MOLECULAR SPECTROSCOPY

Thermo Scientific Microscopy Handbook

High-Performance Infrared Microscopy

Part of Thermo Fisher Scientific

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Table of Contents

Definition of Microscopy

Microscopy is the art and science of making fine details visible. This definition also applies to infrared and Raman microscopes, since the goal of each is to collect spectra free of spectral contributions of the surrounding matrix from the fine details of a sample. Visible light design considerations of a microscope involve magnification, resolution and contrast. The most important visible light consideration is resolution. Without high-resolution capability, the fine details are not visible at higher magnifications. Infrared considerations for a microscope involve aperturing, sample focus and detector sensitivity; each of which is an equally important component contributing to the final spectrum, making any deficiency glaringly obvious in the end result.

The Thermo Scientific brand offers several quality infrared and Raman microscopes that allow you to obtain spectra and visible images from the sample. The Thermo Scientific Nicolet™ Continuμm™ FT-IR microscope provides many features normally found on quality light microscopes, allowing collection of high-quality visual images of samples using a variety of contrast-enhancement techniques.

These features allow more complete analyses on the infrared microscopes. The Continuμm has many patented features that provide the best spatial resolution, ease of use and configuration flexibility in the industry. Throughout this handbook, several will be presented, illustrating the capabilities of high-quality infrared microscopes with exceptional visible-light characteristics.

Thermo Scientific Nicolet Continuμm FT-IR microscope

Unparalleled Technology

Our Nicolet FT-IR and Raman microscopes use exclusive technologies, such as true infinity corrected optics from objective to viewer, simultaneous sample view collection and redundant aperturing. Infinity correction provides high-quality optical and infrared performance since the image information is sent in a collimated beam of light, unaffected by optical elements, such as filters and polarizers. The simultaneous sample view/collect feature allows you to preview the spectrum while observing the sample, ensuring accurate sample placement and quality spectra. The redundant aperture allows you to collect data on extremely small samples without interference from the surrounding matrix. The Continuμm offers multiple infrared and visible light objectives that can be mounted on a removable nosepiece, providing an efficient way to configure the microscope for most sampling conditions. In the pages that follow, infrared and Raman sampling, contrast enhancement, hardware selection and microscopy terms will be discussed to provide a better understanding of quality FT-IR and Raman microscopy.

Thermo Scientific Nicolet Centaurμs™ FT-IR microscope

Thermo Scientific Nicolet Almega™ XR microscope

Sampling Methods

The advantages and disadvantages of various infrared sampling techniques are highlighted in this section. Although no one technique can be applied to every sample, each approach has distinct advantages that can be exploited for a given sample.

Transmission

Definition

Transmission analysis involves passing the energy through the sample and detecting that portion that is not absorbed or that is transmitted. The energy is then focused on the sample by the objective, and collected below the sample by the condenser. On the stage, the sample may be self supporting – such as larger films or plaques. The sample may be in the form of particles or fibers that require support in an infrared transparent window. Typically, two such windows are used, with the sample placed between. Since the window materials above and below the sample introduce boundaries between layers of different refractive indices, spherical aberrations can blur the sample. Simply adjusting the objective or condenser compensation ring to the proper window thickness restores sharp image viewing and accurate infrared sampling, free of spherical aberration.

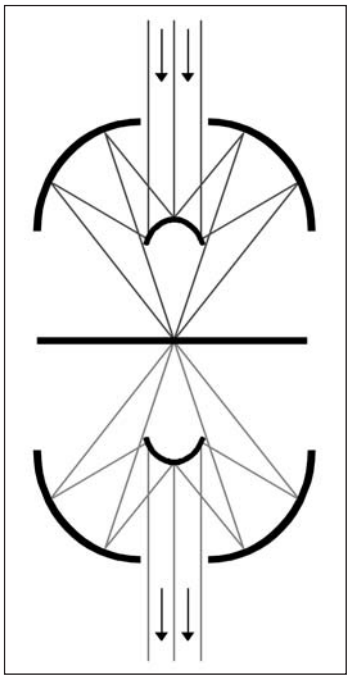

Figure 1: Transmission ray trace

Considerations

Transmission samples require preparation usually in the form of flattening or cutting very thin sections. This not only creates a larger area for the infrared aperture – a mask used to define area of the sample to be analyzed – but also reduces the thickness of the material thereby decreasing the intensity of the spectral bands. The spectral intensity of the bands of interest should be less than 0.7 absorbance units in order to avoid non-linear response of the infrared detector. Compression cells speed the analysis by combining sample support and compression in one step.

Reflection

Definition

Reflection analysis is an optically simpler technique that involves reflecting the infrared light off of the sample. In this mode, the objective serves to focus light on the surface, and to collect the light from the sample as well. This mode of analysis requires that the sample have certain properties that allow the infrared radiation to be reflected in one of several ways.

