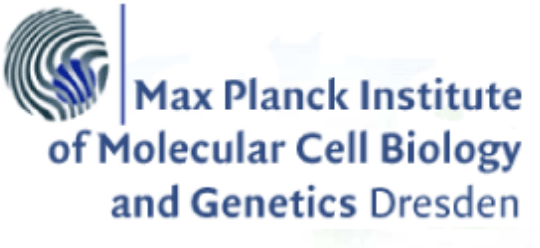


Fluorescence Resonance Energy Transfer (FRET) Microscopy

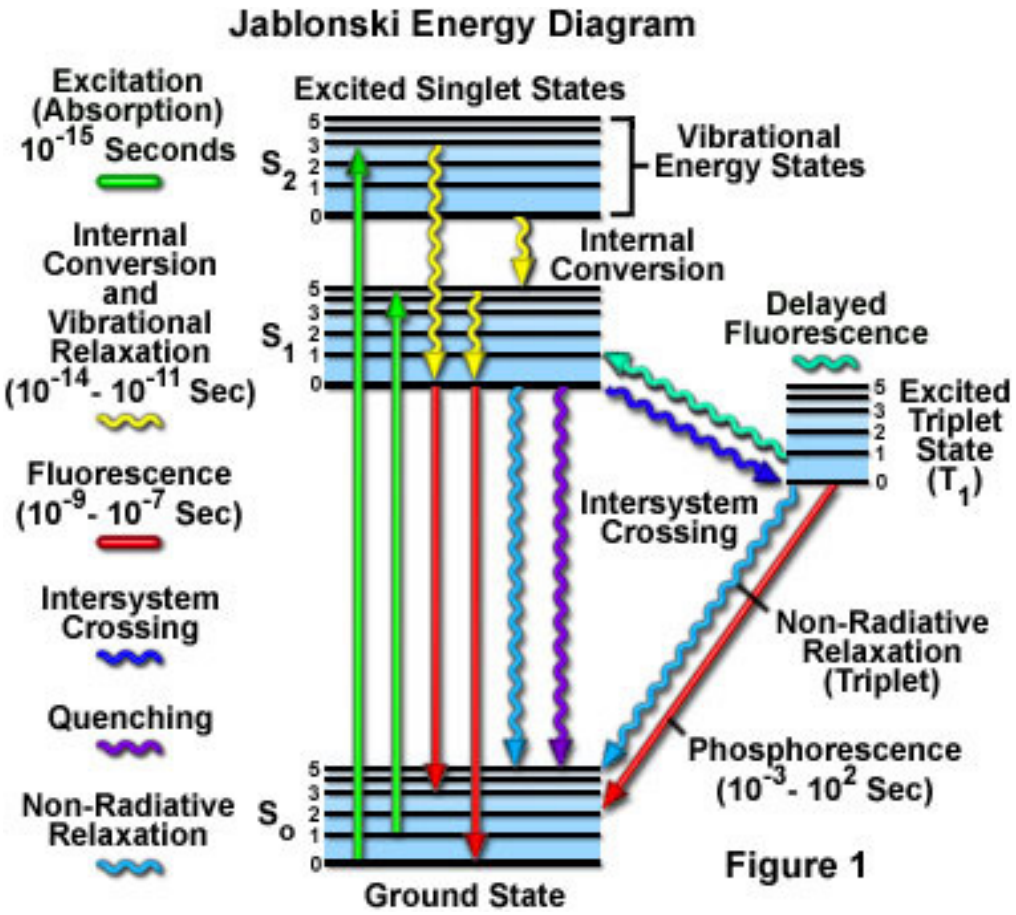
Mike Lorenz

Optical Technology Development

mlorenz@mpi-cbg.de

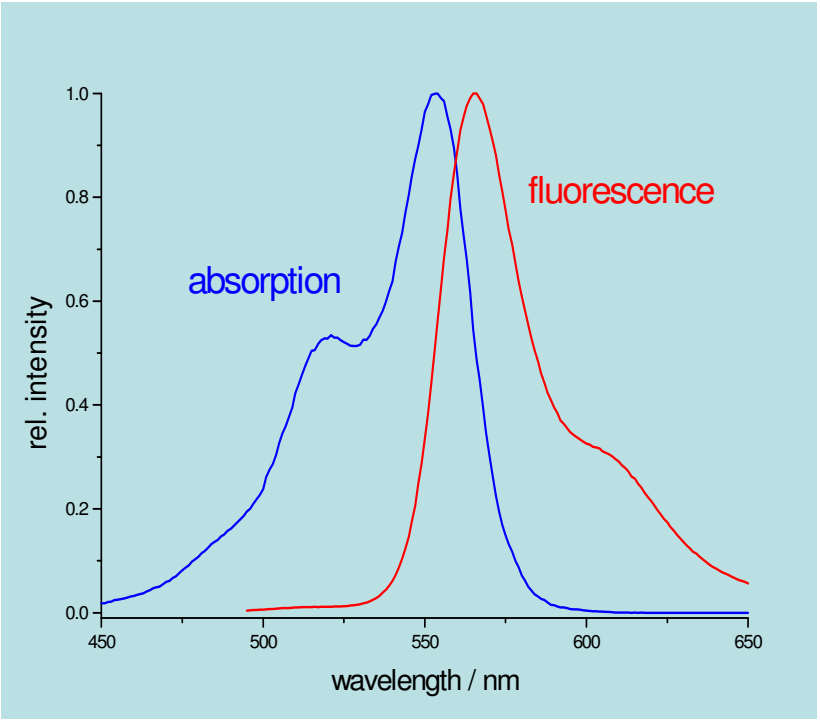
FRET-FLIM course, May 2009

What is fluorescence ?



Stoke's shift

Fluorescence light is always red-shifted!!!



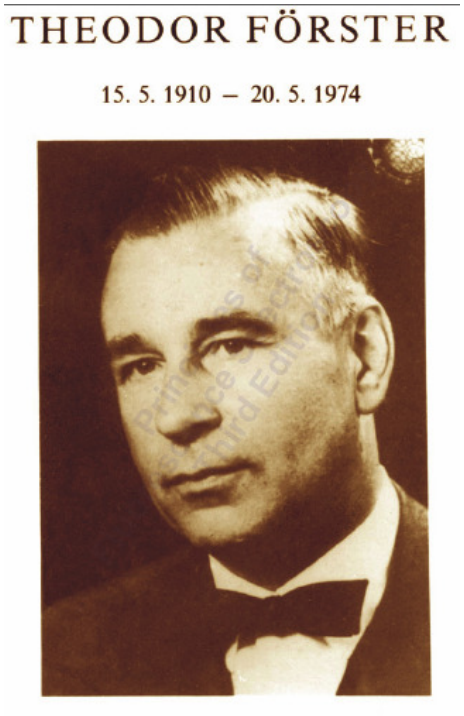
Quantum yield

Ratio of emitted to absorbed photons

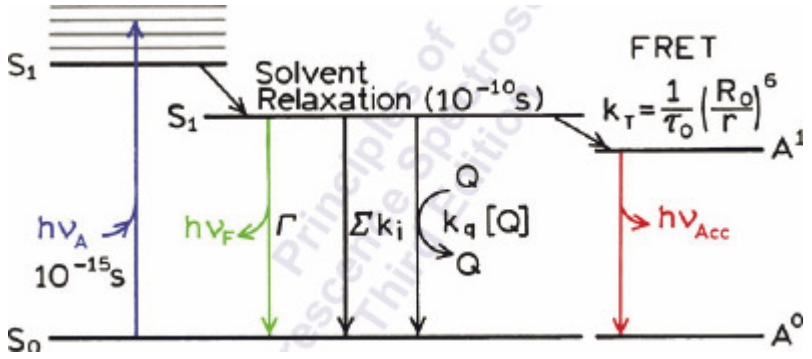
Lifetime

Average time the fluorophore remains in the excited state

Spectroscopic principles of FRET



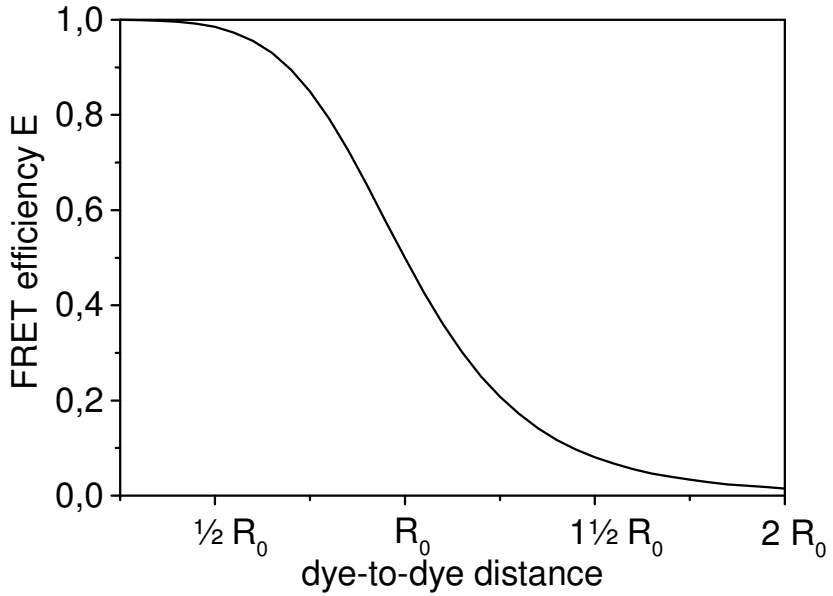
1948 / 49



$$E = \frac{k_{FRET}}{k_{FRET} + k_f + k_x} = \frac{R_0^{\,6}}{R_0^{\,6} + R^6}$$

$$R_0 = 9790 \cdot \left(\kappa^2 \, Q_D \, J(\lambda) \, n^{-4}\right)^{\frac{1}{6}} \text{Å}$$

