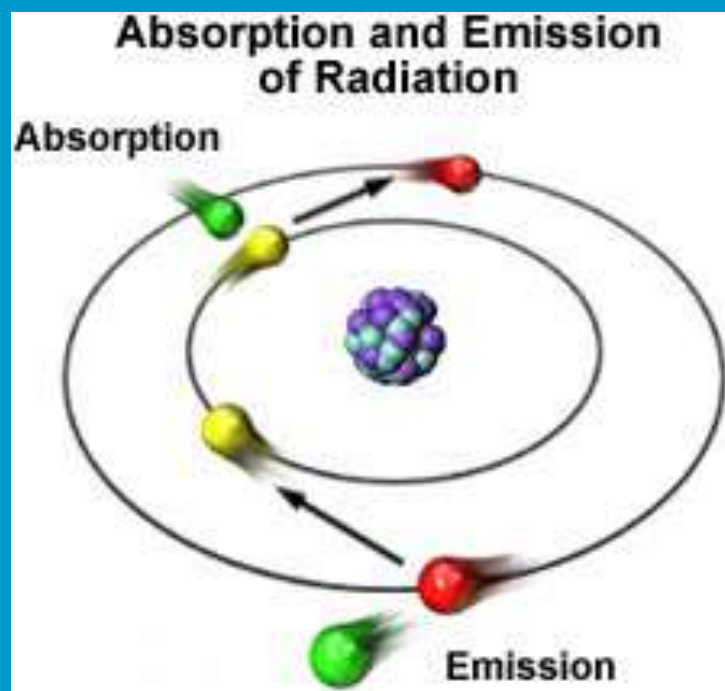




Agilent Technologies

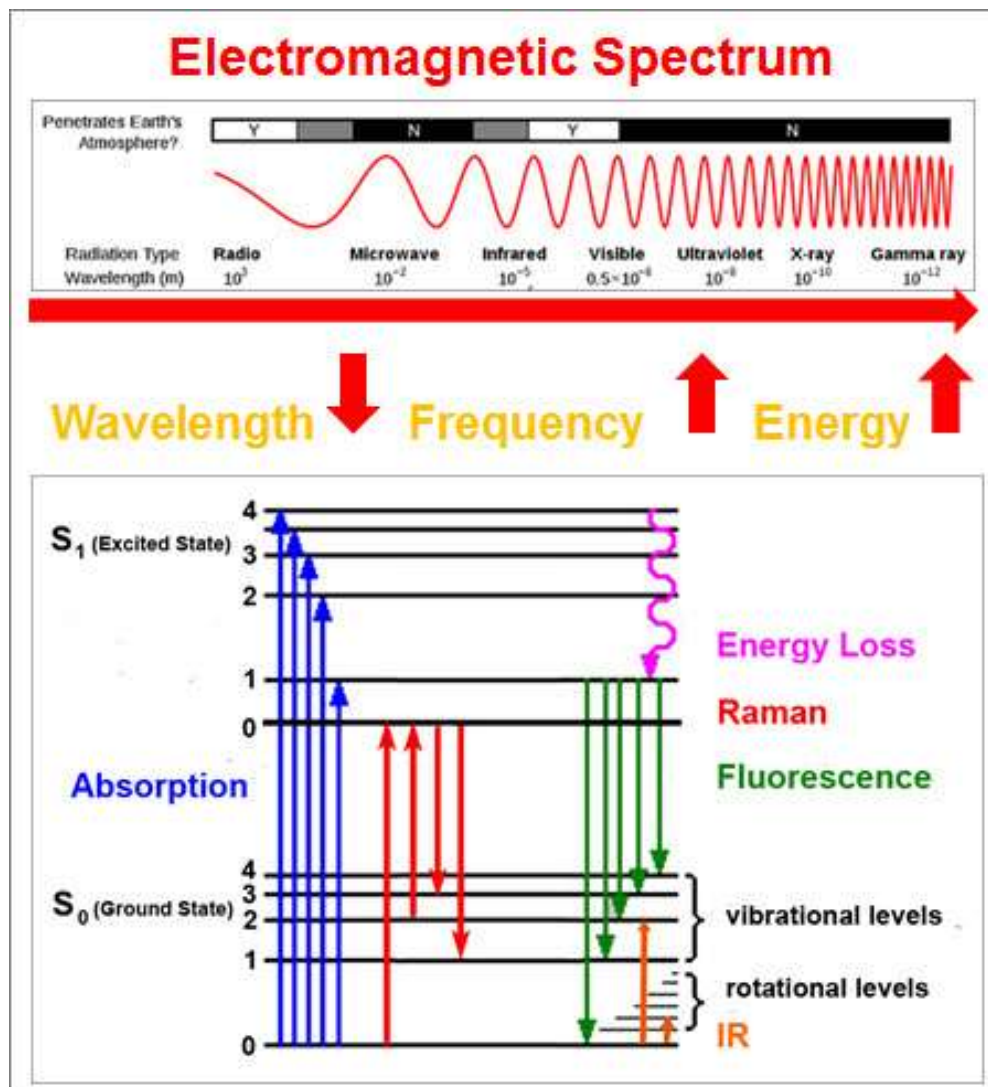


## Molecular Spectroscopy:

Comprehensive Approach to  
UV-Vis & Fluorescence –  
Navigating why we test, how  
we test, and how to choose a  
system

[dean.brown@agilent.com](mailto:dean.brown@agilent.com)

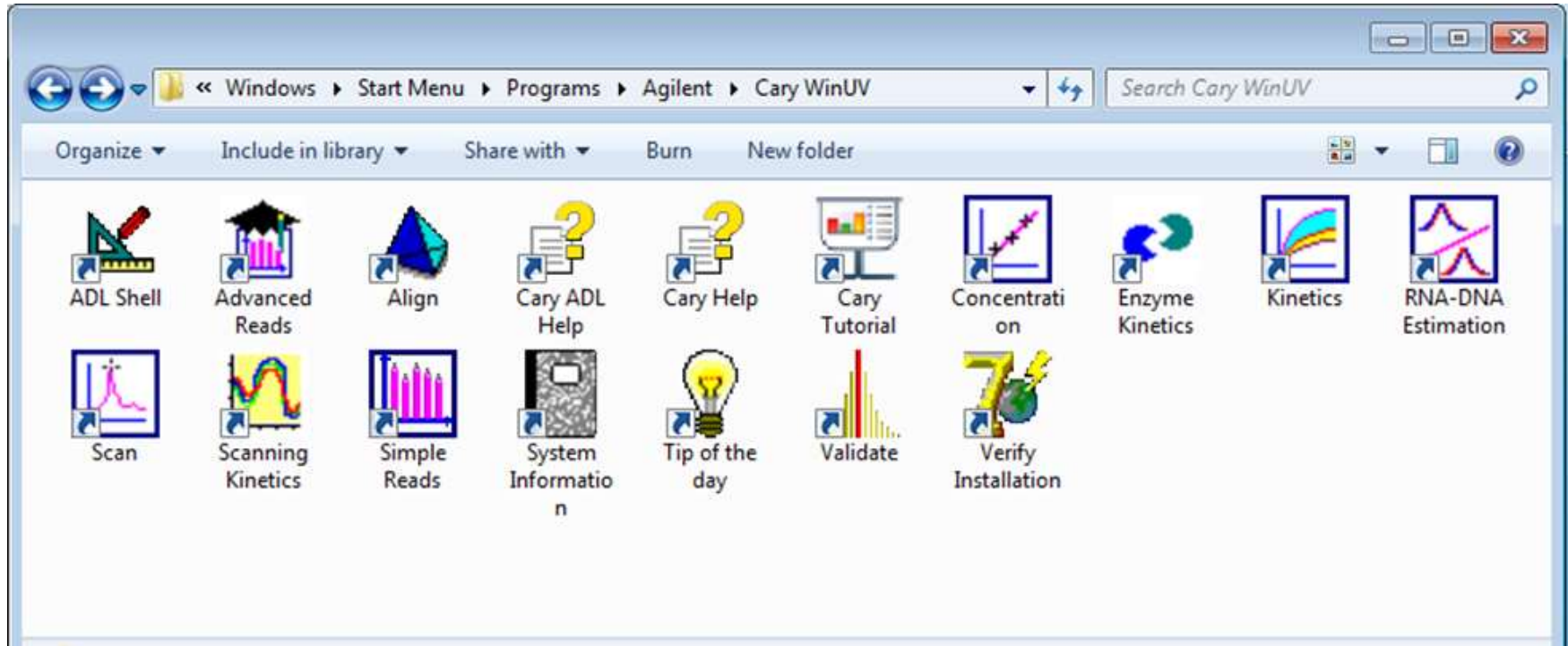
# The Absorption Process





## The Measurement

# Software – For Every Application



This is an individual preference as to whether one likes the software or not. It depends a lot on ease of use and the types of spectral functionality required. Some individuals prefer individual modules others prefer it all in one application.



# Questions to consider

What question am I trying to answer?

- What is it ?
- How much is there?

What level of uncertainty do I need to make a decision?

- +/- 5%, +/- 1%, +/- 0.1%

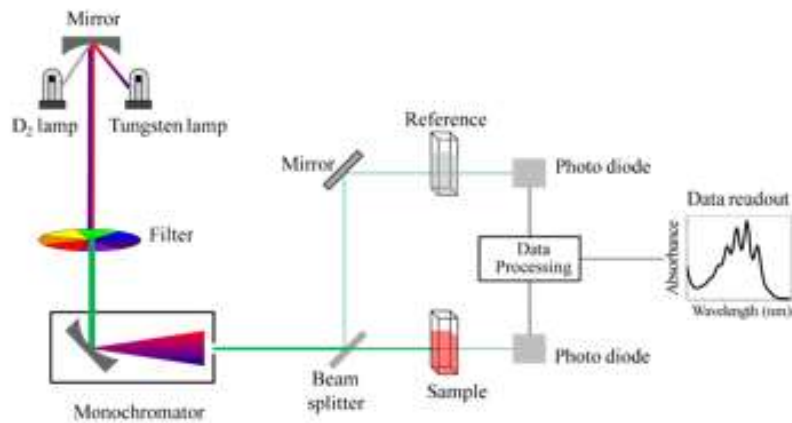
What limitations, restrictions, obstacles do I have to overcome?

- Concentration
- Physical form
- Amount of sample
- Etc.



**We are not at the mercy of the machines (Instruments)**



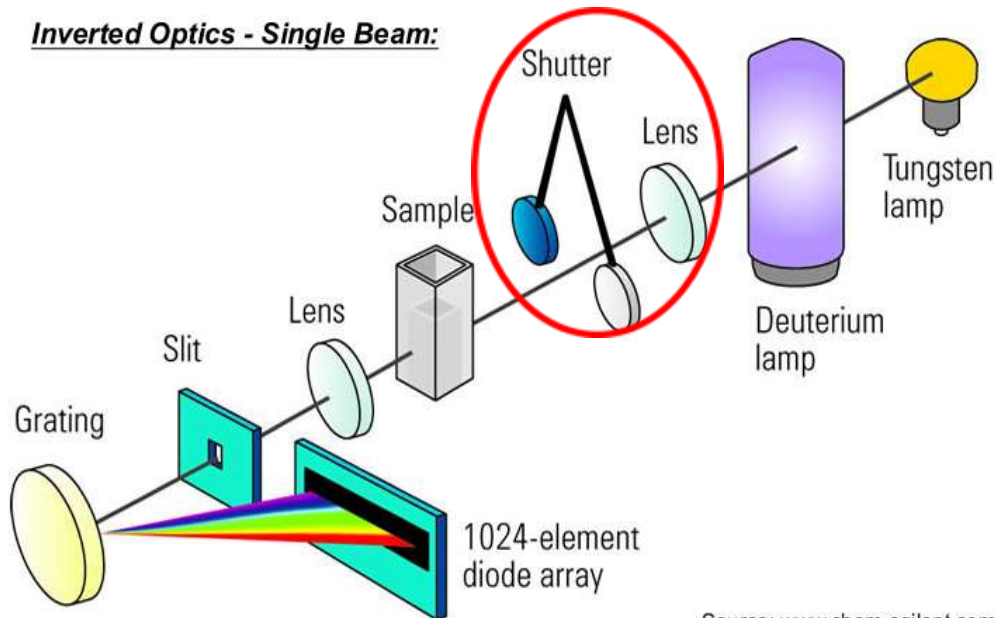


# Instrument Platforms

# Diode Array

- Simple optical and mechanical design ensures durability.
- Reliable. Only one moving part!
- Fast. Collect the full spectrum “instantly”
- Wavelength range 190 – 1100 nm
- Single Beam – Greater Drift than double beam instrument (4X)
- White light hit sample – Possible fluorescence interference

Inverted Optics - Single Beam:



Source: [www.chem.agilent.com](http://www.chem.agilent.com)



# Fast Kinetics – Simultaneous $\lambda$ Collection

## Challenges:

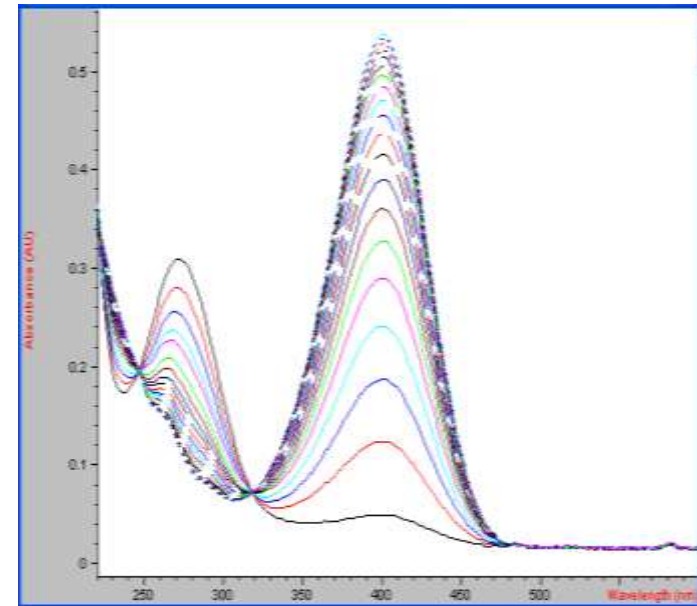
- Need to measure reactions that occur over seconds at more than one wavelength
- Need accurate temperature control to monitor biological reactions
- Use in multi-user laboratory. Must be easy to use and keep clean

## Features

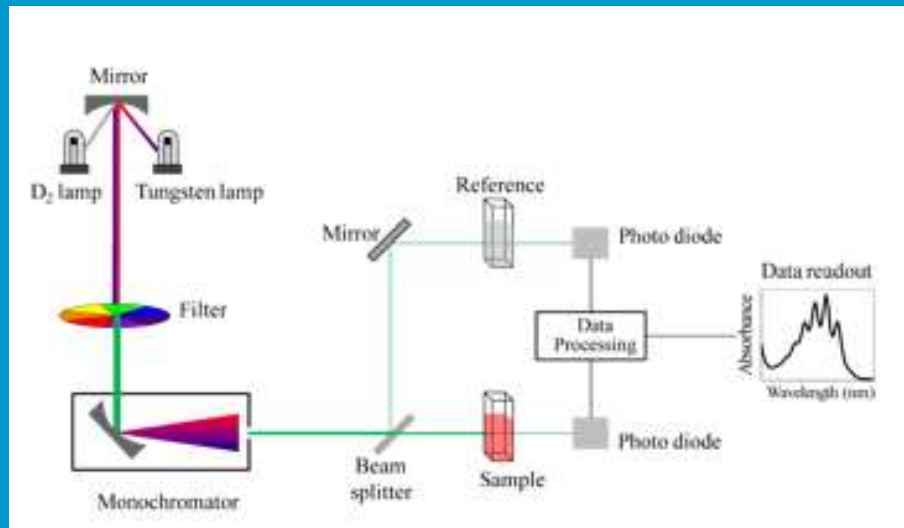
1. Photodiode array detector
2. No moving parts
3. Open sampling area
4. Easy to operate

## Benefits:

1. Fast: measure the full spectrum in a single step and in <1 second.
2. Accurate and reliable results
3. Monitor multiple wavelength kinetics reactions





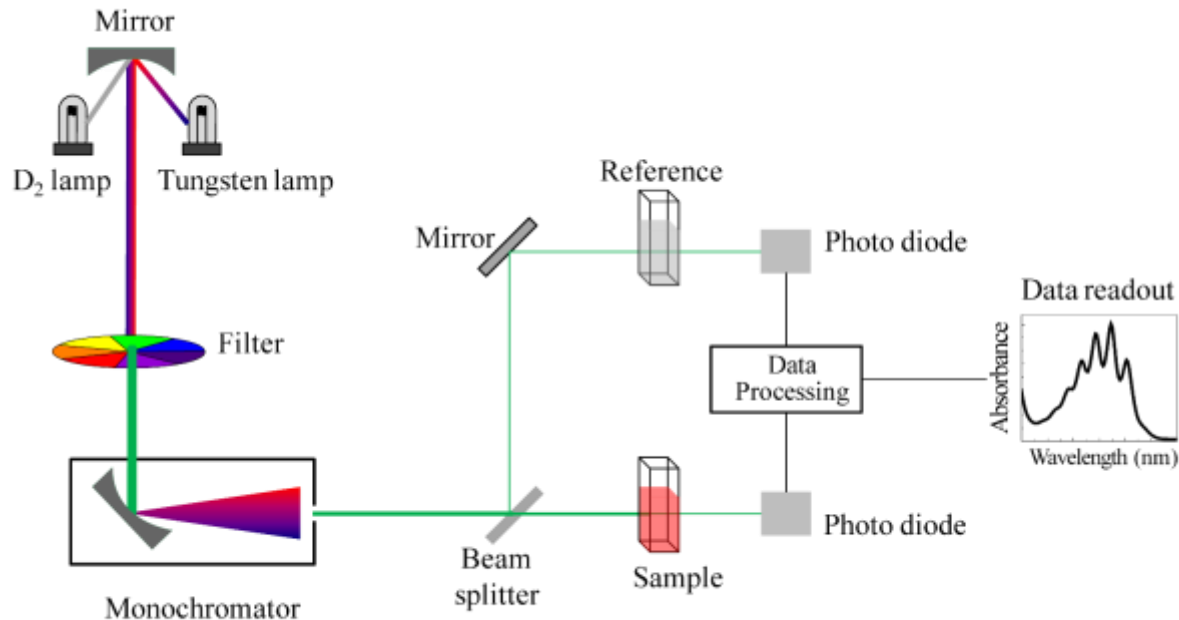


# Double Beam UV-Vis

## Single Monochrometer

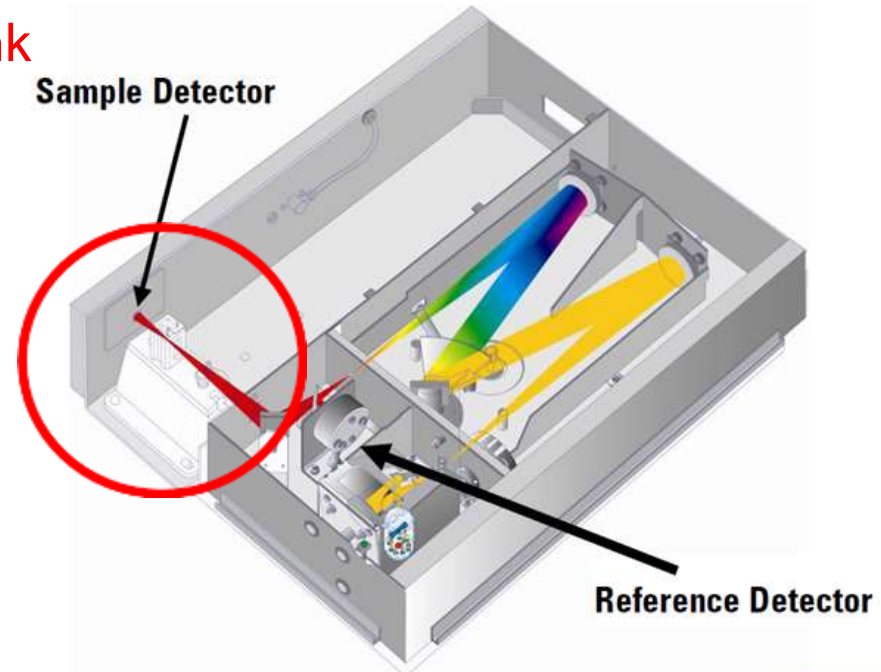
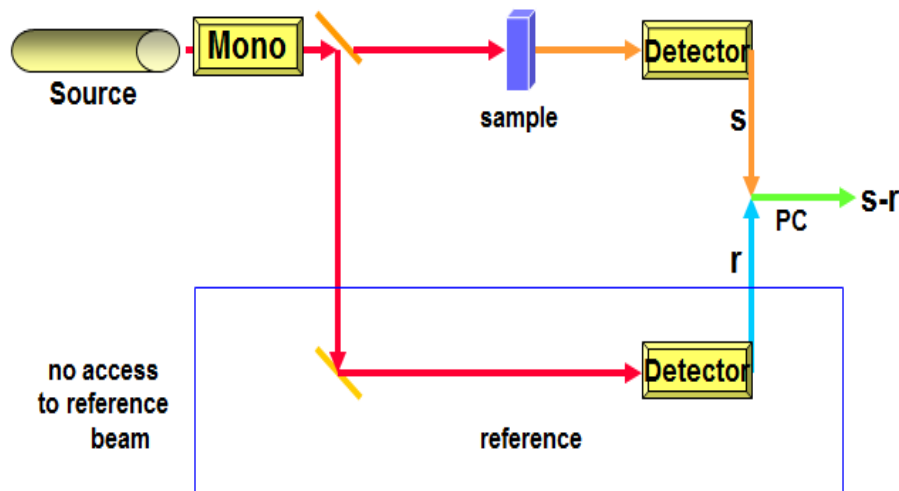
# Double Beam – Split Dual Beam

