Multimodal Spectroscopy Combining Time-of-Flight-Secondary Ion Mass Spectrometry, Synchrotron-FT-IR, and Synchrotron-UV Microspectroscopies on the Same Tissue Section

ARTICLE *in* ANALYTICAL CHEMISTRY · APRIL 2010

Impact Factor: 5.64 · DOI: 10.1021/ac100581y · Source: PubMed

Anal. Chem. **2010,** *82,* 3963–3968

Multimodal Spectroscopy Combining Time-of-Flight-Secondary Ion Mass Spectrometry, Synchrotron-FT-IR, and Synchrotron-UV Microspectroscopies on the Same Tissue Section

Vanessa W. Petit,[†] Matthieu Réfrégiers,[‡] Catherine Guettier,[§,||,⊥] Frédéric Jamme,[‡,⊗] Kumaraparithy Sebanayakam,[§,||] Alain Brunelle,[†] Olivier Laprévote,[†,○] Paul Dumas,[‡] and François Le Naour*[,§,||]

Centre de Recherche de Gif, Institut de Chimie des Substances Naturelles, CNRS, Avenue de la Terrasse, F-91198 Gif-sur-Yvette, France, Synchrotron SOLEIL, F-91192 Gif-sur-Yvette, France, Inserm U785, F-94807 Villejuif, France, Université Paris-Sud 11, Institut André Lwoff, F-94807 Villejuif, France, Service d'Anatomie Pathologique, Hôpital Paul Brousse, F-94807 Villejuif, France, Cepia, INRA, F-44316 Nantes, France, and Chimie-Toxicologie Analytique et Cellulaire, IFR IMTCE, Faculté des Sciences Pharmaceutiques et Biologiques, Université Paris-Descartes, 4, Avenue de l'Observatoire, 75006 Paris, France

Mass spectrometry and spectroscopy-based approaches can provide an overview of the chemical composition of a tissue sample. This opens up the possibility to investigate in depth the subtle biochemical changes associated with pathological tissues. In this study, time-of-flight secondary ion mass spectrometry (TOF-SIMS) and synchrotron-FT-IR and -UV imaging were applied to the same tissue section by using the same sample holder. The tested sample involved liver cirrhosis, which is characterized by regeneration nodules surrounded by annular fibrosis. A tissue section from a cirrhotic liver was deposited on a gold coated glass slide and was initially analyzed by FT-IR microspectroscopy in order to image the distribution of lipids, proteins, sugars, and nucleic acids. This technique has identified collagen enrichment in fibrosis whereas esters were mostly distributed into the cirrhotic nodules. The exact same section was investigated using TOF-SIMS demonstrating that some molecular lipid species were differentially distributed into the fibrosis areas or cirrhotic nodules. Spectra of UV microspectroscopy obtained from the same section allowed visualizing high autofluorescence from fibrous septa confirming the presence of collagen. Altogether, these results demonstrated that TOF-SIMS and FT-IR/UV microspectroscopy analyses can be successfully performed on the same tissue section.

Spectroscopy-based approaches and mass spectrometry are essential in addressing the chemical composition and distribution of components across a biological tissue sample. This opens the possibility to investigate the chemical changes occurring in the course of disease-associated lesions of the tissue.[1−5] For the recent past years, infrared microspectroscopy and other imaging techniques based on mass spectrometry such as TOF-SIMS were used in various biological and clinical investigations.[6−11]

Infrared spectroscopy is based on the determination of absorption of infrared light due to resonance with vibrational motions of functional molecular groups. Biological tissue is essentially made up of proteins, nucleic acids, carbohydrates, and lipids all of which have specific absorption bands in the infrared frequency domain. As such infrared spectroscopy is a very valuable tool for biochemical investigations. Fourier transform-infrared (FT-IR) combines IR spectroscopy and microscopy for determining the chemical composition in small sample area. Application of synchrotron radiation as a high brightness source of infrared photons has brought the technique to achieve analysis at the diffraction limit (typically, half the wavelength of the vibrational frequency) while preserving a high spectral quality.[6,7] Imaging techniques based on mass spectrometry allow the mapping of compounds present at the surface of a tissue section. Time-of-flight secondary ion mass

* Corresponding author. François Le Naour, INSERM U785, 14, Avenue Paul Vaillant Couturier, 94807 Villejuif Cedex, France. Phone: (33) 1 45 59 60 75. Fax: (33) 1 45 59 60 90. E-mail: francois.le-naour@inserm.fr.

[†] Institut de Chimie des Substances Naturelles, CNRS.

[‡] Synchrotron SOLEIL.

[§] Inserm U785.

[||] Université Paris-Sud 11.

[⊥] Hôpital Paul Brousse.

[⊗] Cepia, INRA.

[○] Université Paris-Descartes.

(1) Cornett, D. S.; Reyzer, M. L.; Chaurand, P.; Caprioli, R. M. *Nat. Methods* **2007**, *4*, 828–833.