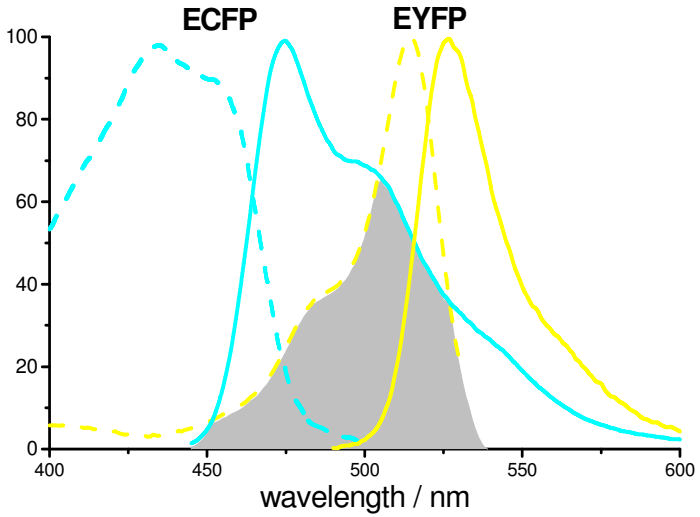
n: refraction index
Φ_D: donor quantum efficiency
J: spectral overlap integral
κ: dipole orientation factor



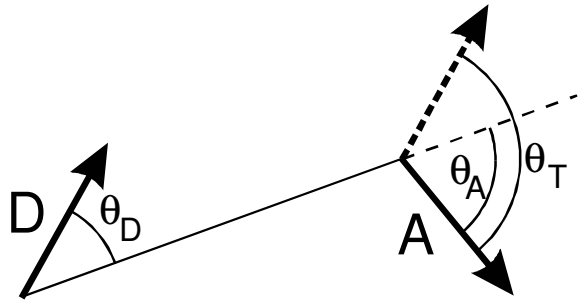
Förster distance R_0

$$R_0 = 9790 \cdot \left(\kappa^2 \; Q_D \; J(\lambda) \; n^{-4}\right)^{\frac{1}{6}} \text{ Å}$$

κ^2	donor-acceptor orientation factor
Q_D	donor quantum yield
$J(\lambda)$	overlap integral
n	refraction index



$$J(\lambda) = \int_0^{\infty} \left(F^D(\lambda)\,\varepsilon(\lambda)\,\lambda^4\right) d\lambda$$



