
Practical issues in FTIR spectroscopy
Lab/demo section - Warwick CD Workshop

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FTIR optical layout – static polarization

- Source
- Aperture
- Interferometer
- Moving mirror
- Fixed mirror
- Detector
- Air Bearing
- HeNe laser alignment
- Voice coil
- Sample
- Polarizer (rotate $\perp$, $\parallel$ - typically wire grid)

Digilab schematic
Alternate, simpler FT-IR : Bomem Michelson

Design often found in routine instruments, no air needed, highly stable
Acquisition of an Infrared Spectrum

Fast Fourier transform done in computer

Convert Interferogram to Spectrum (single beam)
**Acquisition of an Infrared Spectrum**

**Single beam sample**

![Spectrum of source & sample](image1)

**Single beam background**

![Spectrum of source only](image2)

Divide by:

\[
\frac{I}{I_0}
\]

i.e. \(\frac{I}{I_0}\) gives

**Transmittance**

![Transmittance spectrum of the sample](image3)

**Absorbance**

![Absorbance spectrum](image4)

- \(\log_{10}T\)

Transmittance old (negative peak), Absorbance (positive) ~ concentration
Some Useful Terminology and Relationships

• Resolution:
  – Resolution is the measured full width at half the maximum (FWHM) absorbance intensity of a spectral line which is inherently very narrow
  – Resolution in FT-IR depends on 3 factors:
    • *Distance traveled by the scanning mirror*
    • *Size of image transmitted at the sample focus*
    • *Apodization function applied*

• Apodization Function
  – Computationally modifying the interferogram before Fourier Transformation to eliminate artificial oscillations on sides of peaks and improve the line shape. Causes loss of resolution
  – No apodization, called “boxcar” apodization, has oscillations but also the best peak resolution
  – This is not a major issue for broad bio-molecular IR spectra
Spectral Range:

- At low frequency, the beamsplitter and window optics of the FT-IR become opaque. Below this point there is no intensity available. The cutoff point depends on the optical material.

- At high frequency source intensity and beamsplitter efficiency diminish gradually so that a cutoff point is less obvious.

- The free spectral range is the range beyond which no data can be collected. It is dependent on the frequency (periodicity) at which the interferogram is digitally sampled as the mirror scans.

- 31600 measurements per cm of mirror travel provides 0 to 7900 cm\(^{-1}\) – corresponds to measuring every other zero crossing of HeNe (UDR=2)

- 63200 measurements per cm of mirror travel provides 0 to 15800 cm\(^{-1}\) – corresponds to measuring every zero crossing of HeNe (UDR=1)
Going beyond normal IR spectra – FT variations

- **Spectral Range**: $27,000 - 20 \text{ cm}^{-1}$
- **Spectral Resolution**: $128 - 0.09 \text{ cm}^{-1}$
- **Step-Scan**: +/- 0.2 nm position accuracy
- **Continuous-Scan**: $0.0158 - 8.22 \text{ cm/s}$

**Rapid Scan Spectroscopy**
- Chemical Kinetics (ms)
- IRRAS

**Dual Channel Polarization Modulation Spectroscopy**
- VLD
- VCD

**Time-Resolved Spectroscopy**
- Polymer Stretching
- Liquid Crystal Dynamics
- Chemical Kinetics (μs-ns)

**Phased-Resolved Spectroscopy**
- Amplitude Modulation
  - Emission
  - PAS Depth Profiling
- Sample Modulation
  - Polymer Stretching
  - PAS Depth Profiling
- Phase Modulation
  - Liquid Crystal Dynamics

**Space-Resolved Spectroscopy**
- Imaging
- Spectro-Electro Chemistry
Advantages of Raman Spectroscopy--Comparison

- Non-destructive
- Flexible sampling - any phase or size - no preparation
- 1µm sample area - Raman microscopy possible
- Glass cells - good medium for cell design, low cost
- Fiber optics - up to 100m, routine
- Water - weak scatterer - excellent solvent
- Enhanced by resonance, surface interactions
‘Disadvantages’ of Raman Spectroscopy

• Sensitivity to $\pi$-systems means side-chain contributions and DNA base contributions are large, water is small

• Fluorescence can be a problem, sometimes it can be “burned” out of the sample, but excitation with red laser can typically avoid it

• Particulate scatter ($\sim \nu^4$) can be a significant problem, filtering (both chemical and optical) helps
Low Wavenumber Bands

Low Wavenumber Region: IR vs. Raman

Raman provides:
Low wavenumber vibrational bands

IR Transmission Spectrum

Raman Spectrum

Slide Courtesy Renishaw Inc.
Dispersive Raman - Single or Multi-channel

Eliminate the intense Rayleigh scattered & reflected light
- use filter or double monochromator
  – Typically \(10^8\) stronger than the Raman light

Disperse the light onto a detector to generate a spectrum

Sample

Laser – \(v_0\)

Polarizer

Lens

Filter

Scattered Raman - \(v_s\)

