TECHVIEW: MICROSPECTROSCOPY

Imaging Molecular Chemistry with Infrared Microscopy

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Fourier transform infrared (FTIR) microspectroscopy uses infrared radiation to detect and quantify the molecular chemistry of microscopic samples. By combining spectroscopy with microscopy, molecular information can be obtained with great spatial resolution at the microscopic level. Samples can be analyzed directly, in air, at room temperature and pressure, wet or dry, without destroying the sample.

The capability of performing spatially resolved chemical analyses of microscopic regions of samples in situ has been applied to forensic science, materials science, art restoration, and the biological sciences. For example, individual parts of natural or man-made materials, such as layers of a cross section of a paint chip from an art object or from an automobile involved in a hit-and-run accident, have their own unique chemistry. FTIR microspectroscopy is an exceptional tool for investigating the chemistry of these layers, because it does not rely on homogenization, extraction, or dilution, but rather each structure is analyzed in situ.

Defects and contaminants in materials and trace evidence in forensics are investigated routinely with FTIR microspectroscopy. Analysis of a single fiber from fabric or a single human hair is possible using either the transmission or internal reflectance mode. Not only can a fiber's chemical composition be identified with infrared, but analysis of the fiber by polarized infrared radiation reveals the molecular orientation imposed on the fiber during processing (*1, 2*). In a forensic investigation, such analyses narrow the source of fiber evidence found at the scene of a crime and enable comparison of the fiber with a fiber from the suspect.

Laminates such as plastic packaging materials, paint chips, or layers of paint found on an art object under restoration are analyzed routinely layer by layer. For example, analyzing the cross section of a chip of an antique painting for the presence of cholesterol can reveal whether an egg-based glue or a liquid-hide glue was used. Recently, the depth of penetration of ultraviolet rays (from 3 years of Florida sun plus irradiation from a xenon source), which cause photodegradation of acrylic films such as those found in automobile clear-coat paint, was determined by producing a line map of spectra from the edge (exposed surface) to the interior (*3*). Also, with this technology, in the micro-attenuated total reflectance (ATR) mode, nylon marker fibers in U.S. paper currency can be readily identified with this technology.

In the biological sciences, FTIR microspectroscopy has been used to demonstrate differences between diseased and normal tissue, changes in chemical composition that are dependent on the state of differentiation, and metabolic differences between cellular layers. Examples of pathological tissues that have been examined are: (i) white matter from multiple sclerosis patients (*4*) and from twitcher mice with globoid cell leukodystrophy; (ii) extravasated blood in brain tissue (*5*); (iii) Alzheimer's disease plaques; (iv) cancerous tissues (breast, colon, cervical, skin) (*6*); (v) diseased arteries; (vi) gallstones (*6*); and (vii) foreign material in tissue, such as drugs of abuse in hair (*7, 8*) and silicone or polyester implants in the breast (*8*). FTIR microspectroscopy has been used to characterize regions of bone (*9*) at different stages of differentiation and to demonstrate that different layers of the rat cerebellum have different metabolic rates. In the latter example, deuterated water was administered to rats, and the incorporation of deuterium (D) in place of hydrogen (H) enabled the detection of CD, ND, and OD functional groups in the tissue samples (*10*).

The infrared microscope differs from microscopes used for other regions of the spectrum in that it has exclusively front-surface (Schwartzschild) optics for both the objective and condenser. Once the sample is centered in the field of view an image plane mask (an opening between the source and the objective) is projected onto the field and adjusted to limit the area through which infrared radiation will pass. Masking the microbeam preferably should be done both before and after the specimen to reduce diffraction, thus maintaining the desired spatial resolution. A high-quality infrared spectrum is obtained (by averaging data from a reasonable number of scans) in less than 2 min. A ratio of the single-beam spectrum at each wavelength to background is obtained under the same conditions for the same size aperture. This ratio enables presentation of the spectrum in the form of either transmittance or absorbance. Instrumentation worthy of this technique was developed in the last decade with some very recent enhancements.