(2) Zimmerman, T. A.; Monroe, E. B.; Tucker, K. R.; Rubakhin, S. S.; Sweedler, J. V. *Methods Cell Biol.* **2008**, *89*, 361–390.

(3) Franck, J.; Arafah, K.; Elayed, M.; Bonnel, D.; Vergara, D.; Jacquet, A.; Vinatier, D.; Wisztorski, M.; Day, R.; Fournier, I.; Salzet, M. *Mol. Cell. Proteomics* **2009**, *8*, 2023–2033.

(4) Faria, S. C.; Ganesan, K.; Mwangi, I.; Shiehmorteza, M.; Viamonte, B.; Mazhar, S.; Peterson, M.; Kono, Y.; Santillan, C.; Casola, G.; Sirlin, C. B. *Radiographics* **2009**, *29*, 1615–1635.

(5) Solon, E. G.; Schweitzer, A.; Stoeckli, M.; Prideaux, B. *AAPS J.* **2010**, *12*, 11–26.

(6) Miller, L. M.; Dumas, P. *Biochim. Biophys. Acta* **2006**, *1758*, 846–857.

(7) Dumas, P.; Sockalingum, G. D.; Sulé-Suso, J. *Trends Biotechnol.* **2007**, *25*, 40–44.

(8) Brunelle, A.; Touboul, D.; Laprévote, O. *J. Mass Spectrom.* **2005**, *40*, 985–999.

(9) Brunelle, A.; Laprévote, O. *Curr. Pharm. Des.* **2007**, *13*, 3335–3343.

(10) Brunelle, A.; Laprévote, O. *Anal. Bioanal. Chem.* **2009**, *393*, 31–35.

(11) Fletcher, J. S.; Vickerman, J. C. *Anal. Bioanal. Chem.* **2010**, *396*, 85–104.

10.1021/ac100581y
Published on Web 04/13/2010

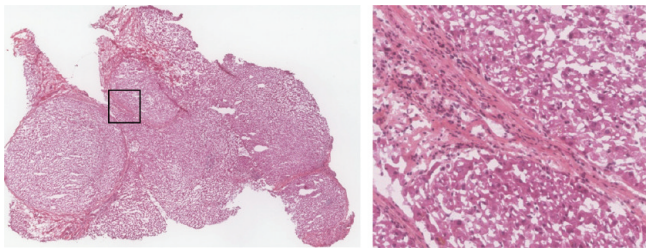

Figure 1. Histological features of cirrhosis. Tissue sections of 6 μm thickness were cut and stained with HES (hematoxylin, eosin, and safran). Cirrhosis is characterized by abnormal liver nodule formation surrounded by collagen deposition leading to fibrosis scarring of the liver (×5) (left). A close up section is shown with a higher magnification (×200) (right).

spectrometry (TOF-SIMS) uses a pulsed and focused primary ion beam (often clusters of heavy metals) to desorb and ionize species from the sample surface. The resulting secondary ions are extracted toward a mass spectrometer, where they are mass-analyzed by measuring their time-of-flight (TOF) from the sample surface to the detector. Ions are identified according to their mass-to-charge (m/z) ratio. The accessible mass range is about 1500 Da, which makes this technique very suitable for the analysis of small molecules such as lipids. Chemical images are generated by collecting a mass spectrum at every pixel as a finely focused primary ion beam is rastered across the sample surface. The mass spectra and the secondary ion images are then used to determine the composition and distribution of sample surface constituents.[8−10] On the other hand, ultraviolet (UV) microspectroscopy can be employed for investigating autofluorescent components of cells and tissues. Indeed, many metabolic markers do provide characteristic fluorescence emission spectra when excited in UV (NADH, collagen, tryptophan, lipo-pigments, elastin, pyridoxins).[12] Their localization can be easily determined, and the changes in molecular environment or oxidation are often associated with modifications of the local fluorescence emission spectrum.

A major advantage of synchrotron-FT-IR and -UV and TOF-SIMS imaging techniques is that they do not necessitate any matrix deposition, chemical treatment, or staining of the sample to acquire images. In addition, the spatial resolution of these imaging techniques allows working at the cellular and subcellular levels. However, only a few studies have been performed with the combination of synchrotron-based FT-IR and TOF-SIMS

