A New Capability for Light Microscopes: Mid Infrared Molecular Analysis

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Background

The merger of molecular spectroscopy with microscopy is certainly not a new concept. Microscope attachments for FT-IR spectrometers have been available for nearly two decades and there are literally thousands of FT-IR spectrometers with these devices currently installed.[1] The vast majority of them are applied to contaminant or forensic oriented problems. As such, the microscopes are often used as sophisticated beam condensers, enabling the spectrometer to focus infrared radiation on the samples that are typically larger than 10 microns.

Thus, virtually all infrared molecular microscopes are thought of, and are used as, accessories to FT-IR spectrometers. In this respect, at least two significant problems arise. First, though the infrared spectra produced are of good quality, the imaging capabilities and functions of the microscope are often compromised. This is a result of the necessary engineering trade-offs required to mate a microscope to an analytical FT-IR spectrometer. Secondly, scientists using FT-IR microscopes have some level of expertise in spectroscopy but little training in microscopy. The complexities of using a microscope attachment to a spectrometer requires a fair degree of knowledge of both technologies to attain useful data and for in-depth interpretation. The expertise necessary to effectively use FT-IR microscopy is not typically in the domain of the occasional user.

Figure 1 – The IlluminatIR, a miniaturized FT-IR spectrometer is mounted between the eyepiece and body of an infinity corrected microscope. The normal imaging functionality of the microscope is not affected by the addition of the spectrometer.

With this backdrop, an alternate approach to obtaining molecular information on microscopic samples is proposed. Rather than adding a microscope to a spectrometer, technology is described that integrates a miniaturized FT-IR spectrometer to an infinity corrected microscope. This approach adds the useful capability of molecular analysis to the arsenal of tools for the microscopist.

Merging optical microscopy and FT-IR microanalysis

Mid-infrared FT-IR spectroscopy covers the classic "fingerprint" region of the electromagnetic spectrum, so-called because the information provided is highly specific to the structure of a particular compound. A mid-infrared spectrum is a precise pattern of absorbance bands arising from the vibration of atom against atom in a chemical molecule. These intra-molecular vibrations have specific energies dependent on the chemical bonds that are present in the compound and this pattern of energy vibrations is represented by the infrared spectrum. Because of the specificity, and because each molecule has its own unique "signature" (or fingerprint), the mid-infrared spectrum is highly diagnostic.

Successful integration of light microscopy and mid-IR spectroscopy implies that the capabilities of the light microscope are retained, and high-information content mid-IR spectral analysis is added as an additional feature. Compromising the performance of the light microscope to meet the requirements of the spectroscopic measurement is not desirable. Thus, a number of potentially competing factors must be considered:

- The spectrometer must be exceedingly compact and should not significantly affect the footprint or ergonomics of the light microscope.

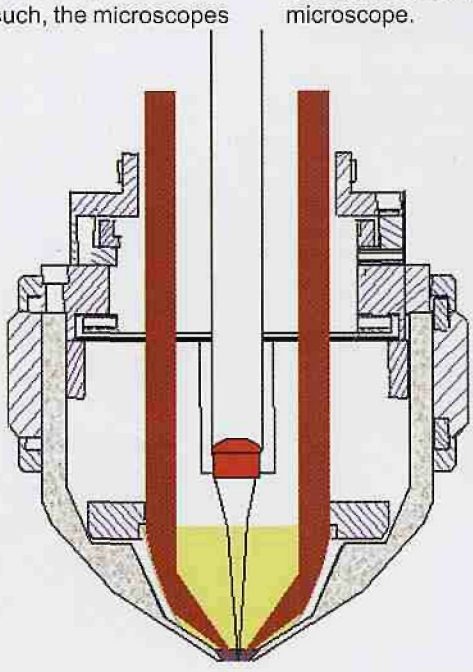
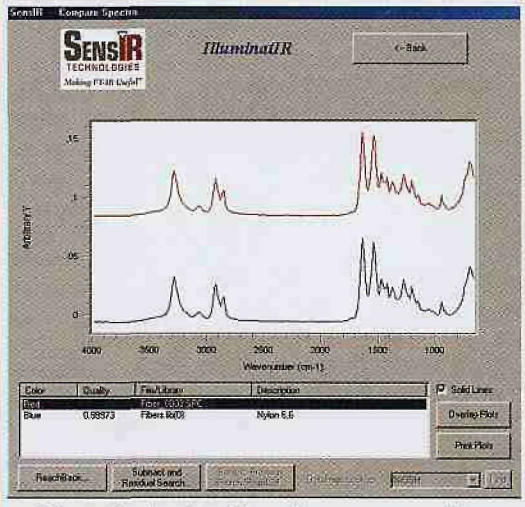


