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

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Chapter 1

General Introduction
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 $1.5 \text{ m}^2 - 2 \text{ m}^2$ 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](image)

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

<table>
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<th>Non-invasive diagnostic modalities</th>
<th>Principle involved</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td>Tape-strip mRNA analysis</td>
<td>Diagnosis based on mRNA analysed from epithelial cells adhering to the tape after stripping it off from lesions.</td>
<td>- Sensitive for early melanomas.</td>
<td>- Only few studies to confirm specificity and sensitivity of diagnosis.</td>
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<td></td>
<td>- Maybe able to detect genomic changes before morphologic changes.</td>
<td>- No studies on NMSC.</td>
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<tr>
<td>Tetrahertz Imaging</td>
<td>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.</td>
<td>- Able to distinguish between cBCC and normal skin.</td>
<td>- Limited studies.</td>
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<td></td>
<td></td>
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<td>- All studies on cBCC.</td>
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<td></td>
<td></td>
<td></td>
<td>- Thermal effects (increase in temperature)</td>
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<td></td>
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<td>- Limited depth penetration for imaging.</td>
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<td></td>
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<td>- No useful cellular details for pathologists.</td>
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<tr>
<td>Electrical bio-impedance analysis</td>
<td>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.</td>
<td>- Able to detect cMM, cBCC and cSCC from normal skin.</td>
<td>- Since the modality relies on electric impedance spectra, no visual/cellular details are available for analysis.</td>
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<td>- High intra-group variance, leading to inability to differentiate one cancer type from another.</td>
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<td>Photodynamic diagnosis</td>
<td>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.</td>
<td>- Application of photosensitisers like aminolevulinic acid (ALA) has been found useful in detecting cBCC and cSCC.</td>
<td>- Requires an exogenous photosensitiser to provide contrast.</td>
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<td></td>
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<td>- Useful in detecting recurrence or local spread of the lesions in skin cancer.</td>
<td>- Highly sensitive to the pharmacokinetics and tissue distribution of the photosensitiser.</td>
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<tr>
<td>Non-invasive diagnostic modalities</td>
<td>Principle involved</td>
<td>Advantages</td>
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| 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 type. | – 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