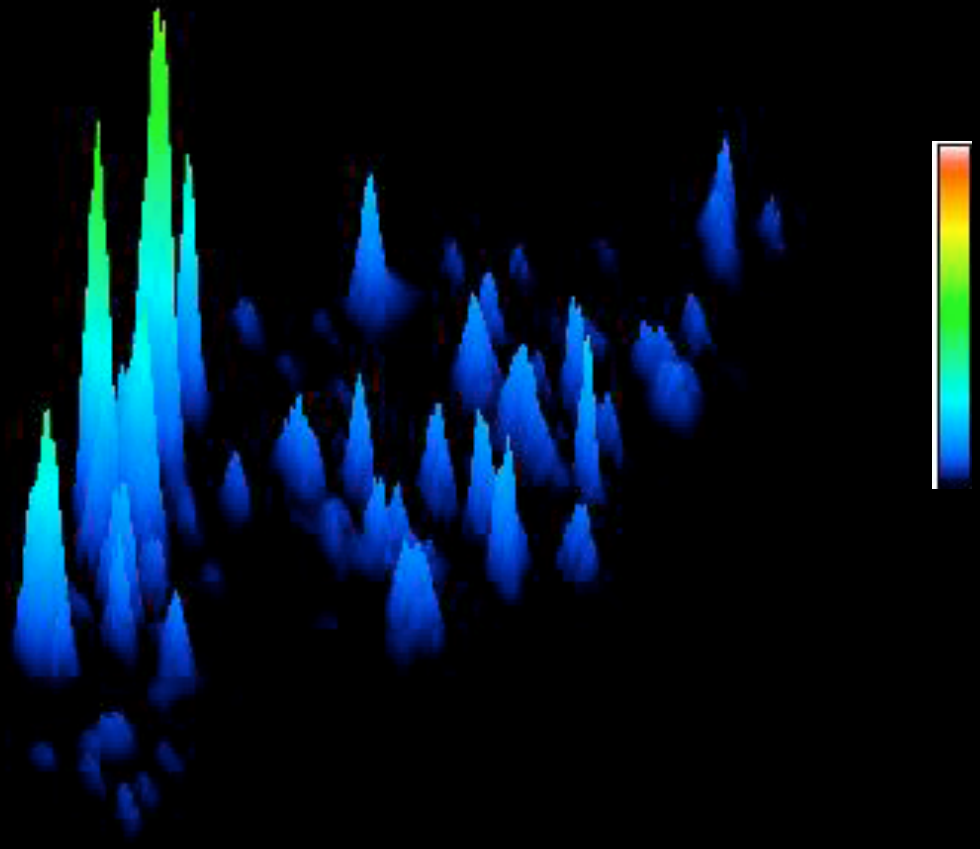
Cell imaging using fluorescence probes



Quantification of molecular dynamics in cells: the "F" words for kinetic microscopy

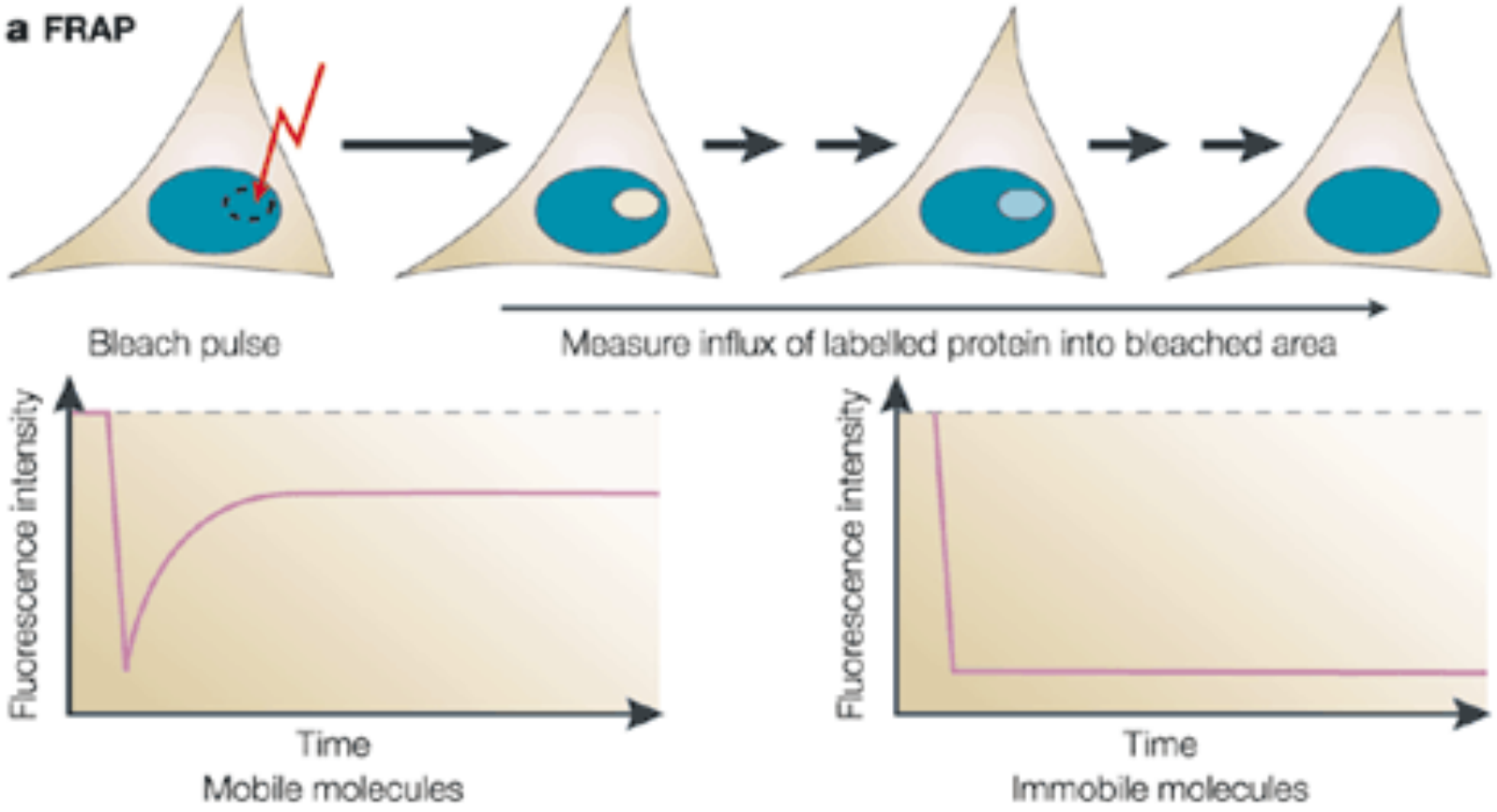
- **FRAP**: Fluorescence Recovery After Photobleaching
- **FLIP**: Fluorescence Loss of Intensity after Photobleaching
- **FLAP**: Fluorescence Localization After Photobleaching

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.

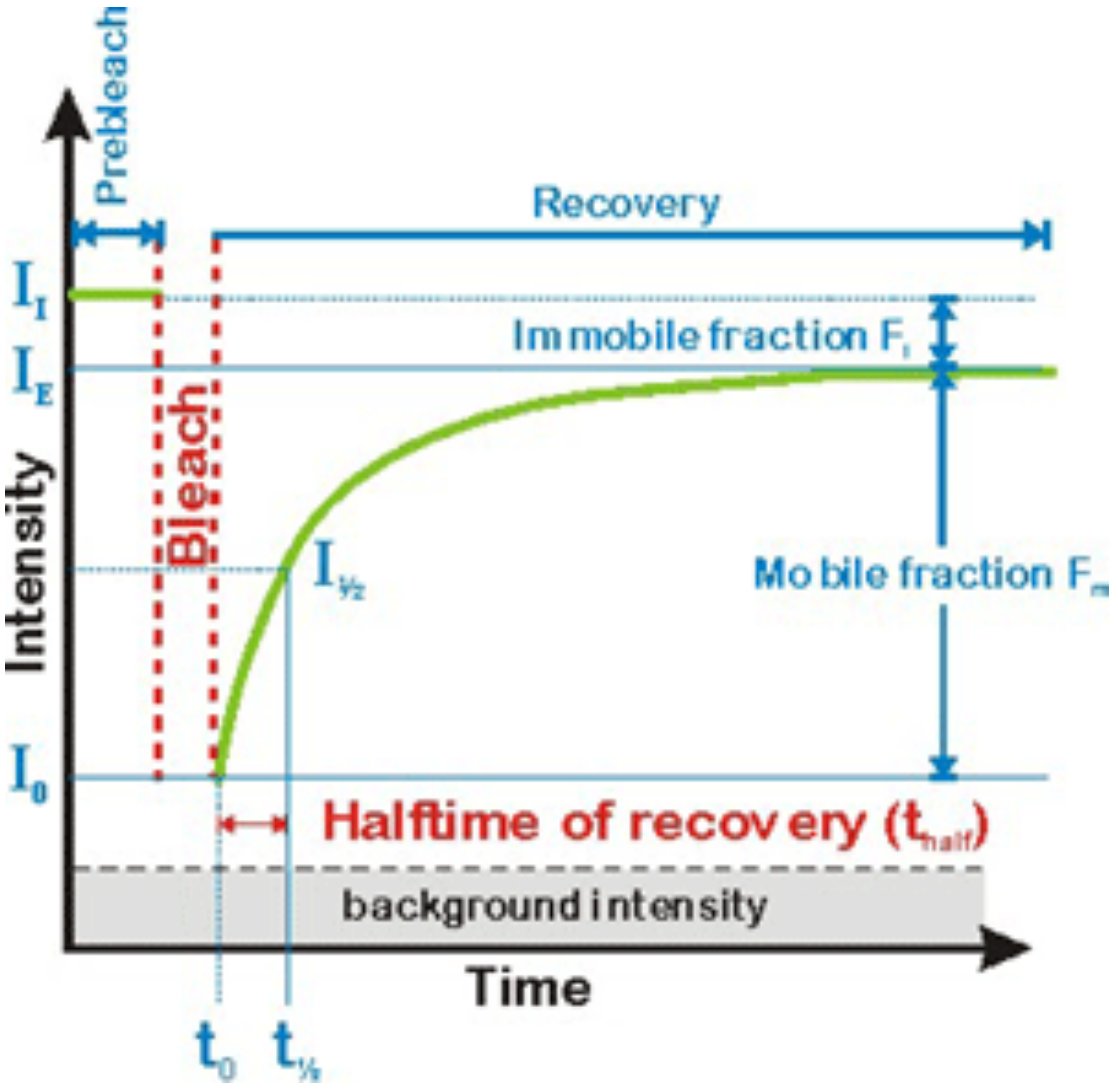
FRAP: Fluorescence Recovery After Photobleaching



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.

The recovery of fluorescence results from the movement of unbleached fluorophores from the surroundings into the bleached area.

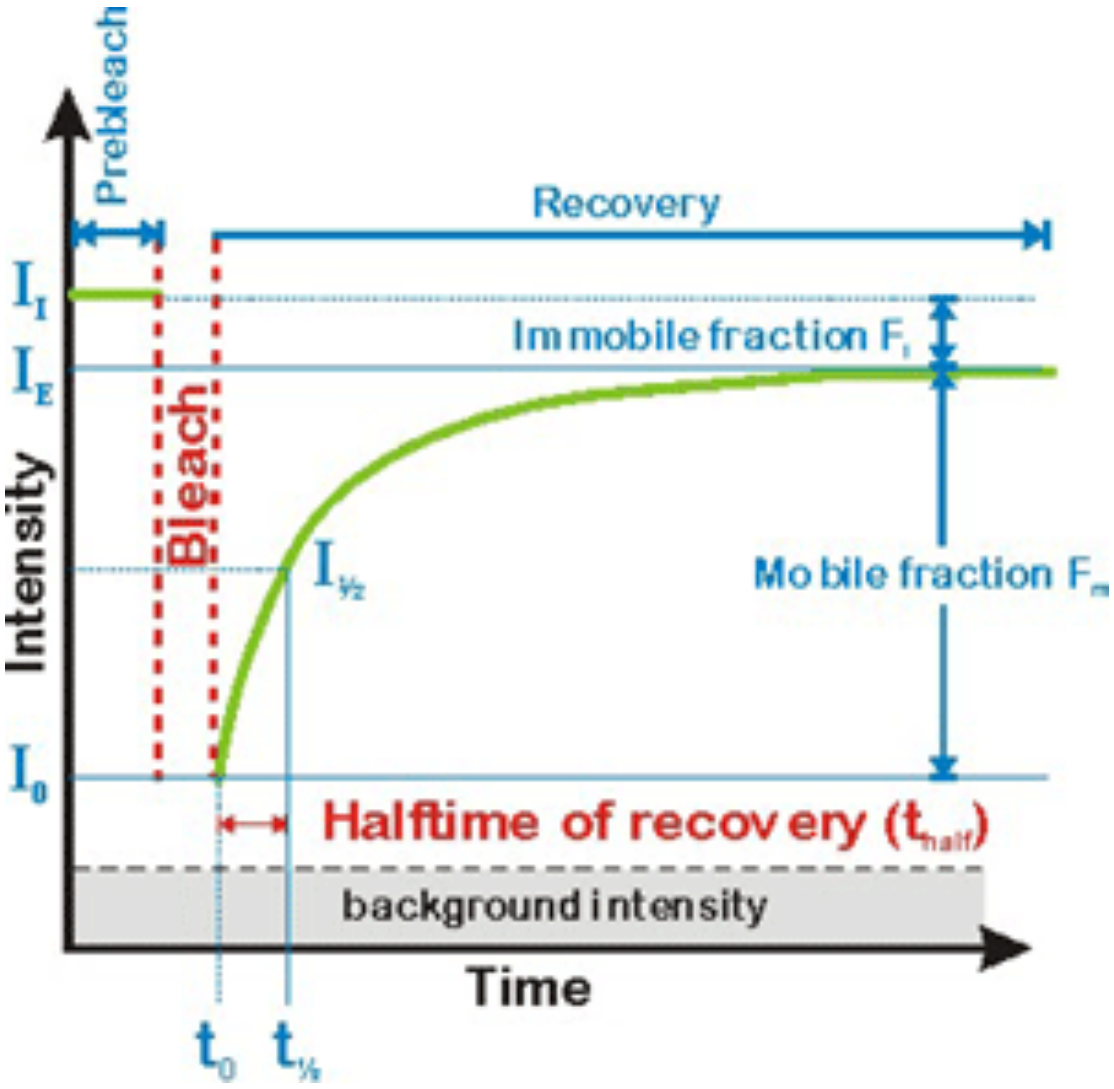
The mean intensity in the ROI is plotted versus time, where the recovery time (half-time) indicates the speed of this mobility, e.g. diffusion time, and the level of fully recovered intensity gives information on mobile/immobile species of the fluorescent molecule.



The Mobile Fraction:

Fraction of fluorescent protein that can diffuse into the bleached area during the time course of the experiment

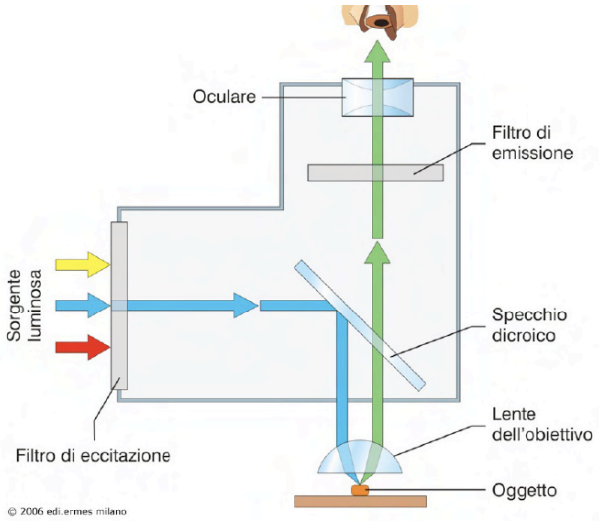
$Fm = I_E / I_I$



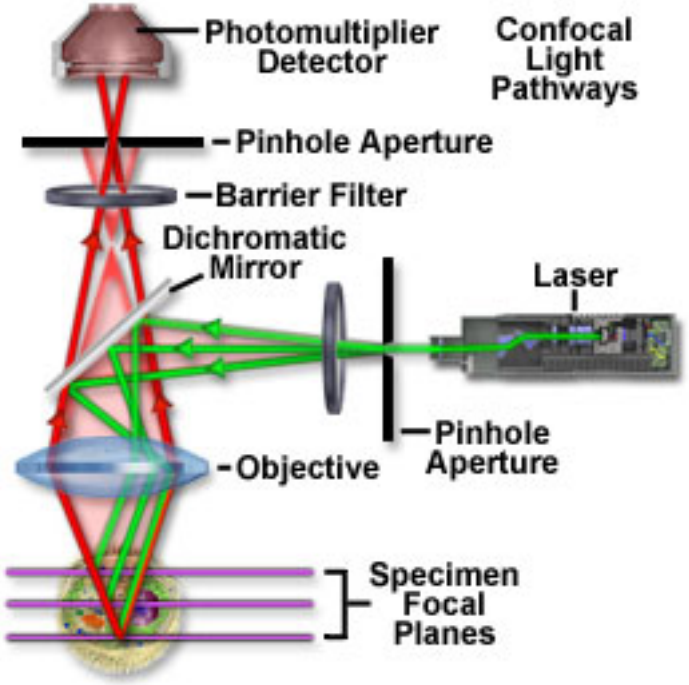
Experimental Setup

The basic apparatus comprises an optical microscope, a light source and some fluorescent probe. Confocal LSM equipped with AOTF are suitable for studies by FRAP.

epifluorescence



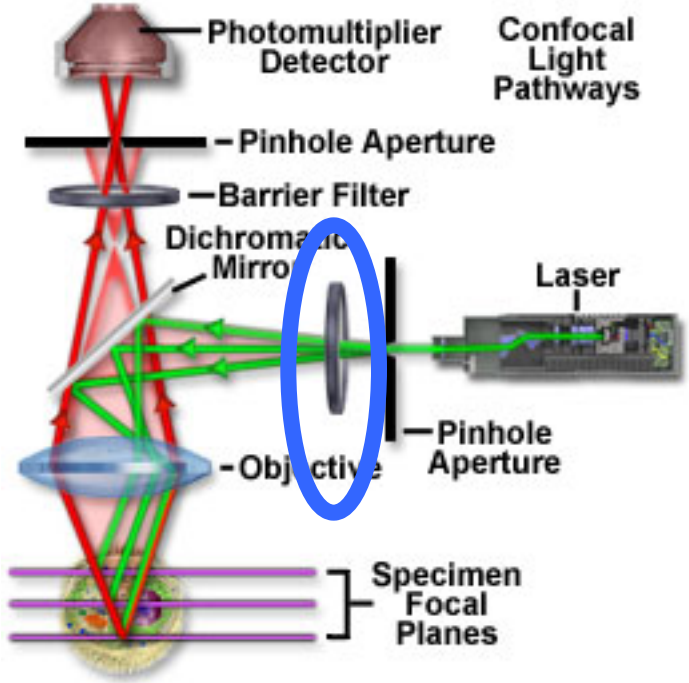
Laser Scanning Confocal Microscpoy

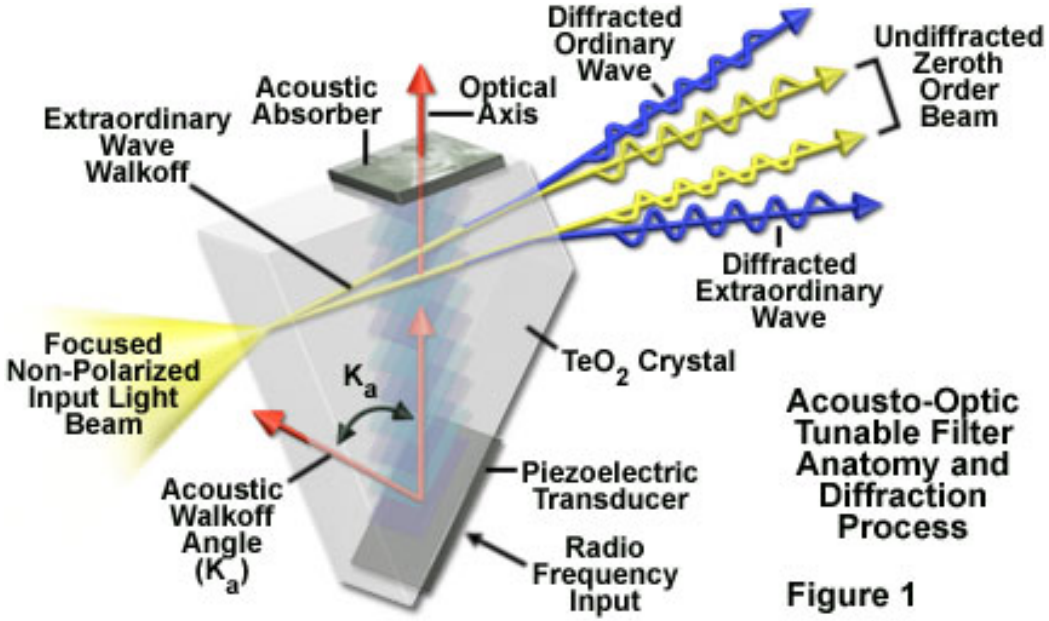


http://www.olympusconfocal.com/theory/

Experimental Setup

AOTF: he acousto-optic tunable filter (AOTF) is an electro-optical device that functions as an electronically tunable excitation filter to simultaneously modulate the intensity and wavelength of multiple laser lines from one or more sources.

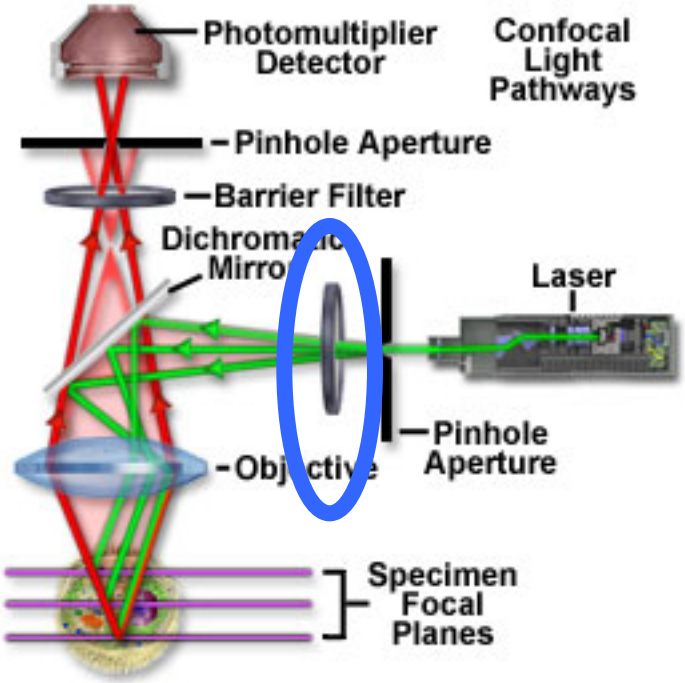




In response to the application of an oscillating radio frequency (RF) electrical signal, the transducer generates a high-frequency vibrational (acoustic) wave that propagates into the crystal. The alternating ultrasonic acoustic wave induces a periodic redistribution of the refractive index through the crystal that acts as a transmission diffraction grating or Bragg diffracter to deviate a portion of incident laser light into a first-order beam, which is utilized in the microscope (or two first-order beams when the incident light is non-polarized; see Figure 1). Changing the frequency of the transducer signal applied to the crystal alters the period of the refractive index variation, and therefore, the wavelength of light that is diffracted. The relative intensity of the diffracted beam is determined by the amplitude (power) of the signal applied to the crystal

Experimental Setup

AOTF: he acousto-optic tunable filter (AOTF) is an electro-optical device that functions as an electronically tunable excitation filter to simultaneously modulate the intensity and wavelength of multiple laser lines from one or more sources.



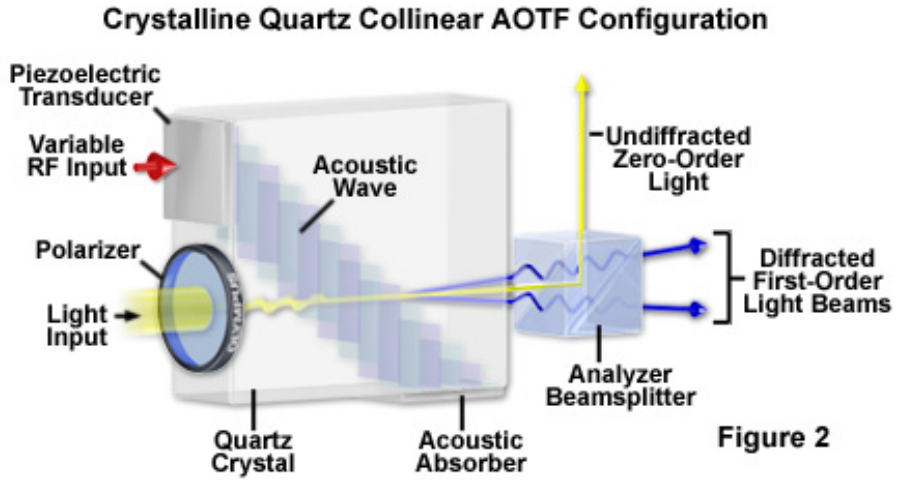


Figure 2

http://www.olympusconfocal.com/theory/

Experimental Setup

The basic apparatus comprises an optical microscope, a light source and some fluorescent probe. Confocal LSM equipped with AOTF are suitable for studies by FRAP.

