



Confocal Raman Microscopy General Overview

Origins – a battle with scattered light

The first confocal scanning microscope was invented in 1955 and patented in 1957 by the Harvard graduate Marvin Minsky. He was obsessed with resolving the mystery of the human nerve system anatomy which aids in performing an outstandingly complex set of cognitive functions. Though at that time the common shapes of nerve cells were generally known, the connection schemes between them were not mapped. The critical obstacle was that the tissue of the central nervous system was solidly packed with interwoven parts of cells. Individual cells were practically indistinguishable from each other by conventional wide-field optical microscopy because of the excessive scattered light blurring the image. Large amount of scattered light was generated in the wide-field microscopy because the whole sample had to be illuminated at once (Figure 1). Solving the scattered light problem became the driving force for creation of the first confocal microscope [1].

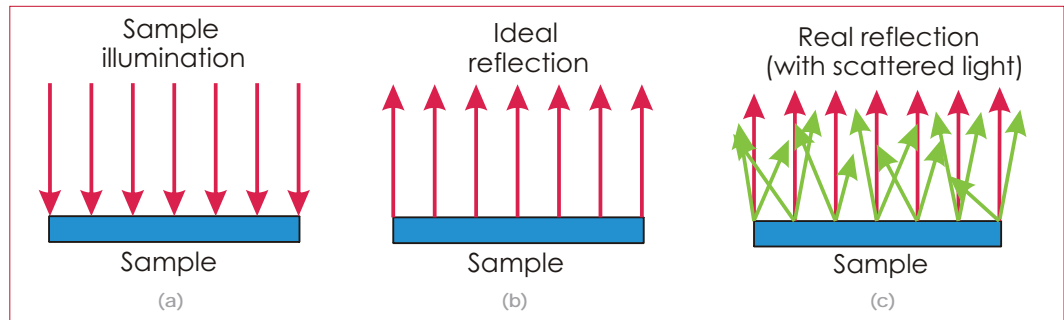


Figure 1. Scattered light problem in the wide-field optical reflection microscopy. Instead of the ideal reflection (b) most of the real samples scatter light (c). Multiple scattering rays are fairly collected by the microscope objective and add to the “blur” in the resulting 2D image of the sample.

Optical design principles

Prof. Minsky hypothesized that all the scattered light could be avoided by preventing it from entering the microscope objective in the first instance. According to his vision an ideal microscope would examine each point of the specimen at a time and measure the amount of light scattered or absorbed just by that point [1]. This can be easily achievable by setting a pinhole aperture in front of the light source (Figure 2). In this case the small pinhole aperture refocused by the microscope objective illuminates only a single point on a sample. Since scattered light from all other parts of the sample does not exist any more, the total amount of scattered light is reduced by a few orders of magnitude without affecting the focal brightness.

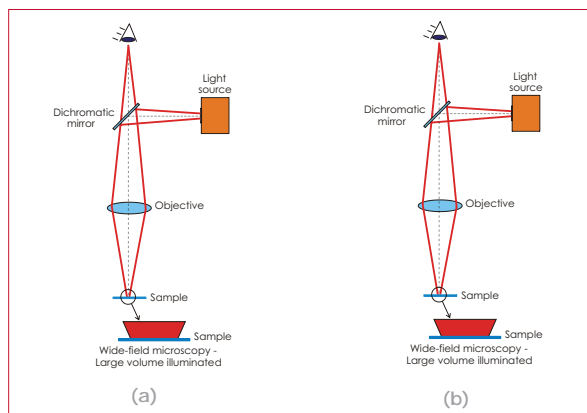


Figure 2. Difference between conventional wide-field microscopy (a) where large volume of sample is illuminated at once and the confocal microscopy (b) where only a single spot on a sample is illuminated.



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The second pinhole aperture placed between the microscope objective and the image plane rejects the residual scattered rays originated from any out-of-focus points on the sample and functions as a spatial filter (Figure 3). Figure 4 shows the simplified schematic diagram of reflection confocal microscope with the two pinhole apertures.

