Real-time calibrating polarization-sensitive diffuse reflectance handheld probe characterizes clinically relevant anatomical locations of oral tissue in vivo

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**Abstract:** We report on the development of a unique real-time calibrating polarization-sensitive diffuse reflectance (rcPS-DR) handheld probe, and demonstrate its diagnostic potential through in-depth characterization and differentiation of clinically relevant anatomical locations of the oral cavity (i.e., alveolar process, lateral tongue and floor of mouth that account for 80% of all cases of oral squamous cell carcinoma) in vivo. With an embedded calibrating polytetrafluoroethylene (PTFE) optical diffuser, the PS-DR spectra bias arising from instrument response, time-dependent intensity fluctuation and fiber bending is calibrated through real-time measurement of the PS-DR system response function. A total of 554 in vivo rcPS-DR spectra were acquired from different oral tissue sites (alveolar process, n = 226, lateral tongue, n = 150 and floor of mouth, n = 178) of 14 normal subjects. Significantly (P < 0.05, unpaired 2-sided Student’s t-test) different spectral ratio (I\textsubscript{540}/I\textsubscript{575}) representing oxygenated hemoglobin contents were found among the alveolar process, lateral tongue and floor of mouth. Further partial least squares discriminant analysis (PLS-DA) and leave-one-out, cross validation (LOOCV) show that, synergizing the complementary information of the two real-time calibrated orthogonal-polarized PS-DR spectra, the rcPS-DR technique is found to better differentiate alveolar process, lateral tongue, and the floor of mouth (accuracies of 88.2%, 83.9%, 84.4%, sensitivities of 80.5%, 75.8%, 78% and specificities of 93.5%, 87.7%, 86.8%) than standard DR (accuracies of 80.8%, 72.9%, 68.5%, sensitivities of 63.2%, 41.5%, 81.3% and specificities of 92.9%, 87.7%, 63.8%) without PS detection. This work showed the feasibility of the rcPS-DR probe as a tool for studying oral cavity lesions in real clinical applications.

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1. Introduction

With an annual incidence of 377,713 cases and 177,757 deaths, oral cancer is one most common malignancy worldwide [1]. Patients diagnosed with advanced stage oral cancer have a 5-year survival rate of only 30%, while the patient’s survival can be improved up to 80% to 90% if the oral cancer can be detected and diagnosed early for appropriate treatments [2]. However, current routine screening of oral cancer relying on medical, social and familial history documentation, risk factor (i.e., tobacco and alcohol usage) evaluation, visual inspection and palpation were deemed insufficient to detect oral cancer early. Invasive random biopsy followed by H&E histopathology are therefore recommended and remain the gold standard for oral cancer detection, but found with limitations. For instance, the H&E slide preparation may distort the oral tissue features, whilst the slide interpretation is highly subjective and depends heavily on the experiences of the pathologists. In particular, the biopsy strategy may not be suitable for patients having multiple suspicious lesions. Therefore, there exists unmet clinical need to develop more advanced...
diagnostic techniques for rapid, objective and enhanced diagnosis of cancer and early cancer in the oral cavity.

Several techniques have been developed and implemented to fill the unmet need, including the established imaging modalities (e.g., computed tomography, magnetic resonance imaging, and positron emission tomography etc. [3]), ultrasound imaging [4], together with a range of different optical imaging/spectroscopy techniques (e.g., OCT: optical coherence tomography [5], photoacoustic tomography [6], hyperspectral imaging [7], auto-fluorescence spectroscopy [8], Raman spectroscopy [9], diffuse reflectance (DR) spectroscopy [10] etc.) under development. Compared with established imaging modalities and/or ultrasound imaging, the optics based oral cancer detection techniques are found with multi advantages. First, optics-based techniques reduce patient exposure to harmful radiation. Second, they offer high resolution (on the order of micrometers or/and sub-micrometers). Third, they are good at visualizing soft tissues [11].

