IR Spectromicroscopy in Biology and Biochemistry: Methodology and Data Processing

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Outline

- Infrared Spectromicroscopy in Biochemistry and Biology
- Sampling and Optical Configurations
- Data Processing for Imaging and Microspectroscopy

Scientific Issues in Biochemistry and Biology

Structure-Function Relationships:

Distribution of Atomic and Molecular Components (scale of nm, µm, mm, …)

Molecular Structure

How does all this relate to the macroscopic functional properties of a system?

Role of FTIR Spectro(micro)scopy

Broad Specificity:

Everything gives a Signal

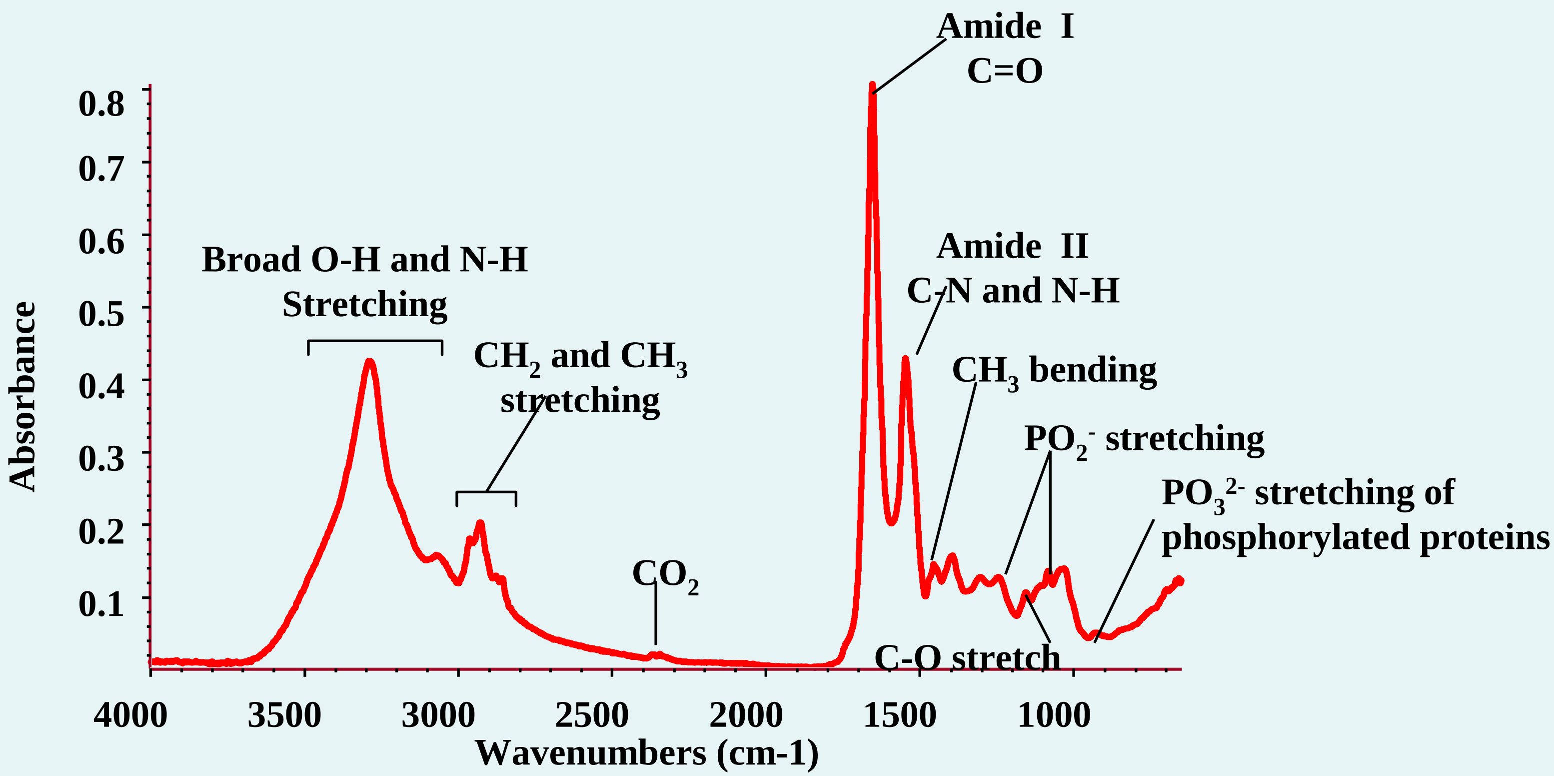
Little or No Need for Pretreatment of Sample

Some Structural Information:

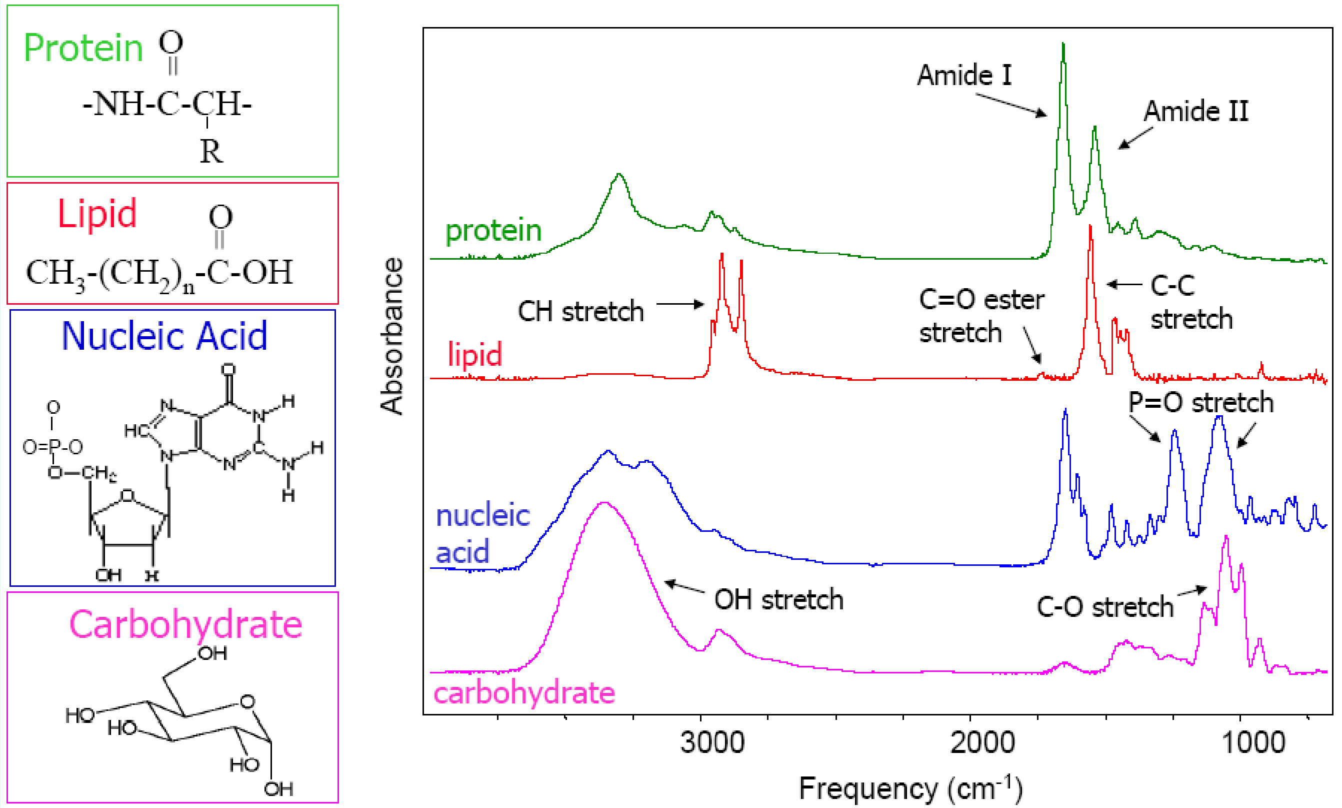
Can Quantify the Presence of Specific Structural Units

(Functional Groups, Conformers, Orientation of Chromophore in Anisotropic Samples, …)

IR Spectrum of Biological Materials: Complex!



Biologically Significant Chromophores

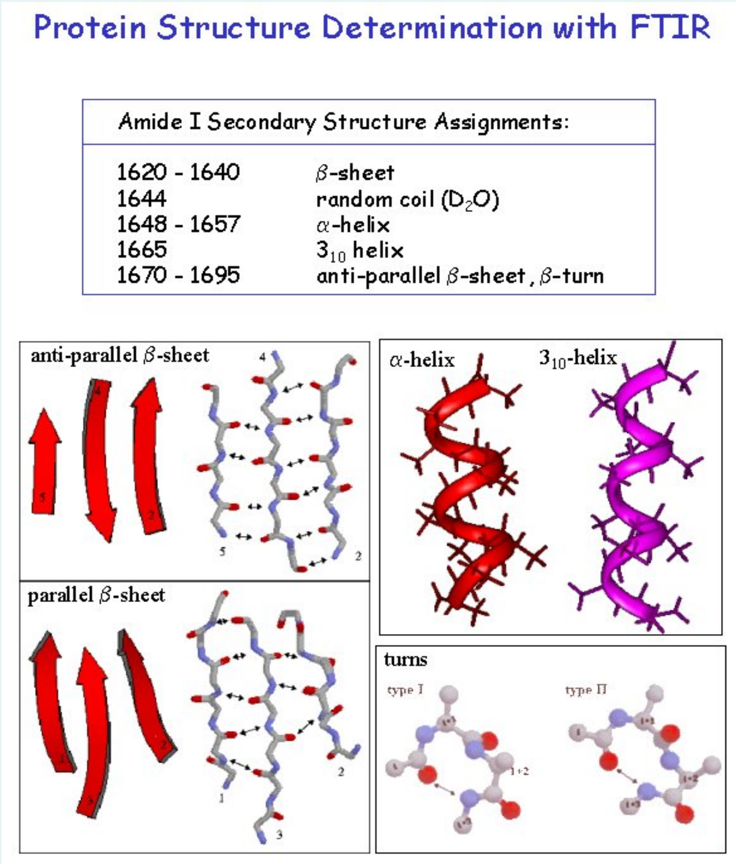


But Also: **Amino Acid Side Chains**

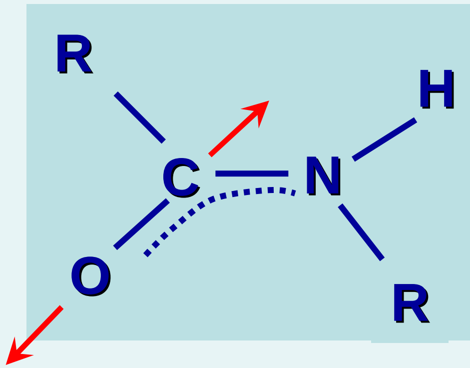
Small Organic Molecules

Organic and Inorganic Ions

Example: Protein Secondary Structure



Lineshape of Amide I allows determining the relative secondary structure composition



