The Weber Conference on Advanced Fluorescence Microscopy Techniques

December 12-17, 2011

Buenos Aires

Basic Instrumentation: David Jameson

(many of these slides were prepared by Theodore “Chip” Hazlett and Joachim Mueller)

The Basics

Light Source

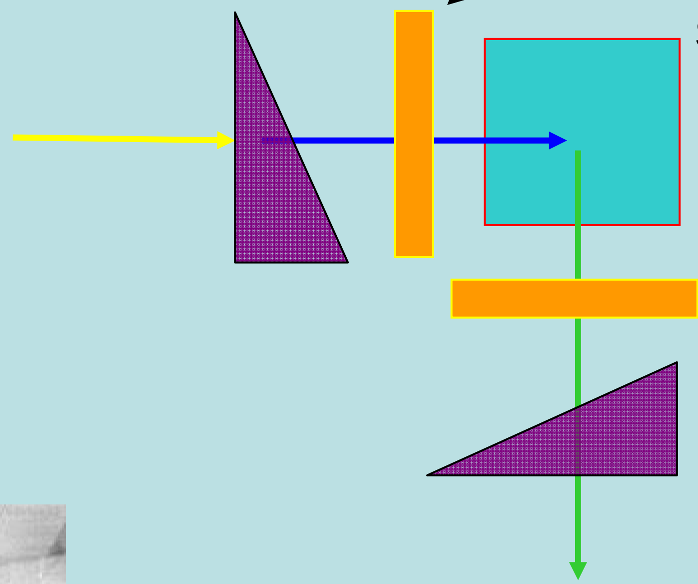
Wavelength Selection

Polarizer

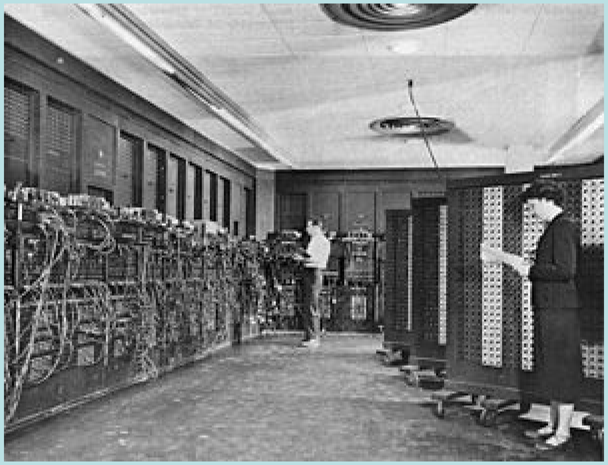
Sample

Polarizer

Wavelength Selection

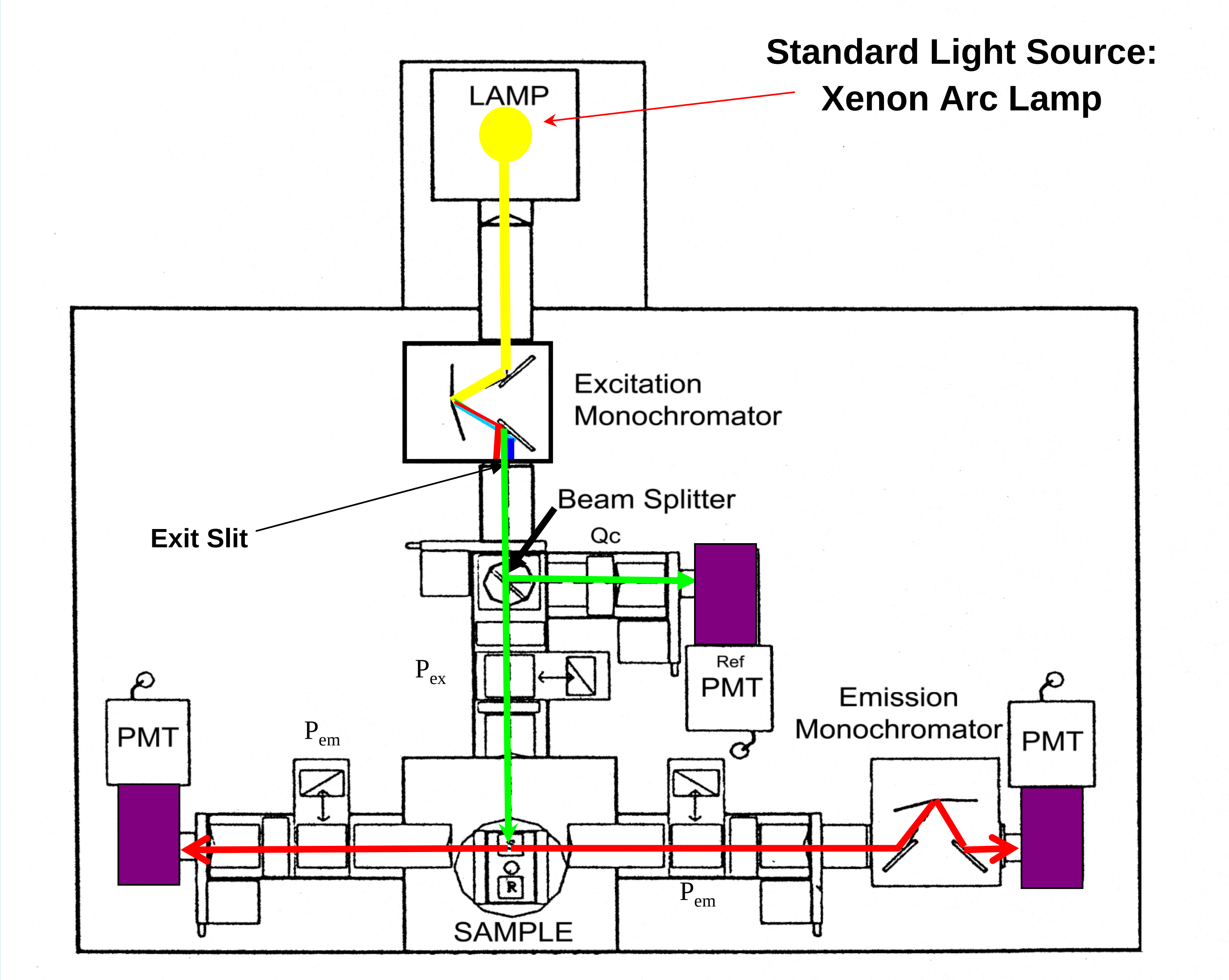


Detector



computer

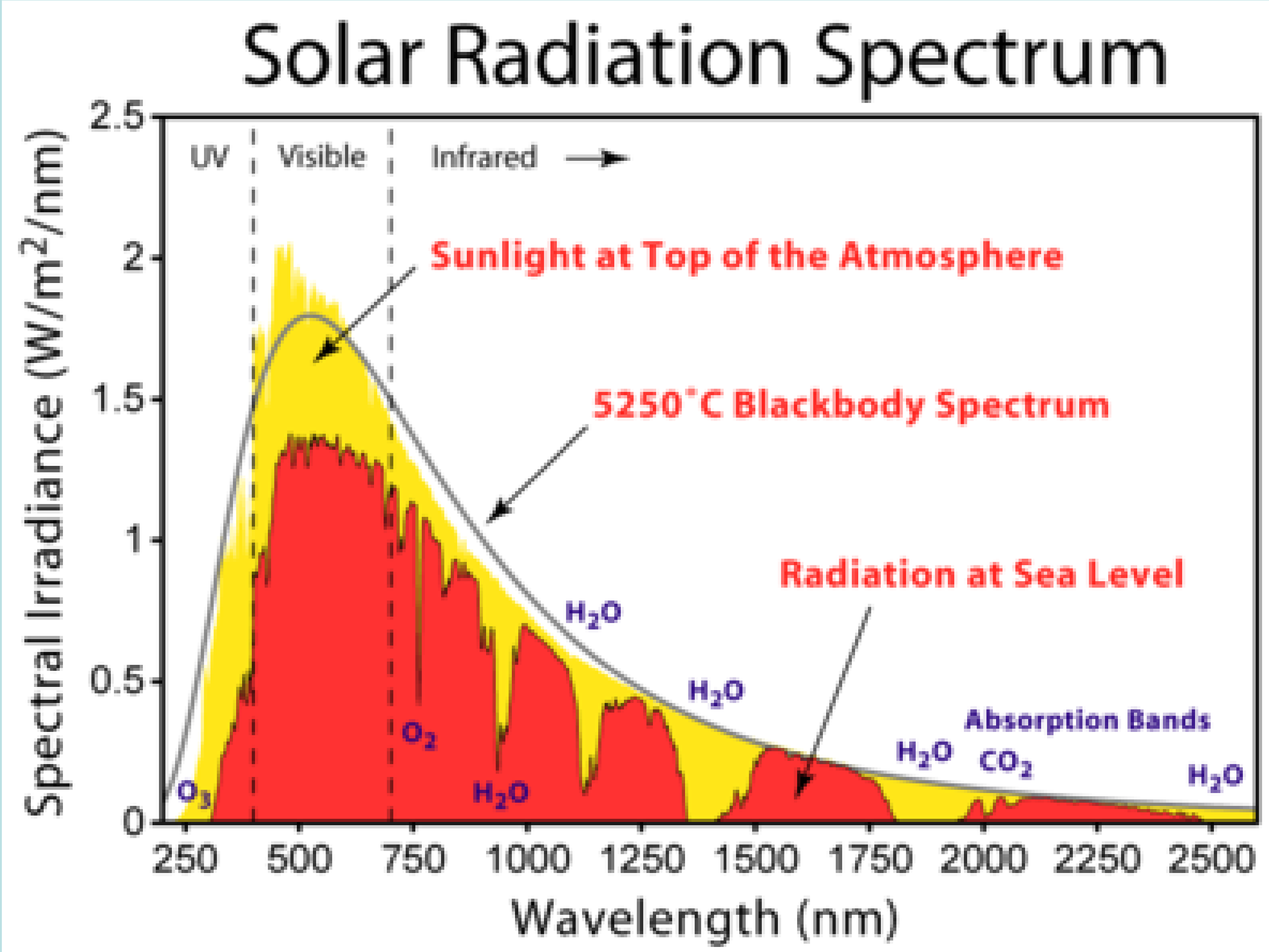
The Laboratory Fluorimeter



ISS (Champaign, IL, USA) PC1 Fluorimeter

Light Sources

Light Sources



http://en.wikipedia.org/wiki/Solar_radiation

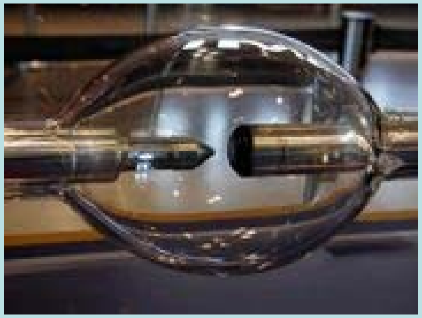
Lamp Light Sources

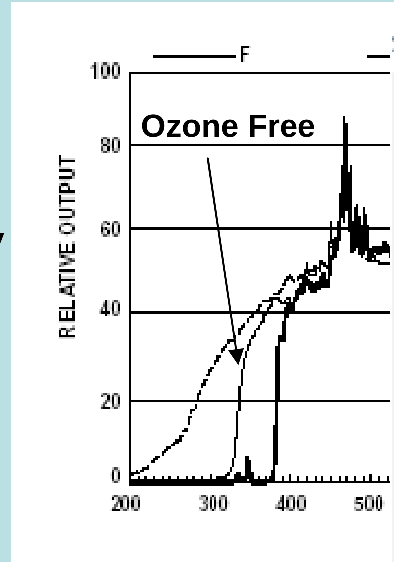
Xenon Arc Lamp Profiles

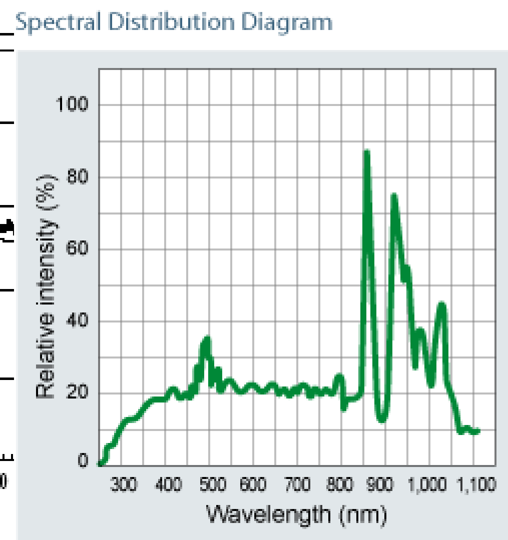
Gas discharge lamps

Xenon Arc Lamp (wide range of wavelengths)

Introduced in 1951 by the Osram Company

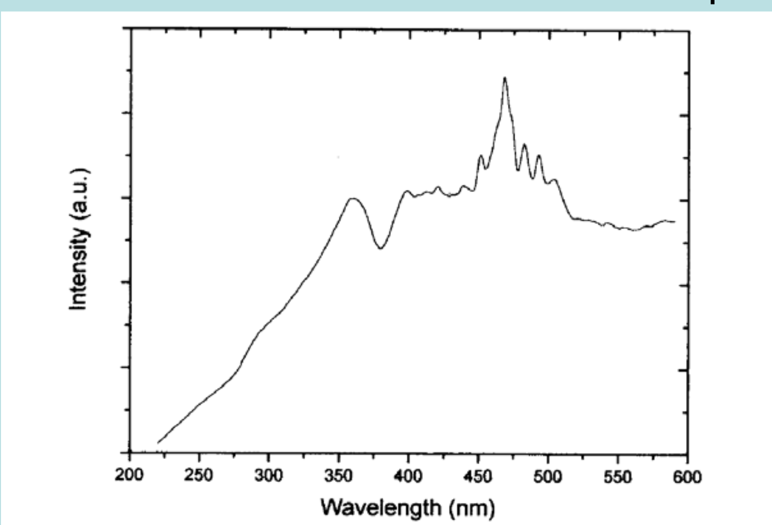


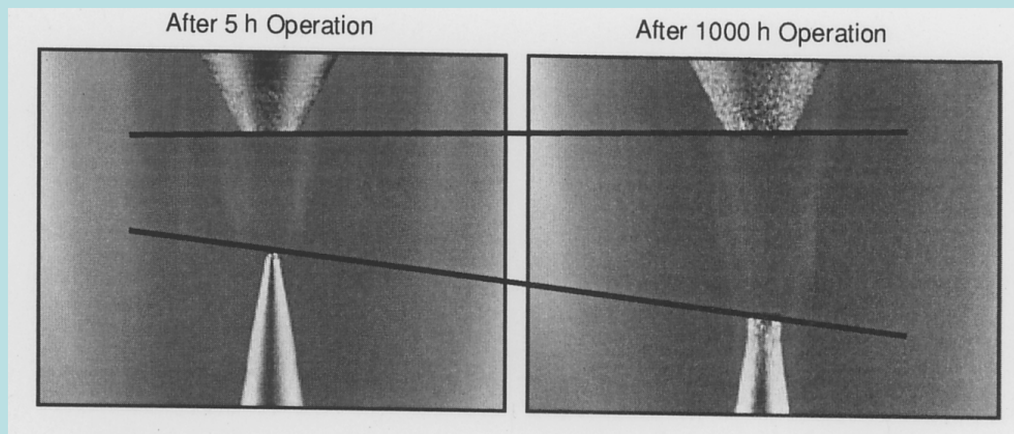




These lamps use tungsten electrodes and xenon gas at pressures up to 25 atmospheres

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



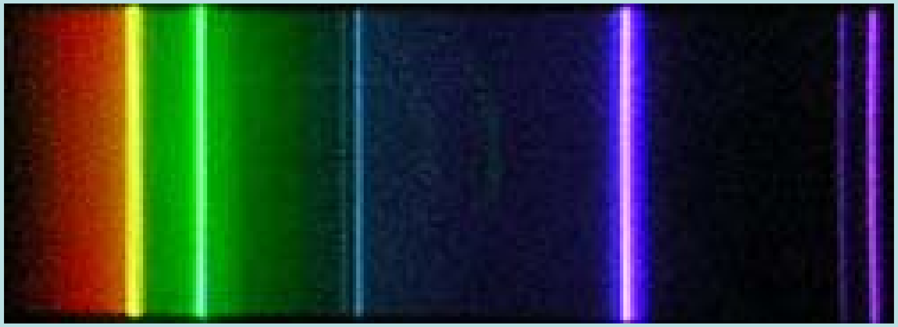


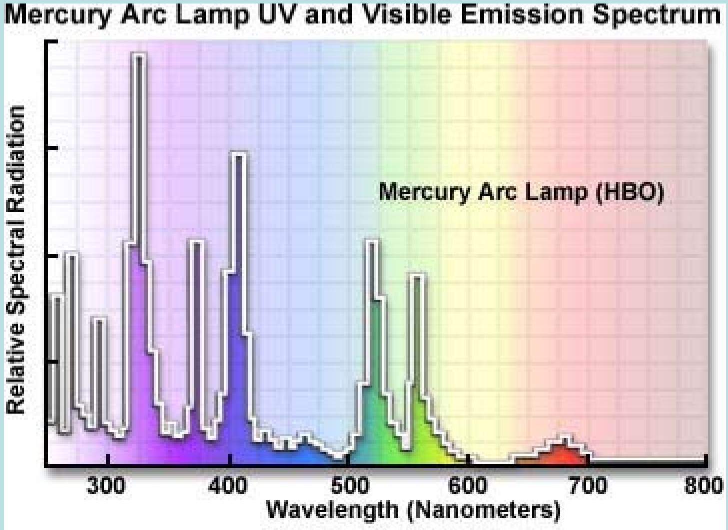
http://jp.hamamatsu.com/resources/products/etd/eng/image/xe_hgxe_003.jpg

Lamp Light Sources

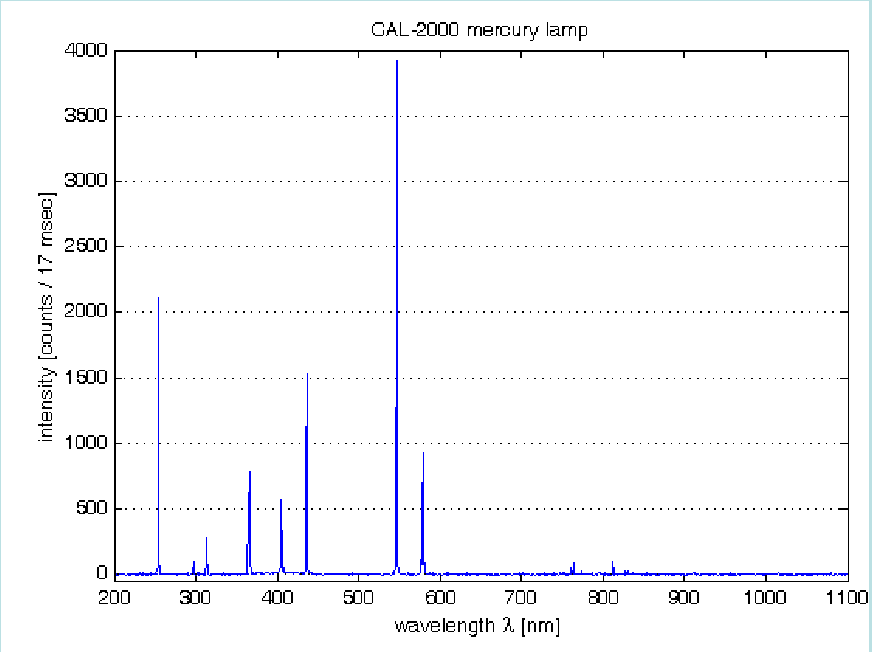
Gas discharge lamps

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





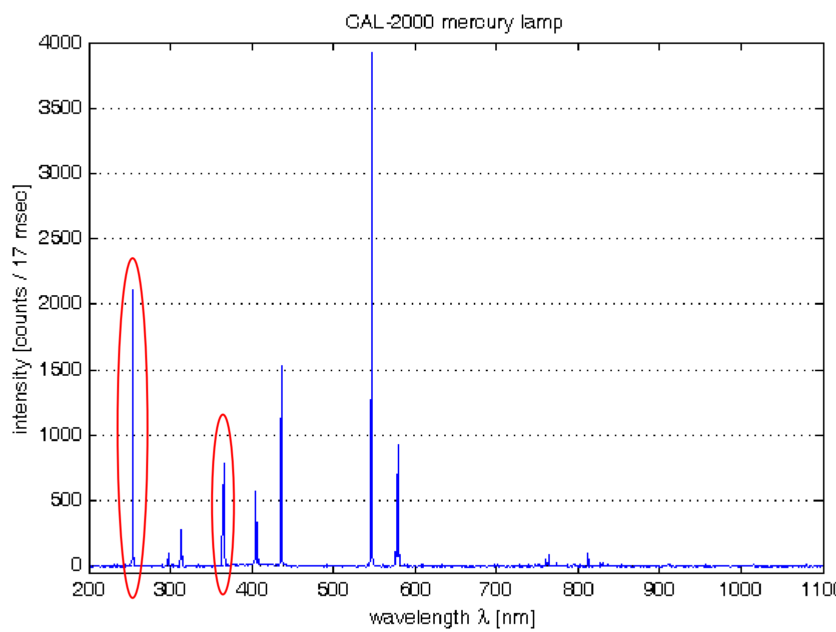
There are strong lines near 254nm, 297nm, 333nm, 365nm, 405nm, 436nm, 546nm and 568nm



