Depth sensitive Raman spectroscopy for skin wounds in rodents

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ABSTRACT

Raman spectroscopy has demonstrated its great potential in skin wound assessment. Given that biochemical changes in skin wound healing is a layer dependent process, depth sensitive Raman spectroscopy could enhance the power of Raman spectroscopy in this application. Considering the critical importance of rodent studies in the field of skin wound assessment, it is necessary to develop and validate a system that can perform depth sensitive measurements in rat skin with a proper target depth range. In this manuscript, we report the design, optimization and evaluation of a new snapshot depth-sensitive Raman instrument for rat skin measurements. The optical design and optimization process are presented first. The depth sensitive measurement performance is characterized on *ex vivo* rat skin samples with wounds. Raman signal emitted by the *ex vivo* rat skin from different target depths were simultaneously acquired. The feasibility of using the measured Raman spectra to differentiate between the wound edge and healthy skin was validated using PLS-LDA with leave-one-out. The accuracy of the classification improves monotonically as more data from new depths are used, which implies that each depth offers additional information useful for classification. This instrument demonstrates the ability to perform snapshot depth sensitive Raman measurements from rat skin, which paves the way towards *in vivo* preclinical studies of rat skin wounds.

Keywords: Raman spectroscopy, depth sensitive, instrumentation, skin, wound, axicon, snapshot, medical optics

1. INTRODUCTION

Highly sensitive, label-free and non-invasive, Raman spectroscopy is an optical technique exceedingly well-suited for *in vivo* measurements of biological tissue. Laser illumination of the biological tissue causes excitation of a multitude of molecular bonds and subsequent inelastic scattering of light. This Raman scattering provides a unique spectral signature based on the biochemical bonds that can be used as a diagnostic tool. For circumstances in which the tissue is layered, such as skin or carcinoma, a depth-sensitive approach to Raman spectroscopy is often more appropriate. Spatially offset Raman spectroscopy (SORS) is one such approach[1]. In SORS, the illumination fiber probe and collection fiber probe are some distance apart, thus Raman scattering from the deeper depths enters the collection fiber. Keller *et al.* took depth sensitive measurements from artificial human tissue and demonstrated the potential of SORS for breast cancer diagnosis[2]. However, while SORS has excellent surface noise removal, it has poor depth resolution as the illumination light is not focused and the collection efficiency is poor. We developed an axicon lens based non-contact Raman spectroscopy instrument with improved depth resolution[3]. By focusing an annular beam onto the sample, a focal line from the extension of the psf is created[4]. Scattered Raman light along the focal line is collected and transformed by an axicon lens into a ring. A ring-to-linear fiber bundle collects this Raman light and projects it into the spectrograph. We have previously demonstrated this instrument on *ex vivo* porcine tissues and *in vivo* human thumbnail[5]. Our intent is to eventually begin more involved *in vivo* testing on humans. Since rodent studies are an important stepping stone towards *in vivo* testing on humans, we further develop and test our instrument specifically for use evaluating rat skin wounds.

The epidermis of rat skin ranges from 76 to 167 μm thick[6]. The previous setup used a focal line of over 2000 μm for human and porcine skin testing, but this is not appropriate for rat skin as the depth resolution would be too poor to distinguish the thinner layers of rat skin. Instead, we chose a focal line 181 μm in length that could capture changes in

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the epidermis and dermis of rat skin. During wound healing, the changes occurring are often layer-dependent. For example, there is re-epithelization in the epidermis and angiogenesis in the dermis[7]. Here, we will present our system capable of capturing these depth-dependent biochemical fingerprints in a single snapshot and present furthermore, results distinguishing the rat skin wound edge from healthy skin.