D || A: $\kappa^2 = 4$

D ⊥ A: $\kappa^2 = 0$

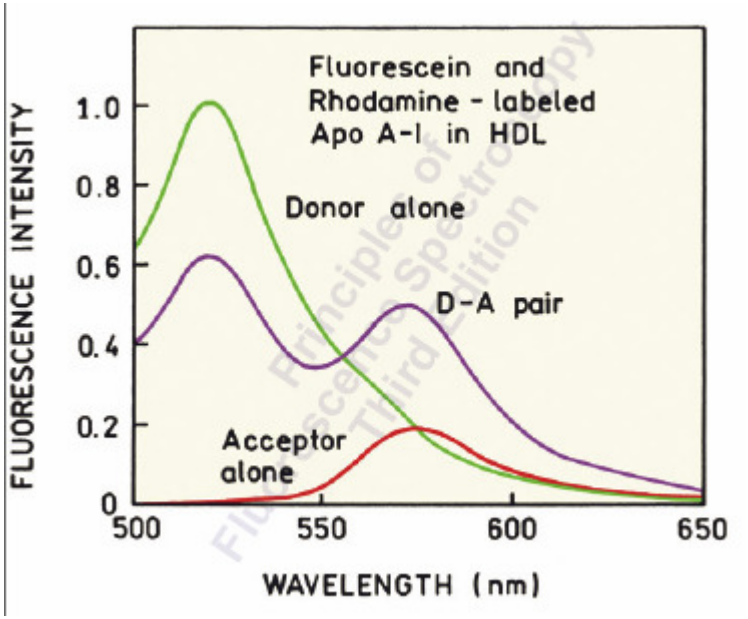
random: $\kappa^2 = 2/3$

$$\kappa^2 = \left(\cos\theta_T - 3\cos\theta_D \cos\theta_A\right)^2$$

For most D-A pairs is R_0 = 1.5-7.0 nm

FRET can be measured via

Intensity
in both channels



$$E = 1 - \frac{I_{FRET}}{I_D}$$

Fluorescence lifetime
of the donor

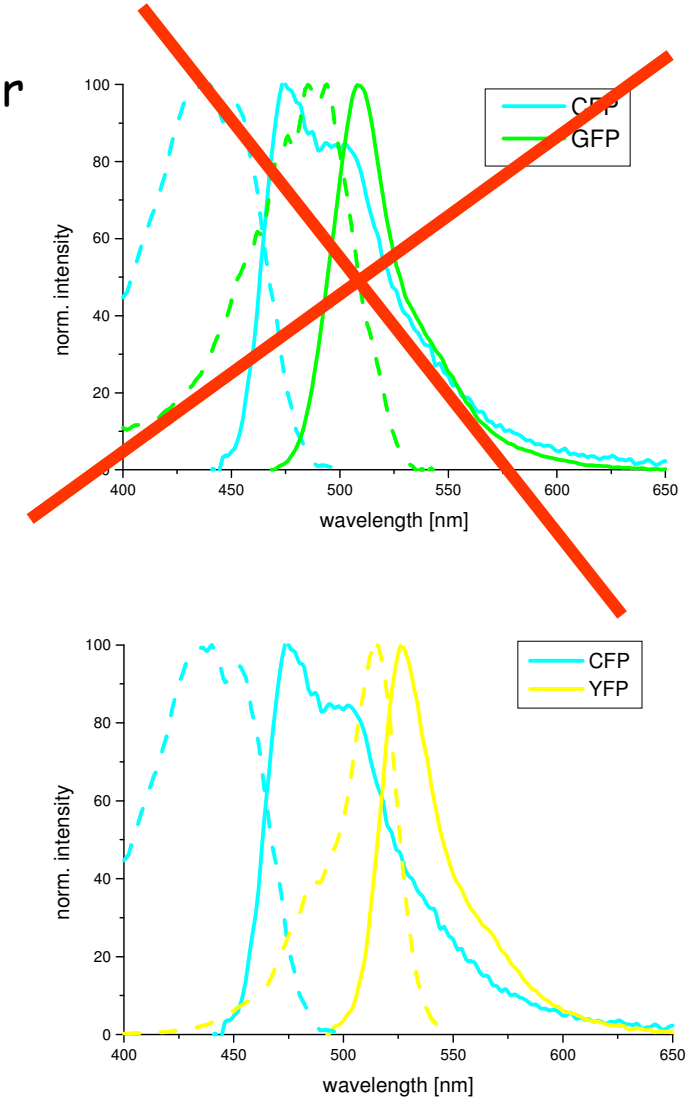
$$\tau_D = \frac{1}{k_f + k_x}$$

$$\tau_{DA} = \frac{1}{k_f + k_x + k_{FRET}}$$

=> $$E = 1 - \frac{\tau_{FRET}}{\tau_D}$$

Requirements for a good FRET pair

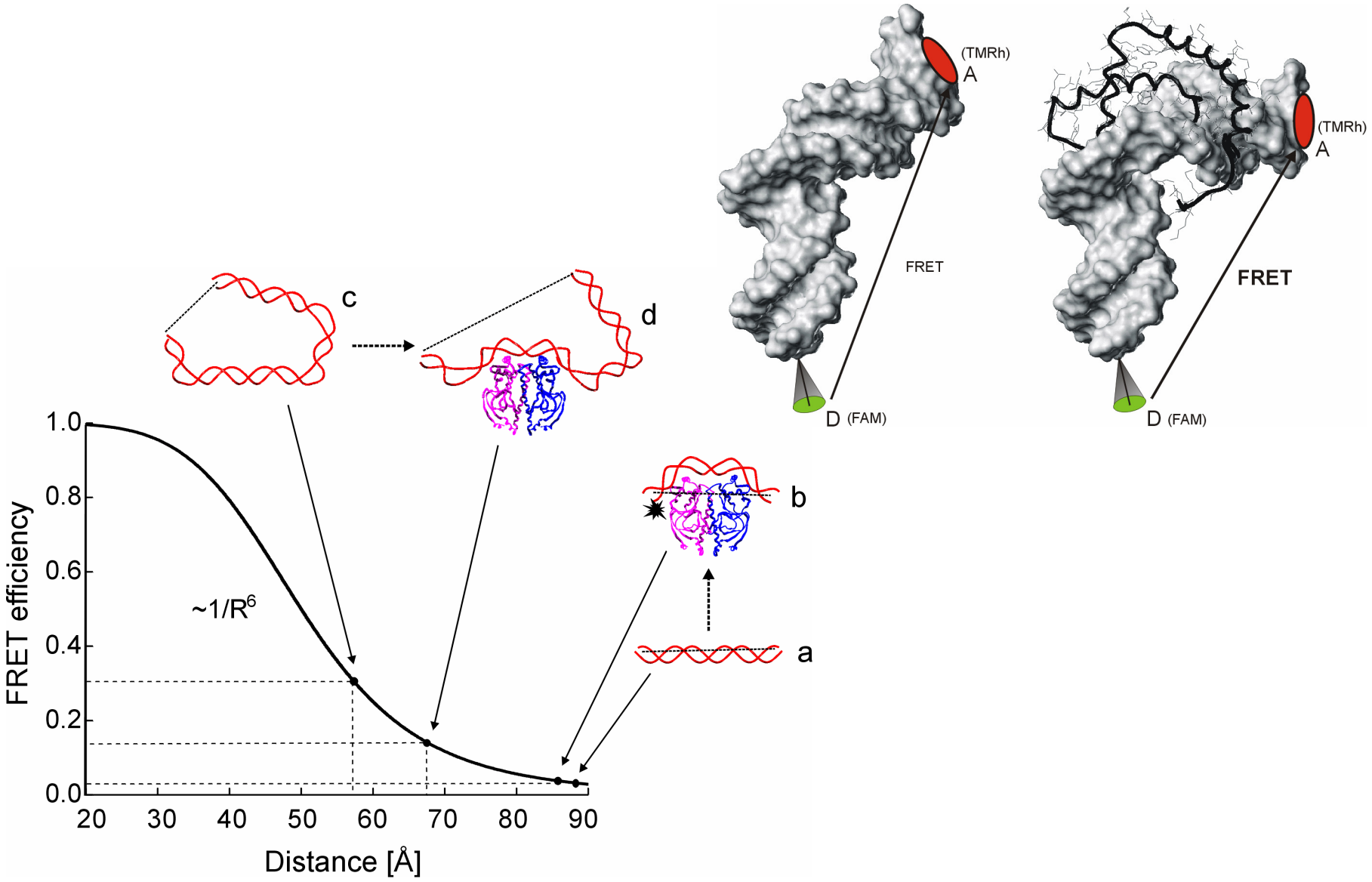
- Maximal overlap of donor emission and acceptor excitation
- High quantum yield of the donor
- Good spectral separation
  - Minimal direct excitation of the acceptor at the excitation maximum of the donor
  - Minimal emission of the donor with the acceptor fluorescence (bleed-through)



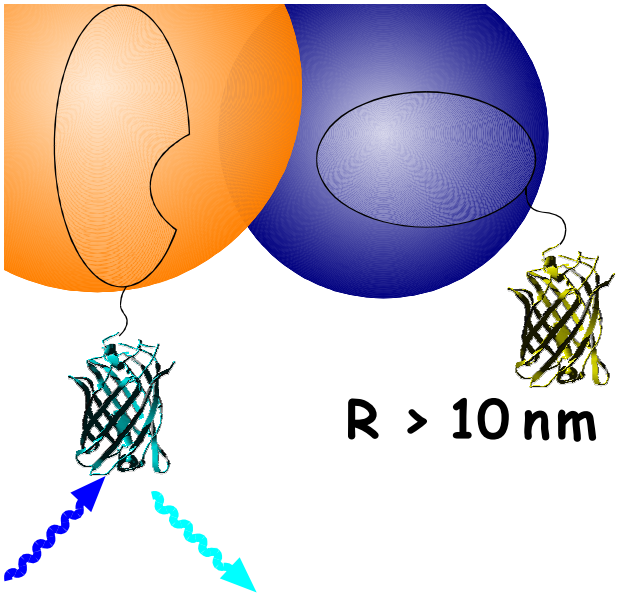
Common donor/acceptor pairs

Donor (Em.)		Acceptor (Exc.)		R_0 (κ^2=2/3)
FITC	(520 nm)	TRITC	(550 nm)	~ 5 nm
Cy3	(566 nm)	Cy5	(649 nm)	~ 5.7 nm
EGFP	(508 nm)	Cy3	(554 nm)	
CFP	(477 nm)	YFP	(514 nm)	~ 5 nm
EGFP	(508 nm)	YFP	(514 nm)	~ 5.7 nm
EGFP	(508 nm)	Cherry	(588 nm)	~ 5.3 nm

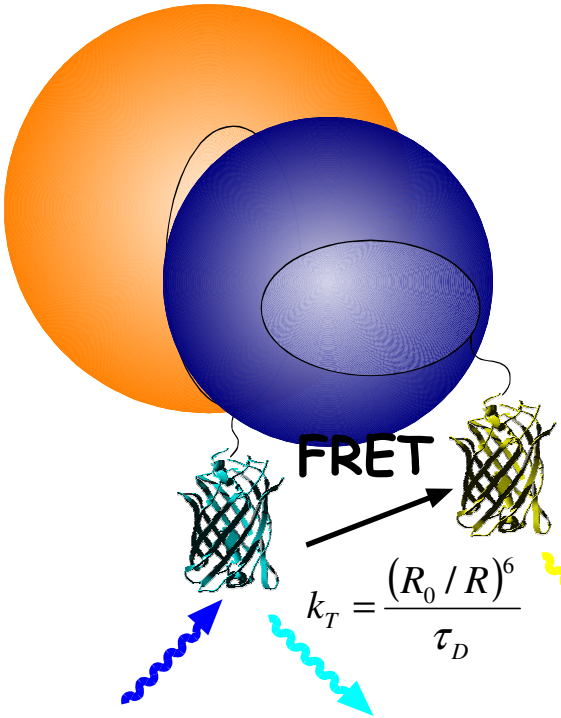
DNA bending measured by FRET



FRET Microscopy



R > 10 nm



FRET

$$k_T = \frac{(R_0/R)^6}{\tau_D}$$

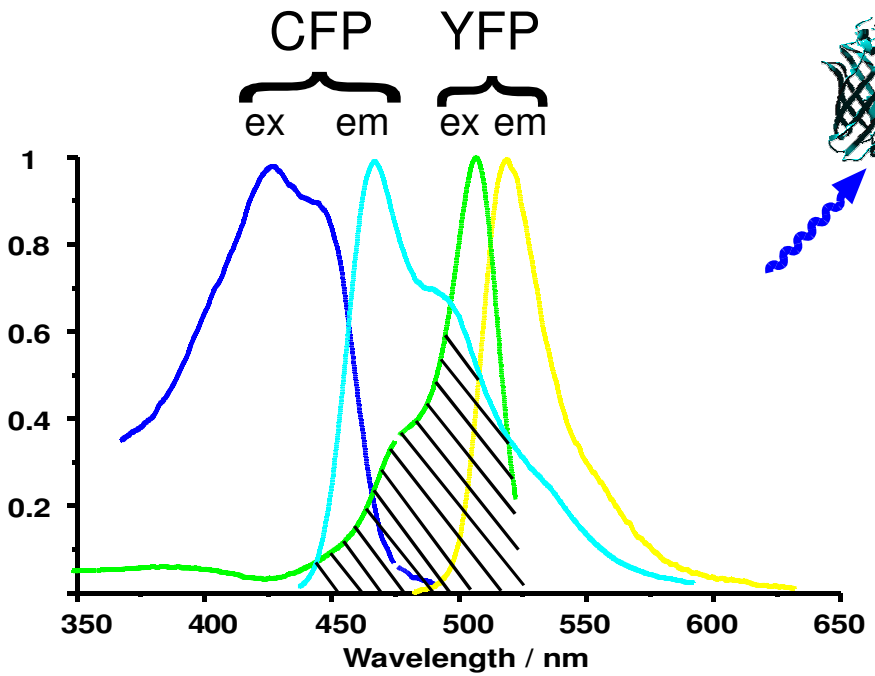
R ~ 2-8 nm

!!!