Lamp Light Sources

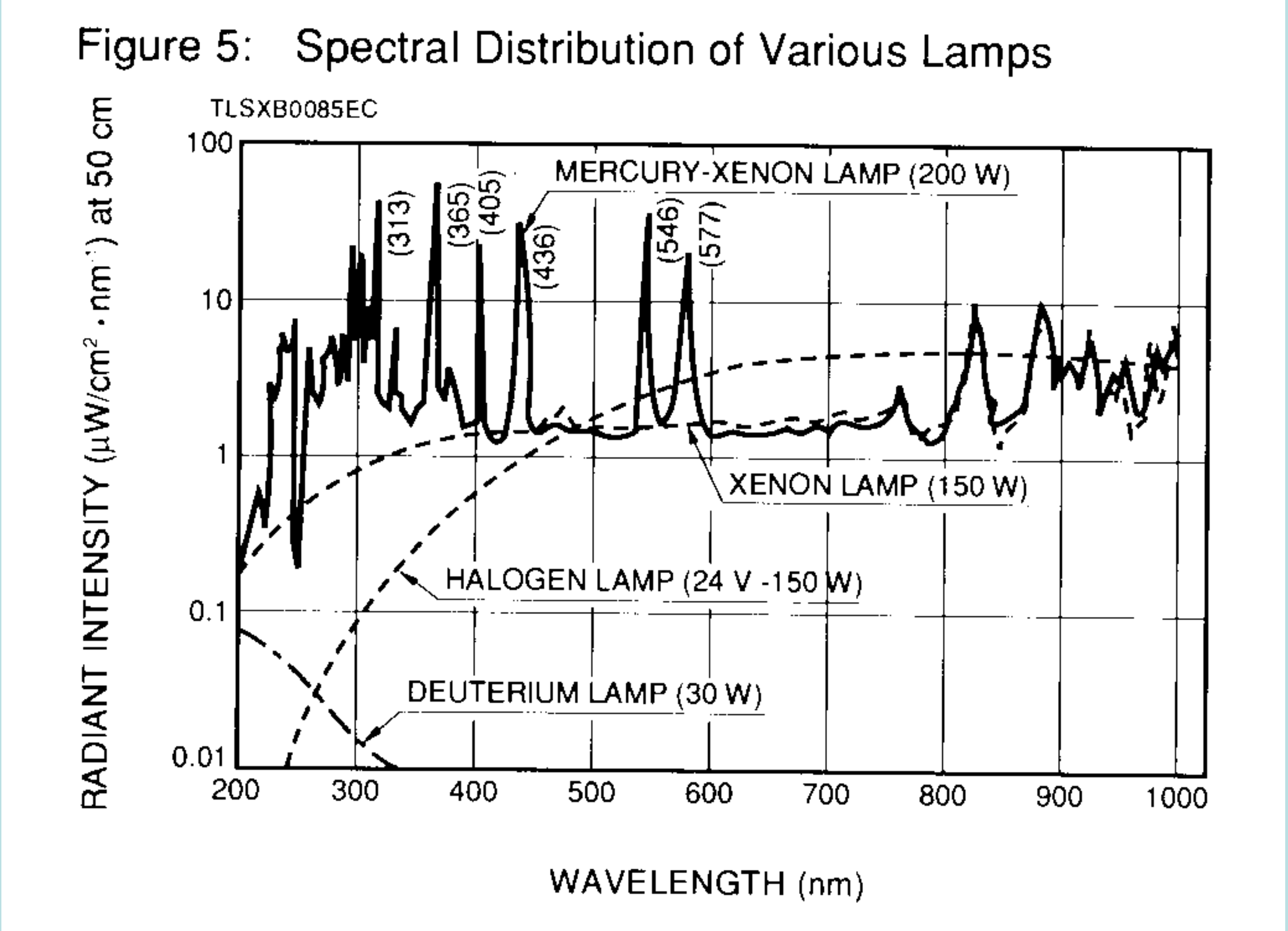
Gas discharge lamps

UV Handlamps usually provide for “short – 254nm” or “long – 365nm” illumination



Lamp Light Sources

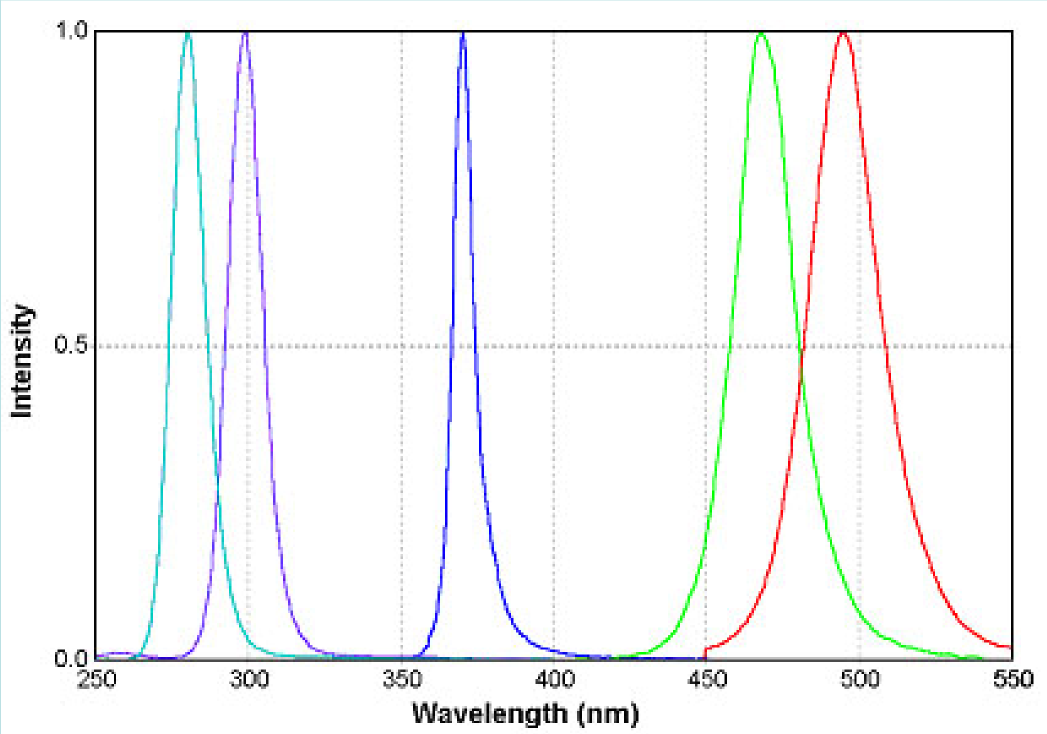
Mercury-Xenon Arc Lamp (greater intensities in the UV)



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

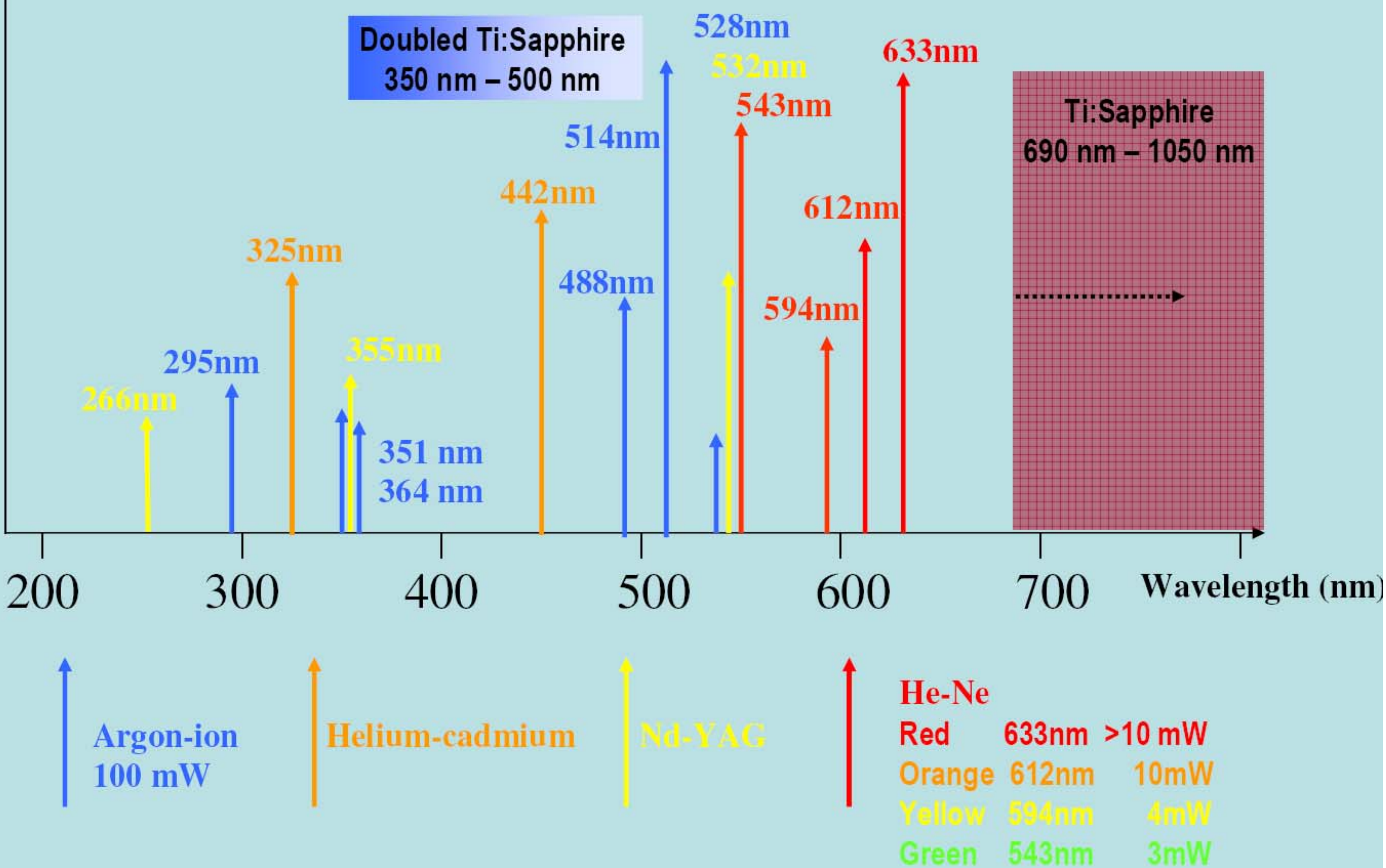
Laser Light Sources

Quiz: What does LASER stand for?

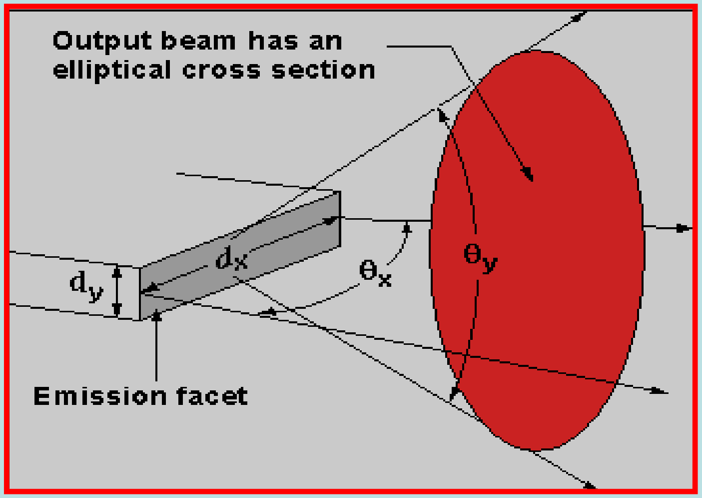
Light **A**mplification by **S**timulated **E**mission of **R**adiation

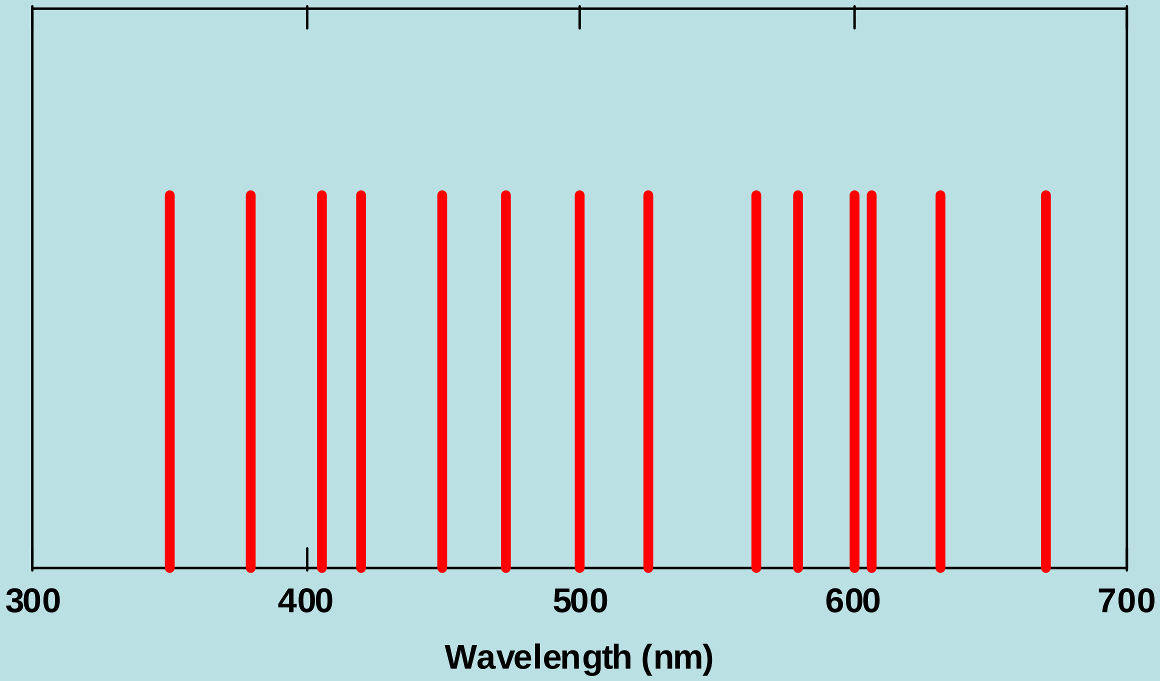
Argon Ion:

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

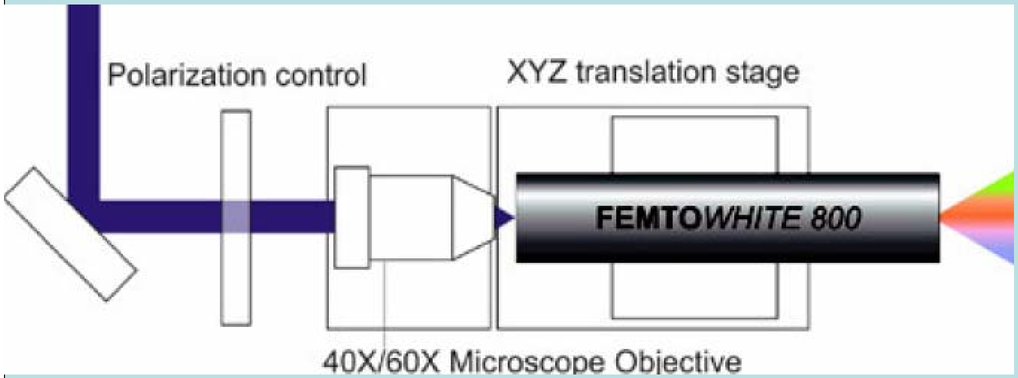


Laser Diodes

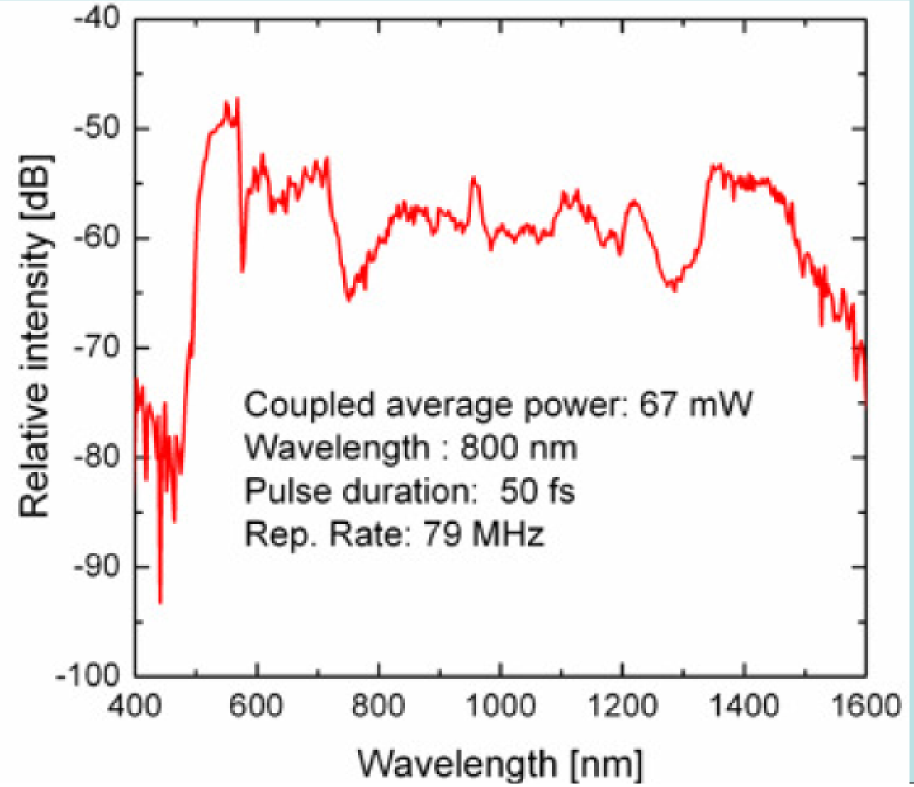


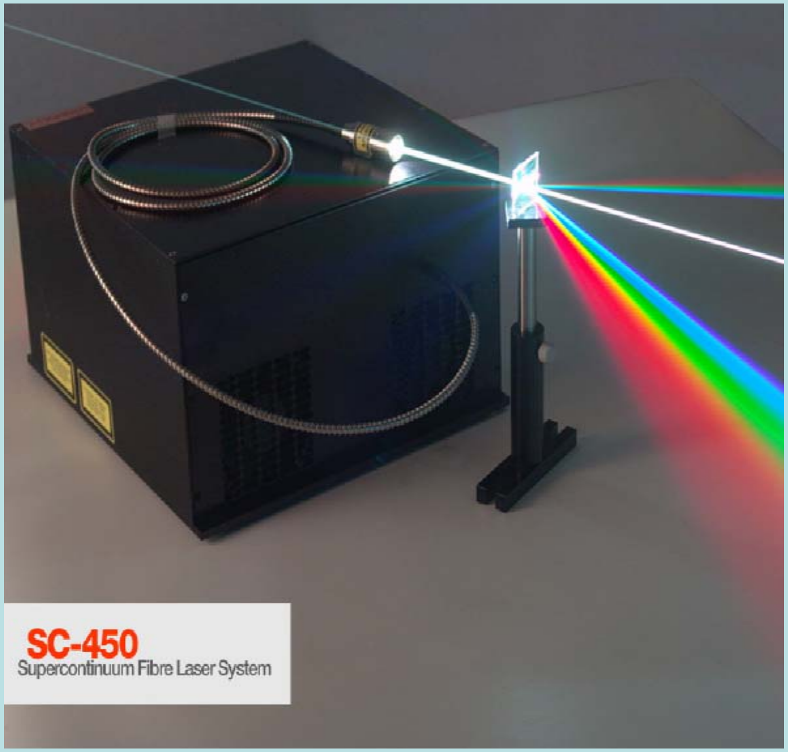


"White" lasers



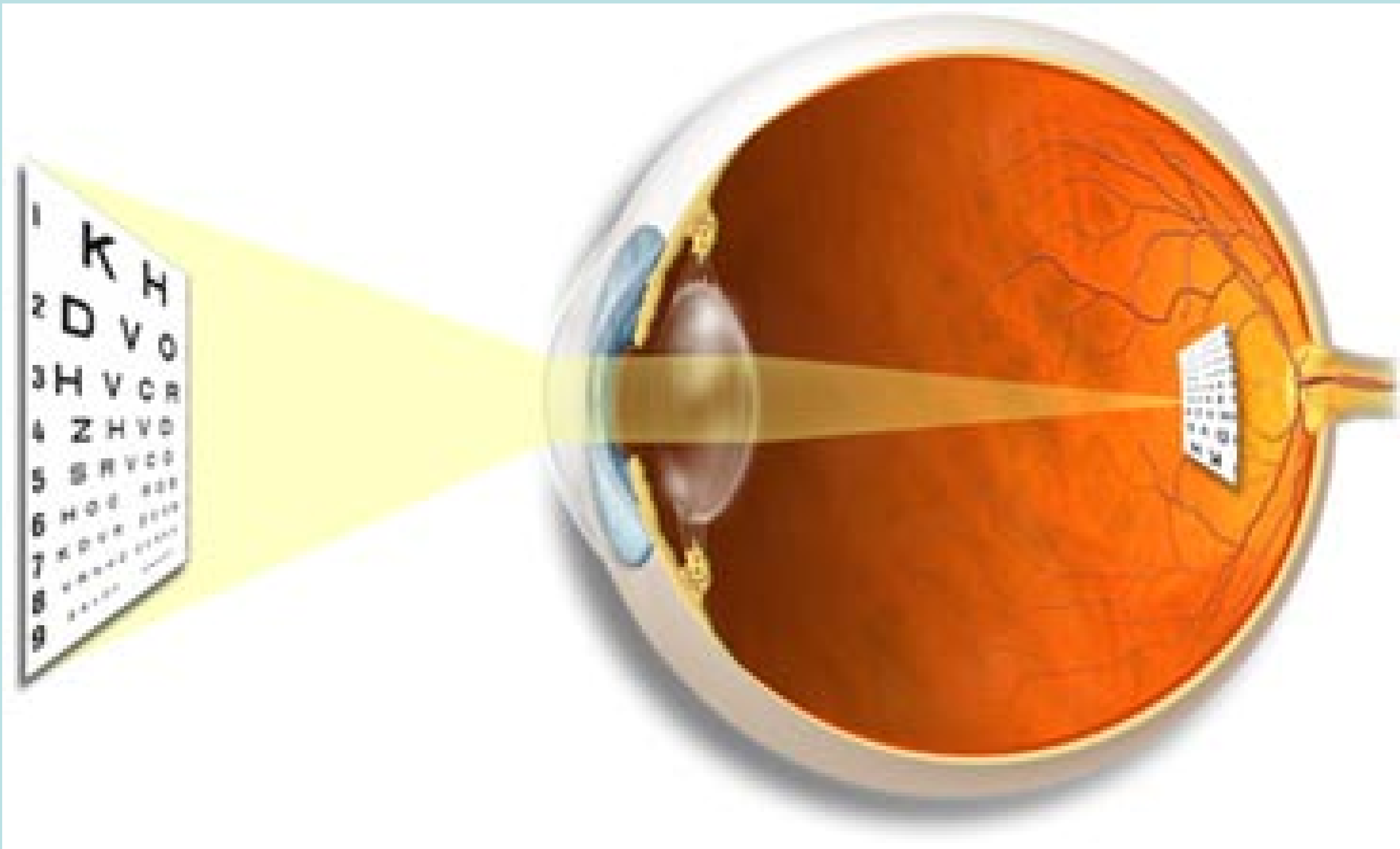
Ultrashort pulsed light is focused into a photonic crystal fiber



