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In vivo measurement of non-keratinized squamous epithelium using a spectroscopic microendoscope with multiple source-detector separations

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Abstract

In the non-keratinized epithelia, dysplasia typically arises near the basement membrane and proliferates into the upper epithelial layers over time. We present a non-invasive, multimodal technique combining high-resolution fluorescence imaging and broadband sub-diffuse reflectance spectroscopy (sDRS) to monitor health at various tissue layers. This manuscript focuses on characterization of the sDRS modality, which contains two source-detector separations (SDSs) of 374 μm and 730 μm , so that it can be used to extract *in vivo* optical parameters from human oral mucosa at two tissue thicknesses. First, we present empirical lookup tables (LUTs) describing the relationship between reduced scattering (μ_s') and absorption coefficients (μ_a) and absolute reflectance. LUTs were shown to extract μ_s' and μ_a with accuracies of approximately 4% and 8%, respectively. We then present LUTs describing the relationship between μ_s' , μ_a and sampling depth. Sampling depths range between 210–480 and 260–620 μm for the 374 and 730 μm SDSs, respectively. We then demonstrate the ability to extract *in vivo* μ_s' , μ_a , hemoglobin concentration, bulk tissue oxygen saturation, scattering exponent, and sampling depth from the inner lip of thirteen healthy volunteers to elucidate the differences in the extracted optical parameters from each SDS (374 and 730 μm) within non-keratinized squamous epithelia.

Keywords

diffuse; reflectance; spectroscopy; microendoscopy; phantoms; human; oral; optical; properties

1. INTRODUCTION

Within the non-keratinized squamous epithelia, dysplasia typically arises at the basement membrane between the epithelium and underlying connective tissue [1–4]. In this situation, severity of dysplasia is typically characterized based on how far the dysplastic tissue has spread from the basement membrane towards the apical epithelial surface. For example, early-stage dysplasia confined at the basement membrane may be considered mild, whereas later-stage dysplasia that has spread to the epithelial surface may be considered severe or even carcinoma in situ. Thus, dysplasia can occur at various layers within the tissue geometry at various thicknesses [1–4].

Our goal is to design instrumentation that can quantify structural and functional changes within the tissue environment at various layers. To do this, we have designed a multimodal

imaging and spectroscopy microendoscope with multiple source-detector separations. This microendoscope employs two modalities within a single, fiber-bundle probe: high-resolution microendoscopy and broadband sub-diffuse reflectance spectroscopy (sDRS) [5]. The high-resolution microendoscopy modality is a well-characterized imaging modality that can image fluorescently labeled, apical cell nuclei within a small field-of-view, typically around 1 mm in diameter [5–12]. The benefit of this modality is the ability to non-invasively monitor the apical epithelial tissue environment and quantify parameters such as nuclear-cytoplasmic ratio or cells-per-area, which could elucidate changes associated with dysplasia. This modality has been studied in a range of endoscopically accessible organs, including the oral cavity [6, 12], esophagus [7, 9], and lower gastrointestinal tract [10, 11]. However, this modality is limited to viewing only the topmost cell layers, and dysplastic changes occurring beneath the apical surface may be missed [6–12]. Therefore, we employ a broadband sDRS modality that has the potential to provide clinically relevant quantitative information at various thicknesses within the epithelium and underlying connective tissue. Clinically relevant quantitative information includes tissue hemoglobin concentration and bulk tissue oxygen saturation, and other optical properties, including reduced scattering coefficient (μ_s'), absorption coefficient (μ_a), can be quantified as well [5, 13–19].

Within our broadband sDRS modality, we use two center-to-center source-detector separations (SDSs) of 374 and 730 μm to sample multiple tissue thicknesses. In this manuscript, we seek to characterize our sDRS modality and establish baseline optical parameter values within human *in vivo* oral mucosa as a model for non-keratinized squamous epithelia. We first present three-dimensional (3D) lookup tables (LUTs) describing the relationship between optical properties (μ_s' and μ_a) and diffuse reflectance [18], as well as 3D LUTs describing the relationship between μ_s' , μ_a , and sampling depth [20] for each SDS (374 and 730 μm) using phantoms. We then apply our technique to human *in vivo* oral mucosa in 13 volunteers to model non-keratinized squamous epithelium. This work will help elucidate the differences we should expect in the extracted optical parameters from each SDS (374 and 730 μm) within human non-keratinized squamous epithelia and guide future development of this technique.

