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Basic Fluorescence Instrumentation

Beniamino Barbieri

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Summary

- The Discovery and Understanding of the Phenomenon
- Applications
- Instrumentation for Fluorescence
- Instrumentation for Microscopy

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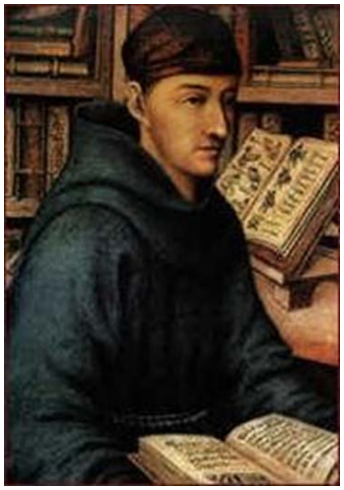
Nicolás Monardes (1493-1588), a Spanish physician and botanist publishes in 1565 the "*Historia medicinal de las cosas que se traen de nuestras Indias Occidentales"* in which he describes the bluish opalescence of the water infusion from the wood of a small Mexican tree. When made into cups and filled with water, a peculiar blue tinge was observed.

Around the same time, **Bernardino de Sahagún** (1499-1590), a Franciscan missionary, independently described the wood – called "coatli" by the Aztecs - in the Florentine Codex.

> *Coatli .....patli, yoan aqujxtiloni, matlatic iniayo axixpatli*.. "it is a medicine, and makes the water of blue color, its juice is medicinal for the urine"
>
> Sahagún, Florentine Codex Vol. III f. 266; CM-RAH, f. 203v.

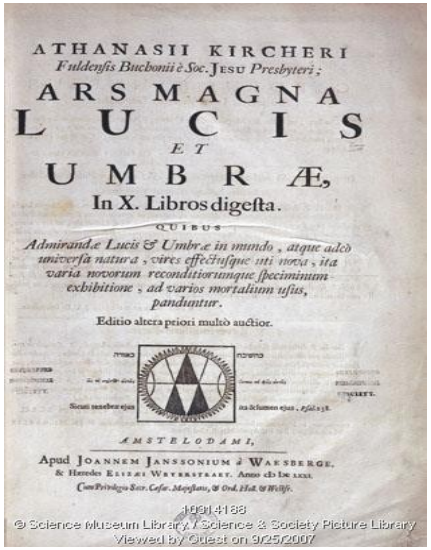


An early Latin translation (1574) of Monardes' work by the influential Flemish botanist **Charles de L'Écluse** (1526-1609), in which the wood's name is given as *Lignum Nephriticum (kidney wood),* helped to extend awareness of its strange optical properties in Europe.

This wood was very popular in XVI - XVII Europe, because of its medicinal virtues for treating kidney ailments.

An Englishman, **John Frampton**, translated Monardes description in 1577 as "*.. white woodde which gives a blewe color*" when placed in water that was good "*for them that doeth not pisse liberally and for the pains of the Raines of the stone..*"

The German Jesuit priest **Athanasius Kircher**, among his numerous achievements, wrote a book in 1646 called *Ars Magna Lucis et Umbrae* in which he described his observation of the wood extract *Lignum nephriticum*.



ATHANASII KIRCHERI
Fuldensis Buchonii è Soc. JESU *Presbyteri;*
ARS MAGNA
LUCIS
ET
UMBRÆ,
In X. Libros digesta.
QUIBUS
Admirandæ Lucis & Umbræ in mundo, atque adeò universa natura, vires effectusque uti nova, ita varia novorum reconditiorumque speciminum exhibitione, ad varios mortalium usus, panduntur.
Editio altera priori multò auctior.

AMSTELODAMI,
Apud JOANNEM JANSSONIUM à WAESBERGE,
& Heredes ELIZÆI WEYERSTRAET. Anno cIↃ IↃC LXXI.
Cum Privilegio Sacr. Cæsar. Majestatis, & Ord. Hol. & Wellfr.

1991 4188
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Viewed by Guest on 9/25/2007

Light passing through an aqueous infusion of this wood appeared more yellow while light reflected from the solution appeared blue.

In the ensuing centuries the wood was no longer used and the botanic identity of the LN was lost in a confusion of several species.

Safford, in 1915, succeeded in disentangling the botanic problem and identified the species which produced the Mexican LN as *Eynsemhardtia polystachia*.

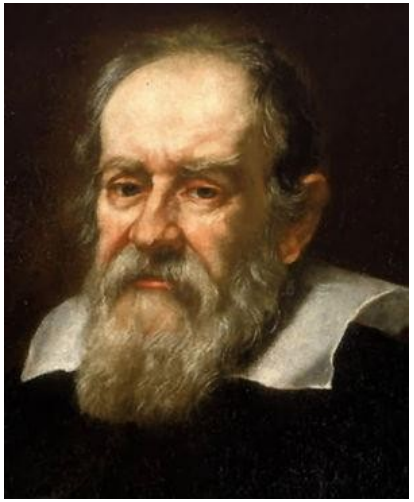
More recently, several highly fluorescent glucosyl-hydroxichalcones were isolated from this plant.

Recent studies by **Ulises Acuña** indicate that the original blue emission observed by the Aztecs was probably due to the conversion of Coatline B, under mildly alkaline conditions, to a strongly blue-emitting compound - resembling fluorescein - with an emission maximum near 466nm and with a quantum yield near 0.8

In 1603, **Vincenzo Casciarolo**, a Bolognian shoemaker who was dreaming of producing gold, discovered that a stone, after being baked, emitted a purple-blue light in the dark.

Most likely, the stone, named lapis solaris, was barium sulfate. The discovery starts a lively debate between scientists at the time.

Galileo Galilei (1612) described the emission of light (phosphorescence) from the famous Bolognian stone, "*It must be explained how it happens that the light is conceived into the stone, and is given back after some time, as in childbirth*."

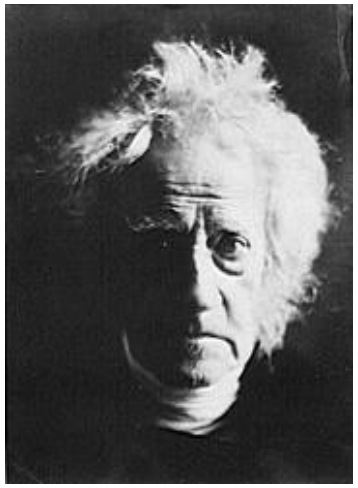


Robert Boyle (1664) was inspired by Monardes' report and investigated this system more fully.

He discovered that after many infusions the wood lost its power to give color to the water and concluded that there was some "essential salt" in the wood responsible for the effect.

He also discovered that addition of acid abolished the color and that addition of alkali brought it back.

David Brewster (1833) described that when a beam of white light passed through an alcohol solution of leaves a red beam could be observed from the side (which was of course chlorophyll fluorescence).
He considered the effect due to "dispersion".



John Herschel (1845) made the first observation of fluorescence from quinine sulfate - he termed this phenomenon "epipolic dispersion".

IV. *Ἀμόρφωτα*, No. I.—*On a Case of Superficial Colour presented by a homogeneous liquid internally colourless. By Sir* John Frederick William Herschel, *Bart., K.H., F.R.S., &c. &c.*

Received January 28, 1845,—Read February 13, 1845.

Edmond Becquerel (1842) reports the emission of light from Calcium sulphate upon excitation in the UV.

He notes that the emission occurs at a wavelength longer than that of the incident light.

Later on (1858) builds the first phosphoroscope enabling him to measure the decay times of phosphorescence.

XXX. *On the Change of Refrangibility of Light.* *By* G. G. Stokes, *M.A., F.R.S., Fellow of Pembroke College, and Lucasian Professor of Mathematics in the University of Cambridge.*

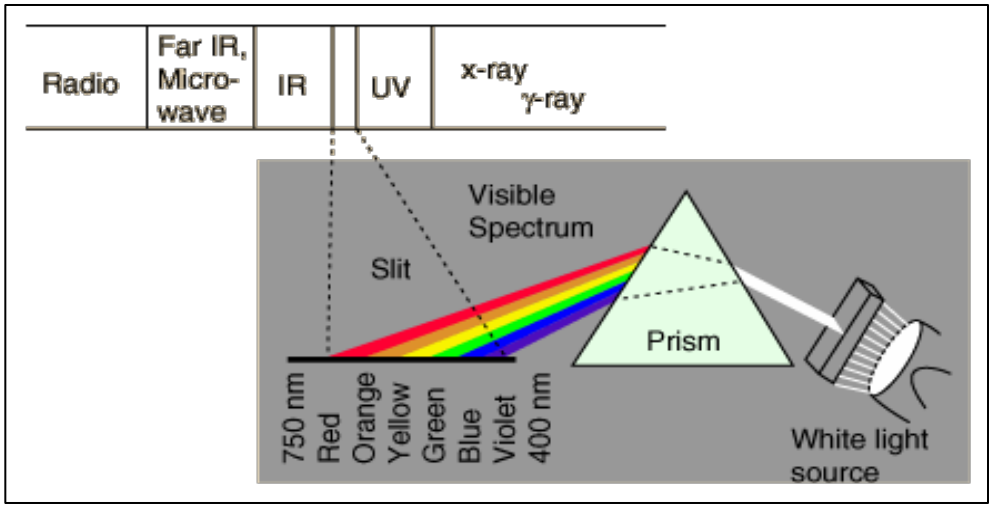
Received May 11,—Read May 27, 1852.

1. The following researches originated in a consideration of the very remarkable phenomenon discovered by Sir John Herschel in a solution of sulphate of quinine, and described by him in two papers printed in the Philosophical Transactions for 1845, entitled 'On a Case of Superficial Colour presented by a Homogeneous Liquid internally colourless,' and 'On the Epipolic Dispersion of Light.' The solution of quinine, though it appears to be perfectly transparent and colourless, like water, when viewed by transmitted light, exhibits nevertheless in certain aspects, and under certain incidences of the light, a beautiful celestial blue colour. It appears from the experiments of Sir John Herschel that the blue colour comes only from a stratum of fluid of small but finite thickness adjacent to the surface by which the light enters.

George Gabriel Stokes (1852) published his massive treatise "*On the Change of Refrangibility of Light*" – more than 100 pages. He initially used the term "dispersive reflection" to describe the phenomenon presented by quinine sulphate.

* I confess I do not like this term. I am almost inclined to coin a word, and call the appearance *fluorescence*, from fluor-spar, as the analogous term *opalescence* is derived from the name of a mineral.

Stokes used a prism to disperse the solar spectrum and illuminate a solution of quinine. He noted that there was no effect until the solution was placed in the ultraviolet region of the spectrum.



It was certainly a curious sight to see the tube instantaneously lighted up when plunged into the invisible rays : it was literally *darkness visible*. Altogether the phenomenon had something of an unearthly appearance.

This observations led Stokes to proclaim that fluorescence is of longer wavelength than the exciting light, which led to this displacement being called the **Stokes Shift.**

He also seems to have been the first to propose, in 1864, the use of fluorescence as an analytical tool, in a lecture "*On the application of the optical properties to detection and discrimination of organic substances.*"

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The Discovery Of Fluorescent Compounds

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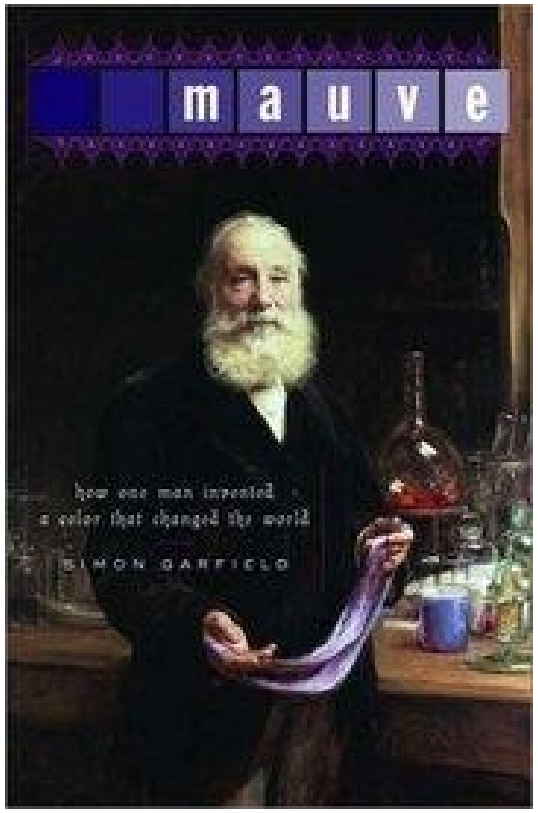
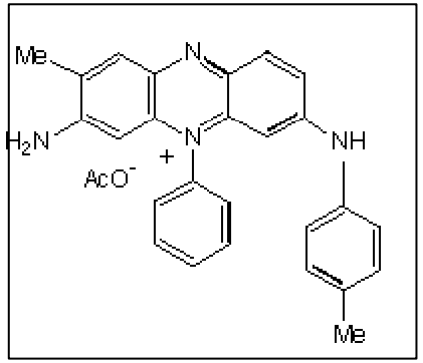
William Henry Perkin: the search for Quinine Sulfate!





In 1856, at the age of 18, William Henry Perkin set out with idea of making *quinine* by oxidizing *allytoluidine* –instead he accidentally produced the synthetic dye, *mauve,* a derivative of coal tar with an aniline base.

Adolph Von Baeyer (1871) a German chemist, synthesized Spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one, 3',6'-dihydroxy.

He apparently coined the name “fluorescein”, from “fluo” and resorcin, (resorcinol) which he reacted with phthalic anhydride.

In 1905 he was awarded the Nobel Prize in Chemistry "in recognition of his services in the advancement of organic chemistry and the chemical industry, through his work on organic dyes and hydroaromatic compounds".

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Applications and Instrumentation

One of the first uses of fluorescein was in 1877 in a major ground-water tracing experiment in southern Germany.

The results of this experiment showed that the River Danube and Rhine are connected by underground streams.

Fluorescein was placed in the Danube and about 60 hours later it appeared in an affluent of the Rhine.

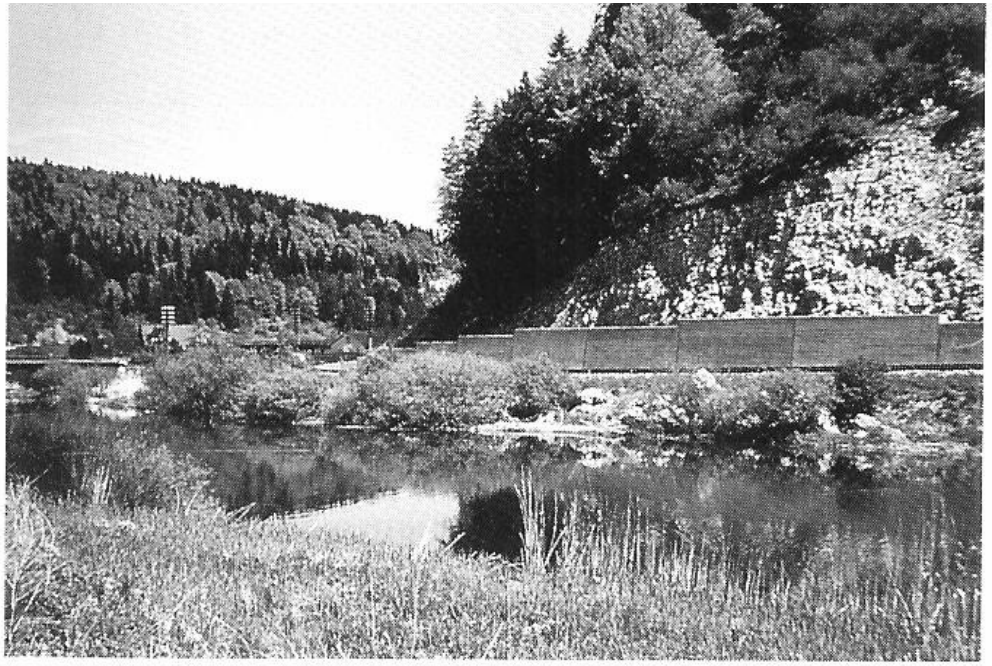

Fig. 4 The Danube at the Immendingen weir with sinkholes on the right bank and the well-stratified Oxfordian limestone behind

10 Kilograms of fluorescein were used!

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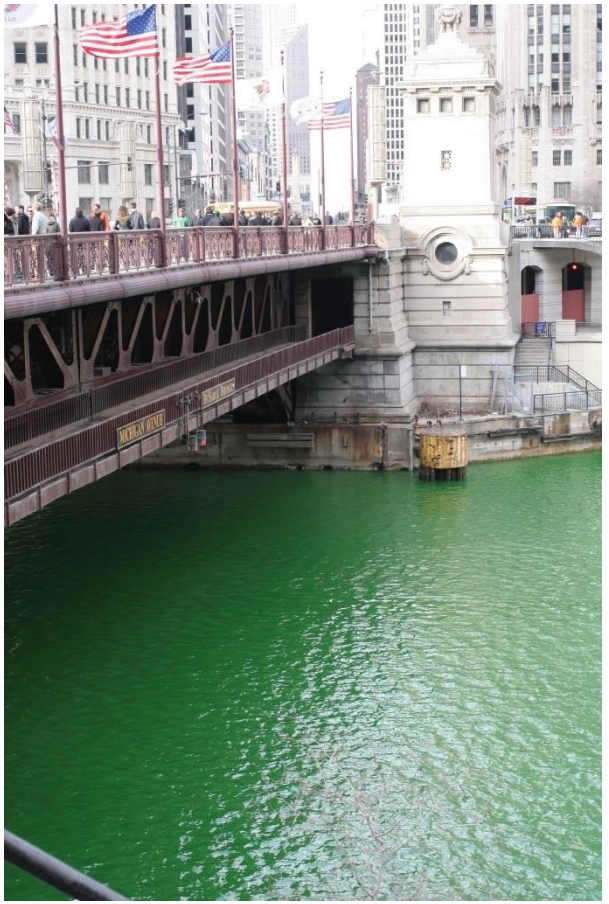
Two miracles appear that day, the river turns a perfect shade of green something that many other cities have tried but have not been successful at doing, and the second miracle by starting with the color orange giving the impression that river will be orange only to convert the river to that true Irish green. We believe that is where the leprechaun comes in.

