

BioMolecular Optical Spectroscopy:
Part 1: Infrared and Raman
Vibrational Spectra Background

Special Lectures for Chem 344
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Vibrational Spectroscopy - Biological Applications

There are many purposes for adapting IR or Raman vibrational spectroscopies to the biochemical, biophysical and bioanalytical laboratory

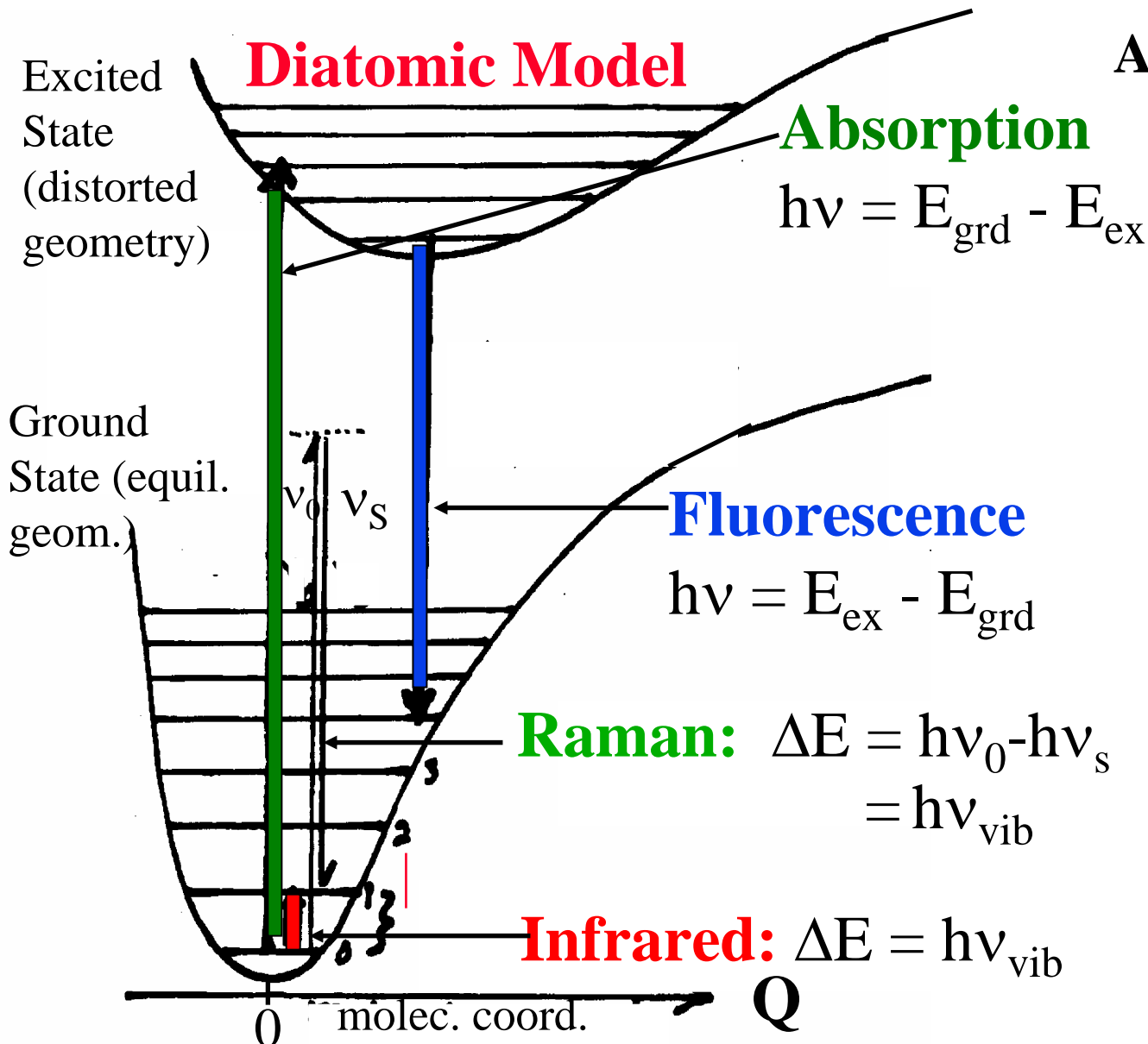
- Prime role has been for determination of **structure**. We will focus early on secondary structure of peptides and proteins, but there are more – especially DNA and lipids
- Also used for **following processes**, such as enzyme-substrate interactions, protein folding, DNA unwinding
- More recently for **quality control**, in pharma and biotech
- New applications in **imaging** now developing, here sensitivity and discrimination among all tissue/cell components are vital

Spectral Regions and Transitions

- **Infrared radiation** excites molecular vibrations, i.e. stretching of bonds and deformation of bond angles. Molecule has $3N-6$ internal degrees of freedom, N atoms. States characterize the bound ground state.
- Radiation in the **visible (Vis) and ultraviolet (UV) regions**, will excite electrons from the bound (ground) state to more weakly bound and dissociative (excited) states, involving **valence electrons**.
 - Changes in both the vibrational and rotational states of the molecule can be associated with this, causing the spectra to become broadened or have fine structure.
- Radiation in vacuum UV and x-ray correspond to changes in electronic structure to either very high excited states or from core electrons (ionization)

Optical Spectroscopy - Processes Monitored
 UV/ Fluorescence/ IR/ Raman/ Circular Dichroism

Diatomic Model



Analytical Methods

UV-vis absorp. & Fluorescence.

move e^- (change electronic state)
 high freq., intense

CD – circ. polarized absorption, UV or IR

Raman – nuclei, inelastic scatter
 very low intensity

IR – move nuclei
 low freq. & inten.

Absorption

$$h\nu = E_{\text{grd}} - E_{\text{ex}}$$

Fluorescence

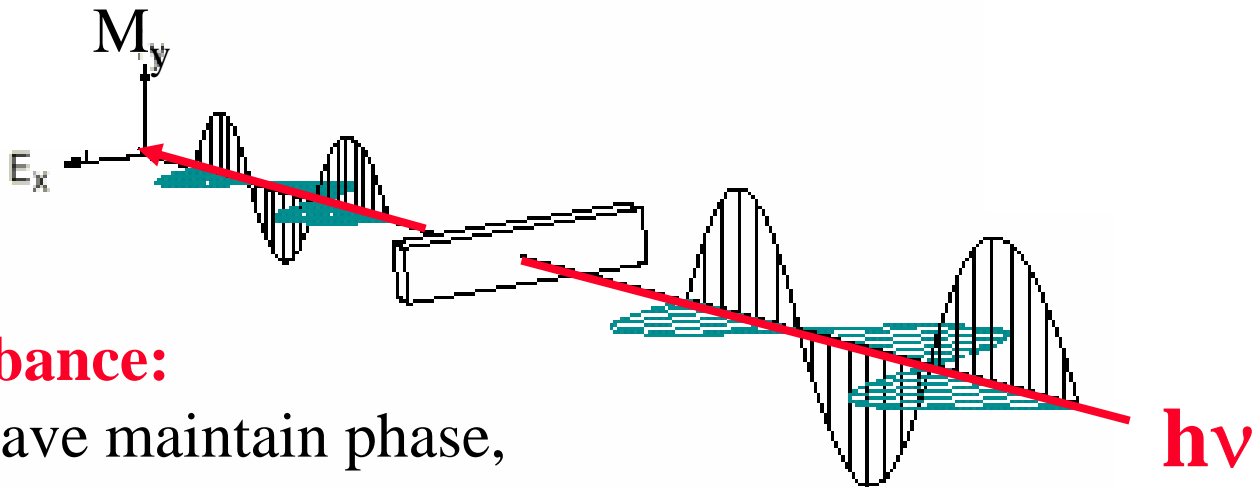
$$h\nu = E_{\text{ex}} - E_{\text{grd}}$$

Raman: $\Delta E = h\nu_0 - h\nu_s$
 $= h\nu_{\text{vib}}$

Infrared: $\Delta E = h\nu_{\text{vib}}$

Spectroscopy

- Study of the consequences of the interaction of electromagnetic radiation (**light**) with **molecules**.
- **Light beam characteristics** - wavelength (frequency), intensity, polarization - determine types of transitions and information accessed.



Absorbance:

E-M wave maintain phase,
attenuate intensity in sample

Optical Spectroscopy – Electronic, Example Absorption and Fluorescence

Essentially a probe technique sensing changes in the local environment of fluorophores

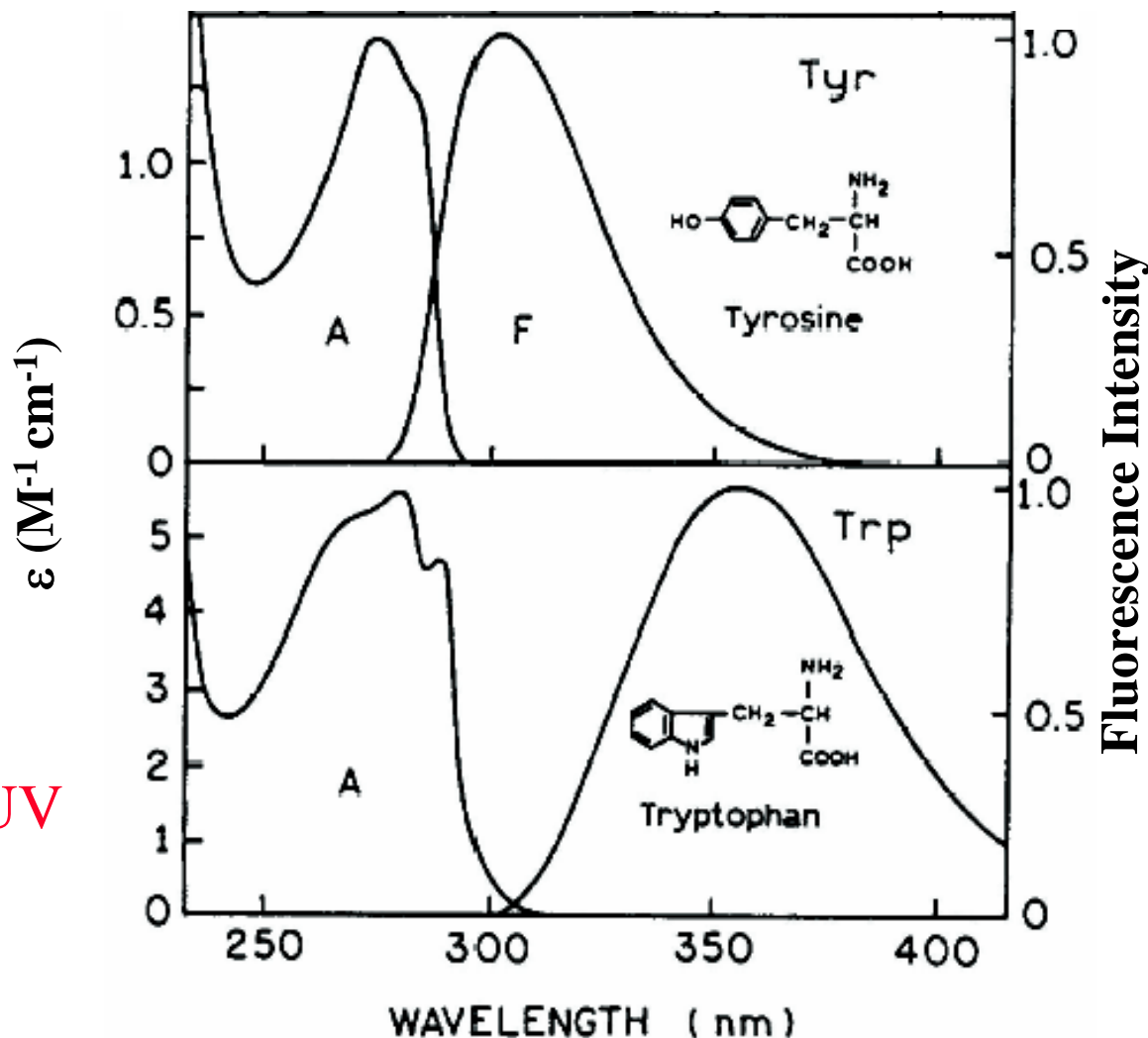
What do you see?
(typical protein)