1. Split the beam so that 50% of the source energy goes to the reference side and the other to the sample side
2. Simultaneous sample and reference measurement
3. Simultaneous measure of sample and blank
4. Wavelength range 190 – 1100 nm
5. **Looses half source energy to sample**

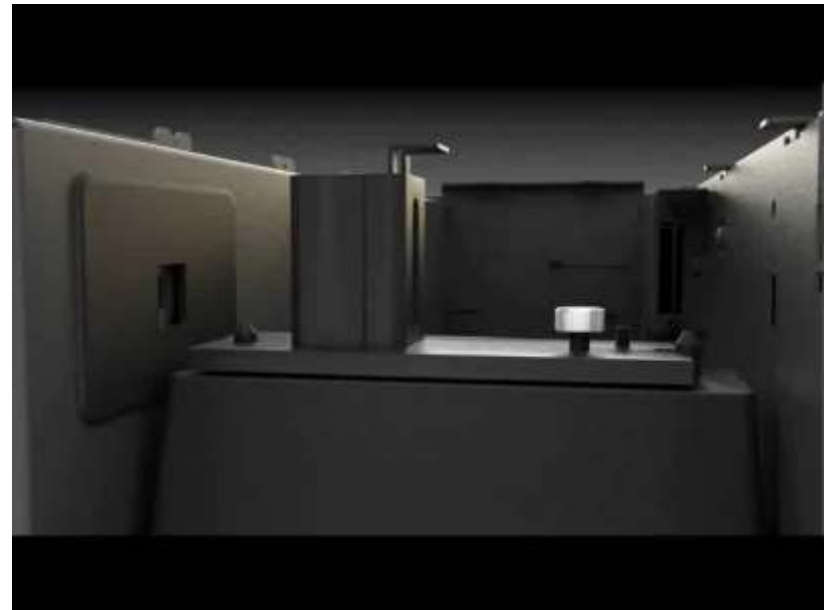
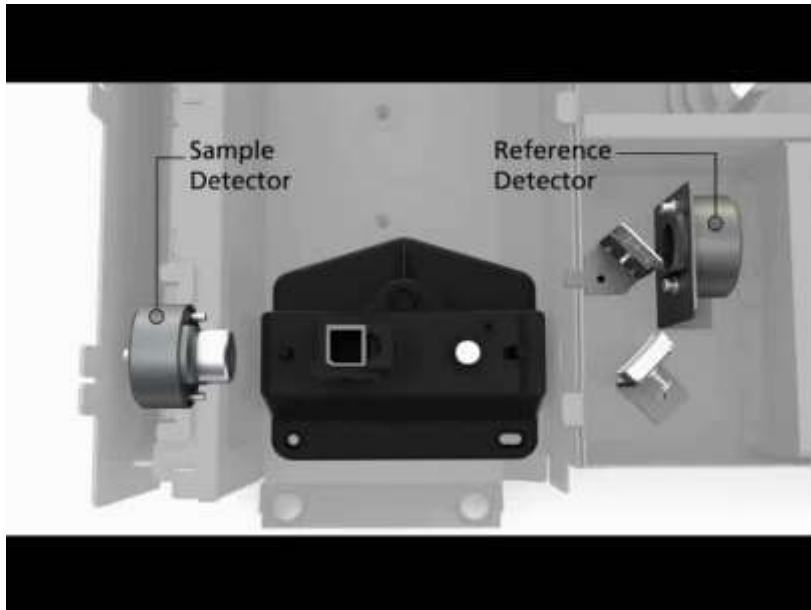


# Ratio Double Beam

1. Pulsed lamp – long life, reduced cost of owners
2. Only about 5% of the energy diverted to reference detector
3. Higher energy through-put to the sample – lower detection limit
4. Focused beam for small sample volumes and fiber optics
5. Room Light Immunity
6. Fast data collection (80 pts/sec)
7. Wavelength range 190 to 1100 nm
8. No reference beam to monitor blank



# Ratio Beam, Pulsed Lamp, Focused Beam



# Fiber optics measurements for small sample volumes



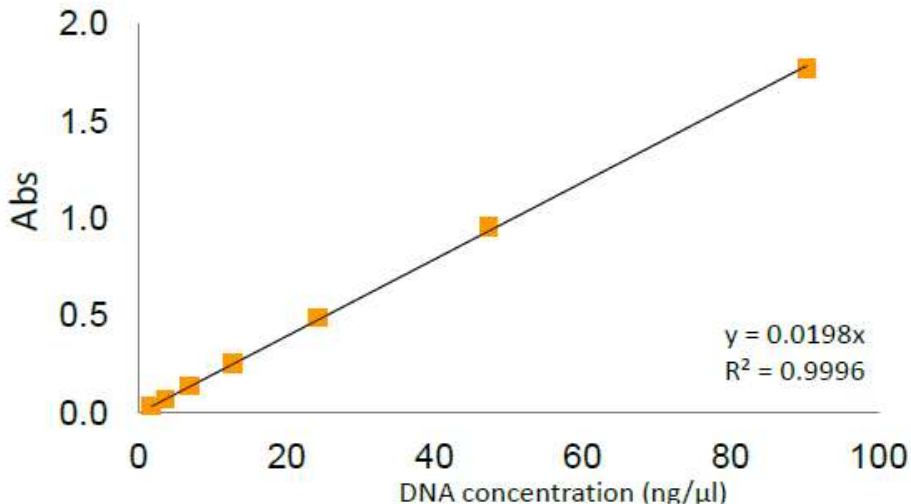
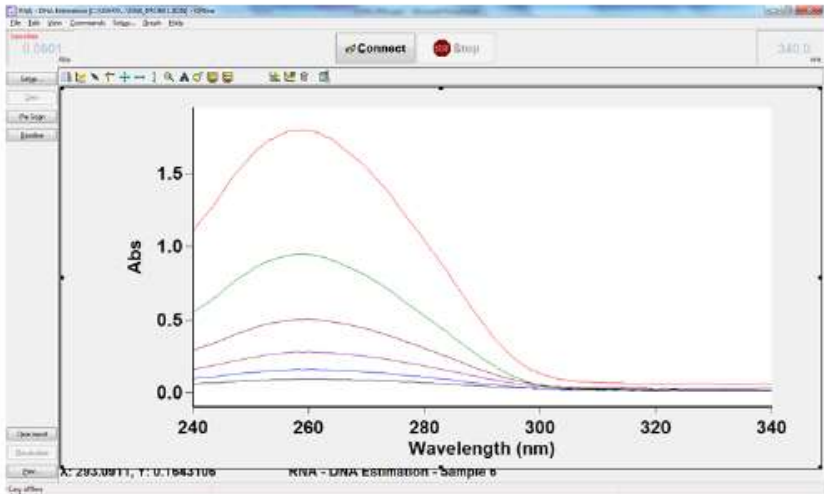
Improve workflow and minimize sample preparation

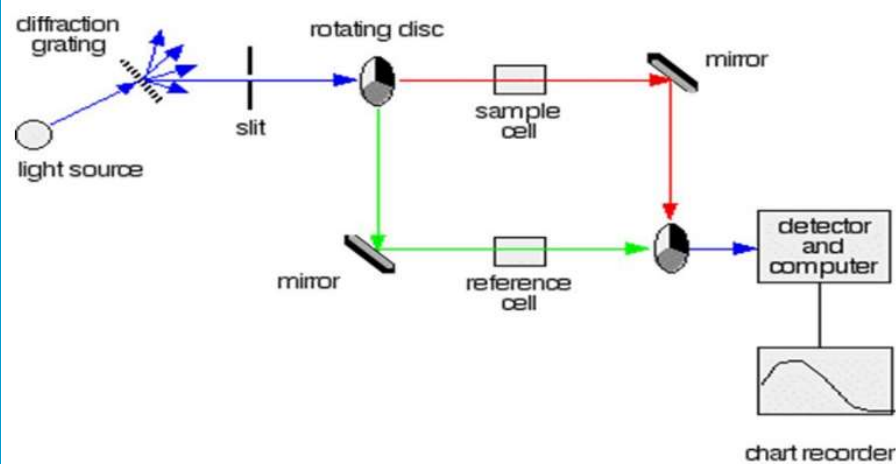


Measure cold samples directly from the refrigerator!



Remotely and safely measure hot, cold, radioactive noxious samples



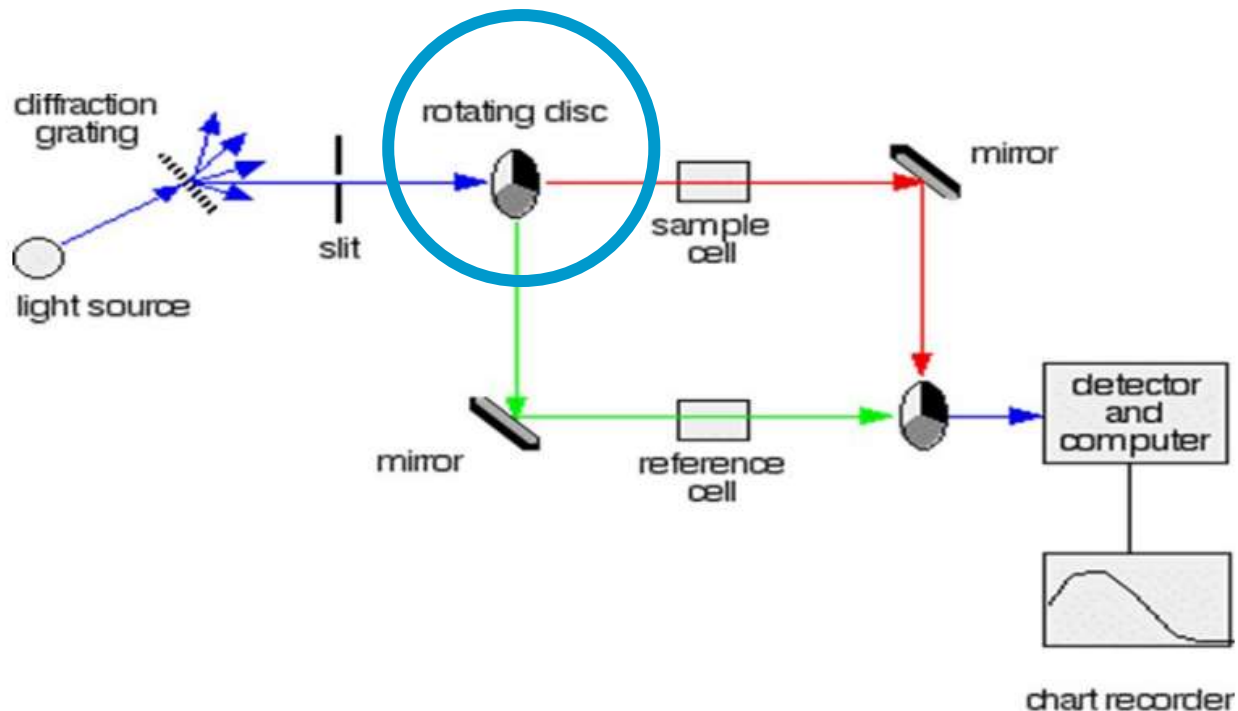


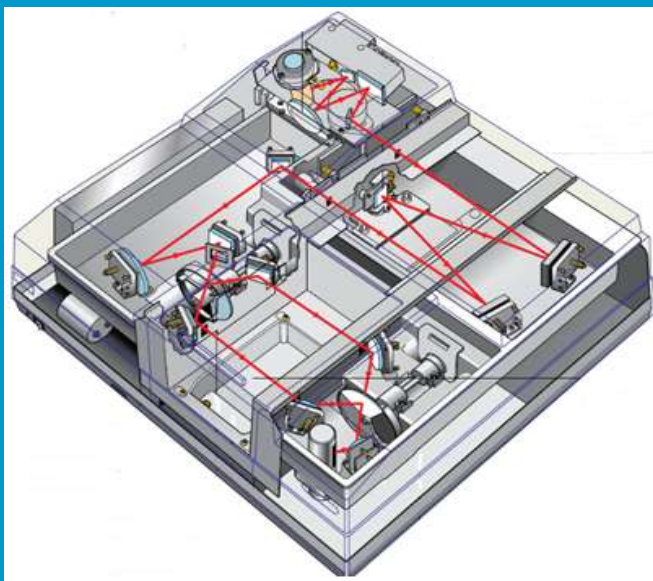
# Double Beam UV-Vis

## Chopper System

# Double Beam – Time Shared

1. Uses a chopper to alternate the beam between sample and reference
2. Most have higher ordinate capabilities due to reduced stray light due and PMT detector
3. Usually have variable slits when better resolution is required
4. Wavelength range 190 – 900 nm





**Double Beam UV-Vis**

**Pre-Grating Monochromator**



# Transmitted light with Increasing Absorbance

<b>Abs</b>	<b>%T</b>
<b>0</b>	<b>100</b>
<b>1</b>	<b>10</b>
<b>2</b>	<b>1</b>
<b>3</b>	<b>0.1</b>
<b>4</b>	<b>0.01</b>

The amount of light transmitted with increasing absorbance.

# Effect of Stray Light Photometric Accuracy

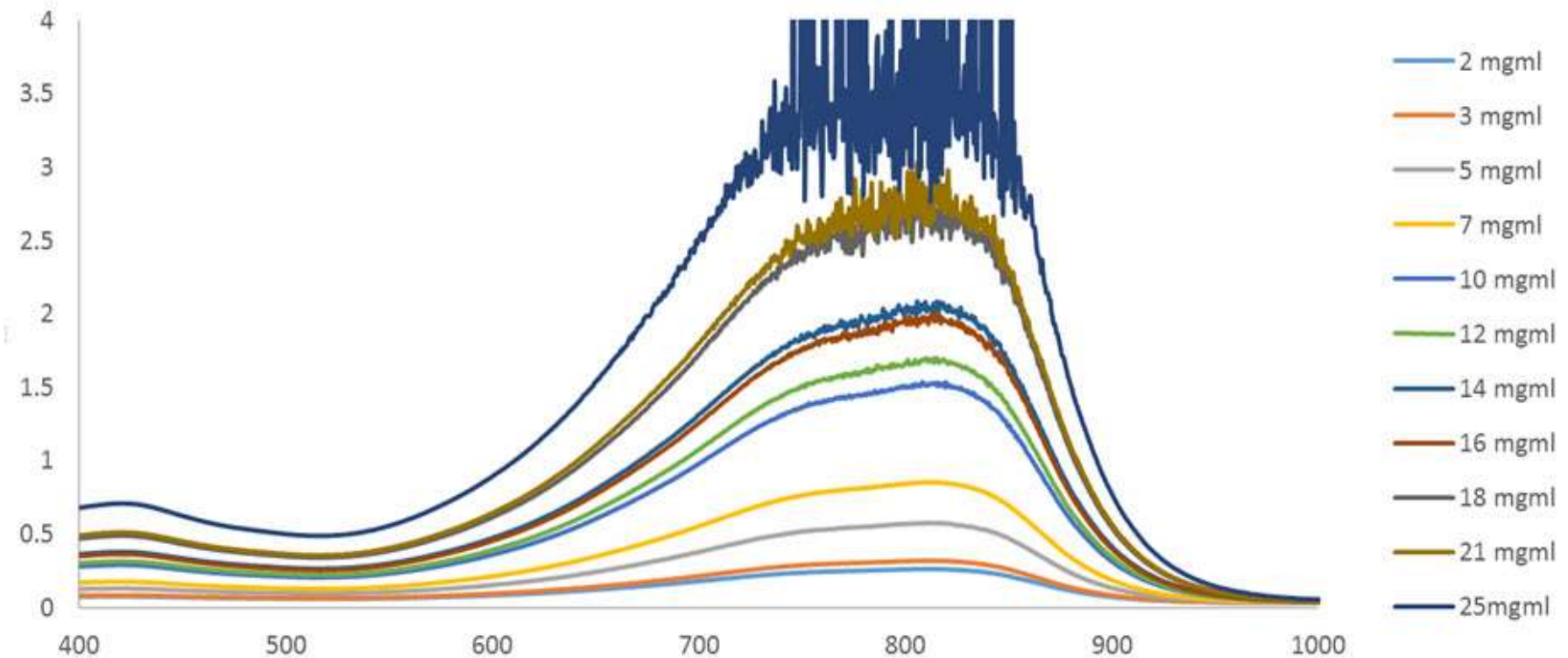
Abs.	Absorbance Observed							
	Level of Stray							
	DM		DGM		SM		SM	
Abs.	.0001	%E	.0005	%E	0.02	%E	0.05	%E
1.0	1.000	.004	1.000	0.002	0.999	0.078	0.998	0.194
2.0	2.000	.002	1.997	0.017	1.991	0.425	1.979	1.048
3.0	2.999	.010	2.997	0.072	2.920	2.636	2.824	5.862
4.0	3.995	.110	3.978	0.529	3.522	11.92	3.222	19.44
5.0	4.958	.827	4.823	3.521	3.677	26.44	3.292	34.14

Where DM=Double Monochromator, DGM=Double Grating Monochromator, SM=Single Monochromator



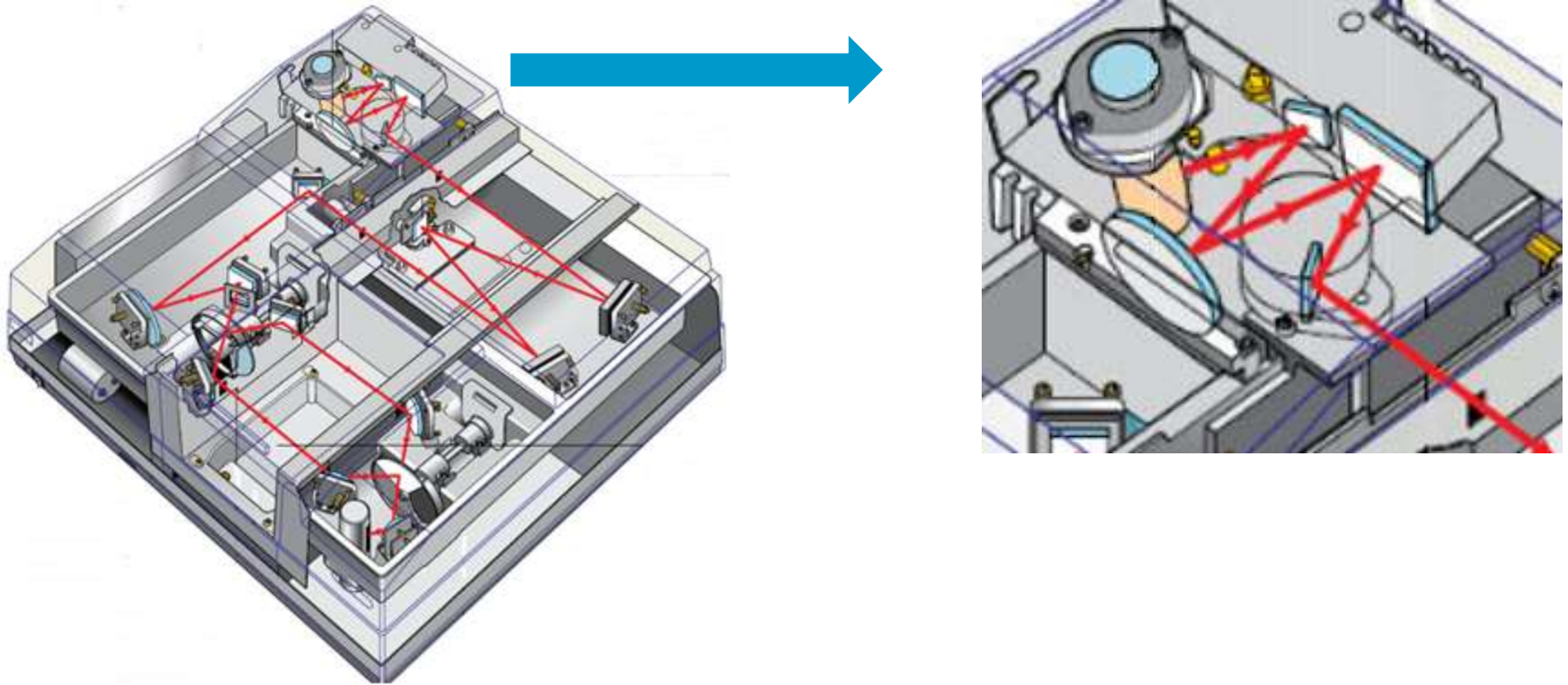
# Exceeding the Linear Range of the Instrument

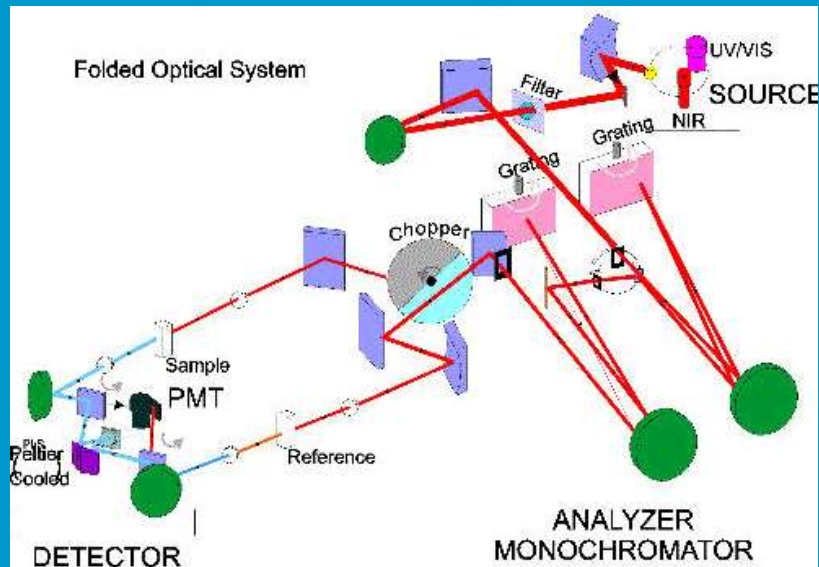
The “grass like” fringe at peak indicates the linear range of the instrument has been exceeded.



