Model-based quantitative optical biopsy in multilayer *in vitro* soft tissue models for whole field assessment of nonmelanoma skin cancer

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Abstract. Optical techniques such as fluorescence and diffuse reflectance spectroscopy are proven to have the potential to provide tissue discrimination during the development of malignancies and hence treated as potential tools for noninvasive optical biopsy in clinical diagnostics. Quantitative optical biopsy is challenging and hence the majority of the existing strategies are based on a qualitative assessment of the concerned tissue. Light–tissue interaction models as well as precise optical phantoms can greatly help in the former and here we present a pilot study to assess the optical properties of a multilayer tissue-specific optical phantom with the help of a database generated using multilayer-Monte Carlo (MCML) models. A set of optical models mimicking the properties of actual and diseased conditions of tissues associated with nonmelanoma skin cancer (NMSC) were devised and MCML simulations of fluorescence and diffuse reflectance were performed on these models to generate the spectral signature of identified biomarkers of NMSC such as hemoglobin, flavin adenine dinucleotide, and collagen. A model library was generated and with the extracted features from modeled spectra, classification of normal and NMSC conditions were tested using the *K*-nearest neighbor (KNN) classifier. Using an in-house assembled scan-based automated bimodal spectral imaging system with reflectance and fluorescence modalities of operation, a layered, thin, tissue equivalent phantom, fabricated with controlled optical properties mimicking normal and NMSC conditions were tested. The spectral signatures corresponding to the NMSC biomarkers were acquired from this phantom and extracted features from the spectra were tested using the KNN classifier and classification accuracy of 100% was achieved. For further quantitative analysis, the experimental and simulated spectra were compared with respect to the light intensity at the emission peak or absorption dips, spectral line width, and average intensity over a range of wavelength of interest and observed to be analogous within specified and systematic error limits. This methodology is expected to give a better quantitative approach for estimation of tissue properties by correlating the experimental and simulated data.

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1 Introduction

Nonmelanoma skin cancer (NMSC) is one of the most common forms of human malignancy with millions of new cases being diagnosed each year across the globe. The cause of NMSC is multifactorial and complex and it occurs most commonly in sun-exposed areas, such as the face, neck, scalp, forearms, hands, legs, and feet.¹ The incidence of NMSC is high in white populations of Celtic heritage and low in Hispanics, Asians, and Blacks. Even though there are many approaches to the management of NMSC, there is no definitive standard of care for the treatment of this type of cancer. A better understanding of the etiological factors is very essential for the prevention of NMSC.²

In the current clinical scenario, NMSC is diagnosed through biopsy, which is a painful procedure involving different sample preparing procedures for histopathology, slow in diagnostic results, limiting the information content in the test sample, and is unpleasant for the patients.^{3–5} On the other hand, optical

spectroscopic techniques are becoming increasingly important in the clinical management of such malignancies where the diagnosis of the tissue is done based on the optical measurements with the help of biomarkers.⁶ The intent of these systems is to provide diagnostic signatures, *in situ*, noninvasively, and in real-time and could offer the potential for fast, noninvasive early detection providing a valuable aid to the clinician and increased comfort to the patient.

Specific to the present context, several research groups have worked on the development of several spectroscopic systems for skin cancer diagnosis. A custom-made fiber optic probe-based multimodal spectroscopic setup with multiple modalities including diffuse reflectance, fluorescence, and Raman spectroscopy was developed to classify normal and NMSC lesions using principal component analysis. The results from the study demonstrated the ability of these modalities to qualitatively assess NMSC with a sensitivity and specificity of ~95% and 85%.⁷ The same multimodal spectroscopic setup was upgraded including miniature spectrometers, fiber-coupled broadband light source, diode laser, and a revised optical probe, which resulted

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in a ~50% reduction in system footprint resulting in a more clinic-friendly system.⁸ An instrument combining Raman, fluorescence, and reflectance spectroscopic modalities was developed to interrogate cutaneous lesions associated with skin cancer. It was observed that the system was able to measure physiological quantities such as relative fluorophore concentration, oxygen saturation, blood volume fraction, and mean vessel diameter.^{9,10}

