Basic Instrumentation

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Principles of Fluorescence Spectroscopy
Genova, Italy
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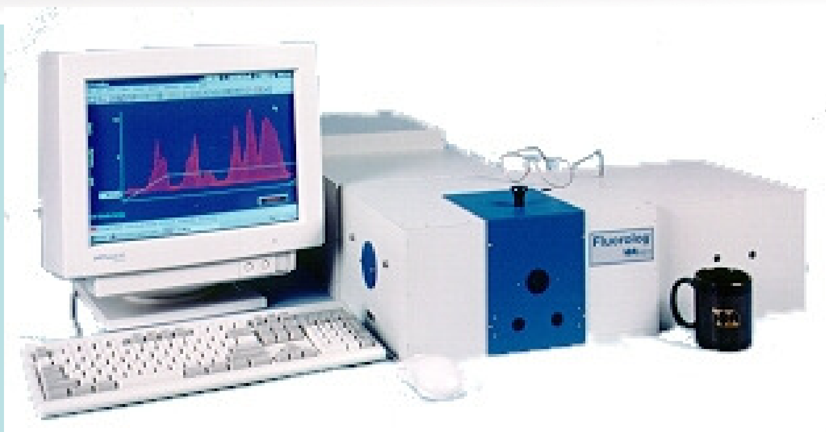
Figure and slide acknowledgements:
Theodore Hazlett

Fluorometer

ISS PC1 (ISS Inc., Champaign, IL, USA)

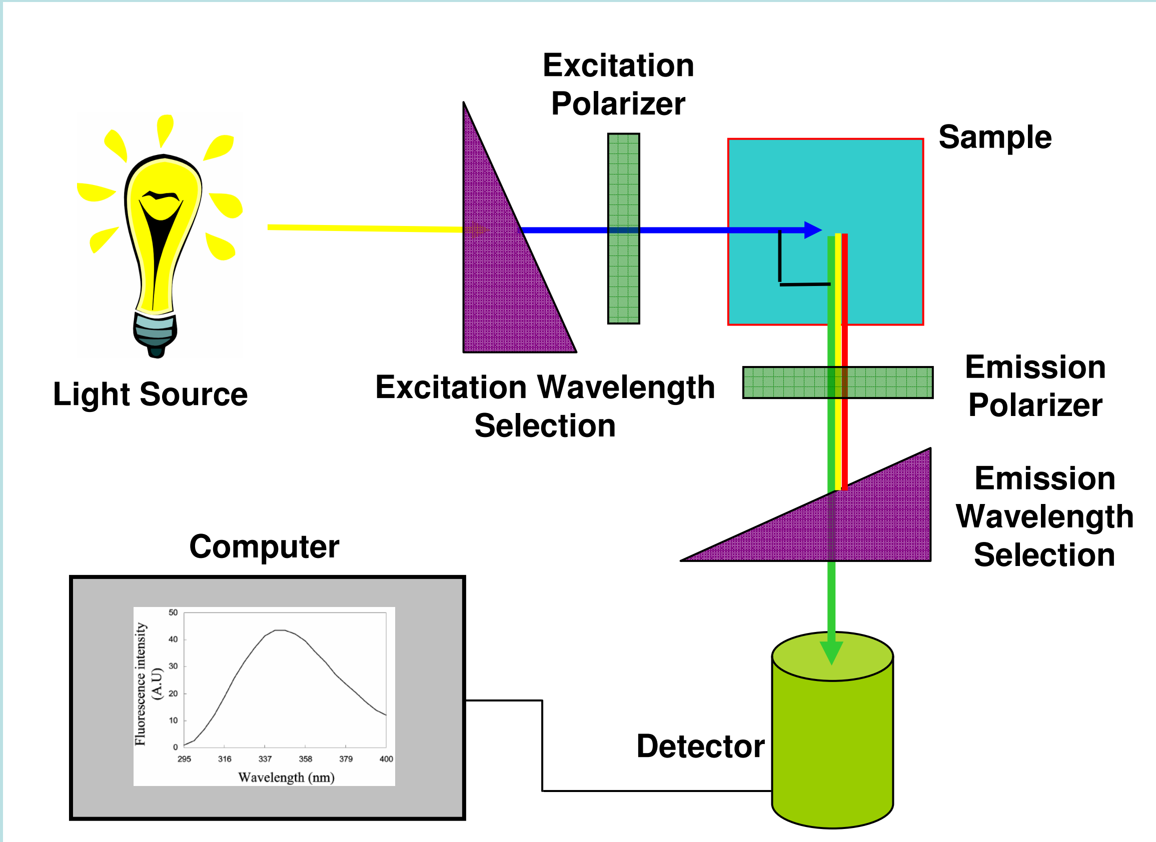


Fluorolog-3 (Jobin Yvon Inc, Edison, NJ, USA)

ISA JOBIN YVON · SPEX HORIBA Group

QuantaMaster (OBB Sales, London, Ontario N6E 2S8)

Fluorometer Components



Note: Both polarizers can be removed from the optical beam path

Fluorometer Components

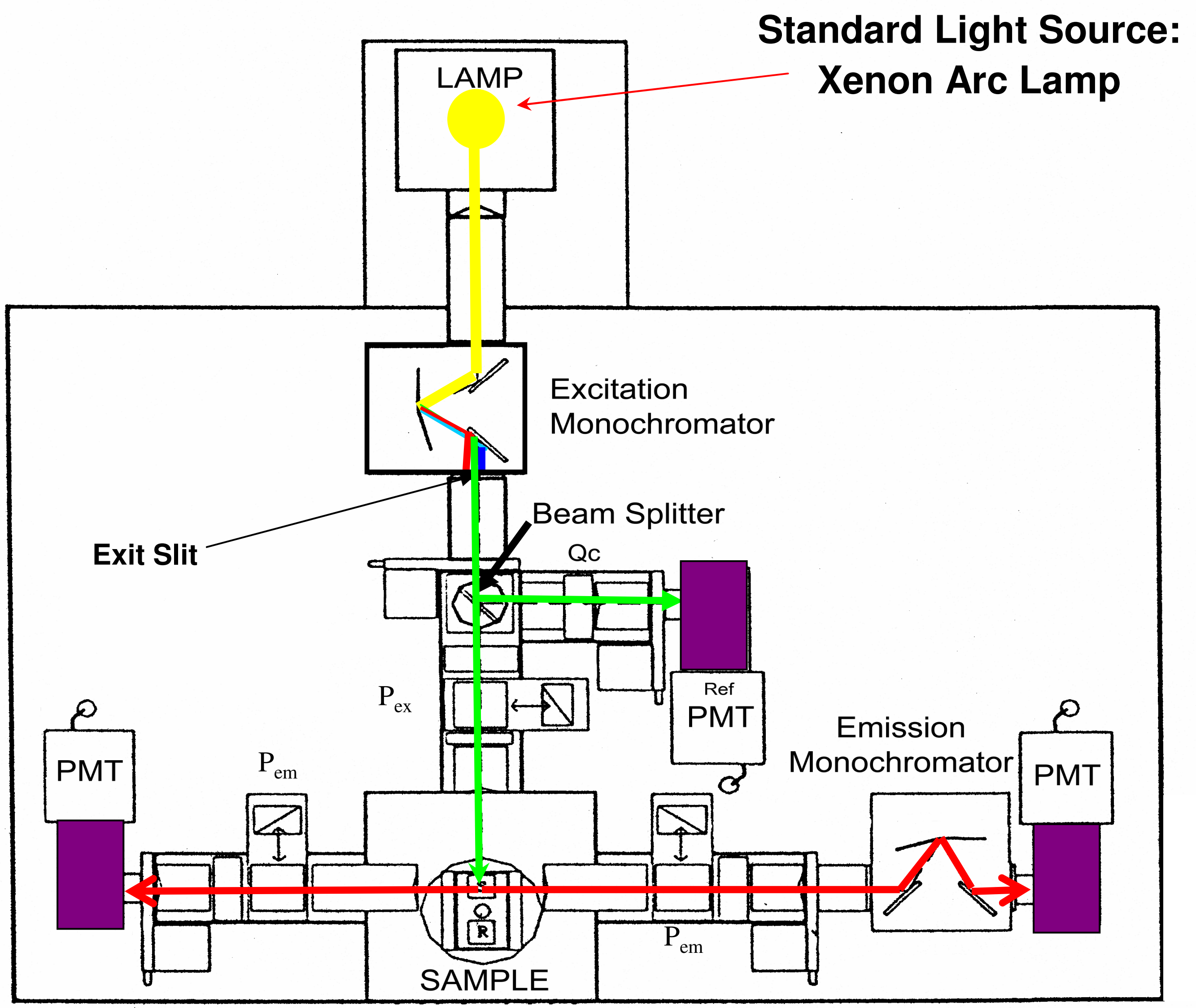
Light Source

Detectors

Wavelength Selection

Polarizers

The Laboratory Fluorometer



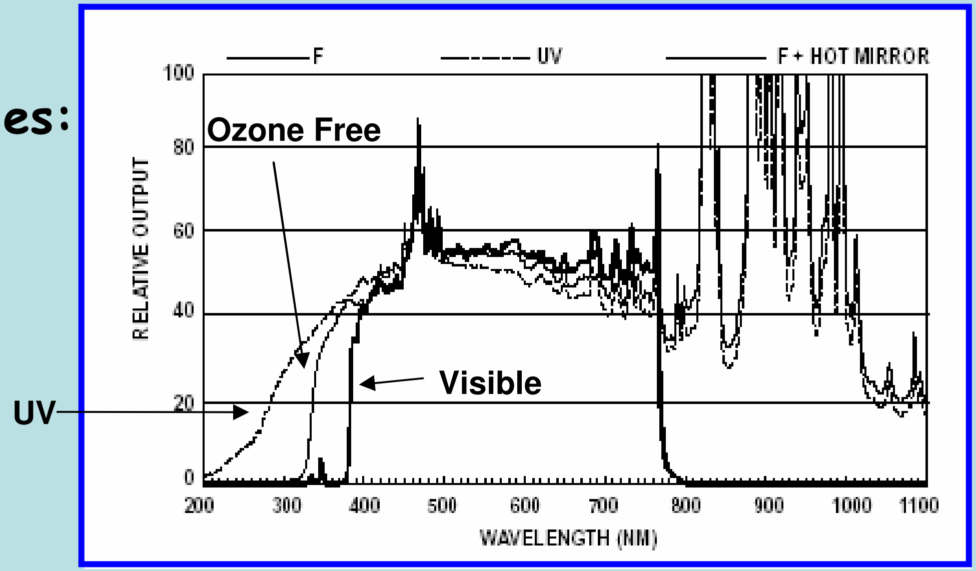
ISS (Champaign, IL, USA) PC1 Fluorometer

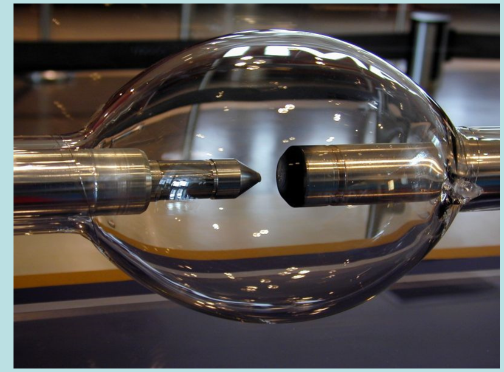
Light Sources

Lamp Light Sources: Arc Lamps

1. Xenon Arc Lamp (wide range of wavelengths)

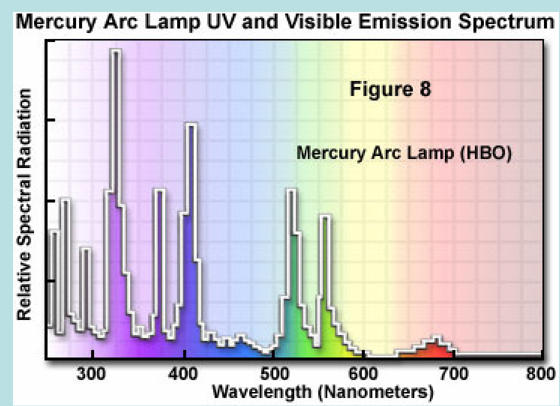
Lamp Profiles:





15 kW Xenon arc lamp

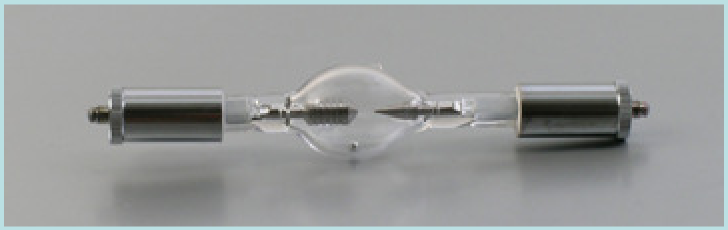
2. High Pressure Mercury Lamps (High Intensities but concentrated in specific lines)

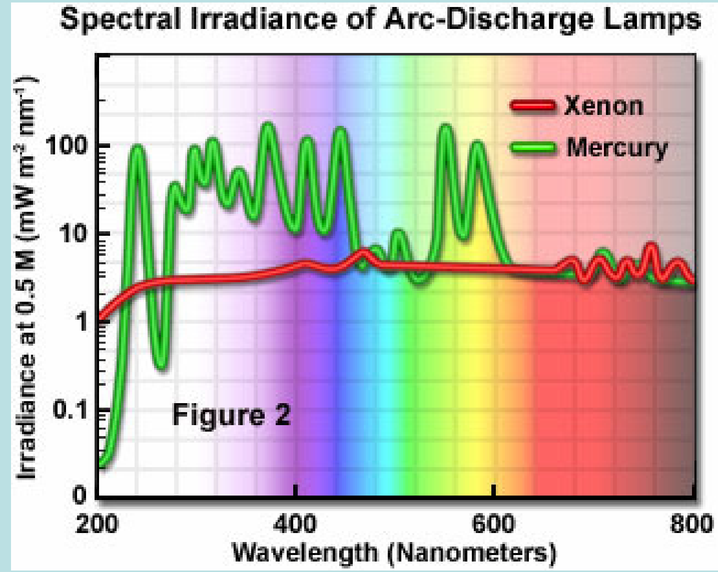


http://microscopy.fsu.edu/primer/anatomy/lightsources

Lamp Light Sources: Arc Lamps

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





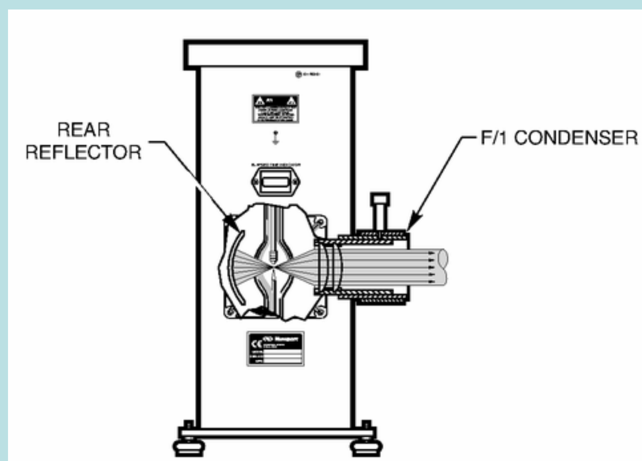
http://microscopy.fsu.edu/primer/anatomy/lightsources

ARC LAMP ISSUES:

- Limited Lifetime
- Stability (flicker + drifts)
- Safety
  - high internal gas pressures
  - hot
  - never stare into burning lamp
  - do not touch with bare hands

LAMP HOUSING + OPTICS :

Conventional OR Compact



Cermax lamp

Lamp Light Sources: Incandescent

4. Tungsten-Halogen Lamps

A Tungsten-Halogen lamp with
a filter to remove UV light.

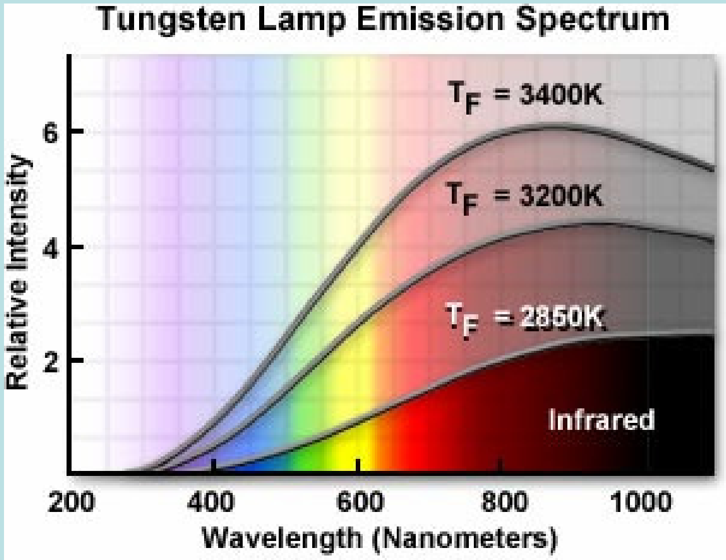
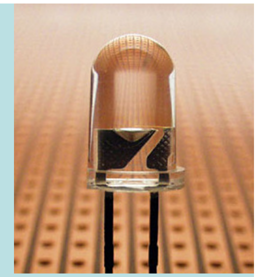


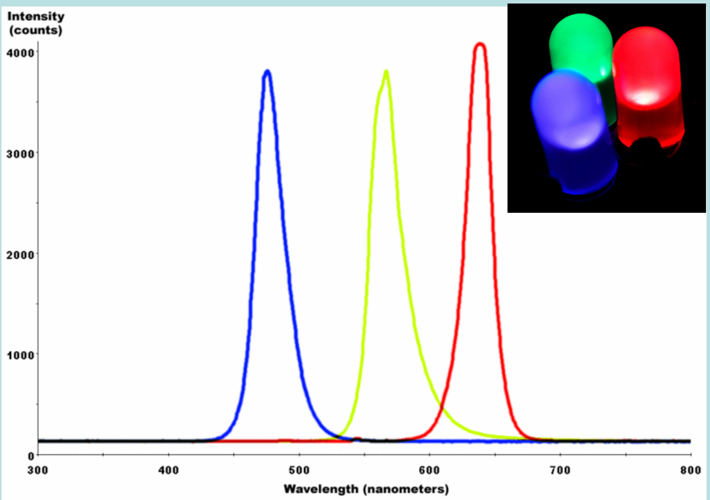
Figure 2

The **color temperature** varies with the applied voltage (average values range from about 2200 K to 3400 K).

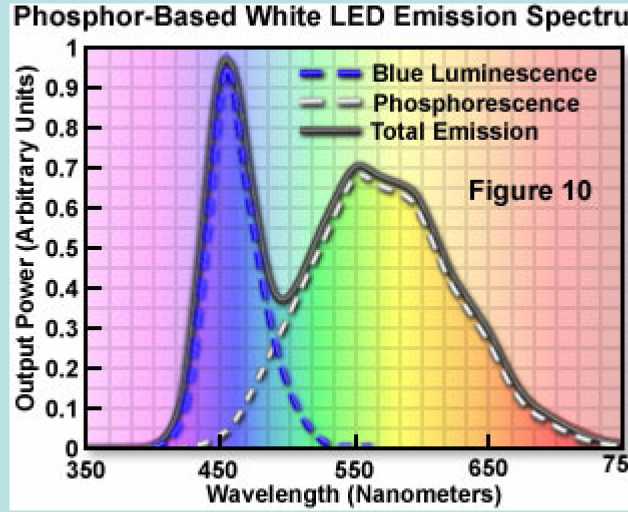
Lamp Light Sources: Semiconductor



5. Light Emitting Diodes (LEDs)



Spectra for blue, yellow-green, and red LEDs. FWHM spectral bandwidth is approximately 25 nm for all three colors.



