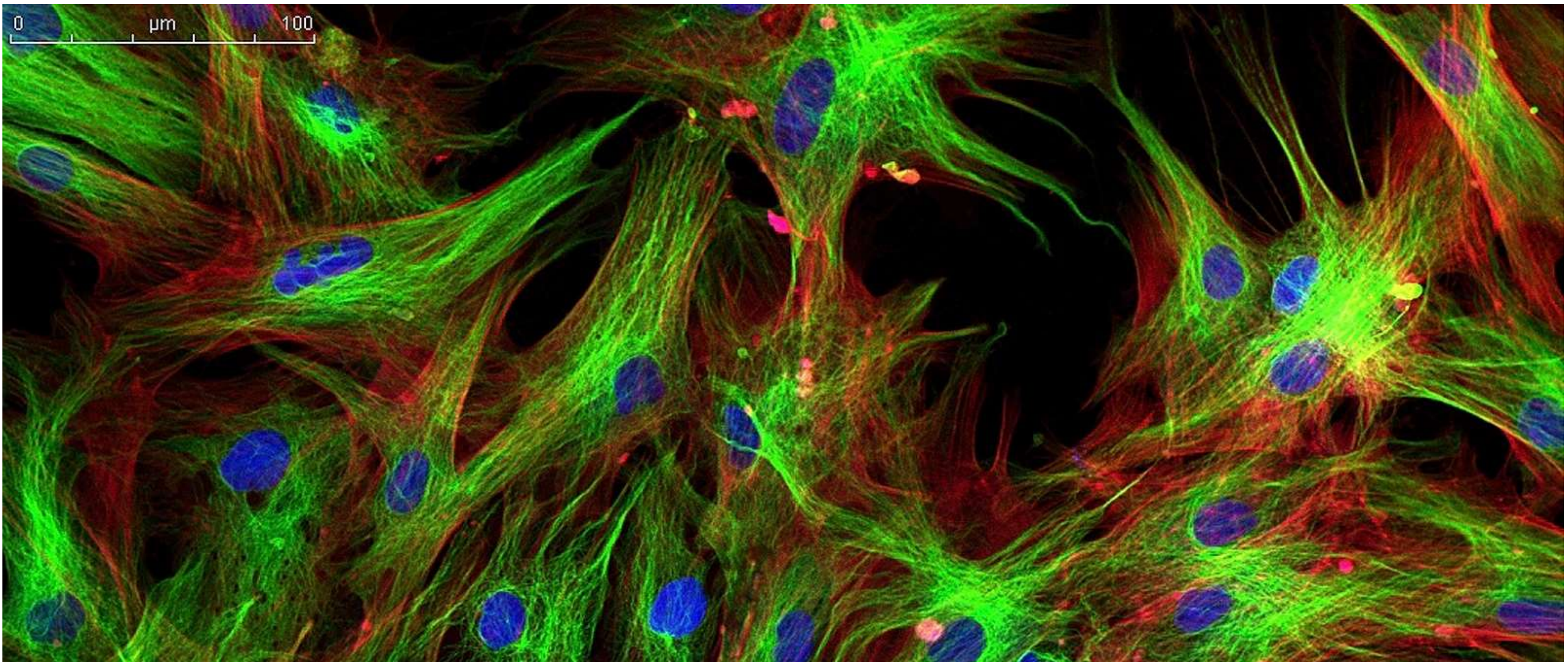
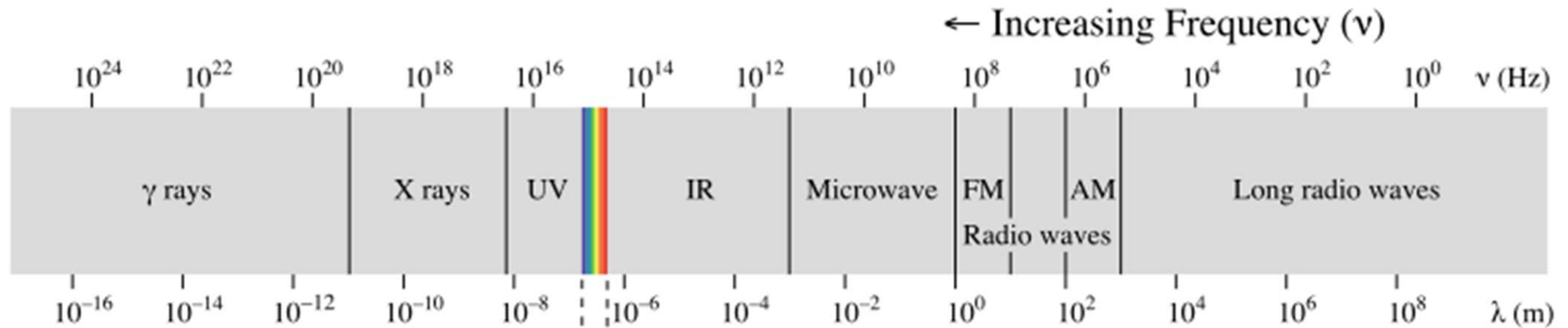
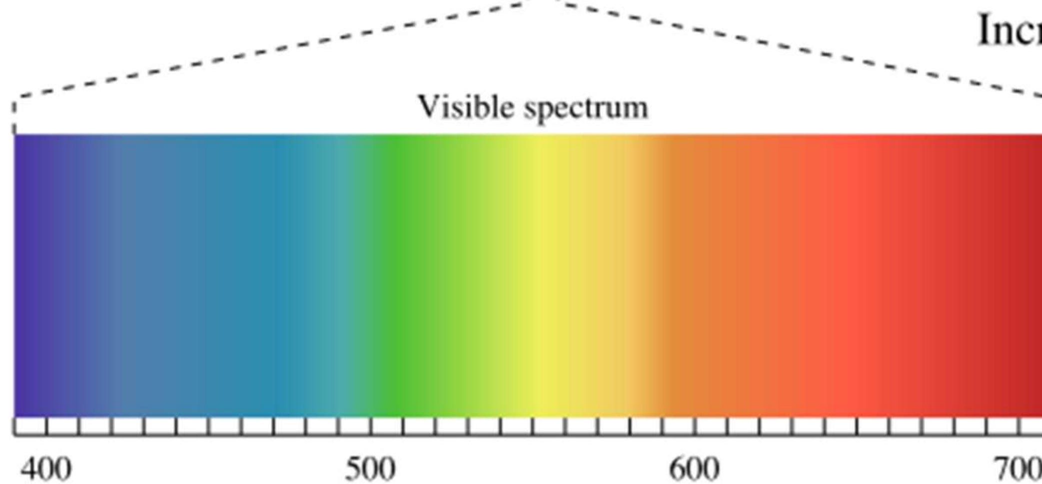
Fluorescence Microscopy



EM spectrum



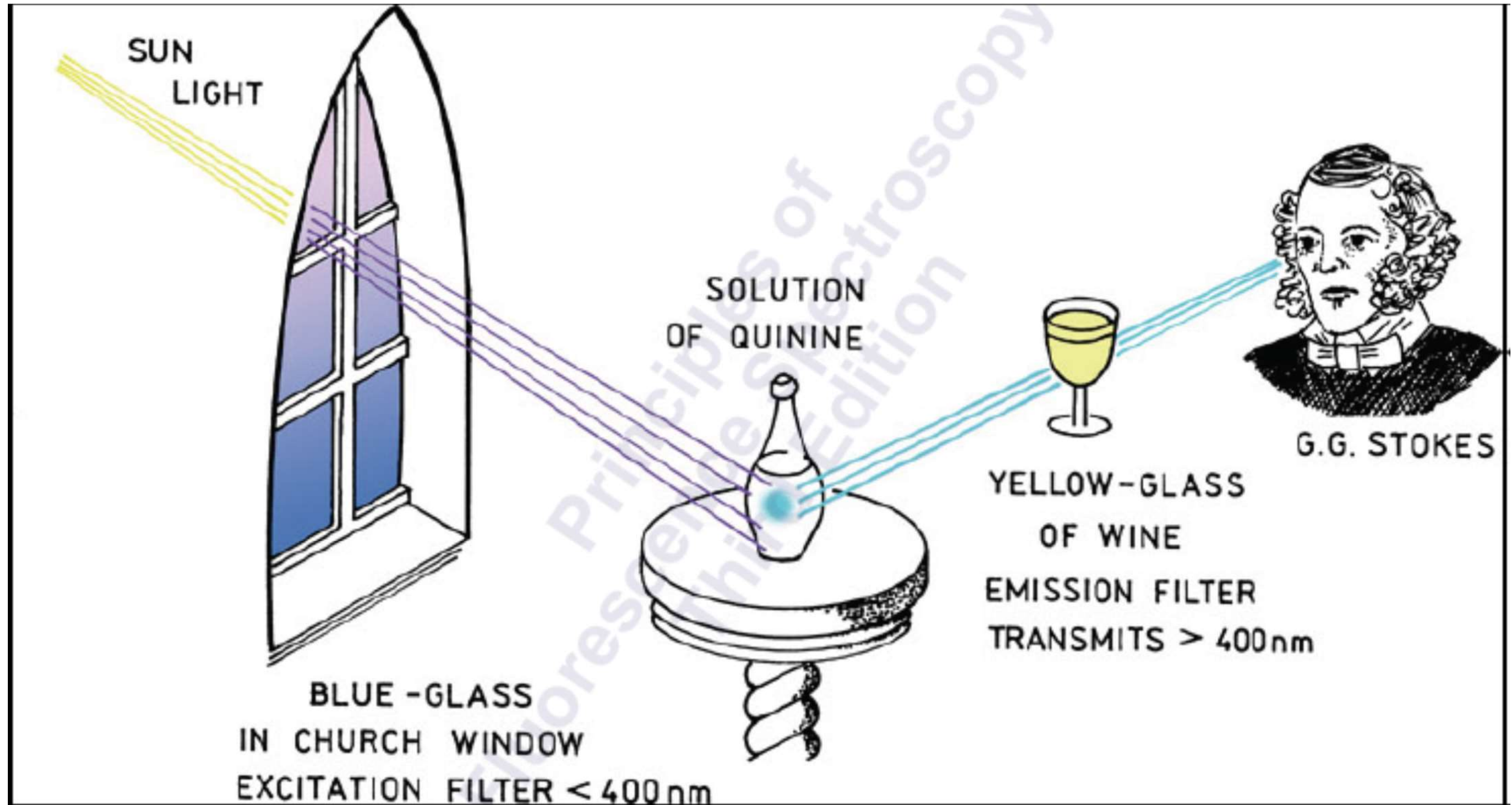


Increasing wavelength (λ) →
← Increasing Energy (E)
← Increasing frequency (ν)
←Increasing wavenumber (1/λ)

