Design and characterization of a novel multimodal fiber-optic probe and spectroscopy system for skin cancer applications

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The design and characterization of an instrument combining Raman, fluorescence, and reflectance spectroscopic modalities is presented. Instrument development has targeted skin cancer applications as a novel fiber-optic probe has been specially designed to interrogate cutaneous lesions. The instrument is modular and both its software and hardware components are described in depth. Characterization of the fiber-optic probe is also presented, which details the probe's ability to measure diagnostically important parameters such as intrinsic fluorescence and absorption and reduced scattering coefficients along with critical performance metrics such as high Raman signal-to-noise ratios at clinically practical exposure times. Validation results using liquid phantoms show that the probe and system can extract absorption and scattering coefficients with less than 10% error. As the goal is to use the instrument for the clinical early detection of skin cancer, preliminary clinical data are also presented, which indicates our system's ability to measure physiological quantities such as relative collagen and nicotinamide adenine dinucleotide concentration, oxygen saturation, blood volume fraction, and mean vessel diameter. © 2014 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4890199>]

I. INTRODUCTION

Spectroscopic techniques utilize the interaction of light with biological tissue to study tissue optical properties, which change with disease progression and can be used for diagnosis. Deployment of spectroscopic-based devices has the potential to significantly augment clinical diagnosis. Three common spectroscopic techniques are Raman spectroscopy (RS), diffuse reflectance spectroscopy (DRS), and laser-induced fluorescence spectroscopy (LIFS). These techniques have been applied – either individually or in combinations – throughout the entire human body to investigate a wide range of pathologies including: atherosclerosis,^{1,2} osteoporosis,³ brain edema,⁴ cataract formation,⁵ kidney stones,^{6,7} and diabetes⁸ and cancer of the breast,^{9–11} cervix,¹² esophagus,^{13,14} gastrointestinal tract,^{15,16} brain,¹⁷ lungs,^{18,19} ovaries,²⁰ and bladder.²¹ Here, we present a novel multimodal spectroscopy (MMS) device, combining RS, LIFS, and DRS, for the purpose of fast and non-invasive early detection of skin cancer which uses a variety of instrumentation and a custom contact probe capable of delivering and collecting light for all three modalities.

A. Skin cancer

Skin cancer, in both its melanoma and non-melanoma forms, has the largest reported incidence of all cancers in the United States. North America has the 2nd highest age-standardized melanoma incidence rate in the world.²² Early identification of skin cancer is paramount for its effect upon patient survival; the five-year “regional” survival rate of melanoma is 62%, dropping to 16% for “distant” detection. Significant ambiguity exists for the clinical distinction via vi-

sual inspection between dysplastic nevi and melanoma, raising issues of considerable practical and financial importance. Currently, the only reliable method of distinguishing between dysplastic nevi and melanoma is via stained biopsies, which is invasive and expensive. Clinician specificity for melanoma is approximately 4%,^{23,24} which means that roughly 25 more times biopsies are performed than required, translating to an estimated cost of $\$6 \times 10^9$ to the US health care system. In 2007, the mean wait time for new patients in urban areas to see a dermatologist was 33 days; in rural areas, the number rises to 46 days.²⁵ Furthermore, delay in diagnosis due to “off site” analysis imparts an emotional cost to the patient.

Skin cancer patient care is currently limited by the invasive nature of biopsies and the high cost associated with biopsies due to inadequate screening techniques. Motivated by improving this degree of patient care, we present a noninvasive, real-time spectroscopic-based technology to significantly improve lesion pathology specificity for the early detection of skin cancer. Ultimately, this device will save lives through early detection and its improved differentiation between skin pathologies will translate directly to lower costs and morbidity.

B. MMS

In short, MMS characterizes the tissue microenvironment via morphological changes observed through DRS and bio-chemical information via RS and LIFS. The DRS measurement is a function of tissue scattering and absorption properties, which in turn are dependent upon tissue morphological changes. Hence, analysis yields information about tissue blood fraction, oxygen saturation, tissue scattering

coefficient, nuclear morphology, and collagen structure. LIFS is bio-chemically sensitive as it interrogates endogenous fluorophores such as nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), and collagen. Their fluorescence levels change with neoplastic progression that is associated with altered cellular metabolic pathways (NADH, FAD)²⁶ or an altered structural tissue matrix (collagen).^{26–29} Raman spectroscopy exploits the inelastic scattering (so-called “Raman” scattering) phenomena to detect spectral signatures of important disease progression biomarkers, including lipids, proteins, and amino acids. Raman spectroscopy is more constituent-specific than fluorescence and is capable of spectrally “breaking down” the biochemical composition; however, the two techniques are complementary as they probe different bio-molecular species.

As different techniques provide complementary information, it has been shown that an instrument combining multiple techniques offers a more precise description of disease status. Specifically for skin cancer, it has been demonstrated that a modal combination improves malignant melanoma³⁰ and non-melanoma (basal and squamous cell carcinomas) diagnosis.³¹ Furthermore, MMS has been successfully applied for the early detection of atherosclerotic plaque.^{32,33} The successful application of MMS in non-cancer related pathologies indicates that it has considerable potential and its efficacy would be further tested by applying it to skin cancer.

C. Fiber-optic probes

Biomedical optical spectroscopy measurements very commonly use fiber-optic probes, which serve as the optical interface between the sample and the spectroscopic equipment. The fiber-optic probes contain fiber bundles that are responsible for both delivering and collecting light from the sample. Many types of probes have been used within the research community and cannot all be summarized here; however, comprehensive reviews of DRS, LIFS, and RS probe technology are available.³⁴ Briefly, combination LIFS-DRS probes have been demonstrated by past studies,^{35,36} as well as a probe combining all three techniques.³²

There are several important, and challenging, design and functional considerations for a probe that combines reflectance, fluorescence, and Raman signals. First, due to the very low probability of Raman scattering (one inelastically scattered photon for every 10^9 elastically scattered photons), the optical design must be fine-tuned to maximize RS signal-to-noise ratios. Second, the traditional source-detector geometry employed for reflectance measurements needs to be preserved such that tissue scattering and absorption properties can be separated and quantified.

