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School on Synchrotron and Free-Electron-Laser Sources and their Multidisciplinary Applications

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Infrared spectroscopy and microscopy for biology and biochemistry

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Infrared spectroscopy and microscopy for biology and biochemistry

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SISSI beamline @ Elettra

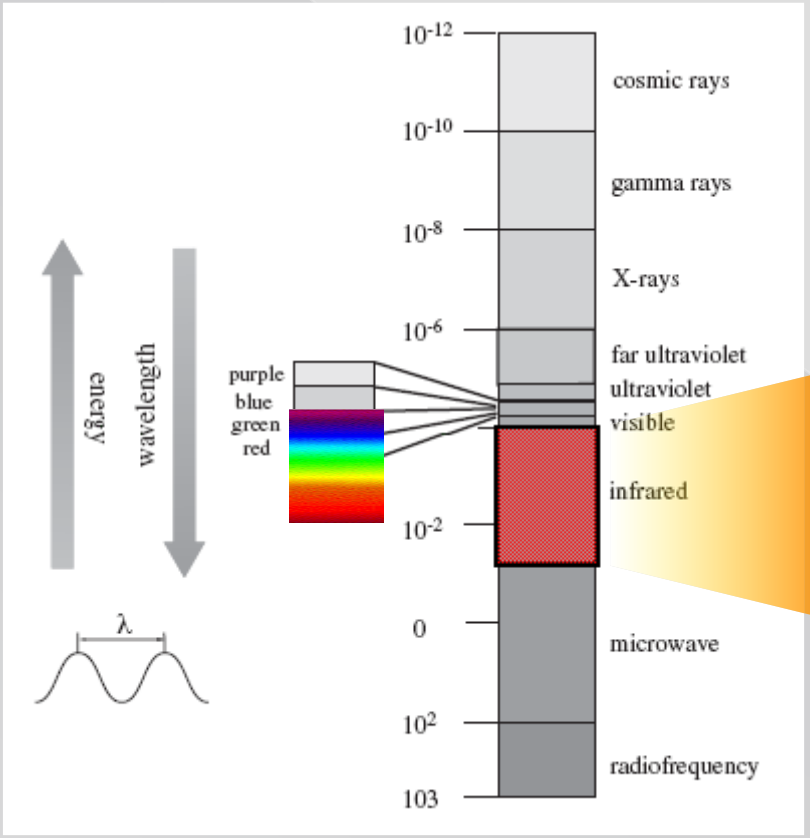
Synchrotron Infrared Source for Spectroscopy and Imaging

School on Synchrotron and Free-Electron-Laser Sources and their Multidisciplinary Applications
ICTP 2010

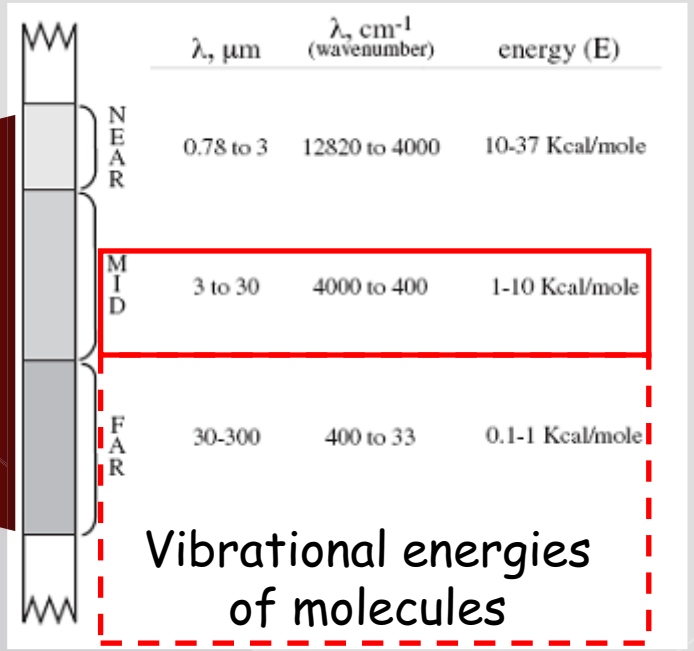
Outline

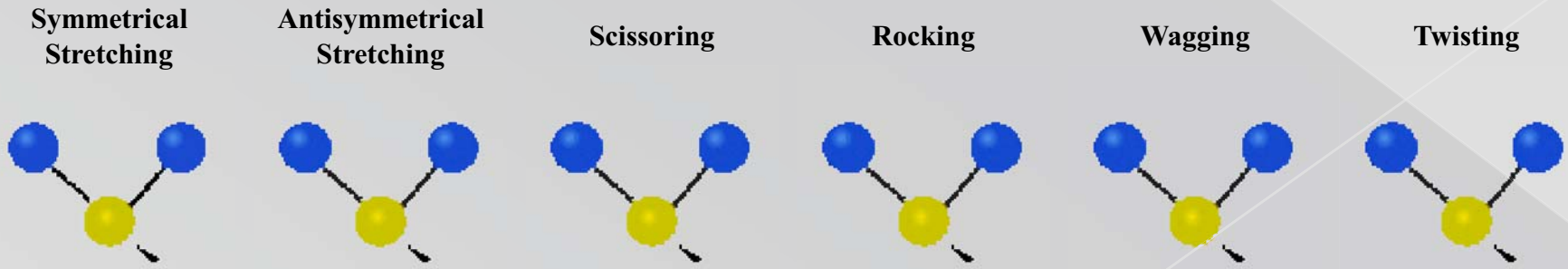
- Introduction: IR fingerprints relevant to the biological applications of IR microspectroscopy (IRMS)
  - Biology and biochemistry: the spectroscopic point of view
- Biological applications of IR microspectroscopy (IRMS)
- An infrared bio-experiment step by step
  - Sample Preparation
  - Data collection
  - Data Analysis
    - Pre-processing of data
    - Spectra comparison
- Selected examples of biological applications of SR IRMS
  - SR IRMS and prion research
  - Adaptive immunity modulated by AMPs (hBD2)
  - Mechanobiology of leucocytes
- Conclusive remarks

IR fingerprints relevant to the biological applications of IR Microspectroscopy (IRMS)



$$E = h\nu = h\,(c/\lambda) = h\,c\,\overline{\nu}$$

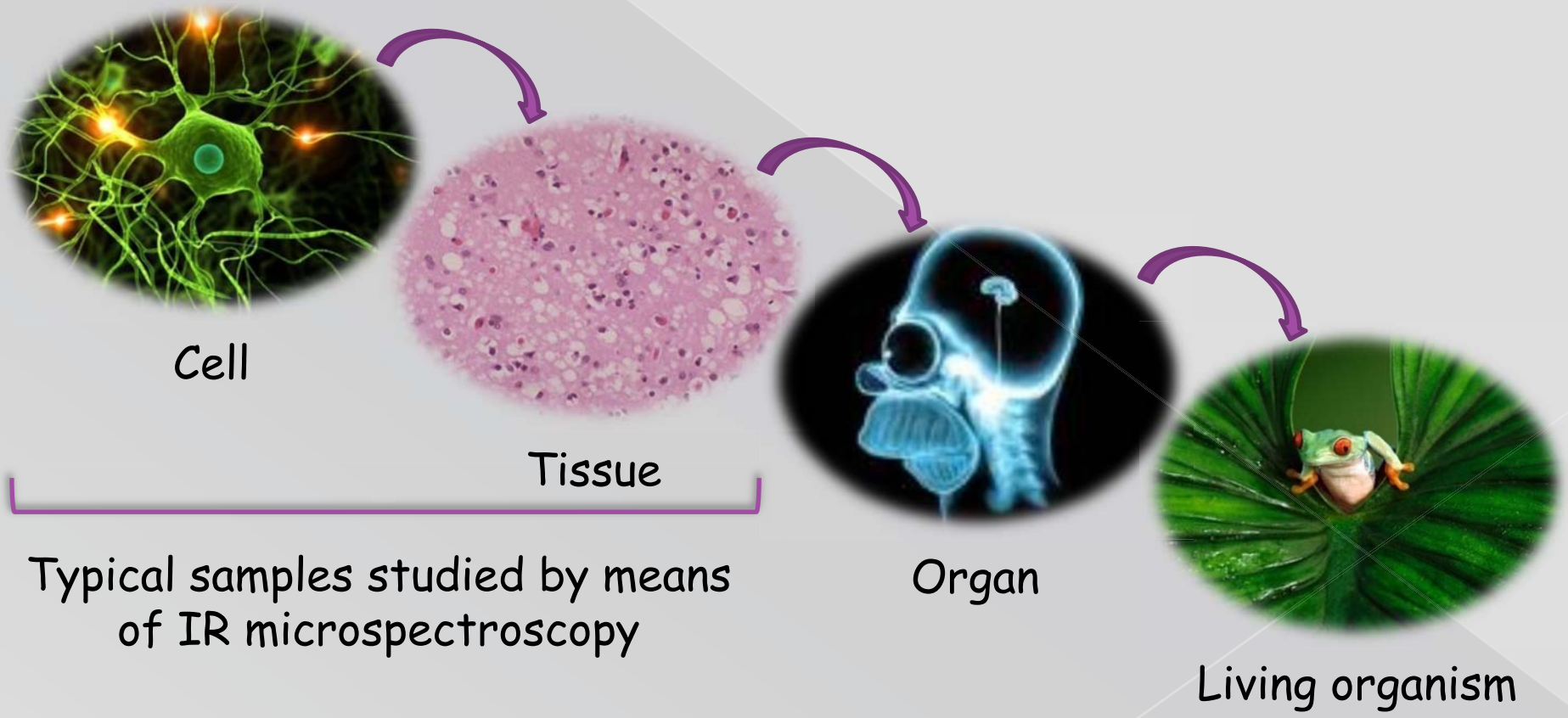




Biology and Biochemistry: The spectroscopic point of view_1

Biology is the branch of natural science that studies life and living organisms.

The building blocks of life are the **cells**.



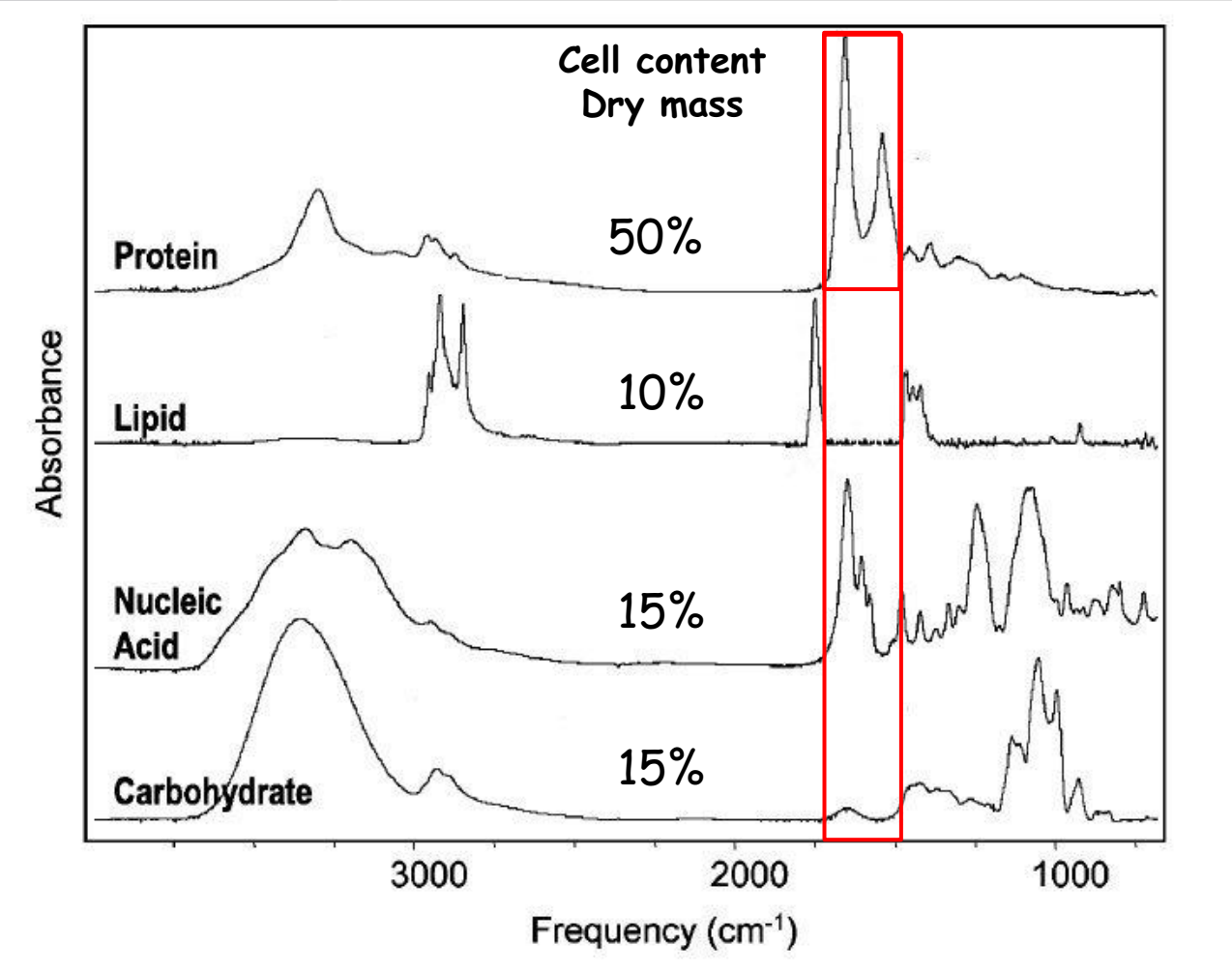
Biology and Biochemistry: The spectroscopic point of view_2

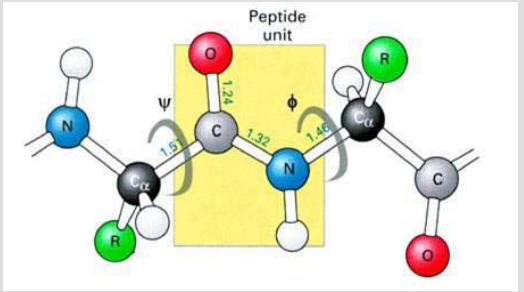
The study of the chemical processes in living organisms is the subject of **Biochemistry**.

It deals with the structures and functions of cellular components such as **proteins**, **carbohydrates**, **lipids**, **nucleic acids** and other **biomolecules**.