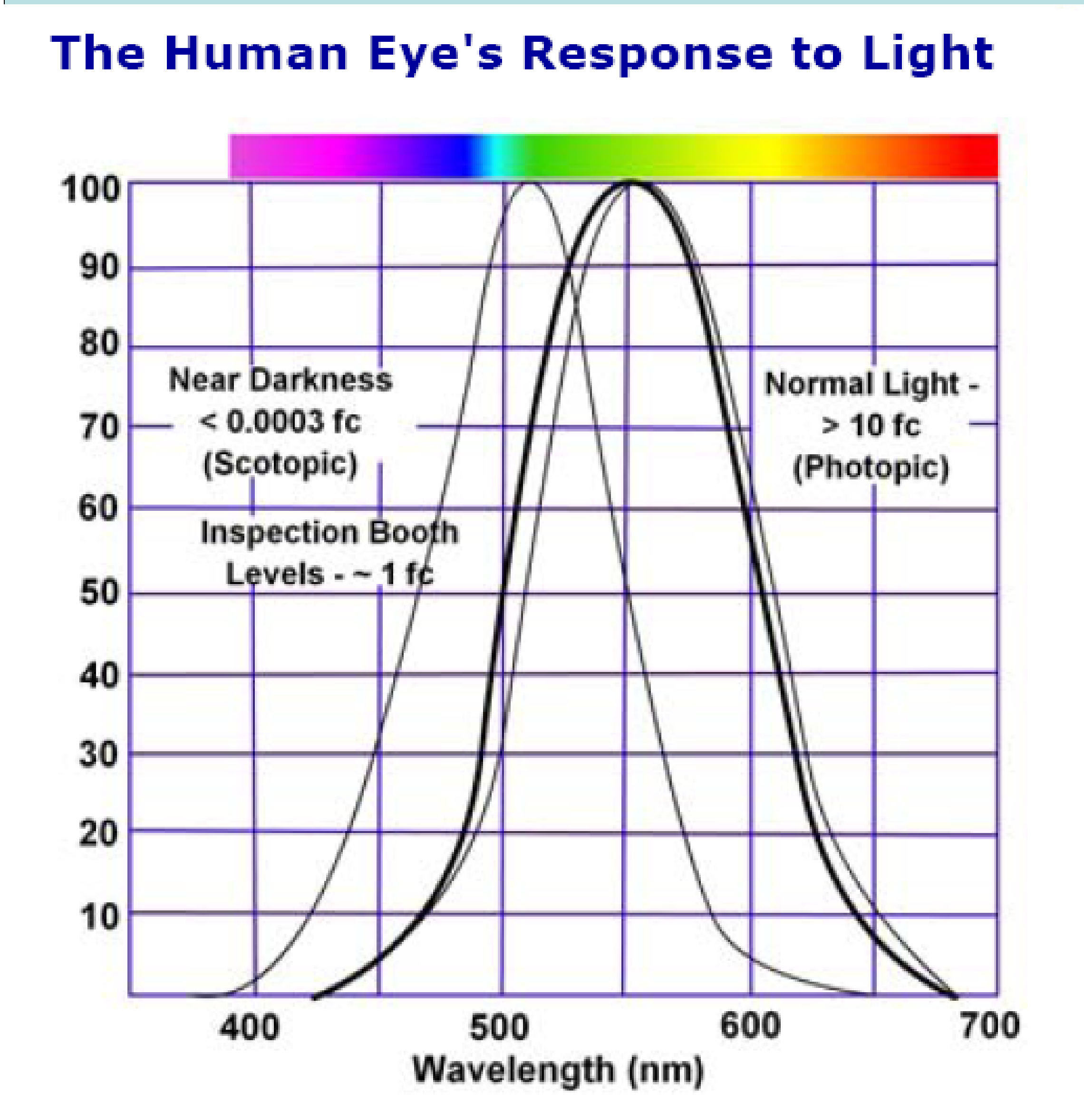


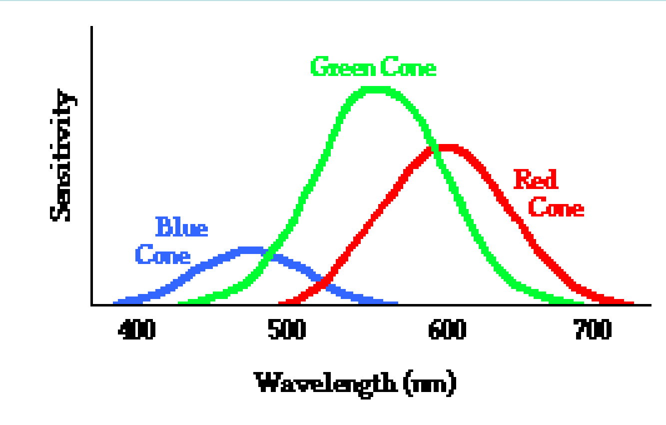
New light source being tested in Hawaii

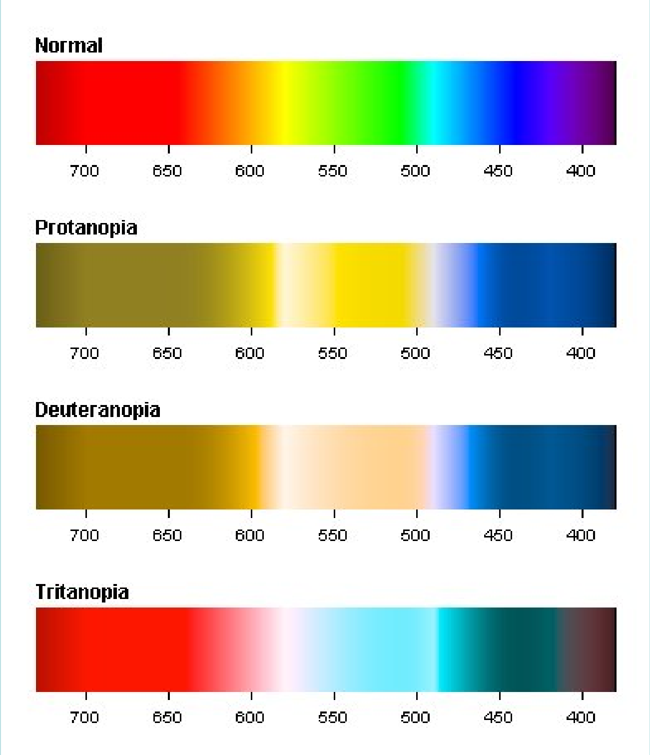
Detectors



Detectors





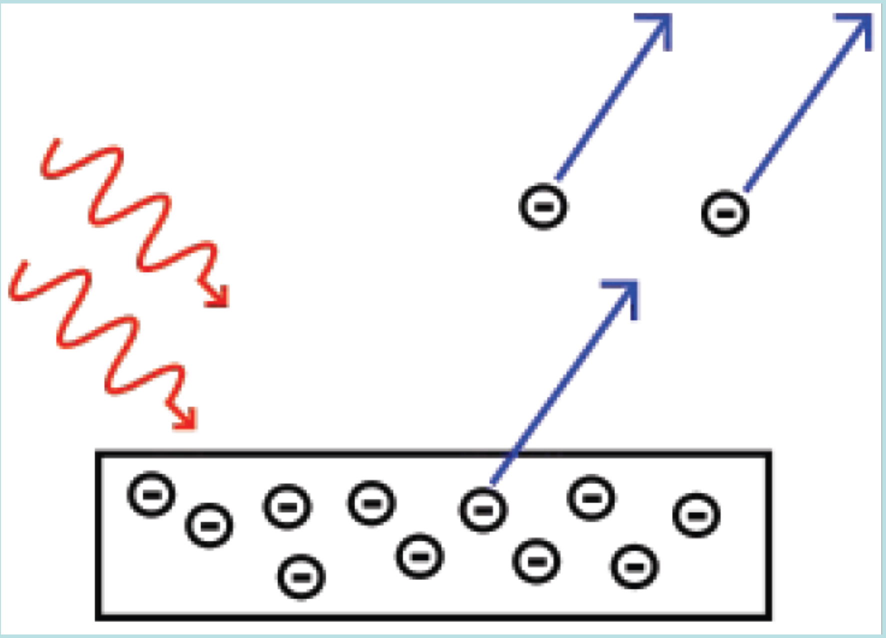


http://www.ndt-ed.org/EducationResources/CommunityCollege/PenetrantTest/Introduction/lightresponse.htm

Detectors

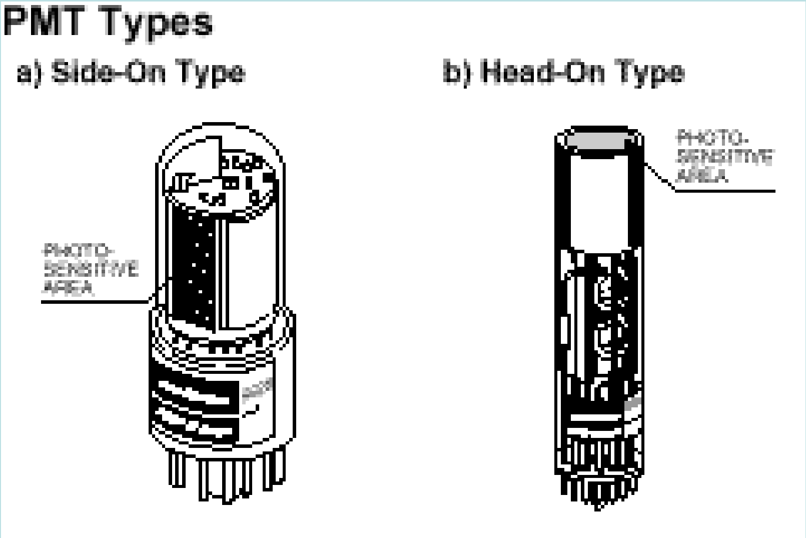
The photoelectric effect was discovered by Heinrich Hertz in 1886

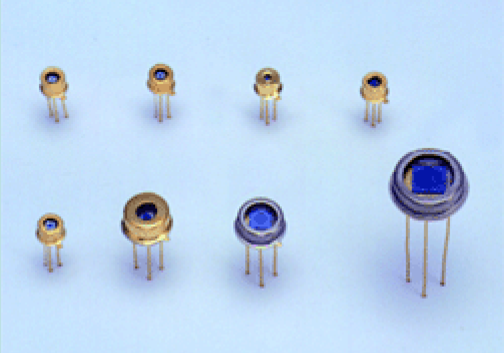
Specifically he noticed that a charged object loses its charge more readily when it is illuminated by UV light



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



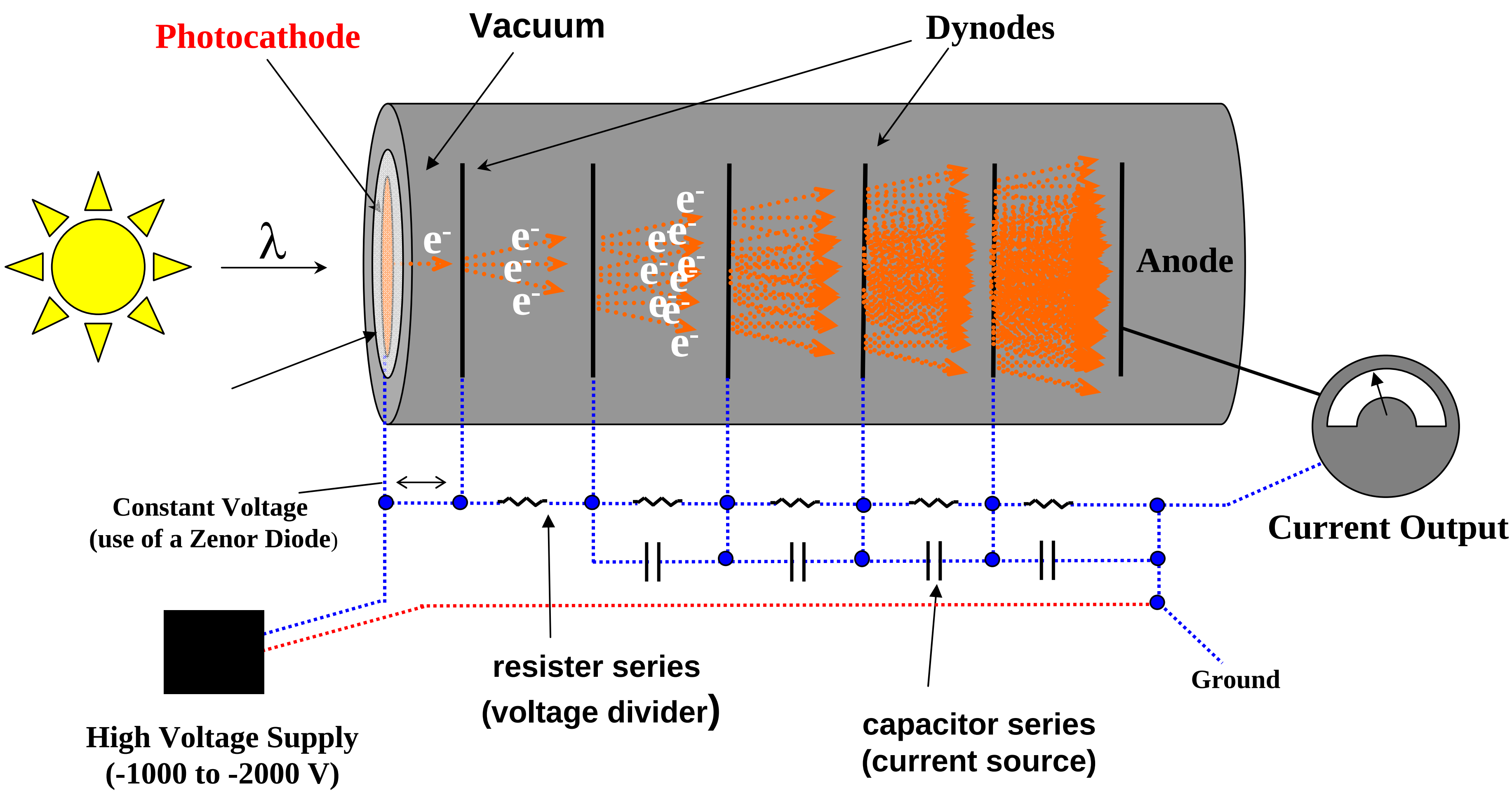


APD

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 | **Head-On PMT**

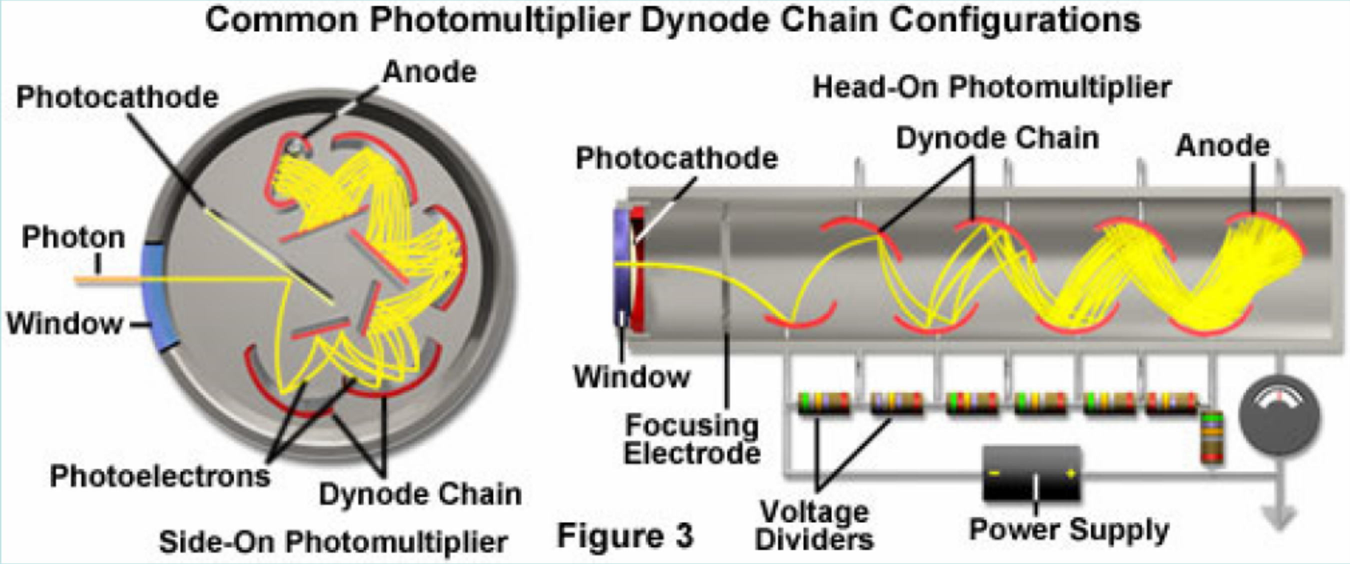
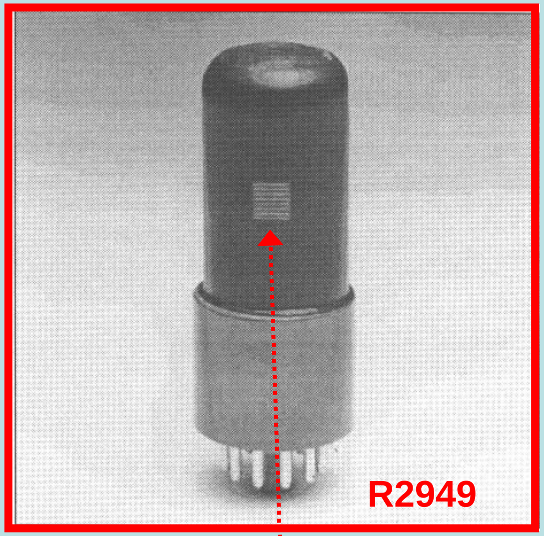


Figure 3

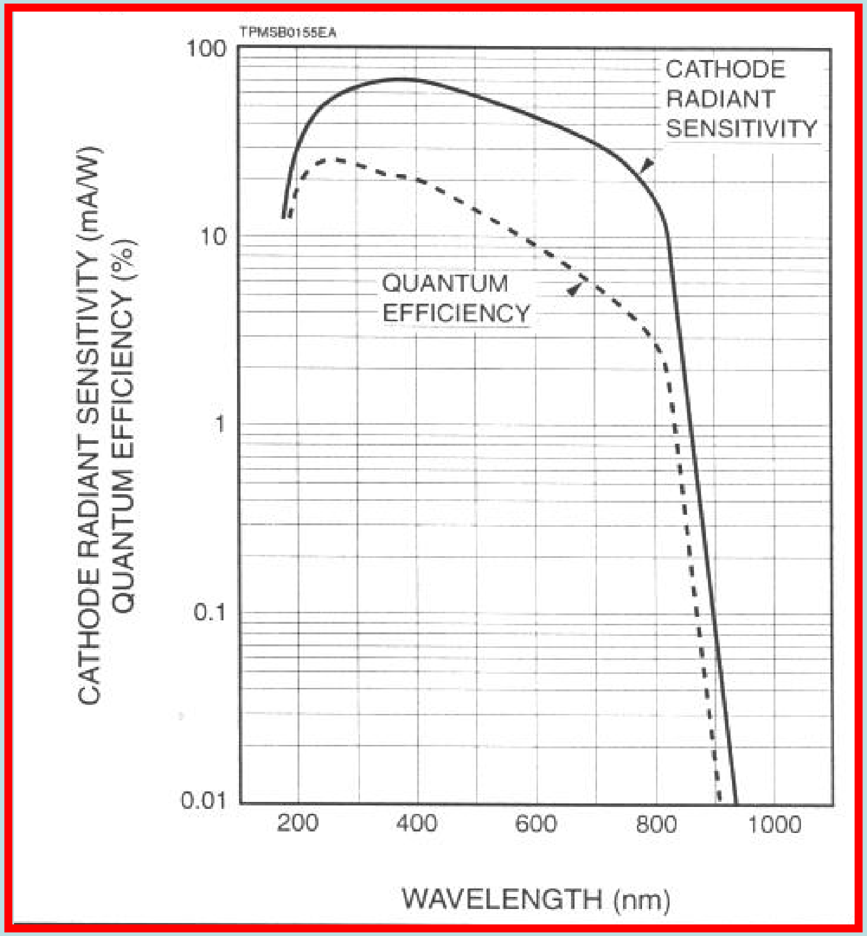
Opaque photocathode | **Semitransparent Photocathode**