Figure 2 – Schematic of diamond internal reflection objective for micro-ATR spectral data collection. The red area represents the infrared beam path through the objective. The beam from the interferometer enters on one side and reflects from the diamond-sample interface, returning to the detector. The yellow feature represents the ZnSe paraboloid-focusing element. The refracting element is positioned on the central axis of the paraboloid and is focused on the sample-diamond interface. By lowering the refractive lens, it can be focused up to 2-mm below the diamond surface. This enables the sample surface to be in visible focus either during sample survey or data collection.



10X Glass Objective | 15X Diamond ATR | In Contact with Diamond

Figure 3 – Unknown fiber viewed through 10x glass objective, the IR Diamond ATR objective, and then the IR objective in contact with the sample. The report shows the identity of the fiber and quality of the search hit.

- The optical path of the microscope must be preserved in order to retain all of its routine capabilities and to facilitate techniques such as polarized light, fluorescence, Nomarski Differential Interference Contrast, image analysis, etc.
- The method and technology for analyzing microscopic samples by IR spectroscopy must be simplified (while preserving the high quality optical information).
- The merged technology must be easy to use and the resultant IR data must be rapidly reduced to pertinent information.

Integration design considerations

To achieve complete integration of the spectrometer and the microscope, the size of the spectrometer must be reduced to the point that it becomes a component of the overall microscope package.

The heart of an FT-IR spectrometer is the interferometer. In order to reduce the spectrometer size, it is necessary to reduce the size of this important sub-system. A reasonable approach is to design a monolithic interferometer that locates all critical components (source, beamsplitter, and laser) in a single compact housing. In addition, one of the elements of a conventional FT-IR spectrometer that contributes to a larger overall size is the HeNe gas reference laser. Gas lasers cannot be made too small because their useful lifetime becomes compromised. A preferable approach is to use a solid-state laser as the spectrometer reference, since it is approximately 1/10 the size of a gas HeNe laser.

A stabilized solid-state laser and monolithic interferometer enables the size of the spectrometer to be significantly reduced. Thus, a spectrometer specifically designed to be an integral part of an optical microscope can be sufficiently compact to fit in between the eyepiece and the microscope body, without changing the fundamental light path of the microscope (figure 1). With this miniaturized spectrometer, the attachment to the light microscope is straightforward and can take less than a minute to complete.

Thus, size and optical design of the infrared system are important factors in preserving the functionality of the light microscope. Another key factor is the fundamental technique of how the infrared spectrum of the microsample is actually measured. Most infrared microscope systems are designed for transmission measurements. This implies that the infrared beam is passed through the sample, requiring infrared reflecting optics below the microscope stage. This is fine for infrared data but compromises the microscope's optics, resulting in reduced visual performance and functionality.

One approach to avoid the necessity of modifying the visible light path of the microscope is to design an IR light path that does not cross the horizontal plane of the stage. Instead, the IR beam could impinge on the sample and be reflected upwards towards the IR detector. This approach would eliminate the necessity to modify the visible path optics in order to gather the IR light after it has interacted with the sample.

In reality, this is accomplished by using two novel IR reflecting objectives that act as the interface between sample and spectrometer. These two objectives, as well as conventional objective lenses can be mounted on a six position rotating nosepiece. Since there is no need to modify the microscope optics below the stage, the full transmitted light functionality of the microscope is retained. Depending on sample type and configuration, one or the other of these IR objectives is chosen, thus enabling the analysis of virtually all types of solids.