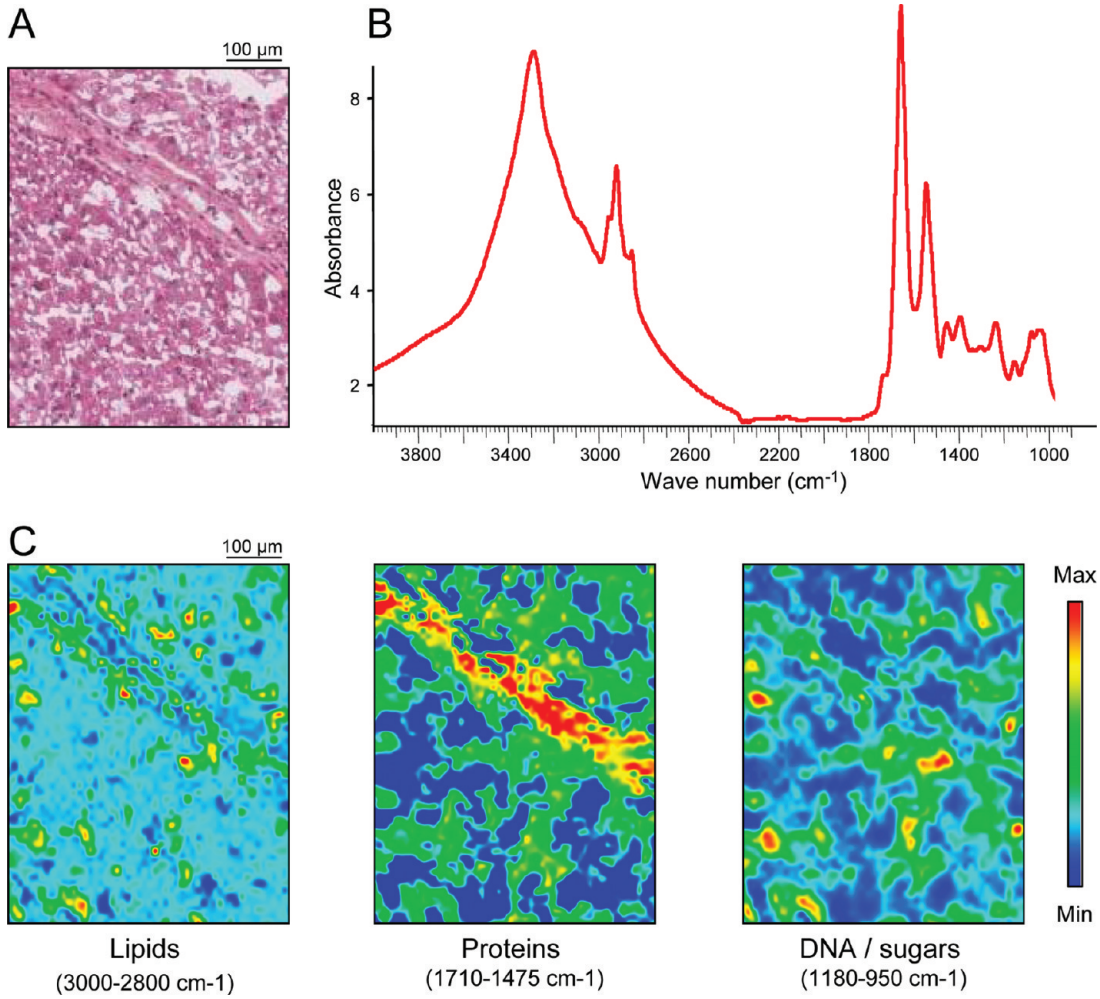

Figure 2. Synchrotron-FT-IR microspectroscopy on liver cirrhosis: (A) An area of liver cirrhosis exhibiting fibrosis and cirrhotic hepatocytes is shown after HES staining. Scale bar: 100 μm. (B) Spectroscopic analysis was performed using synchrotron-FT-IR. A representative spectrum corresponding to 10 × 10 μm^2 is shown. (C) The distribution of some bands corresponding to lipids, proteins, nucleic acids, and sugars were visualized.

