INTRODUCTION TO FLUORECENCE MICROSCOPY

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- *Teacher for Intro to qbio, Imaging Biological systems*
- *Responsible for Lab1*

- Draw and describe a Jablonsky-Perrin diagram
- Why is the fluorescence emission wavelength always larger than the excitation wavelength? What is the Stokes shift?
- Notions about the excited state lifetime and how to measure it
- Pro and cons of fluorescent proteins and dyes.
- What is the maximum resolution attainable in a widefield microscope? Calculate in terms of NA and wavelength.
- Draw the optical path of a fluorescence microscope. Descibe the components

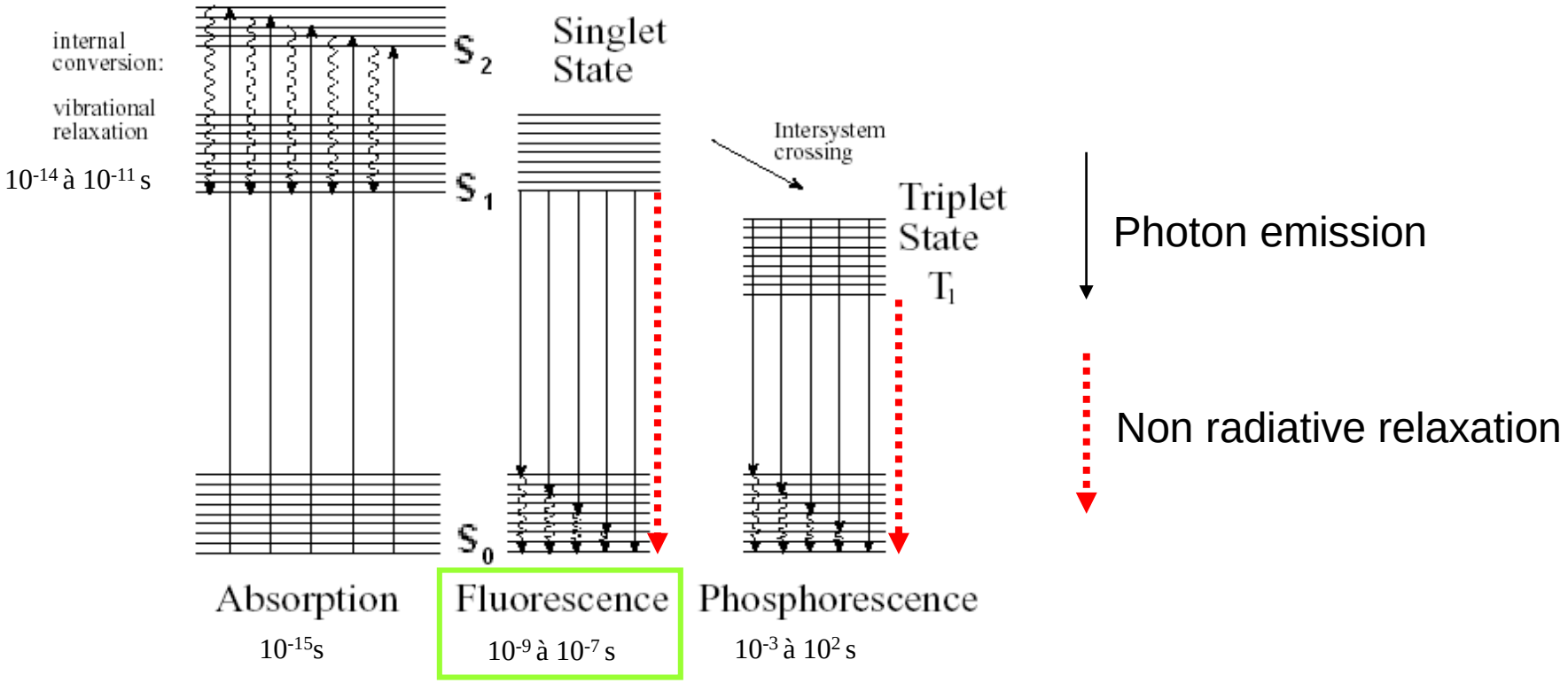
JABLONSKI – PERRIN DIAGRAM

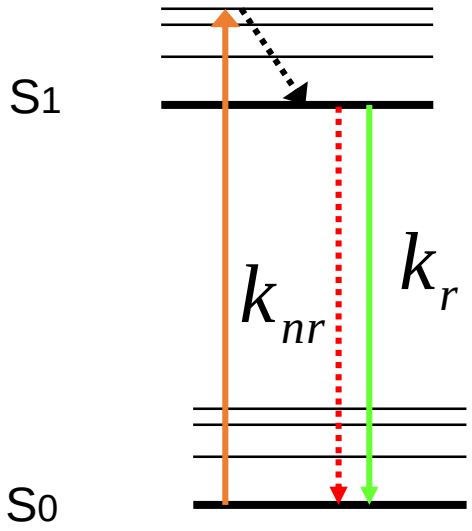
- Singlet States & Triplet State
- Vibrational states
- Vibrational relaxation
- Internal convertion
- Intersystem crossing
- Radiative and non-radiative decays & rates
- Absorption & timescale
- Fluorescence & timescale
- Phosphorescence & timescale
- Quantum yield equation
- Excited state lifetime equation

- Origin of the excitation spectrum
- Origin of the emission spectrum
- The Stokes shift

- The case of FRET
  - FRET transition & rate
  - Lifetime equation in the presence of FRET
  - FRET equations

