

NOVEL USE OF FLUORESCENCE ILLUMINATION WITH AN INFRARED MICROSCOPE

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It has become increasingly obvious that infrared microspectroscopy can be the analysis tool of choice when determining the chemical composition of biological and biomedical samples. Frequently, fluorescence illumination is required for sample characterization, which previously required the use of a separate optical microscope. There has also been a need in the semiconductor manufacturing industry for a single tool for visualizing particle contaminants on integrated wafers as well as the ability to chemically determine their nature. There is now a single microscope platform for conducting rapid Nomarski differential interference contrast and fluorescence illumination sample visualization as well as infrared analysis. This novel infrared microscope has applicability to many fields of investigation, including pharmacology, forensics, cell biology, histology, gemology, and geology.

INTRODUCTION

Historically, there have been separate fields of investigation involving microscopes. Optical microscopes have been well utilized for many years for visual characterization of samples of interest. Infrared microscopes have also been used over the last 20 years to perform chemical analysis on virtually any sample of interest. For several years, refracting optics based optical microscopes have incorporated infinity corrected optics, Nomarski differential interference contrast (DIC), phase contrast and other advanced techniques. While visual observation has been possible with infrared microscopes, the performance has fallen short of the comparable state-of-the-art optical microscope. However, a new infrared microscope platform has been established which incorporates many of the features and benefits of currently available optical microscopes. These features include visible polarization, an infinity corrected beam path, DIC, sample substrate compensation, Koehler illumination, and simultaneous visual observation and infrared data collection. And these features were accomplished without degradation of infrared performance. This accomplishment involved the incorporation of fluorescence illumination into an infrared microscope for the first time. There are many areas of application which would benefit from the dual use of infrared and fluorescence microscopy including forensics, histology, cell biology, pathology, geology, gemology, biomedicine, and many others.

Fluorescent compounds absorb light and immediately emit light at wavelengths longer than the absorbed wavelength. Primary fluorescence is known to occur in plant cell walls, wool, and many pharmaceutical products. Secondary fluorescence is the use of fluorescent dyes or fluorochromes to illuminate samples which do not exhibit native fluorescence. The dyes typically are bound to the compounds of interest. Fluorescence is particularly useful for visualizing samples against a dark background and can have a lower detection limit of 50 molecules per square μm . By the 1950's, the use of secondary fluorescence made fluorescence microscopy a common analytical tool.

Biomedical studies

Recently, applications of infrared microspectroscopy to the study of biomedical and biological species has increased dramatically. The infrared analysis provides necessary chemical

information for fundamental tissue characterization and metabolic activity. In contrast, fluorescence illumination has been a commonly used technique in optical microscopy for many years. To combine these two techniques, it has been necessary to perform microscopic analyses with two separate microscopes. For state of the art optical microscopy investigations involving the use of Koehler illumination, Nomarski differential interference contrast (DIC), fluorescence illumination, and other features, a dedicated optical microscope was required. Frequently, the visible characterization step was followed by infrared chemical analysis using an infrared microscope. Infrared analysis required the user to somehow mark the sample to ensure that the area of inspection using DIC or fluorescence illumination was properly delimited and masked to allow infrared data collection from only the area of interest. The optical design (finite tube length) of earlier infrared microscopes did not allow the ready insertion of accessory optics, and therefore precluded the use of DIC optics.

For the first time, we were able to use a *single* microscope for the analysis of female monkey bone tissue utilizing DIC, fluorescence illumination and infrared analysis. The purpose of this study is to examine the chemical composition of bone remodeled after menopause or ovariectomy in order to gain a further understanding of bone fragility in osteoporosis.

Semiconductors

The microelectronics industry continues to be focused on miniaturization of integrated components on silicon wafers. For the integration of microcomponents to be effective, the wafer must be clean to the parts per billion level. In fact, the initial steps in wafer fabrication are solely devoted to cleaning of the wafer. After component integration takes place, individual components can suffer damage when conducting surface contaminants are present. Typically, quality control is performed at many steps in the manufacturing process to ensure that the product is free of contaminants. When contaminants are found, it is vital that chemical characterization of the particles takes place to determine the source of the contaminants.