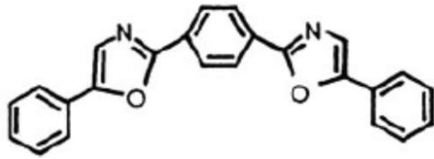
$E=h\nu$

$c=\lambda\nu$

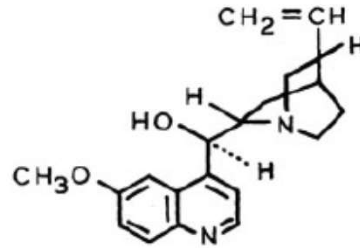
Stokes shift



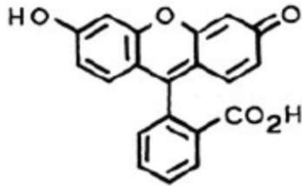
Fluorescenza



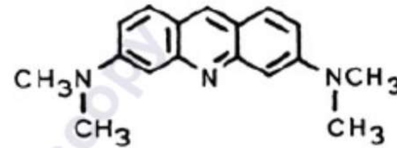
POPOP



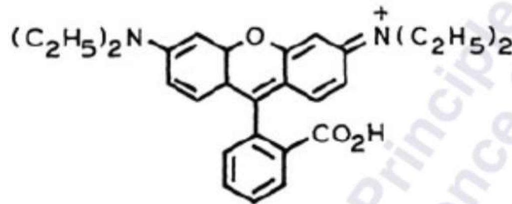
Quinine



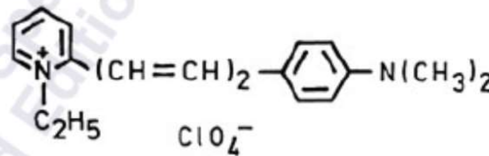
Fluorescein



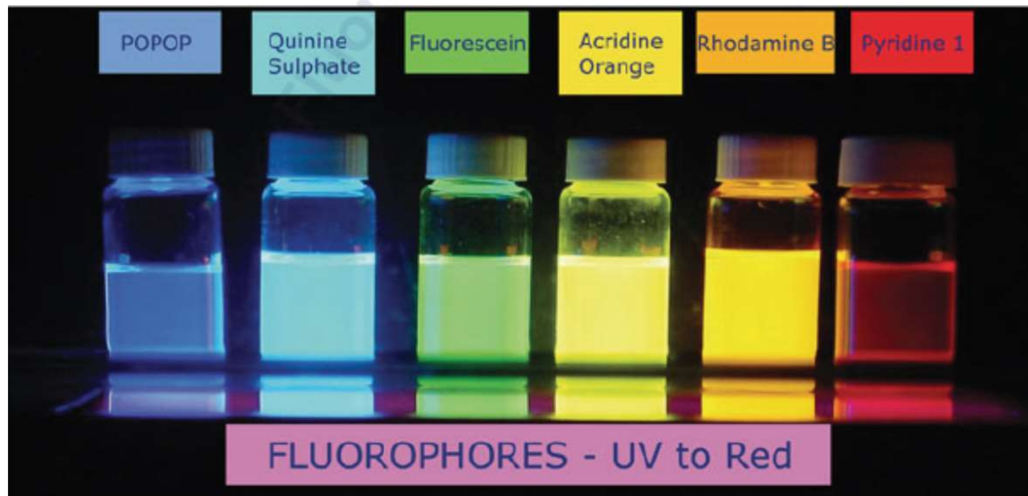
Acridine Orange



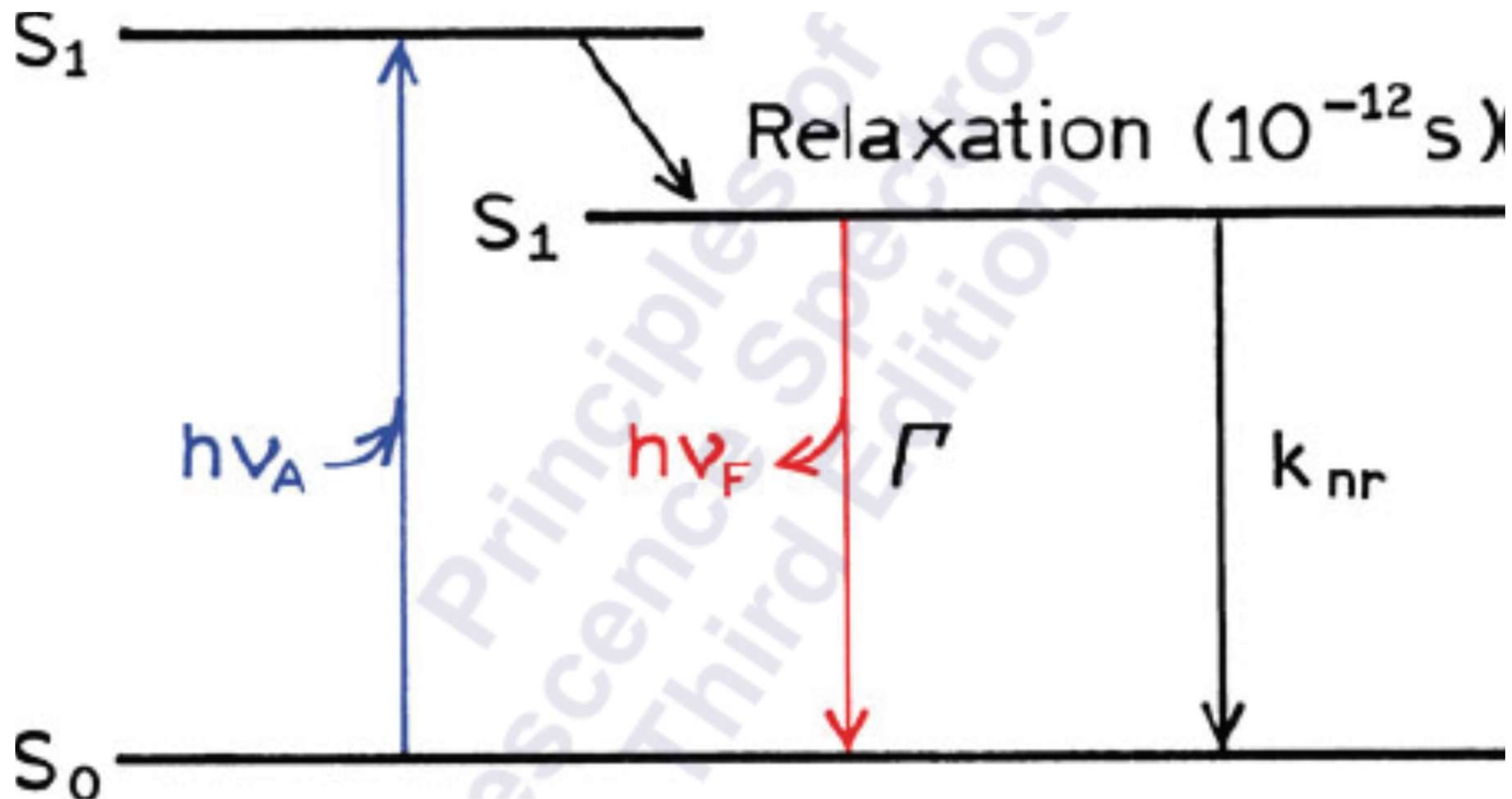
Rhodamine B



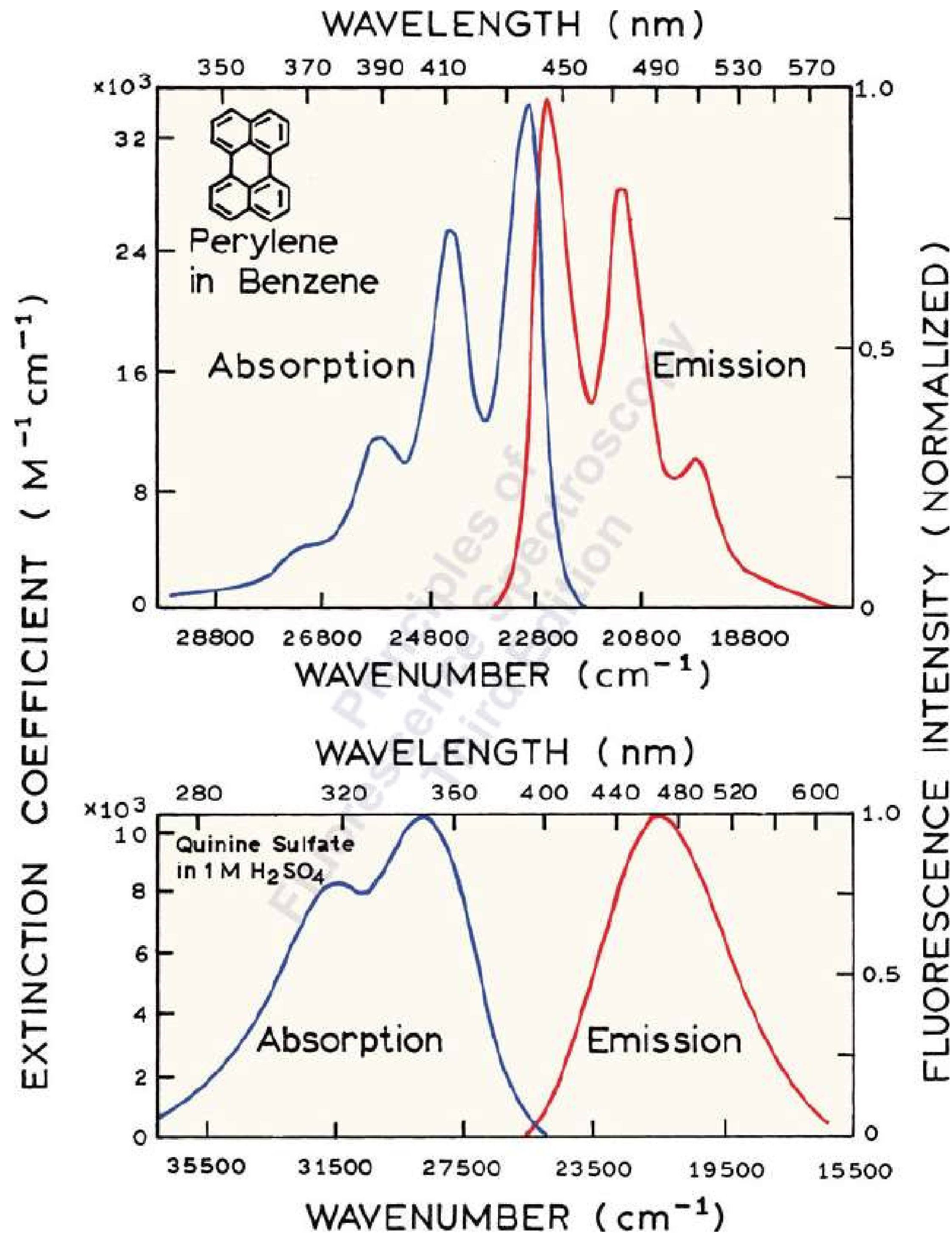
Pyridine 1



Fluorescence Lifetime (≈ns)



Emissione ed assorbimento



Fluorescence microscopy

Excites and observe fluorescent molecules

The most commonly used microscopy

High resolution, sensitive with low background, multi-channel…

comes with variations (fancy names).

- deconvolution, OMX, deltavision
- confocal, spinning disc, two photon
- TIRF, FRAP, FRET, FLIM, iFRAP, FCS …
- PALM, STED, STORM, SIM, (super-resolution)

still in development

What can you do with a fluorescence microscope?

For example:

Determine the localisation of specific (multiple) proteins

Determine the shape of organs, cells, intracellular structures

Examine the dynamics of proteins

Study protein interactions or protein conformation

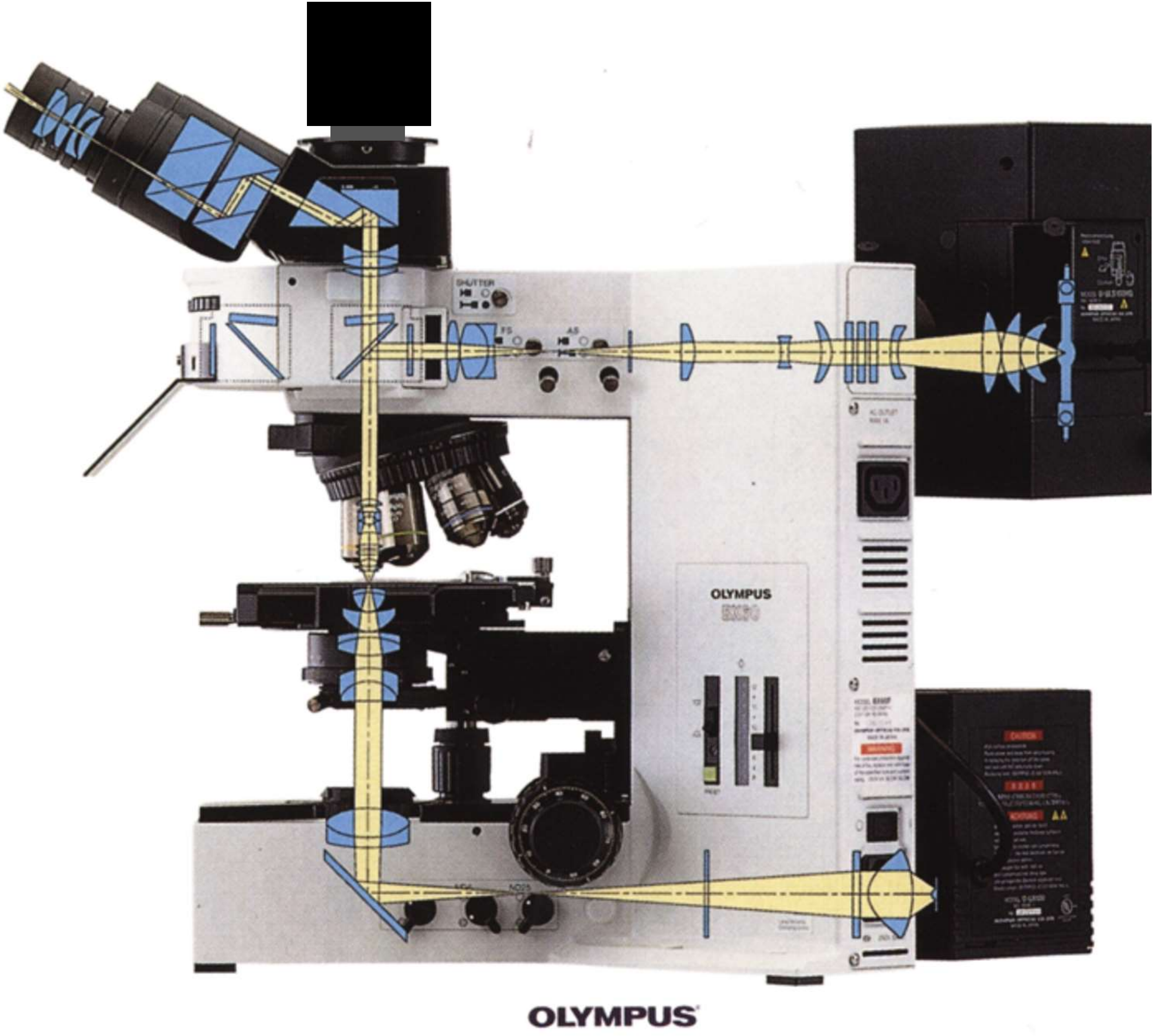
Examine the ion concetration etc.

