

## Modern Microscopy On The Light Side The FTIR Microscope

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The attachment of devices to the eyepiece of a microscope is a long accepted practice among microscopists; almost as soon as a new technical advance appears, someone is adapting it to the study of microscopic specimens. Photomicrography was in use from almost the earliest days of photography, while the microspectroscope was developed and described by Sorby as early as the 1860's<sup>1</sup>. As advances in technology provided improved means for recording data and collecting radiation invisible to the human eye, these were rapidly adapted to the microscope as add-on accessories constructed as one-of-a-kind instruments or in a few cases as commercial products. The development by Barer and his coworkers of an infrared microscope/spectrophotometer and the demonstration of its practical utility paved the way for others who would follow (although, as with most inventions which are ahead of their time, not in droves). One of the most notable of these is Vincent Coates, who went from constructing an infrared microspectrophotometer for Perkin Elmer, to the development of the Nanospecs; first the UV Visible instrument and finally the infrared version. The timing of the later development was not opportune, however, since the first of the modern FTIR spectrometers appeared on the market at the same time. This heralded the decline of dispersive instruments available until then.

Dr. Robert Muggli at McCrone Associates (assisted by Howard Humecki and Mark Andersen) used one of these instruments and the reflecting optics from an old Perkin Elmer instrument designed by Coates to build the first (as far as I can tell) "modern" microscope/FTIR in the late 1970's. This instrument was in daily use in the laboratory for several years before the first commercial instrument appeared on the market. In the 13 or 14 years since then, these instruments have gone from curiosity to essential equipment for the analytical microscopist of today. The infrared microscope of today (as they come to be called - causing no little confusion with microscopes which image with infrared radiation) are already third generation instruments, having evolved rapidly since their introduction.

Not all are created equal, however, and the prospective purchaser is well advised to beware. This writer is aware of a number of infrared microscopes which lay idle under accumulated dust at this moment due to the failure of purchaser and salesman to help each other in seeing that the proper instrument was purchased and the correct training obtained. While I expect a great deal of argument on this point (almost all of it from spectroscopists), the best person to select a microscope FTIR is not a spectroscopist or ordinary chemist, but a microscopist, and more specifically an analytical light or chemical microscopist. The former regard the microscope as an attachment or, more specifically, a micro-sampling device. Salesmen and even the technical support staff from most (not all) FTIR manufacturers are chemists/spectroscopists who have neither academic training nor practical experience in microscopy. At worst, they discount the microscope altogether, treating it with the same disdain as the high school biology teacher who commands his students to direct the light on the mirrors of their instrument, put their eye to the ocular and draw what they see, (after passing out photocopies - mimeographs in my day - of the parts and ray path of a microscope). At best they have done some reading and/or taken a short course or two and now set themselves up as microscopists when they are neither by training, discipline, attitude nor experience anything of the sort. The chemical microscopist, on the other hand, regards the FTIR spectrometer as an accessory, not unlike the camera or other photometric collection attachments with which he or she is well acquainted. Having an academic background and, more than likely, practical experience in IR spectroscopy, they are aware of

KBr pelleting techniques and ATR, for example, as well as other sampling methods and devices. The microscopist recognizes that in the vast majority of cases involving micro samples, however, the methods so well time-tested and known to the spectroscopist are worthless or nearly so in this application.

The reason is that the spectrometer views the sample not as the spectroscopist is used to seeing it, but as a microscopist does. For a microscopist, viewing a sample to obtain the information it holds is a function of three factors:

- 1) Quality of the instrument. While the mechanical construction is ultimately of concern as well, the main factor of concern is optical quality.
- 2) Proper adjustment of illumination and aperturing of the specimen.
- 3) Sample preparation. This is by far the most important, since it is the least understood, even (it pains one to say) by many so-called microscopists.