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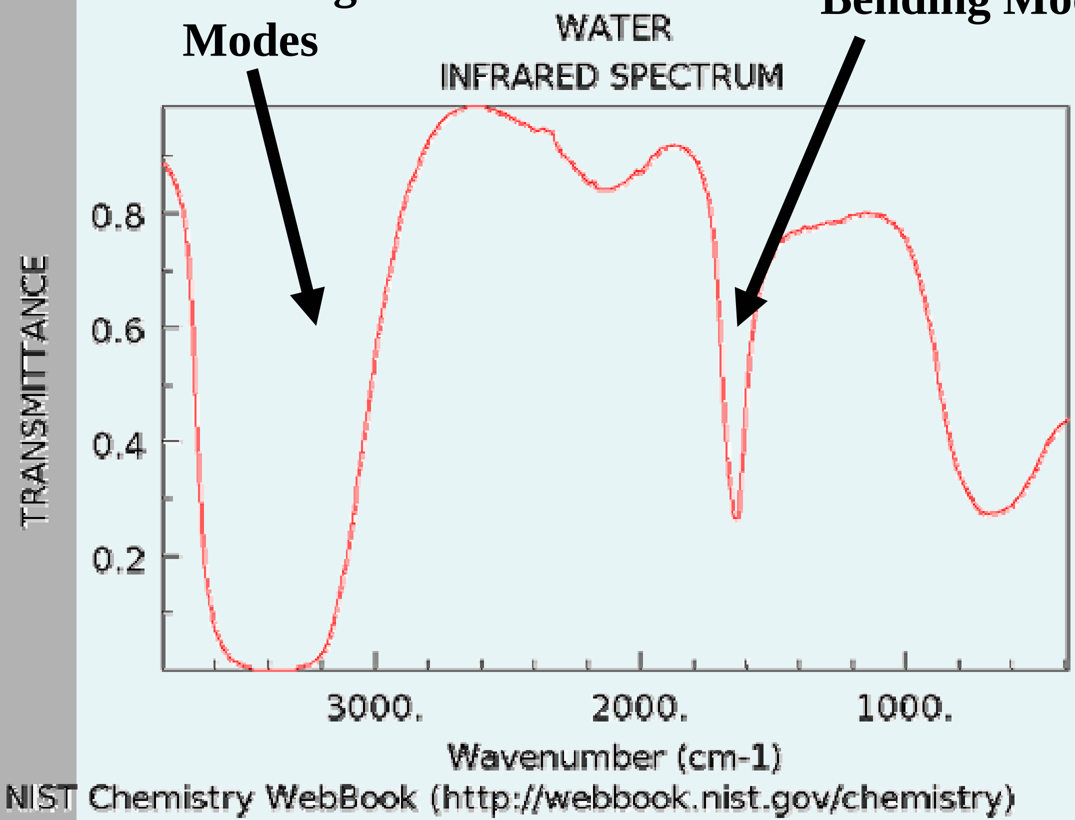
Working with Water

15 µm give ~10 % Transmittance

At 1650 cm^{-1}

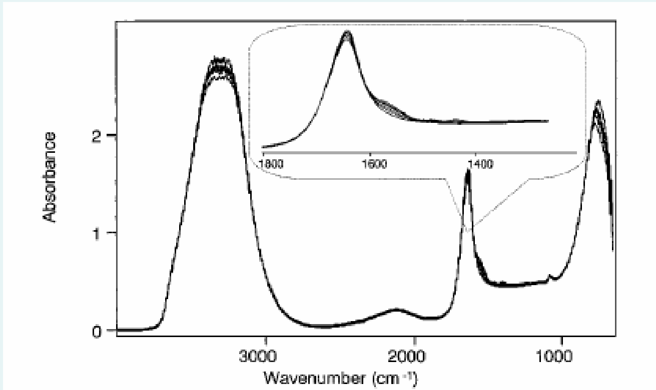
Stretching Modes

Bending Mode



NIST Chemistry WebBook (http://webbook.nist.gov/chemistry)

Most Problematic Issue in IR Spectromicroscopy of Biological Samples



PROTEIN IN WATER

Solving the Water Problem

THREE OPTIONS

Option I: Remove Water

- **Use D_2O**
- **Dry Sample**

Problem:

Sample Survival or Significance

Option III: Measure Spectrum of Interface

Problem:

Complex Configuration

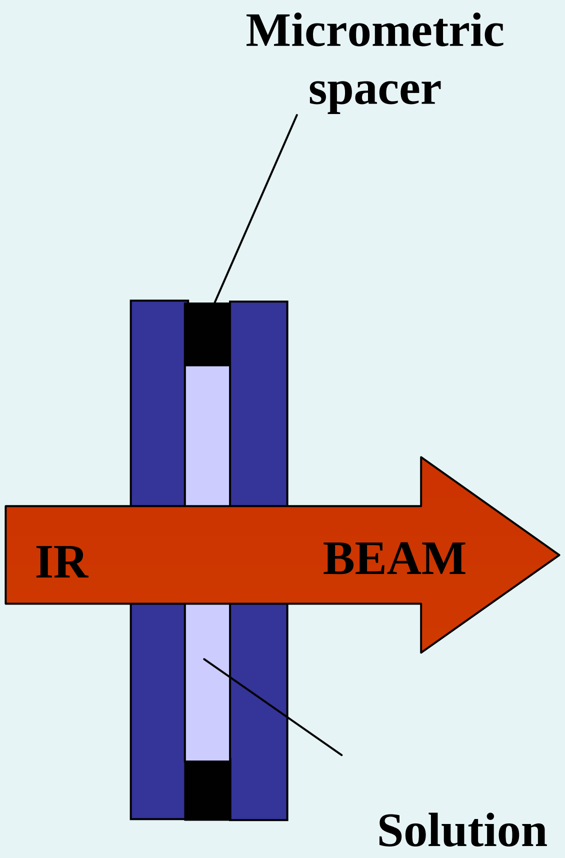
Option II: Reduce Sample Thickness

Problems:

Difficult Sample Handling

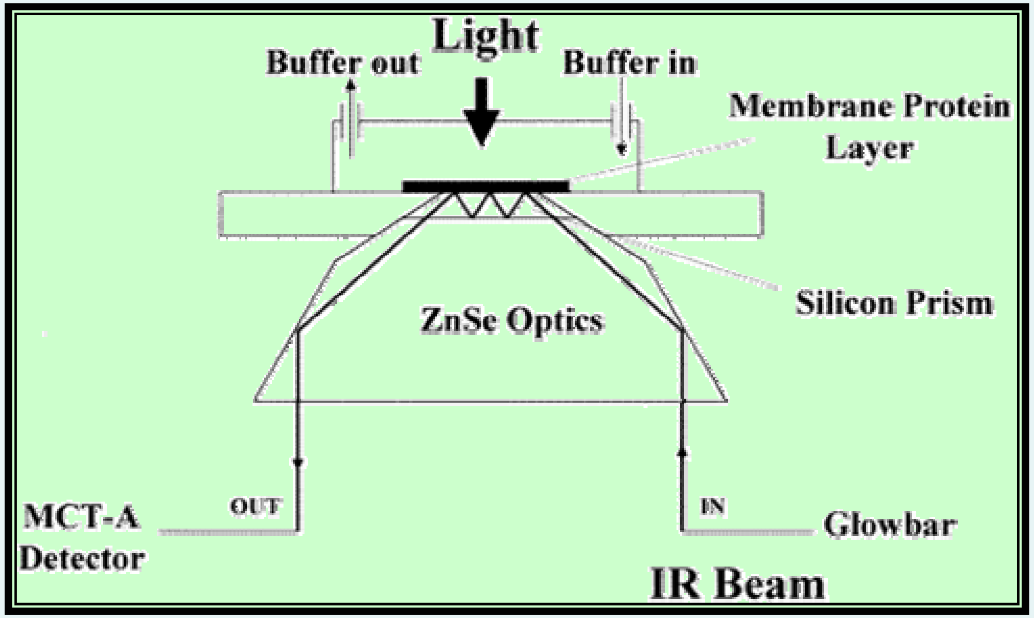
Need Concentrated Sample

Transmission Measurements

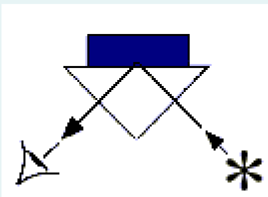


Use THIN Layers of Solution:
Need < 15 µm

ATR: Attenuated Total Reflectance

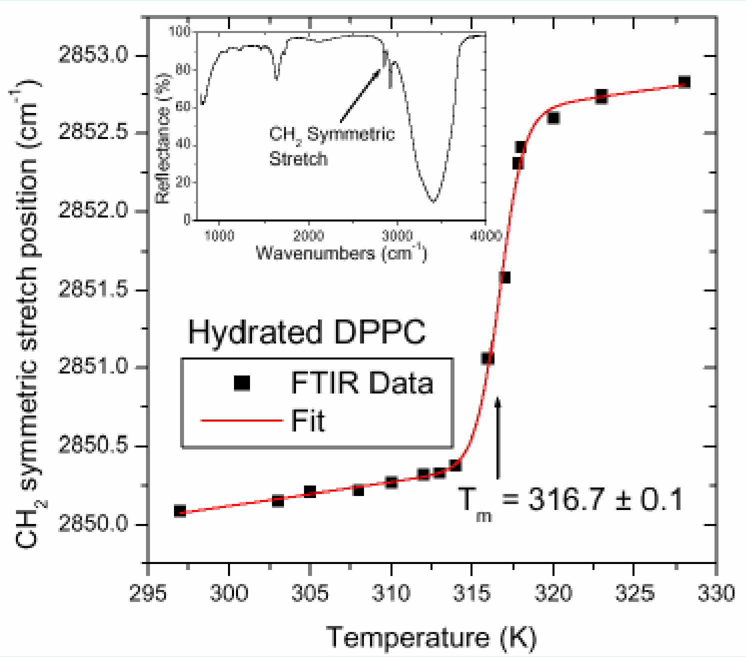


Evanescent Field Sensitive to Interface: Phospholipid Bilayers; Cellular Membranes



Also as Microscope Objectives!!!

