to baseline values within 72 hours following a single UVB exposure [54]. Similarly Rebel et al. demonstrated that cleaved caspase 3 expression could be observed for 6 – 48 hours after UV irradiation, but was not detectable after 7 days [55]. Therefore it is probable that the early cellular stress response via p53 and cleaved caspase 3 expression would have been missed in this study where the earliest time point of assessment was as late as 4 weeks after the first DMBA application. On the other hand, Kemp et al. demonstrated that tumour suppressor p53 played no role in the early initiation and promotion stage of multistage chemical carcinogenesis, and played an important role only in the malignant progression stage of skin cancer [56]. This theory may also explain the minimal p53 expression in early stages and increase in p53 only in later stages following malignant progression observed in this study. However, it would be informative to study if the first DMBA application on skin elicits a response within 24 – 72 hours in a manner similar to UV irradiation as described in earlier studies [54,55].

6.5 | Conclusion

In this particular study a murine skin cancer model with the complete spectrum of skin carcinogenesis was established in hairless and immunocompetent mice strain (SKH1-hr mice). This was accomplished using a simple carcinogenesis protocol that utilised only weekly DMBA applications. This cancer model would be extremely useful for the purposes of refining diagnostic imaging or for chemotherapeutic and chemopreventive drug trials. Upon evaluating keratinocyte differentiation markers like p-Erk1/2 and keratin 14, it was observed that in normal SKH1-hr mice epidermis, p-Erk1/2 was generally expressed in terminally differentiated keratinocytes, while in malignant lesions p-Erk1/2 was localised to the proliferating keratinocytes. In contrast, keratin 14 was retained in undifferentiated actively proliferating keratinocytes in normal skin as well as skin lesions progressing towards cSCC. Tumour suppressor p53 and apoptotic marker cleaved caspase 3 was evidently increased only in malignant tumours. Moreover the study also demonstrated that repeated application of DMBA caused an increase in Ki67+ basal keratinocytes and eventually epidermal hyperplasia, suggesting that multiple DMBA exposure can directly cause epidermal proliferation in mice skin. However additional studies are needed to verify if these epidermal changes are reversible for validating the specific role of DMBA as a classic promoter.
References

42. Z. Y. Wang, et al., Cancer Research 54(13), 3428-3435 (1994).
Chapter 7

*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 cytostructural changes in various stages of carcinogenesis in vivo, in hairless mice skin. It was unique as it also evaluated
if NLOI relying on TPEF and SHG could non-invasively detect microscopic transformations from the preclinical stages of skin cancer itself (i.e. in clinically normal mice skin prior to tumour appearance). This study also distinctly attempted to examine these transformations in mice skin by performing axial (x-z) scans that are more comparable to standard histopathology. Furthermore, the TPEF-SHG index that has been investigated in different forms previously [19, 20], was applied as an indicator of cancer related changes in the skin.

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 Image J 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:

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

(1)

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.
In vivo NLOI to monitor early microscopic changes in a murine cSCC model

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.
In vivo NLOI to monitor early microscopic changes in a murine cSCC model

Table 1: Comparison of TPEF-SHG index values for different skin pathological conditions.

<table>
<thead>
<tr>
<th>Condition</th>
<th>TPEF-SHG Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Mice Skin</td>
<td>0.3 ± 0.05</td>
</tr>
<tr>
<td>Murine Skin with Preclinical</td>
<td>0.6 ± 0.03</td>
</tr>
<tr>
<td>Changes</td>
<td></td>
</tr>
<tr>
<td>Early Clinical Mice Skin Lesions</td>
<td>0.8 ± 0.02</td>
</tr>
<tr>
<td>Late Clinical Mice Skin Lesions</td>
<td>0.9 ± 0.01</td>
</tr>
</tbody>
</table>

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 in vivo NLOI in evaluating cytostructural 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 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 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, 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 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 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 0.045% 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 intra-nuclear 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\(^{3+}\)-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, \textit{in vivo} NLOI would indeed be a suitable non-invasive alternative to conventional histopathology.

7.5 Conclusion

The findings of this study suggest that \textit{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, \textit{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

In vivo NLOI to monitor early microscopic changes in a murine cSCC model

Chapter 8

In vivo nonlinear spectral imaging as a tool to monitor early spectroscopic and metabolic changes in a murine cutaneous squamous cell carcinoma model

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Abstract

Timely detection of cutaneous squamous cell carcinoma with non-invasive modalities like nonlinear spectral imaging (NLSI) can ensure efficient preventive or therapeutic measures for patients. In this study, in vivo NLSI was used to study spectral characteristics in murine skin treated with 7, 12-dimethylbenz(a)anthracene. The results show that NLSI could detect emission spectral changes during the early preclinical stages of skin carcinogenesis. Analysing these emission spectra using simulated band-pass filters at 450 nm – 460 nm and 525 nm – 535 nm, gave parameters that were expressed as a ratio. This ratio was increased and thus suggestive of elevated metabolic activity in early stages of skin carcinogenesis.
8.1 | Introduction

The global incidence of cutaneous squamous cell carcinoma (cSCC) affecting the Caucasian population has increased considerably in the last few decades [1]. Increase in sun seeking behaviours, popularity of tanning salons and more efficient reporting of new cSCC cases in current health systems could be considered as the etiology of this observed upward trend [2]. If left undiagnosed, cSCC arising in face, neck or scalp can turn invasive and reduce the patient’s quality of life. The solution to this problem would be to enable earliest detection of cancer related skin changes such that preventive or therapeutic intervention is effective for the individual.

Suspect skin lesions are often detected by the general practitioner at a routine clinical examination. However the general practitioner is often left in a dilemma to decide whether a patient needs to be referred to a dermatologist, who later sends skin biopsies to histopathology for confirmation. Secondly, needless biopsies performed on benign skin lesions are not beneficial to the patient and leaves behind cosmetic scarring and waste pathologist’s time. Furthermore, diagnostic reports are not immediate. However these problems may be solved by exploring the potential of non-invasive diagnostic modalities in these fields. The most commonly used non-invasive instrument by the dermatologist since the last two decades is the dermatoscope that aided in better clinical diagnosis of cSCC [3]. However dermatoscopes cannot clearly differentiate between benign and malignant lesions as it cannot provide subcellular details that are needed to do so. These subcellular details can be provided with high resolution non-invasively by nonlinear optical imaging.

Nonlinear optical imaging depends on the following optical processes a) Two-photon Excitation Fluorescence (TPEF) [4] and b) Second Harmonic Generation (SHG) [5]. As a result, this imaging modality possesses various advantages over other conventional imaging techniques such as confocal laser scanning microscopy. This includes (a) minimal out-of-focus images, (b) deeper tissue imaging (c) ability to perform 3-D optical sectioning in the imaged tissue and (d) reduced phototoxicity [6]. Nonlinear optical imaging also provides subcellular resolution without relying on exogenous fluorescent labels. Due to these merits as a label-free non-invasive modality, the applications for nonlinear optical imaging in cancer diagnostics has burgeoned significantly in the last two decades [7-9].

Nonlinear optical imaging has been successfully used to detect tissue structural changes and differentiate between normal and cancerous tissues in various organs with high sensitivity and specificity [10-13]. Besides altered tissue architecture, cancerous tissues have also been discovered to have modified biochemical composition, such as altered production of proteins, lipids or other subcellular components [14-16]. This could affect the concentrations of endogenous fluorophores which may in turn change the tissue spectral characteristics. These spectral changes may be detected by the combination of nonlinear optical imaging with spectroscopy, better known as nonlinear spectral imaging (NLSI). Due to this beneficial aspect, NLSI has already been used to evaluate the morphology and biochemistry of skin [17-19] and
other organs [20,21]. But those studies were mainly restricted to normal or non-diseased tissues. Studies that used nonlinear optical imaging for skin cancer have so far relied mainly on fluorescence lifetime measurements [22-24], instead of emission spectral characteristics. Among the few studies that did use NLSI for skin cancer diagnosis, the experiments had been performed on ex vivo human skin biopsies [25-27]. The only notable study that employed in vivo NLSI for cancer characterisation was performed in the hamster oral cavity cancer model by Edward et al. [28]. The avenue of in vivo NLSI for skin cancer diagnostics, especially cSCC remains largely unexplored at present.

Another key component that has been reported in cancerous tissue is its intrinsic metabolism profile. Detection and analysis of cellular metabolism is often based on the fluorescence measurements of endogenous metabolic biomarkers like nicotinamide adenine dinucleotide (NADH) in relation to flavin adenine dinucleotide (FAD) in a ratiometric form known as the metabolic redox ratio [29]. The use of redox ratio as a marker in cancer diagnostics is based on the hypothesis that increased cellular metabolism in cancer cells may cause the route of energy production to shift from the oxidative phosphorylation pathway to the glycolytic pathway [30,31]. This can happen either in absence of oxygen (called diminished Pasteur effect) or in presence of oxygen (called aerobic glycolysis or Warburg effect) [32,33]. This transition causes decrease in the rate of oxidative phosphorylation resulting in reduced FAD production and/or increased accumulation of NADH from glycolysis leading to altered intracellular dynamics between NADH and FAD. Various studies have tested this hypothesis of altered cellular metabolism or the metabolic redox ratio to differentiate cancer affected tissues from normal tissues in different organs such as oral cavity, breast, colon and ovaries [34-37]. However the metabolic activity in epidermal cells during skin cancer progression remains relatively unexplored.

This study primarily utilised in vivo NLSI that relied on TPEF, SHG and spectroscopy to uniquely detect any alteration in the spectral characteristics in carcinogen treated mice skin during various stages of skin cancer. This study was distinct as it mainly focussed on observing spectroscopic changes from the preclinical stages of skin cancer (i.e. in clinically normal mice skin prior to tumour appearance). In addition, the acquired emission spectra were further analysed using simulated band-pass filters to indirectly estimate NADH and FAD levels in the epidermis. The quantified parameters were expressed as a ratio that was then compared during the various stages of skin carcinogenesis.