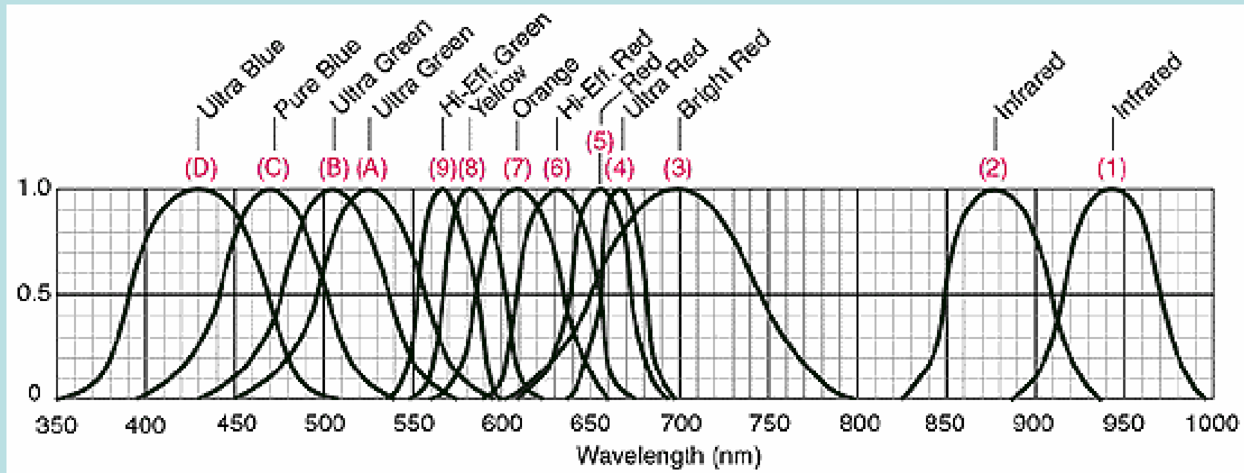
White LED: typical emission spectrum

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

Lamp Light Sources: Semiconductor

5. Light Emitting Diodes (LEDs)

Wavelengths from 260 nm to 2400 nm

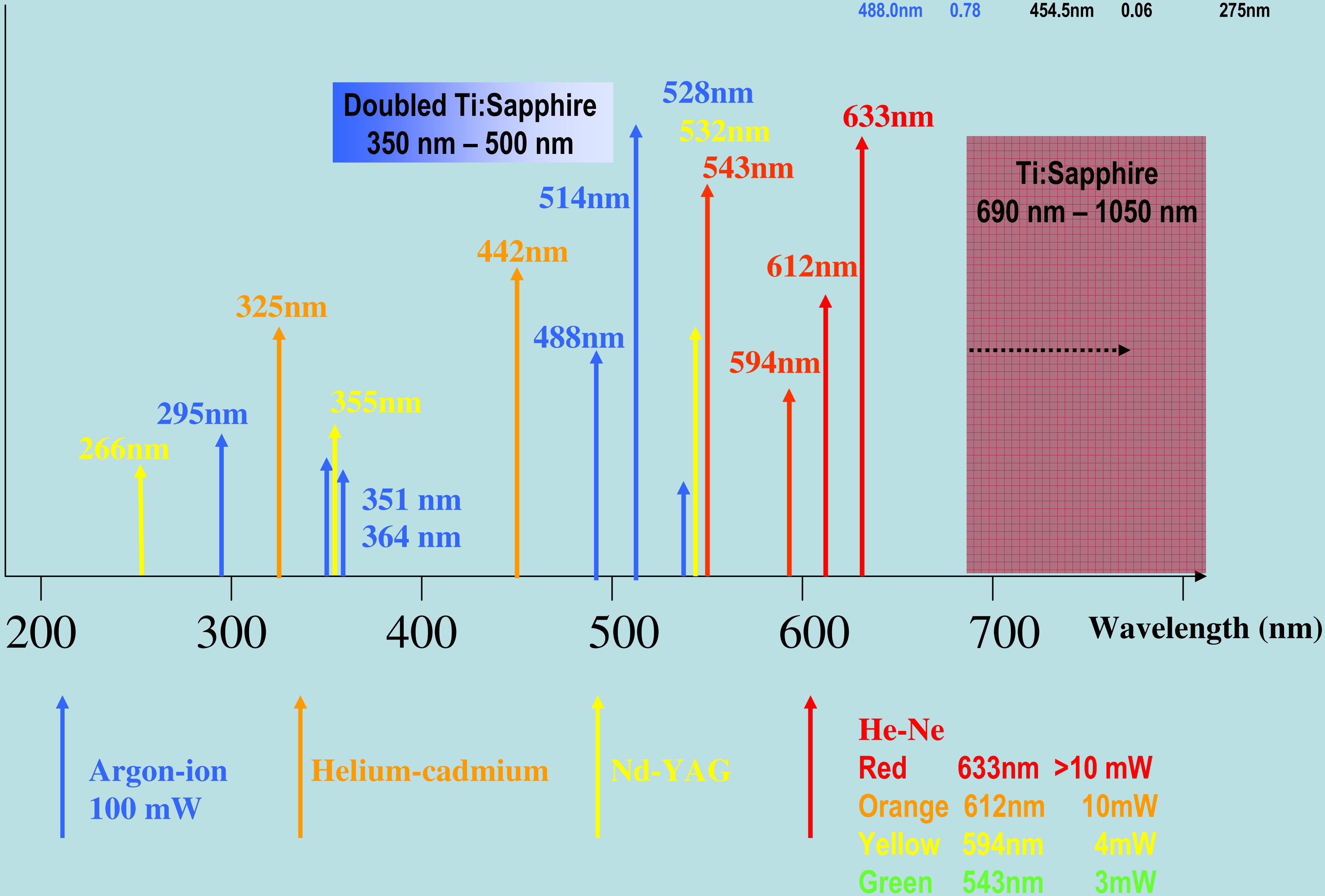


Deep – UV LEDs $\lambda \approx 260 \text{ nm}$

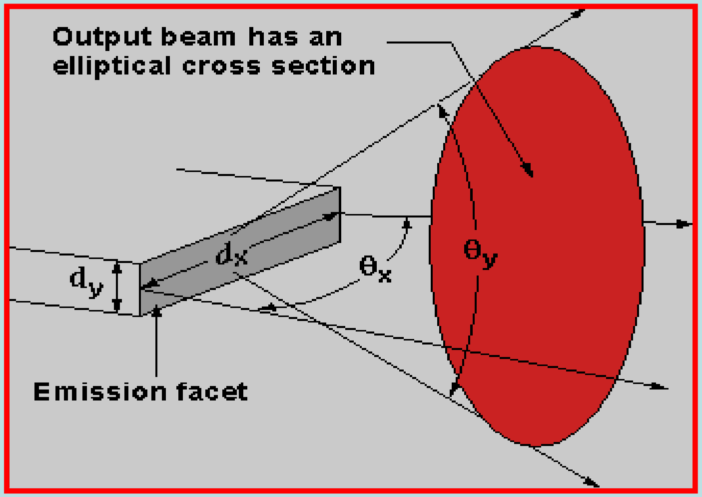
Laser Light Sources

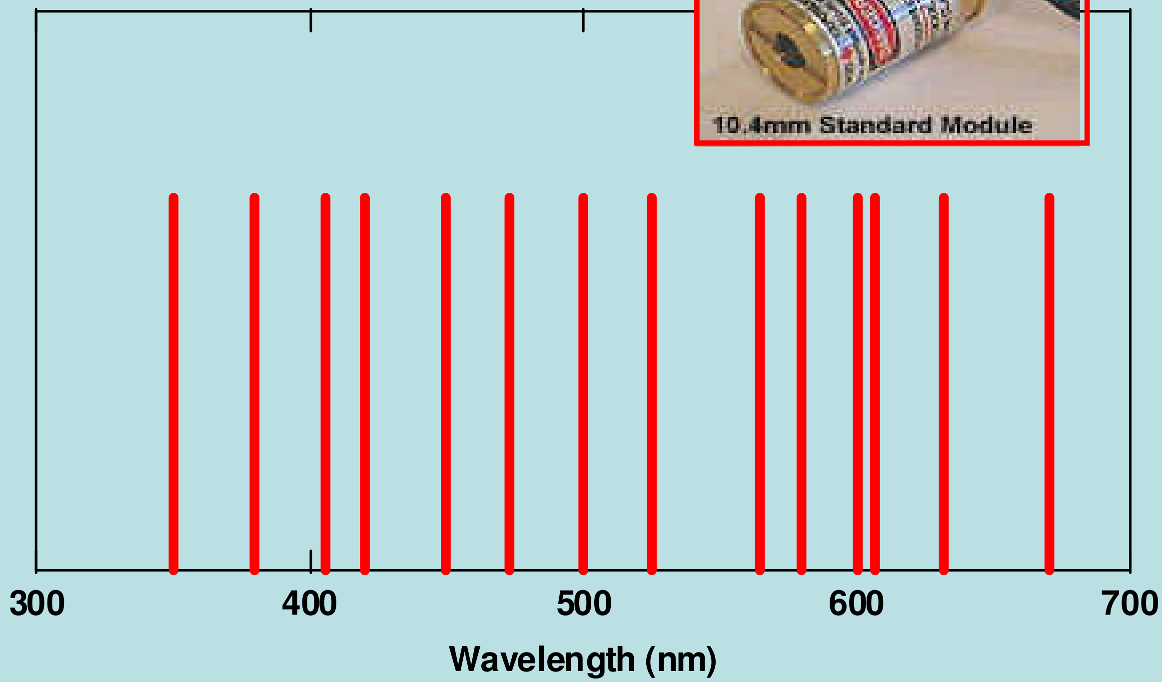
Argon Ion:

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

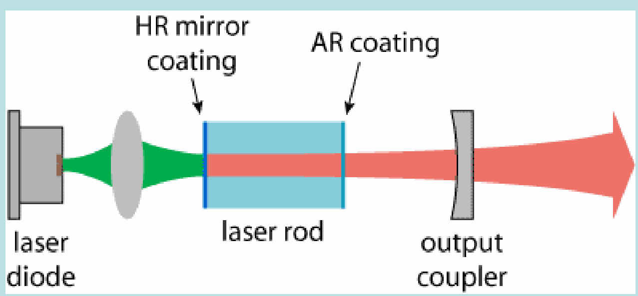


Laser Diodes





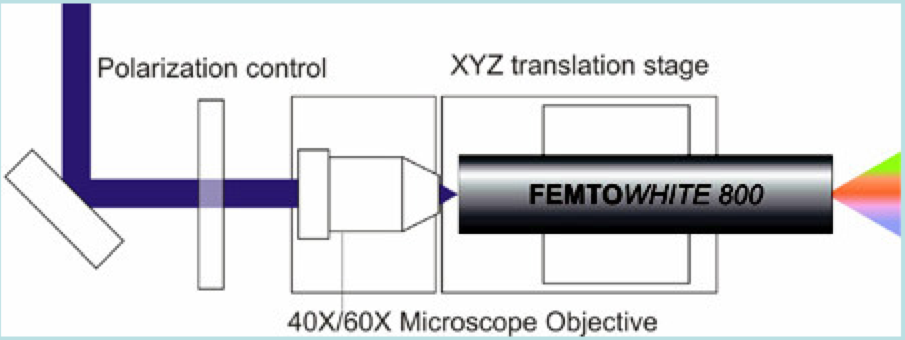
(DPSS) Diode pumped solid state laser



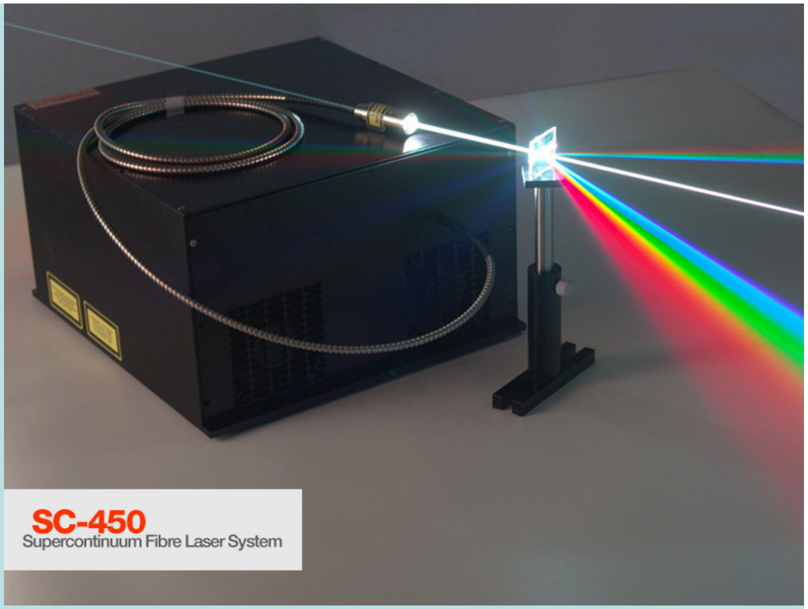
Wavelengths (nm):

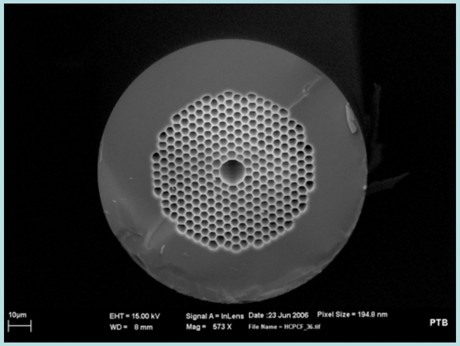
262, 266, 349, 351, 355, 375, 405, 415, 430, 440, 447, 473, 488, 523, 527, 532, 542, 555, 561, 584-593, 638, 655, 658, 671, 685, 785, 808, 852, 946, 980, 1047, 1053, 1064, 1080, 1313-1342, 1444, 1550

Supercontinuum Light

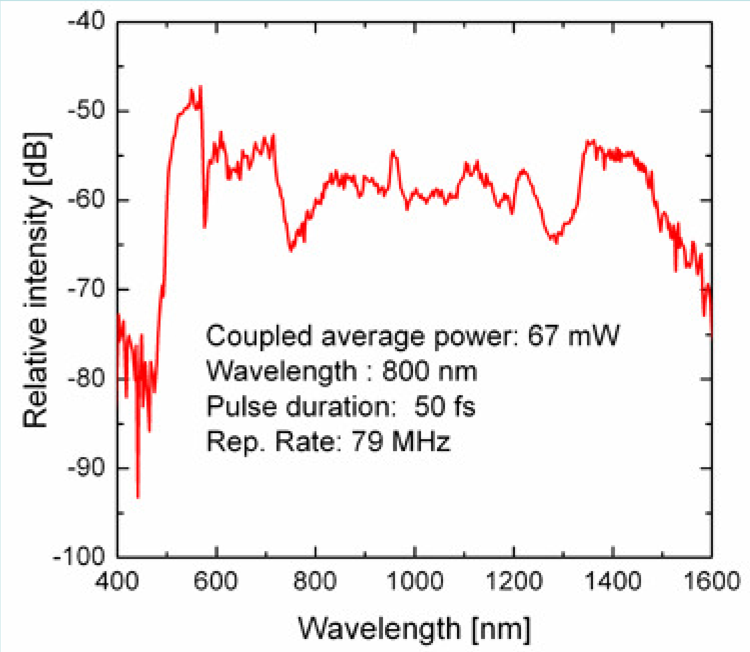


Focus ultrashort pulsed light into photonic crystal fiber

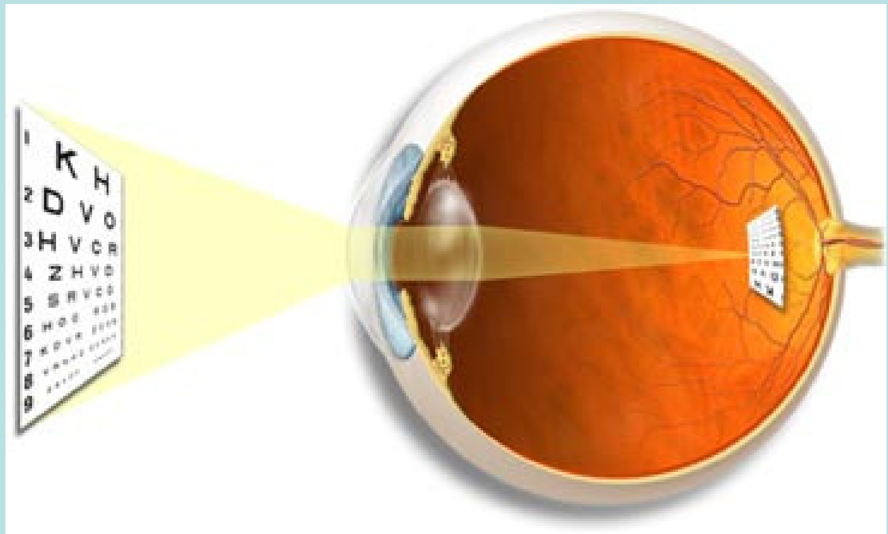




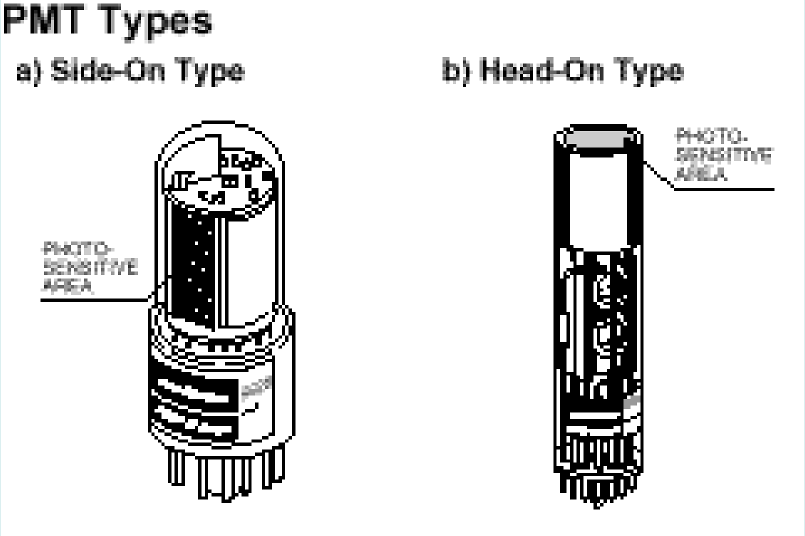
Photonic crystal fiber



Detectors



Photon Multiplier Tube

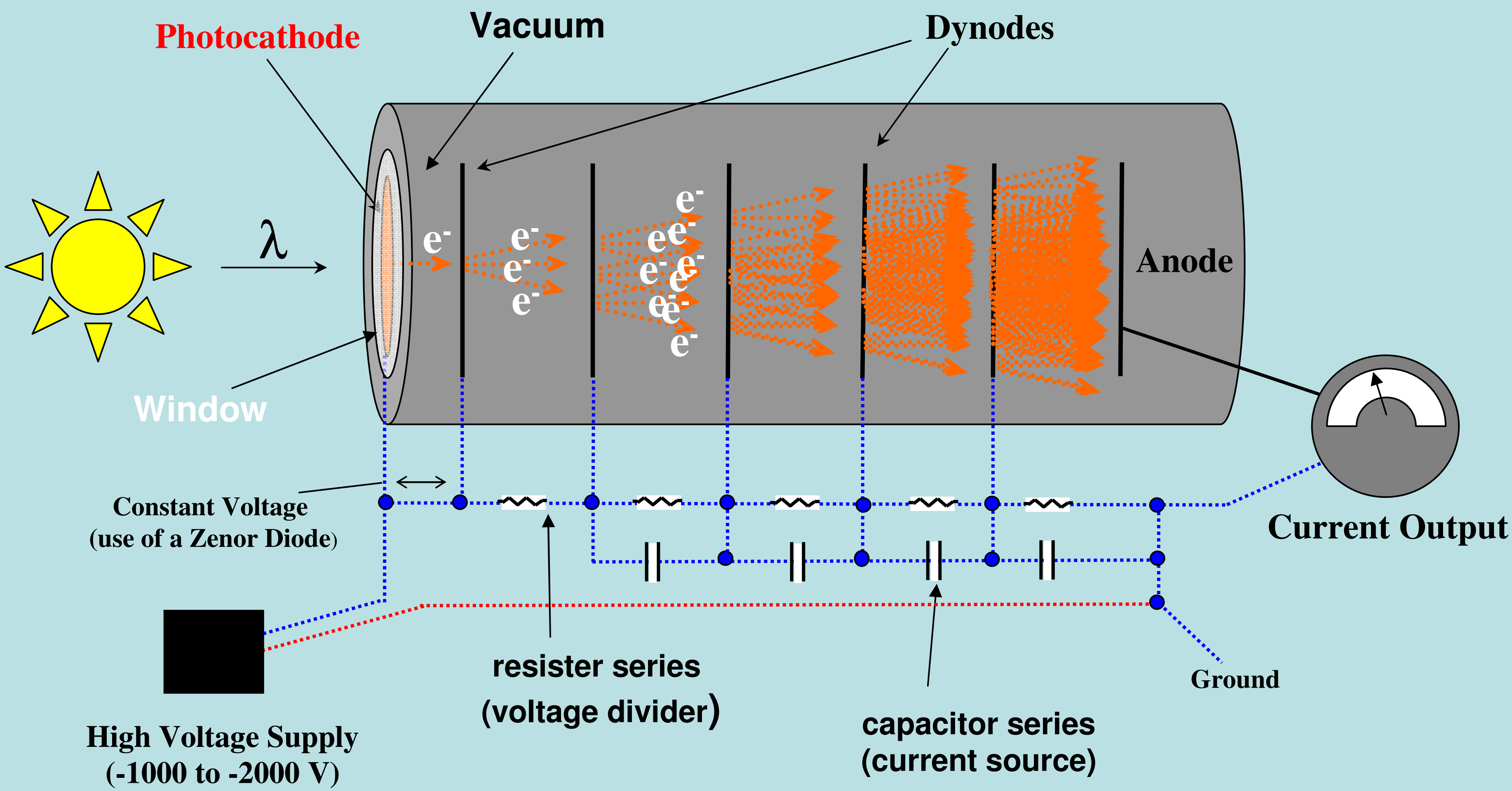


Microchannel Plate Detector (MCP)

