By Barbara Foster and John Reffner

Focus on microscopy

CONVENTIONAL OPTICAL MICROSCOPY VERSUS FTIR MICROSCOPY: SAMPLE PREPARATION FOR BOTH SIDES OF THE COIN, PART 1

MANY YEARS ago, a master microscopist was asked, "What are the three most important things in microscopy?" Without a moment's hesitation he answered, "Sample preparation, sample preparation, and sample preparation." Today, when light microscopy has become the front end for a range of analytical techniques, sample preparation is even more critical and the ultimate outcome less forgiving: a well-prepared sample sets the stage for both high-quality microscopical observation as well as validity of any subsequent measurement.

Interestingly, light microscopy and the related image analysis are on a collision course with the very different realm of Fourier transform infrared (FTIR) spectroscopy, creating a need for more flexible approaches to sample preparation. Practitioners of each technology rarely, if ever, collaborate with the other, yet there is much to be learned by combining the two techniques. Below is a collection of some of our favorite tricks of the trade to bridge the gap.

Ms. Foster is President of Microscopy/Marketing & Education, 53 Eton St., Springfield, MA 01008, U.S.A.: tel.: 413-746-6931. Dr. Reffner is Director of Research, Spectra-Tech Corp., Shelton, CT, U.S.A.

Where are you headed?

In a famous scene from *Alice Through the Looking Glass,* Alice asks the Caterpillar, "Which way should I go?" He responds by asking her, "Which way do you *want* to go?" to which she replies that she doesn't really know. His answer has telling implications for microscopy: "Then it doesn't really matter."

Before beginning any analysis, creating a brief analytical map can save time and reduce the risk of either losing a sample entirely or having to make multiple sample preparations. For example, in the fiber sections below, the conventional light microscopy can be completed, then the fiber rolled and remounted for FTIR analysis.

The first step in any microscopical analysis is selecting a proper sample from the bulk. Faced with analyzing a failure in a 30-gal plastic trash barrel, a colleague once estimated the time necessary to microtome the entire sample. The result could only be plotted on a geological time scale. A better approach is to carefully examine the barrel by eye, determine the problem area, then take a quick look at a large "chunk." that includes that problem area under a hand lens and/or stereo microscope. This first look will provide a general sense of the context.

The next step is to take a smaller, representative sample and prepare it for a more detailed inspection under a conventional compound microscope to evaluate heterogeneity and details of the structure, perhaps followed by some image analysis. If the material or contaminant is organic, the next logical step is chemical fingerprinting using FTIR microscopy. Depending on other needs, the sample might also be reviewed with electron or scanning probe microscopy.

Since each microtechnique provides different information, the approach to sample preparation will also differ. For example, conventional light microscopy differentiates between phases and identifies morphological and optical properties. These parameters require that the sample be kept intact, maintaining sharp edges with well-defined angles and features, and crisp contrast. If there has been some sort of coating, processing, or epitaxial growth, the approach also needs to leave fine structure undisturbed.

Image analysis adds still further constraints. In this scenario, a less-than-human "eye–brain" combination in the camera-computer system tries to separate, identify, then measure features of interest. In addition

to the issues cited above, sample preparation for image analysis is required to produce well-defined phases for high-quality discrimination and segmentation and, wherever possible, sharp, clean, unshaded edges.

Information gleaned from FTIR microscopy is complementary to conventional light microscopy and image analysis. Rather than focusing on edges, angles, shapes, and heterogeneity, the FTIR microscope probes the internal, chemical nature of specific domains within the sample. Because it uses much longer infrared waves than conventional light microscopy (4000–625 cm^{-1} is equivalent to the range between 2.5 µm and 16 µm), it relies on minimum areas on the order of 5–10 µm in diameter. As a result, every effort must be made to create a large, cohesive sample spot for chemical analysis, free of edge effects. Because 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.

Understanding the similarities and differences in these techniques provides better direction for planning a series of sample preparations. The next step is to assemble a collection of functional sample prep tools.