From the existing methodologies, it can be observed that assessment and staging of skin cancer is possible by exploring different modalities of spectral analysis for extraction of biomarkers, which are relevant in the case of skin cancer. In all the works detailed above, the measurements of optical properties over a physiologically relevant range were demonstrated using tissue-simulating liquid phantoms.^{7–10} But liquid phantoms cannot mimic the multilayer nature of real skin tissue with respect to the geometry and dimensions and hence such methods lead to loss of sensitivity in disease diagnosis. Also, matching with the simulated spectra with the real multilayer optical model will be erroneous if the physical phantom does not follow a similar trend and would affect the results of quantification of optical properties.

In most of the studies, single point fluorescence/reflectance measurements have been used to discriminate diseased and nondiseased tissues.^{11,12} Although, these point measurement techniques have the ability to diagnose malignancies, the results may lead to false-positive findings due to the insufficiency of data. In order to study the extent to which the abnormality has spread in the tissues and to define margins of resection for various surgical procedures, it is necessary to collect spectroscopic measurements and images from the region under study. Several groups have developed endoscopic^{13–18} and nonendoscopic-based fluorescence and reflectance imaging systems^{19–21} for these applications. These systems were based on multipixel illumination and detection. Multipixel illumination requires the use

of high-powered light sources to illuminate a large tissue area such that there is sufficient power density/pixel. Multipixel detection is achieved using a charge-coupled device (CCD) camera. The requirement for high-powered light sources and CCD cameras renders these systems expensive.²² In this work, we have developed an automated scan-based bimodal spectroscopic setup that has the ability to record fluorescence and reflectance images/signals in the *in vivo* environment, which will be of clinical interest with less cost.

The goal of the present work is to devise a set of optical models mimicking normal and NMSC conditions with NMSC-specific biomarkers such as hemoglobin (Hb), flavin adenine dinucleotide (FAD), and collagen. A model library is generated with the spectral features extracted from the modeled spectra with regard to fluorescence and diffuse reflectance modalities and discrimination of normal and NMSC conditions is tested using a classification algorithm. Validation studies are conducted using realistic layered, solid-tissue equivalent phantoms with embedded NMSC-specific biomarkers fabricated using spin-coating methodology. The experimental and modeled spectra are compared to obtain qualitative and quantitative information about the biomarkers for better assessment of NMSC.

2 Materials and Methods

The development of automated scan-based bimodal spectroscopic setup for assessment of tissue malignancy will be briefed in the section followed by the optical modeling, numerical simulations, feature extraction, and phantom fabrication.

2.1 Development of Scan-Based Automated Spectroscopic Setup

A schematic of the bimodal spectral acquisition setup developed is shown in Fig. 1. The setup was highly portable, simple in arrangement, and consisted of two sources for each of the two modalities: a Xenon flash lamp (Comtek Scientific,

Fig. 1 Schematic of the bimodal spectroscopic setup.

Bangalore, India), which provides broadband illumination for reflectance measurements, a compact 450-nm LED light source (AvaLight-LED450, Avantes, The Netherlands) to excite FAD and a 325-nm filter aligned with the xenon flash lamp (Comtek Scientific, Bangalore) to excite collagen for fluorescence studies. In the detector part, the setup consists of a spectrometer (USB4000, Ocean Optics), a computer, and associated software (Spectra suite, Ocean Optics) to display the spectrum. The fibre probe (Comtek Scientific, Bangalore) consists of six illumination fibers in a circular arrangement and a single collection fiber located at the center, bundled together into a steel tube. The six-fiber leg of the probe connects to the light source and the single-fiber leg connects to spectrometer. All optical fibers used in the probe were of 750 μm core diameter with a numerical aperture of 0.22. The probe consisted of a stainless steel ferrule with a polyvinyl sleeve at the tip, and all experiments maintained a fixed probe distance of 0.5 mm from the sample surface.

