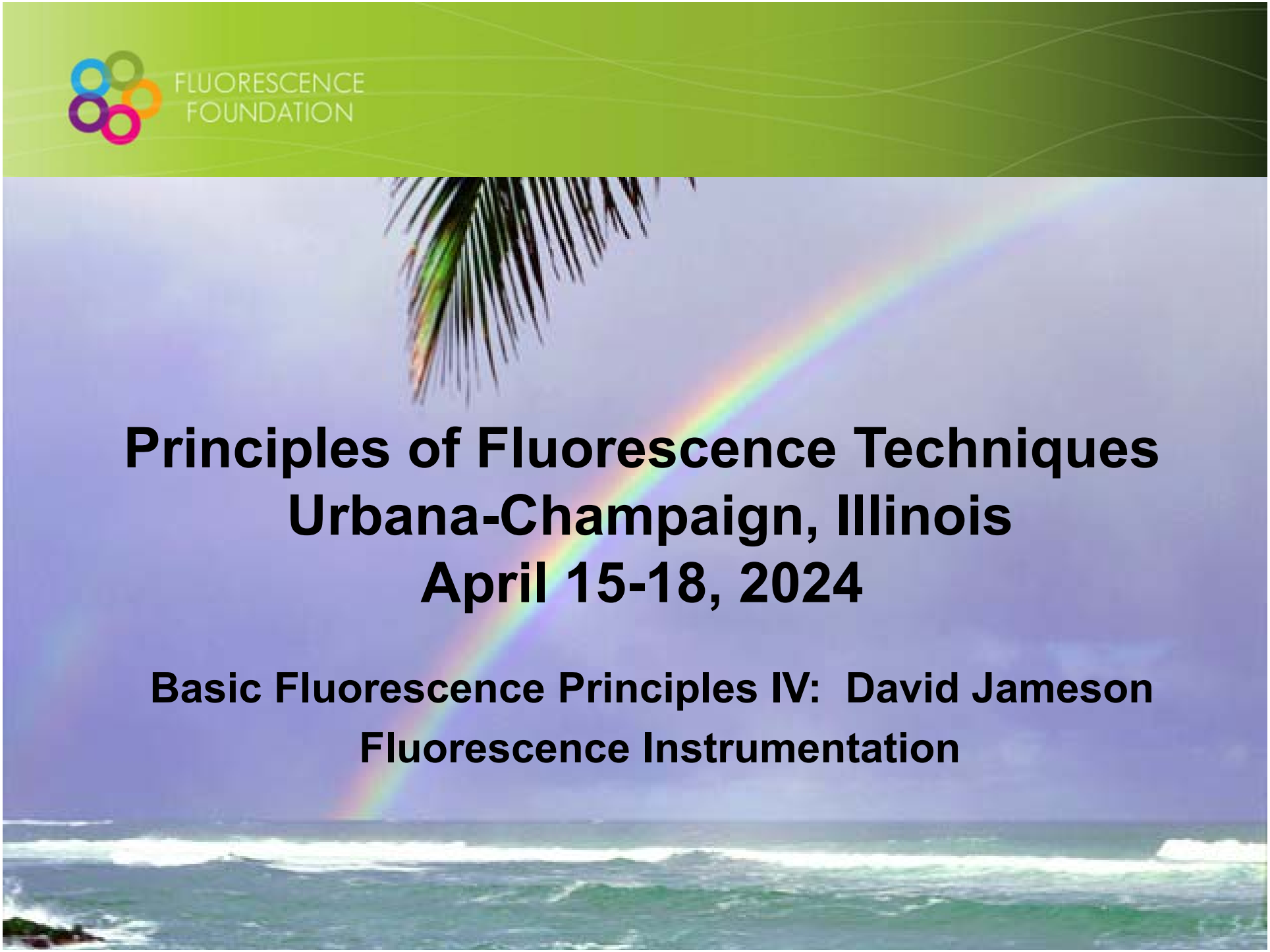
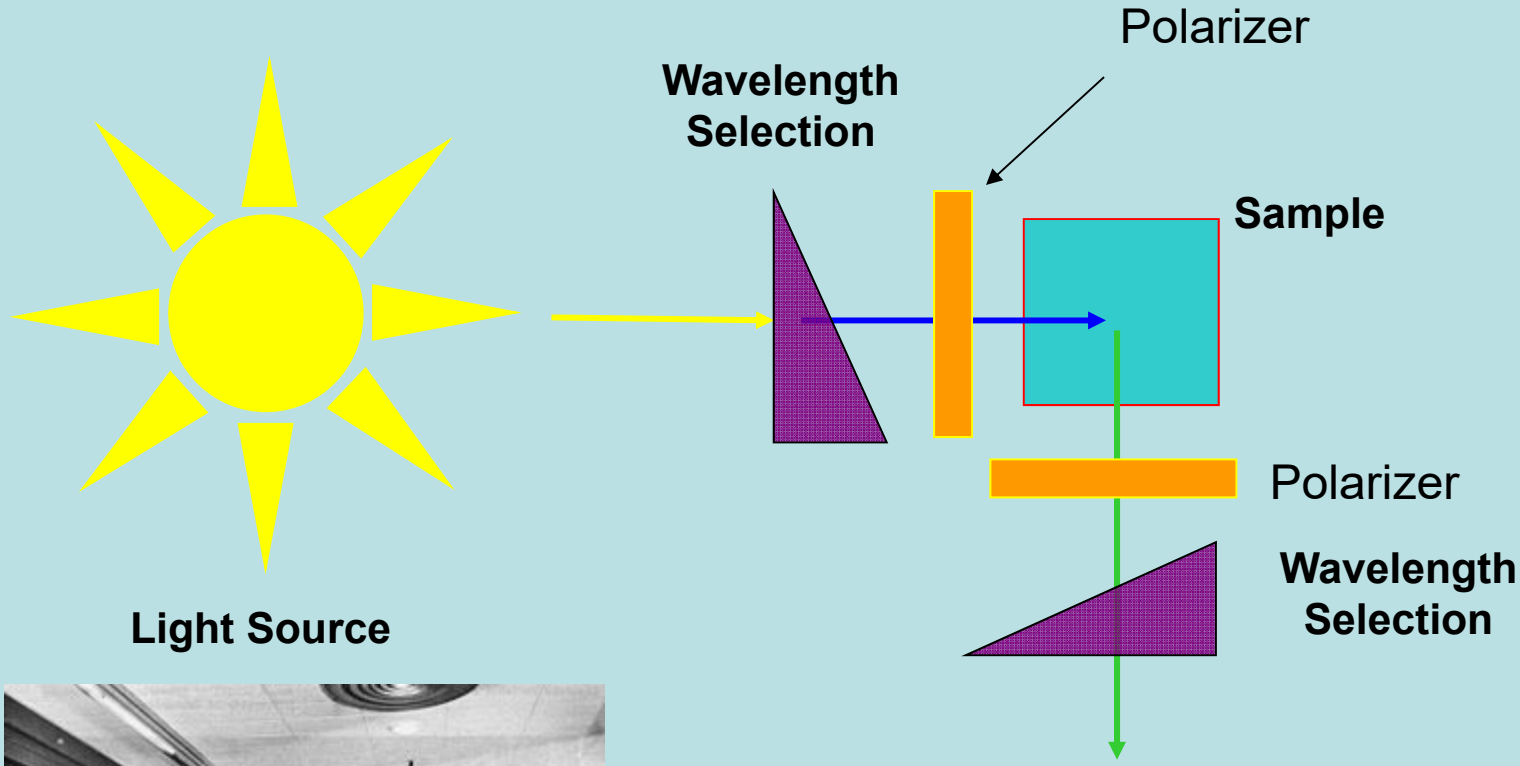


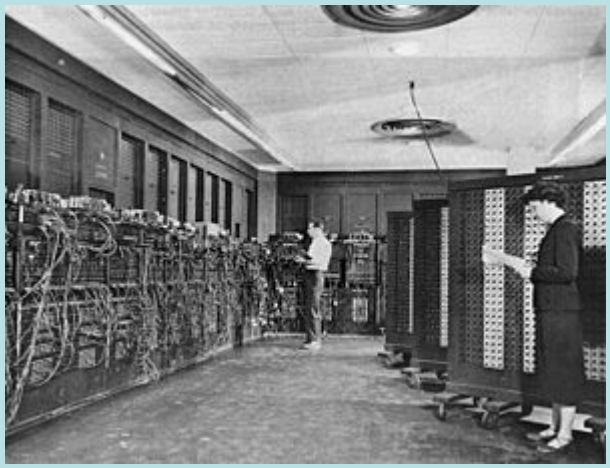
Principles of Fluorescence Techniques
Urbana-Champaign, Illinois
April 15-18, 2024

Basic Fluorescence Principles IV: David Jameson

Fluorescence Instrumentation

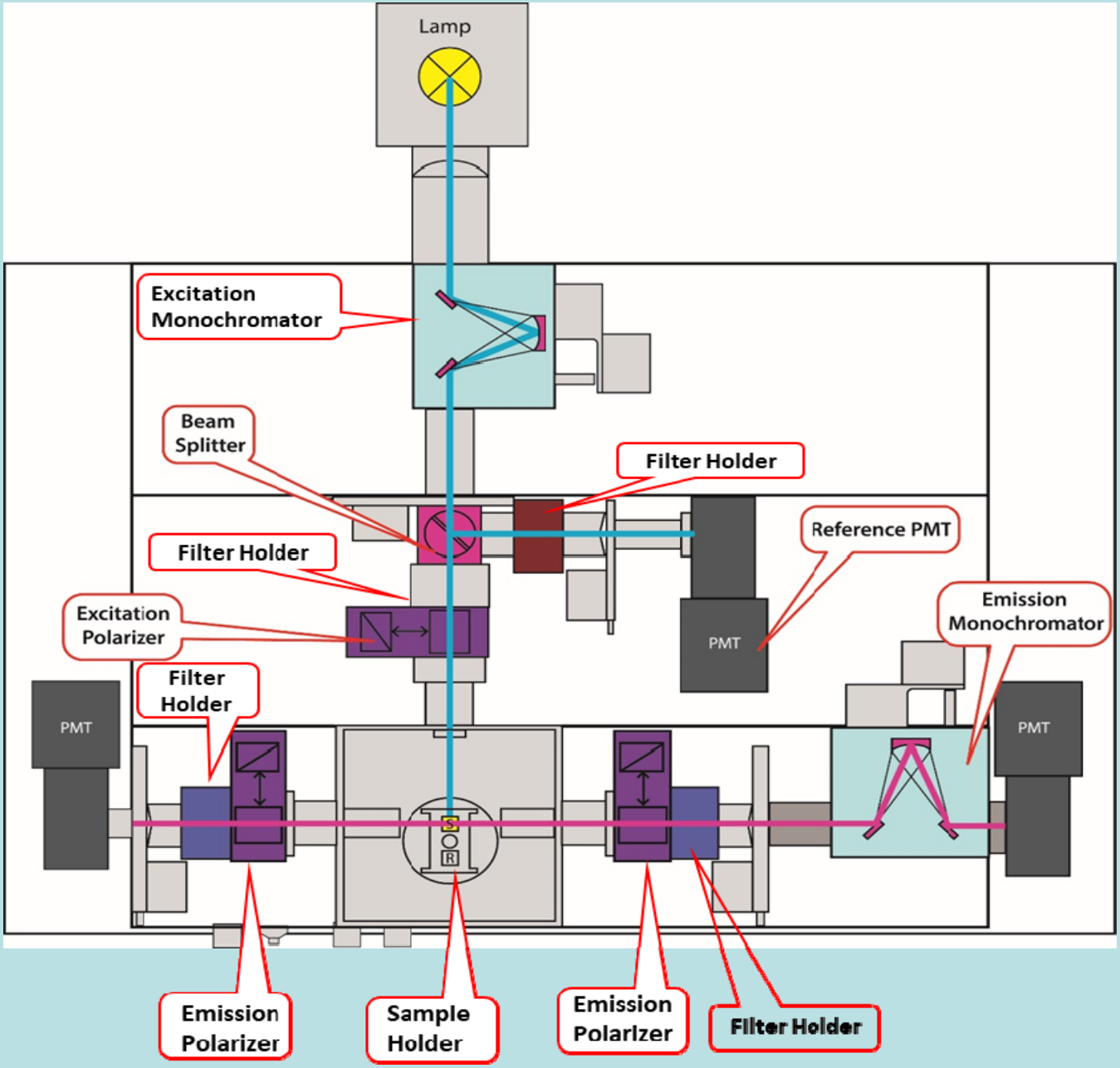
The Basics





computer

The Laboratory Fluorimeter



Light Sources

Light Sources

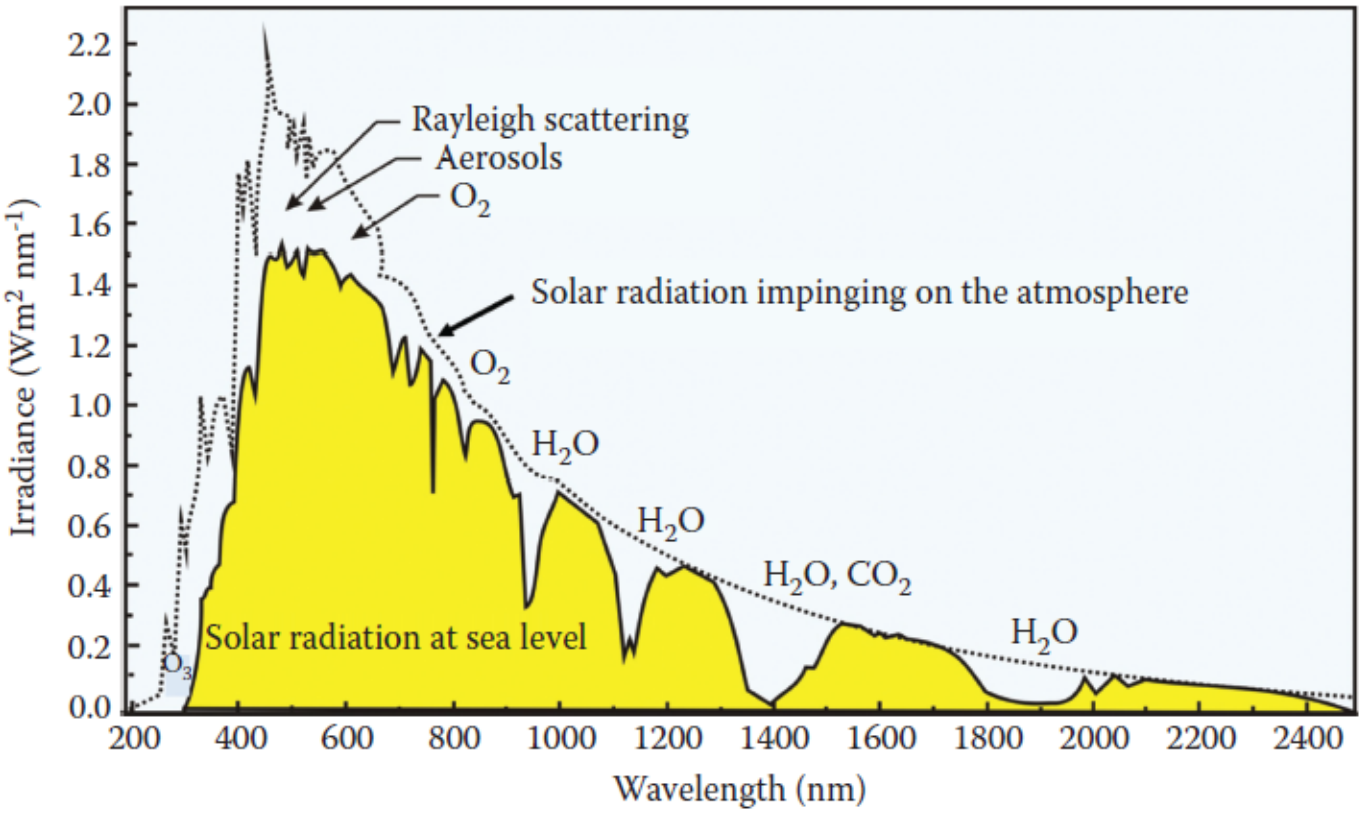


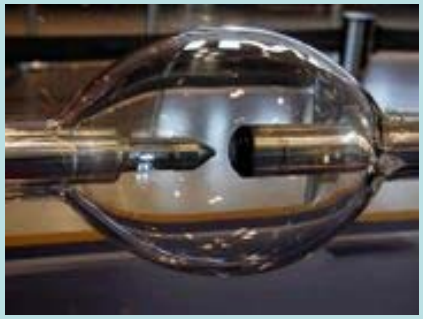
FIGURE 3.3 Sketch illustrating the wavelength distribution of solar radiation at the top of our atmosphere and reaching the earth's surface (sea level). (Modified from http://www.newport.com/Introduction-to-Solar-Radiation/411919/1033/content.aspx. The author would like to thank Leonel Malacrida for redrawing this figure.)

Lamp Light Sources

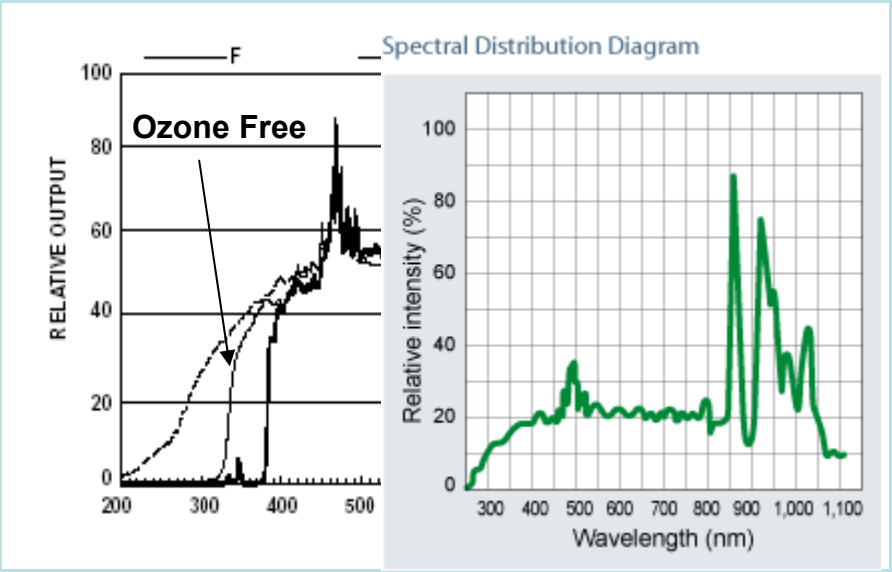
Gas discharge lamps

Xenon Arc Lamp (wide range of wavelengths)

Introduced in 1951 by the Osram Company

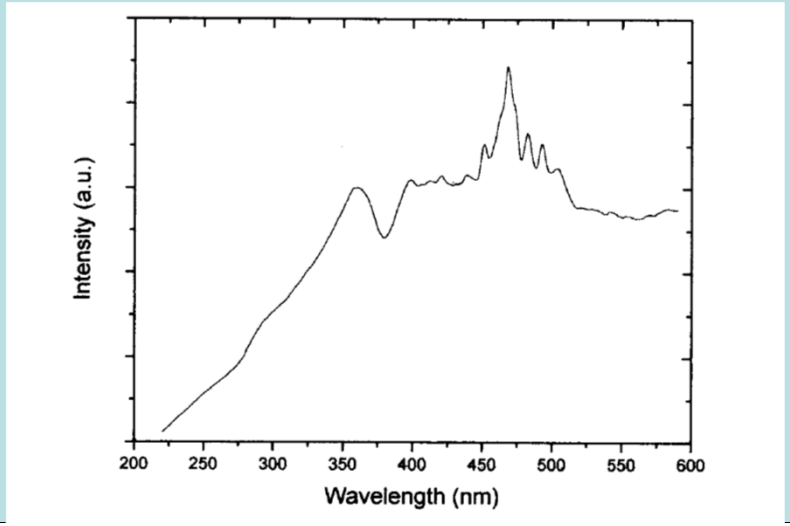


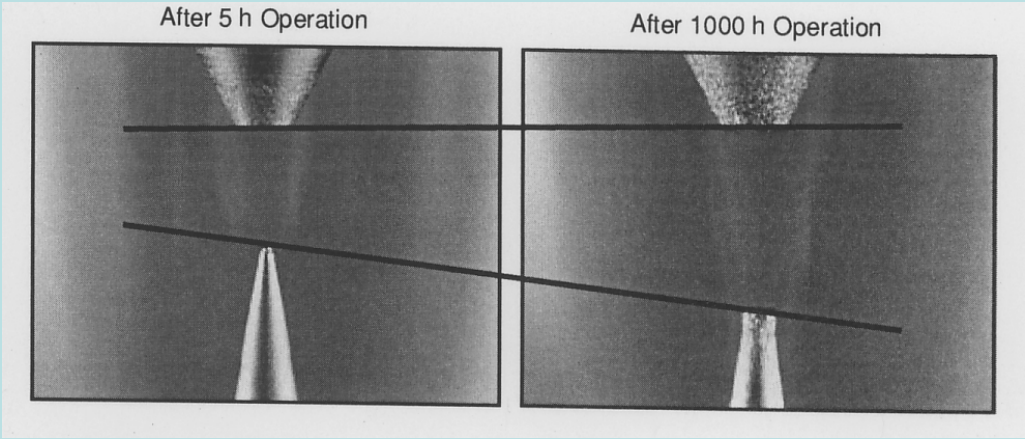
Xenon Arc Lamp Profiles



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

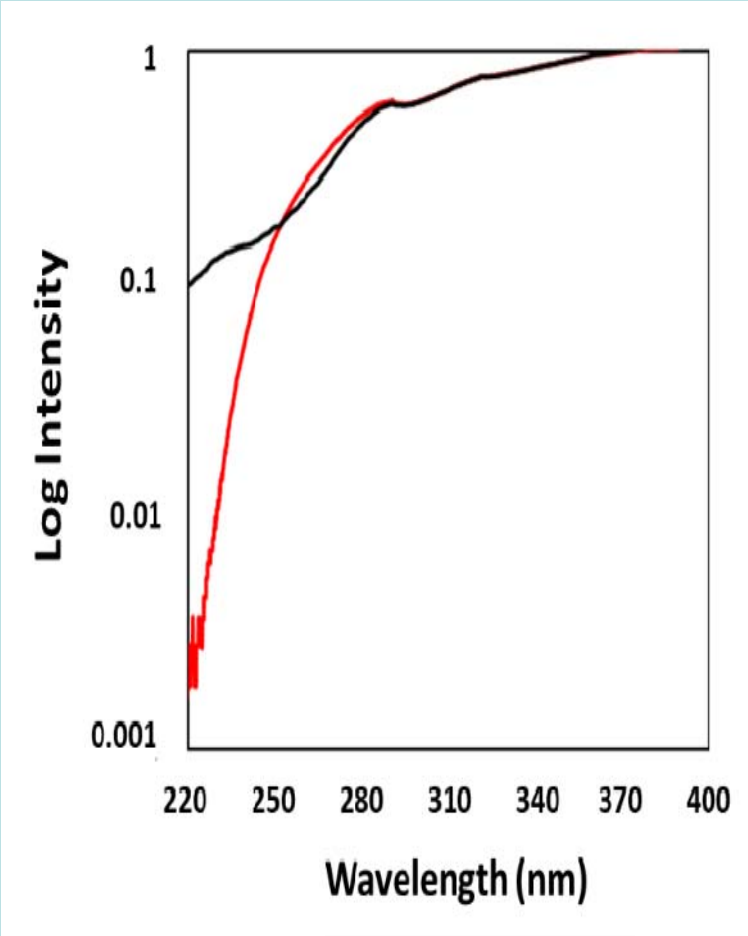
A UV-blocking material can be used to coat the interior of the bulb envelope which prevents the production of ozone outside of the lamp housing





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

Ozone free Lamps

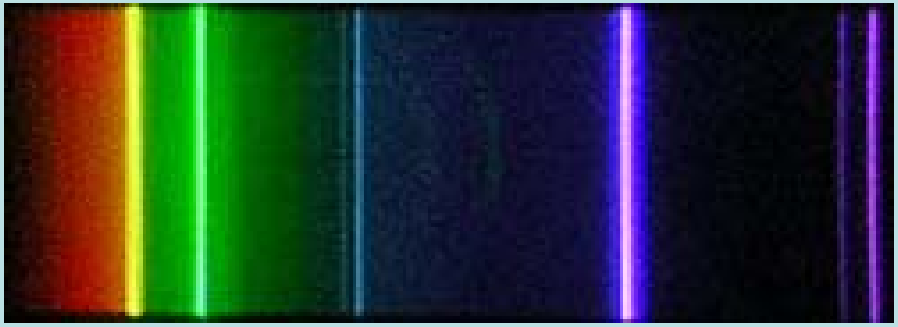


Light intensity emitted from a regular ozone xenon arc lamp (black) compared to that from an ozone-free xenon arc lamp (red). I thank Samantha Redes from Edinburgh Instruments for the data and Leonel Malacrida for drawing the figure.

