



**Principles of Fluorescence Techniques**  
**2008 Chicago**  
**April 9-11, 2008**

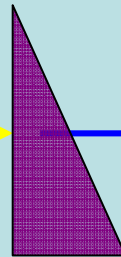
**Basic Instrumentation David Jameson**  
**(many of these slides were prepared by**  
**Theodore “Chip” Hazlett and Joachim Mueller)**

# The Basics



Light Source

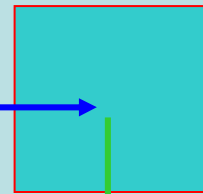
Wavelength Selection



Polarizer



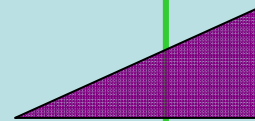
Sample



Polarizer



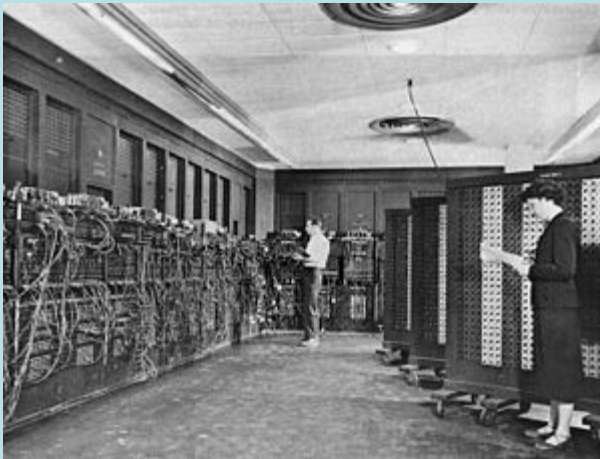
Wavelength Selection



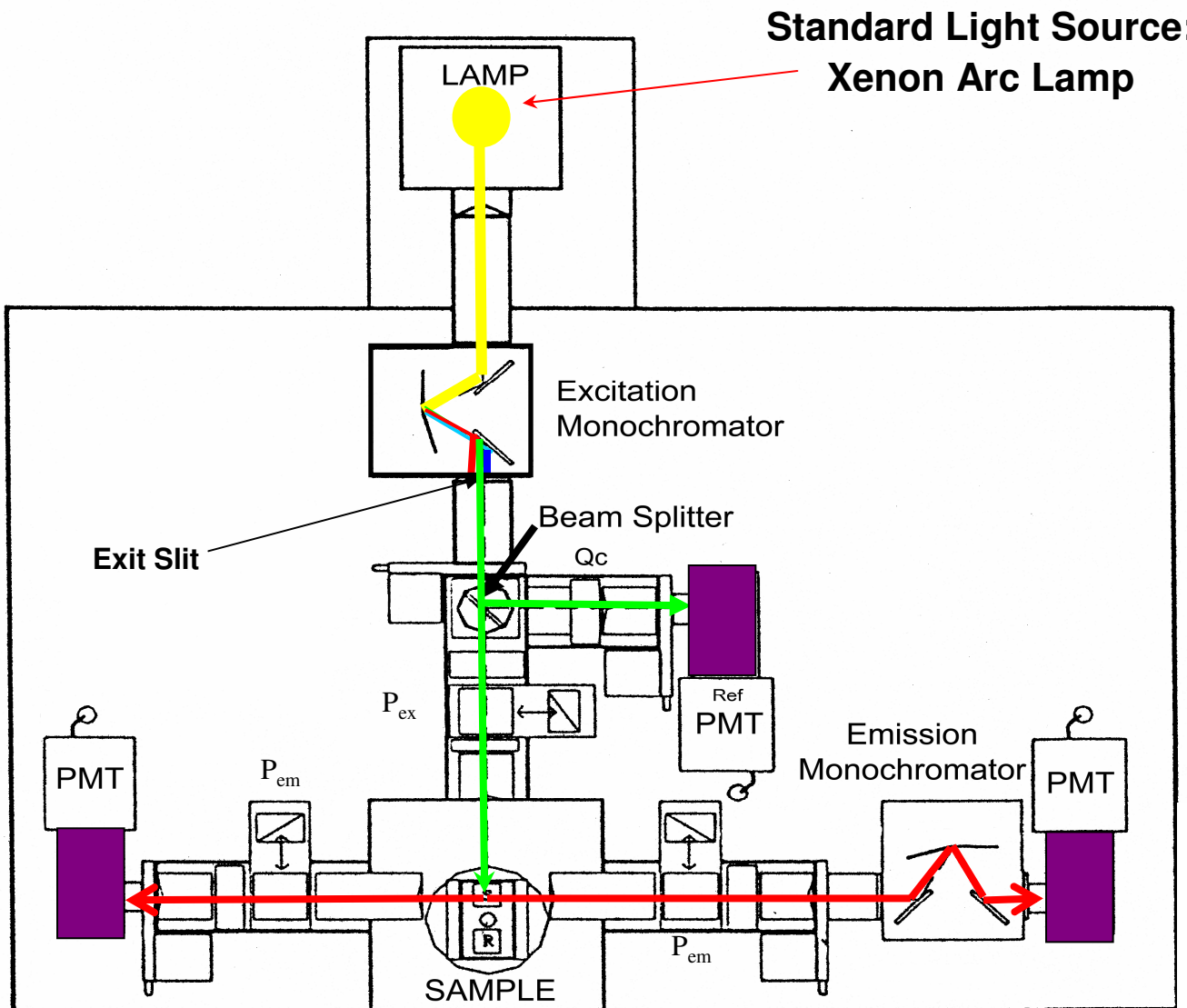
Detector



computer



# The Laboratory Fluorimeter



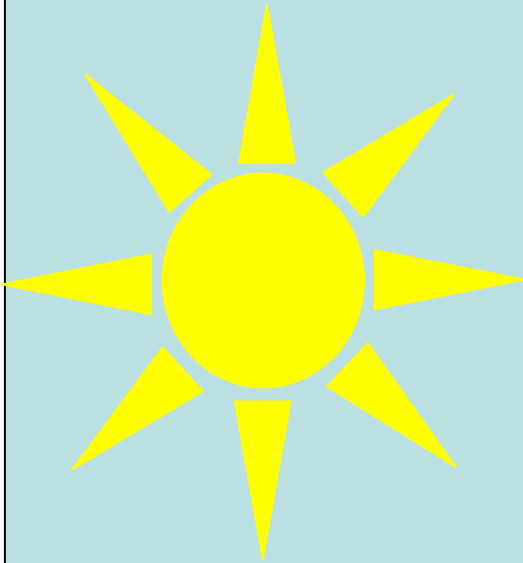
Standard Light Source:  
Xenon Arc Lamp

ISS (Champaign, IL, USA) PC1 Fluorimeter

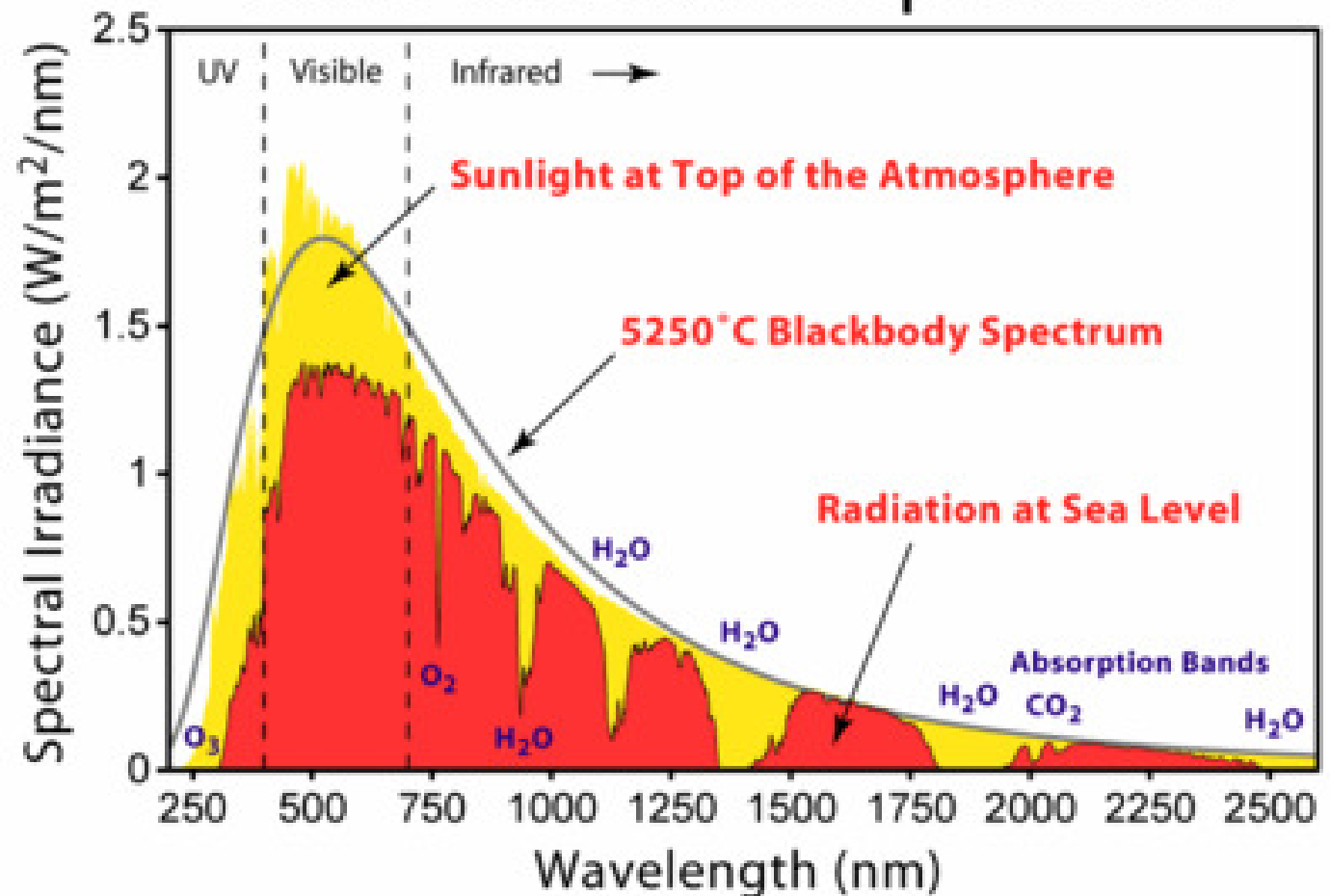
# Light Sources



# Light Sources



## Solar Radiation Spectrum



# Lamp Light Sources

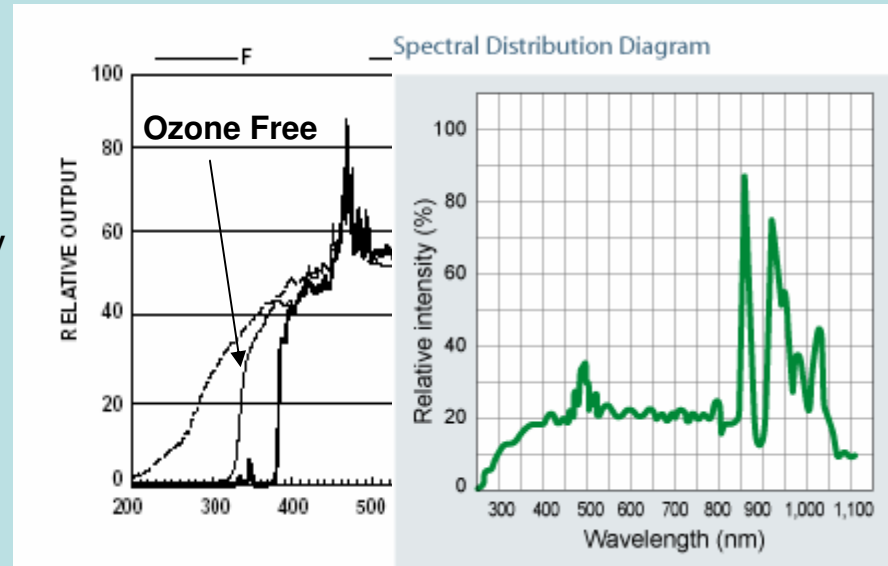
## Gas discharge lamps

### Xenon Arc Lamp (wide range of wavelengths)

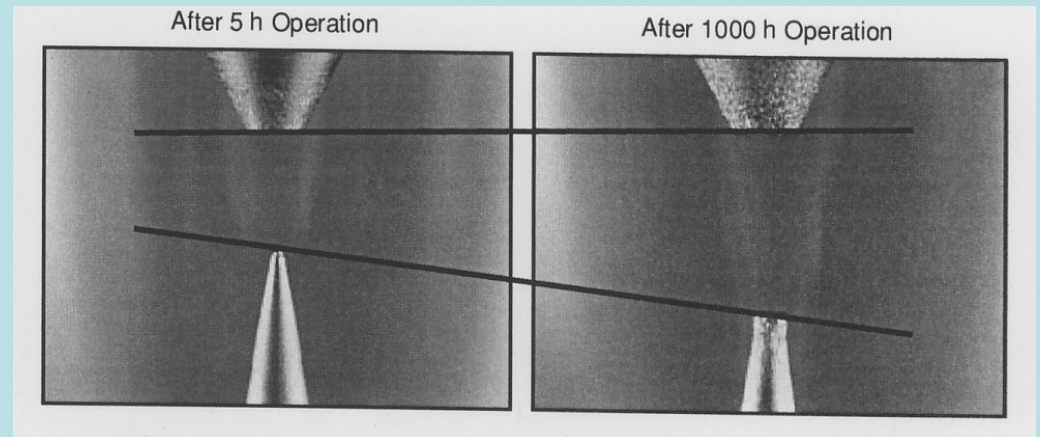
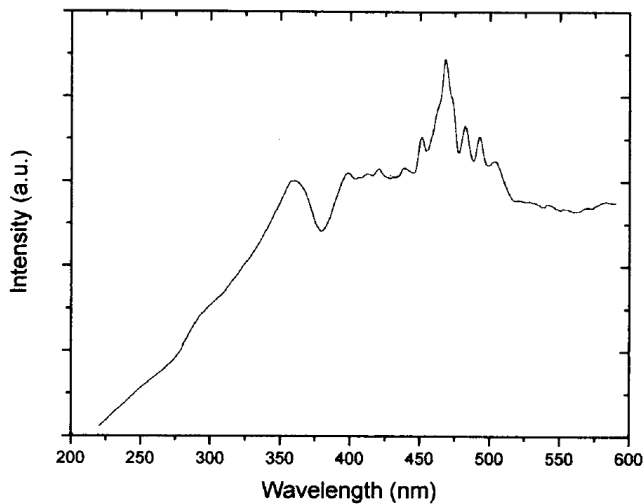
Introduced in 1951 by the Osram Company



## Xenon Arc Lamp Profiles



These lamps use tungsten electrodes and xenon gas at pressures up to 25 atmospheres. A UV-blocking material can be used to coat the interior of the bulb envelope which prevents the production of ozone outside of the lamp housing.

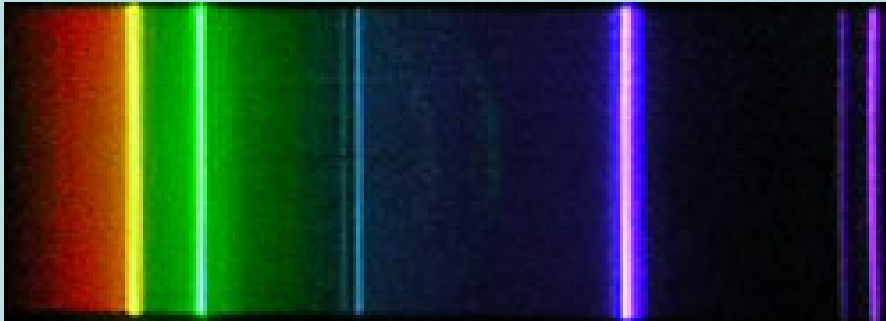


[http://jp.hamamatsu.com/resources/products/etd/eng/image/xe\\_hgxe\\_003.jpg](http://jp.hamamatsu.com/resources/products/etd/eng/image/xe_hgxe_003.jpg)

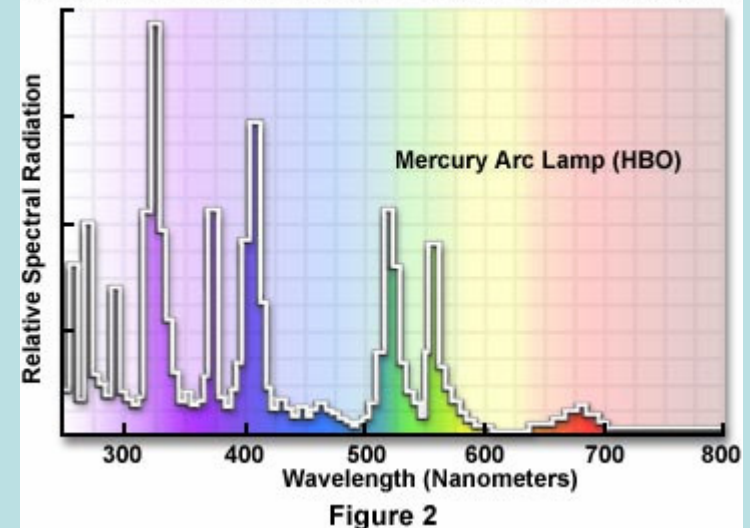
# Lamp Light Sources

## Gas discharge lamps

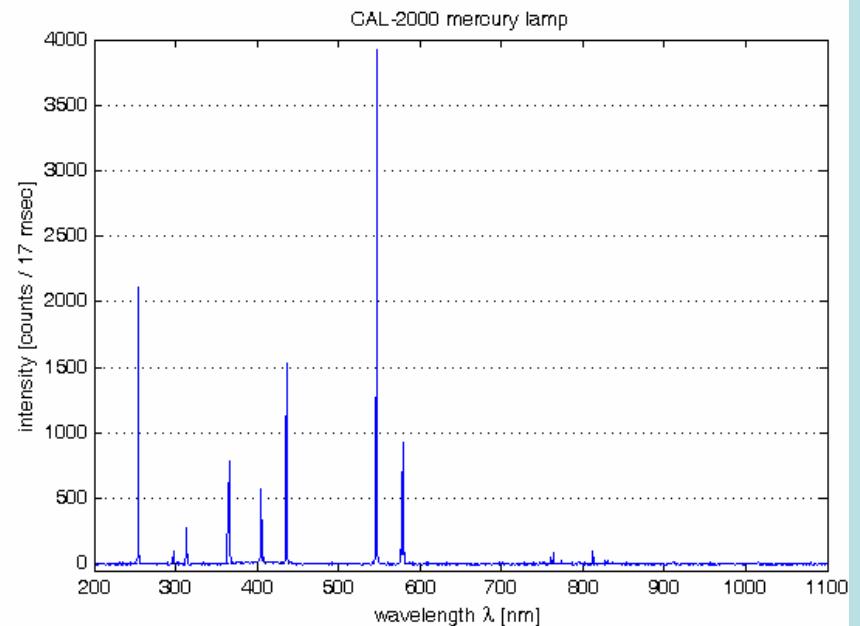
**High Pressure Mercury Lamps**  
(High Intensities concentrated in specific lines)



Mercury Arc Lamp UV and Visible Emission Spectrum



There are strong lines near 254nm, 297nm, 333nm, 365nm, 405nm, 436nm, 546nm and 568nm

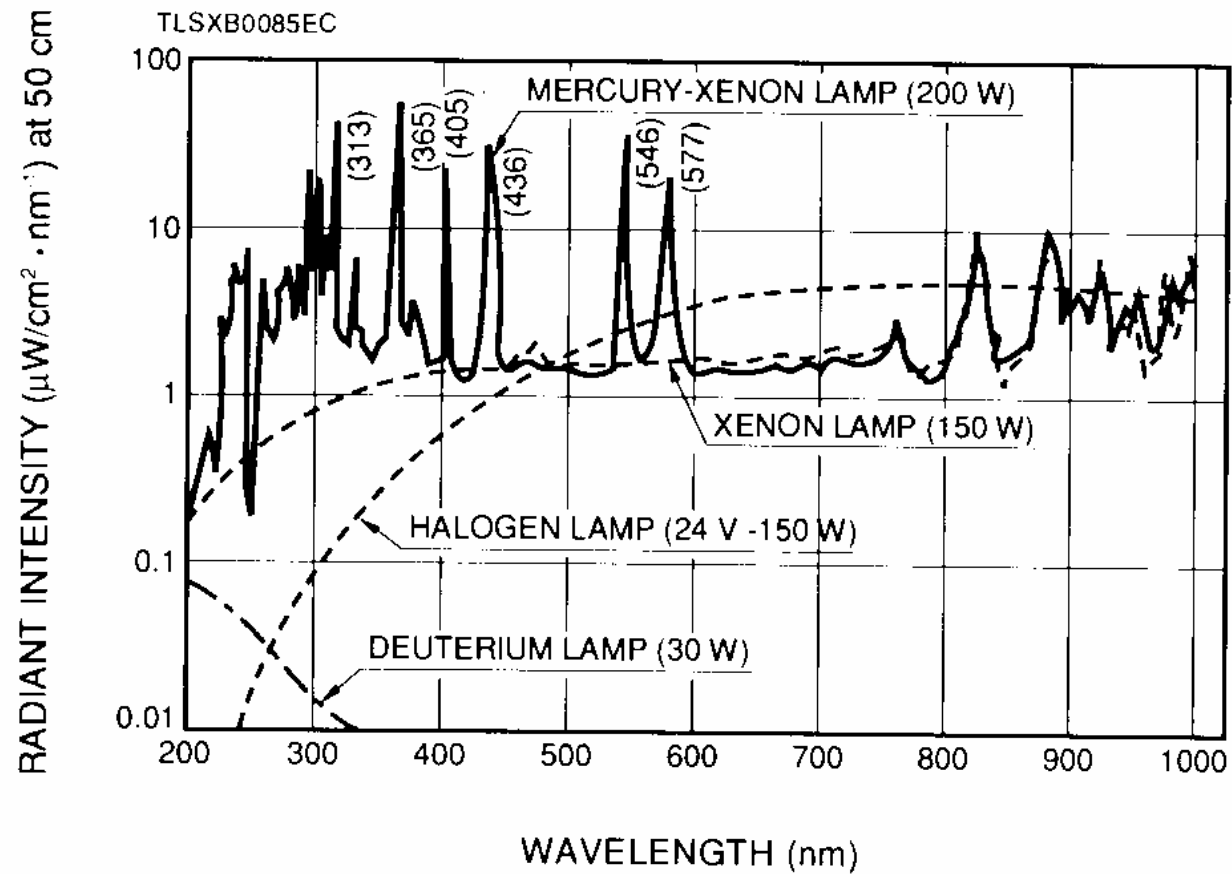




# Lamp Light Sources

Mercury-Xenon Arc Lamp (greater intensities in the UV)

