Instrumentation for the detection of absorption and fluorescence

Fluorescence methods in life sciences: a journey from a molecule to cell

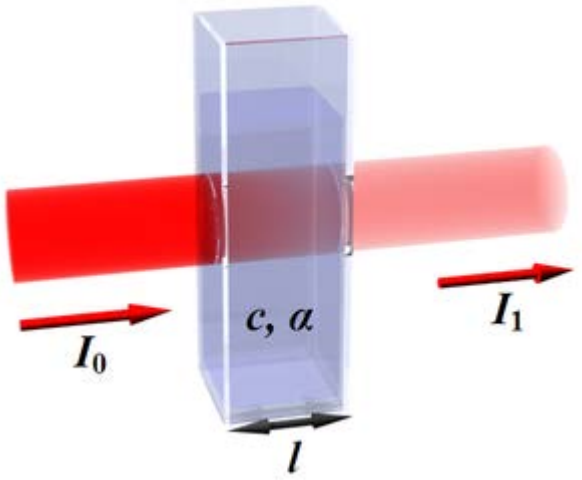
Ctirad Hofr

Overview

- What light source and when should we use?
- How to select light of a given wavelength?
- How can we detect light?
- What is basic setup of all instruments that measure fluorescence?

Absorption

- Light is absorbed in material
- For the absorption of mono-chromatic light
- **Lambert-Beer law:**
  The absorbance is directly proportional to the concentration and thickness of the solution layer



$$A = \varepsilon \cdot c \cdot l = \log_{10} \frac{I_0}{I} \qquad I = I_0 \cdot 10^{-\varepsilon \cdot c \cdot l}$$

ε = molar extinction coefficient, c - concentration, l - optical path-length

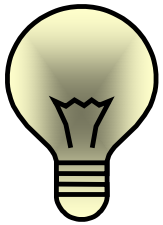
3

SpectroPHOTOmeter

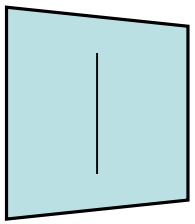
- Instrument for measuring the amount of light absorbed by the sample

Absorbance is measured at different wavelengths

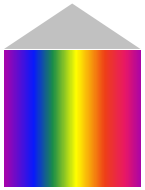
The result is an absorption spectrum of the sample



Light source



Slit



Selection of wavelength

Sample

Detector

The light sources and their use

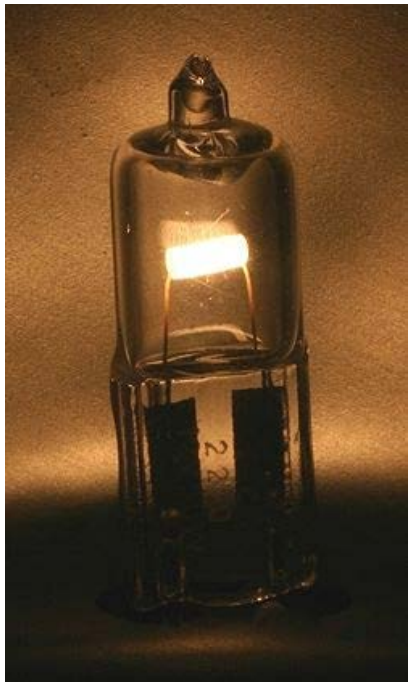
- **Tungsten lamp** (measuring absorption in the visible spectrum)
- **Deuterium lamp** (measuring absorption in the UV spectrum)
- **Xenon arc lamp** (source for time-steady fluorescence)

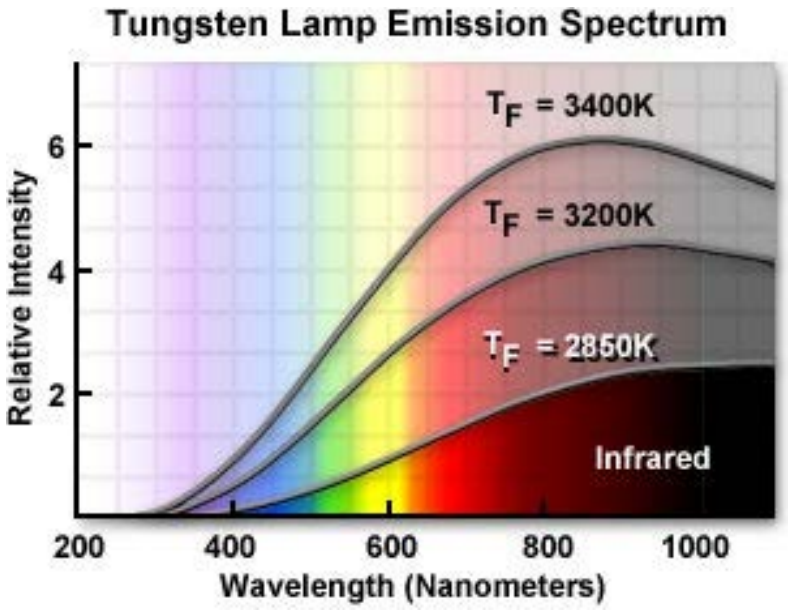
Pulse sources for time-resolved fluorescence

- Laser
- LD a LED diodes

Tungsten lamp – a visible spectrum

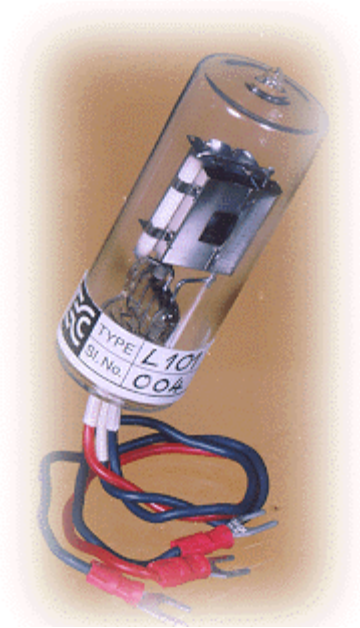
The glass bulb filled with an inert gas. The tungsten filament is inside, which is heated by direct current. It produces a large amount of heat. Only 5-10% of the energy is released as light.

