Basic Fluorescence Instrumentation

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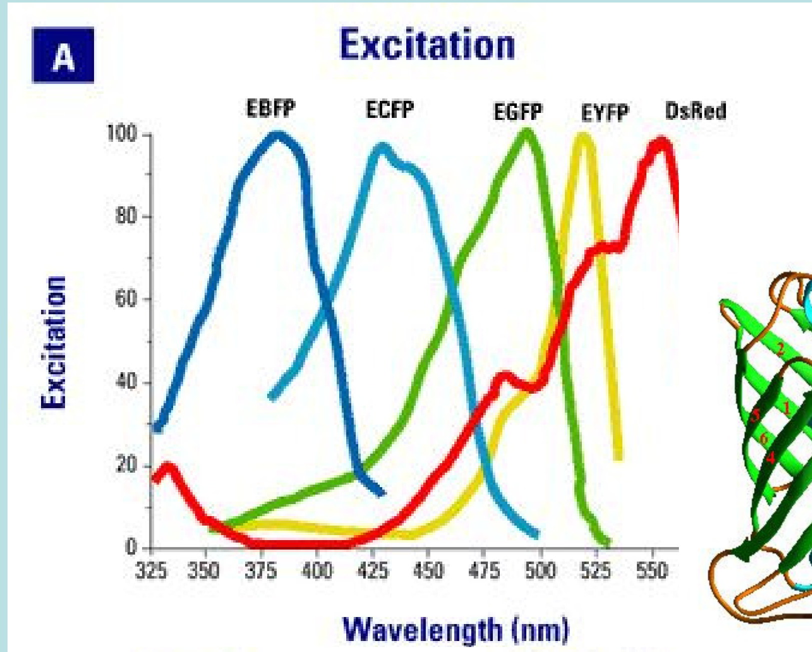
Slide acknowledgements Dr. Theodore Hazlett, Dr. Joachim Müller

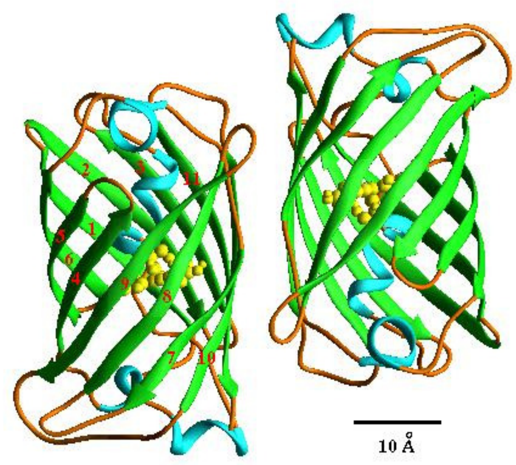
Create Fluorescence Contrast

	Color	Alexa Fluor Dye	Abs*	Em*
1		Alexa Fluor 350	346	442
2		Alexa Fluor 430	433	539
3		Alexa Fluor 488	495	519
4		Alexa Fluor 532	532	554
5		Alexa Fluor 546	556	573
6		Alexa Fluor 568	578	603
7		Alexa Fluor 594	590	617
8		Alexa Fluor 633 †	632	647
9		Alexa Fluor 660 †	663	690
10		Alexa Fluor 680 †	679	702

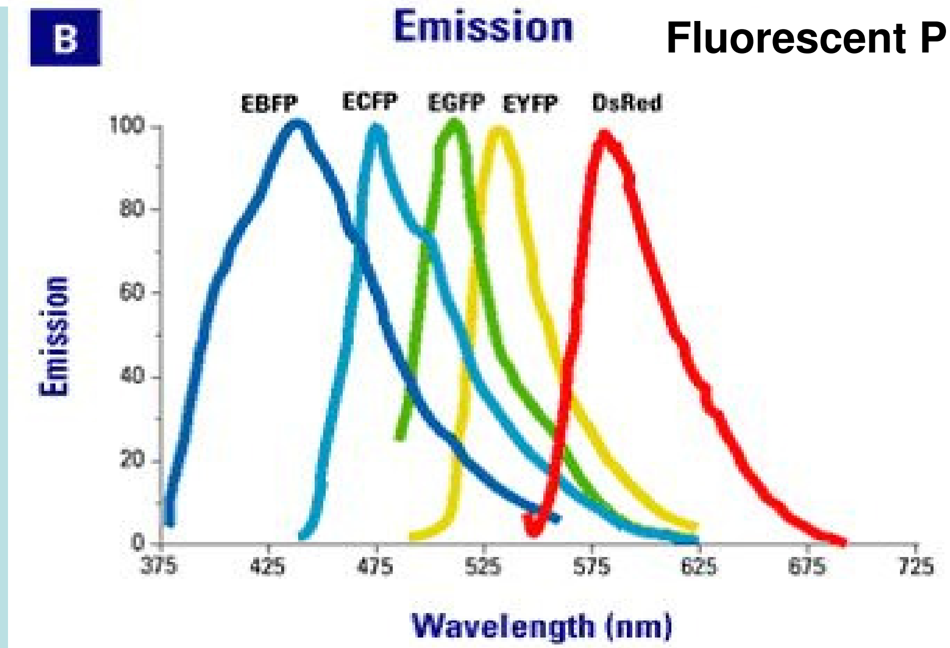
* Approximate absorption (Abs) and fluorescence emission (Em) maxima for conjugates, in nm.
† Human vision is insensitive to light beyond ~650 nm, and therefore it is not possible to view the far-red–fluorescent dyes by looking through the eyepiece of a conventional fluorescence microscope.
Colors in this table match the emission colors in the spectra to the right.

Bright robust dyes

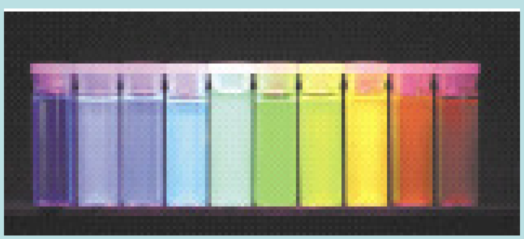




Fluorescent Proteins



Quantum dots



Functionalized Nanoparticles

Fluorometry

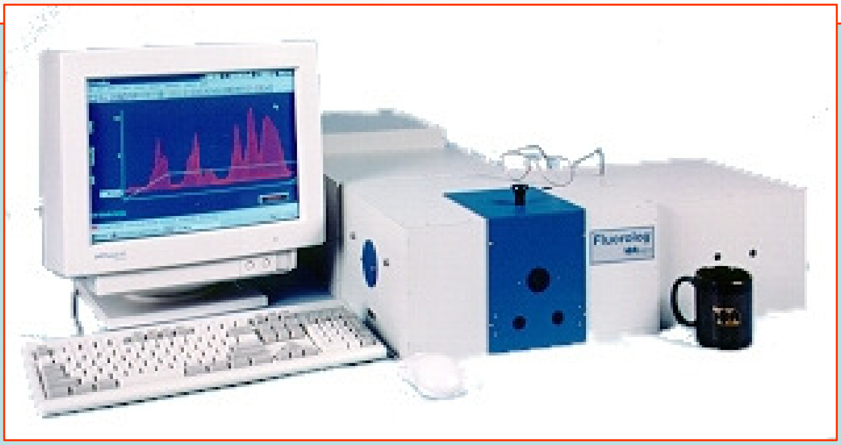
Collecting Spectra, Polarization, Kinetics, Lifetimes …

- Instrument functioning and ageing
- Verify sample identity and integrity
- Verify optimum excitation and emission wavelengths
- Verify levels of scattered excitation and Raman signals
- Impurities in solvent, buffer or sample
- Preparation and validation for FCS, Lifetime and FLIM …

- Elucidate solvent, temperature, pH, aggregation effects … surfaces, films, substrates, molecule orientation …

Fluorometers

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



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

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

More Examples of Fluorescence Based Instrumentation

Tecan ULTRA Evolution Plate Reader (Tecan Trading AG, Männedorf / Zürich, Switzerland)

TECAN.

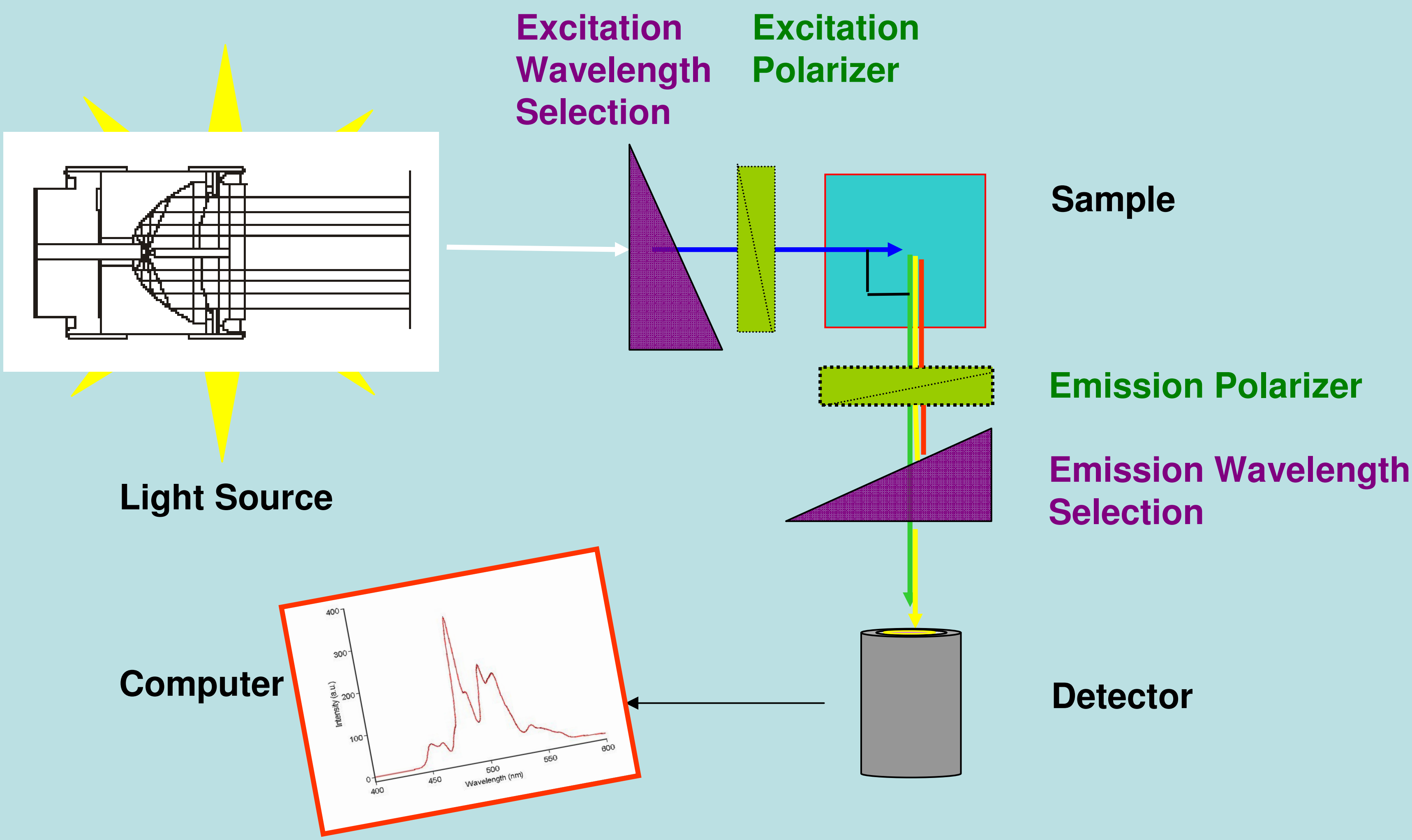
Zeiss LSM 510 META Optical Confocal Microscope (Carl Zeiss AG, Jena, Germany)

ZEISS

Becton Dickinson BD FACSCanto Fluorescence Assisted Cell Sorter (FACS)

BD

Main Fluorometer Components



Note: Polarizers can slide in and out of the optical path

Fluorometer Components

Light Source

Sample Compartment

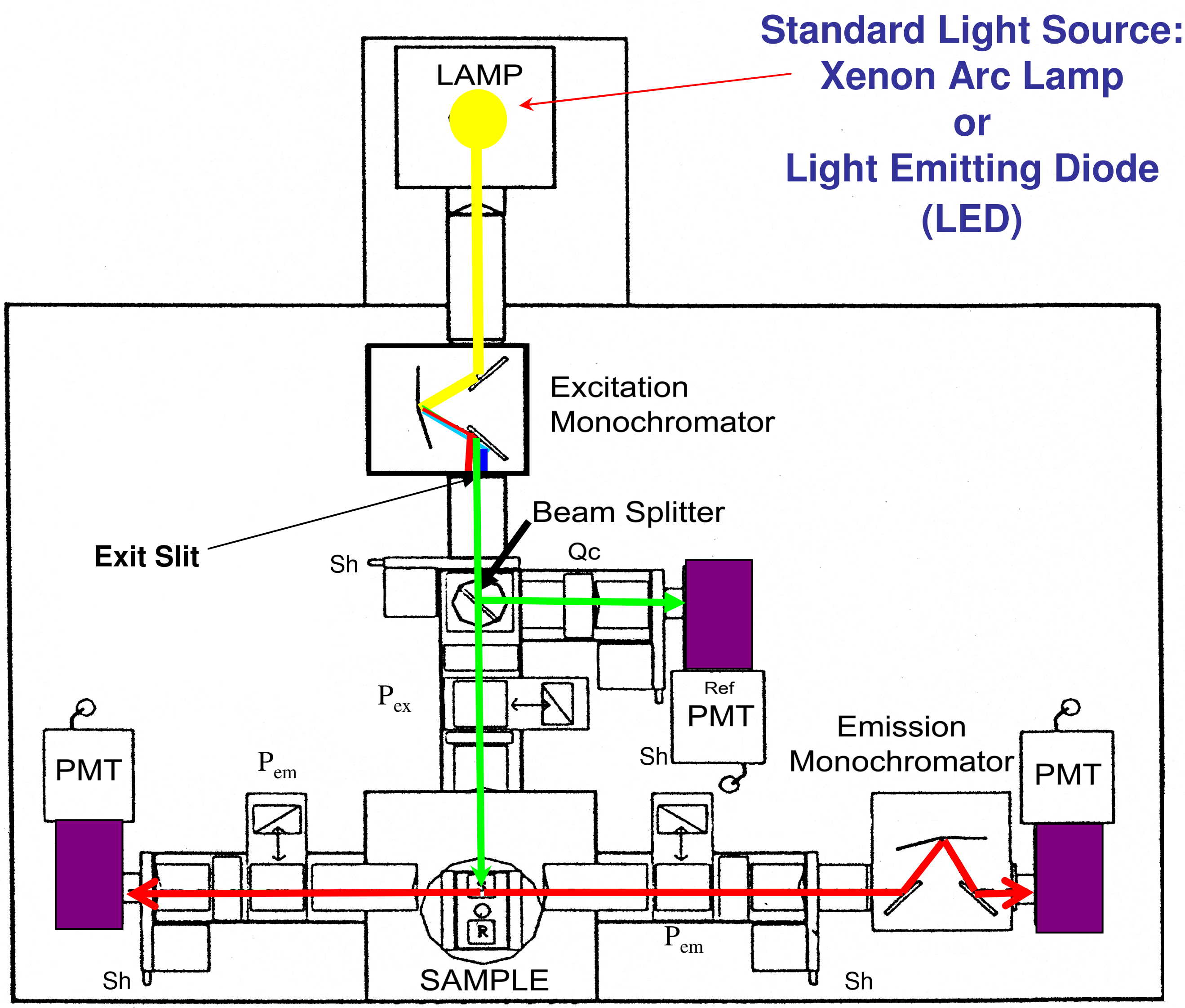
Detectors

Wavelength Selection

Polarizers

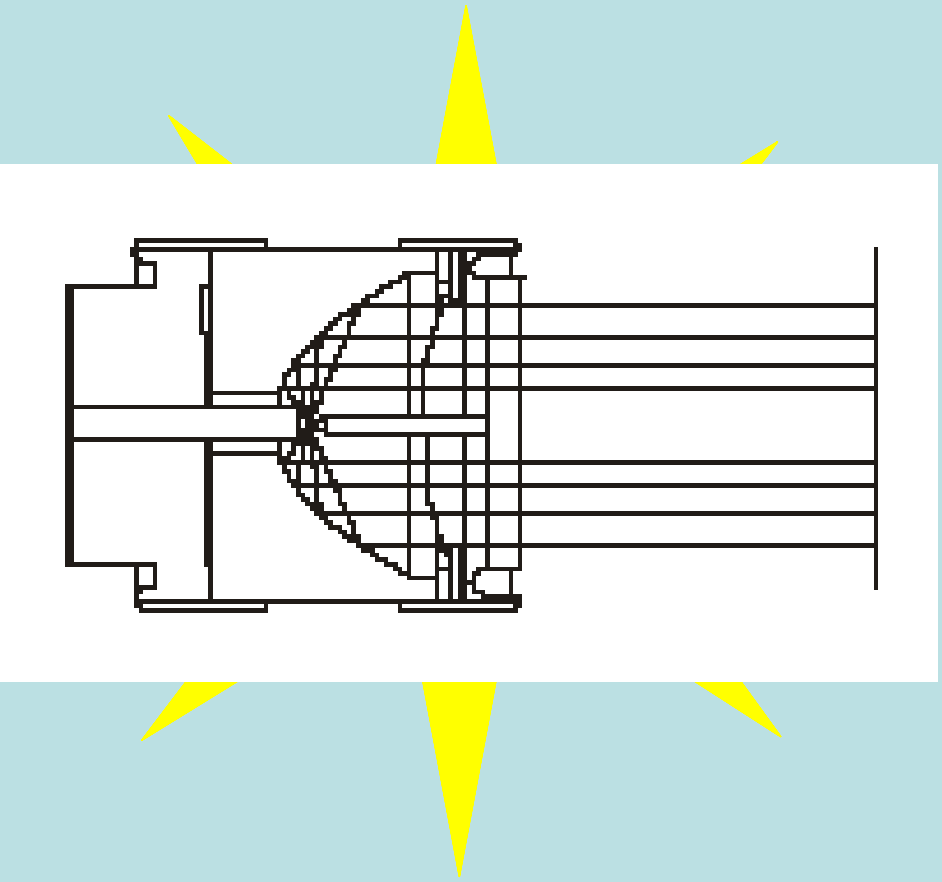
Computer & Software

The Laboratory Fluorometer



ISS (Champaign, IL, USA) PC1 Fluorometer

LIGHTSOURCES

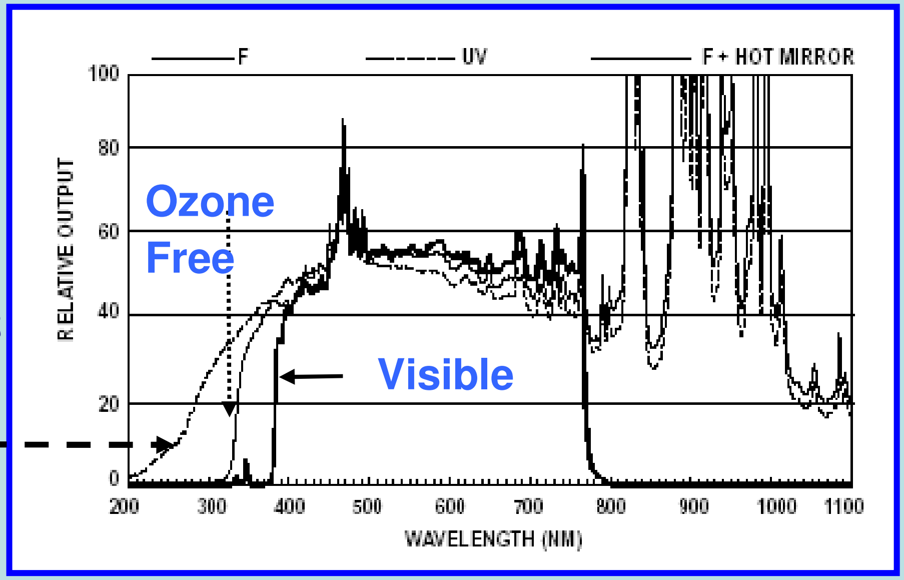


Lamp Light Sources: Arc Lamps (1)

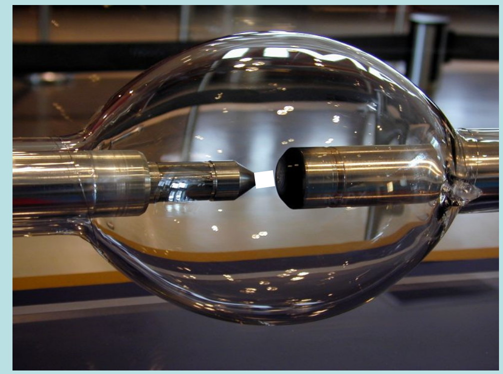
1. Xenon Arc Lamp

Lamp Emission Spectra:

UV

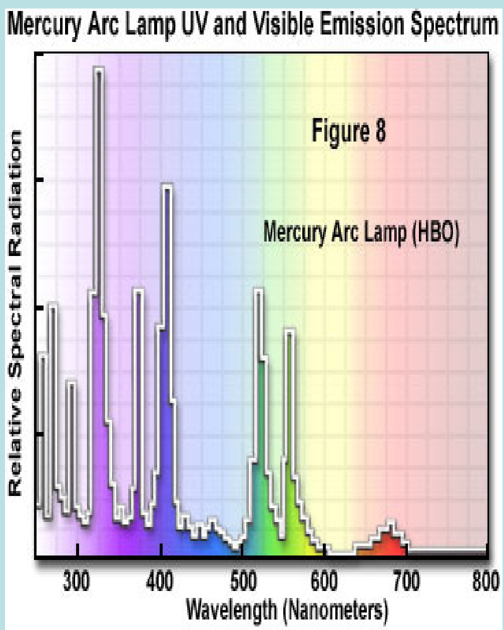


(wide range of wavelengths)



15 kW Xenon arc lamp

2. High Pressure Mercury Lamps

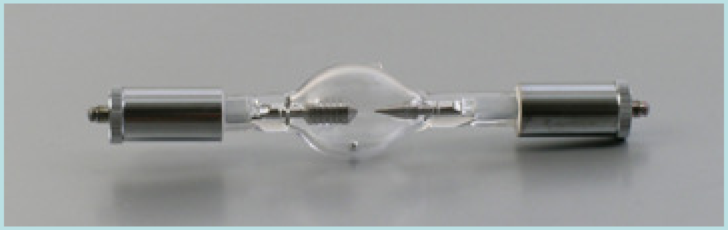


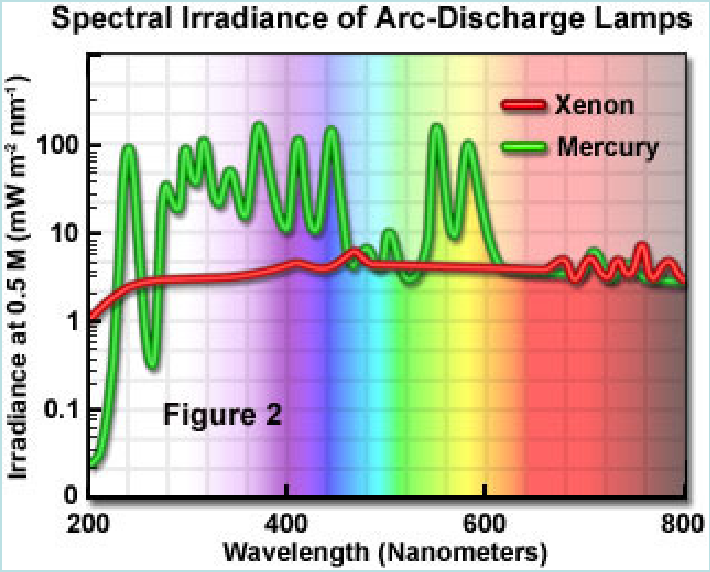
(High Intensities but concentrated in specific lines)

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

Lamp Light Sources: Arc Lamps (2)

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





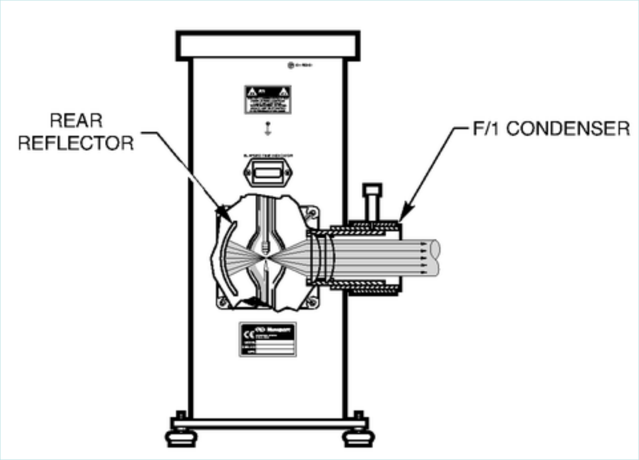
http://microscopy.fsu.edu/primer/anatomy/lightsources

ARC LAMP ISSUES:

- Lifetime
- Stability (flicker + drifts)
- Safety
  - •high internal gas pressures (potential eye damage)
  - •hot
  - •never stare into burning lamp
  - •do not touch with bare hands (fingerprints on quartz lamp envelope)