Lamp Light Sources

Gas discharge lamps

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



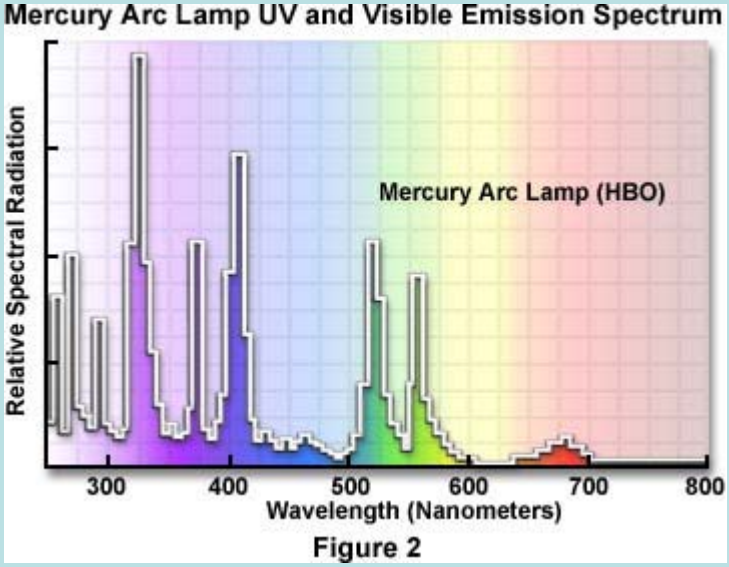
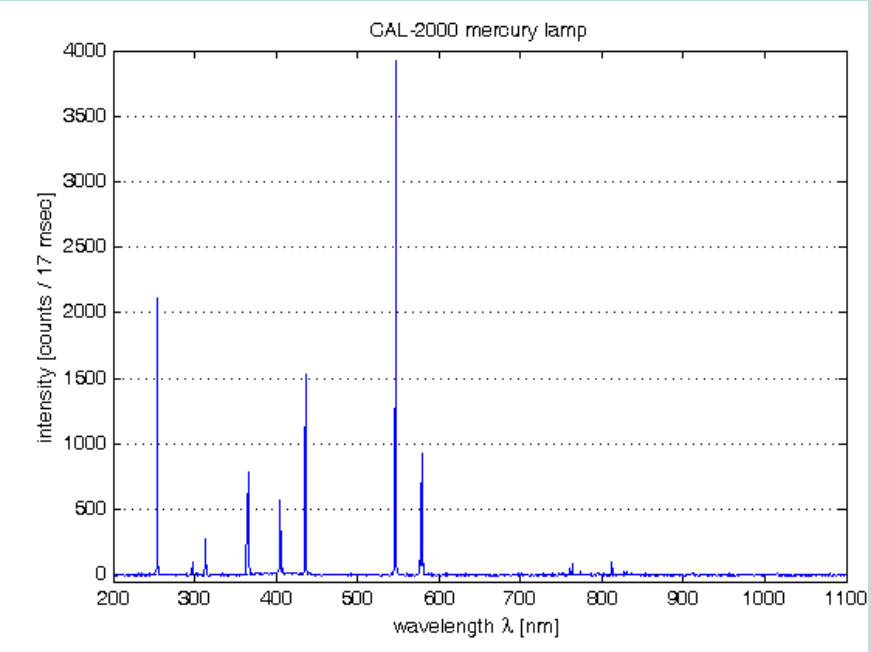


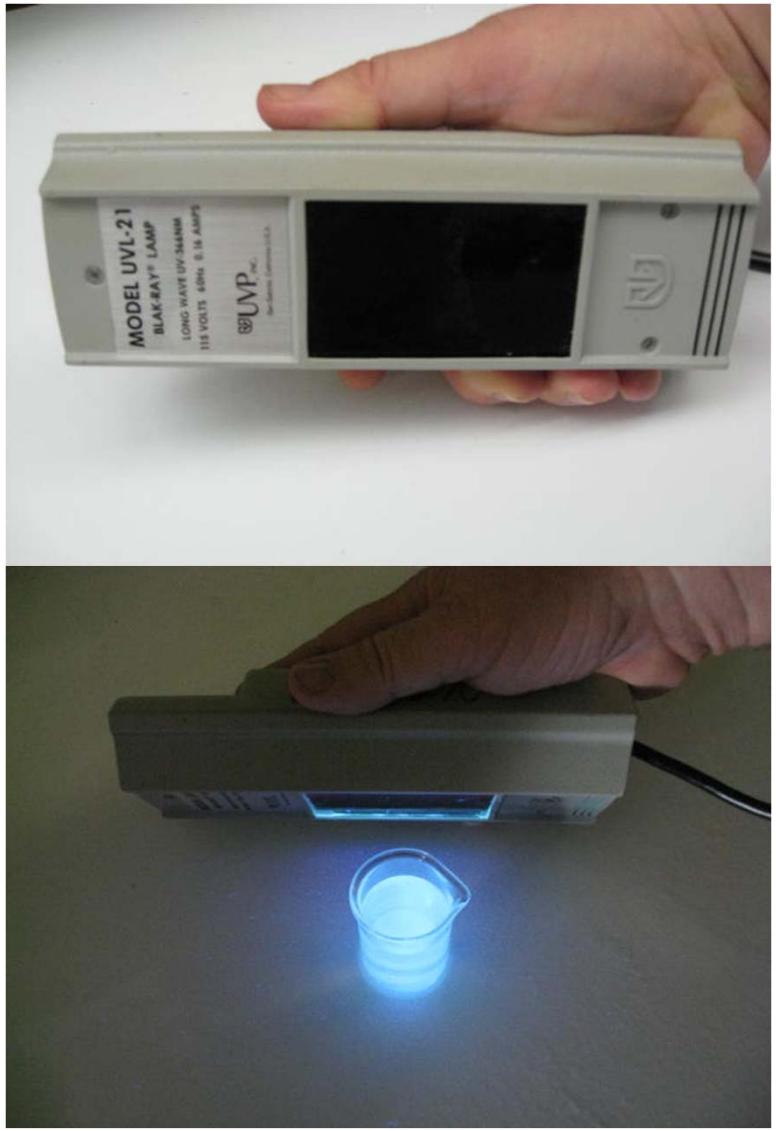
Figure 2

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

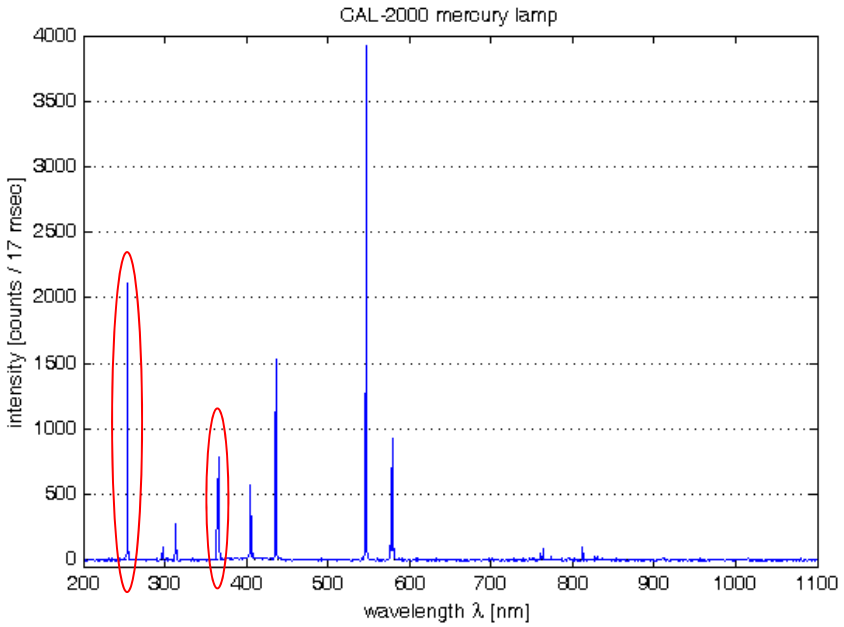


Lamp Light Sources

Gas discharge lamps



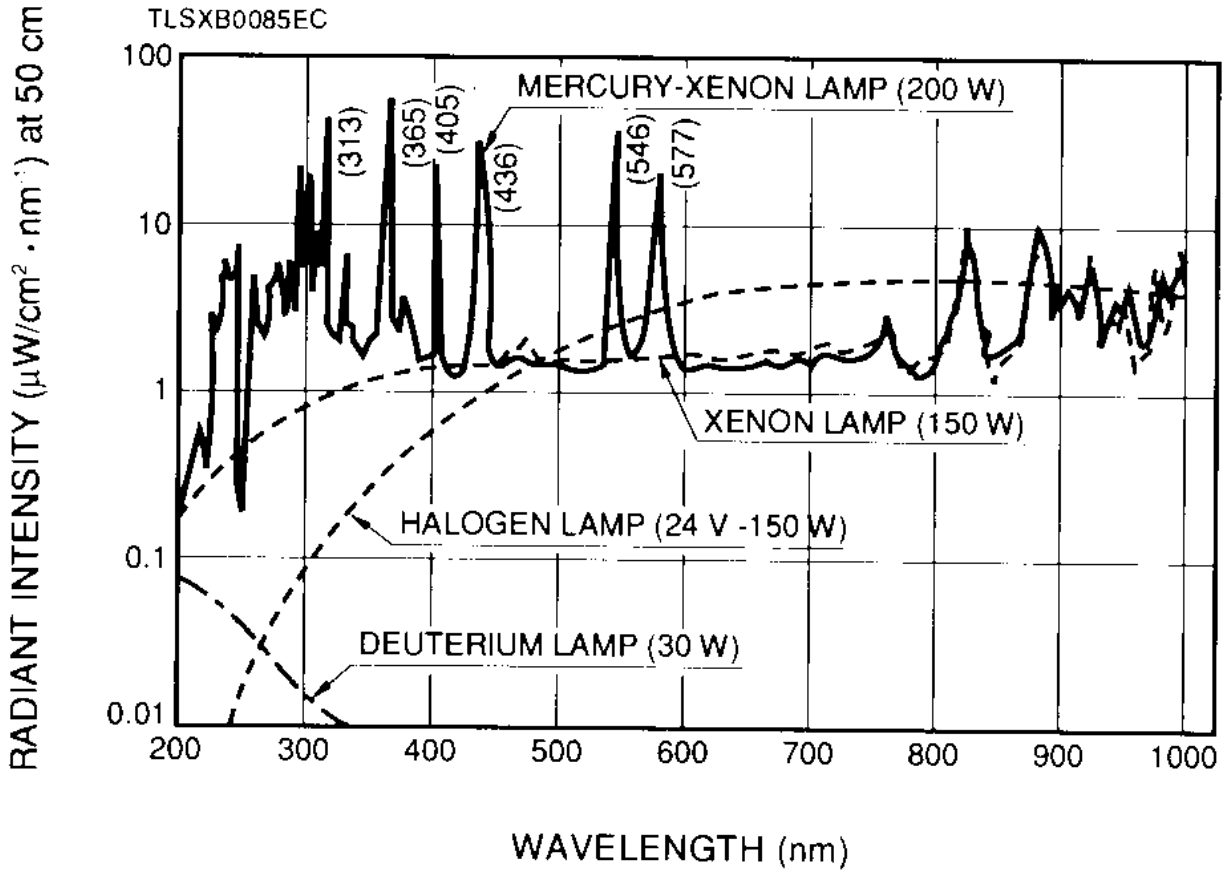
UV Handlamps usually provide for “short – 254nm” or “long – 365nm” illumination



Lamp Light Sources

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

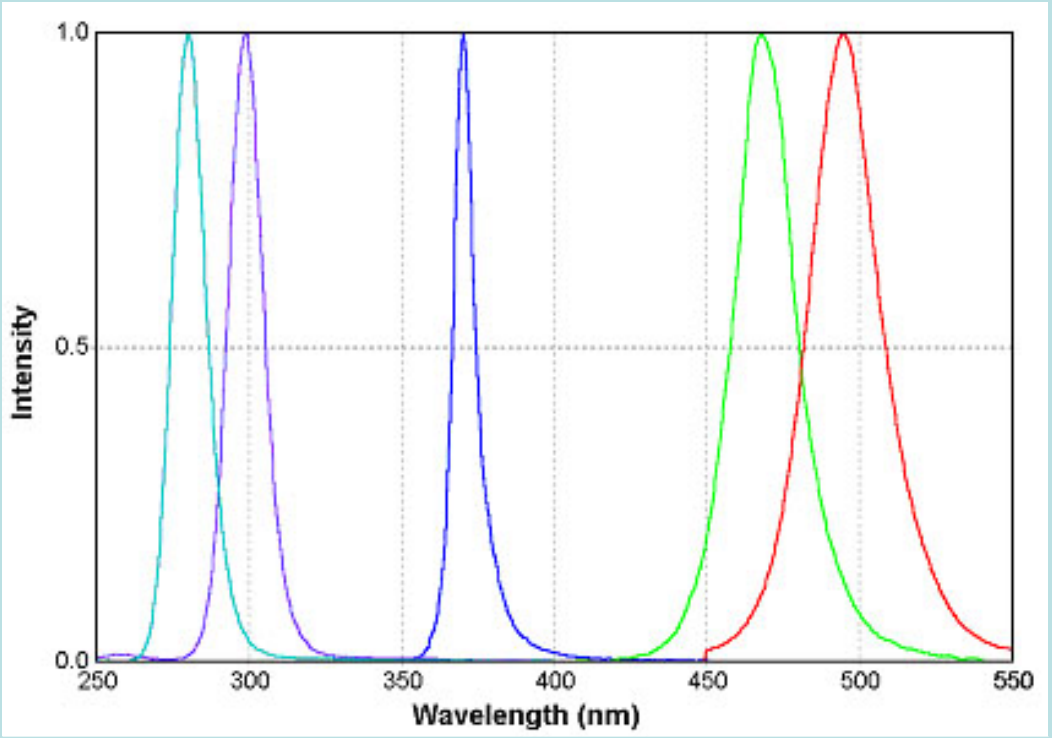
Figure 5: Spectral Distribution of Various Lamps



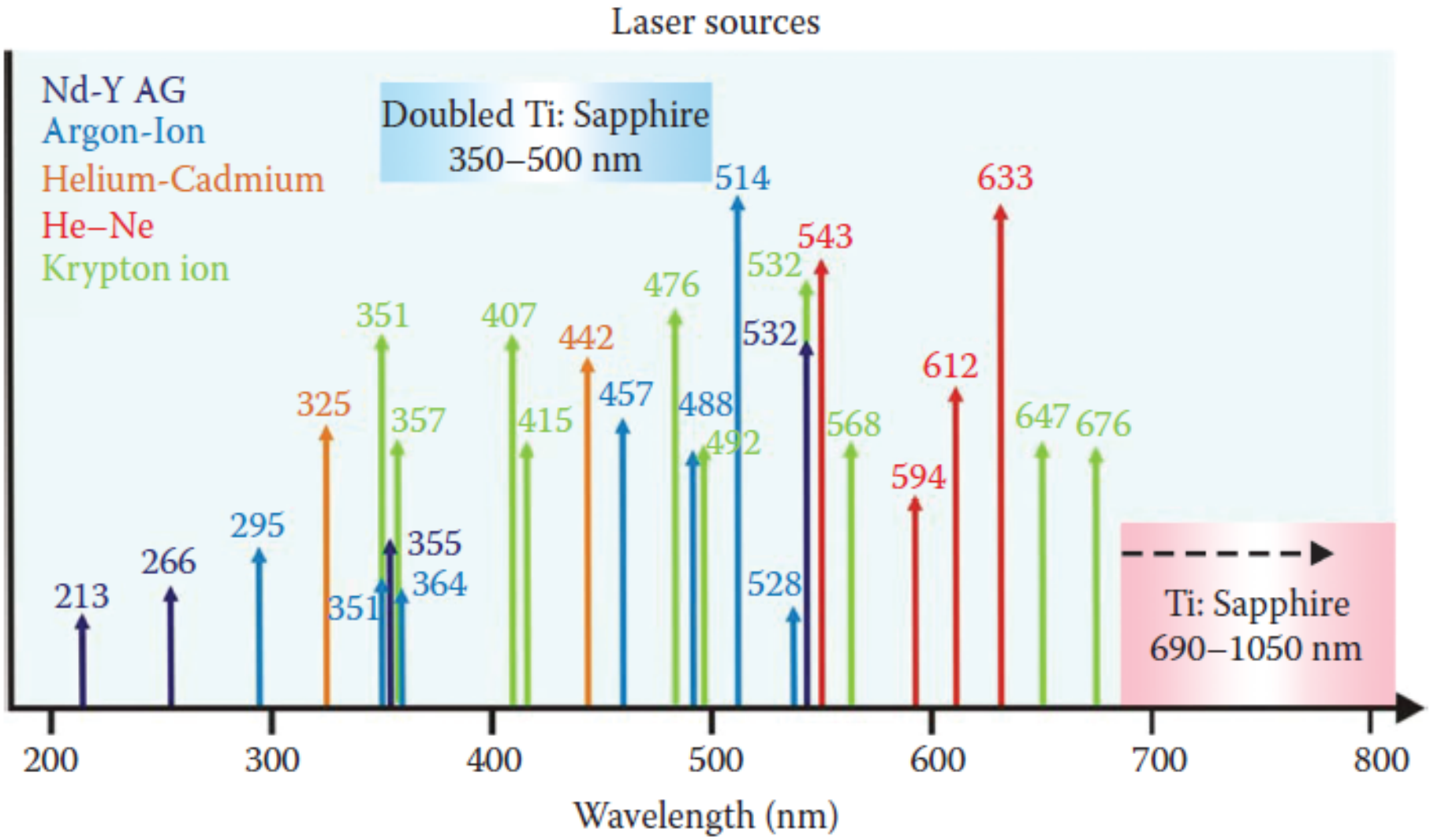
Light Emitting Diodes (LED)

Electroluminescence from a semiconductor junction

Wavelengths from 260 nm to 2400 nm



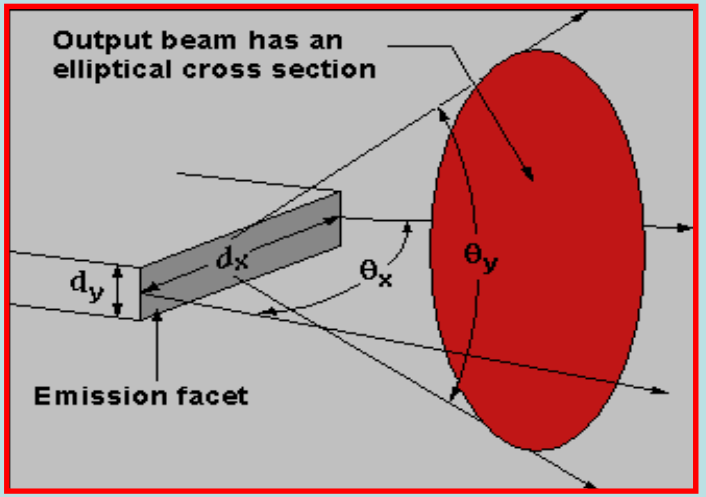
Lamp	Luminous Flux (Lumens)	Spectral Irradiance (Milliwatt/Square Meter/Nanometer)
HBO 100 Watts	2200	30 (350-700 nm)
XBO 75 Watts	1000	7 (350-700 nm)
Tungsten 100 Watts	2800	< 1 (350-700 nm)
LED (Blue, 450 nm)	160	6

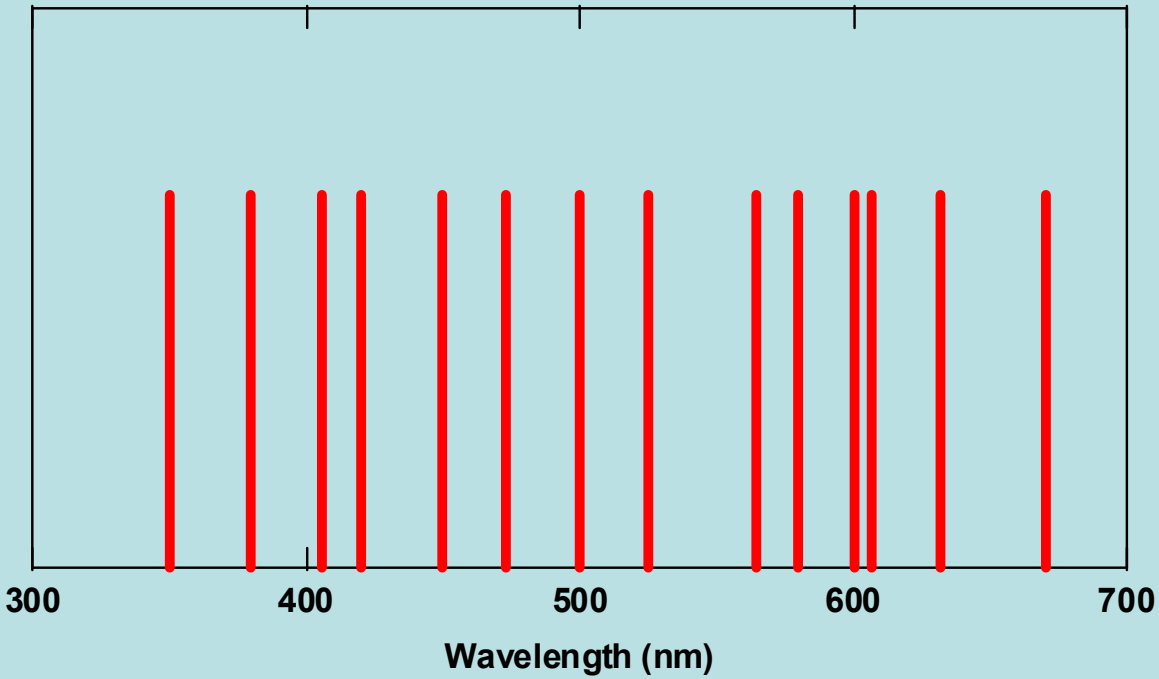


Quiz: What does LASER stand for?

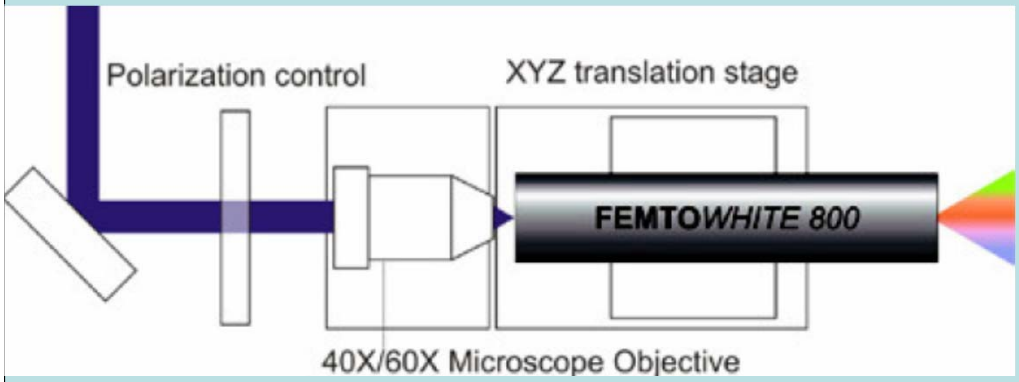
Light **A**mplification by **S**timulated **E**mission of **R**adiation

Laser Diodes

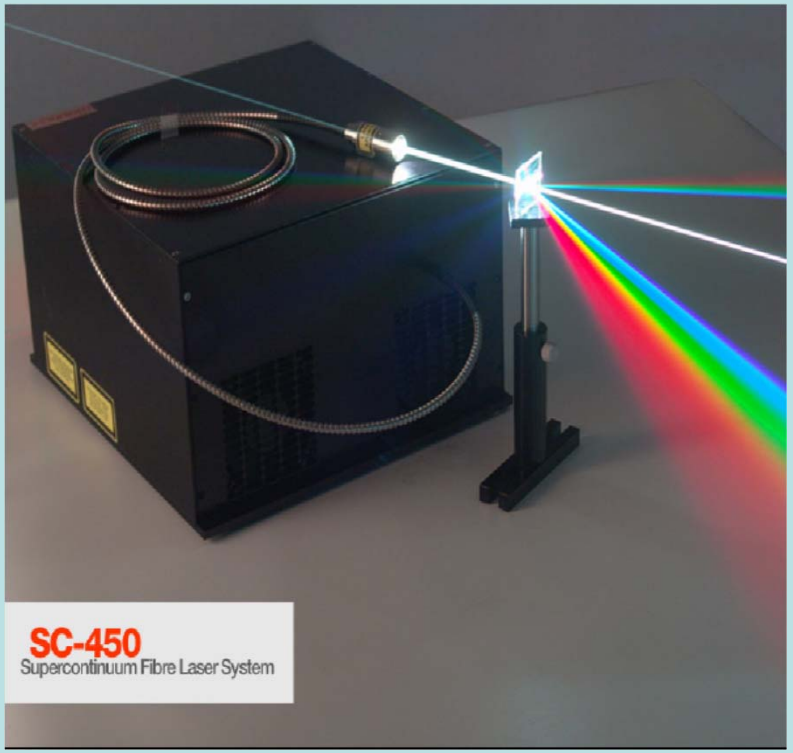


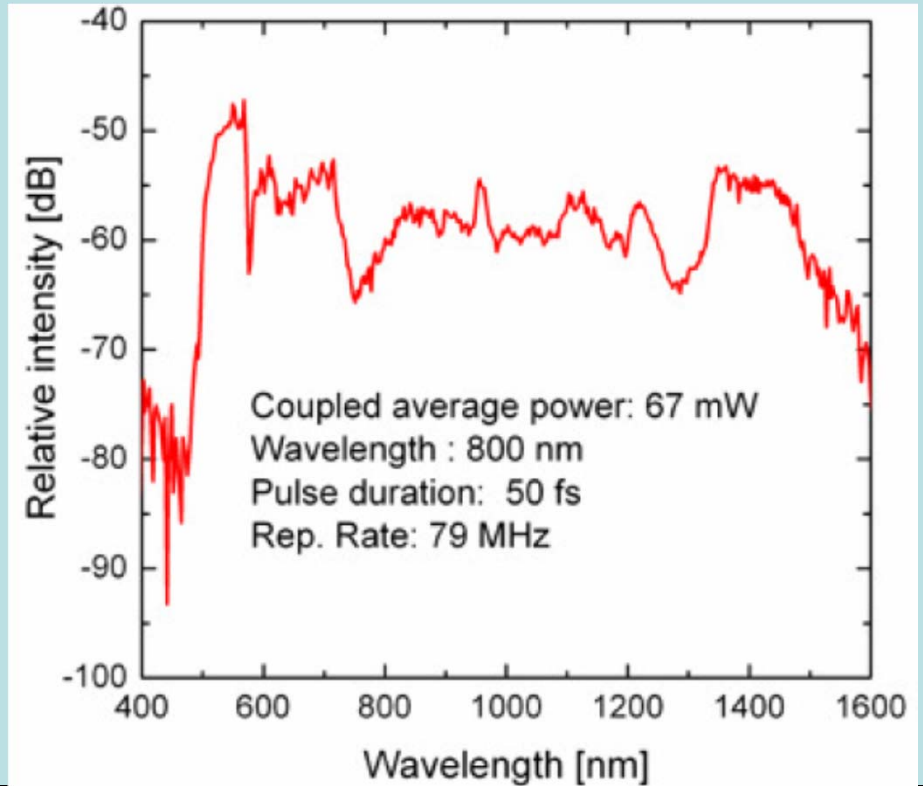


“White” lasers



Ultrashort pulsed light is focused into a photonic crystal fiber

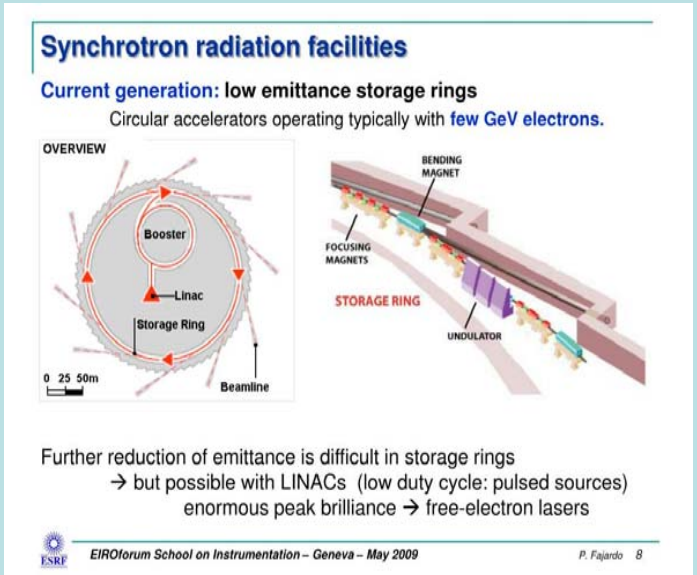


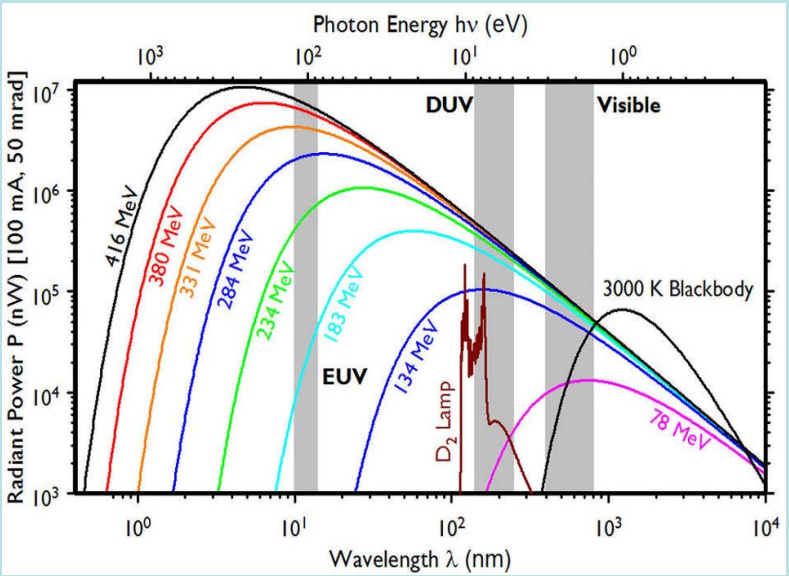


Synchrotron Radiation

In 1054, Chinese and Japanese chroniclers described the appearance of a bright new object in the heavens. We now know that this astronomical event was the explosion of a supernova, the remnants of which we identify with the Crab Nebula. Astrophysicists agree that the light emitted by the Crab Nebula cannot be accounted for in terms of thermal radiation. They believe that this electromagnetic radiation originates from the acceleration of electrons in the magnetic fields associated with the nebula.