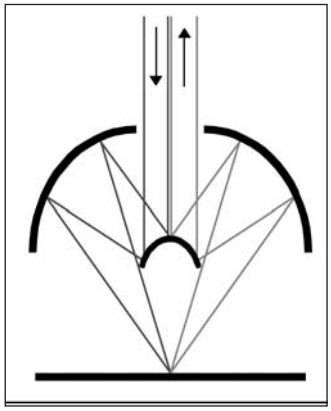

Figure 2: Reflection ray trace

Considerations

There are several forms of reflectance that can occur depending on the surface characteristics of the sample. No changes to the microscope or software are needed. However, there are several software corrections and conversions that may be applied to the collected spectra to make them more compatible with transmission spectra. Specular reflectance occurs when the thick (a few mm) sample has a flat, smooth and glossy surface causing the infrared energy to reflect off the front surface of the sample at the same angle as the incident light. Diffuse reflectance occurs when the sample has a rough surface, causing the IR energy to reflect at angles other than the incident energy and from different locations within the sample. Reflection absorption experiments involve mounting the sample on a reflective surface. The infrared energy passes through the sample, reflects off of the reflective substrate and passes back through the sample effectively, approximating a double pass transmission experiment. Most samples analyzed via reflection produce combinations of specular, diffuse or reflection absorption. This complexity can be overcome by additional sample preparation by choosing another technique.[1,2] The advantages of reflection include little or no sample preparation and fast sampling.

Attenuated Total Reflectance

Definitions

Attenuated total reflectance (ATR) is the easiest mode of analysis, in which the sample is placed in physical contact with the ATR crystal. The infrared energy passes through the crystal at an angle that is greater than the critical angle of incidence for the specific crystal material. This causes the IR energy to reflect off the internal surface of the crystal and return to the detector. At the reflection point in the crystal an evanescent (standing) wave is created, which interacts with the sample that is compressed against the crystal. An infrared spectrum results from the interaction at the interface. The depth of penetration into the sample varies as a function of the wavelength of the infrared energy, the incident angle, the refractive index of the crystal, and the refractive index of the sample. The depth of penetration can be calculated by the following formula:

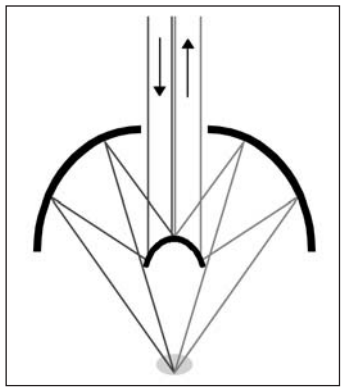

Figure 3: ATR ray trace

$$d_p = (\lambda/2\pi n_0\sqrt{(\sin^2\Theta - n^2/n_0^2)})$$

Where λ is the wavelength of light, Θ is the angle of incidence, $\mathbf{n}$ is the refractive index of the sample, and $\mathbf{n_0}$ is the refractive index of the ATR crystal. By choosing from a variety of crystal types, depth of sample penetration can be controlled. A choice of a dedicated ATR objective and efficient slide-on crystal assemblies for standard objectives are available to suit a variety of needs.

Considerations

The dedicated ATR objective offers direct viewing of the sample when using a zinc selenide or diamond crystal. Since the sample is visible, this ensures accurate sample placement and optimum interaction with the ATR crystal. Alternatively, the Slide-on ATR objective is offered. Installation and removal of the crystal is provided by a prealigned mount that allows the objective to be used without the crystal in place for sample positioning. The ATR crystal is there installed for subsequent contact and analysis or simply by moving the crystal slide from "view" mode to "collect" mode.

Figure 4: Dedicated ATR objectives

Silicon and germanium Slide-On ATR crystals are available allowing quick change of the depth of penetration. A germanium tip conical shape crystal permits analysis of residuals inside depressions. Slides are easily removable from their mount, making the cleaning of the crystal very convenient, while their locking system guarantees a precise alignment and reproducible sample positioning. The Slide-On crystal design, despite the small size, provides exceptional durability and years of operation with no need for replacement. The Reflachromat 15X objective equipped with the Slide-On uniquely combines high visual quality (Reflachromat compensation), high numerical aperture optics and reflection, transmission and micro ATR infrared collection capabilities.

Calculating ATR Sampling Area

Normally, the sample is placed in contact with the crystal face of the ATR objective. As pressure is applied, the sample spreads out and the dimensions increase. In most applications involving ATR analysis, the microscope aperture is fully opened to allow the maximum amount of light to interact with the sample. This large aperture illuminates most of the crystal surface, allowing the infrared light to interact with the entire sample that is in contact with the crystal. If it is desirable to adjust the sampling area to a specific dimension, the infrared aperture can be closed down. It should be understood that the ATR crystal has a lensing effect that reduces the effective aperture area, making the sampling area smaller than the indicated aperture area. The lensing effect can be calculated by dividing the indicated aperture area by the refractive index of the crystal. For instance, when an aperture dimension of 100 microns is used with a diamond ATR crystal having a refractive index of 2.4, the effective area is approximately 42 microns, not 100 microns. This application of the infrared aperture allows reduction of the spectral contributions of the surrounding matrix in which the sample is embedded.

Figure 5: Slide-on ATR

Contrast Techniques

Contrast techniques make it possible to extract rich, visible-light images from the sample. These images complement the infrared data and can be used in conjunction with the infrared data to provide more complete sample analysis. The techniques are chosen on the basis of the sample properties such as opacity, color, isotropy and fluorescence. Though sample preparation techniques differ greatly for visible light analysis as compared to infrared analysis, many of these contrast-enhanced images can be captured while collecting infrared data.

