Non-invasive Raman spectroscopy of human tissue in vivo

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Introduction

One of the most important and at the same time elusive goals of analytical sciences in biomedical research is the provision of a safe non-invasive method for monitoring the chemical composition of deep layers in living tissue or other turbid media. Such information is crucial, for example, in disease diagnosis. At present, available techniques that possess the required degree of chemical specificity have low penetration through tissue. The most attractive techniques are infrared and Raman spectroscopy that provide superb chemical information but these are restricted to interrogating only several hundreds of micrometers below the surface. Thus many tissue components, such as veins, bones and subsurface cancerous tissue are inaccessible without invasive methods. Naturally, there is a great motivation to extend the penetration depth of this technique and perform analysis non-invasively.

Recently our collaborative team has proposed a new approach, Spatially Offset Raman Spectroscopy (SORS)[1] to obtain spectral information from subsurface layers. The SORS approach utilises safe, low power continuous wave laser beams compatible with in vivo probing of tissue and is based on collecting a set of Raman spectra from the surface regions of a sample that are at set distances, Δs, away from the point of illumination by the laser (see Figure 1). Raman spectra obtained in this way exhibit a variation in relative intensities between the contributions from the surface and sub-surface layers and this difference can be utilised to yield the pure Raman spectra of the individual sub-layers.

Coupling the SORS concept with fibre optic technology[1,2,3], as reported here, in combination with current high sensitivity CCD detectors gives a dramatic enhancement in sensitivity, by more than two orders of magnitude. The concept is based on arranging fibres on annuli of different radii, with each annulus being presented to different horizontal sections of a two-dimensional CCD detector. Recently, Morris et al.[4] demonstrated for the first time the feasibility of using a fibre optic probe with global illumination. This permits overall much higher powers to be applied to the sample although there are some limitations in terms of the degree of SORS suppression of the surface Raman and fluorescence signals
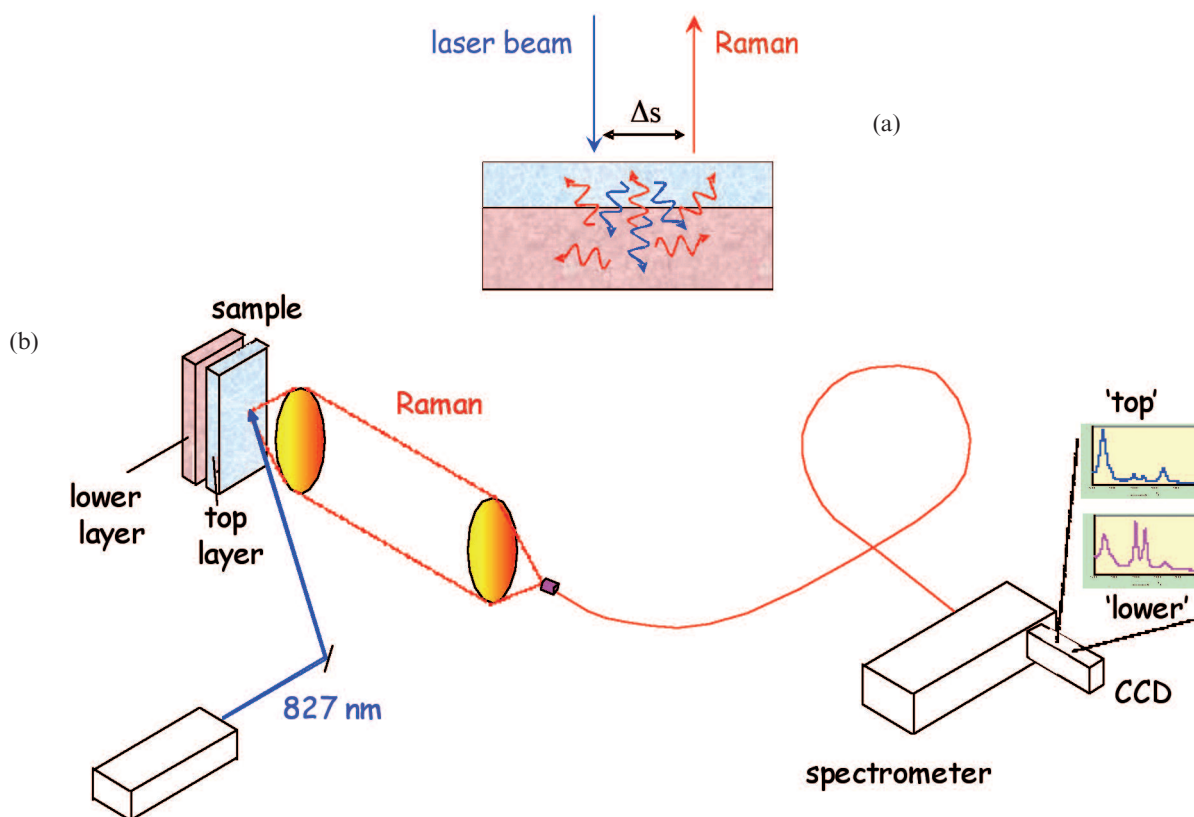