Among the various optics-based techniques developed for cancer detection, DR spectroscopy analyzing the sample optical (including scattering and absorption coefficients [12]), biochemical (water, lipid, protein, glucose [13], hemoglobin and blood oxygen concentration changes [14], etc.) properties and the changes thereof is gaining popularity for tissue characteristion in a number of organs including oral cavity [15–18]. In particular, polarization-sensitive (PS) DR spectroscopy, including that is used in combination with Mie theory calculations to further extract morphological information about epithelial tissue [19,20], that is used to enable depth-selective DR spectroscopy [21], that is used to extract full polarization properties (i.e., depolarization, diattenuation, and retardation et.al.) [22,23] and that is used with machine learning for detection of skin complications caused by diabetes [24], show promise for enhancing the performance of standard DR spectroscopy (without PS detection). Routine application of DR spectroscopy requires only one-time calibration to compensate for lamp intensity fluctuations, wavelength-dependent instrument response, inter-device variations, and fiber bending losses. However, clinical DR spectroscopy utilizes optical fiber that will be unavoidably and frequently twisted by the clinicians, resulting in changing DR system-response and introducing DR spectra bias. To tackle this challenge, Bing Yu et. al. [25–28] innovates a ‘self-calibrating’ DR probe capable of measuring and calibrating the DR spectroscopy system-response in real-time, resulting in better consistency among DR spectra measured across variant DR spectroscopy systems. In addition, the reported PS-DR probe is inherently capable of generating system-response independent spectroscopic ‘depolarization ratio’ [20]. Nevertheless, the two raw orthogonal polarized DR spectra are not system independent. To maximize the diagnostic advantages of PS-DR spectroscopy [23], system-response independent PS-DR spectra carrying more comprehensive diagnostic information are highly needed. To fill the need, we report, in this work, on the development of a unique real-time calibrating PS-DR (rcPS-DR) handheld probe. With an embedded calibrating polytetrafluoroethylene (PTFE) optical diffuser, the PS-DR spectra bias arising from instrument response and time-dependent intensity fluctuation is removed through real-time measurement of the PS-DR system-response function. The diagnostic advantages of rcPS-DR probe are confirmed through its enhanced differentiation of the three clinically relevant anatomical locations (i.e., alveolar process, lateral tongue and floor of mouth that account for 80% of all cases of oral squamous cell carcinoma [29]) of the oral cavity.

2. Material and methods

2.1. Real-time calibrating polarization-sensitive diffuse reflectance spectroscopy (rcPS-DR) system

Figure 1 shows the schematic of the rcPS-DR system developed for tissue measurements. The rcPS-DR system consists of an LED source (Solis-3C, Thorlabs, NJ, USA) for illumination, customized spectrometer array for PS-DR spectra measurements, and a unique rcPS-DR handheld probe. The three spectrometers (SunShine, CNILaser, ChangChun, China) of the customized
spectrometer array are of the same specs, resulting in minimum inter-spectrometer variation and resultant interferences on the PS-DR spectra measured. Further, the spectra acquisition of the three spectrometers were synchronized by an external trigger box (EX-TA-Box, CNILaser, ChangChun, China). The rcPS-DR probe features five multimode fibers (FG200LEA, Thorlabs, NJ, USA) for light delivery. Three of the fibers (Exc. Fiber, Det. Fiber (∥), and Det. Fiber (⊥), as shown in Fig. 1) consisted of PS-DR spectra acquisition channel and the other two fibers (Calib. Exc. Fiber, Calib. Det. Fiber, as shown in Fig. 1) calibration channel used for real-time PS-DR spectra calibration. Along the PS-DR spectra acquisition channel, there exists paired and custom cut polarizing films (Polarizer (∥), and Polarizer (⊥), as shown in Fig. 1. #86-178, EdmundOptics, NJ, USA) with their fast axes positioned orthogonal to each other, enabling polarization-sensitive DR excitation and collection. The excitation-collection fiber spacing of both channels were the same and kept as 1 mm, resulting in a PS-DR spectra interrogation diameter of 0.5 mm and a predicted (by Monte Carlo simulations [30]) interrogation depth ∼ 0.4-0.7 mm, as consistent with previous reports [17]. To enable real-time calibrated PS-DR spectra measurements, a 10 mm thick PTFE diffuser (Calib. PTFE, as shown in Fig. 1) was embedded in the calibration channel of the probe. The thickness (∼10 mm) of the diffuser was the same as the DR standard (WS-1 diffuse reflectance standard, Ocean Insight, FL, USA), enabling PS-DR system response approaching to that of the DR standard (as will be shown below). One notes that the polarization films incorporated are custom cut into 1 mm by 1 mm, and the relative positioning of the probe components (i.e., beam delivery fibers, polarization films, and PTFE diffuser, etc.) were ensured through a 3D-printed component (Boston Micro Fabrication, Shenzhen, China). The overall probe tip diameter is 8 mm.