JABLONSKI – PERRIN DIAGRAM





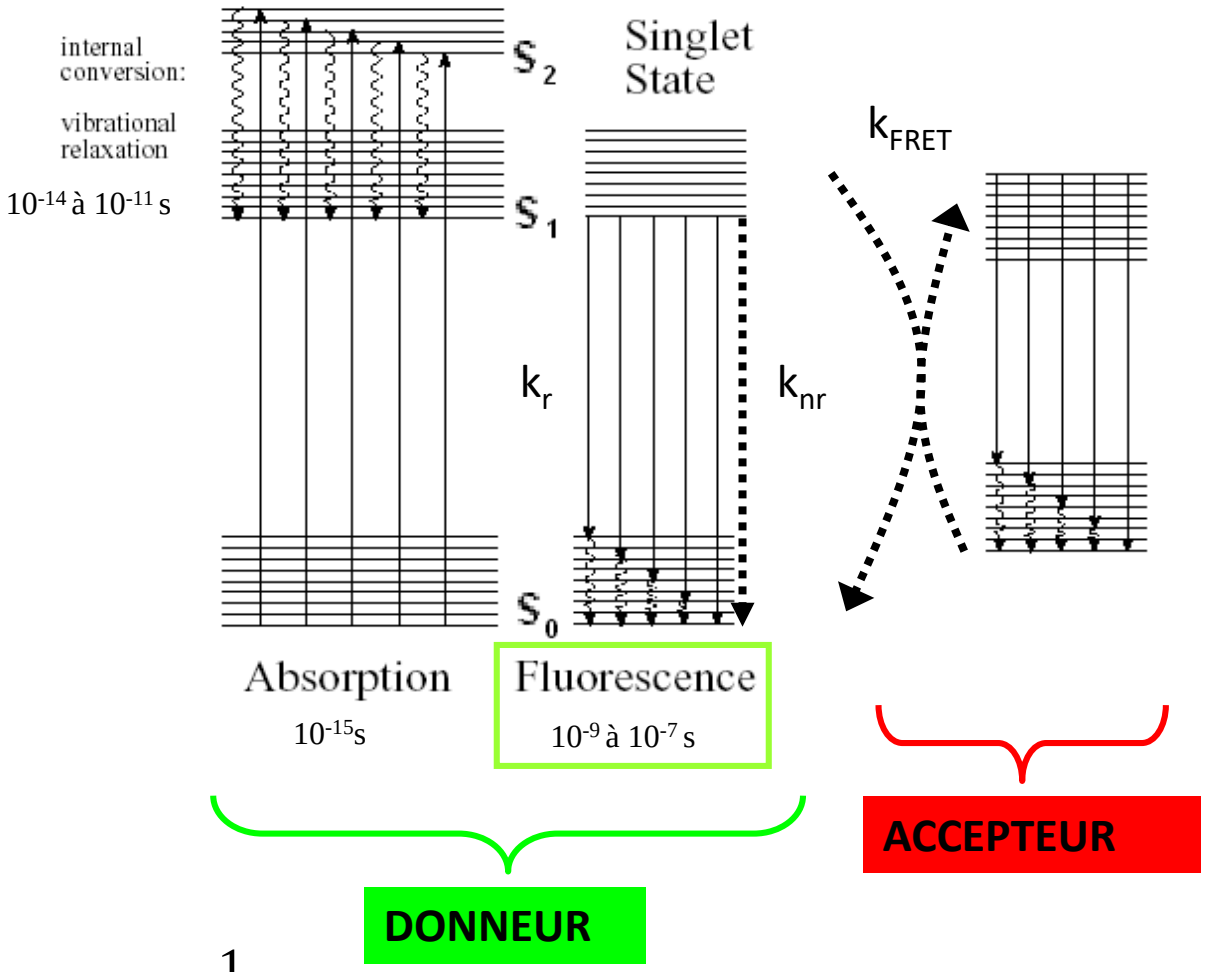
QUANTUM YIELD

$$Q = \frac{k_r}{k_r + k_{nr}}$$

EXCITED STATE LIFETIME

$$\tau = \frac{1}{k_r + k_{nr}}$$

Jablonski diagram in the presence of FRET

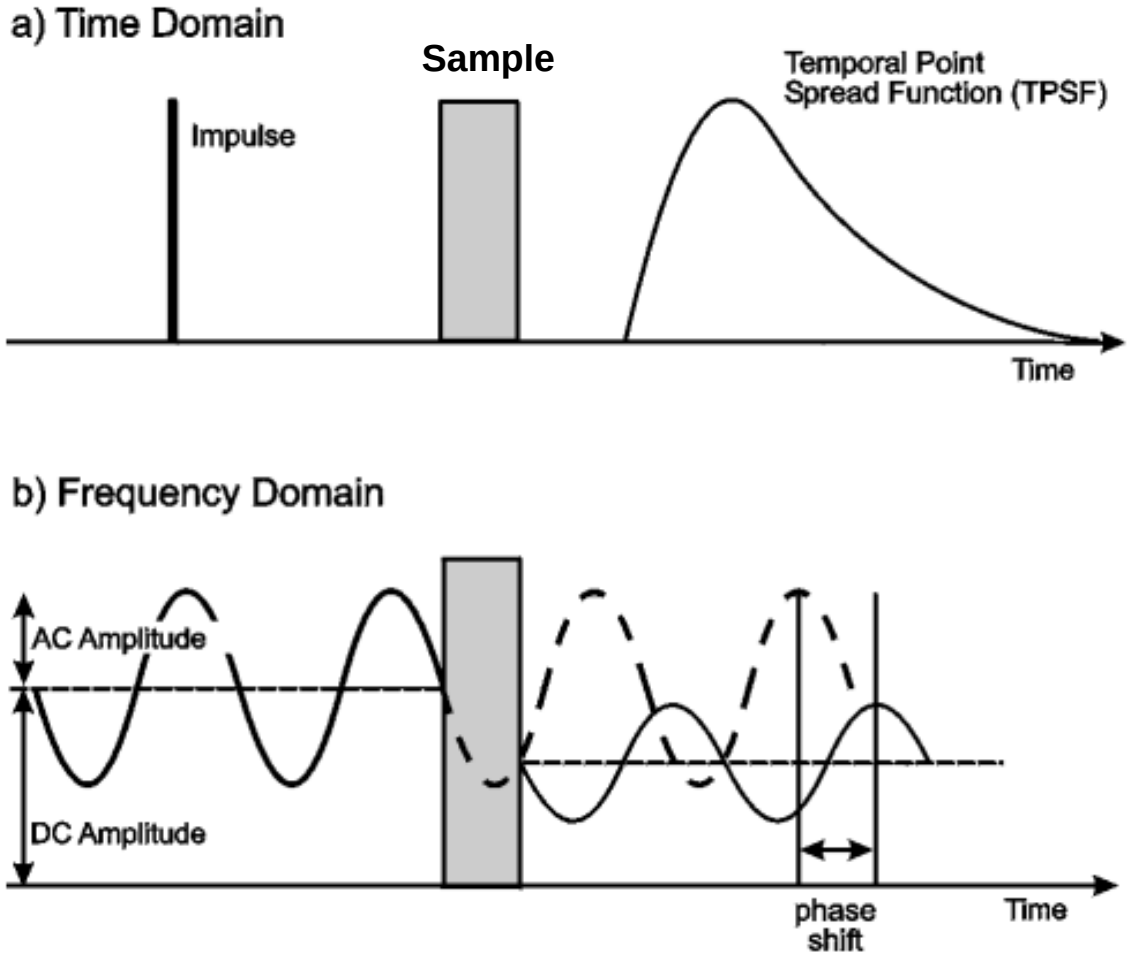


$$\tau_D = \frac{1}{k_r + k_{nr}}$$

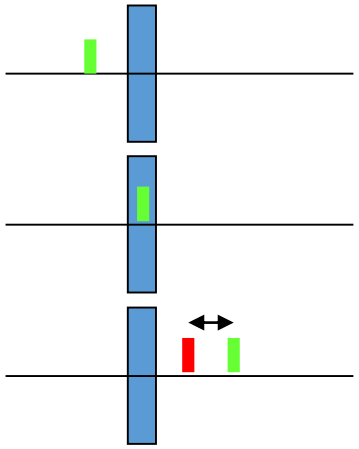
$$\tau_{D,A} = \frac{1}{k_r + k_{nr} + k_{FRET}}$$

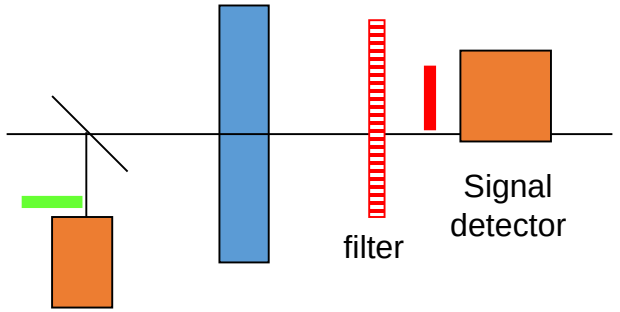
$$E = \frac{k_{FRET}}{k_r + k_{nr} + k_{FRET}} = 1 - \frac{\tau_{D,A}}{\tau_D}$$

