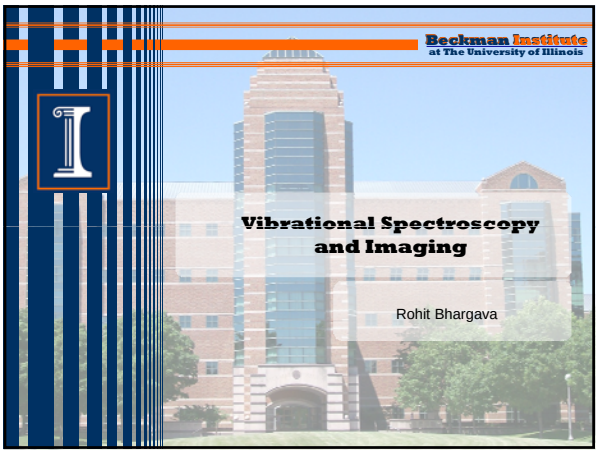
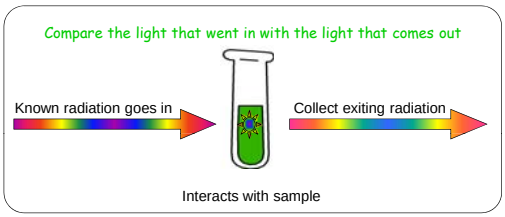


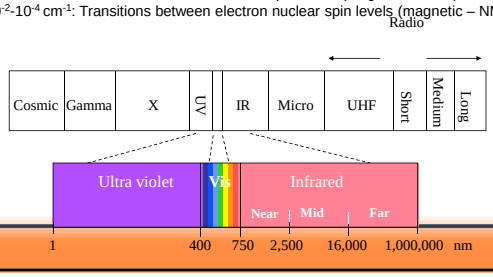
Spectroscopy generalization



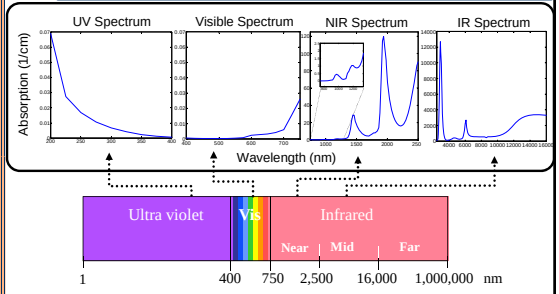
A general definition of spectroscopy is the study of the interaction between radiation and an analyte as a function of wavelength.

Spectroscopy (Radiation)

10^{10}-10^{8} cm^{-1}: Re-arrangement of elementary particles (gamma ray)
10^{8}-10^{6} cm^{-1}: Transitions of inner electrons (X-ray)
10^{6}-10^{4} cm^{-1}: Transitions of valence electrons (UV-vis spectroscopy)
10^{4}-10^{2} cm^{-1}: Transitions between vibrational levels (IR and Raman)
10^{2}-10^{0} cm^{-1}: Transitions between rotational levels (Microwave, IR, Raman, THz)
10^{0}-10^{-2} cm^{-1}: Transitions between electron spin levels (magnetic – ESR)
10^{-2}-10^{-4} cm^{-1}: Transitions between electron nuclear spin levels (magnetic – NMR)



Spectroscopy of liquid water



Why is water blue?

Gas	Assignment	liquid
3651	ν_1	3400
3755	ν_3	
5332	$\nu_2+\nu_3$	5150
7251	$\nu_1+\nu_3$	6900
8807	$\nu_1+\nu_2+\nu_3$	8400
10613	$2\nu_1+\nu_3$	10300
13831	$3\nu_1+\nu_3$	13160 (760 nm)
14319	$\nu_1+3\nu_3$	13510 (740 nm)
15348	$3\nu_1+\nu_2+\nu_3$	15150 (660 nm)
15832	$\nu_1+\nu_2+3\nu_3$	15150 (660 nm)
16822	$3\nu_3+2\nu_2+\nu_1$	weak

- Gas and liquid have different positions
- Combinations and overtones are weaker
- Significant example of absorption determining color
- What is the effect of cold water? Ice?

So, what are these vibrational modes

Symmetrical stretching	Antisymmetrical stretching	Scissoring	Rocking	Wagging	Twisting
					

Simplest Model of a Diatomic Molecule: beads and spring harmonic oscillator

- Hooke's Law Restoring force is proportional to displacement (K~105 dynes
- Newton's law: force if mass x acceleration

$$\vartheta_0 = \frac{1}{2\pi}\sqrt{\frac{K}{\mu}} \sim 10^{12}\text{-}10^{14}\ \text{Hz}$$

Spectroscopy : Molecular Basis

Raman Scattering

$Incident\ field: E = E_0 cos(2\pi\nu_0 t)$

$Dipole\ moment\ in\ the\ molecule: P = \alpha E = \alpha E_0 cos(2\pi\nu_0 t)$

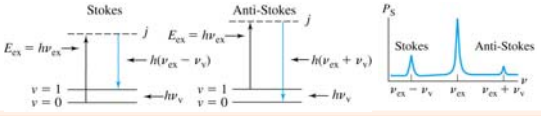
$Nuclear\ displacement: q = q_0 cos(2\pi\nu_m t)$

$For\ small\ amplitudes: \alpha = \alpha_0 + \left(\frac{\partial\alpha}{\partial q_0}\right) q_0 + \ \ldots\ldots$

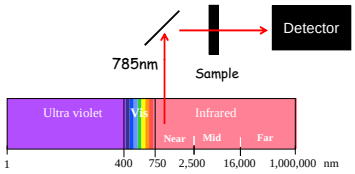
$Resulting\ P = \alpha E = \alpha_0 E_0 cos(2\pi\nu_0 t) + \frac{1}{2}\left(\frac{\partial\alpha}{\partial q_0}\right)_0 q_0 E_0 [cos(2\pi(\nu_0 + \nu_m)t) + cos(2\pi(\nu_0 - \nu_m)t)]$

$\alpha = polarizability: vibrational\ frequency = \nu_m$

Note: - change in polarizability, excitation frequency and shift by vibrational frequency
- Two types of shifts to lower (Stokes) and higher (anti-Stokes): which population is favored?