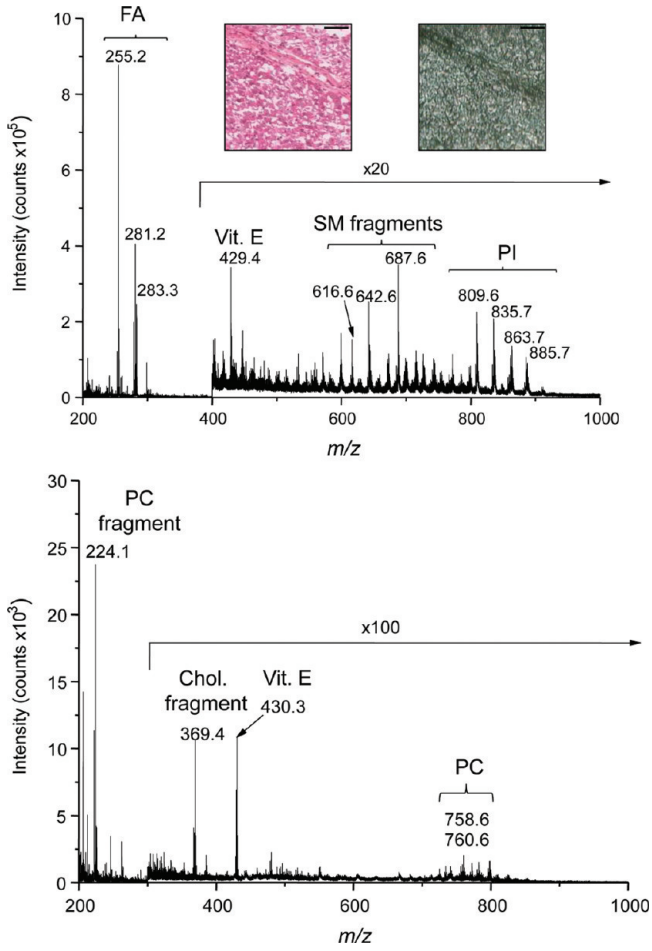

Figure 3. TOF-SIMS mass spectra recorded from surface of the tissue section deposited on a gold coated glass slide. The histological serial tissue section of liver cirrhosis stained with HES or the video image are shown (upper panel) (scale bar: 100 μm). TOF-SIMS mass spectra were extracted from the total area of the cirrhosis liver section in negative ion mode (upper spectrum) or in the positive ion mode (lower spectrum). FA, fatty acid; PI, phosphatidylinositol; SM, sphingomyelin; Vit. E, vitamin E; Chol., cholesterol; PC, phosphatidylcholine.

microspectroscopies and none has been performed yet in combination with UV microspectroscopy. Recently, we have combined synchrotron-FT-IR and TOF-SIMS microspectroscopies for investigating the *in situ* chemical composition on liver steatosis.[13] Although the analyses were focused on the same biopsies, the acquisitions of spectra using synchrotron-FT-IR or TOF-SIMS were performed on serial tissue sections because of the different sample holders employed in these techniques. In this technical note, we have addressed the possibility of coupling TOF-SIMS and synchrotron-FT-IR and -UV microspectroscopies for investigating the same tissue section.

MATERIALS AND METHODS

Samples and Tissue Section. Liver specimens were obtained from the Centre de Ressources Biologiques Paris-Sud, Paris-Sud 11 University, France. Access to this material was in agreement with French ethical laws. Tissues were fixed in formalin for routine pathological assessment, and one specimen was immediately snap frozen in liquid nitrogen and stored at −80 °C until use. Serial sections were cut 6−10 μm thick at −20 °C with a CM3050-S cryostat (Leica Microsystèmes SAS, France) and alternately deposited on a glass slide for histological control, on a gold coated glass slide (Tientascience, Indianapolis, IN) for microspectroscopy analysis, and on a silicon wafer (2 in. diameter polished silicon wafers, ACM, Villiers-Saint-Frédéric, France) for TOF-SIMS. Sections for histology were stained with hematoxylin eosin saffron (HES). Sections for microspectroscopy were dried at room temperature under a pressure of a few hectopascals for 15 min for TOF-SIMS imaging and were further used for FT-IR and UV microspectroscopies.

TOF-SIMS Imaging. A standard commercial TOF-SIMS IV (Ion-Tof GmbH, Münster, Germany) reflectron-type TOF mass spectrometer was used for mass spectrometry imaging experiments. The analysis was performed as previously described.[13,14] Briefly, the primary ion source was a bismuth liquid metal ion gun. Bi_3^+ cluster ions were selected. The ion column focusing mode ensured a spatial resolution of 1−2 μm and a mass resolution $M/\Delta M = 10^4$ (full width half-maximum, fwhm) at m/z 500. A low-energy electron flood gun is activated between two primary ion pulses to neutralize the sample surface with the minimum damage. The mass calibration was always internal, and the signals used for initial calibration were those of H^+, H_2^+, H_3^+, C^+, CH^+, CH_2^+, CH_3^+, and $C_2H_5^+$ for the positive ion mode and C^-, CH^-, CH_2^-, C_2^-, C_3^-, and C_4H^- for the negative ion mode. Structure attributions or assignments of ion peaks were made according to the instrument resolution, accuracy and the valence rule, and the biological relevance of the attribution (according to the tissue type for instance). Ion images were recorded for each selected area with a primary ion fluence of 3×10^{11} ions cm^{-2}. Images were recorded with a field of view of 500×500 μm^2 and 256×256 pixels, giving a pixel size of 2×2 μm^2. Under these experimental conditions, each spectrum was acquired in 0.1 s and the whole map in 6 min. Image reconstruction was done by integrating signal intensities at desired m/z values across the data set. A color scale bar, for which the amplitude, in counts, is given for each image, is placed to the right of the ion images. The data acquisition and processing softwares were IonSpec and IonImage (Ion-Tof GmbH, Münster, Germany).

Synchrotron-FT-IR Microspectroscopy. Synchrotron infrared microspectroscopy was performed at the SMIS beamline at the SOLEIL synchrotron radiation facility (Saint-Aubin, France) as previously described.[13] The beamline is exploiting the bending magnet radiation of the synchrotron radiation, which is coupled to a Thermo Fischer NEXUS FT-IR spectrometer Nicolet 5700. Attached to the spectrometer is a microscope CONTINUUM XL (Thermo Scientist, CA). The detector of the infrared microscope is a liquid nitrogen cooled mercury cadmium telluride (MCT-A) detector (50 μm). The microscope was operating in confocal mode, using a 32× infinity corrected Schwarzschild objective (NA = 0.65) and a matching 32× condenser. All spectra were obtained using a double path single masking aperture (confocal arrangement) size to 10×10 μm^2. The spectra were collected in the 4000−800

(12) Wagnieres, G. A.; Star, W. M.; Wilson, B. C. *Photochem. Photobiol.* **1998**, *68*, 603–632.
(13) Le Naour, F.; Bralet, M. P.; Debois, D.; Sandt, C.; Guettier, C.; Dumas, P.; Brunelle, A.; Laprévote, O. *PLoS One* **2009**, *4*, e7408.
(14) Debois, D.; Bralet, M. P.; Le Naour, F.; Brunelle, A.; Laprévote, O. *Anal. Chem.* **2009**, *81*, 2823–2831.