can observe in live cells

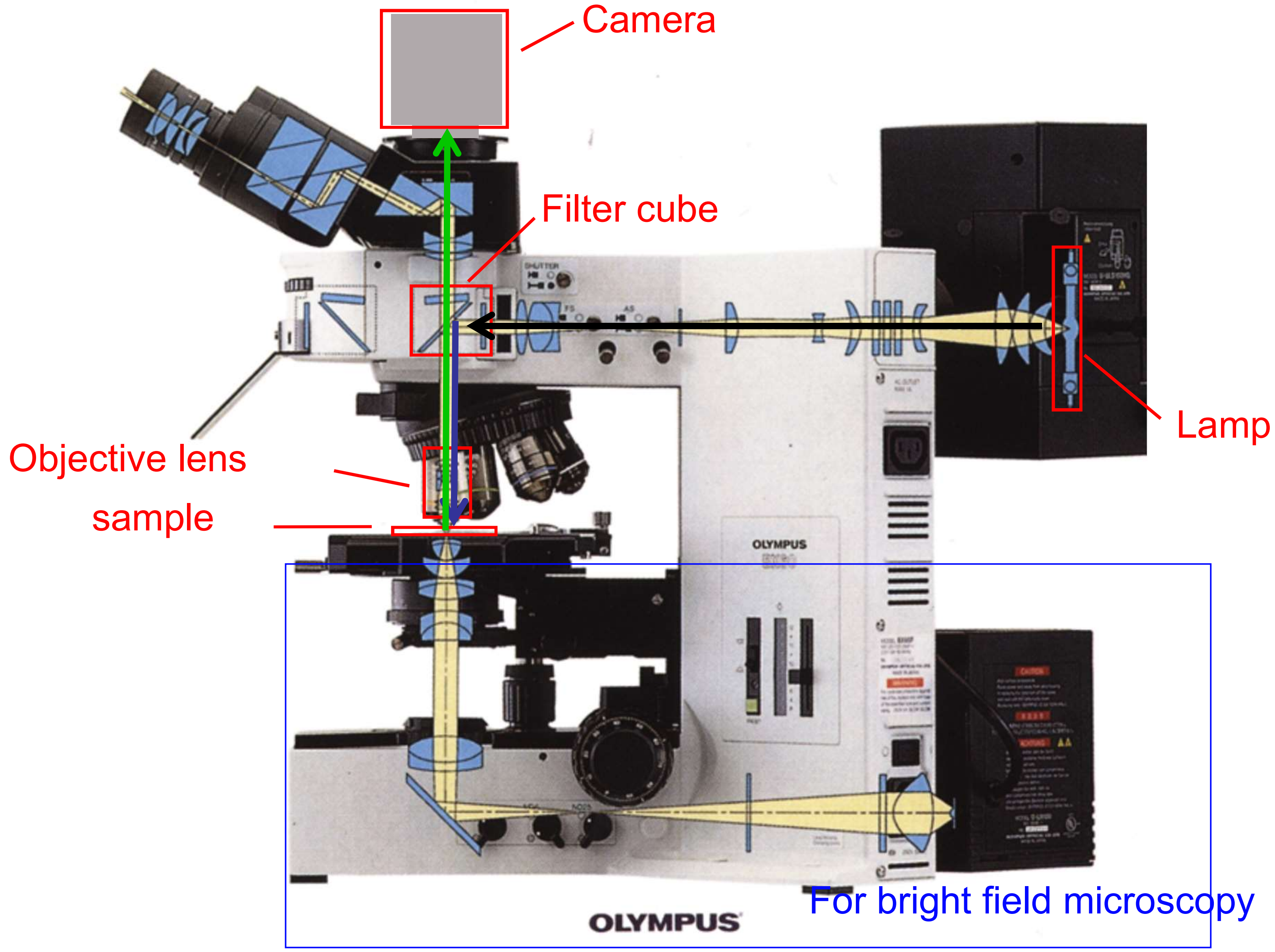
Principle of fluorescence microscopy

–How do a fluorescence microscope work?

upright microscope light path



upright microscope light path

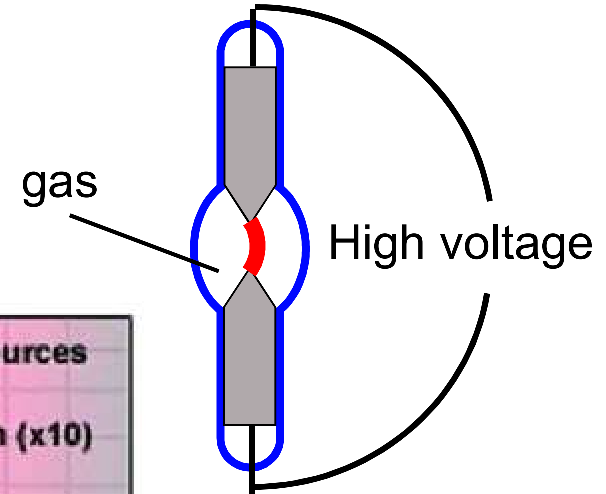


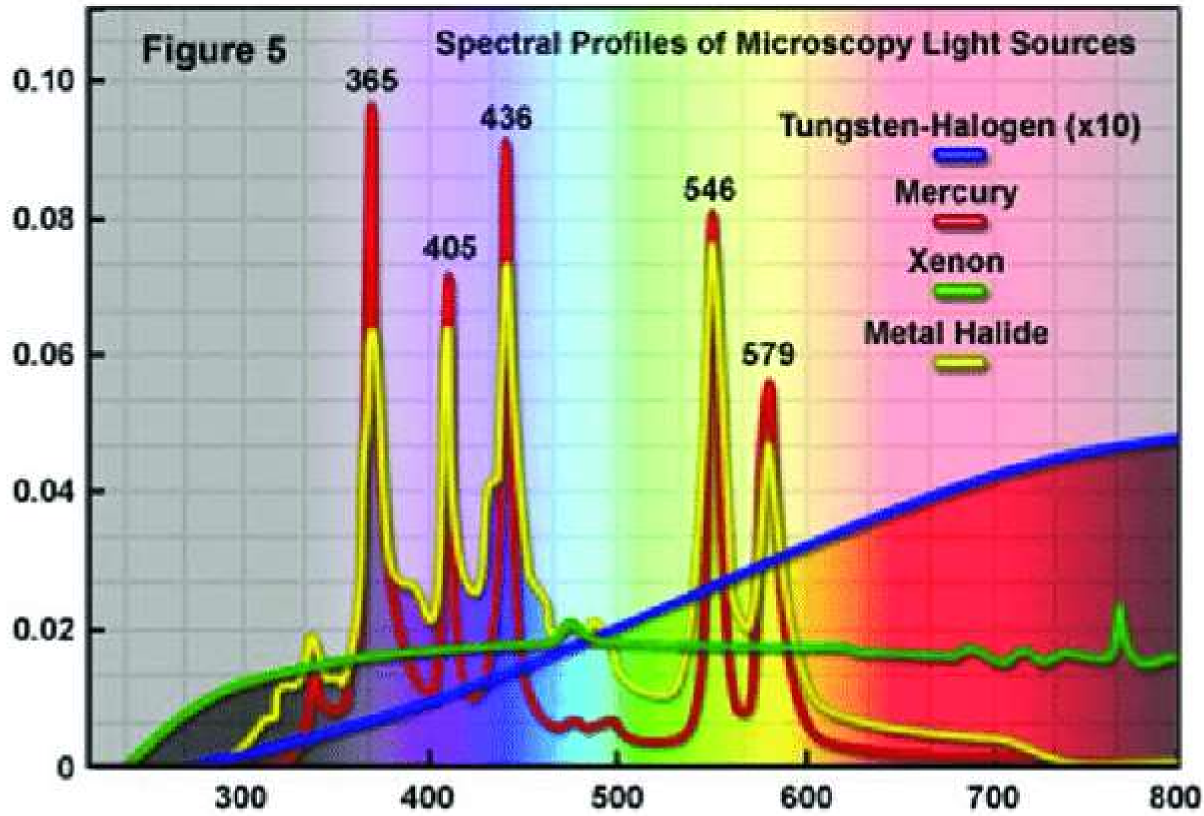
Lamp

– where it starts

Arc lamp

Mercury lamp　　Xenon lamp





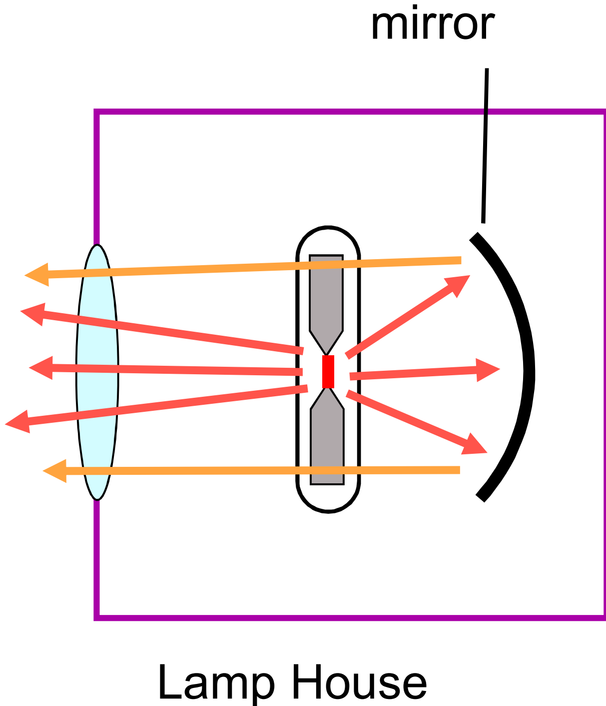
To obtain uniform illumination

"centering or alignment"

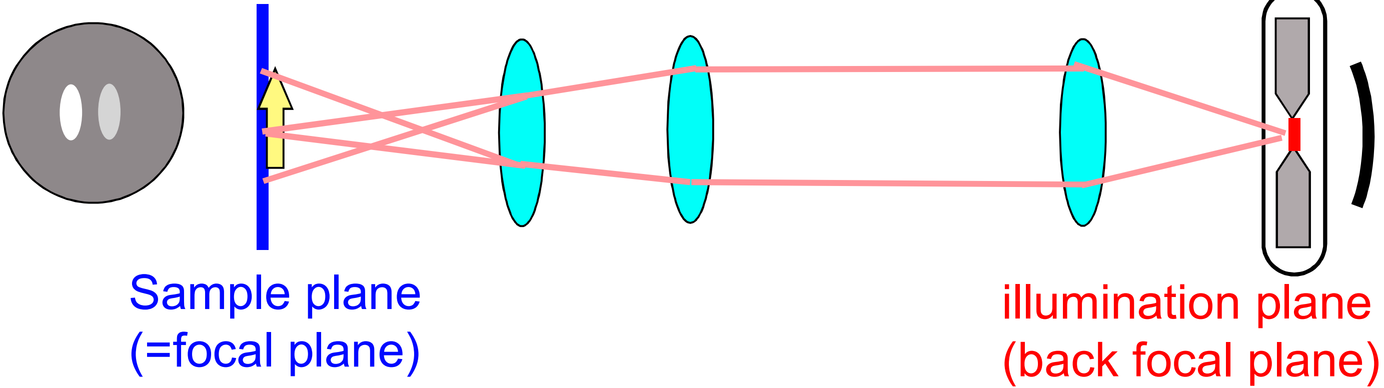
(both lamp and mirror)

= Koeller illumination

Objective lens works as condenser



(Remove objectives to look at back focal plane)



