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A New Sample Substrate for Imaging and Correlating Organic and Trace-Metal Composition in Biological Cells and Tissues

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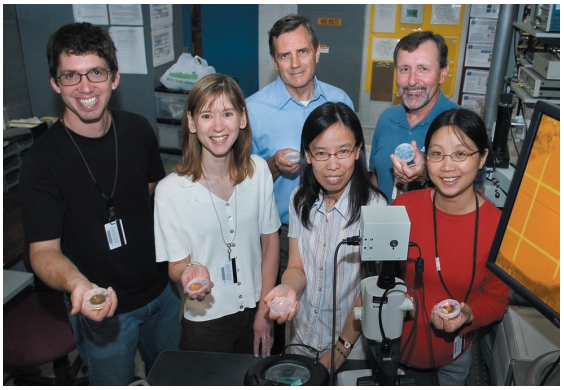
Synchrotron-based infrared (FTIRM) and x-ray fluorescence (XRF) microscopes are complementary tools for imaging the organic and trace metal composition of biological and environmental materials, respectively, without the need for extrinsic labels or stains. However, in order to directly correlate organic composition and trace metal content, it is important to precisely overlap the IR and XRF images. We have developed a gold-patterned sample substrate, where the grid pattern is sensitive to both x-ray and infrared light, and the resulting images can be used as fiducial markers for spatially overlapping the FTIRM and XRF images from the tissue. We show that FTIRM and XRF images can be correlated precisely. By combining FTIRM and XRF microprobe imaging on the same sample utilizing this sample substrate, a more complete picture of many disease states and exposure to environmental contaminants can be achieved by directly correlating the organic and trace metal ion distribution in the tissue.

In many biological and environmental systems, organic composition and trace metal content and distribution are often highly correlated. For example, plaques in Alzheimer's diseased brain consist of both aggregates of the misfolded amyloid protein and the accumulation of metal ions such as copper and zinc. In the environment, metal-reducing bacteria and hyperaccumulating plants represent promising methods for remediation of contaminated soils.

Synchrotron-based Fourier transform infrared microspectroscopy (FTIRM) and x-ray fluorescence (XRF) microprobe are becoming increasingly popular for imaging the organic and trace metal composition of biological and environmental materials without the need for extrinsic labels or stains. FTIRM provides chemical information on the organic components of a material at a diffraction-limited spatial resolution of 2-10 μm in the mid-infrared region. XRF microprobe is a complementary technique used to probe trace element content in the same systems with a similar spatial resolution. However, most published studies utilize either FTIRM or XRF microprobe, but not both, and therefore examine only one aspect of the problem, resulting in the missing information about the relationship between the alterations of the organic and metal contents. This is likely due to technical difficulties such as sample preparation, sample substrates, and image registration.

In this highlight, we describe the development of a sample substrate that contains a gold grid pattern on its surface, which can be imaged with both the IR and XRF microscopes. The substrate consists of a metal-free glass slide that has a gold grid patterned on its surface, where the major and minor parts of the grid contain 25 and 12 nm gold, respectively. Alternate substrates can also be used including polypropylene, mylar, or silicon nitride. The sample is placed on top of the patterned substrate.



Authors (from left) Randy Smith, Lisa Miller, John Warren, Qi (Adele) Wang, Don Elliott, and Hui Zhong

The Au grid pattern can be imaged with the IR microscope because the reflectivity of gold differs as a function of thickness. The relationship between IR reflectivity and Au thickness can be seen in **Figure 1A**. Between 10 and 20 nm of Au, there is a sharp increase in reflectivity from 10% to 90%. Thus, the Au thickness for the grid pattern was chosen to be 12 – 15 nm, to provide high enough reflectivity to collect FTIRM spectra, but low enough re-

flectivity to differentiate the thick (25 nm, 100% reflectivity) from the thinner Au.

The pattern can also be imaged with the XRF microprobe because the Au fluorescence intensity is proportional to the thickness of the gold (**Figure 1B**). The integrated gold fluorescence intensity of the grid (12 nm thick gold) was approximately half of the integrated intensity from the remainder of the substrate (25 nm thick gold).

Figure 1C shows the light micrograph (top), infrared reflectivity (middle), and Au XRF fluorescence images of the substrate. From the images, it can be seen that the grid pattern's IR reflectivity image and the gold SXRF image can be used as fiducial markers for spatially overlapping the IR and XRF images from the same sample.