# Pre-Grating Double Beam

Reduces the stray light of the instrument and eliminates the filter wheel. However, system needs a more sensitive detector (PMT) to realize the benefit of the reduced stray light and higher ordinate readings (5A).



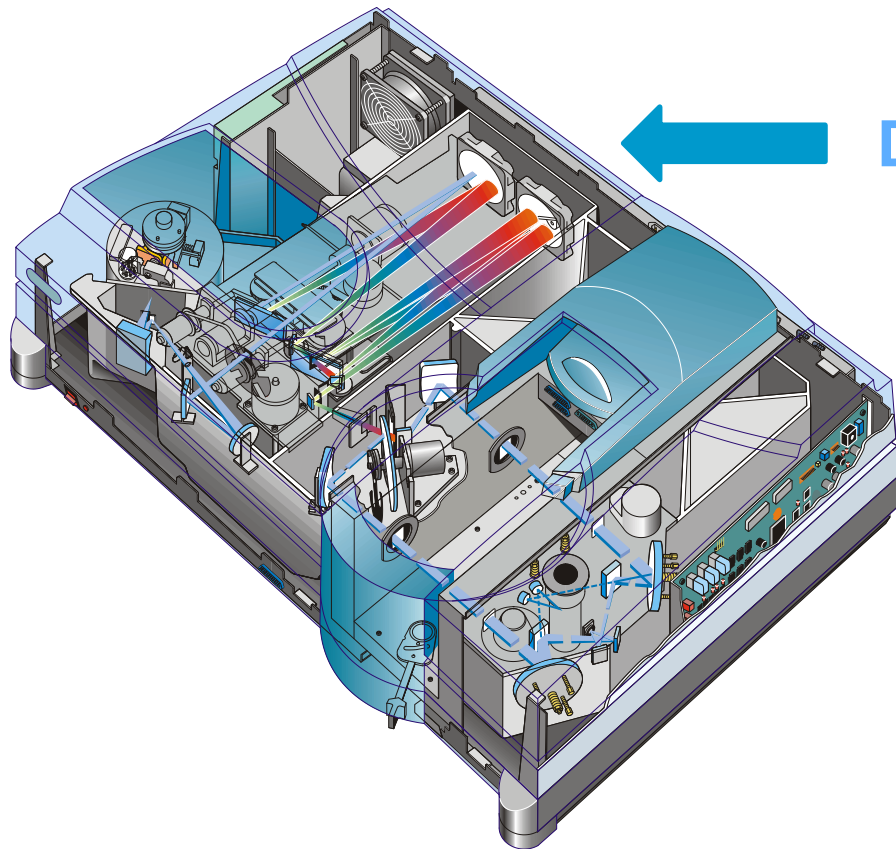


# Double Beam UV-Vis

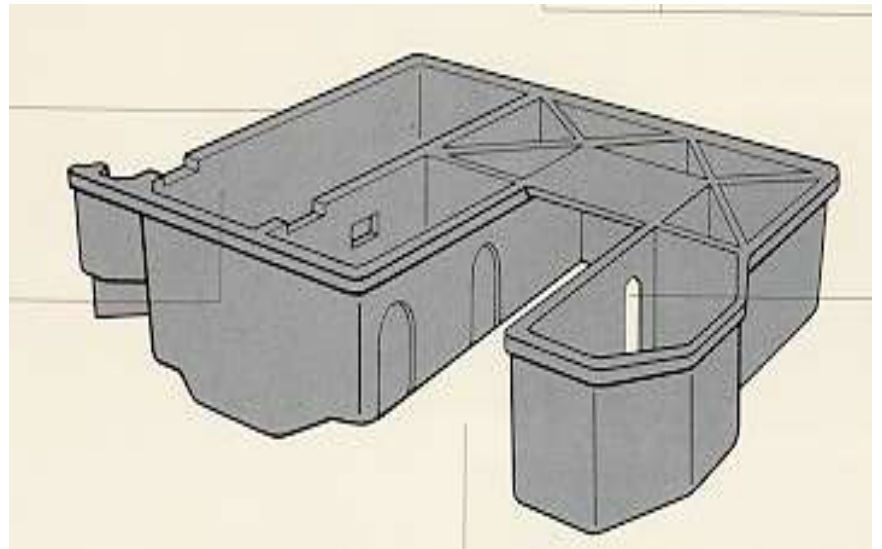
## Double Monochromator

# Double Beam Double Monochromator

1. Wavelength range 175 – 3300 nm
2. Absorbance range 8A



**Double Monochromator**



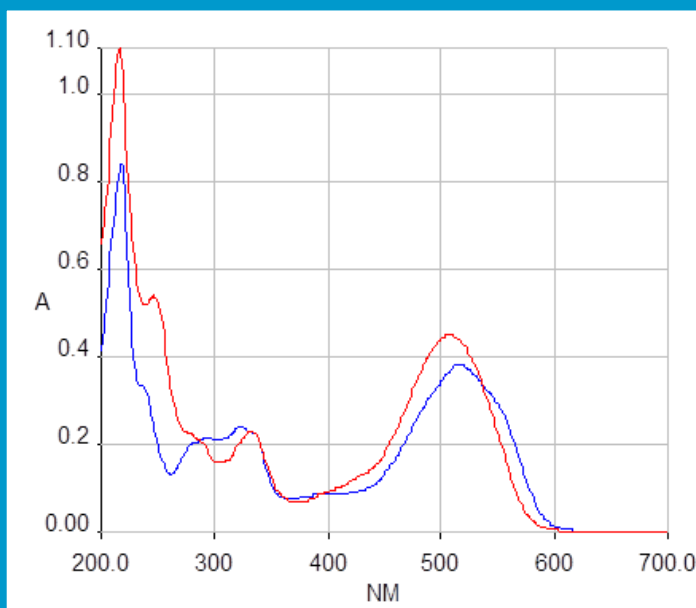
Double monochromator and optical Isolation reduces stray light







Agilent Technologies

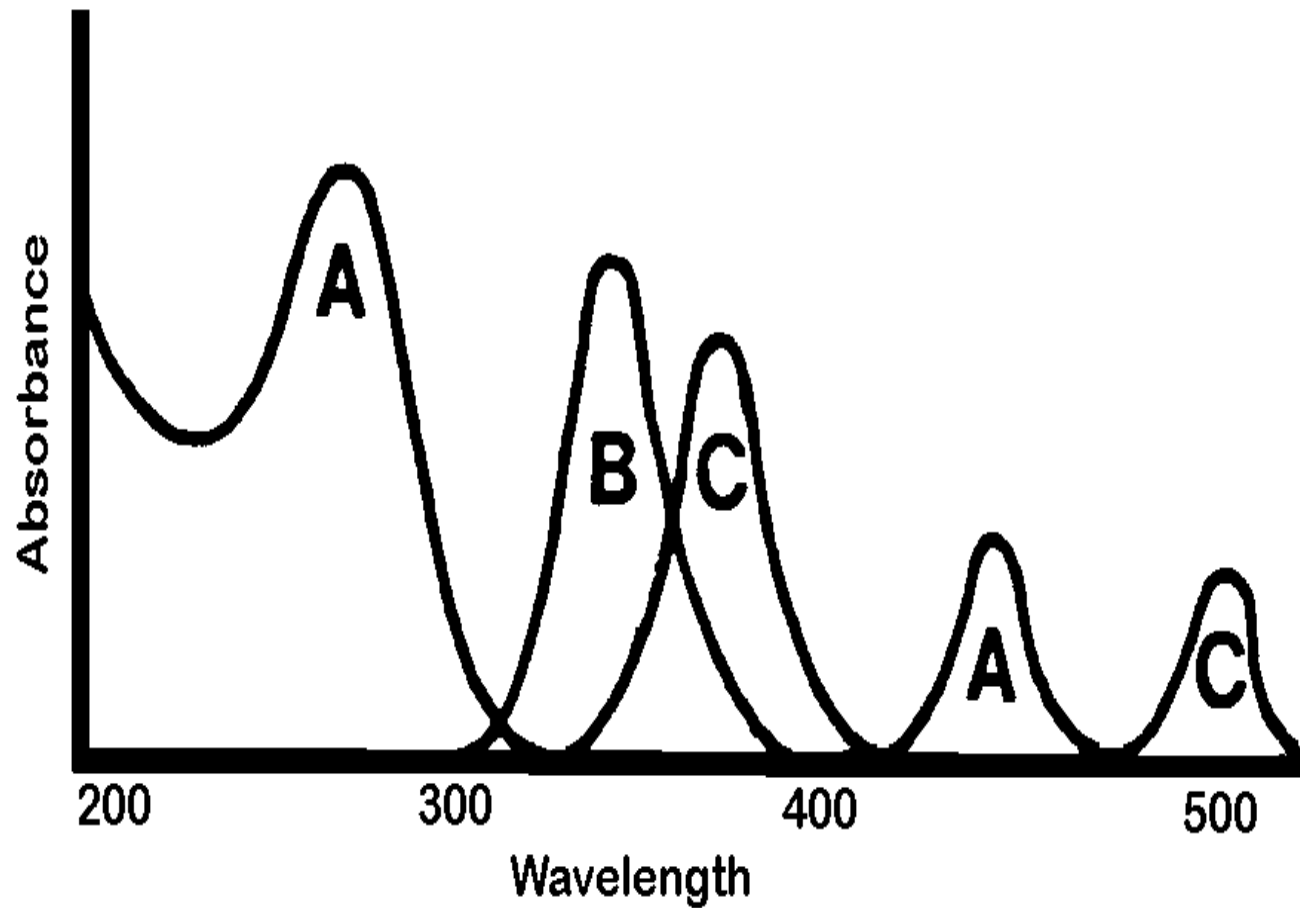


## Qualitative Analysis

### Trading Rules of Spectroscopy

Applies to Uv/Vis and Fluorescence

# Selection of Optimal Wavelength



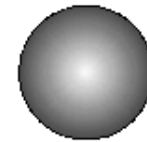
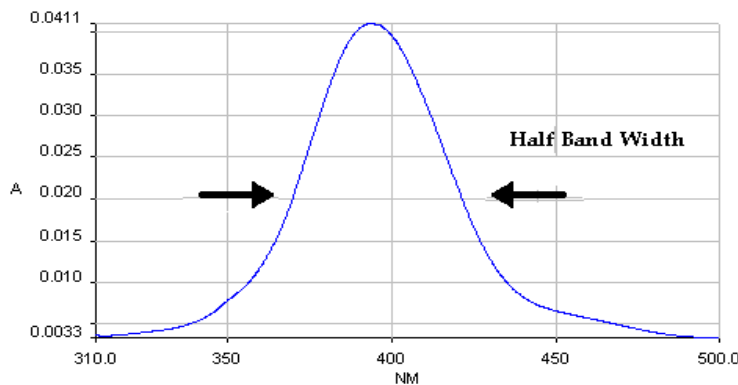


# Resolution

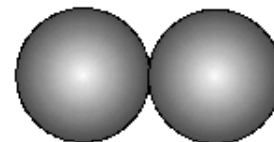
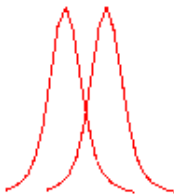
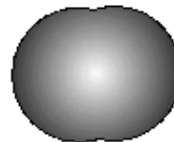
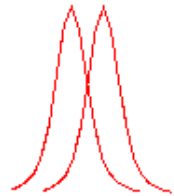
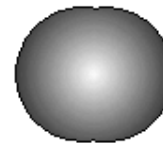
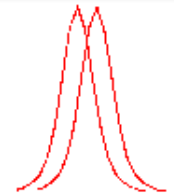
**Resolution** - Ability to detect the presence of two spectral features next to one another.

For qualitative analysis you want to resolve the spectral peaks as much as possible to give the best spectral characterization.

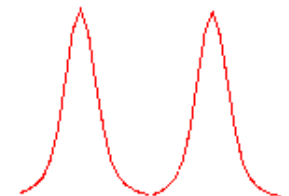
For quantitative you want to resolve the spectral peaks as much as possible to eliminate spectral overlap.



Unresolved



Resolved

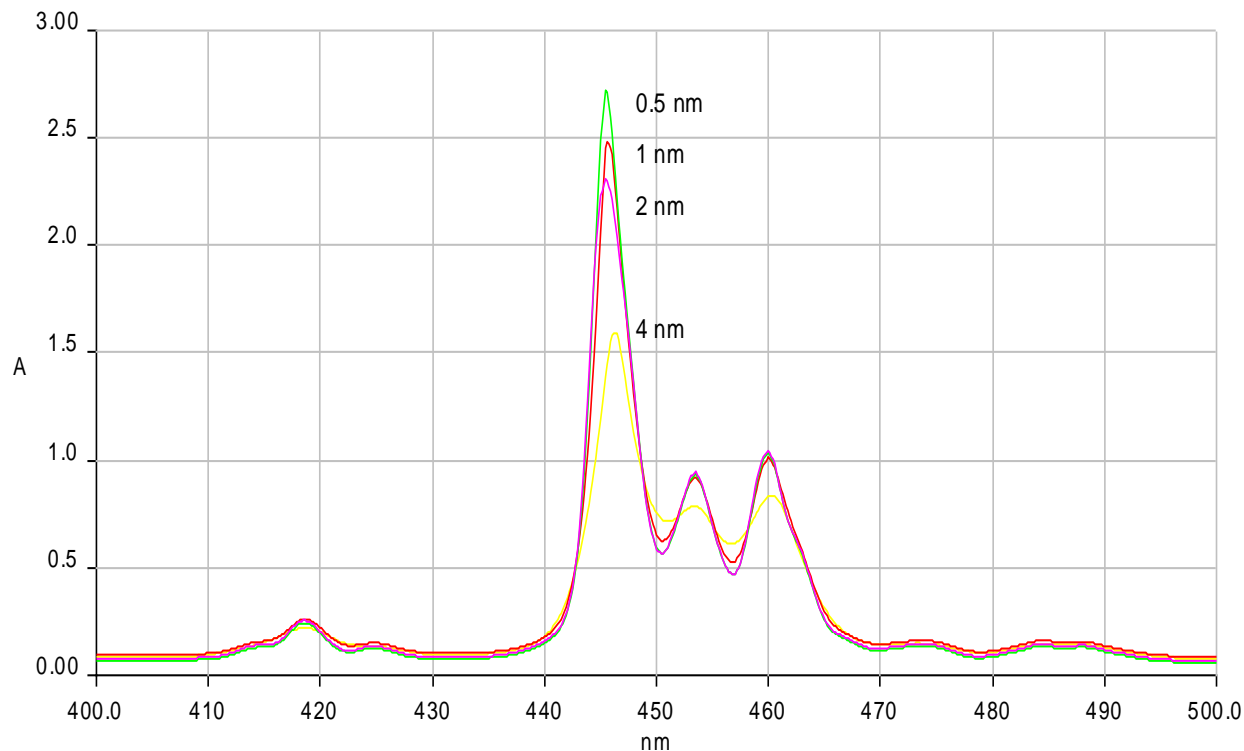


The slit should be 1/10 the half band width of the spectral peak



# Effect of Slit Width on Spectral Resolution

## Holmium Oxide Spectra

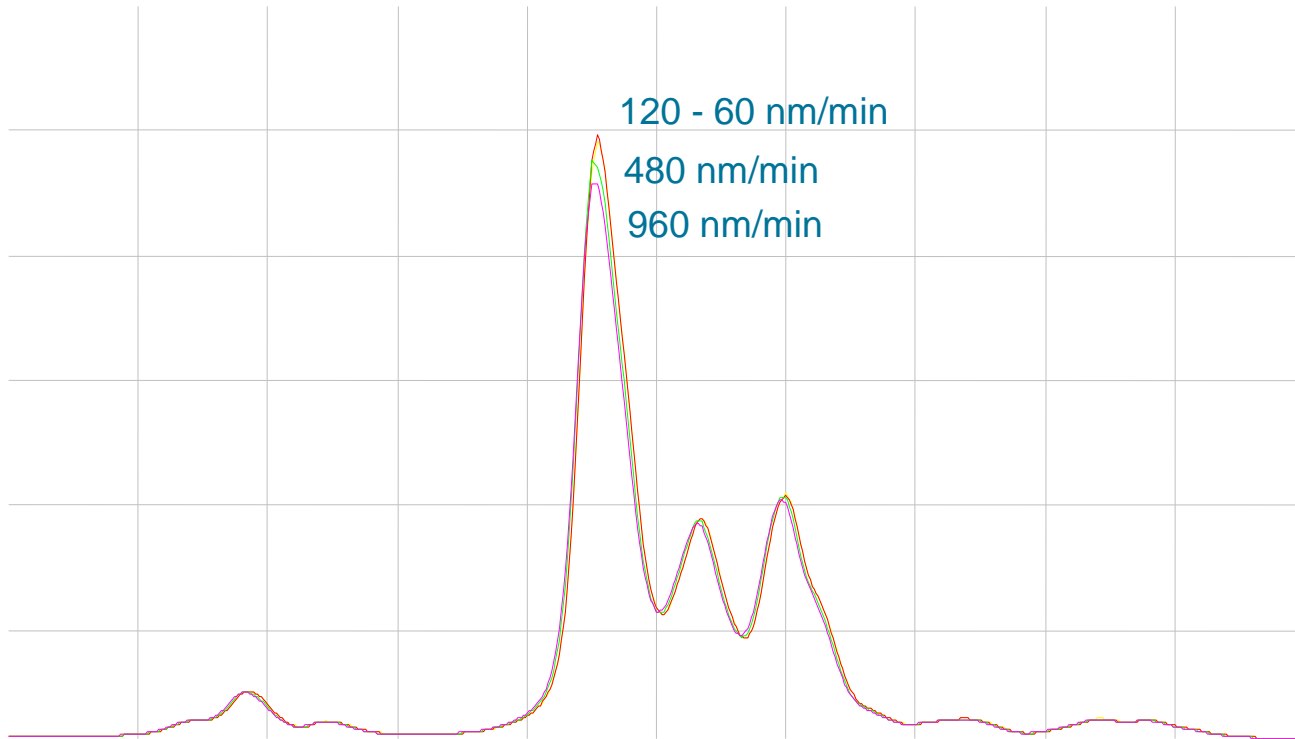


Insufficient resolution can cause a reduction of the analytical peak and a spectral shift



# Effect of Scan Speed on Spectral Peak Shape

## Holmium Oxide Spectra

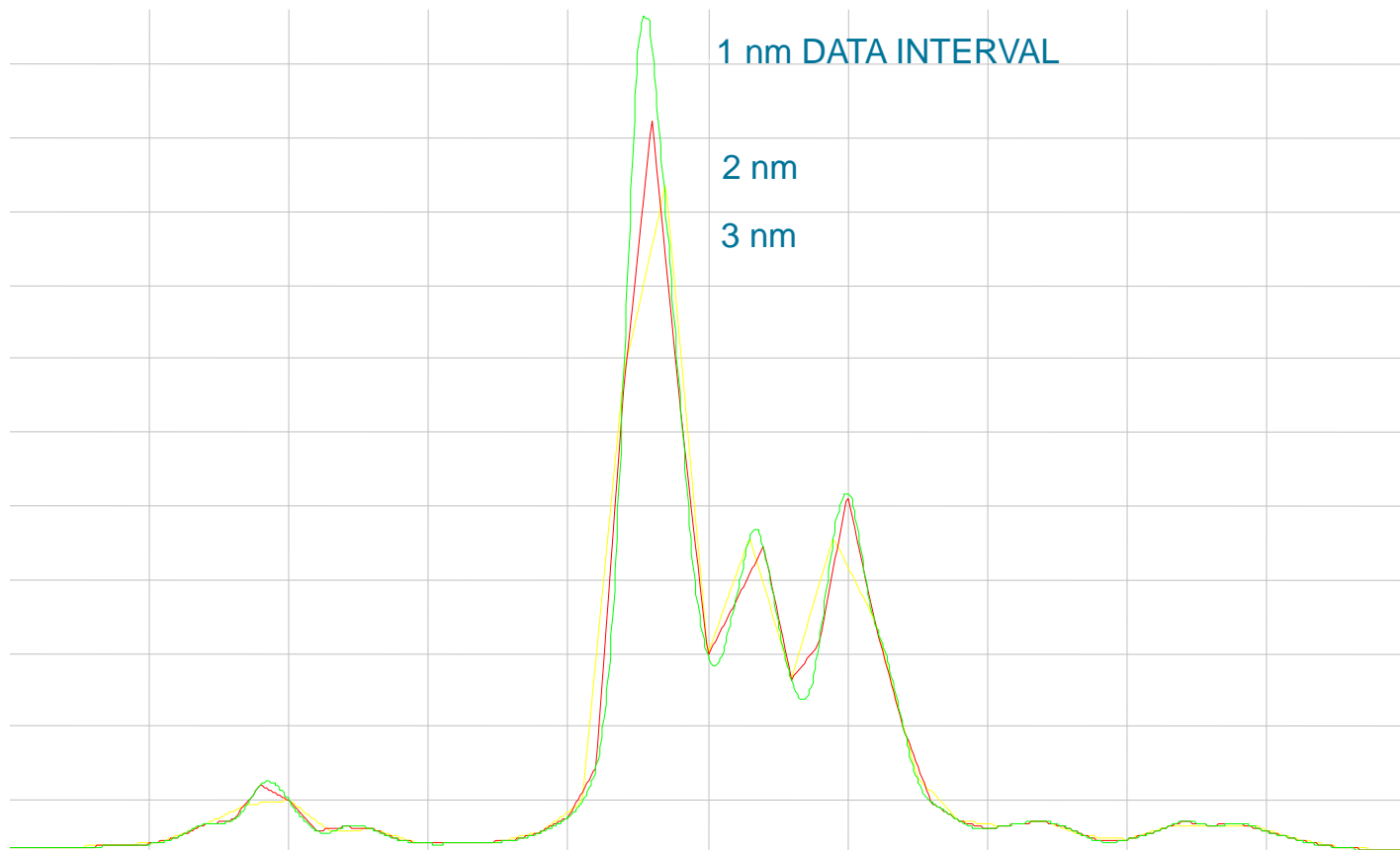


The scan speed and data interval are linked. Faster scan speeds limit the data interval that can be taken, affecting resolution and signal/noise since the instrument is measuring the signal for a shorter period of time.