2. MATERIALS AND METHODS

2.1 Design of the multimodal microendoscope

The multimodal imaging and spectroscopy probe consists of a high-resolution fluorescence imaging modality and a broadband sub-diffuse reflectance spectroscopy (sDRS) modality contained within a single 2.5 mm-diameter probe. For the high-resolution fluorescence imaging modality, a 455 nm LED (20 FWHM) light source (Philips, USA) is coupled through a 1 mm-diameter image fiber (FIGH-50-1100N, Myriad Fiber Imaging, USA) after passing through a 460 nm short pass filter and being reflected by a 475 nm dichroic mirror. Signal from the 455 nm LED excites the fluorescent contrast agent proflavine, and fluorescent signal (peak of 515 nm) travels back through the 1 mm-diameter image fiber. The fluorescent signal passes through the 475 nm dichroic mirror, reflects off a 590 nm dichroic mirror, and passes through a 525/40 nm bandpass filter and 50 mm tube lens into a

CMOS camera to project an image of fluorescently stained tissue on a monitor. This imaging modality has been described previously [5].

For the broadband sDRS modality, a tungsten-halogen lamp (HL-2000-LL, Ocean Optics, USA) delivers broadband light through an off-axis multimode 200 μm (NA = 0.22) delivery fiber (FBP200220240, Molex Inc., USA) to a sample. Two adjacent 200 μm (NA = 0.22) collection fibers (FBP200220240, Molex Inc., USA) with center-to-center source-detector separations (SDS) of 374 and 730 μm , respectively, collect the broadband light, pass through a custom-built optical switch, and into a single spectrometer (USB2000+UV-VIS, Ocean Optics, USA) [5]. Figure 1 shows a schematic of the multimodal imaging and spectroscopy instrumentation. Data from the spectroscopy modality is later post-processed to extract important tissue optical parameters.

2.2 Design of look-up tables for optical parameter and sampling depth extraction

Lookup tables were designed to determine the relationship between optical properties (μ_s' and μ_a) and reflectance. To determine this relationship, a set of 10 calibration phantoms was designed with known μ_s' and μ_a . The 10 calibration phantoms were constructed using distilled water as the solvent, 1 μm -diameter polystyrene microspheres as the scattering agent, and a combination of yellow, red, and blue dye (McCormick & Company, USA) as the absorbing agent. Across all 10 calibration phantoms, μ_s' spanned a continuous range from 4.4–40.0 cm^{-1} and μ_a spanned a continuous range from 0.0–45.0 cm^{-1} . The μ_s' was calculated using Mie Theory to determine the percentage of 1 μm -diameter polystyrene microsphere solution in the liquid phantom. The μ_s was calculated using Beer's Law to determine the percentage of each food dye color in the liquid phantom [5, 18]. Table 1 shows the range of μ_s' and μ_a for the calibration phantoms, as well as the percentage of each of the optical agents used for each phantom.

A 2 mL solution of distilled water, polystyrene microspheres, and dye were gently mixed inside a 7 mL scintillation vial. The tip of the multimodal imaging and spectroscopy probe was dipped inside the liquid phantom 2 mm beneath the surface such that the distance between the probe-tip and bottom of the vial could be considered semi-infinite. Broadband light was delivered and collected by both the 374 and 730 μm SDS with power at the sample plane of 0.35 mW and spectrometer integration time of 500 ms. Five spectra were sequentially averaged together for each phantom, yielding a total of 2.5 seconds of data collection per phantom. Broadband sDRS data was collected for the 10 calibration phantoms. The raw sDRS data was corrected for background noise and converted to absolute reflectance by calibrating with a Spectralon® 20% diffuse reflectance standard (SRS-20-010, Labsphere, USA). Absolute reflectance was then related to the phantoms' wavelength dependent μ_s' and μ_a and was visually demonstrated as a three-dimensional LUT for both the 374 and 730 μm SDSs.