To acquire the PS-DR spectra, light output from the LED is first coupled into the excitation fibers (Exc. Fiber, Calib. Exc. Fiber, as shown in Fig. 1) of both the acquisition and the calibration channel. On the one hand, the light input to the acquisition channel is further linearly-polarized (by Polarizer (∥), as shown in Fig. 1) and shined onto the sample. Part of the backscattered DR spectra from the sample passed through the same polarizer enabling polarized illumination, whilst the remaining backscattered DR spectra passed through the other orthogonally-polarized polarizer. All backscattered PS-DR spectra were sent to the spectrometer array through the detection fibers in the acquisition channel. On the other hand, the light input to the calibration channel was backscattered by the embedded PTFE diffuser and passed to the spectrometer array. One notes that since the PS-DR system response function is measured through the calibration channel of the rcPS-DR probe, in real-time and in synergy with the PS-DR spectra, the PS-DR spectra bias arising from instrument response, time-dependent LED intensity fluctuation and fiber bending can be removed (as will be shown below).

To quantify the polarization property of the sample under investigation, the spectroscopic degree of linear polarization (DLP) was further extracted from the two orthogonal-polarized and real-time calibrated PS-DR spectra as below [31]:

\[ DLP = \frac{I_{\parallel}(\lambda) - I_{\perp}(\lambda)}{I_{\parallel}(\lambda) + I_{\perp}(\lambda)} \]  

where \( I_{\parallel}(\lambda) \) and \( I_{\perp}(\lambda) \) are the back-scattered PS-DR spectra with polarization parallel and perpendicular to the excitation light, respectively, and \( \lambda \) represents the light wavelength.

2.2. Statistical analysis

The unpaired two-sided Student’s t-test was used to evaluate the rcPS-DR spectra differences among alveolar process, lateral tongue and floor of mouth [32]. A criterion of \( P \) value less than 0.05 was used to consider differences as statistically significant. Partial least squares (PLS) discriminant analysis (DA) was applied on the rcPS-DR spectra to classify the different sites of the oral cavity [33]. Leave-one-out, cross-validation (LOOCV) was further used to assess
Fig. 1. (a) Schematic of the rcPS-DR system developed for tissue measurements. (b) distal tip of the probe. Light emission diode (LED); spectrometer (Spec); excitation Fiber (Exc. Fiber); detection fiber (Det. Fiber); excitation fiber in the calibration channel (Calib. Exc. Fiber); detection fiber in the calibration channel (Calib. Det. Fiber); polytetrafluoroethylene for calibration (Calib. PTFE).

and optimize the PLS-DA model complexity, while reducing the risk of over-fitting [33]. The adopted cross validation strategy was leave one tissue site out [33]. One notes that one-way analysis of variance (ANOVA) with a Fisher post hoc least significant difference (LSD) test was used to evaluate which wavelength range of the PS-DR spectra contributes most to the PLS-DA
analysis before PLS-DA model development [34]. The above multivariate statistical analysis was performed using both in-house written scripts and open-source PLS-DA tool [33] in the Matlab programming environment (Mathworks. Inc., Natick, MA).