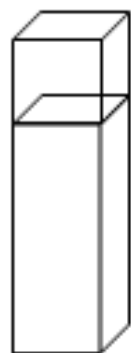
# Data Point Resolution

## Holmium Oxide Spectra

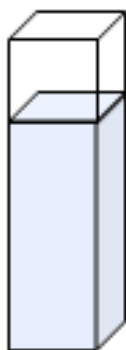




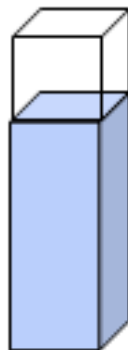
Agilent Technologies



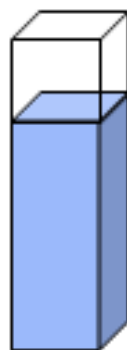
Blank



10 ppm



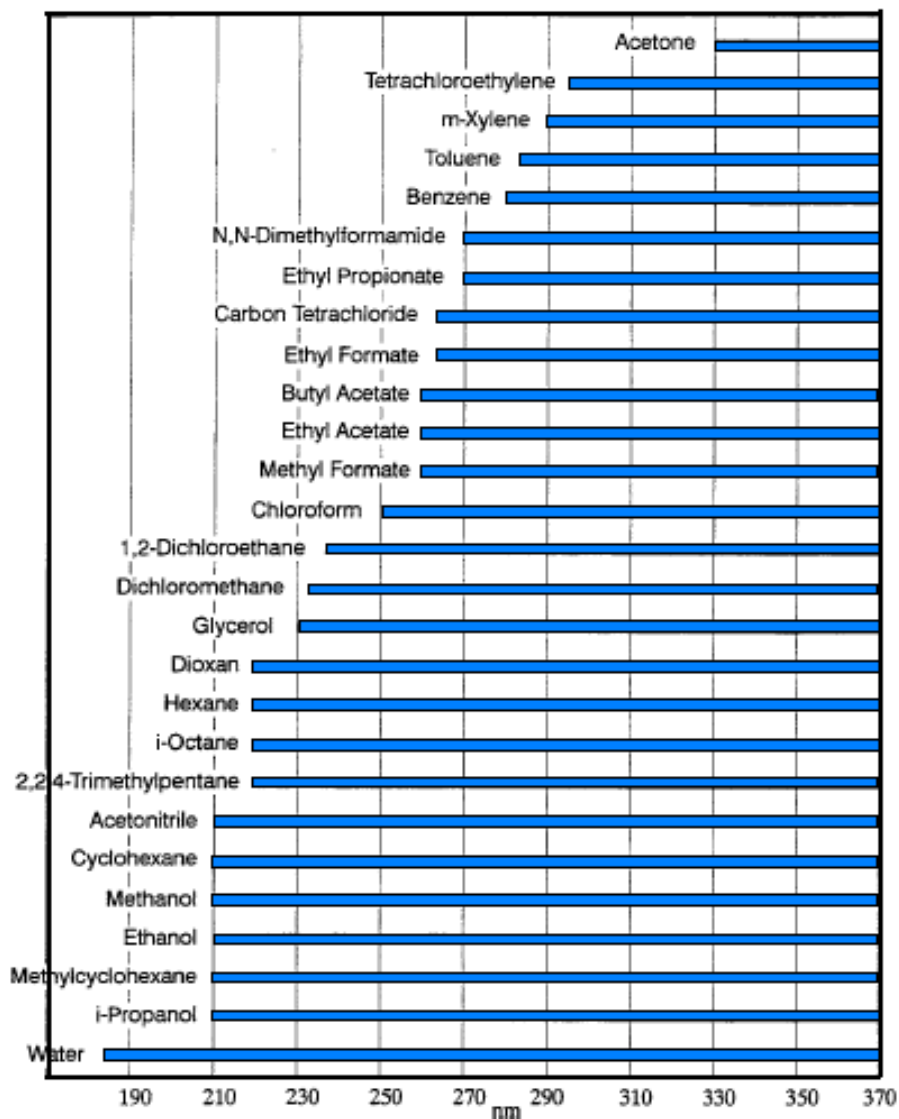
50 ppm



100 ppm

## Sample Handling Considerations

# Common Solvent Cut-offs (based on a 10mm cuvette filled with solvent reading 1A/ 10%T)

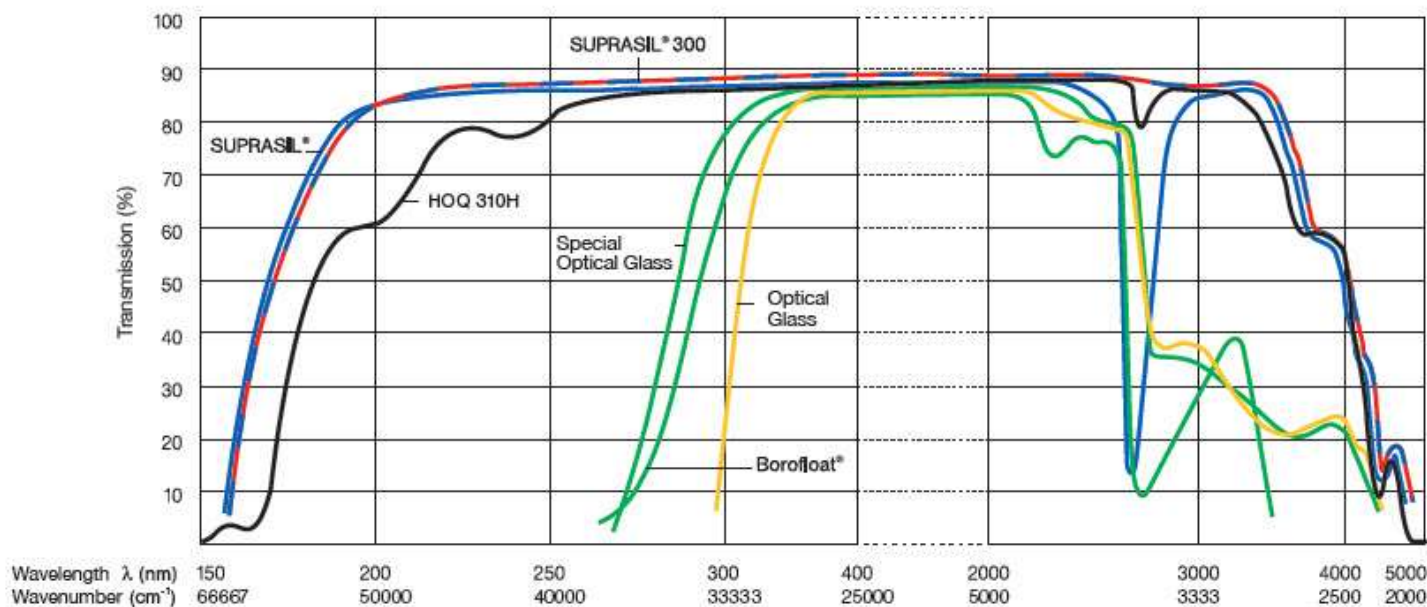


# Cuvette Transmission Characteristics

Material	Trademarks	Wavelength
Optical glass	OG	360 nm – 2500 nm
Borofloat®	BF	330 nm – 2500 nm
Special optical glass	OS	320 nm – 2500 nm
HOQ 310H	UV	260 nm – 2500 nm
Quartz SUPRASIL®	QS	200 nm – 2500 nm
Quartz SUPRASIL® 300	QX	200 nm – 3500 nm



Transmission of empty cells made of different materials



# Sample Handling & Cuvettes

## Materials

Quartz sample cells (cuvettes) or sample cells with **quartz face plates** are required if you want to use the full **190 to 1100 nm** wavelength range of your spectrophotometer. If you plan on working only in the visible and/or short-wave near-infrared range of **350 to 1100 nm**, you can use good quality **glass cells**.



## Aperture cuvettes

When using aperture cuvettes it is important that the walls of the cell are black so that light passes only through the sample itself and light does not pass through the walls of the cuvette.



## Disposable plastic

The quality of these cells varies and should not be used below ~ 325 nm. For better uniformity, some manufacturers place an arrow on the front of the cell so they are measured with the same orientation.





# Sample Handling & Cuvettes

## Flow Cells

Using a flow cell eliminates the necessity of removing the cell between sample measurement. The design of the flow cell should minimize entrapment of bubbles and flow channeling to provide the most reliable results. Small volume and ease of cleaning are also desirable features



## Cleanliness

The oils in fingerprints are significant absorbers in the UV region and, if left on optical surfaces, can cause erroneous results. Wipe off all fingerprints and contaminants before using a sample cell. Lens tissues for glasses or other uses often contain detergents or lubricants which can affect your measurements. Once a blank measurement has been made, however, do not clean the faces of your cell unless you plan on taking a new blank measurement.



# Sample Handling & Cuvettes

## Working with Cells

A sample cell should be rinsed three to five times with your intended solvent before you fill it with the pure solvent that will be used in the measurement. Turning the cell upside down on a small stack of absorbent tissues will help remove any residual solvent. This treatment will minimize contamination from previous experiments.

**Always install a cell so that it faces the same direction to minimize problems with cell non-uniformity.**

## Stirring and Temperature Control

Solution uniformity can be a problem, especially for viscous solutions. There are cases where, due to convection induced gradients, rapid absorbance changes may invalidate data. To minimize convection effects keep the temperature of your sample the same as the cell holder or environmental temperature.



# Sample Handling & Cuvettes

## Mixing

A similar effect can occur in cases of incomplete mixing. This is especially true where the specific gravities or miscibilities of the solvent and analyte are quite different. Again, stirring is a way to prevent this kind of problem.

## Photodegradation

In an unstirred cell, it is sometimes possible to observe local photodegradation of sensitive analytes. Because the actual volume of the sample in the light path is very small, stirring the sample will reduce the time any given analyte molecule is in the light path. This minimizes the photodegradation and increases uniformity.



# Beer-Lambert Law

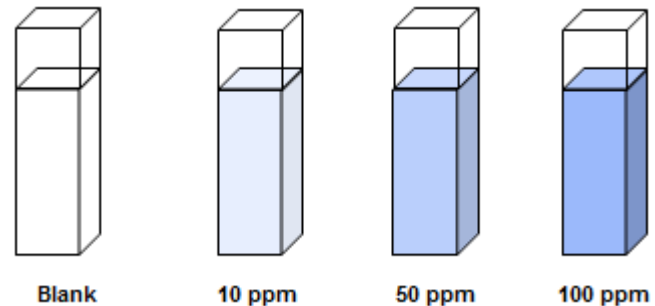
What parameter can we change to effect better quantitative analysis?

$$\text{Absorbance} = \epsilon c \ell$$

$\epsilon$  = Molar Absorptivity

$c$  = Concentration  
(moles/L)

$\ell$  = Path length (cm)



# What can we do?

You are presented with the following sample situations:

1. Small sample volume (1 - 2 Abs, ~ 0.5 ml )



2. High concentration sample (~ 5 Abs, > 10 ml)



# What can we do?

## 3. Multiple Samples ( > 100, Abs 0.1 to 2, < 2 ml)



176.200 10 mm  
176.200 50 mm  
176.202 10 mm



176.210 10 mm  
176.210 50 mm  
176.211 10 mm



176.210 10 mm  
176.210 50 mm  
176.211 10 mm  
176.212 10 mm



## 4. Low concentration sample (< 0.01 Abs, > 10 ml)



120  
1 mm



120  
10 mm



120  
50 mm



Long Pathlength Cells up to 100 mm

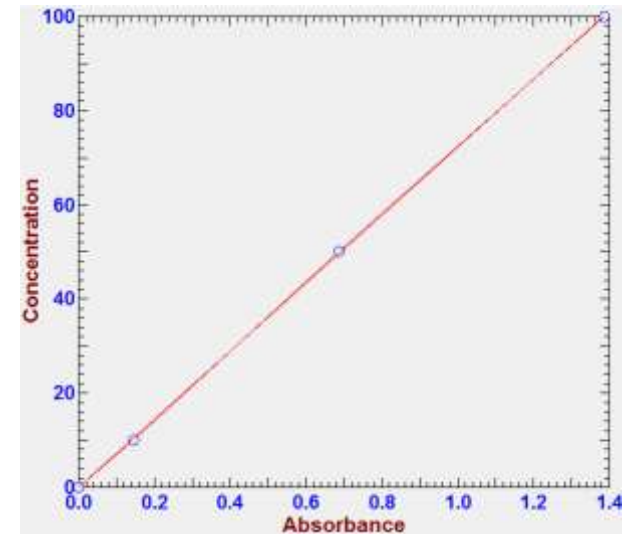
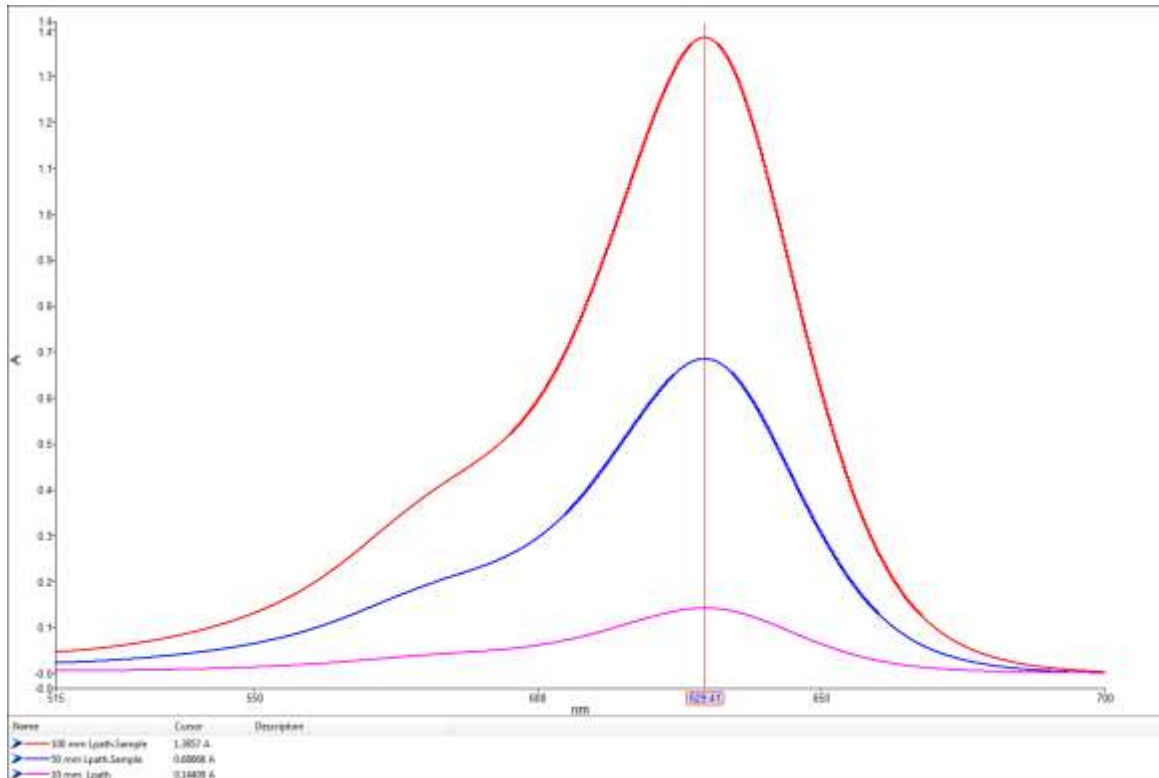
Rectangular Long Pathlength Cell



Agilent Technologies

# Effect of Pathlength on Absorbance – Long Path Cells

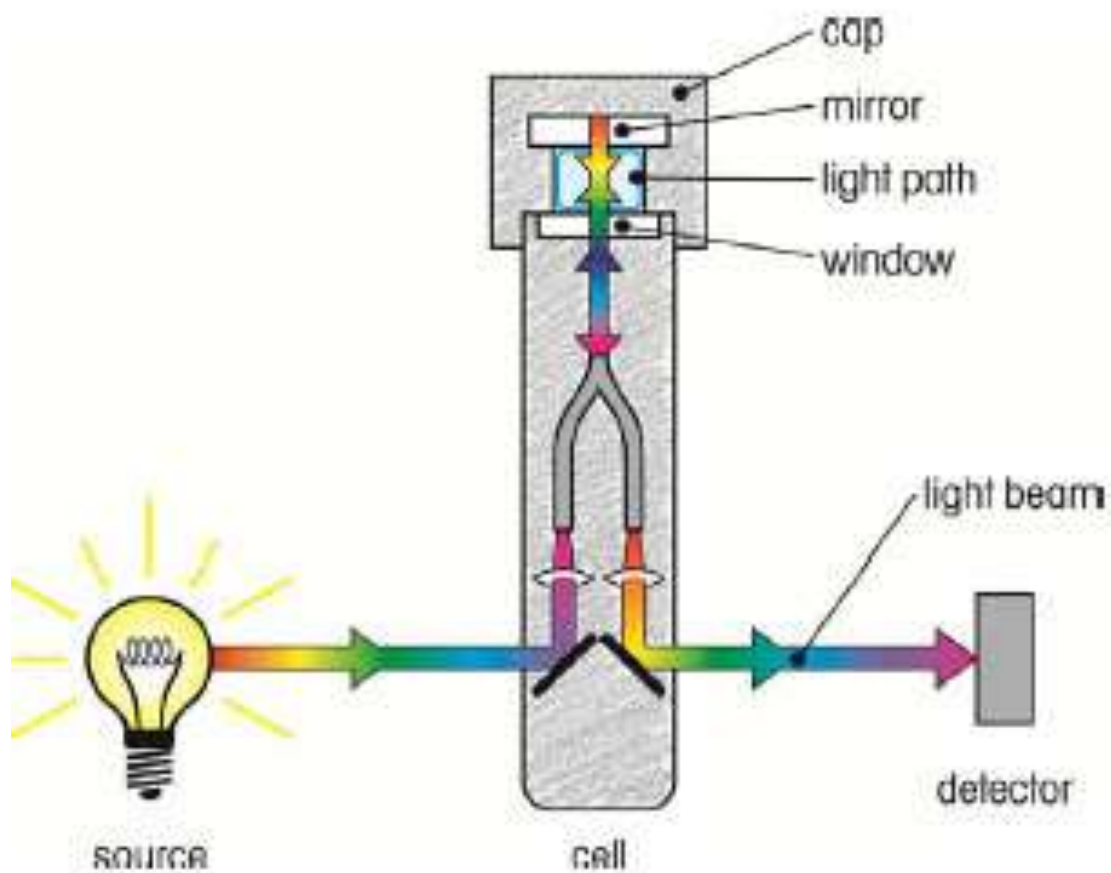
➤ 100 mm Lpath.Sample	1.3857 A
➤ 50 mm Lpath.Sample	0.68666 A
➤ 10 mm Lpath	0.14409 A



R: 0.999974395     $\Sigma \delta^2$ : 0.31749848



# Hellma Tray Cell for Nucleic Acid Analysis



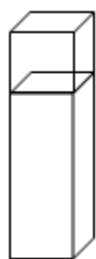
The Tray Cell can read samples as small as 1-2  $\mu\text{l}$



