Cellular and Molecular Biophysics

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CFU 5 LM Biotecnologie Industriali- 6 LM Fisica - A.A. 2024/25

Corso di laurea in LM Biotecnologie Industriali- LM Fisica

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Life Sciences
and Systems Biology

FLUORESCENCE

I. Introduction to Fluorescence

II. Fluorochromes

III. Biological and biomedical applications

FLUORESCENCE

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BIOLOGICAL APPLICATIONS

- **TECHNIQUES IN FLUORESCENCE FOR LIVE CELL IMAGING**
  - **Studies of molecular interactions in live cells (FRET)**
  - **Studies of molecular dynamics in live cells (FRAP-FLIP-FLAP)**

BIOMEDICAL APPLICATIONS

- **DIAGNOSTIC TOOLS FOR *IN VIVO* IMAGING**
- **PHOTODYNAMIC THERAPY**

TECHNIQUES IN FLUORESCENCE FOR LIVE CELL IMAGING

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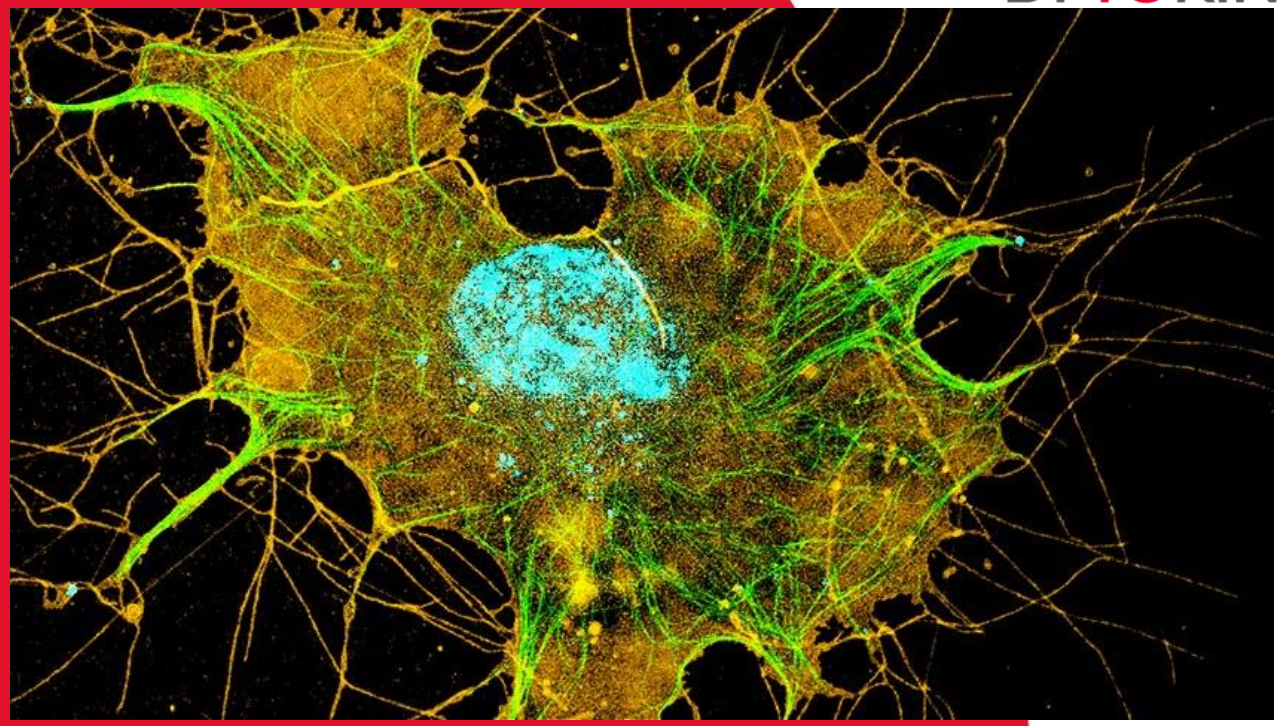


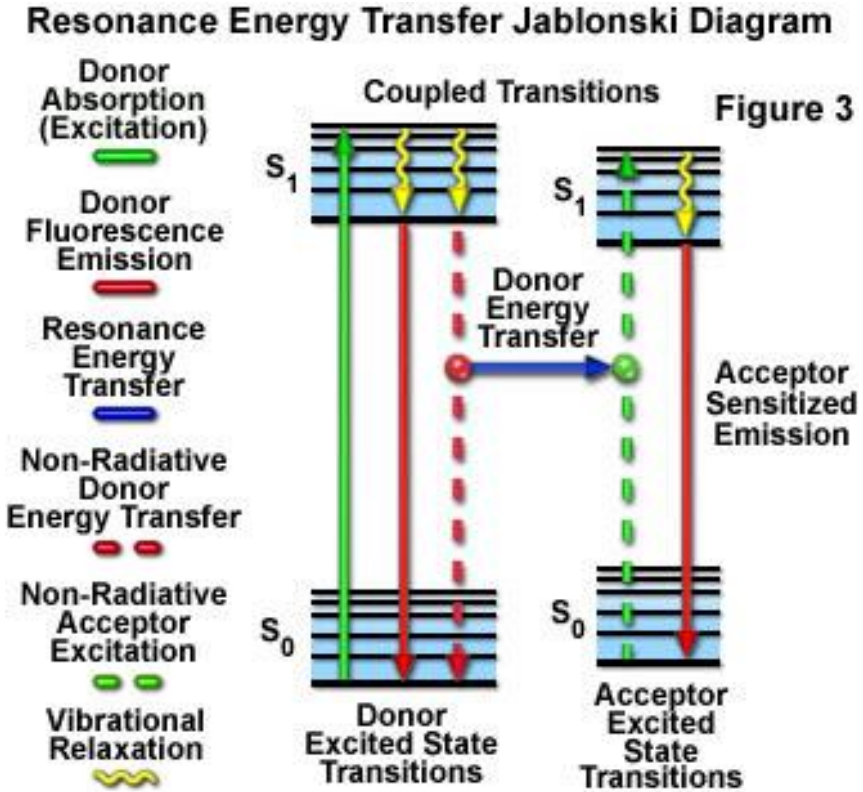
III. Biological and biomedical applications

- **Studies of molecular interactions in live cells**

FRET: FÖster Resonance Energy Transfer

FRET is a quantum-mechanical phenomenon that occurs when two fluorochromes are in molecular proximity. It describes an energy transfer mechanism between two chromophores.

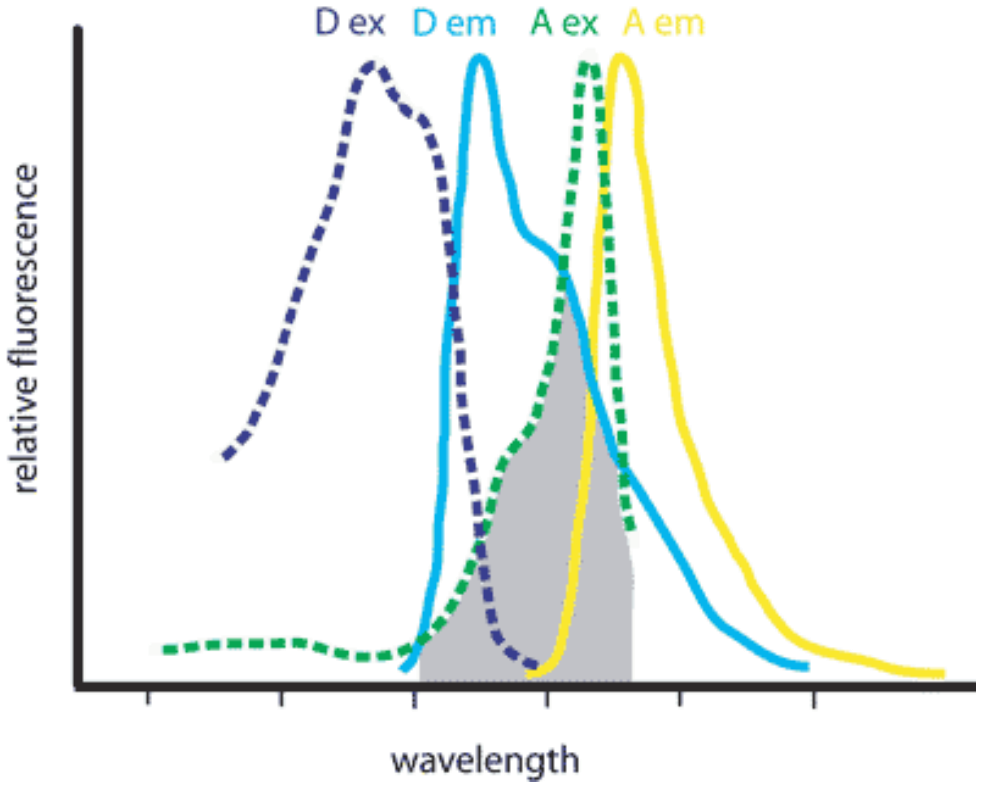
A **donor chromophore** in its **excited state** can **transfer energy** by a non radiative, long-range dipole-dipole coupling mechanism **to an acceptor chromophore in close proximity** (typically < **10 nm**). This energy transfer mechanism is termed "Föster resonance energy transfer" (FRET), named after the German scientist: Theodor Förster.

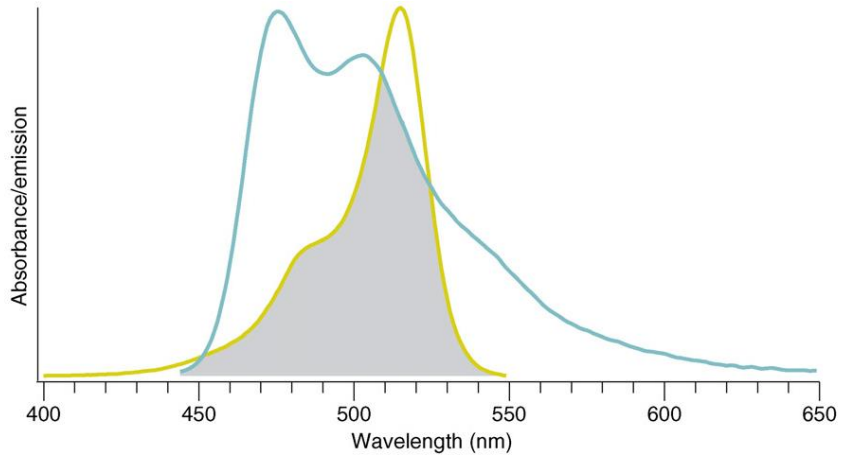


http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/Technical-Notes-and-Product-Highlights/Fluorescence-Resonance-Energy-Transfer-FRET.html

FRET: Föster Resonance Energy Transfer

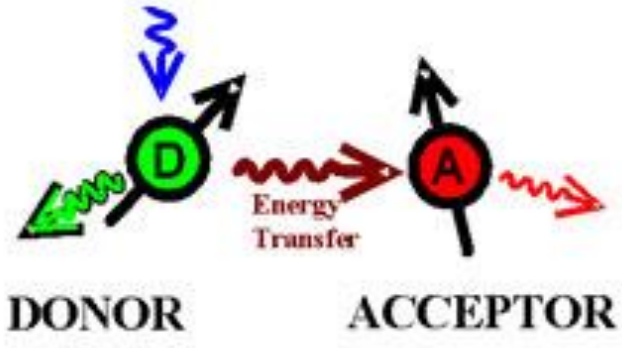
The emission spectrum of the donor fluorophore should overlap the excitation spectrum of the acceptor fluorophore.





FRET: Föster Resonance Energy Transfer

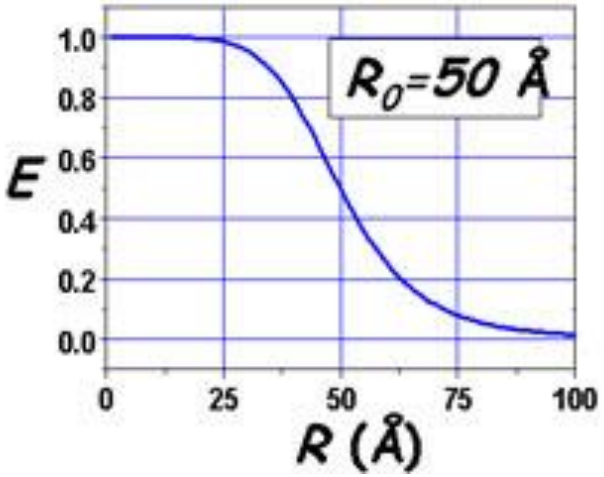
FRET is well-suited to the study of protein protein interactions, which occur on a similar spatial scale.



