Microscale chemical imaging using vibrational spectroscopy methods

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By combining a light microscope with an infrared or Raman spectrometer both chemical and spatial information may be obtained simultaneously from complex samples. This is of special interest not only for biological samples, but also for novel functional materials, where many properties strongly depend on the spatial distribution of their chemical components.

An overview will be given of modern instrumental designs of FT-IR and Raman microscopes with fast mapping techniques or focal plane array (FPA) detectors to obtain 2D and 3D spectral images of multicomponent samples. The complementary informative value of IR and Raman microscopy will be assessed. Spatial resolution of the methods will be discussed and their imaging power compared with other microscopic systems (light and electron microscopy).

Examples from polymer (industrial and biological) and semiconductor characterisation will be presented to show the potential of these methods. Some of these will also deal with radiation damage of soft and photosensitive materials caused by focused ion beam (FIB) preparation or electron radiation.

Keywords FT-IR microscopy; Raman microscopy; chemical imaging; spatial resolution

1. Introduction

One of the most challenging areas in materials science today is the design of new high performance materials. The nearly unlimited field of functional materials is growing very fast. Modern applications demand material properties, which may only be obtained by special processing methods (e.g. compounding, stabilising, and coating) and a careful selection of basic raw materials.

The interdependence of performance, properties, synthesis and microstructure (often referred to as the “materials tetrahedron” [1]) reflects the four fundamental principles of modern materials science. Those may be treated as individual disciplines for elementary issues, but a comprehensive approach to the materials tetrahedron is usually required for complex problems. The whole potential of new materials systems, whether they might be manufactured of a single compound or be a composite, can only be evaluated, if the microscopic details, even down to the molecular or atomic level, are fully understood.

In analytical investigations, microscopic characterisation methods play a fundamental role. A nice introduction to the application of these methods in polymer characterisation is given in reference [2]. Heterogeneous samples can be composed of different types of materials, both inorganic and organic (e.g. co-polymers, textiles, mineral fillers, pigments), and incompatibility or miscibility issues of the different components can be a problem. The physical and chemical properties of complex samples are strongly influenced by the spatial distribution of their chemical components. Thus, analytical methods providing information of both the molecular nature and the localisation of their components are essential for the design and fabrication of advanced heterogeneous materials. Vibrational spectroscopic methods, such as Fourier transform infrared (FT-IR) and Raman spectroscopy, are particularly attractive, as they may not only be used for bulk or point analyses, but also as imaging methods providing both chemical and spatial information simultaneously. The spectrally and spatially resolved chemical information can be quickly collected, analyzed and visualised – even for systems that do not have much inherent visible contrast in conventional light or electron microscopy.

We will start our considerations with Raman microscopy, because the optical setup of a Raman microscope corresponds to that of a conventional light microscope. After a short survey of sampling techniques (point measurement, line scanning, mapping and imaging) we will discuss spatial resolution in the microscope as the limiting factor for the expressiveness of these methods. Then we will continue with IR microscopy, the spectroscopic method giving the richest chemical information from complex molecules, particularly organic ones. Two-dimensional detectors with a high number of detector elements allow the creation of images based on vibrational spectroscopic information with micrometer resolution. Practical examples will mostly deal with polymer characterisation (the main field of activity of the authors), and shall try to illustrate the possibilities and limitations of these methods for the characterisation of heterogeneous materials. Raman and IR microscopy in the context of this survey will be limited to conventional microscopy, disregarding the range of near field and tip enhanced techniques requiring a special instrumental setup and sophisticated sample preparation.
2. Raman Spectroscopy

Vibrational spectroscopy is based on the interaction between light and matter causing different vibrational states of the investigated molecules. A number of excellent books describing the principles, instrumentation and applications are available [e.g. 3, 4], also on micro spectroscopy [5, 6].