A well-rounded tool box

Probes and tweezers

No matter what type of microscopy you are doing, it is always helpful to have a needle probe and a good pair of tweezers handy for teasing single fibers or crystals out of a mass and transporting materials from one location to another. While tweezers are best purchased from standard sources, very sharp probes can easily be homemade in the laboratory. Materials include a standard mechanical pencil that uses 0.5-mm lead (preferably one that has a rubberized barrel and feels comfortable in your hand), a short (2 in./5 cm) length of 0.5-mm tungsten wire, a small blowtorch, a small ceramic crucible of sodium nitrite crystals, and protective goggles. Remove the lead from the pencil and replace with the wire. While wearing the goggles, heat the exposed end of the wire until it is glowing, then plunge it into the crucible of sodium nitrite crystals. The resulting exothermic reaction is signaled by the expansion of the glow from the wire through the crystals and will result in a finely sharpened probe. A quick rinse with distilled water will remove any crystalline residue. While you have everything assembled, sharpen the other end for future use. To resharpen, repeat the process. (This technique is derived from one used by Professor E. D. Müller at

Penn State University [University Park, PA] for developing probes for field emission microscopes.)

Slicers, dicers, and rollers

In many instances, it is helpful to cross-section or slice off a representative section. For nonmicroscopists, it may be difficult to understand the difference in dimensions between the conventional world and the microrealm and resulting critical need for subsampling. Consider a tiny 3-mm (⅛-in.) cube. Converting that cube into a 10-µm thin film for FTIR analysis would spread it into a 52 mm × 52 mm square, requiring measurements on roughly 2500 microscopic fields of view, even at a modest magnification of 200×!

There are many microtomes available on the market, ranging from the sliding sledge microtomes for very large samples, to manual and automatic rotary microtomes, to freezing microtomes and elaborate ultramicrotomes. However, one of the simplest cutting tools is a diamond knife, available with flat blades for scraping or angled blades for cutting. Several tools designed especially for FTIR microscopy will also be of interest to conventional microscopists: a small, handheld microplane and a microroller, each mounted on handles reminiscent of Exacto knives (Spectra-Tech, Shelton, CT). The plane is especially effective for rapid sectioning of thicker, polymeric materials such as vinyl siding and golf ball coverings while the roller is especially effective in preparing powders, fibers, and slivers of plastic for FTIR analysis. These samples can be placed directly on a mirrored slide and flattened with the roller. Still less-expensive alternatives to the roller are the old ball-tipped rubbing tools used in predesktop-publishing days for dry transfer lettering.

In the less-expensive microtome class is a microtome constructed from two glass slides and a single-edged razor blade. Place the sample on the first slide and hold it firmly in place with the second slide so that only a very small section protrudes under its edge. Using the edge of the second slide as a guide, slice the thin section with the razor blade.

Slides and other supports

Regular glass slides can serve a myriad of functions in both the conventional optical laboratory and the FTIR facility. For example, to break up agglomerates or large-grained powders, just place the material between two glass slides and press. For FTIR work, the fragments can be transferred to a transparent infrared window.

A similar approach can be used to prepare a thinner section of a meltable plastic. Start by gently heating two slides by resting them