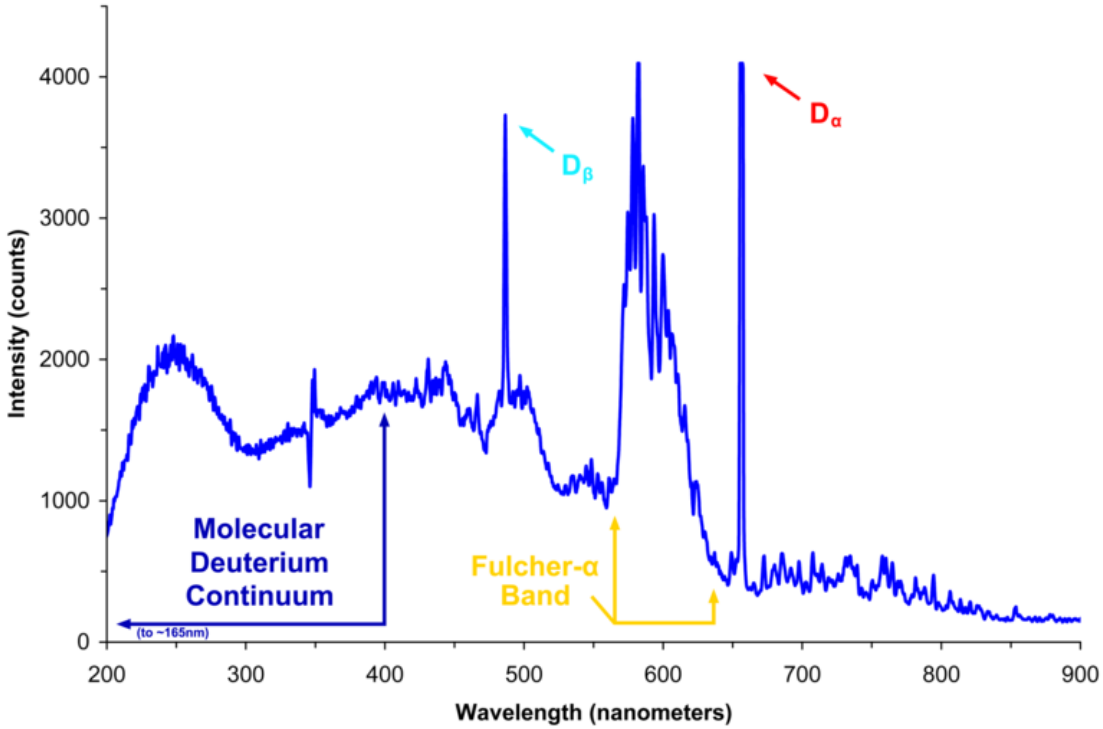




Deuterium lamp

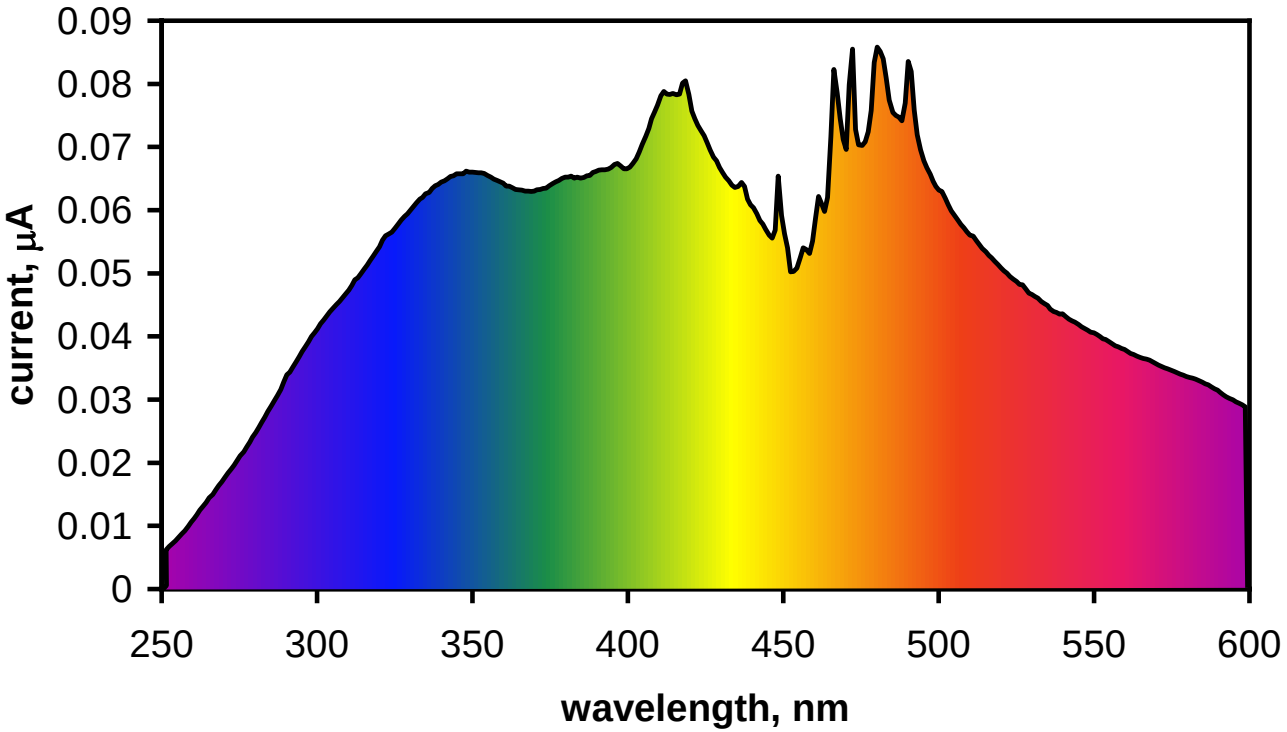
- Low pressure source particularly suitable for UV spectrum(160-400 nm)
- Filled with deuterium in gaseous state





Xenonon lamp

Xenon Arc Lamp (XB0), relatively smooth spectrum
220nm – 1000nm

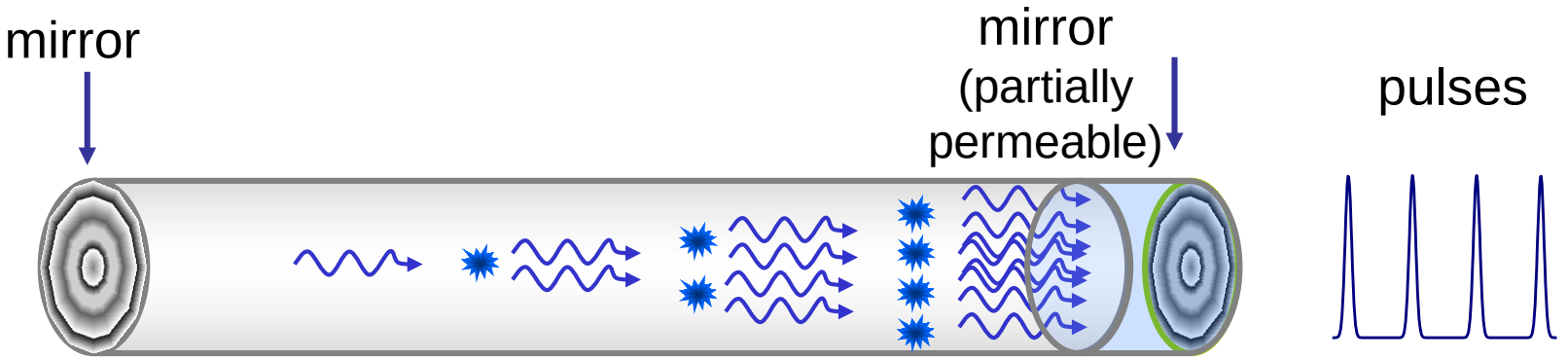


Provided by HORIBA Jobin Yvon

LASER

***L**ight **A**mplification by **S**timulated **E**mission of **R**adiation*

- Most of the molecules of the substance must be in the excited state
- After absorption of light, the molecule will be stimulated and it emits a photon
- Photons subsequently causes the emission of doubling the number of photons
- All photons have the same energy and wavelength and the emitted light has the same color – is monochromatic and is also coherent



- The substance is continuously supplied with energy, that the molecules are still in the excited state
- Photons are reflected inside the space between two mirrors
- Photons in pulses passing through partially permeable mirror
- The distance between the pulses is given by the size of the space between the mirrors and the speed of the cycle
- Spectrum is a line - only one wavelength

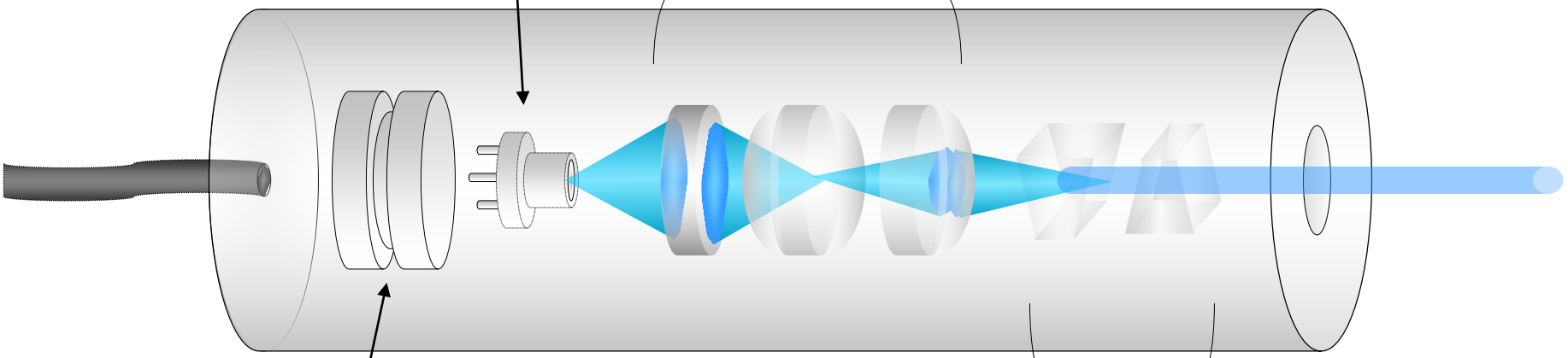
http://www.edumedia-sciences.com/a392_l2-laser.html

LD - Laser Diode

Laser diode housing

Collimating lens assembly

Beam Collimation & Temperature Stabilization



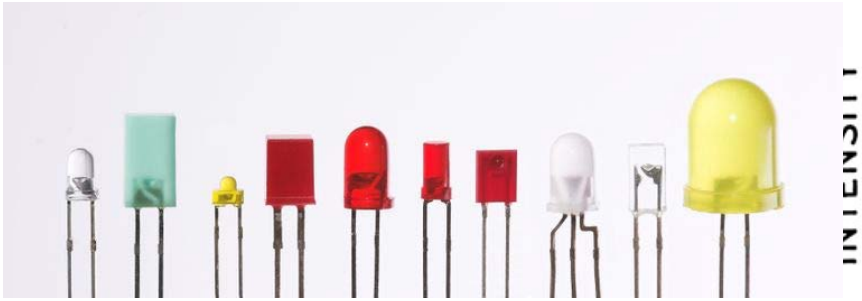
Thermoelectric cooler

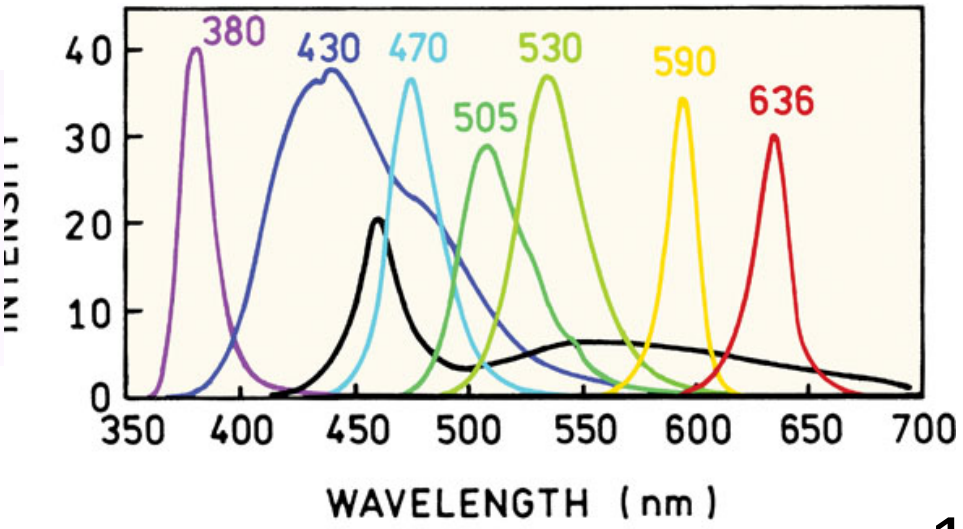
Anamorphic Prism pair

Provided by HORIBA Jobin Yvon

LED
Light Emiting Diode

- low power consumption
- high efficiency
- sufficiently narrow spectral range





The light sources and their use

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Pulse sources for time-variable fluorescence:

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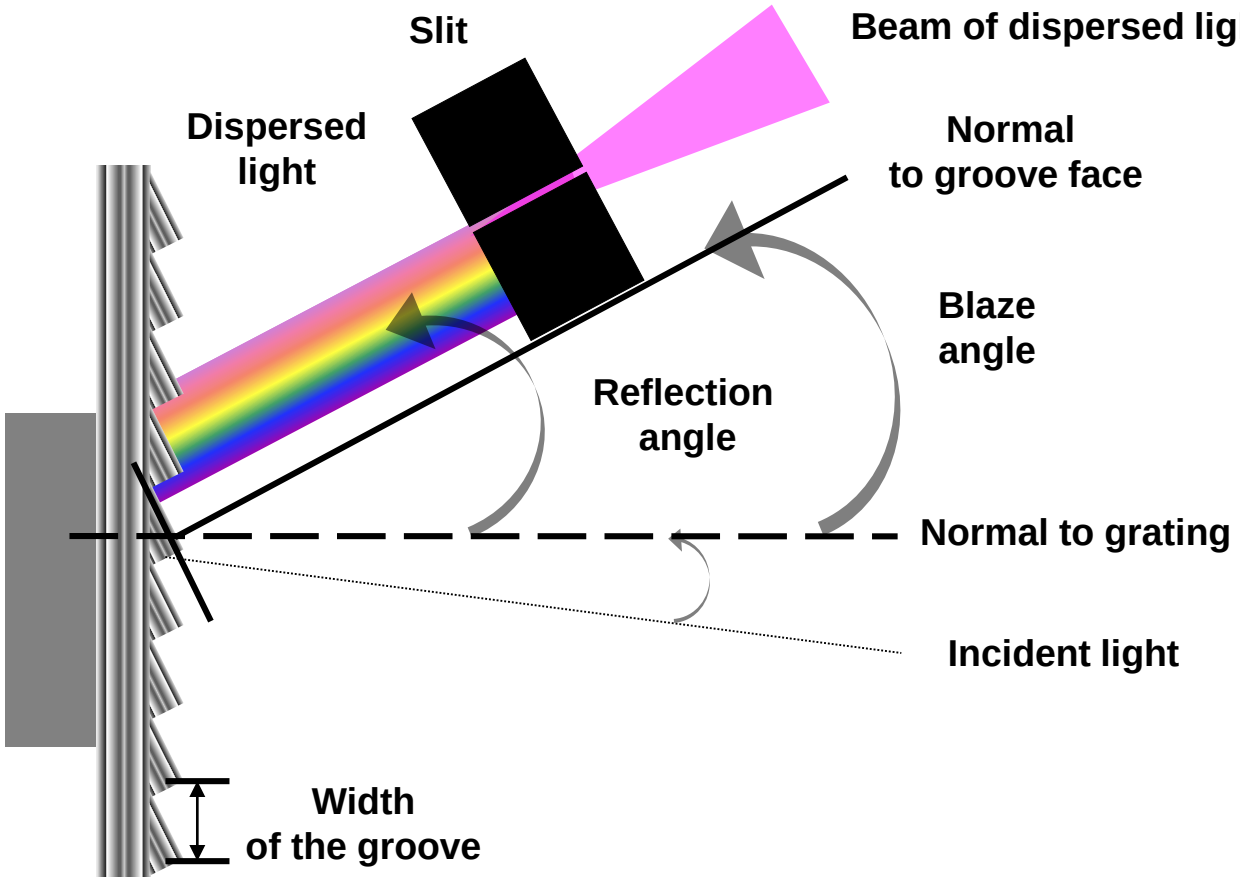
Monochromator

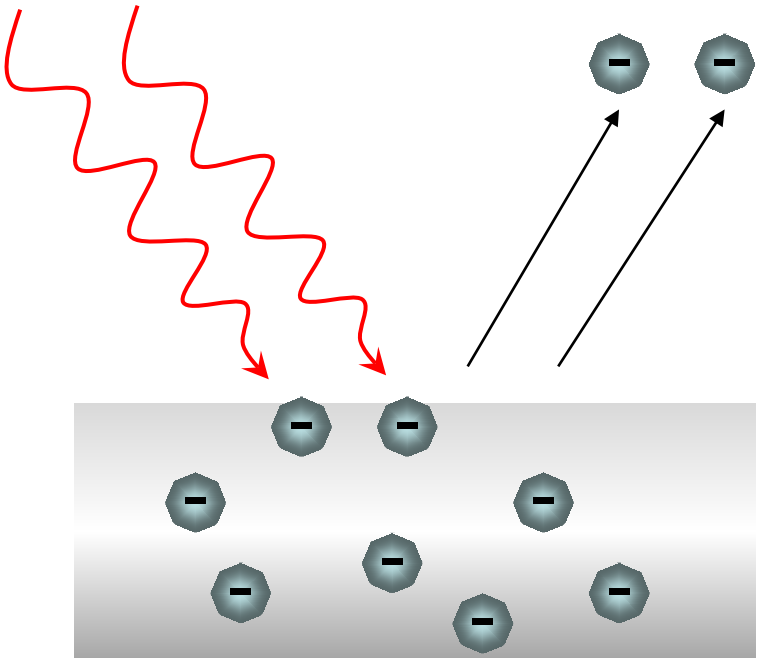
It uses light dispersion on a grid

the wavelength range is selected by the slit



3 http://www.shsu.edu/~chm_tgc/sounds/Czechdir/Grating%20Czech.swf Provided by HORIBA Jobin Yvon

Photoelectric effect



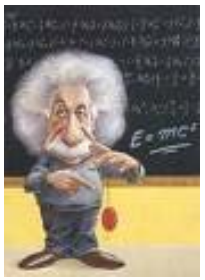
$$hf = W + E_k$$

W – the energy required for ejection of the electron

E_k - The kinetic energy of a free electron after the ejection

Nobel prize

A. Einstein, 1921



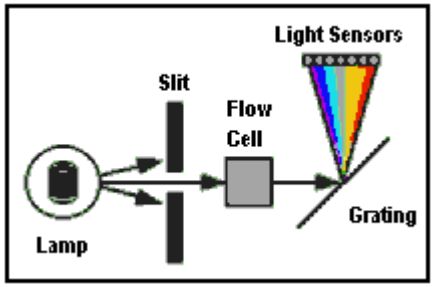
http://www.youtube.com/watch?v=v5h3h2E4z2Q

Detector – photomultiplier tube (PMT)

Light Detectors: **P**hoto**M**ultiplier **T**ube (R928 Side-on) Provided by HORIBA Jobin Yvon

Diode array detector

- Array of photosensitive diodes
- Each diode absorbs light of a specific wavelength range
- Advantage: the whole spectrum is scanned simultaneously



http://www.youtube.com/watch?v=zbTM36_7jIg&feature=related

CCD detektor
Charge Coupled Device

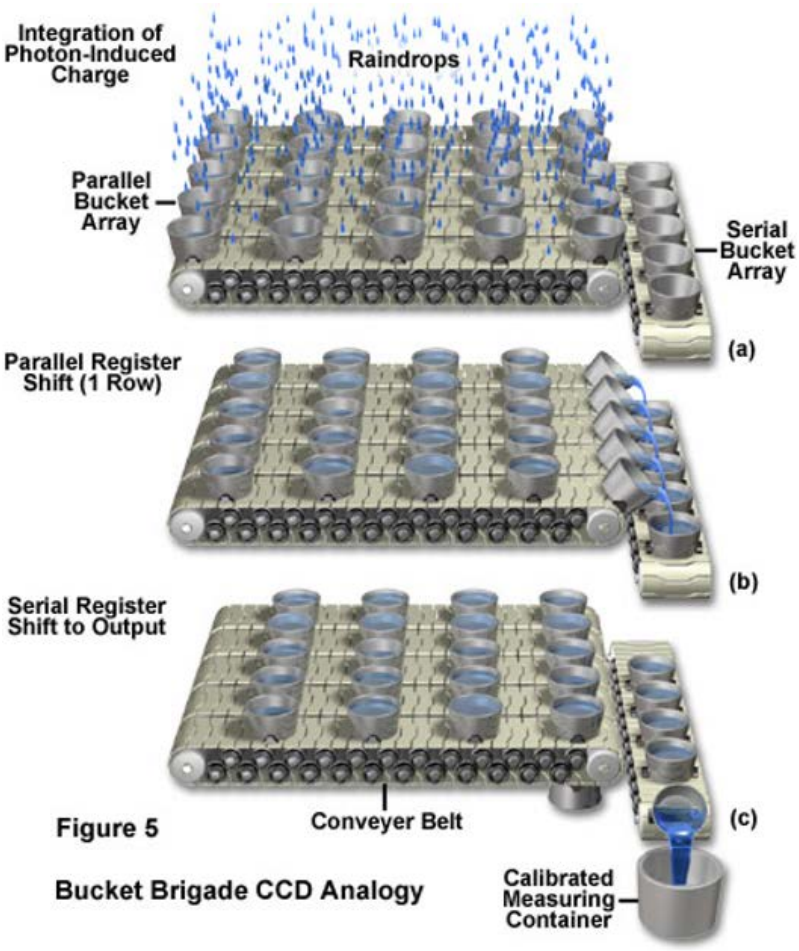


Figure 5

Bucket Brigade CCD Analogy

a) light = photons (raindrops) hit electrodes and release electrons that are accumulated as charge

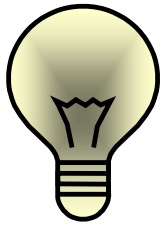
b) after a certain exposition time (integration time) a partial charge from each pixel is accumulated from each pixel (point at the detector). The charge is transferred gradually in rows to the outer row (register) where the charge is measured

c) The charge collected for each pixel is then determined and converted to voltage.

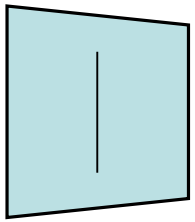
Resulting image is created from bright and dark pixels according to voltage values i.e. number of photons that hit the detector in a given point.