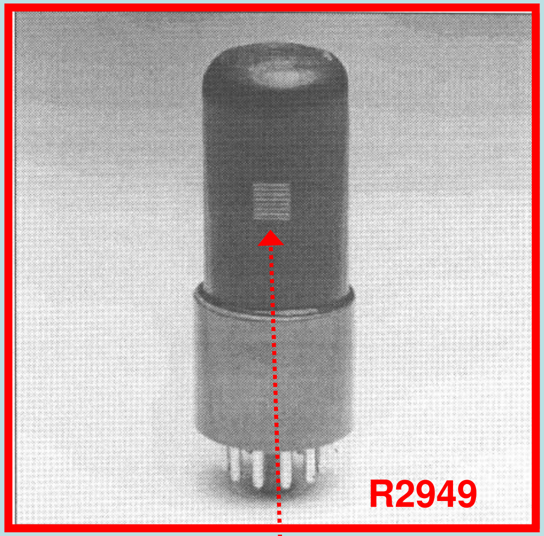
MCP & Electronics
(ISS Inc. Champaign, IL USA)

For fast modulation f > 500 MHz

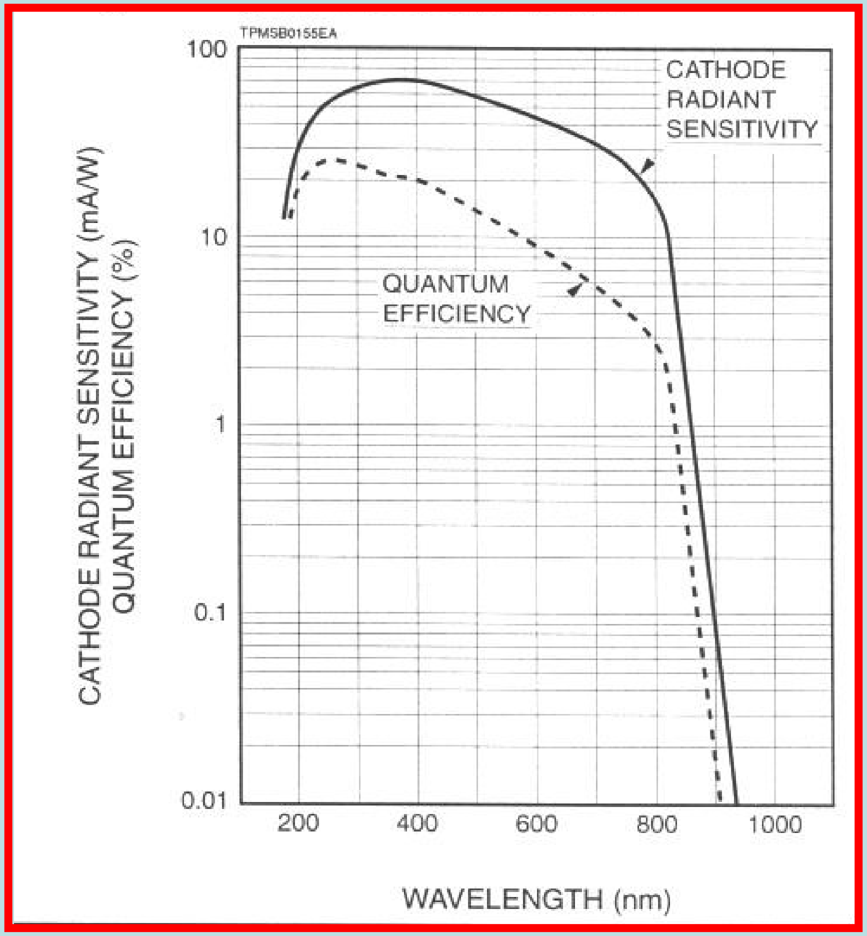
The Classic PMT Design



Hamamatsu R928 PMT Family

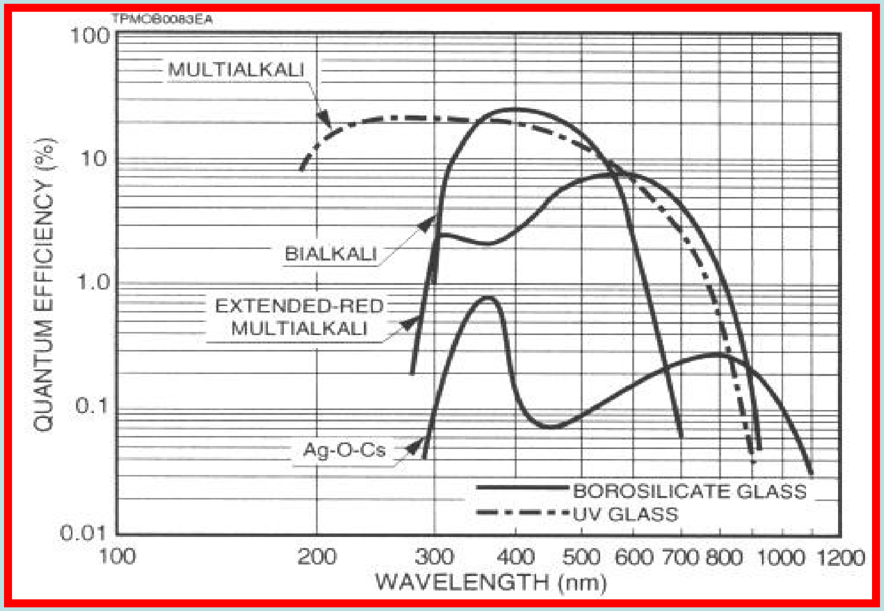


Window with Photocathode Beneath

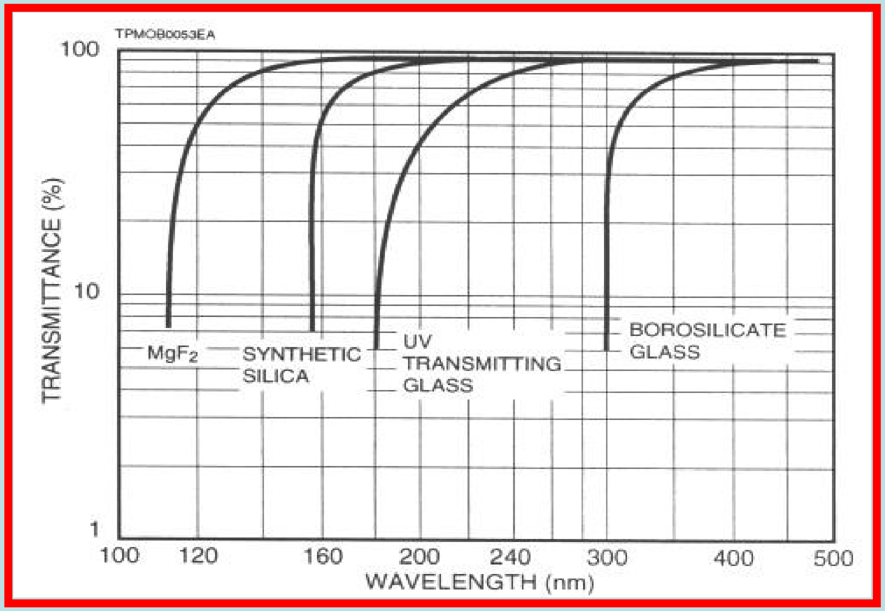


PMT Quantum Efficiencies

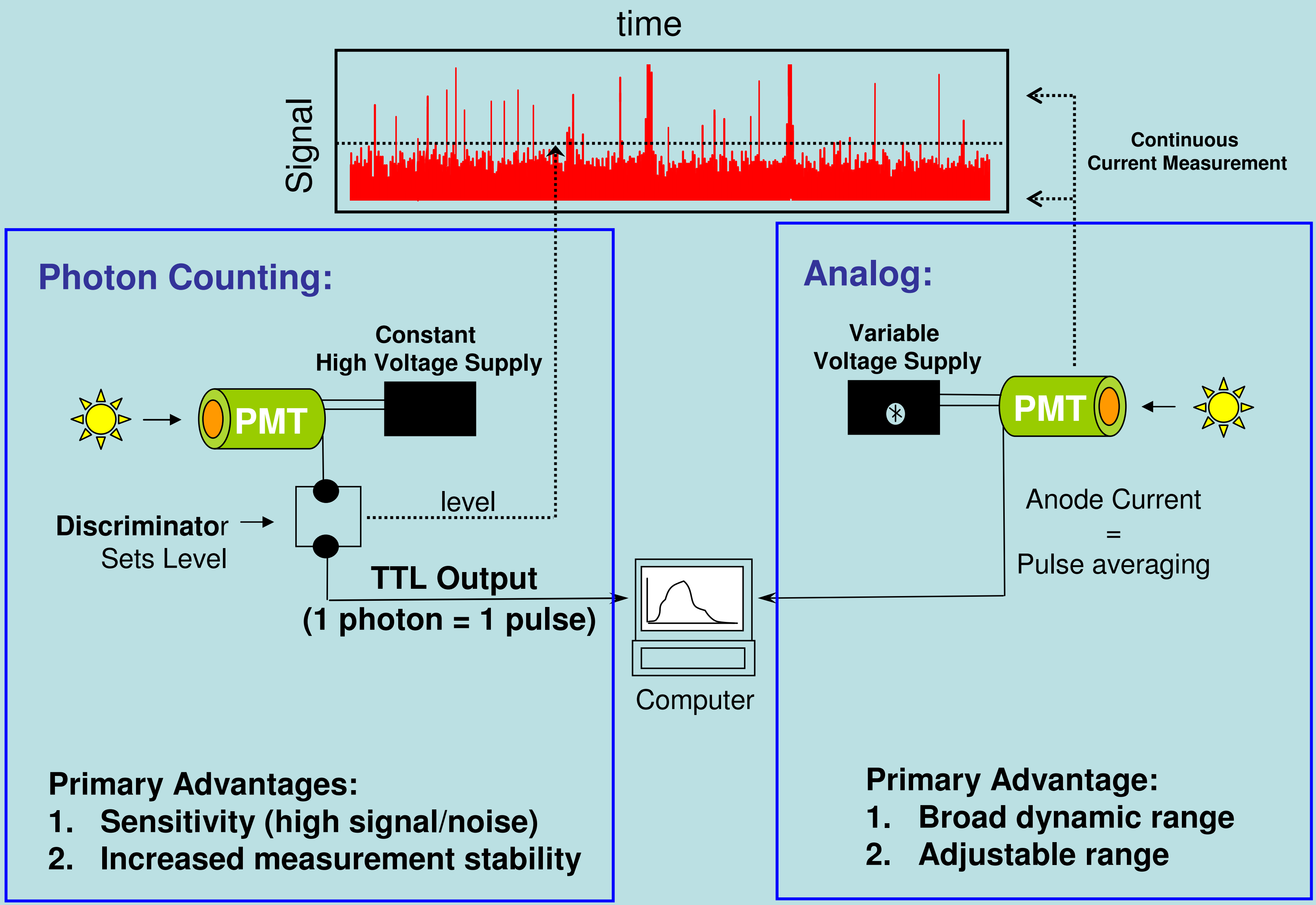
Cathode Material



Window Material

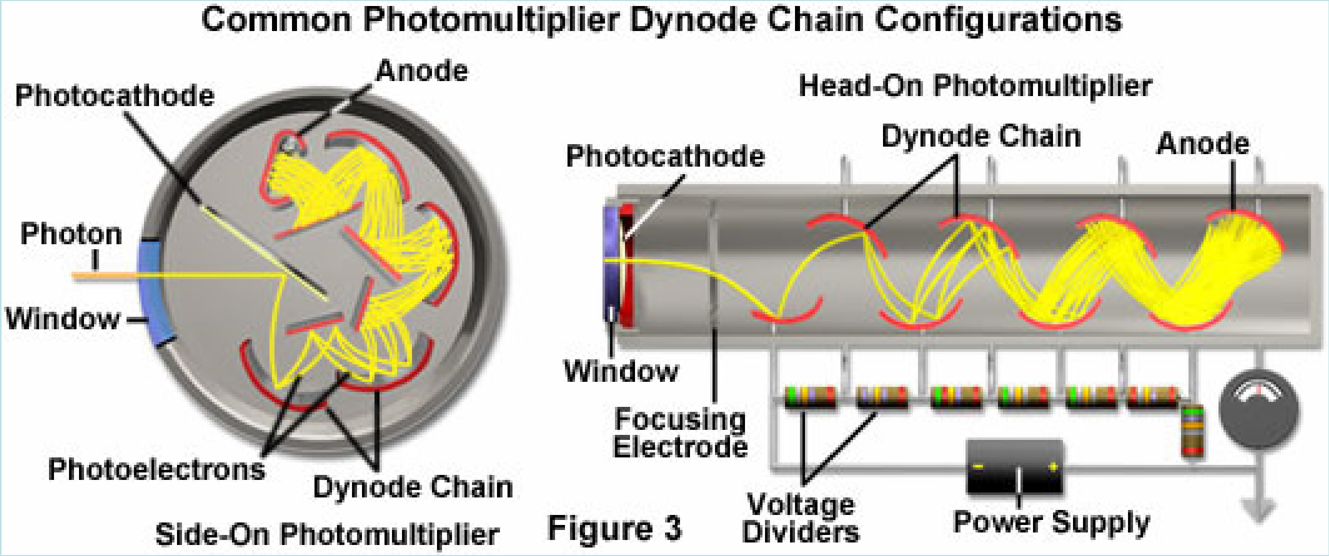


Photon Counting (Digital) and Analog Detection



PMT Geometries

Side-On PMT **Head-On PMT**

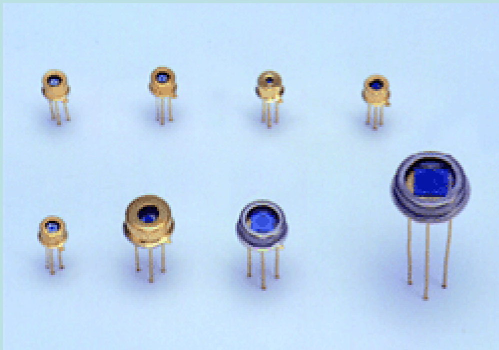


Opaque photocathode **Semitransparent Photocathode**

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

Avalanche Photodiode (APD)