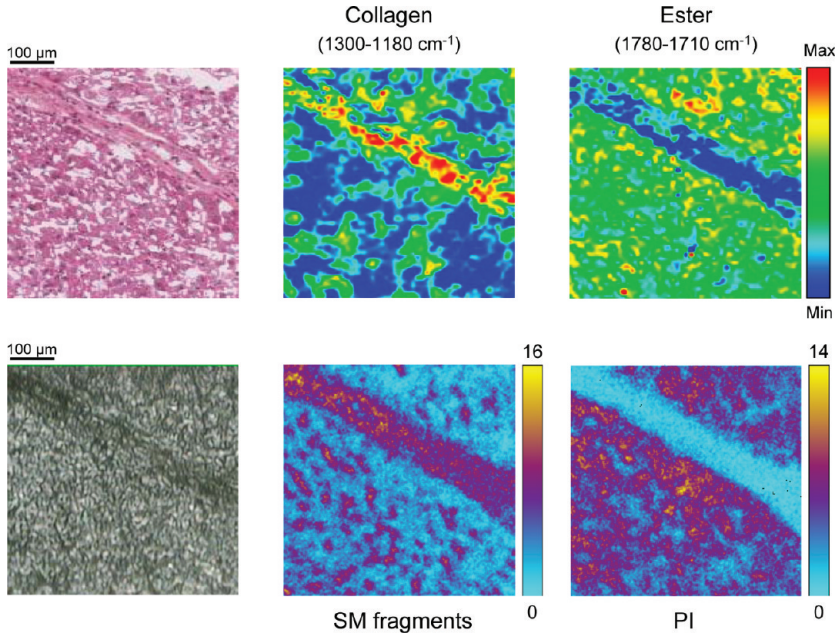

Figure 4. Combination of synchrotron-FT-IR and TOF-SIMS microspectroscopies: The histological serial tissue section of liver cirrhosis stained with HES is shown (upper left). Synchrotron-FT-IR microspectroscopy experiments were performed on liver cirrhosis and the distribution of bands corresponding to collagen or ester were visualized (upper panel). Mass spectrometry images using TOF-SIMS were subsequently recorded on the same tissue section. The video image is shown (lower left). This allowed imaging sphingomyelin fragments or phosphatidylinositol (PI) (lower panel).

cm^{-1} mid-infrared range at a resolution of 8 cm^{-1} with 16 coadded scans. Each spectrum was recorded in approximately 10 s. The sensitivity of infrared microspectroscopy is on average 10^{-4} M (10^{-12} g of molecules). Data analysis of IR spectra and chemical images were performed using OMNIC software (Thermo Scientific).

Synchrotron-UV Microspectroscopy. Synchrotron UV microspectroscopy was performed at the DISCO beamline[15] at the SOLEIL synchrotron radiation facility (Saint-Aubin, France). UV monochromatized light (typically between 270 and 330 nm) was used to excite tissue sections through a 40× ultrafluar objective (Zeiss, Germany). The fluorescence spectrum arising from each excited pixel is recorded. Rastering of the sample allows one to record x, y, λ, I maps of interest. Mapping of 50 × 50 μm^2 was performed with 2 μm spatial resolution with 2 s acquisition time per spectrum. The sensitivity of UV microspectroscopy on tissue is on the order of micromolar. Autofluorescence spectra were deconvoluted using Labspec software (Jobin-Yvon, France), and images of each individual fluorescent component were produced. The use of synchrotron light as a source of UV permits one to finely tune the excitation light to the absorption of endogenous fluorochromes.[12]

RESULTS AND DISCUSSION

Coupling several imaging techniques on the same tissue section can provide a much more complete characterization of the chemical composition of such a sample than each of the methods undertaken separately. To this end, we have implemented a strategy combining three types of spectroscopy such as TOF-SIMS, synchrotron-based FT-IR and synchrotron-based UV microspectroscopies. FT-IR allows investigating the global chemical composition whereas TOF-SIMS can provide the molecular composition in particular small molecules and lipids. In addition, UV microspectroscopy can give information on the content in some autofluorescent molecules such as collagen or tryptophan.

The biological model was focused on liver cirrhosis. Indeed, cirrhosis is characterized by regeneration nodules surrounded by annular fibrosis (Figure 1). Thus, cirrhotic liver parenchyma constitutes a peculiar architecture that represents an excellent model for investigating the combination of imaging techniques.