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

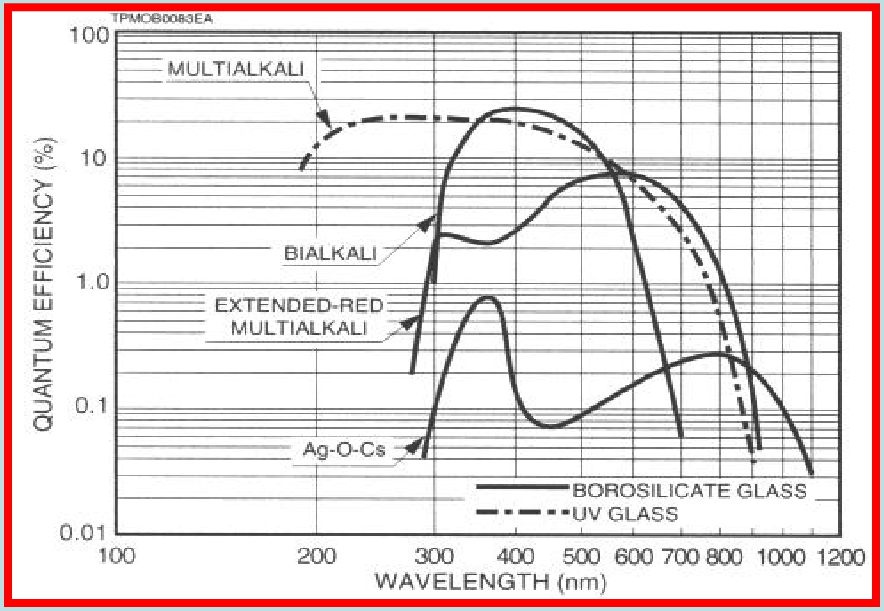


Window with Photocathode Beneath

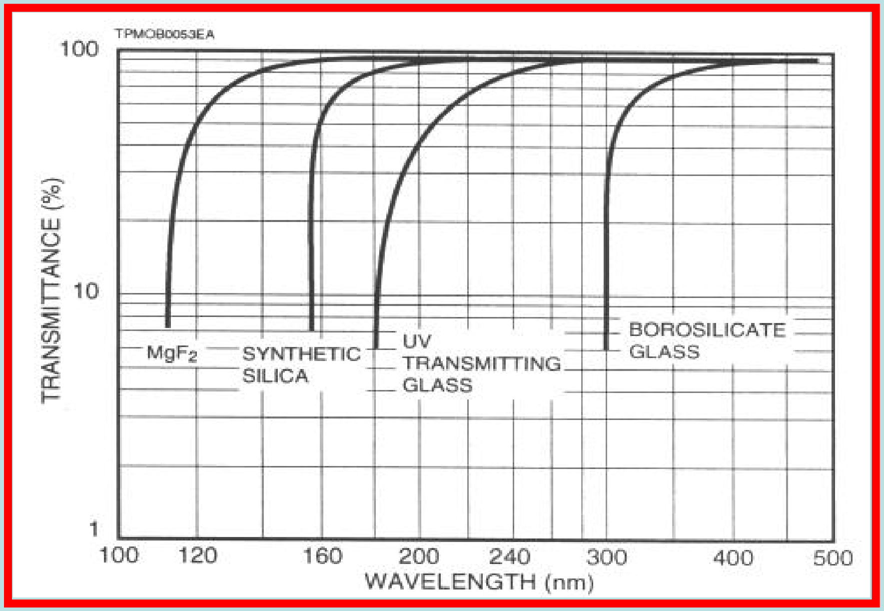


PMT Quantum Efficiencies

Cathode Material



Window Material



Detectors

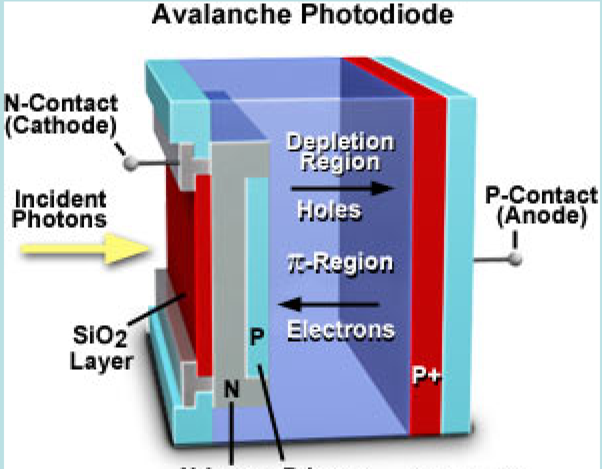
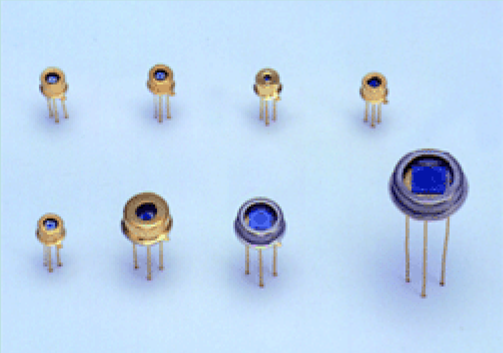