Brightfield

Definition

Brightfield illumination is the traditional illumination scheme used in the setup of an infrared analysis. In this approach the sample is illuminated against a bright background. In brightfield, all of the light is directed down the full center of the objective and focused on the sample. The light coming through at a near-normal angle illuminates the full field, while the rays coming in at greater angles provide the edge contrast. Objectives with higher numerical aperture capture more of the extreme angle rays than objectives with a lower numerical aperture. The matched numerical aperture of the objective and the condenser used in transmission analysis provides brightfield illumination.

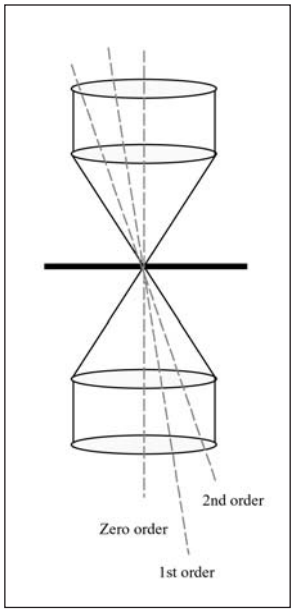

Figure 6: Brightfield ray trace

Application

Brightfield illumination is best used with samples that contain color or high-contrast features. Contrast can be improved by using the aperture stop and field stop controls which condition the sample illumination. As the aperture stop is closed, more coherent light is directed to the sample improving the contrast, but decreasing the illumination intensity. As the field stop is reduced, illumination from the surrounding area is eliminated reducing glare to the viewer.

Darkfield

Definition

In darkfield, light passing down the center of the objective at near-normal angles is blocked, so only the high angle rays hit the sample. This provides high contrast due to interactions with the fine structure of the sample. The resulting "false" image is darker but highlights sample contrast. Special objectives are normally required for darkfield analysis, but the Continuum can perform the transmission darkfield-contrast technique with a 4X or 10X refractive objective with the 15X condenser. The mismatch in numerical apertures between the objective and condenser, and the presence of the secondary mirror in the condenser blocks the direct light.

Application

Darkfield illumination is best used with samples that are colorless or lack high contrast features. Contrast in darkfield illumination is less affected by the aperture stop and field stop controls. In fact, the field stop must be fully opened, allowing higher angles of incident light to interact with the sample.

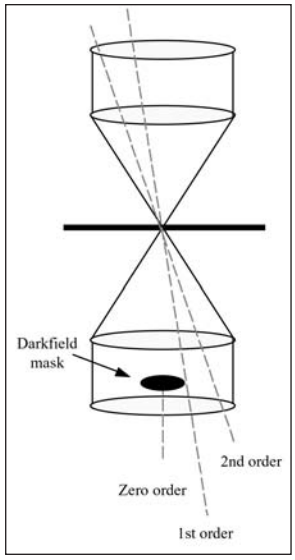

Figure 7: Darkfield ray trace

Polarized Light

Definition

Polarized light studies allow observation of anisotropic samples that change color or intensity under plane polarized light. Anisotropy is the difference in the refractive index of the sample based upon the orientation of the material to plane polarized light. Anisotropy can occur naturally or be imparted to the sample through a stretching process that orients the molecular structure. Plane polarized light passes through the sample faster when the low refractive index orientation is positioned parallel to the light. Conversely, plane polarized light passes through slower when high refractive index orientation is positioned parallel to the light. Thus, the terms slow and fast sample orientation are used. The sample thickness creates a lag in the slow ray as compared to the fast ray – the greater the thickness, the greater the lag.[1] If plane polarized light is directed at the sample where neither orientation is aligned, there is no difference in intensity versus polarization.

Polarized light studies require two identical filters – one placed before the sample (polarizer), and one after the sample (analyzer) in the illumination path. The polarizer is designed to isolate a particular polarization, while the analyzer is fixed in orientation normally horizontal to the field of view. When the analyzer and polarizer are crossed 90 degrees in relation to each other, no light is passed to the viewer. The sample is then placed upon a special rotatable stage that allows the sample to rotate about the optical axis of the microscope. When the sample is placed at 45 degrees in relation to the polarizer, equal contributions of the plane polarized light interact with both the fast and the slow sample orientation. The analyzer sums the fast and slow contributions of the light together, rendering color to the otherwise transparent sample.

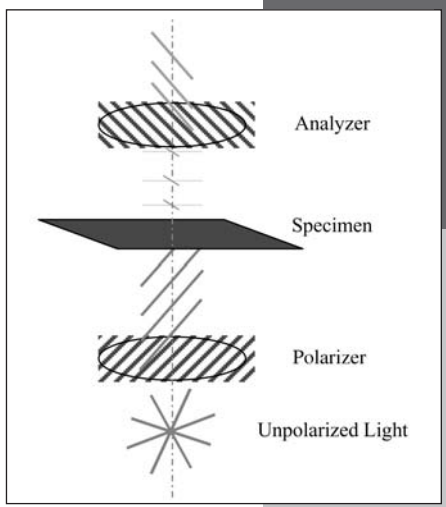

Figure 8: Polarized light ray trace

Application

Polarized light is employed in many areas, such as pharmaceutical and polymer studies to differentiate particles or layers. Once isolated by polarized light, an infrared spectrum is collected without removing the polarizers, allowing rapid location and identification of these constituents.

Differential Interference Contrast

Definition

Differential interference contrast (DIC) is a technique complementary to polarized light, allowing the collection of vivid images of colorless isotropic samples. Isotropy is defined as a material having the same refractive index in the presence of plane-polarized light, regardless of its orientation. DIC consists of two optical prisms that are inserted into the optical path just after the objective and just before the condenser. With crossed visible polarizers installed, tuning the adjustable prisms create false, three dimensional or richly contrasting colors in the sample.

