Raman spectroscopy of biological samples
Gratings, OEM Spectrometers, VUV
Optical Spectroscopy
Spectroscopic Ellipsometry
Raman Spectroscopy
Fluorescence
SPRi
Inductively Coupled Plasma Spectrometry
RF Glow Discharge Emission Spectrometry
Carbon/Sulfur and Oxygen/Nitrogen/Hydrogen Analyzers
Sulfur and Chlorine in Hydrocarbons Analyzers
Particle Size Analysis
Forensics

Explore the future
### Session 1 - Molecular Characterization

<table>
<thead>
<tr>
<th>Title</th>
<th>Presenter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Use of fluorescence to study aggregation of biopharmaceuticals</td>
<td>Tudor Arvinte, University of Geneva and Therapeutic Basel (Switzerland)</td>
</tr>
<tr>
<td>Size and charge characterization of nanoparticles used in bio-applications</td>
<td>Christelle Mégier, HORIBA Scientific (France)</td>
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<tr>
<td>Title to be confirmed</td>
<td>Maríte Cárdenas Gómez, University of Copenhagen (Denmark)</td>
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### Session 2 - Clinical Applications

<table>
<thead>
<tr>
<th>Title</th>
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<tbody>
<tr>
<td>SPRi-MS coupling for the identification and characterization of clinical biomarkers</td>
<td>Wilfrid Boireau, FEMTO-ST Institute Besançon (France)</td>
</tr>
<tr>
<td>Analysis of sperm DNA quality by Raman microspectroscopy</td>
<td>Victoria Sanchez, University of Münster (Germany)</td>
</tr>
</tbody>
</table>

### Demonstration of the Instruments

- Particle Characterization
- Raman
- SPRi
- Fluorescence
- Spectroscopic Ellipsometry
- Electrochemistry, micro volume measurement
Raman spectroscopy

High selectivity to chemical species, molecular bondings
From micron spot analysis to big areas fast mapping
From Research to Analytical applications

Within the last 10 years,

12 European Research Projects
23’000 Scientific Publications
6 new Raman spectrometers
Applications in Biology

Biology is a natural science concerned with the study of life and living organisms, including their structure, function, growth, origin, evolution, distribution.

In biology, Raman Spectroscopy is mainly used to study:

- DNA Analysis
- Lipids, proteins & amino acids
- Bacteria classification and recognition
- Drug / Cell interaction
- Diagnosis & prognostic for cancer
- Dental prostheses
Advantages of Raman spectroscopy in Biology

- **Sensitivity to many different functional groups**: access to C=C, S-S, C-S bonds (that are weak in IR)

- **Highly selective fingerprint**: similar compounds can be discriminate

- **Non-invasive and non-destructive method, no sample preparation**

- **Compatibility with aqueous solutions**

- **High spatial resolution**: Single cell level analysis, intracellular imaging are achievable

- **Sensitivity to Molecular orientation**: polarization measurements

- **Measurements can be done in vivo or in vitro**

- **SERS, TERS and Resonance effects**: can highly increase sensitivity
Raman analysis of proteins

E(bending) < E(stretching)

Raman Intensity

Wavenumber/cm⁻¹
Raman analysis of proteins

- Size of proteins makes spectrum complex
  - Polypeptide backbone
    - Secondary structure
  - Amino acids in side chains
    - H-bonding
    - Environment
    - Intermolecular interactions

- Raman spectroscopy provides unique information about :
  - Chemical composition
  - Conformational structure

- Reactions can be monitored by Raman to study the mechanisms of the reaction and derive bio-physical properties
Raman analysis of proteins

- Amide bonds involved in H-bonding
  - Strongly dependent on $2^\circ / 3^\circ$ structure

<table>
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<th>Wavenumber Range</th>
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<th>Secondary Structure</th>
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</table>
Raman analysis of proteins
Biomolecules analyses: proteins

- Example of the disulfide bridge breaking in proteins
- Disulfide bridge:
  - stabilization of the protein structure
  - denaturation of the protein when broken

- Protein used: albumine (BSA)
  - BSA reacts with reducing agents (DTT, phosphine) to break the S-S bonds.
  - Depending on the conditions, the reaction is equilibrated or total
  - The kinetic of the reaction is monitored by measuring Raman spectra over time
Biomolecules analyses: proteins