The coupling of several spectroscopy-based approaches on the same tissue section is highly dependent on the possibility to perform the acquisitions on a single holder. We first focused on FT-IR microspectroscopy. Thus, tissue sections were deposited on gold coated glass slides for better IR reflection followed by synchrotron-FT-IR microspectroscopy that allowed investigating the global composition and distribution of lipids, proteins, nucleic acids, and sugars. Imaging of the bands of amides I and II demonstrated high signal in fibrosis area that corresponds mostly to enrichment of proteins in such a region. Interestingly, the distribution of lipids according to imaging of bands in the frequency range 2800−3000 cm^{-1} was partially concentrated in the fibrosis area as well as on its edge. By contrast, the distribution of bands usually attributed to nucleic acids and sugars was quite homogeneous (Figure 2). The acquisition of images on the same tissue section was further performed by TOF-SIMS leading one to obtain detectable and interpretable mass spectra in the negative and positive ion modes (Figure 3). The mass resolution ($M/\Delta M$, fwhm) and sensitivity (area in number of counts of several characteristic ion peaks) of the signal obtained on the gold coated glass slide were comparable to those obtained on the regular support, a silicon wafer, usually used for TOF-SIMS. These

(15) Giuliani, A.; Jamme, F.; Rouam, V.; Wien, F.; Giorgetta, J. L.; Lagarde, B.; Chubar, O.; Bac, S.; Yao, I.; Rey, S.; Herbeaux, C.; Marlats, J. L.; Zerbib, D.; Polack, F.; Réfregiers, M. *J. Synchrotron Rad.* **2009**, *16*, 835–841.

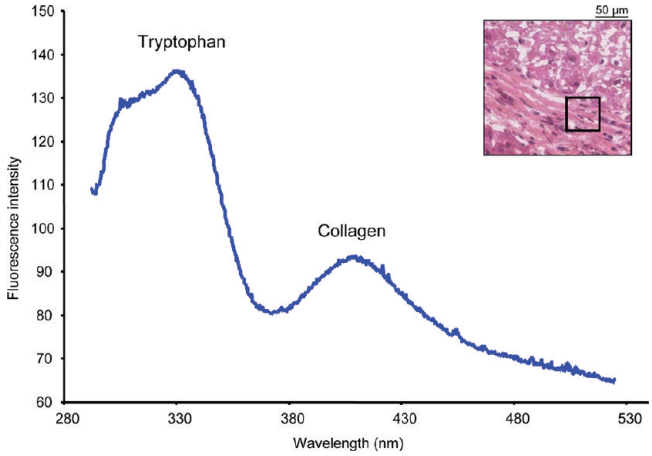

Figure 5. Synchrotron-UV microspectroscopy on liver cirrhosis. The histological tissue section of liver cirrhosis stained with HES is shown. The square represents the region investigated. Synchrotron-UV microspectroscopy experiments were performed leading one to observe high signals coming from autofluorescence of tryptophan and collagen.

observations demonstrated that TOF-SIMS mass spectrometry analysis can be performed on a gold coated glass slide. In addition, since the gold coated glass slide is a conductive surface, the settings of the secondary ion extraction and of the TOF analyzer are similar as for the silicon wafer (Figure S-1 in the Supporting Information). It should be noted that the tissue sections deposited on each holder were not exactly identical since they were serial tissue sections. That may explain slight variability in terms of intensity for some ions. The distribution of some chemical components was subsequently investigated by combining FT-IR and TOF-SIMS microspectroscopies. Thus, the distribution of collagen in fibrosis was imaged since it is characterized by a specific band in FT-IR (1180−1300 cm^{-1}). Interestingly, fibrosis is also selectively enriched in some lipid species such as sphingomyelin as observed by mass spectrometry imaging. By contrast, FT-IR showed the selective distribution of esters into the cirrhotic nodules according to the imaging of the band 1710−1780 cm^{-1}, and TOF-SIMS revealed a correlation with the distribution of ester lipid species such as phosphatidylinositol (Figure 4). Further experiments were performed using UV microspectroscopy on the same area of the tissue section of liver cirrhosis. Spectra allowed visualizing the high signal of autofluorescence of tryptophan and collagen, in particular in the fibrosis area confirming the enrichment of collagen in such a region (Figure 5). Altogether, these results demonstrated that coupling FT-IR, TOF-SIMS, and UV microspectroscopies can be performed on the same tissue section yielding complementary information on the *in situ* composition.

