# Probing live cellular responses to freezing by infrared spectromicroscopy

Fernanda Fonseca, J. Gautier, Stéphanie Cenard, Stéphanie S. Passot, Frederic Jamme, P. Dumas

# CellNanoSpec 2011
# Workshop 11 - 14 sept. 2011
## *IGESA - Porquerolles Island*

Organized by the Laboratory of Physical Chemistry (LCP)
joint research unit CNRS - Université Paris-Sud 11

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# Abstracts book

Cell Nano Spec 2011

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Infrared and vibrational spectromicroscopy has made decisive progresses thanks to the brillant infrared sources now available at synchrotron and free-electron facilities and to recent developments applicable to bench top in- struments : detector arrays, confocal Raman and near-field microscopies allowing a spatial resolution well below the diffraction limit (« nanoscopy »). The goal of this workshop is to gather specialists of infrared spectromicroscopy with biologists and biochemists that are users or potential users of these facilities in order to discuss the best strategies taking profit of these recent advances in cell imaging and to identify the best challenging biological questions with such an improved spatial resolution. Cell imaging with techniques in visible and near-UV will also be the subjects of a few review papers in order to analyze the complementarity of all these methods.

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J.-M. Ortega (LCP - Orsay)
Paul Dumas (Synchrotron SOLEIL)
Peter Lasch (Robert Koch-Institut)

**Local organizing committee (LCP – Orsay)**
Abdelfattah Halim
Marie-Françoise Lecanu
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Ariane Deniset (LCP - Orsay)
Gian-Piero Gallerano (ENEA)
Peter Lasch (Robert Koch-Institut) - co-chair
Clotilde Policar (ENS – Paris)

**Invited speakers**
A. Dazzi - LCP Université Paris-Sud (France)
P. Lasch - Robert Koch Institute (Germany)
V. Deckert - Institute of Photonic Technology JENA (Germany)
M.-P. Fontaine-Aupart - CLUPS Université Paris-Sud (France)
Peter Gardner - University of Manchester (UK)
C. Hirschmugl - Department of Physics, University of Wisconsin- Milwaukee (USA)
R. Prazeres - LCP Université Paris-Sud (France)
C. Policar—ENS Paris (France)
Josep Sulé-Suso - University Hospital of North Staffordshire (UK)
Ali Turhan - Poitiers University (France)
J. Tobin (Australian Synchrotron)
L. Vaccari - Elettra synchroton light source (Italy)

**MONDAY 12 SEPTEMBER**
**I. Near-field vibrational spectroscopy (AFMIR) (1)**
***Chair : J.-M. Ortega***
8:50- 9:00 Welcome
9:00-9:30 A. Dazzi (Invited) - AFMIR, a way to make nanoscale infrared spectroscopy and microscopy
9:30-10:00 R. Prazeres (Invited) - Data treatment on near field microscopy AFMIR at CLIO
10:00-10:20 C. Prater - Nanoscale chemical composition mapping with AFM-based IR spectroscopy

## AFMIR, a way to make nanoscale infrared spectroscopy and microscopy

A. Dazzi, A.Deniset, C. Mayet, R. Prazeres, F. Glotin, J-M. Ortega
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For 25 years, near-field techniques have quickly progressed and have taken a large place in Nanoscience microscopy. Whatever, in the infrared range, the near-field microscopes have been not so numerous to work properly. We can count up only two different ways to make infrared studies : the optical technique measuring the transmitted signal coming from the nano-object and the photothermal approach using a tiny thermometer to link temperature to absorption measurements.
Considering these optical methods limitations (mixing of topography and absorption in the signal), we have developed an innovative infrared spectromicroscopy technique, called AFMIR, based on the coupling between a tunable infrared laser and an AFM (Atomic Force Microscope). This coupling allows us to perform ultra-local infrared spectroscopy and chemical mapping at the nanometric scale. The principle1 is based on detecting the local thermal expansion of the sample, irradiated at the wavelength of its absorption bands. This expansion is detected by the AFM tip in contact mode. As the duration of expansion and relaxation of the sample is always shorter than the response time of the cantilever in contact, the excitation transmitted to the cantilever is close to a delta function, so that it always oscillates at its own resonance frequencies. By recording the amplitude of these oscillations we can measure the corresponding infrared absorption as a function either of space or wavelength.

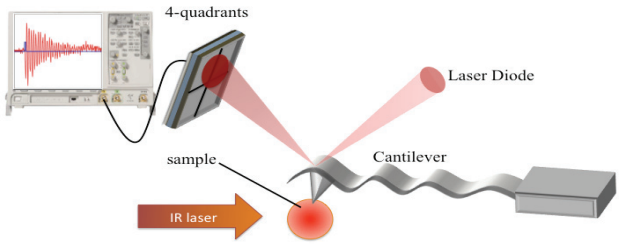


***figure n°1 : scheme of the AFMIR technique***

After validating this technique, we have obtained exciting results in different domains like semiconductor2,3,4 or biology5,6,7,8. For four years, the AFMIR technique associated to the CLIO infrared FEL facility (http://clio.lcp.u-psud.fr/clio_eng/clio_eng.htm) is proposed to users as a standard beam line (also called AFMIR).
To understand and to explain more precisely the complete phenomenon involved into the AFMIR technique, we have developed an analytical model able to make the link between the signal recorded by the AFM and the absorption of the sample. The sample studied is assimilated to a sphere surrounding by an homogeneous media. Even if the real setup is using a prism to support the sample, it is always possible to find effective conductivity to fit well the experimental condition. The sample is illuminated by a pulsed laser of two different types : a fast pulse (25 ns) to simulate the OPO laser characteristic and a long pulse (10 µs) to describe the CLIO free electron laser. We will see that the duration of the pulse can

have some repercussions on AFM oscillations and of the mode excited by the thermal expansion. The tip of the AFM is in contact on the sample with a static force during the laser illumination (fig.n°1).

The thermal expansion is calculated by solving the heat equation and using thermoelasticity properties of the sample. The resolution of the motion equation of the cantilever is done in function of the different illuminations of the sample to express the signal recorded by the 4-quadrants photodetector of the AFM. By the this model, we are able to describe correctly all the physical phenomenon involved into the technique and able to demonstrate that the recorded signal is proportional to the absorption of the sample9, whatever its size and whatever the laser temporal structure (provided that it is shorter than the period of cantilever oscillation, i.e. around 20 µsec).

The advantage of this method is that the signal is directly proportional to the local infrared absorption and that the spatial resolution is typically better than 100 nm. Indeed, one would expect that the heating induced in the sample by the absorption would have a tendency to be diluted in the sample, thus providing signal outside the absorption zone. In fact, due to the pulsed character of the laser, sample expansion takes place only on a zone very close to the absorption zone. This due also to the fact that the heat is evacuated by the surrounding medium in an extremely short time, depending on the size of absorbing zone and thermal properties of the substrate (fig. 2). As a consequence there is no theoretical limit, as in far-field, to the spatial resolution other than the AFM tip size (10 – 20 nm). However, there are limits due to the experimental conditions : sample thickness, thermal properties of the substrate, presence of polymer for fixing the cells, etc. We discuss these various parameters and some examples showing cases where the resolution is affected by sample preparation.

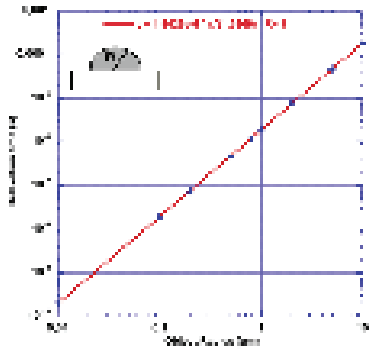

**Fig. 2 : Simulation of cooling of an hemispherical object**

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[8] C.Mayet, A.Dazzi, R.Prazeres, F.Allot, F.Glotin, J.M.Ortega, Opt. Lett. 33, 1611-1613 (2008).
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## Data treatment on near field microscopy AFMIR at CLIO

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The technique AFMIR of near field microscopy, using an AFM tip as sensor for infrared spectroscopy, has given very good results. The data acquisition and treatment represents an important part of this experiment. The real time analysis of tip vibration by Fast Fourier Transform, which is performed at same time as the AFM scan of the sample, allows to measure both topography and infrared absorption spatial distribution. This system requires high rate acquisition and fast data treatment. This paper makes a description of such system.

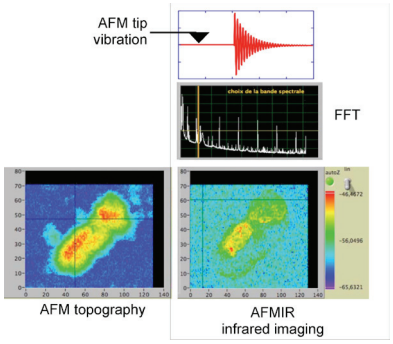


**Fig. 1 : Images obtained with AFMIR of a sample of Capsulatus bacterium : left is standard AFM topography, and right is infrared image of the sample at 1660 cm-1, resonance of amid component.**

## Nanoscale chemical composition mapping with AFM-based infrared spectroscopy

C.B. Prater [1], M. Lo [1], D. Cook [1], A. Dazzi [2], C. Marcott [3] and K. Kjoller
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We have employed AFM-Based infrared spectroscopy (AFM-IR) [1-5] to map nanoscale chemical, structural and mechanical variations in polymers and other samples. The AFM-IR technique irradiates the sample with light from an infrared laser and measures the absorption of this light on a sub-wavenlengh scale using the tip of an AFM by detecting local thermal expansion of the sample. AFM-IR can be used both to obtain point spectra at arbitrary points and to spatially map IR absorption at selected wavenlenghs. Simultaneous measurement of the cantilever's contact resonance frequency as excited by the IR absorption provides a complimentary measurement of relative mechanical properties.
We have used these techniques to chemically identify individual chemical components in polymer nanocomposites and multilayer films. Using self-heating probes we have been able to locally modify the state of a semicrystalline polymer and observe the resulting change in absorption spectra on the nanoscale. We have also used the AFM-IR technique to perform subcellular spectroscopy and chemical imaging on biological cells.

Figures
Figure 1. AFM-based IR spectroscopy is enabled by illuminating a sample with a tunable laser and measuring the resonant response of an AFM in response to IR absorption.
Figure 2. Chemical domains identified in polycarbonate/polymethylmethacrylate blend by their AFM-IR absorption spectra on a ~ 100 nm scale.
Figure 3. Subcellular chemical imaging. A) Composite image of
streptomyces bacteria topography overlaid with AFM-IR chemical image
shows internal locations of energy storage polymer. B) AFM-IR chemical image
of microfibrilated cellulose (sample courtesy of Doug Gardner, University of Maine).
Figure 4. Crystallinity changes in a degradable polymer blend induced with a self-heating AFM tip.

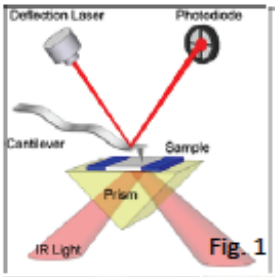

Fig. 1

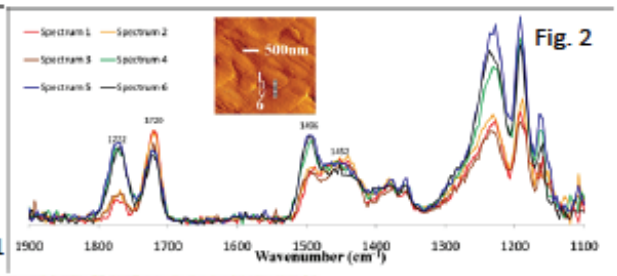

Fig. 2

Fig. 3A

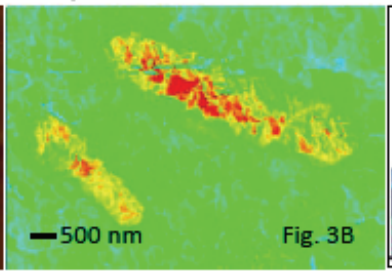

Fig. 3B

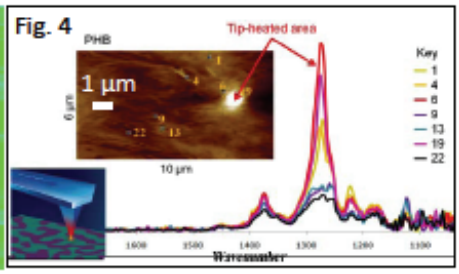

Fig. 4

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3. A. Dazzi, in Biomedical Vibrational Spectroscopy, edited by J. Kneipp and P. Lash (Wiley, Hoboken, NJ, USA, 2008), pp. 291-312
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## II. Near-field vibrational spectroscopy (SERS/TERS)

***Chair : P. Lasch***

10:50-11:20 V. Deckert (Invited) - *Molecular investigation of bio interfaces with nanoscale Raman spectroscopy*
11:20-11:40 A. Hermelink - *Correlation of spectroscopic information and nanoscale imaging techniques*
11:40-12:00 D. Drescher - *Silver nanoparticles in cultured cells as viewed from a combined SERS, bioassay, and ultrastructural approach*

### Molecular investigation of bio interfaces with nanoscale Raman spectroscopy

V. Deckert, M. Richter, T. Deckert-Gaudig, B. Wood

Tip-enhanced Raman scattering (TERS) allows structural investigations with nanometer resolution with detection limits down to single molecules. The high lateral resolution allows to discriminate features that standard IR or Raman spectroscopy detect only as an average signal. For instance a discrimination between protein and lipid structures on a cell or organelle membrane requires a spatial resolution well bellow 10 nm. We will present applications where membrane interfaces are investigate with TERS to discriminate either between specific molecular compositions or even to identify structures that were either postulated or even unknown. In particular the investigation of cancer cells shows a clear distinction of lipid and protein dominated regions on a sub diffraction limited scale. Multivariate data analysis tools are an important tool to extract the data of spectral arrays and allow a fast distinction of major components. In specific cases like the purple membrane the distinction of specific structures, for example bacteriorhodopsin with and without the prosthetic group, is possible and will be discussed.