8.2 | Materials and methods

8.2.1 | DMBA skin carcinogenesis protocol
This experimental animal protocol was discussed and approved by the Animal Research Committee of the Erasmus University, Rotterdam. 36 female albino SKH1-hr strain hairless mice were ordered from Charles River Laboratories, Someren, the Netherlands. The mice
were housed under standard housing conditions and fed ad libitum on a chlorophyll-free diet (Hope Farms BV, Woerden, The Netherlands). This diet was followed to prevent the autofluorescence emission from pheophorbide-a in mice skin at 674 nm [38]. The mice were randomly and equally distributed over three groups. In the first control group, the mice skin remained untreated, while mice in the second control group were treated with weekly topical application of acetone (Sigma Aldrich Chemie, Zwijndrecht, the Netherlands) on skin for 20 weeks. The test group mice were treated similarly with a weekly topical application of 0.15 mg 7,12-dimethylbenz(a)anthracene (DMBA) (Sigma Aldrich Chemie, Zwijndrecht, the Netherlands) dissolved per ml of acetone [39]. The mice were clinically examined every week post the first topical DMBA application, to assess skin changes and onset of tumour formation.

8.2.2 | In vivo imaging protocol with the animal model

The imaging protocol was performed on a self assembled setup that has been described elaborately earlier in Chapter 3 [17]. The reference emission spectral values were obtained from ex vivo rat tail tendon, 8.1 g/ml aqueous solution of human epidermal keratin, 0.2 mg/ml FAD in phosphate buffered saline (PBS) solution (pH 7.4) and 2.5 mM NADH (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) in 1 ml of 3-(N-morpholino)propanesulfonic acid (MOPS) buffer solution (pH 7.4). Prior to imaging, a combination of ketamine (75 mg/kg of mouse body weight) and medetomidine (1 mg/kg of mouse body weight) in physiological saline solution (Janssen Pharmaceutica, Tilburg, the Netherlands) was administered intraperitoneally to the mice for anesthesia. Black ink was later used to pre-mark the skin area to be imaged on the mice.

The femtosecond laser functioned at a pulse repetition frequency of 80 MHz and pulse duration of 90 femtoseconds. Wavelength of 765 nm was selected as the excitation light source while spectral imaging was performed with an infinity corrected water-immersion objective (40×, Numerical Aperture = 0.8, Nikon, Japan). In every imaged region, emission spectra was obtained from an axial x-z scan (field of view: 100 μm × 60 μm) and a series of transverse x-y scans (field of view: 100 μm × 100 μm) that had been performed at intervals of 3 μm extending down to a depth of 60 μm from the surface. The average laser power ranged from 17 mW – 45 mW at the mice skin surface, and was gradually raised with increasing tissue depths. The pixel dwell time was kept at 128 µs, and scanned over 224 × 224 pixels. The acquisition time was 6.5 seconds/scan and ~ 2.5 minutes per skin spot. These steps were repeated for at least two spots per mouse. All mice from untreated, acetone treated and DMBA treated groups underwent the same imaging protocol.

In vivo NLSI was performed on clinically normal skin or DMBA induced lesions preferentially located near the thigh region of the mice to (a) minimise motion artefacts from breathing and (b) eliminate anatomical variation in skin from different regions. Following imaging anaesthetised mice were euthanised by cervical dislocation. After euthanasia, skin biopsies from the pre-marked regions were taken and fixed in 10% buffered formalin solution and sent for histopathologic evaluation by a certified veterinarian pathologist. Based on the clinical
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Macroscopic appearance and its corresponding histopathological evaluation, murine skin were later categorised as: (a) Normal: No microscopic or clinical skin changes, (b) Preclinical (hyperplastic): Positive microscopic changes of hyperplasia with no clinical skin change and (c) Clinically visible tumours (preneoplastic/neoplastic): Positive microscopic changes with presence of clinically visible lesion. All experiments were performed in compliance with the relevant laws and institutional guidelines in accordance with the ethical standards of the Declaration of Helsinki.

2.4 Data analysis of NLSI experimental data

Spectral width of SHG obtained in vivo from mice skin was broader than that obtained from the reference sample. This was also observed by Palero et al. [19] and was attributed to spectral resolution degradation of the setup due to increased scattering of detected light. This generally occurs more during deeper in vivo imaging of thicker specimens like skin, as compared to ex vivo imaging of relatively thinner monolayers of reference samples. This spectral broadening of SHG is a setup artifact and was found to be inconsequential for the data analysed in this study.

Transverse x-y scans deeper than 48 µm were not evaluated due to marked signal attenuation and decreased signal-to-noise ratio beyond this depth. In addition, spectral scans that were affected heavily by breathing artefacts from the anaesthetised mice were excluded. Moreover NLSI had also been performed on skin lesions that had a diameter > 4 mm and were extensively keratinised. These types of lesions gave no detectable signal from any fluorophores besides keratin throughout the imaged depth and were therefore omitted from further data analysis. Eventually a sample size of 32 skin regions was considered, comprising of 17 normal skin (from 6 untreated and 6 acetone treated mice), 8 skin with preclinical hyperplasia (from 6 DMBA treated mice) and 7 skin with clinically visible tumours (from 6 DMBA treated mice). Analysis was performed for one axial x-z scan and a stack of transverse x-y scans for each skin region. For each individual scan, spectral analysis was performed by averaging over the entire image. Microscopic and spectral information of scans deeper than 36 µm were similar to that obtained from scans at 36 µm and therefore did not contribute any additional valuable information.

Spectral data acquisition and processing into RGB images was performed by software written in V++ (Digital Optics, Auckland, New Zealand) [17,40]. The microscopic features seen in NLSI RGB images were then compared with the findings seen in haematoxylin and eosin (H&E) stained slides that had been evaluated by the pathologist. The acquisition of spectral details from the image data file was done on the ImageJ software (available at http://imagej.nih.gov/ij/) by using the approach mentioned in previous studies [17,18]. The acquired emission spectra was obtained from axial x-z scans and then normalised to the maximal intensity. The normalised emission spectra were categorised under the assigned clinico-pathologic grade,
averaged and then compared. This process was also repeated for emission spectra obtained from transverse x-y scans at varying depths from skin surface, to observe spectral variation with depth in all three clinico-pathologic grades of mice skin.

The original (un-normalised) emission spectral plot values of axial x-z scans were then divided by the square of the average laser power used during imaging. This was done to compensate for nonlinear fluctuation in fluorescence intensities that arises due to differences in laser powers used during the imaging experiments. Following this, the area under curve (AUC) for the spectral plot was integrated from 410 nm – 650 nm to generate total autofluorescence intensity and from 370 nm – 410 nm for SHG intensity for the imaged skin region. These two parameters were averaged to obtain the mean total autofluorescence intensity and the mean total SHG intensity for each clinico-pathologic grade, which were then compared later.

In order to estimate NADH and FAD from the epidermis, we used an indirect method by applying simulated band-pass filters on the emission spectra of transverse x-y scans. As described earlier, the emission spectra of transverse x-y scans were also divided by the square of the average laser power to compensate for laser power variation. This method later involved integrating AUC of the emission spectra from 450 nm – 460 nm to provide a relative estimate for NADH in the epidermis and denoted as $AUC_{450nm-460nm}$. Similarly the emission spectra were integrated from 525 nm – 535 nm to obtain a relative estimate of FAD in the epidermis and indicated as $AUC_{525nm-535nm}$. The simulated band-pass filters had a bandwidth of 10 nm and were based on emission peak values of NADH and FAD obtained from literature [41]. The ratios were calculated as $\frac{AUC_{450nm-460nm}}{AUC_{525nm-535nm}}$ and then averaged for the corresponding clinico-pathologic grade and compared. To keep $AUC_{450nm-460nm}$ and $AUC_{525nm-535nm}$ as a reliable indicator for NADH and FAD respectively, we tried to minimise autofluorescence from other fluorophores such as keratin (stratum corneum and hair follicle), elastin and collagen (dermis) at 450 nm – 460 nm, and lipids and lipofuscin (stratum corneum) at 525 nm – 535 nm. This was done by performing the analysis in a spatially discriminated and depth specific manner in the transverse x-y scans. Therefore ratio calculations were limited to epidermal cells at depth of 6 – 12 μm for normal skin, 6 – 24 μm for skin with preclinical hyperplasia and 9 – 36 μm for clinically visible tumours. Statistical analysis was performed for all data while comparison, using two-sample t test of unequal variance with a p-value < 0.05 being considered statistically significant.

It should additionally noted that analysis of the reference NADH (2.5mM) and FAD (0.2 mg/ml) solutions showed spectral overlap at the imaging wavelength 765 nm used in this study. The spectral overlap was about 11% and 29% at the simulated band-pass filter of 450 nm – 460 nm and 525 nm – 535 nm respectively. It is known that the degree of spectral overlap between two fluorophores also depends on the individual fluorophore concentration. Therefore it is not known if the degree of spectral overlap observed ex vivo in reference solutions of known fluorophore concentrations can be extrapolated reliably for an ‘in vivo’ environment, where the fluorophore concentrations are unknown. Therefore for the simplicity of calculations in an in vivo scenario, the spectral overlap between NADH and FAD at 450 nm – 460 nm and 525 nm – 535 nm shall be considered negligible, while calculating the ratio $\frac{AUC_{450nm-460nm}}{AUC_{525nm-535nm}}$. 
8.3 | Results

8.3.1 | Clinical and histopathological findings of DMBA induced skin carcinogenesis

Untreated and acetone treated mice exhibited no skin related symptoms or signs throughout the experiment. In DMBA treated mice the first skin lesion was observed after around 11 weeks of topical DMBA application. Following its onset, DMBA induced lesions presented initially as non keratinised lesions of diameter < 1 mm and > 2 mm. These lesions increased in diameter and got heavily keratinised in the successive weeks.