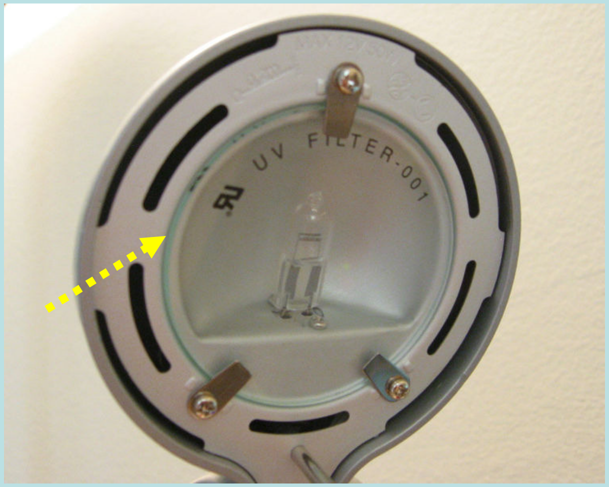
LAMP HOUSING + OPTICS :

Conventional OR **Compact**



Lamp Light Sources: Incandescent

4. Tungsten-Halogen Lamps



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

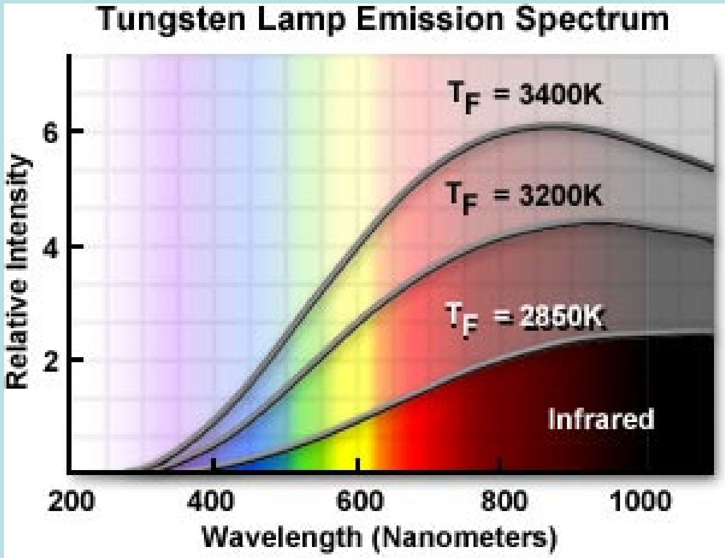
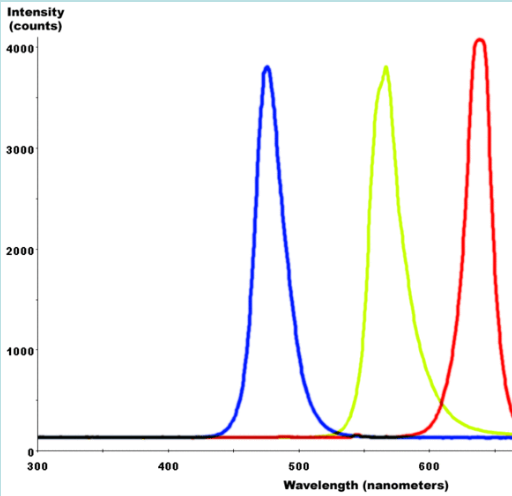


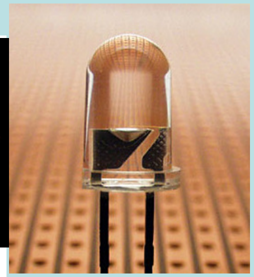
Figure 2

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

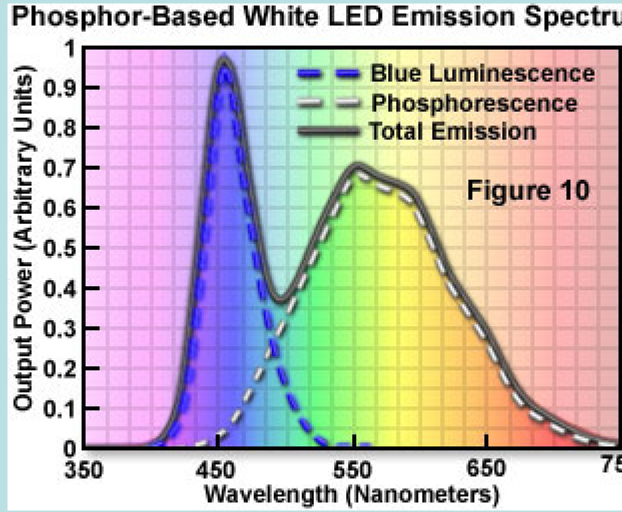
Lamp Light Sources: Semiconductor (1)

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

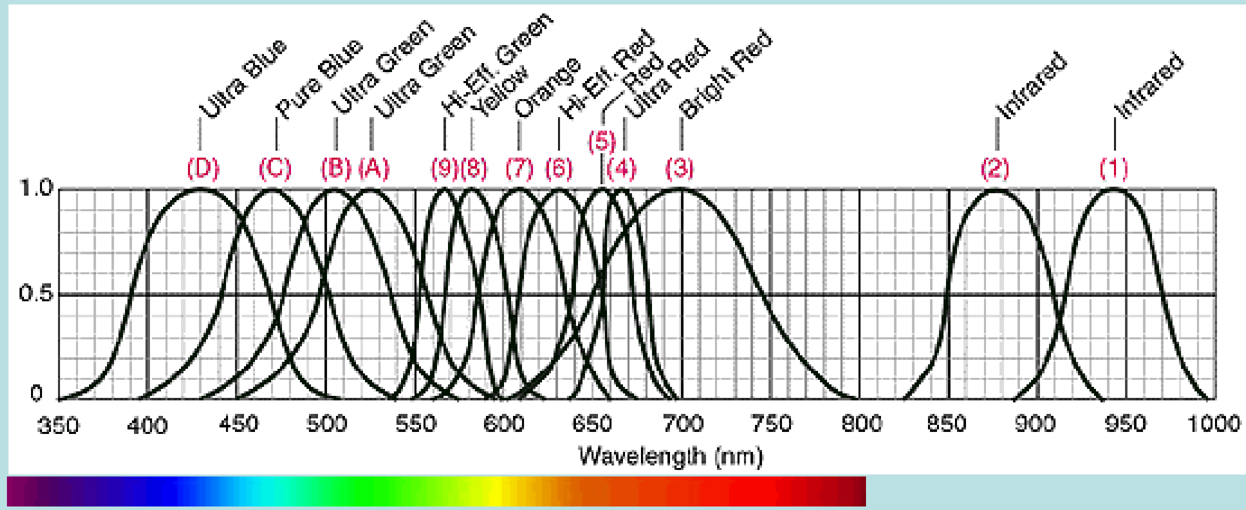
Superbright LED

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

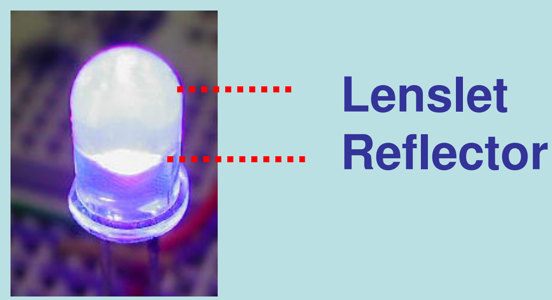
Lamp Light Sources: Semiconductor (2)

5. Light Emitting Diodes (LEDs)

Wavelengths from 260 nm to 2400 nm

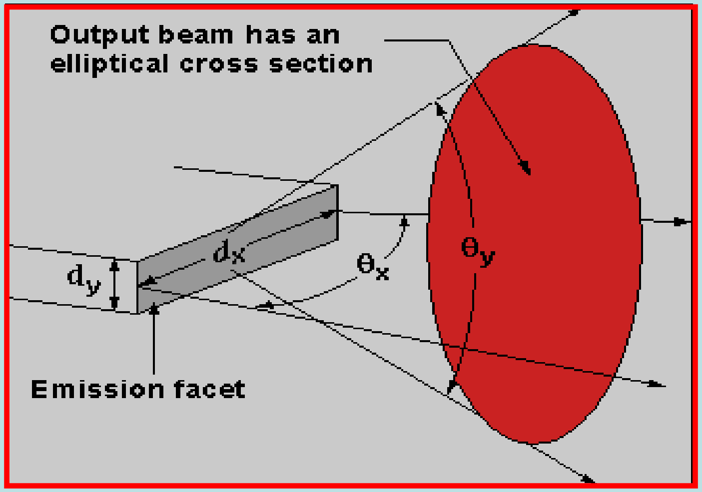


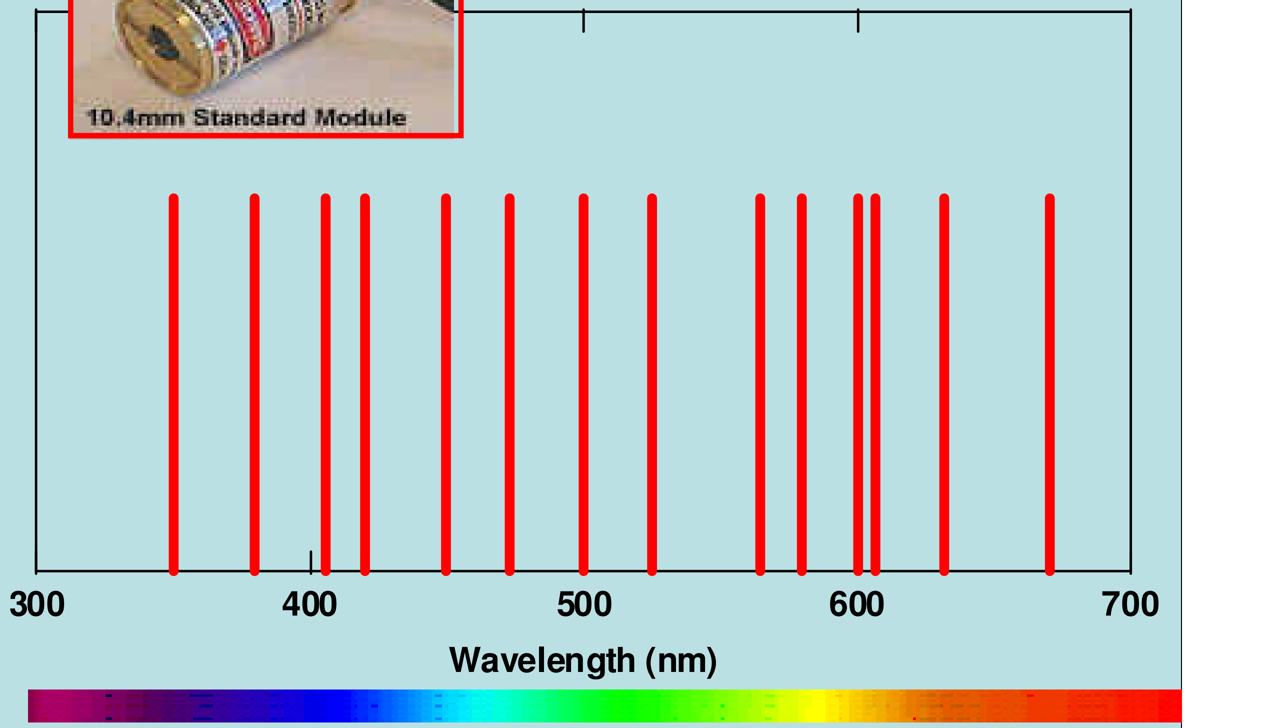
Deep – UV LEDs $\lambda \approx 260\ \mathrm{nm}$



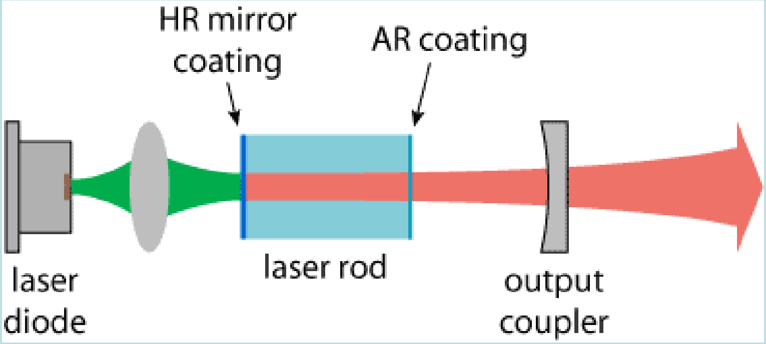
Near UV LED

Laser Light Sources: Diode Lasers





(DPSS) Diode-pumped solid state laser



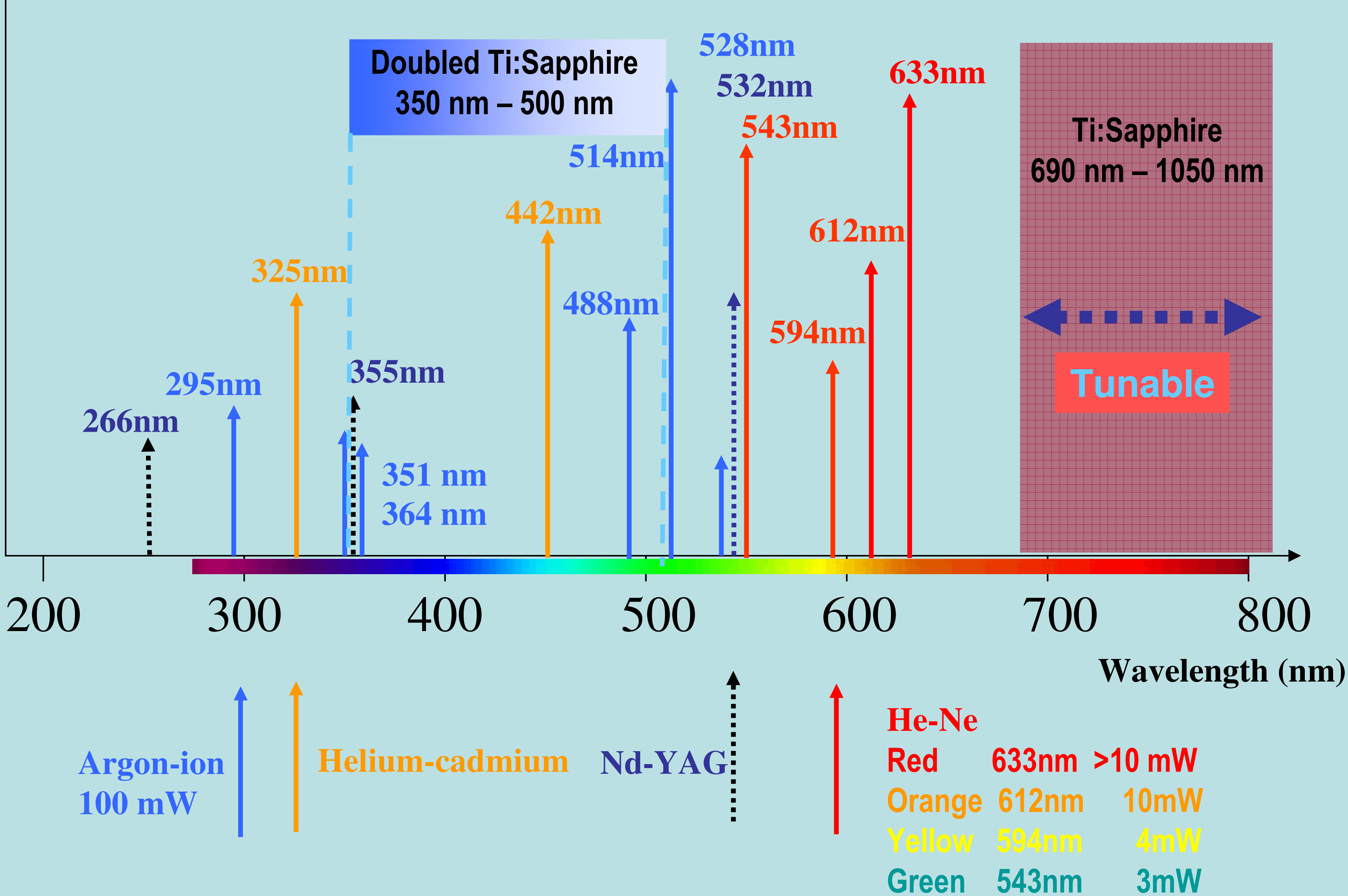
Many Wavelengths (nm) Available:

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

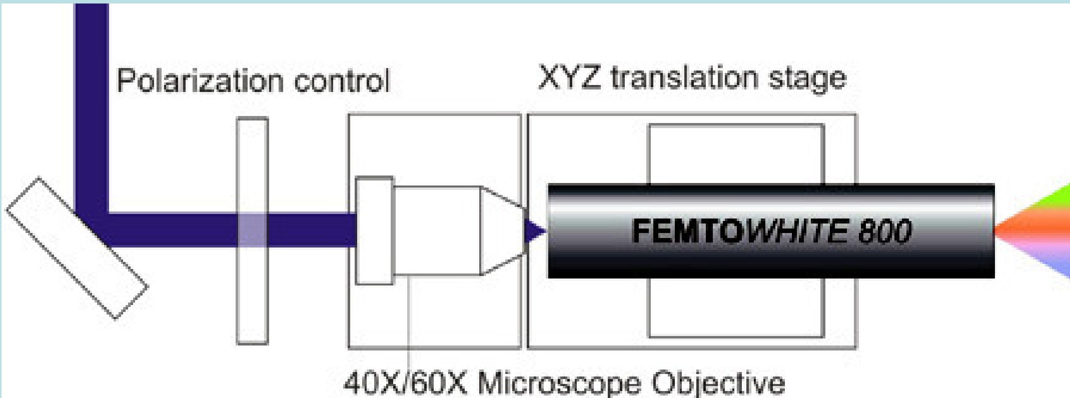
Laser Light Sources

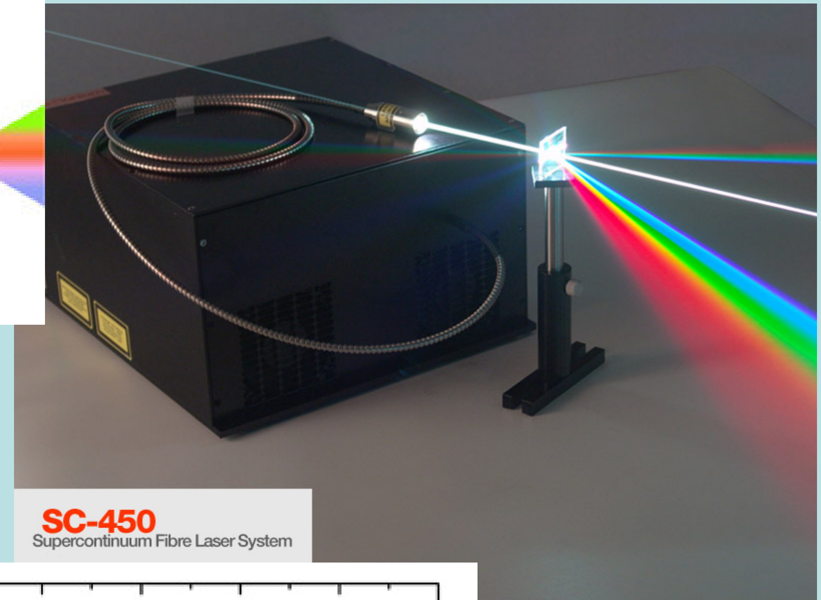
Argon Ion:

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

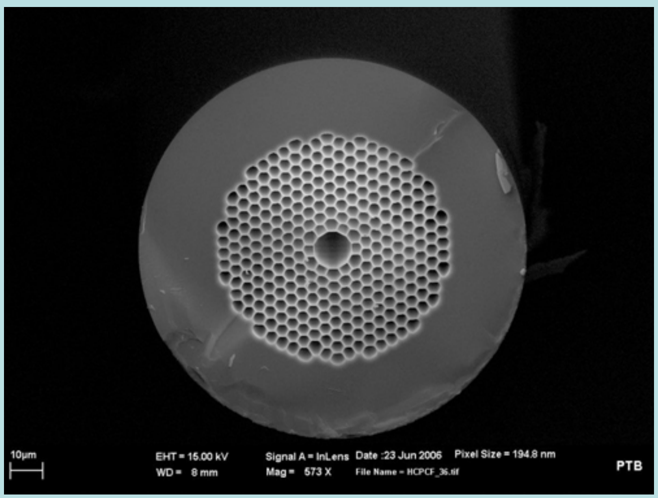


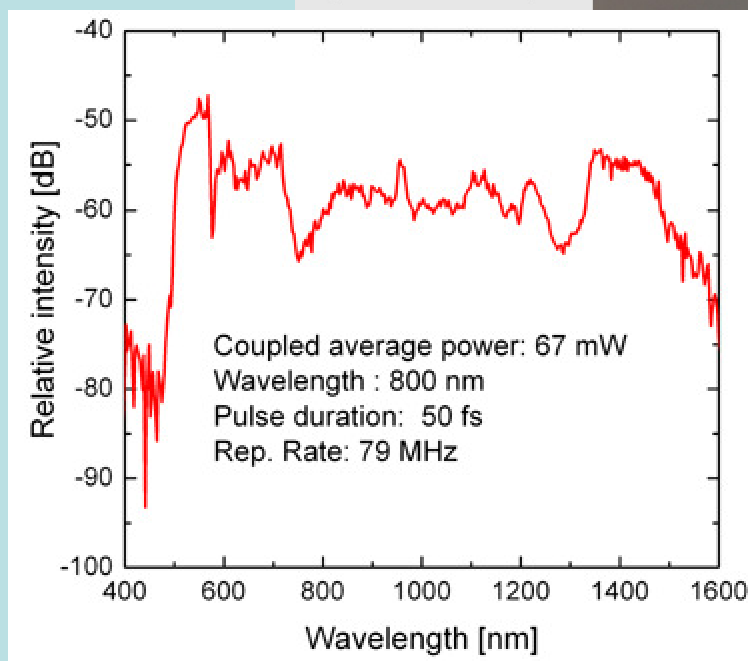
Supercontinuum White Light





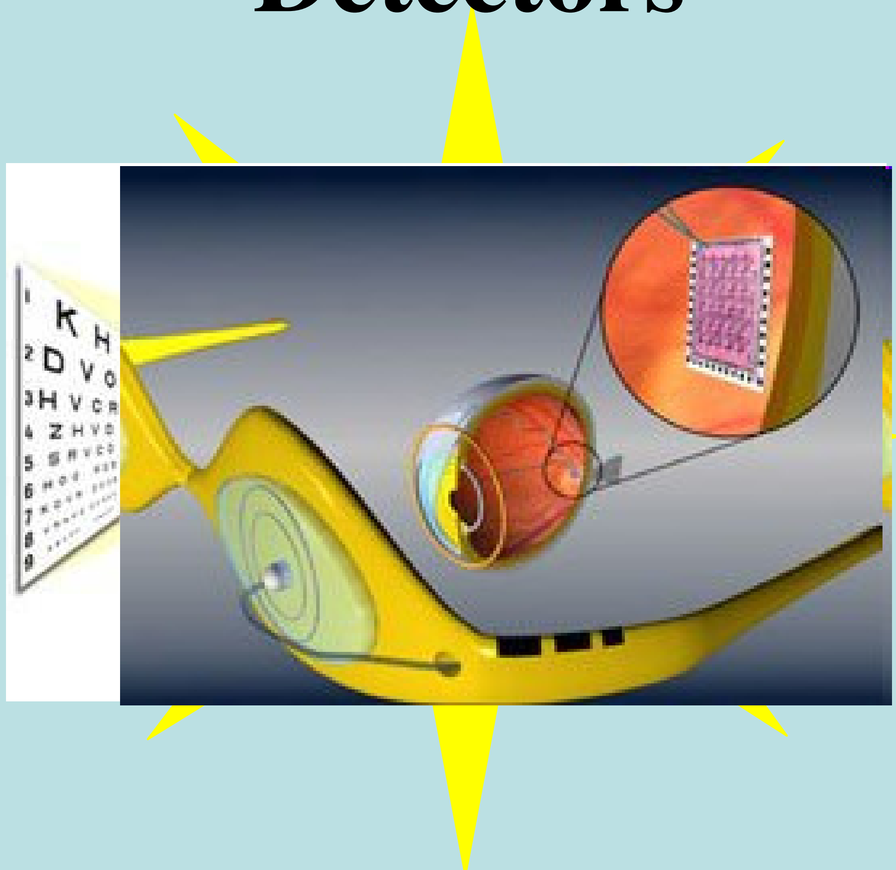
Ultrashort pulsed light focused into photonic crystal fiber





