Synchrotron-Based Infrared Spectral Imaging at the MIRIAM Beamline of Diamond Light Source

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Introduction to imaging on MIRIAM

Infrared (IR) spectral imaging is a quantitative scientific technique for measuring both the molecular composition and its spatial distribution across large areas of materials. Synchrotron radiation (SR) enhances IR imaging with a broad spectral bandwidth unobtainable with a conventional laboratory source or laser (covering 10000 to 5 cm$^{-1}$), while the high collimation and small size of the SRIR source provide a diffraction-limited microbeam. The use of a few micron-sized aperture for IR imaging has a clear advantage in SR in terms of spectral quality, due to the high spectral flux reaching microspots of the sample even at the lowest wavenumbers. In this article, we present IR imaging examples developed mostly in collaboration with the user community and the staff of the IR beamline MIRIAM (Multimode InfraRed Imaging And Microspectroscopy) at Diamond Light Source [1]. The layout of the MIRIAM beamline (B22) is shown in Figure 1. Optically, this is composed of a UHV vacuum vessel including a periscope system each with two metal mirrors, allowing refocusing and overcoming a midway shield wall (not shown). From the right, the bending magnet source illuminates a first flat mirror (with a horizontal slot rejecting X-rays) that reflects the SRIR fan onto an ellipsoidal mirror. The SRIR is focused midway and collected by another ellipsoidal mirror that redirects it down onto a double flat mirror; this can be translated laterally to send the focused SRIR to either of the two end stations (or part of the beam to both) through wedged diamond windows. The two experimental end stations are composed of Bruker Vertex 80V in-vacuum Fourier Transform IR (FTIR) interferometers with Hyperion 3000 IR microscopes. The IR detectors are broadband or high-sensitivity MCT (mercury cadmium telluride; 100 μm or 50 μm pitch) for point-by-point microscopy, and photovoltaic MCT focal plane array (FPA) 64 × 64 pixel detectors for full-field imaging.

In the present contribution, we highlight the impact of the method as well as its versatility in a variety of applications, ranging from biology and medical research to geology.

Metabolism of living cells

The major application of SRIR at Diamond is biomedicine; in particular, IR spectral imaging, which allows the probing of individual cells as well as tissues at sub-cellular resolution, collecting molecular information fast and with excellent spectral quality at the highest magnification. This insight is important because it reveals the cell-by-cell differences (e.g., due to cell cycle or biological variability) which are averaged together in conventional IR imaging. A typical science case in cancer studies is to quantify the effectiveness of new molecules on the cellular metabolism; e.g., time-dose response to new drug candidates. The research frontline has moved from fixed specimens to living cells. In fact, ex vivo biochemical information can be achieved by SRIR imaging at the micron spatial scale and few-minute timescale.

A key development recently achieved is moving from fixed and dried samples to ex vivo—i.e., living conditions in the natural aqueous environment—allowing for time-dependent studies of biological processes. The combined requirements of high spatial resolution, rapid data acquisition, and high photon flux (due to strong IR absorption by water) make SR an invaluable microanalysis tool.

Figure 1: Schematic of the optical path and vacuum vessel of MIRIAM beamline (B22). The SR source is on the right (bending magnet source in green) with the two endstations on the left.
The first experiment of this type at Diamond’s MIRIAM beamline (B22) was a collaboration with the Paul Scherrer Institut (PSI, Switzerland): fibroblast cells were grown in Diamond’s Cell Culture Laboratory and analyzed in a liquid cell sample holder with IR-transparent windows. The growth medium surrounding the fibroblasts was exchanged and isotopically enriched with heavy water (>90%). The use of a 20-μm-thick D₂O layer had the double purpose of: (1) tracking isotopic pathways in and out of the cell; and (2) shifting the strong water absorption out of the IR fingerprint spectra. To study the biochemical processes inside/outside fibroblast cells via isotopic exchange, synchrotron illumination with an FPA detector was successfully used. The FPA detector was employed to collect molecular images of living cells consecutively over a period of five hours under the IR microscope [2].

The optical set-up of a 15x condenser and a 36x objective gave a field of view area ~70 × 70 μm² (64 × 64 pixels FPA), with the 40 μm single pixel effectively 1.1 μm at the sample. Within the diffraction limit (equal to the wavelength for NA = 0.5 and in apertureless mode), this set-up provided an IR image with oversampling greater than three times across the whole mid-IR, while the SRIR gave a signal per pixel up to six times better than a Globar. In fact, the signal-to-noise ratio achieved in this proof-of-principle experiment on a living cell, shown in Figure 2, was good enough to allow second derivative analysis per pixel spectrum, without noticeable noise enhancement.

This study shows that synchrotron-based IR imaging on living cells can be performed which allows visualization and quantification of specific cellular properties with both spatial and temporal resolution. It is therefore a useful tool to study both metabolic turnover of cells and the properties of cellular proteins in vivo.