In 1898, Alfred-Marie Liénard demonstrated that electrons rotating in a circular orbit, and hence subject to a centripetal acceleration, should emit electromagnetic radiation. On April 24, 1947, at the General Electric research laboratory in Schenectady, New York, Floyd Haber observed visible radiation through the glass walls of the 70MeV synchrotron. Such radiation is now termed Synchrotron Radiation. For several decades, synchrotron radiation has been used as a bright X-ray source for crystallography studies. It also is an excellent light source for time-resolved fluorescence studies due to its exceptional ultraviolet intensity and its inherent pulsed nature.





Excimer Lasers

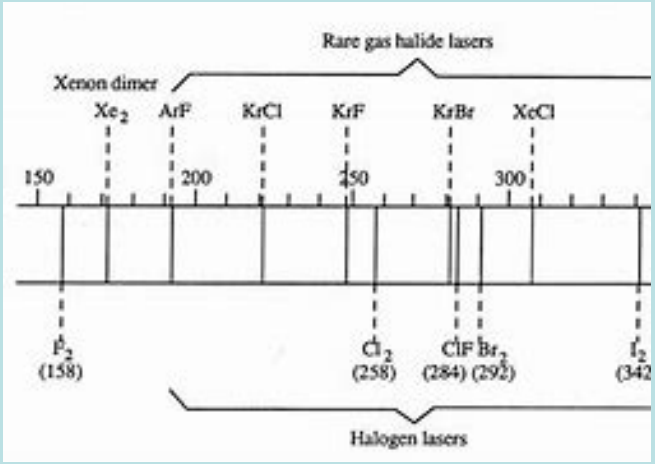
The excimer (excited dimer) laser (also sometimes known as exciplex laser for excited complex) is based on the combination of two gases: a noble gas and halogen.

Both of these are generally stable in their normal low-energy state. When a high-voltage electrical discharge is delivered into the laser cavity containing these gases, the gases combine to form a higher energy excited-gas state compound. Upon the dissociation of this high-energy compound, a photon of energy is released that corresponds to the bond energy of the noble gas-halogen molecule.

This wavelength of light energy is amplified in the laser system, resulting in the production of a discrete high energy pulse of laser energy. The specific wavelength of an excimer laser depends on the composition of the gases used in the laser system

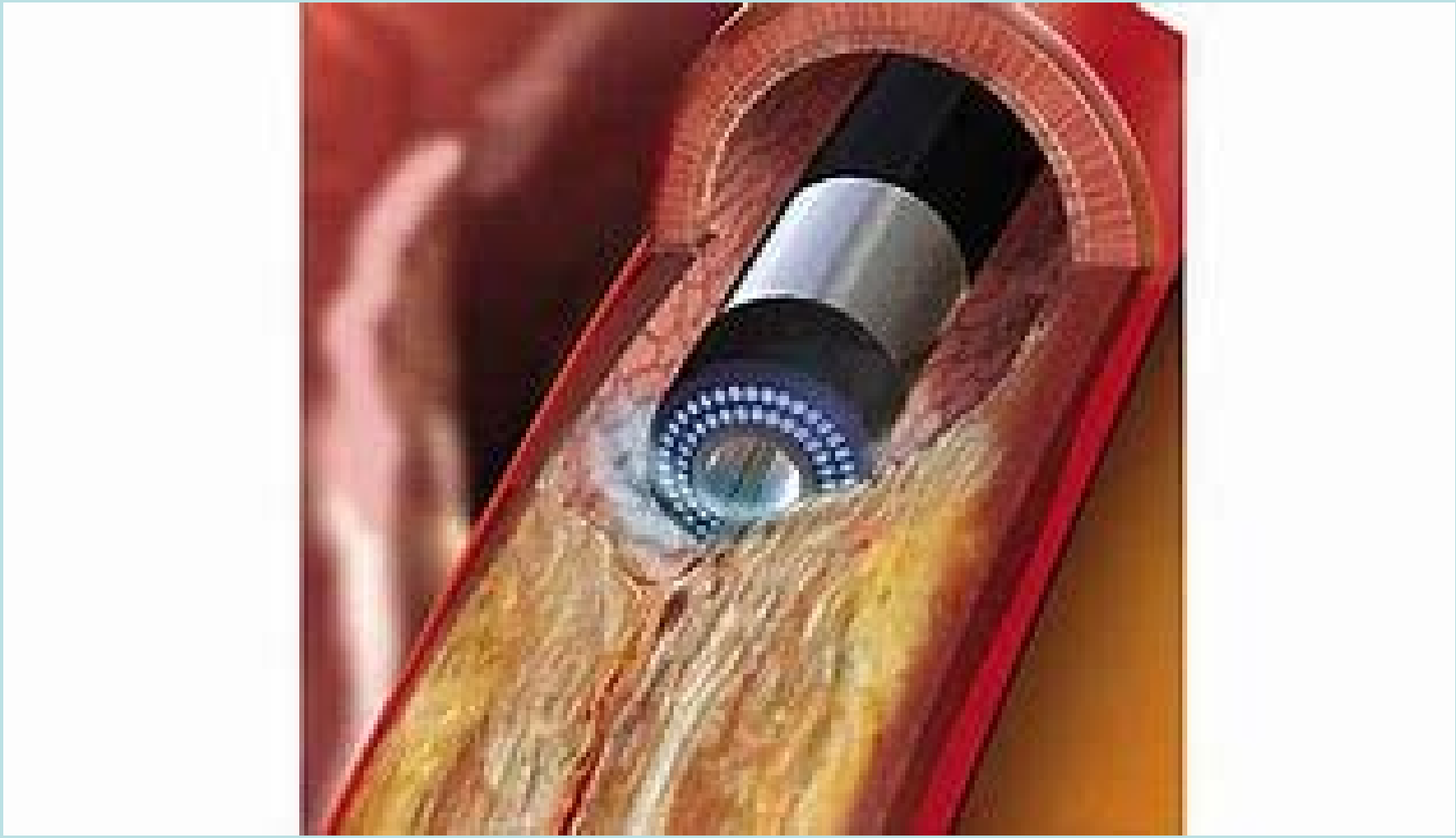
Excimer lasers are commonly used in photolithography of semiconductor based integrated circuits as well as Lasik surgery. The US Food and Drug Administration (FDA) approved the use of laser therapy dentistry in 1990, specifically for use in intraoral gingival and mucosal tissue surgery.

Excimer laser systems in current clinical use rely on argon and fluorine gases. The argon-fluorine excimer lasers emit energy at a wavelength of 193 nm. The krypton-fluoride excimer laser used in early laboratory studies emits a wavelength of 248 nm. Laser energy at 193 nm is very well absorbed by the proteins, glycosaminoglycans and nucleic acids comprising the cornea because of its sufficient photon energy (6.4 eV) and precision (only penetrating the superficial layer; 0.3 μm).



In Lasik surgery, the goal is to reshape the cornea so that rays of light that enter the eye are focused clearly onto the retina.

Excimer lasers are also now being used for coronary angioplasty

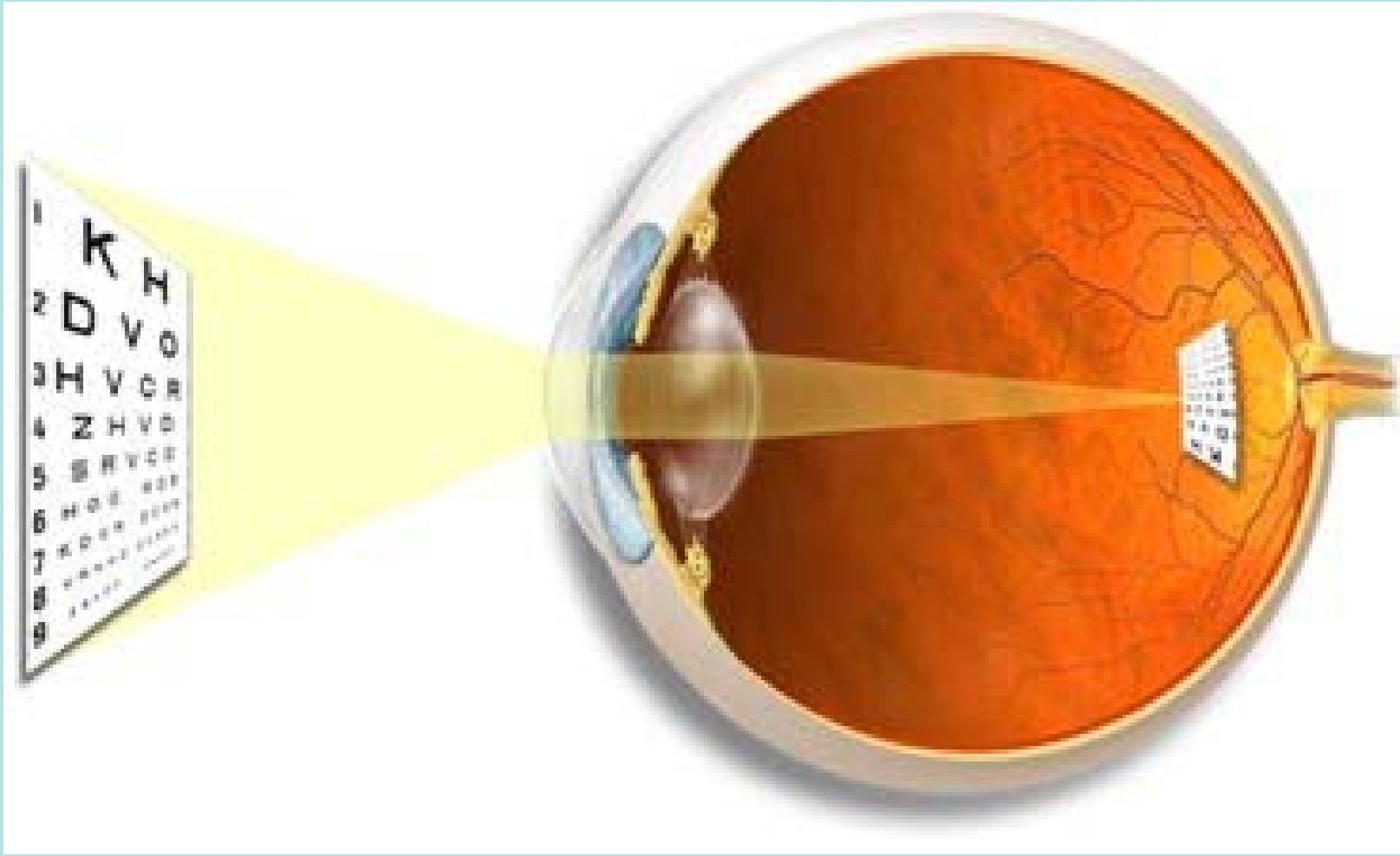


Fluorescent Lighting

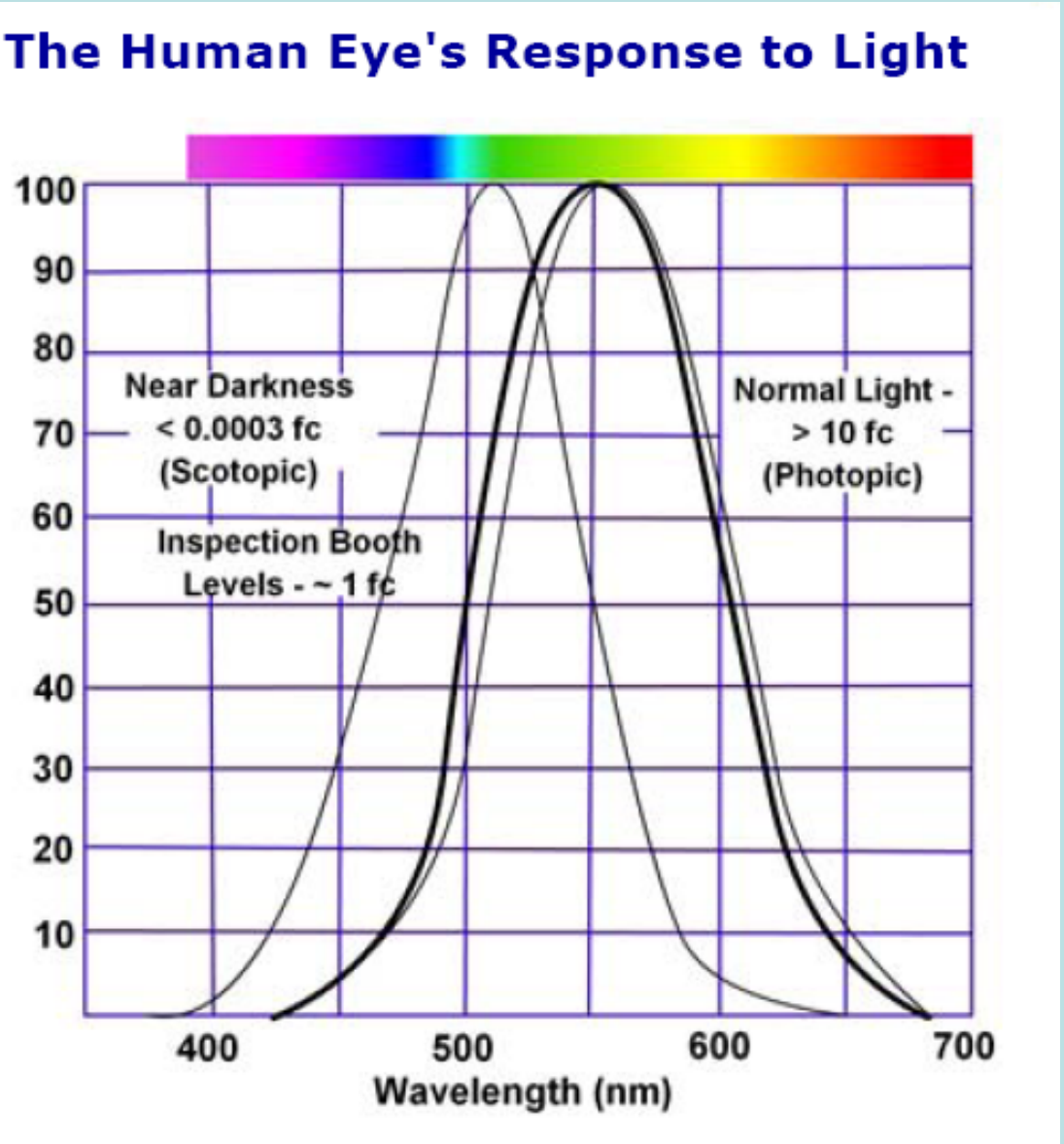
Alexandre Edmond Becquerel, an important person in the development of the field of phosphorescence, was the first to suggest that the inside of electric discharge lamps could be coated with fluorescent material.

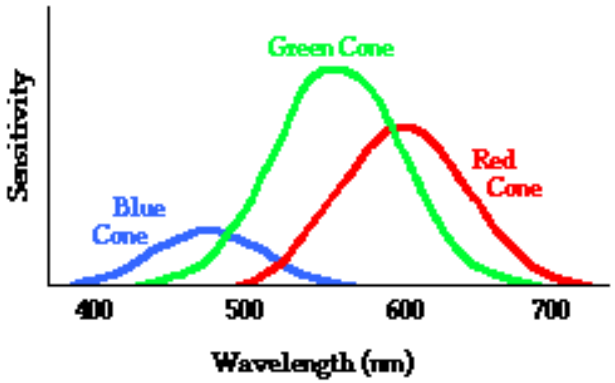
In modern fluorescent lighting, a glass tube is usually filled with a noble gas (e.g., argon, krypton or neon) along with a few drops of mercury. Only a small fraction of the mercury atoms need to be in the gas phase so that when an electron (from the electrical discharge) strikes them they will fluorescence, for example at 254nm. This UV light will then hit the layer of fluorescent powder (called the phosophor) coating the inside of the tube and give rise to visible fluorescent light. Note that the 254nm mercury emission is not hazardous since it will be absorbed by the glass tubing. These types of fluorescent lights are, however, considered to be environmental hazards since breakage can release mercury.

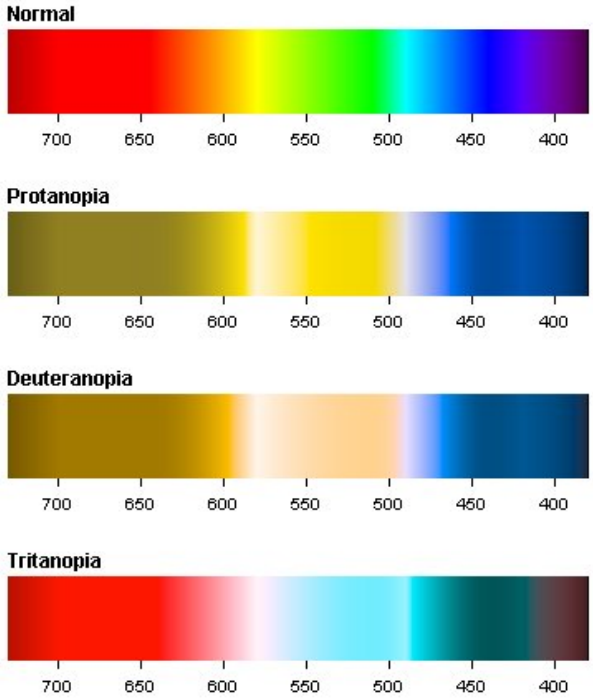
Detectors



Detectors





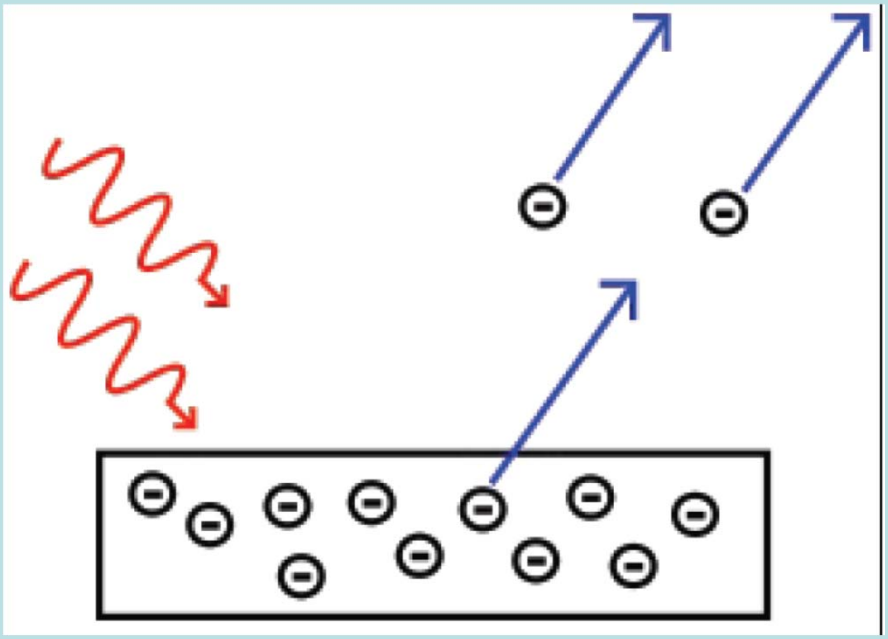


http://www.ndt-ed.org/EducationResources/CommunityCollege/PenetrantTest/Introduction/lightresponse.htm

Detectors

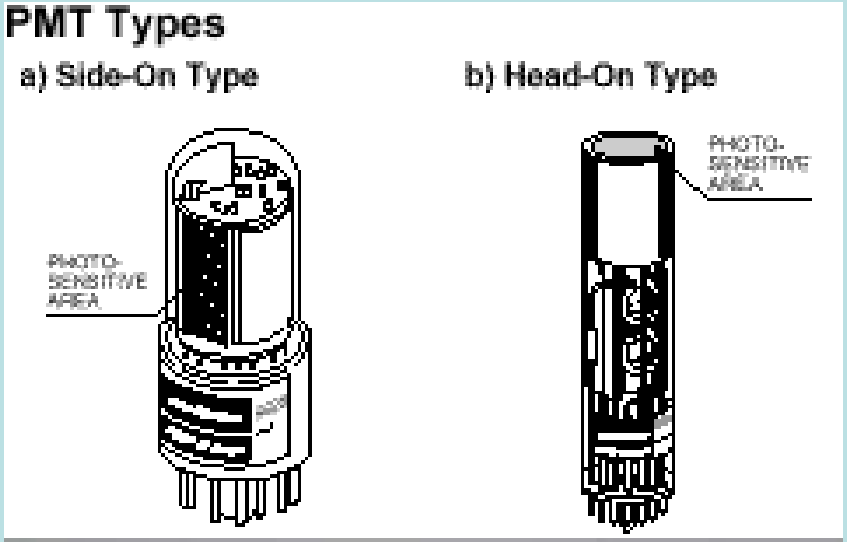
The photoelectric effect was discovered by Heinrich Hertz in 1886

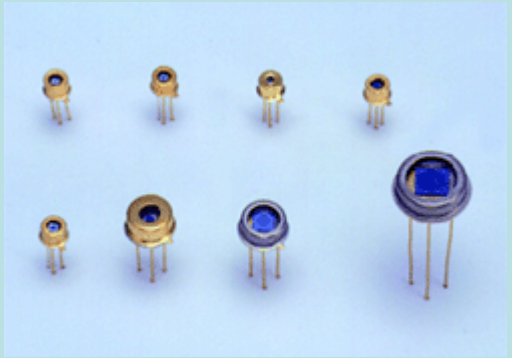
Specifically he noticed that a charged object loses its charge more readily when it is illuminated by UV light