## Correlation of Spectroscopic Information and Nanoscale Imaging Techniques

Antje Hermelink[1], Peter Hermann[1], Janett Piesker[2], Michael Laue[2], and Dieter Naumann[1]

[1] Biomedical Spectroscopy (P25), [2] Center for biological Safety (ZBS4), Robert Koch Institute, Nordufer 20, 13353 Berlin, Germany

The correlation of optical microscopy and Raman spectroscopy is nowadays a widely used method for investigating cells, spores and other samples in the µm-range [1]. The resolution of optical techniques is however diffraction limited, thus preventing investigations of structures smaller than λ/2. For correlative characterization of nanostructures the applied microscopic and spectroscopic techniques must provide a resolution in the nm-range and a significantly improved sensitivity simultaneously. Recent electron microscopic techniques like high-resolution transmission electron microscopy (HR-TEM) offer a spatial resolution in the sub-nm range, thus enabling even the resolution of single atoms in crystalline samples [2]. The application of surface-enhanced Raman scattering (SERS) increases the Raman scattering cross section by several orders of magnitude, allowing detection down to nearly single molecule level [3]. This high sensitivity is exploited in tip-enhanced Raman scattering (TERS), where a metalized AFM tip is used to gain topographic and spectroscopic information from small surface features. The combination of electron microscopy [4] and tip-enhanced Raman spectroscopy [5, 6] would provide not only morphological but also spectral information on a nano-scale, thus enabling a more reliable identification of unknown single biological particles.
As a first step different TEM grids were evaluated for the applicability for AFM and TERS measurements and a method is presented to acquire TERS spectra from virus particles on TEM grids.

References:
[1] A. Hermelink, A. Brauer, P. Lasch and D. Naumann, Analyst, 134, 1149-1153 (2009)
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[6] P. Hermann, A. Hermelink, V. Lausch, et al., DOI: 10.1039/c0an00531b (2011)

Silver nanoparticules in cultured cells as viewed from a combined SERS, biossay, and ultrastructural approach

[1,2] D. Drescher, [3] G. Laube, [4] P. Guttmann,

# Silver nanoparticles in cultured cells as viewed from a combined SERS, bioassay, and ultrastructural approach

D. Drescher,[1,2] G. Laube,[3] P. Guttmann,[4] G. Schneider,[4] and J. Kneipp[1,2]

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[2] *BAM Federal Institute for Materials Research and Testing, Berlin, Germany*
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The thorough investigation of chemical and physical properties of nanoparticles and the processes from uptake to potential apoptosis are very important for the understanding and evaluation of nanoparticle cytotoxicity. To date a large number of uncertainties concerning toxicological effects and in particular the associated mechanisms at the molecular level still remain.

Several techniques were utilized to investigate i) the distribution and agglomeration state, ii) the molecular vicinity and iii) the cytotoxic behaviour of silver nanoparticles in a eukaryotic cell model. Silver nanoparticles represent an interesting and expedient reference system with respect to their known cytotoxic properties and *surface-enhanced Raman scattering* (SERS) capability. The latter enables spatially resolved investigations of the uptake and transport mechanism of silver nanoparticles inside living cells and give chemical information about the local environment in the proximity of the SERS nanostructures. In additon to the SERS measurements, the cytotoxic behaviour of the nanoparticles was determined by XTT assay. In this context, the stability of silver and also of silica nanoparticle suspensions in aqueous media depending on the environmental conditions was evaluated by UV-vis, DLS and TEM measurements.

To correlate the cytotoxic effects of silver nanoparticles with their uptake mechanism and intracellular localization, we analyze the ultrastructure of nanoparticle-incubated fibroblast and macrophage cells by X-ray tomography at BESSY. In parallel, transmission electron micrographs give evidence of an endocytotic uptake mechanism. Both microscopic methods prove a different accumulation and agglomeration behaviour of silver nanoparticles within the cellular matrix in comparison to gold and silica nanostructures. By combination of these complementary methods we are able to get valuable insight into the biological reaction pathways of nanoparticles in living cells, starting from endocytosis, to vesicular transport, accumulation and apoptosis. These systematic investigations have implications also for other nanomaterials and can improve the knowledge of particle / protein and particle / cell interactions in general.

**III. Raman vibrational microspectroscopy and imaging**

***Chair : V. Deckert***
**14:00-14:30** J. Sule Sulo (Invited) - *Nanoscale imaging in medicine: is there a niche?*
**14:30-14:50** H. Byrne - *Raman micro-spectroscopy of nanoparticles in cells*
**14:50-15:10** D. A. Schmidt - *Raman microspectroscopy: applications in life sciences*
**15:10-15:30** S. F. El-Mashtoly - *Label-free imaging of colon cancer tissues and cells using Raman microscopy*

## NANOSCALE IMAGING IN MEDICINE. IS THERE A NICHE?

Josep Sulé-Suso
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Over the last few years, a lot of work has been carried out to exploit the capability of vibrational spectroscopy into medicine. While this research has included both malignant and non-malignant diseases, most of it has been aimed at improving cancer diagnosis and to better assess the effects of drugs on cancer cells.

Cancer diagnosis entails obtaining a biopsy and identifying cancer cells in tissue and/or cytology samples. However, it is not uncommon for a pathologist to identify cells that are suspicious but not diagnostic of cancer. In these cases, patients need to undergo further biopsies with all the side effects, risks, and costs this entails, and delaying the treatment. Therefore, any technique that could better characterize these abnormal cells would be highly welcomed.

On the other hand, once the diagnostic of cancer has been made, patients might undergo a course of chemotherapy. The selection of a drug or drugs to be used for each type of cancer is based on many clinical studies including hundreds if not thousands of patients giving an indication of which could be the best regime for each type of tumour. However, due to tumour variability, that does not mean it would be the best regime for each individual patient. Several spectroscopy techniques have been used to better clarify these issues. However, in spite of all the work carried out so far, the techniques have not made it yet into clinical practice, probably because there is still a need of fundamental research in such cases.

Vibrational spectroscopy has concentrated in the study of single cells and/or tissues. In the case of FTIR spectroscopy, most of the research has been carried out studying whole cells (several microns size) with fewer groups reporting work at subcellular level (down to micron range resolution). This is not the case for Raman spectroscopy which is able to study cells at subcellular level down to 1 µm. On this basis, several authors have shown these techniques can differentiate between normal and malignant cells. However, the study of cells at subcellular level for both cancer diagnosis and effects of drugs on cells needs a good understanding of the biological properties of cancer cells when compared to their normal counterparts. Many of the differences between malignant and non-malignant cancer cells reside in the cell nucleus, which could be detected if the probing technique might resolve submicron units, ideally at the nanoscale. In this scale, we can study, as an example, DNA composition which is most relevant in biomedicine. In fact, pathologists use, amongst other, nuclear changes to differentiate between cancer and normal cells. Some of these include amongst other changes in nuclear morphology and size, chromatin, and number and size of nucleoli. These fundamental studies are necessary to help improving out knowledge making the far field infrared micro-spectroscopy a potential tool into clinical practice.
Regarding the possibility to figure out whether a tumour could be sensitive of not to a certain chemotherapy drug, many of the chemotherapy agents used in the management of cancer act on the cell nucleus. Therefore, the possibility of studying just in the nucleus following exposure to chemotherapy drugs might help to better assess tumour cells sensitivity to chemotherapy agents, specially following changes in the targeted nuclei, and the modification of the drug chemistry. However, it is very important to understand the action of chemotherapy drugs on cells as this varies according to the drug used and include, amongst other, formation

of new DNA bonds, DNA breakage, and inhibition of DNA synthesis and cell division.
Finally, even if these and other techniques are able to study cells at subcellular level and provide robust data,

an important issue needs to be tackled before a full application of these techniques into medicine is achieved. This is standardization, and, in my opinion, should be applied in the following cases:
1. Sample preparation. The way samples are prepared could alter the final results, therefore, work needs to be done to assess which is the best way to prepare samples (cells and tissues) when studied with different techniques.
2. Data collection and analysis. The parameters (number of scans, resolution, aperture, …) to collect data should be standardized. Furthermore, if subcellular structures are to be studied, it is imperative to standardize the way the area to be studied is located. This might be sometimes difficult when studying unstained cells and/or tissues.
It is my personal view that any techniques that could bring more information at subcellular level will help towards a quicker and more robust cancer diagnosis, and to assess the effects of drugs on cancer cells. However, this will require a strong collaboration between spectroscopists and clinicians.

## RAMAN MICROSPECTROSCOPY OF NANOPARTICLES IN CELLS

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Understanding the uptake and trafficking of nanoparticles in cells has become fundamental to their toxicity as well as their potential in biomedical areas as for example drug delivery or diagnostic contrast agents and thus, the ability to visualize the particles on a subcellular level is paramount. Visualisation is commonly achieved via functionalisation of the particles with luminescent moieties and imaging using confocal fluorescence microscopy, often with co-staining for sub-cellular organelles such as lysosomes or endosomes. However, such functionalisation is not always chemically feasible, and it must be assumed that the luminescent moieties do not change the effective size or surface functionality of the nanoparticles.
As an alternative, Raman mapping of model nanoparticles in cells is demonstrated. Initially, the technique is validated using fluorescently tagged polystyrene particles of ~50nm, which have been demonstrated to be endocytosed and trafficked through early endosomes and lysosomes. A549 human lung cells are initially employed and Raman profiling is performed at 785nm, whereas the fluorescence profiling is performed at 532nm. The Raman signatures of the nanoparticles are shown to be spatially correlated with the fluorescence profile, in situ, using a selection of data mining processes, and the relative sensitivities of the techniques are explored. Beyond localization, Raman spectroscopy is employed to probe the local environment of the nanoparticles. The ultimate objective or a probe for the simultaneous localisation of nanoparticles, characterisation of their sub-cellular environment and evaluation of the cellular metabolisms is discussed.

# Raman Microspectroscopy: Applications in Life Sciences

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The newly developed, multimodal microscopy station at the ANKA-IR2 beamline combines far-field optical microscopy with other microscope modes, such as atomic force (AFM) microscopy [1]. Far-field methods include fluorescence microscopy and confocal laser and Raman microscopies. Complementary near-field methods including aperture-based scanning near-field optical microscopy (SNOM) and scattering SNOM for infrared radiation have also been demonstrated.

We present applications in life sciences, emphasizing the power of Raman microscopy for imaging living cells with sub-cellular resolution approaching the diffraction limit. Compared to other highly sensitive microscopy techniques, such as electron microscopy, x-ray imaging, or secondary ion-mass spectroscopy, Raman microspectroscopy is a chemically sensitive and non-invasive technique capable of revealing both chemical composition and structural information with lateral resolutions of ~300 nm. Examples include tracking designer drug delivery molecules in living cells [2] and monitoring the status of sub-cellular compartments in human spermatozoa [3] for picturing infertility, featured as a Highlight in Chemical Biology of the Royal Society of Chemistry.

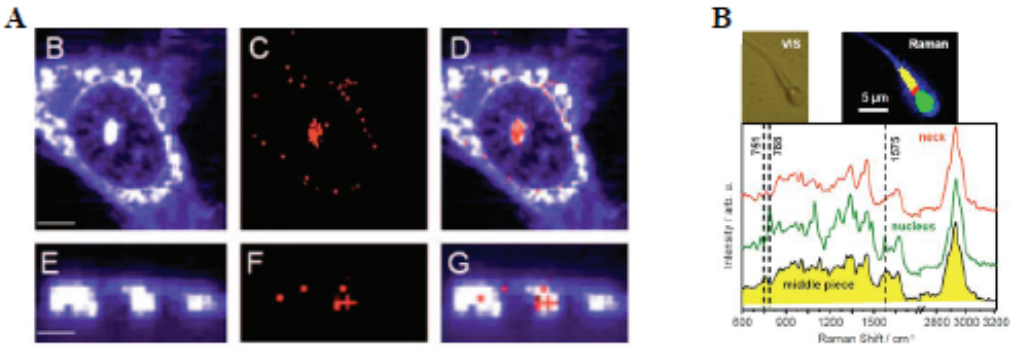


**Figure.** (A) Tracking drug delivery in living cells; sub-panels (B) & (C) Raman images constructed from integrating the C-H stretch and C≡O bands, respectively; overlay of sub-panels (B) & (C) shown in sub-panel (D) ; sub-panels (E-G) show *x-z* cross sections of the same cell. Scale bar in sub-panels (B) & (E) is 6 µm. (B) Sub-cellular details of spermatozoa measured by confocal Raman microscopy and spectra of neck, nucleus, and middle piece.

In principle, the combination of a multimodal microscope with broadband synchrotron radiation will open up new possibilities to comprehensively study biological samples with optical and IR radiation in the future.

This project is developed in close collaboration with the ANKA-IR2 team, and we especially thank B. Gasharova, Y.-L. Mathis, M. Süpfle, and A.-S. Müller. M. H. thanks BMBF grants 05KS7PC2 and 05K10PCA for support.

**References**

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[3] K. Meister, D. A. Schmidt, E. Bründermann, M. Havenith, Analyst 135, 1370 (2010).

# Label-Free Imaging of Colon Cancer Tissues and Cells Using Raman Microscopy

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Raman microscopy is a non-invasive imaging technique that combines vibrational spectroscopy with imaging. It has an intrinsic advantage of providing spatially resolved information based on the molecular compositions and structures without introducing any external labels or dyes. Raman microscopy is exceptionally well suited for differentiating distinct tissue types and for identifying tissue pathology. In addition, Raman microscopy can be used for the identification of subcellular components of single cells [1-3]. Here, we performed Raman imaging of HCT-116 cells to resolve these components. We have also investigated the effect of chemotherapeutic agents on colon adenocarcinoma.

