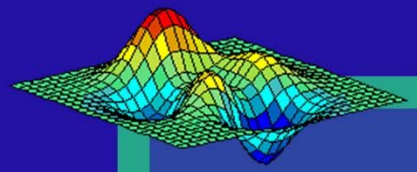

FRET in flow and image cytometry

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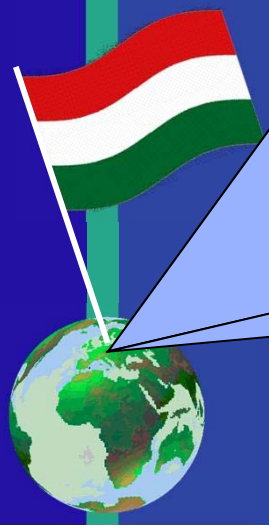
University of Debrecen



Debrecen, Hungary

The importance of FRET in biology

FRET = fluorescence resonance energy transfer

described by the German physical chemist ***Theodor Förster***, therefore FRET can also be the acronym for Förster resonance energy transfer

- FRET is a sensitive distance measuring tool → it is suitable for measuring the association state and conformation of molecules
- FRET is quantitative ↔ many molecular biological methods used for the investigation of the interaction between molecules are semi-quantitative at best
- microscopic and flow cytometric implementations of FRET make single cell measurements possible
- FRET measurements are usually cheap and relatively easy

- P. Nagy, G. Vereb, S. Damjanovich, L. Mátyus, J. Szöllősi: Measuring FRET in Microscopy and Flow Cytometry, Unit 12.8, Current Protocols in Cytometry
- J. Szöllősi, S. Damjanovich, P. Nagy, G. Vereb, L. Mátyus: Principles of Resonance Energy Transfer, Unit 1.12, Current Protocols in Cytometry
- P. Nagy et al.: Novel Single Cell Fluorescence Approaches in the Investigation of Signaling at the Cellular level. Chapter 2 In: Biophysical aspects of transmembrane signaling (Ed.: S. Damjanovich), Springer, 2005
- EA. Jares-Erijman, TM. Jovin: FRET imaging, Nat. Biotechnol., 21:1387 (2003)
- J. Szöllősi, S. Damjanovich, L. Mátyus: Application of fluorescence resonance energy transfer in the clinical laboratory: routine and research. Cytometry, 34: 159 (1998)

The FRET phenomenon

- After excitation the molecule returns to the lowest vibrational level of the first excited state. Every subsequent process starts from this level.

- In FRET an acceptor molecule close to the donor receives the excitation energy of the donor by means of radiationless energy transfer.

- FRET is manifested in the fluorescence of the acceptor after exciting the donor.

The rate constant of FRET is described by the following equation:

$$k_{FRET} = const \cdot Jn^{-4} k_f R^{-6} \kappa^2$$

In **most** experiments every parameter other than R can be considered to be constant → FRET only depends on the donor-acceptor distance.

R – donor-acceptor distance

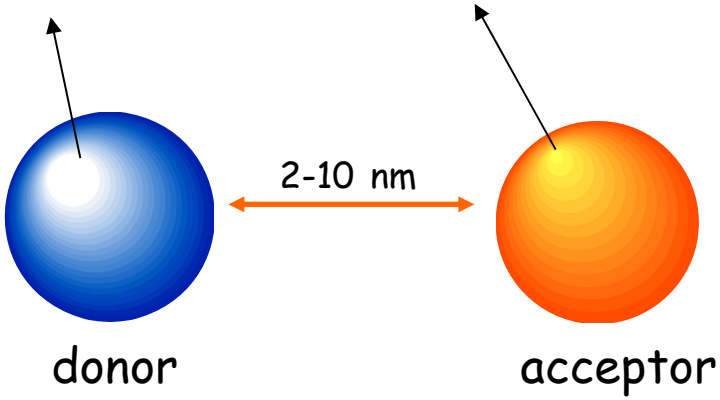
κ^2 – orientation factor

J – overlap integral

k_f – donor fluorescence quantum yield in the absence of acceptor

n – index of refraction

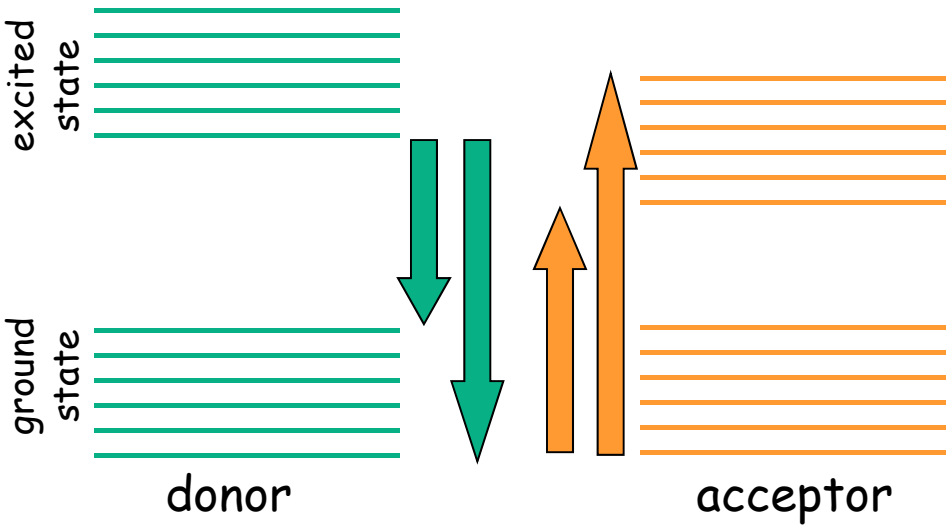
Conditions of FRET

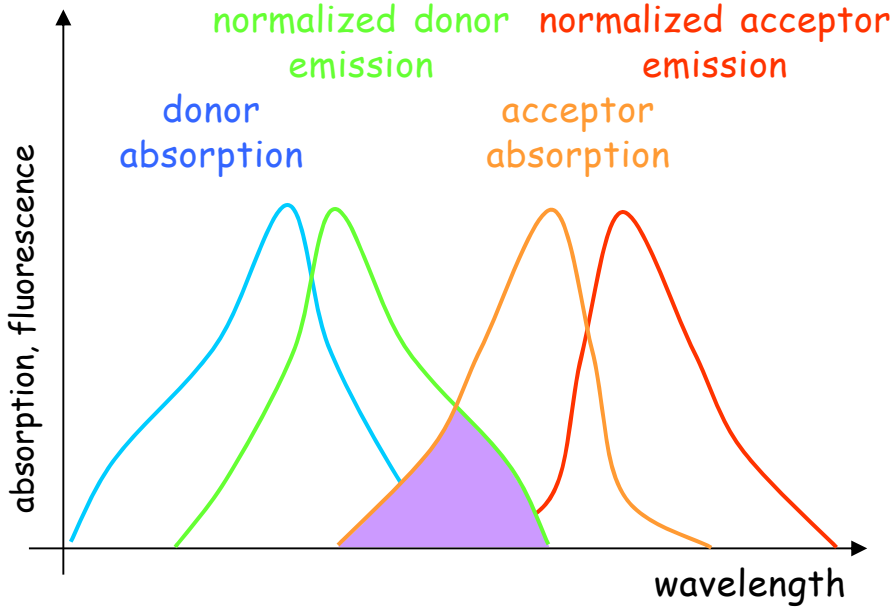


- the distance between the donor and the acceptor is 2-10 nm
- the orientation of the donor relative to the acceptor is *favorable*
- the difference between the energies of the ground and excited states of the donor and the acceptor are comparable → the emission spectrum of the donor overlaps with the absorption spectrum of the acceptor

$$k_{FRET} = const\ J\ n^{-4} k_f R^{-6} \kappa^2$$

J – overlap integral between the emission spectrum of the donor and the absorption spectrum of the acceptor





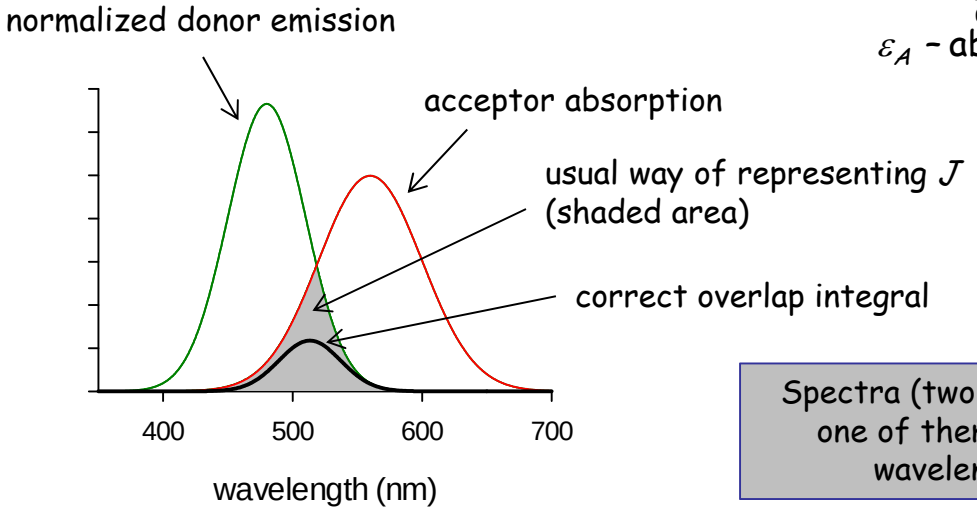
The overlap integral (J)

$$J = \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda = \frac{\int_0^\infty f_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int_0^\infty f_D(\lambda) d\lambda}$$

F_D – normalized emission spectrum of the donor (area normalized to unity)

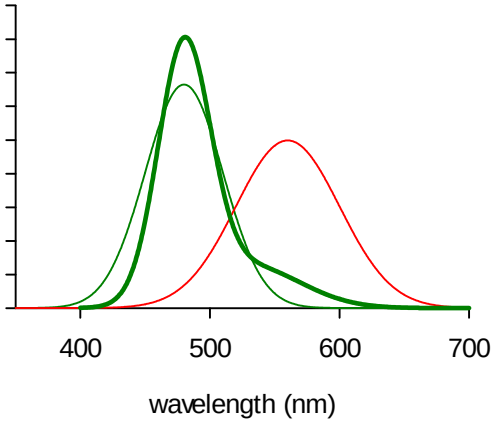
f_D – non-normalized emission spectrum of the donor (which is normalized by dividing with $\int_0^\infty f_D(\lambda) d\lambda$)

ε_A – absorption coefficient of the acceptor

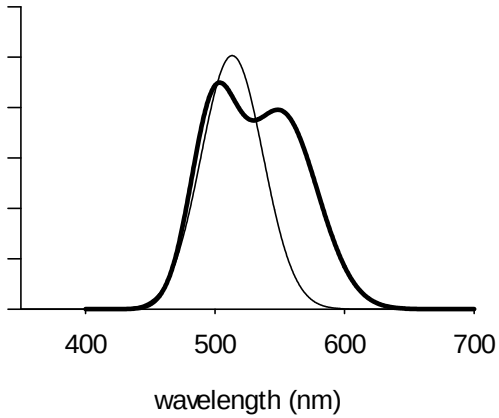


Long wavelengths are weighted more heavily in the overlap integral (due to λ^4).

Spectra (two donor spectra, one of them with a long wavelength tail)



Overlap integrals

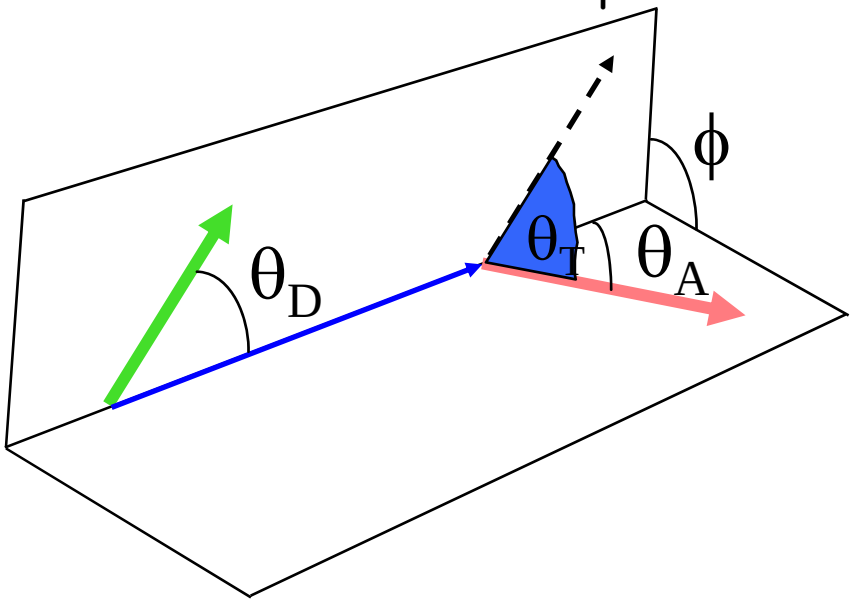


The role of the orientation of the donor relative to the acceptor

$$k_{FRET} = const \cdot Jn^{-4} k_f R^{-6}$$

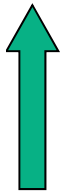
$$\kappa^2 = \left(\cos\theta_T - 3\cos\theta_D \cos\theta_A\right)^2$$

κ^2 describes the orientation of the donor relative to the acceptor.

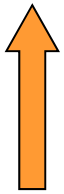


During the excited state lifetime of the donor the relative orientation of the donor and acceptor changes rapidly.

all possible relative orientations are averaged (dynamic averaging)



donor

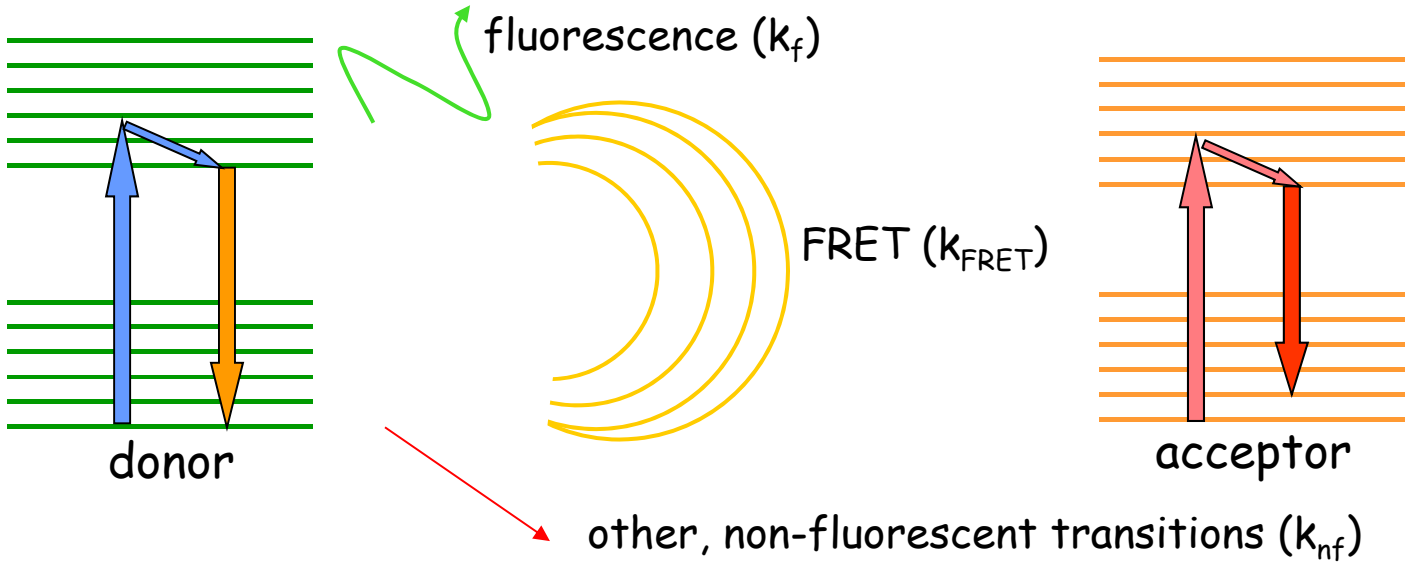


acceptor

In this case κ^2=2/3, and the FRET efficiency is independent of the relative orientation of the donor and the acceptor

FRET is the function of the donor-acceptor distance only

Interpretation of FRET efficiency



- FRET competes with all other relaxation mechanisms.
- The probability that an excited donor undergoes FRET is given by the rate constant of FRET relative to the rate constants of all relaxations mechanisms:

$$E = \frac{k_{FRET}}{k_{FRET} + k_f + k_{nf}}$$

It is preferable to determine FRET efficiency instead of uncalibrated FRET intensity or enigmatic FRET parameters!