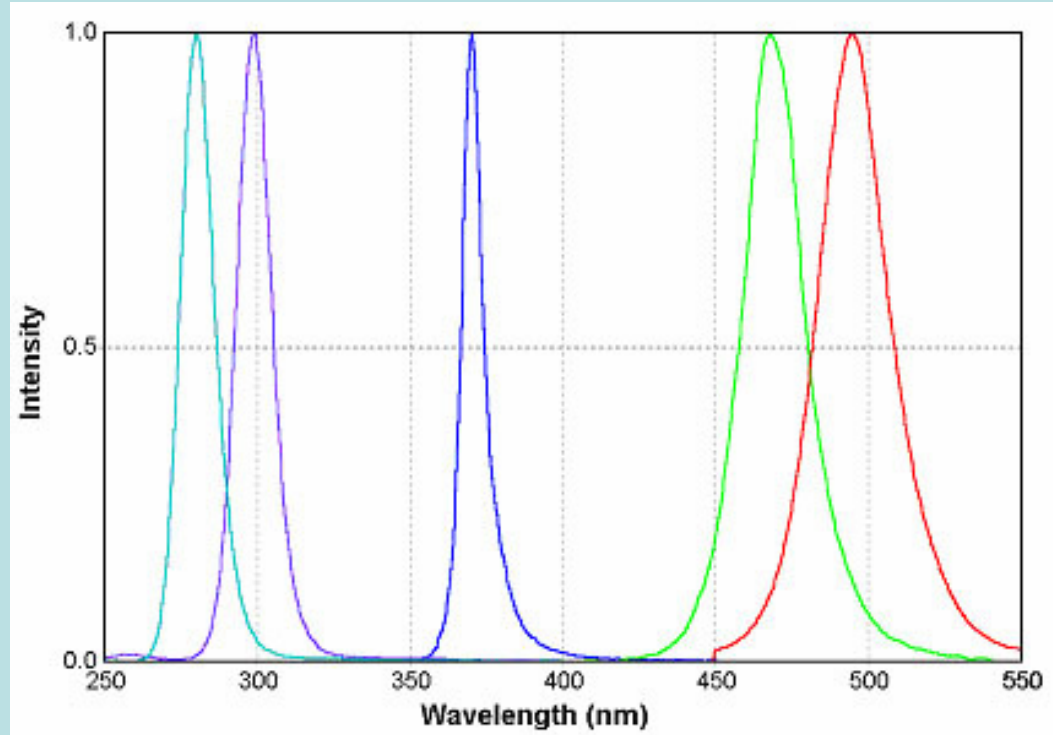
Figure 5: Spectral Distribution of Various Lamps



# Light Emitting Diodes (LED)

Electroluminescence from a semiconductor junction

Wavelengths from 260 nm to 2400 nm



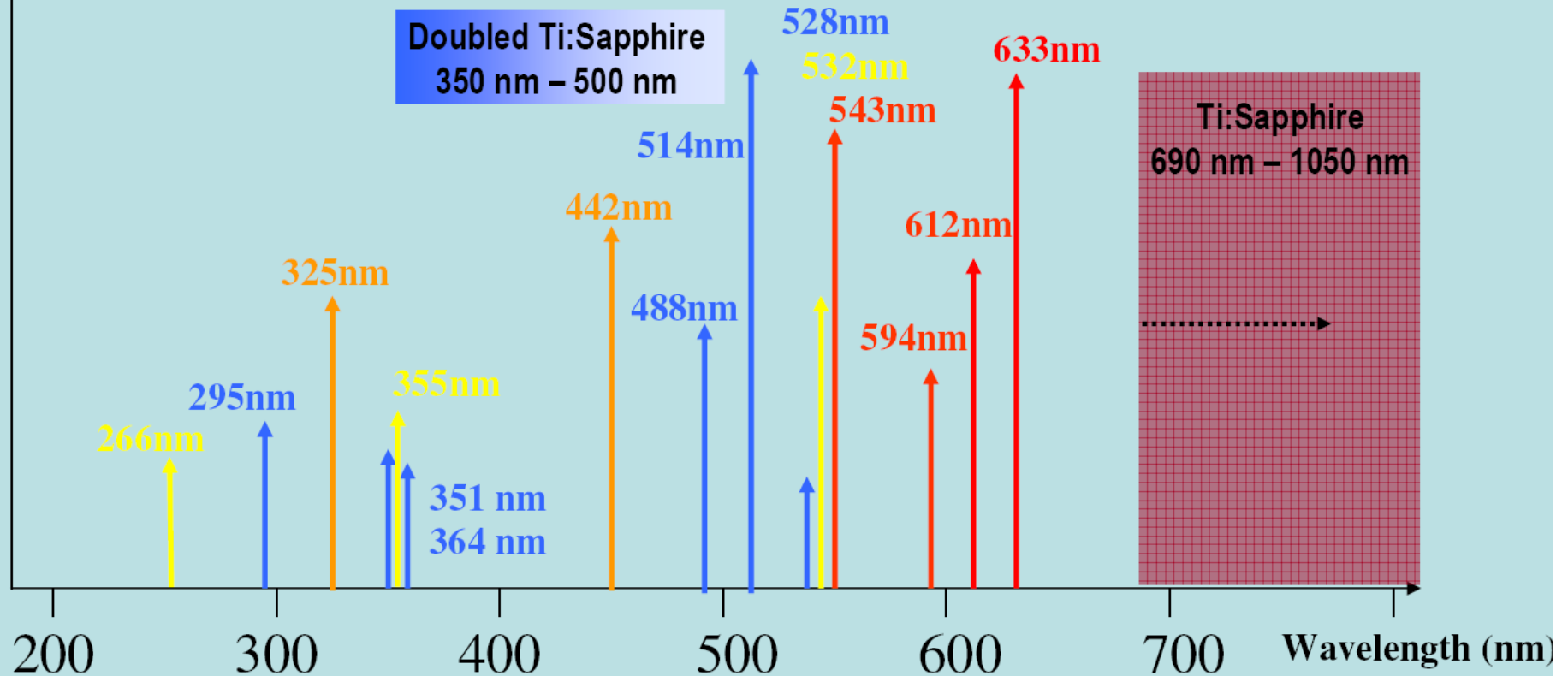
Lamp	Luminous Flux (Lumens)	Spectral Irradiance (Milliwatt/Square Meter/Nanometer)
HBO 100 Watts	2200	30 (350-700 nm)
XBO 75 Watts	1000	7 (350-700 nm)
Tungsten 100 Watts	2800	< 1 (350-700 nm)
LED (Blue, 450 nm)	160	6

# Laser Light Sources

Quiz: What does LASER stand for?

Light Amplification by Stimulated Emission of Radiation

Argon Ion:					
Wavelength	Rel Pwr	Wavelength	Rel Pwr	Wavelength	
528.7nm	0.16	476.5nm	0.29	437nm	
514.5nm	1.0	472.7nm	0.10	364nm	
501.7nm	0.2	465.8nm	0.07	351nm	
496.5nm	0.35	457.9nm	0.18	....	
488.0nm	0.78	454.5nm	0.06	275nm	



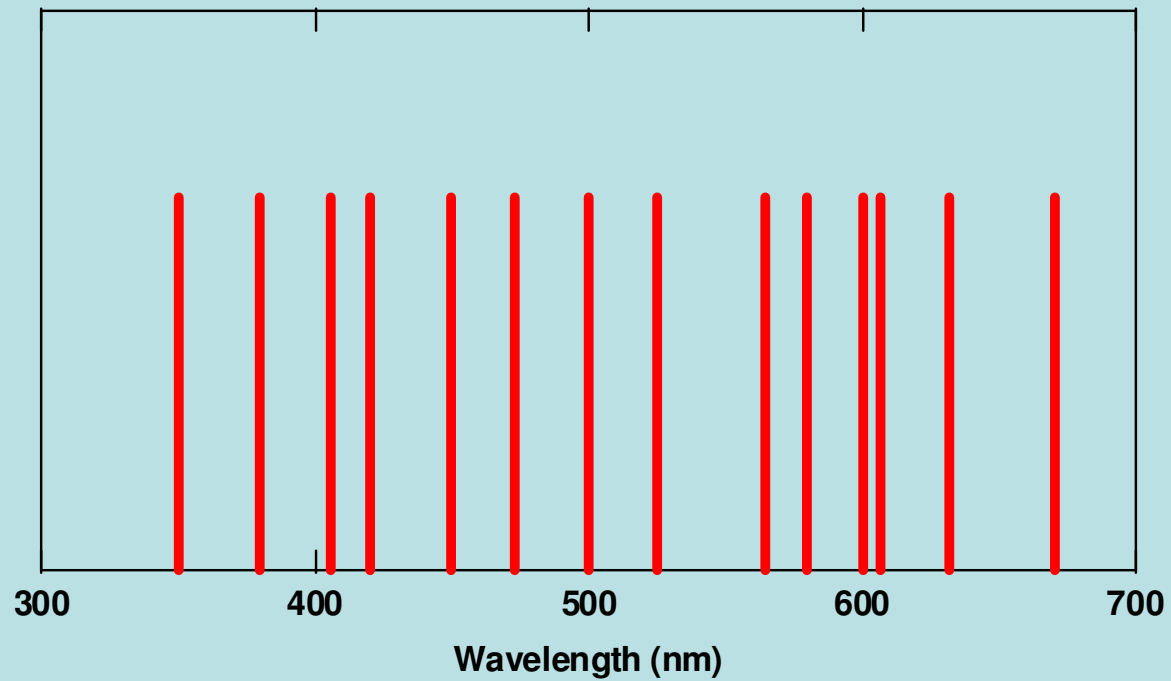
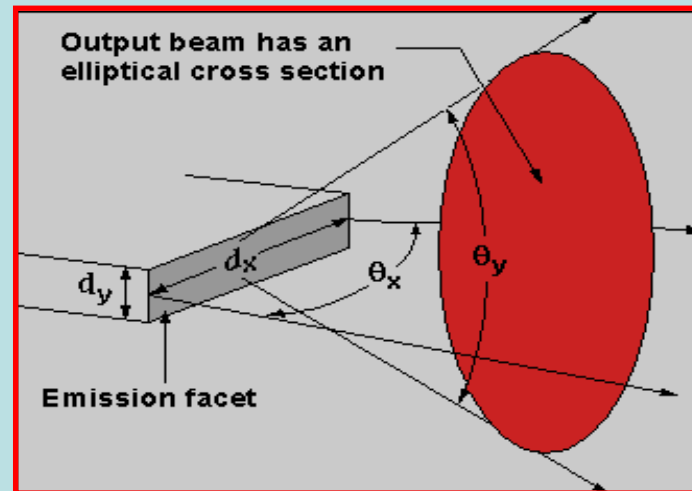
↑  
Argon-ion  
100 mW

↑  
Helium-cadmium

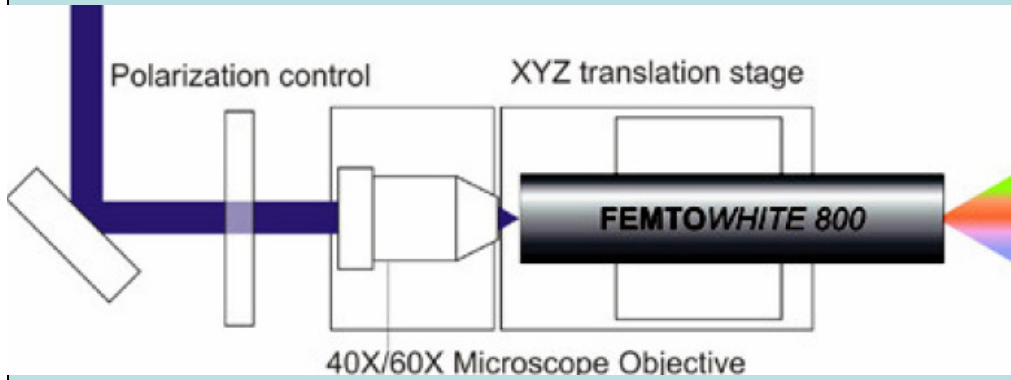
↑  
Nd-YAG

↑  
He-Ne  
Red 633nm >10 mW  
Orange 612nm 10mW  
Yellow 594nm 4mW  
Green 543nm 3mW

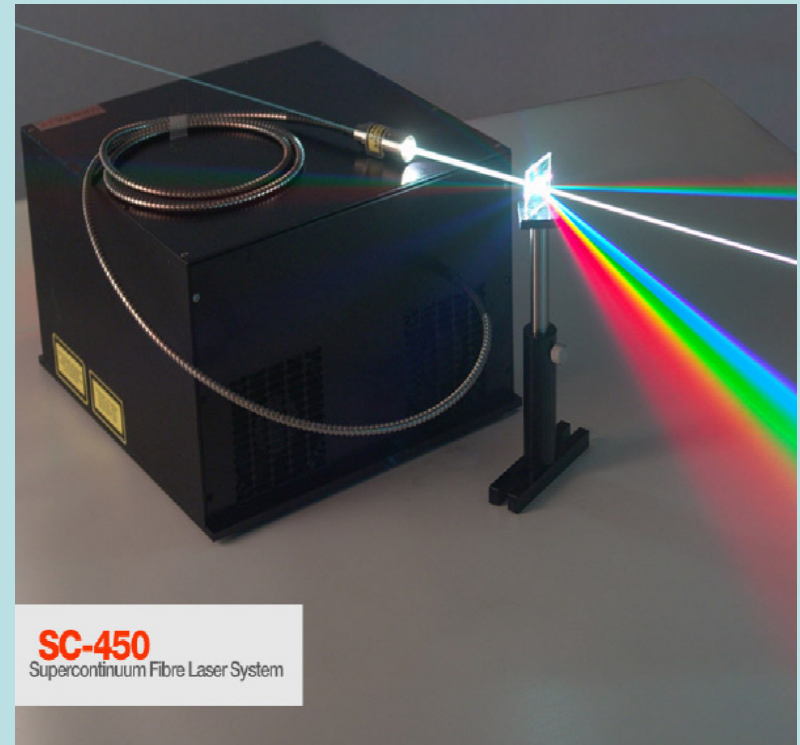
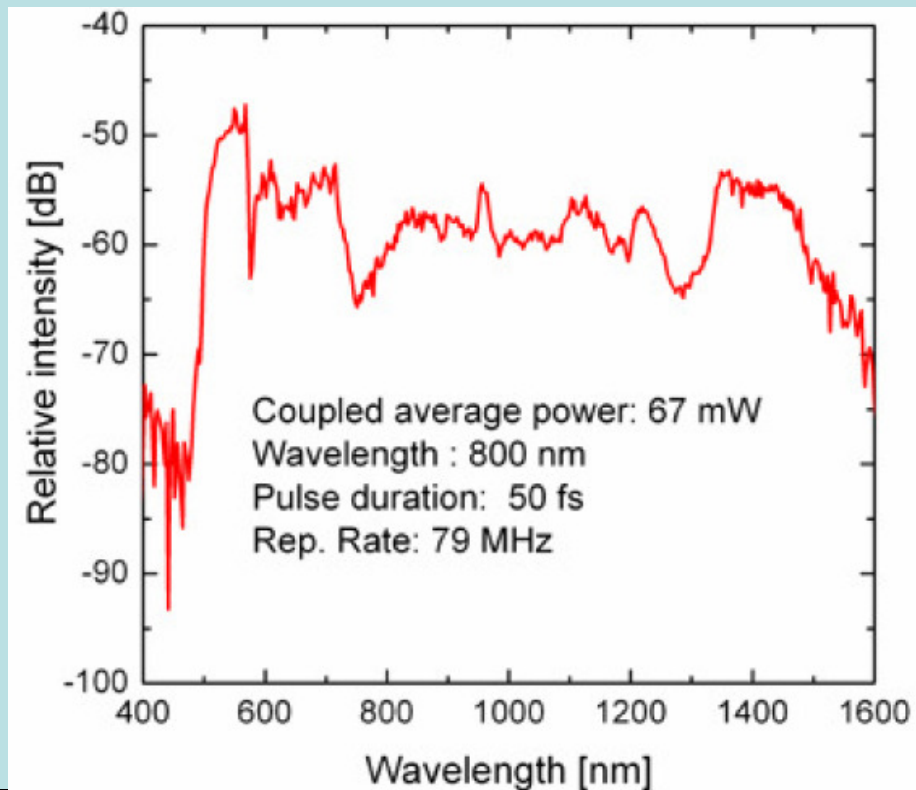
# Laser Diodes



# “White” lasers



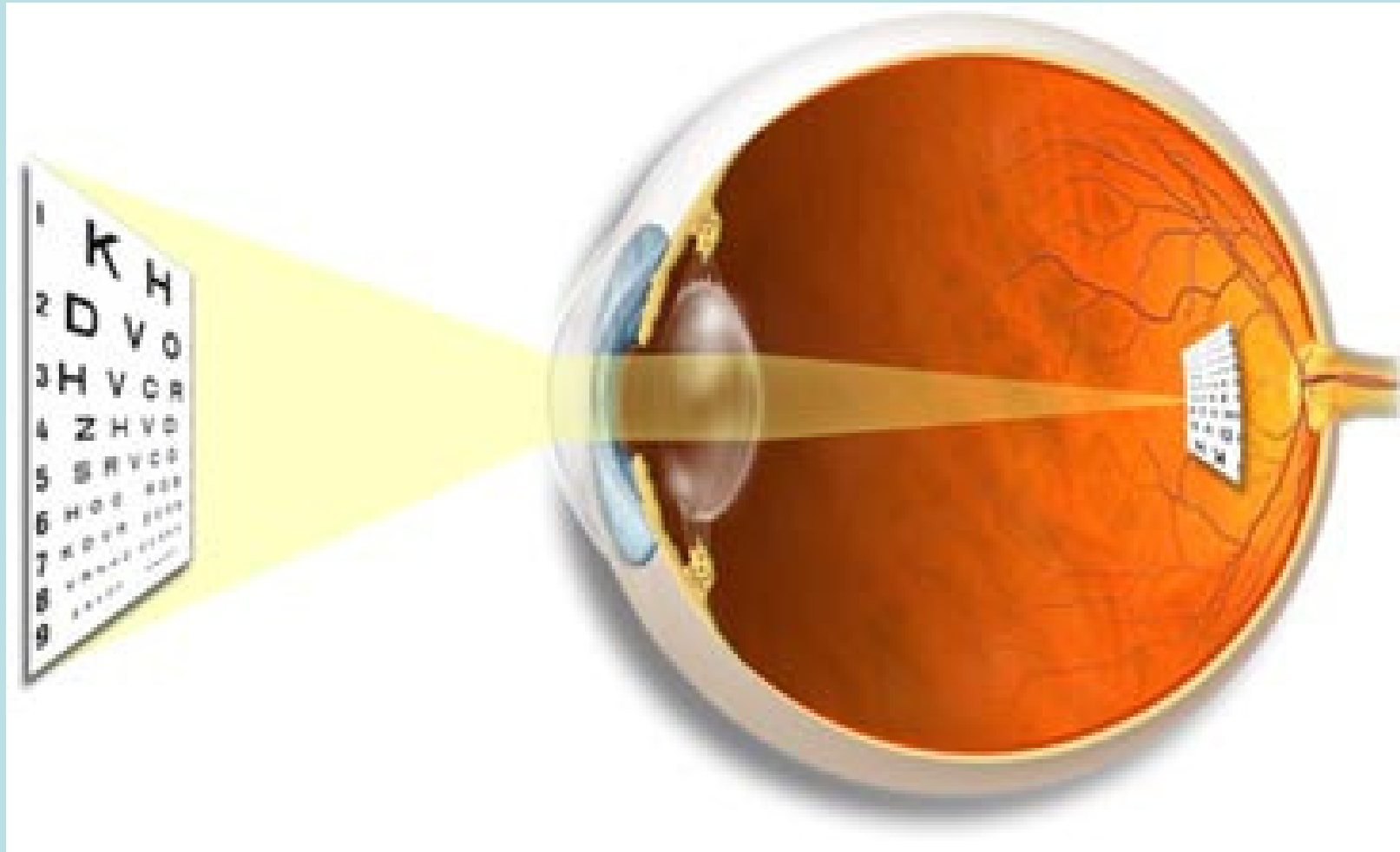
Ultrashort pulsed light is focused into a photonic crystal fiber



# New light source being tested in Hawaii

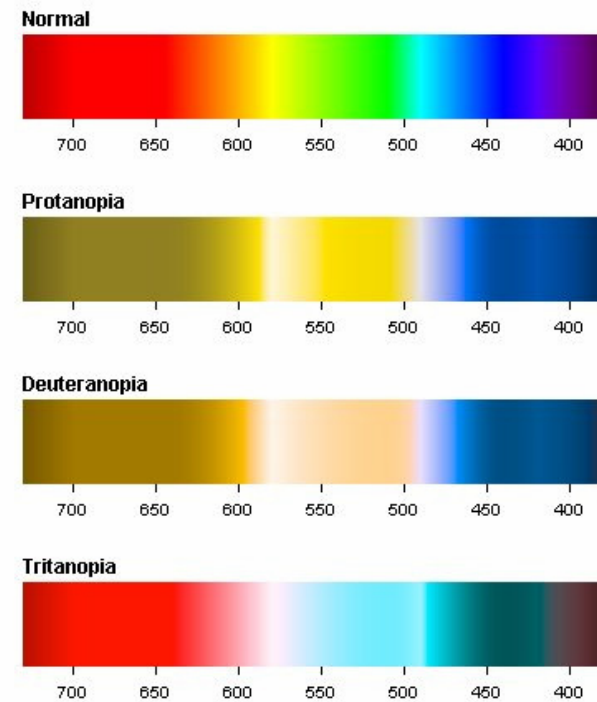
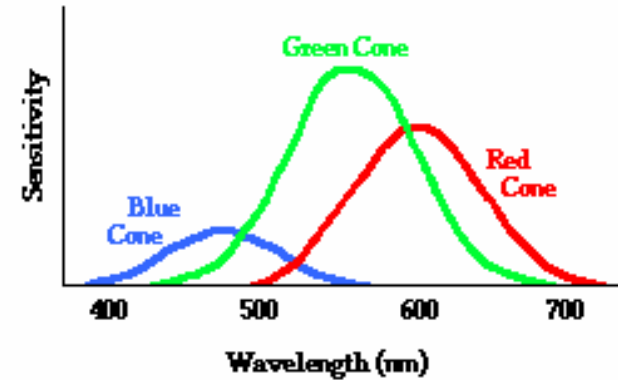
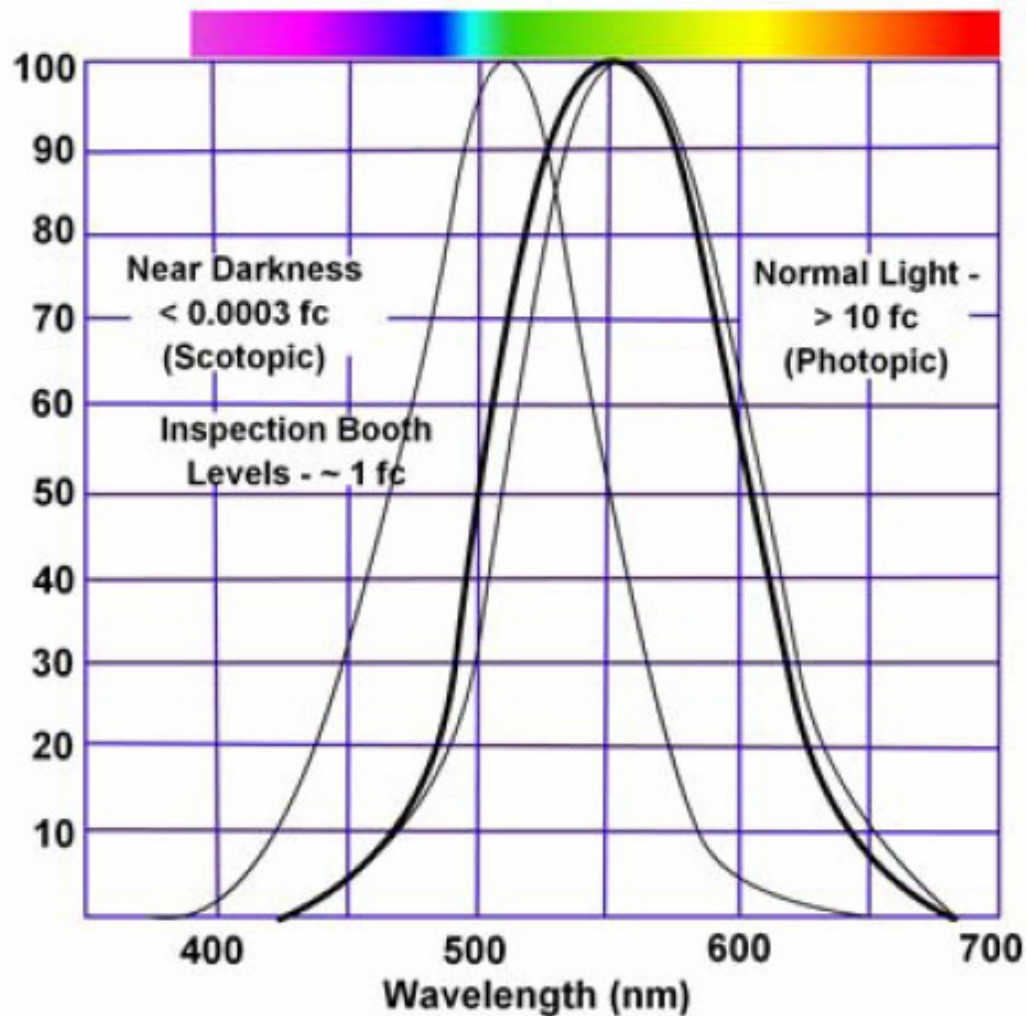


