
UV-Visible-NIR Microspectroscopy of Trace Evidence

Dr. Paul Martin

Trace Evidence Symposium

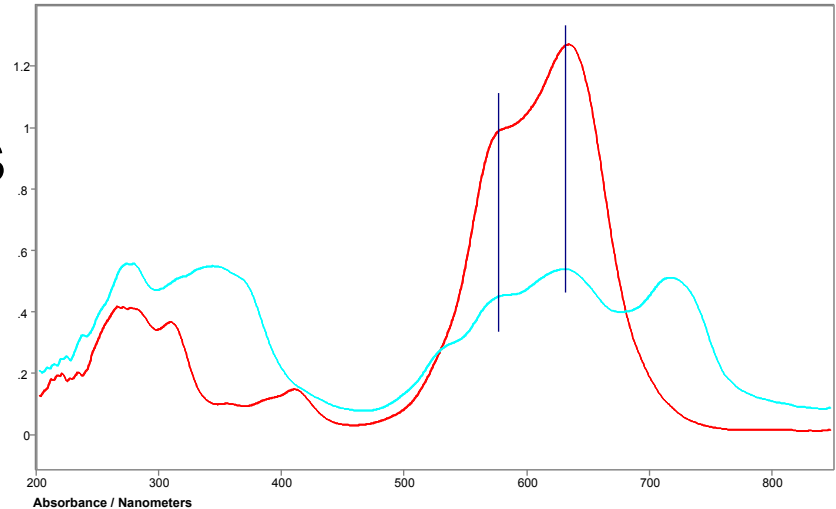
August 2007

Why UV-Visible microspectroscopy?

- Ultraviolet-visible-NIR range microspectroscopy provides information on dyes, pigments and additives in trace evidence such as paint, fibers and glass
- Microspectroscopy also provides information on pigments, dyes and additives in inks & paper
- Comparing known and questioned can be done easily, quickly and more accurately than by eye

Advantages of UV-Visible microspectroscopy

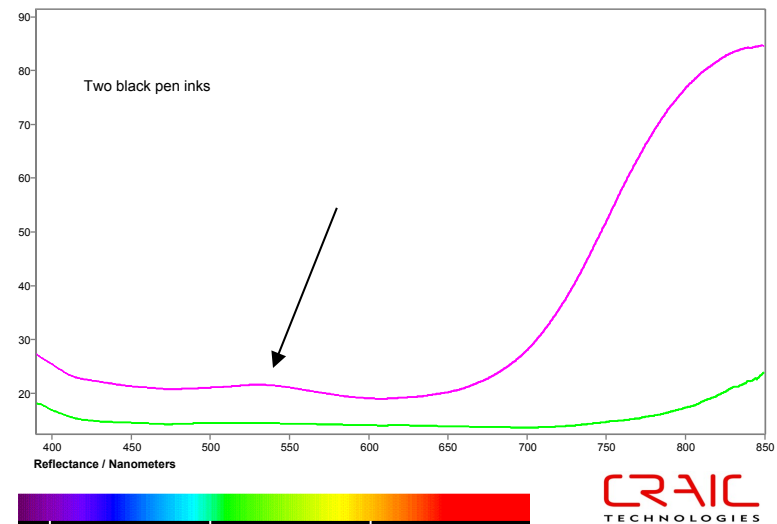
- UV-visible-NIR microspectroscopy is a *non-destructive* test of pigments and dyes
 - Chemical tests destroy evidence
- Analyzes dyes & pigments: FTIR lacks sensitivity for trace components



Absorbance microspectra of two blue inks

Advantages of UV-Visible microspectroscopy

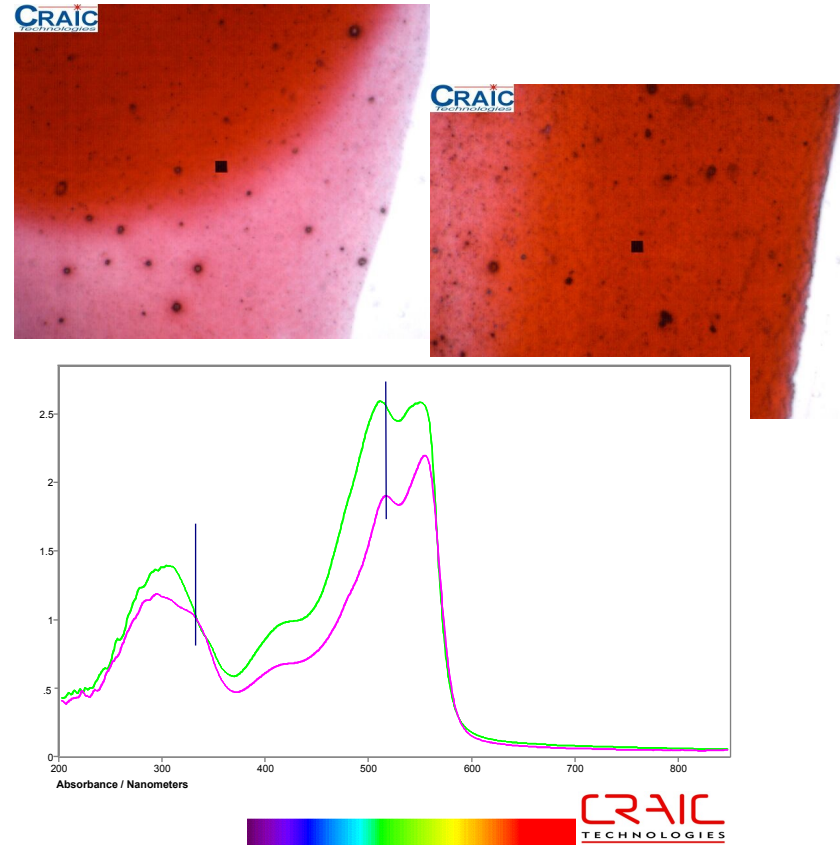
- UV-visible-NIR microspectroscopy is can “see” beyond the range of the human eye
 - Ultraviolet and NIR
- Microspectroscopy can “see” subtle variations human eye cannot



Reflectance microspectra of two black pen inks

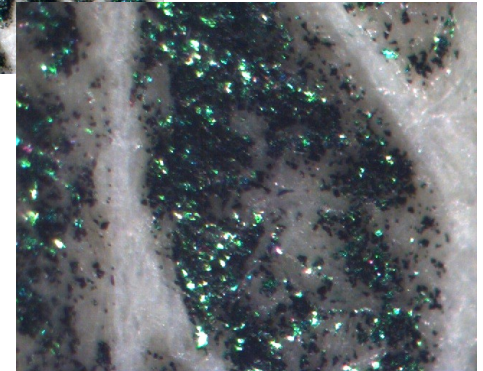
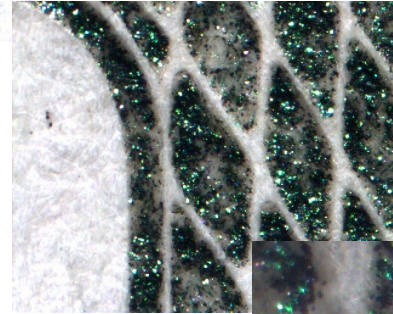
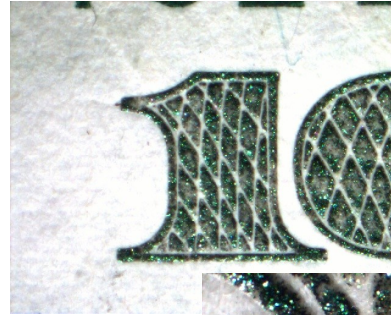
Advantages of UV-Visible microspectroscopy

- Easier and more accurate to compare linear data than colors
 - Easier to explain in court too



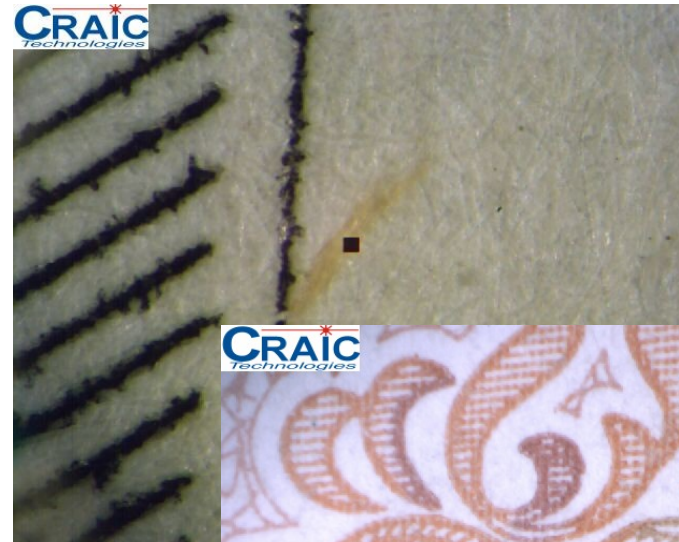
Advantages of UV-Visible *microspectroscopy*

- Macroscopic UV-visible-NIR spectrometers cannot analyze microscopic areas
 - Trace evidence too small
 - Fibers, paint chips, soil, glass fragments, etc.



Drawbacks of UV-visible microspectroscopy

- Does not provide easily identifiable “fingerprints”
 - Most textiles dyes made in very small batches
 - Spectra usually of mixtures
- Lack of databases designed for forensics
 - Comparative tool



Currency

Organization of Talk

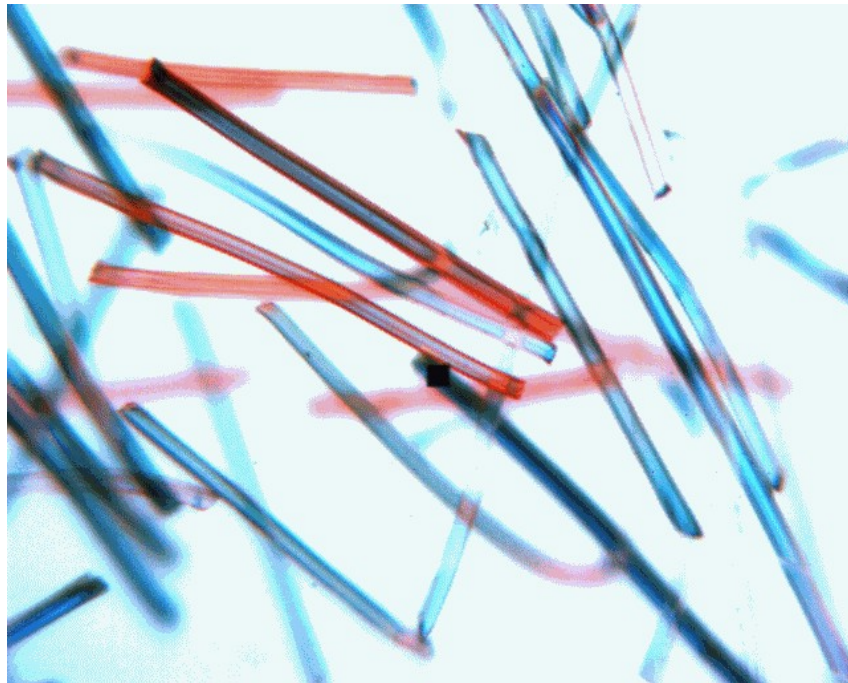
- Science of UV-visible-NIR microspectroscopy
- Overview of microspectrometers
- Interpretation of Spectra
- Preparation techniques for common samples
- Validation of microspectrometer
- Calibration of microspectrometer
- Microspectroscopic examination techniques

Definitions

- Spectrum (n): series of colors, including those beyond the visible, produced by dispersion of radiated energy.
- Microspectroscopy (n): The branch of science dealing with the theory and interpretation of spectra obtained from microscopic samples. It is the study of the interaction of electromagnetic radiation with the quantized energy states of matter.

Physics of microspectroscopy

Physics is the key to understanding the data...



Theory of Spectroscopy

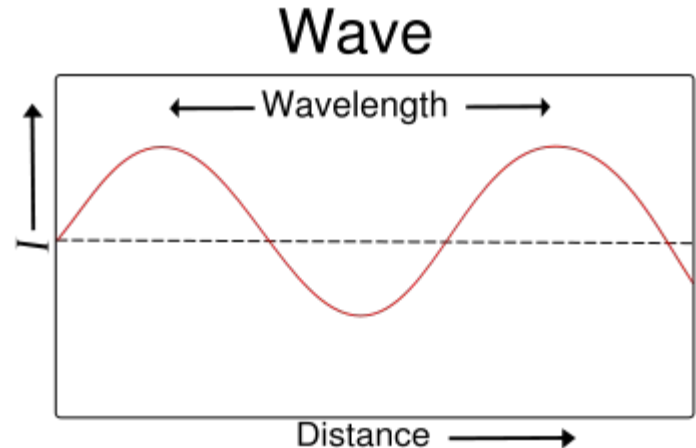
- Spectroscopy is based upon the fact that there is a quantized energy transfer between radiation and matter
- The energy of light involved in this transfer reveals information about the structure and environment of the matter in question

Wave Property Terms

Radiation “waves” are described by:

- Wavelength (λ): distance between wave crests
- Velocity of light (c) in a vacuum
- Frequency (ν): number of waves passing a point in unit time

$$\lambda = \frac{c}{\nu}$$



Einstein-Planck Relation

- E is energy in joules
- “h” is Plancks constant (6.62 x 10⁻³⁴ J-s)
- “v” is frequency of radiation in hertz

$$E = h\nu = \frac{hc}{\lambda}$$

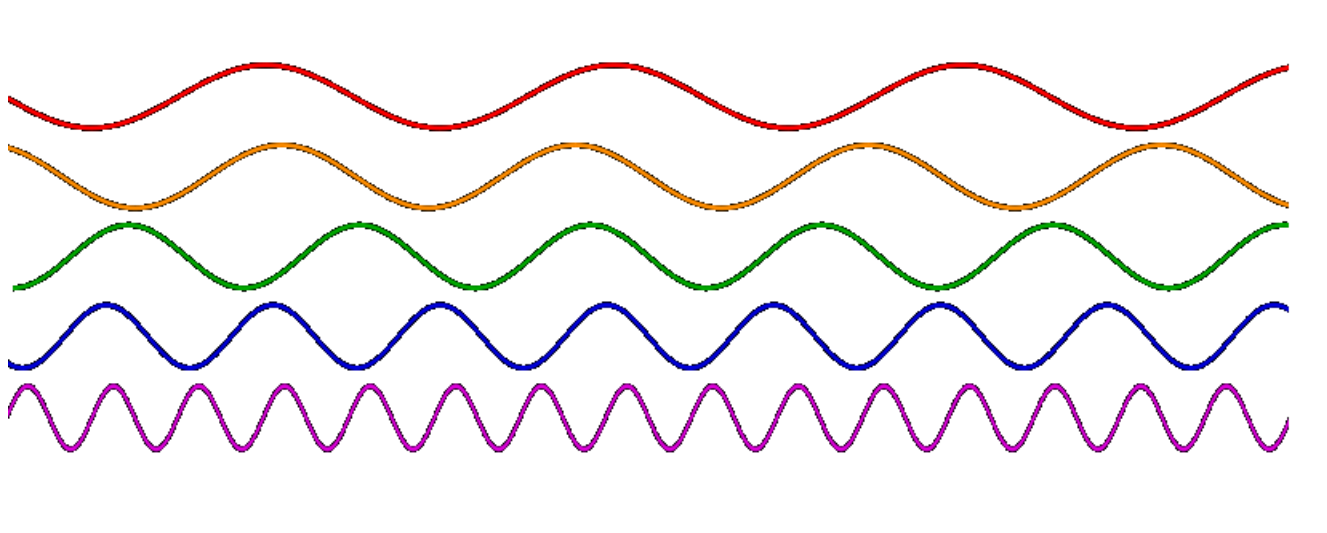
Einstein-Planck Relation

- Quantum energy of a single photon depends only on its wavelength
- *Intensity* of radiation depends on number of photons per unit area and time