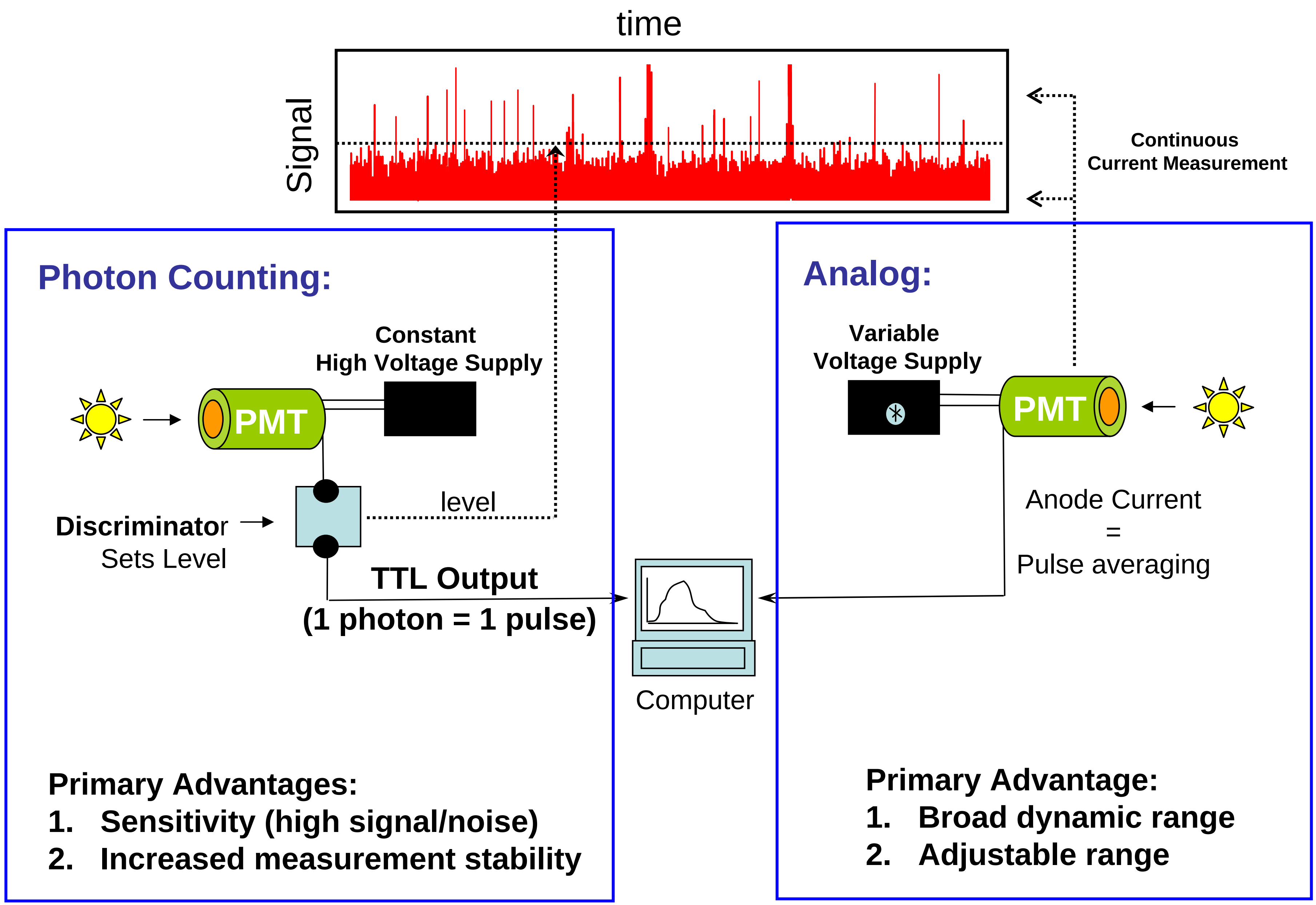
Figure 1

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

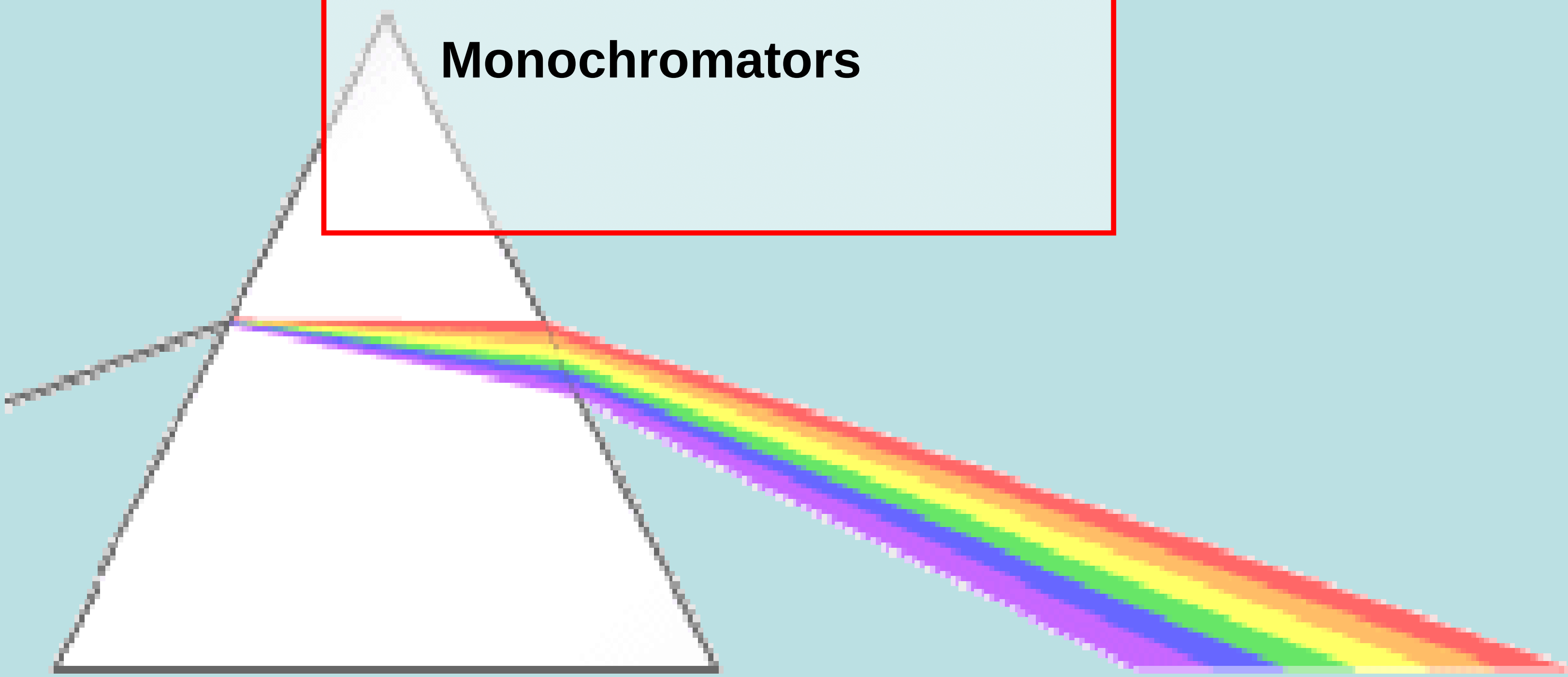


Wavelength Selection

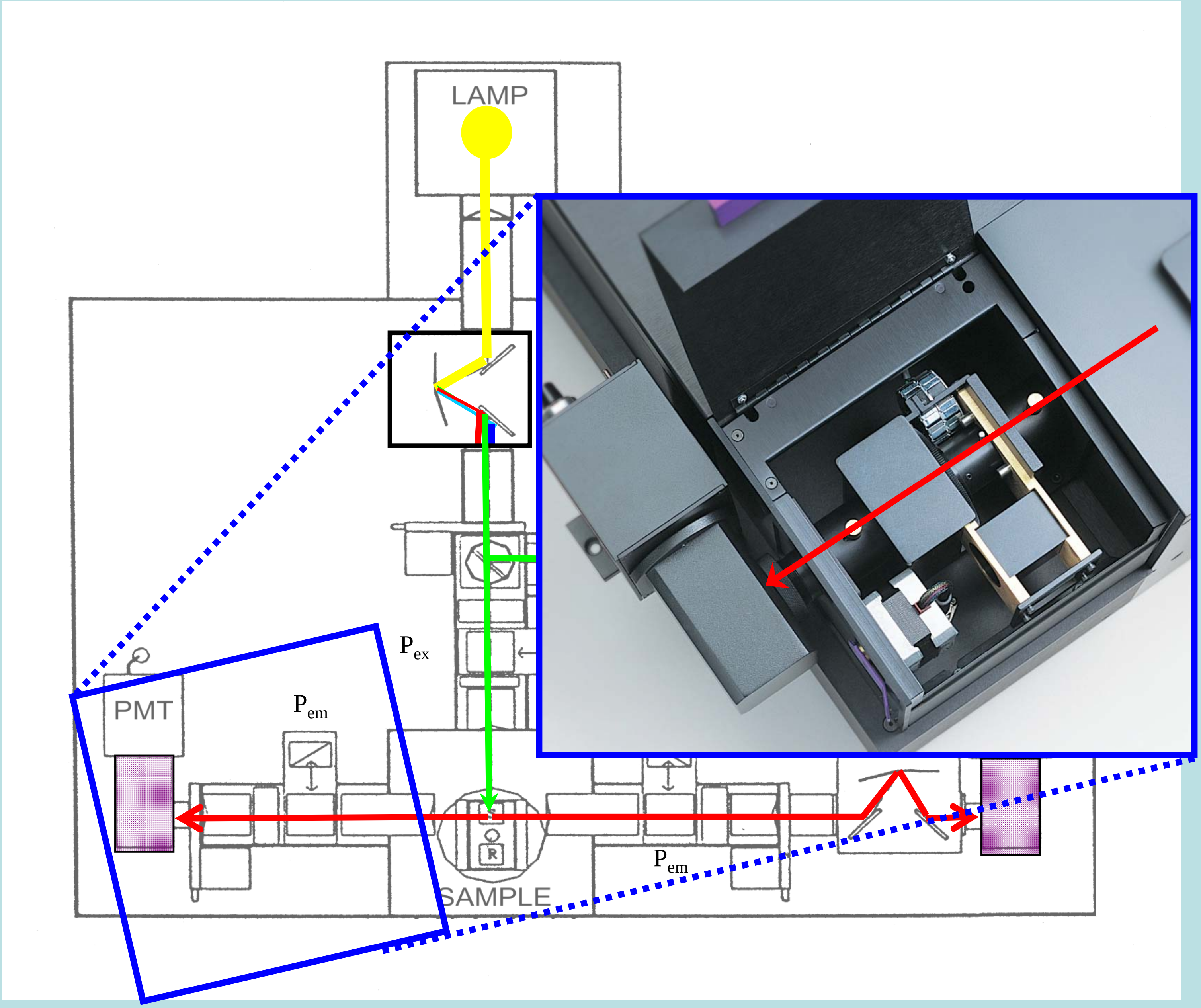
Fixed Optical Filters

Tunable Optical Filters

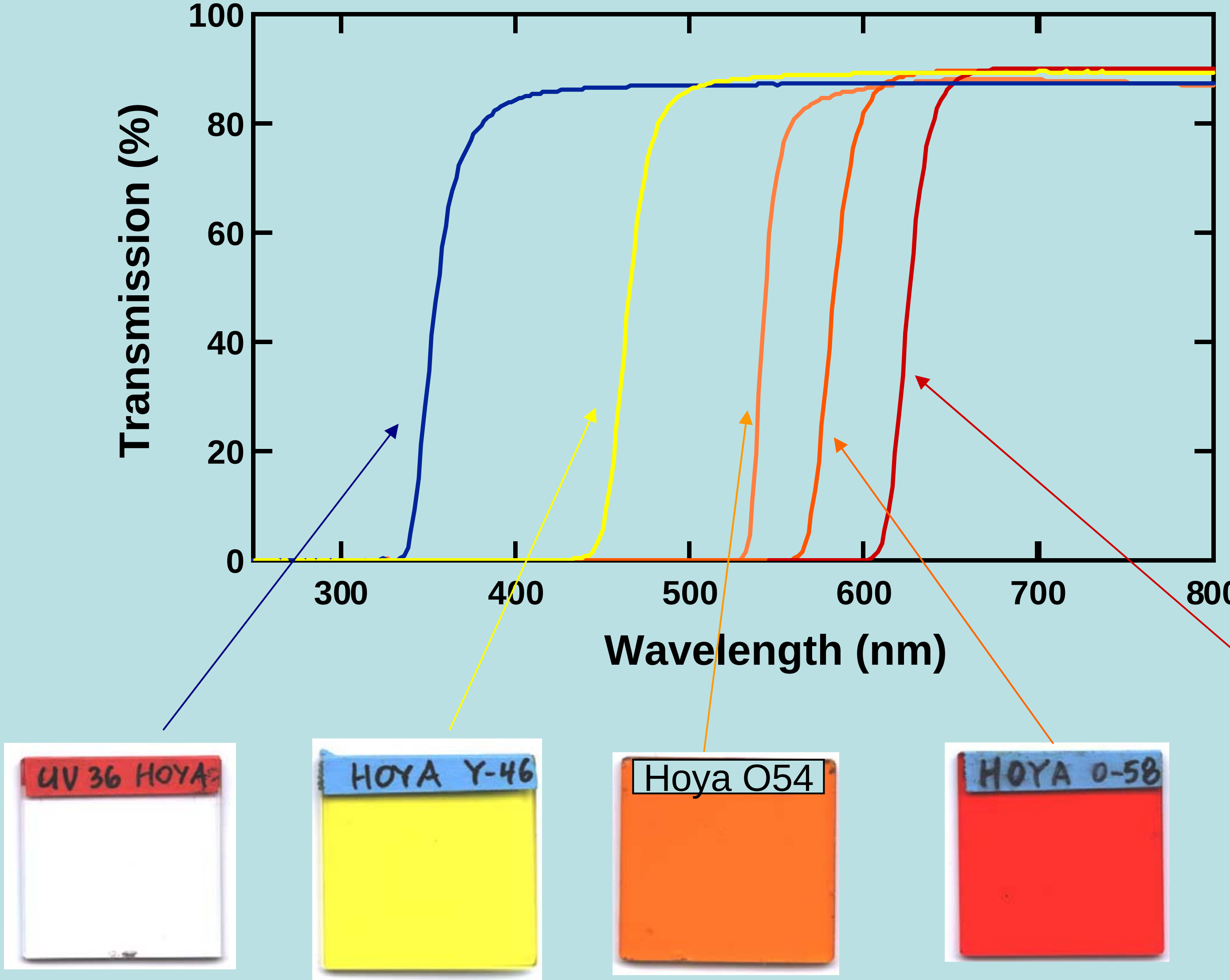
Monochromators



Optical Filter Channel

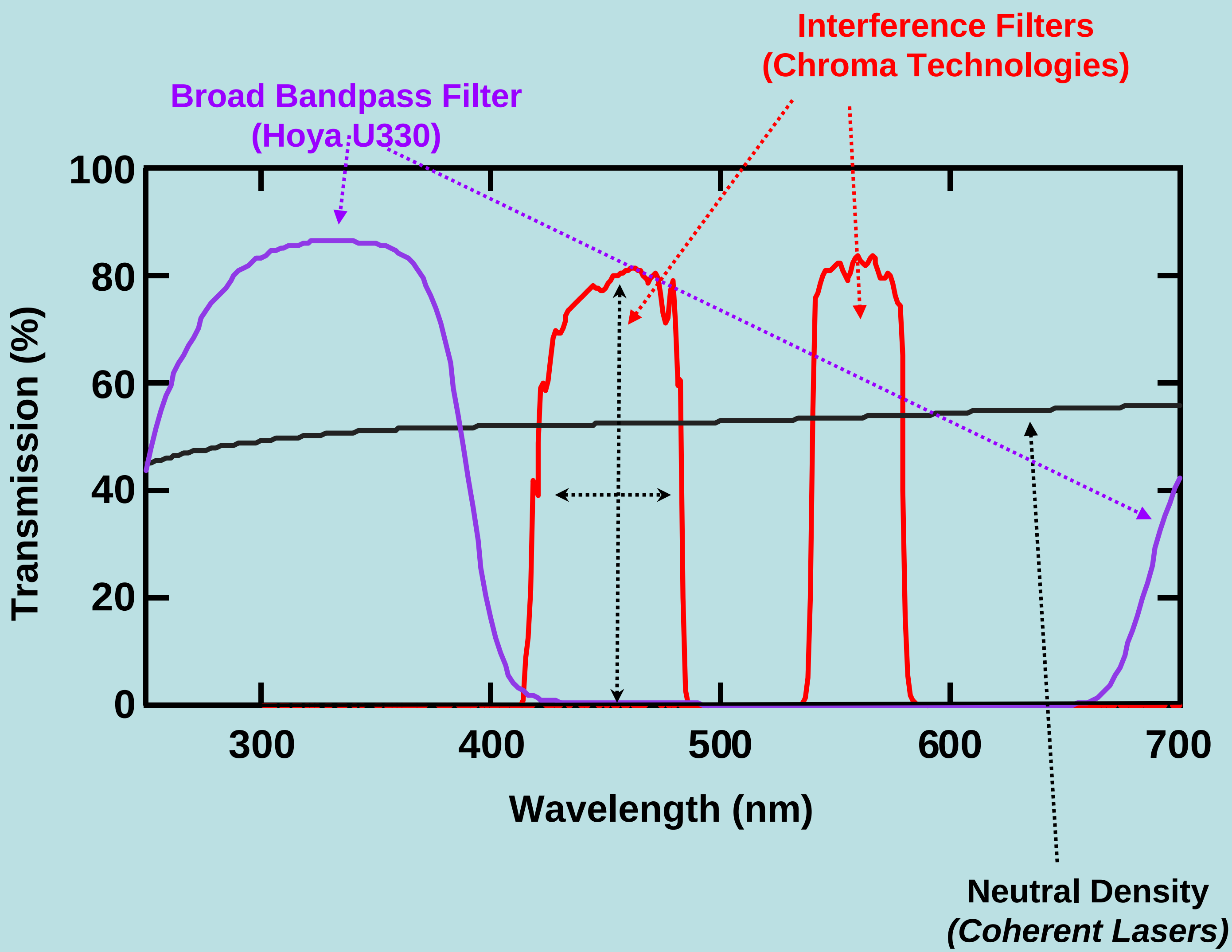


Long Pass Optical Filters



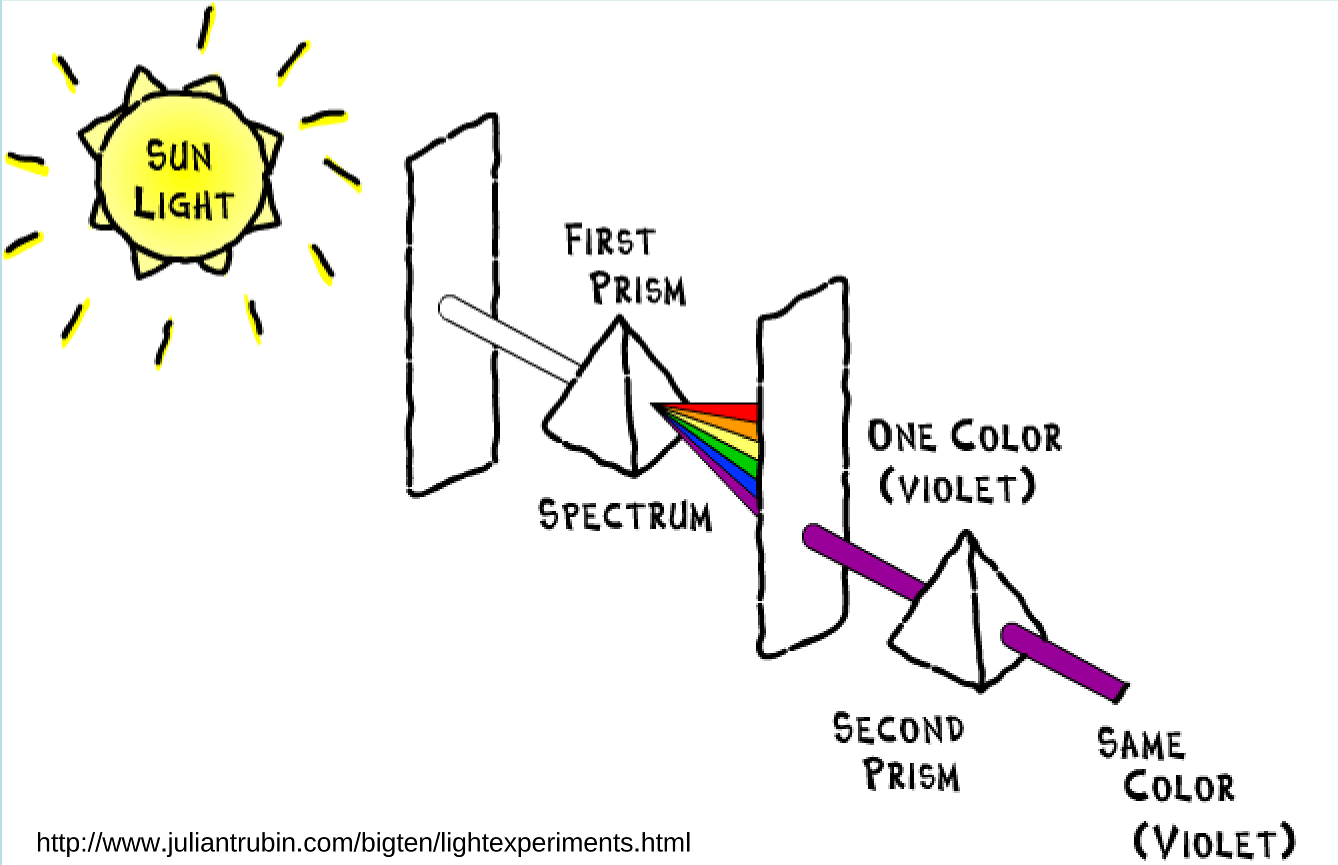
Spectral Shape
Thickness
Physical Shape
Fluorescence (!?)

More Optical Filter Types…



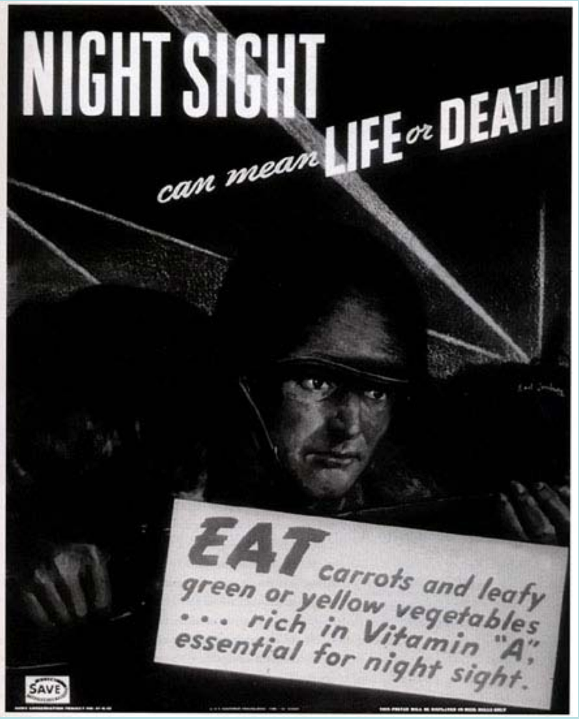
Monochromators

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 lght – namely that it was composed of many colors.

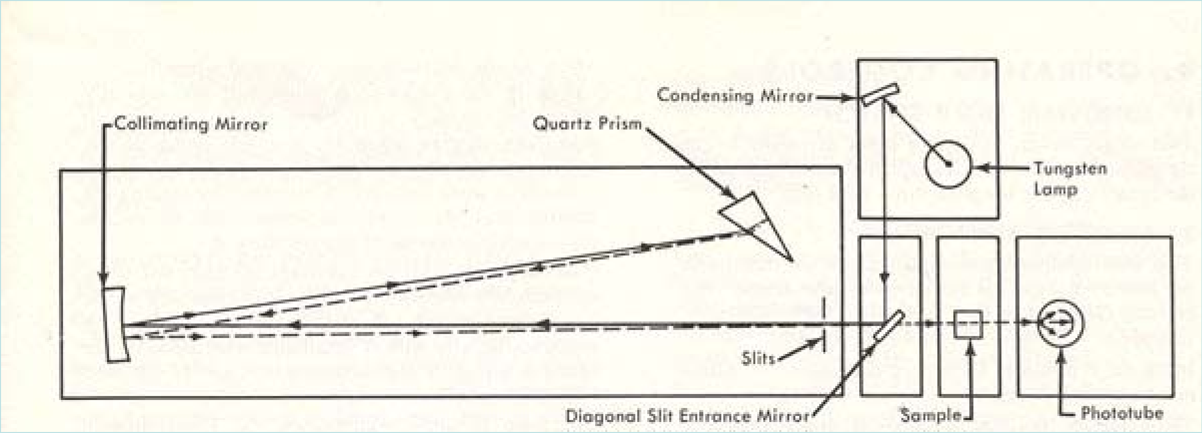


http://www.juliantrubin.com/bigten/lightexperiments.html

Monochromators



An important impetous 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**



http://www.wooster.edu/chemistry/is/brubaker/uv/uv_landmark.html#1

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).

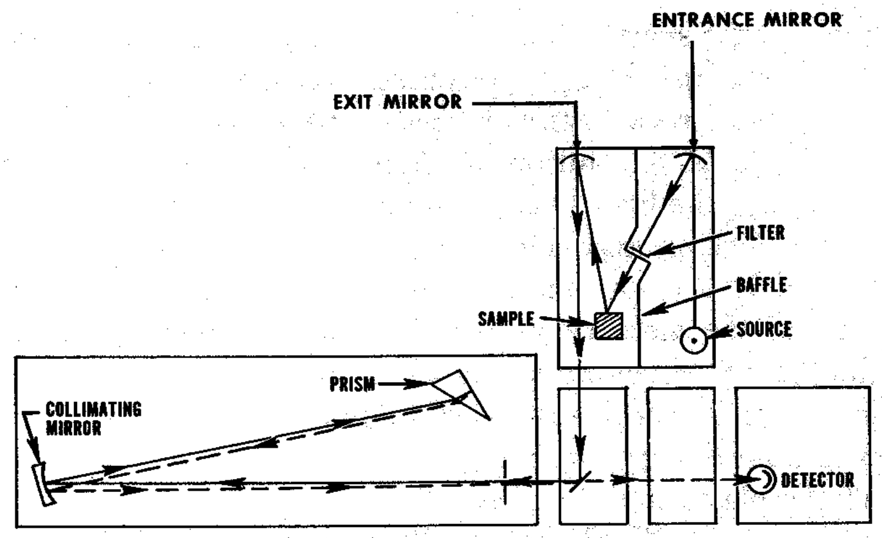
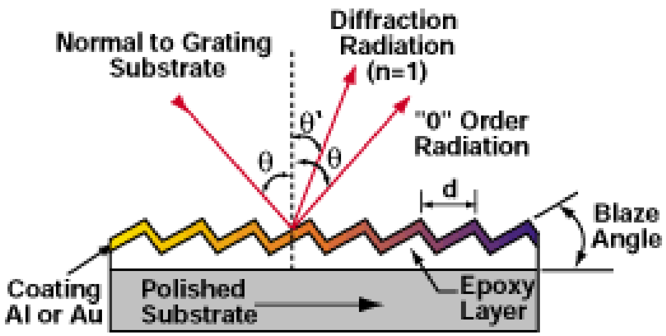


FIG. 20. Attachment for measuring fluorescence spectra with the Beckman Model DU and DK spectrophotometers.

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

Ruled Grating Replica



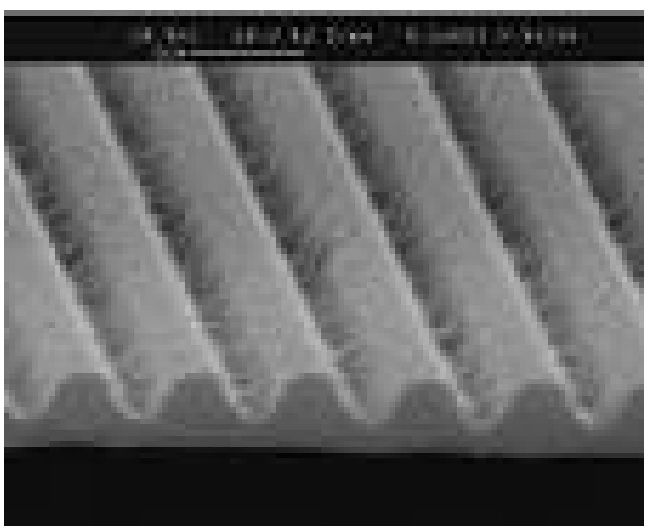
GRATING EQUATION

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

n=order of diffraction

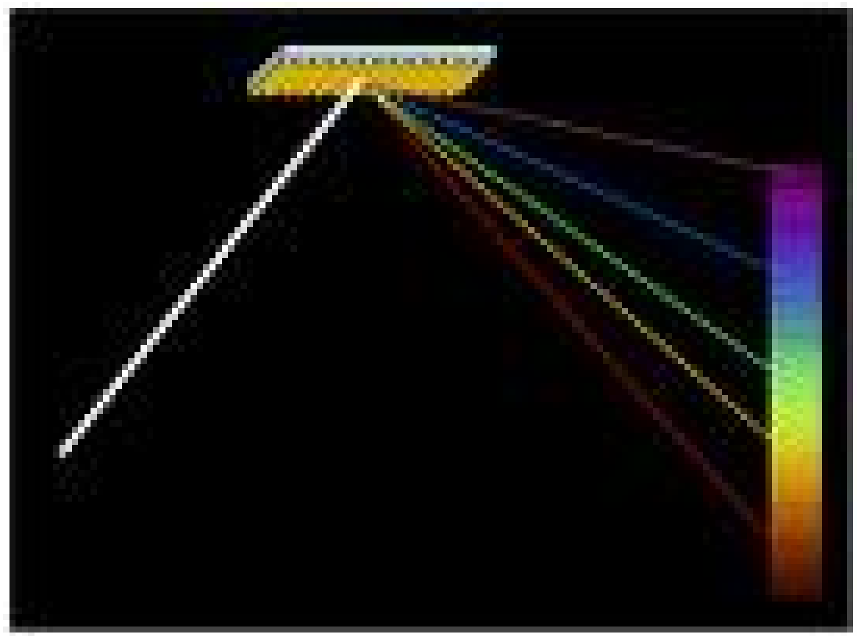
d=grating constant

λ = diffracted wavelength



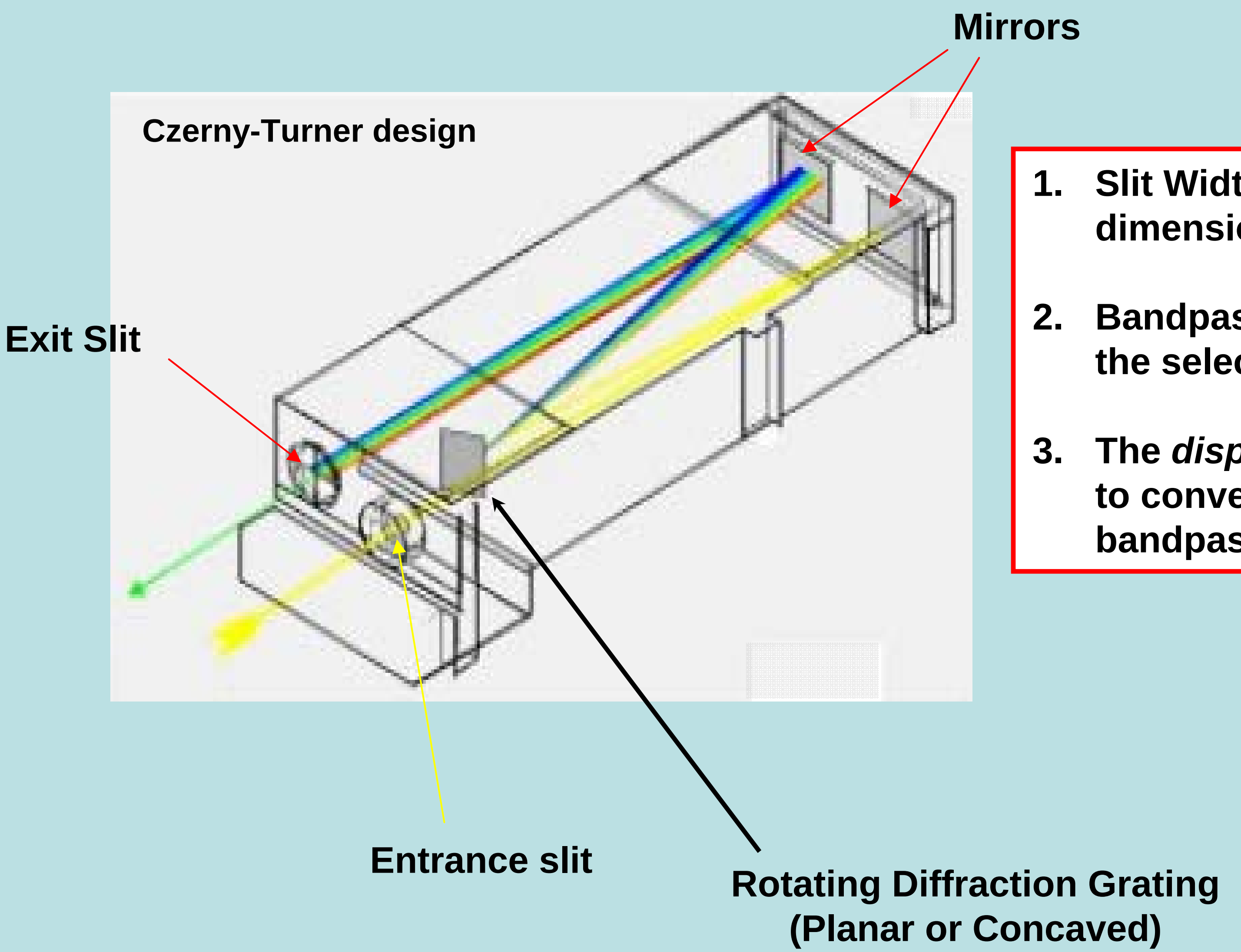
Formerly ruled with diamond-tipped instruments

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



http://gratings.newport.com/products/supplemental/types.asp

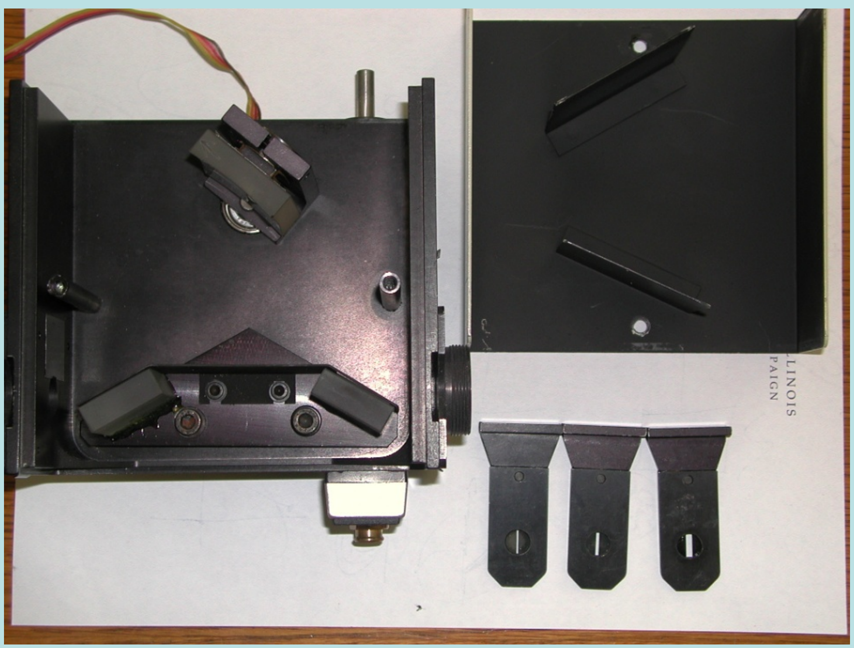
Monochromators

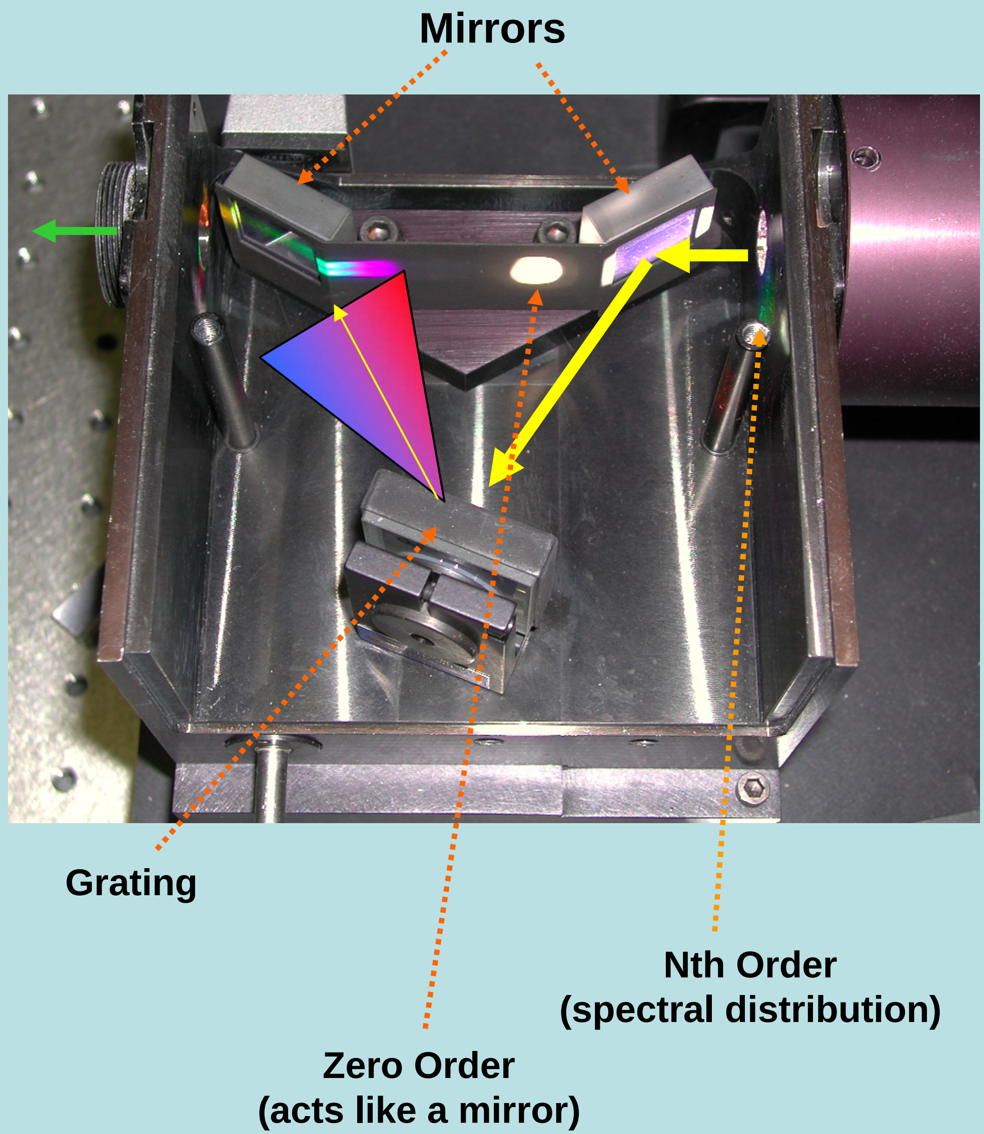


1. Slit Width (mm) is the dimension of the slits.
2. Bandpass is the FWHM of the selected wavelength.
3. The *dispersion* is the factor to convert slit width to bandpass.