Point spectral measurements were obtained by directing the light from the source to the sample surface by means of the illumination fibers of the optical probe and the collection fiber collects the backscattered/emitted light. For DRS studies, the probe was held in upright position and illumination to the sample was provided at an angle of 90 deg to the phantom surface, whereas for fluorescence studies, illumination to the sample was provided at an angle of 45 deg to the phantom surface for backscattering elimination. The emitted fluorescence/reflectance is collected by the probe and is directed to a PC integrated spectrometer, which is processed for further analysis.

Whole field imaging is achieved by raster scanning of the sample in X and Y directions using a programmable translation stage (MTS-90-90-2, Holmarc Opto Mechatronics Pvt. Ltd., India) equipped with a microposition controller (HO-MPC-2L, Holmarc Opto Mechatronics Pvt. Ltd., India). The micro-translational stage in the setup moves the sample in XY plane in steps to collect the entire sample fluorescence/reflectance from a given number of sample positions by means of point by point scanning. Acquired spectral data are processed for further analysis using a suitable algorithm to generate the images.

2.2 Optical Modeling of NMSC Tissue and Numerical Simulations

A set of 50 optical models mimicking normal and NMSC conditions (25 each) were devised consisting of epidermis and papillary dermis with a predefined thickness of 100 μm for epidermis and 300 μm for papillary dermis, which corresponds to human skin.²³ Tissue models mimicking normal conditions were devised with their compositions ranging from 5 to 20 μM Hb, 70 to 120 μM FAD, and 15 to 25 mg collagen and abnormal models were devised with 30 to 46 μM Hb, 150 to 200 μM FAD, and 5 to 10 mg collagen. Each layer of

the model is characterized by its intrinsic optical properties such as absorption coefficient μ_a , scattering coefficient μ_s , anisotropy factor g , and refractive index η as a function of wavelength λ expressed on the order of nanometers. The volume fraction of melanosomes, which corresponds to the optical absorption coefficient of the epidermis, is taken as 1% for the simulation study.²⁴ The absorption coefficient of dermis is determined primarily by the absorption of Hb in blood and minor baseline skin absorption.^{25,26} Typical ranges of Hb concentration variation mimicking normal and abnormal tissues were obtained from the literature.²⁷ Scattering coefficient μ_s and anisotropy factor g of normal and abnormal conditions were approximated using the relations given in the literature.^{28–30} High and low concentrations of FAD representing normal and abnormal conditions²¹ were chosen for the study, and collagen fibers were taken as the major fluorophore and scatterer in the dermal layer. Quantum yield of FAD was taken as 0.03 and collagen was taken as 0.4³¹ in the model. The refractive index of epidermis was taken as 1.4 and dermis as 1.36.³² Table 1 provides the values of absorption coefficient (μ_a) of FAD, collagen, and Hb chosen for a normal and NMSC model obtained using a UV–VIS–NIR spectrophotometer (Varian Cary 5E).

MCS of diffuse reflectance^{33–35} and fluorescence^{36,37} were performed on the devised optical normal and NMSC models to generate the spectral signature of the specified biomarkers of interest namely Hb, FAD, and collagen. A cylindrical coordinate system was used to orient the photons and to map the spatial distribution of excited and emitted photons at different depths and radial distances. Spectra were simulated for a wavelength range from 370 to 650 nm and optical parameters such as number of fluorescence photons emitted, spatially resolved diffuse reflectance and total re-emitted fluorescence were obtained for the particular probe geometry.

2.3 Feature Extraction and Classification

A model library was generated from the normal and abnormal modeled spectra obtained from Sec. 2.2 with the extracted first-order statistical features from spectra, which include signal mean, signal peak, variance, skewness, kurtosis, maximum to minimum difference, root mean square value, and entropy. The K -nearest neighbor (KNN) classifier was used to distinguish normal and NMSC conditions due to its simplicity and transparency in machine learning modalities.³⁸ It is a method for classifying objects based on closest training examples in the feature space: an object is classified by a majority vote of its neighbors, with the object being assigned to the class most common among its KNNs (k is a positive integer, typically small). In this work, the optimum value of k was set to 1 in order to allow for the closest training samples of the class. Selection of appropriate features is an important precursor, which helps to avoid dimensionality issues and to balance accurate and robust

Table 1 Absorption coefficient of FAD and collagen at their excitation and emission wavelengths and Hb at its absorption peaks.