By Dan O'Leary, courtesy of
www.ChicagoStPatsParade.com



St. Patrick's Day 2014, Chicago
(courtesy of Darcy Lear)

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The Instrumentation: Quinine sulfate again!

During World War II, the United States government issued a desperate call to scientists and doctors: find a treatment for malaria! Since Japan had taken over most of the world's supply of quinine-the best known treatment-Allied forces in the Pacific Theater needed a new drug, and fast.

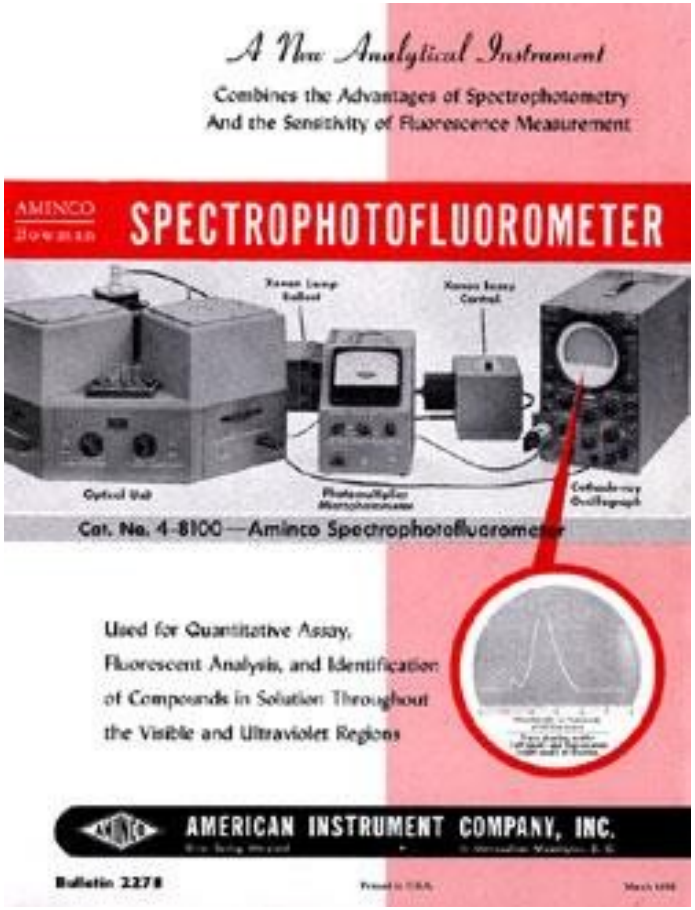
With an instrument called a fluorometer, Brodie and Udenfriend could measure how much of the drug was in a patient's plasma sample.

1956

Aminco-Bowman (Silver Spring, MD)

Farrand Optical Co. (Walhalla, NY)

Cost: over $8,000 – which is about $120,000 in 2016 !



Fluorometers

(courtesy of ISS)

(courtesy of Agilent)

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Other Applications/Instruments

Flow Cytometry

(courtesy of Sony-ICyt)

Genome Sequencing

(courtesy of Pacific Biosciences)

Microwell Plate Readers

(courtesy of Molecular Devices)

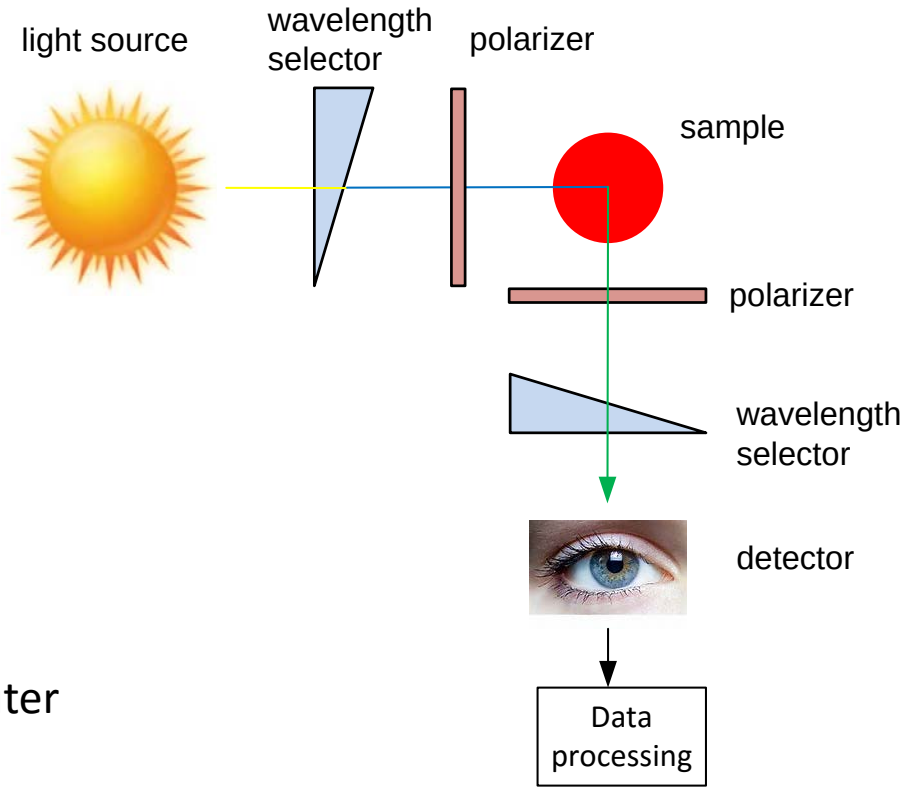
Immunoassay Chemistry Analyzer

(courtesy of Abbott Diagnostics)

A fluorometer measures the following five fluorescence parameters:

- Excitation spectrum
- Emission spectrum
- Anisotropy (polarization)
- Quantum yield
- Decay times of fluorescence

Instrument schematics



- Light source
- Wavelength selector
- Polarizers
- Detector
- Data processing computer

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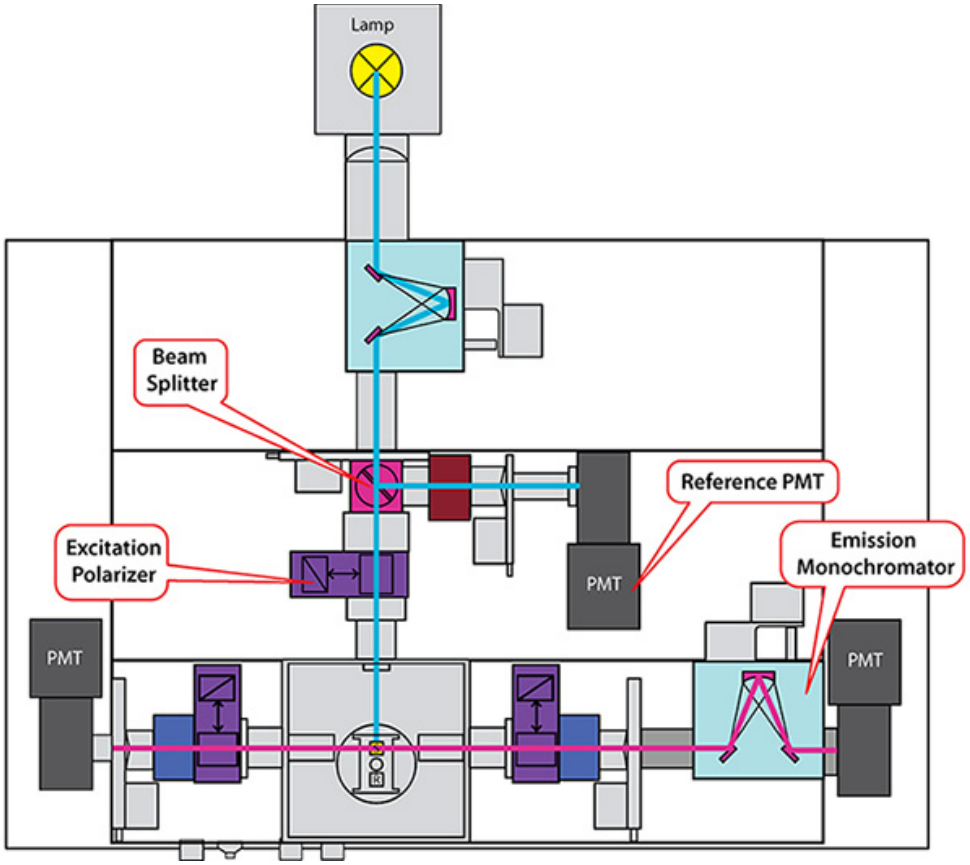
The PC1 Photon Counting Spectrofluorometer



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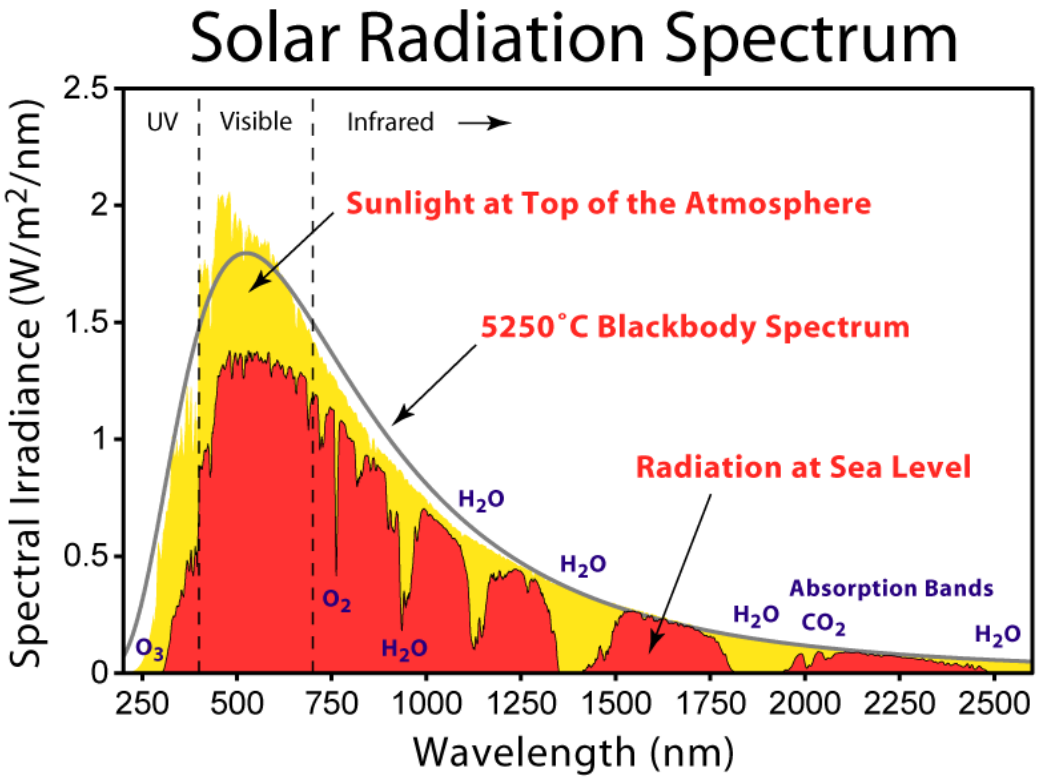
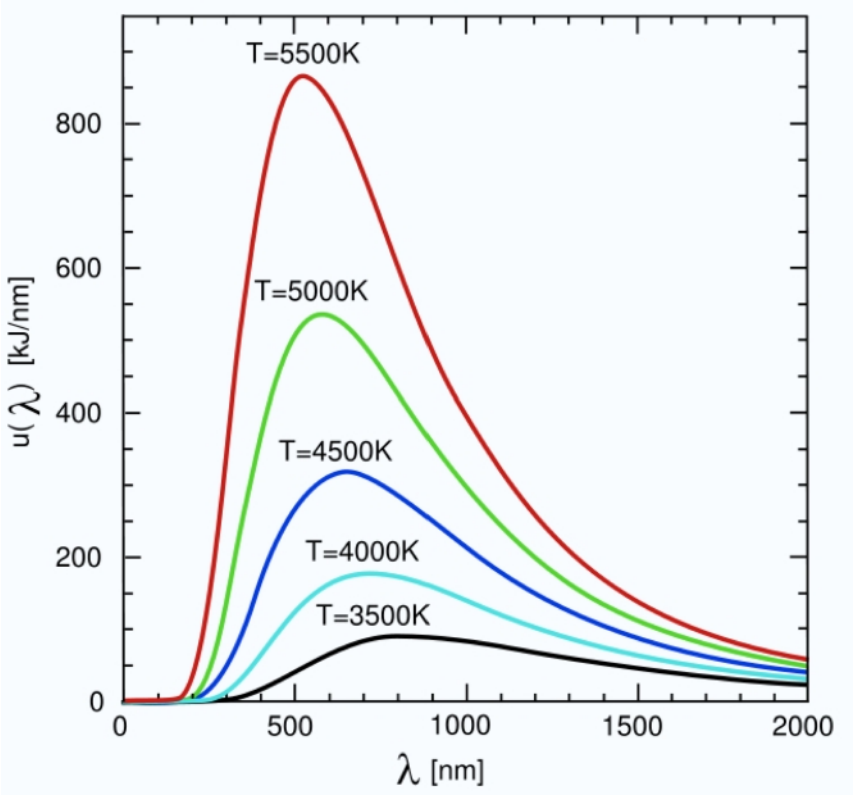
Light Sources: O sole mio ...

Che bella cosa è na jurnata 'e sole,
n'aria serena doppo na tempesta!
Pe' ll'aria fresca pare già na festa...
Che bella cosa na jurnata 'e sole.