While IR spectroscopy requires radiation to be absorbed by the investigated sample, Raman spectroscopy is based on inelastic scattering ("Raman scattering") of monochromatic light, usually from a laser in the visible, near infrared or ultraviolet range. While the laser photons are predominantly scattered elastically ("Rayleigh scattering"), the Raman effect occurs when light interacts with the electron cloud, resulting in an energy shift of the photons. According to the selection rules, Raman spectroscopy (change of polarizability) and IR spectroscopy (change of polarisation) yield similar - but complementary - information on the chemical nature of the molecule.

The following considerations will only deal with dispersive, not with FT Raman spectroscopy. In a dispersive Raman instrument the sample is illuminated with a laser beam; the scattered light from the illuminated spot is collected with a lens and sent through a monochromator. Wavelengths close to the laser line (Rayleigh scattering) are filtered out, and the rest of the collected light (Raman scattering) is dispersed onto a detector.

While IR spectroscopy has been used as a routine tool in analytical laboratories for a long time, Raman spectroscopy was largely confined until the 1990s to only a few experts, until advances in instrumentation (lasers, Notch filters, CCD cameras) facilitated a broader acceptance and opened up many new fields of application in analytical investigations and quality control. In particular, the combination of Raman spectroscopy with a light microscope has proved to be an essential tool for materials analyses [6]. To assess the spatial resolution of a dispersive Raman microscopy system (excitation lasers in the visible range) the same relations as for light microscopy may be applied.

2.1 Single point spectra

In the simplest case the laser beam is focused through the microscope objective onto the surface of the sample, and the scattered light is collected with the same optics in 180° backscattering geometry. The spectrum obtained by this procedure provides information of the probed sample volume defined by the magnification power of the microscope objective and its confocality (see below). Using an excitation source in the visible range of the spectrum and a standard 50x objective on the microscope, the diameter of the laser spot on the sample will be about 2 micrometers in diameter. The penetration of the laser beam into the sample (transparency, opacity) and the so-called "depth of focus" have to be considered, describing the region around the theoretical focal point in which the laser beam waist diameter remains unchanged. The depth of focus (dof) can be calculated with the following equation (λ: wavelength of the excitation laser, α: half of the opening angle of the objective):

\[ \text{dof} = \frac{4 \cdot \lambda}{(\sin \alpha)^2} \]

This equation is only valid, if the beam is focused on the sample surface. In opaque or transparent samples, where the laser light can penetrate the surface and be scattered into deeper regions, Raman light from deeper zones also contributes to the collected signal. This is of particular relevance with non-homogeneous samples (e.g., multilayer systems or blends). Confocal Raman microscopy is a very useful technique to probe such samples on and below their surface (depth profile). This non-destructive method (no microtome sectioning necessary) may be used for studies on thin layers, inclusions and impurities buried within a matrix, and will be discussed below.

2.2 Line scans

The conventional way of line scanning consists in collecting a number of spectra at equidistantly spaced points along a line. But taking full advantage of a two-dimensional CCD detector can save time. A computer-controlled optical scanner focuses the laser beam as a line on the sample plane. The Raman scattering generated from that line on the sample is subsequently projected onto the entrance slit of the spectrometer and imaged onto a row of the CCD. This approach provides simultaneous acquisition of Raman signals from each point of the sample illuminated by the scanned laser line [7]. Furthermore, by moving the sample perpendicular to the laser line, even two-dimensional Raman images may be obtained rapidly.

2.3 Confocal Raman microscopy

To get both high lateral and depth resolution, Raman microscopy has to be done in confocal mode, the fundamental optical arrangement being the same as in confocal light microscopy. Small pinholes or apertures are placed in the back focal plane of the microscope in order to isolate a small sample volume, from which the emerging Raman light is focused onto the detector. The pinholes used in the original approach (also referred to as "true confocal", [8]) may be replaced by one mechanical slit aperture and the two-dimensional CCD as a second electronic aperture (sometimes referred to as "pseudo confocal"), mainly to facilitate the necessary optical alignments ("easy confocal", cf. [9]). The
fundamentals and theory for application, and practical limitations of confocal Raman microscopy have been discussed by N.J. Everall [10].