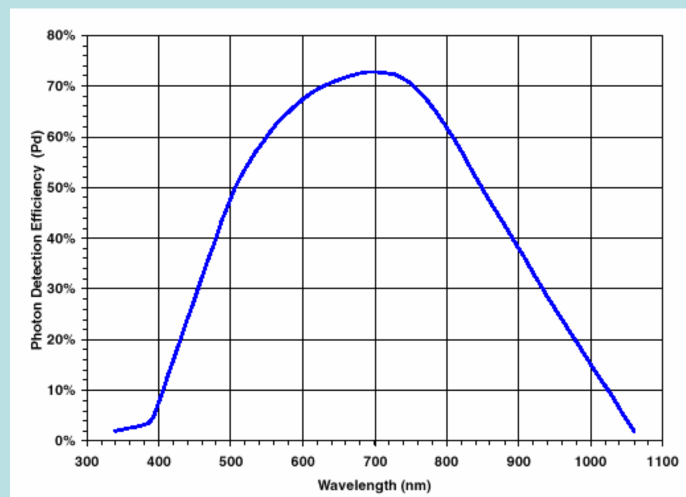
APD for analog detection



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

APD for photon counting

Single photon counting module (SPCM) from Perkin-Elmer

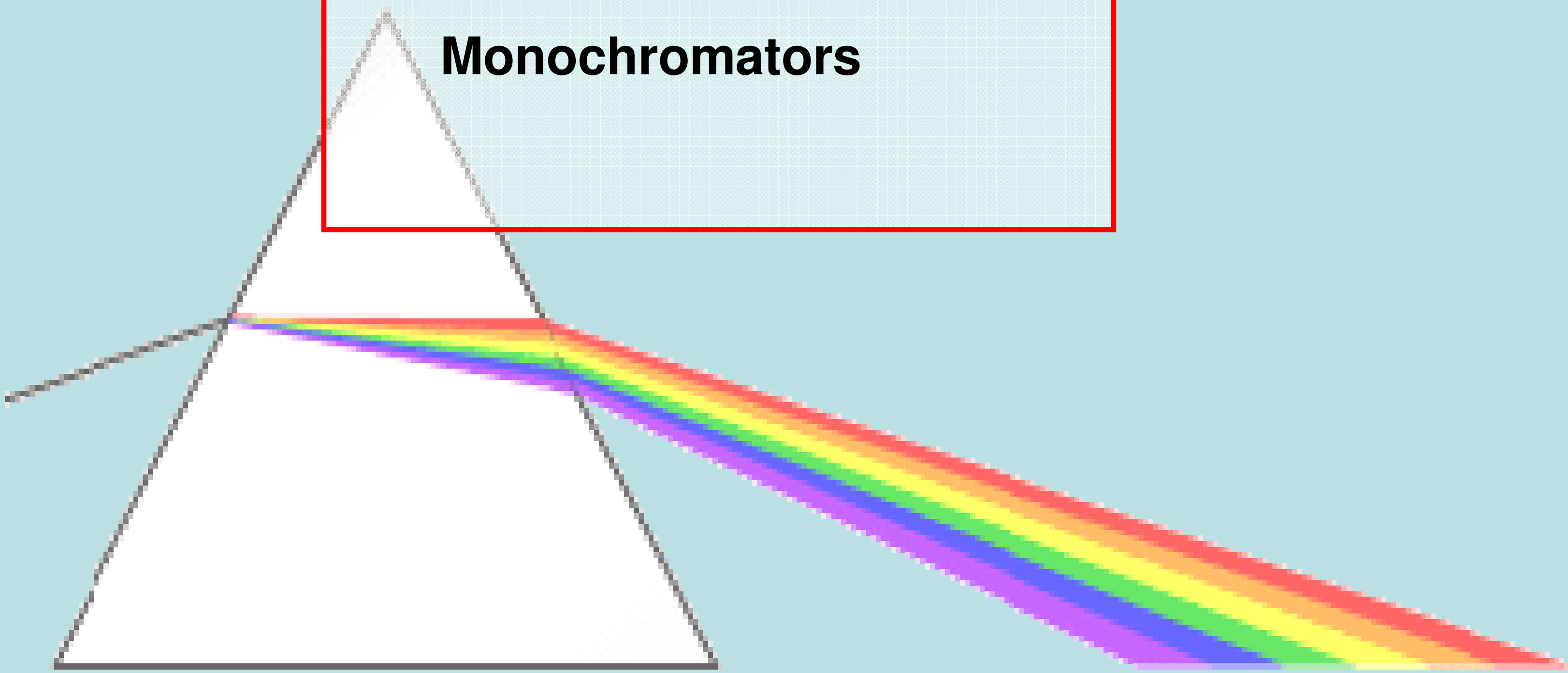


Wavelength Selection

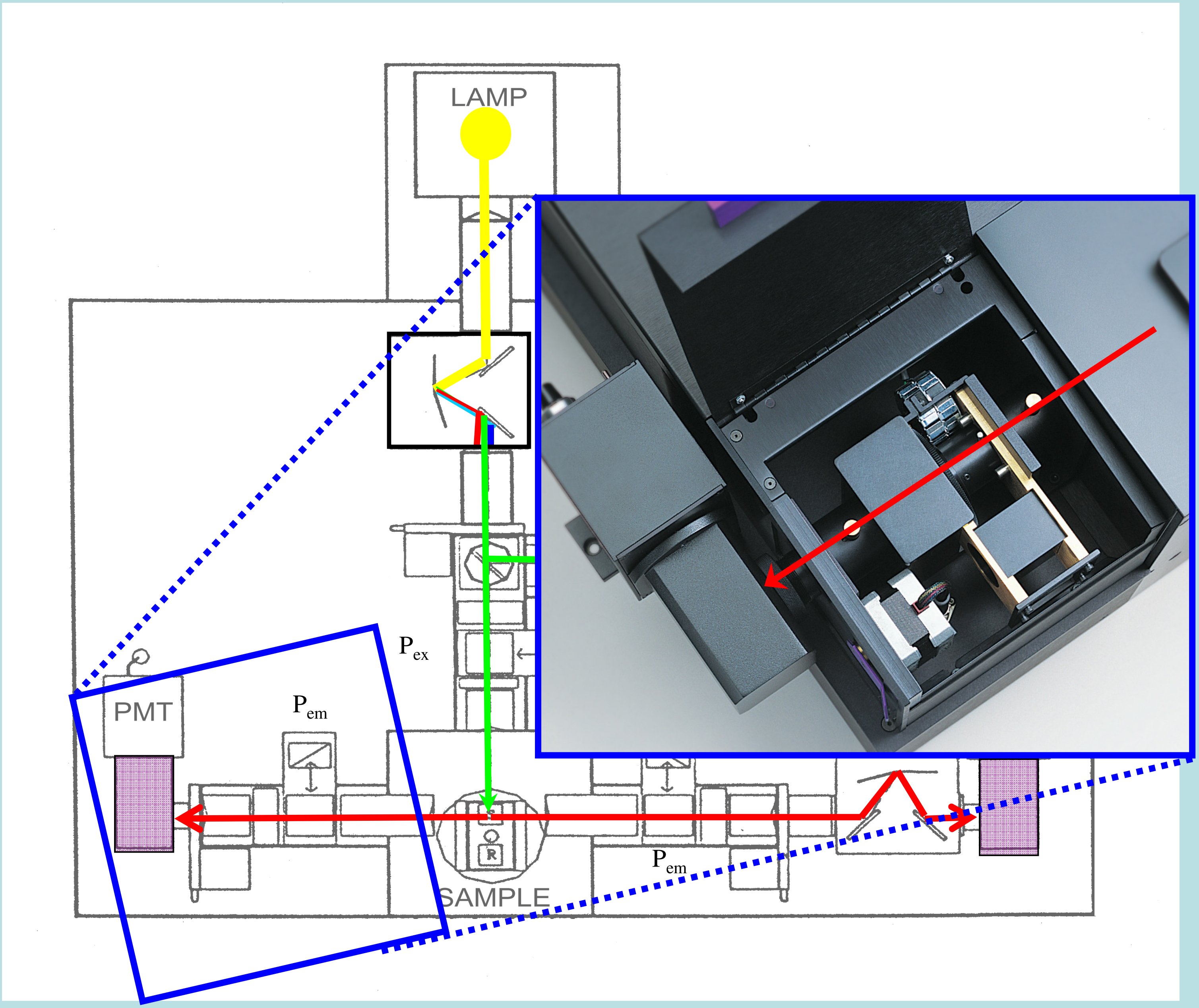
Fixed Optical Filters

Tunable Optical Filters

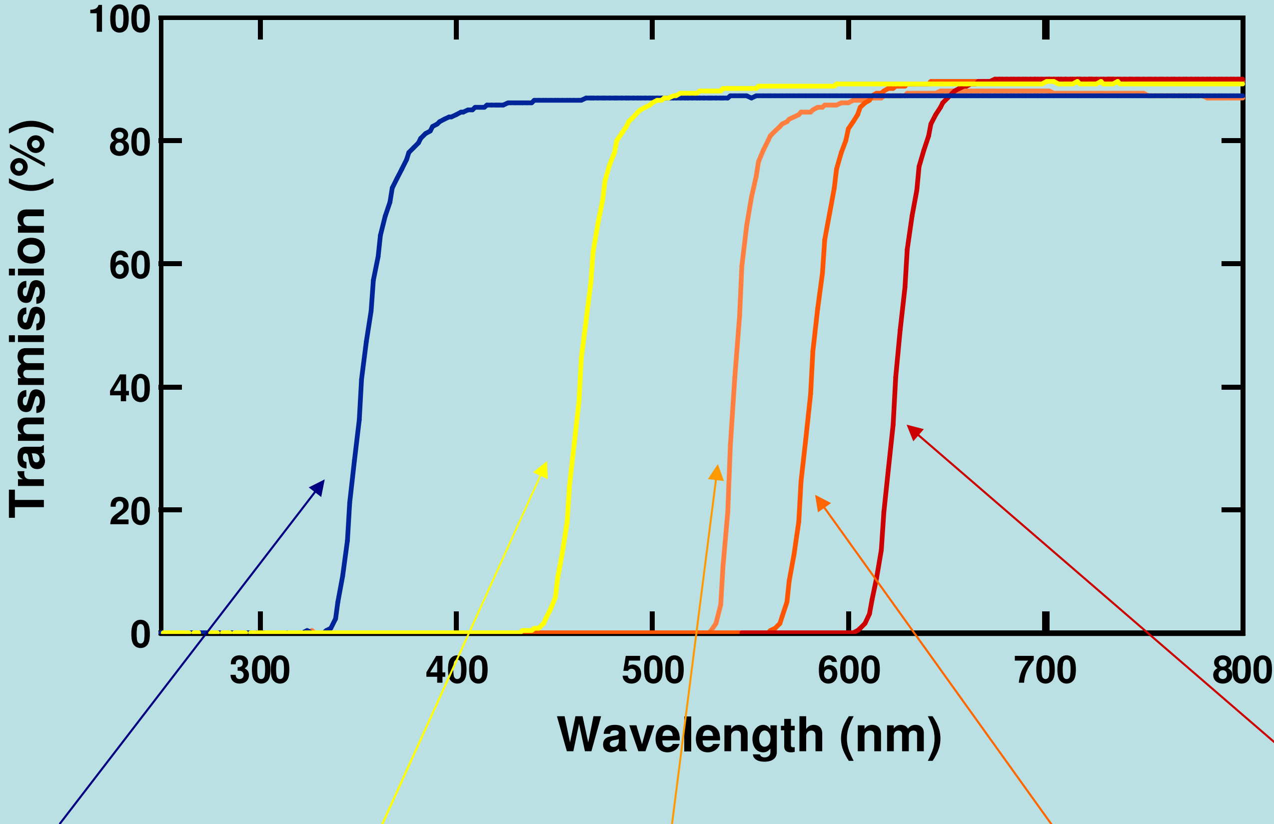
Monochromators



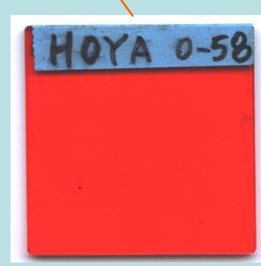
Optical Filter Channel



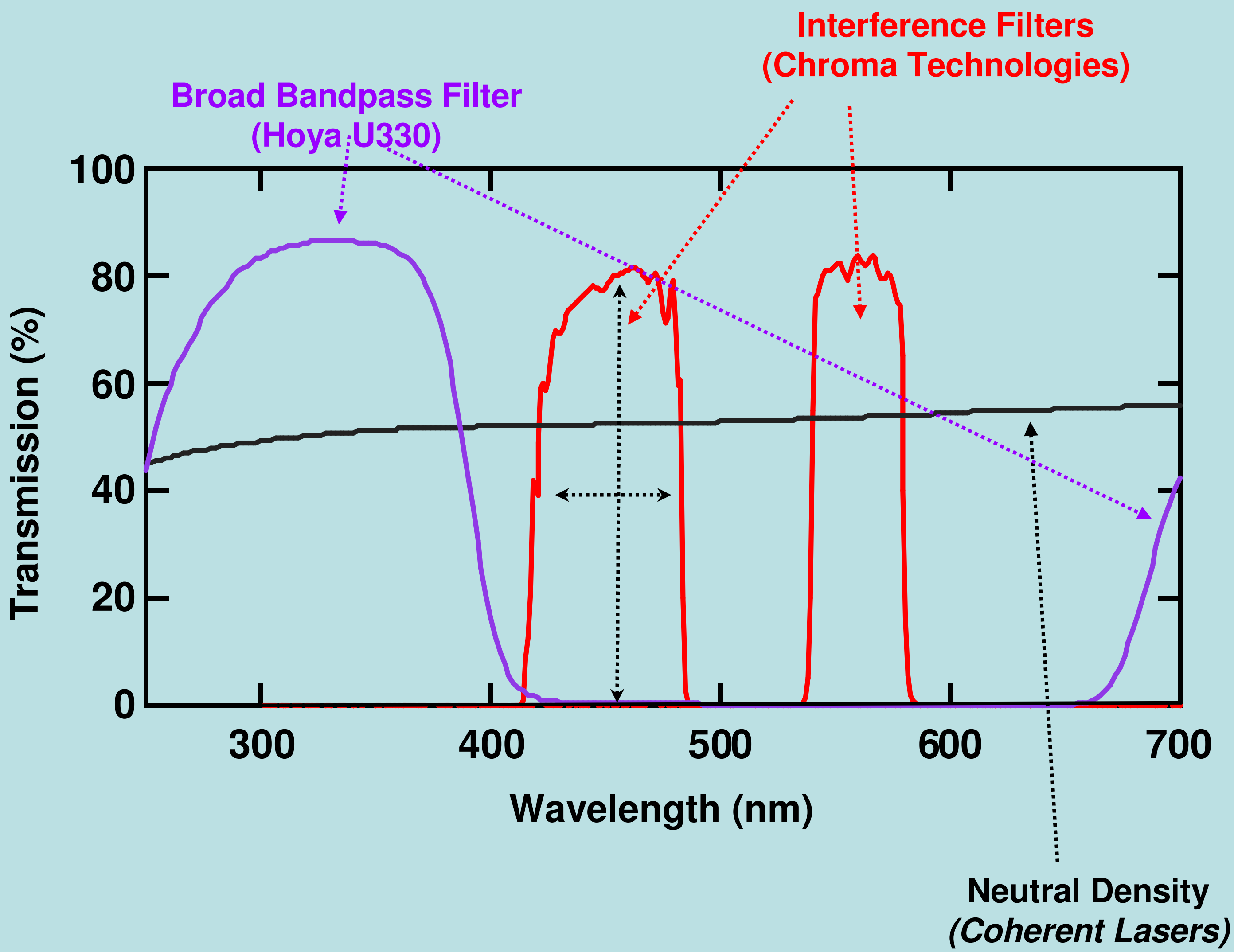
Long Pass Optical Filters



Spectral Shape
Thickness
Physical Shape
Fluorescence (!?)



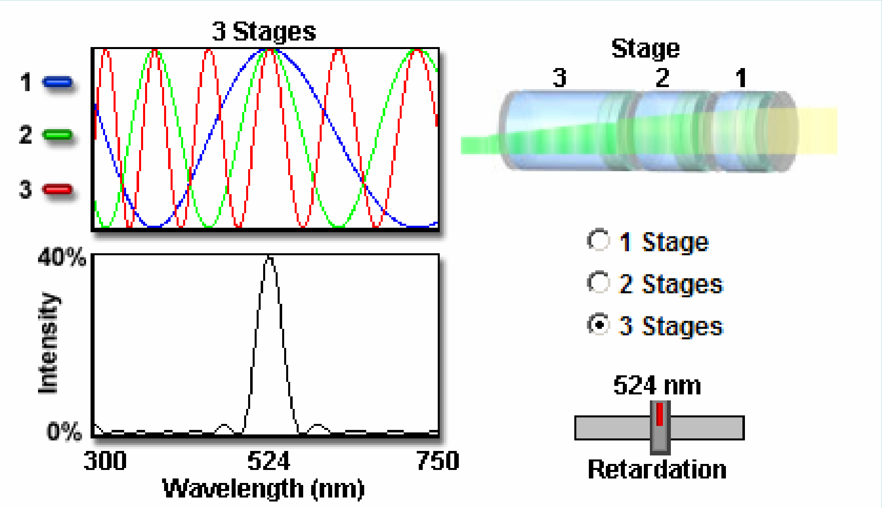
More Optical Filter Types…



Tunable Optical Filters

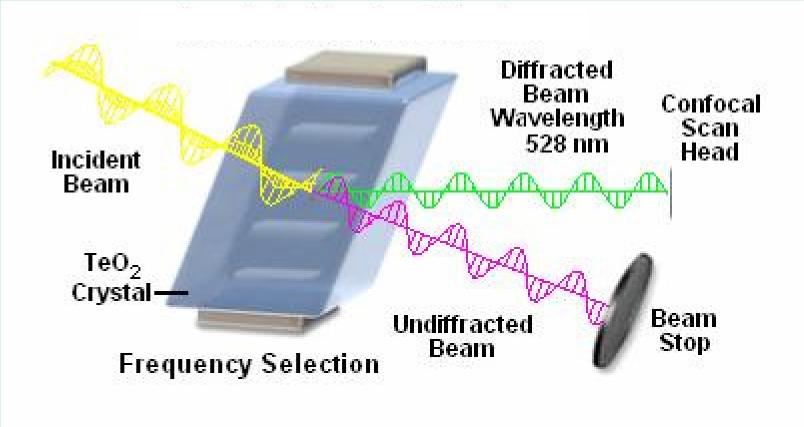
Liquid Crystal Filters:

An electrically controlled liquid crystal elements to select a specific visible wavelength of light for transmission through the filter at the exclusion of all others.

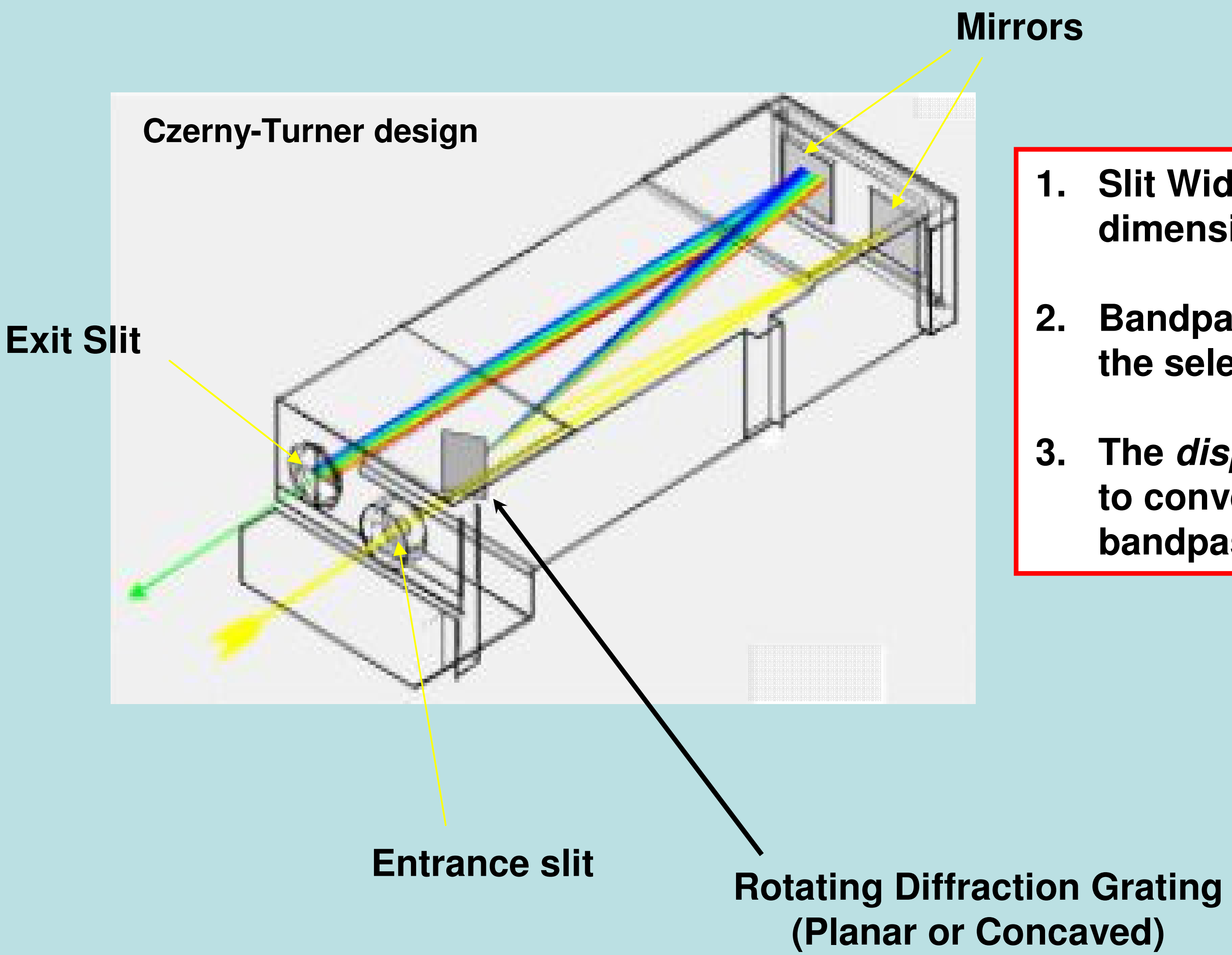


AO Tunable Filters:

The AOTF range of acousto-optic devices are solid state optical filters. The wavelength of the diffracted light is selected according to the frequency of the RF drive signal.



Monochromators



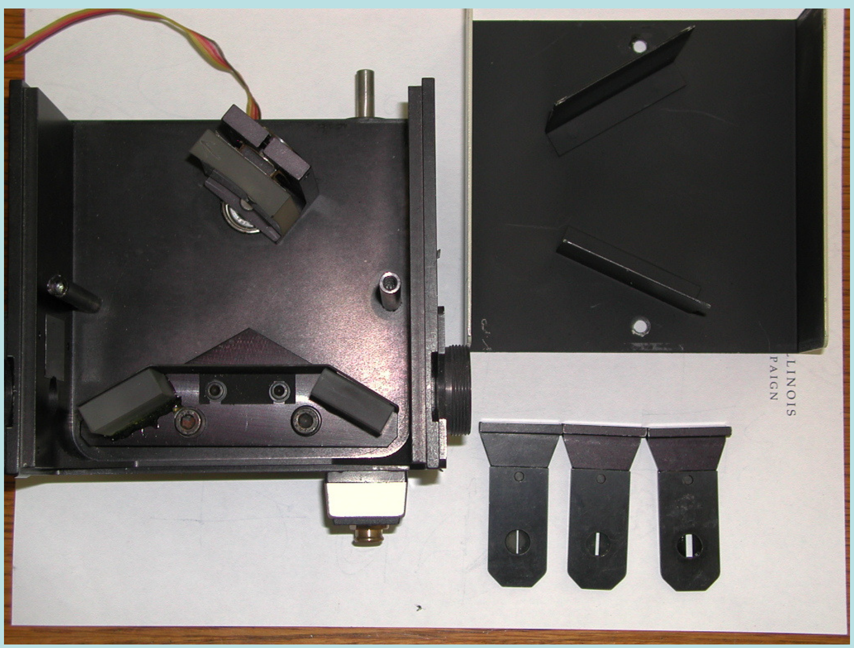
1. Slit Width (mm) is the dimension of the slits.