Blackbody spectrum



Light sources

Lamps

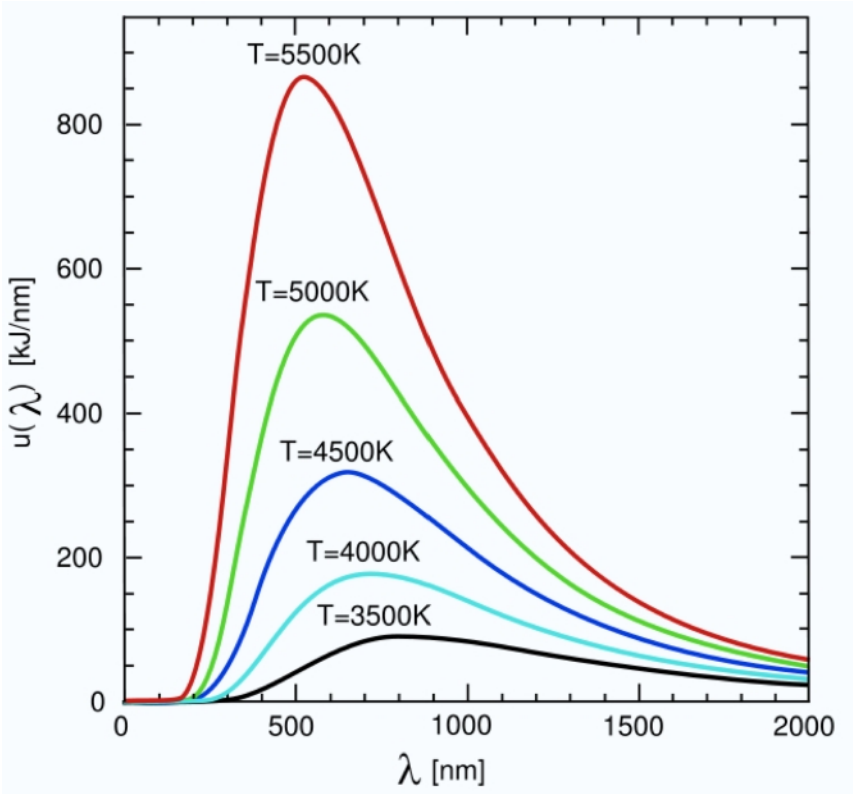
- Xenon arc
- Hollow cathode
- Halogen
- Tungsten

Light Emitting Diodes (LEDs)

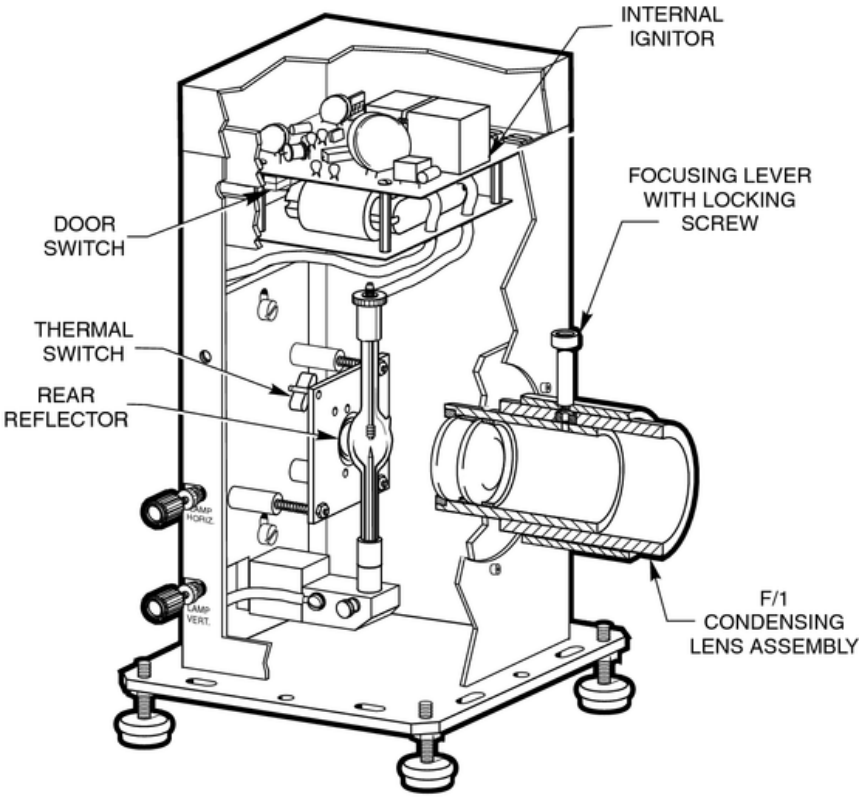
Lasers

- Laser diodes
- Ti:Sapphire
- supercontinuum

Synchrotron radiation



Xenon arc lamps



Courtesy of Newport Corp.

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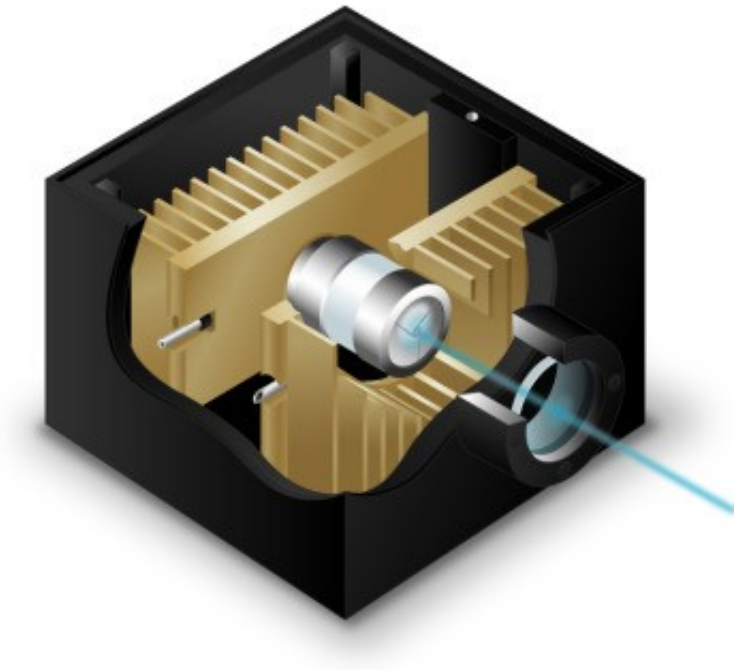
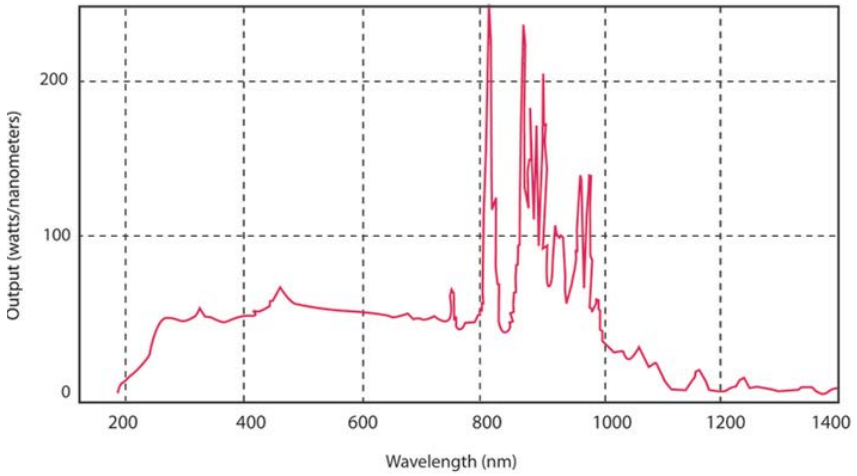
Cermax xenon arc lamp

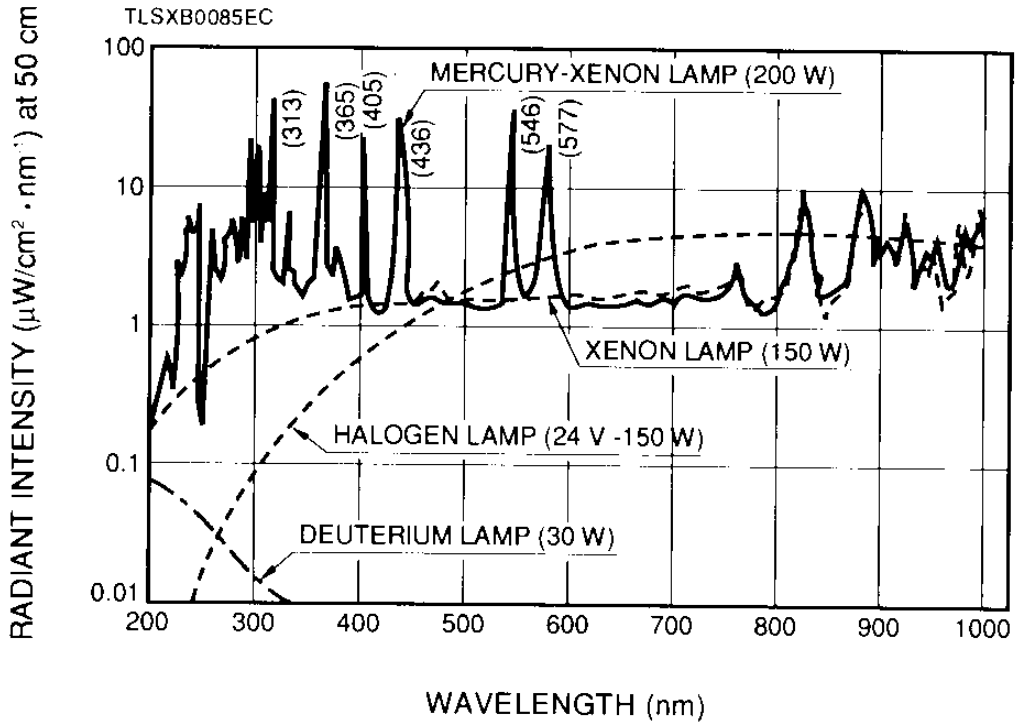




- 300W power
- horizontal electrodes with internal mirror
- 90% of the optical energy collected
- replaceable by the user
- 2000 hours lifetime

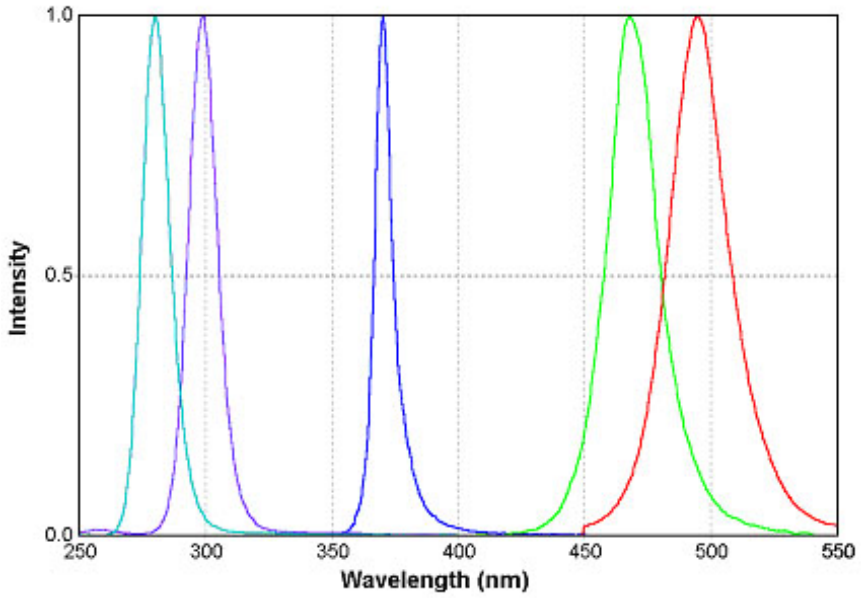
Spectra of various lamps

Figure 5: Spectral Distribution of Various Lamps



Light Emitting Diodes (LEDs)

Wavelengths from 260 nm to 2400 nm



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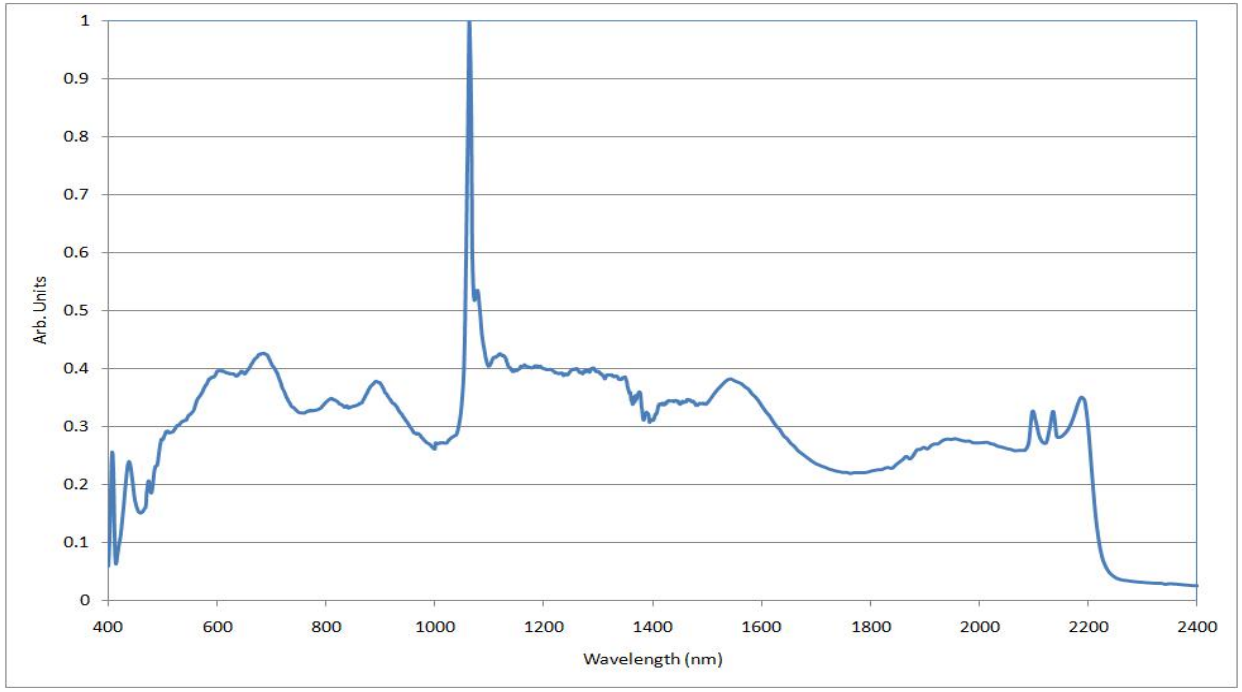
Lasers (Light Amplification by Stimulated Emission of Radiation)

Light source	Lines (nm)
Laser diodes	370, 405, 440, 473, 488, 514, 532
OPSL	552, 561, 568, 588
supercontinuum	Tunable in the range 390-2000
Ti:Sapphire	Tunable in the range 700-1000

White laser (fiber laser)

- Short-wavelength cut-in ~405nm
- Long wavelength edge >2200nm
- 6ps pulsewidth
- Up to 6W total power



(Courtesy of Fianium)