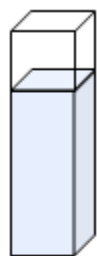


Agilent Technologies

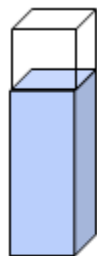
## Quantitative Analysis



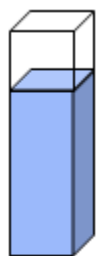
Blank



10 ppm

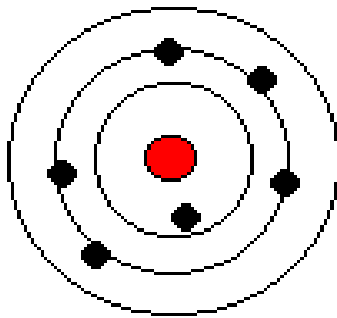


50 ppm

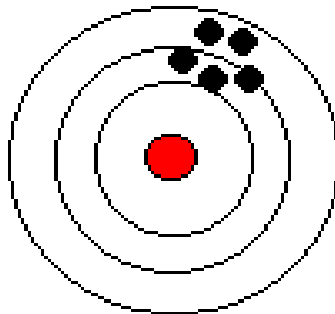


100 ppm

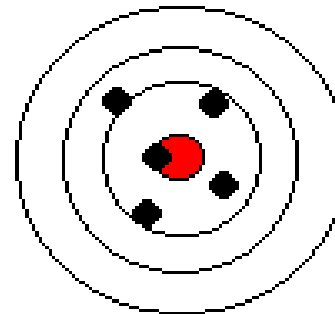
# Accuracy and Precision



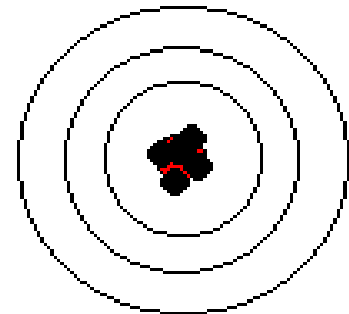
**Inaccurate &  
Imprecise**



**Inaccurate but  
Precise**



**Accurate but  
Imprecise**

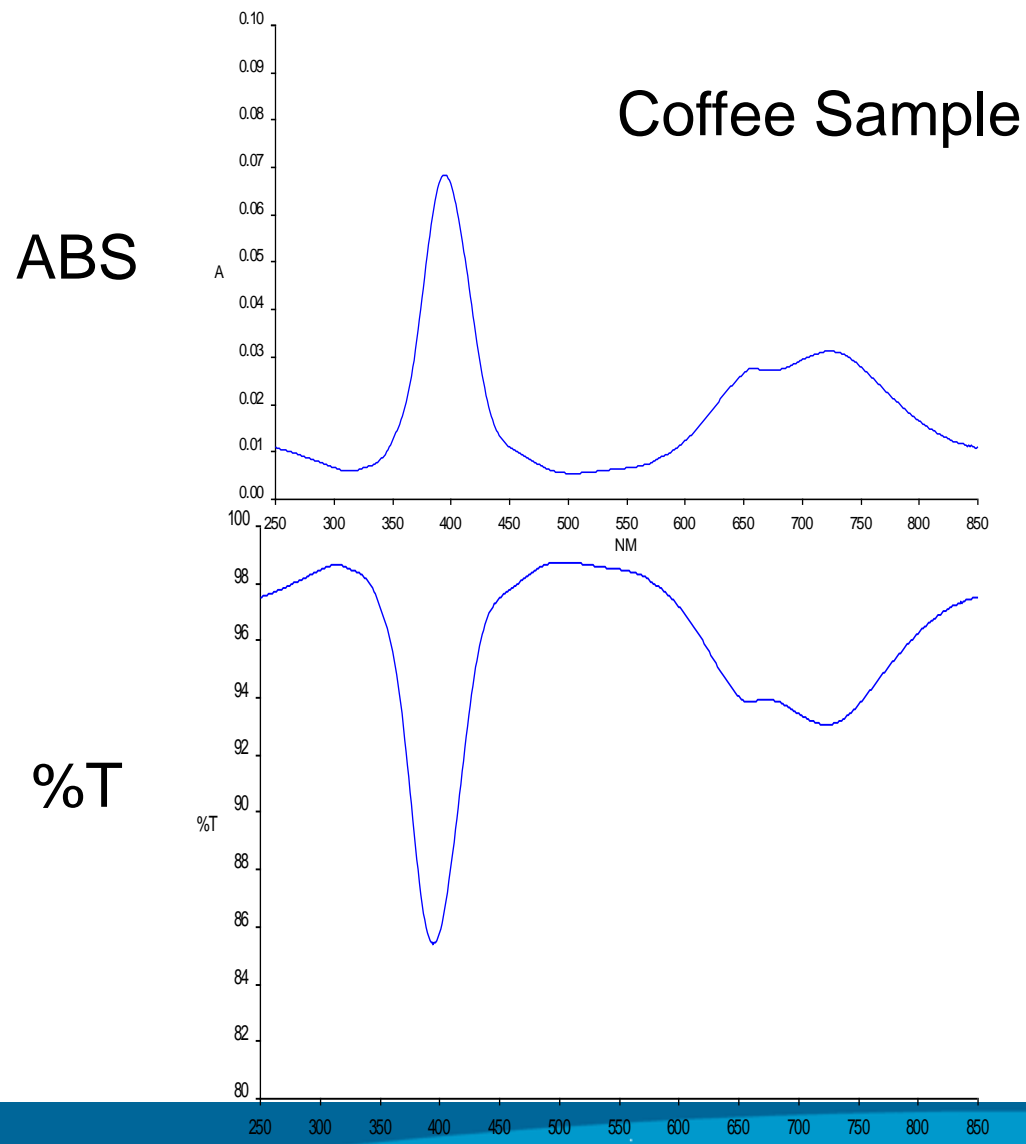


**Accurate &  
Precise**

**One can be precisely wrong!**

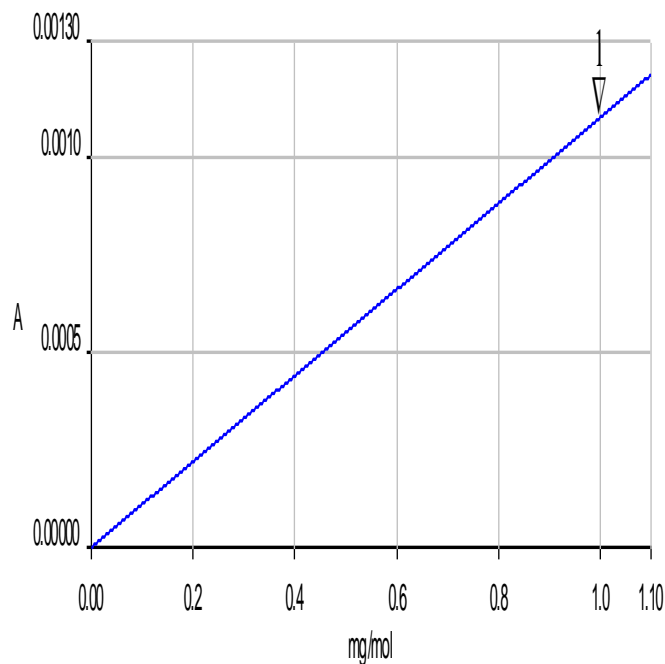


# Absorbance Vs Transmittance



# Why do we quantitative in absorbance when the instrument measures %T?

- Concentration is linear with absorbance not % T
- Historically, a straight line relationship was easier to plot than a polynomial
- With computers, we are able to fit the curve to a number of curve fitting models



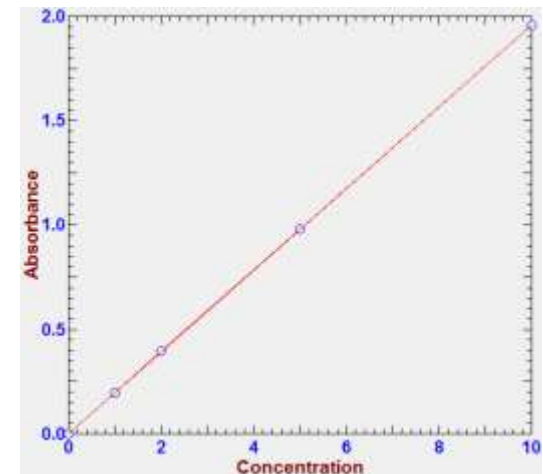
$$Y=mx+b$$

# Steps in Developing Standard Concentration Curve

1. Scan the solvent to identify any cut-offs
2. Scan the sample in the solvent to confirm the analytical wavelength
3. Initially, run a serial dilution to establish the upper and lower detection limits
4. Keep the absorbance range of the standards between 0.2 and 2.0 A
5. Make up individual standards from stock solution and bracket sample range of unknowns
6. Use at least three standards to construct the regression curve (Linear, Quadratic, Polynomial, Cubic, Multicomponent Analysis, Etc.)
7. Validate the method



$$Y = mx + b$$



# Factors Causing Deviation From Beer's law

**Gravimetric / Diametric  
Errors**

**Incomplete Spectral  
Resolution**

**Ionizable Species**

**Temperature Effects**

**Stray Light**

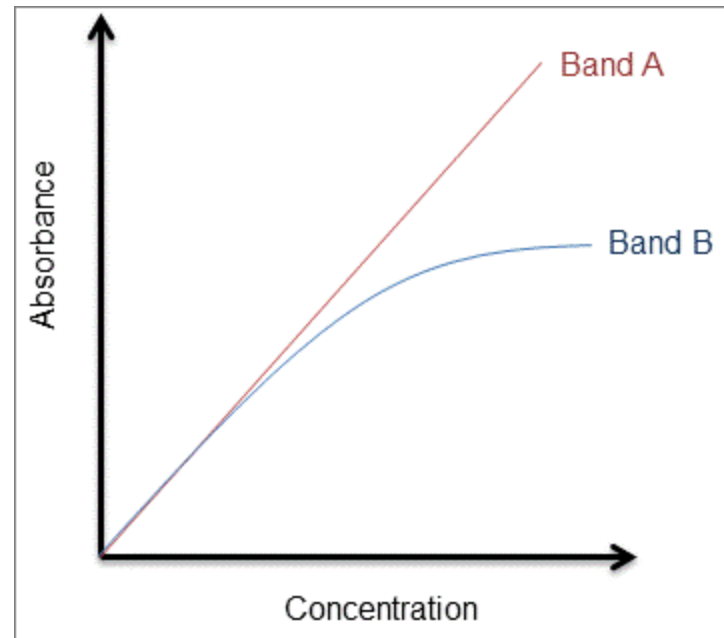
**Solvent Absorption**

**Aggregation**

**Sensitivity**

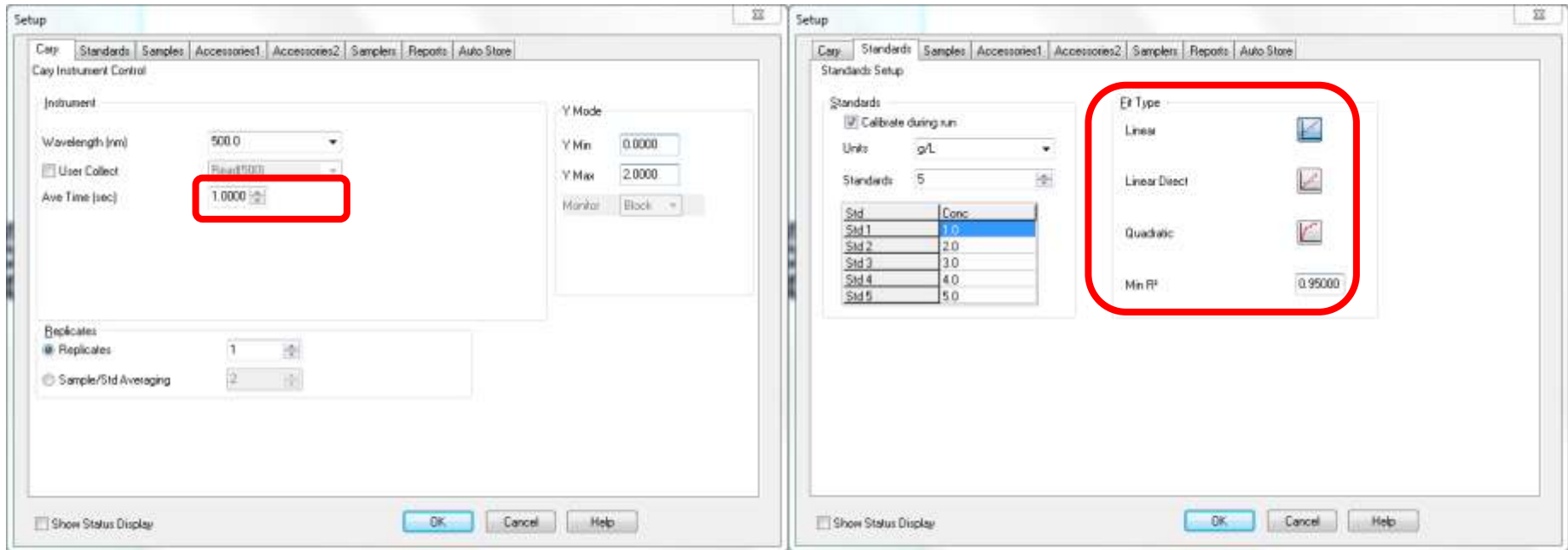
**Scattering**

**Adsorption**



# Response Time and Curve Fitting Algorithms

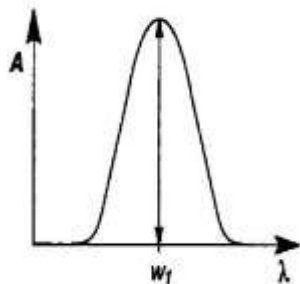
**Noise is random and self cancels while the signal is constant. A longer response time will reduce the noise in the measurement yielding better precision.**



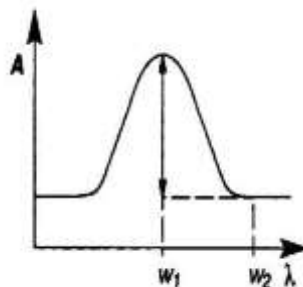
**Curve fitting algorithms can give better results for non-linear systems**

# Peak Measurement – Minimizing Matrix Interferences

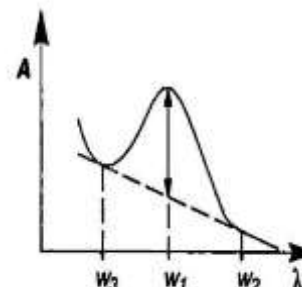
Peak Height



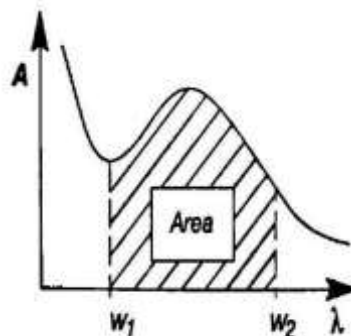
1 point



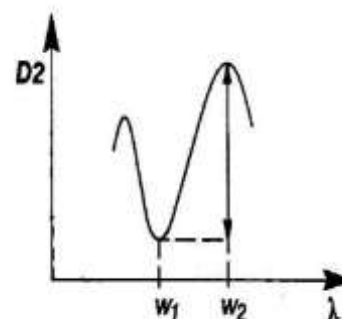
2 point



Area



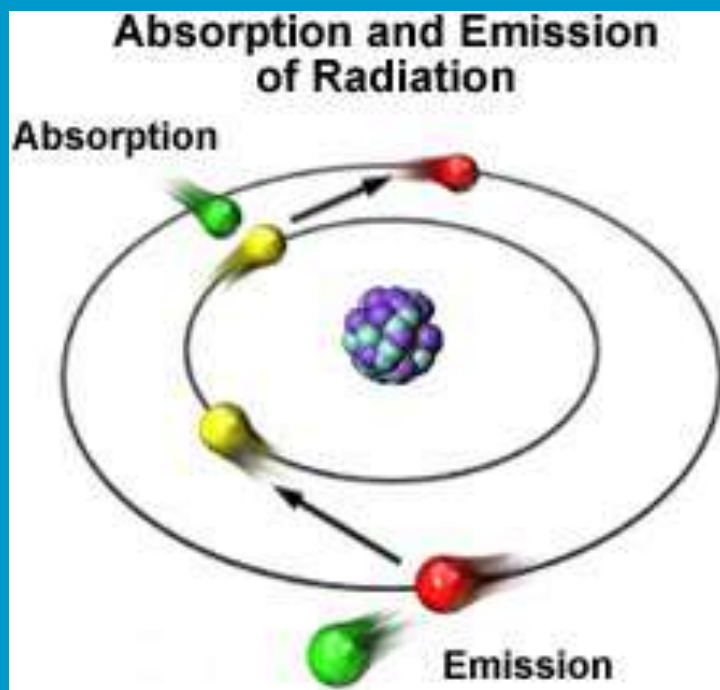
Derivative (Matrix Interference)







Agilent Technologies



## Fluorescence Spectroscopy



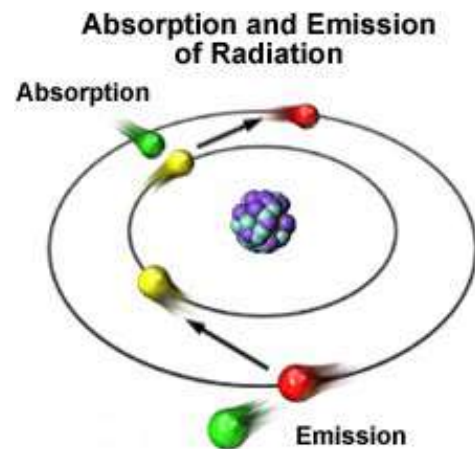
# Fluorescence and the Particle Nature of Light

The absorption of light increases the velocity of an electron primarily by changing its direction, not its speed.

Thus the electron will undergo an electronic transition from the ground state to an excited state

Three things can happen while it is in this excited state:

1. It may undergo a radiationless loss (vibrational relaxation) of electronic energy through collisions and other interactions.
  - a. Most molecules do not fluoresce because of this deactivation process.
2. It can emit a photon of light (fluoresce).
3. It can undergo a transition to a metastable state (triplet) and phosphoresce.



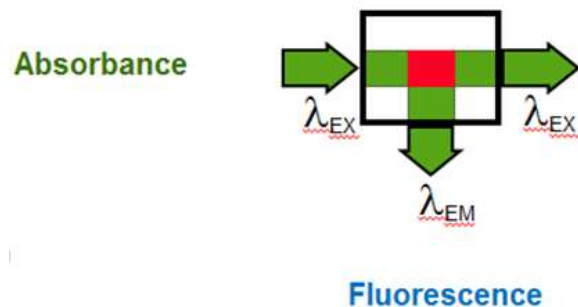
# Why Fluorescence?

## The benefits of fluorescence spectroscopy

- **Sensitivity** - Measure target species with extremely high sensitivity (pb, ppt)
- **Selectivity** – Measure an individual component in a complex matrix without the need for chromatography
- **High Information** – Information on emitting species, concentration, reaction kinetics, interactions, molecular motion, etc.
- **Non Destructive** – fluorescence allows one to run preliminary tests then reanalyze the same sample with a complimentary technique



# Fluorescence vs. Absorbance - Sensitivity



Absorbance measurements are made by detecting small changes in large amounts of light passing through a sample

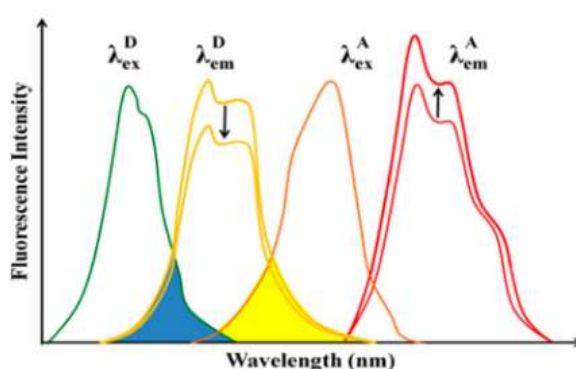
- A calculated is obtained which has units
- The value is the same on any instrument
- Optimal range .5 to 2.5 A

Fluorescence measurements are made by detecting a relatively small amount of light emitted by the sample

- The value is relative and without units
- The value can be different on every instrument
- Optimal Range < .05 Abs



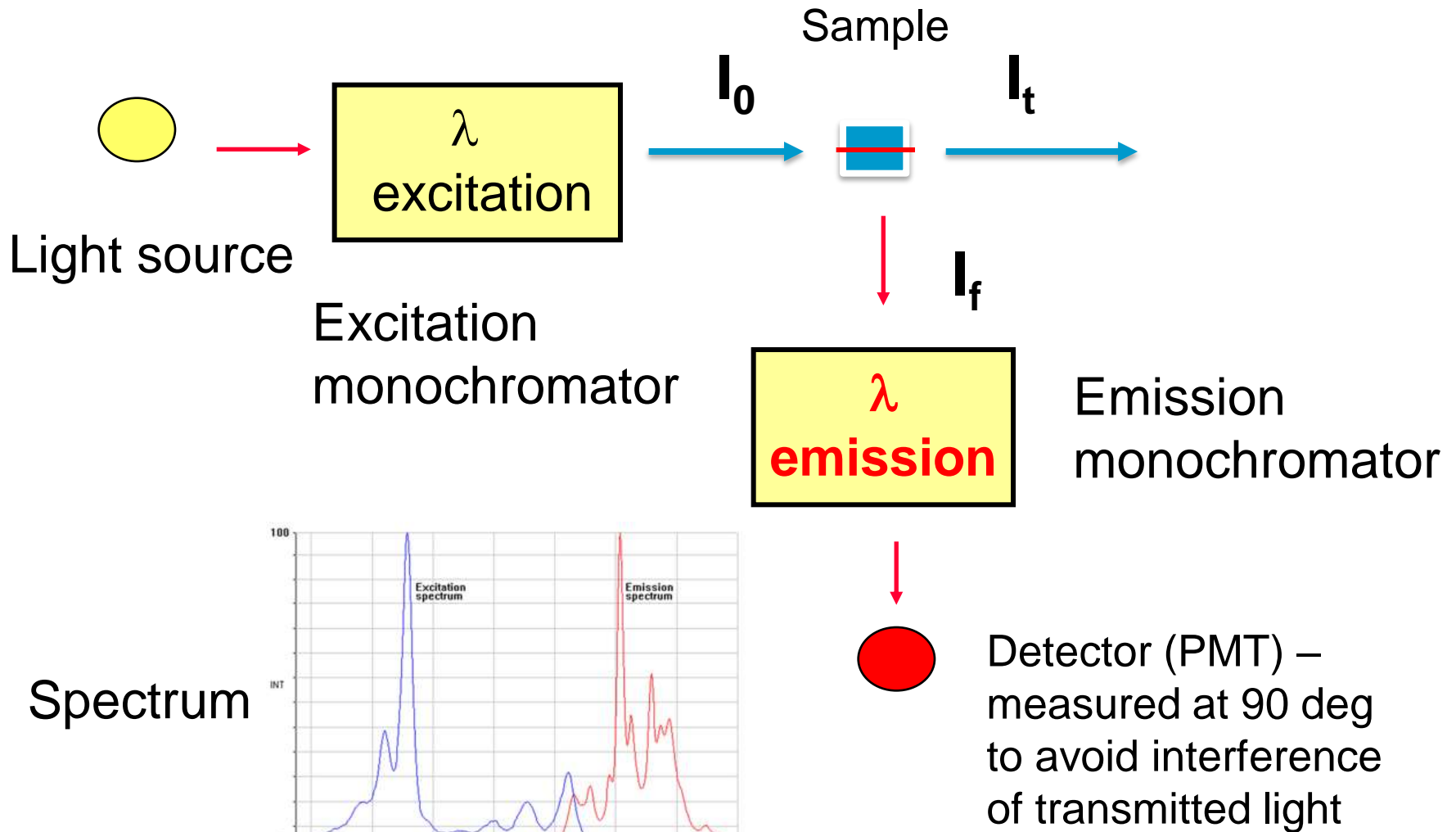
# Fluorescence Advantage - Selectivity



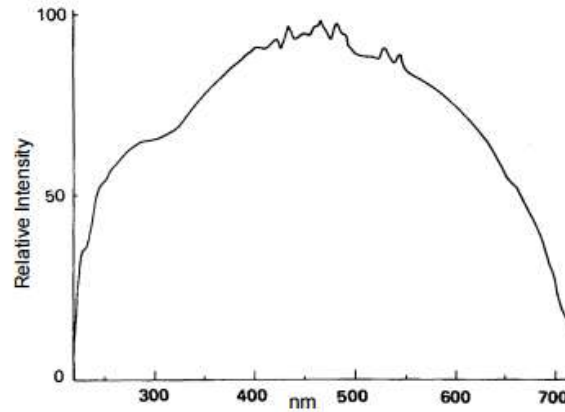
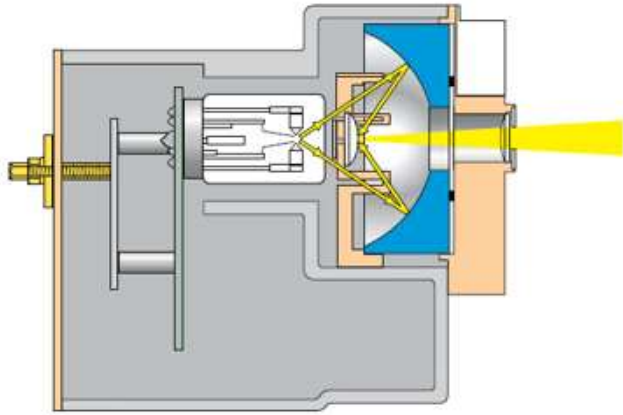
- Specificity in luminescence arises from two factors, Only about 10% of all substances that absorb radiation re-emit it as light and there are two selectable wavelengths (excitation and emission) in luminescence as opposed to only one in absorbance.
- It is highly unlikely that two substances will share both a similar excitation and emission. This is because the difference between excitation and emission peaks can range anywhere from several nanometers to hundreds of nanometers.
- No separation chemistry needed.