Two techniques for excited state lifetimes measurements

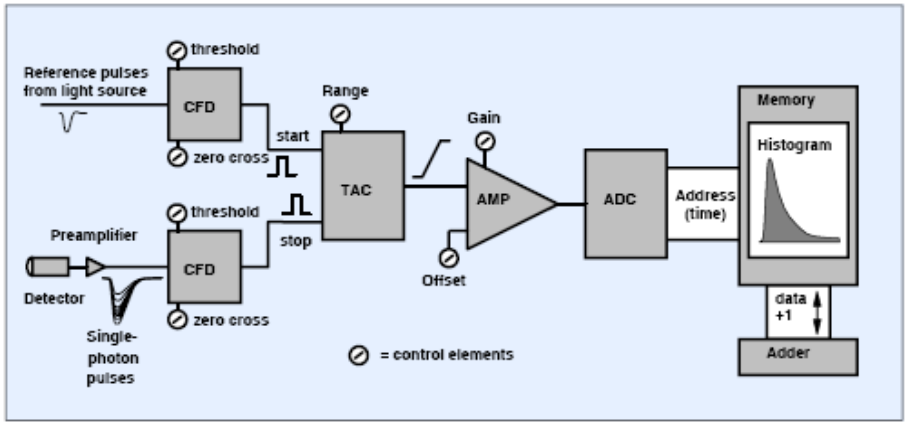


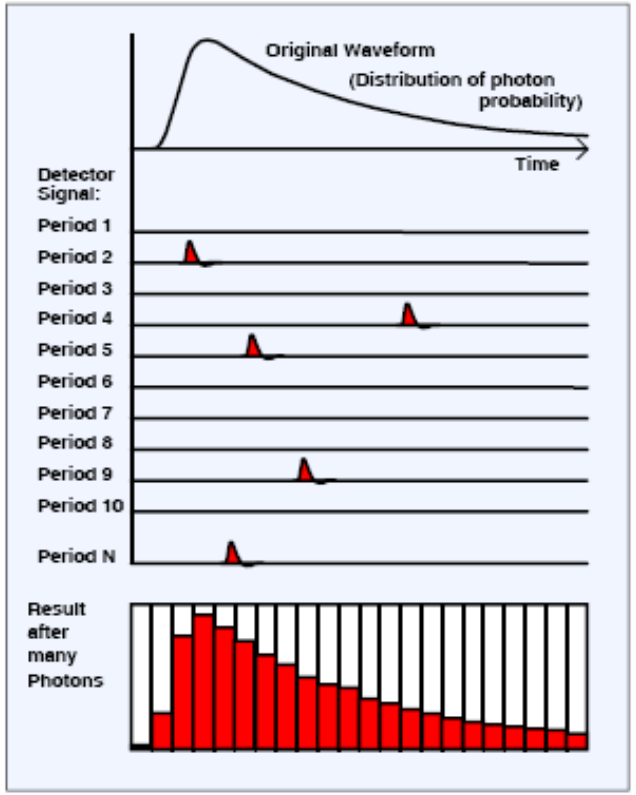
Time domain : Time Correlated Single Photon Counting