Execution of FRAP experiment

1. Saving some background images of the sample before photobleaching.
2. 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

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

$$D = w2 / 4t1/2$$

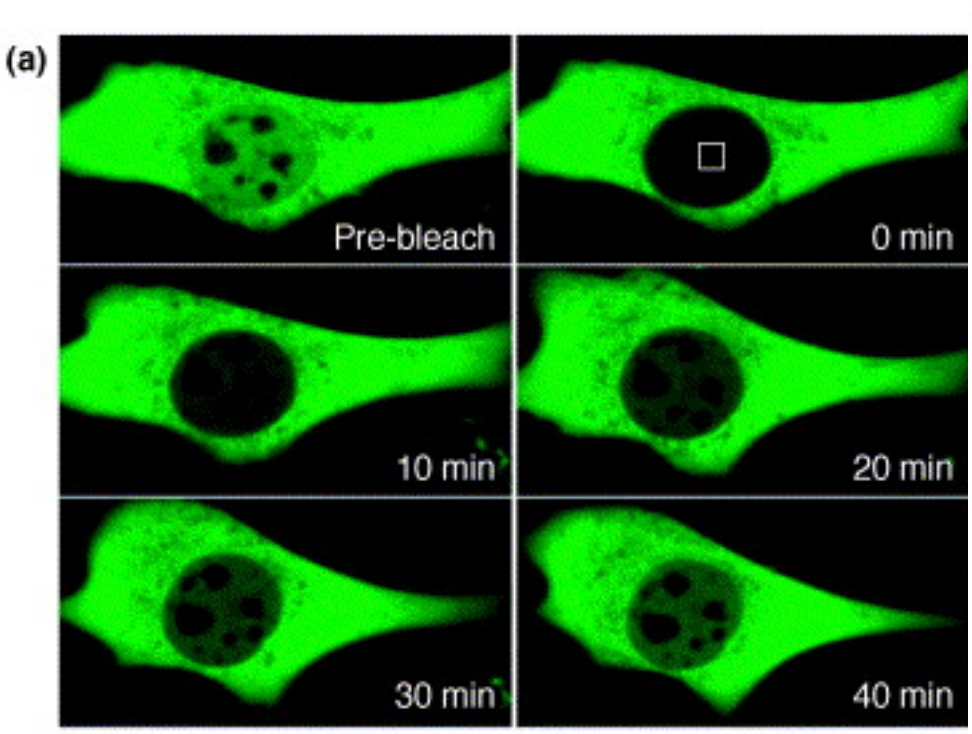
where w is the width of the beam and t1/2 is the time required for the bleach spot to recover half of its initial intensity.

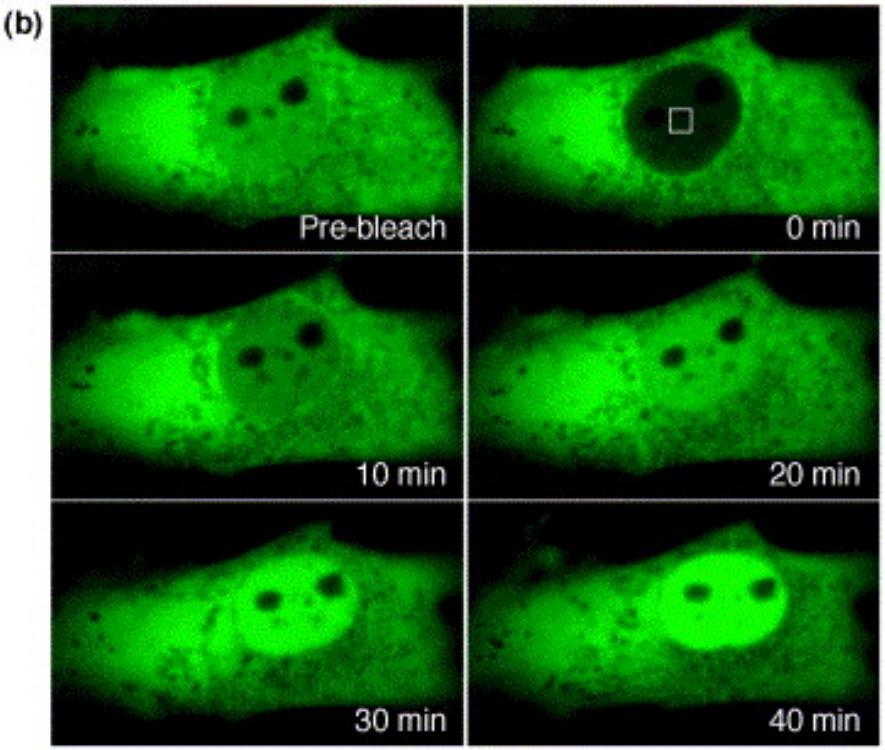
APPLICATION OF FRAP TO NUCLEOCYTOPLASMIC SHUTTLING

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

CNTRL

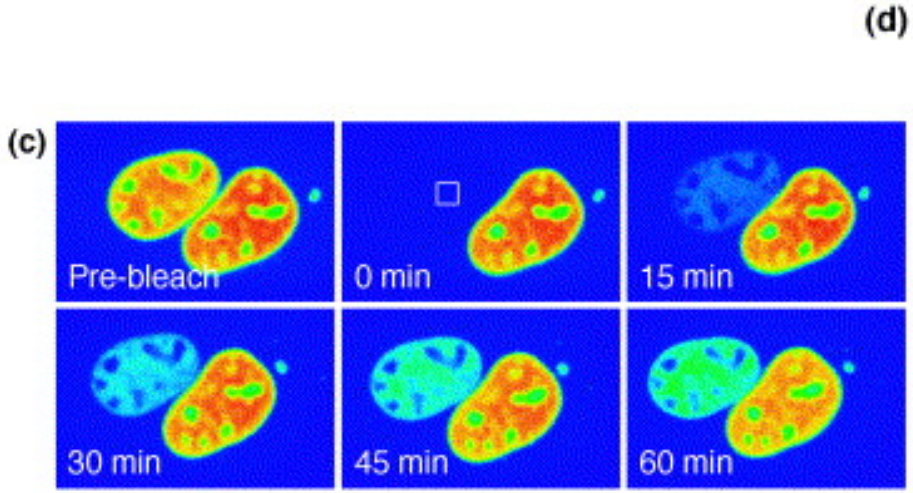
IFN stimulation

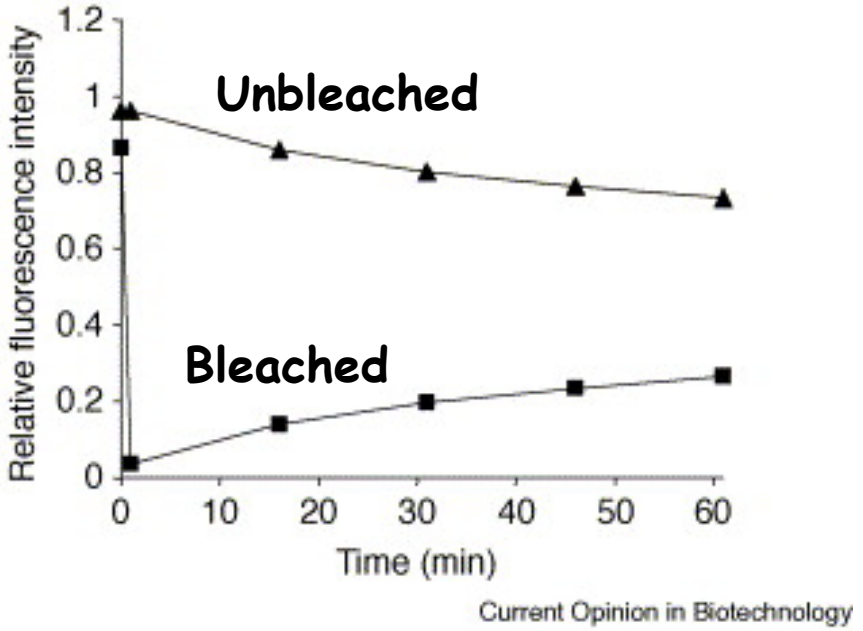




Koster M et al, Curr Opinion in Biotech, 2005, 16: 28-34

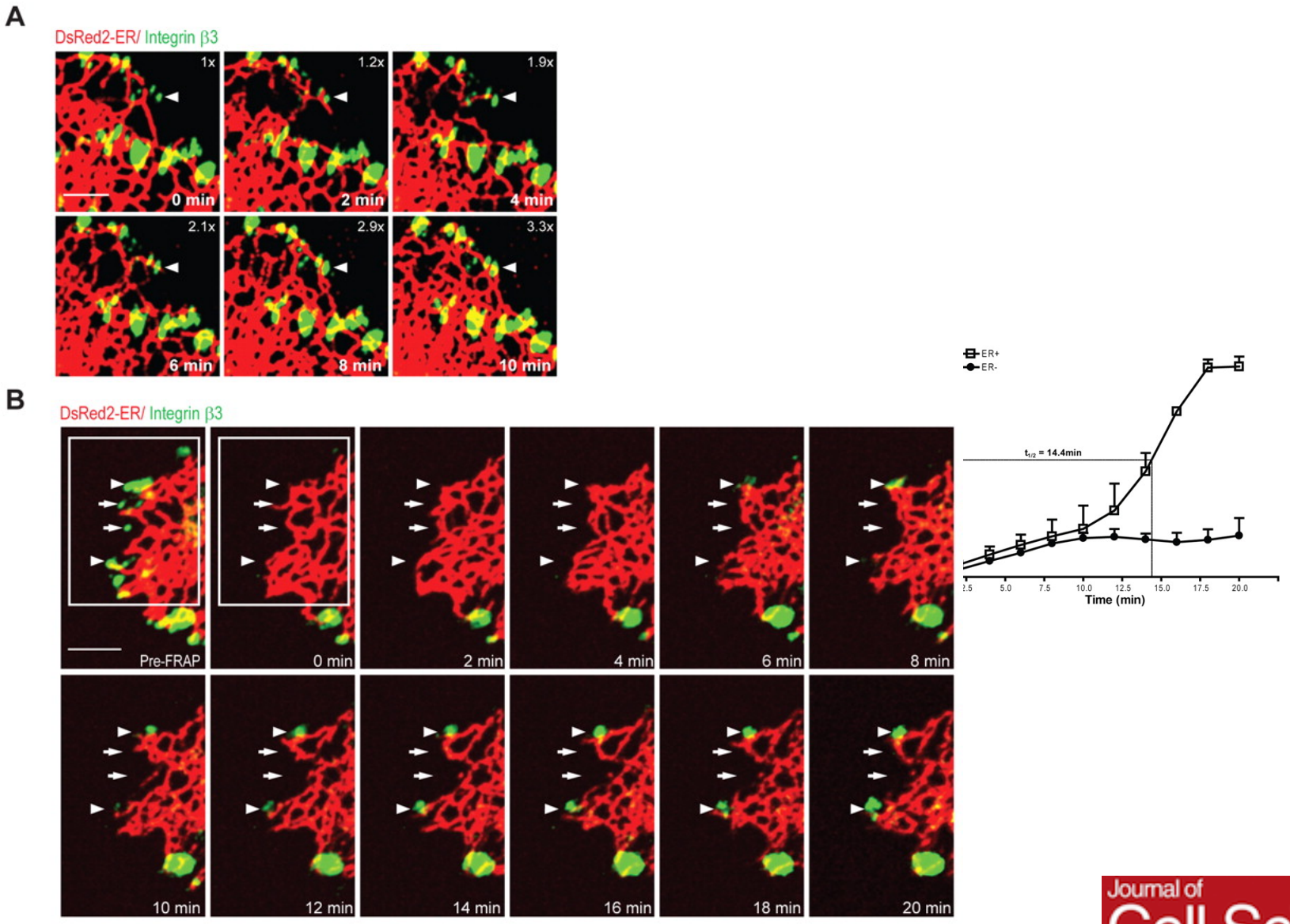
Selective FRAP to demonstrate repeated nucleocytoplasmic shuttling of activated STAT1–EGFP. After bleaching of one nucleus the recovery of STAT1–EGFP during ongoing signaling was monitored for 60 min. Fluorescence intensities of the entire unbleached and bleached nucleus were measured and corrected for background intensity.





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

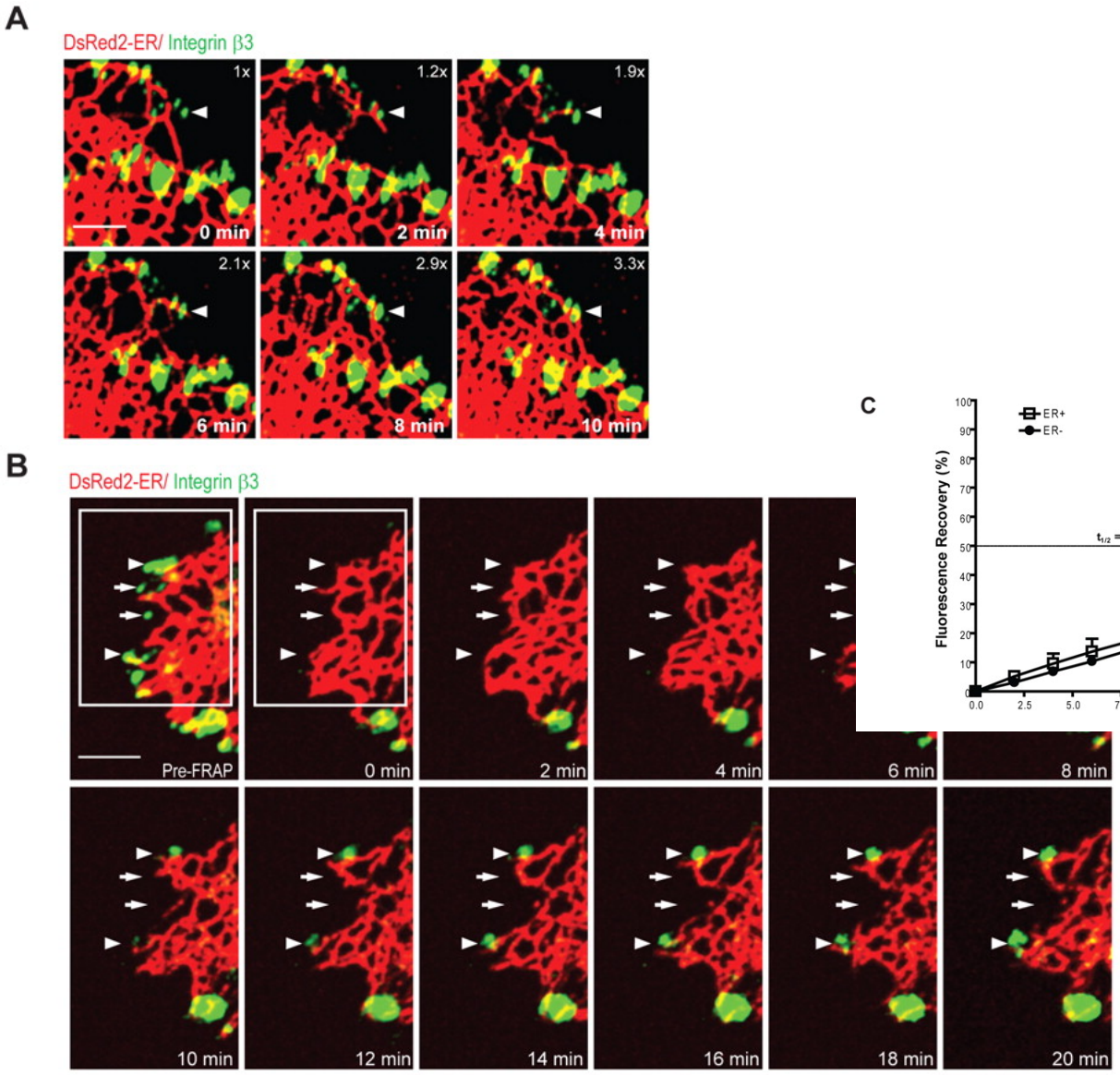
ER extension to FAs is required for integrin β3 protein recruitment.



Zhang X et al. J Cell Sci 2010;123:3901-3912

Journal of Cell Science
jcs.biologists.org

ER extension to FAs is required for integrin β3 protein recruitment.



Zhang X et al. J Cell Sci 2010;123:3901-3912

Journal of Cell Science
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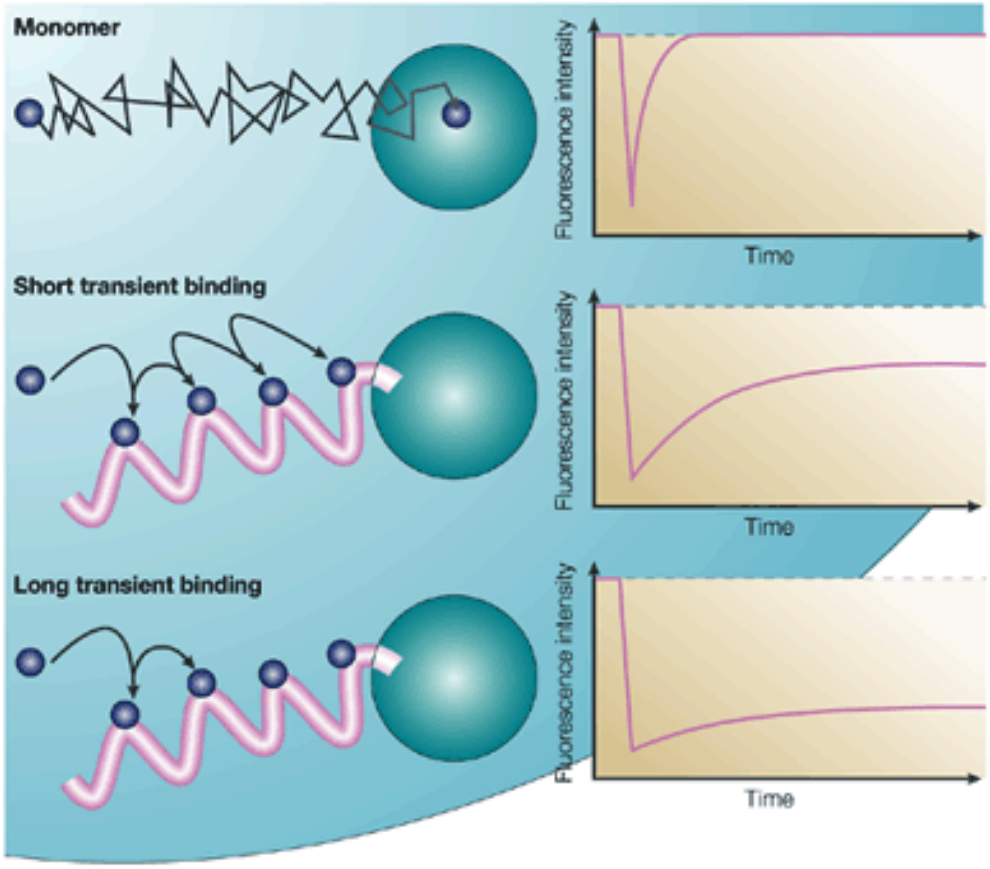
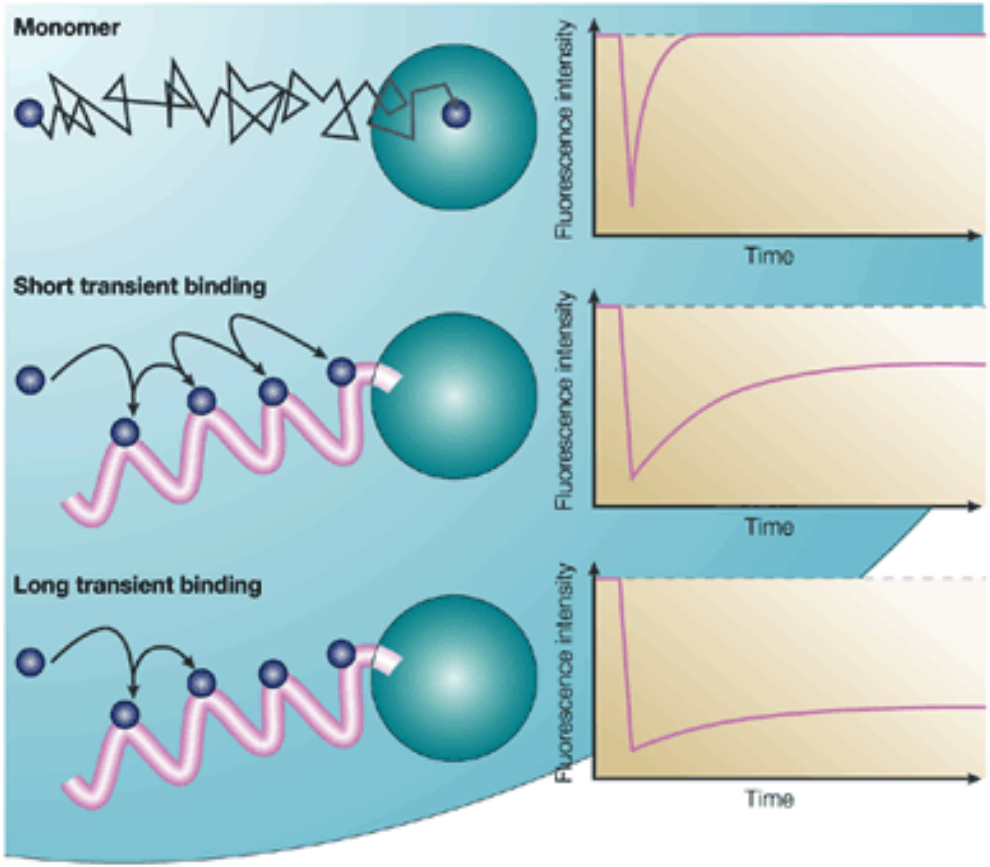
FRAP for the measurement of protein binding versus free diffusion *in vivo*.

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



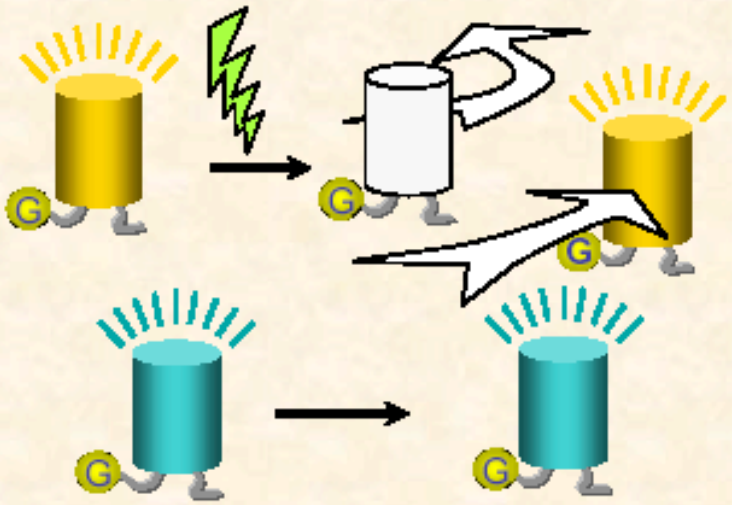
FRAP for the measurement of protein binding versus free diffusion *in vivo*.

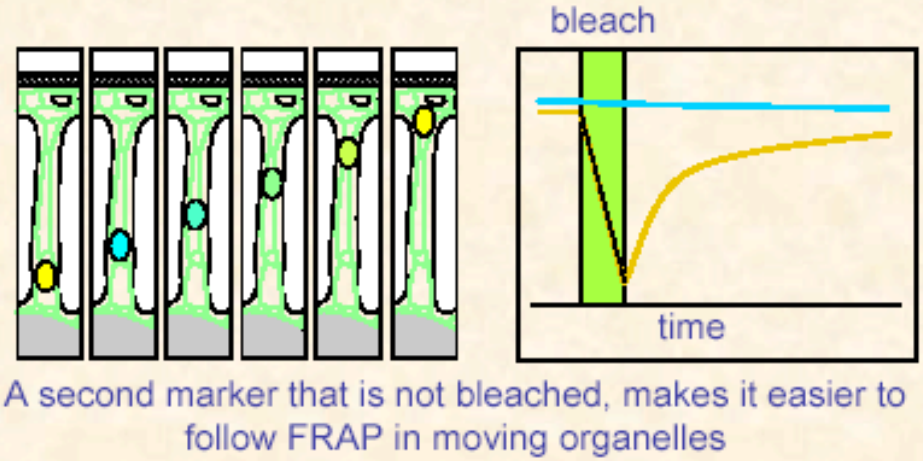
The fact that the interaction properties of a protein are reflected in the mobility measurement complicates the analysis of photobleaching data



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

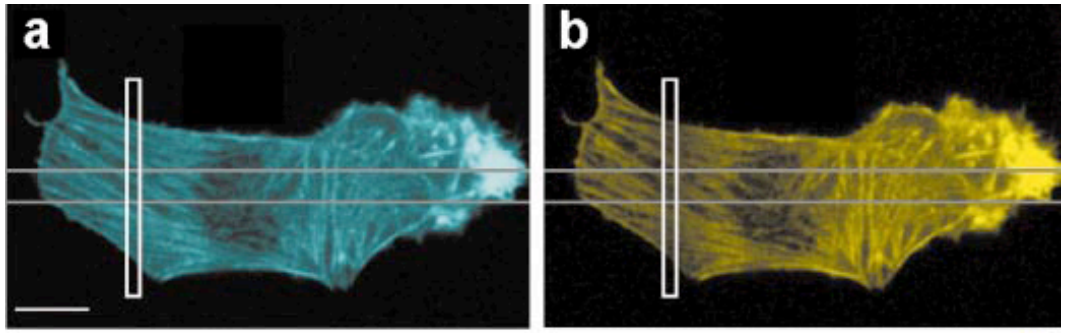
FLAP: Fluorescence Localization After Photobleaching

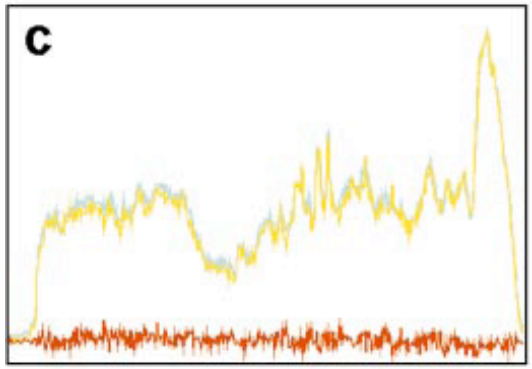


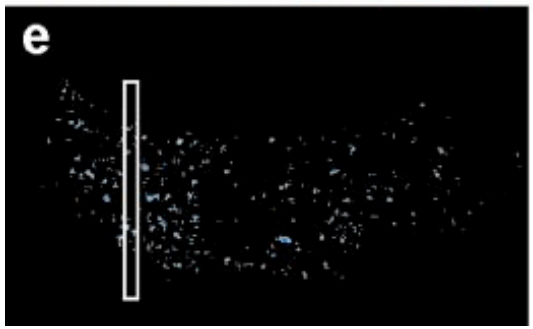


The molecular species to be located carries two different fluorophores that can be imaged independently but simultaneously by fluorescence microscopy. One of the fluorophores YFP (the target fluorophore) is then rapidly photobleached at a chosen location. The unbleached CFP (reference) fluorophore remains 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.