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



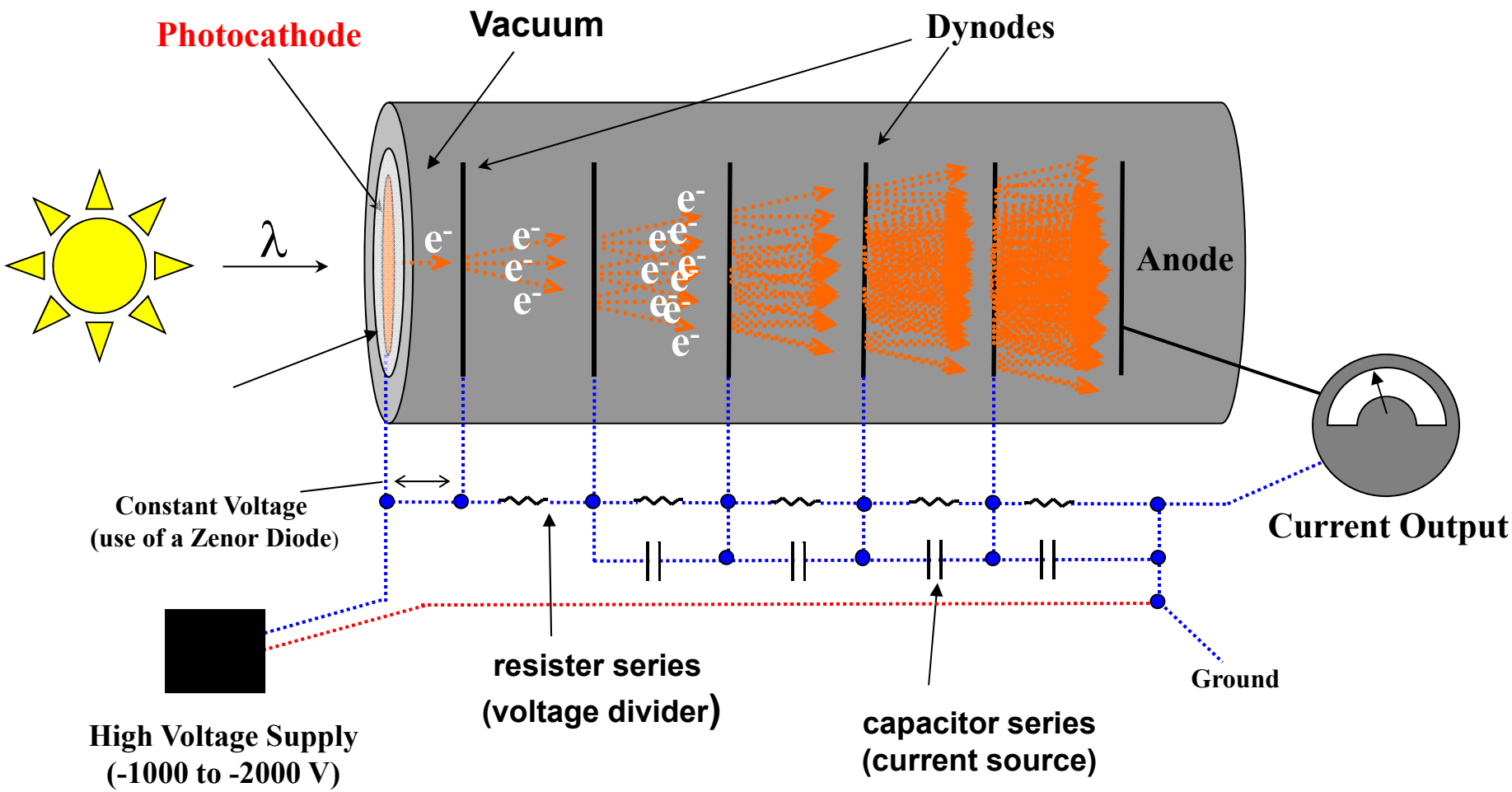


APD

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

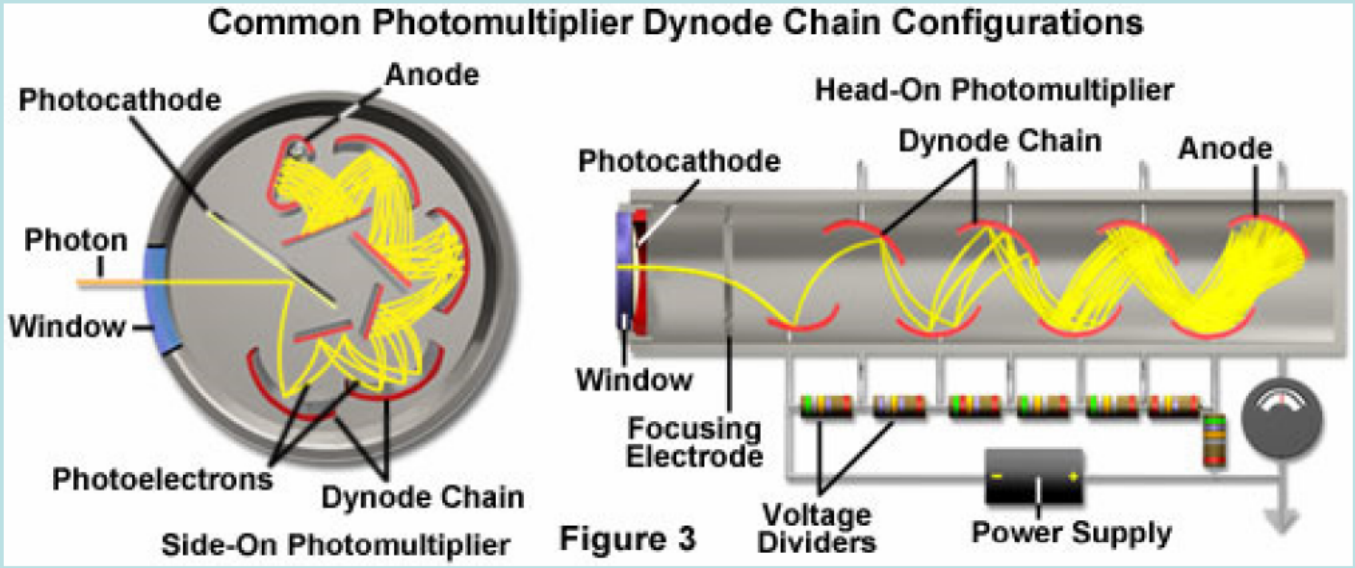


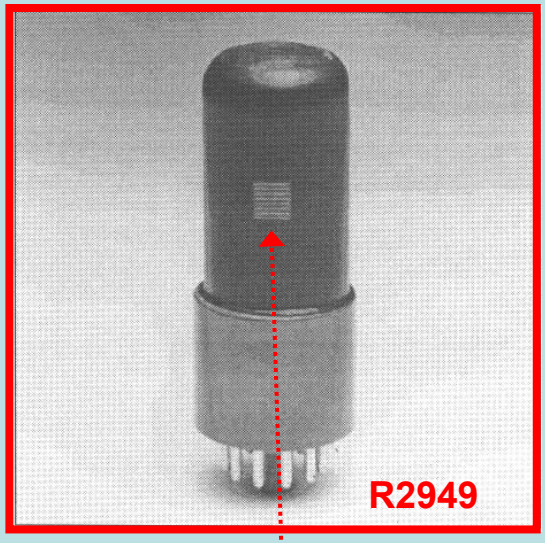
Figure 3

Opaque photocathode

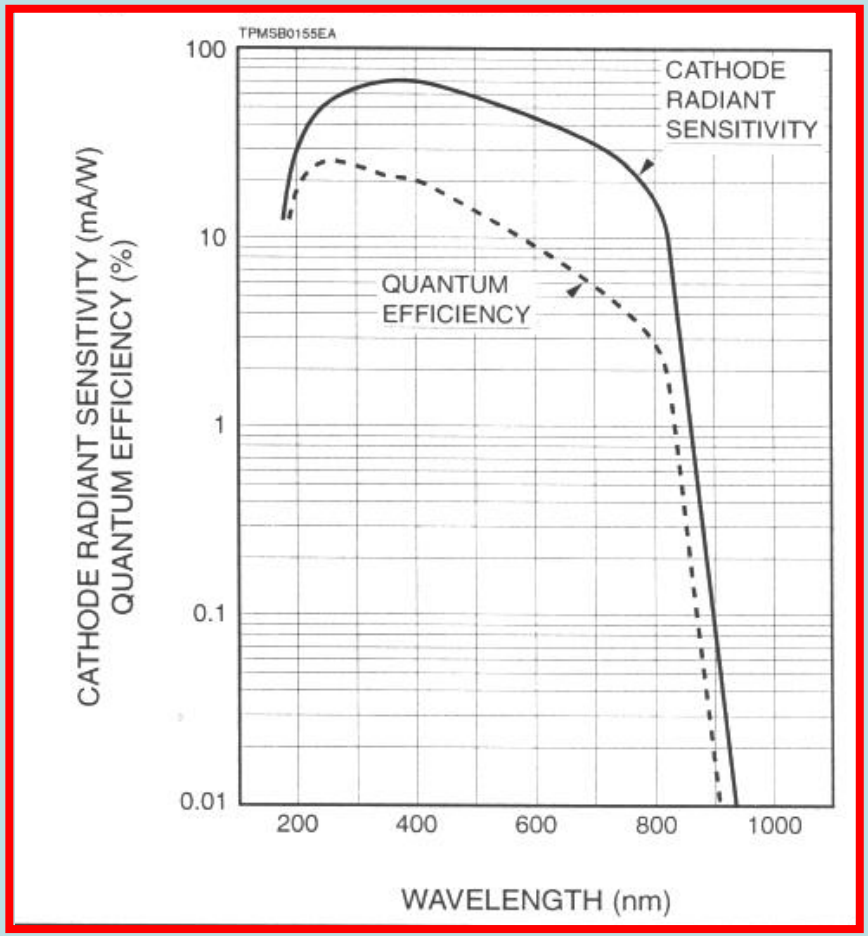
Semitransparent Photocathode

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

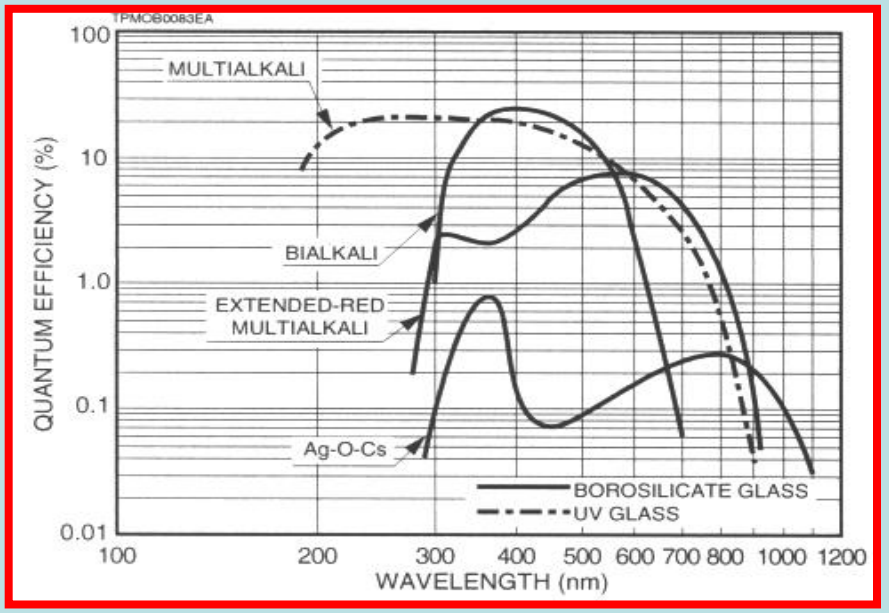


Window with Photocathode Beneath

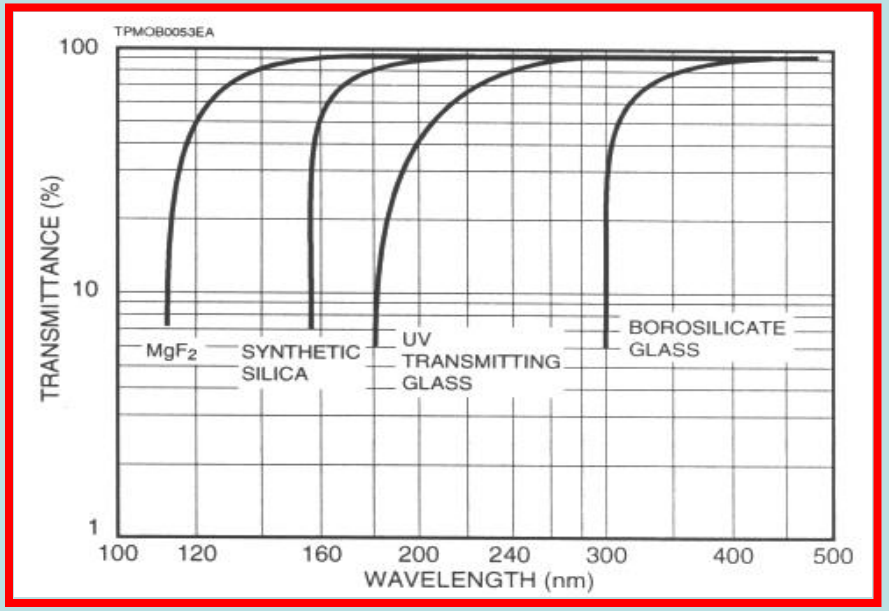


PMT Quantum Efficiencies

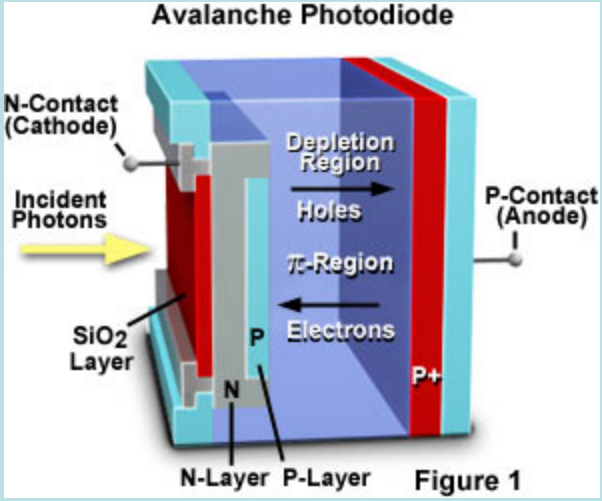
Cathode Material



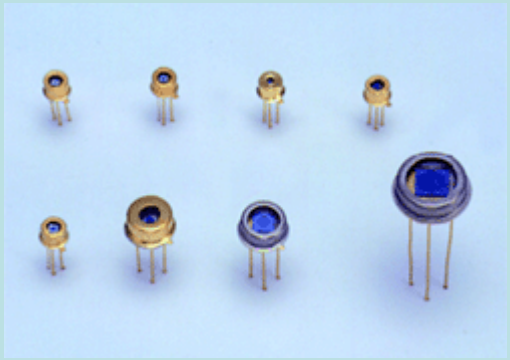
Window Material



Detectors

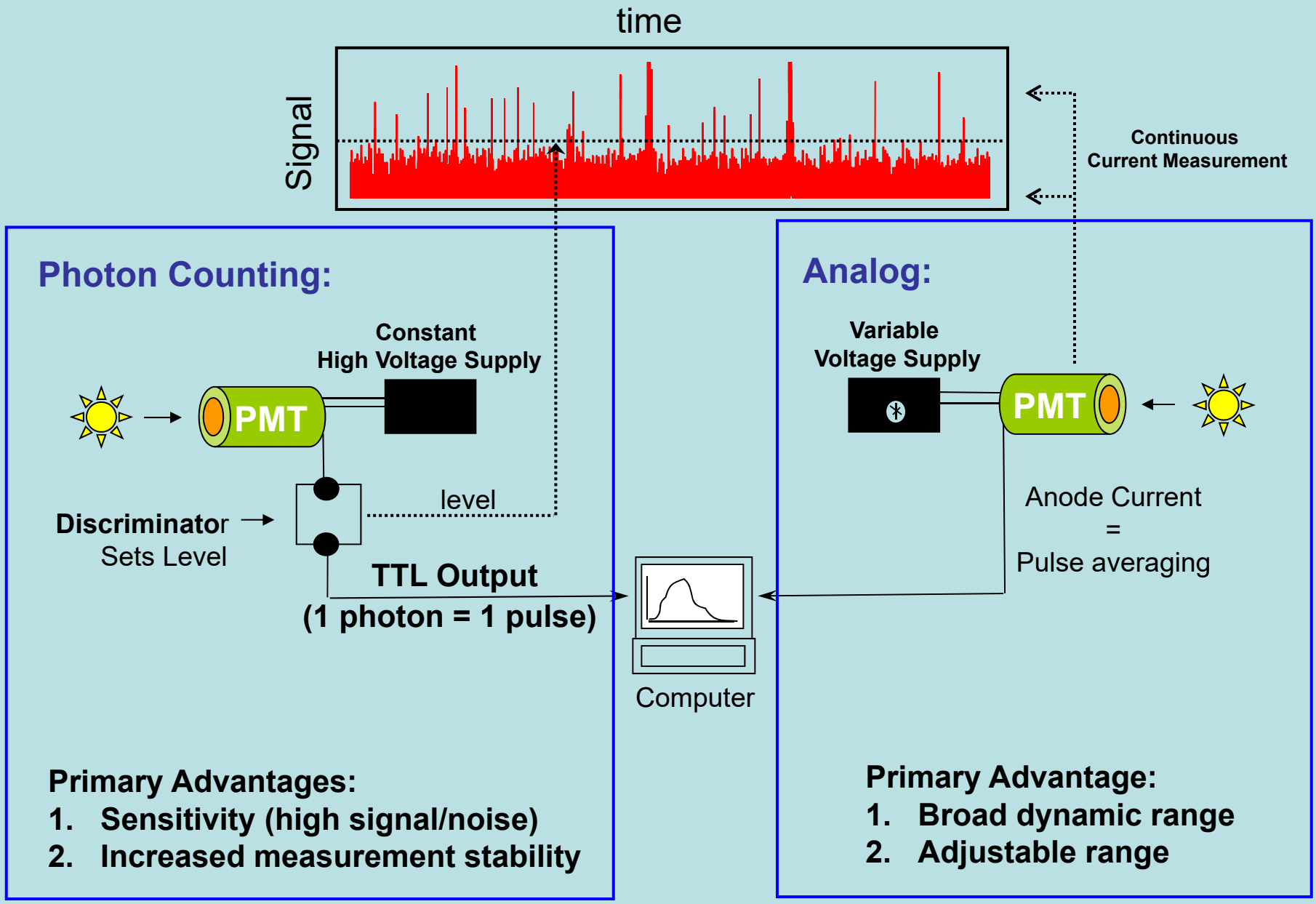


APDs are usually used in applications characterized by low light levels



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

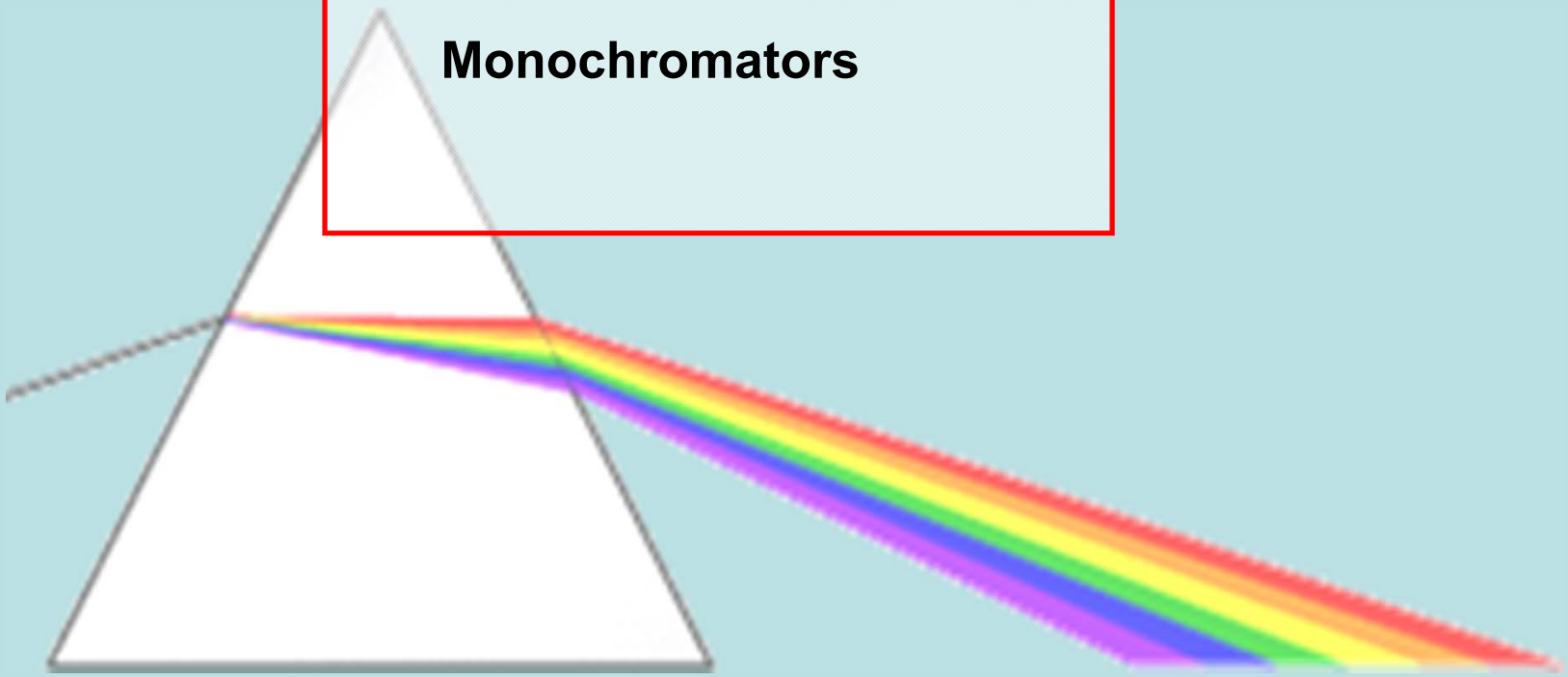


Wavelength Selection

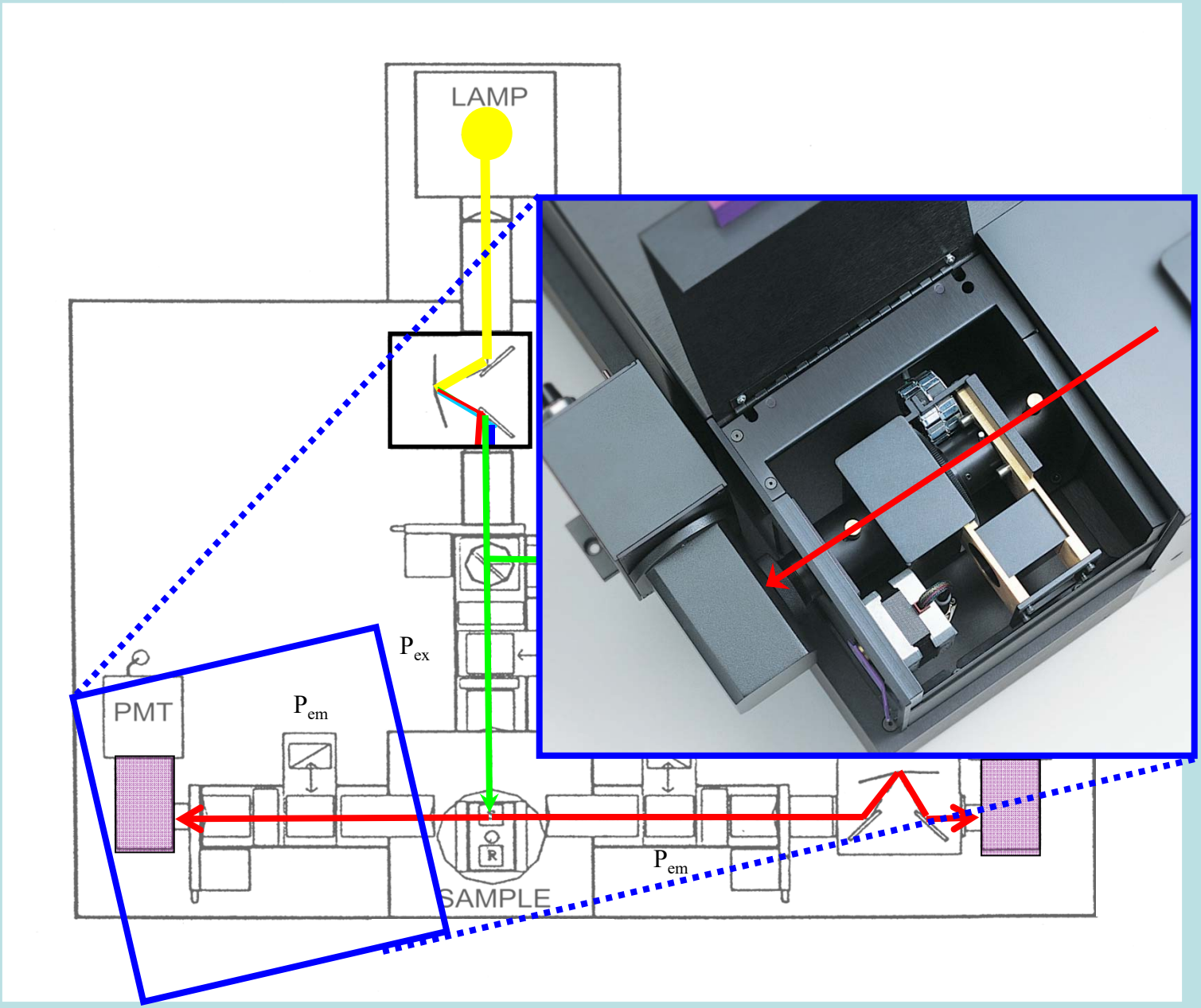
Fixed Optical Filters

Tunable Optical Filters

Monochromators



Optical Filter Channel



Long Pass Optical Filters

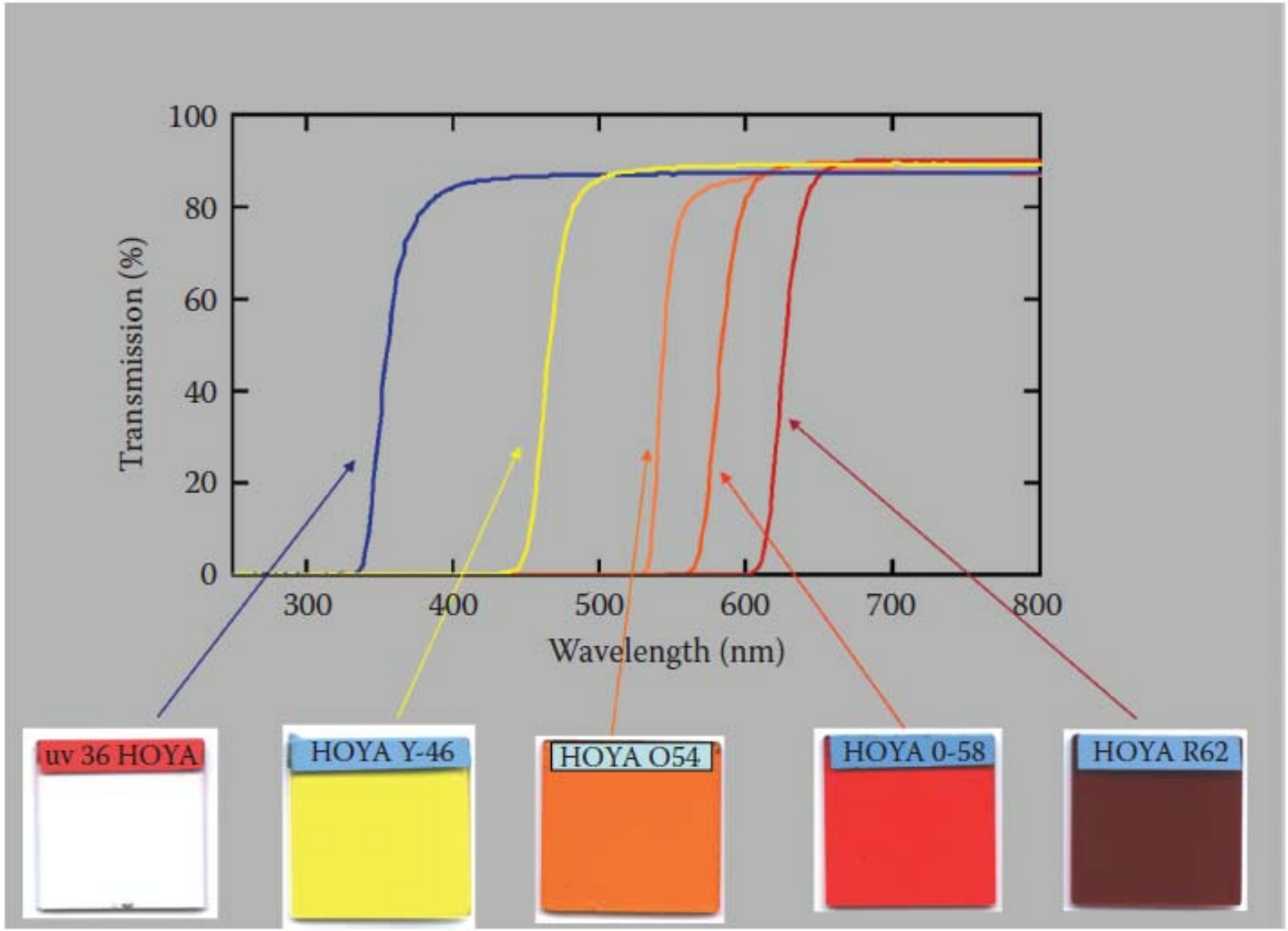
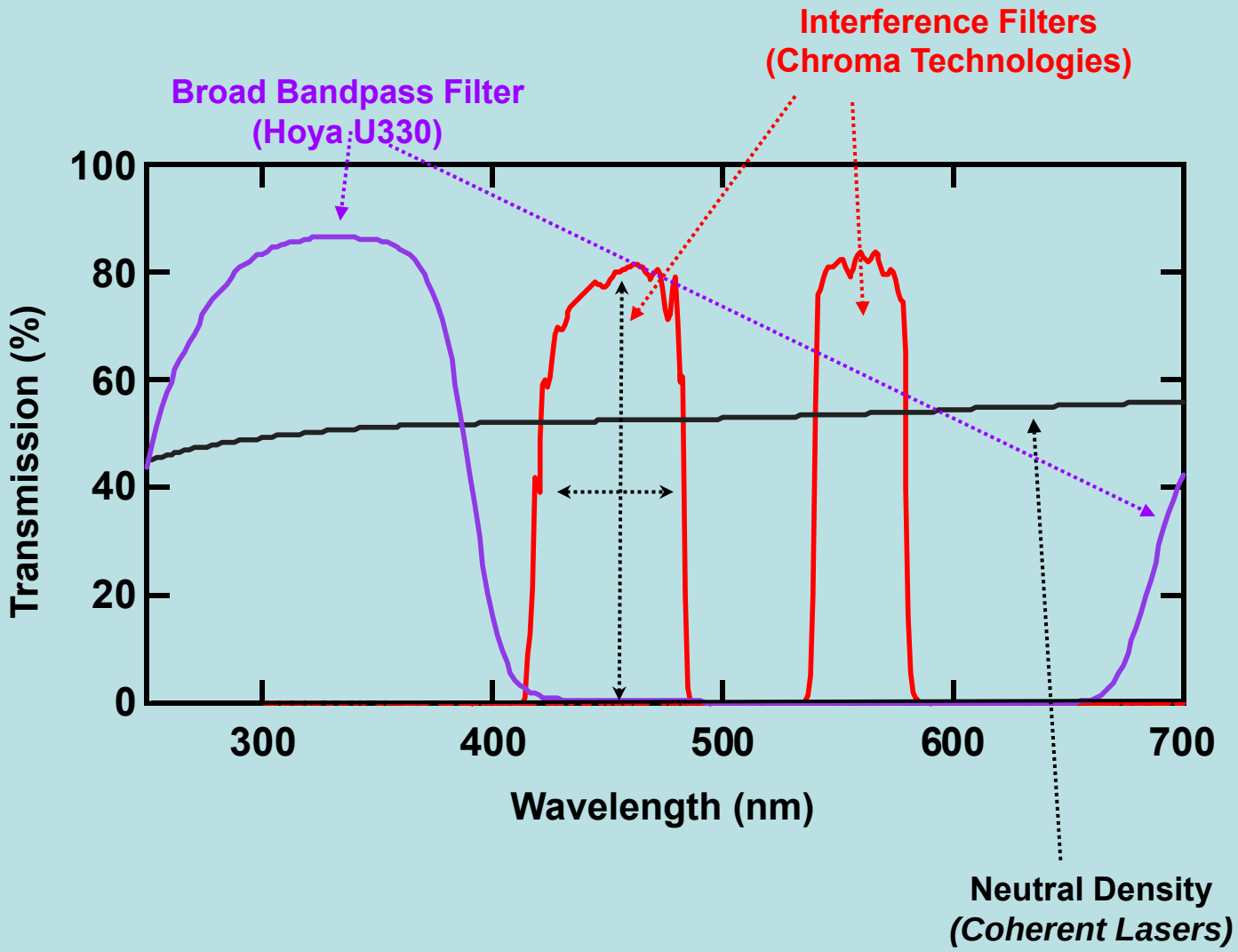


FIGURE 3.10 Depiction of five longpass filters and their associated transmission spectra. (The author would like to thank Theodore Hazlett for this figure.)