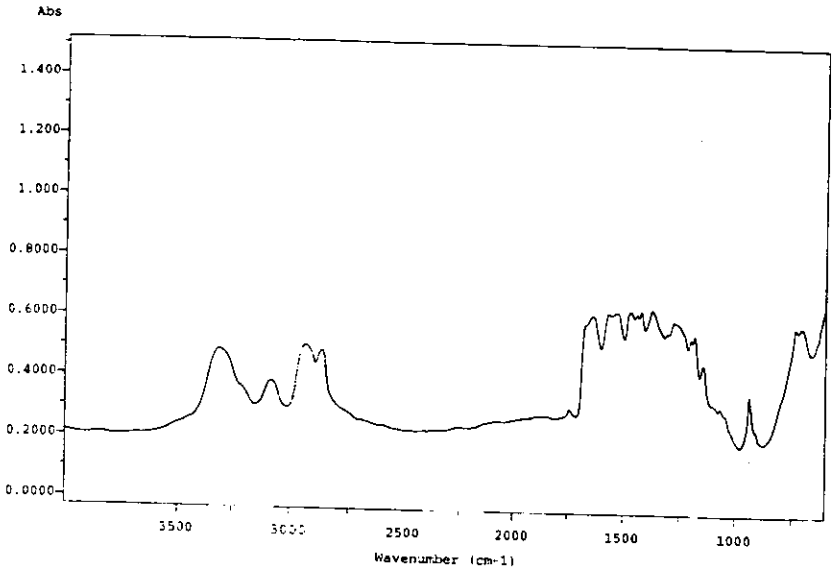

Figure 1 *Effect of sample mounting: nylon fiber, as received (no preparation); 8 cm^{-1} spectral resolution.*

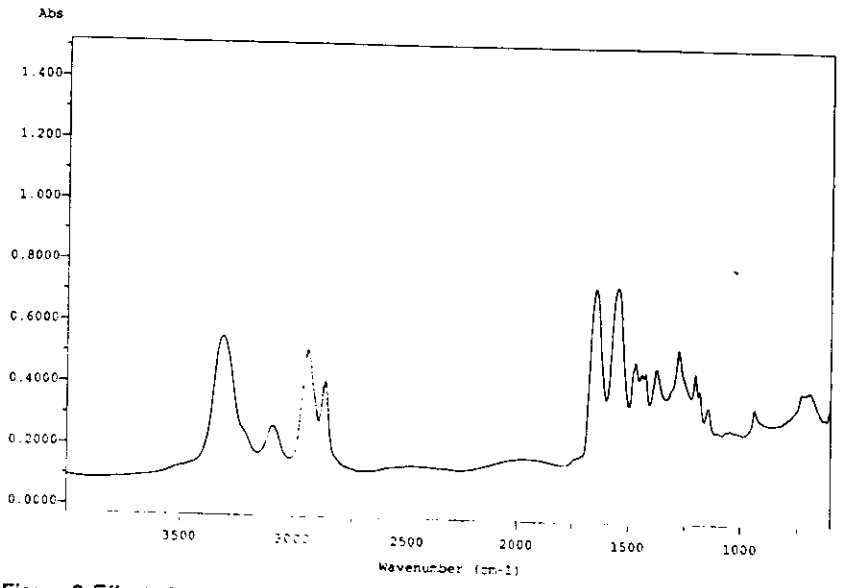

Figure 2 *Effect of sample mounting: nylon fiber, flattened; 8 cm^{-1} spectral resolution.*

on the edge of a hotplate. When they are warm (experience is the best guide as to how hot they need to be for your specific material), place the sample between them, and again, press gently to flatten and thin it.

The actual support used depends, again, on where your analysis is headed. For reflection-absorption spectroscopy and for solvent extractions of heterogeneous samples, gold- or aluminum-coated slides are now available. Designed to hold up to 12 samples in numbered areas, they can improve sample throughput considerably. To reduce glare from slides when looking at multilayer cross-sections such as film or paint in reflected light, try a regular microscope slide painted on the reverse side with a very flat, black spray paint.

FTIR often requires its own sample holders, ranging from open holes in plates across which fibers can be stretched, to compression cells designed to flatten and stabilize samples. The improvement on the quality of the spectrum can be dramatic, as shown by the spectra in *Figures 1–3*. In Figure 1, a strand of nylon 6.6 has been mounted in air. Note that the section of the spectrum between about 2500 and 1500 cm^{-1} lacks definition, especially on the left shoulder and across the top. In the spectrum in Figure 2, the fiber has been flattened using the roller described above, resulting in greater definition on the left shoulder, but revealing two artifact fringes across the top curve, derived from edge effects at the fiber–air interface. Finally, rolling the fiber to flatten it, then mounting it in a compression cell with a small amount of fluid (Figure 3), produces a clean, crisp spectrum with sharp shoulders and no residual edge artifacts.