Figure 1. a) Principal of the SORS concept showing how photons migrate within tissue. b) Schematic diagram of the SORS experimental setup.

one can achieve [2]. Recently, this technique was also successfully used by Morris *et al.* [5] in non-invasive measurements of human bones in cadavers.

Our annular fibre probe design makes it possible to take the full advantage of the SORS effect as each of group of fibres exhibits a clean, well defined SORS spatial offset from the point of illumination. This enables sensitive probing of deep layers of tissue even at the low illumination powers required for safe non-invasive probing of human bones in vivo with laser light as we show for the first time. A full account of this work is given in reference [6].

Experimental

Raman probe light was generated using a temperature stabilised diode laser for Raman spectroscopy operating at 827 nm (Micro Laser Systems, Inc, L4 830S-115-TE). The laser beam was attenuated to yield 2 mW at the sample, well below the maximum permissible exposure as defined by the laser safety standard. The collimated beam was passed through a 1 mm diameter pinhole aperture placed about 10 cm before the sample to define precisely the beam diameter and provide a near-uniform illumination across the beam profile. The transmitted part of the beam was incident on the sample at ~45 degrees.

The Raman light was collected in backscattering geometry using a 50 mm diameter lens with a focal length of 60 mm. The scattered light was collimated and passed through a 50 mm diameter holographic notch filter (830 nm, Kaiser Optical Systems, Inc) to suppress the elastically scattered component of light. The second lens, identical to the first, was then used to image, with magnification 1:1, the sample interaction zone onto the front face of the annular fibre probe. The laser incident spot was imaged in such a way so that it coincided with the centre of the probe. Two more filters (25 mm diameter holographic notch filter, 830 nm, Kaiser Optical Systems, Inc, and an edge filter, 830 nm, Semrock) were used just before the fibre to suppress any residual elastically scattered light that passed through the first holographic filter. The Raman light was then propagated through the SORS annular fibre systems of

length ~1 m to the linear fibre end which was oriented vertically and placed in the input image plane of a Kaiser Optical Technologies Holospec f# = 1.4 NIR spectrograph with its slit removed. In this orientation the fibres themselves acted as the input slit of the spectrograph. The Raman spectra were collected using a deep depletion liquid nitrogen cooled CCD camera (Princeton Instruments, SPEC10 400BR LN Back-Illuminated Deep Depletion CCD, 1340 x 400 pixels) by binning the two regions vertically to produce two spectra, one corresponding to the zero spatial offset Raman scatter and the other to the 3 mm spatially offset Raman scatter. The Raman spectra presented here are not corrected for the variation of detection system sensitivity across the active spectral range.