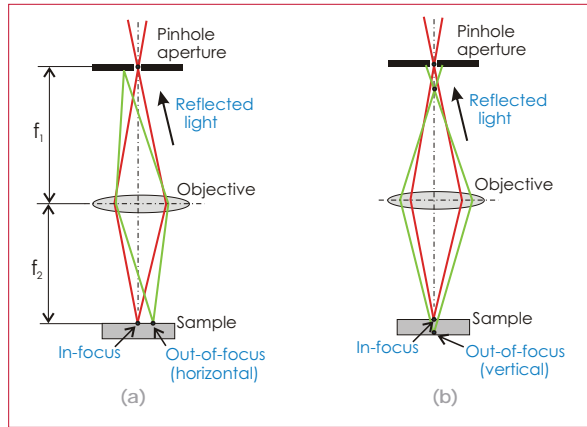


Figure 3. Spatial filtering with the second pinhole aperture of the horizontal (a) and vertical (b) out-of-focus rays.

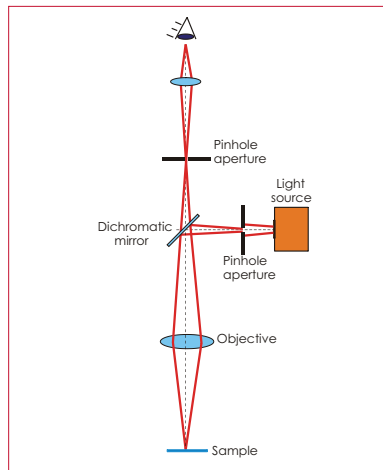


Figure 4. Simplified scheme of the reflective confocal microscope with two pinhole apertures.

Such an optical design offers a few distinctive benefits. The first pinhole aperture greatly reduces scattered (stray) light and immensely improves the image quality while the second aperture eliminates any image degrading out-of-focus information, allows for controllable depth of field and gives the ability to collect series of optical sections from bulk specimens. With this optical design, Prof. Minsky was able to realize his dream. Figure 5 demonstrates the stunning difference in image quality between the wide-field and confocal types of microscopy [2].

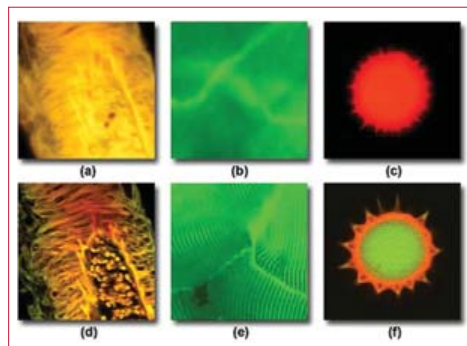


Figure 5. Difference in quality of micro-images obtained with conventional wide-field (a - c) and confocal (d - f) microscopy: fluorescently stained human medulla (a, d); whole rabbit muscle fibers stained with fluorescein (b, e); autofluorescence in a sunflower pollen grain (c, f). Images are courtesy of Olympus America Inc [2].



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Mapping the sample

All the significant improvements of image quality in confocal microscopy come with a price – the necessity to map a sample to obtain the full set of data. The mapping can be arranged in two ways - by scanning the light beam along the sample with a system of oscillating mirrors or by moving the sample itself using an X-Y or X-Y-Z scanning stages. There are a few distinctive techniques of sample mapping.

X-Y optical sectioning (Surface scan)

This simple technique characterizes flat samples by mapping a thin 2D layer on the sample surface which is called a single optical X-Y section (Figure 6a). The technique is useful for biological and pharmaceutical samples laid under a cover glass or in geology and material science for structural and chemical analysis of polished thin-sections and cut-off sections.

X-Z cross-sectioning (Depth profiling)

Confocal microscopy can be used as a powerful non-destructive tool to obtain information about a sample's 2D cross-section in X-Z direction (Figure 6b). Applications of this technique include in vivo biopsy tests and non-destructive analysis of material internal structure and integrity.