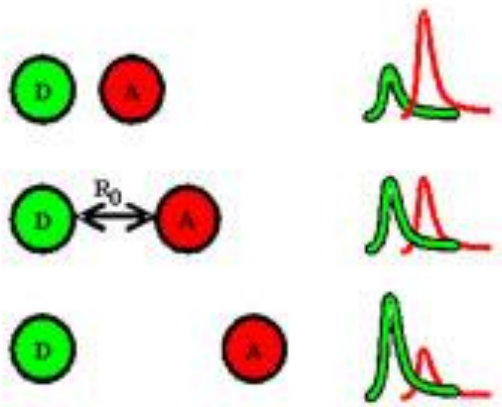
Energy Transfer Efficiency

$$E = \frac{1}{1+(R/R_0)^6}$$

R_0=50% transfer efficiency distance
3nm~7nm



"Spectroscopic Ruler"



FRET: Föster Resonance Energy Transfer

R_0 can be calculated for any pair of fluorescent molecules.
The Förster distance **depends on the overlap integral of the donor emission spectrum with the acceptor absorption spectrum and their mutual molecular orientation** as expressed by the following equation:

$$R_0^6 = 8.8 \times 10^{-28} k^2 n^{-4} Q_0 J$$

k^2 = dipole orientation factor (often assumed as 2/3 when both the dyes are freely rotating and can be considered to be isotropically oriented during the excited state lifetime)
n = refractive index of the medium
Q_0 = fluorescence quantum yield of the donor in the absence of the acceptor
J = spectral overlap integral

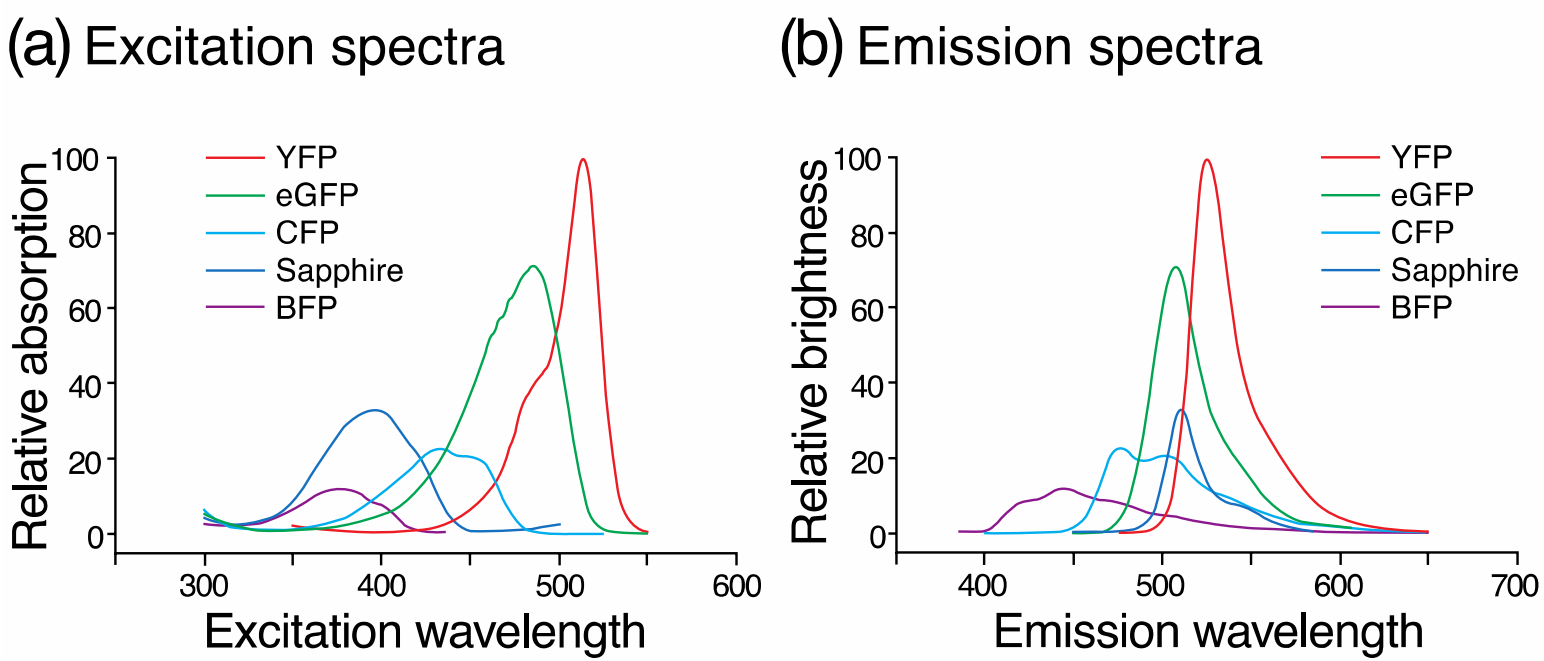
$$J = \int f_D(\lambda)\, \epsilon_A(\lambda)\, \lambda^4\, d\lambda$$

f_D = normalized donor emission spectrum
ε_A = acceptor molar extinction coefficient

Characteristics to take in account when choosing GFPs for FRET

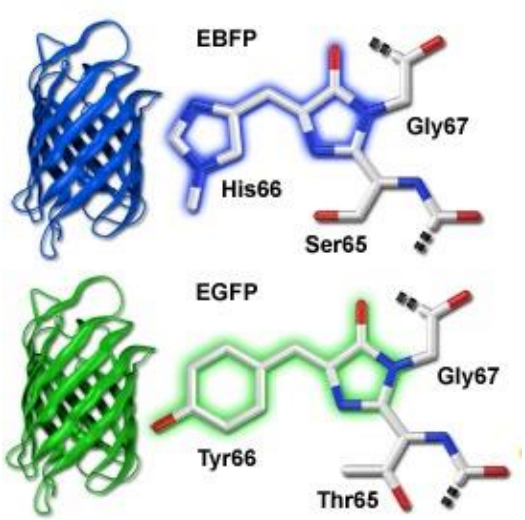
1) **Excitation spectra** of the two dyes have to be **sufficient separated** in order to stimulate the donor selectively
2) **The emission spectrum of the donor fluorophore should overlap the excitation spectrum of the acceptor** fluorophore in order to obtain efficient energy transfer
3) **Reasonable separation** in **emission spectra** between donor and acceptor GFPs is required to allow the fluorescence of each chromophore to be measured independently

Characteristics to take in account when choosing GFPs for FRET



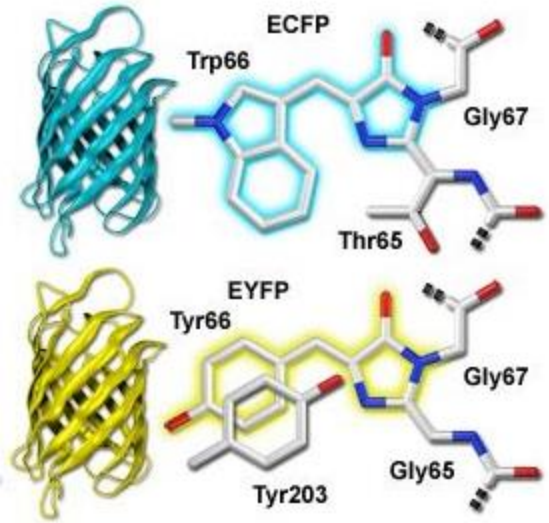
Characteristics to take in account when choosing GFPs for FRET

EBFP-EGFP



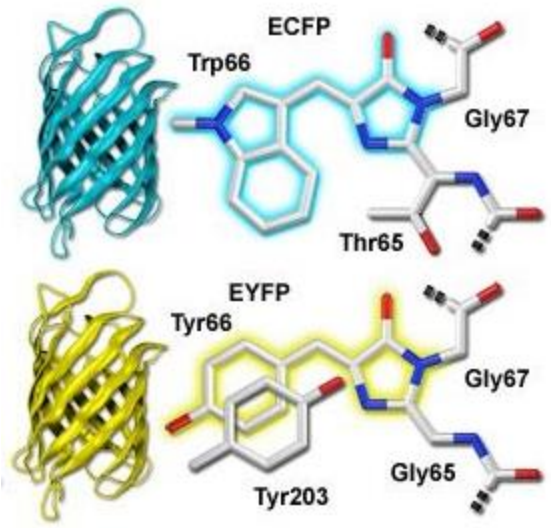
Poor photophysical properties of EBFP is a serious limitation to the use of this couple for FRET

ECFP-EYFP

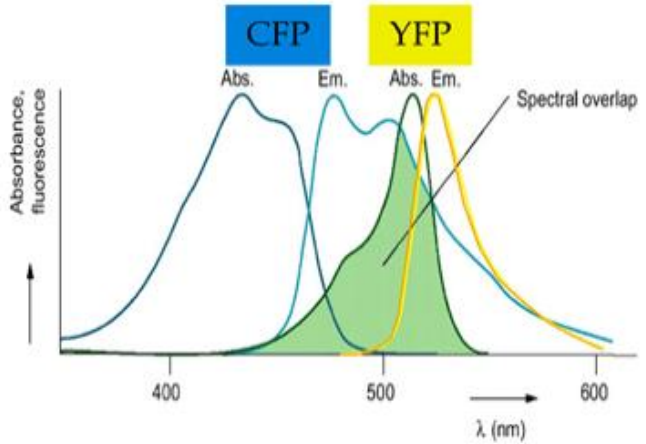


CFP-YFP is the best couple for FRET

Characteristics to take in account when choosing GFPs for FRET



CFP-YFP



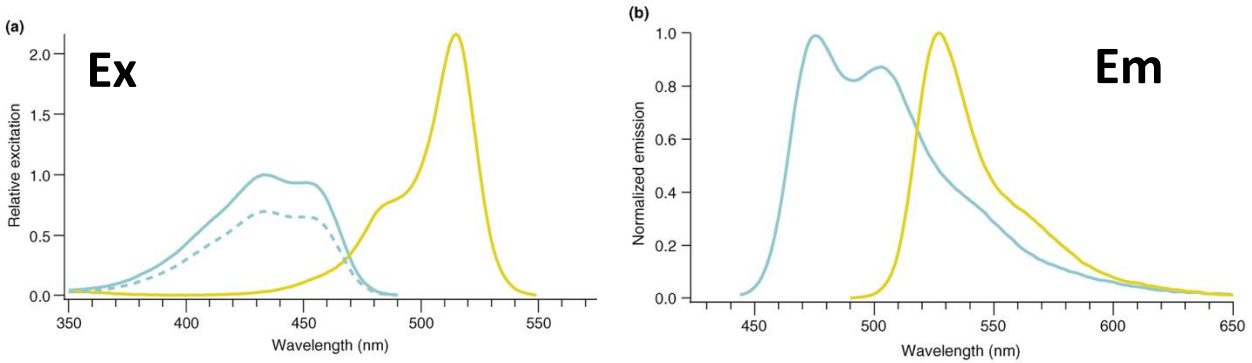
…although the significant cross-talk between both the excitation and emission spectra

λ_{ex} CFP = 405 nm

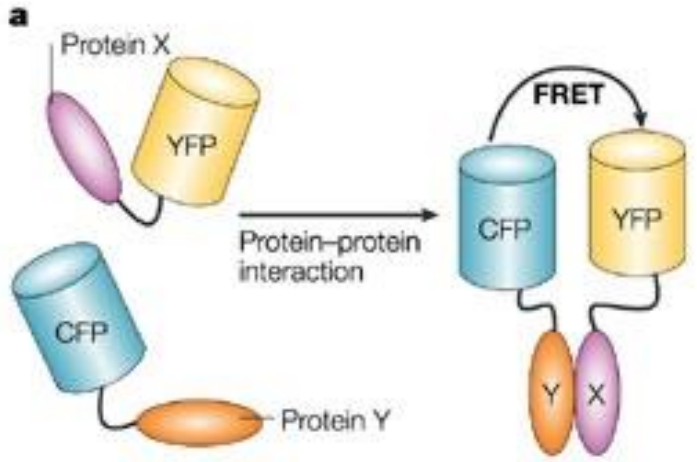
λ_{em} CFP = 485 nm

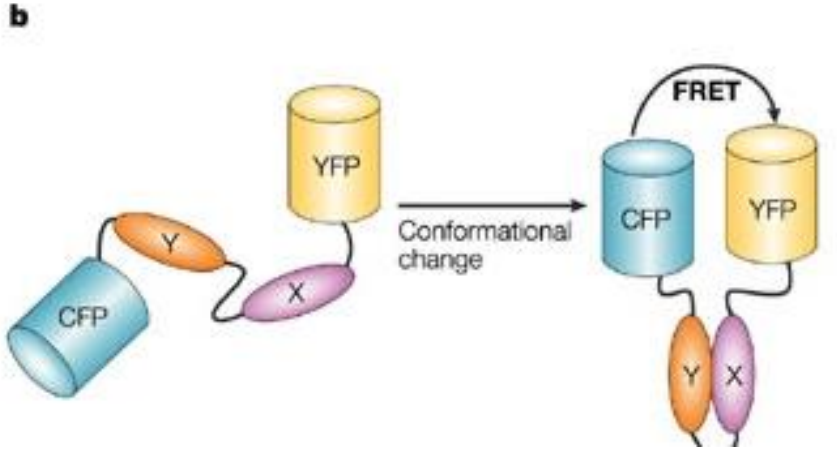
λ_{ex} YFP = 514 nm

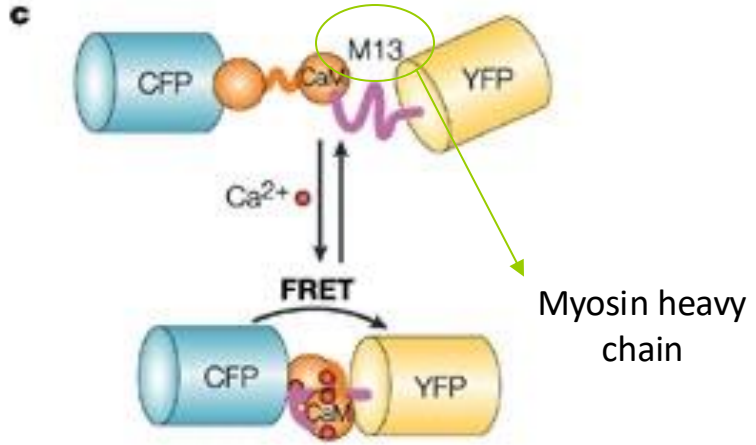
λ_{em} YFP = 527 nm



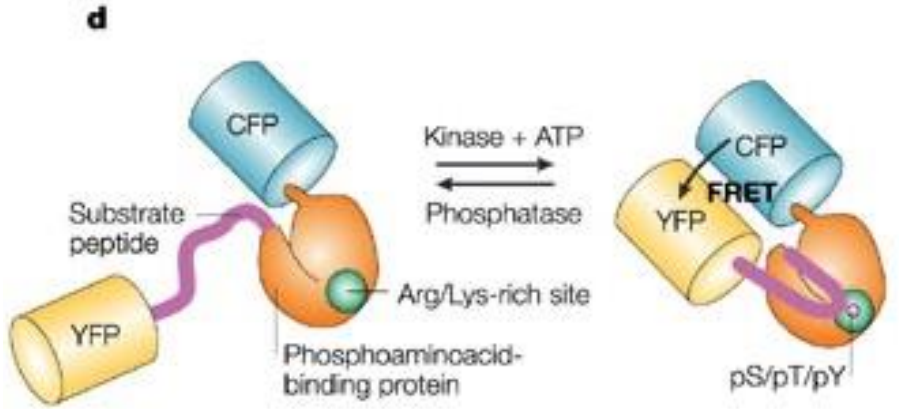
General design of FRET-based fluorescent probes