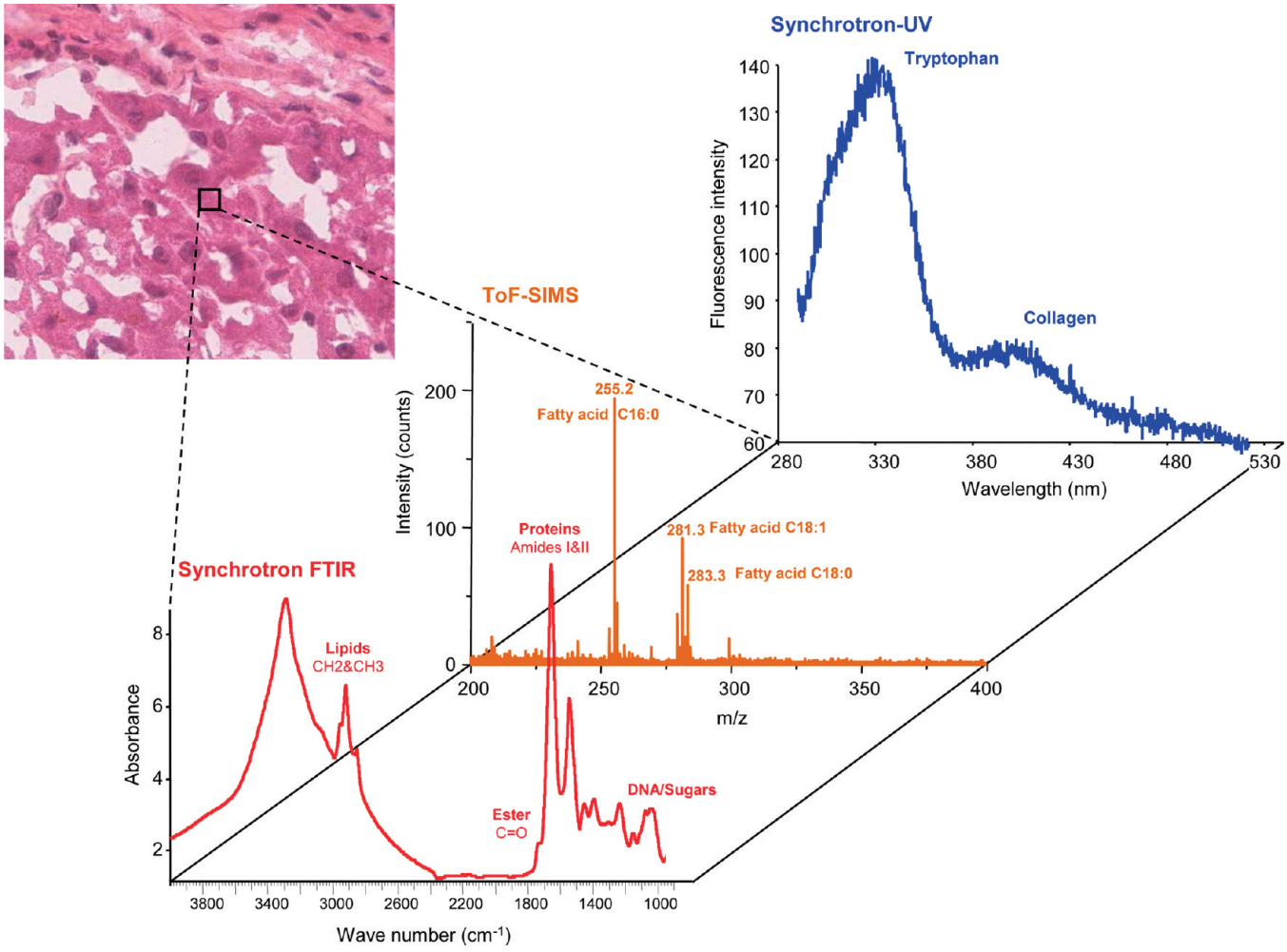

Figure 6. Combination of microspectroscopies with high spatial resolution: The histological tissue section of liver cirrhosis stained with HES is shown. The square represents the 10 × 10 μm^2 region investigated. Sequential acquisitions were performed using synchrotron-FT-IR, TOF-SIMS, and synchrotron-UV microspectroscopies. The corresponding spectra are shown.

Finally, we sought to address the highest spatial resolution for investigating a biological sample by using the combination of the three microspectroscopies. The spatial resolution of FT-IR microspectroscopy is limited by the diffraction limit which is half the wavelength of the vibrational frequency (1−10 μm). TOF-SIMS and synchrotron-UV acquisitions were performed with 2 μm spatial resolution, and the corresponding spectra were extracted. However, TOF-SIMS necessitates summing 25 pixels that correspond to 10×10 μm^2 to obtain an intense spectrum in the mass range 0−400 Da and a larger area for analysis molecular species with higher masses, although only a few counts per pixel are enough to build an ion image. These experiments demonstrated that TOF-SIMS and synchrotron-FT-IR and -UV microspectroscopies can be combined at the spatial resolution up to 10 μm (Figure 6), thus allowing investigations at the cellular level.

CONCLUSIONS

This is the first report combining TOF-SIMS mass spectrometry with synchrotron-FT-IR and synchrotron-UV microspectroscopies. The major advantages of combining these three types of spectroscopies for investigating biological tissue is that they do not require any treatment, labeling, or staining of the sample, and they exhibit similar spatial resolution. With the use of a single sample holder, our study has opened the multidimensional spectroscopy analysis on the same tissue section at the cellular level. The different spectroscopies yield complementary information on the chemical composition of cells and tissues. Thus, the multidimensional spectroscopy analysis of normal and pathological tissues may provide a better understanding in chemical changes occurring in pathologies and may open new avenues for the characterization of spectral and molecular markers.