$$E = h\nu = \frac{hc}{\lambda}$$

Electromagnetic Spectra

Longer Wavelength = Lower Energy

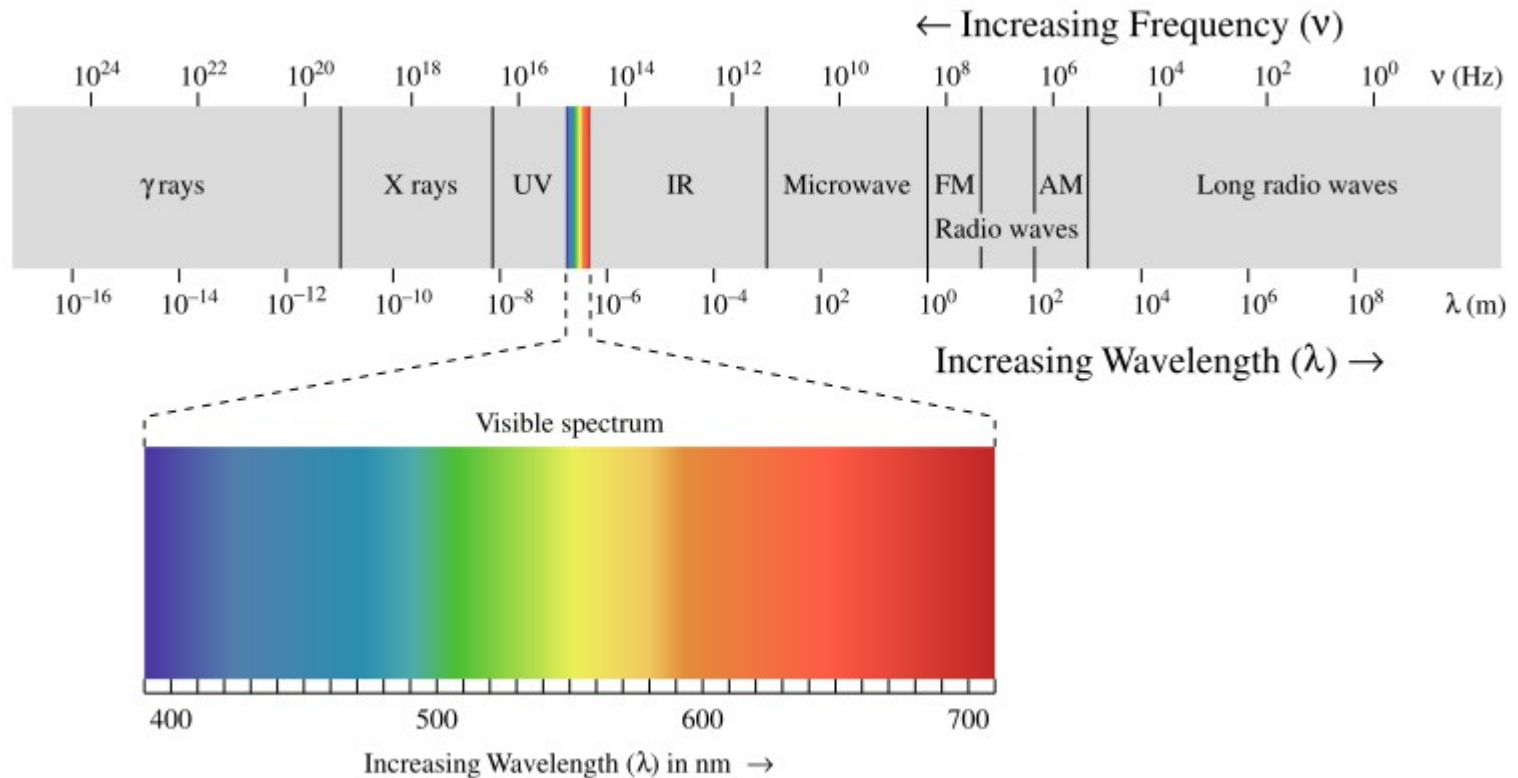


Shorter Wavelength = Higher Energy

Electromagnetic Spectra

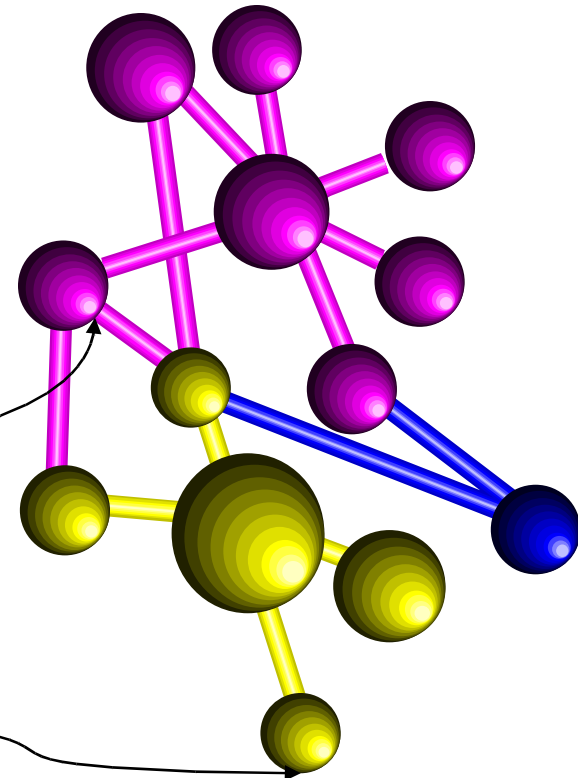
Higher Energy

Lower Energy



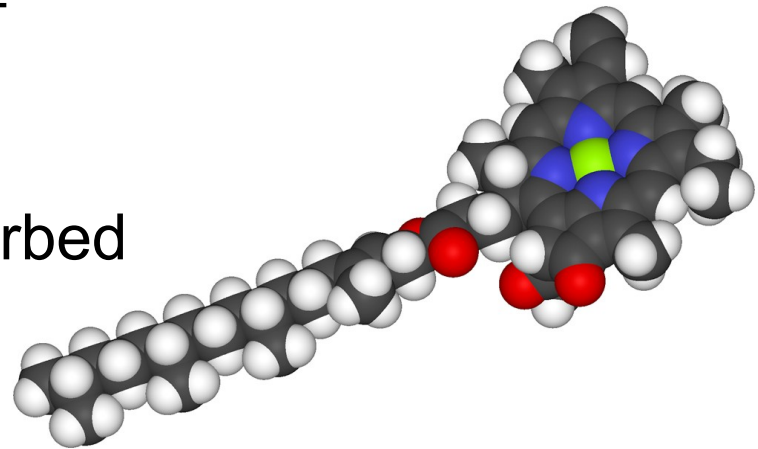
Color and Structure

- Molecules containing systems of conjugated bonds which absorb specific wavelengths of light are called **Chromophores**
- Side groups which donate or accept electrons, called **Auxochromes**, can be attached



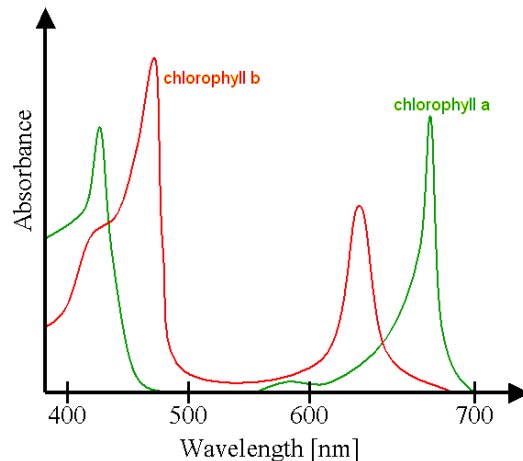
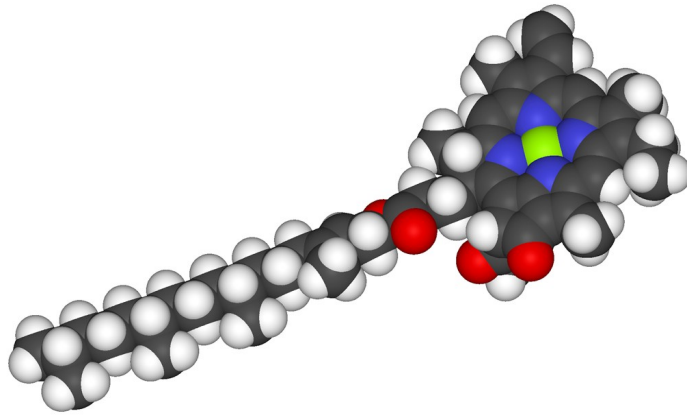
Molecules “make” color

- Molecules absorb light of specific energy or wavelength
- Wavelength of light absorbed depend on electronic structure of molecule
- Pigments and dyes are designed to absorb in visible range
- Some pigments and dyes also absorb in UV or NIR range



Chlorophyll

Effect of Molecule's Structure



- Spectrum is function of structure of molecule and it's surroundings
- Molecule's atoms affect spectrum
 - Molecule's structure affects spectrum
 - Surrounding molecules affect spectrum

Transmittance & Absorbance

- $T = I / I_0$
 - I_0 is the intensity of radiant energy striking sample
 - I is the intensity of energy emerging from sample
- $A = -\log T = \log (I_0 / I)$
- Beer Lambert Law: relates transmittance, sample thickness, and concentration of absorbing species
 - $A = \epsilon bc$
 - Where ϵ is the molar absorptivity, b is path length, and c is concentration

Reflectivity

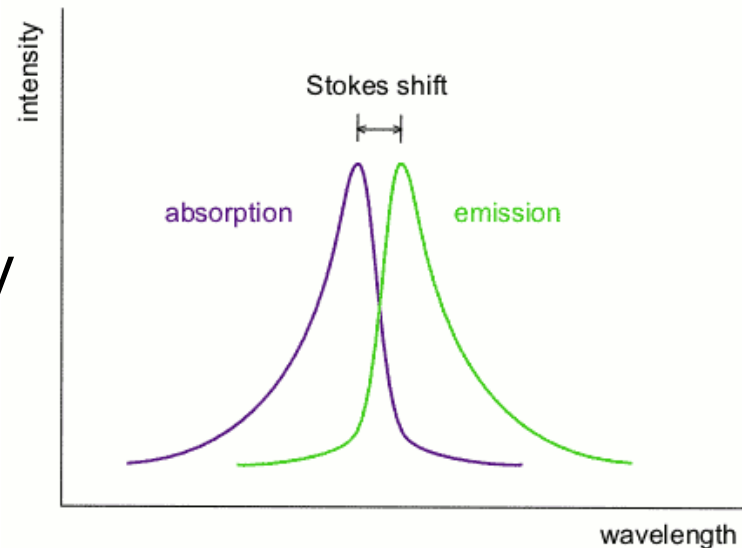
- **Relative Reflectance:** Final spectrum of sample contains spectral components of reference material
 - $R = I / I_0$
 - I_0 is the intensity of energy reflected from the reference sample
 - I is the intensity of energy reflected from the sample
- **Absolute reflectance:** Final spectrum of sample is independent of the reference material

$$R_A = R_m \frac{R_m^o}{R_A^o}$$

Fluorescence Spectra

Spectra contain information about:

- Light-absorbing species
- Occurs mainly with aromatic molecules
- Can be quenched by many means
- Stokes shift means lower energy emission maxima than absorbance
 - Franck-Condon Principle



Fluorescence

- Measured in counts
- Not corrected to any standard
- $F = I$ where I is the radiant energy

What is a microspectrometer?



- A microspectrophotometer combines a microscope and an UV-visible-NIR range spectrophotometer.
- Also called a microspectrometer, microscope spectrophotometer, or microscope spectrometer

What is a microspectrometer?



- Microscope (*n*): an instrument that produces enlarged images of small objects, allowing them to be studied at a scale convenient for examination and analysis.
- Spectrophotometer (*n*): a device that measures the intensity of *each* color (or wavelength) of light in the UV, visible, and NIR spectral region.

Capabilities of a microspectrophotometer



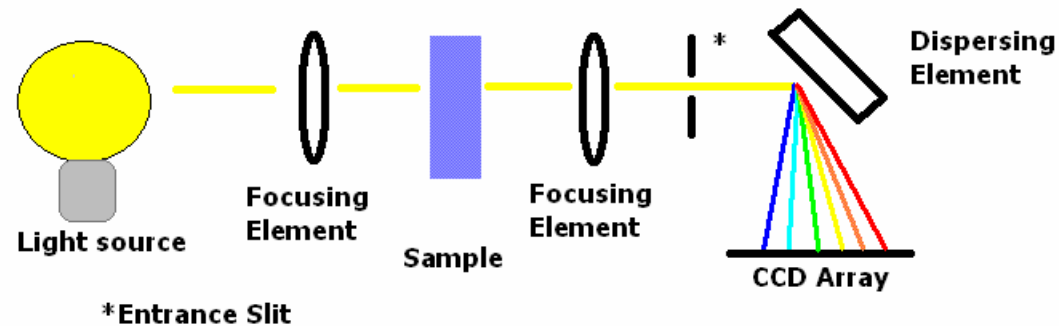
- UV-visible-NIR range spectra
- Transmission / Absorbance
- Reflectance
- Fluorescence
- Polarization
- Areas can be less than a micron

Microspectrometer

- Interface optics, spectrophotometer, digital imaging, electronics
- Eyepieces
- Objectives
- Sample stage
- Condenser
- Light source



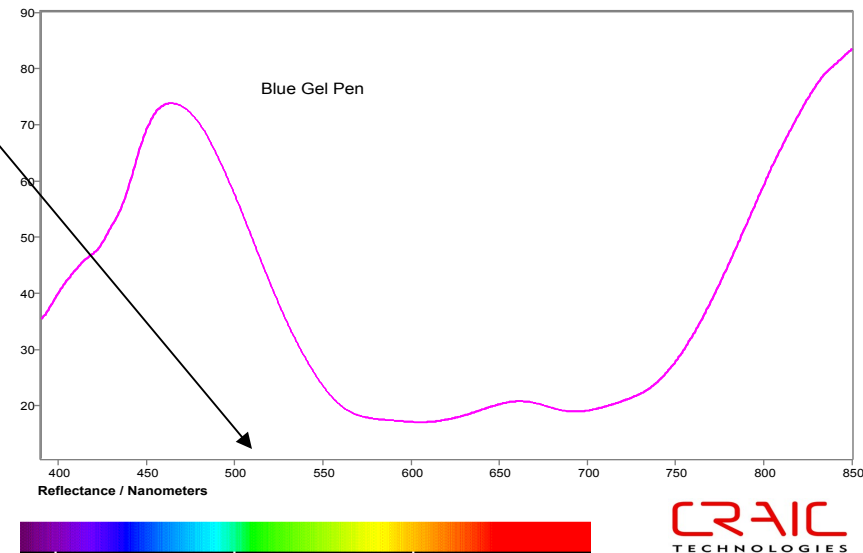
How a spectrophotometer works



- White light is focused on sample
- Depending upon it's molecular composition, sample absorbs some wavelengths of light
- Monochromator separates wavelengths of light transmitted through sample
- CCD array measures intensity of light at each wavelength

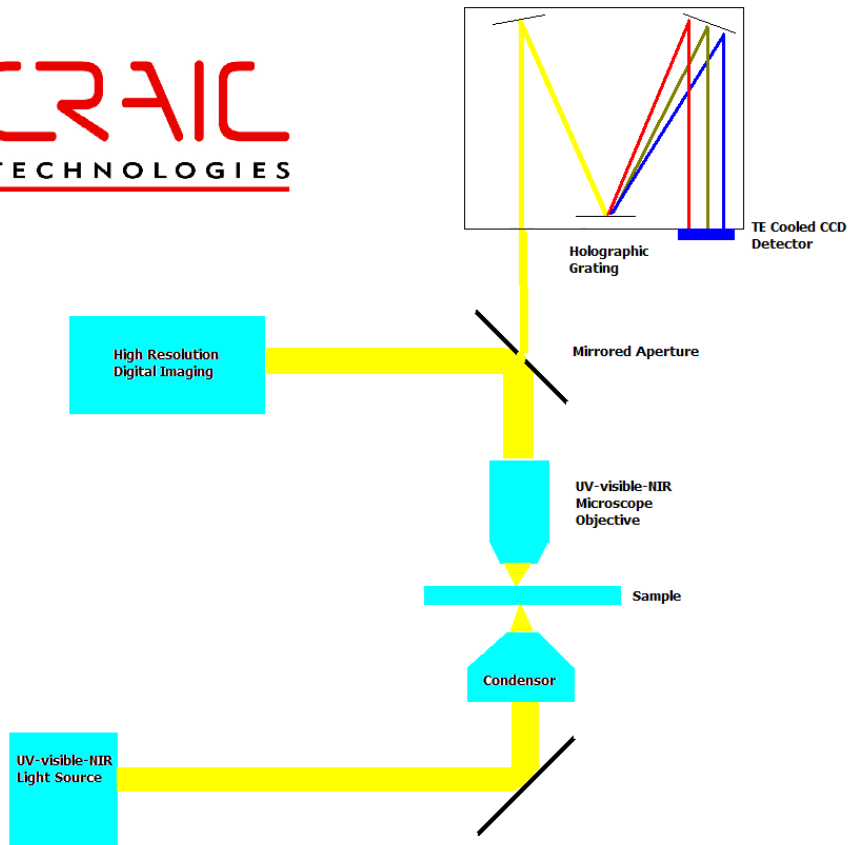
How a spectrophotometer works

- The measured spectrum is then plotted as an XY chart
- X axis is wavelength
- Y axis can be:
 - Transmission (or Absorbance)
 - Reflectance
 - Fluorescent Intensity



Transmission Microspectroscopy

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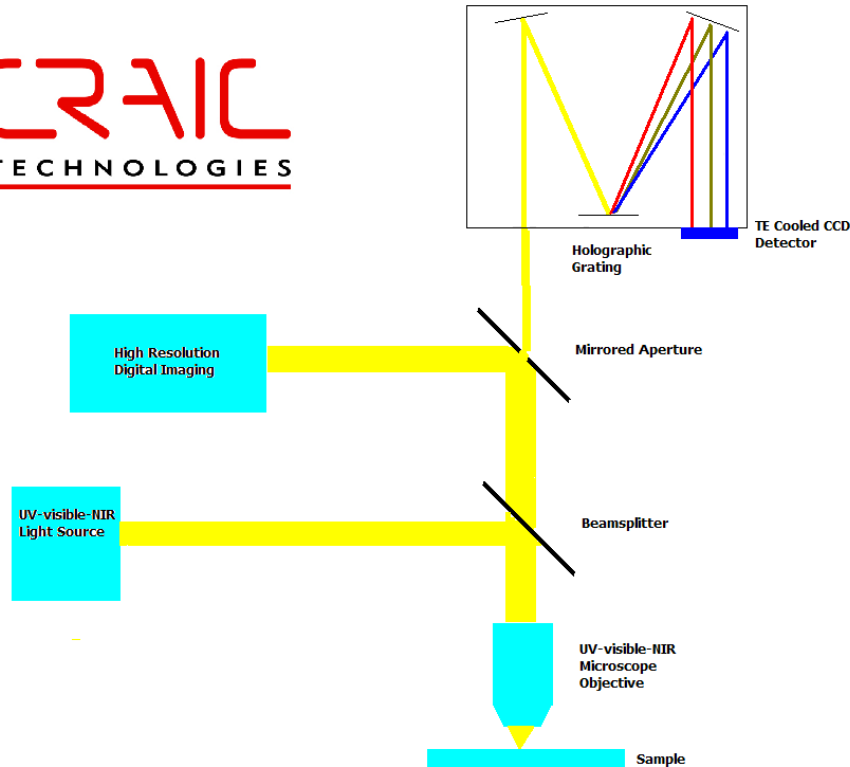