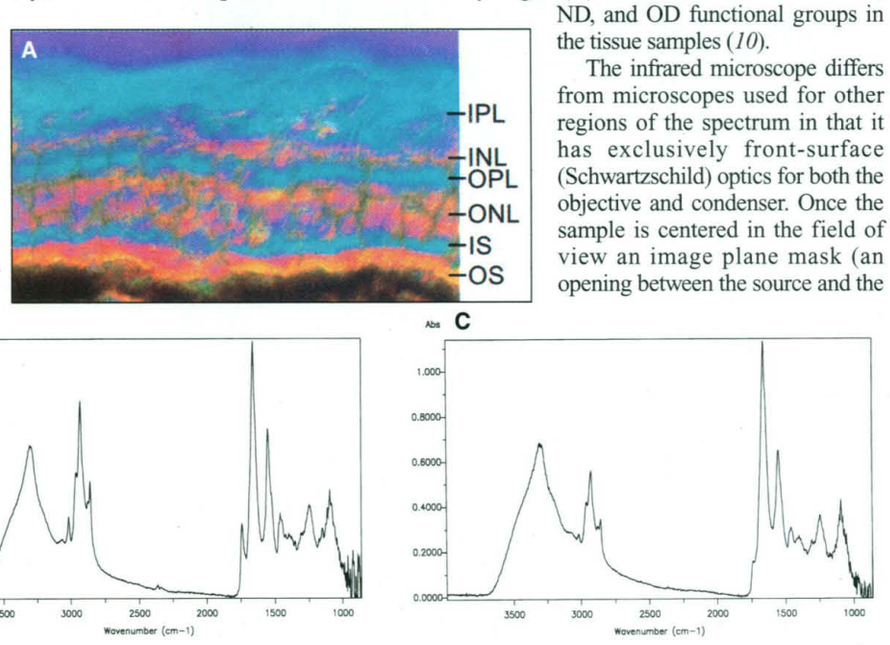

Fig. 1. (**A**) A photomicrograph from a Nomarski DIC image of layers of the rat retina. The spectrum of (**B**) was obtained from the outer segment that is rich in lipid material. (**C**) is from the outer nuclear cell layer. The presence of the nuclei are no doubt responsible for the strong P=O band at 1235 cm^{-1}. Lipid chain length, branching, and glycolipids are inferred by comparing contributions of C=O, CH_3 stretch, CH_2 stretch, and H-C-OH groups. The amount of unsaturation is in evidence from the CH absorption band at 3015 cm^{-1} on the carbon that is attached to the C=C bond.

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Sample preparation often involves sectioning specimens to approximately 8 μm. For tissue, fresh-frozen specimens are used in order not to alter the chemical constituency. Although fixed or stained tissue can be examined, the chemical changes that these steps cause must be considered. Advances in instrumentation since 1993 have included substituting a synchrotron source of infrared radiation for a conventional globar source. The extremely bright, nondivergent, and low-noise beam of synchrotron radiation enables collection of good quality spectra from microscopic regions (routinely 6 μm by 6 μm and 3 μm by 3 μm in special cases). On the other end of the instrument is a liquid nitrogen–cooled HgCdTe focal plane array (infrared camera with a flat grid of microdetectors) that allows simultaneous collection of spectra at 4096 pixels. This form of instrumentation, a spin-off from military technology, is particularly useful when infrared imaging is the primary objective. In 1998, a research-grade infrared microscope equipped with Nomarski DIC (differential interference contrast) was introduced. This feature is composed of infinity corrected optics for both viewing with visible light and interrogating the sample with infrared.

Organic functional groups (in the specimen) are identified by their select pattern of absorption wavelengths in the infrared spectrum, and the relative amounts of these groups are determined by the magnitude of absorption at each wavelength. Functional group maps or "chemical images" can be generated pixel by pixel for each individual absorption band from a series of spectra collected in a grid pattern. These chemical maps can be superimposed onto photomicrographic images, adding a microchemical dimension.

Mapping a specimen by running a series of spectra in a one-dimensional (1D) row or in a 2D grid allows comparison of any one point with surrounding points. An early application of this technique was mapping the boundaries of gray matter, white matter, and the basal ganglia (*11*). In these studies, all of the distinguishing bands of white matter were assigned, and the composition was reconciled with data obtained by other biochemical methods. Maps of various portions of cross sections of grain such as wheat and corn have been investigated extensively. The mapping of a single cell from a wheat cross section (aleurone cell from a row of cells between the endosperm and seed coat) was achieved as early as 1993 (*12*). Subsequently, with the synchrotron source, a sharper aleurone cell map in greater spatial resolution was accomplished, and ATR surface analysis spot size was reduced (*13*). Other synchrotron studies have included mapping individual living cells (*6*, *14*), some of which are mitotically active, and detecting the presence of CO_2 and organic materials in rocks that have been thinly sawed with a diamond knife to allow transmission of infrared radiation (*15*). An investigation is in progress in our laboratory to map the different layers of the rat retina (Fig. 1).

Unlike FTIR microspectroscopy, imaging by light microscopy does not provide chemical information. Other methods of contrast such as use of x-rays, magnetic resonance, and ultrasound do not reveal as much about the underlying chemistry as FTIR does. A disadvantage of FTIR is that not all materials will fit into this device. Samples must be made very thin; thus, this methodology does not facilitate the chemical analysis of most living subjects. Near-infrared spectroscopy has a much deeper penetration and has been used in certain experimental situations on living subjects. A second difficulty is that identification of individual molecules may be challenging because a spectrum represents a combination of all absorbing molecules in the infrared path.

