Published in final edited form as:

Lasers Surg Med. 2011 February ; 43(2): 143–151. doi:10.1002/lsm.21041.

A Clinical Instrument for Combined Raman Spectroscopy-Optical Coherence Tomography of Skin Cancers

Chetan A. Patil, Ph.D.,

Department of Biomedical Engineering, Vanderbilt University, Nashville TN, 37235

Dermatology Service, Veterans Affairs Tennessee Valley Healthcare System, Nashville, TN, 37212

Harish Kirshnamoorthi, B.A.,

Department of Biomedical Engineering, Vanderbilt University, Nashville TN, 37235

College of Arts and Sciences, Vanderbilt University, Nashville TN, 37235

Darrel L. Ellis, M.D.,

Division of Dermatology, Department of Medicine, Vanderbilt University, Nashville TN, 37235

Dermatology Service, Veterans Affairs Tennessee Valley Healthcare System, Nashville, TN, 37212

Ton G. van Leeuwen, Ph.D., and

Department of Biomedical Engineering and Physics, Amsterdam Medical Center, Amsterdam, The Netherlands

Biophysical Engineering Group, Faculty of Science and Technology, University of Twente, Enschede, The Netherlands

Anita Mahadevan-Jansen, Ph.D.

Department of Biomedical Engineering, Vanderbilt University, Nashville TN, 37235

Dermatology Service, Veterans Affairs Tennessee Valley Healthcare System, Nashville, TN, 37212

Abstract

Background and Objective—The current standard for diagnosis of skin cancers is visual inspection followed by biopsy and histopathology. This process can be invasive, subjective, time consuming, and costly. Optical techniques, including Optical Coherence Tomography (OCT) and Raman Spectroscopy (RS), have been developed to perform non-invasive characterization of skin lesions based on either morphological or biochemical features of disease. The objective of this work is to report a clinical instrument capable of both morphological and biochemical characterization of skin cancers with RS-OCT.

Materials and Methods—The portable instrument utilizes independent 785 nm RS and 1310 nm OCT system backbones. The two modalities are integrated in a 4" (H) × 5" (W) × 8" (L) clinical probe. The probe enables sequential acquisition of co-registered OCT and RS data sets. The axial response of the RS collection in the skin was estimated using scattering phantoms. In addition, RS-OCT data from patients with cancerous and non-cancerous lesions are reported.

Results—The RS-OCT instrument is capable of screening areas as large as 15 mm (transverse) by 2.4 mm (in depth) at up to 8 frames/sec with OCT, and identifying locations to perform RS. RS signal is collected from a 44 μm transverse spot through a depth of approximately 530 μm . RS-OCT data sets from a superficial scar and a nodular BCC are reported to demonstrate the clinical potential of the instrument.

Conclusion—The RS-OCT instrument reported here is capable of morphological and biochemical characterization of cancerous skin lesions in a clinical setting. OCT can visualize microstructural irregularities and perform an initial morphological analysis of the lesion. The images can be used to guide acquisition of biochemically specific Raman spectra. The two data sets can then be evaluated with respect to one another to take advantage of the mutually complimentary nature of RS and OCT.

Introduction

Skin cancer is the most commonly occurring of all cancers, accounting for more than a 1.7 million incidences in the United States annually(1). The two most common types of skin cancer, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), are highly curable if detected at an early stage. The most dangerous skin cancer, malignant melanoma, can have a 5 year survival rate of only 16% at it's most advanced stage, however if detected *in situ*, the survival rate improves dramatically to 99%(2). Early diagnosis and complete resection are the keys to achieving a favorable outcome; however diagnosis of skin cancers can be difficult. Clinicians are presented with the initial challenge of deciding which and how many skin lesions to biopsy, usually relying on visual inspection and palpation. Non-cancerous lesions can visually resemble malignancies, and even well trained dermatologists can find the process to be subjective and painstaking. It can be particularly easy to overlook disease in patients with numerous questionable lesions. This is not uncommon amongst elderly patients, who often present with numerous benign lesions but also suffer from increased skin cancer incidence.. Preemptive removal may seem to be a simple solution, however it can result in unnecessary costs and patient discomfort. A reliable positive diagnosis requires biopsy and subsequent histo-pathological examination, a process that can take up to 2 weeks depending on the provider. This protocol for skin lesion diagnosis is the gold-standard; however it is also subjective, invasive, time-consuming, and costly. These limitations have motivated the development of non-invasive techniques capable of direct characterization of potentially cancerous skin lesions. Such tools currently have the potential to assist in biopsy guidance, and in the future possibly even circumvent the need for biopsy and histo-pathology altogether.