II. SYSTEM DESCRIPTION

Our MMS system design is modular, consisting of 2 main “sub-units”: hardware to control the LIFS and the DRS measurements and separate hardware for RS acquisition.

A. Sources

The MMS system has three sources for each of the three modalities: a pulsed Xenon flash lamp (L7684, Hamamatsu Photonics, Bridgewater, NJ), which provides broadband 375–700 nm illumination for DRS; a pulsed 337-nm nitrogen laser (NL-100, Stanford Research Systems, Sunnyvale, CA) to induce NADH and collagen fluorescence; and a 830-nm diode laser (Lynx, Germany) for Raman excitation. The Raman diode laser is gated by a mechanical shutter which is controlled by triggering software written in MATLAB and LabVIEW. In order to prevent second-order dispersion contaminating the reflectance spectra, the Xenon white light is first passed through a 340-nm long-pass filter (Asahi Spectra, Torrance, CA) and then coupled into a fiber. The Xenon lamp provides a pulse of full width half maximum (FWHM) $2.9 \mu\text{s}$. For LIFS, the nitrogen laser has been configured to provide approximately $160 \mu\text{J}$ per pulse for a pulse FWHM of 3.5 ns. The LIFS signal-to-noise ratio could be increased by increasing the pulse power; however, the value of $160 \mu\text{J}$ strikes an effective balance between sufficient signal strength and laser cartridge lifetime. The output power of the Raman diode laser can be controlled by adjusting the supplied current through the custom software; for this application, 56 mW output power (0.198 A supplied current) at 830-nm is delivered from the laser engine. The Raman laser is housed inside the Raman module, which is completely shielded by specially constructed blackened material (Thorlabs, NJ, USA) to prevent any stray light getting in or leaking out.

B. MMS probe

1. Probe design

The multi-spectroscopy probe is similar in design to the probe described in detail elsewhere (US patent no. 8,175,423 and US patent application no. 20120236303). The probe is designed to be used in contact with the skin. The distal end of the probe is a polished, flat surface to ensure that the contact is as uniform as possible across the probe diameter to prevent measurement irregularities arising from gaps between the skin and probe and non-zero contact angles. Illustrations of the probe design are shown in Figure 1, where Figure 1(a) is a front-on view of the probe distal end and Figure 1(b) is an exploded assembly view to show all the components. As seen in Figure 1(a), seven $300 \mu\text{m}$ Raman collection fibers and a DRS/LIFS “triangle” concentrically surround a $200 \mu\text{m}$ core Raman delivery fiber.

The DRS/LIFS triangle contains two low OH $200 \mu\text{m}$ core visible light collection fibers and a high OH $200 \mu\text{m}$ core DRS/LIFS delivery fiber. The low and high OH cores are chosen for collection and delivery, respectively, because of the wavelength dependent attenuation characteristics of silica fiber optic cables: high OH content fibers have lower losses in the UV (hence, the selection for the LIFS delivery fiber) while low OH content fibers have lower losses in the visible (hence, the selection for the collection fibers where all the collected light is in the visible). The triangle of fibers pass through holes drilled in the long pass donut filter and the front lens as

FIG. 1. Schematics of the MMS probe. The left panel, (a), shows the probe distal end front view. The right panel, (b), shows the assembly exploded view with optical elements such as the filters and front lens identified along with the collection and delivery fibers for all three modalities.

illustrated in Figure 1(b) in order to bypass these elements. Without the front lens bypass, it was seen that the source-detector geometry, necessary for extracting optical properties from the reflectance signal, was not sufficiently preserved due to spectral aberrations and focusing effects introduced by the front lens. The current design avoids these issues and allows the DRS and LIFS data to be collected in the same fashion as the standard bundle probe configuration. The trade-off is that there is not perfect overlap between the Raman and LIFS/DRS collection spots; however, ray tracing simulations confirm that the overall delivery spot diameter (spanning all three modalities) is approx. $600\text{ }\mu\text{m}$ and this overlap is sufficient.

The Raman portion of the probe uses seven low hydroxyl (OH) content $300\text{-}\mu\text{m}$ core, 0.22 NA collection fibers. A donut shaped 830 nm long pass filter is positioned in front of these seven fibers, which rejects the 830 nm laser light and passes the Raman light from the sample. These seven fibers surround a stainless steel tube inside which is the laser delivery fiber assembly. The laser delivery fiber is a $200\text{-}\mu\text{m}$ core low OH, 0.22 NA fiber which has a small 830 nm band-pass filter positioned in front of it. The choice of fibers and filtering of Raman probes has been discussed by many sources previously.^{34,37,38} The two-piece converging front lens is made of a plano convex 2 mm diameter curvature sapphire back portion (the high refractive index bends the light sharply) and a flat front portion of 1 mm thick plano Magnesium Fluoride which has virtually no Raman signature. Epoxy is used to bond the required individual components together. The fibers, lens, and other components are placed inside a stainless steel 14 gauge extra thin wall needle tube (0.072 in. ID, 0.083 in. OD 2.1 mm OD).

2. Coupling

The MMS probe has 2 input connections: one for the 830-nm Raman laser and one port for both the N_2 laser and the Xenon lamp. Raman and LIFS/DRS ports are separated as near infrared (NIR) light for the Raman modality has different

optical design requirements (fiber material, filters, transmission, etc.) than the ultraviolet and visible wavelengths used in DRS and LIFS.

The white light and laser pulses (DRS and LIFS modes) are coupled into optical fibers and guided into a 3×1 fiber optic switch (FSM-13, Piezosystems Jena, Germany). The switch is a microelectromechanical (MEMS) device, which uses micropisms to control and open different optical ports to ensure that the 377-nm laser light and broadband Xenon light are separated and coupled sequentially into the MMS probe without any overlap. The switch is controlled via transistor-transistor logic (TTL) pulse trains initiated within the custom software. Light from the switch's output is passed to the dual LIFS/DRS input port of the MMS probe via a subminiature version A (SMA) nipple fitting; roughly 30% loss in signal is measured due to the optical switch and SMA fitting. The 830-nm Raman laser light is delivered without the optical switch and is triggered after the LIFS and DRS pulses.