CFP-actin YFP-actin



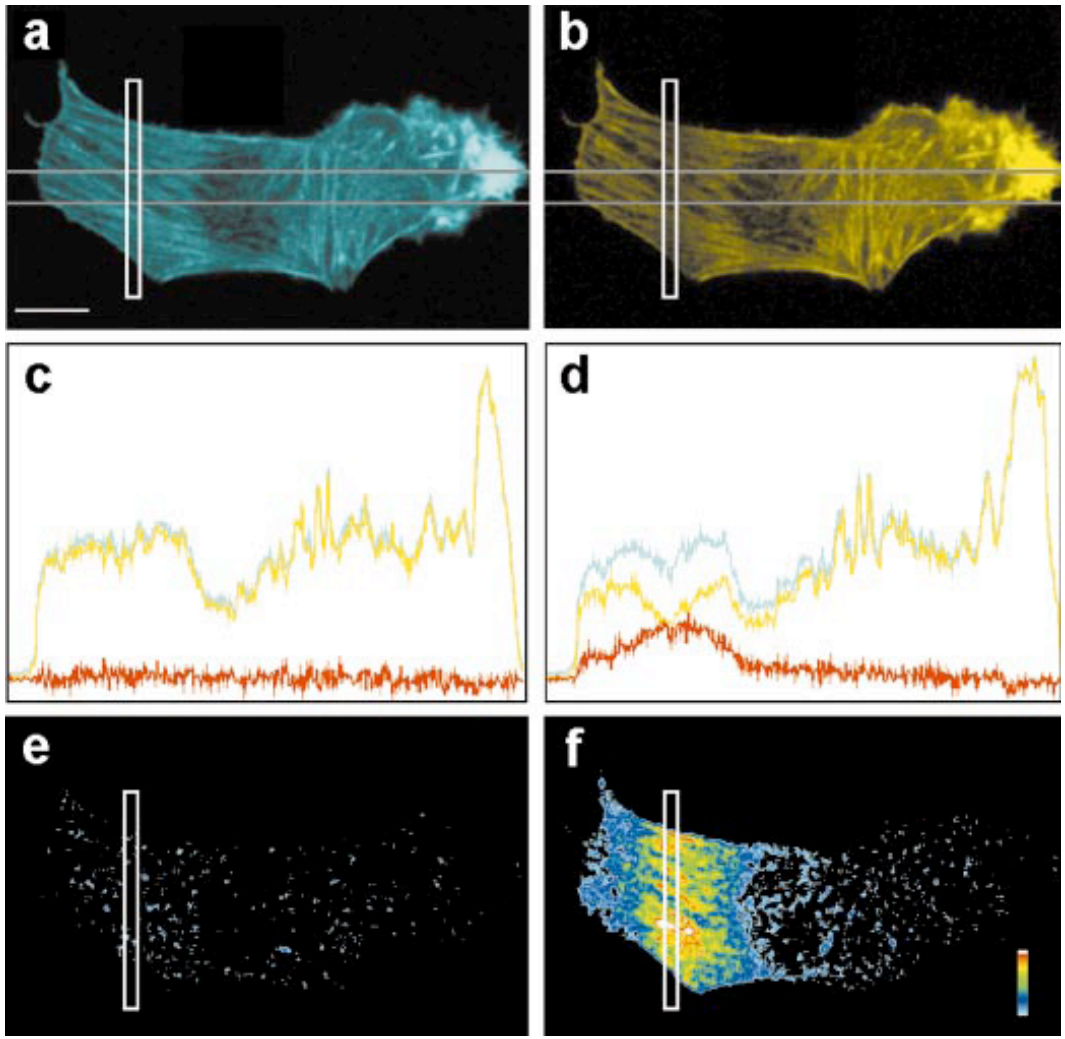




Confocal microscopy revealed that the two fluorophores are accurately co-localized within the cells and thus that the intensity difference – the FLAP signal – is close to zero throughout the image

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

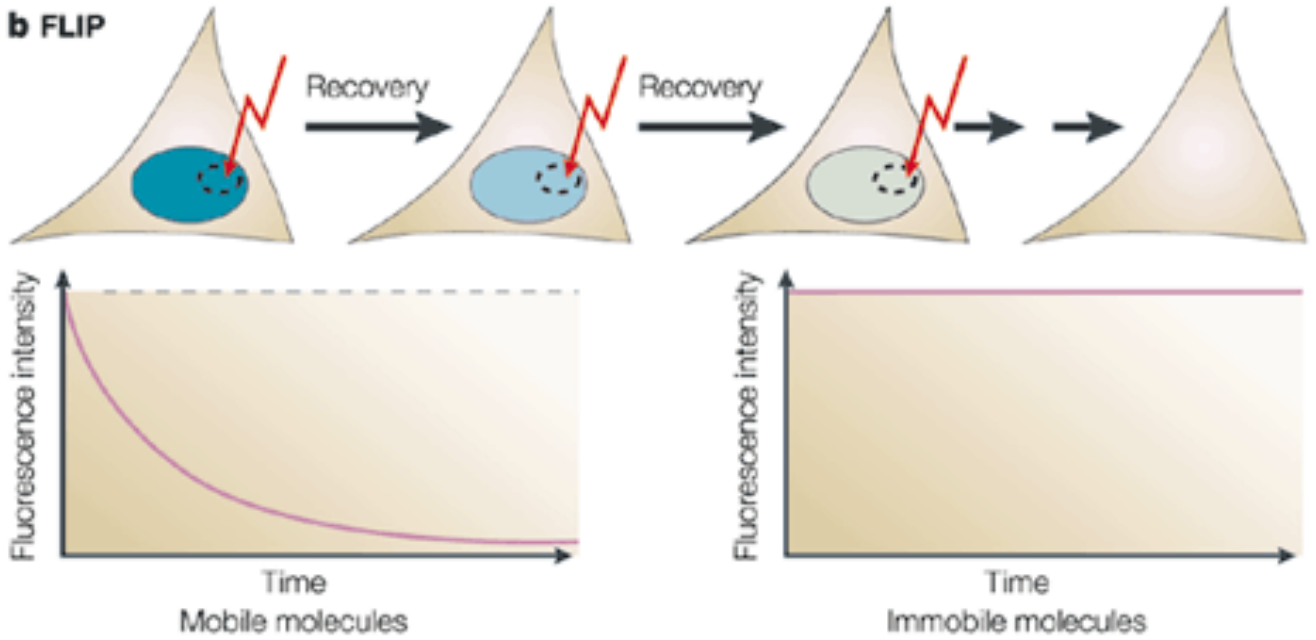
CFP-actin YFP-actin



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

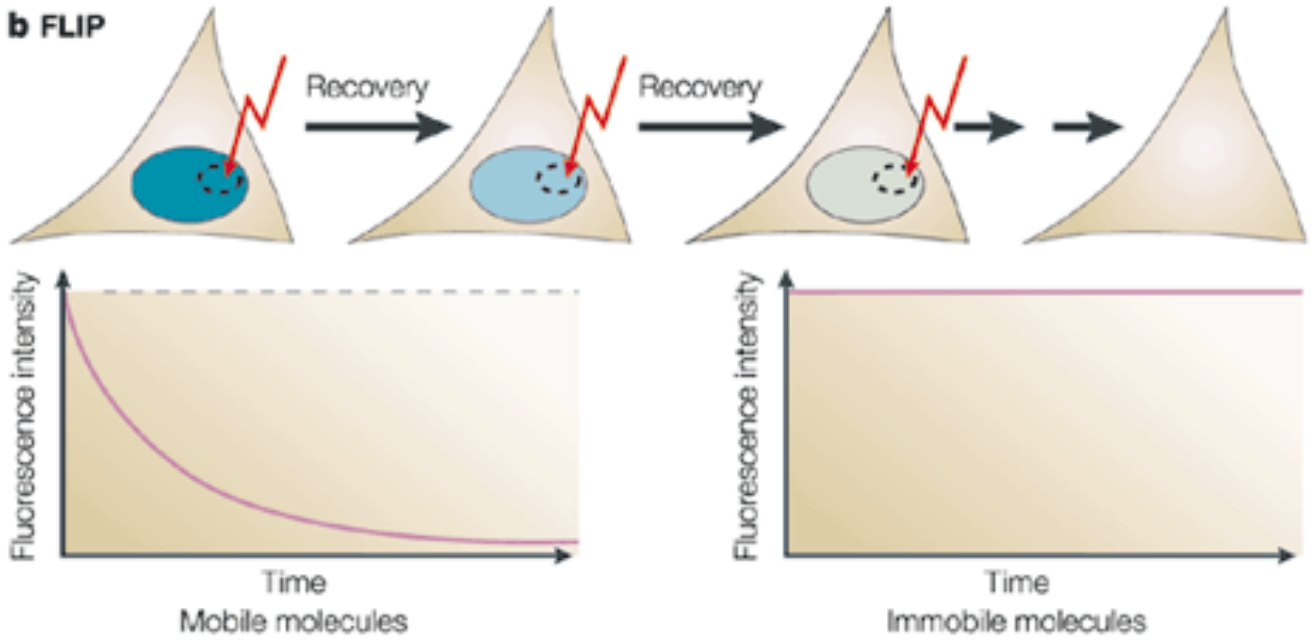
Dunn et al., J Microscopy. 2002; 205:109-112.

FLIP: Fluorescence Loss In Photobleaching



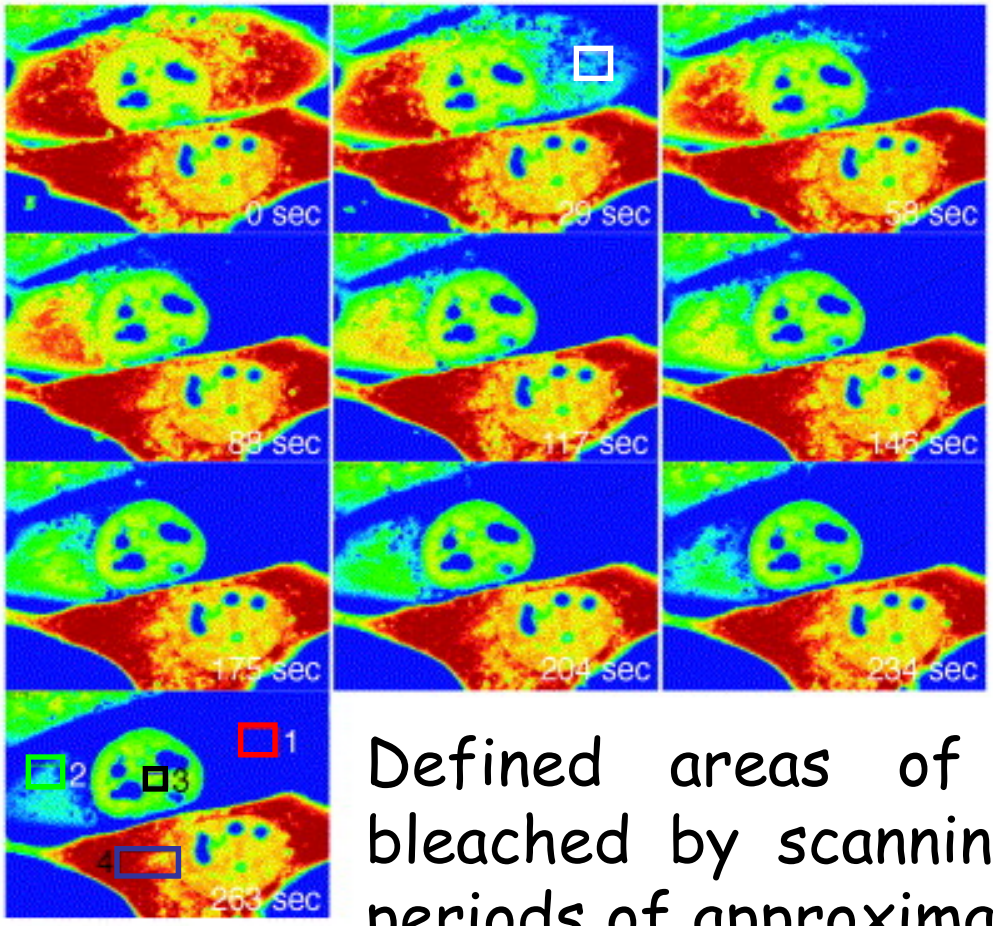
An area within the cell is repeatedly bleached and the loss of fluorescence in areas that are distant from the bleach area is monitored

FLIP: Fluorescence Loss In Photobleaching



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.

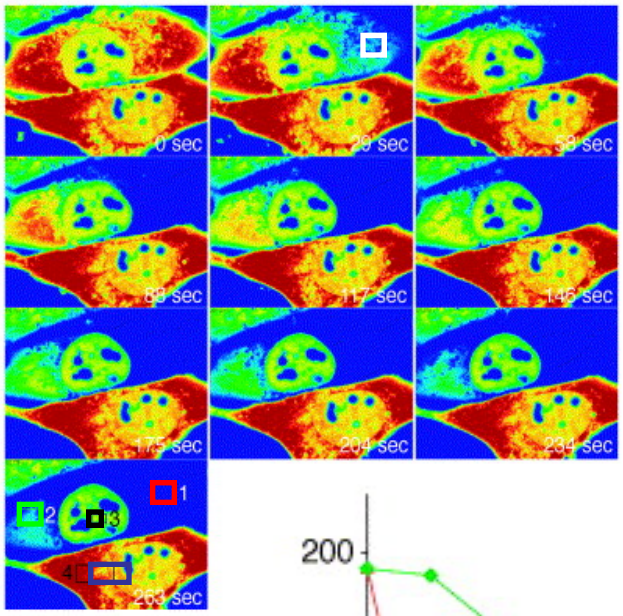
APPLICATION OF FLIP TO NUCLEOCYTOPLASMIC SHUTTLING



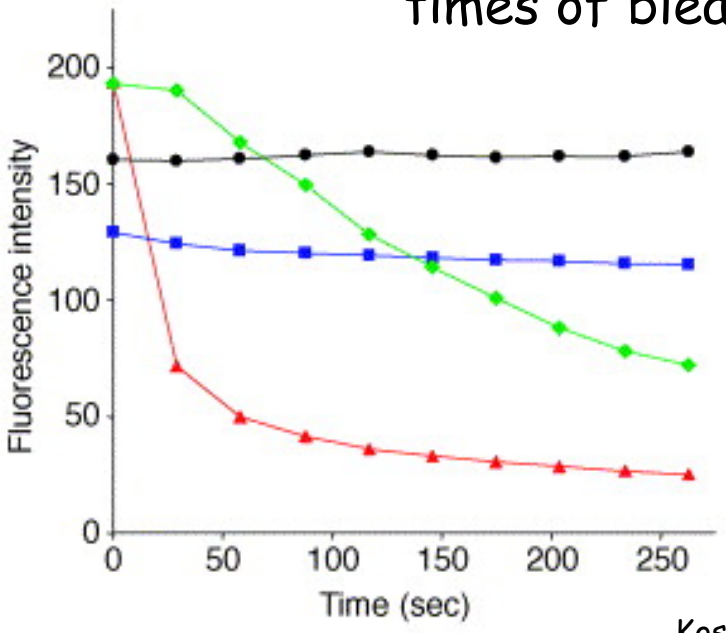
Defined areas of the cytoplasm were bleached by scanning for nine consecutive periods of approximately 30 s with maximum laser intensity.

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

APPLICATION OF FLIP TO NUCLEOCYTOPLASMIC SHUTTLING



To correct the acquired fluorescence intensities for a generalized bleaching effect, which results from the imaging scan, an unbleached neighboring cell in the same window was monitored. The total fluorescence of the bleached cell and of a neighboring cell was monitored between the times of bleaching.



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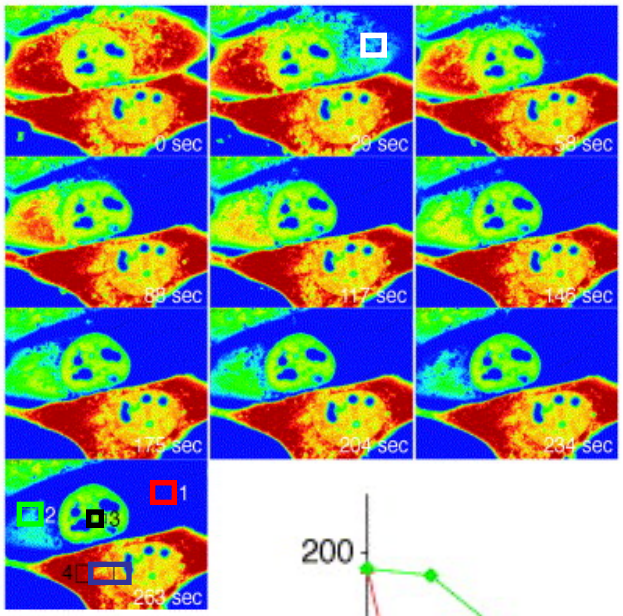
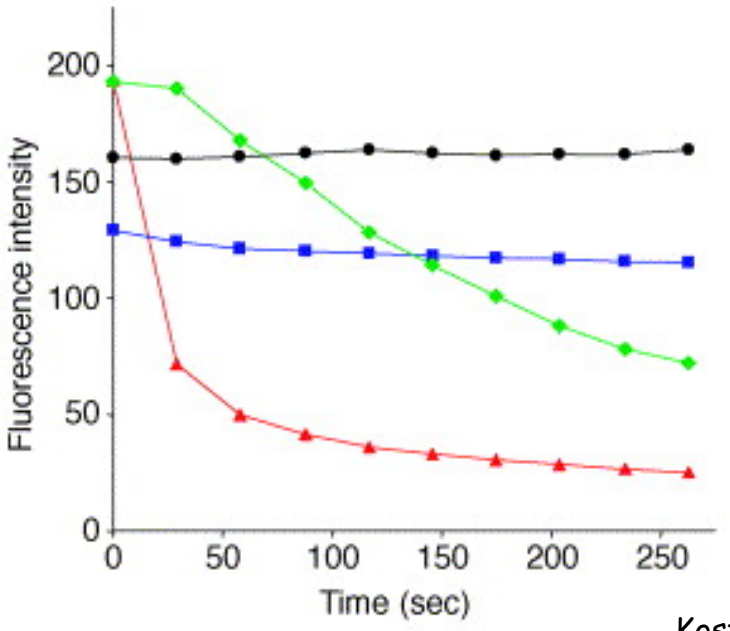
APPLICATION OF FLIP TO NUCLEOCYTOPLASMIC SHUTTLING



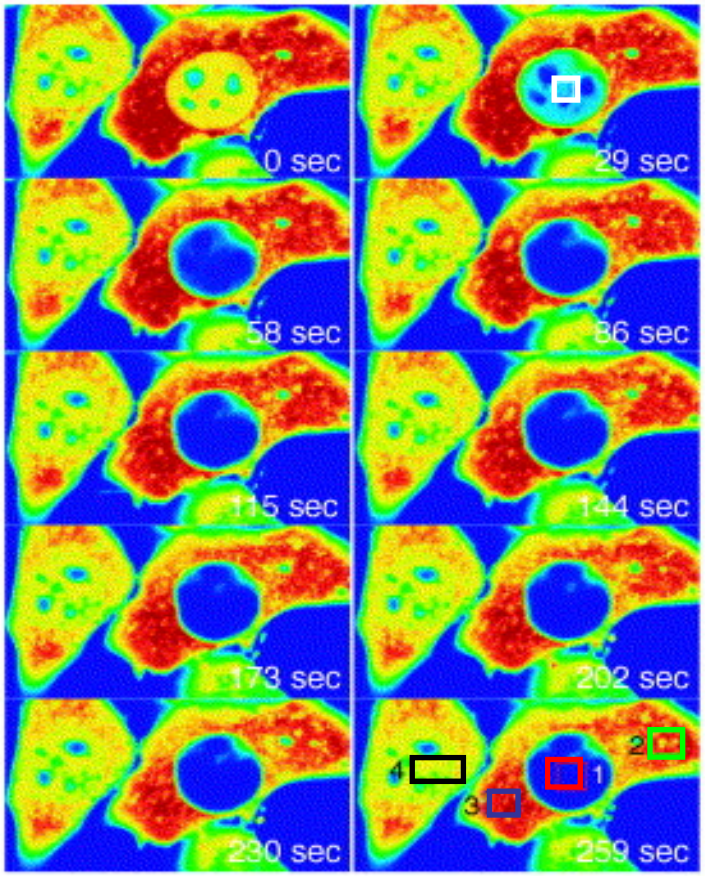
The fluorescence population within the nucleus is not affected.

During this short time period, no significant export of STAT1-EGFP form the nuclear compartment take place.

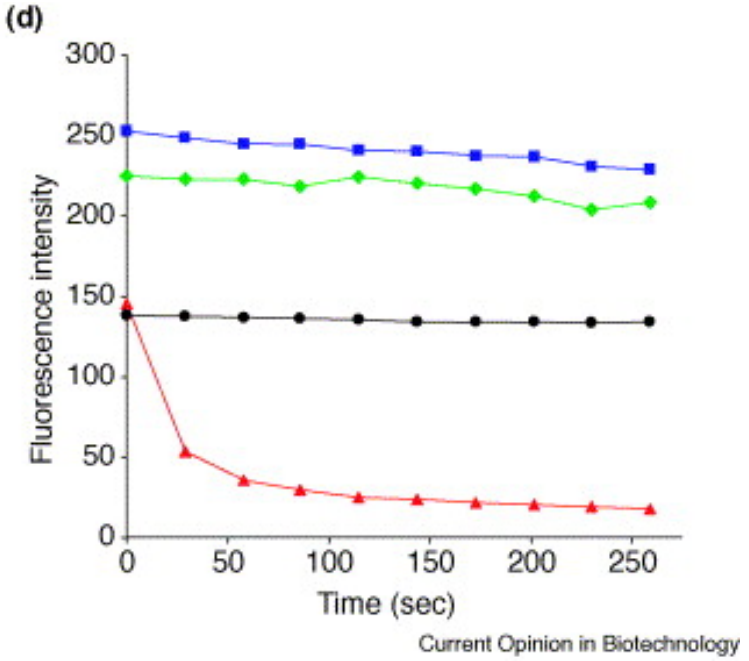


Koster M et al, Curr Opinion in Biotech, 2005, 16: 28-34

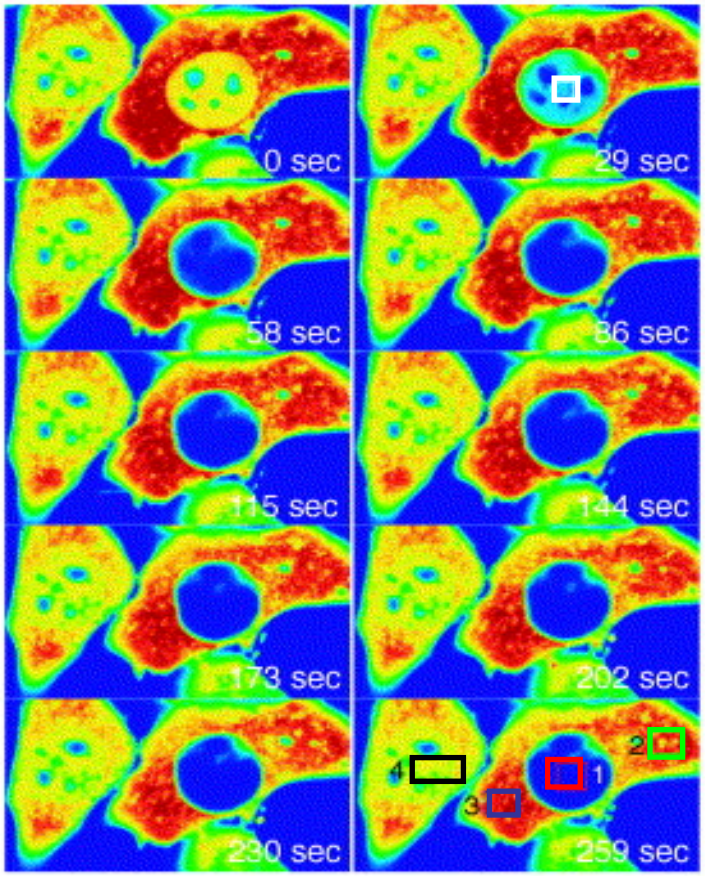
APPLICATION OF FLIP TO NUCLEOCYTOPLASMIC SHUTTLING



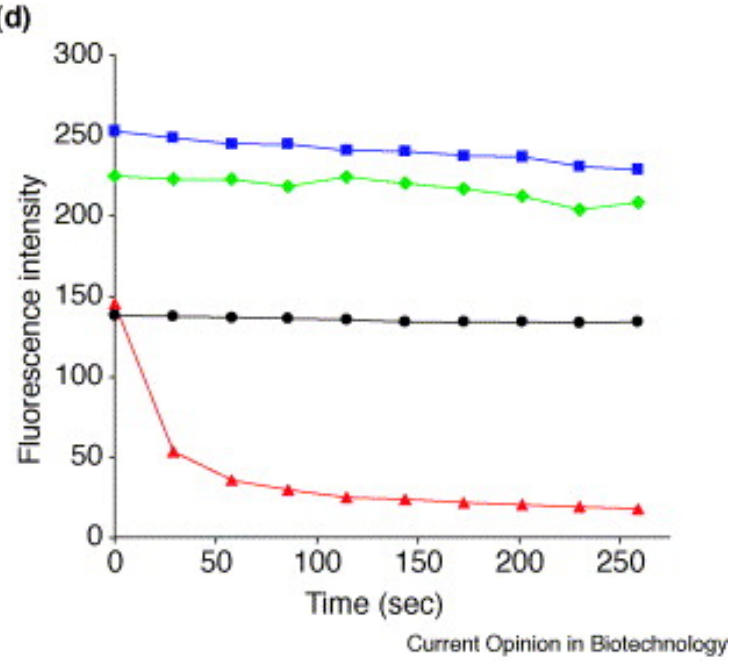
To analyze nucleocytoplasmic exchange of cytoplasmasmically localized proteins bleaching is carried out in a nuclear area.