# Detectors



# Detectors

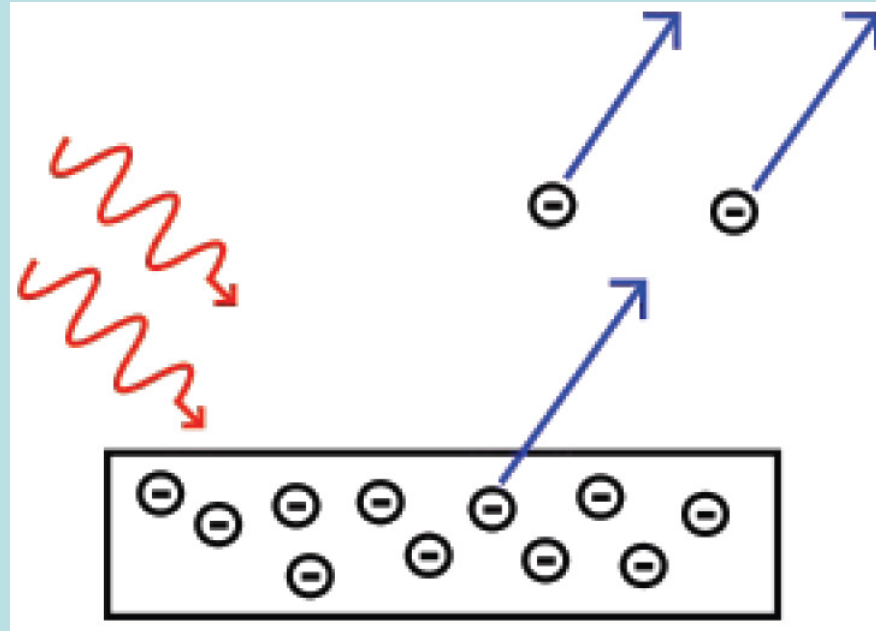
## The Human Eye's Response to Light



# Detectors

The photoelectric effect was discovered by Heinrich Hertz in 1886

Specifically he noticed that a charged object loses its charge more readily when it is illuminated by UV light



It was soon discovered that the energies of the ejected electrons were independent of the intensity of the illuminating light, whereas this energy increased with the frequency of the light. This phenomenon as explained by Einstein in 1905 as being due to the quantum nature of light, i.e., photons. Einstein received his Nobel Prize for this work in 1921.

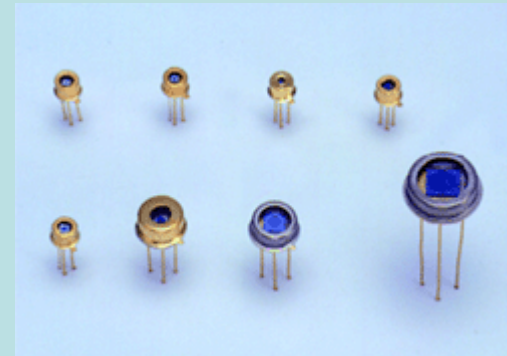
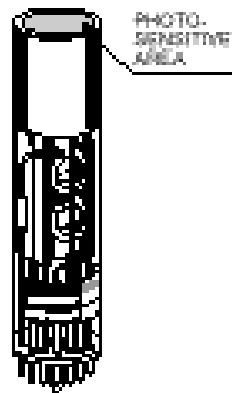
# Detectors

## PMT Types

a) Side-On Type



b) Head-On Type

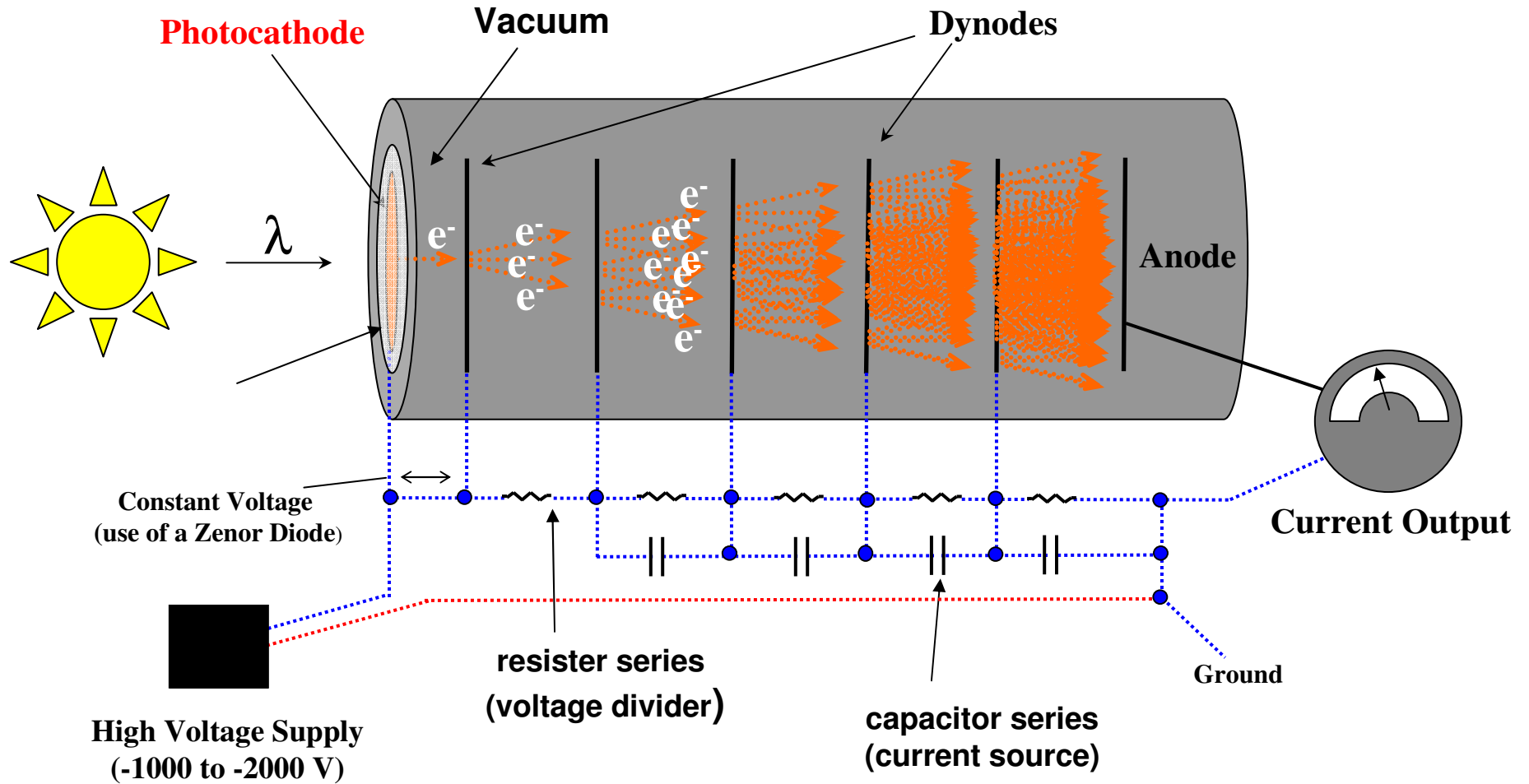


## APD

The silicon avalanche photodiode (Si APD) has a fast time response and high sensitivity in the near infrared region. APDs can be purchased from Hamamatsu with active areas from 0.2 mm to 5.0 mm in diameter and low dark currents (selectable). *Photo courtesy of Hamamatsu*

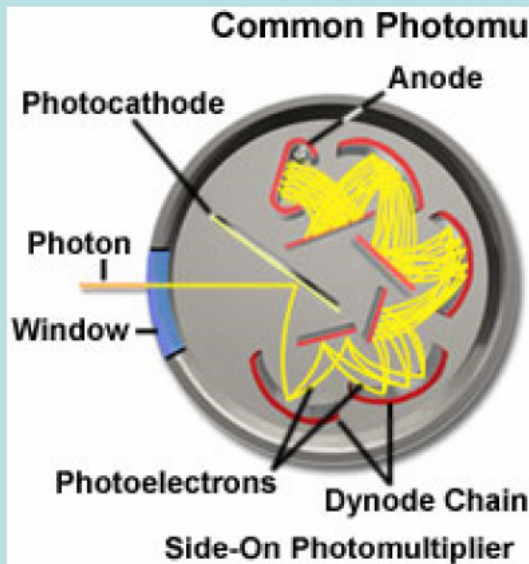
Photomultipliers were developed in the 1930's but not generally adopted for research until after WWII

## The Classic Photomultiplier Tube (PMT) Design



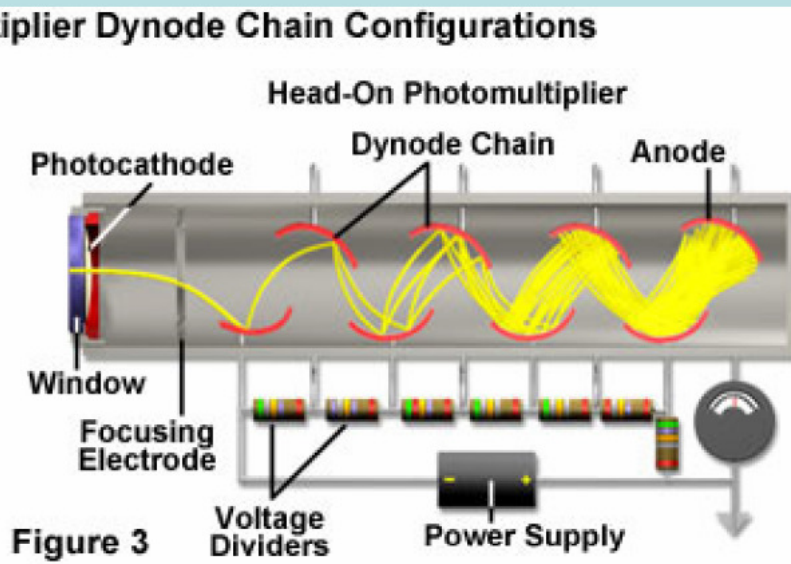
# PMT Geometries

## Side-On PMT



Opaque photocathode

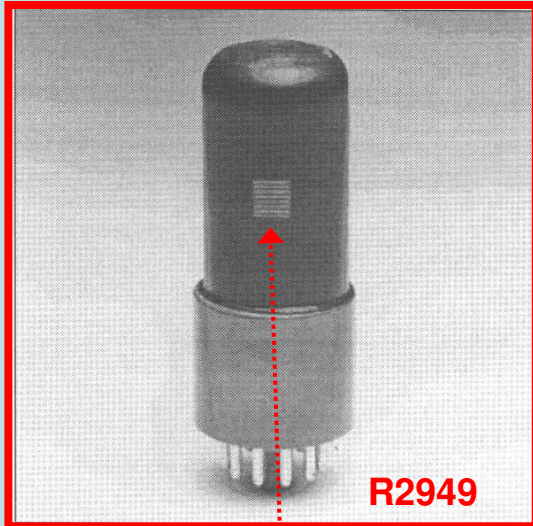
## Head-On PMT



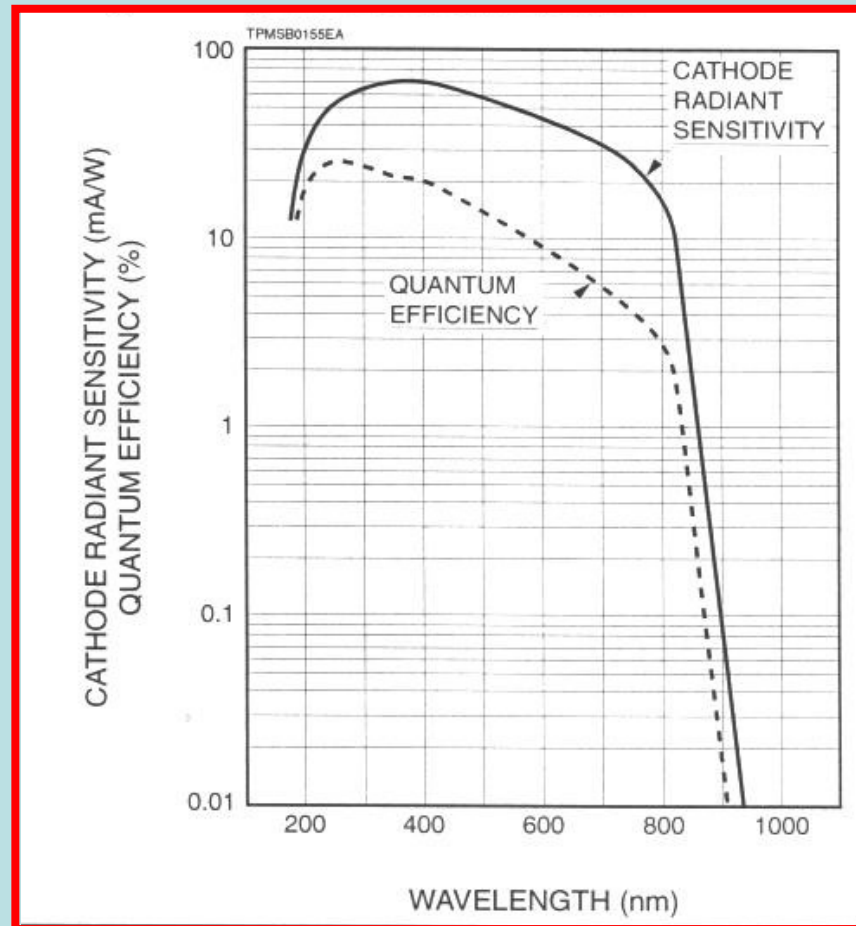
Semitransparent Photocathode

- Side-on PMTs have slightly enhanced quantum efficiency over Head-on PMTs
- Side-on PMTs often have larger afterpulsing probabilities than Head-on PMTs
- Side-on PMTs count rate linearity less than for Head-on PMT
- Head-on PMTs provide better spatial uniformity than Side-on PMTs
- Side-on PMTs have faster response time than Head-on PMTs (compact design)
- Side-on PMTs are less affected by a magnetic field than Head-on PMTs

# Hamamatsu R928 PMT Family

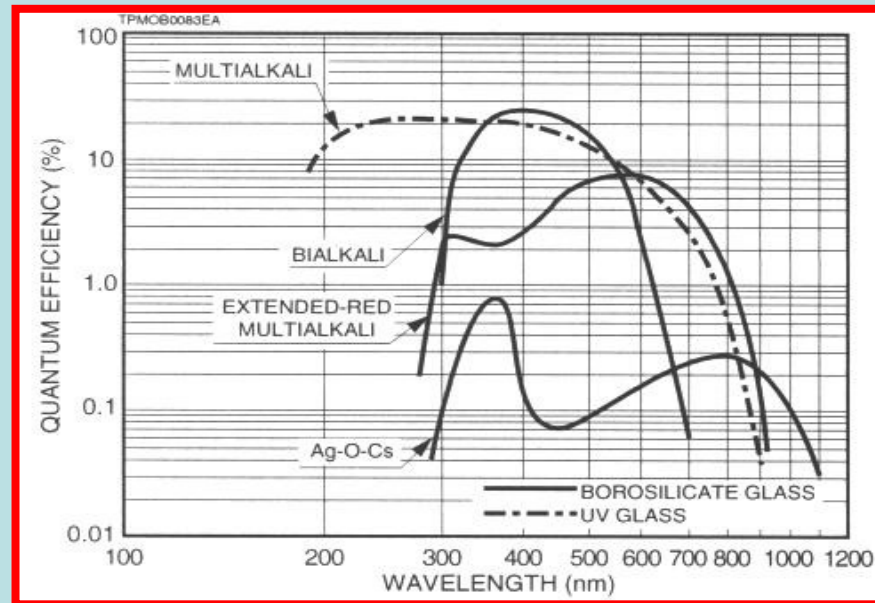


Window with  
Photocathode Beneath

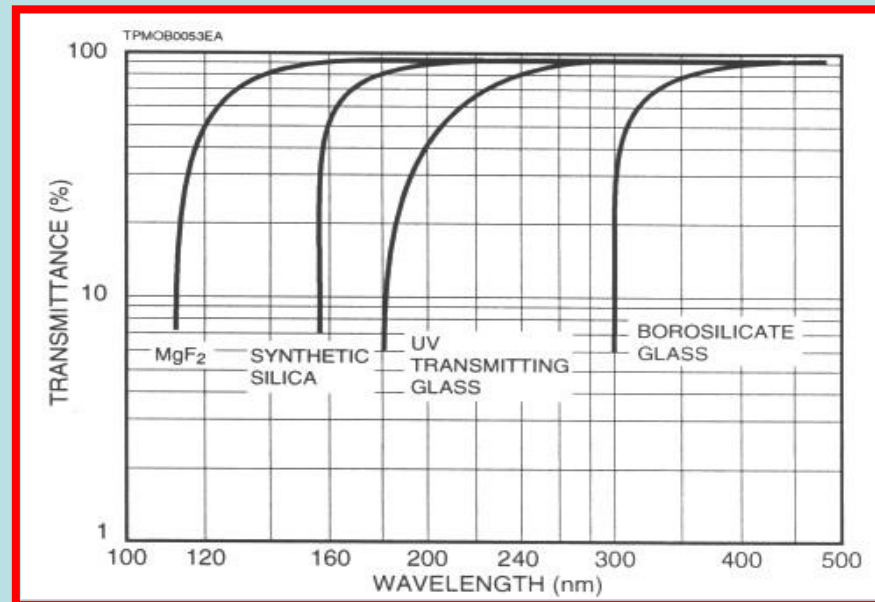


# PMT Quantum Efficiencies

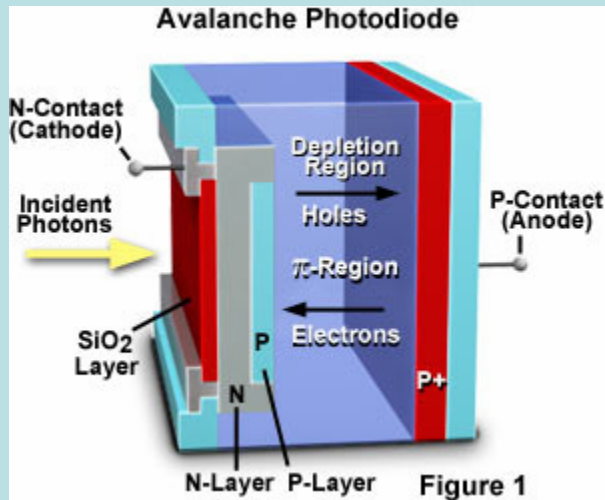
Cathode Material



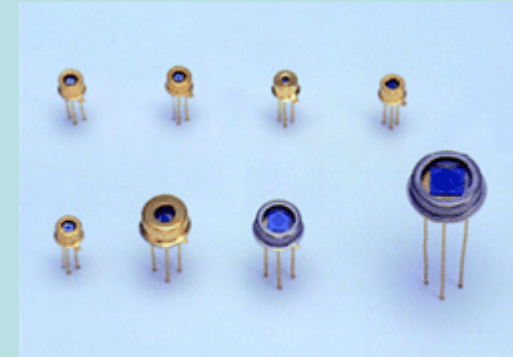
Window Material



# Detectors

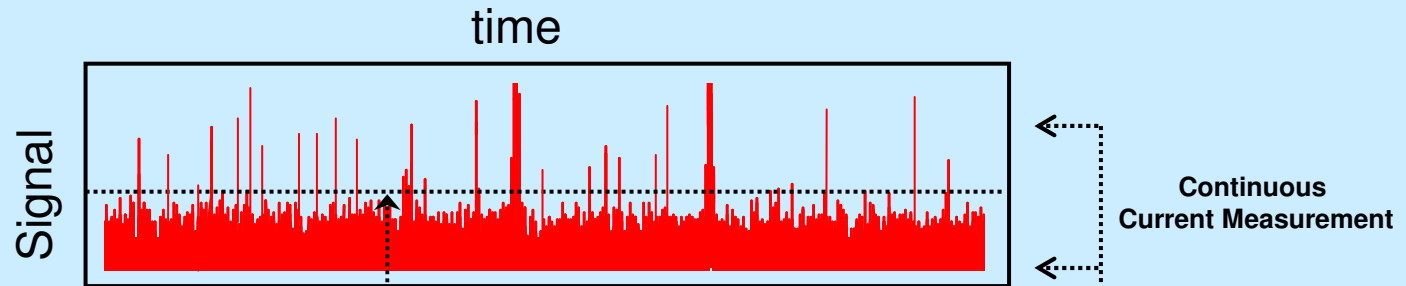


APDs are usually used in applications characterized by low light levels

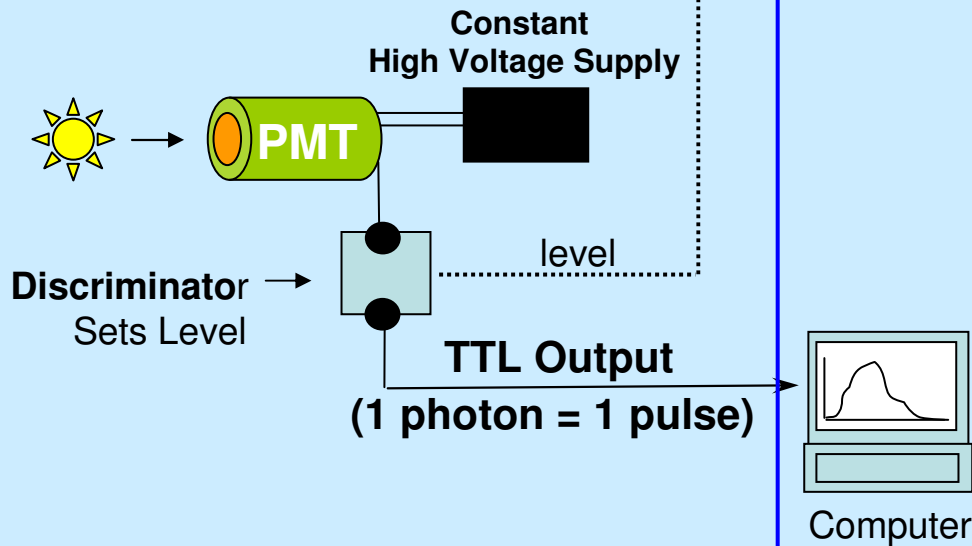


The silicon avalanche photodiode (Si APD) has a fast time response and high sensitivity in the near infrared region. APDs can be purchased from Hamamatsu with active areas from 0.2 mm to 5.0 mm in diameter and low dark currents (selectable). *Photo courtesy of Hamamatsu*