- Focus on sample
- Take dark scan (D)
- Take reference scan (R)
- Take sample scan (S)
- Analyze data

$$C = \frac{S}{R} \frac{D}{D}$$

Reflectance Microspectroscopy

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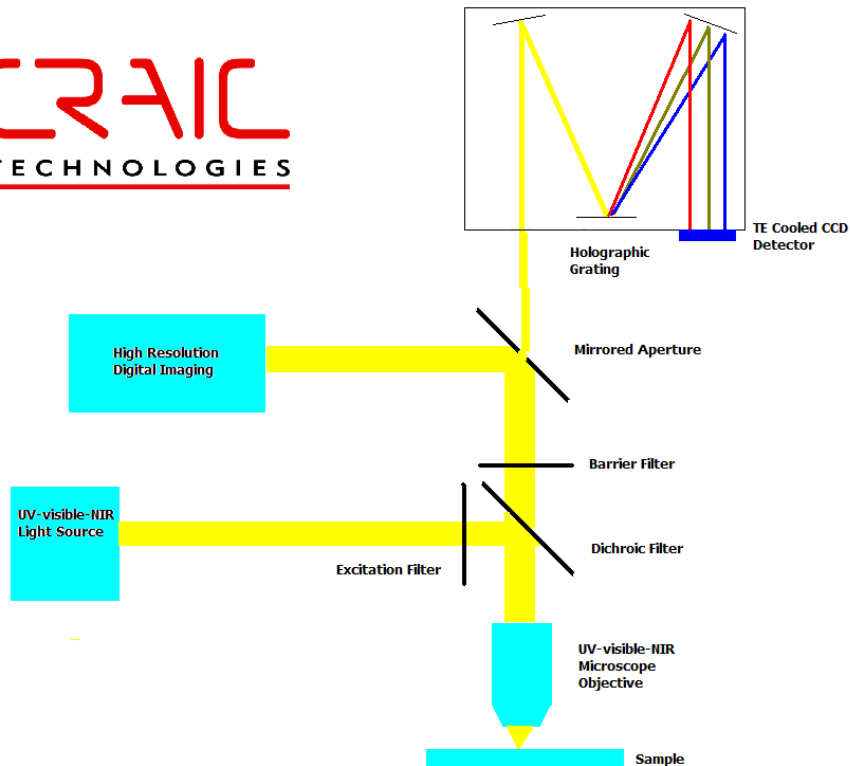


- Focus on sample
- Take dark scan (D)
- Take reference scan (R)
- Take sample scan (S)
- Analyze data

$$C = \frac{S}{R} \frac{D}{D}$$

Microfluorometry

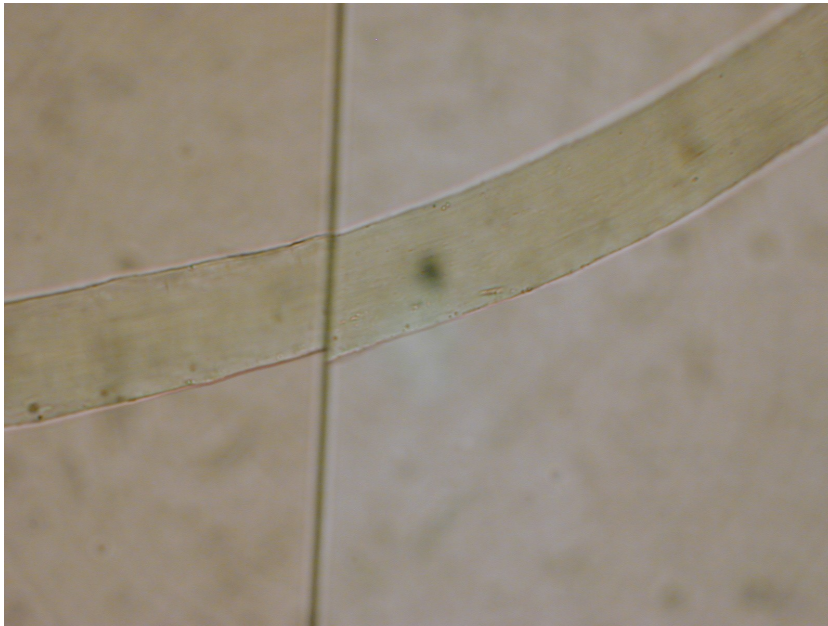
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- Focus on sample
- Take dark scan (D)
- Take sample scan (S)
- Analyze data

C S D

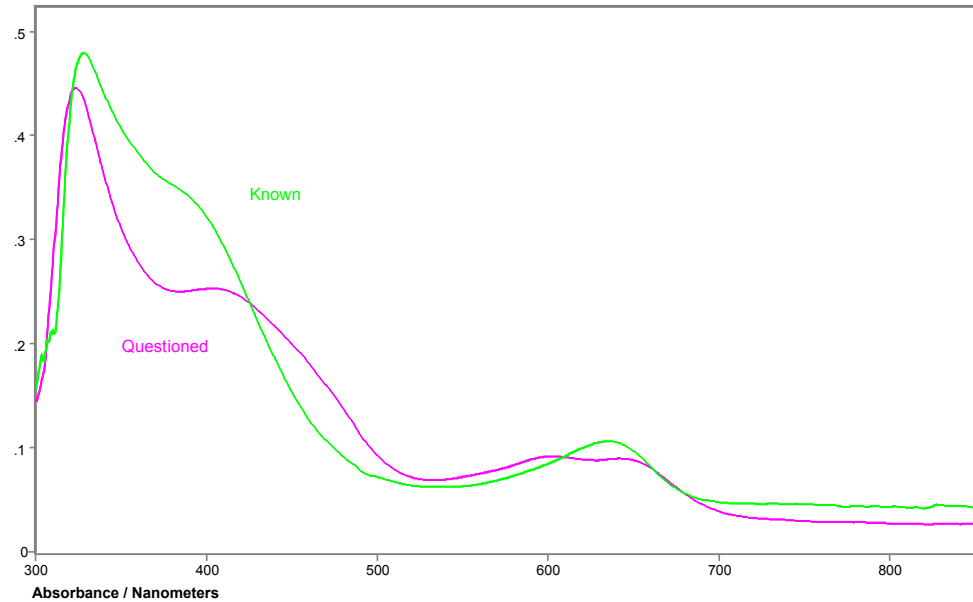
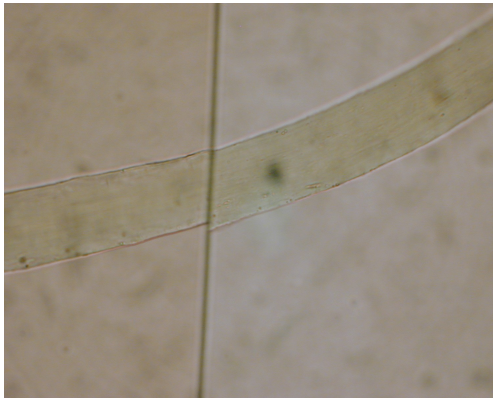
Power of microspectroscopy



- Two green wool fibers
- Appear physically identical
- Appear to have the same color

From the personal collection of Ronald Wojciechowski, Washington State Patrol

Power of microspectroscopy



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From the personal collection of Ronald Wojciechowski, Washington State Patrol

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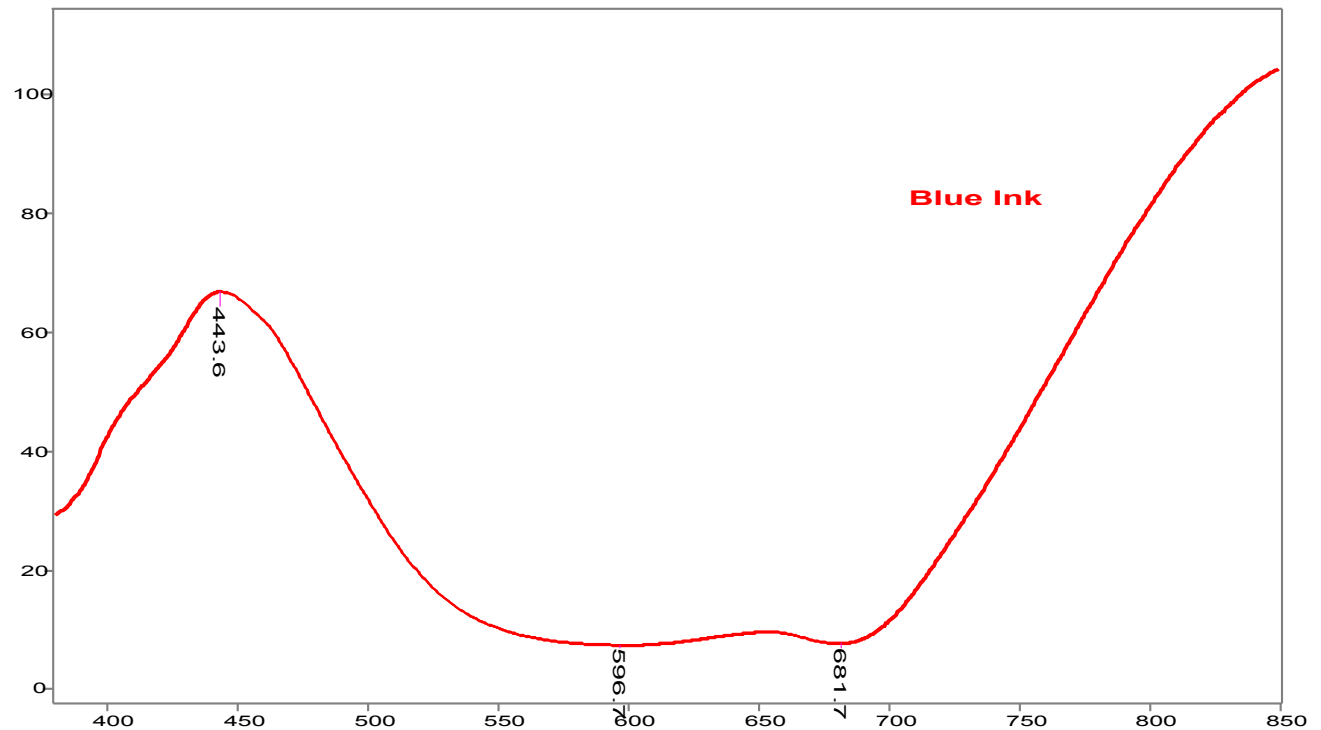
Spectral Interpretation

Transmission, Reflectance, and
Fluorescence

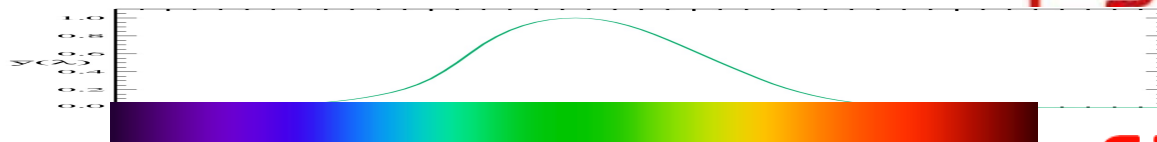
Spectral Interpretation

- Acquiring spectra is easy... understanding them, that can be hard
- Look for peaks and shoulders
- Compare relative intensities of peaks to one another
- Look for peak wavelengths
- And always, *always* read the axes units i.e. is it reflectance or transmission or....

How to read a transmission spectrum



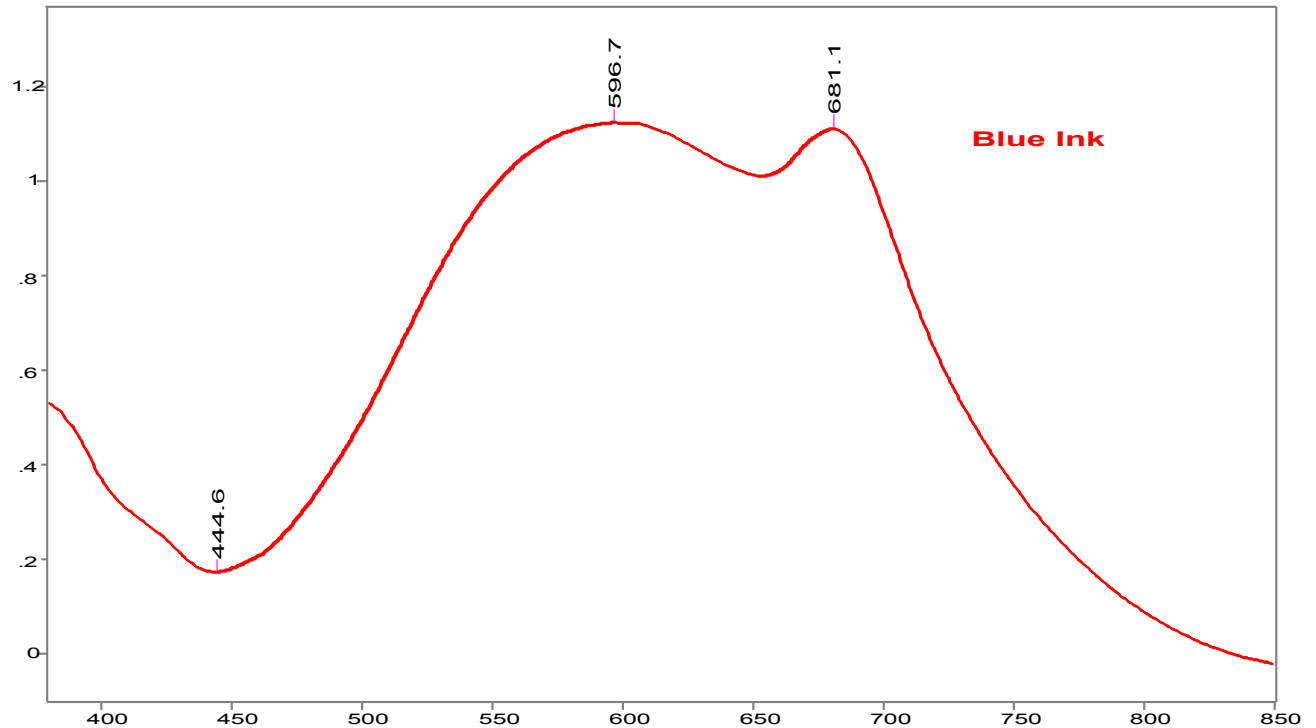
Transmission / Nanometers



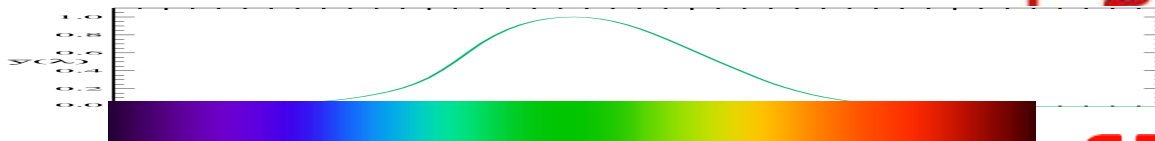
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How to read an absorbance spectrum



Absorbance / Nanometers



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Absorbance vs. Transmittance

