Biological
Applications of
Synchrotron
Infrared in
Europe

1st BASIE Workshop

Karlsruhe, 11th - 12th September 2003

Programme
Abstracts
List of Participants

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Programme

Wednesday 10 September

14:00-18:00 Executive board meeting - open to all
20:00 Welcome reception and registration at the Hotel Löwen

Thursday 11 September

Welcome address

09:00-09:05 **Dr. Peter Fritz, Executive Board of the Forschungszentrum Karlsruhe**

Chairman's opening address

09:05-09:30 **David Moss, ANKA, Karlsruhe, Germany**
What biologists need to know about synchrotrons - and what beamline scientists need to know about biology

Session 1: Synchrotron infrared radiation

09:30-10:00 **Ulrich Schade, BESSY, Berlin, Germany**
Spectroscopy and near-field microscopy at BESSY using coherent THz synchrotron radiation

10:00-10:30 **Yves-Laurent Mathis, ANKA, Karlsruhe, Germany**
Synchrotron IR spectroscopy and microscopy with edge radiation at ANKA

10:30-11:15 Coffee break and poster session/trade show

11:15-11:45 **Oleg Chubar, SOLEIL, Saclay, France**
Wave-optics methods for infrared beamline simulation / optimization

11:45-12:15 **Claudio Marcelli, DAPHNE, Frascati, Italy**
Development of custom spectroscopy instrumentation for synchrotron infrared beam lines

12:30-13:30 Lunch

Session 2: Biomolecules and biomembranes

13:45-14:15 **Marco Colombatti, University of Verona, Italy**
Characterization of redox systems in RIPs-II dependent cell intoxication mechanisms

14:15-14:45 **Andreas Barth, University of Stockholm, Sweden**
Infrared spectroscopy as a tool to study the binding of molecules to proteins - ATP binding to the Ca^{2+}-ATPase as an example

14:45-15:15 **Gianfranco Menestrina, CNR-ITC, Institute of Biophysics, Trento, Italy**
Investigation of amyloid peptides configurations by FTIR and synchrotron radiation

15:15-15:45 **Pascale Roy, LURE, Orsay, France**
Reverse micelles and lamellar systems - new tools for studying the role of water in biological systems

15:45-16:30 Coffee break and poster session/trade show

16:30-17:00 **Karsten Hinrichs, ISAS, Berlin, Germany**
FT-IR ellipsometry for studying organic layers

17:00-17:30 **Alessandro Paciaroni, Univeristy of Perugia, Italy**
The nature of low-frequency vibrations in proteins

17:30-18:00 **Erik Goormaghtigh, Free University of Brussels, Belgium**
Hydrogen deuterium exchange in membrane proteins monitored by FTIR spectroscopy: new tools to resolve protein structure and dynamics

18:15 Bus transfer to the hotels

19:00 Bus transfer from hotels to Ettlingen

19:30 Short stroll through Ettlingen to the Workshop Dinner

Programme continued

Friday 12 September

Session 3: Cells, tissues and diagnostics

09:00-09:30 **Peter Lasch, Robert Koch Institute, Berlin, Germany**
Detection of protein structural changes in prion-infected tissue

09:30-10:00 **Michel Manfait, University of Reims, France**
Characterization of cells and tissues by optical microspectroscopy

10:00-10:30 **Max Diem, City University of New York, USA**
High resolution spectroscopy of living cells with a conventional IR source - how good is the competition?

10:30-11:00 Coffee break and poster session/trade show

11:00-11:30 **Mark Tobin, SRS, Daresbury, UK**
Synchrotron infrared microscopy of cancer cells in whole tissues and in tissue culture

11:30-12:00 **Ioanna Anastassopoulou, National Technical University of Athens, Greece**
Micro-FT-IR studies of human bones

12:30-13:00 **Marine Cotte, Research Laboratory of the Museums of France, Paris, France**
Applications of synchrotron IR microscopy on archaeological cosmetics and biological samples

12:45-13:45 Lunch

Special concluding address

14:0 0-14:45 **Hoi-Ying Holman, Lawrence Berkeley National Laboratory, USA**
Biological applications of synchrotron IR at ALS - what can Europe learn from the Californian experience?

14:45-15:15 Concluding remarks and end of the meeting

15:15 Bus transfer to the train station

15:30-16:00 Coffee break

16:00-17:45 Optional tour of ANKA

18:00 Bus transfer to the hotels and the train station

Posters

P1: **Michael Gensch, ISAS, Berlin, Germany**
Instrumentation for FT-IR synchrotron ellipsometry

P2: **Luca Quaroni, ELETTRA, Trieste, Italy**
The new IR beamline at ELETTRA

P3: **Josefa Rodriguez Baena, Technical University of Vienna, Austria**
Synchrotron IR monitoring of chemical events in microfluidic mixing devices and CE microchips

P4: **Mariangela Cestelli Guidi, University of Rome "La Sapienza", Italy**
First experiments at SINBAD, the infrared beamline at DAFNE

P5: **Bojidar Jordanov, Bulgarian Academy of Sciences, Sofia, Bulgaria**
Measurement of infrared circular dichroism by means of totally reflecting retarders

P6: **Rainer Pepperkok, EMBL, Heidelberg, Germany**
Synchrotron IR spectroscopy of single living human cells at ANKA-IR

P7: **P. Mariani, University of Marche, Ancona, Italy**
X-ray and Neutron Small Angle Scattering shape analysis in assessing protein structures obtained by computational prediction techniques

Abstracts

What biologists need to know about synchrotrons - and what beamline scientists need to know about biology

D. A. Moss

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With the sequencing of the human genome completed, the life sciences are entering a new era. Relationships between sequence, structure and function of gene products, their mechanisms of action at the cellular and tissue levels, and their role in health and disease are the new issues.

FTIR spectroscopy is emerging as one the key tools in this area, particularly for its ability to provide structural-functional information on biological processes at multiple levels of organization - from individual atomic bonds to global protein folding and conformational changes, from single living cells in culture to tissue studies and diagnostics.

Synchrotron-based FTIR spectroscopy, with its extended spectral range and high spatial resolution, brings such studies to the cutting edge. The new era of life science research coincides with an unprecedented period of development in European synchrotron infrared capabilities, with a total of 8 new facilities recently opened, under construction or planned in France, Germany, Italy, Switzerland and the U.K.

The aim of the BASIE Network is to bring infrared beamline scientists from European synchrotron facilities together with European life sciences expertise, in order to promote the application of synchrotron-based FTIR spectroscopy to current issues in biological and biomedical science.

In this introductory address, I will describe the outline, aims and scope of this 1st BASIE Workshop, and will discuss how we can get synchrotron physicists, spectroscopists and biologists to pool their expertise, understand each other and work synergistically.