Lasers

= Light Amplification by Stimulated Emission of Radiation

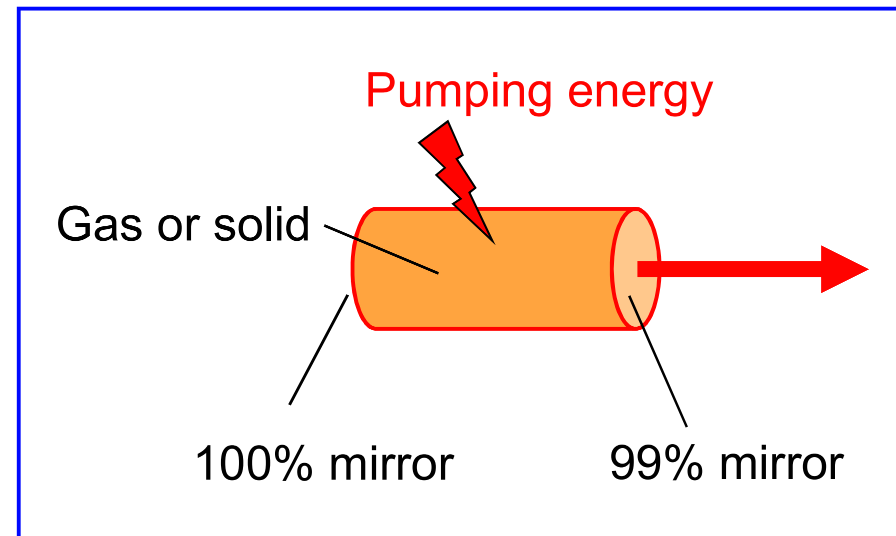
Used for confocal microscopy or FRAP etc.