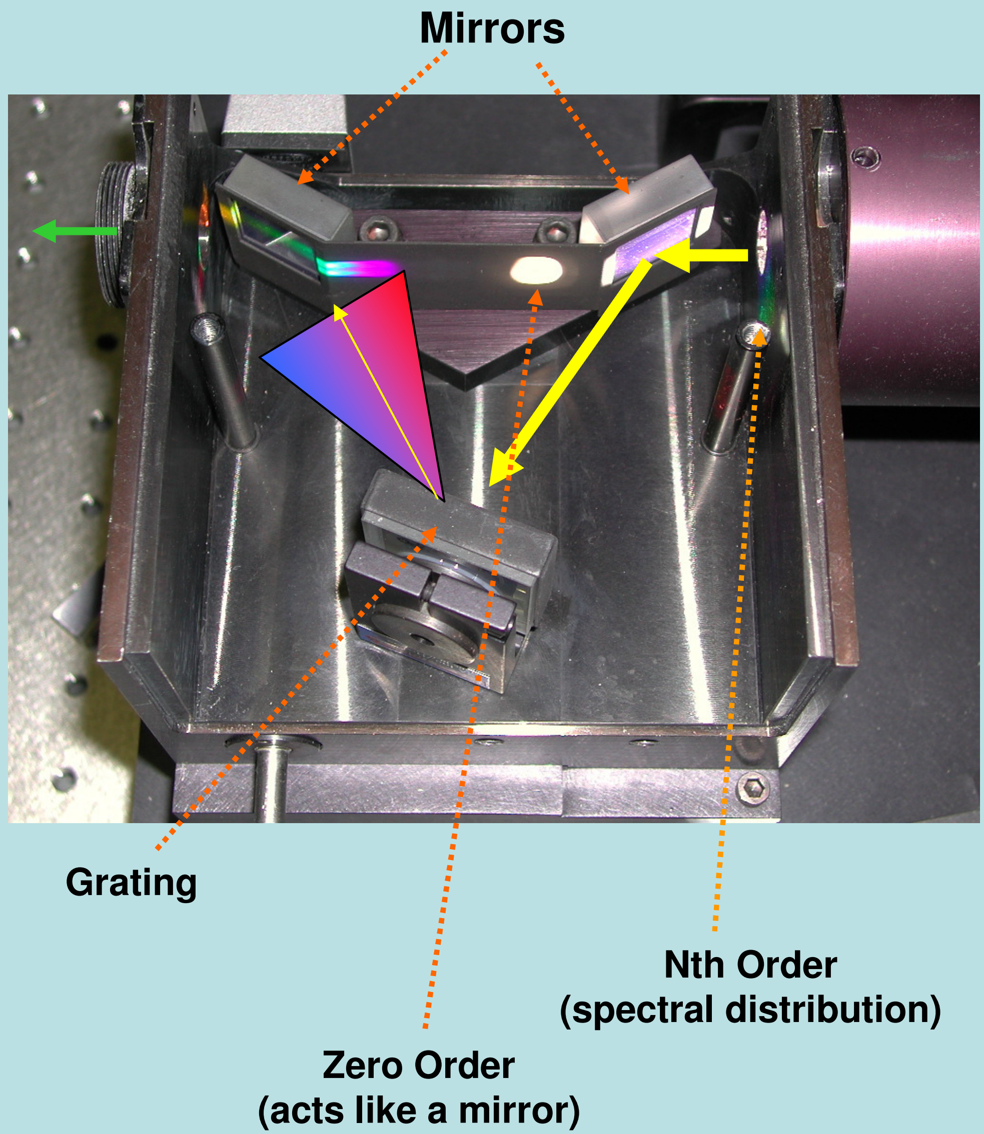
2. Bandpass is the FWHM of the selected wavelength.

3. The *dispersion* is the factor to convert slit width to bandpass.

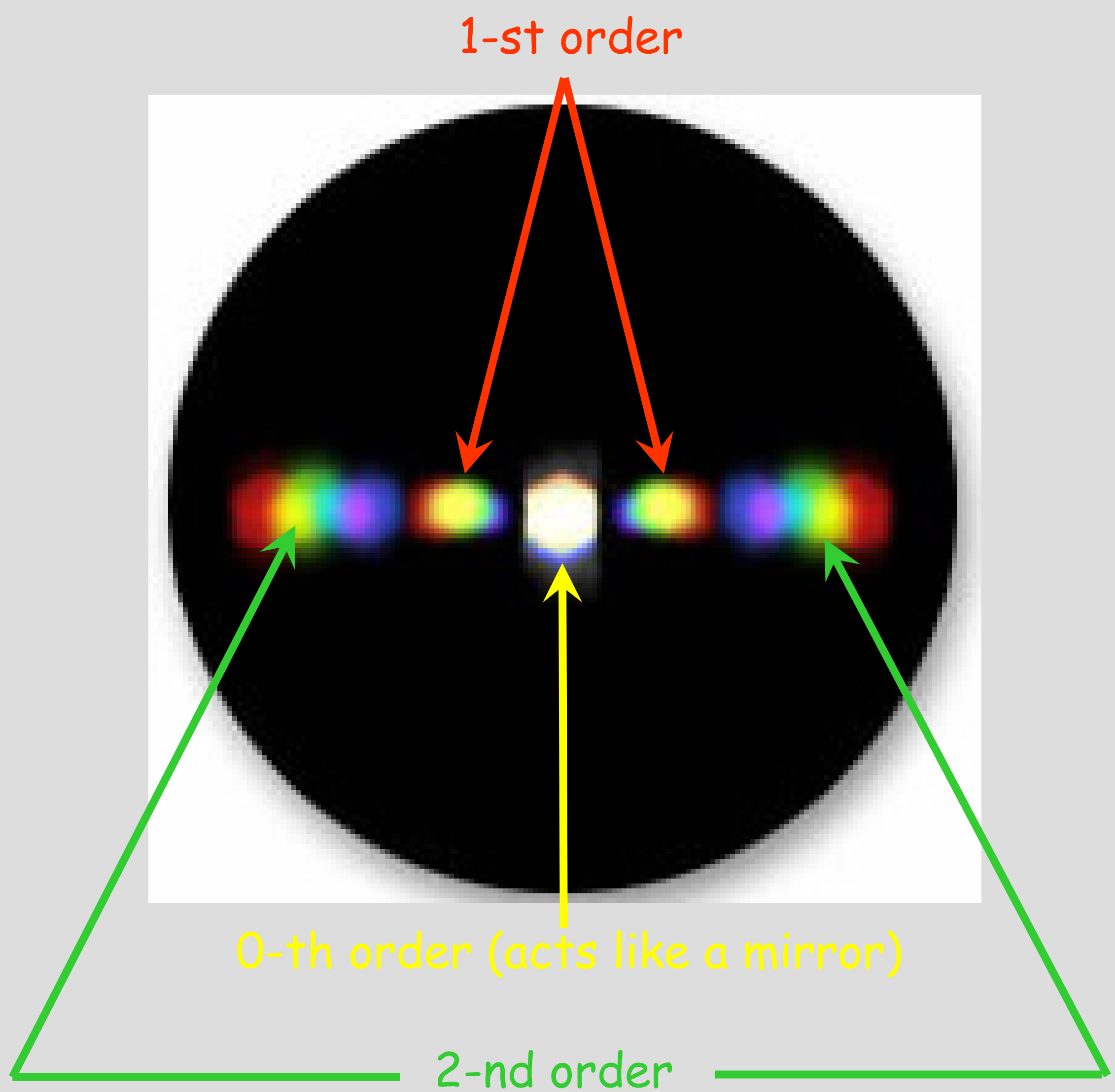
The Inside of a Monochromator

H10 Monochromator





Order of diffraction

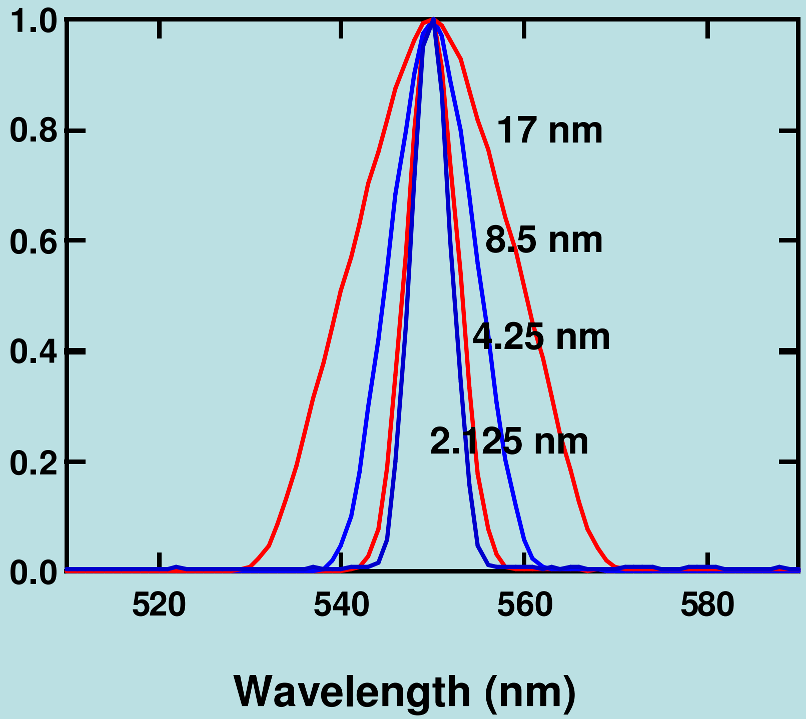


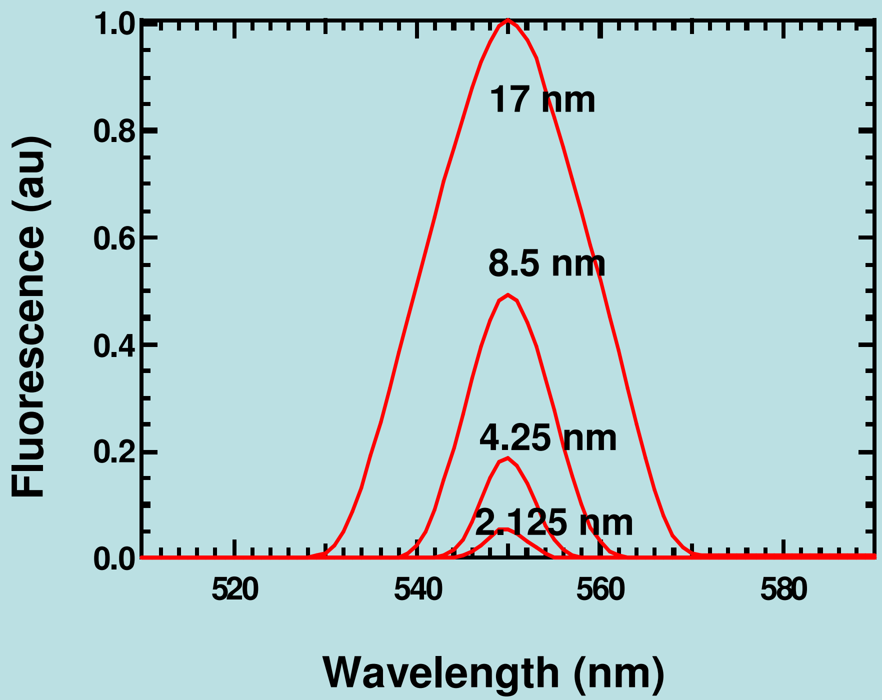
Changing the Bandpass

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

Fixed Excitation Bandpass =
4.25 nm

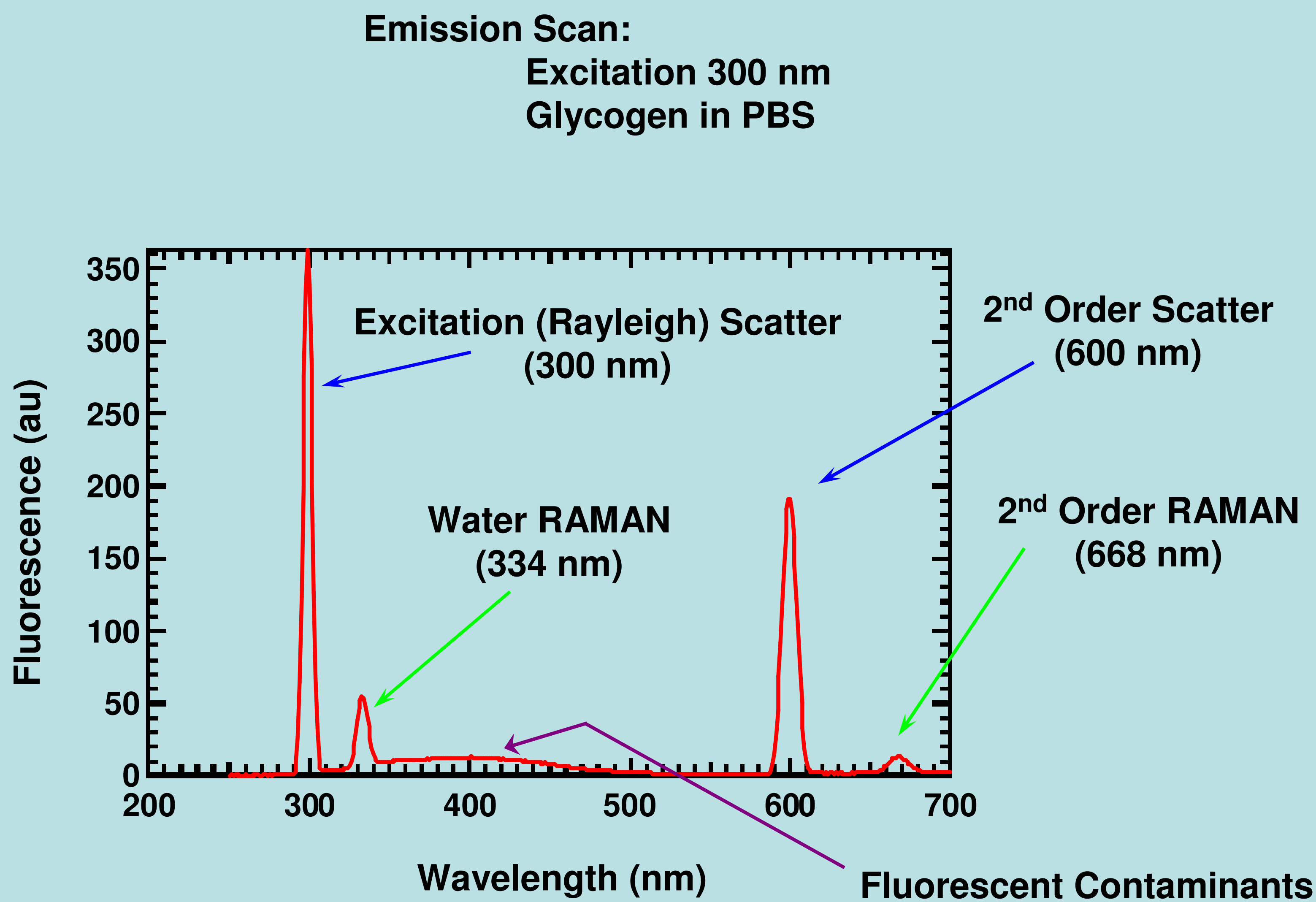
Changing the Emission Bandpass



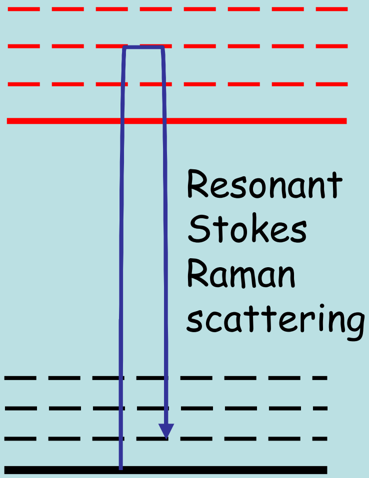


Collected on a SPEX Fluoromax - 2

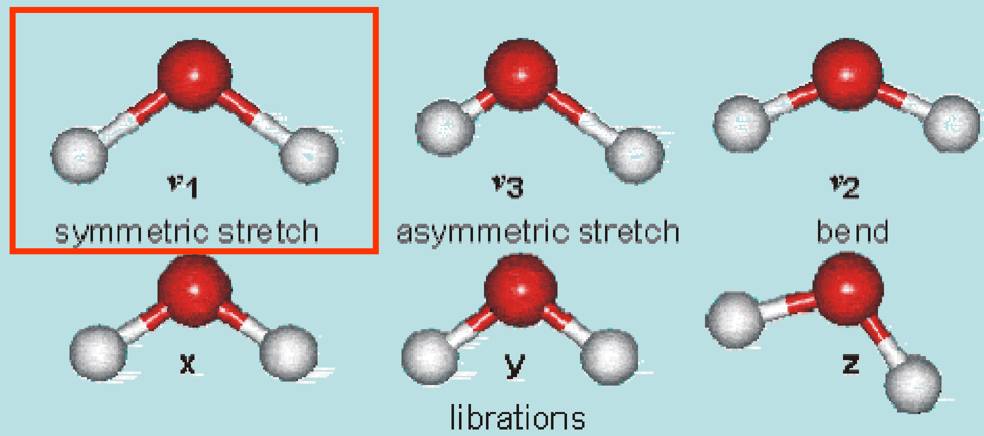
Higher Order Light Diffraction



Raman scatter of water



Vibrational modes of water



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

Simple formula to calculate the wavelength of the Raman peak:

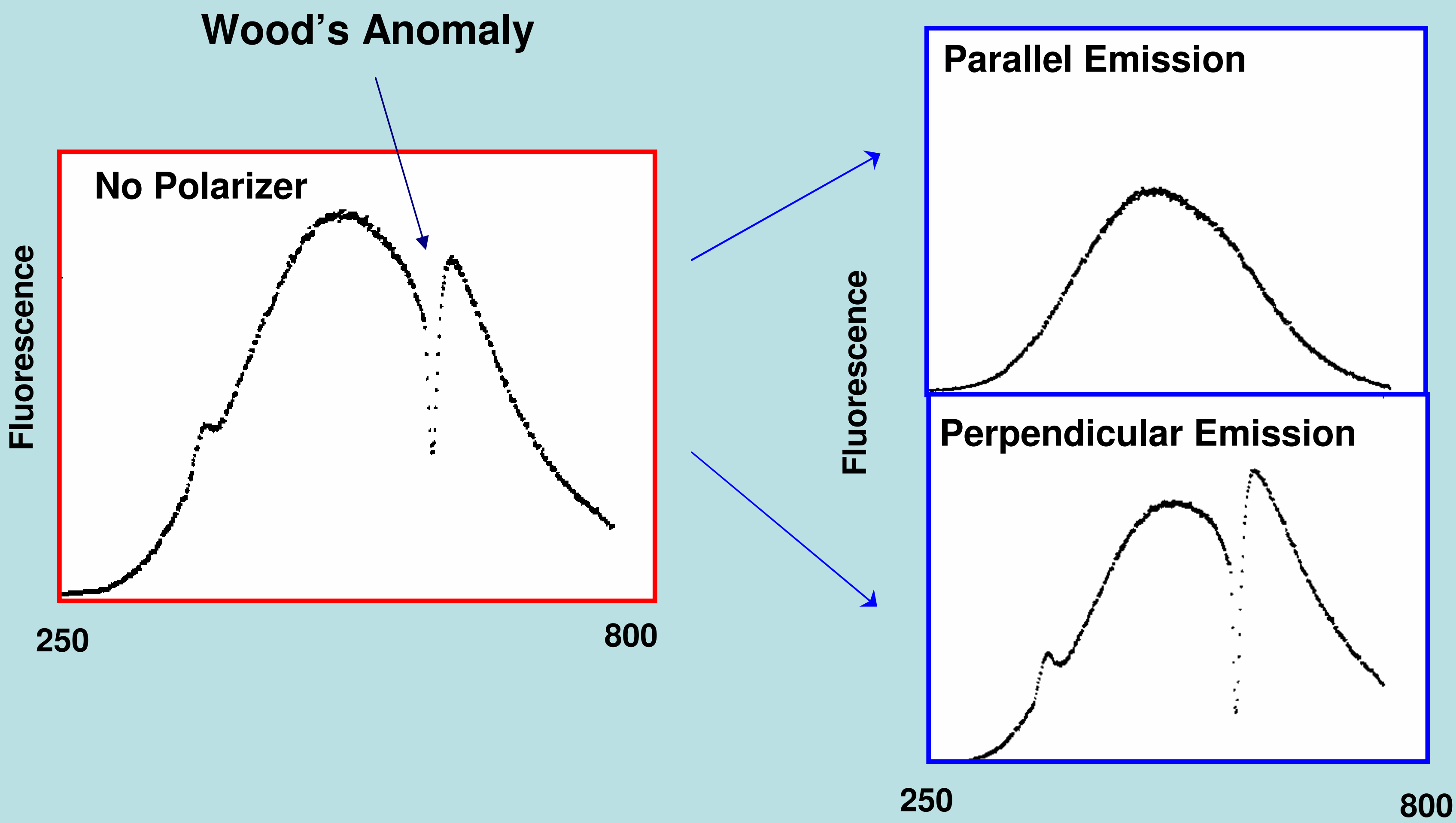
(1) Take the excitation wavelength (say 490 nm) and insert in the following equation:

(2) The result specifies the position of the raman peak in nanometers (i.e. the raman peak is at 587nm for an excitation wavelength of 490nm.

$$\frac{10^7}{\frac{10^7}{490} - 3400} = 587$$

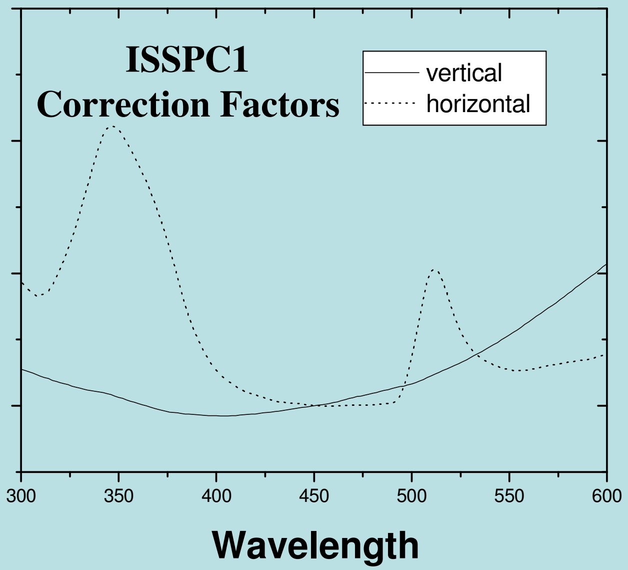
Monochromator Polarization Bias

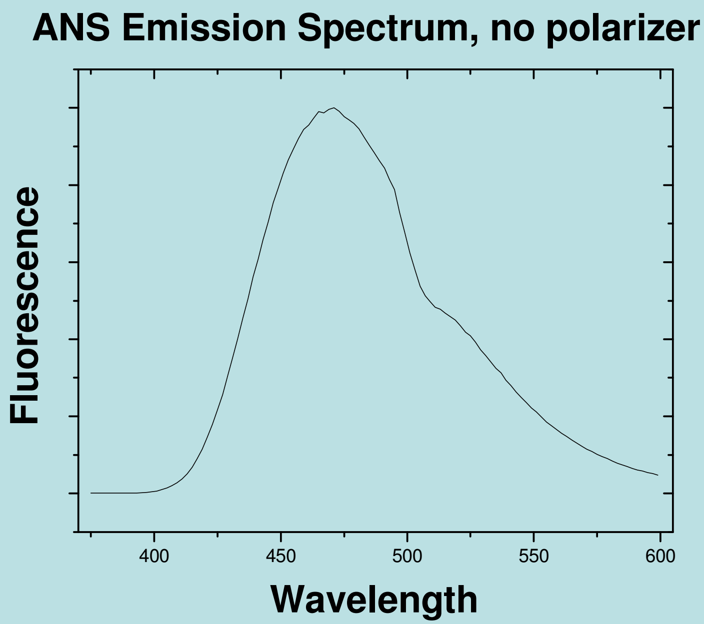
Tungsten Lamp Profile Collected on an SLM Fluorometer

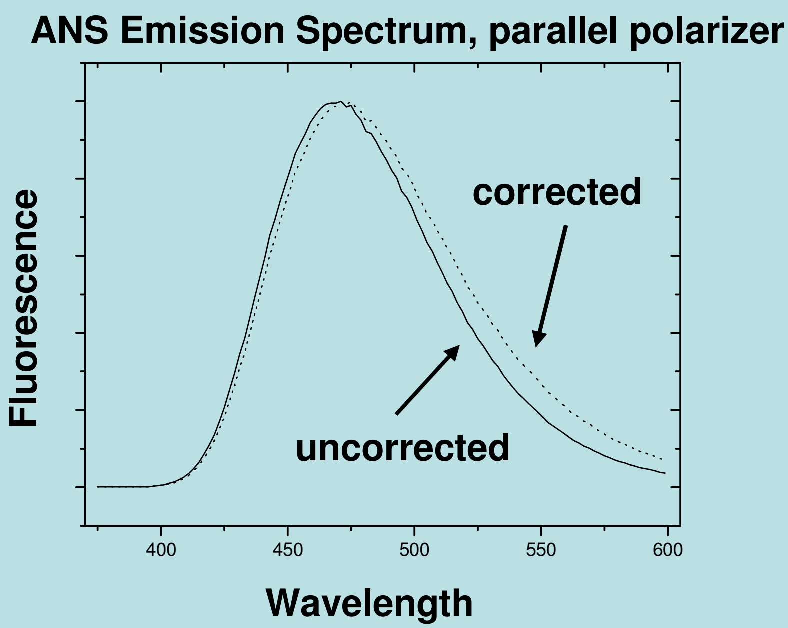


Adapted from Jameson, D.M., Instrumental Refinements in Fluorescence Spectroscopy: Applications to Protein Systems., in Biochemistry, Champaign-Urbana, University of Illinois, 1978.

Correction of Emission Spectra

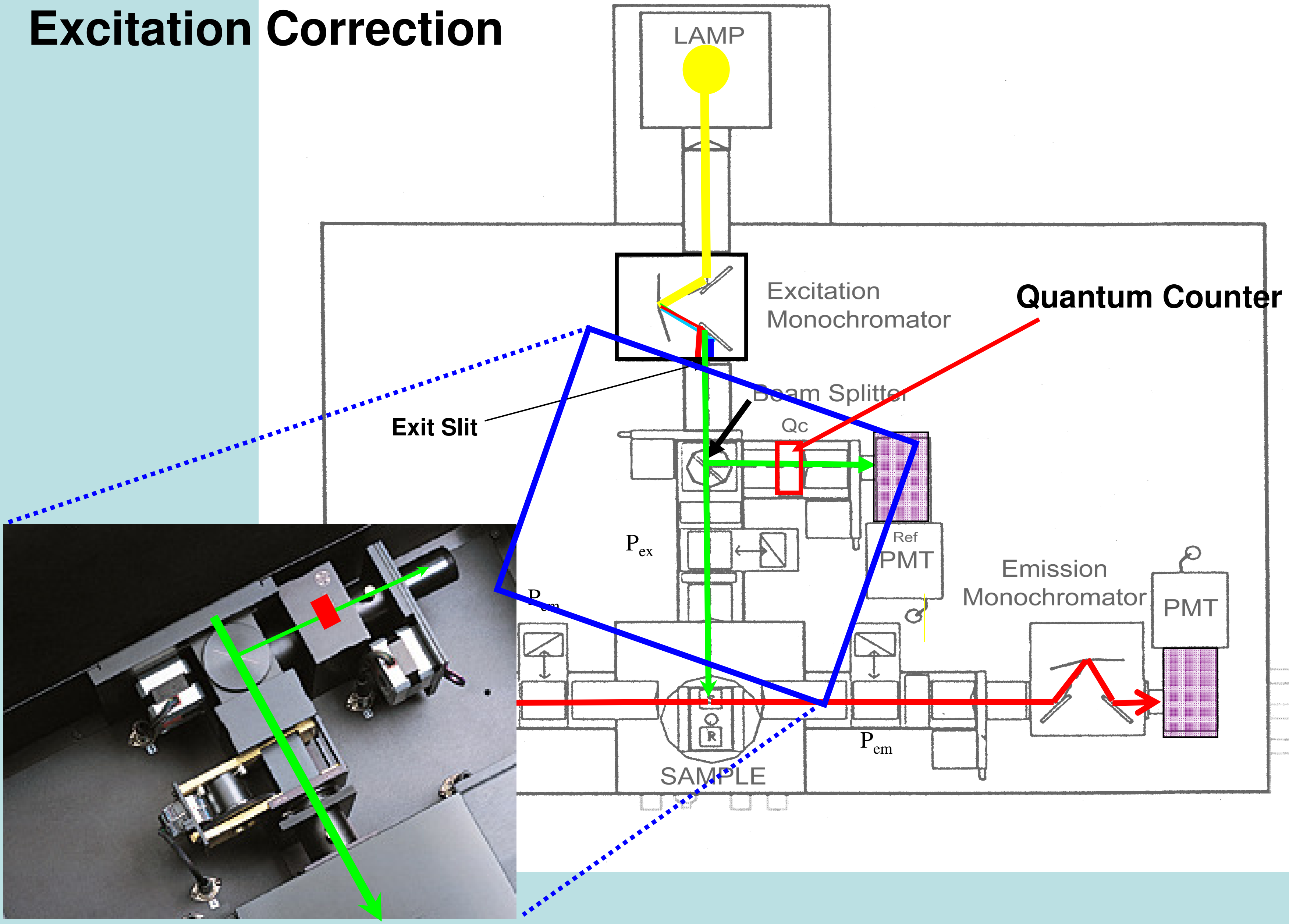






from Jameson et. Al., Methods in Enzymology, 360:1

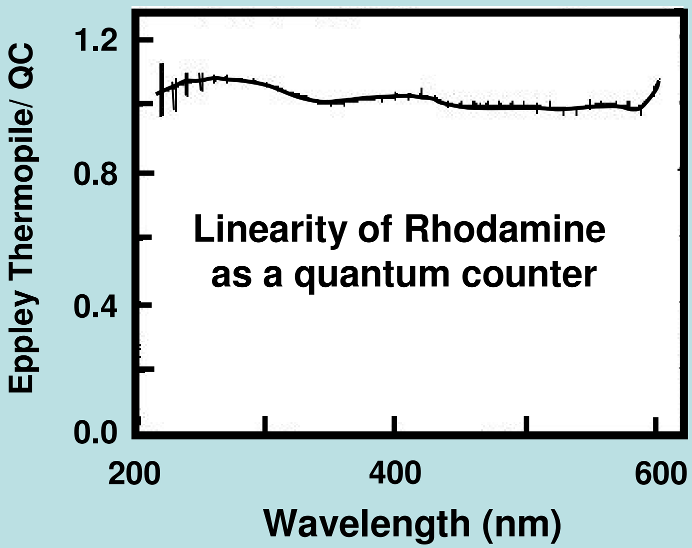
Excitation Correction

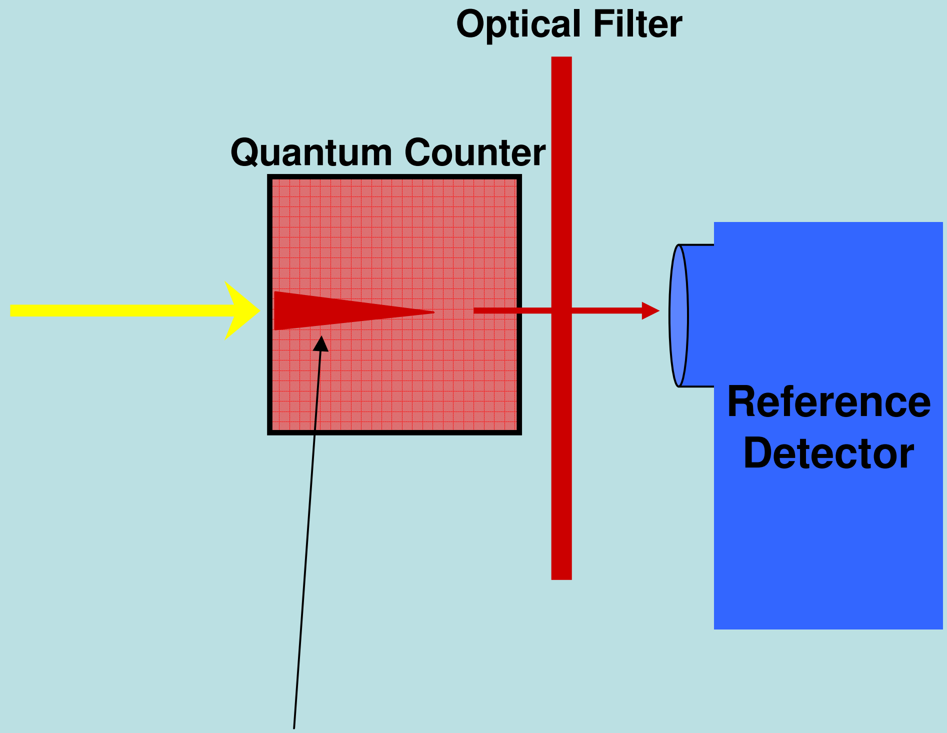


The Instrument Quantum Counter

Common Quantum Counters (optimal range)*

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





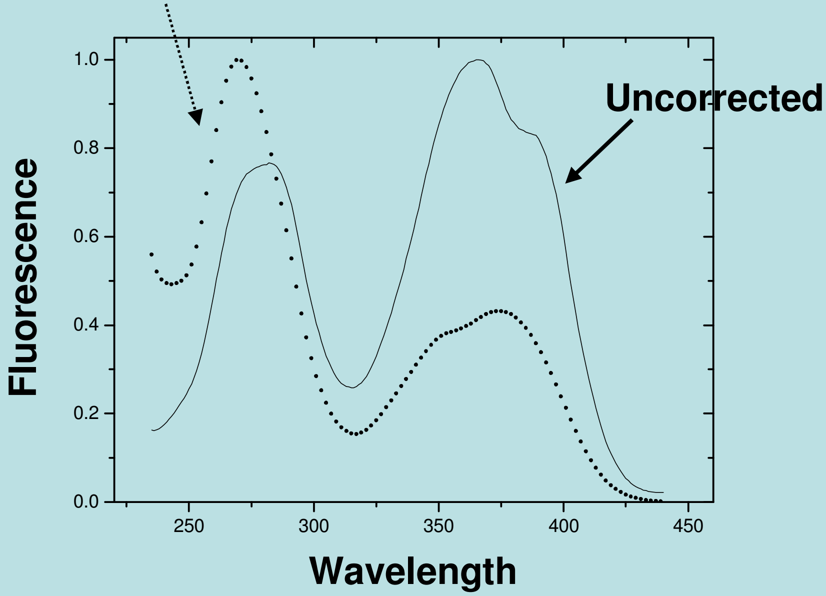
Fluorescence

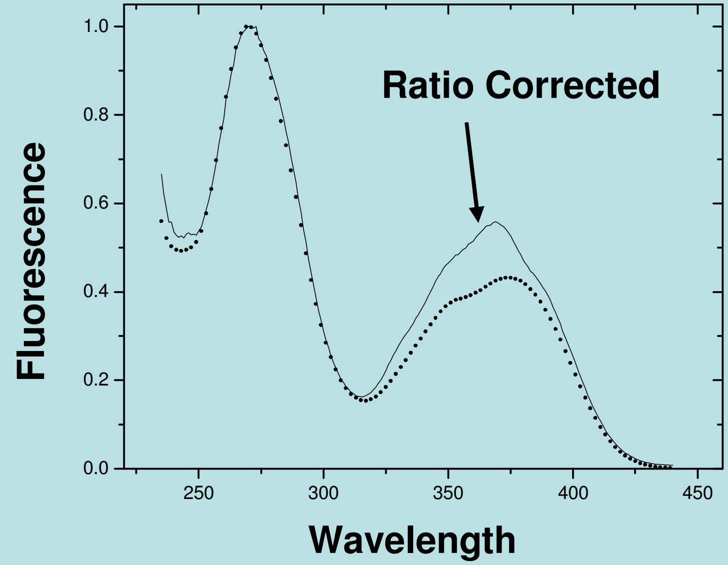
Here we want the inner filter effect!

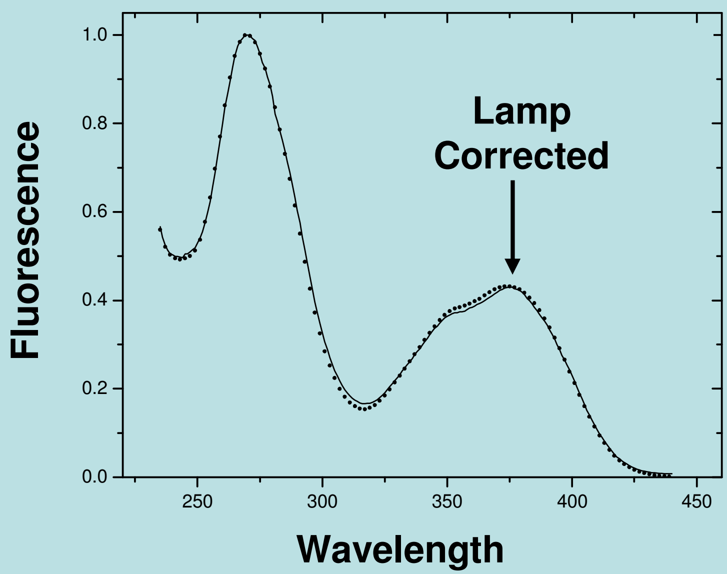
** Melhuish (1962) J. Opt. Soc. Amer. 52:1256*

Excitation Correction

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







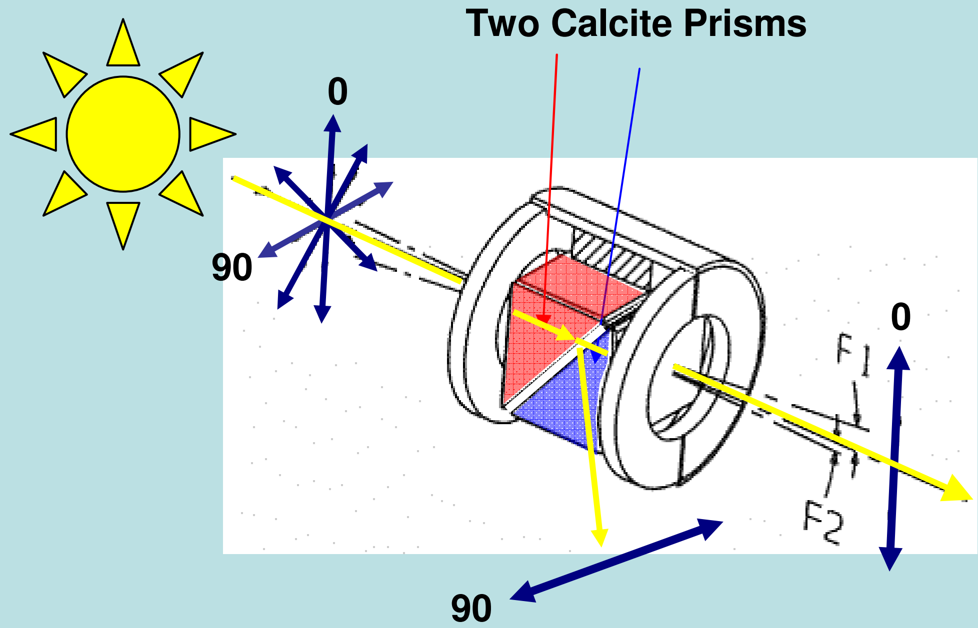
from Jameson et. Al., Methods in Enzymology, 360:1

Polarizers

Common Types:

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

The *Glan Taylor* prism polarizer



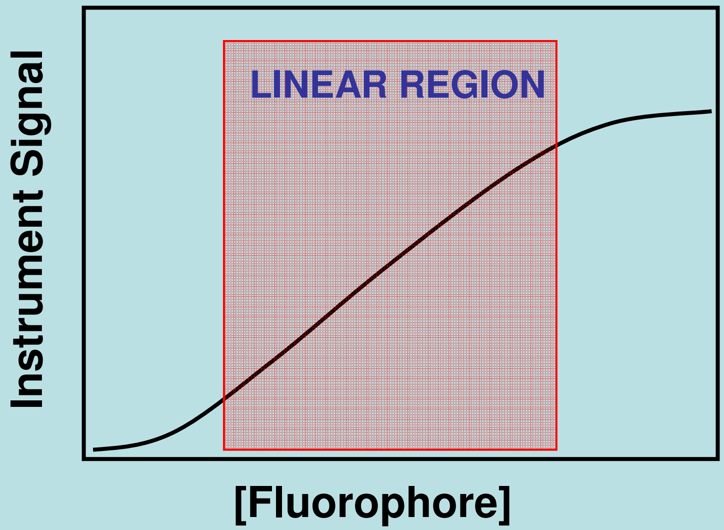
Sheet polarizer

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.