optical resolution

~ 200 - 250 nm

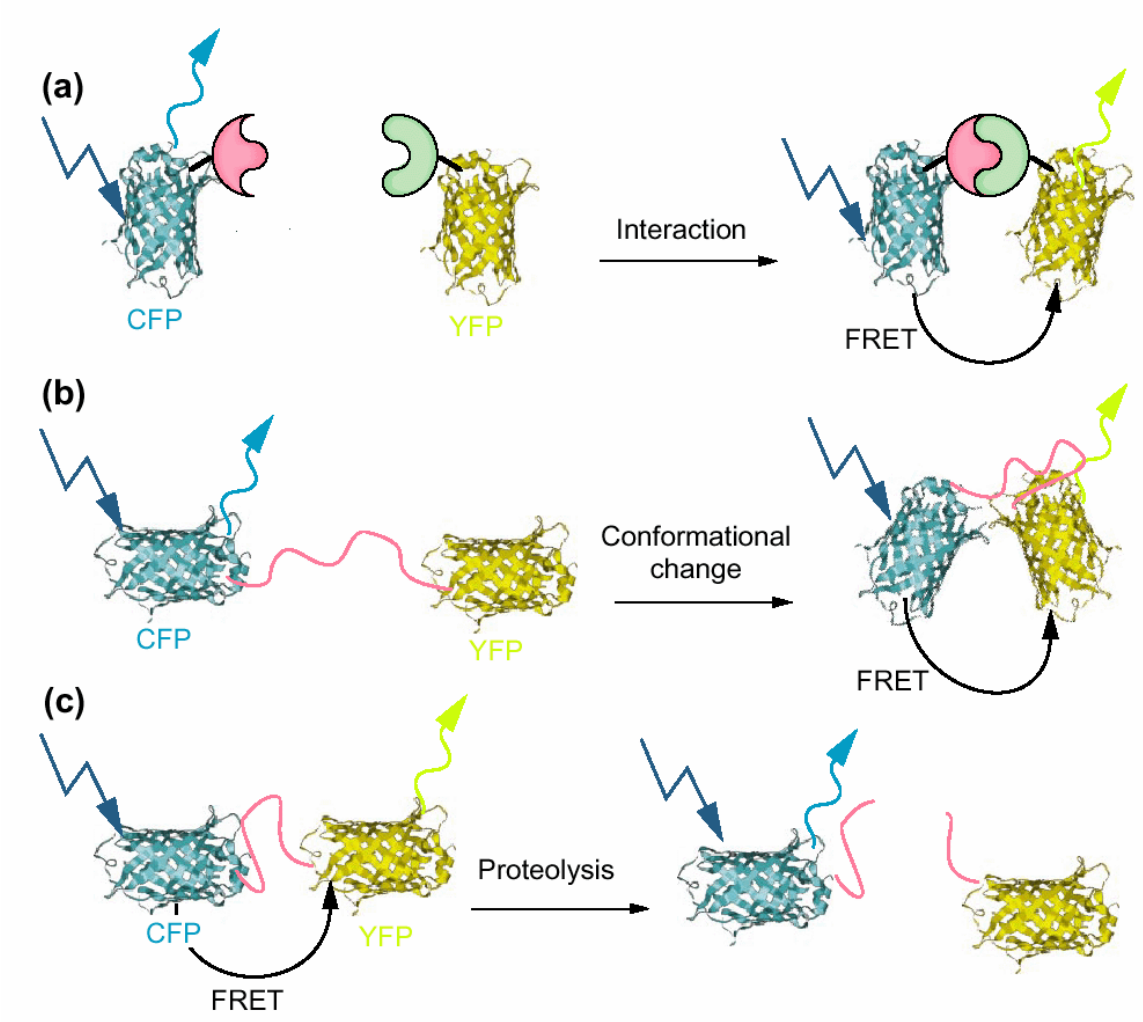
$$\left(\Delta\chi \geq 0.61\frac{\lambda}{N_A}\right)$$



FRET efficiency

$$E = \frac{k_T}{k_D + k_T} = \frac{R_0^6}{R_0^6 + R^6}$$

Applications of FRET microscopy



FRET detection methods

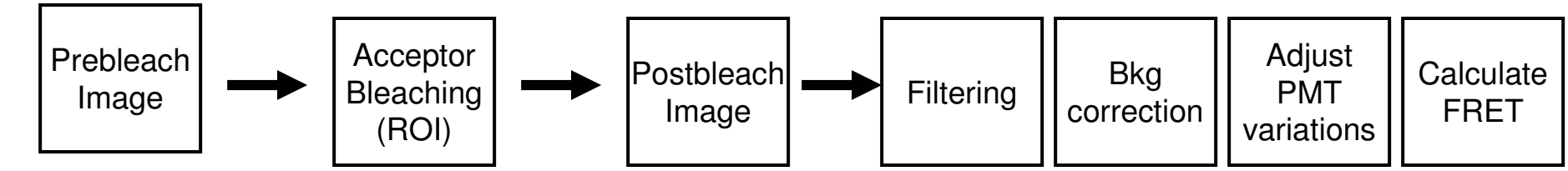
- **Donor Photobleaching**
- **Acceptor Photobleaching**

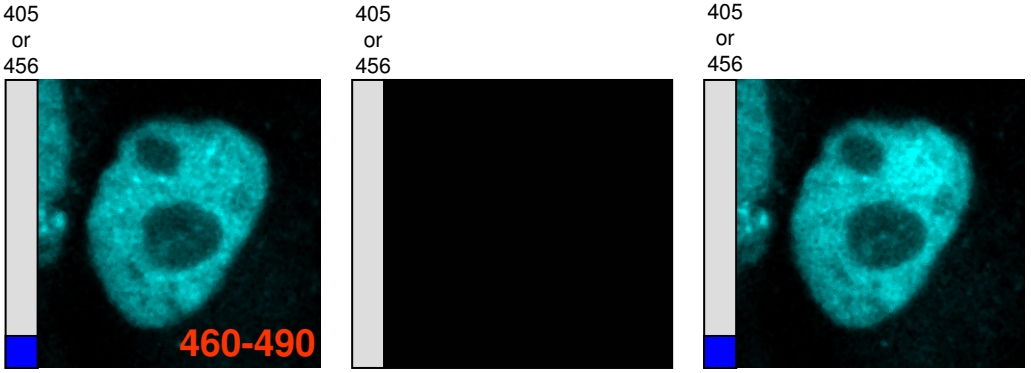
=> fixed samples

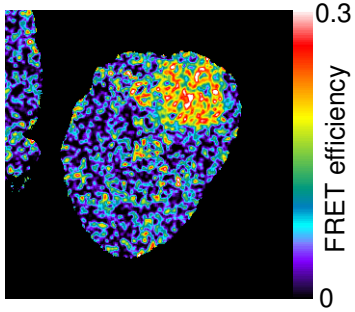
- **Sensitized Emission**
- **Ratio Imaging**
- **Fluorescence Lifetime**
- **Polarization / Anisotropy**

=> in vivo

FRET by Acceptor Photobleaching





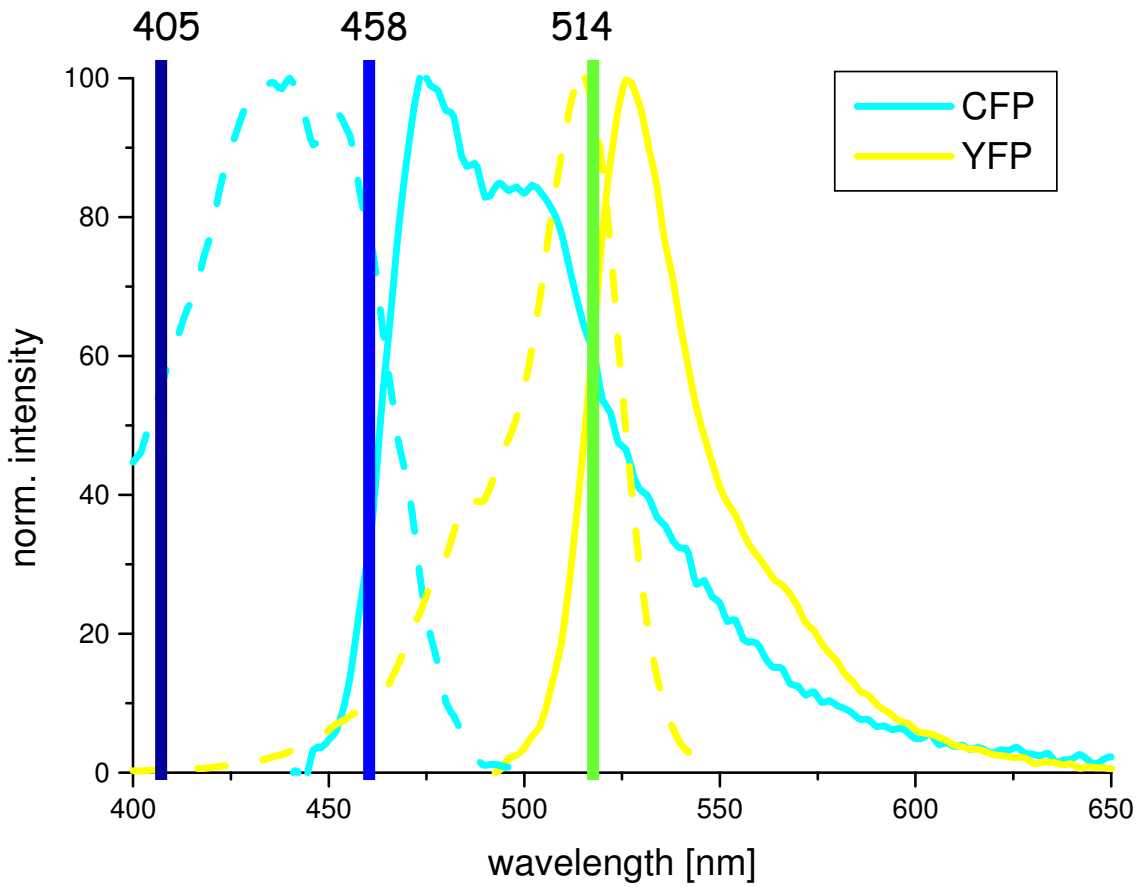


$$E = 1 - \frac{I_D^{prePB}}{I_D^{postPB}}$$

Acquisition

Processing

CFP-YFP: An excellent pair for CLSM



- All laser scanning microscopes have the necessary laser lines for CFP and YFP (458 & 514 nm Argon-Laser).
- 514 nm excites the acceptor only allowing a selective photobleaching
- Tuneable emission filters can collect most of the CFP fluorescnce without any contribution of the acceptor YFP (460 – 495 nm)

Recommended CLSM:

- Leica SP2 or SP5 (with AOBS)
- Olympus FV1000
- Zeiss LSM 710

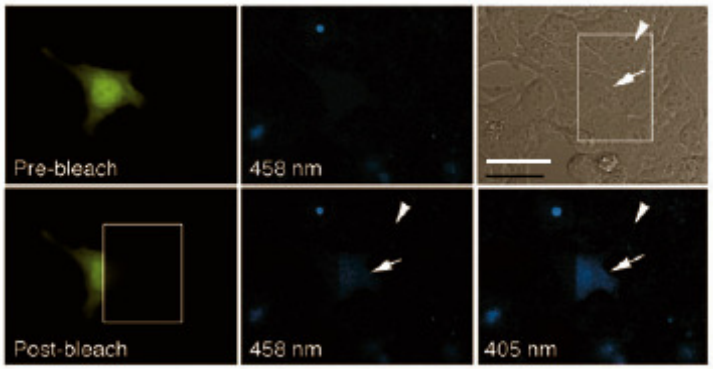
Problems with CFP-YFP in pbFRET?