Property of light from lasers

- High intensity
- uniform wavelength, phase, polarity
- can be tightly focused

Gas
- HeNe, Argon, Krypton

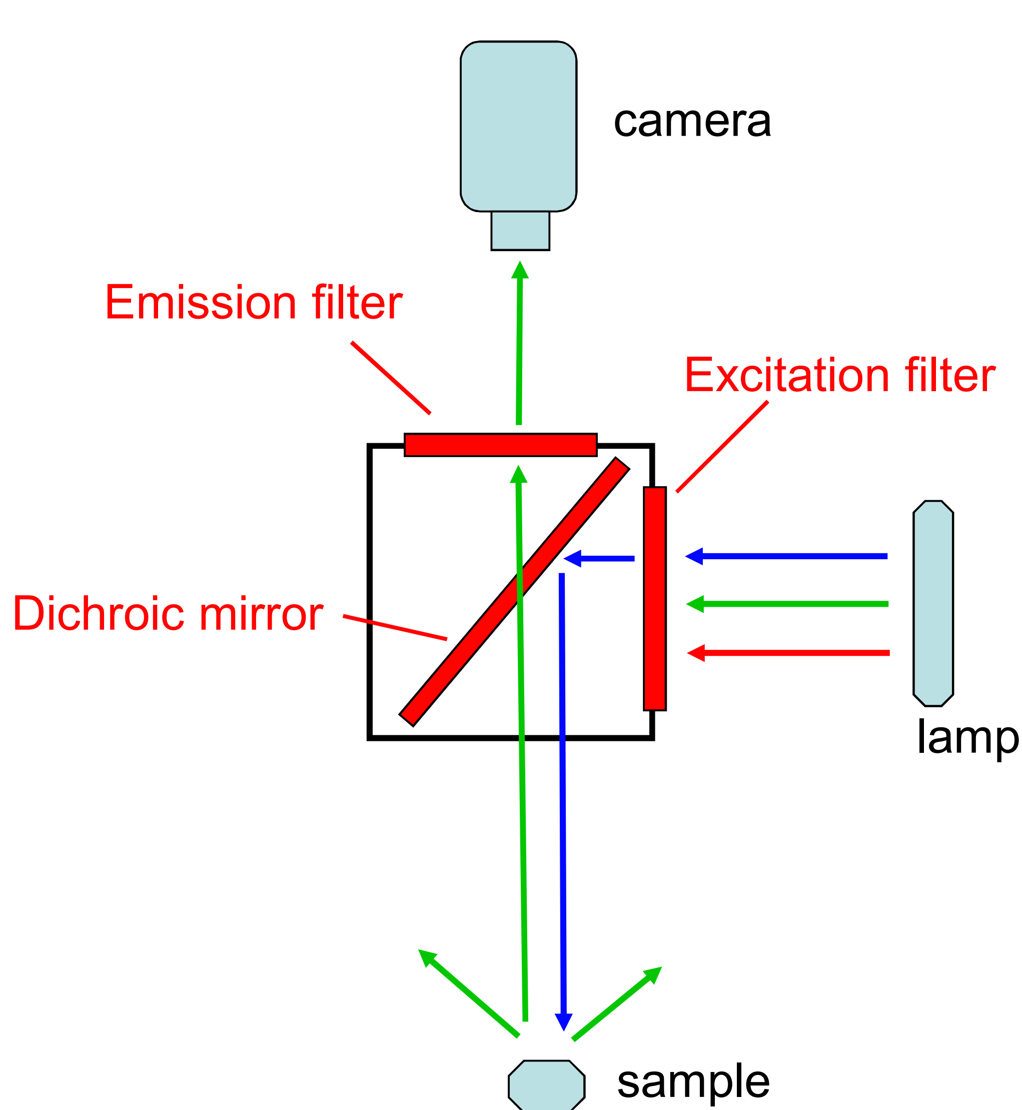
Solid diode

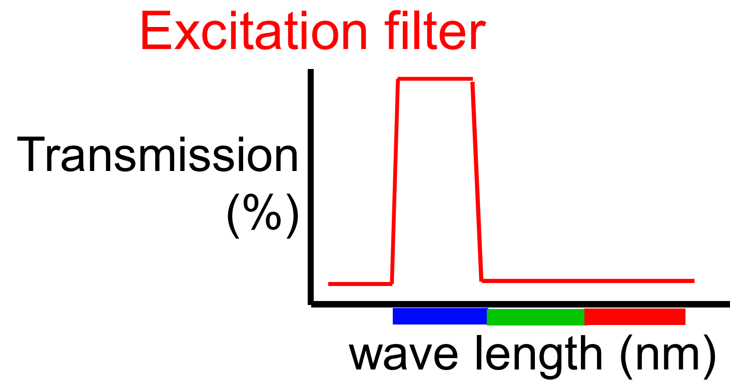


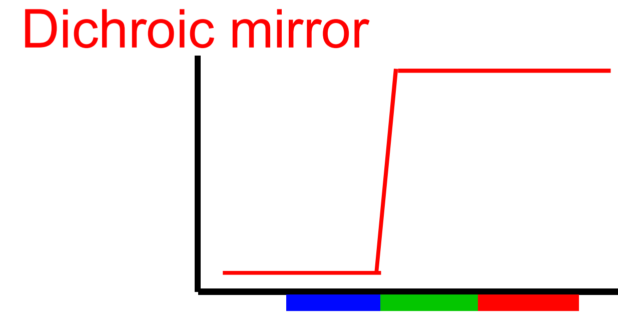
Filters

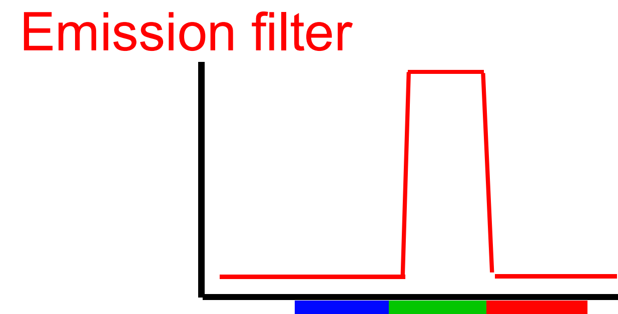
–the heart of fluorescence microscopy

Filter cube contains three filters

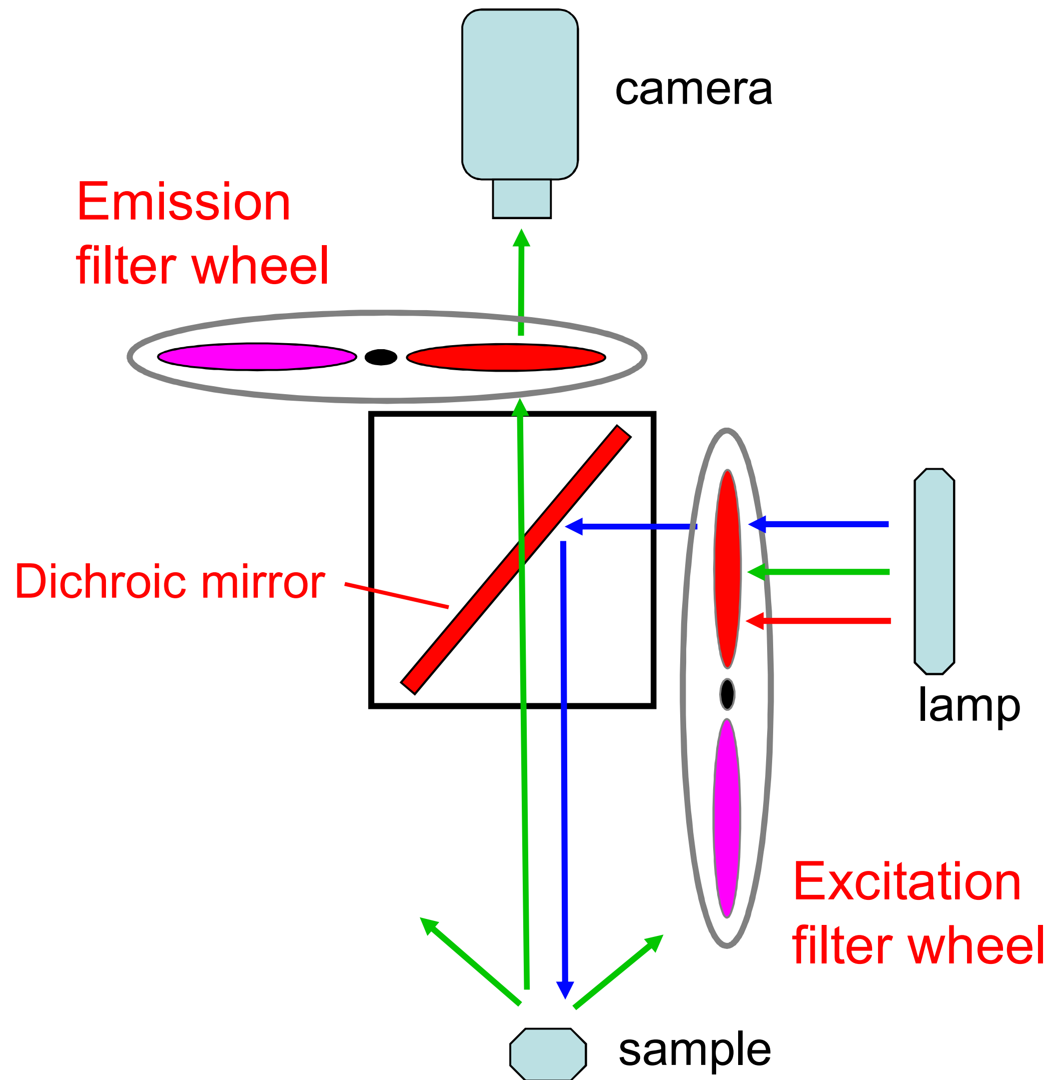


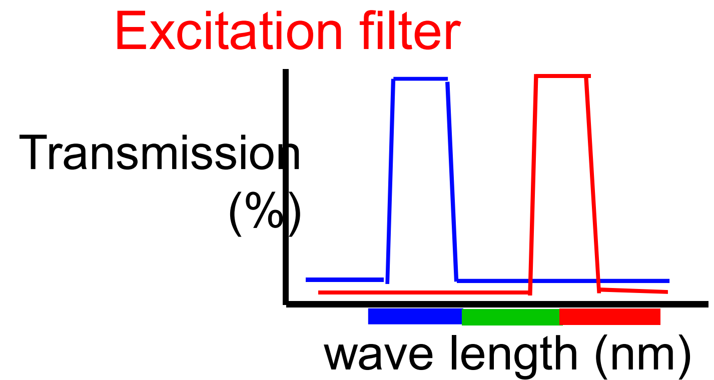


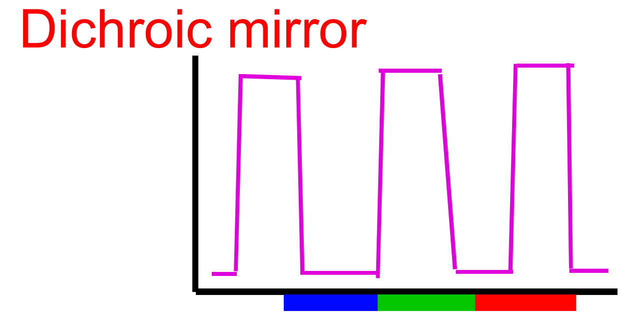


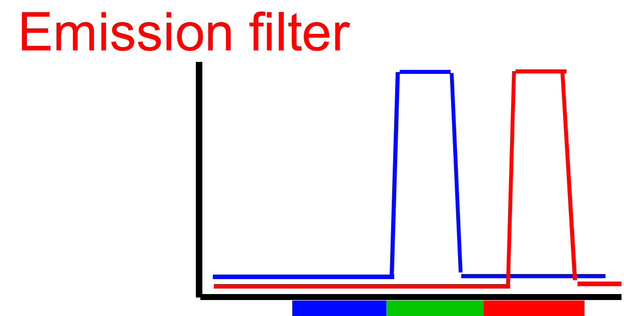


Filter wheels are often used for speed

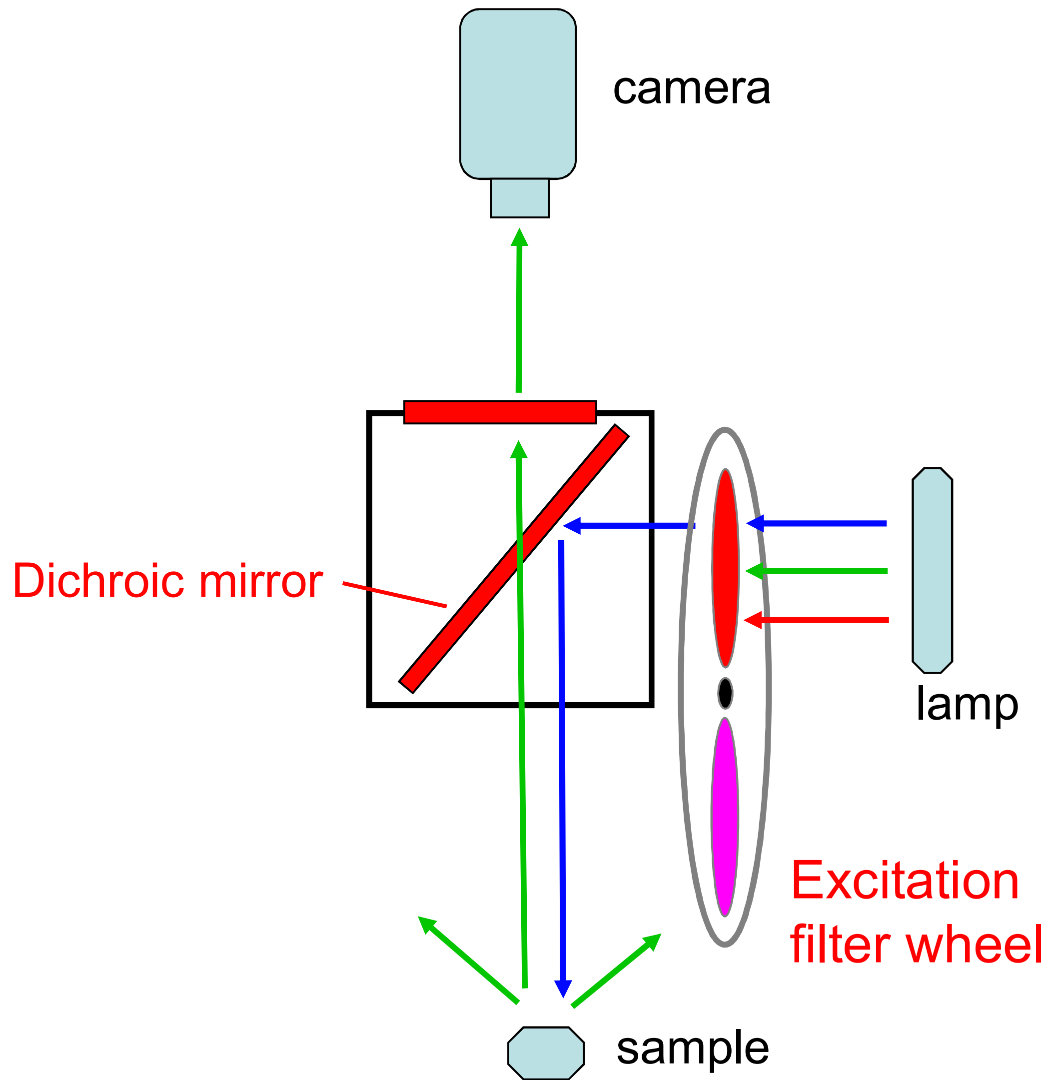


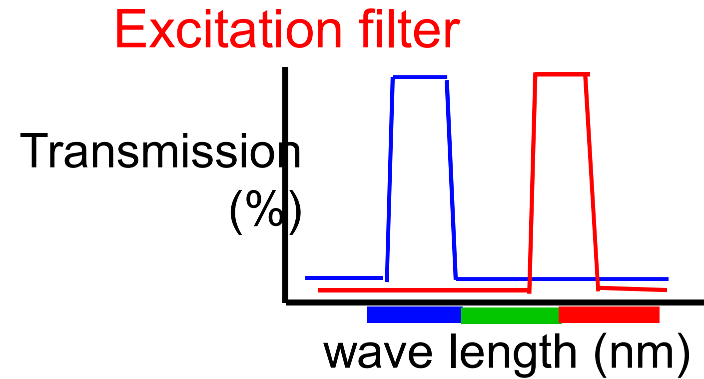


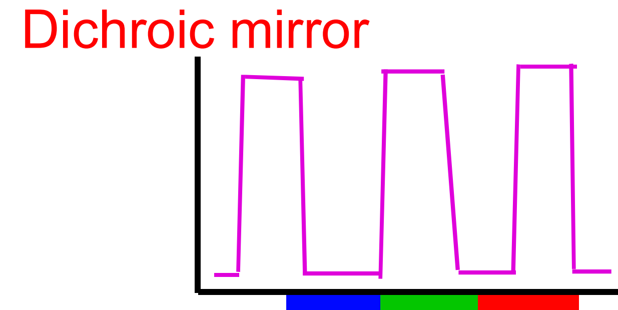


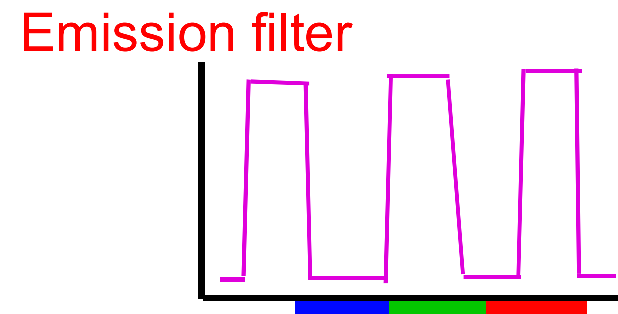