Optical techniques have demonstrated the potential to perform non-invasive characterization of skin cancers. Imaging modalities such as confocal microscopy(3) and optical coherence tomography (OCT)(4) are advantageous because they allow real-time, non-invasive visualization of tissue structure with micron-scale resolution. Although confocal reflectance microscopy is typically capable of visualizing cellular-level detail, the high resolution ($< 5 \mu\text{m}$) comes at the expense of a limited transverse field of view (FOV) ($\approx 500 \mu\text{m} \times 500 \mu\text{m}$). This spatial scale can limit the practical utility of the instrument for screening larger areas, thereby complicating clinical application. The benefit of OCT is that the axial resolution is

independent of the transverse FOV, so axial resolutions of $<20\ \mu\text{m}$ can be achieved over a large (i.e. 10 mm) transverse scan. The larger FOV allows for easier screening and better visualization of the microstructural features of skin cancers at the expense of sub-cellular level detail. Although OCT has demonstrated the ability to visualize morphological features such as the tumor cell nests in BCC and architectural disarray seen in melanocytic lesions(5,6), interpretation of OCT images can be fairly difficult in a clinical setting. This is highlighted in a recent study in which dermatologists and pathologists trained in the interpretation of OCT images of skin lesions had difficulty separating BCC and actinic keratosis (AK, a pre-malignancy that can develop into SCC)(7). Since OCT images are simply cross-sectional maps of tissue reflectivity, it is possible that characterization of the biochemical or molecular composition of the tissue could assist in the difficult task of classification based on morphology alone.

Spectroscopic techniques including autofluorescence(8) and Raman spectroscopy (RS)(9), have demonstrated the ability to perform disease classification based on the molecular composition of skin lesions. In comparison to autofluorescence spectroscopy, which detects broad spectral features originating from a limited number of intrinsic fluorophores in the skin, RS detects the sharp spectral features originating from a wide range of molecular moieties in the skin(10). In a direct comparison with autofluorescence and diffuse reflectance spectroscopy, RS has been shown to achieve superior classification performance(11). In the skin, RS has demonstrated the ability to perform direct multi-class identification of malignant lesions, non-malignant lesions, and normal skin with overall accuracies of $>90\%$ (9,12). Spectral classification is typically performed with automated, statistical algorithms that provide objective correlation of spectra to pathological state. Unfortunately, it is not feasible to collect 2-D maps of tissue biochemistry in a clinically relevant time frame due to the inherently weak nature of Raman scattering. Clinical implementations of RS are limited to point-wise measurements which cannot directly determine the spatial extent of disease, and can also be susceptible to sampling error. While OCT excels at imaging tissue microstructure with limited biochemical specificity, RS excels at characterizing the biochemical composition of tissue with limited spatial information. The uniquely complimentary strengths and limitations of RS and OCT make the two modalities well suited for combination into a single instrument for characterization of skin lesions.

Development of dual-modal instruments combining imaging and spectroscopy is a straightforward approach to realize the potential benefits of both morphological and biochemical analysis. For instance, an instrument performing laser induced fluorescence and OCT demonstrated the advantages of combining imaging with spectroscopy to characterize the cancer in the colon of a rodent model(13). In the skin, a confocal RS-confocal reflectance microscopy probe demonstrated the ability to acquire images with cellular level resolution along with the micron scale biochemical analyses of normal tissue structures, *in vivo*(14). The combination of RS and OCT can facilitate high-resolution imaging of the microstructural features of skin disease with an expanded FOV, along with highly specific biochemical characterization of tissue composition. The potential advantages of combined RS-OCT have previously been reported in two different non-portable benchtop configurations(15,16), which described how OCT can be used to guide positioning of point-

wise RS, while ambiguous structures within OCT could be molecularly evaluated with RS. Here, we describe the design and application of a portable combined RS-OCT instrument with a sample probe amenable to *in vivo* imaging and spectroscopy of skin cancers.