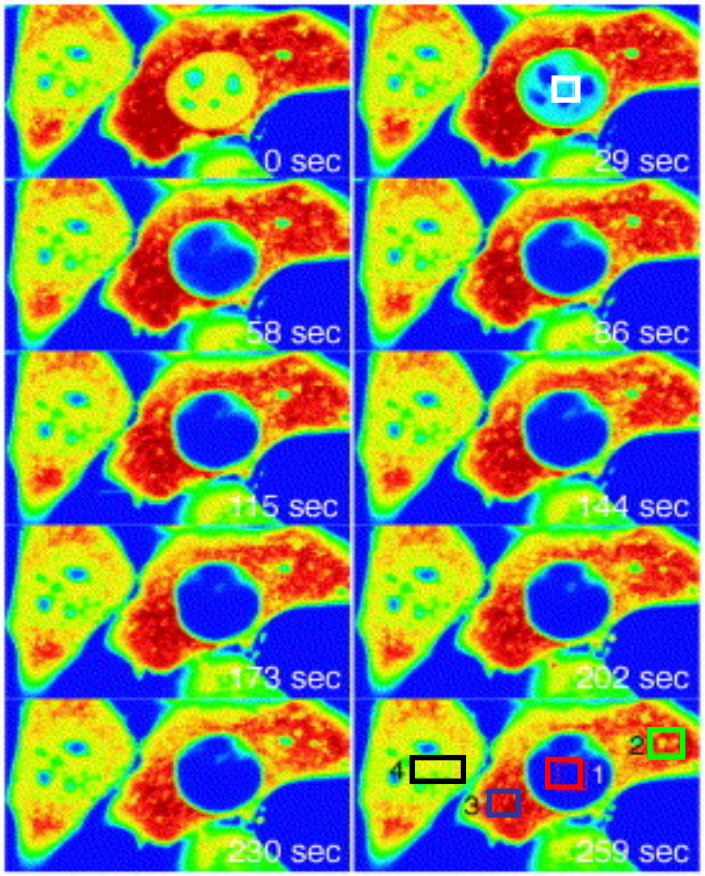
APPLICATION OF FLIP TO NUCLEOCYTOPLASMIC SHUTTLING



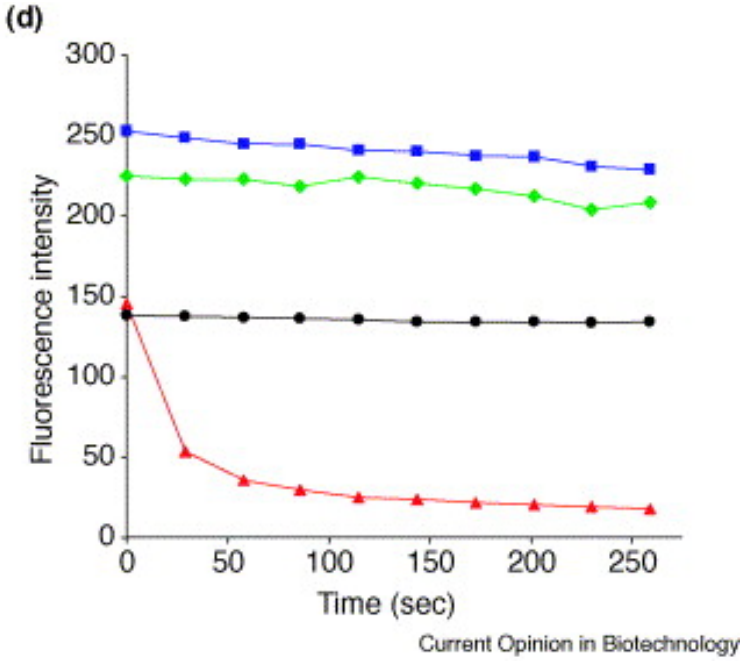
An additional correction of the row data for background bleaching of the cytoplasmic pool is necessary



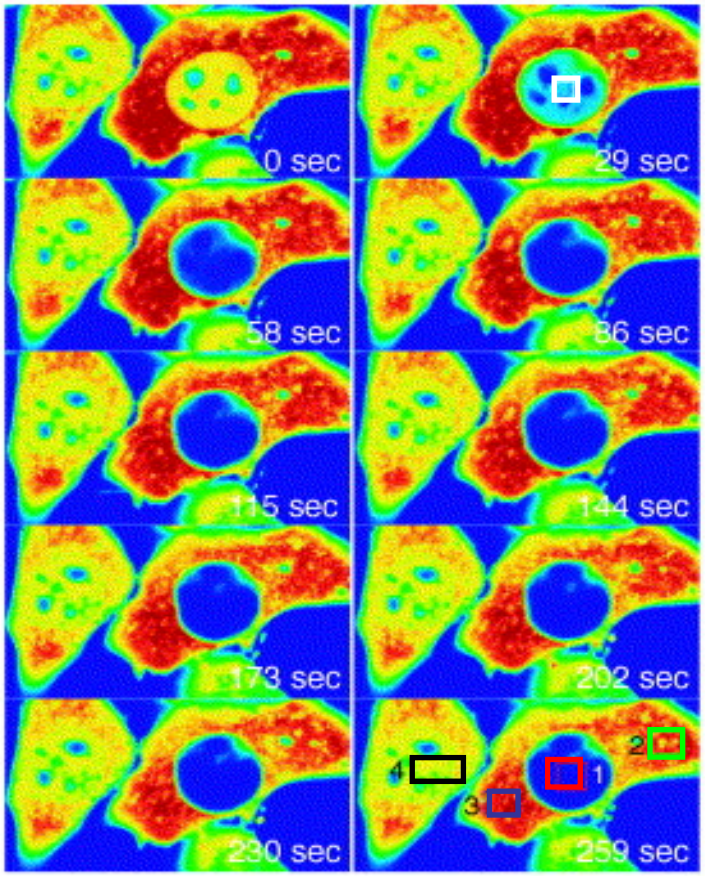
APPLICATION OF FLIP TO NUCLEOCYTOPLASMIC SHUTTLING



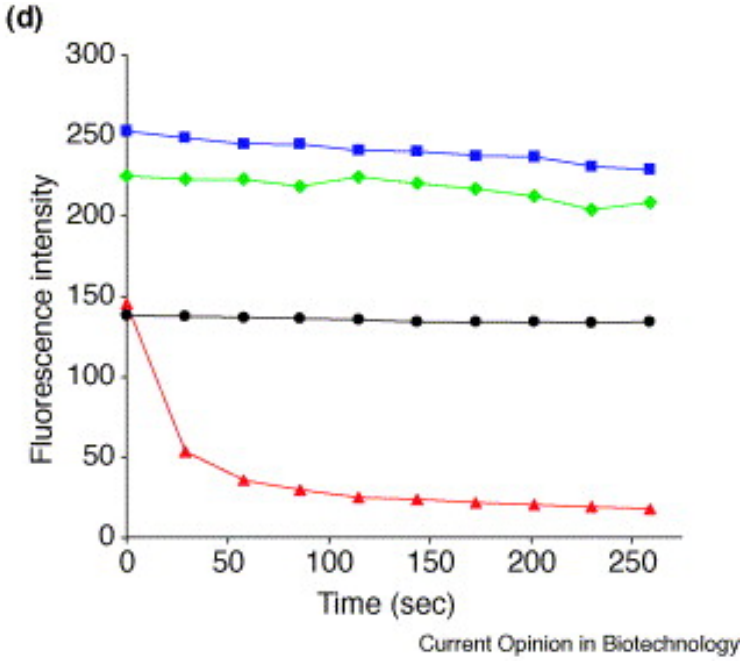
The decrease of fluorescence in the cytoplasm monitors the exchange of molecules between both compartments.



APPLICATION OF FLIP TO NUCLEOCYTOPLASMIC SHUTTLING



The results show that a loss of fluorescence intensity in the cytoplasmic fraction due to the bleaching through the vertical axis is negligible.



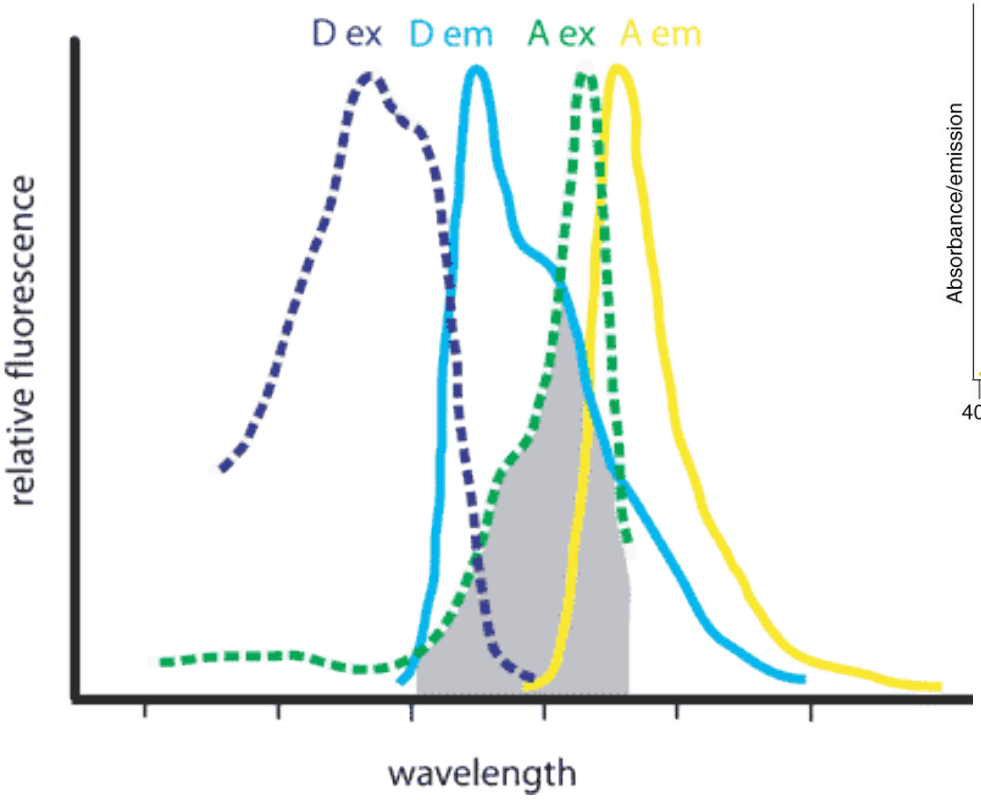
FRET: Föster Resonance Energy Transfer

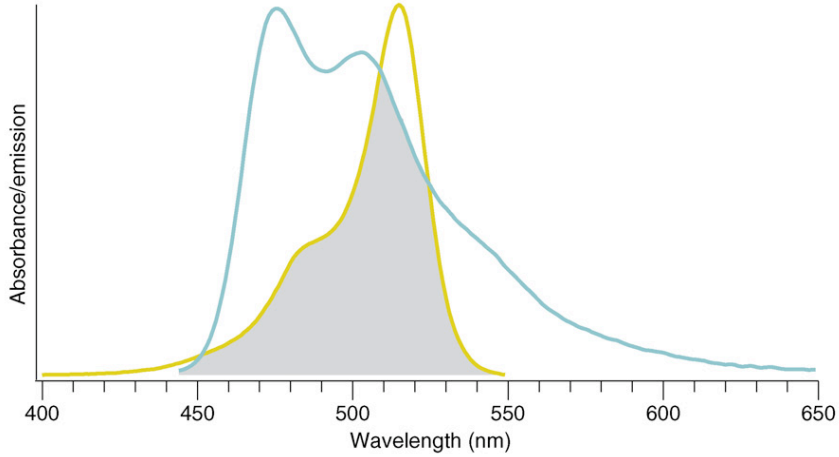
FRET is a quantum-mechanical phenomenon that occurs when two fluorochromes are in molecular proximity. It describes an energy transfer mechanism between two chromophores.

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

FRET: Föster Resonance Energy Transfer

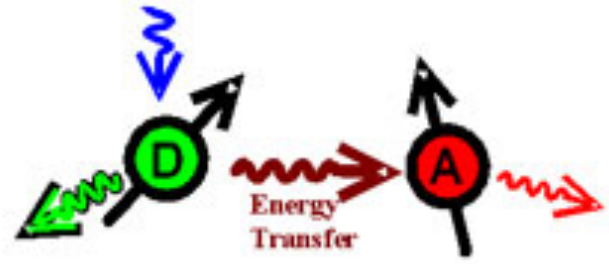
The emission spectrum of the donor fluorophore should overlap the excitation spectrum of the acceptor fluorophore.

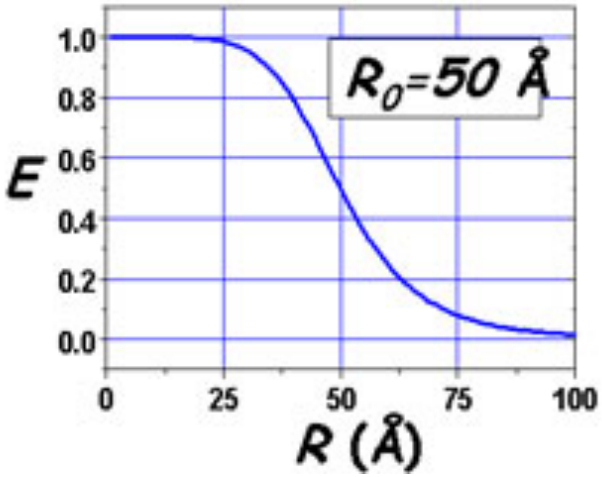




FRET: Föster Resonance Energy Transfer

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



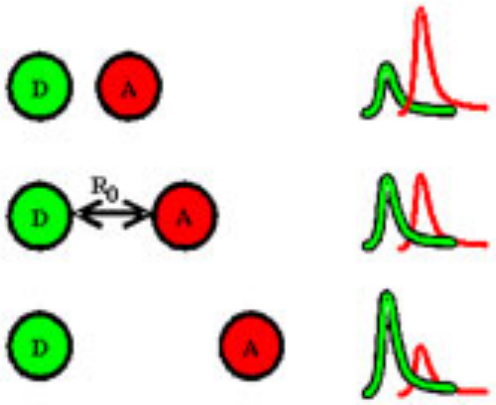


Energy Transfer Efficiency

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

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

"Spectroscopic Ruler"



FRET: Föster Resonance Energy Transfer

R0 can be caclulated 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:

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

where k^2 is the dipole orientation factor, n is the refractive index of the medium, Q0 is the fluorescence quantum yield of the donor in the absence of the acceptor, and J is the spectral overlap integral calculated as

FRET: Föster Resonance Energy Transfer

$$J = \int f_{\mathrm{D}}(\lambda)\, \epsilon_{\mathrm{A}}(\lambda)\, \lambda^4\, d\lambda$$

fD is the normalized donor emission spectrum, and ε A is the acceptor molar extinction coefficient.

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

k^2 =2/3 is often assumed. This value is obtained when both dyes are freely rotating and can be considered to be isotropically oriented during the excited state lifetime. If either dye is fixed or not free to rotate, then k^2 =2/3 will not be a valid assumption.

FRET: Föster Resonance Energy Transfer

$$J = \int f_{\mathrm{D}}(\lambda)\, \epsilon_{\mathrm{A}}(\lambda)\, \lambda^4\, d\lambda$$

fD is the normalized donor emission spectrum, and ε A is the acceptor molar extinction coefficient.

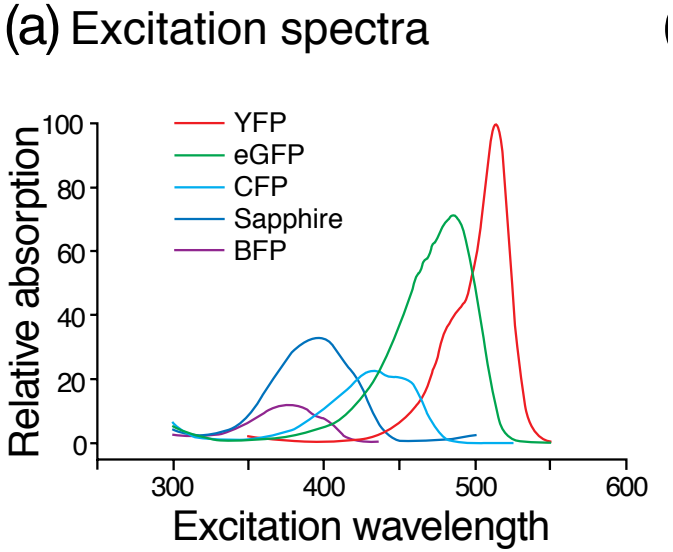
$$R_0{}^6 = 8.8 \times 10^{-28}\, \kappa^2\, n^{-4}\, Q_0\, J$$

Even when k^2 is quite different from 2/3 the error can be associated with a shift in R0 and thus determinations of changes in relative distance for a particular system are still valid. Fluorescent proteins do not reorient on a timescale that is faster than their fluorescence lifetime. In this case $0 \leq k^2 \leq 4$.

Charachteristics to take in account when choosing GFPs for FRET

When selecting GFPs to use as workable FRET pairs, three spectroscopic properties of the donor and acceptor GFPs should be considered.

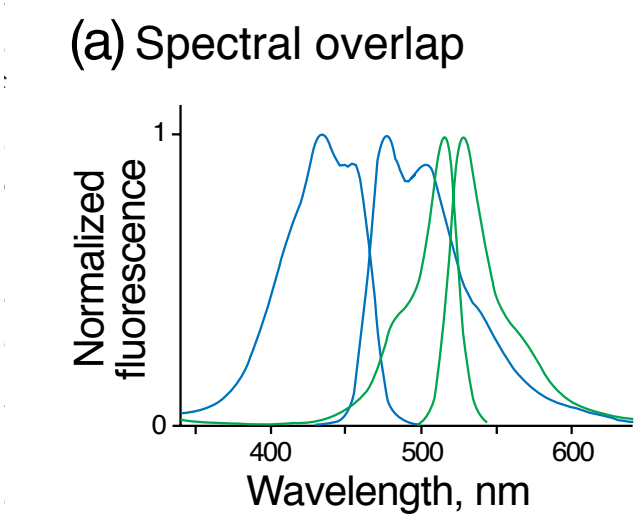
- First, the need to be **sufficient separation** in **excitation spectra** if the donor GFP is to be stimulated selectively.



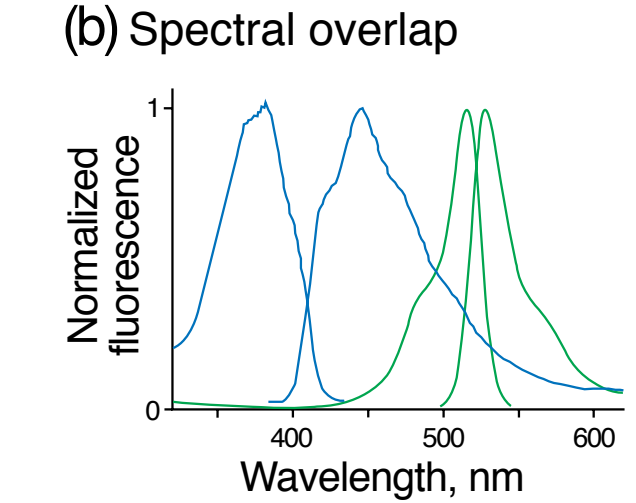
Charachteristics to take in account when choosing GFPs for FRET

- Second, the need to be an **overlap** between the **emission spectrum of the donor and the excitation spectrum of the acceptor** to obtain efficient energy transfer.

CFP-YFP

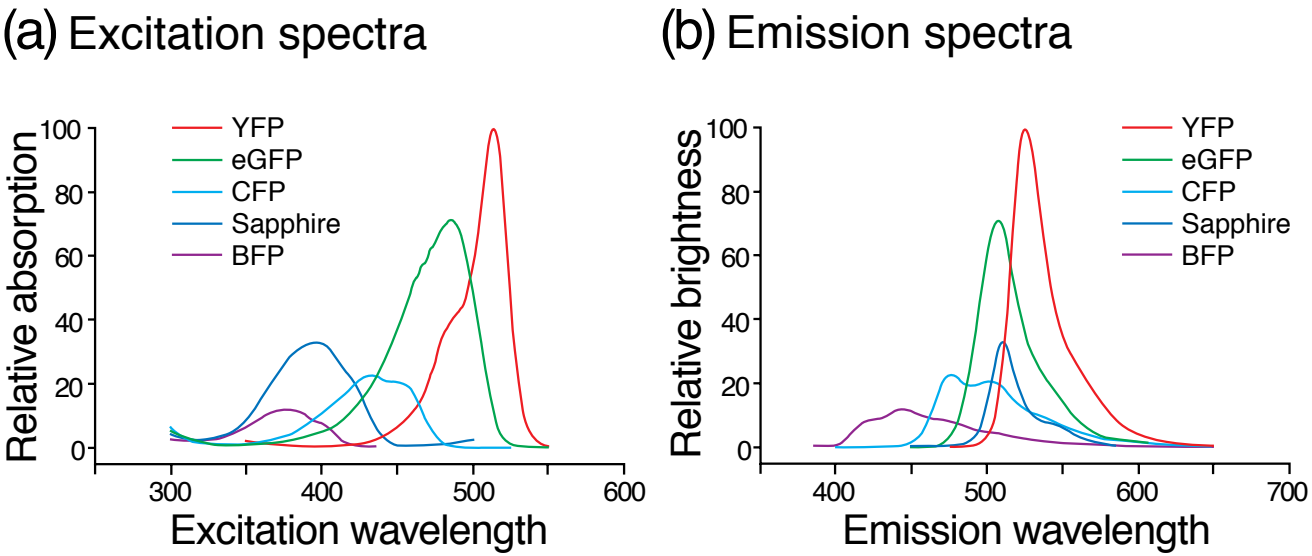


BFP-YFP



Charachteristics to take in account when choosing GFPs for FRET

- Third, **reasonable separation** in **emission spectra** between donor and acceptor GFPs is required to allow the fluorescence of each chromophore to be measured independently.



Fluorescent Proteins and FRET: EBFP-EGFP

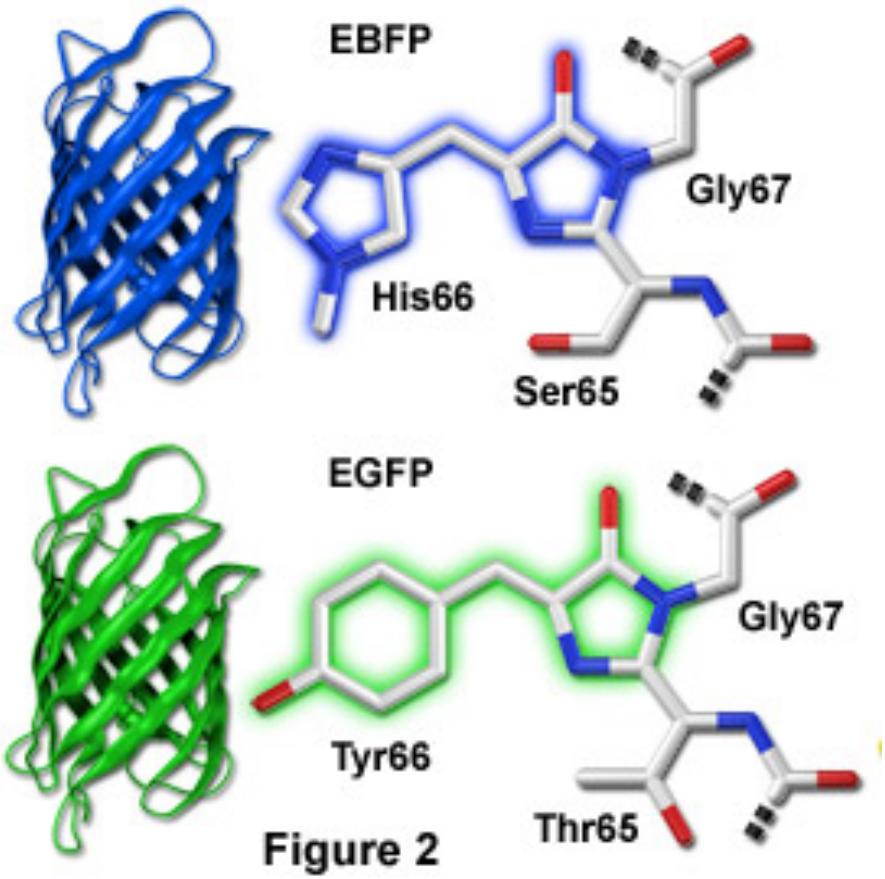
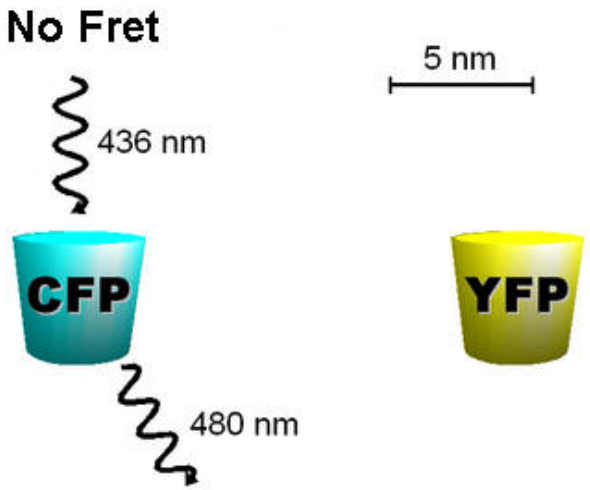