Electronic card to measure the delay between two pulses



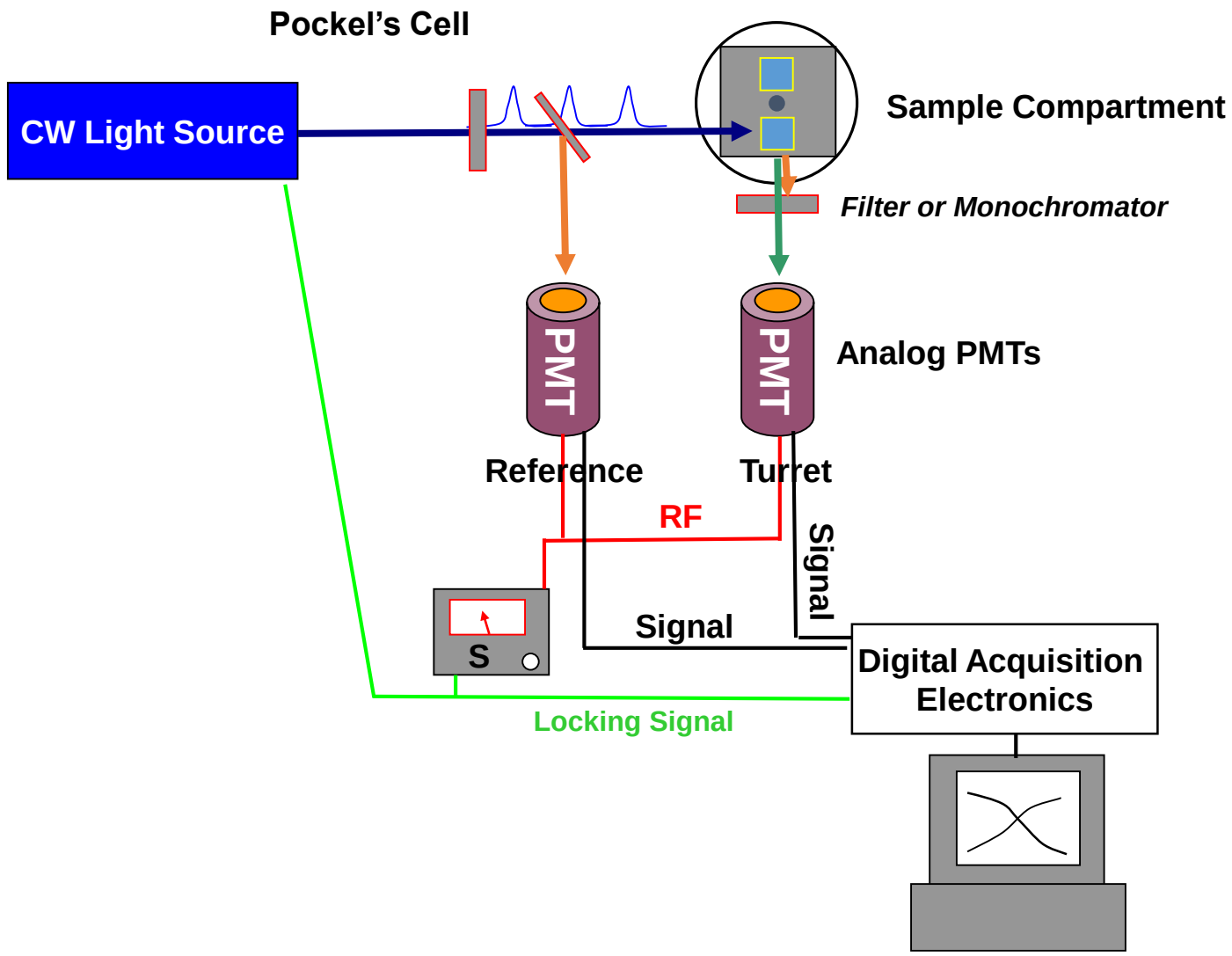


- CFD : constant fraction disciminator

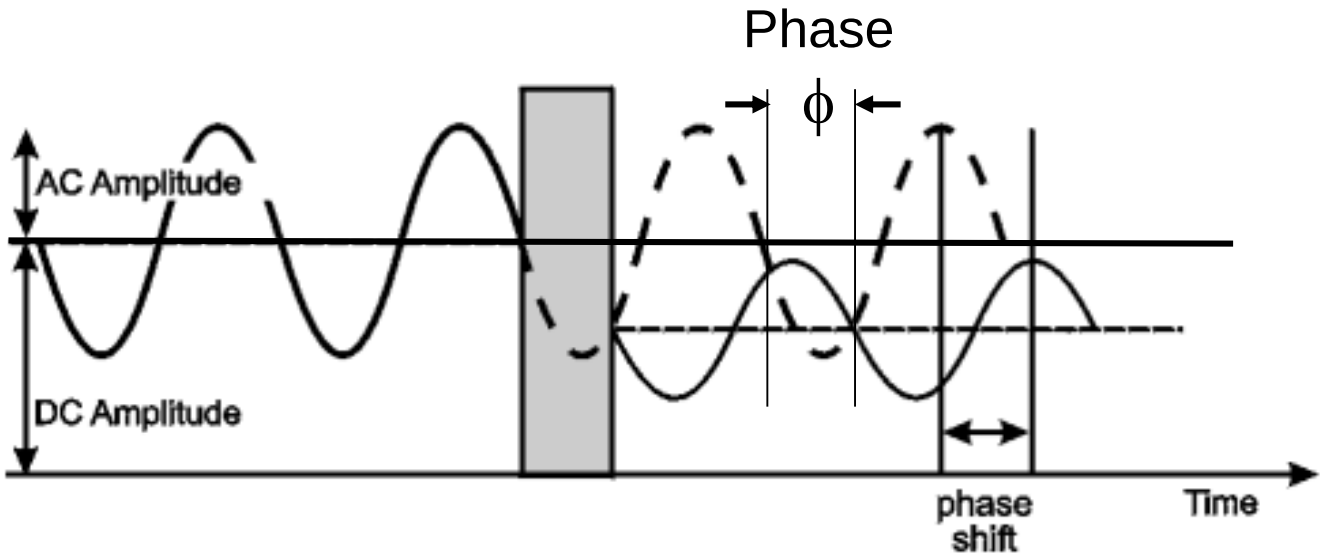
- TAC : Time to amplitude converter

- ADC : analog to digital converter

Frequency domain : time / modulation detection



Frequency domain : time / modulation detection



$$M_{ex} = AC_{ex} / DC_{ex}$$

$$M_{em} = AC_{em} / DC_{em}$$

$$m = M_{em} / M_{ex}$$

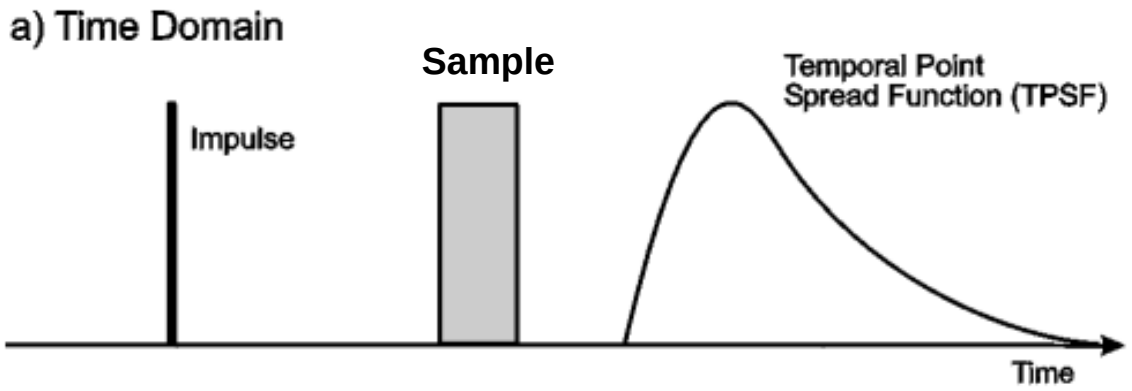
For a certain excitation frequency ω, we can show

$$\tan \phi = \omega \tau_{\phi}$$

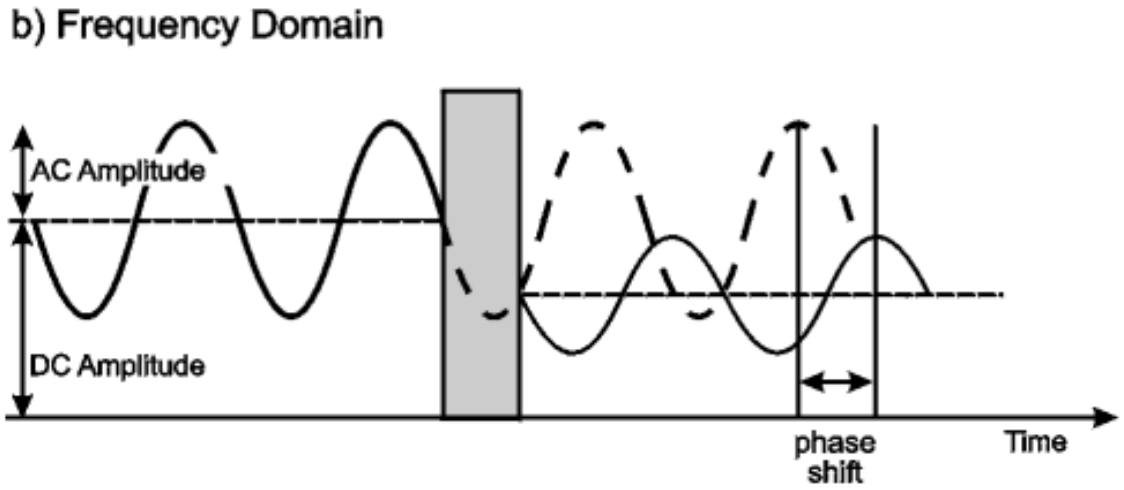
τ can be determined as well from the ratio of modulations *m* :

$$m = \frac{1}{\sqrt{1 + (\omega \tau_m)^2}}$$

Time domain vs frequency domain



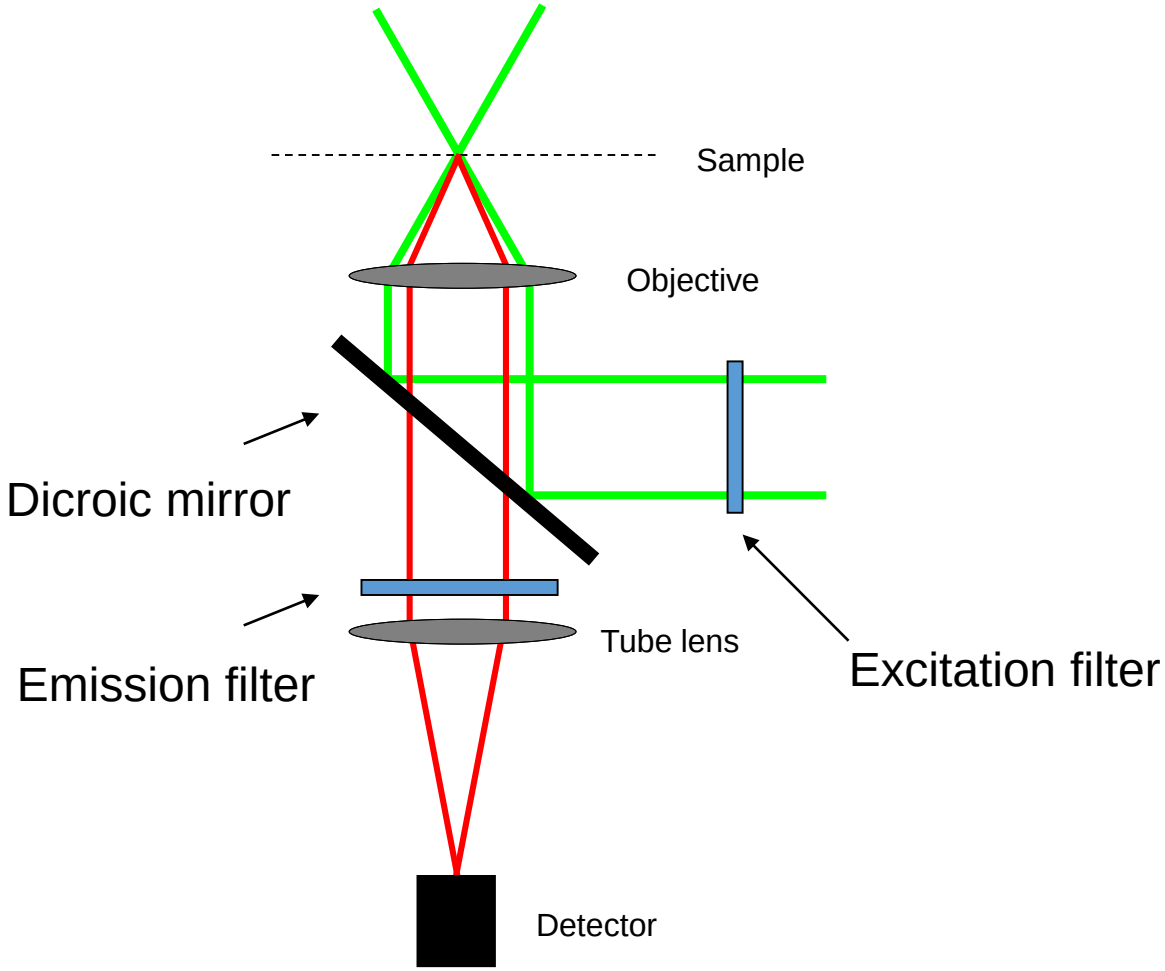
→ Useful for low light levels (single photon counting) : single molecules