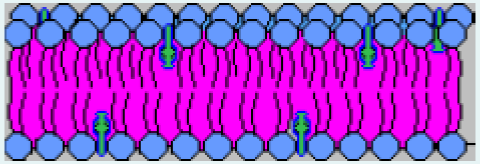
Sample Stability and Heating



M. Martin et al. ALS

Synchrotron source: Measured 1 mW which is focused onto ~10 μm^2

DPPC = dipalmitoylphosphatidylcholine, a phospholipid bilayer undergoes a gel to liquid-crystalline phase transition at T= 316 K



Also: No Evidence for Cytotoxic Effects on Living Cells

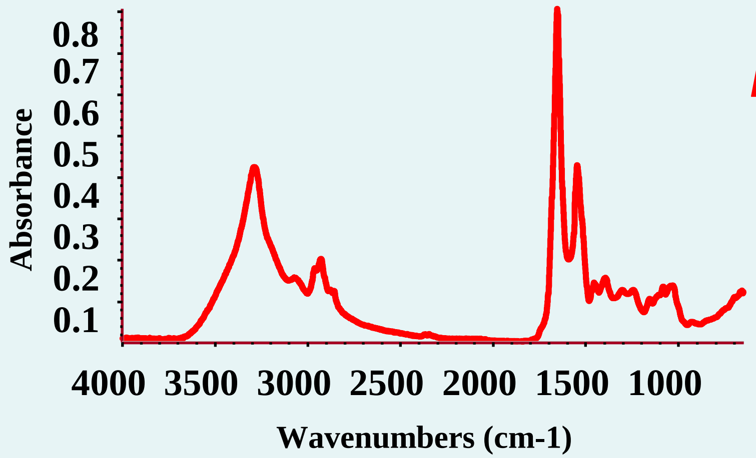
Optical Materials

MATERIAL		REFRACTIVE INDEX	FREQUENCY RANGE	SOLUBILITY (g/100g water)
Calcium Fluoride	T	1.39	66,666-1110 cm-1	0.00151 (20^{0}C)
Zinc Sulfide	T	2.2	10,000-715 cm-1	Insoluble
Zinc Selenide	T	2.4	10,000-500 cm-1	Insoluble
Cadmium Telluride	T	2.67	5,000-320 cm-1	Insoluble
Silver Chloride	T	1.98	25,000-435 cm-1	0.00015 (20^{0}C)
Silver Bromide	T	2.2	20,000-285 cm-1	0.000012 (20^{0}C)
IR Quartz	T	1.42	50,000-2500 cm-1	Insoluble
Amtir	T	2.5	11,000-725 cm-1	Insoluble
Sapphire	T	1.75	50,000-1780 cm-1	Insoluble
Silicon		3.4	10,000-1540 cm-1, 500-30 cm-1	Insoluble
Germanium		4.0	5,000-600 cm-1	Insoluble
Diamond	T	2.4	45,450-2325 cm-1, 1665-285 cm-1	Insoluble

Au or Al Mirrors

MirrIR Slides

Complexity of Spectra: Experiment Design



Everything gives this spectrum!!

Difficult to Quantify and Difficult to Interpret

Possible Solution: Work with Difference Spectra

Complexity of Spectra: Band Assignment

Traditional Approach in Vibrational Spectroscopy of Small Molecules:

1) Calculate Band Positions Based on a Structural Model

2) Calculate Isotope Shifts for Some Bands

3) Perform Isotopic Substitution and Measure Spectrum

$$\tilde{\omega} = \frac{1}{2\pi}\left(k\frac{m_1+m_2}{m_1 m_2}\right)^{1/2}$$

Pair of Vibrating Atoms

Isotopic Substitution in a Biological System?

Substitute a Bulk Component:

$H_2O \rightarrow D_2O$

$H_2O \rightarrow H_2{}^{18}O$

$^{16}O_2 \rightarrow {}^{18}O_2$

Grow an Organism in Enriched Medium:

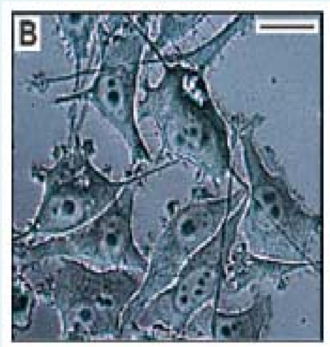
^{13}C, ^{15}N, ^{57}Fe, ...

Engineer an Organism:

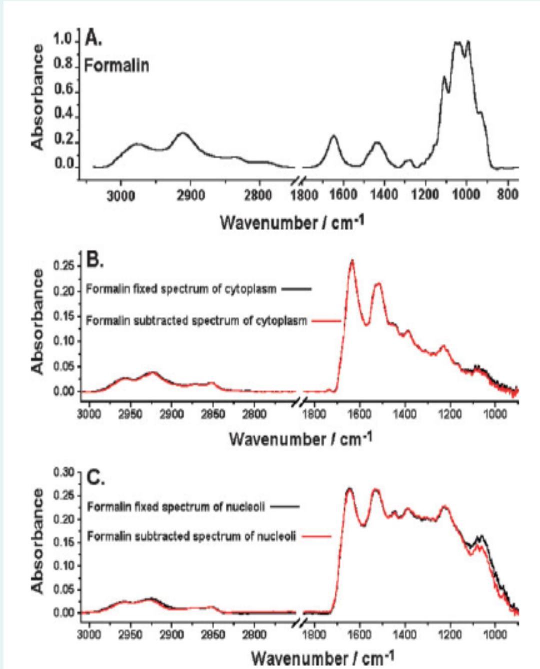
Delete a gene for biosynthesis and supply the synthetic component

Fixation Protocols for Cells and Tissues

Need to Preserve Distribution of Components and Spectral Purity



Formalin Fixed Cells



E. Gazi, J. Dwyer, N. P. Lockyer, J. Miyan, P. Gardner, C. Hart, M. Brown, N. W. Clarke; Biopolymers, Vol. 77, 18–30 (2005)

Cell Adhesion on Support

Grow Cells on Support

(CaF_2, ZnSe, MirrIR)

And Wash

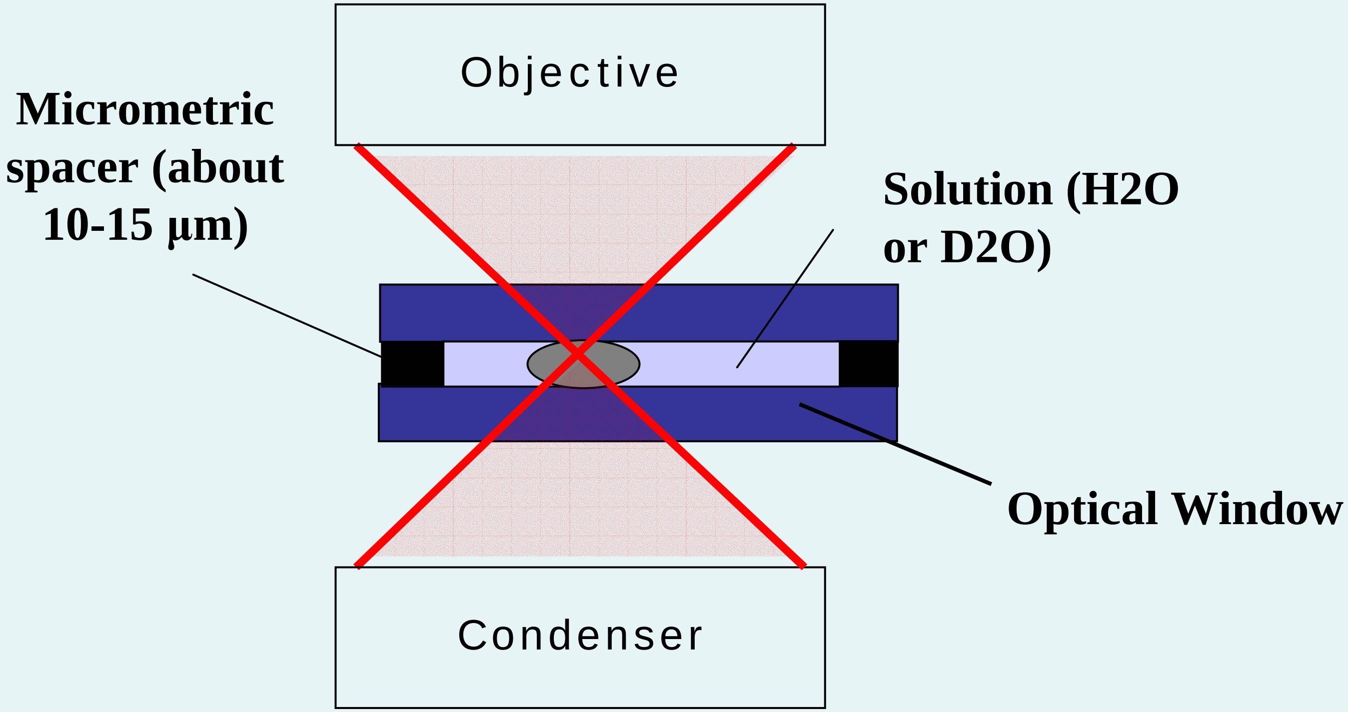
Coat Support with Adhesive Layer:

Charged polymers

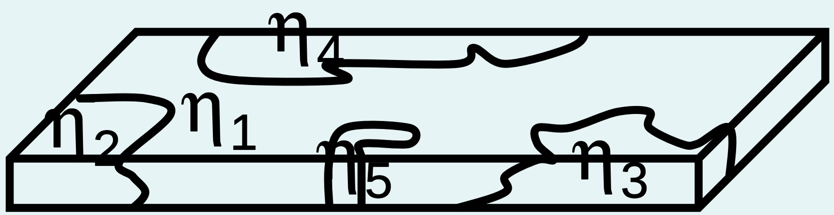
Linkers (chemical conjugation)

Beware of IR absorption of adhesive!