Photonic crystal fiber optic

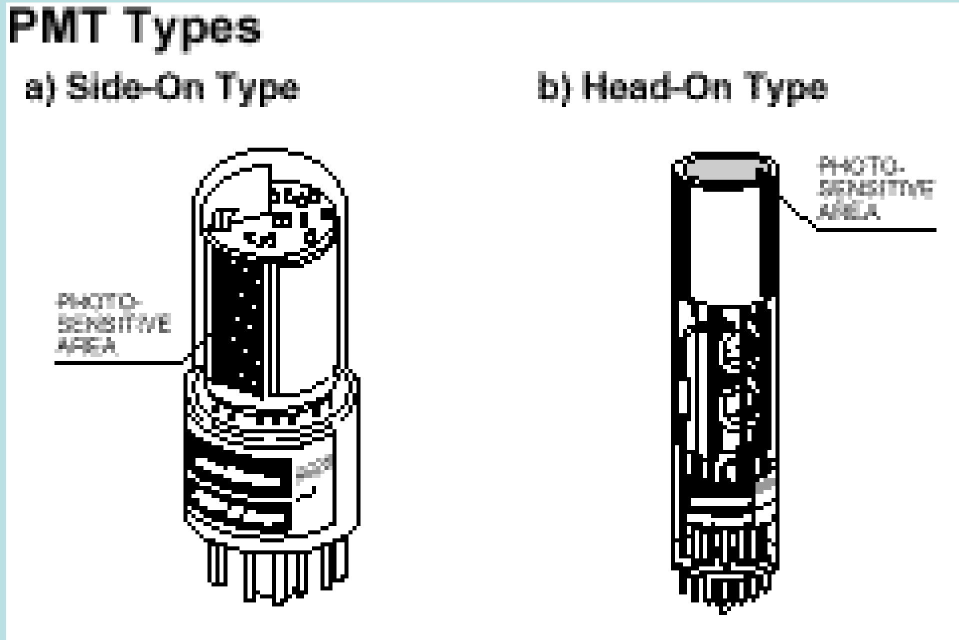
Detectors



Conversion of Light into an Electrical Signal

Non-Imaging Detector:

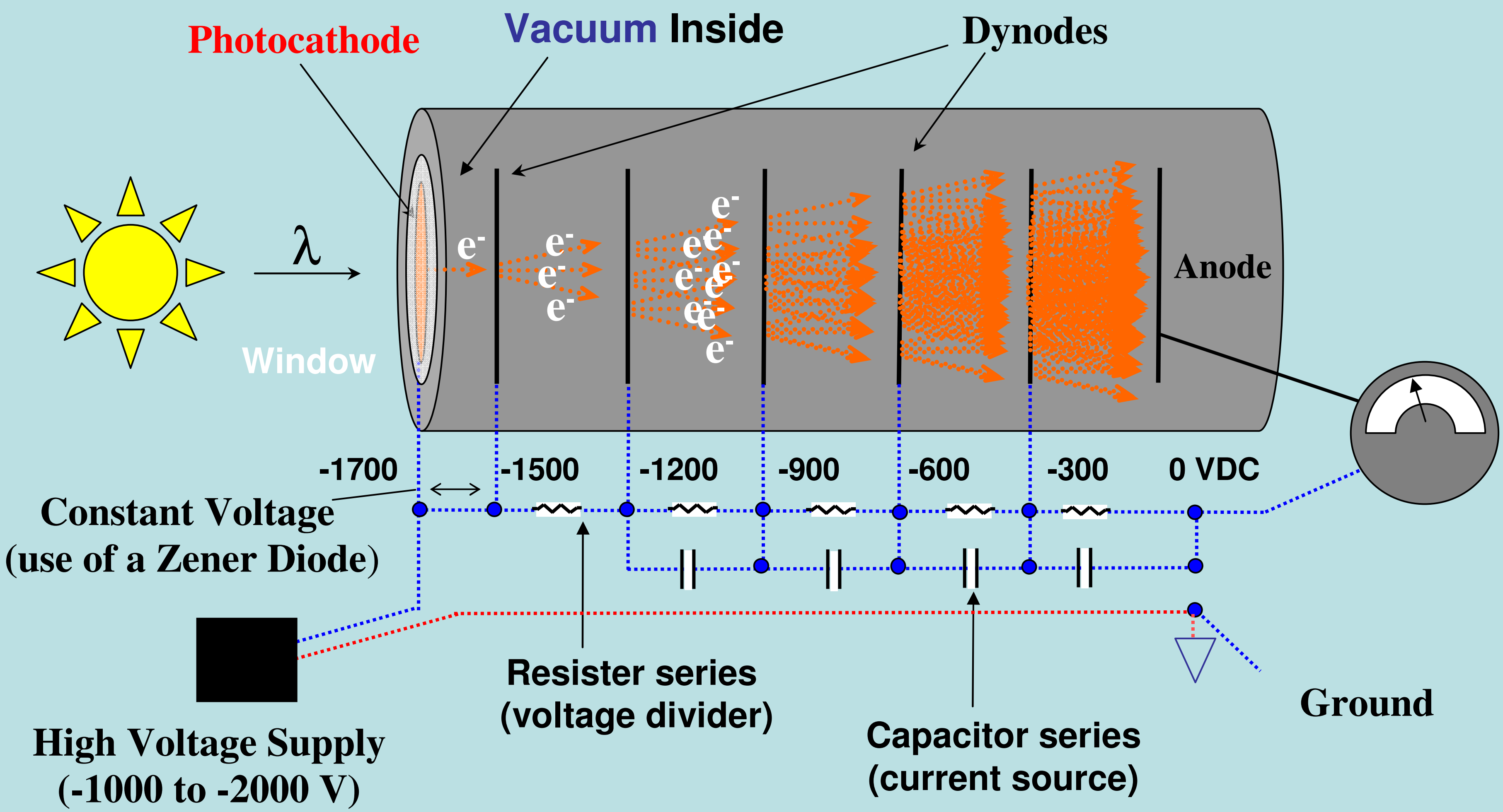
Photomultiplier (PMT)



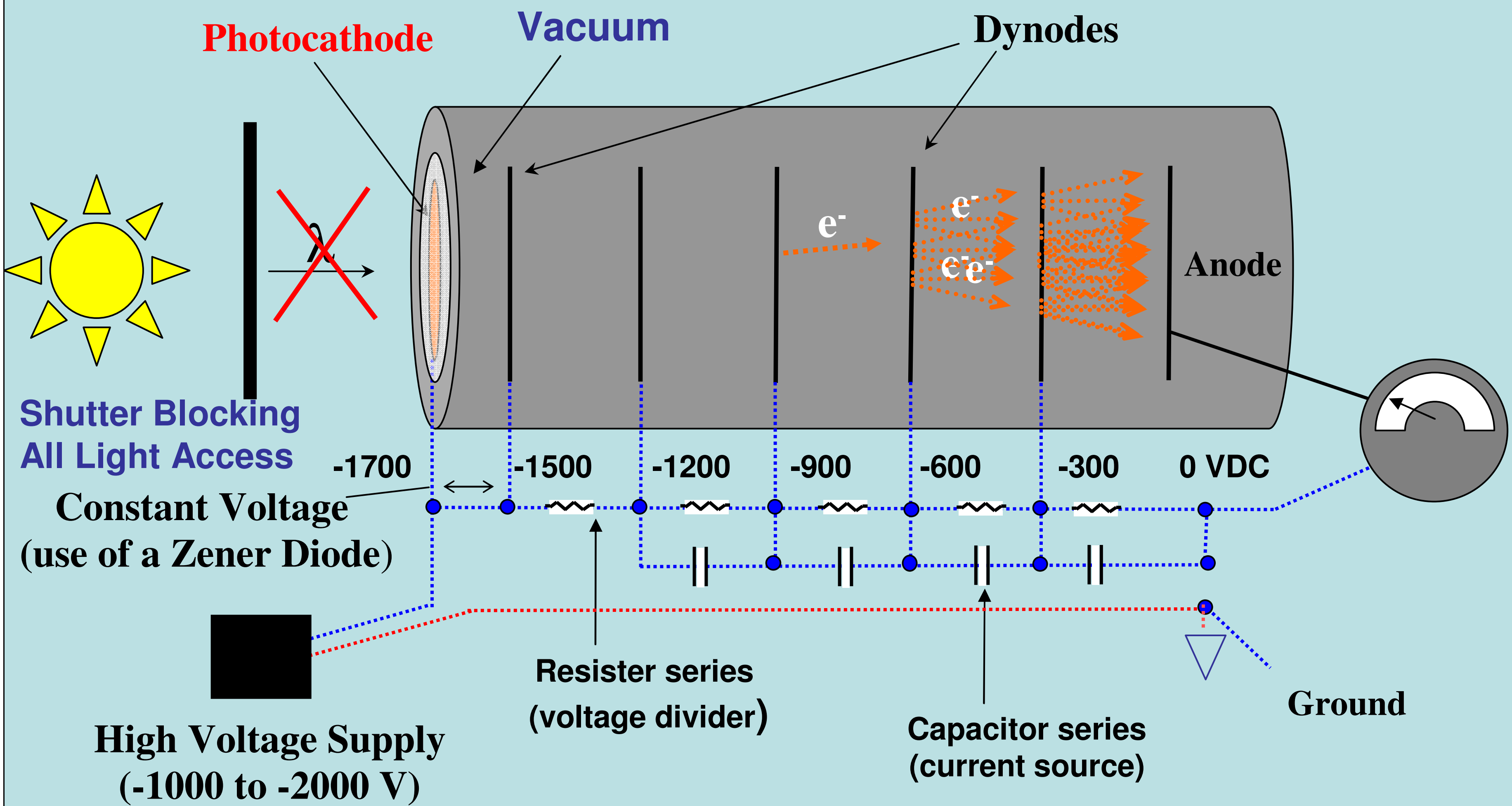
Imaging Detector: Microchannel Plate (MCP) PMT

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

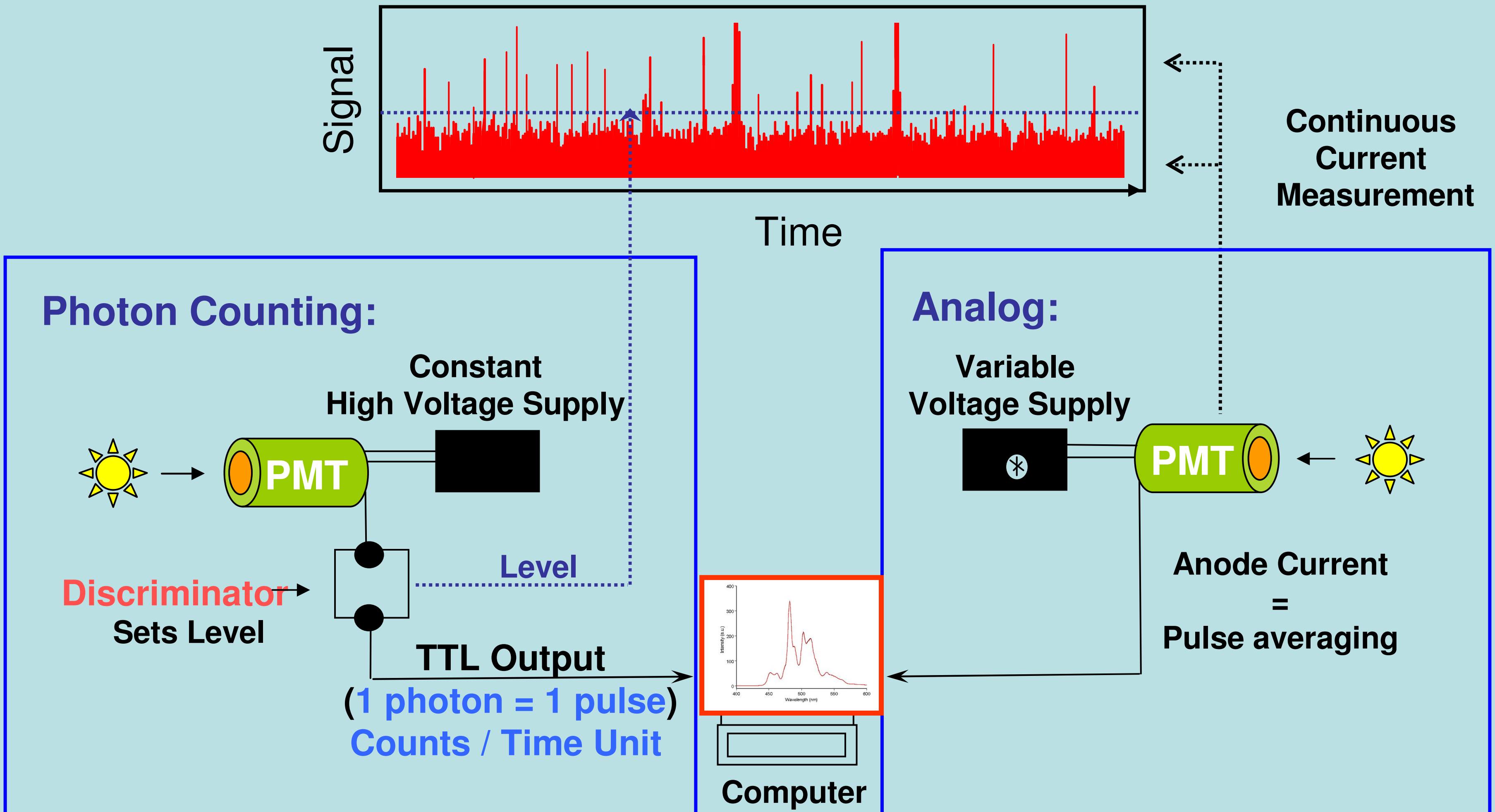
The Classic PMT Design
End-On Tube



The Detector Dark Signal



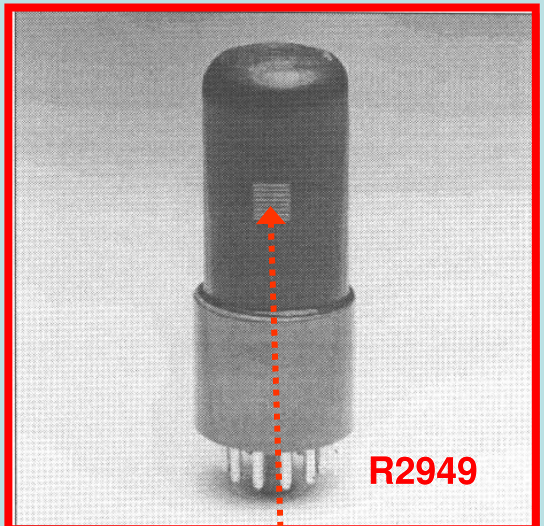
Photon Counting (Digital) and Analog Detection



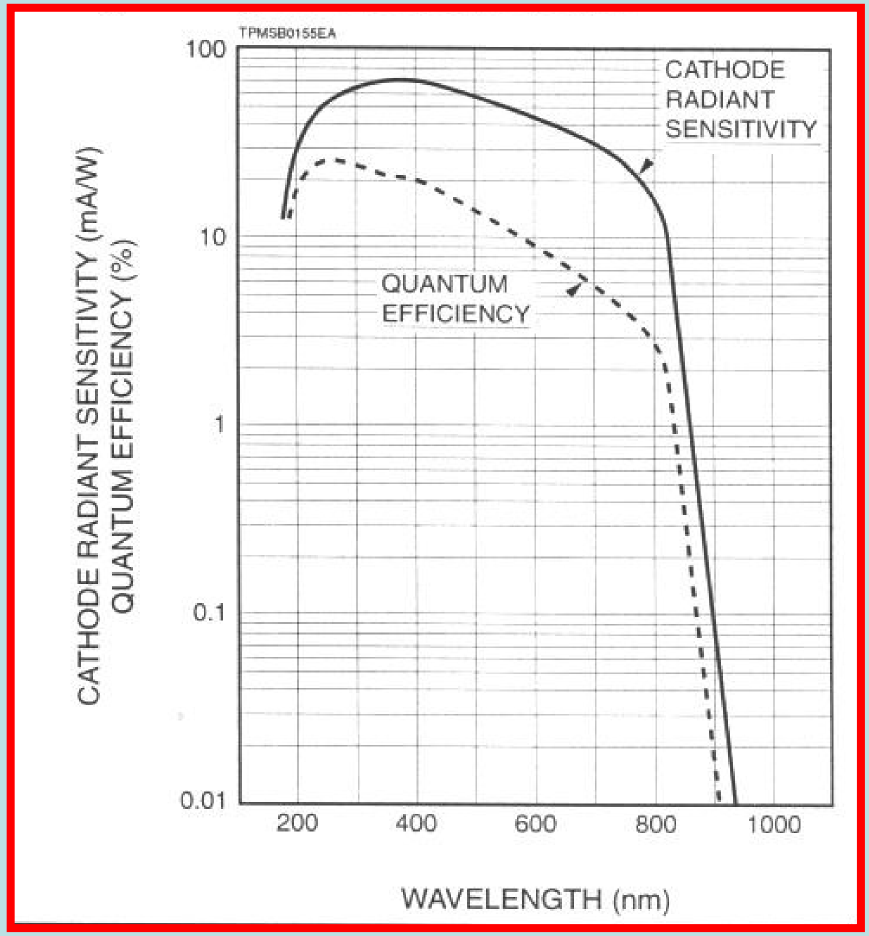
Primary Advantages:
1. Sensitivity (high signal/noise)
2. Increased measurement stability
3. Digital signals

Primary Advantage:
1. Broad dynamic range
2. Adjustable range

Hamamatsu R928 PMT Family
Side-On Tube



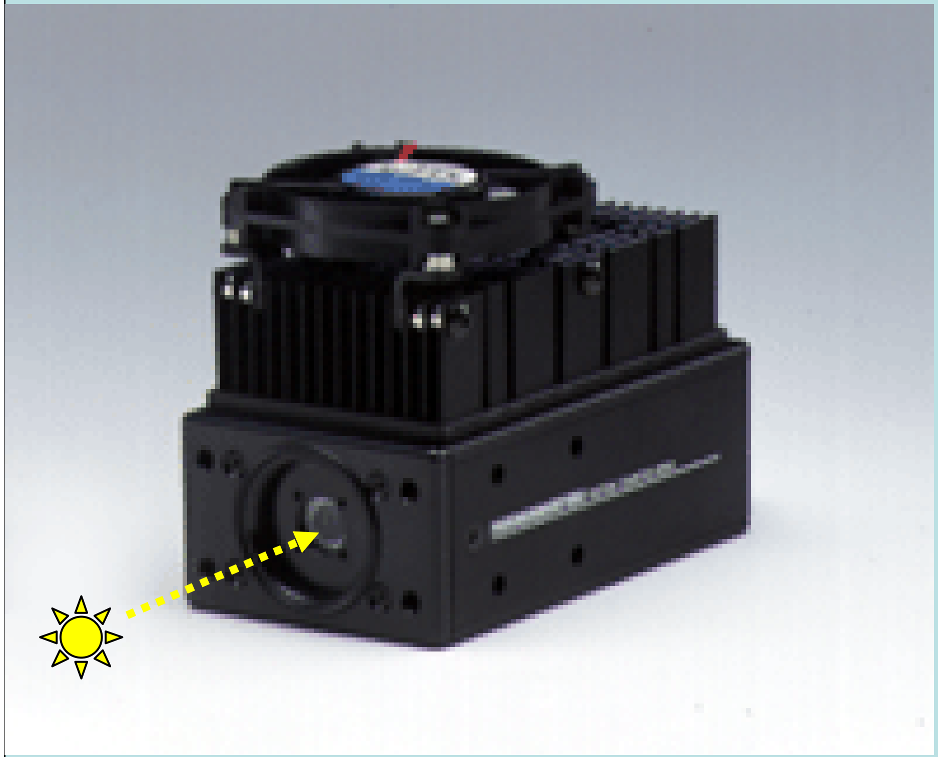
Quartz Window with Photocathode Beneath



Wavelength Dependent Quantum Efficiency

Hamamatsu H7422P-40 PMT

P : selected for photon counting



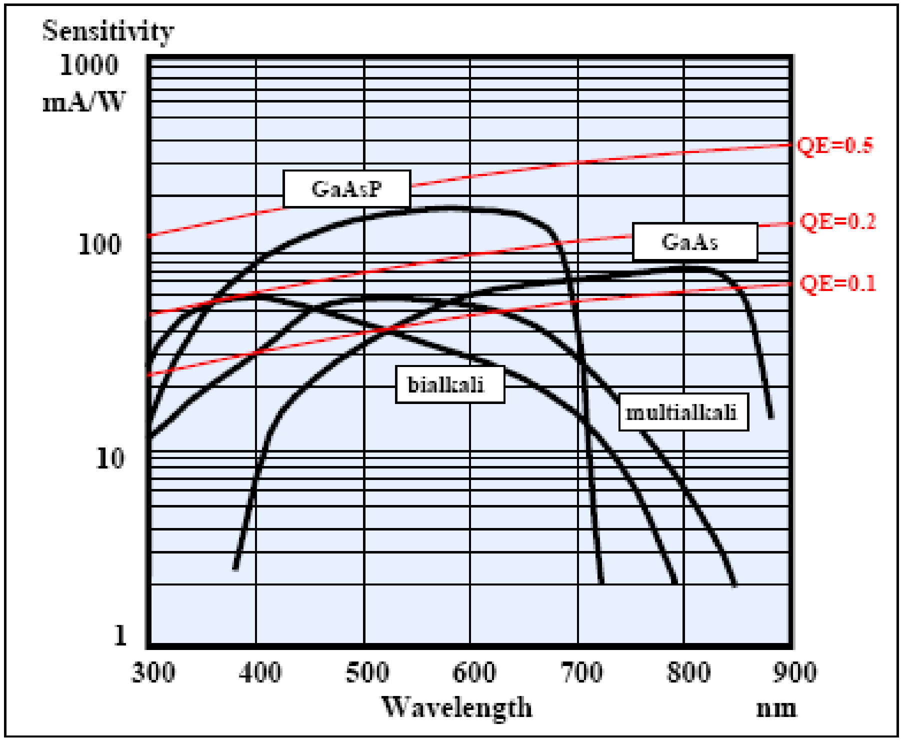


Fig. 13: Sensitivity of different photocathodes [34]

40% Quantum Efficiency

300 – 720 nm GaAsP spectral response

Time resolution 150 – 250 psec

After-pulsing at highest gain

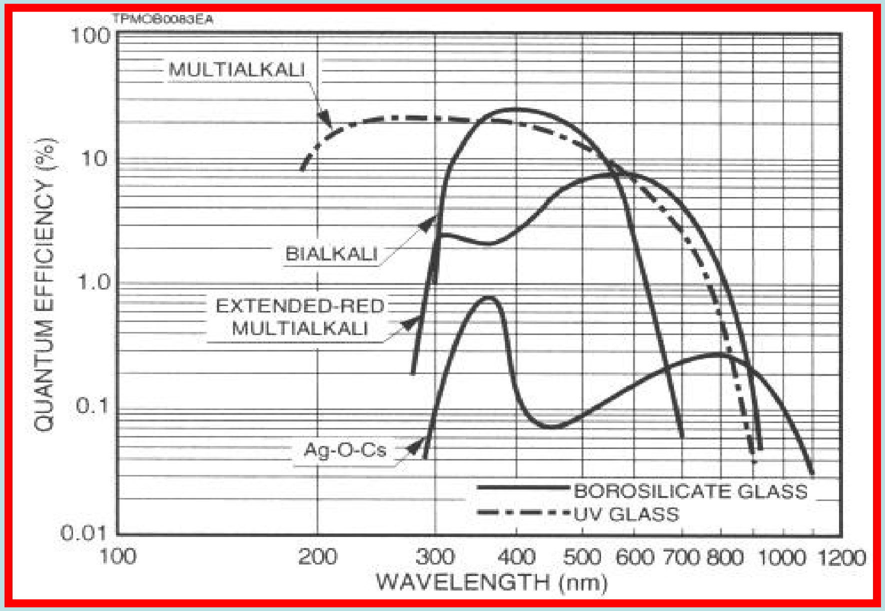
http://usa.hamamatsu.com/hcpdf/parts_H/H7422_series.pdf

http://www.hpk.co.jp/Eng/products/ETD/pmtmode/m-h7422e.htm

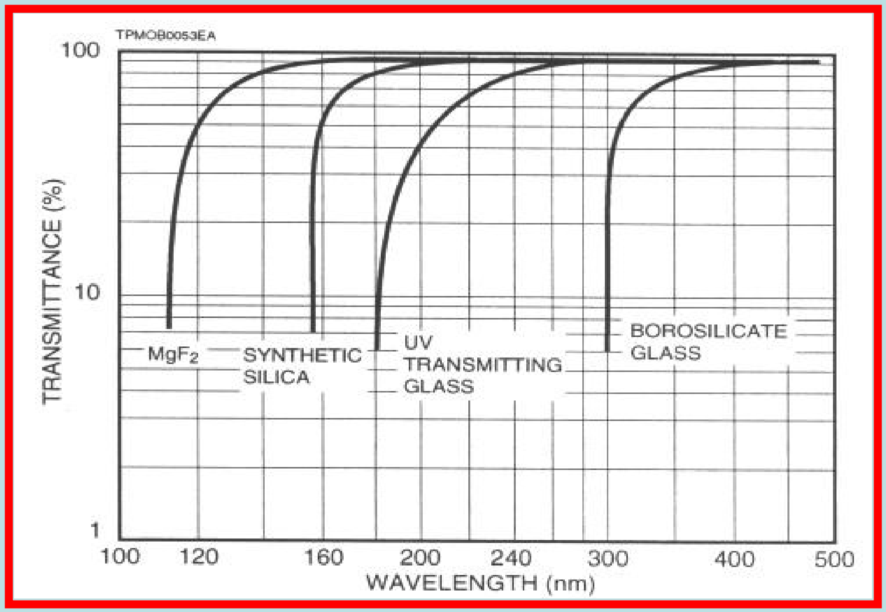
http://www.becker-hickl.de/pdf/tcvgbh1.pdf

