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Focus on microscopy

CONVENTIONAL LIGHT MICROSCOPY VERSUS FTIR MICROSCOPY: SAMPLE PREPARATION FOR BOTH SIDES OF THE COIN, PART 2

FOURIER TRANSFORM infrared (FTIR) and regular light microscopy are optical techniques providing complementary information. Part 1 of this article[1] spelled out some of the similarities and differences in sample preparation for both of these technologies, indicating that, with a little advanced planning, many samples could be first observed with light microscopy and then analyzed with micro FTIR.

Part 1 also differentiated between the objectives of each technique and their influence on sample preparation. For example, conventional light microscopy distinguishes one phase from another and identifies morphological and optical properties, requiring that the sample be kept intact and maintain sharp edges with well-defined angles and features and crisp contrast. Image analysis adds an additional need for clearly definable phases that can be segmented by the computer and, wherever possible, sharp, clean, unshaded edges. FTIR microscopy, on the other hand, requires a cohesive sample spot for chemical analysis, free of edge effects. Since a combination of reflectance and absorption spectroscopy is often used, it also helps if the sample can be prepared as a thin (10-μm) film.

While Part 1 of this series suggested a number of generic tools, Part 2 zeros in on more specific, commonly encountered applications, notably particles, fibers, films, composites, and foams, examining both light and FTIR sample preparation, imaging options, and image analysis tips.

Crystals, particles, and pigments

In both conventional light microscopy and FTIR microscopy, too many layers of crystals can obscure information. Both techniques benefit from a gentle sprinkle of particles, avoiding depth. A good support for conventional microscopy is a piece of double-sided sticky tape or a gentle coating of any nonreactive tacky adhesive (e.g., artist's fixative or even a gentle swipe with the coater from instant film) laid down on the slide. To prepare the sample, gently sprinkle the powder over the surface, using a probe where necessary to separate aggregates.

For identification, either visually or with the spectrometer, bigger single crystals may be better. Growing crystals under the microscope is not difficult. First, check the *Chemical Handbook*[2] for solubility information. If the material is soluble in warm water, prepare it as indicated above; then, gently blow a breath of warm, moist air from deep in the lungs. Frequently, this moist vapor is enough to dissolve and then recrystallize the particles into larger, better-shaped crystals.

If the sample contains a mixture of particles, chances are that the components will each exhibit a different solubility, allowing preferential growth of one species over another. This approach was very effective in separating and ultimately identifying a contaminant in an artificial sweetener. The contaminant was soluble in warm water and recrystallized into larger, more readily identified domains when the warm-breath method described above was applied.

If the material is soluble in something other than warm water, try recrystallizing it in a small chamber made from a coverslip and a well slide. Cut a small strip of filter paper, and bend it into a "Z" shape so that it just touches the bottom of the well. Sprinkle a small amount of the powder to be examined on a second slide; then, gently rub the back of a coverslip with a piece of nylon to build up enough static charge to encourage the coverslip to pick up a collection of crystals when held over the sprinklings. Gently place the coverslip with the crystals clinging to its underside over the well, and add a drop of solvent to the exposed end of the filter paper. The paper will act as a wick, drawing solvent into the well. The crystals clinging to the underside of the coverslip will dissolve slightly as the solvent evaporates and then will recrystallize in place, in larger crystals, with cleaner, better-defined shape, making them easier to inspect in both the light and the FTIR microscopes.

Remember that, for both techniques, edge information can be adjusted through judicious use of immersion oils. As discussed in Part 1,

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to remedy a sample that exhibits too much contrast, find an immersion medium with a closer refractive index, and vice versa.

Image analysis of these samples

For computer analysis or measurements of these samples, especially to a highly heterogeneous mixture of particles, try circularly polarized light microscopy. This derivation of conventional polarized light involves placing a standard polarizer below the sample and an analyzer (a second polarizer, set with its direction of vibration at right angles) above the sample. Next, insert a pair of quarter-wave (146-nm retardation) plates. The first plate can be the regular quarter-wave plate slider available from a microscope manufacturer, positioned at 45° in the accessory slot of a routine Pol scope. The second plate should be situated below the sample but above the polarizer, "crossed" to the first plate so that, when the user looks through the microscope without any sample in the beam path, the background will still be black. Since the human eye is extremely sensitive to changes in intensity, the system can be fine-tuned by gently rotating this lower plate to a position of minimum intensity.

With conventional polarized light, most crystals having a refractive index parallel to the direction of permitted vibration for either the polarizer or analyzer will appear dark. The direction of permitted vibration is a key optical property of polarizing components and is frequently marked on these components, either by using a fiduciary mark or by rotating a vernier scale to 0° or 90°. While there are a few exceptions, crystals oriented at ±45° to these directions will typically exhibit maximum brightness, and crystals at intermediate orientations will have varying intensities. Circularly polarized light removes all these orientation effects, producing uniform brightness for all orientations. A second benefit of this technique is its ability to detect changes in thickness as well as orientation. The resulting shifts in color provide excellent discriminators for segmentation.