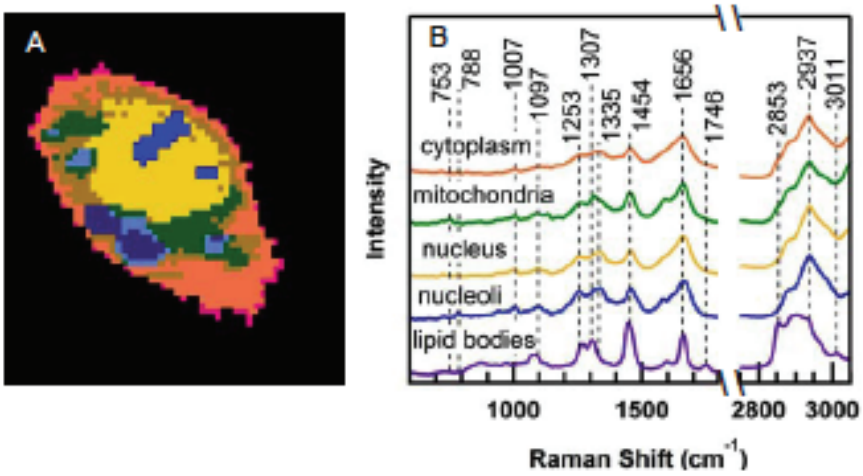


Figure 1. HCA image constructed from Raman hyperspectral data set of HCT-116 cell (A) and average Raman spectra of subcellular organelles of HCT-116 cell (B).

Colon cancer is the third most common type of cancer and the second most frequent cause of cancer death. To elucidate the biological action of any drug candidate for this disease, it is necessary to obtain a detailed picture of the intracellular drug distribution and how it evolves with the time and its effect on subcellular components. In order to set the stage for observing such processes in single living cells, we have constructed the Raman images of colon cancer cells, HCT-116, via multivariate hierarchical cluster analysis (HCA) from Raman hyperspectral data set as shown in Figure 1A. In addition, the average Raman spectra of subcellular components such as cytoplasm, mitochondria, nucleus, nucleoli, and lipid bodies are shown in Figure 1B. These results demonstrate the feasibility of using Raman spectroscopy coupled with HCA to visualize the subcellular organelles and its distribution in the cell. We have also investigated the effect of chemotherapeutic agents on colon cancer cells and the results have shown large changes in the DNA and proteins.

Furthermore, we have performed Raman imaging of intestinal mucosa using 532 nm excitation. False color images of tissues were constructed via HCA analysis from Raman hyperspectral data set and were compared with those obtained after hematoxylin and eosin staining of the tissues. The results demonstrate the feasibility of using 532 nm excitation for Raman imaging of cancerous tissues.

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[2] C. Matthäus, B. Bird, M. Miljković, T. Chernenko, M. Romeo, and M. Diem, *Methods Cell Biol.* **89**, 275-308 (2008).
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**IV. Posters session 16:00-18:30** (11 + 2 booth)

*1. Analysis of type I and IV collagens by Raman microspectroscopy* (T.T. Nguyen)
*2. Synchrotron near-field absorption microspectroscopy at Diamond* (J. Filik)
*3. Classification of anticancer drugs by classification of FTIR spectroscopy according to their mode of action* (A. Derenne)
*4. The NanoWizard® 3 – The most flexible, high resolution AFM with true optical integration* (M. Richter)
*5. Infrared microspectroscopy: refine breast cancer prognosis and tissue discrimination (A. Benard)*
*6. Sub-100 nm infrared spectromicroscopy of living cells* (A. Dazzi)
*7. Infrared nanoscopy AFMIR used for the production analysis of PHB vesicles by Rhodobacter capsulatus* ( R. Prazeres)
*8. Imaging live cells grown on a three dimensional collagen matrix using Raman microspectroscopy* (H. Byrne)
*9. The chemical composition profile of skin dissected with Raman microscopy* (M. Mischo)
*10. Sub-cellular imaging of a metal-carbonyle exogenous compound using AFM-IR or Synchrotron-FTIR* (S. Clède)
*11. Analysis of skin cancer biopsies by IR spectral imaging combined to automated fuzzy clustering (D. Sebiskveradze, C. Gobinet, V. Vrabie, M. Manfait, E. Ly, P. Jeannesson, O. Piot)*
*12. Stand ORIEL - ANASYS*
*13. Stand AGILENT*

## ANALYSIS OF TYPE I AND IV COLLAGENS BY RAMAN MICROSPECTROSCOPY

T.T. Nguyen, J. Feru, S. Brassart-Pasco, M. Manfait, O. Piot
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Type I and IV collagens are important constituents of the skin. Type I collagen is found in all dermal layers in high proportion and participates to the great tensile strength and allows to withstand deformation of the dermis. Type IV collagen is localized in the basement membrane of the dermo-epidermal junction (DEJ); it is crucial to maintain the integrity, the stability and the functionality of this very thin membrane. Type IV collagen structure is different from type I collagen. The collagen IV N-terminus domains are associated in tetramer, through disulfide bonds and lysine-hydroxylysine crosslinks, to constitute the 7S domain. Another characteristic feature of type IV collagen is the presence of several interruptions (between 21 and 26) in the collagenous triple helix. The aim of our research is to study the correlation between the molecular conformations of the type I and IV collagens and their Raman features.
Raman spectroscopy is based on a non-destructive interaction of the light (visible or near infrared) with the matter. This technique permits to probe the intrinsic molecular composition of the samples without any staining nor particular preparation. Data were collected using a Labram microspectrometer (Horiba Jobin Yvon, Lille, France), equipped with a CCD detector, and using a 785 nm Titanium-Sapphire laser as excitation source. A 100X objective (NA=0.8, Leica) permits to focus the incident laser beam and to collect the Raman

scattering. Our analysis was focused at the S-S stretching spectral range (500-550 cm-1) and on amide I and amide III bands. Type I and IV isolated collagens were analyzed. The S-S vibrations of type IV collagen appear at 510 cm-1 and 540 cm-1, these vibrations seem to be correlated with the C-S stretching vibration at 722 cm-1. These signals, specific of the type IV collagen, are associated to the disulfide bridges of the 7S domains; they indicate that these bonds are in the gauche-gauche-gauche (510 cm-1, very stable) and the trans-gauche-trans conformation (540 cm-1). This conformational information at the level of the 7S domain could be used as in situ markers the stability of the collagen IV macromolecular network.
The amide I (1590-1740 cm-1) and the amide III (1200-1300 cm-1) bands of type I and IV collagens were analyzed by mathematical decomposition and curve-fitting, in order to determine the secondary structure of the proteins. The results show that the α-helix conformation and the triple helix content are more important in type I collagen than in type IV collagen; these results are in accordance with the interruption in the Gly-X-Y repeat sequence observed in type IV collagen.
Raman microspectroscopy appears as an appropriate technique to characterize the conformational changes of biological macromolecules such as collagenous materials as shown here on purified products. Further works will be devoted to in situ analysis, on skin sections samples.

## SYNCHROTRON NEAR-FIELD INFRARED ABSORPTION MICROSPECTROSCOPY AT DIAMOND

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The long wavelengths and large source sizes required in Fourier Transform Infrared (FTIR) Microspectroscopy means that it suffers from a poor spatial resolution compared to techniques which work in the visible, such as Raman Spectroscopy. The spatial resolution is only limited by diffraction if a source with high brightness, such as a synchrotron, is used instead of a conventional Globar, but this still only allows the study of micron sized features rather than nanometer sized.

It has been shown that the spatial resolution of FTIR microspectroscopy can be improved beyond the diffraction limit by using a near-field method to detect the absorption rather than the traditional far-field optics [1]. Working in the near-field general requires an atomic force microscope (AFM) to illuminate the sample, collect the transmitted light or detect a physical effect of the absorption. Recently there have been many new innovations in this field, using both monochromatic [2] and broadband IR sources [3].

At Diamond we are currently developing a new near-field infrared microscope, designed to take full advantage of the high brightness of the synchrotron IR radiation source.

The near-field system is based on an infinity-corrected dual optical microscope and AFM with excellent optical access. The system has been designed to deliver the maximum flux of light to the area of sample under the tip. The AFM has been customized to allow the use of high magnification, high numerical aperture Cassegrain objectives with minimal obscurations to the infrared beam. The dual microscope base for the AFM is very flexible, allowing illumination of the sample from either above or below.

The system is also capable of working with multiple feedback mechanisms (4-quadrant photodiode "beam-bounce" system and normal force "tuning fork" modes) and a diverse range of IR detection mechanisms.

The state of the project is going to be discussed, in terms of design and actual development, in view of having the near-field infrared microscope as an additional experimental station on the B22 beamline at Diamond.
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## INFRARED MICROSPECTROSCOPY: REFINE BREAST CANCER PROGNOSIS AND TISSUE TYPE DISCRIMINATION

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D. Larsimont3, C. Sotiriou2 and E. Goormaghtigh1
1 Laboratory for the Structure and Function of Biological Membranes, Université Libre de Bruxelles, Brussels, Belgium
2 Functional Genomics and Translational Research Unit, Department of Medical Oncology
3 Department of Pathology, Institut J. Bordet, Brussels, Belgium

The aim of this study is to develop a new diagnostic and prognostic tool for breast cancer. Clinical guidelines for breast cancer prognosis are currently based on tumor size, histological grade, lymph node status as well as expression of various cellular receptors. Yet, current predictions remain unsatisfactory to identify the best treatment for the individual patient. Although gene expression profiling based on DNA microarrays may help improve decision making by the oncologist (1), this approach does not take into account the cellular heterogeneity of the tumor sample. The recently developed IR imaging systems allow the analysis of the different components of a tumor sample at the cell level. In this study, IR microspectroscopy was applied to breast cancer samples.
IR microspectroscopy has become a new innovative opportunity to study tissue specimens for diagnosis/prognosis of disease and progression monitoring. As an alternative to manual histopathological examinations, computer-based pattern recognition approaches could provide more accurate and reproducible diagnoses. IR image segmentation-based methodologies produce false colour maps of the sample which can be compared to histopathologic gold standard and help pathologists making better decisions. As IR pattern can be fully automated, non trained users can interpret these false colour images.
Here is highlighted the potential of IR microspectroscopy for histological differentiation on FFPE breast tissue sample. Databases of different cellular types present in breast tissue have been constructed by picking individual spectra in the sample region characteristic of the cell type. A partial separation of cell signature can be achieved by a non supervised statistical tool (Principal Component Analysis). Our data resulted in the development of these spectral databases and the construction of an algorithm translating spectral data into histopathological information helpful for the management of the disease. Five clinical cases and more than 5 cell types have been used for the training of the diagnostic tool. Sensitivity, accuracy and specificity of the diagnostic tool will be assessed on a external validation set of tissue.
We show here that discrimination of breast tissular structures can be achieved on FFPE tissues [2], which gives accesss to a large tumor tissue bank and allows direct comparison with histopathologic gold standard procedures. The use of that kind of preserved tissues allows perfoming retrospective studies as enough hindsight on patient outcome is reported.

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2. A.Bénard and E.Goormaghtigh, Discrimination of breast tissular structure by FTIR FPA imaging, 2010 In Press

## SUB-100 NM INFRARED SPECTROMICROSCOPY OF LIVING CELLS

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We have performed infrared spectromicroscopy of cells immersed in liquid water, with a lateral resolution better than 100 nm. Here, we use the motion of an atomic force microscope tip, probing the local transient deformation induced by an infrared pulsed laser tuned at a sample absorbing wavelength. By a Fourier analysis of the vibration of the cantilever tip, we can discriminate frequencies that are characteristic of the object, thus eliminating the influence of the water absorption. This opens the door of chemical imaging of living species "in vivo", with spatial resolution of the order of the size of cell components.

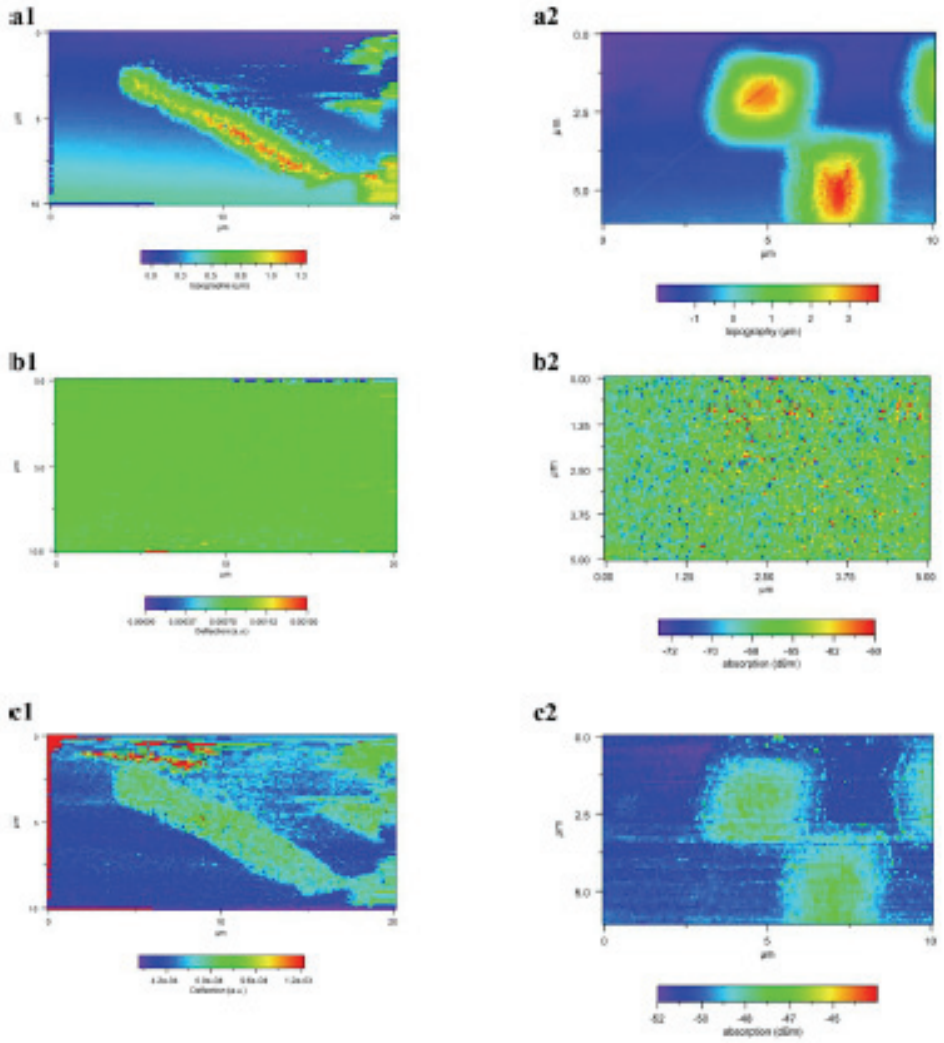


Fig.1: Study of an hyphae (1) and a blastospore (2) immersed in water. The data are taken by irradiating at 1080 cm-1
a - AFM topography
b – IR mapping at cantilever frequency of 80 kHz, characteristic of the surrounding medium
c - IR mapping at cantilever frequency of 31.6 kHz, characteristic of the cells

In situ identification and localization of bacterial polymer nano-granules by infrared spectromicroscopy.