One of the IR reflecting objectives uses the principle of internal reflection to obtain the spectrum (figure 2).[2] The beam of infrared energy is directed from the interferometer into a small wafer of diamond located in the objective. The diamond acts as a "wave guide," and the beam of infrared light internally reflects (known as attenuated total reflection or ATR) inside the diamond wafer. Though all of the infrared light is internally reflected (i.e., does not leave the diamond crystal), a standing wave forms on the diamond surface creating an electro-magnetic field that penetrates into a sample that is placed in contact. Any chemical substance in contact with the diamond's surface is probed by the evanescent infrared wave, and an ATR spectrum, or

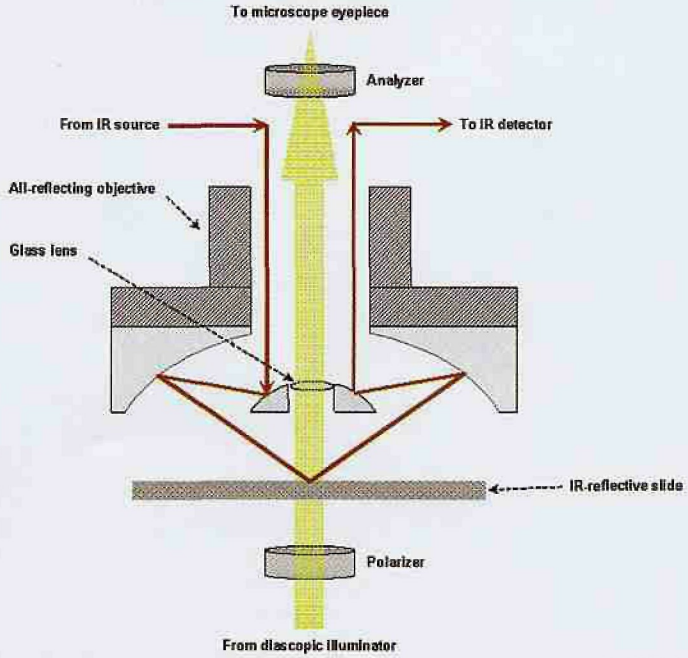


Figure 4 – A modified Schwarzschild all-reflecting objective with a refracting optical system mounted in the secondary mirror for visible light viewing of large fields. The refracting optic has the same magnifying power as the reflecting lens and they are also parfocal and concentric. Slide is a dichroic reflector; it transmits visible light and reflects mid-infrared radiation.

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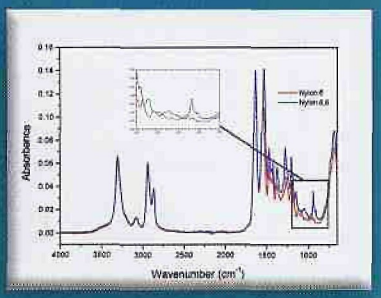

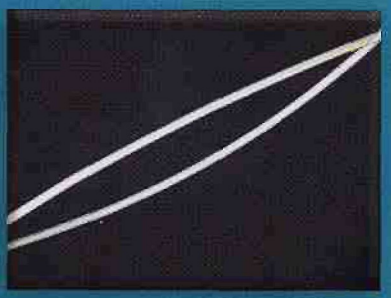

When samples are visually identical, FT-IR microscopy can show you the difference.
Visually these two nylon fibers are identical. However, when FT-IR analysis is run on each of the fibers and their spectra is overlaid the difference in the two fibers becomes clear. The top fiber was identified as Nylon 6 and the bottom fiber to be Nylon 6,6.