PMT Quantum Efficiencies

Cathode Material

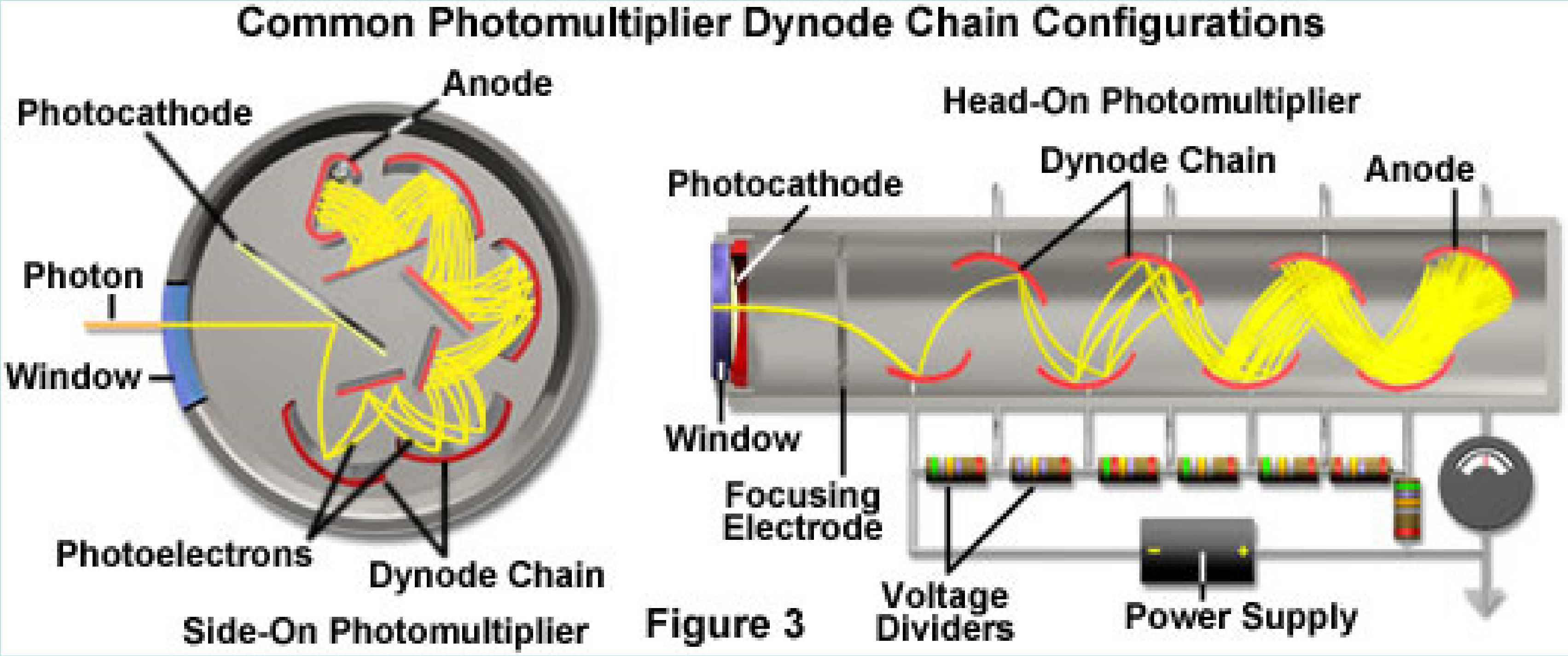


Envelope Window Material



PMT Geometries

Side-On PMT — **Head-On PMT**



Opaque photocathode

Semitransparent Photocathode

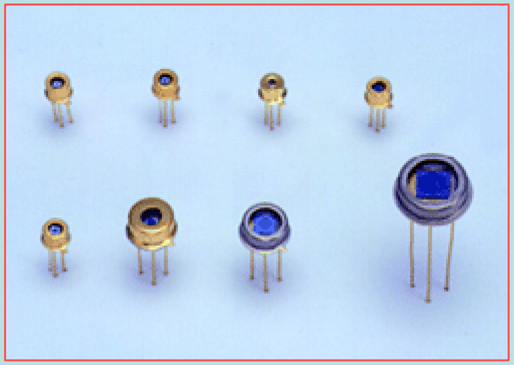
Slightly enhanced quantum efficiency

Smaller afterpulsing
Count rate linearity better
Better spatial uniformity

Faster response time (compact design)
Less affected by a magnetic field

Avalanche Photodiodes (APDs)

APD for analog detection

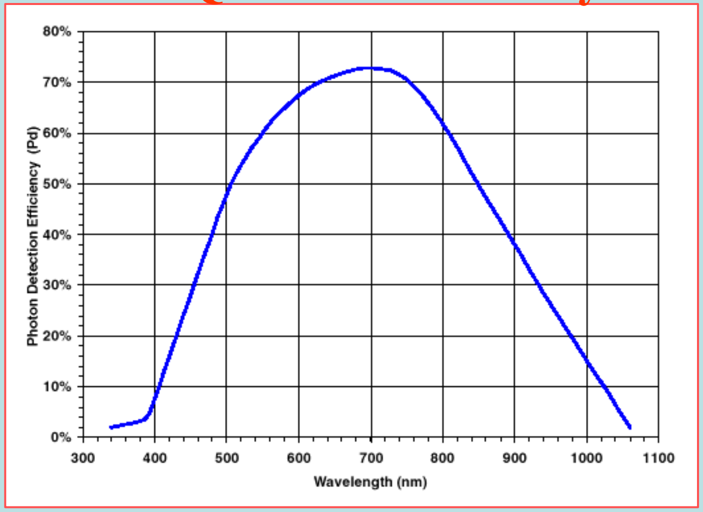


The silicon avalanche photodiode (Si APD) has a fast time response and high sensitivity in the near infrared region. APDs are available with active areas from 0.2 mm to 5.0 mm in diameter and low dark currents (selectable). *Photo courtesy of Hamamatsu*

APD for photon counting

Single photon counting module (SPCM) from Perkin-Elmer

70% Quantum Efficiency

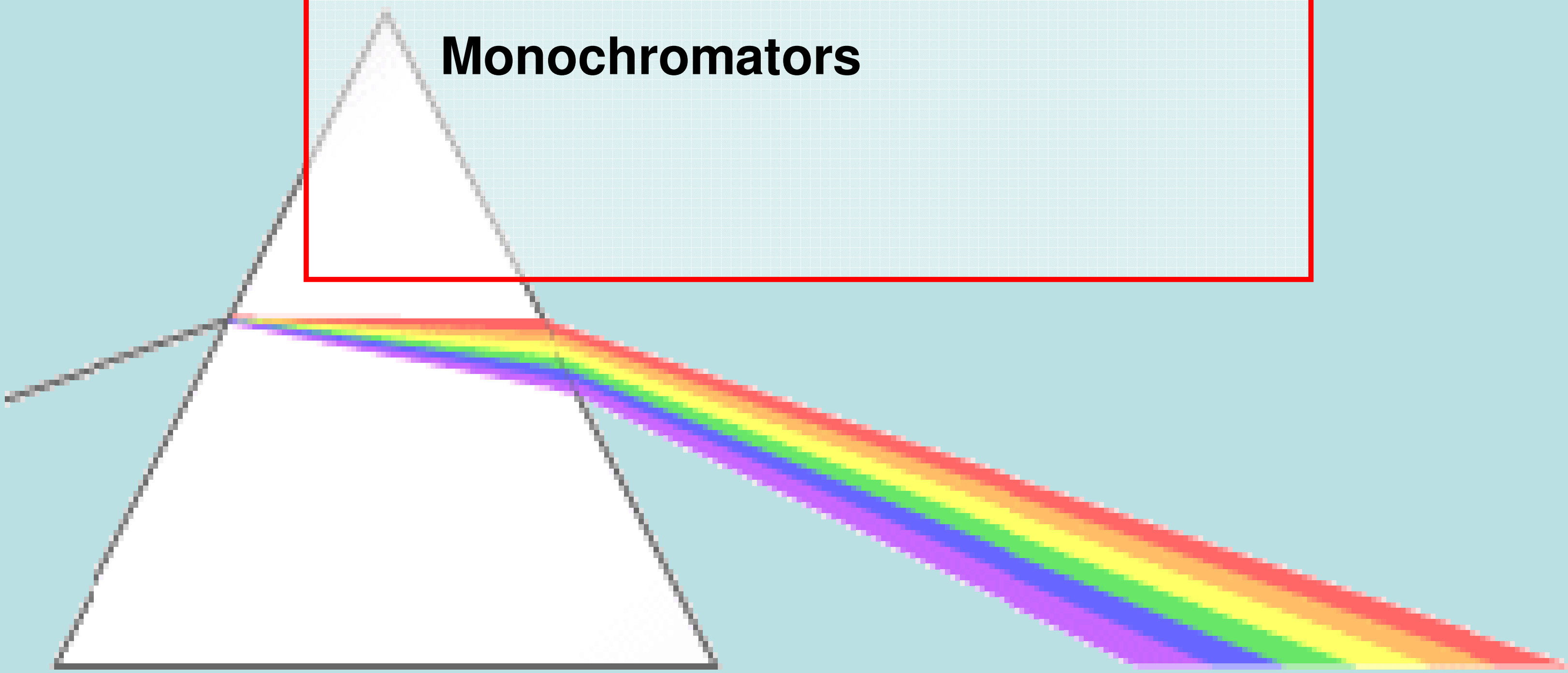


Wavelength Selection

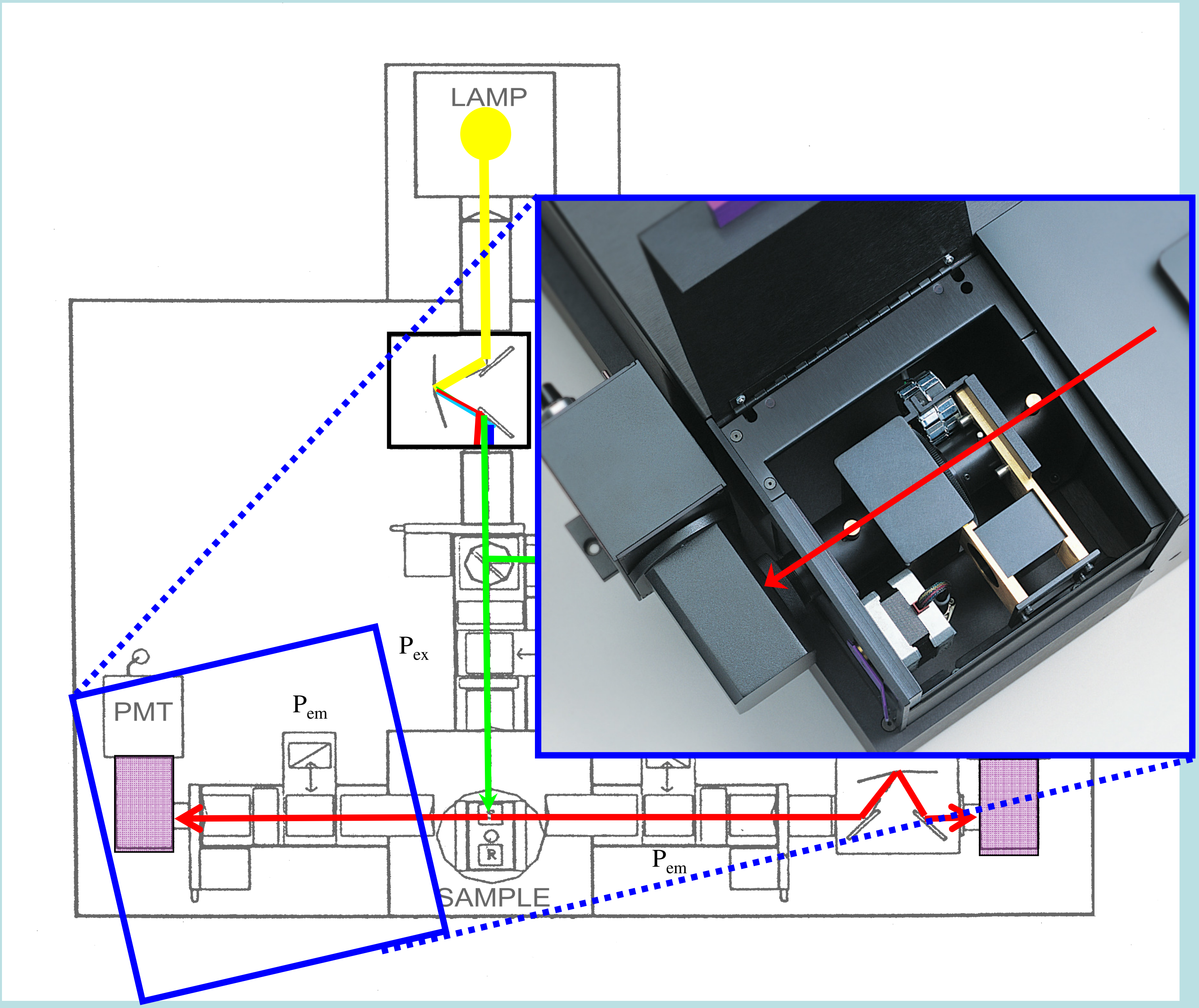
Fixed Optical Filters

Tunable Optical Filters

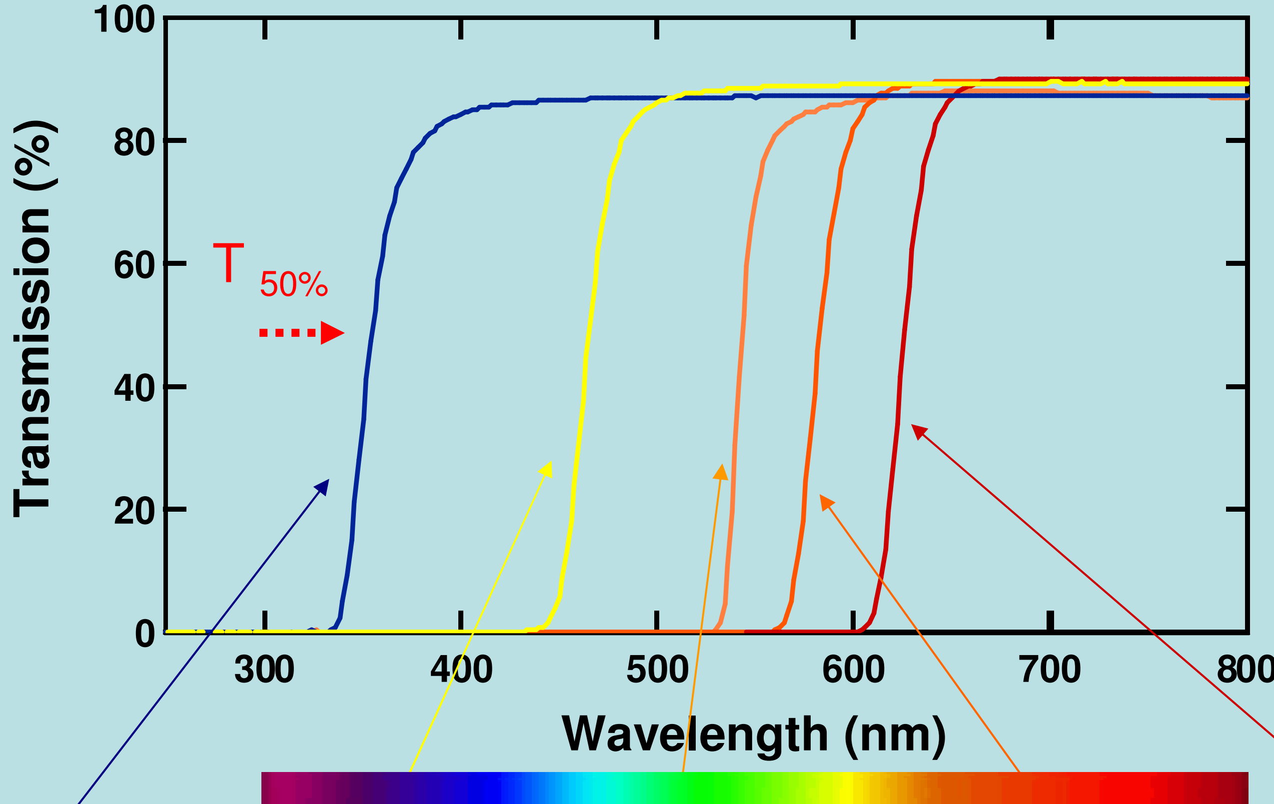
Monochromators



Optical Filter Channel

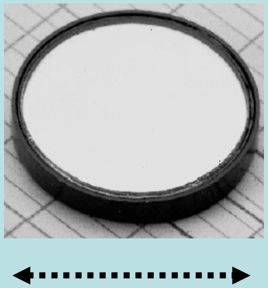


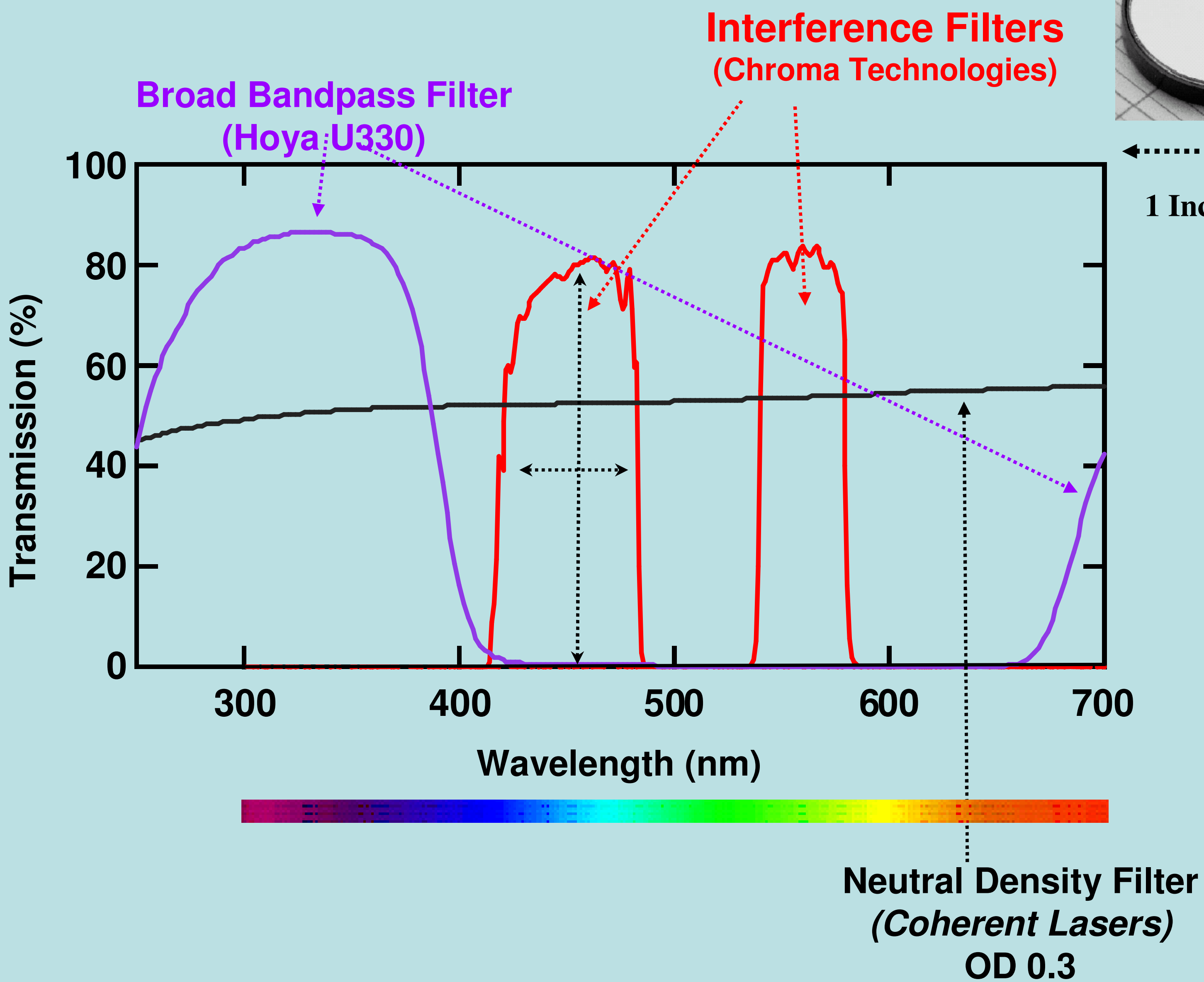
Long Pass Optical Filters Based on Absorption of Light



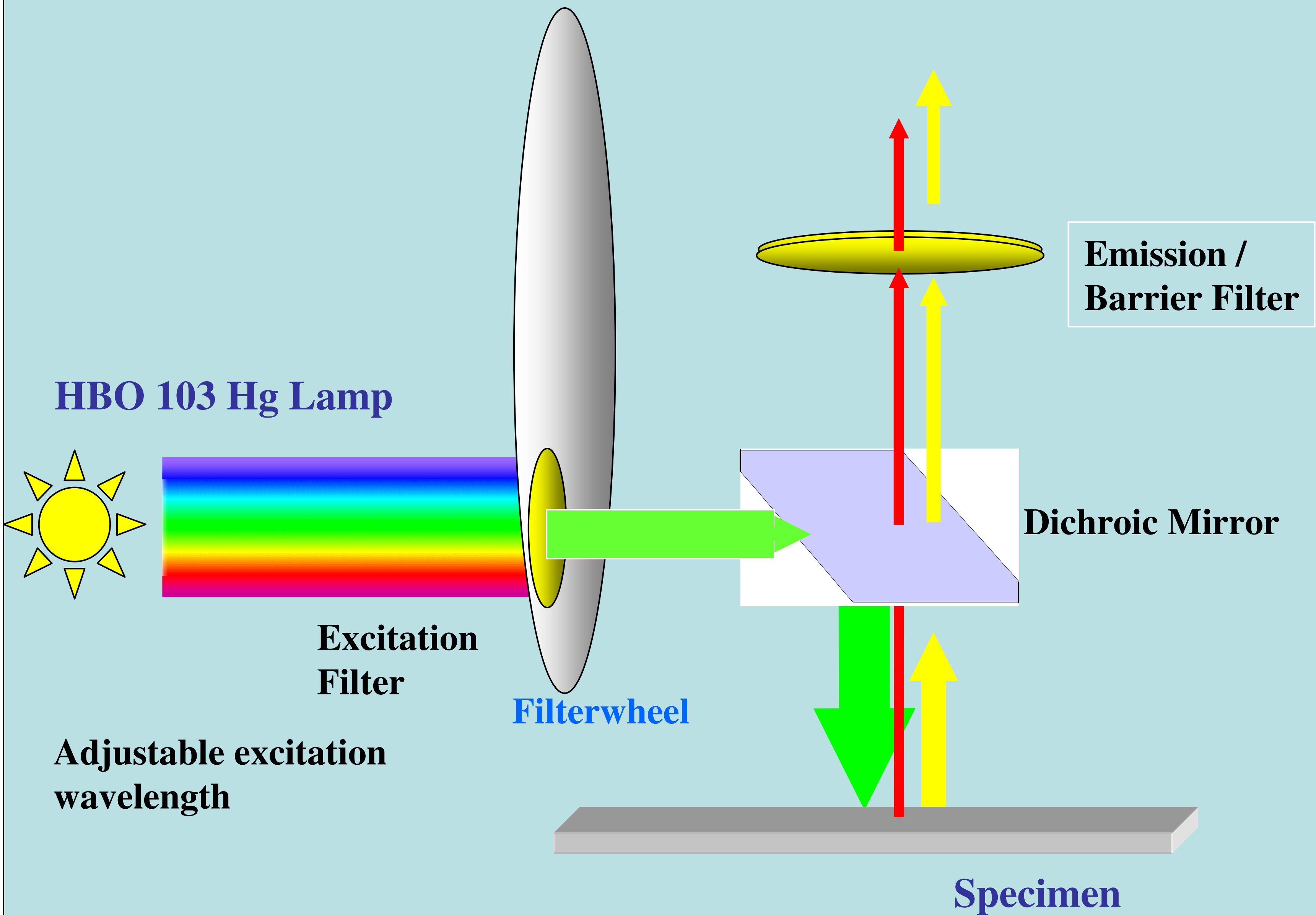
Spectral Shape
Thickness
Physical Shape
but also possibly
Substrate Fluorescence (!?)