Cells in Water (Deuterated Water)

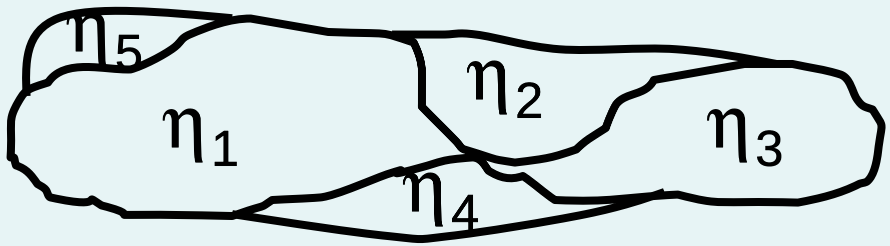


Ideal Sample



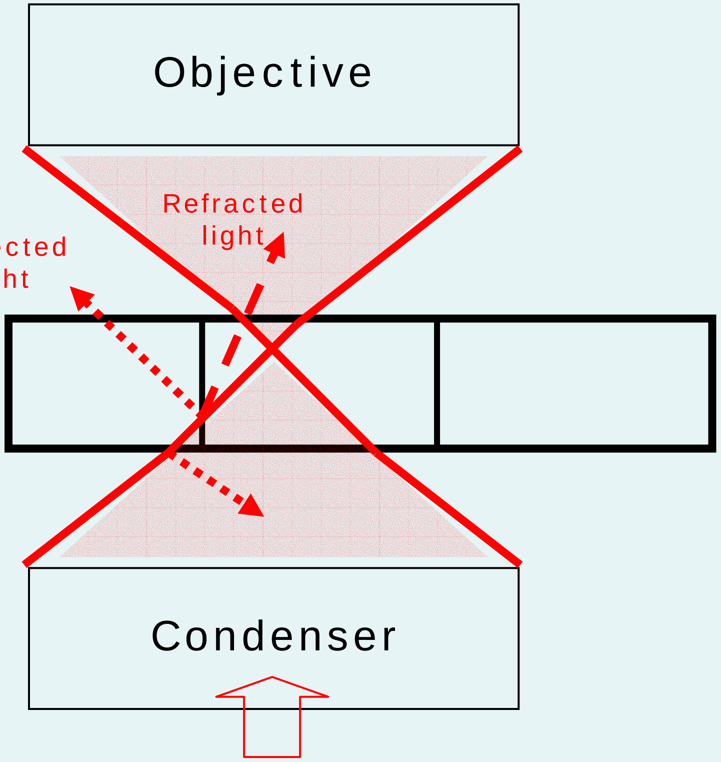
Refractive Indexes η_l are Similar in the Real Part

Real Sample

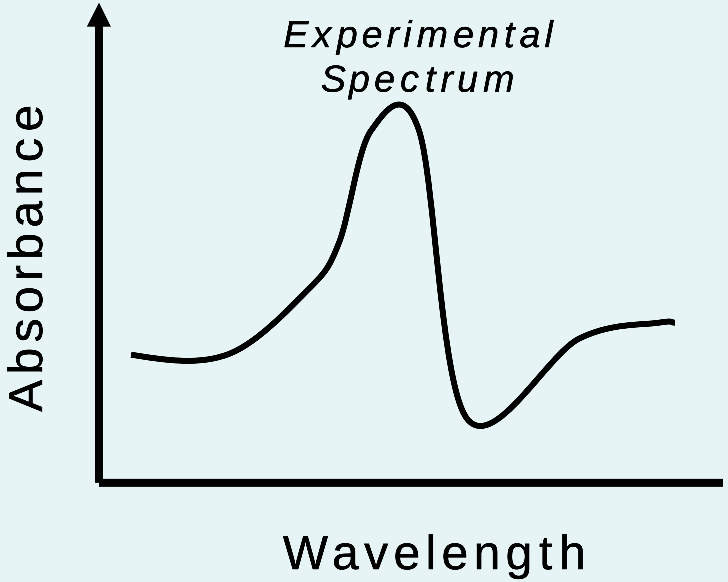


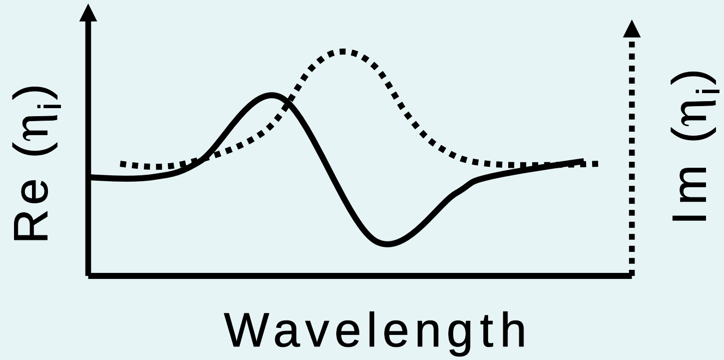
Refractive Indexes η_l are Different (from 1 to 1.7 mostly)

Refraction and Reflection Effects



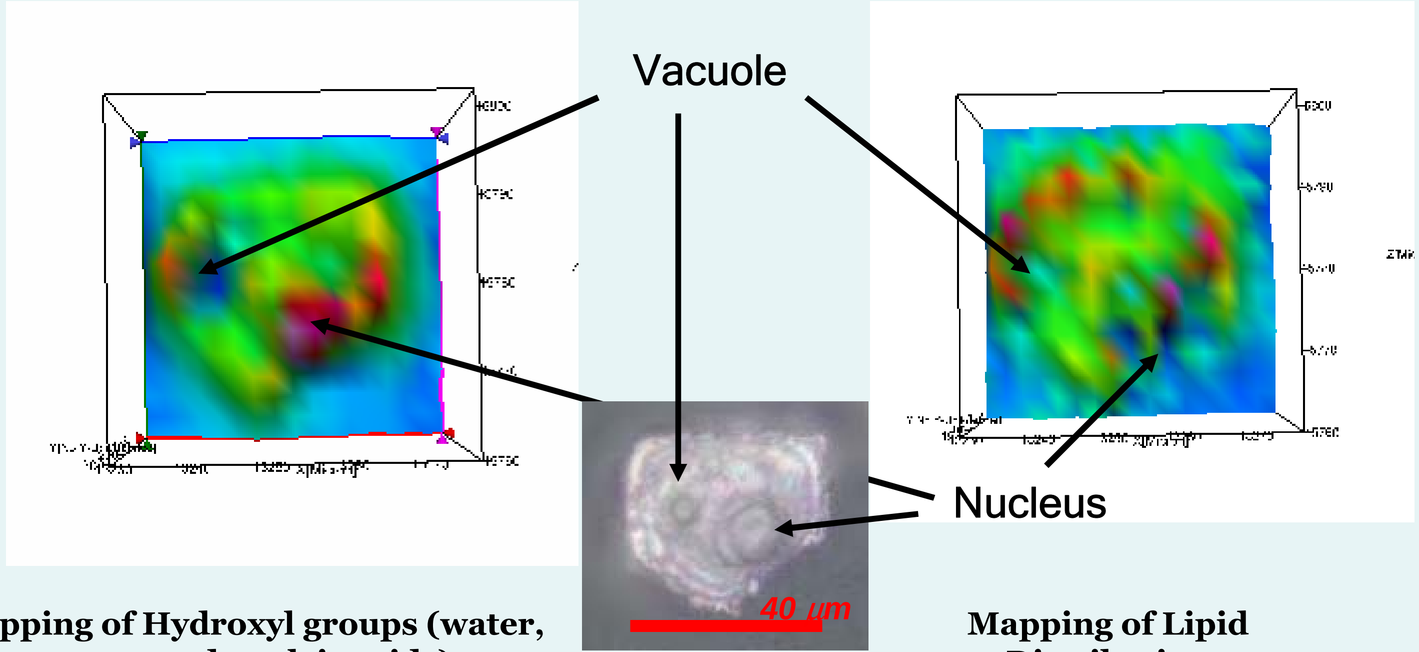
Worse for synchrotron light





Sub-cellular Mapping

Aperture: 3 µm x 3 µm

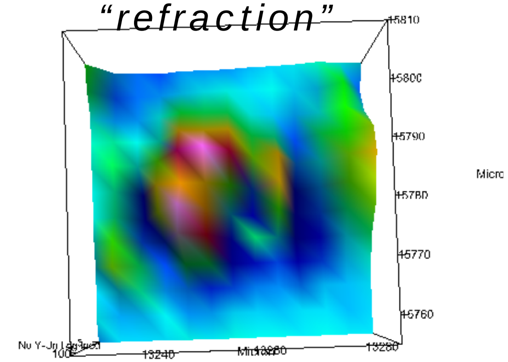


Mapping of Hydroxyl groups (water, sugars and nucleic acids)

Formalin Fixed Cell

Mapping of Lipid Distribution

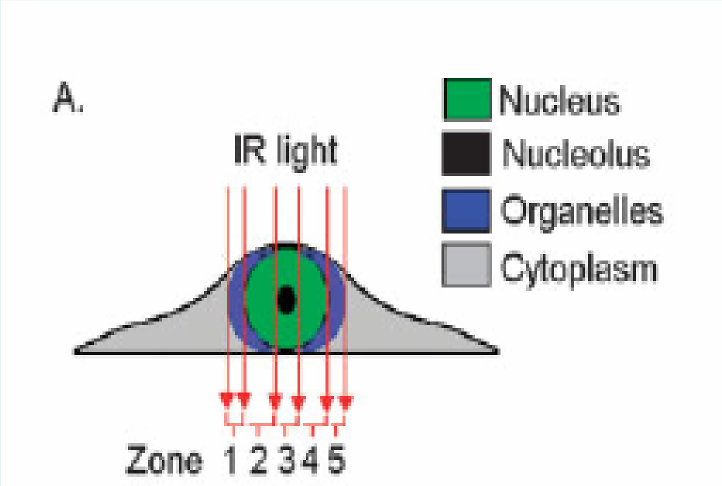
"refraction"



L. Quaroni, M. Campagna, O. Burrone

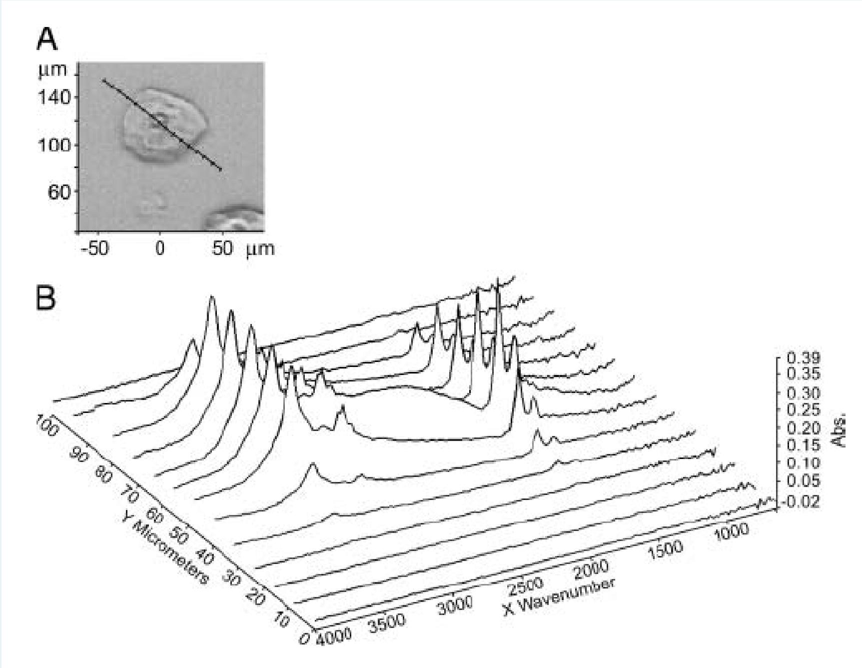
Thickness Effects

Absorption increases with sample thickness



Introduce Effect of Topography in IR Maps

Scattering Effects



Brian Mohlenhoff,* Melissa Romeo,* Max Diem,* and Bayden R. Woody; Biophysical Journal Volume 88 May 2005 3635–3640