- Absorbance is related to transmittance by the equation

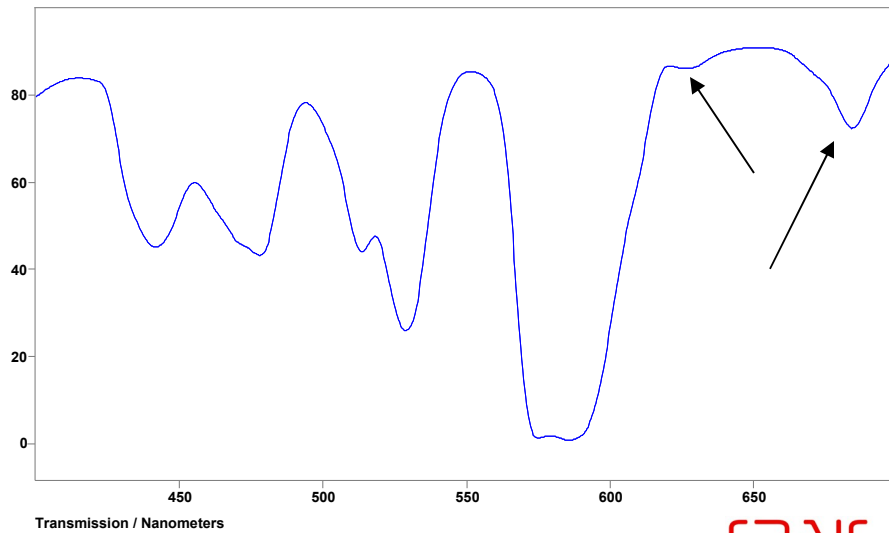
$$A = -\log T$$

- Absorbance is linearly proportional to concentration by

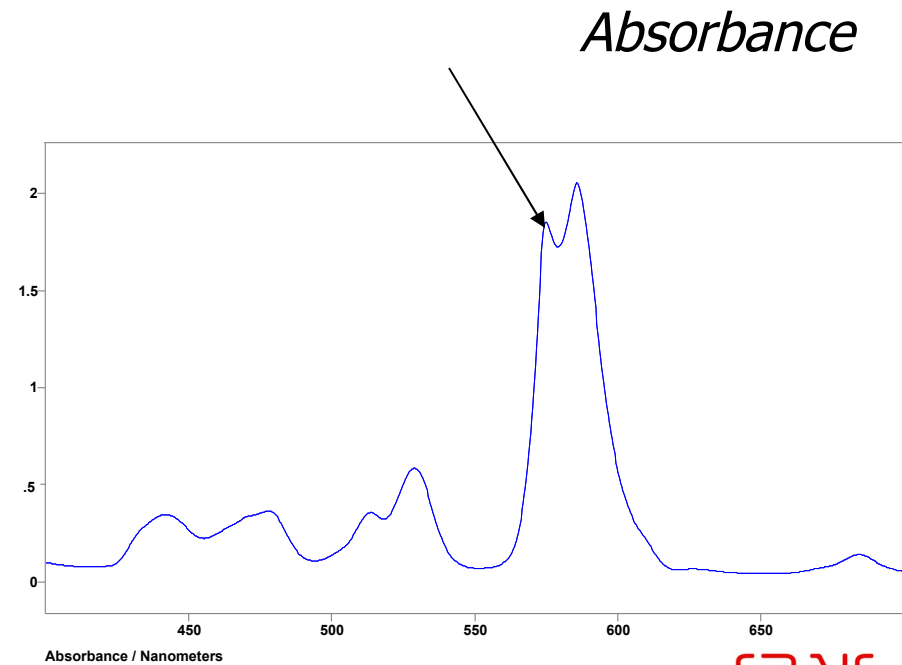
$$A = \epsilon bc$$

- Transmittance emphasizes lower concentration peaks
- Absorbance emphasizes high concentration detail

Absorbance vs. Transmittance



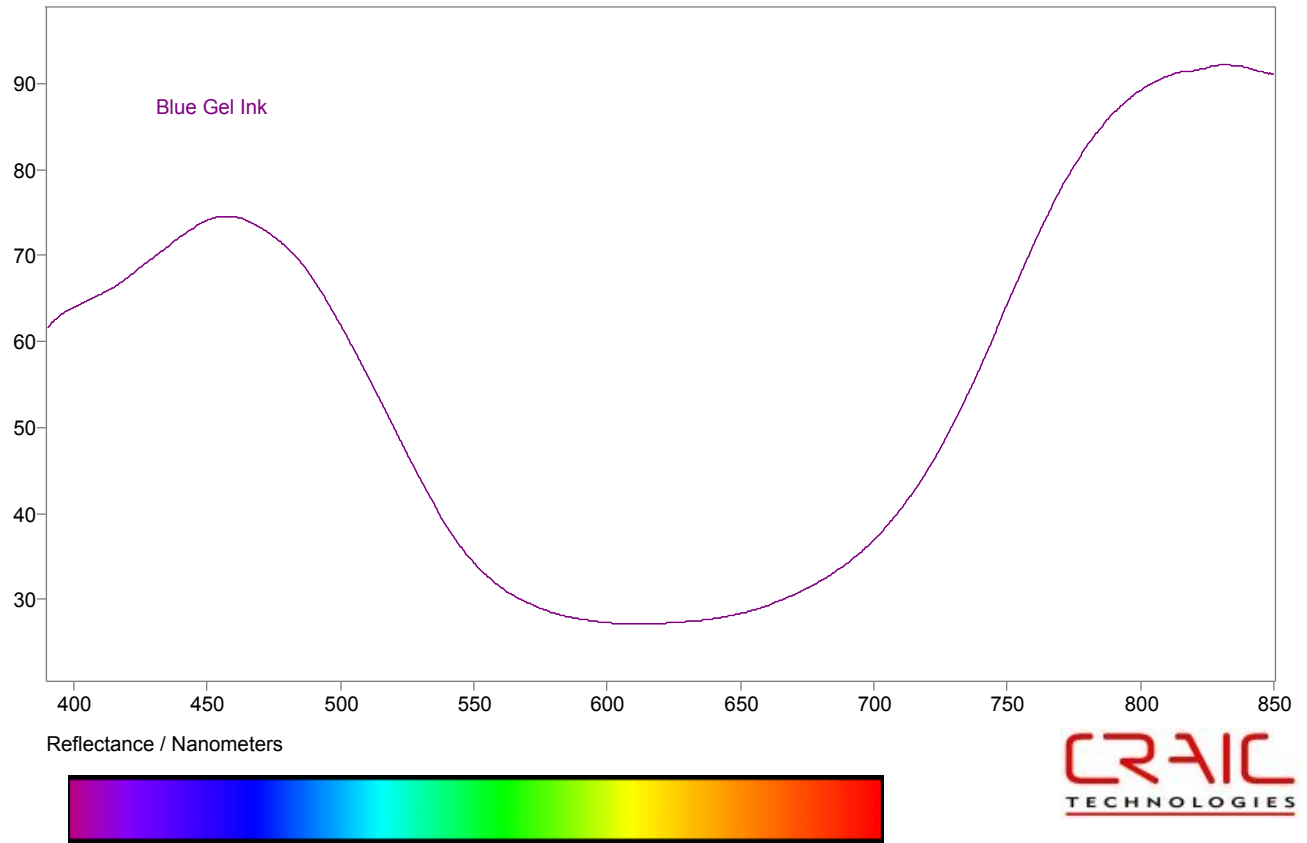
Transmission



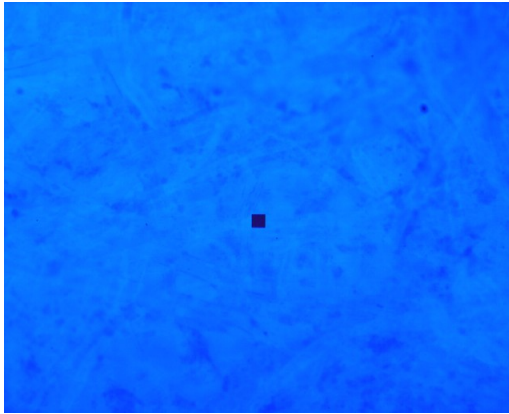
Absorbance



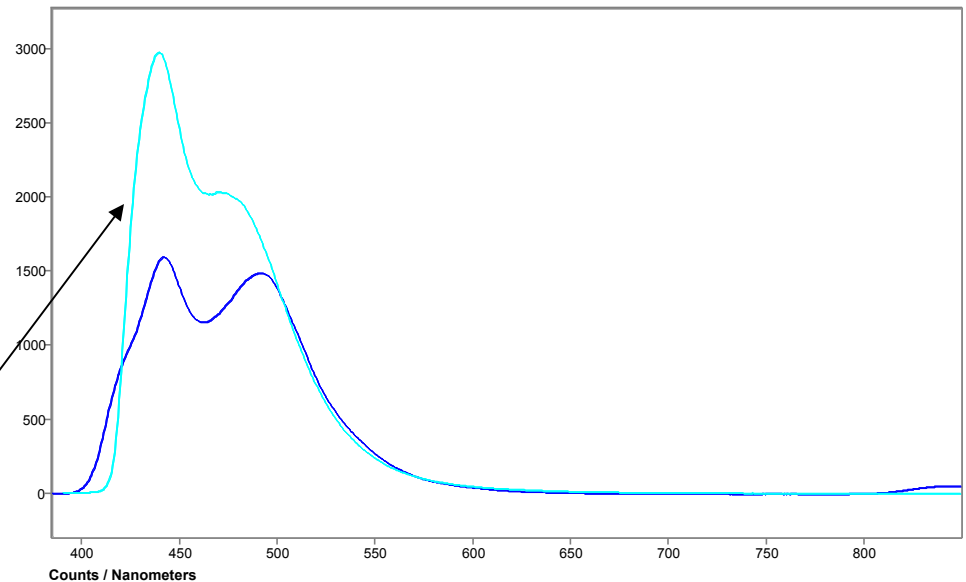
How to read a reflectance spectrum



How to read a fluorescence spectrum



Bleach fluorescence spectrum



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Sample Preparation

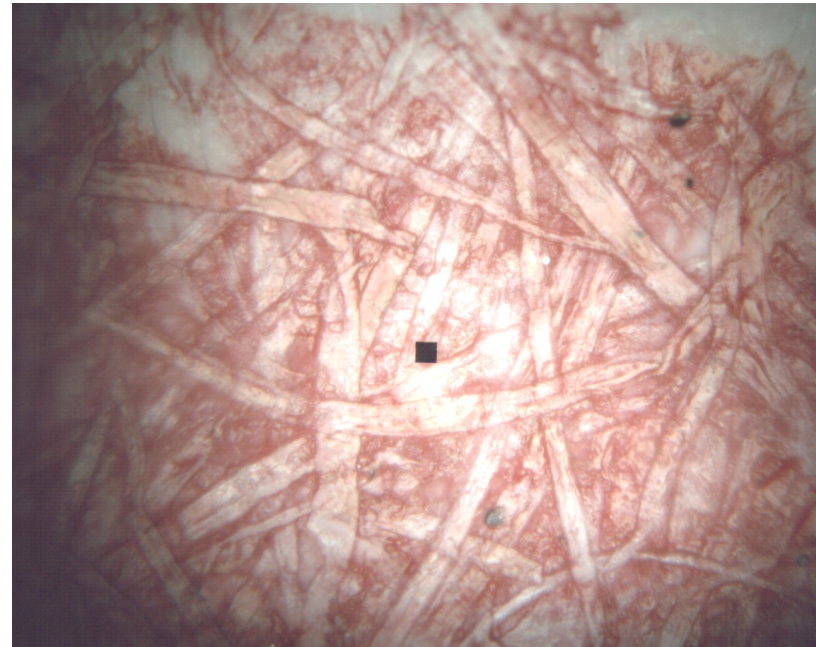
- When to use which type of microspectroscopic analysis
- How to prepare samples for for chosen analysis

Sample Preparation

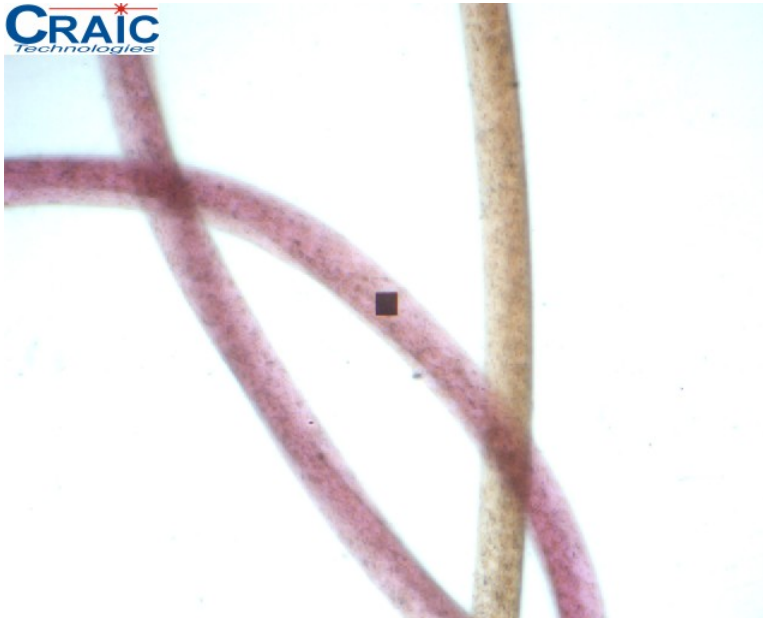
Sample preparation is very important as better preparation means less ambiguous microspectral data

Different spectral techniques require different preparation

Samples determine type of microspectroscopy

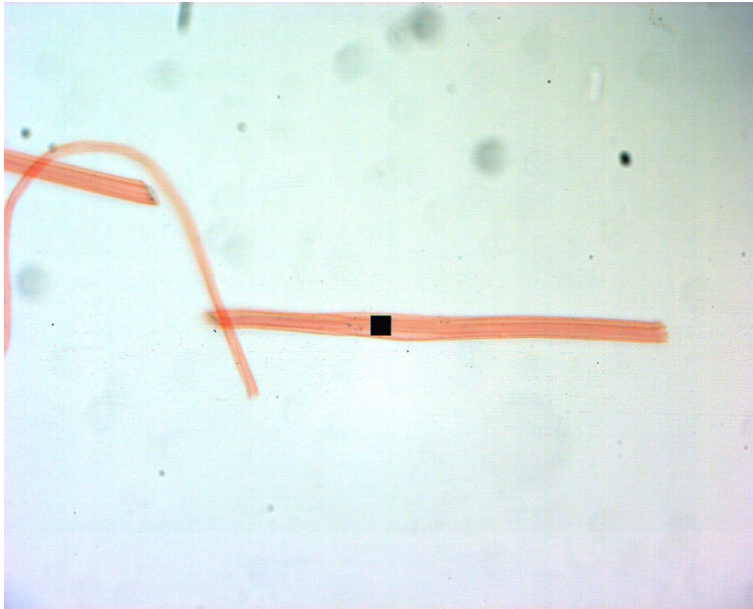


Sample Preparation: Fibers



- Fibers always measured by transmission & fluorescence
- Remove twists from fiber to avoid spectral artifacts

Sample Preparation: Fibers



- Place on slide so that fiber is visible, straight, and not twisted
- Flatten if too dark
- Add drop of mounting medium
- Cover with cover slip

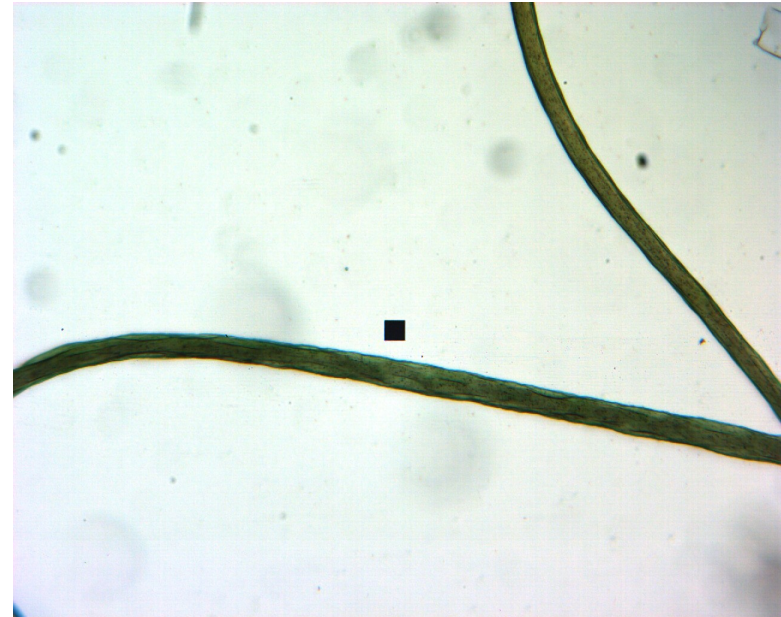
Sample Preparation: Fibers

- If measuring UV spectra, must mount samples on quartz slides with quartz coverslips and glycerin as a mounting media
 - Glass and common mounting medias absorb all light below 320 nm
- If doing low level fluorescence, don't use fluorescing mounting media i.e. Permout
 - Uncontaminated Glycerin works
 - Glass slides sometimes fluoresce

Sample Preparation: Fluorescence of Fibers

Fluorescence spectroscopy can be used for fibers

- Non-destructive
- Simple sample preparation
- *Caveat:* Fiber may not be fluorescent
- *Caveat:* Detergent brightener may fluoresce more intensely than fiber

