Surface-sensitive polarized Raman spectroscopy of biological tissue

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In a two-layer diffusing medium, polarized light directly backscattering off the superficial layer will partially retain its sense of polarization, whereas deeper-probing light will be increasingly depolarized by diffusion. This effect has been studied in both elastic scattering and fluorescence contexts. We apply this method to Raman scattering in two two-layer models with a highly diffusing lower layer of glucose powder and an upper layer of either clear plastic or chicken skin. We employ detection of orthogonal polarization states to generate a Raman spectrum of only the superficial layer by combining the orthogonal signals. © 2005 Optical Society of America

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Epithelial tissue has a layered structure. Skin, for example, consists of the stratum corneum, the epidermis, and the dermis. There are many biomedical applications, such as detecting superficial skin cancers, in which only the near-surface portion of the epithelium is of interest, with lower-lying layers providing unnecessary or potentially even confounding signals. Raman spectroscopy of such a layered medium will in general detect chemical signatures from multiple layers, if they are thin enough. If the goal is to distinguish between healthy and diseased tissue in the uppermost layer, the multilayer probing reduces the diagnostic potential of the measured Raman spectrum. One usually performs classification by feeding the spectrum into a multivariate regression algorithm (e.g., partial least squares), a neural network, or a discriminant analysis routine. In all cases it is the variability between spectra that is examined by the algorithms to produce maps of spectra to disease state or chemical concentration. For such processes to be robust in biological systems it is desirable to remove as many interfering substances as possible, such that the spectra fed to the prediction algorithms will vary primarily in ways that have predictive value.Removing subsurface signals from systems for which only the surface is of interest will help to reduce the interfering variations in spectral data, with an eye to improving the accuracy of using the data to predict a tissue’s state of health.

Single Raman scattering events are partially depolarizing, to an extent determined by the spherical asymmetry of the change in polarizability within the molecule involved in the interaction. Even when one considers an ensemble of randomly oriented molecular oscillators, the singly backscattered light produced by many polarized photons incident onto this ensemble will still retain some knowledge of its incident polarization state. The lower limit to the amount that such singly scattered light can be depolarized by such an ensemble was given by Wilson et al. as 25% polarized output for 100% polarized input. This applies to all Raman vibrational modes, although different modes even within the same molecule may depolarize light by different amounts. Therefore, an ensemble of photons, each experiencing a single Raman scattering event, will at least partially retain its incident sense of polarization, no matter what Raman mode of a molecule is probed.

Because skin is turbid, it will scatter and diffuse light traveling through it. Therefore, polarized light incident upon a biological sample will be quickly depolarized by elastic scattering as it diffuses deeper into the tissue. When a measurement in reflectance mode is made, only those photons that have traveled ballistic paths in and out of the tissue will fully retain their sense of polarization. Because the probability of a photon’s entering and leaving a diffusing medium with only one scattering event decreases exponentially as the photon probes deeper into the sample, the singly scattered photons will be generated overwhelmingly within the first few scattering lengths. We can isolate the singly scattered photons and thus obtain a surface-sensitive signal by utilizing polarized detection. To illustrate this procedure we define our detected signal:

\[ I_{\text{tot}} = I_{\text{SS}} + I_{\text{MS}}, \]

where \( I_{\text{SS}} \) is the singly scattered light and \( I_{\text{MS}} \) is the multiply scattered, or diffuse, light collected by the system. Here we note that \( I_{\text{SS}} \) and \( I_{\text{MS}} \) have each undergone a single Stokes-shifting event, with \( I_{\text{MS}} \) suffering additional elastic scattering events while \( I_{\text{SS}} \) experiences none. We make the approximation here that the light becomes completely depolarized after two scattering events, and thus \( I_{\text{MS}} \) is a polarization-independent quantity. If the illumination light is linearly polarized, we can define two polarization states in the system as those states that are parallel and perpendicular to the illumination’s polarization state. We can then employ polarized detection to isolate the singly scattered signal in the following fashion:

\[ I_{\perp} = \frac{1}{2} I_{\text{MS}} + (1/2 - \alpha/2) I_{\text{SS}}, \]

\[ I_{\parallel} = \frac{1}{2} I_{\text{MS}} + (1/2 + \alpha/2) I_{\text{SS}}, \]

where \( I_{\perp} \) and \( I_{\parallel} \) are intensities detected at a spectrograph–CCD array placed after an analyzer oriented either perpendicular or parallel to the laser.
polarization, $\alpha(\vec{v})$ is the degree of polarization preservation for a particular mode ($0.25 < \alpha < 1$; $\alpha = 1$ implies complete preservation), and $\vec{v}$ is the Stokes shift in wave numbers. It is clear from Eqs. (2) and (3) that we can generate the spectral line shape of either $I_{\text{tot}} = I_1 + I_\perp$ or the surface-sensitive, singly scattered signal $\alpha_{\text{SS}}I_{SS} = I_1 - I_\perp$.