Upon histopathological evaluation, normal skin was identified in biopsies from all three groups of mice. However skin regions with preclinical changes or clinically visible tumours were graded only from skin biopsies of DMBA treated mice. It was further observed that DMBA treated mice skin exhibited normal, preclinical hyperplasia and clinical pre-/neoplastic stages concurrently in the same mouse at all evaluated time points starting from week 11. Skins with preclinical changes were basically characterised by epidermal or squamous hyperplasia. Clinically visible early tumours (1 – 4 mm diameter) were identified as: (a) squamous cell papilloma (with or without atypia) and (b) exophytic and endophytic acanthoma (with or without atypia). The highly advanced and heavily keratinised skin tumours (diameter > 4 mm; not considered for further spectral analysis as mentioned earlier) were found to be (a) keratoacanthoma (with or without invasion), (b) squamous cell carcinoma in-situ (Bowenoid lesion) or (c) squamous cell carcinoma (well or moderately differentiated).
8.3.2 | In vivo nonlinear spectral images compared with standard H&E stained images

In vivo nonlinear spectral image from axial x-z scan

H&E stained images from comparable mice skin

Figure 1 | Comparison between axial NLSI x-z scans and H&E stained sections for normal skin, skin with preclinical hyperplasia and clinically visible tumours (top to bottom). Axial x-z scans clearly displays stratum corneum (green layer), the deeper epidermis (blue layer) and the dermis (violet layer) (2a). The RGB colours in the images were obtained by multiplying the emission spectrum from skin with the spectra for the red, green and blue sensitivities of the human eye. The RGB colors in NLSI scans correspond to the wavelengths in tissue emission spectra shown. Epidermal proliferation can be clearly observed with in vivo NLSI in 2c and 2e. The corresponding H&E stained images show normal SKH1-hr mice skin (b), hyperplasia with no atypia (d) and severe hyperplasia with atypia (part of an acanthoma) (f). The corresponding axial H&E obtained from biopsy of the imaged spot may not represent the exact position of the imaged spot. (x-z scan scale – 100 µm × 60 µm, 224 pixels × 224 pixels).
Since in vivo NLSI were taken from a microscopic field of view (100 × 100 μm), it was not possible to match the exact imaged spot with the corresponding H&E stained section of the skin biopsy. Hence, our analysis relies on the assumption that there are no major histological changes between the imaged spot and adjacent regions. As shown in Figure 1, NLSI scan of normal mice skin provided RGB images (Figure 1a) that were quite comparable to the corresponding H&E stained section as seen in Figure 1b. In normal skin, stratum corneum was visualised as the greenish-yellow colored layer in the NLSI scan. The underlying stratum spinosum and basale appears as a blue layer, while SHG from the dermis was observed as the bright violet layer in the RGB image.

On the other hand compared to normal skin, H&E stained sections of DMBA treated mice skin with preclinical hyperplasia showed an evident increase in epidermal thickness as observed in Figure 1d. This change was clearly seen in NLSI scan as well (Figure 1c) characterised by an increased thickness of the blue coloured layer (stratum spinosum and basale). In contrast, SHG from dermis was visibly diminished as compared to normal skin. In skin with clinically visible tumours, H&E stained section (Figure 1f) showed a greater degree of epidermal proliferation as compared to the preclinical stage. In the corresponding NLSI, only epidermal autofluorescence was observed with no dermal SHG as seen in Figure 1e.

**8.3.2 | Tissue spectral changes and mean spectral emission intensities in different stages of DMBA induced skin carcinogenesis**

![Figure 2](image.png)

Figure 2 | (a) Reference emission spectra (normalised) from rat tail tendon collagen, free NADH, keratin and FAD, (b) Normalised emission spectra from axial x-z scans in normal mice skin (z = 17, s = 17, n = 12), mice skin with preclinical changes (z = 8, s = 8, n = 6) and clinically visible mice skin tumours (z = 7, s = 7, n = 6). The corresponding spectrum is an average of the emission spectra obtained from ‘z’ axial x-z NLSI scans (100 μm × 60 μm) in ‘s’ regions from ‘n’ mice for each clinco-pathologic grade.

The rat tail tendon reference sample gave a characteristic emission spectrum as seen in Figure 2a, which was composed of a strong SHG component that peaks at 382 nm which corresponds
to collagen in the rat tail and an autofluorescence component that peaks at about 450 – 480 nm. This autofluorescence component could be contributed by (i) collagen cross-links (digestible by collagenase) [42] or (ii) elastin [43] present in the rat tail tendon. Emission spectra from reference sample solutions of keratin, free NADH and FAD gave characteristic peaks at 480 nm, 460 nm and 535 nm respectively (Figure 2a), which was in agreement with literature for the corresponding fluorophores [41,44].

As seen in Figure 2b, the emission spectra of normal mice skin characteristically possessed (a) an SHG component from 370 nm to 410 nm and (b) a broadband autofluorescence component from 410 nm to 650 nm. Comparing the emission spectra from reference samples obtained ex vivo with that of normal mice skin obtained in vivo gave the following findings – (a) The SHG component obtained from normal mice skin in vivo had a similar emission peak with that of the rat tail tendon sample ex vivo, indicating that source of SHG in mice skin is obviously dermal collagen. (b) The ex vivo emission spectra of reference sample solutions indicate that the broad autofluorescence component obtained in mice skin in vivo can be attributed to contributions from fluorophores like NADH, keratin and FAD in epidermis and collagen and elastin in dermis.

From the normalised tissue spectral plots of axial x-z scans in normal skin, the epidermal autofluorescence intensity was found to be only one tenth of the dermal SHG intensity (as plotted in continuous blue line in Figure 2b. In contrast, the emission spectra of DMBA treated mice skin with preclinical hyperplasia (plotted in dashed green line in Figure 2b showed the epidermal autofluorescence peaked at around 450 nm – 460 nm and its intensity was roughly as high as half the dermal SHG intensity. This suggested that relative contribution of the epidermal autofluorescence component in the spectral plot increased by about 5 times in skin with preclinical hyperplasia when compared to normal mice skin. Unlike normal mice skin and preclinical hyperplastic skin, the tissue emission spectra from axial x-z scans obtained from clinically visible skin tumours (plotted in continuous red line in Figure 2b) showed the epidermal autofluorescence component (emission peak about 450 nm – 460 nm) to be the sole contributor to the spectral graph, while the relative contribution of dermal SHG was negligible.

Upon evaluating the variation in emission spectral plots for normal skin, skin with preclinical hyperplasia and clinically visible skin tumours at different skin depth (3 μm – 36 μm), it was seen that each clinico-pathologic grade had its own distinctive pattern. As seen in Figure 3a, at a depth of 3 μm, all the three grades of skin regions had a common emission peak at around 480 nm that corresponds to keratin. Interestingly there was another notable peak at 540 nm – 550 nm for normal skin and skin with preclinical hyperplasia, while clinically visible skin tumours had a unique peak near 570 nm. The dermal SHG component was absent for all three grades at this depth. As the scans approached a depth of 6 μm, the emission peak shifted to 450 nm – 460 nm for all three grades (Figure 3b), suggesting a dominant presence of NADH from this point onwards. While the peak at 540 nm – 550 nm begins to clearly diminish for normal mice skin and skin with preclinical hyperplasia, the unique emission peak at 570 nm is quite evident for clinically visible skin tumours at this depth.
Figure 3 | Normalised emission spectra for normal mice skin (y = 17, s = 17, n = 12), skin with preclinical hyperplasia (y = 8, s = 8, n = 6) and clinically visible skin tumours (y = 7, s = 7, n = 6) at depths of (a) 3 μm, (b) 6 μm, (c) 12 μm, (d) 18 μm, (e) 24 μm and (f) 36 μm from skin surface. The depicted spectrum is an average of the emission spectra obtained at the corresponding depth from ‘y’ transverse x-y NLSI scans (100 μm × 100 μm) in ‘s’ regions from ‘n’ mice for each clinico-pathologic grade.

From the depth of 12 μm downwards, the dermal SHG was the dominant component of the spectral plot for normal mice skin, while the epidermal autofluorescence decreased relatively and remained at its lowest level from 24 μm downwards as displayed in Figure 3c – Figure 3f. However in skin with preclinical hyperplasia, the spectral contribution from epidermal...
autofluorescence remained high up to a depth of 24 μm, following which it gradually decreased. Despite this decrease, the relative contribution of epidermal autofluorescence to tissue emission spectra in mice skin with preclinical hyperplasia remained higher than that of normal mice skin even at 36 μm (Figure 3f). In contrast, the dermal SHG spectral component in mice skin with preclinical hyperplasia kept rising with increasing skin depth, albeit at a relatively slower rate than normal skin. Clinically visible skin tumours on the other hand, exhibited a consistent spectral profile with no alterations from 12 μm to 36 μm, characterised by the predominant epidermal autofluorescence peak at 450 nm – 460 nm and absence of dermal SHG. Another notable point was that the spectral peaks present at 540 nm – 550 nm for normal and skin with preclinical hyperplasia and at 570 nm for clinically visible skin tumours were not observed anymore at depths below 12 μm.

Figure 4 | Comparison of mean total autofluorescence intensity (410 nm – 650 nm) and mean total SHG intensity (370 nm – 410 nm) for normal mice skin (z = 17, s = 17, n = 12), skin with preclinical hyperplasia (z = 8, s = 8, n = 6) and clinically visible skin tumours (z = 7, s = 7, n = 6). The intensities were calculated and averaged from ‘z’ axial x-z NLSI scans (100 μm×60 μm) in ‘s’ regions from ‘n’ mice for each clinico-pathologic grade. Y-bars stand for standard error of the mean (* - p-value < 0.05 with respect to normal skin, § - p-value < 0.05 for skin with preclinical hyperplasia with respect to clinically visible skin tumours).

The mean total autofluorescence and SHG intensity quantified from the area under the curve of the spectral plots for each clinico-pathologic grade of mice skin is depicted in Figure 4. There was no significant difference in the mean total autofluorescence intensity between normal mice skin, mice skin with preclinical hyperplasia or clinically visible skin tumours. On the other hand, the mean total SHG intensity was significantly decreased in skin with preclinical
hyperplasia by about a factor of 3.77 compared to normal skin (p-value < 0.05). The mean total SHG intensity measured from visible skin tumours was almost negligible.

The ratio formulated as $\frac{\text{AUC}_{450\text{nm}-460\text{nm}}}{\text{AUC}_{525\text{nm}-535\text{nm}}}$ was observed to be the lowest for normal mice skin as seen in Figure 5. On the other hand, this ratio was found to be increased by about 12% for skin with preclinical hyperplasia and 9% for clinically visible skin tumours (p-value < 0.05). There was no significant difference in the ratio obtained from skin with preclinical hyperplasia and clinically visible skin tumours.