The lecture will illustrate recent achievements of SR InfraRed MicroSpectroscopy (IRMS) in studies of biochemical features of complex biological systems (tissues and cells)

Biology and Biochemistry: The spectroscopic point of view_3





1700-1600

1705-1690 : ν C=O RNA

1660-1650 : ν C=O DNA

1660-1650 : ν C=O RNA

1700-1600: Amide I

80% ν C=O + 10% ν C-N +10% δ N-H

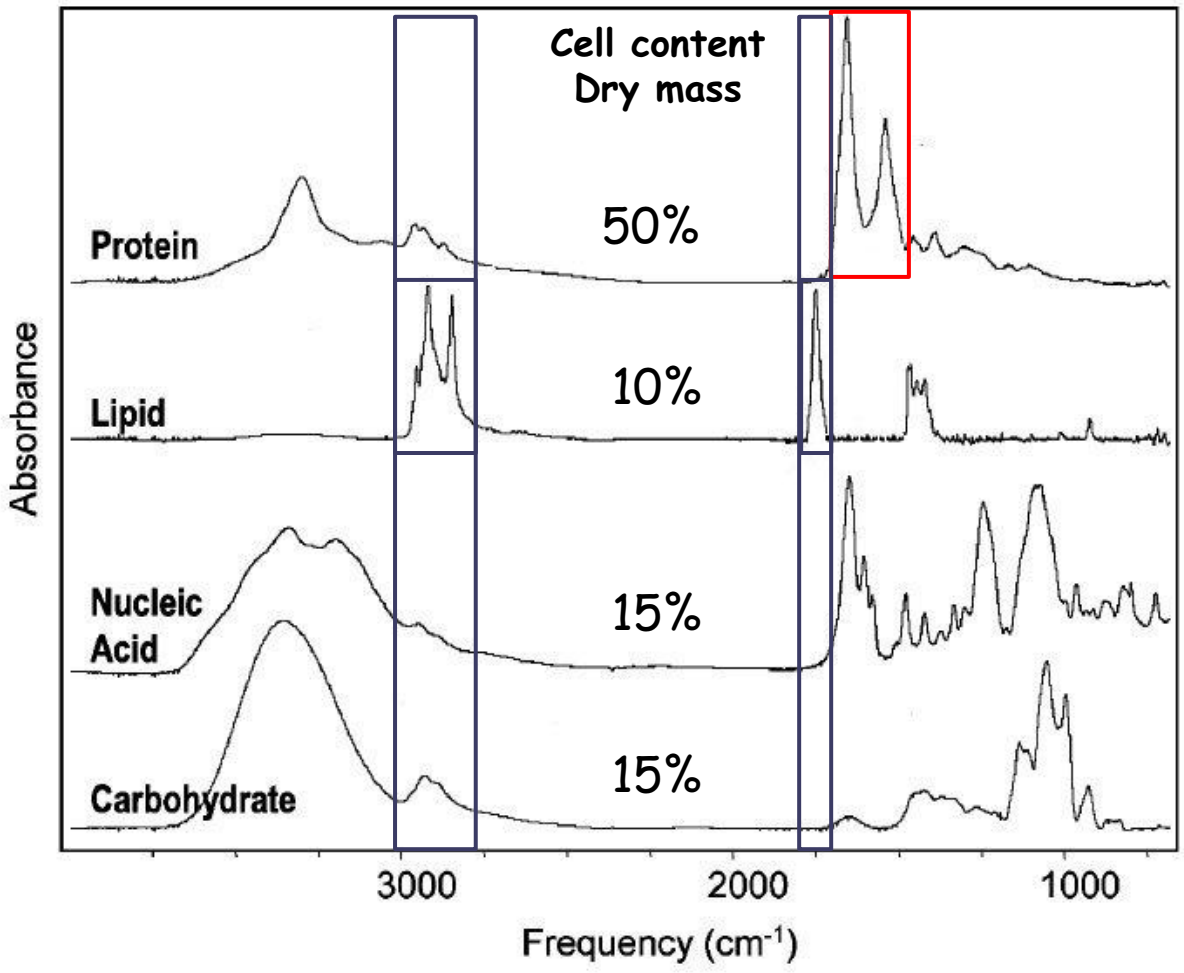
1600-1500

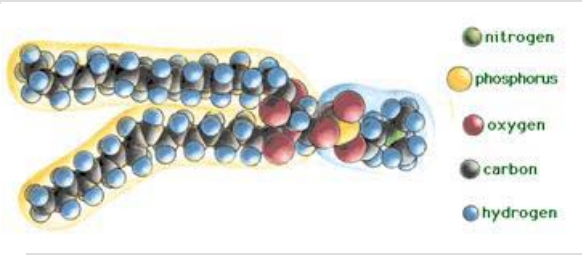
1600-1500: Amide II

40% ν C-N + 60% δ N-H

Adapted from: L. M. Miller, G.D. Smith and G. L. Carr, *Journal of Biological Physics*, 29 (2-3), 219-230, **2003**

Biology and Biochemistry: The spectroscopic point of view_4





Saturated Acyl chains

2950-2960 : ν_{as} (CH_3)

2915-2925 : ν_{as} (CH_2)

2867-1877 : ν_s (CH_3)

2845-2855 : ν_s (CH_2)

Unsaturated Acyl chains

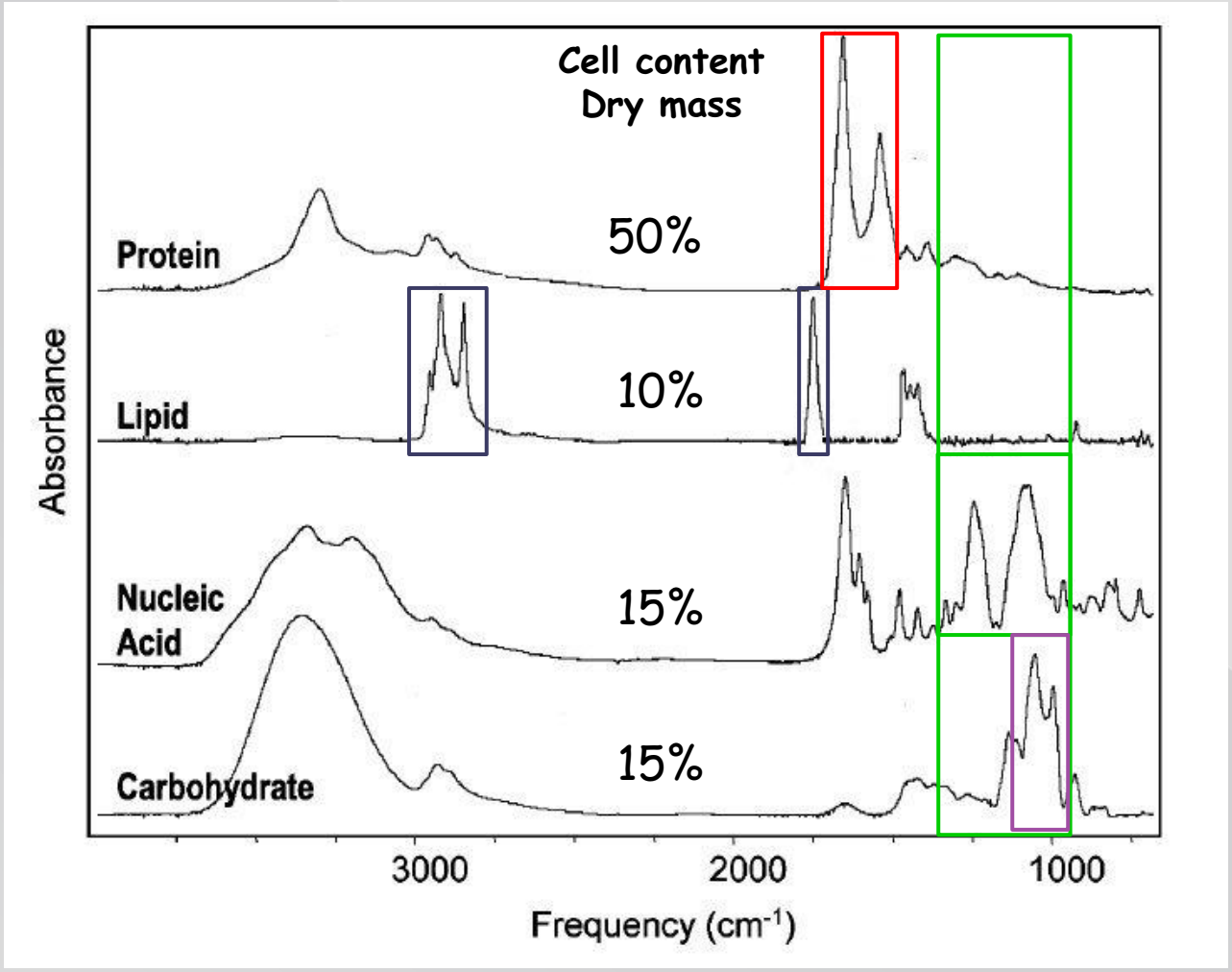
> 3000 : ν (=CH)

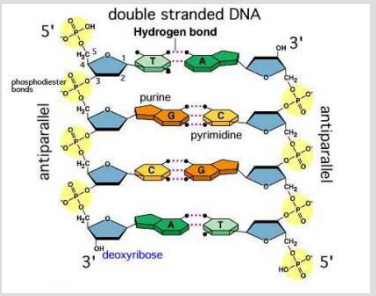
Ester Band

1700-1750 : ν (C=O)

Adapted from: L. M. Miller, G.D. Smith and G. L. Carr, *Journal of Biological Physics*, 29 (2-3), 219-230, **2003**

Biology and Biochemistry: The spectroscopic point of view_5





1250-1200

1244 : ν_{as} PO^{2-} RNA

1230 : ν_{as} PO^{2-} DNA

1100-1050

1089 : ν_{s} PO^{2-} DNA

1084 : ν_{s} PO^{2-} RNA

1100-1000

DNA and RNA ribose

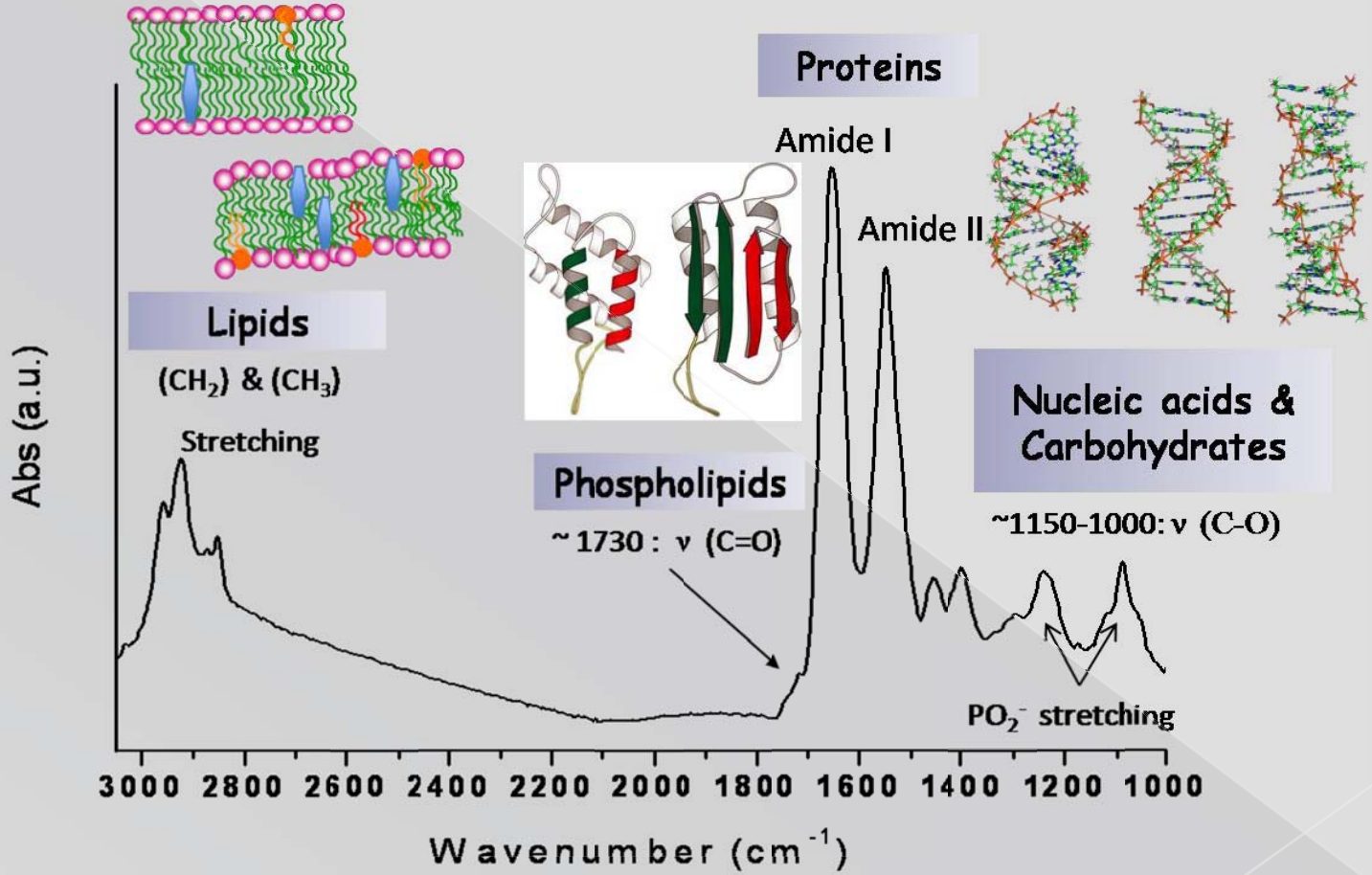
ν C-O

Complex network of carbohydrate bands

Adapted from: L. M. Miller, G.D. Smith and G. L. Carr, *Journal of Biological Physics*, 29 (2-3), 219-230, **2003**

Biology and Biochemistry: The spectroscopic point of view_6

Band intensity, position, width and shape (band components) are sensitive to subtle biochemical changes of bio-specimens.



Compositional and structural information at tissue, cellular and sub-cellular level can be achieved by exploiting SR brightness advantage

Biological applications of IR microspectroscopy (IRMS)

Infrared spectroscopy and microspectroscopy of plant, animal or human cells, tissues and body fluids as well as of microbial species can be exploited for :

1- Characterizing and differentiating microbial cells and strains: **rapid method for identifying micro-organisms responsible for infections.**

2- Differentiation of healthy and diseased tissues and/or cells: **new tool in medical diagnostic.**

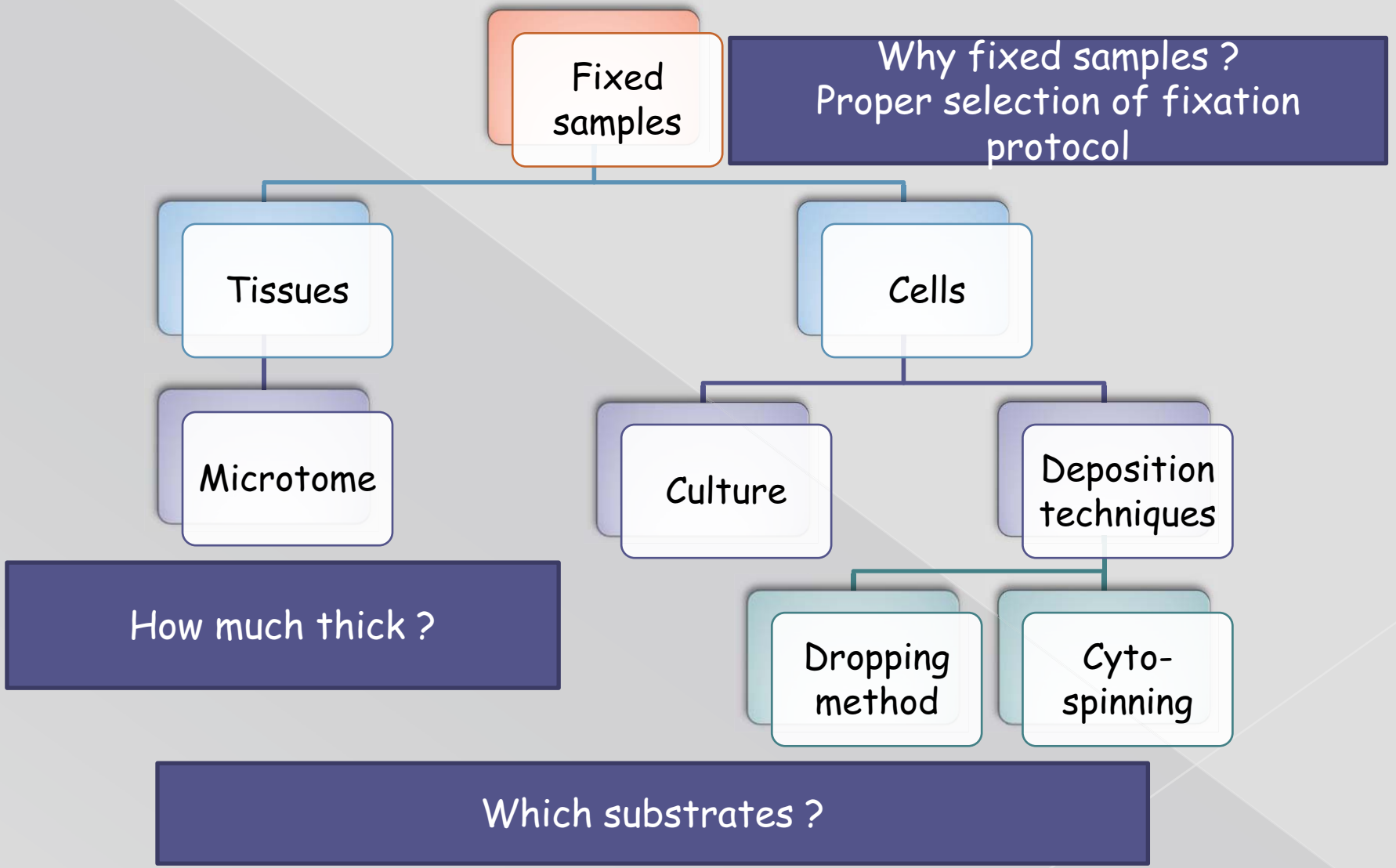
3-Charatherizing cell growth dependent phenomena and cell interaction with different agents such as drugs, pollutants, poisons, chemotactic agents: **a complementary tool to conventional biochemical assays.**

4- Analyzing blood and urine: **fast and new clinical chemistry tool.**

5- Great variety of other less conventional applications.