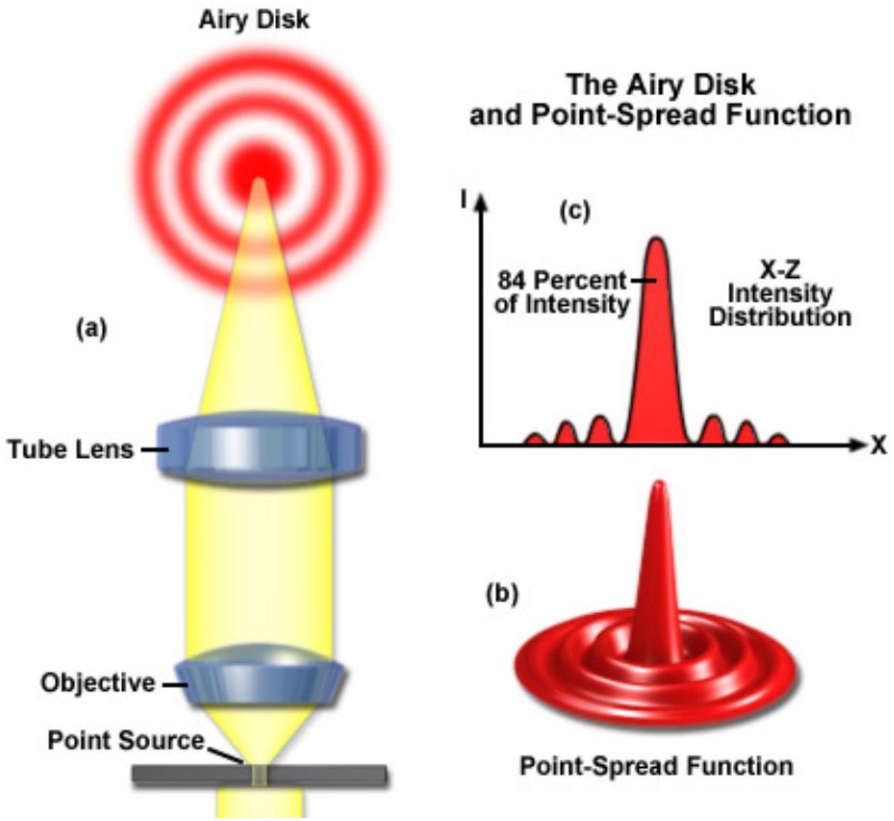


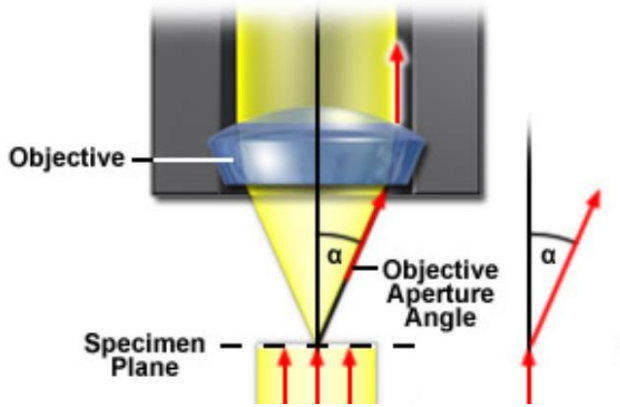
→ Very fast measurements if only one frequancy is used (average lifetime)

The fluorescence microscope

- Like in a spectrofluorimiter, it is necessary to separate and emission wavelength, ad well as optical paths







$$\text{Resolution}_{x,y} = \lambda / 2[\eta \bullet \sin(\alpha)]$$

$$\text{Resolution}_{z} = 2\lambda / [\eta \bullet \sin(\alpha)]^2$$

Where η.sin(α)=NA (Numerical aperture)