Better Optical Filter Types…





Filterwheels

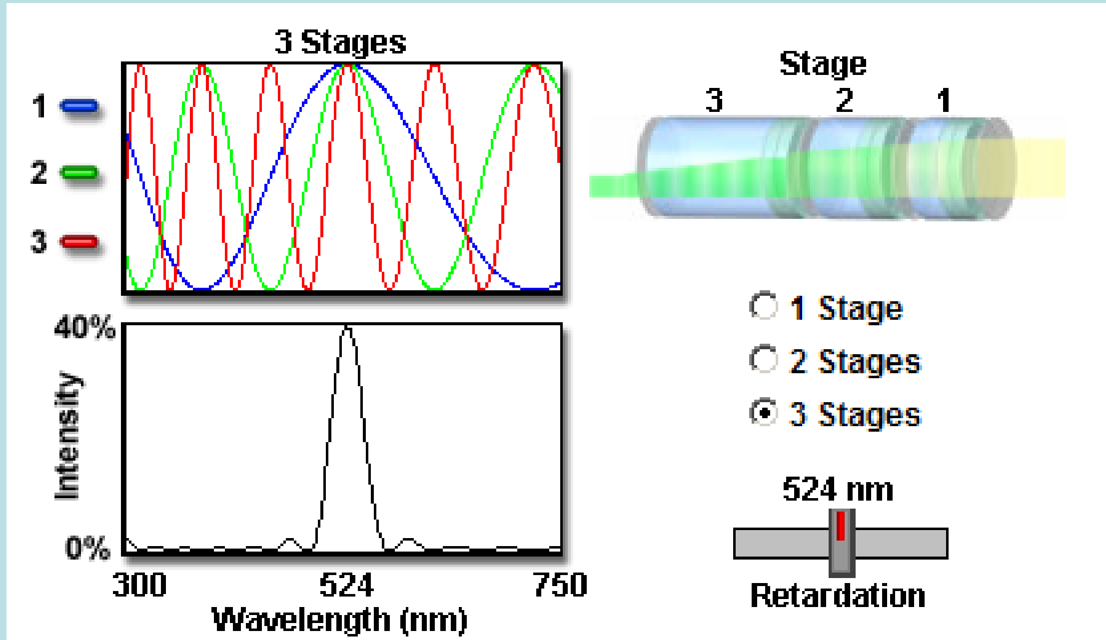


Adjustable excitation wavelength

Tunable Optical Filters

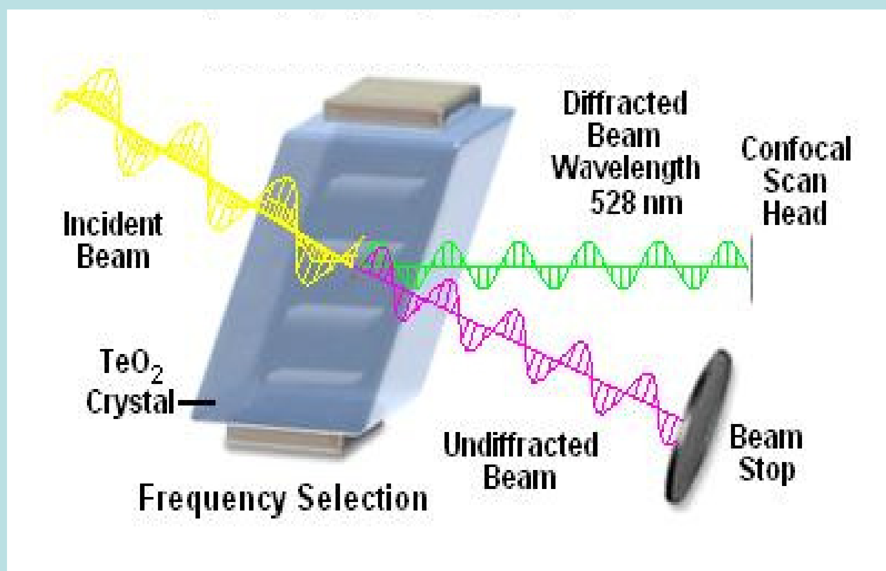
Liquid Crystal Filters:

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.



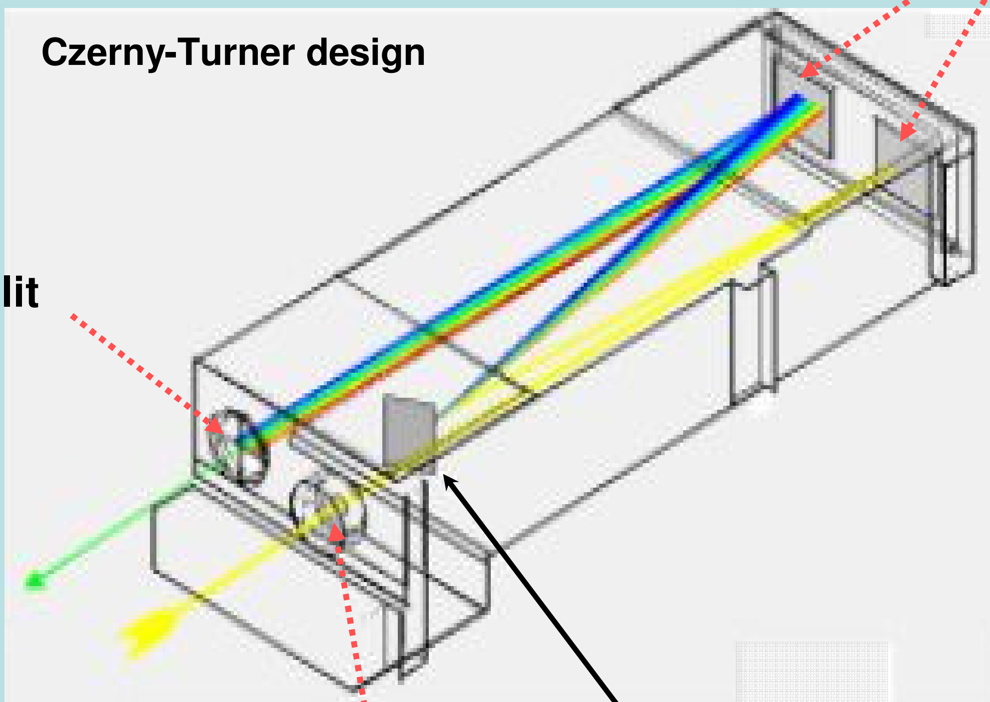
AO Tunable Filters:

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



Confocal Microscopy

Monochromators

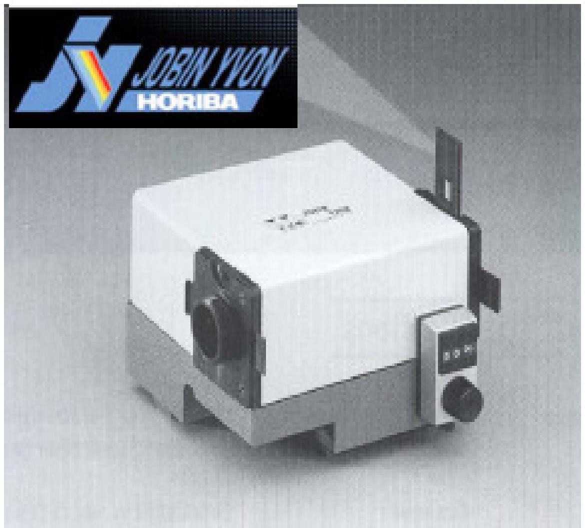


1. Slit Width (mm) is the dimension of the slits.

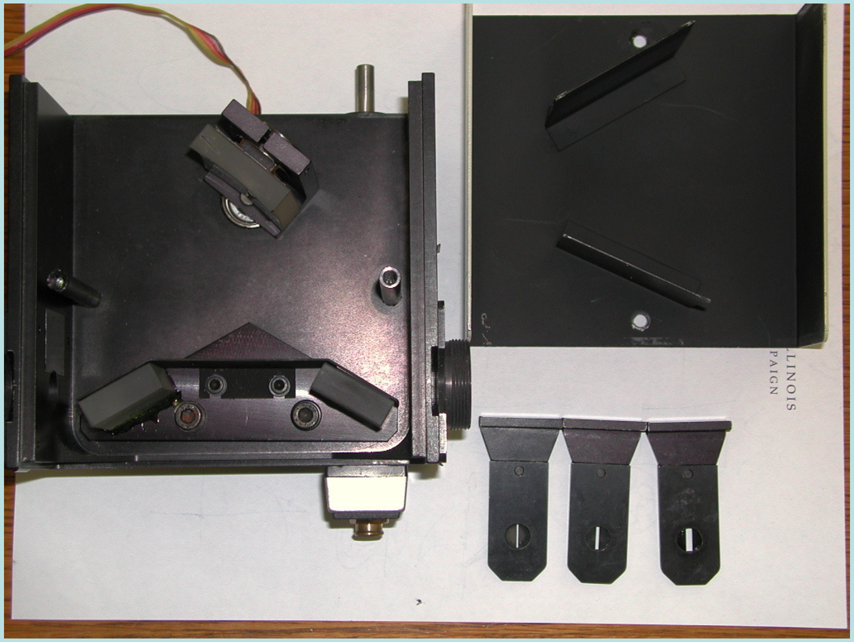
2. Bandpass is the FWHM at the selected wavelength.

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

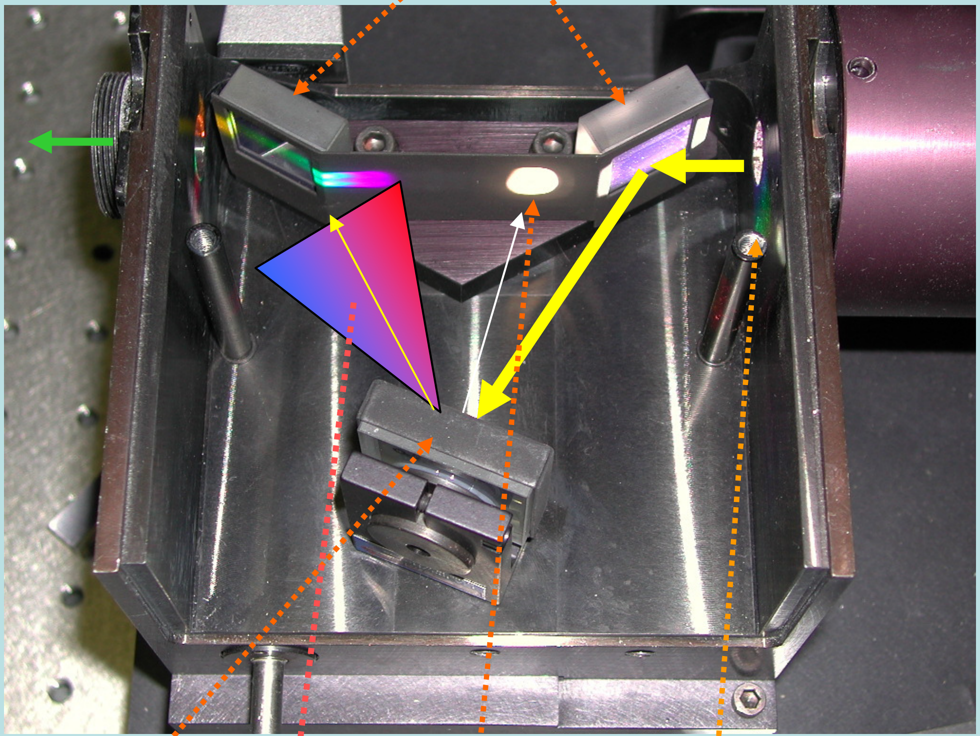
The Inside a Monochromator: Tunable Wavelengths



H10 Monochromator



Mirrors



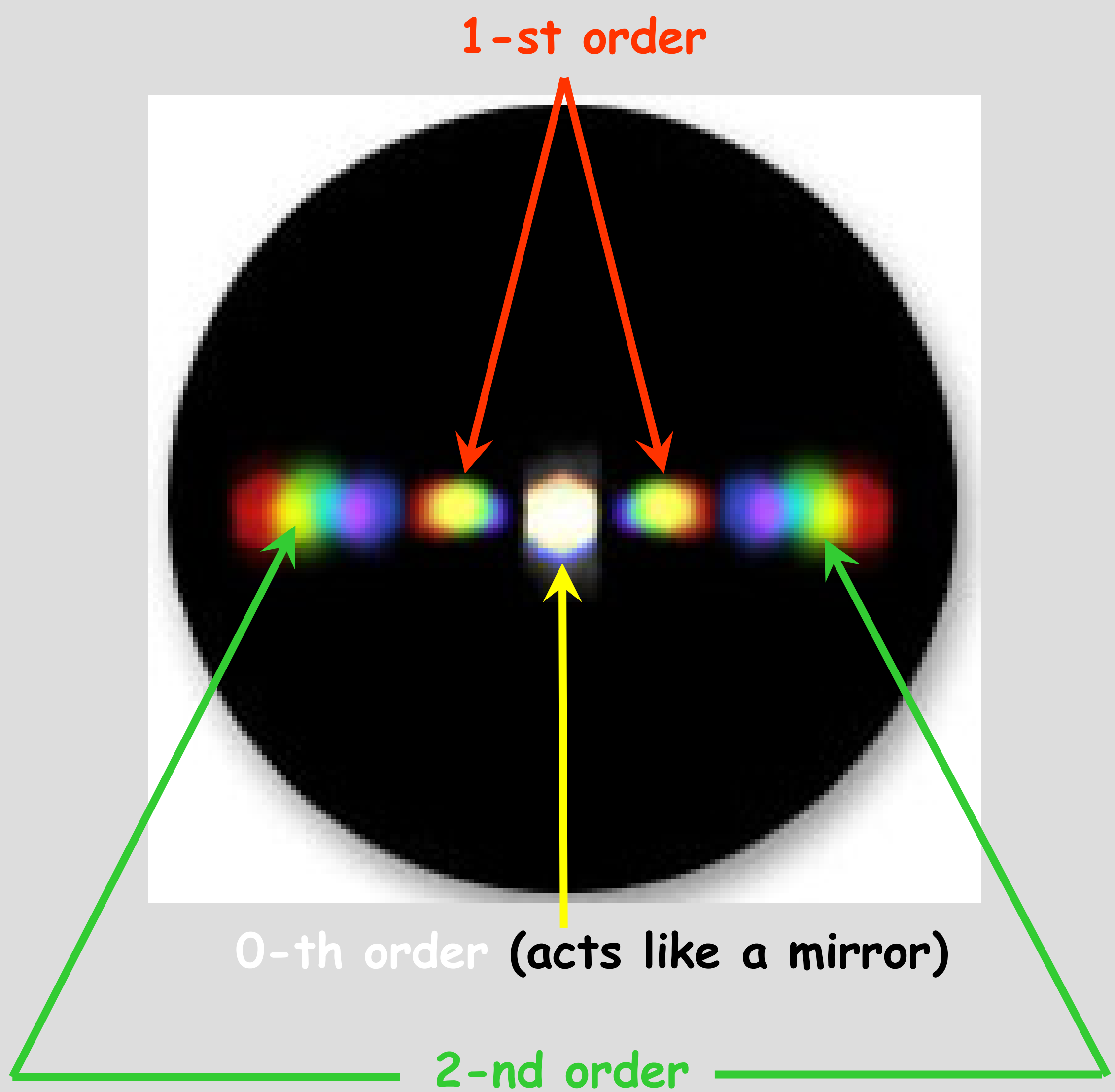
Grating

1^{st} Order spectrum

Nth Order
(spectral distribution)

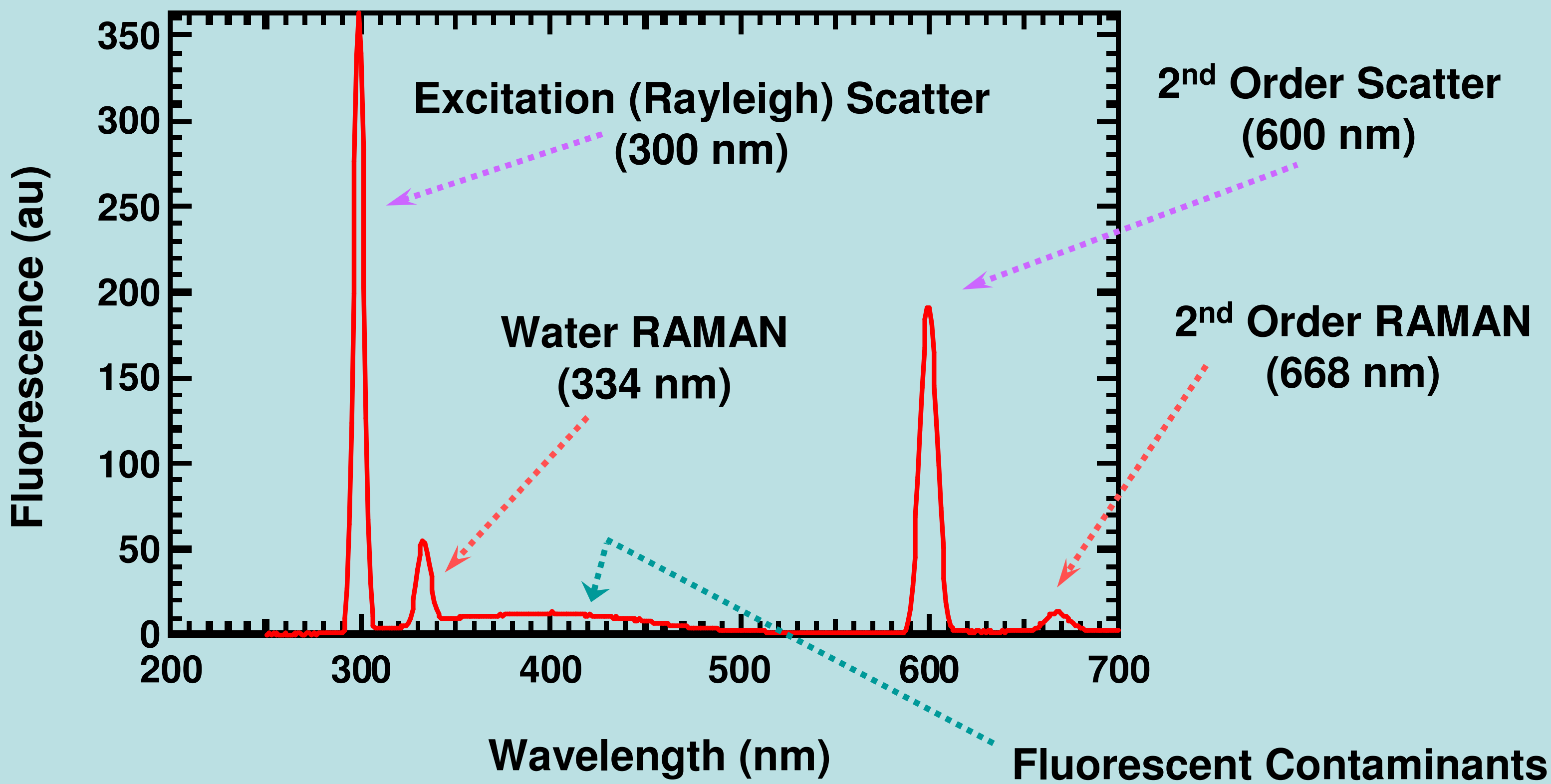
Zero Order
(acts like a mirror)

Order of Diffraction



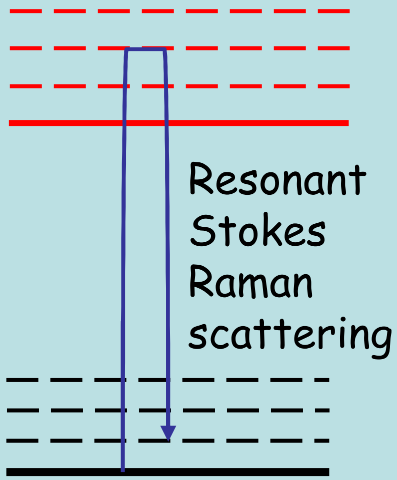
Higher Order Light Diffraction & Spectral Features

Emission Scan:
Excitation 300 nm
Glycogen in PBS

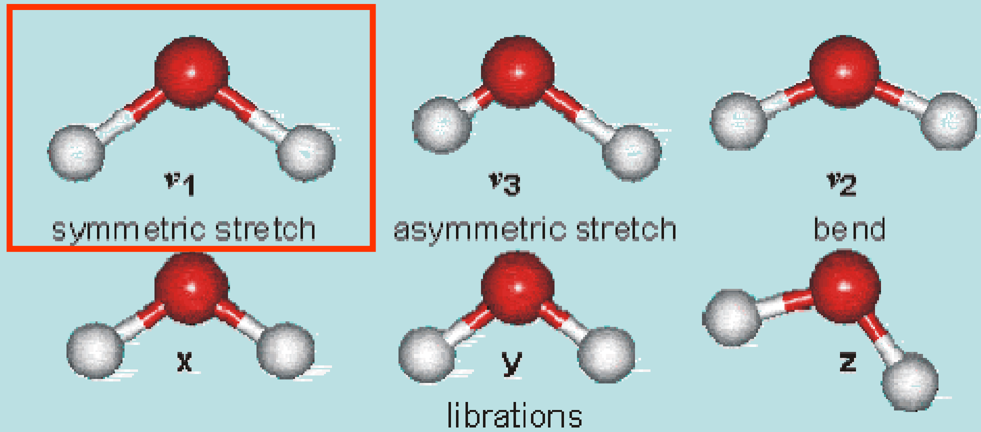


Spectrum 1   Spectrum 2 .....

Raman Scatter of Water



Vibrational modes of water



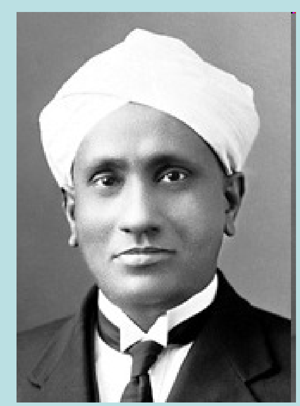
Energy for the OH stretch vibrational mode in water (expressed in inverse wavenumbers): 3200 cm^{-1}

Simple formula to calculate the wavelength of the Raman peak:

(1) Insert the excitation wavelength (eg. 490 nm) in the following equation:

$$\frac{10^7}{\frac{10^7}{490} - 3200} = 581 \text{ nm}$$

(2) The result specifies the position of the Raman peak in nanometers (i.e. the Raman peak of water is located at 581 nm for this excitation wavelength of 490 nm.