More Optical Filter Types…



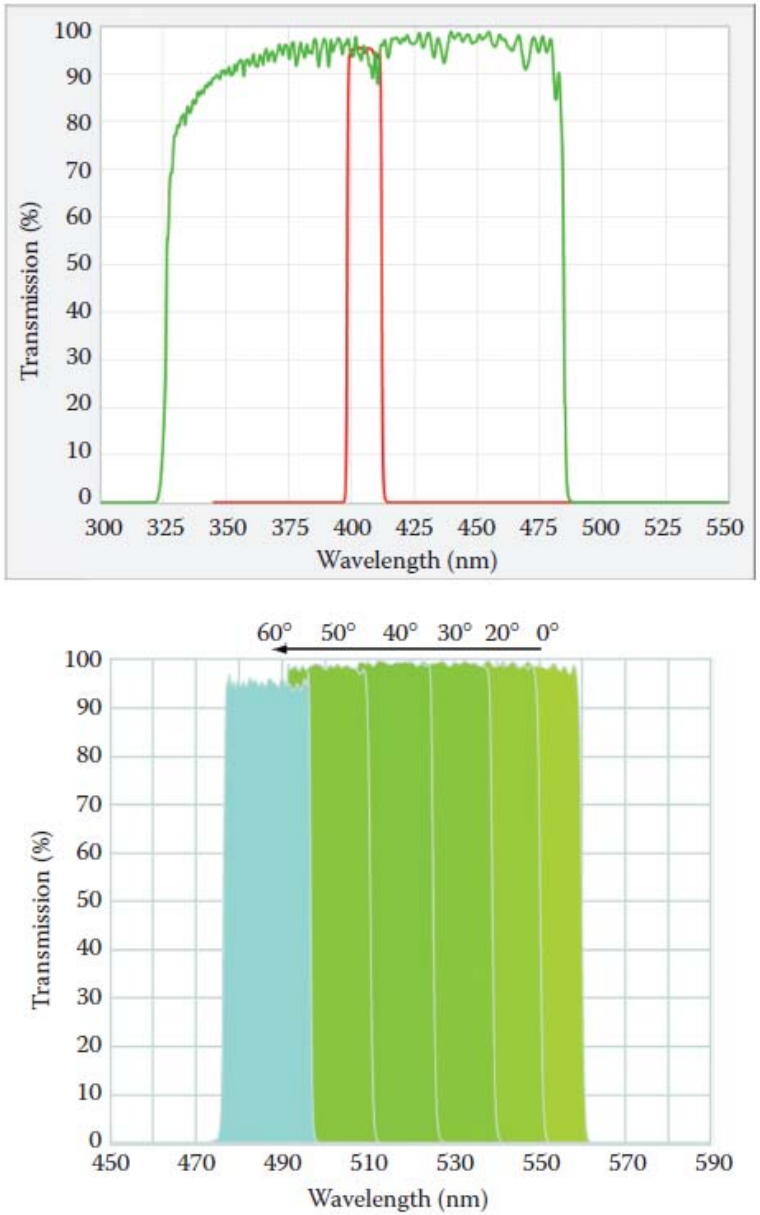
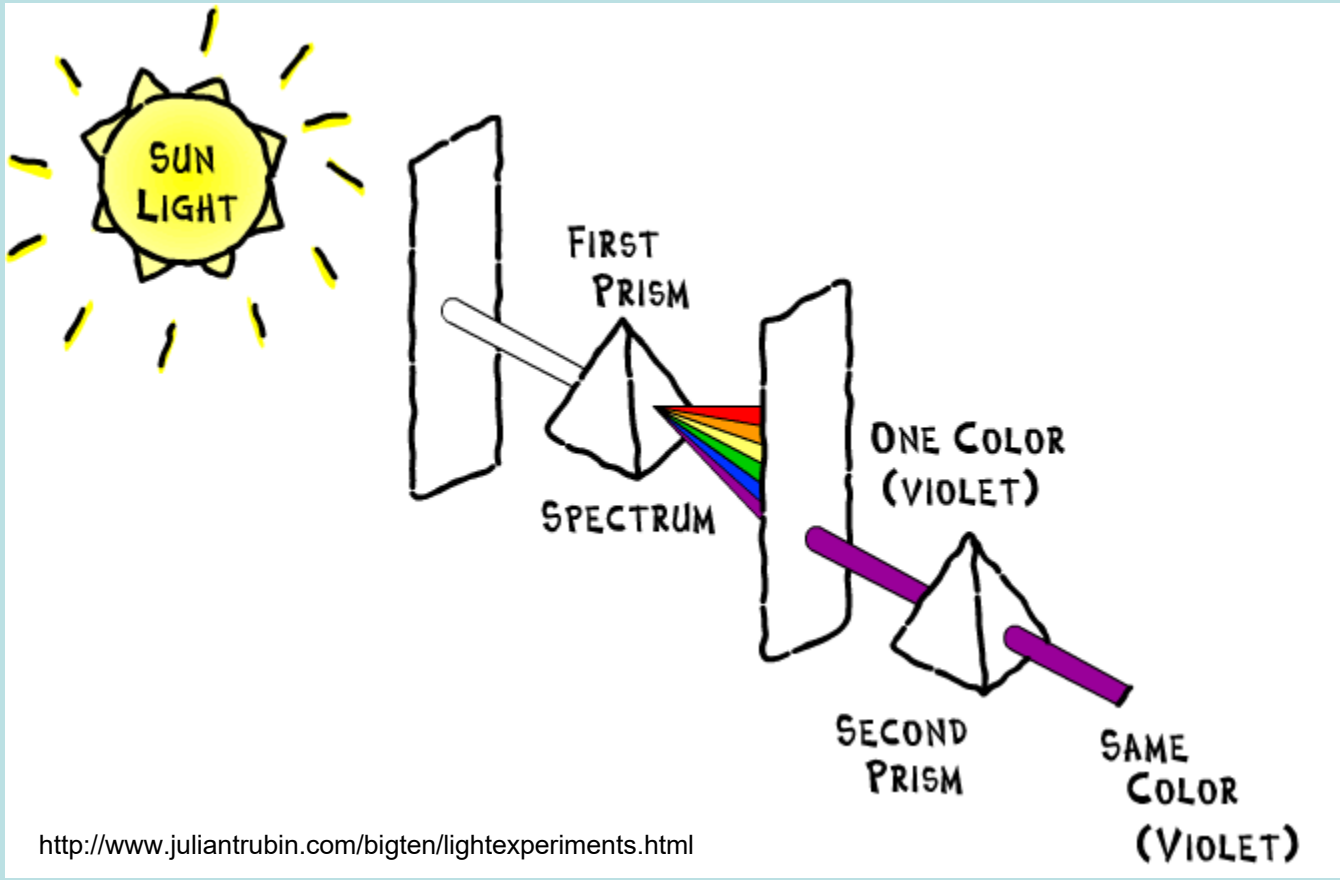


FIGURE 3.11 Transmission spectra for two bandpass filters and a tunable filter. Both bandpass filters are centered at 405 nm, with FWHM of 150 nm (green) and 10 nm (red) (the author would like to thank Jim Passalugo of Semrock, Inc. for these spectra).

Monochromators

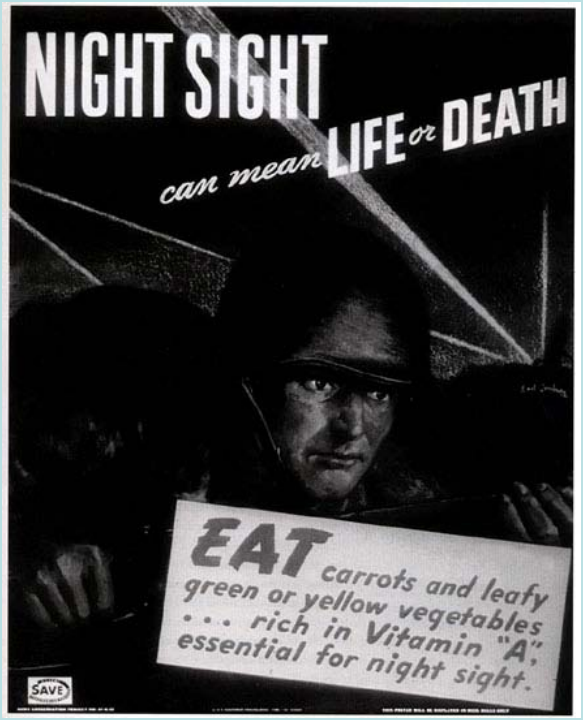
People had experimented with prisms and light before Newton – but generally it was thought that the prism somehow “colored” the light. Newton was the first to clearly state that the prism revealed an underlying characteristic of white lght – namely that it was composed of many colors.

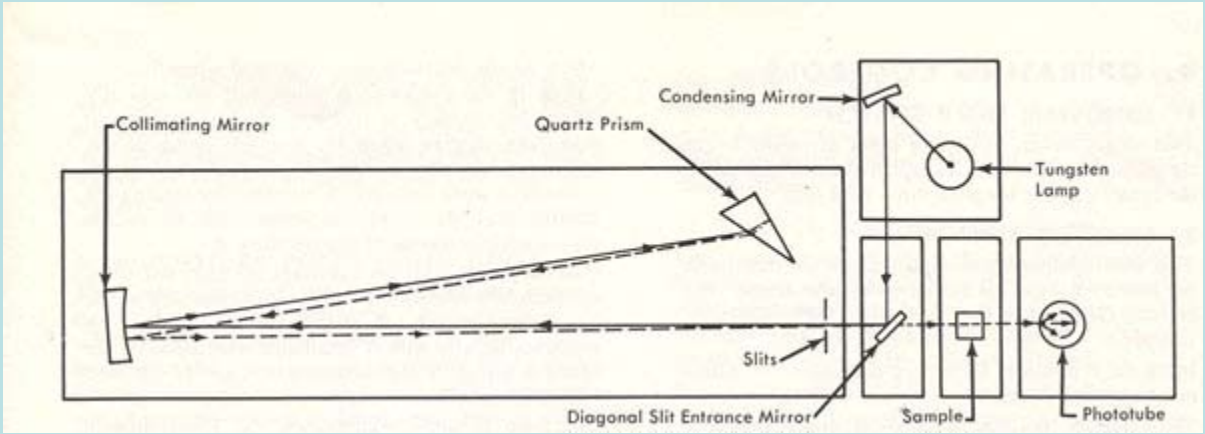


http://www.juliantrubin.com/bigten/lightexperiments.html

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).

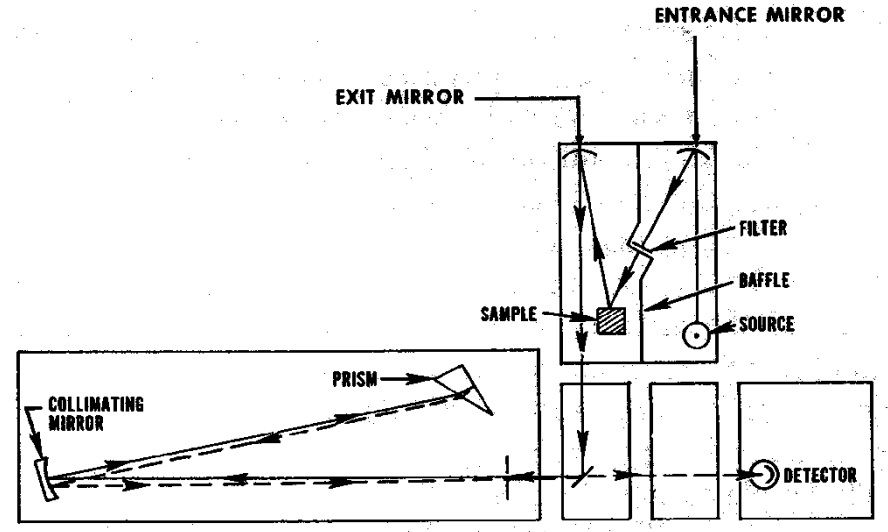
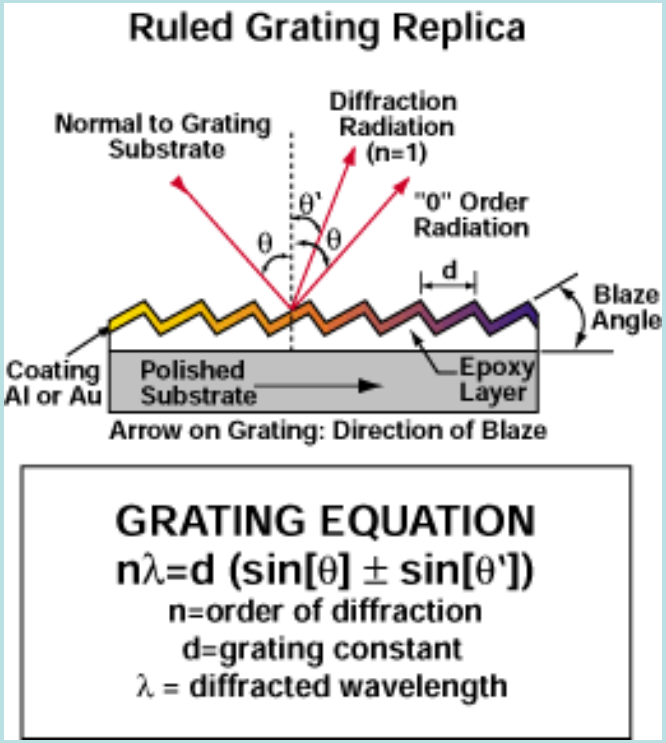


FIG. 20. Attachment for measuring fluorescence spectra with the Beckman Model DU and DK spectrophotometers.

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



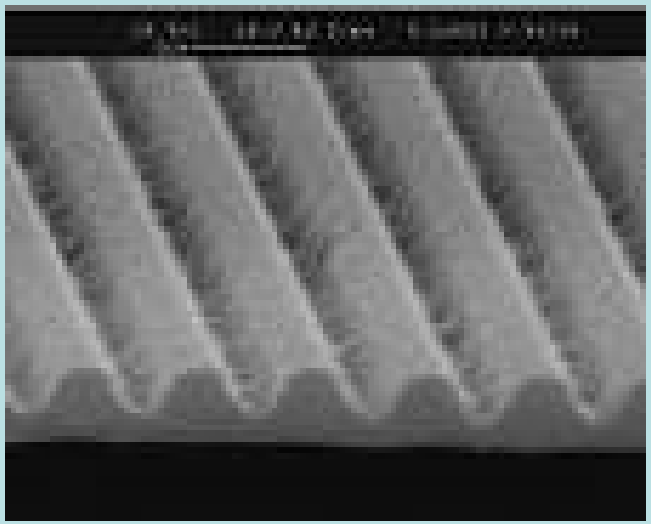
GRATING EQUATION

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

n=order of diffraction

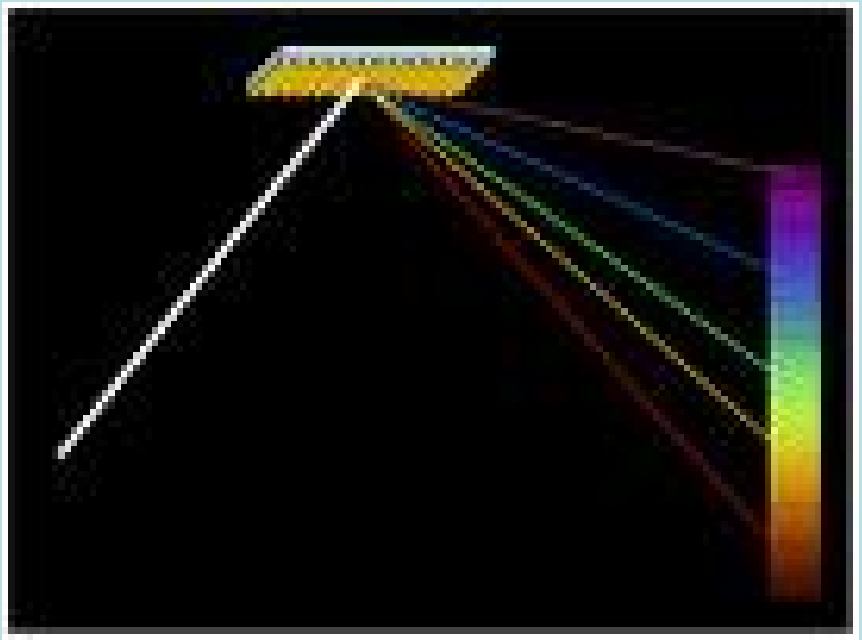
d=grating constant

λ = diffracted wavelength



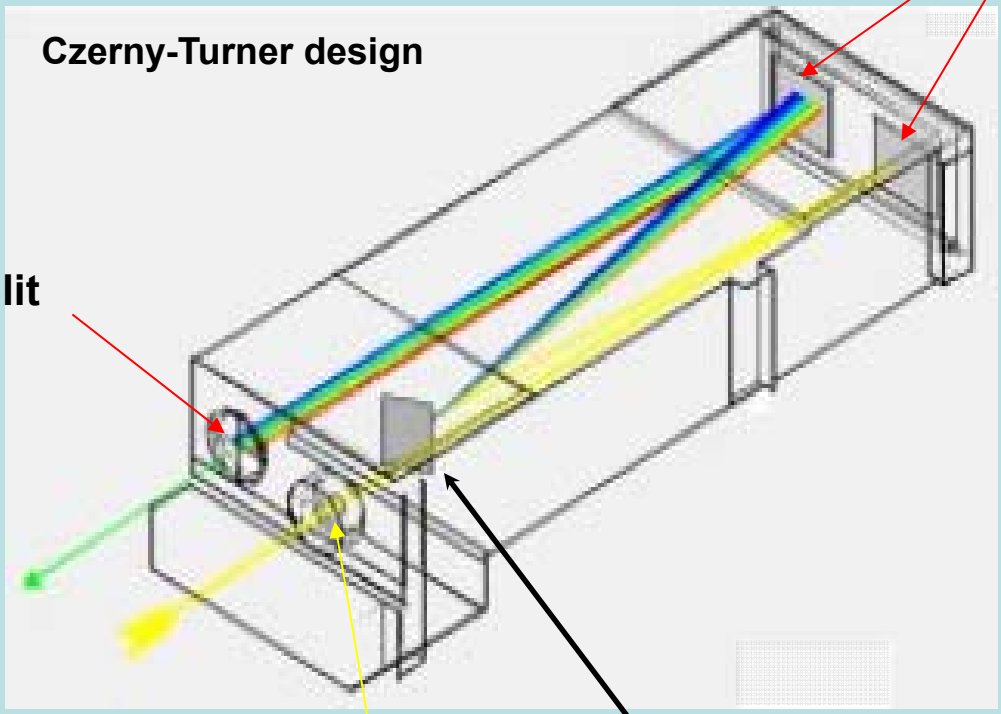
Formerly ruled with diamond-tipped instruments

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



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

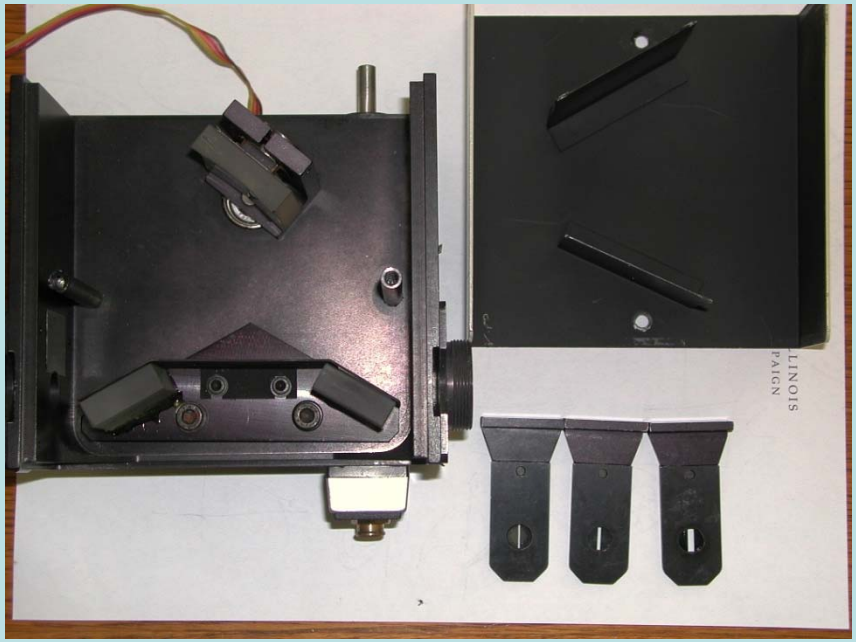
Monochromators

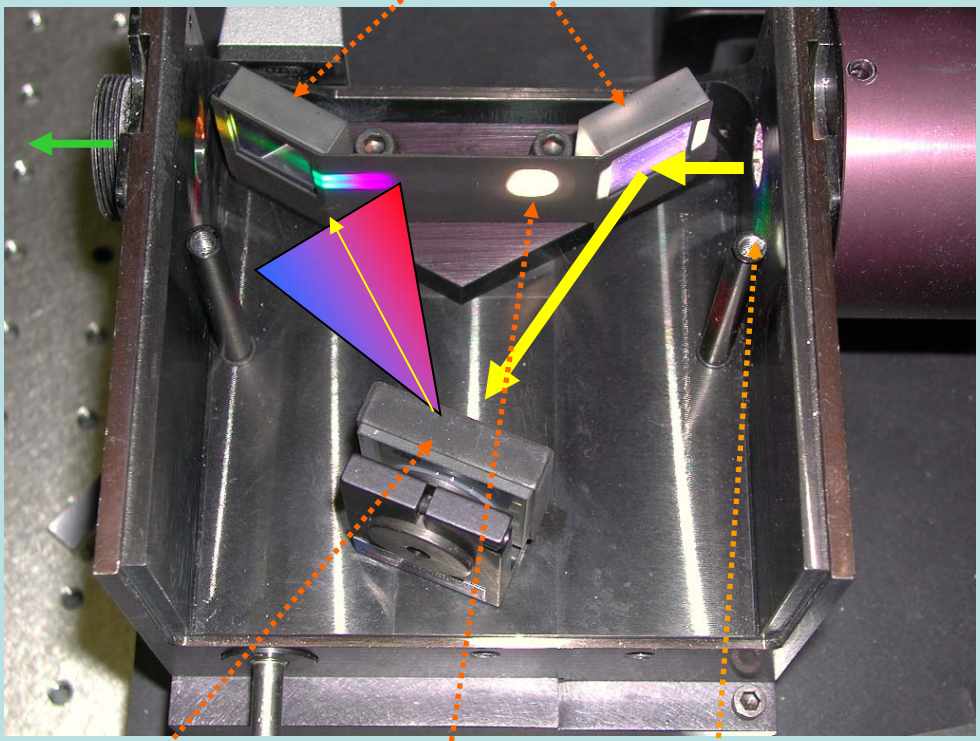


1. Slit Width (mm) is the dimension of the slits.
2. Bandpass is the FWHM of the selected wavelength.
3. The *dispersion* is the factor to convert slit width to bandpass.