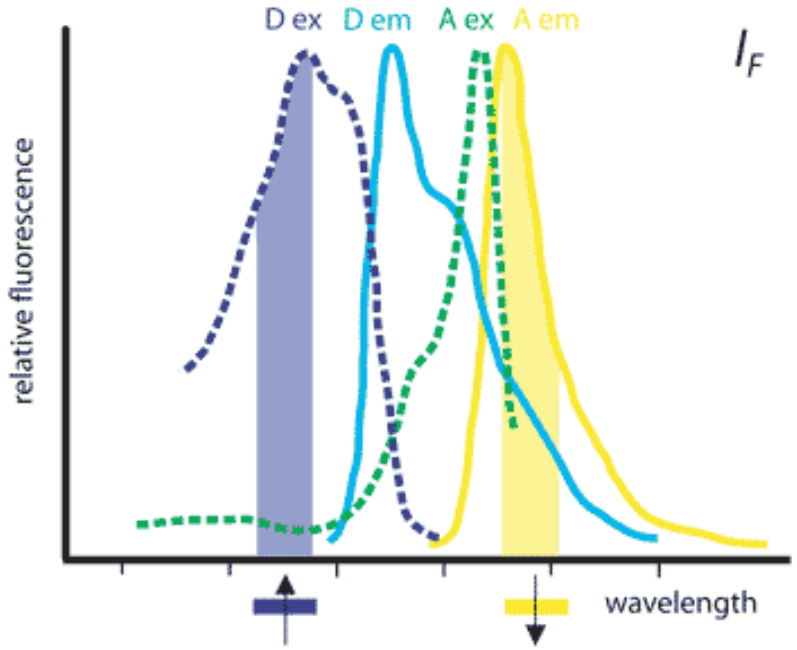
CHAMALEON

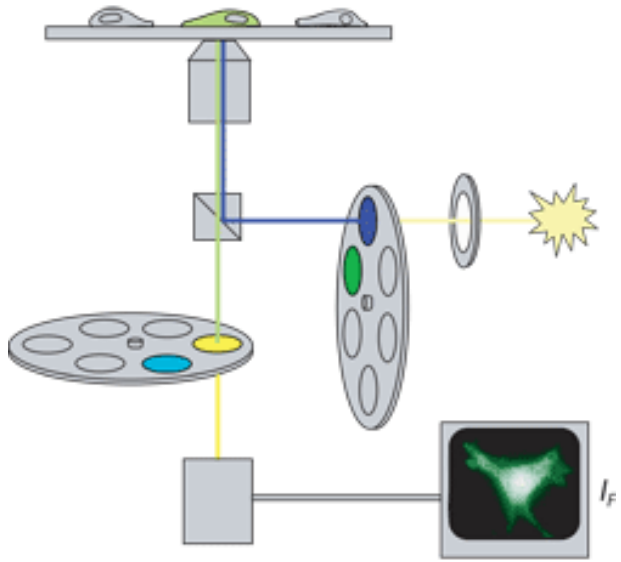


KAR

Methods for imaging FRET

1. SENSITIZED EMISSION

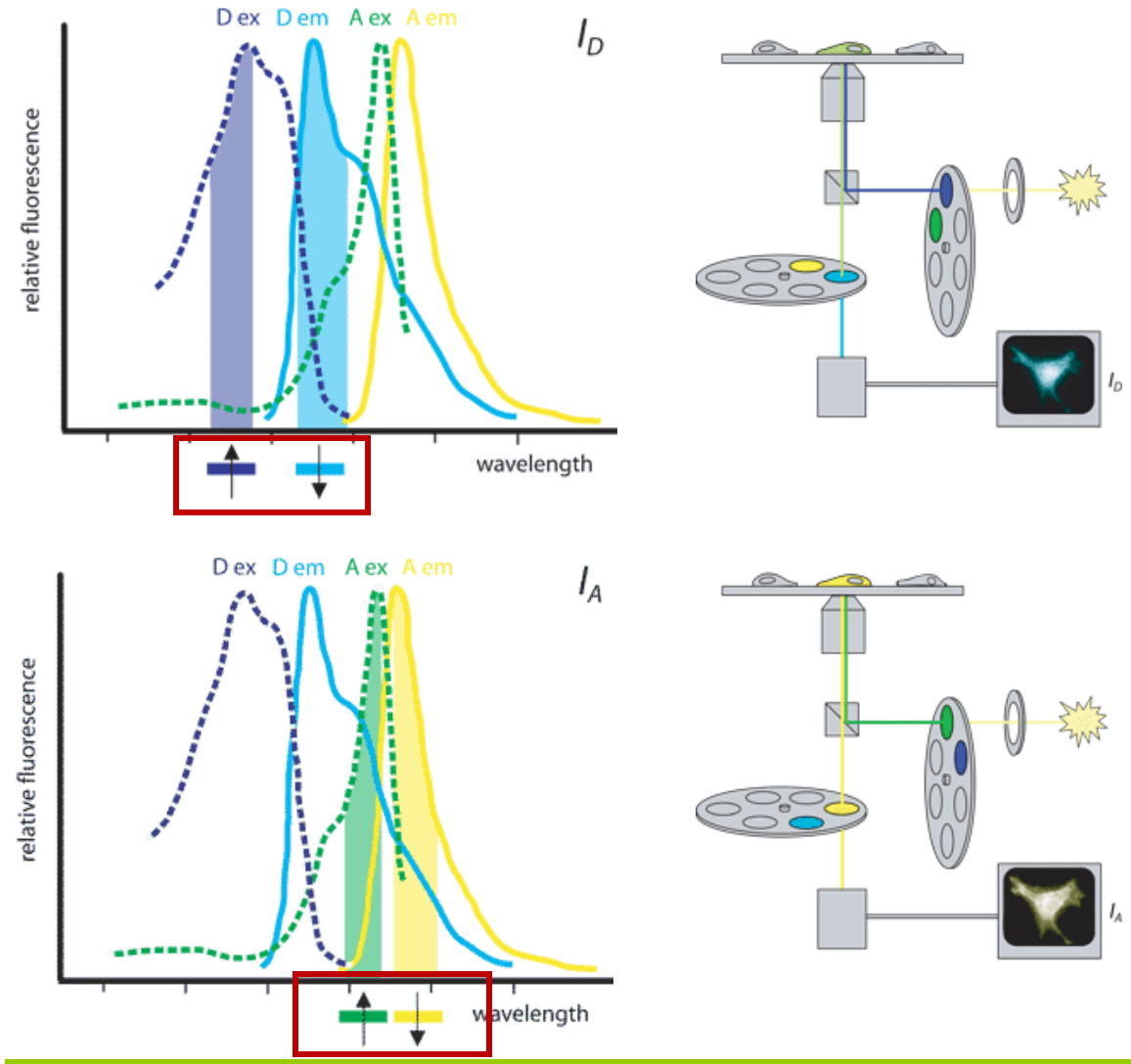




Donor is excited by a specific wavelength and the signal is collected by using emission filters chosen for the donor and the acceptor fluorescence

Methods for imaging FRET

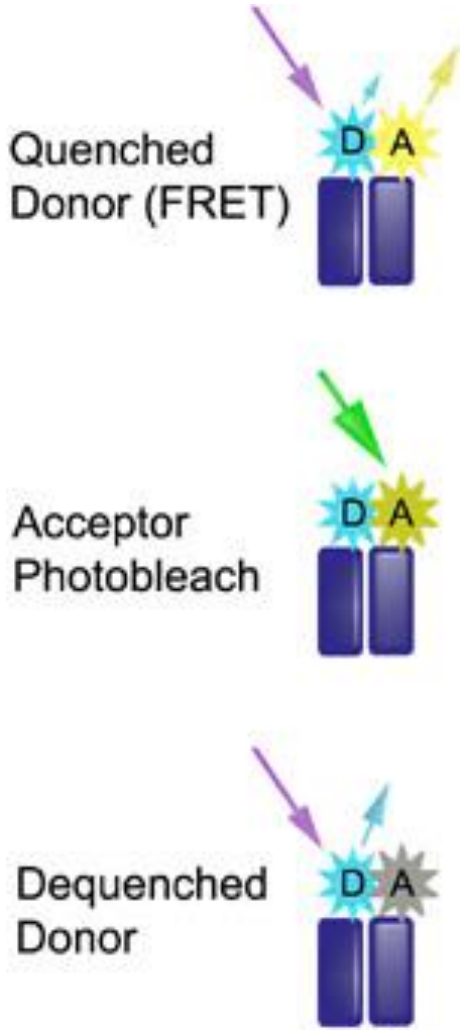
1. SENSITIZED EMISSION



Appropriate **<u>controls</u> to correct for the cross-talk between excitation and emission** of the fluorophores

Methods for imaging FRET

2. ACCEPTOR PHOTOBLEACHING



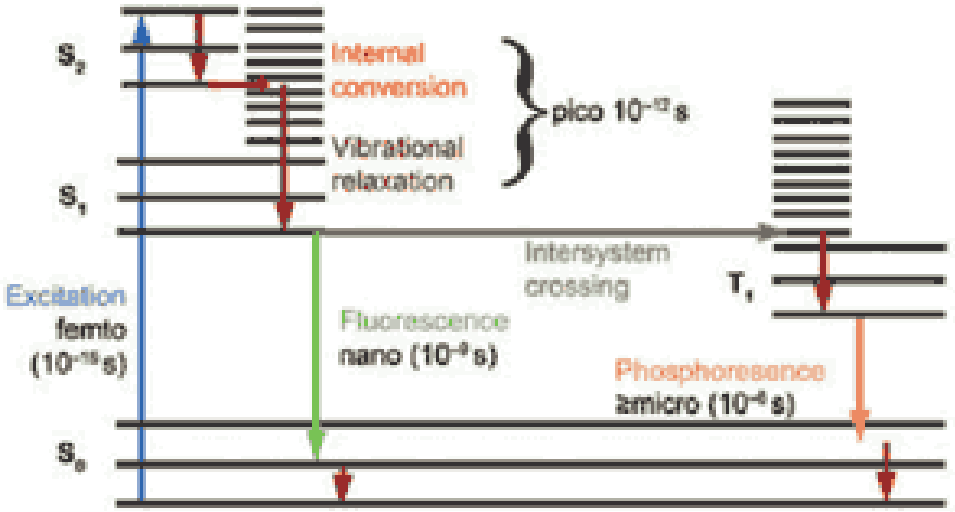
The donor fluorescence is quenched owing to FRET because some of the donor fluorescence energy is used to make acceptor fluorescence.

Photobleaching the acceptor fluorophore releases this quenching and **increases the donor fluorescence**

For these experiments it is important to ensure that the acceptor photobleaching does not degrade the donor

Methods for imaging FRET

3. FLIM (Fluorescence Lifetime Imaging Microscopy)

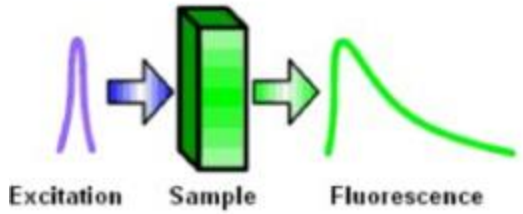


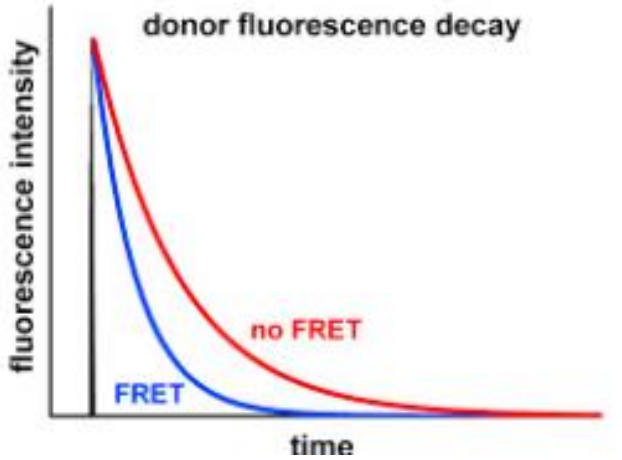
Fluorescence lifetime:
time it takes an electron in an excited energy level of the dye molecule to return to its ground level while emitting fluorescence light

It depends on molecule environment: **if there are alternative routes to fluorescence to depopulate the excited energy state, the measured lifetime is reduced**.

Methods for imaging FRET

3. FLIM (Fluorescence Lifetime Imaging Microscopy)





The donor fluorescence is quenched by FRET and the amount of quenching can be determined by measuring the shortening of fluorescence decay of the donor in the presence of FRET.

Advantages: less prone to crass-talk artifact because it looks only at the donor fluorescence. FLIM is not so sensitive to direct acceptor excitation artifacts.

Disadvantages: expensive instrumentation; low temporal resolution (potentially several min/image)

- **Studies of molecular dynamics in live cells**

✓ **FRAP**: Fluorescence Recovery After Photobleaching

✓ **FLAP**: Fluorescence Localization After Photobleaching

✓ **FLIP**: Fluorescence Loss of Intensity after Photobleaching

Photobleaching

Photobleaching is the photochemical destruction of a fluorophore.

Photobleaching occurs when a fluorophore permanently loses its ability to fluoresce due to photon-induced chemical damage and covalent modification.