As an example, the sample substrate was used to study trace metal uptake in human hair. FTIRM has been shown to be a unique method for examining the chemical makeup of hair. Especially with the high-resolution of a synchrotron-based IR source, it is possible to separately analyze the different regions of the hair cross-section, i.e. the cuticle, cortex, and medulla. It has been used to study drug abuse by examining the uptake of drugs into the medulla, i.e. central core, of human hair. This technique eliminates the question of externally contaminated hair by analyzing only that portion of the hair that is formed from within the root where ingested material would be transported. FTIRM has also been used to study the effects of bleaching and coloring on different regions of the hair, and the process of ancient mummification.

Hair analysis has also frequently been used to study environmental metal contaminants such as methyl mercury uptake from eating contaminated fish, lead poisoning in children from drinking water pipes, and lead accumulation in smelter workers. Yet to date, very little work has been done comparing the organic composition of the hair with the propensity for metal uptake. By combining FTIRM and SXRF microprobe, this can be accomplished.

Figure 2A shows a cross-section of a human hair that has been embedded in paraffin wax, microtomed to a thickness of 7 µm, and deposited onto a gold-patterned substrate. The grid pattern is clearly visible under the light microscope. FTIRM analysis of the Amide I protein band (1600 – 1700 cm^{-1}) can be seen in **Figure 2D**. The protein distribution is lower in the medulla and cuticle and relatively uniform throughout the cortex of the hair. For the SXRF microprobe images, the Cu image shows an elevated amount of Cu in the cuticle of the hair (**Figure 2E**). By overlaying the FTIRM and XRF images, results show that the elevated ring of Cu is outside of the cortex where protein concentration is lower. These findings indicate that the protein content is inversely correlated with Cu content in the hair. Since the cuticle is the outmost layer of the hair, this suggests that the elevated copper content in the cuticle is due to exogenous copper binding. Prior studies on copper content in hair have shown that damage to the cuticle of the hair can increase the cysteic acid and anionic sulfonate groups in keratin, which enhance copper adsorption. Conversely, if the metabolic levels of copper were elevated in the body, this would most likely be transported to the hair through the medulla, which was not observed in this study.

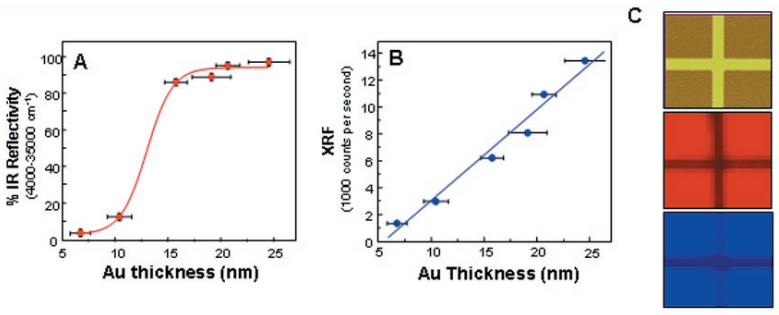


Figure 1. (A) *Infrared reflectivity of gold as a function of thickness. The substrate was produced with a bar thickness of 12 nm (~85% IR reflectivity), and the remaining part of the grid was 25 nm (100% reflectivity).* ***(B)*** *XRF intensity of gold as a function of thickness. The Au XRF intensity from the bar was approximately half the intensity from the remaining part of the grid.* ***(C)*** *visible light micrograph (top), FTIRM reflectivity image (middle), and Au XRF intensity image (bottom) from the sample substrate.*

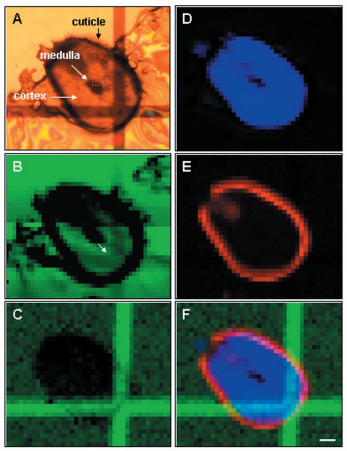


Figure 2. (A) *Cross-section of human hair under visible light illumination. The cuticle, cortex, and medulla are indicated.* ***(B)*** *FTIRM reflectivity image of the Au grid. The white arrow indicates the grid intersection, used for the image correlation.* ***(C)*** *Au SXRF microprobe image of the Au grid.* ***(D)*** *FTIRM image of the protein content in the hair, generated by plotting the Amide I peak area from 1600 – 1700 cm^{-1}.* ***(E)*** *XRF microprobe image of copper content in the hair.* ***(F)*** *A red-green-blue (RGB) color image illustrating the correlation of the protein from FTIRM (blue channel) and Cu content from XRF microprobe (red channel) in the tissue. The gold grid pattern from SXRF microprobe is placed in the green channel. Scale bar for all images is 20 µm.*