Extracting Raman spectra from highly fluorescent samples with "Scissors" (SSRS, Shifted-Subtracted Raman Spectroscopy)

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Introduction

Raman spectroscopy is increasingly being used for non-destructive, qualitative and quantitative analysis of materials that range all the way from high-purity tableted pharmaceuticals through to tissue samples and recycled polymers. In principle, Raman measurements are easy to perform: an intense monochromatic light source (invariably a laser) irradiates a sample and the scattered light is collected. The Raman scattered photons are then separated from the more numerous but unwanted elastically-scattered photons and are then detected and displayed on a cm^{-1} scale. Since studying a sample is as simple as directing a laser onto the area of interest, little or no sample preparation is required, which can have significant time and cost advantages in industrial applications and/or make possible the non-destructive *in situ* characterisation of rare, fragile or inaccessible samples.[1]

The main challenges in Raman spectroscopy arise from the fact that only a tiny fraction of the photons incident on the sample are Raman scattered. For many years this meant that obtaining good quality Raman data demanded skill and expertise. This situation is now changing and several commercial manufacturers now produce reasonably compact and easy-to-use, integrated Raman instruments at affordable prices. These instruments, like many of the home-built systems currently in use, typically use the combination of compact and reliable (typically diode) laser sources, very efficient optical (typically holographic) filters to separate the intense elastically-scattered laser light from the weaker Raman signal and, finally, sensitive and robust CCD detectors. Although spectrometers of this type can now readily provide the high sensitivity demanded in Raman experiments they cannot circumvent the problem that any system that captures the Raman scattering efficiently will be equally sensitive to background fluorescence emanating from the sample.

Unfortunately, many industrial, archaeological and medical samples (and indeed unpurified materials of any type) fluoresce when irradiated by a focused visible laser. The absolute intensity of this fluorescence may not be very high but, because Raman spectrometers are designed to collect and detect weak optical signals, even what would normally be regarded as very weak fluorescence will give a strong signal. This in turn means that any Raman bands will appear as small, narrow peaks on a broad (normally featureless) high intensity background. Since it is usually not possible to remove the source of the fluorescence (either because it is an unknown impurity or because it is the compound of interest that is fluorescent) some other method to reduce the background is needed. Currently, the most widely used strategy is to use an excitation wavelength where the fluorescent molecules do not absorb (if there is no absorption then there is no fluorescence). Many commercial Raman instruments, for example, use far-red lasers; typically 1064 nm in Fourier transform Raman instruments or 785 nm for dispersive instruments (which keeps the Raman signal within the sensitivity range of normal CCDs). While the widespread adoption of red excitation has been a major step forward in fluorescence minimisation, it is not a universal solution to the problem because some samples fluoresce even when excited at 785 or even 1064 nm.

This short article outlines an alternative approach where, instead of trying to record signals without fluorescence, we live with the fluorescence and look for a way to cope with signals that have weak Raman peaks on a large background. A convenient illustration of the power of this approach comes from our work on ancient Chinese papers so it is useful to outline the nature of the problems associated with these specific samples before discussing the general solution that we have developed.

Ancient Chinese documents

The Stein Collection in the British Library contains the *Diamond Sutra* (frontispiece inset in Figure 1), the world's oldest, dated, printed document which is part of a large hoard of documents that were discovered in a cave in China and brought to London by Sir Marc Aurel Stein at the end of the 19th century. The paper of the *Diamond Sutra,* and other documents from the Stein collection, is believed to be dyed yellow by a natural extract from the bark of a small native tree, *Phellodendron amurense*, which contains two predominant yellow dye compounds: berberine and palmatine.[2] Conservation of these documents requires definitive information on the chemical composition of the dyes, so the analytical problem is to detect the compounds (believed to be berberine