In practical applications it has been shown that depth resolutions around 2–3 µm collected with a 100x microscope objective are possible. However, the depth resolution will degrade when probing deeper into the sample; this is a consequence of refraction caused by refractive index changes at the sample surface and boundaries within the sample. At least it is possible to do nondestructive Raman depth profiling (including subsurface imaging, as will be shown below) with good spatial resolution and sensitivity, provided care is taken to minimise spherical aberration by a properly corrected objective. In special cases aberrations may be minimised by using an oil immersion objective [11]. Otherwise, depth profiles will be badly distorted with degraded depth resolution, and signal intensity will fall rapidly with depth. The working spatial resolution is also affected by the transparency of the sample, because weak spectral contributions are detected from out-of-focus regions, which may become quite significant in some cases.

2.4 Mapping and imaging

Whenever large sample areas have to be investigated with regard to the distribution of different species, mapping will be the method of choice. Single spot Raman spectra are collected in equidistant steps within a grid pattern on the sample surface (or subsurface exploiting the confocal power of Raman microscopy). Both, the spatial and chemical information recorded, form a “data cube”, as illustrated in Fig. 1. Using normalised band intensities, integrated areas of single bands (characteristic for the presence of a certain chemical species in the composite material), or band ratios, a number of different maps can be created. By superimposing these maps with the light microscopy picture of the area analysed, selected regions of the sample may be related to the presence of spectroscopically identified species.

All optical considerations for the collection of single spot Raman spectra also hold for mapping. The spatial resolution in a map depends not only on the measured volume on the sample, but also on the distance between the single points. By increasing the distance, the spatial resolution in the map worsens, but larger sample areas can be probed in a shorter timescale. This is especially advantageous when structures within the sample are large compared to the resolution of a single spot spectrum; experiment times get longer if the investigated area is extended or the number of grid points increased. Technically, the area that can be probed is only limited by the dimension of the moveable microscope stage. Companies producing Raman microscopes have come up with manifold ideas to speed up spectroscopic acquisition of large areas, e.g. by expanding the laser spot to lines or areas using cylindrical lenses or mirrors. Quick spectral surveys are possible with these sophisticated instruments, to discern gradual variations of components, or to locate very small features within a large area [7,9,11]. On the other hand, by making the distance between two measuring spots smaller than the laser size, the best spatial resolution for the current configuration may be achieved (known as “oversampling”).

In Fig. 1 the data cube of a two-dimensional map is shown. One element (row) of the cube has been taken out and enlarged on the right side of the figure. It is visible that each row contains the information of a whole spectrum. In the z-direction (depth-direction) the resolution is determined by the confocal instrument settings. While it is essential to be aware of the limitations of confocal measurements, as mentioned above, it is possible to create 3-dimensional maps by probing a sample in x, y and z-direction.

A practical example is shown in Fig. 2, which shows a Raman map of a logo (Graz University of Technology), produced on the surface of a silicon plate by the Focused Ion Beam (FIB) technique [12]. Due to the implantation of Gallium ions, the FIB-etched details are not Raman active (dark structures in the figure, where no silicon signal can be detected). The size of the whole logo is approximately 11 µm x 7 µm. A red laser (633 nm) has been used. The step width was 0.2 µm, and the spectra were collected with an acquisition time of 2x0.5 seconds. Preparing such types of samples may help in evaluating the spatial resolving power of Raman instruments (because the dimensions of the etched structures are precisely known). Otherwise, silicon stress may be detected and mapped in correlation to the processing of silicon wavers (by recording changes of position and shape of the Raman silicon line).
Fig. 1: Data cube generation in mapping and imaging. The 4-dimensional hyper-spectral cube contains the full spectral information, signal intensity vs. wave numbers (cm$^{-1}$), for each x,y pixel from the imaged area. A horizontal slice through that cube contains a chemical image (band intensity at a selected wave number for each x,y pixel of the image, as shown below).