2. METHODS

2.1 Optical Setup

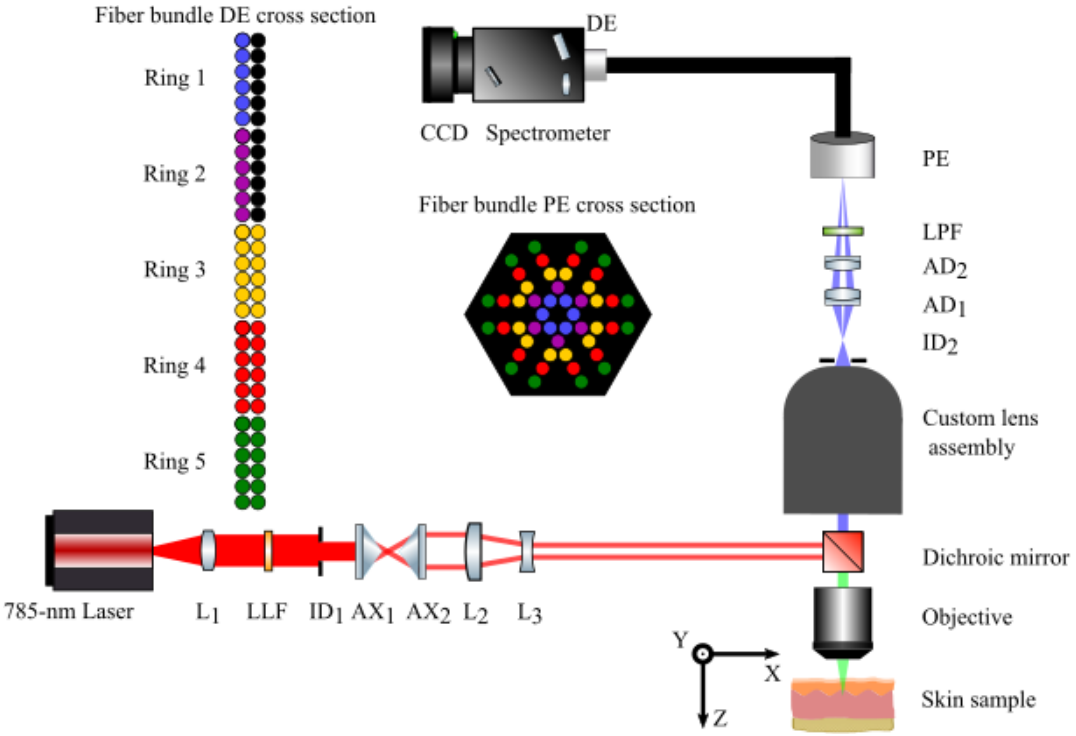

Figure 1. Schematic of the depth sensitive Raman spectrometer for rat measurements. L1: collimation lens; LLF: laser line filter; ID1, ID2: iris diaphragm; AX1, AX2: axicon; L2, L3: beam shrinker; AD2: achromatic doublet; LPF: long pass filter; PE: proximal end of fiber bundle; DE: distal end of fiber bundle; Ring 1-5: DE linear fiber bundles corresponding to PE rings.

Axicon lenses, AX_1 and AX_2, transformed the 785-nm laser beam into a ring-shaped beam. The beam was reflected into the objective causing the interference pattern of the ring of light to form a focal line from the axial extension of the point-spread function. The focal line stimulated the emission of Raman photons along the depth of the sample. A custom lens assembly mapped the Raman signals acquired from different depths onto the corresponding rings of the PE. Five spectra were collected by the spectrograph from each ring of the DE.

2.2 *Ex vivo* rat skin wound model

Biopsy punches with a diameter of 6 mm were used to create full-thickness excisional wounds on the back of each male Sprague Dawley rat. The wounds were harvested 24 hours later and immersed in 4% paraformaldehyde overnight prior to Raman measurement.

2.3 Data processing and PLS analysis

Spectral processing steps occurred as follows: cosmic ray removal, summation of frames, system background subtraction, wavelength calibration, system spectral response correction, fluorescence estimation and separation, and Savitsky-Golay smoothing filter. Partial least square (PLS) analysis and linear discriminant analysis (LDA) were used to create classifiers for spectral analysis. Leave-one-out validation across both PLS and LDA was used to quantify the accuracy, sensitivity and specificity of the classifier.

3. RESULTS

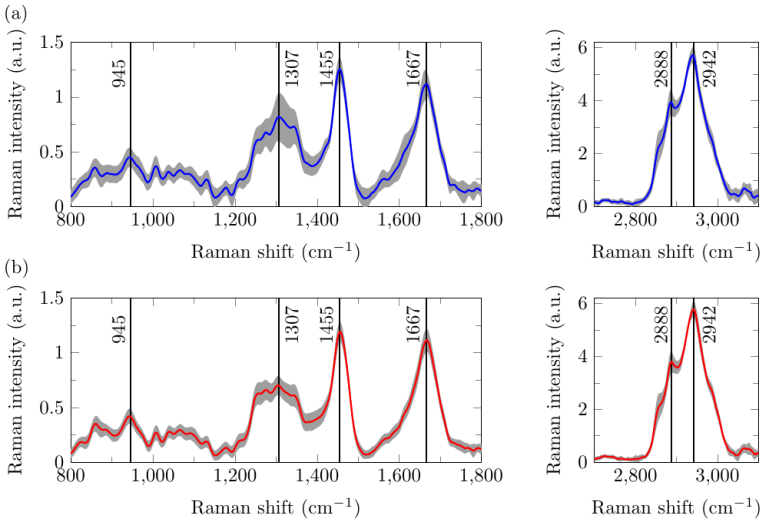

Figure 2. Raman spectra from rat skin (a) proximal and (b) distal to the wound site acquired by Ring 1. Averaged Raman spectra is shown in the blue and red colored lines while the variation in each spectrum is shown in grey, in which the upper and lower bound of each point in the shaded region indicates mean ± standard deviation. Black lines with text highlight specific Raman shifts (cm^{-1}).

Representative Raman spectra taken from the rat skin region distal to the wound site (DWS, >5 mm) and those from the rat skin region proximal to the wound site (PWS, <1 mm from the scab and wound edge) are shown in Figure 2. Rat skin has peaks at 945 cm-1 (C-C stretching of protein backbone), 1307 cm-1 (CH2 stretching), 1455 cm-1 (CH2), 1667 cm-1 (Amide I), 2888 cm-1 and 2942 cm-1 (asymmetrical CH2 and symmetrical CH3 stretching respectively). By visual inspection, skin PWS has a peak at 1307 cm^{-1} slightly higher than skin DWS, although it is inconclusive as the standard deviation for the spectra at that wavenumber is high. This indirectly suggests the thickening of the epidermis PWS as the epidermis has a stronger peak at 1307 cm^{-1} compared to the dermis[8].

A PLS-LDA classifier using the leave-one-out validation method was evaluated in terms of accuracy, sensitivity and specificity. The average accuracy in distinguishing skin PWS from skin DWS improved with increasing number of rings used, from 78.3% for one ring to 86.5% for all five rings. The sensitivity showed a 15% improvement from 76.7% using just one ring to 91.7% when all five rings were included. The specificity remained steady however, at around 80%.

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