E: the fraction of excited donor molecules relaxing by FRET

Interpretation of FRET efficiency

A common way to define FRET efficiency:

$$E = \frac{R_o^6}{R_o^6 + R^6} \qquad \left(k_{FRET} \sim R_o^6\right)$$

R_o is the distance at which FRET efficiency is 50% for a given donor-acceptor pair. R_o is characteristic of the donor-acceptor pair. When selecting a donor-acceptor pair, it is usually advisable to **maximize R_o**.

$$k_{FRET} = \frac{1}{\tau_D}\left(\frac{R_o}{R}\right)^6$$

$$R_o^6 = const \cdot \kappa^2 Jn^{-4} \Phi_D = const \cdot \kappa^2 Jn^{-4} k_f \tau_D$$

$$k_{FRET} = \frac{1}{\tau_D} \frac{const \cdot \kappa^2 Jn^{-4} k_f \tau_D}{R^6} = \frac{const \cdot \kappa^2 Jn^{-4} k_f}{R^6}$$

$$\frac{k_f}{k_{FRET}} = \frac{R^6}{const \cdot \kappa^2 Jn^{-4}} = \Theta$$

Φ_D – fluorescence quantum yield of the donor in the absence of FRET

τ_D – fluorescence lifetime of the donor in the absence of FRET

- k_f/k_{FRET} is a parameter only related to fundamental variables describing the FRET interaction, BUT in most cases it is **impossible to measure**
- Θ is related to the FRET efficiency (E) according to the following equation:

$$\Theta = \Phi_D \frac{1-E}{E}$$

Roberti, M. J., L. Giordano, T. M. Jovin, and E. A. Jares-Erijman. 2011. FRET Imaging by k(t) /k(f). Chemphyschem 12:563-566.

Jares-Erijman, E. A., and T. M. Jovin. 2003. FRET imaging. Nat Biotechnol 21:1387-1395.

Interpretation of FRET efficiency

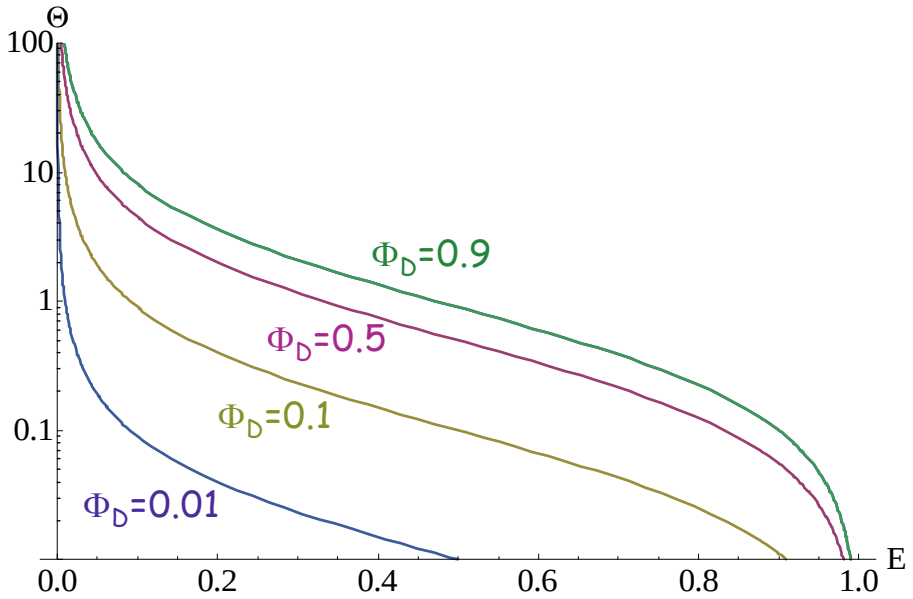
$$\Theta = \Phi_D \frac{1-E}{E}$$

- 'E' is measurable ($\Leftrightarrow \Theta$, k_{FRET}), but it is related to factors not fundamentally related to the FRET process, e.g. Φ_D.

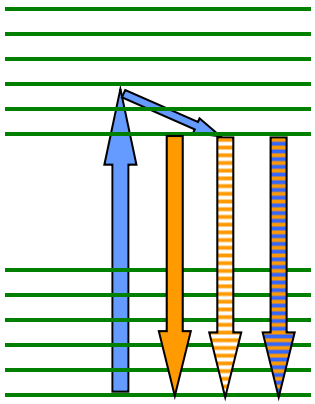
It is advisable to use donor with high quantum yield (Φ_D), so that

- Ro is large
- the dynamic range of Θ is large

The variation in Θ at large values of E is low (log scale!). High FRET efficiencies and changes thereof have to be interpreted with caution.



Manifestations of FRET, methods for measuring FRET: donor quenching



fluorescent transition (k_f)

non-fluorescent transition (k_{nf})

excitation

FRET (k_{FRET})

Fluorescence quantum efficiency/yield (Φ): the fraction of excited molecules emitting a fluorescence photon.

Fluorescence quantum efficiency in the absence of FRET:

$$\Phi_D = \frac{k_f}{k_f + k_{nf}}$$

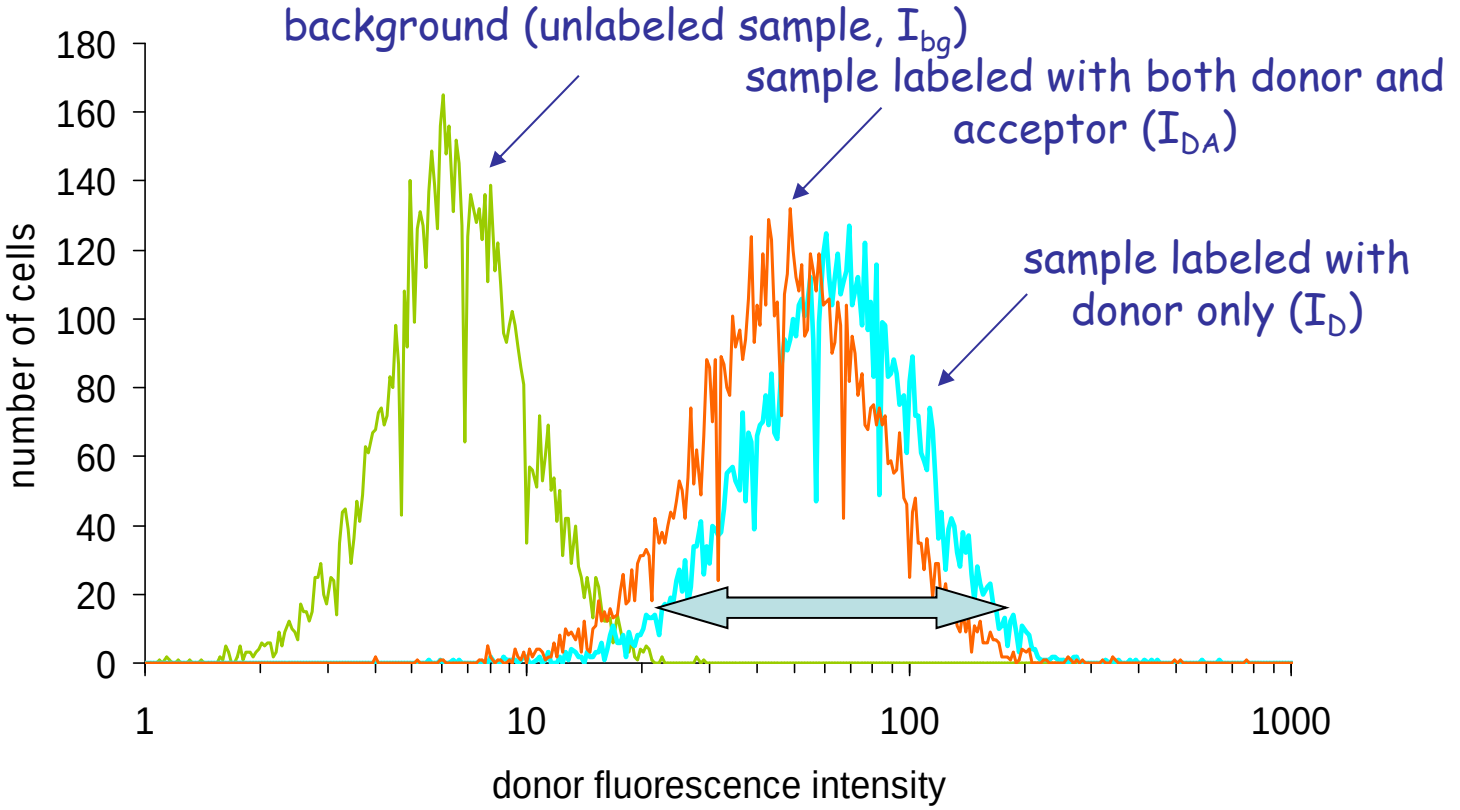
Fluorescence quantum efficiency in the presence of FRET:

$$\Phi_{DA} = \frac{k_f}{k_{FRET} + k_f + k_{nf}}$$

The fluorescence quantum yield is decreased by FRET, since FRET competes with fluorescence.

$$\frac{\Phi_{DA}}{\Phi_D} = \frac{k_f + k_{nf}}{k_{FRET} + k_f + k_{nf}} = \frac{k_f + k_{nf} + k_{FRET} - k_{FRET}}{k_{FRET} + k_f + k_{nf}} = 1 - E \quad \Rightarrow \quad E = 1 - \frac{\Phi_{DA}}{\Phi_D} = 1 - \frac{I_{DA}}{I_D}$$

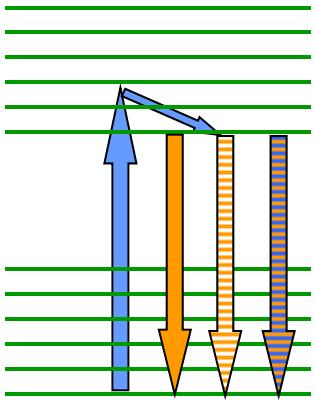
Manifestations of FRET, methods for measuring FRET: donor quenching



$$E = 1 - \frac{I_{DA} - I_{bg}}{I_D - I_{bg}}$$

- Donor quenching can only be measured using flow cytometry due to the large variability of cells.
- Disadvantage: I_{DA} and I_D are measured on different samples, so differences between the donor-only and donor-acceptor double-labeled sample (other than FRET) lead to errors in the FRET calculation.

Manifestations of FRET, methods for measuring FRET: decreased donor fluorescence lifetime



fluorescent transition (k_f)

non-fluorescent transition (k_{nf})

excitation

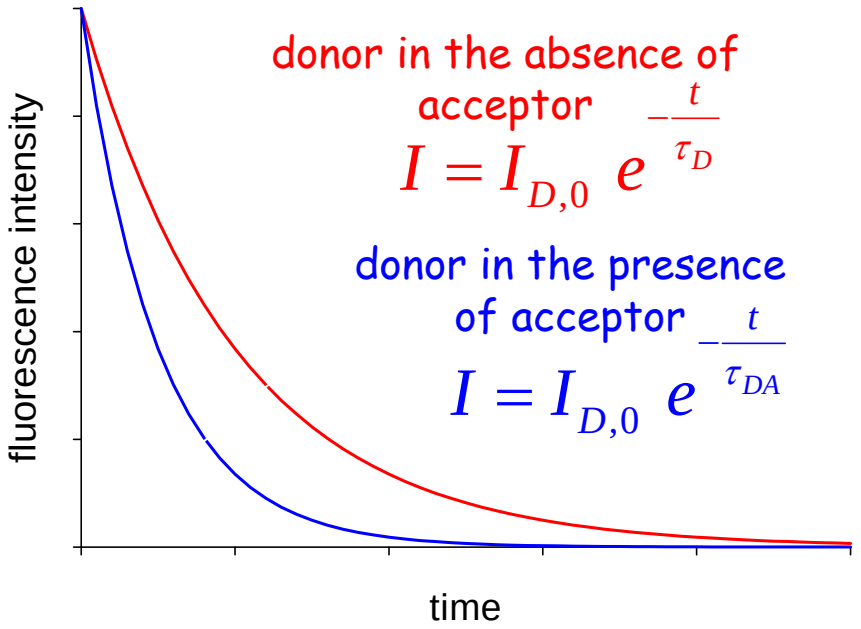
FRET (k_{FRET})

Since the fluorescence lifetime of the donor is in the nanosecond time range, the measurement is complicated and expensive.

FRET, as an extra mechanism of relaxation, increases the rate of donor relaxation.

The fluorescence lifetime of the donor decreases in FRET: $\tau_{da}<\tau_d$

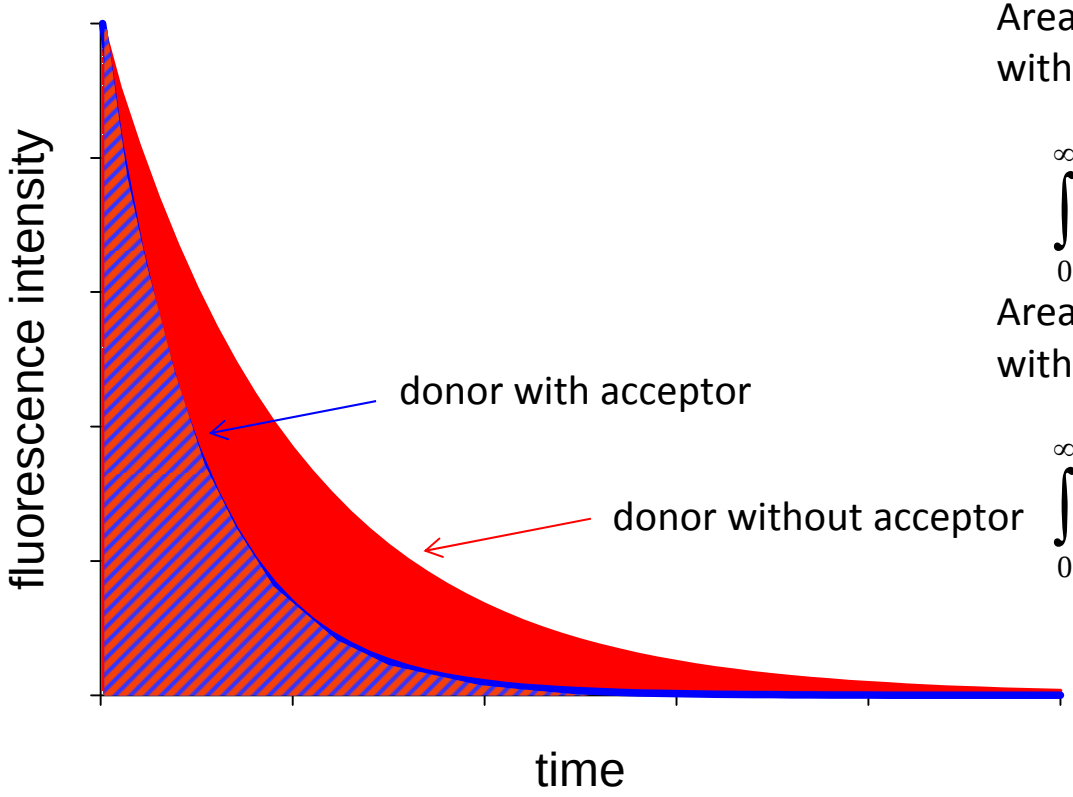
$$E = 1 - \frac{\Phi_{DA}}{\Phi_D} = 1 - \frac{I_{DA}}{I_D}$$



donor in the absence of acceptor $I = I_{D,0}\, e^{-\frac{t}{\tau_D}}$

donor in the presence of acceptor $I = I_{D,0}\, e^{-\frac{t}{\tau_{DA}}}$

Decreased steady-state donor intensity is the consequence of the shortened donor fluorescence lifetime



Area under the curve of the red line (donor without acceptor):

$$\int_0^\infty I_{D,0} e^{-\frac{t}{\tau_D}} = I_{D,0}\tau_D$$

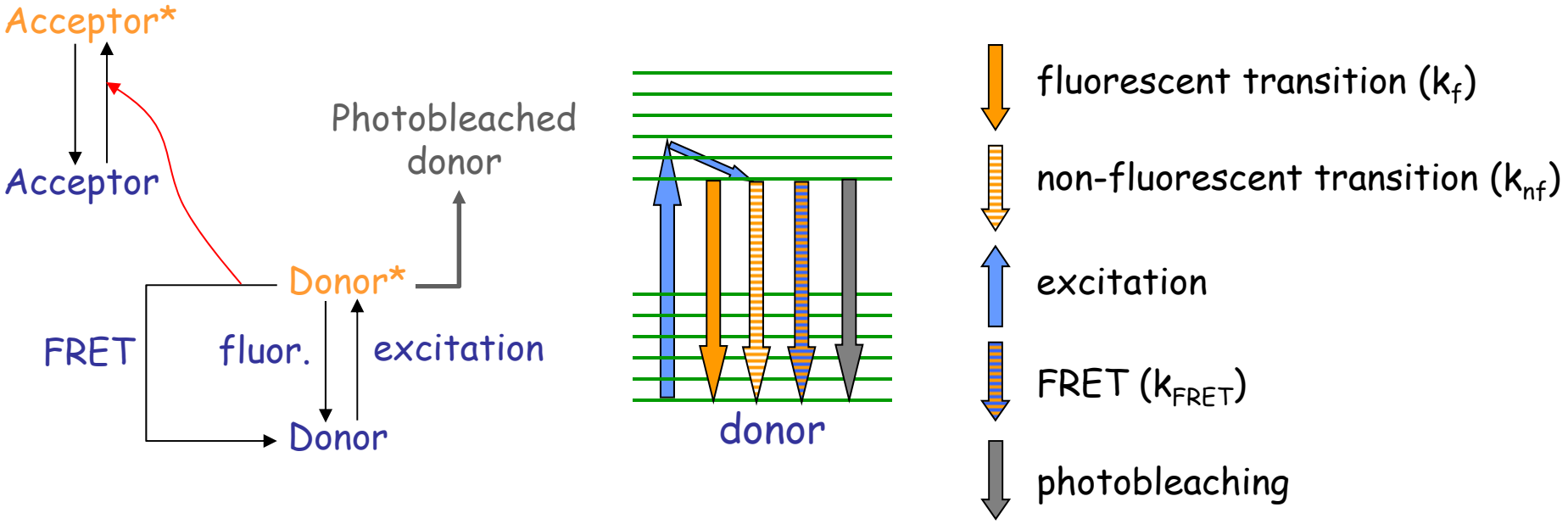
Area under the curve of the blue line (donor with acceptor):

$$\int_0^\infty I_{D,0} e^{-\frac{t}{\tau_{DA}}} = I_{D,0}\tau_{DA}$$

Steady-state intensity is proportional to the area under the curve of the time-dependent fluorescence. Therefore:

$$\frac{I_{DA}}{I_D} = \frac{I_{D,0}\tau_{DA}}{I_{D,0}\tau_D} = \frac{\tau_{DA}}{\tau_D}$$