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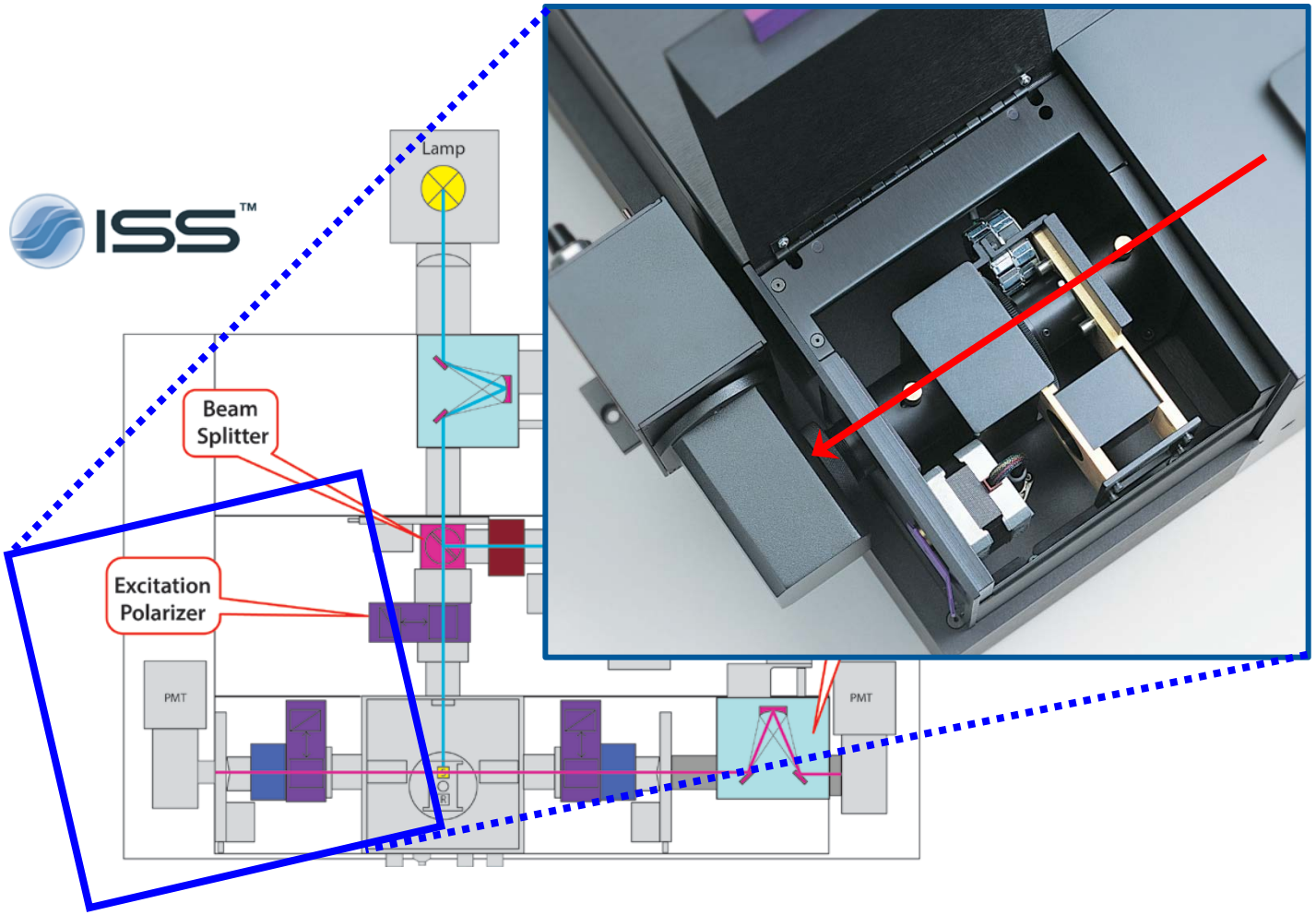
Filter channel



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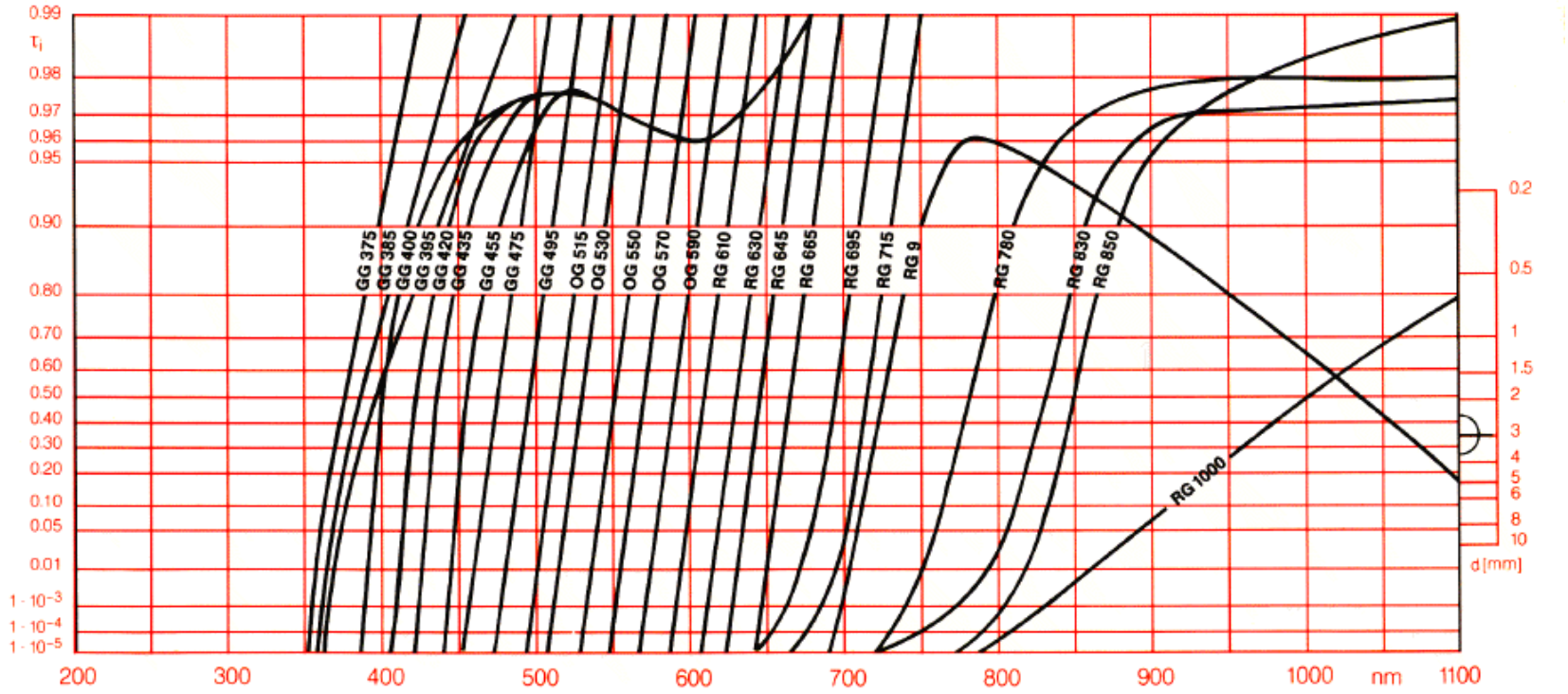
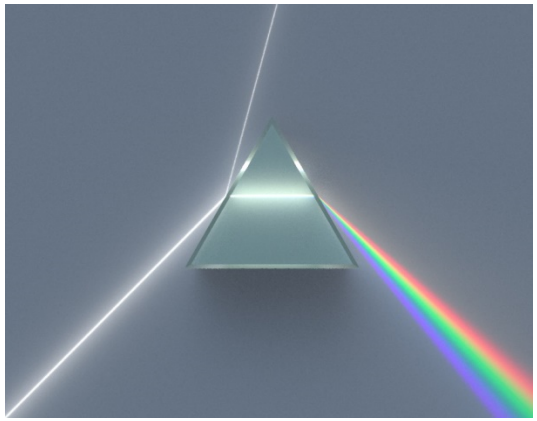
High-pass filters



Wavelength selector - prism



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).

Problems with prisms:

- the light dispersion is not linear with the wavelength
- normal glass prisms does not pass UV light – so expensive quartz prism has to be used

α_i

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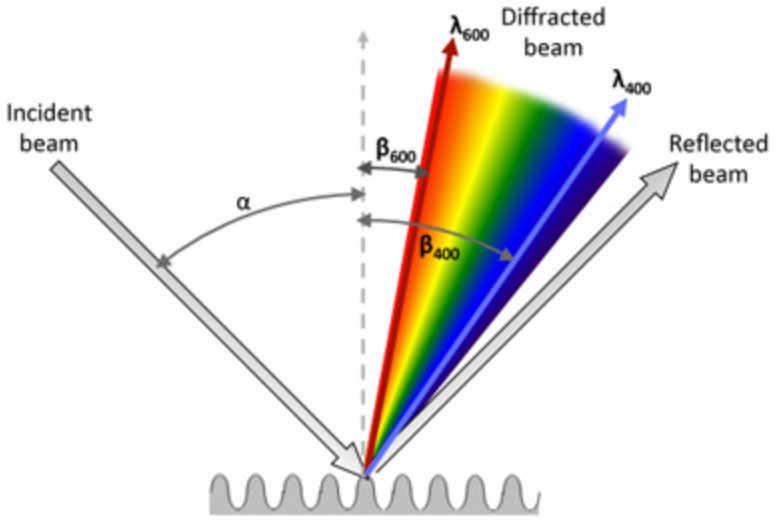
Wavelength selector: diffraction grating



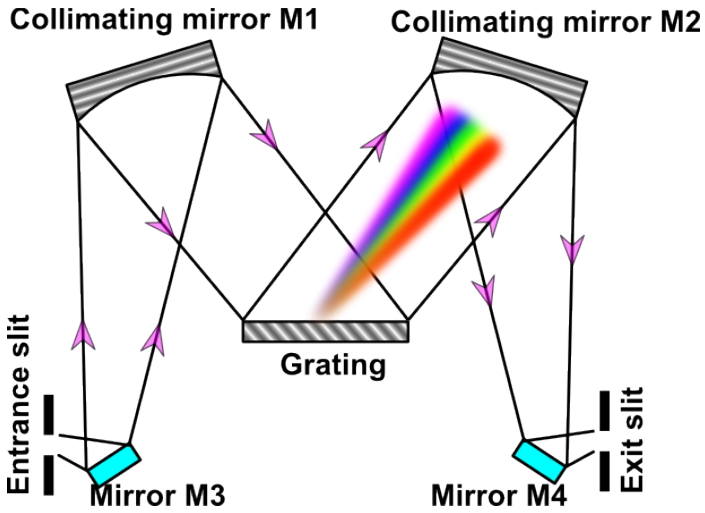
Types of gratings
- ruled
- holographic

Geometries of gratings
- plane
- concave

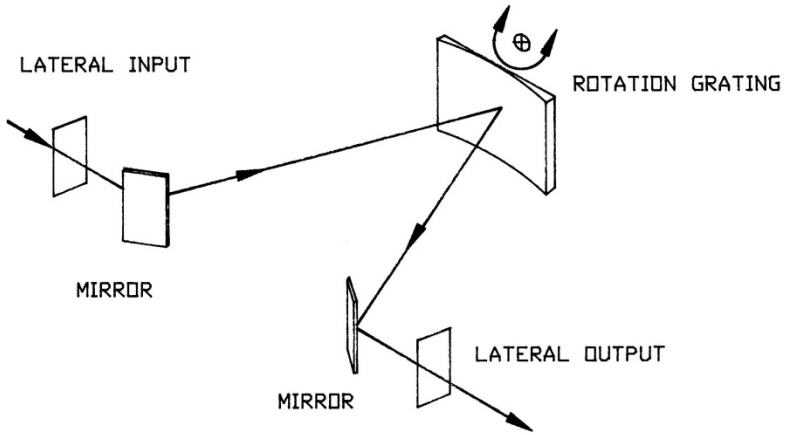
$$d\left(\sin\alpha_i + \sin\beta_m\right) = m\lambda$$

[α_i angle of incidence]

Monochromators: gratings mounts

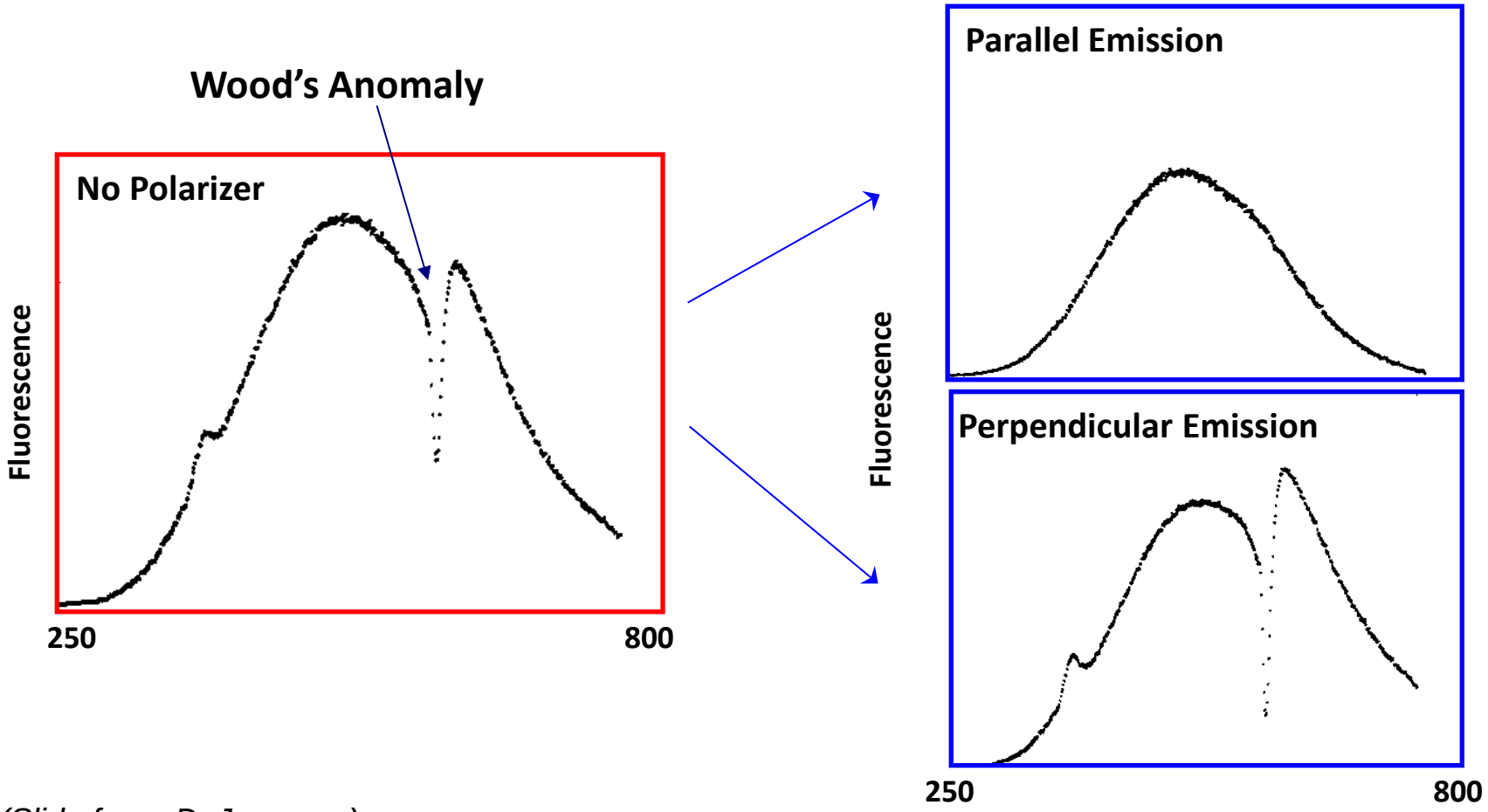


Czerny-Turner

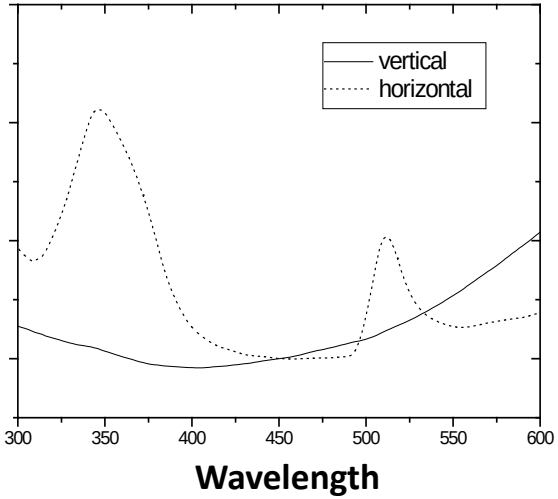


Seya-Namioka

Wood's Anomaly: Tungsten Lamp Profile Collected on an SLM Fluorometer

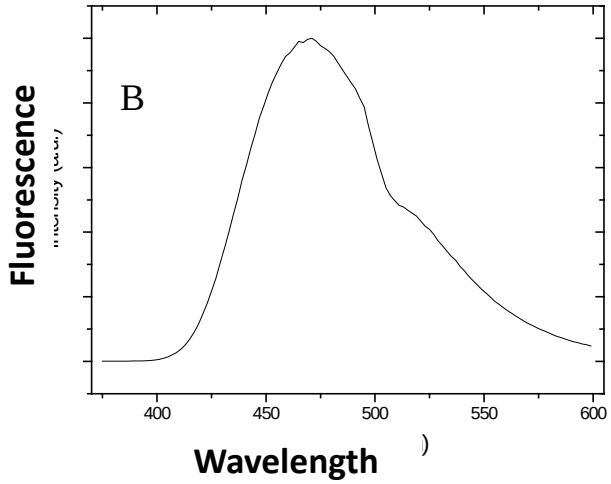


(Slide from D. Jameson)