- Example of the disulfide bridge breaking in proteins

![Graph showing S-S bond before/after reaction](image1)

![Graph showing Amide I bond before/after reaction](image2)

- Contributions of the different peaks are calculated after deconvolution
  - calculation of physical parameters, such as reaction rate, free enthalpy, activation energy
  - information about the structure of the denatured protein
Biomolecules analyses: DNA

- Example of DNA in semen
  - Male infertility is often not well diagnosed, thus therapeutic options are limited
  - Nuclear DNA damage of semen is one of most crucial cause, but it currently lacks of reliable method to assess the status of the sperm’s DNA

- Mapping of a sperm head with spectra obtained every 50 nm. The colours represent the different parts of the head, based on spectral information.
Biomolecules analyses: DNA

- Example of DNA in semen
  - Semen collected from 8 donors. 200 sperm/sample/treatment were analyzed by Raman
  - Half of the population is irradiated with UV-B; the other half remain untreated
  - UV-B causes damages on nuclear DNA

- 2 populations are clearly identified in the PCA plot: the untreated one vs the UV-bleached one
- Averaged spectra of both population effectively show significant differences, assigned to modifications of nucleotide bases (phosphate band) due to UV-B irradiation
Biomolecules analyses: DNA

- Example of DNA in semen
  - Distribution of damaged and undamaged DNA within the sperm nucleus

![Image of sperm nucleus with damaged and undamaged DNA]

- Using the scores of the PCA (principal component analysis), the distribution of the damaged DNA can be visualized
- It is mainly located in the periphery (under the acrosomal cap)
Cells analysis: sorting and imaging
Cells analyses

- Raman: interesting tool for cells analysis
  - Spatial resolution
  - Confocality
  - Complementary to fluorescence

- Used to analyse single cells for identification purposes

- Used to analyse cells content
  - Drug location and interaction in cells
  - SERS to enhance the signal of low concentrated compounds
Disease diagnostic and prognostic

- IHMO 2008-2010
  - Hybrid Imaging Microscopy for Oncology
  - Diagnostic and prediction of tumors
  - Blood smear analysis

- DIAGRAM 2009-2010
  - Detection and Identification of bacteria by Raman spectroscopy and SERS on nanostructured surfaces
Cells analyses: bacteria

- Bacteria identification and classification
  - More than 3800 spectra of different species / strains
  - Classification according to the species and strain

<table>
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<tr>
<th></th>
<th>Total number of spectra</th>
<th>Number of wrongly classified strain spectra</th>
<th>Recognition rate for strains (%)</th>
<th>Number of wrongly classified species spectra</th>
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</table>

- 96% identification @ specie level
- 83 % identification @ strain level
- Use of chemometrics to build up classification models
Cells analysis: lymphocytes

Lymphocytes classification

- Raman signature is used to lymphocytes classification
Cells analyses

Video capture

Band-pass confocal microscopy mode

Spectral imaging mode: fluorescence, Raman

Bovin embryo incubated with Nile Red fluorochrome

Fast selection of slice of interest

Spectral analysis reveals lipidic content of the sample

400x400 points
4 sec per slice

~35000 spectra recorded in 3 min,
(SWIFT™ acquisition mode, Horiba Jobin Yvon)

20 slices, with 400 x 400 data points
(over a 203um x 185um area)
Cells analyses: chemotherapy optimization

**XploRA INV** confocal fluorescence spectral imaging

**Drug (doxorubicine) delivery in breast cancer cell**

- **Video capture**
- **Band-pass confocal microscopy mode**
- **Spectral imaging mode: fluorescence, Raman**

- MCF-7 cancer cell Treated with DOX
- Most intense DOX fluorescence is in the nucleus

Spectral analysis reveals drug molecular interactions
Tissues imaging
Diagnosis, pronostic for cancer
Diagnosis and tissue analysis

- Raman provides detailed information on cell/tissue biochemistry
- Probes DNA, RNA, lipids, proteins, carbohydrates etc
- Clustering of spectra identifies tissue type
- Two classifications of Brain cancer tumour
- Red tumour areas match identification by pathologist
- Green areas are likely to be ‘early’ stage tumours
Characterization of human thyroid tumor tissue

- Raman spectroscopy allows to distinguish between tissue corresponding to non-tumorous **goiter**, benign **adenoma** and malign **carcinoma**.
Diagnosis and tissue analysis

Characterization of human thyroid tumor tissue

- Peak identification comparison: goiter and carcinoma.