The Inside of a Monochromator

H10 Monochromator



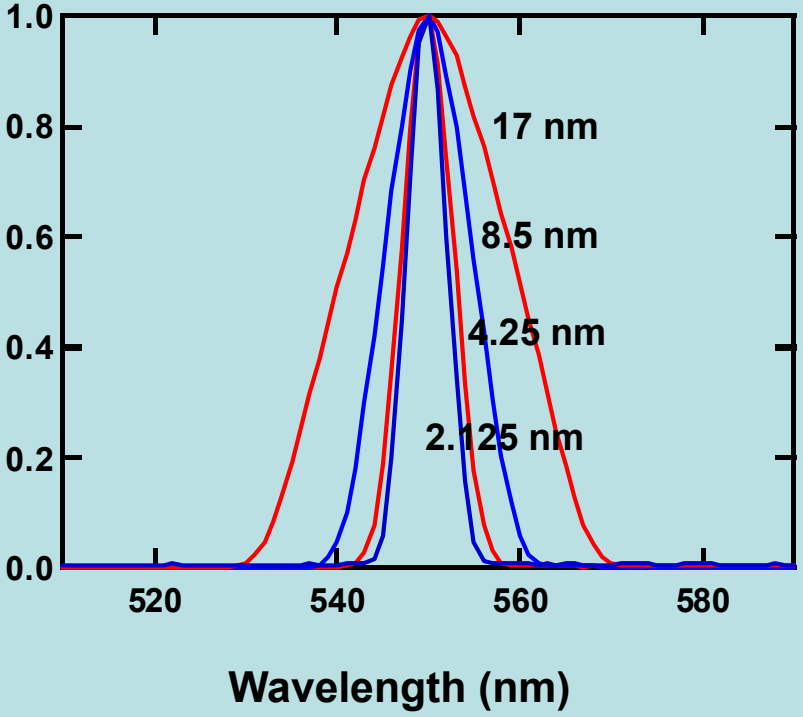


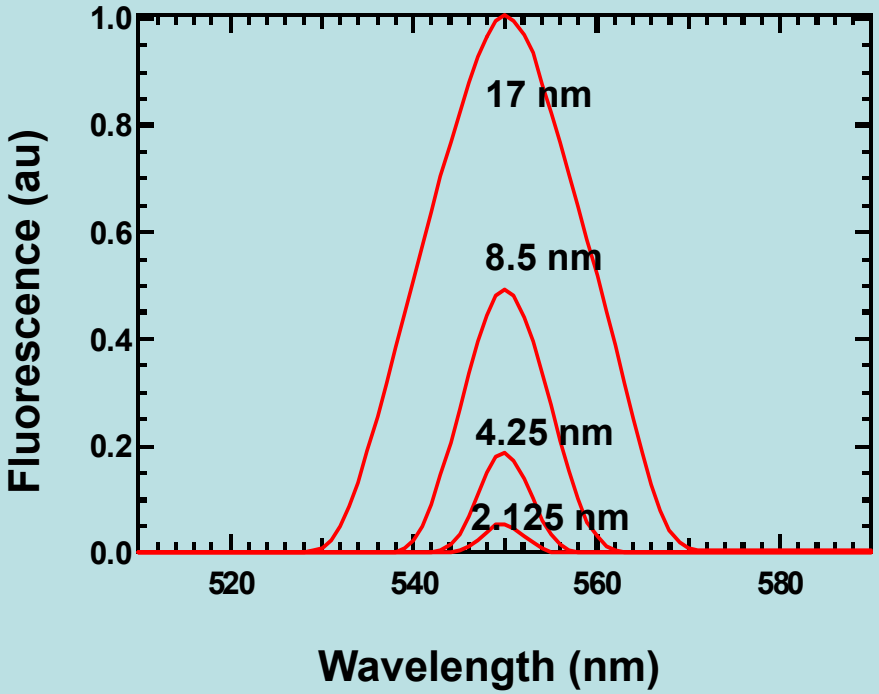
Changing the Bandpass

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)

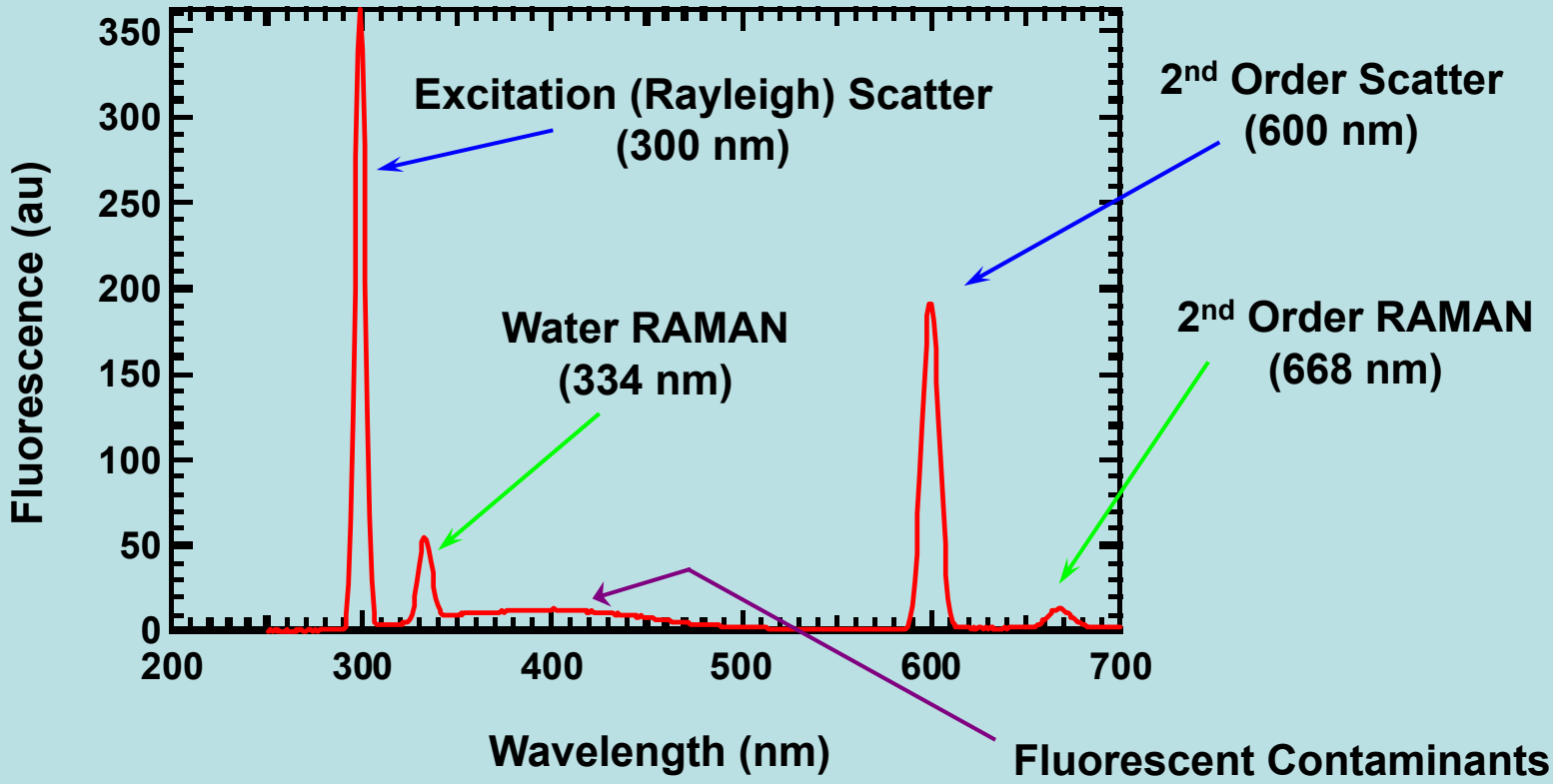




Collected on a SPEX Fluoromax - 2

Higher Order Light Diffraction

Emission Scan:
Excitation 300 nm
Glycogen in PBS



Initial emission spectra are typically *uncorrected,* i.e., not corrected for the instrument response characteristics.

But to get the true "corrected" emission spectrum one must take into account:

1) The solvent background, including Raman scattering (and Rayleigh scatter if present)

2) The response characteristics of the monochromator

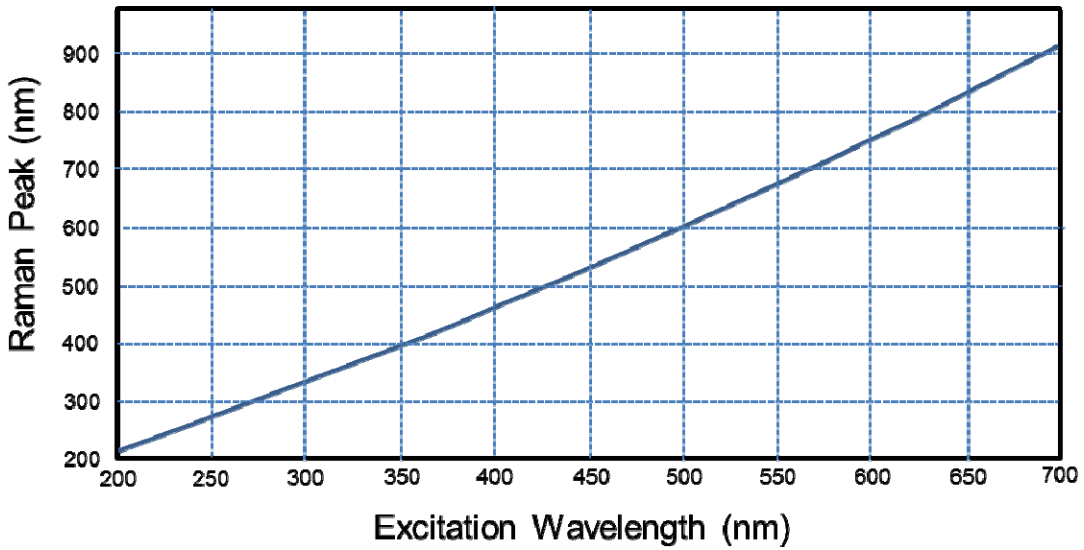
3) The response characteristics of the detector

Correcting for solvent background is usually not required unless the sample fluorophore has a very low quantum yield or the concentration is very low. In the case of turbid samples, such as membrane systems or large aggregates, Rayleigh scatter may be an issue (especially for polarization measurements which will be covered later).

Raman peaks **– Water molecules exhibit two O-H stretching frequencies (symmetric and asymmetric) and one bending frequency. These motions result in absorption of electromagnetic energy in the infrared region of the spectrum over a narrow range near 3400 cm^{-1}. Although the absorbance of these frequencies is weak, given the fact that water is so concentrated (55M), the total absorption is not negligible, which results in a slight loss of energy in the scattered light from an aqueous solution. This energy loss results in an "inelastic scattering," known as Raman scatter (after Chandrasekhara Venkata Raman), shifted around 3400 cm^{-1} to lower energy from the elastic scattering known as Rayleigh scatter (after John William Strutt who, upon his father's death, became the 3rd Baron Rayleigh). A simple expression to estimate the position of the Raman scatter from water is:**

$$\frac{1}{\lambda_r} = \frac{1}{\lambda_e} - 0.00034$$

where λr is the wavelength of the Raman scatter and λe is the wavelength of the excitation.



If the concentration of the fluorophore is low than the Raman peak for water may appear

Let's look at an extreme case: the emission spectrum of 10^{-11} M quinine sulfate

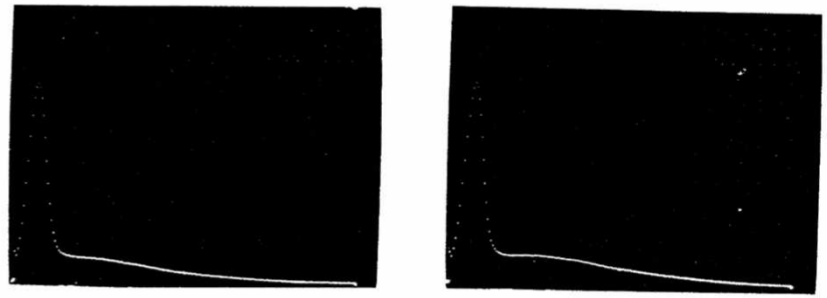
A. Background of 0.2N H_2SO_4 scanned from 371 to 560nm. Excitation 340nm. The Raman peak appears at 384nm

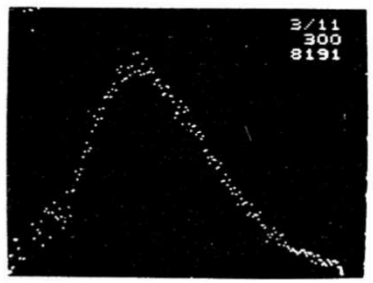
B. Background plus 10^{-11} M quinine sulfate

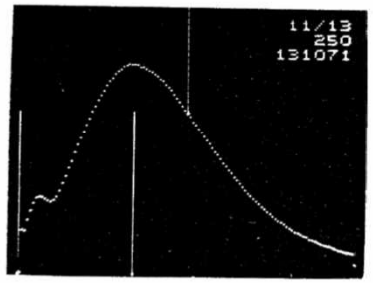
C. Spectrum B minus Spectrum
A. Vertical scale expanded

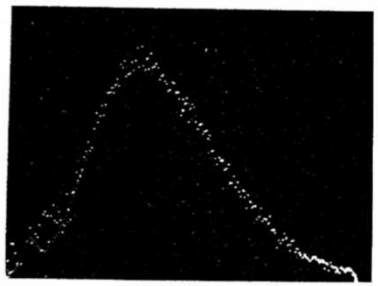
D. 10^{-8} M quinine sulfate

E. Overlay of Spectra C and D

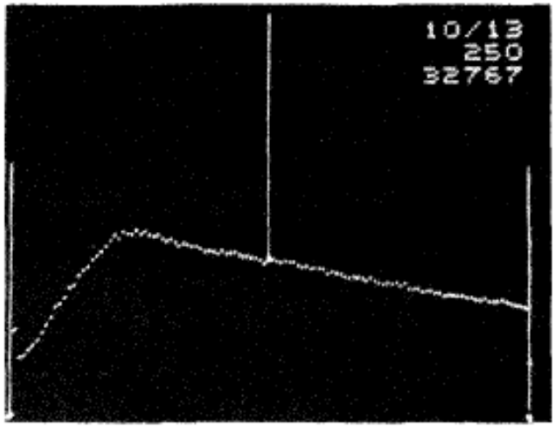




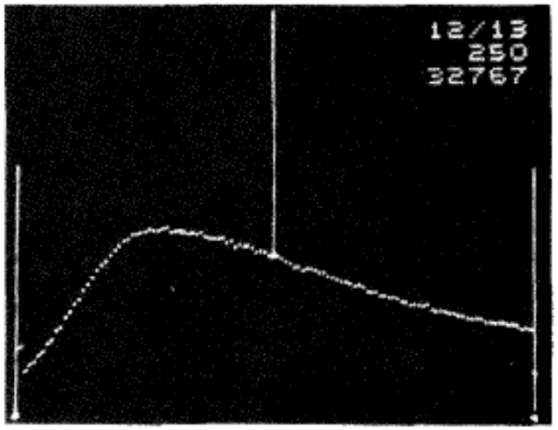




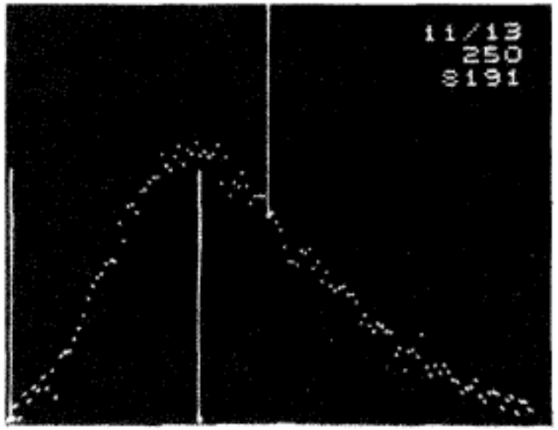
In some cases the Raman peak can be eliminated using optical filters



(a)



(b)

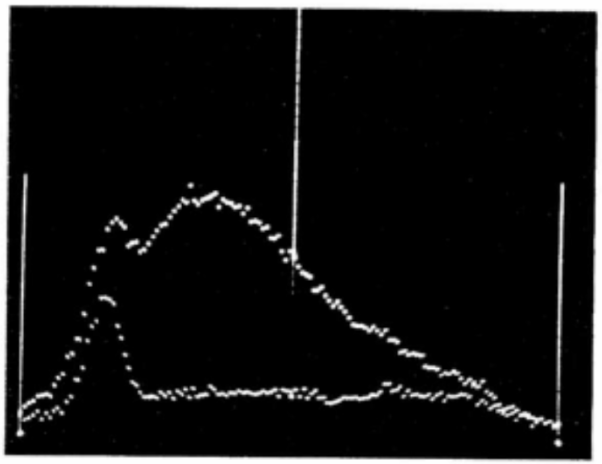


(c)

FIG. 4. (a) Background emission of 0.01 *M* phosphate buffer p_H 7.0 scanned from 301 to 400 nm. Excitation at 280 nm. Corning 0-54 filter cuts out most of Raman. (b) Background plus 5.0×10^{-10} *M* HLADH. (c) Spectrum *B* minus spectrum *A*. Note change in vertical scale from 32767 counts full scale to 8191 CFS.

Consider the case where the fluorophore concentration is relatively high, but the quantum yield is very low.

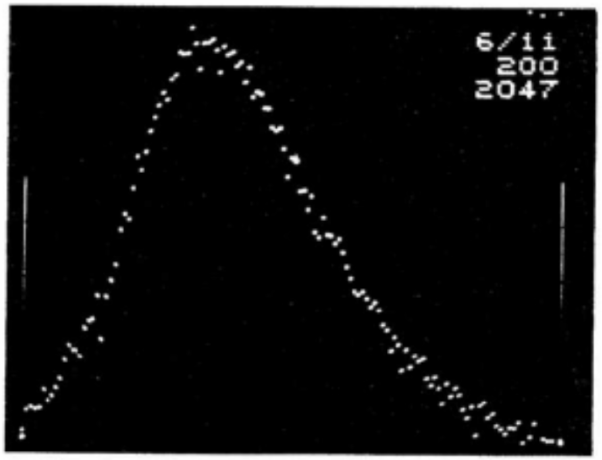
Figure A shows the emission from 2μM of the α subunit of hemoglobin (which has one tryptophan residue) along with the spectrum of the buffer. The optical density of the protein at the excitation wavelength (270nm) was 0.1



A

The intrinsic fluorescence of hemoglobin is very low due to energy transfer from the tryptophan to the heme moiety.

Figure B shows the α subunit emission minus the buffer signal.



B

Although the Raman peak can lead to confusion in fluorescence studies, it can provide some service as an emission standard. Specifically, the strength of the water Raman scatter can serve as a measure of the light intensity from a light source, e.g., a xenon arc lamp, to indicate when the lamp is delivering less light than expected. For example, a log book can be kept near the spectrofluorimeter and every week or so one can monitor the Raman scatter intensity, near 397nm, from a water solution excited at 350nm, under the same conditions of slitwidths and lamp current settings.

After background correction one must then obtain correction factors for the monochromator wavelength and the photodetector wavelength response.

One common way to obtain these factors is to use a standard lamp. Table 1 shows the output of a standard lamp from the National Bureau of Standards.

Table 2 shows the correction factors obtained, using the standard lamp, for an SLM monochromator

TABLE I

Spectral Irradiance of Lamp L-209 in Microwatts per (cm^2 nanometer) at a Distance of 50 cm When Operated at 6.50 Amperes

Wavelength (nm)	Spectral Irradiance
250	.000304
260	.000531
270	.000947
280	.00155
290	.00246
300	.00364
320	.00767
350	.0189
370	.0307
400	.0563
450	.123
500	.219
550	.332
600	.457
650	.571
700	.678
750	.758

TABLE II

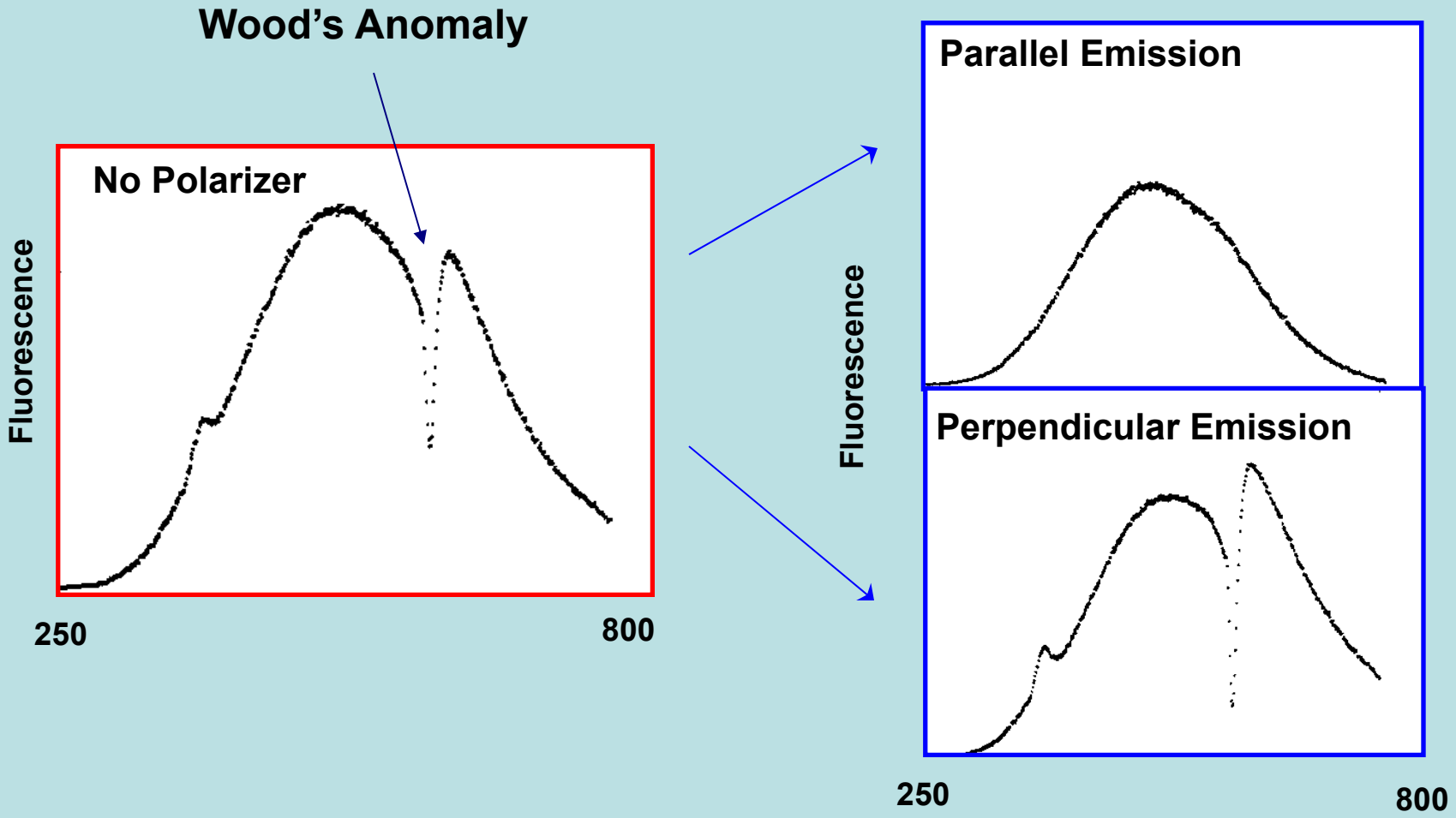
Multiplicative Correction Factors with Emission Polarizer Parallel*

Wavelength (nm)	Factor	Wavelength (nm)	Factor
280	1.119	530	2.548
290	1.111	540	2.837
300	1.111	550	3.185
310	1.096	560	3.541
320	1.081	570	4.007
330	1.052	580	4.511
340	1.037	590	5.067
350	1.022	600	5.711
360	1.015	610	6.400
370	1.007	620	7.348
380	1.015	630	8.563
390	1.015	640	10.022
400	1.037	650	11.830
410	1.067	660	13.874
420	1.111	670	16.637
430	1.148	680	19.748
440	1.207	690	23.778
450	1.267	700	28.593
460	1.363	710	34.630
470	1.452	720	41.481
480	1.570	730	49.993
490	1.711	740	61.274
500	1.859	750	74.200
510	2.074	760	91.133
520	2.296	770	110.407
		780	129.630

*Normalized to 1.000 at 377 nm.

Monochromator Polarization Bias

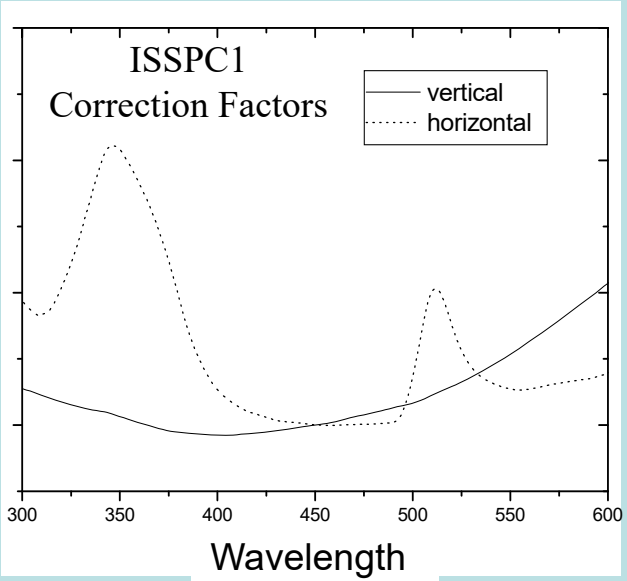
Tungsten Lamp Profile Collected on an SLM Fluorometer

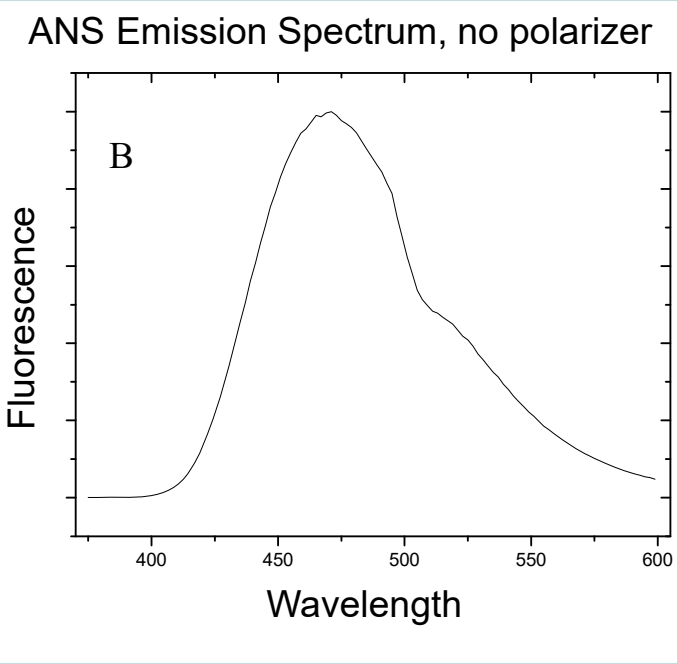


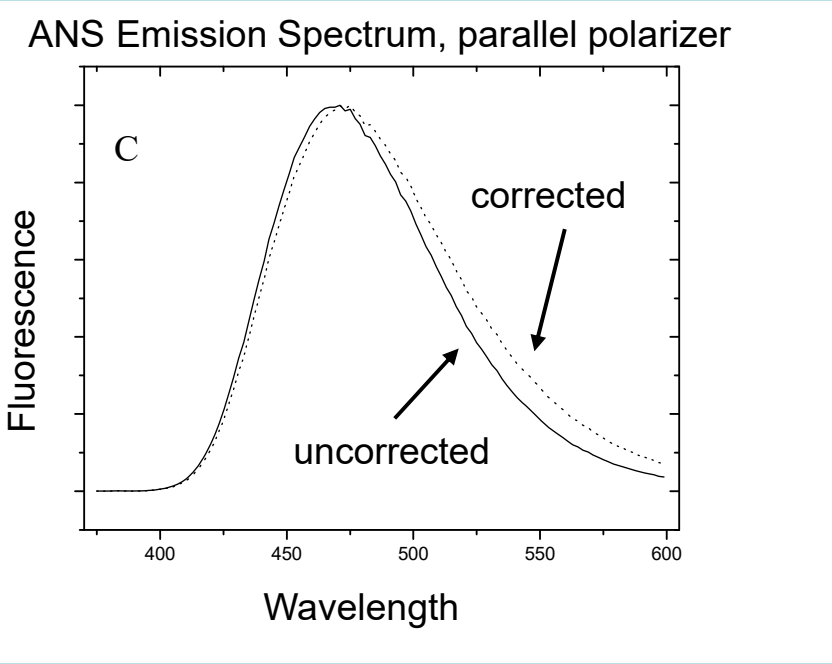
Adapted from Jameson, D.M., Instrumental Refinements in Fluorescence Spectroscopy: Applications to Protein Systems., in Biochemistry, Champaign-Urbana, University of Illinois, 1978.