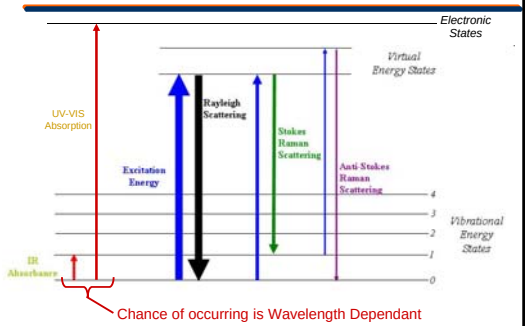


Raman Spectroscopy: Basic Concept



First observed by Sir C. Venkata Raman in 1928 using sunlight and photographic filters (won Nobel price in physics in 1930).

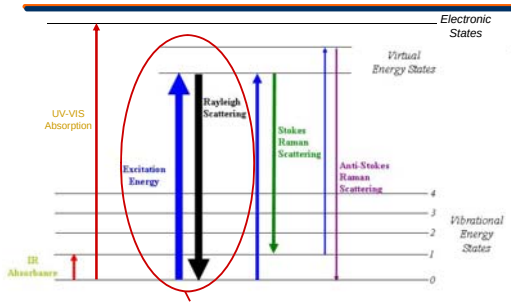
Raman Spectroscopy: Basic Concept



Chance of occurring is Wavelength Dependant
Resonance Raman Scattering?

http://en.wikipedia.org/wiki/File:Raman_energy_levels.jpg

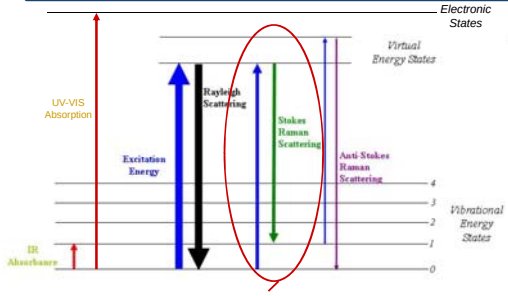
Raman Spectroscopy: Basic Concept



If light is not absorbed… the majority of the photons pass through the sample by means of Rayleigh Scattering

http://en.wikipedia.org/wiki/File:Raman_energy_levels.jpg

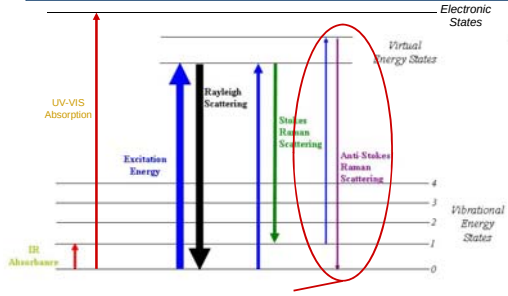
Raman Spectroscopy: Basic Concept



Some of the photons (~1/10,000,000) that are not absorbed will pass through the sample and undergo Stokes Raman Scattering

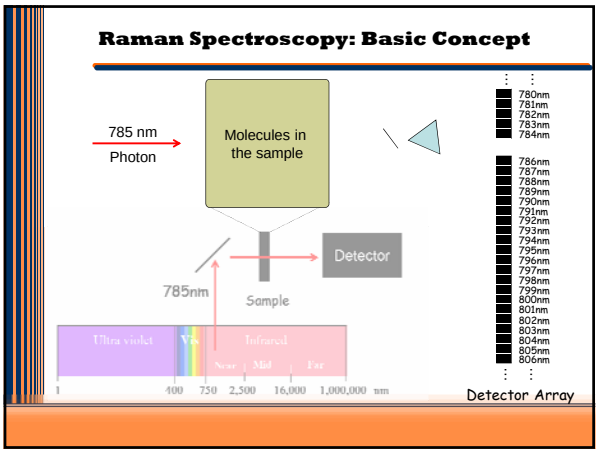
http://en.wikipedia.org/wiki/File:Raman_energy_levels.jpg

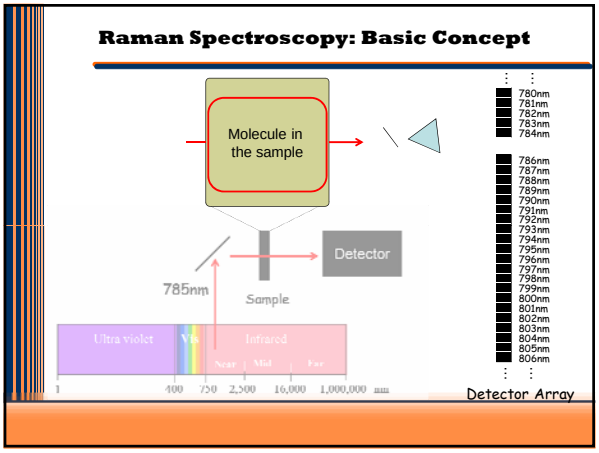
Raman Spectroscopy: Basic Concept



Even fewer photons (temperature dependant) that are not absorbed will pass through the sample and undergo Anti-Stokes Raman Scattering

http://en.wikipedia.org/wiki/File:Raman_energy_levels.jpg


Raman Spectroscopy: Basic Concept
785 nm
Photon
Molecules in the sample
Detector
785nm
Sample
Ultra violet
Vis
Infrared
Near
Mid
Far
1
400
750
2,500
16,000
1,000,000
nm
780nm
781nm
782nm
783nm
784nm
786nm
787nm
788nm
789nm
790nm
791nm
792nm
793nm
794nm
795nm
796nm
797nm
798nm
799nm
800nm
801nm
802nm
803nm
804nm
805nm
806nm
Detector Array