![Figure 5](image)

**Figure 5** | Comparison of mean ratio $\frac{\text{AUC}_{450\text{nm}-460\text{nm}}}{\text{AUC}_{525\text{nm}-535\text{nm}}}$ (AUC- Area under curve) between normal mice skin (y = 49, s = 17, n = 12), skin with preclinical hyperplasia (y = 42, s = 8, n = 6) and clinically visible skin tumours (y = 63, s = 7, n = 6). The ratio was calculated and averaged from 'y' transverse x-y NLSI scans (100 µm×100 µm) in ‘s’ regions from ‘n’ mice for each clinico-pathologic grade. Y-bars stand for standard error of the mean (* - p-value < 0.05 with respect to normal mice skin).

### 8.4 | Discussion

#### 8.4.1 | Spectroscopic changes monitored by in vivo nonlinear spectral imaging during DMBA induced skin carcinogenesis

This study attempted to determine whether *in vivo* NLSI based on TPEF and SHG could monitor morphological, spectroscopic and metabolic changes in mice skin at different stages of DMBA induced carcinogenesis. When *in vivo* NLSI was applied on various clinico-pathologic grades of mice skin, distinct microscopic changes could be observed. These observations mainly included DMBA treated mice skin region with preclinical changes displaying a clear increase
in the area with epidermal autofluorescence when compared to control normal mice skin. This increase in area with epidermal autofluorescence could be due to a) increased keratin deposition and elevated cornocyte turn-over that lead to thickened stratum corneum (hyperkeratosis) and b) increase in area with intracellular NADH fluorescence due to epidermal proliferation. The degree of epidermal proliferation was even more severe in clinically visible skin tumours such that the dermal SHG could not be visualised anymore.

In conjunction with the microscopic observations, NLSI was able to generate additional spectral details that gave an insight of the biochemical changes during different stages of skin carcinogenesis. The acquired emission spectra varied distinctively for each clinico-pathologic grade of mice skin. At 3 μm, the tissue emission spectra for all three grades of mice skin shared a common peak at 480 nm that corresponded to keratin present in stratum corneum. There were additional peaks at 540 nm for normal mice skin and DMBA treated skin with preclinical hyperplasia which is probably due to high concentration of lipids in stratum corneum, especially phospholipids that have a similar emission peak [41,45]. On the other hand, clinically visible skin tumours exhibited a unique peak at 570 nm which may be due to lipofuscin or lipofuscin-like pigments which have emission around the same range [46]. Possibly the increased generation of lipofuscin in the non-dividing post-mitotic cells is due to lipid oxidation with time and ageing [47]. Due to rapid proliferation in these tumours, these post-mitotic cells are rapidly extruded towards the skin surface leading to lipofuscin accumulation in these layers. This hypothesis is however based on the emission peaks of lipofuscin obtained from literature [46]. Since reference samples for lipofuscin were not commercially available to evaluate the corresponding emission spectra, this lipofuscin hypothesis needs further validation in future studies.

As the x-y scans were performed deeper below the stratum corneum, there was a clear blue-shift in the emission spectra towards 450 nm – 460 nm for all three clinico-pathologic grades. This is probably attributed to intracellular NADH present in metabolically active epidermal cells (stratum spinosum and basale) that lie below the metabolically inert stratum corneum. However beyond a depth of 12 μm, NADH associated fluorescence diminished in normal mice skin. At the depth below 12 μm, dermal SHG dominated in the spectra for normal skin. This spectral transition from epidermis to dermis was comparable with the findings in the in vivo study by Palero et al. on normal hairless mice skin [18,45]. Interestingly, this spectral pattern was altered for DMBA treated skin with preclinical hyperplasia and clinically visible skin tumours with NADH associated fluorescence being present as deep as 24 μm and 36 μm respectively. This was clearly indicative of actively dividing epidermal cells extending deeper and thence resulting in a thicker epidermis.

Another characteristic feature observed was the reduction of dermal SHG signal for mice skin with preclinical hyperplasia and its complete absence in clinically visible skin tumours. This finding was confirmed upon quantifying the mean total SHG intensity from the spectral plots which also showed a similar trend for skin with preclinical hyperplasia and visible skin tumours. This is obviously due to downward displacement of dermis caused by epidermal
proliferation. This in turn leads to reduced detection of SHG signal from skin surface due to signal attenuation. Another possible cause for diminished SHG signal with advancing stages of carcinogenesis could be dermal collagen degradation or lysis during epidermal invasion into dermis as observed by Xiong et al. [26]. However this possibility seems improbable in the present study for the following reasons – a) advanced skin tumours such as SCC and carcinoma-in-situ had been excluded from the present data set and b) no dermal invasion was observed histopathologically in the corresponding H&E stained sections of the assessed skin with preclinical hyperplasia and clinically visible early skin tumours, which rules out epidermal invasion into dermis.

Our findings distinctly showed that NLSI could effectively visualise microscopic and spectroscopic differences between normal skin and skin with preclinical hyperplasia, both of which would appear clinically normal to the dermatologist. The remarkable aspect of these findings was that – a) NLSI was able detect these early cancer related changes without any exogenous labels and b) this was achieved in real-time, in vivo and in a non-invasive manner. This property would also be very useful for surgeons to distinguish between normal and high risk tissue around the margins of a cancerous lesion during its excision, such as during Moh's surgery [48]. Thus the findings of this study demonstrates the immense potential of in vivo NLSI to monitor structural and biochemical changes during carcinogenesis in skin and possibly in other organs that have high incidence of cancer such as lungs, breast and colon.

8.4.2 | Metabolic changes monitored during DMBA induced skin carcinogenesis by in vivo NLSI

Cellular metabolism has often been assessed quantitatively by calculating the redox ratio calculated on the basis of the concentrations of endogenous metabolic fluorophores such NADH and FAD, derived from their fluorescence intensities. The redox ratio was originally formulated to study oxidation-reduction changes in brain and kidney in rats under anoxia by Chance et al., who found that the redox ratio was affected by vascular oxygen supply and cellular metabolism rate [29,49-51]. The recent discovery that cancer progression maybe associated with tumour hypoxia [52], led to a newer perspective on the redox ratio as being a potential indicator of ongoing carcinogenesis. Skala et al. had observed the redox ratio (formulated as FAD/FAD+NADH) to decrease in precancerous stages in an oral cancer model of hamster cheek in vivo [53]. Similarly Varone et al. [54] demonstrated fluctuation in the metabolic redox ratio between engineered normal and precancerous squamous epithelial tissues ex vivo. Numerous ex vivo or in vivo studies that measured the redox ratio either found a decrease in the redox ratio or an increase in the inversed redox ratio (formulated as NADH/FAD) in neoplastic samples, suggestive of hypoxia in cancerous cells [9,35]. Most cancer based redox studies generally relied on fluorescence lifetime measurements to assess the redox ratio, with the exception of a few studies where the redox ratio was assessed from the acquired emission spectra [28,36,37,55,56]. However till date, there have been no in vivo studies that
utilised NLSI to assess metabolic activity in epidermis of skin during ongoing carcinogenesis in an animal model.

In the present study, we utilised simulated band-pass filters at 450 nm – 460 nm and 525 nm – 535 nm to indirectly estimate NADH and FAD respectively from the epidermis. To minimise the contribution of other fluorophores (keratin, lipids, lipofuscin, elastin and collagen) in the mentioned wavelength ranges, the analysis was performed in a spatially discriminatory manner exclusively on epidermal cells in the deeper layers. As a result, autofluorescence from keratin (from stratum corneum and hair follicle), lipids (from stratum corneum), lipofuscin (from ageing cells in the superficial layers), elastin and collagen (from dermis) were minimised. The ratio \( \frac{\text{AUC}_{450\text{nm}-460\text{nm}}}{\text{AUC}_{525\text{nm}-535\text{nm}}} \) can therefore be considered an indirect approximation to the ratio NADH/FAD that had been utilised in earlier studies. Although it should be noted that calculating AUC at 450 nm – 460 nm and 525 nm – 535 nm only provides a relative measure of NADH and FAD respectively in the epidermis and is not an absolute measure of NADH and FAD concentration in the epidermis. It is difficult to assess the absolute concentration of fluorophores like NADH and FAD in an in vivo scenario, as it is not possible to have an ‘in vivo’ reference of known concentration. In contrast, ex vivo quantification of fluorophores is feasible as demonstrated in the study of Keyes et al. [57], but this approach is not applicable for an in vivo study. Moreover, for absolute quantitative measurement of fluorophores in tissues ‘in vivo’, factors like attenuation of laser power needs to be corrected. This can be problematic, as rates of laser power absorption and scattering could differ between normal and cancerous tissue. By expressing the parameters \( \text{AUC}_{450\text{nm}-460\text{nm}} \) and \( \text{AUC}_{525\text{nm}-535\text{nm}} \) in a ratiometric form as described above, the effects of laser power attenuation can therefore be eliminated.

The ratio formulated as \( \frac{\text{AUC}_{450\text{nm}-460\text{nm}}}{\text{AUC}_{525\text{nm}-535\text{nm}}} \) was found to be increased for mice skin with preclinical hyperplasia and clinically visible skin tumours. If this ratio is considered analogous to the NADH/FAD ratio used in earlier studies, the findings suggest that there might be an elevated epidermal metabolism in these early stages of skin carcinogenesis. A possible explanation may be that NADH production could be raised because of increased glycolysis in the proliferative epidermal cells due to either the diminishing Pasteur effect (reduced oxygen) or the Warburg effect (in presence of oxygen) [32,33]. However it should be noted that despite the difference being statistically significant (\( p < 0.05 \)), the margin of difference was relatively low for the three clinico-pathologic grades of mice skin, when compared to the redox ratio difference observed in cancerous and normal samples of other organs analysed in earlier studies [10,28,56]. One possible explanation is that epidermal cells during skin carcinogenesis may not be affected by hypoxia as much as compared to other visceral organs, because of ambient oxygen that can easily diffuse into skin from the atmosphere. In the study by Stücker et al. [58], it was estimated that total thickness of human skin supplied by external atmospheric oxygen was about as deep as 266 – 375 \( \mu \)m. Another reason could be that the \( \frac{\text{AUC}_{450\text{nm}-460\text{nm}}}{\text{AUC}_{525\text{nm}-535\text{nm}}} \) ratio assessments were primarily performed during the early stages of skin carcinogenesis – preclinical hyperplasia and clinically visible early skin tumours. It would have been informative to assess the ratio in advanced stages of DMBA-induced skin
carcinogenesis such as keratoacanthomas, squamous cell carcinoma in-situ and squamous cell carcinoma in skin. Unfortunately since all these lesions were > 4 mm in diameter and had very thick layers of keratin above, it was not possible to image and assess the ratio from the deeply located proliferative and metabolically active zone of these lesions using the current NLSI setup. However, it must be noted that in vivo NLSI was sensitive to even small alterations in the ratio during early stages of mice skin carcinogenesis.