C. Mayeta, A. Dazzia, R. Prazeresa, F. Glotina, J.M. Ortegaa, P. Sebbana, V. Derriena, D. Jaillardb
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b Centre Commun de Microscopie Electronique, Université Paris-Sud, FRANCE.

The photothermal technique, called Atomic Force Microscope InfraRed (AFMIR), combines an atomic force microscope in contact mode with a pulsed infrared laser. Its very good lateral resolution depending essentially on the curvature radius of the AFM tip (that is, dozens of nanometers), AFMIR allows to make "ultra-local" infrared spectroscopy and to perform highly accurate chemical mappings. The principle of this process is based on the photothermic effect: when the sample absorbs the laser pulse, it heats and expands. The dilatation being very fast, it is seen as a shock wave by the cantilever, which starts to oscillate on its own modes. So, by measuring the amplitude of the AFM deflection, we are able to record the absorption of the sample. This setup is now proposed to CLIO users as the beamline called AFMIR and has been improved specifically to biological studies.
One of the relevant research topics of the team is the study of the bacterium Rhodobacter Capsulatus. It's a purple nonsulfur photosynthetic bacterium, which has the particularity to produce a polymer, the polyhydroxybutyrate (PHB), for its energy storage. PHB is used for some years for the production of plastics having similar properties to those of polyethylene and polypropylene but with the advantage to imply no renewable resources and besides to entail no pollution. Thanks to its biodegradable and biocompatible

character, it can be used in a lot of domains such as the food industry or medicine. In bacteria, PHB is contained in granules with variable size and it can be used by the bacterium when its nutritive resources in the middle become insufficient.
This product has several typical infrared bands including at 1740 cm-1 (stretching bond C=O), which is perfectly distinct from other bacterium's bands (Amide I at 1660 cm-1, Amide II at 1550 cm-1). Thus, by imaging bacteria at 1740 cm-1, we are able to locate PHB vesicles [1]. Growing conditions having an influence on the production of PHB, three different culture media have been tested: i) an usual medium, ii) a medium enriched with glucose and iii) a medium containing acetone. The sizes and quantity of vesicles observed on chemical mappings show that Rhodobacter Capsulatus produce more PHB in the presence of acetone [2]. FTIR spectra and pictures obtained by transmission electron microscopy confirmed our results.
[1] C. Mayet et al. Analyst, 135, 2540-2545 (2010)

## IMAGING LIVE CELLS GROWN ON A THREE DIMENSIONAL COLLAGEN MATRIX USING RAMAN MICROSPECTROSCOPY

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The potential of Raman spectroscopic microscopy for the analysis of biological systems and disease diagnostics is well established. Using optical wavelengths, its potential reaches further to sub cellular analysis of biochemical function on a molecular level, and changes to that function as a result of disease or external agents such as radiation, chemotherapeutic agents or toxicants. While analysis of fixed cells has demonstrated the potential of such hyperspectral imaging, live cell imaging remains a challenge.
In this study, live cell imaging in three dimensional collagen gels by Raman spectroscopy is demonstrated. The study is conducted on a human lung adenocarcinoma (A549) and a spontaneously immortalized human epithelial keratinocyte (HaCaT) cell line. The lateral resolution of the system has been estimated to be <1.5 µm making it possible to access the subcellular organization. Using K-means clustering analysis, it is shown that the different subcellular compartments of individual cells can be indentified and differentiated. The biochemical specificity of the information contained in the Raman spectra allows the visualization of differences in the molecular signature of the different sub-cellular structures. Furthermore, to enhance the chemical information obtained from the spectra, principal component analysis has been employed, allowing the identification of spectral windows with a high variability. The comparison between the loadings calculated and spectra from pure biochemical compounds enables the correlation of the variations observed with the molecular content of the different cellular compartments.
The study paves the way for real time spectroscopic analyses of cellular metabolism in a 3D environment.

**TUESDAY 13 SEPTEMBER**

**V. Alternatives methods**

***Chair : C. Policar***
**9:00-9:30** M.-P. Fontaine-Aupart (Invited) - *Correlative dynamic confocal and non linear fluorescence microscopy to decipher biosystem reactivity*
**9:30-9:50** F. Jamme - *Synchrotron UV fluorescence microscopy uncover new probes in cells*

# CORRELATIVE DYNAMIC CONFOCAL AND NON LINEAR FLUORESCENCE MICROSCOPY TO DECIPHER BIOSYSTEM REACTIVITY

Marie-Pierre Fontaine-Aupart1,2, Karine Steenkeste1,2, Romain Briandet3, Sandrine Lécart2, Samia Daddi Oubekka1,2, Ariane Deniset-Besseau4

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During the last decade, the emergence of innovative fluorescence microscopy techniques in combination with original fluorescent probes has radically transformed the imaging research, giving the possibility to non-invasively investigate the dynamic mechanisms of formation and reactivity of many different biosytems (cells, tissues, bacteria, biofilms, etc…).at time and spatial nanoscale resolution.

Confocal laser scanning microscopy (CLSM) was the first major improvement in laser microscopy. The optical sectioning due to the introduction of a "pinhole" in the path of the fluorescence emitted light, ensures a submicronic resolution compatible with observation of subcellular organization or single eukaryotic or prokaryotic cells. It is also now possible to add two other dimensions to these image stacks: time (t) for 2D or 3D dynamics analysis (time lapse microscopy), and wavelength (λ) for spectral imaging, for example to unmix multi-fluorescent labels or subtract interference from a fluorescent background. Therefore, the introduction of CLSM has led to considerable progress in studying the architecture, physiology and molecular interactions within a biosystem without prior chemical fixation (in situ observations in aqueous or culture medium).

The widespread use of lasers has elsewhere led to the emergence of new imaging methods, including approaches based on nonlinear optics. Hence, the limited depth penetration of CLSM can be overcome by employing two-photon laser scanning microscopy (2PLSM). Experimentally, two-photon excitation of fluorescent molecules is obtained by the quasi-simultaneous absorption of two photons of half the energy of the photons used in CLSM, typically using near-infrared lasers. Another advantage is that two-photon effect occurs only in the focal plane, thereby protecting out-of-focus areas from bleaching.

These fluorescence imaging methods under one or two photon excitation are constantly evolving to improve both image acquisition rate (fast confocal Spinning-disk microscopes, multiphoton multifocal microscopy) and spatial resolution (structured illumination, total internal reflection fluorescence microscopy (TIRF), stimulated emission depletion microscopy (STED), photoactivation localisation microscopy (PALM), stochastic optical reconstruction microscopy (STORM), 4pi microscopy).

Besides fluorescence intensity imaging techniques, more advanced fluorescence-based approaches can be implemented for in situ molecular diffusion/reaction studies within "live" biosystems, including FRAP (Fluorescence Recovery After Photobleaching), FCS (Fluorescence Correlation Spectroscopy) and FLIM (Fluorescence Lifetime IMaging).

FRAP is a simple method that is now routinely implemented on commercial CLSMs. Its principle is based on brief excitation of fluorescent molecules by a very intense light source in the volume defined by the confocal microscope objective to irreversibly quench their fluorescence (photobleaching). Fluorescence redistribution is then observed if the fluorophores are allowed to diffuse in the sample. The analysis of the time course of fluorescence intensity recovery associated to proper mathematical models gives thus access to the quantitative mobility of the fluorescent molecules (diffusion coefficient and/or molecular association rates determination). However, FRAP typically requires micromolar molecular concentrations of fluorescent tracers, and consequently gives access only to average diffusion coefficients, potentially masking the effects

of local heterogeneity; FRAP is not sensitive enough to analyze diffusion-reaction processes with single molecule resolution.

For this purpose, FCS is the most appropriate technique. It is based on monitoring the emission intensity fluctuations due to a small number of molecules (nanomolar concentration) passing through a confocal or a biphotonic excitation volume (<1 fL). These fluctuations can be quantified in their amplitude and duration by temporally autocorrelating the recorded intensity signals. They reflect not only the diffusion of fluorescent molecules through the 3D structure of a biosystem, but also the photophysical and photochemical reactions that quench the fluorescence (charge transfer transition to the triplet state of the molecule), or conformational changes of molecules (molecular complexation, aggregate formation). Thus the FCS signal analysis quantifies a range of reaction parameters on a large time scale from microseconds to several seconds.

Both FRAP and FCS methods can highlight diffusion inhibition related to a fluorophore interaction with its biological environment but only fluorescence lifetime analysis is well-suited for characterising such molecular interaction processes. The fluorescence lifetime of a molecule is an intrinsic property of a fluorophore, independent of its concentration that may locally vary depending on its reactivity with the biological environment. It is hence possible to build fluorescence lifetime images (FLIM) that allow assessment of the reactivity of a fluorophore at the molecular level throughout a 3D biological structure.

These extensive exploitations of CLSM and multiphotonic microscopy are also intrinsically related to the development of dedicated specific fluorophores. Available fluorophores allow visualisation of different components of the 3D-biosystems, of individual cells and their local physiology or patterns of gene expression, or of the extracellular organic matrix and the existence of heterogeneous microdomains.

This presentation provides a short outline of some recent applications of our group using correlative dynamic confocal and non linear fluorescence microscopy for studies of interest in biomedical and environmental domains [1-5]. The performances and limits of fluorescence imaging microscopy were discussed and related to infrared and vibrational spectromicrosocpy.

1- Ex vivo fluorescence imaging of Papanicolaou-stained urothelial cells to enhance early diagnosis. Karine Steenkeste, Sandrine Lécart, Ariane Deniset, Pascal Pernot, Pascal Eschwege, Sophie Ferlicot, Sandrine Leveque-Fort, Romain Briandet, Marie-Pierre Fontaine-Aupart. Photochem Photobiol., 83 (2007), 1157-1166.
2-Three-dimensional time-resolved fluorescence imaging by multifocal multiphoton microscopy for a photosensitizer study in living cells. Ariane. Deniset-Besseau, Sandrine Lévêque-fort, Marie-Pierre Fontaine-Aupart, Gérard Roger, Patrick Georges. Appl. optics, 46 (2007), 8045-8051.
3-Diffusion measurements inside biofilms by image-based fluorescence recovery after photobleaching (FRAP) analysis with a commercial confocal laser scanning microscope. François Waharte, Karine Steenkeste, Romain Briandet, Marie-Pierre Fontaine-Aupart. Appl Environ Microb., 76 (2010), 5860-5869.
4-Deciphering biofilm structure and reactivity by multiscale time-resolved fluorescence analysis. Arnaud Bridier, Ekaterina Tischenko, Florence Dubois-Brissonnet, Jean-Marie Herry, Vincent Thomas, Samia Daddi-Oubekka, François Waharte, Karine Steenkeste, Marie-Pierre Fontaine- Aupart, Romain Briandet. Adv Exp Med Biol,, Bacterial adhesion, biology, chemistry and physics 715 (2011) 333-351.
5-Ex vivo assay base on drug fluorescence to predict the response to chemotherapy in patients with urothelial or breast cancer. Ariane Deniset-Besseau, François-Alexandre Miannay, B.A. Lwaleed, Pascal Eschwege, Philippe Vielh, Corinne Laplace,-Builhé, Sandrine Lécart, Marie-Pierre Fontaine-Aupart. Bristish Journal of Cancer. (2011) soumis.

# Synchrotron UV fluorescence microscopy uncover new probes in cells

Frederic Jamme[1,2], Slavka Kascakova[1], Deborah Bourquin[1], Sandrine Villette[3], Alexandre Giuliani[1,2], Valerie Rouam[1] and Matthieu Réfrégiers[1]

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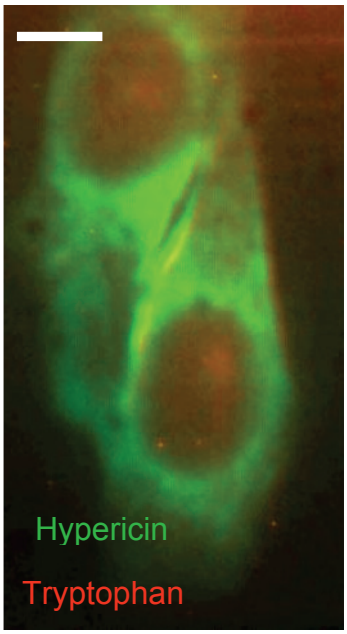
[2]Cepia, Institut National de la Recherche Agronomique (INRA), BP 71627, 44316 Nantes, France
[3]Centre de Biophysique Moléculaire, CNRS UPR4301, Rue Charles Sadron, 45071 Orléans, France

Use of deep ultraviolet (DUV, below 350 nm) fluorescence opens up new possibilities in cell biology because, it does not need external specific probes or labeling, but instead allows taking profit of the intrinsic fluorescence that exist for many biomolecules when excited in this wavelength range. Indeed, observation of label free biomolecules or active drugs ensures that the label will not modify the biolocalisation or any of its properties. UV monophotonic excitation does still present real spectral excitation, leading the way to excitation imaging and a better selectivity of the chromophores. Moreover, our detection setup is true DUV compatible allowing measurements down to 200 nm.