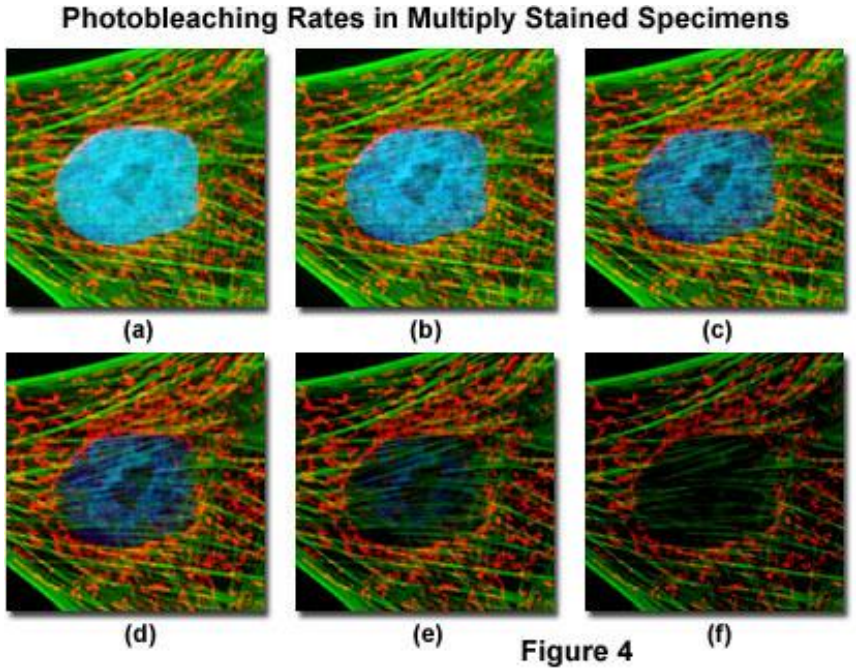
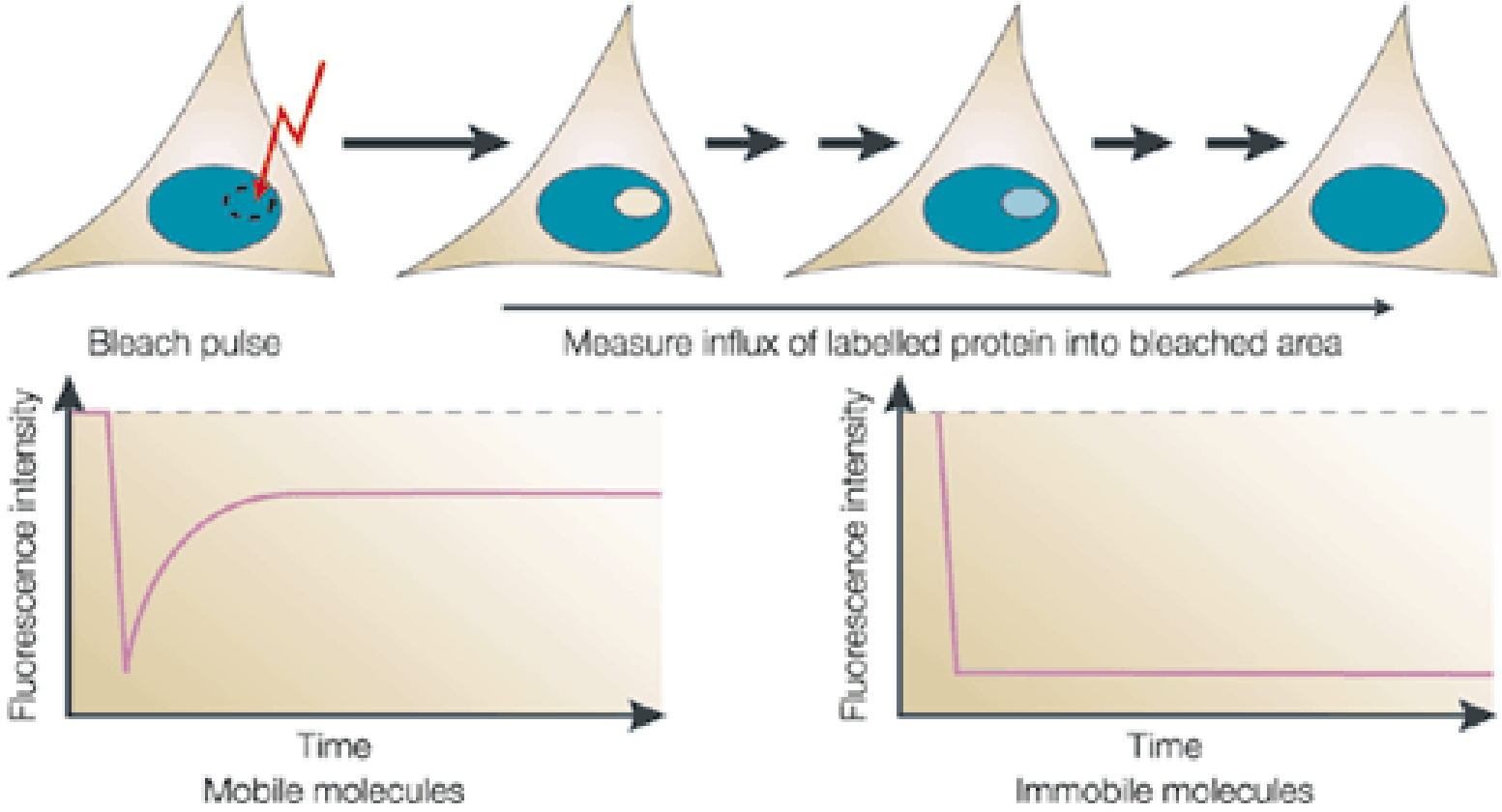


Figure 4

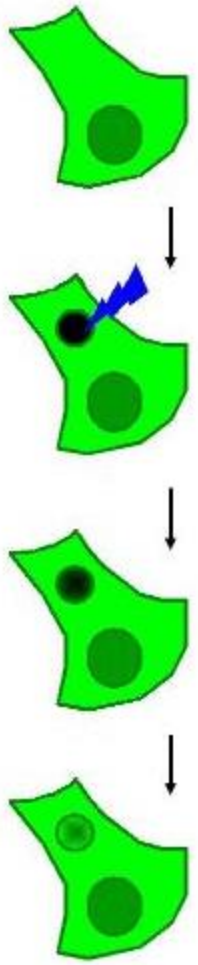
FRAP
Fluorescence Recovery After Photobleaching

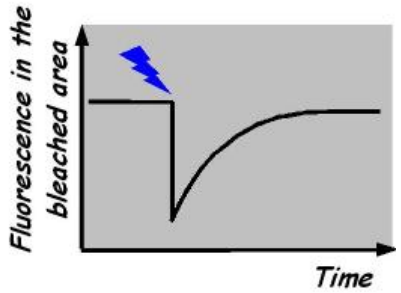


FRAP is used to measure the dynamics of 2D or 3D molecular mobility e.g. diffusion, transport or any other kind of movement of fluorescently labeled molecules in living cells.

Execution of FRAP experiment

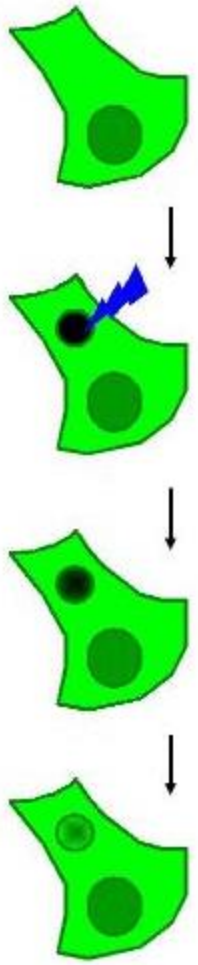
1. Label the protein of interest with a fluorescent probe (e.g. GFP)
2. Choose a ROI and save some background images of the sample before photobleaching
3. Apply short high intensity local bleach: the fluorophores in this region receive high intensity illumination which causes their fluorescence lifetime to quickly elapse. Now the image in the microscope is that of a uniformly fluorescent field with a noticeable dark spot

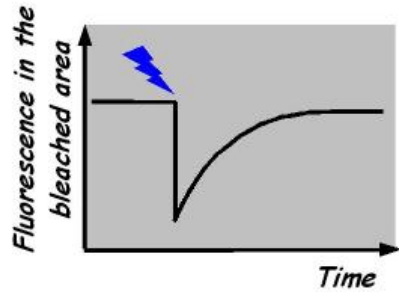




Execution of FRAP experiment

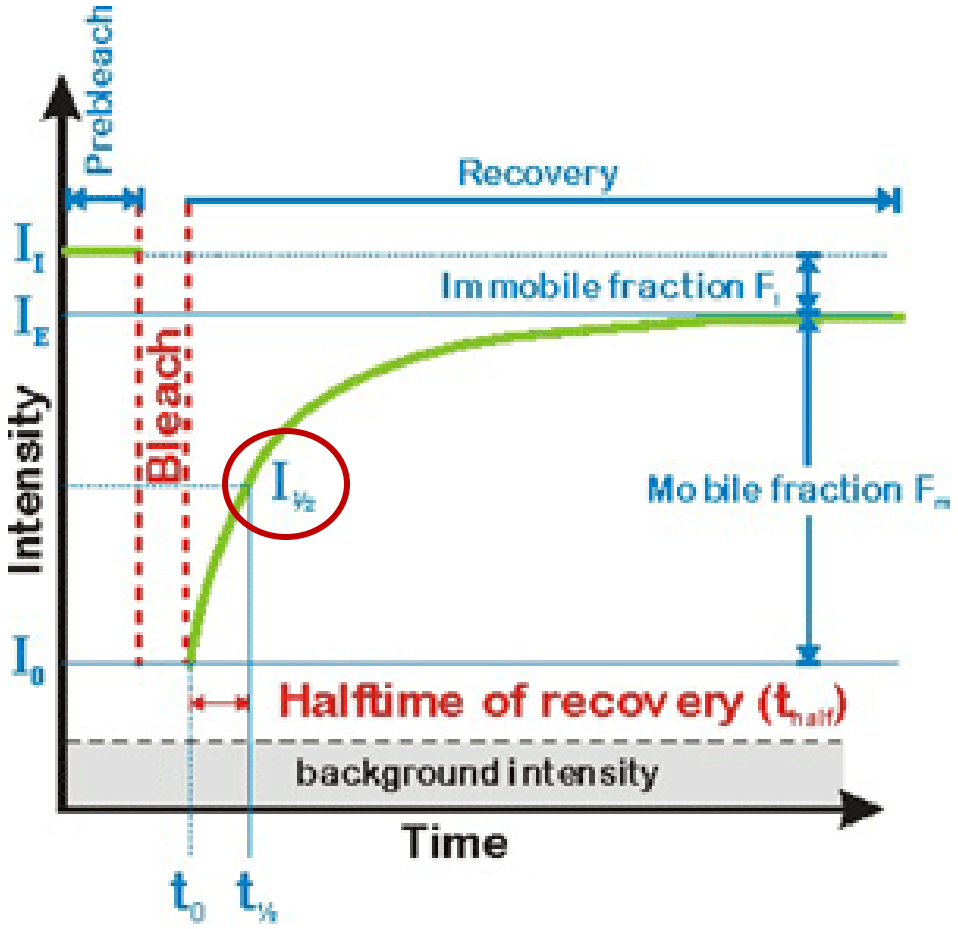
4. Take images after bleaching until the recovery in the bleach area reach a plateau. As Brownian motion proceeds, the still-fluorescing probes will diffuse throughout the sample and replace the non-fluorescent probes in the bleached region.





The mean intensity in the ROI is plotted versus time, where the level of fully recovered intensity gives information on mobile/immobile species of the fluorescent molecule.

Mobile Fraction: fraction of fluorescent protein that can diffuse into the bleached area during the time course of the experiment $F_m = I_E / I_I$

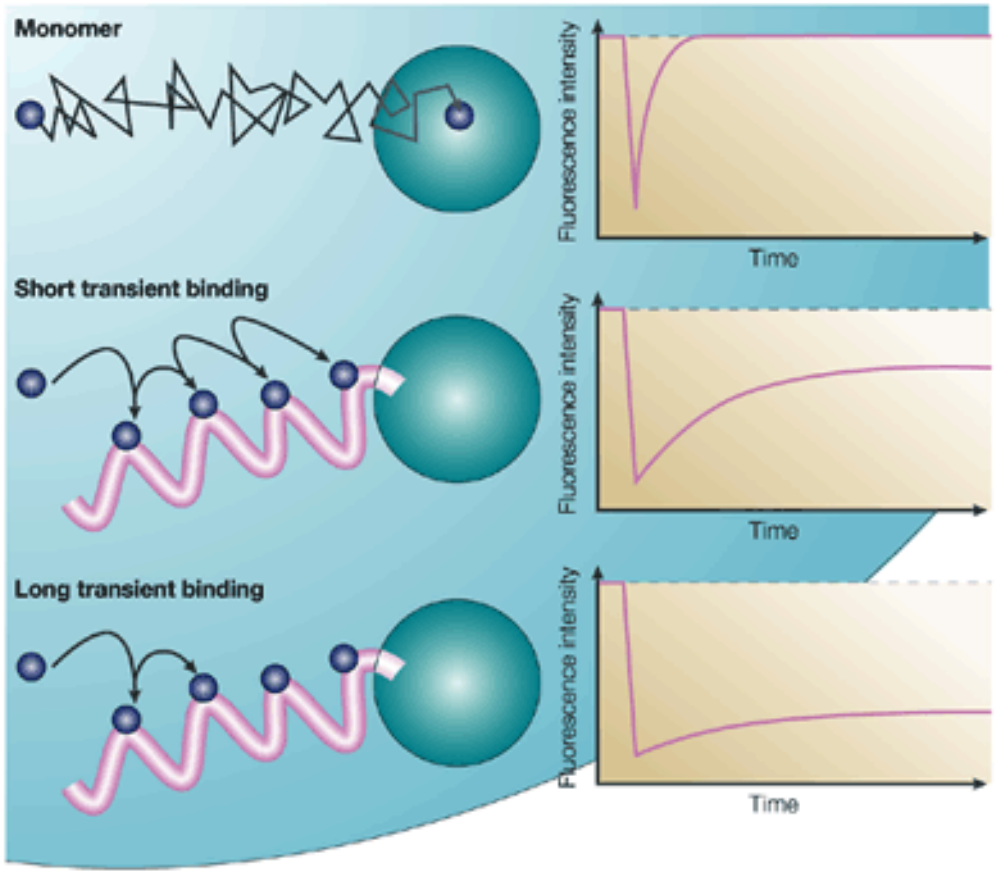


The recovery time (half-time) indicates the speed of this mobility, e.g. diffusion time;
This diffusion proceeds in an ordered fashion, analytically determinable from the diffusion equation. Assuming a gaussian profile for the bleaching beam, the diffusion constant D can be simply calculated from:

$$D = \frac{w^2}{4t_{1/2}}$$

w = width of the beam
$t_{1/2}$ = time required for the bleach spot to recover half of its initial intensity

The fluorescence intensity versus time plot can give us information about diffusion properties of the molecule of interest.