Intrinsic fluorophores

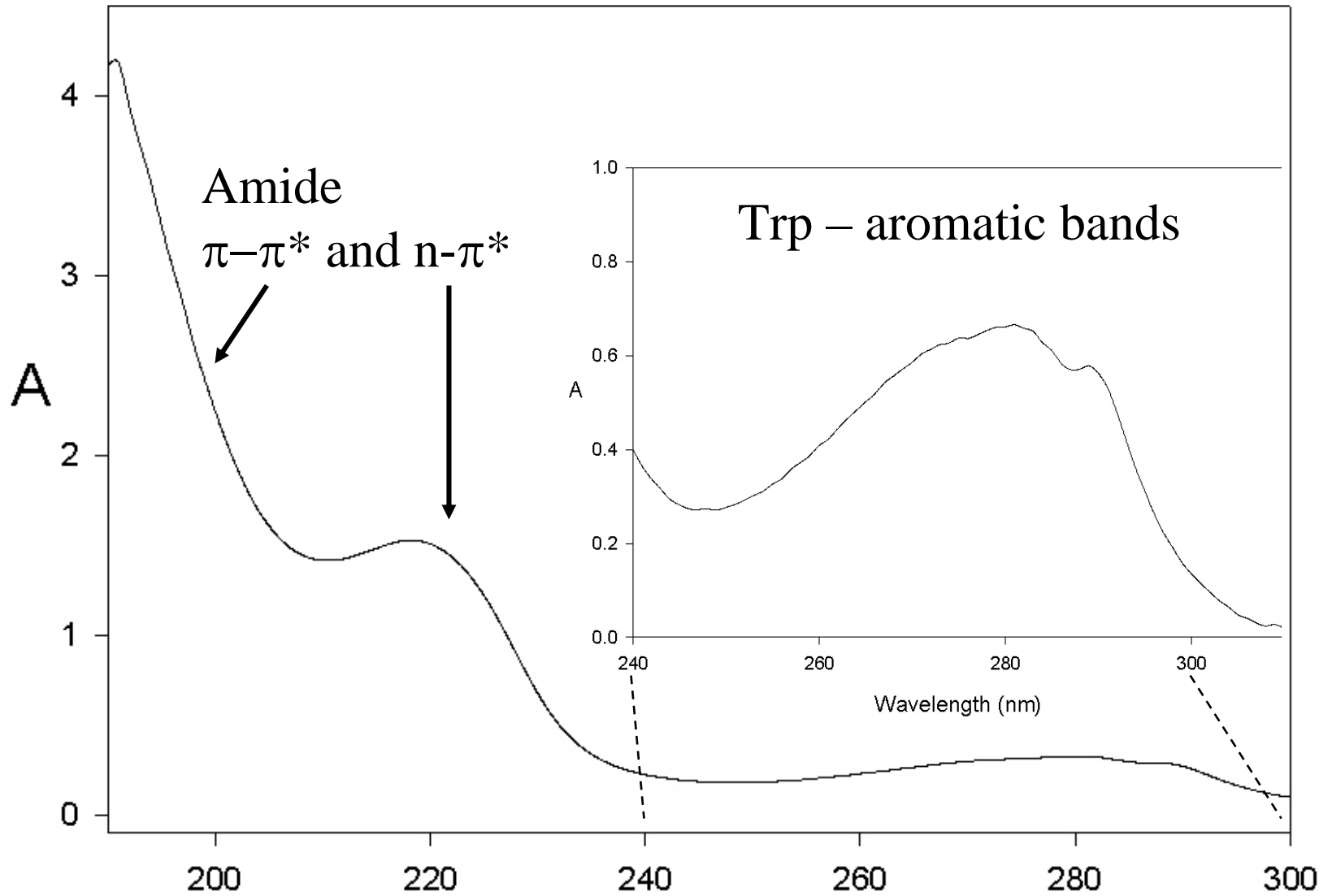
eg. Trp, Tyr

Change with tertiary
structure, compactness

Amide absorption broad,
Intense, featureless, far UV
~200 nm and below



UV absorption of peptides is featureless --except aromatics



TrpZip peptide in water
Rong Huang, unpublished

Wavelength (nm)

Time out—states and transitions

Review of Vibrational Spectra Theory and Characteristics

Spectroscopy—transitions between energy states of a molecule excited by absorption or emission of a photon

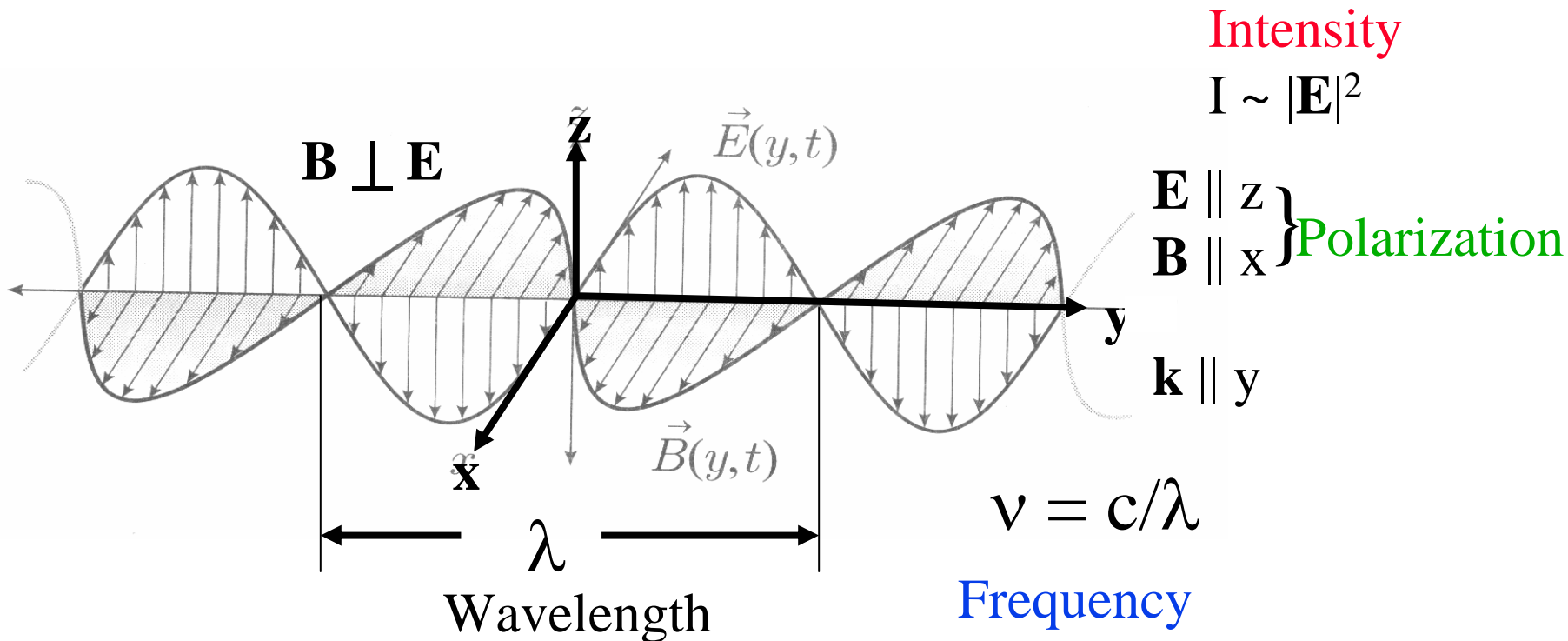
$$h\nu = \Delta E = E_i - E_f$$

Energy levels due to interactions between parts of molecule (atoms, electrons and nuclei) as described by **quantum mechanics**, and are

characteristic of components involved, i.e. electron distributions (orbitals), bond strengths and types plus molecular geometries and atomic masses involved

Spectroscopy

- Study of the consequences of the interaction of electromagnetic radiation (**light**) with **molecules**.
- **Light beam characteristics** - wavelength (frequency), intensity, polarization - determine types of transitions and information accessed.



Properties of light – probes of structure

- Frequency matches change in energy, type of motion

→ $E = h\nu$, where $\nu = c/\lambda$ (in sec^{-1})

- Intensity increases the transition probability—

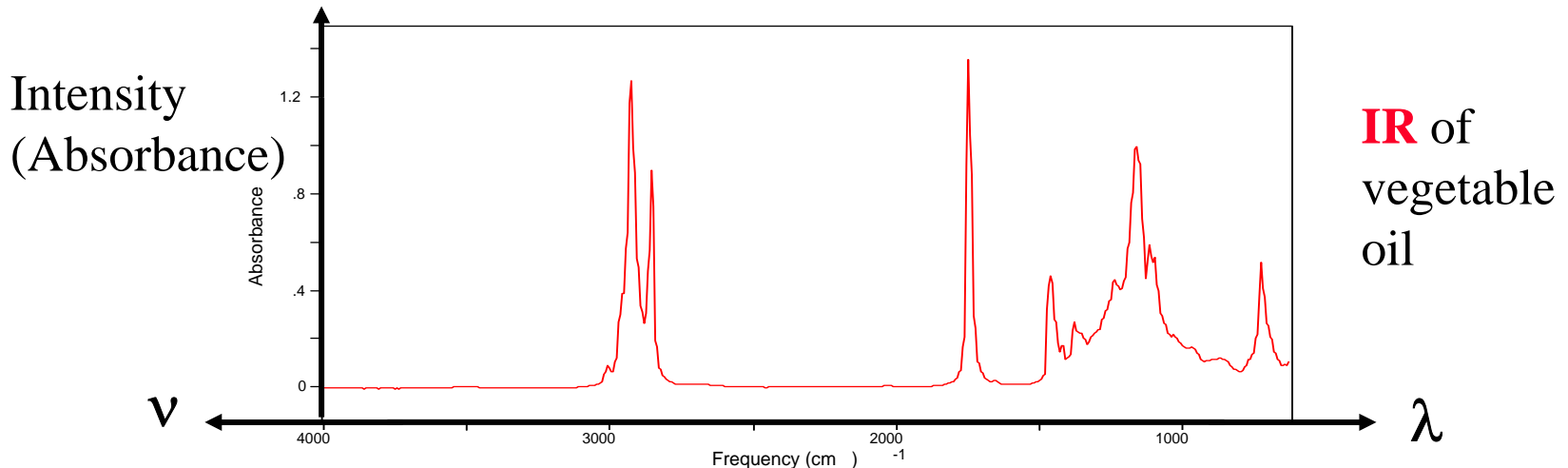
→ $I \sim \epsilon^2$ —where ϵ is the radiation Electric Field strength

Linear Polarization (absorption) aligns with direction of dipole change—
(scattering to the polarizability)

→ $I \sim [\delta\mu/\delta Q]^2$ where Q is the coordinate of the motion

Circular Polarization results from an interference:

→ $\text{Im}(\mu \cdot m)$ μ and m are electric and magnetic dipole



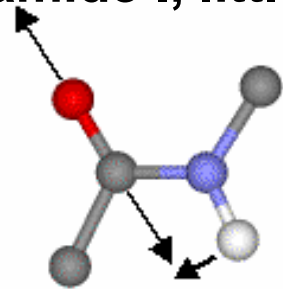
Optical Spectroscopy - IR Spectroscopy

Protein and polypeptide secondary structure obtained from vibrational modes of amide (peptide bond) groups

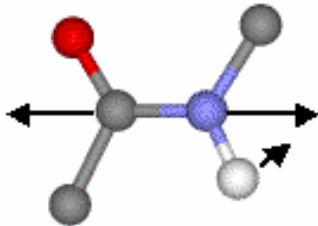
Aside: Raman is similar, but different amide I, little amide II, intense amide III

What do you see?