Co-localisation fluorescence image of a single living cell. The scale bar is 10 µm.

We have developed two DUV fluorescence microscopes set-up for cell biology coupled to a synchrotron beamline[1], providing fine tunable excitation from 200 to 600 nm and full spectrum acquired on each point of the image, to study DUV excited fluorescence emitted from nanovolumes directly inside live cells[2]. In addition, to record full field UV fluorescence images of living cells, a full field DUV microscope with only one transmission optic has been installed on the DISCO beamline imaging branch. Due to diffraction limit the lateral resolution is always increased when looking in the UV range allowing nanometric spatial resolution[3].

1. Giuliani, F. Jamme, V.Rouam, F. Wien, J.L. Giorgetta, B. Lagarde,O. Chubar,S. Bac, I. Yao,S. Rey, C. Herbeaux, J.L Marlats, D. Zerbib,F. Polack and M. Réfrégiers, J. Synchrotron Rad. 2009, 16 : 835-841.
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3, Tawil, G., Jamme, F., Réfregiers, M., Viksø-Nielsen, A., Colonna, P., & Buléon, A. . Analytical Chemistry, 2011, 83(3): 989–993

**VI. Infrared far-field microscopy: impact in biology (1)**

***Chair : P. Dumas***

**9:50-10:20** C. Hirschmugl (Invited) - *High-resolution Fourier-transform infrared chemical imaging with multiple synchrotron beams (tentative title)*

**10:20-10:50** M. Tobin (Invited) - *Enhancing the spatial resolution of FTIR microspectroscopy for the study of processes at the subcellular level.*

**11:20-11:50** P. Gardner (Invited) - *Synchrotron based infrared spectroscopy at the single cell level: The case for scattering correction.*

**11:50-12:10** J. Filik - *Optical artifacts in infrared absorption microspectroscopy*

**12:10-12:30** M. Kansiz - *Large area FT-IR Imaging at high spatial resolution with biological samples - how differing levels of spatial resolution can influence interpretation*

## HIGH-RESOLUTION FOURIER-TRANSFORM INFRARED CHEMICAL IMAGING WITH MULTIPLE SYNCHROTRON BEAMS

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A new mid-infrared beamline (IRENI) extracting 320 hor. x 25 vert. mrads2 to homogeneously illuminate a commercial IR microscope equipped with an infrared Focal Plane Array (FPA) detector has recently been commissioned at the Synchrotron Radiation Center in Stoughton, WI. [1] The swath of radiation from the SRC is extracted as 12 beams and recombined into a 3 x 4 bundle of beams that is refocused onto a sample plane of an infrared microscope illuminating 40 x 60 micron2 sample area. This new facility provides the opportunity to obtain chemical images with diffraction-limited resolution, for all wavelengths in the mid-IR concurrently, in minutes. The design of this facility and initial applications of biomedical interest will be presented.

The optical arrangement of the microscope at IRENI is based on a Bruker Optics Hyperion Microscope that is equipped with a 20x Schwarzschild condenser (modified from a GAO) and 74x Schwarzschild objective achieving effective geometric pixel sizes of 0.54 x 0.54 mm2. This effective pixel size is approximately l/4 for even the shortest wavelength of 2 µm, providing adequate information for point spread function (PSF) deconvolutions of the chemical images to obtain high fidelity images and high quality spectra for each pixel.

Examples of biological interest will be shown, demonstrating the advantages of the high definition imaging, focusing on results at the sub cellular scale and in vivo studies.

This work has been done with support from an NSF Major Research Instrumentation grant (DMR-0619759) and the Synchrotron Radiation Center, which is also supported by NSF (DMR-0537588).

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## ENHANCING THE SPATIAL RESOLUTION OF FTIR MICROSPECTROSCOPY FOR THE STUDY OF PROCESSES AT THE SUBCELLULAR LEVEL.

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Introduction

The infrared beamline at the Australian Synchrotron (AS) currently operates a conventional Bruker Hyperion 2000 infrared microscope with all-reflecting optics, in common with many other synchrotron facilities. Led by our own research needs and by an increasing requirement from our user

community for improved spatial resolution, we are currently working with several methods for either optimizing the resolution in the far field, or overcoming the far-field diffraction limit, both at the AS and at other facilities. Three methods will be discussed here, and outcomes from each technique will be presented at the workshop.

Scientific challenges

Our scientific interest relates to the study of cell migration and adhesion, in particular the membrane and cytoskeletal changes that are involved in the remodelling of cells during migration, or under the influence of growth hormones. Our desire is to be able to extract relevant chemical information at sub-cellular spatial resolution, without the use of large marker molecules, and in time to be able to do this in living systems.

Cell migration and adhesion are central to a multitude of biological events including embryonic development, axonal guidance, inflammation and wound healing. The morphology of cells undergoing migration varies considerably, but in most environments involves the production by the cells of protrusions, either in the form of membrane "blebs" [1] or more often as highly ordered structures including spike-like "filopodia" and broader, flatter "lamellipodia" [2, 3]. Development of these protrusions is often accompanied by, or results in, a polarisation of the cell body leading to a clear directionality of the cell, thus enabling migration. This polarisation, and the production of lamellipodia and/or filopodia can be observed in many cell types including fibroblasts, T-cells, B-cells and epithelial cells, and may either be present under normal laboratory growth conditions, or can be induced by external stimuli such as growth factors , chemotactic gradients, or mechanical stimuli [4, 5]. The structural "driving force" behind this directional extension of the cell body is a combination of events, including: 1. the polymerisation of actin in a branching network within the cytoplasm, close to the leading edge of the cell protrusion, 2. the attachment, via specialised structures, of the actin fibres to the cell substratum, and 3. The contraction of actin fibres through interaction with myosin chains further back in the cell body. Several recent reviews have dealt with the complex interaction of the many molecules responsible for this process, and in particular the connections between the actin fibres and the extracellular matrix, referred to as "focal adhesions"

[2, 6], and much work has been devoted to understanding the workings of this complex protein network [7, 8]. Restructuring of cells in this manner also involves great extension of the cell membrane, and relatively little attention has been paid to the role of the membrane's structure and composition in enabling these extended structures to be produced.

It has long been recognised that the cellular membrane is not simply a uniform and passive bilayer behind which a protein-based ultra-structural mechanism operates independently. It is now accepted, though not universally, that a degree of phase separation occurs in the cell membrane of many cell types, both as a result of structural changes to integral membrane proteins, and as a potential mechanism of "chaperoning" important signalling proteins. These areas of separation are generally referred to as "membrane rafts", and are domains rich in cholesterol and sphingomyelin, though their size in any cell type, and during rest or migration is not well understood [9, 10]. It has also been shown that when certain cell lines become polarised that there is a concentration and aggregation of these domains at the leading edge of structures such as lamellipodia. However, studying the role of the membrane in important events such as cell protrusion has proven difficult since many of the methods employed, such as cholesterol depletion, and the insertion of fluorescent analogues, have been shown to either interfere with the membrane structure, or to alter the localisation of membrane constituents themselves (such as sphingomyelin or cholesterol) into one or other of different phases. Much of what has been shown, such as localisation of rafts in polarised

cells, is dependent on the inferred location of specific membrane proteins that are known to be extracted from cell membranes along with cholesterol for example [9]. Other recent studies have relied on fluorescence techniques such as resonant energy transfer (FRET) between fluorescent analogues inserted into cell membranes to infer intermolecular distances on the nm scale, and have given clues to the size of membrane rafts in non migrating cells or fluorescence recovery after photobleaching (FRAP) to study cell polarisation [10,11].
Vibrational spectroscopy has the potential to provide new information regarding the composition and distribution of membrane lipid and protein components within important cellular structures such as the lamellipodium, without the use of significantly altered probe molecules such as fluorescent analogues. Advanced imaging methods such as Coherent Anti Stokes Raman (CARS) microscopy have shown potential in tissue imaging, but have not yet been able to shed light on the native state of the cell membrane with higher spatial resolution [12] and we therefore propose exploring the potential of three developments of IR microspectroscopy to probe the structure of the lamellipodium of migrating cells.

1. Synchrotron Focal Plane Array (FPA) FTIR Imaging
The IRENI beamline at SRC Wisconsin incorporates a Bruker Hyperion 3000 FPA microscope into a beamline in which twelve separate beams extracted from a total horizontal synchrotron aperture of 300 mrad are coupled into the interferometer, and through the microscope [13]. This level of illumination, when combined with a high magnification, high numerical aperture objective (74x 0.65 NA), permits a small, well illuminated field of the sample to be transferred to the detector with an effective pixel projection of 0.54 x 0.54 µm. While this is significantly beyond the theoretical diffraction limit in the mid-IR for an objective of this NA, the high level of oversampling permits spatial deconvolution to be more effectively performed on the resulting data sets. At this workshop we will report on the outcomes of our beamtime at IRENI studying cultured cells following growth factor stimulation.

A further challenge at this "optimised" spatial resolution is the ability to study live cells. We are currently developing a flow chamber suitable for maintaining living cells in a stable environment and which is specifically designed for operating with the short working distance 74x objective in use at IRENI and also at the Australian Synchrotron.

2. Total internal reflection microspectroscopy
Attenuated total internal reflection (ATR) microscopy is ideal for the microscopic analysis of samples that cannot be presented as a thin section for transmission measurement and cannot be highly polished for reflectance studies. It also has the advantage that the focal spot of the beam within the ATR crystal, and hence the probing evanescent wave, is reduced in dimensions by the refractive index of the ATR crystal material. Common ATR crystal materials include germanium (n = 4.0) and zinc selenide (n = 2.4). While germanium has the advantage of a very high refractive index, zinc selenide has the advantage of being transparent in the visible wavelength range, enabling small features in a sample to be located for analysis. The method of ATR Microspectroscopy usually involves pressing the ATR crystal into contact with the sample, either using a fine-pointed crystal, or the underside of a hemispherical crystal. Alternatively biological materials such as cells can be cultured directly onto the ATR crystal and observed using a focal plane array detector [14]. We have developed an alternative micro ATR method whereby the sample substrate, such as a zinc selenide window, is coupled to a separate focusing optical element, enabling ATR measurement to be made on interchangeable samples. This method is being used to probe the lamellipodia of fibroblast cells and breast adenocarcinoma cells cultured onto ZnSe windows. To date, ATR maps of single fixed cells have been achieved with excellent sub-cellular spatial resolution.

3. AFMIR-PTIR

In order to achieve sub-micron spatial resolution in the mid IR, we are currently exploring the potential of Atomic Force Microscopy IR (AFMIR) microspectroscopy to probe the structure of the lamellipodium of migrating cells. This is being achieved through access to the AFMIR beamline at the CLIO Free Electron Laser, which utilizes the technique of photothermal induced resonance (PTIR) to obtain infrared absorption information at spatial resolution of the order of 100 nm or better. The lamellipodium of migrating cells, which is of interest to us, can extend from a few microns to 10s of microns from the main body of mammalian cells, but is typically only between 70 and 180 nm in thickness [8]. The flat nature of the sample and its adhesion to the substrate therefore make it an ideal candidate for investigation using AFMIR-PTMS.

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## SYNCHROTRON BASED INFRARED SPECTROSCOPY AT THE SINGLE CELL LEVEL: THE CASE FOR SCATTERING CORRECTION.

Peter Gardner - The University of Manchester

The coupling of infrared microscopes to synchrotron radiation sources in the 1990s enabled infrared spectra to be obtained from single intact biological cells, and offered the potential for a new powerful analytical tool for the cell biologist [1]. This, however, proved to be a false dawn in that the spectra subsequently measured were often highly distorted by scattering, (causing changes in peak shape and peak position) such that meaningful interpretation was difficult and often impossible. Thus, for a decade, although measurement was possible, research in this field was stilted with only a few papers being published. In 2009 the underlying causes of the scattering phenomenon were finally understood and termed resonant Mie scattering (RMieS) [2] and in 2010 a correction algorithm (RMieS-EMSC) was published that enables the absorption component of the spectra to be separated and extracted from the measured spectrum [3]. Using this correction algorithm now means that chemical interpretation of the spectra can be undertaken with confidence. In this paper examples will be given where extraction of the pure absorption

spectrum has enabled subtle chemical differences to be observed which were previously obscured by scattering. These include high resolution single cell data showing sub cellular resolution and also drug-cell interaction studies that demonstrate the subtle difference in the cells response to dugs operating by different modes of action [4].

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## OPTICAL ARTIFACTS IN INFRARED ABSORPTION MICROSPECTROSCOPY

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Fourier Transform Infrared (FTIR) spectroscopy is often used for routine sample analysis because of its non-destructive nature and the relative ease in which the normal mode "finger print" region can be interpreted.

Despite the apparent simplicity of the technique, complicated correction operations are often required to transform the collected data into an absorbance spectrum that would be comparable to the spectrum obtained in a tradition transmission measurement. The most common of these operations are the Kramers-Kronig [1] and Kubelka-Munk [2] transforms for specular and diffuse reflection measurements, as well as the ATR depth correction [3].

It is therefore crucial to recognize when FTIR spectra have been affected by artifacts, and to know how to correct them or how to tailor an experiment to avoid them.

Two artifacts that are particularly relevant to microspectroscopy are the Electric Field Standing Wave (EFSW) effect [4] and Resonant Mie Scattering (RMieS) [5].