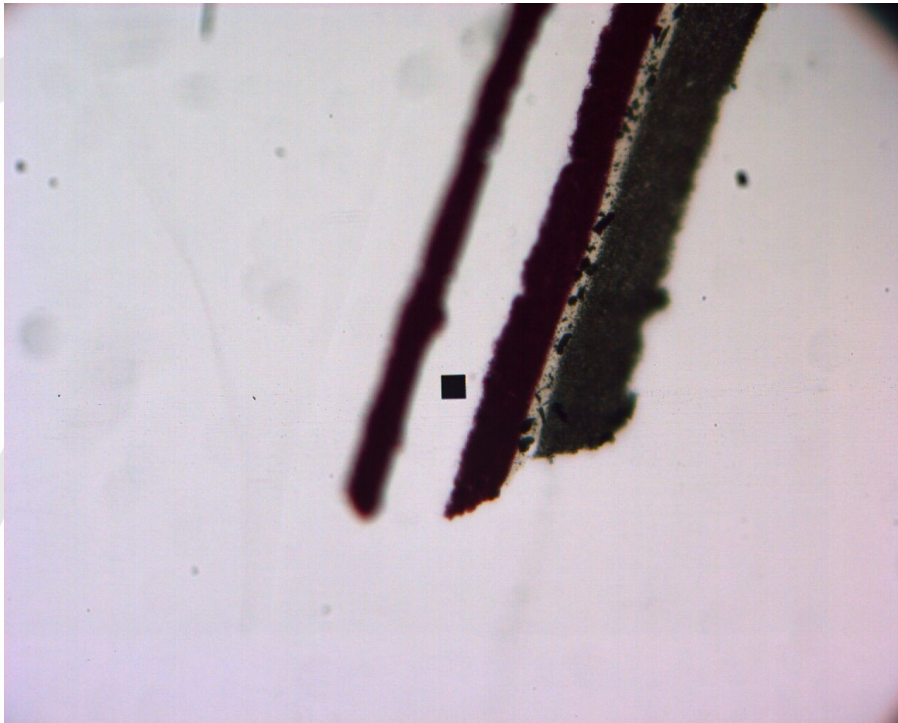


Sample Preparation: Fluorescence of Fibers



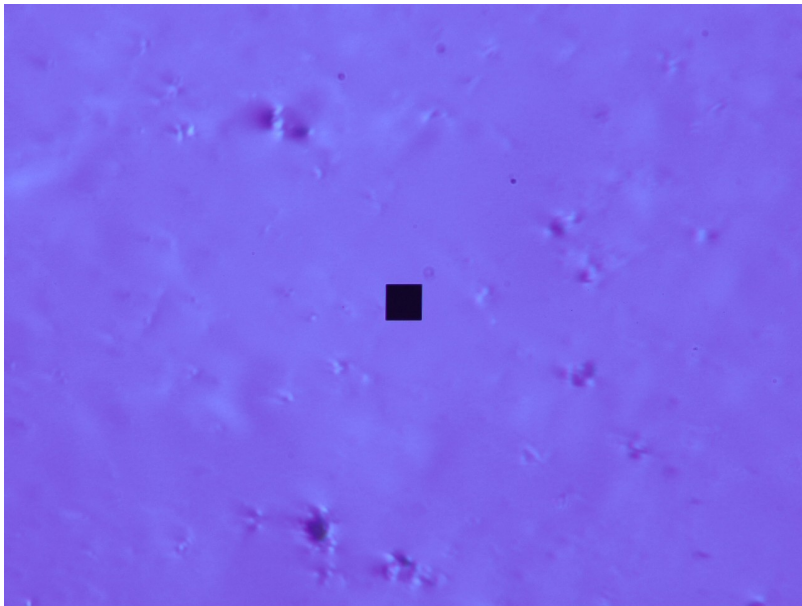
- Use samples as mounted for transmission microspectroscopy
- Must use non-fluorescing mounting media

Paints: Transmission



- Advantages
 - More light energy
 - Well defined reference and absolute spectra
 - No interference from opaque impurities
 - Simpler interpretation and comparison
- Disadvantages
 - Sample preparation required

Paints: Reflectance



- Advantages
 - No sample preparation needed
 - Non-destructive
- Disadvantages
 - Never get absolute spectra so can be difficult to compare
 - Less spectral signal

Paints: Fluorescence



- Advantages
 - Completely different spectral analysis on same instrument
 - No sample preparation... use reflectance and/or transmission samples
- Disadvantages
 - Sample may not fluoresce
 - Sample fluorescence may be overwhelmed by mounting media fluorescence

Sample Preparation: Sectioning Paint by Microtome



- Thickness of known and questioned will be the same
 - Can then compare pigment concentration
- Mount K and Q side by side to eliminate microtome variations
- Make sections ~3 microns thick
 - Pigment or dye concentration variations may require different thicknesses

Sample Preparation: Sectioning Paint by Microtome

- Multiple paint sections can be made at once
 - Add thin layer to mold and *partially* cure
 - Add paint chip, noting ID of chip
 - Add thin layer to mold and *partially* cure
 - Repeat steps 2 and 3 until complete
 - Fully cure mold
 - Section with microtome at ~3 microns

Sample Preparation: Sectioning Paint by Microtome

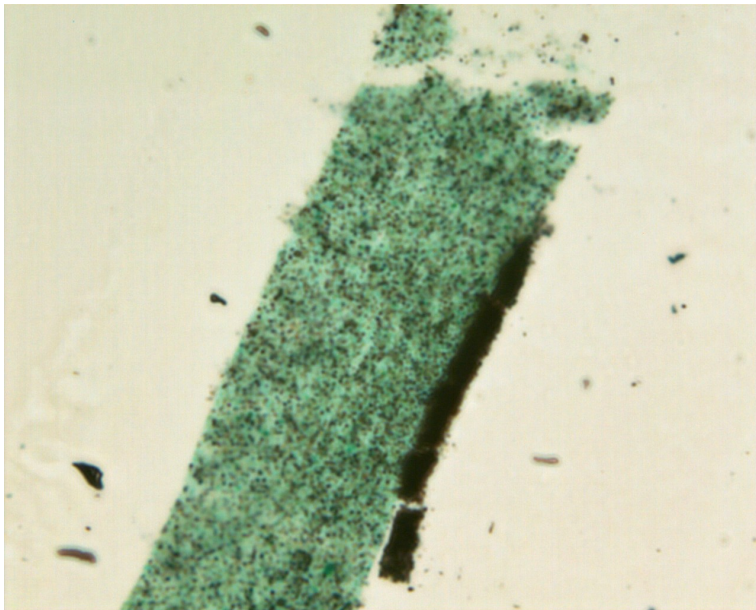
- This technique creates many cross-sections that can be easily analyzed spectroscopically
- Sections will have all the same thicknesses to allow for comparison of concentration variations
- Another technique, used by art conservators, is referenced: JAIC 1991, Volume 30, Number 2, Article 4 (pp. 163 to 177)

Sample Preparation: Sectioning Paint by Hand



- If you do not have a microtome, the next best technique is to section by hand
- Thickness will differ so not be able to compare pigment concentrations
- Can still compare locations and shapes of spectral peaks
- Can still compare relative intensities of peaks

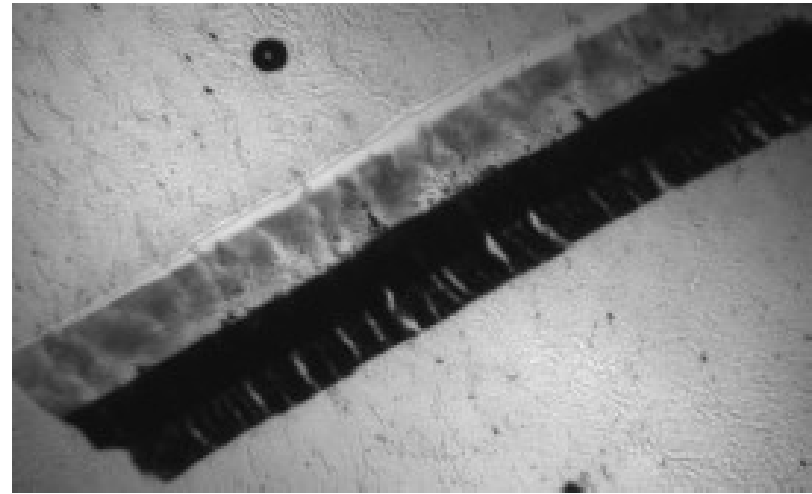
Sample Preparation: Sectioning Paint by Hand



- Hold paint chip on end under stereoscope (use clay or Play-Doh).
- Drag a fresh scalpel over edge to make a thin cross section
- Try again until you get it right
- Roll flatter if needed
- Should be able to see through under compound scope.

Sample Preparation: Paint

- Retrieve paint cross-section
- Place on slide so that each paint layer is visible
- Add drop of mounting medium
- Cover with cover slip



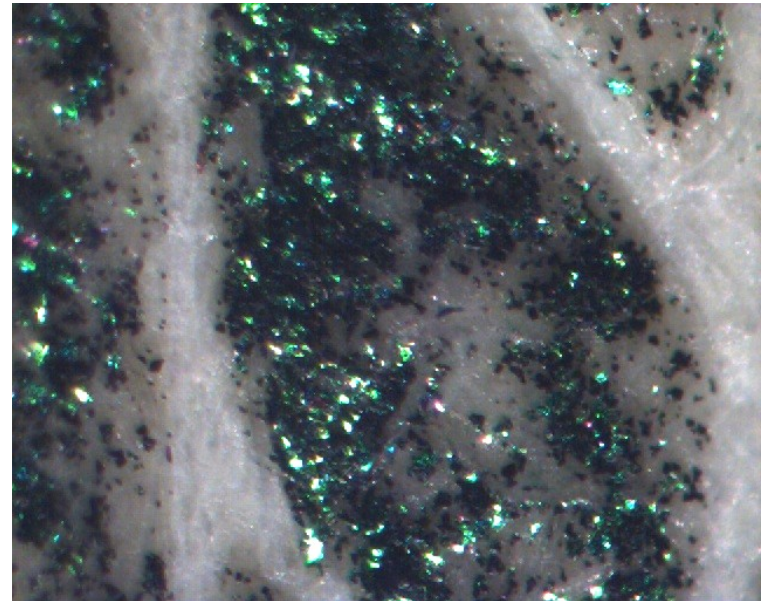
Sample Preparation: Paint

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 - Glass and common mounting medias absorb all light below 320 nm
- If doing low level fluorescence, don't use fluorescing mounting media i.e. Permout
 - Uncontaminated Glycerin works
 - Glass slides sometimes fluoresce

Sample Preparation: Reflectance of Paint

Some paint pigments can only be analyzed by reflectance

- Optically variable pigments work by light interference and diffraction
 - Spectraflair
 - Chromaflair
- Will not transmit light



Sample Preparation: Reflectance

- Multiple paint chips can be analyzed
- Add thin layer to mold and *partially* cure
- Add paint chip, noting ID of chip
- Add thin layer to mold and *partially* cure
- Repeat steps 2 and 3 until complete
- Fully cure mold
- Saw mold in half so all chips have exposed face
- Polish face to analyze
- Mount on slide with glue or clay

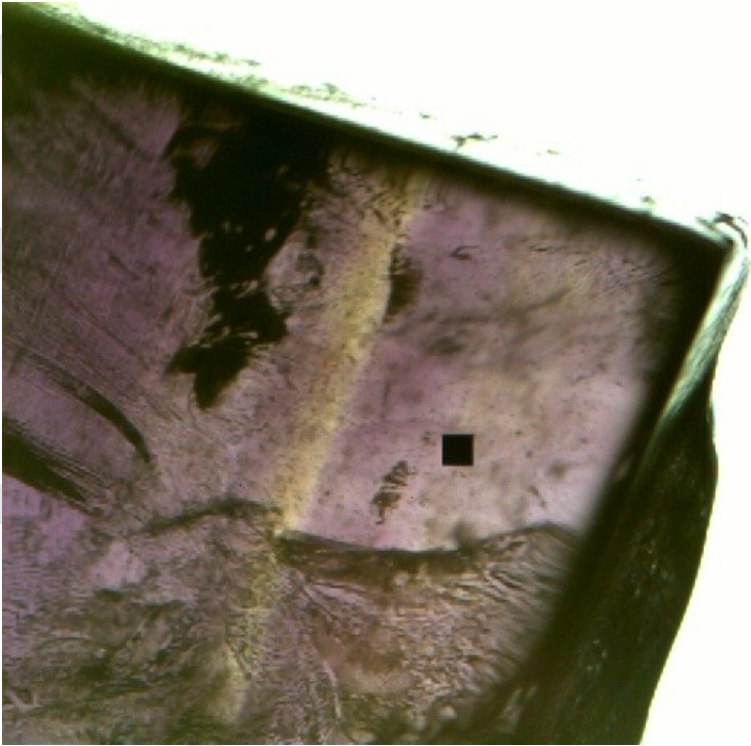
Sample Preparation: Fluorescence of Paints

Fluorescence
spectroscopy can be
used for paints

- Non-destructive
- Simple sample preparation: used that already prepared
- *Caveat:* Paint may not fluoresce



Sample Preparation: Glass



Quartz with no index matching liquid

- Glass fragments always measured by transmission
- Immerse fragment in liquid with similar RI as glass in quartz well slide
- This will eliminate optical artifacts caused by irregular glass surfaces

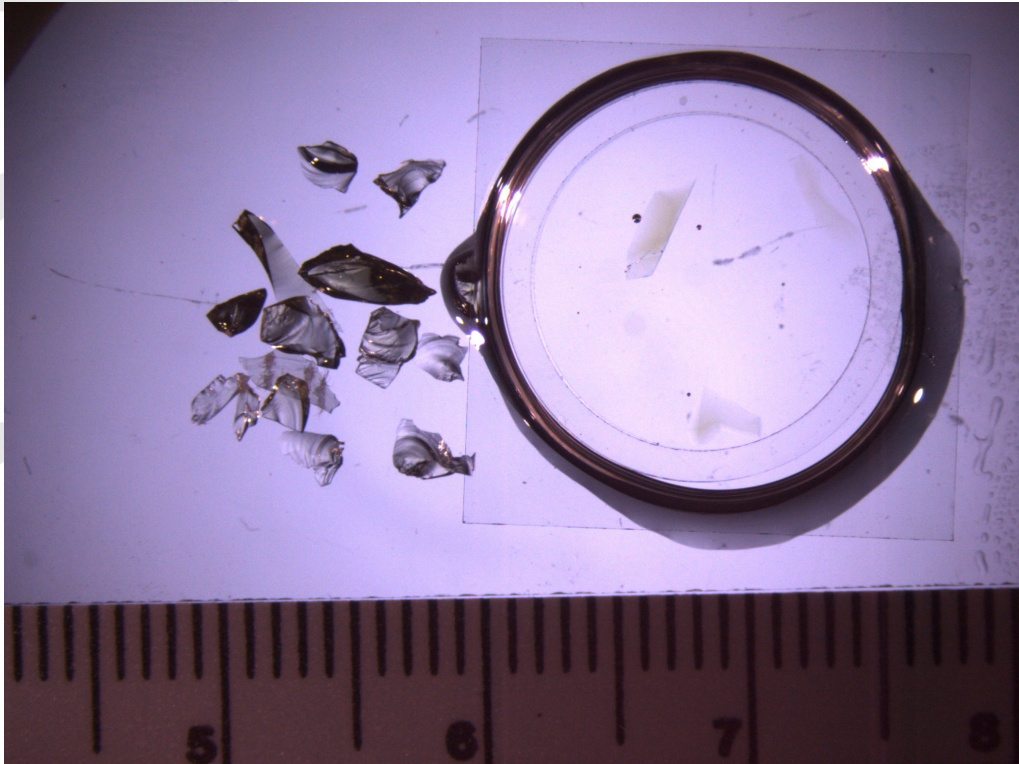
Refractive Indices of Materials

Crown Glass	1.52
Flint Glass (29% lead)	1.569
Flint Glass (55% lead)	1.669
Pyrex	1.474
Lucite	1.495
Plexiglass	1.488
Fused Silica	1.459
Nylon	1.53

Refractive Index Liquids Used

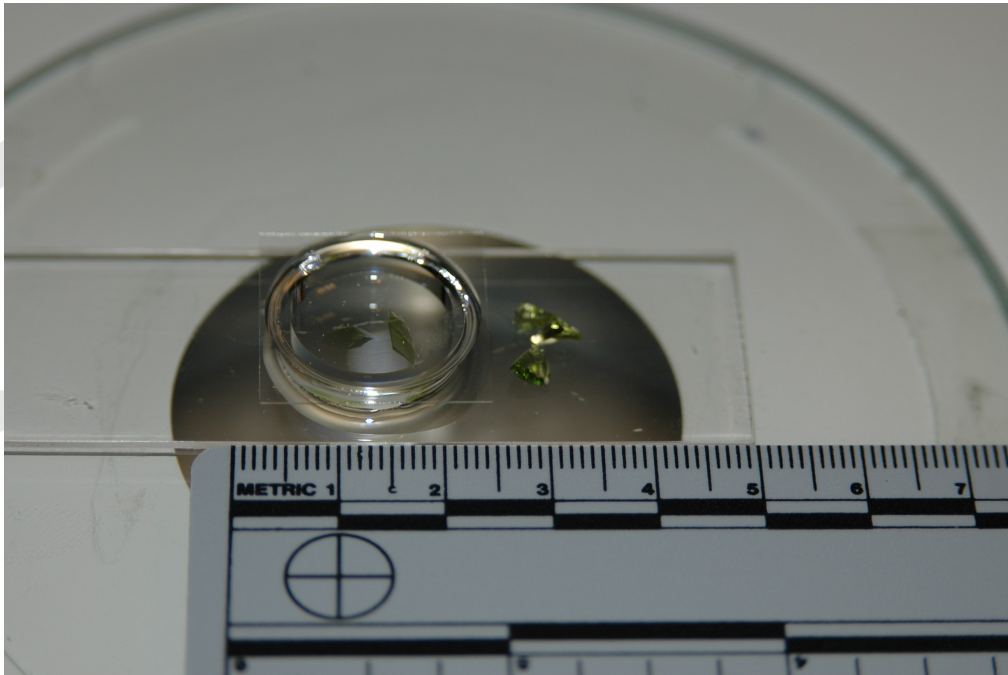
Manufacturer	Many	Cargille
Type	Glycerol	DF Series Oil
Refractive Index	1.473	1.515
Fluorescence	None	Very low