Materials and Methods

System Instrumentation

The combined RS-OCT system schematic is shown in figure 1(a). The backbone of the OCT subsystem is a fiber-based Michelson interferometer that generates A-scans in the time-domain. A 1310 nm optical amplifier source ($\lambda_{FWHM} = 60$ nm, AFC technologies) is fiber coupled to a 2×2 fiber splitter (50:50 coupling ratio). The reference arm consists of a rapid scanning optical delay line that facilitates real-time imaging (< 8 frames/sec)(17). The OCT subsystem is fiber coupled to a custom designed RS-OCT probe that directs the light onto the sample (described below). The light returning from the sample probe interferes with the light returning from the reference arm at the fibersplitter. The OCT signal is then detected by a balanced detector that collects interference signal from both source and detection arms of the interfereometer to improve detection sensitivity (18). The analog signal is then band pass filtered and digitized by a multi-function analog input / output data acquisition device (National Instruments). The Raman subsystem consists of a wavelength stabilized laser diode source located entirely within the probe and operated via an external control module (Innovative Photonics Solutions, Inc.). The Raman scatter is collected, fiber coupled, and sent to a high throughput spectrograph (Kaiser Optical Systems) that disperses the light onto a back illuminated, deep depletion, thermo-electrically cooled CCD (Andor Technologies). All the components of the OCT and RS subsystems are housed with in a mobile cart (fig. 1(b)) that enables transportation to the clinic, and mobility from one exam room to another.

Combined RS-OCT Sample Probe

The combined RS-OCT sample probe is depicted in figure 2. The Raman source is a 785 nm diode laser device with a 1" × 1" footprint that contains the diode and thermo-electric cooler (Innovative Photonics Solutions). The source outputs a collimated beam that is band pass filtered and co-aligned with the OCT beam at dichroic mirror 1. The objective lens and galvanometer (Cambridge Technologies) are configured to generate a telecentric scan for OCT image generation. The OCT system's axial resolution was measured at 14 μ m in air. The objective lens focuses the OCT beam to a 25 μ m lateral spot ($P_{OCT \text{ sample}} = 7$ mW). During Raman acquisition, galvanometer scanning is stopped, and the Raman spectrum is acquired from the central optical axis of the objective lens. The Raman beam is weakly focused to a 44 μ m lateral spot ($P_{Raman \text{ sample}} = 40$ mW). A thin (100 μ m) layer of polyethylene was translated axially through the sampling range to estimate the point-spread-function (PSF) of the Raman collection in the absence of scattering. The full width at half max (FWHM) of the response function was measured at 1.8 mm, and focused at a depth that corresponded to $z=1.0$ mm in the OCT image frame. In order to verify co-alignment of the RS beam with the central axis of the OCT scan, a gelatin sample was irradiated with a stationary RS beam until a small indentation had been formed due to dehydration. OCT imaging verified the apex of the indentation corresponded to the center of the OCT scan. The Raman scatter band from 800 – 950 nm is separated from the Raman excitation and

OCT by dichroic mirror 2 (Chroma Technologies, Inc.), long pass filtered (Semrock, Inc. $\lambda_{\text{cutoff}} = 818 \text{ nm}$) to further reject elastic scatter, and coupled into a $100 \mu\text{m}$ low-OH fiber for detection. The overall size of the probe is $4'' (\text{H}) \times 5'' (\text{W}) \times 8'' (\text{L})$. The probe is mounted to a manually manipulated mechanical arm (seen in fig. 1(b)) that supports the probe's weight and provides stability over the duration of Raman acquisition.

Data Pre-processing and Software Interface

A custom designed software interface (LabView) performs instrument control, data acquisition, data processing, and display for both the OCT and Raman systems. The software performs demodulation of the full interferometric OCT signal, and real-time processing and display of both the OCT image and Raman spectra.

Calibration of the Raman spectrograph dispersion is performed daily prior to data collection using the atomic emission lines of a neon-argon lamp. Variations in spectral throughput of the system are calibrated using a NIST-certified quartz-tungsten-halogen lamp. Relative Raman wavenumber shift is calibrated with acetaminophen and naphthalene standards. Background spectra are acquired to remove laser-induced fluorescence from the sampling optics. Spectral pre-processing includes binning the data to $\frac{1}{2}$ the spectral resolution and noise filtering with a 2nd order Savitzky-Golay filter. The window size of filter is twice the spectral resolution. Finally, background tissue autofluorescence is subtracted with a modified polynomial fitting algorithm(19), and the resulting spectra are area normalized to account for variations in the intensity of the detected signal.