The EFSW effect [4] occurs in transflection measurements, where a transparent sample is measured on a reflective surface. Reflection by a metal surface causes an 180o phase shift in the electric field of the light causing the incident and reflected beams to interfere. This creates a standing wave pattern above the mirror, with node at the surface and a node-antinode spacing that is dependent on the wavelength. Since absorption is proportional to the electric field strength, a material at a node in the standing wave contributes less to the absorption spectrum than a material at an antinode. This effect can make the absorption coefficient for a homogenous material have dramatic, non-

Beer Lambert dependence on wavelength and sample thickness.

Using a simple protein gel structure as a phantom for a biological sample, we discuss the results of FTIR microscopy transmission and transflection measurements and how the differences seen are reasonably consistent with the EFSW effect. The results of this test reinforce how important using standard, well understood samples are important for checking for artifacts when measuring complex samples in different optical configurations.

The other artifact, RMieS [5], is a particularly difficult problem and is observed when a sample has structure on the order of the wavelength of the probing light (several microns for the IR). This structure causes Mie scattering, the efficiency of which is dependent on the refractive index of the scattering material. Since the refractive index changes over an absorption band RMieS can result in specular reflectance-like artifacts around strong absorption bands. The physics behind this artifact have been well studied and explained by measuring polymer microspheres of various sizes [5].

Numerical algorithms have been developed by other researchers [6,7] to correct for these effects but it is important that these corrections be thoroughly tested before being applied to real world systems. We discuss the results of these correction algorithms on simulated data created from protein standards and the equations for RMieS. We also show results of mixed protein gel/polymer microstructure phantoms and how the data affects the potential for algorithmic correction.

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## LARGE AREA FT-IR IMAGING AT HIGH SPATIAL RESOLUTION WITH BIOLOGICAL SAMPLES – HOW DIFFERING LEVELS OF SPATIAL RESOLUTION CAN INFLUENCE INTERPRETATION

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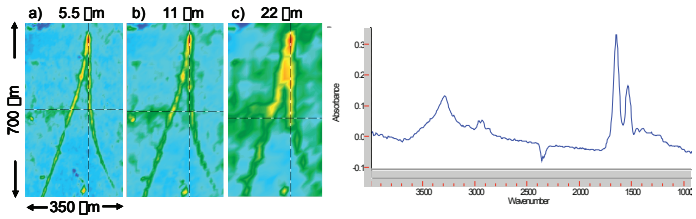
FT-IR Imaging using a 2-D Focal Plane Array (FPA) has established itself as a mainstream technique. With its unique ability to provide spectral (chemical), and spatial (positional) information, application areas are growing substantially.
One application of particular importance and popularity are biological samples, including biomedical samples. The resulting infrared spectra and images acquired represent the total chemical composition of the sample under measure. Generally speaking, the most prominent spectral features are those arising from macromolecular components, such as proteins, lipids, carbohydrates and nucleic acids.
It is this unique ability, to provide spectral and spatial information in parallel that makes FT-IR FPA imaging a powerful technique in biological analyses. The premise that proceeding the morphological changes (often used as markers for the onset of disease), subtle chemical changes occur, allows for the possibility of using FT-IR for earlier detection of these chemical changes and hence possible earlier disease diagnosis. The ability to detect and locate exactly where these subtle chemical changes are occurring provides biomedical

researchers with a powerful analytical tool. Such subtle differences often necessitate the use of multivariate image processing techniques, such as cluster analysis to highlight regions of similarity or difference.
These subtle morphological changes can occur across relatively large areas of samples and in very small (<10 microns) localized areas. It is for this reason, that in order to obtain the most accurate information, collecting data in "high spatial resolution mode" (<10 microns) rather than "low spatial resolution mode"(>20 um) as used in survey scans is more preferred.
The parallel data collection capabilities of FPA imaging, allow for the collection of high spatial resolution (5.5 microns) images across a relatively large field of view (1-2 mm squared) within minutes.
Examples from various biological samples analysed over relatively large areas at differing spatial resolutions will be presented to demonstrate the benefits of high spatial resolution imaging.




Rat brain cross-section images were collected on an Agilent Cary 670 FTIR spectrometer and Cary 620 infrared microscope equipped with a 64x64 MCT FPA. All images were acquired at 8 cm-1 spectral resolution with
a) 16 scans at 5.5 µm spatial resolution. Time of collect = 44 sec.
b) 4 scans at 11 µm spatial resolution. Time of collect = 20 sec.
c) 1 scan at 22 µm spatial resolution. Time of collect = 13 sec

**VII. Near field vibrational spectroscopy (AFMIR) (2)**
Chair : A. Dazzi
14:30-15:00 C. Policar (Invited) - Photothermal IR-spectromicroscopy for subcellular imaging: an example of cellular mapping of a metal-carbonyl exogenous compound
15:00-15:20 A. Deniset-Besseau - The dynamics of lipid storage in Streptomyces – a study by the AFMIR technique
15:20-15:50 P. Lasch (Invited) - AFMIR and confocal Raman microspectroscopy of bacterial endospores

# Photothermal IR-Spectromicroscopy for Subcellular Imaging: an Example of Cellular Mapping of a Metal-Carbonyl Exogenous Compound

Clotilde Policar*,[a]

Sylvain Clède,[a] François Lambert,[a] Marie-Aude Plamont,[b] Jenny Waern,[c] Céline Mayet,[d] Ariane Deniset,[d] Rui Prazeres,[d] Jean- Michel Ortega, [d] Anne Vessières,[c] Alexandre Dazzi [d]

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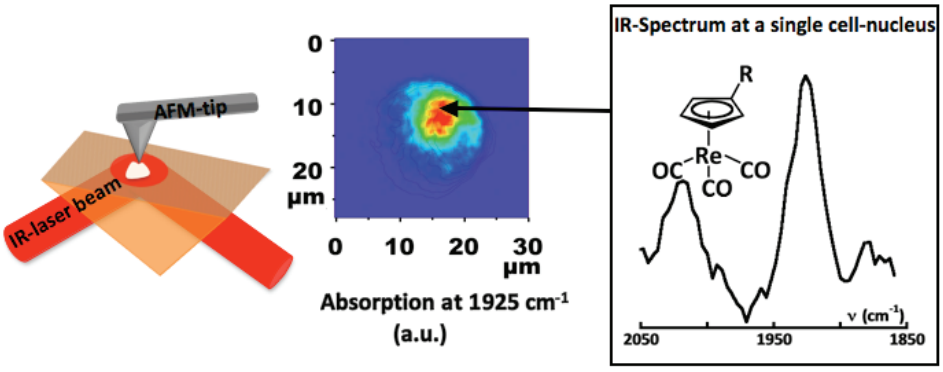
The IR-energy range is particularly attractive for chemical-imaging[1] as vibrational excitation in the IR induces no photo-bleaching. However, in classical optical microscopy, sub-cellular —that is sub-micrometric— resolutions are no attainable in the IR-range, as the diffraction criteria imposes a resolution higher than the µm. Sub-micrometri resolution are possible using near-field techniques. AFM-IR, a technique based on the PTIR effect (phototherma induced resonance) is an emerging and challenging technique using a set-up patented by Dr. A. Dazzi (Paten US11/803421) coupling an AFM and a tunable infrared laser to record spatially resolved absorption measurements i the IR-range. A. Dazzi et al. have successfully mapped a single air-dried *E. coli* cell by irradiation in the amide I and I bands.[2] The question of the possible extrapolation of the technique to exogenous dilute species arose.

As a proof of principle, we have recently shown[3] that P89, an hydroxy-tamoxifene hormone conjugated with a Re-tris-carbonyl [4] is providing, after cellular incubation (1h, 10 µM, MDA-MB231 cells, non-hormono-dependent breast-cancer), an intense signal in classical FTIR and allows an efficient mapping inside cells. A colocalisation of P89 and a region rich in amide and phostate was evidenced.

We will present in this communication both the results of cellular content quantification by FTIR and the chemical imaging of P89 inside MDA-MB231 cells using PTIR.[3]

Interestingly, PTIR enables microspectroscopy, that is to record spectra at a definite location of the AFM-tip. This is o utmost importance to validate the images and check that the PTIR signal is actually due to the molecule of interest Spectra in and outside hot spot will be presented.

Recent results obtained using other imaging techniques and other probes will also be presented.




On the left, prism illuminated by the IR-beam with deposited cell and AFM-tip in contact. In the middle, chemical mapping at one CO-vibration band IR-spectra recorded at the nucleus

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# THE DYNAMICS OF LIPID STORAGE IN STREPTOMYCES - A STUDY BY THE AFMIR TECHNIQUE

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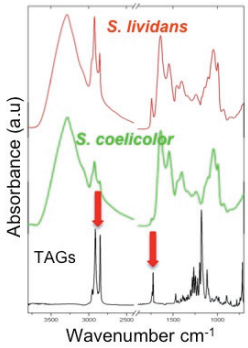
Streptomyces are filamentous Gram + bacteria living in the superficial layers of the soil where they contribute to the degradation of organic compounds. These bacteria produce many secondary metabolites with various chemical structures and useful biological activities like antibiotics, anticancer drugs, herbicide, fungicid.... The synthesis of these molecules is triggered by nutritionnal deficiences, specially in phosphate, and takes place when growth of the bacteria slows down or stops. The wild type strain of Streptomyces lividans possesses in its genome biosynthetic pathways able to direct the biosynthesis of various secondary metabolites but does not express them. Interestingly, a mutant of this strain interrupted for a single gene (ppk) over-expresses these biosynthetic pathways as does the natural strain, Streptomyces coelicolor. Recently, electron microscopy observations revealed the presence of numerous lipid vesicles in the cytoplasm of the wild strain of S. lividans whereas these vesicles were much less abundant in the cytoplasm of the ppk mutant and indetectable in S. coelicolor. These observations established an inverse correlation between the presence of lipid vesicles and antibiotics production that suggests that the carbon stored in these lipid vesicles might be used for antibiotics biosynthesis. These lipid vesicles in S. lividans contain mainly triacylglycerols (TAGs) that posses several absorption bands in IR. In particular we can easily distinguish the band of the C=O stretching of the esters at 1740 cm-1 from the amide I band of the bacterium. By recording the IR spectra by FTIR technique, we are able to observe that during the growth of S. lividans this vibrational band increases, proving the constitution of lipid vesicles.

A.




B.

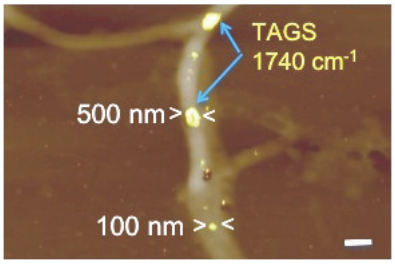



Figure : A. ATR-FTIR spectra of two Streptomyces wild strains (Lividans and coelicolor) compared to TAGs, B. Merging of AFM topography and AFMIR chemical mapping at 1740 cm-1 of S. lividans (the absorption wavenumber of esters C=O stretching, vibrational band typical of the TAGs).

From these results, a first study of the dynamics of lipid storage in Streptomyces in connection with the production of antibiotics was performed by IR nanospectroscopy AFMIR (1-3) on the three different strains mentionned above. This technique (Atomic Force Microscope technique coupled with a IR OPO laser tuned at the specific absorption wavelength of the TAGs) is an extremly useful quick and quantitative tool to monitor the constitution and/or degradation of storage lipids in different strains of Streptomyces that produce or not antibiotics. Indeed, considering the size of the vesicles (≈ 200nm) and their IR properties (C=O stretching of the esters at 1738 cm-1), the in vivo counting and determination of the size of these vesicles can only be easily done with our AFMIR technique.

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# AFMIR and Confocal Raman Microspectroscopy of Bacterial Endospores

Peter Lasch

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Rapid and objective characterization of microorganisms by spectroscopic techniques (IR, Raman, MS) is of increasing interest for applications in clinical microbiology, food monitoring, environmental research and also for detection and identification of highly pathogenic (biosafety level 3, BSL-3) microorganisms. In this context, the research group "*Biomedical Spectroscopy*" (P25) at the Robert-Koch-Institute (RKI) has initiated a series of studies in which MALDI-TOF MS and vibrational (IR, Raman) spectroscopy were used to establish spectral databases of highly pathogenic microorganisms, which allow - in combination with advanced methods of chemometrics - unambiguous identification at the genus, species and sometimes even at the strain level.

In all studies with BSL-3 microorganisms the main challenge was to test and optimize inactivation methods that ensure complete microbial inactivation, but permit at the same time comprehensive spectroscopic analysis of the microbial samples. Within the context of these efforts, our group has established a MALDI-TOF MS based microbial identification system for BSL-3 microorganisms in which inactivation is achieved by sample treatment with trifluoroacetic acid (TFA). The TFA inactivation procedure is now routinely applied for MS-based microbiological identification at the RKI's Center for Biosafety. Although mass spectrometry has been identified as a highly specific microbial identification technique, it has the drawback of a relatively low analytical sensitivity. For example, MS analyses require between $10^4$ and $10^5$ colony forming units (CFU) which frequently impedes direct analysis of environmental samples. Thus, for most analyses an additional time-consuming cultivation step is required. To avoid this cultivation step and to enable direct analysis of filed samples we have tested vibrational spectroscopic methods with the potential for single cell analysis, among them confocal Raman microspectroscopy (CRM) and the AFMIR technique. As in the case of MS, the applicability of CRM or AFMIR for identification of highly pathogenic microorganisms evidently depends on the availability of a reliable inactivation method that is compatible with the given spectroscopic modality. Within the context of these studies we have thus tested several inactivation methods among them inactivation by peracetic acid (PAA) and inactivation by gamma ray irradiation.

The results of these tests will be presented at the meeting. In the presentation we will show CRM and AFMIR spectra of viable and inactivated single bacterial endospores which were acquired at a lateral spatial resolution of less than 500 (CRM), or 100 nm (AFMIR). Furthermore, the spectra will be compared with respective far field IR and NIR FT-Raman spectra obtained from standard spore preparations. The presentation will additionally include a discussion of the pros, cons and trade-offs of the CRM- and AFMIR-based microbial identification techniques.

