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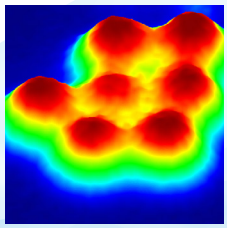
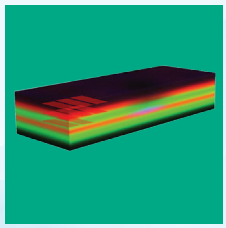
Raman

The Importance of Confocality in Optical Microscopy and Raman Microscopy





Technical Note
Confocality
RA TN-15

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When performing optical microscopy and/or Raman microscopy, you may have come across the term "confocal," and wondered how it varies from standard widefield microscopy or conventional Raman microscopy, why it is important, and what its advantages are. Confocal is defined as having the same focus or foci, and in the context of optical microscopy, a confocal microscope is one with an aperture (called a confocal hole) in a conjugate focal plane, whose imaging system only collects light from the sample focal plane, giving greater spatial resolution. The same concept is applied to confocal Raman microscopy, in the sense that a confocal hole is placed in the conjugate focal plane and only the Raman signal from the sample focal plane is detected at the spectrometer, resulting in greater axial and lateral spatial resolution. The easiest way to visualize confocality and what it means for an image is to compare it with depth of field in photography. A wide depth of field will allow the photographer to capture everything in his/her field of view in focus. A narrow depth of field, however, will allow the photographer to focus on one particular plane or object in his field of view, which will be in focus, while all other objects are out of focus, as is illustrated in Figure 1 below.

Figure 1: Visualization of a narrow depth of field in which only the wasp and the words "depth of field" are in focus, and all other text in the foreground and background is blurred. (Photo 157016 © Alptraum | Dreamstime.com)

Note that the wasp and the words "depth of field" are all in crisp, clear focus, while all other text in the foreground and background is blurred. Confocal images are similar in the sense that the focus is placed on the plane of interest and all other information outside of that plane is not included in the final image. This results in high spatial resolution in depth, which allows for thin optical sectioning of a thick sample in order to obtain a complete, high resolution 3D image.

In a widefield optical microscope design, as shown in Figure 2(a) below, all the light from various depths in the sample is collected and imaged onto the detector. In a confocal optical microscope design, as shown in Figure 2(b), a confocal pinhole is added in the conjugate focal plane, in order to block light which emerges from planes other than the selected plane of focus. The result is higher spatial resolution and contrast as a function of depth. Visually, this can be related to the photographic image shown previously in Figure 1. Imagine that Figure 1 represents a confocal microscope view where only a part of the image, in this case the focal plane containing the wasp and the words "depth of field" is in focus at one time.

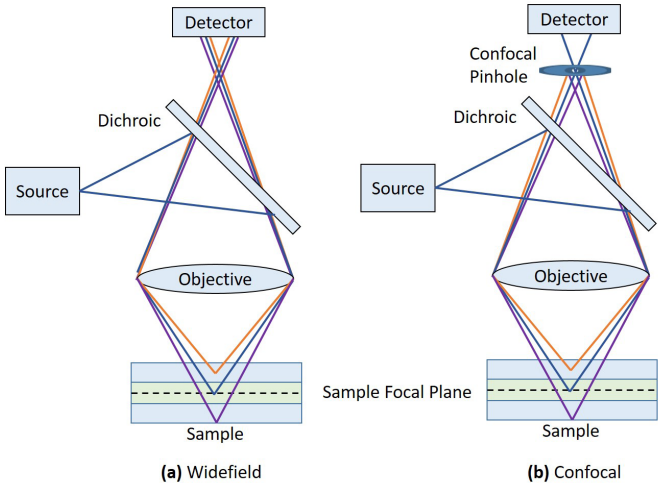

Figure 2: (a) Widefield microscope design (b) confocal microscope design which includes a confocal pinhole in the conjugate focal plane in order to block out-of-focus light from the sample focal plane. The light from above (orange) and below (purple) the sample focal plane will be blocked by the pinhole and hence not collected or imaged at the detector; only the light at the sample focal plane (blue) is collected and imaged.

So far, the discussion about confocality has been focused only on optical microscopy, but the concept can also be applied to Raman microscopy. Just like in optical microscopy, when performing Raman measurements with a confocal Raman microscope, only the Raman signal from the sample focal plane will be detected, as shown in Figure 3(b). (For comparison, a conventional Raman microscope design is shown in Figure 3(a)). It is important to note that for a confocal Raman microscope, the concept of confocality only applies to the Raman signals and not the optical image. Confocal Raman microscopy allows for high resolution depth profiling and 3D Raman imaging. Sample types which benefit from a confocal Raman microscope design include transparent, multilayer polymers, inclusions in a transparent matrix, and any other sample (usually translucent or transparent) that varies as a function of depth.