The only competing technology that truly gives the same type of microbeam molecular information is Raman microspectroscopy. In this technique, the spatial resolution is defined by the laser profile used to excite a particular part of the tissue. Raman spectra give molecular vibrational information that complements but does not overlap with that obtained from FTIR.

A prime example of an exciting use of FTIR is the investigation of pathogenesis in different diseases. Traditional biochemistry studies often fail to detect compounds associated with pathology because of homogenization and dilution of the tissue sample. FTIR can zero in on microscopic areas associated with pathology. Detection of compounds in individual cells before they die could reveal insights into mechanisms of some diseases such as leukodystrophies.

Some of the work on single fibers analyzed by polarized FTIR microspectroscopy could be the basis for important advances in forensic science. Additionally, subsurface irregularities in solid-state devices can be detected by mapping material in a transmission mode. In the future, additional studies with deuterium to examine brain or tissue metabolism may provide an alternative to using radioisotopes. FTIR microspectroscopy might be valuable as a complementary tool for diagnostic pathology. The application of infrared for the objective evaluation of pap smears of cervical cells has already been suggested and is under development.

Since the field of modern FTIR microspectroscopy began about 10 years ago with the introduction of a high-performance infrared microscope, three augmentations of this technique have become available. The first is the use of a synchrotron radiation source (*16*). Currently, there are at least 10 sites in the world where synchrotrons are being outfitted with infrared ports specifically for this purpose. The second is the use of a focal plane diode array (*17*): initially, the array was composed of an InSb diode in the shorter wavelength region but more recently HgCdTe focal plane arrays have been introduced. In terms of imaging, the focal plane array certainly is a fast way to obtain a chemical overview of the specimen. The field will probably be divided up into imaging and spectroscopy. Many scientists who have a modest background in spectroscopy will be able to get a lot of information through built-in imaging processing diagnostics and false color images to allow sorting of healthy material from defective or diseased material. The third is infinity corrected reflection optics to maximize image quality and permit interference contrast (*18*).

As for spectroscopy itself, there has been a race to achieve smaller and smaller pixel sizes with greater and greater spatial resolution. Unfortunately, when the pixel size approaches the wavelength size, the spatial resolution becomes diffraction limited. In spite of this limit, pure spectra of thin layers can be obtained by taking small step sizes across the layer and by spectral subtraction to remove the contributions of the neighboring material and thus reveal the spectrum of the pure layer that, in fact, may have dimensions below that of the diffraction limit.

References and Notes

1. L. Cho *et al.*, *J. Forensic Sci.*, **44**, 275 (1999); *ibid.*, 283 (1999); L. Cho *et al.*, *Mikrochim Acta* **14**, 349 (1997).
2. L. Cho *et al.*, in *FT-IR Spectroscopy*, J. A. de Haseth, Ed. CP430 (American Institute of Physics, Woodbury, NY, 1998), pp. 539–542.
3. D. L. Wetzel and R. O. Carter III, in (*2*), p. 567.
4. S. M. LeVine and D. L. Wetzel, *Free Radical Biol. Med.* **25**, 33 (1998).
5. ———, *Am. J. Pathol.* **145**, 1041 (1994).
6. Special issue on FT-IR Microspectroscopy, *Cell. Mol. Biol.* **44**, (Feb. 1998).
7. D. L. Wetzel and G. P. Williams, in (*2*), pp. 302–305.
8. V. Kalasinsky, in (*6*), pp. 141–144; K. Kalasinsky, in (*6*), pp. 81–88.
9. L. Miller, in (*6*), pp. 117–128; C. Marcott *et al.*, in (*6*), pp.109–116.
10. D. L. Wetzel *et al.*, *Cell. Mol. Biol.* **44**, 15 (1998).
11. D. L. Wetzel and S. M. LeVine, *Spectroscopy* **8**, 40 (1993).
12. D. L. Wetzel and J. A. Reffner, *Cereal Foods World* **38**, 9 (1993).
13. D. L. Wetzel *et al.*, in (*6*), pp. 145–168; in (*2*), pp. 354–357.
14. N. Jamin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 4837 (1998).
15. N. Guilhaumou *et al.*, *Appl. Spectrosc.* **52**, 1029 (1998).
16. J. A. Reffner *et al.*, *Rev. Sci. Instrum.* **66**, 1298 (1995).
17. E. N. Lewis *et al.*, *Anal. Chem.* **67**, 3377 (1995); C. Marcott *et al.*, *Vibr. Spectros.* **19**, 123 (1999).
18. J. A. Reffner and S. H. Vogel, U.S. Patent 5,864,137 (1999).
19. A more thorough discussion of FT-IR microspectroscopy, with 135 references, can be found in D. L. Wetzel and S. M. LeVine, in *Infrared and Raman Spectroscopy of Biological Specimens*, H.-U. Gremlich and B. Yan, Eds. (Dekker, New York, in press).