Raman Spectroscopy: Basic Concept
Molecule in the sample
Detector
785nm
Sample
Ultra violet
Vis
Infrared
Near
Mid
Far
1
400
750
2,500
16,000
1,000,000
nm
780nm
781nm
782nm
783nm
784nm
786nm
787nm
788nm
789nm
790nm
791nm
792nm
793nm
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795nm
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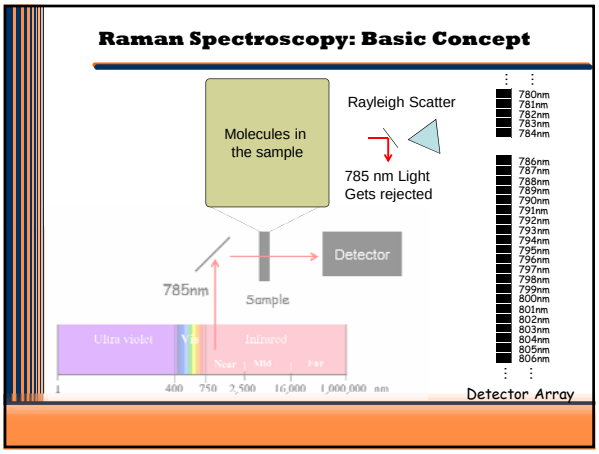
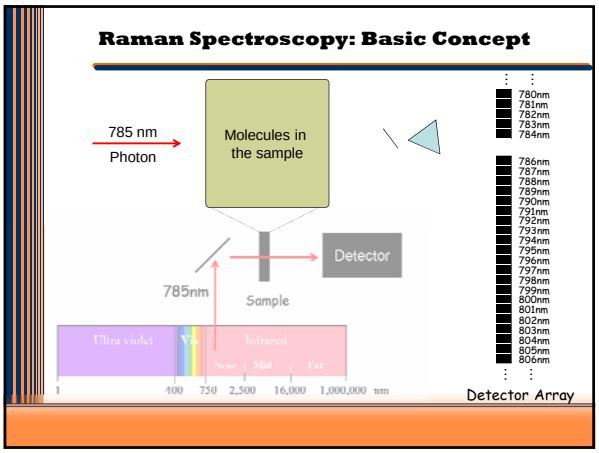
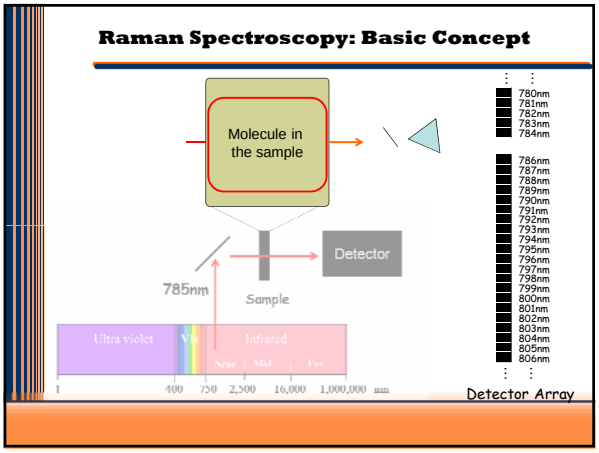
797nm
798nm
799nm
800nm
801nm
802nm
803nm
804nm
805nm
806nm
Detector Array


Raman Spectroscopy: Basic Concept
Molecules in the sample
Rayleigh Scatter
785 nm Light
Gets rejected
Detector
785nm
Sample
Ultra violet
Vis
Infrared
Near
Mid
Far
1
400
750
2,500
16,000
1,000,000
nm
780nm
781nm
782nm
783nm
784nm
786nm
787nm
788nm
789nm
790nm
791nm
792nm
793nm
794nm
795nm
796nm
797nm
798nm
799nm
800nm
801nm
802nm
803nm
804nm
805nm
806nm
Detector Array


Raman Spectroscopy: Basic Concept
785 nm
Photon
Molecules in the sample
Detector
785nm
Sample
Ultra violet
Vis
Infrared
Near
Mid
Far
1
400
750
2,500
16,000
1,000,000 nm
780nm
781nm
782nm
783nm
784nm
786nm
787nm
788nm
789nm
790nm
791nm
792nm
793nm
794nm
795nm
796nm
797nm
798nm
799nm
800nm
801nm
802nm
803nm
804nm
805nm
806nm
Detector Array


Raman Spectroscopy: Basic Concept
Molecule in the sample
Detector
785nm
Sample
Ultra violet
Vis
Infrared
Near
Mid
Far
1
400
750
2,500
16,000
1,000,000 nm
780nm
781nm
782nm
783nm
784nm
786nm
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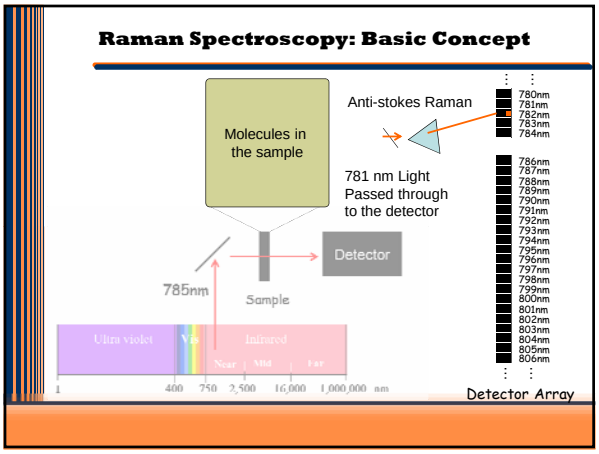
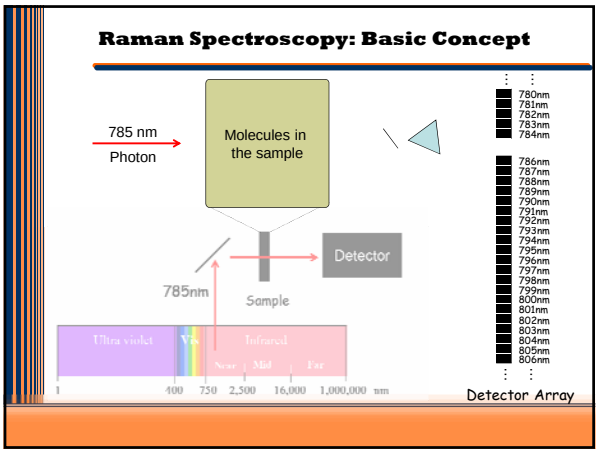
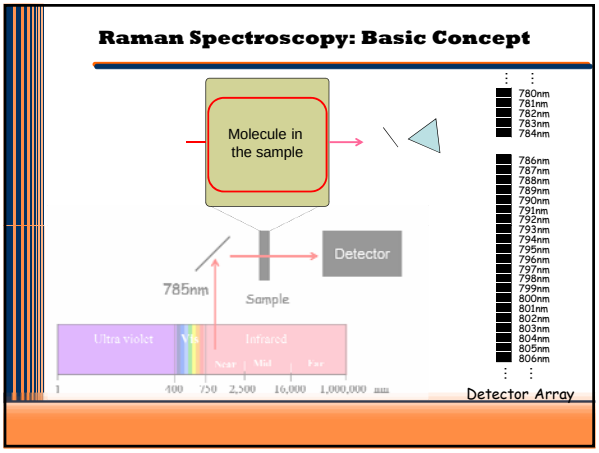
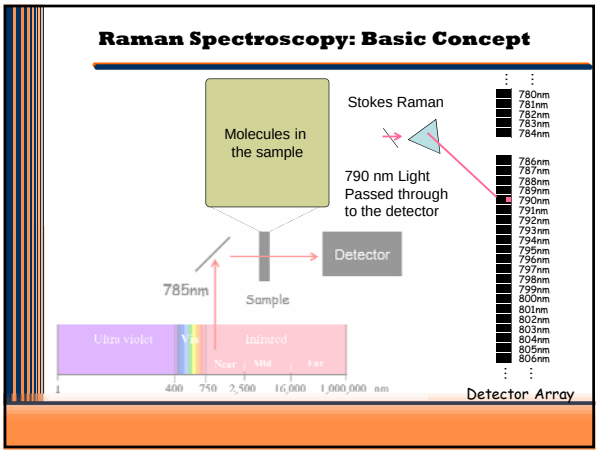
797nm
798nm
799nm
800nm
801nm
802nm
803nm
804nm
805nm
806nm
Detector Array