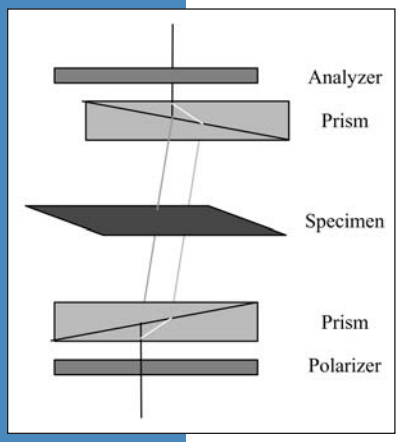

Figure 9: DIC ray trace

Application

DIC works with many forms of transparent or colorless isotropic samples, serving as a form of optical staining that allows differentiation of barriers. DIC also creates the illusion of surface contours that can be attributed to changes in sample thickness or refractive index. When used with high numerical aperture objectives, DIC provides a means of optically sectioning thin, transparent samples whereby the top, middle and bottom of the sample can be brought into focus and imaged. DIC optics must be removed prior to infrared data collection.

Fluorescence

Definition

Fluorescence provides an alternative way to visualize otherwise invisible particles in the sample based on the way they respond to specific wavelengths of high-intensity light. The sample absorbs high-intensity energy and spontaneously reemits energy in all directions. Most of the emitted energy is of the same wavelength, but a small amount is emitted at longer wavelengths. This longer wavelength energy is the desired fluorescence signal. By placing appropriately chosen optical filters in the light path after the sample, the fluorescence can be seen and used to identify previously invisible features. Fluorescence is normally used in biological studies where the sample is stained with one or more fluorophores (dyes that provide known fluorescence) designed to attach themselves to a particular structure. However, many polymer and pharmaceutical samples fluoresce naturally, allowing them to be quickly located visually, and then setup for the infrared analysis.

Figure 10 is a diagram of the fluorescence option available for the Continuμm microscope. A mercury arc source provides the excitation energy, and cubes containing a beam splitter, emission filter, and barrier filter provide specific wavelength ranges of energy to be passed to the sample. These cubes are mounted on a turret that allows up to three different cubes to be installed.

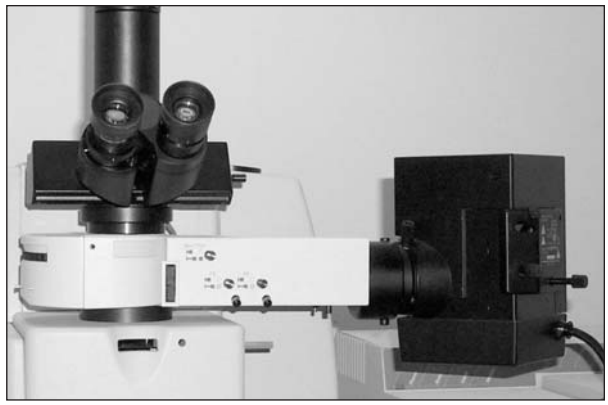
Figure 10: Thermo Scientific Nicolet Continuμm with fluorescence capability

Application

By using various filters, you may choose from UV, UV-blue, blue and green excitation frequencies from a single mercury-arc lamp source. Fluorescence may be used to locate contaminants that are colorless or difficult to detect under normal light conditions. Since spatial resolution is frequency dependent, the shorter wavelength UV excitation conceivably allows detection of particles smaller than the resolution limit of normal visible-light observations.

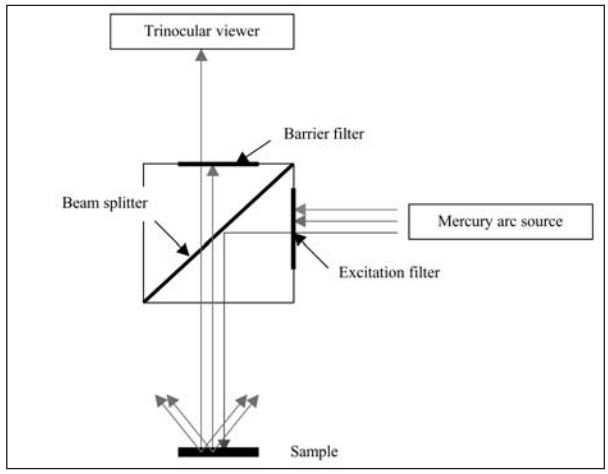

Figure 11: Fluorescence ray trace

Microscope Components

Objectives

The heart of a good microscope lies in the objective – the light-gathering optical component of the microscope. The quality of the objective dictates the data quality collected because it is responsible for capturing sample detail. In a transmission arrangement, a complementary optic, known as a condenser, is located beneath the sample stage. The condenser focuses light from the source onto the sample. Objectives are defined as either refractive or reflective in design. Refractive objectives use high-quality glass lenses stacked in a barrel configuration to provide the magnified image.

Reflective Objective

Since glass absorbs infrared energy below 2000 cm^{-1}, refractive lenses are commonly used in visible light and Raman applications. Reflective objectives use stainless steel mirrors to provide the magnified image and are used in infrared microscopes because they do not absorb infrared energy. Reflective objectives are available for a variety of applications, differing in magnification and numerical aperture.