Fig. 2: Raman map of a FIB-modified silicon sample (area ca. 11 µm x 7 µm; see electron micrograph in the upper right corner). The cleft at position 2.66 (arrow) is clearly visible in the intensity diagram (lower graph). The small picture right of centre is the Raman image after processing with PCA (see below, chapter 3.5).
Another Raman imaging technique sometimes used, is the so called "Global imaging", a wide-field illumination technique. It is faster than both the point- and line-scanning (mapping) methods, but might be more sensitive to artefacts arising from scattering effects due to the topography of the investigated surface [13]. The area is limited, due to decreasing illumination density on the sample surface when defocusing the laser. The technique has higher spatial, but worse spectral resolution, compared to mapping. Possibilities for data treatment are limited, since this image is only a slice through the hyperspectral data cube, but not a set of spectra. An image is generated in one single step, essentially at one particular narrow wave number region (typically about 20 wave numbers, selected by filters), using the CCD detector as a camera. Area selection on the specimen is done by defocusing the laser spot onto the image plane over the desired size. Defocusing the laser beam means parallel (instead of convergent) illumination. The foci of the laser beam and the microscope objective are not in the same plane anymore. The diameter of the probed area is determined by the magnification of the objective and the degree of defocusing (with a standard 50x objective a circle with about 50 µm in diameter). The selected wave number region should be free of any other band that could interfere with the Raman signal of interest. A background image has to be collected from exactly the same imaged area, but in a spectral region that has no Raman bands from the observed sample. This approach will also account for inhomogeneities in laser intensity distribution and features arising from fluorescence. Global Raman imaging can be a fast and simple technique, providing high lateral resolution (down to the diffraction limit corresponding to the excitation laser wavelength) images of the specimen, but - of course - no depth resolution.

As an example, Fig. 3 shows an image of a polymer blend consisting of polyamide (PA) and poly(tetrafluoroethylene) (PTFE). Such materials are used for friction bearings, and the interesting question was to determine the size and shape of the PTFE clusters forming within the PA matrix [14].

![Raman image of PTFE clusters](image.png)

Fig. 3: Raman image of PTFE clusters in a PA matrix (imaged area 20 µm x 20 µm; high intensities of PTFE signal are displayed in bright color; left), and spectral regions used for imaging (right). The area around 731 cm⁻¹ is selective for PTFE [a]. The second area around 790 cm⁻¹ [b] was acquired to obtain an image for background subtraction (no contributions from PTFE or PA; right).

### 3. Infrared microscopy

IR spectroscopy is based on an absorption process. A broad band source, covering the whole mid- infrared region (and beyond) is used in a typical FT-IR spectrometer. The mid-infrared region of the electromagnetic spectrum refers to wavelengths between 25 000 and 2500 nm (4000 cm⁻¹ to 400 cm⁻¹). In this chapter the terms “infrared” or “IR” will be used to indicate the mid-infrared region. The IR radiation can excite vibrations, and photons of the appropriate frequency for certain molecular excitations will be absorbed. The absorbed part of the infrared beam can be expressed in terms of absorbance or percentage loss in transmitted light compared to a reference beam (i.e. without the absorbing sample). While Raman spectra can be acquired without any further preparation (180° backscattering geometry), samples for infrared transmission spectroscopy have to be of appropriate thickness (typically a few µm). Taking transmission spectra from solid samples often requires a microtome for sample preparation. For mapping and imaging transmission and attenuated total reflection (ATR) will be the most interesting sampling techniques.

#### 3.1 IR transmission spectra

In transmission mode a spatial resolution of about 15-20 µm can be achieved with infrared microscopes [5]. This is generally sufficient to identify small regions, inclusions etc. Similar to Raman spectroscopy, line profiles or maps over larger sample areas can be performed.
In an IR microscope Cassegrainian objectives are used for focusing the beam, instead of glass lenses (which are impenetrable for IR radiation). The objective illuminates the sample, and, after passing through the specimen, the infrared light is collected by the condenser (when working in transmission mode). To define the interesting area, in most cases rectangular apertures are used, usually located in a remote image plane. Because the spatial resolution of IR microscopes is limited by diffraction (see below) they cannot be made arbitrarily small (minimum about 10 - 20 µm).