# Fluorescence Spectrophotometer - Sensitivity



# Pulsed Lamp vs Continuum



- Fluorescence intensity is directly proportional to the incident light.
- Large Uv output where most fluorophores absorb
- The Schwarzschild collection optics capture a large portion of the light from the powerful xenon flash lamp and directs it through the sample,
- There is no warm-up time for the lamp

# Other Advantages of a Pulsed Lamp

Multiple measurement modes

Fluorescence

Phosphorescence

Lifetimes

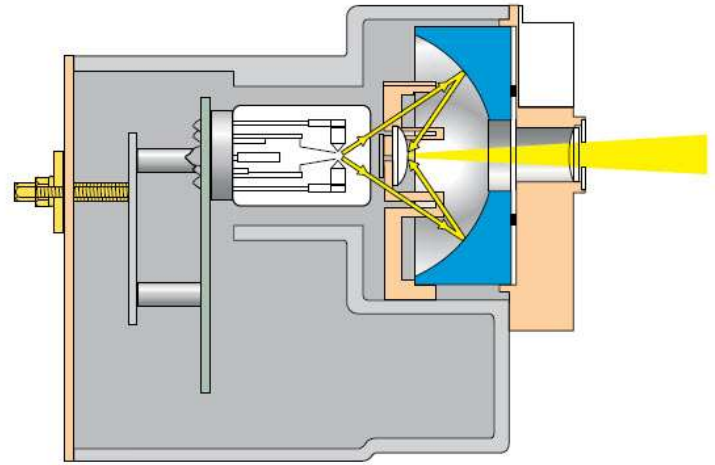
Bio & Chemi-luminescence

Time Delayed Fluorescence

Minimizes Photodegradation

Room-Light Immunity

There is no warm-up time for the lamp



## **Superior optics**

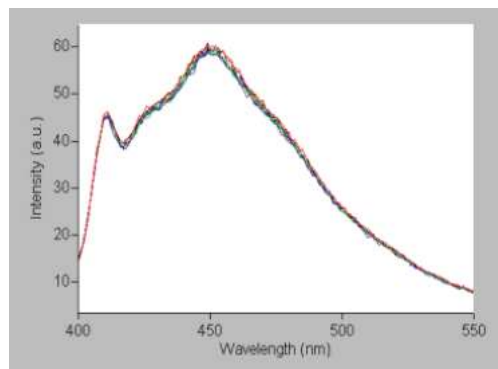
*The Schwarzschild collection optics capture a large portion of the light from the powerful xenon flash lamp and direct it through the sample, resulting in excellent sensitivity and low signal noise.*



Agilent Technologies

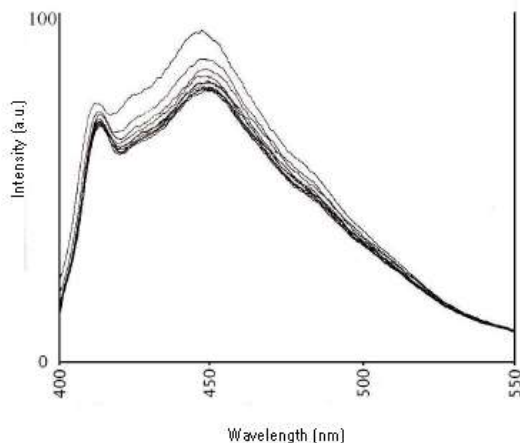


# What If My Sample Is Photosensitive



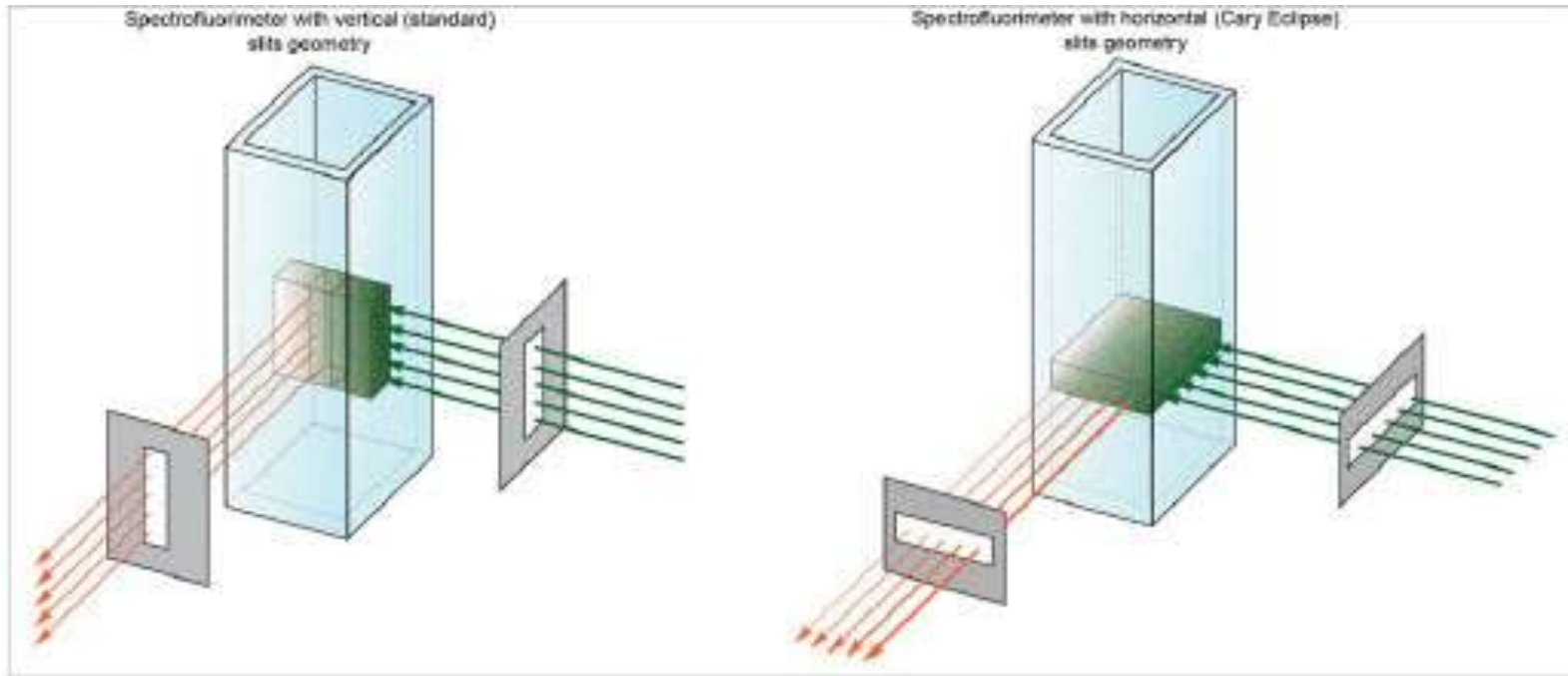
The pulsed lamp reduces the exposure time of the fluorophore to the incident light, eliminating or reducing any photodecomposition of the sample. The lamp only flashes when a measurement is being made.

Some pulsed lamp units pulse the lamp continually.



**Figure (a) and (b).** Emission wavelength vs intensity for BFP following 370 nm excitation. A negligible drop in peak BFP emission (450 nm) was recorded after 10 successive scans at a scan rate of 120 nm/min (total exposure time 12 min 30 s) using the Cary Eclipse (a), whereas photobleaching of approximately 20% was observed using a commercially available instrument fitted with a xenon arc lamp (b)

# Horizontal Beam Image



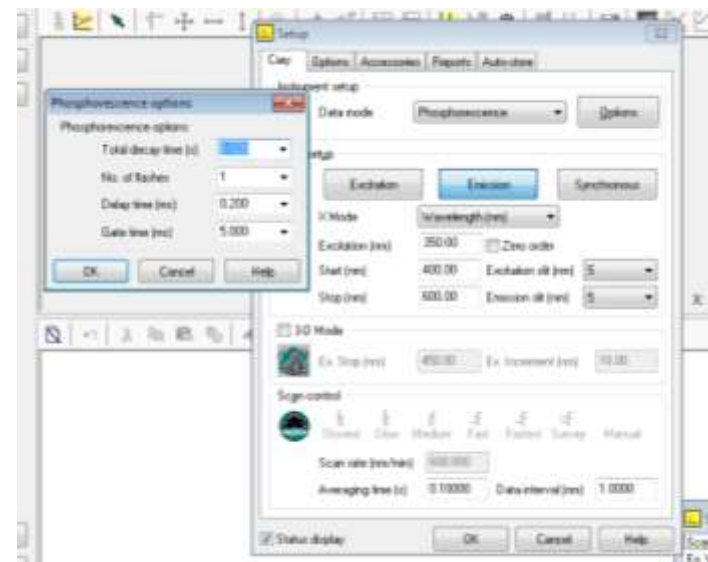
If one is sample limited, the horizontal beam allows one to use far less sample while having the full beam image impinging on the sample for maximum sensitivity and minimal amount of sample.

# Phosphorescence

Fluorescence is a nanosecond phenomena in that fluorescence occurs as soon as the light goes on and disappears as soon as the light goes off.

The advantage of delayed fluorescence and phosphorescence is that the lamp is off when the measurement is made

So there is no background light, and it eliminates any competing fluorescence in the system by other fluorophores.

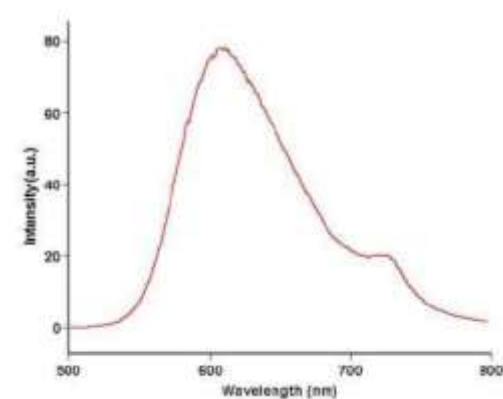


# Chemiluminescence

Emission resulting from a chemical reaction

No instrument light source required

Capable of measuring emission well into the red region of the spectrum

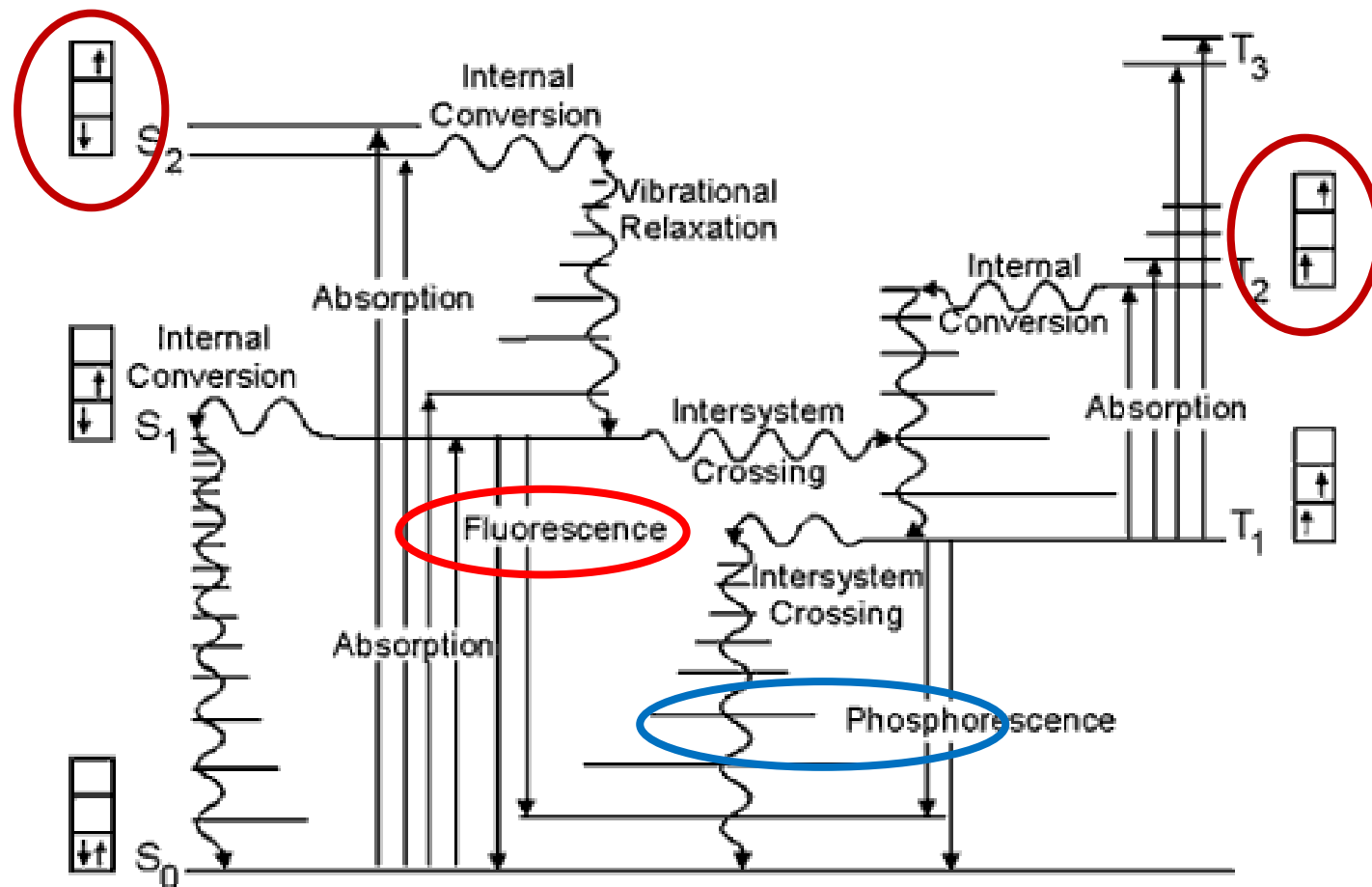


Application note: [Measuring chemiluminescence of a ruthenium complex](#)



Agilent Technologies

# Time Delayed Fluorescence



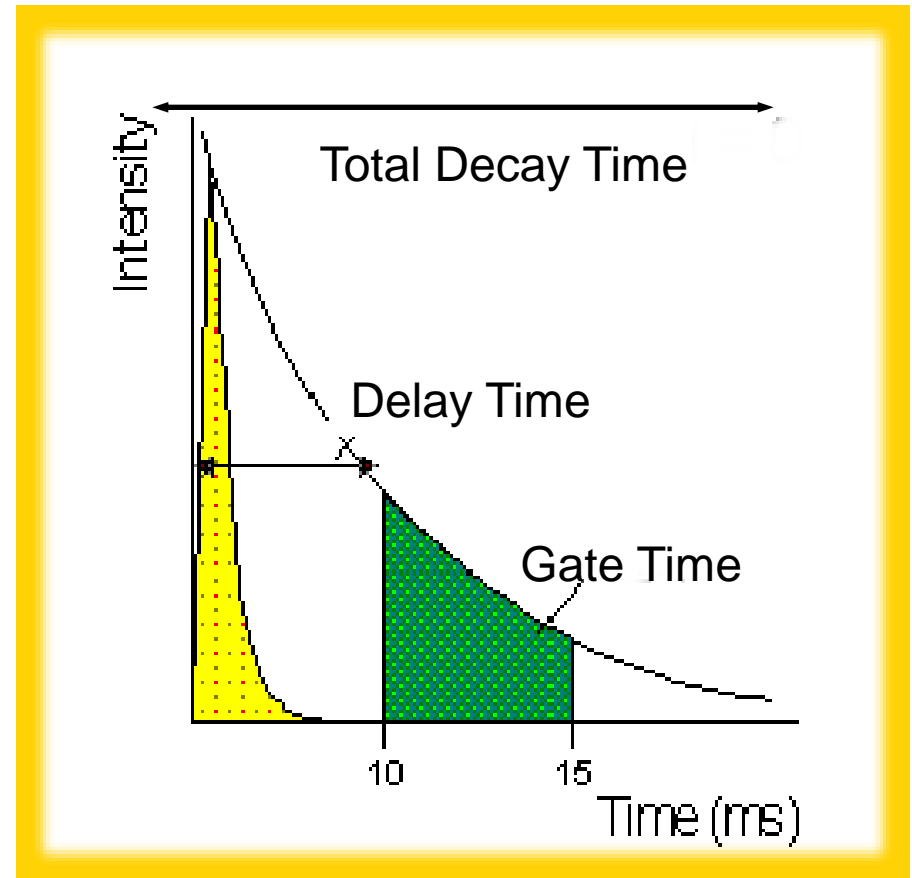
# Time Delayed Fluorescence & Phosphorescent Lifetimes

- best performance  $>100$  ms lifetime, can as low as do 50 ms

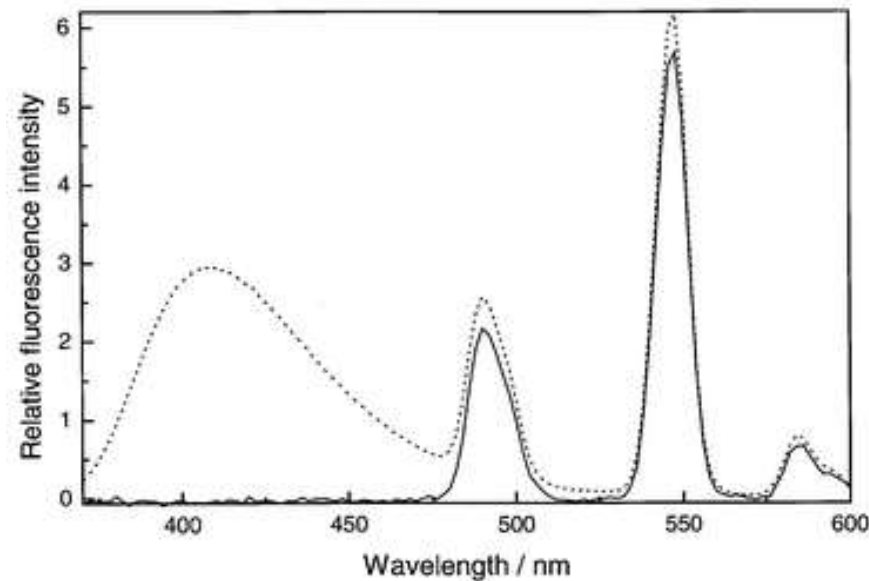
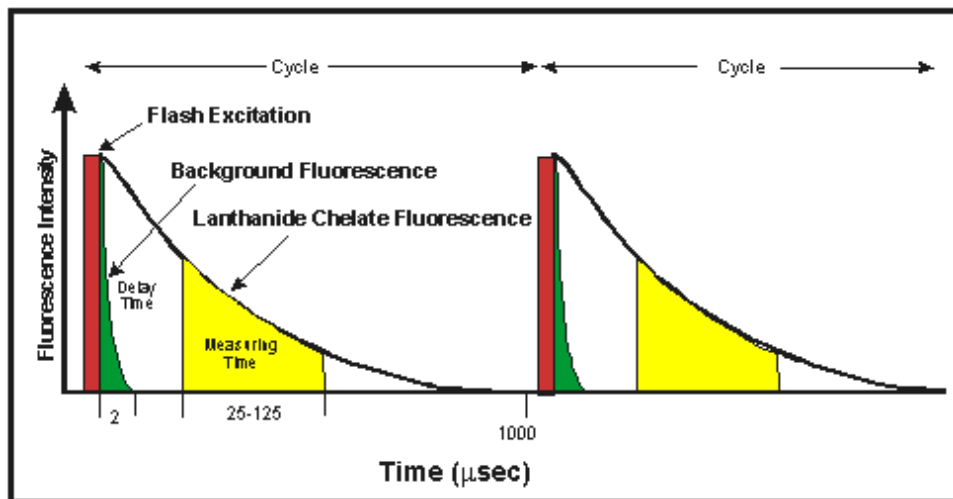
Total decay time (TDT) represents the time over which the decay will be measured

Delay Time (DT) represents the time elapsed between the flash the lamp and the emission measurement

Gate Time (GT) represents the overall reading time of the emission signal, where the fraction of light collected depends on the GT