Figure 12: Reflective objective

The barrel of an objective has several markings that indicate the performance and operational environment. Figure 12 illustrates an objective with a linear magnification of 32, a numerical aperture (N.A.) of 0.65, infinity-correction and variable compensation. An objective with a low N.A. will create a blurrier image of fine structure as compared to a high N.A. objective of similar magnification. A high N.A. objective collects more of the light diffracted by the sample, thus capturing fine structural detail. Figure 13 depicts a reflective objective with the minimum and maximum rays indicated by lines.

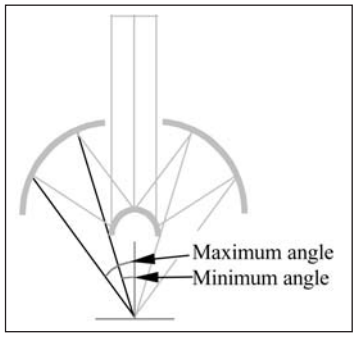

Figure 13: Objective ray trace

The secondary convex mirror, held in place at the focal length of the larger primary mirror, limits the minimum angle. The maximum angle is defined by the diameter of the primary hemispherical mirror, the working distance of the optic, and the medium between the optic and the object.

The standard 15X objective is most commonly used for routine analysis, providing excellent performance, working distance, and sampling flexibility. This objective is an excellent choice for larger samples ranging from 20 microns and larger, when paired with any 250-micron element detector. For extremely small samples, routinely 20 microns or smaller in size, the 32X objective is a better choice. The high magnification allows accurate sample positioning and easier observation of fine sample detail, while the high numerical aperture provides improved spatial resolution over the 15X objective.

Grazing Angle Objective

The grazing angle objective (GAO) is used to analyze sub-micron thick layers on metallic substrates. This objective has a shallow working distance and a large numerical aperture – 0.996 – providing the maximum interaction with very thin samples. The GAO provides a viewing mode for sample positioning and a grazing mode for spectrum collection. Figure 15 illustrates the performance advantage of the GAO as compared to the 15X and 32X Reflachromat objectives for analyzing a thin film on gold. The GAO's high angle of incidence allows the infrared beam to interact with extremely thin sample layers producing excellent spectra.

Figure 14: Grazing angle objective

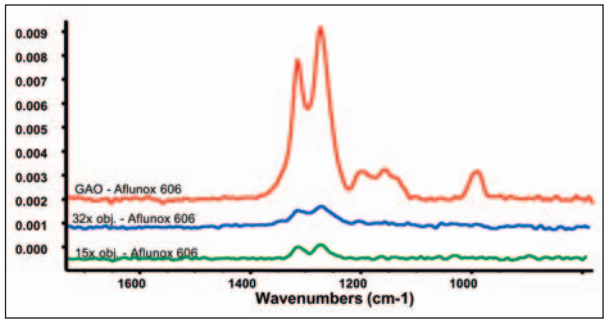

Figure 15: Grazing angle objective comparison to 15X and 32X objective

Stages

The stage, which provides support and fine-movement control of a sample, is placed between the objective and condenser and can position samples manually or with the use of motors. Manual stages are available in the form of traditional X-Y motion, or circularly rotatable stages. Rotating stages are commonly used in concert with contrast techniques where the sample rotation on the optical axis is required.

The motorized stage is controlled through a joystick and software applications. The advantage of the motorized stage is unattended operation when the analysis of a large sample is required. The data are collected in the form of an array of spectra vs. distance.

Detectors

Detectors provide response to the infrared energy after it has been directed through the sample. They take the form of single infrared elements and multiple infrared element arrays. We offer two single element microscopes – the Centaurμs and the Continuμm – as well as the array-based microscope – the Almega. The single element detectors collect one spectrum from the entire masked sample area. The array in the Almega collects a full spectrum at once from a given point in the sample.

Array Detectors

Array detectors are spatially separated, individual detectors on a common chip that respond to light directly from a specific area of the sample. The advantage of arrays is the ability to collect spectra quickly and simultaneously from many discrete points in the entire field of view.

Single Element Detectors

The most common single element detectors used in IR microscopy are the mercury cadmium telluride (MCT) detectors available in a variety of forms, each with a different purpose. MCTA detectors have a narrower spectral range but offer higher sensitivity, while MCTB detectors have a wider spectral range but lower sensitivity than MCTA. The TE-cooled indium gallium arsenide (InGaAs) detector provides spectral information in the near-infrared spectral range, allowing observation of overtone and combination bands. The choice of detector is based on the sensitivity and spectral range desired.

Mid-infrared Detectors

The MCT detectors are defined by their sensitivity to weak contributions or the spectral range to which they respond. The sensitivity is indicated by the D* ratings, where larger numbers indicate higher sensitivity. Figure 16 compares the noise level of three MCT detectors and their spectral range on a common Y axis scale. These detectors, with a 250-micron square detector element, are defined as narrow band, medium band, and wide band. The MCTA 50-micron detector is optimized for samples less than 20 microns in size. This small element-narrow-band MCTA detector provides better infrared sensitivity for small samples than detectors with larger elements, but is not as useful for larger sample dimensions (> 20 µm). Figure 17 illustrates the performance advantages of the 50-micron detector with small sample sizes, and Table 1 shows the performance data of each detector.

