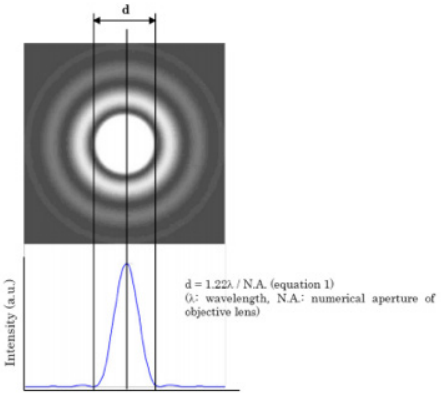
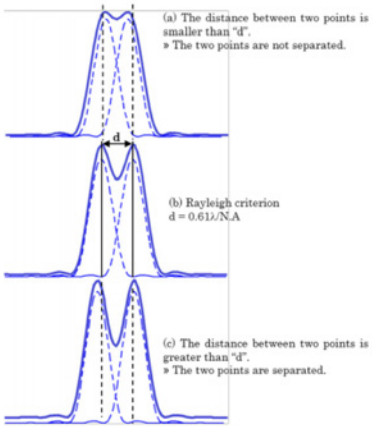
RAMAN IMAGING MICROSCOPY

TIPS & TRICKS

1



Airy-disk resulting from diffraction with a circular aperture



Spatial resolution defined by the Rayleigh criterion

Spatial Resolution

In Raman microscopy, spatial resolution is extremely important to be able to discriminate small structural differences in the sample. Optical microscope objective lenses (up to x100 magnification) are used to observe the sample and pass the Raman scatter to the spectrograph. When light with a constant energy distribution is introduced into the objective lens, the diffraction pattern shown below occurs. The bright enter area is called an Airy-disk, and its size (d) can be determined from the wavelength (l) and the numerical aperture (N.A.) (see the equation, above left). The “d” term refers to the diffraction limit that determines the spatial resolution of an optical lens.

The definition of spatial resolution on an XY plane is based on the distance between two points close to each other — the Rayleigh criterion. In the standard configuration of an NRS-5000/7000 (532 nm laser, using a x100 objective lens with N.A. = 0.90), the laser spot size at the diffraction limit is calculated as d = 720 nm. The spatial resolution, according to the Rayleigh criterion, is then equal to 360 nm.

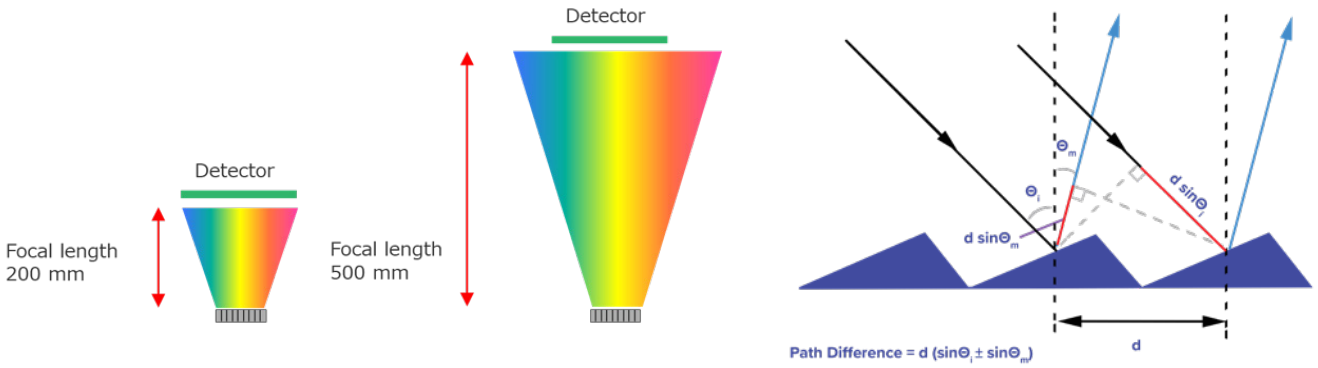
If the laser wavelength is decreased, the spatial resolution is then increased. Additionally, if an oil-immersion objective lens providing a larger N.A. is used, a comparative improvement in spatial resolution can also be achieved.

However, the definition based on FWHM (full width at half maximum) of the intensity of the interference ring has to also be recognized.

2

Spectral Resolution

Spectral resolution in a Raman microscope system is defined by several factors - focal length of the spectrograph, number of lines on the grating and resolution of the elements in the detector. Focal length is the distance between focusing mirror and the detector, longer focal length provides greater resolution. The grating is an optical component that consists of periodic grooves at a specific angle (blaze angle) to match the wavelength of the excitation laser. Resolution increases with a greater numbers of lines. The specification is typically described by the lines or groove per millimeter on the surface (a common grating is 900 gr/mm) and the blaze wavelength. It is not possible to keep increasing the number of grooves to increase the spectral resolution, there is a physical limitation. Light striking the grating surface is dispersed following the Huygens–Fresnel principle.