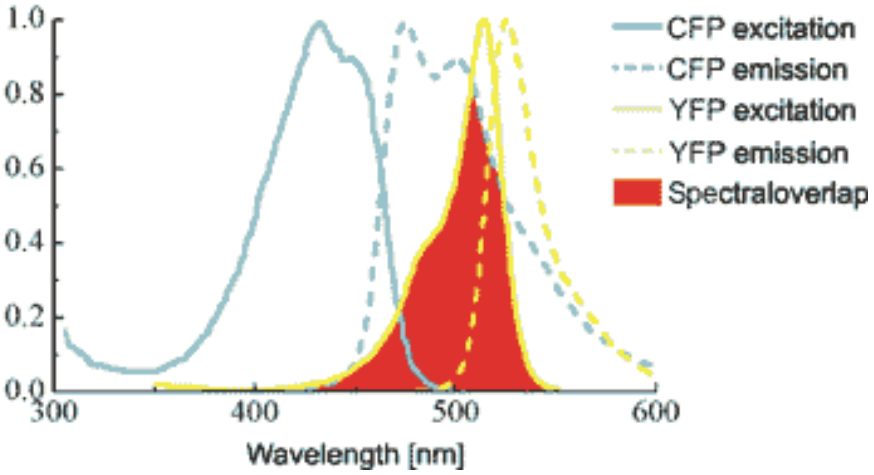
Figure 2

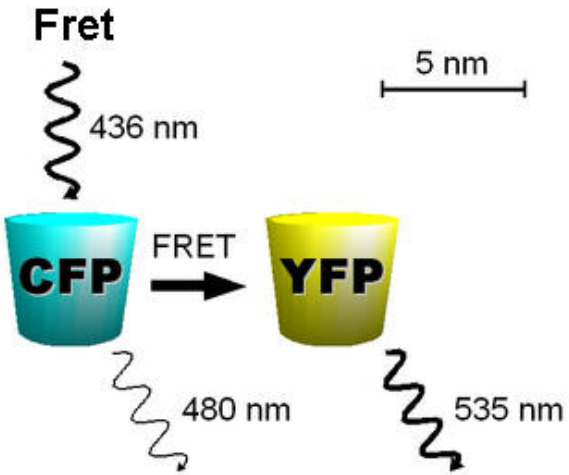
Poor photophysical properties of EBFP is a serious limitation to the use of this couple for FRET

http://zeiss-campus.magnet.fsu.edu/articles/probes/fpintroduction.html

Fluorescent Proteins and FRET: CFP-YFP





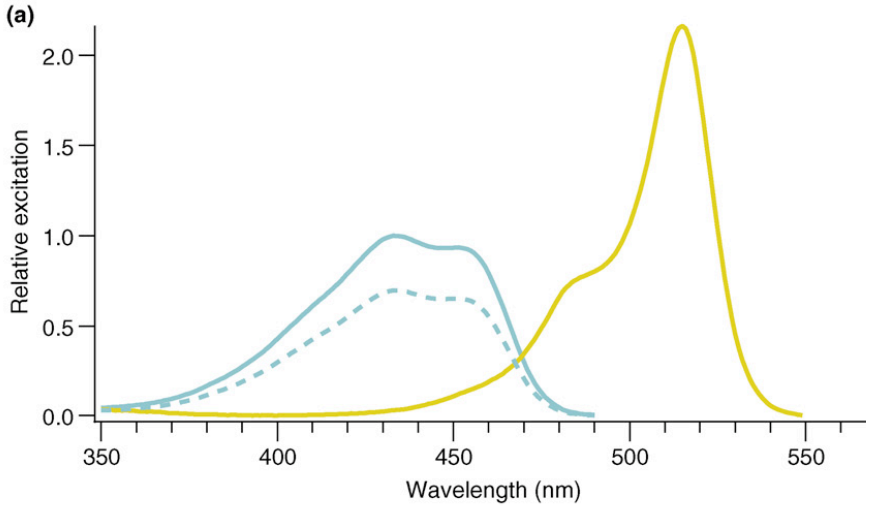


limitation of the CFP-YFP FRET system is the extenuated tail for the right end of the CFP emission spectrum

http://www.microscopyu.com/tutorials/java/fluorescence/fpfret/

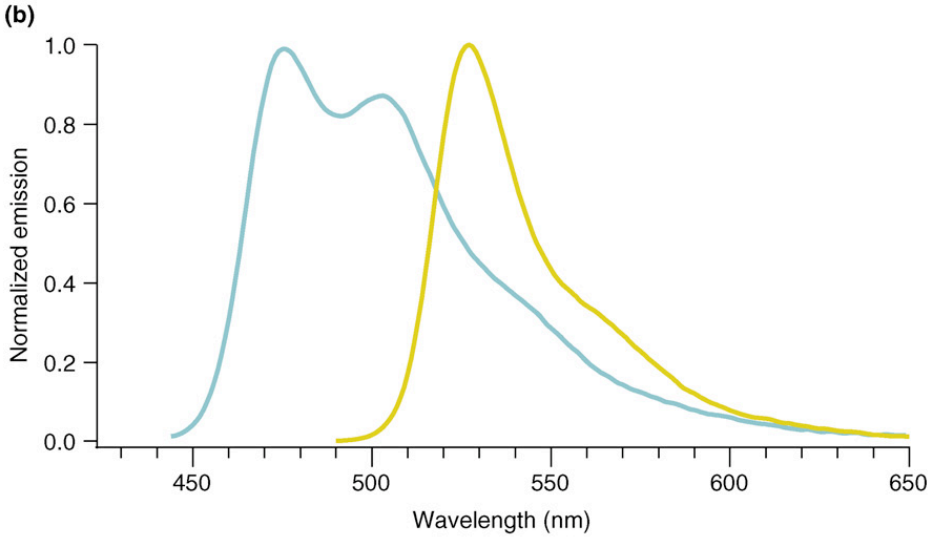
Fluorescent Proteins and FRET: CFP-YFP

CFP and YFP remain the best couple although the significant cross-talk between both the excitation and emission spectra



Bleeding of the CFP emission into the emission spectrum of YFP compromises the independent measurement of fluorescence derived from the donor and acceptor GFPs, thus decreasing the efficiency of detecting FRET changes. Judicious choice of filter sets for fluorescence detection helps to minimize this problem.

limitation of the CFP-YFP FRET system is the extenuated tail for the right end of the CFP emission spectrum



Fluorescent Proteins and FRET:other combinations

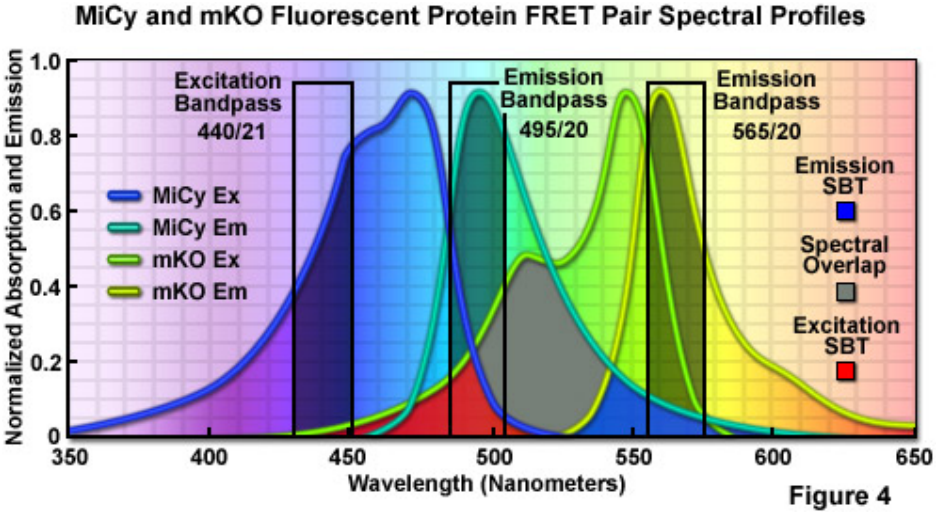


Figure 4

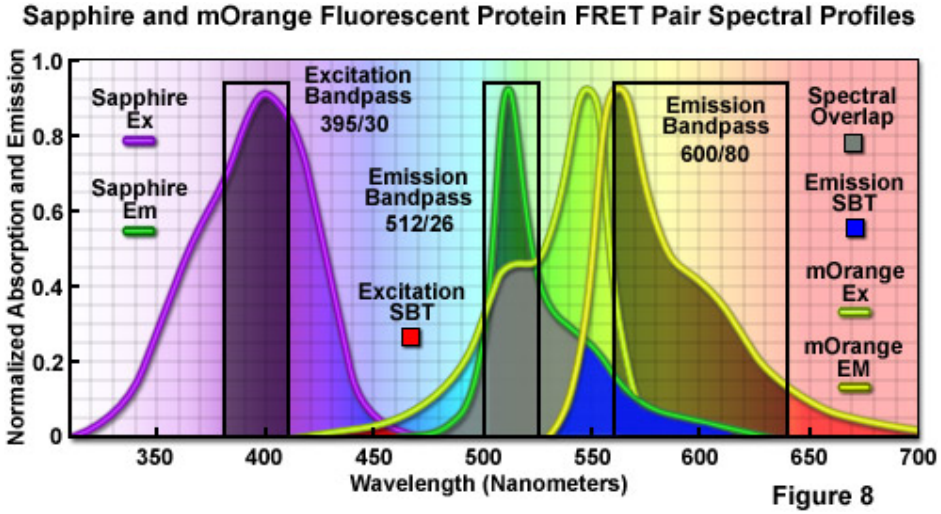
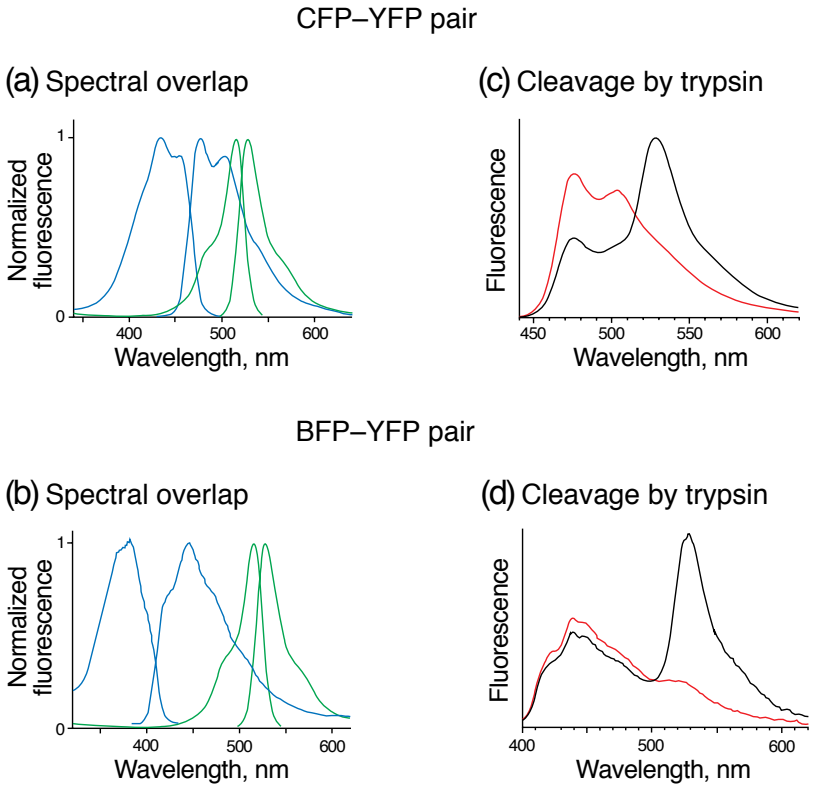


Figure 8

Tandem GFP FRET pairs used as protease substrates

The earliest use of GFP as a FRET donor and/or acceptor was in the genetic construction of FRET-based endoprotease substrates.

Heim and Tsien demonstrated that a BFP (Y145F, Y66H) donor and an eGFP (S65C or S65T) acceptor could engage in FRET when they were linked by a 25-amino-acid sequence.
Complete cleavage within this linker sequence by trypsin destroyed FRET, leading to a 4.6-fold change in the ratio of fluorescence emission from the respective GFPs (535 nm for eGFP, 440 nm for BFP).

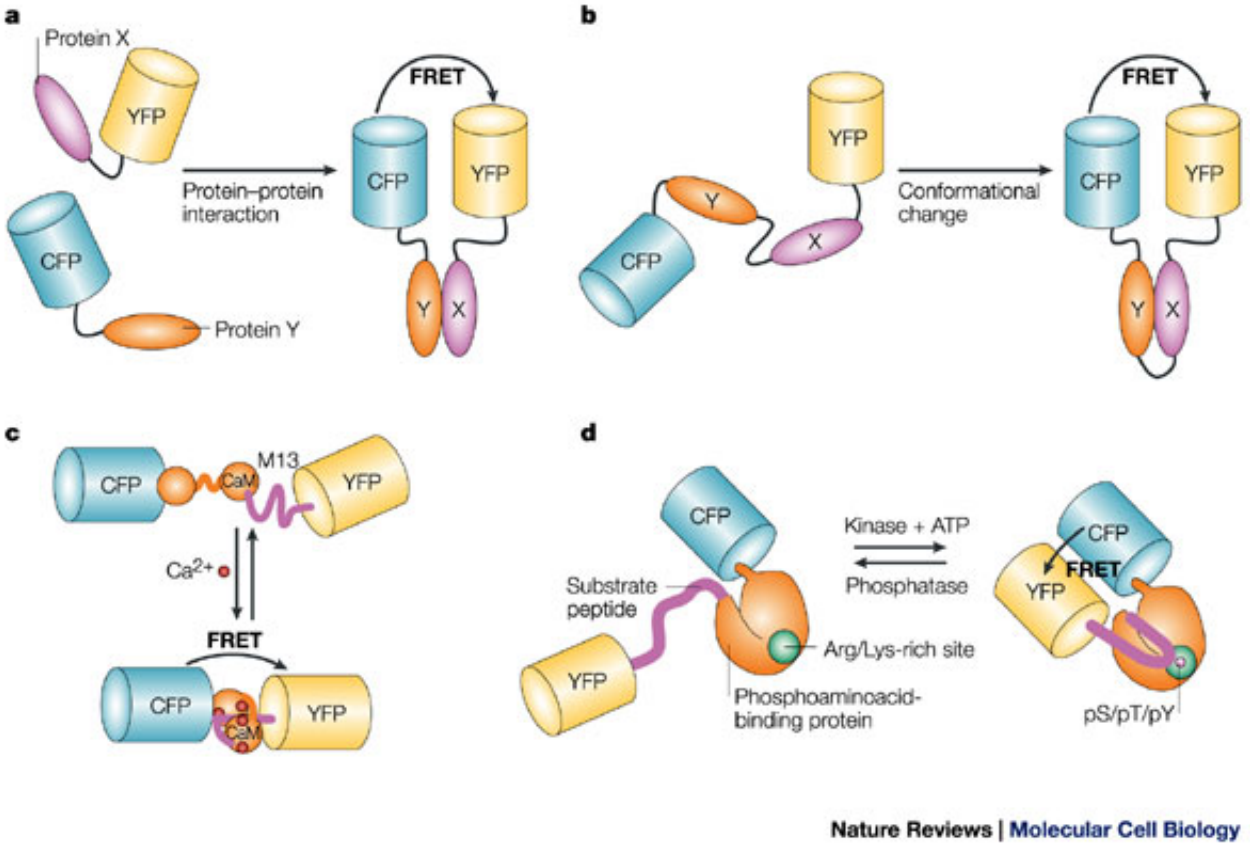


Tandem GFP FRET pairs used as protease substrates

Because GFP can be expressed in a variety of mammalian cell types, both authors postulated that tandem GFP molecules connected by a cleavable linker sequence for a particular protease could serve potentially as intracellular reporters for cytoplasmic- or Golgi-localized proteases.

Recent work by Xu *et al* has demonstrated the feasibility of this approach. They showed that FRET was lost in apoptotic cells transiently expressing a tandem BFP–eGFP

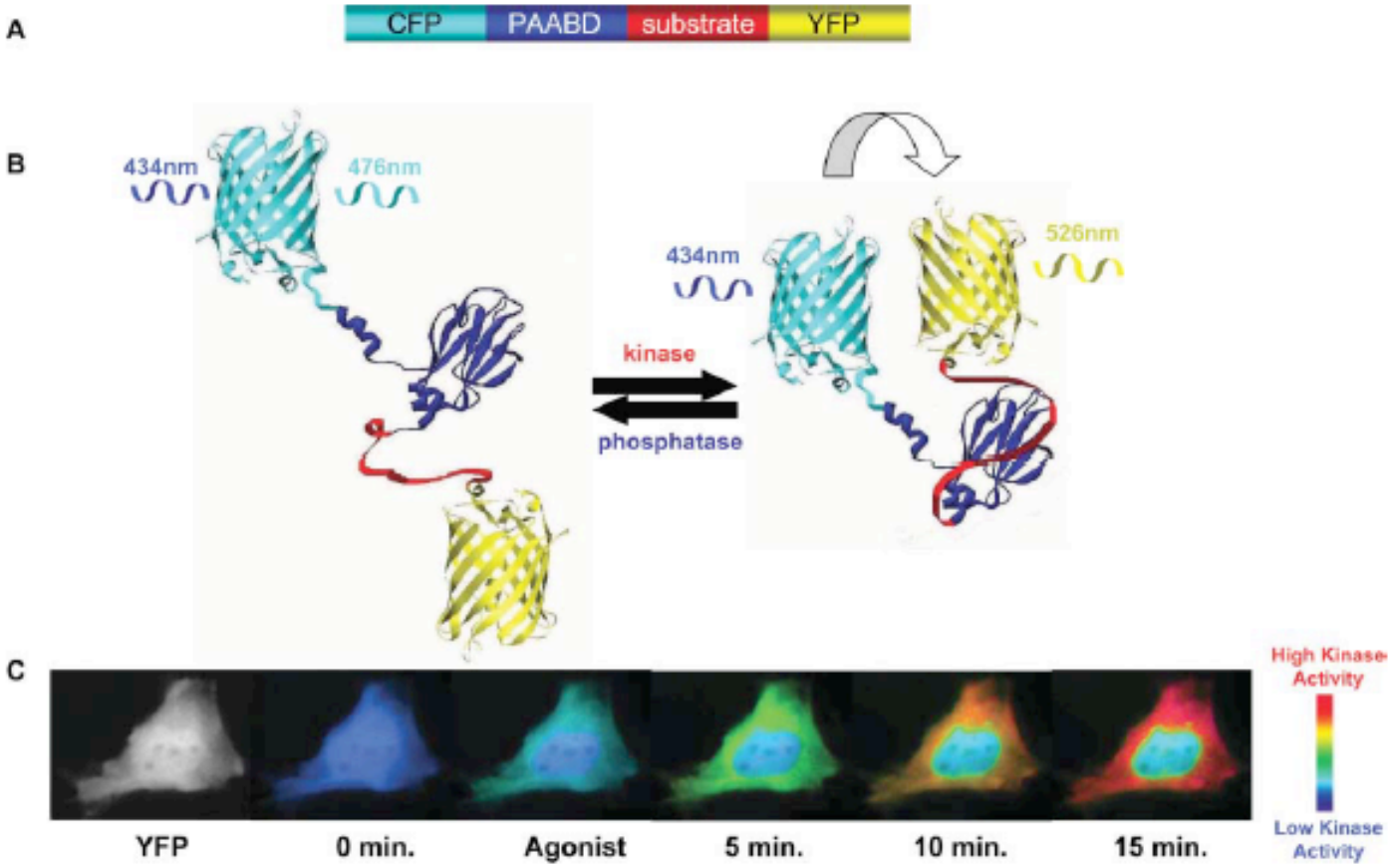
General design of FRET-based fluorescent probes



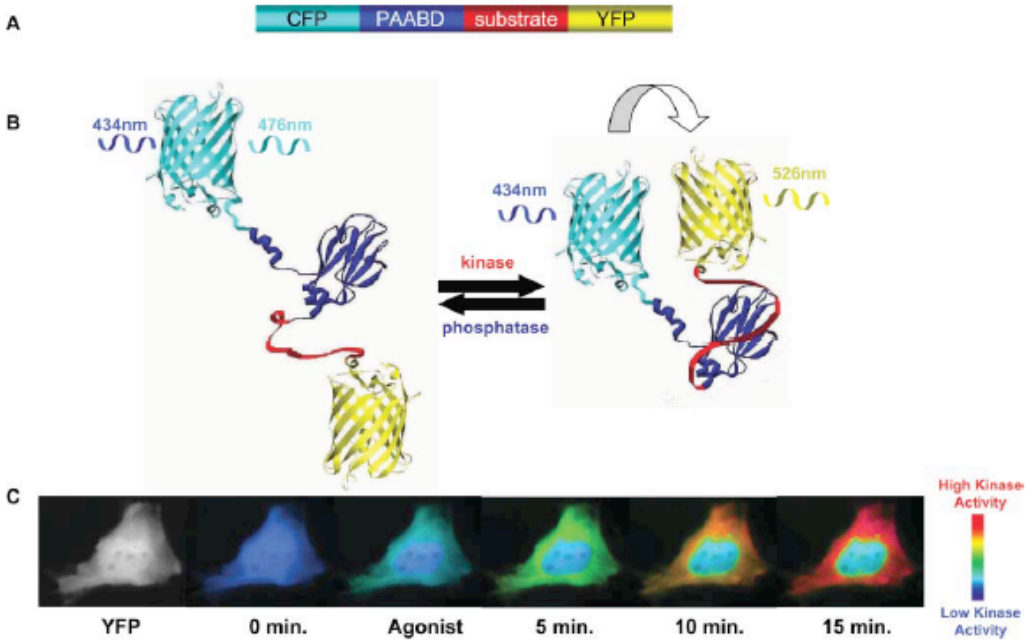
Nature Reviews | Molecular Cell Biology

FRET-based biosensors for protein kinases: illuminating the kinome

Kinase activity reporters serve as surrogate substrates for kinases and report the dynamics of phosphorilation by FRET changes



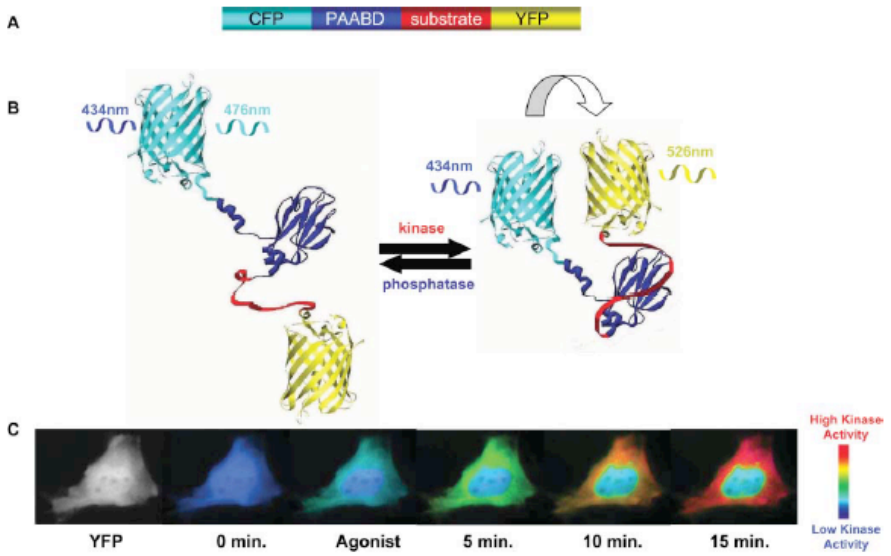
FRET-based biosensors for protein kinases: illuminating the kinome



Such reporters have been developed for various serine/threonine and tyrosine kinases. These reporters utilize, as a molecular switch, a kinase substrate domain attached to a phosphoamino acid binding domain (PAABD). Upon phosphorylation of the substrate, the PAABD binds the phosphopeptide, altering the distance and/or orientation between donor and acceptor fluorophores, resulting in a detectable change in FRET

FRET-based biosensors for protein kinases: illuminating the kinome

The substrate domain must be efficiently and specifically phosphorylated by the desired kinase, and once phosphorylated, readily recognized by the PAABD.



FRET-based biosensors for protein kinases: illuminating the kinome

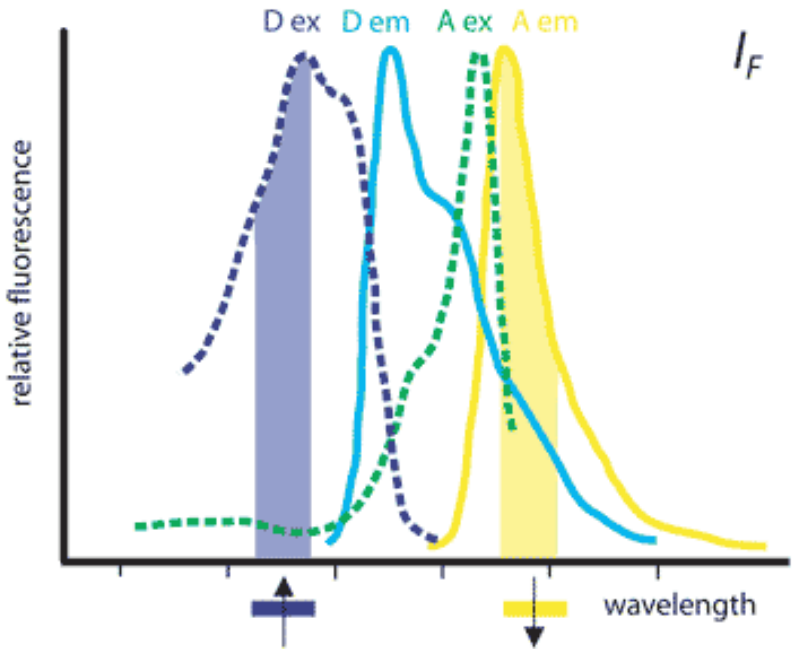
Table 1 FRET-based kinase activity reporters (KAR) that utilize the modular design of a phosphoamino acid binding domain and kinase-specific substrate as the molecular switch

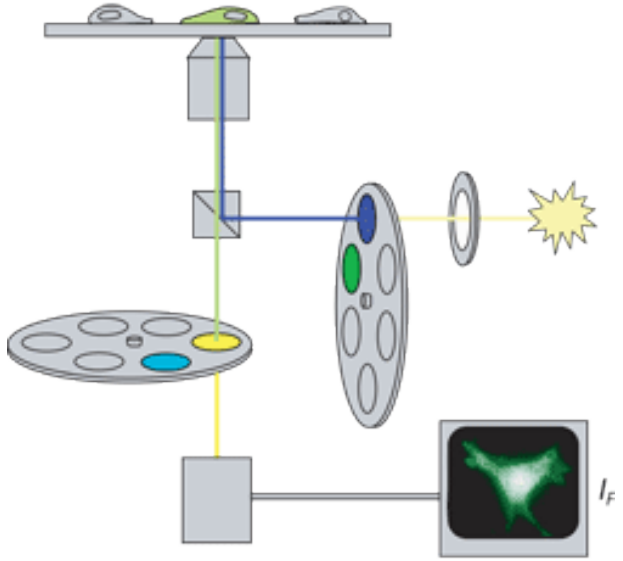
Reporter	Target	FRET pair	Signal change (%)[a]	Reference
AKAR	PKA	ECFP/cpVenus	40 ↑	13,20,28,47
Aktus	PKB	CFP/YFP	10 ↑	17
ATOMIC	ATM kinase	CFP/YFP	10 ↓	21
BKAR	PKB	ECFP/Citrine	10–25 ↓	18
CKAR	PKC	ECFP/Citrine	15–20 ↓	16
CrkII-based reporter	Abl	ECFP/Citrine	15–30 ↑	14
DKAR	PKD	CFP/YFP	10–20 ↓	22
EGFR reporter	EGFR	ECFP/Citrine	25–35 ↑	14
Erkus	Erk1	CFP/YFP	10 ↓	24
Phocus	IR	CFP/YFP	15–20 ↑	15
Picchu	Abl/EGFR	CFP/YFP	60 ↑	23
Src reporter	Src	ECFP/Citrine	30–35 ↓	14,19

[a] Best dynamic ranges are shown when different generations of reporters exist, with arrows representing the directions of FRET responses when plotted as changes in yellow over cyan emission ratio.