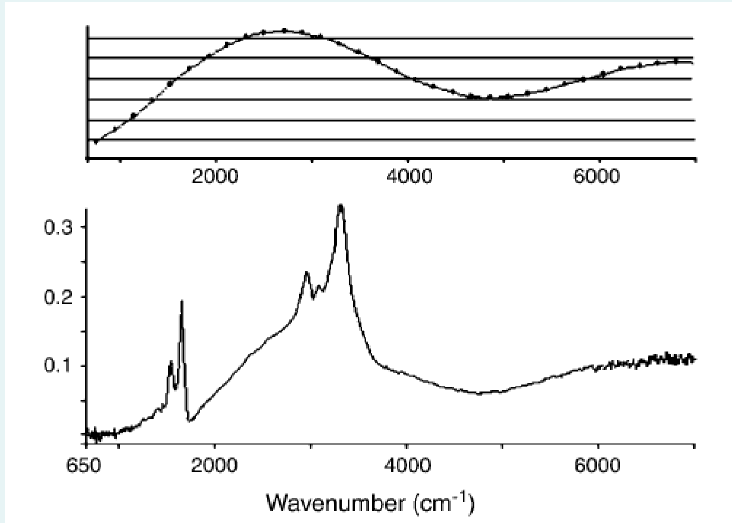
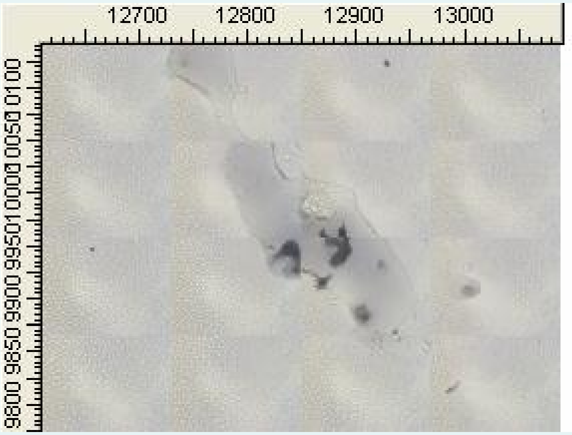
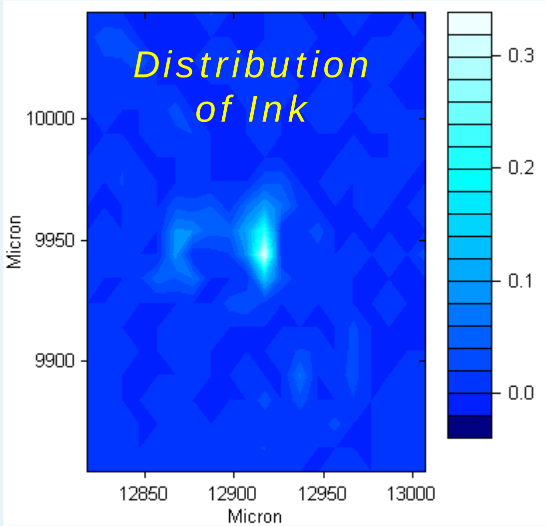
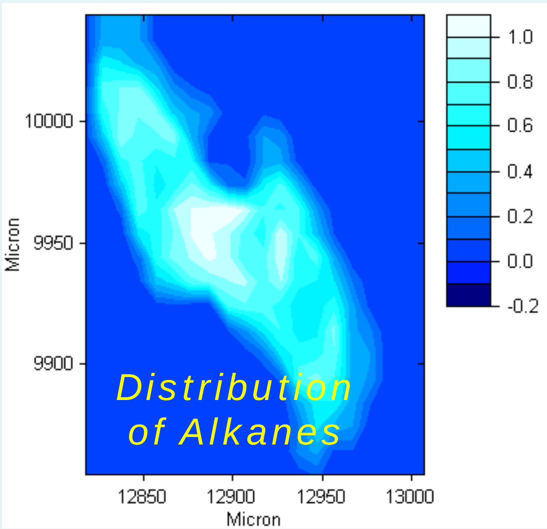


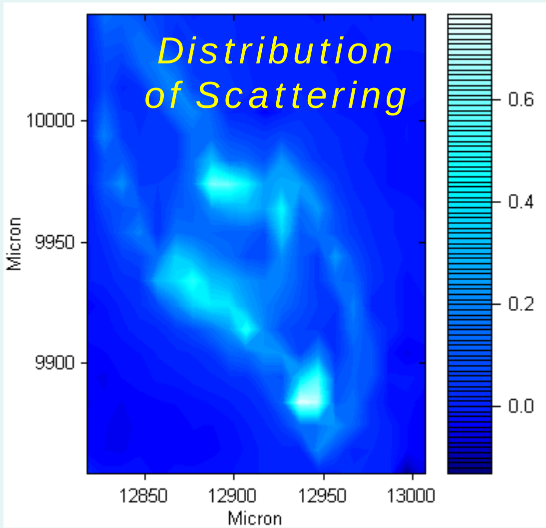
FIGURE 5 IR spectrum of oral mucosa cell (*bottom*), and scattering spectrum for a dielectric sphere with $a = 4.2$ μm (*top*).

Example: grease deposit on reflective surface









Z. Arsov, L. Quaroni,

Need to Simplify Sample Topography

Microtome Samples

Measure Small Portions

Care with choice of background position

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Techniques for Automated Spectral Classification