Manifestations of FRET, methods for measuring FRET: donor photobleaching



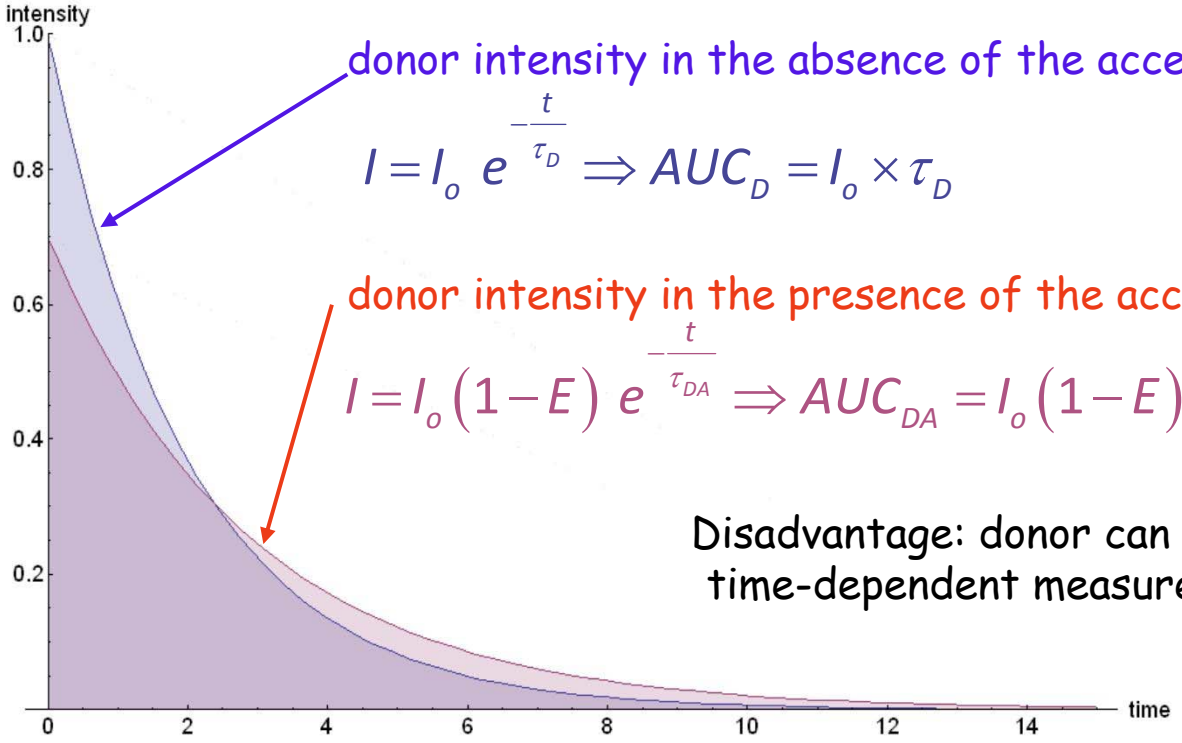
Photobleaching:

- a process starting from the excited state in which the fluorophore loses its absorption capability
- since it starts from the excited state, the more time the fluorophore spends in the excited state, the faster photobleaching is
- since FRET decreases the donor fluorescence lifetime, the rate of photobleaching is decreased by FRET

Manifestations of FRET, methods for measuring FRET: donor photobleaching

Alternative explanation for the effect of FRET on donor photobleaching:

- the total number of photons a donor can emit before it is photobleached is constant, i.e. it is independent of the presence of the acceptor
- the total number of photons is equal to the area under the curve (AUC) of the intensity-time plot
- since the donor intensity starts from a lower value in the presence of the acceptor, the curve must decrease more slowly so that the AUC is the same in the presence as in the absence of the acceptor



donor intensity in the absence of the acceptor

$$I = I_o\, e^{-\frac{t}{\tau_D}} \Rightarrow AUC_D = I_o \times \tau_D$$

donor intensity in the presence of the acceptor

$$I = I_o\left(1-E\right) e^{-\frac{t}{\tau_{DA}}} \Rightarrow AUC_{DA} = I_o\left(1-E\right)\times \tau_{DA}$$

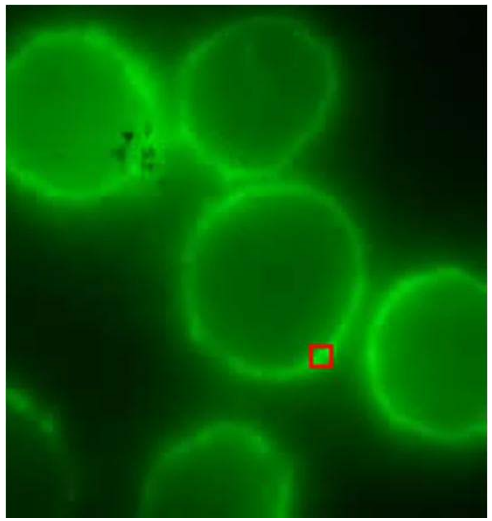
$$AUC_D = AUC_{DA}$$

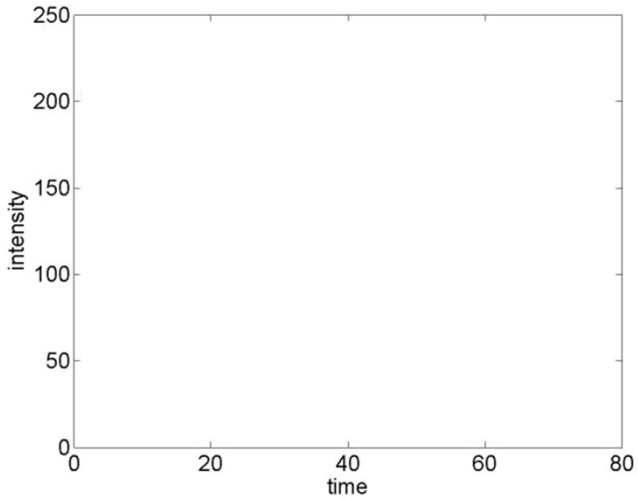
$$I_o \times \tau_D = I_o\left(1-E\right)\times \tau_{DA}$$

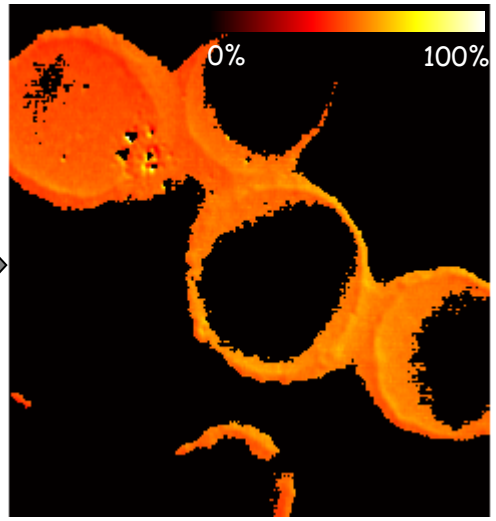
$$E = 1 - \frac{\tau_D}{\tau_{DA}}$$

Disadvantage: donor can only be bleached once → time-dependent measurements are not possible.

Manifestations of FRET, methods for measuring FRET: donor photobleaching







FRET image

- select region of interest (ROI) or do the analysis on a pixel-by
- analyze the intensity as a function of time
- fit the intensity values to exponential functions:

$$I = I_1 e^{-\frac{t}{\tau_1}} + I_o$$

$$I = I_1 e^{-\frac{t}{\tau_1}} + I_2 e^{-\frac{t}{\tau_2}} + I_o$$

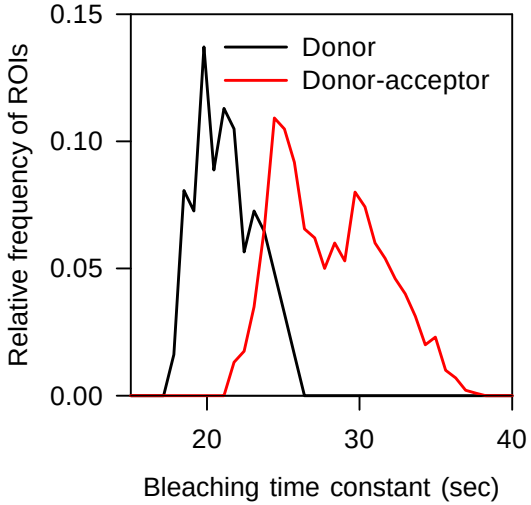
$$\tau_{eff} = \frac{I_1\tau_1 + I_2\tau_2}{I_1 + I_2}$$

determine on donor-only sample

$$E = 1 - \frac{\quad}{\quad}$$

determine on donor-acceptor double-labeled sample

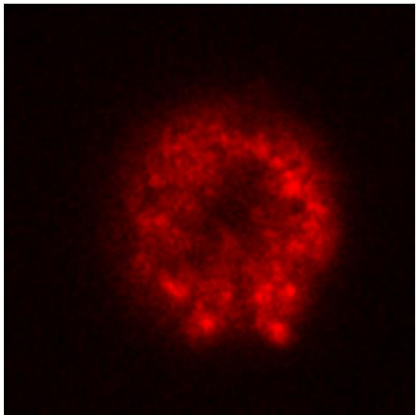
Caution: τ_d and τ_{da} are measured on different samples

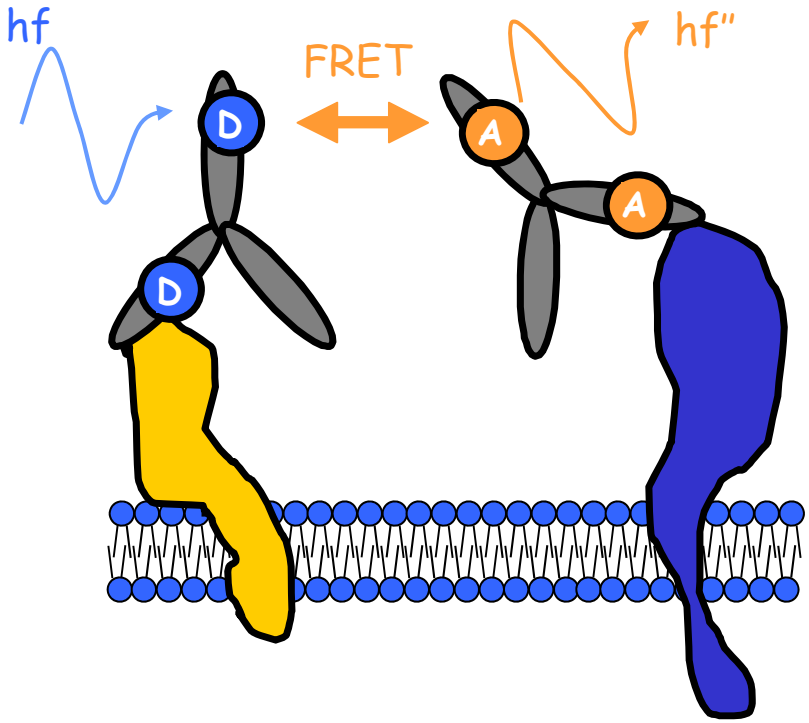


RM. Young et al.: Biophys. J. 67: 881 (1994)

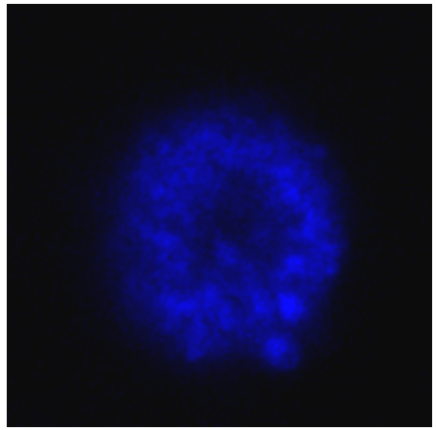
Manifestations of FRET, methods for measuring FRET: acceptor photobleaching

donor



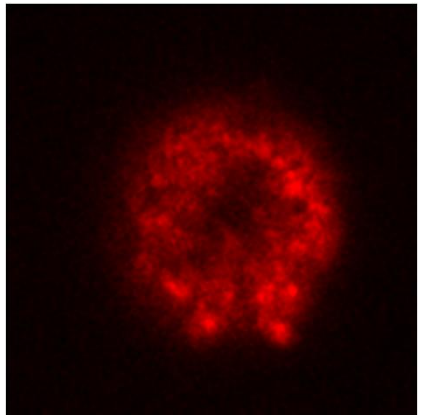


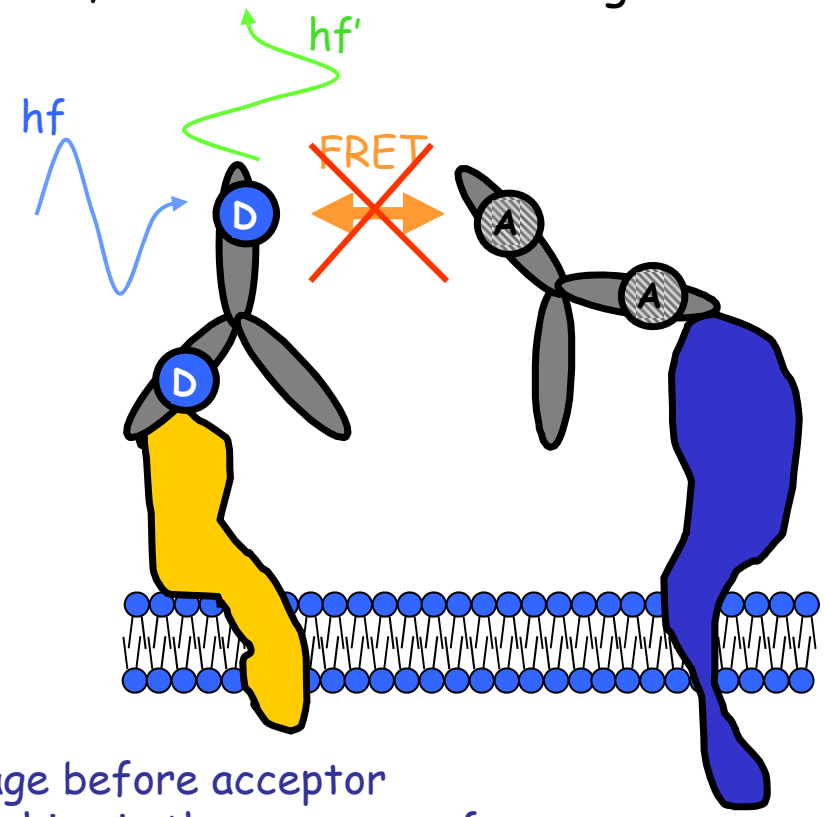
acceptor

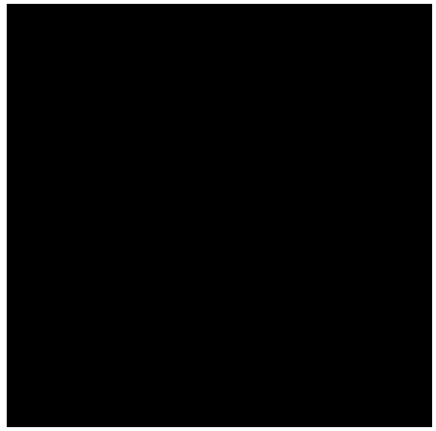


PI. Bastiaens et al.: EMBO J. 15: 4246 (1996)

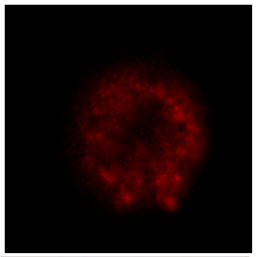
Manifestations of FRET, methods for measuring FRET: acceptor photobleaching

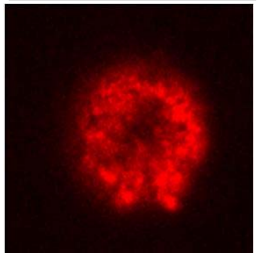






$$E = 1 - \frac{I_{DA}}{I_D} = 1 - \frac{I_{pre}}{I_{post}}$$





Donor image before acceptor photobleaching in the presence of FRET, $I_{pre}=I_{DA}$

Donor image after acceptor photobleaching, i.e. after "turning FRET off", $I_{post}=I_D$

NO TEMPORAL RESOLUTION (BLEACHING IS IRREVERSIBLE)

Be careful: acceptor photobleaching can cause funny things with the donor and acceptor molecules!
E.g. photoconversion of YFP to a CFP-like species artificially increasing the intensity in the donor channel → "negative" FRET, Nat. Methods 4:767 (2007)

Careful controls!!
TS. Karpova et al: J. Microsc., 209:56 (2003)