# Photon Counting (Digital) and Analog Detection



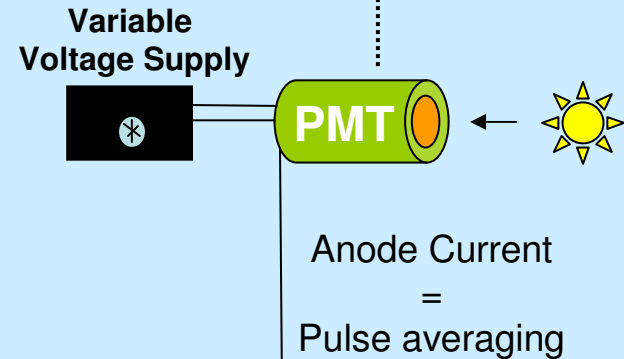
## Photon Counting:



### Primary Advantages:

1. Sensitivity (high signal/noise)
2. Increased measurement stability

## Analog:



### Primary Advantage:

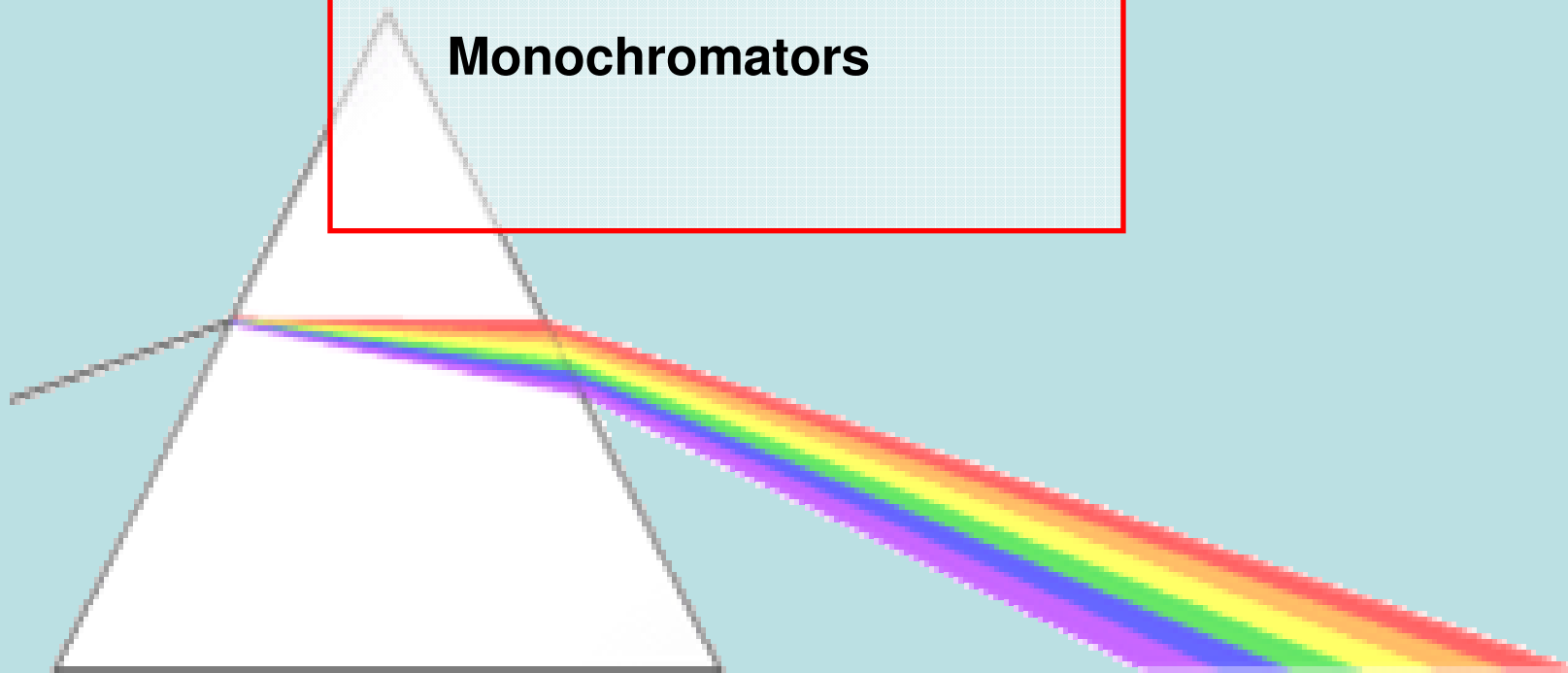
1. Broad dynamic range
2. Adjustable range

# Wavelength Selection

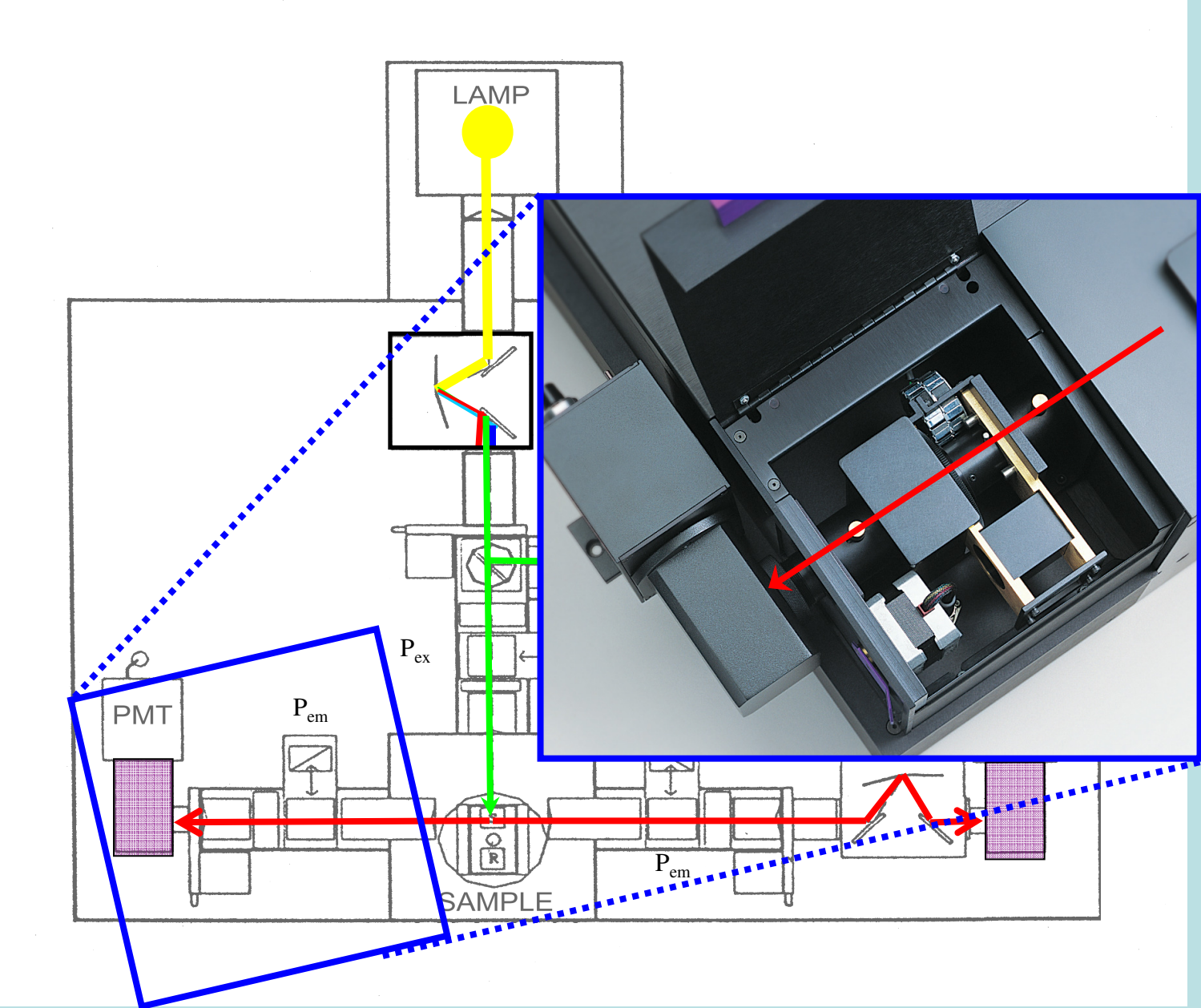
**Fixed Optical Filters**

**Tunable Optical Filters**

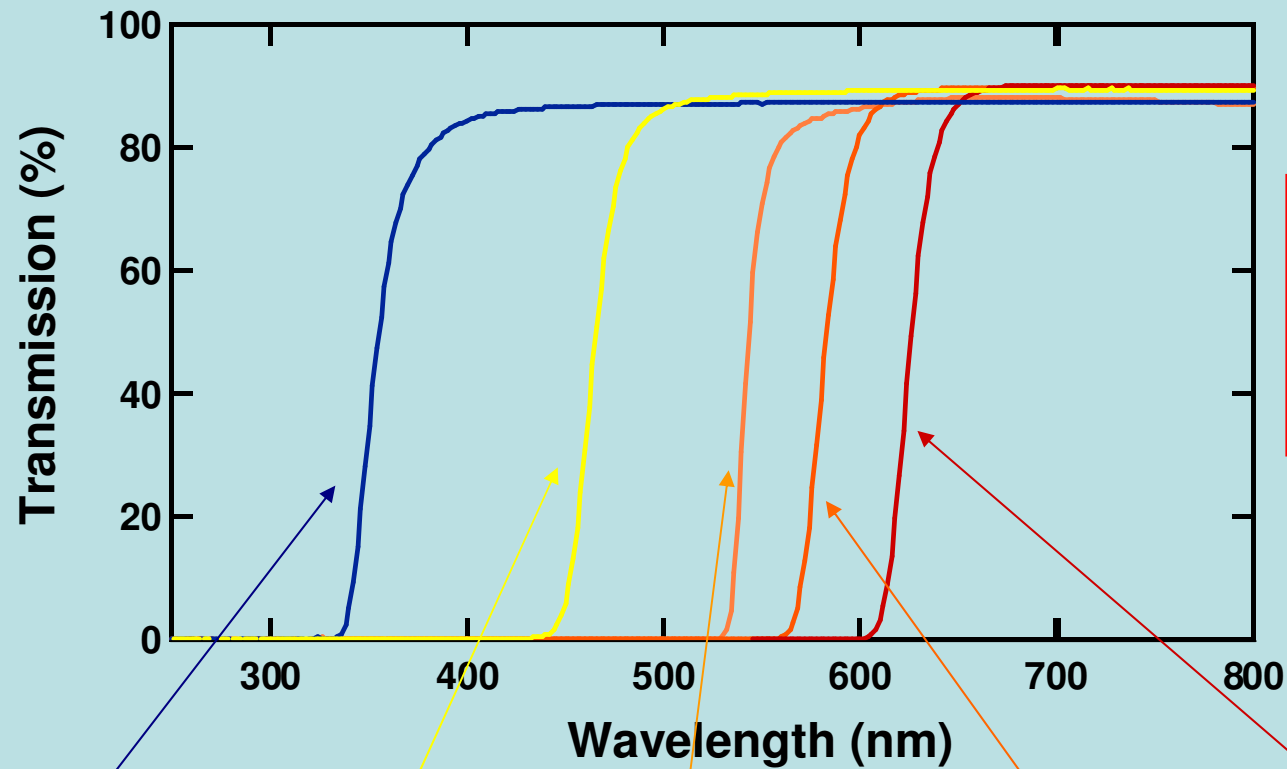
**Monochromators**



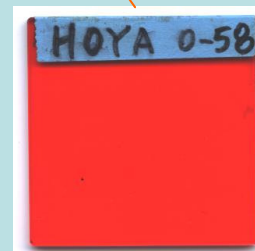
# Optical Filter Channel



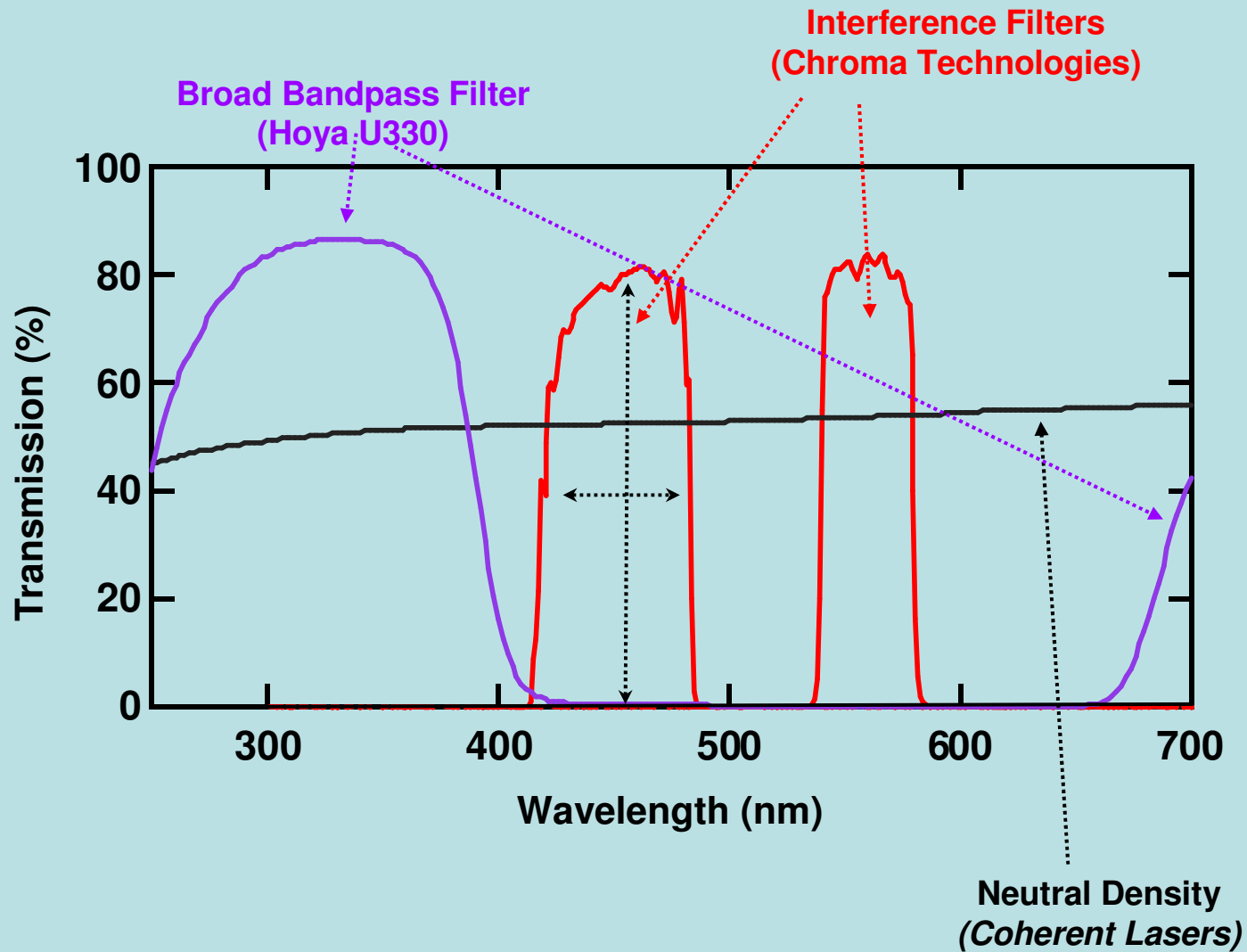
# Long Pass Optical Filters



Spectral Shape  
Thickness  
Physical Shape  
Fluorescence (!?)

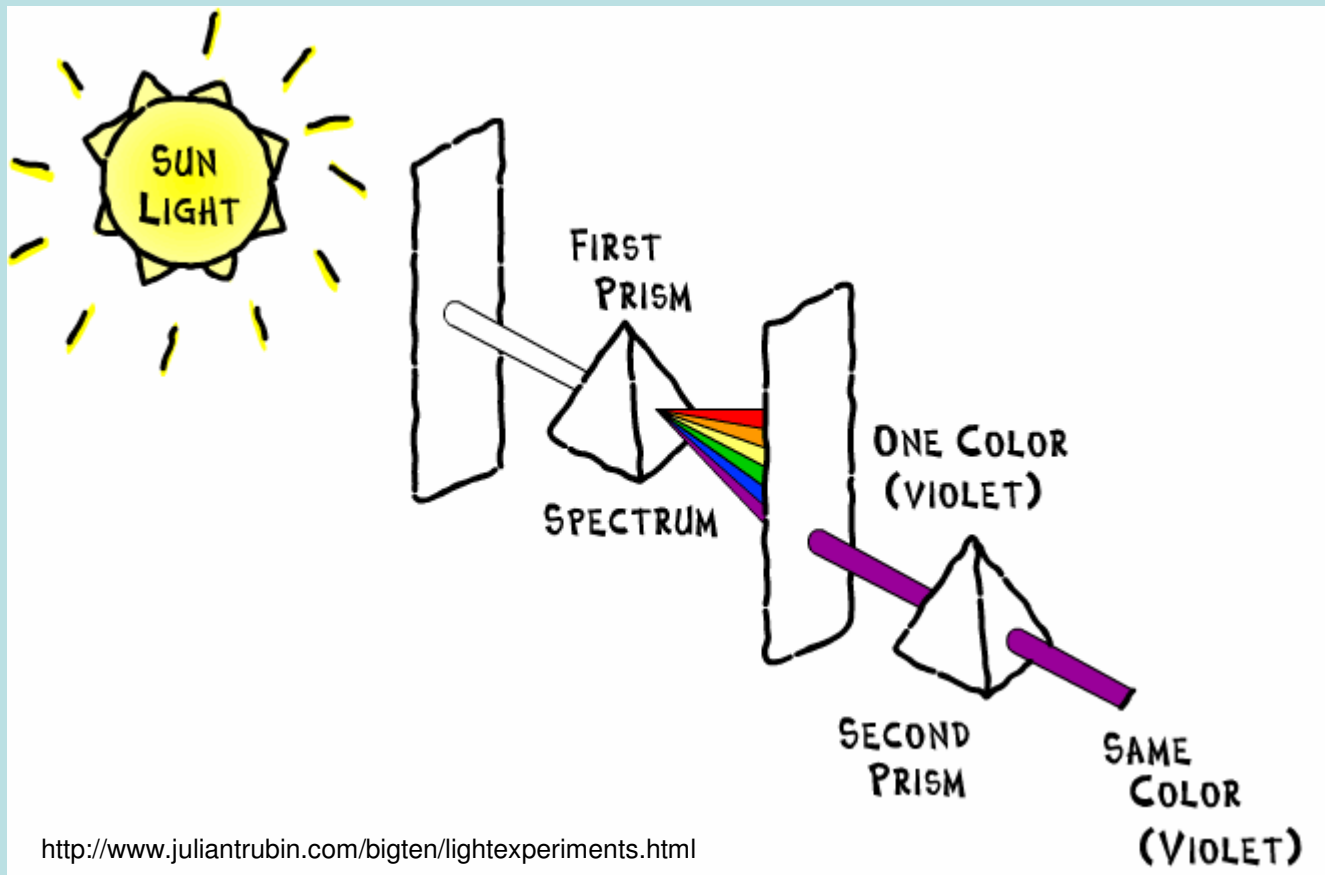


# More Optical Filter Types...



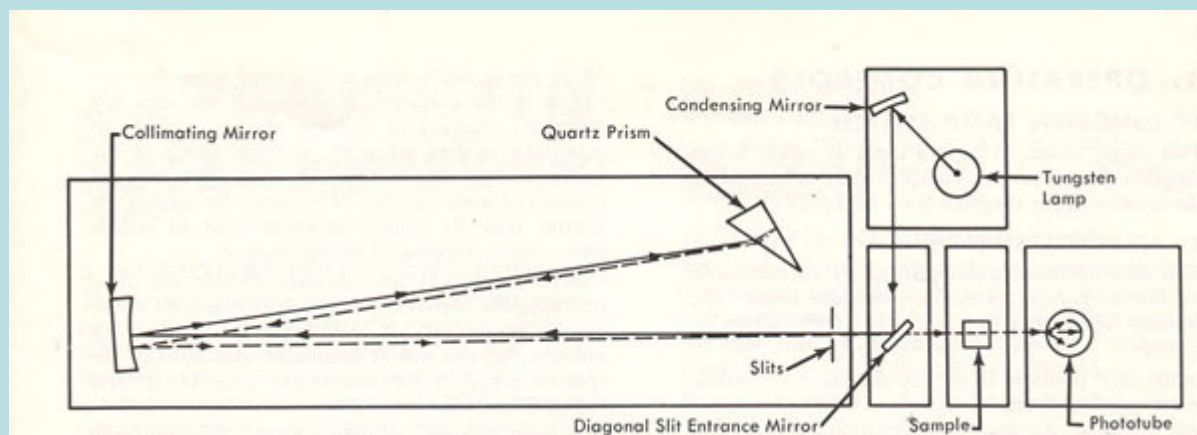
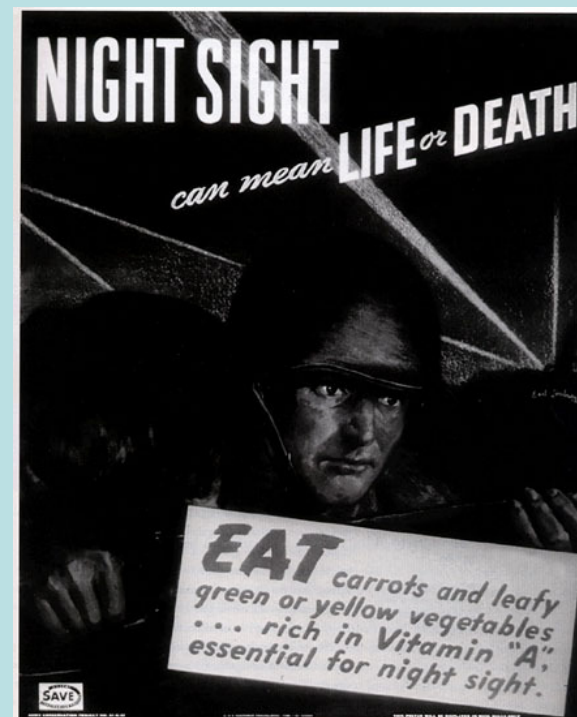
# Monochromators

People had experimented with prisms and light before Newton – but generally it was thought that the prism somehow “colored” the light. Newton was the first to clearly state that the prism revealed an underlying characteristic of white light – namely that it was composed of many colors.