There are several advantages to using a confocal microscope design in both optical and Raman microscopy. A confocal design allows for control of depth of field in order to achieve the highest axial and lateral spatial resolution. Confocal microscopes also help eliminate or reduce background or unwanted information. For example, from Figure 2(b), only the light of interest from the sample focal plane (blue) will be detected, and the unwanted light from above (orange) and below (purple) the focal plane, will not interfere with the overall image, as that light is not collected at the detector. This is similar for Raman microscopy, as shown in Figure 3(b), where only the Raman signal from the sample plane of focus is detected at the spectrometer and all other background signal is reduced or eliminated. In this sense, confocal microscopy can be used to produce thin optical sections from thick samples for better spatial resolution. It can also be useful for measuring Raman signals through coverslips or other transparent media with little to no interference from the coverslip/transparent medium.

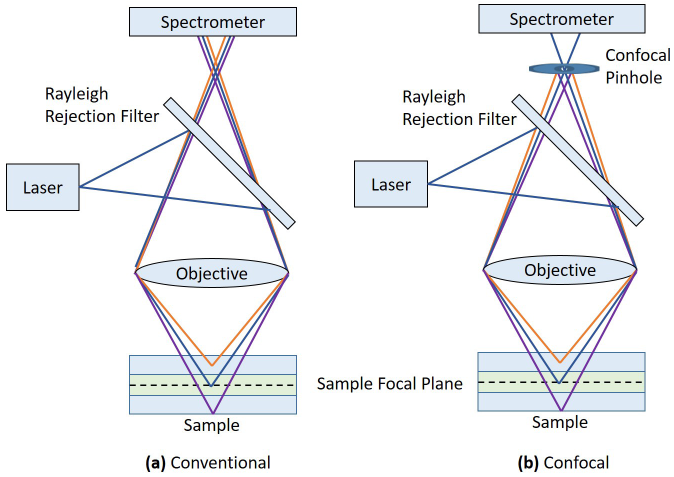

Figure 3: (a) Conventional Raman microscope design (b) Confocal Raman microscope design which includes a confocal hole in the conjugate focal plane. The Raman signal from above (orange) and below (purple) the sample focal plane will be blocked by the pinhole and hence not collected at the spectrometer; only the light at the sample focal plane (green) is collected.

As an example, for Raman microscopy, shown below in Figure 4(a), is a transparent, multi-layered stack of polymer films, which was measured as a function of depth, with different confocal hole sizes. Figure 4(b) shows the depth profile of the sample using a confocal hole size of 1000 µm, which essentially mimics the widefield microscope design and results in all the light/Raman signal being collected at the detector. In Figure 4(c), the confocal hole size was reduced to 100 µm, which helps to block out some of the out of focus light as the sample is probed in depth. In comparing Figure 4(b) to Figure 4(c), a clear difference in the sharpness of each film interface becomes apparent; the layers are more distinctly defined when the confocal hole size is reduced. The observed reduction in intensity as a function of depth is simply due to aberrations stemming from the objectives and the expansion of the focal volume as the beam is focused deeper into a sample of higher