The light collection end of the SORS fibre probe was constructed with 7 fibres placed at the centre of the probe and 26 fibres distributed around the ring of 3 mm radius (see Figure 2). At the fibre exit the two fibre bundle groups were organised into a linear array with one group placed above the other. The individual fibres were made of silica with a core diameter of 200 μm, cladding diameter of 230 μm and numerical aperture of 0.37. The bundle was custom made by C Technologies Inc.

Results and Discussion

The SORS device has been applied to the transcutaneous measurement of human bones. Figure 3 shows in vivo Raman spectra obtained from the thumb distal phalanx bone of a volunteer with acquisition time of 200 s. The spectra shown are raw SORS spectra with only the fluorescence background subtracted. It can be seen that the spectrum with the zero spatial offset has substantially larger noise due to a high fluorescence background masking the underlying Raman signal. The fluorescence originates predominantly from the melanin component of skin located at the very surface regions of the probe sample. This fluorescence level has decreased substantially relative to the subsurface signal by collecting Raman light through the 3 mm offset fibre annulus. Furthermore, when using the 3 mm spatial offset the Raman spectra show a substantial
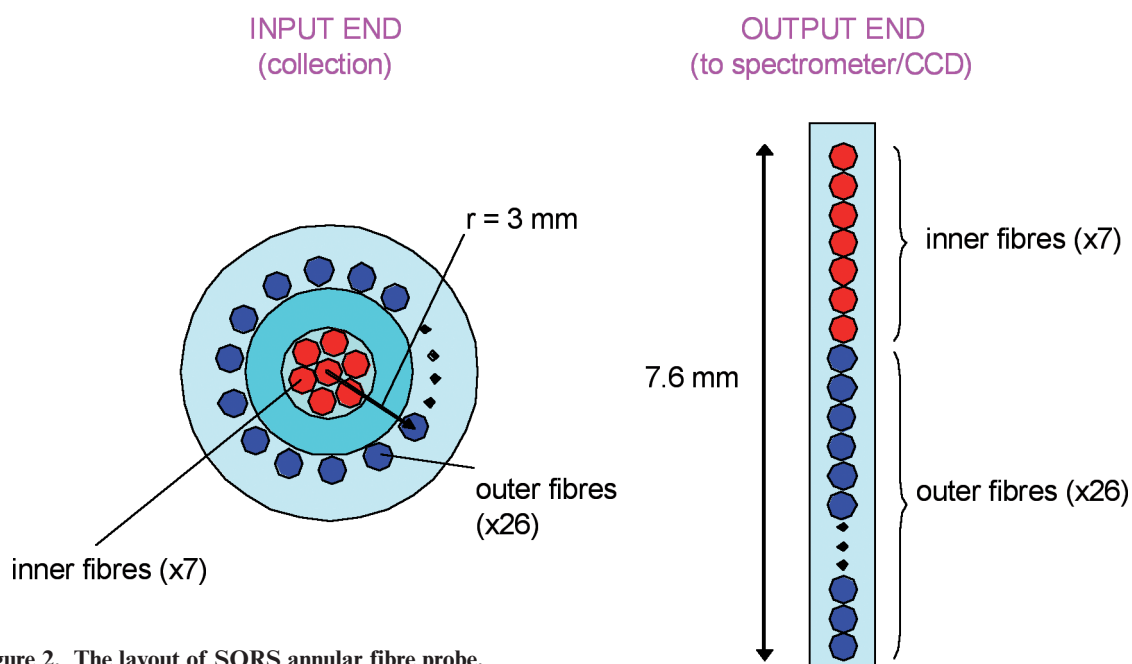

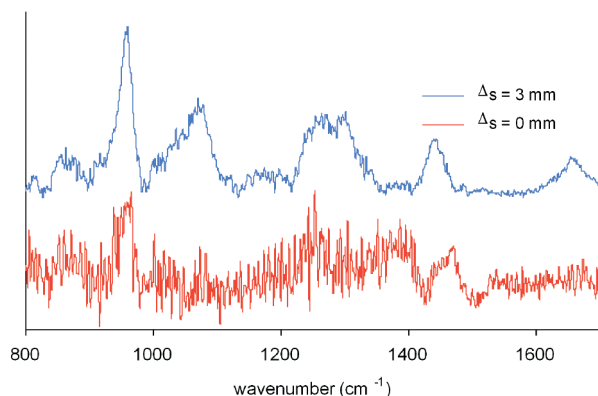Figure 2. **The layout of SORS annular fibre probe.**