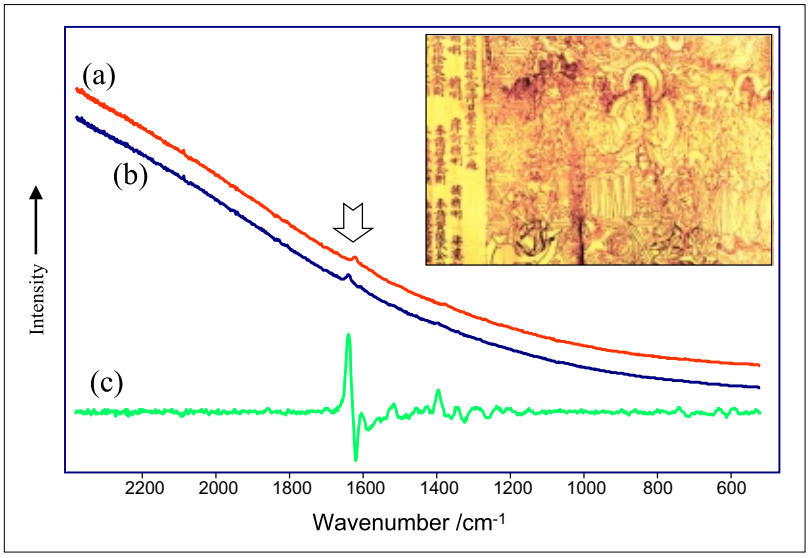

Figure 1. (a) and (b) the resonance Raman spectrum (λ_{ex} = 363.8 nm) of an ancient Chinese paper sample taken at two slightly shifted spectrometer positions (upper traces). The spectrum resulting from subtraction of the two shifted spectra is shown as the lower trace, (c). Insert is a photograph of the frontispiece of the *Diamond Sutra*.

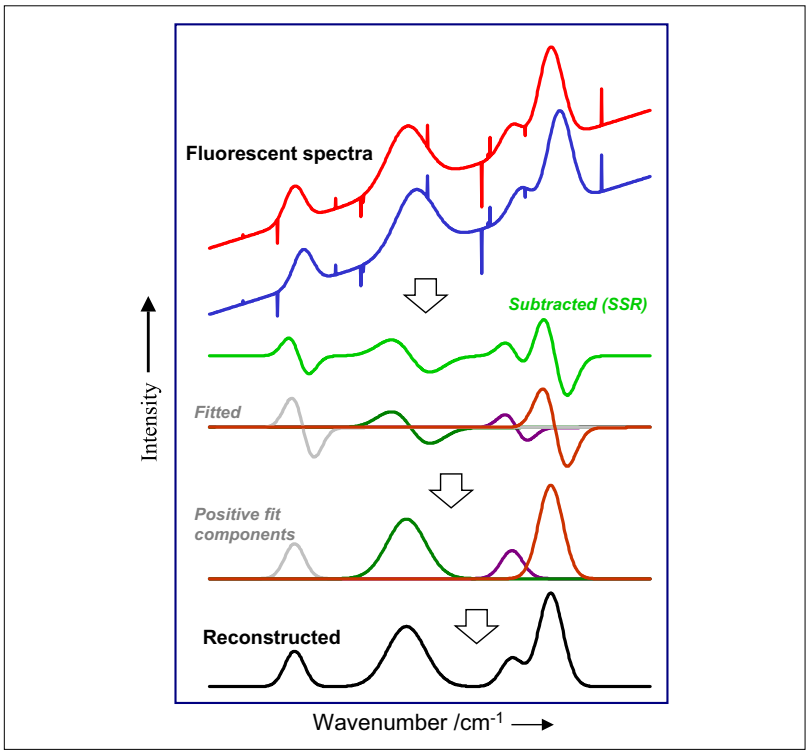

Figure 2. A cartoon illustration of how useful Raman data can be obtained from fluorescent samples by shifting, subtracting and reconstructing spectra. The top two spectra (red and blue) are recorded at two slightly shifted spectrometer positions. Note that the Raman bands move but the sharp features caused by defects in the CCD detector stay in the same position. Subtraction of these two spectra generates the shifted, subtracted Raman (SSR) spectrum (shown in green), which looks like a complex waveform but has no features due to the defects because they cancel in the subtraction. As an additional bonus, much of the fluorescence also cancels in the subtraction. The SSRS trace can be fitted by the four, double Lorentzian functions shown below the SSRS trace. Each of these is a Raman band and its negative echo. Turning off the negative echoes gives the bands that can be added to produce the final reconstructed spectrum, shown in black.