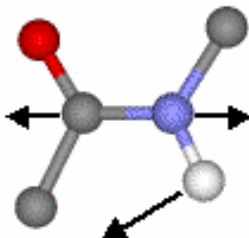
Model peptide IR



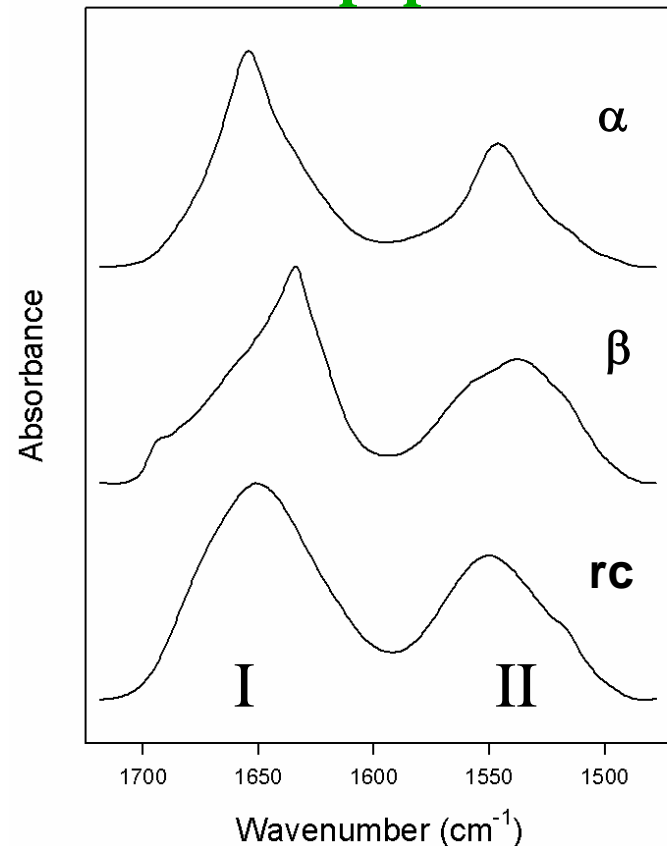
Amide I
(1700-1600 cm^{-1})



Amide II
(1580-1480 cm^{-1})



Amide III
(1300-1230 cm^{-1})



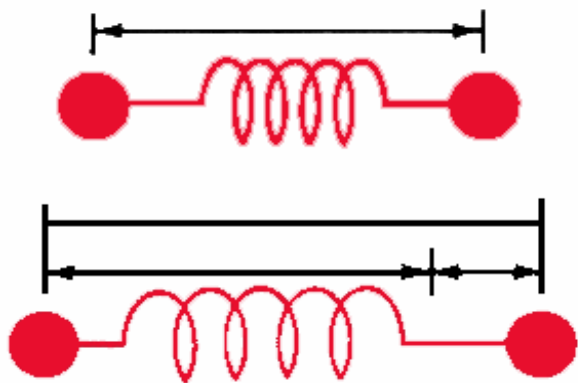
Vibrational States and Transitions

- The simplest case is a diatomic molecule. Rotations (2) and translations (3) leave only one vibrational degree of freedom, the bond length change, Δr , a one dimensional harmonic oscillator
- One can solve this problem exactly as a classical Hook's law problem with
 - a restoring force: $F = -k\Delta r$ and
 - potential energy: $V = 1/2 k(\Delta r)^2$
- Quantum mechanically: $E_v = (v+1/2)h\nu$,

ν is the vibration frequency

Harmonic Oscillator

Model for vibrational spectroscopy



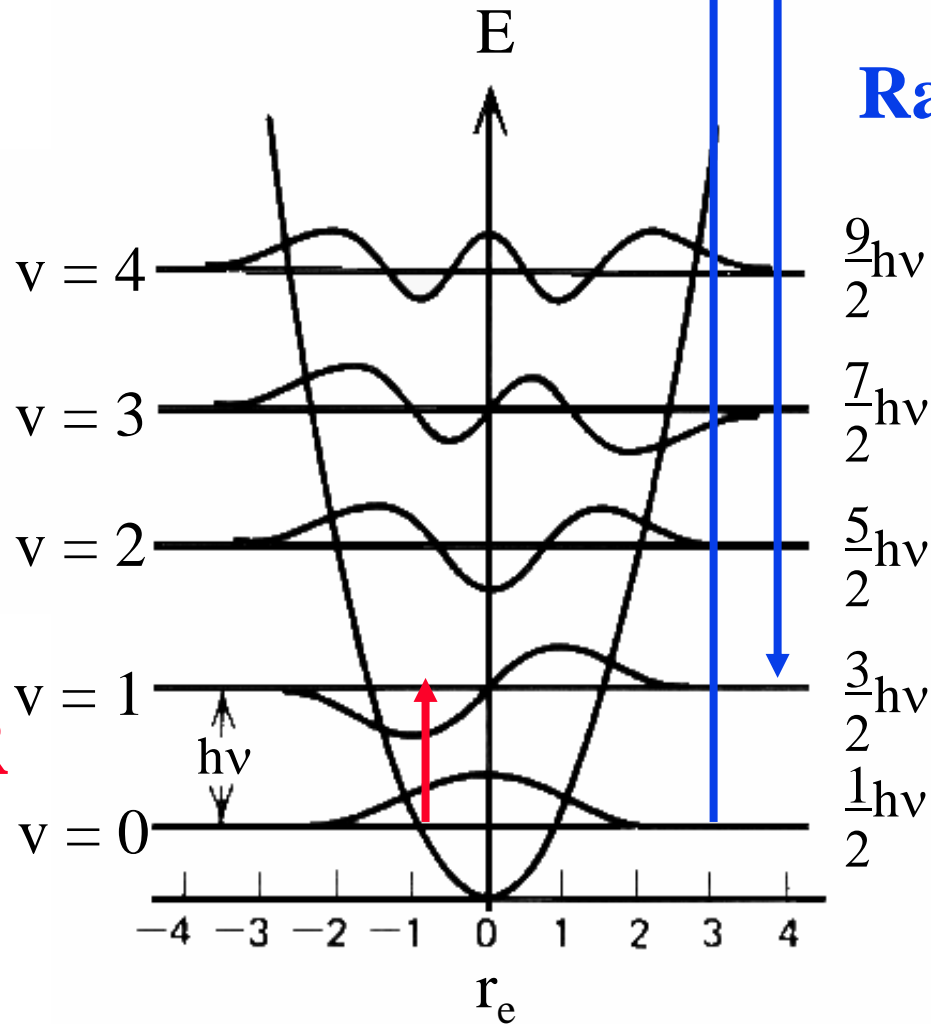
$$E_v = (v + 1/2)h\nu$$

$$\Delta v = \pm 1$$

$$\Delta E = h\nu$$

$$\nu = (1/2\pi)(k/\mu)^{1/2}$$

IR



(virtual state)

Raman

Vibrational States and Transitions

- for the simplest harmonic oscillator case (diatomic):
- $\nu = (1/2\pi)(k/\mu)^{1/2}$

where k is the force constant (d^2V/dq^2).

- In practice, **stronger** bonds have sharper (more curvature) potential energy curves, **result:** higher k , and **higher** frequency.

and μ is the reduced mass $[m_1m_2 / (m_1+m_2)]$.

- In practice, **heavier** atom moving, have **lower** frequency.

Thus vibrational frequencies reflect structure, bonds and atoms

Vibrational States and Transitions

- Summary: high mass \implies low frequency

strong bond \implies high frequency

- Some simple examples (stretches in polyatomics):

-C-C- $\sim 1000 \text{ cm}^{-1}$

C-H $\sim 2800 \text{ cm}^{-1}$

-C=C- $\sim 1600 \text{ cm}^{-1}$

C-D $\sim 2200 \text{ cm}^{-1}$

-C \equiv C- $\sim 2200 \text{ cm}^{-1}$

C-N $\sim 1300 \text{ cm}^{-1}$

Polyatomic Vibrational States

- For a molecule of **N** atoms, there are **(3N-6) vibrational degrees of freedom**. This complex problem can be solved the harmonic approximation by transforming to a new set of **normal coordinates** (combinations of the internal coordinates, q_i) to simplify the potential energy V —**method unimportant for this course**
 - $V = V_0 + \sum (dV/dq_i)_0 q_i + (1/2) \sum (d^2V/dq_i q_j)_0 q_i q_j + \dots$
- This results in a molecular energy that is just the sum of the individual vibrational energies of each normal mode:
 - $E = \sum E_i = \sum (v_i + 1/2) h\nu_i$
- As a result we have characteristic IR and Raman frequencies, ν_i , which are reflect bond types in the molecule. The **frequency pattern forms a “fingerprint” for the molecule and its structure.**
- Variations due to conformation and environment give structural insight and are the prime tools for Protein - Peptide IR and Raman.

Normal Mode Analysis

- In the Harmonic approximation it is possible to define:
 - normal coordinates: $Q_j = \sum c_{ji} q_i$
 - simplify potential: $V_{\text{harm}} = (1/2) \sum (d^2V/dQ_j^2) Q_j^2$
- This summed potential is solved by a simple product wavefunction:
$$\Phi = \prod \phi_i(Q_i)$$
- This results in an energy that is just the sum of the individual vibrational energies of each normal mode:

$$E = \sum E_i = \sum (v_i + 1/2) h\nu_i$$

Vibrational States and Transitions

- Each $\phi_i(Q_i)$ gives a unique frequency, ν_i . The general mass and bond strength characteristics still come through.
- As a result we have IR and Raman group frequencies, ν_i , which are characteristic of bond types in the molecule. The frequency pattern forms a “fingerprint” for the molecule and its structure.
- The variations due to its conformational and environmental conditions can give added structural insight and are the prime tools of IR and Raman spectra of Proteins and Peptides.

Vibrational Transition Selection Rules

Harmonic oscillator: only one quantum can change on each excitation

$$\Delta v_i = \pm 1, \Delta v_j = 0; i \neq j.$$

These are *fundamental* vibrations

Anharmonicity permits overtones and combinations

Normally transitions will be seen from only $v_i = 0$, since most excited states have little population.

Population, n_i , is determined by thermal equilibrium, from the Boltzman relationship:

$$n_i = n_0 \exp[-(E_i - E_0)/kT],$$

where T is the temperature (°K) – (note: kT at room temp $\sim 200 \text{ cm}^{-1}$)

Dipole Moment

- Interaction of light with matter can be described as the induction of *dipoles*, μ_{ind} , by the light electric field, E :

➤ $\mu_{\text{ind}} = \alpha \cdot E$ where α is the polarizability

- **IR absorption** strength is proportional to

➤ $A \sim |\langle \Psi_f | \mu | \Psi_i \rangle|^2$, transition moment between Ψ_i Ψ_f →

- To be observed in the IR, the molecule must change its electric dipole moment, μ , in the transition—leads to selection rules

$$d\mu / dQ_i \neq 0$$

- **Raman intensity** is related to the polarizability,

➤ $I \sim \langle \Psi_b | \alpha | \Psi_a \rangle^2$, similarly $d\alpha / dQ_i \neq 0$ for Raman observation

Raman Selection Rules

Any mode which **expands the molecule** ,

particularly any delocalized bonds, will be intense.

Aromatic groups and disulfides in protein side chains have strong Raman bands and the C=O and C-N stretches in a peptide are relatively strong, O-H and N-H and water are weak (strong IR).

Bases in DNA are strong Raman scatterers

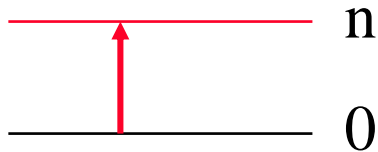
IR vs. Raman Selection Rules

- At its core, Raman also depends on dipolar interaction, but it is a **two-photon process**, excite with ν_0 and detect ν_s , where $\nu_{\text{vib}} = \nu_0 - \nu_s$, so there are two μ 's.

$$\langle \Psi_0 | \mu | \Psi_i \rangle \cdot \langle \Psi_i | \mu | \Psi_n \rangle \sim \alpha$$

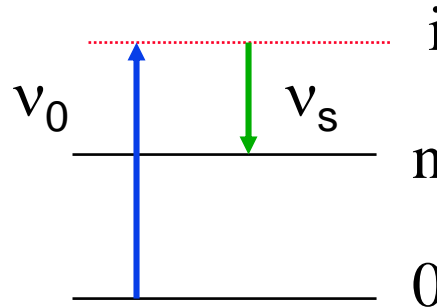
=> need a change in POLARIZABILITY for Raman effect

IR



$$\nu_{\text{vib}} = \nu_n - \nu_0 = \Delta E / h$$

Raman



$$\nu_{\text{vib}} = \nu_0 - \nu_s$$

Symmetry Selection Rules (Dipole, etc.)