2.3. Subjects

A total of 14 normal healthy subjects (10 females and 4 males, median age of 26) were recruited for in vivo rcPS-DR spectra measurements from the oral cavity. Informed consent forms were obtained from all participating subjects. Exclusion criteria included smokers, regular alcohol consumers and subjects suffering from systemic or oral mucosal diseases. Before in vivo rcPS-DR spectra measurements, all subjects underwent extensive mouthwash to reduce confounding factors (e.g., food debris, microbial coatings). A total of 6 anatomic locations (i.e., left and right sides of alveolar process: left and right sides of lateral tongue; left and right sides of the floor of mouth, as illustrated in https://visualsonline.cancer.gov/details.cfm?imageid=9259) were predefined for rcPS-DR spectra measurements; and a total of 554 in vivo rcPS-DR spectra (alveolar process, \( n = 226 \), lateral tongue, \( n = 150 \) and floor of mouth, \( n = 178 \), as summarized in Table 1) were acquired from different oral tissue sites (alveolar process, \( n = 84 \), lateral tongue, \( n = 70 \) and floor of mouth, \( n = 84 \)) of the recruited subjects.

| Table 1. The detailed tissue types break down and sample distribution. AP: alveolar process. FM: floor of mouth; LT: lateral tongue. |
|-----------------|------|------|------|
|                 | Left | Right| Overall |
| AP              | 112  | 114  | 226    |
| LT              | 78   | 72   | 150    |
| FM              | 88   | 90   | 178    |

3. Results and discussion

3.1. Real-time calibrating capability evaluation of the rcPS-DR probe

To evaluate the real-time calibrating capability of the rcPS-DR probe, the system response functions (SRFs) determined by the rcPS-DR embedded diffuser and the DR standard (WS-1 diffuse reflectance standard, Ocean Insight, FL, USA) were measured and compared (Fig. 2(a)). As shown in Fig. 2(a), both SRFs were consistent and close to each other, suggesting that the rcPS-DR probe is capable of measuring the PS-DR SRF accurately. Further, we mimicked the fiber bending that is usually encountered and unavoidable during the clinical applications of the fiber-optic DR spectroscopy system. Using mirror (PF10-03-G01, Thorlabs, NJ, USA) positioned 20 \( \mu \)m away from the rcPS-DR probe as sample, different fiber bending radius were tested (5, 10, 15, 20 mm, Fig. 2(b)) with corresponding SRFs measured, demonstrating the different SRFs that would otherwise be neglected by the generally adopted one-time calibration strategy. Figure 2(c) shows the calibrated and therefore consistent PS-DR spectra in the detection channel, suggesting the real-time calibrating capability of the rcPS-DR probe developed. We also calculated the DLP (degree of linear polarization) when using the mirror (PF10-03-G01, Thorlabs, NJ, USA) as sample (Fig. 2(d)). We found the DLP measured deviates from but close to 1, validating the polarization detection capability of the rcPS-DR probe. One notes that the deviation is likely caused by the imperfection of the linear polarizer films used (Polarizer (∥), Polarizer (⊥), as shown in Fig. 1).

In a separate experiment, we further investigate how the rcPS-DR probe functions for real-time calibration of the PS-DR SRFs. The intensity variations of the LED source were simulated.
through gradual reduction of the LED intensity through driving current adjustment using the LED driver (DC20, Thorlabs, NJ, USA). The raw PS-DR spectra in both polarization detection channels were measured (Figs. 3 a-b) in concurrence with the SRF of the calibration channel. While the raw PS-DR spectra were found with intensity variations arising from the LED intensity variations, the calibrated PS-DR spectra were shown (Figs. 3(c-d)) with intensity variation less than ±5%, confirming the real-time calibrating capability of the rcPS-DR probe developed. The remaining ±5% variation could be attributed to the variant coupling between the LED source and the rcPS-DR probe when tuning the LED intensity. The DLP measured was calculated to be close to 1 (Fig. 3(e)), revalidating the polarization detection capability of the rcPS-DR probe.