Raman Spectroscopy: Basic Concept
Molecules in the sample
Anti-stokes Raman
781 nm Light
Passed through
to the detector
Detector
785nm
Sample
Ultra violet
Vis
Infrared
Near
Mid
Far
1
400
750
2,500
16,000
1,000,000 nm
780nm
781nm
782nm
783nm
784nm
786nm
787nm
788nm
789nm
790nm
791nm
792nm
793nm
794nm
795nm
796nm
797nm
798nm
799nm
800nm
801nm
802nm
803nm
804nm
805nm
806nm
Detector Array


Raman Spectroscopy: Basic Concept
785 nm
Photon
Molecules in the sample
Detector
785nm
Sample
Ultra violet
Vis
Infrared
Near
Mid
Far
1
400
750
2,500
16,000
1,000,000 nm
780nm
781nm
782nm
783nm
784nm
786nm
787nm
788nm
789nm
790nm
791nm
792nm
793nm
794nm
795nm
796nm
797nm
798nm
799nm
800nm
801nm
802nm
803nm
804nm
805nm
806nm
Detector Array


Raman Spectroscopy: Basic Concept
Molecule in the sample
Detector
785nm
Sample
Ultra violet
Vis
Infrared
Near
Mid
Far
1
400
750
2,500
16,000
1,000,000 nm
780nm
781nm
782nm
783nm
784nm
786nm
787nm
788nm
789nm
790nm
791nm
792nm
793nm
794nm
795nm
796nm
797nm
798nm
799nm
800nm
801nm
802nm
803nm
804nm
805nm
806nm
Detector Array


Raman Spectroscopy: Basic Concept
Molecules in the sample
Stokes Raman
790 nm Light
Passed through
to the detector
Detector
785nm
Sample
Ultra violet
Vis
Infrared
Near
Mid
Far
1
400
750
2,500
16,000
1,000,000 nm
780nm
781nm
782nm
783nm
784nm
786nm
787nm
788nm
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790nm
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792nm
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795nm
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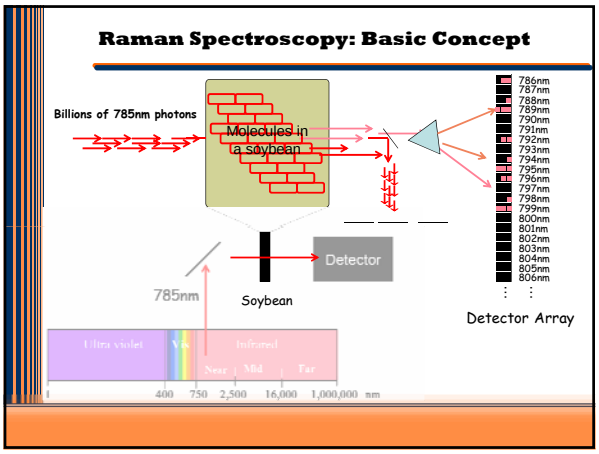
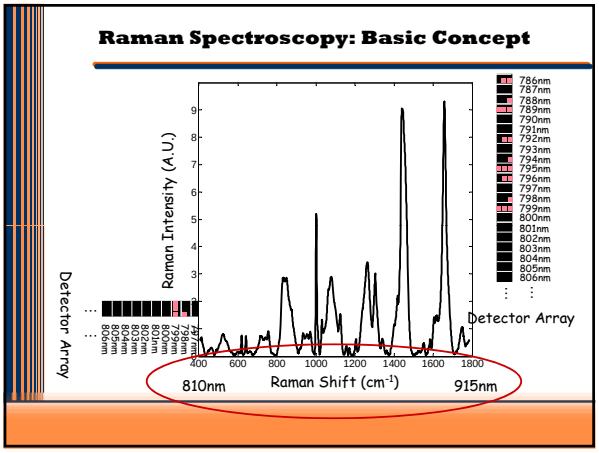
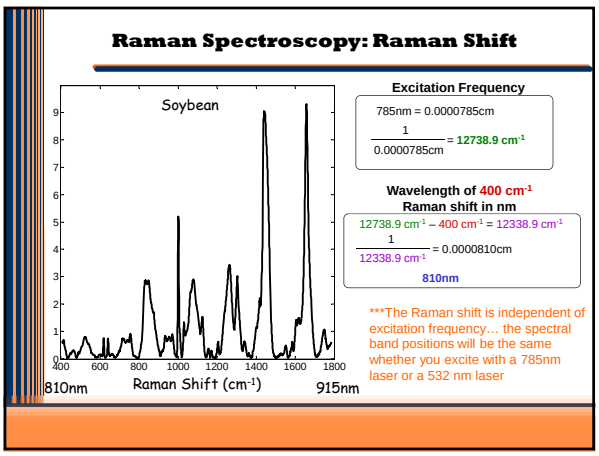
797nm
798nm
799nm
800nm
801nm
802nm
803nm
804nm
805nm
806nm
Detector Array

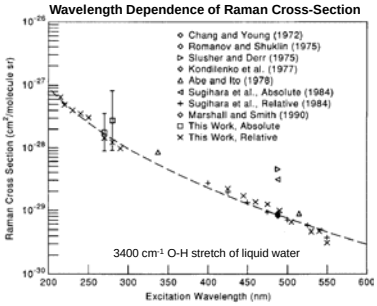

Raman Spectroscopy: Basic Concept
Billions of 785nm photons
Molecules in a soybean
Detector
785nm
Soybean
Detector Array
786nm
787nm
788nm
789nm
790nm
791nm
792nm
793nm
794nm
795nm
796nm
797nm
798nm
799nm
800nm
801nm
802nm
803nm
804nm
805nm
806nm
Ultra violet
Visible
Infrared
Near
Mid
Far
400
750
2,500
16,000
1,000,000
nm