How CCD works

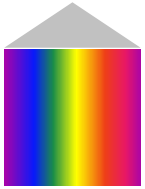
SpektroPHOTOmeter



Tungsten lamp (VIS) /deuteruim lamp (UV)



Slit



Grating monochromator

Cuvette made of UV/Vis transparent glass

Photomultiplier

http://www.youtube.com/watch?v=pxC6F7bK8CU

Nanodrop

NanoDrop 1000 Specifications

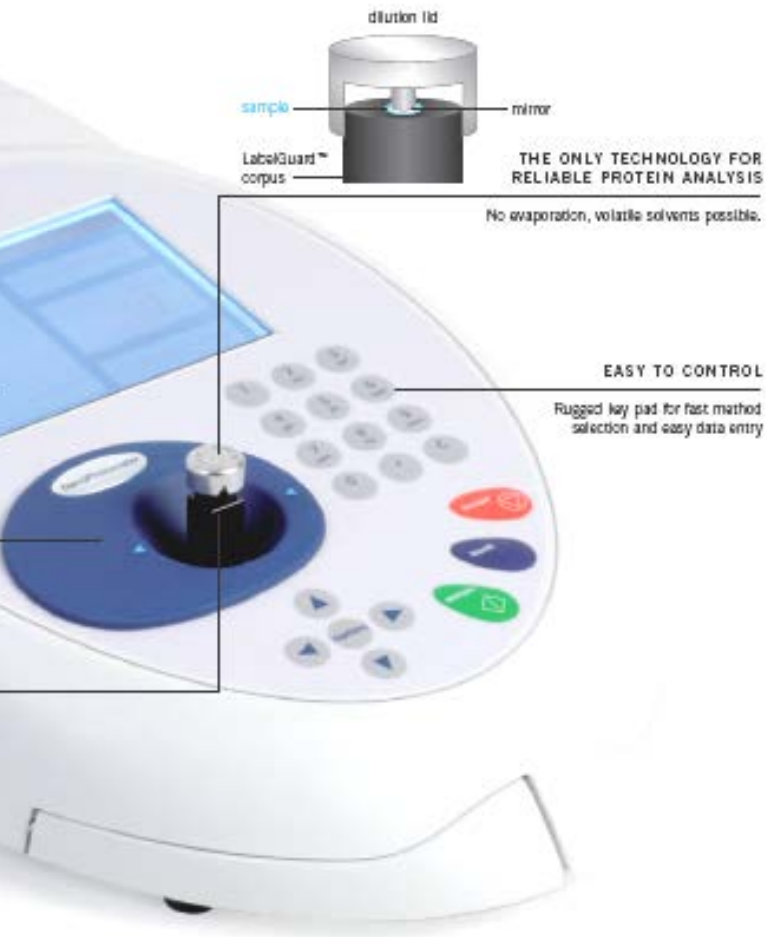
Sample size	1 microliter
Path lengths	1mm and 0.2mm
Light Source	Xenon flash lamp
Detector Type	2048-element linear silicon CCD
Wavelength range	220-750 nm
Wavelength accuracy	1nm
Wavelength resolution	3nm (FWHM at Hg 546 nm)
Absorbance Precision	0.003 absorbance (1mm path)
Absorbance Accuracy	2% (at .76 absorbance at 257 nm)
Absorbance Range	0.02-75 (10mm equivalent absorbance)
Detection Limit	2 ng/microliter (dsDNA)
Maximum Concentration	3700 ng/microliter (dsDNA)
Measurement Cycle Time	10 seconds
Dimensions (footprint)	20cm X 14cm
Weight	1.6 kg

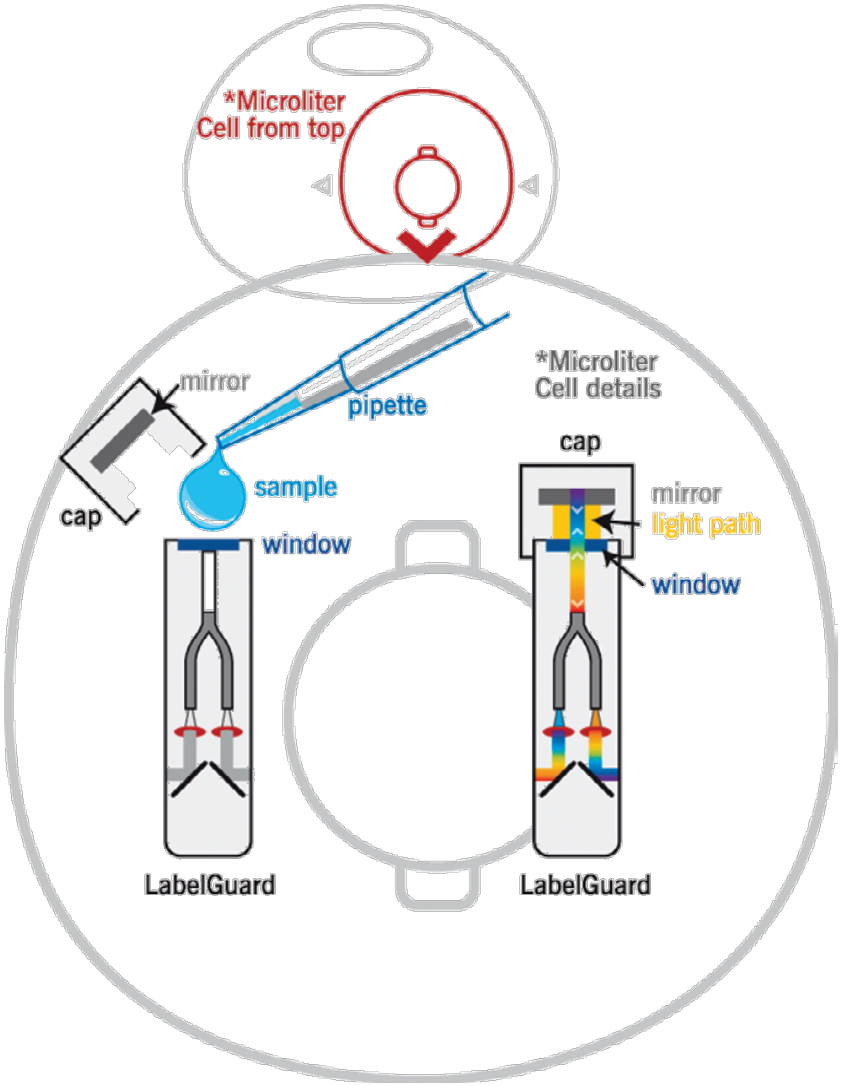
http://www.nanodrop.com/LikeItsHotVideo.aspx

The NanoPhotometer™

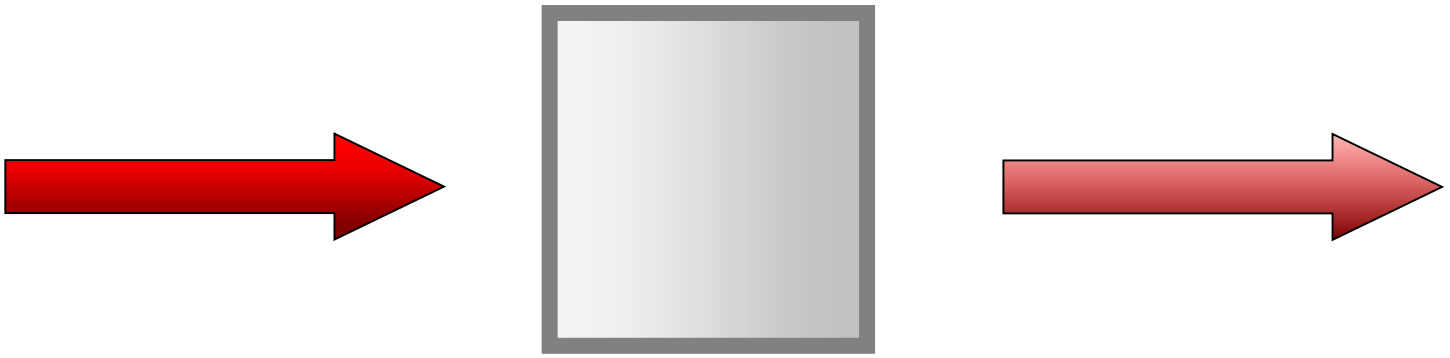
Complete solution for submicroliter and standard volume applications