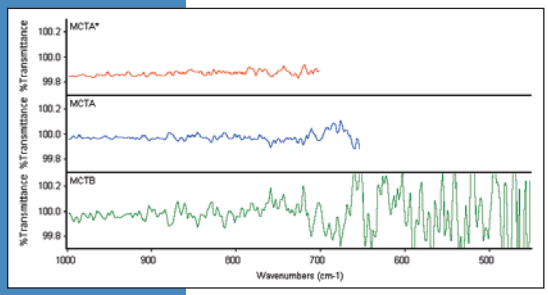

Figure 16: Spectral range vs. spectral noise for several MCT detectors

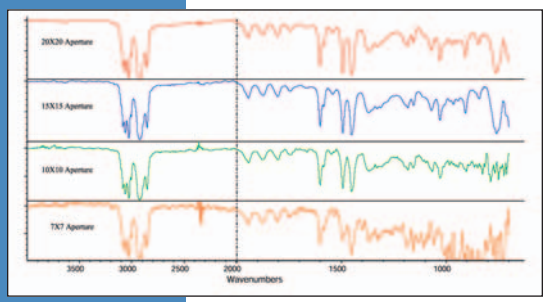

Figure 17: Performance advantages of the 50-micron detector with small sample sizes

DETECTOR TYPE	APPROXIMATE SPECTRAL CUTOFF (cm^{-1})	D* RATING
MCTA*	700	6.5 E10
MCTA	650	4.5 E10
MCTB	450	8.5 E9
MCTA 50-micron	700	8.0 E10

Table 1: Performance data of several MCT detectors

Samples rich in spectral information in the fingerprint region, can be analyzed with an infrared microscope configured with an MCTB detector. To maximize flexibility of the microscope, both an MCTA and an MCTB can be installed to provide the optimum system configuration for organic and inorganic sample analysis. Figure 18 shows spectra of automotive paint samples collected on a Continuµm microscope via transmission analysis using a diamond compression cell.

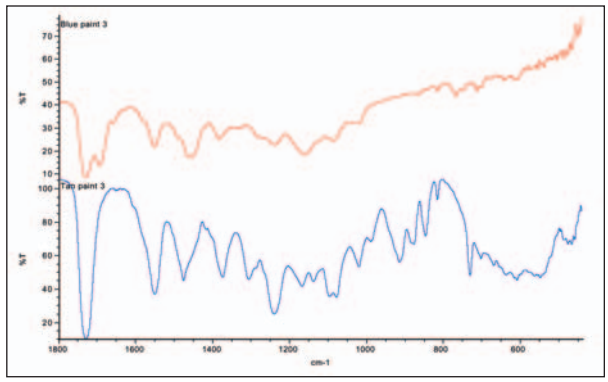

Figure 18: Automotive paint samples collected on Continuµm with MCTB

Infrared Aperture

The infrared aperture provides a mask that confines the IR energy to a specific area of the sample. Apertures are adjustable blades of metal or glass which control the spatial extent of sampling. As the aperture is closed, infrared energy bends around the blades, interacting with sample area beyond the borders of the aperture. This diffracted energy appears as bands of spectrum not attributed to the desired area, as shown in Figure 19.

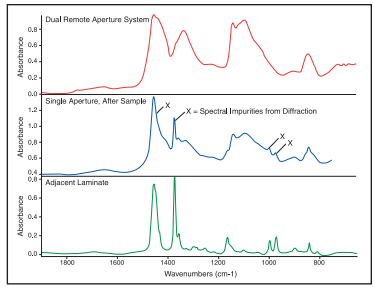

Figure 19: Upper spectrum collected on Continuµm microscope with Reflex aperture. Middle spectrum collected from another vendor's IR microscope system with single aperture showing interfering data from the surrounding sample area. Lower spectrum collected from surrounding sample medium

We hold two patented technologies that significantly improve the quality of spectra. The Centaurµs uses Thermo Scientific Targeting™, which provides an apertured IR beam before the sample and the Continuµm uses Thermo Scientific Redundant Aperturing™, which provides an aperture before and after the sample to effectively eliminate diffraction effects introduced by the aperture. Figure 20 illustrates the infrared path through the patented Reflex aperture system, which combines the benefits of Redundant Aperturing with the ease of an automated single aperture.

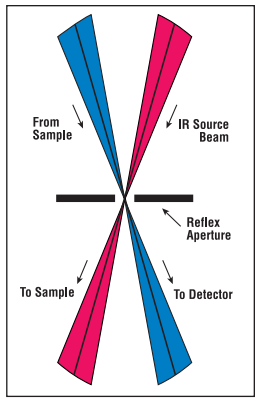

Figure 20: The automated Reflex aperture provides dual masking with a single aperture for the highest spectral quality and maximum ease-of-use

Sample Preparation Tools

Sample preparation is often necessary to either fit the sample onto the scope or to optimize the spectral band intensity. For transmission and reflection absorption microscopy, a sample thickness of 5 to 15 microns is typical as long as the largest peaks in the spectrum are no greater than 0.7 absorbance units. Reflection and ATR analyses usually only involves preparing the sample to fit on the stage.