C. Photodetection

The MMS detection hardware consists of components optimized for visible (LIFS and DRS) and NIR (RS) detection.

1. Reflectance and fluorescence

The LIFS/DRS spectral system comprises a interline CCD camera (CoolSNAP HQ, Princeton Instruments, Trenton, NJ) cooled to $-30\text{ }^\circ\text{C}$. For each of the LIFS and DRS pulses, the CCD is gated at $50\text{ }\mu\text{s}$. The distal ends of the two DRS/LIFS fibers are aligned with the vertical axis of the spectrograph (SpectraPro 2150i, Princeton Instruments, Trenton, NJ) using software provided by the manufacturer (WinSpec, Princeton Instruments, Trenton, NJ). A 150 grooves/mm grating, blazed at 500 nm, is used in order to capture the entire visible spectrum needed for LIFS (385–650 nm) and DRS (375–700 nm). A slit width of $200\text{ }\mu\text{m}$ is used. To improve the SNR, we bin every three pixels for a spectral dispersion of

0.78 nm/pixel and a resulting spectral resolution FWHM of 5.6 pixels (4.32 nm).

2. Raman

The Raman system consists of a 1024×1024 camera (IMG, Finger Lakes Instrumentation, NY) cooled to -30°C which is coupled to an $f/1.8$ spectrograph (Holospec, Kaiser Optical Systems, MI) utilizing a low-frequency Stokes grating for 830 nm excitation. The longer Raman excitation wavelength reduces tissue autofluorescence by a factor of four compared to 785 nm excitation. A custom 200 μm width slit was installed. This slit width was selected as it strikes an effective balance between ensuring sufficient resolution for spectral analysis and signal strength. The measured spectral dispersion is 1.79 cm^{-1} per pixel and the spectral resolution is a FWHM of 13.43 cm^{-1} .

D. Data acquisition hardware and software

Custom software has been written in LabVIEW (National Instruments, Austin, TX) for single-click operation of the entire MMS system. The software executes MMS data collection by sequentially capturing DRS, LIFS, and RS spectra. For the DRS and LIFS modalities, the sources are triggered for data acquisition via TTL pulses provided by a timer-counter board (NI 2121, National Instruments, Austin, TX) while for RS this same timer-counter board triggers the mechanical shutter to open (as the diode laser is a continuous source and therefore always on). The DRS and LIFS camera is controlled by a PCI card (PCI-6602, National Instruments, Austin, TX) and operated, in part, by pre-written software (R3 Software, Princeton, NJ). At the basic software architecture level, the Raman instrument components (laser and camera) are controlled via drivers written in C++ and incorporated into a MATLAB code; however, these codes and drivers are called and user inputs implemented within LabVIEW. Spectra are displayed for instant user feedback via onboard binning and background subtraction. Finally, for the DRS and LIFS modalities, an extra step is required for optical switch operation.

E. Calibration

To achieve day-to-day consistency and accurate spectral measurements, calibration procedures must be performed. The Raman and LIFS/DRS wavelength calibrations are performed by measuring known spectral lines from a solid 4-acetamidophenol (Tylenol) capsule and a mercury-argon pencil lamp (Hg-1, Ocean Optics, FL), respectively. To account for how the quantum efficiency of the detectors alter the fluorescence and Raman spectra (system response), the spectrum of a calibrated Tungsten light source (LS1-Cal, Ocean Optics, FL) shone onto a 20% reflectance standard is recorded; the system response is not required for the DRS measurement as it is inherently normalized. To account for white light source day-to-day variations, the reflectance amplitude is measured by recording the spectrum of a solid titanium dioxide stan-

dard. This step ensures that all reflectance measurements are calibrated to the LUT model before extraction of optical properties. Background calibration, which accounts for stray light and dark current, is performed for all three modalities by taking spectra with the lights off. With external triggering, the shot-to-shot variation from the N_2 laser was measured at 12%. Therefore, to account for these fluctuations, a beam splitter was installed to create a power measurement arm and each fluorescence spectra is then normalized by this measured power (3A-P, Ophir Optics, Israel). These calibration procedures were performed prior to every clinical data collection day (to account for day-to-day variations) and also whenever any optical alignment took place such as distal end reinsertion into a spectrometer, slit width adjustment, and camera or lens alignment.

F. Clinical safety and transportation

As our system is used in the clinic, stringent safety protocols had to be followed. The output energy of the N_2 laser is measured at $6.5 \mu\text{J}$, which is considerably lower than the maximum permissible levels ($53 \mu\text{J}$) of a Class 1 device.³⁹ The mechanical shutter, which blocks the 830-nm laser diode laser, opens when triggered through the software, closes immediately after the acquisition and remains closed until the next acquisition.

For clinical portability, all MMS components are mounted to a two-level utility cart (4546-10, Rubbermaid, Winchester, VA) as shown in Figure 2. The utility cart was specially outfitted with 6-in. pneumatic caster wheels to prevent vibration and increase transportation ease. To ensure electrical line isolation and prevent electrical damage, all powered MMS components (spectrometers, cameras, etc.) are connected to the main power via an isolation transformer power conditioner (IS250, Triplite, Chicago, IL). Furthermore, in the event of main power loss and to allow the system to be transported between clinical rooms, the MMS system is also connected to a battery supply (CP1500AVR, CyberPower, Shakopee, MN) which provides approximately 10 min of external power.