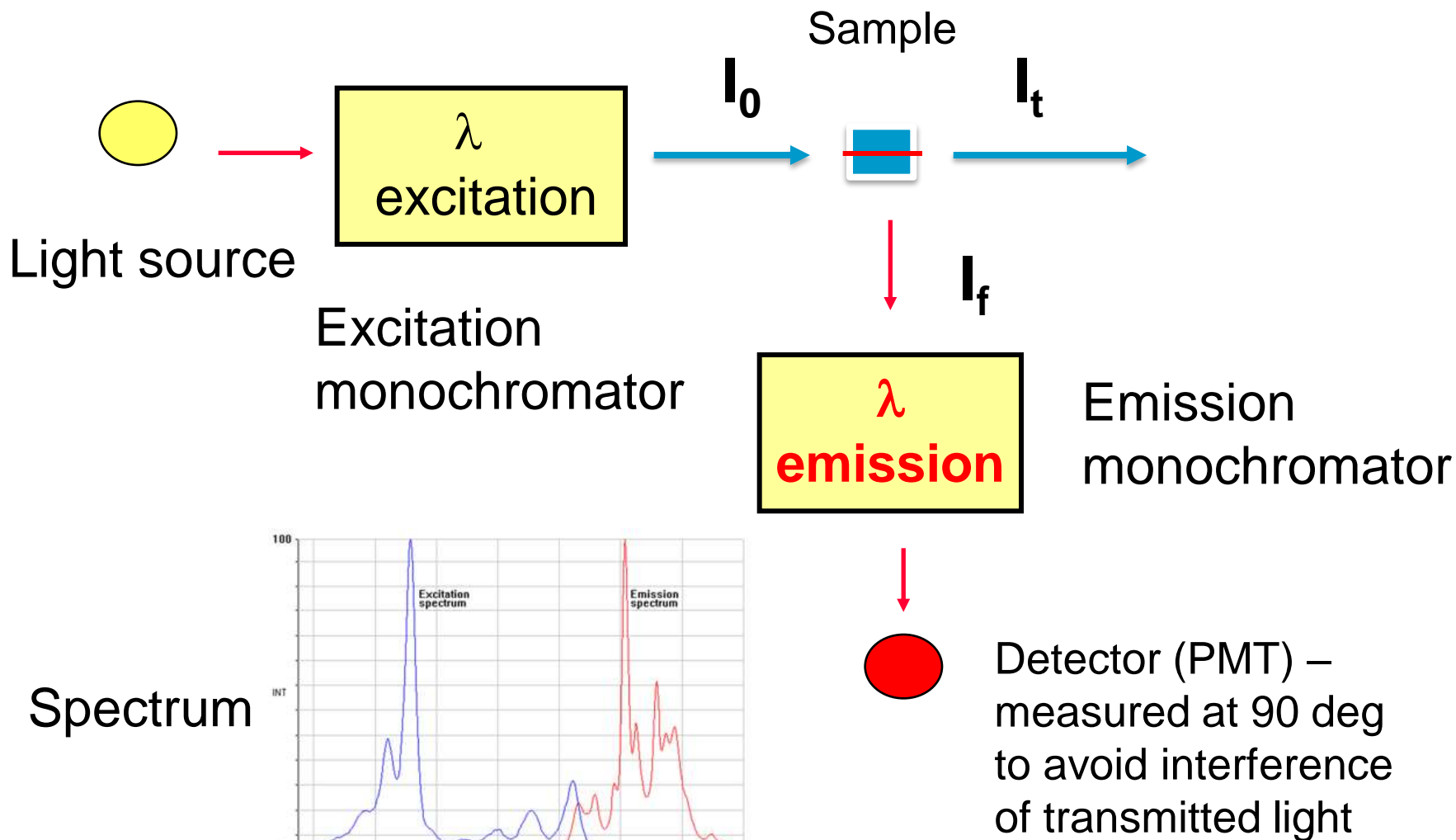
# Time Delayed Fluorescence - Peroxidase enhanced lanthanide luminescence



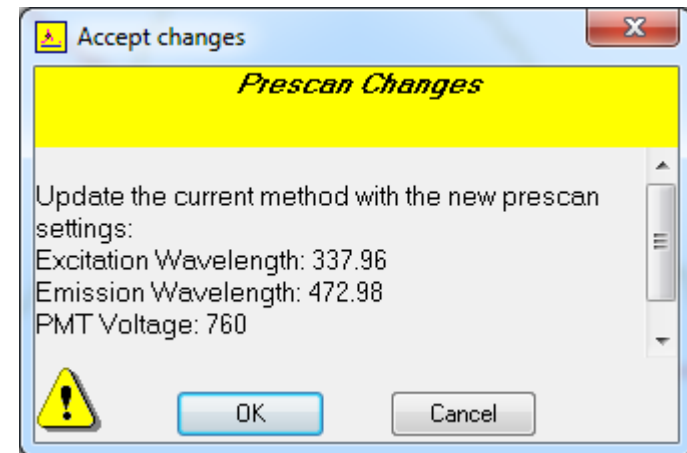
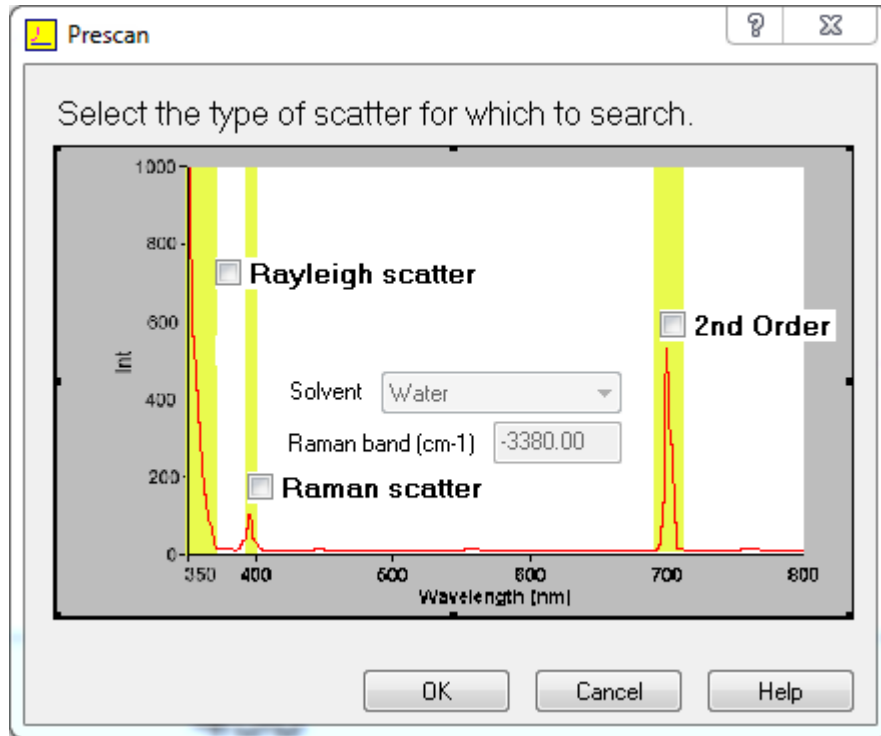
**Dotted line: standard fluorescence spectrum. Solid line: time resolved fluorescence spectrum (delay time) .**



# The Measurement

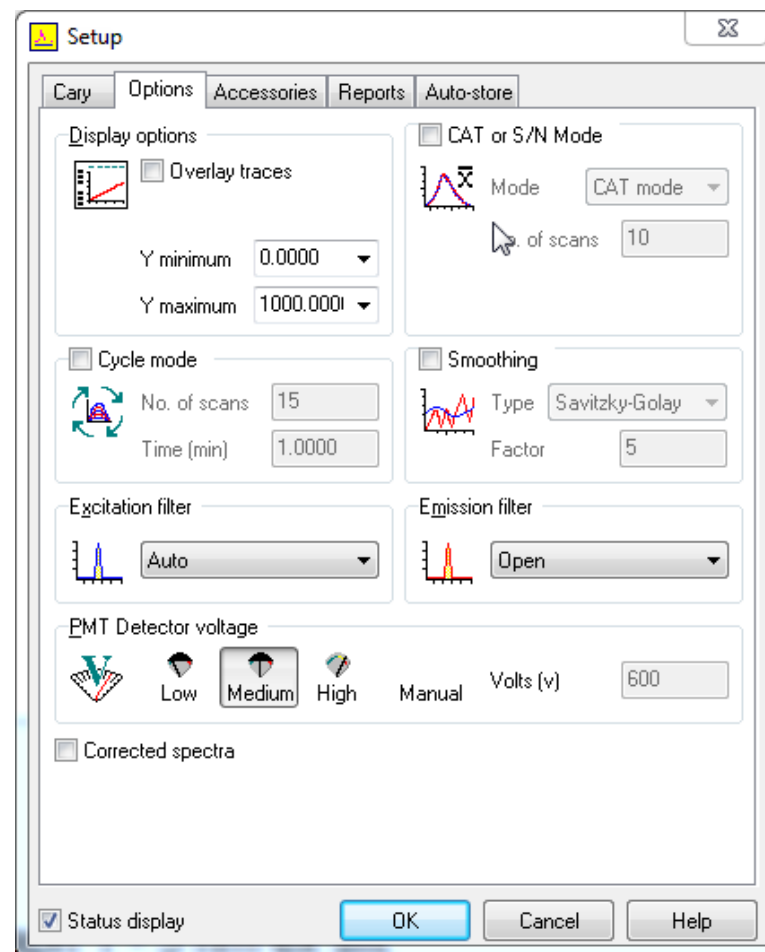
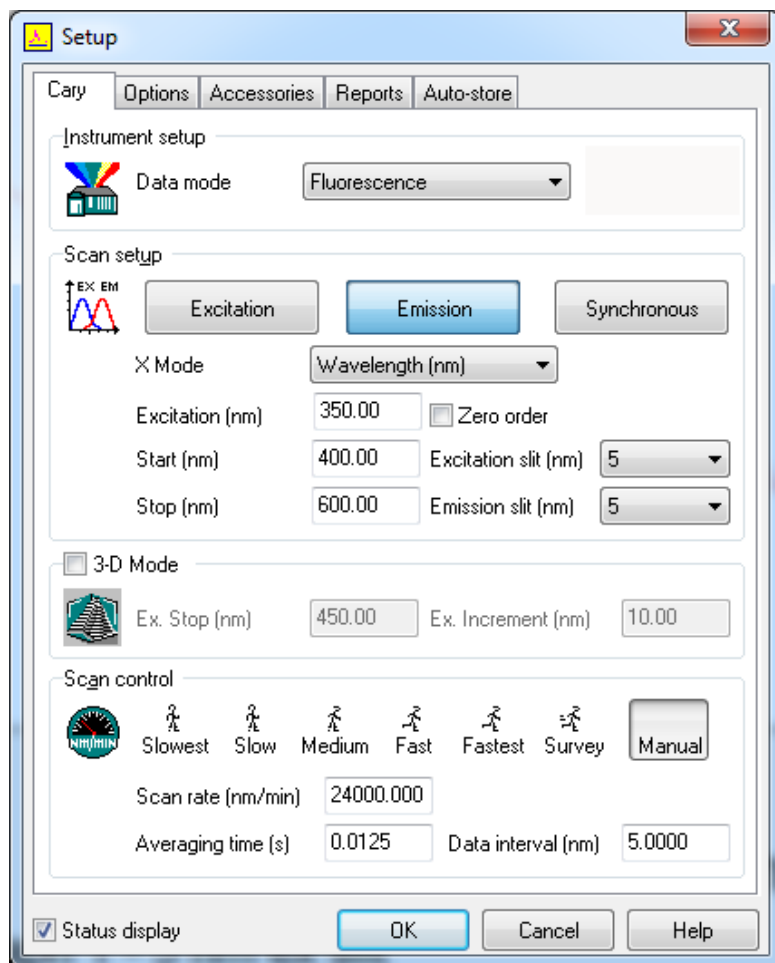


# Prescan Function





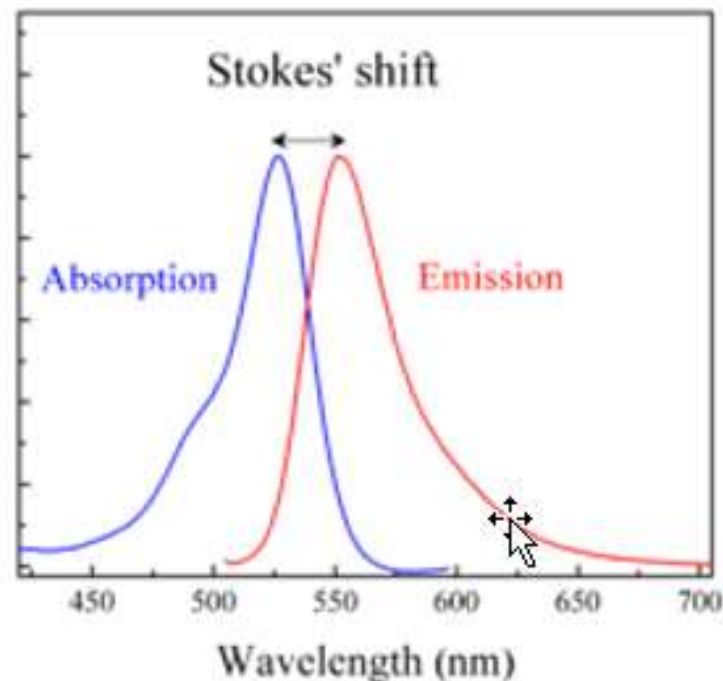
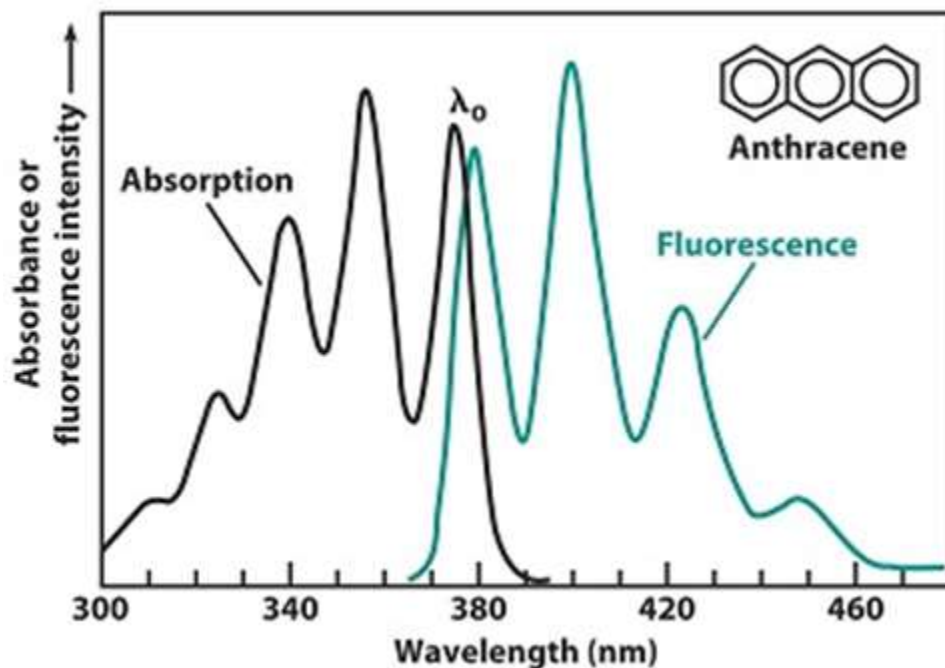
# The Software



# Fluorescence "Rules-Of-Thumb"

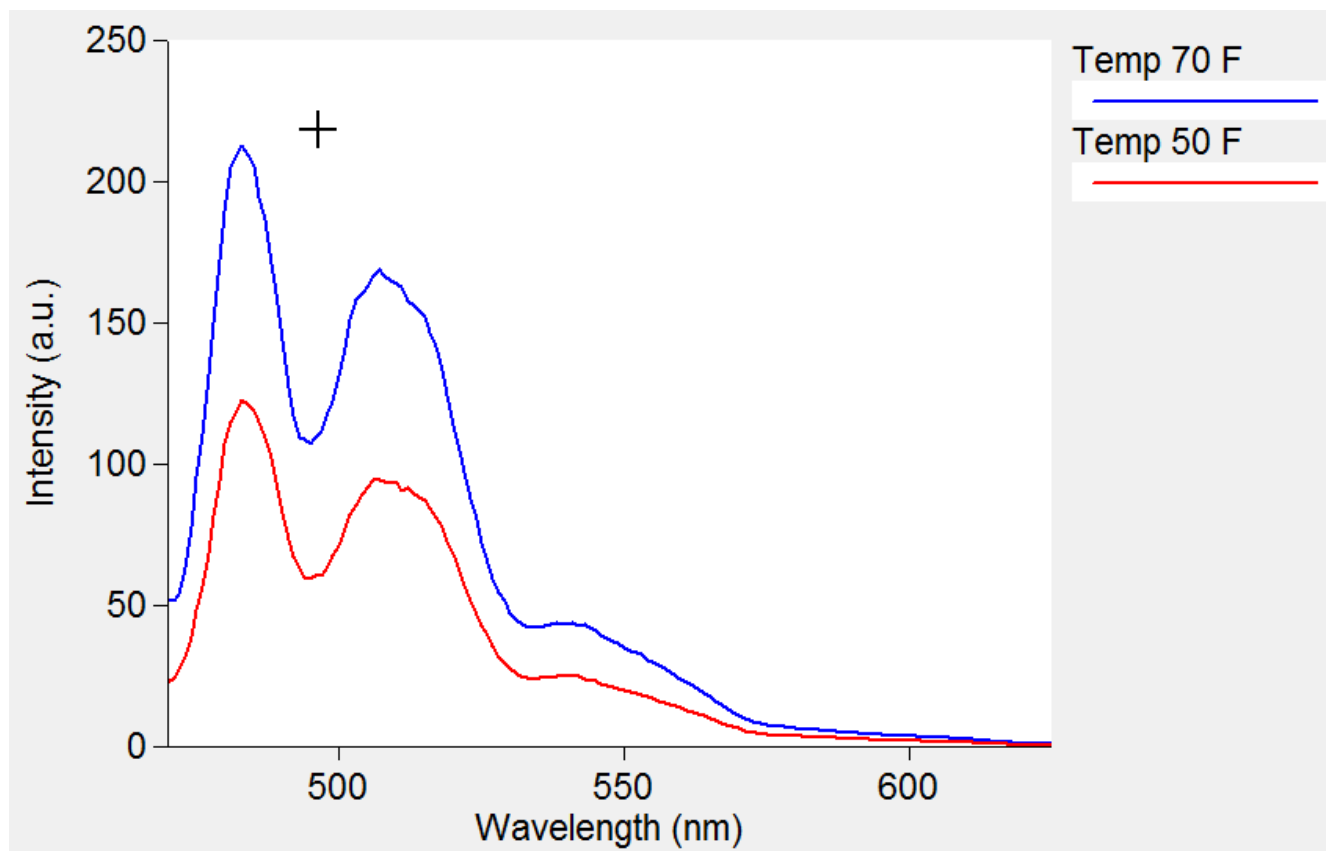
The excitation spectrum is identical in shape to the absorbance spectrum.

The emission spectrum is the mirror image of the longest wavelength absorption band and occurs at wavelengths longer than the absorption spectrum.

