Application Note AN M133

# Infrared Microspectroscopic Imaging of Biological Samples

The analysis of biological tissues typically is performed using microscopic methods. Due to the lack of visual contrast, staining procedures are required in order to highlight any structures of interest. Moreover, for the specific visualization of components (e.g., proteins, carbohydrates, lipids) and of patterns indicating diseases (e.g., cancer, neurodegeneration) simple staining methods using dyes are usually not sufficient and immunohistochemical (IHC) staining is used. This staining method applies antibodies, which specifically bind to antigens in the tissue sample. In principle, this method is quite effective and very sensitive. However, the sample preparation procedure is very time consuming and complex resulting in many potential sources of errors. Serious drawbacks are cross-reactions leading to strong background staining and a weak staining of the target antigen both of which lead to an insufficient contrast.

FT-IR microscopy constitutes a very fast and efficient alternative to the well-established staining methods. It allows to analyze biological samples with minimal sample preparation and without the need for any agents for contrast enhancement. The measured spectrum is a result of the interaction of IR-light with the sample and reflects its biochemical composition.

| Keywords | Instruments and software |
|---|---|
| FT-IR imaging | HYPERION 3000 |
| Focal plane array | INVENIO |
| Cancer tissue | OPUS spectroscopic software |
| Denatured proteins | OPUS/3D |
| Plant tissue | OPUS/VIDEO |

By using multi-element focal plane array (FPA) detectors it is possible to measure thousands of spectra simultaneously with a lateral resolution only limited by the diffraction of light. The result of an FPA measurement is a spectral matrix bearing complex information about the chemical composition of the measured sample area. For the analysis, evaluation methods like integration, cluster analysis or principal component analysis are applied. The resulting so called "chemical images", visualize the distribution of individual chemical components or certain spectral characteristics of the tissue and can be superimposed with the visual microscopic image.

FT-IR imaging reveals the chemical nature of biological structures and identifies proteins, lipids, polysaccharides etc. In many scientific studies, it has been shown that the method allows to differentiate between cancer cells and normal cells. Additionally, different structural forms of proteins (α-helix, β-sheet and random coil) and fatty acids (degree of unsaturation) can be discriminated. Thereby valuable information about neurodegenerative diseases like Alzheimer is generated, since the histological patterns of cell-damage can be precisely localized and identified. So far, IR-imaging is used exclusively for research purposes. In the future, the scientific community expects the method to become a powerful tool in medical diagnostics, providing faster results that are more objective when compared to the current staining techniques.



Figure 1: HYPERION 3000 microscope with INVENIO

### Instrumentation

The HYPERION FT-IR microscope utilizes state-of-the-art optics for optimal sample visualization and infrared data collection. Many contrast enhancement tools and a wide variety of dedicated objectives enable an appropriate visual inspection of any sample. Even at the highest spatial resolution, the HYPERION exhibits an outstanding sensitivity in all the measurement modes transmission, reflection, and attenuated total reflectance (ATR).

Equipped with a multi-element FPA-detector with up to 128x128 elements the HYPERION 3000 microscope (see Figure 1) allows measuring thousands of spectra simultaneously. High-resolution chemical images can be collected in a matter of just a few seconds. By assembling subsequently acquired IR images using the motorized xy-stage large sample areas can be measured. Because the pixel resolution in the HYPERION 3000 is very high for all available objectives, its resolving power is only limited by the diffraction of light.

Data acquisition using the HYPERION is performed in a very easy manner as the software guides the user through the different steps of microanalysis from visual sample inspection via selection of the measurements settings to the final measurement. Many univariate and multivariate algorithms are implemented in OPUS to extract the relevant information from the measured single or 3D data. Resulting IR images can be displayed in different 2D and 3D perspectives on top or beside the visible image.

### Differentiation of healthy and cancerous areas in breast tissue

The following example shows the analysis of breast tissue that contains a cluster of cancer cells. To allow the IR-microscopic analysis of tissues the sample has to be cut with a microtome to a thin section (typically <10 µm thickness) which then is placed on an IR-transparent window ($CaF_2$) and dried before being measured in transmission. In this example, an area of about 1 x 1mm was imaged with a spectral resolution of 4 $cm^{-1}$ and a pixel resolution of 10.8µm.