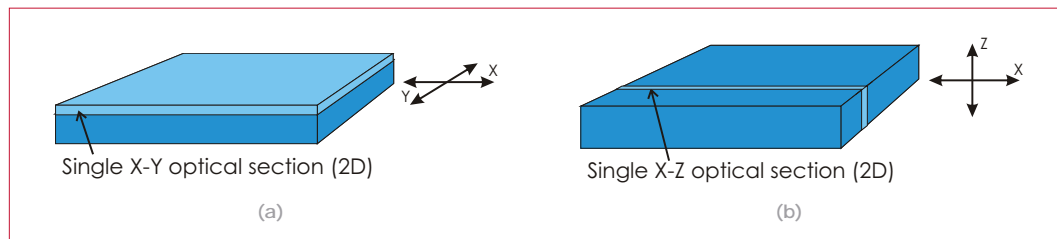


Figure 6. 2D mapping: single X-Y (a) and X-Z (b) optical sections on a sample.

Z-series (X-Y-Z scan)

When a 3D structure of a bulk sample should be revealed one can engage in a tedious task of taking multiple X-Y optical cross-sections along the Z-axis (Figure 7a). The resulting information is transferred into software to reconstruct the detailed 3D image. The technique is the backbone of confocal microscopy and is extremely useful for analysis of polymers, nano-materials, integrated circuits, pharmaceutical samples and biological objects. The first 3D image of a human neural tissue was successfully obtained by Prof. Minsky at the end of the 1950s.

Surface profiling (Auto-focusing)

In principle it is possible to reconstruct a 3D surface of a sample from the X-Y-Z scan. Though, if one is interested in obtaining only a surface profile it can be done much faster using an auto-focusing technique. While scanning the sample surface the focal point microscope objective is moving up and down to focus exactly at the sample surface (Figure 7b). The technique resembles a profilometer functioning but confocal surface profiling can reveal not only morphological but also detailed chemical information about the sample.

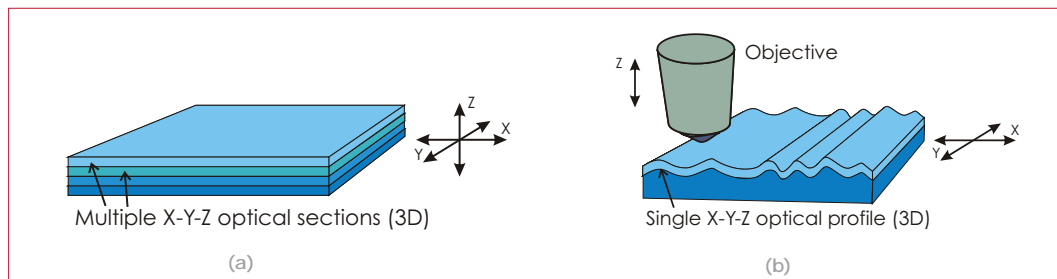


Figure 7. 3D mapping: multiple X-Y-Z optical sections (a) and a single X-Y-Z profile (b) obtained in auto-focus mode.



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The power of confocal spectral measurements

One of the ultimate goals of any analytical technique is to achieve the best possible differentiation. In terms of microscopy this means the ability to distinguish between sample details and features of interest as clearly as possible, by somehow differentiating them from the ambient background. Microscopy has a rich history of finding and developing numerous dyes, fluorophores and labels to “stain” target sample features in order to differentiate them from the rest of the sample. However, the arsenal of staining agents is limited and some properties and structures are difficult to study with conventional analytical techniques due to their inability to chemically differentiate materials with sufficient spatial resolution, without damage or preferential solvent washing. This is where the true power of confocal micro-spectroscopy unleashes itself.

It is possible to collect a signal from a single point on a sample, disperse it into a spectrum using a spectrometer and detect the spectrum using a multi-channel detector such as CCD or PDA (Figure 8). In this case, instead of obtaining trivial cumulative information about the spot signal intensity we can obtain a signal spectrum which can be transformed into detailed information about the chemical composition of the given spot on a sample. In other words, we just added a principally new dimension in our microscopy measurements giving us an opportunity to differentiate the structures never detectable before, based on new information contained in the spectra.

