

Supplementary Material

Quartz-Background Correction

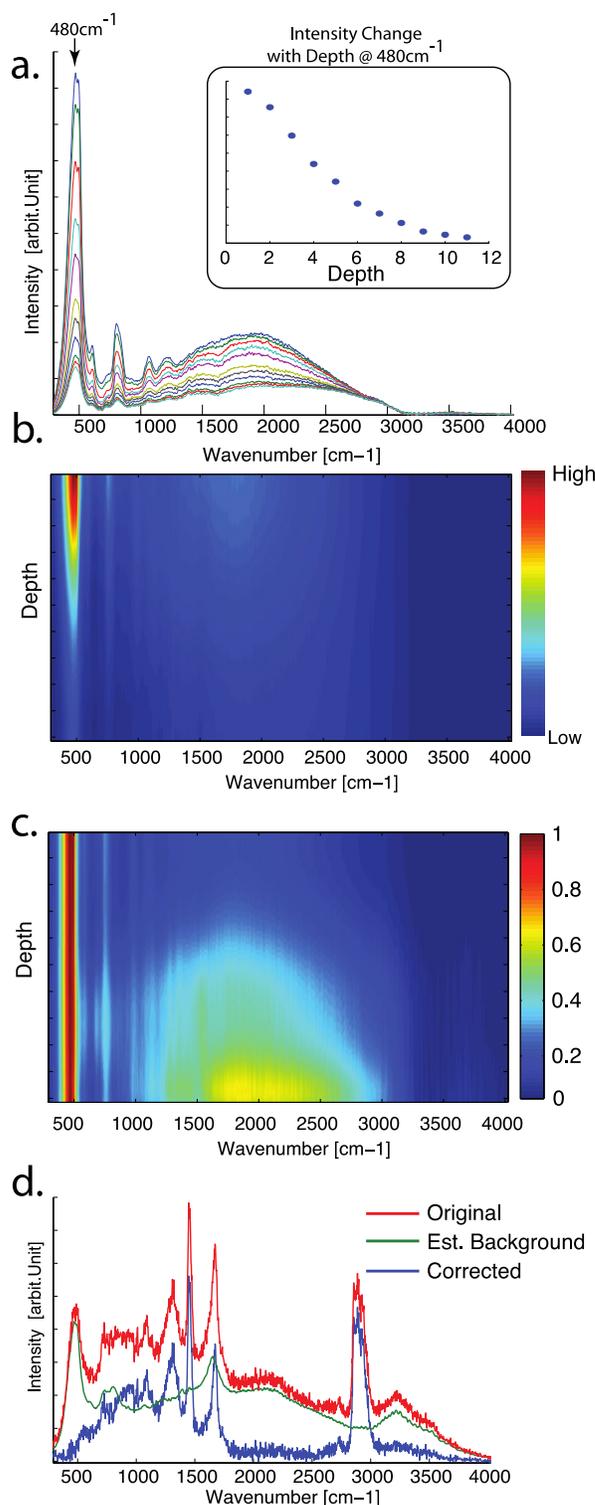


Figure 1S (a). Dependence of quartz background signal acquired by gradually varying the focus. The signal changes as a function of the focal depth. The signal is highest when the laser is focused into the quartz substrate. (b). 5th order polynomial fit to interpolate the intensities at different depths of the eight quartz background spectra shown above. (c). By plotting the intensity of Fig. S1b, normalized to the maximum at the respective wavenumber the signal intensity of the quartz contribution changes individually for each wavenumber and depth. (d). Typical Raman spectrum before the background correction, blue, the background estimate for quartz and water, green, and the background corrected spectrum, red. The quartz and the water background are effectively removed.

Several substrates are popular for Raman microspectroscopy at 785 nm excitation, with MgF₂, CaF₂ and quartz being frequently used. While VUV-grade CaF₂ has very low background contributions it is brittle and cannot be easily used in inverted setups. MgF₂ can be produced very thin and suitable for inverse Raman spectroscopy, but are also very expensive. Here, quartz substrates provide a good alternative. These substrates are thin and inexpensive compared to CaF₂ and MgF₂. However, one common problem using quartz coverslips in Raman spectroscopy is the noticeable background contribution that can easily overshadow the sample signal. Usually, quartz background is removed by acquiring a spectrum of the substrate in close vicinity to the sample, following a simple subtraction of background signal from the sample signal. The subtraction of quartz contributions using background spectra, which were not acquired in close vicinity to the sample, does not always seem to be suitable. However, acquiring a background spectrum every time is quite tedious, time consuming, and not suitable for high-throughput applications. While it can be assumed that the quartz signal is identical for any position in the sample, the absolute intensity of the quartz background appears to be depth dependant and will influence the correction, at least for a simple subtraction of the background. *Beier and Berger* have proposed an approach of fitting spectra of known contaminants to remove background contributions, but also this does not lead to acceptable results. To solve this conundrum we have acquired Raman spectra of dried quartz substrate at eight different depths. Fig. 1a shows that, as expected, the Raman signal intensity of the quartz substrate depends on the depth of the focus.