Non Supervised

•Agglomerative Hierarchical Cluster Analysis

•Fuzzy C-Means Clustering Analysis

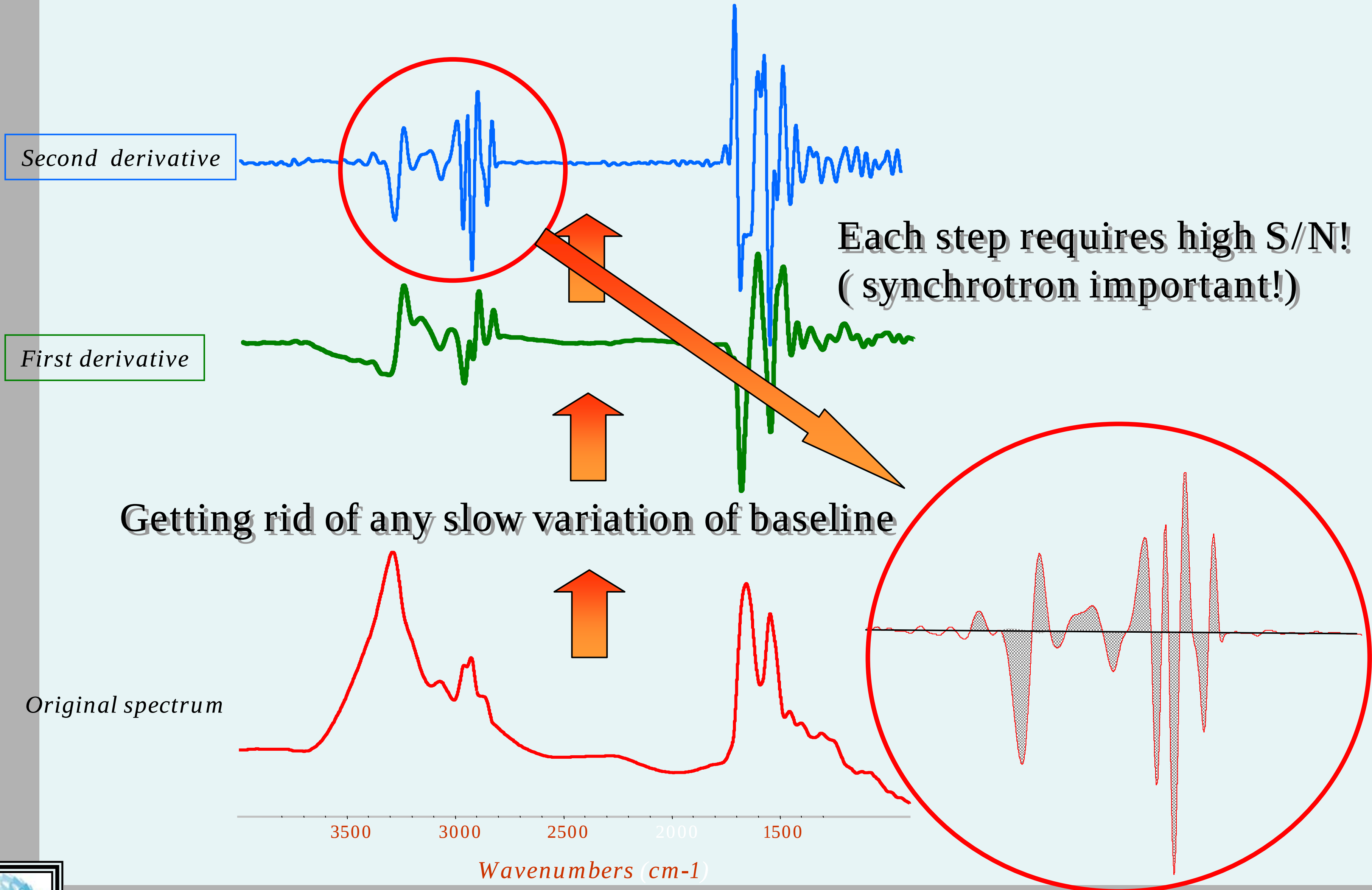
•K-Means Clustering Analysis

Supervised

•Artificial Neural Networks

……

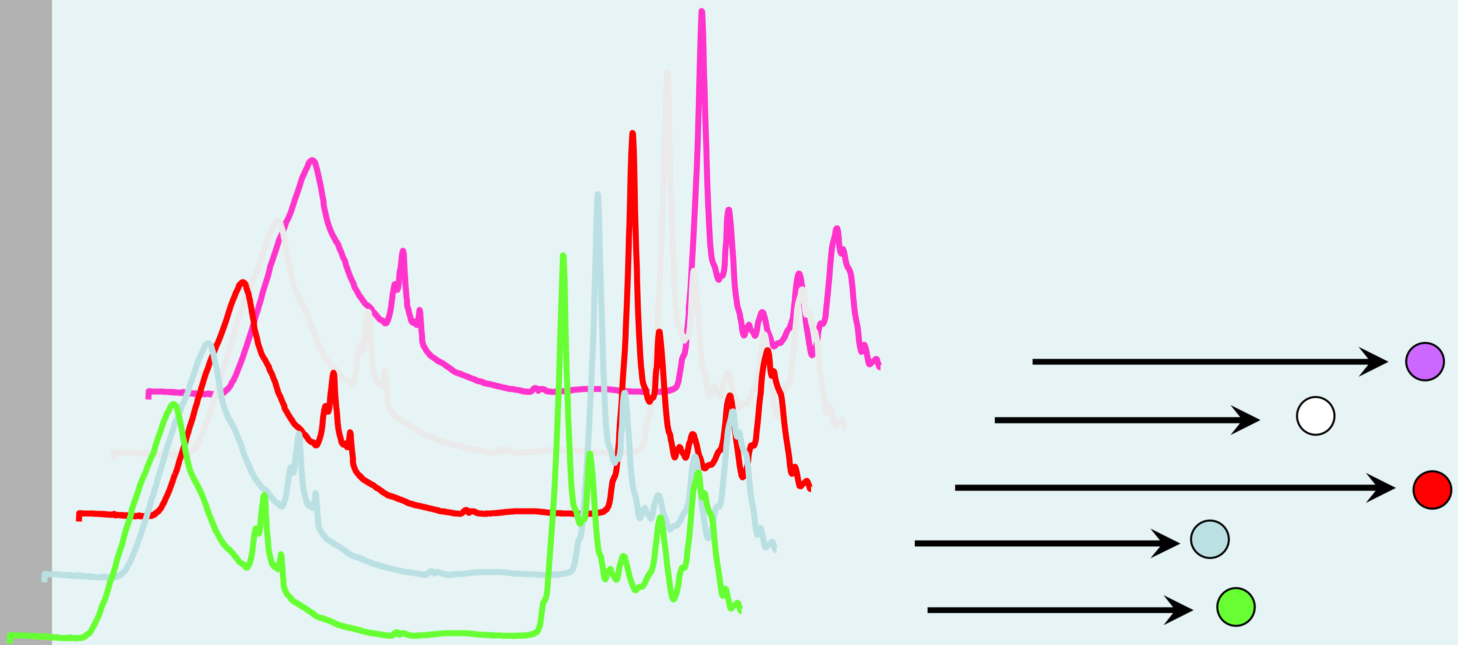
Statistical treatment proceeds as follows...



Each step requires high S/N!
(synchrotron important!)

Then vector normalized

Data reduction:
each spectrum is given a value

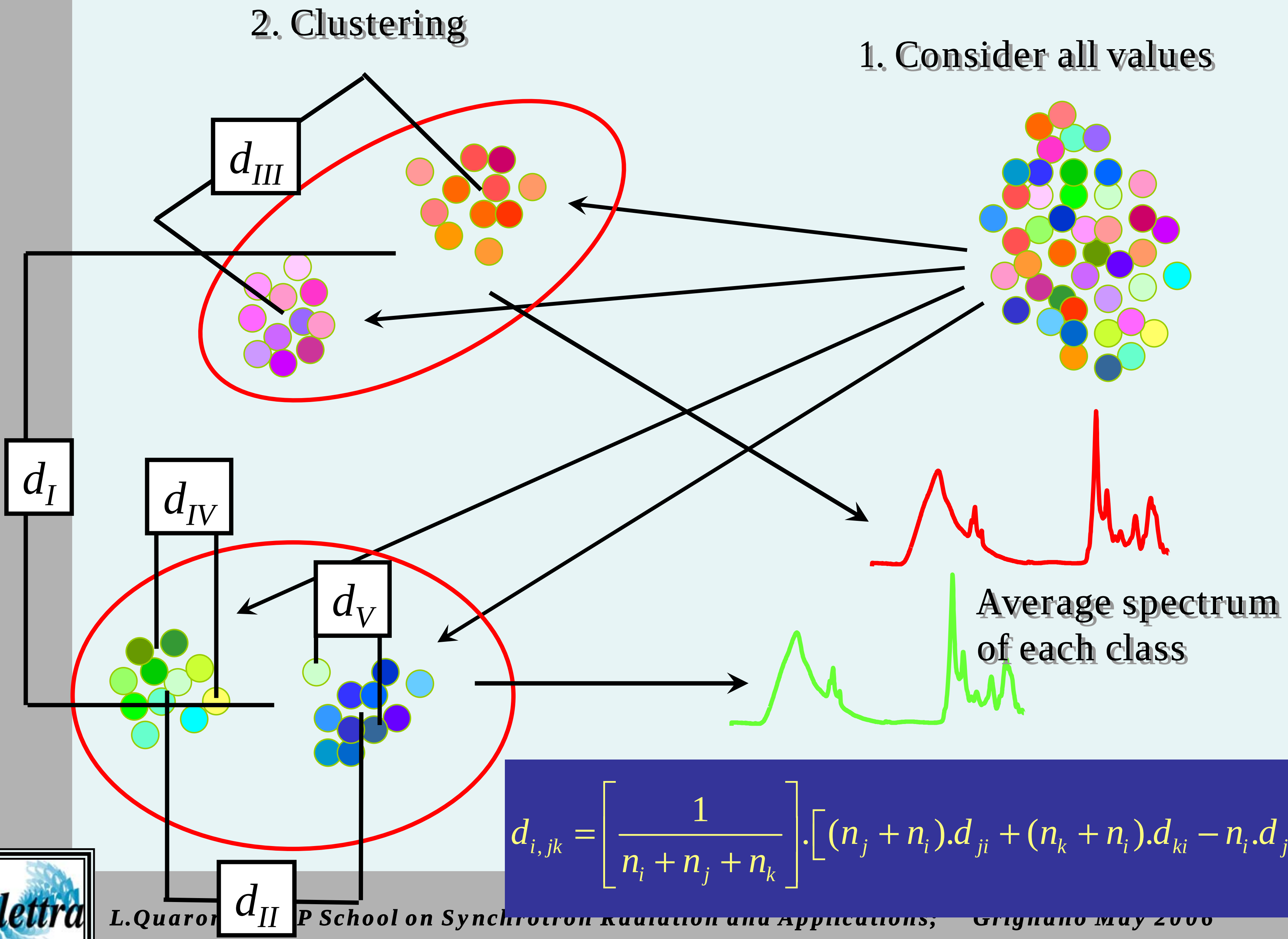


$$r_{jk} = \frac{\left(\sum_{i=1}^{n} x_{ji}.x_{ki}\right) - n.\bar{x}_j.\bar{x}_k}{\sqrt{\left(\sum_{i=1}^{n} x_{ji}^2 - n.\bar{x}_j^2\right).\left(\sum_{i=1}^{n} x_{ki}^2 - n.\bar{x}_k^2\right)}}$$

Pearson’s correlation coefficient

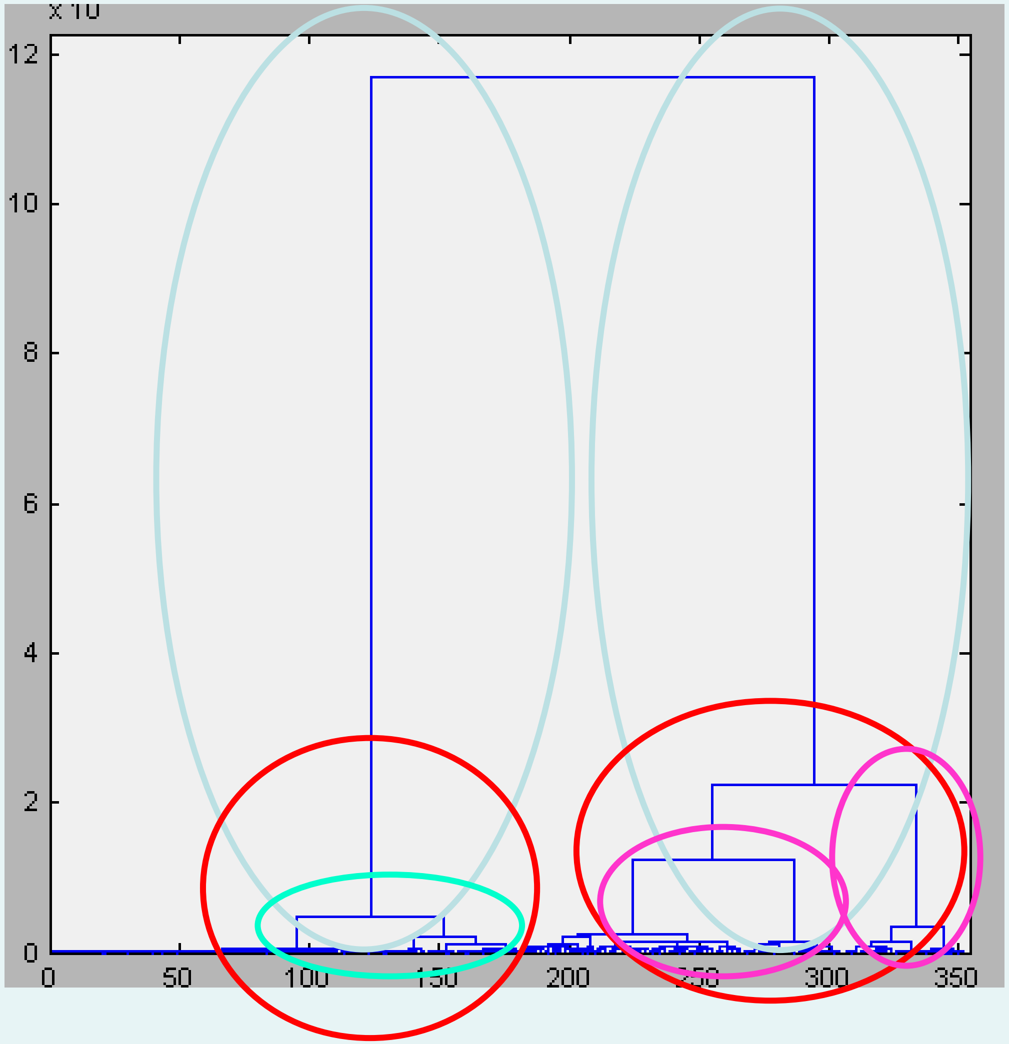
$$d_{jk} = \left(1 - r_{jk}\right).1000$$

D-values



$$d_{i,jk} = \left[\frac{1}{n_i + n_j + n_k} \right] . \left[(n_j + n_i).d_{ji} + (n_k + n_i).d_{ki} - n_i.d_{jk} \right]$$