Immersion liquids

No micro laboratory would be complete without a collection of immersion liquids. Whether your facility is involved in regular light microscopy or FTIR analyses, whether you look at fibers, films, foams, or particles, careful matching of the refractive index of the mounting medium to the sample will improve edge definition, reduce distracting edge artifacts (such as halos, diffrac-

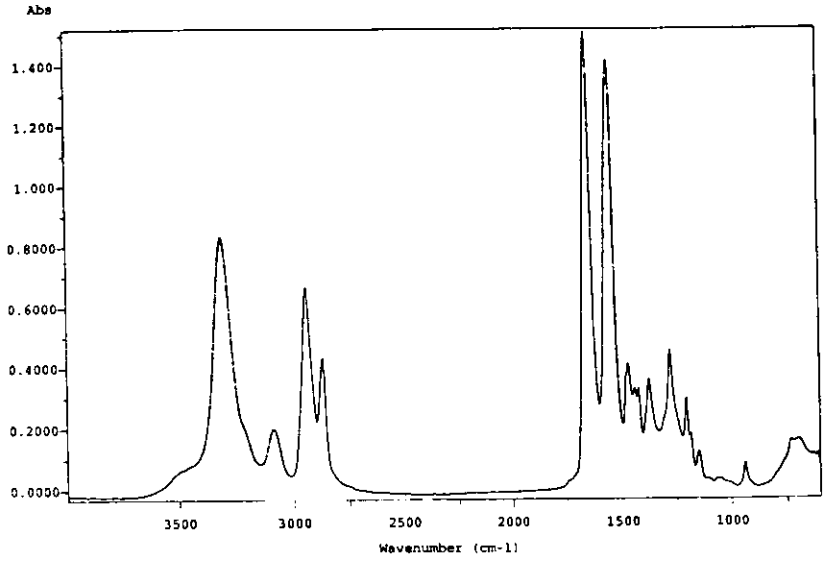

Figure 3 *Effect of sample mounting: nylon fiber, mounted in compression cell; 8 cm^{-1} spectral resolution.*

Table 1
Refractive indices of commonly available liquids

Material	Refractive index
Air	1.00
Water	1.333
Glycerin	1.455
Standard immersion oil	1.51
Nujol or mineral oil	1.5

tion ringing, and lens effects) in both images and spectra, and generally enhance the contrast balance between the object and its surroundings. A quick experiment can verify these findings: Collect a small sample of sugar crystals. Sprinkle several on a regular glass slide, cover with a coverslip, and observe them just mounted in air, using a 10× or 20× objective, in regular brightfield on a compound microscope. Next, place a small drop of standard immersion oil at the edge of the coverslip near the crystals. Capillary action should draw the oil under the coverslip and around the crystals. Observe them again and note the difference in both edge information and surface defects.

Table 1 provides a range of materials and their refractive indices. An important point to remember: The closer the match, the less apparent the boundary between the sample and its surrounding.

More expensive but very valuable is a set of standardized immersion liquids of known refractive index such as those available from Cargille Laboratories (Cedar Grove, NJ). A quick glance at the complex of numbers shown on the sides of each bottle indicates that these oils need to be treated with care (used at correct temperature, with correct illumination/filtration, and guarded against contamination). They are carefully engineered oils that degrade over time and should be replaced at reasonable intervals (annually).

Immersion liquids can present specific problems for FTIR analyses because many of them contain chemical bonds that generate secondary spectra, overshadowing detail from the sample under investigation. One answer is Fluorolube, which has no C-H absorption. It is especially effective when used in a very thin layer surrounding the sample, in a compression cell. Nujol (mineral oil) or Nujol mixed with 10% vol/vol deuterated heptane (C7D16) also provides a nonintrusive mountant. The approach requires standard procedures: taking the spectrum of just the mountant, then the spectrum of sample in mountant, and finally, subtracting the spectrum of the immersion medium to obtain the clear spectrum of just the sample. Another useful compound is fully deuterated heptodecane (available from Cambridge Isotope Labs, Woburn, MA). In both cases, the C-D absorption bands in the 2000-cm^{-1} range rarely interfere with other hydrocarbons.

Part 2

The next "Focus on microscopy" column will apply many of these more general preparation techniques to specific applications ranging from crystals and fibers to multilayer laminates and biological samples.