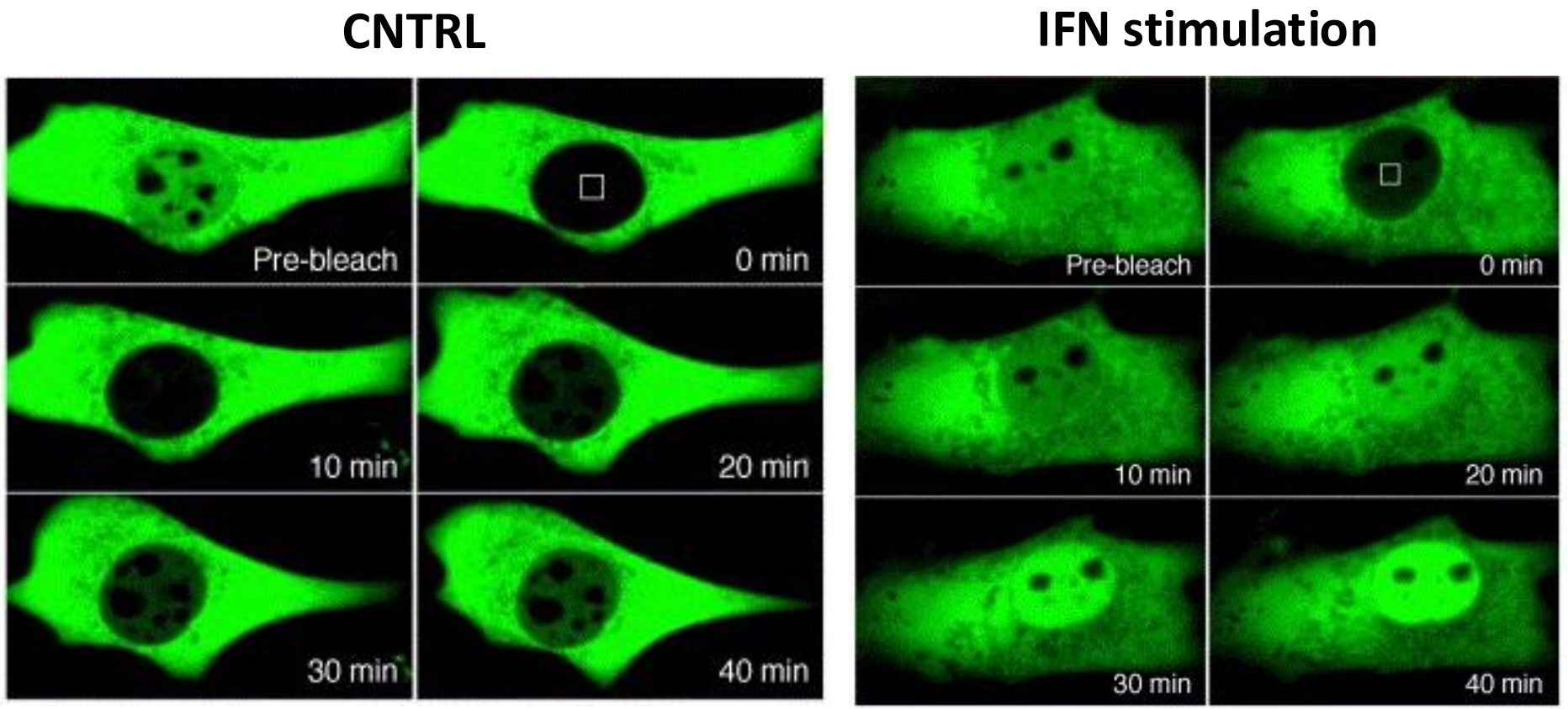
protein binding versus free diffusion *in vivo*.

As all proteins readily interact with many partners *in vivo*, it is virtually impossible to measure pure diffusional mobility of a protein in a living cell.

The fact that the interaction properties of a protein are reflected in the mobility measurement complicates the analysis of photobleaching data.

Kinetic modelling allows us to extract information about the binding properties from mobility measurements.

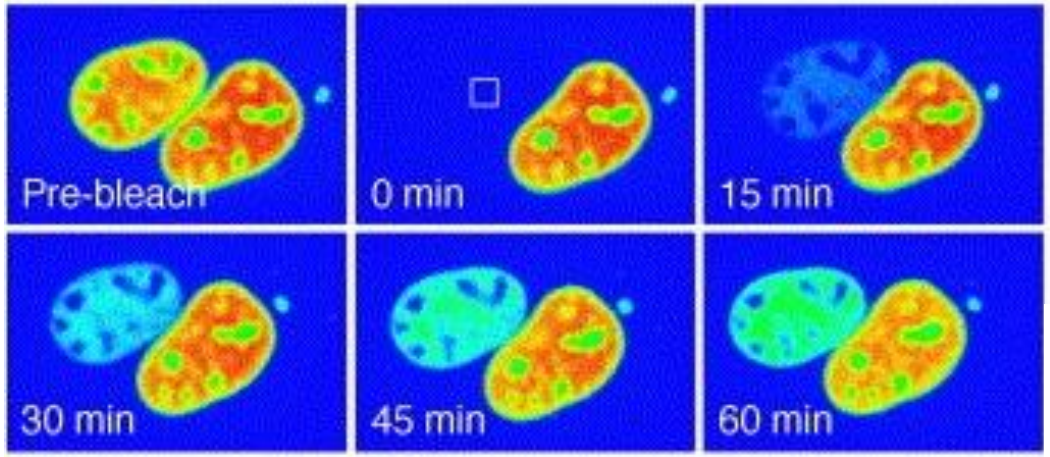
Application of FRAP to nucleocytoplasmic shuttling



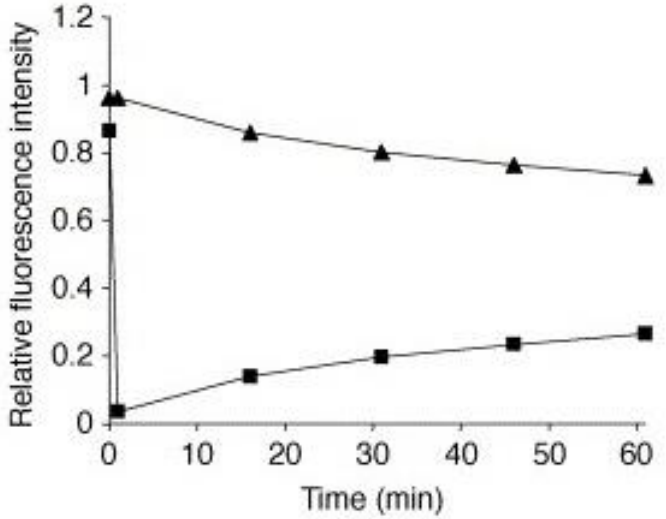
Koster M. *et al.*, Curr Opinion in Biotech, 2005, 16: 28-34

Qualitative FRAP analysis to determine the kinetics of constitutive and stimulated nucleocytoplasmic transport of STAT1–EGFP in NIH3T3 cells

Application of FRAP to nucleocytoplasmic shuttling

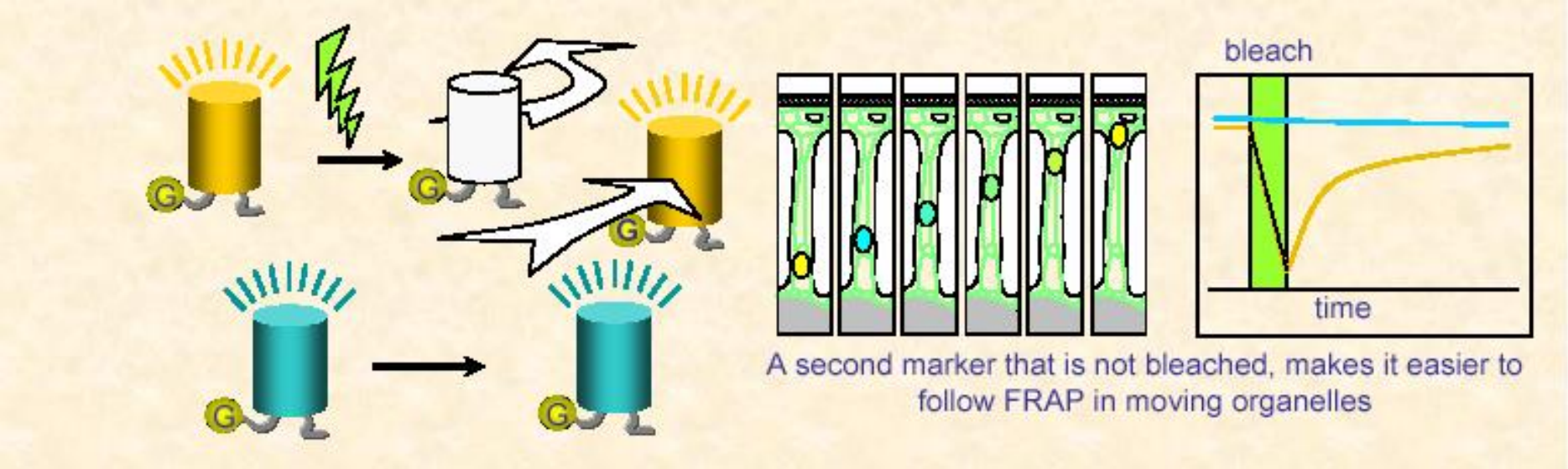


After bleaching of one nucleus the recovery of STAT1–EGFP during ongoing signaling was monitored for 60 min. Fluorescence intensities of the entire unbleached and bleached nucleus were measured and corrected for background intensity.



Koster M. *et al.*, Curr Opinion in Biotech, 2005, 16: 28-34

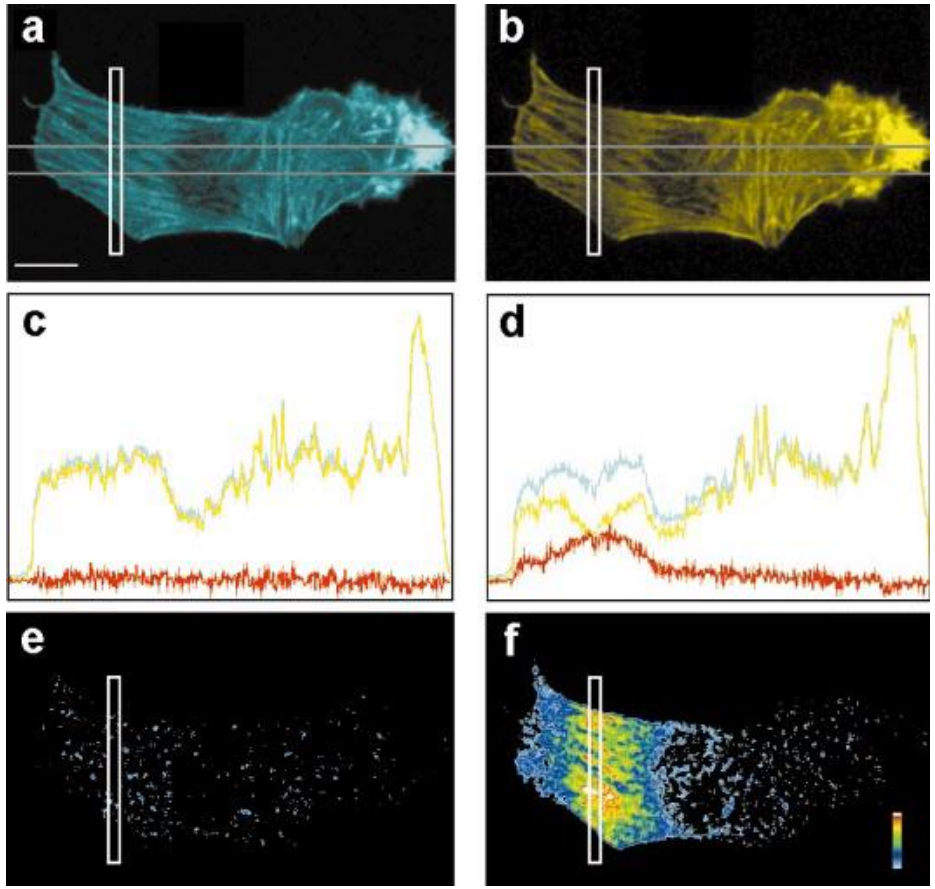
FLAP
Fluorescence Localization After Photobleaching



The molecular species to be located carries two different fluorophores that can be imaged independently but simultaneously by fluorescence microscopy. One of the fluorophores YFP (the **target fluorophore**) is then rapidly photobleached at a chosen location. The unbleached CFP (**reference**) fluorophore remains colocalized with the target fluorophore; thus, the subsequent fate of the photobleached molecules can be revealed by processing simultaneously acquired digital images of the two fluorophores.