While the quartz signal is strongest when the beam is focused into the substrate, it decreases when the beam is above the quartz surface, see inset in Fig. 1a. Because the Raman signal intensity from quartz is expected to be a continuous function along the axial positions, the depth-dependent intensities for each wavenumber were fitted using a 5th order polynomial function. The resulting depth-dependent profile of the quartz signal is shown in Fig. 1b. Hence, when simply trying to subtract the quartz background a slight differences in the focal position of the background acquisition and sample signal acquisition will result in overestimated or underestimated quartz backgrounds. This, however, should not be a point of concern if the intensities for all wavenumbers change at the same rate, because it should still be possible to estimate the background spectrum using a least squares fitting approach, as suggested by Baier and Berger. This, however, is not the case. By plotting the depth-dependent intensity profile of Fig. 1b, normalized to the maximum intensity at the 480 cm⁻¹ wavenumber it can be shown that the signal intensity does not change at the same rate for every depth, Fig. 1c. While the quartz bands remain unchanged, a broad scattering contribution occurs between ca. 1000 cm⁻¹ and 3000 cm⁻¹ probably due to laser scattering on the water-substrate interface. Hence, fitting a quartz background spectrum, which was acquired at a different focal depth than the Raman spectrum of the sample, will result in over or under estimation for different wavenumbers and a poor background removal. To properly remove the full background, i.e. Raman spectrum of quartz and scattering contribution, it is important to use background spectra from many different depths, such as the depth-profile in Fig. 1b. To account for the contributions of water a Raman spectrum of water was added to the depth dependent quartz background profile. Raman spectra of cells were fitted with the combined background using asymmetric least squares (AsLS). In Fig. 1d, a typical Raman spectrum, in blue, is shown before the background subtraction; the estimated background spectrum is shown in green; the spectrum corrected for the quartz and water background is shown in red. After the subtraction the quartz background and the water background are properly removed.

Spectral-Noise Contribution

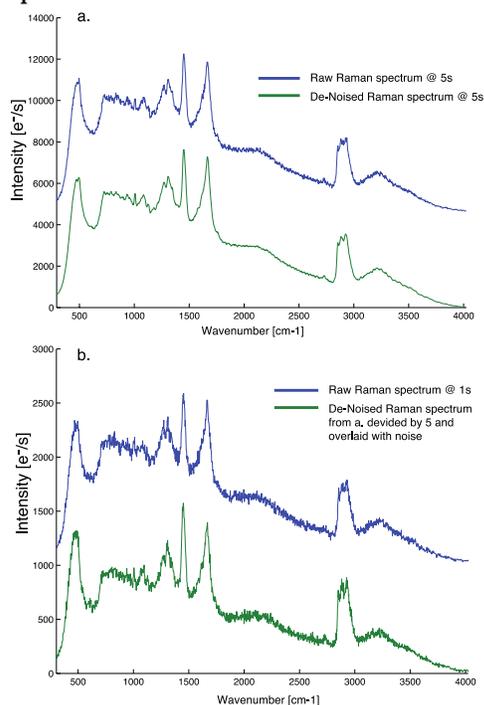


Figure S2a. Original Raman spectrum acquired with the integrated Raman approach at 5s acquisition time, and the same spectrum de-noised. (b). The spectrum from Fig.S2a. down-scaled 5 times, and with addition of noise, corresponding to 1 s acquisition time. For comparison a spectrum acquired at 1 s acquisition time is included.

Integrated Raman spectra were corrected for the constant offset bias of the CCD-camera, and de-noised, using singular value decomposition (SVD), as outlined in the Methods section. S2a shows a typical Raman spectrum acquired at 5 s acquisition time, in blue. The same spectrum de-noised is shown in green. The intensity of the de-noised spectrum was divided by five, because Raman is a linear method, this corresponds to the number of photons detected at an acquisition time of 1 s. Spectral noise on Raman spectra is typically governed by 3 main parameters: dark noise, read-noise, and shot-noise. Because the dark noise for the Pixis eXelon 400x1340 BB-DD camera (Princeton Instruments, USA) @75°C is typically 0.03 e⁻/p/s it is negligible, even at an acquisition of 5 s. Therefore, 2 main sources for noise remain: read noise and shot noise. The typical read noise at a read speed of 100 kHz for the same CCD is 3 e⁻, described by a Gaussian process; and the shot noise is statistical noise dependent on the quantized nature of photons emission. This process goes with square root number of photons, and is described by a Poisson distribution. Noise was generated according to the mentioned specifications and overlaid with the de-noised and by five divided spectrum from S2a. This spectrum is plotted in S2b in green. For comparison a typical Raman spectrum acquired with 1s acquisition time and point spectral acquisition is plotted offset, in blue. It can be seen, the noise levels of the spectra are comparable. This noise-overlay was performed for an entire batch and used as the spectral data in Fig. 4.