Spectroscopy and near-field microscopy at BESSY using coherent THz synchrotron radiation

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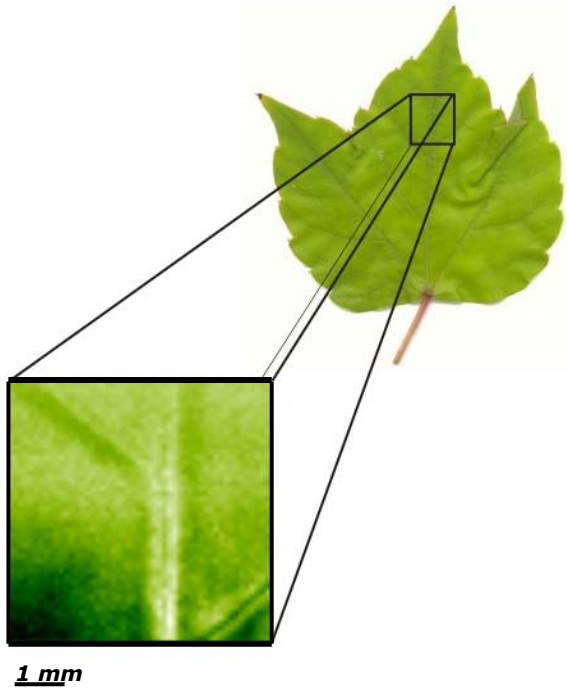
The recent reports on a new brilliant coherent far infrared broadband source at BESSY have found considerable international interest. The spectral range at around 1 mm wavelength (0.3 THz) which can be only poorly accessed by conventional sources is now covered by operating BESSY in a special machine mode. Here, up to 10^8 more brilliance than from a black body source has been achieved. At least for the next few years BESSY will remain the only synchrotron radiation source worldwide being able to offer that brilliant light within this spectral range. Other storage rings e.g. CIRCE (Coherent Infrared Center) at the ALS (Berkeley) are planned but, by now, not yet funded. Other sources like radiation from a LINAC at Jefferson Lab could demonstrate high power pulsed radiation as expected from LINAC bunch compressors, but they are not yet operating a far infrared user facility.

Recent results at BESSY using coherent synchrotron radiation demonstrated that the radiation source is stable and covers the spectral range between 2 and 60 cm^{-1} showing a gain in brilliance up to 10^5 compared to the normal incoherent Synchrotron radiation in a stable mode and a gain of 10^8 in a pulsed mode (single bunch at a repetition rate of 1.25 MHz).

The first mode being feasible for *Fourier transform IR spectroscopy* was already successfully used for High-T_c-superconductors, semiconductors and living biological tissue. Since the wavelength range covers collective modes of biological objects such as in



Near-field image of a *parthenocissus* leaf (130 µm spatial resolution).

polymembranes or heart cells, new scientific results in these fields can be expected.

Using the second mode *Near Field Spectro-Microscopy* in the sub-cm and mm wavelength range down to resolutions of 1/38 of the wavelength was established at BESSY for the first time at a storage ring source. Strongly absorbing and 'wet' biological samples can be imaged here as demonstrated by the figure showing a transmission near field image acquired at 10 cm^{-1}. One can clearly recognize the inner structure of veins in a living leaf as cut from a bush few minutes before the measurement.

Encouraged by the above results, we are heading for a new THz end station upgrading the existing infrared beamline IRIS. The radiation shall be accepted by an additional pre-mirror being focused through a large aperture quartz window in order to overcome diffraction losses into a Martin-Puplett spectrometer with a resolution < 0.1 cm^{-1}.

Synchrotron IR spectroscopy and microscopy with edge radiation at ANKA

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ANKA is a new synchrotron radiation facility at the Forschungszentrum Karlsruhe, a large government research center in the southwest of Germany. The acronym stands for Angstrom Source Karlsruhe. The electron storage ring is 110.4 m in circumference and was designed to store a 2.5 GeV electron beam at a nominal current of 400 mA. The machine has been in operation since September 2000 and is now running routinely at full energy and 200 mA current.

On modern synchrotron light source optimized for the production of x-rays, extraction of infrared radiation from a dipole poses a particular challenge: the natural vertical divergence of synchrotron radiation emitted from particles circulating in the constant magnetic field part of a bending magnet increases with the wavelength of the radiation, while the maximum acceptance angle for extraction of the radiation is limited by design constraints.

The solution to this conflict implemented at the ANKA-IR beamline is to use edge radiation rather than conventional bending magnet radiation as the source. Edge radiation is emitted as the electrons enter and leave a magnetic field, and is characterized by a much tighter spatial distribution than bending magnet radiation. This makes it possible to extract all of the radiation across the entire infrared range down to 100 cm^{-1} through a vertical acceptance angle of only 15 mrad: with a conventional bending magnet source, the natural divergence would exceed this acceptance angle at frequencies below 2500 cm^{-1}.

In this presentation we will detail the beamline design and experimental set-ups, compare the theoretical predictions and measurements of the photon beam characteristics, and report on the most recent experiments performed.

References:

[1] A. Boris et al, *Phys. Rev. Lett*. **89**, 277001 (2002)

[2] Y.-L. Mathis, B. Gasharova and D. A. Moss, *J. Bio. Phys*. **29**, 313-318 (2003)

Wave-optics methods for infrared beamline simulation / optimization

O. Chubar

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An overview of the main features of spontaneous infrared synchrotron radiation, including the emission from central parts and from edges of bending magnets, will be presented.

After this, the basic wave-optics principles of wave front propagation, as well as their application to synchrotron radiation, will be discussed.

The wave-optics approach is particularly useful for the simulation of wave fronts from diffraction-limited sources, because it takes into account all special features of the emitted radiation, and treats correctly the diffraction and interference phenomena, as well as wave aberrations, taking place in a beamline.

This enables realistic predictions of radiation intensity distributions at different longitudinal positions of the beamline (including waists and wide wave front parts), and allows for various beamline optimizations. A number of numerical simulation examples of wave front propagation through optical schemes corresponding to realistic infrared beamlines, which are currently in operation and/or planned in second- and third-generation synchrotron radiation sources (Super-ACO, ANKA, ESRF, SOLEIL) will be presented.

Development of custom spectroscopy instrumentation for synchrotron infrared beam lines

A personal view of what has been done and what we have to do to match IRSR source properties and new experimental demands

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In the last few decades important developments, in particular those connected with Fourier transform spectroscopy, enabled one to improve the spectroscopic methods in the infrared region of the spectrum for basic and applied research in physics, chemistry, biology and medicine. Fourier-transform spectroscopy requires broad-band sources and synchrotron radiation emission not only fulfills the requirement, it exhibits a high brilliance over a "white" spectrum, polarization properties and the absence of thermal fluctuations.

Synchrotron radiation sources are present all around the world and nowadays there are more than fifteen dedicated IRSR beamlines all around the world. Most of them have been installed in the USA in a context of growing interest of industrial users in particular for infrared microscopy. With their high-brilliance, polarized and broad-band radiation one may perform experiments that are out of the range of conventional sources from the near-IR up to the far-IR range. Moreover, modern spectroscopies and applications demand for high source stability, like in differential spectroscopy, and/or a small spot, as in infrared microspectroscopy and in experiments at high-pressure.