The interface between IR spectra, statistical treatment and imaging



Dendrogram

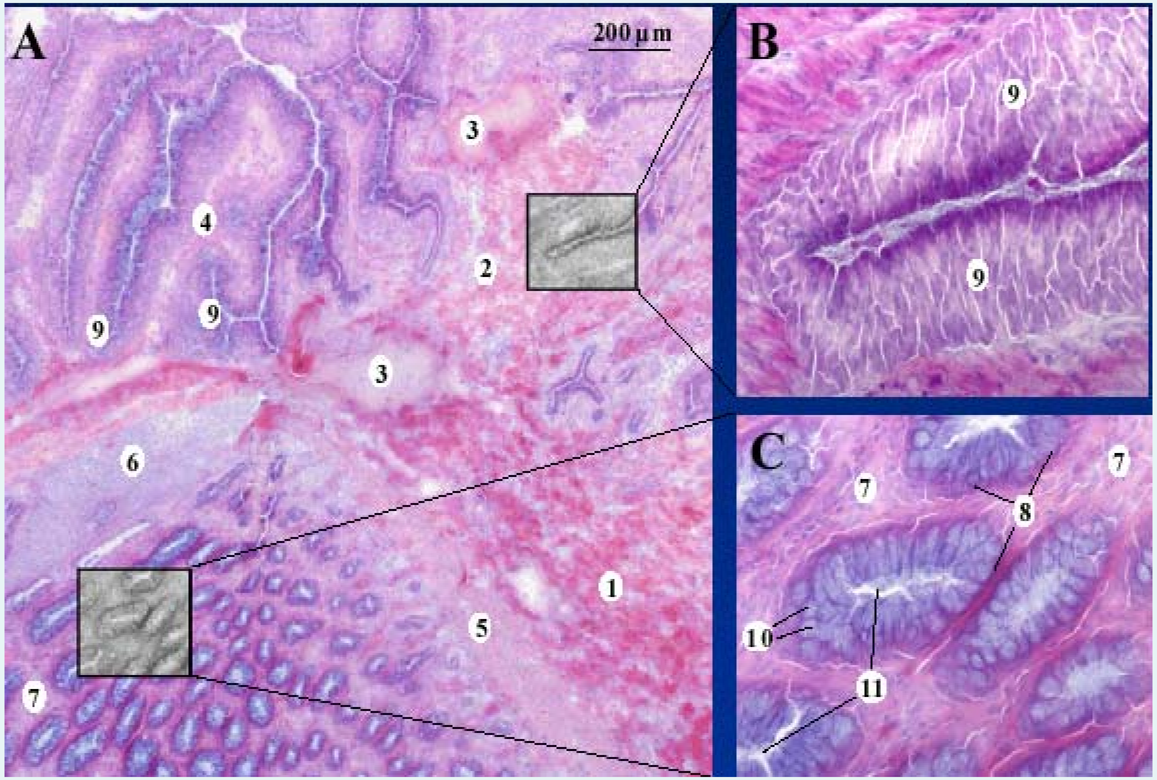
2 classes

+2= 4 classes

+2= 6 classes

+1= 7 classes

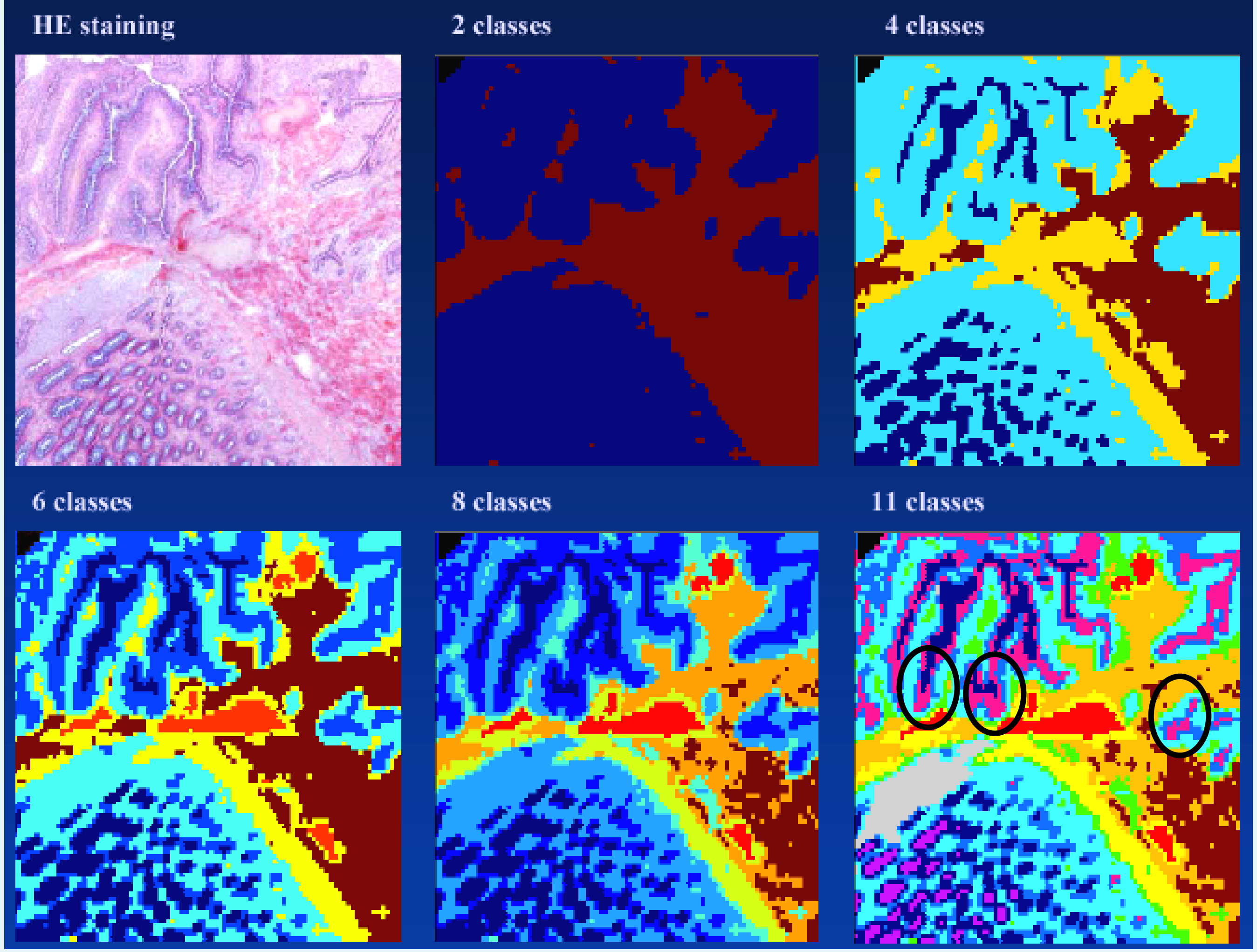
Illustration: colorectal adenocarcinoma



1- submucosa
2- submucosa and remnants of tunica muscularis (infiltrated by the carcinoma)
3- lymph vessel
4- fibrovascular stock and other connective tissues
5- lamina muscularis mucosae
6- inflammatory cells
7- lamina propria mucosae
8- colonocytes
9- adenocarcinoma (cancerous epithelium)
10- goblet cells
11- central lumen of the crypts

From. Peter Lasch and D. Naumann (Berlin)

IR Imaging: Hierarchical Clustering

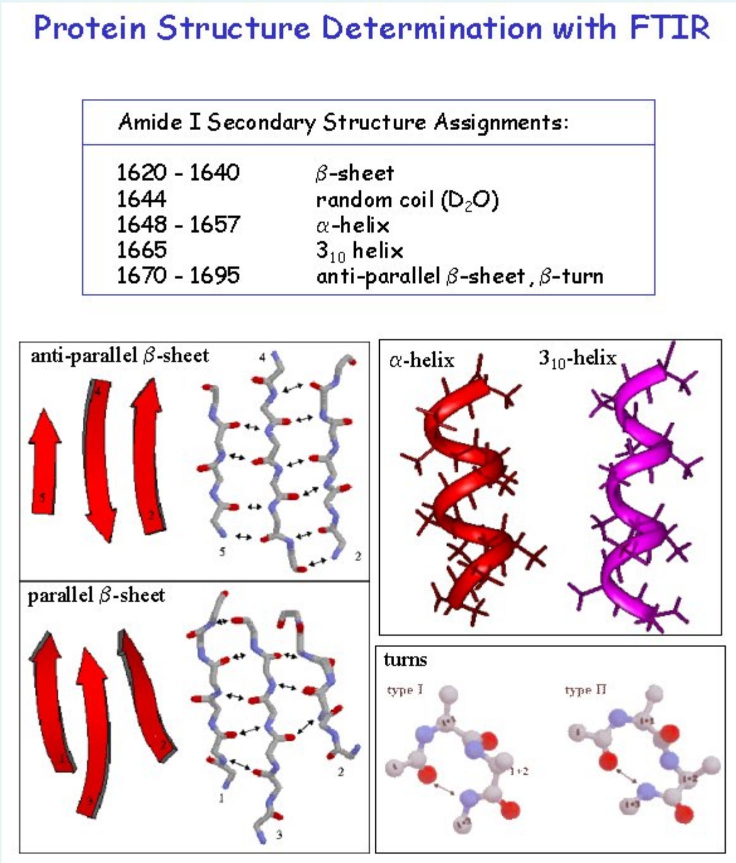


Each « class » , displays a characteristic average spetrum
... including adenocarcinoma region

Curve Fitting Analysis of Bands (Deconvolution)