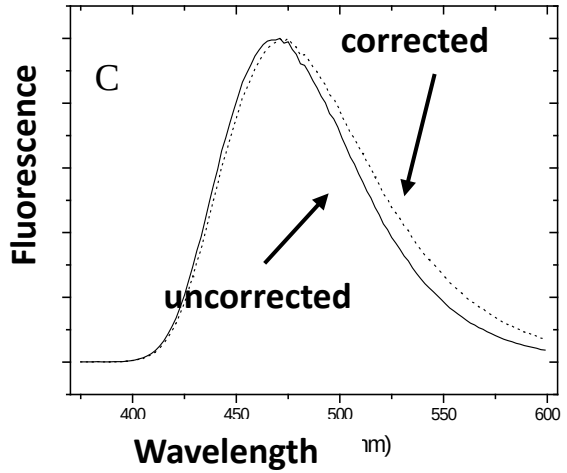


ISSPC1
Correction Factors

ANS Emission Spectrum, no polarizer



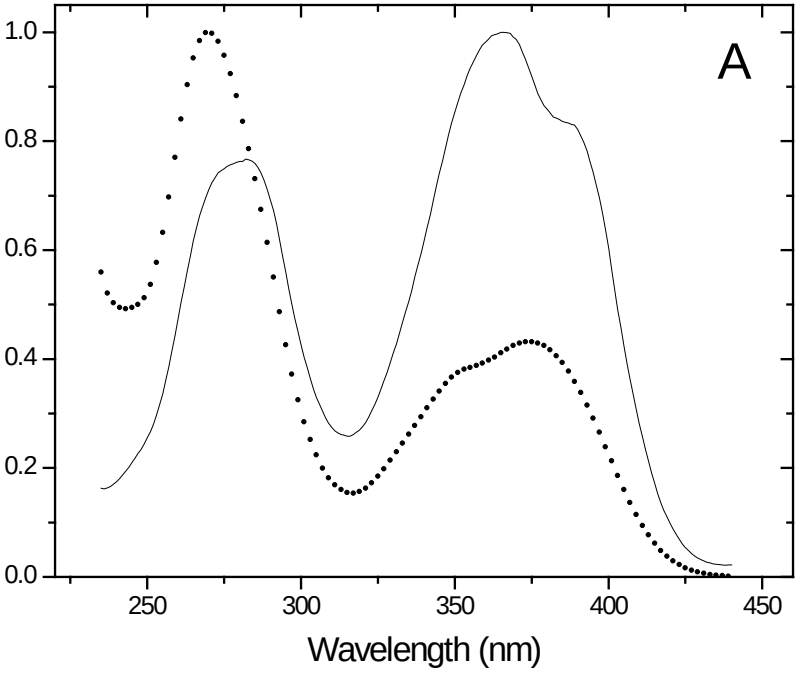
ANS Emission Spectrum, parallel polarizer



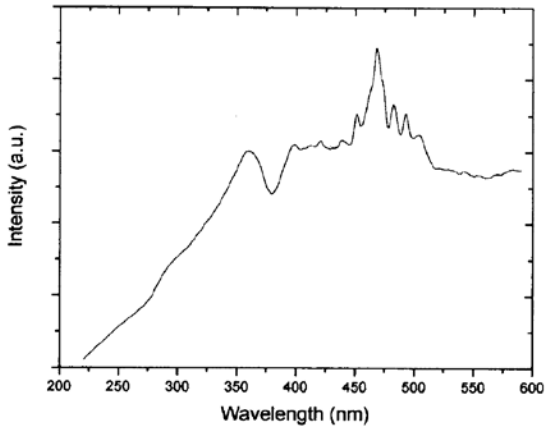
(from Jameson et. Al., Methods in Enzymology, 360:1)

Correction of Excitation Spectra

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

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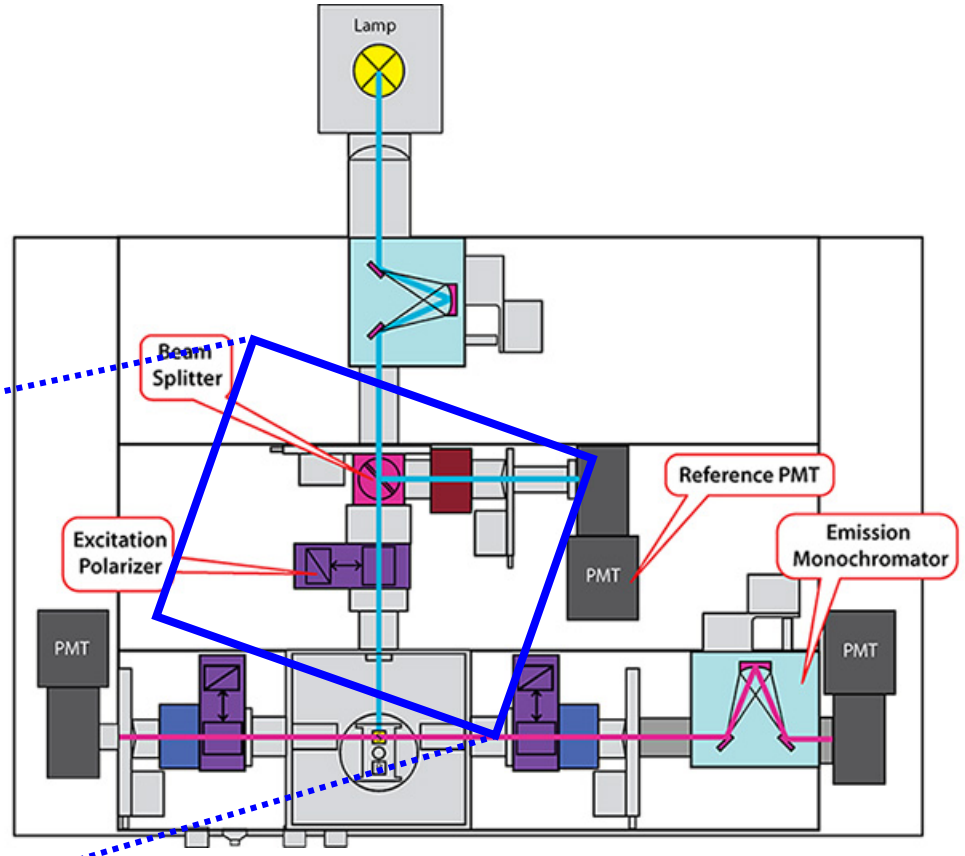
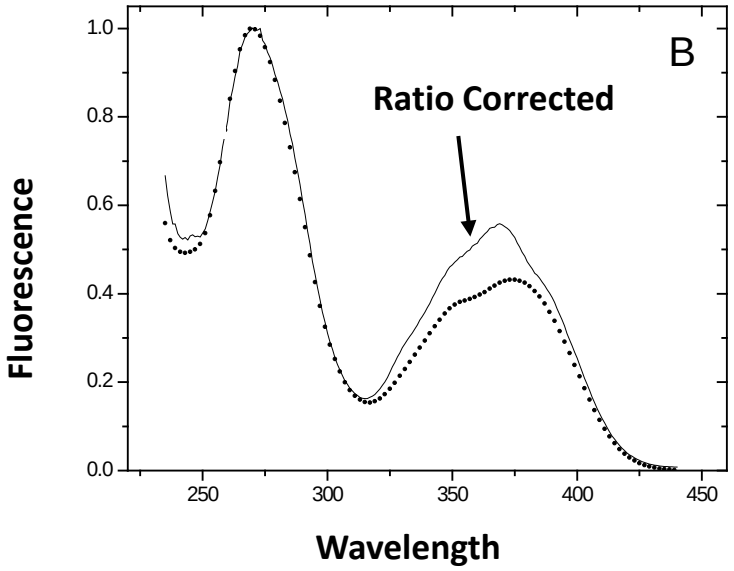
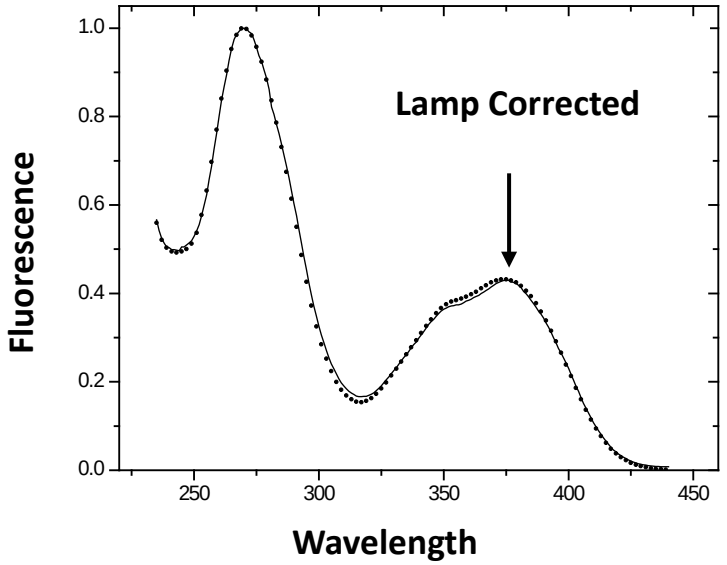
Excitation correction



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

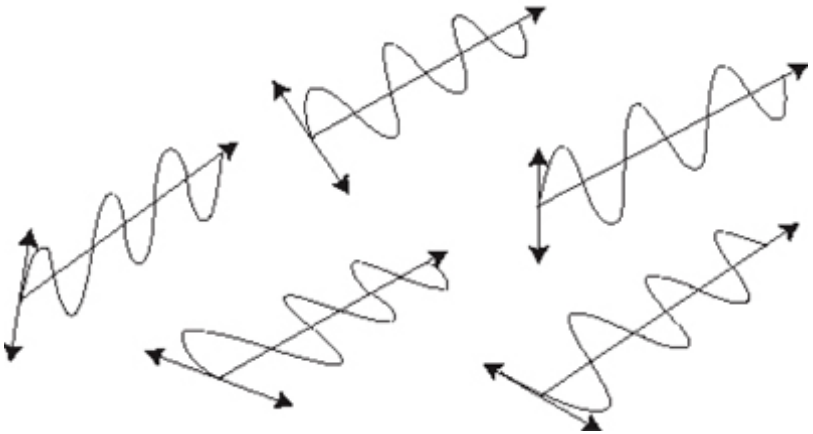


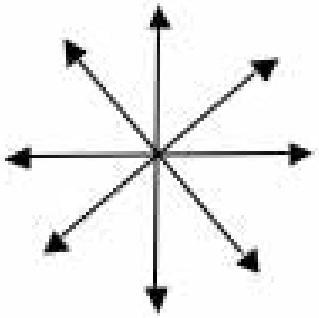
If we determine the lamp curve at the sample position and then divide the sample excitation spectrum by this curve we can get excellent agreement



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

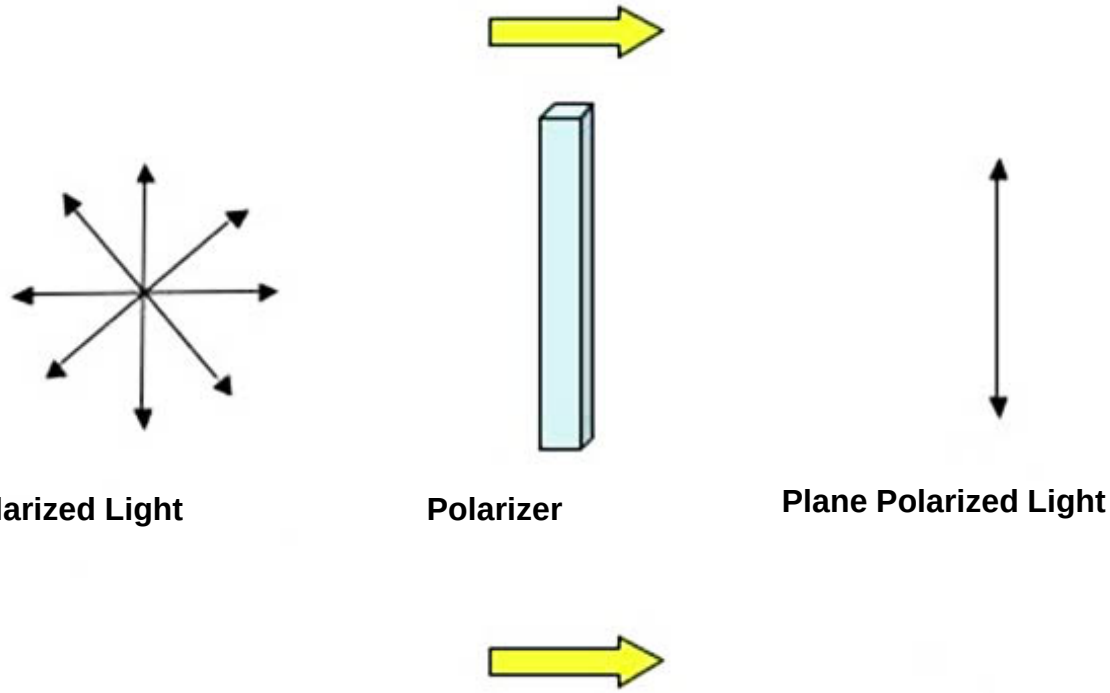
Natural Light





Unpolarized Light

Polarization



(1) dichroic devices, which operate by effectively absorbing one plane of polarization (e.g., Polaroid type-H sheets based on stretched polyvinyl alcohol impregnated with iodine)

(2) $CaCO_3$ crystal polarizers - which differentially disperse the two planes of polarization

Polarization

Etienne-Louis Malus (1775-1812)

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

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

He published his findings in 1809:
"*Sur une propriété de la lumière réfléchie par les corps diaphanes*" (*Bull. Soc. Philomat.* I:16)

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

(*courtesy of D. Jameson)*

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

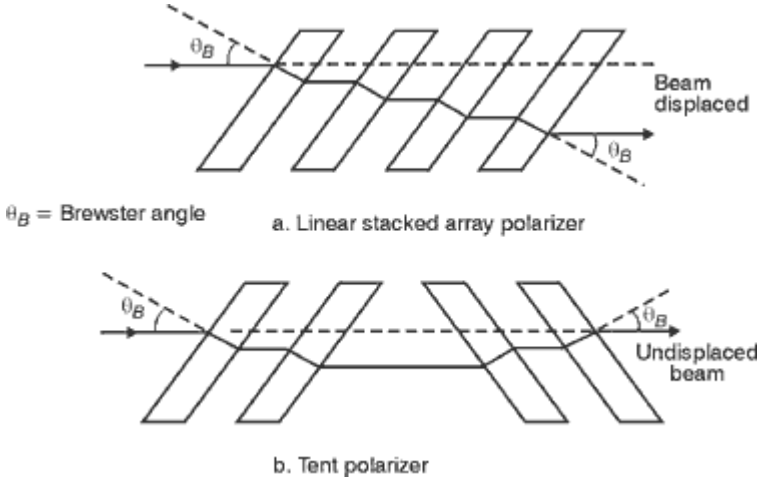
III. *On the law of the partial polarization of light by reflexion.* By DAVID BREWSTER, *LL.D. F.R.S. L. & E.*

Read February 4, 1830.

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

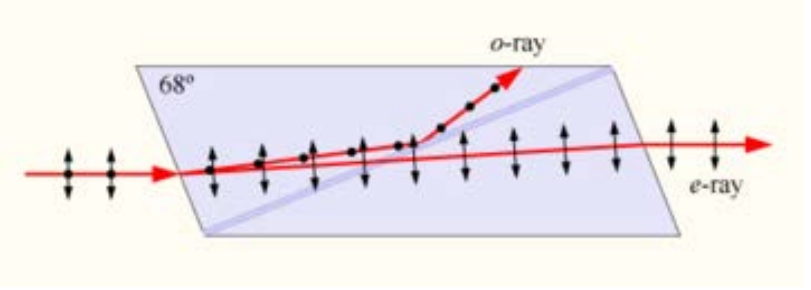
$$\theta_B = tan^{-1}\left(\frac{n_2}{n_1}\right)$$

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



(courtesy of D. Jameson)

Crystal Polarizers



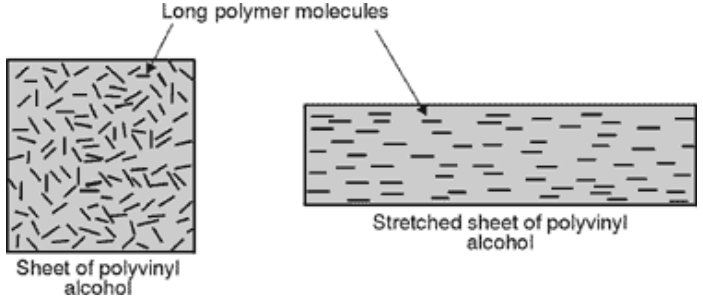
In 1828, Nicol joined two crystals of Iceland spar, cut at an of 68° angle, using Canada balsam.