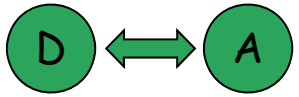
PI. Bastiaens et al.: EMBO J. 15: 4246 (1996)

Homo-FRET vs. hetero-FRET

hetero-FRET: "conventional" FRET between a donor and a different acceptor molecule

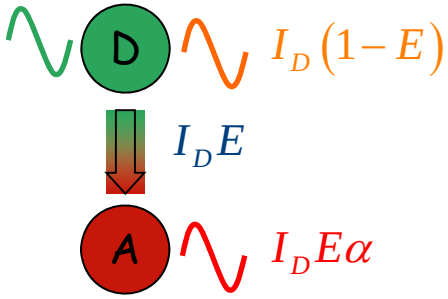
Manifestations:

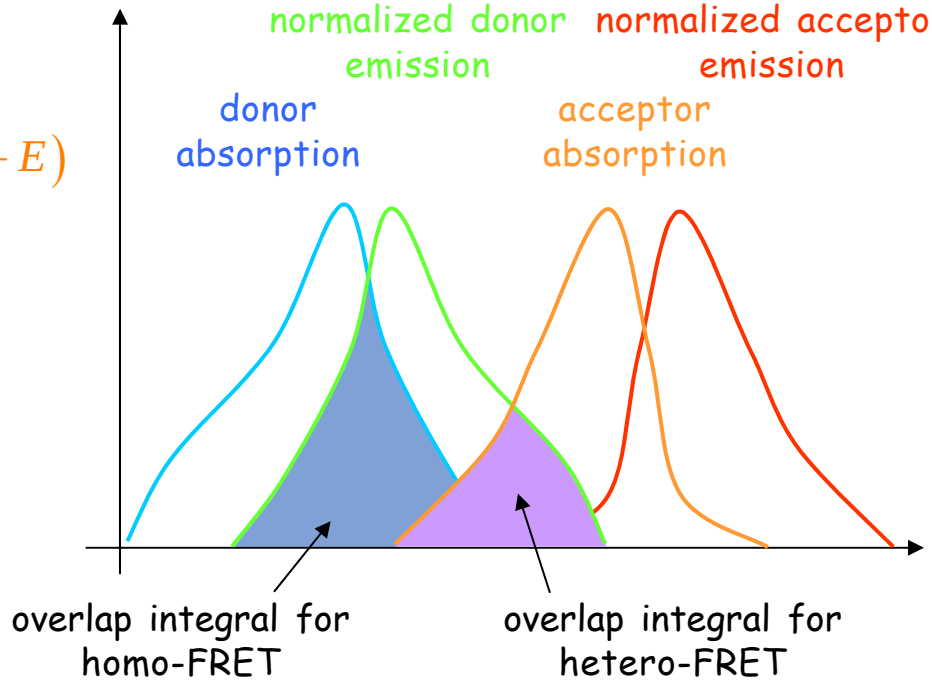
- donor quenching
- sensitized acceptor emission
- **increased donor anisotropy** due to shortened donor lifetime



homo-FRET: FRET between two, spectroscopically identical molecules. It goes back and forth.

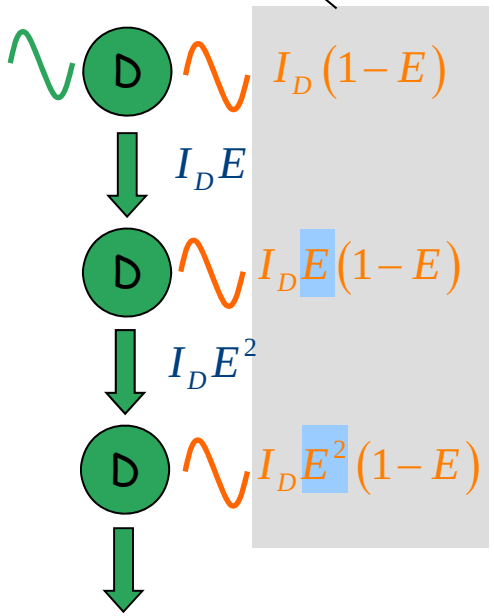
Since the donor and acceptor molecules are identical (i.e. their fluorescence cannot be separated from each other), the only manifestation of homo-FRET is **decreased donor anisotropy**.



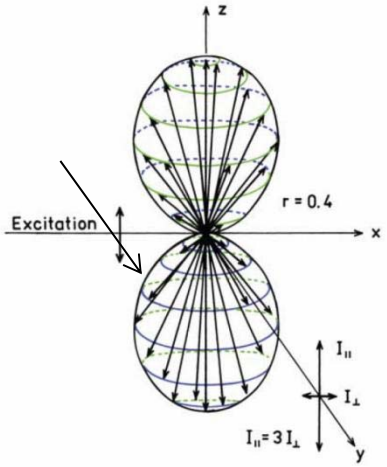


sum of these emissions (infinite geometrical series):

$$\frac{I_D(1-E)}{1-E} = I_D$$

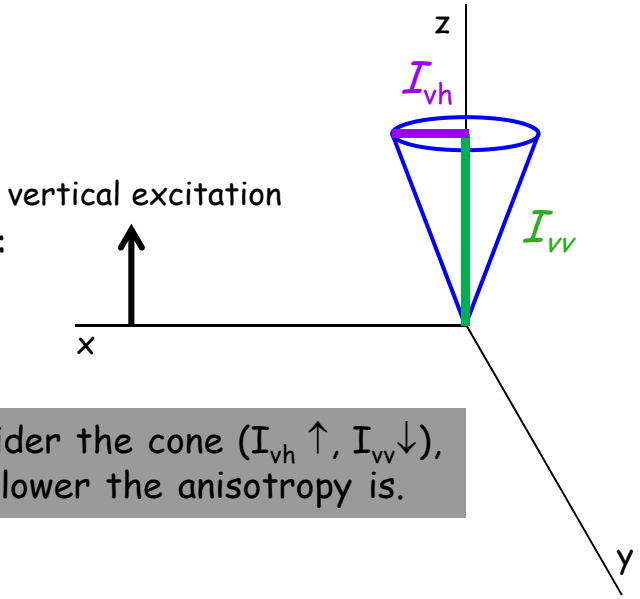


Fluorescence anisotropy: excitation photoselection, rotation during the excited-state lifetime

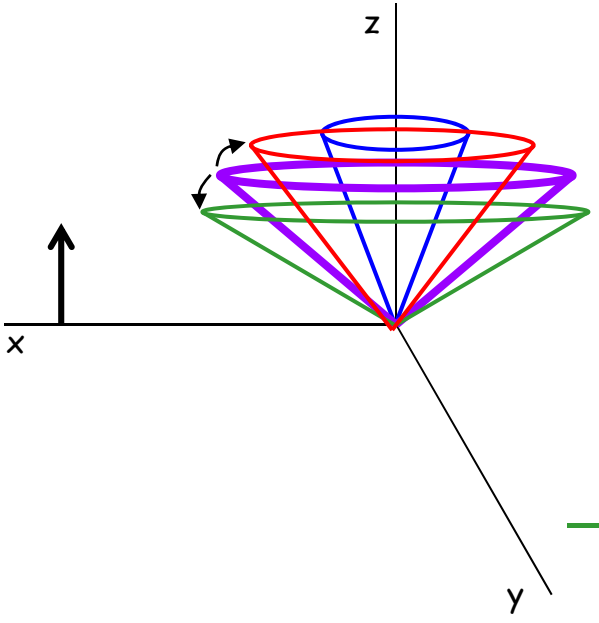


Photoselection: The more parallel a molecule is to the *z* axis, the more likely it is to be excited.

Usually displayed like this:

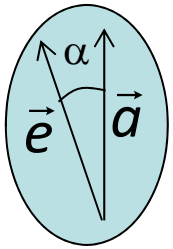


The wider the cone (I_{vh} ↑, I_{vv}↓), the lower the anisotropy is.



immobile fluorophore, absorption and emission are parallel r=0.4

immobile fluorophore in which emission is rotated by an angle of α relative to excitation:



$$r = \frac{2}{5}\frac{3\cos^2\alpha - 1}{2} = \frac{3\cos^2\alpha - 1}{5}$$

This is called the **limiting anisotropy** of the fluorophore.

any other effect decreasing anisotropy (e.g. rotational diffusion, homo-FRET)

effects decreasing anisotropy (hindered rotation, decreased fluorescence lifetime of the donor in hetero-FRET)

Fluorescence anisotropy: how to measure it in practice?

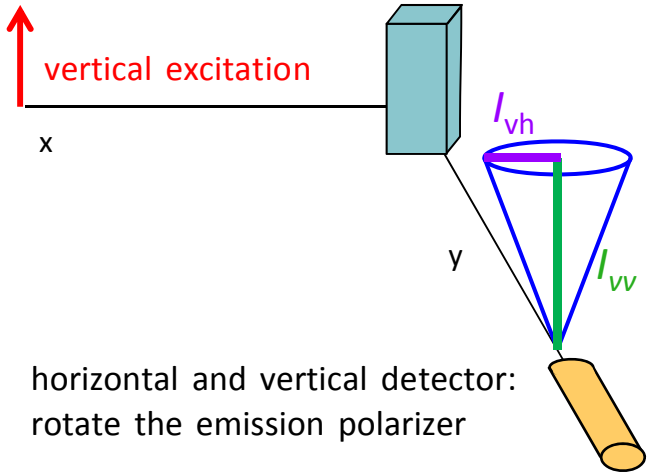
$$r = \frac{I_{vv} - I_{vh}}{I_{tot}} = \frac{I_{vv} - I_{vh}}{I_{vv} + 2I_{vh}}$$

anisotropy if the device is equally sensitive for detecting vertically and horizontally polarized photons

$$r = \frac{I_{vv} - GI_{vh}}{I_{tot}} = \frac{I_{vv} - I_{vh}}{I_{vv} + 2GI_{vh}}; \; G = \frac{I_{hv}}{I_{hh}}$$

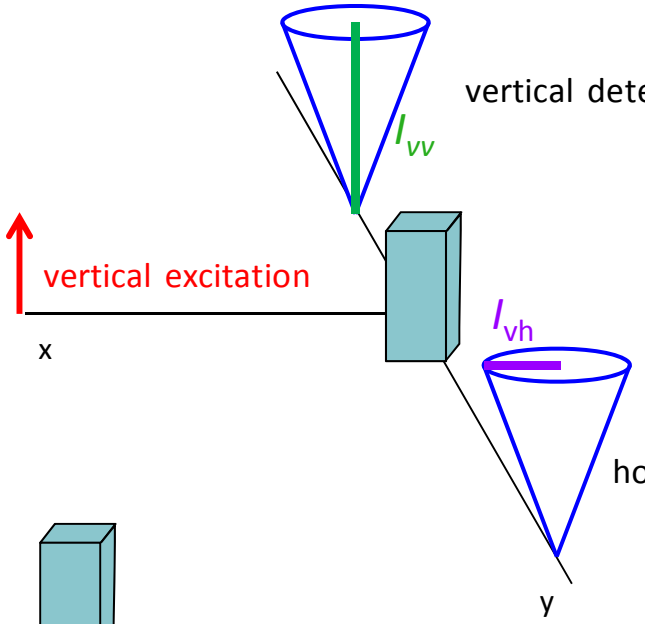
anisotropy if the device is NOT equally sensitive for detecting vertically and horizontally polarized photons

With one detector at a time: L-format

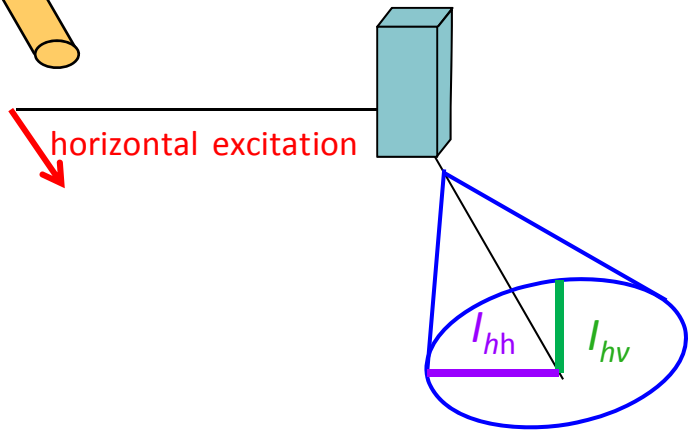


horizontal and vertical detector: rotate the emission polarizer

With two detectors at a time: T-format



Determination of the G factor

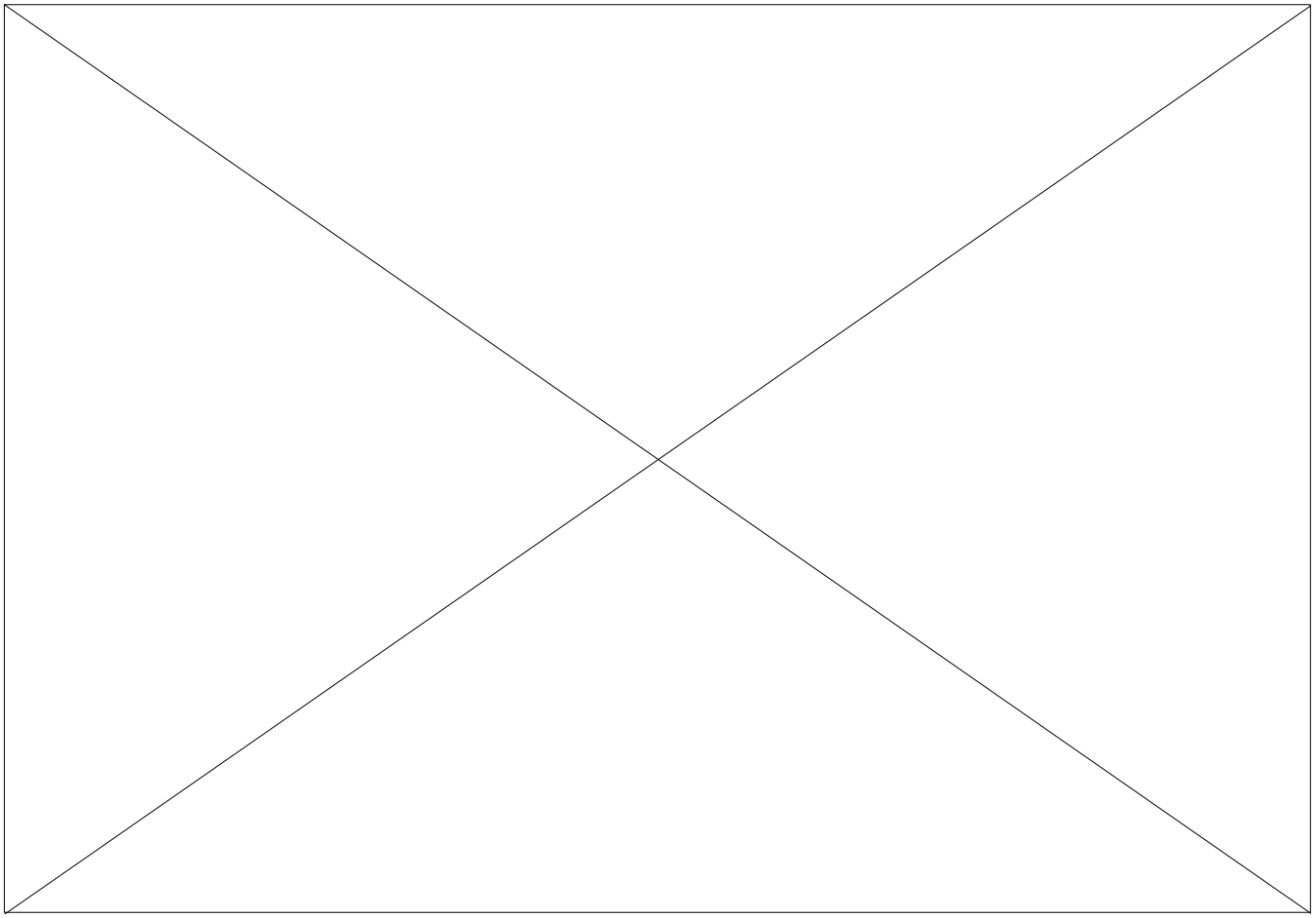


Due to the symmetry of rotation around the *y* axis the horizontal and vertical emission components are equal. Any difference between the ***measured*** I_{hh} and I_{hv} are due to instrumental factors.