How can all this information be deduced from a series of infrared spectra?

An infrared bio-experiment step by step
Sample preparation_1

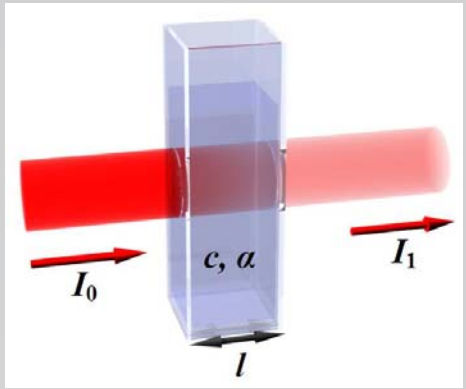


An infrared bio-experiment step by step
Sample preparation_2

Why fixed samples?

- *In-vivo* MIR imaging of internal organs (and cells) at the actual stage of the technique is still not possible
- Fiber-optic based ATR methods in the mid-infrared have been used for studying epithelial surfaces and surface skin contaminants *in-vivo* [Refs]. All the other tissues have to be analyzed *ex-vivo*.
- Conventional tissue slice thickness ranges from 5 to 20 microns, depending on the tissue type, in order to avoid signal saturation, that will make impossible the interpretation of IR data [Lambert-Beer Law].
- Single cell thickness is usually too small for inducing IR signal saturation.

The Lambert-Beer Law



$$A = -\log_{10}(I_1/I_0) = \varepsilon l c$$

$$[\varepsilon] = L \cdot mol^{-1} \cdot cm^{-1}$$

$$[c] = mol \cdot L^{-1}$$

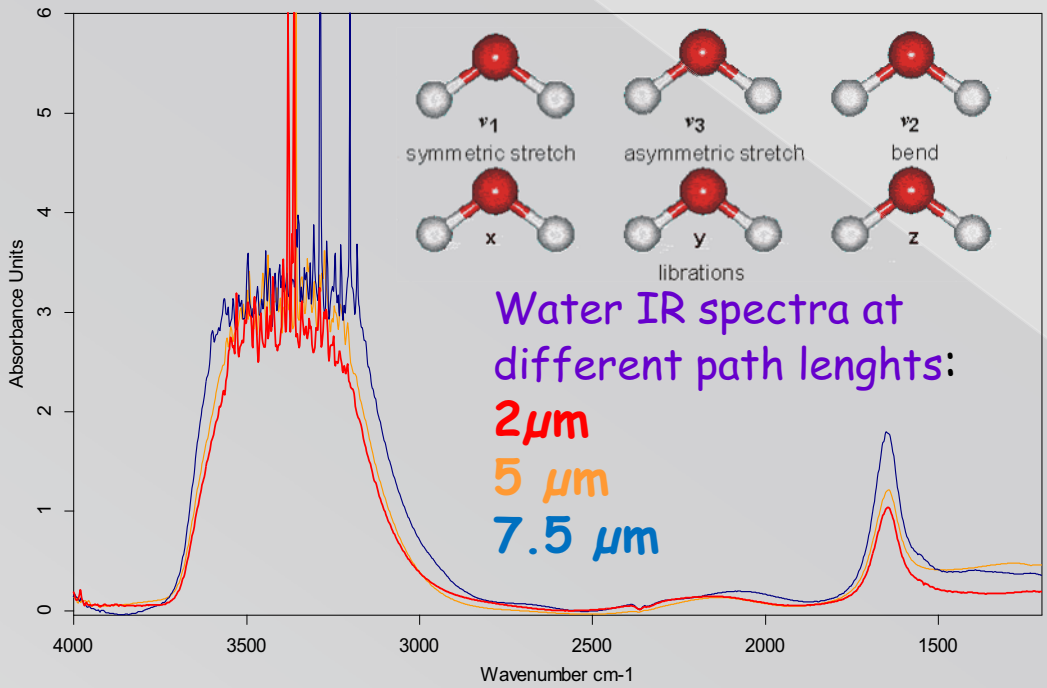
$$[l] = cm$$

- "Fiber-optic Probes for Mid-infrared Spectrometry", Peter J. Melling and Mary Thomson in *Handbook of Vibrational Spectroscopy,* John M. Chalmers and Peter R. Griffiths (Editors), John Wiley & Sons Ltd, Chichester, 2002
- L. Brancoleon, M.P. Bamberg and N. Kollias, *Appl. Spectrosc.*,54, 1175 (2000).

An infrared bio-experiment step by step
Sample preparation_2

Why fixed samples?

• *In-vitro* experiments under physiological conditions are limited by the strong water absorption bands in the Mid-IR regime.



In particular, the bending band of water centered at 1650cm^{-1}, completely hides the Amide I band of proteins, one of the most important for biologic application of IRMS.

New strategies are under development for allowing to perform *in-vitro* cellular experiments -> See "Mechanobiology of leucocytes"

An infrared bio-experiment step by step
Sample preparation_3
Proper selection of fixation protocol

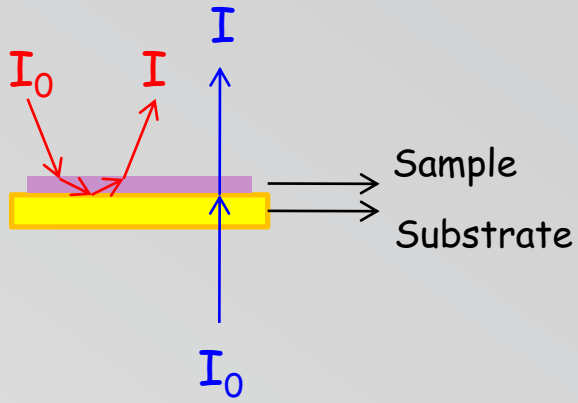
The aim of fixation is to preserve the structural and biochemical constituents of cells in as close to in vivo conditions as possible

- *Air-drying* can cause collapse of internal cellular structures and activation of cell autolysis (dramatic variation of osmotic pressure within the cells).
- *Flash-freezing* followed by cell lyophilisation (freeze-drying) can not be applied to most common FTIR materials, such as CaF_2 or BaF_2, since too brittle and with poor thermal contact.
- *Alcohol fixation* results in water extraction and decrease of the cellular volume. Water is displaced from proteinaceous material, resulting in protein denaturation and organelle disruption. Alcohol extracts lipids from cells but has little effect on carbohydrates.
- *Formalin fixative* has bands potentially overlapping with cellular constituents bands (the most intense peak occurs at 1000 cm^{-1}); however it preserves most lipids and has little impact on carbohydrates. Formalin also appears to preserve protein secondary structure.

No definite solution since direct comparison with living cell IR spectra under physiological conditions has not been performed yet.

An infrared bio-experiment step by step
Sample preparation_4
Which substrates?

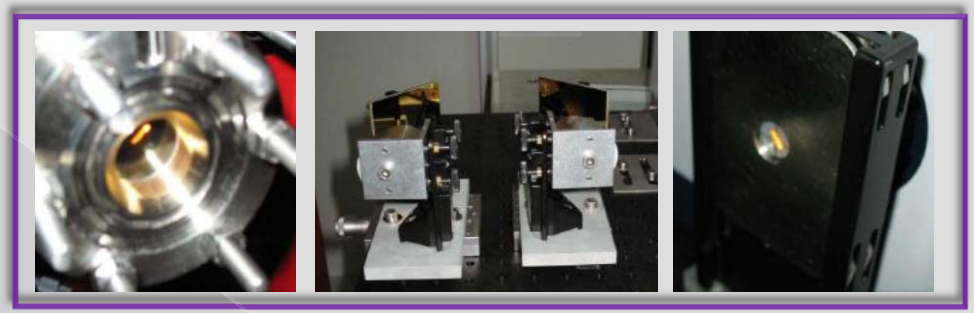
Sampling technique	Material	Vis-Transparent	MIR-Transparent	MIR-Reflective	Biocompatibility	Other
Transflection	MirrIR-slides	No	No	Totally	Yes	Cheap
Both	Si, Ge	No	Partially	Partially	Yes	Cheap
Transmission	Diamond	Yes	Partially	No	Yes	Expensive +
	BaF_2	Yes	Totally	No	No	Expensive
	CaF_2	Yes	Partially	No	Possibly	Expensive
	ZnSe	Slightly	Partially	No	No	Expensive
	Si_3N_4	Slightly	Partially	No	Yes	Fragile
	TEM grids	Yes	Totally	No	Yes	Fragile

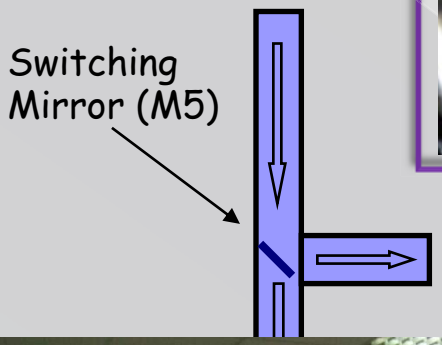


The optimal IR substrate

- Biocompatible, for performing cell culture on it
- IR transparent/reflective in the MIR
- Vis transparent/reflective, to easily match Vis and IR data
- Cheap and/or recyclable
- Exploitable also for other investigation techniques

An infrared bio-experiment step by step
Data collection_1





Coupling box

VERTEX 70

Hyperion 3000

elettra

2nd Branch *(Elettra)*
Life Sciences
Optimized for MIR microspectroscopy

Open to users since October 2006

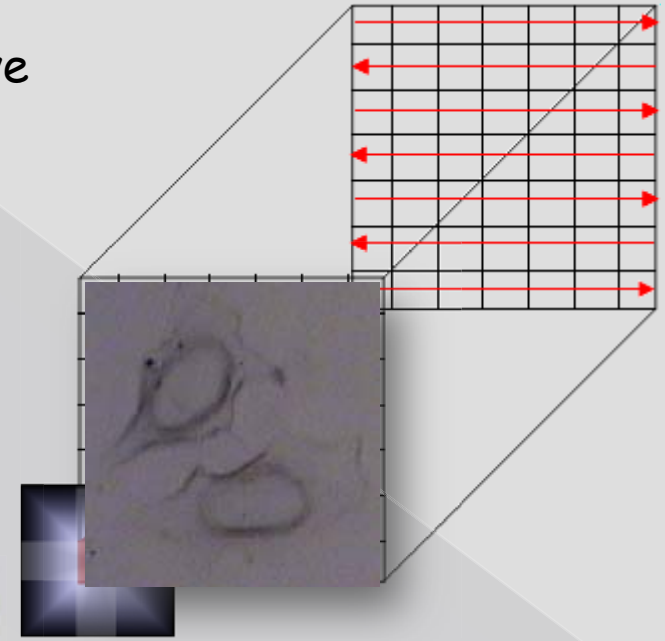
1st Branch *(CNR-INFM)*
Solid State Physics & High Pressures
Broad working range (2-20000 cm^{-1})

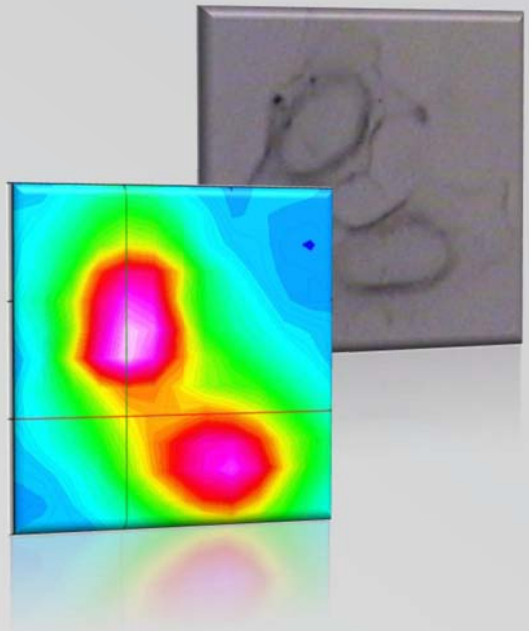
Open to users since January 2006

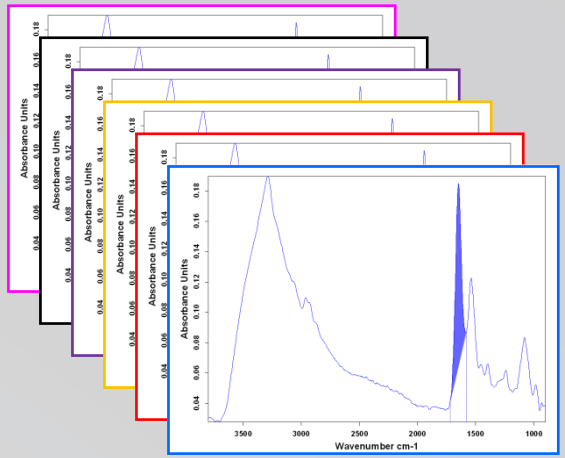
COHERENTIA
INFM

An infrared bio-experiment step by step
Data collection_2

Schwarzschild objective







Acquisition parameters

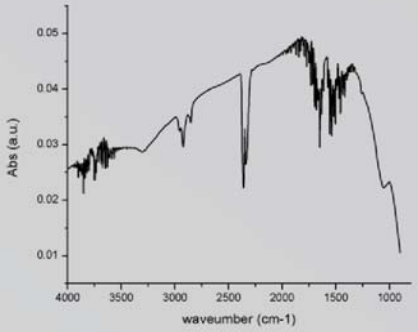
- **Knife edge apertures**, in order to match the desired experimental spatial resolution avoiding diffraction artifacts
- **Number of scans per spectral point** in order to enhance S/N ratio without dramatically increasing the acquisition time