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

Edwin Herbert Land (1909-1991)

(courtesy of D. Jameson)

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

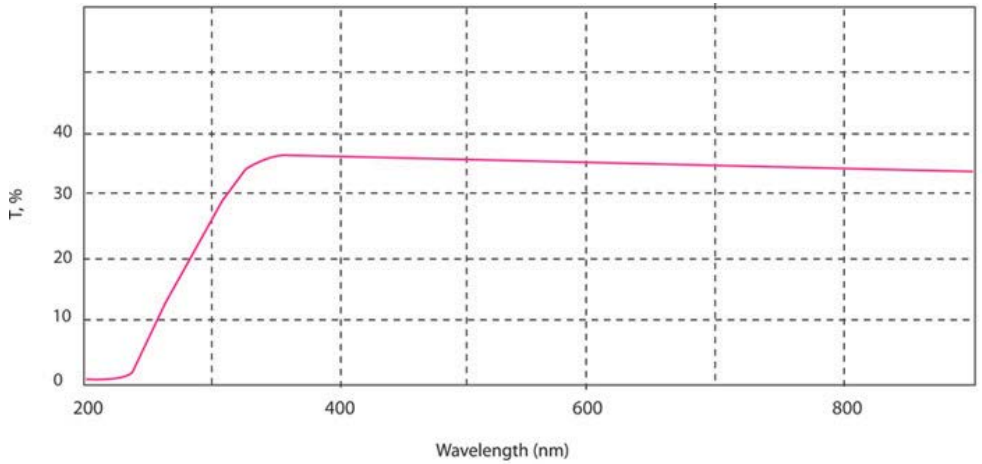


Polarizers used in fluorescence

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

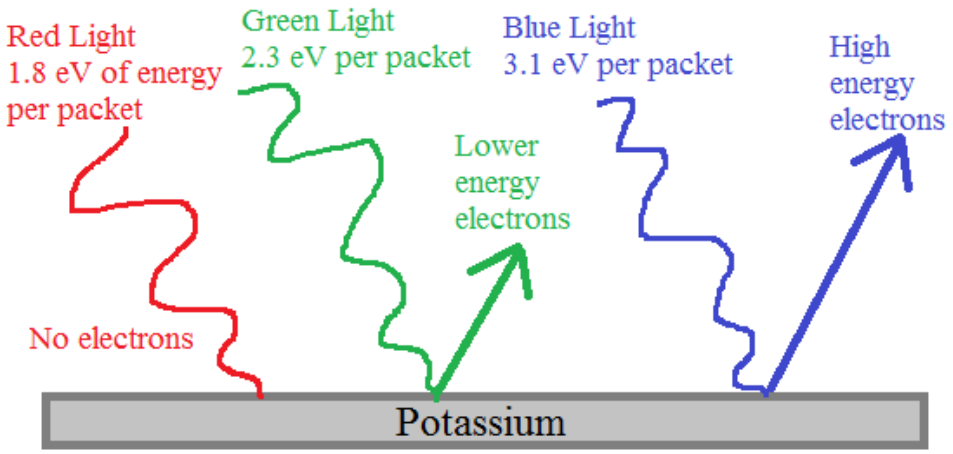
Factors to look for in polarizers:

- Transmission range
- Extinction ratio



Detectors: Photomultiplier tubes (PMTs)

The working principle is based upon the photoelectric effect, discovered by Hertz in 1887. An electron is ejected if the surface is illuminated with photons having energy > E_0 with $E_0 = h\nu_0$ depending upon the specific material.



Potassium electrons need 2 units of energy to escape (2 eV).

The effect was explained by Einstein in 1905; he got the Nobel prize in 1921 for this work.

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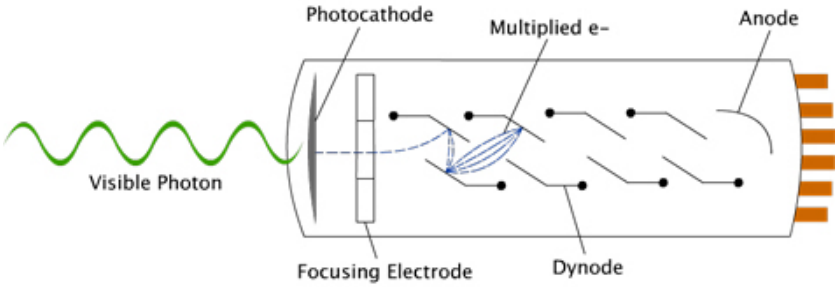
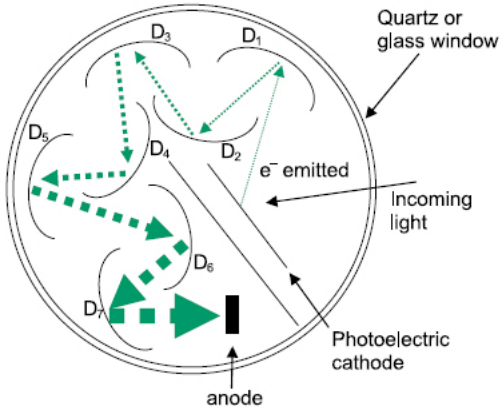
Schematics of a PMT



R9880

R928



H7422P

Parameters characterizing PMTs

Model	Detector type	STT (ps)	SER (ps)	TTS/IRF (ps)	Anode rise time (ps)	Application
H5773 (PMC-100)	multialkali	5400	1500	140-200	780	lifetimes
H7422P-40	GaAs	6500		170-220	780	lifetimes
R10467U	Hybrid PMT		850	130	400	lifetimes
R3809U-50	MCP	550	360	25-30	150	lifetimes
R928	multialkali	22000	5000	1200	2200	Utilized for steady-state measurements

Table 6.7 Light detectors for the ChronosBH

- SST- Transit time of the electron through the PMT
- SER - Single Electron Response
- TTS – Transit Time Spread (Total Transit Time)
- Anode Rise Time – Overall Time response of the detector

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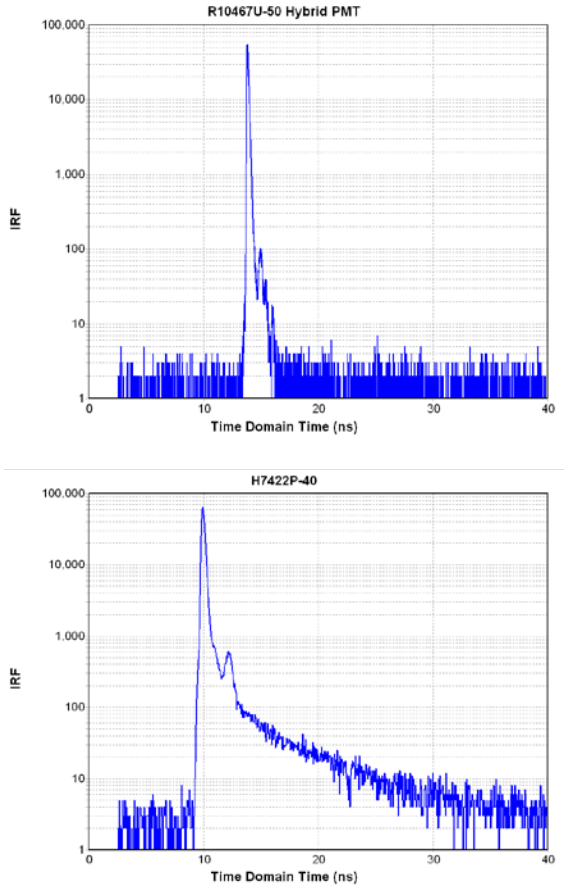
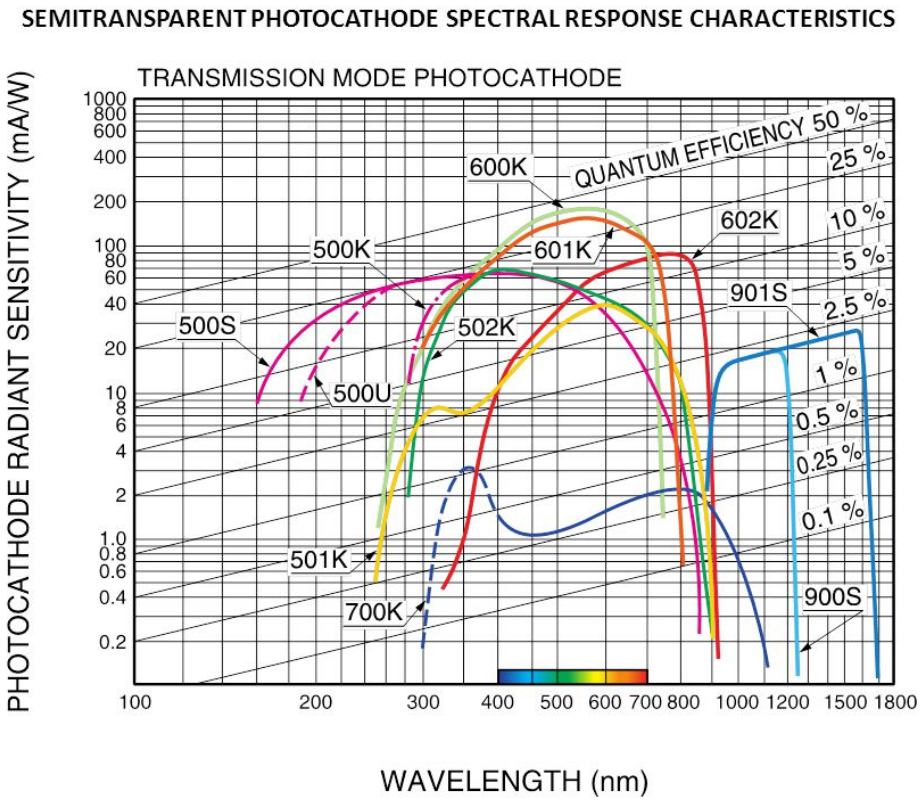
What to look for in a PMT





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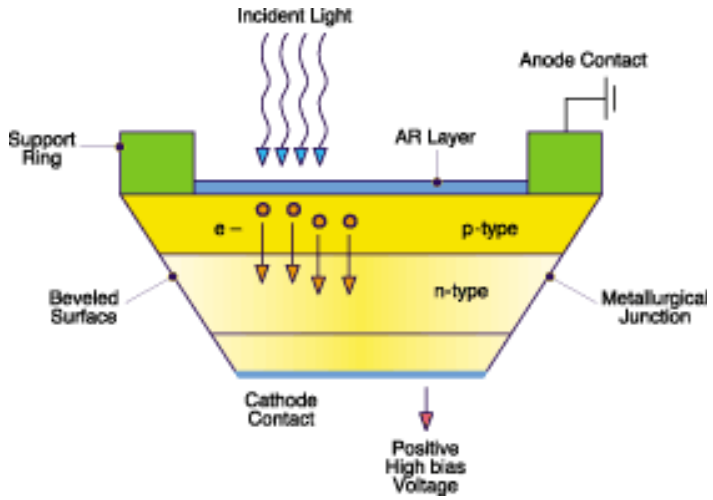
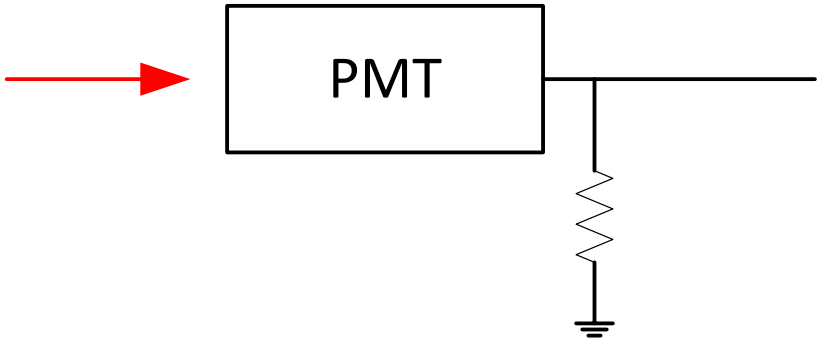
Detectors: Avalanche Photodiodes (APDs)

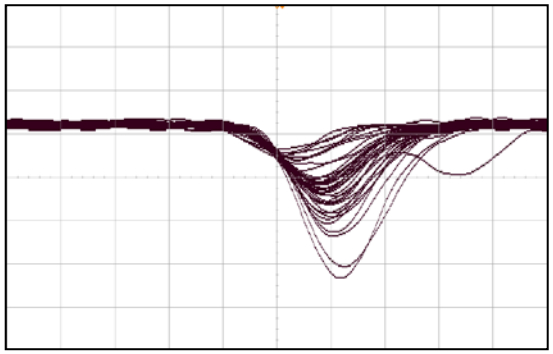
APD are photodiodes working in high reverse bias (they converts light to current using a semiconductor material)

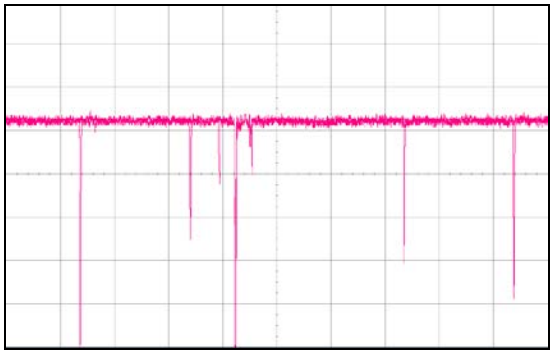


- High quantum efficiency
- Low noise
- Good wavelength range

Signal processing





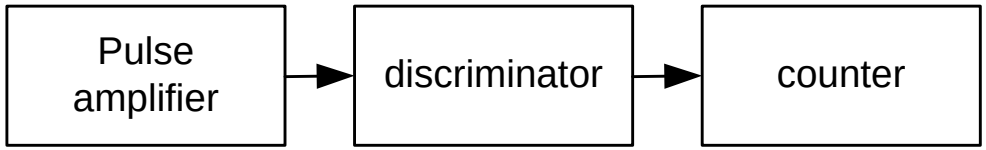


Pulses from a H7422P-40 on a 1ns/div scale (left) and a 100 ns/div scale (right). The number of pulses detected is about 100,000 per second.