III. SPECTRAL MODELING AND ANALYSIS

A. Reflectance

The diffusion approximation to the radiative transport equation, which is most commonly used in order to extract optical property information from reflectance spectra,^{40,41} is only valid when the separation between the excitation fiber (source) and collection fibers (detector) are less than the mean free path and in absorption-dominated tissues (high albedo absorption $\gg$ scattering). MMS work conducted by other researchers, who studied atherosclerosis, used this diffusion approximation to calculate reflectance using their MMS probe. However, the diffusion approximation is not valid for our application as skin cancers originate in shallow cutaneous layers, which requires relatively short source-detector separations to confine the light only to these shallow layers, and the lesions of interest (i.e., dysplasia) can have low albedo. As

FIG. 2. Photo of the MMS clinical system (left) and the MMS probe (right).

the diffusion approximation is not valid, we use a look-up table (LUT) inverse model approach that is appropriate for our conditions (short source-detector separation and low albedo) and accurately measures tissue optical properties such as the reduced scattering coefficient, μ'_s , and absorption coefficient, μ_a . It has been shown by previous researchers⁴² that the diffuse reflectance will be the same for any combination of μ_s , and g that result in the same μ'_s . We have previously shown in our laboratory – by performing detailed parametric studies – that the extracted absorption and reduced scattering coefficient values have less than 10% error when the anisotropy is greater than 0.7 when using the LUT method.⁴³ The LUT is essentially a database of reflectance values across a range of scattering and absorption values. The database is generated by measuring reflectance spectra from a matrix tissue simulating phantoms with known optical properties and then interpolating between these values to generate a topography in R , μ_a and μ'_s space. Any reflectance spectra, obtained from a sample with unknown optical properties, can be fit to this database in order to determine its optical properties. The tissue phantoms are created by using polystyrene beads with nominal 1 μm diameter and 2.6% solids by volume (Polysciences, Warrington, PA) and black India ink (Speedball, Statesville, NC) as the scattering and absorption media, respectively. Mie theory was used to calculate the μ'_s of the polystyrene beads (and therefore the amount of volume to add for a desired μ'_s) and a spectrophotometer (DU720, Beckman Coulter, CA) to measure the μ_a of a stock India ink solution. In total, 21 phantoms were used to generate the LUT, spanning physiological relevant values of μ'_s (0.44–4.74 mm^{-1}) and μ_a (0–2.5 mm^{-1}). Raw spectra were then collected by the probe and reflectance spectra calculated using the following equation:

$$R_{\text{diffuse}}(\lambda) = \frac{I_{\text{sample}}(\lambda) - I_{\text{background}}(\lambda)}{[I_{\text{standard}}(\lambda) - I_{\text{background}}(\lambda)] \times 100/R_{\text{standard}}}, \quad (1)$$

where $I_{\text{sample}}(\lambda)$ is the raw spectrum from the phantom, $I_{\text{background}}(\lambda)$ is the background spectrum, $I_{\text{standard}}(\lambda)$ is the spectralon standard spectrum and $100/R_{\text{standard}}$ is a factor used to account for the calibrated reflectance level of the spectralon standard (throughout this paper all results were obtained with a 20% spectralon reflectance standard). Spectra are presented in terms of wavelength by performing the wavelength calibration procedure discussed above. Validation of the LUT is discussed below. This approach follows previous work conducted in our laboratory^{43,44} and the successful application of the LUT approach for skin cancer diagnosis.⁴⁵

A nonlinear optimization fitting routine is employed to minimize the difference (χ^2) between the database LUT reflectance spectra and the measured reflectance spectra between 400 and 650 nm. For the LUT validation (see Sec. IV A), the reduced scattering coefficient and absorption coefficient are constrained to the following forms:

$$\mu'_s(\lambda) = \mu'_s(\lambda_0) \left(\frac{\lambda}{\lambda_0} \right)^{-B}, \quad (2)$$

$$\mu_a(\lambda) = \frac{2.303yA(\lambda)[\text{absorber}]}{L}, \quad (3)$$

where λ_0 is 630 nm, $A(\lambda)$ is the absorbance spectra of the dye when measured using a spectrophotometer, L is the path length of the spectrophotometer measurement, $[\text{absorber}]$ is the concentration of absorber used for the spectrophotometer measurement, and y is a scaling factor to account for the dilution of the dye solution used in the spectrophotometer. For the validation study, the fitting outputs are $\mu'_s(\lambda_0)$, B , and $[\text{absorber}]$, from which $\mu'_s(\lambda)$ and $\mu_a(\lambda)$ can be calculated. For clinical fitting (see Sec. IV B), the reduced scattering coefficient is constrained in the same fashion (Eq. (2)), however, the physiological absorption coefficient is calculated using the following equations described by van Veen:⁴⁶

$$\mu_{a(\text{blood})}(\lambda) = 150v[\alpha\varepsilon_{HbO_2}(\lambda) + (1 - \alpha)\varepsilon_{Hb}(\lambda)], \quad (4)$$

$$C_{pack} = \left[\frac{1 - e^{(-2\mu_{a(bl)}(\lambda)D_{vessel})}}{2\mu_{a(bl)}(\lambda)D_{vessel}} \right], \quad (5)$$

$$\mu_{a(tissue)}(\lambda) = C_{pack}\nu\mu_{a(bl)}(\lambda) + [mel]\varepsilon_{mel}(\lambda), \quad (6)$$

where ν is the blood volume fraction sampled by the light (assuming a hemoglobin concentration of 150 mg/ml in the bloodstream), α is the oxygen saturation (ratio of HbO₂ to total Hb), $\epsilon_{HbO_2}(\lambda)$, $\epsilon_{Hb}(\lambda)$, and $\epsilon_{mel}(\lambda)$ are the extinction coefficients of oxygenated hemoglobin, deoxygenated hemoglobin, and melanin, respectively, D_{vessel} is the mean vessel diameter and $[mel]$ is the concentration of melanin. These equations are used to account for the inhomogeneous distribution of blood vessels in tissue. For clinical data, the fit outputs are $\mu'_s(\lambda_0)$, B , $[mel]$, ν , α , and D_{vessel} .

B. Fluorescence

Fluorescence spectra are first background corrected by subtracting a dark spectrum (lights off, probe pointing upwards) from the raw spectrum. Next, a wavelength calibration is performed by using a peak fitting algorithm to find the pixel locations of HgAr lines, fitting a 3rd order polynomial (as 4 strong lines are seen in the visible) from these pixel locations to the known wavelengths of these lines and then converting the entire pixel array to wavelength space. The intensity calibration is performed by scaling the measured blackbody spectrum to the measured values provided by the manufacturer; the measured spectrum will be altered due to the wavelength dependence of the detector's quantum efficiency and this step is necessary in order to correct for this instrument response.