**T**: thymine  
**Tyr**: tyrosine  
**Phe**: phenylalanine  
**Trp**: tryptophan  
**A**: adenine  
**G**: guanine
Probing tumour tissues in skin basal cells carcinoma using polarized Raman microspectroscopy

- Polarized Raman has been used mostly for oriented macromolecules such as fibers (silk, hair...), DNA, viruses, bones and isolated cells
- Applied to skin tissues, it was evidenced that polarized Raman can bring complementary information
- Setup was the following:

www.skinscience.fr
Probing tumour tissues in skin basal cells carcinoma using polarized Raman microspectroscopy

Basal cell carcinoma (N=5)

4 areas were studied
- Peritumoral dermis (=close to the tumour)
- Healthy dermis (=far from the tumour)
- Healthy epidermis
- Tumour

3 spectra were acquired for each area
- Standard Raman
- Polarized Raman I//
- Polarized Raman I⊥

Tumour invasion analysis

Collagen triple helix

Skin typical Raman spectrum
Probing tumour tissues in skin basal cells carcinoma using polarized Raman microspectroscopy

- degradation of collagen in 3 alpha chains
- transient state of collagen degradation?
  (middle peak might be related to double alpha chain)
Tips and tricks to optimize your Raman measurements of biological samples

- Get a dedicated fully automated system with
  - multiple lasers & fast mapping
    (fluorescence, photosensitivity issues…)
  - immersion objectives
- Use appropriate microscope slides in CaF$_2$ or fused quartz
- Hold your sample with optical tweezers
- Enhance your signals through plasmonic resonance effects (SERS, TERS)
NEW in 2010 Innovation & Performance

Product release

- XploRA Inverted:
  - Inverted Nikon Ti-U microscope for applications in biology, lifesciences, and nanomaterials
  - Mid-range spectral resolution (<2cm⁻¹)
  - Fully automated system
  - 3 lasers in Vis-NIR (473 to 785nm), 4 gratings
  - High throughput microscope without compromise on microscopy visualisation
  - Compatible with Ultra fast imaging
LabRAM HR Evolution:

- Fully achromatic system from 200nm to 2.1μm
- Multiple microscopes available, including double microscope configuration (upright, inverted)
- Highest Spectral resolution for single stage spectrograph
  ✓ down to 0.5cm⁻¹ FWHM in visible with 2400g/mm grating
- Fully automated system
  ✓ Up to 6 lasers from 229nm to 1064nm
  ✓ Up to 3 simultaneous detectors
- Improved spatial resolution
  ✓ Down to <400nm FWHM in XY
  ✓ Down to <1μm FWHM in Z
NEW in 2012 Innovation & Ease of use

LabSpec 6 Spectroscopy Suite

✓ Advanced automation and customization
✓ Fully Integrated Multivariate Analysis module
✓ Particle Finder module
✓ On-the-fly automated fluorescence removal “FLAT”
✓ Real 3D imaging, XYZ and chemical information
Select appropriate microscope slides

- Ban plastic or glass microscope slides which give a strong fluorescence signal when excited in visible and/or NIR

- Go for fused quartz (10$) or best Calcium fluoride slides (35$)
  - Crystran (UK)
  - GM Associates (USA)
Raman combined with Laser tweezers for bacteria analysis

- Photons exert a force on any material to which they are incident
- A focussed laser beam exerts sufficient force to be able to hold and move small objects (typically in the size range 0.1-10 μm)
- Combination of Raman and laser tweezers allows individual bacteria to be:

  - Held in place
  - Interrogated using Raman
  - Classified according to species / age / $^{13}$C labelling
  - Moved to a storage point
  - Dropped
**SERS: improve weak Raman signals**

- Surface Enhanced Raman Scattering (SERS) has long been used to **enhance weak Raman signals** by means of surface plasmon resonance, allowing detection of chemical species at **very low concentration**