Photoconversion of YFP into a CFP-like species during acceptor photobleaching FRET experiments

Guillaume Valentin[1], Céline Verheggen[1], Tristan Piolot[2], Henry Neel[1], Maïté Coppey-Moisan[2] & Edouard Bertrand[1]

[1]IGMM-CNRS UMR 5535; 1919, route de Mende; 34293 Montpellier Cedex 5; France. [2]IJM-CNRS Université Paris VI/VII; 2, pl. Jussieu; 75251 Paris Cedex 5; France.
e-mail: edouard.bertrand@igmm.cnrs.fr

NATURE METHODS | VOL.2 NO.11 | NOVEMBER 2005 | 801



YFP photoconversion revisited: confirmation of the CFP-like species

Michael T Kirber[1], Kai Chen[2] & John F Keaney Jr[2]

[1]Section of Molecular Medicine, Department of Medicine and Whitaker Cardiovascular Institute Multi-photon Microscopy Core, Boston University School of Medicine, Boston, Massachusetts 02118, USA. [2]Division of Cardiovascular Medicine, Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01655, USA.
e-mail: mkirber@bu.edu

NATURE METHODS | VOL.4 NO.10 | OCTOBER 2007 | 767

Photobleaching of YFP does not produce a CFP-like species that affects FRET measurements

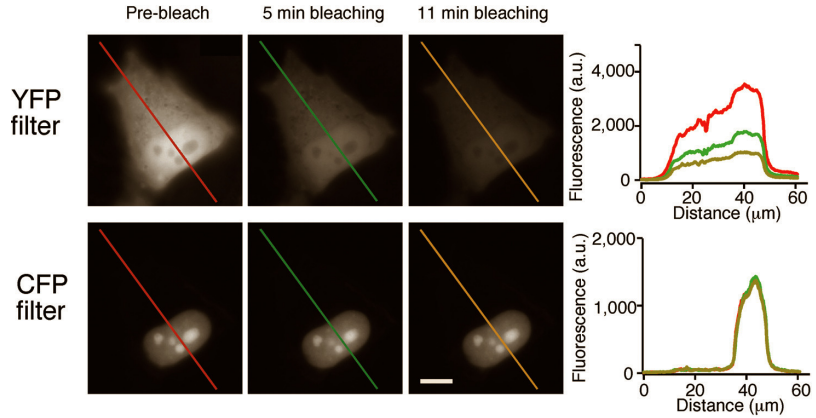
Christopher Thaler[1], Steven S Vogel[1], Stephen R Ikeda[2] & Huanmian Chen[2]

Laboratory of Molecular Physiology, Sections on [1]Cellular Biophotonics and [2]Transmitter Signaling, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland 20892, USA.
e-mail: huanchen@mail.nih.gov

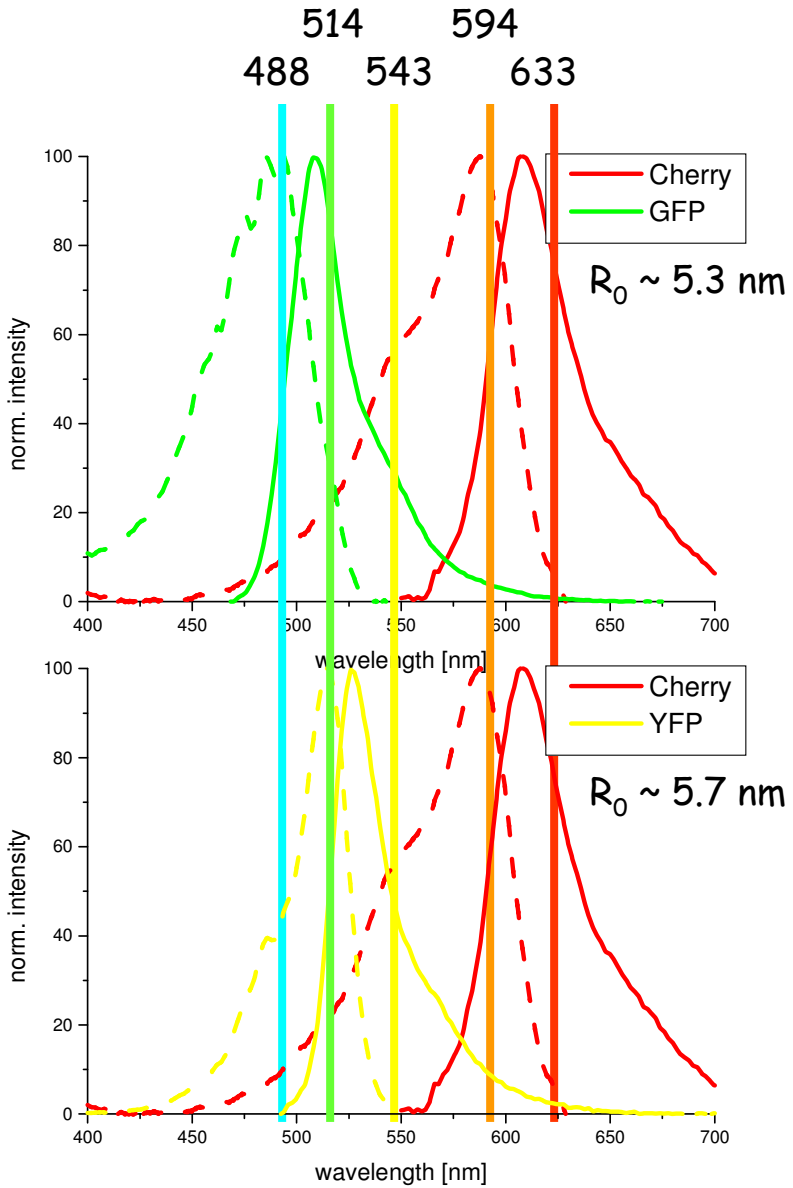
Sophie E Verrier[1] & Hans-Dieter Söling[1,2]

[1]Max Planck Institute of Biophysical Chemistry, Department of Neurobiology, Am Fassberg 11, D-37077 Göttingen, Germany. [2]Deceased.
e-mail: sophie.verrier@curie.fr

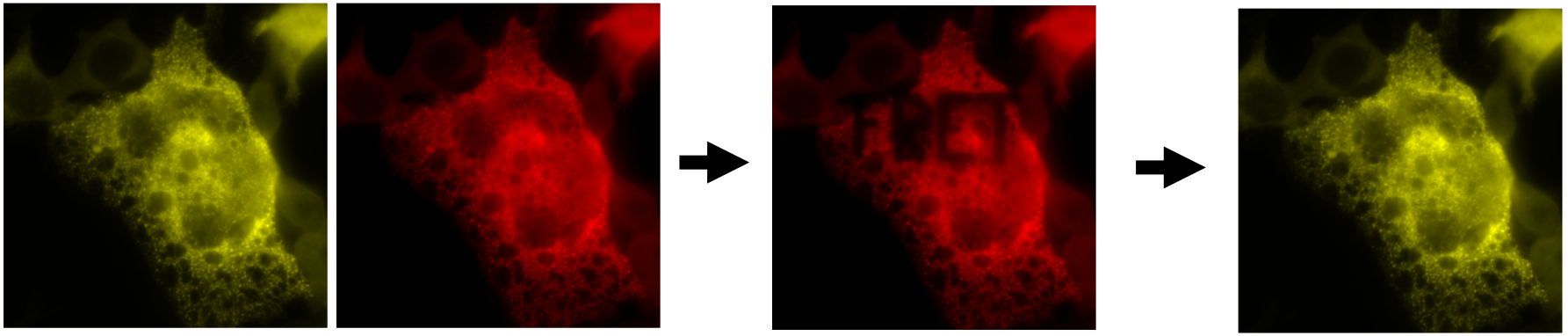
NATURE METHODS | VOL.3 NO.7 | JULY 2006 | 491



Can green-red work as a FRET pair?