Single, double or triple monochromator

Detector: PMT or CCD for multiplex
Conventional Raman Instrumentation

- Excitation source (generally a CW laser)
- Sample illumination and scattered light collection system
- Sample holder
- Monochromator or spectrograph
- Detection system (PMT, Photodiode array, CCD)

- Scattering efficiency for Raman can be 1 in $10^{10}$ photons!
- Need extremely high intensity source, spectrometer with high degree of discrimination against stray light, extremely sensitive detection system able to detect small numbers of photons over dark background
Detection: CCD (now most typical)

The CCD (charge-coupled device) is an optical array detector consisting of a two-dimensional array of silicon diodes. CCDs have low read-out noise and high quantum efficiency and sensitivity over wide wavelength range (100-1000 nm).
Fourier Transform Raman Schematic

(Schrader & Simon, 1987)

Use near-IR laser (typ. YAG) to avoid Fluorescence
Useful for materials and poorly purified samples
Same measurement approach as FTIR
Sampling biological molecules, solution and solid state (film, powder, suspension)
Liquid cells--examples

Liquid samples, biopolymers in solution, concentrated in H2O or more dilute in D2O – two windows typically CaF2 or BaF2. Separate by spacer 25-100 microns.
Variable path liquid cell

Works best with organic solvents, min 50 micron, max. long ~5 mm
Beam condensor for micro sampling

Useful for micro cells or for small crystals or solids

Lens based

Mirror based--achromatic
Steps in Measurement, Processing FTIR

- Measure → single beam spectra
- empty cell or cell holder
- cell + buffer/solvent
- cell + buffer/solvent + protein
  - Ratio each to empty cell, take log to yield absorbance, subtract blank from sample with variable coefficient

Data processing

Subtractions: Buffer/solvent, Atmospheric Vapor, Sidechain

Analysis techniques

FSD (Fourier Self-Deconvolution), Derivative, Bandshape
Protein Studies: Experimental

H-O-H bending mode at ~ 1645 cm\(^{-1}\) overlaps the amide I band of peptides/proteins

- Substitute D\(_2\)O – moves band, but ‘problem’:
  - need to deuterate protein
  - can cause different frequency shifts
  - possible conformational changes
Buffer / Solvent Subtraction

Protein

Buffer

Protein-Buffer

File # 2: BV1227A
Buffer for 2cab, carbonic anhydrase, 2 time, 12/27/96
Must eliminate the sharp vapor transitions by subtraction, direct overlap suggests study of wings (high wavenumber)
Subtract vapor spectrum until protein spectrum is flat (featureless)
SOLID STATE IR Sampling: EXPERIMENTAL

• IR spectroscopy is the most flexible method capable of studying proteins/peptides in the solid state

• Solid state spectra can be collected using several techniques:
  
  • *KBr* pellets
  
  • *Deposited film in transmission*
  
  • *Attenuated Total Reflectance (films and solution)*
  
  • *Diffuse reflectance*

  • In Biology, can even study tissue and cells by using microscopy for imaging

  • Film studies can encompass membranes and mixed systems, membrane – peptide interactions and orientations
Reflectance methods

- Attenuated Total Reflectance
- Diffuse Reflectance
- Specular Reflectance
Solid Sample Technique: Diffuse Reflectance:

IR energy penetrates into the powdered sample and then emerges in all directions. The optics collect scattered radiation and direct it to the detector.

(This is the trick, mirrors that “surround the surface.)

Sometimes called DRIFTS
Internal Reflectance

- Light is Focused Upon Crystal of High Refractive Index Material
  - Crystals typically ZnSe, Diamond, Silicon, or Germanium
  - Light Refracts Towards Upper Surface

- What Happens When Light Encounters an Internal Surface?
  - Depends upon the crystal’s critical angle, $\theta_c$
  - If $\theta < \theta_c$ light refracts and exits the crystal (dashed line)
  - If $\theta > \theta_c$ light reflects off of internal surface, “Internal Reflection” (solid line)

“Single bounce” concept, if external reflection, then IRRAS (later)
Conventional multi-reflection ATR

IR beam totally reflects inside crystal. Multiple bounces (~10-20) inside until emerge. Sample surface (~1 μm) provides absorption.

For liquids and solids:

- Use with materials which are either too thick or too strongly absorbing to be analyzed by transmission spectroscopy or when only the surface of the material is of interest.