An infrared bio-experiment step by step Data Analysis_1

Pre-processing of data

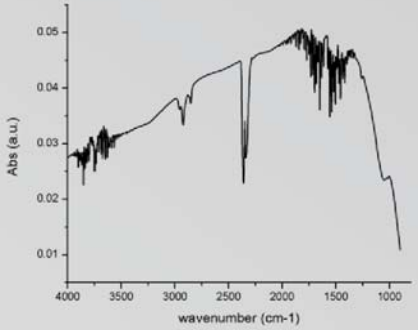
Original Spectrum

Sample Single channel

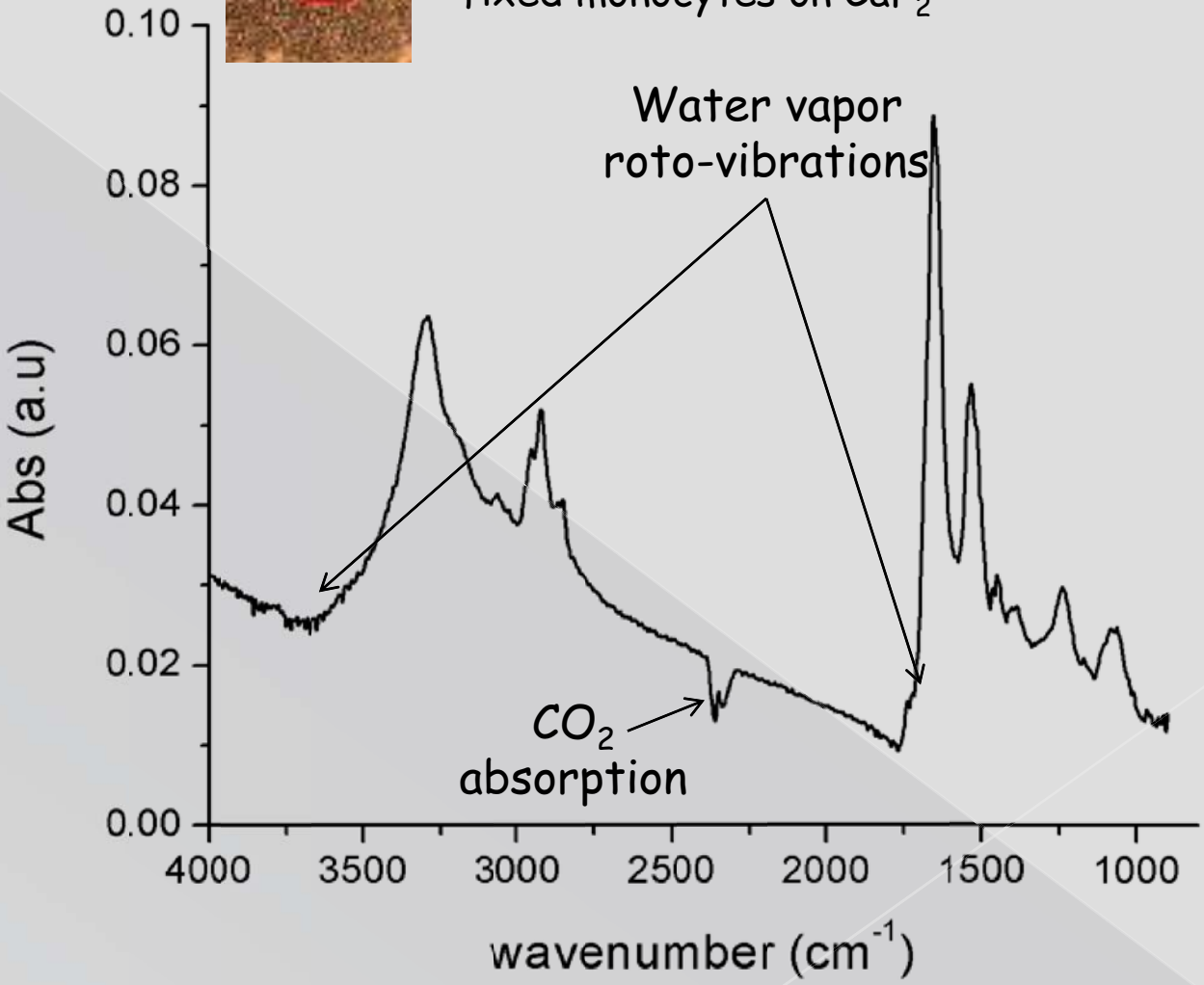


$$Abs = -\log\left(\frac{SSC}{RSC}\right)$$

Reference Single channel

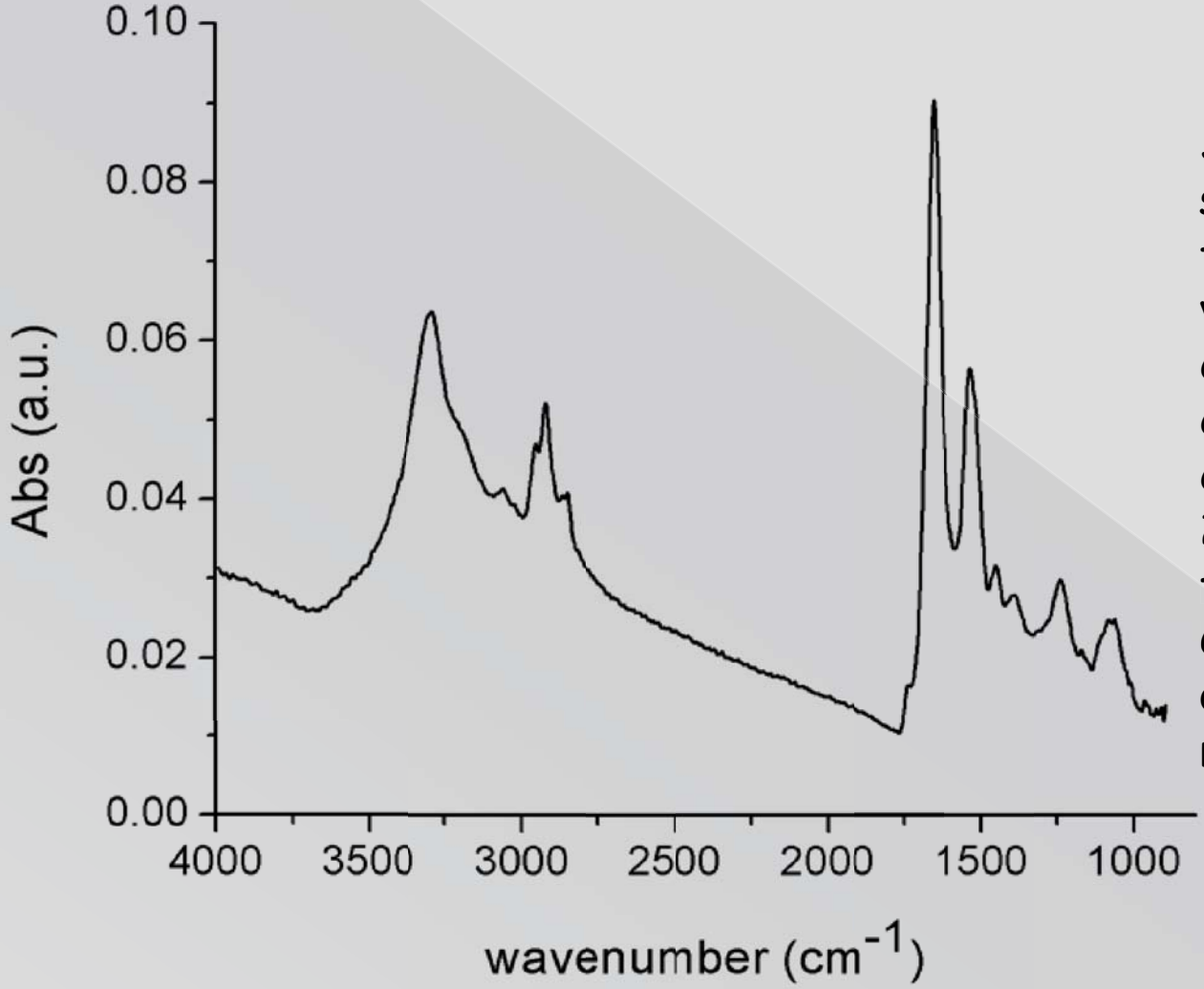


Homogenous monolayer of formalin fixed monocytes on CaF_2



An infrared bio-experiment step by step
Data Analysis_1

Pre-processing of data

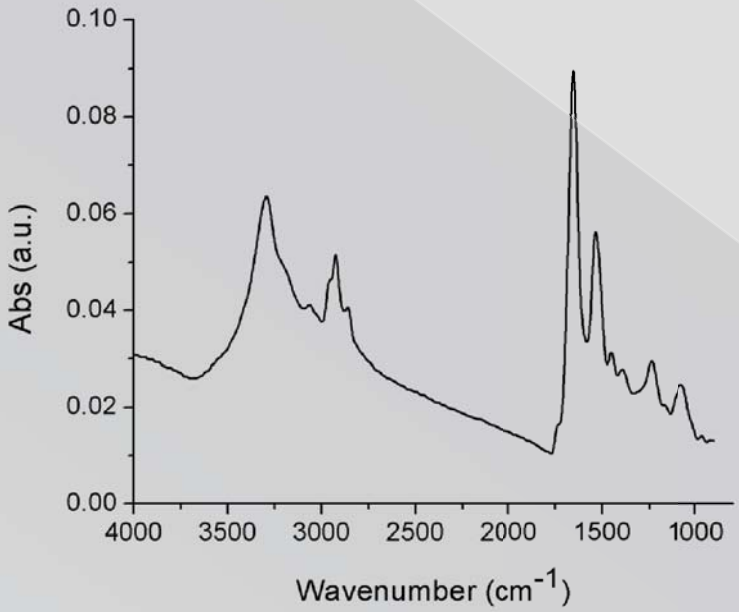


Atmospheric compensated Spectrum

Spectral regions free of sample bands are used for evaluating water vapor and carbon dioxide content: 3600 to 4000 cm^{-1} region for H_2O compensation, and 2300 to 2400 cm^{-1} region for CO_2.
Correction is extended over the entire spectral range.

An infrared bio-experiment step by step Data Analysis_1

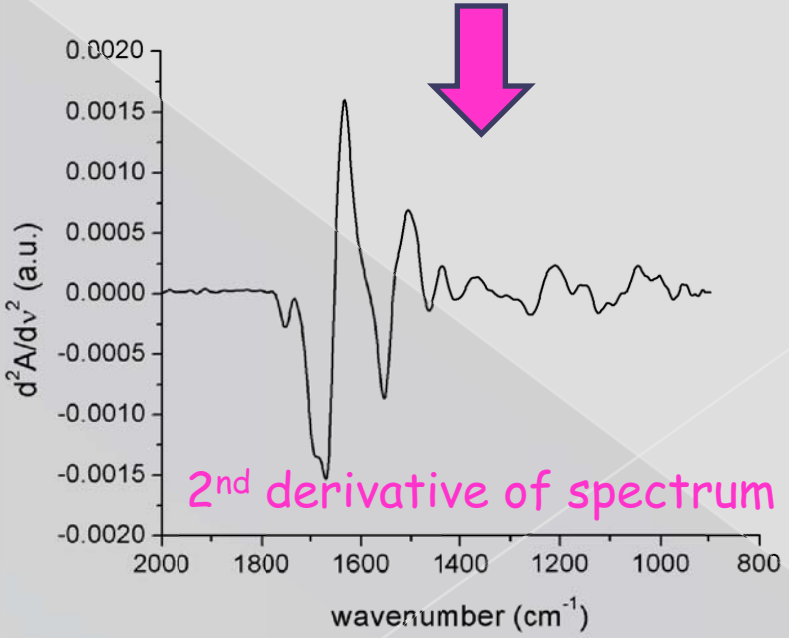
Pre-processing of data



Smoothed Spectrum
Savitzky-Golay method, K+1 smoothing points

The method essentially performs a local polynomial regression on a series of values (k+1 points, equally spaced in the series) to determine the smoothed value for each point. This method is also provided for calculating the first up to the fifth derivatives of spectra.

First or second derivatives of spectra are often used for data analysis, in order to minimize baseline variations and maximize spectral resolution.



An infrared bio-experiment step by step
Data Analysis_1

Pre-processing of data

Water vapor and carbon dioxide contributions to bio-specimen spectra as well as the spectral noise can be greatly reduced:

- Purging the interferometer with N2/dry air or operating in vacuum
- Purging the microscope stage environment with N2/dry air
- With a good conditioning of the laboratory environment

Too many people around the microscope!

- Enhancing the S/N spectral ratio
    - Increasing the number of scans
    - Increasing the signal -> Brighter Sources -> Synchrotron Radiation
    - Reducing any possible source of noise: vibrations, electronic noise, …..

An infrared bio-experiment step by step
Data Analysis_2

Spectra comparison

Spectral analysis "by visual inspection"

For small data sets (few spectra), spectra can be compared "visually" in order to highlight spectral similarities and/or differences affecting:

- Band position (band shifts), width (band broadening) and shape (band components)
- Ratios of peak areas (different proportion of most fundamental tissue-cell constituents).

The reliability of biological conclusions drawn out from an experiment relies on the measurements of a statistically relevant number of samples.

Statistical analysis

Univariate and multivariate statistical analysis methods allow to compare a huge amount of spectra simultaneously, classifying them on the base of spectral similarities, affinities.

Univariate Methods: Average, standard deviation, regression techniques (PLS,….)

Multivariate Methods: **Cluster Analysis**, Principal Component analysis (**PCA**), ….

An infrared bio-experiment step by step Data Analysis_2

1- Cluster Analysis

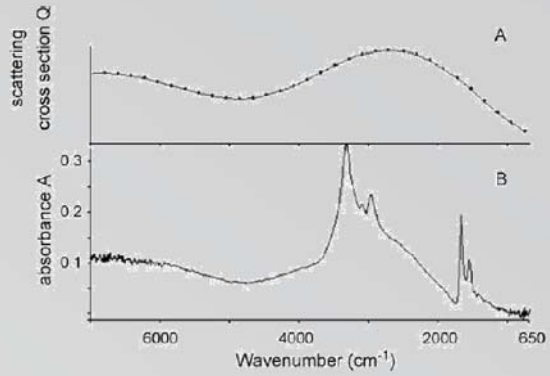
1. Data pre-processing

Spectra are **normalized** in order to process an homogenous data set. Commonly employed normalization methods (alone or in combination) are:

1. Vector Normalization

$$a_m = \frac{\sum_k a(k)}{N} \quad \text{average value;} \quad a'_k = a(k) - a_m \quad \text{subctracted spectrum;} \quad a''_k = \frac{a'_k}{\sqrt{\sum_k (a'(k))^2}} \quad \text{normalized spectrum}$$

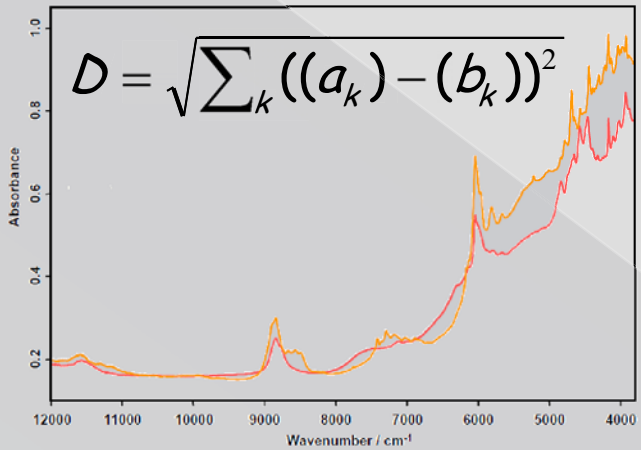
2. (Extended) Multiplicative Scattering Correction (E)MSC



Single cell spectra often present slow oscillations of the baseline. The origin is that the interaction of electromagnetic radiations with dielectric spheres of dimensions close to that of the wavelength of light induces a strong scattering that can be modeled with the **Mie Scattering theory**. EMSC correct for this effects modeling the scattering object as a non-absorbing dielectric sphere. New algorithms are under development that consider the absorbing characteristics of the scattering object.

3. Derivative (1st or 2nd)

An infrared bio-experiment step by step
Data Analysis_2
1- Cluster Analysis



2. Spectral distance calculation

Distance between spectra *a* and *b* can be calculated with many algorithms.

Euclidean spectral distance between *a* and *b* spectra is calculated over the all sampled *k* points.

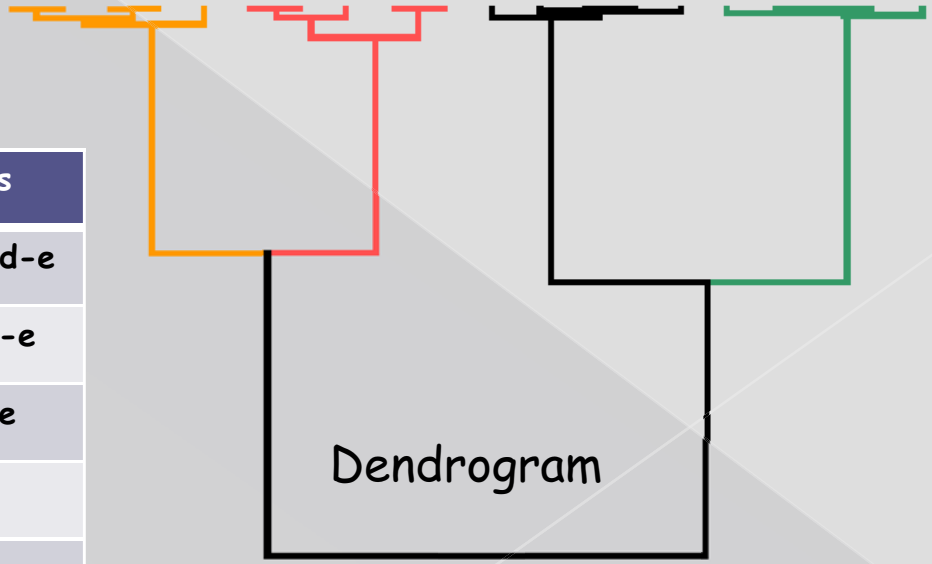
3. Spectral distance matrix

	a	b	c	d	e
a	0				
b	44	0			
c	11	54	0		
d	100	68	97	0	
e	120	92	115	21	0

There are many methods available to calculate spectral distances between a newly-created cluster and all the other spectra or clusters.