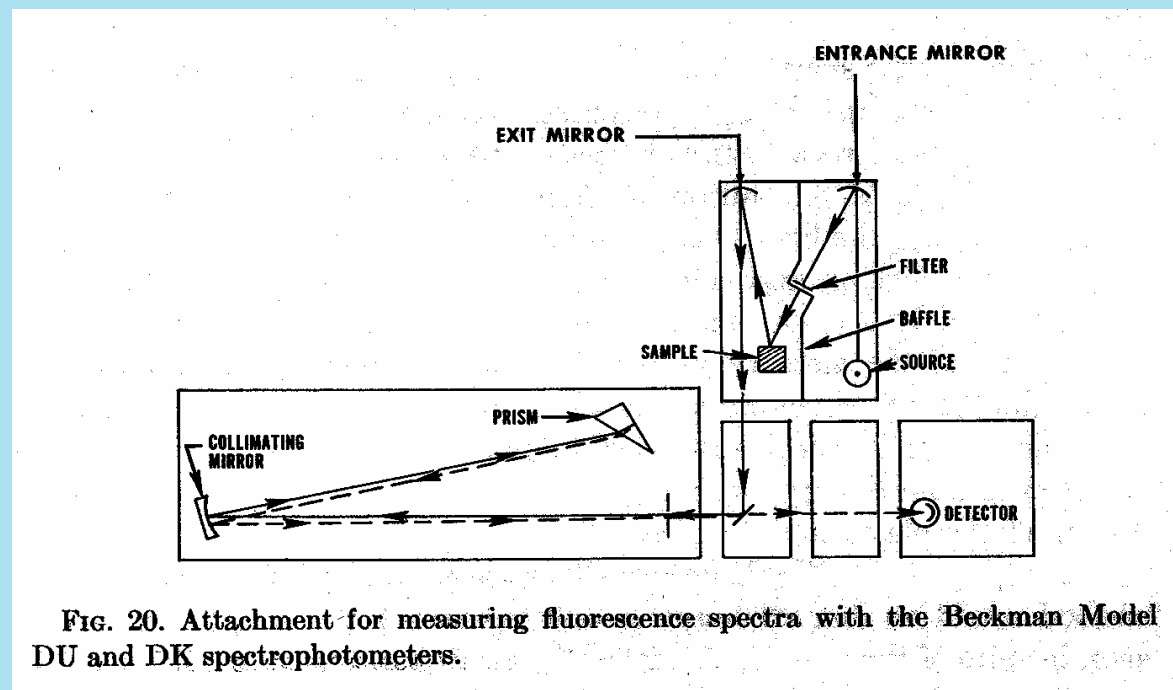


# Monochromators

An important impetus to the development of optical spectroscopy was the discovery that vitamin A had a characteristic absorption in the ultraviolet region of the spectrum. The Government was very interested in the development of methods to measure and characterize the vitamin content of foods. This initiative eventually led to the **Beckman DU UV-vis spectrophotometer**

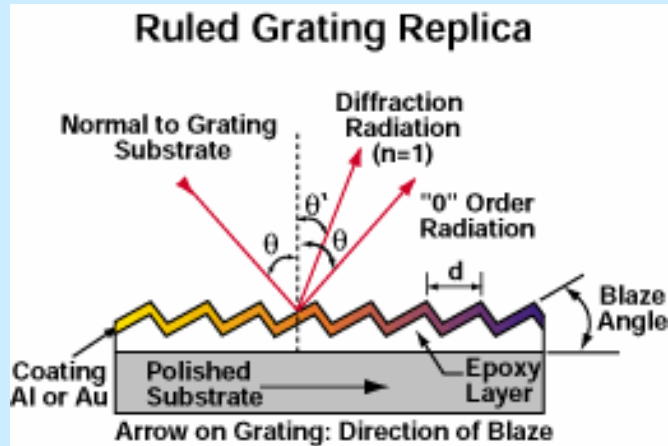


The earliest commercial fluorescence instruments were essentially attachments for spectrophotometers such as the Beckman DU spectrophotometer; this attachment allowed the emitted light (excited by the mercury vapor source through a filter) to be reflected into the spectrophotometer's monochromator. The first description of this type of apparatus was by R.A. Burdett and L.C. Jones in 1947 (J. Opt. Soc. Amer. 37:554).



The problem with prisms, however, was that the light dispersion was not linear with wavelength and normal glass prisms did not pass UV light – so expensive quartz prism had to be used. For these reasons grating based systems became more popular.

# Diffraction Gratings



## GRATING EQUATION

$$n\lambda = d (\sin[\theta] \pm \sin[\theta'])$$

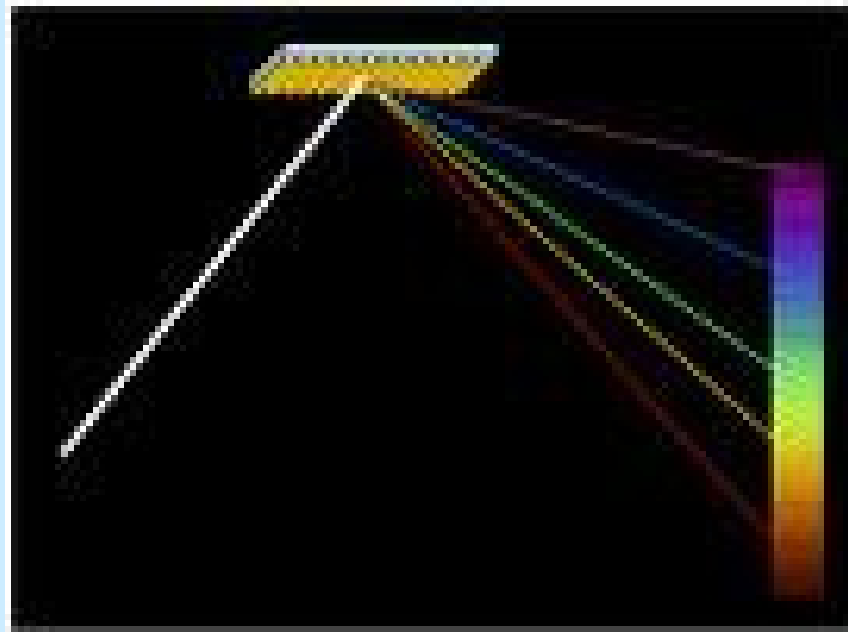
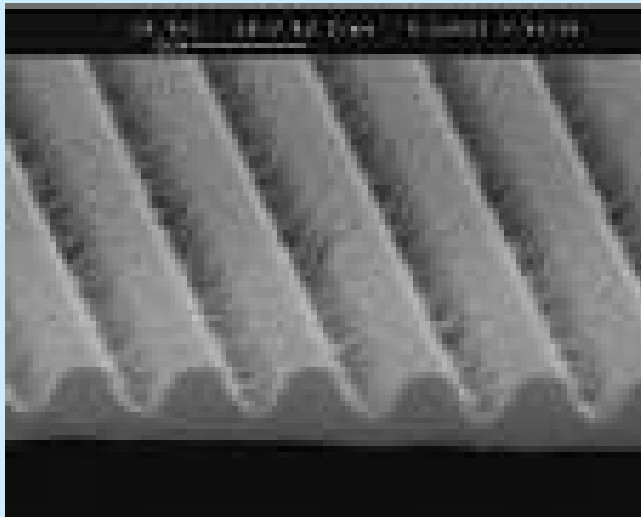
$n$  = order of diffraction

$d$  = grating constant

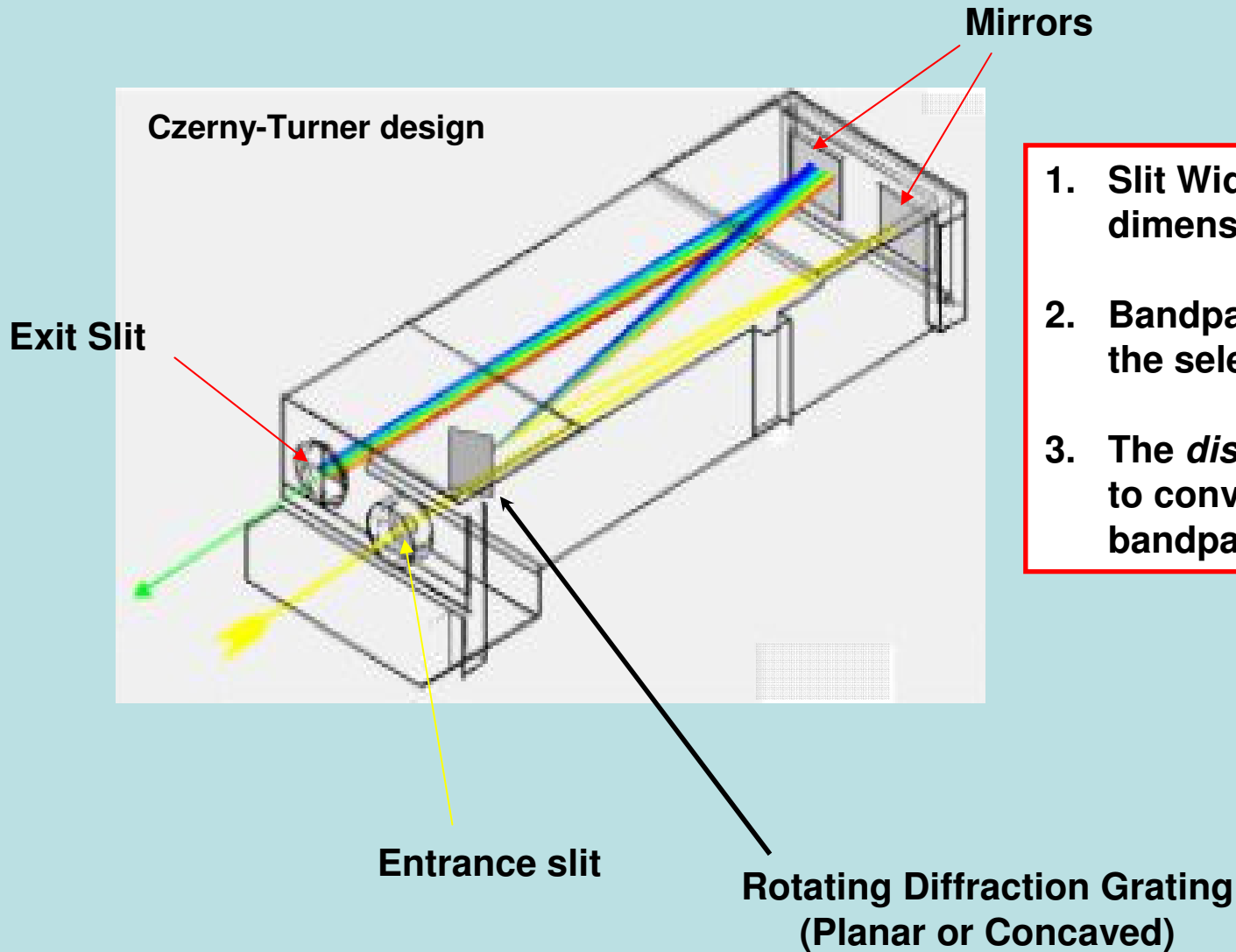
$\lambda$  = diffracted wavelength

Formerly ruled with diamond-tipped instruments

Now almost always made using a holographic, photolithographic technique or a photosensitive gel method



# Monochromators

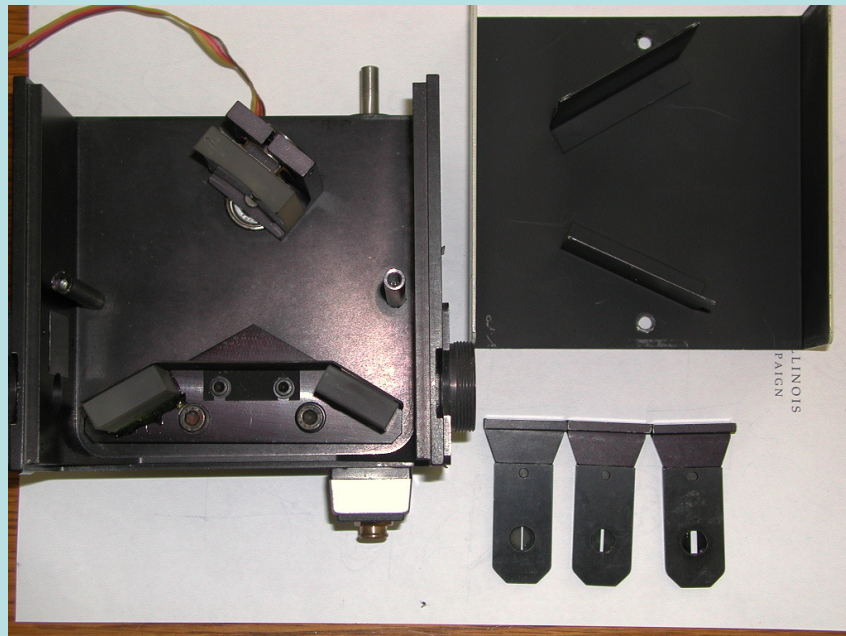
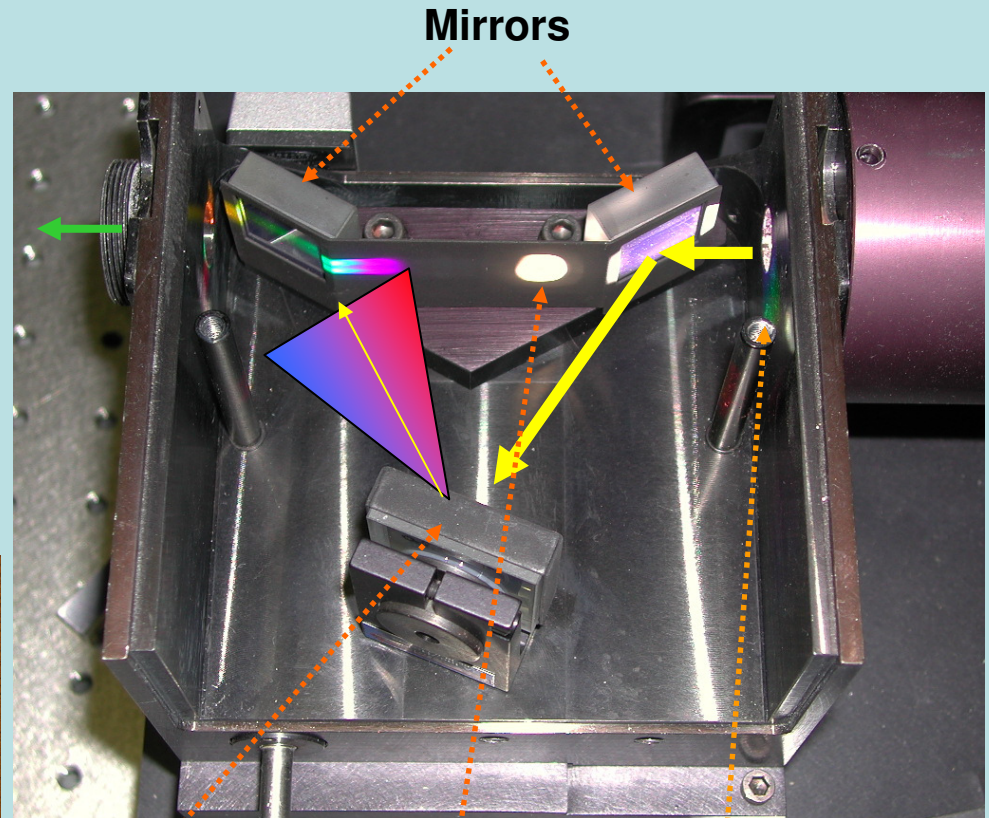


1. **Slit Width (mm)** is the dimension of the slits.
2. **Bandpass** is the FWHM of the selected wavelength.
3. The *dispersion* is the factor to convert slit width to bandpass.

# The Inside of a Monochromator



H10 Monochromator

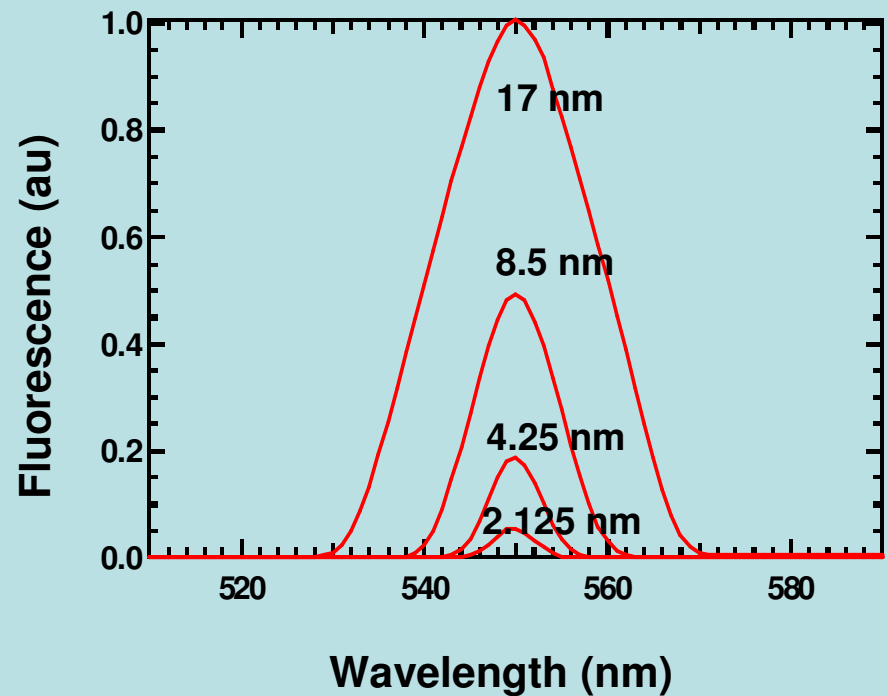
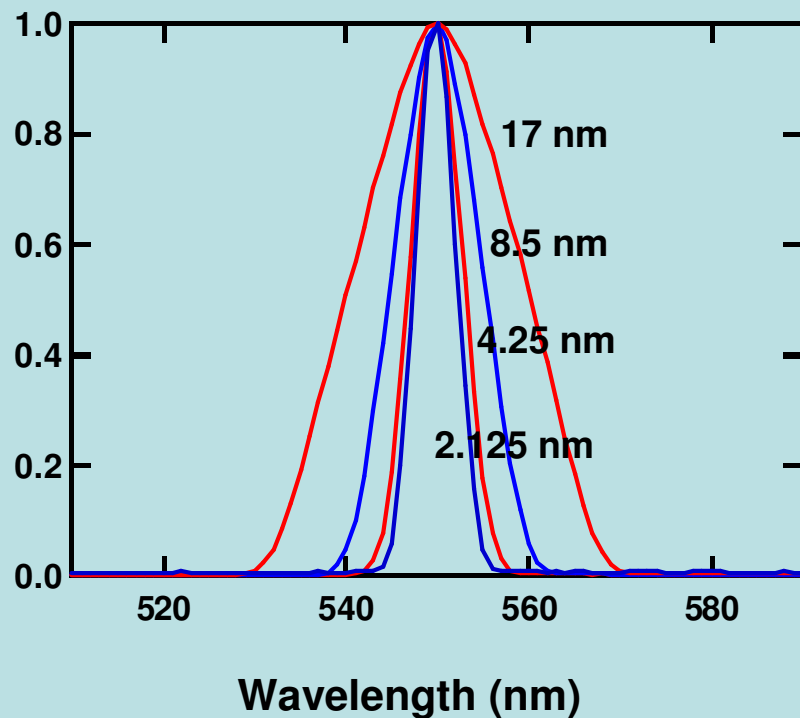


# Changing the Bandpass

1. Drop in intensity
2. Narrowing of the spectral selection

Fixed Excitation Bandpass = 4.25 nm

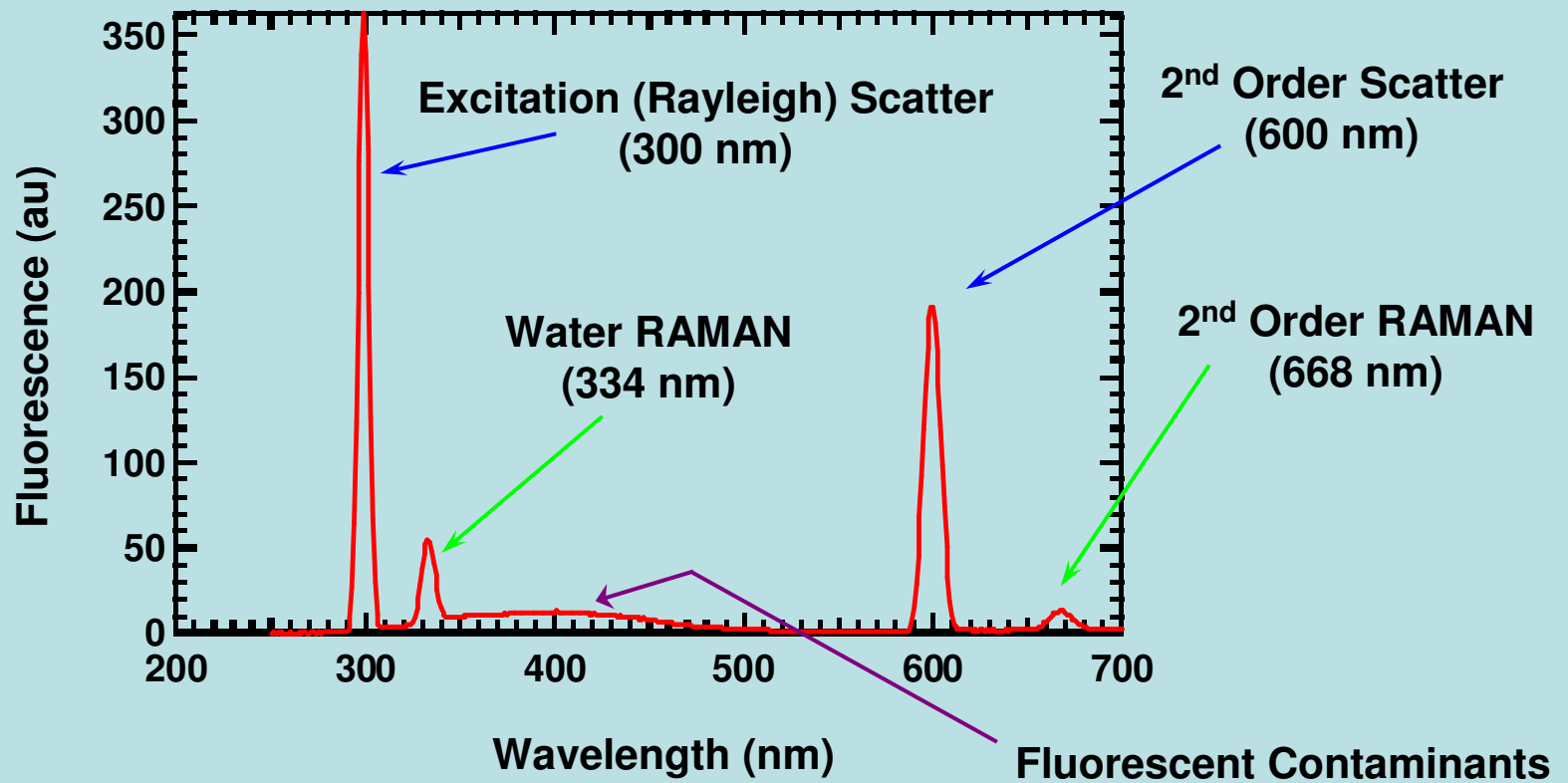
## Changing the Emission Bandpass Full Width Half Maximum (FWHM)



