Multi-beam microscopy free of sample-induced phase distortions

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When imaging biological samples it is commonly assumed that the point spread function of the microscope is not perturbed by the sample itself. Quantitative phase imaging studies indicate that typically studied cells can induce strong phase shifts, sufficient to disturb the complex point spread function. Even a relatively small phase shift of half a wavelength can completely change the point spread function of a multi-beam microscope. Here we propose a method to render the point spread function insensitive to sample-induced phase shifts.

Introduction

Biological samples, such as living cells and bacteria, are referred to as weak phase objects. Consequently, it is commonly assumed that the light beam is not perturbed in any manner by the sample itself [1]. Numerous studies indicate the opposite, i.e., typically studied cells can often induce phase shifts of more than a few wavelengths [2, 3]. A phase shift of this magnitude will alter the Point Spread Function (PSF) of the microscope.

In multi-beam microscopy, a phase shift as small as half the wavelength can completely change the PSF. In a 4π microscope [4], two counter-propagating beams are focused in a common focal spot to produce the PSF shown in Fig.1.(a), where a main peak is formed at the center due to constructive interference. The phase shift induced by the sample itself can change the relative phase between beams so that the interference becomes destructive, as shown in Fig.1.(b). Here we propose a method, depicted in Fig.1.(c), to render the multi-beam microscope insensitive to sample-induced phase shifts. It uses the sample itself, namely the interface between the sample and the substrate it lies on, as the phase reference of the microscope.

Methodology

The multi-beam optical setup employed for these experiments is depicted in Fig.2. We used it previously for 4π Raman measurements [5]. On the right side we have a microscope with a spectrometer to measure the Raman signal from the sample. On the left side an interferometer measures and controls the phase shift between both pump beams at the sample plane. Connecting both parts, interferometer and microscope, we use polarization maintaining fibers which introduce phase noise in the system due to environmental fluctuations, indicated as ϕ_T and ϕ_B. There are mainly four beams going

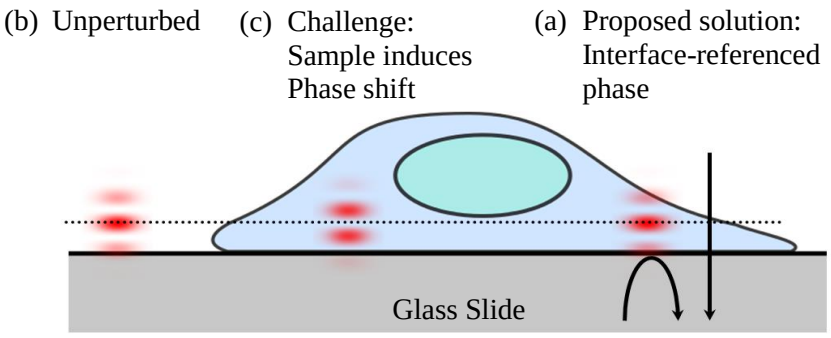

Fig.1. (a) Unperturbed 4π point spread function with constructive interference at the center. (b) The presence of a cell perturbs the interference pattern. **(c)** We propose to use the slide interface as the phase reference. The interference between a transmitted beam and a reflected beam, indicated by arrows, allows measuring the phase state at that interface. The interference pattern is depicted in red in all three cases. The dashed line indicates where the interference pattern should be the maximum for constructive interference at the center. Drawings are approximately to scale, where the cell width is ~20 μm and the width of the main peak of the interferogram is ~0.4 μm.

through the setup, two beams transmitted through the sample, and their two reflections happening at the sample plane. We block two of them, indicated by dashed red arrows, with an isolator. The remaining two beams give access to the phase shift between pump beams at the sample plane. We realized that the reflection at the sample plane can sometimes be too weak to be useful. There are two main cases when the reflection this can happen: when the sample is immersed in water the index contrast between substrate and water is very weak, reducing the reflection significantly, and when we simply measure a part of the sample that is far away from that interface. In those cases we need to use an external reflector to obtain a sufficient signal to noise ratio at the detector, on the condition that there is no phase noise between the sample plane and the external reflection plane.

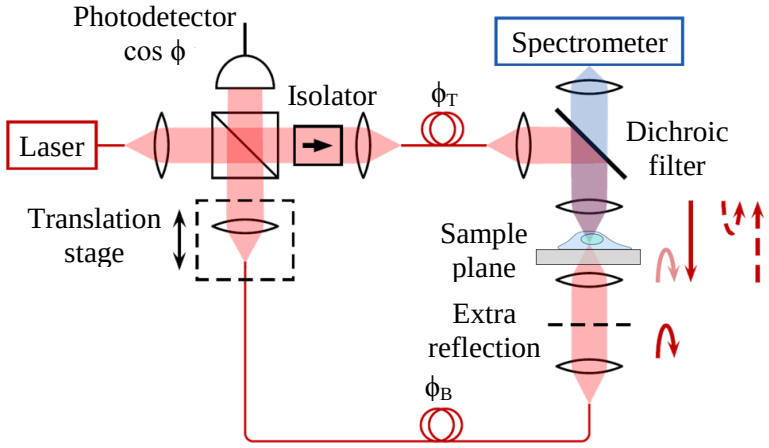

Fig.2. Optical schematic of the 4π Raman microscope used in the experiments. Red arrows indicate different beams travelling through the setup. Dashed arrows are filtered out by the isolator. The extra reflection is optional, depending upon the strength of the sample plane reflection in light red.