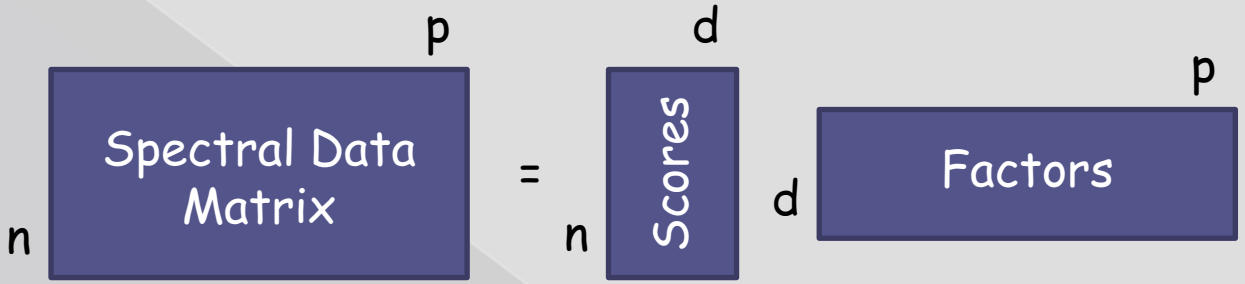
N	Clusters
1	a-b-c-d-e
2	ac-b-d-e
3	ac-b-de
4	abc-de
5	abcde

4. Spectra clustering

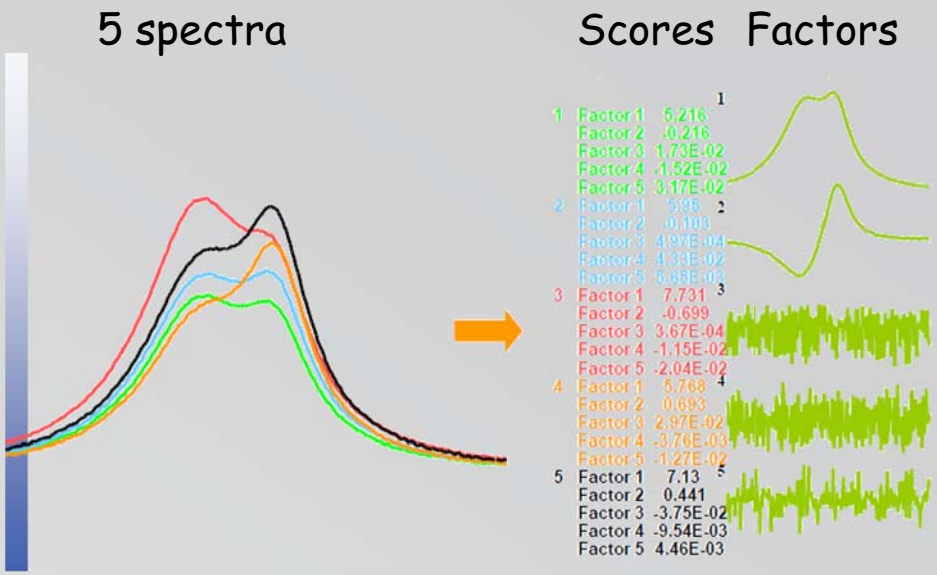


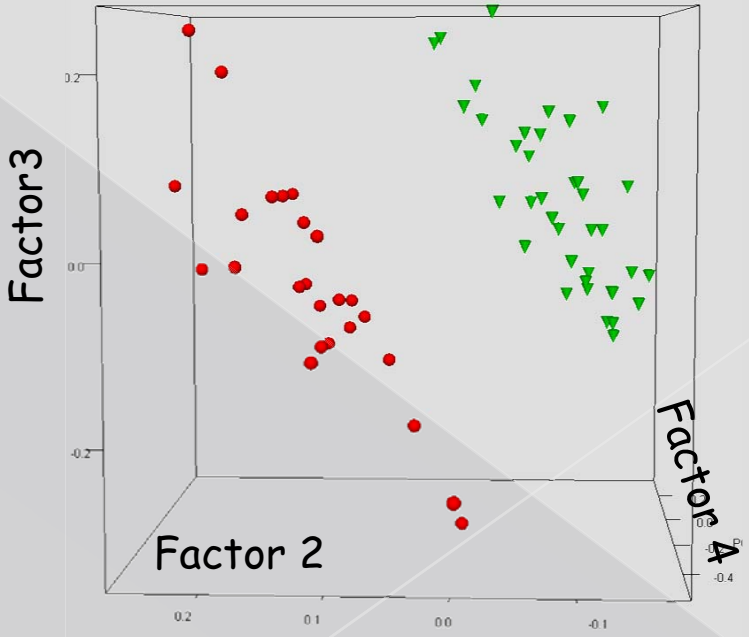
An infrared bio-experiment step by step Data Analysis_2

2- PCA – Principal component Analysis



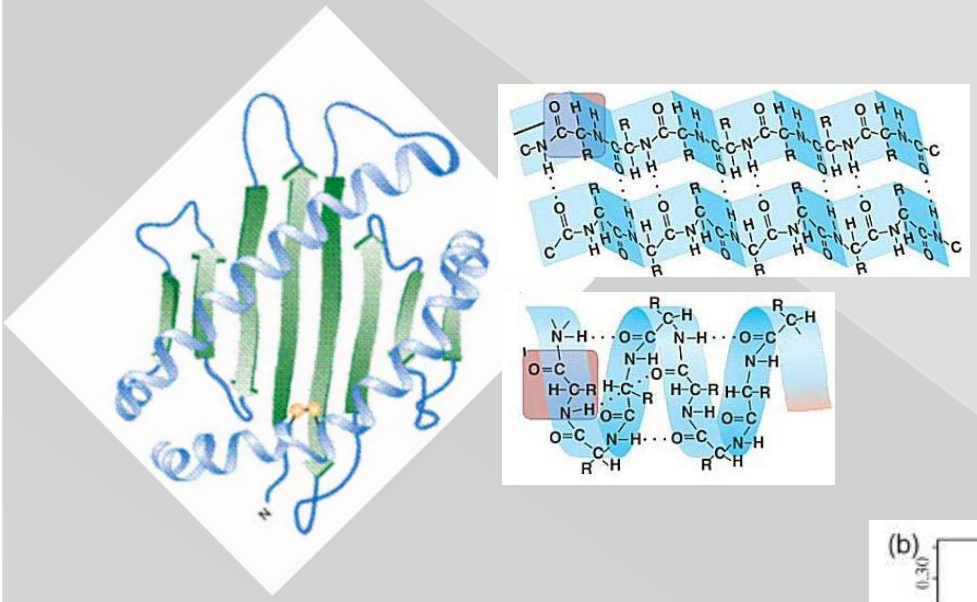
n spectra with p data points; d scores for each spectrum (d<n); d factors with p data points (d<n)





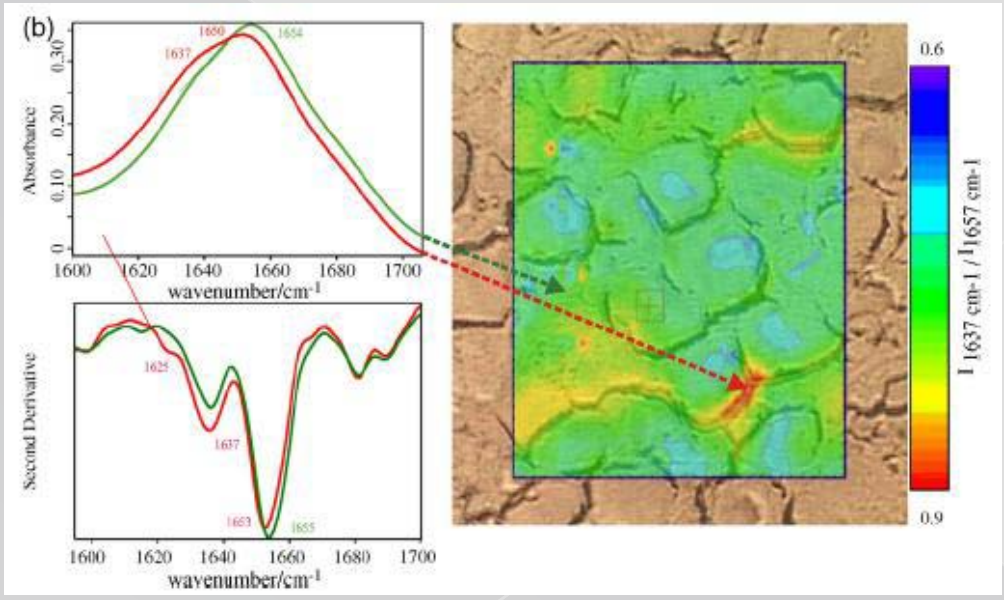
SR IRMS and Prion research

Protein structural information from Amide I band



Amide I band – 1700-1600 cm^{-1}	
1695-1675	Antiparallel β-sheet/Aggregated strands
1670-1660	3_{10} - Helix
1660-1648	**α-helix**
1648-1640	Random coil
1640-1625	**β-sheet**
1628-1610	Aggregated strands

… exploited for studying Transmissible spongiform encephalopathies (TSEs, also known as prion diseases)

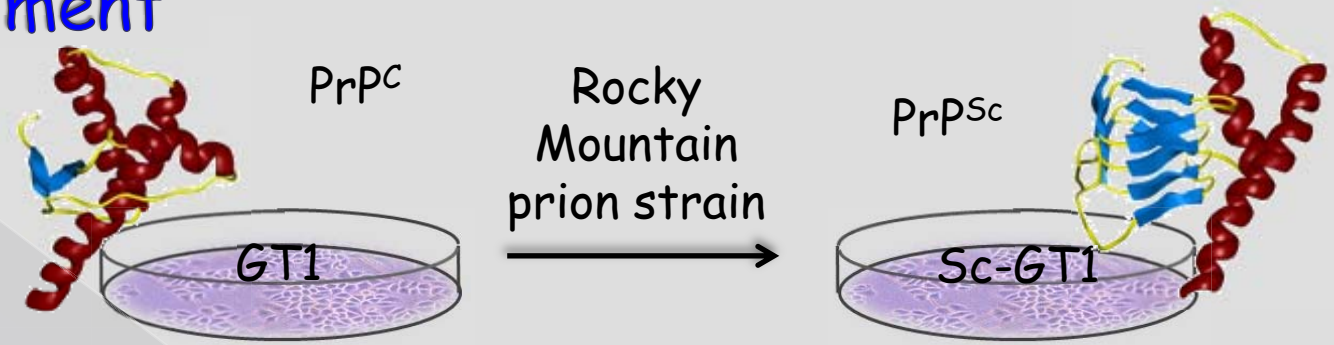


M. Beekes et al., Analytical applications of Fourirer transform-infrared (FT-IR) spectroscopy in microbiology and Prion research, Veterinary microbiology (2007), 123:305-319

SR IRMS and Prion research

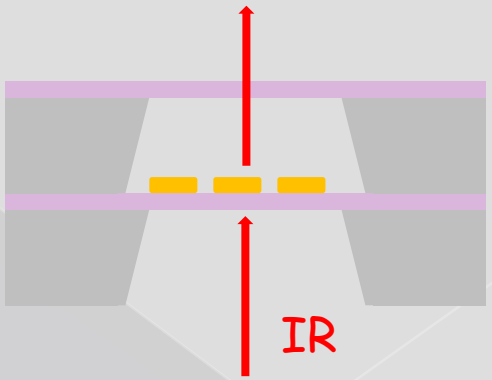
Design of experiment

- Sample preparation



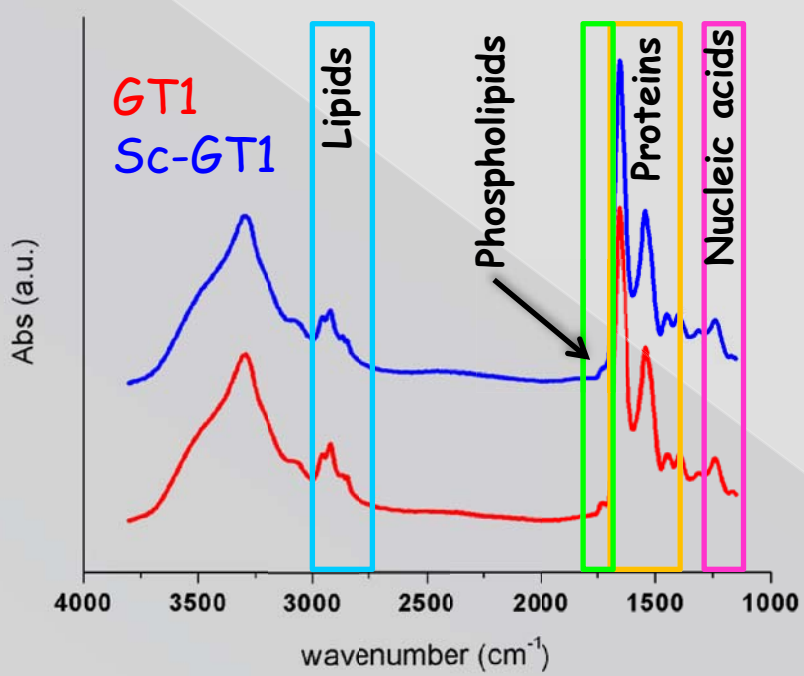
Cells were grown on silicon nitride 100nm membranes (GT1 cells do not preserve vitality when cultured on CaF_2 windows) and fixed in formalin 4%. An IR measurement chamber was then realized gluing a second window onto the first to guarantee the **biological safety**. Once concluded the experiment, samples are disposed (Si_3N_4 membranes are quite cheap).

- Data acquisition (TR mode)
- Individual whole-cell analysis
- Intra-cellular analysis
- Conventional biochemical assays



Prion Laboratory , Neurobiology Sector, SISSA
Prof. G. Legname, A. Didonna

Individual whole-cell analysis_1



Acquisition parameters

Spectra have been averaging 1024 scans. Knife edge apertures have been set at 30X30 μm in order to match an entire cell

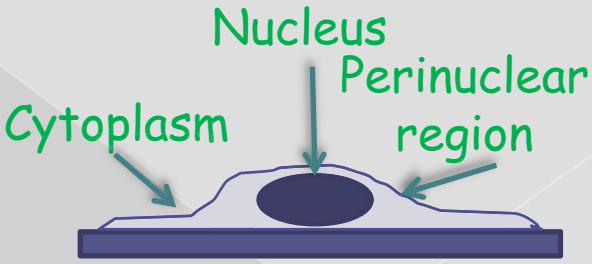
Data preprocessing

- Atmospheric compensation
- Cut :3800-1150 – Spectra have been cut at 1150 cm^{-1} since (i) absorption band of silicon nitride is hardly compensate below 1200 cm^{-1} (ii) the more intense band of formalin fixative is falling between 1100-1000 cm^{-1}. With this choice, both problems are avoided.

Is IR spectroscopy a quantitative analytical technique?