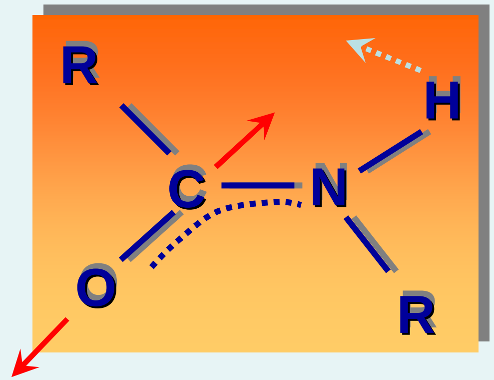
2D Correlation Analysis

Structural Information from an IR Spectrum

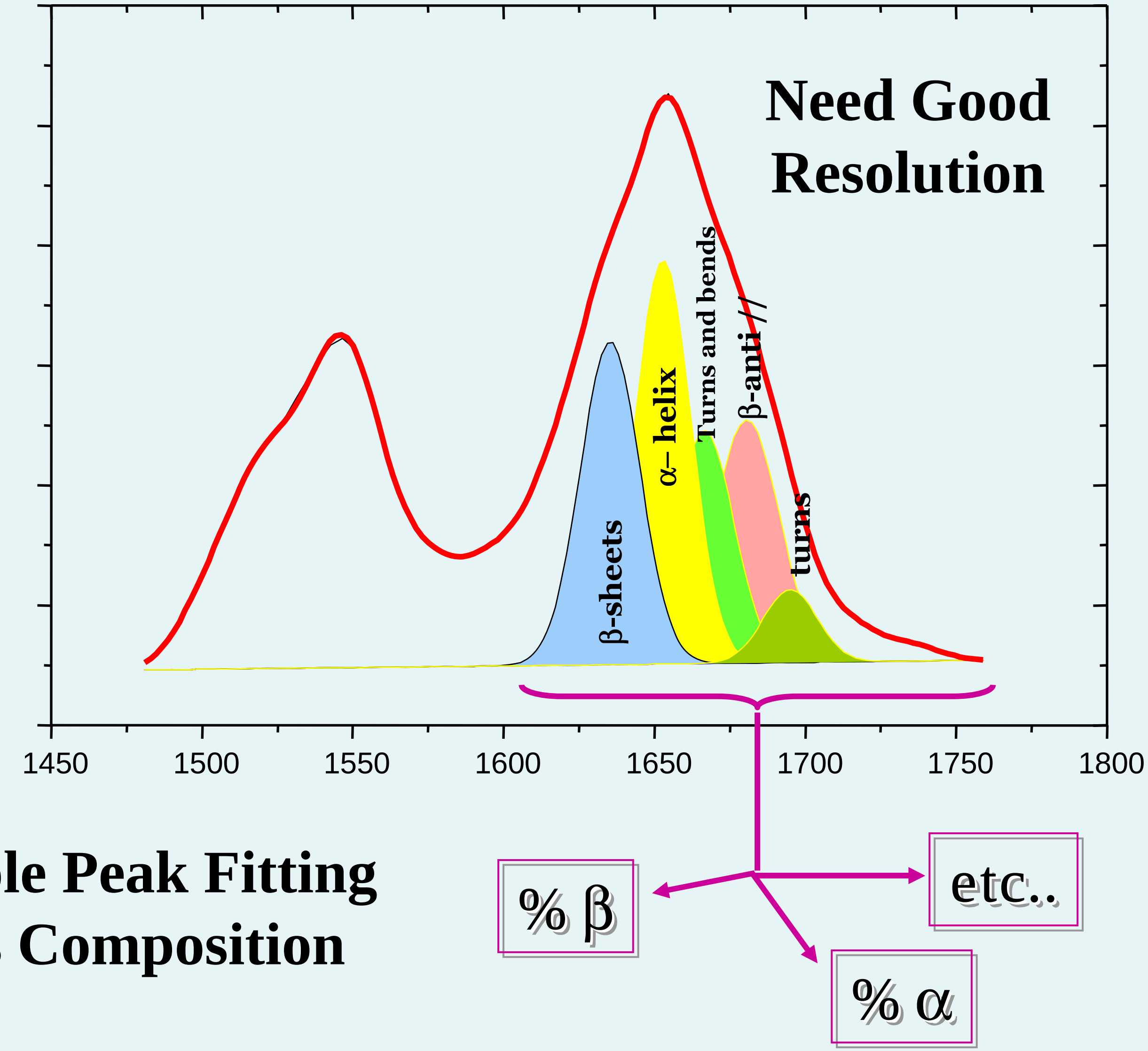


Example: Secondary Structure in Proteins

Lineshape of Amide I allows to determine the relative secondary structure composition



Band Deconvolution



Courtesy of Dr Paul Dumas, SOLEIL

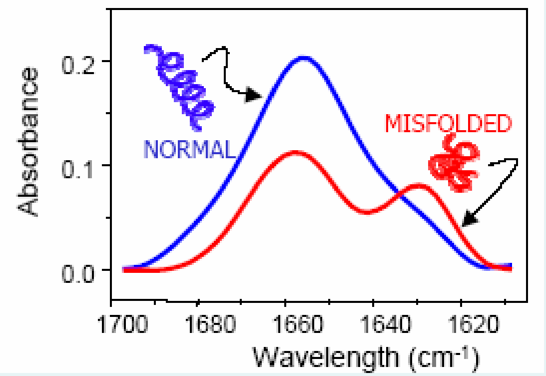
Multiple Peak Fitting Gives Composition

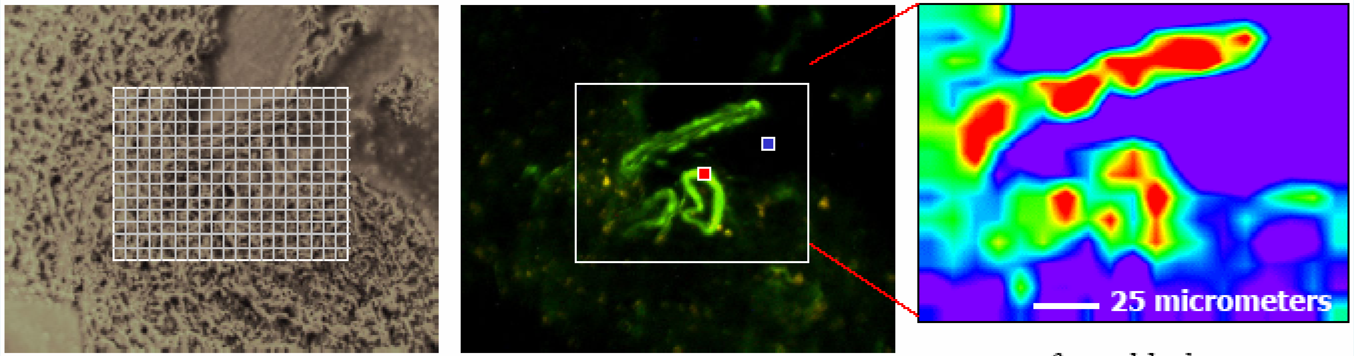
Imaging AND Spectroscopy

IR Microspectroscopy of Alzheimer's Plaques

Alzheimer's plaques are misfolded Abeta protein

- α-helical protein cleaved and folds to β-sheet format
- metal accumulation occurs
- plaques are thought to damage surrounding neurons





visible light image *fluorescent light image* *infrared light image*

L.M. Miller, P. Dumas, N. Jamin, J.-L. Teillaud, J. Miklossy, L. Forro (2002). *Rev. Sci. Instr.*, **73**: 1357-60.

Limitations of Deconvolution

Limited Capability to Resolve Complex Multiplets

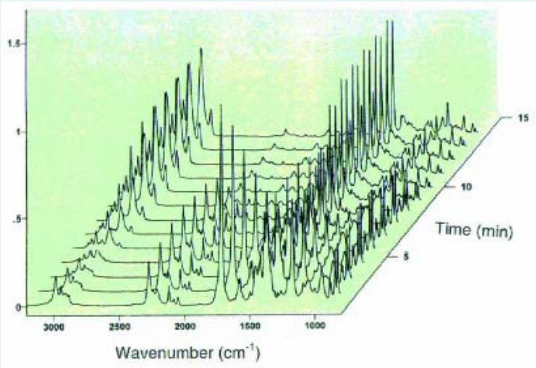
Limited Accuracy for Complex Systems and/or Unknown systems

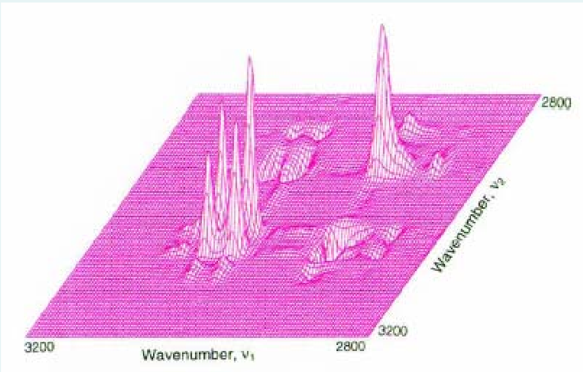
Strongly Affected by Noise

Difficult to assign different bands to same compound

2D-Correlation Analysis

Vary One Parameter (Temperature, Pressure, Time, whatever you want …)





2D Spectrum

See Effect on Spectral Components

Process Mathematically: Correlation Analysis

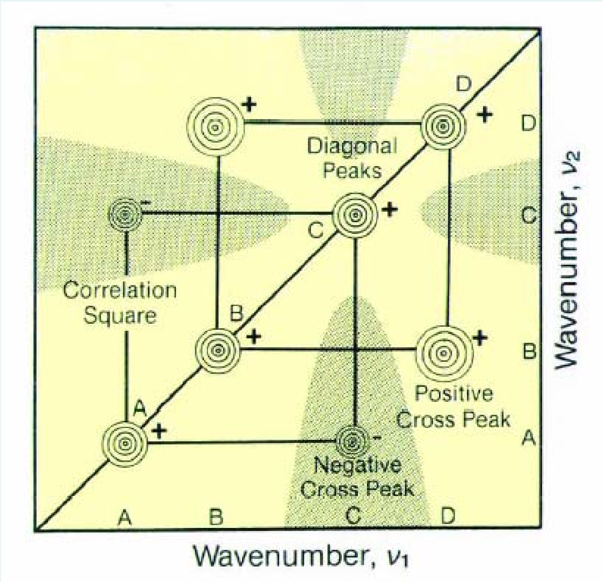
$$X(\nu_1, \nu_2) = \Phi(\nu_1, \nu_2) + i\Psi(\nu_1, \nu_2)$$

Synchronous Spectrum

Asynchronous Spectrum

2D-Correlation Analysis

$$X(\nu_1, \nu_2) = \Phi(\nu_1, \nu_2) + i\Psi(\nu_1, \nu_2)$$



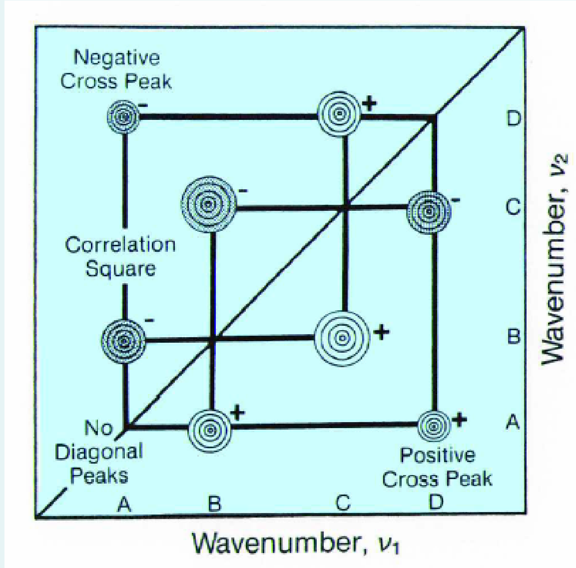
Synchronous Spectrum

Correspondence of changes

Less Affected by Noise

Allows Identification of Spectral Components Associated to Same Molecule

Separation of Poorly Resolved Components



Asynchronous Spectrum

Sequence of changes

Final Considerations

Techniques and Methods discussed are also used in IR spectromicroscopy with conventional sources

Advantages when using synchrotron

- **Better Spatial Resolution for Imaging**
- **Better S/N**
- **Pulsed Emission (for T-R)**
- **Far IR Emission**

Bibliography

Just write me!

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