8.4.3 | Limitations
Earlier studies calculated the metabolic redox ratio by performed imaging on tissues with two different excitation wavelengths to minimise spectral overlap between NADH and FAD and obtain separate emission intensities, [28,36,55]. In this study we performed imaging with a single wavelength and attempted to monitor the metabolic status of the epidermis by evaluating the ratio $\frac{\text{AUC}_{450\text{nm}-460\text{nm}}}{\text{AUC}_{525\text{nm}-535\text{nm}}}$ which may serve as a ballpark estimate of the NADH/FAD ratio. This approach has its advantages as it employed a single wavelength excitation that allowed a) quicker image acquisition per spot and therefore shorter imaging duration per mouse and b) minimising motion artifacts that arise during the wavelength switch. However this technique has its limitations, as the spectral overlap between NADH and FAD is higher when using a single near infrared (NIR) wavelength of 765 nm as compared to use of two different imaging NIR wavelengths. For the sake of measuring the ratio $\frac{\text{AUC}_{450\text{nm}-460\text{nm}}}{\text{AUC}_{525\text{nm}-535\text{nm}}}$ in a simple manner, the spectral overlap between NADH and FAD was not taken into consideration. In order to compensate for this spectral overlap, it should be noted that spectral overlap depends on (a) fluorophore concentration and (b) the imaging wavelength. When the imaging wavelength is kept constant and the fluorophore concentration is known, the amount of spectral overlap and hence the overlap correction factor can be determined ex vivo as in the case of the reference NADH and FAD solutions used in this study. As mentioned earlier, the spectral overlap between NADH and FAD measured ex vivo in reference solutions was 11% at the simulated band-pass filter of 450 nm – 460 nm, while it was 29% at 525 nm – 535 nm. The corresponding spectral overlap correction factor for ratio $\frac{\text{AUC}_{450\text{nm}-460\text{nm}}}{\text{AUC}_{525\text{nm}-535\text{nm}}}$ would therefore be 1.25 (i.e. $1 - \frac{0.11}{1 - 0.29} = 1.25$). If this correction factor was to be applied to the ratios obtained from in vivo measurements, the revised results would still show elevated $\frac{\text{AUC}_{450\text{nm}-460\text{nm}}}{\text{AUC}_{525\text{nm}-535\text{nm}}}$ values for early preclinical and clinical stages of carcinogenesis. This is because a correction factor of 1.25 would only further increase the existing difference shown presently in the results. However it would be inaccurate to extrapolate this spectral overlap correction factor from ex vivo reference samples of known fluorophore concentration to an in vivo environment with unknown fluorophore concentration.

Another factor to be noted is that in order for $\text{AUC}_{450\text{nm}-460\text{nm}}$ and $\text{AUC}_{525\text{nm}-535\text{nm}}$ to provide a reliable estimate of NADH and FAD in epidermis, the spectral contribution from other fluorophores such as keratin, lipids, lipofuscin, elastin and collagen needed to be minimised. This was achieved by performing the ratio analysis in a spatially discriminatory and depth
specific manner exclusively in epidermal cells, thus excluding superficial dead layers such as stratum corneum, hair follicles and dermis. However, the mentioned fluorophores still exist in epidermal cells as well, albeit in significantly lesser amounts than NADH and FAD. Keratinocytes contain intracellular keratin intermediate filaments that may affect reliable estimation of NADH in epidermis from $\text{AUC}_{450\text{nm}-460\text{nm}}$ while lipofuscin and lipids present due to ageing or waste product accumulation [59] could similarly interfere with FAD estimation from $\text{AUC}_{525\text{nm}-535\text{nm}}$. Future studies that use single wavelength imaging for NLSI, should therefore try to unmix the contribution of all fluorophores in the epidermis by adopting more complex algorithms such as classical linear unmixing, principal component analysis (PCA), parallel factor analysis (PARAFAC), non negative matrix factorisation (NMF) and spectral phasor analysis (SPA) [60]. The ratio calculated after unmixing with the aforementioned methods would be a more reliable indicator of the metabolic status in the epidermal cells.

### 8.5 Conclusion

From this study, it can be seen that *in vivo* NLSI could readily detect spectroscopic alterations that were present in the preclinical stages of skin carcinogenesis. The acquired spectral RGB images clearly revealed the changes in structural layers of skin during early carcinogenesis, which were confirmed by histopathology. The emission spectra revealed distinctive spectral shapes for normal skin, skin with preclinical hyperplasia and clinically visible skin tumours. This distinction between the spectral profiles of the three clinico-pathologic grades was observed the best at depth of 12 – 36 μm from mice skin surface. The results also showed the mean total SHG intensity decreased as the clinico-pathologic grade progressed from normal to preclinical hyperplasia and eventually clinically visible tumours. Furthermore, the ratio $\text{AUC}_{450\text{nm}-460\text{nm}} / \text{AUC}_{525\text{nm}-535\text{nm}}$ which is an indirect approximation of NADH/FAD in epidermal cells was found to be higher in skin with preclinical hyperplasia and clinically visible early skin tumours, when compared to normal skin. This suggests that the epidermal cells may have elevated cellular metabolism in the early preclinical stage of hyperplasia itself. Therefore based on all the results obtained in this study, it can be concluded that *in vivo* NLSI would be a useful tool to screen for early preclinical stages of cSCC.
References

37. N. D. Kirkpatrick, et al., Cancer Epidemiology Biomarkers & Prevention 16(10), 2048-2057 (2007).
Chapter 9

Discussion and Outlook
9.1 | Discussion and Outlook

This thesis provides the readers a perspective on how nonlinear optical imaging (NLOI) can potentially be applied for skin cancer diagnostics. The focus of this thesis can be broadly categorised into three objectives:

(a) To evaluate the potential biosafety risk that could be caused by \textit{in vivo} NLOI to minimise laser radiation associated hazards for patients.

(b) To establish a useful and easily replicable skin cancer model for biomedical imaging purposes. An attempt was made to establish this model in SKH1-hr strain mice as this particular strain of mice is hairless and immunocompetent. Therefore skin carcinogenesis in SKH1-hr mice would be more translatable for human subjects due to the comparable immune status and hair density, as compared to other mice skin cancer models. Due to low hair density, this mice skin cancer model can be extremely useful for dermatologic research using NLOI as there is minimal interference from hair during the imaging process.

(c) To recognise and discover optical and spectral signatures during different stage of skin carcinogenesis using \textit{in vivo} NLOI in the developed murine skin cancer model.

This chapter will focus on the findings presented in the previous chapters and discuss the implications these findings could have on clinical translation of NLOI for cancer diagnostics.

9.2 | Biosafety risk associated with \textit{in vivo} NLOI

For successful translation of NLOI as a diagnostic tool in the clinic, it is essential that the biosafety aspects and laser associated radiation hazard for NLOI be examined, especially considering the potential long-term effects of NLOI. In Chapter 4, it was seen that femtosecond laser irradiation used during NLOI could generate cyclobutane pyrimidine dimers (CPDs) in DNA which can trigger carcinogenesis if left unrepaired. In this chapter, it was shown that CPD formation during NLOI occurred due to simultaneous two- and three-photon absorption. It was also observed that the three-photon process was the primary mechanism for CPD production. It was found that modulating various imaging parameters such as laser power (peak irradiance), wavelength, pulse duration and pixel dwell time affected the level of CPDs produced significantly. To minimise CPD formations, the imaging regime of NLOI in the clinic should be customised accordingly. The optimal peak irradiance required for NLOI could be as high as 0.1 – 1 TWcm\(^{-2}\). Yet, even with peak irradiances as low as 250 GWcm\(^{-2}\), CPD formation still takes place in the DNA [1]. However, it was considered more relevant to estimate the risk of cutaneous squamous cell carcinoma (cSCC) induction from NLOI relative to regular solar UV exposure, which is a natural source of CPDs in humans. In Chapter 5, a unique risk model was developed with which we showed that the increase in CPD-induced cSCC risk from NLOI
biopsy is insignificant compared to the existing risk from sunlight. For instance, 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 (20 mW average laser power at focal plane) in that individual’s lifetime. In addition, the risk model indicated that the risk for cSCC significantly increased upon increasing the NLOI laser power or energy fluence and the number of biopsies in an individual’s lifetime. In contrast, use of longer NIR wavelength for imaging and reduced number of NLOI scans decreased the risk of cSCC. These findings reported in Chapter 5 again imply that NLOI biopsies should be performed with a well-planned protocol.