Raman Spectroscopy: Basic Concept
Raman Intensity (A.U.)
Detector Array
Raman Shift (cm-1)
810nm
915nm
400
600
800
1000
1200
1400
1600
1800


Raman Spectroscopy: Raman Shift
Soybean
Raman Shift (cm-1)
810nm
915nm
Excitation Frequency
785nm = 0.0000785cm
1 / 0.0000785cm = 12738.9 cm-1
Wavelength of 400 cm-1 Raman shift in nm
12738.9 cm-1 – 400 cm-1 = 12338.9 cm-1
1 / 12338.9 cm-1 = 0.0000810cm
810nm
***The Raman shift is independent of excitation frequency… the spectral band positions will be the same whether you excite with a 785nm laser or a 532 nm laser

Raman Spectroscopy: Raman Shift

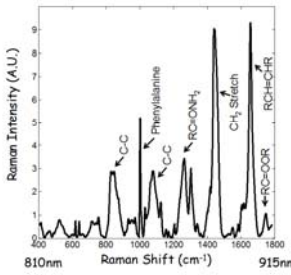
Wavelength Dependence of Raman Cross-Section



Important advantage of Raman… wavelength select ability to avoid light absorption and fluorescence… **however** the higher the wavelength the lower the Raman efficiency (Raman Cross Section).

Appl. Opt. **36**, 2686-2688 (1997)

Raman Spectroscopy: Selection Rules

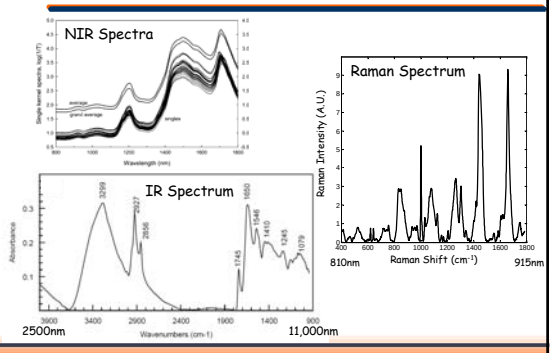


Raman Bands result from the Vibrations of specific chemical Bonds.

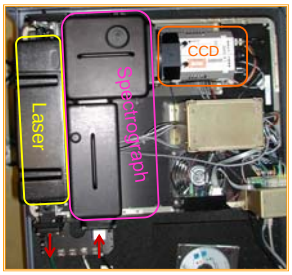
Soybean band assignments:

Wave number	Molecule	Assignment
800-900	C-C	Backbone
1003	Phe	Ring breathing
1010-1200	C-C	Backbone
1265	$RC=ONH_2$	Amide III
1450	CH_2	Stretch
1660	RCH=CHR	Cis
1670	RCH=CHR	Trans
1750	RC=OOR	Ester

Raman Spectroscopy: Examples



Raman Spectroscopy: Instrumentation



Kaiser Optical

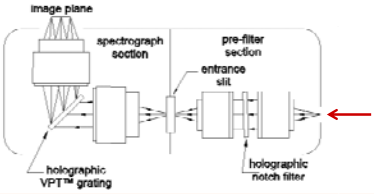
Raman Spectroscopy: Instrumentation

Spectrograph

-This is a axial dispersive spectrograph

-Reflective spectrographs are also common

-Interferometers are used for FT-Raman



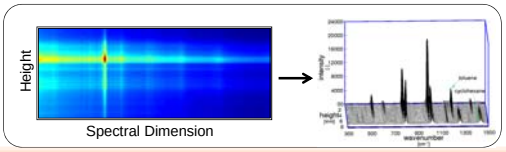
Kaiser Optical

Raman Spectroscopy: Instrumentation

Detector

-Charged-coupled devices (CCDs) are the most common detectors (256x1024 pixel array)

-FT Raman is used for frequencies where CCDs have low quantum efficiency



Raman Spectroscopy: Advantages and Disadvantages

Advantages:

-Little sample preparation (Polishing and fixing to a slide is common)

-Not sensitive to water (Good for biological samples)

-High chemical specificity (Narrow spectral bands)

-Qualitative and Quantitative information

-Non-destructive (A measurement does not chemically or physically change the sample)

-Can take measurements on solids, liquids, or gases

-Measurements are taken without touching the sample (Remote sensing)

-Easily coupled with fiber-optics

Raman Spectroscopy: Advantages and Disadvantages

Disadvantages

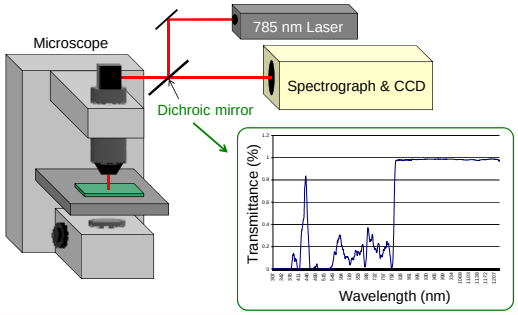
-Acquisition times tend to be longer than other techniques (real time measurements have been demonstrated… but something like video rate imaging is not yet a reality)

-Raman signal tends to be weak

-Raman signal is often mixed with a fluorescent background signal, which can make signal processing difficult.

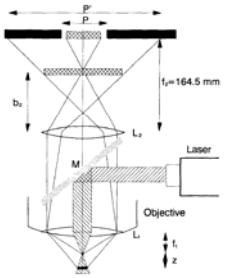
-High laser powers and burn delicate samples