The first pioneering observation of the IR emission was performed with basic commercial instrumentations using (small in term of solid angle) existing exit ports originally designed for xray. Following attempts were performed at UVSOR, BESSY and NSLS collecting large solid angles, still using commercial FT interferometers. Special attentions to the development of optical systems to transfer the synchrotron radiation emission to the instrumentations installed at the end of beamlines and the first accurate analysis of the IRSR source properties were also perfomed.

Only in 90's new IRSR beamlines were built taking advantage of accurate ray tracing simulations, as usually performed in x-ray beamline design, and of new optical systems specifically designed and tested for IR applications (e.g., CDV wedged diamond windows, supermirrors, etc.,). At that time also up-to-date theoretical analysis of the IR emission including the edge emission become available.

A standard IRSR experimental apparatus includes a Michelson interferometer (in some facility suitably modified for vacuum operation), an infrared microscope, and several IR detectors to cover the entire domain. However almost none of the existing facility developed dedicated instrumentations for IRSR, as a consequence important improvements and benefits are expected from optimized design of interferometers and detectors for IR applications in the mid and far IR range.

Among the several properties of the synchrotron radiation emission, that holds true also for the IR domain, we have to underline here the time structure. Actually, the pulsed structure of IRSR represents a feature of particular relevance for experiments in the IR domain. Light pulses are suitable to investigate time-dependent phenomena in different systems on a very short time scale. In addition to time resolved and/or Fast-scan spectroscopy, IRSR can be used for pump-probe experiments using standard FTIR spectroscopic techniques. Nowadays time resolved Fourier Transform spectroscopy may be (partially) applied in the mid-IR region while it will be fully exploited at far infrared wavelengths only when fast detectors will be available (typical cut-off frequency of a liquid-helium-cooled bolometer is presently 300 Hz) opening new fields of application to spectroscopists.

A comprehensive (but certainly partial) review of what have been done will be presented and discussed outlining issues were improvements are possible (and strongly required by experiments).

Characterization of redox systems in RIPs-II dependent cell intoxication mechanisms

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Verona, Italy

Many toxins are heterodimers composed of a binding subunit B covalently linked by a disulfide bond to the toxic enzymatic subunit A. This group of A-B toxins includes bacterial toxins such as shiga toxin, diphtheria toxin (DT), pseudomonas exotoxin A (PEA), cholera toxin (CT) as well as the plant toxin ricin. Ricin chain A (RTA), the catalytic subunit of ricin, can be chemically cross-linked with a disulfide bridge to a vehicle molecule (i.e. antibody, growth factor, cytokine) obtaining Immunotoxins (IT), a new class of pharmacologic agents that find application in human diseasesFollowing binding at the cell surface and endocytosis ricin travels backwards along the secretory pathway to the Golgi apparatus and endoplasmic reticulum (ER) where it is unfolded, the inter-chain disulfide bond is reduced and the enzymatic subunit (RTA) is released and transferred across the ER membrane to the cytosol where it inhibits protein synthesis at the ribosomal level. Although a role of Protein Disulfide Isomerase (PDI) has been proposed in these processes, its involvemenet in the mechanism of ricin reduction and dislocation to the cytosol still needs to be elucidated. In eukaryotic cells two main systems operate the reduction of protein disulfide bonds: the GSH system where Glutaredoxin (GrX) acts in concert with NADPH and GSH and the Thioredoxin system in which Thioredoxin (TrX) operates together with Thioredoxin Reductase (TrXR) as an NADPH-dependent protein disulfide reductase. The redox state and activity of TrX are in turn controlled by TrXR.

We studied the role of PDI and thioredoxin systems in reducing inter-chain disulfides of ricin and RTA-based immunotoxins. We will here present evidence that: (i) ricin can be reduced to RTA and RTB by DTT pre-activated PDI or more efficiently by pre-incubating PDI with TrXR in the presence of NADPH; (ii) GSH is not essential in reducing ricin to RTA and RTB; (iii) reduction of disulfide in ricin and in RTA-based ITs can be efficiently carried out by the NADPH-dependent TrX/TrXR system; (iv) the kinetcs of disulfide reduction are only slightly accelerated by PDI; (v) TrXR activity plays a pivotal function both in vitro and in vivo and (vi) the efficiency of inter-chain disulfide bond cleavage of ricin is much greater than that of ITs. Taken together, our results suggest that TrXR plays a central role in modulating TrX and PDI thiol-disulfide oxidoreductase activities and that proteins involved in the reduction of inter-chain disulfides of toxins must be further characterized in different sub-cellular organelles.

Infrared spectroscopy as a tool to study the binding of molecules to proteins - ATP binding to the Ca^{2+}-ATPase as an example

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Infrared spectroscopy emerges as a new marker-free technique for drug optimisation. It provides more than just a "binding" or "no-binding" answer: Also monitored are the change of protein conformation upon binding and the environment of molecule and side chains in the binding pocket. Even large membrane proteins can be investigated at "atomic resolution".

As an example, binding of the substrate ATP to the Ca^{2+}-ATPase has been studied. The conformational changes of the protein depend in a characteristic way on the structure of the substrate molecule, as shown for several ATP analogues (ADP, ITP, 2´-dATP, 3´-dATP). This mapping of the binding site identified those groups of ATP that interact with the protein.

Investigation of amyloid peptides configurations by FTIR and synchrotron radiation

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[2]Dip. di Fisica "E. Amaldi", Università di Roma Tre, Rome, Italy
[3]Insto Química-Física "Rocasolano", CSIC, Madrid, Spain
[4]European Molecular Laboratory, c/o DESY,Hamburg, Germany
[5]Dip. di Fisica, Università di Roma "Tor Vergata" and INFM, Rome, Italy

Alzheimer and prion disease are distinct in clinical and neuropathological aspects, but may be based on a similar molecular event, the formation of an altered protein conformer that self-aggregates into long fibers of predominant β-structure. In both cases, a role of metal ions, in particular Cu(II), has been envisaged for the normal function and for the degeneration.

Both the Alzheimer's amyloid beta peptide (Aβ) and the prion protein (PrP), contain histidines that may be involved in specific binding of divalent metal ions like Cu and Zn. In the case of human PrP, four sequential copies of a conserved octarepeat sequence (PHGGGWGQ) are present in the N-terminal region. In the case of Aβ peptides three histidines appear in both pathogenic forms, $A\beta^{40}$ and $A\beta^{42}$, which derive from the proteolytic cleavage of the amyloid precursor protein (APP). We constructed synthetic peptides corresponding to the first 16, 28, 40 and 42 residues of the Aβ peptide (called $A\beta^{16}$, $A\beta^{28}$, $A\beta^{40}$ and $A\beta^{42}$ respectively), or formed by a variable number of PrP octarepeats (1, 2 or 4 repeats in the peptides called PrP-P1, -P2 and -P4 respectively) and a hydrophilic flanking motif. Fourier transform infrared spectroscopy (FTIR) was used to investigate their structure, in particular via the amide I absorption band.