Following generation of the LUTs, the LUTs were validated with a second set of liquid phantoms, referred to as validation phantoms. These validation phantoms were prepared with distilled water, 1 μm -diameter polystyrene microspheres, and red food dye to yield known values of μ_s' and μ_a . 24 validation phantoms were created spanning a range of μ_s' from 5.7–21.1 cm^{-1} and μ_a' from 0.0–18.5 cm^{-1} , which correspond to the key optical

property ranges of the LUTs that can be expected in human *in vivo* tissue. Broadband sDRS data of the 24 validation phantoms was collected in an identical manner to the calibration phantoms. Optical properties (μ_s' and μ_a) at each SDS (374 and 730 μm) were then extracted from the reflectance data of the validation phantoms using the LUTs and compared to their known μ_s' and μ_a values based on Mie Theory and Beer's Law. Comparison between extracted and known optical properties are presented as percent errors and thus the accuracy of the LUTs could be elucidated. Percent errors for μ_s' and μ_a extracted were calculated as follows:

$$Error_{\%,\mu_s'} = \left| \frac{\mu_{s,extracted}' - \mu_{s,theoretical}'}{\mu_{s,theoretical}'} \right| \bullet 100\%, \quad (1)$$

$$Error_{\%,\mu_a} = \left| \frac{\mu_{a,extracted} - \mu_{a,theoretical}}{\mu_{a,theoretical}} \right| \bullet 100\%. \quad (2)$$

Finally, the five calibration phantoms containing the absorbing agent (C.P. #2, 4, 6, 8, 10 from Table 1) were used to quantify sampling depth for the 374 and 730 μm SDSs [20]. To quantify sampling depth, a 10 mL beaker was sufficiently coated with flat black paint (1602, Krylon, USA) such that all light reaching the painted layer would be completely absorbed. The five calibration phantoms were dispensed inside the beaker. Then, the probe-tip was placed in direct contact with the blackened beaker bottom and sDRS data was taken with a spectrometer integration time of 500 ms. Then, the probe-tip was vertically translated 50 μm from the blackened beaker bottom and new sDRS data was taken. Per phantom, this process was repeated 40 times until the probe-tip had been translated 2000 μm away from the blackened beaker bottom. With these 40 measurements, sampling depth was quantified as the depth in which there was the greatest rate-of-change of intensity as a function of depth [20]. This process was repeated for all five calibration phantoms and for each SDS (374 and 730 μm). Sampling depth was then related to the phantoms' wavelength dependent μ_s' and μ_a and was visually demonstrated as a three-dimensional LUT for both the 374 and 730 μm SDSs. Thus, for any sample, including *in vivo* tissue as described in the following section, a sample's μ_s' and μ_a can be extracted by the absolute reflectance LUTs and then sampling depth can be quantified from the sampling depth LUTs.

2.3 Optical parameter extraction from *in vivo* oral mucosa

Once the reflectance LUTs and sampling depth LUTs were created and validated, as described previously, our method for optical property extraction could be applied to *in vivo* tissue. Oral mucosa was chosen as the model tissue in our study because of its ease-of-access and containing only a single absorbing agent, hemoglobin. Therefore, this technique was demonstrated in the inner lip of thirteen healthy volunteers between the ages of 18 and 35. All procedures were approved by the University of Arkansas Institutional Review Board (IRB #15-09-149), and informed consent was obtained from all volunteers. For each volunteer, the probe-tip was placed directly in contact with the inner lip and broadband sDRS data was collected for both the 374 and 730 μm SDSs with a spectrometer integration

time of 500 ms. Power of the broadband light from the halogen lamp was 0.35 mW at the sample plane.

To extract optical properties (μ_s' and μ_a) from *in vivo* data, we implement custom software based in MATLAB. For μ_s' extraction, this software determines μ_s' at 630 nm ($\mu_s'(\lambda_0)$) from Eq. 3) and the scattering exponent ($-B$ from Eq. 3) to determine μ_s' . For μ_a extraction, this software imports well-established hemoglobin absorption data, including the gram molecular weight of hemoglobin (MW , Eq. 4)) which is 64,500 g/mole [21], the weight of hemoglobin in whole blood (x , Eq. 4) which is 150 g/L [21], and the wavelength-dependent molar extinction coefficient ($\text{cm}^{-1}\text{M}^{-1}$) of oxygenated hemoglobin (Hb_{oxy} , Eq. 4) and deoxygenated hemoglobin (Hb_{de-oxy} , Eq. 4) [21]. Based on these known values [21], the software determines the clinically important values for tissue oxygen saturation (α , Eq. 4) and tissue hemoglobin concentration ($[Hb]$, Eq. 4). Thus, after *in vivo* data collection, the LUT-based inverse model was used to extract five optical parameters to elucidate the differences between the extracted parameters for each SDS (374 and 730 μm) including reduced scattering coefficient (μ_s'), absorption coefficient (μ_a), hemoglobin concentration ($[Hb]$), tissue oxygen saturation (SaO_2), and scattering exponent (B). Additionally, once μ_s' and μ_a were known, sampling depth (D) was quantified.

