APPLICATIONS OF MICROSPECTROSCOPY, HYPERSPECTRAL CHEMICAL IMAGING AND FLUORESCENCE MICROSCOPY IN CHEMISTRY, BIOCHEMISTRY, BIOTECHNOLOGY, MOLECULAR AND CELL BIOLOGY

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Abstract

Chemical imaging is a technique for the simultaneous measurement of spectra (chemical information) and images or pictures (spatial information)[1][2] The technique is most often applied to either solid or gel samples, and has applications in chemistry, biology[3][4][5] [6][7][8], medicine[9][10], pharmacy[11] (see also for example: Chemical Imaging Without Dyeing), food science, biotechnology[12][13], agriculture and industry. NIR, IR and Raman chemical imaging is also referred to as hyperspectral, spectroscopic, spectral or multispectral imaging (also see microspectroscopy). However, other ultra-sensitive and selective, chemical imaging techniques are also in use that involve either UV-visible or fluorescence microspectroscopy.

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0. Introduction

Chemical imaging techniques can be used to analyze samples of all sizes, from the single molecule[14][15] to the cellular level in biology and medicine[16][17][18], and to images of planetary systems in astronomy, but different instrumentation is employed for making observations on such widely different systems.

Chemical imaging instrumentation is composed of three components: a radiation source to illuminate the sample, a spectrally selective element, and usually a detector array (the camera) to collect the images. When many stacked spectral channels (wavelengths) are collected for different locations of the microspectrometer focus on a line or planar array in the focal plane, the data is called hyperspectral;

fewer wavelength data sets are called multispectral. The data format is called a hypercube. The data set may be visualized as a three-dimensional block of data spanning two spatial dimensions (x and y), with a series of wavelengths (lambda) making up the third (spectral) axis. Such a data hypercube can be visually and mathematically treated as a series--or stack-- of spectrally resolved images (each image plane corresponding to the image at one wavelength) or a stack of spatially resolved spectra. An analyst may choose to view the spectrum measured at a particular spatial location for a special reason; this approach can be very useful for chemical identification of impurities, trace molecules, identifying microscopic labels, etc. Alternatively, one may select an image plane at a particular wavelength to highlight the spatial distribution of selected sample components, provided that their spectral signatures are different at that selected wavelength.

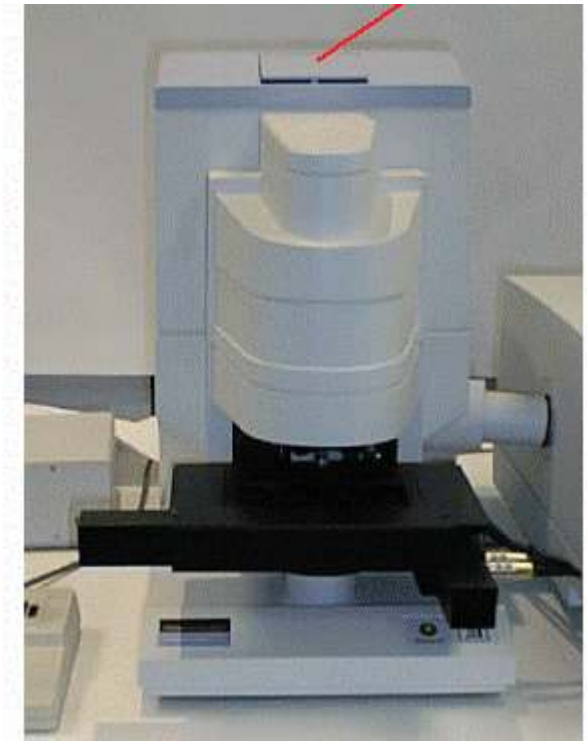

Figure 1. *NIRS Chemical Imaging Microscope, model AutoImage*

Many materials, both manufactured and naturally occurring, derive their functionality from the molecular structure and spatial distribution of sample components, or "texture". For example, the slow extended-release of pharmaceutical formulations can be achieved by using an appropriate coating that may act as a barrier layer, or layers. The slow release of the active ingredient is achieved, or controlled, by the presence of this barrier, and imperfections in the coating, such as discontinuities, can result in significant performance losses. In the semi-conductor industry, irregularities or contaminants in silicon wafers or printed micro-circuits can lead to failure of such components, as well as an entire circuit or board. The functionality of biological systems is similarly dependent upon the chemical gradients in a single cell, tissue, and even whole organs function because of the very specific arrangement of components and their specific interactions. It has been often the case that tiny changes in chemical composition and/or distribution of certain bioactive molecules may be an early step in diseases such as