	Absorption coefficient $\mu_a(\lambda)$ (cm^{-1})			Absorption coefficient $\mu_a(\lambda)$ (cm^{-1})		Hb (μM)	Absorption coefficient $\mu_a(\lambda)$ (cm^{-1})	
	325 nm	405 nm	FAD (μM)	450 nm	526 nm		542 nm	578 nm
Collagen (mg)								
20	3.48	3.66	100	2.21	0.03	15	1.84	1.89
10	1.73	1.84	200	4.39	0.05	46	5.65	5.80

classification by identifying the more predictive features of the category. Among the eight features extracted from the spectra, signal peak, signal mean, and variance showed a considerable difference in both sets of data and were chosen to test the performance of the classifier.³⁹ Extracted features from 35 models (20 normal and 15 abnormal) were used for training and 15 models (5 normal and 10 abnormal) were used for testing to discriminate the normal and NMSC conditions.

2.4 Fabrication of Tissue Equivalent NMSC Phantom

Following the simulation study, a bilayered tissue equivalent phantom with epidermal (100 μm) and dermal (300 μm) layers mimicking the normal and NMSC condition was fabricated using spin-coating methodology previously developed by our group.⁴⁰ Gelatin (Product No. ME9M591041; Merck Chemicals) was used as the host material. Water-soluble FAD (Product No. 064829; Sisco Research Laboratories, Mumbai, India) (100- μM normal, 200- μM abnormal) was taken as the major fluorophore for study in the epidermal layer and collagen from bovine achilles tendon in fiber form (Product No. C9879; Sigma Aldrich, St. Louis, Missouri) (20 mg-normal, 10 mg abnormal) was used as the fluorophore and scatterer in the dermal layer. Coffee solution was chosen as the absorber-mimicking melanin (1%) in epidermal layer and lyophilized powder human Hb (Product No. H0267; Sigma Aldrich, St. Louis, Missouri) (15 μM normal, 46 μM abnormal) was used as the major absorber in the dermal layer. The scattering property of aqueous suspension of polystyrene beads with 0.3 μm diameter (Product No: L9654; Sigma Aldrich, St. Louis, Missouri) (0.45% v/v) was found to match with the scattering property of the epidermal layer and was used as the major scatterer in the epidermal layer. These compositions were chosen to study the effects of changes in fluorescence and reflectance from epidermal and dermal layers that occur in tissues during its transformation from normal to NMSC state.

3 Results

This section details the diffuse reflectance and fluorescence spectral signatures obtained from the optical model and layered tissue equivalent phantom.

3.1 DRS Measurements

MCS of DRS was performed on the devised NMSC models described in Sec. 2.2 to simulate the reflectance spectra. Figure 2(a) shows a simulated DRS spectra corresponding to a model mimicking a normal (15- μM Hb, 100- μM FAD, and 20-mg collagen) and NMSC condition (46- μM Hb, 200- μM FAD, and 10-mg collagen) in the wavelength range from 500 to 600 nm. Diffuse reflectance measurements were also obtained from the fabricated phantom mentioned in Sec. 2.4 using the spectroscopic setup briefed in Sec. 2.1 by illuminating the phantom using the broadband source. Figure 2(b) show the DRS spectra obtained from the normal and abnormal regions of the phantom.

Hb dips were clearly observed in both experimental and simulated spectra at 542 and 578 nm in Figs. 2(a) and 2(b) and it is noticeable from the spectra that as the concentration of Hb increases in the abnormal region, diffuse reflectance decreases indicating increased absorption.