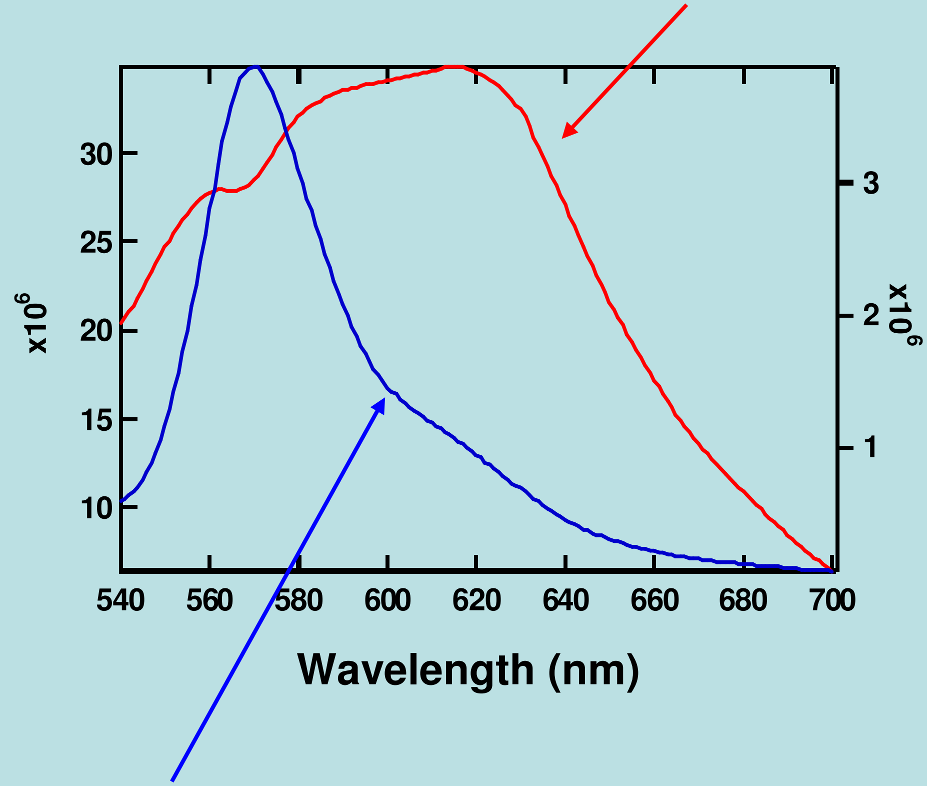
Sample Issues

Signal Attenuation of the Excitation Light
PMT Saturation

Fluorescence vs Signal



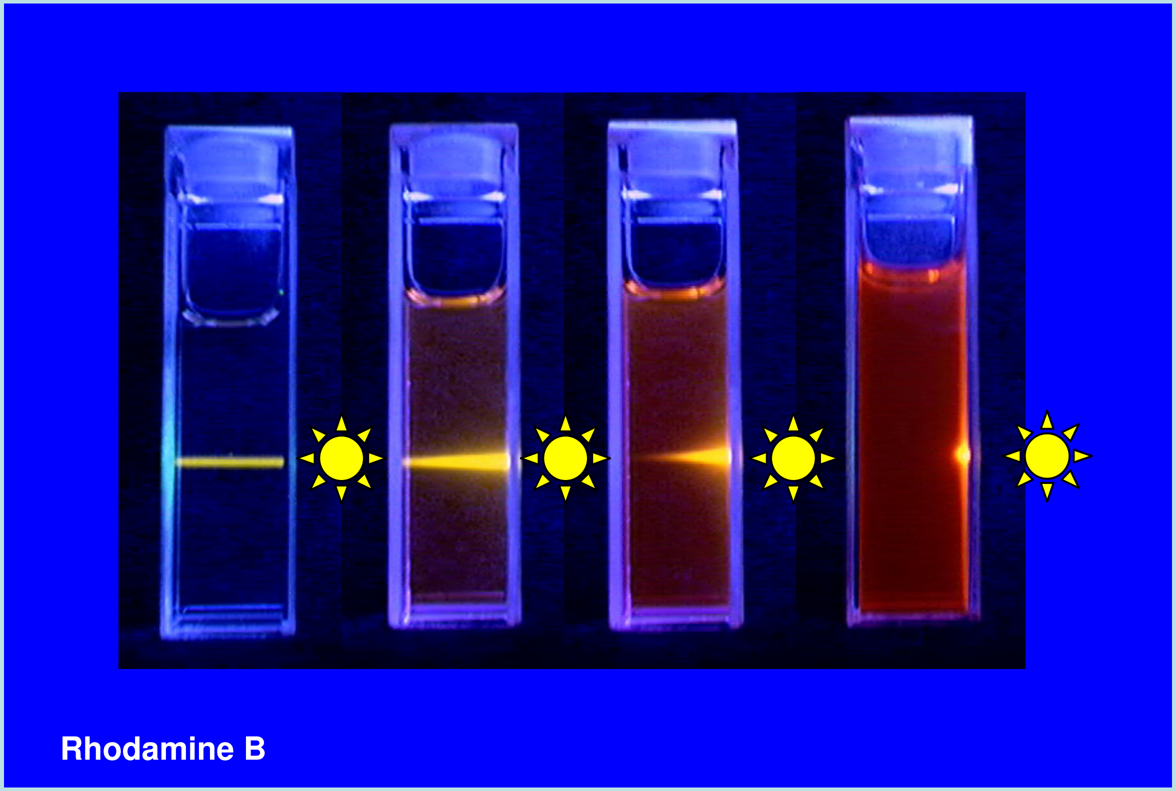
Excess Emission



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

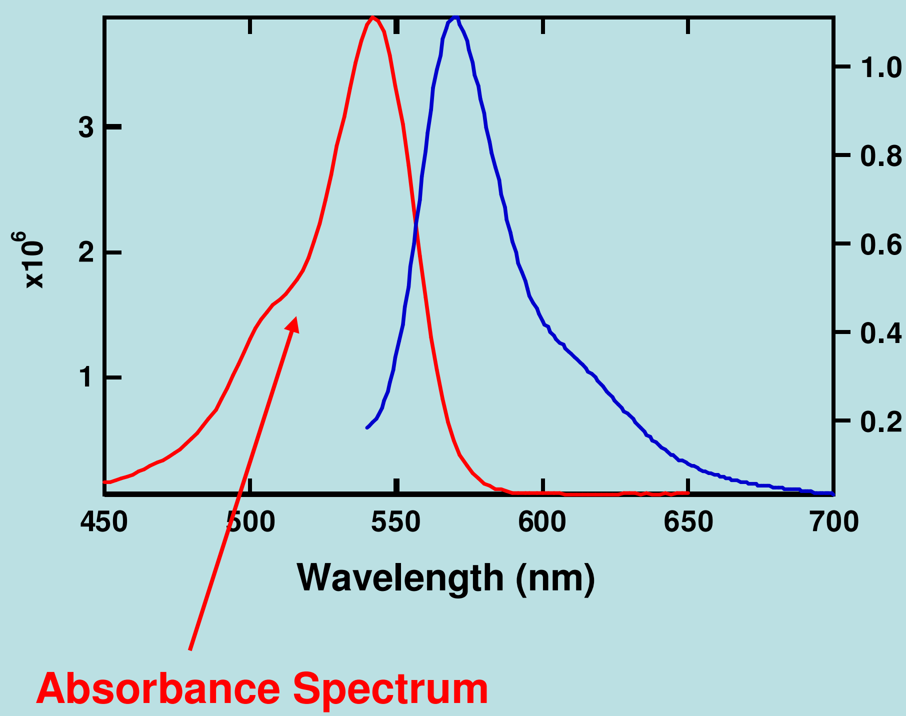
Attenuation of the Excitation Light through Absorbance

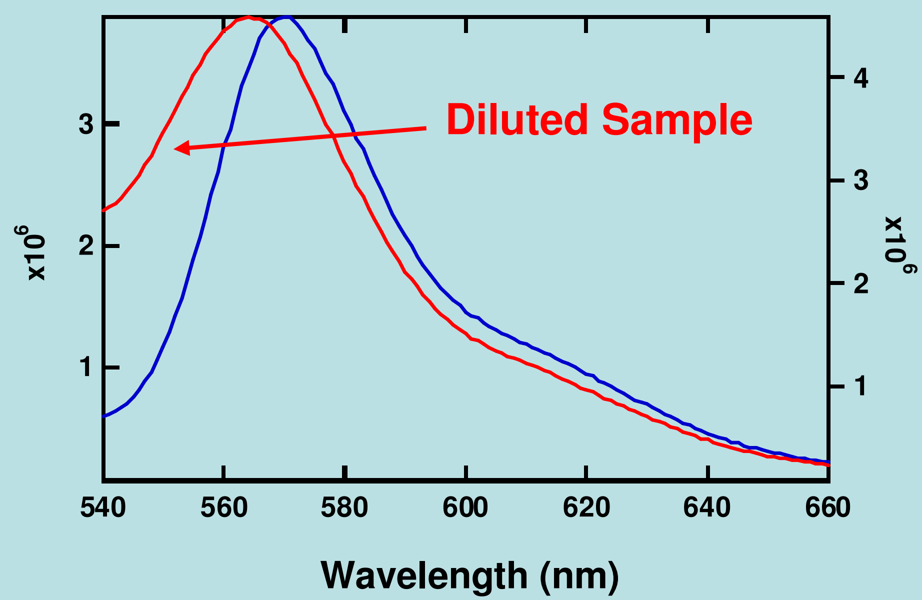
Sample concentration & the *inner filter effect*



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

The second half of the *inner filter effect*: <u>attenuation of the emission signal.</u>

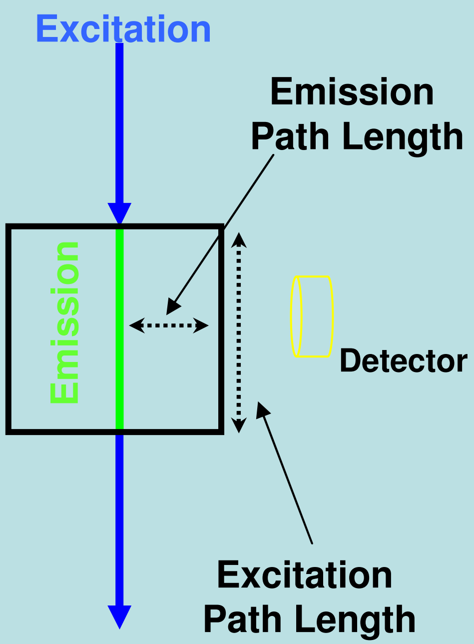


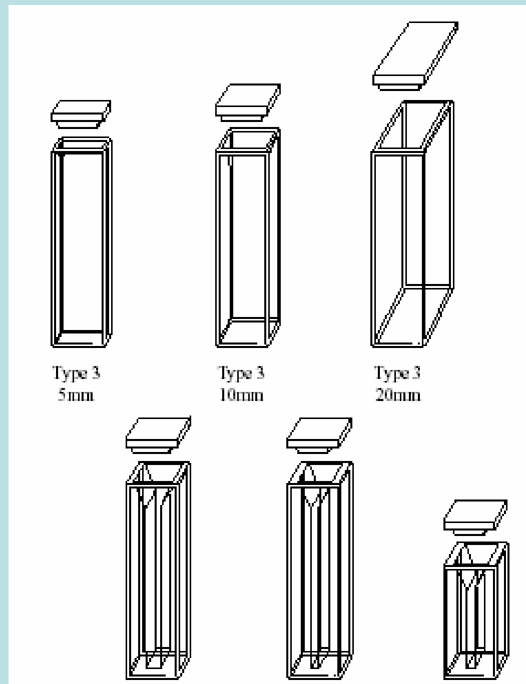


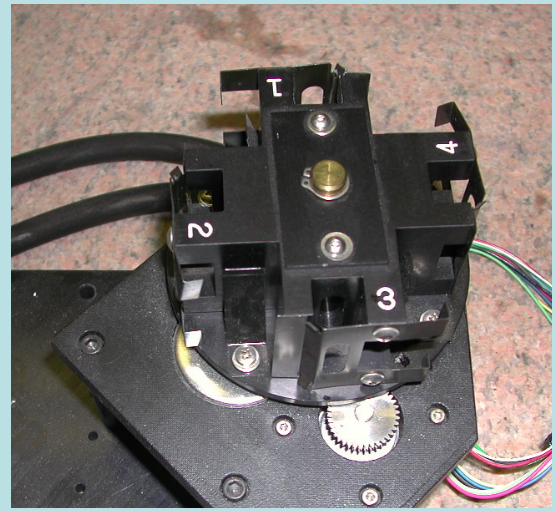
(1) Spectral Shift
(2) Change in Spectral Shape

How do we handle highly absorbing solutions?

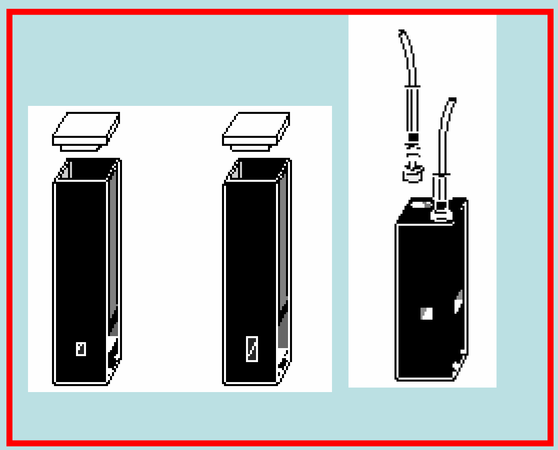
Quartz/Optical Glass/Plastic Cells

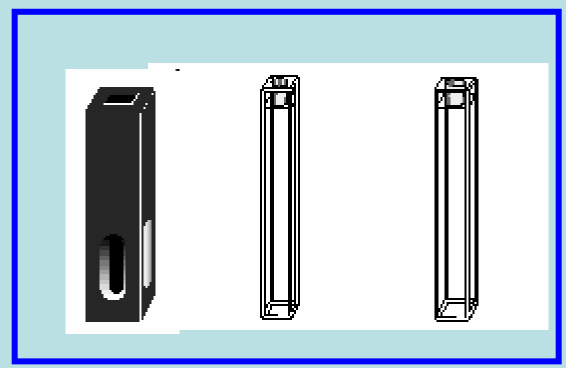






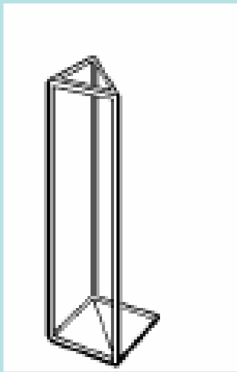
4 Position Turret
SPEX Fluoromax-2, Jobin-Yvon

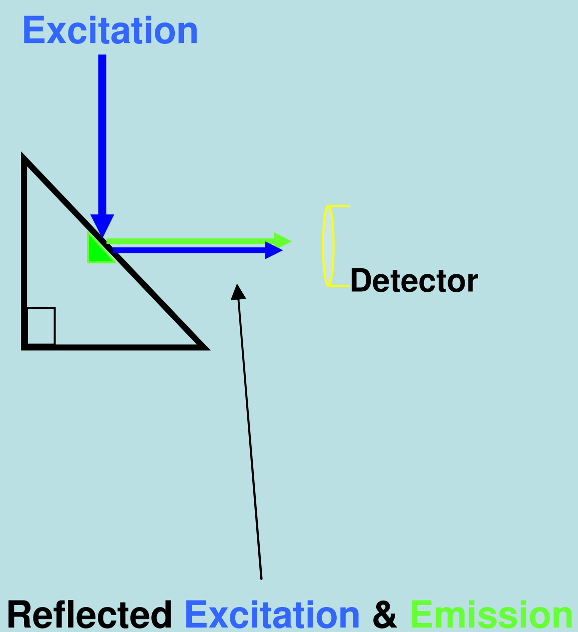




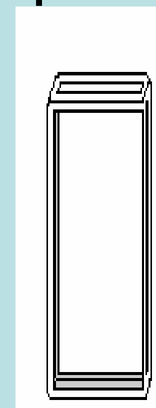
Front Face Detection

Triangular Cells





Thin Cells & Special Compartments



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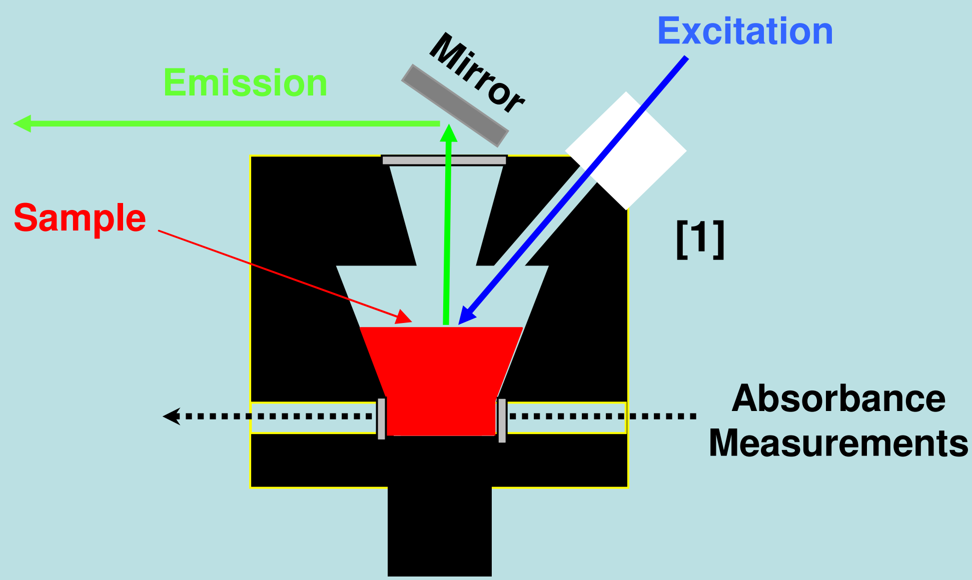
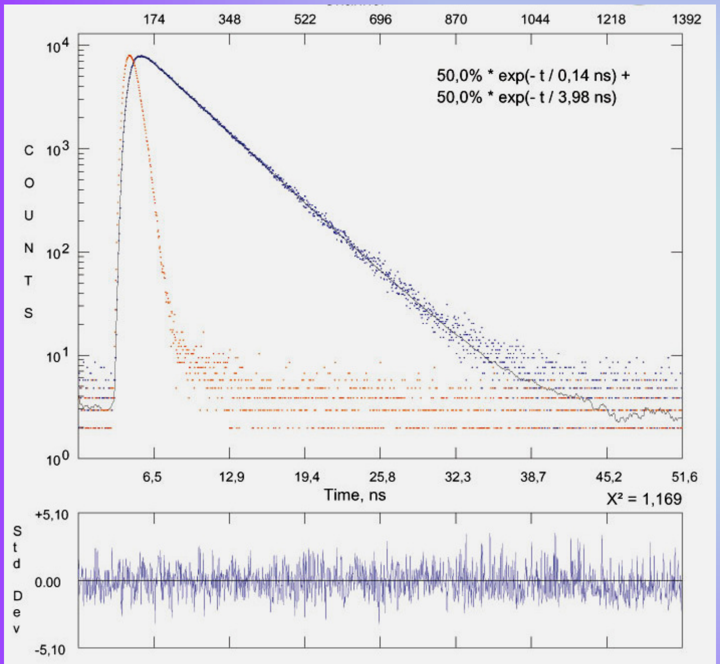
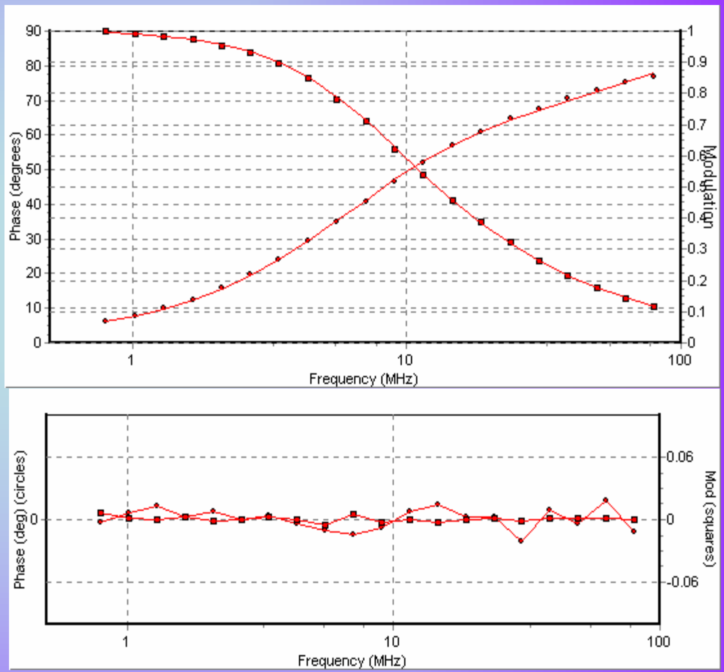