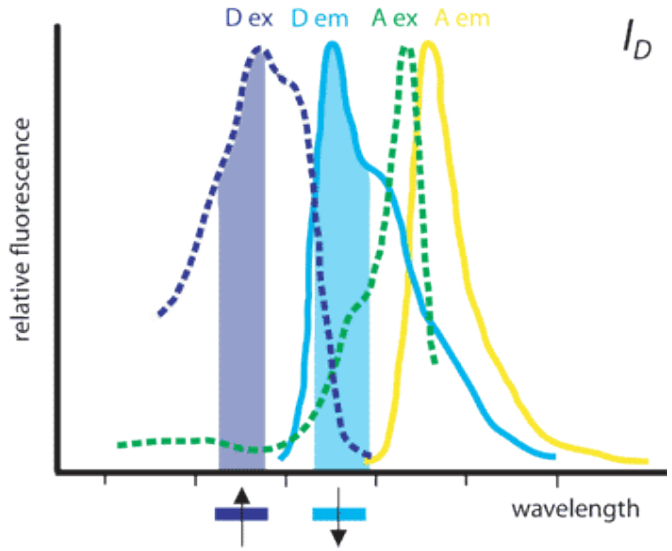
Methods for imaging FRET
1. Sensitized emission

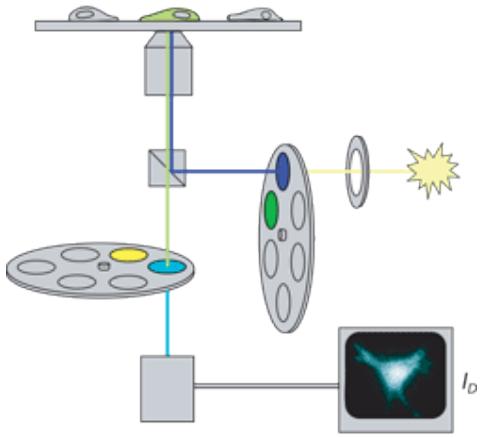
Donor is excited by a specific wavelength and the signal is collected by using emission filters chosen for the donor and the acceptor fluorescence

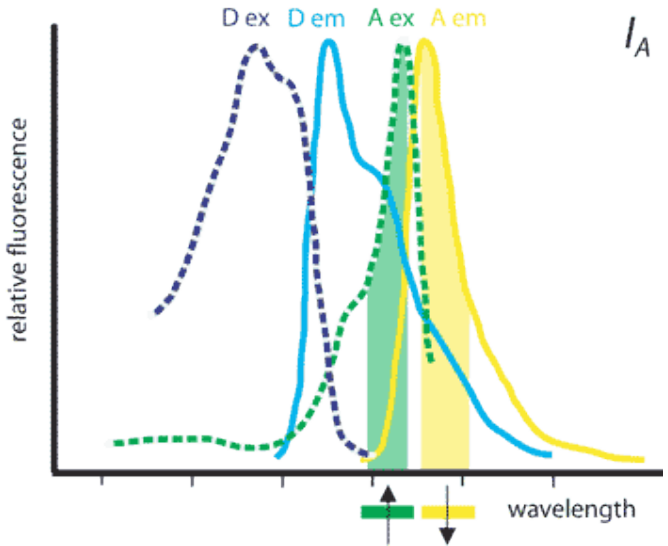


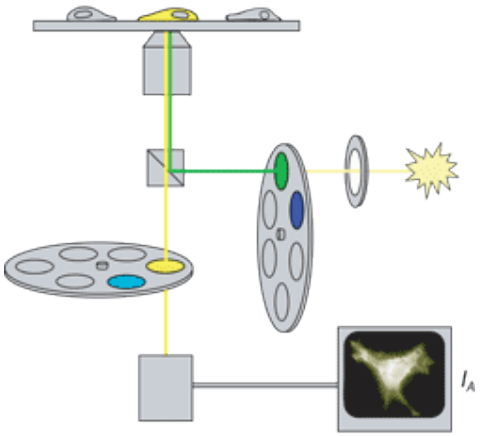


Methods for imaging FRET
1. Sensitized emission



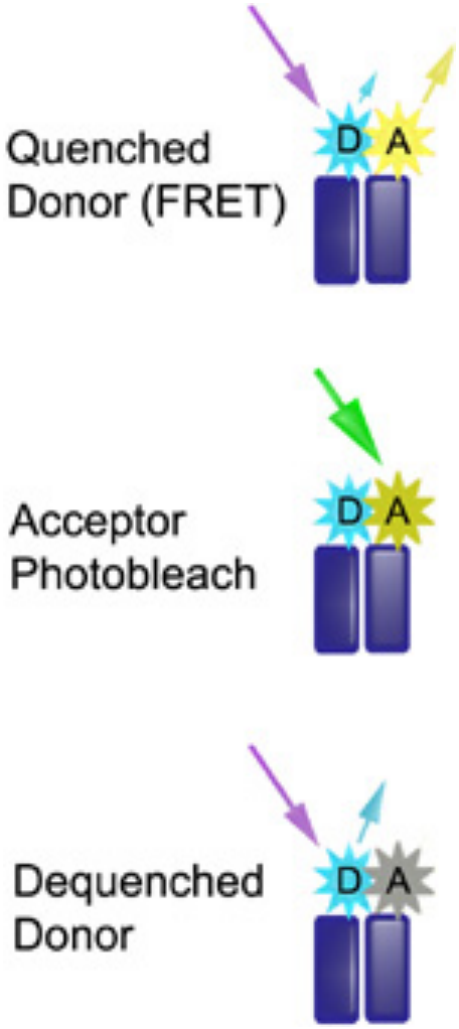






Appropriate controls to correct for the cross-talk betwwen excitation and emission of the fluorophores

Methods for imaging FRET
2. Acceptor photobleaching

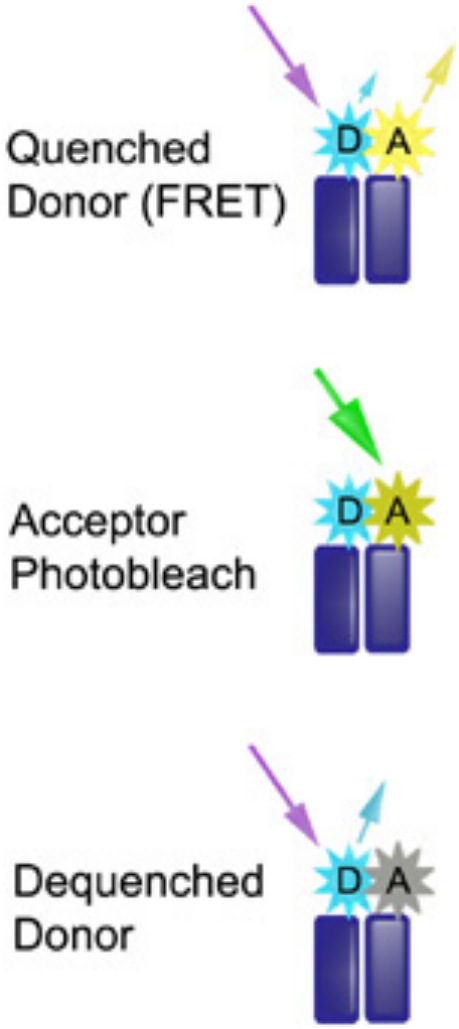


The donor fluorescence is quenched owing to FRET because some of the donor fluorescence energy is used to make acceptor fluorescence.

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

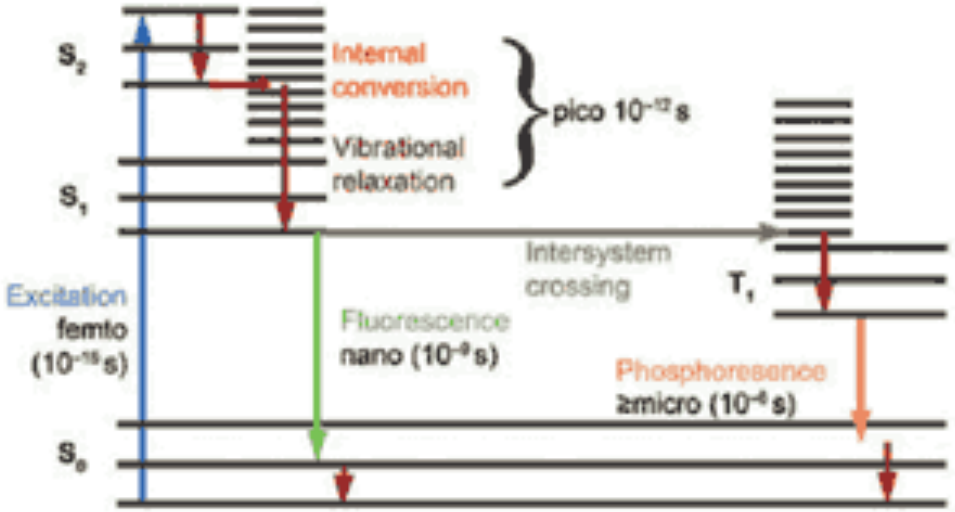
Methods for imaging FRET
2. Acceptor photobleaching



Disadvatages: usable only once
Not suitable for dynamic measuremets

Neverthless it is useful to perform an acceptor photobleaching at the end of an experiments regardless of what methods are being used

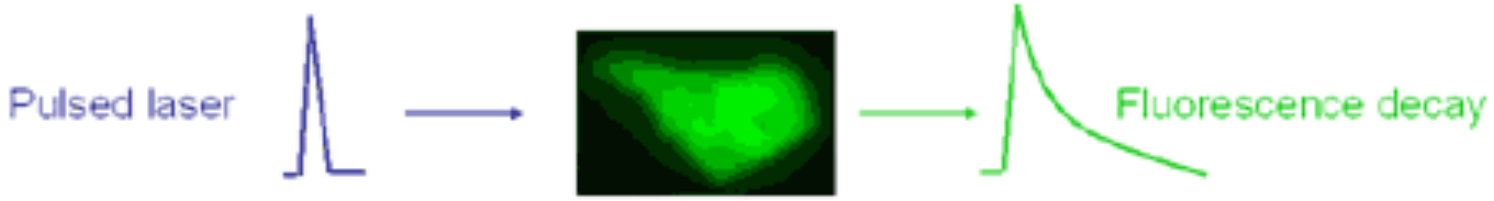
Methods for imaging FRET
3. Fluorescence Lifetime Imaging Microscopy FLIM



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

The fluorescence lifetime of a molecule depends on its environment. If there are alternative routes to fluorescence to depopulate the excited energy state, the measured lifetime is reduced.

Methods for imaging FRET
3. Fluorescence Lifetime Imaging Microscopy FLIM

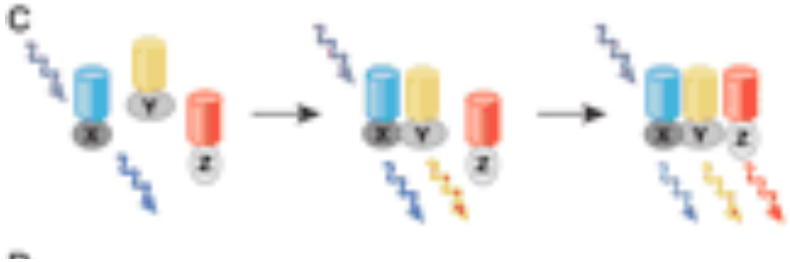


The donor fluorescence is quenched by FRET and the amount of quenching can be determined by measuring the shortening of fluorescence decay of the donor in the presence of FRET.

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

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

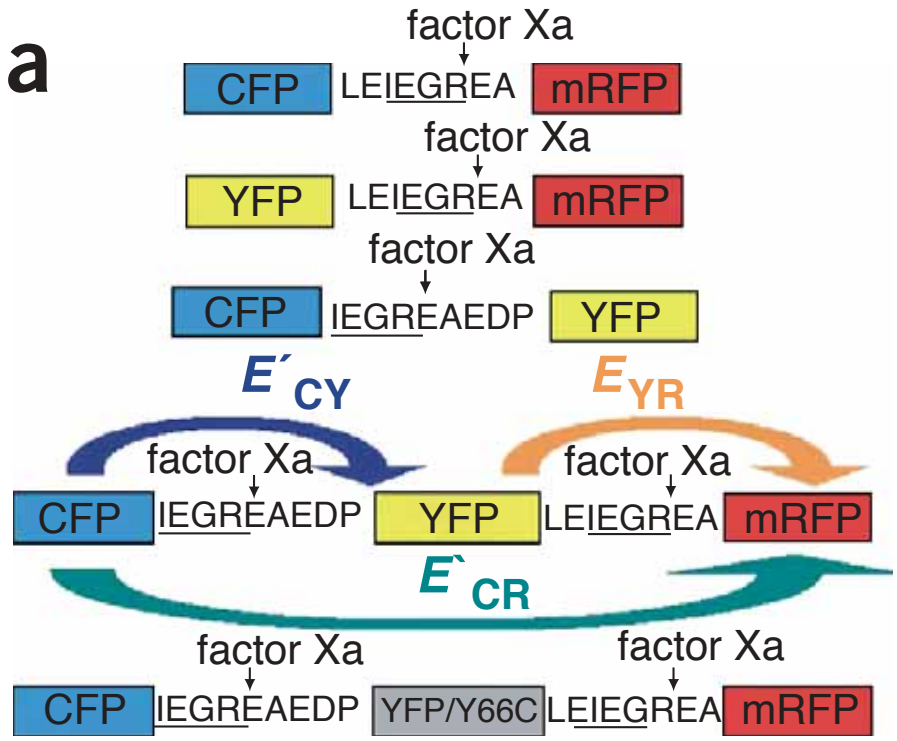
Intermolecular FRET/3FRET



Recently, the possibility of using three fluorescent proteins to study higher order complexes has been addressed by adding a monomeric red fluorescent protein (mRFP) to the CFP/YFP pair. In trimeric complexes, CFP is the FRET donor for YFP; subsequently, YFP can act as a FRET donor for mRFP. 3-FRET has been shown in multiprotein complexes and in protein trimerization. Further optimization of a higher wavelength FRET pair, as well as spectral deconvolution, might improve the 3-FRET technique.

Three-chromophore FRET microscopy to analyze multiprotein interactions in living cells

Emilia Galperin[1,2], Vladislav V Verkhusha[1,2] & Alexander Sorkin[1]



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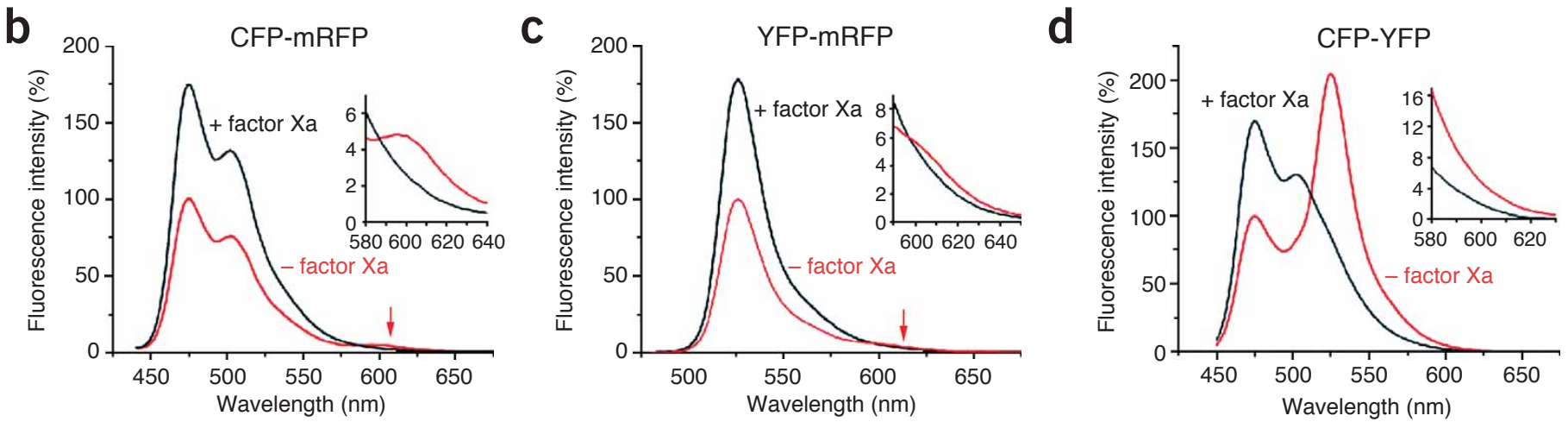
NATURE METHODS | VOL.1 NO.3 | DECEMBER 2004 | 209

Spettri di emissione prima e dopo il clivaggio con Xa



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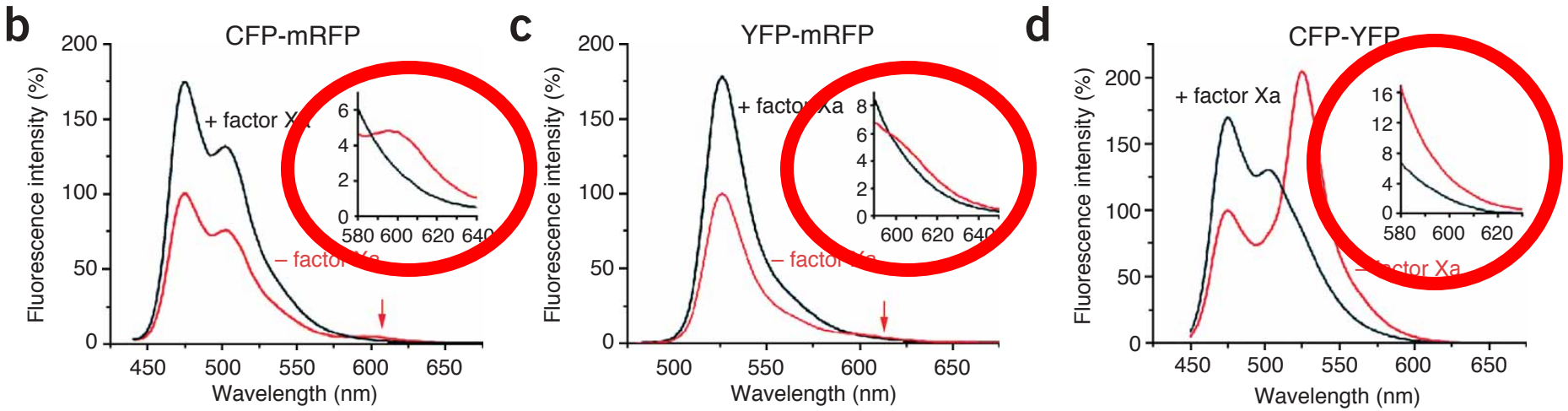
NATURE METHODS | VOL.1 NO.3 | DECEMBER 2004 | 209

http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-SpectraViewer.html?fileId1=CFPp74



The insets in the panels (b–f) zoom in the mRFP emission range.

Three-chromophore FRET microscopy to analyze multiprotein interactions in living cells

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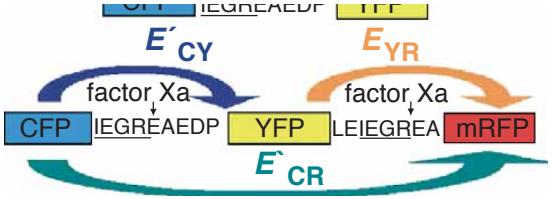
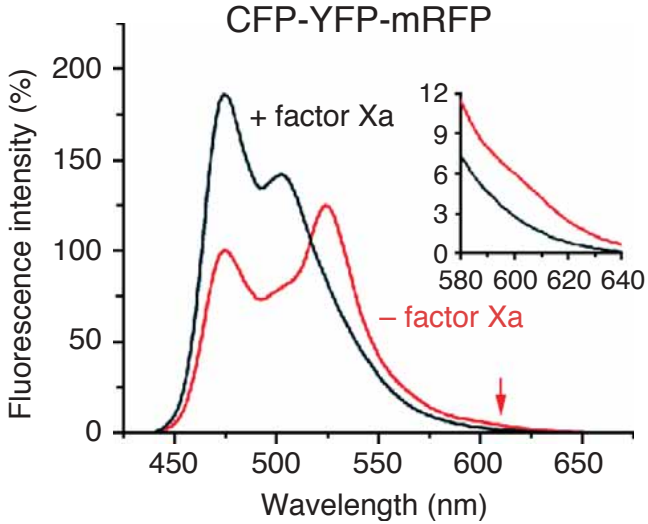
NATURE METHODS | VOL.1 NO.3 | DECEMBER 2004 | 209

e





E_{CYR} (0.46) > E_{CY} (0.42)

energy from CFP is transferred not only to YFP but also directly to mRFP in the triple-fusion protein.

Three-chromophore FRET microscopy to analyze multiprotein interactions in living cells

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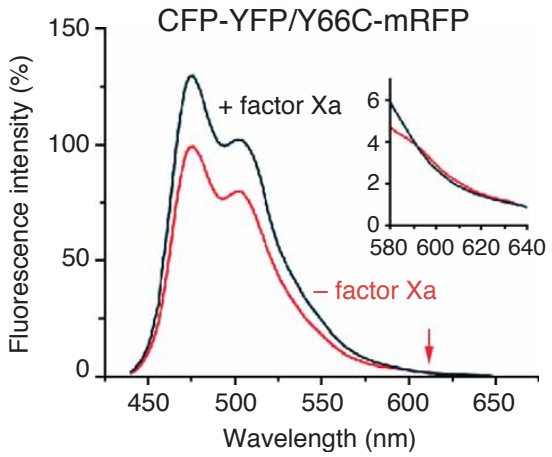
NATURE METHODS | VOL.1 NO.3 | DECEMBER 2004 | 209

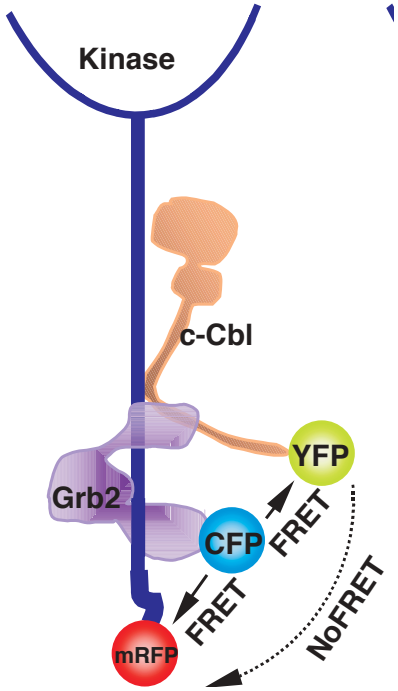
control construct containing the YFP Y66C mutant lacking the chromophore

f



CFPYFP/Y66C-mRFP fusion exhibited E_{CR} efficiency of 0.22, which reflected the increase in distance between CFP and mRFP in the triple-fusion as compared with that in the CFP-mRFP double fusion protein.

Analysis of EGFR interactions with Grb2 and Cbl



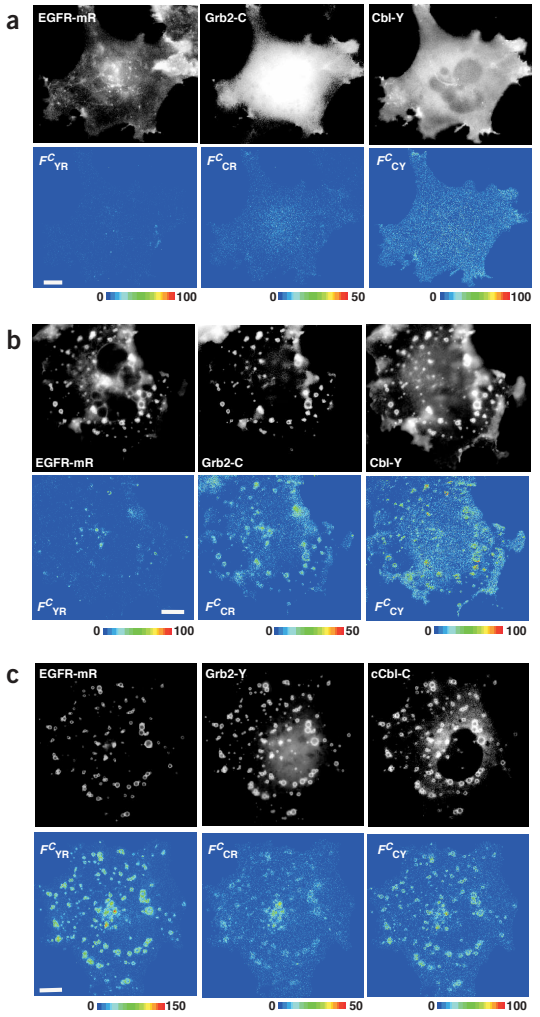


Figure 4 | 3-FRET analysis of EGFR-mRFP interactions with a pair of Cbl-YFP and Grb2-CFP, or a pair of Cbl-CFP and Grb2-YFP (**a**,**b**). EGFR-mRFP was coexpressed with Cbl-YFP and Grb2-CFP. Six-filter FRET analysis was carried out on serum-starved cells (**a**) or cells treated with 17 nM EGF at 4 °C and then for 30 min at 37 °C (**b**). The cells were treated with nocodazole that was added for the last 15 min of incubation at 37 °C. (**c**) EGFR-mRFP was coexpressed with Cbl-CFP and Grb2-YFP and six-filter FRET analysis was carried out in cells treated with EGF as described in **b**. $FRET^C$ images in **a**–**c** are presented in pseudocolor. Scale bars, 10 μm.