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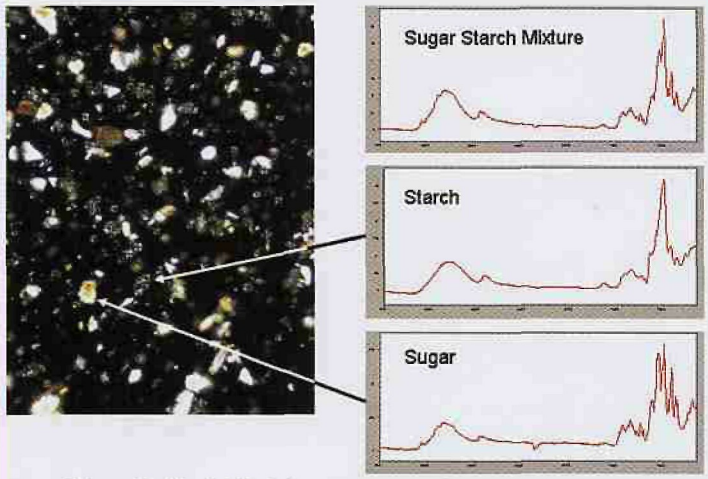

Figure 5 – The individual components of a heterogeneous mixture can be determined by selecting the components one-by-one using PLM imaging and then identifying them with mid-IR spectroscopy.

"fingerprint" of the substance is obtained.

Because all IR optics used in the internal reflecting objective are visibly transparent materials, the internal reflection IR objective permits the user to visibly see the specific area of the sample during the collection of the IR data.[3] An integrated near infrared video imaging system allows direct observation of the IR beam and ensures that IR light is actually striking the correct sample point. The ability to see the sample area through the IR optics (figure 3) provides the assurance that the IR data arises from the correct portion of the sample.

In actual use, the sample area is sighted through the visible optics, and the internal reflection optic is rotated into position. The sample area can now be seen through the IR internal reflection objective at which point the microscope stage is raised until the diamond internal reflection element makes contact with the sample. The diamond internal reflection element has a curved surface allowing reliable spectra to be recorded from samples that are not flat, or may have rough or pitted surfaces. A high sensitivity mercury-cadmium-telluride detector and efficient optics combine to permit the infrared spectra of samples as small as 10 microns to be recorded.

The other IR objective (figure 4) employs Schwarzschild all-reflecting optics that both focus IR energy on the sample and then collects the reflected light. With this objective, there is no physical contact between the optics and the sample. The all-reflecting objective is used in conjunction with a microscope slide that is coated with a commercial IR reflecting material. The infrared light from the objective passes through the thin sample, is reflected from the microscope slide, passes through the sample a second time and then is collected by the objective. The double pass reflected light provides an infrared spectrum that is similar to a transmission measurement, but the IR beam is not required to cross the horizontal plane of the microscope stage. Because the glass slide on which the sample is placed is both transparent to visible light and reflects IR energy, all normal light microscope functionality remains available.

A significant quality-of-data and ease-of-use advantage is that both the visible and IR data can be gathered using the same microscope. With conventional IR microscopes, the visible light performance and functionality limitations may force the analyst to examine the sample with a separate microscope and then bring the sample to the IR microscope for analysis. This is time consuming and risky since transporting the sample makes it difficult to relocate the measurement area as well increases the chance the sample can be lost altogether.

Reducing Data to Information

The use of infrared microspectroscopy by occasional users or microscopists not trained in IR spectroscopy requires a user-interface and data analysis software system that maximizes the information content obtained from the IR spectrum. At the same time, the more expert user will require more advanced capabilities. One approach to solving the issue of breadth of user experience and overall degree of use is a bi-level software user interface and data analysis package. The goal is to provide the level of software that is needed to solve the type of problem that the user will face.

The first level of software is specifically focused on the task of rapid and intuitive identity determination of unknown microsamples. The user interface enables data to be rapidly acquired, and the resultant data is reduced to the informational content by the use of closely linked spectral libraries. The quality of the spectral libraries is very important to ensure the accuracy of the identification. The reference spectra in the database libraries must be recorded using the same reflection technique as the unknown sample being investigated. The available libraries must cover the wide range of samples that the user might encounter such as white powders, pharmaceuticals, illicit drugs, polymers, explosives, paints and pigments, as well as other chemical and biological compounds.