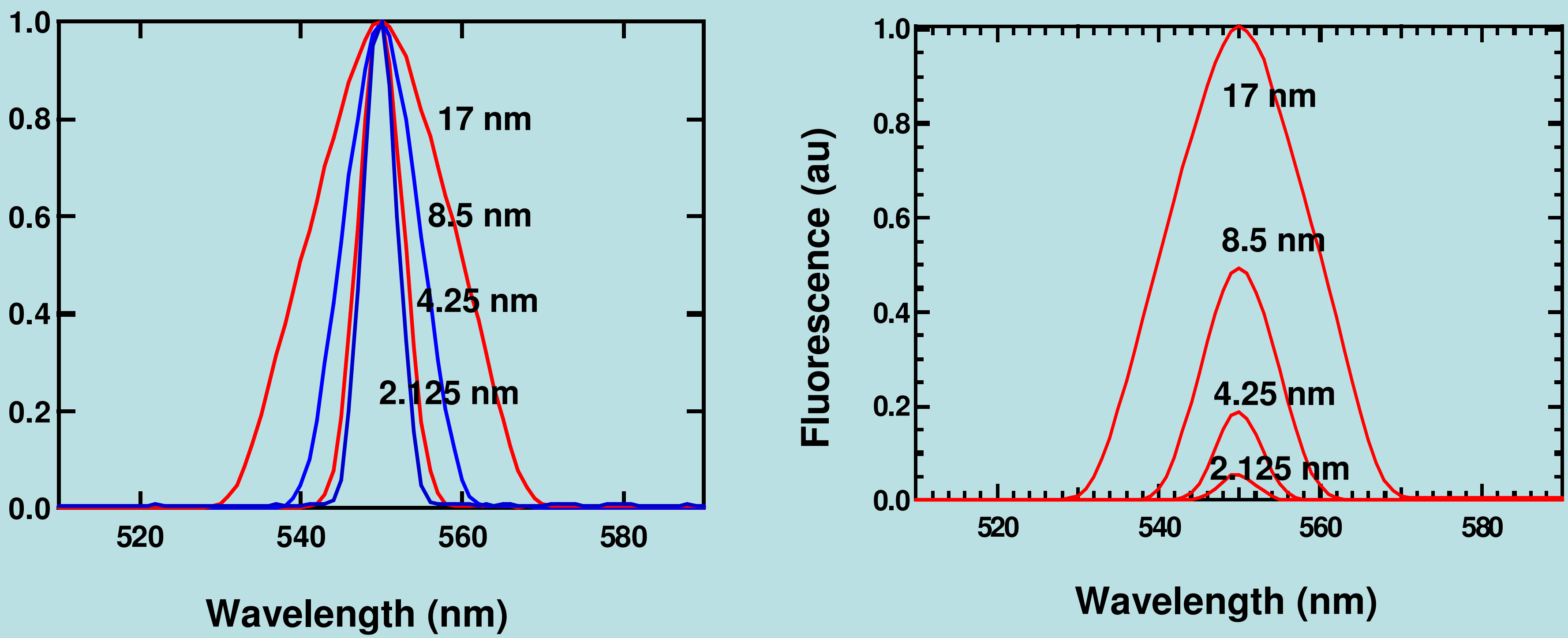


Changing the Bandpass

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

@ Fixed Excitation Bandpass = 4.25 nm

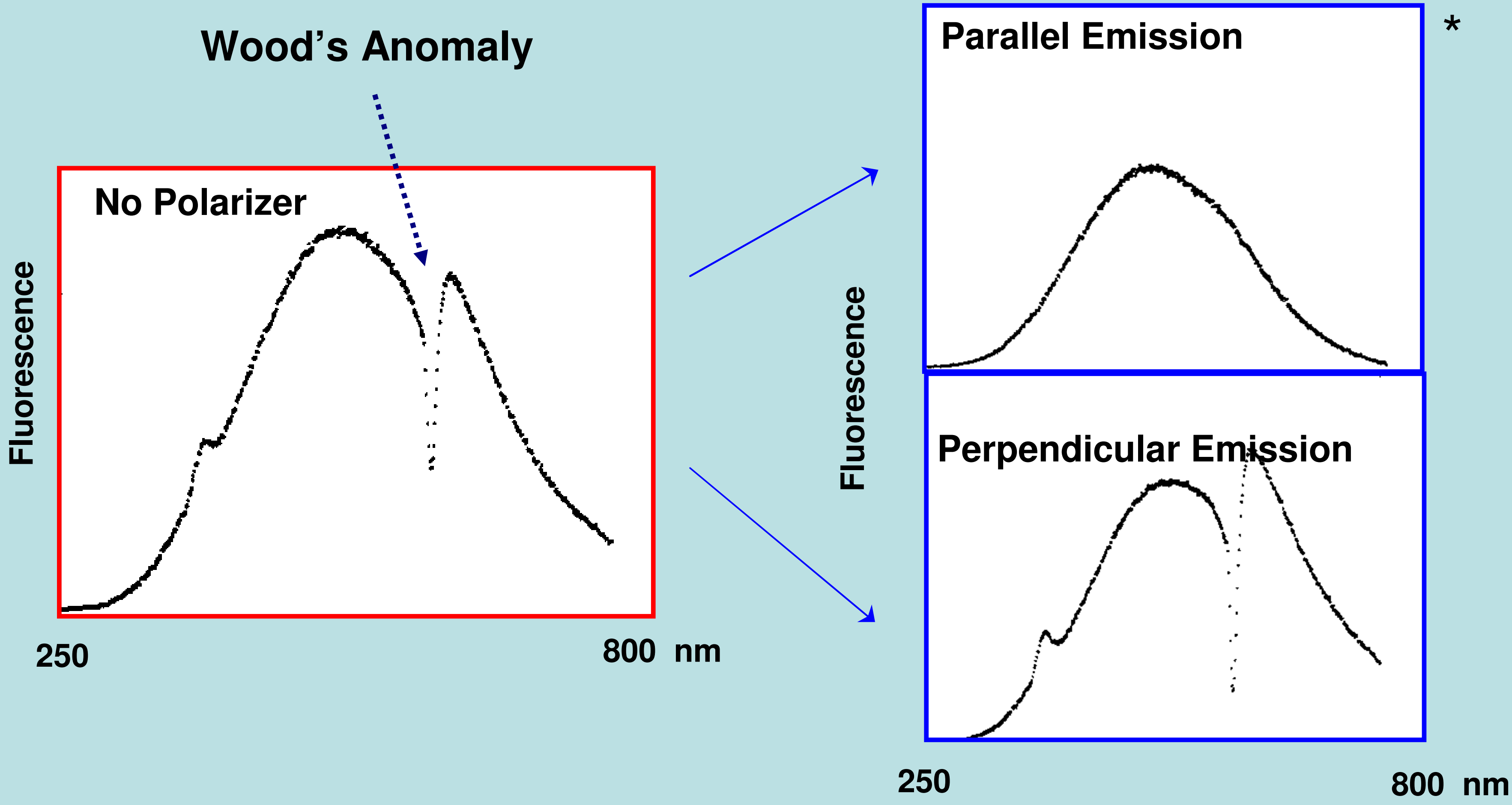
Changing the Emission Bandpass



Collected on a SPEX Fluoromax - 2

Monochromator Polarization Bias

Tungsten Lamp Profile Collected on an SLM Fluorometer

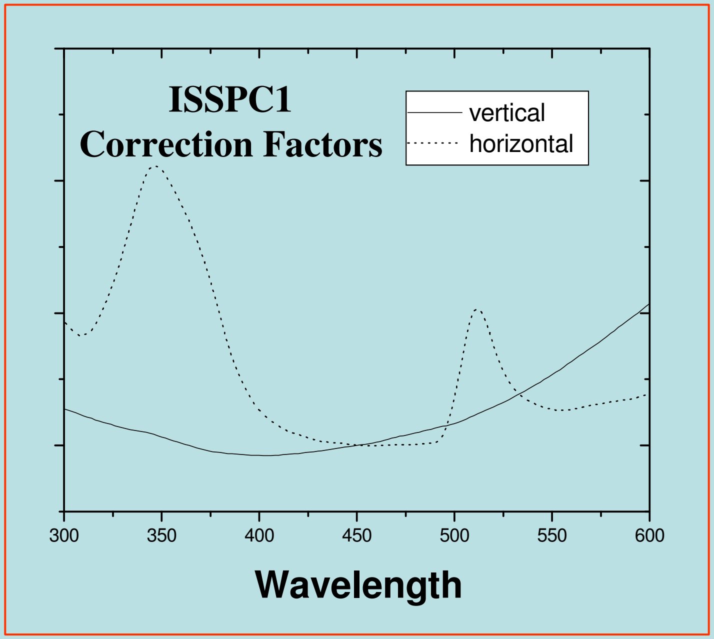


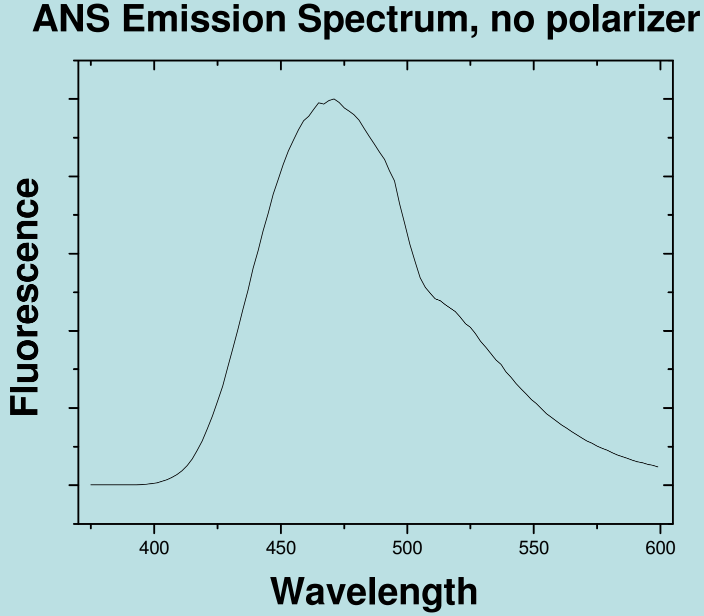
Technical vs. Absolute spectra

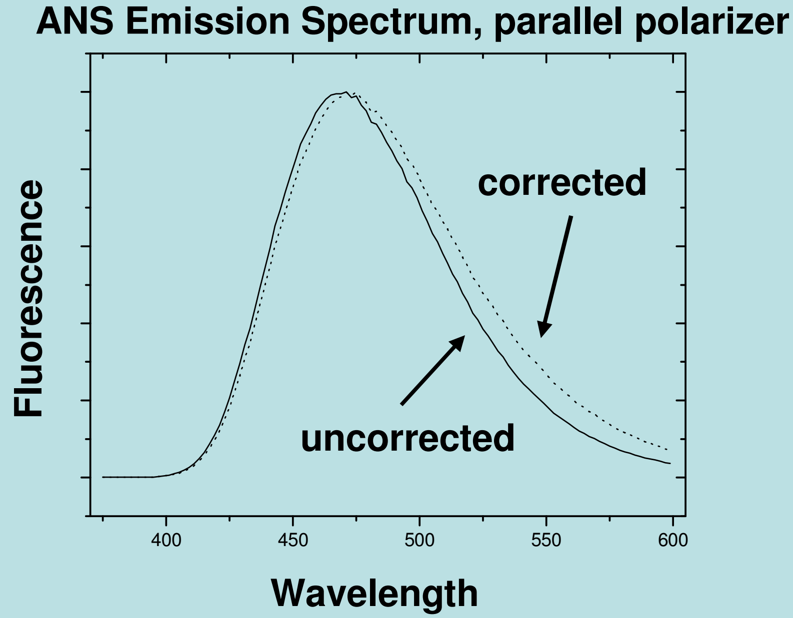
Jameson et. al.,
Methods in Enzymology (2002), 360:1
for more on the correction
of (emission) spectra

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

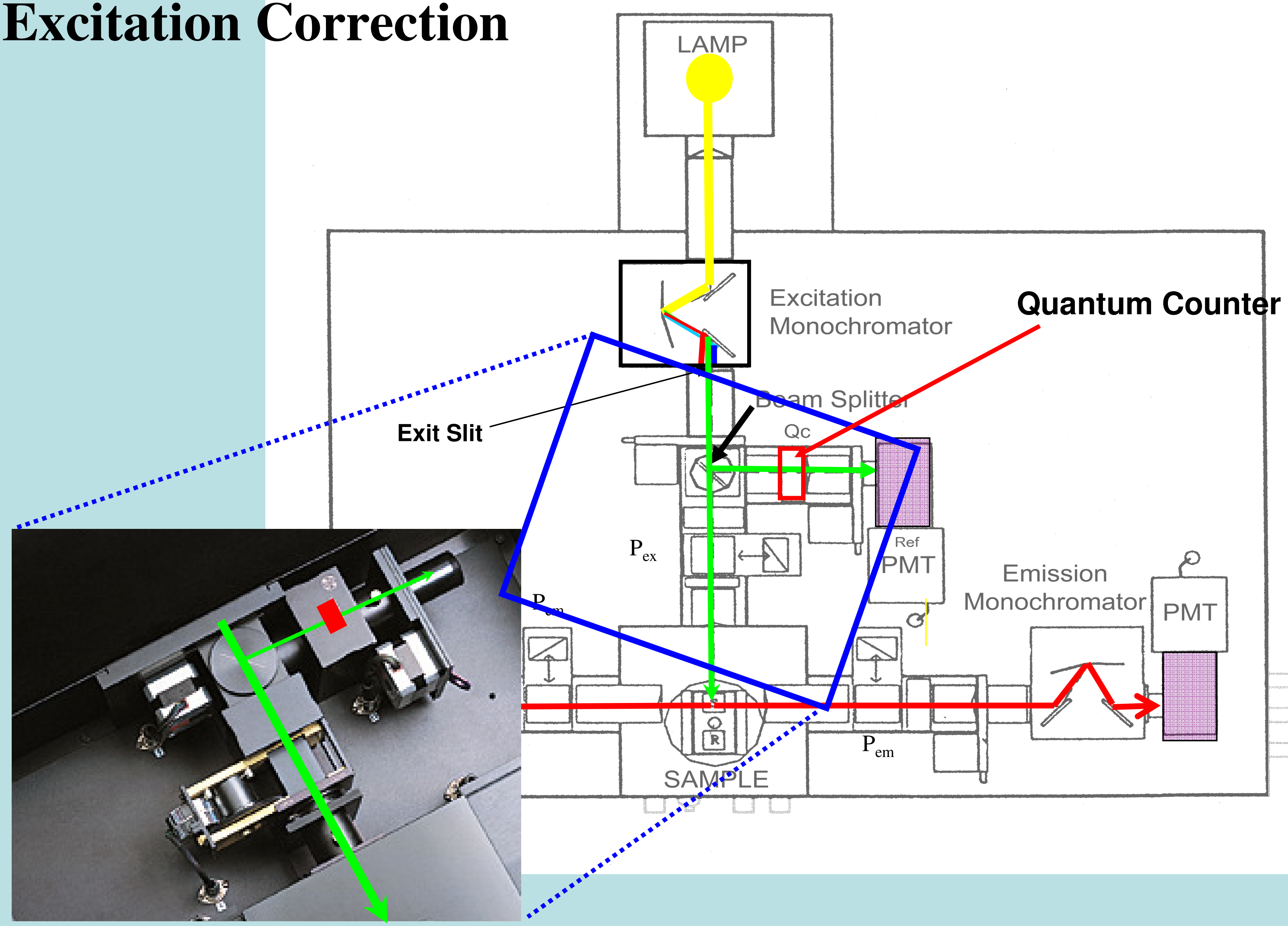






from Jameson et. al., Methods in Enzymology, 360:1

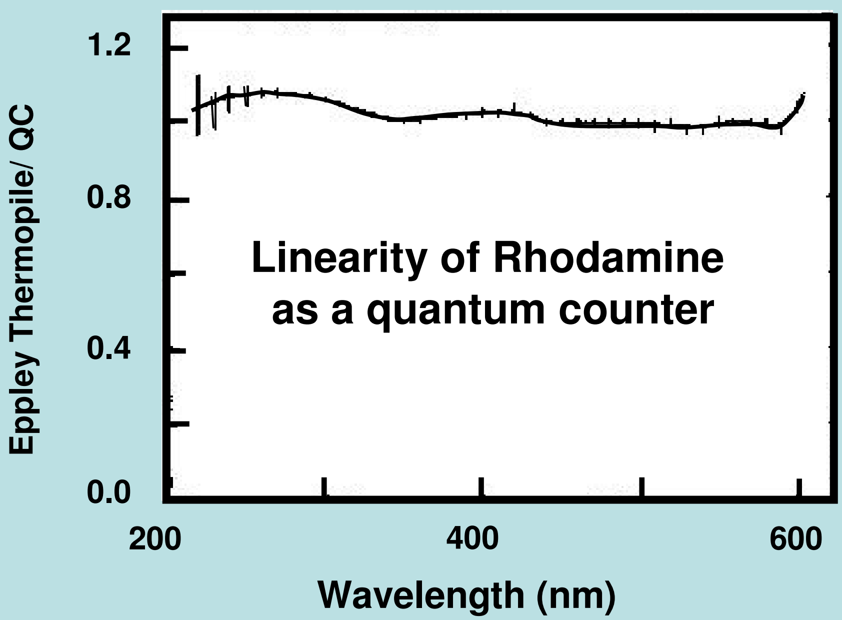
Excitation Correction

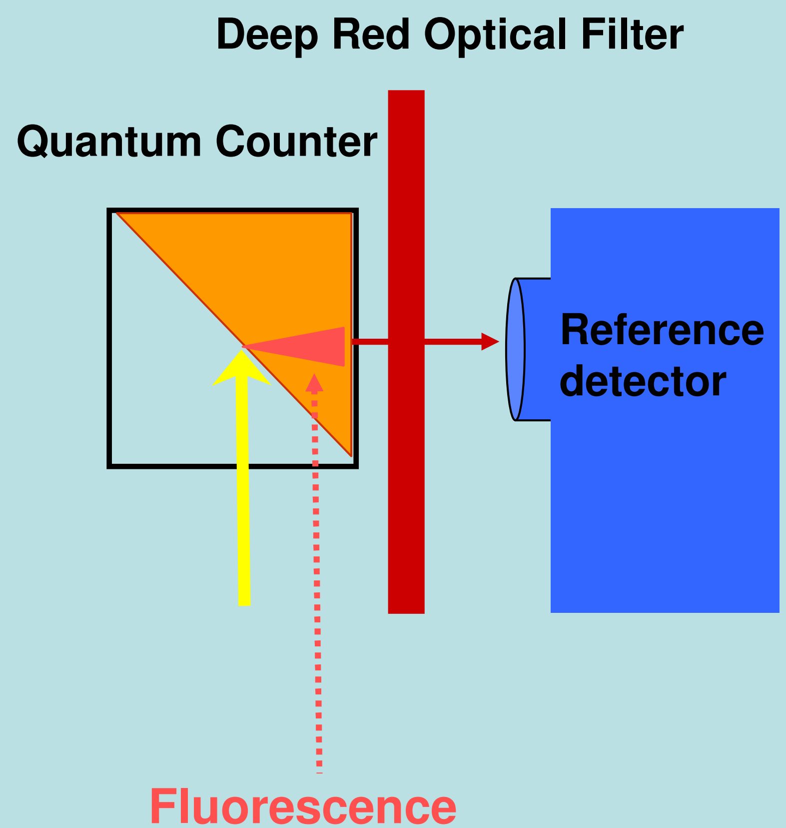


The Instrument Quantum Counter

Common Quantum Counters (optimal range) *

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

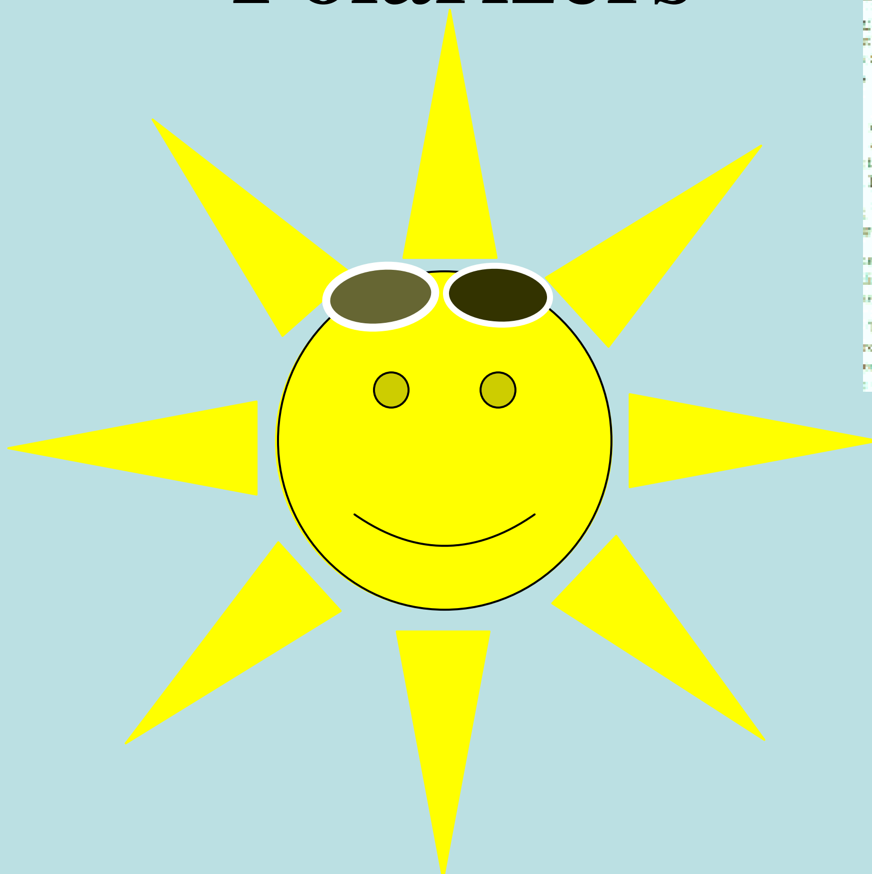


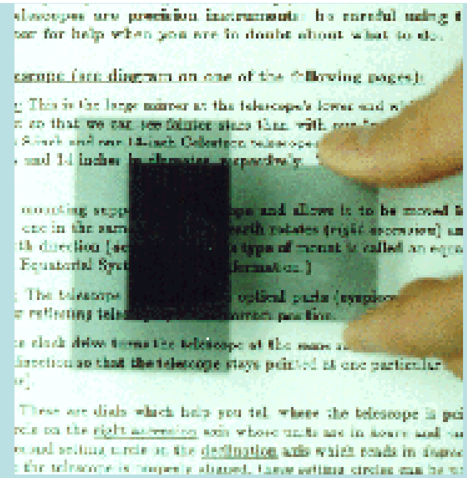


The maximum inner filter effect needed !

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

Polarizers





Polarizers

Common Types:

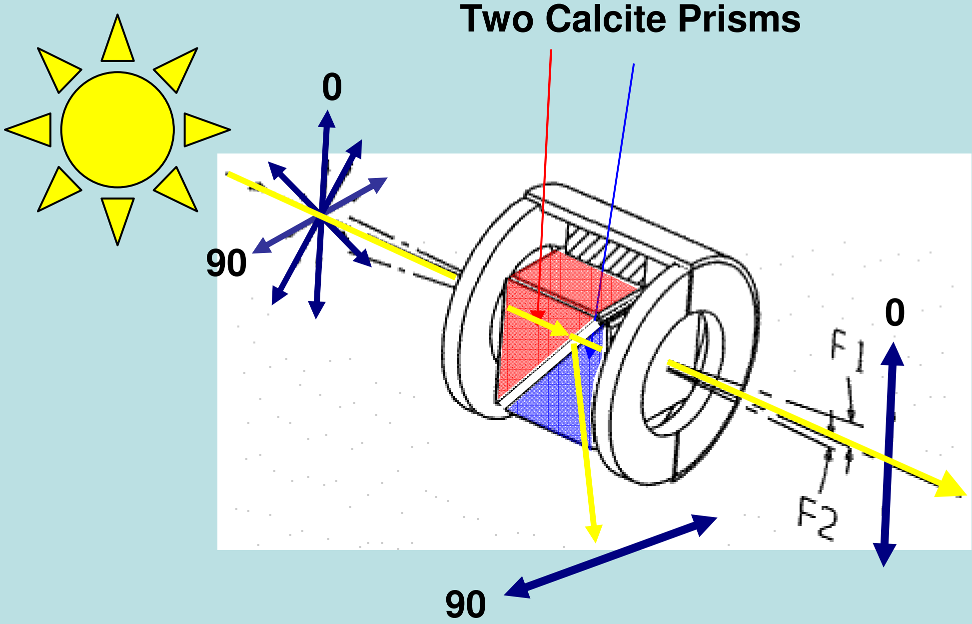
Glan Taylor (air gap)

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.

Filter Choice For Steady-State as well as Time-Resolved Polarization Measurements

Make sure absolutely no scattered excitation light is detected !

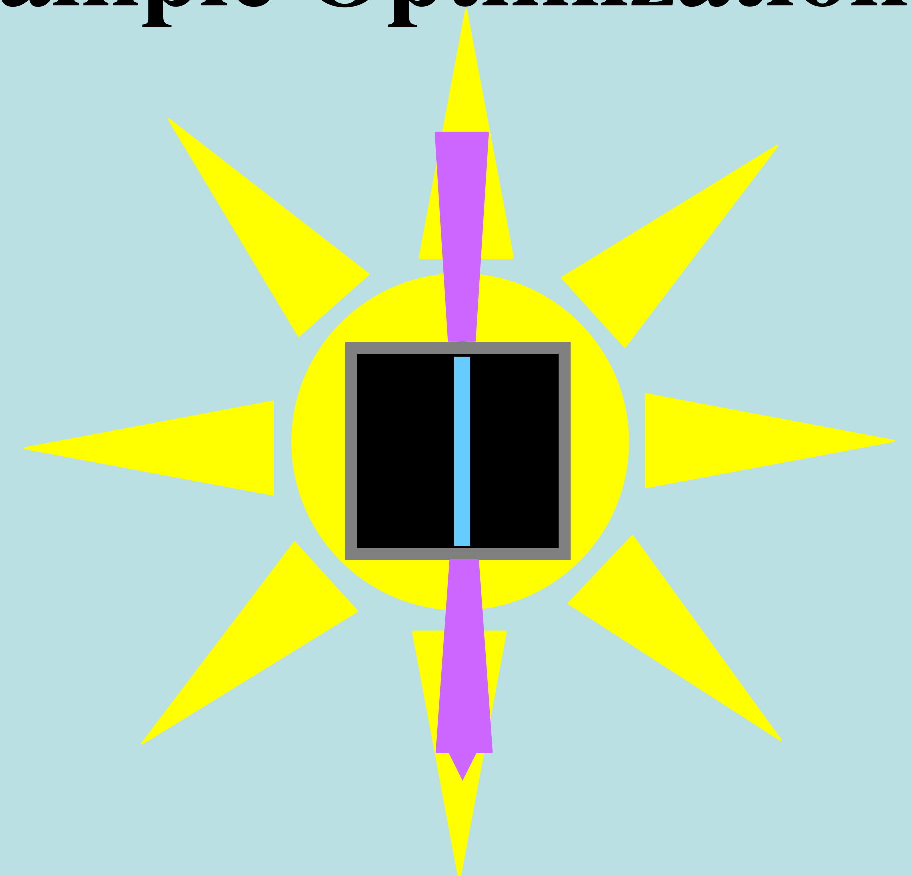
An inserted emission filter should block the excitation very well

Why ?

$$P = \frac{I_{//} - I_{\perp}}{I_{//} + I_{\perp}}$$

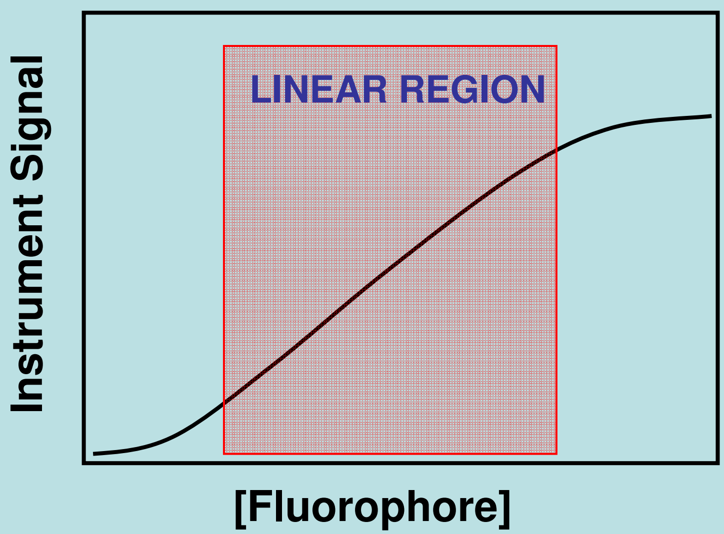
Scattered excitation light influences $I_{//}$