Currently, there are no satisfactory techniques for performing quality control on semiconductor wafers and circuit boards. A technician typically uses an optical microscope to scan for contaminant particles. This can be a tedious process and is particularly difficult when investigating integrated circuits. Integrated circuits contain so many small features that the observation of dust and other contaminant particles is time consuming and requires much patience. Infrared spectroscopy has been shown to be most helpful in identifying the source of these contaminants.

It has been found that fluorescence illumination provides a dark background for rapid observation of contaminant particles, which frequently exhibit fluorescence. After visual observation of the particles, the corresponding infrared spectra are collected allowing chemical identification of the contaminants.

EXPERIMENTAL

Tibia bone tissue from an ovariectomized female monkey (*Macaca fascicularis*) was analyzed using a Continuum infrared microscope (Spectra-Tech, Inc., Shelton, CT) attached to a Nicolet (Madison, WI) Magna 560 infrared spectrometer. The monkeys were administered fluorochrome labels at various time points after ovariectomy, which were deposited into the newly made bone. Calcein (green) and alizarin complexone (orange) were administered one year and two years after ovariectomy, respectively. At necropsy, the tibia was embedded in poly-methyl

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methacrylate (PMMA) and subsequently cryogenically microtomed to 5 μm thicknesses.

Violet (400-410 nm) and green (510-550 nm) excitation wavelengths were used to visualize the fluorochrome labels present in the bone tissue. The excitation source was a mercury arc lamp, where wavelengths were selected by utilizing Olympus America (Melville, NY) fluorescence cubes U-MNV and U-MWG, respectively. The visual images were captured by an Olympus DP-10 video camera.

A single dual remote masking aperture was used to define the samples for infrared data collection and minimize diffraction. The aperture was set to 30x8 μm in the μView software package (Spectra-Tech, Inc.) for transmission microanalysis. The final format of the bone tissue data was absorbance, where the background was collected open beam. The infrared spectra were the average of 128 scans collected at 4 cm^{-1} with triangular apodization. Finally, the collected spectra were baseline corrected.

A scrap integrated circuit semiconductor wafer was analyzed using violet fluorescence illumination using the same instrumentation as described above. Contaminant particles were remotely masked while under fluorescence illumination for subsequent infrared data collection. The data collection parameters were the same as above. A gold mirror was used as a background for the semiconductor infrared data, where the resultant data was reported in $\log(1/R)$.

RESULTS AND DISCUSSION

Biomedical Studies

Figure 1 (upper) shows a micrograph of a female monkey tibia tissue, which has not been enhanced. Note that it is very difficult to resolve any structure in the tissue despite the employment of the aperture stop available in the Koehler beam path. The use of an aperture stop in the Koehler beam path is usually the first step taken to enhance sample contrast and visualization. Figure 1 (lower) shows the Nomarski DIC image of the bone tissue, where structure is clearly evident. The dark gray area is the PMMA embedding compound. It is now possible to navigate around the bone tissue and identify bone sub-structure, but not possible to perform