[1] Adapted from Gryczynski, Lubkowski, & Bucci Methods of Enz. 278: 538

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

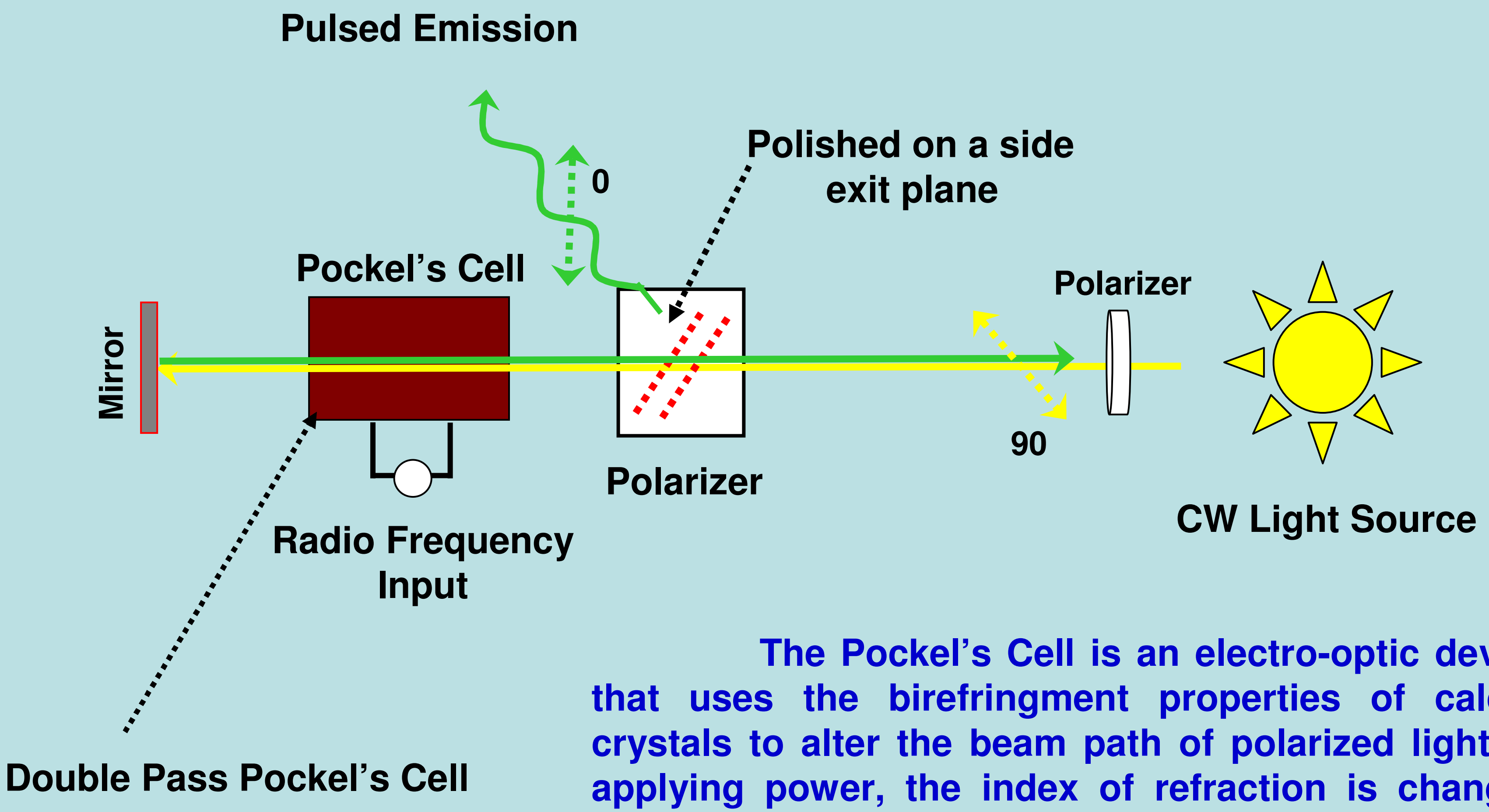
Directly Modulated Light Sources

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

Flash Lamps

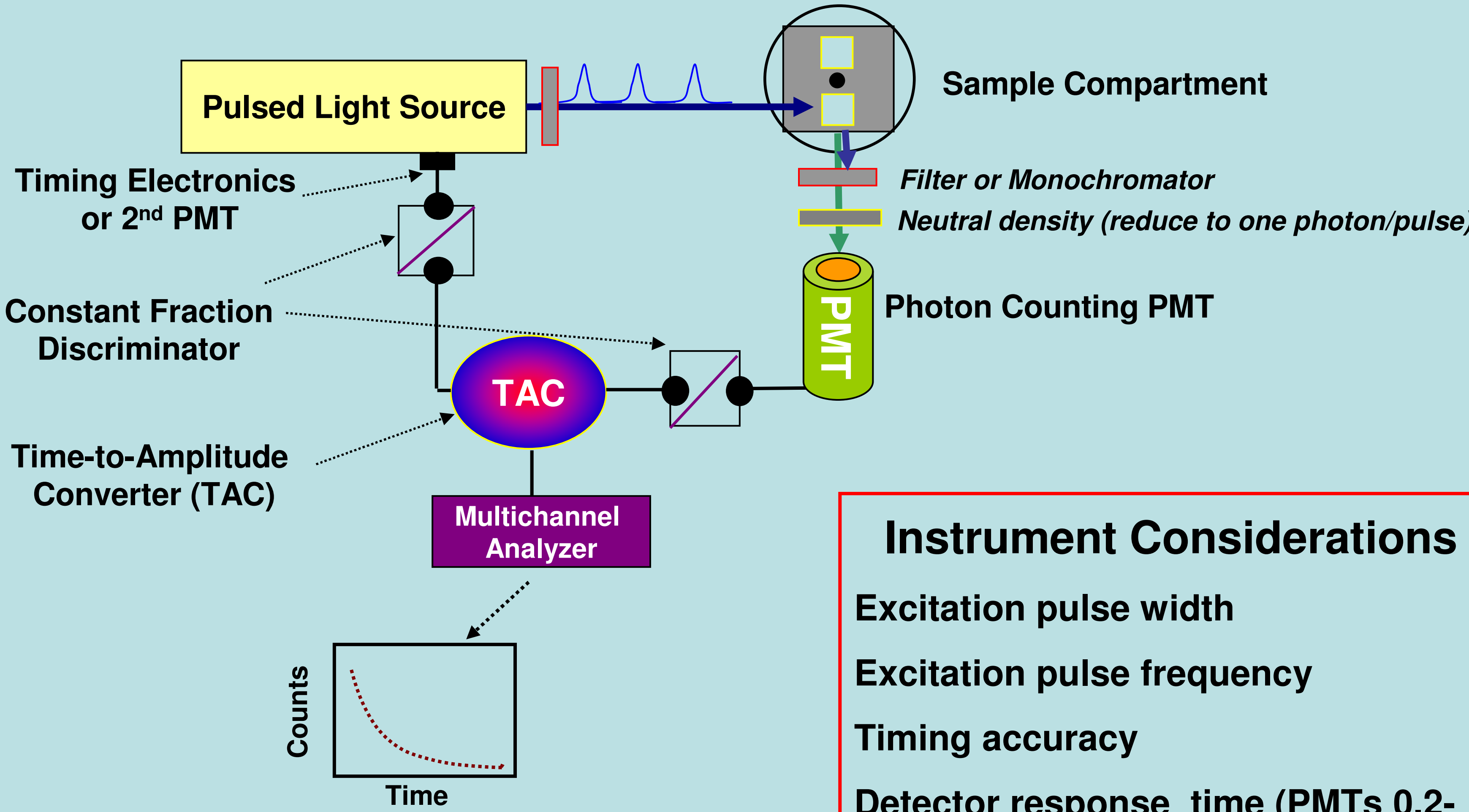
- Thyratron-gated nanosecond flash lamp (PTI), 25 KHz, 1.6 ns
- Coaxial nanosecond flashlamp (IBH), 10Hz-100kHz, 0.6 ns

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

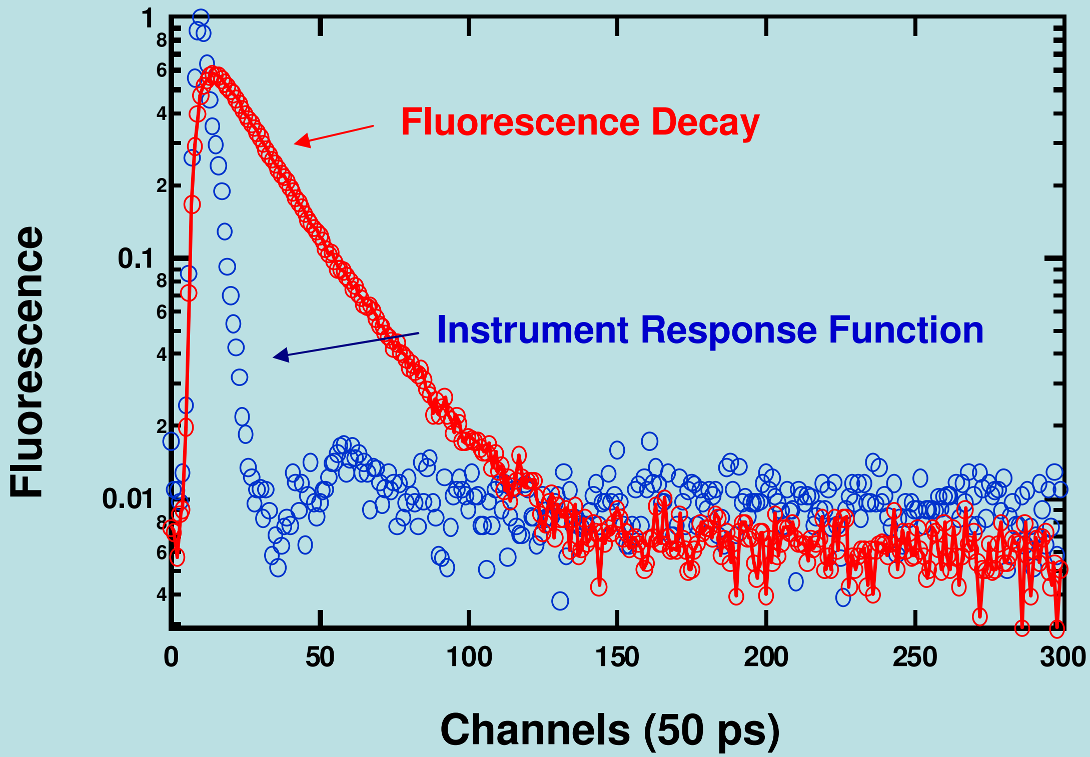
Excitation pulse width

Excitation pulse frequency

Timing accuracy

Detector response time (PMTs 0.2-0.9 ns; 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.

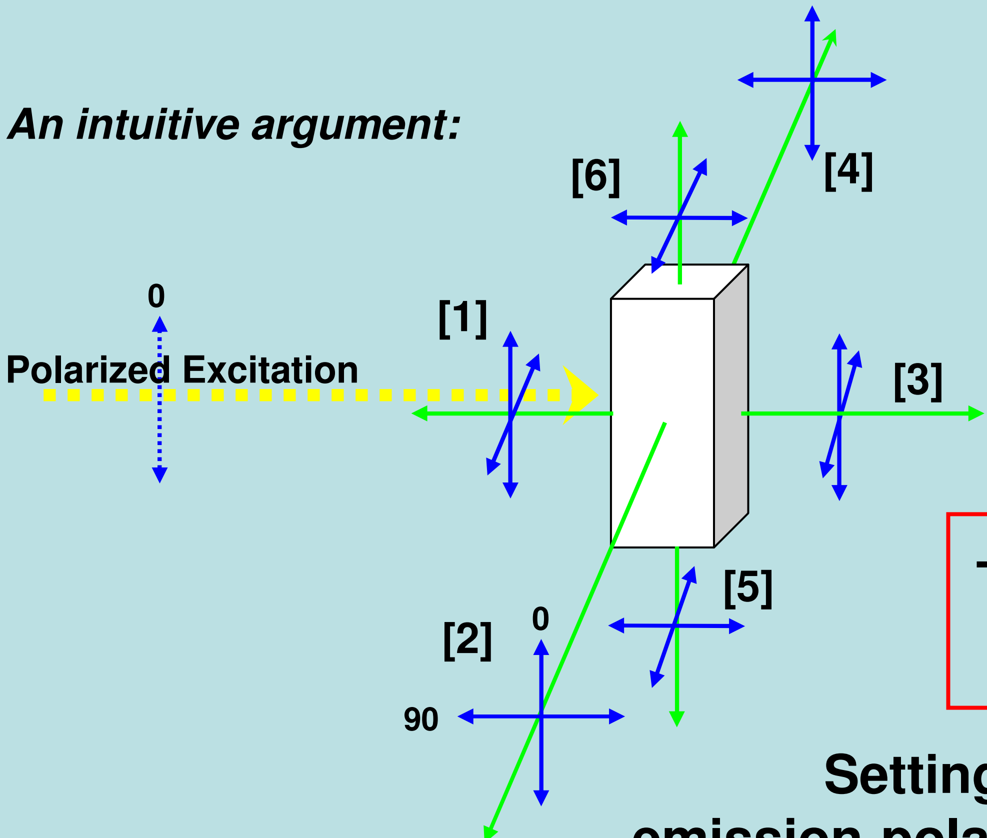
(2) The pulse frequency also influences the time window. An 80 MHz pulse frequency (Ti:Sapphire laser) would deliver a pulse every 12.5 ns and the pulses would interfere with photons arriving later than the 12.5 ns time.

Polarization Correction

There is still a polarization problem in the geometry of our excitation and collection (even without a monochromator)!!

Will the corrections never end ???

An intuitive argument:



$[1] = I_0 + I_{90}$
$[2] = I_0 + I_{90}$
$[3] = I_0 + I_{90}$
$[4] = I_0 + I_{90}$

$[5] = 2 \times I_{90}$
$[6] = 2 \times I_{90}$

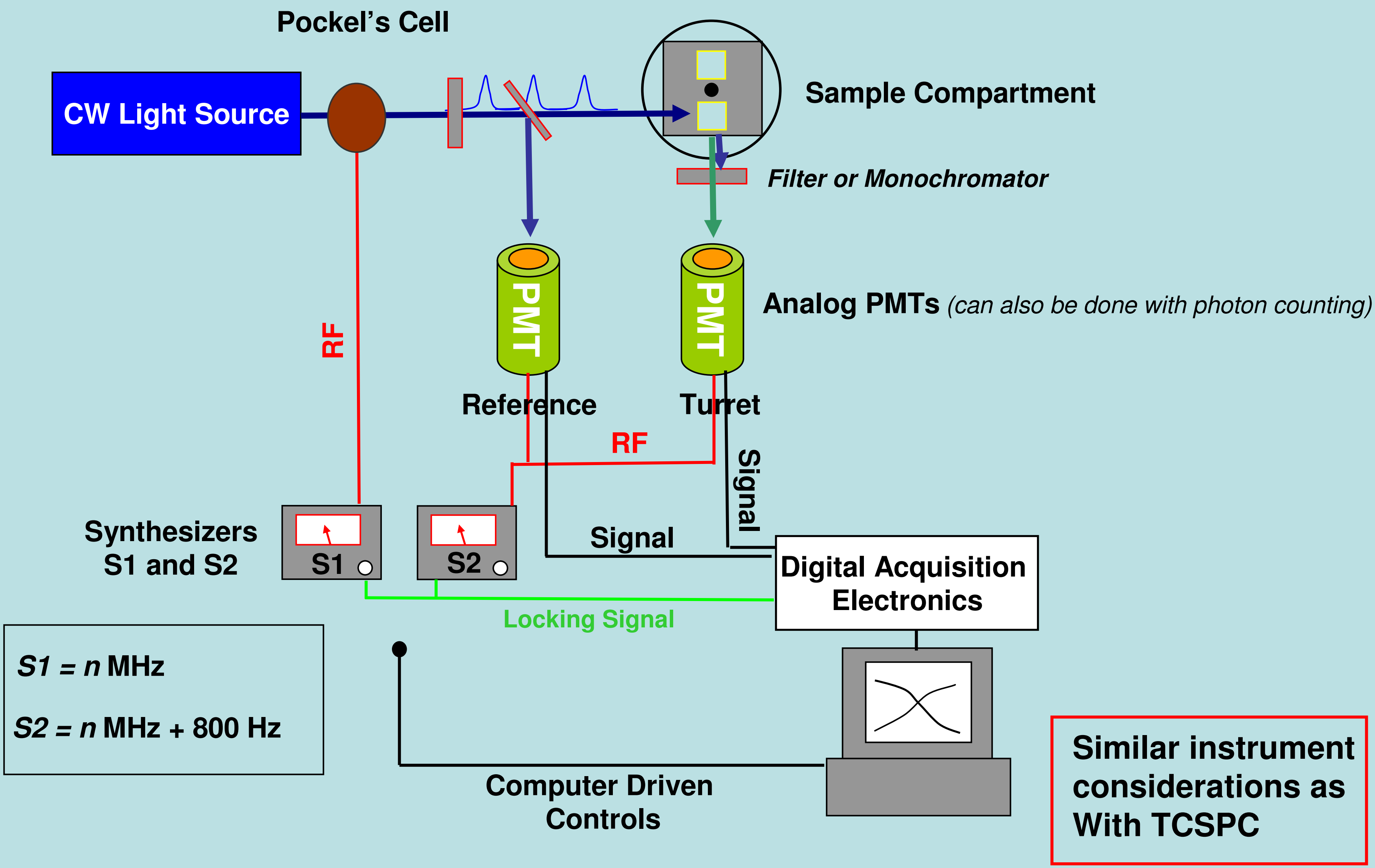
$\text{Total} = 4 \times I_0 + 8 \times I_{90}$

The total Intensity is proportional to:
$I_0 + 2 \times I_{90}$

Setting the <u>excitation angle to 0</u> and the <u>emission polarizer to 54.7</u> the proper weighting of the vectors is achieved.*

*Spencer & Weber (1970) J. Chem Phys. 52:1654

Frequency Domain Fluorometry



Lifetime Station #3, LDF, Champaign IL, USA