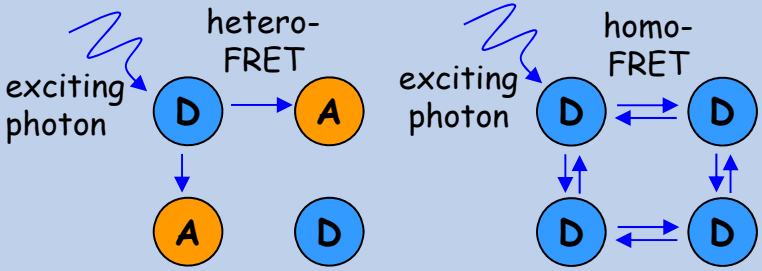
Manifestations of FRET, methods for measuring FRET: FRET-induced change in donor fluorescence anisotropy

Anisotropy is a measure of fluorescence polarization: $r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}}$

where I_{VV} and I_{VH} are the vertically and horizontally, respectively, polarized fluorescence intensities of the fluorophores excited by vertically polarized light



Advantages of homo-FRET



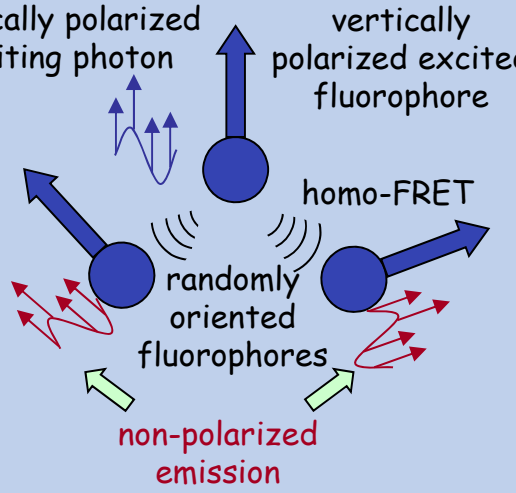
Advantages of homo-FRET

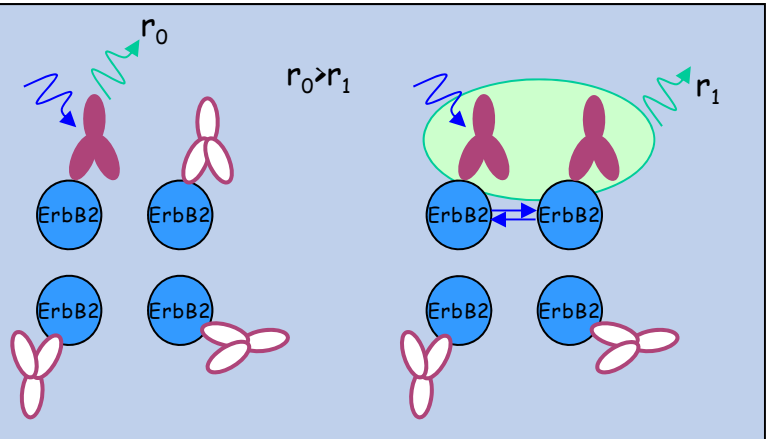
- the excitation energy is spread in the ensamble of fluorophores → it is possible to derive the cluster size (the number of molecules taking part in FRET)
- the sample has to be labeled by only one fluorophore

Disadvantage of homo-FRET:

- requires special, but relatively simple and cheap instrument

Homo-FRET depolarizes fluorescence (the lower anisotropy is, the more homo-FRET takes place).



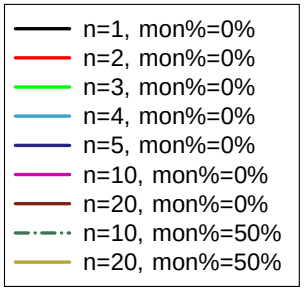


If cells are labeled with an increasing concentration of fluorescent antibodies, anisotropy decreases as a function of saturation.

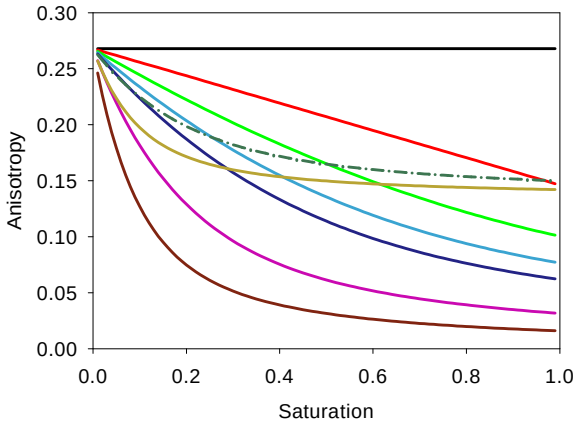
the probability that an N-mer contains *k* labeled antibodies

anisotropy of cluster containing *k* labeled antibodies

$$r_{s,N} = \frac{(1-mon)}{Ns}\sum_{k=0}^{N}\left[\binom{N}{k}s^k(1-s)^{N-k}\,k\left(r_1\frac{1+d^6}{1+kd^6}+r_{FRET}\frac{(k-1)d^6}{1+kd^6}\right)\right]+mon\cdot r_1$$



The shape of the function depends on cluster size.

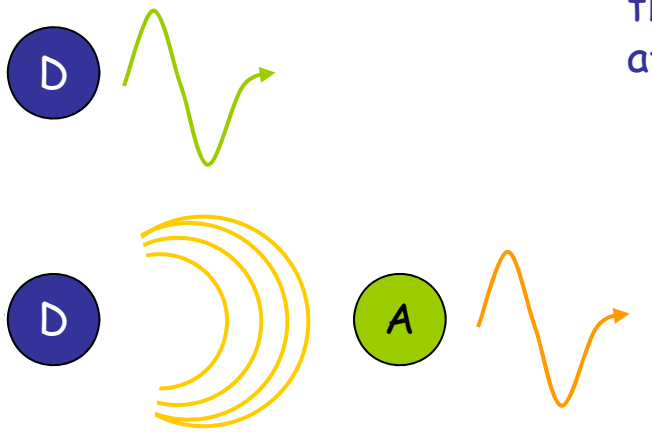


The number of proteins in a cluster can be determined.

- Szabó et al, Biophys J 95:2086-2096.
- Bader, et al, Biophys J 97:2613-2622.

Runnels and Scarlata, Biophys. J., 69:1569 (1995)

Manifestations of FRET, methods for measuring FRET: sensitized emission of the acceptor



sensitized emission: excitation of the acceptor through the donor (the acceptor fluoresces after donor excitation)

$$E = \left(\frac{F_{AD}}{F_A} - 1 \right) \frac{\varepsilon_A c_A}{\varepsilon_D c_D}$$

F_{AD} – acceptor fluorescence in the presence of the donor

F_A – acceptor fluorescence in the absence of the donor

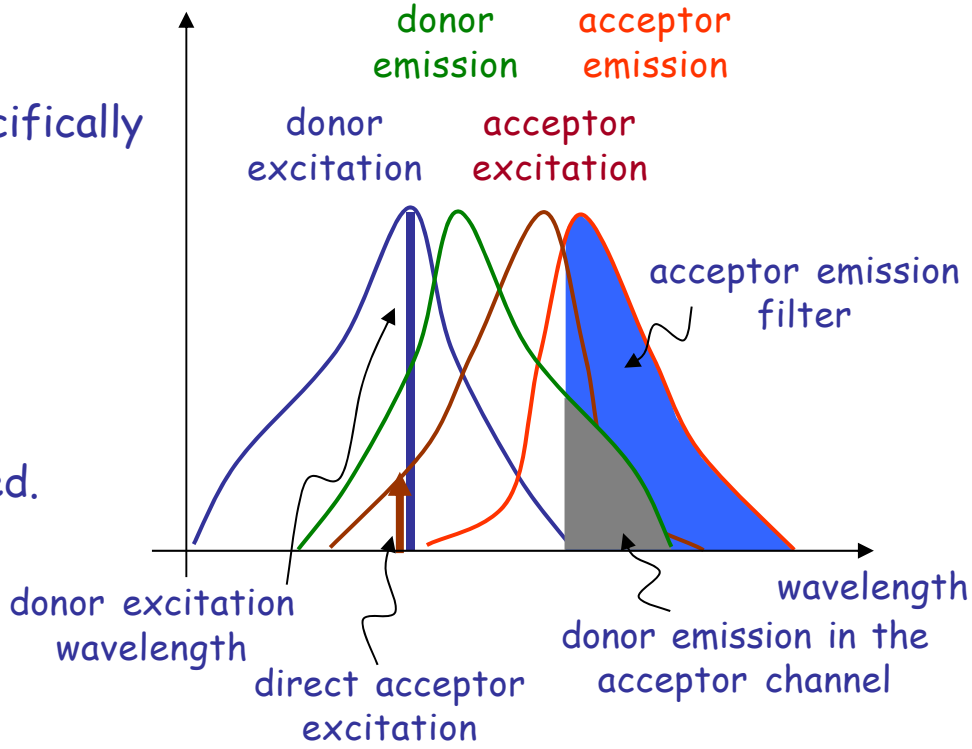
ε – molar absorption coefficients

c – molar concentrations

Problem:

- it is not possible to excite the donor specifically
- it is not possible to detect acceptor fluorescence specifically

Spectral spillover has to be compensated.



Manifestations of FRET, methods for measuring FRET: sensitized emission of the acceptor

$$I_1(\lambda_{ex,D},\lambda_{em,D}) = I_D(1-E) + I_A S_4 + I_D E\alpha \frac{S_4}{S_2}$$ donor channel

$$I_2(\lambda_{ex,D}\ ) = I_D(1-E)S_1 + I_A S_2 + I_D E\alpha$$ FRET channel

$$I_3(\lambda_{ex,A},\ ) = I_D(1-E)S_3 + I_A + I_D \cdot E \cdot \alpha \cdot \frac{1}{S_2} \cdot \frac{\varepsilon^D_{\lambda A} \cdot \varepsilon^A_{\lambda D}}{\varepsilon^D_{\lambda D} \cdot \varepsilon^A_{\lambda A}}$$ acceptor channel

donor signal — acceptor signal — FRET signal

spectral spillover factors

α: the fluorescence intensity of an excited acceptor molecule relative to an excited donor molecule in the FRET channel

$$E = \frac{S_2\left(I_1(S_1 - S_2S_3) + I_2(S_3S_4 - 1) + I_3(S_2 - S_1S_4)\right)}{\alpha\left(\frac{\varepsilon^D_{\lambda A} \cdot \varepsilon^A_{\lambda D}}{\varepsilon^D_{\lambda D} \cdot \varepsilon^A_{\lambda A}} - 1\right)(I_1S_2 - I_2S_4) + S_2\left(I_1(S_1 - S_2S_3) + I_3(S_2 - S_1S_4) + I_2(S_3S_4 - 1)\right)}$$

$$\frac{E}{1-E} = A = \frac{1}{\alpha}\left[\frac{S_2\left(I_2(S_3S_4 - 1) + I_3(S_2 - S_1S_4) + I_1(S_1 - S_2S_3)\right)}{\left(\frac{\varepsilon^D_{\lambda A}\varepsilon^A_{\lambda D}}{\varepsilon^D_{\lambda D}\varepsilon^A_{\lambda A}} - 1\right)(I_1S_2 - I_2S_4)}\right]$$

If E is large, we divide with a small number (1-E) → **low reliability**. Interpret high FRET efficiency with caution. This can be considered to be the consequence of high donor quenching → low donor fluorescence is measured.

Manifestations of FRET, methods for measuring FRET: sensitized emission of the acceptor

$$I_1(\lambda_{ex,D},\lambda_{em,D}) = I_D(1-E) + I_A S_4 + I_D E\alpha \frac{S_4}{S_2}$$ donor channel

$$I_2(\lambda_{ex,D}\ ) = I_D(1-E)S_1 + I_A S_2 + I_D E\alpha$$ FRET channel

$$I_3(\lambda_{ex,A},\ ) = I_D(1-E)S_3 + I_A + I_D E\alpha \frac{S_3}{S_1}$$ acceptor channel

donor signal — acceptor signal — FRET signal

spectral spillover factors

α: the fluorescence intensity of an excited acceptor molecule relative to an excited donor molecule in the FRET channel

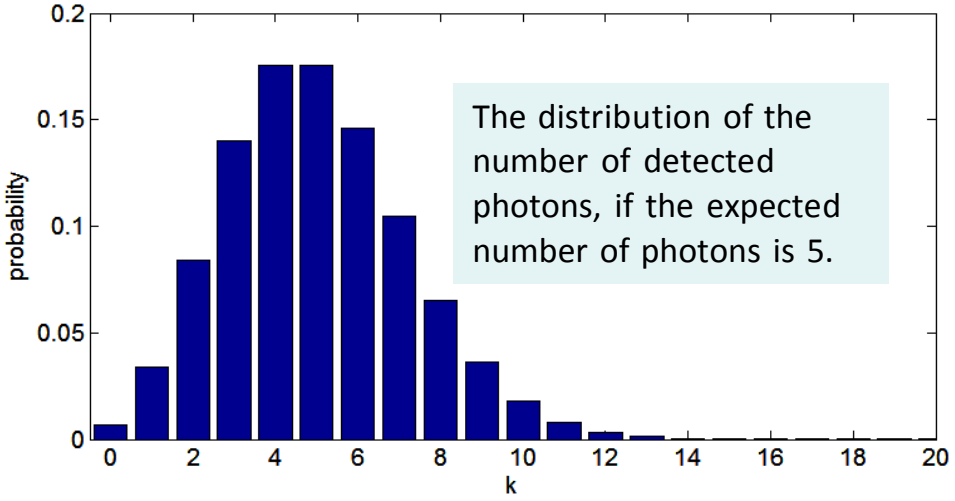
$$E = \frac{S_1S_2(I_2 - I_1S_1 - I_3S_2 + I_1S_2S_3 + I_3S_1S_4 - I_2S_3S_4)}{\alpha(S_1 - S_2S_3)(I_1S_2 - I_2S_4) + S_1S_2(I_2 - I_1S_1 - I_3S_2 + I_1S_2S_3 + I_3S_1S_4 - I_2S_3S_4)}$$

$$\frac{E}{1-E} = A = \frac{1}{\alpha}\left[\frac{S_1S_2(I_2 - I_1S_1 - I_3S_2 + I_1S_2S_3 + I_3S_1S_4 - I_2S_3S_4)}{(S_1 - S_2S_3)(I_1S_2 - I_2S_4)}\right]$$ ⇒

If E is large, we divide with a small number (1-E) → **low reliability**. Interpret high FRET efficiency with caution. This can be considered to be the consequence of high donor quenching → low donor fluorescence is measured.

Noise generated by photon detection

The number of detected photons (*n*) follows a Poisson distribution: $P(n=k)=\frac{N^k}{k!}e^{-N}$



The distribution of the number of detected photons, if the expected number of photons is 5.

Expected number of photons: N

SD of photon number: $\sqrt{N}$

Relative error of photon number: $\frac{\sqrt{N}}{N}=\frac{1}{\sqrt{N}}$

The larger the expected photon number, the smaller the relative error is.

N=5

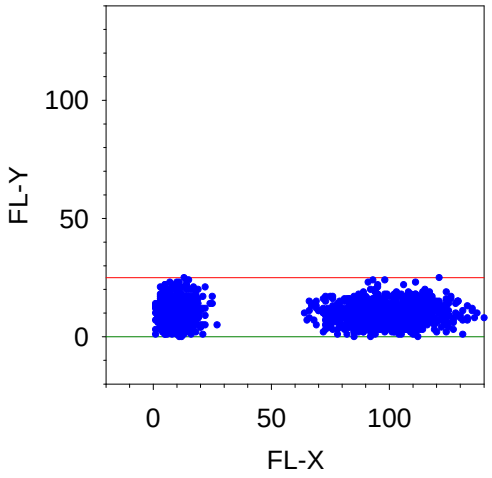
The images are displayed linear stretched, this is why the images on the left look equally bright.

N=50

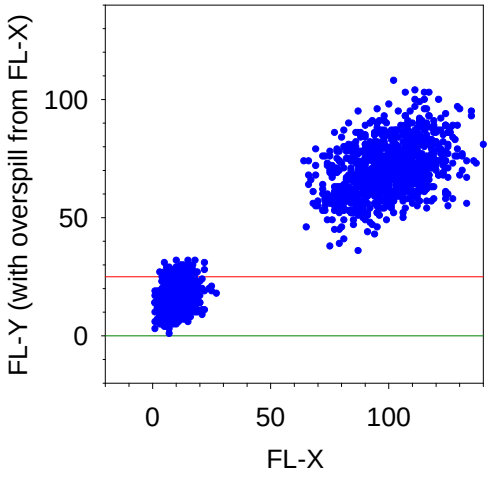
Original image (without noise)

with Poisson noise added

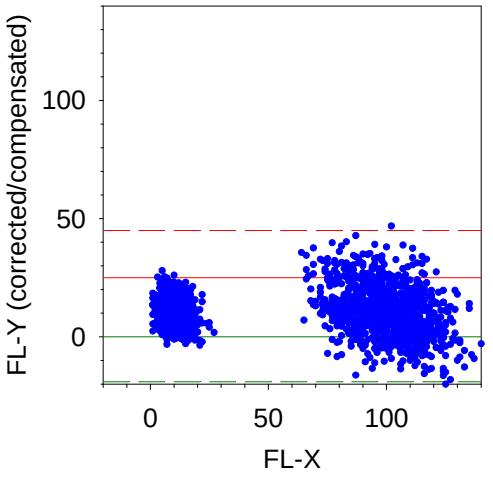
Problems arising from spectral compensation