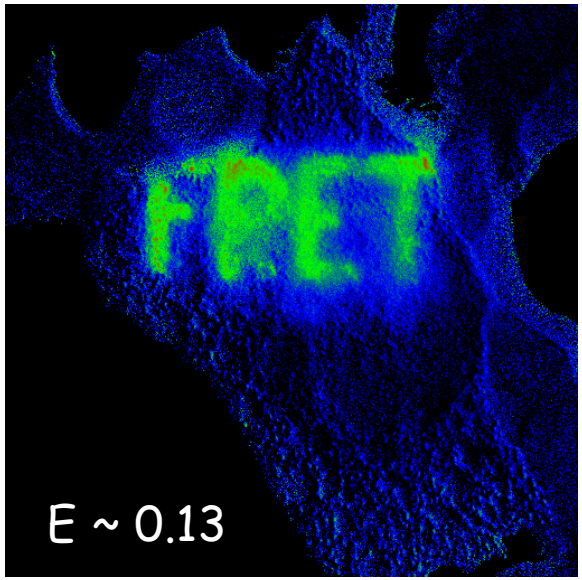
FRET by Acceptor Photobleaching



1. Take donor & acceptor image

2. partially pb acceptor

3. Take donor image



Microscope: Confocal

Advantages:

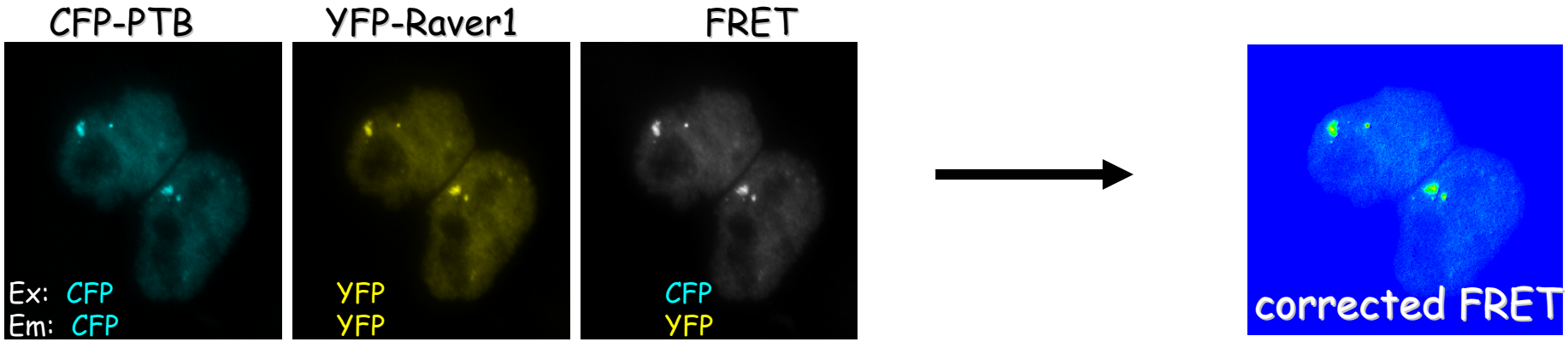
- Can be used for all FRET experiments
- Easy quantitative measurements

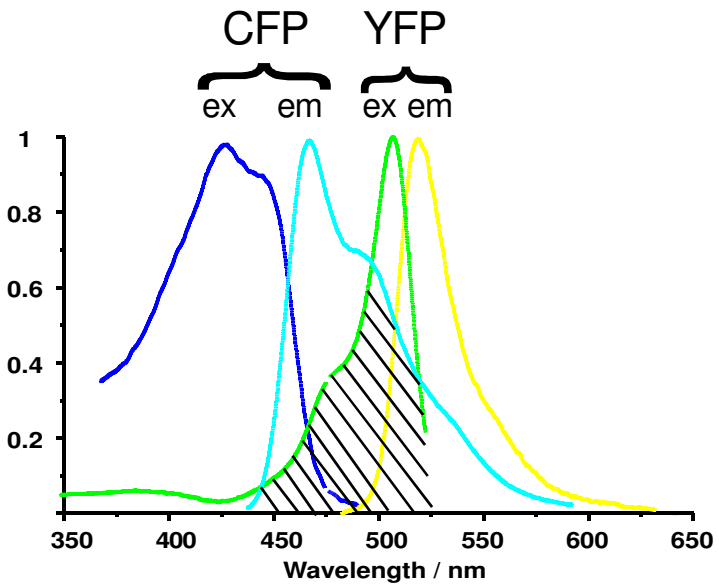
Disadvantages:

- Destructive
- Fixed samples only

FRET by Sensitized Emission

$$nF = I_{FRET}^{DA} - \frac{I_{FRET}^{D}}{I_{Donor}^{D}} \cdot I_{Donor}^{DA} - \frac{I_{FRET}^{A}}{I_{Acceptor}^{A}} \cdot I_{Acceptor}^{DA}$$





Microscope: Widefield / Confocal

Advantages:

- Non-destructive => live cell imaging

Disadvantages:

- Not quantitative
- Requires correction for bleedthrough etc.
- Sensitive to photobleaching

FRET by Sensitized Emission

nF can be affected by several factors:
- Donor and acceptor intensity (or concentration) of the pixels
- FRET efficiency
- Ratio of complexes to free donor and acceptor

=> nF should be normalized to be intensity independent

Normalization

1. $$\frac{nF}{I_{Donor}}$$

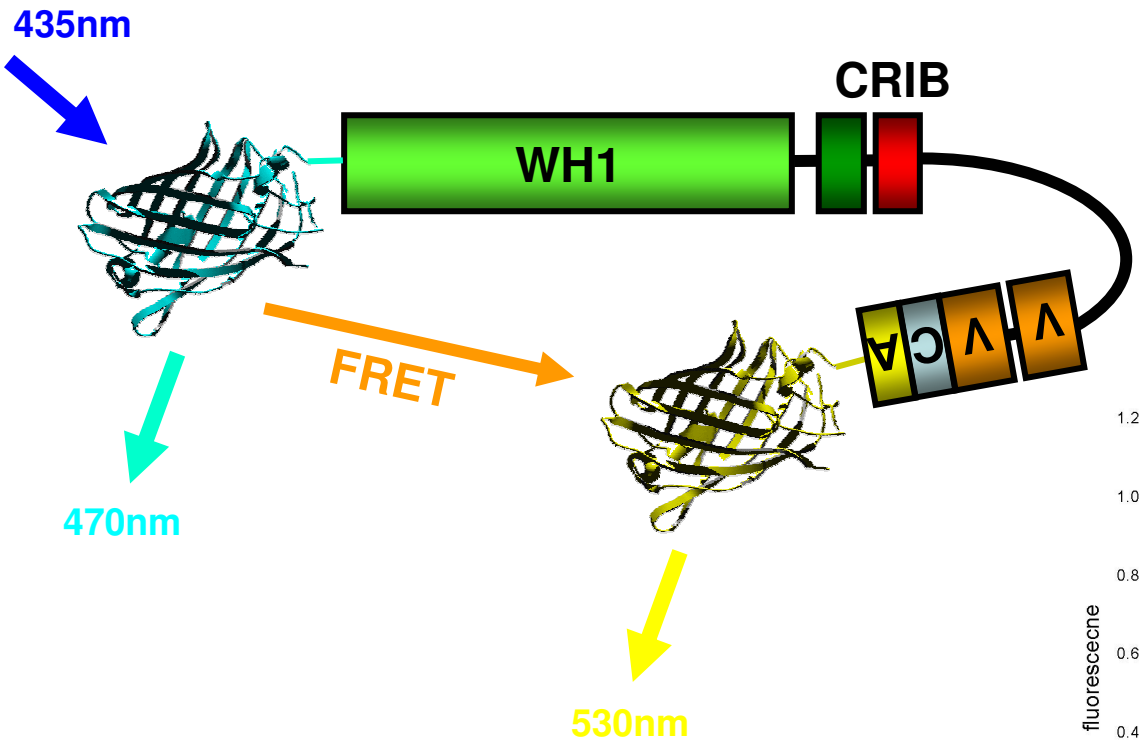
2. Gordon et al. (1998) $$FRETN = \frac{nF}{I_{Donor} \cdot I_{Acceptor}}$$

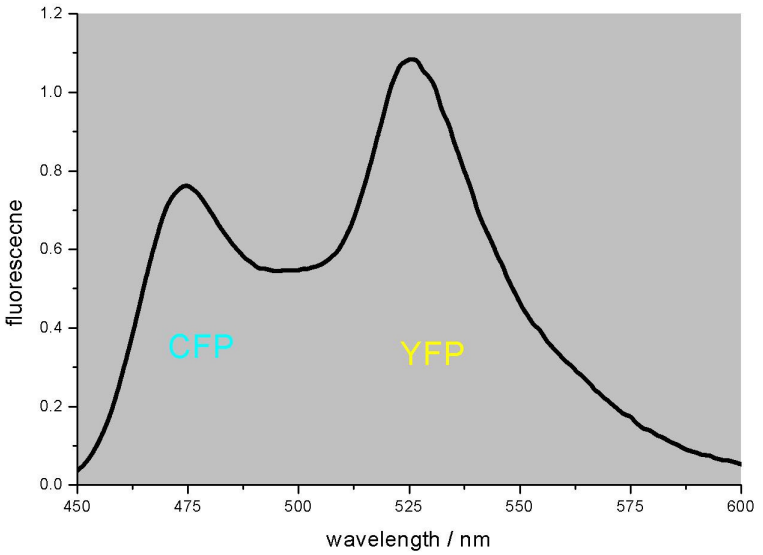
3. Xia & Liu (2001) $$N_{FRET} = \frac{nF}{\sqrt{I_{Donor} \cdot I_{Acceptor}}}$$

- Normalization by Gordon (2) is not intensity independent
- (1) and (3) are, but only Xia takes both concentrations into account