FTIR microscopy for these samples

Remember from Part 1 that FTIR microscopy uses longer wavelengths and therefore requires larger, more consistent and cohesive samples. One method for meeting this requirement, outlined in Part 1, was to mount the crystals or particles on the reflective substrate of a gold or aluminum slide and then to use a microroller to flatten the particles.

Thermoplastic resin chips create their own challenges for FTIR. To form a thin film from the chips that is easily read in the FTIR microscope, place a small amount of the chips on a small square of aluminum foil; then, touch a hot soldering iron to the back of the foil.

A variation of this technique also works well for the chemical analysis of letters on suspect documents. Place the foil on top of the letter, and touch it with the soldering gun. The letter will melt and transfer to the foil, which can be used as the FTIR substrate.

Today, many materials are colored dark gray or black. If the colorant is organic, it will produce its own spectrum, readily analyzed and then subtracted from the general spectrum. However, if the colorant is carbon black, it will mask other signals, interfering with the analysis. Experimenting with the sample is worthwhile.

Fibers and fabrics

Conventional light microscopy

As discussed in Part 1, keep in mind that this is a microscopy preparation: A little goes a long way. Use a probe and tweezers to isolate individual fibers or small groups of fibers for analysis. If the fibers or fabrics will not lie flat on a microscopy slide, try stretching them between small pieces of tape, then anchoring them to the slide. Since fibers and fabrics are usually very three-dimensional, they present very strong lens effects at their edges. These effects can be minimized by thoughtful use of immersion medium.

For enhanced contrast, try polarized light, fluorescence, or the older color staining of Rheinberg illumination. To image surface effects, especially those resulting from milling, drawing, or other processing, try Hoffman modulation contrast (HMC) or differential interference contrast (DIC, often referred to as Nomarski/DIC).

Finally, to make inexpensive cross sections, try the following technique. Use a conventional but large-diameter sewing needle to bore a hole from top to bottom through a small laboratory cork. Next, thread a small amount of fiber (several strands) through the needle; then, pull the fiber through the hole in the cork. Section the cork using a new, single-edged razor blade; then, mount the whole section, cork and all, on a microscope slide. The cork will hold the fiber firmly in place and will also guide the user in finding the cross section quickly and easily. This method works exceedingly well with both round and trilobed fibers.

FTIR

These systems come equipped with special sample holders for fiber analysis consisting of an open aperture in a convenient slider, making sample preparation easy and convenient. Simply suspend a fiber between two pieces of cellulose adhesive tape over the opening as follows: Secure one end of the

fiber with a piece of tape, oriented so that the tape stretches vertically over the right quarter of the opening but the fiber stretches horizontally across the opening. Catch the end with a second piece of tape; then, stretch the fiber taut across the opening, and firmly attach the second piece of tape, again mounted vertically, to the far side of the support. At each step, use a probe to firmly press the fiber into the tape.

In general, 10-μm-thick cross section and millimeter lengths are ideal for micro FTIR analysis. As with conventional microscopy, fibers may be too thick and/or too wavy for FTIR analysis, creating distortion from edge and lens effects. Both problems are easily addressed by rolling the fiber before mounting.

An alternative to the free-hanging fiber preparation is to flatten the fiber and then mount it in a pressure cell with a small drop of immersion medium. As shown in Part 1, this approach often produces the cleanest spectra.

There are several methods for optimizing these analyses. First, use an unpigmented fiber as a reference. Second, FTIR works best on pigmented fibers in which the colorant is not soluble in the fiber, versus dyed materials in which the organic molecules actually penetrate the fiber. Finally, fibers respond uniquely to the incoming FTIR beam. Acrylics, for example, tend to be low in infrared absorption; therefore, acrylic samples can be very thick and still produce credible spectra. Wool, on the other hand, is strongly absorbing and requires much thinner preparations.

Films, plastic slabs, and multilayered laminates

Conventional light microscopy

These samples are usually observed in cross sections made in conventional microtomes, small handheld microtomes, or the handheld plane mentioned in Part 1.

Multilayered laminates can also be microtomed, but do need support. One approach is to construct supports from thin pieces of polystyrene or PMMA (polymethyl methacrylate) plate. A drop of methylene chloride will soften the PMMA, creating an all-in-one support/mounting medium.