Example : λ=550nm, NA=1.4 R_{xy}=305nm, R_z=560nm

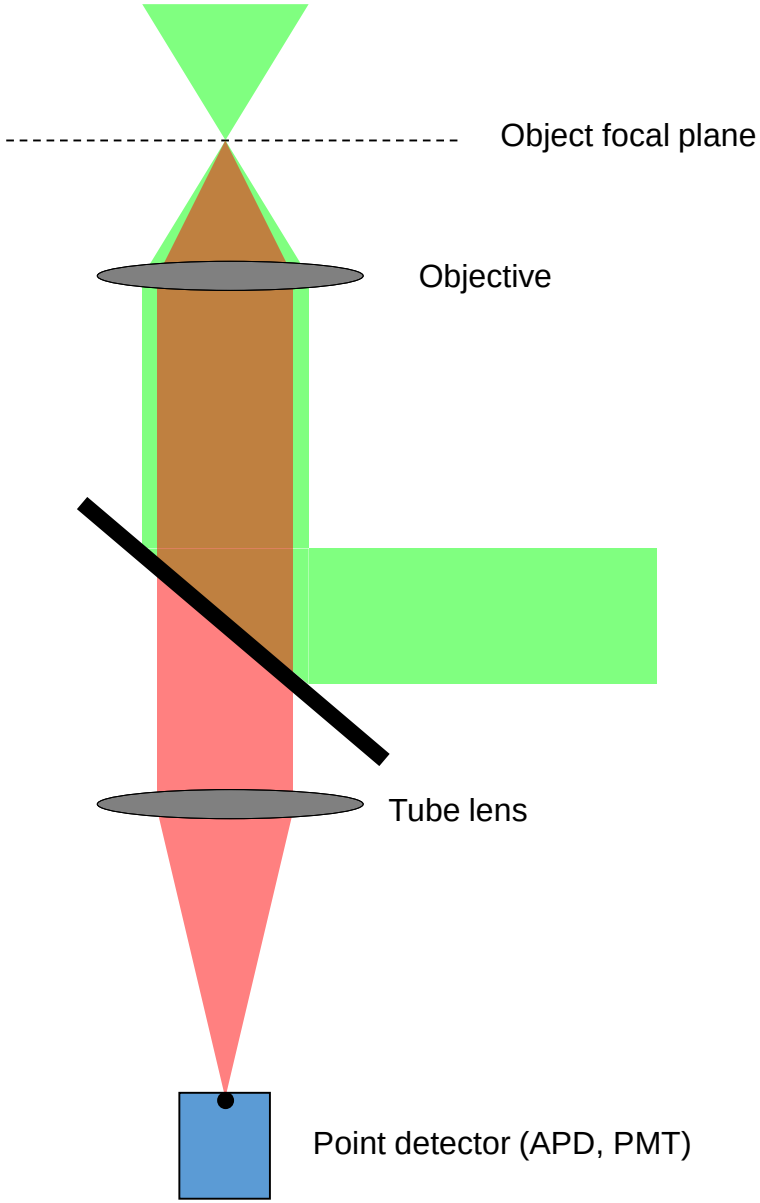
Illumination and collection modes

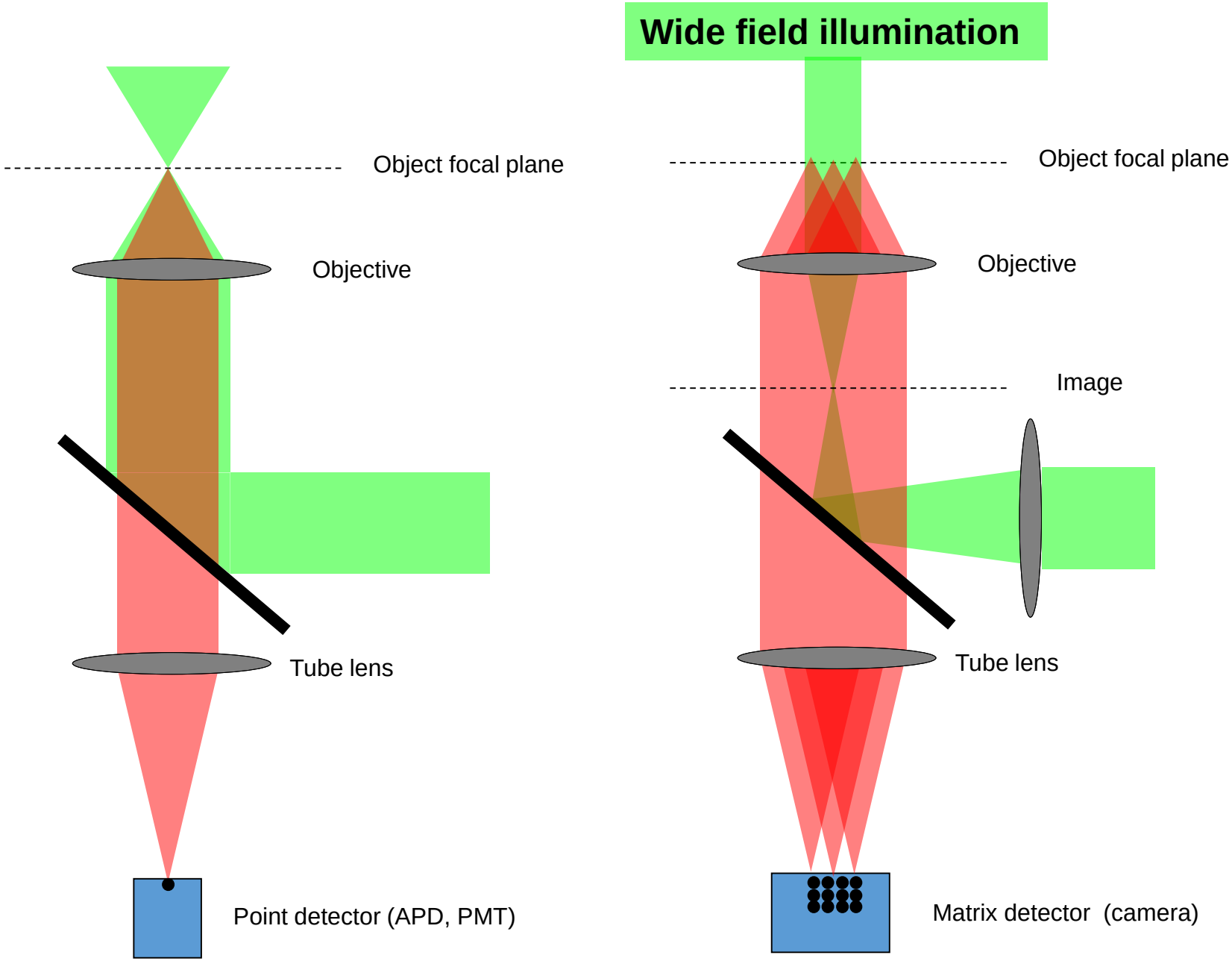
- EPISCOPIC ILLUMINATION

- CONFOCAL MICROSCOPY

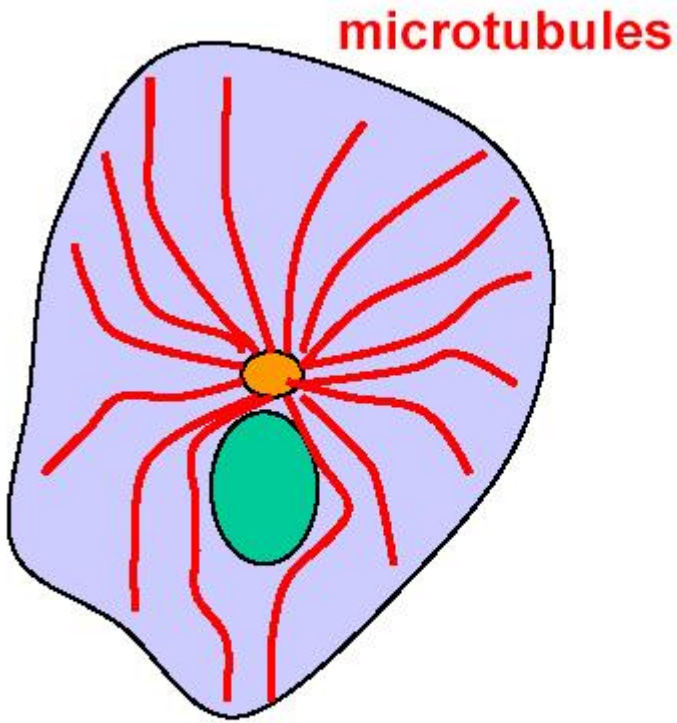
- TOTAL INTERNAL REFLEXION MICROSOPY

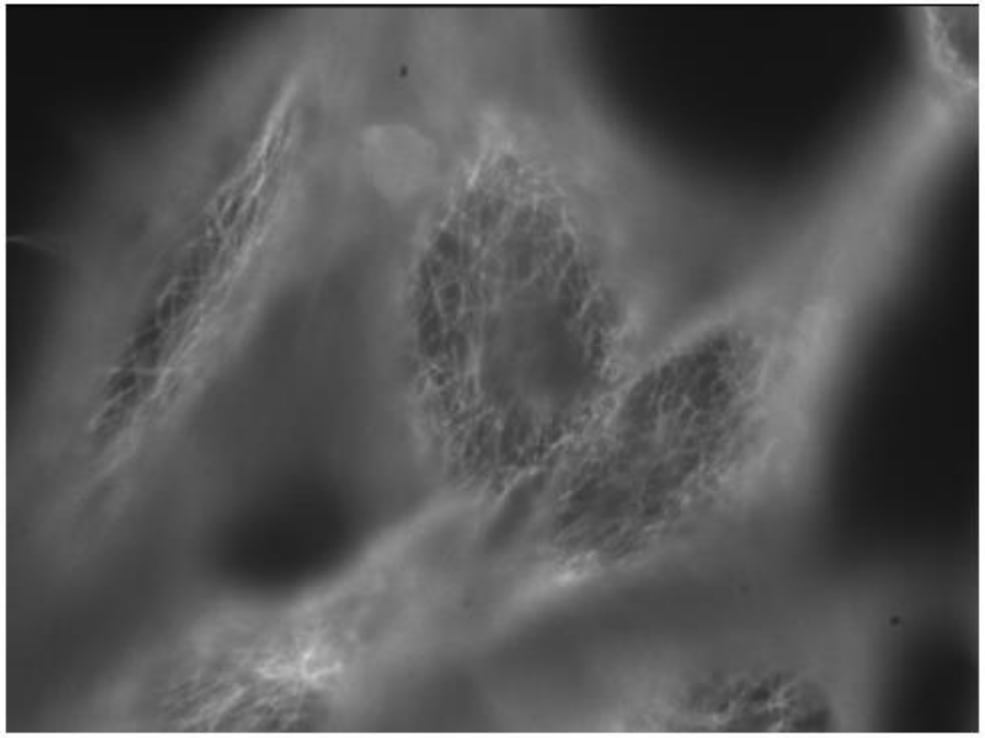
- MULTIPHOTON MICROSCOPY


Object focal plane
Objective
Tube lens
Point detector (APD, PMT)