**VIII. Round table discussions**

**16:20-17:10** Round table 1- Near field approch to biologically-relevant case:where are the needs? What is the relevant scale? Volume samples? Imaging or spectroscopy ? Artifacts ? Ease of operation for biologists ? What level of skill is needed ?
**Chairmen: P. Lasch and P. Dumas**
**17:10-18:00** Round table 2 - Next steps in near-field microscopy: sources, devices
Chairmen: M. Tobin, A. Dazzi, V. Deckert
**18:00-18:30** Round table 3 - Discussion of where to discuss near-field experiments in the future ? (ICCAVS, CellNanoSpec20nn…) ?
Chairmen: P. Lasch, J.-M. Ortega

**WEDNESDAY 14 SEPTEMBER**

**IX. Infrared far-field microscopy impact in biology (2)**

***Chair : C. Hirschmugl***
**9:00-9:30** L. Vaccari-Invited - *SR-IRMS of living single cells in microfluidic devices*
**9:30-10:00** A. Turhan-Invited - *Characterization of spectral signatures of pluripotent and leukemic stem cells by FT-IR microspectroscopy*
**10:00-10:15** F. Fonseca - *Probing Live Cellular Responses to freezing by Infrared Spectromicroscopy*
**10:45-11:00** M.P. Andersson - *Coccolith polysaccharides examined by IR and Raman microspectroscopy*
**11:00-11:15** T. Wrobel - *Endothelial cells imaging with FT-IR-ATR spectroscopy*
**11:15-11:30** A. Engdahl - *Recent work at the Infrared Beamline 73 at MAX-lab*
**11:30-11:45** H. Byrne - *The EuroBioImaging ESFRI program*
**11:45-12:00** Chairmen of RT - *Report of round tables*
**12:00-12:30** Chairmen - Final discussion

## SR-IRMS OF LIVING SINGLE CELLS IN MICROFLUIDIC DEVICES

Lisa Vaccari 1, Giovanni Birarda 1, Gianluca Grenci 2, Luca Businaro 3
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Non-invasive, label-free analytical methodologies for real-time monitoring of living cells are the new frontier of cell-based assays, opening new opportunities to cellular biology. Fast, sensitive and non-radiation damaging, IRMS can now be operated at diffraction-limited spatial resolution achieving sub-cellular details, thanks to the high brilliance of IR-SR sources, but it is almost exclusively employed for studying fixed cellular samples. The well known spectroscopic constrains on working under physiological conditions are the main reason for that, joined with the poor expertise in manufacturing the most common IR transparent materials.
This talk will present the fabrication strategies for development of CaF2 microfluidic devices suitable for IRMS of living cells, developed by the scientists of the SISSI beamline at Elettra in collaboration with the micro-nanofabrication team at IOM-CNR. The report will be focused on the sealing step, which is making the difference between conventional demountable liquid cells and a Lab-On Chip (LOC) in the strict sense of the word.
Some selected examples from the SISSI in-house research activity will highlight the biological advantages of studies using living cells compared to those using fixed cellular samples. The possibility to effectively follow temporal biochemical rearrangements by monitoring characteristic spectroscopic features of living cells will be illustrated by the results obtained with both U937 monocyte apoptosis induction by CCCP (Carbonyl cyanide m-chlorophenyl hydrazone) and U937 chemokine stimulation by fMLP (n-formyl-Met-Leu-Phe).

However, the affirmation of SR-IRMS as a mature label-free cell-based tool is not free from hurdles that will also be highlighted in the presentation.

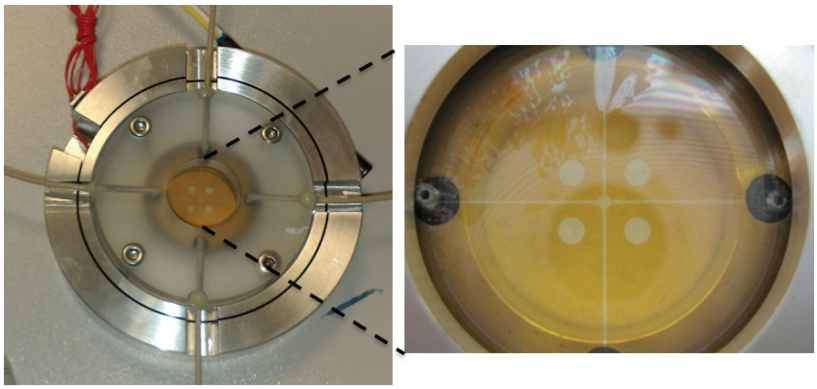

Figure 1: Example of a 3D microfluidic device made in XARP resist on CaF2 allocated within the chip holder, manufactured in order to allow i- the connections with syringe pumps through microcapillaries for supply/ waste of cells and chemical ii- the chip thermalization through an heater, made by a resistor embedded in an aluminium cylinder.

## « CHARACTERIZATION OF SPECTRAL SIGNATURES OF PLURIPOTENT AND LEUKEMIC STEM CELLS BY FT-IR MICROSPECTROSCOPY»

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## PROBING LIVE CELLULAR RESPONSES TO FREEZING BY INFRARED SPECTROMICROSCOPY

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One of the great challenges, presently, is to investigate the living cell behaviour of micro organisms at the nanoscale. For elucidating the molecular mechanism involved in their degradation in food products, near field infrared micros-spectroscopy would be the key experimental technique. This abstract is attending to provide the context in which such approach would be required.

Concentrates of lactic acid bacteria (LAB) are widely used as starters for manufacturing cheese, fermented milk, meat, vegetables, bread and health benefit products. Lactic acid bacteria (Lactobacillus delbrueckii subsp. bulgaricus) are small and non spherical sized (cylinder of 1 µm of diameter and 5-6 µm of length) cells Freezing and storage at low temperatures (-40°C) are common procedures to preserve the viability of concentrates while maintaining their technological properties upon thawing (acidification activity, production of aroma compounds, and contribution to product texture). But freezing is a critical step in the production of LAB concentrates, as it affects both the viability and acidification activity upon thawing. All cell systems do share common cryobiological responses described by the so-called Mazur's two-factor hypothesis. Two different processes are supposed to occur depending upon the rate at which the cooling ramping is performed.

At high cooling rates, cells are damaged due primarily to the intracellular ice formation At low rates of cooling, dehydration predominates, and cell damage is induced by osmotic injury due to solute effects. Optimization of cryopreservation requires thus a quantitative understanding of the biophysical response in the lactic acid bacterial cell during freezing.

Our previous works have showed the effect of the composition of the extra-cellular medium and the freezing kinetics on the degradation of lactic acid bacteria (Fonseca et al,.,2006). Furthermore by using transmission electronic microscopy, we have demonstrated that freezing injury at high cooling rates cannot be ascribed to the formation of intracellular ice but to cell plasmolysis occurring during thawing. Lactic acid bacteria survival during freezing application is thus highly dependent on the cellular biophysical event of cell dehydration. Importantly, cell membrane appears as one of the most critical site for cell injury, since it has been established that cooling alters the physical state of lipids, thus altering lipid organization and membrane fluidity (Balasubramanian et al., 2009, Oldenhof et al., 2010).

In situ and in conditions close to the industrial ones, FTIR spectroscopy has allowed investigating lipid phase transition of fresh and dehydrated LAB (Oldenhof et al. 2005). Modifications of the physical state of bacterial lipids of whole cell populations of Lactobacillus delbrueckii subsp. bulgaricus during freezing and thawing were recently characterized for two different physiological states, corresponding to different behaviours in terms of resistance to freezing and thawing processes.

A pioneer work on LAB has been recently conducted by using the synchrotron infrared beamline SMIS (Synchrotron SOLEIL), with a high reflective index hemisphere (ZnSe) for investigating the chemical response of some individual lactic acid bacterial cells. The aim of this study was to quantify the population heterogeneity and the individual cell biophysical and chemical response. ATR analysis of bacterial suspension dehydrated made possible the mapping of individual or at least a small group of cells. The impact of freezing conditions on cell biophysical behaviour was investigated by analysing samples before and after each freezing and storage conditions (data treatment in progress)

When studying bacterial suspensions in real time freezing–thawing processes, water absorption bands make extremely difficult the identification and quantification of protein conformational changes. AFMIR experiences, could make possible this challenge of chemical imaging living LAB in vivo, with spatial resolution of the order of the size of cell components (Mayet et al., 2008), .

Such approach should enable to differentiate the dynamic behaviour (during the process) of cell membrane from the cytoplasmic components (AND, proteins, …). To meet this challenge, a promising emerging in situ technique combines a classical infrared approach with a brilliant synchrotron and an appropriate microfluidic platform (Holman et al 2009), that should, in our case, allow freezing . As AFMIR can perform nanoscale analysis and imaging in solution, combining AFMIR with a microfluidic device would be tremendously potential for such study.

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## COCCOLITH POLYSACCHARIDES EXAMINED BY IR AND RAMAN MICROSPECTROSCOPY

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Coccolithophores are single-cell marine algae that surround themselves with biomineralized calcite platelets called coccoliths, Fig 1. In cultured samples, they are known to have polysaccharides associated with them. The polysaccharides that the algae produce are thought to be unique to each species and there is a multitude of species of coccolithophores alive today. Chalk is composed of the remains of ancient coccoliths such as Fig. 2. This sample contains the remnants of algae that lived 60 million years ago. This project was aimed at investigating similarities and differences in the polysaccharide composition for the modern and ancient samples, in an attempt to see if particular functional groups are consistently present.

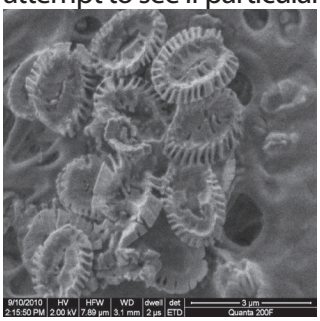

**Figure 1. Scanning electron microscopy (SEM) image of coccoliths from *G. Oceanica*.**

We have used infrared and raman microspectroscopy to study three species of cultured coccoliths and compared the results with those from fossil coccoliths extracted from samples of white chalk. We used epoxy to glue single samples to the end of an atomic force microscopy cantilever. IR spectroscopy identified organic material on all three species of the cultured coccoliths and the spectra were consistent with polysaccharides. We were unable to extract further details about the identity of the organic material from the data. Present day coccoliths are very small compared to those produced by species that lived during the Cretaceous.

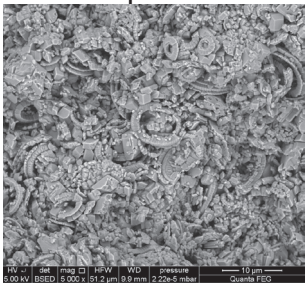

**Figure 2. SEM of chalk from the North Sea.**

On the fossil samples, Raman microspectroscopy confirmed the presence of organic material, but no further identification of the compounds was possible from either Raman or IR spectroscopy. The size of the ancient coccoliths (~5 µm) induced severe scattering for the infrared spectra in the 600-2000 cm-1 region, making identification from the fingerprint region impossible. We tried applying a Mie scattering correction with two

different calcite reference spectra but no significant improvements could be achieved.

We then proceeded by dissolving the coccoliths on a silicon nitride window in deionized water and studied the residue using IR microspectroscopy. The spatially resolved infrared spectra at the micrometer scale allowed for monitoring different points on the dissolved sample. We were able to measure at several snapshots along the path toward complete dissolution, from intact to fully dissolved coccoliths as determined by the intensity of the asymmetric carbonate stretch vibration for calcite. Part of the polysaccharides associated with the coccoliths is insoluble in deionized water and the dissolution procedure concentrates this insoluble material in some areas on the sample window. This made it possible to obtain high quality infrared spectra of the undissolved residue from all three species of cultured coccoliths. This procedure also made it possible to dissolve an ancient coccolith and obtain an infrared spectrum from its residue.

The residue from the dissolved coccoliths is similar, in that they contain highly methylated polysaccharides. This helps explain their low solubility in water compared with non-methylated polysaccharides, because many hydrophilic -OH groups have been replaced by more hydrophobic -OCH3 groups. There are important differences in composition between the three species of cultured coccoliths. In addition to alcohol hydroxyls and ethers from methylation, E. Huxleyi coccoliths contain a large amount of sulfate esters. P.Cartereae coccoliths contain a high proportion of carboxylic acids. We could identify no such functional groups on the G. Oceanica coccoliths, but they also have noticeably less polysaccharide than the other two species. Our results confirm that polysaccharide composition is highly species dependent, consistent with the broad variety of environments occupied by coccolith producing algae. The similarities in the chemical signature between the organic material from fossil coccoliths in chalk and that of E. Huxleyi is remarkable. It shows that the bond between the calcite surface and a highly methylated polysaccharide including a large amount of sulfate esters is stable enough to have survived, at least to some extent, in the geological record for more than 60 million years.

We used IR and Raman microspectroscopy to conclude that cultured coccoliths of the E. Huxleyi could serve as a very good model system to represent the fossil coccoliths in chalk. This is particularly true with respect to the type of surface interaction that binds the polysaccharides to the calcite surface.