No overspill from FL-X to FL-Y



Overspill from FL-X to FL-Y



Overspill compensated

Threshold for positive cells in the FL-Y channel

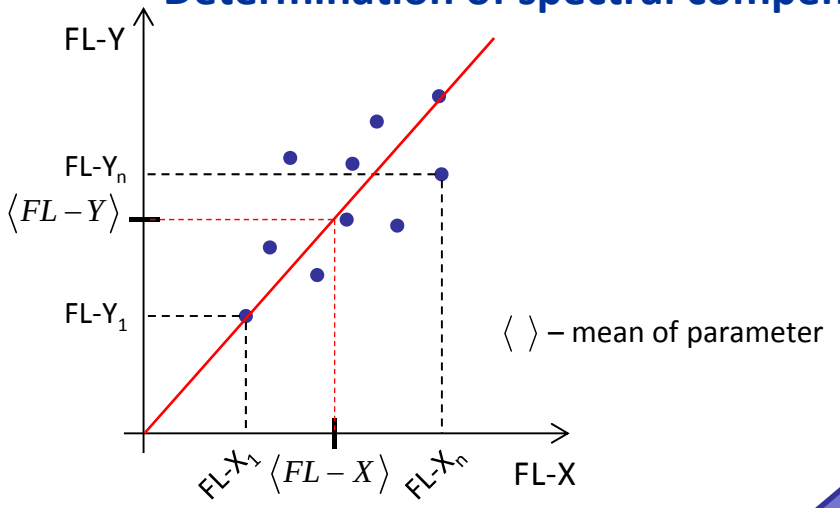
Zero intensity in the FL-Y channel

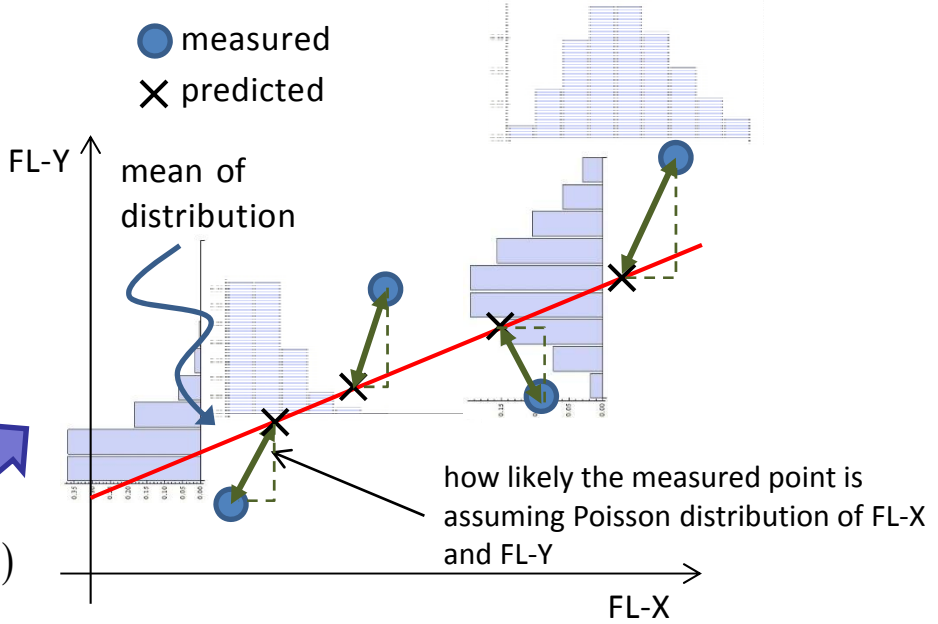
As a result of compensation (*S* factors in the FRET equations)

- the spread of the compensated channel (FL-Y) becomes larger
  - higher threshold for detecting positive cells
  - appearance of negative values

These phenomena are caused by noise (e.g. Poisson noise of photon detection).

Determination of spectral compensation factors (*S* factors, ratio parameters)





$$likelihood = \prod_i Pois_{FL-X_{pred,i}}\left(FL - X_i\right) \cdot Pois_{FL-Y_{pred,i}}\left(FL - Y_i\right)$$

Method 1: mean of ratios	Method 2: ratio of means	Method 3: fitting
$S_1 = \frac{FL - Y_1}{FL - X_1} \quad \ldots \quad S_n = \frac{FL - Y_n}{FL - X_n}$ $S = \langle S_i \rangle$	$S_2 = \frac{\langle FL - Y \rangle}{\langle FL - X \rangle}$	S_3=slope of the fitted line
$E(S_1) = \frac{\mu_{FL-Y}}{\mu_{FL-X}} + \mathrm{var}(FL - Y)\frac{\mu_{FL-X}}{\mu_{FL-Y}^3} - \frac{\mathrm{cov}(FL - X, FL - Y)}{\mu_{FL-Y}^2}$	$E(S_2) = \frac{\mu_{FL-Y}}{\mu_{FL-X}} + \frac{1}{n}\left(\mathrm{var}(FL - Y)\frac{\mu_{FL-X}}{\mu_{FL-Y}^3} - \frac{\mathrm{cov}(FL - X, FL - Y)}{\mu_{FL-Y}^2}\right)$	both FL-X and FL-Y have uncertainty ⇒ classical regression methods give a biased estimation
biased estimator of *S*	asymptotically unbiased estimator of *S*	MLE is a reliable and robust estimator of *S*

About method 1 and 2: van Kempen GM and van Vliet LJ (2000) Mean and variance of ratio estimators used in fluorescence ratio imaging. *Cytometry* **39**(4):300-305.

Maximum likelihood estimation (MLE) of ratio parameters (*S*)

- Likelihood of the measured intensities:

$$P=\prod_{k=1}^{n}\left(\frac{\left(S\,I_{xp,k}+b\right)^{I_{y,k}}}{I_{y,k}!}e^{-\left(S\,I_{xp,k}+b\right)}\frac{I_{xp,k}^{\;I_{x,k}}}{I_{x,k}!}e^{-I_{xp,k}}\right)$$

likelihood of measured I_y | likelihood of measured I_x

Subscript *p* designates predicted intensities.

- Log-likelihood of the measured intensities:

$$L=\ln(P)=-b\,n-\sum_{k=1}^{n}\ln\left(I_{x,k}!\right)-\sum_{k=1}^{n}\ln\left(I_{y,k}!\right)+\sum_{k=1}^{n}\ln\left(I_{xp,k}\right)I_{x,k}-\sum_{k=1}^{n}I_{xp,k}-S\sum_{k=1}^{n}I_{xp,k}+\sum_{k=1}^{n}\ln\left(b+S\,I_{xp,k}\right)I_{y,k}$$

- Determination of the predicted intensities:

  Partial derivatives of the log-likelihood with respect to the predicted I_x intensity ($I_{xp,k}$):

$$\frac{\partial L}{\partial I_{xp,k}}=\frac{S\,I_{y,k}}{S\,I_{xp,k}+b}-S+\frac{I_{x,k}}{I_{xp,k}}-1$$

  The predicted intensity ($I_{xp,k}$) is equal to the value where the partial derivative of the log-likelihood is zero:

$$\frac{\partial L}{\partial I_{xp,k}}=0\;\Rightarrow\;I_{xp,k}=\frac{-b(1+S)+S\left(I_{x,k}+I_{y,k}\right)+\sqrt{4bS(1+S)I_{x,k}+\left(b(1+S)-S\left(I_{x,k}+I_{y,k}\right)\right)^{2}}}{2S(1+S)}$$

Maximum likelihood estimation (MLE) of ratio parameters (*S*)

- Substitute the predicted intensities into the log-likelihood and find the maximum in *Mathematica* by modifying parameters *S* and *b*.
- MLE of ratio parameters is as good as the ratio of the means method, but pixelwise calculations perform poorly:

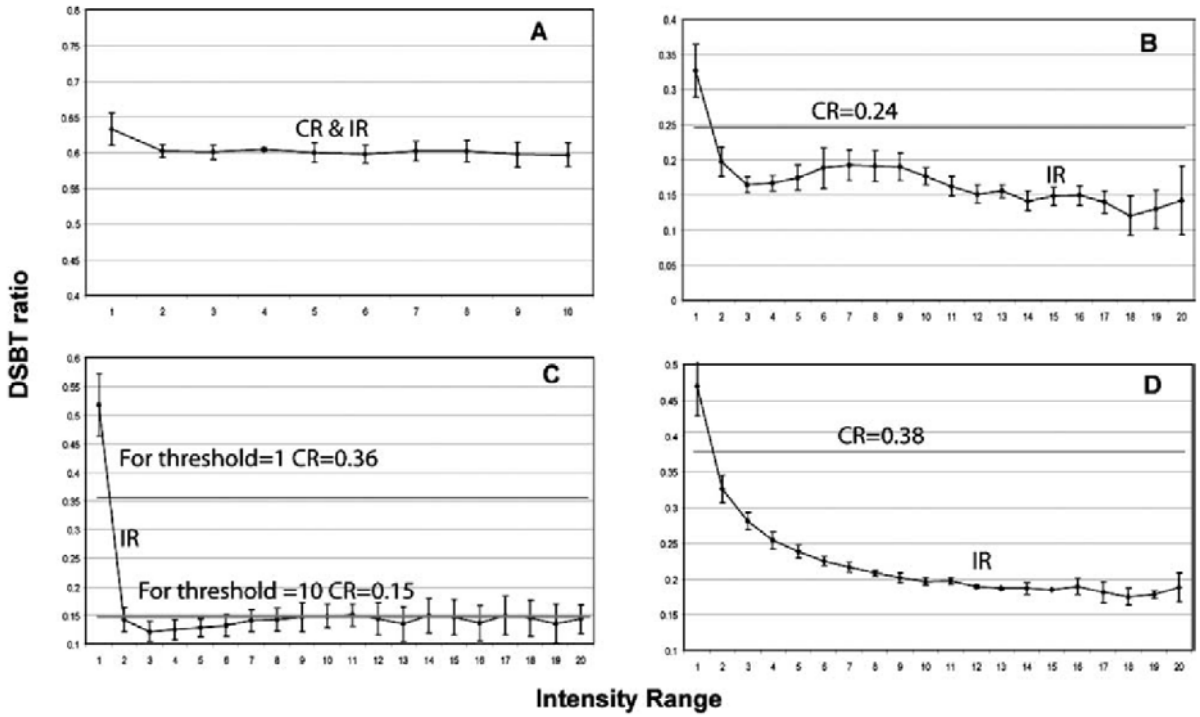
1. Generate random, normally distributed I_x with a mean and SD of 10.
2. Generate I_y assuming S=0.25 and b=0.
3. Generate photon numbers with Poisson distribution using the above intensities as mean values of the Poisson distribution.
4. Repeat steps 1-3 100-times to test the reproducibility of the method.

	S
MLE	0.274±0.011
mean of pixelwise FRET	0.432±0.012
trimmed mean of pixelwise FRET	0.409±0.013
median of pixelwise FRET	0.312±0.015
S from summed intensities	0.27±0.005

- MLE outperforms the ratio of the mean method if there are outlier pixels: normally distributed noise was added to every second pixel with mean and SD of 10 and 3, respectively.

	S
MLE with thresholding	0.288±0.019
mean of pixelwise FRET	0.573±0.013
trimmed mean of pixelwise FRET	0.536±0.011
median of pixelwise FRET	0.493±0.011
S from summed intensities	0.482±0.009

Intensity-dependent ratio parameters (*S* factors)



DSBT=donor spectral bleed-through

As a result of

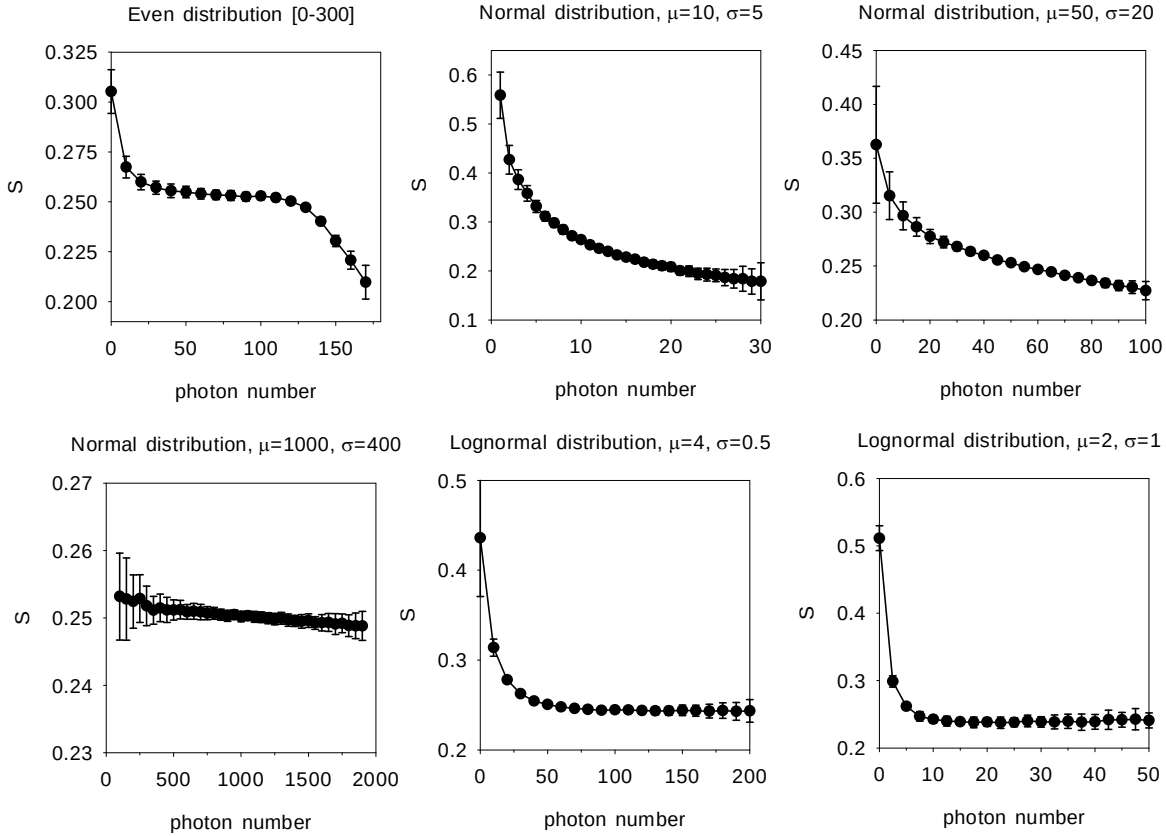
- detector non-linearity
- wavelength-dependent point spread function (PSF) and influence of pixel intensities by neighboring pixels
- statistical nature of photon detection (see next slide)

the compensation factors (ratio parameters, *S* factors) can be intensity dependent.

- Sun Y and Periasamy A (2010) Additional correction for energy transfer efficiency calculation in filter-based Forster resonance energy transfer microscopy for more accurate results. *J Biomed Opt* **15**(2):020513.
- Chen Y and Periasamy A (2006) Intensity range based quantitative FRET data analysis to localize protein molecules in live cell nuclei. *J Fluoresc* **16**(1):95-104.

Intensity-dependent ratio parameters (*S* factors)

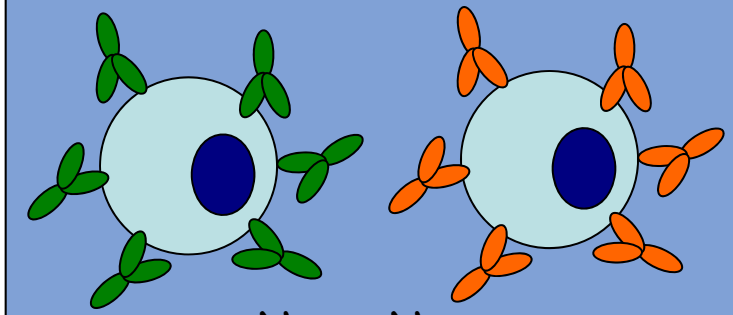
1. Generate 10000 random data for I_x according to the specified distributions.
2. Calculate I_y according to the following equation: $I_y=0.25*I_x$
3. Generate the detected I_x and I_y intensities using the above values as means of the Poisson distribution.
4. Calculate intensity-dependent *S* factors
5. Repeat the above 100x to calculate SD.



The statistical nature of photon detection may give rise to intensity-dependent *S* factors.

Determination of α

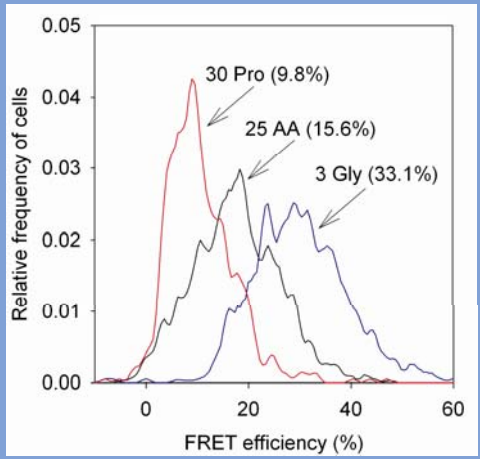
Antibody-labeled cells



$N_{donor}=N_{acceptor}$

label the first sample with donor-antibody, the second sample with acceptor-antibody against the same epitope.

$$\alpha = \frac{I_2}{I_1}\frac{\varepsilon_D L_D}{\varepsilon_A L_A}$$

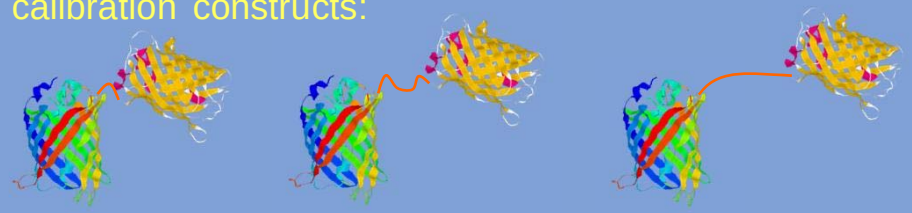


Cells labeled with GFP or its variants:

Problem: it is not possible to transfect cells with an equal number of donor and acceptor molecules.

Solution: create tandem conjugates in which a donor (CFP) and an acceptor (YFP) are separated by linkers of different lengths.