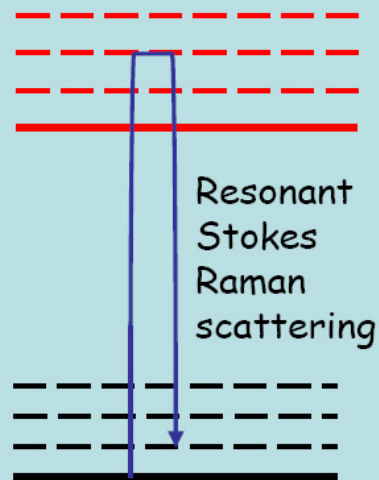
Collected on a SPEX Fluoromax - 2

# Higher Order Light Diffraction

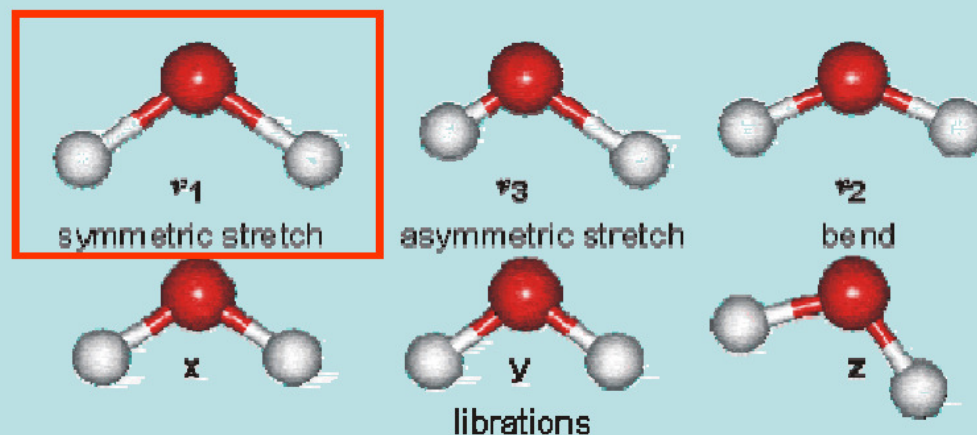
Emission Scan:  
Excitation 300 nm  
Glycogen in PBS



# Raman scatter of water



## Vibrational modes of water



Energy for the OH stretch vibrational mode in water (expressed in inverse wavenumbers):  $3400 \text{ cm}^{-1}$

$$\frac{1}{\lambda_R} = \frac{1}{\lambda_{EX}} - 0.00034$$

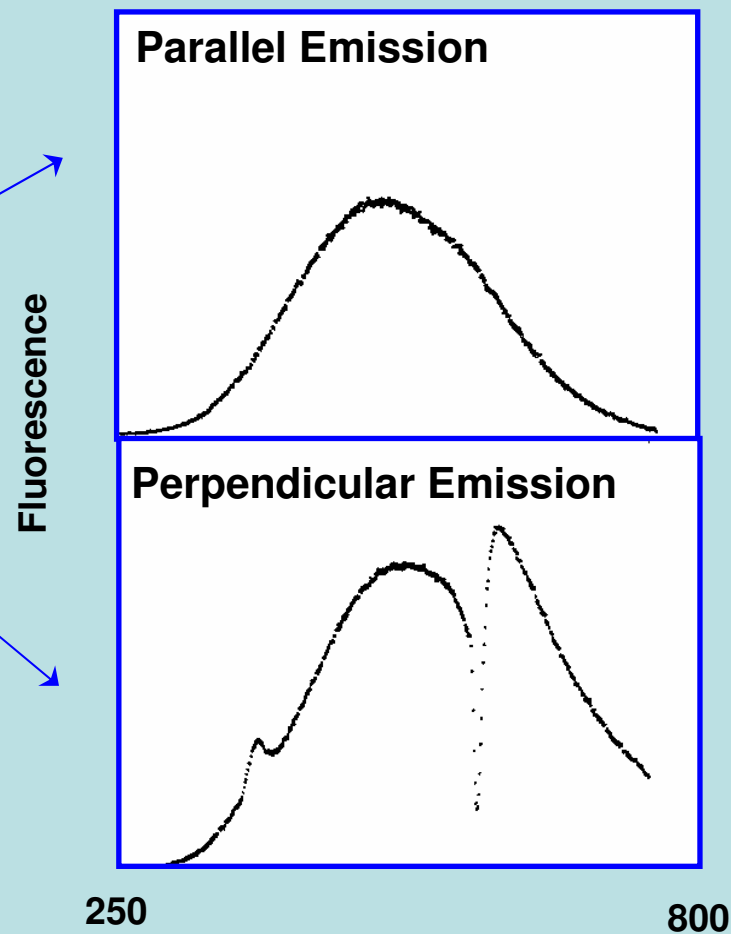
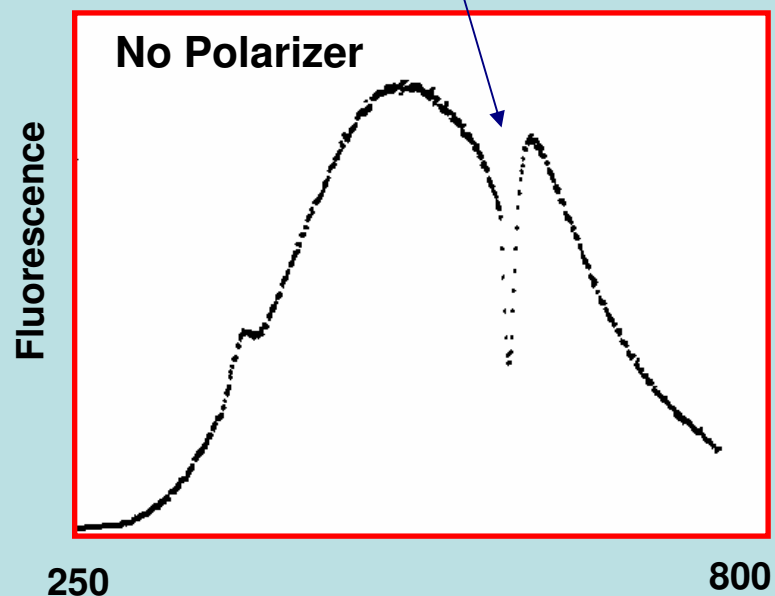
The approximate position of the water Raman peak can be calculated with this formula

For example:	Exc	Raman
	280	309
	350	397
	480	574

# Monochromator Polarization Bias

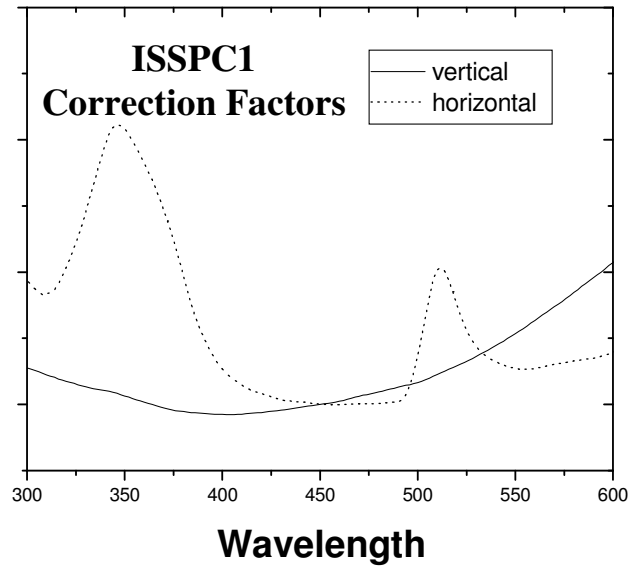
Tungsten Lamp Profile Collected on an SLM Fluorometer

Wood's Anomaly

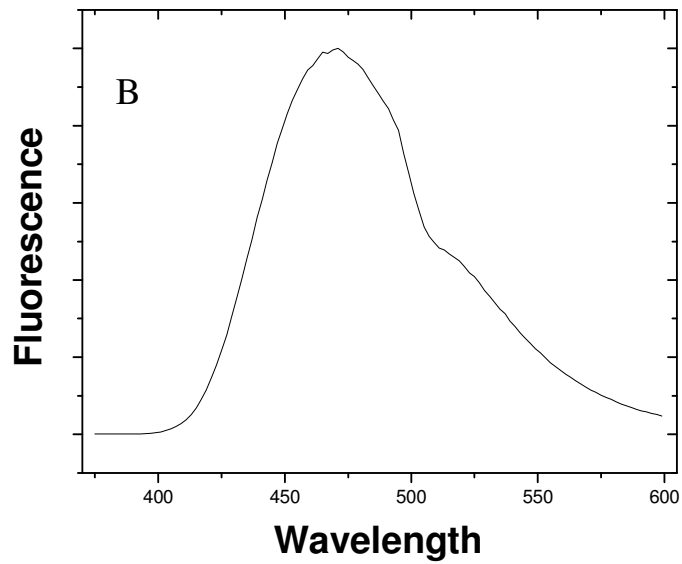


*Adapted from Jameson, D.M., Instrumental Refinements in Fluorescence Spectroscopy: Applications to Protein Systems., in Biochemistry, Champaign-Urbana, University of Illinois, 1978.*

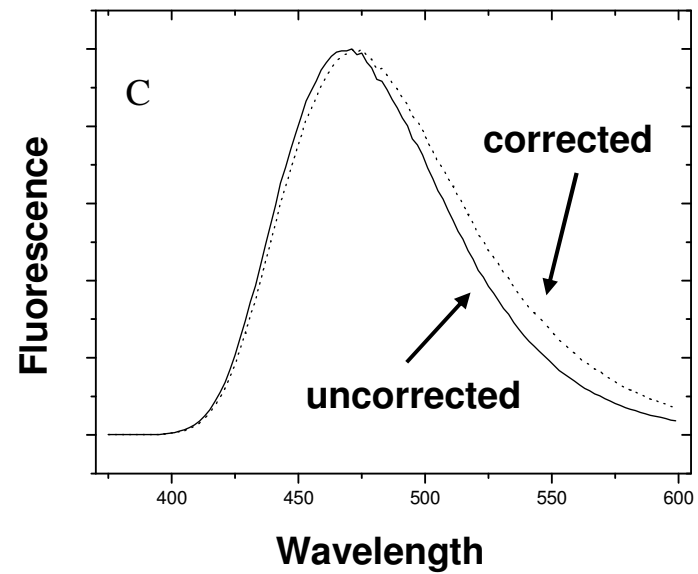
# Correction of Emission Spectra



**ANS Emission Spectrum, no polarizer**



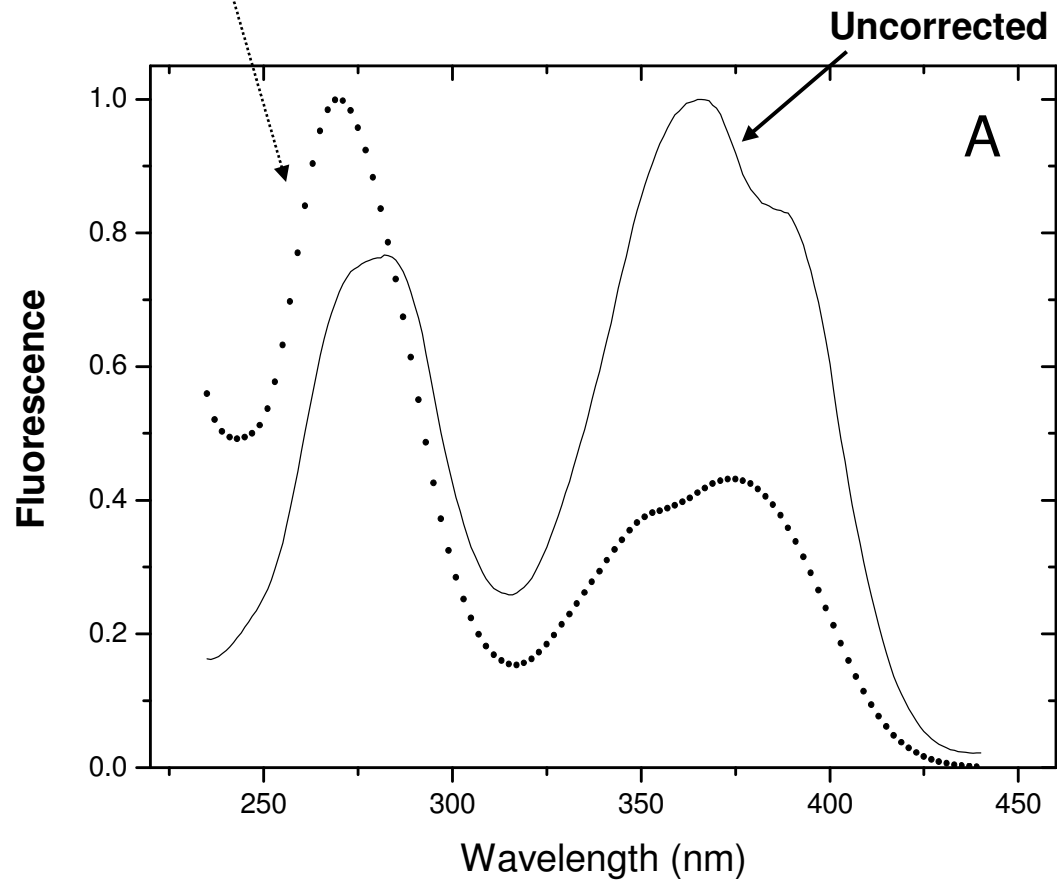
**ANS Emission Spectrum, parallel polarizer**



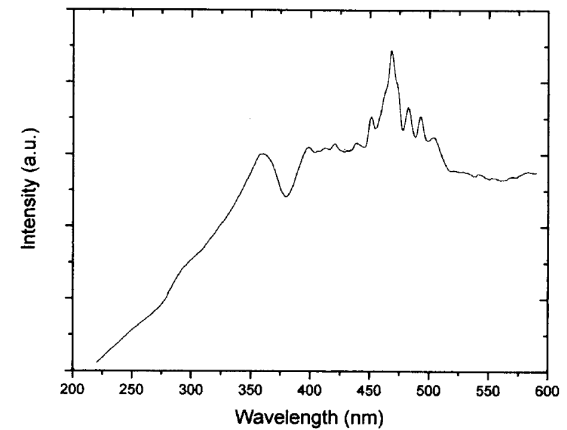
*from Jameson et. Al., Methods in Enzymology, 360:1*

# Excitation Correction

Absorption (dotted line) and Excitation Spectra (solid line) of ANS in Ethanol



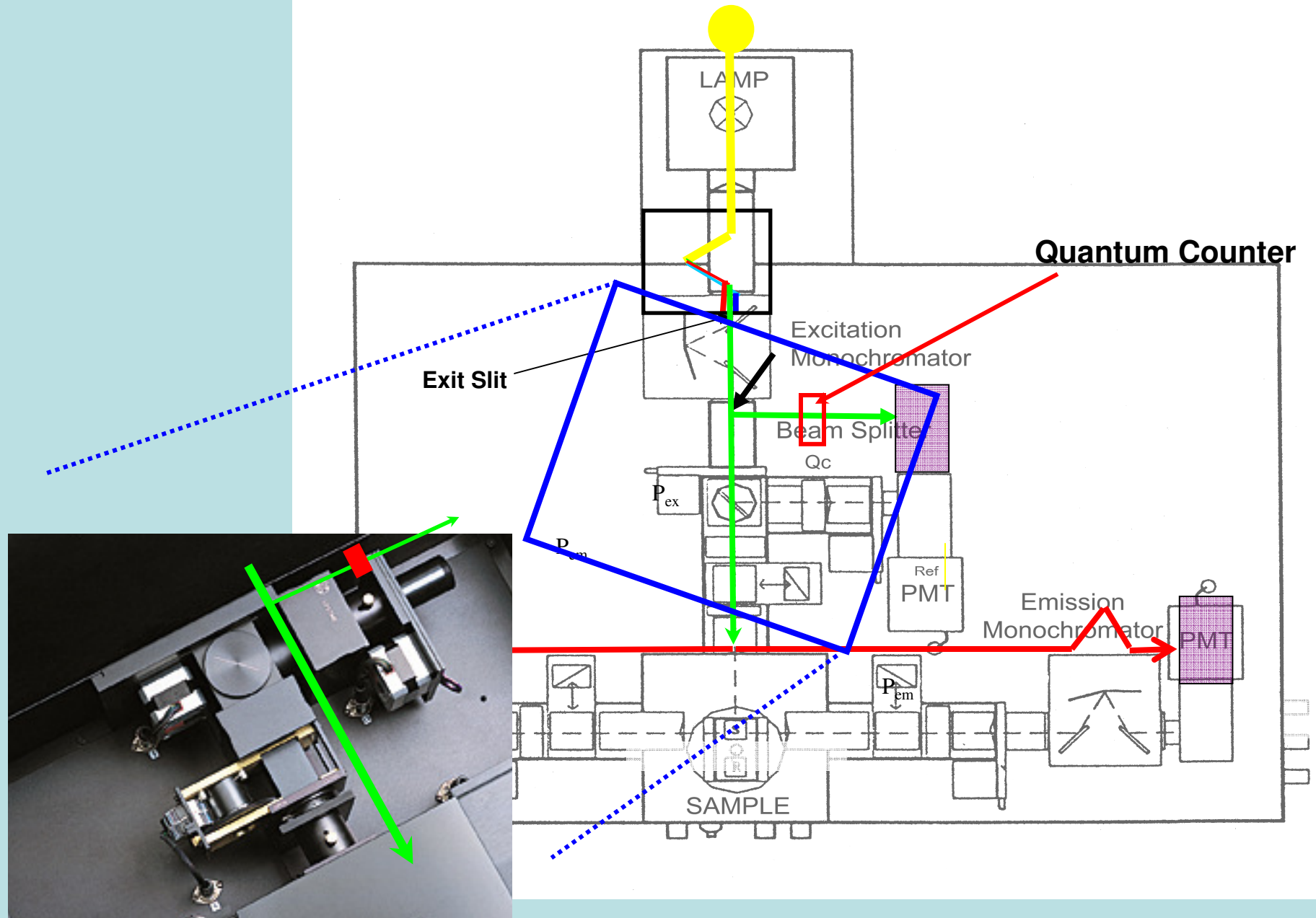
Recall the output of the xenon arc



Note the huge difference between the absorption spectrum and the excitation spectrum

*from Jameson, Croney and Moens, Methods in Enzymology, 360:1*

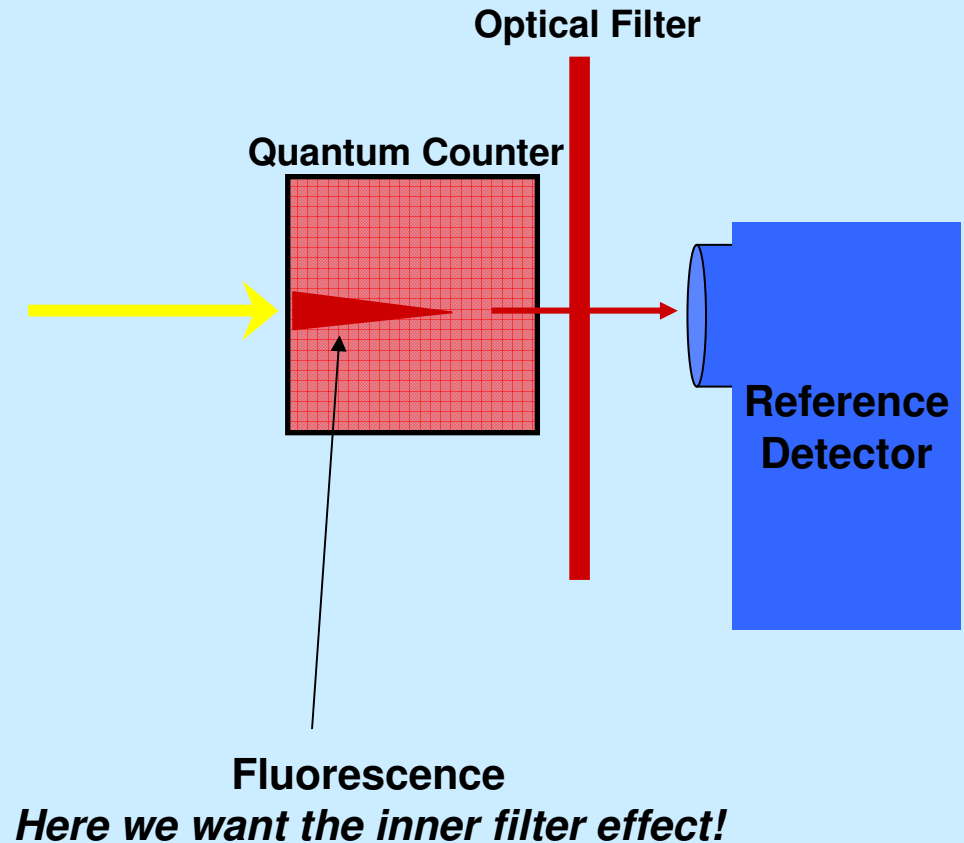
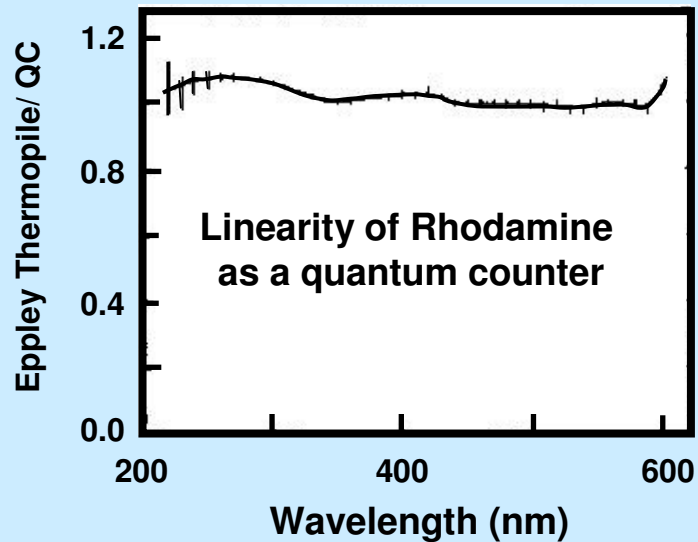
# Excitation Correction



# The Instrument Quantum Counter

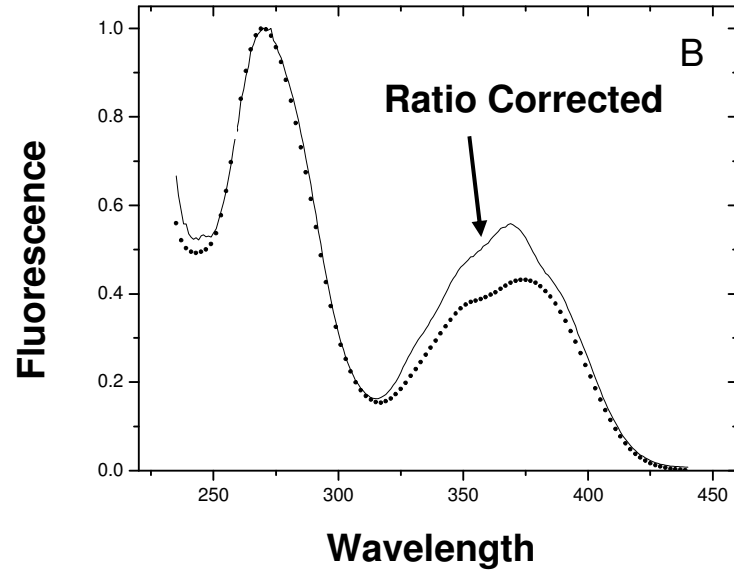
## Common Quantum Counters (optimal range)\*

Rhodamine B	(220 - 600 nm)
Fluorescein	(240 - 400 nm)
Quinine Sulfate	(220 - 340 nm)

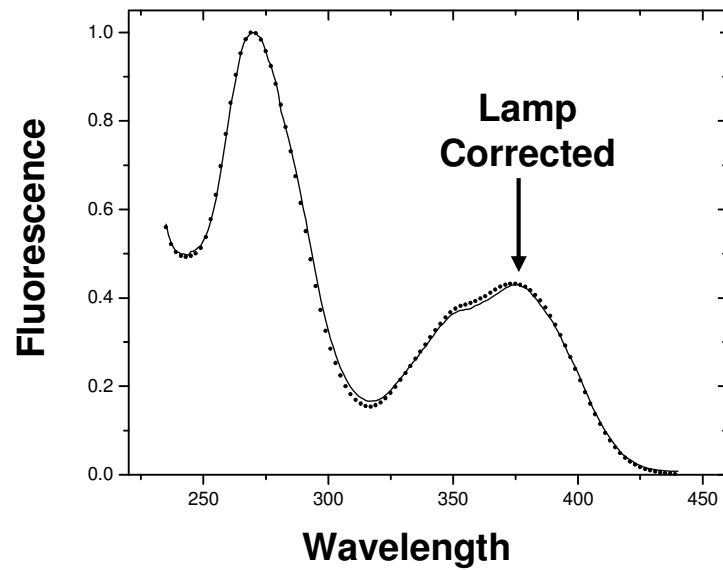


\* Melhuish (1962) *J. Opt. Soc. Amer.* 52:1256

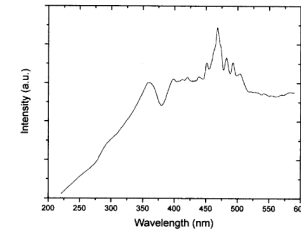
# Excitation Correction



Still not perfect since the quartz reflector to the quantum counter has a polarization bias.