Figure 2 shows color coded chemical images that were created by integrating certain band positions of the IR imaging spectra which are characteristic for individual biological components or special properties (as in the case of the cancer cells). The color coding shows the intensity of the respective band: Black stands for the lowest values followed by green, blue/violet, red, and white for the highest values.
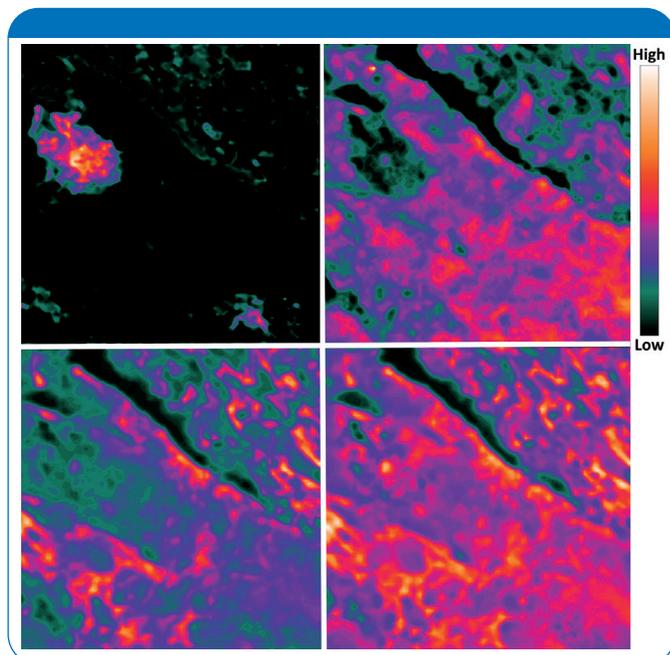


Figure 2: Color coded chemical images showing the distribution of individual biological components or certain properties: Cancerous region (upper left), carbohydrates (upper right), collagens (lower left), and proteins (lower right).

| Substance class | Integration range | Comment |
|---|---|---|
| Proteins | 1720 – 1480 cm⁻¹ | Amide I and Amide II band |
| Carbohydrates | 1150 – 1000 cm⁻¹ | C-O stretching vibration |
| Collagen | 1150 – 1000 cm⁻¹ | Amide III band + side chains |
| Lipids | 1770 – 1721 cm⁻¹ | C=O stretching vibration |
| Cancerous tissue | 3298 – 3180 cm⁻¹ | Region typical for N-H and O-H stretching vibrations |

Table 1 shows the respective integration ranges of the different tissue components.

To combine the information of several chemical images each single image/component is assigned to a certain color. In the "Winner Takes It All"-mode, the dominant component is determined for each individual pixel and the respective color is assigned. This type of image is especially useful to illustrate more than one component in one image. Figure 3 shows the combined information where the single components are color coded: Healthy tissue is shown in blue, cancerous region are shown in red, carbohydrate are shown in green, lipids are orange and collagen is shown in turquoise.
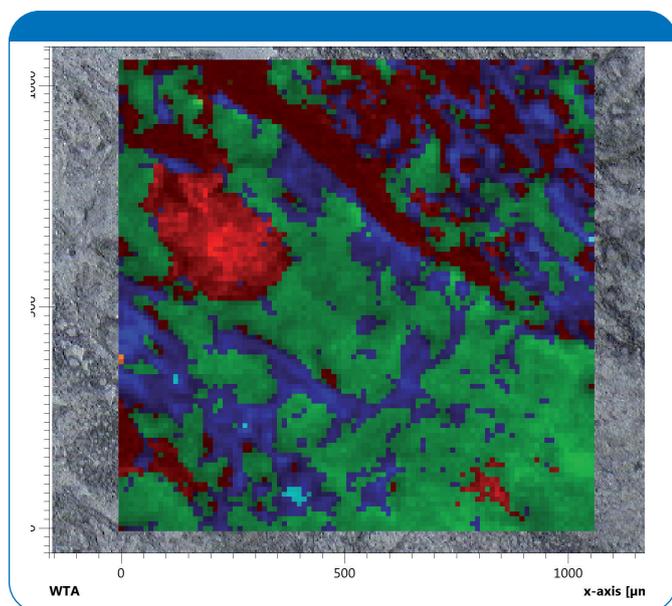


Figure 3: WTA image showing the four main biological components in one picture: Cancer cells (red), carbohydrates (green), healthy tissue (blue), lipids (orange, barely visible on the left) and collagen (turqoise).