MSP Sample Holder



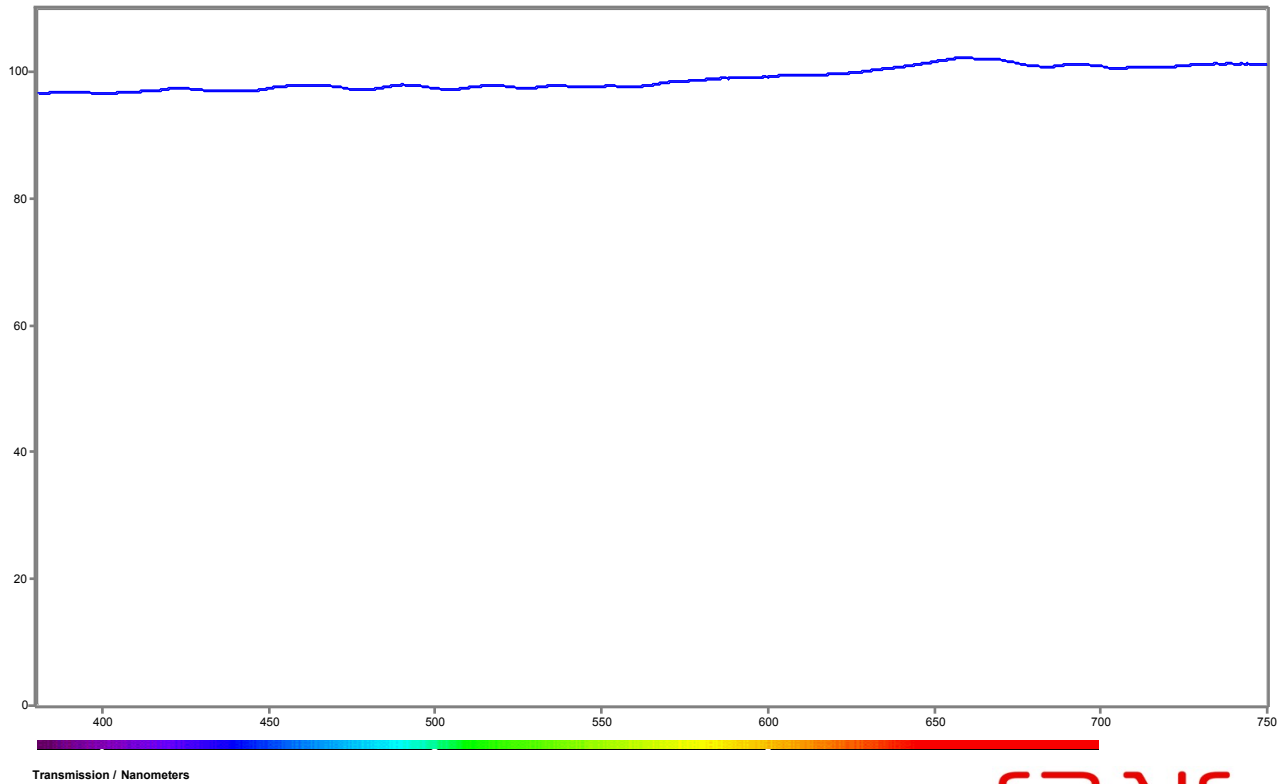
1. Due to irregular shape and refractive index, glass fragments are difficult to analyze
2. Refraction causes spectral artifacts in terms of:
 1. Wavelength
 2. Intensity

MSP Sample Holder



- Use a well slide made out of quartz to hold DF immersion oil *and* glass fragments
- DF oil has similar RI to that of glass
- DF oil is the spectral reference
- Eliminates most spectral artifacts for good color analysis of glass

Spectra of DF Immersion Oil



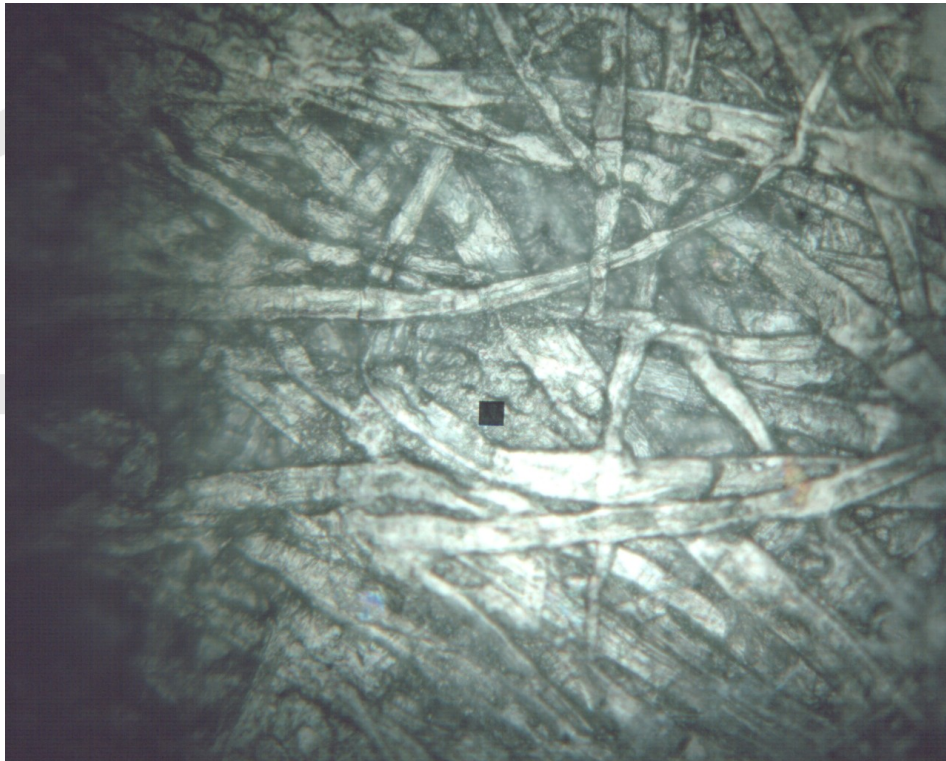
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Sample Preparation: Glass

- If measuring UV spectra, must mount samples on quartz well slides and glycerol
- Since RI of glycerin is 1.47 and glass ranges from 1.54 (Crown) to 1.65 (Flint) there will be optical artifacts

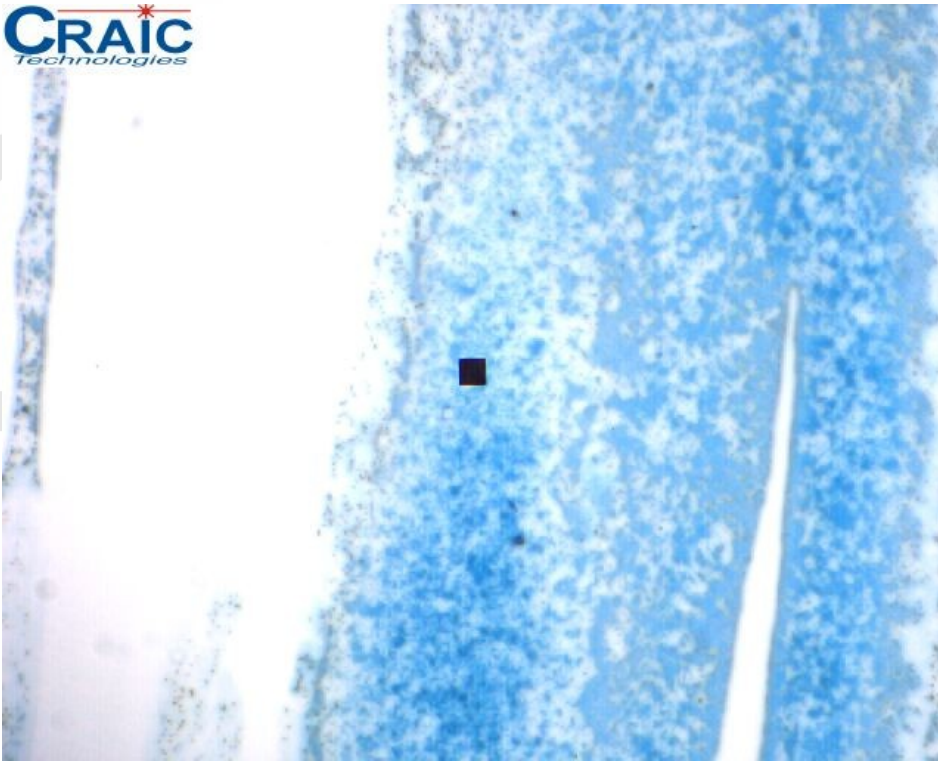
Inks: Reflectance



- Advantages
 - No sample preparation needed
 - Non-destructive
- Disadvantages
 - At high magnifications, paper unreliable as reference
 - Less signal

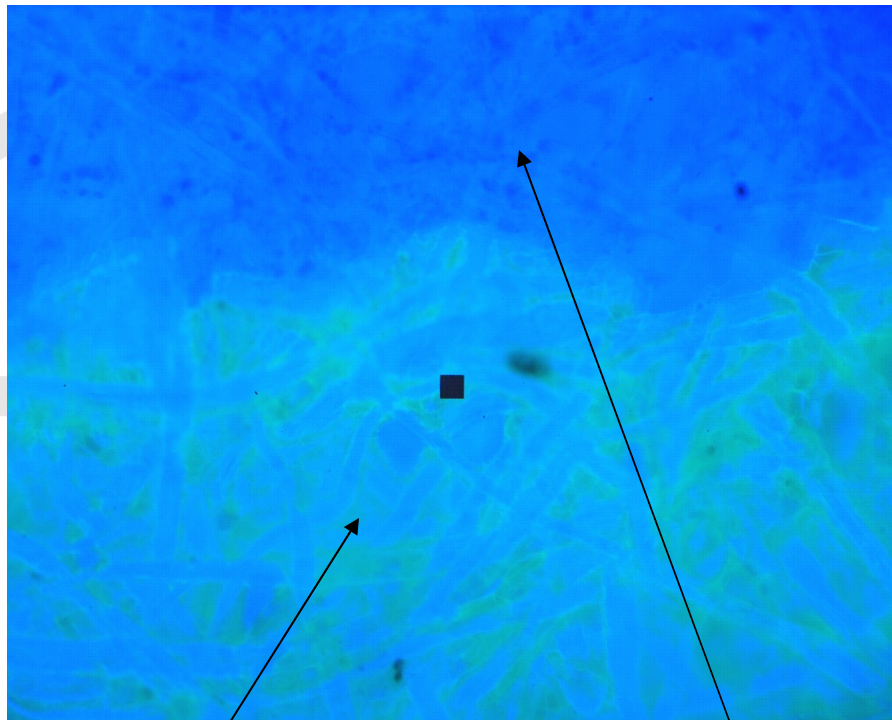
Inks: Transmission

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- **Advantages**
 - More light energy
 - Well defined reference
 - No interference from opaque impurities
 - Simpler interpretation and comparison
- **Disadvantages**
 - Evidence can be damaged
 - Sample preparation required

Inks: Fluorescence



Ink

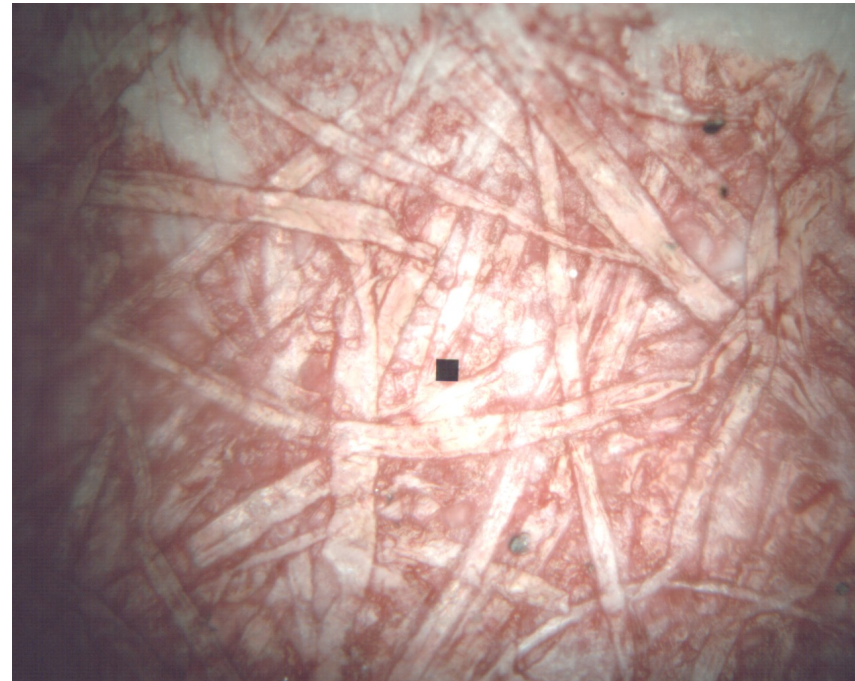
Paper

- Advantages
 - Completely different spectral analysis on same instrument
 - No sample preparation... use reflectance and/or transmission samples
- Disadvantages
 - Sample may not fluoresce
 - Sample fluorescence may be overwhelmed by paper fluorescence

Sample Preparation: Reflectance of Ink

Reflectance microspectroscopy of ink:

- No sample preparation required
- Make sure free from contaminants though (blood, etc.)
- Must hold flat on stage and eliminate focal plane shifts
- Some pigments can only be analyzed by reflectance
 - Optically Variable Pigments



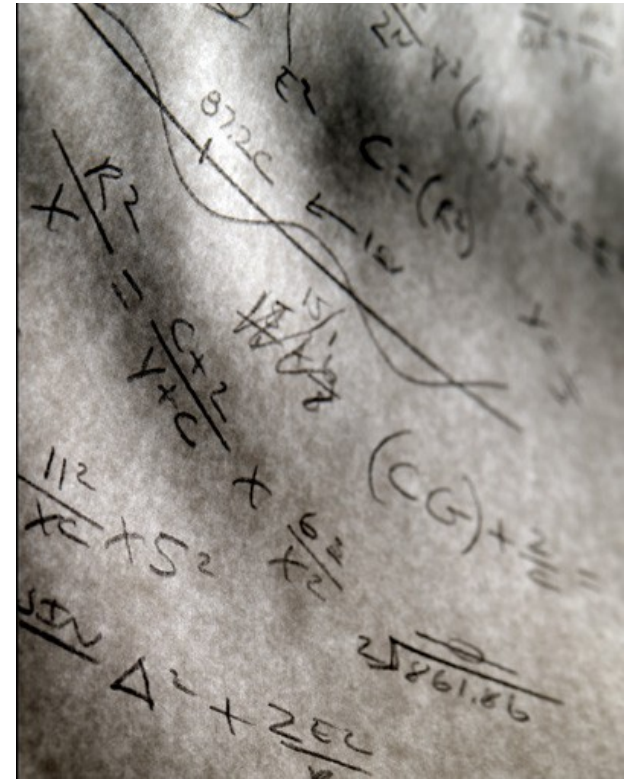
Sample Preparation: Transmission of Ink

- Transmission
microspectroscopy
of ink must meet
one of these
conditions:
- Paper thin enough to transmit light
 - Must be able to remove ink from paper so that it transmits light

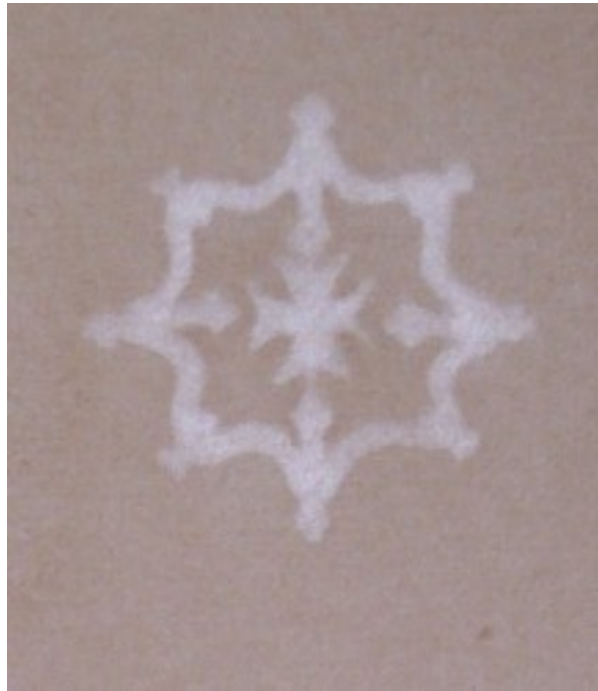


Sample Preparation: Transmission of Ink

- If going to do UV-visible-NIR microspectroscopy, must use quartz slides and coverslips and glycerin mounting media
- Glass slides and coverslips and most mounting medias absorb UV light

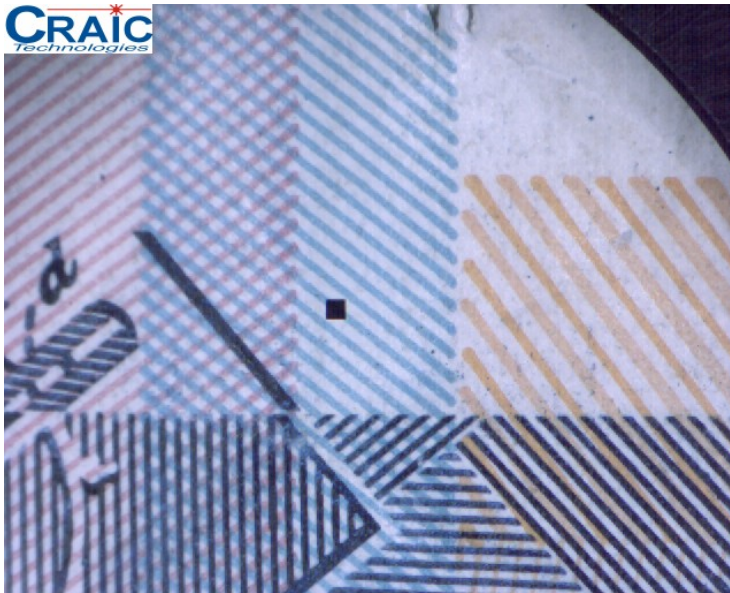


Transmission: Thin paper



- No sample preparation required
- Make sure free from contaminants though (blood, etc.)
- Must hold flat on stage and eliminate focal plane shifts