The model described in Chapter 5 can however be only applied for cSCC. Since the described risk model is based on cumulative sun exposure during one’s lifetime, it would be erroneous to extrapolate the risk prediction for cutaneous basal cell carcinoma (cBCC) and cutaneous malignant melanoma (cMM) which have a more complex multifactorial aetiology [2]. Nonetheless, investigating the risk of cBCC and cMM from NLOI biopsy is equally necessary, as BCC is the most common form of skin cancer while MM is the most fatal. Another factor to be borne in mind is that the measured risks in Chapter 5 apply mainly to Caucasian skin types. The described risk model does not account for non-Caucasians with darker skin who have a lower risk of cSCC from sun exposure due to UV protection from melanin. As a result of increased light absorption by melanin, NLOI procedures in these individuals may require higher laser powers. Consequently the relative risk for cSCC from NLOI maybe higher in non-Caucasians compared to their Caucasian counterparts. Therefore NLOI should be performed even more cautiously in the non-Caucasian population. Additionally, future studies should investigate the role of other DNA mutations that can also be caused by NLOI besides CPDs, such as oxidative lesions (8-oxo-guanine), DNA single strand breaks, and DNA double strand breaks [3]. Since NLOI potentially could have diagnostic applications in other organs besides skin, different risk models should be developed to assess the biosafety risk in these organs. This is essential as these organs may be more susceptible to radiation induced DNA damage unlike skin which is comparatively better protected due to melanin.

9.3 | Development of a hairless murine skin cancer model for in vivo NLOI research

SKH1-hr mice are the most popularly employed for translational skin cancer research using UV induced carcinogenesis. Multistage chemical carcinogenesis which gives a deeper insight into the stages of skin carcinogenesis has only rarely been employed for SKH1-hr mice. This may be because in order to initiate skin carcinogenesis, chemical carcinogens like DMBA require presence of active hair follicles with stem cells [4-6] which are sparse or abnormal in hairless mice. In chapter 6, a simple and easily reproducible murine skin cancer model was established in SKH1-hr mice using DMBA. The results show that complete carcinogenesis was possible in SKH1-hr mice with just weekly DMBA topical applications. The possible explanation could
be that DMBA may still be able to initiate carcinogenesis from the interfollicular epidermis [4] or rudimentary pilosebaceous appendages [7] despite the scarcity of active hair follicles in SKH1-hr mice. The results in Chapter 6 further showed that the mouse tumour load is highly sensitive to the frequency of DMBA application as well as concentration of DMBA per application. Therefore these two parameters must be customised accordingly to establish a successful murine skin cancer model. This particular cancer model was successful in producing tumours after around 10-11 weekly DMBA applications with malignant transformation occurring after just 17 weekly applications. Therefore, all the stages of skin carcinogenesis could be demonstrated in this particular murine model in about 17 – 20 weeks of DMBA application, which is comparable to conventional UV skin cancer models and quicker than DMBA-TPA skin cancer models. We believe that this model will therefore stand to benefit biomedical researchers aiming to study all stages of skin carcinogenesis in a shorter duration.

Among the other important observations in Chapter 6, it was seen that frequent DMBA applications produced epidermal hyperplasia in SKH1-hr mice skin. This was further validated by assessing the expression of cellular proliferation marker Ki67 which was also increased in DMBA treated skin with hyperplasia. However, at present it is not known if DMBA induced skin changes are reversible. Further studies are required to monitor skin response upon cessation of DMBA applications. The role of H-ras activation marker p-Erk1/2 was not very clear from this study as it was predominantly expressed in differentiated keratinocytes for normal and early benign lesions, while it was localised in proliferating keratinocytes in the malignant lesions. It may be possible that p-Erk1/2 serves a pro-differentiation role in keratinocytes in normal skin or early tumour stages and may get altered towards anti-differentiation and hyper proliferation of keratinocytes upon malignant transformation. In contrast, keratin 14, a marker for basal (undifferentiated) keratinocytes was present in stratum basale and the proliferating keratinocytes in all stages of skin carcinogenesis, while its expression was markedly reduced in the differentiated keratinocytes. Expression of tumour suppressor p53 and apoptotic marker cleaved caspase 3 was present only after the stage of malignant progression. This may be because unlike UV carcinogenesis, p53 may not have a prominent role in early stages of multi-stage chemical carcinogenesis such as initiation and promotion as shown in the study of Kemp et al. [8]. This shows a clear difference in the molecular mechanisms involved for UV induced carcinogenesis and DMBA induced carcinogenesis. Therefore, there is a dire need to clearly delineate the differences and overlap in features of carcinogenesis involved in these two types of skin cancer models. In addition, it should be noted that skin in SKH1-hr mice is generally associated with dermal inflammation [9]. It is important to evaluate if this pre-existing dermal inflammation makes SKH1-hr mice more susceptible for skin carcinogenesis when compared to other murine strains.
9.4 | In vivo NLOI to monitor early microscopic changes of skin cancer

NLOI that relied on two photon excitation fluorescence (TPEF) and second harmonic generation (SHG) has been tested as a skin cancer diagnostic tool in various studies [10-12]. Only few studies have explored the potential of in vivo NLOI to evaluate the early skin cancer changes. In chapter 7, in vivo NLOI was utilised to observe microscopic changes in all stages of skin carcinogenesis, which included the preclinical (hyperplasia), early clinical (preneoplasia/neoplasia) and late clinical (neoplasia) stages. Furthermore, early changes in skin carcinogenesis could be visualised using axial x-z NLOI scans, which are more comparable to standard H&E slides and thus more convenient for observation by a pathologist. The corresponding transverse x-y NLOI scans were able to provide finer complimentary details such as cell size, cell shape, nuclear cytoplasmic ratio and presence of mitotic cells. In this chapter, it was demonstrated that in vivo NLOI could clearly detect an increase in the epidermal thickness when performed on mice skin regions with preclinical or early clinical changes. The transverse x–y scans were additionally sensitive to detect cells with enlarged nuclei and actively dividing cells whenever present in the examined skin, suggestive of epithelial atypia and increased mitotic activity respectively. The quantitative index expressed as TPEF/TPEF+SHG in the imaged skin increased considerably in the early preclinical stage and then continued to rise for the further clinical stage of skin carcinogenesis. This rise in the index can be attributed to (a) increased TPEF from a proliferating epidermis and (b) decreased SHG detection due to downward displacement of dermal collagen by a thicker epidermis.

The significant aspect of the findings described in Chapter 7 was that in vivo NLOI could detect early microscopic changes in the preclinical stage of carcinogenesis itself, even when the skin regions appeared clinically normal. The results therefore showed that in vivo NLOI has immense potential as a non-invasive tool to visualise preclinical and early clinical changes in skin and other organs as well. 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 or dermatopathologist, in vivo NLOI can be utilised to track skin changes in patients at high risk or follow through in patients with recurring or a previous history of skin malignancies. Additionally, during Mohs surgery [13], in vivo NLOI could aid the surgeon to precisely excise the unhealthy or precancerous margins of malignant skin lesions to minimise recurrence.
9.5 | Spectroscopy and Metabolic assessment using NLSI

In Chapter 8, *in vivo* nonlinear spectral imaging (NLSI) based on TPEF and SHG was used to assess spectroscopic and metabolic changes in the DMBA induced skin cancer model in SKH1-hr mice. The acquired emission spectra were distinctive for normal skin, skin with preclinical hyperplasia and early neoplastic skin tumours. The spectra mainly differed between the three categories in the following ways:

(a) Compared to normal skin, the NADH emission at 460 nm predominated in the tissue spectra for skin with hyperplasia and early clinically visible skin tumours. This increase in NADH emission probably resulted from intracellular NADH being present more abundantly following epidermal proliferation that was observed in these stages as mentioned earlier in Chapter 7.

(b) Signal for dermal SHG at 382 nm was markedly reduced for skin with preclinical hyperplasia and completely absent in early clinically visible skin tumours when compared to normal skin. This is again probably due to SHG signal attenuation caused by downward dermal displacement as a result of epidermal proliferation, as hypothesised in Chapter 7.

Chapter 8 also demonstrated that *in vivo* NLSI can be used to indirectly assess to the metabolic changes during early stages of mice skin carcinogenesis. Simulated band-pass filters were applied at 450 nm – 460 nm and 525 nm – 535 nm and areas under curve (AUC) were measured in these described wavelength bandwidth. The quantified parameters AUC$_{450\text{nm}-460\text{nm}}$ and AUC$_{525\text{nm}-535\text{nm}}$ served as an estimate to NADH and FAD respectively, as they are the predominant fluorophores in skin epidermis at 450 nm – 460 nm and 525 nm – 535 nm. The influence of other fluorophores such as keratin, lipids, lipofuscin, elastin and collagen were minimised as the evaluation was performed exclusively on deeper epidermal cells, while hair follicle, stratum corneum and dermis were excluded from the data analysis. Therefore the ratio AUC$_{450\text{nm}-460\text{nm}}$/AUC$_{525\text{nm}-535\text{nm}}$ could be considered analogous to the ratio NADH/FAD. Upon evaluation, a notable increase was observed in the ratio AUC$_{450\text{nm}-460\text{nm}}$/AUC$_{525\text{nm}-535\text{nm}}$ for skin with preclinical hyperplasia and clinically visible early skin tumour when compared to normal mice skin. Since, the ratio is an indirect estimate of the ratio NADH/FAD, the increase in ratio AUC$_{450\text{nm}-460\text{nm}}$/AUC$_{525\text{nm}-535\text{nm}}$ could be explained by an increase in NADH in early stages of epidermal proliferation associated with hyperplasia and neoplasia. The increase in NADH could have occurred because of upregulation of glycolysis in proliferative epidermal cells due to either reduction of the Pasteur effect (reduced oxygen) or the presence of Warburg effect (in presence of oxygen) [14,15].
9.6 | Limitations in this thesis

The biggest limitation associated with *in vivo* NLOI described in chapters 7 and 8 of this thesis was the difficulty to obtain clear NLOI scans deeper than 60 µm in mice skin. There was a significant loss of signal intensity at depths below 60 µm upon using the NLOI setup described in Chapter 3. Therefore it was only possible to visualise the superficial layers of early and smaller clinical lesions. Due to extensive keratinisation present in advanced and larger lesions, it was not possible to image beyond the keratin layers that could extend beyond 100 – 200 µm deep in these lesions. As a result, no microscopic changes could be visualised at the epidermo-dermal junction of the advanced neoplastic skin lesions. Since this zone was located at a depth beyond the imaging limit of the NLOI setup described in this thesis, it could not be determined if the lesion was benign or malignant, as visualising the epidermo-dermal junction of the lesion is essential for a pathologist in order to establish the invasiveness of a lesion. It should however be borne in mind that performing *in vivo* NLOI non-invasively on larger skin tumours offers no additional benefit to the individual. This is plainly because in such an advanced state of carcinogenesis, a classical biopsy followed by appropriate treatment of the tumour is unavoidable.