FRET by Donor-Acceptor Ratio Imaging

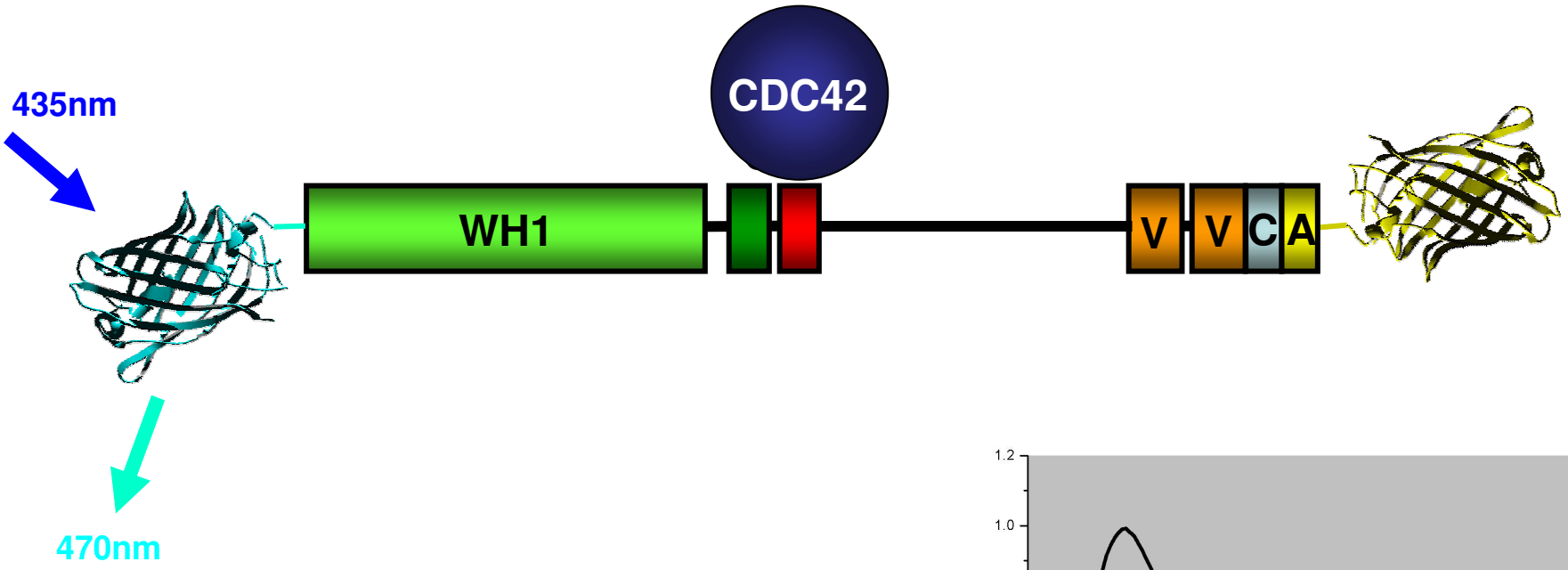
$$ratioFRET = \frac{FRET - Ch}{Donor - Ch}$$

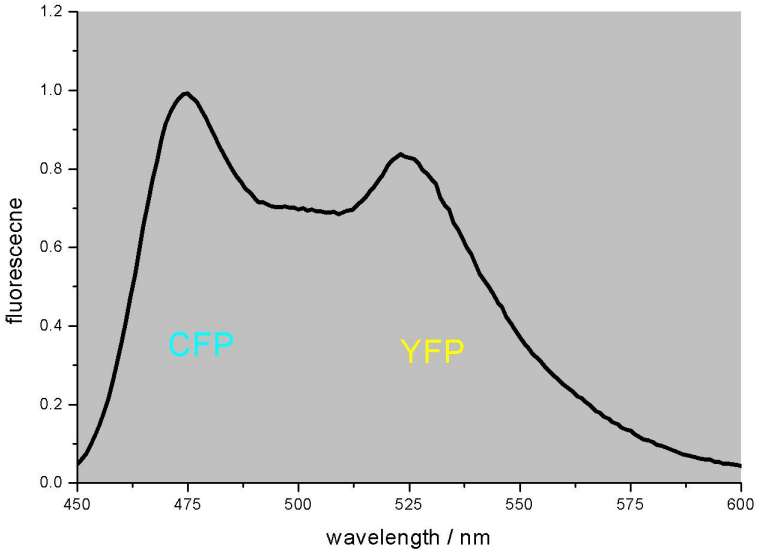




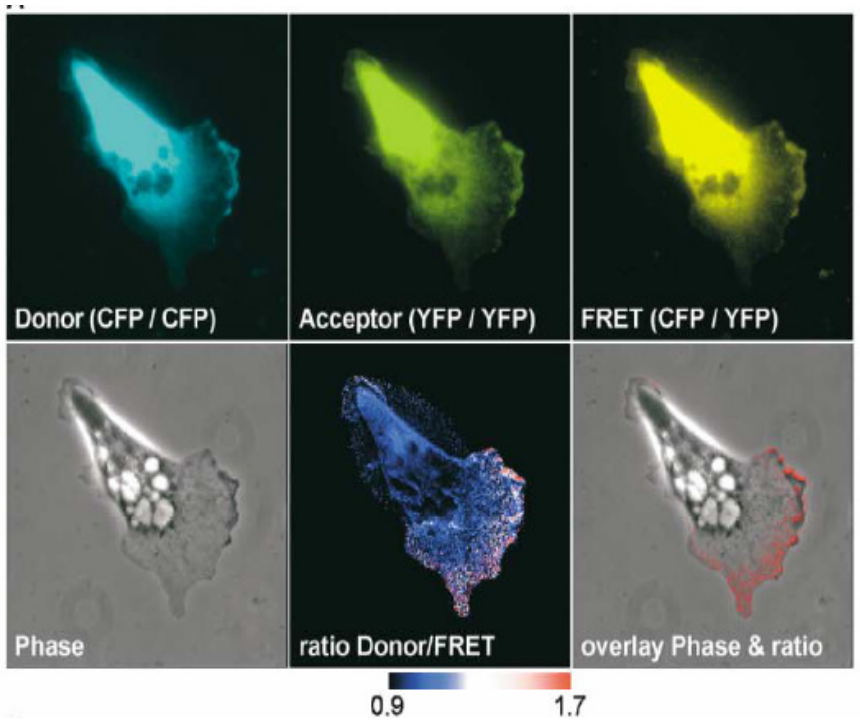
FRET by Donor-Acceptor Ratio Imaging

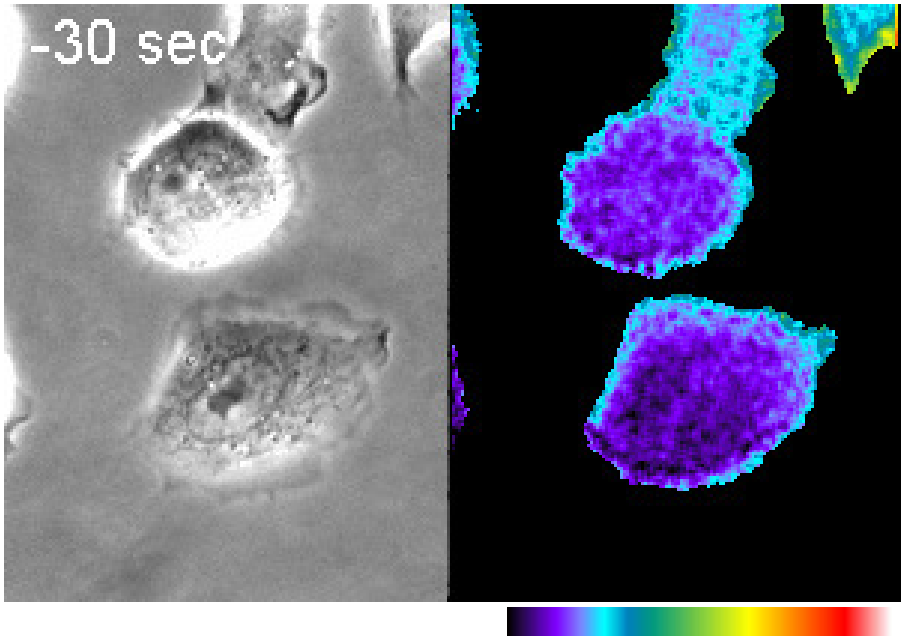
$$ratioFRET = \frac{FRET - Ch}{Donor - Ch}$$

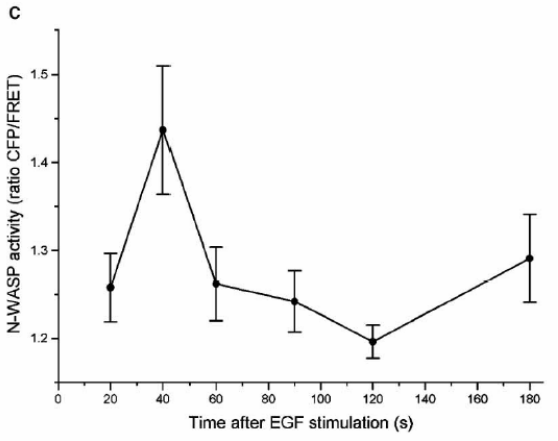




FRET by Donor-Acceptor Ratio Imaging







Microscope: Widefield / Confocal

Advantages:

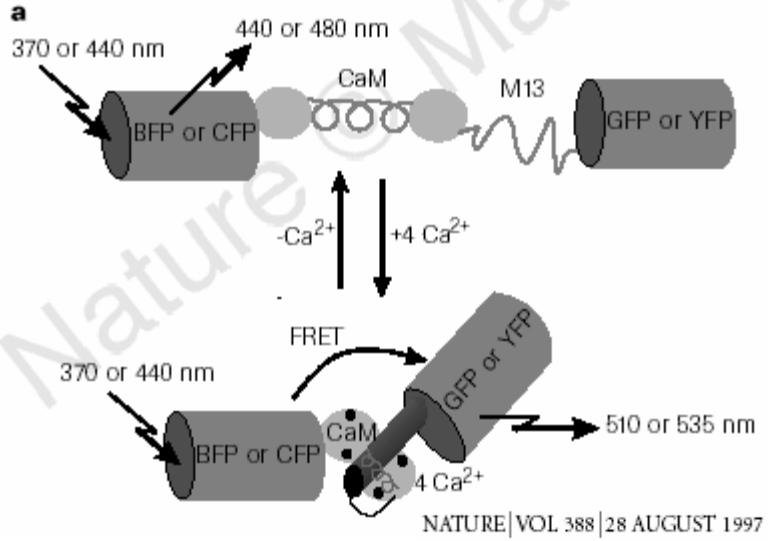
- Non-destructive => live cell imaging
- Easy qualitative measurements
- Images can be taken simultaneously