Analysis of EGFR interactions with Grb2 and Cbl

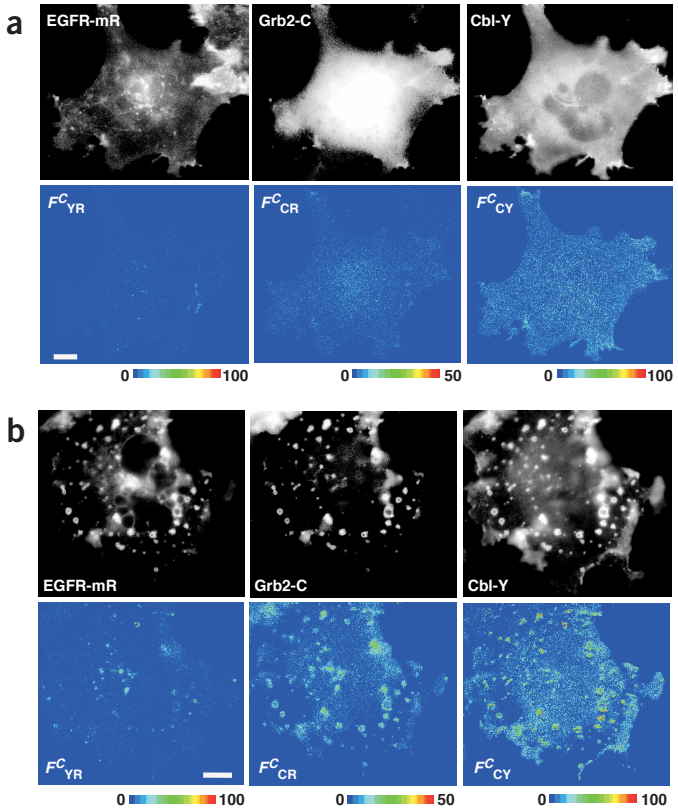


Figure 4 | 3-FRET analysis of EGFR-mRFP interactions with a pair of Cbl-YFP and Grb2-CFP, or a pair of Cbl-CFP and Grb2-YFP (**a**,**b**). EGFR-mRFP was coexpressed with Cbl-YFP and Grb2-CFP. Six-filter FRET analysis was carried out on serum-starved cells (**a**) or cells treated with 17 nM EGF at 4 °C and then for 30 min at 37 °C (**b**). The cells were treated with nocodazole that was added for the last 15 min of incubation at 37 °C. (**c**) EGFR-mRFP was coexpressed with Cbl-CFP and Grb2-YFP and six-filter FRET analysis was carried out in cells treated with EGF as described in **b**. $FRET^C$ images in **a**–**c** are presented in pseudocolor. Scale bars, 10 μm.

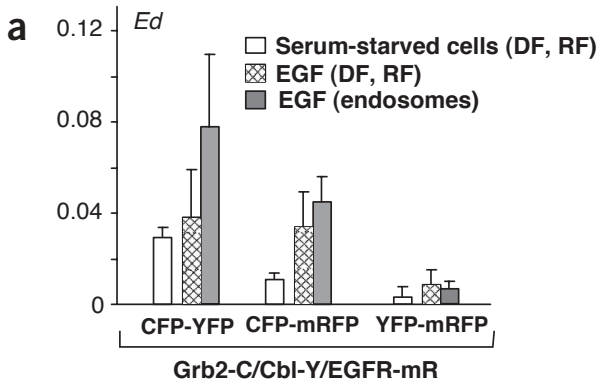
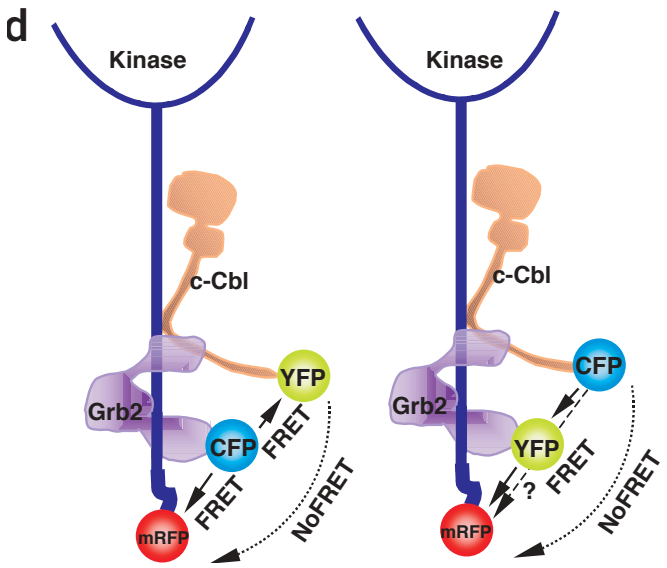


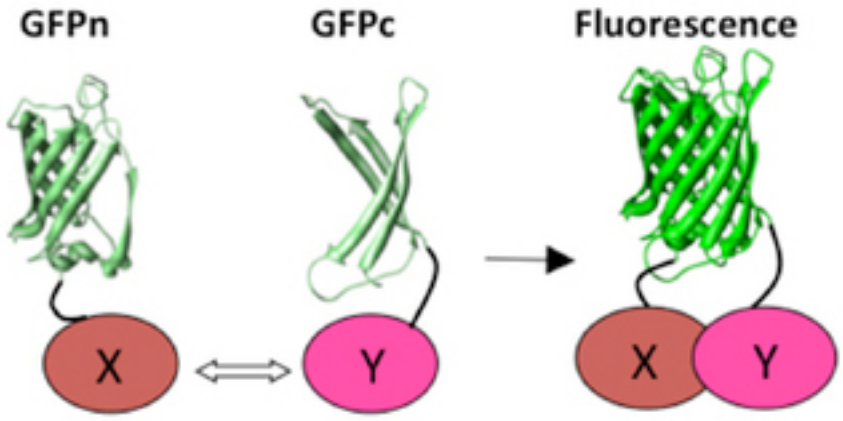
Figure 5 | Comparative 3-FRET analysis of EGFR interactions with differentially tagged Cbl and Grb2 proteins. Bar graphs show mean *Ed* values (± s.d.) obtained for diffuse fluorescence areas (DF) and plasma membrane ruffles (RF) in serum-starved untreated cells (white bars) or EGF-treated cells (cross-hatched bars), and endosomes of EGF-treated cells (gray bars) in experiments presented in **Figure 4a**,**b** (**a**) and **Figure 4c** (**b**). The scatter plots of *Ed* values vs. acceptor intensity are presented in **Supplementary Figure 4** online.

Analysis of EGFR interactions with Grb2 and Cbl



- FRET from Cbl-CFP to EGFR-mRFP required the presence of the intermediate adaptor, Grb2 tagged with YFP and, therefore, occurred due to sequential CFP-YFP-mRFP energy transfer.
- Taken together, the data obtained in the 3-FRET analysis provide direct evidence to **support the previously proposed model that although Cbl is capable of direct binding to EGFR, it preferentially binds receptors indirectly through Grb2**

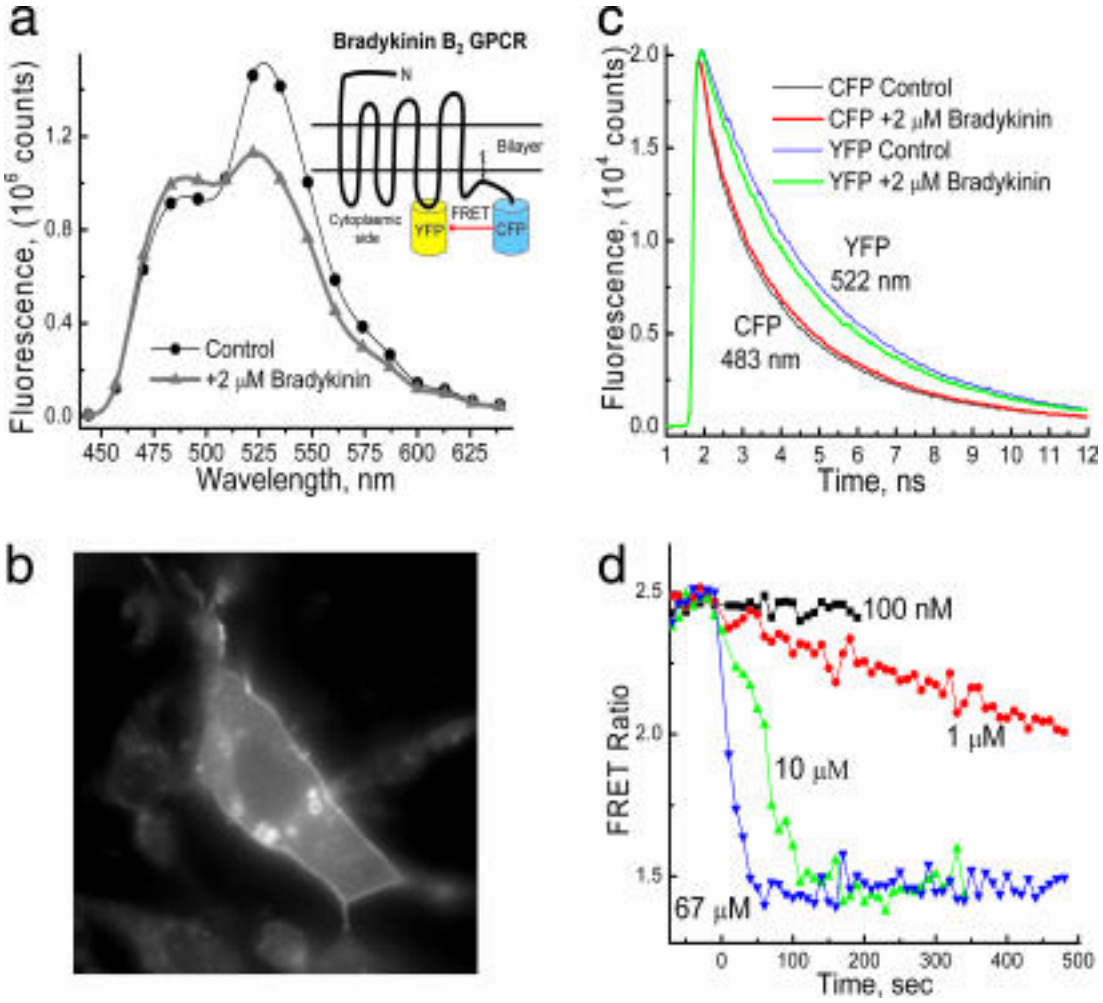
BIFC



GFPs split at appropriate sites can fold and reconstitute the chromophore when the two halves are fused to interacting partners, a two-hybrid system termed bimolecular fluorescence complementation (BiFC). Self-assembling fragments of GFP have also been reported, in which the two fragments only have to exist in the same compartment to generate fluorescence, without requiring splinting by other protein-protein interactions. BiFC can be used to study gene expression of at least two promotors, as has been demonstrated in Caenorhabditis elegans. BiFC has a high signal-to-background ratio, because it creates new fluorescence rather than modulating existing fluorescence. Multiple protein protein interactions can be studied in parallel using spectrally distinct split FPs.

However, BiFC is slow (hours to days) and irreversible, and the geometrical and affinity requirements for the protein-protein interaction have not yet been characterized.

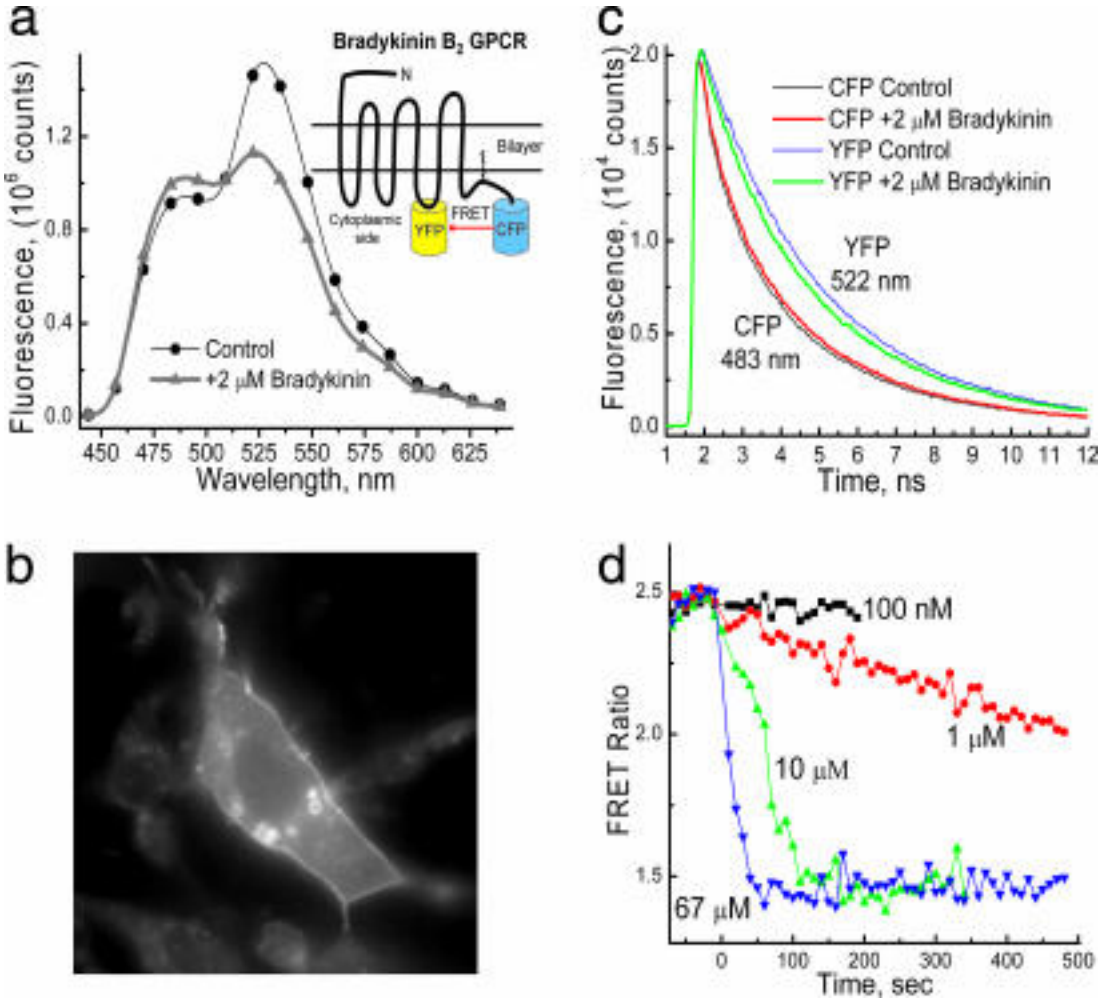
Detection and Characterization of Ligand-Induced GPCR Conformational Change.



To monitor conformational dynamics of the GPCR, a B2 receptor chameleon (B2K) with yellow fluorescent protein (YFP) inserted into the third cytoplasmic loop and cyan fluorescent protein (CFP) fused to the C terminus was constructed.

Chachisvilis M, et al.,PNAS 2006 Oct 17;103(42):15463-8

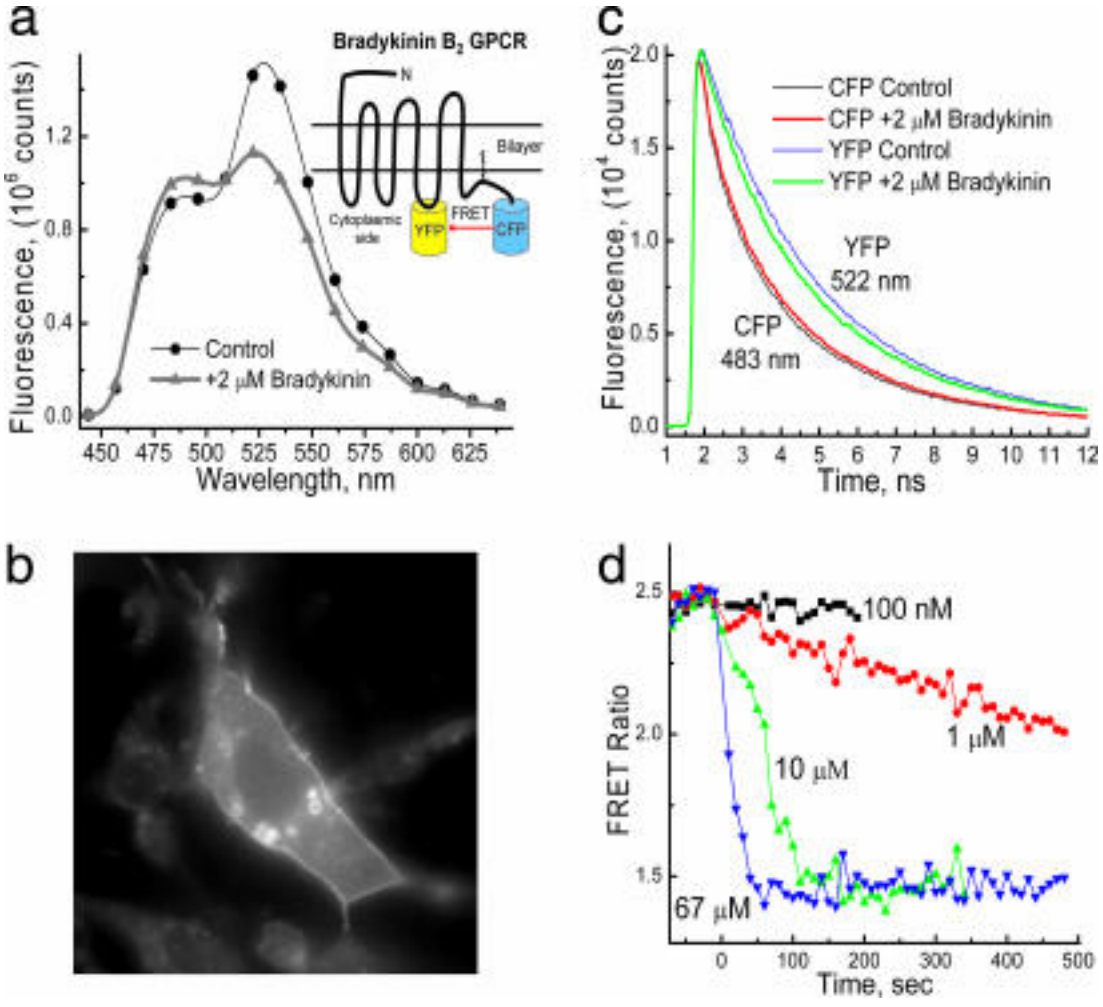
Detection and Characterization of Ligand-Induced GPCR Conformational Change.



The B2K chameleon expressed in bovine aortic endothelial cells (BAECs) displays mostly membrane localization (Fig. 1b) and a dual fluorescence spectrum with characteristic CFP and YFP emission peaks at ≈485 and 525 nm, respectively.

Chachisvilis M, et al.,PNAS 2006 Oct 17;103(42):15463-8

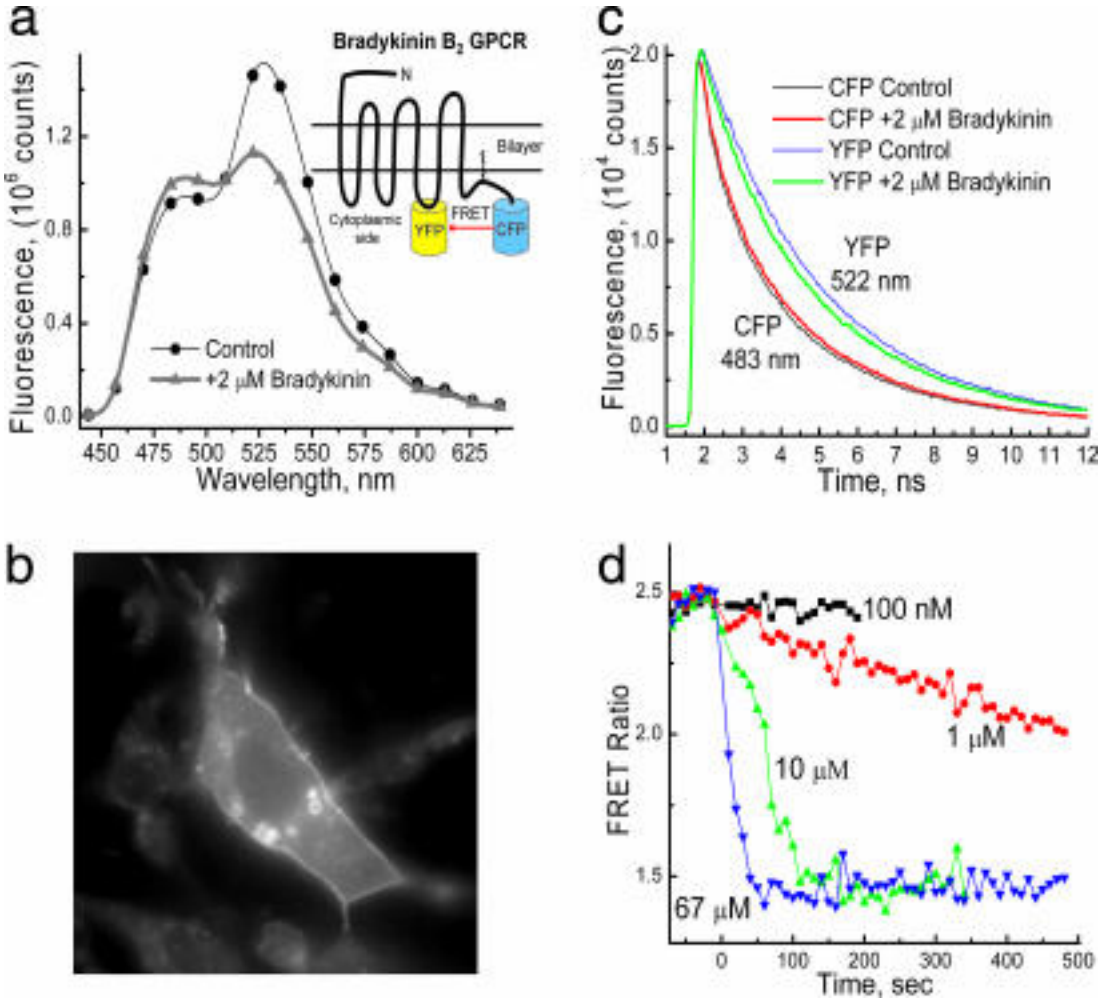
Detection and Characterization of Ligand-Induced GPCR Conformational Change.



exposure of BAECs expressing B2K chameleon to their natural agonist bradykinin results in a pronounced spectral change characterized by a decrease in YFP emission and an increase in CFP emission

Chachisvilis M, et al.,PNAS 2006 Oct 17;103(42):15463-8

Detection and Characterization of Ligand-Induced GPCR Conformational Change.



corresponding fluorescence emission kinetics show that the fluorescence decay of CFP becomes slower, whereas fluorescence decay of YFP becomes faster (Fig. 1c), suggesting that conformational change leads to lower FRET efficiency.

Chachisvilis M, et al.,PNAS 2006 Oct 17;103(42):15463-8

Total Internal Reflection Microscopy (TIRFM) or Evanescent wave microscopy

TIRFM provides a means to selectively excite fluorophores in an aqueous or cellular environment very near a solidsurface (within < 100nm) without exciting fluorescence from regions farther from the surface.

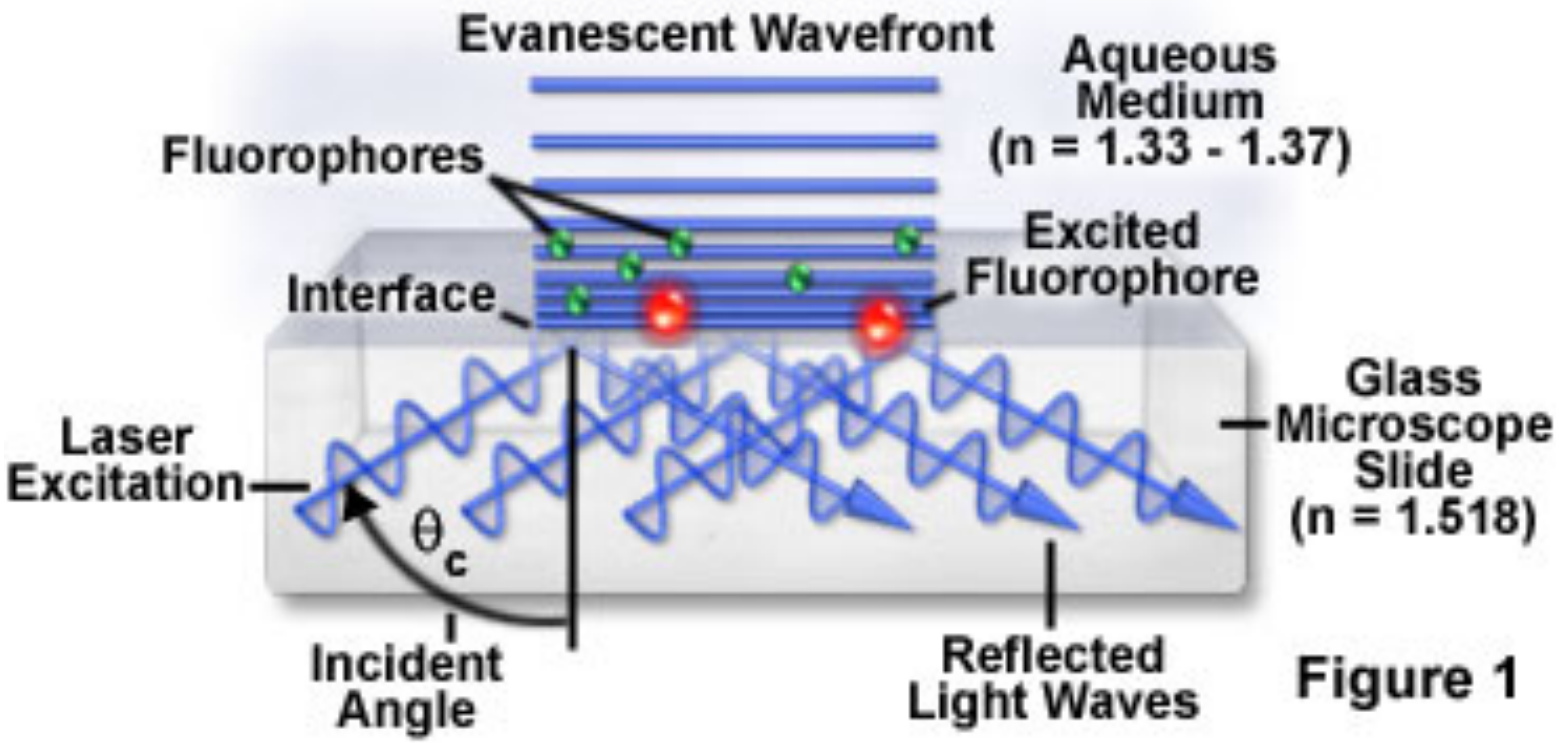


Figure 1

The physical phenomenon of total internal reflection (TIR)

A light beam propagating through one medium and reaching an interface is either refracted as it enters the second medium, or reflected at the interface, depending upon the incident angle and the difference in refractive indices of the two media.