Example:

symmetric stretch

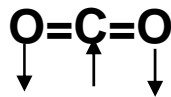


Raman Intense $\delta\alpha/\delta Q \neq 0$

asymmetric stretch $\rightarrow \text{O}=\text{C}=\text{O} \rightarrow$ Infrared active $\delta\mu/\delta Q \neq 0$

Raman *inactive* $\delta\alpha/\delta Q = 0$

bending



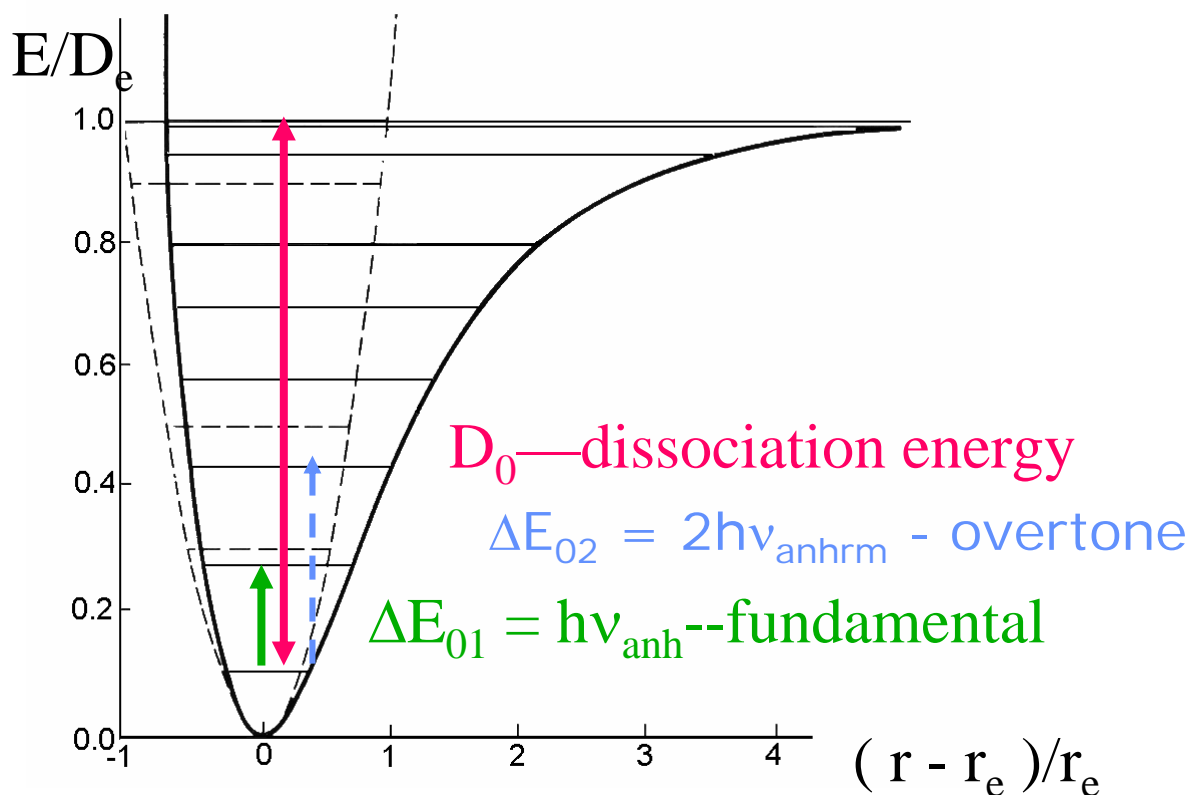
Infrared active $\delta\mu/\delta Q \neq 0$

Raman *inactive* $\delta\alpha/\delta Q = 0$

Note: in biomolecules generally there is little symmetry, however, the residual, approximate symmetry has an impact. Raman and IR intensities often are complementary, for example amide II and III

Anharmonic Transitions

Real molecules are anharmonic to some degree so other transitions do occur but are weak. These are termed *overtone* ($\Delta v_i = \pm 2, \pm 3, \dots$) or *combination bands* ($\Delta v_i = \pm 1, \Delta v_j = \pm 1, \dots$). [Diatomic model]



Complementarity: IR and Raman

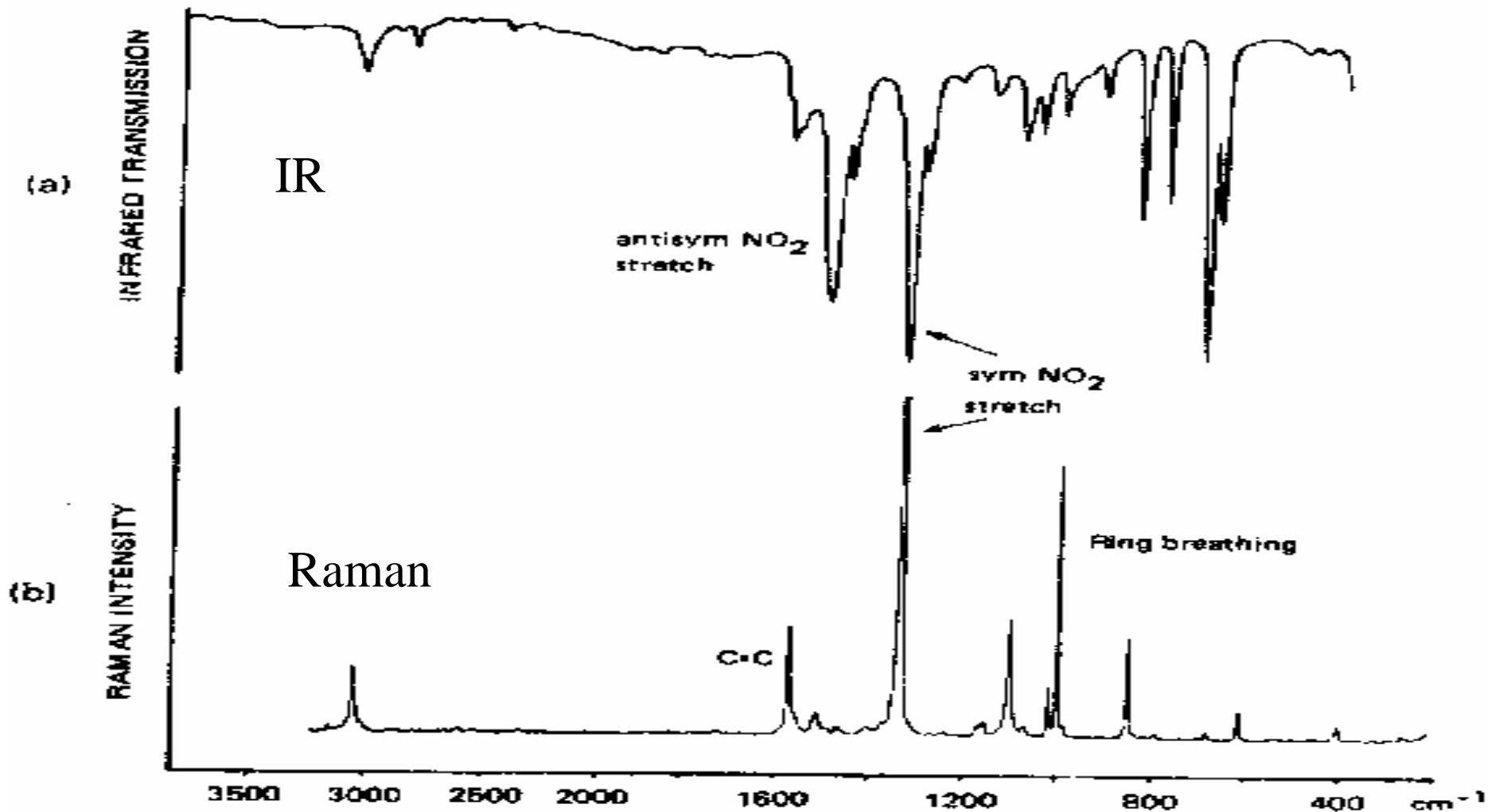


Figure 9-13 (a) Infrared and (b) Raman spectra of nitrobenzene.

If molecule is centrosymmetric, no overlap of IR and Raman

Comparison of Raman and IR Intensities

Table 1.1. Characteristic Wavenumbers and Raman and Infrared Intensities of Groups in Organic Compounds

| Vibration ^a | Region(cm^{-1}) | Intensity ^b | |
|--|----------------------------|------------------------|----------|
| | | Raman | Infrared |
| $\nu(\text{O—H})$ | 3650–3000 | w | s |
| $\nu(\text{N—H})$ | 3500–3300 | m | m |
| $\nu(\equiv\text{C—H})$ | 3300 | w | s |
| $\nu(=\text{C—H})$ | 3100–3000 | s | m |
| $\nu(\text{—C—H})$ | 3000–2800 | s | s |
| $\nu(\text{—S—H})$ | 2600–2550 | s | w |
| $\nu(\text{C}\equiv\text{N})$ | 2255–2220 | m–s | s–0 |
| $\nu(\text{C}\equiv\text{C})$ | 2250–2100 | vs | w–0 |
| $\nu(\text{C}=\text{O})$ | 1820–1680 | s–w | vs |
| $\nu(\text{C}=\text{C})$ | 1900–1500 | vs–m | 0–w |
| $\nu(\text{C}=\text{N})$ | 1680–1610 | s | m |
| $\nu(\text{N}=\text{N})$, aliphatic substituent | 1580–1550 | m | 0 |

Table 1.1. (Continued)

| Vibration ^a | Region(cm ⁻¹) | Intensity ^b | |
|---|---------------------------|--|----------|
| | | Raman | Infrared |
| $\nu(\text{N}=\text{N})$, aromatic substituent | 1440–1410 | m | 0 |
| $\nu_a((\text{C}-)\text{NO}_2)$ | 1590–1530 | m | s |
| $\nu_s((\text{C}-)\text{NO}_2)$ | 1380–1340 | vs | m |
| $\nu_a((\text{C}-)\text{SO}_2(-\text{C}))$ | 1350–1310 | w-0 | s |
| $\nu_s((\text{C}-)\text{SO}_2(-\text{C}))$ | 1160–1120 | s | s |
| $\nu((\text{C}-)\text{SO}(-\text{C}))$ | 1070–1020 | m | s |
| $\nu(\text{C}=\text{S})$ | 1250–1000 | s | w |
| $\delta(\text{CH}_2)$, $\delta_a(\text{CH}_3)$ | 1470–1400 | m | m |
| $\delta_s(\text{CH}_3)$ | 1380 | m-w, s, if at C=C | s-m |
| $\nu(\text{CC})$, aromatics | 1600, 1580 | s-m | m-s |
| | 1500, 1450 | m-w | m-s |
| | 1000 | s (in mono-; m-; 1,3,5- derivatives) | 0-w |
| $\nu(\text{CC})$, alicyclics, and aliphatic chains | 1300–600 | s-m | m-w |

| | | | |
|--|-----------|------|-----|
| $\nu_a(\text{C}=\text{O}-\text{C})$ | 1150–1060 | w | s |
| $\nu_s(\text{C}=\text{O}-\text{C})$ | 970–800 | s–m | w–0 |
| $\nu_a(\text{Si}-\text{O}-\text{Si})$ | 1110–1000 | w–0 | vs |
| $\nu_s(\text{Si}-\text{O}-\text{Si})$ | 550–450 | vs | w–0 |
| $\nu(\text{O}-\text{O})$ | 900–845 | s | 0–w |
| $\nu(\text{S}-\text{S})$ | 550–430 | s | 0–w |
| $\nu(\text{Se}-\text{Se})$ | 330–290 | s | 0–w |
| $\nu(\text{C}(\text{aromatic})-\text{S})$ | 1100–1080 | s | s–m |
| $\nu(\text{C}(\text{aliphatic})-\text{S})$ | 790–630 | s | s–m |
| $\nu(\text{C}-\text{Cl})$ | 800–550 | s | s |
| $\nu(\text{C}-\text{Br})$ | 700–500 | s | s |
| $\nu(\text{C}-\text{I})$ | 660–480 | s | s |
| $\delta_s(\text{CC})$, aliphatic chains | 400–250 | s–m | w–0 |
| C_n , $n = 3, \dots, 12$ | 2495/ n | | |
| $n > 12$ | | | |
| Lattice vibrations in molecular crystals (liberations and translational vibrations) | 200–20 | vs–0 | s–0 |

Source: Reprinted from B. Schrader, *Angew. Chem.* **12**, 882 (1973), with permission of Verlag Chemie, GMBH, Weirheim, Germany.