The Inside of a Monochromator

H10 Monochromator



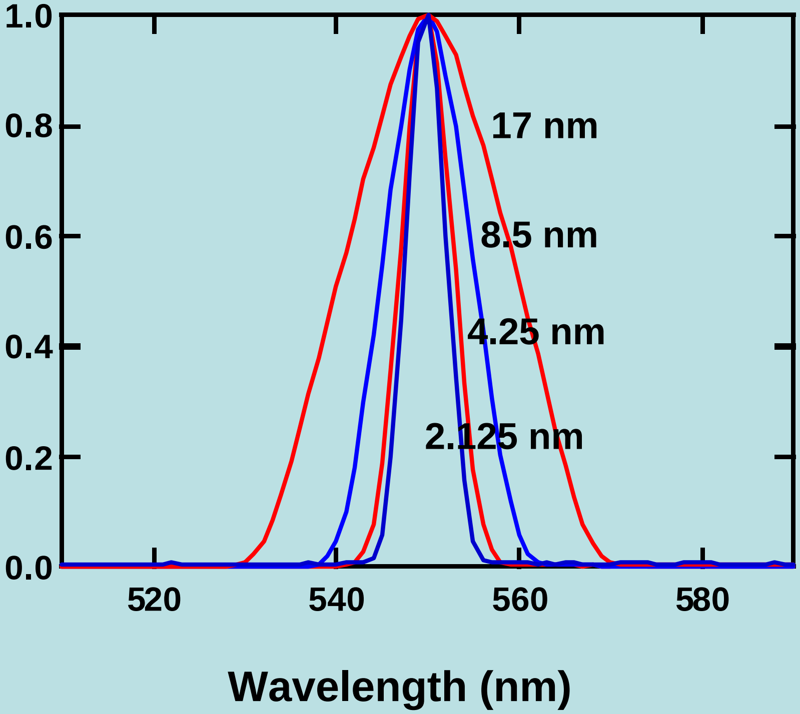


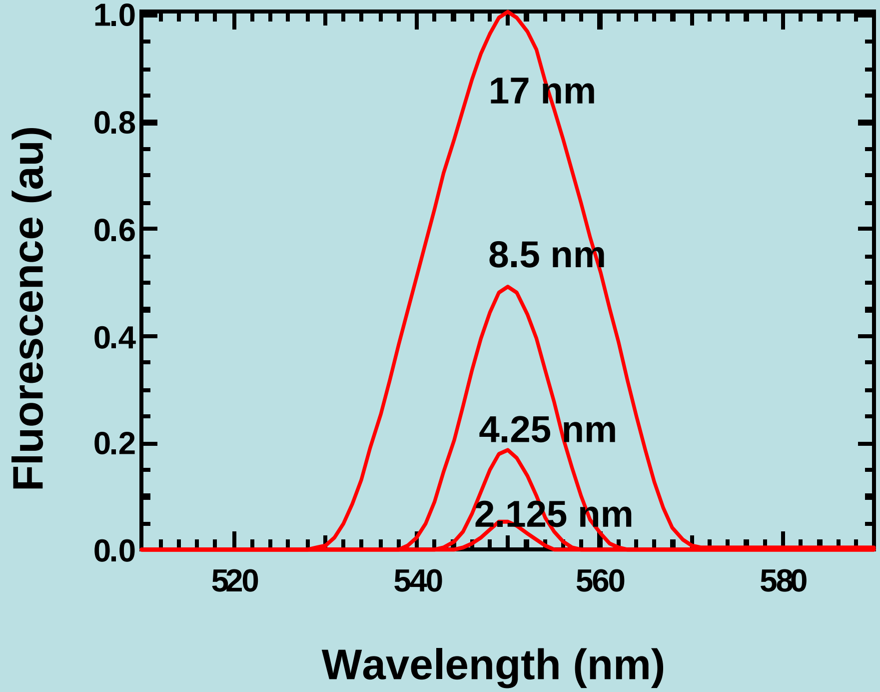
Changing the Bandpass

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)

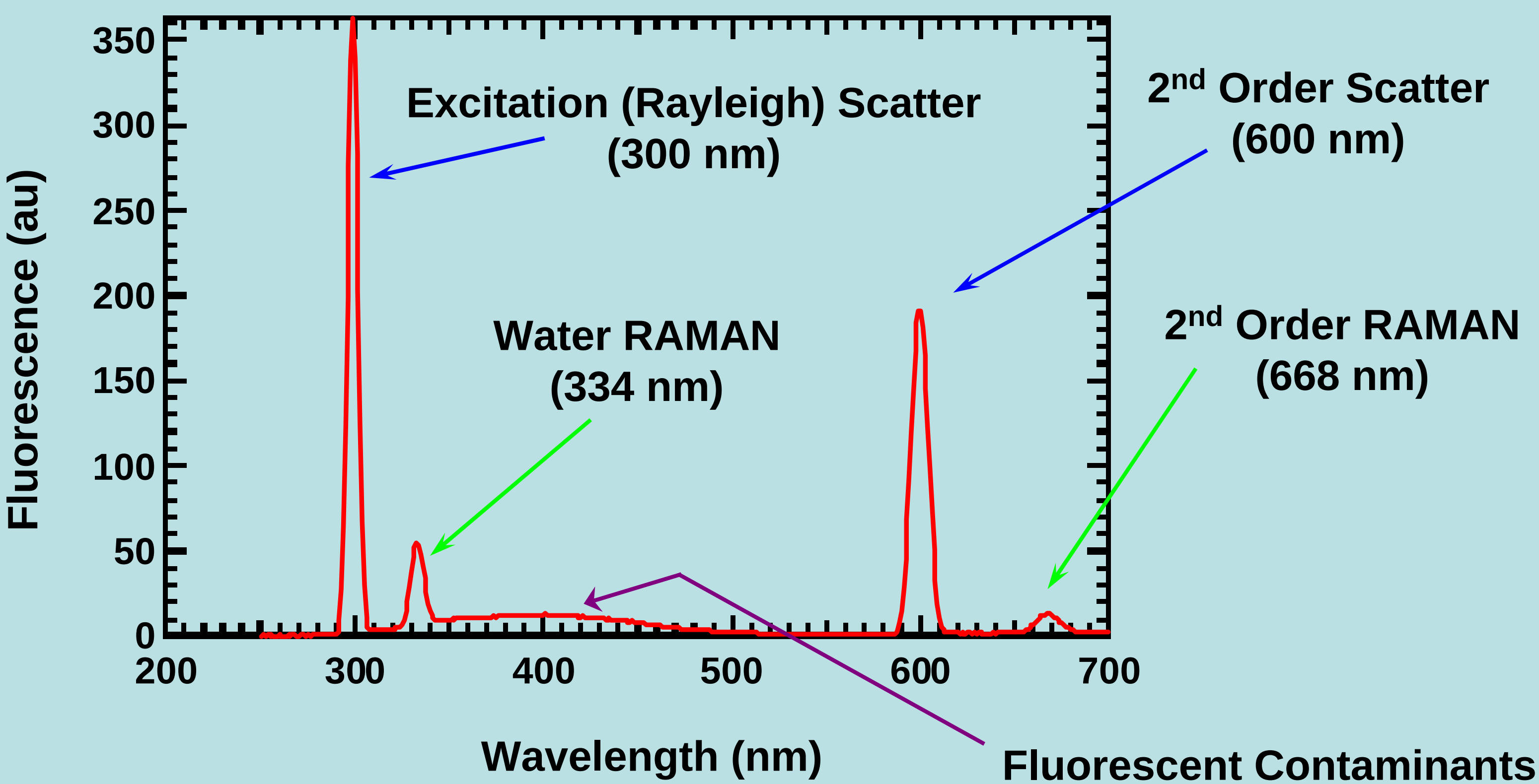




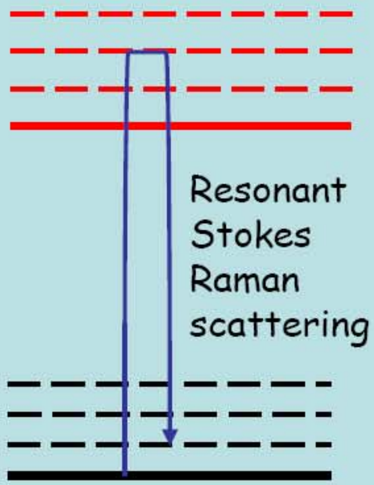
Collected on a SPEX Fluoromax - 2

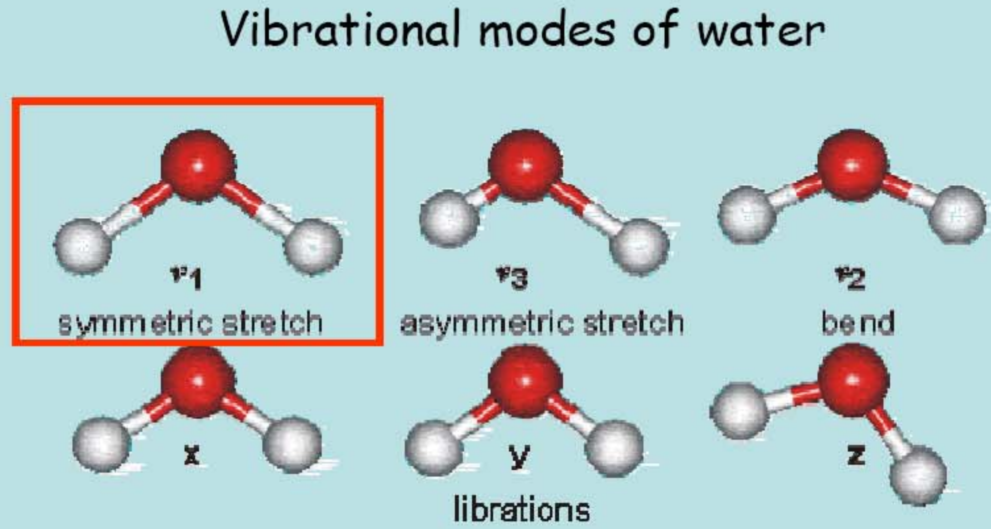
Higher Order Light Diffraction

Emission Scan:
Excitation 300 nm
Glycogen in PBS



Raman scatter of water





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

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

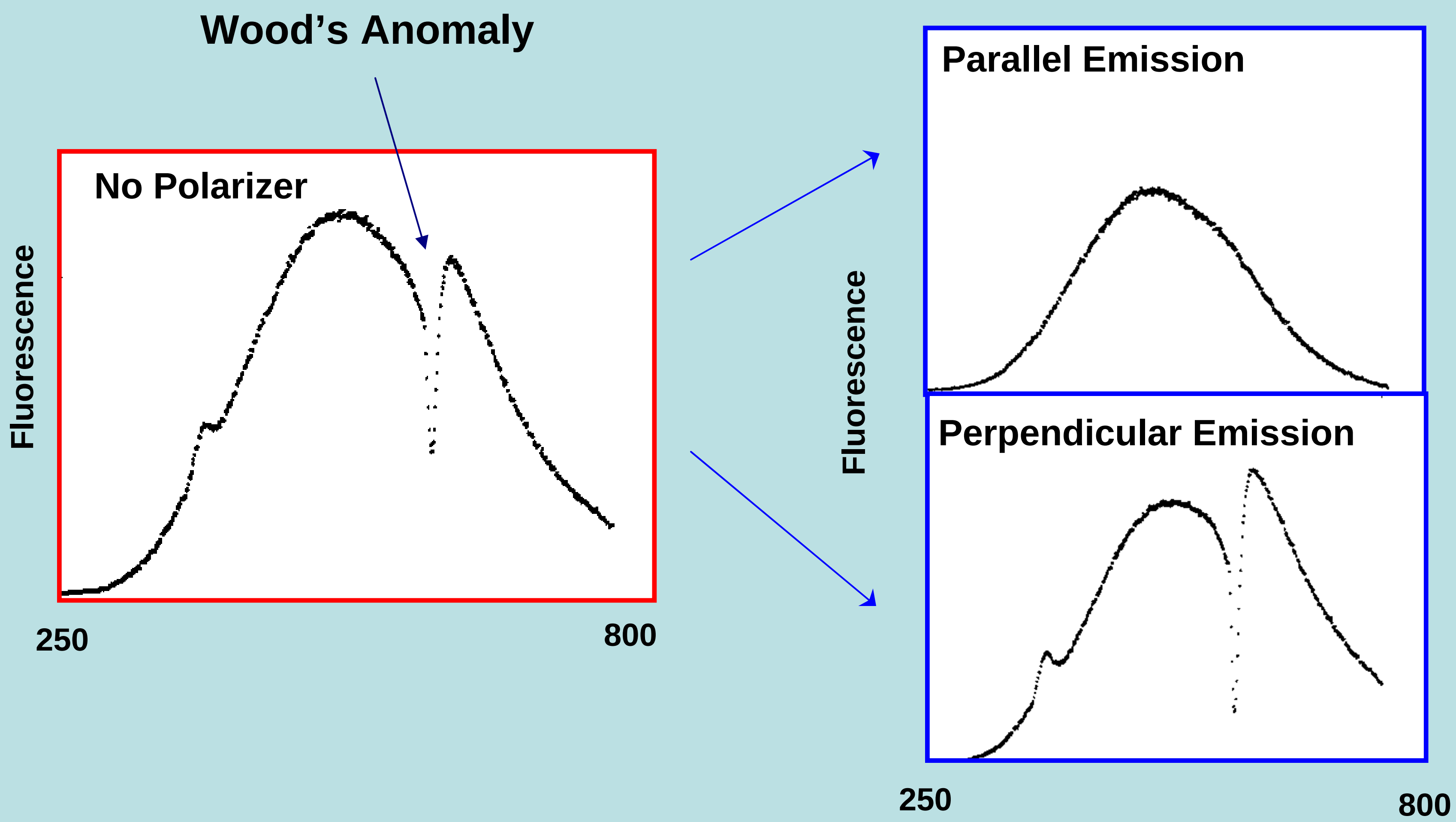
The approximate position of the water Raman peak can be calculated with this formula

For example:

Exc	Raman
280	310
350	397
480	574

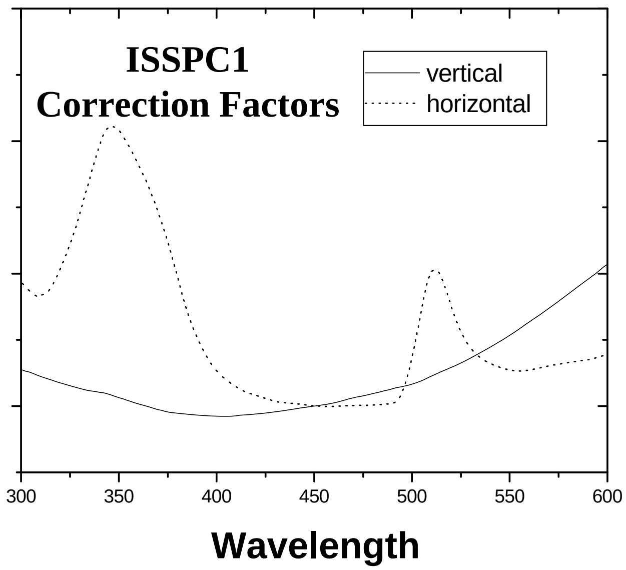
Monochromator Polarization Bias

Tungsten Lamp Profile Collected on an SLM Fluorometer

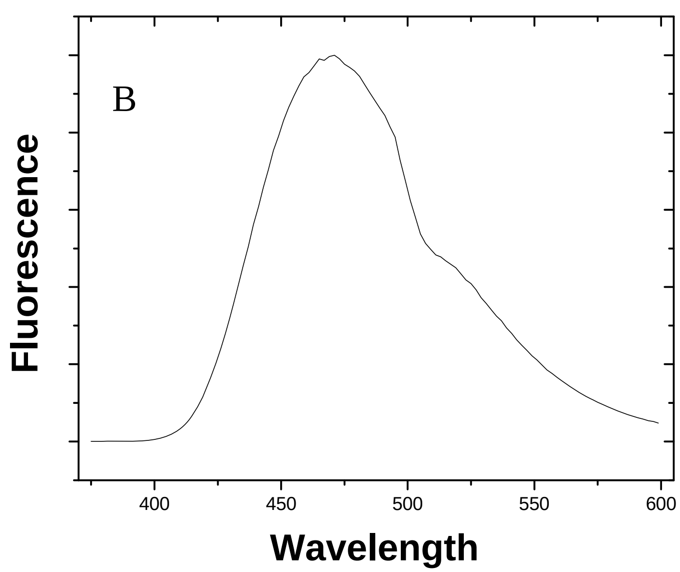


Adapted from Jameson, D.M., Instrumental Refinements in Fluorescence Spectroscopy: Applications to Protein Systems., in Biochemistry, Champaign-Urbana, University of Illinois, 1978.

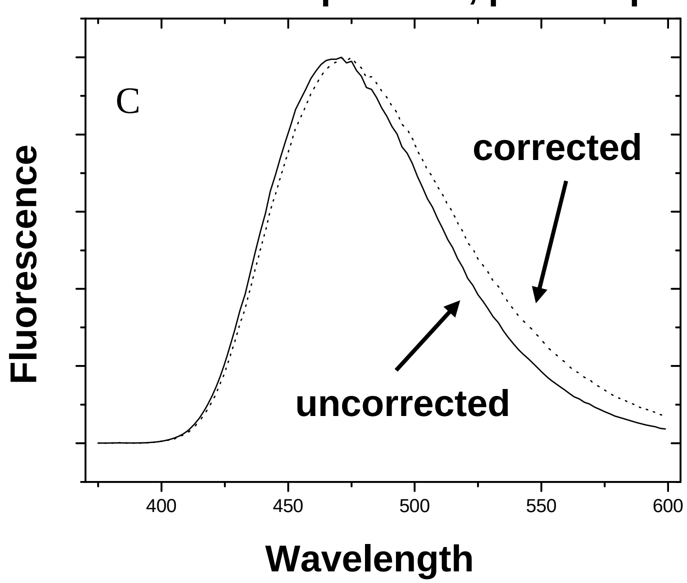
Correction of Emission Spectra



ANS Emission Spectrum, no polarizer



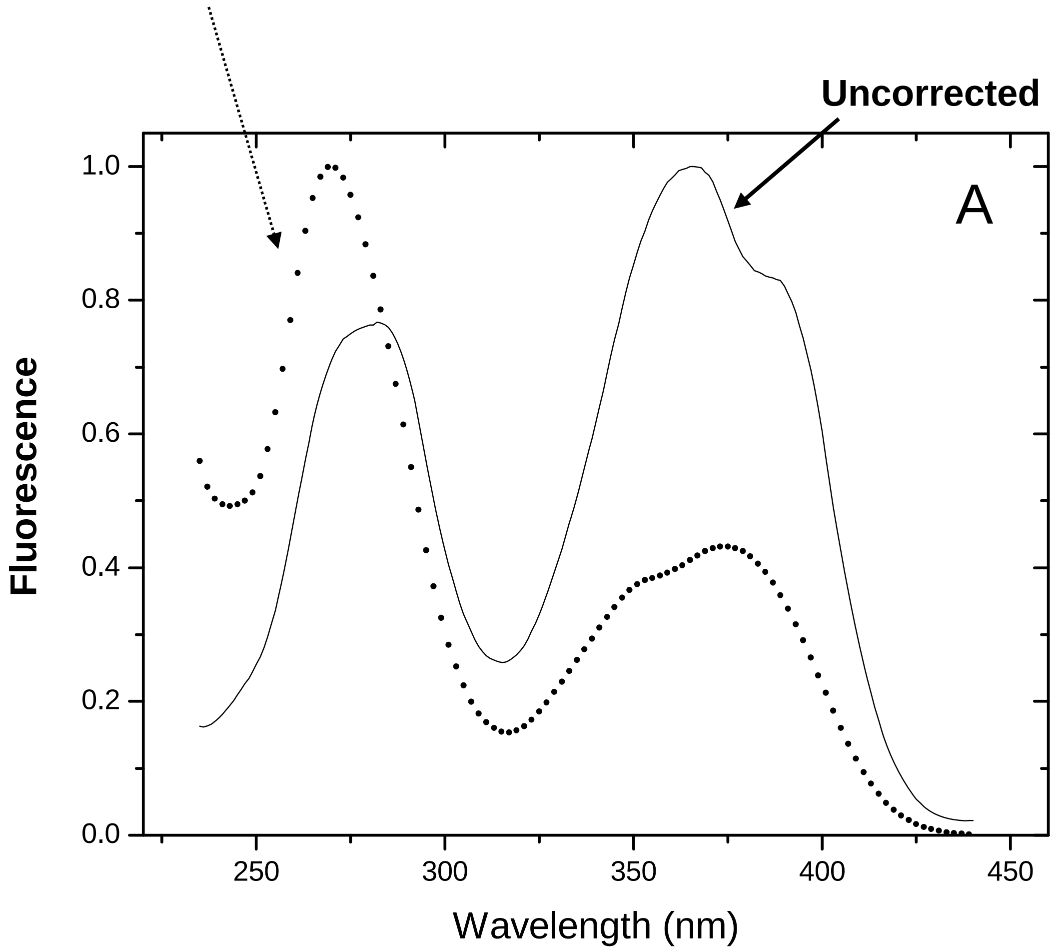
ANS Emission Spectrum, parallel polarizer