Yes, it is $A = -\log_{10}(I_1/I_0) = \varepsilon lc$

BUT the cell thickness is not homogenous and it differs from cell to cell, even within the same cell type

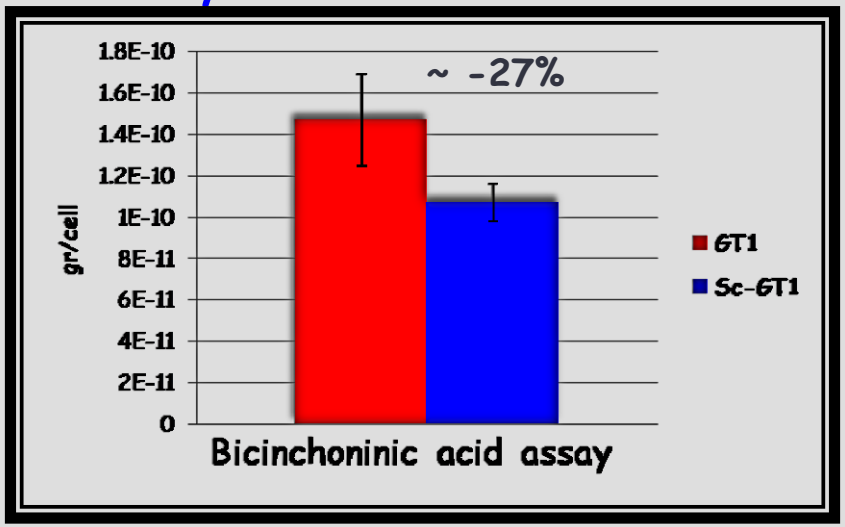


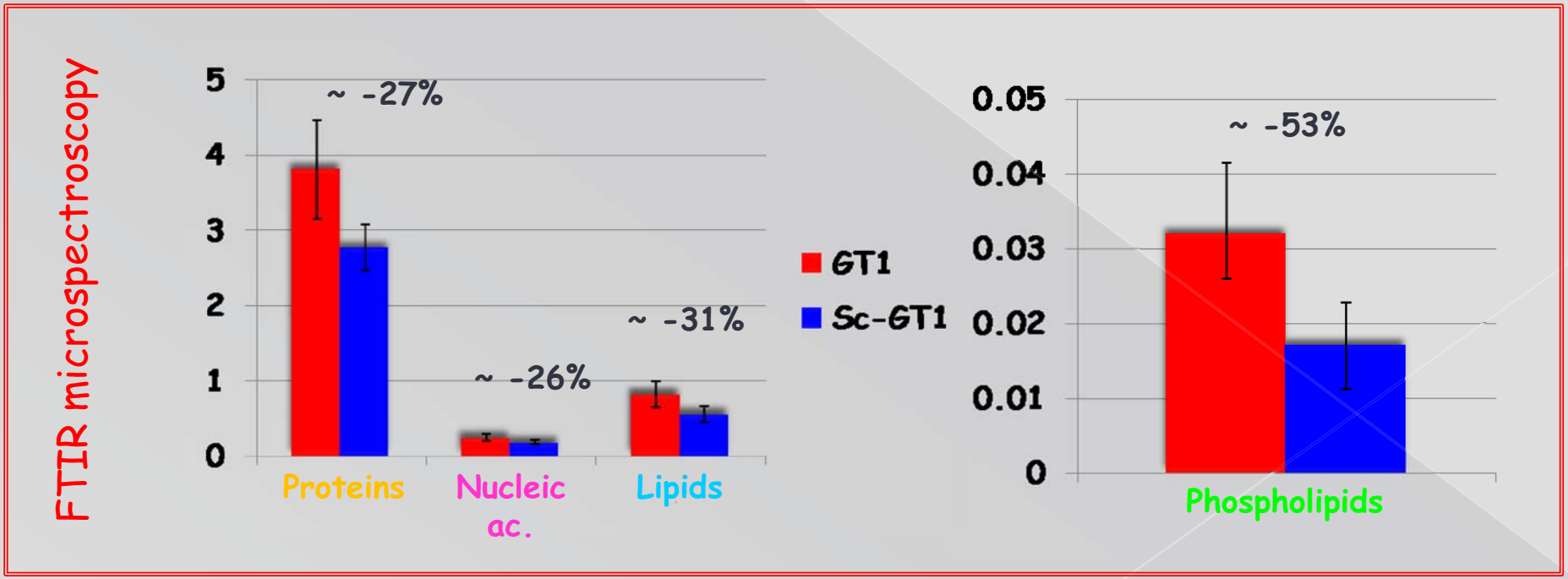
Only relative variations in concentrations of most fundamental cellular macromolecules can be deduced (such as Protein to Lipid ratio) by rationing associated IR band areas (or heights) unless some additional information are available!

Individual whole-cell analysis_1

Semi-quantitative analysis

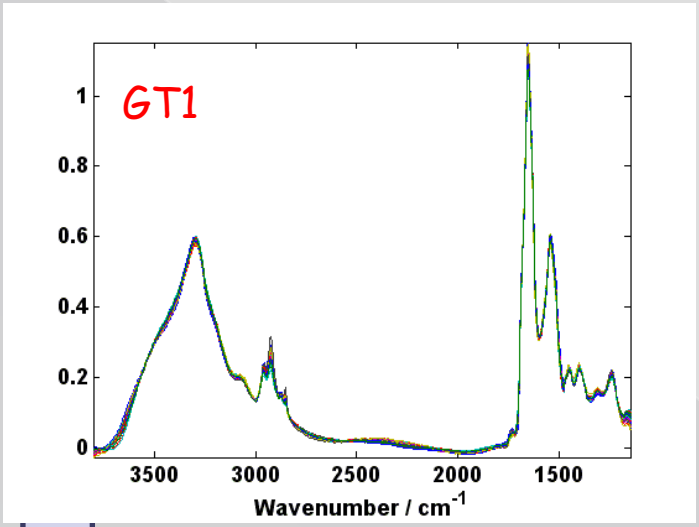
1. GT1 and Sc-GT1 cells are indiscernible from a morphological point of view as known from the scientific literature and AFM (Atomic Force Microscopy) investigations .
2. A conventional biochemical assay (Bicichoninic acid) was performed in order to verify the protein cellular content variation upon infection, giving results comparable with IR analysis.

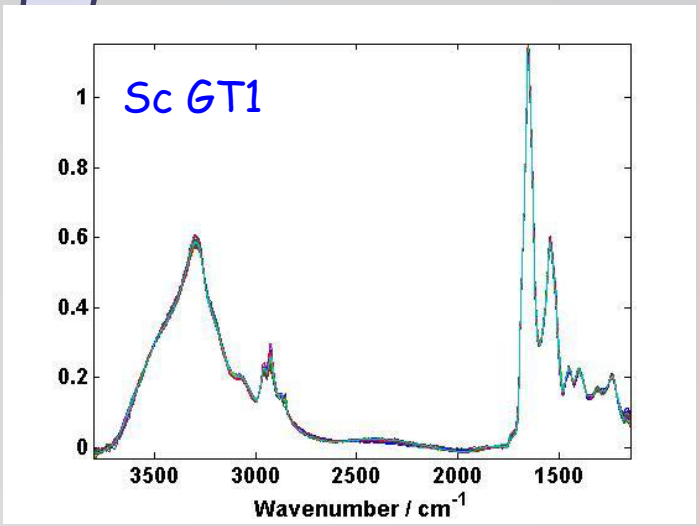




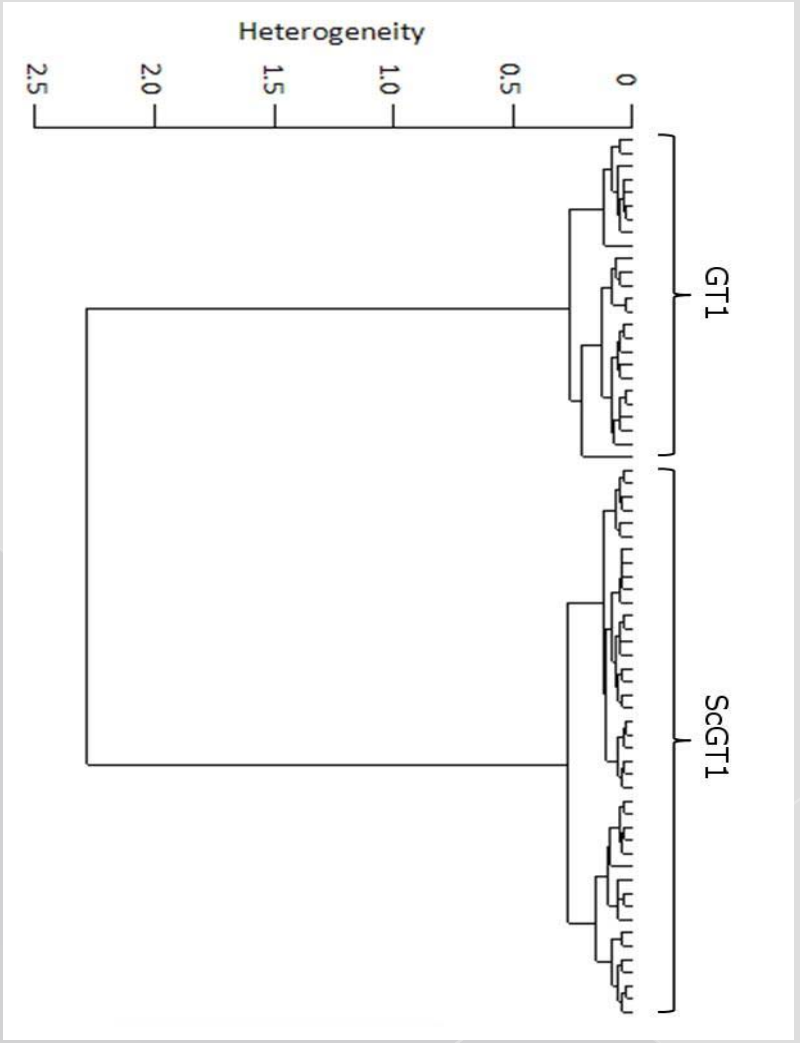
Individual whole-cell analysis_2

Classification of spectra





Cluster Analysis

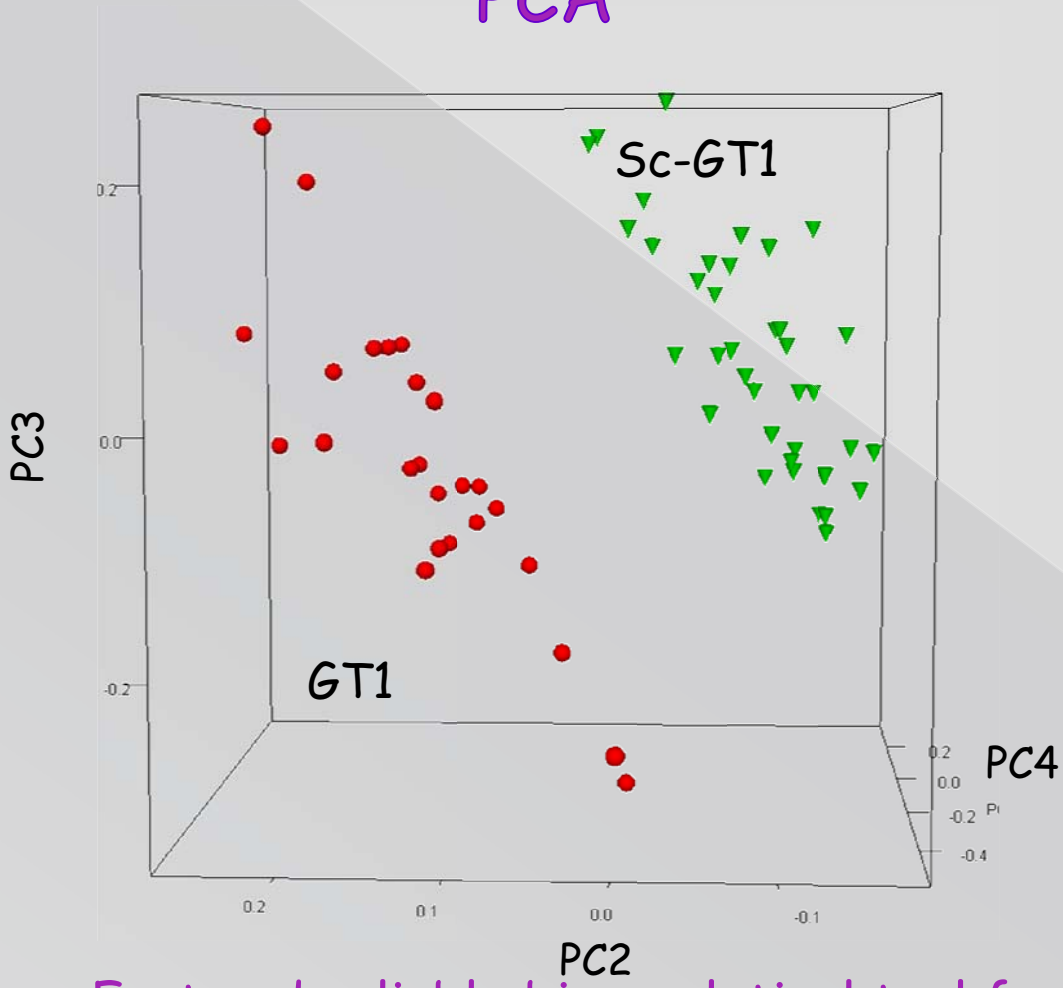


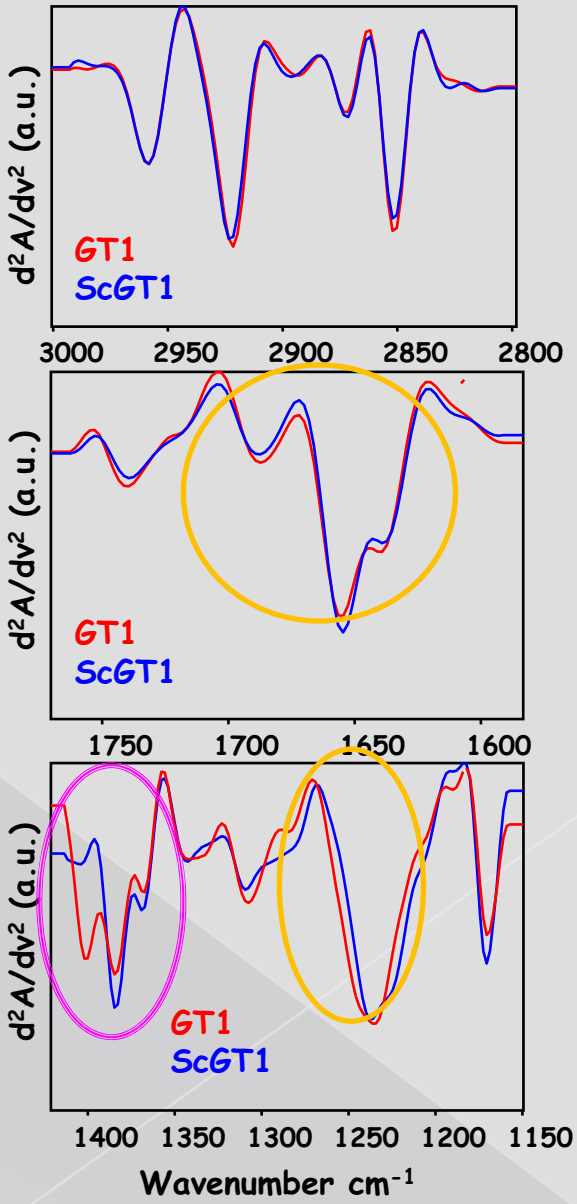
First derivative of spectra
Euclidean distances & Ward's algorithm

Individual whole-cell analysis_2

Classification of spectra

PCA





Fast and reliable bio-analytical tool for discriminating between healthy and infected cells