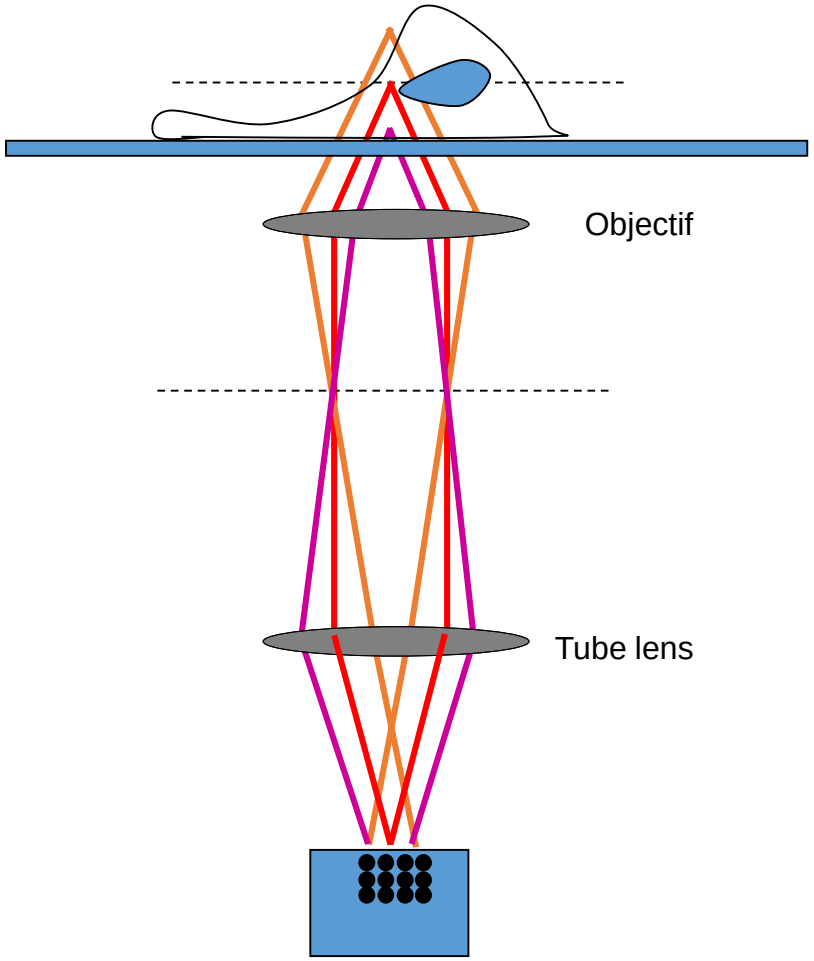

Wide field illumination
Object focal plane
Objective
Tube lens
Point detector (APD, PMT)
Object focal plane
Objective
Image
Tube lens
Matrix detector (camera)

ILLUMINATION EPISCOPIQUE





In a thick sample, light is collected from all sample planes



How to reject out-of-focus light ?