Figure 3. The SORS spectra for the zero and 3 mm spatial offsets from the non-invasive transcutaneous measurement of human bone at the distal phalanx of the thumb. The spectra were obtained using a continuous wave laser operating at 827 nm with skin-safe laser power (2 mW). The spectra are offset for clarity.
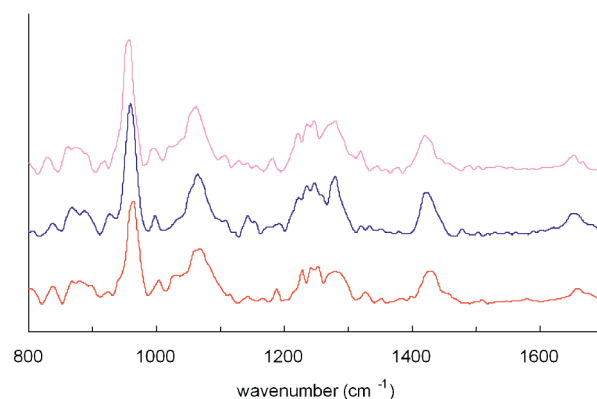

Figure 4. The estimates of pure Raman spectra of bones measured from the distal phalanx of the thumb from three different measurements decomposed from the raw SORS spectra exemplified in figure 3.

contribution from the underlying bone as evident from the phosphate and carbonate bands present in bone but absent in the overlaying tissue. [7] The bands greater than 1100 cm^{-1} are predominantly due to proteins (mainly collagen) and originate from both bone and the overlaying tissue. Even without mathematical processing the raw spectrum readily enables one to draw qualitative and quantitative conclusions about the bone mineral composition. For instance the phosphate band profile and its relative intensity to the carbonate band can be clearly monitored without any further decomposition of the layers contributing to this spectrum. This ability to measure relative differences in ratios of bone specific peaks can provide information on the material quality of the bone matrix and has implications for potential diagnosis of metabolic bone diseases. The experiments were performed on 5 human subjects. The measurements were taken from the proximal region of the distal phalanx of the thumb from depths of approximately 2 mm as estimated radiographically.

A pure bone Raman spectrum, without any surface (skin tissue etc.) signatures for the analysis of bone collagen quality can be obtained by performing a scaled subtraction of the lower (offset) spectra from the upper (non-offset) spectra. The result of such subtraction is shown in figure 4. This procedure effectively removes interference caused by the Raman signal generated from the collagen present in skin. The spectra were obtained by binning the pixels horizontally in groups of 5 and then scaled and subtracted utilising non-zero band constraints to reveal estimates of pure bone components. [7] The fluorescence backgrounds have been subtracted for clarity.

Conclusions
The new method paves the way for developing an array of safe screening techniques for disease diagnosis of tissue components in depths of several millimetres. Through further improvements of the collection and laser beam delivery techniques, we envisage the penetration depth to increase to 5 mm or more; limited only by the ability to detect sufficient photons.

This work demonstrates that the SORS annular fibre probe concept can be applied to obtain Raman spectra of bone transcutaneously. However, the applicability of this generic

instrument stretches well beyond this area. We foresee the development of SORS for the diagnosis of various diseases where molecular details within tissue can provide indicators of health problems, for example in the non-invasive diagnosis of cancer of subsurface tissue [8], monitoring tooth dentin for disease or periodontal disease below accessible areas. Other analytical applications include the probing of drugs through layers, packaging, biofilms or paints to monitor their adhesion to substrates and food products.

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