The turbid nature of raw tissue alters the fluorescence signal such that the measured fluorescence spectral shape is altered and its intensity attenuated. Therefore, the intrinsic fluorescence – the true endogenous fluorescence without scattering or absorption distortion – must be calculated in order to accurately model physiological fluorescence. The intrinsic fluorescence is calculated by using the photon migration model of Zhang *et al.*,⁴⁷ which uses the measured reflectance of the sample (with a particular μ_a and μ'_s) and probe specific parameters in order to correct the fluorescence

$$IF(\lambda) = \frac{F(\lambda)}{\frac{R(\lambda_x)}{\mu_s(\lambda_x)lR_0(\lambda_x)} \sqrt{\frac{R_0(\lambda_x)R_0(\lambda)}{\varepsilon(\lambda_x)\varepsilon(\lambda)} \left[\frac{R(\lambda)}{R_0(\lambda)} + \varepsilon(\lambda) \right]}}, \quad (7)$$

where λ_x is the excitation wavelength, $\mu_s(\lambda_x)$ is the value of the scattering coefficient at the excitation wavelength, $IF(\lambda)$ is the intrinsic fluorescence spectrum, $F(\lambda)$ is the measured fluorescence spectrum, $R(\lambda)$ is the measured reflectance spectrum, $R(\lambda_x)$ is the value of the measured reflectance at the excitation wavelength, $R_0(\lambda)$ is the measured reflectance spectrum for no absorption, and $R_0(\lambda_x)$ is the value of the reflectance at the excitation wavelength for no absorption. $F(\lambda)$ and $R(\lambda)$ are directly measured via experiment and $R(\lambda_x)$ is a constant determined from the $R(\lambda)$ spectrum. $R_0(\lambda)$ is calculated by first fitting the measured spectrum, $R(\lambda)$, to the LUT to obtain μ_a and μ'_s . The corresponding reflectance spectrum with this μ'_s is then numerically evaluated via two-

dimensional interpolation of the LUT space by constraining $\mu_a = 0$. Additionally, $\varepsilon(\lambda)$ is calculated using $\varepsilon = e^\beta - 1$, where $\beta = S(1-g)$ for a probe specific constant, S , and the anisotropy, g ; $\varepsilon(\lambda_x)$ is the value of $\varepsilon(\lambda)$ at the excitation wavelength. The last remaining term, l , is also a probe specific constant.

As discussed above, S and l are probe specific parameters and they are determined empirically. These were calculated as follows. First, the fluorescence spectra of 0.26 μ M molar Stilbene 420 (Exciton, OH) dissolved in ethanol was measured. Stilbene-420 was chosen as its peak emission wavelength is similar to that of NADH. The heavily diluted 0.2 μ M solution was chosen in order to ensure that the Stilbene itself was not contributing to the scattering signal while guaranteeing sufficient fluorescence SNR. Without any scattering or absorption, this spectra corresponds to $IF(\lambda)$. Next, a spectra was taken with polystyrene beads added to the 0.26 μ M Stilbene solution to create a solution with $\mu'_s = 1.5 \text{ mm}^{-1}$. As this spectra is only influenced by scattering (no absorption), it is equal to $R_0(\lambda)$ and from which $R_0(\lambda_x)$ can be determined. In sequential order, 4 volumes of red dye were added to the solution to create solutions with μ_a values of 0.29, 0.57, 0.86, and 1.14 mm^{-1} . These spectra corresponded to four different $R(\lambda)$. An optimization routine was written to calculate S and l by minimizing the difference between the four $IF(\lambda)$ spectra when calculated using Eq. (2). The values of S and l , specific to our particular MMS probe, are 1.83 and 0.163.

In tissue, 337 nm excites both NADH and collagen.⁴⁸ We assume that the measured intrinsic fluorescence is a linear combination of fluorescence from NADH and collagen, which can be expressed mathematically as

$$IF(\lambda) = A_1 IF_{NADH}(\lambda) + A_2 IF_{collagen}(\lambda), \quad (8)$$

where $IF_{NADH}(\lambda)$ and $IF_{collagen}(\lambda)$ are the intrinsic spectra of NADH and collagen, respectively, and $\chi_{nadh} = A_1 / (A_1 + A_2)$ and $\chi_{collagen} = A_2 / (A_1 + A_2)$ represent the relative concentrations of NADH and collagen, respectively. The values of A_1 and A_2 are determined by a fitting routine.

C. Raman

Raman spectra is collected in the “fingerprint” region ($\sim 400\text{--}1800 \text{ cm}^{-1}$), because it is a rich source of Raman bio-markers useful for skin properties and skin cancer diagnosis.^{49–55} The first processing step involves background subtraction whereby a dark spectrum is subtracted from the raw spectrum. Second, the wavelength and intensity calibrations are performed in a very similar manner as described for the fluorescence spectra; the only difference is that the peak fitting algorithm is used to find Tylenol pixel locations (instead of HgAr lines as needed for the fluorescence). Raman incident laser light can cause tissue autofluorescence, which has practical implications as the fluorescence swamps the Raman signal. This autofluorescence is most likely due to the morphological make-up of the epidermis, which absorbs light up to 1000 nm and can re-emit it as fluorescence.⁴⁸ Tissue autofluorescence is removed by fitting the Raman spectra to a

FIG. 3. Comparison between fitted (LUT) and expected (experimental) for the reduced scattering coefficient (left) and absorption coefficient (right).

5th order polynomial^{37,38} and subtracting the fit from the raw spectrum, revealing the desired endogenous Raman signals.