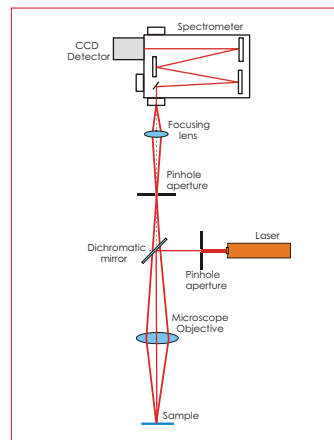


Figure 8. Optical scheme for confocal spectroscopy including confocal microscope, spectrometer and multi-channel CCD detector.

Data Cubes

The collection of spectra taken from multiple spots on the same sample is called a “Data Cube” because for a simple 2D X-Y scan the data is represented by a 3D array (cube) where X and Y dimensions hold spatial coordinates and the Z-dimension stores the spectral information (Figure 9). A 3D spectral scan is represented by a 4D Data Cube which includes three spatial dimensions plus one spectral dimension. The additional spectral dimension in Data Cubes provides a powerful tool for differentiation of internal sample structure.

Human mind is unable to comprehend dense 3D and 4D arrays of data. Therefore, the reconstructing software handling the Data Cubes should have a potent data mining functionality. The useful information from the multi-dimensional data structures is extracted by slicing them along any of spatial or spectral coordinates.

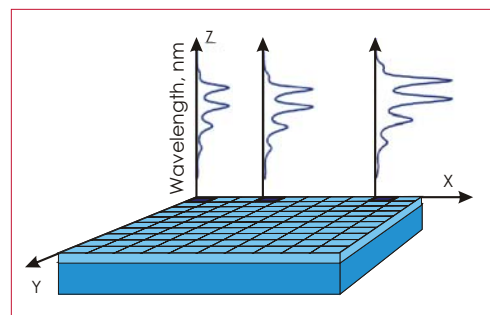


Figure 9. Structure of a 3D Data Cube: X and Y dimensions store spatial information, while Z-dimension stores spectral information from each spatial spot.



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Confocal Raman

The spectral information in confocal microscopy can be obtained through different techniques such as Absorption, Reflection, Transmission, Emission, Photoluminescence, Fluorescence or Raman spectroscopy. Among those techniques confocal Raman holds a special place. Raman spectroscopy as such is based on the inelastic scattering of monochromatic light when the frequency of photons changes upon interaction with a sample. The photons of the laser light are absorbed by the sample and subsequently reemitted. Frequency of the reemitted photons is shifted up or down in comparison with the original monochromatic frequency, which is known as the Raman effect. The Raman shift provides information about vibrational and rotational energies of molecular bonds.

It was realized that Raman spectroscopy was a convenient probe of the vibrational energy levels within a molecule which easily provides molecular fingerprints. Another unique advantage of Raman spectroscopy is it can be used to selectively excite a needed portion of the molecule by changing the excitation wavelength. On top of that Raman spectroscopy doesn't require any sample preparation, samples are not destroyed, water bands are usually small and easily subtracted and Raman spectra usually contain sharp bands that are characteristic of the specific molecular bonds in the sample. The intensity of the bands in a Raman spectrum is proportional to the concentration of the corresponding molecules and thus can be used for quantitative analysis.

In biological samples, such as cells and tissues, infrared spectra often show broad spectral features which can give information regarding cellular components. However, Raman spectra give this information as well as more detailed information regarding the constituents of these specific components allowing good specificity for qualitative analysis and for discrimination among similar materials.

The latest trend in high-end confocal Raman microscopy instrumentation is modular systems designed for easy customization of their components and therefore providing the best possible results in terms of chemical differentiation and sensitivity. One of the best examples is Princeton Instruments modular system. It includes confocal microscope which can be coupled directly or fiber-optically to the entrance slit of a Triple spectrometer equipped with a multi-channel CCD, ICCD or InGaAs PDA detector (Figure 10). The system is designed to handle a wide range of excitation wavelengths including tunable laser sources. The 3D Data Cube obtained with this system is shown in Figure 11.