Figure 21: Sample preparation tools

Microtomy is the process of preparing thin sections of a sample. With a microtome, a substrate such as wax or a polymer is used to mount the sample perpendicular to a blade. The blade slices thin cross-sections of the material that can be mounted in a compression cell for transmission analysis. A simpler method used to prepare thin cross-sections shown in Figure 22 involves clamping the sample between two glass microscope slides or plates of metal. A razor blade is used to prepare an initial straight edge of the sample. Drawing the top slide back very slightly exposes a small wedge of the sample that can be cut with a second pass of the razor blade. This multi-layer wedge can then be placed into a compression cell on edge and analyzed. Alternatively, most non-laminated materials can be placed directly into a compression cell. When preparing a sample with a compression cell, a background material, typically a single crystal of KBr powder, is also placed between the windows.

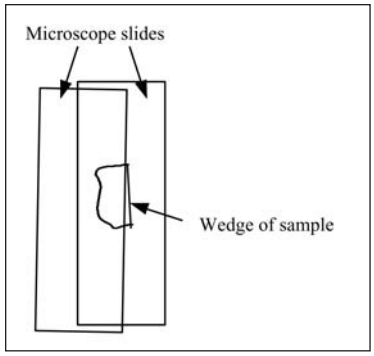

Figure 22: Simple microtome using glass slides

Figure 23: Microcompression cell

Microscopy Terms

This section will discuss various terms that relate to the science of microscopy.[2] Understanding these terms helps with optimizing the experiment for the best result, taking full advantage of all features of the microscope.

Magnification

Linear magnification relates to the size of the image as compared to the size of the object. Magnification provides an image large enough to be observed. Low-magnification refractive objectives, also known as scanners, are used to rapidly locate areas of interest. Once the sample is located, higher magnification objectives are brought into position via a multi-objective nosepiece. Higher magnification objectives provide the larger image needed to observe the fine structure of the sample, set up apertures and focus for the infrared experiment. Quality microscopes offer multiple objectives that are aligned to provide a focused image of the sample with minimal stage adjustment. Total image magnification can be calculated by multiplying the objective magnification by the ocular (eyepiece) magnification. In a typical Continuμm, a 15X objective with the standard 10X oculars provides a total visible-light magnification of 150X. Since infrared energy does not pass through the eyepieces, infrared energy is only magnified by the objective, in this case 15X.

Numerical Aperture

Numerical aperture (N.A.) is a measure of the light collection efficiency of an objective. A numerical aperture of 1.0 would be considered perfect for a dry objective, but many oil immersion optics have a numerical aperture greater than 1.0. N.A. is calculated by the following formula:

Numerical Aperture = n sin (μ)

Where **n** is the refractive index of the medium between the objective and the sample and **μ** (angular aperture) defines the greatest angle of light scattered from the sample as measured from the optical axis of the optic. The numerical aperture is used to calculate many other parameters of the objective.

Infinity Correction

Microscopes are identified as either infinity-corrected or finite tube length. Economy microscopes use finite tube length optics that are typically 140 to 170 mm. This number corresponds to how far behind the objective the image comes into focus. Since the light from the objective converges to this point, the introduction of optical filters would disturb the focus, causing a poorly defined image. To prevent focal point disturbances, high-quality microscopes use infinity-corrected designs. Infinity-corrected microscopes send the light through the entire instrument in a collimated beam that never converges until the objective focuses on the sample or a mirror focuses on the detector. This beam remains undisturbed by the introduction of optical filters and polarizers. After the optical filters and just before the oculars, a tube lens is required to converge the light to the primary image plane. As a result, sharply contrasting images of the sample can be captured using a variety of contrast enhancement optics. This infinity corrected design allows the Continuμm, and Almega to offer many enhancement techniques commonly found on light microscopes.

Compensation

As light is passed from the sample to the objective, support windows and cover slips around the sample interfere with the light, causing aberrations due to the refractive properties of the covering window. In light microscopy, it is common to use objectives that are corrected for standard thickness (0.17 mm) glass cover slips. Infrared sample preparations commonly use transparent windows that are one to three millimeters in thickness. Quality microscopes provide variable compensation for the use of various sampling accessories while maintaining a sharp visual image and accurate sample masking.

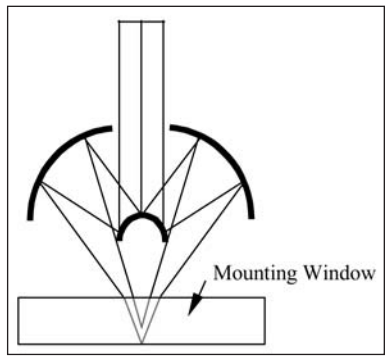

Figure 24: Spherical aberration causes a blurred image if not compressed

Working Distance

Working distance is defined as the distance between the objective and the sample, when in focus. Working distance is different for each objective, and typically decreases as the objective N.A. increases. Short working distances limit the use of a number of specialized sampling accessories available for the microscope.

Field of View

Field of view is defined as the diameter, in millimeters, that is visible in the viewer across the field of the sample. As objective magnification increases, the field of view decreases. The diameter of the field of view can be calculated, allowing an estimate of the sample size. The calculation requires information from the eyepiece, known as the field number, which is the diameter of a ring inside the eyepiece that limits the field of view. The field number is usually printed on the side of the eyepiece. For the eyepiece in Figure 25, the field number is 22.

Figure 25: Eyepieces

The calculation for field of view is:

FOV = field number/objective magnification

Thus, when using a 15X objective and an eyepiece with a field number of 22 millimeters, the field of view is 1.4 millimeters.