It should be duly noted is that the inability to image deeper than 60 µm is more likely a limitation of the NLOI setup described in Chapter 3 and not a limitation of NLOI in general. Other studies that utilised NLOI for skin cancer diagnostics have successfully imaged as deep as 135 – 320 µm in human skin [11, 16]. In those studies, NLOI could be performed at a much greater depth because of the following reasons: a) use of an objective with higher numerical aperture (NA) [16] resulted in more efficient emission signal collection at greater depths, b) use of an oil-immersion objective [16] instead of a water-immersion objective (used in the Chapter 7 and 8) resulted again in more effective collection of autofluorescence signals at increasing depths, as the former has a lower refractive index mismatch compared to the latter [17] and c) use of higher laser power (peak irradiance) [11] provides a higher signal-to-noise ratio at greater depths.

Although axial x-z NLOI scans can provide images analogous to standard H&E slides noninvasively, it still suffers from poor visualisation of finer details such as cellular cohesiveness and cytonuclear morphology. An alternative to this disadvantage would be to implement an imaging protocol which involves combination of axial x-z scans and transverse x-y scans. In such a protocol, a quick axial x-z NLOI scan could be performed to identify the suspect region of interest in skin. Following this, finer tissue cellular details that are essential for pathologists could be obtained by performing a series of transverse x-y NLOI scans in the region of interest to identify the cytonuclear features. Since multiple scans are required per spot to obtain useful diagnostic information, it would be useful to have rapid image acquisition per scan to keep NLOI less time consuming for the patient. In such a scenario, a single axial x-z scan or a transverse x-y scan takes about 6.5 seconds with the setup described in this thesis. If an elaborate imaging protocol is followed that would involve one axial x-z scan and a complimentary stack of 20
transverse x-y scans for the finer cytonuclear details, the process may take about 2 – 2.5 minutes which is clinically feasible for the patient. Although the NLOI protocol may last only a couple of minutes, it could result in images that contain motion artefacts arising from the patient. This can interfere with image analysis and therefore needs to be resolved.

It is also quite challenging to arrive at a specific histopathological diagnosis for a skin tumour or to differentiate one cancer subtype from the other by solely using in vivo NLOI. This is essentially because in vivo NLOI provides very poor intra-nuclear details at present [18, 19]. 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 or the best location of an invasive biopsy on a suspect region, rather than to provide an accurate pathology diagnosis. This would still help in reducing the number of unnecessary invasive biopsies considerably benefiting the patient, the dermatologist and the pathologist.

Another factor to be considered while utilising NLOI to assess the metabolic status of epidermal cells in the skin, is that $A_{450nm-460nm}$ and $A_{525nm-535nm}$ only represent indirect approximations of NADH and FAD respectively in the epidermis. To obtain a more precise estimation of the epidermal metabolic status, more complex algorithms should be utilised to unmix NADH and FAD from other fluorophores in the epidermis. Future studies should try to utilise algorithms such as classical linear unmixing, principal component analysis (PCA), parallel factor analysis (PARAFAC), non negative matrix factorisation (NMF) and spectral phasor analysis (SPA) to quantify NADH and FAD levels in normal and cancerous tissues more accurately [20].

9.7 | Future outlook

Despite the limitations observed in this thesis, it is possible that in the near future technological advancements could deliver:
(a) 3-photon based NLOI that may enable deeper tissue penetration [21,22].
(b) Compact NLOI based micro-endoscopy that could detect cancerous changes in visceral organs [23,24].
(c) Spatial overlap modulation NLOI that can further reduce background noise [25].
(d) Advanced motion compensations during optical imaging that may minimise motion artefacts [26].
(e) Photon counting detection that could improve signal-to-noise ratio [27].

These advancements could substantially enhance the quality of NLOI images that may then be able to provide histopathological diagnosis in not just skin but other organs as well. These advances would also ensure more efficient cancer detection in a non-invasive manner with improved sensitivity and specificity. In addition, there are studies where NLOI...
has been efficiently combined with other emerging optical imaging techniques such as optical coherence tomography (OCT) [28-30] and coherent anti-Stokes Raman's spectroscopy [16,31-34]. As a result, anatomical, physiological as well as biochemical details can now be jointly obtained from these multimodal imaging techniques with increased sensitivity. In this manner, the limitations of one particular optical imaging technique are countered by the other complimentary imaging modality.

With regard to clinical feasibility, it should be noted that NLOI setups are quite complex and expensive for diagnostic or surveillance purposes at present. This is primarily because NLOI devices are often home built mainly only for academic or research purposes at the moment, with the exception of Dermainspect and MPTflex developed by Koenig et al. [16]. Of late, there have however been attempts to build NLOI in a more cost-effective manner as shown by Kieu et al. [35]. At the moment, NLOI still needs to be successfully established through clinical trials with further sensitivity and biosafety evaluation. Only then commercial healthcare industries would invest in manufacturing simpler and more affordable instrumentation NLOI for primary or specialist clinics. Once cost-effectiveness and improved sensitivity is achieved for in vivo NLOI, it would indeed be a highly suitable non-invasive alternative to conventional histopathology.
References

Summary

The objective of this thesis was to investigate the potential of in vivo nonlinear optical imaging (NLOI) as a diagnostic tool in a mice skin cancer model. To achieve the goals of this thesis, the carcinogenesis risk associated with femtosecond laser radiation arising from the NLOI setup was initially evaluated. This was followed by developing a simple and easily reproducible skin cancer model in hairless mice for the purpose of diagnostic skin cancer research. The core of the thesis involved identifying diagnostic signatures using in vivo NLOI to differentiate normal mice skin from skin in different stages of carcinogenesis.

In Chapter 2, an overview is presented regarding the advances and challenges in label-free in vivo NLOI for translational cancer research. This chapter first covers the basic concepts related to label-free NLOI, such as two-photon excitation fluorescence (TPEF), second harmonic generation (SHG) and endogenous optical fluorescence. This review continues with the accomplishments of various label-free NLOI based studies in cancer diagnostics and the challenges faced for successful clinical translation of label-free NLOI as a cancer diagnostic tool. This is followed by Chapter 3, where a brief description is provided for the NLOI setup utilised for the experiments described in this thesis.

Chapter 4 and Chapter 5 mainly deal with the evaluation of biosafety and carcinogenic risk associated with NLOI. Chapter 4 shows that DNA mutations such as cyclobutane pyrimidine dimers (CPDs) can be produced in vitro in Chinese Hamster Ovary (CHO) cells by femtosecond laser irradiation during NLOI. The results further show that these CPDs were generated as a result of concurrent two- and three-photon absorption and demonstrate that CPD production increased upon use of higher peak irradiance and longer pixel dwell time, while it decreased with use of longer near infrared (NIR) imaging wavelength and broader pulse width. In Chapter 5, the findings obtained in Chapter 4 are extrapolated towards a unique cancer risk model to assess the risk of cutaneous squamous cell carcinoma (cSCC) following NLOI biopsies. The chapter demonstrates that the additional risk of cSCC arising in skin following NLOI is negligible above the pre-existing risk for cSCC from regular UV exposure from the sun. The study also indicates the need to delineate an efficient imaging protocol for potential NLOI procedures in the clinic, as the risk for cSCC from NLOI could increase if in vivo NLOI biopsies are conducted haphazardly by carrying out excessive NLOI biopsies in the same individual or using high peak irradiances.

In Chapter 6, a skin cancer model we have developed is described exhibiting all stages of skin carcinogenesis in the hairless SKH1-hr mice. This was achieved by using a simple and easily reproducible protocol that involved low dose weekly topical application of 7, 12-dimethylbenz(a)anthracene (DMBA). This cancer model when established in SKH1-hr mice provided malignant conversion and carcinoma development at a rate comparable to UV skin cancer mice models. The investigations also revealed that repeated DMBA applications can directly cause significant epidermal proliferation in mice skin, without the aid of another tumour promoter. The role of markers for Ras activation (p-Erk1/2), basal or undifferentiated
keratinocytes (keratin 14), proliferation (Ki67), tumour suppression (p53) and apoptosis (cleaved caspase 3) were additionally investigated to understand the underlying mechanism of DMBA induced carcinogenesis in SKH1-hr mice skin. Expression of p-Erk1/2 was found to be localised in differentiated keratinocytes in normal skin and early cancer stages, while it was predominantly present in the proliferating keratinocytes in the malignant stages. On the contrary, the expression of keratin 14 and Ki67 was present in mainly proliferating keratinocytes and was increased with hyperplasia and subsequent neoplastic stages. Tumour suppressor p53 and apoptotic marker cleaved caspase 3 were significantly expressed only in the malignant stages of skin carcinogenesis.

Chapter 7 and Chapter 8 describe our efforts to identify diagnostic cancer signatures by applying in vivo NLOI in the developed cSCC mice models. In Chapter 7, the results show that axial x-z NLOI scans clearly detect abnormal epidermal proliferation in skin during different stages of DMBA induced skin carcinogenesis. Complimentary transverse x-y NLOI scans visualise cytonuclear features such as size and shape of epidermal cells and its nuclei that makes it useful to determine the presence of epithelial atypia. In addition, the TPEF-SHG index was found to be a reliable indicator of epidermal proliferation as it was significantly increased in skin with preclinical hyperplasia and in neoplastic skin lesions. In chapter 8, we show that spectral imaging performed with in vivo NLOI could detect spectroscopic alterations that were present right in the preclinical stages of skin carcinogenesis itself. The emission spectra were found to be distinctive for normal skin, skin with preclinical hyperplasia and neoplastic skin lesions. Following DMBA treatment, the mean total SHG intensity measured from the spectral RGB images decreased as normal skin progressed to hyperplasia and eventually neoplasia, while the mean total autofluorescence intensity remained unaffected. The ratio in epidermal cells, formulated as \( \frac{\text{AUC}_{450-460\text{nm}}}{\text{AUC}_{525-535\text{nm}}} \), which is an indirect approximation of NADH/FAD, was found to be higher in skin with preclinical hyperplasia and early clinically visible skin tumours, when compared to normal skin. This suggests that the epidermal cells expressed higher cellular metabolism in the early preclinical stage of carcinogenesis itself.