Intracellular analysis

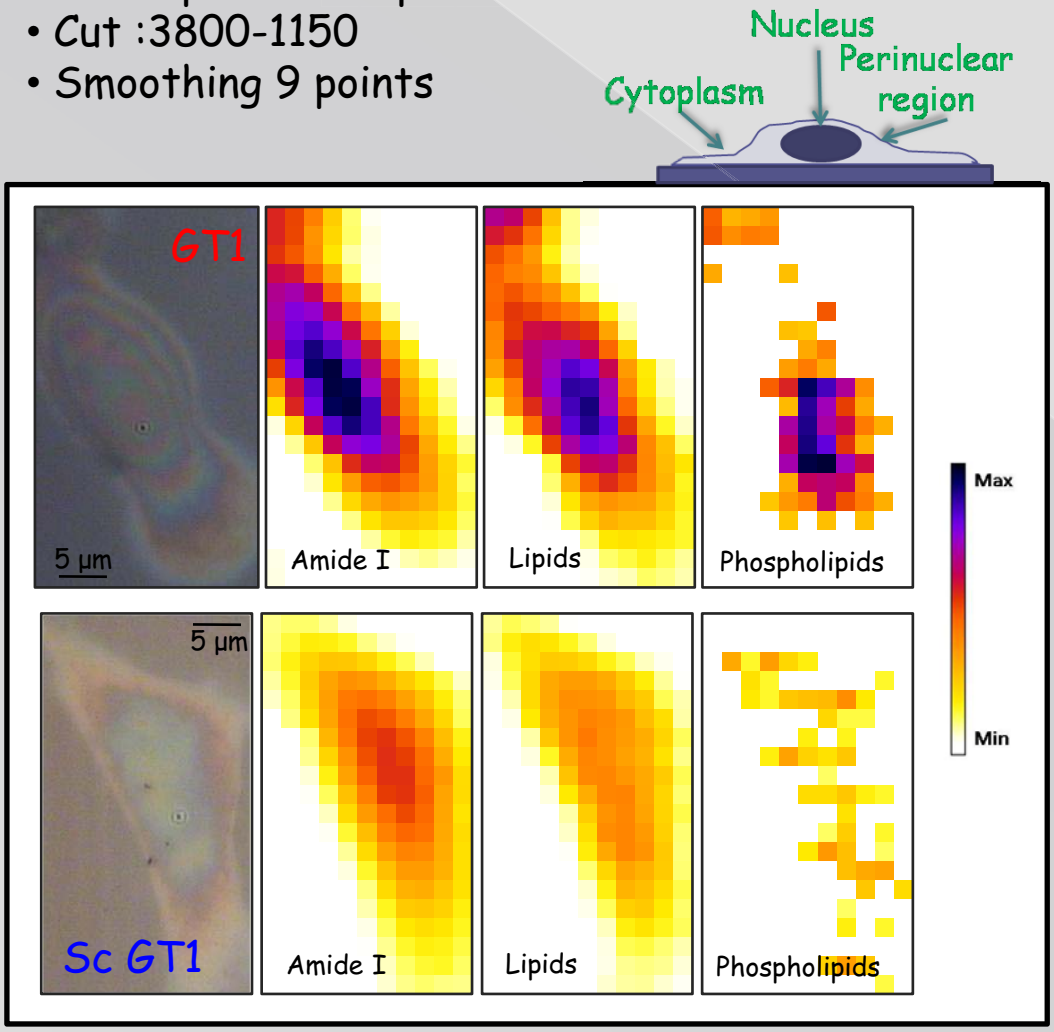
Acquisition parameters

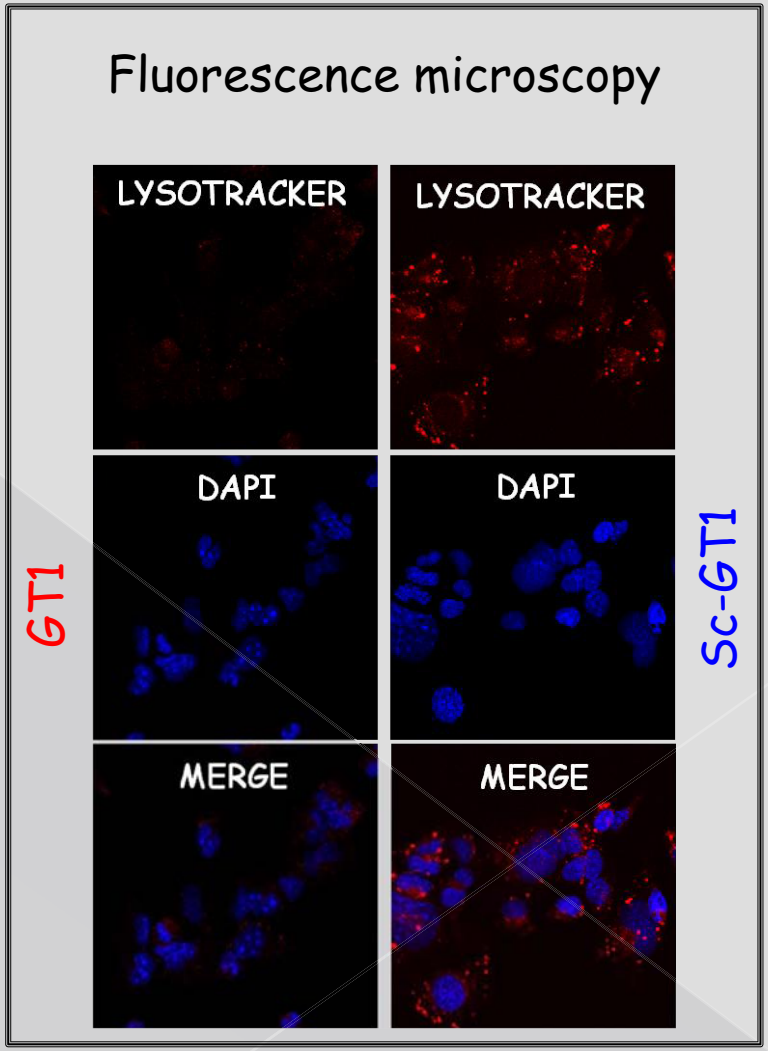
512 scans; Knife edge apertures set at 6X6

Amide I band ~ 6 µm; Phospholipid band ~ 5.7 µm; Lipid band 3.4 µm

Data preprocessing

- Atmospheric compensation
- Cut :3800-1150
- Smoothing 9 points





Adaptive immunity modulated by hBD2

Host Defense Peptides (HDPs): short introduction

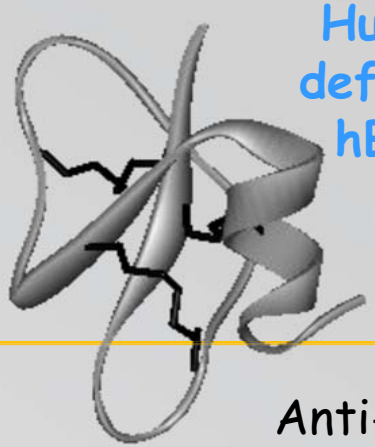
✓ Evolutionarily ancient component of INNATE IMMUNITY in multicellular organisms

✓ DIRECT KILLING of invading pathogens and/or MODULATION of IMMUNE and healing responses of the host.

✓ Huge DIVERSITY in sequence and structure

✓ Two major families: ***Cathelicidins*** and ***Defensins***

Human cathelicidin LL37



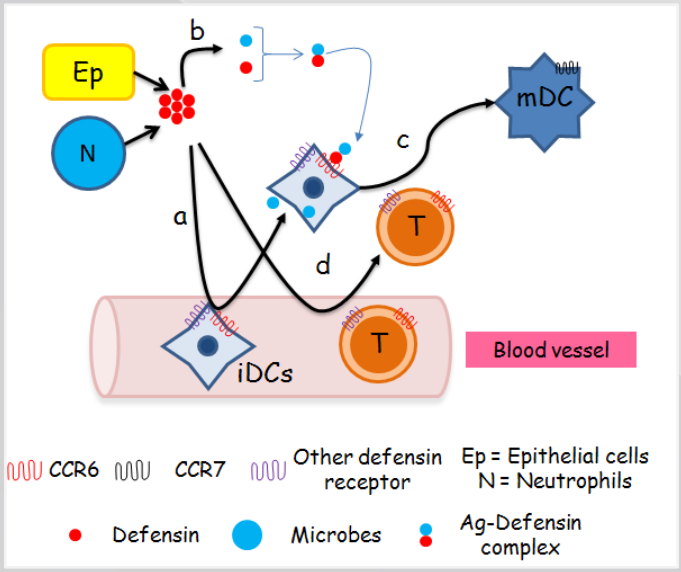
Human defensin hBD2

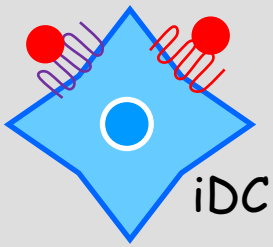
TEMPLATE
for development of
antimicrobial compounds
and/or
immunomodulatory agents

Anti-infective Laboratory
Life Sciences Dept., University of Trieste
Prof. A. Tossi, Prof. R. Gennaro and Prof. Sabrina Pacor
PhD thesis of Francesca Morgera

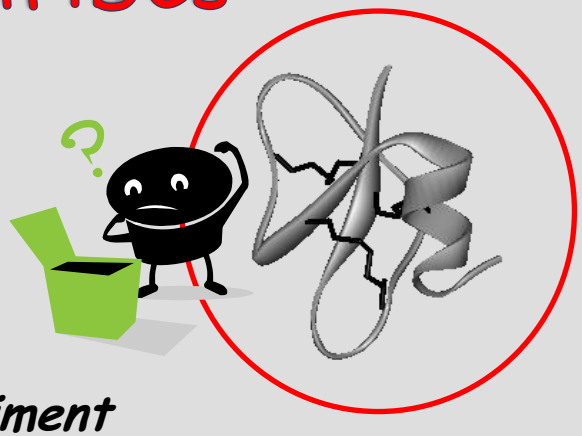
Life Sciences Dept

Short term effects of hBD2 on iDCs





Short term →



Design of experiment

iDC generation from peripheral blood monocytes

hBD2 1uM 30'

Control: Ct, T

Treated: Ct, T

Fixation
Dehydration (EtOH)