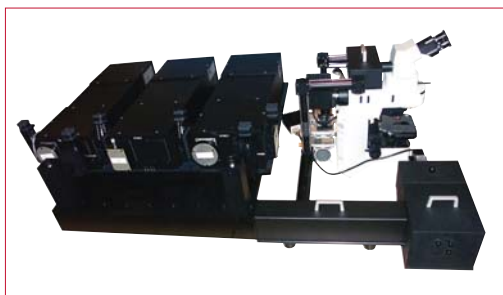


Figure 10. Modular confocal Raman system with Triple spectrometer from Princeton Instruments (detector is not shown): 1 – microscope; 2 – X-Y stage; 3- microscope adaptation optics; 4 – excitation beam optics; 5 – emission beam optics; 6 – macro-sample chamber; 7 – switch between micro- and macro- options, includes optional optical elements; 8 - triple spectrometer Acton TriVista777; 9 - entrance slits; 10 – switch between triple and single modes.

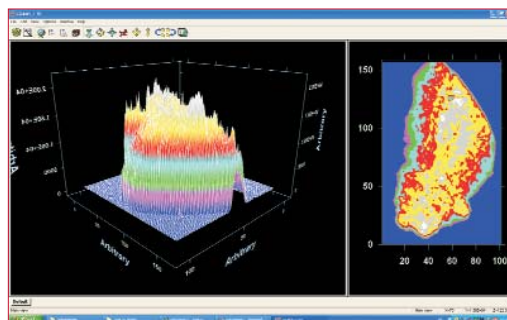


Figure 11. 3D Data Cube of a solid micro-particle obtained with Princeton Instruments confocal Raman system.



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Confocal Raman Applications

Clear advantages of confocal Raman microscopy assured its wide-spread proliferation into multiple areas of industry and academic research. Partially, it has happened due to explosive growth of Nanoscience. Characterization of heterogeneous nano-systems continues to grow in importance and impacts key applications in the field of nanotechnology (molecular electronics, nanosensors, nanotubes and nanowires), material science (phase segregated systems) and catalysis (single site catalysts). One of the hot subjects in this area is characterization of carbon nanotubes (CNT) – the unique nanostructures with remarkable mechanical and electrical properties and significant potential for future innovations. Confocal Raman microscopy is able to capture the space distribution of CNT with different electronic structures helping to reveal mechanisms controlling the batch synthesis of preferentially metallic or semiconductor nanotubes. This type of analysis can not be done with traditionally used SEM or TEM microscopy.

Bio-science research and pharmaceutical industry is another rapidly growing application area for confocal Raman. As the instrumentation evolves towards a dedicated biologist's tool, the variety and scope of biological applications will only widen. The unique advantage that confocal Raman microscopy needs no sample preparation and its ability to give improved axial and spatial resolution over conventional microscopy make it possible to perform extremely detailed analysis of cells in their natural state. Macroscopic resolution allows investigating the chemistry of individual cells and mapping of generated images. These images can contain full spectral information at each pixel so that the distribution of components within the cell can be visualized based upon their Raman signature. This is extremely valuable to researchers as biochemical changes can be observed during a cell's life cycle or when a cell becomes damaged or cancerous. Using confocal Raman, the changes in a variety of cells, including bacteria and eukaryotes can be monitored over time [3] and comparison between healthy and diseased tissue states can be easily analyzed.

Pharmaceutical industry has specific challenges for confocal Raman in characterizing the structure and distribution of the active components within surface coatings of medical devices, high-resolution chemical mapping of commercially distributed pills and tablets and homogeneity tests of creams and ointments.

Conclusion

Confocal Raman microscopy has proven to be an extremely popular analytical technique with the set of unique advantages such as:

- No preparation of the sample required.
- Great spatial resolution.
- Clear image quality.
- Outstanding chemical differentiation.
- Ability to perform 3D mapping of bulk samples.

The technique is useful in multiple application areas of physical and life sciences as well as industry and has great perspectives for long-term growth.

The latest trend in high-end confocal Raman microscopy instrumentation is modular systems designed for easy customization such as the one from Princeton Instruments. Such systems provide the best possible chemical differentiation and sensitivity.

References

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