Scattering Phantom for Characterization of Raman Axial Response Function

Although the axial PSF of the RS collection was measured to be 1.8 mm in the absence of scattering, the axial response in a highly scattering sample such as the skin is likely quite different. Therefore, a layered scattering phantom was constructed in order to approximate the axial response function of the RS sub-system in the skin. The phantom consisted of a thin layer with a distinct RS signature sandwiched between scattering layers with varying thickness. The RS target layer was a $100 \mu\text{m}$ thick polyethylene sheet with a set of 3 peaks (1062 , 1129 , and 1262 cm^{-1}) with spectral features distinct from that of the scattering layers. The scattering layers were $300 \mu\text{m}$ in thickness and consisted of a mixture of silicone and 0.28 weight percent TiO_2 powder (20). The corresponding optical attenuation coefficient at 850 nm , μ_t , was 6 mm^{-1} , which is on the order of measured optical properties of skin in the near-infrared (21,22). Three models were then constructed to measure the collected RS signal intensity at depths of 0 (no scattering layer above the RS target layer), $300 \mu\text{m}$ (1 scattering layer), and $600 \mu\text{m}$ in depth (2 scattering layers). In each model, the RS active polyethylene layer was placed above a semi-infinite set of 10 scattering layers with a thickness of 3 mm . Spectral measurements were made with the RS target layer placed at the focus of the RS collection ($z = 1.0 \text{ mm}$ in the OCT image frame). The RS collection depth was then defined as the $1/e^2$ falloff of the axial response function.

Human Subjects Measurement Protocol

Combined RS-OCT measurements of skin lesions were made from volunteers presenting at the Veterans Affairs – Tennessee Valley Health Care System (VA-TVHS) dermatology

service for complete lesion resection. All measurements were performed in accordance with a protocol approved by the VA-TVHS institutional review board. OCT images of the lesion were acquired to provide an overview of its microstructural features. OCT imaging was then suspended and Raman spectra were acquired from the center of the lesion for biochemical characterization. Due to the inherent heterogeneity of skin, spectra and images were then acquired from regions directly adjacent to the lesion (within 3–4 cm's) that appeared visibly normal for comparison. For each spectral acquisition site, three independent measurements were made and then averaged to produce a single mean spectrum of the location. The acquisition time for individual Raman spectra was 30 seconds. After data acquisition, a 2 mm punch biopsy was taken from the center of the lesion for histopathological correlation.

Results

Characterization of Raman Axial Response Function

A representative OCT image of the scattering phantom with the RS target layer beneath 2 scattering layers is shown in figure 3(a). The mean intensity of the polyethylene spectral lines (normalized to the surface RS intensity) is plotted as a function of the thickness of the overlying scattering layers in figure 3(b). The data indicates that the RS signal intensity decays exponentially with increasing depth. This relationship was supported by the high correlation coefficient ($R^2 = 0.994$) that is obtained when the data is fit to an exponential decay. The fit was then used to calculate the $1/e^2$ falloff depth of 531 μm .

Representative Clinical Measurements

An OCT image of normal skin from the forearm of a 62 year old male is shown in figure 4(a). The epidermal layer can be observed as a slightly darker band that is most apparent near the black arrow on the far right of the image. The OCT image from the adjacent lesion is shown in figure 4(b). The image from the lesion site shows a number of hyper-reflective (green arrows) and hypo-reflective (asterisk) features that contrast with the relative homogeneity of the normal skin image, and could suggest the presence of malignancy. Histological analysis revealed the skin had a loss of dermal papillae and the presence of some inflammatory infiltrate, which indicates the lesion was primarily a scar with some residual inflammation (fig. 4(c)). The corresponding mean Raman spectra from the two sites are shown in figure 4(d). The approximate Raman acquisition axes are overlaid in red on the OCT images. Overall the spectra are fairly similar, with an increase in the RS intensity in the scar tissue spectra in the band from 1250 to 1350 cm^{-1} .

Figure 5 depicts the RS-OCT data collected from a 67 year old male with a BCC over the right temple. Again, the OCT image of the adjacent normal skin (fig. 5(a)), shows a generally homogenous subsurface morphology, although some subsurface hypo-reflective features can be seen (green arrows). The white arrow identifies a region where the epidermal-dermal boundary is the most clear. The OCT image of the lesion (fig. 5(b)), however, clearly shows the presence of dark hyper-reflective features that suggest a potential BCC. In comparison to the normal image, the epidermal-dermal boundary is much more clearly defined in the vicinity of the lesion. The histopathology (fig 5(c)) depicts the presence of tumor cell nests (arrows) that are characteristic of nodular BCC. The Raman

spectra (fig. 5(d)) show a similar overall line shape, but there are distinct differences in the bands centered at 1090 cm^{-1} , 1340 cm^{-1} , and 1440 cm^{-1} (shaded areas).