In actual use, the infrared spectrum of a microsample is measured and is automatically searched against the integral database containing thousands of compounds. The identity of the unknown, as well as a numerical indicator of the quality of the match, is presented to the analyst in less than one minute.

At times, an IR spectrum can be too rich in information and the ability to reduce this to a specific microsample identity is compromised. An example of this problem is in samples that are heterogeneous mixtures. An integral advantage of infrared microsample analysis is that individual particles in a mixture can be physically separated and the spectrum recorded for each of the different types of particles present (figure 5). Each of these different spectra can then be searched against the database to reveal the identity of the specific particles in the mixture.

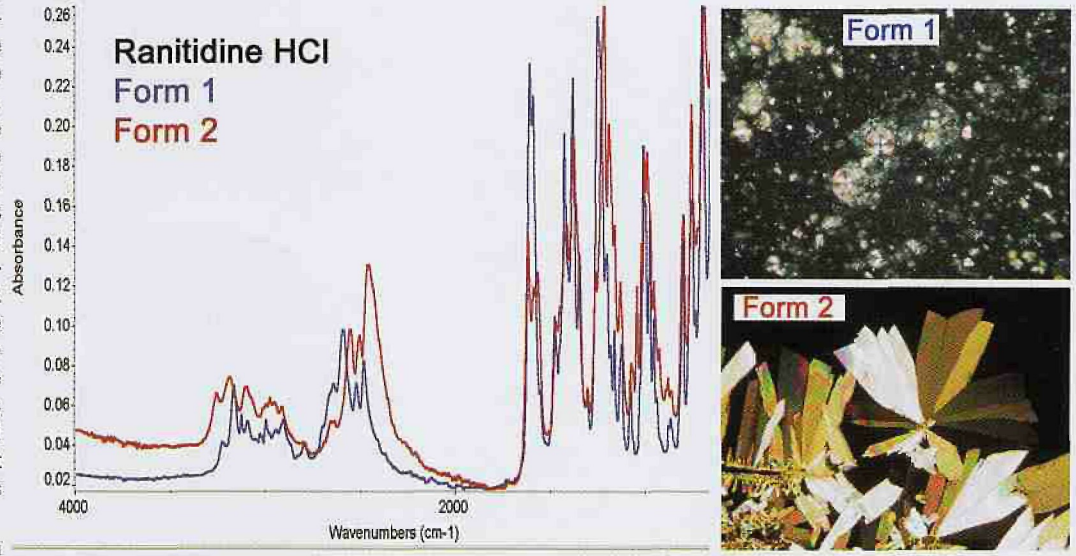

Figure 6 – PLM images and mid-infrared spectra captured using an infinity corrected microscope with miniaturized spectrometer attachment. Both techniques confirm the presence of crystal polymorphism.

For more experienced users or particularly demanding applications, a second level of software provides the full depth of FT-IR spectroscopy data processing. The user can send his data into the widely used GRAMS software for advanced spectroscopic reduction and analysis.

Merging IR spectroscopy and optical microscopy – Areas of Use

When traditional techniques of optical microscopy are combined with infrared microanalysis, the amount of information obtained on the microsample is enhanced. The historic issue with conventional infrared microspectroscopy has been the difficulty associated with obtaining this data, and the ease of converting data to information. The development of an FT-IR attachment for light microscopes is an approach to address these issues. The goal is to increase the potential range of applications and users.

Thus, the FT-IR attachment for light microscopes will have its greatest impact in those applications where visual examination and molecular information are of significant importance. For example (figure 6), the combination of polarized light microscopy and IR spectroscopy shows how information obtained from one of the more commonly used techniques in optical microscopy is enhanced with molecular spectroscopy. The range of applications that meet the aforementioned definition include:

- solid state characterization of pharmaceuticals
- forensic analysis including evidentiary information
- identification of unknown samples in public health and safety labs
- polymer and materials analysis
- cell, tissue and related biomedical studies ■

References:

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3. Barbara Foster, American Laboratory, November 2001