ACKNOWLEDGMENT

We are grateful to Mathieu Wavelet and Benoit Trousselle for skillful technical assistance. This work was supported by Univer-Sud Paris. It was also supported by beam time allocations at SOLEIL synchrotron (Proposals 20090088 and 20090115). V.W.P. is indebted to the Institut de Chimie des Substances Naturelles for a postdoctoral grant.

SUPPORTING INFORMATION AVAILABLE

Negative ion TOF-SIMS mass spectra on a silicon wafer or gold coated glass slide. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review March 4, 2010. Accepted April 2, 2010.

AC100581Y

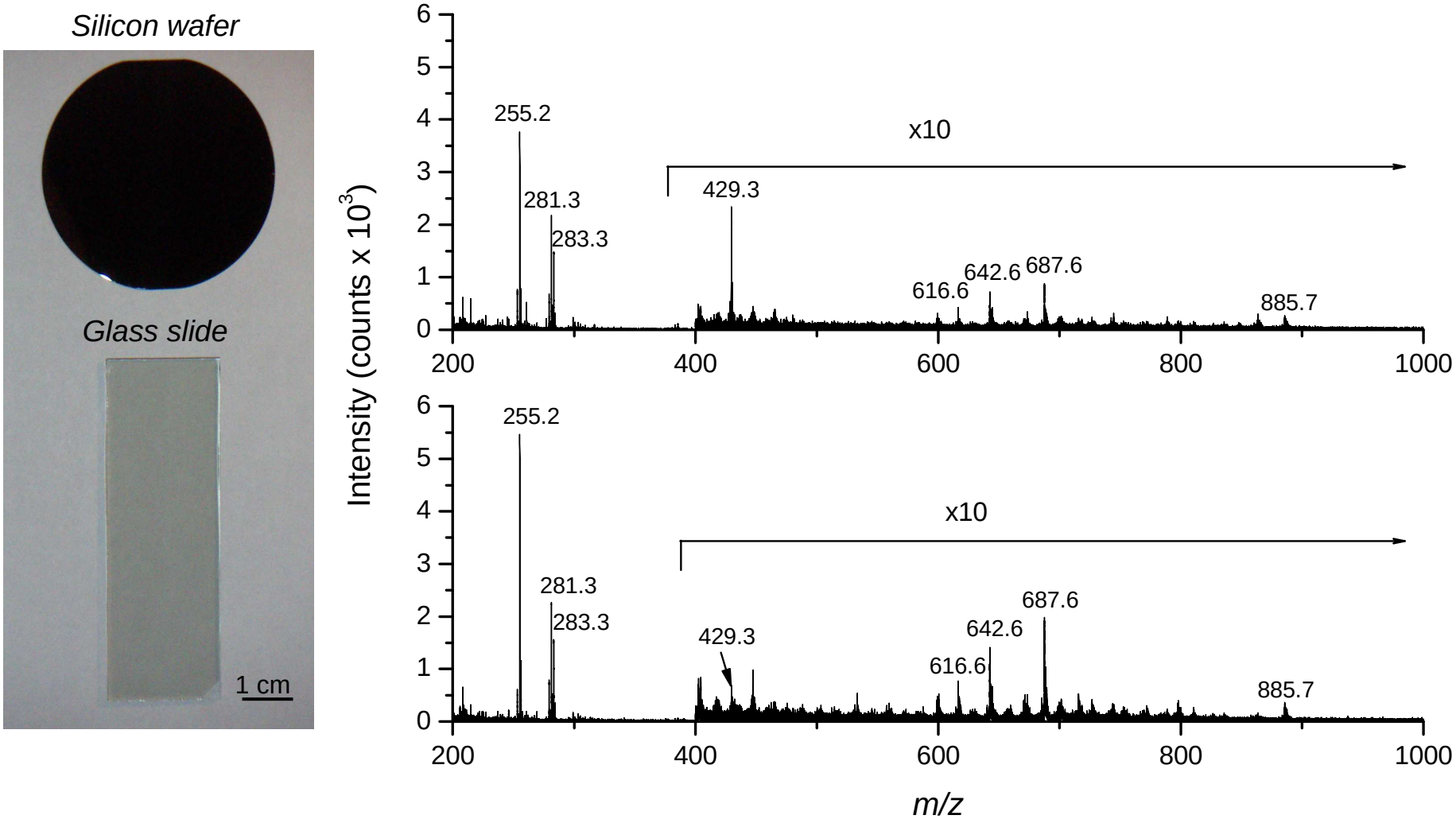

Figure S-1. Negative ion ToF-SIMS mass spectra on silicon wafer or gold coated glass slide. ToF-SIMS acquisitions were performed on serial tissue sections from liver cirrhosis deposited on silicon wafer or gold coated glass slide. Similar spectra were observed in terms of sensitivity and resolution on the two sample holders.