IV. MMS SYSTEM AND PROBE PERFORMANCE

A. LUT validation

The LUT was validated by fitting the LUT topography to spectra obtained from 47 validation phantoms with known optical properties. The validation phantoms were fabricated by using the polystyrene beads and colored food dye (red, blue, and green) to simulate scattering and absorption, respectively. These phantoms spanned ranges of $0.72\text{--}4.31\text{ mm}^{-1}$ and $0\text{--}2.42\text{ mm}^{-1}$ for μ'_s and μ_a , respectively, which covered approximately 90% of the LUT surface. For each validation phantom, spectra were averaged across 5 measurements. Using the LUT fitting approach described above, the optical properties were extracted with normalized root-mean-square errors of 7.19% and 9.81% for μ'_s and μ_a , respectively, as shown in Figures 3(a) and 3(b); for a particular optical property, the errors were calculated by averaging across all wavelength and all 47 phantoms.

B. Raman *in vivo* performance

To demonstrate the Raman performance, *in vivo* Raman spectra were obtained at different locations on the body of a healthy Caucasian male volunteer, Figure 4. Each location was measured 5 times. Tissue autofluorescence was removed using the polynomial fitting procedure described previously. Encouragingly, bands identified by previous researchers as being important for skin cancer diagnosis^{52,53,56} – the Amide I, Amide III, and CH_2 scissor – show very good signal to noise ratios.

C. MMS clinical performance

The MMS system is currently being used for clinical testing for the detection of non-melanoma and melanoma skin cancers. Clinical data acquisition times are roughly 4.5 s in to-

tal, comprising a 4 s Raman exposure and the 3 Xenon flashes, N_2 laser pulse, and optical switching making up the remaining 500 μs . The clinical data acquisition procedure was as follows: (1) Dermatologist identified suspicious lesion, (2) 3 repeat measurements made on each lesion, (3) 3 repeat measurements of corresponding normal skin as close to the lesion as possible, and (4) lesion is biopsied and lesions classified using histopathology. Sample MMS spectra are presented in Figure 5 for a basal cell carcinoma. Fitting to Eqs. (2)–(6) and (8) above gives physiological quantities consistent with a non-melanoma skin cancer clinical study previously conducted by our group.

D. Repeatability

As our final long-term goal is to apply this technology in the clinic, two major evaluations would be the specificity/sensitivity of the instrument and its repeatability. As the clinical data collection is ongoing, specificity/sensitivity data are not presented at this stage. However, bench top and clinical data have been collected to quantify the repeatability of the MMS system. In order to quantify the repeatability of the system, each modality was characterized separately. For Raman, the standard deviation as a percentage of the mean for 50 peak intensity measurements of a major Tylenol peak (at $\sim 1324\text{ cm}^{-1}$) was calculated to be 1.6% and the variation in Scissor band peak ($\sim 1450\text{ cm}^{-1}$) intensity from 5 measurements (from Sec. IV B) was calculated to be 6%. It is expected that the *in vivo* measurement displays greater variation than the bench top measurement due to patient movement. For DRS, the repeatability was measured to be 3.2% for μ'_s and 3.4% for μ_a . These values were determined by averaging across 3 sets of 20 measurements each (total of 60 measurements) for the reflectance values at maximum absorption and at 630 nm for the 3 most highly absorbing and highly scattering liquid phantoms (blue, green, and red with μ'_s (630 nm) = 4.31 and $\mu_a = 2.32\text{ mm}^{-1}$). From 5 measurements of the volar forearm, the variation was 7.0% and 8.2% for values at 630 nm and 417 nm (Hemoglobin Soret band peak). For LIFS, the repeatability was assessed by calculating the

FIG. 4. Sample *in vivo* Raman spectra obtained using the MMS probe for various body locations. Exposure time is 4 s.

variance in 100 measurements of the fluorescent peak of a heavily diluted Coumarin sample (Exciton, OH) at 337 nm excitation. Without accounting for the laser fluctuation, the variance is 17% and when normalizing the signal by the power arm measurement (see Sec. II E) this variance dropped to 4%, which clearly demonstrates the need for the power arm measurement in order to account for laser fluctuations.

V. DISCUSSION

A. Validation studies

The addition of an absorber to a phantom involves extra pipetting steps in order to complete the spectrophotometer measurement, which is not needed for a purely scattering phantom. It is proposed that the lower extraction accuracy of μ_a compared to μ'_s is due to the extra steps which represent more sources of measurement error due to instrument resolution and calibration (spectrophotometer, pipettes) and also human error. At this stage, we have no definitive explanation for the μ_a trend of increasing divergence between fitted and expected values (as seen in Figure 3) with increasing

μ_a values. However, the extra μ_a measurement steps could offer an explanation as equipment error (spectrophotometer, pipettes) could be proportional to volume or concentration, which would result in the systemic divergence observed.

B. Clinical feasibility

There is a clear discrepancy between the intrinsic fluorescence fit (used to determine the collagen and NADH relative concentrations) and the extracted measured intrinsic fluorescence, Figure 5. This discrepancy is primarily due to the fact that our intrinsic fluorescence model does not fully capture the physics. We assume linear superposition of the two fluorophores, which implies that their measured fluorescence is independent of each other. In reality, these fluorescence signals are coupled as NADH has non-negligible absorption within the visible and will therefore absorb some of collagen's fluorescence emission – the opposite situation will also occur (collagen absorbing some of NADH's fluorescence). Therefore, the measured flux from both NADH and collagen fluorescence will be attenuated due to this fluorescence

FIG. 5. MMS clinical data of a basal cell carcinoma for Raman (left), diffuse reflectance (middle), and intrinsic fluorescence (right) modalities. Physiological quantities obtained from DRS spectral fitting were $\mu'_s(\lambda_0) = 1.7 \text{ mm}^{-1}$, $\nu = 2.87$, $\alpha = 0.46$, and $D_{\text{vessel}} = 0.48 \text{ } \mu\text{m}$ and $\chi_{\text{nadh}} = 0.55$ and $\chi_{\text{collagen}} = 0.45$ from the intrinsic fluorescence spectral fitting.

emission-absorption overlap and will spectrally manifest itself as attenuated signal and a slightly different peak location. A more accurate model would include second-order, nonlinear terms to describe this fluorescence emission-absorption overlap; however, previous researchers^{13,33} have successfully applied this linear superposition approach for clinical diagnosis as it still does a very good job of capturing the endogenous fluorescence physics.