Transmission: Thick paper



Ink must be removed from paper that is blocking the light. This can be done by:

- Placing micro-plug of document in solvent and dissolving ink
- Teasing a fiber (with ink) free of document
- Removing ink particle free from document
- Taping of ink on the paper

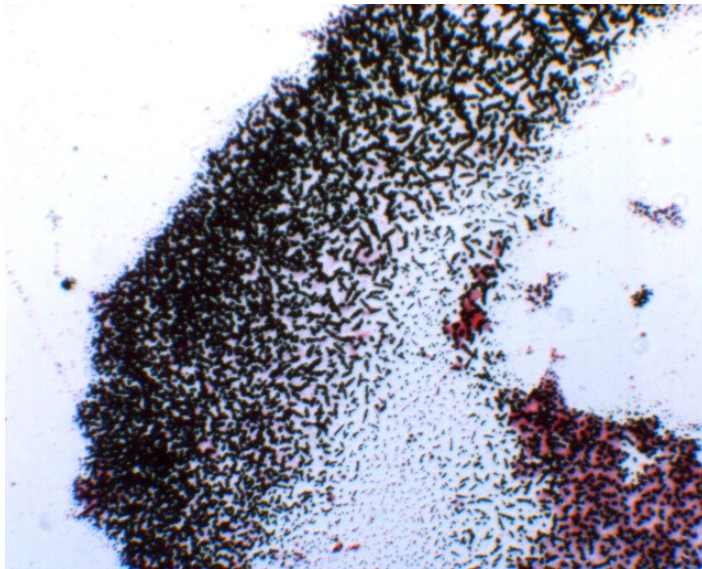
Transmission: Solvation of Ink



Dissolving the ink

- Take micro-plug of document and place in small amount of appropriate solvent
- Evaporate solution on slide
- Take spectra

Transmission: Solvation of Ink

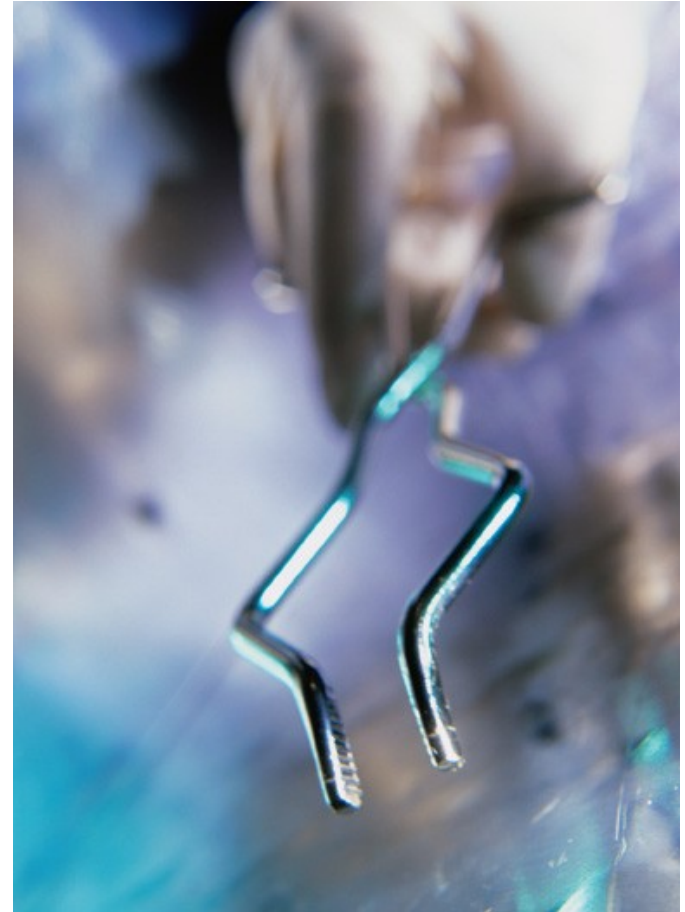


Dissolving the ink

- Must find solvent that dissolves ink and readily evaporates
- Destructive of document
- Very good quality spectra

Transmission: Teasing of Ink

- Requires stereomicroscope, scalpel, needles, tweezers
- Must be able to extract pulp fibers from paper
 - Very difficult with some papers
 - Good for inks that soak into pulp fibers

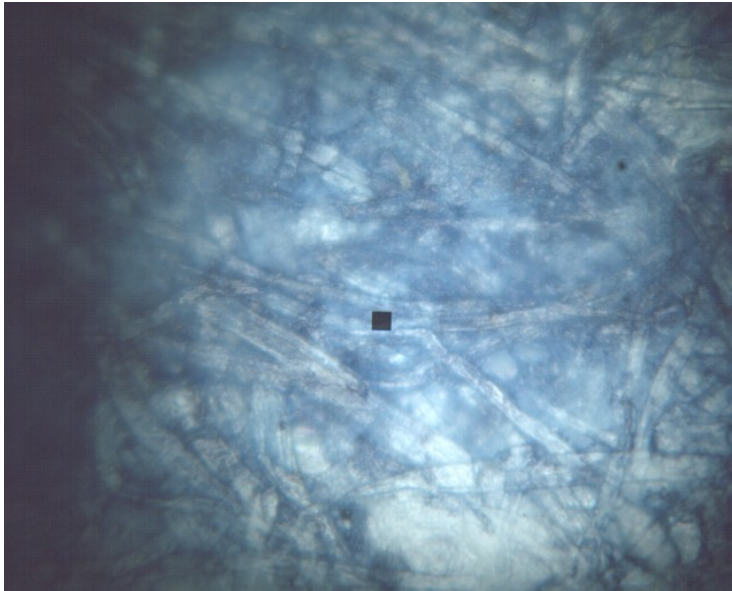


Transmission: Teasing of Ink

- Extract fiber with ink from document
- Place on slide, add drop of mounting media, cover with coverslip, let media cure.
 - Minimal damage to evidence

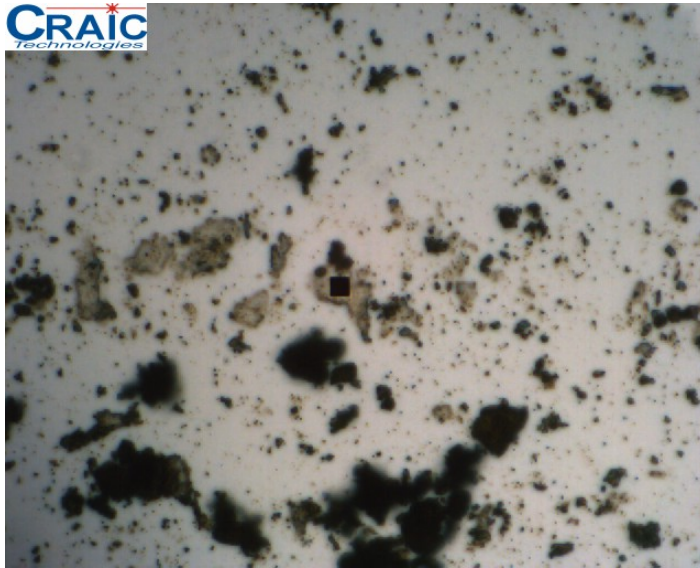


Transmission: Removal of ink



- Requires stereomicroscope, scalpel, needles, tweezers
- Must be able to remove ink particles from paper
 - Good for inks that cover pulp fibers like paint
 - Ink must be dry

Transmission: Removal of ink



- Extract ink particles from document
- Place on slide, roll flat if too thick, add drop of mounting media, cover with coverslip, let media cure.
 - Minimal damage to evidence

Transmission: Taping of Ink

- Place evidence collection tape over ink line
 - Must not be too sticky or will rip paper
- Pull tape up and then inspect for ink particles



Transmission: Taping of Ink

- Extract ink particles from tape. Wash if needed
- Place particles on slide, roll flat if too thick, add drop of mounting media, cover with coverslip, let media cure.
 - Minimal damage to evidence

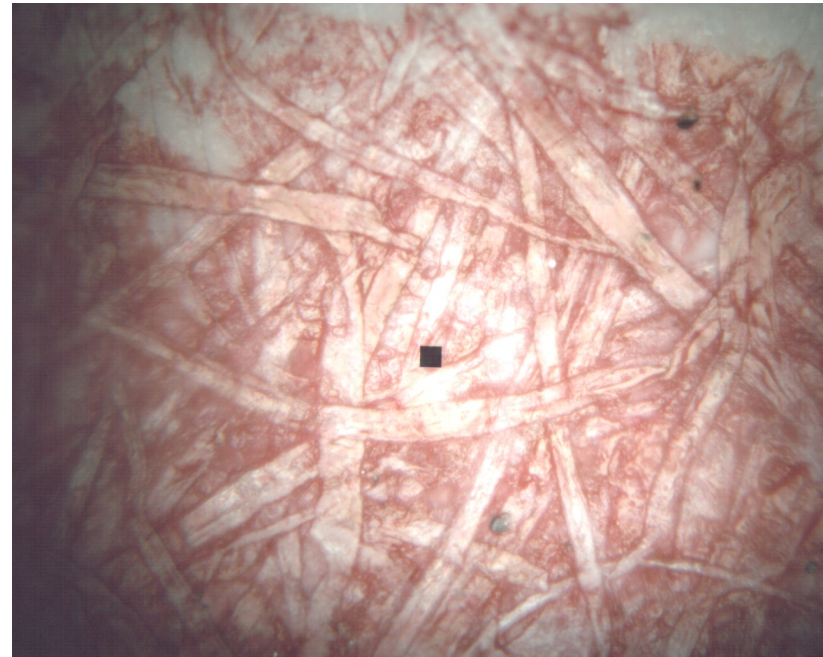


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Sample Preparation: Fluorescence of Ink

Fluorescence spectroscopy can be used for inks and paper

- Non-destructive
- Simple sample preparation
- *Caveat:* Ink may not fluoresce
- *Caveat:* Paper may fluoresce more intensely than ink



Sample Preparation: Fluorescence of Ink



- Use samples as mounted for reflectance or transmission microspectroscopy
- Must use non-fluorescing mounting media

Microspectrophotometer Operation

Microspectrophotometer



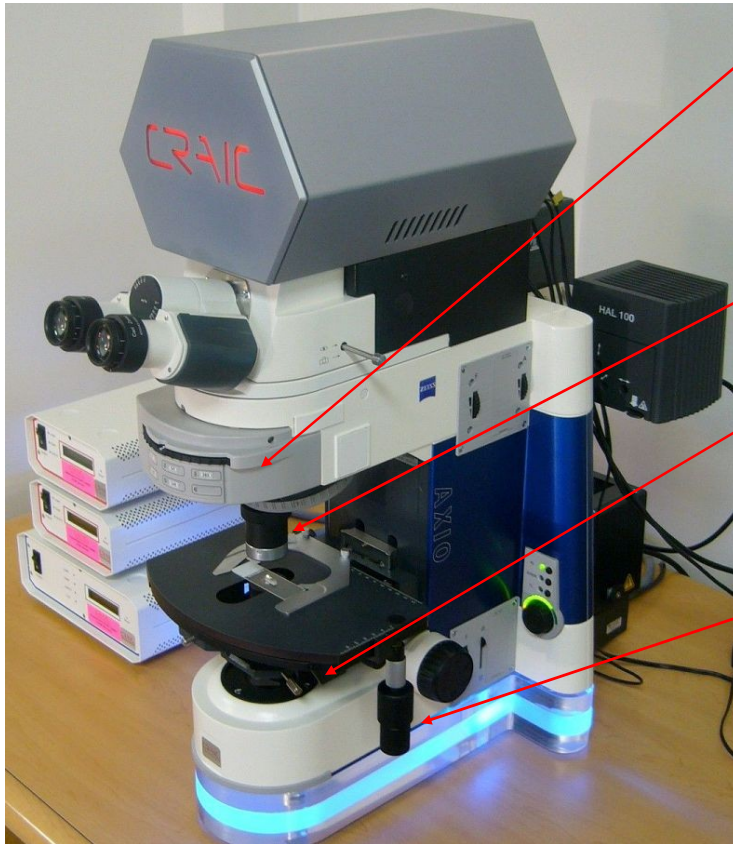
Microspectrometer has

- UV-visible-NIR range

Microspectrometer can take

- Transmission
- Reflectance
- Fluorescence
- Polarization
- Spectra of microscopic samples

Microspectrometer



1. Rotating control for reflectance and fluorescence
2. Objective
3. Condenser centering controls
4. Manual stage control

Microspectrometer Front



Setting for:

2. Transmission
3. Reflectance
4. Fluorescence 365 nm
5. Fluorescence 420 nm
6. Fluorescence 546 nm

Instrument Validation

- Why? To verify proper functioning of equipment and provide baseline
- When? When new equipment is installed
- How? Multiple measurements with NIST Traceable MSP Standards
- How much? Depends on lab's ASCLD or ISO protocols

Instrument Validation

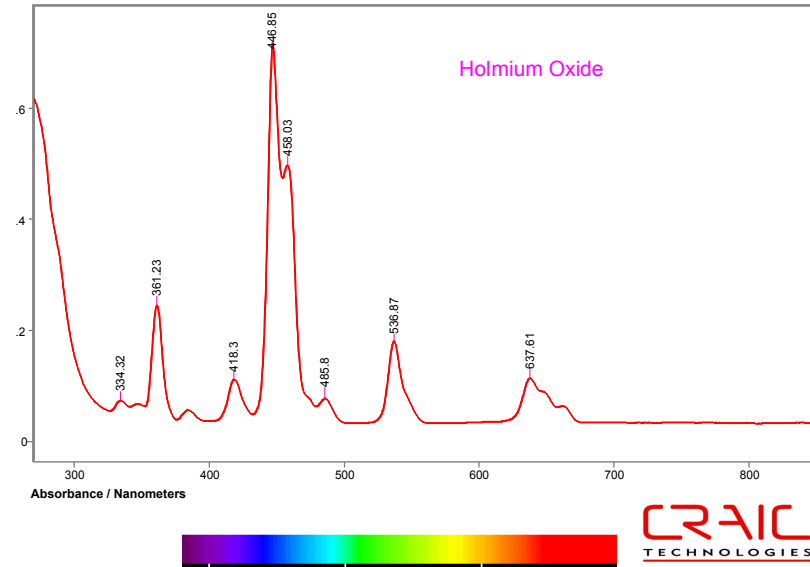
- ASCLD only requires wavelength validation
- Some labs take 50 measurements
 - Builds good statistical sampling for baseline
- Suggestion: Take 50 measurements over two weeks (5 a day).
 - Software makes it very easy & fast (it checks the values for you!)
 - Check both wavelength and photometric accuracy
 - Store printouts in notebook for inspectors

Instrument Calibration

- Should be done each day of use
 - In addition to any requirements per your ASCLD or ISO protocols
- Check wavelength accuracy
- Check photometric accuracy
- Should use NIST Traceable MSP Standards
 - Internationally recognized standards agency
 - Must be recertified every two years

Traceable MSP Standards

- Wavelength standards: Holmium Oxide & Didymium
- Photometric standards: 3 ND filters
- Used to check MSP wavelength & photometric calibration



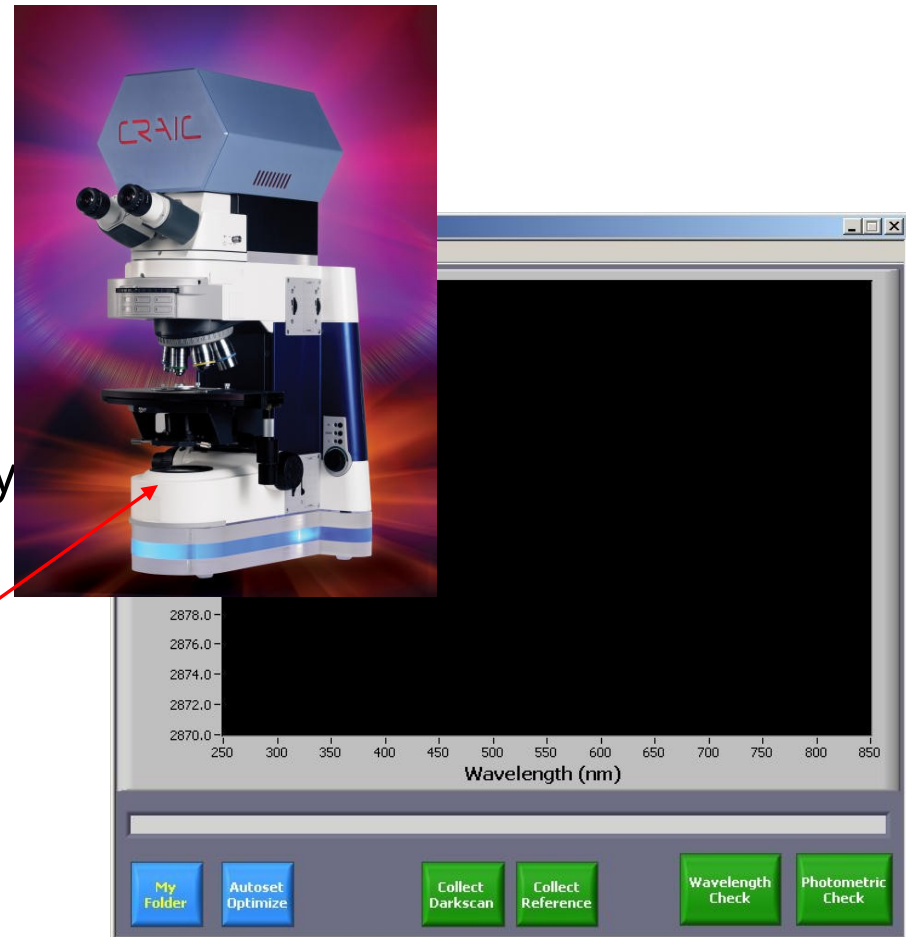
Instrument Calibration



- Place reference slide on microscope stage
 - This will not be moved during test procedure.
- Rotate to %T position
- Focus on ink dot on slide
- Set Koehler illumination:
 - Closing F stop
 - Centering condenser
 - Open F stop