3 calibration constructs:



3 amino acids | 25 amino acids (flexible) | 30 amino acids (rigid)

Calculate FRET using two different approaches:

$$\left.\begin{aligned} &\frac{E}{1-E} = \frac{1}{\alpha}\left(\frac{I_2 - S_2 I_3}{\left(1-\frac{S_2 S_3}{S_1}\right)I_1} - S_1\right) = \frac{1}{\alpha}R_I \qquad E = \frac{R_I}{\alpha + R_I} \\ &\frac{F_{AD}}{F_A} = 1 + \frac{\varepsilon_D c_D}{\varepsilon_A c_A}E = R_F \qquad E = \frac{R_F - 1}{\frac{\varepsilon_D c_D}{\varepsilon_A c_A}} \end{aligned}\right\}$$

Adjust α so that the difference between the FRET values determined by the two approaches is minimal.

$$\sum_{j=2,3..n}\left(\frac{R_{F,1}-1}{R_{F,j}-1} - \frac{R_{I,1}}{\alpha + R_{I,1}}\frac{\alpha + R_{I,j}}{R_{I,j}}\right)^2 \Rightarrow \min$$

Determination of α

Method 1: EGFP-mRFP1 fusion construct

- $N_{donor}=N_{acceptor}$
- $E\neq 0$ and E is unknown
- α is unknown

FRET equations:

$$I_1 = I_D(1-E) + Bg_1$$
$$I_2 = I_D(1-E)S_1 + I_A S_2 + I_D E\alpha + Bg_2$$
$$I_3 = I_A + Bg_3$$

1. Assume E=0.
2. Determine α:

$$\alpha = \frac{(I_3 - Bg_3)S_2}{\frac{I_1 - Bg_1}{1-E}} \frac{\varepsilon_{488}^{EGFP}}{\varepsilon_{488}^{mRFP}}$$

3. Using this α calculate E:

$$E = 1 - \frac{1}{1 + \frac{1}{\alpha}\left(\frac{(I_2 - Bg_2) - S_2(I_3 - Bg_3)}{I_1 - Bg_1} - S_1\right)}$$

Using this E redetermine α and iterate until convergence.

Method 2: ECFP-EYFP fusion construct

- $N_{donor}=N_{acceptor}$
- $E\neq 0$ and E is unknown
- α is unknown

FRET equations:

$$I_1 = I_D(1-E) + I_A S_4 + I_D E\alpha \frac{S_4}{S_2}$$
$$I_2 = I_D(1-E)S_1 + I_A S_2 + I_D E\alpha$$
$$I_3 = I_D(1-E)S_3 + I_A + I_D E\alpha \frac{\varepsilon_{488}^{ECFP}\varepsilon_{405}^{EYFP}}{S_2\varepsilon_{405}^{ECFP}\varepsilon_{488}^{EYFP}}$$
$$\alpha = \frac{I_A S_2}{I_D}\frac{\varepsilon_{405}^{ECFP}}{\varepsilon_{405}^{EYFP}}$$

These equations constitute a system of four equations with four unknowns (I_D, I_A, E, α). Solve for α:

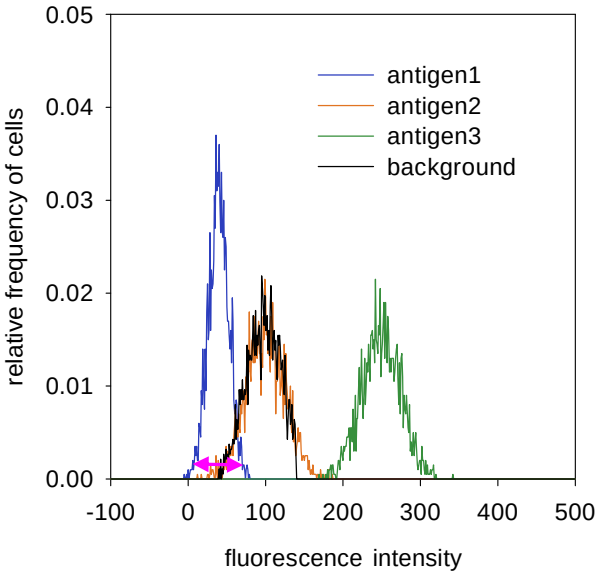
$$\alpha = \frac{S_2\left(I_1\left((1+\varepsilon_2)S_2S_3 - (1-\varepsilon_2\varepsilon_4)S_1\right) - (1+\varepsilon_2)I_3(S_2 - S_1S_4) + I_2\left(1 - S_3S_4 + \varepsilon_2(\varepsilon_4 - S_3S_4)\right)\right)}{(\varepsilon_4 - 1)(I_1S_2 - I_2S_4)}$$

where $\varepsilon_2 = \frac{\varepsilon_{405}^{ECFP}}{\varepsilon_{405}^{EYFP}}$, $\varepsilon_4 = \frac{\varepsilon_{488}^{ECFP}\varepsilon_{405}^{EYFP}}{\varepsilon_{405}^{ECFP}\varepsilon_{488}^{EYFP}}$

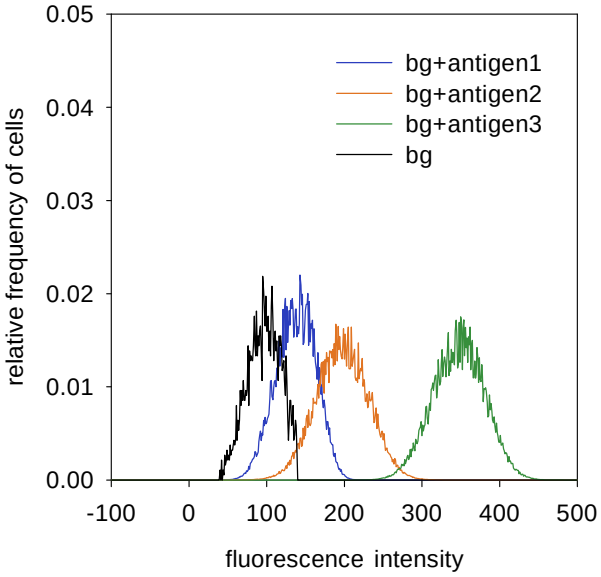
Method 1: Vámosi G et al., *Biophys J* **94**(7):2859-2868.
Method 2: Szaloki N et al., *Cytometry A* **83**(9):818-829.

Autofluorescence correction for FRET measurements

pure signals (not possible to measure)



mixed (measured) signals

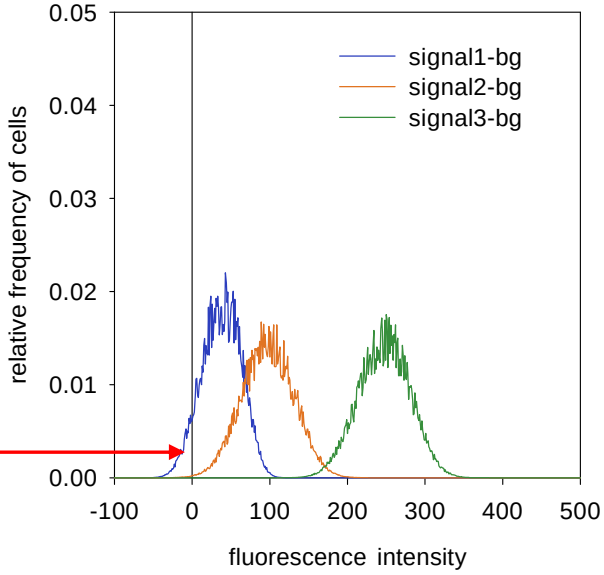


constant background subtraction

Problem:

If the signal is too low, subtraction of a constant background from the intensity of each cell leads to

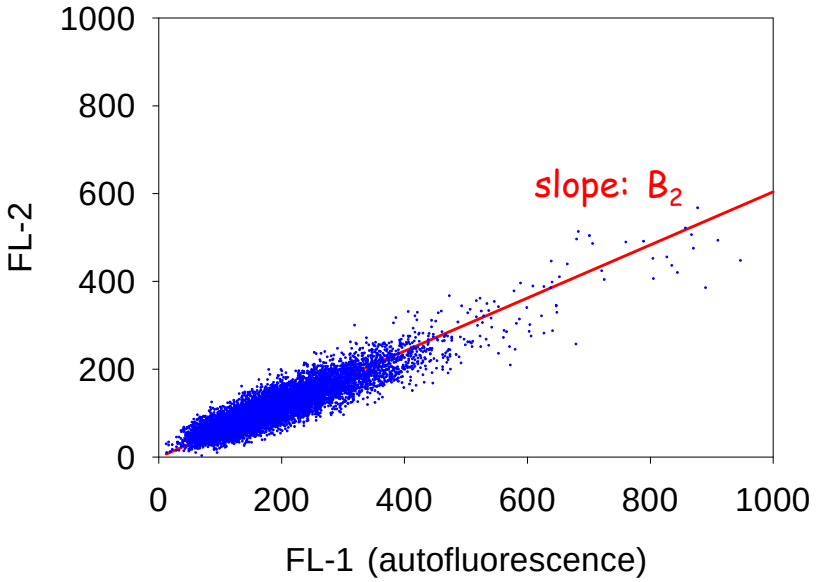
- widening of the distribution
- negative fluorescence intensities

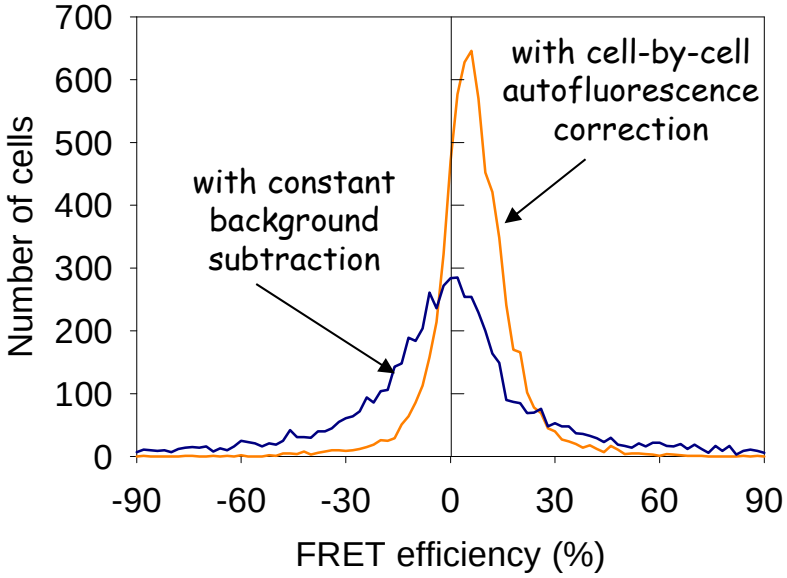


Autofluorescence correction for FRET measurements

Solution:

- Measure the autofluorescence of cells in a channel in which the fluorescent labels don't fluoresce.
- Determine spectral spillover factors (B_2-B_4) to compensate for autofluorescence





$$FL1(488,530) = AF + I_D(1-E)\cdot S_5 + I_A \cdot S_6 + I_D \cdot E \cdot \alpha \cdot \frac{S_6}{S_2}$$

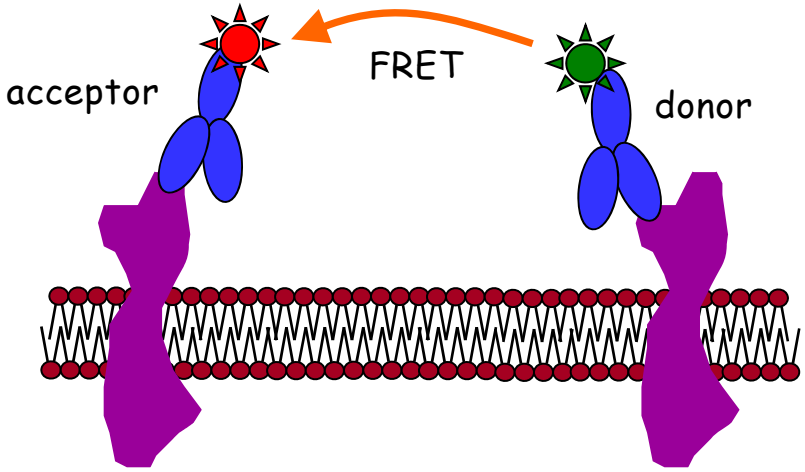
$$FL2(488,585) = AF \cdot B_2 + I_D(1-E) + I_A \cdot S_4 + I_D \cdot E \cdot \alpha \cdot \frac{S_4}{S_2}$$

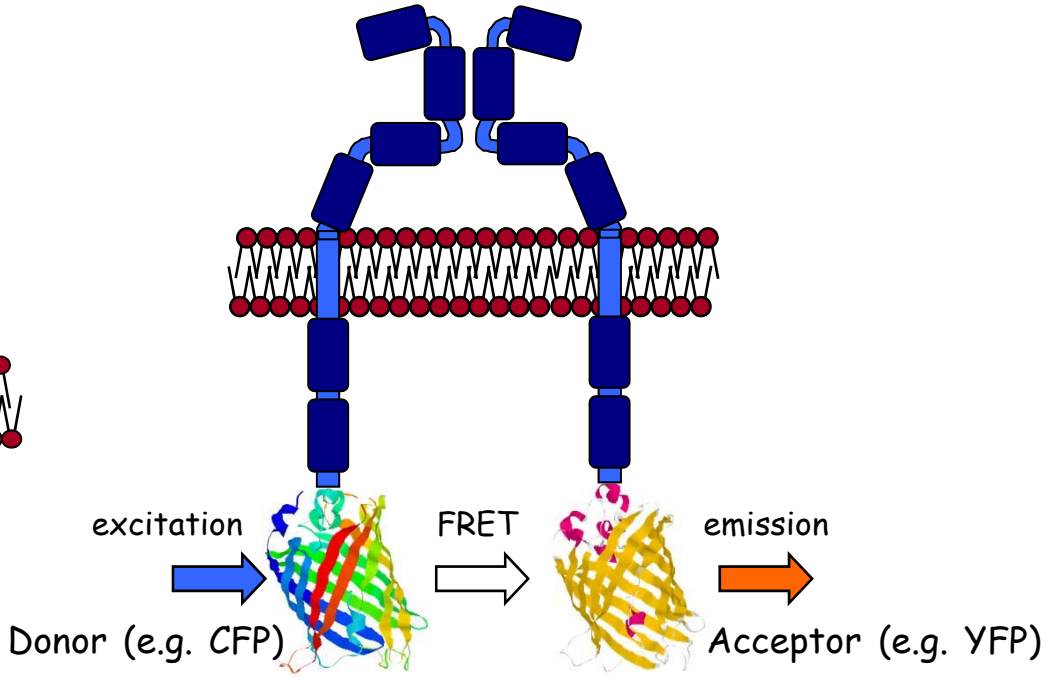
$$FL3(488,>670) = AF \cdot B_3 + I_D(1-E)\cdot S_1 + I_A \cdot S_2 + I_D \cdot E \cdot \alpha$$

$$FL4(635,661) = AF \cdot B_4 + I_D(1-E)\cdot S_3 + I_A + I_D \cdot E \cdot \alpha \cdot \frac{1}{S_2} \cdot \frac{\varepsilon_{635}^D \cdot \varepsilon_{488}^A}{\varepsilon_{488}^D \cdot \varepsilon_{635}^A}$$

$$A = \frac{E}{1-E} = \frac{1}{\alpha} \cdot \frac{FL1\cdot(B_2S_1 + B_4S_2 - B_3) + FL2\cdot(B_3S_5 - S_1 - B_4S_2S_5) + FL3\cdot(1-B_2S_5) + FL4\cdot S_2\cdot(B_2S_5 - 1)}{FL2 - FL1\cdot B_2}$$

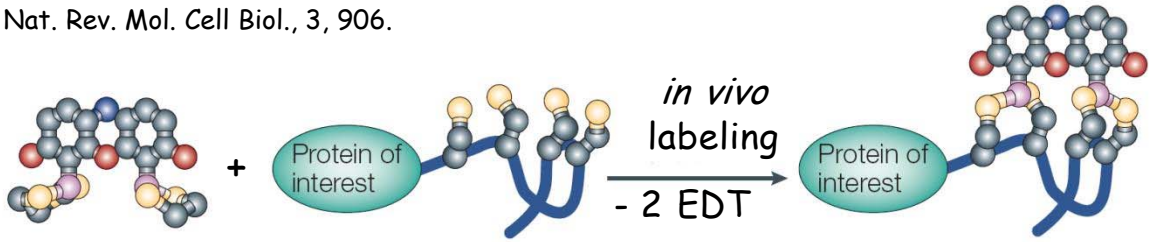
Labeling of biological molecules for FRET measurements





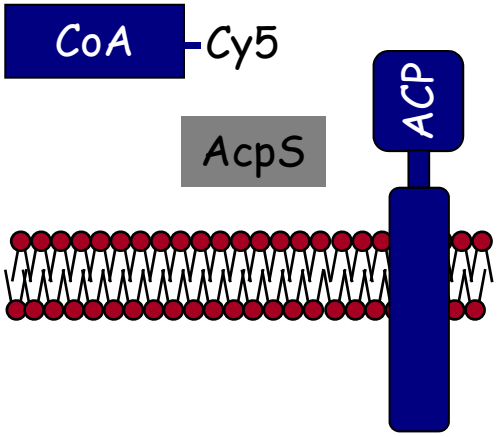
Tetracysteine motif

Nat. Rev. Mol. Cell Biol., 3, 906.