Nanophotometer





Geometry in absorbance measurement



Incident light

cuvette from above

Transmitted light

The range for measuring the absorbance

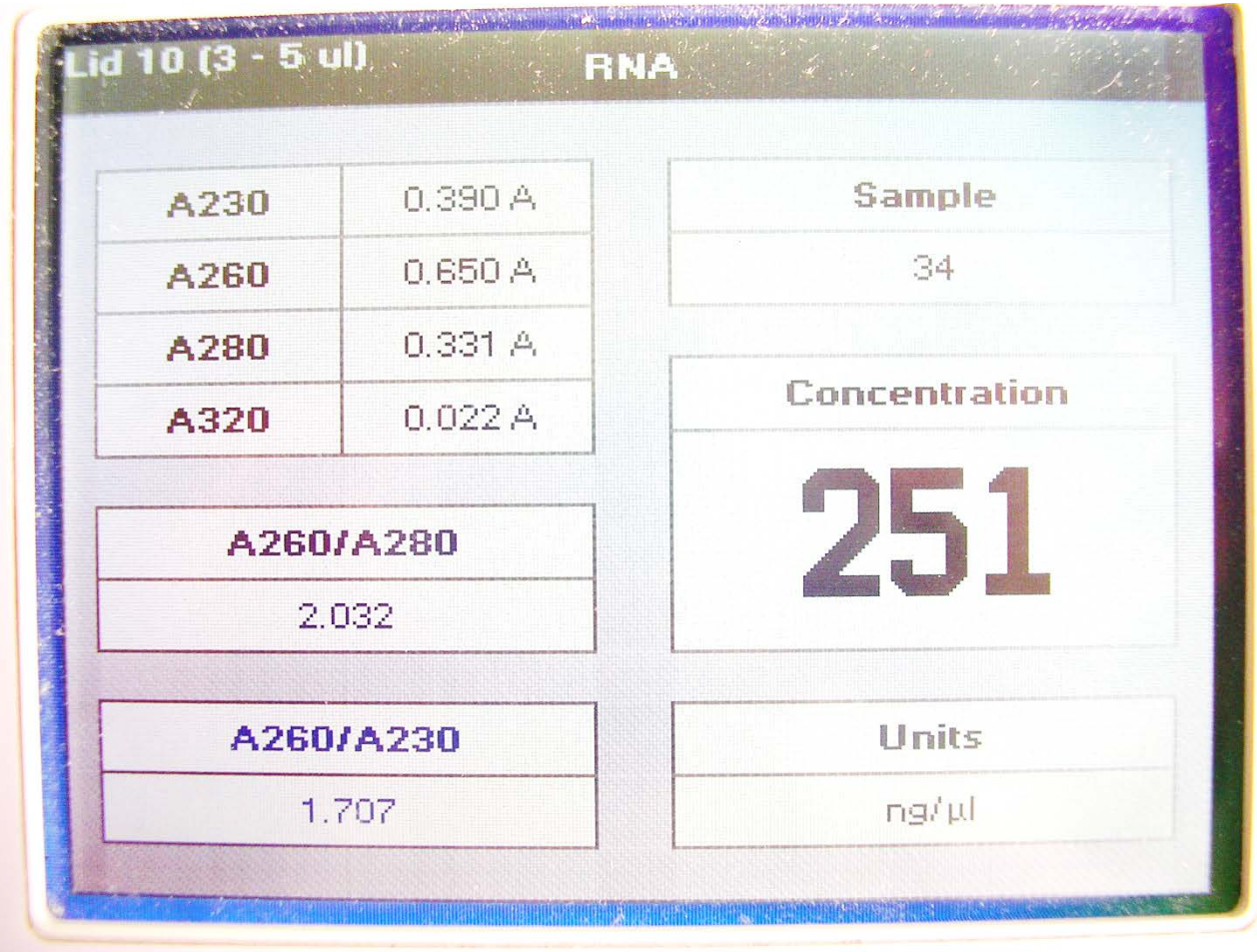
Range with linear response: i.e. that $ABS = c\varepsilon l$

ABS = 0.1 – 1.0

The most accurate area: i.e. the values are the most reliable in the interval

ABS = 0.3 - 0.7

What to watch when measuring?



Instruments for measuring the fluorescence

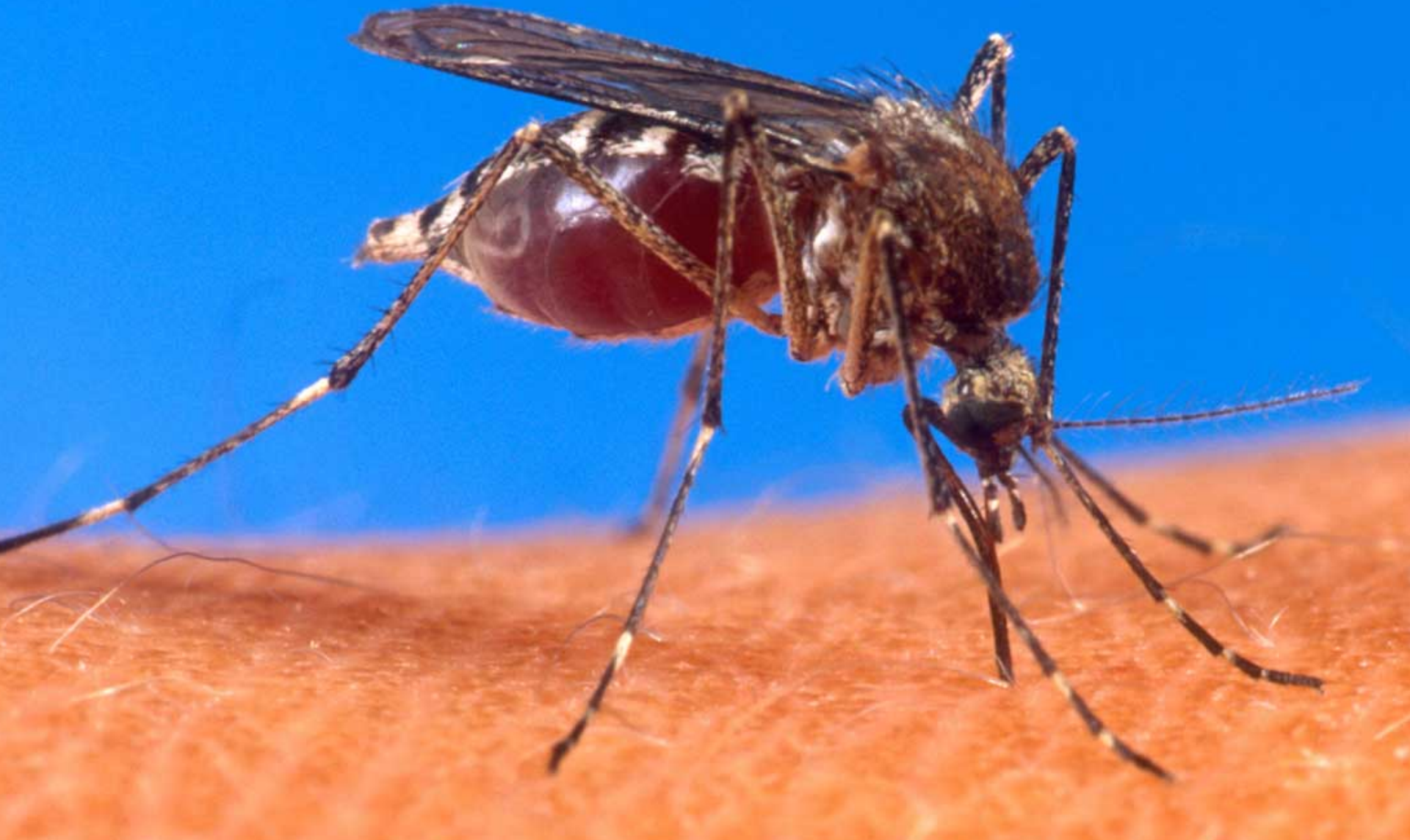
1. **Spectrofluorometers** – measure the middle signal of the whole sample usually placed in the cuvette or in the microplates well

2. **Fluorescence scanners** (including microplate readers) – measure the fluorescence of two-dimensional macroscopic objects (electrophoretic gels, blots, chromatograms)

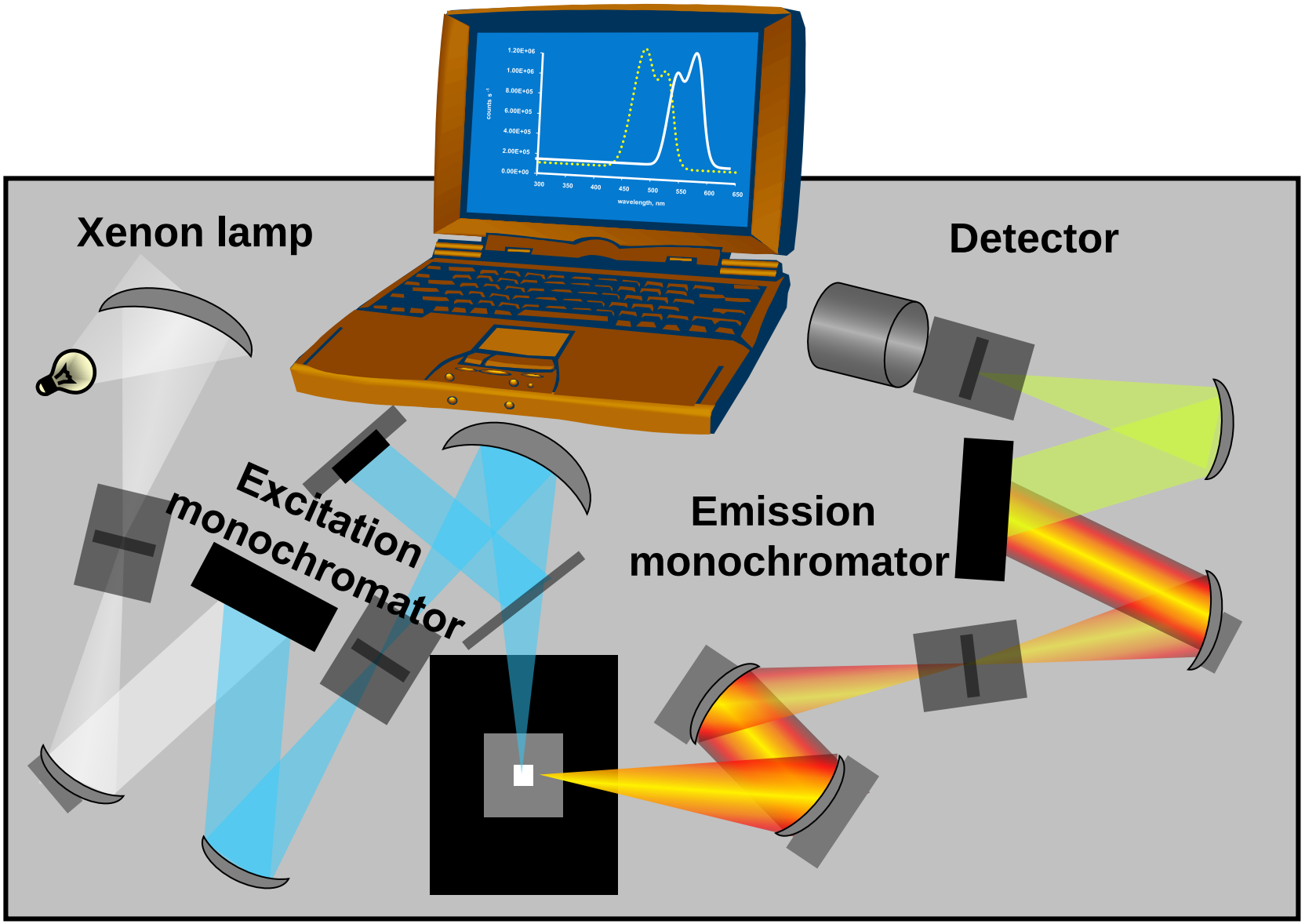
3. **Fluorescence microscopes** – to observe the fluorescence of two- or three-dimensional microscopic objects

4. **Flow cytometers –** measure the fluorescence of a large number of individual cells and allow the identification and separation of their subpopulations

How a mosquito contributed to the development of instruments?