Ct T → Ct T

Resin inclusion

→ **TEM**

ETOH 70% fixation

FITC/PI staining

Ct T → Ct T

→ **Flow cytometry & Fluorescence microscopy**

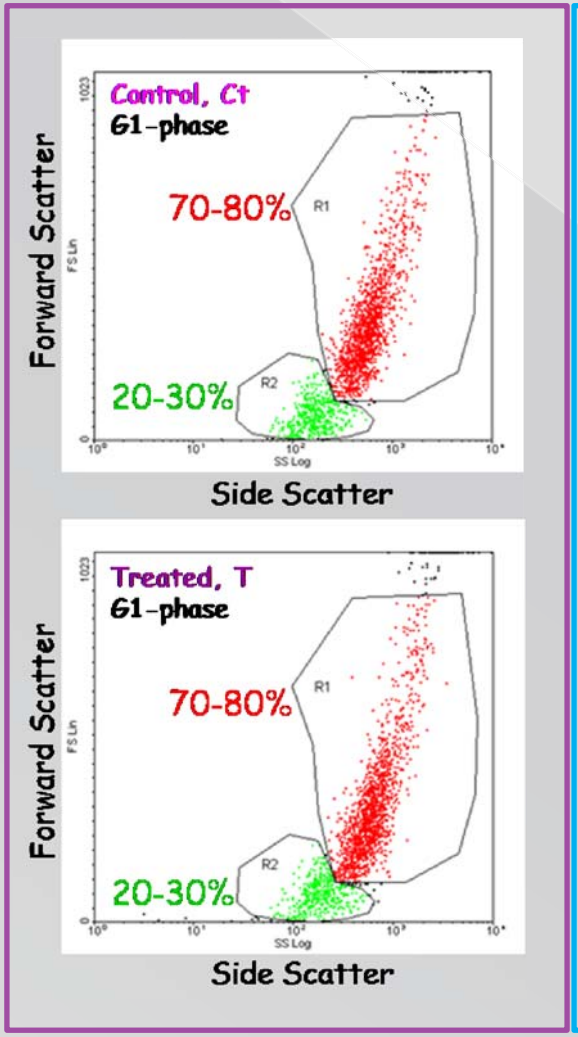
Deposition on CaF_2 window or SiN_3

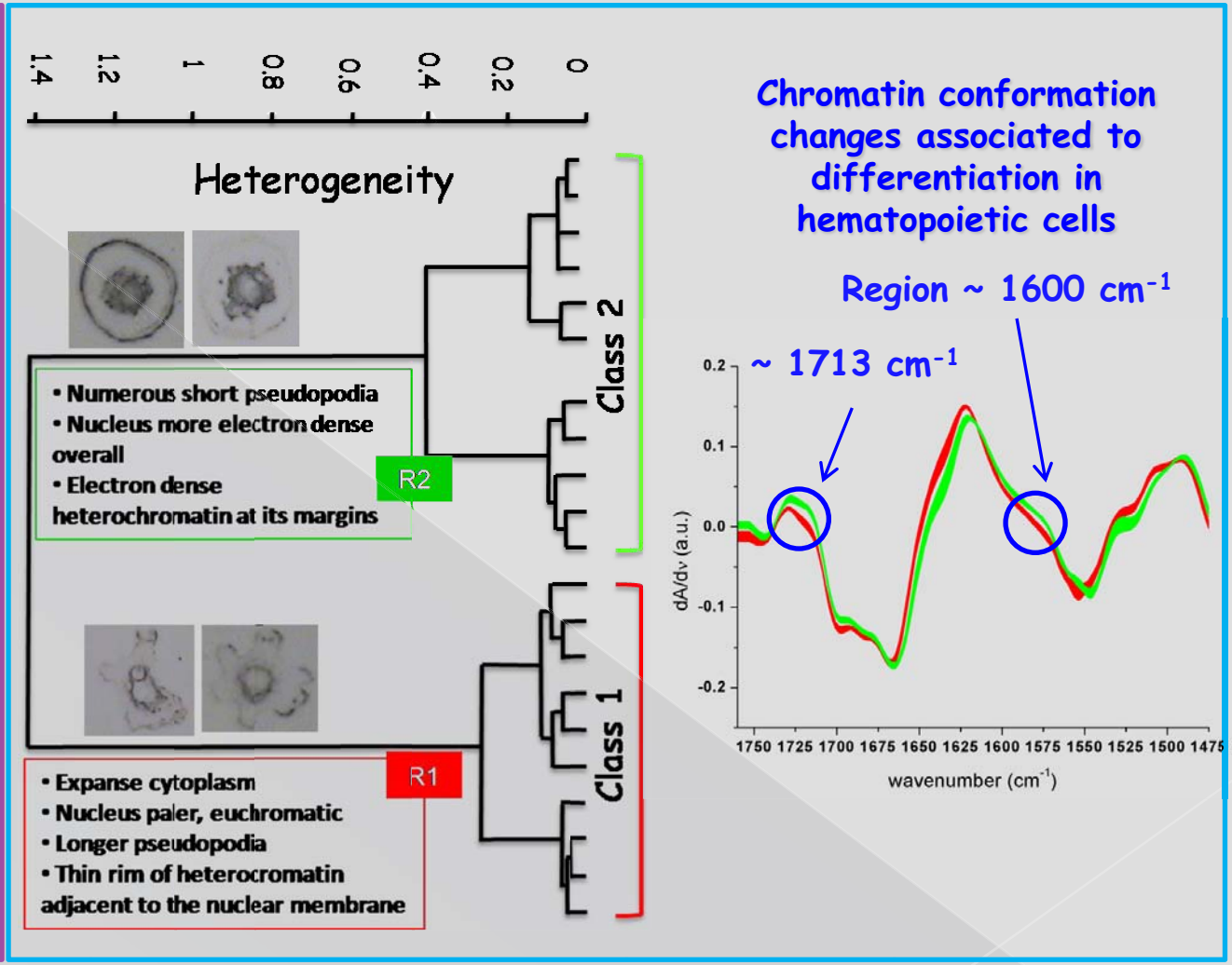
4% formalin fixation 20 min RT

Ct T → Ct T

→ **μ -FTIR**

Flow cytometry & single cell μ-FTIR

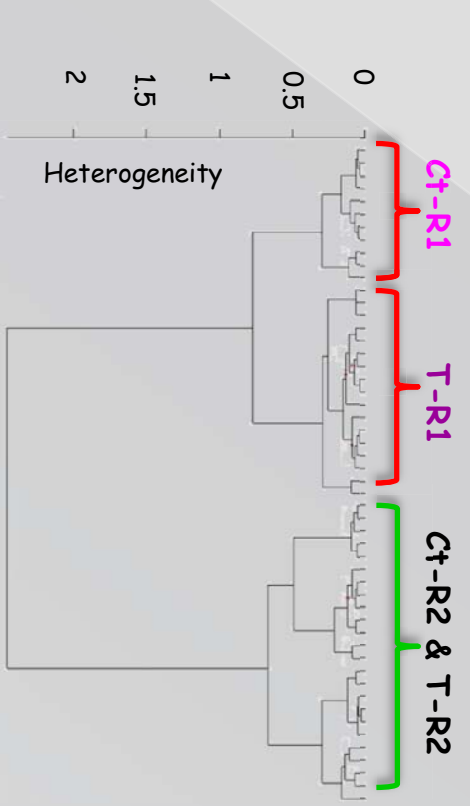


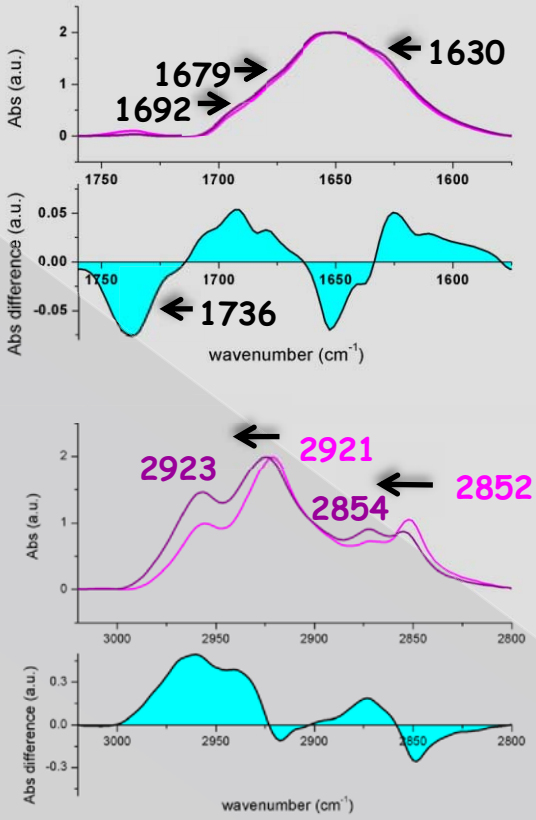


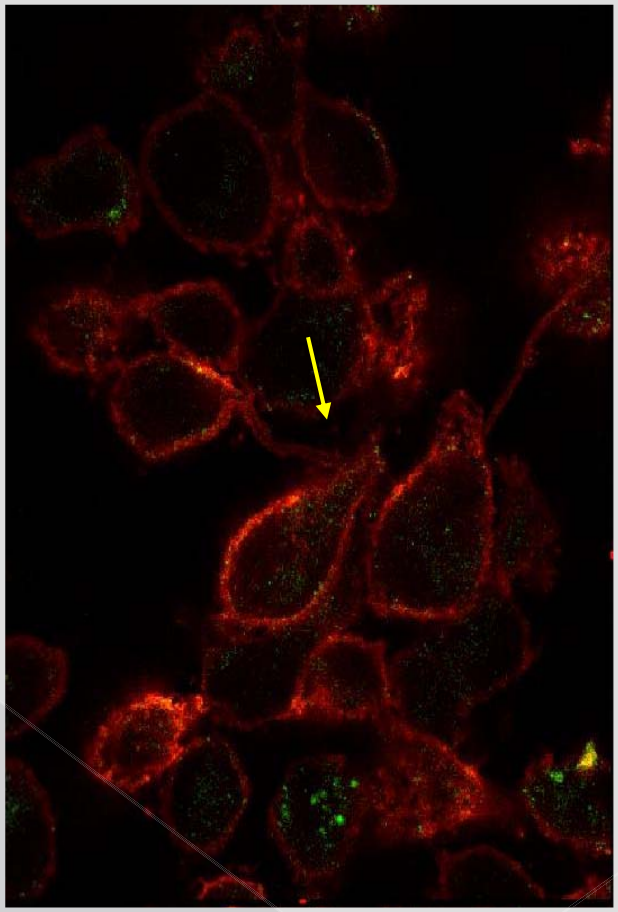
R1=completely differentiated, responsive iDC

R2= non completely differentiated, unresponsive cells

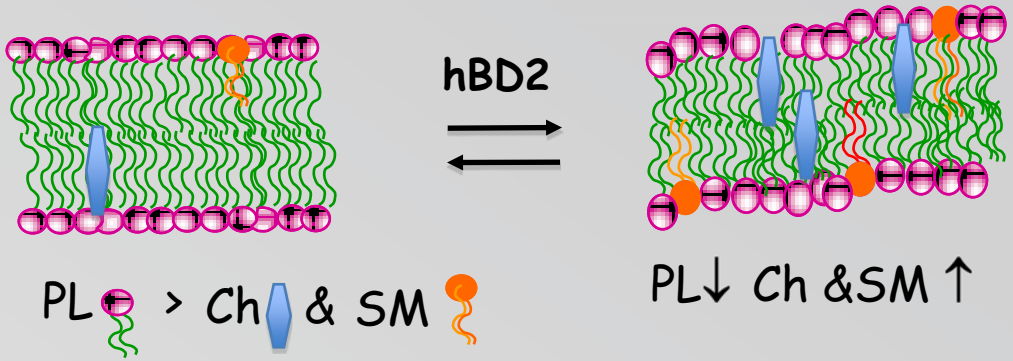
Single cell μ-FTIR & Fluorescence microscopy





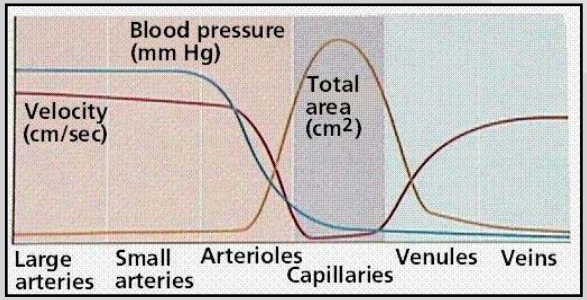


Plasma membrane Ch depletion affects hBD2 internalization

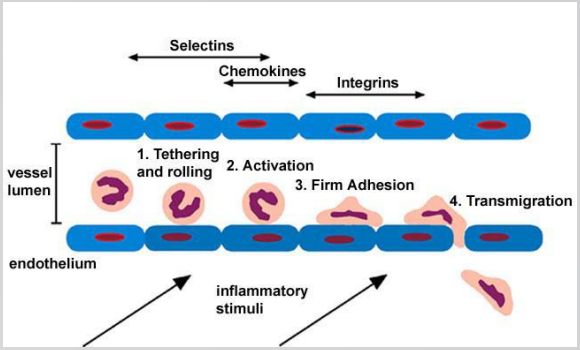


Living cell μ-FTIR measurements
Mechanobiology of leucocytes

Pressure-driven deformation



Chemically-driven deformation



- **Fluidic cells with a carefully and reproducible path length (<9μm) for a precise and reliable water subtraction**
  - **Amide I band disclosure**
- **Fluidic devices shaped with narrow channels (few microns wide and thick) in order to mimic microcapillaries and/or epithelium interstices**

Microfabrication is needed

Microfabrication facilities

L. Businaro, G. Grenci, B. Marmiroli

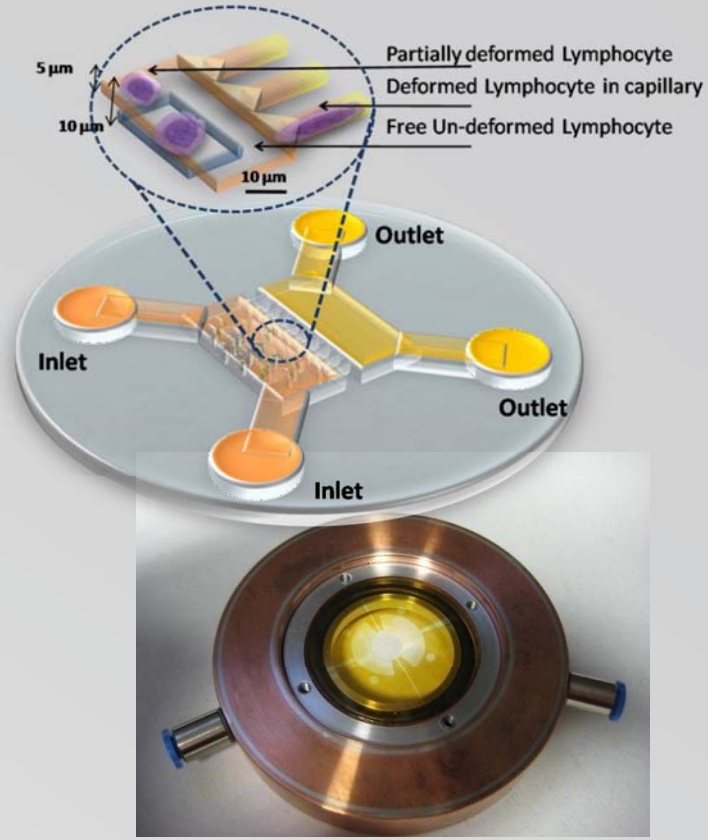
Prof. Sabrina Pacor

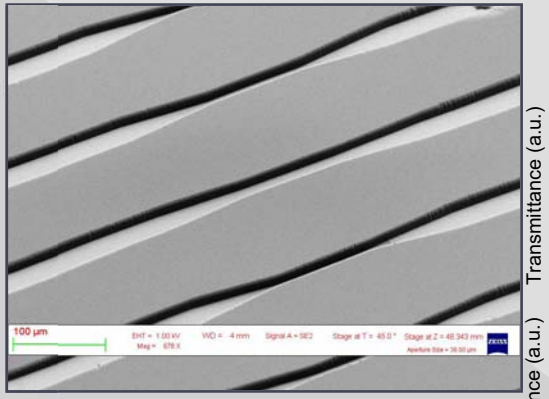
PhD activity of Giovanni Birarda

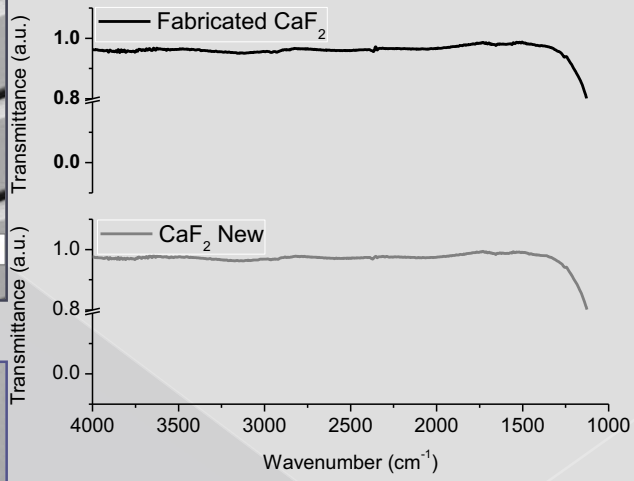
The microfabrication issues

Calcium fluoride: new fabrication strategies

+ Vis-IR transparent
+ Lower water solubility (→ citotoxicity) than BaF_2
- High sensitivity to thermal shocks
- Low reactivity towards common reagents for wet etching

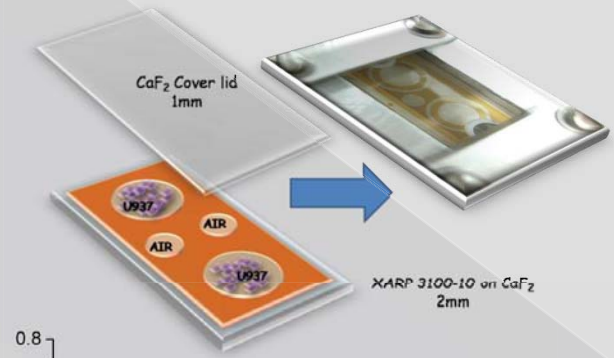




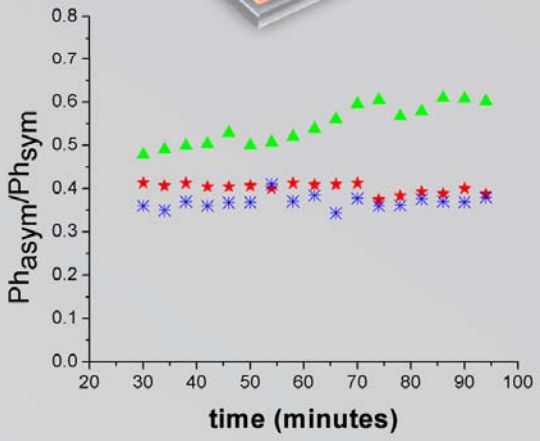


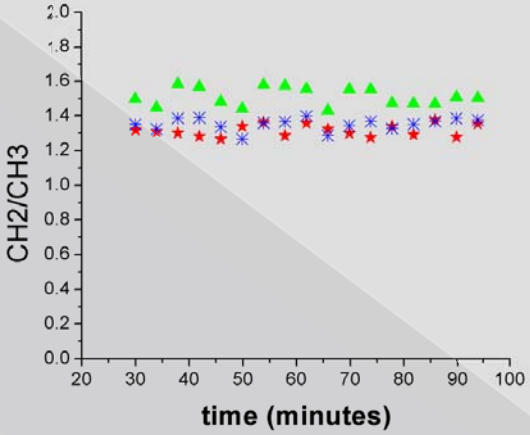
IR properties of CaF2 are preserved

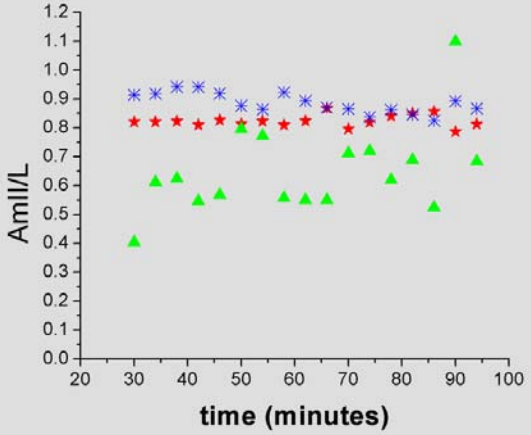
The spectroscopic approach



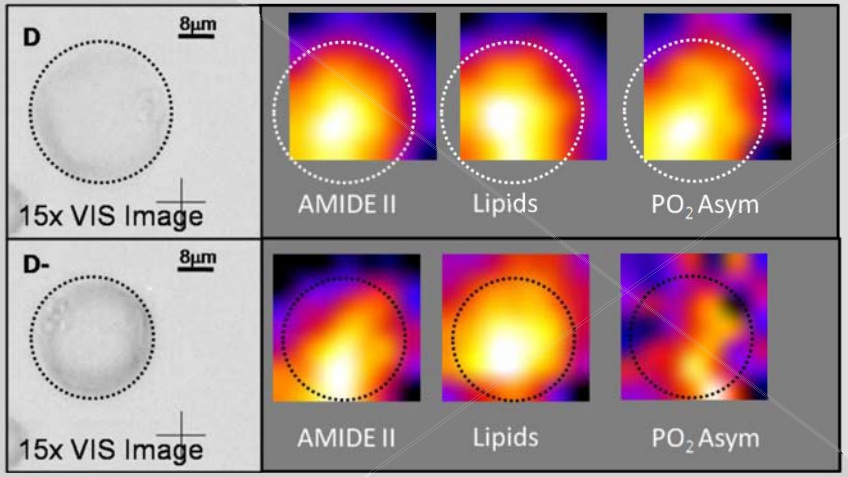
U937 monocytic (average diameter 8-10 microns)
D- Gently deformed cells (★) [9 µm device]
D Strongly deformed cells (✖) [5 µm device]
D+ Extremely deformed (▲) [3 µm device]







The synergic match between µ-fabrication and µ-FTIR could extend the frontiers of FTIR microspectroscopy to almost unexplored fields such as mechanobiology.



Summary and Conclusions

- SR IR microspectroscopy: highly sensitive non-radiation damaging technique for the characterization of subtle biochemical changes in biological matter with sub-cellular spatial resolution.
- To unveil biological divergences associated to spectral differences, a good experimental design has to be done: sample preparation, data collection and data analysis.
- The present limitations for studying living systems in physiological environment can be overcome developing microfabrication approaches suitable for IR-transparent materials.
- The complexity of biological word can be addressed only by a multidisciplinary/multi-technique approach

Selected literature

Infrared Spectroscopy: Fundamentals and Applications
by Stuart, Barbara H.
John Wiley & Sons Ltd, 2004

Biological Applications of Infrared spectroscopy
by Stuart, Barbara H.
John Wiley & Sons Ltd, first edition 1997

Handbook of vibrational Spectroscopy (5 Volumes)
John Chalmers (Editor), Peter Griffiths (Editor)
John Wiley & Sons Ltd, 2002

Infrared Spectroscopy of Biomolecules
Henry H. Mantsch (Editor), Dennis Chapman (Editor)
Wiley-Liss, first edition 1996

Biomedical Vibrational Spectroscopy
Peter Lasch PhD (Author), Janina Kneipp (Author)
Wiley-Interscience, 2008