^a ν , stretching vibration; δ , bending vibration; ν_s , symmetric vibration; ν_a , antisymmetric vibration.

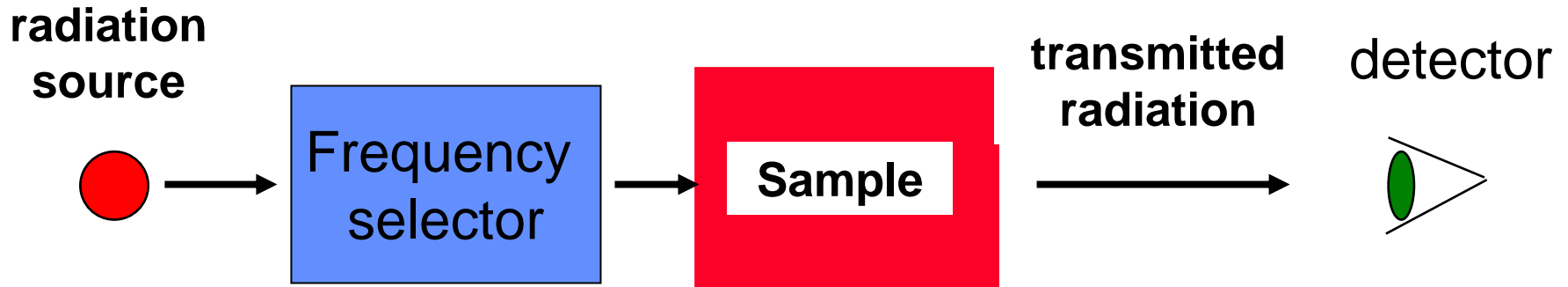
^bvs, very strong; s, strong; m, medium; w, weak; 0, very weak or inactive.

IR & Raman Instrumentation - Outline

- Principles of infrared spectroscopy
- FT advantages
- Elements of FTIR spectrometer
- Acquisition of a spectrum
- Useful Terminology
- Mid-IR sampling techniques
 - Transmission
 - Solids
- Raman instrumentation comparison
- (Note—more on sampling variations later)

Techniques of Infrared Spectroscopy

Infrared spectroscopy deals with **absorption of radiation**--
detect attenuation of beam by sample at detector



Dispersive spectrometers (old) measure transmission as a function of frequency (wavelength) - sequentially--same as typical UV-vis

Interferometric spectrometers measure intensity as a function of mirror position, all frequencies simultaneously--**Multiplex advantage**

Major Fourier Transform Advantages

- **Multiplex Advantage**

- All spectral elements are measured at the same time, simultaneous data acquisition. **Felgett's advantage.**

- **Throughput Advantage**

- Circular aperture typically large area compared to dispersive spectrometer slit for same resolution, increases throughput. **Jacquinot advantage**

- **Wavenumber Precision**

- The wavenumber scale is locked to the frequency of an internal He-Ne reference laser, +/- 0.1 cm⁻¹. **Conne's advantage**

Typical Elements of FT-IR

IR Source (with input collimator)

- Mid-IR: Silicon Carbide glowbar element, $T_c > 1000^\circ\text{C}$; 200 - 5000 cm^{-1}
- Near IR: Tungsten Quartz Halogen lamp, $T_c > 2400^\circ\text{C}$; 2500 - 12000 cm^{-1}

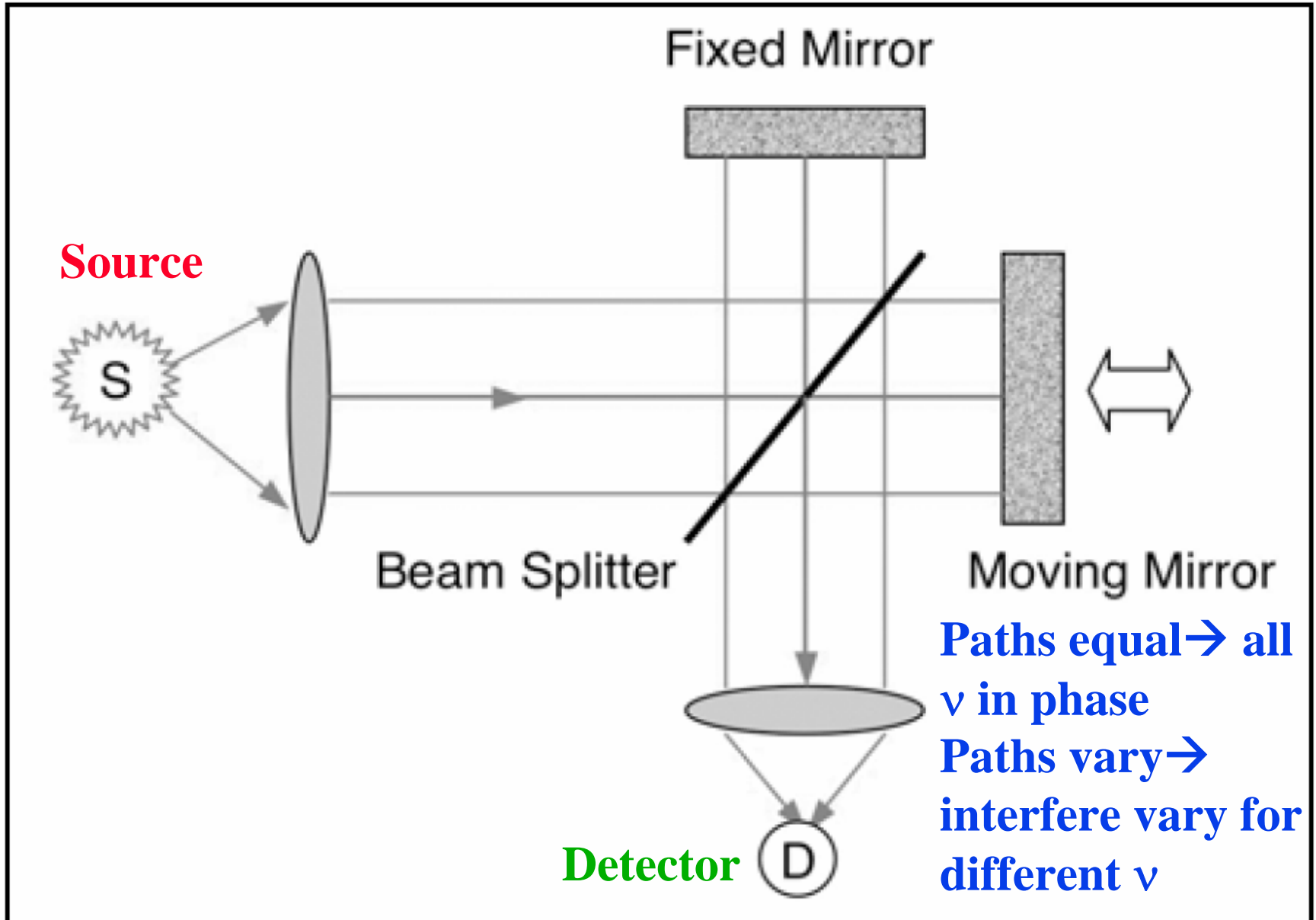
IR Detectors:

- DTGS: deuterated triglycine sulfate - *pyroelectric bolometer (thermal)*
 - Slow response, broad wavenumber detection
- MCT: mercury cadmium telluride - *photo conducting diode (quantum)*
 - must be cooled to liquid N_2 temperatures (77 K)
 - mirror velocity (scan speed) should be high (20Khz)

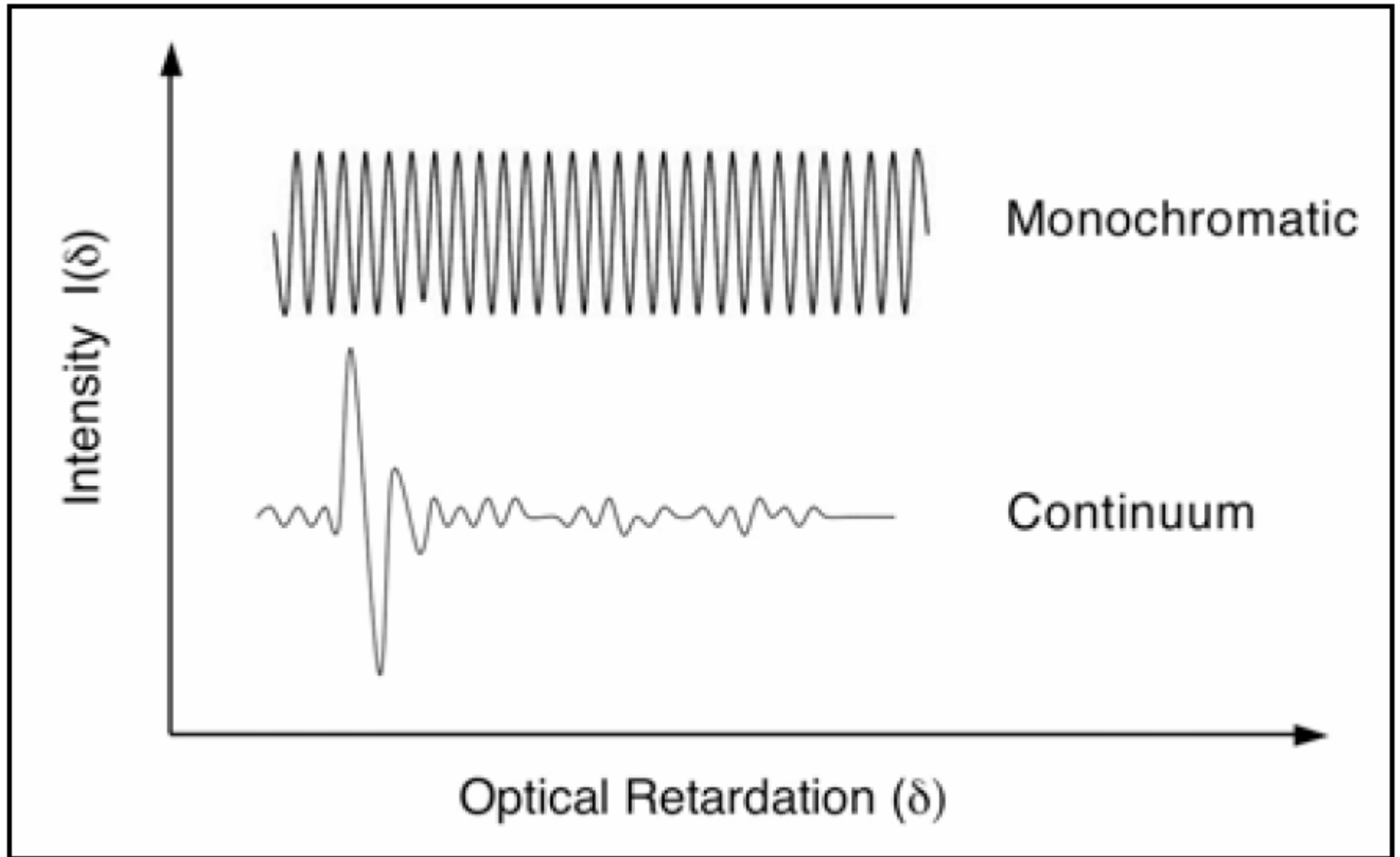
Sample Compartment

- IR beam focused (< 6 mm), permits measurement of small samples.
- Enclosed with space in compartment for sampling accessories