Application of FLAP to actin replacement dynamics

CFP-actin YFP-actin

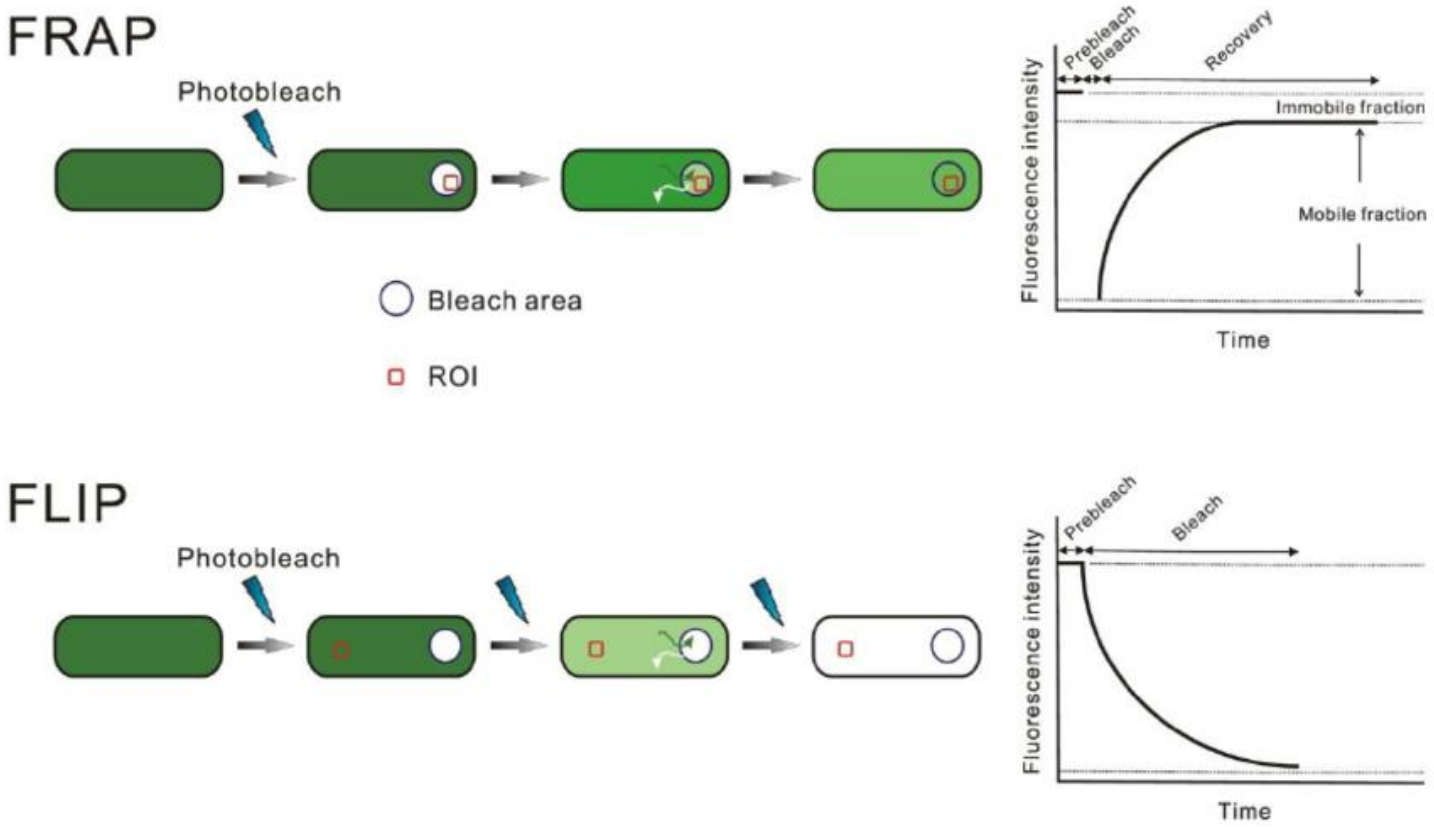


Dunn *et al.*, J Microscopy. 2002; 205:109-112.

Confocal microscopy revealed that **the two fluorophores are accurately co-localized within the cells** and thus that the intensity difference (**the FLAP signal**) **is close to zero** throughout the image

Photobleaching YFP give rise to a strong FLAP signal.
Immediately after bleaching a diffuse low-level signal had uniformly filled the local cytoplasm of the cell but not the cell nucleus

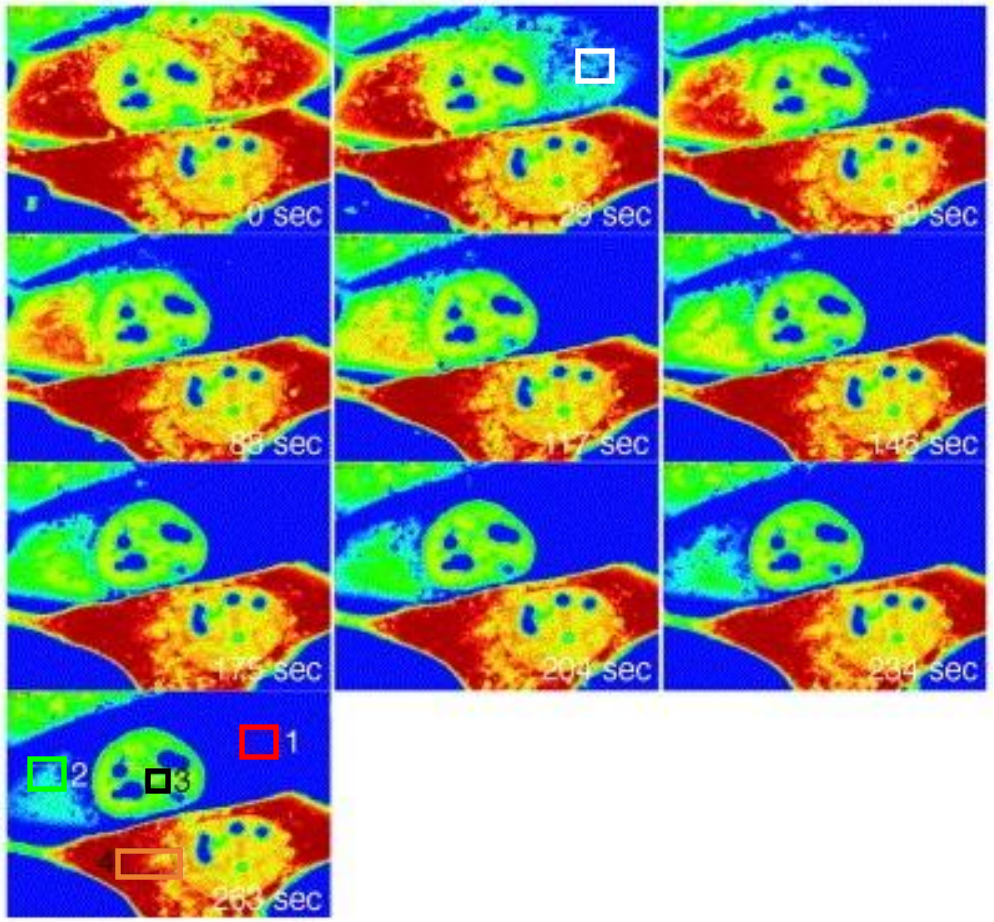
FLIP
Fluorescence Loss of Intensity after Photobleaching



An area within the cell is repeatedly bleached and the loss of fluorescence in areas that are distant from the bleach area is monitored.

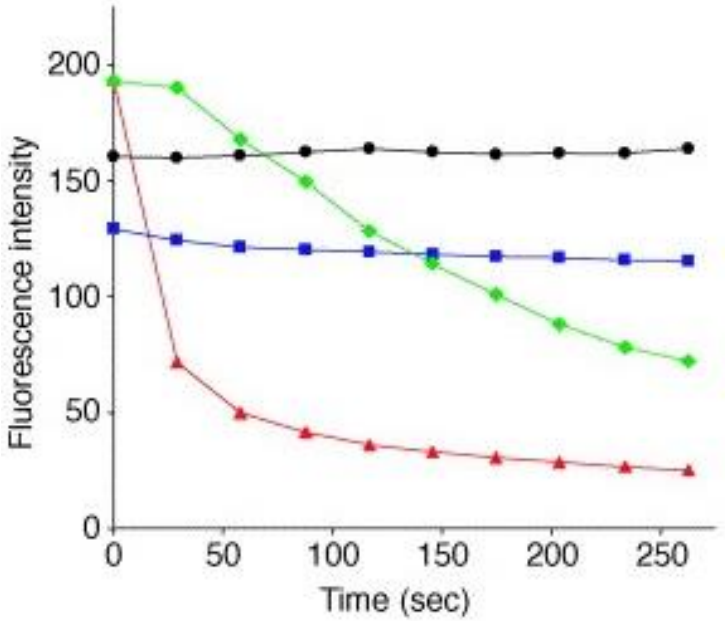
FLIP largely **eliminates the concern that the recovery properties are due to damage at the bleach spot**, as all measurements are made in areas that are never bleached.

Application of FLIP to nucleocytoplasmic shuttling



Koster M. *et al.*, Curr Opinion in Biotech, 2005, 16: 28-34

STAT1-EGFP



bleached cytoplasmatic area

unbleached cytoplasmatic area

nucleus

reference cell

BIOMEDICAL APPLICATIONS

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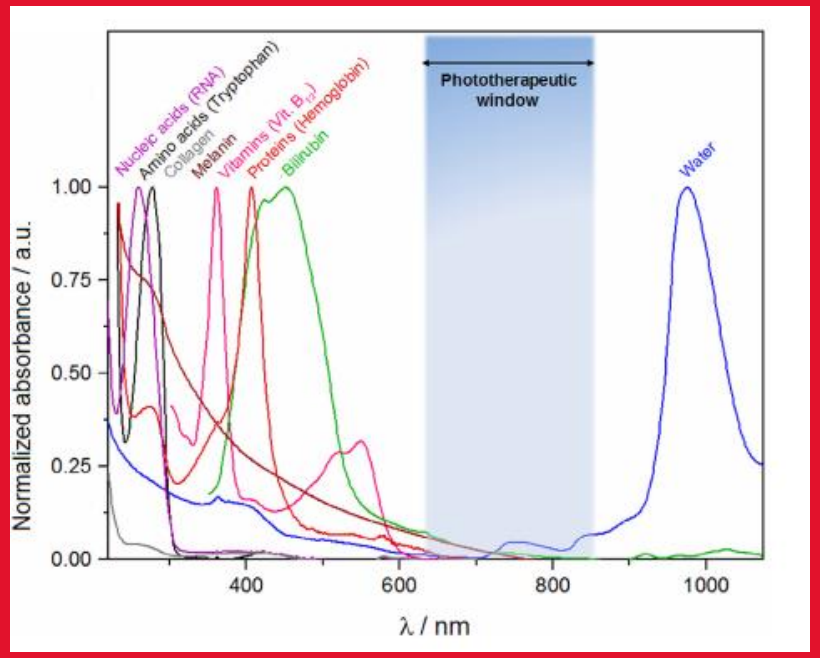
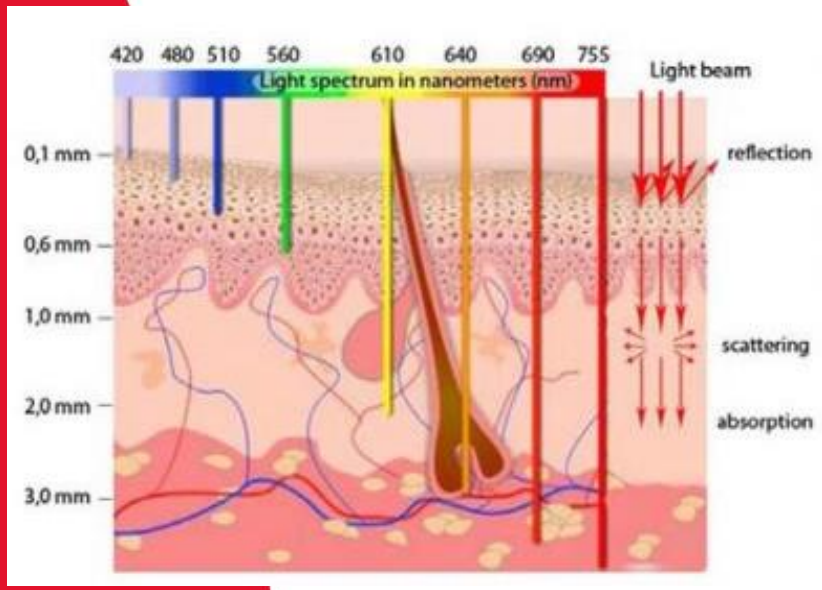
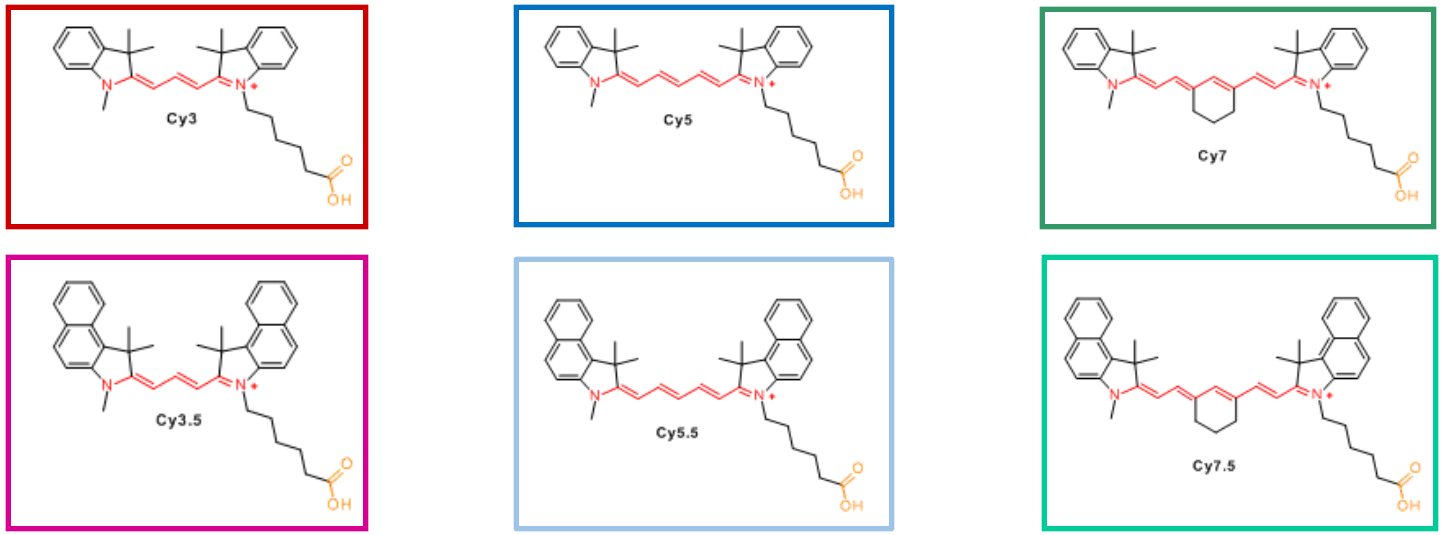