SpectroFLUOROmeter



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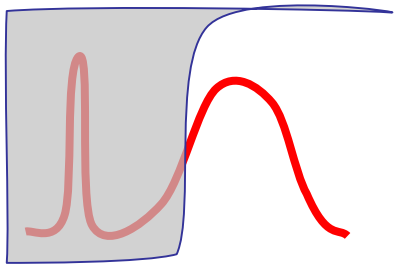
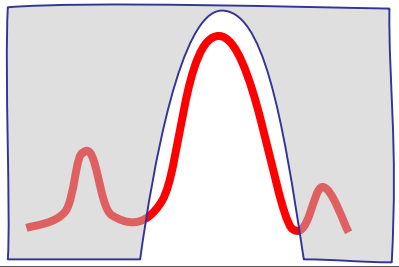
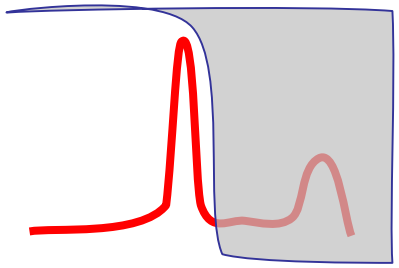
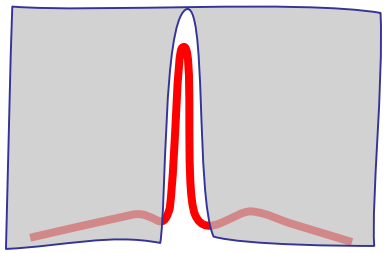
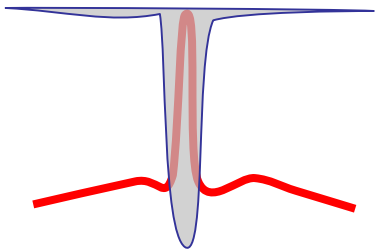
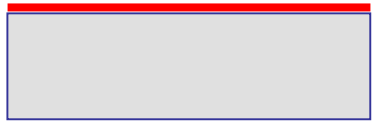
Filters

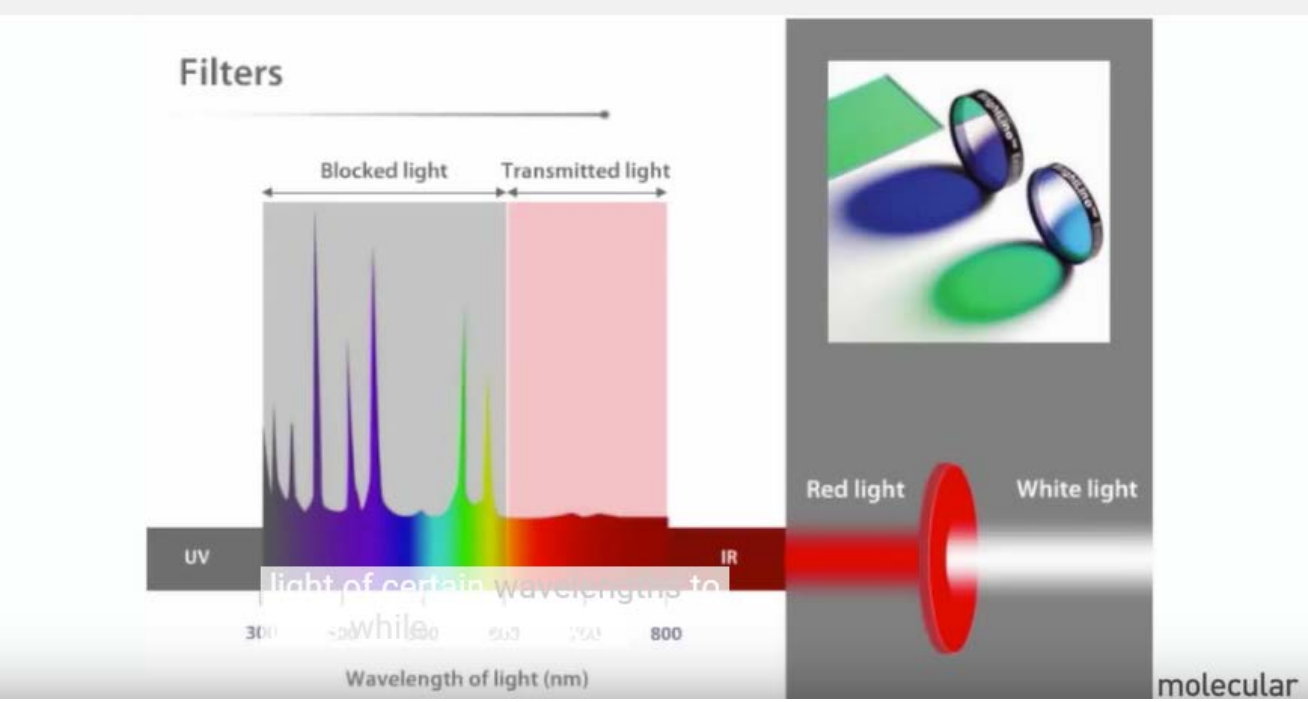
Advantages in comparison with the monochromator :

Cheap, compact, high-efficiency, high-throughput

Disadvantages: not adjustable, often with the specific requirements, an instrument can hold a limited umber of fliters (carousel), have internal fluorescence

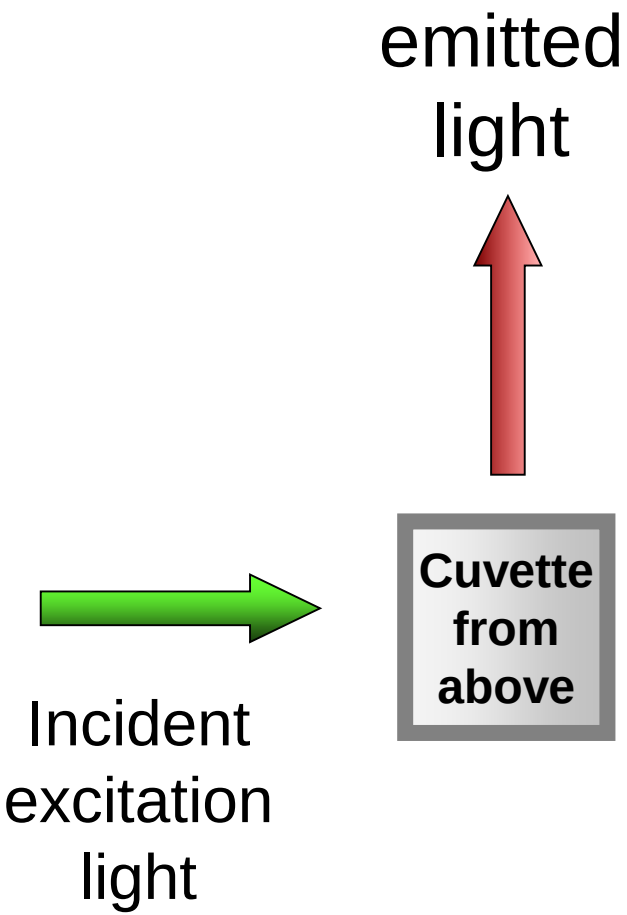
Cut-off or long pass	**Band-pass**	**Short-pass**
		