3.2 IR ATR Spectra

With materials investigations surface sensitive techniques are of special interest. The major contribution of infrared spectroscopy to this field is internal reflection spectroscopy (IRS), often called the “attenuated total reflection” (ATR) technique [15].

In practice a crystal of a material with high refractive index $n_1$ (the ATR crystal), such as diamond ($n=2.4$), zinc selenide ($n=2.4$), silicon ($n=3.4$), or germanium ($n=4.0$) is brought into contact with the surface of a specimen with lower refractive index $n_2$ (for polymers $n$ is approximately 1.5). The infrared beam passing through the ATR crystal is directed to this crystal-sample interface at an angle greater than the critical angle $\alpha_c$ to ensure that only internal (total) reflection occurs at the crystal-sample boundary, and that the infrared throughput is as high as possible. The critical angle is given by: $\sin \alpha_c = (n_2/n_1)$.

Simply speaking, the infrared beam penetrates (about 0.5 - 2.5 µm) just beyond the crystal-specimen boundary before it is reflected back and makes its way through the crystal to the detector. On this short path (the "evanescent wave") into the sample surface layer, light is absorbed, and the reflected beam carries characteristic spectral information of the sample. The depth of penetration depends on the incident angle, the refractive indices and the wavelength of the radiation. As a consequence of lower penetration at higher wave number (shorter wavelength), bands are relatively weaker compared to a transmission spectrum, but surface specificity is higher.

It has to be mentioned that the ATR technique may be potentially destructive to samples. Because the ATR crystal has to be brought in contact with the sample, both the sample (especially with soft materials) and the ATR crystal may be damaged, when not carefully handled. Pressing too hard will also affect dramatically the penetration depth into the sample, and, when used in imaging mode (next chapter), may even distort the resulting images.

3.3 IR imaging

With IR imaging band intensities and/or position are evaluated by appropriate software algorithms (e.g. integration) in order to display chemical and physical parameters of the sample in a multi-colour or grey-scale diagram (as discussed above with Raman imaging). Modern sophisticated instruments have overcome the drawbacks of mapping experiments (time-consuming, poor spatial resolution) by using focal plane array (FPA) detectors, consisting of a large number of mercury cadmium telluride (MCT) detector elements (typically 64 x 64 elements, but smaller and larger array sizes are offered by the instrument manufacturers, [16]). Detectors of this kind acquire a large set of complete infrared spectra simultaneously, which can then be used for image creation. Acquisition time is typically in the range of several minutes. Other systems are equipped with a linear MCT detector array, and images are generated by moving the sample stage or the ATR crystal [17]. When used for transmission measurements, an FPA detector with 64 x 64 elements can probe an area of about 270 x 270 µm$^2$ (using a standard 15x Cassegrainian objective). Specimen thickness needs to be a few microns (5 - 10 µm) thick. Detailed information about instrumentation and applications may be found in two books published recently, covering both IR and Raman imaging [18, 19].

An increase in lateral and depth resolution can be obtained by recording images in the ATR mode, exploiting the higher refractive index of the medium between the sample and the objective (ATR crystal instead of air). An excellent introduction to IR micro- and macro-ATR imaging has been published recently [20].

With mapping radiation passing through apertures is diffracted (due to its wave nature), and the diffraction increases with decreasing aperture size. The resulting diffraction pattern of a uniformly illuminated circular aperture has a bright region in the centre, known as the "Airy disc" or "Airy pattern" (named after the British astronomer George Biddell Airy), which is surrounded by concentric rings (Fig. 4). The diameter of this disc is related to the wavelength of the illuminating light and the size of the circular aperture. The angle from the centre at which the first minimum occurs is: $\sin \theta = 1.22 (\lambda/d)$, where $\lambda$ is the wavelength of the light and $d$ is the diameter of the aperture [21].
The resolution or “resolving power” of a light microscope is usually specified as the minimum distance between two lines or points in the imaged object, at which they will be perceived as separated by the observer. The Rayleigh criterion [22] determines the resolution of optical microscopes and imposes a resolution limit. The criterion is satisfied, when the centre of the Airy disc for the first object occurs at the first minimum of the Airy disc of the second. This minimum distance \( r \) can then be calculated by:

\[
\frac{1.22 \cdot \lambda}{n \cdot \sin \alpha}
\]