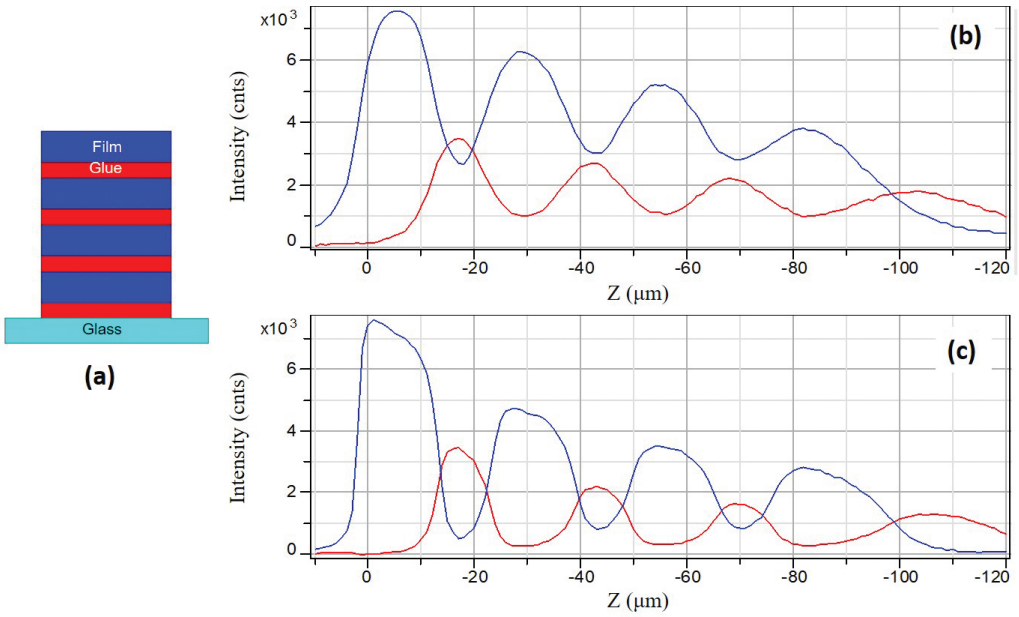

Figure 4: (a) Multi-layered polymer film stack (b) Raman intensity vs. depth (z) for a confocal hole size of 1000 µm (c) Raman intensity vs. depth (z) for a confocal hole size of 100 µm.

refractive index. Air objectives have been designed for best focus in air, not in a material with a different index of refraction, such as a polymer (n ~ 1.3-1.7).

In order to show the differences between measurement with an air objective versus a water immersion objective (index of refraction ~1.3), a similar transparent, multi-layered stack of polymer films, shown in Figure 5(a), was measured as a function of depth with the same confocal hole size of 100 µm, but with different objectives. Figure 5(b) shows the depth profile of the sample using a 100x LWD air objective and Figure 5(c) shows the depth profile of the sample using a 100x water immersion objective, where the index of refraction of water is similar to that of the sample (n ~ 1.5). In comparing Figure 5(b) to 5(c), not only are the interfaces more clearly defined when using a water immersion objective, but also, the intensity stays relatively constant throughout the depth of the sample. This means that for optimum depth resolution, the choice of objective is an important factor to consider when performing confocal Raman spectroscopy. For more information about the effects of sampling optics on Raman depth profiling, please refer to the following Spectroscopy article: "Effect of Sampling Optics on Raman Depth Profiling," Spectroscopy Online. **https://www.spectroscopyonline.com/view/effect-of-sampling-optics-on-raman-depth-profiling**

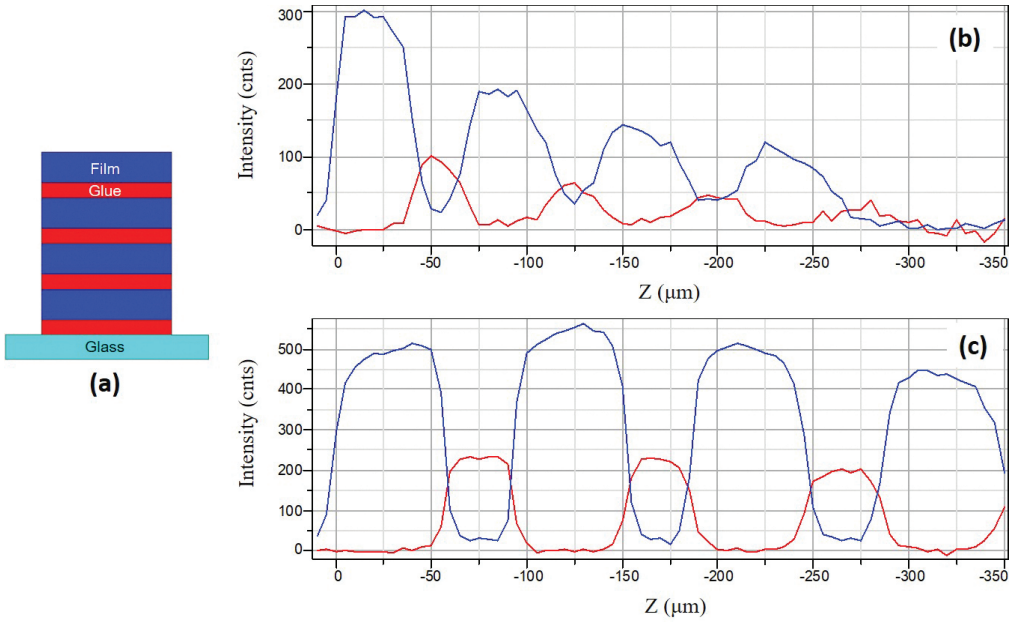

Figure 5: (a) Multi-layered polymer film stack (b) Raman intensity for a 100x LWD air objective and a 100 µm confocal hole size; (c) Raman intensity for a 100x water immersion objective and a 100 µm confocal hole size.