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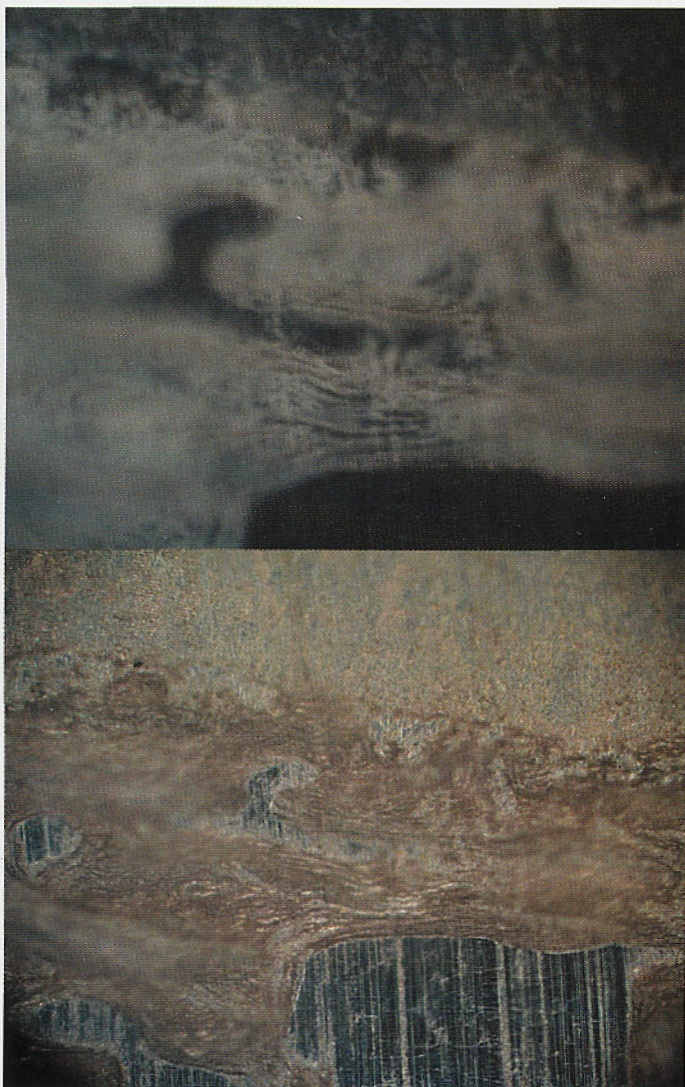


Figure 1. Photographic images of a 5 mm-thick cross-section of a female monkey tibia are shown under different illumination conditions. Upper shows the tissue under normal brightfield illumination and Lower is the same tissue utilizing Nomarski DIC.

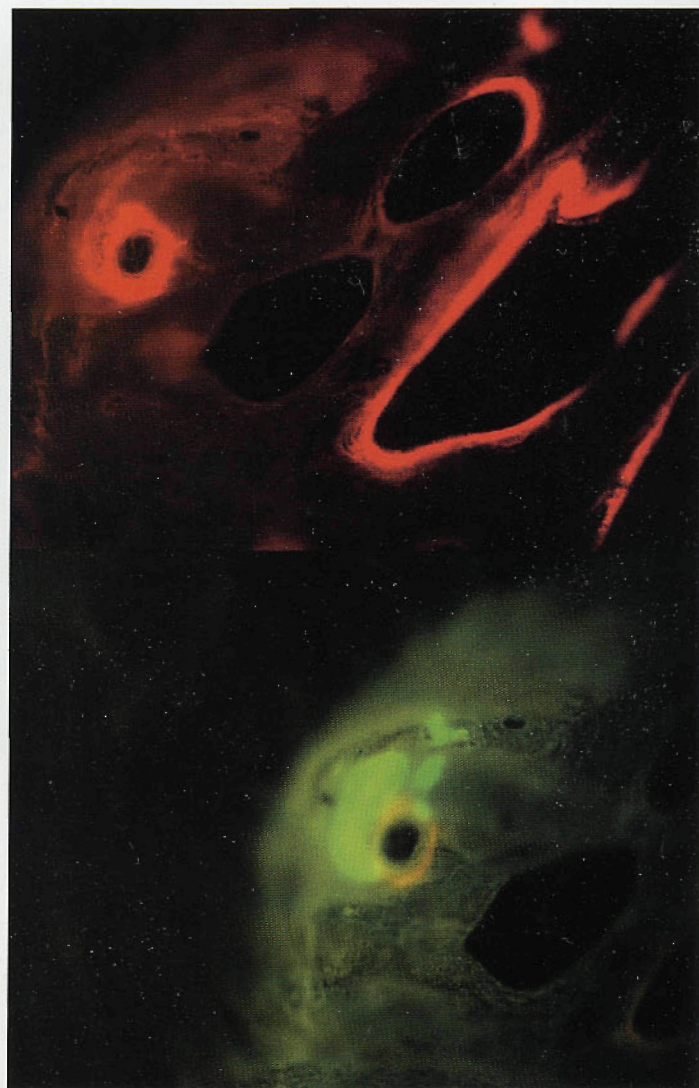
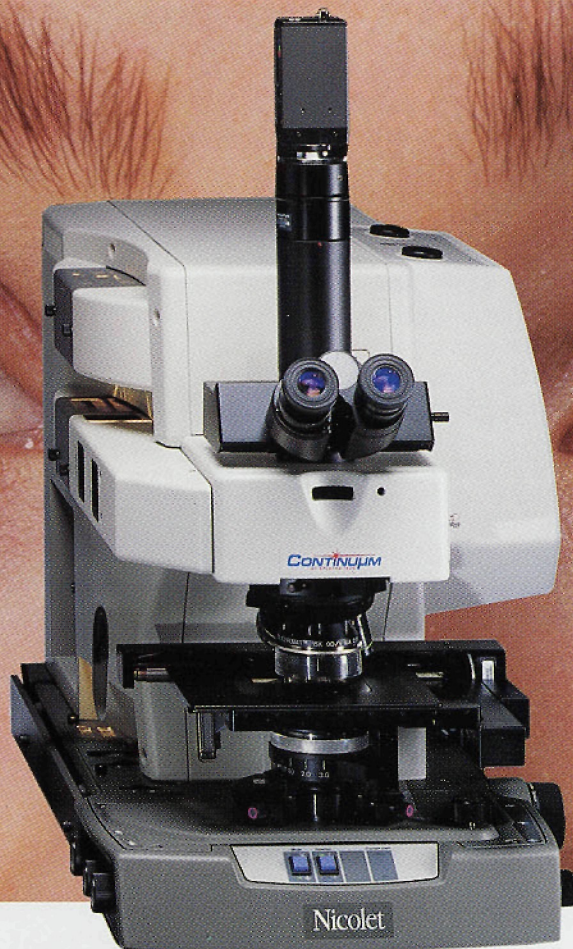