Another inexpensive solution is to cut squares from the flat bottoms of disposable Petri dishes and use gel instant glue as the mountant. Another quick-setting cement can be made by dissolving foam cups in methylene chloride. If the laminate contains softer layers, try freezing before cutting, using the technique described in the "Foams" section. In any of these methods, the resulting cross sections serve double duty since they can be used for both conventional light microscopy and FTIR.

Clever use of a range of contrast enhancement techniques can define a number of properties in these materials. For example, HMC (or DIC if the material does not respond to polarized light) produces information about pits, scratches, and other surface information. In cross sections, they can also define the boundaries between layers and elucidate interface information. Polarized light is critical for establishing stress-strain relationships but can also be used to image inclusions. Similarly, darkfield illumination can be used to highlight both surface information and inclusions, even those far below the resolving power of the microscope. Finally, for highly scattering samples, try a variety of immersion media to reduce haze and glare.

FTIR

An underlying principle to all these discussions has been the thickness of the sample. If it is rubbery, use diamond windows to compress it. Alternatively, try ATR (attenuated total reflectance). The advent of this accessory has greatly simplified analysis of many polymeric materials since it makes sample preparation unnecessary. Simply focus, bring the sample into contact with the ATR objective, and record the spectrum.

For combination samples such as lottery tickets comprised of printed letters on foil, leave the sample as is; then combine reflection and absorption FTIR.

Synchrotron FTIR emissions have been used successfully for analyzing very thin samples, such as 6 × 6 μm square of 2–4 μm thick layers of instant photographic film.

These particle accelerators put out 70% of traditional intensity in 5 sec with a 200:1 signal-to-noise (S/N) ratio, due in part to their ability to be dual apertured. They produce 100–1000 times the intensity of standard sources but over very small areas. The FTIR microscope is simply mounted at the synchrotron portal and analysis conducted as usual. For example, in the photographic film, the first run was used to identify the major components in the top layer. The data were then subtracted from the general composite spectrum, and the system focused on the next lower level to identify secondary components. There are a number of synchrotron sites across the country, and the time is available free for research and at nominal costs for other analyses.

Foams

The imaging problems inherent in foam analyses derive from their excessive three-dimensionality and the resulting scatter. A longstanding but not always satisfactory approach involves freezing the foam in liquid nitrogen, then microtoming it. To construct an inexpensive, homemade freezing microtome, try a copper block surrounded by a PTFE shroud. Use liquid nitrogen to freeze the sample to the block; then quickly place the block in a conventional microtome and section it.

Another older method involves simply cutting the foam with any sharp blade, then dry brushing the surface with India ink to enhance the surface structure.

While both of these techniques work with some foams, there are still a number of laboratories that have expressed dissatisfaction with producing clean surfaces or sections free of artifacts. A new technology, the confocal microscope, reduces many of these problems by using a point-by-point scanning mechanism and aperturing the detector to eliminate glare. Samples can be cut in much thicker sections (even 1 × 3 in. blocks) and scanned with the confocal microscope. The resulting three-dimensional data are fed into a computer for reconstruction and analysis. Since many foams autofluoresce, the confocal's high-definition images are amazingly clear, displaying the full foam structure for hundreds of microns in depth.

FTIR

To prepare foams for FTIR analysis, either cut small sections and treat them as fibers or go directly to the ATR.

Composites

For conventional microscopy, treat composites like any slab or film, including using polarized light or circularly polarized light for imaging either the matrix or the fillers. For example, one mystery concerning the distribution of reinforcing carbon fibers mounted in rubber sheeting was solved using crossed polarizers with a first order red plate. The sheeting produced a soft, magenta glow in response to the polarized light, while the carbon fibers remained black.

A new approach for tracing glass fibers through the plastic matrix was described last September at Micro '94.[3] The fibers were treated with a fluorescent coating, then imaged in three-dimensions using confocal microscopy.

FTIR

As with many bulk samples, the best approach for FTIR is to use the ATR.

Biological samples

Since the FTIR spectrometer analyzes a broad range of carbon, hydrogen, nitrogen, and oxygen bond types, it is ideal for mapping and tracking biological events. The sample preparation is similar to conventional methods. Begin by embedding the sample in carboxymethyl cellulose, then microtoming in 5-µm-thick sections. Next, mount the sample on a mirrored slide and analyze with the micro FTIR. This technique has been used successfully to analyze cross sections of whole mouse embryo, track the changes in calcification between the bone and cartilage in the head of the femur of a chicken, analyze storage characteristics of wheat, and map brain tissue.

Summary

A blending of conventional light microscopy with micro FTIR analysis provides a winning combination of tools in both material and biological laboratories. Each technique brings its own information, and with a little advanced thought, sample preparation can be optimized to go from one to the other quickly, easily, and effectively. The resulting dual-faceted analysis provides a much broader and more accurate picture of any analytical problem.

References

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