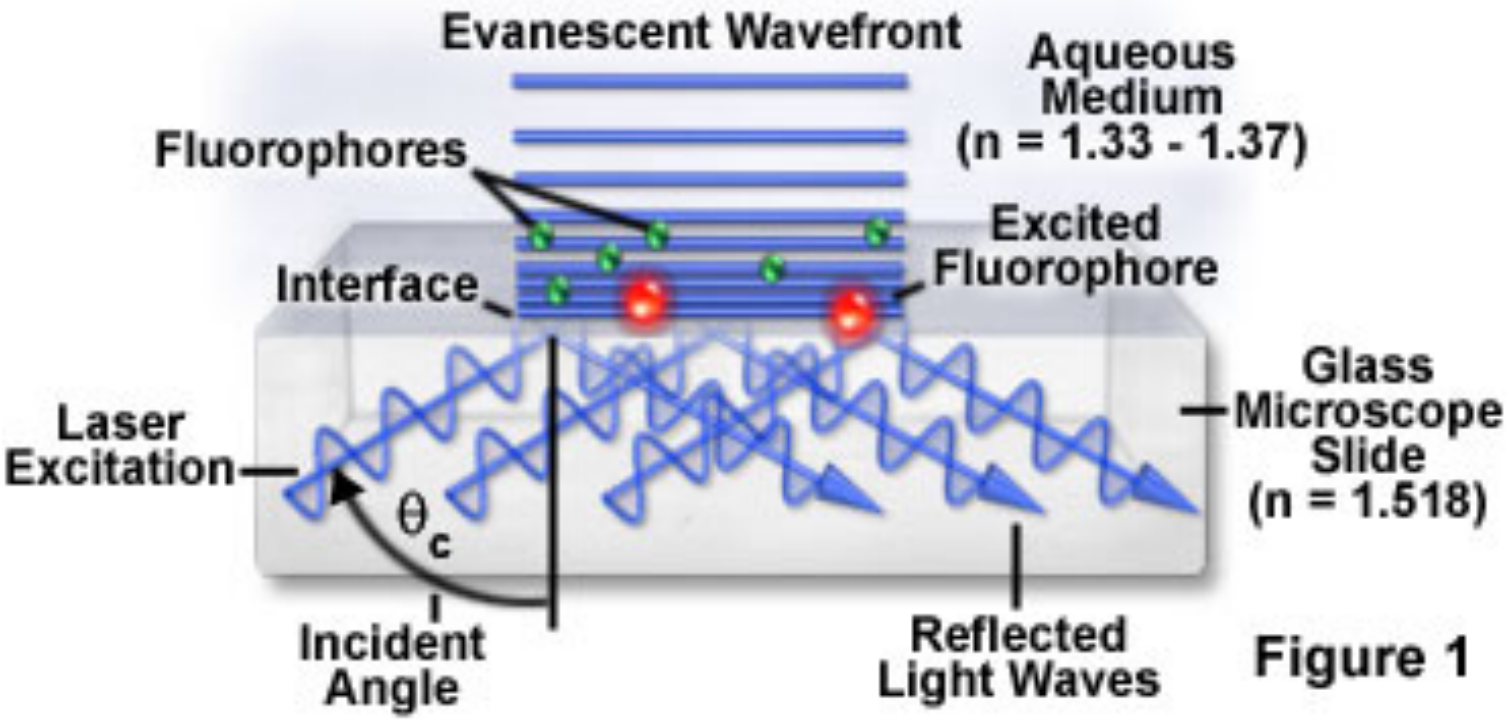


Figure 1

The physical phenomenon of total internal reflection (TIR)

When light strikes the interface of the two materials at a sufficiently high angle, termed the critical angle (q(c)), its refraction direction becomes parallel to the interface (90 degrees relative to the normal), and at larger angles it is reflected entirely back into the first medium

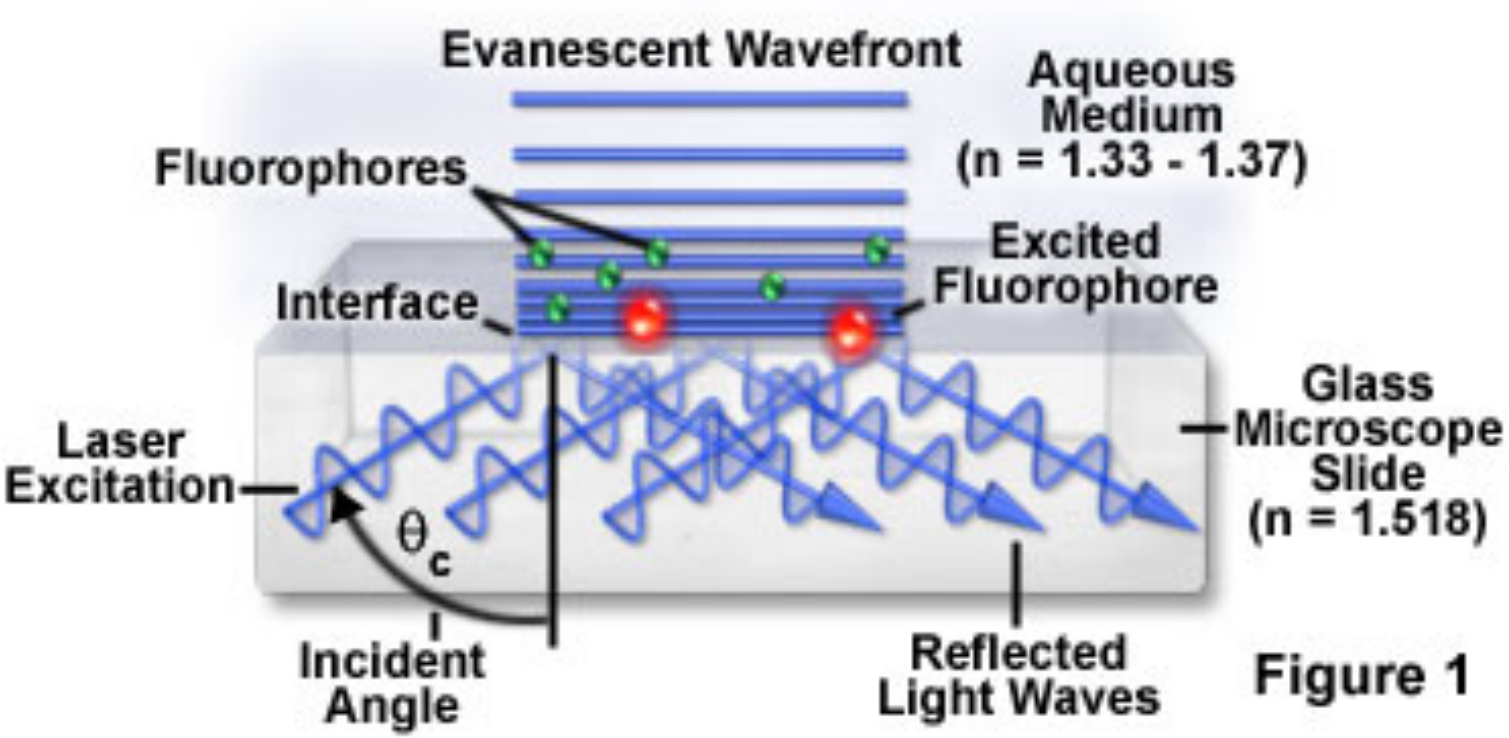


Figure 1

The physical phenomenon of total internal reflection (TIR)

The electromagnetic field of the total internal reflected light extends into the sample beyond the interface, extending only a few hundred nanometres into the second medium of lower refractive index - essentially in the z direction. Only the section of the specimen located within the evanescent field undergoes fluorescence excitation.

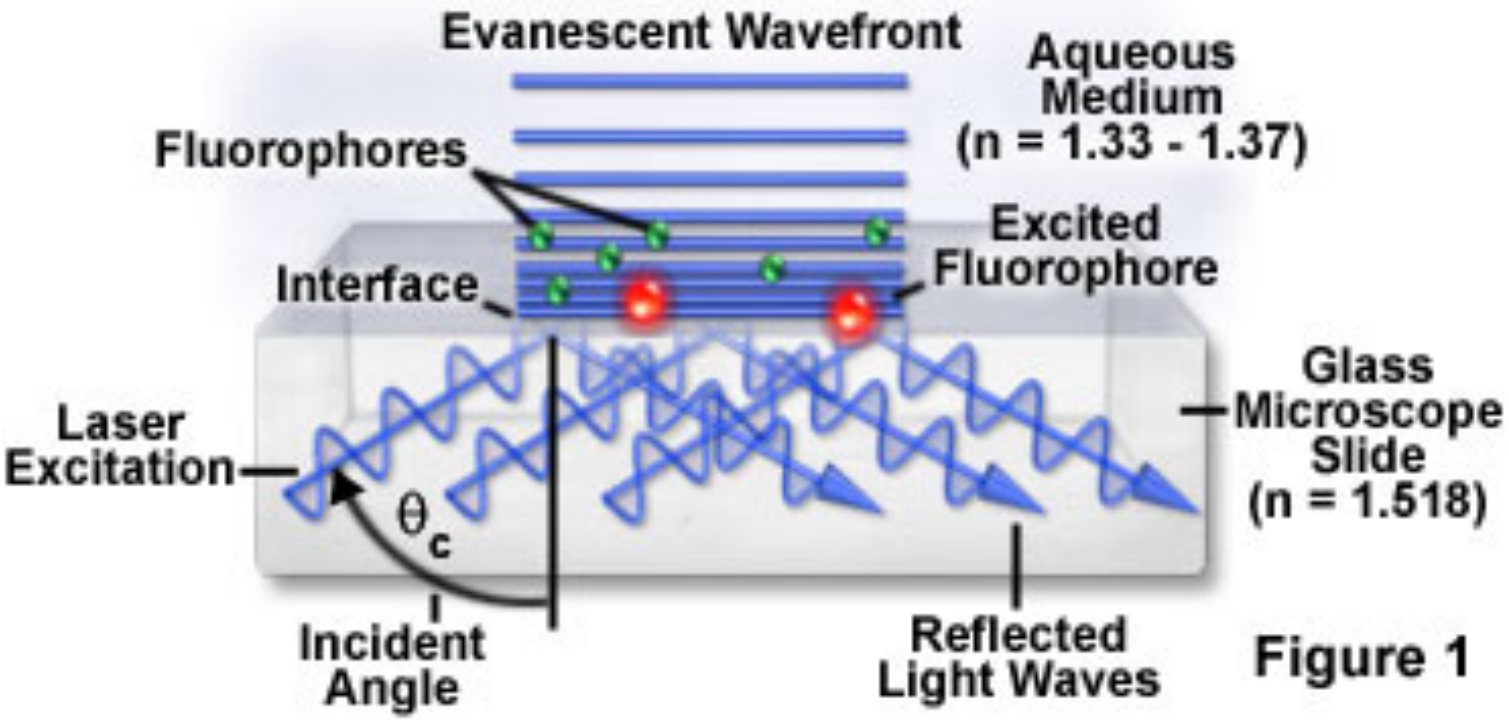


Figure 1

The physical phenomenon of total internal reflection (TIR)

Since only a very thin sliver of excitation is being produced, we only detect photons that are created within that excitation volume, which has the effect of significantly improving signal to background.

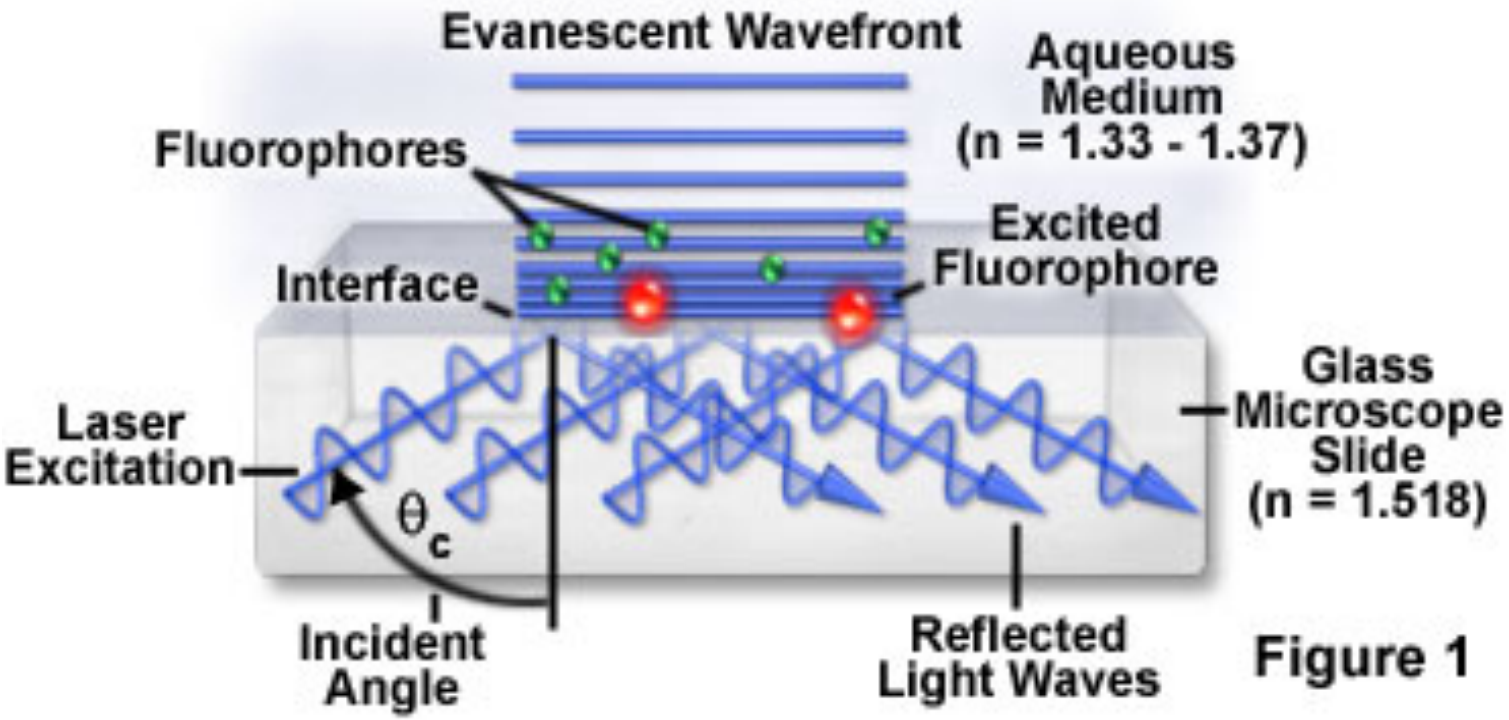


Figure 1

The physical phenomenon of total internal reflection (TIR)

TIRFM is ideal for capturing high resolution, high Signal to Noise (S/N) kinetic series of membrane events, such as exocytosis and membrane receptor/transporter lateral dynamics. TIRFM is also a common technique for imaging single molecules dynamics, covered more thoroughly in the Single Molecule Detection area of the Biology Applications section.

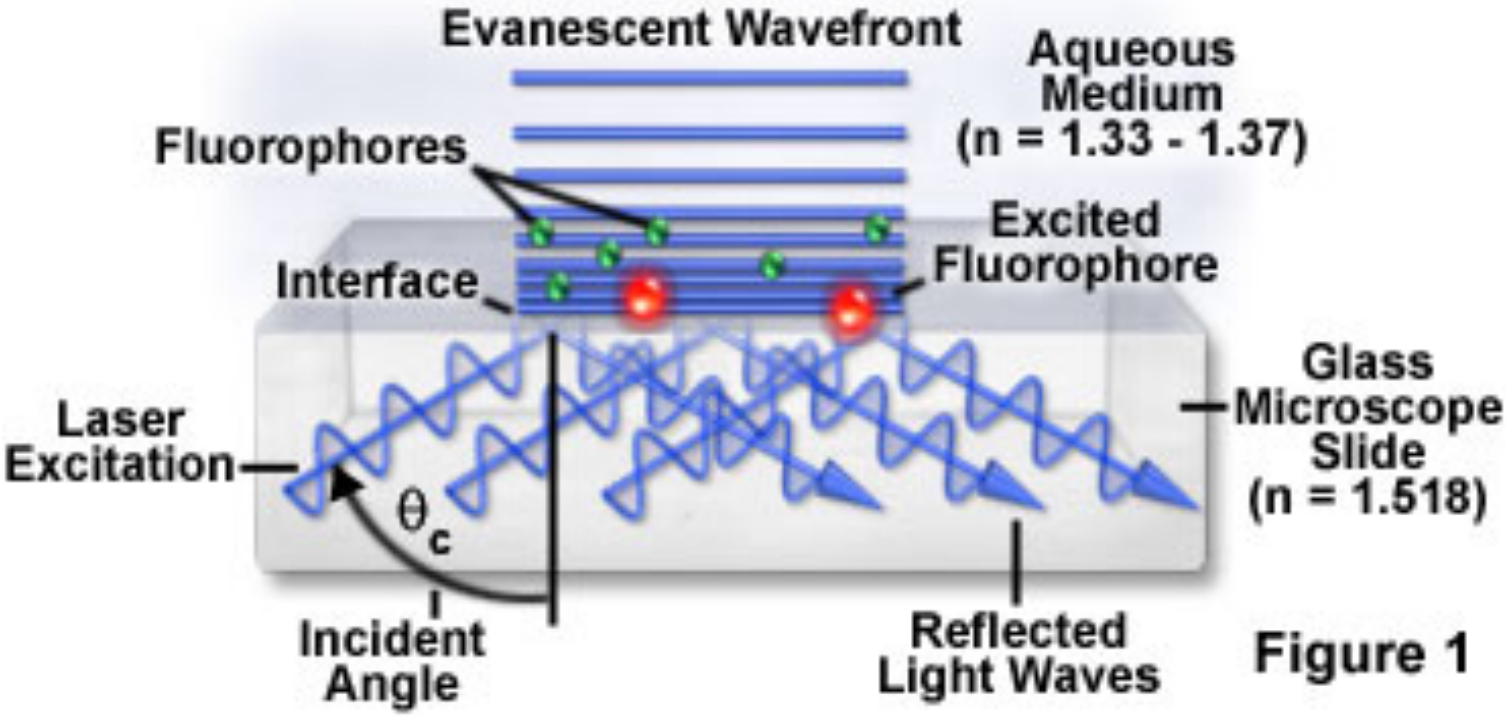
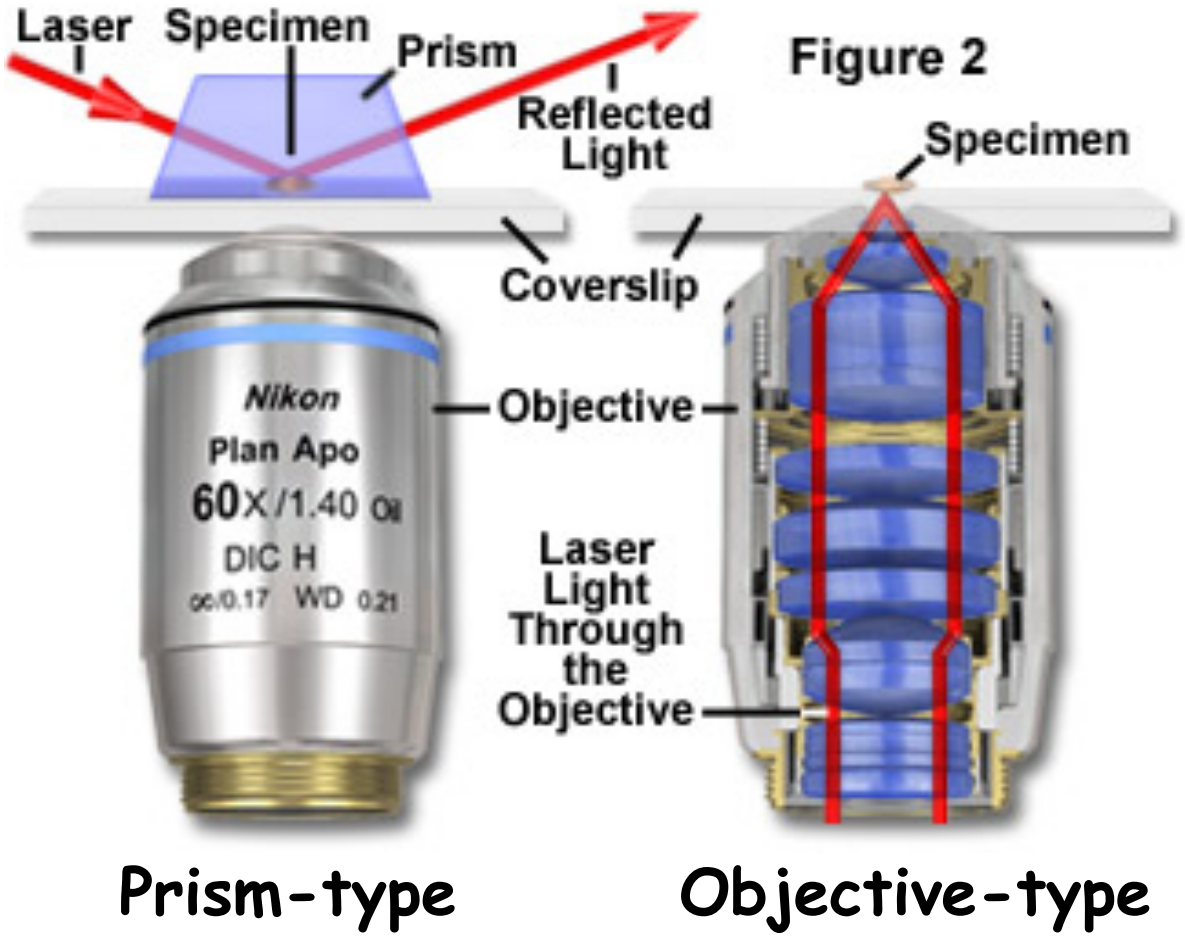


Figure 1

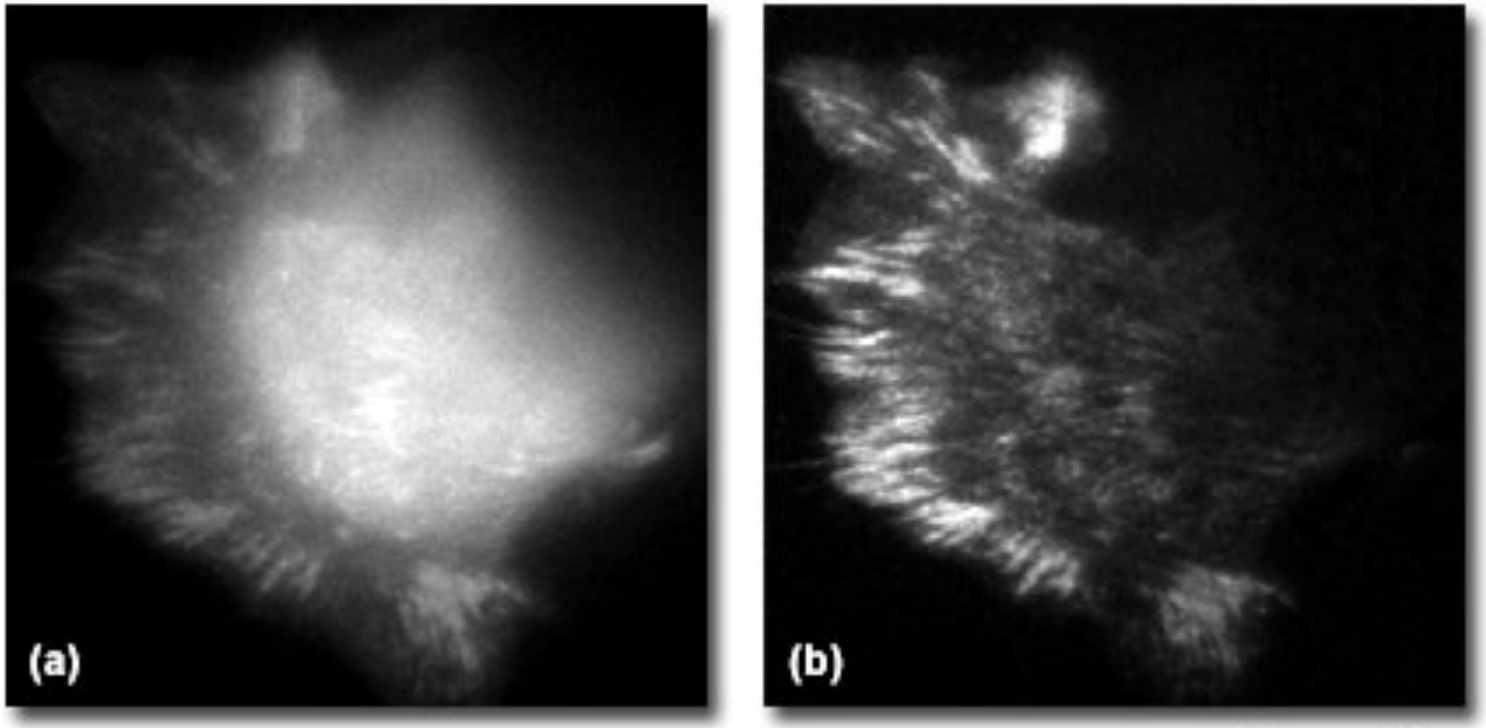
TIRFM specimen illumination configurations



Cell Focal Adhesion in Widefield and TIRF

A live cell microscopy set-up that enables one to switch rapidly between TIRFM and widefield epi-fluorescence microscopy, the latter for deeper penetration of the excitation into the bulk of the sample, represents a flexible and powerful approach for studying intracellular transport mechanisms.

GFP-vinculin expressing epithelial cells



Widefield epi-fluorescence

Evanescent wave illumination

Time Lapse sequence of Protein Dynamics

GFP-Rac trafficking along thin filopodia