Figure 2. Photographic images of a 5 mm-thick cross-section of a female monkey tibia are shown using fluorescence illumination. Upper and Lower images show the tissue under violet and green illumination, respectively. The dark area to the bottom right in both images is the sample holder, which does not exhibit fluorescence.

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a careful chemical analysis on the tissue. It is desirable to detect chemical differences in the newly remodeled bone tissue with respect to the old bone tissue following ovariectomy. Changes in the bone chemistry might reveal important information pertaining to the cause of bone fragility in osteoporosis and how it might be prevented and/or treated.

Green illumination reveals the presence of tissue emitting orange fluorescence (alizarin complexone label), shown in Figure 2 (upper). The orange tissue is on the perimeter and is the newest modeled tissue. Since the newly remodeled bone tissue is clearly evident, this area can be remotely masked for collecting an infrared spectrum from just this region, shown in Figure 3a. Infrared absorption bands present at 850-880 ($-\text{CO}_3^{2-}$), 900-1200 ($-\text{PO}_4^{3-}$), and 1415 ($-\text{CO}_3^{2-}$) cm^{-1} indicate the presence of mineral in the bone. Bands between 1375 and 1450 ($-\text{CH}_2-$) cm^{-1} indicate the presence of lipids. Lastly, the bands at 1660 and 1550 cm^{-1} reveal amide I and amide II absorption bands, respectively, indicating the presence of collagen (protein). There are no infrared absorption bands present in the spectrum resulting from the fluorochrome label, alizarin complexone. Secondary fluorescing dyes are typically used at concentrations dilute enough to be undetectable in the infrared.

Figure 2 (lower) shows a micrograph of the bone tissue sample under violet illumination. The bone tissue remodeled only one year after ovariectomy is evidenced by the green colored bone tissue. The green color is due to the presence of calcein. The respective infrared spectrum is shown in Figure 3b. There are two band differences present in this spectrum with respect to Figure 3a. With respect to the protein (amide I) band intensity, the intensities of bands attributed to carbonate ($-\text{CO}_3^{2-}$) (850-880 and 1415 cm^{-1}) are clearly greater in tissue remodeled after one year versus two years. The presence of carbonate is directly related to the amount of mineral present in the bone. The phosphate bands, 900-1200 cm^{-1} , are too strong in absorption intensity to be considered in this investigation. Thinner sections would be required for evaluation of the phosphate content. It is clear from the data that the degree of mineralization is reduced after the onset of osteoporosis resulting from ovariectomy. Figure 3c shows the infrared spectrum from adjacent cartilage tissue for comparison. There is a complete absence of bands attributed to phosphate or carbonate, indicating a lack of mineral content in the cartilage.