Amyloid peptides. Precipitation experiments indicated that $A\beta^{42}$ and $A\beta^{40}$ form aggregates, whereas the shorter $A\beta^{28}$ and $A\beta^{16}$ do not. Furthermore, $A\beta^{42}$ and $A\beta^{40}$ adopt the characteristic beta-sheet configuration whereas $A\beta^{28}$ and $A\beta^{16}$ have increasingly different configuration. Cu induces a conformational change of all peptides, rearranging internal hydrogen bonds at least in a portion of the peptide, and slightly promoting aggregation. Zn instead has a quite smaller effect on peptide conformation, but a much stronger effect on aggregation. We hypothesize that Cu undergoes mainly intrapeptide binding (with larger conformational change) whereas Zn promotes interpeptide binding (with larger aggregation). The binding site is in the N-term half of the peptide, as a

peptide containing residues 17-40 does not bind either Cu or Zn, suggesting that histidines 6, 13 and 14 are involved. FTIR spectra indicated also that the hydroxyl group of tyrosine 10 participates in Cu, but not in Zn, binding.

Prion peptides. PrP-P1, P2 and P4 were able to bind Cu with a maximum stoichiometry of one Cu per repeat, as indicated by electrospray ionization mass spectroscopy (ESI-MS). By FTIR, the peptides had a complex 3D structure which was not much dependent on Cu binding. The geometry of the binding of Cu was directly investigated using X-ray absorption spectroscopy (XAS) at the Cu K-edge. XAS spectra of Cu in solution and in complexes with the peptides were collected at a synchrotron site. The near edge region (XANES) confirms that upon binding Cu(II) preserves its oxidation state. The extended region (EXAFS), analysed by a multiple-scattering approach, shows the occurrence of two types of coordination geometries differing in the number of imidazole rings per Cu atom. For PrP-P4, at a half saturation of cation sites, Cu is bonded to two imidazole nitrogens, whereas in PrP-P1 and -P2 the first Cu shell always contains only one imidazole nitrogen. In PrP-P4, increasing Cu saturation from 0.5 to 0.75 per repeat generates an intermediate situation. The modeled geometries for the alternative Cu coordination sites provide, for the first time, a structural ground for the observed positive cooperativity in cation binding.

Reverse micelles and lamellar systems – new tools for studying the role of water in biological systems

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In many diverse systems, water is not in its bulk state but confined to small cavities where it may play a significant role in the system function and organisation. The confining media can either be biological, porous solids, lamellar systems or micelles. Such a variety of objects infers a diversity of confinement conditions regarding size and/or shape of the confinement volume, osmotic pressure, presence of solvated ions, chemical reactivity of the walls, Therefore, one would expect a wide variety of water behaviours as far as structural and dynamical properties (transient network formation, spatial extent and lifetime of these networks,) are concerned.

Of particular interest are reverse micelle systems, which can be considered as intermediate systems between complex biological water cavities and rigid wall pores in solid media. As in biological objects, the reverse micelles are soft non-rigid cavities, which may host either small or significant amounts of water. Furthermore, micelles are potentially able to host, polar molecules in addition with water. So far the most commonly investigated reverse micelle systems have been Aerosol OT or AOT, i.e. bis (2-ethylhexyl) sulfosuccinate microemulsions as these systems are easily available and well controlled in size.

We will present two reversed micelles systems, ionic (AOT) or not (DMP), and one lamella systems in a range of size between 10 and 100 angstrom implicating respectively water pool of 10 to 130000 water molecules. For these systems, the micelle size has been precisely measured in order to establish the link between the structure and the property of the organisation of the water molecules.

The use of a Fast Fourier Transform Spectrometer because of its extended spectral range is range is well adapted to the study of all the intermolecular and intramolecular mode of vibration. Moreover, when combined with synchrotron radiation in the far infrared, its permits to realise measurements on highly absorbent samples with a good signal to

noise ratio. Accordingly, the first observation of the connectivity band in confined systems is indebted to the high brilliance of the synchrotron radiation.

Future direction includes using reverse micelles with biological objects hydrated with controlled quantities of water.

FT-IR ellipsometry for studying organic layers

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Spectroscopic mid-infrared ellipsometry as a tool for investigations of organic films is presented. The optical constants, the thickness and structural properties of organic films are extracted from the ellipsometric spectra by calculations in layer models [1]. When calculating the optical constants of the different materials, their molecular vibrations are taken into consideration as harmonic oscillators. The evaluation allows the resolution of overlapping bands, recealing parameters of the individual molecular oscillations and the identification of features due to optical effects.

However, many organic samples are of small size or have a heterogeneous structure (e.g. protein crystals, protein films and bio arrays). Unfortunately samples in lateral dimensions smaller than a few mm^2 can often not be investigated with conventional IR ellipsometers as the signal to noise ratio is hampered by the poor brilliance of the commercially available sources in the MIR. This problem can be solved by using a highly brilliant synchrotron radiation source [2, 3].

We succeeded in taking ellipsometric spectra of 1 mm^2 large samples of thin polyimide films [4] with the infrared ellipsometer at the IR beamline at the BESSY II Synchrotron in Berlin. In comparison to a globar source the signal intensity has been improved by more than one order of magnitude for this example. Recently measurements of different spots of a bacteriorhodopsin containing membrance film ("purple membrane") have been performed. The evaluation of the synchrotron measurements shows that the investigated films are laterally inhomogeneous with respect to the "optical" quality.

References:

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[3] M. Gensch, K. Hinrichs, A. Röseler, E.H. Korte, U. Schade, *Anal. Bioanal. Chem.* 376 (2003) 626

[4] K. Hinrichs, M. Gensch, A. Röseler, E.H. Korte, K. Sahre, K.J. Eichhorn, N. Esser, U. Schade, *Appl. Spectrosc.* (2003) in press

The nature of low-frequency vibrations in proteins

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The functionality of proteins depends in a critical fashion on their ability in properly performing the conformational rearrangements necessary to carry out their specific biological action.

In several proteins the conformational path between open and closed forms of such proteins is provided by de-localised low-frequency modes below 3 meV. In this energy region neutron scattering measurements revealed that proteins powders show a well defined bump.

Here we report on the behaviour of such low-frequency excitations on biological systems as a function of the environment around the biomolecule and of the temperature. Some recent results upon unfolding are also presented.