$$\mu_s'(\lambda) = \mu_s'(\lambda_0) \times \left(\frac{\lambda}{\lambda_0}\right)^{-B} \quad (3)$$

$$\mu_a = \left(2.303 \times (x) \times \left(\frac{1}{MW}\right) \times \left[\alpha \left(\varepsilon_{Hb_{oxy}}\right) + (1-\alpha) \left(\varepsilon_{Hb_{de-oxy}}\right)\right] \times \left(\frac{[Hb]}{150 \frac{\text{mg}}{\text{mL}}}\right)\right), \quad (4)$$

3. RESULTS AND DISCUSSION

3.1 Design of look-up tables for optical parameter and sampling depth extraction

Figure 2 shows the results for generation of the reflectance LUTs and sampling depth LUTs. Both LUTs were created by interpolating raw data to create surface fit using MATLAB. The reflectance LUTs were created with a μ_s' range of 4.0–40.0 cm^{-1} and a μ_a range of 0.0–45.0 cm^{-1} , although these LUTs were validated with a μ_s' range of 5.7–21.1 cm^{-1} and a μ_a range of 0.0–18.5 cm^{-1} . Thus, the reflectance LUTs are presented within their validated range. In this range, reflectance ranged between approximately 0–0.30 and 0–0.45 for the 374 and 730 μm , consistent with results from previous studies [18, 22]. The validation phantoms showed that the reflectance LUTs can extract a sample's μ_s' with average percent errors of 4.4% and 3.3% for the 374 and 730 μm SDSs, respectively. Additionally, the reflectance LUTs can extract a sample's μ_a with average percent errors of 9.3% and 7.5% for the 374 and 730 μm SDSs, respectively.

Next, sampling depth was quantified as a function of optical properties and the sampling depth LUTs are presented within the validated range of the reflectance LUT. Sampling depth ranged from 210–480 μm and 260–480 μm for the 374 and 730 μm SDSs, respectively. These values for depth were comparable to other studies [20]. These LUTs, generated from a

set of calibration phantoms, were applied to *in vivo* human tissue data in the following section.

3.2 Optical parameter extraction from *in vivo* oral mucosa

Broadband sDRS data was collected from 13 volunteers using our multimodal technique. From this raw sDRS data, reduced scattering coefficient (μ_s'), absorption coefficient (μ_a), hemoglobin concentration ([Hb]), tissue oxygen saturation (SaO₂), scattering exponent (B), and sampling depth (D) were quantified to elucidate the differences between the extracted parameters for each SDS (374 and 730 μm). Results are shown in Fig. 3. It was found that average μ_s' at 630 nm was higher for the shorter 374 μm SDS (13.1 cm^{-1}) than the longer 730 μm SDS (10.2 cm^{-1}) and this difference was found to be statistically significant (p-value = 0.00). The maximum μ_a , which occurred at the Q-band for hemoglobin (542 nm), was found to be higher for the 730 μm SDS (2.6 cm^{-1}) than the 374 μm SDS (2.2 cm^{-1}) although a significant difference was not observed in within our sample (p-value = 0.21). Additionally, [Hb] and SaO₂ were higher for the 730 μm SDS (2.9 mg/mL and 90.1%) than the 374 μm SDS (2.6 mg/mL and 84.6%). Differences in [Hb] were not statistically significant (p-value = 0.30) whereas differences in SaO₂ were statistically significant (p-value = 0.04). The scattering exponent (B) was higher for the 374 μm SDS (0.71) compared to the 730 μm SDS (0.45) and found to be statistically significant (p-value = 0.00). Finally, consistent with the extracted optical parameters, sampling depth was higher across all wavelengths for the longer 730 μm SDS, as expected. Minimum and maximum sampling depths are presented in Fig. 3. Overall, these average optical parameters help elucidate the differences we should expect in healthy *in vivo* oral mucosa for both tested source-detector separations and are comparable to several studies [14, 23].