Raman Microscopy



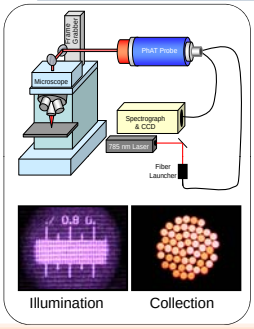
Confocal Raman Spectroscopy

Confocal Raman Microscopy

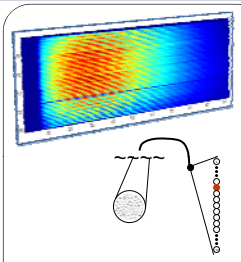


Tabaskblat et al., Appl. Spec 1992

Hyperspectral Raman Mapping

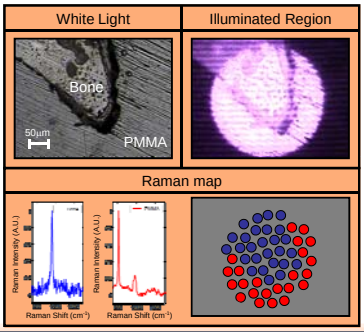


Illumination Collection

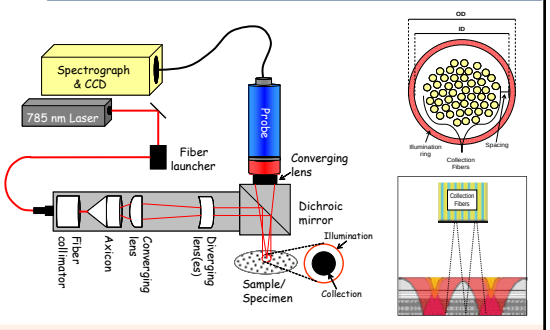


Collection fibers are transposed into a line when to enter the spectrograph

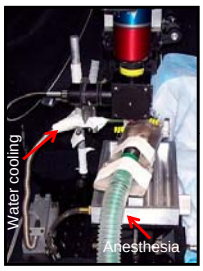
Hyperspectral Raman Mapping

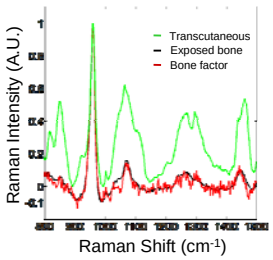


Spatially Offset Raman Spectroscopy



Spatially Offset Raman Spectroscopy





Schulmerich et. al., Appl. Spectrosc., *63(3) 286-295*

Other Raman Techniques

-Surfaced enhanced Raman spectroscopy (SERS)

-Tip enhanced Raman spectroscopy (TERS)

-Resonance Raman spectroscopy

-Raman tomography

-Raman imaging

-Coherent anti-stokes Raman spectroscopy (CARS)

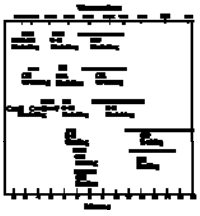
-Stimulated Raman spectroscopy

-Others I can't think of at the moment and much more to come!

Infrared Spectroscopy

Theory of Infrared – Chemical bonds

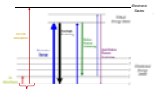
- Infrared spectroscopy :
  - *Principle*: chemical bonds rotate or vibrate at specific frequencies (Group theory basis of IR Spectroscopy)
- Practical Aspects
  - The frequency at which resonance occurs depends on the properties of the chemical bond
  - Factors include shape of molecular bond, energy levels and mass of atoms.
  - Chemical bonds can be divided into those which are IR active and those which are IR inactive



Molecular Basis of IR Absorption

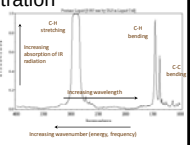
- Direct absorption of light

$$\Delta E = h\nu_m$$



- Beer's law relates absorption to concentration

$$I_s = I_0 e^{-abc} \quad \Rightarrow A = \log\left(\frac{I_0}{I_s}\right)$$



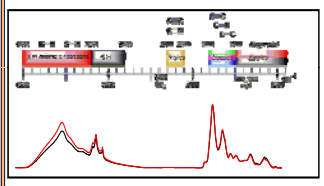
- Selection rule
  - Dipole moment (symmetrical mode: diatom)

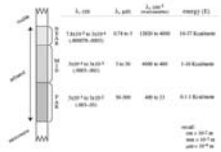
Theory of Infrared – Chemical bonds

- To be an IR active mode, the motion must have a change in the electric dipole moment of the bond
- Implications
  - The intensity of absorbance peaks is related to size of dipole moment.
  - Bonds with higher dipole moments tend to be covalent bonds with highly different electronegativities e.g C=O
  - Symmetrical bonds are typically IR inactive e.g. C-C, N-N
  - The same chemical bond can also have multiple modes of vibration e.g. phosphate has a symmetrical and antisymmetrical modes

Infrared Spectra

• Vibrational frequencies are directly resonant with optical frequencies – vibrational transition is energy matching



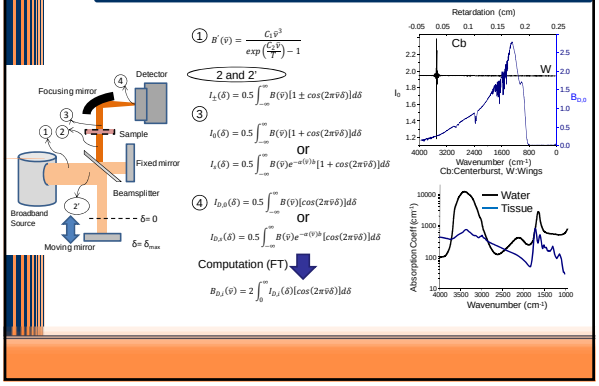


Infrared Spectroscopy

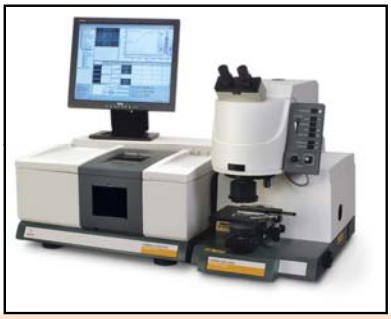
- IR was discovered in 1801 by William Herschel who split the EM spectrum using a prism – noted increase in temperature beyond the red part of visible spectrum
- 1930's began to be exploited for spectroscopy studies

- Two mains types of spectrometer – Dispersive and Fourier Transform
- Dispersive uses monochromatic source of light and changes frequency over time by moving grating, mirror or detector. Originally used prisms and later grating.
- Largely replaced by Fourier Transform – Allows for all frequencies to be measured at once: Fellgetts advantage – No slits: higher throughput

IR Spectroscopy: Theory

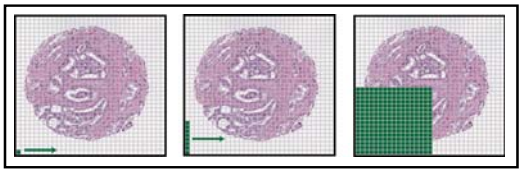


FTIR Microscope



A popular commercial instrument combining the FTIR spectrometer with a microscope

Point spectra or Mapping/Imaging?