Hydrogen deuterium exchange in membrane proteins monitored by FTIR spectroscopy: new tools to resolve protein structure and dynamics

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As more and more high-resolution structures of proteins become available, the new challenge is the understanding of these small conformational changes that are responsible for protein activity. Specialized difference FTIR techniques allow the recording of side chain modifications or minute secondary structure changes. Yet, large domain movements remain usually unsuspected. FTIR spectroscopy also provides a unique opportunity to record H/D exchange kinetics at the level of the amide proton. This approach is extremely sensitive to tertiary structure changes and yields quantitative data on domain/domain interactions [1].

An experimental setup designed for attenuated total reflection and a specific approach for the analysis of the results will be proposed. The study of two membrane proteins, the gastric H^+, K^+-ATPase [2] and the bacterial LmrA [3], will demonstrate the usefulness of H/D exchange kinetics for the understanding of the molecular movement related to the catalytic activity.

A new approach was developed in order to provide information about membrane domain dynamics. Monitoring the infrared linear dichroism spectra in the course of H/D exchange allowed focusing the recording of exchange rates on the membrane-embedded region of the protein only. This approach revealed an unusual structural dynamics, indicating high flexibility in this antibiotic binding and transport region of LmrA.

References:

[1] Grimard,V., C.Vigano, A.Margolles, R.Wattiez, H.W.van-Veen, W.N.Konings, J.M.Ruysschaert, and E.Goormaghtigh, *Biochemistry* **40** (2001), 11876-11886.

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Detection of protein structural changes in prion-infected tissue

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Transmissible spongiform encephalopathies are a group of fatal neurodegenerative disorders characterized by the conversion of the normal prion protein (PrP^{C}) into aggregates of its pathological conformer (PrP^{Sc}).

To analyze the disease-related protein structural changes directly in the tissue environment, we have examined protein structure within the dorsal root ganglia in the 263K scrapie hamster model.

Using synchrotron-based infrared microscopic imaging, individual neurons were scanned for the distribution of protein structure based on the infrared absorption of the protein backbone mode (Amide I: 1700–1600 cm^{-1}). The high brilliance of the synchrotron infrared light source permitted sub-cellular spatial resolution, revealing regions of increased beta-sheet and decreased alpha-helical structure in and/or around scrapie-infected cells.

No evidence of these structural changes was observed in normal neurons. Comparison of the infrared images with PrP^{Sc} immunostaining of the same tissue demonstrated that the elevated beta-sheet regions correspond to the misfolded structure of PrP^{Sc}.

Characterization of cells and tissues by optical microspectroscopy

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The group at the URCA has, from many years, developed spectroscopic applications at the level of single living cell. More particularly, optical microspectroscopies (Raman, SERS Raman, FT-IR, fluorescence) and multispectral imaging analysis are used to study intracellular pharmacokinetics of drug in relation to biological processes (cytotoxicity, differentiation, resistance phenotype, ...). Very recently, in collaboration with clinicians, the research has been focused on the applications of Raman and IR spectroscopies in the field of health engineering (identification of tumor tissues, early diagnosis and prognostic, early identification and characterization of microorganisms).

High resolution spectroscopy of living cells with a conventional IR source - how good is the competition?

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We have collected hundreds of FTIR spectra of individual human cervical cancer (HeLa) cells, using reflection/absorption, ATR and absorption methods. In addition, maps of individual cells were collected both for dried cells, as well as for live cells suspended in buffer solution or cell culture medium.

We find a large variance in the spectra observed for the dried cells. The spectral variation correlates well with the cell size, and thus, with the stage of the cell's division cycle. ATR and absorption measurements on the same cell reveal striking differences, which we interpret in terms of different depth penetration of the IR beam in the two sampling modalities.

The spectra of live cells in an aqueous environment are quite different from those of dried and fixed cells. In particular, the protein amide I and II peaks are significantly narrower in the live cells, indicating protein precipitation and aggregation in the dried cells.

All measurements reported in this contribution were performed on IR micro-spectrometers employing conventional light sources. Mapping experiments, and all absorption measurements, were carried out using a Spectrum One / Spectrum Spotlight 300 spectrometer (Perkin Elmer, Corp. Shelton, CT). All ATR, and most of the reflection/absorption measurements were carried out using an IlluminatIR micro-spectrometer (SensIR Technologies, Danbury, CT). Both these instruments have produced data that rival those based on Synchrotron-based measurements in spectral quality.

Synchrotron infrared microscopy of cancer cells in whole tissues and in tissue culture

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The potential for vibrational spectroscopy to supplement, if not to replace, the conventional methods of histological and cytological characterisation in cancer diagnosis has led to much research in this area, and its application has recently been reviewed by Dukor.[1] Two areas of interest to our group, and which have attracted considerable research effort world-wide, are the screening of cervical cells and oral cells for evidence of malignancy or premalignancy.

The aim of the first area of our work has been to examine the ability of IR microspectroscopy to discriminate between specific cell types present in the normal and malignant oral mucosa. Oral tissue samples known to contain epithelial tumour were frozen on excision and cryo-microtomed to a nominal thickness of 5 microns. Sections were then mounted onto 0.5 mm thick BaF_2 windows and air dried for infrared analysis. Infrared transmission spectra were collected from individual cells within the tissues, at a spatial resolution of 10 microns. The spectra were then analysed using the chemometrics package Pirouette® (Infometrix Inc., USA) by both hierarchical cluster (HC) analysis and principal component (PC) analysis.

HC analysis was able to discriminate between malignant cell and stroma within tumour sections, as well as keratinised tumour cells. By applying Nearest Neighbour classification following PC analysis, the data set built up was found to be suitable for the correct classification of cells within similar tissue regions, including those from other patients.

In the second part of our work we have focused on the study of cellular changes in cultured cervical epithelial carcinoma cells under the influence of an important cell growth regulatory hormone, epidermal growth factor (EGF). EGF-dependent changes in the DNA and protein IR absorption have

been shown in cultured cells, with evidence for down regulation of the EGF signalling mechanism at higher growth factor concentrations. These changes are relevant to the spectroscopy of both cervical and oral epithelia.

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Micro-FT-IR studies of human bones

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FT-IR and micro-FT-IR spectroscopy were used to study human cortical and cancellous bone samples, obtained intra-operatively from paediatric and adult patients undergoing orthopaedic procedures. There were found histologically that bones were normal and osteoarthritic for paediatric and adult patients, respectively. It was observed, that after treatment of the bones with hydrogen peroxide and acetone, the bands at ~2925 cm^{-1} and ~2853 cm^{-1} which correspond to ν_{as} CH_2 and ν_s CH_2 groups, respectively were disappeared from the spectra.

The disappearance of the band at 1746 cm^{-1} in combination with the absence of the band at 1377 cm^{-1}, which are assigned to ν C=O group of the non-ionised carboxyl group (COOH) and ν_s C=O bond in face with OH group of fatty acids, respectively, shows that hydrogen peroxide and acetone eliminates the fat tissue contents. From the reduction in intensity of the band at 1559 cm^{-1}, which is assigned to ionised carboxyl group (-COO-) and the band of the Amide II group at 1540 cm^{-1} is confirmed the presence of non-collagenous proteins in the bones. The peak at 1658 cm^{-1} is assigned to Amide I of collagen in α-helix configuration.