The time required to visualize the sample with and without Nomarski DIC and two different wavelengths of fluorescence illumination was about 10 minutes. The time required to mask the sample areas of interest and collect infrared spectra was another ten minutes. Software control collect infrared spectra was another ten minutes. Software control of the sample position and aperture settings simplified the user interface, saving time. The ability to rapidly visualize and chemically characterize tissue samples provides a new and important capability for biological and biomedical applications.

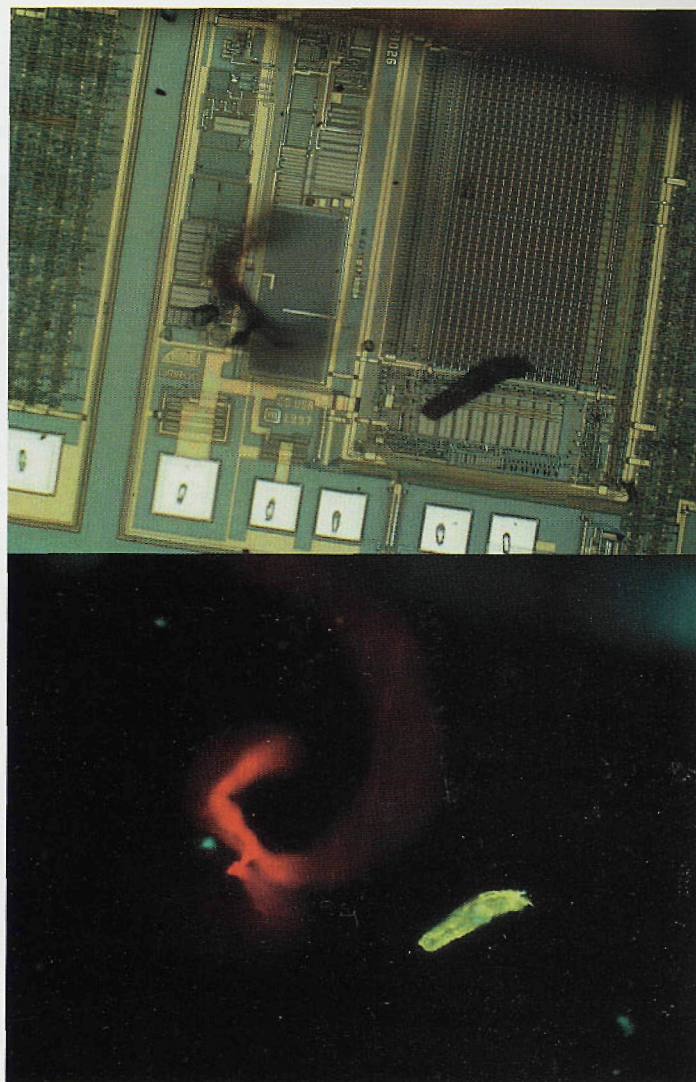


Figure 4. Photographic images of an integrated wafer, where Upper shows the wafer under normal reflection illumination and Lower shows the wafer under violet fluorescence illumination.