Signal processing: two approaches

- Analog

- Photon counting



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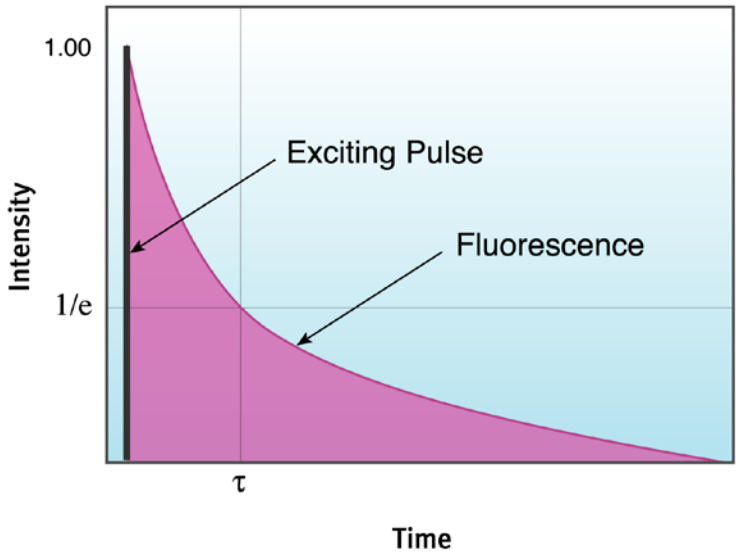
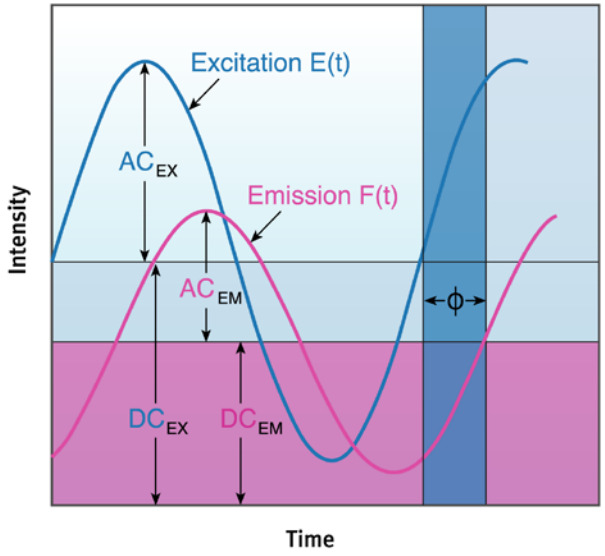
Lifetime instrumentation



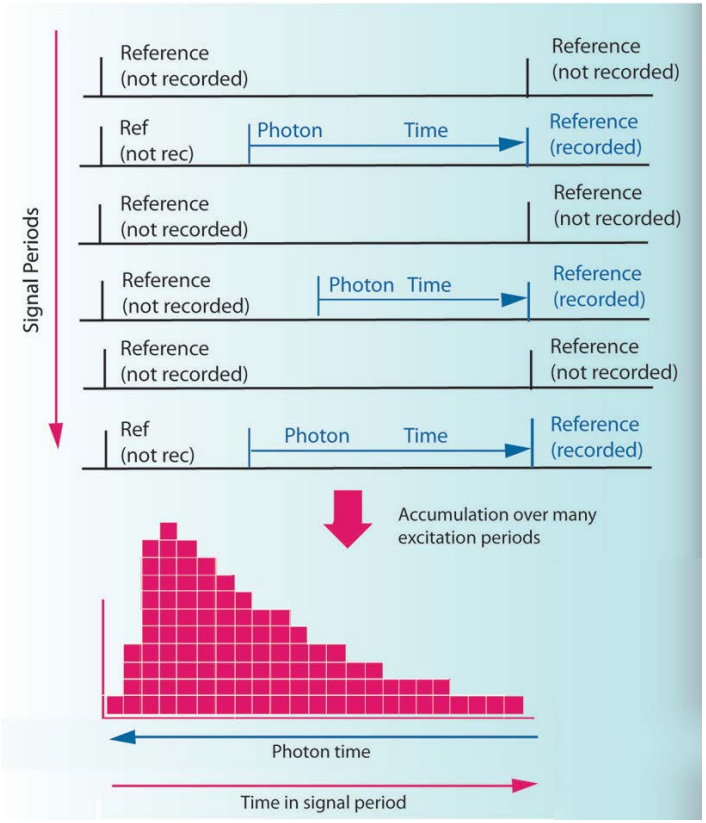


$$I(t) = I_0 \, \mathrm{e}^{-t/\tau}$$

$$\tau_P = \frac{1}{\omega}\tan\phi \qquad \tau_M = \frac{1}{\omega}\sqrt{\frac{1}{m^2}-1}$$

TCSPC

CFD
TAC
ADC


Signal Periods
Reference (not recorded)
Reference (not recorded)
Ref (not rec)
Photon
Time
Reference (recorded)
Reference (not recorded)
Reference (not recorded)
Reference (not recorded)
Photon
Time
Reference (recorded)
Reference (not recorded)
Reference (not recorded)
Ref (not rec)
Photon
Time
Reference (recorded)
Accumulation over many excitation periods
Photon time
Time in signal period

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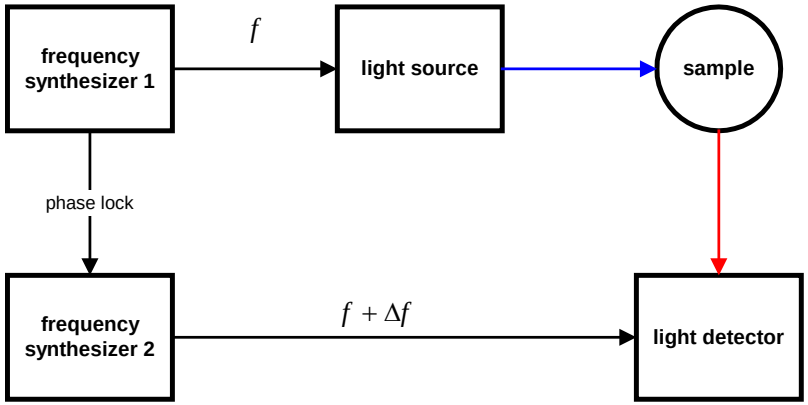
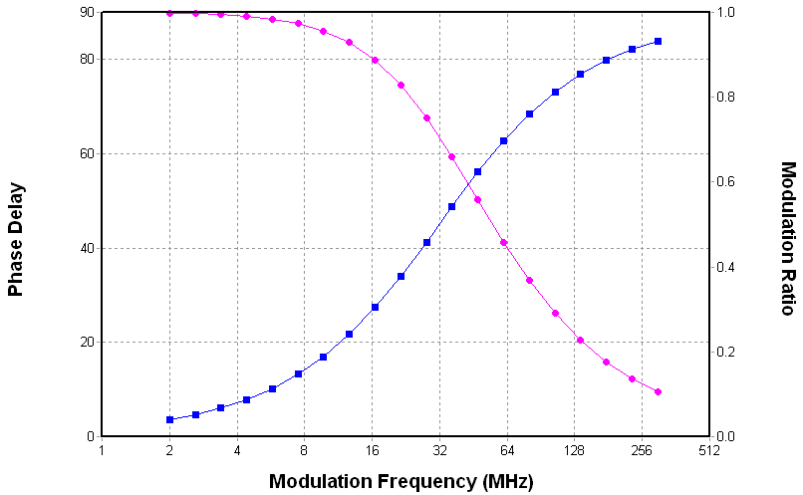
Analog Frequency Domain





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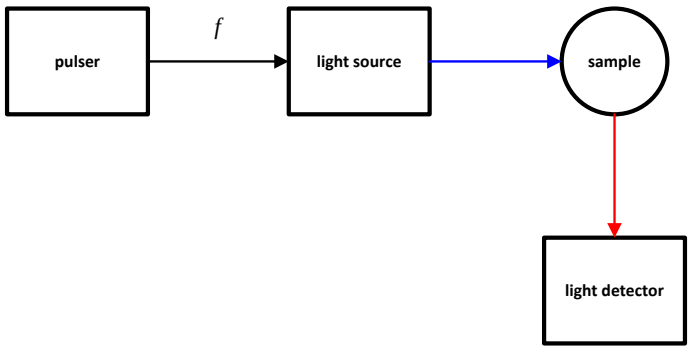
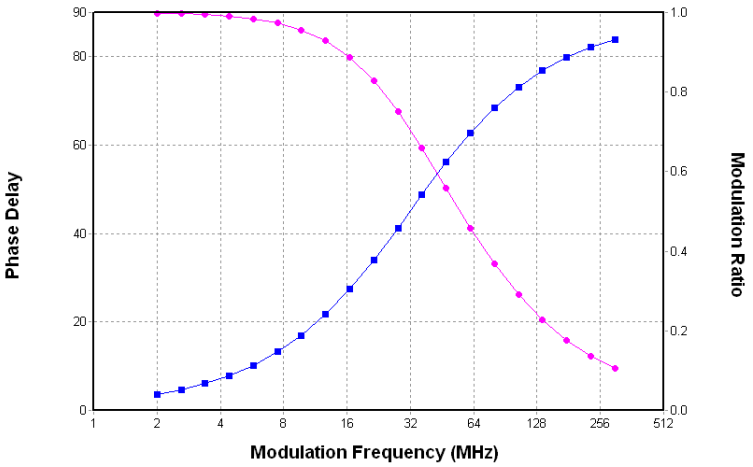
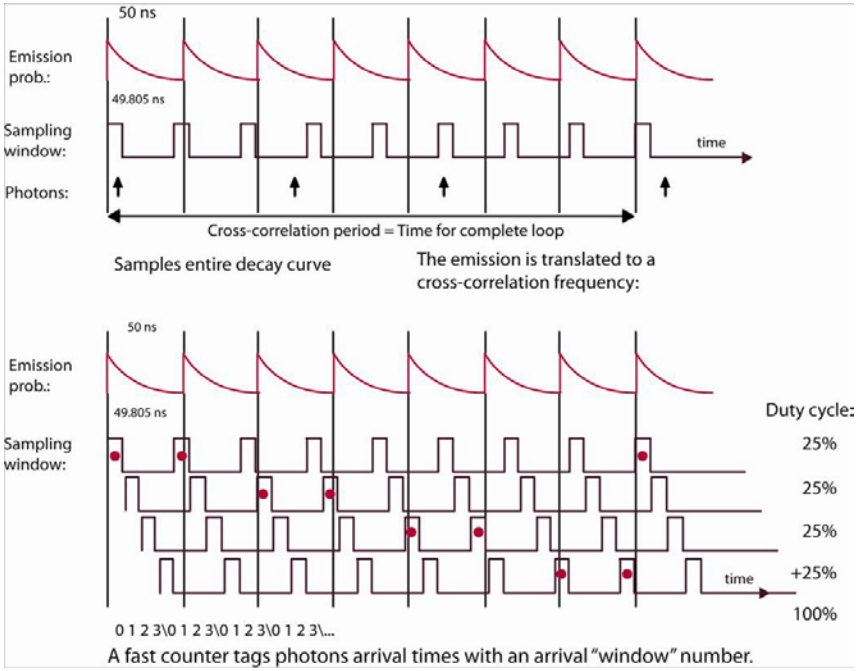
Digital Frequency Domain







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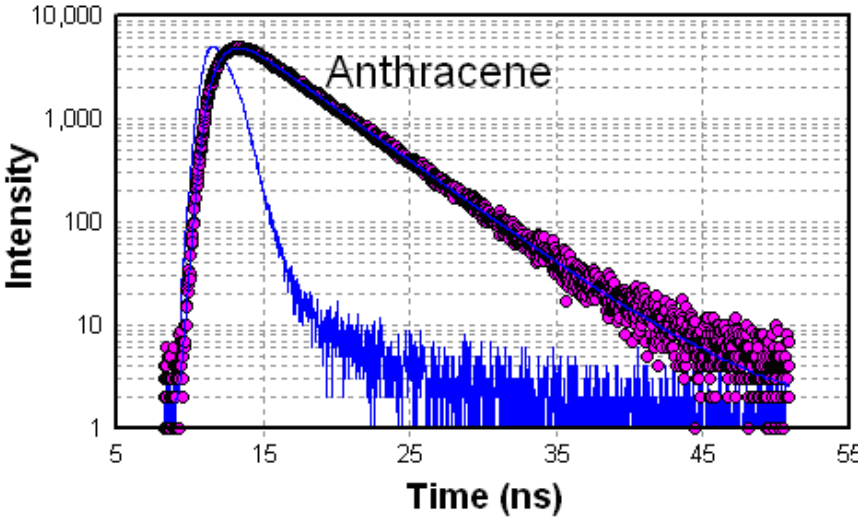
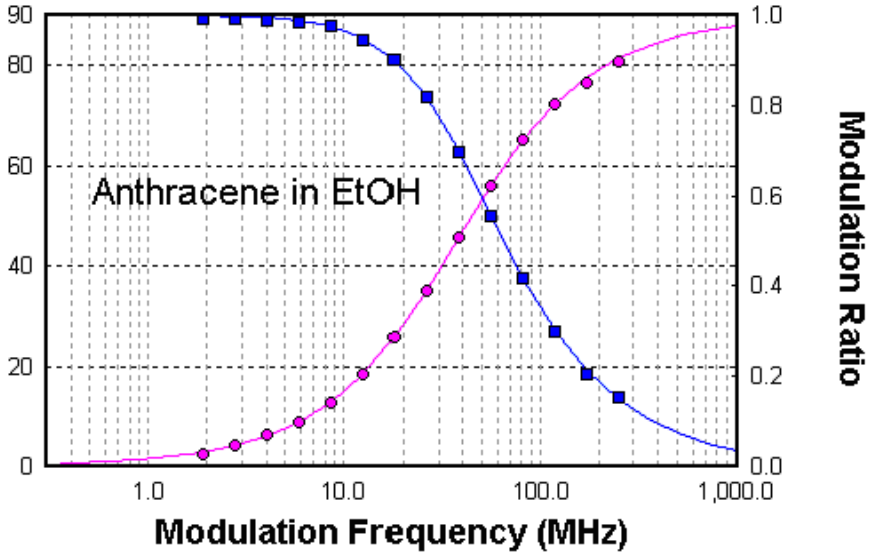
Lifetime standards