Microscopes designed for the acquisition of infrared spectra with reflecting optics vary in quality. Beware of the sales lines that read "designed by spectroscopists for spectroscopists who want to obtain spectra from small samples" or words to that effect. This is akin to the blind building for the sightless an instrument that is supposed to see a concept which neither group, by definition, understands. It makes no sense whatsoever to be able to see a structure clearly on one's own microscope and find that it is barely visible when transferred to the stage of an infrared microscope. Yet several commercial instruments suffer this defect to a high degree.

The proper set-up of illumination is critical in any application involving optical instrumentation and nowhere more so than in microscopy. Spectroscopists are used to introducing their samples into the optical path of their spectrometer without the adjustment, refinement and critical appraisal of the image that falls on the spectrometer slit. To the microscopist these are prime considerations each and every time they place a specimen on the stage. They recognize that proper illumination not only insures a pleasing image, in which details can be seen and structures recognized or numerical constants correctly measured, but that a poor image invites difficulty of recognition and questionable measurements.

John Reffner of Spectra-Tech (chemical microscopist, well recognized among spectroscopists as well) uses a slide in his lecture which sums up the three most important factors of successful infrared microspectroscopy. It lists the phrase "Sample Preparation" three times. There is no question that this is the ultimate and deciding factor in determining the value of the results obtained. The methods of preparation do not need to be the most elaborate or require the most expensive sample preparation equipment, but they do need to be appropriate. This is an aspect too often overlooked in our modern rush for sample through-put. There are many who maintain, however, that "slop results" can be regarded as wasteful of time and, therefore, not at all efficient. Time spent preparing a sample correctly in the first place or at least trying to, is well worth the expense in the long run. Not surprisingly, most of the methods of most value are those of the microscopist and microchemist.

The examination of multilaminar polymer film serves as an example of the ideas expanded here. The advent of micro-FTIR was a boom to analysts desiring to analyze these special purpose polymers. These films require examination in quality control, defect analysis and perhaps most frequently in monitoring of competitors' products. The composition, thickness and order of layers are important factors in the suitability of a product for a specific application. Conventional methods of analysis require mechanical and solvent stripping as well as computer subtraction. These procedures still did not provide precise information about layer order or the thickness of individual layers. The preparation of cross sections with a microtome with subsequent analysis of each layer with a microscope FTIR spectrometer appear to offer an elegant solution. In practice, most scientists (including most microscopists) are not very good microtomists. If the imaging qualities of the microscope chosen are less than optimal, then specific layers and structures can not be properly seen or apertured. This leads, at best, to confusing and super imposed spectra and, at worst, incorrect assignment of layers.

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# The Biological Images Project

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The Biological Images Project is the culmination of the four summers I have spent at Apple Computer. In short it is a software package which allows students to manipulate and explore biological images (i.e. micrographs, CTs, MRIs, etc.). Consisting of four sections - interface, biological images, image processing, and communication software - this package can serve as a powerful educational medium. The interface supports the more traditional forms of teaching - textual notes and brief lectures. The collection of biological images allows students to visualize biological structures in ways they never have before and exposes them to a variety of scientific techniques and tools. Image processing lets the student interact with the images, to manipulate, explore, discover and in some ways gain ownership of the image. Finally the communication package will provide the students a medium to share their discoveries in an interactive manner and submit them to peer review.

The key to this project is the images themselves. In total I am hoping to collect between 40 and 60 high quality biological images from a variety of media - light and electron microscopy, MRIs, CTs, X-rays, ultrasound, PET scans, etc. These images will be used only for a prototype version and will certainly not be distributed without their author's permission. The major requirement for these images is that they can be explored/analyzed through image processing. This can include simply enhancing the image to see structures better, morphometrics, particle counting and analysis, artificial color enhancement and animation. I am looking into possible software packages for three dimensional reconstruction but at this point only two dimensional data or animation of serial sections will be supported. Although aesthetic images are certainly a plus the most important characteristic is that some manipulations can be done to them. Examples of such images include - using particle analysis to find the number and area of food vacuoles in a protozoa, measuring DNA length of a virus which underwent osmotic shock, coloring and animating a collection of confocal laser scanning microscope images of a cell in metaphase to measure chromosome size and see if an antibody tag cross reacts, a freeze fracture preparation of a nucleus to estimate the size and number of nuclear pores, a serial collection of MRIs that when animated produces a "movie" of the brain, enhancing and rearranging chromosomes in a karyotype to check for chromosome abnormalities, etc. The rule of thumb to see if an image is appropriate for this project is to determine if more information can be gained through using a computer to view and analyze the image than by simply seeing the image in a text book.