Applications of this technique include developing spectral biomarkers for disease diagnosis (particularly cancer research), location of stem cells within tissues, following effects of natural and synthetic chemicals on stem-cell differentiation, and quantifying drug sensitivity.

**Novel diffusion mechanisms in minerals**

Another area where chemical and spectral information is desirable is mineralogy and material characterization. Microscale CO₂-doped beryl single-crystals were examined to investigate the diffusion and distribution of carbon dioxide and water across the mineral matrix by Della Ventura et al. [3] at the MIRIAM beamline with polarized IR imaging. Cordierite and beryl are two isostructural microporous minerals known to trap significant amounts of water and carbon dioxide within their structural cages. As shown in Figure 3(c), type II water (where the H-H vector is parallel to the channel axis, labelled c in Figure 3(a)) is distributed homogeneously, except along the hourglass boundary. CO₂ is close to the basal edges of the crystal and also, surprisingly, revealed along the hourglass boundaries for the first time.

Preliminary data showed that the crystal is structurally homogeneous and the hourglass texture is due to the distribution of chromium across the grain. A mismatch in the periodic structure may be the mechanism responsible for the formation of such sector zoning. It is evident that the hourglass texture/zoning controls the diffusion of CO₂ within the matrix, with the defects arising from the structural mismatch likely speeding up diffusion. The images displayed in Figure 3(b) and 3(c) suggest that the role of the hourglass zoning on the H₂O diffusion is extremely different from that of CO₂. The spectroscopic data and high-resolution SR-FTIR imaging in Figure 3(e) highlight the insight available by per-
forming SRIR imaging in scanning mode in a selected key area (red square in top figure). The improved spatial resolution disentangles the hourglass zone line from the fracture, suggesting the possible combination of a defect-controlled diffusion, a fracture-induced diffusion, and a structural channel diffusion, which are a novelty in the field.

Compositional variation in minerals of biological origin

Amorphous calcium carbonate (CaCO$_3$) is unstable when synthesized in the laboratory, rapidly forming crystalline phases such as calcite. Instead, many organisms produce calcium carbonate in a highly stable form which is used for skeletal building or, for example, in the case of earthworms, for pH regulation. The reason for the remarkable stability in nature of biomineral CaCO$_3$ is not known. This study used earthworm-secreted CaCO$_3$ granules and synchrotron micro-FTIR analysis, together with several other techniques, to look for spatial correlations with chemical/elemental species present in the thin sections.

Both amorphous CaCO$_3$ (ACC) and crystalline phases have a strong IR band ($\nu_2$) at around 785 cm$^{-1}$. In calcite, this is accompanied by a band ($\nu_4$) at 705 cm$^{-1}$ known to be absent in ACC. The ratio of intensity of these bands ($\nu_2/\nu_4$) is therefore used to locate the different phases in the sample. Figure 4 shows visible and micro-FTIR chemical images of two sections of different granules. In the first example, a complete section of the granule (Figure 4(a)) approximately 1 mm across was imaged by SR-FTIR in mapping mode at spatial resolution 20 µm. Absorbance images for $\nu_2$ and $\nu_4$ are portrayed in Figure 4(b) and 4(c) and the ratio in Figure 4(d) clearly identifies extended regions of ACC-rich material. A high-resolution analysis by 6 µm per point is shown for a second granule in Figures 4(e) to 4(h), where now amorphous regions as small as about 10 µm can be resolved. Typical spectra from regions of each phase are plotted in Figure 4(i) showing the marked difference in the relative strength of the two IR bands [4].

The use of SRIR in this imaging example has major advantages over benchtop measurements: high signal-to-noise data can be obtained rapidly from small spatial regions allowing high-spatial-resolution maps over large areas. Commercial FPA imaging detectors are currently limited to the spectral range above about 900 cm$^{-1}$ so, while highly effective for fast IR imaging over large areas, they cannot be used for this work.

Spectroscopic differentiation of layers in human aorta valves

Diseases of the aortic valve cause serious health problems; there is a need for studies of pathomorphological processes in human valves.
Compositional variation in minerals of biological origin—specifically, to obtain distribution of the middle layer—allows high-resolution analysis by SR-FTIR microscopy on such samples. Amorphous calcium carbonate (ACC) is unstable when synthesized in the laboratory, and is not formed from the crystalline biomineral CaCO3 that is used for skeletal building. Carbonate in a highly stable form which is used for skeletal building is not known. This study used earthworm-secreted CaCO3 granules and calcium carbonate on CaF2 substrates. Serial sections were deposited onto glass slides and onto CaF2 substrates. Serial sections were deposited onto glass slides for further histopathological analysis. The map size was about 875 × 500 μm², which corresponds to a total of 2800 spectra. On the parallel sections, four different stains were used for the histological comparison: (1) Hematoxylin & Eosin (H&E) to highlight distribution of collagen; (2) Alcian Blue to distinguish the layers of the valve; more specifically, to obtain distribution of the middle layer—spongiosa—by staining the glycosaminoglycans (GAGs); (3) Oil Red (O) to highlight the distribution of lipids; and (4) Alizarin Red to study the presence of calcific depositions. In order to determine the spatial distribution of interesting chemical components in the SR-FTIR tissue maps, spectral bands were integrated for GAGs and acetyl amino groups as well as for the distribution of lipids and nucleic acids.