In the end, Chapter 9 provides a concise discussion of the results obtained in the studies presented in this thesis. The implications of the presented findings for future NLOI research in cancer diagnostics are further discussed. This chapter concludes with a perspective on how current technological advancements may improve the efficiency, sensitivity and cost-effectiveness of NLOI in order to turn it into a clinically viable technology for screening and aiding biopsy procedures, or maybe even providing a non-invasive alternative to conventional histopathology.
Samenvatting

Doel van het onderzoek gepresenteerd in dit proefschrift was om te onderzoeken of het mogelijk is om met behulp van niet lineaire optische imaging (NLOI) huidkanker in een vroeg stadium te detecteren. Daartoe is eerst onderzocht wat het carcinogene risico is van het gebruik van NLOI op de menselijke huid. Daarna is een huidkanker model in de haarloze muis ontwikkeld. Het belangrijkste werk in het proefschrift betreft de bestudering van de spectrale signaturen gemeten in vivo met NLOI gedurende de ontwikkeling van huidtumoren.

Hoofdstuk 2 geeft een overzicht van de voortgang en uitdagingen in NLOI toegepast in vivo bij translationeel kankeronderzoek. In dit hoofdstuk wordt ingegaan op de basisconcepten van NLOI, zoals twee-foton fluorescentie (TPEF) second harmonic generation (SHG) en endogene fluorescentie. Vervolgens wordt een overzicht gegeven van wat reeds bereikt is met NLOI bij de diagnose van kanker en wat samen wat de uitdagingen zijn om te komen tot klinische translatie. Daarna wordt in hoofdstuk 3 een beschrijving gegeven van de apparatuur die gebruikt is voor de in dit proefschrift beschreven experimenten.

Hoofdstukken 4 en 5 gaan voornamelijk over de veiligheidsaspecten van het gebruik van NLOI. In hoofdstuk 4 wordt in vitro aangetoond dat er mutaties kunnen optreden in DNA zoals cyclobutaan pyrimidine dimeren (CPDs) t.g.v. bestraling met femtoseconde laserpulsen. De resultaten laten verder zien dat de CPDs veroorzaakt worden door zowel twee- als drie-foton absorptie en dat de effectiviteit toeneemt met toenemende piekintensiteit. In hoofdstuk 5 worden deze resultaten toegepast op een uniek risicomodel dat de toename van het risico op huidkanker in een menselijke populatie voorspelt in relatie tot het risico van door zonlicht geïnduceerde huidtumoren. Hiermee laten we zien dat de toename van het relatieve risico bij verantwoord gebruik van NLOI verwaarloosbaar is.

In hoofdstuk 6 wordt een huidkankermodel beschreven dat we speciaal hiervoor hebben ontwikkeld. Dit model maakt gebruik van haarloze SKH1-hr muizen die wekelijks een lage dosis van de carcinogene stof 7, 12-dimethylbenz(a)anthracene (DMBA) op de huid krijgen toegediend. Dit model laat alle stadia van kankerontwikkeling in de huid zien, inclusief de transformatie naar invasieve maligniteit en in een tempo vergelijkbaar met UV geïnduceerde huidtumoren. Het model werd in detail gekarakteriseerd: de rol van markers voor Ras activering (p-Erk1/2), basaal of ongedifferentieerde keratinocyten (keratin 14), proliferatie (Ki67), tumor suppression (p53) en apoptose (cleaved caspase 3). De marker p-Erk1/2 kwam met name tot expressie in gedifferentieerde keratinocyten in normale huid en vroege stadia van kanker terwijl het in het maligne stadium beperkt bleef tot de prolifererende keratinocyten. Dit in tegenstelling tot de expressie van keratine 14 en Ki67 dat aanwezig was in de prolifererende keratinocyten en toenam in hyperplasie en de daaropvolgende neoplastische stadia. Tumor suppressor p53 en apoptotische marker cleaved caspase 3 kwamen alleen noemenswaardig tot expressie in de maligne stadia van huidkanker.
Hoofdstukken 7 en 8 beschrijven de zoektocht naar spectrale signaturen in NLOI geschikt voor kanker detectie in het muismodel. In hoofdstuk 7 is te zien dat axiale x-z scans duidelijk de verschillende stadia van DMBA geïnduceerde huidtumoren kunnen onderscheiden. Aanvullende transversale x-y scans geven duidelijke cytonucleaire informatie zoals vorm en grootte van cellen en kernen weer, zodat het mogelijk is hiermee de aanwezigheid van epitheliale atypie te bepalen. Bovendien kon een TPEF-SHG index gedefinieerd worden waarmee op een betrouwbare manier epidermale proliferatie kon worden gekwantificeerd. Deze was significant verhoogd in huid met preklinische hyperplasie en in neoplastische huid. In hoofdstuk 8 laten we zien dat het mogelijk is om met NLOI spectroscopische veranderingen te detecteren in de preklinische stadia van huidkanker. De spectra van normale huid, huid met preklinische hyperplasie en neoplastische huid waren duidelijk verschillend. Gedurende de transformatie van normaal naar hyperplasie en uiteindelijk neoplasie ging de totale SHG systematisch omlaag, terwijl de totale autofluorescentie toe nam. Het quotiënt $\frac{\text{AUC}_{450-460\text{nm}}}{\text{AUC}_{425-535\text{nm}}}$, een indirecte schatting van de redox ratio NADH/FAD, bleek verhoogd te zijn in huid met preklinische hyperplasie en in vroege klinisch zichtbare laesies, t.o.v. normale huid. Dit suggereert dat het cel metabolisme in de preklinische stadia hoger is dan in de fase dat het een klinisch herkenbare tumor is.

Hoofdstuk 9 worden alle resultaten op een rijtje gezet en de implicaties voor verdere ontwikkeling van NLOI bediscussieerd. Het hoofdstuk eindigt met een visie op de noodzakelijke technologische ontwikkelingen die noodzakelijk zijn om tot een effectieve, sensitieve en kosteneffectieve toepassing van NLOI te komen, zodat het gebruikt kan worden voor het verbeteren van diagnostische procedures, of mogelijk als een niet invasief alternatief boven conventionele histopathologie.
Appendices

Acknowledgements
List of publications
PhD portfolio
About the Author
Acknowledgements

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List of publications

Peer reviewed publications


Conference proceedings


### PhD Portfolio

**Name of PhD student**: Giju Thomas  
**PhD period**: 2009 – 2014  
**Promotor**: Prof. dr. A.G.J.M. van Leeuwen  
Prof. dr. ir. H.J.C.M. Sterenborg  
**Co-promotor**: Prof. dr. H.C. Gerritsen

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### PhD Training

#### Courses
- Basic and translational oncology  
  2009  
- Animal imaging workshop by AMIE  
  2010  
- Article 9 (Experimental Animals Act) course on laboratory animal science  
  2011  
- Biophotonics 2011, Summer school in Ven, Sweden  
  2011  
- Biomedical English writing and communication  
  2012  
- Molecular Diagnostics  
  2014  
- Workshop on Grant Proposal Writing  
  2014

#### Seminars and research meetings
- Department research meetings  
  Oral presentations  
  2009 – 2013  
- STW – Project meetings  
  Oral presentations  
  2009 – 2014  
- Biophotonics 2011, Ven, Sweden  
  Poster presentation  
  2011  
- Open Seminar – Molecular Biophysics group, Utrecht  
  Oral presentation  
  2012  
- Networking and negotiation workshop for final year PhDs and postdocs  
  2013 – 2014

#### International conferences
- ECBO, Munich, Germany  
  Oral presentation  
  2011  
- SPIE, Photonics West, San Francisco, USA  
  Poster presentation  
  2012  
- SPIE, Photonics Europe, Brussels, Belgium  
  Poster presentation  
  2014
Giju Thomas was born on November 25th, 1982 in Fujairah, United Arab Emirates. He completed his primary and high school education at Our Own English High School, Sharjah, United Arab Emirates. He was placed in the top 0.1% of candidates who participated in the Mathematics Olympiad organised by the Central Board of Secondary Education, India on a national level in 1997. He had also secured the highest aggregate during his sophomore (10th grade) and senior high school (12th grade – Science Stream) examinations at his school. After finishing his high school in 2000, he began his Medical Doctor’s degree (MBBS) at Father Muller Medical College, Mangalore, India under Rajiv Gandhi University of Health Sciences. During the course, he attained proficiencies in subjects such as Physiology (in 2001), Pharmacology (in 2003) and Microbiology (in 2003). He graduated in 2006 with the award of “The Best Outgoing MBBS Student – 2006” being conferred to him.

After his medical doctor studies, he functioned as a Junior Neurosurgery Resident for a brief period at Pushpagiri Medical College Hospital, Tiruvalla, India. In order to pursue his interests in cancer research, he later enrolled for a Masters in Technology program in Nanomedical Sciences at Amrita Centre for Nanosciences, Kochi, India under Amrita University in 2007. His Master’s thesis focused on the cytocompatibility of surface-modified titanium implants. The thesis involved evaluating protein adsorption, primary osteoblast proliferation and released cytokine levels in response to titanium surfaces modified with titanium dioxide nanotubes of different dimensions. In 2009, he obtained his Master’s degree and was awarded a Gold Medal for securing the highest cumulative grade point average during the course.

Following his Master’s course, Giju joined as a PhD student under the supervision of Professor dr. ir. H.J.C.M. Sterenborg at the Centre for Optical Diagnostics and Therapy in Erasmus Medical Centre, Rotterdam, the Netherlands. This PhD project was in collaboration with Professor dr. Hans C. Gerritsen from the Molecular Biophysics Department at the Utrecht University, Utrecht, the Netherlands. In the initial phase of the PhD project, Giju’s work involved assessing the biosafety and cancer risk from nonlinear optical imaging. In the next stage, he managed to develop skin cancer models in hairless immunocompetent mice using chemical carcinogens. Eventually he used these skin cancer models for in vivo nonlinear optical and spectral imaging to identify diagnostic optical signatures for skin cancer detection.