4. CONCLUSION

We have developed a multimodal imaging and spectroscopy technique comprising of a conventional fluorescence fiber-bundle microendoscopy platform coupled with two off-axis broadband spectroscopy (sDRS) channels [5]. In this manuscript, we characterize the sDRS modality and show its applicability in a pre-clinical *in vivo* study of human oral mucosa. This multimodal imaging and spectroscopy platform may be capable of collecting a wealth of information about the structural (imaging) and functional (spectroscopy) properties of tissue at various imaging sites in *ex vivo* and *in vivo* models. Most importantly, we have shown that we can quantify the clinically important parameters, hemoglobin concentration and tissue oxygen saturation, which can be correlated with high-resolution images of apical non-keratinized squamous epithelial tissue architecture.

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Figure 1.

A schematic of the multimodal imaging and spectroscopy microendoscope showing a 455 nm LED delivering light through a 460 nm short pass (S.P.) filter, which reflects off a 475 nm dichroic mirror (D.C.M.), passes through a 10X/0.30 NA objective and into a 1 mm-diameter image fiber. Proflavine-stained tissue fluoresces and emits peak 515 nm light which passes back through the 1 mm-diameter image fiber, 10X/0.30 NA objective, and 475 nm D.C.M., before being reflected by a 590 nm D.C.M., and passing through a 525/40 nm bandpass (B.P.) filter, 50 mm tube lens, and finally into an 8-bit monochrome CMOS camera which projects an image onto a monitor for data collection. For the sub-diffuse reflectance spectroscopy (sDRS) modality, a halogen lamp delivers broadband (450–750 nm) light through an off-axis 200 μm multimode fiber within the same probe. Two additional 200 μm multimode fibers at increasing source-detector separations (374 and 730 μm) collect the broadband light that has traveled through a sample/tissue, pass through a custom optical switch, and finally into a single spectrometer which projects a spectrum onto a monitor for data collection.

Figure 2.

Lookup tables for optical property and sampling depth extraction. The figure shows the relationship between optical properties (μ_s' and μ_a) and (a) reflectance for the 374 μm SDS, (b) reflectance for the 730 μm SDS, (c) sampling depth for the 374 μm SDS, and (d) sampling depth for the 730 μm SDS.

Figure 3.

Optical parameter extraction for *in vivo* human oral mucosa from 13 volunteers. This figure shows the optical parameter differences between the 374 μm SDS (dark gray) and 730 μm SDS (light gray) for (a) reduced scattering coefficient (μ_s') at 630 nm, (b) maximum absorption coefficient (μ_a) at 542 nm, (c) hemoglobin concentration ([Hb]), (d) bulk tissue oxygen saturation (SaO_2), (e) scattering exponent (B), and (f) minimum and maximum sampling depths (D).

Table 1

Specifications for the calibration phantoms for generation of the reflectance lookup table (LUT)

Calibration Phantom	μ_s' Range (cm ⁻¹)	% Scattering Agent (1.00 μ m-diameter polystyrene microspheres)	μ_a Range (cm ⁻¹)	% Absorbing Agent (Yellow/Red/Blue food dye)
1	4.4 – 7.0	6.30 %	0.0 – 0.0	0/0/0 %
2	4.4 – 7.0	6.30 %	0.0 – 45.0	1.00/0.25/0.10 %
3	6.6 – 10.5	9.45 %	0.0 – 0.0	0/0/0 %
4	6.6 – 10.5	9.45 %	0.0 – 45.0	1.00/0.25/0.10 %
5	10.0 – 16.2	14.50 %	0.0 – 0.0	0/0/0 %
6	10.0 – 16.2	14.50 %	0.0 – 45.0	1.00/0.25/0.10 %
7	15.7 – 25.3	22.70 %	0.0 – 0.0	0/0/0 %
8	15.7 – 25.3	22.70 %	0.0 – 45.0	1.00/0.25/0.10 %
9	24.9 – 40.0	39.95 %	0.0 – 0.0	0/0/0 %
10	24.9 – 40.0	39.95 %	0.0 – 45.0	1.00/0.25/0.10 %