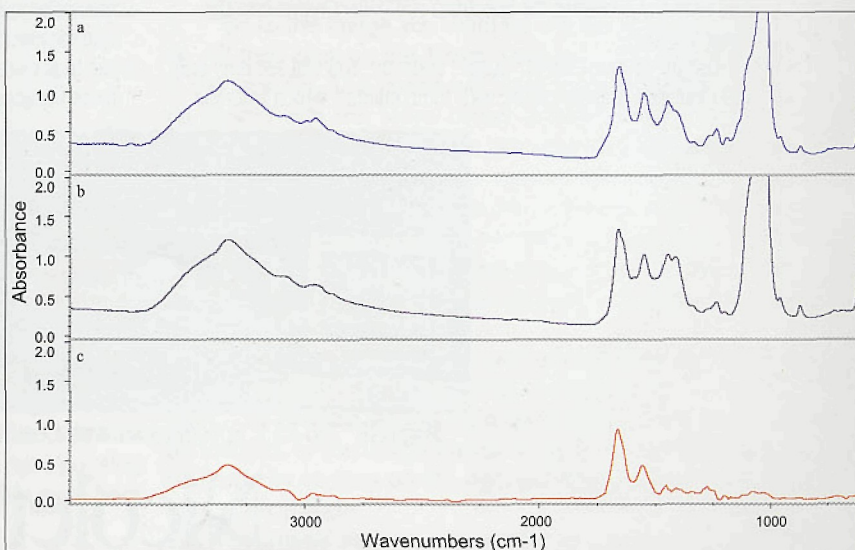
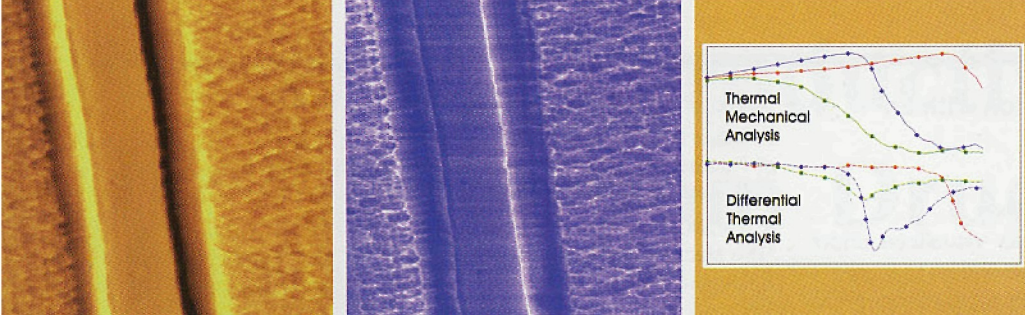
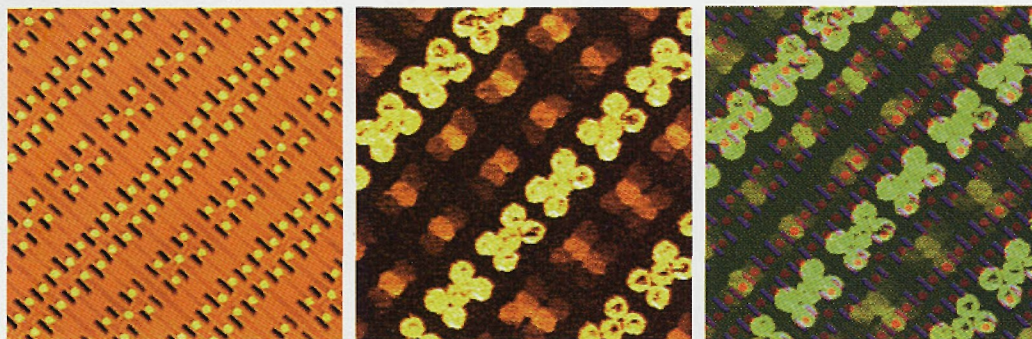


Figure 3. Infrared spectra collected from the newly remodeled bone tissue, where 3a corresponds to tissue remodeled two years and 3b one year after ovariectomy. Figure 3c is collected from cartilage in the tibia

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Using topography, thermal conductivity mapping and micro thermal analysis identifies layers in a polymer film. Courtesy of Duncan Price, Loughborough.



Topography and electrostatic force images combine to show misalignment of contacts and implants in SRAM.