Interference - Moving Mirror Encodes Wavenumber



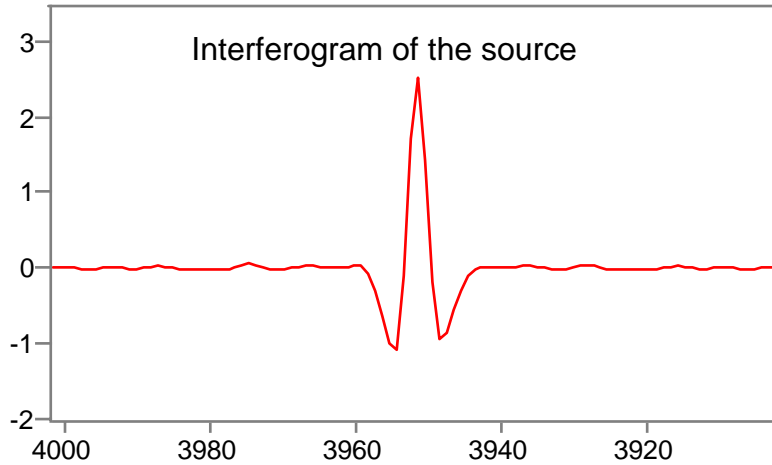
Interferograms for different light sources



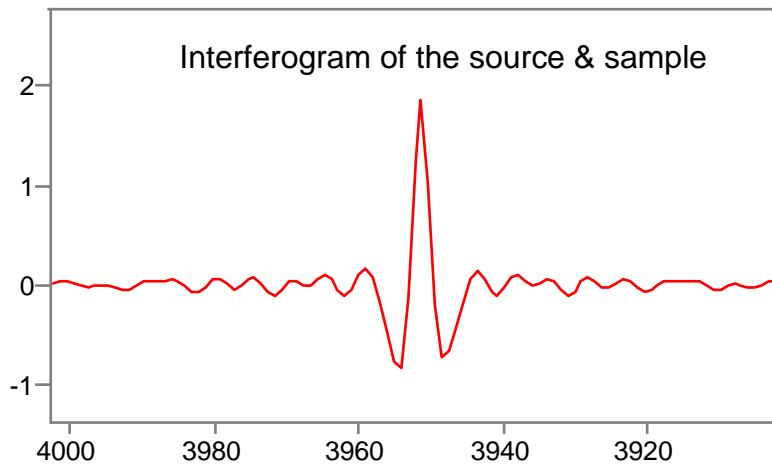
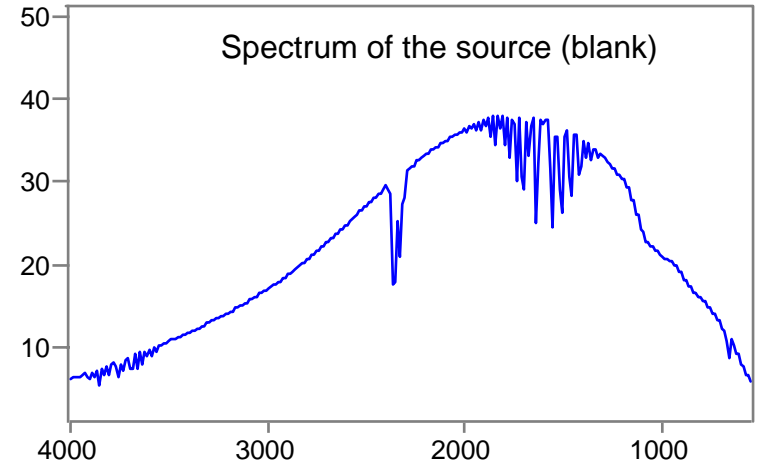
Acquisition of an Infrared Spectrum

- The light coming from the source goes through the interferometer; the movement of the mirrors causes an interference pattern which is called the **Interferogram**
- Next the light passes through the sample; the Interferogram of the source is modified as the light is absorbed by the sample
- The Interferogram is measured by the detector
- The Interferogram is transformed into a spectrum by a mathematical operation called the **Fourier Transform** algorithm
- **Measurements are Single Beam:** To get useful spectrum must correct for the light throughput, divide **Sample response by Background** response (better by blank, cell plus solvent)
- Take log of transmission, **$A = -\log_{10}(I/I_0)$**

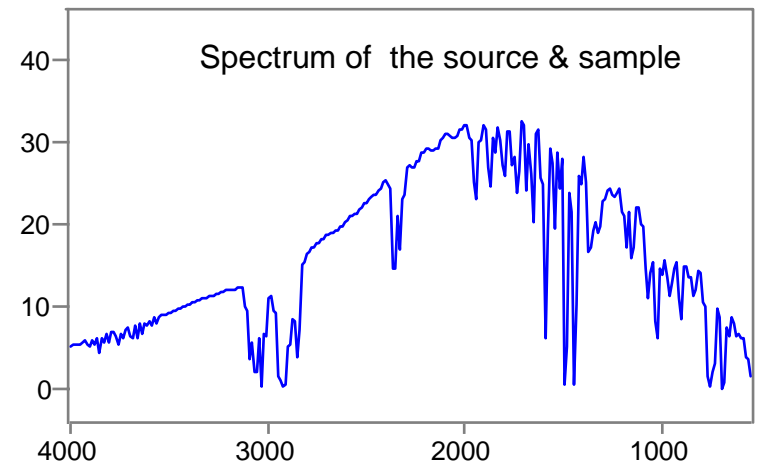
Acquisition of an Infrared Spectrum



FT →



FT →

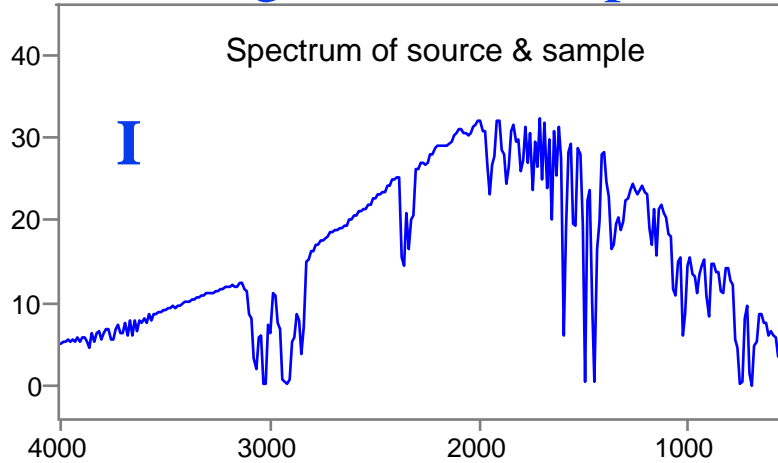


Fast Fourier transform done in computer

Convert Interferogram to Spectrum (single beam)

Acquisition of an Infrared Spectrum

Single beam sample

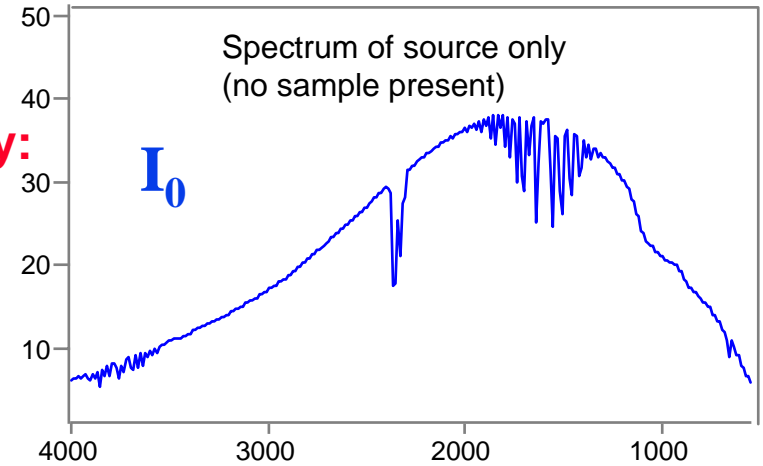


Divide by:

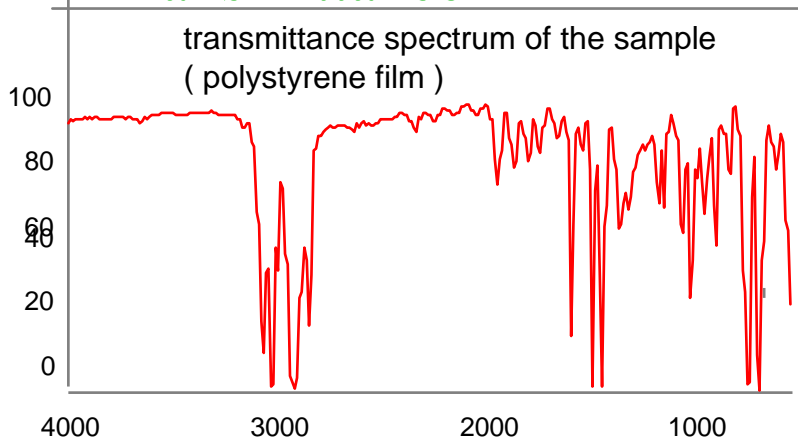
i.e.
 I/I_0

gives

Single beam background



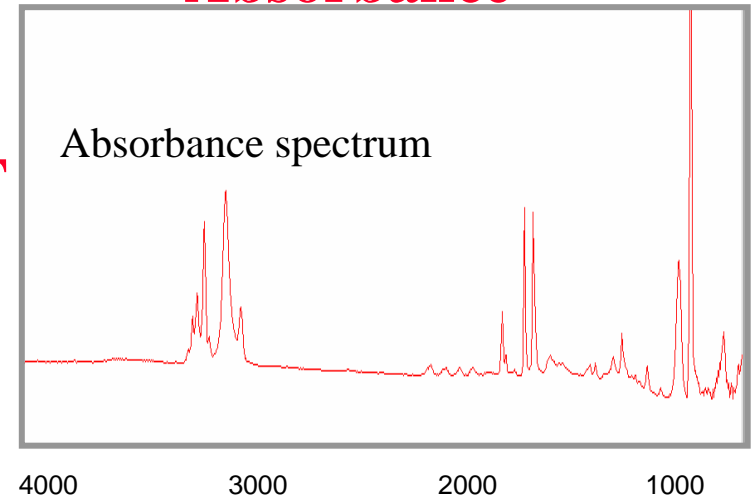
Transmittance



$-\log_{10} T$

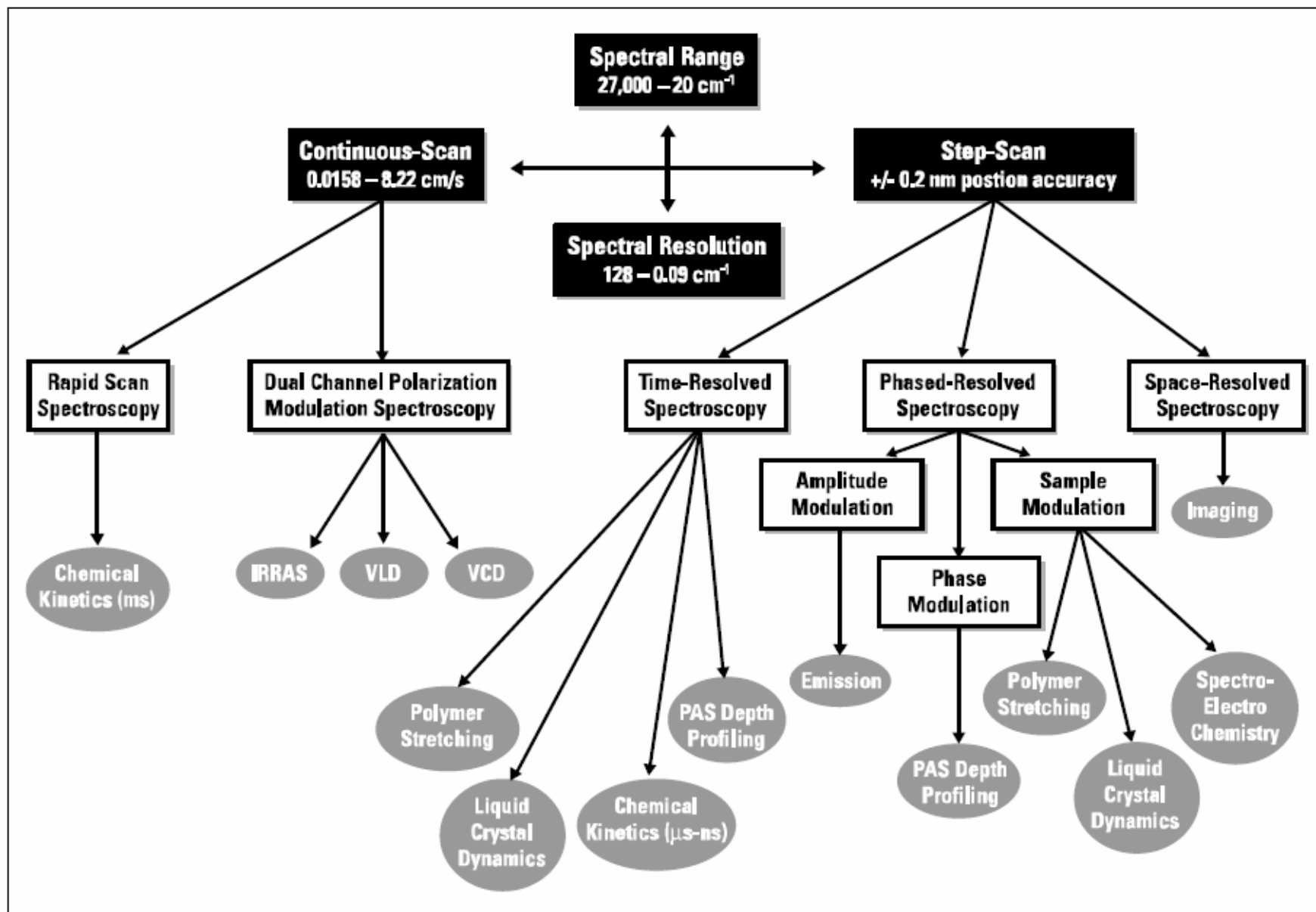


Absorbance



Transmittance old (negative peak), Absorbance (positive) ~ concentration

Going beyond normal IR spectra – FT variations



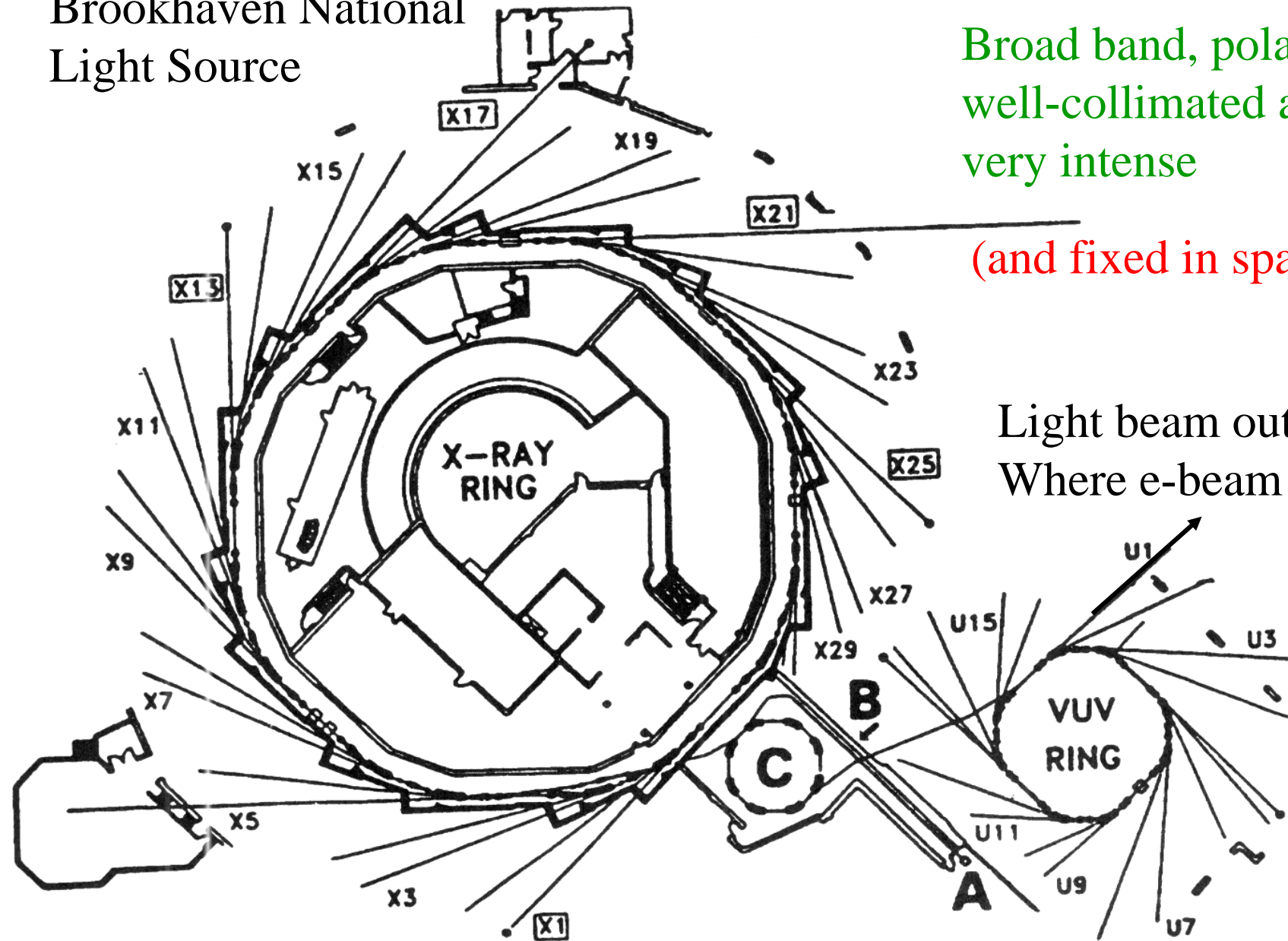
Synchrotron Light Sources – the next big thing

Brookhaven National
Light Source

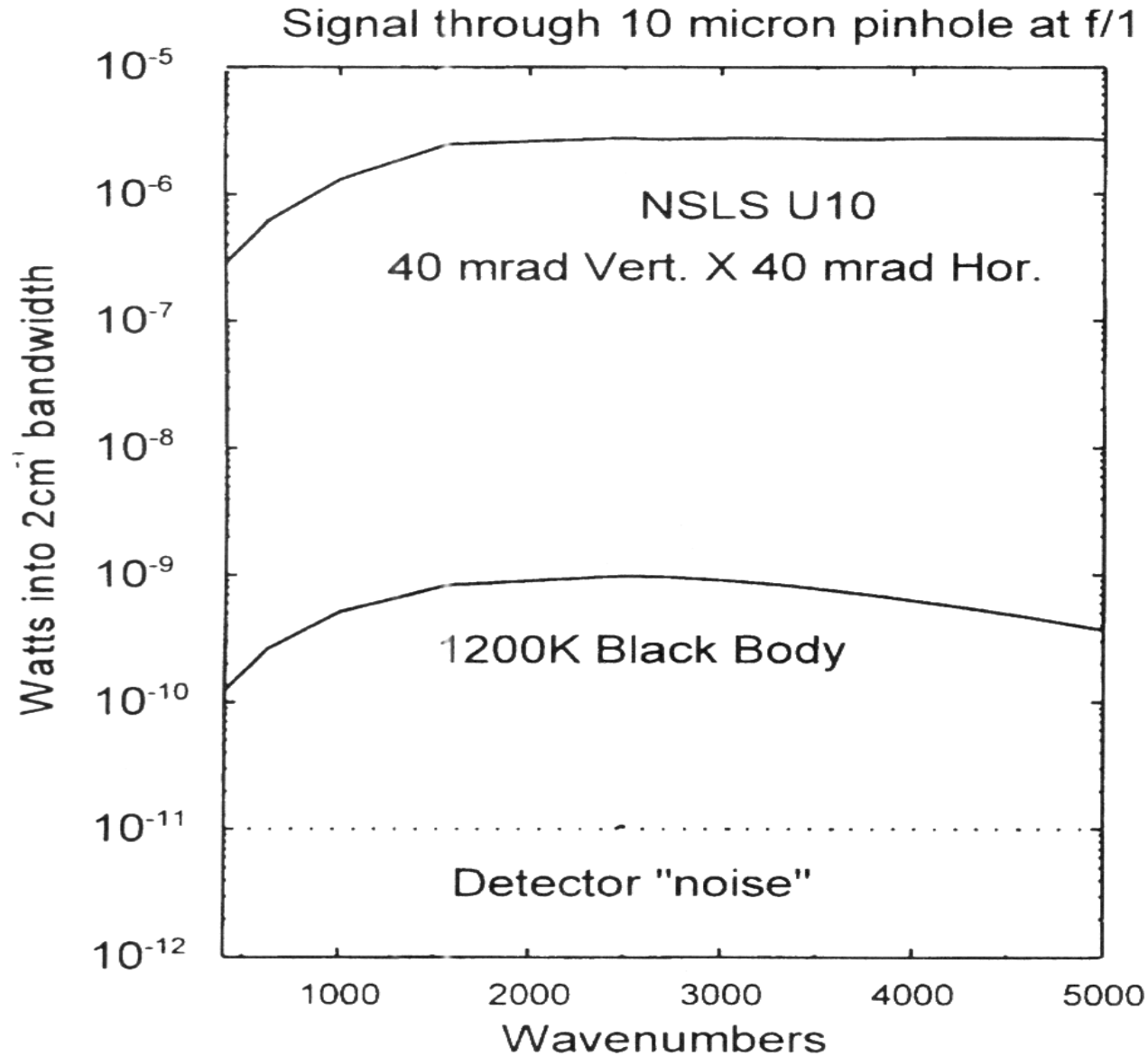
Broad band, polarized
well-collimated and
very intense

(and fixed in space!)

Light beam output
Where e-beam turns



Synchrotron advantage – high brightness



Advantages of Raman Spectroscopy--Comparison

- **Non-destructive**
- **Flexible sampling - any phase or size - no preparation**
- **1 μ m 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**

Raman and IR Spectra - Complementary

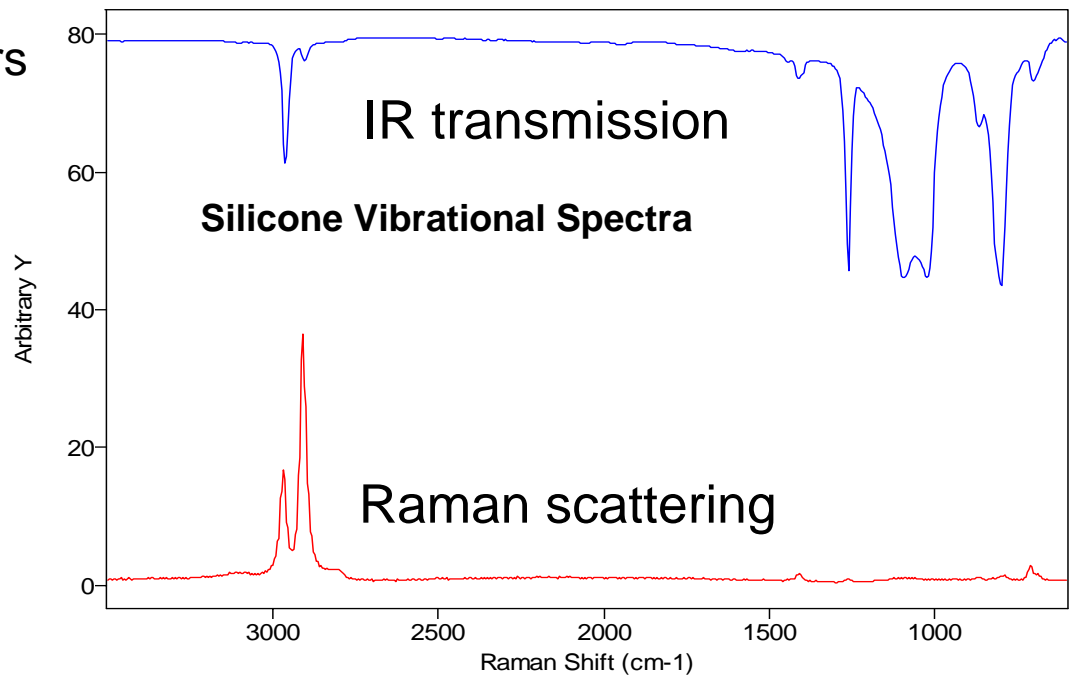
Raman and IR are Complementary - similar transitions, different sensitivities

Raman and IR both provide chemical bond information.