Sample Optimization

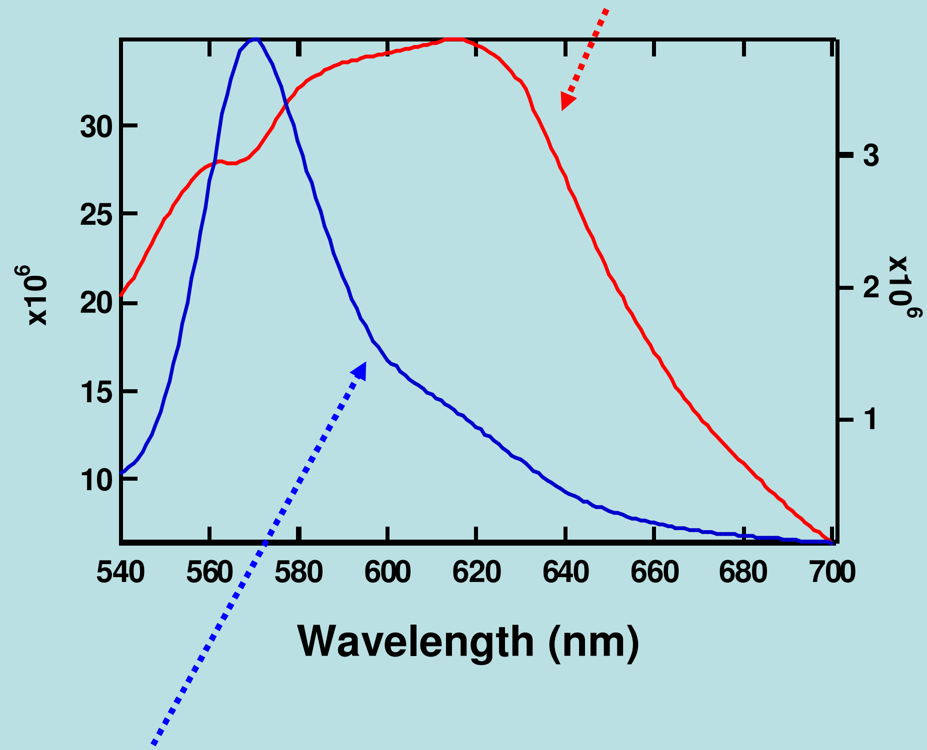


Signal Attenuation of the Excitation Light
PMT Saturation

Fluorescence vs. Signal



Excess Detection Saturating Emission



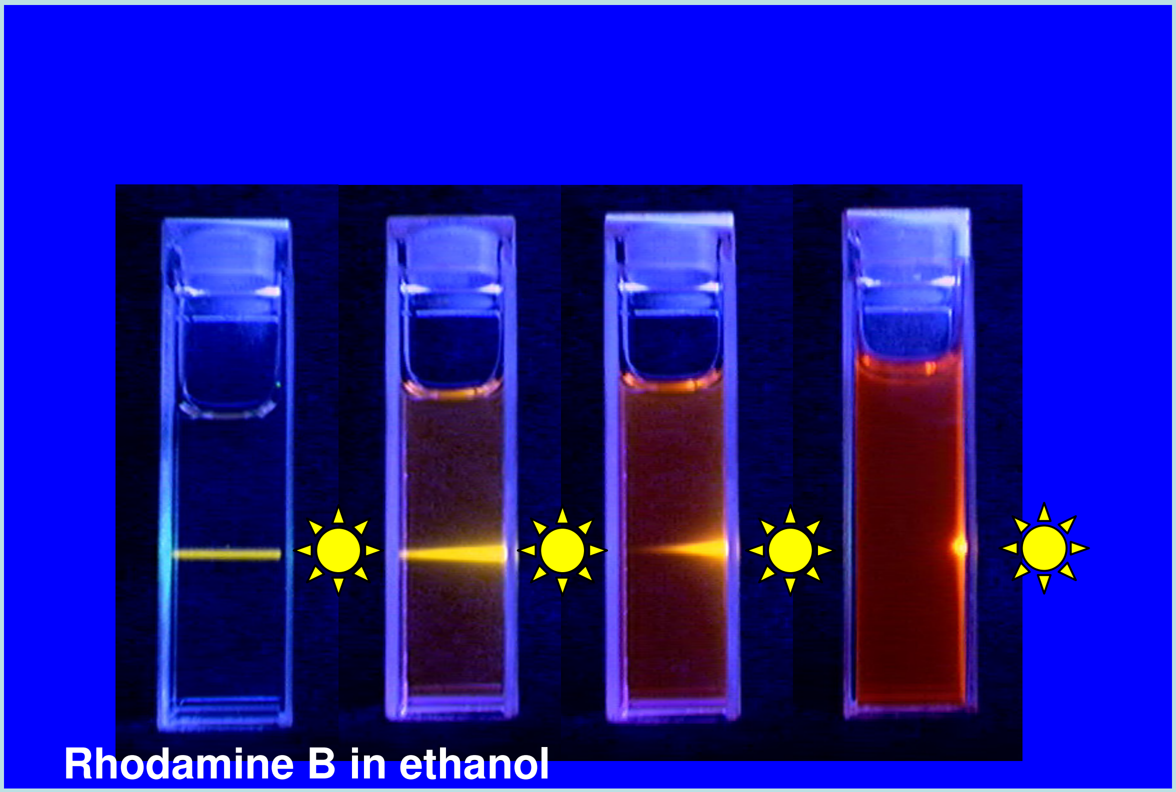
Reduced emission intensity

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

Concentration

Attenuation of the Excitation Light through Absorbance

Sample concentration
& the *inner filter effect*



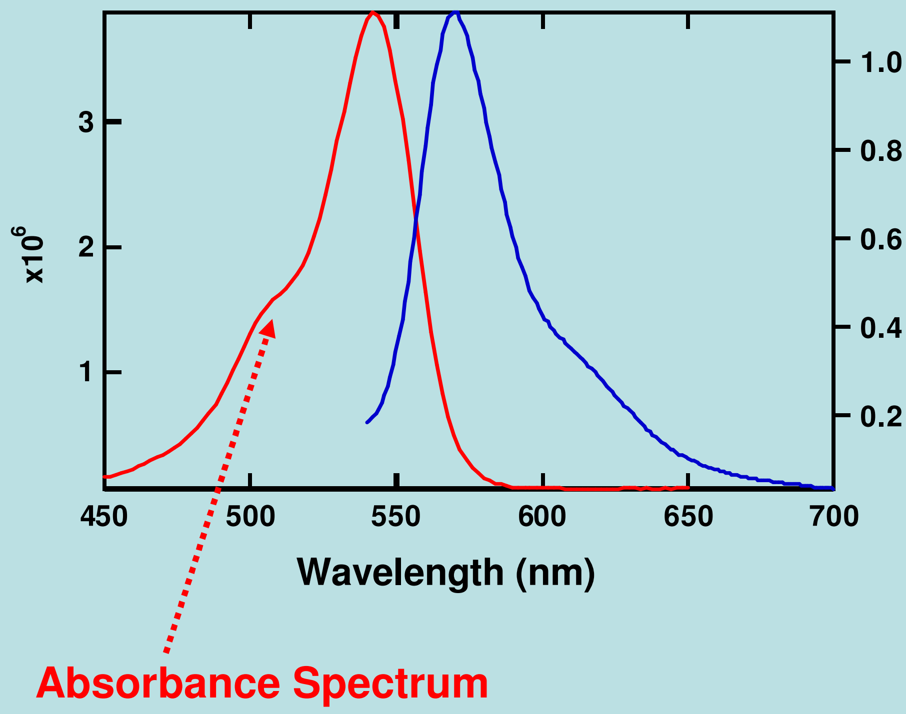
OD_{532} 0.04 1 3 >30

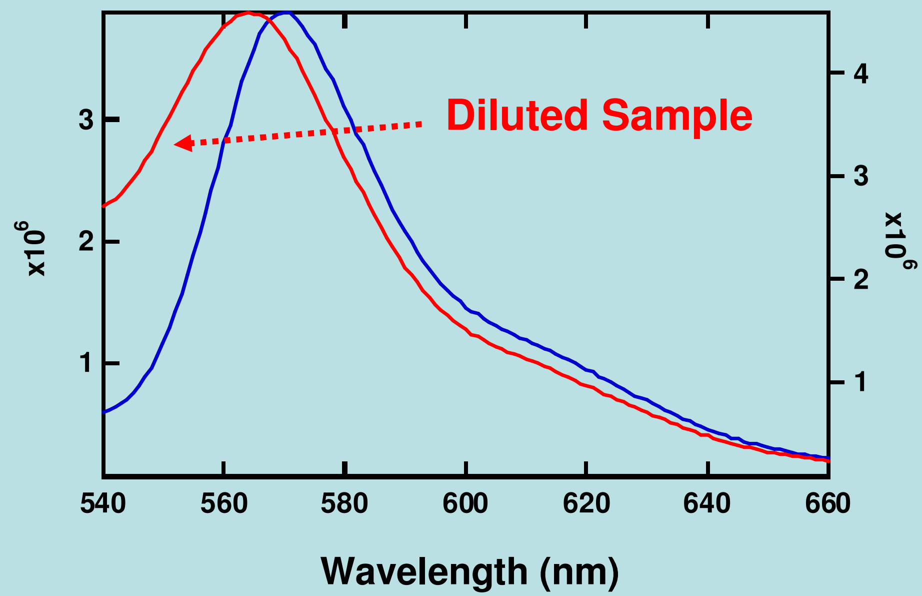
Correct Optical Density (OD)

Look down into sample cuvette and check by eye how the beam looks like

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

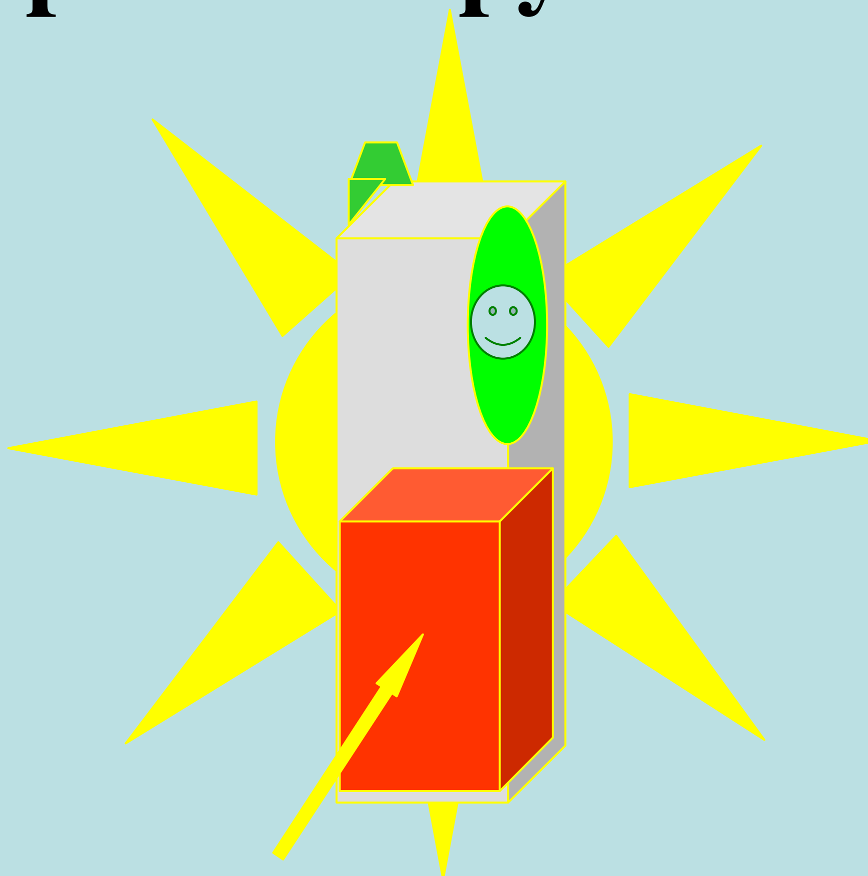
The Second Half of the *Inner Filter Effect* : <u>Attenuation of the Emission Signal</u>





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

Spectroscopy Cuvettes

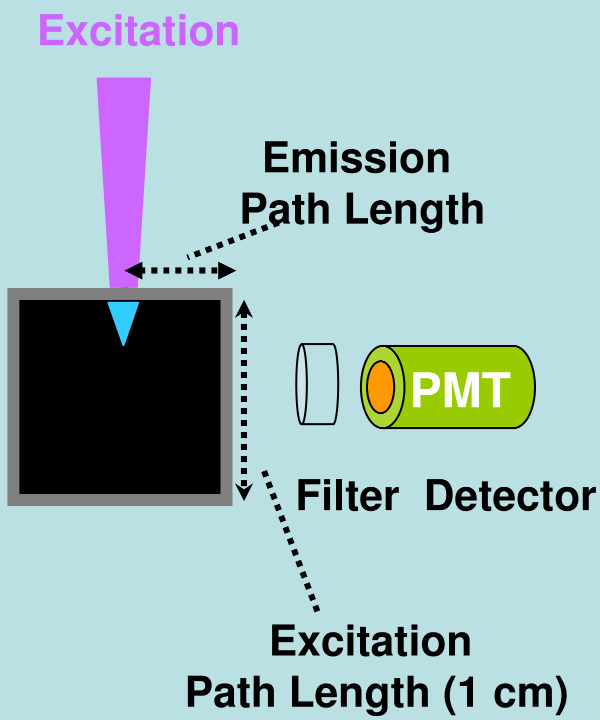


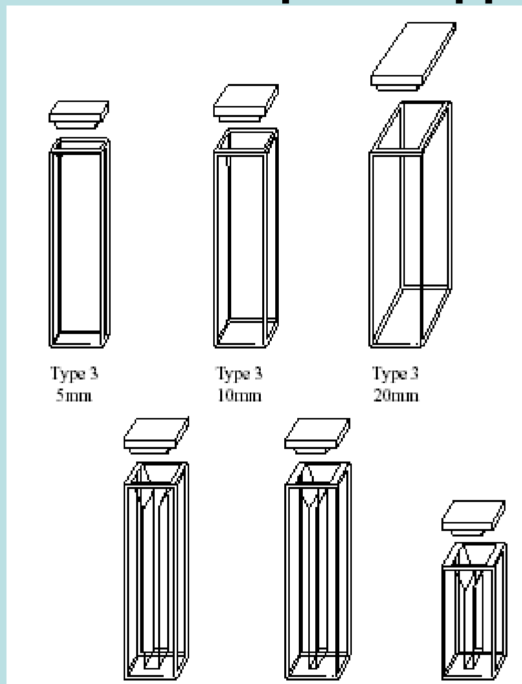
Handling Highly Absorbing Solutions

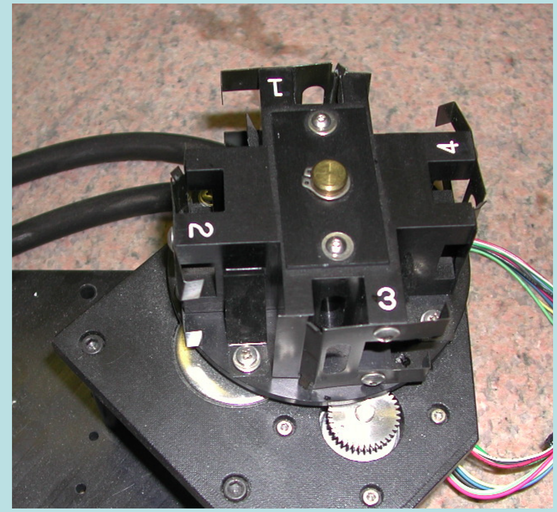
Use smaller optical pathlengths for excitation and emission

Quartz/Optical Glass/Plastic Cells with Caps / Stoppers

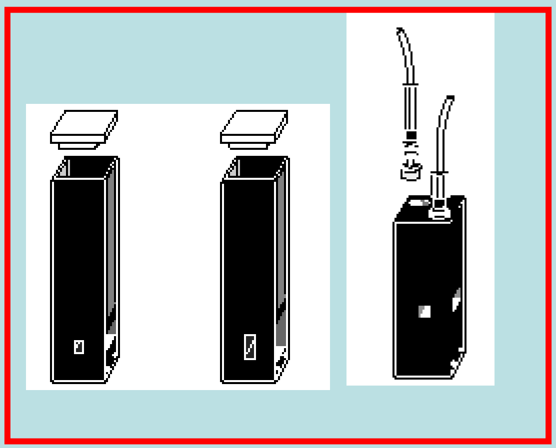
O.D >> 0.04

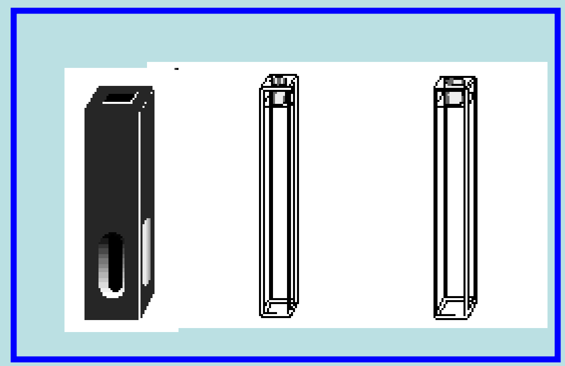






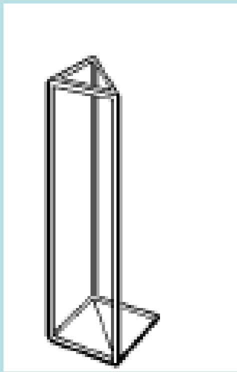
4 Position Turret
SPEX Fluoromax-2, Jobin-Yvon



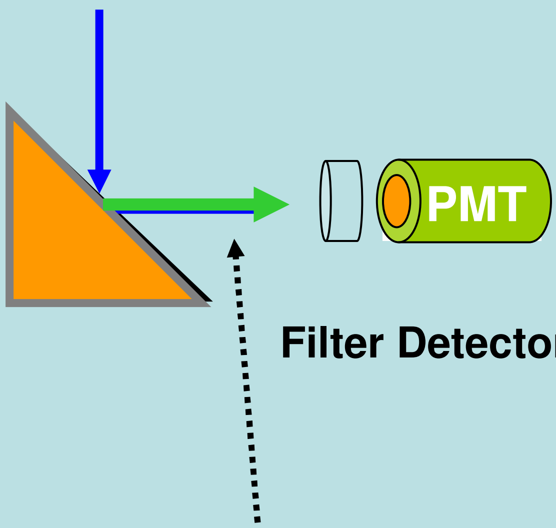


Front Face Detection

Triangular Cells



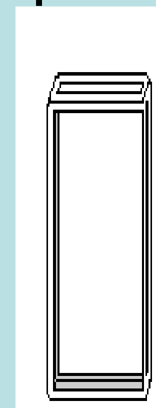
Excitation



Filter Detector

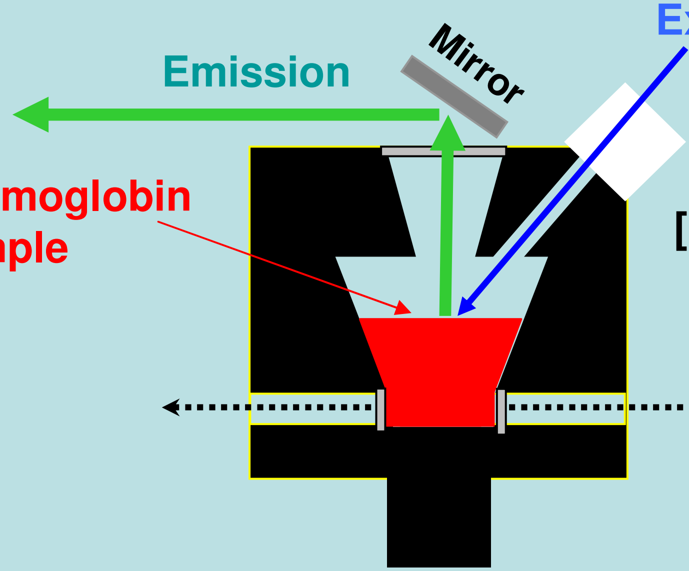
Reflected Excitation & Emission

Thin Cells & Special Compartments



IBH, Glasgow G3 8JU
United Kingdom

Excitation

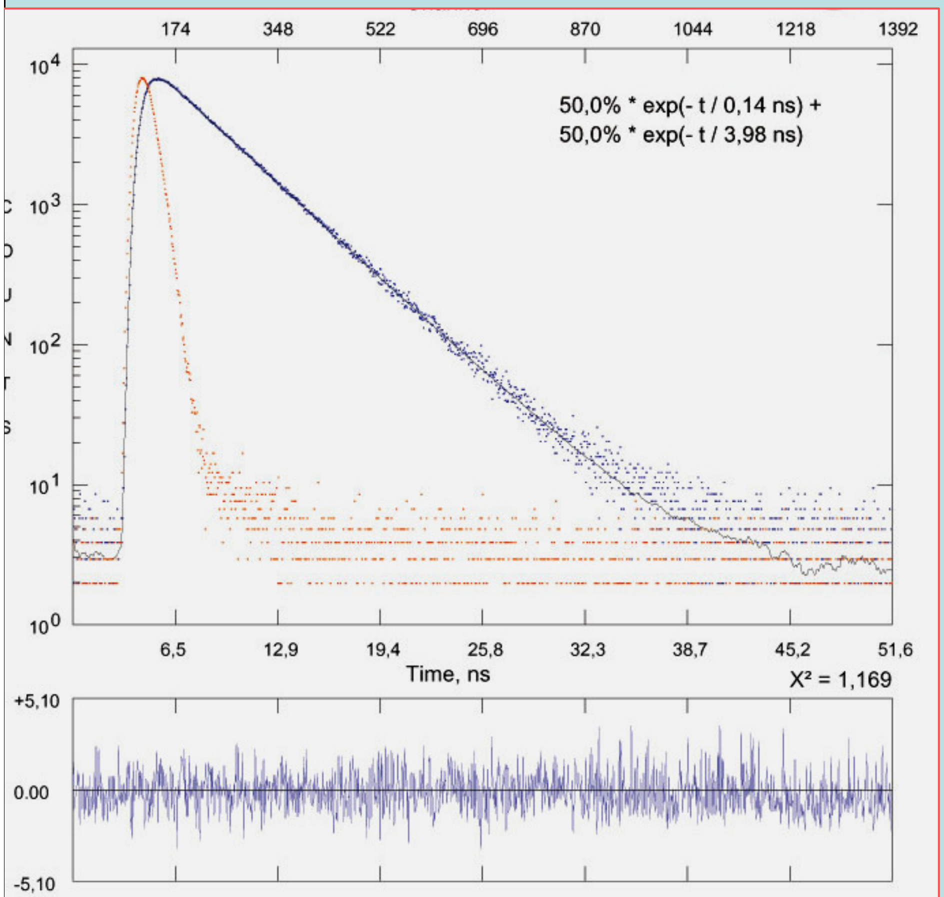


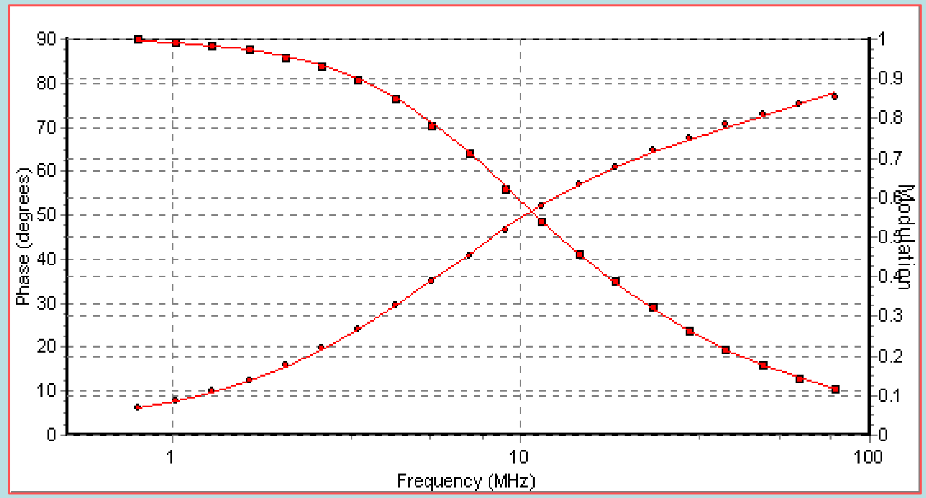
[1]