and/or palmatine) that give rise to the yellow colour of the paper but to do this non-destructively and with sufficiently high sensitivity to detect small amounts of organic dye in the presence of an overwhelming mass of paper. This is a considerable challenge and only made possible through the use of a simple variant of the normal Raman method: resonance Raman spectroscopy.

In resonance Raman experiments the excitation laser is tuned to a wavelength where the sample of interest has a strong absorption band. Under these conditions, the Raman scattering from the absorbing material (the chromophore) is increased over its normal value. This gives an increase in both the sensitivity and selectivity, since the Raman signal due to the chromophore will grow larger than the signal of the surrounding matrix, even if this matrix is present in much larger quantities. The classic use of this effect is in studies of enzymes, where the Raman spectrum of even relatively small, coloured prosthetic groups such as a porphyrins can be detected, even though they sit within a huge protein that itself is in an aqueous solvent.[1] In the ancient paper samples when a blue excitation laser wavelength is chosen it falls within the absorption region of the yellow compounds of interest so that the Raman spectrum due to the yellow dye should be enhanced and rise above the signal due to the surrounding paper matrix.

Unfortunately, blue laser excitation of the ancient paper samples or of model samples (which can be samples of pure dye, modern paper treated with this dye or even samples of the tree bark that is believed to be the dye's principal source) give signals which are completely dominated by fluorescence (see Figure 1). In the spectra, the largest Raman bands are just visible as tiny features on top of an apparently overwhelming fluorescence background and the weaker features are completely lost. It is not possible to reduce the fluorescence by moving the excitation wavelength away from the strong absorption bands of the dye because this would also remove the dye's resonance enhancement, so that the Raman signal will be completely dominated by the paper matrix.

Fluorescence and noise

At first glance, it might appear that fluorescence in Raman spectra is simply cosmetically undesirable and, if some form of fitting procedure was used, the broad featureless background