If we determine the lamp curve at the sample position and then divide the sample excitation spectrum by this curve we can get excellent agreement





## Etienne-Louis Malus (1775-1812)

In 1808, Malus observed sunlight reflected from the windows of the Luxemburg Palace in Paris through an Iceland spar (Calcite) crystal that he rotated.



(Erasmus Bartholin (1625-1698) discovered the double refraction of light by Iceland spar in 1669)

Malus discovered that the intensity of the reflected light varied as he rotated the crystal and coined the term “**polarized**” to describe this property of light.

He published his findings in 1809:

“Sur une propriété de la lumière réfléchie par les corps diaphanes” (*Bull. Soc. Philomat.* 1:16)

Malus also derived an expression for calculating the transmission of light as a function of the angle ( $\theta$ ) between two polarizers. This equation (Malus’ Law) is now written as:  $I_{\theta} = I_0 (\cos^2\theta)$

# Sir David Brewster (1781-1868)



David Brewster studied the relationship between refractive index and angle of incidence on the polarization of the reflected light

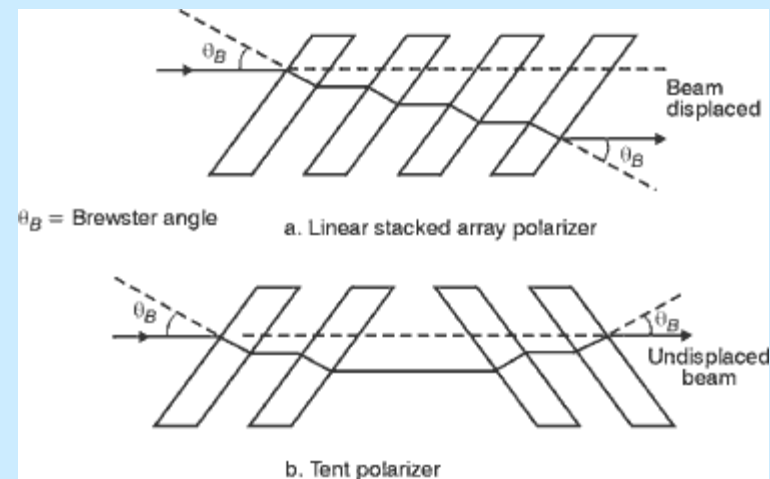
III. *On the law of the partial polarization of light by reflexion.* By  
DAVID BREWSTER, LL.D. F.R.S. L. & E.

Read February 4, 1830.

He discovered that for normal glass and visible light, an incidence angle of ~56 degrees resulted in total reflection of one plane of polarization – this angle is now known as *Brewster's Angle*

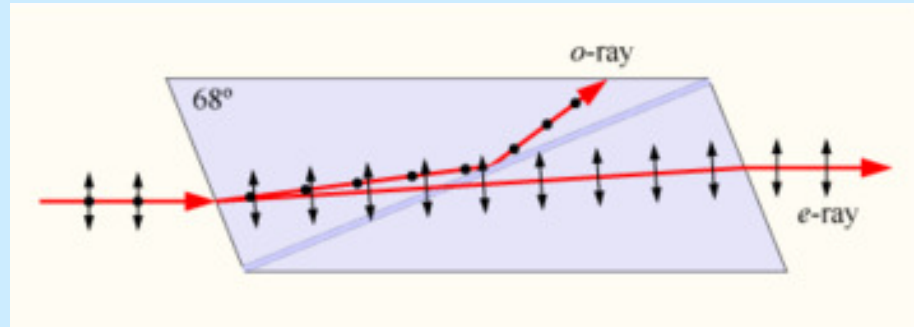
$$\theta_B = \tan^{-1} \left( \frac{n_2}{n_1} \right)$$

This discovery allowed Brewster to construct a polarizer composed of a “pile of plates”



## William Nicol (1770-1851)

In 1828, Nicol joined two crystals of Iceland spar, cut at an angle of  $68^\circ$ , using Canada balsam.



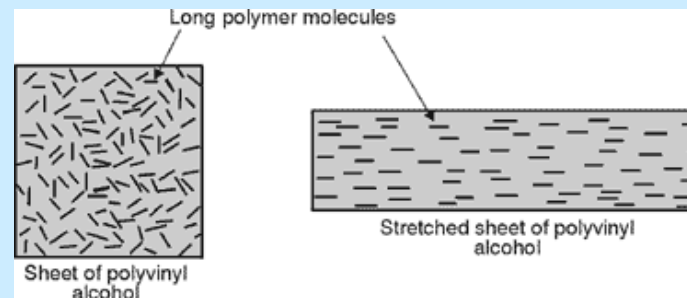
Other important calcite polarizers developed around this time include: Glan-Foucault; Glan-Thompson; Glan-Taylor; Wollaston; Rochon

But the Henry Ford of polarizers was.....

## Edwin Herbert Land (1909-1991)



In 1929 Edwin Land patented the sheet polarizer (the J-sheet), consisting of crystals of iodoquinine sulfate embedded in nitrocellulose film followed by alignment of the crystals by stretching which led to dichroism. In 1938 he invented the H-sheet which was comprised of polyvinyl alcohol sheets with embedded iodine.



# Polarizers

## The *Glan Taylor* prism polarizer

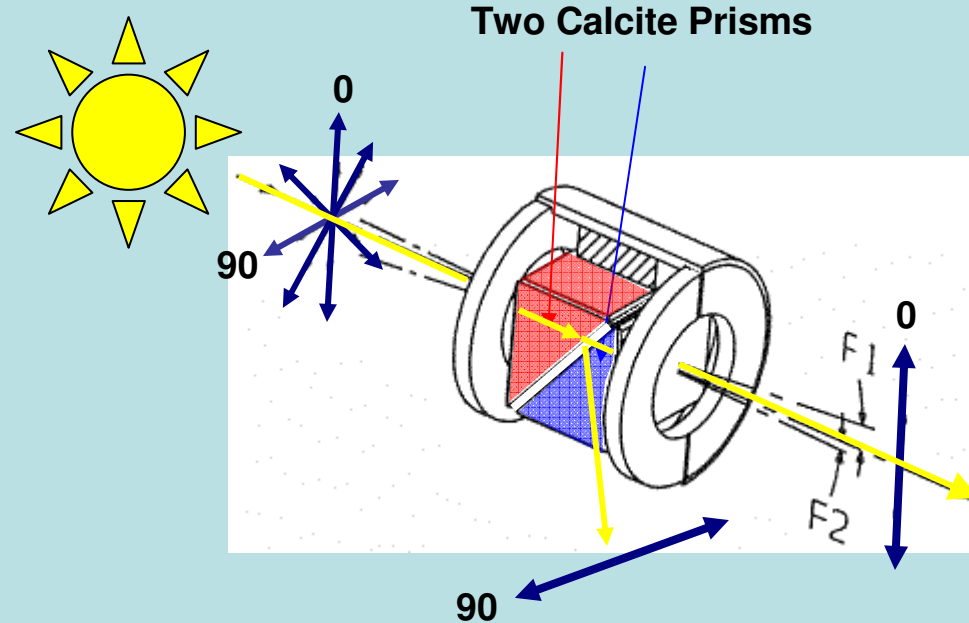
### Common Types:

Glan Taylor (air gap)

Glan Thompson

Sheet Polarizers

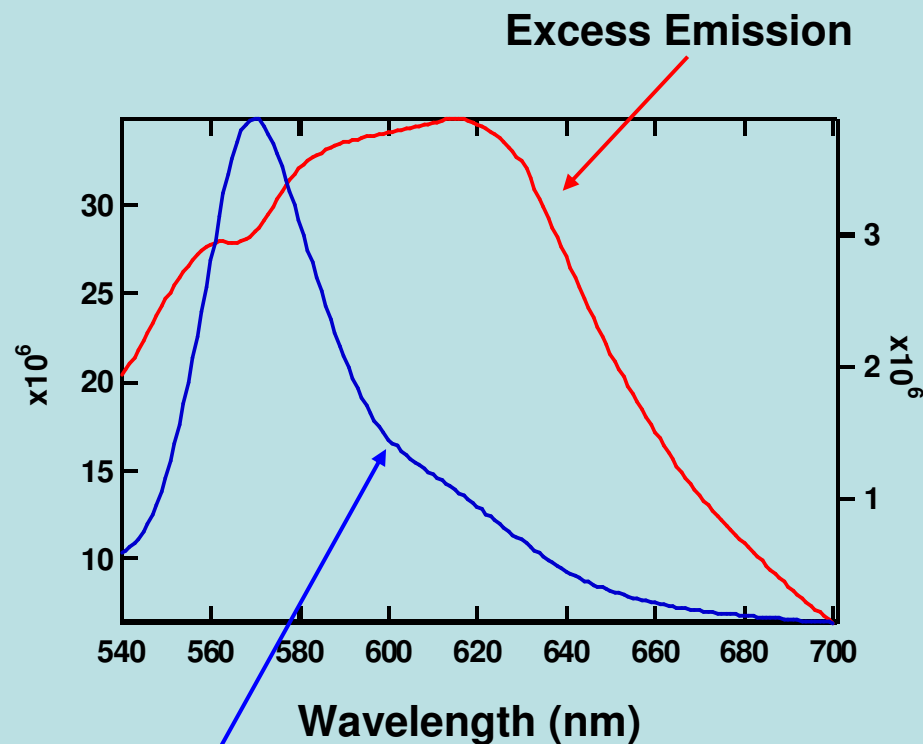
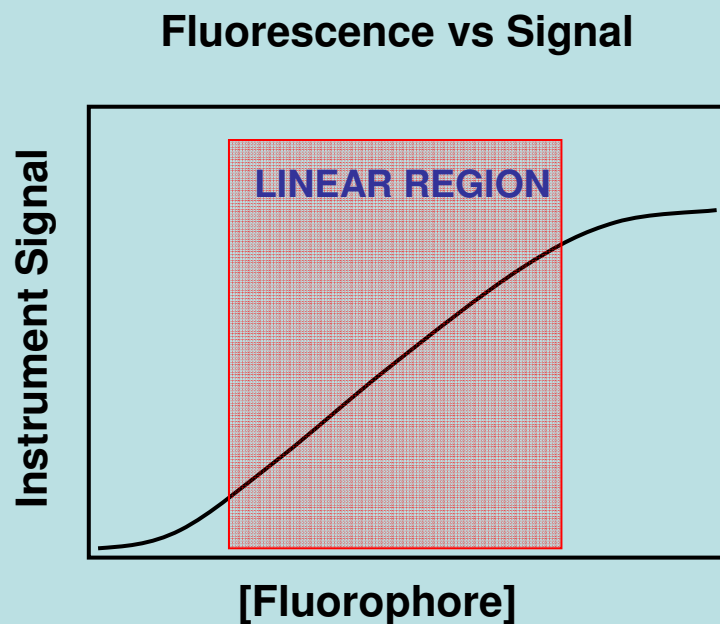
### Sheet polarizer



Two UV selected calcite prisms are assembled with an intervening air space. The calcite prism is birefringent and cut so that only one polarization component continues straight through the prisms. The spectral range of this polarizer is from 250 to 2300 nm. At 250 nm there is approximately 50% transmittance.

# Sample Issues

## Signal Attenuation of the Excitation Light *PMT Saturation*

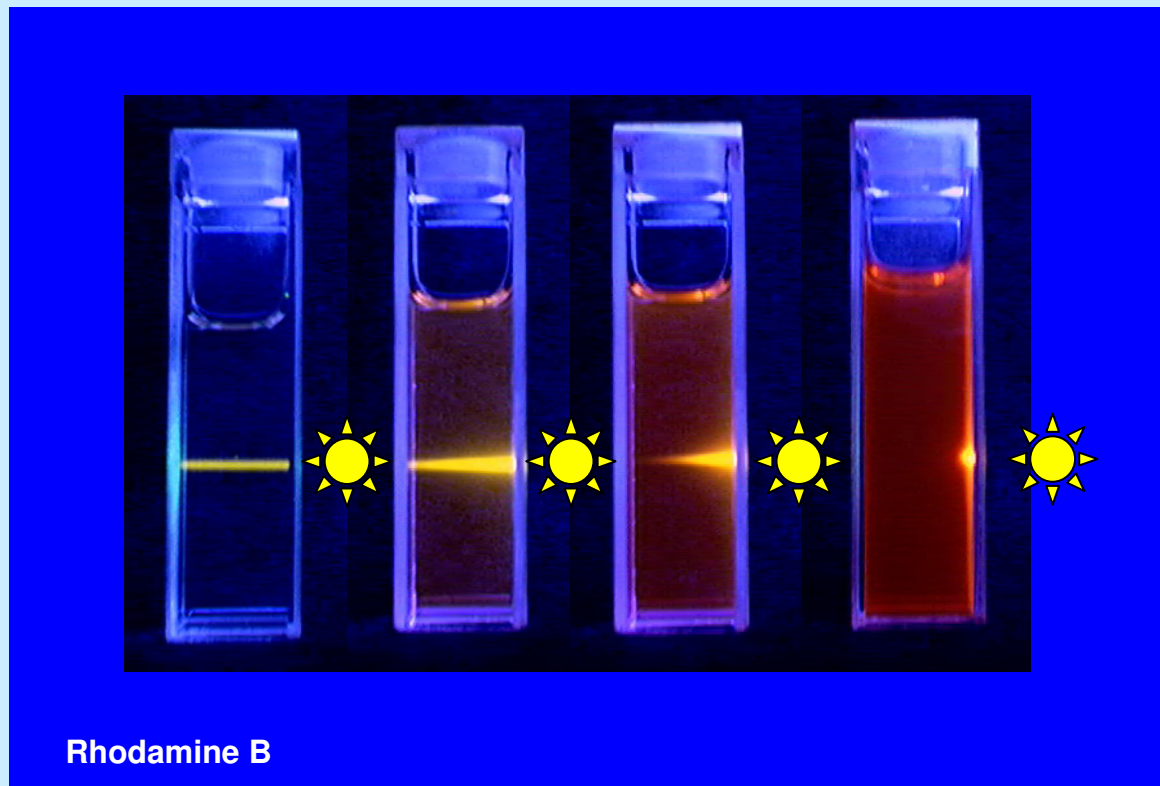


Reduced emission intensity

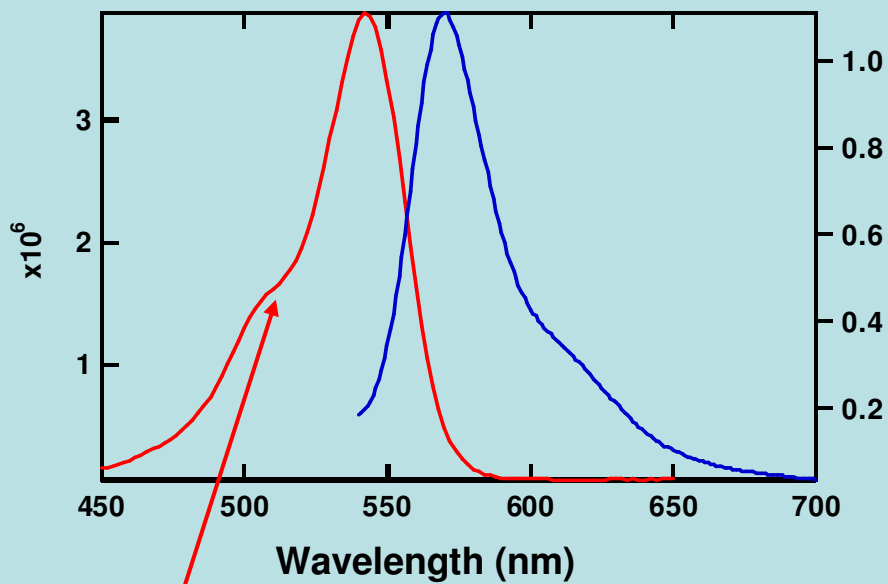
1. ND Filters
2. Narrow slit widths
3. Move off absorbance peak

# Attenuation of the Excitation Light through Absorbance

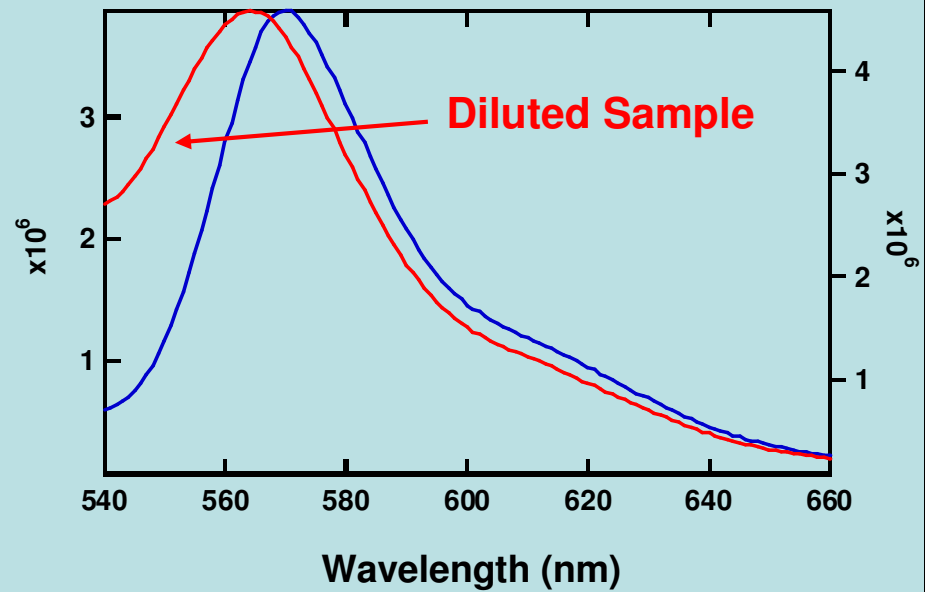
Sample concentration  
& the *inner filter effect*



**The second half of the *inner filter effect*:  
attenuation of the emission signal.**



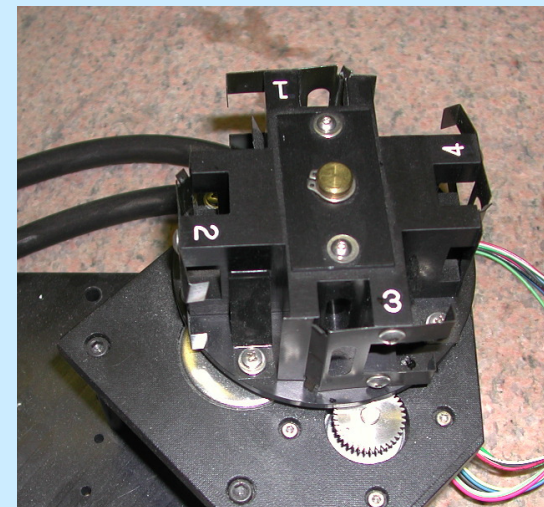
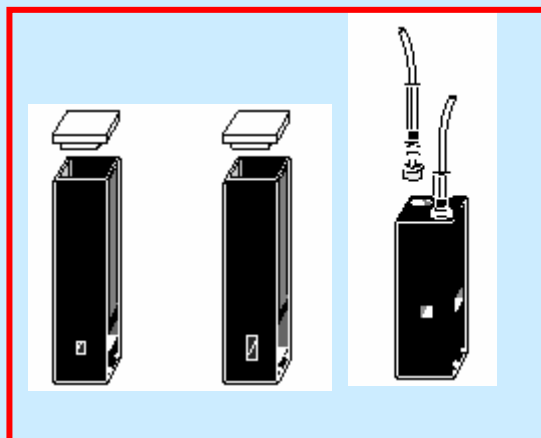
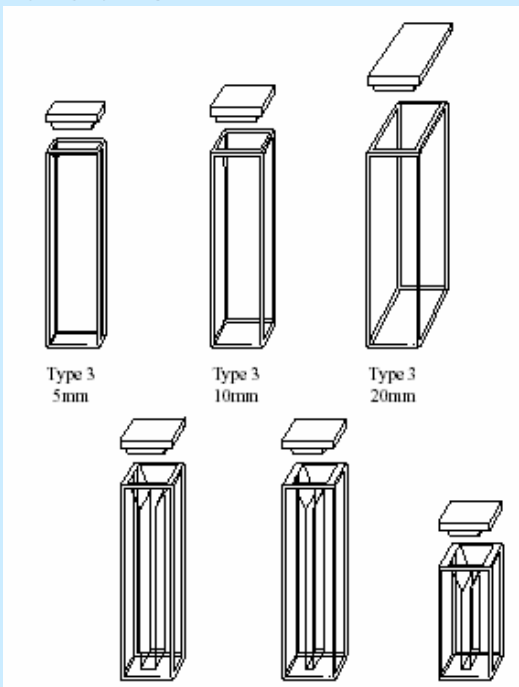
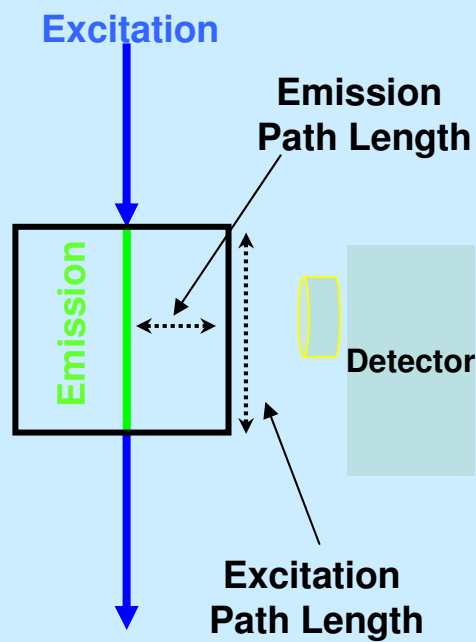
**Absorbance Spectrum**