Correction of Emission Spectra

Note that the correction factors for the horizontal polarizer position must take the Wood's anomaly into account



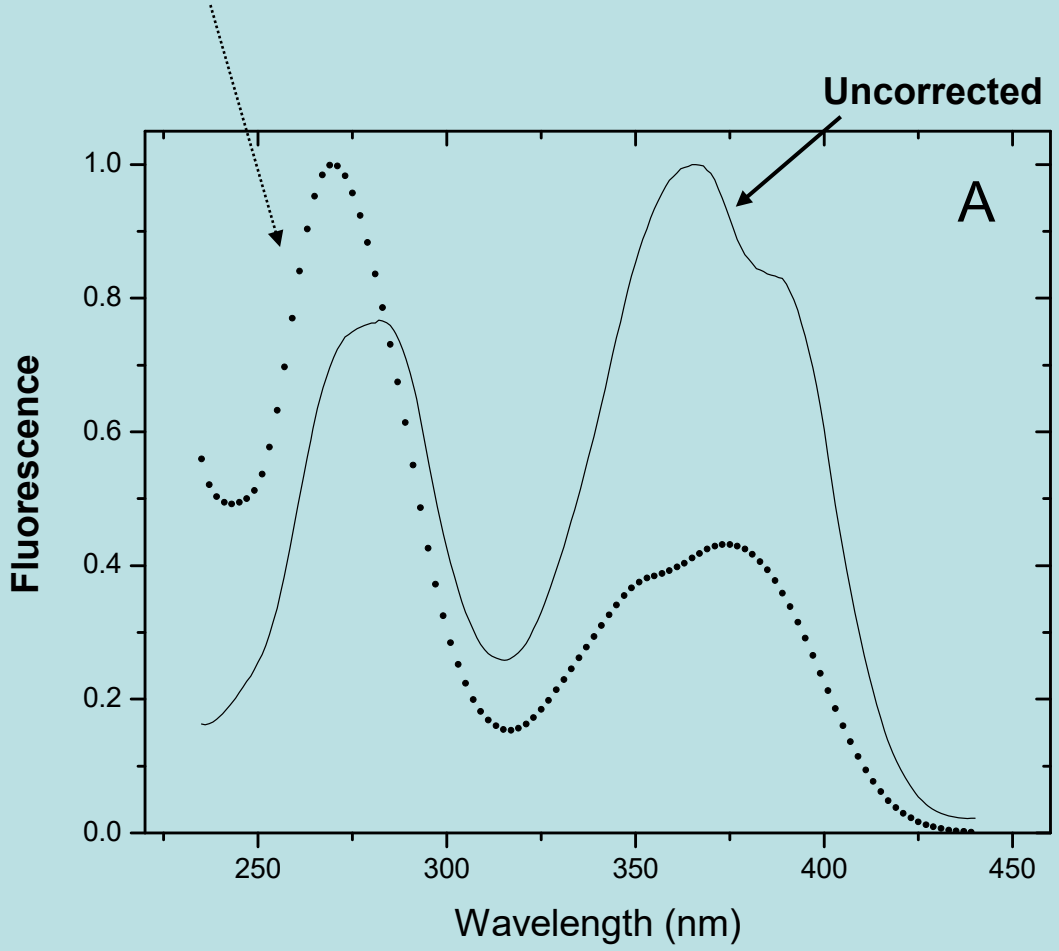




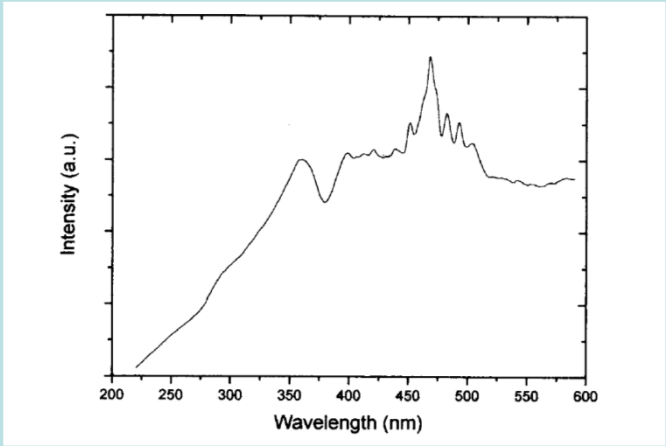
from Jameson et. Al., Methods in Enzymology, 360:1

Excitation Correction

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



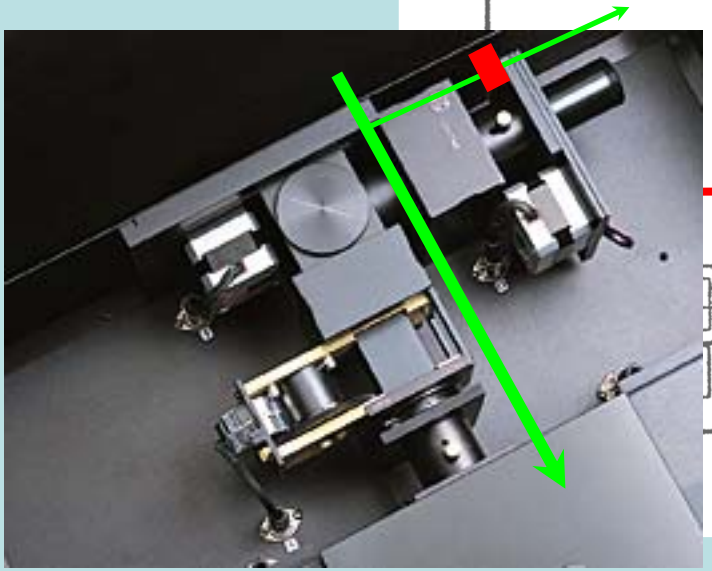
Recall the output of the xenon arc

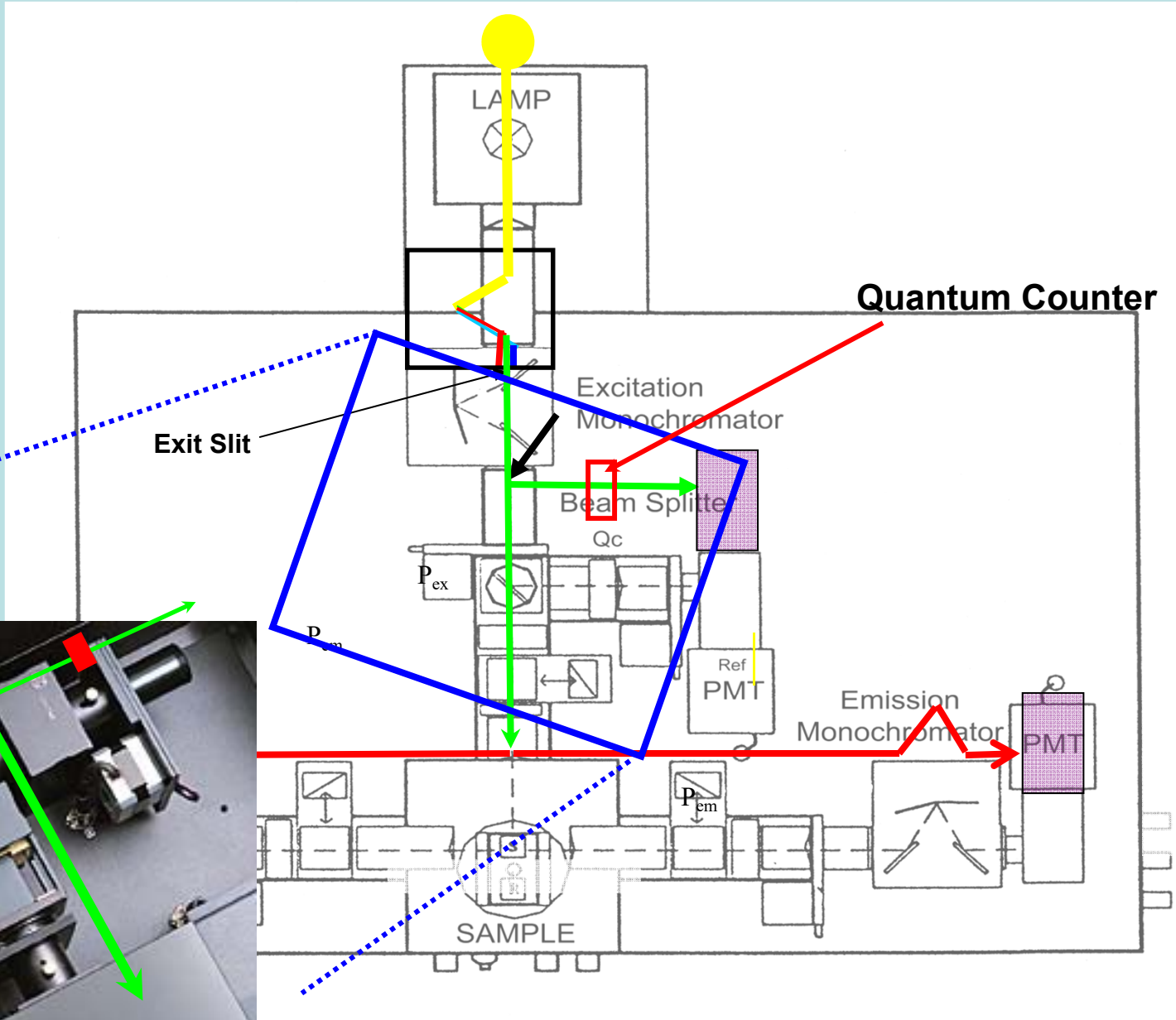


Note the huge difference between the absorption spectrum and the excitation spectrum

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

Excitation Correction

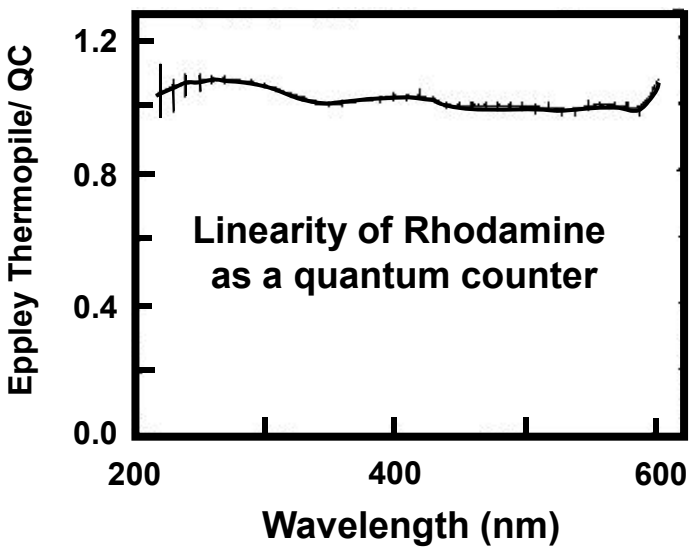


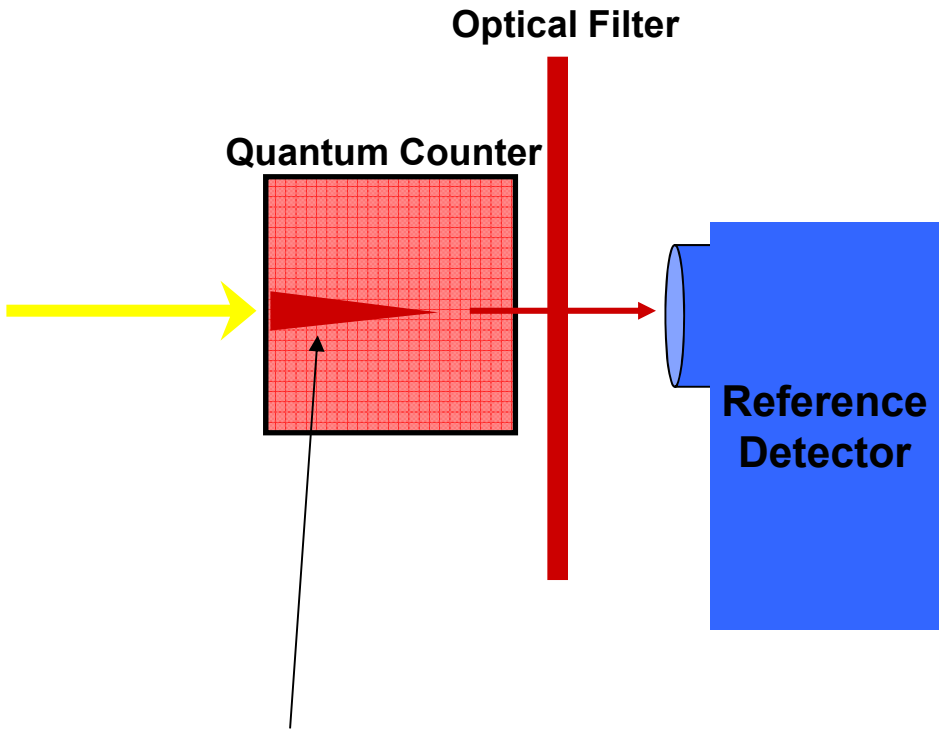


The Instrument Quantum Counter

Common Quantum Counters (optimal range)*

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

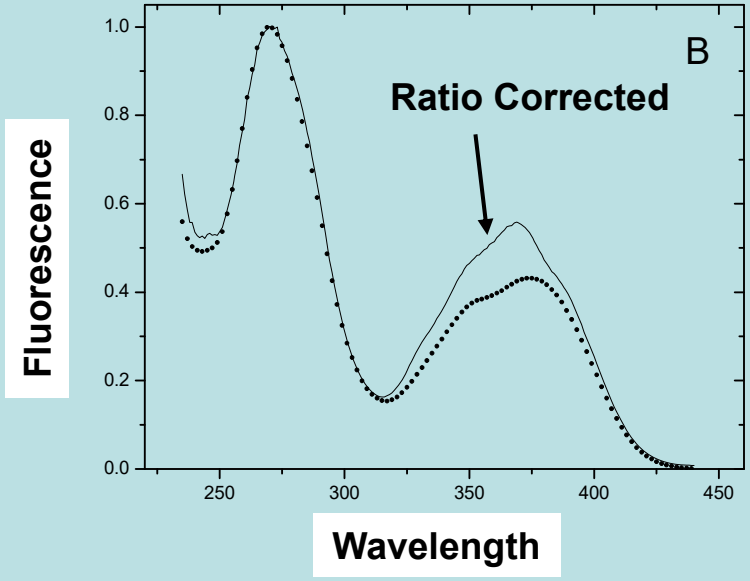




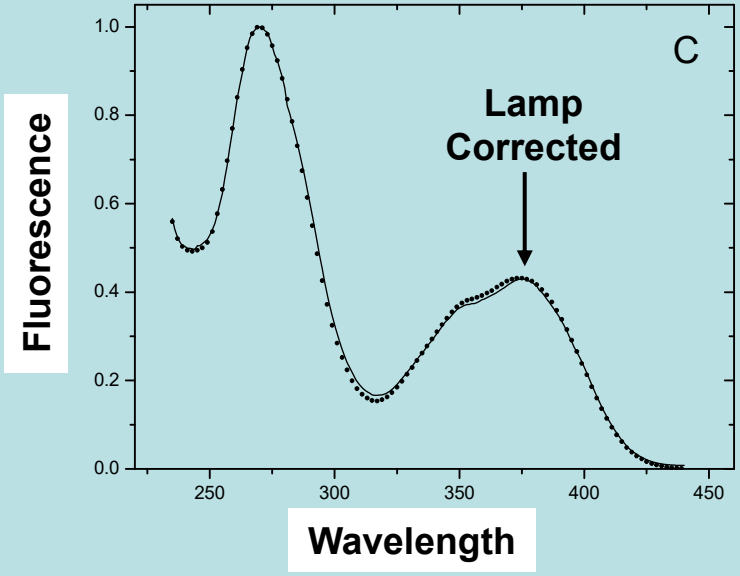
Here we want the inner filter effect!

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

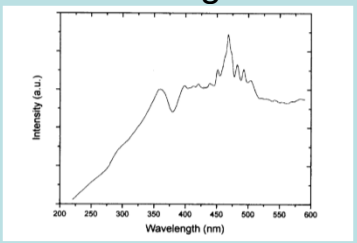
Excitation Correction



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

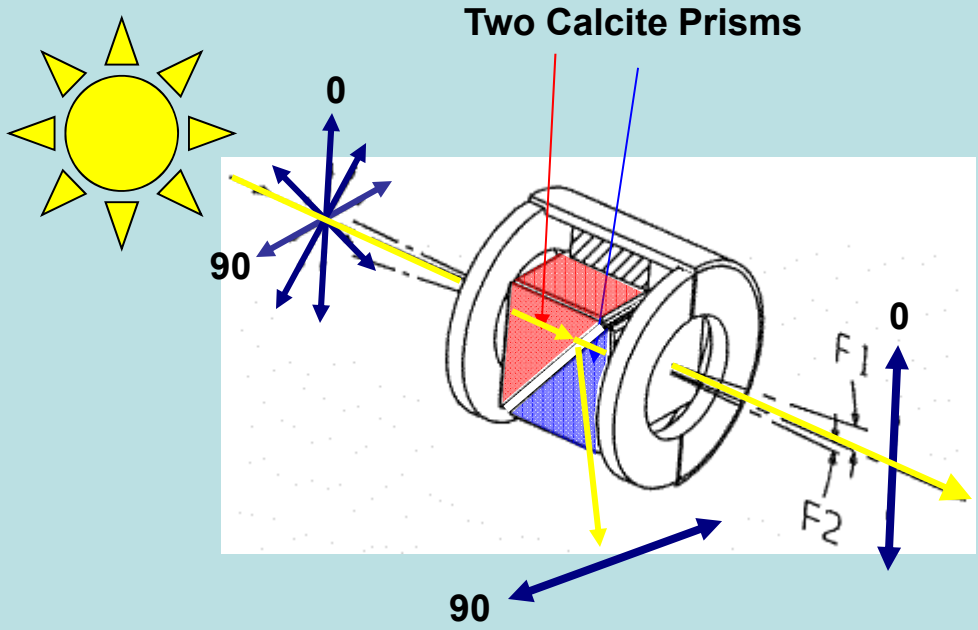
Polarizers

Common Types:

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

Sheet polarizer

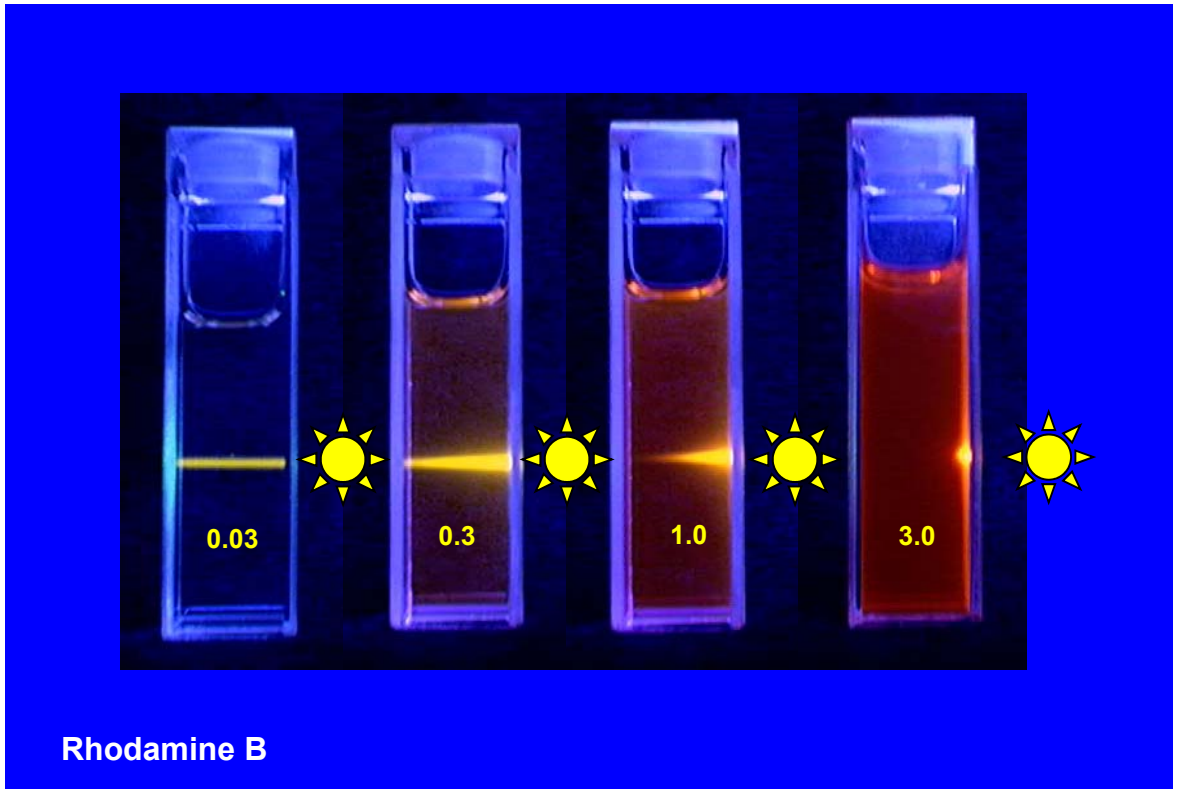
The *Glan Taylor* prism polarizer



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.

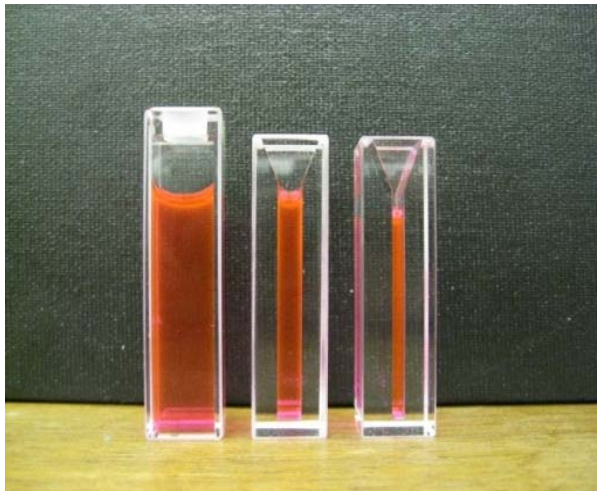
Attenuation of the Excitation Light through Absorbance

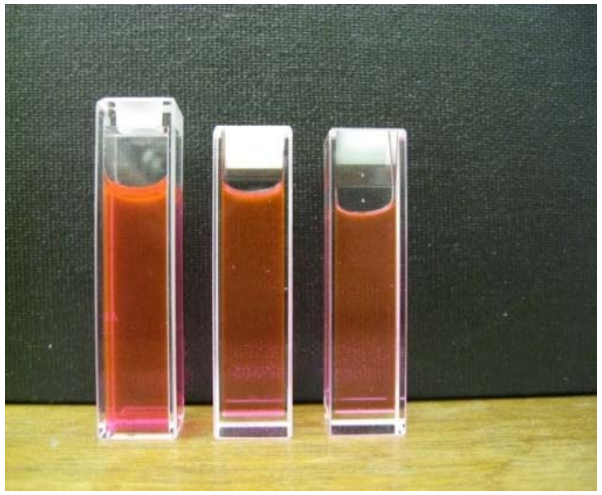
Sample concentration
& the *inner filter effect*

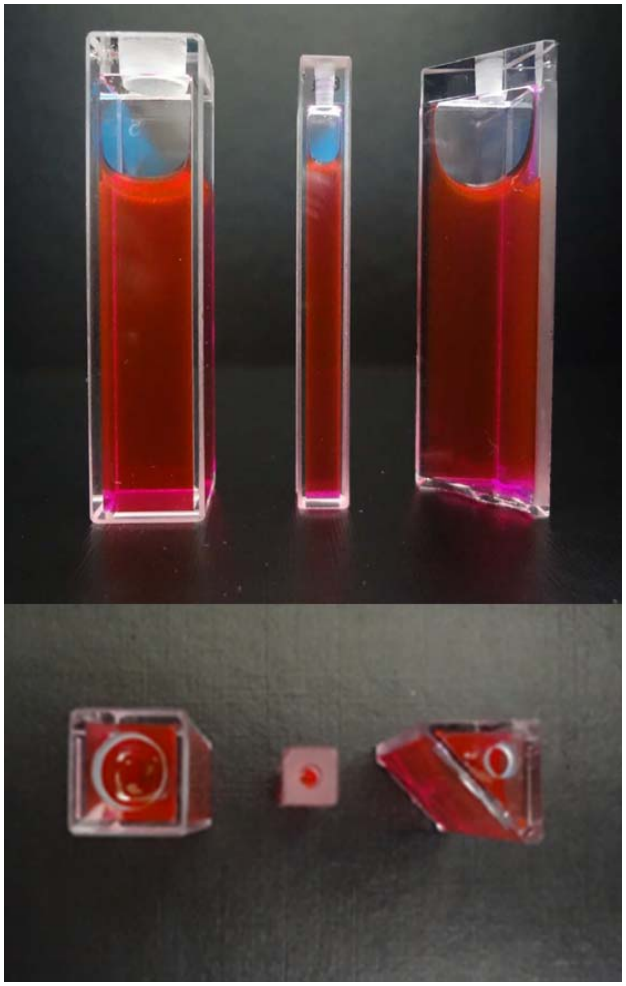


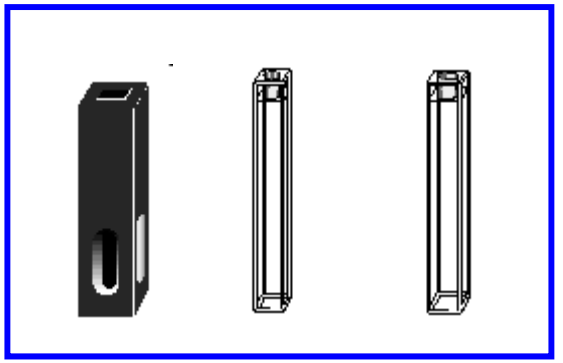
from Jameson et. al., Methods in Enzymology (2002), 360:1

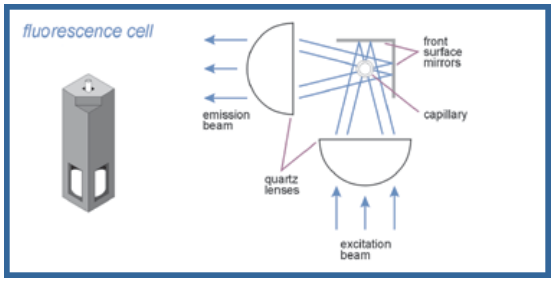
How do we handle highly absorbing solutions?