3.2 Fluorescence Measurements

Fluorescence measurements were obtained from the fabricated phantom mentioned in Sec. 2.4 using the developed bimodal spectroscopic setup by exciting the phantom at 450 nm to observe the peak emission. Figure 3(a) shows the FAD spectra obtained from the normal and abnormal regions of the phantom.

The spectra were normalized with respect to the spectrum corresponding to the abnormal region (200- μM FAD + 46- μM Hb).

MCS of fluorescence was performed on the devised NMSC model described in Sec. 2.2 to simulate the emission spectrum of FAD. The NMSC model was excited at 450 nm and the FAD emission spectrum was simulated for the wavelength range from 500 to 650 nm. Figure 3(b) shows the simulated FAD fluorescence spectra corresponding to normal and NMSC conditions. It is noticeable from the spectrum that as the concentration of FAD increases, fluorescence emission intensity also increases indicating abnormal conditions.

The collagen spectral signature was obtained in a similar way by exciting the fabricated bilayer phantom mentioned in Sec. 2.4 at 325 nm to obtain the emission peak at 405 nm using the bimodal experimental setup described in Sec. 2.1. Figure 4(a)

Fig. 2 Diffuse reflectance spectra from the NMSC sample: (a) simulated and (b) experimental.

Fig. 3 FAD fluorescence spectra: (a) experimental and (b) simulated.

Fig. 4 Collagen fluorescence spectra: (a) experimental and (b) simulated.

shows the normalized fluorescence from the normal and abnormal sections of the phantom. Both the spectra are normalized with respect to the spectrum corresponding to the normal region of the phantom (with 20-mg collagen).

Figure 4(b) shows the simulated collagen spectrum obtained by exciting the NMSC model described in Sec. 2.2 using MCS of fluorescence. Simulated collagen spectra were also normalized with respect to the spectrum corresponding to the normal section of the model.

3.3 Classification of Normal and NMSC Conditions

Figures 5(a)–5(c) clearly show the classification of normal and NMSC condition with selected spectral features using the KNN classifier for a trained data set generated using multilayer-Monte Carlo (MCML) modeling as mentioned in Secs. 2.2 and 2.3.

3.4 Fluorescence and Reflectance Images

Applicability of the developed instrumentation and adopted methodology was tested for optical imaging of the fabricated phantom mentioned in Sec. 2.4. Spectral data of the specified

biomarkers namely FAD, collagen, and Hb in the phantom sample were collected at each sample positions by raster scanning using the automated scan-based spectral imaging setup. Acquired data were processed and enhanced using a suitable algorithm^{41,42} to generate the intended fluorescence and reflectance image of the biomarkers highlighting the normal and abnormal regions of sample as shown in Figs. 6(a)–6(d).

4 Discussion

4.1 Assessment of Hb

Comparing Figs. 2(a) and 2(b), the experimental and simulated spectra show good correlation in their spectral shapes and agreeable correlation in their intensities. The difference between experimental and simulated spectra was quantified by calculating the mean of differences for a wavelength range 500 to 600 nm as

$$M_{\text{error}} = \frac{1}{N} \sum_{i=1}^N \left| \frac{F_{\text{exp}}(\lambda_i) - F_{\text{sim}}(\lambda_i)}{F_{\text{exp}}(\lambda_i)} \right|. \quad (1)$$

Fig. 5 Classification of normal and NMSC conditions using KNN classifier for (a) DRS data, (b) FAD fluorescence data, and (c) collagen fluorescence data.

$F_{\text{exp}}(\lambda i)$ and $F_{\text{sim}}(\lambda i)$ represent the experimental and simulated spectrum and N is the number of curve points in the spectra. The mean percentage of error was estimated to be 10% for normal and 11% for abnormal phantom samples. However, for estimating the chromophore concentration, DRS intensities at specific absorption wavelengths (542 and 578 nm) are required. For single wavelength points, the error between experimental and simulated profiles was around 30%. As expected, these errors were greater than the average error computed using Eq. (1). Considering only these specific values, the percentage change between DRS curve points at 542 nm (experimental spectra) for differentiating normal and abnormal samples was quantified using Eq. (2) and was found to be 29%.