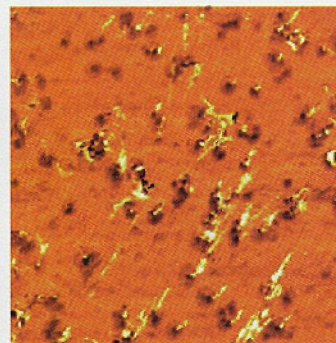
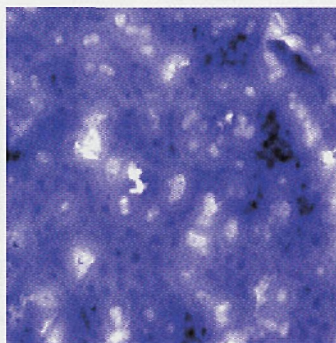
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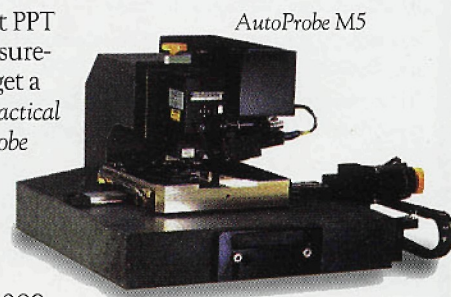


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Semiconductor applications

An integrated semiconductor wafer was visualized under normal bright field observation conditions, as shown in Figure 4 (upper). Note the difficulty in discerning the presence of small contaminant particles with the presence of integrated components. Figure 4 (lower) shows the same wafer under ultraviolet fluorescence illumination. The dark field used for fluorescence microscopy cloaks the non-fluorescing integrated components, making it a trivial matter to identify and mask each particle for infrared data collection. It is also possible to quantify the degree of contamination present on the wafer. The infrared spectrum of the yellow particle is shown in Figure 5a. It is apparent that absorptions due to low molecular weight polystyrene are present in the spectrum. Figure 5b shows a spectrum of molecular weight 800 polystyrene and 5c shows the result of mathematically subtracting the polystyrene spectrum from the raw spectrum. Figure 5d shows a reference spectrum of an alkyd urea resin, which closely matches the subtraction result found in 5c. The identification of the low molecular weight polymer (polystyrene) resin contaminant would allow for precise identification of where the contaminant originates.

Figure 6a shows the infrared spectrum of the red ribbon like particle as shown in Figure 4 (lower). A reference infrared spectrum of purified Zein, a common organic resin, is shown in Figure 6b. The spectra correlate very well, with intensity differences in the 1455 and 1405 cm^{-1} absorption bands. As with the first contaminant, the origin of this contaminant would be easily determined by a technician familiar with the manufacturing process. These particles are representative of contaminants which might be manifest. Analysis of other areas of the circuit board indicated the presence of water residuals near solder joints. These water residuals are a common trail left by flux, which is used in the soldering process. The ability to quickly locate and identify these organic contaminants clearly demonstrates that fluorescence illumination coupled with infrared spectroscopy is a powerful new tool for analyzing semiconductors, integrated wafers, and circuit boards. ■

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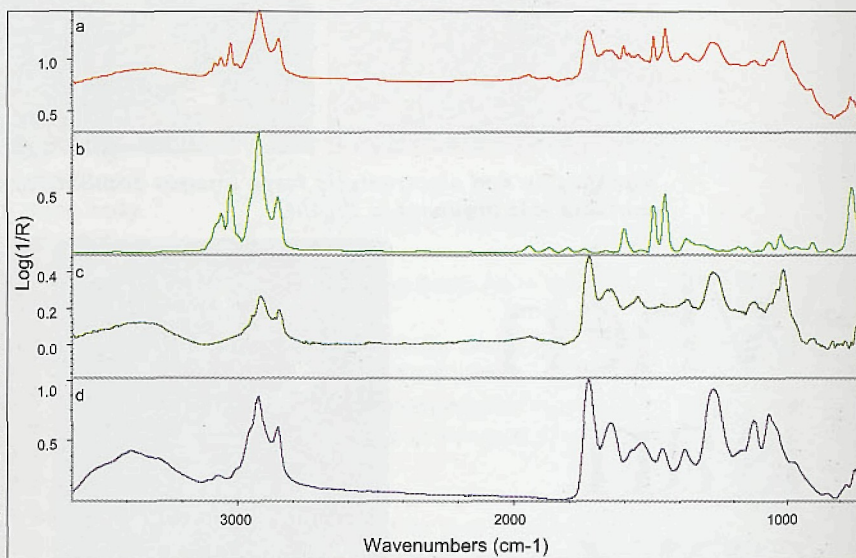


Figure 5. Spectrum 5a is the infrared spectrum collected from the yellow particle found in Figure 4 Lower.. 5b shows a reference spectrum of 800 MW polystyrene from the Hummel Polymer Reference Library (Nicolet Instrument Corp.). Spectrum 5c is the infrared spectrum resulting from the subtraction of 5b from the raw spectrum, 5a. 5d shows the spectrum of an alkyd urea resin reference spectrum also from the Hummel Polymer Library.