One wheel + multiband pass filter









Light may leak to other channels

Selecting filter sets is critical

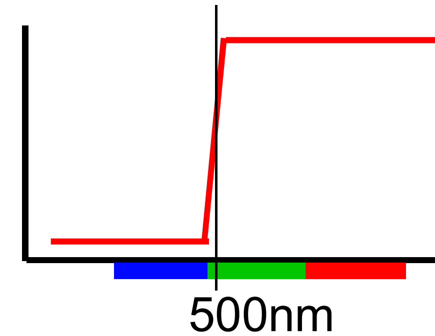
for sensitivity, colour separation

(dealt in the next lecture)

How to tell the property of filters

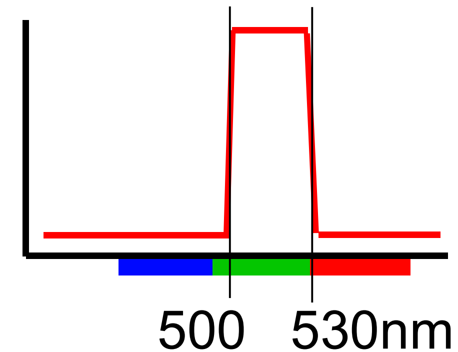
Long pass (LP) filter

LP500



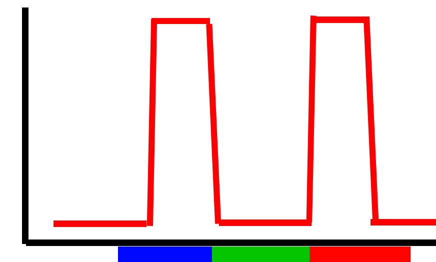
Band pass (BP) filter

BP500-530 or BP515/30



Short pass (SP) filter

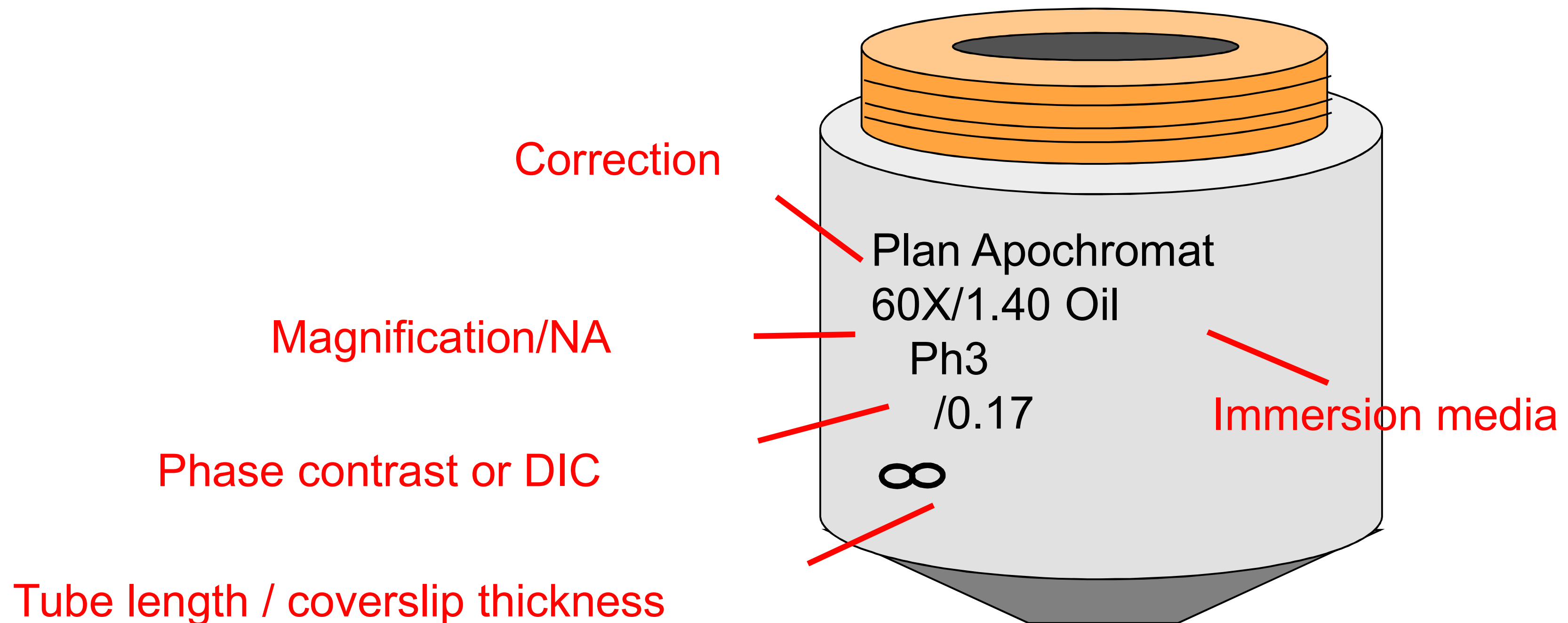
Multiband pass filter



Objective lens

– making it bigger

Objective lens



Magnification /numerical aperture (NA)

Resolution: propotional to $1/NA$

Brightness: propotional to $(NA)^4 / (magnification)^2$

Correction of optical aberration

Spherical aberration