Discussion

Since the Raman scattering phenomena itself is inherently weak, collection efficiency is an important factor in the design of clinical RS probes. In the skin, both confocal and non-confocal approaches have been utilized for *in vivo* measurements(9,23). Although confocal RS facilitates high axial resolution, it comes at the expense of collection efficiency, since a great deal of the out-of-focus light is rejected by the confocal aperture. Moreover, it remains unclear as to whether high spatial resolution is necessary to perform accurate diagnosis of skin cancer. In a study by Lieber et. al, confocal RS spectra acquired in $20\text{ }\mu\text{m}$ increments from the surface down to $100\text{ }\mu\text{m}$ were combined together to represent a RS instrument with decreased axial selectivity(24). The authors reported classification of skin cancer in these spectra with $> 90\%$ accuracy, which suggests that reliable diagnosis is possible without a confocal collection geometry. This finding motivated the weakly focused RS illumination and collection geometry in the current RS-OCT probe in order to prioritize collection efficiency over spatial resolution. Although the PSF of the RS axial response in the absence of scattering was estimated at 1.8 mm , this figure is likely not representative of the probe's performance in highly scattering media, such as the skin. The scattering phantom experiments were therefore designed to develop a better understanding of the RS axial profile of the lesions measured in the clinic. The results show that the RS signal is surface weighted. It is important to understand that the depth-integrated spectra reported by Lieber et. al assigned an equal weight to spectra from each individual depth. Direct comparisons between data sets is therefore not warranted, nor is direct application of the classification algorithms developed by Lieber et. al.

Calculation of the $1/e^2$ falloff point of the axial response allows a quantitative characterization of the RS penetration depth at roughly $500\text{ }\mu\text{m}$ (fig. 3(b)). This figure will certainly vary from patient-to-patient as well as from lesion-to-lesion, however $500\text{ }\mu\text{m}$ serves as a good initial approximation in the skin and provides an important frame of reference when attempting to interpret spectra acquired in the clinic. Although the use of glycerol as an optical clearing agent has been shown to increase the depth from which signals can be collected in both OCT and RS(25), the RS signature of glycerol(26) overlaps many of the bands previously identified as relevant to skin disease(9,12,24,27). Since the ultimate objective of the device reported here is to perform classification of both malignant and non-malignant lesions with subtly different RS signatures, organic optical clearing agents, such as glycerol, were not applied due to concern that the clearing agent could skew the measured spectral signatures and confound the performance of classification algorithms. However, more work is needed before any definitive conclusion can be reached.

The images shown in figure 4 (a,b) demonstrate the ability of the OCT imaging subsystem to visualize the stratified nature of the epidermis and dermis (fig. 4(a)) in normal skin, along with the appearance of irregular features associated with the scar lesion (fig. 4(b)). The data acquired in figure 4 was taken from a patient who was returning to the VA dermatology service for complete resection of a lesion previously biopsied and determined to be

cancerous. After examination of the histology we obtained, it was clear that the initial scrape biopsy had removed the tumor in its entirety, and the measured lesion simply consisted of residual scar tissue. The image of the scar contrasts with normal skin due to distinct hyper-reflective and hypo-reflective features in the dermis. Based on the histopathology, the hyper-reflective regions (arrows) likely are a result of the reported increase in scattering intensity of reorganized collagen fibers in scar tissue(28), however these features have also been reported in association with actinic keratoses(29), a pre-malignant lesion that is capable of developing into SCC. A hypo-reflective region (right of asterisk), is also seen adjacent to the center of the lesion. Hypo-reflective features have been attributed to inflammation(30), sebaceous glands(31), vasculature, and tumor cell nests(5). These structures encompass a wide range of normal and cancerous morphology, and their presence complicates classification of the lesion based on the OCT alone. Nevertheless, this image demonstrates the high sensitivity of the OCT subsystem to atypical skin microstructure, a valuable performance feature when screening large areas for the presence of disease.