$$\text{Change}(\%) = \left| \frac{I_{\text{abnormal}} - I_{\text{normal}}}{I_{\text{abnormal}}} \right| \times 100. \quad (2)$$

I_{abnormal} and I_{normal} represent the intensity values from abnormal and normal regions, respectively. This percentage error remained the same for the 578-nm data point. The percentage change calculated for simulated spectra under similar conditions was 28% giving rise to a percentage change of 6% in the DRS intensity values for disease discrimination for both experiments and simulations. This percentage change between experiments and simulations was found to be minimal as compared to the computed error over a range of wavelengths. Table 2 provides a summary of the tabulated error values computed for the DRS data.

4.2 Assessment of FAD

Referring to Fig. 3(a), the FAD emission spectra obtained from the abnormal region of the phantom was found to have its emission peak at 524 nm with a peak normalized intensity value of 1.

Fig. 6 (a) Photograph of phantom (top view), (b) experimental FAD image from phantom, (c) collagen image from phantom, and (d) reflectance image from phantom.

Table 2 Summary of calculated error values for DRS data.

Wavelength (nm)	Percentage error (1)		Percentage change (2)	
	Normal	Abnormal	Experiment	Simulation
500 to 650	10%	11%	29%	28%
542, 578	30%	30%		

The spectra from the normal region of the phantom had its emission peak at 525 nm with a peak intensity value of 0.67. The corresponding percentage change between the normalized peak emission intensity from abnormal and normal spectra was calculated using Eq. (2) and it was found to be 33%.

For 200- μ M FAD, the emission from the abnormal region of the sample was found to be blueshifted by 1 nm (from 525 to 524 nm). This may be because of the increase in absorber concentration (Hb) in the dermal layer from 15 to 46 μ M corresponding to NMSC. Full width at half maximum (FWHM) values were calculated for the phantom and it was found to be 81.43 nm for normal and 83.39-nm for the abnormal region

of the phantom. The normalized percentage change in FWHM between the abnormal and normal regions of the phantom computed using Eq. (2) was found to be around 2.35.% and this increase in percentage line width could be attributed to the depth dimension of the scatterers in the dermal layer facilitating predominant multiple scattering. Such effects of spectral broadening accompanied with the changes in sample scattering concentration was previously studied by our group conducting spectroscopic studies on thin skin-mimicking bilayer solid tissue phantoms.⁴⁰

Referring to Fig. 3(b), the FAD emission spectra obtained from the abnormal region of the model was found to have its emission peak at 524 nm with a peak normalized intensity value of 1. The spectra from the normal section of the NMSC model had its emission peak at 525 nm with a peak intensity value of 0.59 with a corresponding percentage change of 41% calculated using Eq. (1). For 200- μ M FAD, the emission from the abnormal region of the model was found to be blueshifted by 1 nm (from 525 to 524 nm). This may be because of the increase in absorber concentration (Hb) in the dermal layer from 15 to 46 μ M corresponding to NMSC. FWHM values were computed for the devised model and it was found to be 87.92 nm

Table 3 Summary of error values for FAD fluorescence data.

Wavelength (nm)	Percentage error (1)		Percentage change (2)	
	Normal	Abnormal	Experiment	Simulation
500 to 650	20%	22%		
At emission peak		24%	33%	41%
FWHM		34%	2.35%	3.15%

for normal and 90.78 nm for the abnormal region in the model. The normalized percentage change in FWHM between the abnormal and normal regions of the model was found to be 3.15% and this increase in percentage line width could be attributed to the decrease in scatterer concentration in the dermal layer and depth dimension of the scatterers in the dermal layer facilitating predominant multiple scattering.

Comparing the experimental and simulated results, the percentage change in normalized peak emission intensity of FAD and FWHM value between the abnormal and normal section of the devised model was found to be 41% and 3.15% whereas for the physical phantom the value of the parameters was observed to be 33% and 2.35%. The difference between experimental and simulated spectra is quantified by calculating the mean of differences for a wavelength range 500 to 600 nm as mentioned in Eq. (1) and the percentage of error was found to be 22%. Similarly, the error percentage in normalized intensity alterations between experiment and simulation at the single peak wavelength is around 24% and the error calculated at the single peak wavelength was higher than the average error. Table 3 summarizes the mean percentage error and percentage change computed for FAD fluorescence data.