Clean-up	**Notch**	**N.D** **neutral density**
		

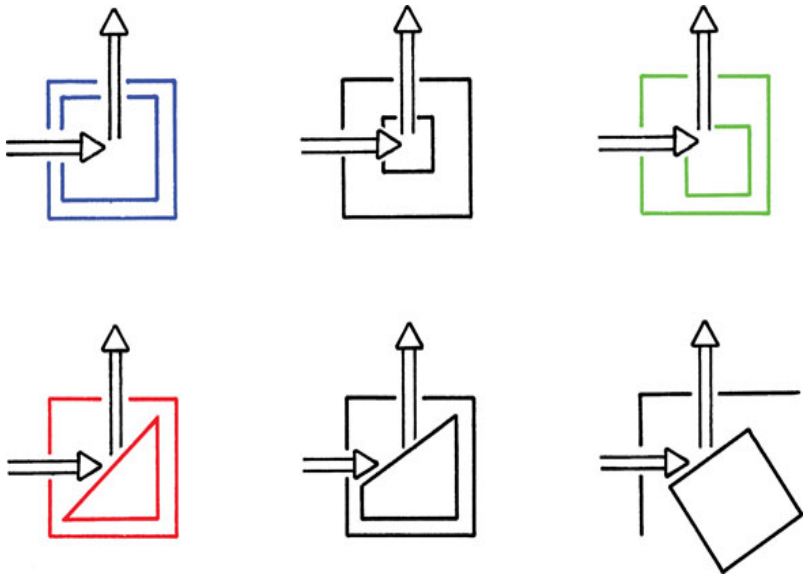
Sources and filters



https://www.youtube.com/watch?v=xJGmARfBasU&t=208s

L-geometry of fluorescence measurements

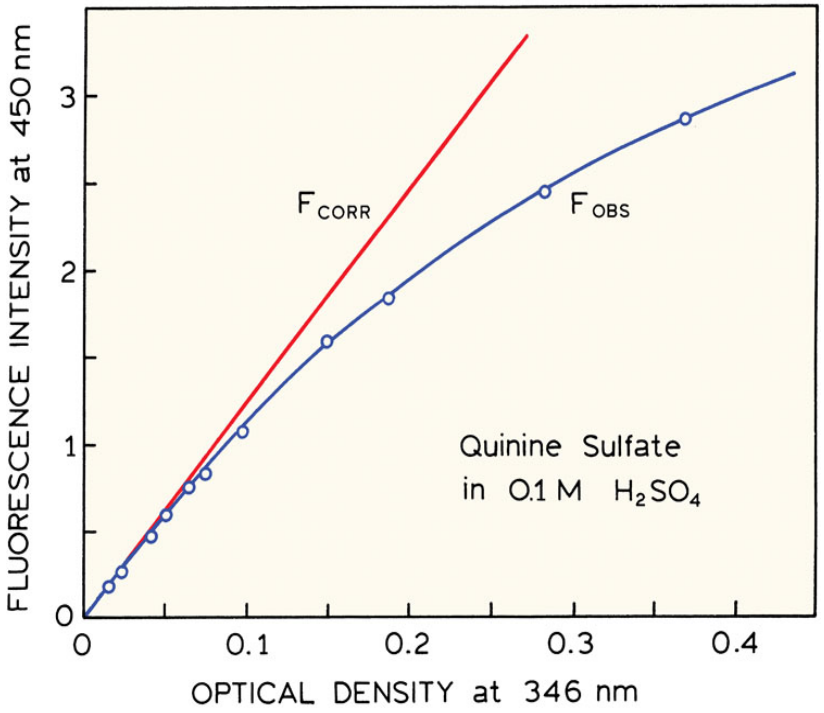




Taken from J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Third Edition,Springer, 2006

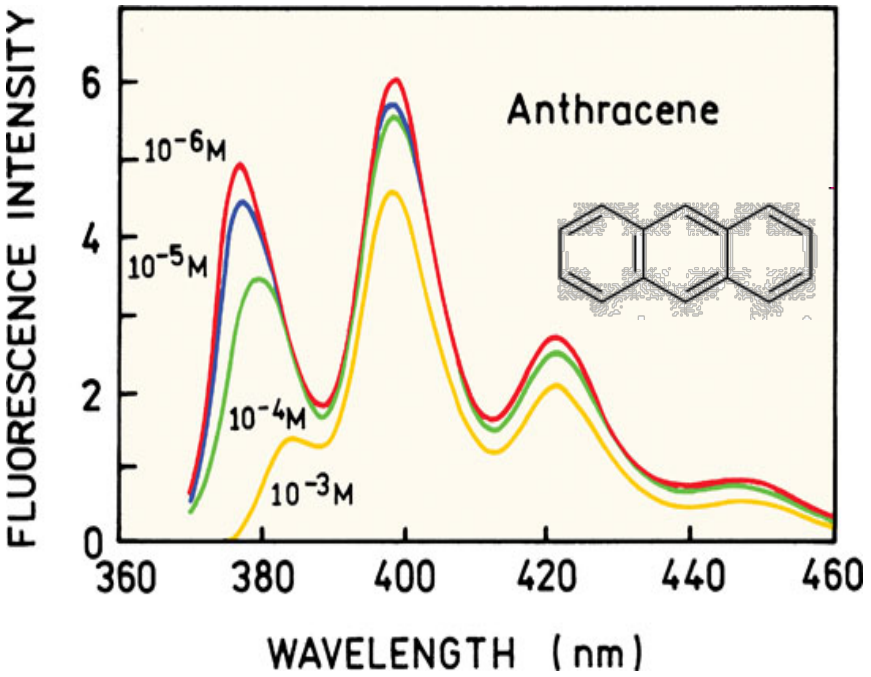
Effect of inner filter

The fluorescence intensity at a certain value of the absorbance (usually from about 0.1 Abs) becomes nonlinear. This is caused by the fact that the layers of the sample distant from plane of incidence of the excitation light on the sample are excited by lower light intensity, because part of the excitation light is absorbed by the surface layers. This error appears only in strongly absorbing solutions, but it has to be always considered and corrected for the precision measurements of quantum yields.

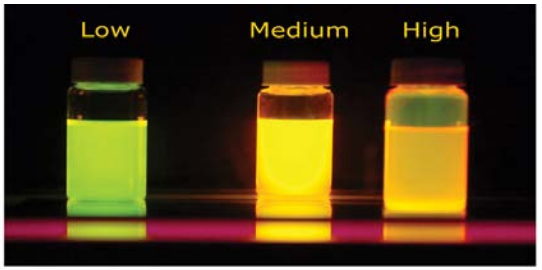


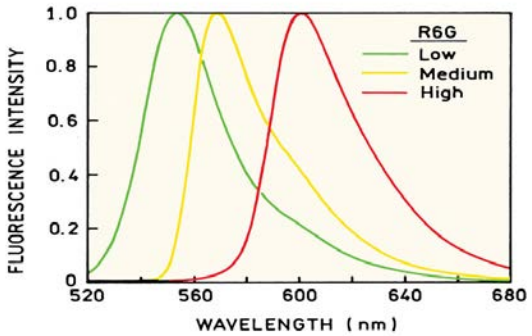
Taken from J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Third Edition,Springer, 2006

Effect of concentration of the fluorophore to the shape and position of its emission spectrum



High concentration of anthracene causes a reduction of emission signal at lower wavelengths. It is caused by reabsorption of fluorescence by anthracene in the area where absorption and emission spectrum is most overlapped, i.e. in 370 - 400 nm.

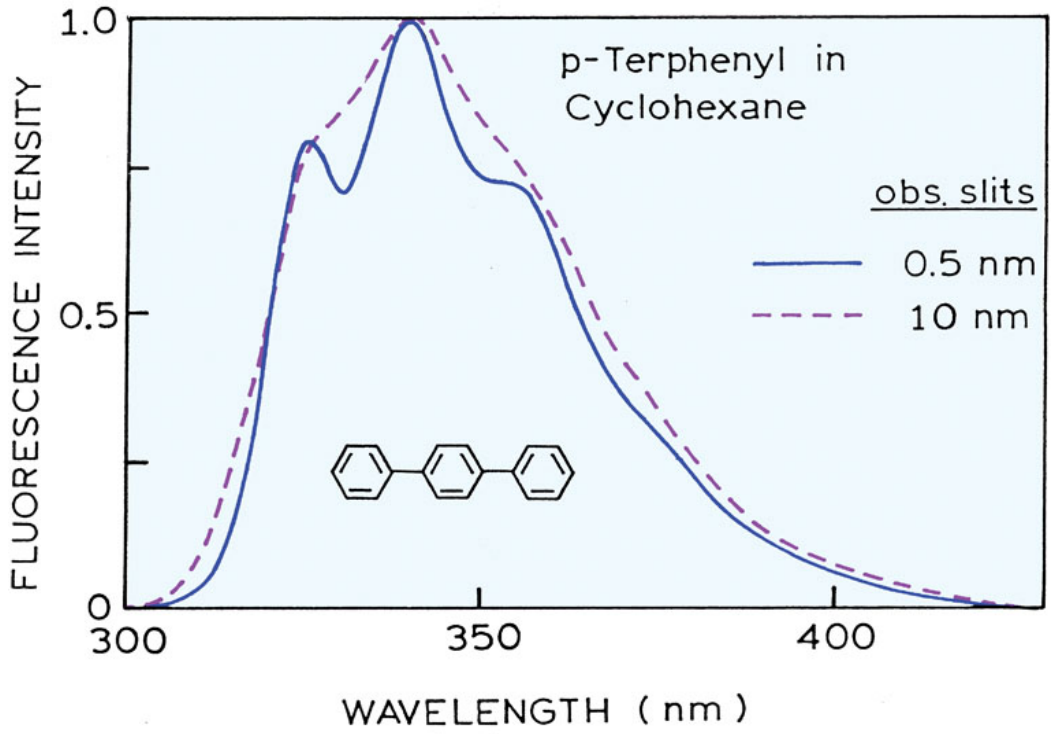




The largest effect of he concentration on the emission spectrum is observed in fluorophore with a small Stokes shift – a large overlap of absorption and emission spectrum. The figure shows three concentrations of Rhodamine 6G increasing from left to right and the corresponding change in the color of emitted light. This effect is due to reabsorption of shorter wavelengths of emission spectrum. This shifts the whole spectrum to longer wavelengths at higher concentrations of fluorophore.

Taken from J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Third Edition,Springer, 2006

Effect of setting the width of slits on the shape of the spectrum



Taken from J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Third Edition,Springer, 2006

Common mistakes when preparing fluorescent samples

- Fluorophore concentration is too high

  - Use a dilute solution of Abs under 0.05

- Contamination of solvents or cuvette

  - For the unknown sample always validate background, ie. measure the fluorescence spectrum of the cuvette itself only with the solvent

- Solid particles in solution - Filter the solution

Documentation system

„ Intelligent darkroom“

It allows to determine the local optical absorption and fluorescence.