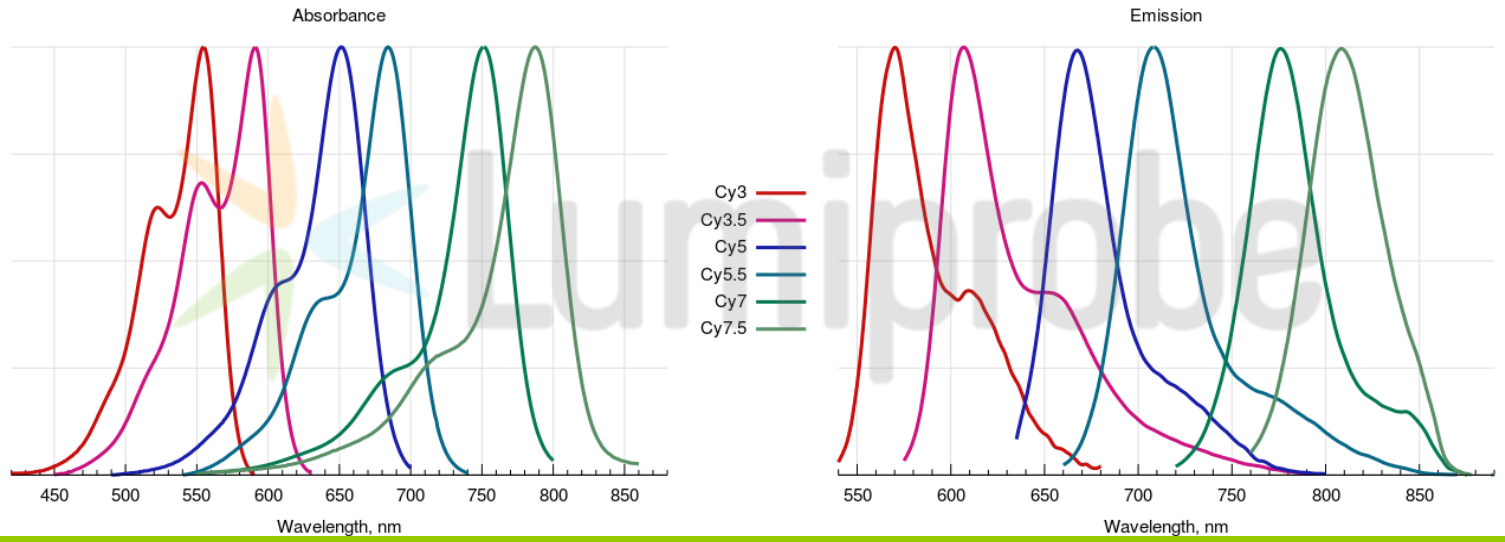
- high molar extinction coefficient (ε)
- absorption in the red and near infrared region (650-850 nm)



- stability and solubility in physiological environment
- optimal ADME (Absorption, Distribution, Metabolism, Excretion)

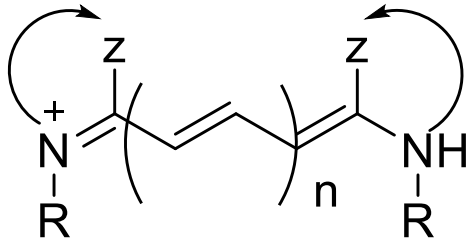
POLYMETHINE DYES





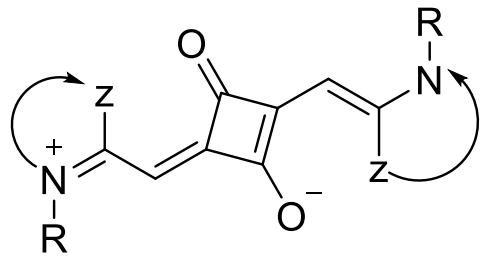
POLYMETHINE DYES

- Excellent optical properties (high ε and ϕ)
- High structural versatility
- Tunable spectral properties
- Wide application fields



Cyanines

- Good biocompatibility at effective concentrations
- Good cellular uptake
- Preferential accumulation in tumor tissues



Squaraines

DIAGNOSTIC TOOLS FOR *IN VIVO* IMAGING

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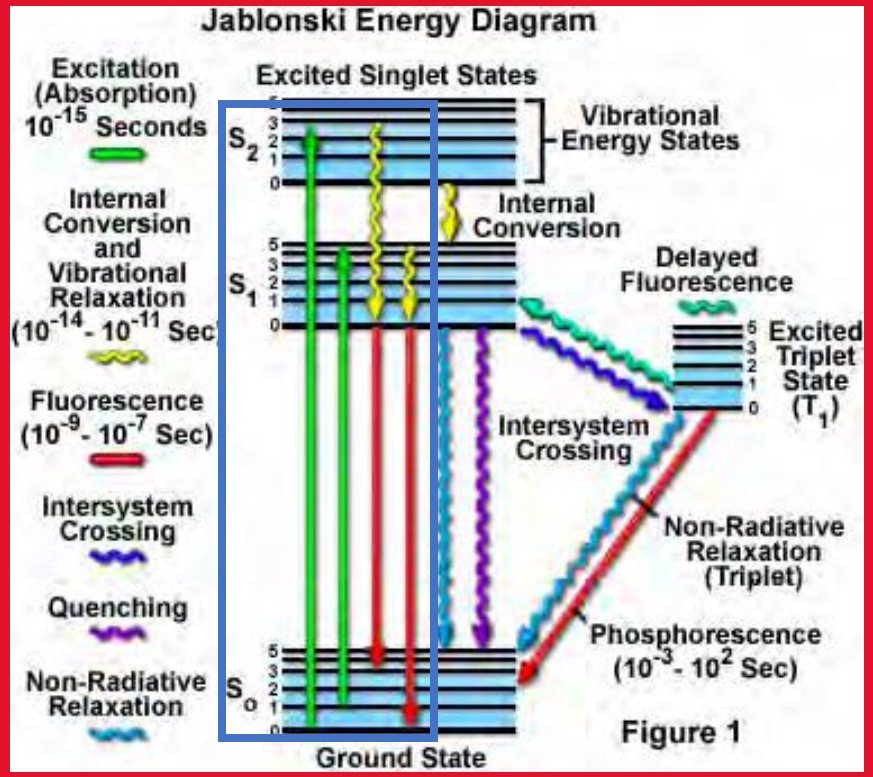
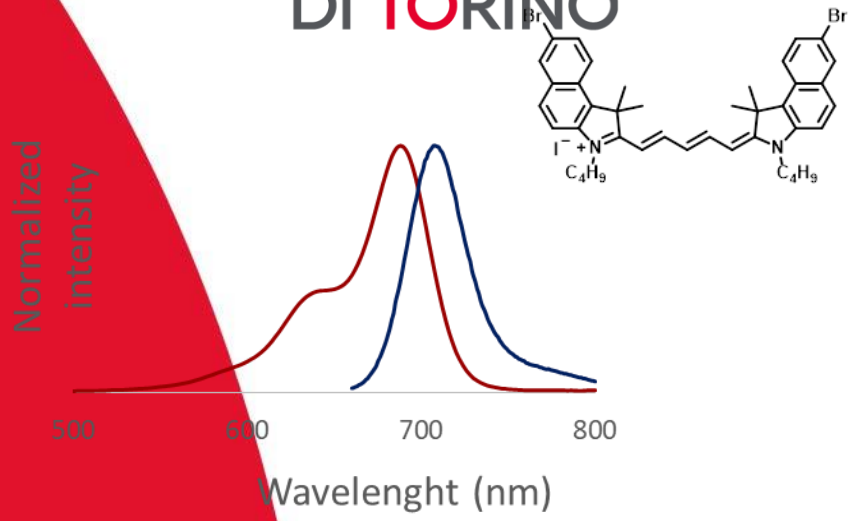
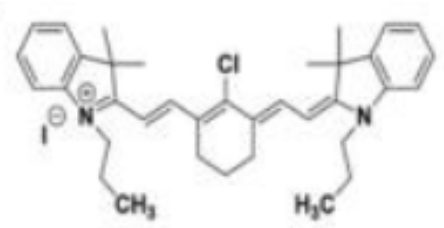


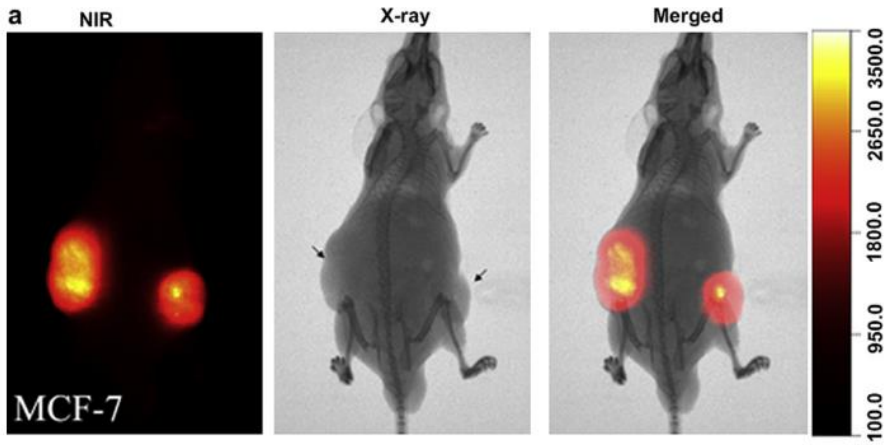


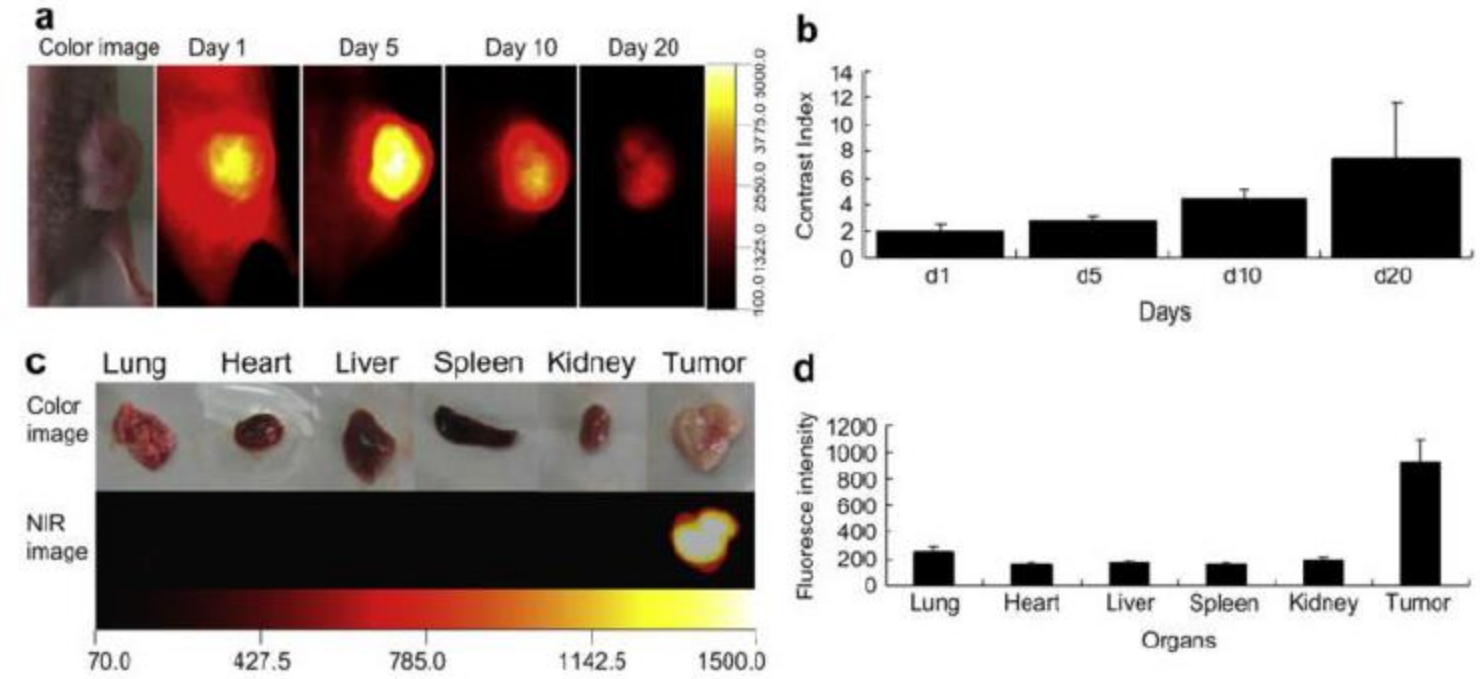
ε_{EtOH} = 208 538 $M^{-1}cm^{-1}$

ϕ_{DMSO} = 36,6 %



IR-780





Tan *et al.*, *Biomaterials* **33**, 2230-2239 (2012)

PHOTODYNAMIC THERAPY

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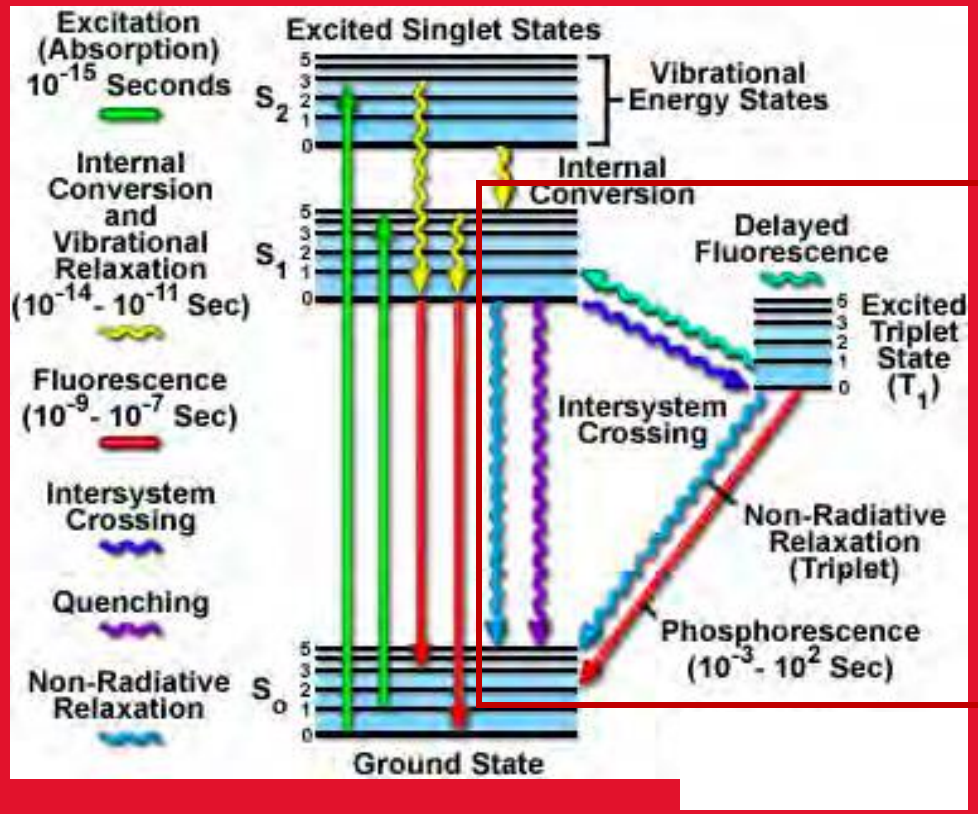
INTERSYSTEM CROSSING