Software packages available for Nicolet FT-IR and Raman microscopes provide onscreen tools for measuring sample size and dimensions with great accuracy, replacing the need to calculate sample size visually.

Depth of Field

The depth of field is defined as the vertical distance through the sample that is in focus at any given point. Objectives with higher N.A. have a smaller depth of field, while objectives with lower N.A. have a larger depth of field. For infrared transmission experiments the depth of field is irrelevant, since the infrared energy interacts with the sample or sample substrate through the entire thickness of the sample. For visible-light experiments, depth of field provides the ability to "optically section" a sample, bringing into focus only the sample depth of interest within a relatively transparent sample. In Raman experiments, this depth of field allows confocal analysis (vertical mapping) of optically transparent samples with little interaction from the adjacent sample matrix. Depth of field (z) can be calculated as:

$$z = 4\lambda/(N.A.)^2$$

Table 2 illustrates the infrared objective specifications available on the Continuμm.

OBJECTIVE	WORKING DISTANCE	N.A.	FOV (mm)	DOF (MICRONS AT 18181 cm^{-1})
15X	11 mm	0.58	1.44	6.5
32X	7 mm	0.65	0.68	5.2
10X	12 mm	0.71	2.2	4.4

Table 2: Infrared objective specification on the Nicolet Continuμm (15X and 32X), and Nicolet Centaurμs (10X).

Spatial Resolution

Spatial resolution determines the minimum distance that two closely positioned objects can be seen as separate images. Since spatial resolution is directly related to the numerical aperture of an optic, higher numerical aperture optics provide better resolution.

The term spatial resolution is defined as:

$$\text{Spatial Resolution} = 1.22\lambda/2N.A.$$

where λ is the wavelength of light, and N.A. is the numerical aperture of the optic. This can be simplified to 0.61λ/N.A.

Microscopes with redundant aperturing offer spatial resolution better that what is given by the equations above. Spatial resolution is a fundamental aspect in either typical infrared microscopy or infrared imaging.

Hyperspectral imaging provides physical and chemical information from the sample and is rapidly growing as a preferred technique in many applications. The infrared collection of large sample areas is greatly improved by current mapping stage technology, especially the high speed they can achieve, and by using MCT arrays capable of simultaneous collection of multiple spectra. In most applications the speed of acquisition is the fundamental requirement, but the spatial resolution must be sufficient to extract the required chemical information from the sample. Spatial resolution is wavelength and numerical aperture dependent and not related to the pixel size of the array detector at the focal plane.

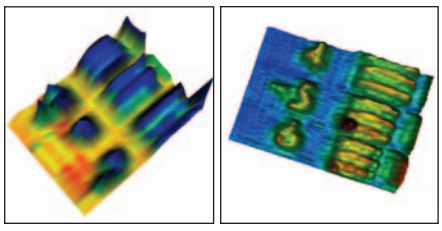

Figure 26: Three-dimensional renderings of (a) standard image and (b) oversampled high-definition image

Optical Magnification

The 32X Reflachromat objective and condenser provide a higher magnification than the 15X optics, and their higher numerical aperture offers the highest possible spatial resolution achievable through infrared optics.

Oversampling

In standard sampling mode, the image of the pixel at the focal plane is the smallest "frame" from which spectral information is collected. Oversampling allows the collection of multiple "frames" within the same area and the recombination of the complete information to obtain a more detailed infrared image. The clarity of any infrared image can be improved by applying oversampling using factors of 4X, 9X or 16X, depending on the amount of detail required, either with standard 15X optics or with higher magnification 32X optics.

Diffraction

Diffraction is the bending of light as it passes by an edge. In an infrared microscope, the aperture provides the edge used to limit the area that is illuminated by the infrared energy. Diffraction is a frequency dependent phenomenon that is more pronounced at the longer wavelengths of infrared energy. As the aperture blades close, diffraction becomes more significant, until a point is reached where the spectrum below a certain wavelength is void of features. The diffraction limit can be calculated as:

$$\text{Diffraction Limit} = 1.22\lambda/N.A.$$

Table 3 provides the minimum aperture size achievable before the effects of diffraction are observed with given detector cut-off limits.

WAVENUMBERS	WAVELENGTH (MICRONS)	DIFFRACTION LIMIT (MICRONS) 15X OBJECTIVE	32X OBJECTIVE
700.00	14.29	30.05	26.81
600.00	16.67	35.06	31.28
450.00	22.22	46.74	41.71

Table 3: Calculating minimum spot size using the formula d = (1.22* wavelength) / N.A.

Clearly, diffraction will erode the performance of the low wavenumber response, assuming a "perfect world" scenario, without regard for the sample-scattering effects or the spectral response of the detector at these longer wavelengths.

Summary

This handbook has defined common terms used in microscopy, methods of infrared and Raman microscope sampling, contrast enhancement of visual images, and hardware selection. The intent was to illustrate the performance advantages of FT-IR and Raman microscopes from Thermo Fisher Scientific. The Continuμm, and Almega XR offer unparalleled design features that provide the best infrared, Raman and visible information from your sample. Optional components, such as different detectors, stages and contrast accessories, are available whenever your sampling requires additional capabilities.

References

1. R. Saferstein, Forensic Science Handbook, Prentice-Hall. Cc 1982.

2. B. Foster, Optimizing Light Microscopy for Biological and Clinical Laboratories, Kendall/Hunt Publishing Co.

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