### 3.2. Polarization functionality validation of the rcPS-DR probe

Whilst the results in Figs. 2–3 confirm the real-time calibrating performance and validate the polarization detection capability of the rcPS-DR probe, further experiment was conducted to determine the polarization functionality of the rcPS-DR probe. External polarized light was launched into the rcPS-DR probe. As shown in Fig. 4(a), light delivered from an external multimode fiber was firstly collimated, linear polarized, and then was incident onto the rcPS-DR probe after passing through a half waveplate (HWP, AHWP20-VIS, LBTEK, Shenzhen, China). The HWP was rotated from 0 to 360 degrees, changing the polarization by 360 degrees. According to Malus Law, the intensities of HWP-tuned polarized light \( I_\parallel(\lambda) \) that passes through the acquisition channel of the rcPS-DR probe \( (I_\parallel(\lambda) \text{ and } I_\perp(\lambda)) \) vary as the square of the cosine of the angle between the HWP fast axis and those of both polarizer films \( (\phi \text{ and } \phi + \pi/2) \) within rcPS-DR probe, i.e., \( I_\parallel(\lambda) = I_0(\lambda) \cos(\phi)^2 \) and \( I_\perp(\lambda) = I_0(\lambda) \cos(\phi + \pi/2)^2 \). Besides, the DLP changes were calculated to be: \( \cos(\phi)^2 - \sin(\phi)^2 \). The experimentally collected PS-DR spectra in both polarization acquisition channels (Figs. 4 b-d) and the relevant DLP (Fig. 4(e)) versus the HWP rotation angles were also shown, which were consistent with the predicted intensities by Malus Law. The results in Fig. 4 confirm the polarization detection capability of the rcPS-DR probe developed.
3.3. Clinical performance evaluation of the rcPS-DR probe

In light of the above-confirmed real-time calibration (Figs. 2–3) and polarization detection (Fig. 4) capabilities of the rcPS-DR probe, we further sought to investigate the potential benefits of the rcPS-DR probe for real clinical applications. Figure 5(a) shows the real-time calibrated PS-DR spectra (mean ± SE, standard error) of alveolar process, lateral tongue and floor of mouth. The measured PS-DR spectra were consistent with previous studies [15–17], showing clearly identified dips around 540 nm and 575 nm that could be attributed to oxygenated hemoglobin absorption. Further look into the PS-DR spectra reveals subtle width and amplitude differences of the dips among the three clinically relevant sites. Quantitatively, the intensity ratios of the two dips were also shown (Fig. 5(b)), demonstrating significant ($P < 0.05$, unpaired 2-sided Student’s t-test) differences. In addition, the resulting DLP (Fig. 5(c)) differs among the three sites. We also found that the DLP (Fig. 5(c)) does not show a change corresponding to the dips of oxygenated hemoglobin absorption as in Fig. 5(a). This observation is consistent with previous reports [20], and warrants further investigations. The PS-DR spectra differences observed (Fig. 5) are likely caused by the significant structural differences among the alveolar process, lateral tongue and floor of mouth as revealed by our previous OCT study [35]. For instance, the alveolar processes investigated consists of $\sim 200 \, \mu m$ thick gingival layer on top of the underlying bone. The floor of mouth is comprised of clearly identified $\sim 240 \, \mu m$ thick non-keratinized epithelium above the lamina propria rich in collagen fibers, resulting in a significantly higher DLP (Fig. 5(c)) compared with the other two anatomical locations. Unlike the alveolar processes and floor of
mouth, the lateral tongue is lacking of layering structure. However, how the structural differences cause the PS-DR spectra differences observed warrant further investigations. We are currently developing co-registered PS-DR spectroscopy and OCT imaging systems (i.e., PS-OCT and OCT angiography) to correlate and explain the PS-DR spectra findings of the current work.

To elucidate the diagnostically important PS-DR wavelength range, Fig. 6(a) shows a logarithmic plot of the calculated $P$-values (ANOVA Fisher post hoc LSD test at the 0.05 level.) for each of the PS-DR spectra intensities in the entire wavelength range. We find the PS-DR spectra over the entire wavelength range shows statistically different differences ($P < 1 \times 10^{-4}$), PLS-DA and LOOCV were therefore implemented on the entire PS-DR spectra measured (Fig. 5(a)), allowing quantitative evaluation of the inter-anatomical PS-DR spectra differences of the oral cavity. Fig. 6(b) shows the 2-dimensional ternary plot of the posterior probabilities of each PS-DR prediction using PLS-DA-LOSCV. The prediction results are also summarized in Table 2. Figure 6 and Table 2 elucidates that the 3 tissue sites can generally be well separated with varying sensitivities (alveolar process: 80.5%, floor of mouth: 75.8%, lateral tongue: 78%), and specificities (alveolar process: 93.5%, floor of mouth: 87.7%, lateral tongue: 86.8%) (Table 2) by rcPS-DR, which are superior than those standard DR without PS detection (sensitivities of
Fig. 5. (a) Real-time calibrated PS-DR spectra (mean ± SE) measured from alveolar process, floor of mouth, and lateral tongues. (b). Box plot of the intensity ratios (mean ± SE) between $I_{540}$ and $I_{575}$ owing to oxygenated hemoglobin absorption. ◦ Par and ◇ Perp correspond to the parallel- and perpendicular- polarization detection channels of the rcPS-DR probe. * (c) DLP (mean ± SE, standard error) of the alveolar process, floor of mouth, and lateral tongues. * $P<0.05$.