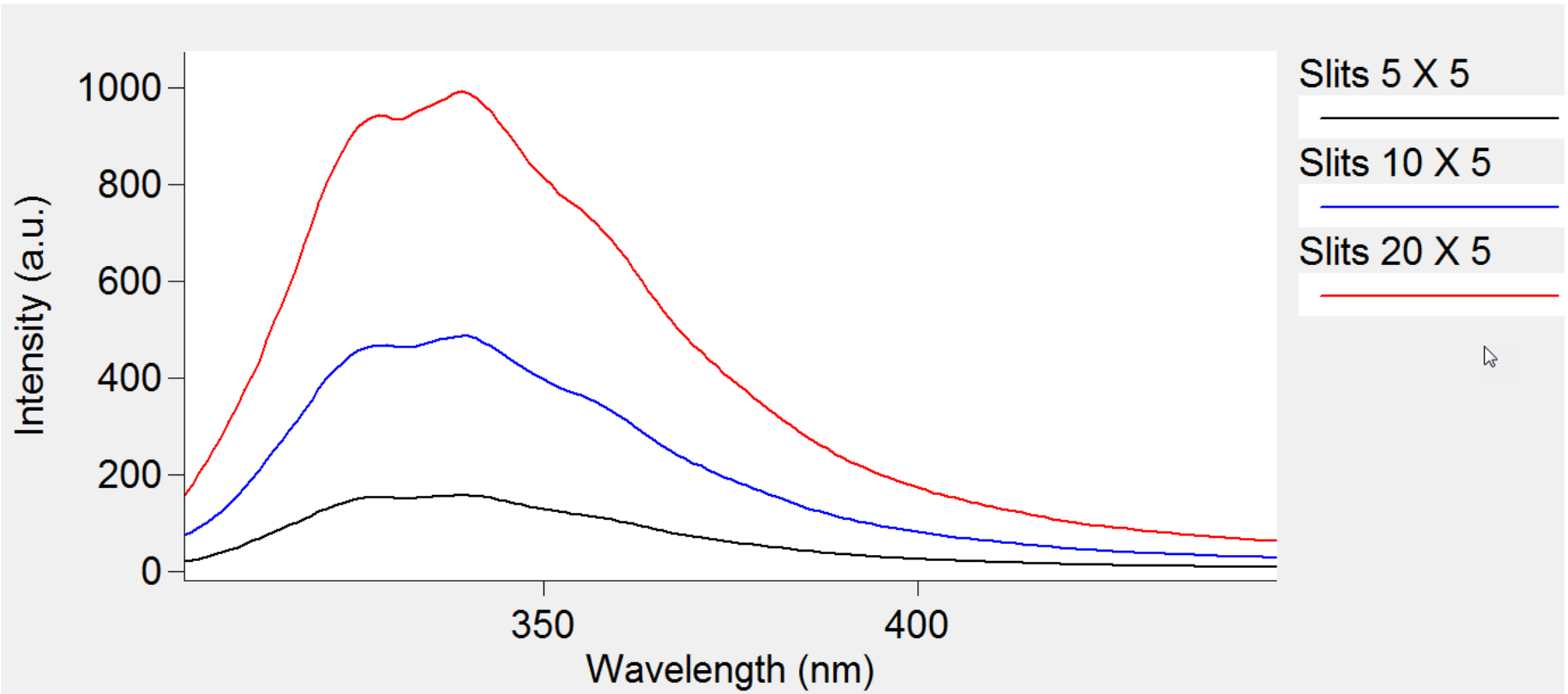


# Characteristics of Fluorescence Analysis

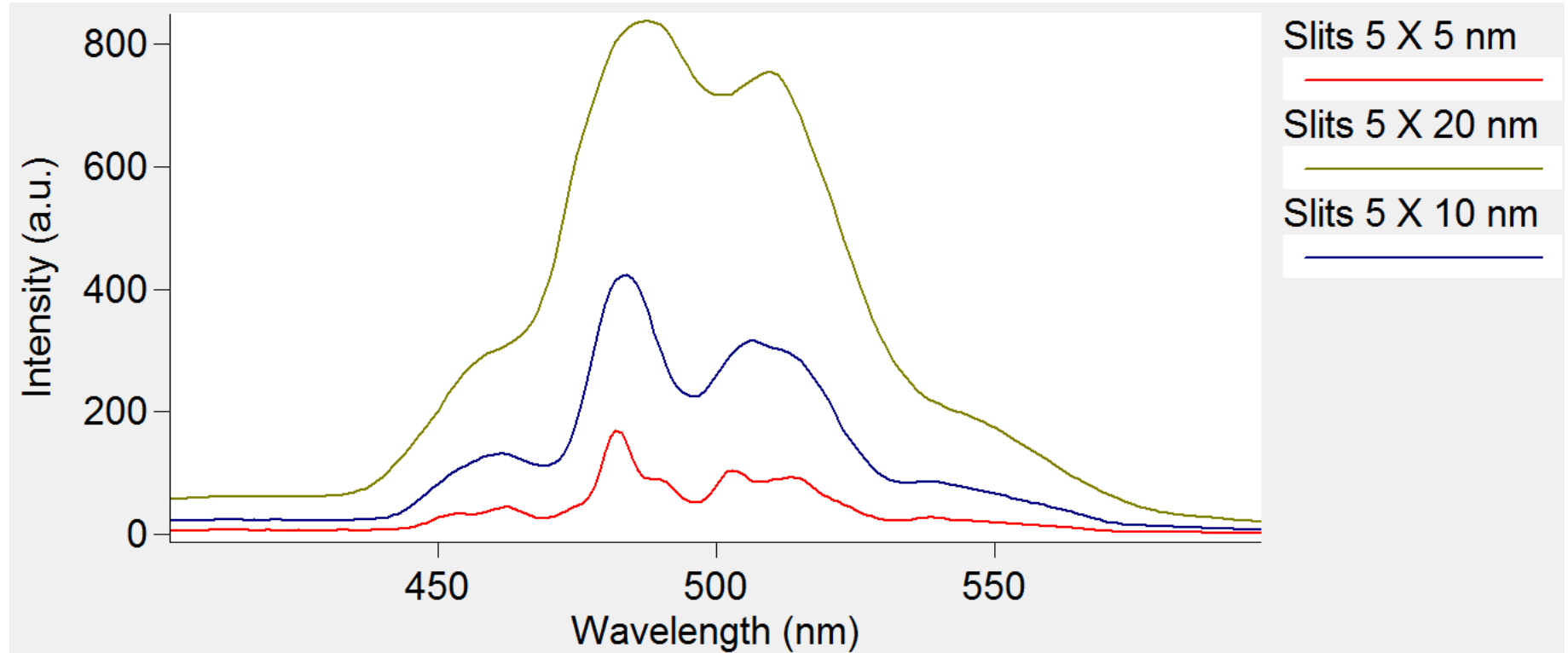
**Inversely Proportional to  
Temperature**



# Fluorescence Intensity vs Excitation Slit Width

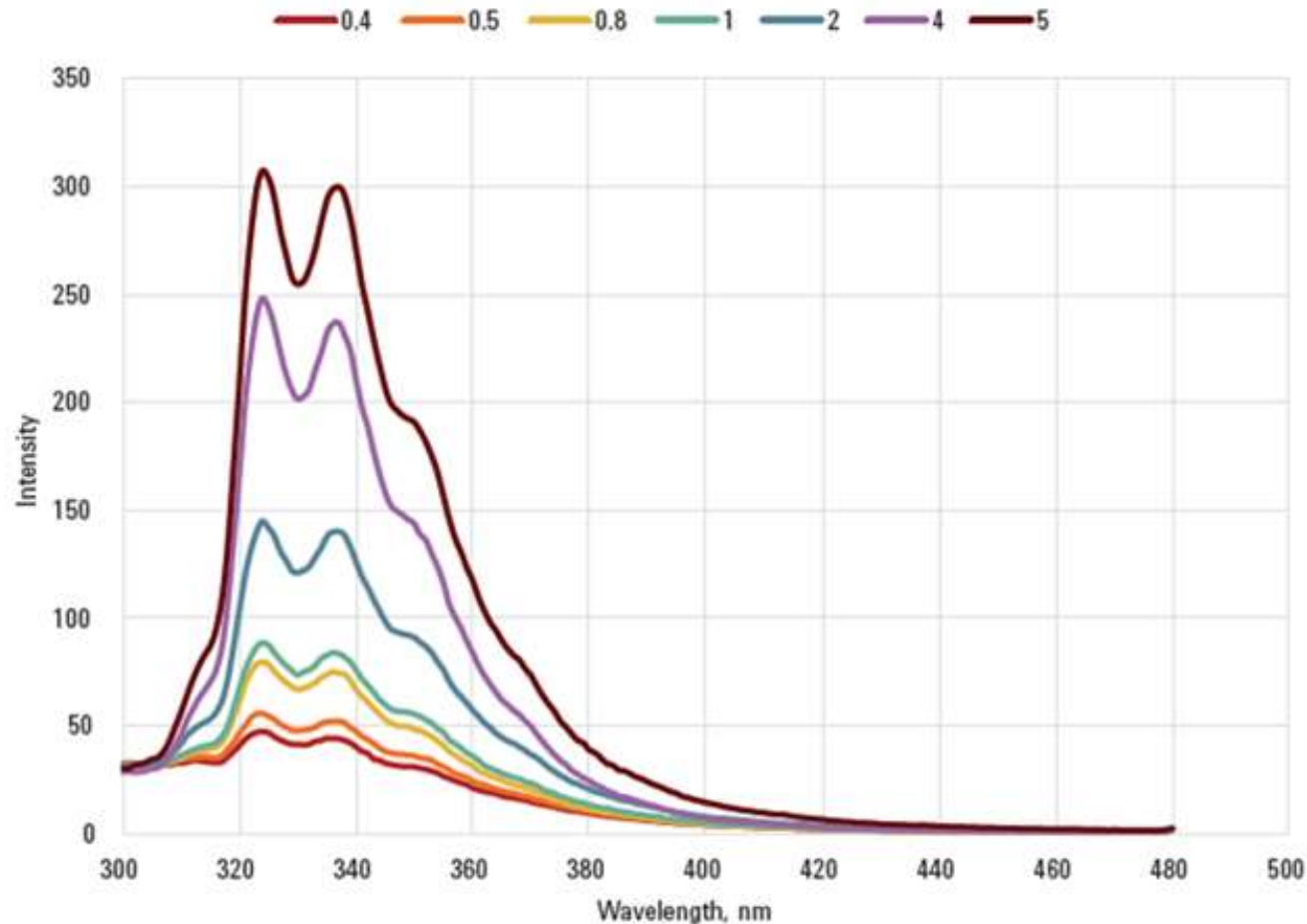


# Effect of Emission Slit on Resolution



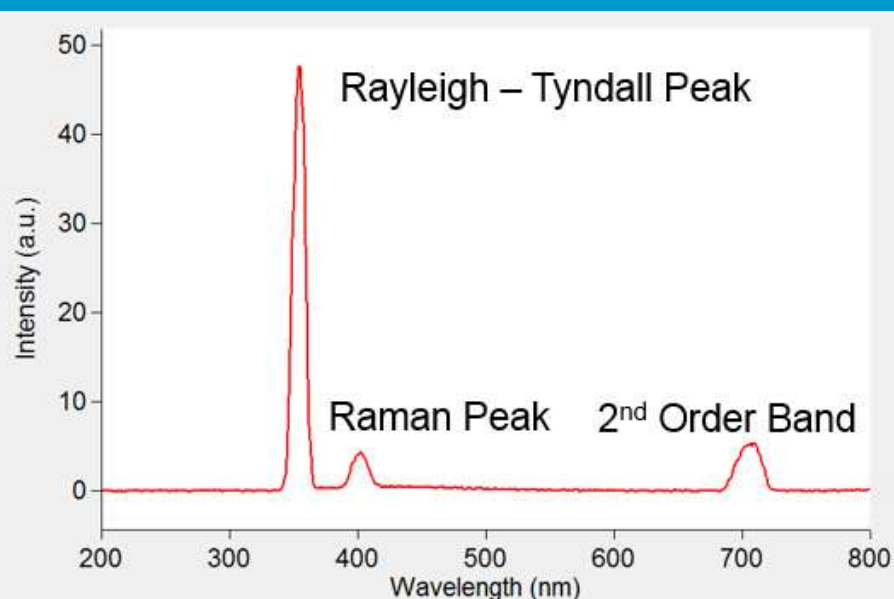
# Quantitative Analysis of a Fluorophore

**Fluorescence is a relative measurement while  
Uv/Vis is an absolute measurement**



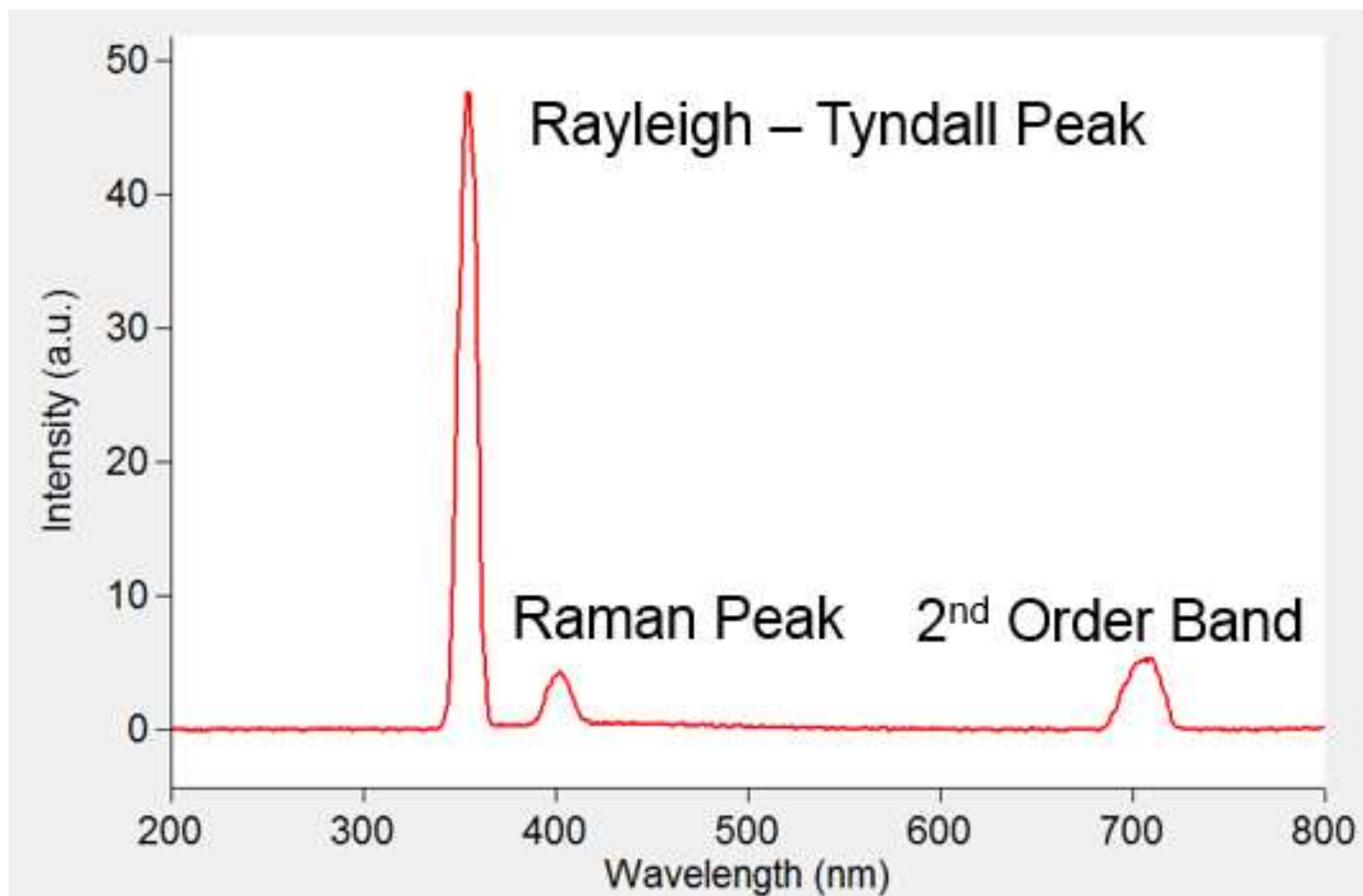


Agilent Technologies



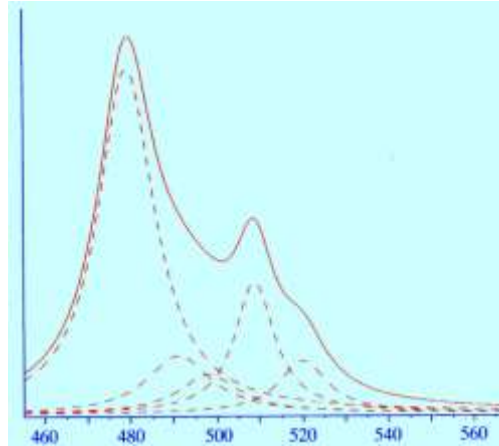
## Interferences in Fluorescence Analysis

# Interferences in Fluorescence

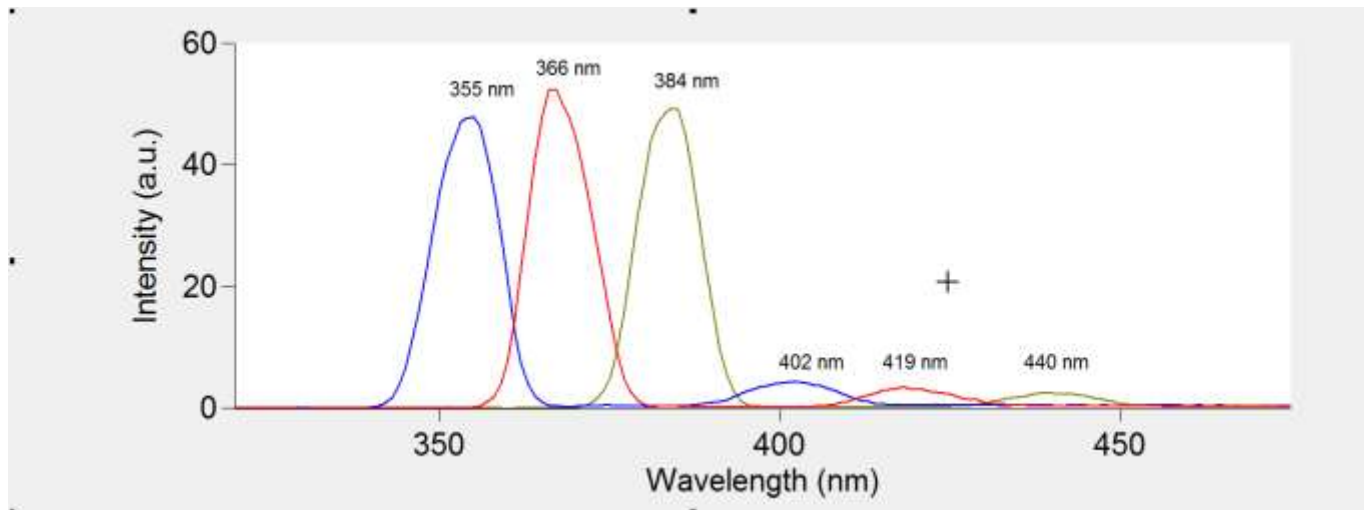




# Eliminating the Raman Peak Interference



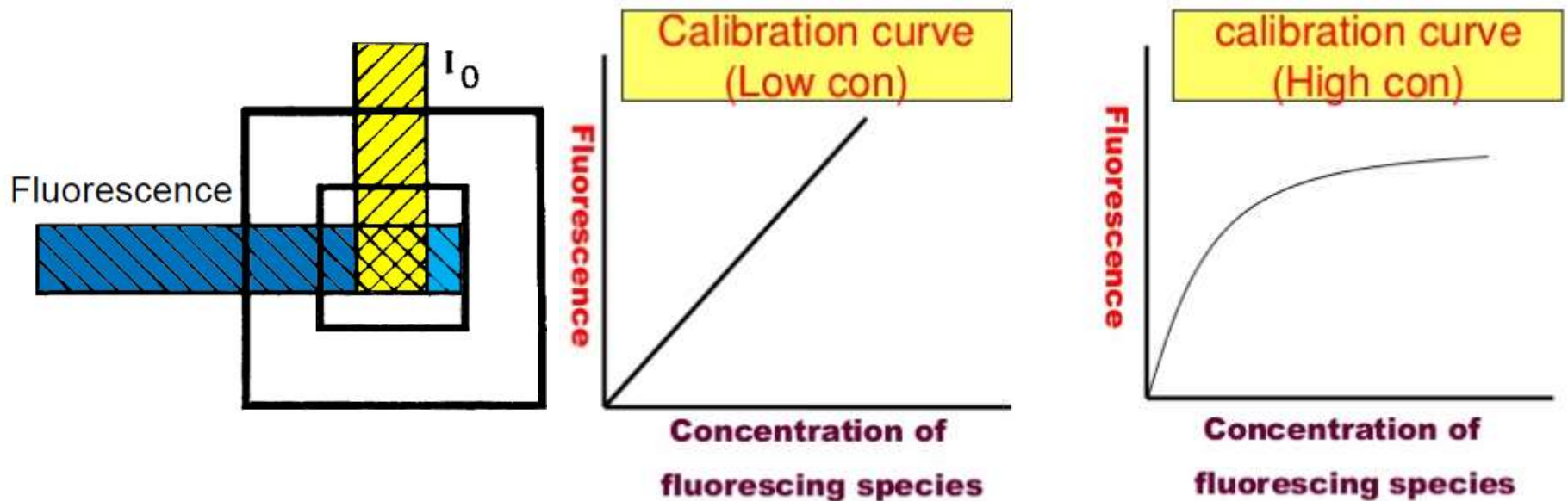
How to eliminate Raman band interference



# Concentration Quenching

Quenching refers to any process that reduces the fluorescence intensity of a given substance

This may occur due to various factors like pH. Concentration, temperature, viscosity, presence of oxygen, heavy metals or, specific chemical substances etc..



# Other Factors Effecting Fluorescence Intensity

Concentration

Quantum yield of fluorophore

Intensity of Incident light

Adsorption

Oxygen

Ph

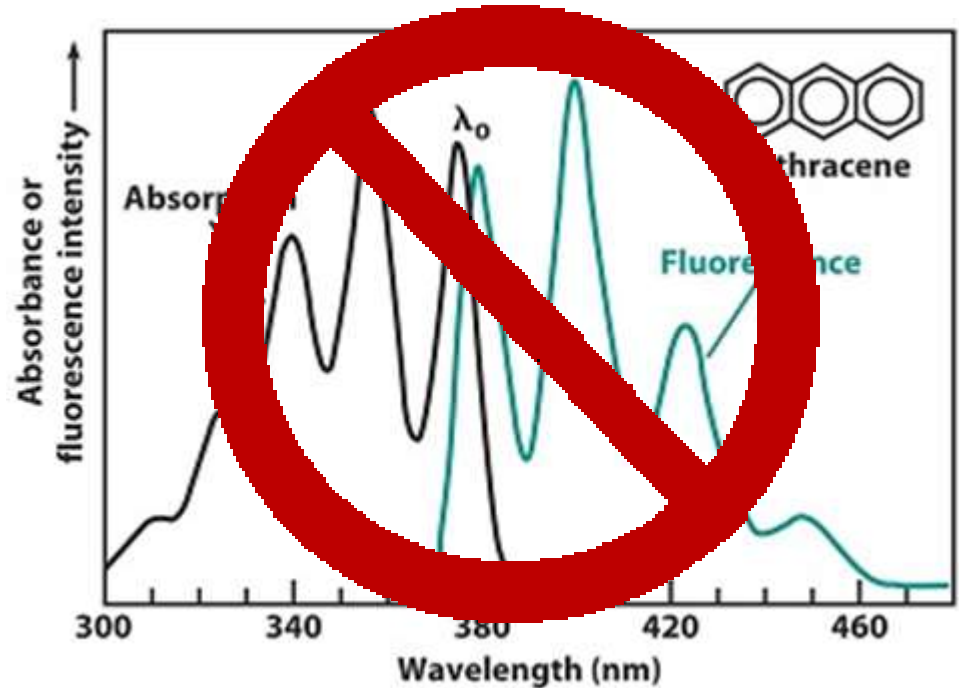
Temperature

Viscosity

Photodecomposition

Quenchers

Scatter





**The End**

**Questions?**