\( \lambda \) is the wavelength of the radiation, \( n \) the refractive index of the medium between sample and objective (in most cases air), and \( \alpha \) half the opening angle of the objective. Often the term \( n \cdot \sin \alpha \) is called the numerical aperture (NA) of the system. The theoretical spatial resolution, as deduced from Rayleigh’s criterion, however, is heavily deteriorated by diffraction effects at the edges of apertures, as in conventional IR microscopy (a practical example is presented in [19]).

Taking into account the above considerations, spatial resolution (in contrast to conventional mapping techniques) may be improved by three ways:

1. Apertures should be avoided, and FPA detectors used instead.
2. Characteristic absorption bands at higher wave number (shorter wavelength) should be used for evaluation, when possible. (However, O-H and C-H absorptions are not always really helpful, unless they are really specific for the problem).
3. ATR technique should be preferred to transmission measurements, if applicable.

Table 1: Values for \( r \) (Rayleigh criterion) estimated for three characteristic wave numbers; typical instrument parameters for transmission and ATR experiments assumed.

<table>
<thead>
<tr>
<th>Absorption band (Wave numbers)</th>
<th>NA = 0.4 (x15 Cassegrainian objective, air) Transmission</th>
<th>NA = 2.4 (x20 ATR objective, Ge) ATR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 cm(^{-1}) (C-O, Si-O)</td>
<td>15 ( \mu )m</td>
<td>3 ( \mu )m</td>
</tr>
<tr>
<td>1700 cm(^{-1}) (C=O)</td>
<td>9 ( \mu )m</td>
<td>2 ( \mu )m</td>
</tr>
<tr>
<td>3000 cm(^{-1}) (C-H)</td>
<td>5 ( \mu )m</td>
<td>1 ( \mu )m</td>
</tr>
</tbody>
</table>

Table 1 shows some approximate \( r \) (Rayleigh criterion) values calculated for frequently used absorptions. NA values are assumed for customary Cassegrain objectives (for transmission and ATR, respectively). Several examples from the field of polymer characterisation are discussed in [19,23,24], where the authors tried to confirm the conclusions drawn from these theoretical considerations, and to correlate IR and Raman imaging results with the results of other microscopic techniques (e.g. electron microscopy).

3.4 IR ATR image of a multilayer film

In multilayer films, e.g. such as used in food packaging, each layer has a different function contributing to the desired properties of the final product (flexibility, stability, permeability, visual appearance, etc.). In order to characterise the individual layers in such a film, a cross section has to be made by microtomy (or cryo-microtomy, especially for soft materials), after embedding the film in an appropriate embedding medium.
A light microscopy picture of a multilayer film (taken in reflexion) is shown in Fig. 5a. At least 5 different layers varying in thickness and appearance can be distinguished. From that cross section an IR ATR image has been taken (measuring area 50 µm x 50 µm), to analyse the chemical species (Fig. 5b). For the images the ester band has been integrated, bright colours representing areas of high ester intensity. The following layers have been detected:

A: Polyethylene terephthalate (PET); single IR spectrum shown to the right of Fig. 5b.
B: A polymer similar to PET (but more C-H and less ester bands), thickness about 8 µm. Strictly speaking, this layer consists of three different species, one of them containing a pigment. Due to the chemical similarity of the 3 parts of this layer and possibly also because of smearing effects (during microtomy), these layers cannot be resolved reliably by IR spectroscopy.
C: An aluminium barrier layer (no IR spectrum); thickness about 8 µm;
D: A polyester (single spectrum shown to the right); thickness about 4 µm;
E: Polyethylene (spectrum shown to the right).