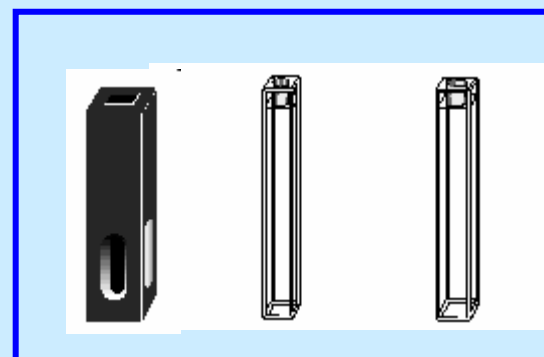
- (1) Spectral Shift
- (2) Change in Spectral Shape

# How do we handle highly absorbing solutions?

## Quartz/Optical Glass/Plastic Cells

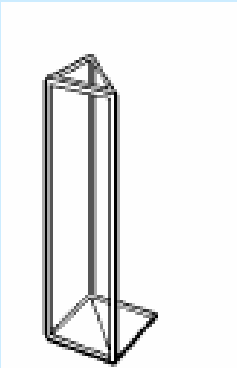


**4 Position Turret  
SPEX Fluoromax-2, Jobin-Yvon**

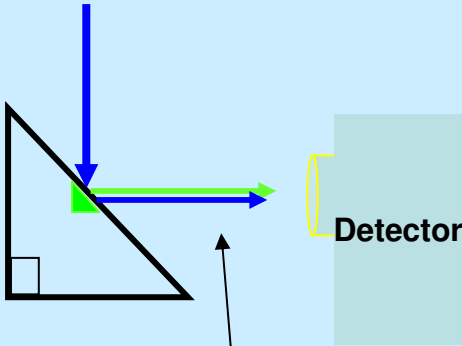


# Front Face Detection

## Triangular Cells



Excitation

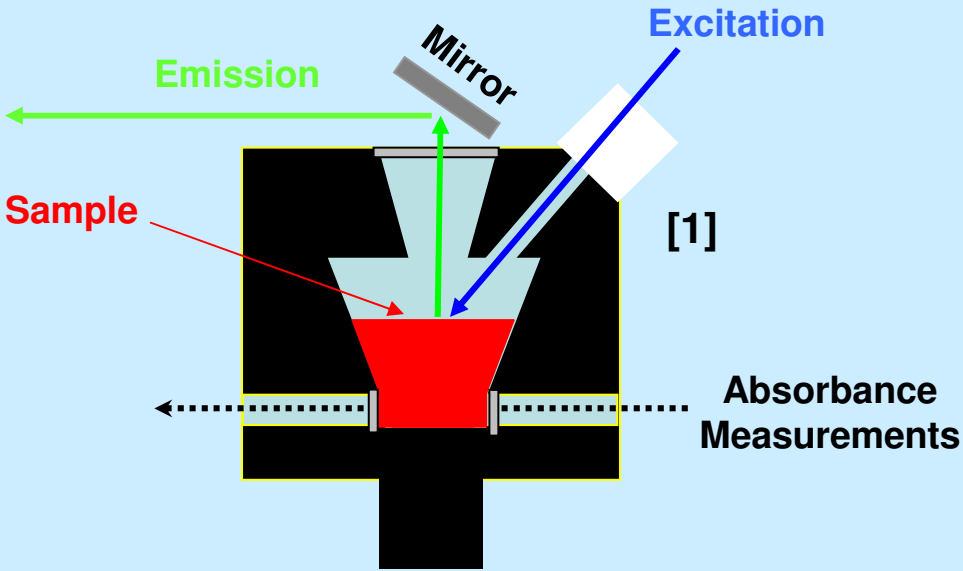
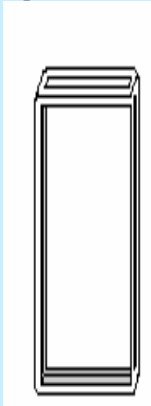


Reflected **Excitation** & **Emission**

## Thin Cells & Special Compartments

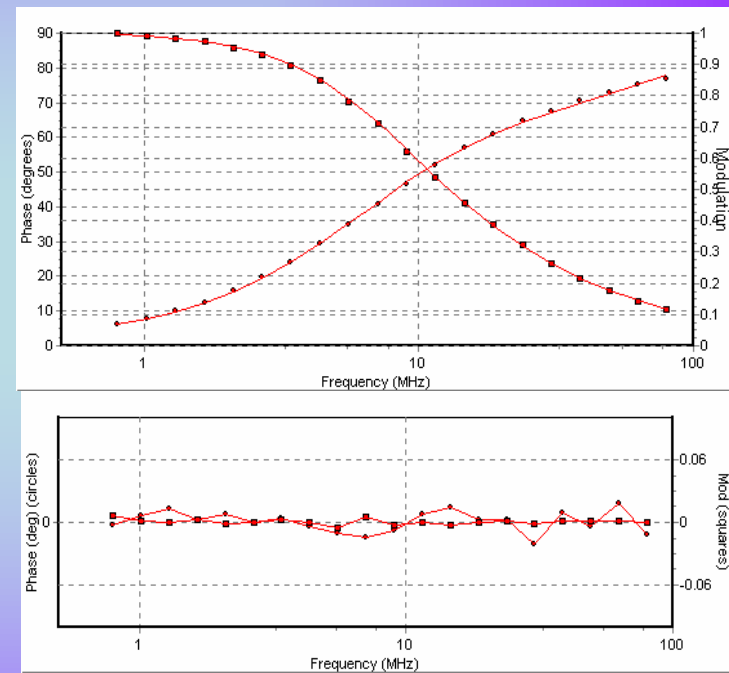
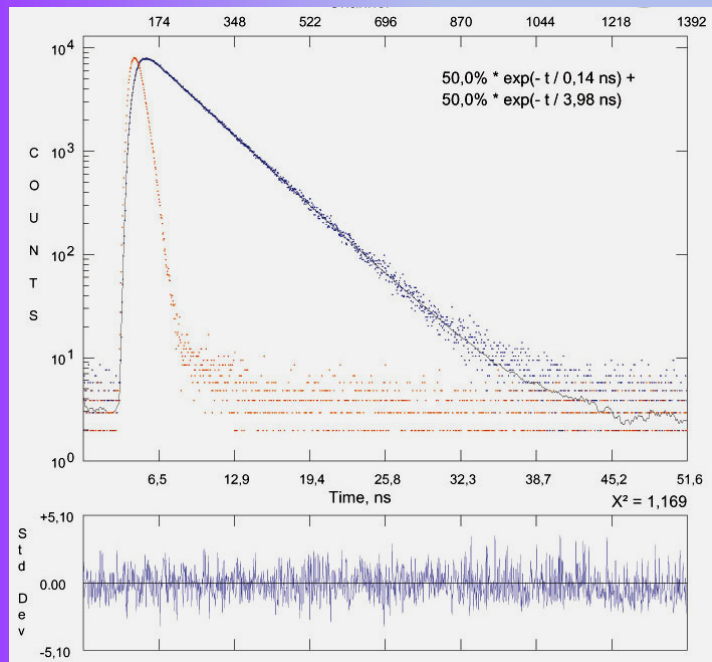


*IBH, Glasgow G3 8JU  
United Kingdom*



[1] Adapted from Gryczynski, Lubkowski, & Bucci *Methods of Enz.* 278: 538

# Lifetime Instrumentation



# Light Sources for Decay Acquisition: Frequency and Time Domain Measurements

## Pulsed Light Sources (frequency & pulse widths)

### Mode-Locked Lasers

ND:YAG (76 MHz) (150 ps)

Pumped Dye Lasers (4 MHz Cavity Dumped, 10-15 ps)

Ti:Sapphire lasers (80 MHz, 150 fs)

Mode-locked Argon Ion lasers

### Directly Modulated Light Sources

Diode Lasers (short pulses in ps range, & can be modulated by synthesizer)

LEDs (directly modulated via synthesizer, 1 ns, 20 MHz)

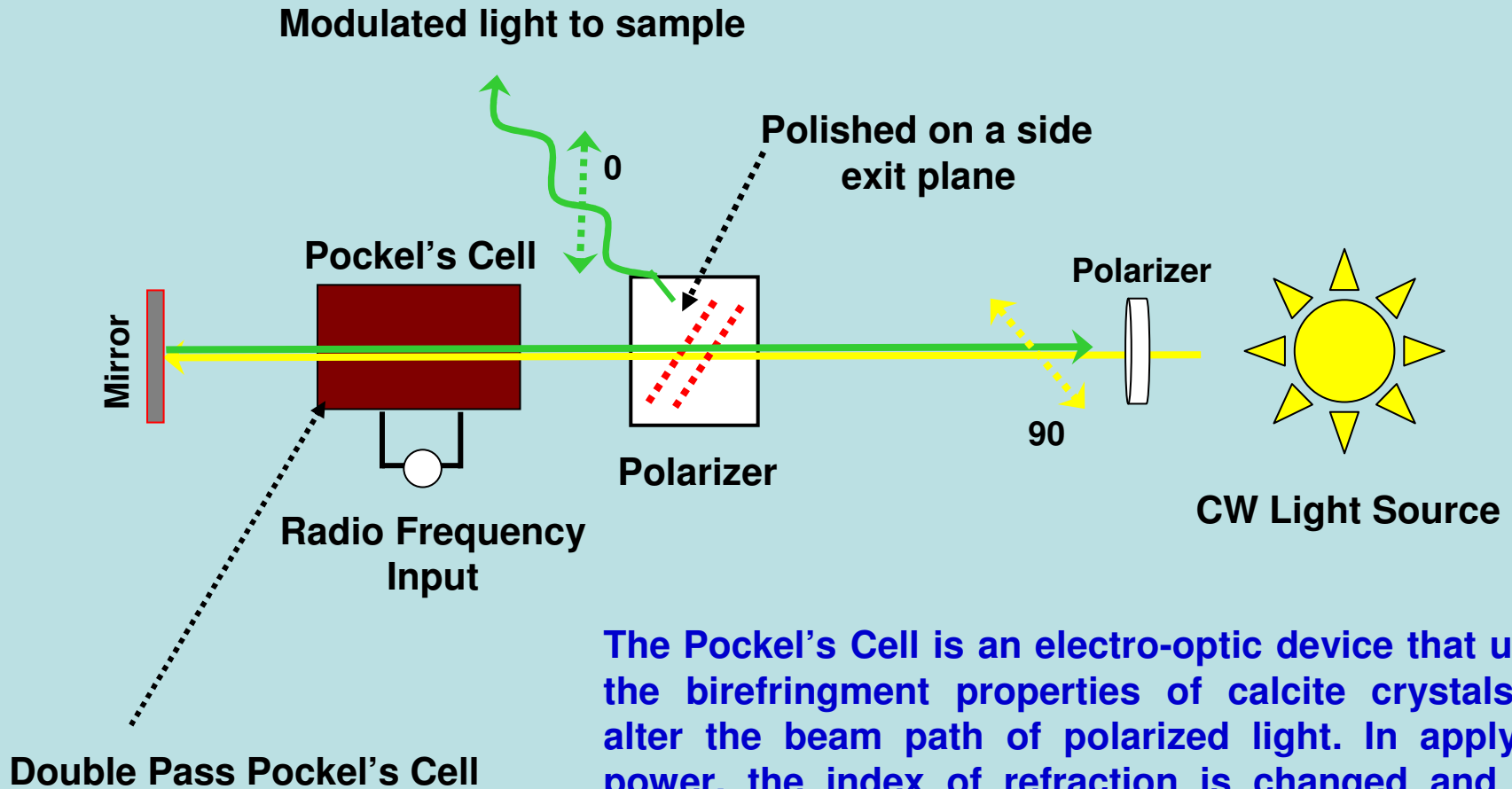
Synchrotron Radiation

### Flash Lamps

Thyratron-gated nanosecond flash lamp (PTI), 25 KHz, 1.6 ns

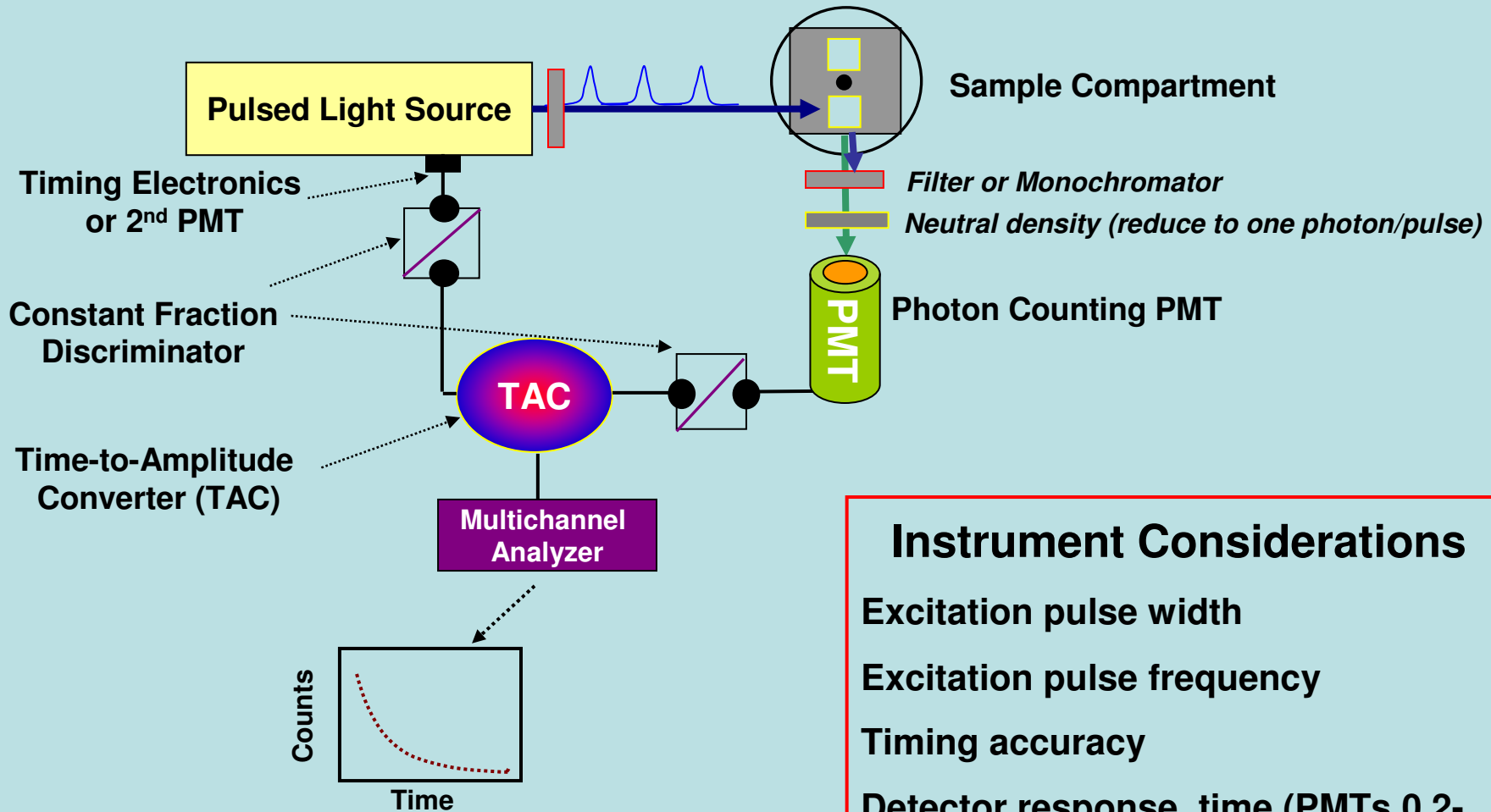
Coaxial nanosecond flashlamp (IBH), 10Hz-100kHz, 0.6 ns

# Modulation of CW Light Use of a Pockel's Cell



The Pockel's Cell is an electro-optic device that uses the birefringent properties of calcite crystals to alter the beam path of polarized light. In applying power, the index of refraction is changed and the beam exiting the side emission port (0 polarized) is enhanced or attenuated. In applying RF the output becomes modulated.

# Time Correlated Single Photon Counting



## Instrument Considerations

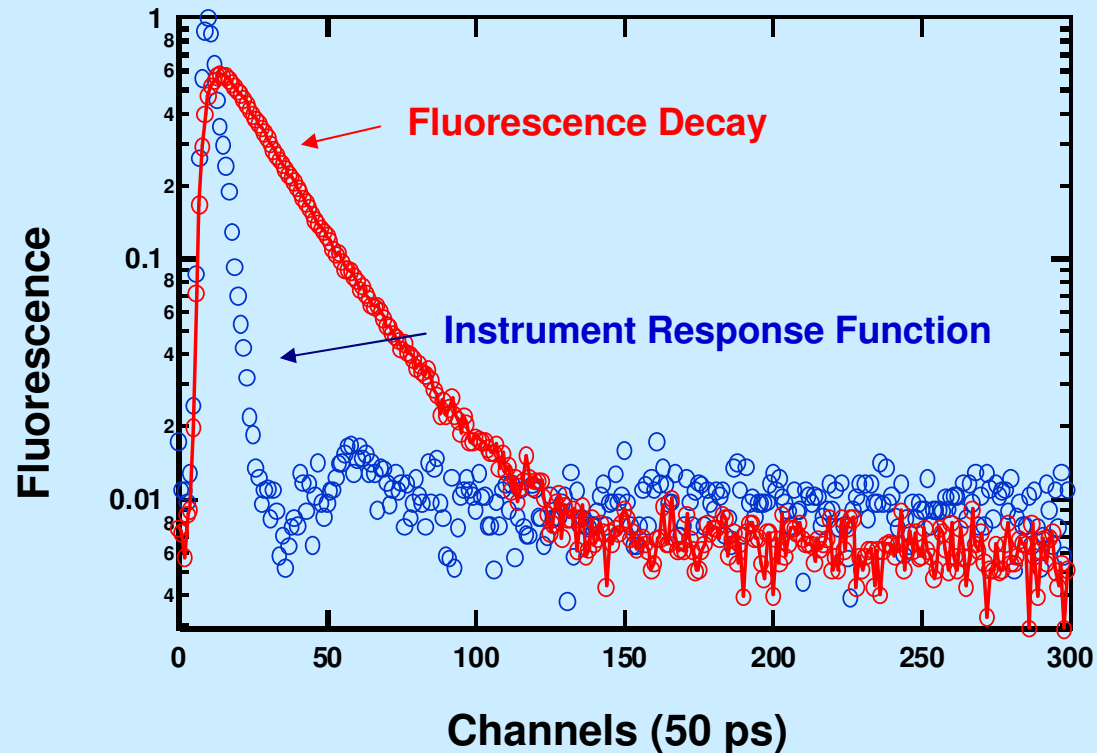
Excitation pulse width

Excitation pulse frequency

Timing accuracy

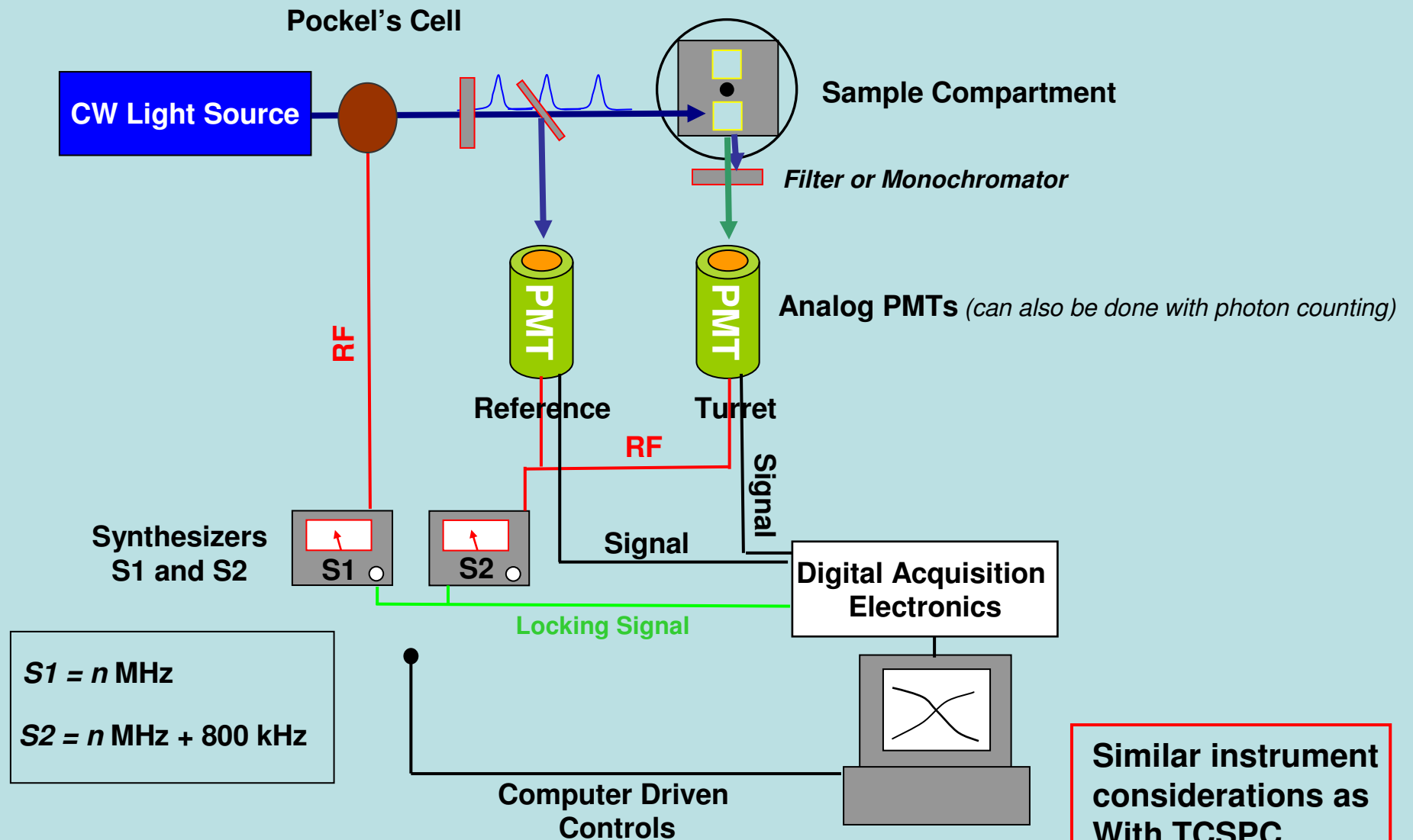
Detector response time (PMTs 0.2-0.9; MCP 0.15 to 0.03 ns)

## Histograms built one photon count at a time ...



- (1) The pulse width and instrument response times determine the time resolution.
- (2) The pulse frequency also influences the time window. An 80 MHz pulse frequency (Ti:Sapphire laser) would deliver a pulse every 12.5 ns and the pulses would interfere with photons arriving later than the 12.5 ns time.

# Frequency Domain Fluorometry



That's all!!!