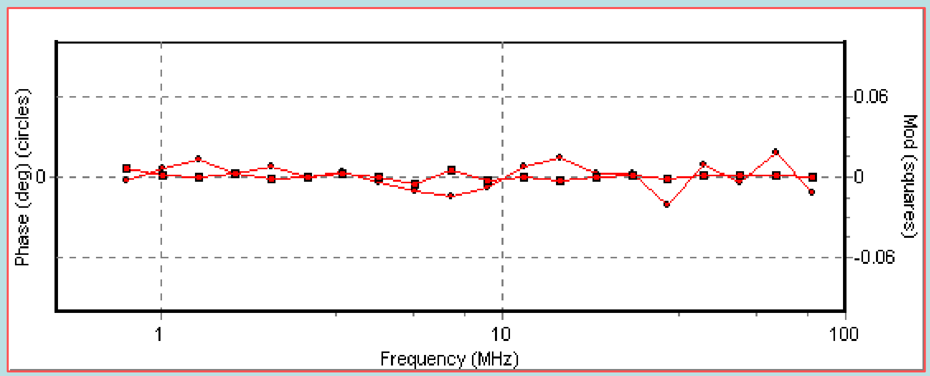
Absorbance
Measurements

[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

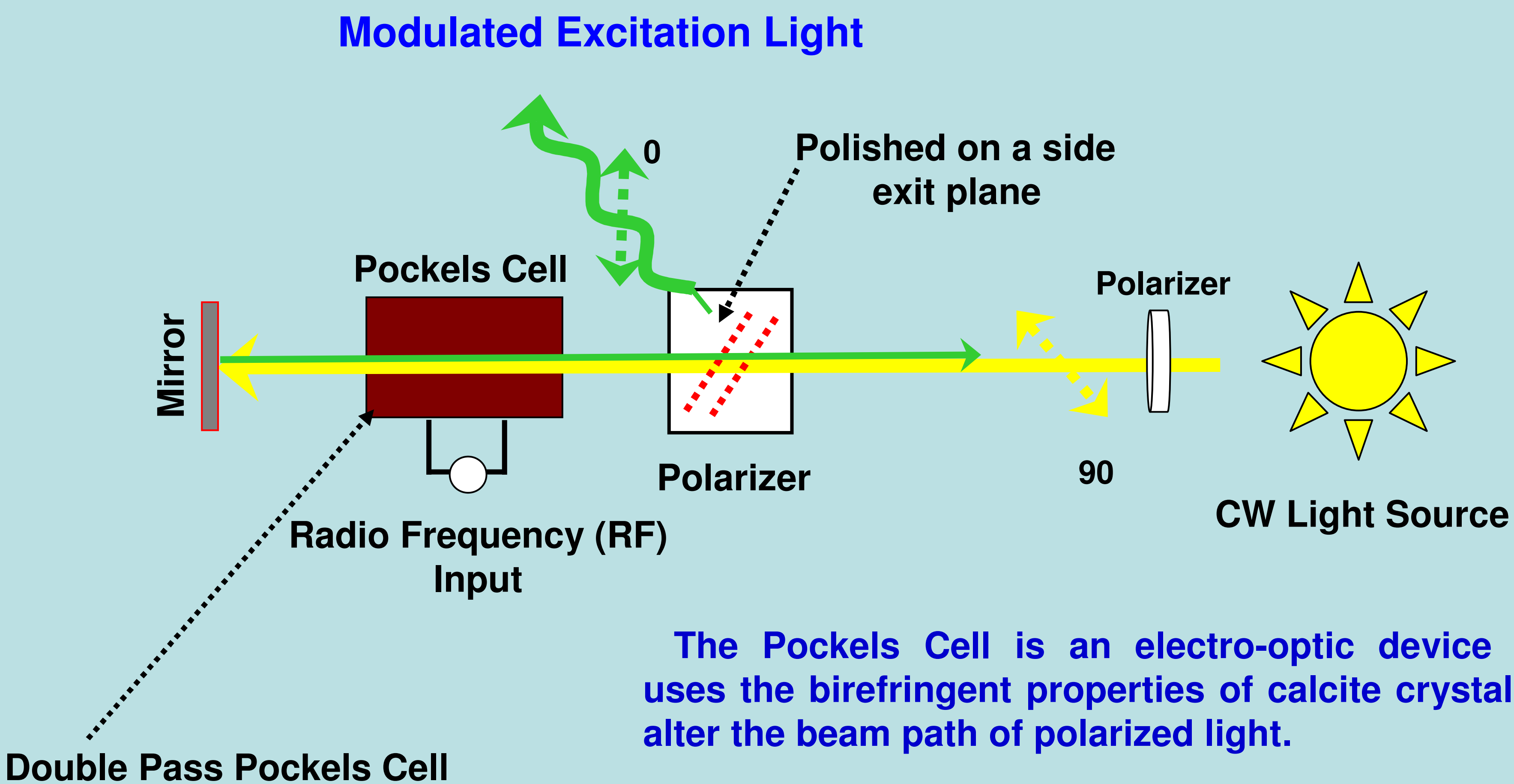
Directly Modulated Light Sources

- 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

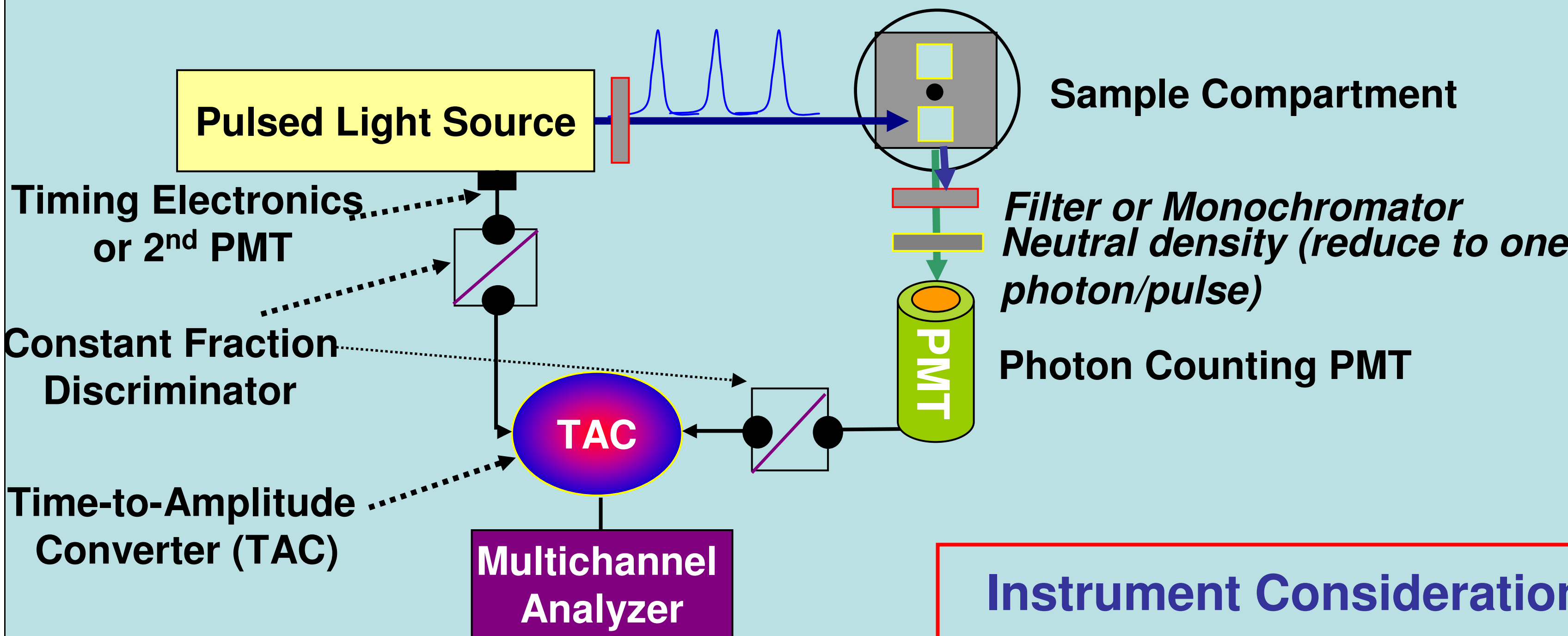
Modulation of Continuous Wave Light
Use of a Pockels Cell Modulator

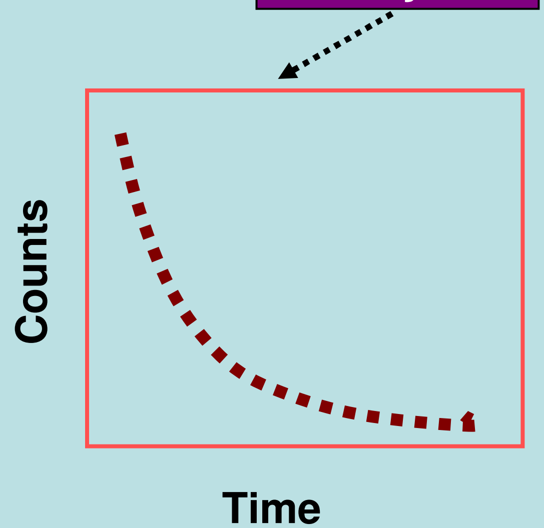


The Pockels Cell is an electro-optic device that uses the birefringent properties of calcite crystals to alter the beam path of polarized light.

In applying RF 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 light becomes modulated.

Time Correlated Single Photon Counting





Instrument Considerations

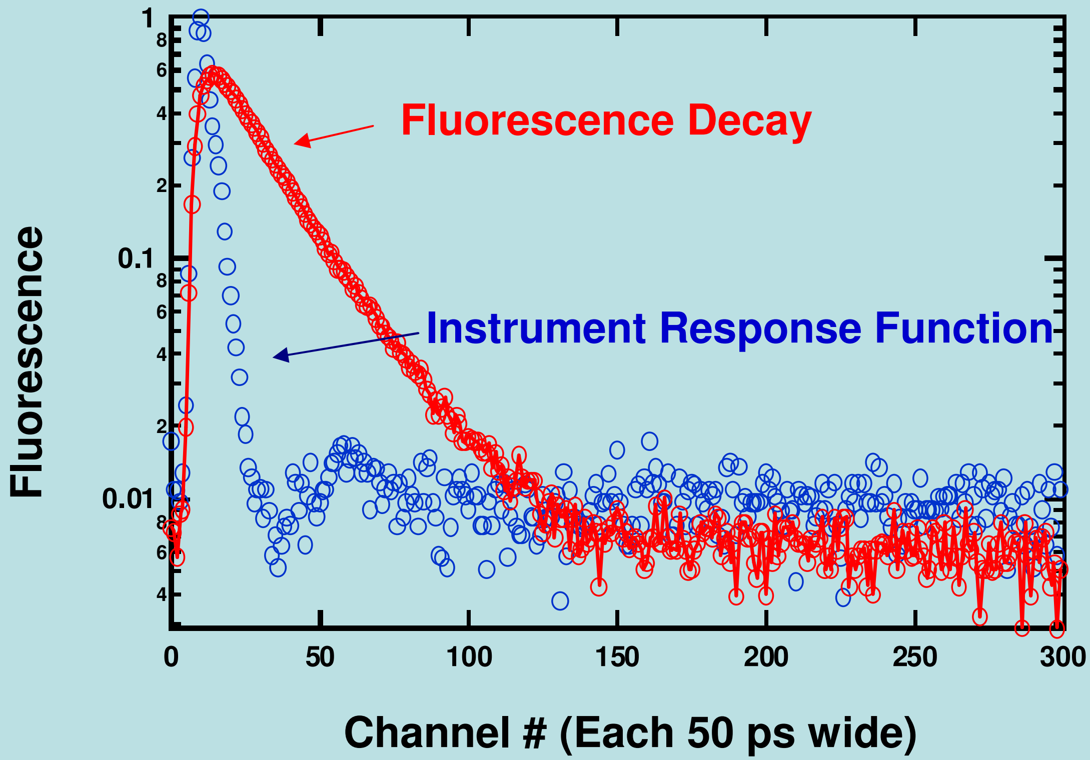
Excitation pulse width

Excitation pulse frequency

Timing accuracy

Detector response time (PMT 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.

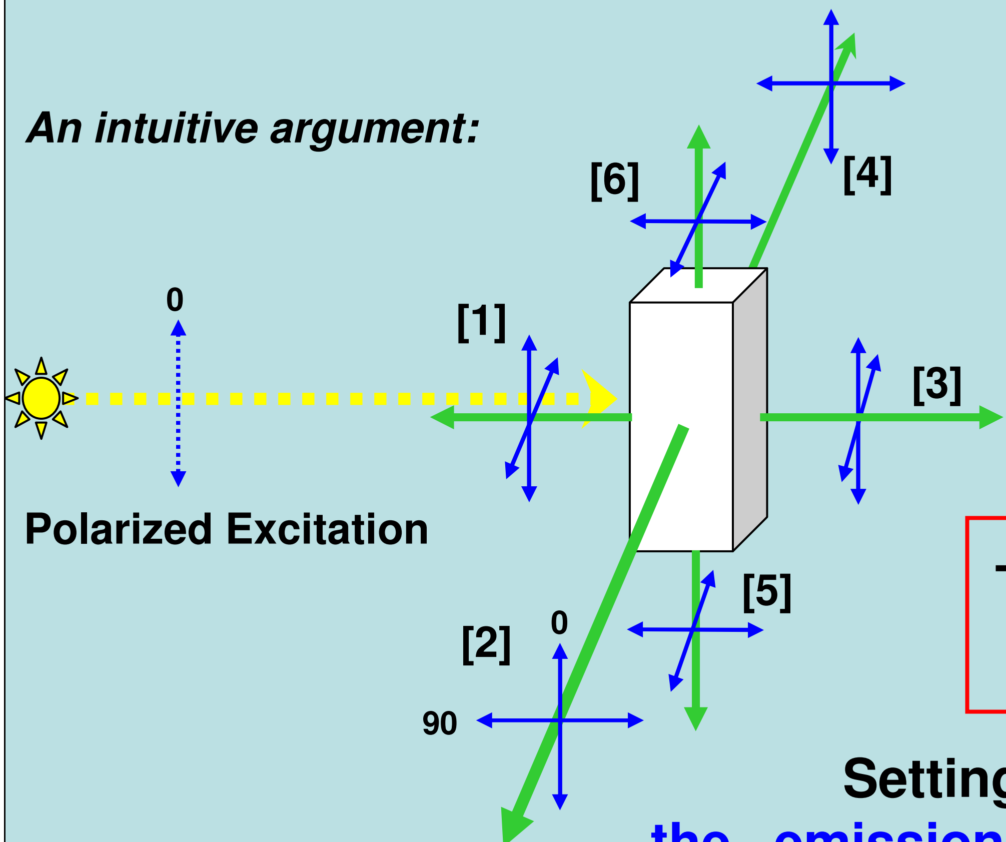
(2) The pulse frequency also influences the time window. An 80 MHz pulse frequency (Ti:Sapphire laser) would deliver a pulse every 12.5 ns and the pulses would interfere with photons arriving later than the 12.5 ns time.

Polarization Correction

There is still a polarization bias due to the geometry of our excitation and collection (even without a monochromator) !!

Corrective polarizer settings

An intuitive argument:



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

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

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

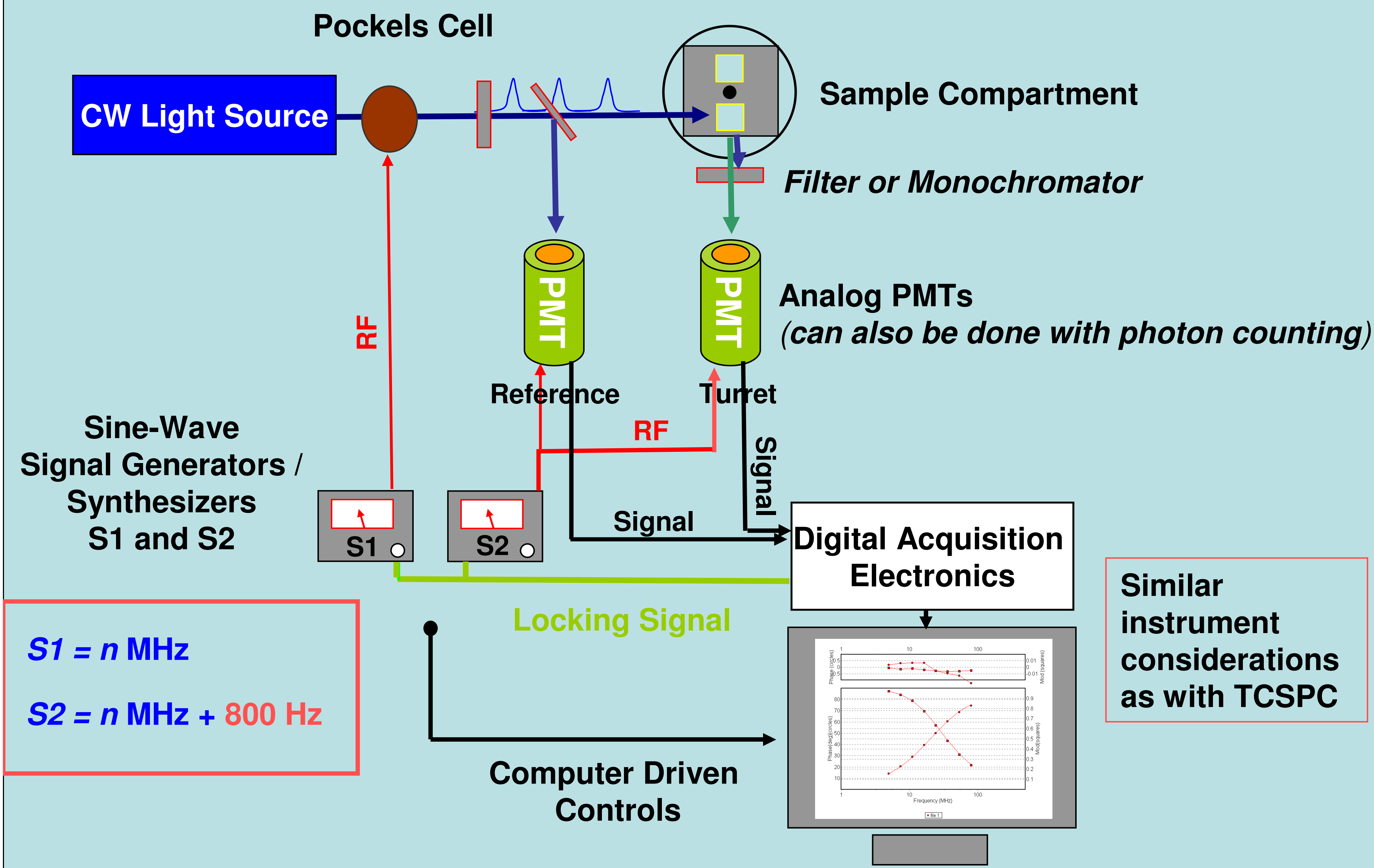
The total Intensity is proportional to:
$I_0 + 2 \times I_{90}$

Setting the excitation angle to 0° and the emission polarizer to 54.7° the proper weighting of the vectors is achieved.*

$\sin^2 54.7° = 2/3$

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

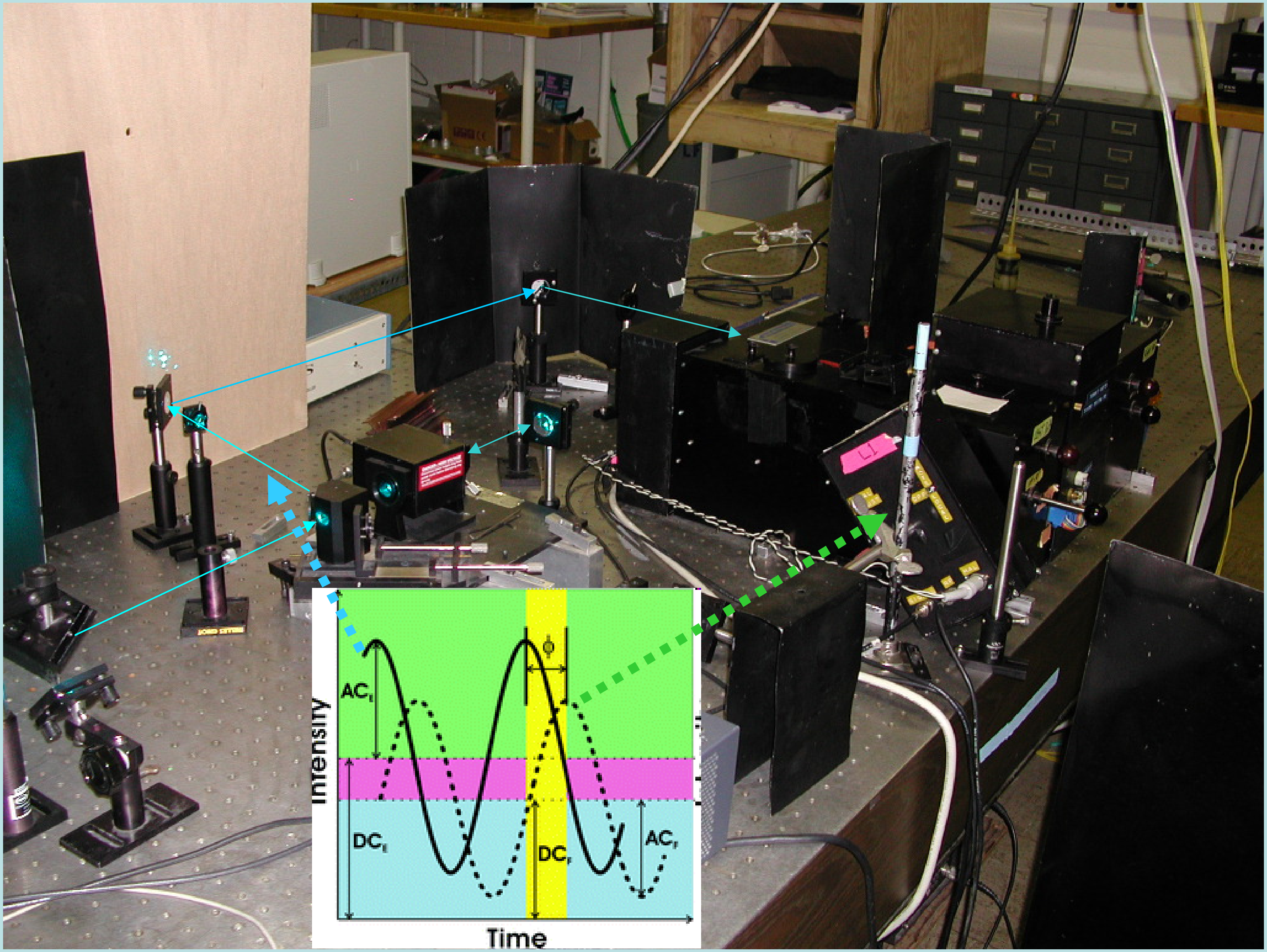
Frequency Domain Fluorometry



S1 = n MHz

S2 = n MHz + 800 Hz

Similar instrument considerations as with TCSPC



Modulated intensity figure, Source : Ross & Jameson, Photochem. Photobiol. Sci., 2008, 7, 1301 - 1312

THE FLUORESCENCE FOUNDATION

Instrument Validation through Fluorescent Standards

Tab. 6.2. Lifetime of various compounds in deoxygenated fluid solutions at 20 °C. Averages of the values measured by eight laboratories by either pulse fluorometry (four laboratories) or phase fluorometry (four laboratories)[a]

Compound[b]	*Solvent*	*Lifetime* $\bar{\tau}$ (ns)[c]	*100* $s/\bar{\tau}$	λ^{ex} (nm)	λ^{em} (nm)	*d*	*e*
NATA	Water	3.04 ± 0.04	1.2	295–325	325–415	5	4
Anthracene	Methanol	5.1 ± 0.3	6.4	300–330	380–442	6	6
	Cyclohexane	5.3 ± 0.2	3.0	295–325	345–442	5	5
9-Cyanoanthracene	Methanol	16.5 ± 0.5	6.0	295–325	370–442	6	5
	Cyclohexane	12.4 ± 0.5	4.1	295–325	345–380	4	3
Erythrosin B	Water	0.089 ± 0.002	2.5	488, 514, 568	515–575	5	4
	Methanol	0.48 ± 0.02	5.0	488, 514	515–560	5	5
9-Methylcarbazole	Cyclohexane	14.4 ± 0.4	2.5	295–325	360–400	5	4
DPA	Methanol	8.7 ± 0.5	5.9	295–344	370–475	7	7
	Cyclohexane	7.3 ± 0.5	6.2	295–344	345–480	7	6
PPO	Methanol	1.64 ± 0.04	2.4	295–330	345–425	7	7
	Cyclohexane	1.35 ± 0.03	2.5	295–325	345–425	6	6
POPOP	Cyclohexane	1.13 ± 0.05	4.3	295–325	380–450	4	4
Rhodamine B	Water	1.71 ± 0.07	4.1	488–514	515–630	5	4
	Methanol	2.53 ± 0.08	3.1	295, 488, 514	515–630	6	5
Rubrene	Methanol	9.8 ± 0.3	2.6	300, 330, 488, 514	530–590	5	5
SPA	Water	31.2 ± 0.4	1.4	300–330	370–510	5	5
p-Terphenyl	Methanol	1.16 ± 0.08	7.0	284–315	330–380	6	6
	Cyclohexane	0.99 ± 0.03	2.9	295–315	330–390	4	4

a) Data collected by N. Boens and M. Ameloot.
b) Abbreviations used: NATA: N-acetyl-L-tryptophanamide, DPA: 9,10-diphenylanthracene, POPOP: 1,4-bis(5-phenyloxazol-2-yl)benzene, PPO: 2,5-diphenyloxazole, SPA: N-(3-sulfopropyl)acridinium. All solutions are deoxygenated by repetitive freeze–pump–thaw cycles or by bubbling N_2 or Ar through the solutions.
c) The quoted errors are sample standard deviations

$$s = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(\tau_i - \bar{\tau})^2}.$$

d) Number of lifetime data measured.
e) Number of lifetime data used in the calculation of the mean lifetime $\bar{\tau}$ and its standard deviation s. The difference between columns d and e gives the number of outliers.

•B. Valeur (2002) *Molecular Fluorescence. Principles and Applications*, Wiley-VCH, Weinheim.
•Boens et al. Anal Chem. 2007 Mar 1;79(5):2137-49. Epub 2007 Feb 1.


Thank you
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Thank you
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