Chromatic aberration

Better correction (↓)

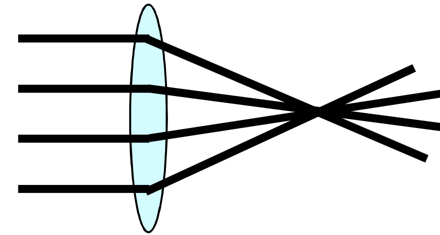
- Achromat
- Fluorite
- Apochromat

Curveture of field

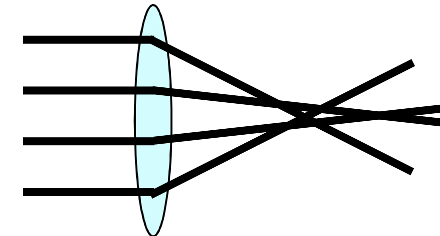
- Plan

Plan Apochromat is the best corrected
(may not be the brightest)

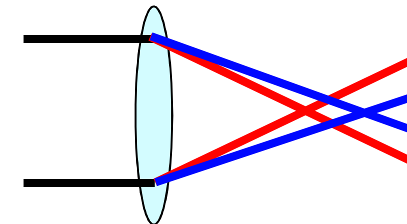
Ideal lense



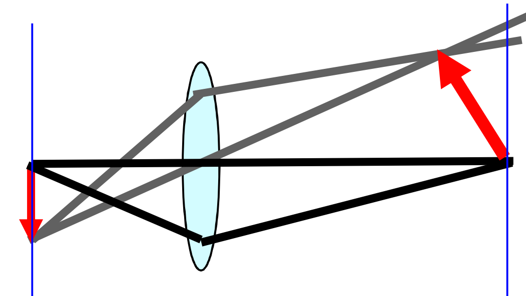
Spherical aberration



Chromatic aberration



Curveture of field



Other considerations of correction

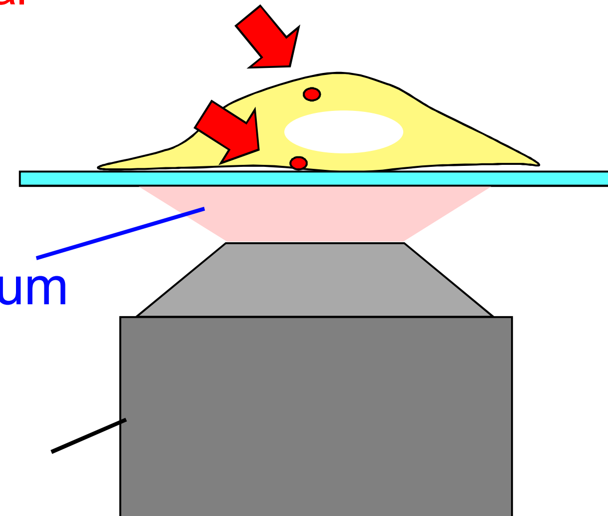
Thick sample

Not corrected for this signal

Corrected for this signal

Immersion medium

objective



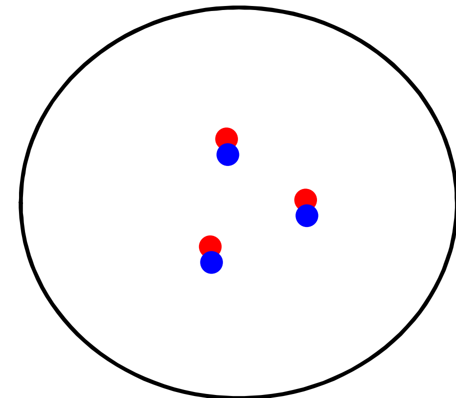
Use a water-immersion lens (for live samples)
Use immersion oil with different reflactive index
Use a lens with a movable internal lens.