- ATR is a technique for obtaining infrared spectra of samples that are difficult to deal with, such as solids limited solubility, films, threads, pastes, adhesives, and powders.
A ‘Few’-bounces ATR - Types available

• Often diamond over ZnSe
  – Provides very hard surface
  – Can apply pressure with rod above

• 1-3 bounces, signal can be small – solution difficult
  – Good for membranes, solids
  – Easy to clean

• 9-bounce dip to hold liquid

• Design to fit your FTIR

• Several manufacturers
Relative Intensities Affected by ATR

- **Top**: ATR Spectrum of Sucrose
- **Bottom**: DRIFTS Spectrum of Sucrose

- **High cm\(^{-1}\)** peaks have less relative intensity in ATR spectrum than DRIFTS spectrum.
Pathlengths Vary with ATR Crystal

- ATR Spectra of Sucrose Obtained with Diamond and Ge Crystals
  - Diamond $n_c = 2.4$, Germanium $n_c = 4.0$
  - Beam penetrates further into diamond, hence more intense absorb
External reflection IR, Air-Water Interface

Design of Mendelsohn and coworkers, Appl. Spectr. 2001
‘Perfect’ protein FT-IR spectrum: H₂O solution

Empirical formula for success

**Spectrum “Must” Have**

a. Amide I/II ratio: 1.2-1.7
b. Presence of Amide III bands
c. Presence of C-H stretching modes
d. Flat baseline between 1800-2200 cm⁻¹
e. Gradual baseline rise below 1800 cm⁻¹
f. No vapor bands
Transmission vs. ATR: ‘Danger’ of ATR measurements

Published spectrum of aqueous solution measured using ATR (dashed line) and transmission (solid line):

- notice incorrect ratio of Amide I/II intensities =>

mistake due to protein adsorption to the surface of ATR crystal
Spectral features become more evident, band shape variations increase allowing discrimination between similar spectra.
Fourier Self-Deconvolution
how it works—mathematical manipulation of spectra

FT original absorption, multiply by increasing exponential
apodize result

0
1.0
0

exp(-t/τ) cos ω₀t

0
1.0
0
t

exp(+ t/τ)

0
1.0
0
t

exp(- a² t²)

0
1.0
0
t

0
1.0
0
t

Reverse

Broad original spectrum

FT results in narrowed deconvolved spectrum

Interferogram now favors high res. components

FT original absorption, multiply by increasing exponential
apodize result

0
1.0
0

exp(-t/τ) cos ω₀t

0
1.0
0
t

exp(+ t/τ)

0
1.0
0
t

exp(- a² t²)

0
1.0
0
t

0
1.0
0
t

F.T.
Fourier Self deconvolved Amide I – Ribonuclease S
Band fit result to Lorentzian shapes, assign, analyze

More sheet than helix,
helix probably 2 types,
turns not quantiative

(Byler& Susi, Biopolymers 19
2nd derivative and deconvolution: get same number of bands, same position
Overlaid 22 protein FTIR spectra used for 2D correlation analysis (top) Correlation coefficients of the 6th order even polynomial fit of the FTIR spectral intensity as the function of α-helical FC (bottom)
Synchronous correlation map for α-helical FC (top) and corresponding disrelation (absolute value) map (bottom). Contours positive: yellow/white; negative: red/pink.
3D surface obtained by fitting Raman spectra with a polynomial. Correlation coefficients of the polynomial fit of the Raman spectral intensity as the function of α-helical FC(bottom).
Corrected (disrelations subtracted) synchronous correlation map of the protein Raman spectra with respect to α-helix FC perturbation.

Positive contours : white/cyan, negative contours: red/pink.
Factor (Principle Component) Analysis

• Approach is functionally equivalent to Principle Component Analysis - Singular Value Decomposition
  - No curve fitting, no Band assignments, Method is general

• Method:
  - *treat set of protein spectra as basis set of functions, [ϕ]*
  - Diagonalize the co-variance matrix to
    • *find most common elements*- $\psi_1$
    • *find most common deviation*- $\psi_2$
    • *continue*
  - Reconstruct Spectra: $[ϕ] = [ψ][α]$, where $[α]$ is a matrix of coefficients, $c_{ij}$ for $i^{th}$ protein and $j^{th}$ subspectrum
  - Use vector of $c_{ij}$ for protein $i$ to characterize protein. *Note $ψ_i$ depends on training set, construct to be orthogonal*
Standard deviations -Secondary Structure prediction with Variable Selection (proteins eliminated indicated)

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Helix</th>
<th>Sheet</th>
<th>Turns</th>
<th>Bends</th>
<th>“Other”</th>
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<tbody>
<tr>
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<td>(SOD, CYT, CAN)</td>
<td>(CYT, MYO, LYS)</td>
<td>(LYS, CYT, HEM)</td>
<td>(REI, CAN, GRS)</td>
<td>(STI, LDH, CYT)</td>
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<td>(LDH, LYS, MYO)</td>
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<td>(MYO, RNS, GRS)</td>
<td>(SOD, LYS, CYT)</td>
<td>(TIM, RNA, CYT)</td>
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<tr>
<td>FTIR H/D exchange</td>
<td>4.41 – 4.83</td>
<td>5.31 – 5.62</td>
<td>2.86 – 3.15</td>
<td>2.35 – 2.56</td>
<td>4.77 – 4.84</td>
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<td>(MYO, LYS, CAN)</td>
<td>(LYS, SBT, CYT)</td>
<td>(SOD, TIM, CYT)</td>
</tr>
</tbody>
</table>

Variable selection optimizes the fit to the unknown eliminates the outliers, sensitive to spectra shape