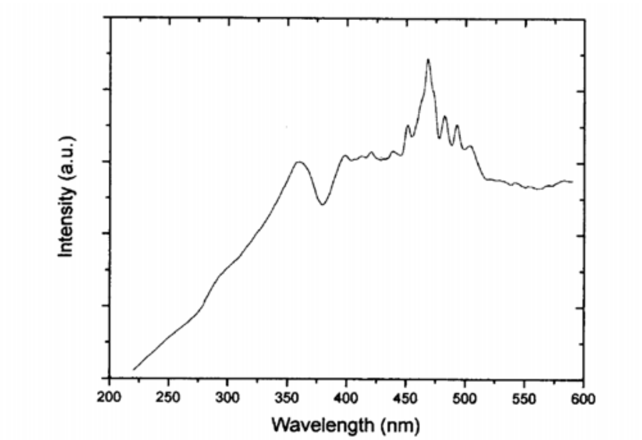
from Jameson et. Al., Methods in Enzymology, 360:1

Excitation Correction

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



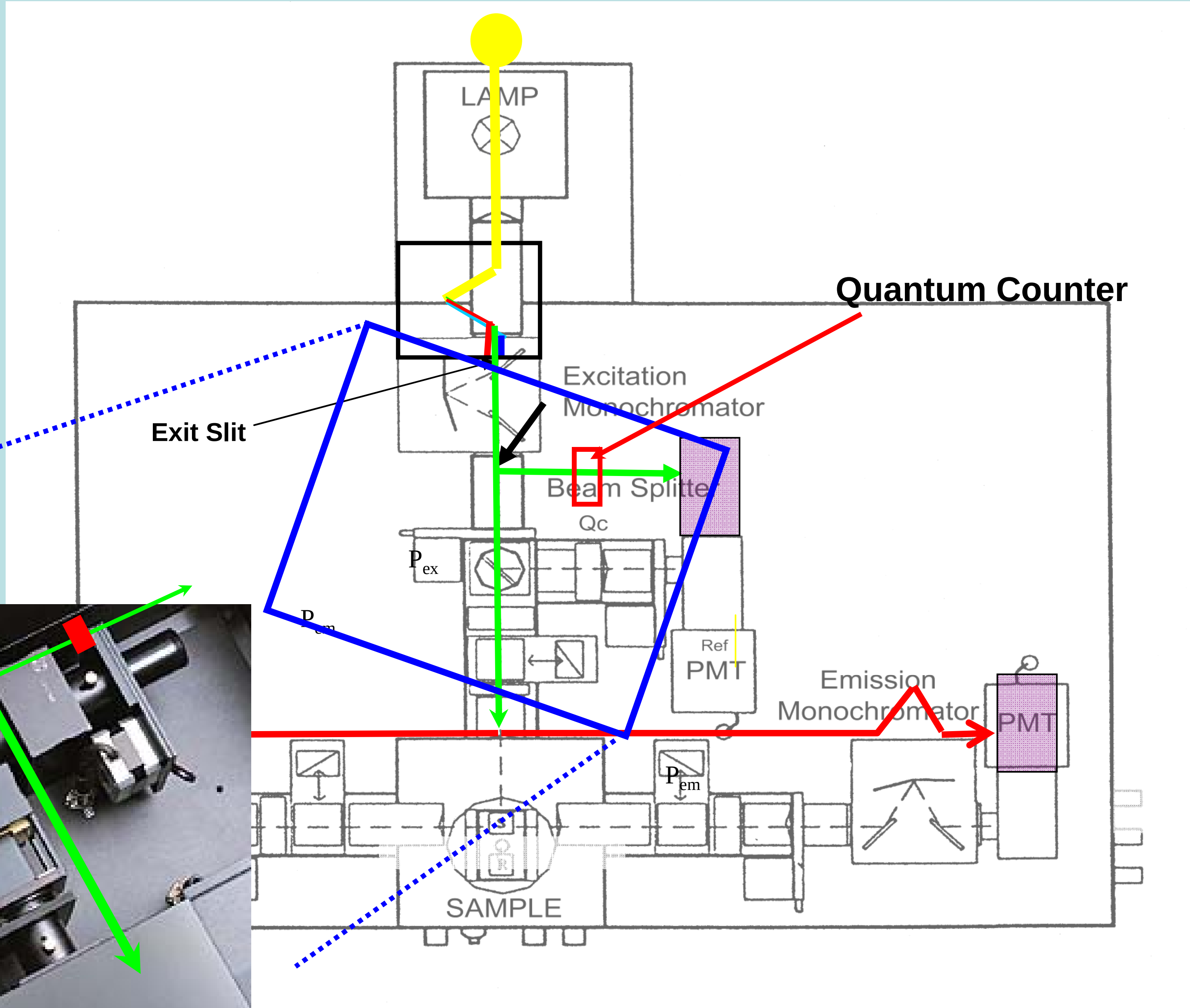
Recall the output of the xenon arc

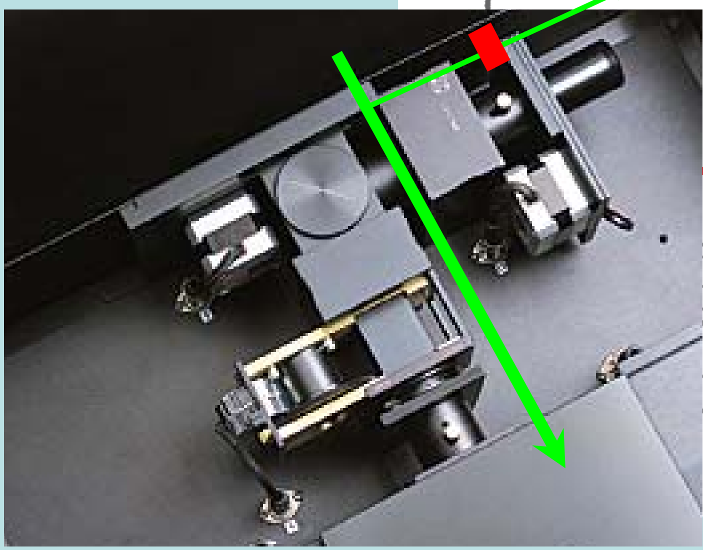


Note the huge difference between the absorption spectrum and the excitation spectrum

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

Excitation Correction

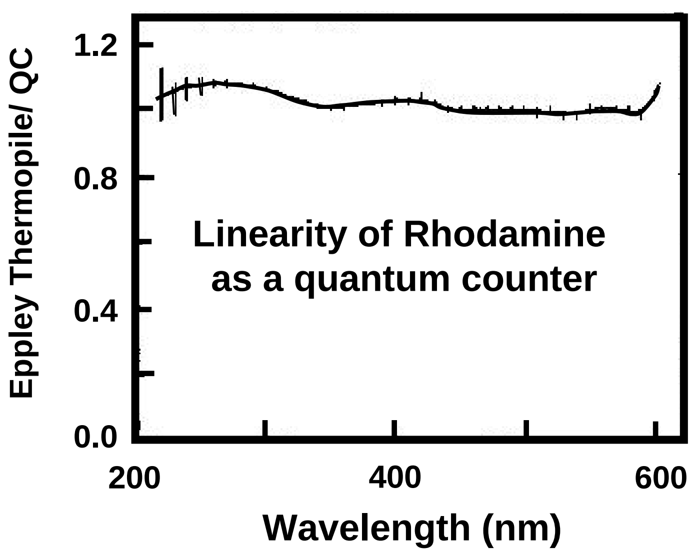


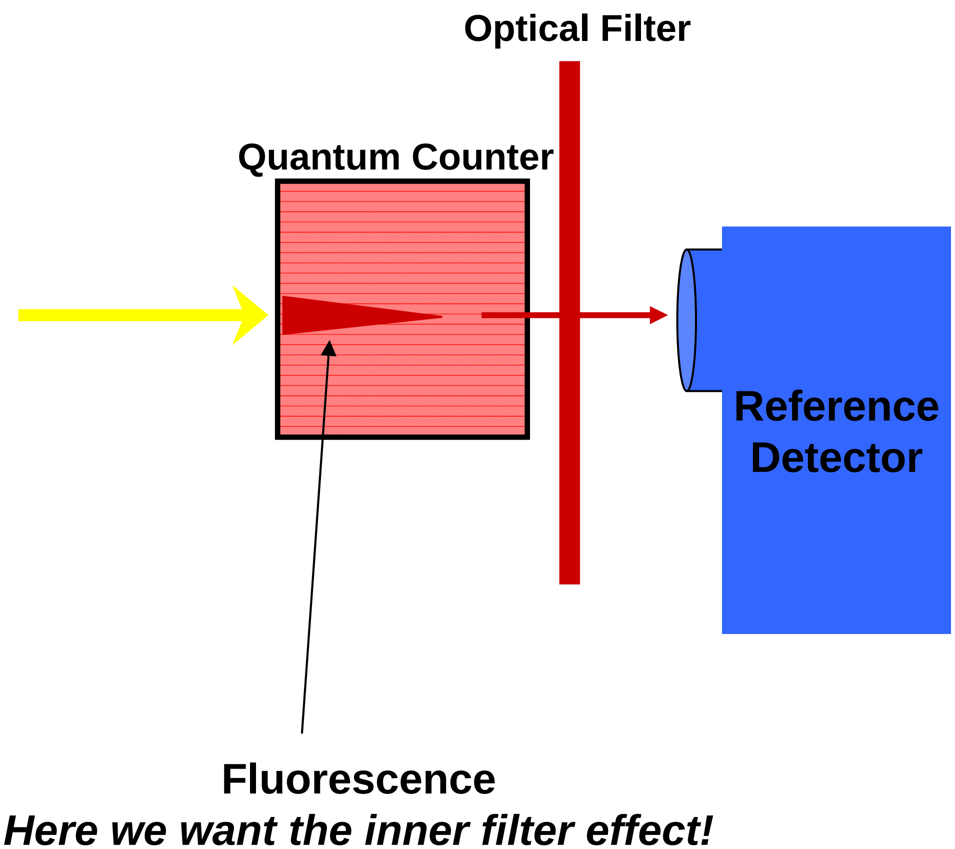


The Instrument Quantum Counter

Common Quantum Counters (optimal range)*

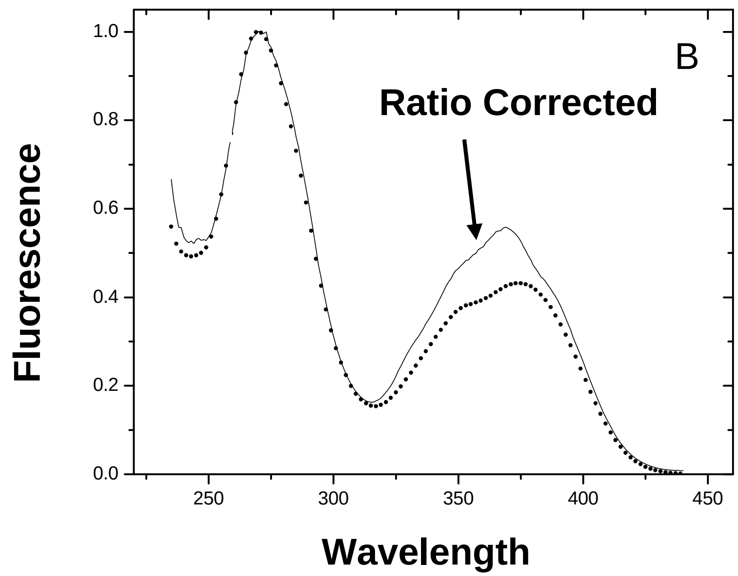
Rhodamine B	(220 - 600 nm)
Fluorescein	(240 - 400 nm)
Quinine Sulfate	(220 - 340 nm)



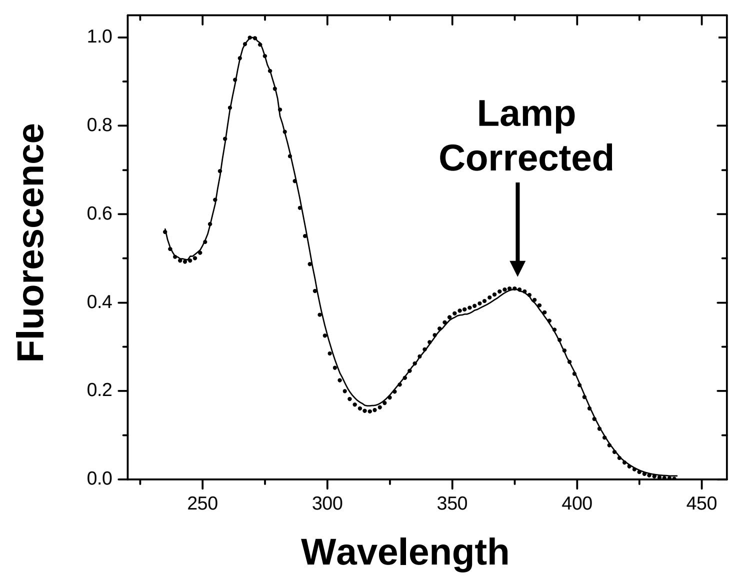


** Melhuish (1962) J. Opt. Soc. Amer. 52:1256*

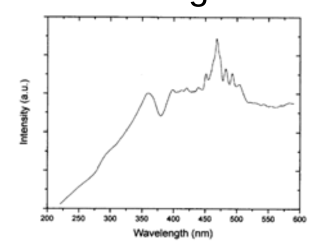
Excitation Correction



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



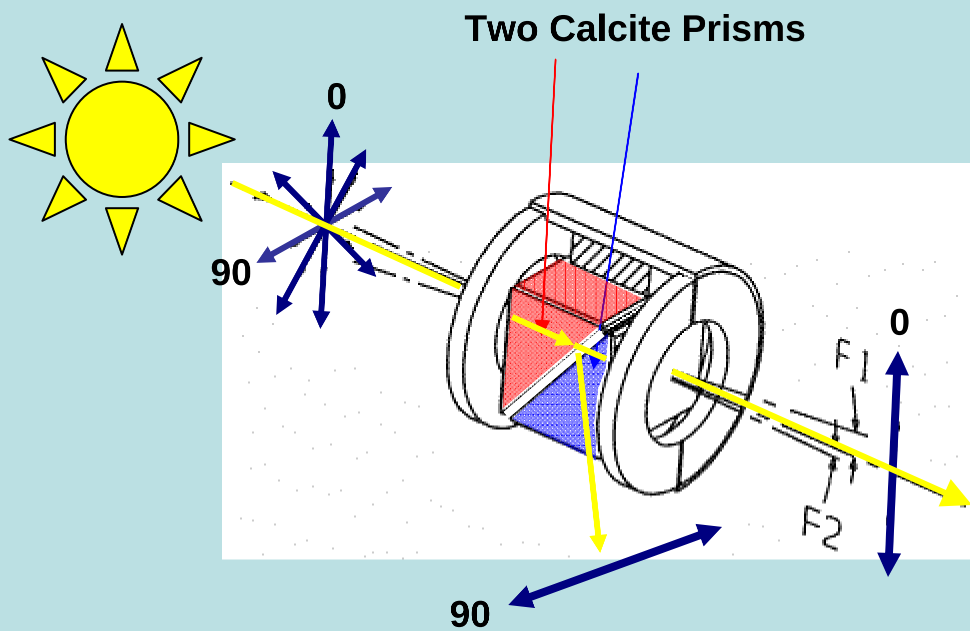
from Jameson, Croney and Moens, Methods in Enzymology, 360:1

Polarizers

The *Glan Taylor* prism polarizer

Common Types:

- **Glan Taylor (air gap)**
- **Glan Thompson**
- **Sheet Polarizers**

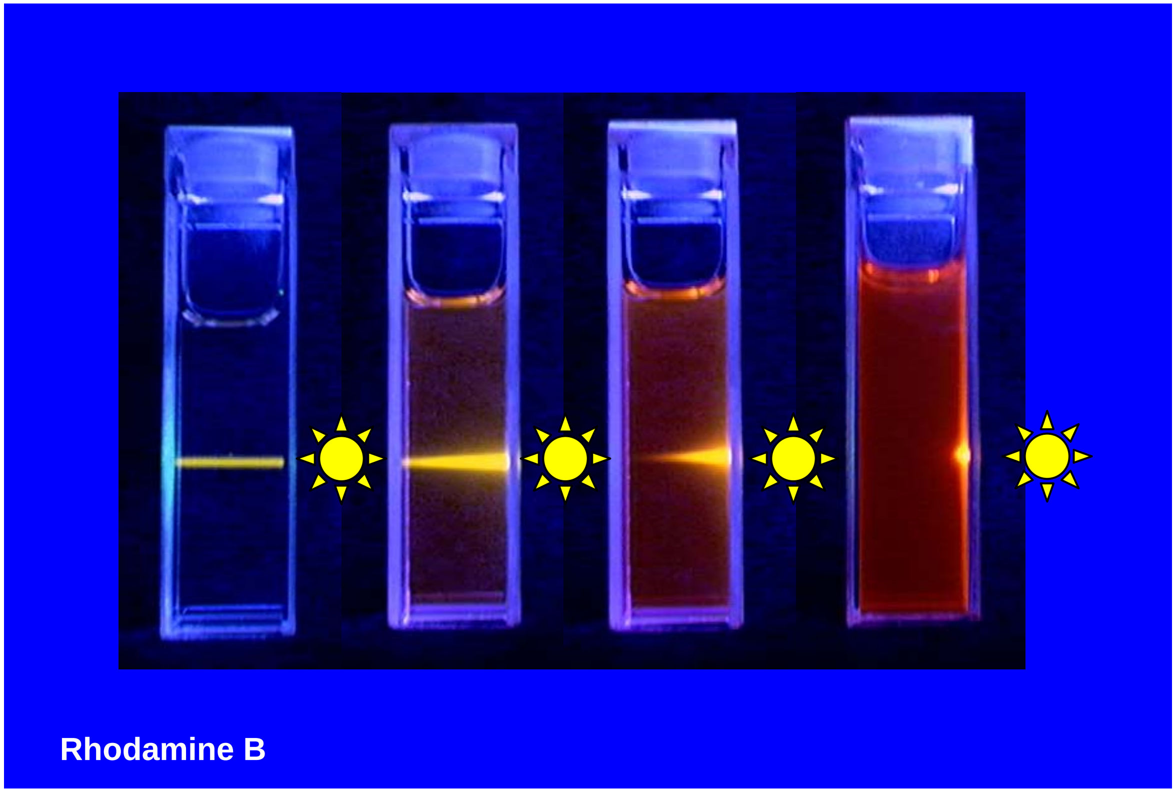


Sheet polarizer

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.

Attenuation of the Excitation Light through Absorbance

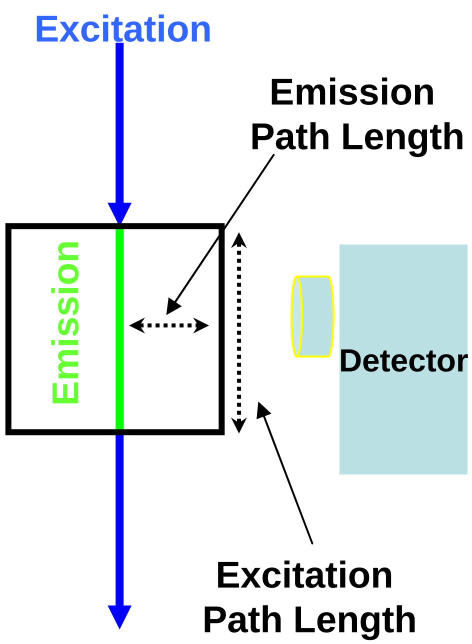
Sample concentration
& the *inner filter effect*

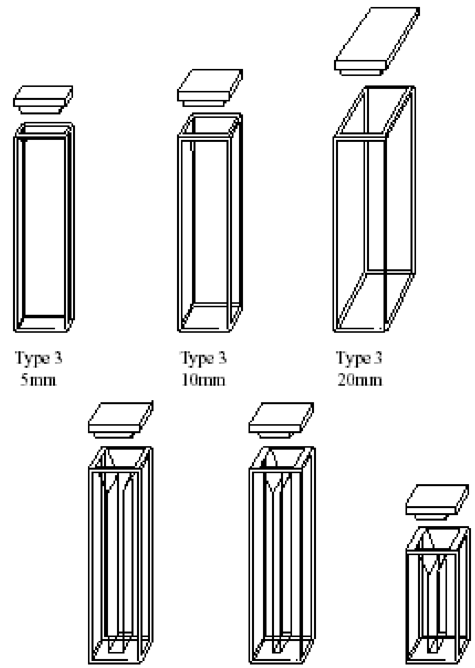


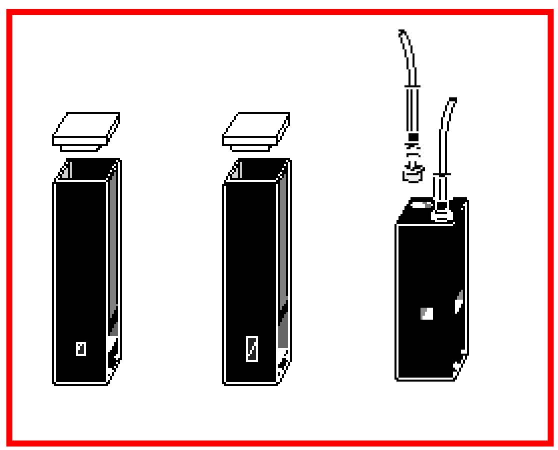
from Jameson et. al., Methods in Enzymology (2002), 360:1

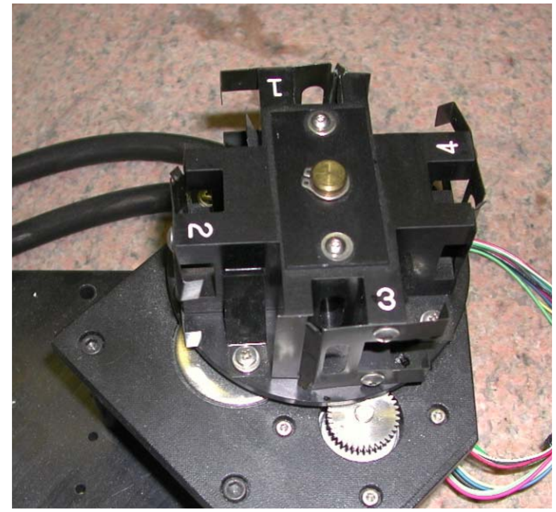
How do we handle highly absorbing solutions?