Instrument Calibration

- Optimize spectrophotometer with reference slide
- Take dark scan
- Take reference scan
- Automatically check wavelength calibration by following software prompts and placing filters on transmission field stop
- Automatically check photometric calibration with same procedure

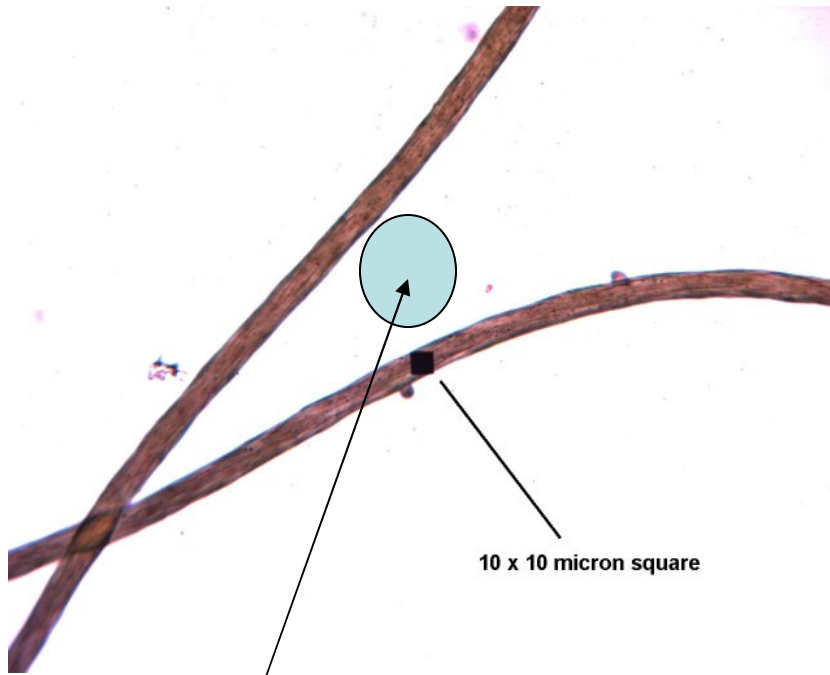


Taking Transmission Spectra



- Rotate to %T position
- Place sample on stage and focus
- Set Koehler illumination:
 - Closing F stop
 - Centering & focusing condenser
 - Open F stop

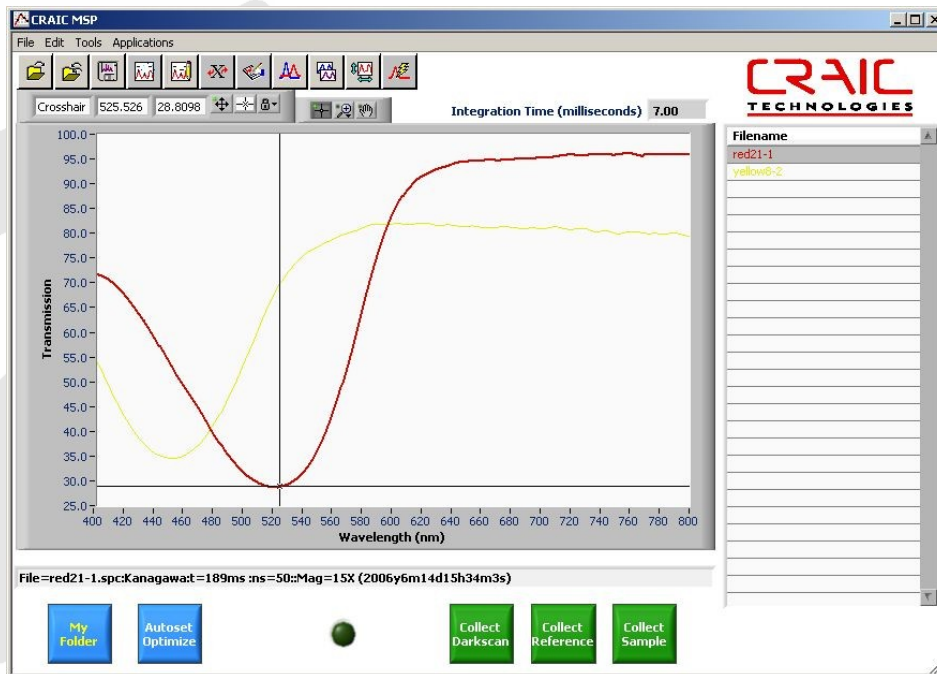
Taking Transmission Spectra



This is a good reference area

- Reference will be taken at location just off of sample in an area containing only slide, mounting media and coverslip

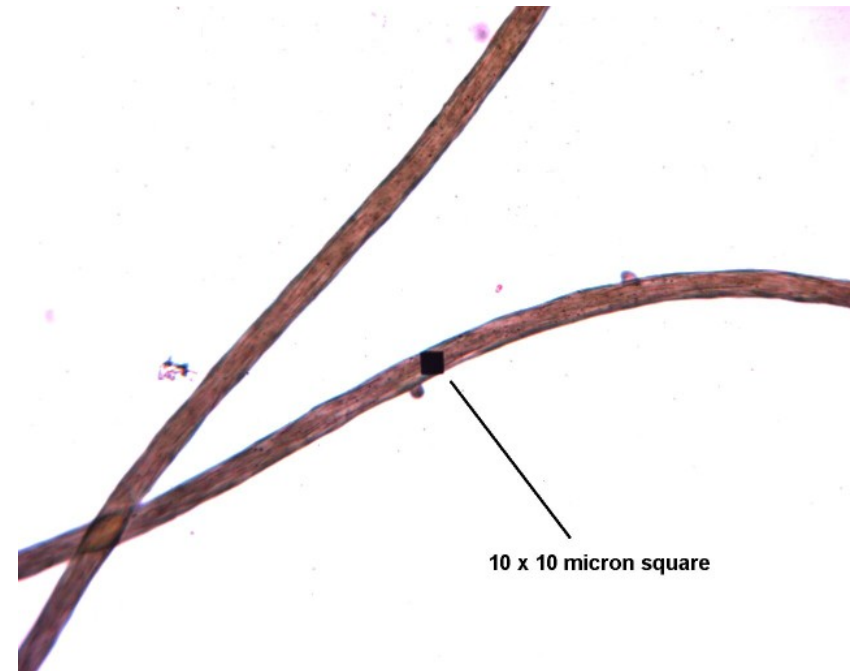
Taking Transmission Spectra



- Optimize MSP
- Close transmission shutter
- Software: Collect Darkscan
- Open transmission shutter
- Software: Collect Reference
- Now ready to take absorbance or transmission spectra

Taking Transmission Spectra

- Move stage so that aperture is over sample to be measured
- Software: Collect Sample
- Result is an absolute transmission or absorbance spectrum.

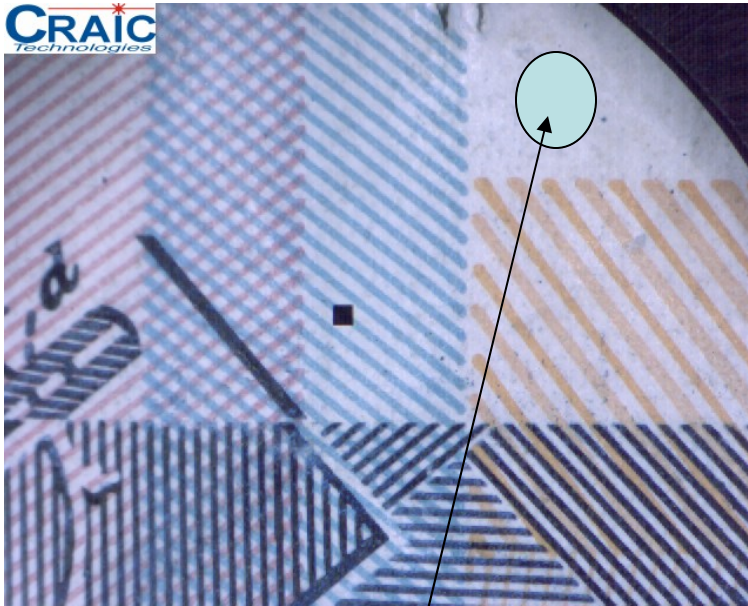


Taking Reflectance Spectra



- Rotate to %R position
- *If installed*: Select reflectance lamp with switching mirror in the rear of microscope

Taking Reflectance Spectra



This is a good reference area

- Place reference material on stage and focus
- With documents, the paper substrate is a possible reference site

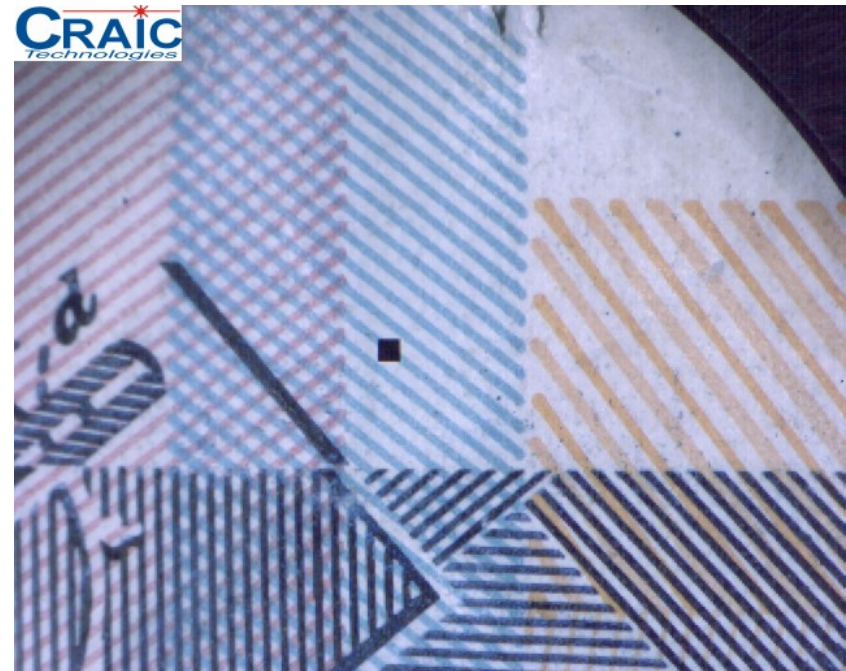
Taking Reflectance Spectra



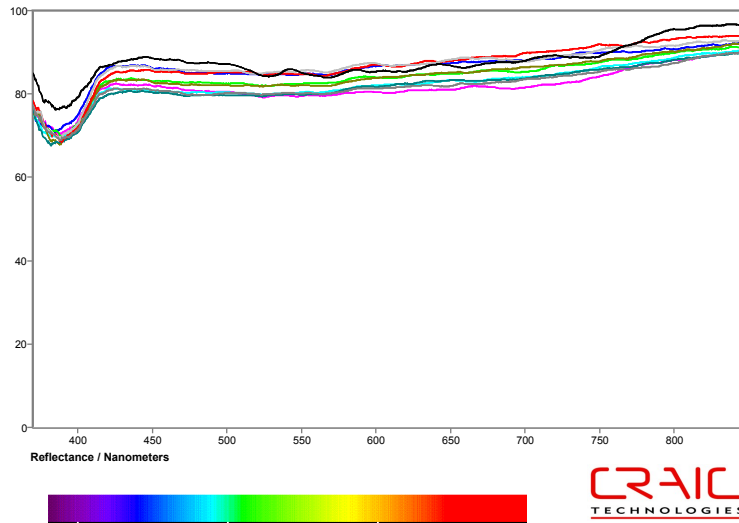
- Optimize MSP
- Close reflectance shutter
- Software: Collect Darkscan
- Open reflectance shutter
- Software: Collect Reference
- Now ready to take relative reflectance spectra

Taking Reflectance Spectra

- Move stage so that aperture is over sample to be measured
- Software: Collect Sample
- Result is a reflectance spectrum relative to reference.



Taking Reflectance Spectra



Ten locations on same sheet of paper

Documents:

- Paper reference
 - Convenient
 - NOT reproducible at small scale
- White standard
 - Reproducible
 - Easy
 - Relative spectra of ink...not absolute

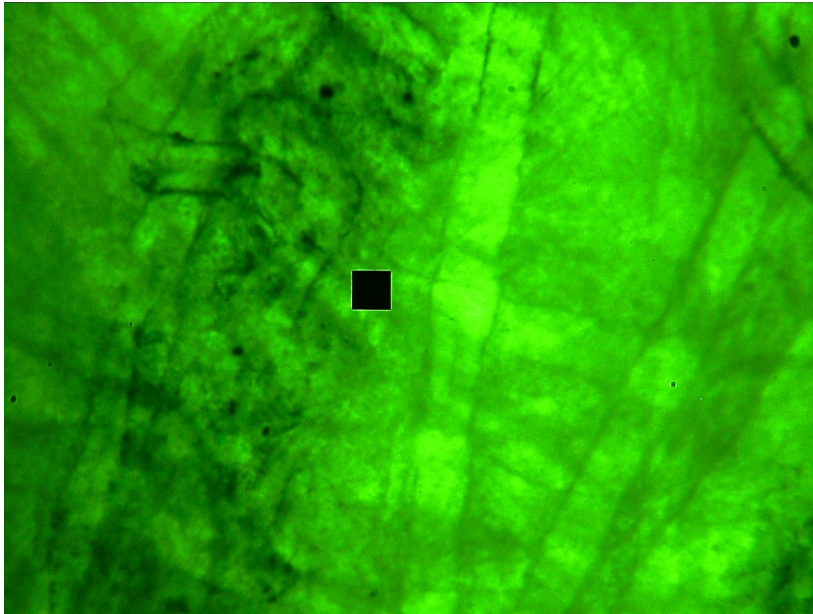
Taking Fluorescence Spectra



- Rotate to select fluorescence excitation wavelength
- *If installed:* Select fluorescence lamp with switching mirror in the rear of microscope



Taking Fluorescence Spectra



Security thread in currency

- Place sample material on stage and focus
- Move aperture over sample to be measured
- There is no reference needed for fluorescence

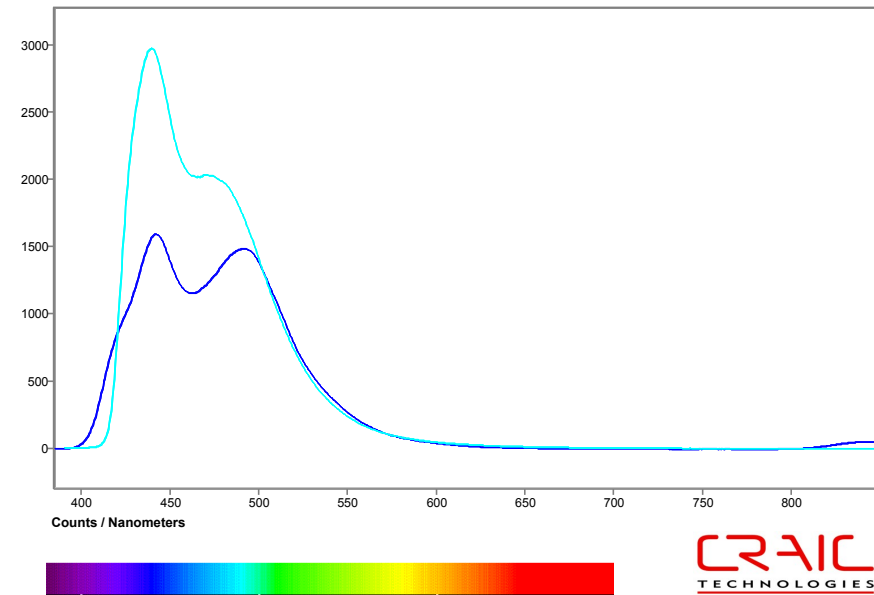
Taking Fluorescence Spectra



- Software: Manually set integration time.
 - Automatic optimization routines not designed for fluorescence
- Close reflectance shutter
- Software: Collect Darkscan
- Open reflectance shutter
- Now ready to take fluorescence spectra

Taking Fluorescence Spectra

- Move stage so that aperture is over sample to be measured
- Software: Collect Sample
- Result is a fluorescence emission spectrum.



Fluorescence spectra of brighteners of two different papers

Thank you



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