cancers. Any material which depends on chemical gradients for functionality could be amenable to detailed studies by chemical imaging used as an analytical technique which couples a chemical composition with the spatial distribution or localization of a specific chemical or biochemical. Moreover, to efficiently and effectively design and manufacture some materials, both the chemistry/molecular composition and the location of specific molecules must both be determined at the same time. The demand for this type of analysis is increasing continuously as manufactured materials become more complex. Chemical imaging techniques not only permit visualization of the spatially resolved chemical information which is critical to our understanding of modern manufactured products, but it is also a noninvasive technique so that samples are preserved for further testing, or such that it can be used noninvasively, and rapidly, for medical diagnosis.

High quality, commercially available laboratory-based chemical imaging systems emerged after the year 2000 (refs. 1 to 5). In addition to economic factors, such as the need for sophisticated electronics and extremely high-end computers, a significant barrier to commercialization of infrared imaging was that the focal plane array (FPA) needed to read IR images were not readily available as commercial items, but have been extensively used for non-commercial uses. As high-speed electronics and sophisticated computers became more commonplace, and infrared cameras became readily commercially available, laboratory chemical imaging systems were rapidly introduced along with the appropriate software to handle rapidly very large families of hyperspectral data sets.

Initially used for advanced chemical imaging research in a few specialized laboratories, hyperspectral chemical imaging became a more commonplace analytical technique used for general R&D, quality assurance (QA) and quality control (QC) in less than five years. The rapid acceptance of such complex technology in a variety of industries (foods, pharmaceutical, polymers, semiconductors, security, forensics and agriculture) rests in the wealth and specificity/selectivity of information characterizing both the chemical composition and microscopic or molecular structure and morphology. The parallel nature of chemical imaging data makes it possible to analyze multiple samples simultaneously in all applications that require high throughput analysis in addition to characterizing a single sample.

1. **Physical and Chemical Principles of Chemical Imaging/Microspectroscopy**

Chemical imaging shares the fundamentals of vibrational spectroscopic techniques, but provides additional information by way of the simultaneous acquisition of spatially resolved spectra. It combines the advantages of digital imaging with the attributes of spectroscopic measurements. Briefly, vibrational spectroscopy measures the interaction of light with matter. Photons that interact with a sample are either absorbed or scattered; photons of specific energy are absorbed, and the pattern of absorption provides information, or a fingerprint, on the molecules that are present in the sample.

On the other hand, in terms of the observation setup, chemical imaging can be carried out in one of the following modes: (optical) absorption, emission (fluorescence), (optical) transmission or scattering (Raman). A consensus currently exists that the fluorescence (emission) and Raman scattering modes are the most sensitive and powerful, but also the most expensive.

In a transmission measurement, the radiation goes through a sample and is measured by a detector placed on the far side of the sample. The energy transferred from the incoming radiation to the molecule(s) can be calculated as the difference between the quantity of photons that were emitted by the source and the quantity that is measured by the detector. In a diffuse reflectance measurement, the same energy difference measurement is made, but the source and detector are located on the same side of the sample, and the photons that are measured have re-emerged from the illuminated side of the sample rather than passed through it. The energy may be measured at one or multiple wavelengths; when a series of measurements are made, the response curve is called a spectrum.