This project depends upon the voluntary support of the scientific and educational community; I greatly appreciate your time, support and input. If you I have any images which you feel would be appropriate for this project, think you know people who would, or have any comments regarding my work, I can be reached at (408)974-3210 or electronically at MPenn@Applelink, Apple.Com.

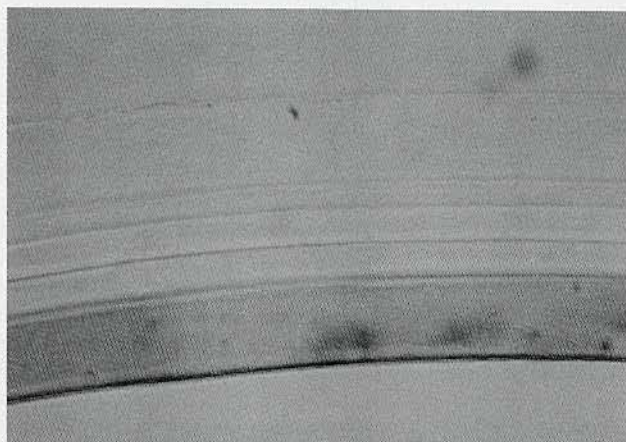


Figure 1

In practice a great deal can be learned about these films with relatively little expenditure of effort. The five layer film shown in Figure 1 is a section of a soft, flexible packaging film. It was cut free-hand<sup>2</sup> by drawing a fresh razor across the surface while supporting the film (cut to a thin strip) between the thumb and forefinger of the other hand and observing with a stereomicroscope. For the less experienced, the strip of film may be placed on the stage and sections obtained using the fingernail as a guide as described elsewhere<sup>3</sup>.

The section shown permits clear recognition of the individual layers and measurement of their thickness. A preliminary indication of the composition of the film can be obtained by the use of stains. The bottom layer of the film shown in Figure 1 has been stained blue by short immersion in a stain specific for polyamides, thus demonstrating that this layer is nylon.

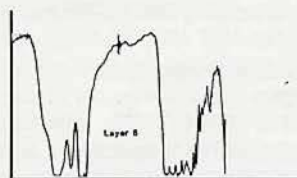


Figure 2

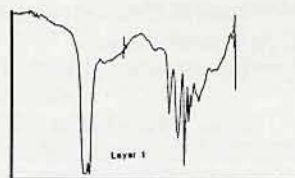


Figure 3

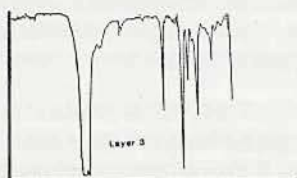


Figure 4

The stain does not interfere with the subsequent spectroscopic examination as seen in Figure 2. Although somewhat thick, the layer is clearly identified as nylon. The color of the layer is helpful in orienting difficult specimens on the FTIR microscope. Layer 1 is Surlin (Figure 3) while layers 2,3, and 4 are thin, but distinct, layers of ethylene vinyl acetate (EVOA). Melting point determination, as seen in Figure 4, revealed that each layer of EVOA has a different melting point indicating differences in composition not seen in the spectra. The entire examination took less than a hour with sample preparation occupying only minutes.

<sup>1</sup> Sorby, H.C. Proceedings of the Royal Society (@ 1867) p.33.  
<sup>2</sup> Dieter Kranter in his excellent Mikroskopie in Alltag (Franck'sche Verlags handlung, Stuttgart Germany 1968) recommends that male microscopists shave with a straight razor to develop skill at freehand sectioning.  
<sup>3</sup> Palenik, S. and Fitzsimons, C., Fiber Cross Sections Part II, Microscope 38 (1990) p. 315.