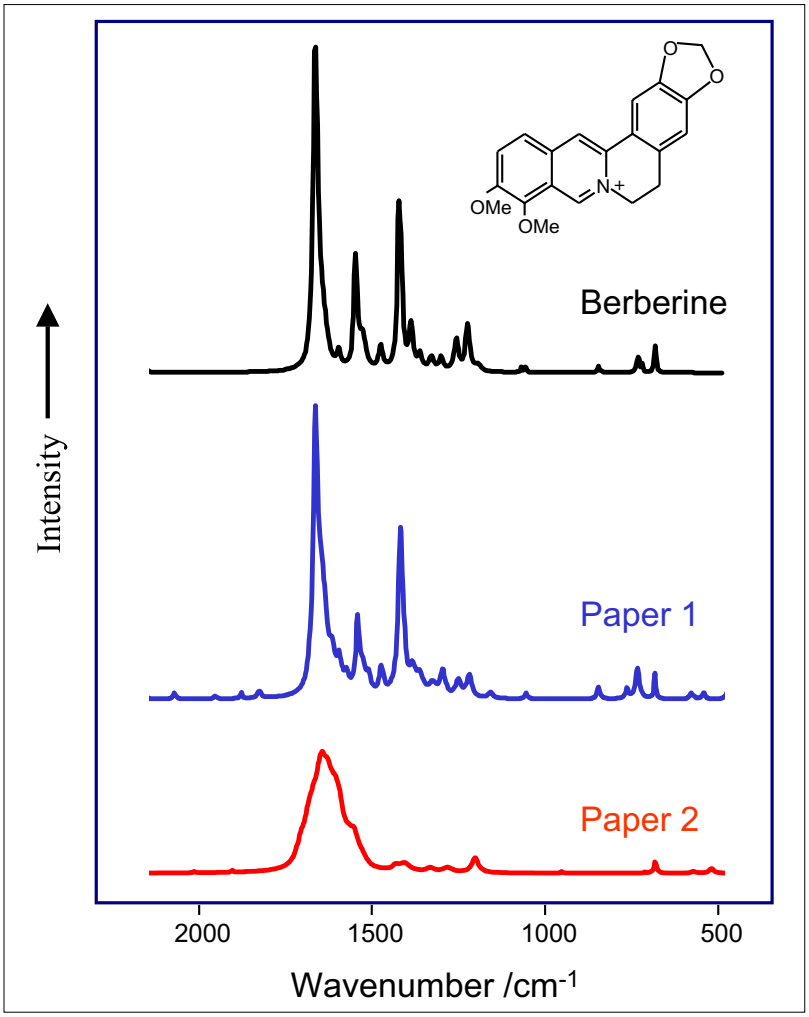

Figure 3. The "reconstructed" resonance Raman spectra of two different ancient Chinese paper samples and of a sample of one of the dyes believed to have been used on such paper. The match between the spectrum of paper sample 1 and that of berberine dye (structure shown on the Insert) is excellent. Paper sample 2, on the other hand, is clearly not dyed with berberine.

could then be subtracted away, leaving the sharper Raman bands behind. Of course it would be necessary to accumulate very large signals to reduce the random shot noise on the background but with modern spectrometers fitted with CCD detectors it is easy to accumulate the high signal levels needed. Unfortunately, CCDs typically display random (but stable) variations in the pixel-to-pixel sensitivity, which means that even a smooth fluorescence background signal will appear to have peaks and dips associated with the high- or low-response pixels. These irregularities can be larger than the actual Raman signal and of course subtracting away a smooth background function will not remove them. However, every spectrum recorded with a given detector under the same experimental conditions will have the same irregularity in response and we have recently implemented a very general method for correcting the irregularity which uses this fact.[3,4] The method is simple; sets of spectra are recorded at two (or more) slightly shifted spectrometer positions, the pairs of shifted spectra are then subtracted. The resulting shifted, subtracted Raman (SSR) spectra that are created have much lower apparent noise than the raw data because the major cause of apparent noise, the irregularity in the detector response, is cancelled in the subtraction (see Figures 1 and 2).

The only problem is that the shifted, subtracted spectra are complex waveforms that resemble first-derivative spectra. To convert them back to the form of conventional spectra it is nec-