## ENDOTHELIAL CELLS IMAGING WITH FT-IR-ATR SPECTROSCOPY

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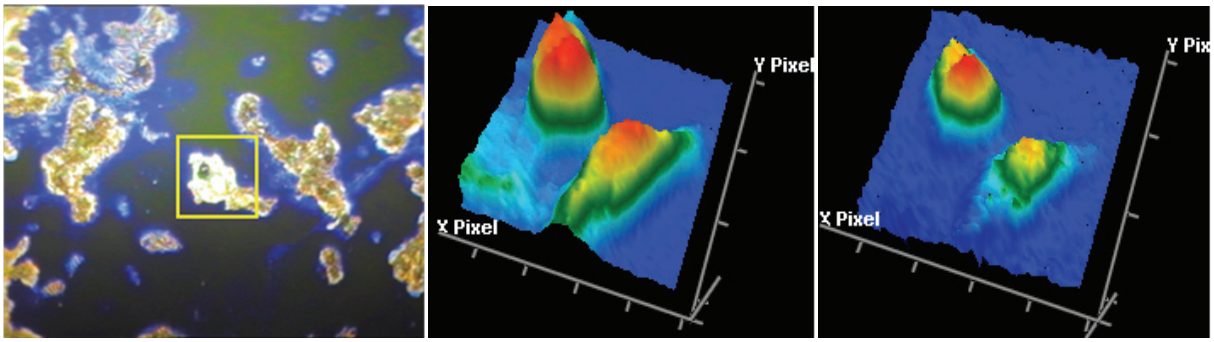
Endothelium, the multifunctional organ building the blood-tissue barrier, is involved in regulation of vascular tone, thrombosis and inflammation of the vascular wall and its malfunction leads to many western world diseases, such as atherosclerosis or diabetes. Today, there is the overwhelming evidence indicating that endothelium by means of various endothelial mediators (e.g. nitric oxide (NO), prostacyclin (PGI2), carbon monoxide (CO), epoksyeikozanotrienoic acids (EETs) defends against vascular inflammation and thrombosis, and protects against the development of atherothrombosis, diabetes and other cardiovascular diseases, while dysfunctional endothelium which is associated with the impairment of the activity of vasoprotective mediators and promotes vascular inflammation and thrombosis leading to cardiovascular diseases. Although there is heterogeneity of endothelium in various organs in general, endothelial cells are flat with a nucleus located centrally, and are about 1-2 μm thick and 10-20 μm in diameter.
In order to study the state of the endothelium in the vascular wall, first a model of cell response to different dysfunctional factors (e.g. TNF-α) needs to be developed. This problem may be addressed

by investigation of cell cultures with FT-IR spectroscopy. The spatial resolution of transmission and reflection modes is approximately 10 µm, which is comparable to the size of the cells. Furthermore, Attenuated Total Reflection (ATR) mode increases the spatial resolution (2-4 µm), but can be used to probe only outer parts of the cells, since the penetration depth is limited up to 2 µm.
The visible picture of the HMEC cell culture (4x magnification) with the labeled (yellow) investigated area




(left) is compared with 3D IR images constructed by integration of the Amide I band (middle) and the phosphate band at 1240 cm-1 (right).
Application of ATR imaging to cell cultures studies has already been reported by Kuimova et al. [1], but surprisingly this technique has not been used very often. As a preliminary results, we demonstrate FT-IR-ATR imaging of human microvascular endothelial cell line (HMEC) grown on the poly-L-lysine layer that shows the ability of this technique to detect a single cell signal. Moreover, a TNF-α treated cell culture is compared with control and spectral changes are reported. Most prominent differences are due to the phosphate band at 1240 cm-1.
The work was performed on the Varian 620-IR system with 128x128 FPA coupled to a 670-IR spectrometer dedicated for fast imaging, with a Ge crystal for ATR imaging.

Acknowledgements: This work was supported by the European Union from the resources of the European Regional Development Fund under the Innovative Economy Programme (grant coordinated by JCET-UJ, No POIG.01.01.02-00-069/09).
[1] M. Kuimova, K. L. A. Chan and S. Kazarian, Chemical Imaging of Live Cancer Cells in the Natural Aqueous Environment, Applied Spectroscopy, Volume 63, Number 2, 2009

## TRACKING CARBON AND NITROGEN ISOTOPE INCORPORATION INTO ALGAL CELLS WITH SYNCHROTRON INFRARED MICROSPECTROSCOPY

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Carbon uptake by algal cells is a critical mechanism of energy input into aquatic ecosystems, and an important pathway for carbon dioxide (CO2) sequestration from the atmosphere. Conversion of inorganic nitrate to protein is influenced by the nutrient availability. Knowing which algal species within a community can best utilize available NO3- and CO2 can help to predict how species alterations caused by environmental changes will alter input of these nutrients into foodwebs, and help refine species selection for maximum nutrient uptake and carbon retention in bioengineering applications. We used synchrotron infrared microspectroscopy to determine which species in a natural algal community incorporated added labeled inorganic isotope of 15N and 13C. Amide II bands show a response to the 15N isotope after four days and two phytoplankton species that rely solely on CO2 as a carbon source showed strong reduction in infrared absorption band intensity within

the amide I, methyl, and methylene groups. Relative concentrations from protein and carbohydrate band frequencies for labeled and unlabeled treatments of algal cells are presented and discussed. Synchrotron infrared microspectroscopy provides a method to track which algal cells within a natural, mixed-species community are incorporating the added nutrients.

## RECENT WORK AT THE INFRARED BEAMLINE 73 AT MAX-LAB.

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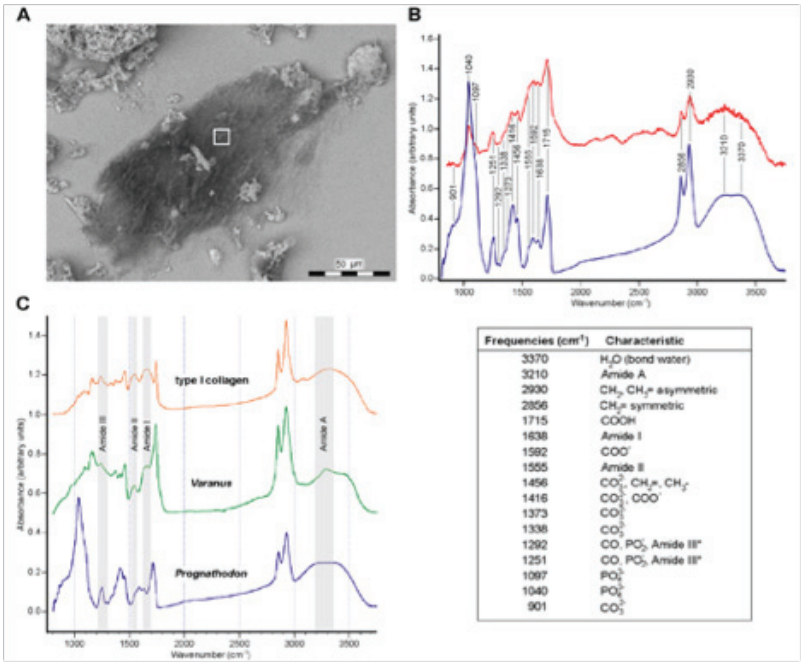
Endogenous Proteins Found in a 70 Million Year Old Giant Marine Lizard.
Fossil – just stone? No, primary biological matter have been discovered in a fossil of an extinct varanoid lizard (a mosasaur) that inhabited marine environments during Late Cretaceous times. Using state-of-the-art technology, it has been possible to link proteinaceous molecules to bone matrix fibres isolated from a 70-million-year-old fossil; i.e., genuine remains of an extinct animal entombed in stone have been found.

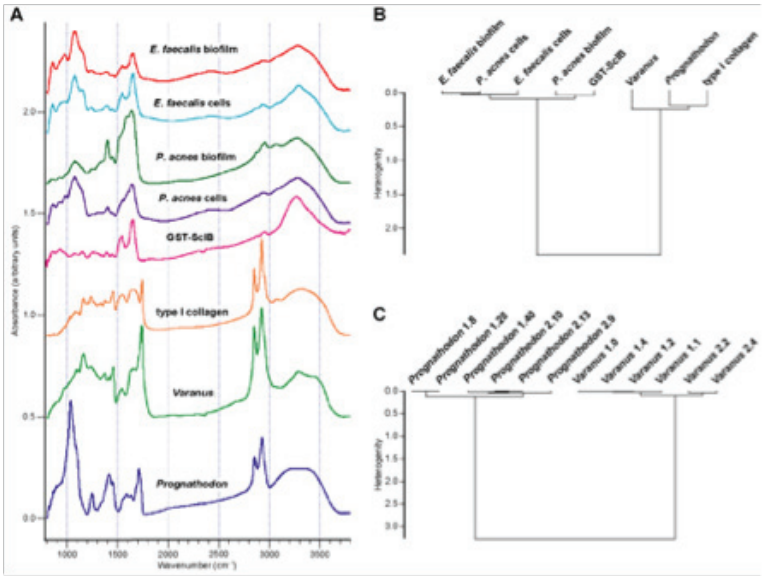
With the discovery, it is demonstrated that remains of type I collagen, a structural protein, are retained in a mosasaur fossil. Methods employed are e.g., synchrotron radiation-based infrared microspectroscopy at MAX-lab in Lund to show that amino acid containing matter remains in fibrous tissues obtained from a mosasaur bone. Previously, other research teams have identified collagen-derived peptides in dinosaur fossils based on e.g., mass spectrometric analyses of whole bone extracts. The present study provides compelling evidence to suggest that the biomolecules recovered are primary and not contaminants from recent bacterial biofilms or collagen-like proteins. Moreover, the discovery demonstrates that the preservation of primary soft tissues and endogenous biomolecules is not limited to large-sized bones buried in fluvial sandstone environments, but also occurs in relatively small-sized skeletal elements deposited in marine sediments.

Facts: :
Mosasaurs are a group of extinct varanoid lizards that inhabited marine environments during the Late Cretaceous (approx. 100-65 million year ago).


Collagen is the dominating protein in bone.
A broad spectrum of sophisticated techniques have been applied to achieve the results. In addition to synchrotron radiation-based infrared microspectroscopy, mass spectrometry and amino acid analysis have been performed. Almost all experiments have been made in Lund. At MAX-lab, the experiments have been conducted at the MAX I ring, beamline 73.




Infrared microspectroscopy of fibres isolated from a mosasaur bone.
(a) SEM-picture of the fibres. The white square marks the area measured by synchrotron light. (b) Absorbance spectra from the fibre bundle reproduced in a (red=synchrotron light; blue=conventional light). (c) Comparison between absorbans spectra from recent typ I collagen, osteoid from a recent monitor lizard (varan), and fibres from a mosasaur (Prognathodon).




(a) Comparison between absorbance spectra from two modern bacterial biofilms, isolated bacteria cells, a bacteria protein, typ I collagen, osteoid from monitor lizard, and fibres from a mosasaur (Prognathodon). (b) Dendrogram that show among other things the similarity i the spectra from typ I collagen, osteoid from a monitor lixard, and fibres from the mosasaur bone (Prognathodon). (c) Dendrogram showing how homogenic different fibre bundles from the monitor lizard and the mosasaur samples are.
Complete paper: http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0019445
About MAX-lab:
MAX-lab is a synchrotron light facility and a part of the MAX IV Laboratory. The MAX IV Laboratory is a national research laboratory comprised of the present MAX-lab and the MAX IV project. It is run by Lund University and the Swedish Research Council, and is situated in Lund, Sweden. www.maxlab.lu.se

# IMAGING LIVE CELLS GROWN ON A THREE DIMENSIONAL COLLAGEN MATRIX USING RAMAN MICROSPECTROSCOPY

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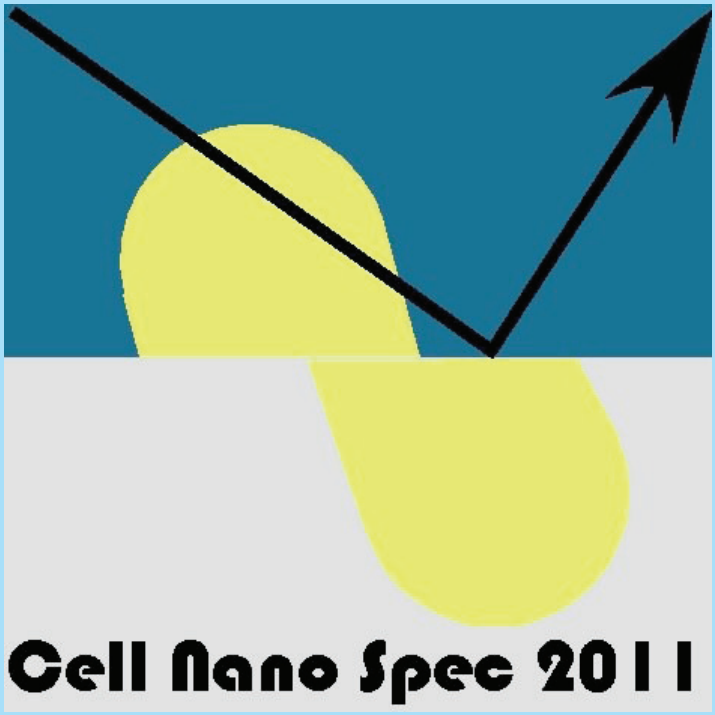
Abstract
The potential of Raman spectroscopic microscopy for the analysis of biological systems and disease diagnostics is well established. Using optical wavelengths, its potential reaches further to sub cellular analysis of biochemical function on a molecular level, and changes to that function as a result of disease or external agents such as radiation, chemotherapeutic agents or toxicants. While analysis of fixed cells has demonstrated the potential of such hyperspectral imaging, live cell imaging remains a challenge.
In this study, live cell imaging in three dimensional collagen gels by Raman spectroscopy is demonstrated. The study is conducted on a human lung adenocarcinoma (A549) and a spontaneously immortalized human epithelial keratinocyte (HaCaT) cell line. The lateral resolution of the system has been estimated to be <1.5 µm making it possible to access the subcellular organization. Using K-means clustering analysis, it is shown that the different subcellular compartments of individual cells can be indentified and differentiated. The biochemical specificity of the information contained in the Raman spectra allows the visualization of differences in the molecular signature of the different sub-cellular structures. Furthermore, to enhance the chemical information obtained from the spectra, principal component analysis has been employed, allowing the identification of spectral windows with a high variability. The comparison between the loadings calculated and spectra from pure biochemical compounds enables the correlation of the variations observed with the molecular content of the different cellular compartments.
The study paves the way for real time spectroscopic analyses of cellular metabolism in a 3D environment.
Keywords: Raman Spectroscopy; Live cell imaging; 3D Collagen gels, K-means clustering, Principal Component Analysis


Cell Nano Spec 2011