Due to the elimination of the fat tissue and non-collagenous proteins contents, new intense bands are observed in the region of 1200-450 cm^{-1}. These bands are assigned to the stretching vibrations of ν_1, ν_3 PO_4^{3-} and ν_2 CO_3^{2-} groups. Micro-FT-IR spectra of the osteons suggest that the presence of organic components, mainly collagen, is intense and that the bone mineral is less mature containing acid phosphate and/or carbonate groups, near the centre. Mineral to matrix ratios were calculated from the integrated areas of the [ν_1, ν_3 PO_4^{2-}]/[Amide I] bands and was found to increase from the centre of the osteon towards the periphery, with a maximum plateau being reached at ~50-60 μm from the centre.

This variation of the bone mineral composition around the osteonal centres was uniformly seen in all cortical bone sections from two groups of patients. The [CO_3^{2-}]/[ν_1, ν_3 PO_4^{2-}] ratios were also calculated from integrated areas of the [ν_2 CO_3^{2-}]/[ν_1, ν_3 PO_4^{2-}] bands. This ratio was found to decrease, as a function of increasing distance from the centre of the osteon in some osteons, whereas it remained essentially unchanged in others.

Applications of synchrotron IR microscopy on archaeological cosmetics and biological samples

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Biological samples such as hair and skin are often found during archaeological excavations. Therefore, it is of particular interest to study the aging effect on the biochemical composition as well as protein secondary structure. Synchrotron IR microscopy applications have flourished during the recent years, and its intrinsic high spatial resolution is of particular importance for a detailed study of such biological samples.

We have used such an analytical technique to study a horse hair, cosmetics and skin. The experiments have been carried out at the MIRAGE beam line, at LURE (France). A string, probably of horse hair origin, was collected on the mummy of a Prince in the Kazakhstan. It was used to stitch up again the skin of the skull, after a trepanation, 24 centuries ago. This example exhibits many similar features to that observed on hair originating from other Chinese mummies. In both cases, the conservation is amazingly good, according to the composition (lipids and proteins) as well as to the protein secondary structures.

Cosmetics compounds have also been studied. They were found in tombs, either on mummy skin or in bottles. The amount of material found is often very little, and we have seeked for the presence and identification of organic matter. By this way, synchrotron infrared microscopy enables us to better understand ancient cosmetical and pharmaceutical practices.

To complete this study, we have undertaken some modelling on modern compounds, considered as representative of antique recipes, in order to analyse their behaviour when interacting with skin. Deuterated species are a good way to follow up the penetration of molecules inside the skin.

Biological applications of synchrotron IR at ALS - what can Europe learn from the Californian experience?

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Lawrence Berkeley National Laboratory
USA

A grand challenge in the life sciences for the coming decade is to develop effective methods to interrogate live cells to obtain real-time biological activity information, building on the wealth of data coming from the advances in genomics and proteomics. Researchers at the synchrotron infrared (SIR) facility at the Advanced Light Source (ALS) at LBNL are using an integrated approach to develop SIR spectromicroscopy of live cells to meet this challenge.

In this presentation, I will give a brief summary of the activities at LBNL's SIR facility and talk about critical issues in applying the SIR technique to live cells. Then several representative examples of applications in the biological and environmental fields will be presented.

Particular attention will be given to the importance of integrating approaches and information across disciplines to form coherent and useful knowledge bases. Close collaborations with scientists in a wide range of specialties in the life sciences, from microbiology to oncology have played an essential role in the development of the SIR technique.

Acknowledgement: Financial support for this research was provided by the Office of Biological & Environmental Research, U.S. Department of Energy (DOE) under Contract No. DE-AC03-76SF00098.

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Poster abstracts

P1

FT-IR Synchrotron Ellipsometry for studying the Anisotropy of small OR HETEROGENEOUS samples

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FT-IR reflectance methods such as infrared spectroscopic ellipsometry (IRSE) provide valuable information about the sample properties by probing the reflectance for radiation differently polarized with respect to the plane of incidence [1]. The analytically relevant information is extracted from the experimental results by quantitative comparison with optical theory on the basis of an optical and geometrical model of the sample.

Ellipsometric measurements require well defined optical conditions since reflectance depends strongly on the angle of incidence and the polarization azimuth of the incident radiation. As a consequence the focussing optic is restricted to rather moderate f-numbers and the signal to noise ratio limits ellipsometric investigations to rather large samples. The typical sample size for infrared ellipsometry is around 100 mm^2 when using a conventional FT-IR ellipsometer.

We present a versatile experimental set-up for infrared ellipsometric investigations of small anisotropic samples with synchrotron radiation. Due to the high brilliance of the synchrotron radiation source, the irradiation of samples smaller than 1 mm^2 is found to be improved by more than one order of magnitude when compared to a globar [2]. The performance of the FT-IR synchrotron ellipsometer is characterized by investigating the optical properties of thin polyimide films on silicon substrates. The derived anisotropic optical constants are used to draw conclusions about the structural properties of the samples.

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P2

The Beamline for IR Microscopy and Spectroscopy at ELETTRA

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This work describes the implementation and configuration of the IR beamline at the Elettra light source. The beamline extracts synchrotron radiation from a bending magnet the chamber of which has been modified to maximize the vertical acceptance angle.

Four plane and elliptical gold coated mirrors deliver IR and visible radiation to a Bruker IFS 66 interferometer and Hyperion microscope. The configuration provides radiation covering the spectral range between 10 and 25000 cm^{-1}, with the option of selecting linear and circularly polarized emission.

The beamline has been conceived as a tool for studies in material and surface science, chemistry, biochemistry, biophysics and biomedical science, including both microscopy and spectroscopy applications.

Accessible experiments include IR and fluorescence microscopy of single cells and surface domains, IR reflection-absorption and attenuated total reflectance of interfacial systems, fast kinetic studies and spectroscopy of reaction intermediates in solution.

P3

Synchrotron IR monitoring of chemical events in microfluidic mixing devices and CE microchips

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This presentation deals with the design and first results obtained with dedicated chip based devices for rapid mixing of solutions for the study of chemical reactions and the electrophoretic separation of mixtures using mid-IR synchrotron radiation.

The developed micromixer allows highly reproducible and fast mixing of two or three liquids in micro-channels. Due to the small overall dimensions of the channels (10-15 µm) the diffusion distance is short which results in fast diffusion based mixing. Following the equation $t=D/L^2$ (t: time required for complete mixing, L: characteristic diffusion length, D. Diffusion constant) reduction of L, as made possible by the chip design and the use of synchrotron light (small measuring spot) allows to increase the time resolution to a maximum. Mixing times in the order of a few milliseconds are achievable. The chemical reactions (bio-ligand interactions, protein folding,etc.) can then be followed on-chip in real time. The spectra obtained provide valuable information about the connection between structure and function in complex biochemical systems. A second advantage resulting form the miniaturisation is the reduction of sample volume required for measurement. This is considered to be of importance especially when investigating biological samples where only minute amounts of sample are available. The combination of the small measuring spot with the chip-design makes this development a promising tool for the study of many different biochemical systems.