Schematic of dispersion with focal length

The last factor that influences spectral resolution is the pixel dimension in the CCD detector, the smaller the pixel size, the greater the spectral resolution.

RAMAN IMAGING MICROSCOPY

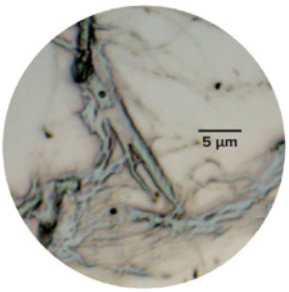
TIPS & TRICKS

3

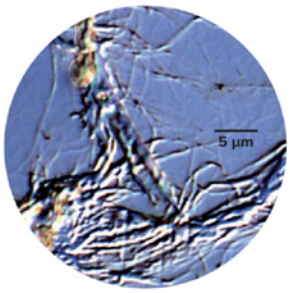
Observation

Samples that are often measured using Raman imaging microscopy may have little or no observable structure, in these cases the image can be improved by making use of some of the visualization techniques developed for light microscopy.

Differential Interference Contrast Observation (DIC) uses polarized light and a Nomarski-modified Wollaston prism to enhance the observation of images with low contrast. DIC uses phase difference in light to stereographically view very small step differences in the submicron order. Nomarski prisms are used to create bright and dark contrast from the differences in the two beams directly reflected at the sample's surface. This technique can be applied equally to low contrast biological and non-biological samples that have small unevenness in the surface.

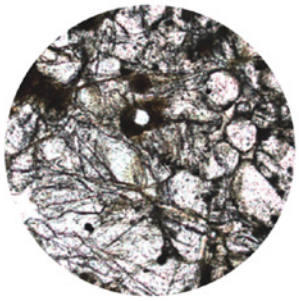


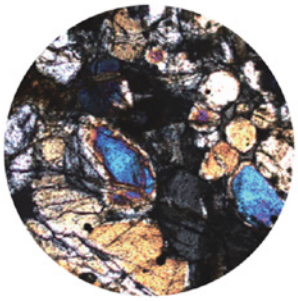
Bright Field Microscopy



DIC Microscopy

Polarized Light Observation (PLO) exploits the differences in anisotropic properties to enhance the observation of materials with low contrast. PLO uses two polarized elements located in the optical path on each side of the sample being observed. It is particularly useful for samples such as biomolecules and biostructures, minerals, ceramics, mineral fibers, extended polymers, liquid crystals, etc.


Parallel Polarizers


Crossed Polarizers

RAMAN IMAGING MICROSCOPY

TIPS & TRICKS

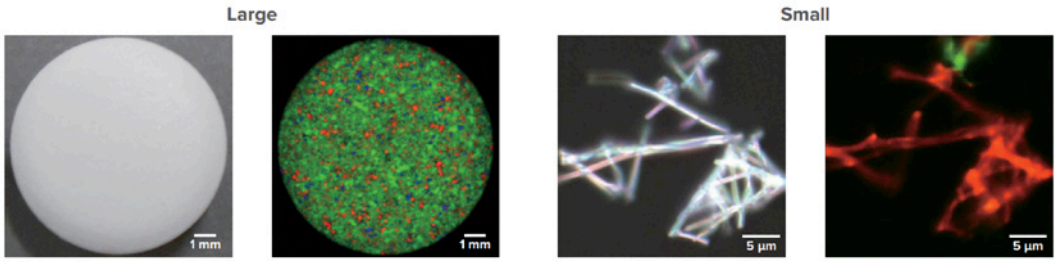
4

Imaging

Raman imaging is a powerful technique that provides information about spatial distribution in 3D as well as chemical identification. Small or large sample areas in the order of micrometers to millimeters can be measured and imaged in times as short as a few minutes. JASCO developed QRi, a technology that increases the data acquisition speed by up to 50 times compared with conventional mapping and also offers a dramatic improvement in sensitivity.

Speed and sensitivity of QRi Raman imaging are achieved with:

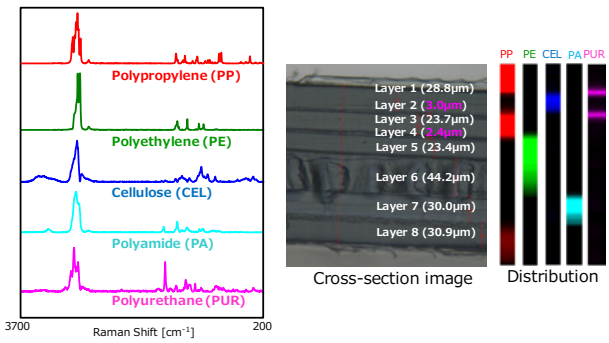
- A high-speed XYZ automated stage with step resolution OF ONLY 0.1 µm
- A high sensitivity electron multiplied EMCCD in place of the conventional CCD



Examples of small and large mapping areas

Mapping

When using conventional software without an intelligent interface, experience is required to create a good color distribution map of the chemical structure of the sample. However, Spectra Manager™ analysis wizard makes it possible to create clear and informative distribution images. The wizard provides recommendations at each step in the data processing to automate the process. The Raman spectral data is truncated to a range of interest, noise reduction and baseline correction are applied, and multivariate curve reduction (MCR) chemometric modeling is used to create the mapped image. Enhanced post processing algorithms for faster noise reduced data analysis includes digital filtration and spectral averaging.