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- ¹H. P. Buschman *et al.*, "Diagnosis of human coronary atherosclerosis by morphology-based Raman spectroscopy," *Cardiovasc. Pathol.* **10**(2), 59–68 (2001).
- ²J. H. Nazemi and J. F. Brennan III, "Lipid concentrations in human coronary artery determined with high wavenumber Raman shifted light," *J. Biomed. Opt.* **14**, 034009 (2009).
- ³B. R. McCreadie *et al.*, "Bone tissue compositional differences in women with and without osteoporotic fracture," *Bone* **39**(6), 1190–1195 (2006).
- ⁴R. Wolthuis *et al.*, "Determination of water concentration in brain tissue by Raman spectroscopy," *Anal. Chem.* **73**(16), 3915–3920 (2001).
- ⁵Y. Ozaki *et al.*, "Nondestructive analysis of biological materials by FT-IR-ATR. Method I: Direct evidence for the existence of collagen helix structure in lens capsule," *Appl. Spectrosc.* **43**(1), 138–141 (1989).
- ⁶V. R. Kodati, A. T. Tu, and J. L. Turumin, "Raman spectroscopic identification of uric-acid-type kidney stone," *Appl. Spectrosc.* **44**(7), 1134–1136 (1990).
- ⁷V. R. Kodati *et al.*, "Raman spectroscopic identification of phosphate-type kidney stones," *Appl. Spectrosc.* **45**(4), 581–583 (1991).
- ⁸A. J. Berger *et al.*, "Multicomponent blood analysis by near-infrared Raman spectroscopy," *Appl. Opt.* **38**(13), 2916–2926 (1999).
- ⁹A. S. Haka *et al.*, "Diagnosing breast cancer by using Raman spectroscopy," *Proc. Natl. Acad. Sci. U.S.A.* **102**(35), 12371 (2005).
- ¹⁰C. Zhu *et al.*, "Diagnosis of breast cancer using fluorescence and diffuse reflectance spectroscopy: A Monte-Carlo-model-based approach," *J. Biomed. Opt.* **13**, 034015 (2008).
- ¹¹Z. Volynskaya *et al.*, "Diagnosing breast cancer using diffuse reflectance spectroscopy and intrinsic fluorescence spectroscopy," *J. Biomed. Opt.* **13**, 024012 (2008).
- ¹²A. Robichaux *et al.*, "In vivo detection of cervical dysplasia with near-infrared Raman spectroscopy," Proceedings of PSIE 4614, Biomedical Vibrational Spectroscopy II, 145, March 28, 2002.
- ¹³I. Georgakoudi *et al.*, "Fluorescence, reflectance, and light-scattering spectroscopy for evaluating dysplasia in patients with Barrett's esophagus," *Gastroenterology* **120**(7), 1620–1629 (2001).
- ¹⁴L. B. Lovat *et al.*, "Elastic scattering spectroscopy accurately detects high grade dysplasia and cancer in Barrett's oesophagus," *Gut* **55**(8), 1078–1083 (2006).
- ¹⁵N. Stone *et al.*, "Near-infrared Raman spectroscopy for the classification of epithelial pre-cancers and cancers," *J. Raman Spectrosc.* **33**(7), 564–573 (2002).
- ¹⁶A. Dhar *et al.*, "Elastic scattering spectroscopy for the diagnosis of colonic lesions: Initial results of a novel optical biopsy technique," *Gastrointestinal Endosc.* **63**(2), 257–261 (2006).
- ¹⁷W. C. Lin *et al.*, "Diffuse reflectance spectroscopy for in vivo pediatric brain tumor detection," *J. Biomed. Opt.* **15**, 061709 (2010).
- ¹⁸M. P. L. Bard *et al.*, "Optical spectroscopy for the classification of malignant lesions of the bronchial tree," *Chest* **129**(4), 995–1001 (2006).
- ¹⁹Z. Huang *et al.*, "Near-infrared Raman spectroscopy for optical diagnosis of lung cancer," *Int. J. Cancer* **107**(6), 1047–1052 (2003).
- ²⁰C. A. Lieber *et al.*, "Diagnostic tool for early detection of ovarian cancers using Raman spectroscopy," *Biomedical Spectroscopy: Vibrational Spectroscopy and Other Novel Techniques, Proc. SPIE* **3918**, 129 (2000).
- ²¹F. Koenig *et al.*, "Spectroscopic measurement of diffuse reflectance for enhanced detection of bladder carcinoma," *Urology* **51**(2), 342–345 (1998).
- ²²D. M. Parkin *et al.*, "Global cancer statistics, 2002," *Ca-Cancer J. Clin.* **55**(2), 74–108 (2005).
- ²³G. Monheit *et al.*, "The performance of MelaFind: A prospective multicenter study," *Arch. Dermatol.* **147**(2), 188 (2011).
- ²⁴D. S. Rigel, J. Russak, and R. Friedman, "The evolution of melanoma diagnosis: 25 years beyond the ABCDs," *CA: Cancer J. Clin.* **60**(5), 301–316 (2010).
- ²⁵A. B. Kimball and J. S. Resneck, Jr., "The US dermatology workforce: A specialty remains in shortage," *J. Am. Acad. Dermatol.* **59**(5), 741–745 (2008).
- ²⁶I. Pavlova *et al.*, "Microanatomical and biochemical origins of normal and precancerous cervical autofluorescence using laser-scanning fluorescence confocal microscopy," *Photochem. Photobiol.* **77**(5), 550–555 (2003).
- ²⁷R. Drezek *et al.*, "Understanding the contributions of NADH and collagen to cervical tissue fluorescence spectra: Modeling, measurements, and implications," *J. Biomed. Opt.* **6**, 385 (2001).
- ²⁸I. Pavlova *et al.*, "Understanding the biological basis of autofluorescence imaging for oral cancer detection: High-resolution fluorescence microscopy in viable tissue," *Clin. Cancer Res.* **14**(8), 2396–2404 (2008).
- ²⁹I. Georgakoudi *et al.*, "NAD (P) H and collagen as in vivo quantitative fluorescent biomarkers of epithelial precancerous changes," *Cancer Res.* **62**(3), 682 (2002).
- ³⁰E. Borisova *et al.*, "Diagnostics of pigmented skin tumors based on laser-induced autofluorescence and diffuse reflectance spectroscopy," *Quantum Electron.* **38**, 597 (2008).
- ³¹N. Rajaram *et al.*, "Pilot clinical study for quantitative spectral diagnosis of non-melanoma skin cancer," *Lasers Surg. Med.* **42**(10), 716–727 (2010).
- ³²O. R. Scepanovic *et al.*, "A multimodal spectroscopy system for real-time disease diagnosis," *Rev. Sci. Instrum.* **80**(4), 043103 (2009).
- ³³O. R. Scepanovic *et al.*, "Multimodal spectroscopy detects features of vulnerable atherosclerotic plaque," *J. Biomed. Opt.* **16**(1), 1–10 (2011).
- ³⁴U. Utzinger and R. R. Richards-Kortum, "Fiber optic probes for biomedical optical spectroscopy," *J. Biomed. Opt.* **8**, 121 (2003).
- ³⁵A. F. Zuluaga *et al.*, "Fluorescence excitation emission matrices of human tissue: A system for in vivo measurement and method of data analysis," *Appl. Spectrosc.* **53**(3), 302–311 (1999).
- ³⁶I. J. Bigio and J. R. Mourant, "Ultraviolet and visible spectroscopies for tissue diagnostics: Fluorescence spectroscopy and elastic-scattering spectroscopy," *Phys. Med. Biol.* **42**(5), 803 (1997).
- ³⁷J. T. Motz *et al.*, "Optical fiber probe for biomedical Raman spectroscopy," *Appl. Opt.* **43**(3), 542–554 (2004).
- ³⁸A. Mahadevan-Jansen *et al.*, "Development of a fiber optic probe to measure NIR Raman spectra of cervical tissue in vivo," *Photochem. Photobiol.* **68**(3), 427–431 (1998).
- ³⁹ANSI, Z136.1-1993, American National Standard for the Safe Use of Lasers, 1993.
- ⁴⁰T. J. Farrell, M. S. Patterson, and B. Wilson, "A diffusion theory model of spatially resolved, steady-state diffuse reflectance for the noninvasive determination of tissue optical properties in vivo," *Med. Phys.* **19**, 879 (1992).
- ⁴¹A. Kienle and M. S. Patterson, "Improved solutions of the steady-state and the time-resolved diffusion equations for reflectance from a semi-infinite turbid medium," *J. Opt. Soc. Am. A* **14**(1), 246–254 (1997).
- ⁴²R. Graaff *et al.*, "Condensed Monte Carlo simulations for the description of light transport," *Appl. Opt.* **32**(4), 426–434 (1993).
- ⁴³B. S. Nichols, N. Rajaram, and J. W. Tunnell, "Performance of a lookup table-based approach for measuring tissue optical properties with diffuse optical spectroscopy," *J. Biomed. Opt.* **17**(5), 057001 (2012).
- ⁴⁴N. Rajaram, T. H. Nguyen, and J. W. Tunnell, "Lookup table-based inverse model for determining optical properties of turbid media," *J. Biomed. Opt.* **13**, 050501 (2008).
- ⁴⁵N. Rajaram *et al.*, "Design and validation of a clinical instrument for spectral diagnosis of cutaneous malignancy," *Appl. Opt.* **49**(2), 142–152 (2010).
- ⁴⁶R. Van Veen, W. Verkruysse, and H. Sterenborg, "Diffuse-reflectance spectroscopy from 500 to 1060 nm by correction for inhomogeneously distributed absorbers," *Opt. Lett.* **27**(4), 246–248 (2002).