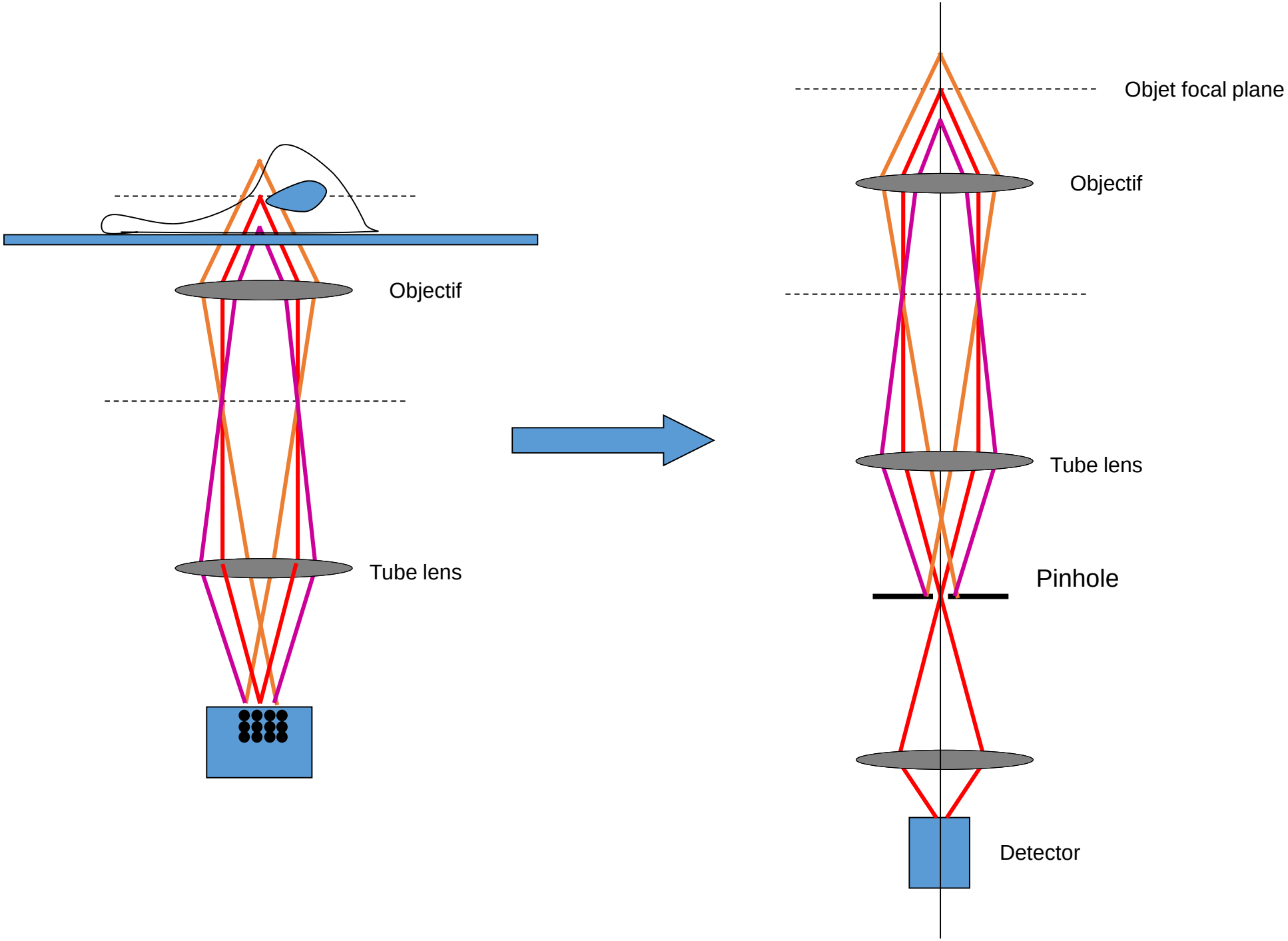
Illumination and collection modes

- EPISCOPIC ILLUMINATION

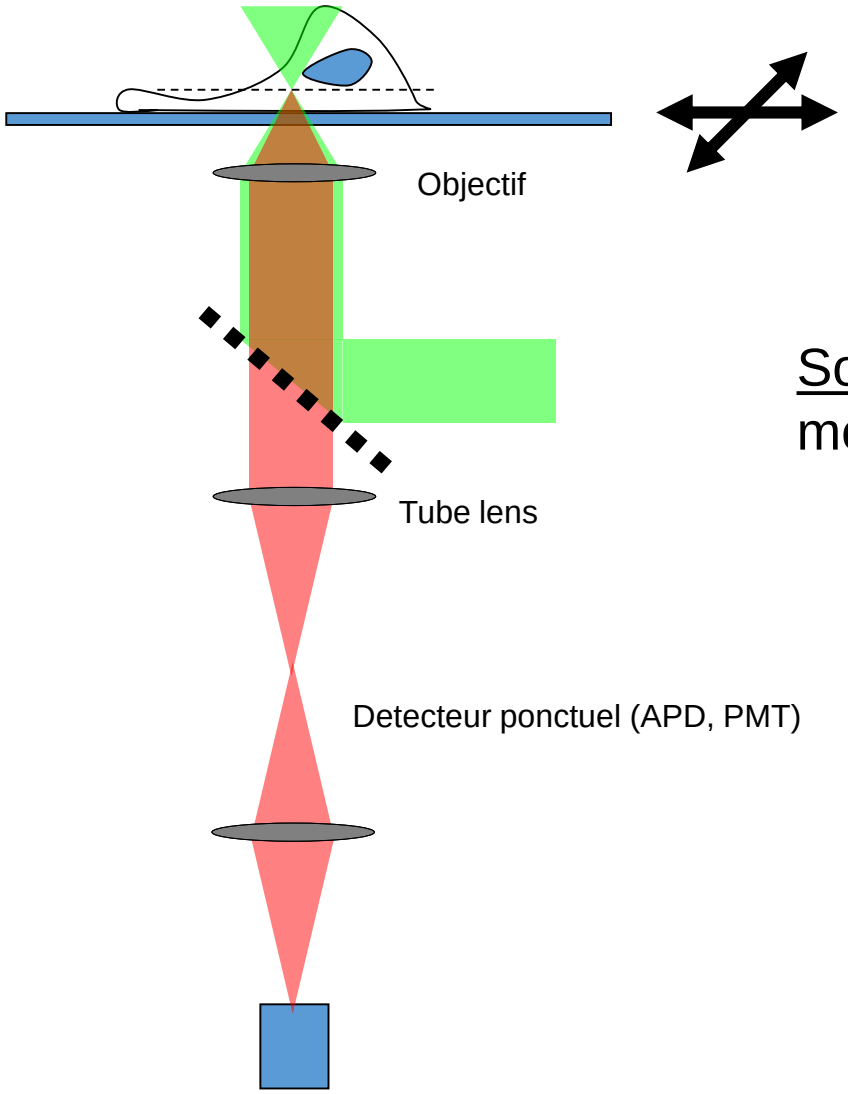
- CONFOCAL MICROSCOPY

- TOTAL INTERNAL REFLEXION MICROSOPY

- MULTIPHOTON MICROSCOPY

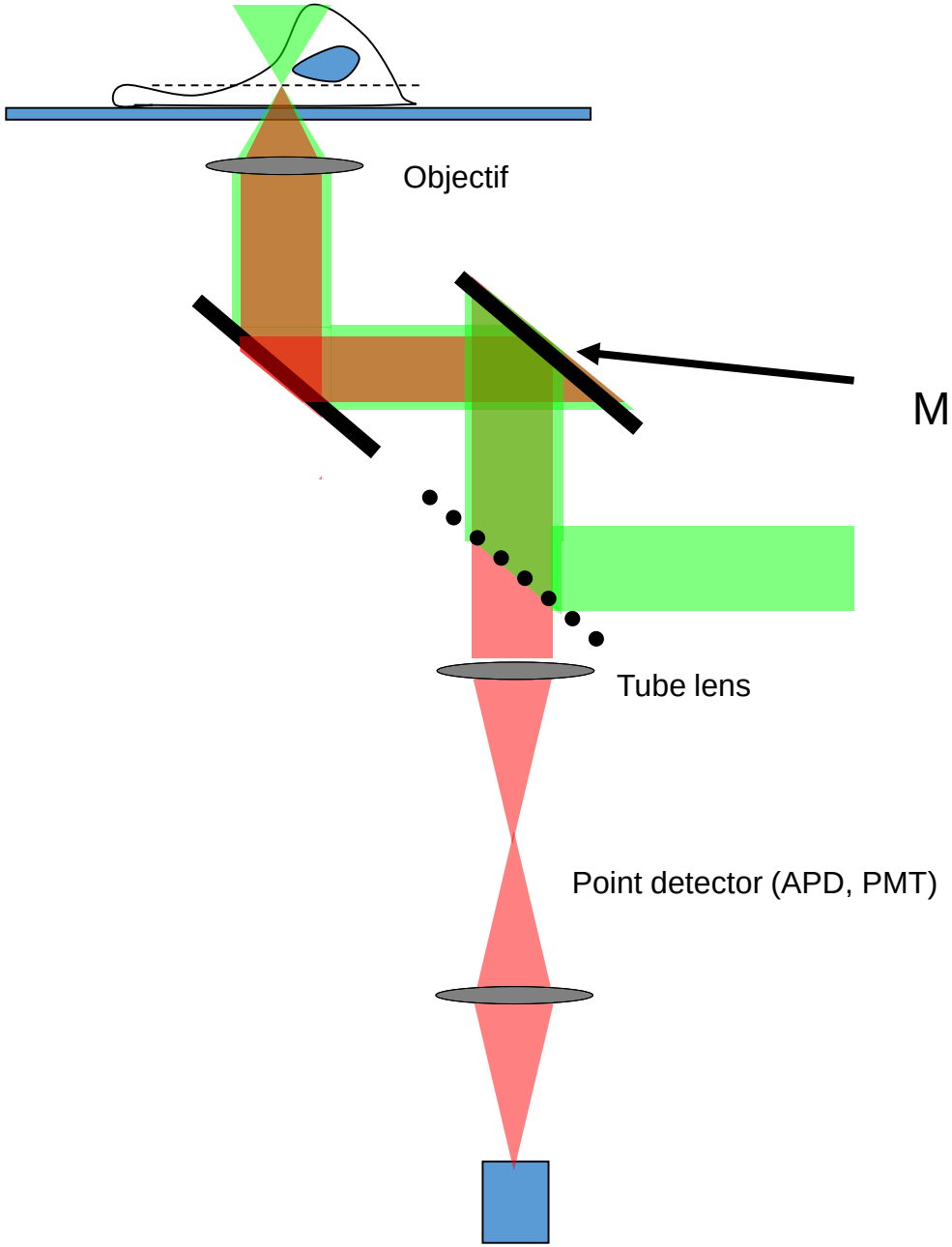

Objet focal plane
Objectif
Tube lens
Pinhole
Detector
Objectif
Tube lens

Then, to get an image, you need to do point scanning



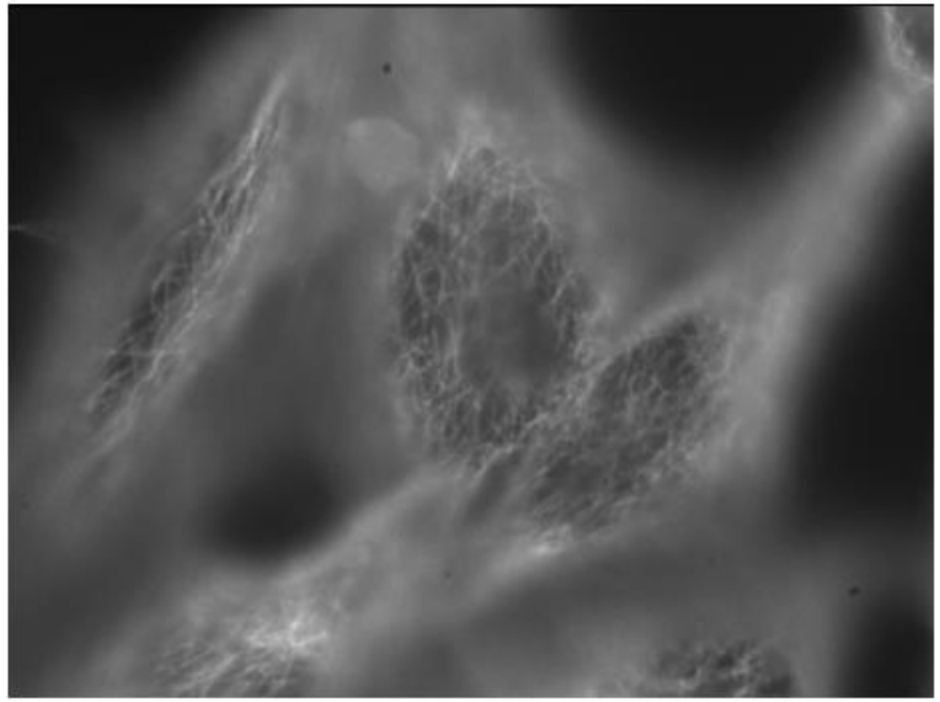
<u>Solution 1 :</u> Laser is fixed, sample is moving

Then, to get an image, you need to do point scanning



Solution 2 : Sample is fixed, laser is scanned

Epifluorescence



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