Depth profile of a packaging laminate with microscopic image and chemical distribution

RAMAN IMAGING MICROSCOPY

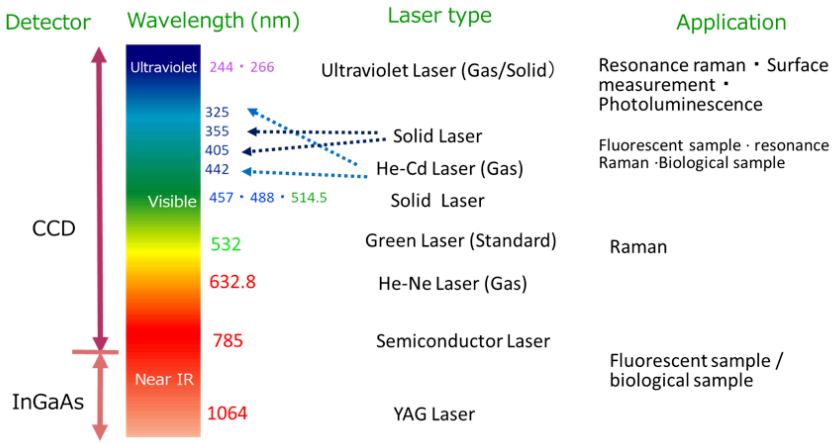
TIPS & TRICKS

5

Laser Selection

Selection of the laser wavelength is important in obtaining meaningful Raman data, the excitation wavelength can be selected from the UV though visible to near IR, and with a range of power options depending on the application. Multiple lasers with a variety of wavelengths can often be installed in a Raman microscope to allow data to be measured for different types of sample. A popular laser wavelength is 532nm because it offers relatively high Raman excitation energy with low fluorescence.

However for greater reduction in fluorescence, longer wavelength lasers such as 785nm and 1064nm are often used. Although less frequently required, UV lasers offer the additional benefit of “resonance Raman”, an excellent choice for carbonaceous materials.



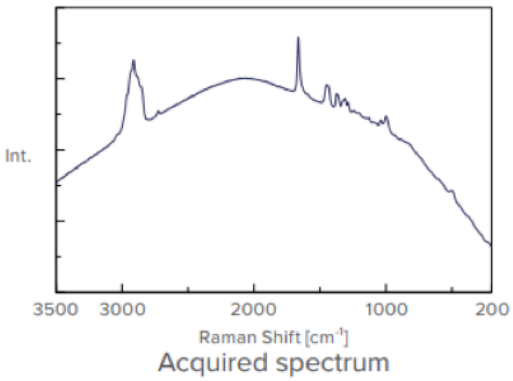
RAMAN IMAGING MICROSCOPY

TIPS & TRICKS

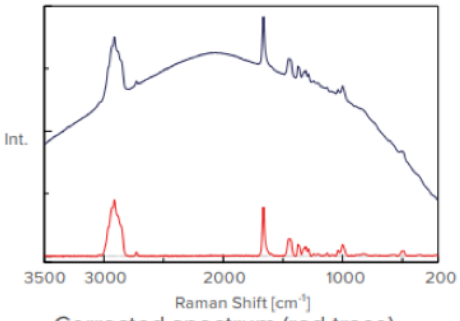
6

Fluorescence

Fluorescence can be a side effect in Raman measurement, it can occur in either the target molecule, or the surrounding matrix. Care has to be taken if the sample is contained in a vial or tube as this can also cause background interference. There are two physical methods for reducing fluorescence. The first is to select a laser wavelength at which fluorescence does not occur, this is typically at longer wavelengths where there is insufficient energy for electron excitation. The second method is to use aperture size and shape to mask as much of the sample matrix as possible to eliminate matrix fluorescence – this is improved with dual spatial filtration (DSF) included with NRS-5000/7000 spectrometers. A third option is the use of a fluorescence rejection algorithm (patented), which is highly effective at removing the broader fluorescence spectrum, leaving the sharper Raman peaks in a clean baseline, as shown below. This feature can be used either automatically during measurement for fast imaging, or post run for more additional data processing. This often works well for samples with strong fluorescence when using shorter wavelength lasers (such as 405 or 457nm), which give a more intense Raman signal.



Acquired spectrum



Corrected spectrum (red trace)