4.3 Assessment of Collagen

It can be observed from Fig. 4(a) that the spectra corresponding to the normal region had a dominant peak at 405 nm with a normalized peak intensity of 1. As the concentration of collagen is reduced (as in the case of NMSC) from 20 to 10 mg, the normalized intensity is reduced from 1 to 0.73. The corresponding percentage change in intensity with respect to abnormal calculated using Eq. (2) was found to be 36.99%. This decrease in peak intensity is attributed to the decrease in collagen concentration and increase in Hb concentration in the abnormal region from (15 to 46 μ M). Hb in the dermal layer has its absorption peak at 418 nm, which absorbs the collagen emission at 405 nm reducing the overall intensity of collagen. The increment in dermal Hb concentration from 15 μ M to 46 μ M also caused an additional blueshift of 1 nm from 405 (normal) to 404 nm (abnormal) in this case.

FWHM as an estimate of the spectral line width was computed for the fluorescence spectra obtained from the normal and abnormal regions of the phantom sample, and it was found to be 49.69 nm for the normal and 53.83 nm for the abnormal regions. The normalized percentage change in FWHM between the abnormal and normal regions of the phantom was found to be 7.69% and this increase in percentage line width indicates the spectral broadening, which could be attributed to the variation in scatterer concentration in the dermal region.

Referring to Fig. 4(b), the spectra corresponding to the normal region (20 mg collagen) had a dominant peak at 405 nm with a normalized peak intensity of 1. As the concentration of collagen is reduced (as in the case of NMSC), the normalized intensity from the abnormal region is decreased from 1 (normal) to 0.71 and a corresponding percentage change with respect to abnormal calculated using Eq. (2) is of 40.85%. Also, a blueshift of 1 nm (from 405 to 404 nm) was observed in the emission peak of collagen from the abnormal section as observed in the case of experiments. FWHM values computed for the fluorescence spectra obtained from the normal and abnormal sections of the model was found to be 46.66 nm for normal and 51.72 nm for abnormal. The normalized percentage change in FWHM between the abnormal and normal regions of the phantom was found to be 9.78% and this increase in percentage line width indicates the spectral broadening, which can be attributed to the decrease in scatterer concentration in the dermal region.

The percentage change in normalized peak emission intensity of collagen and FWHM value between the abnormal and normal sections of the devised model was found to be 40.85% and 9.78% whereas for the physical phantom the value of the parameters was observed to be 36.99% and 7.69%. From the above analysis, it is clear that the optical phantom fabricated in this work is reflective of optical property changes of collagen in the tissue that occur during the progression of NMSC.

It can be observed from Figs. 4(a) and 4(b) that both the experimental and simulated spectra showed an agreeable correlation in their spectral shapes and intensities. The difference between experimental and simulated spectra is quantified by calculating the mean of differences in intensity for a wavelength range 380 to 450 nm as mentioned in Eq. (1) and the percentage of error was found to be 20%. The percentage of error in normalized intensity between experiment and simulation at the single peak wavelength was found to be 10%. However, in the case of collagen, the error associated with single point measurements was found to be less than that of the average error. Table 4 summarizes the mean percentage error and percentage change computed for collagen fluorescence data.

A part of the stated errors between the experimental and simulated curves may be attributed to the inherent simulation errors associated with MCS. As the position of the targeted fluorophores varies in the case of FAD and collagen, the associated errors are also found to vary. This specifies the need for modification in the current simulation/experimental modification to facilitate depth resolved imaging. Further studies are required to optimize these parameters with respect to current simulation/experiments. Also in the case of dermal fluorescence, these

Table 4 Summary of estimated error values for collagen fluorescence data.