Lack of Registration

Light with different wavelengths from the same point
does not focus on the same place

Can be caused by

- objective lens
- filters
- or mechanical



Detectors

– capturing data

Detectors

Eye

Film

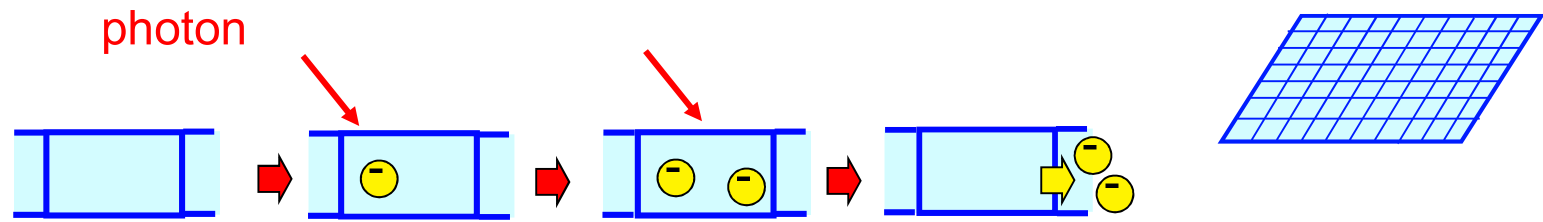
PMT (photo multiplier tube)

no space information
very high time resolution
used for laser scanning confocal microscope

CCD (charge coupled devise) camera

space information
low time resolution
very sensitive
(quantum efficiency: >70% vs 25% (PMT), 2% (film))
most commonly used

CCD camera – how it works



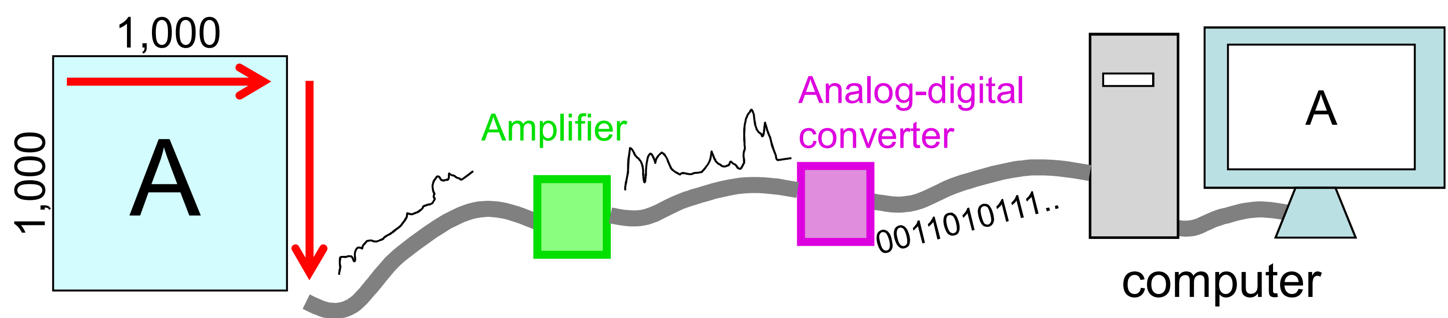
Generate and accumulate charge in response to photon

charge is propotional to the number of photon

can achieve high sensitivity by longer exposure

Readout by transferring charges by one pixel to the next

slow download

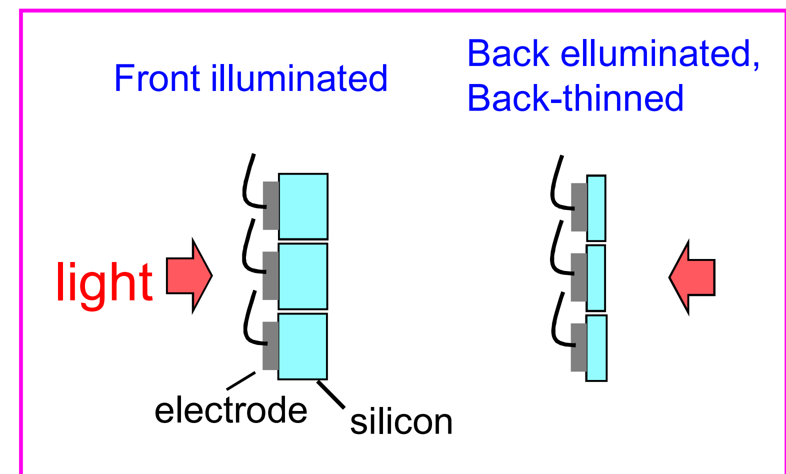


Property of CCD camera

Resolution	pixel size
Field size	pixel number x size
Time resolution	read-out rate (Hz)
Dynamic range	bit (12,14 etc), full well capacity
Sensitivity	quantum efficiency (wave-length dependent), "back-thinned" (QE >90%)
Noise	cooling temperature

Monochrome vs colour

Colour camera is, in general,
less sensitive
less resolution
more expensive.

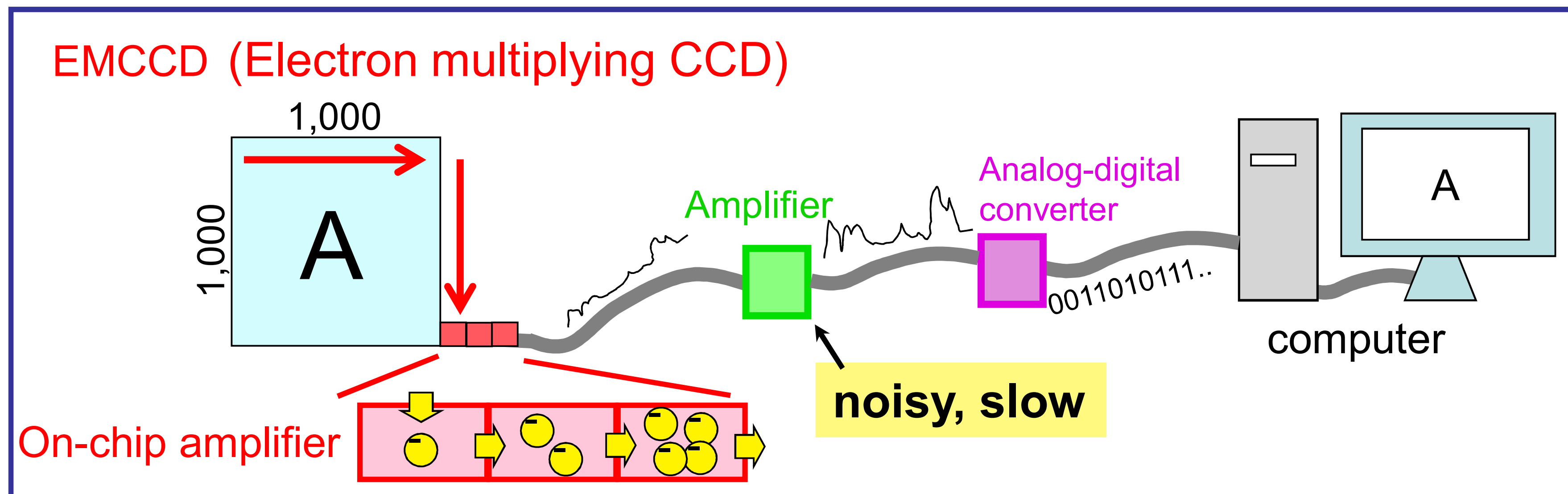


Reducing noise: on-chip amplification

Dark noise: significant at a long exposure.
can be reduced by cooling the chip (-50, -70°C)

Readout noise: significant at a low signal
can be reduced by slow readout, on-chip amplification

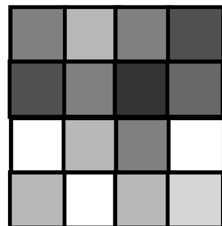
Camera with on-chip amplification: EMCCD, EBCCD, iCCD
(low readout noise, high readout rate)



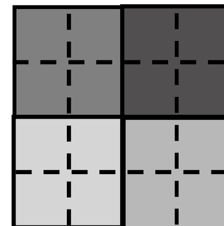
Useful function of CCD camera

Binning

no binning



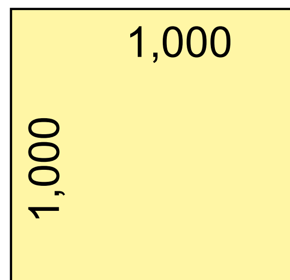
2x binning



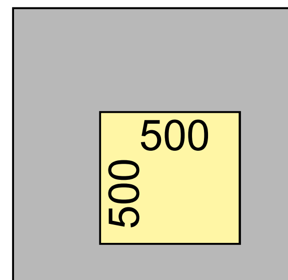
sensitivity
readout rate
resolution

Subarray readout

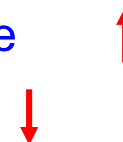
full readout



subarray readout



readout rate
field size



The lecture you miss this round.

I want to improve ….

Colour separation

Sensitivity

Resolution

What can I do?

Further reading

Sites

https://en.wikipedia.org/wiki/Fluorescence_microscope

https://www.microscopyu.com/techniques/fluorescence

Book

"Fundamentals of light microscope and electronic imaging"
by Douglas B. Murphy.