A key element in acquiring spectra is that the radiation must somehow be energy selected either before or after interacting with the sample. Wavelength selection can be accomplished with a fixed filter, tunable filter, spectrograph, an interferometer, or other devices. For a fixed filter approach, it is not efficient to collect a significant number of wavelengths, and multispectral data are usually collected. Interferometer-based chemical imaging requires that entire spectral ranges be collected, and therefore results in hyperspectral data. Tunable filters have the flexibility to provide either multi- or hyperspectral data, depending on analytical requirements. Spectra may be measured one point at a time using a single element detector (single-point mapping), as a line-image using a linear array detector (typically 16 to 28 pixels) (linear array mapping), or as a two-dimensional image using a Focal Plane Array (FPA)(typically 256 to 16,384 pixels) (FPA imaging). For single-point the sample is moved in the x and y directions point-by-point using a computer-controlled stage. With linear array mapping, the sample is moved line-by-line with a computer-controlled stage. FPA imaging data are collected with a two-dimensional FPA detector, hence capturing the full desired field-of-view at one time for each individual wavelength, without having to move the sample. FPA imaging, with its ability to collected tens of thousands of spectra simultaneously is orders of magnitude faster than linear arrays which are can typically collect 16 to 28 spectra simultaneously, which are in turn much faster than single-point mapping.

Some words common in spectroscopy, optical microscopy and photography have been adapted or their scope modified for their use in chemical imaging. They include: resolution, field of view and magnification. There are two types of resolution in chemical imaging. The spectral resolution refers to the ability to resolve small energy differences; it applies to the spectral axis. The spatial resolution is the minimum distance between two objects that is required for them to be detected as distinct objects. The spatial resolution is influenced by the field of view, a physical measure of the size of the area probed by the analysis. In imaging, the field of view is a product of the magnification and the number of pixels in the detector array. The magnification is a ratio of the physical area of the detector array divided by the area of the sample field of view. Higher magnifications for the same detector image a smaller area of the sample.

2. Fundamental Concepts

2.1. Vibrational chemical imaging instruments

Chemical imaging has been implemented for mid-infrared, near-infrared spectroscopy and Raman spectroscopy. As with their bulk spectroscopy counterparts, each imaging technique has particular strengths and weaknesses, and are best suited to fulfill different needs.

2.2. Infrared chemical imaging

Infrared (IR) spectroscopy probes fundamental molecular vibrations, which arise in the spectral range 2,500-25,000 nm. Commercial imaging implementations in the MIR region typically employ Fourier Transform Infrared (FT-IR) interferometers and the range is more commonly presented in wavenumber, 4,000 to 400 cm^-1. IR absorption bands tend to be relatively narrow and well-resolved; direct spectral interpretation is often possible by an experienced spectroscopist. IR spectroscopy can distinguish subtle changes in chemistry and structure, and is often used for the identification of unknown materials. The absorptions in this spectral range are relatively strong; for this reason, sample presentation is important to limit the amount of material interacting with the incoming radiation in the IR region. Most data collected in this range is collected in transmission mode through thin sections (~10 micrometres) of material. Water is a very strong absorber of MIR radiation and wet samples often require advanced sampling procedures (such as attenuated total reflectance). Commercial instruments include point and line mapping, and imaging. All employ an FT-IR interferometer as wavelength selective element and light source. Remote chemical imaging of a simultaneous release of SF6 and NH3 at 1.5km using the FIRST imaging spectrometer[19]. Atmospheric windows in the infrared spectrum are also employed to perform chemical imaging remotely. In such spectral regions the atmospheric gases (mainly water and CO2) present low absorption and allow infrared viewing over kilometer distances. Target molecules can then be viewed using the selective absorption/emission processes described above. An example of the chemical imaging of a simultaneous release of SF6 and NH3 is shown in the image.