## Plant tissue: Analysis of a wheat seed

This example illustrates the analysis of a wheat seed by IR-imaging. The microtome cut thin section of the sample was placed on a CaF₂ window and measured in transmission. Figure 4 shows the schematic structure of a seed. The blue colored outer layer is the so called pericarp that surrounds the seed; it is followed by the seed coat (colored in red) which is mostly made of wax. The next layer (shown in yellow) is the aleuron layer that contains lipids and proteins. The endosperm, shown in green, is the storage tissue of carbohydrates and supplies nutrients to the growing plant in its early growth stages.
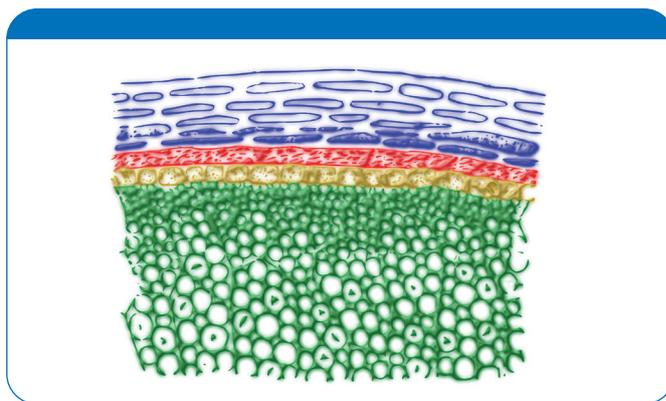


Figure 4: Schematic structure of a seed. Layers from top: Pericarp (blue), seed coat (red), aleuron (yellow) and endosperm (green).