Type I photochemistry:
Electron/hydrogen transfer → RADICAL OXIGEN SPECIES

Type II photochemistry:
Energy transfer
(to molecular oxygen) → $^1O_2$

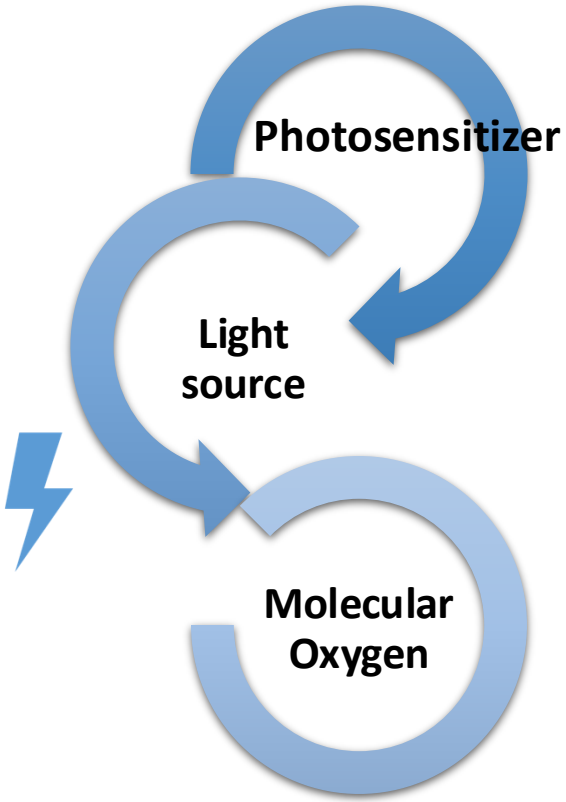
Photochemical reaction

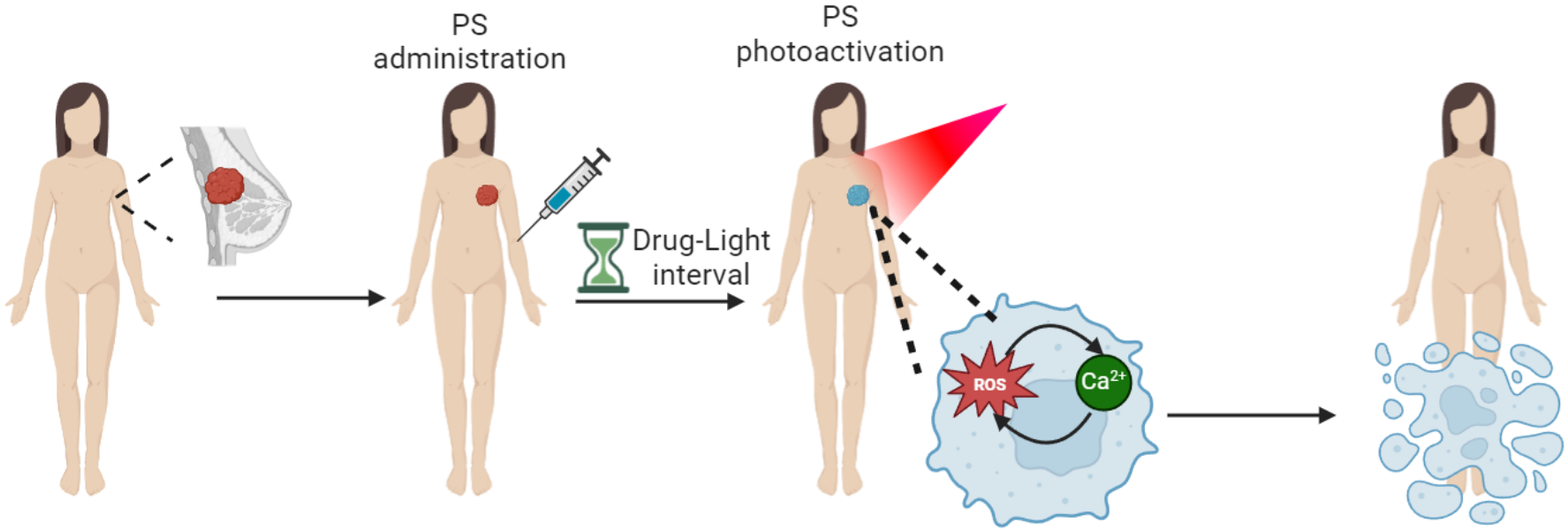
Photodynamic therapy (PDT)

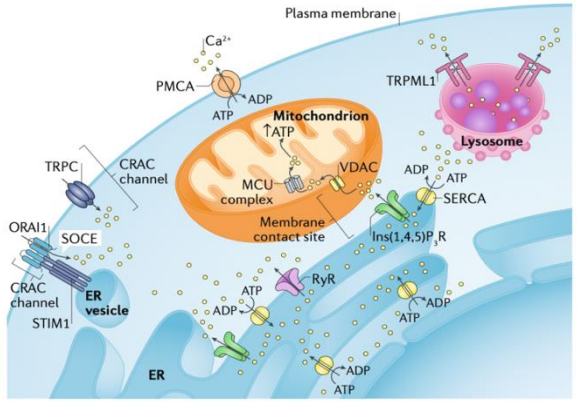
is a minimally invasive and minimally toxic light-based treatment used in medicine to treat a wide range of clinical conditions

Advantages
Technique FDA-approved
Localized therapy
Minimally invasive
Few collateral effects
Possibility to combine it with conventional anti-cancer therapies

How does PDT work?

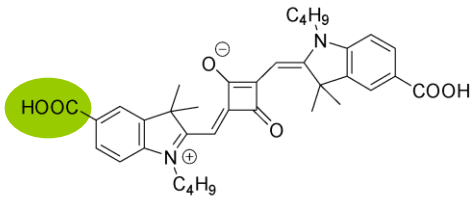




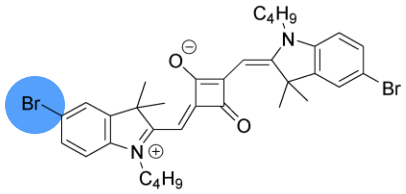


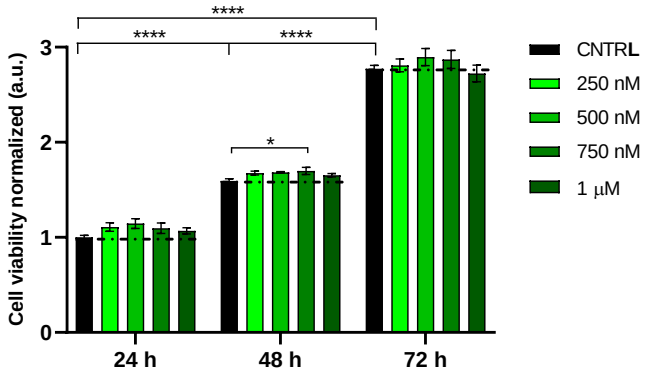
Giorgi C. et al.(2018)

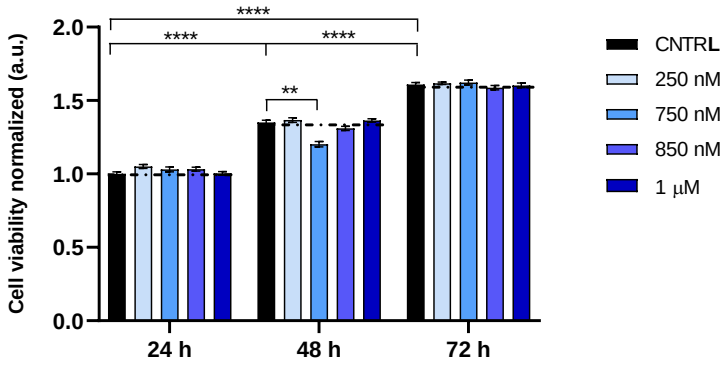
COOH-SQ-C4

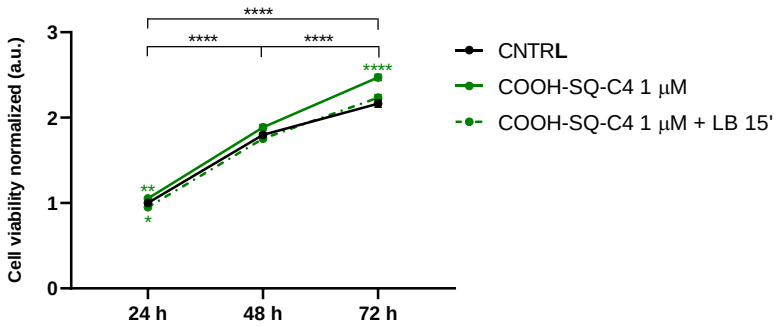


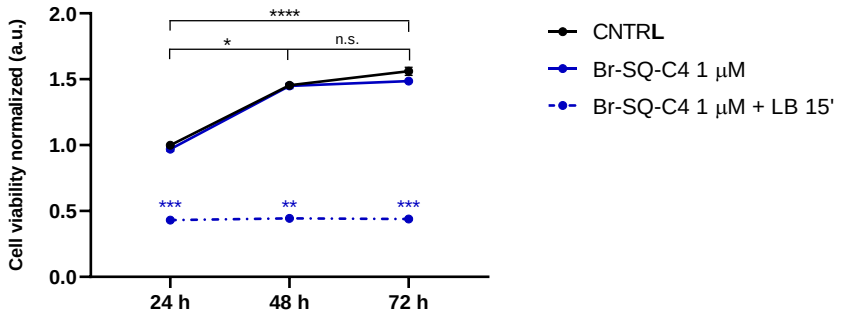
Br-SQ-C4



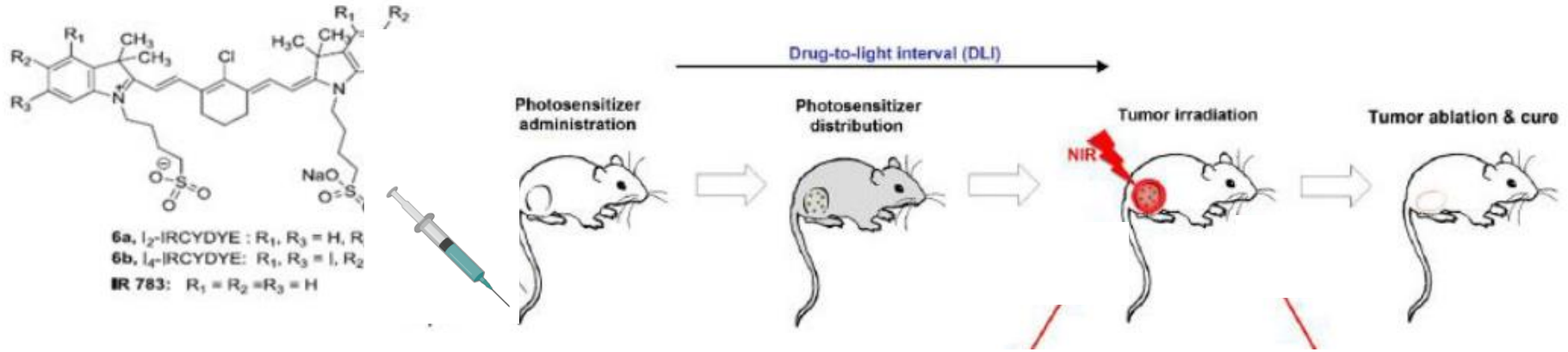


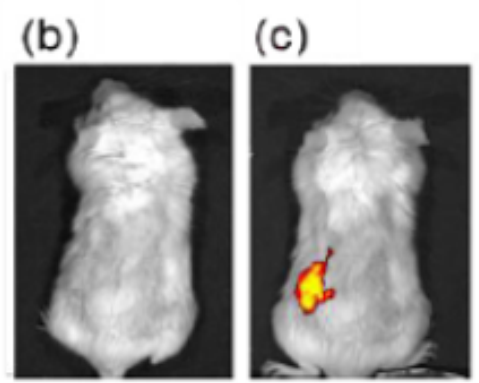


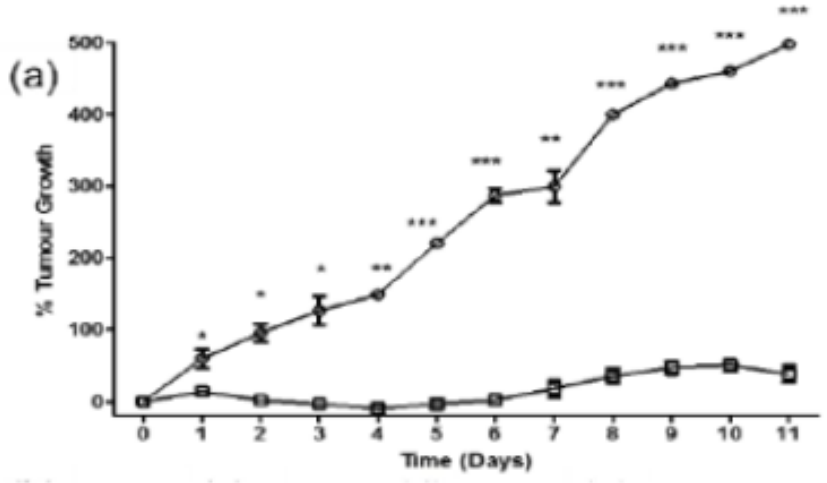




III. Biological and biomedical applications







Atchison *et al., Chem. Commun*. **53**, 2009-12 (2017)

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Thank you