A) Single Point Detector (mapping)

B) Linear Array Detector (imaging)

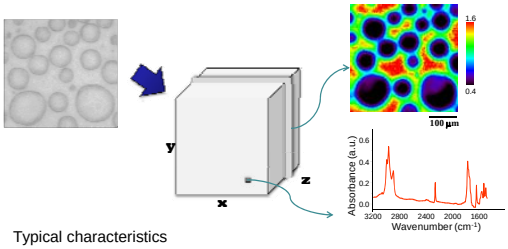
C) Focal Plane Array (imaging)

Example improvements in speed

1	16	16,384
Single point	16 detector linear array	128x128 Focal Plane Array

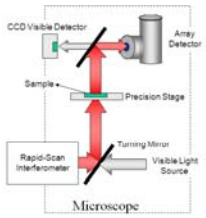
In fact, Imaging is even quicker and allows for diffraction limit measurements

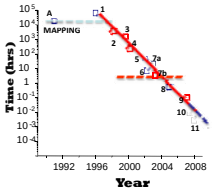
FT-IR spectroscopy → Imaging



- Typical characteristics
  - Wavelengths (2048 elements over 2.5 – 12.5 µm), x, y typically ~1024
- Computation is essential to recover data
  - Manual examination is prohibitive
- Trade-offs: spatial coverage vs. resolution, spectral resolution vs. signal-to-noise ratio, time vs. data quality/size vs. information

FTIR Chemical Imaging





8 cm^{-1}, SNR of 1000:1, 1 Mpix, 6 x6 micron res., 1 cm x 1 cm

Blue – hardware, red – interferometry/software, green – emerging technology

1. E.N. Lewis et al. Anal. Chem. 67, 3377-3386 (1995) ; 2. Snively et al. Appl. Spectrosc. (1998); 3. Bhargava et al. Appl. Spectrosc. 53, 1313-1322 (1999); 4. Snively et al. Opt. Lett., 24; 1841-1843 (1999); 5. Bhargava et al. Appl. Spectrosc. 54, 486-495 (2000); 6. Perkin-Elmer Inc.; 7a. Varian Inc. ; 8. NIH-FBI camera; 9. Reddy et al (To be submitted); 10 & 11 – under development.

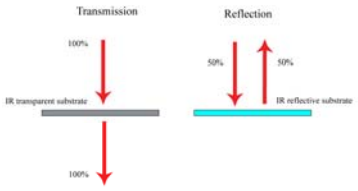
Settings and Parameters

A number of important considerations must be taken into account prior to experiments;

- Sample preparation is critical!
- Signal to Noise – Number of co-additions
- Mirror speed
- Spectral resolution
- Spatial resolution
- Point spectra vs. Point Mapping vs. Imaging

Sample preparation

- Transmission or reflection



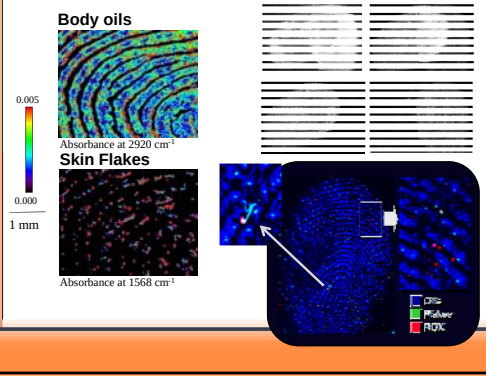
- IR transparent substrates – BaF2, CaF2
- IR reflective substrates – Gold, MirrIR Slides
- Reflection is low-cost, however reduction in spectral quality and increase in scattering artifacts

Signal to noise

- It is important to get high quality noise-free data – This can involve multiple strategies;
  - Better sample preparation (more or less sample)
  - More scans and averaging
  - Reduce Michelson mirror speed
- Spectral resolution is important, this is the number of points within a spectra, the fewer the faster the scans but potential for loss of information
- Spatial resolution is also important, the higher the spatial resolution, the lower the signal.

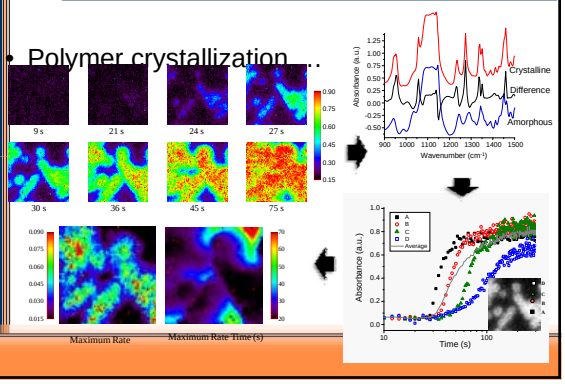
Take home message; Many factors must be adapted depending on what you are interested in

Application 1: Forensic Sciences



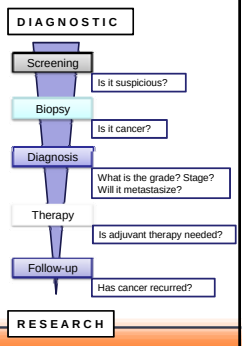
An example of imaging…..

- Polymer crystallization…

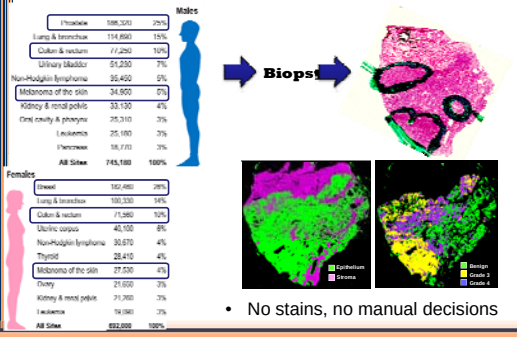


Motivation

- Cancer pathology
  - Prostate cancer as a paradigm – 1 in 6 men
  - Biopsies: >1 million annually with disease ~20%
  - Diagnoses: >200, 000 annually with lethal ~20%
  - Grading is subjective, variable, leads to conflicting therapy routes
  - Prognosis tools are not perfect – 97% undergo therapy
  - "Holy Grail" of oncologic pathology
  - Primary evaluative standard for research
- Manual recognition in stained tissue



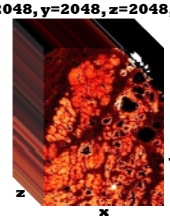
Cancer and Diagnostic Process



- No stains, no manual decisions

From Data to Knowledge

x=2048, y=2048, z=2048, t ~ ms to days



Information

- Model based design of experiments
- Hypothesis driven analysis – supervised data analysis
- Biologically inspired statistical pattern recognition of spectra
- Approach – Model, Train algorithm, Classify, Validate

Analytical Approaches

The types of analysis available to spectroscopists can be divided into two groups;

Un-Supervised — All data has the same weighting and no-prior information about what the spectra corresponds to is known -Useful for interest of discovery