335 nm pulsed LED

WG 385 LP filter

$\tau = 4.24\,ns$

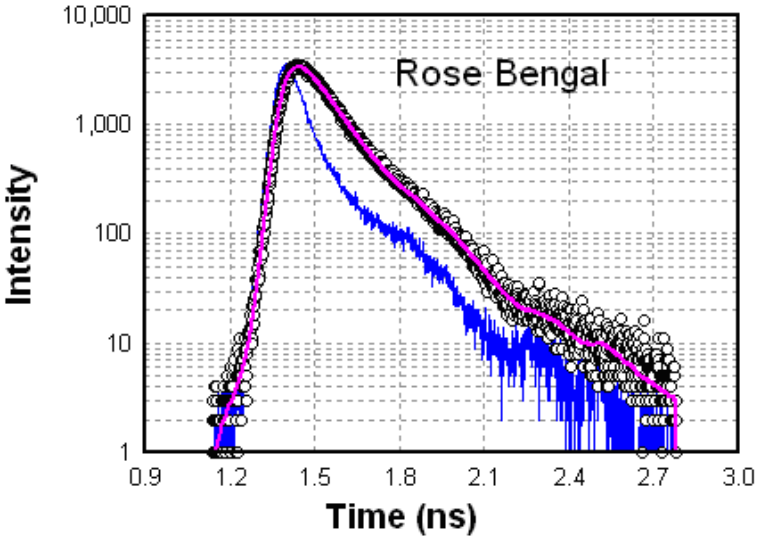


370nm cw LED

WG 389 LP filter

$\tau = 4.25\,ns$

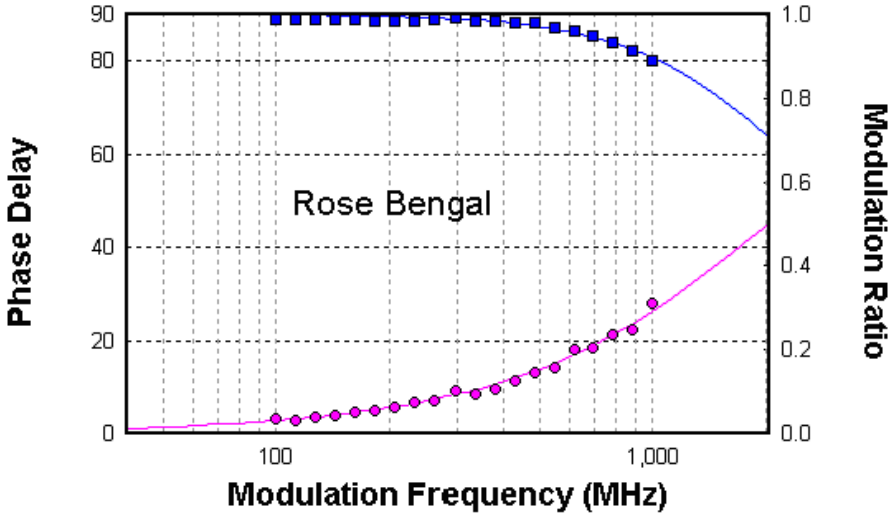
Picosecond standards



473 nm pulsed laser diode

515 nm LP filter

$\tau = 77\,ps$



471 nm cw laser diode

OG530 LP filter

$\tau = 78\,ps$

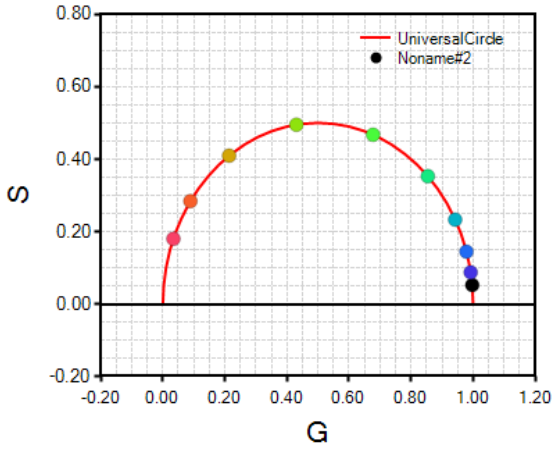
Data Analysis

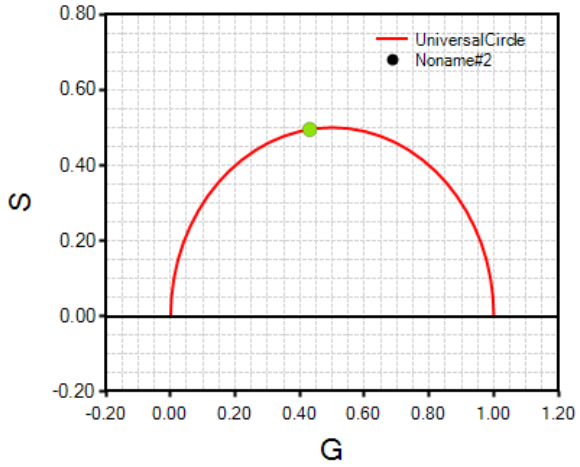
Time domain

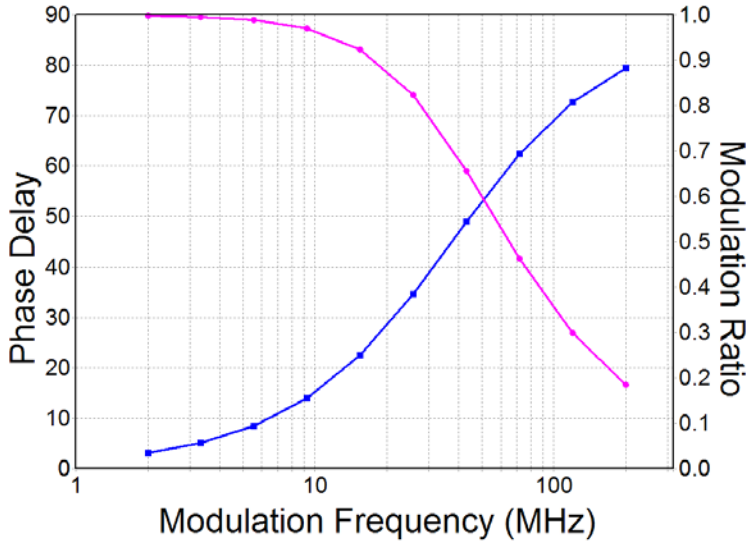
$$\chi^2 = \frac{1}{\nu}\sum_{k=1}^{n}\frac{\left[N(t_k) - N_c(t_k)\right]^2}{\sigma_k^2} = \frac{1}{\nu}\sum_{k=1}^{n}\frac{\left[N(t_k) - N_c(t_k)\right]^2}{N(t_k)}$$

Frequency domain

$$\chi^2 = \frac{1}{\nu}\left\{\sum_{j=1}^{N}\left[\frac{\varphi_\omega - \varphi_{c\omega}}{\sigma_\varphi}\right]^2 + \sum_{j=1}^{N}\left[\frac{M_\omega - M_{c\omega}}{\sigma_M}\right]^2\right\}$$



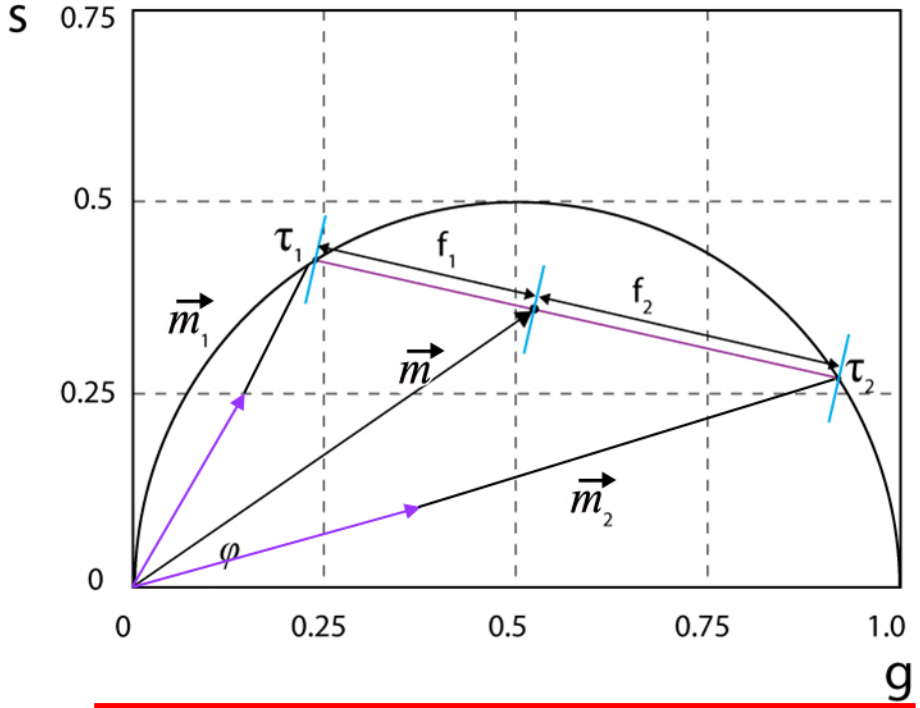




$$\omega_{OP}^2 = \frac{1+\sqrt{3}}{2\,\tau^2}$$

1	ns	186	MHz
4	ns	47	MHz
10	ns	18.6	MHz

Double-exponential



In a double exponential decay, the experimental points fall within the semicircle

$$\vec{m} = f_1 \vec{m}_1 + f_2 \vec{m}_2$$

$$g = \sum_i f_i g_i \qquad \sum_i f_i = 1$$

$$s = \sum_i f_i s_i$$

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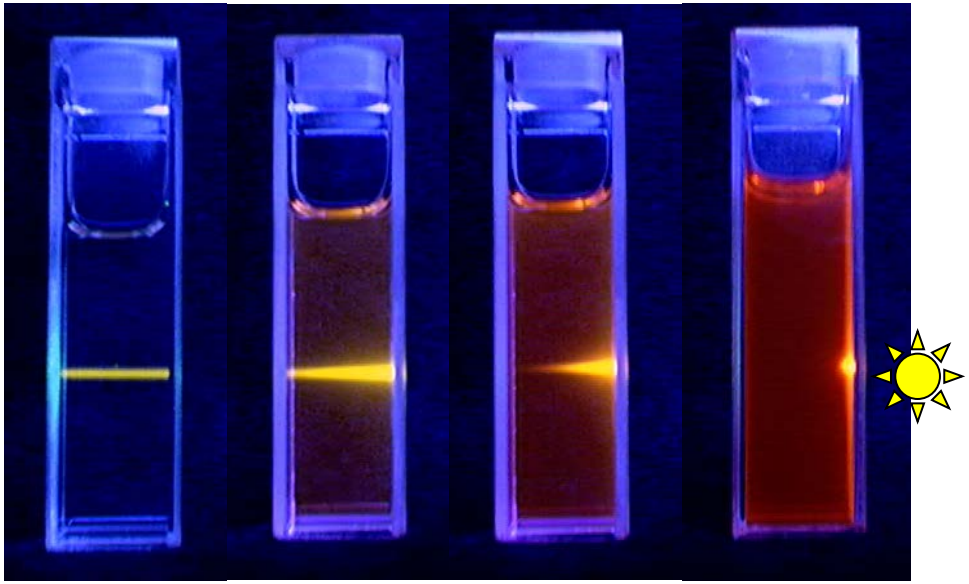
The Sample

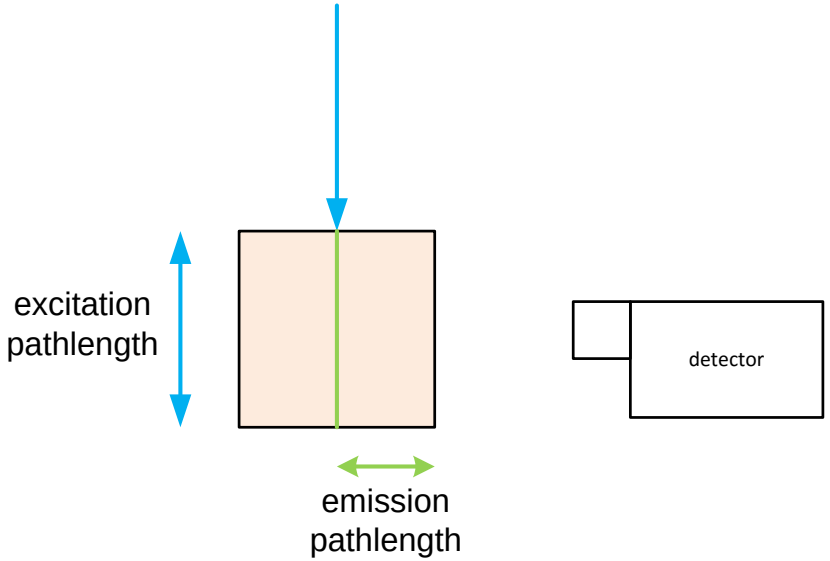
The theory is based upon the assumption of working with optical thin solutions, that is $OD < 0.1$

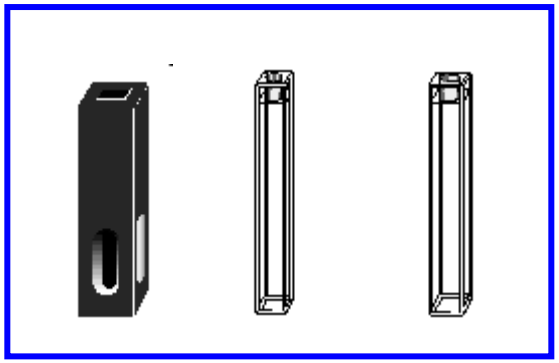


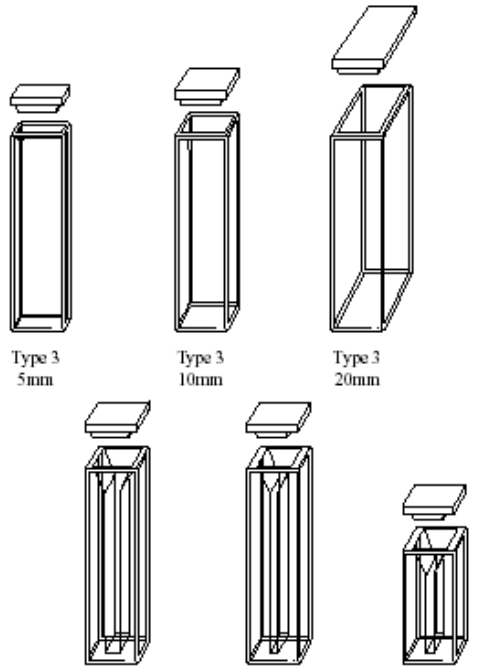
Rhodamine B

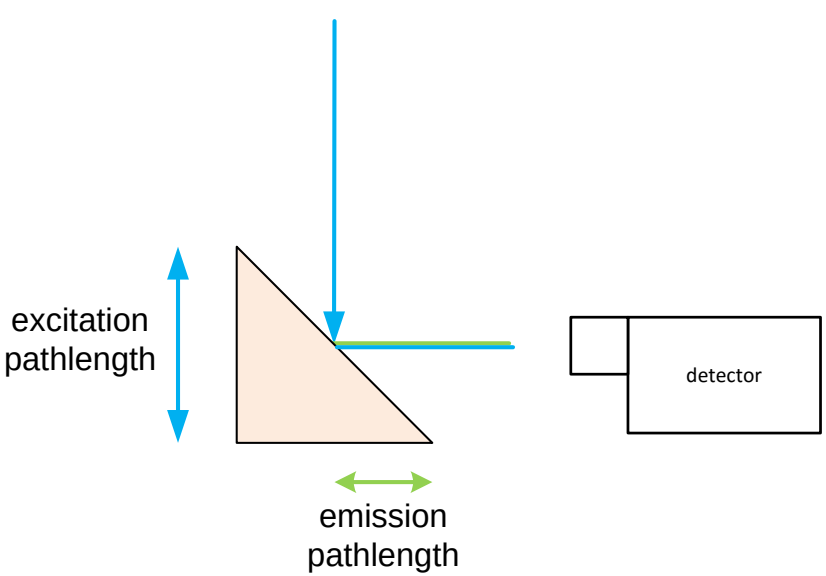
from Jameson et. al., Methods in Enzymology (2002), 360:1

Dealing with concentrated samples





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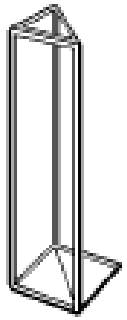
emission
pathlength



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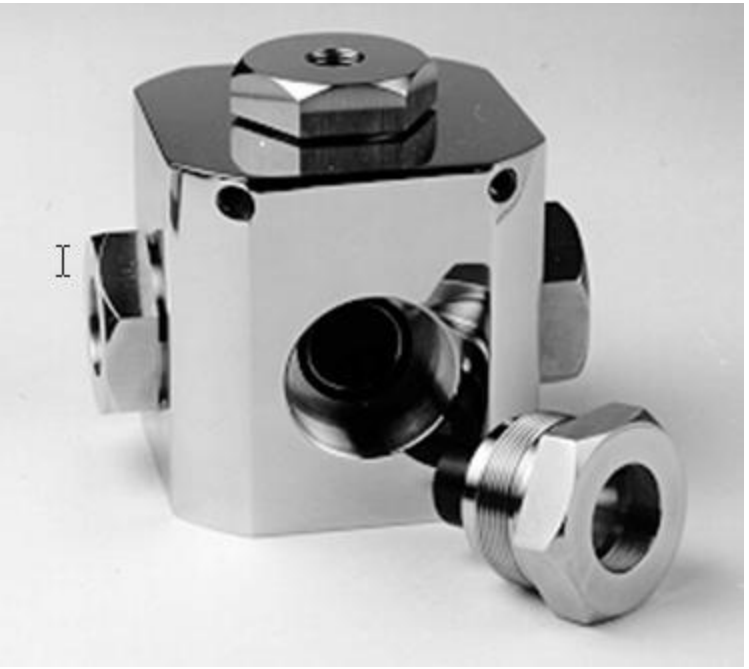
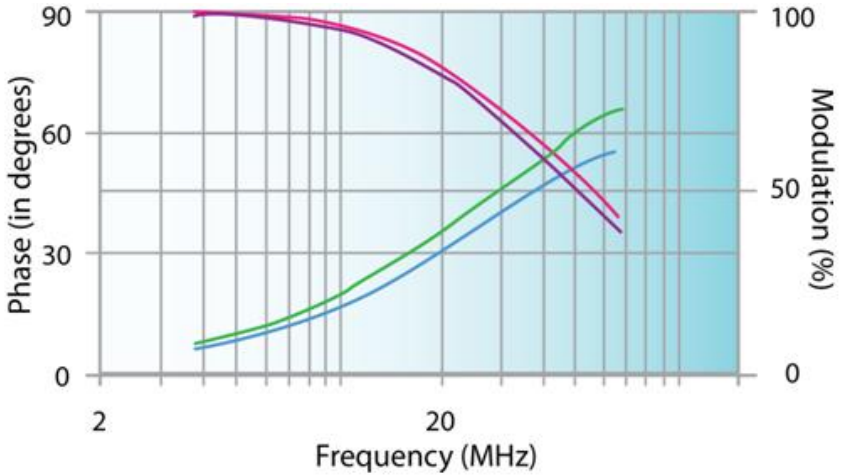
High Pressure cell





Effect of pressure on fluorescence lifetimes of staphylococcal nuclease. Frequency response profiles at atmospheric and high pressure using the HPCell. Excitation wavelength was 295 nm and emission was monitored at 350 nm.
High pressure (2 Kbar) data: blue (phase) and red (modulation) lines
Atmospheric pressure data: green (phase) and purple (modulation) lines
(Courtesy of Prof. Catherine A. Royer, Centre de Biochimie Structurale, Université Montpellier I, Montpellier, France)

Thank you.