2.3. Near-infrared chemical imaging

The near infrared (NIR) region spans the range from approximately 700 to 2,500 nm (or as it is more commonly specified, 14,000 to 4,000 cm^-1. The absorption bands seen in this spectral range arise from the overtones and combination bands of C-H, O-H, N-H, and S-H stretching and bending vibrations (with fundamental, normal modes in the IR region). Absorption is one to two orders of magnitude smaller in the NIR compared to the IR; this characteristic often eliminates the need for powdering samples, or other extensive sample preparation. Even relatively thick, solid samples (less than about 4mm in pathlength) can be often analyzed without any sample preparation, and it is usually possible to acquire NIR chemical images through some plastic or glass packaging materials, and NIR techniques can be employed to study hydrated samples in the reflection mode even at very high hydration levels. Intact samples can be imaged in either transmittance or diffuse reflectance modes, but the latter is often both more convenient also much more accurate provided a properly designed integrated sphere is utilized in conjunction with an FT-NIR spectrometer.

NIR peaks corresponding overtone and combination bands tend to be broader and overlap much more than the fundamental IR absorption bands. Often, multivariate analysis methods are being employed to separate characteristic spectral patterns of selected sample analytes. NIR chemical imaging is thus

especially useful for performing rapid, reproducible and noninvasive analyses of most known materials, with the exception of metals and very highly reflective coatings,[20][21]. The most advanced NIR imaging instruments are typically employing an FT-NIR interferometer coupled to a sophisticated NIR microscope with its own detectors in both visible and NIR, as well as high-reflectivity mirrors so that NIR incident light intensity losses in the microscope are minimized both in the absorption and transmission modes.

2.4. Raman chemical imaging and microscopy

The Raman shift chemical imaging spectral range spans from approximately 50 to 4,000 cm-1, and it is thus in the IR range; the actual spectral range over which a particular Raman measurement is made is determined by excitation frequency of the incident laser beam. The basic principle behind Raman spectroscopy differs from the IR and NIR in that the x-axis of the Raman spectrum is measured as a function of the frequency shift (or wavenumber shift in cm-1) relative to the frequency of the laser used as the source of radiation. Briefly, the Raman spectrum arises from inelastic scattering of incident photons, which requires a change in molecular polarizability associated with specific molecule vibration modes, as opposed to infrared absorption, which requires a change in dipole moments associated with specific vibrations. The consequence is that Raman spectral information is in many cases complementary to that obtained from IR. The Raman effect is 'weak' - only about one in 100 photons incident to the sample might undergo Raman scattering. Both organic and inorganic materials, and even symmetric molecules with no dipole moment changes caused by vibrations, do possess a Raman spectrum; generally, Raman peaks are sharp and, therefore, chemically specific. Both Stokes and anti - Stokes modes are of interest in Raman spectroscopy, whereas the latter were reported to be of special interest for studying or analyzing biosystems.

Fluorescence is a competing phenomenon with Raman scattering, and-- depending on the nature of the sample--it can overwhelm the Raman signal, for both bulk spectroscopy and other imaging implementations. Thus fluorescence imaging techniques are wider spread/ more popular than Raman spectroscopy or microspetroscopy/chemical imaging.

Raman chemical imaging requires little or no sample preparation. However, physical sample sectioning can be used to expose the surface of interest, with special care being taken to obtain a surface that is as flat as possible. The conditions required for a particular measurement dictate the level of invasiveness of the technique, and samples that are sensitive to high power laser radiation may be damaged during analysis. It is relatively insensitive to the presence of water in the sample and is therefore useful for imaging samples that contain large amounts of water such as solutions, frozen solutions, biological materials, ice-cream, textiles, soils, and so on.

3. Fluorescence Imaging – visible and NIR

This emission microspectroscopy mode is the most sensitive in both visible and FT-NIR microspectroscopy, and has therefore numerous biomedical, biotechnological and agricultural applications. There are several powerful, highly specific and sensitive fluorescence techniques that are currently in use, or still being developed; among the former are FLIM, FRAP, FRET and FLIM-FRET;

among the latter are NIR fluorescence and probe-sensitivity enhanced NIR fluorescence microspectroscopy and nanospectroscopy techniques.