In the visual image these layers can only be distinguished due to structural characteristics, the visual contrast is very low (see Figure 5). The IR-spectral data clearly reveals the biochemical differences between the different layers of the seed. Figure 6 shows example spectra of different layers. The spectrum on the top was extracted from the endosperm layer. It shows the bands of a typical starch spectrum. In the middle, an example spectrum of the aleuron layer is shown with characteristic amide bands centered on 1650 cm⁻¹ and 1550 cm⁻¹ respectively. The spectrum on the bottom is from the seed coat and displays spectral features of carbonic acids and esters (C=O valence band at 1733 cm⁻¹, strong C-H valence bands below 3000 cm⁻¹) that can typically be found in wax. By integration of characteristic bands, contrast rich chemical images are generated which have been combined in Figure 7 using again the WTA approach. Endosperm (green, integration range 1070 – 996 cm⁻¹), aleuron (yellow, 1758 – 1740 cm⁻¹), seed coat layer (red, 1744 – 1727 cm⁻¹) and pericarp (blue, 1349 – 1297 cm⁻¹) are clearly discernible.
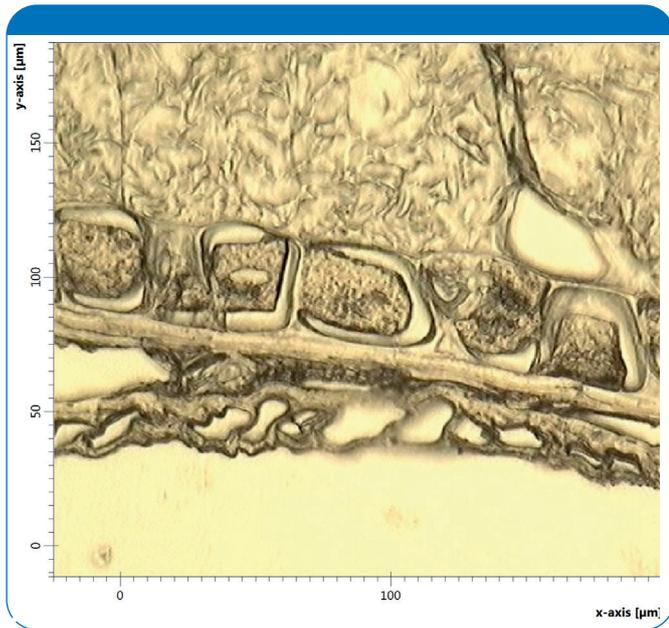
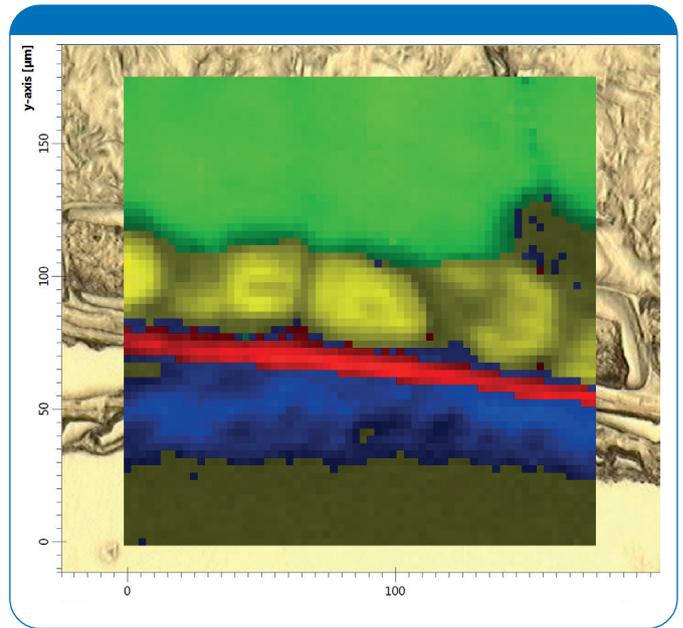Figure 5: Visual transmission image of the wheat seed.



Figure 7: WTA image of the wheat seed: Pericarp (blue), seed coat (red), aleuron (yellow) and endosperm (green).
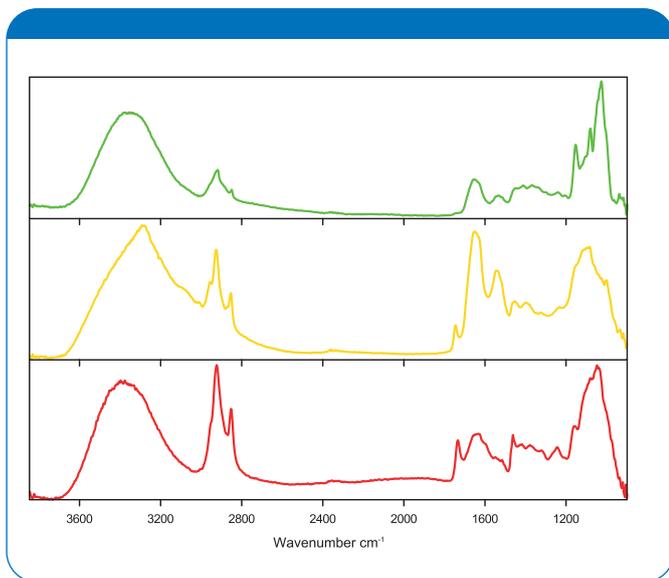


Figure 6: Example spectra: Endosperm (top, green), aleuron (middle, yellow) and seed coat (bottom, red).