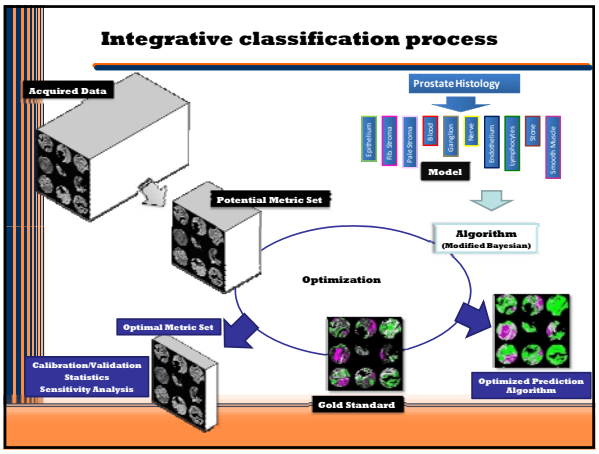
Supervised — Known information about data e.g. classes is used for data analysis -Useful for classification (more amenable to clinical setting?)

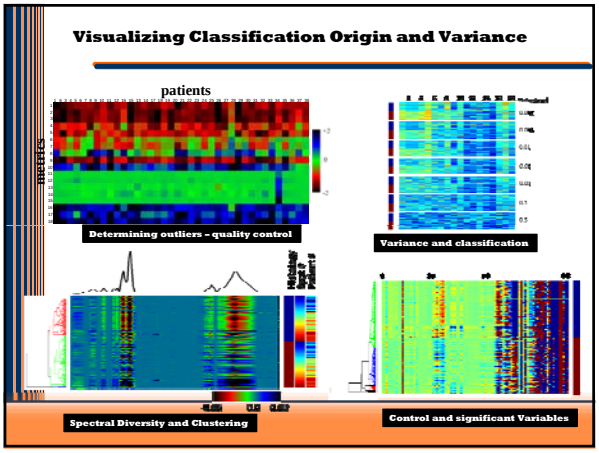
Analytical Approaches

Which MVA approach?

Bayesian, PCA, PLS, SIMCA, HCA, FUZZY, LDA, KNN, ANN

Important to determine which are best for analysis of point spectra and which for images

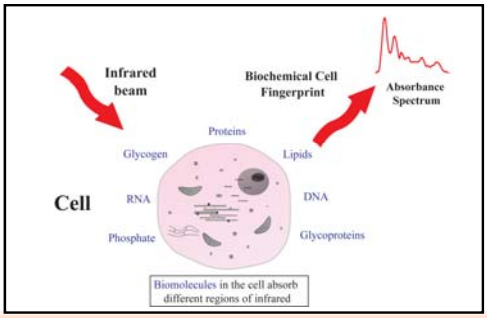




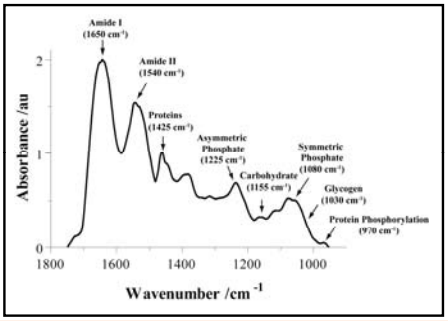
Examples of FTIR for Biomedical applications

- Fundamental biological processes
  - Apoptosis
  - Necrosis
  - Stem cell differentiation
  - Toxicology
  - Disease initiation and progression
- Clinical Applications
  - Disease diagnosis
  - Disease prognosis

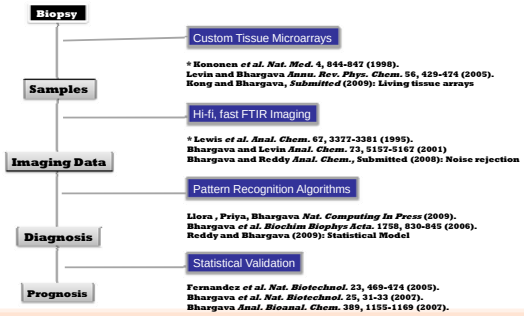
FTIR-Biomedical Applications



Biochemical-cell fingerprint region



Integrative Process and Key Technologies



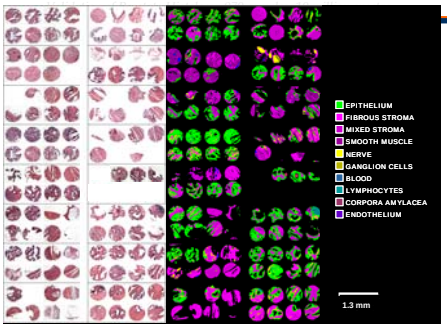
* Kononen *et al. Nat. Med.* 4, 844-847 (1998).
Levin and Bhargava *Annu. Rev. Phys. Chem.* 56, 429-474 (2005).
Kong and Bhargava, *Submitted* (2009): Living tissue arrays

* Lewis *et al. Anal. Chem.* 67, 3377-3381 (1995).
Bhargava and Levin *Anal. Chem.* 73, 5157-5167 (2001)
Bhargava and Reddy *Anal. Chem.*, Submitted (2008): Noise rejection

Llora , Priya, Bhargava *Nat. Computing In Press* (2009).
Bhargava *et al. Biochim Biophys Acta.* 1758, 830-845 (2006).
Reddy and Bhargava (2009): Statistical Model

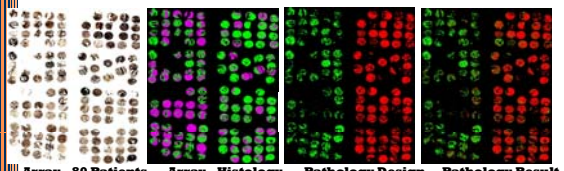
Fernandez *et al. Nat. Biotechnol.* 23, 469-474 (2005).
Bhargava *et al. Nat. Biotechnol.* 25, 31-33 (2007).
Bhargava *Anal. Bioanal. Chem.* 389, 1155-1169 (2007).

Application 1: Prostate Histology



Fernandez, Bhargava, Hewitt and Levin ***Nat. Biotechnol.***, 23, 469-474 (2005)

Prostate Cancer Diagnosis



- Overall pixel accuracy ~ 88.5% ; Heterogeneity in samples?
- 1 cancer sample classified as benign (71)
- 1 benign sample classified as cancerous (69)
- Sensitivity and specificity exceeding human capabilities
- Large validation studies underway

Whats in store?

- Label-free methods
  - Talks on Monday focus on applications
- Lab tours
- More information