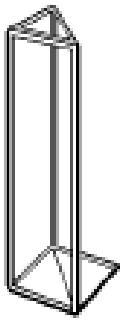


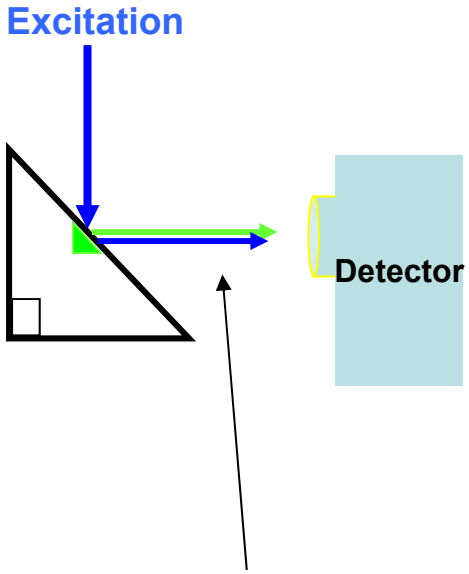




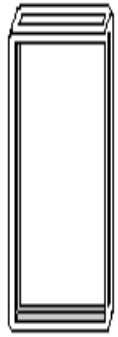
Front Face Detection

Triangular Cells

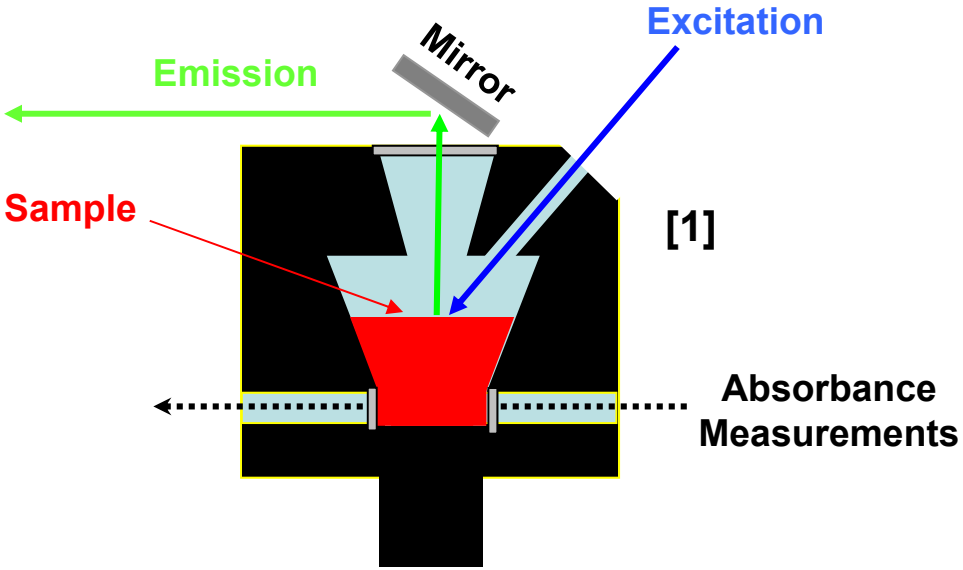




Thin Cells & Special Compartments

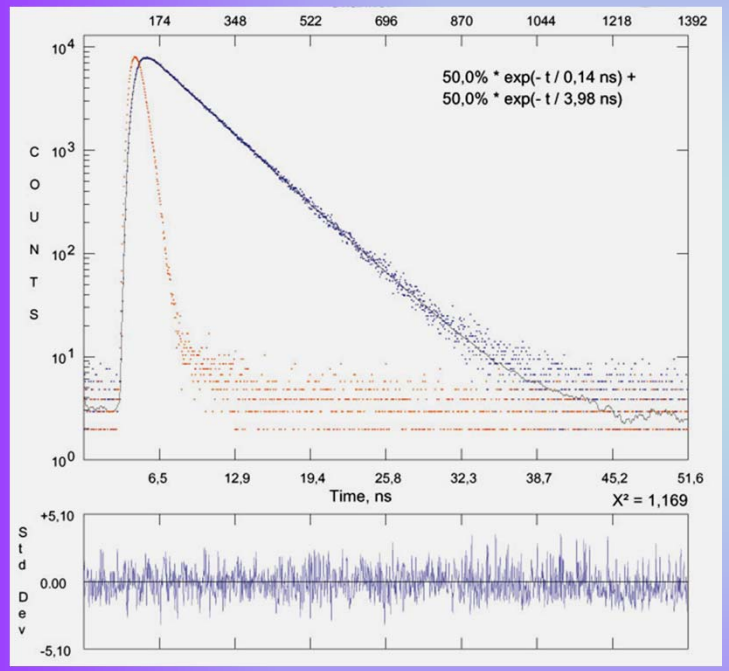


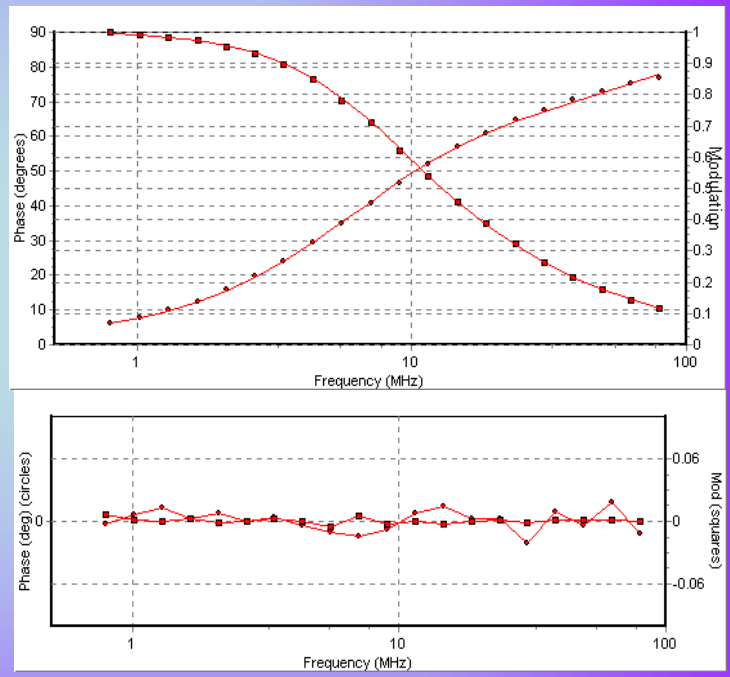
IBH, Glasgow G3 8JU
United Kingdom



[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

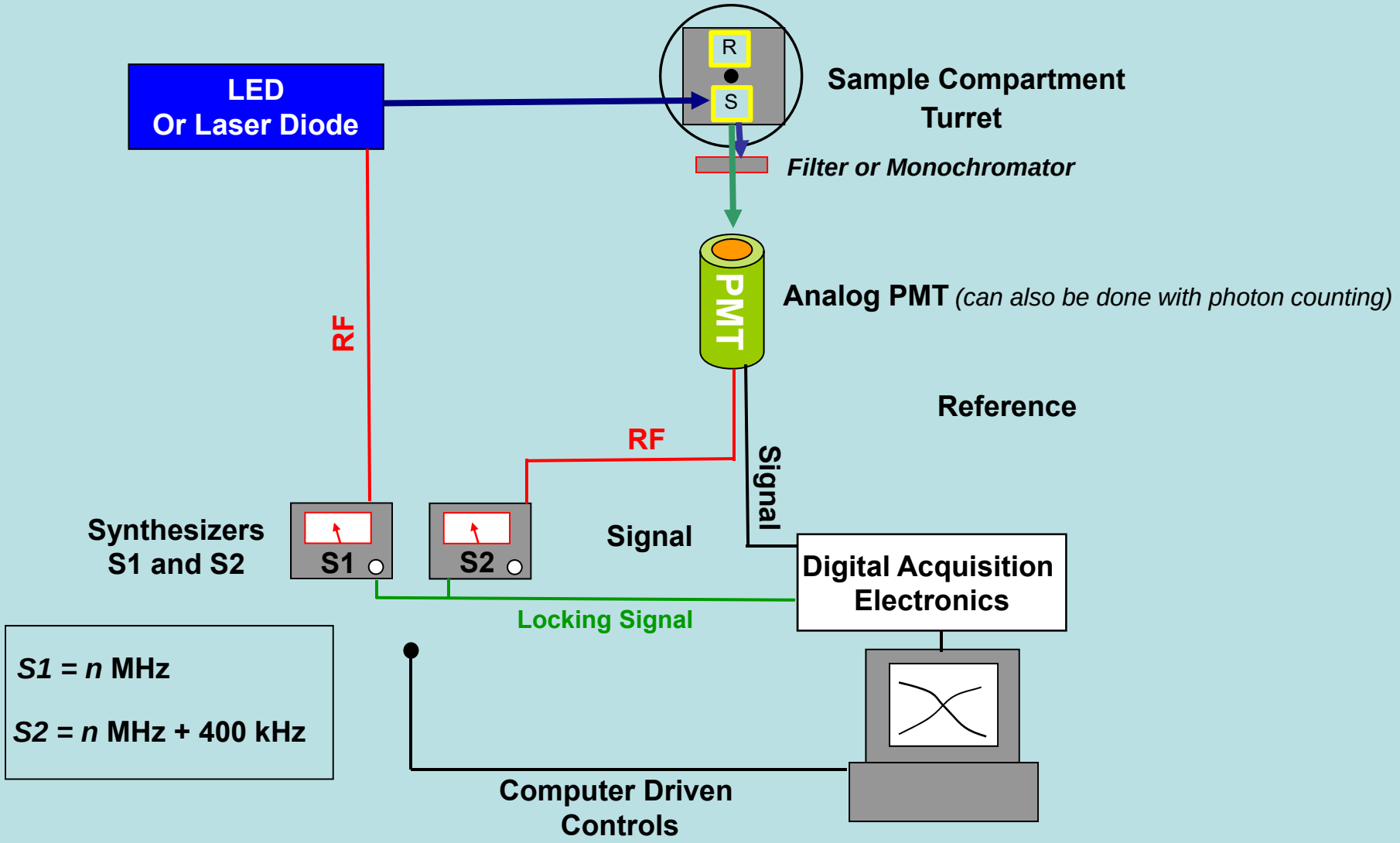
Directly Modulated Light Sources

- Diode Lasers (short pulses in ps range, & can be modulated by synthesizer)
- LEDs (directly modulated via synthesizer, 1 ns, 20 MHz)
- Synchrotron Radiation

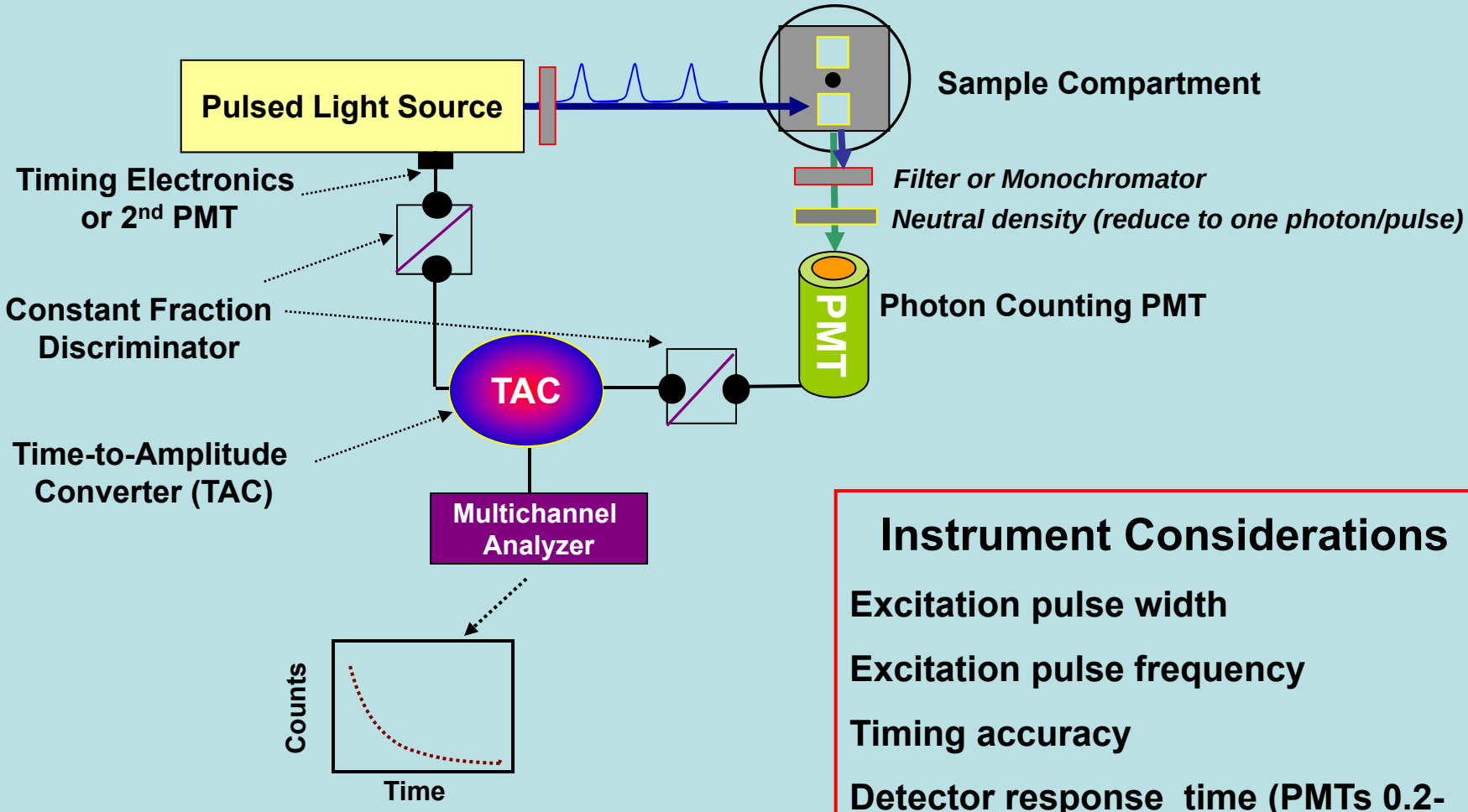
Flash Lamps

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

Traditional Frequency Domain Fluorometry



Time Correlated Single Photon Counting



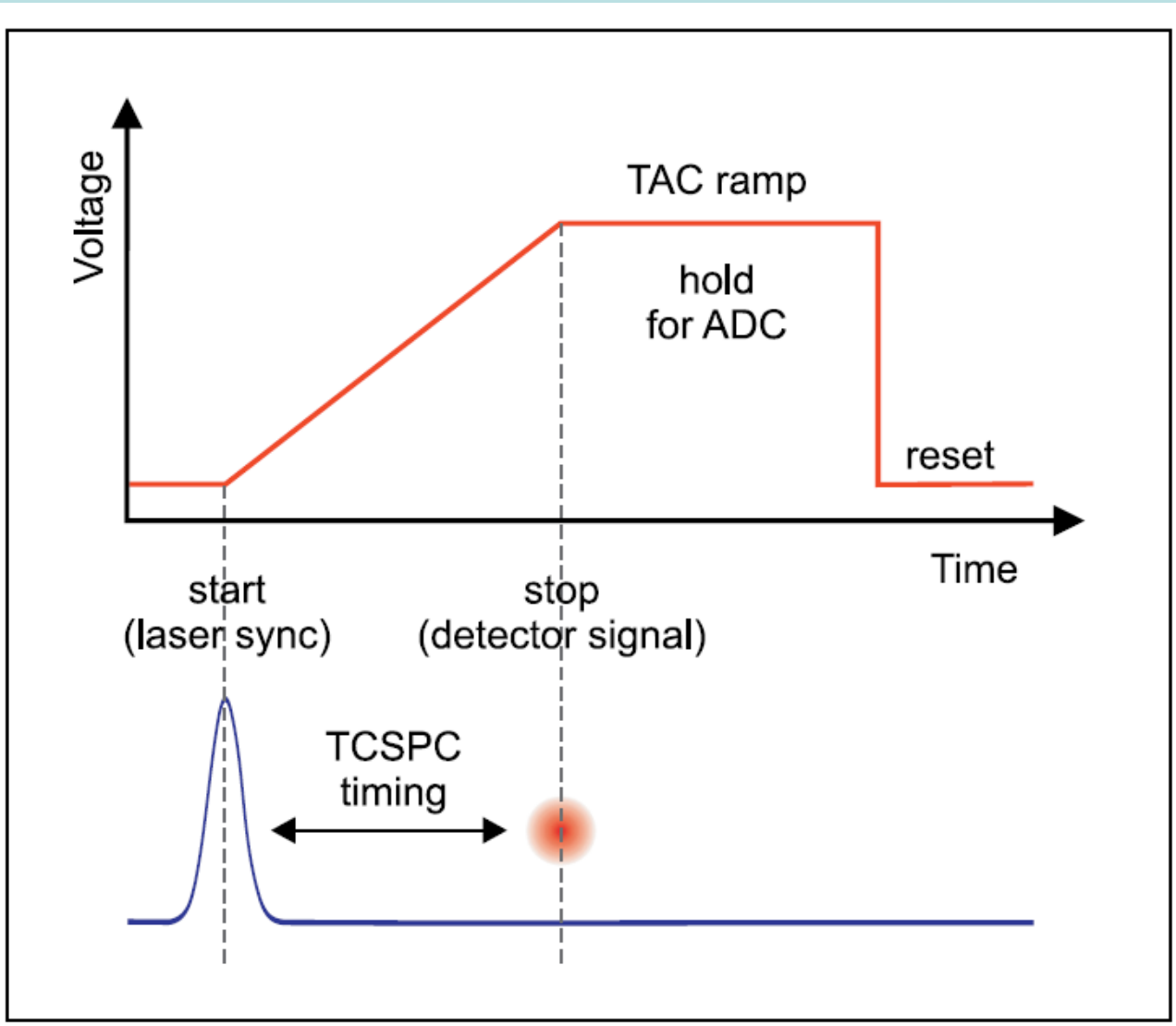
Instrument Considerations

Excitation pulse width

Excitation pulse frequency

Timing accuracy

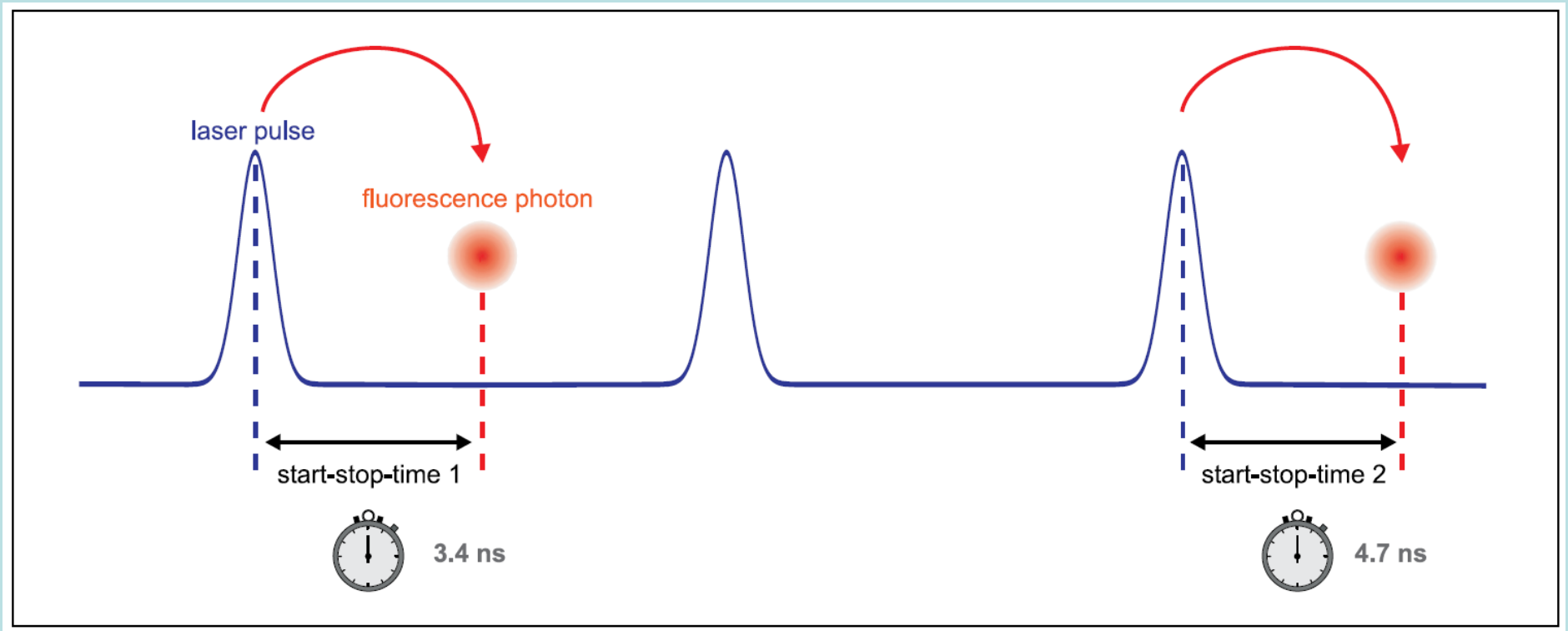
Detector response time (PMTs 0.2-0.9; MCP 0.15 to 0.03 ns)



Time-Correlated Single Photon Counting

Michael Wahl

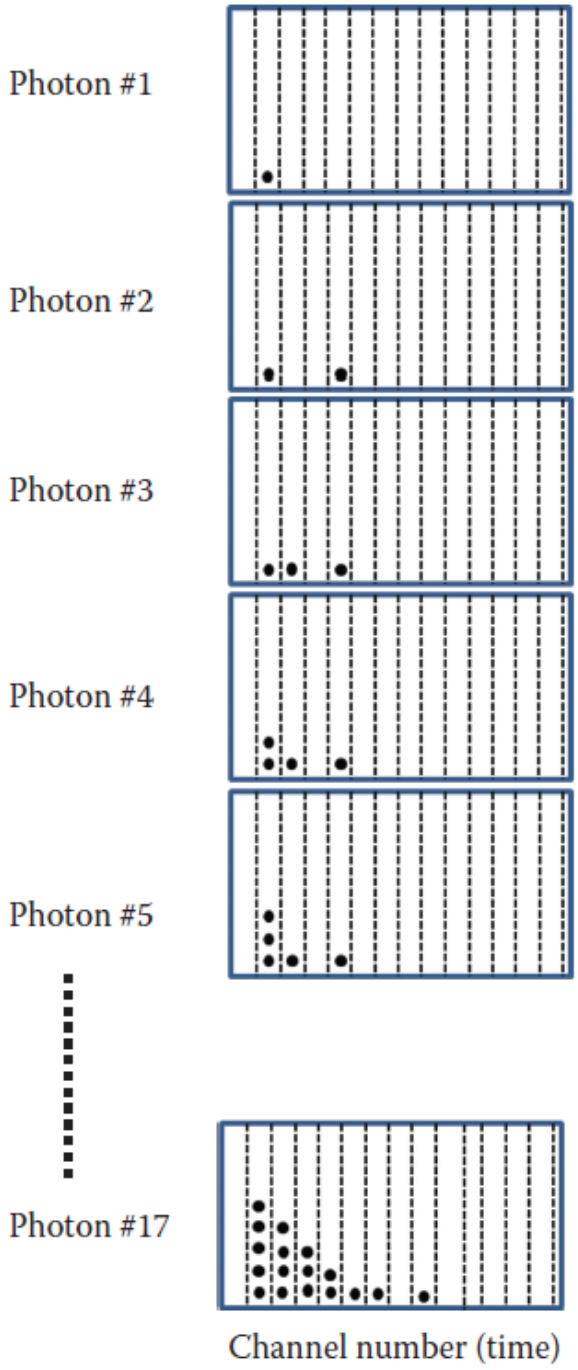
PicoQuant GmbH, Rudower Chaussee 29, 12489 Berlin, Germany, info@picoquant.com



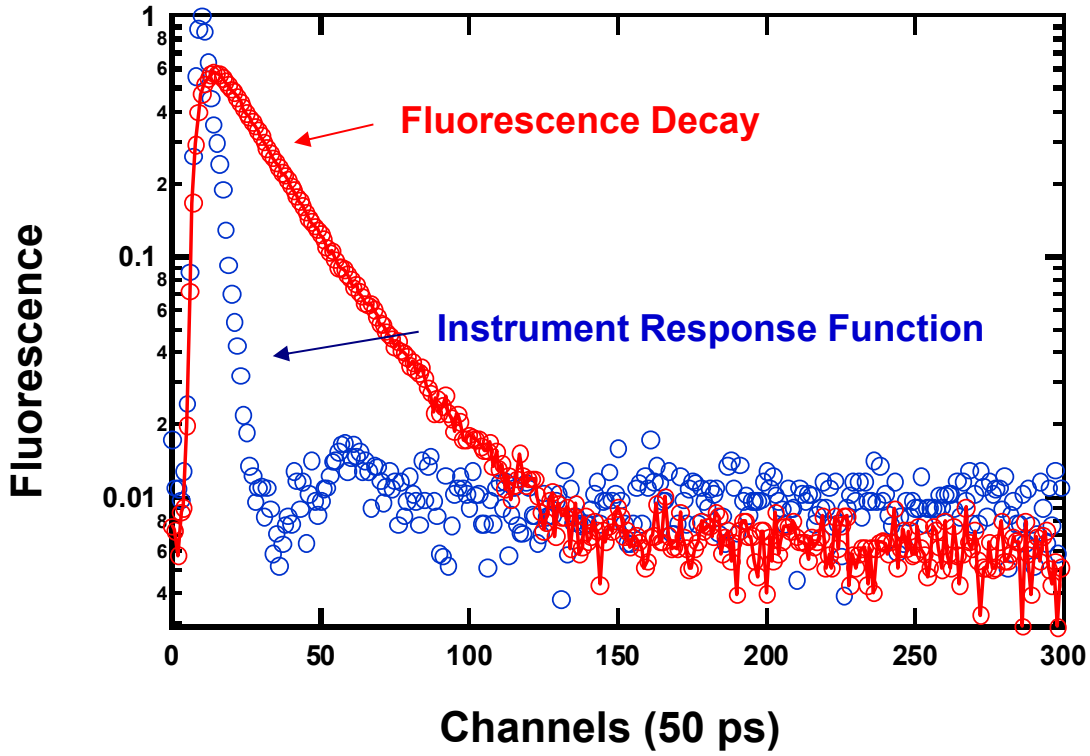
Time-Correlated Single Photon Counting

Michael Wahl

PicoQuant GmbH, Rudower Chaussee 29, 12489 Berlin, Germany, info@picoquant.com


Photon #1
Photon #2
Photon #3
Photon #4
Photon #5
Photon #17
Channel number (time)

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.

That’s all!!!