In the following experiments we use polystyrene nanoparticles of 250 nm, Fig.3.(a), to demonstrate that the phase shift between counter-propagating beams is anchored to the sample plane. We focus on their polystyrene peak indicated in Fig.3.(b). The Raman signal from the nanoparticles at the spectrometer should both follow a general form of interference between two beams $O_f + A\,cos\,\phi$, where $\phi = \phi_T - \phi_B$. At every point we take 4 spectra at different phase shifts between counter-propagating beams to recover all the parameters of the interferogram. To resolve the π-ambiguity introduced by the sinusoidal term on the phase ϕ we need two measurements, plus two other measurements to solve the offset O_f and the amplitude A.

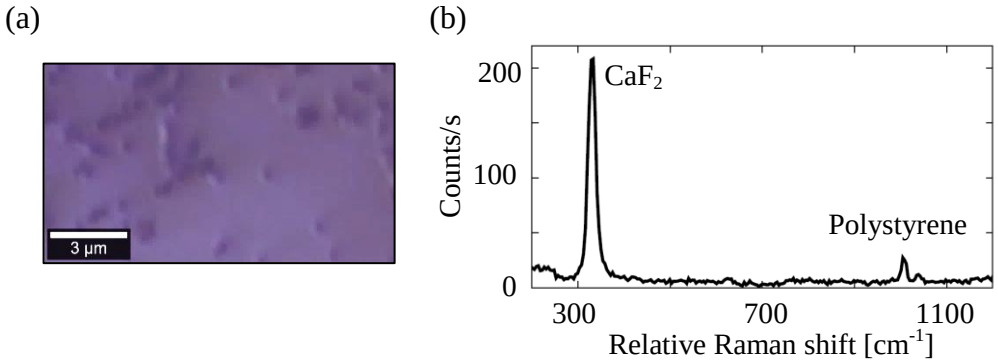

Fig.3. Polystyrene nanoparticles of 250 nm diameter. (a) Visible image. (b) Raman spectrum. The CaF_2 peak comes from the substrate and the polystyrene peak around 333 cm^{-1} corresponds to the particles.

Results

With biological samples we would need to use the external reflector, but in this particular experiment we use dry nanoparticles and we measure very close to the interface, so we do not require it. Since the interface is acting as the phase reference we can say that the phase is anchored to the sample. We investigated how the phase between top and bottom beams changes when we axially scan the sample, expecting a constant value for an anchored phase. We measured how much we have to change the phase between counter-propagating beams in order to get the maximum polystyrene Raman signal for different axial positions of the sample. The result is plotted in Fig.4, where an approximately constant value for a sample position z<0 microns can be seen. After that, the phase changes 3.7 radians in 0.9 microns, which is smaller than the

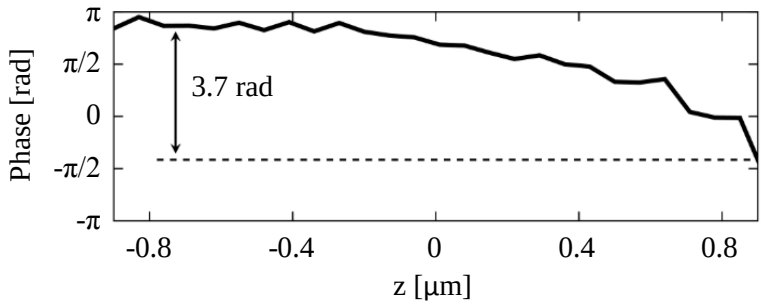

Fig.4. Phase shift needed between top and bottom beams to maximize the Raman polystyrene peak.

optical path difference travelled. Further investigation is necessary to clarify the origin of this unexpected phase shift, but it could be due to the different influence of the sample at different axial positions or even to optical aberrations when the beams travel different lengths of the substrate.

Using the data above we plot in Fig.5 the signal coming from the particles in such a way that emulates the case where the point spread function is static, regardless of the sample position. That is the point spread function of the microscope in the axial direction. For comparison purposes we also plot the point spread function of the microscope when only the top or bottom excitation is used. The characteristic secondary lobes of 4π microscopy can be seen.

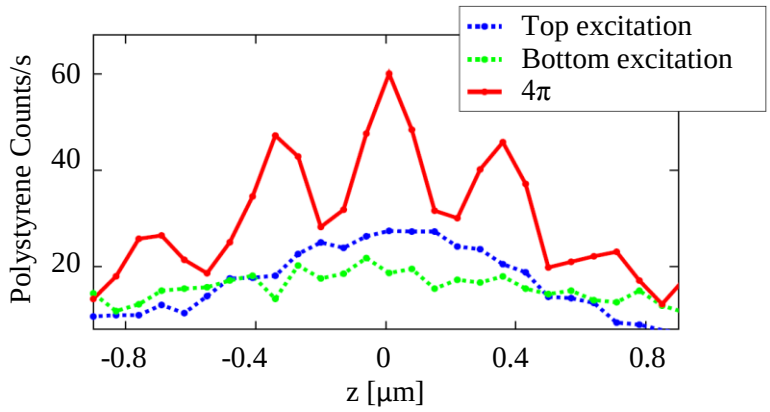

Fig.5. Standard confocal and 4π point spread functions in the axial direction.

Conclusions

The technique described here allows controlling the phase shift between beams in a multi-beam microscope with respect to the sample. It can compensate for the phase shift introduced by the sample itself. In the fluorescence microscopy field, the effect of the sample itself on the 4π point spread function is known, but there is no method to compensate for it. The field of view is therefore restricted to a part of the sample where this effect is negligible. We hope that this technique can be used to increase the field of view of this kind of microscopy techniques.

References

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