Raman uses visible or uv light, so optics and cells can be glass,
detectors can be high sensitivity

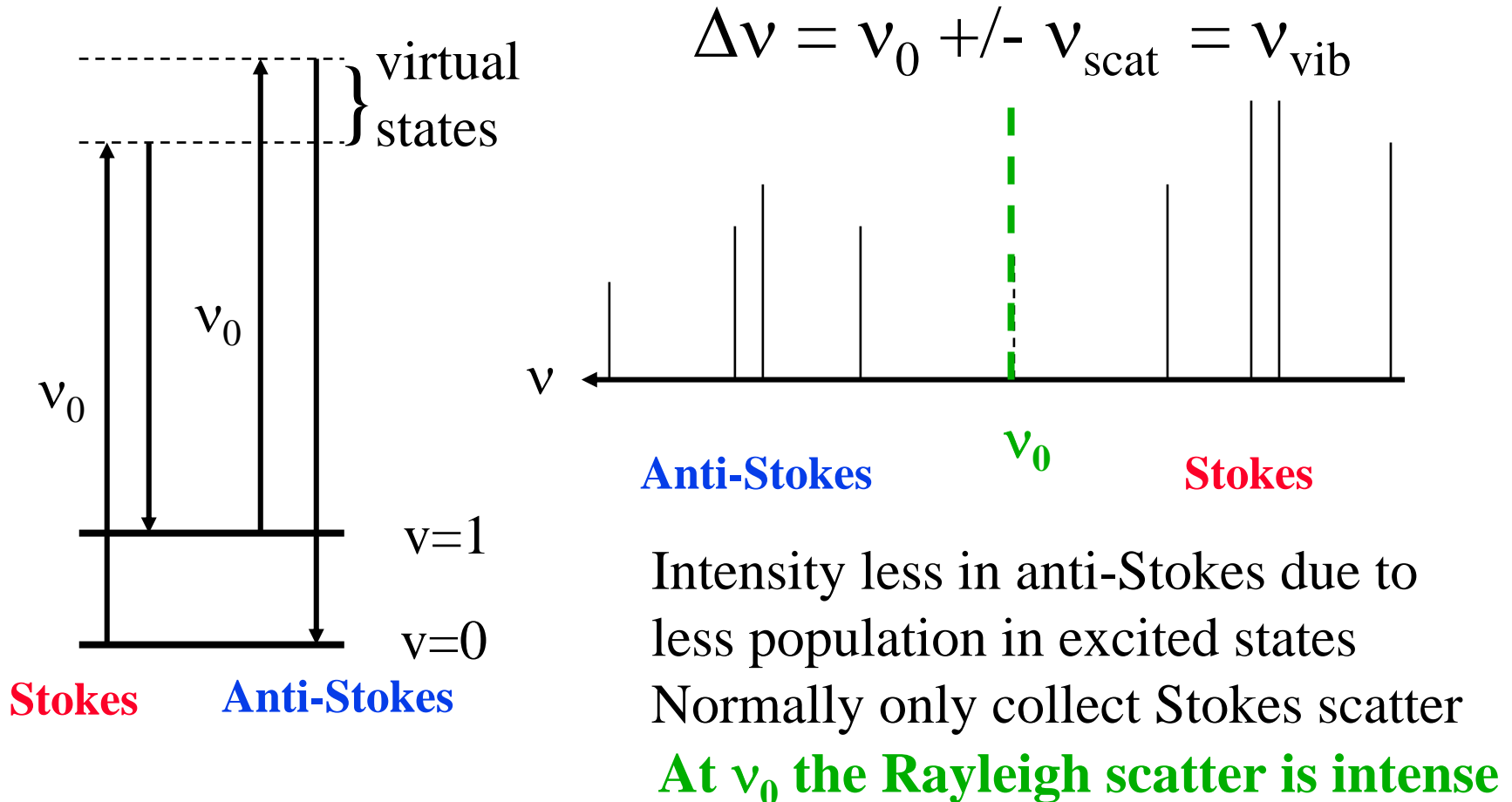
Frequently, Strong IR Absorbers
are Weak Raman Scatterers,
and Vice Versa - Analysis of
biological molecules in water
becomes easier

Variants of Raman,
especially resonance and
surface enhanced Raman
are very sensitive



Raman spectra are result of scattering

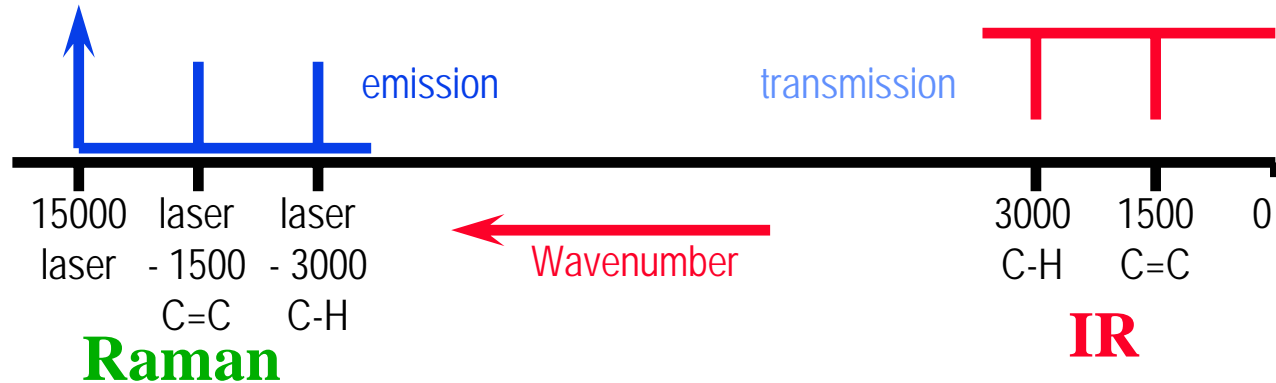
Typically a laser (intense at ν_0) is focussed onto sample and scattered light is collected, often at 90 degree angle
 Like fluorescence, but no real state is excited by the laser



Raman Shift

- IR absorption frequency = Raman shift from laser
- Reported as Raman shift, $\Delta\nu_{\text{Raman}}$ with units of cm^{-1}

$$\Delta\nu_{\text{Raman Shift}} = \nu_{\text{laser}} - \nu_{\text{scattered}}$$



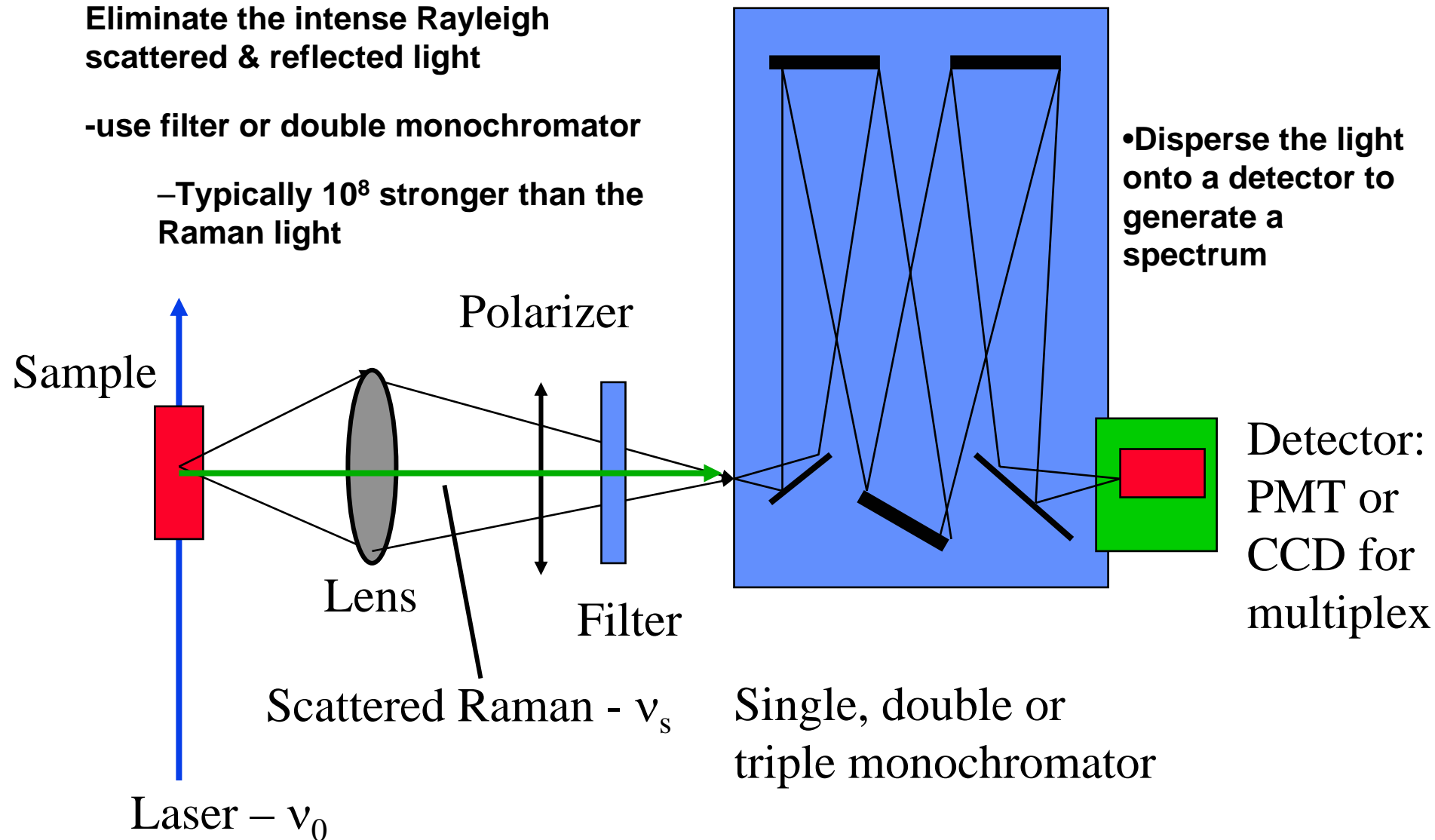
- The **absolute wavenumber** of the Raman scattered light depends on the laser wavelength.
- It's the **Raman Shift**, the Change in Photon Energy, that is Sensitive to Molecular Structure

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



Excitation Sources

- CW gas lasers
(Ar, Kr, Ne, N₂ (337.1 nm UV, pulsed), CO₂ (9-11 μm IR, CS/pulsed), excimer XeCl, 308 nm UV, pulsed)
- Dye lasers
- Solid-state lasers
Ruby (694.3 nm, pulsed), Nd:YAG (1064-nm near-IR, CW/pulsed), Diode (3500-380 cm⁻¹ IR, CW/pulsed)
- Pulsed Nd:YAG
laser harmonics (532 nm, 355 nm, 266 nm) for time-resolved and UV resonance Raman applications