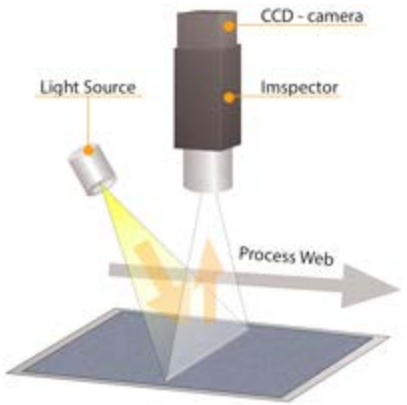
The source of excitation light is usually transilluminator which emits in the UV region.

The source of selective excitation light are LED diodes that are on the sides.

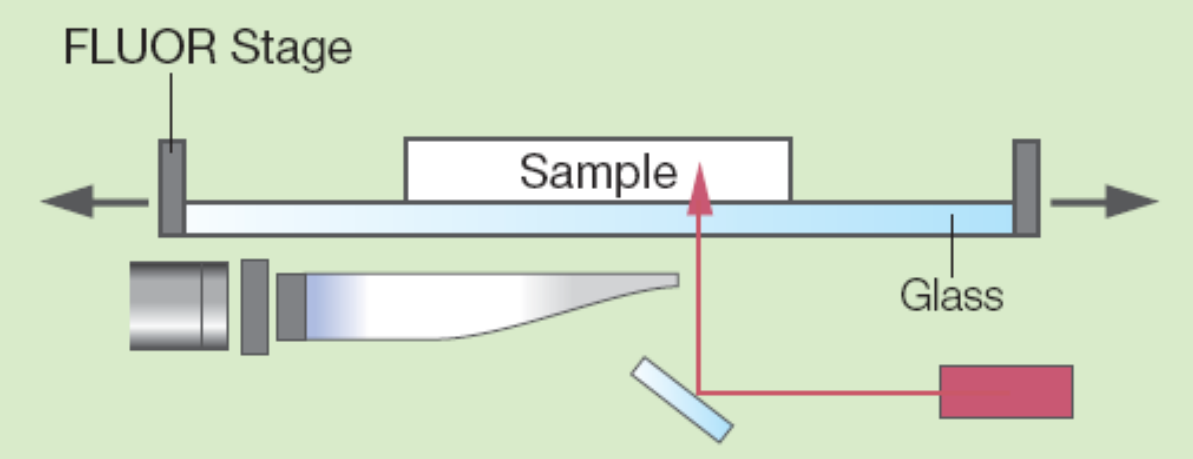
Emission is monitored by (digital) camera or cooled CCD detector

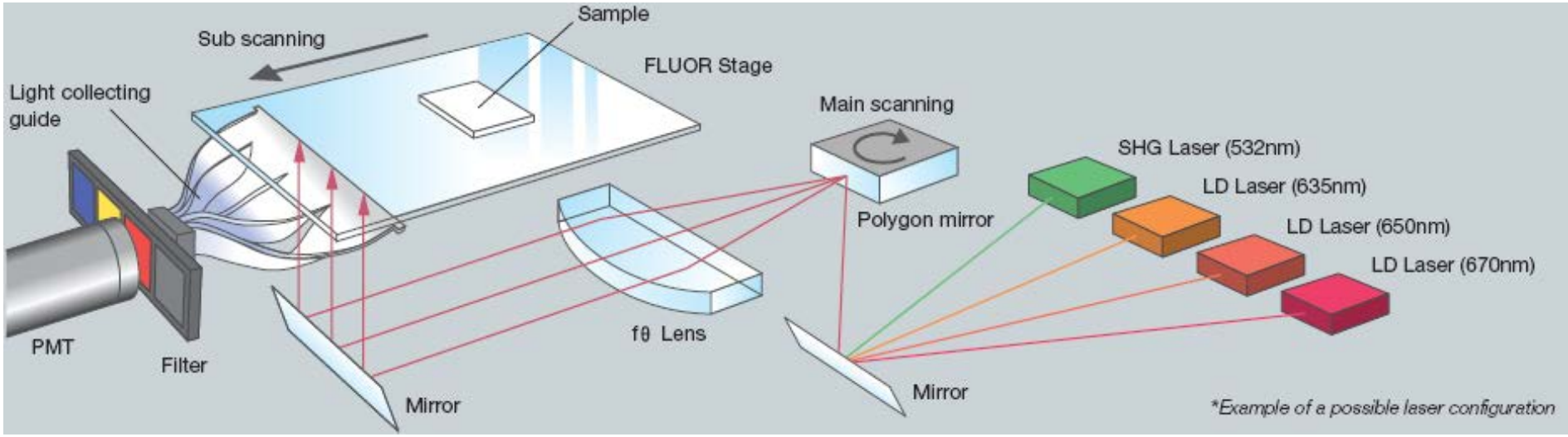
Fluorescence scanner

- Scans area and observes the dependence of fluorescence emission intensity at a given excitation light
- Used for detection of fluorescently labeled and stained molecules after electrophoretic separation



Fluorescence scanner - scheme

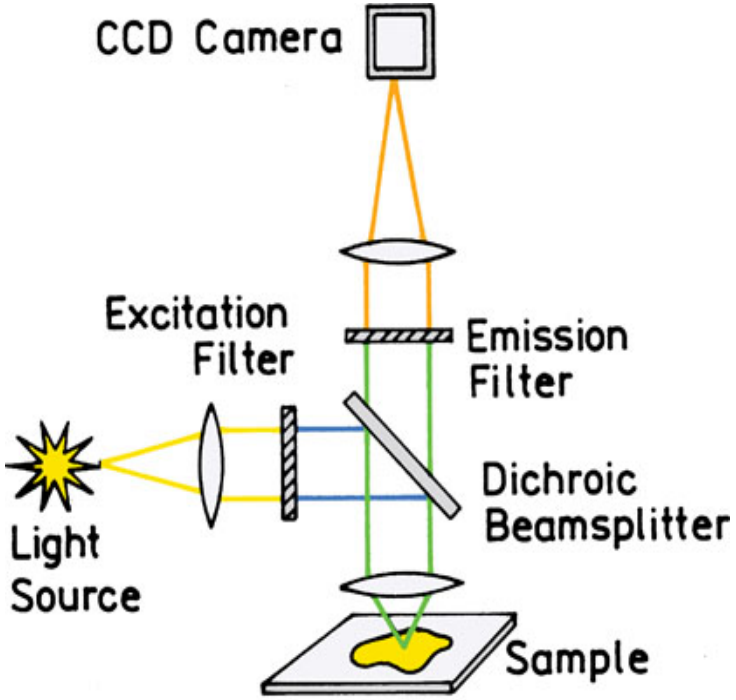




Taken from the product documentation of FUJIFILM

Fluorescence microscope

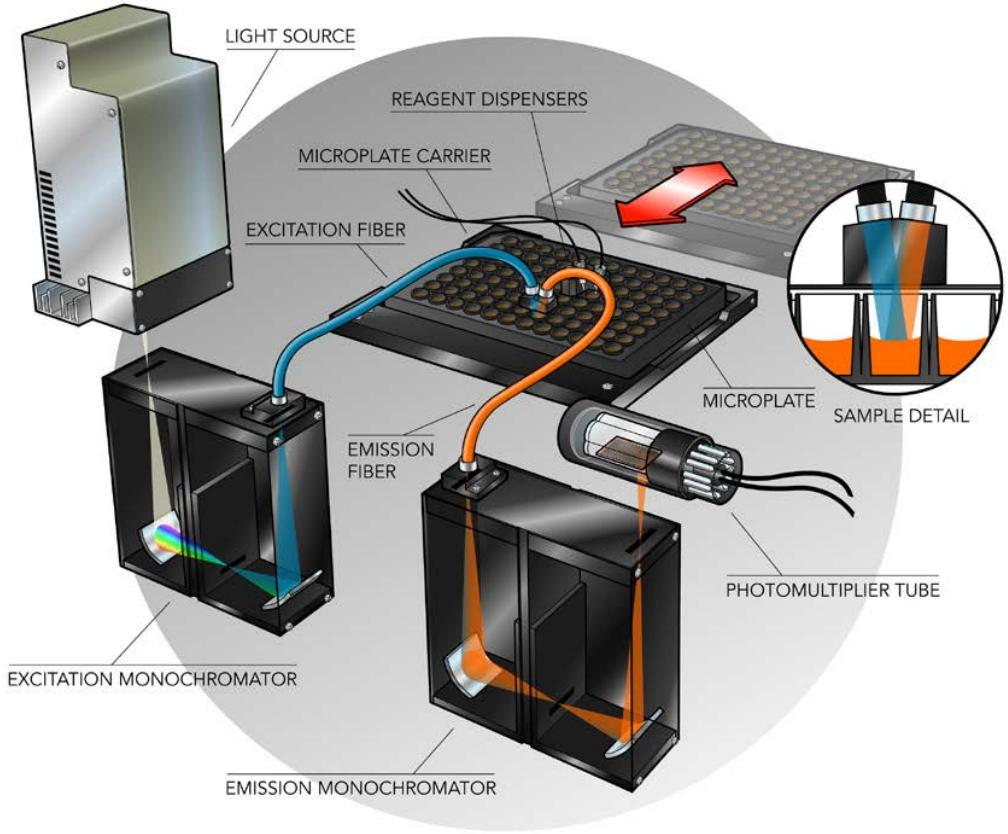
Main difference compared to spectrometers:
does not use monochromators, but **excitation and emission filter**



Taken from J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Third Edition,Springer, 2006

http://micro.magnet.fsu.edu/primer/java/lightpaths/fluorescence/index.html

Microplate reader



Taken from the instrument documentation of Biotek

Next

How to measure steady-state fluorescence?