SR-FTIR mapping allowed spectroscopic differentiation of the three layers of human AV. The most distinguishable was spongiosa, with a clearly different chemical composition from that of the ventricularis and fibrosa layers. The latter layers are mostly composed of collagen and elastin, while spongiosa contains a large amount of GAGs and few fibrous proteins. As seen in Figure 5, SR-FTIR mapping, in combination with histological staining, confirmed that within the spongiosa layer, GAGs (Figure 5(e)) are co-localized with lipids (Figure 5(f), 5(g)). Possible co-localization of lipids (Figure 5(f), 5(g), 5(j)) and calcific depositions (Figure 5(k)) in AV occurred in these samples too. In both fibrosa and spongiosa layers in AV from healthy patients, calcific depositions are observed. This is crucial, especially in the context of studying development of AV stenosis or atherosclerosis, since these phenomena could be caused by high pressure which affects fibrosa more than the ventricularis layer. Importantly, lipid and calcific depositions in tissue are not necessarily caused by disease development, but are part of normal physiological processes in AV. The great spectral quality and spatial resolution obtained with SR-FTIR made it possible to find even subtle spectral differences between three layers of human AV over a very large area; e.g., the submillimeter scale of these samples.

**New imaging capabilities at the MIRIAM beamline**

The IR imaging research examples presented in this article are a selection among the broad panorama of experimental activity carried out at a state-of-the-art IR facility like the MIRIAM beamline. We are currently commissioning two new methods for SRIR imaging that should push beyond the current limitations of IR microscopes, hopefully allowing new classes of IR experiments at Diamond.
The first new facility is a near-field IR microscope, developed in order to beat the diffraction limit and achieve submicron spatial resolution in the mid-IR and, ideally, far-IR/THz ranges. It consists of an atomic force microscope (AFM, Nanonics MV1000) with an in-vacuum modified mechanical chopper coupled to the beamline FTIR. The SRIR is focused through a Cassegrain objective underneath the AFM tip: the sample absorbs the IR beam and undergoes thermal expansion, registered by the AFM tip as an absorbance spectrum via the slow FTIR modulation. The IR beam is also fast-chopped in order to resonantly enhance the cantilever oscillation and therefore the photothermal IR signal from submicron areas. The MIRIAM group was the first to demonstrate that resonance-enhanced (RE) IR-AFM is operable via SRIR [6, 7] and we are expecting to take our first collaborative users on this third end station of the beamline towards the end of 2017/beginning of 2018.

The other new development is dedicated optics to enable ideal coupling between the SR and the FPA detector, allowing beam optimization for each Cassegrain objective/condenser in use (e.g., 15× to 74×). As

Figure 5: μSR-FTIR integrated maps of a human aortic valve combined with histological staining: (a) optical image of the studied tissue section indicating the three different layers; (b), (c) averaged spectra collected from the three different layers of the tissue in the spectral range of 3050–2750 cm\(^{-1}\) in (b) and 1850–1000 cm\(^{-1}\) in (c) showing the major differences on specific IR bands; (d) integration map of 1190–1150 cm\(^{-1}\) GAGs (ν\(_C-O-S\)); (e) 1395-1355 cm\(^{-1}\) GAGs (acetyl amino group); (f) 1760–1720 cm\(^{-1}\) carbonyl ν(C=O); (g) 3000–2750 cm\(^{-1}\) lipids ν\(_{as}(CH_{3}, CH_{2})\), ν\(_s(CH_{3}, CH_{2})\); (h) H&E stain for collagen; (i) Alcian Blue staining for GAGs; (j) Oil Red O stain for lipids; (k) Alizarin Red for calcific depositions.
the source is spatially structured, it is currently causing a limitation for FPA imaging with SRIR. This is because the consequent illumination inhomogeneity at the focal plane is detrimental for full-field imaging of scattering samples. In fact, the analysis of FPA images can be challenging when the spectral intensity as seen at each pixel is very different across the sample versus the background; i.e., leading to potential spatial/spectral artifacts. Based on deformable mirrors, our innovative approach is to reshape and homogenize the synchrotron IR profile for the sake of a uniform illumination at the sample and FPA. Done correctly, this should reduce the pixel-to-pixel illumination difference dramatically, removing the artifacts across IR images. We will finally be able to exploit the order of magnitude spectral advantage in signal-to-noise expected per single FPA pixel at the highest magnification and image oversampling [8, 9]. Currently under commissioning, the aim is to take users with this system within the next year.

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Note
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