This theory was employed previously in analogous problems in elastic scattering spectroscopy and fluorescence. In elastic scattering spectroscopy, polarized detection of singly scattered light has been used to isolate subtle wavelength-dependent Mie spectra created by cell nuclei in the outer lining of esophageal tissue, while the strong and wavelength-dependent contributions of lower layers are suppressed. Fluorescence spectroscopy was applied to skin tissues to isolate the fluorescence of collagen and coenzymes in human skin while eliminating signal variations caused by lower-lying blood vessels. Although Raman scattering typically generates weaker signals than the two previously employed methods, its sharp bands provide a chemical specificity that allows accurate and detailed characterizations of skin’s chemical constituents.

We expect fluorescence, elastic scattering, and Raman scattering from randomly oriented molecules to have different ratios of polarized to depolarized signals. In all cases, the degree of spherical symmetry of the scatterers plays a key role. Backman et al. reported that, in the limit of an optically thin sample (∼1 transport length) of polystyrene beads whose polarizability is spherically isotropic, $I_{SS}/I_{MS}$ can reach 100 or more. For Raman scattering we expect such strong retention of polarization only for groups of molecules whose change in polarizability exhibits high spherical symmetry. For most molecules the ratio of $I_{SS}/I_{MS}$ will range from 0.167 to 4.5, depending on the geometry of the molecule being excited. In addition to symmetry issues, fluorescence has a time delay between the absorption and the reemission of a photon. Therefore the excited molecule may tumble through Brownian motion and thus reorient its induced dipole axis, resulting in reduced polarization.

The experimental setup for polarized Raman detection was a standard confocal Raman microscope, described previously, with the addition of a polarizer after the laser bandpass filter, an adjustable analyzer placed behind the long-pass filter, and a $10\times$ rather than a $50\times$ objective lens at the microscope. In the current implementation of this setup, it is required to take two separate spectra at two different times to obtain both the parallel and the perpendicular spectra. However, with the addition of a polarizing beam splitter it would be possible to measure both spectra simultaneously, removing ambiguities that may arise from time-correlated system drift or quenching of sample autofluorescence.

Two separate two-layer model systems were considered. The first was a plastic cuvette filled with glucose and laid on its side. Powdered glucose was chosen for this experiment because of its extremely high diffusivity and strong, many-peaked Raman spectrum. In highly diffusing samples, such as powdered glucose, $I_{MS}$ becomes so large that the polarized component becomes lost in shot noise, and the two detected signals $I_1$ and $I_\perp$ are essentially equal. Both plastic and glucose are strong Raman scatterers with well-defined peaks and low amounts of fluorescence. Therefore this artificial system is ideal for obtaining high-quality spectra that can be used to clearly illustrate the surface-sensitive nature of the singly scattered signal. We gathered spectra by focusing the laser spot on the upper surface of the cuvette wall, with the glucose lying 1 mm beneath.

The second system considered was locally obtained chicken skin stretched over a bed of glucose such that the glucose and the chicken were in contact with no air gaps between them. Glucose was again chosen for its high scatter level, and chicken skin was chosen for its biological relevance in being a turbid tissue whose primary chemical constituents are similar to those of human skin. We gathered spectra by focusing the laser spot on the surface of the skin, which was approximately 500 μm thick. For each of the two systems, spectra of each of the individual layer components (skin, glucose, plastic) were gathered as well for comparison.

The results of the experiments performed on the two models can be seen from Figs. 1 and 2. In Fig. 1, spectrum A shows $I_{\text{tot}}$ as containing strong glucose bands (labeled with asterisks), whereas C, the polarized spectrum, looks identical to that of pure plastic. We emphasize the strong presence of glucose in the unpolarized spectrum despite the confocal geometry's being optimized for top-layer sensitivity. When polarized detection is added, the interference vanishes. Likewise we can see from Fig. 2 that spectrum B, the total spectrum, is a superposition of chicken skin and glucose signals, whereas the polarized signal, D, contains only peaks that pertain to the surface skin.

![Fig. 1.](Image)
layer. Note that, because of standard biological variability within the chicken skin, the polarized spectrum of the sample consisting of both skin and glucose differs slightly from that of the pure skin sample in its peak ratios (Fig. 2, spectra D and E). This result is due to the variations in densities of skin constituents and is not an artifact of glucose, as the discrepancies do not correspond to glucose peak positions.

As noted, these experiments were made in conjunction with confocal detection, which, of course, provides depth sectioning of its own. Even with confocal detection, however, a clear reduction in the multiply scattered background is seen in the data shown here. Schemes in which polarization effects provide the sole sectioning ability can be envisaged, analogous to the reflectance experiments cited above. An effect specific to Raman scattering is the strong variation in \( \alpha \) (the degree of polarization preservation) across the spectrum. It is possible to envisage situations in which the information in \( \alpha(\nu) \) is exploited to provide an additional dimension of contrast in discriminating between, for example, healthy and diseased tissues.

In conclusion, our study shows that polarization effects studied in elastic scattering and fluorescence can be harnessed in Raman spectroscopy as well, to provide a surface-sensitive Raman spectrum of, for example, a biological tissue layer. This modality could prove useful for investigation of epithelial tissues and other layered systems.

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References