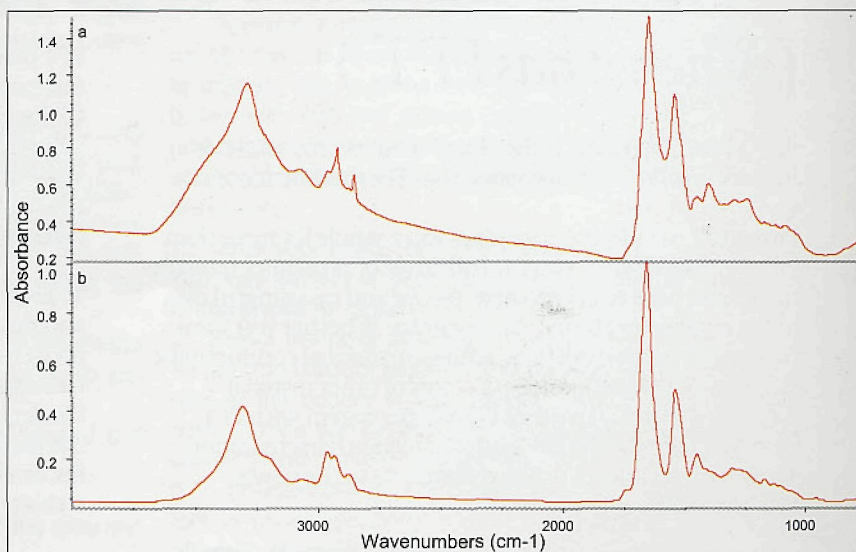
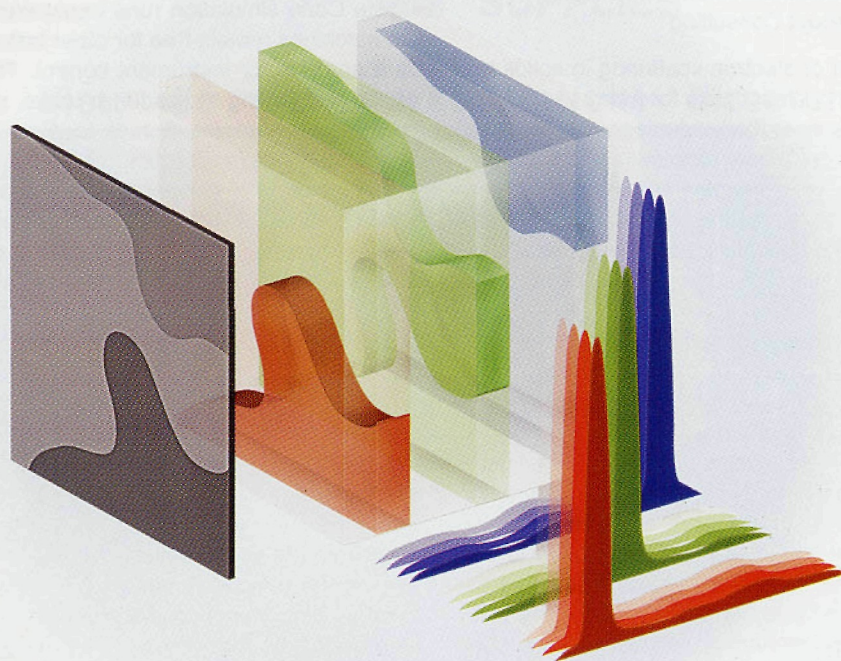


Figure 6. Spectrum 6a is the infrared spectrum collected from the red particle found in Figure 4 Lower. 6b shows a Zein (an organic polymer resin) reference spectrum also from the Hummel Polymer Library.

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