The resolving spectroscopic power is substantially good for these layers. It is, of course, not possible to measure the exact thickness of each layer because of overlapping due to the diffraction limits (discussed above). Valuable complementary information can be obtained by other methods, like Raman and electron microscopy [25].

Fig. 5a: Light microscopy image of multilayer film cross section (microtome section).

Fig. 5b: IR ATR image of a multilayer film (cross section). Imaged area: 50 µm x 50 µm. For explanations see text.
3.5 Processing of images with chemometric tools

As the output from a hyper spectral experiment provides a very large data set, it is important to know about the tools capable to handle such huge data sets and to improve image representation, i.e. to extract any interpretable results. The use of chemometric methods in image analysis is crucial in order to take advantage of the full information contained in the measurement results [18]. PCA (principal component analysis) decomposes a data set into "principal components", so that the relevant information in the hundreds or thousands of spectral channels is contained in a very small number of principal components. Only the first few of them describe spectral variations linked to the chemical species, while the others only account for noise-related signal contributions. Two particular potential benefits of using PCA are the ability to assist with generation of survey images to locate finer details with a minimal number of scans and the ability to assist with interpretation of relatively subtle spectral differences in the image data. Although images are mainly focused on providing information on the chemical composition of the material under investigation (qualitative analysis) and the component distribution, there are chemometric procedures available to extract quantitative information from such images too (multivariate image regression, which includes multivariate calibration methods, [18]). An example of a Raman image refined by PCA processing has been shown in Fig. 2 above.

4. Summary and conclusions

For the spectroscopist today it is very important to choose the right method, to match the demands of the customer or serve the purpose of his research as effective as possible (a cost-benefit analysis). For the characterisation of heterogeneous samples a broad range of methods and instruments is available.

Infrared spectroscopy/microscopy certainly is the method of choice, when organic substances have to be identified. Sample preparation usually is simple for identification purposes, but will be an issue for imaging experiments, and spatial resolution may then well be only in the range of a few micrometers, depending on the used experimental approach (transmission or attenuated total reflection).

Raman microscopy provides a spatial resolution slightly better than IR, and no sample preparation is necessary in many cases. It has advantages with special types of substances (e.g., systems containing conjugated double bonds, oriented systems, amorphous and crystalline carbon, oxides, semiconductors). SNOM techniques (with spatial resolution below 1 µm) have been more popular with Raman than with IR, so far, but as yet are not routinely practiced.

Many other popular and meaningful analytical techniques, like NIR (near infrared), ToF-SIMS (time of flight secondary ion mass spectroscopy), NMR (nuclear magnetic resonance) and ESR (electron spin resonance), XRF (X-ray fluorescence), may also be used in microscopic layouts (with spatial resolution in the micrometer range), and even in imaging mode, with rich analytical profit [19].

The advantages of imaging methods (including fast mapping techniques) are obvious, whenever multicomponent samples have to be investigated:
- Short measuring times, when many spectra can be recorded simultaneously.
- Better spatial resolution, when apertures can be avoided.
- The output of imaging experiments is readily comprehensible to non-spectroscopists (comparable to light and electron microscopy), because the spectroscopic images may directly be compared with light or electron micrographs. (Or, to modify a famous saying: "An image is worth a thousand spectra.")

Careful attention should be paid to sample preparation, however. The materials investigated may be changed by the preparation procedure (e.g. smearing during microtomy of soft materials at room temperature instead of cryo-microtomy) or during the measurement (radiation damage, contact with ATR crystal, etc.).

Analysts may benefit from a smart combination of several spectroscopic methods, e.g. IR and Raman - both methods giving complementary information on the same sample. Interesting results may be obtained by combining micro-spectroscopic with other microscopic techniques (light and electron microscopy, thermal microscopy). Namely scanning electron microscopes can be equipped with a wide range of different spectroscopic probes, like X-ray spectroscopy, and even Raman spectroscopy. Data evaluation exploiting chemometric methods (multivariate data analyses, PCA), already integrated in many commercial spectroscopy software packages, will further improve the results and enhance their reliability.

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