Disadvantages:

- Limited for biosensors

Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin

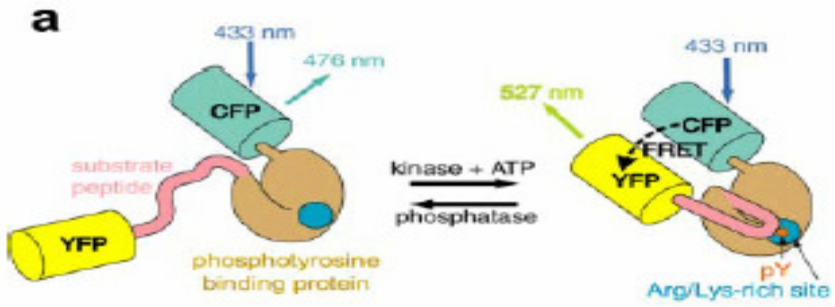
Atsushi Miyawaki*, Juan Llopis*, Roger Heim*†, J. Michael McCaffery‡, Joseph A. Adams§, Mitsuhiko Ikura‖, & Roger Y. Tsien*†



NATURE | VOL 388 | 28 AUGUST 1997

Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells

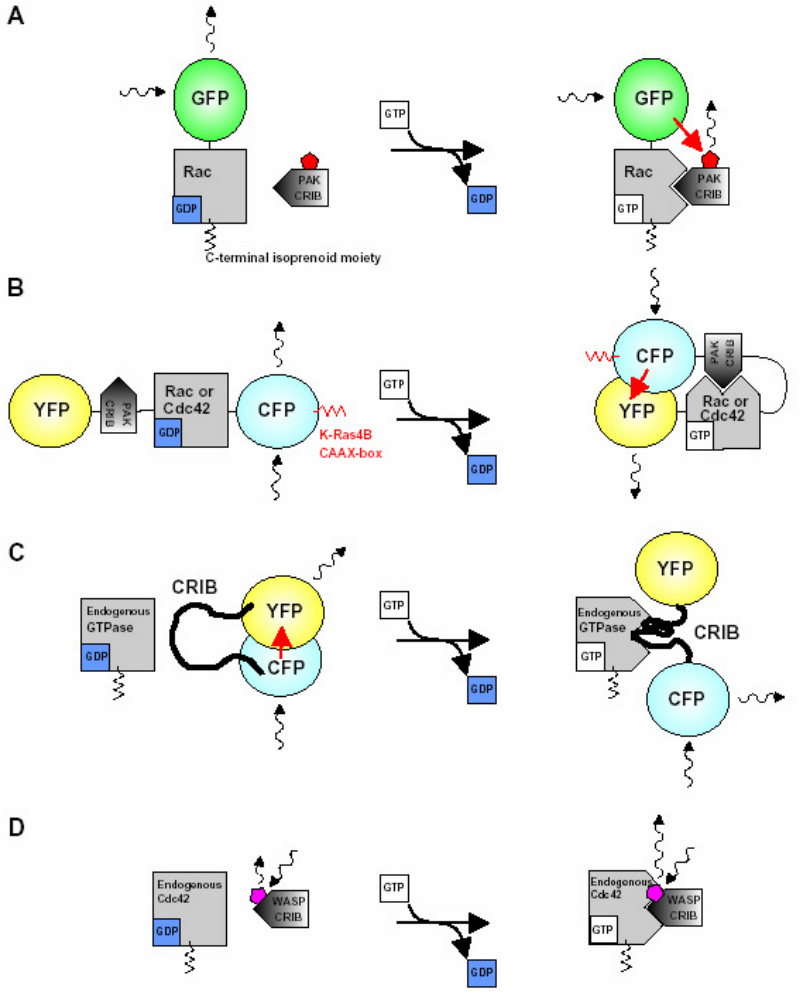
Alice Y. Ting*, Kristin H. Kain†, Richard L. Klemke†, and Roger Y. Tsien*‡§



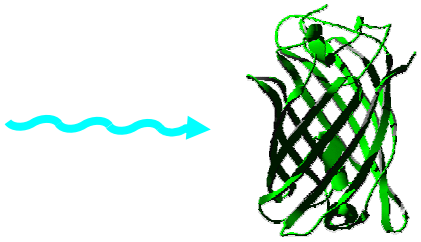
PNAS | December 18, 2001 | vol. 98 | no. 26 | 15003–15008

Designing biosensors for Rho family proteins – deciphering the dynamics of Rho family GTPase activation in living cells

Olivier Pertz and Klaus M. Hahn*

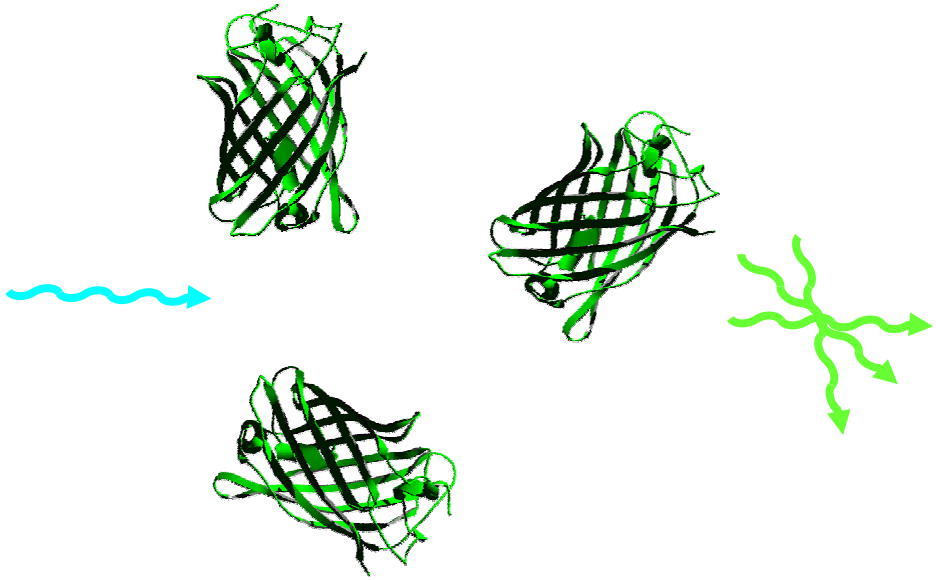


FRET by Anisotropy (Homotransfer)

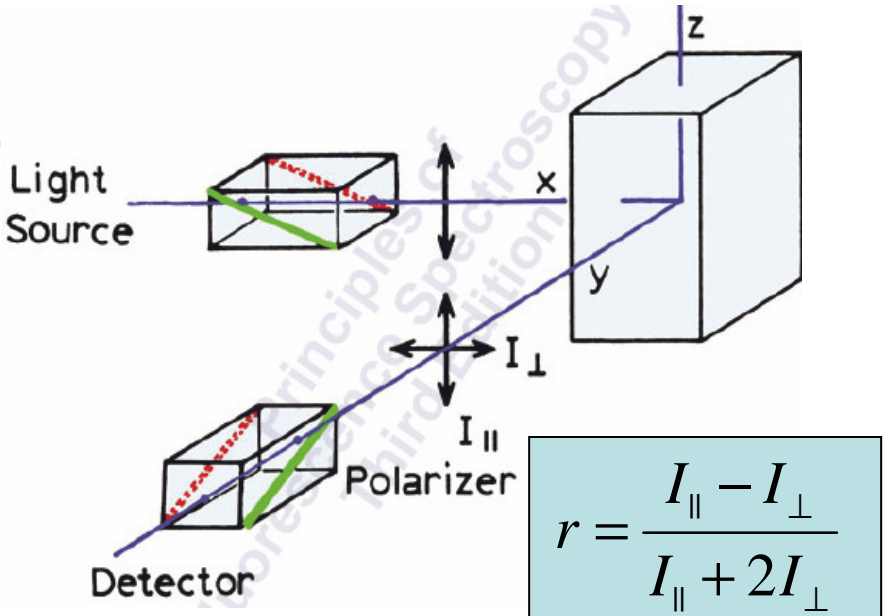


rotational correlation time (~18
>>
fluorescence lifetime (<3 ns)

Emission light is polarized when excited with polarized light !!!



Emission light is depolarized.



$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}$$

Microscope: Widefield / Confocal

Advantages:

- Non-destructive => live cell imaging
- Images can be taken simultaneously
- Useful for dimerization studies (e.g. receptor dimerization)
- Only 1 construct is necessary

Disadvantages: ???

FRET - Microscopy

Method: Detects proximity between donor and acceptor fluorophores (up to ~2-8nm)

Application:

- Protein-protein interactions
- Intramolecular conformational changes
- Biosensors (e.g. Ca^{2+}, GTPases, kinases activity)

Advantages:

- Increases spatial resolution of fluorescence microscopy (~ 200-250nm)

Limitations:

- Absence of FRET is not definitive.
- Due to a long rotational correlation time of GFP (~18ns) no exact distance information can be obtained.

Literature

Review
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