Furthermore, we introduce infrared spectrometry as a novel molecule specific detection technique in chip based capillary electrophoresis (CE). A CE microchip with mid-IR detection has been designed which allows to separate mixtures of analytes (inorganic species, proteins, etc.) and to record their IR spectra after separation. The use of FT-IR as a detection method in CE on a chip allows to detect specific molecular features like the secondary structure of a protein and is therefore a suitable complementary method to standard detection methods.

The microchips were constructed by two IR transparent CaF_2 windows separated by a photoresistant epoxy polymer layer in which different channels were developed according to the structures shown in the figures bellow. In the case of the mixer, 3 channels merge for mixing resulting in a final channel which is 15 μm broad and 10 μm deep. The meander structure allows following the sample for 5 cm. In the case of the CE chip, the separation channel is 10 μm deep, 100 μm broad and 4 cm long.

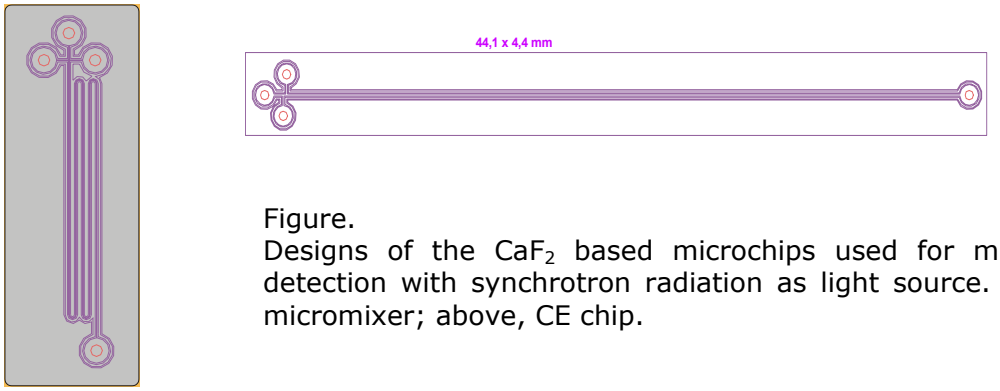

Figure.
Designs of the CaF_2 based microchips used for mid-IR detection with synchrotron radiation as light source. Left, micromixer; above, CE chip.

P4

First experiments at SINBAD, the infrared beamline at DAFNE

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First results from the SINBAD beamline at DAFNE are reported.

The SR brilliance, as measured in the far infrared at the sample position, has been compared with that of the mercury lamp. For l=100 mm and through an aperture of 1 mm, the gain in intensity is larger than 20. The high brilliance of the synchrotron radiation in the Far Infrared is fully exploited by high pressure experiments.

Preliminary experiments with a diamond-anvil cell in the 10 GPa range are reported.

P5

Measurement of infrared circular dichroism by means of totally reflecting retarders

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Circular polarization of IR radiation is obtained by means of linear birefringence, total internal reflection, metal reflection and cholesteric filtering. The first three methods are based converting linear polarization into circular one. The cholesteric filtering converts directly unpolarized radiation into circular one. The widely used method for measurement of IR vibrational circular dichroism (VCD) is achieved by means of photoelastic modulators (PEM) Their function rests on stress induced linear birefringence in an isotropic optical element governed by *ac* pulses thus switching between left and right circular polarization and passing through all possible polarizations – elliptical and linear (*phase modulation*).

The exact circular polarization, expressed as the phase shift δ, certainly refers only to a definite wavenumber ν_0 and is determined by the thickness *d* of the modulator and the induced birefringence Δn produced by its pulse driver ($\delta = 2\pi\nu_0\, d\cdot\Delta n = \pi/2$). This restricts the usable spectral region typically between 900 and 1600 cm^{-1} independently of the IR transperancy of the photoelastic modulator itself.

The method based on total internal reflection utilizes the whole IR region determined only by the transparency of the totally reflecting element since the generated phase shift $tan(\delta/2) = (\cos\varphi\sqrt{\sin^2\varphi - n^{-2}}) / \sin^2\varphi$ at a given angle of incidence φ slightly and smoothly changes with the wavenumber ν through the dependence $n = n(\nu)$ of the refractive index of the totally reflecting element. The switching between left and right circular polarization can be achieved by altering the direction of the linear polarization as an *amplitude modulation*. Some examples are given. A possible scheme based on total internal reflection using the IR synchrotron beam is considered.

P6

Synchrotron IR spectroscopy of single living human cells at ANKA-IR

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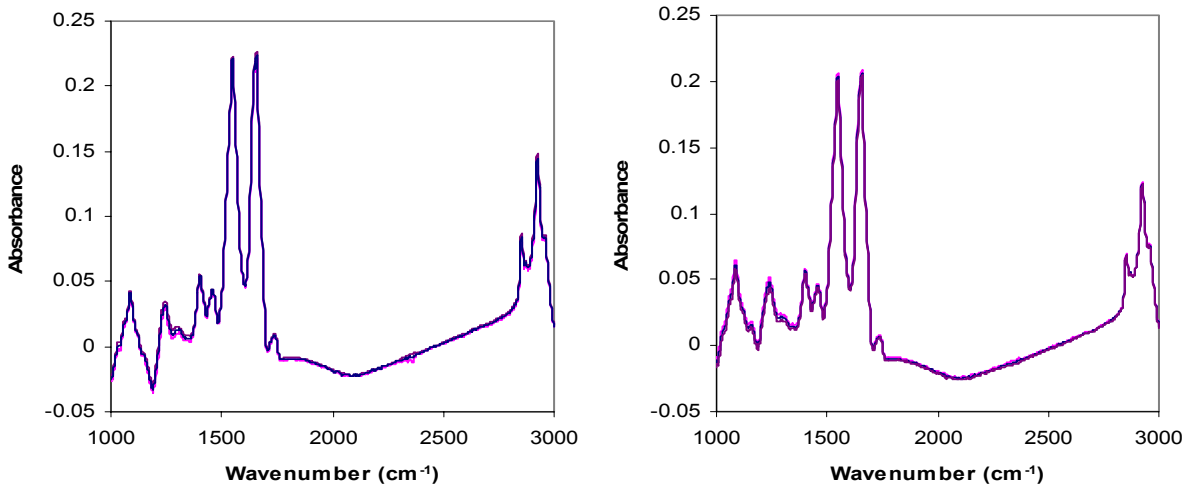
The edge radiation beamline ANKA-IR promises particularly high brilliance compared to conventional synchrotron sources. This should allow us to record infrared spectra of biological materials at a spatial resolution sufficient for the resolution of single cells or even sub-cellular details, with an unprecendently high data quality.