3.1. Sampling and samples

The value of imaging lies in the ability to resolve spatial heterogeneities in solid-state or gel/gel-like samples. Imaging a liquid, or even a suspension, may have limited use in NIR/IR or Raman imaging as constant sample motion serves to average spatial information, unless ultra-fast recording techniques are employed as in fluorescence correlation microspectroscopy or FLIM observations where a single molecule may be monitored at extremely high (photon) detection speed. High-throughput experiments (such as imaging multi-well plates) of liquid samples can however provide valuable information. In this case, the parallel acquisition of thousands of spectra can be used to compare differences between samples, rather than the more common implementation of exploring spatial heterogeneity within a single sample.

Similarly, there is no benefit in imaging a truly homogeneous sample, as a single spectra will generate the same amount of spectral information and may be acquired in a shorter time than hyperspectral chemical images. Obviously, one's definition of homogeneity depends ultimately upon the maximum spatial resolution of the imaging system employed. For IR imaging, where wavelengths span from 3-10 micrometres, objects on the order of 5 micrometres may theoretically be resolved, but practically with FPA's a resolution of 10 microns is routinely obtained. Sampled areas are limited by current experimental implementations because illumination is provided by the interferometer. Raman imaging is able to resolve particles less than 1 micrometer in size, but the sample area that can be illuminated is severely limited. With Raman imaging, it is considered impractical to image large areas and, consequently, large samples, even though diode array (DA) designs may be able to get arround this problem as it is the case for DA NIR instruments.

FT-NIR chemical/hyperspectral imaging usually resolves only larger objects (>10 micrometres), and is better suited for large samples because illumination sources are readily available. However, FT-NIR microspectroscopy was recently reported to be capable of about 1 micron resolution in biological samples [22].

4. Detection Limits for Chemical Imaging techniques

The concept of the detection limit for chemical imaging is quite different than that for bulk spectroscopy, as it depends on the sample itself. Because a bulk spectrum represents an average of the materials present, the spectral signatures of low-level, trace components may be simply overwhelmed by noise. In imaging however, each pixel of about 5 micron x 5 micron has a corresponding spectrum. If the physical size of the trace contaminant is on the order of the pixel size imaged on the sample, its spectral signature will most likely be detectable. If however, the trace component is dispersed homogeneously (relative to pixel image size) throughout a sample, it might not be detectable. Therefore, detection limits of chemical imaging techniques are strongly influenced by particle size, the chemical and spatial heterogeneity of the sample, and the spatial resolution of the image, as well as by

the observation mode employed, as for example in FT-IR transmission vs. reflection imaging, or Raman vs. fluorescence imaging.

5. Other Fluorescence Techniques

FLIM= *Fluorescence Lifetime Imaging Microscopy*: Fluorescence, fluorophore chemical imaging, confocal emission microspectroscopy,

FLIM Applications: "*FLIM is able to discriminate between fluorescence emanating from different fluorophores and autofluorescing molecules in a specimen, even if their emission spectra are similar. It is, therefore, ideal for identifying fluorophores in multi-label studies. FLIM can also be used to measure intracellular ion concentrations without extensive calibration procedures (for example, Calcium Green) and to obtain information about the local environment of a fluorophore based on changes in its lifetime.*" FLIM is also often used in microspectroscopic/chemical imaging, or microscopic, studies to monitor spatial and temporal protein-protein interactions, properties of membranes and interactions with nucleic acids in living cells.

FRET = *Fluorescence Resonant Energy Transfer* (Forster)

FCCS = *Cross-correlation Fluorescence* (microspectroscopy).

6. Data analysis

Data analysis methods for chemical imaging/hyperspectral data sets typically employ mathematical algorithms common to both spectroscopy and image analysis, but they also depend strongly on the observation mode, that is, absorption, emission (fluorescence) or (Raman) scattering mode. The spectrum acquired by each detector is equivalent to a 'single point spectrum'; therefore pre-processing and chemometrics are still being utilized with the similar goal to separate chemical and physical (such as multiple scattering) effects and perform a qualitative or quantitative characterization of individual sample components. In the spatial dimension, each chemical image is equivalent to a digital image and standard image analysis and robust statistical analysis can be used for feature extraction.

Similar terms employed instead of chemical hyperspectral imaging are: Multi-spectral Imaging, Microspectroscopy and Imaging Spectroscopy.

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