The Raman spectra acquired from both the lesions and the adjacent normal skin have spectral lineshapes similar to those typically reported in the skin (27,32). These features include the tyrosine/protein peak near 850 cm^{-1} , phenylalanine ring breathing peak at 1003 cm^{-1} , the PO_2 symmetric stretching peak attributed to lipids as well as the nucleic acid backbone in the $1070\text{--}1090\text{ cm}^{-1}$ band, the amide III band centered around 1304 cm^{-1} , the 1440 CH_2 peak, and the 1670 cm^{-1} amide I peak. The distinction between normal skin spectra and scar/inflammation spectra has previously been associated with increased signal intensity in the band between 1250 cm^{-1} and 1350 cm^{-1} (9). The mean spectra shown in figure 4(d) reflect this same difference, which is in agreement with the histology cross-section and pathology report, which identifies the lesion as residual scar and inflammation. Taken together the RS-OCT datasets demonstrate the value of both morphological and biochemical data sets in assessing a potentially cancerous irregular skin lesion. Although the OCT images did not clearly depict features associated with malignancy, they enabled a morphological context to guide placement of the RS acquisition axes for specific biochemical analysis of the tissue.

The RS-OCT data shown in figure 5 demonstrates the sensitivity of both RS and OCT to features that suggest skin cancer. Although the OCT image of normal skin in figure 5(a) depicts the presence of some subsurface hypo-reflective features, they are very likely to depict hair follicles and sebaceous glands based on their location and morphology (31). Both are superficial and appear to nearly extend out beyond the skin surface, in contrast to the hypo-reflective features seen in figure 5(b) which lie below the epidermal-dermal boundary, and are generally well defined, as has been reported in the literature of OCT images of BCC (5). In addition, the epidermal-dermal junction is sharp and distinct in the region above the tumor cell nests in figure 5(b), indicating a significant difference in the local optical properties. These image features enabled easy positioning of RS acquisition axis, and assist in reducing the likelihood of sampling error.

Although the OCT image features identified above strongly suggest the presence of BCC tumor cell nests, the presence of similar features in both figure 4(b) and figure 5(a) indicates the difficulty in attributing hypo-reflectivity to tumor cell nests. Examination of the

corresponding Raman spectra shown in figure 5(d) also show differences in wavenumber regions that suggest the presence of BCC. Notable differences in the mean spectra include increased RS intensity the 1090 cm^{-1} band, which has been attributed to PO_2 symmetric stretching of nucleic acid backbone (27), and the 1300 cm^{-1} band (amide III, proteins), and 1440 cm^{-1} (also associated with proteins). Previous reports identifying spectral differences between normal skin and BCC have all implicated similar increased scattering intensities in these bands with the presence of BCC (9,12,27). An increase in the spectral contribution from proteins and nucleic acids corresponds with the known increase in nuclear-to-cytoplasm ratio often associated with tumors in histopathology. In this instance, morphological features seen in OCT and biochemical properties measured with RS are both confirmed by the histopathology.

The representative data shown here indicates the ability of the clinical RS-OCT instrument to utilize both imaging and spectroscopy to perform more thorough characterization of questionable skin lesions than using either technique alone. The instrument is able to utilize the sensitivity of OCT to microstructural irregularities to 1) perform an initial morphological analysis of the lesion, and 2) guide the acquisition of biochemically specific Raman spectra. The two data sets can then be evaluated with respect to one another to take advantage of the mutually complimentary nature of RS and OCT. RS-OCT can be utilized to screen potentially cancerous lesions, direct biopsy, and monitor for lesion recurrence. The probe design allows measurements to be made from various locations on the body in a clinical setting, including the limbs, torso, and head.

Conclusion

We have demonstrated a clinical RS-OCT system capable of evaluating potentially cancerous skin lesions, *in vivo*. The images and spectra reflect the potential benefit that combined morphological and biochemical characterization of skin lesions can provide for clinical diagnosis. Moving forward, the primary challenge is interpreting the data sets in the context of a complex clinical problem such as skin cancer detection. The inherent variability in the micro-structure of normal, non-cancerous, and cancerous skin was readily apparent from our clinical experience and in the OCT images shown here. Obtaining meaningful information from these images will require expertise that can only be developed through analysis of an extensive set of images covering the full range of lesions relevant to skin cancer diagnosis. In addition, development and validation of a statistical classification technique is necessary to realize the capability of biochemical analysis with RS. This task will also require an expanded study to construct an adequately sized spectral library for training. Finally, realizing the full potential of combined RS-OCT will require development of analytical techniques that appropriately incorporate features from both the imaging and spectroscopy data sets.

Acknowledgments

The authors acknowledge Dr. Allison Hanlon for her clinical expertise and assistance in patient enrollment, Dr. Mark Mackanos and Dr. Xiaohong Bi for assistance in performing measurements, and the Vanderbilt University Skin Disease Research Center (NIH 5P30 AR041943) for histopathology. This research was funded by NIH R21 CA133477 01/02 and NIH NCI r01 CA114471.

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(a)

(b)

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.