In our five-day allocation of beamtime in August 2003, we carried out the first measurements of biological materials at ANKA-IR. Around 2000 spectra of single living human fibroblasts and adenocarcinoma cells (SW480 cell line) were recorded. These included spatially resolved spectra of single cells or of nucleus/cytoplasm regions, as well as time-resolved monitoring of spectral changes under various treatments. Analysis and evaluation of these spectra will take much longer than the time to record them, but in this poster we will present a preliminary view of our data.



Mean and standard deviation IR spectra of two individual living cells from a confluent human fibroblast culture. Individual spectra were recorded through a 30 µm aperture at 512 scans/67 secs per spectrum, at intervals of 24 mins over a period of 2 hours. Reference for the absorbance calculation was an adjacent cell-free region of the culture plate. The spectra have not been scaled, normalized, smoothed or baseline corrected in any way.

P7

X-ray and Neutron Small Angle Scattering shape analysis in assessing protein structures obtained by computational prediction techniques

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Projects on functional genomics and proteomics would provide information on the specific structure-dynamics-function relations of all proteins encoded in human and other genomes. Major obstacles are the large complexity of the individual proteins and the even more complex interaction of different proteins and other biomolecules to form functional complexes. Moreover, in the modern trend towards high throughput, as well as high resolution structure determination, many proteins will not readily crystallize.

It is then evident that crystallography and NMR of biological molecules, and, in a different range of resolution as well as time scale, small angle scattering (SAS), X-ray absorption spectroscopy and neutron inelastic scattering constitute major components of a concerted action for 3-D structure analysis [1,2]. In particular, the use of solution X-ray and neutron small angle scattering (SAXS and SANS) can offer the chance to determine the fold of a protein in solution, and to characterize its quaternary structure [2-4]. In combination with computational prediction techniques, this has been proved to help in assessing ab-initio protein structure and to evaluate conformational changes modeled by Molecular Dynamics simulations [5-7].

Here, the determination of the 3-D structure of a Sulfolobus solfataricus carboxypeptidase [7] and the analysis of the structure of a human tissular transglutaminase [5] and of its conformational changes occurring after calcium activation and guanosine triphosphate inhibition [6] will be discussed. If the interest in assessing a 3-D structure is straightforward, the conformational rearrangements occurring after ligand binding appear strictly related to the structure-function relationships and to the induced-fit hypothesis that long ago replaced the lock and key view for enzyme substrate interactions.

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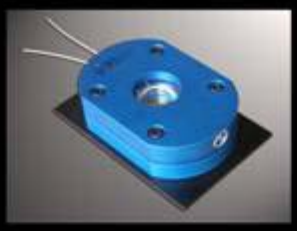
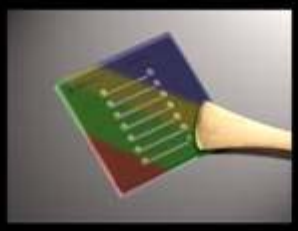

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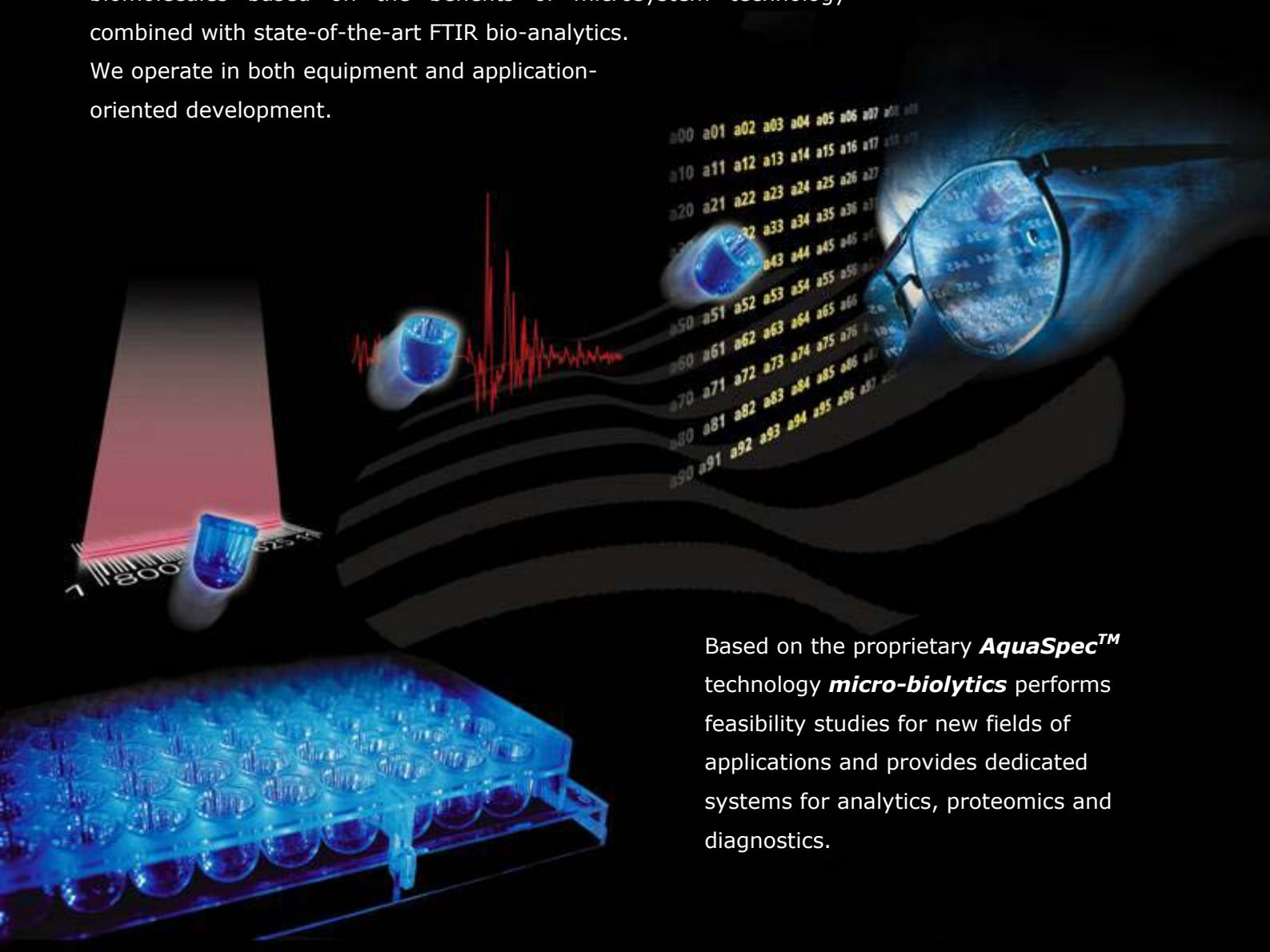

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