## Analysis of denatured proteins in a rat brain sample

This example shows the analysis of a diseased rat brain. Neurodegenerative diseases typically involve the formation of denatured proteins that differ in their structure from native proteins. For instance in Alzheimer's disease the Amyloid beta peptide is misfolded and aggregates in the brain in the form of plaques. For the analysis, a microtome section of the rat brain sample was placed on a gold mirror serving as reflective substrate to allow a measurement in reflection mode. A grid of 3 x 5 FPA measurements was performed covering a sample area of about 500 x 900 µm. Fatty acids were determined by integrating the carbonyl band between 1760 and 1710 cm$^{-1}$. The so called amide I band which results from the peptide bonds in proteins is sensitive to the protein's conformation and was used to differentiate the native from the denatured protein. Whereas native proteins showed a high spectral intensity between 1698 – 1652 cm$^{-1}$, denatured proteins were characterized by a shift of the band maximum into the region 1659 to 1598 cm$^{-1}$. This is a typical band position for proteins aggregated as intermolecular β-sheet. As an example, Figure 8 shows the chemical image for the denatured proteins.
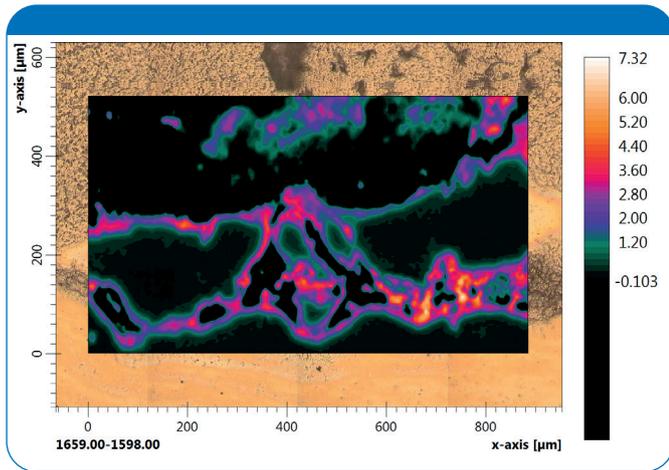
Figure 8: Chemical image for the denatured proteins of a rat brain sample.

In order to combine all the information about the location of the different tissue types a WTA-image was created. The image in Figure 9 shows the fatty acids in red, the native proteins in blue, and the denatured proteins in green. The native protein regions are all surrounded by denatured proteins suggesting that the protein degeneration is proceeding from the outside of the structures.
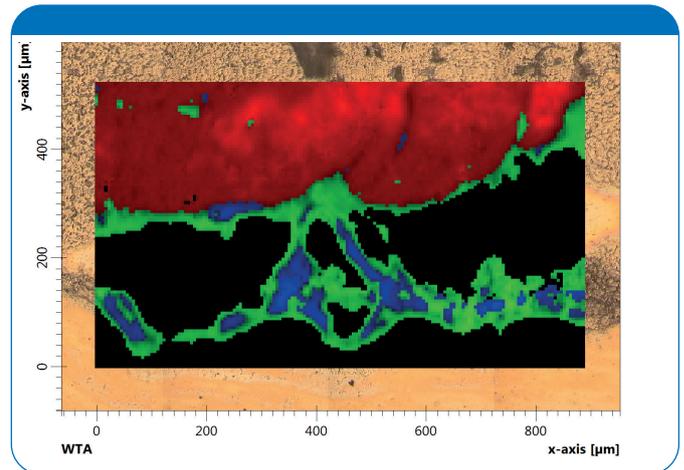


Figure 9: WTA image of the rat brain sample: Showing fatty acids (red), native proteins (blue), and denatured proteins (green).

## Summary

FT-IR imaging is a powerful technique to generate chemical images showing the distribution of the main biochemical components (e.g., lipids, proteins, and polysaccharides) in plant and animal tissue. Moreover, the obtained spectral information allows to differentiate between various properties of the analyzed tissue, including healthy and diseased. As the method does not require any labelling and therefore provides results much faster than current staining techniques, researchers expect it to become a valuable tool for medical diagnostics in future.

The HYPERION 3000 is an FT-IR imaging microscope providing the maximum sensitivity and resolution power that is required for high-end research applications.

**Bruker Scientific LLC**

Billerica, MA · USA
Phone +1 (978) 439-9899
info.bopt.us@bruker.com

**Bruker Optics GmbH & Co. KG**

Ettlingen · Germany
Phone +49 (7243) 504-2000
info.bopt.de@bruker.com

**Bruker Shanghai Ltd.**

Shanghai · China
Tel.: +86 21 51720-890
info.bopt.cn@bruker.com

**www.bruker.com/optics**