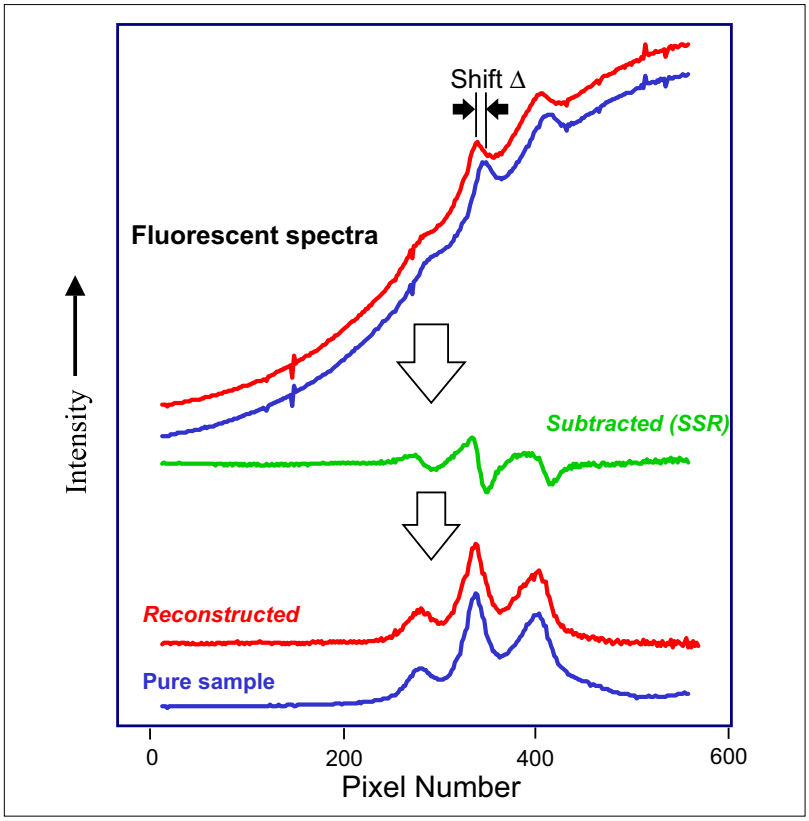

Figure 4. Upper two traces are two slightly offset Raman spectra of the C–H stretching region of an organic solvent that were obtained with an echelle spectrograph. The sample has been contaminated by an impurity that gives strong fluorescence despite the long wavelength (785 nm) excitation. Subtraction of the upper two traces gives the SSRS trace (shown in green), which was then reconstructed using automated software. The lower two traces compare the Raman data obtained for the fluorescent sample (red) with the spectrum of a pure sample of the solvent (blue).

essary to use an algorithm that will convert each peak and its negative-going echo, which is created in the subtraction, to a single positive Raman band. In our earlier work, we fitted the SSR spectra with double Lorentzian peaks and then turned off the negative component of each to generate a representation of what the spectra would have looked like if the shifting and subtraction had not been carried out.[3,4] (This method is illustrated in Figure 2.) More recently, we have developed software that reconstructs the spectra automatically with no operator intervention.

Figure 3 shows the results of reconstructing some data from the very fluorescent ancient Chinese paper samples shown in Figure 1. The detailed interpretation is not important here, although a very cursory inspection certainly demonstrates that the method has easily been good enough to obtain useable spectra from even the most fluorescent samples. In this case, the spectrum of one of the ancient paper samples is clearly almost identical to that of berberine while a second sample shows only a broad feature in the spectral region where the strong berberine bands are found. In fact, the data can even distinguish between berberine and the other significant dye, palmatine, despite their close chemical similarity and we have found evidence that the berberine : palmatine ratio is quite different in the tree bark and in the ancient documents, this difference could be due either to the dying process favouring berberine or to the loss of palmatine over the thousand years since the papers were dyed.[4]

Finally, it is worth emphasising that the SSRS method is not confined to a specific instrument set-up or sample type. For example, Figure 4 shows data taken in the C–H stretching region of a contaminated organic solvent. These spectra were obtained with red (785 nm) excitation and an echelle spectrograph and used the automated software for reconstruction of the SSRS data. Despite the fact that this system is very different from the blue laser, conventional dispersive spectrograph and manual reconstruction that were used for the Chinese paper samples, the same suppression of fixed pattern noise and extraction of the Raman bands is achieved.

Conclusions

The technical advances of the past few years have certainly made Raman spectroscopy much more accessible to the non-specialist user than was the case even ten years ago. Notably, the widespread adoption of red excitation lasers has helped to increase the number of unpurified industrial/biomedical/archaeological samples that can be studied without interference from strong fluorescence. However, in any general purpose Raman laboratory a proportion of samples will continue to exhibit problem fluorescence, for example they may fluoresce even under red excitation or it may be necessary to use a resonant excitation wavelength. The shifting method described here, because it is so general and easy to apply, should help in these situations.

The ancient paper samples are a particularly dramatic illustration of the problems that can be overcome but we hope that the shifting/subtraction method will not be confined to such exotic materials. It can be applied in a wide range of situations and we hope that it will be adopted as a useful method which can be used with samples where fluorescence gives difficulties but purchase of a whole new laser system with a different excitation wavelength would not help or could not be justified on cost grounds.

Acknowledgements

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