Wavelength (nm)	Percentage error (1)		Percentage change (2)	
	Normal	Abnormal	Experiment	Simulation
380 to 450	15%	20%		
At emission peak		10%	36.99%	40.85%
FWHM		27%	7.69%	9.78%

differences were found to be less at the emission peak as compared to the extended wavelength regions (550 to 650 nm) of the spectra. This may be due to the variations in the Mie theory assumptions taken in the simulations at longer wavelength regions and the properties of scattering by the embedded collagen fibrils in the dermal layer.

4.4 Classification Accuracy

Accuracy is calculated as the number of all correct predictions divided by the total number of the test dataset. Classification accuracy of KNN for DRS data obtained from modeled spectra mentioned in Secs. 2.2 and 2.3 was found to be 100% for the feature combinations such as spectral intensity at 542 nm and spectral variance. Accuracy of the KNN classifier for classifying normal and NMSC conditions based on the modeled fluorescence data was also found to be 100% for the distinguishable feature combinations namely signal peak and signal mean for both FAD and collagen fluorescence data.

To validate the proposed method, identified spectral features were extracted from the experimental reflectance and fluorescence data obtained from the layered tissue equivalent phantom fabricated with controlled optical properties mimicking the normal and NMSC conditions mentioned in Secs. 3.1 and 3.2. Classification accuracy of the KNN classifier was found to be 100% for the optimum k value of 1 for the extracted DRS and fluorescence test feature sets. The reason for this classification accuracy could be attributed to the consistent percentages of errors between the experimental and simulated values as detailed in Secs. 4.1 to 4.3.

4.5 Applicability of Whole Field Imaging in NMSC Assessment and Qualitative Assessment

Referring to Figs. 6(b)–6(d), it can be observed that the difference in the fluorophore constituents in the prepared sample was evident in the images collected using the automated spectral imaging system. For the region of the phantom representing malignancy as shown in Fig. 6(a), the image pixel intensities were (i) increased at 524 nm (corresponding to the emission peak of FAD, fluorescence map), (ii) decreased at 404 nm (corresponding to the emission peak of collagen, fluorescence map), and (iii) increased at 542 nm (corresponding to the absorption peak of Hb, reflectance map) compared to their normal counterparts. These intensity changes reflect the change in biomarker concentration variations. Once the abnormality is identified, spectra from the abnormal area can be taken and compared with the modeled spectra to extract the properties. In a clinical setting, such a dual-mode spectroscopic setup along with the methodology and procedures could be undertaken for tissue classification and can be applied to demarcate tumor margins during surgery.

The present work provides an insight for noninvasive assessment of NMSC on a quantitative approach by comparing the experimental spectra from a tissue-mimicking phantom and simulated spectra from the model. Prior to testing the ability of the developed spectroscopic setup and adopted methodology in a real-time clinical setting, a detailed model library has to be created with the spectral parameters from a large number of tissue equivalent models/phantoms with the specific biomarkers of interest. Quantitative analysis can be done on the extracted spectral data using any high end multivariate classification algorithms, which can help in diagnosis by direct comparison.

The next phase of the work is focused on the generation of one such detailed model library and to extend the experimental studies to a real-time clinical environment for quantitative assessment of NMSC and to make an instant diagnosis.

5 Conclusion

This paper represents an improved methodology for assessment of NMSC using the biomarkers Hb, FAD, and collagen on a quantitative scale by comparing the simulated and experimental spectra and images from optical models and a precise, soft, layered phantom using bimodal spectroscopy. The detailed analysis as explained in this article can be carried out following an initial screening and qualitative analysis involving the in-house developed dual-mode optical imaging system. Experimental results combined with demonstrated simulations and classification algorithm suggest that the variations in optical properties of biomarkers could be assessed on a quantitative scale within specified error limits that would help in the assessment of NMSC. Such a Monte Carlo-based semiempirical methodology would help in tissue optical characterization in a whole field environment and in identifying the extent of malignancies, which could be used for future *in vivo* clinical applications.

Disclosures

No conflicts of interests, financial or otherwise are declared by the authors.

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