63.2%, 41.5%, 81.3% and specificities of 92.9%, 87.7%, 63.8%). One notes that four lateral variables were used for the PLS-DA model with PS-DR spectra, while the number is three for that with DR spectra. The enhanced separation capability can be explained by the real-time calibration (Figs. 2–3) and polarization-sensitive detection (Fig. 4) capability of rcPS-DR probe developed. Overall, the results of this study indicate the potential of the rcPS-DR probe as a tool for studying oral cavity lesions in real clinical applications, and the PS-DR spectra variations of different oral tissue sites should be taken into account in algorithm development for accurate tissue diagnosis and characterization in the oral cavity by using rcPS-DR probe. We are currently seeking collaborations with clinicians to assess how the rcPS-DR probe developed could aid to differentiate oral malignant lesions from normal tissue and determine oral cavity tumor margins for surgical operations.

Fig. 6. (a) ANOVA of the three tissue categories over the entire PS-DR spectra wavelength. (b) posterior probabilities of 554 statistically different PS-DR spectra measured by the rcPS-DR handheld probe belonging to alveolar process (n = 226), floor of mouth (n = 178), and lateral tongue (n = 150).

Some limitations of the current work should be pointed out. First, the DR spectroscopy is in general sensitive to the pressure applied, and stepwise changes could occur in tissue DR spectra
Table 2. Confusion matrix detailing the multiclass classification results of PS-DR spectra of different oral tissues using PLS-DA and LOSCV. AP: alveolar process. FM: floor of mouth; LT: lateral tongue.

| PREDICTED | AP | FM | LT |
|-----------|----|----|----|
| TRUE      | 182| 24 | 20 |
|           | 10 | 135| 33 |
|           | 11 | 22 | 117|

Sensitivity (%) 80.5 75.8 78
Specificity (%) 93.5 87.7 86.8
Accuracy (%) 88.2 83.9 84.4

even at subtle pressure [36]. When in use, the rcPS-DR probe developed is in gentle contact with the tissue sites measured, minimizing the pressure-introduced PS-DR spectra variation. However, for real clinical use of the rcPS-DR probe, further integration of a pressure sensor at the rcPS-DR probe tip is needed [27], allowing real-time pressure monitoring and assuring consistent pressure applied. Second, the current rcPS-DR probe is lacking depth-resolved DR spectra interrogation capability as required for epithelial precancer detection [37]. Pressure-sensitive depth-resolved rcPS-DR probe through integration of pressure sensor and focusing optics (i.e., ball lens, or GRIN lens) at the current rcPS-DR probe tip is under development within our lab.

4. Conclusion

In summary, we have developed a unique rcPS-DR probe. Significant different oxygenated hemoglobin contents were observed among different anatomic locations of the oral cavity (i.e., alveolar process, lateral tongue and the floor of mouth). Synergizing the complementary information of the two real-time calibrated orthogonal-polarized PS-DR spectra, the rcPS-DR probe is found to better differentiate alveolar process, lateral tongue, and the floor of mouth. This work demonstrates the potential of using rcPS-DR probe as a clinically useful tool for enhancing real-time in vivo detection and diagnosis of oral disease in the oral cavity.

Funding. Beijing Institute of Technology Research Fund Program for Young Scholars.

Disclosures. The authors declare no conflicts of interest.

Data availability. Data underlying the results presented in this paper are not publicly available at this time but may be obtained from the authors upon reasonable request.

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