- ⁴⁷Q. Zhang *et al.*, “Turbidity-free fluorescence spectroscopy of biological tissue,” *Opt. Lett.* **25**(19), 1451–1453 (2000).
- ⁴⁸T. Vo-Dinh, “Biomedical photonics handbook,” *J. Biomed. Opt.* **9**, 1110 (2004).
- ⁴⁹J. Zhao *et al.*, “Real time raman spectroscopy for noninvasive *in vivo* skin analysis and diagnosis,” in *New Developments in Biomedical Engineering* (InTech, 2010).
- ⁵⁰A. Nijssen *et al.*, “Discriminating basal cell carcinoma from its surrounding tissue by Raman spectroscopy,” *J. Invest. Dermatol.* **119**(1), 64–69 (2002).
- ⁵¹S. B. Cartaxo *et al.*, “FT-Raman spectroscopy for the differentiation between cutaneous melanoma and pigmented nevus,” *Acta Cirurg. Bras.* **25**(4), 351–356 (2010).
- ⁵²M. Gniadecka *et al.*, “Melanoma diagnosis by Raman spectroscopy and neural networks: Structure alterations in proteins and lipids in intact cancer tissue,” *J. Invest. Dermatol.* **122**(2), 443–449 (2004).
- ⁵³M. Gniadecka *et al.*, “Diagnosis of basal cell carcinoma by Raman spectroscopy,” *J. Raman Spectrosc.* **28**(23), 125–129 (1997).
- ⁵⁴Z. Huang *et al.*, “Raman spectroscopy of *in vivo* cutaneous melanin,” *J. Biomed. Opt.* **9**, 1198 (2004).
- ⁵⁵M. Gniadecka, O. F. Nielsen, and H. Wulf, “Water content and structure in malignant and benign skin tumours,” *J. Mol. Struct.* **661–662**, 405–410 (2003).
- ⁵⁶J. Zhao *et al.*, “Real-time Raman spectroscopy for non-invasive skin cancer detection-preliminary results,” *Conf. Proc. IEEE Eng. Med. Biol. Soc.* **2008**, 3107–3109 (2008).