Study of bacterial inner structures with 4π Raman microscopy

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Abstract: 4π Raman microscopy provides better resolution and Raman signal than standard Raman microscopy. We determine the improvement using a silicon layer and show its applicability to biological specimen for the first time.

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1. Introduction

We recently demonstrated the application of 4π Raman microscopy to quantitatively characterize inorganic nanolayers in a multilayer stack [1]. This technique improves the spatial resolution and, in contrast to conventional 4π fluorescence microscopy [2], it does not require any fluorophores. As it is based on Raman signatures, it can spatially resolve the chemical information inside a complex sample in a noninvasive manner.

Here, we first discuss the performance of 4π Raman microscopy when applied to a thin silicon slab. Then, we study the spatial chemical distribution inside a biological specimen. We show that this technique unveils the presence of small and large scale structures inside filamentous *Deltaproteobacteria* of the *Desulfobulbaceae* family – also known as cable bacteria.

4π microscopy improves the axial resolution by adding a second objective to a standard conventional confocal microscope to excite the sample from two opposite directions. Both contra-propagating beams produce an interference pattern in the common focal spot, reducing the effective illuminated volume and yielding an almost isotropic resolution. In order to stabilize both counter-propagating beams at the sample plane, the relative phase between beams has to be probed exactly at that plane. This is achieved,as described in [1], by using a partial reflection, normally provided by the sample itself or, in case of low reflection biological samples, by the substrate. The Raman signal from the sample can then be recorded for different relative phases of the contra-propagating beams, i.e. for different positions of the exciting interference pattern.

2. Silicon nanolayer

To characterize the system, we used a Silicon 215 nm thick free-standing membrane. The refractive index of silicon at the pump wavelength of $\lambda = 785$ nm is 3.7, therefore, its optical length in terms of wavelength is 1.01λ. Since the optical length of the membrane matches the wavelength it acts as a Fabry-Perot cavity at resonance.

Silicon has a strong Raman peak and, due to the high refractive index, it provides a strong reflection. These two facts render the silicon membrane an ideal sample to characterize the 4π Raman microscope.

In Fig. 1(a) the two main Raman peaks of the silicon layer are shown, a peak at 302 cm^{-1} and a much stronger one at 520.4 cm^{-1}. Instead of axially moving the sample we equivalently scanned the optical phase of one of the pump beams, and that is what we call *nominal phase* ϕ. The counts of the strongest peak are plotted and fitted to a sinusoidal curve in Fig. 1(b). We normalized the counts with respect to the standard or normal confocal Raman spectrum. The normalized sinusoidal curve should ideally reach a maximum value of 2 due to the redistribution of energy withing the focal spot. We attribute the lower experimental value of 1.7 mainly to an intensity mismatch between the two pump beams.

As discussed in [1], only an infinitesimally thin feature would have a strong enough contrast to reduce the peak counts to zero but also to have twice as many counts as a standard spectrum. Features thicker than the axial resolution of

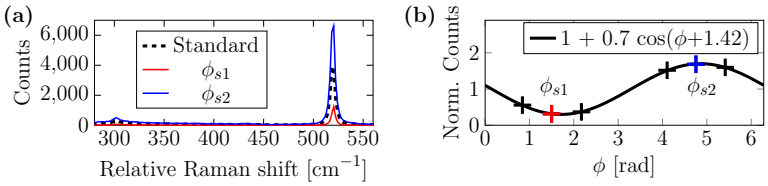

Fig. 1. (a) 4π Raman spectra of the 215 nm silicon layer. (b) Response of the 520.4 cm^{-1} peak versus nominal phase and normalized with respect to the standard response. Crosses indicate experimental data points. Red and blue colors link data from both figures.

a normal Raman microscope fill the confocal volume and therefore the Raman signal remains unchanged for different nominal phases. This arguments do not apply for the silicon layer due to Fabry-Perot cavity effects.

3. Cable bacteria

Biological samples pose additional challenges to the 4π Raman experiments. They typically have much weaker Raman signal than the silicon nanolayer studied above, meaning that the measurement acquisition time has to be longer. Thermal fluctuations and drifts in the system become relevant at this time scales. Thermal vibrations of the sample are also an issue when resolving nanometric features inside the sample. Here we use relatively big bacteria (approximately 4 μm thick) of the *Desulfobulbaceae* family attached to a 1 mm thick and low Raman background CaF_2 substrate to minimize these issues. Such a thick substrate, however, induces aberrations and forces us to use a lower numerical aperture objective lens in the bottom part of the microscope.

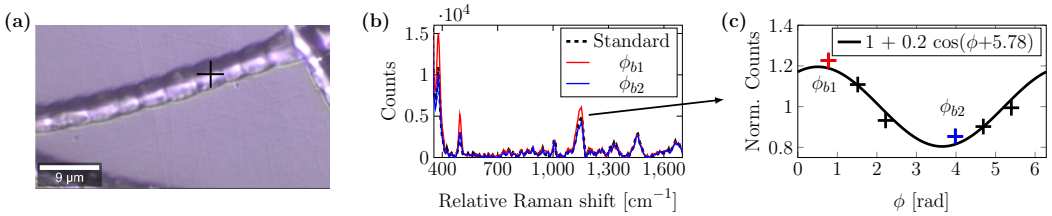

Fig. 2. (a) Bright field image of the cable bacteria. The red cross indicated the point at which the Raman spectrum is taken. (b) 4π Raman spectra of the electric bacteria at different nominal phases, (c) Response of the 1153.2 cm^{-1} peak normalized with respect to the standard response. Crosses indicate experimental data points. Red and blue colors link data from (b) and (c).

Fig. 2(a) shows a bright field image of the cable bacteria. The cross indicates the position at which the Raman spectrum in Fig. 2(b) was taken. We see that the counts of some of the Raman peaks change for different nominal phases. That proves that the 4π Raman microscope can resolve features smaller than what a conventional confocal Raman microscope can. Only the peaks that vary for different nominal phases correspond to features thinner than the resolution of the microscope.

The intensity variations, with a contrast of 1.2 as seen in Fig. 2(c), are not as strong as the variations obtained from the silicon layer. This can be partially explained by the aberrations suffered by one of the pump beams and by the fact that these features are smaller than the conventional resolution but still thick enough to reduce the contrast of the interference pattern. Another factor reducing the contrast could be that the peaks are generated by a combination of both thin and thick sample features.

In conclusion, 4π Raman microscopy is able to resolve features inside biological specimen smaller than the resolution of a conventional confocal Raman microscope.

References

1. A. Diaz Tormo, D. Khalenkow, K. Saurav, A. G. Skirtach, N. L. Thomas, Opt. Lett. **42**, 21 (2017).
2. S. W. Hell, E. H. K. Stelzer, S. Lindek, C. Cremer, Opt. Lett. **19**, 3 (1994). 222 (1994).