Quartz/Optical Glass/Plastic Cells

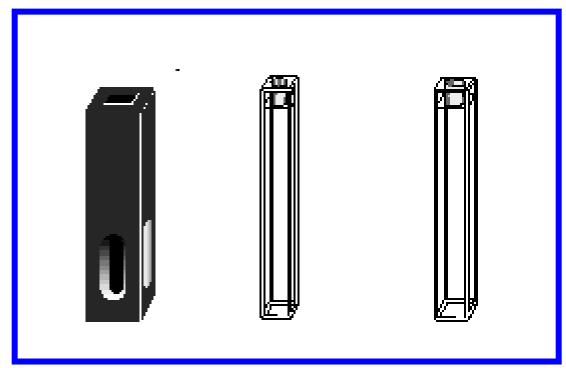






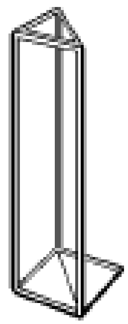


4 Position Turret
SPEX Fluoromax-2, Jobin-Yvon

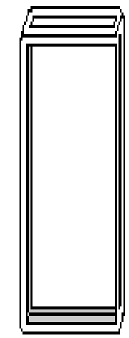


Front Face Detection

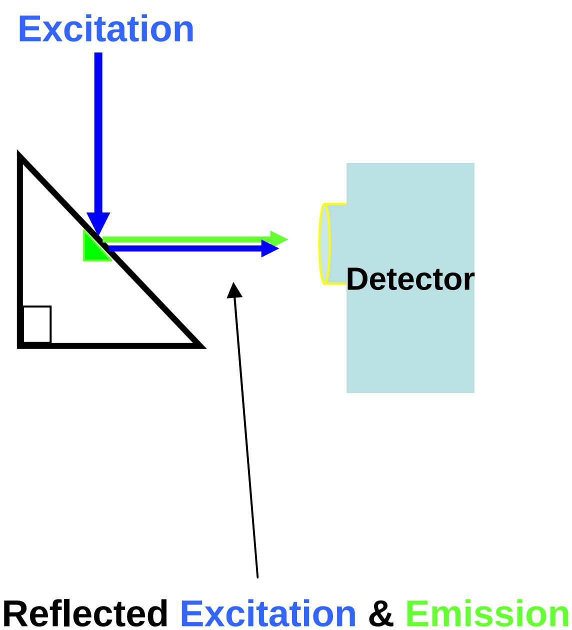
Triangular Cells

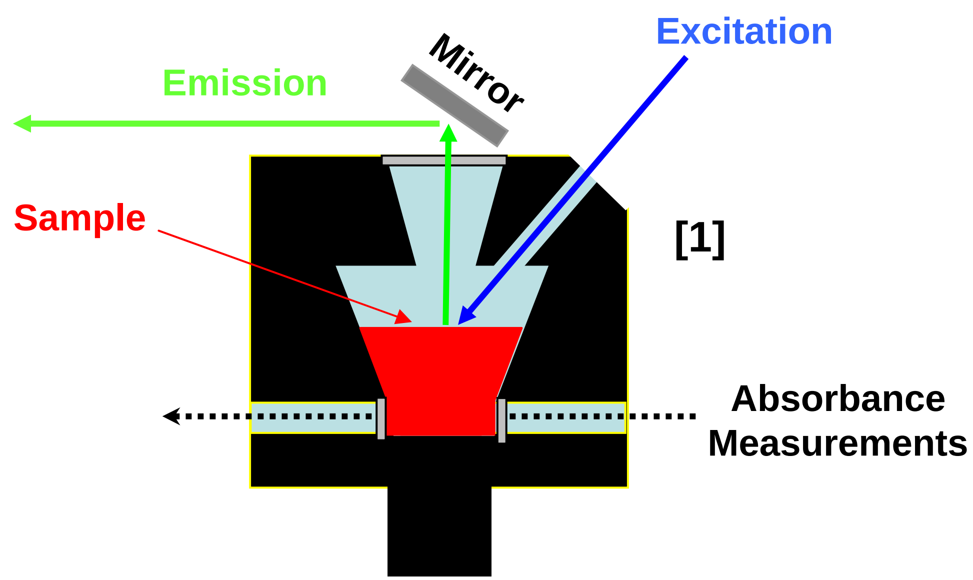


Thin Cells & Special Compartments



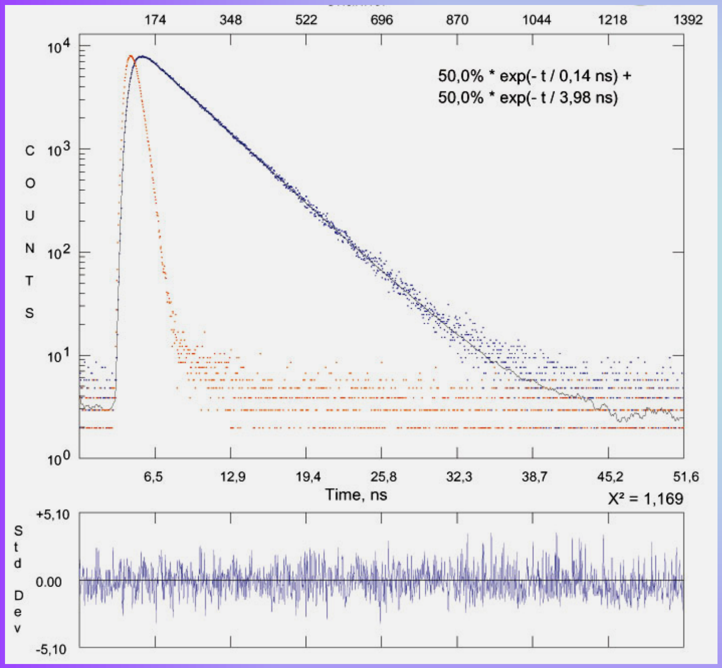
IBH, Glasgow G3 8JU
United Kingdom

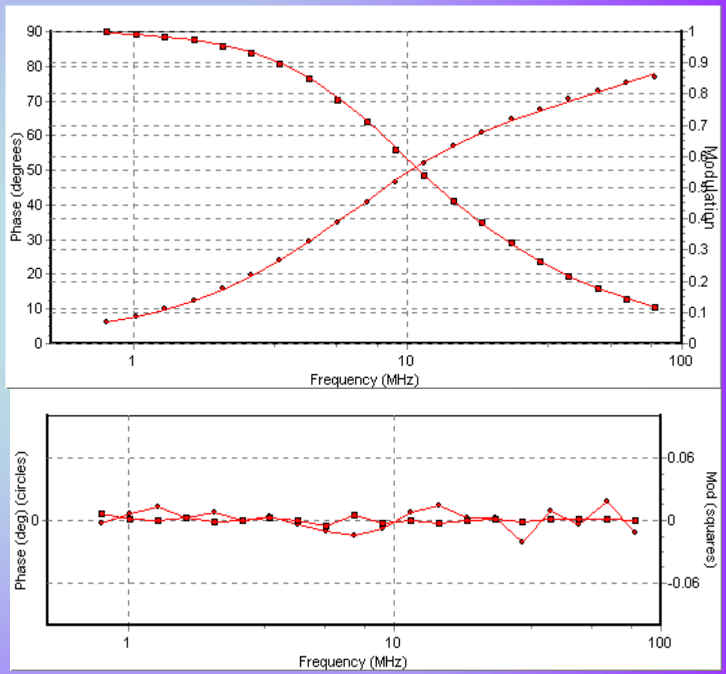




[1] Adapted from Gryczynski, Lubkowski, & Bucci Methods of Enz. 278: 538

Lifetime Instrumentation





Light Sources for Decay Acquisition: Frequency and Time Domain Measurements

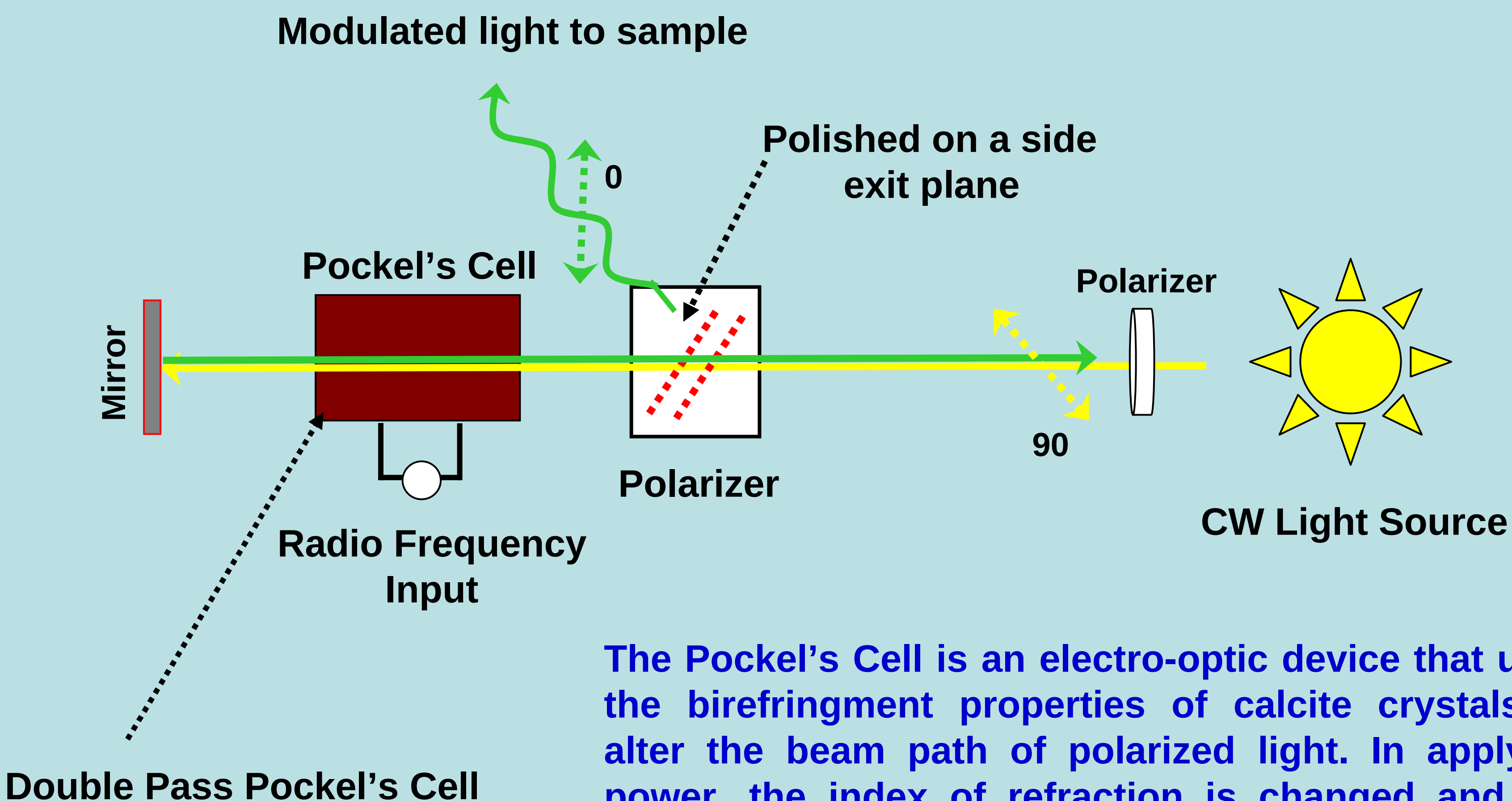
Pulsed Light Sources (frequency & pulse widths)

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

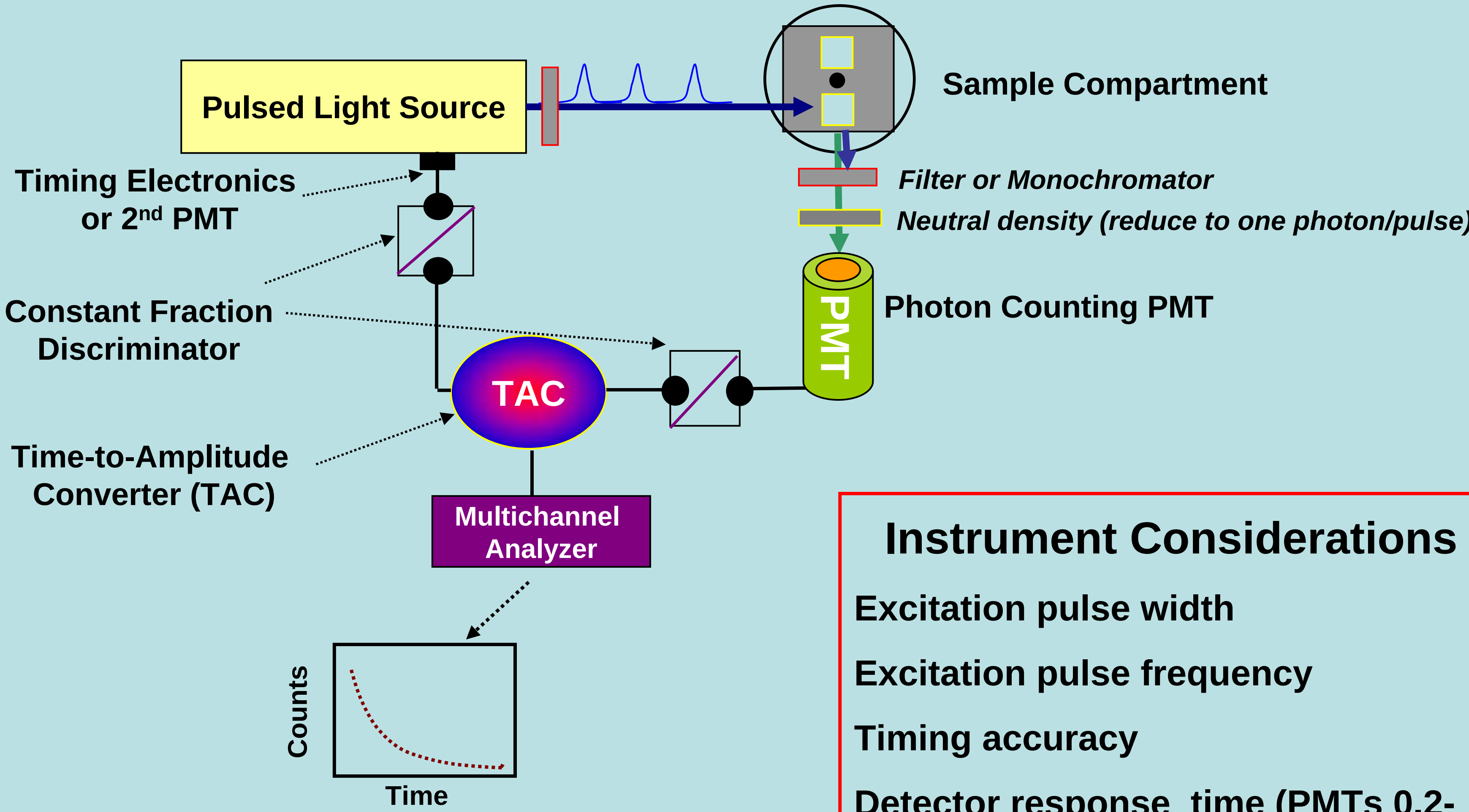
Flash Lamps
- Thyratron-gated nanosecond flash lamp (PTI), 25 KHz, 1.6 ns
- Coaxial nanosecond flashlamp (IBH), 10Hz-100kHz, 0.6 ns

Modulation of CW Light
Use of a Pockel's Cell



The Pockel's Cell is an electro-optic device that uses the birefringment 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

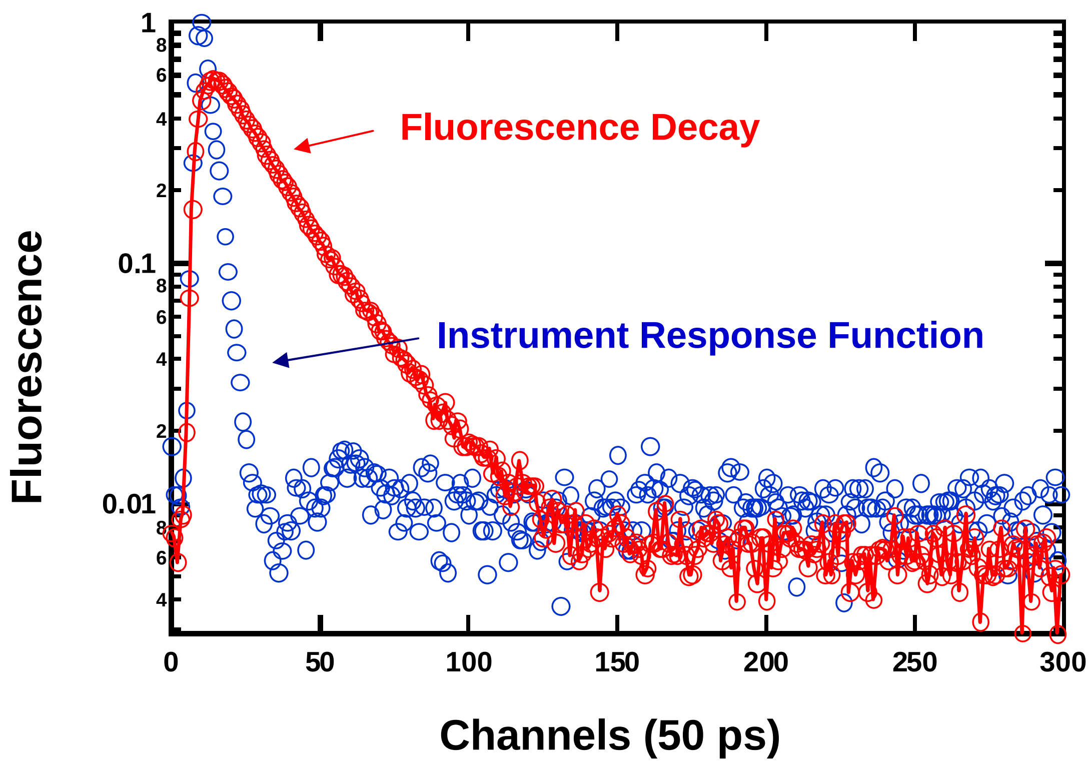
Excitation pulse width

Excitation pulse frequency

Timing accuracy

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

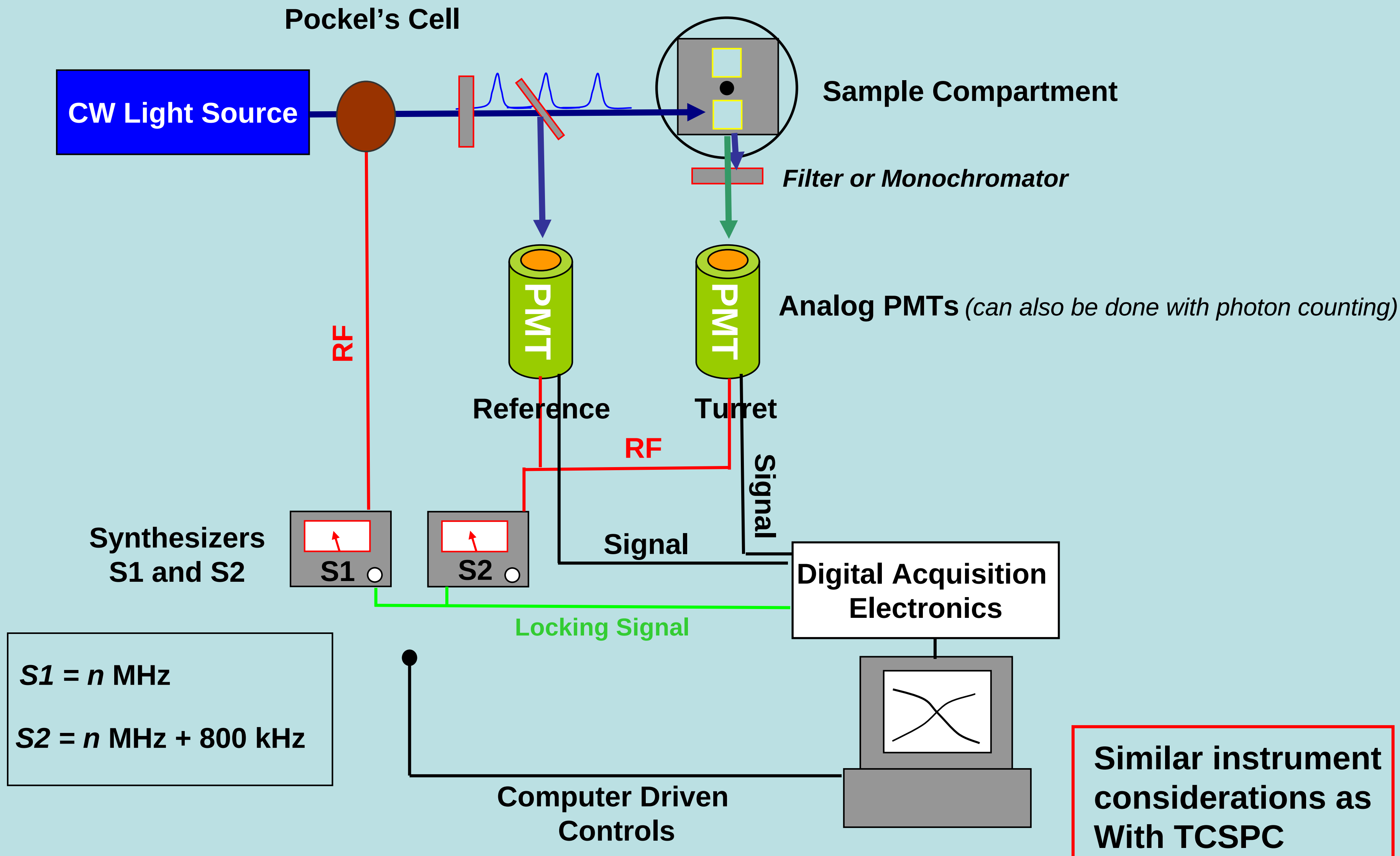
Histograms built one photon count at a time …



(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



Similar instrument considerations as With TCSPC