- SERS exploits the **generation of highly localized fields in the near field of adapted metallic nanostructures** for enhancing spontaneous Raman scattering

- Increases in sensitivity can be by **many orders of magnitude**, improving from $10^{-3}$M for spontaneous (normal) Raman, to $10^{-5}$M for resonance Raman, and **up to $10^{-12}$M** for SERS

- Common metals used for SERS include **gold and silver**, and these can either be used in the form of a **nano-scale roughened surface** onto which the sample is adsorbed, or as a **colloid suspension**
SERS for proteins – Developing a reproducible nanobiosensor

SERS substrates: gold nanocylinders arrays

(a) SERS spectrum measured for RNase-A at 1mM concentration
(b) Raman spectrum of RNase-A in aqueous solution at 1mM
(c) Raman spectrum of RNase-A in powder state

SERS intensity as a function of the position of the plasmon resonance calculated for the RNase-A Raman bands located at 1614 cm\(^{-1}\). The excitation wavelength, \(\lambda_{\text{exc}}\), is 632.8 nm and the considered Raman band \(\lambda_R\) for RNase-A is located at 705 nm.

(C. David et al. Nanotechnology (2010))
Anticancer drug targeting

- External magnetic field
- Vascular permeability, biological recognition of cancer cells

Imaging of anticancer drugs/cancer cells

- Drug (DOX), and/or fluorochrome
- SPION, and/or Au, Ag
- Polymer (PEG, ...)
- Biological ligand (FA, ...)

- MRI
- SERS and/or fluorescence

Methodological coupling: Simultaneous co-detection SERRS-fluorescence on the same spectral image

Courtesy of Pr I. Chourpa, group « Magnetic nanovectors for chemotherapy », EA 4244

Video capture

MCF-7 cancer cell treated with 1 μM MTX and incubated with NP Ag-citrate

Spectral intensity

Spectral analysis:
SERRS and fluorescence

Rem 1:
Aggregates ≤ 1 μm are detectable

Rem 2:
SERRS and fluorescence spectral informations are complementary
A combination of various techniques and effects:

- **Scanning Probe microscopy**
  - spatial resolution (near-field, imaging)

- **Surface plasmon resonance**
  - signal enhancement necessary because Raman is a weak scattering

- **Optical spectroscopy**
  - Raman ‘sensor’
    - excitation
    - collection
    - detection
TERS Imaging results

- DNA: Raman resolution <15nm
- Inverted microscope
- Oil immersion
Tip Enhanced Raman Spectroscopy

- TERS profile across a single amyloid fibril

DOI 10.1002/jbio.201100142
Tip Enhanced Raman Spectroscopy


- TERS profile across a single amyloid fibril.
  - The study of amyloid structure and growth has been motivated by their implication in many human diseases. There are ~20 diseases associated with excessive deposits of amyloid plaques in the affected tissue or organ including Alzheimer’s disease (AD), Parkinson’s disease (PD), type II diabetes, and spongiform encephalopathies.

- Raman bands of the different constituents of amino acids are distinguished

DOI 10.1002/jbio.201100142
Tip Enhanced Raman Spectroscopy

- TERS profile across a single amyloid fibril

- TERS signal intensity tracking of amino acid signals of the fibril, blue: cystine (C), orange: tyrosine (Y), grey: proline (P), green: phenylalanine (F), purple: histidine (H).

- The sequence is clearly visible and shows a resolution better than 2nm

DOI 10.1002/jbio.201100142
Conclusion

- Raman as tool for bio-medical investigations
  - at biomolecules, cells and tissue levels
  - used for medical investigation and diagnosis, pharmacokinetics, biophysics, …
- Complementary technique to traditional ones:
  - Brings chemical specific information on the sample
  - Micron-scale spatial resolution (imaging)
  - Confocality
  - Coupling to confocal fluorescence
  - Non destructive
- Tips and Tricks
  - Use of appropriate substrates/matrix (CaF$_2$ or quartz slides, SERS nanocolloids)
  - Use of optical tweezers combined to Raman to hold the biomolecule during spectra acquisitions
  - SERS Increases highly the sensitivity for low concentrated samples
  - TERS increases both sensitivity and spatial resolution
Happy Researching,

Thank you!

www.horiba.com