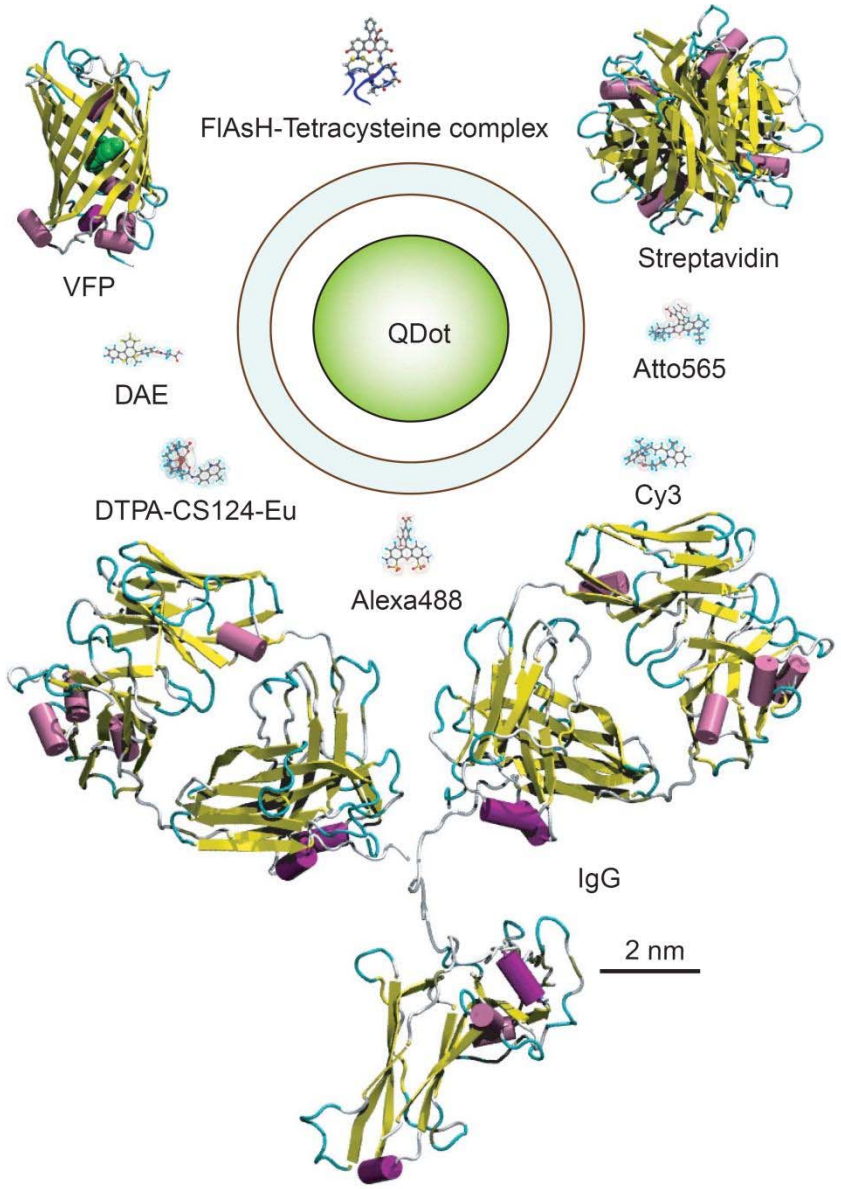


Non fluorescent biarsenical dye-EDT complex

Fluorescent biarsenical-tetracysteine complex

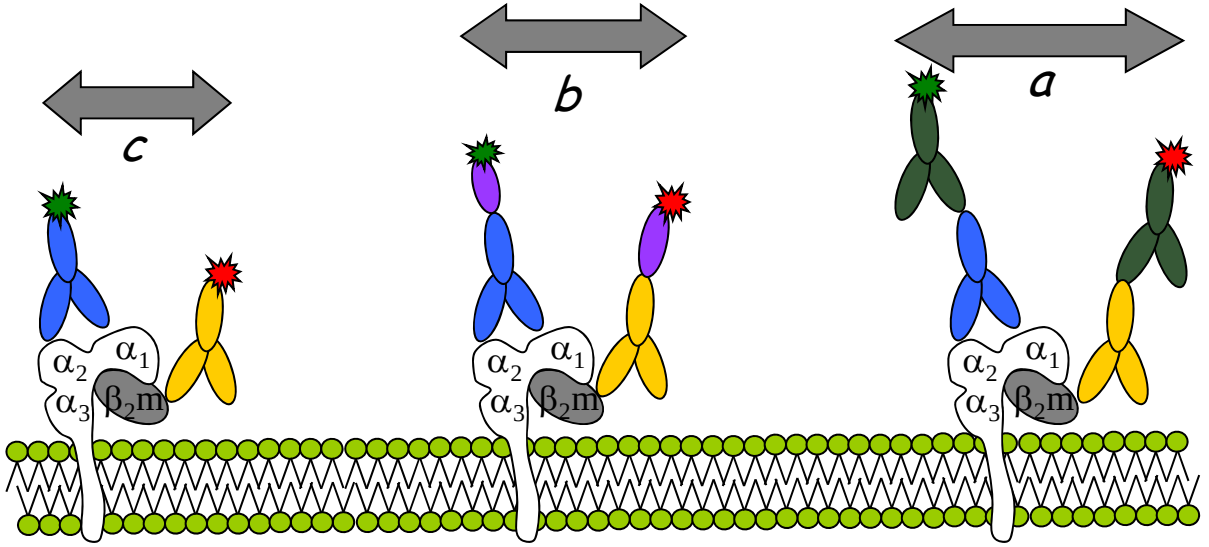


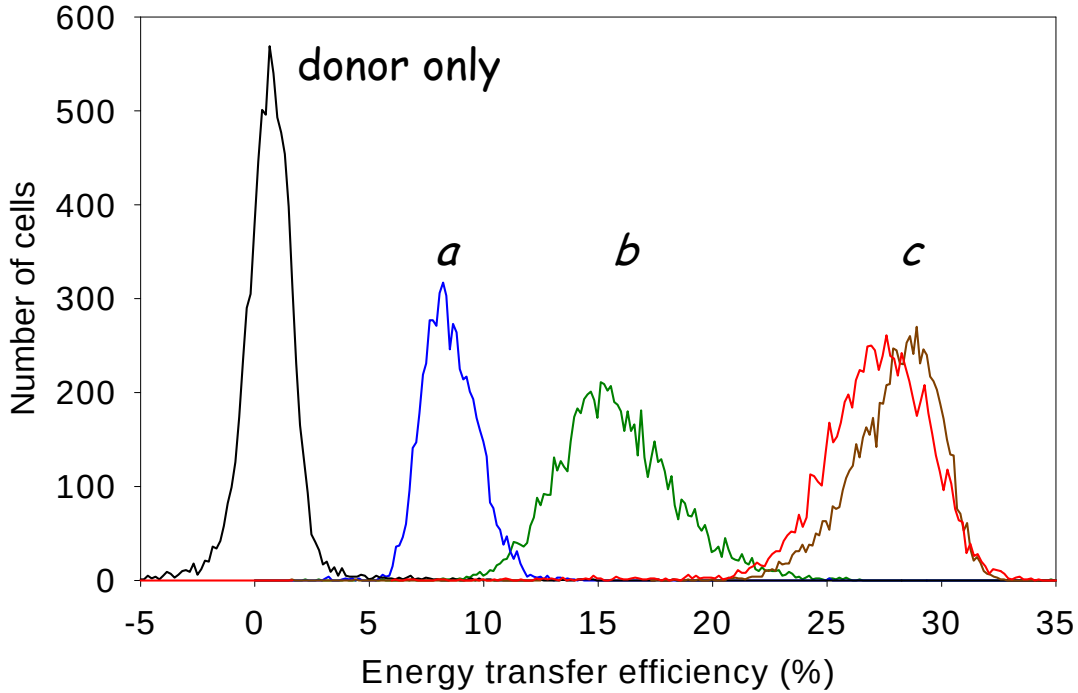
The size of fluorophores used for labeling biological molecules



Nat. Biotechnol., 21, 1387.

Effect of the size of the fluorophore carrier on the FRET efficiency





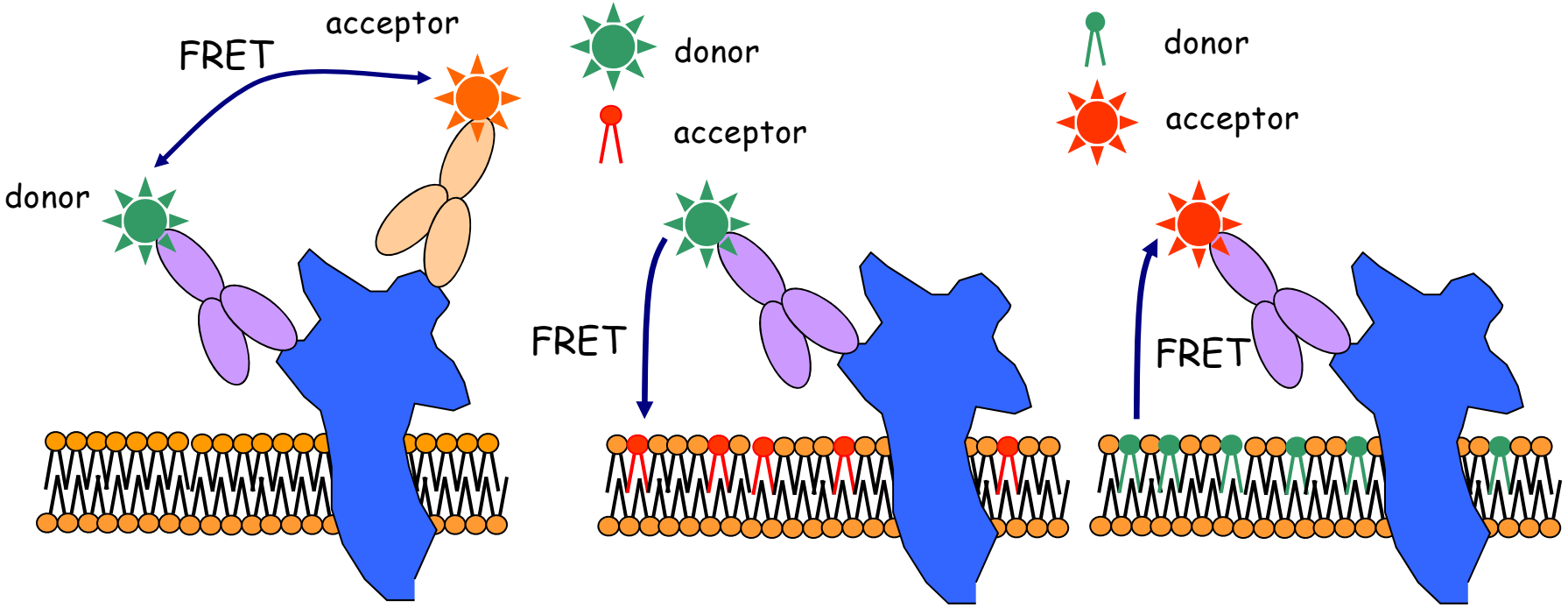
We found an inverse correlation between FRET efficiency and the size of the fluorophore complex.

Sebestyén et al., Cytometry, 48:124 (2002).

Epitope mapping with FRET

The distance between epitopes can be measured.

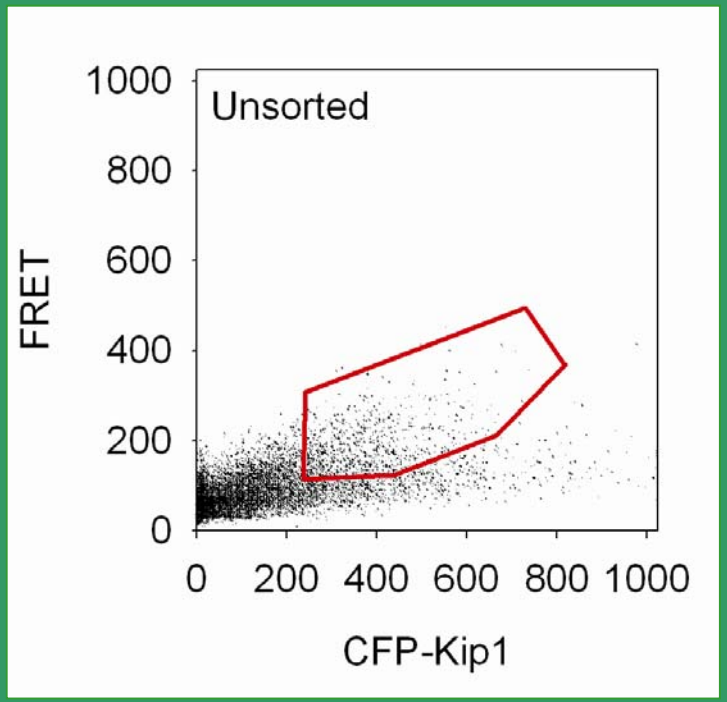
<u>Vertical mapping</u>: the distance of epitopes from the membrane can be measured

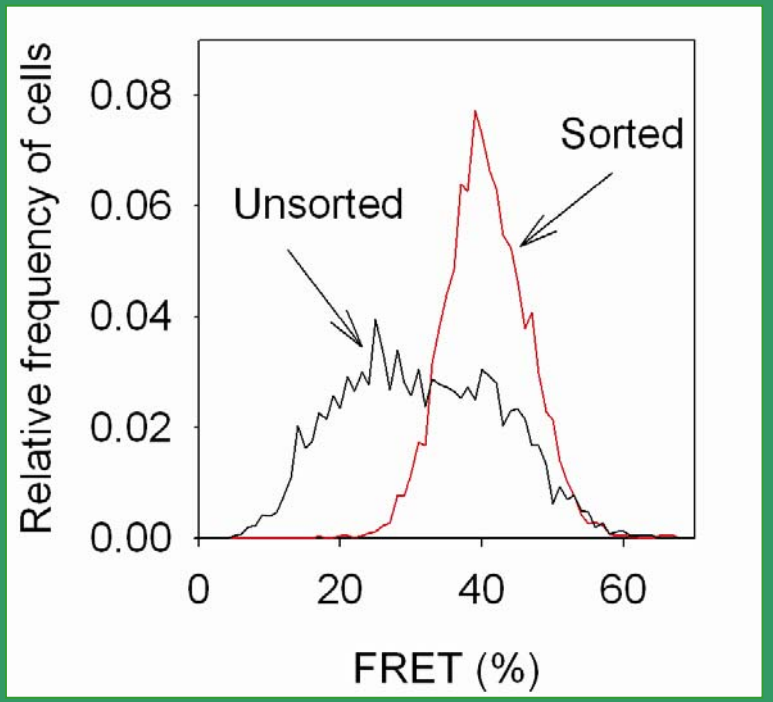


FRET-based sorting

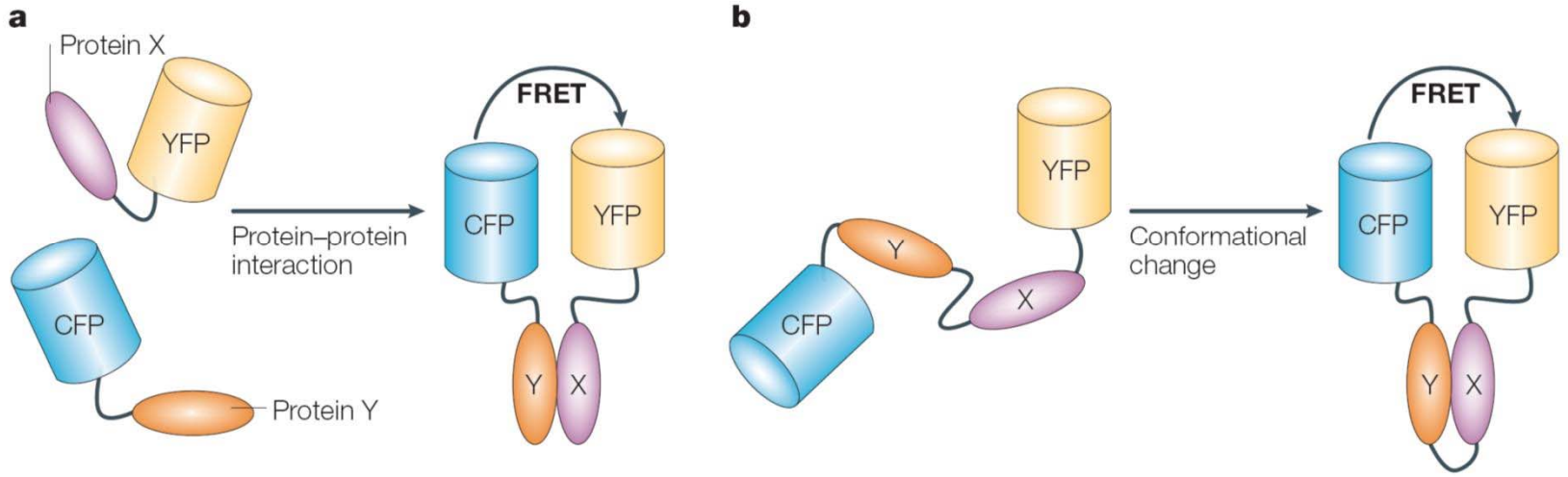
- Yeast cells transfected with a CFP-YFP construct were analyzed.
- The cells which had high intensity in the FRET channel were sorted.
- The FRET efficiency was calculated for the unsorted and sorted populations.
- The sorted population was indeed enriched in cells showing an interaction between CFP and YFP.

Sorting of CFP-YFP expressing yeast cells with high FRET efficiency

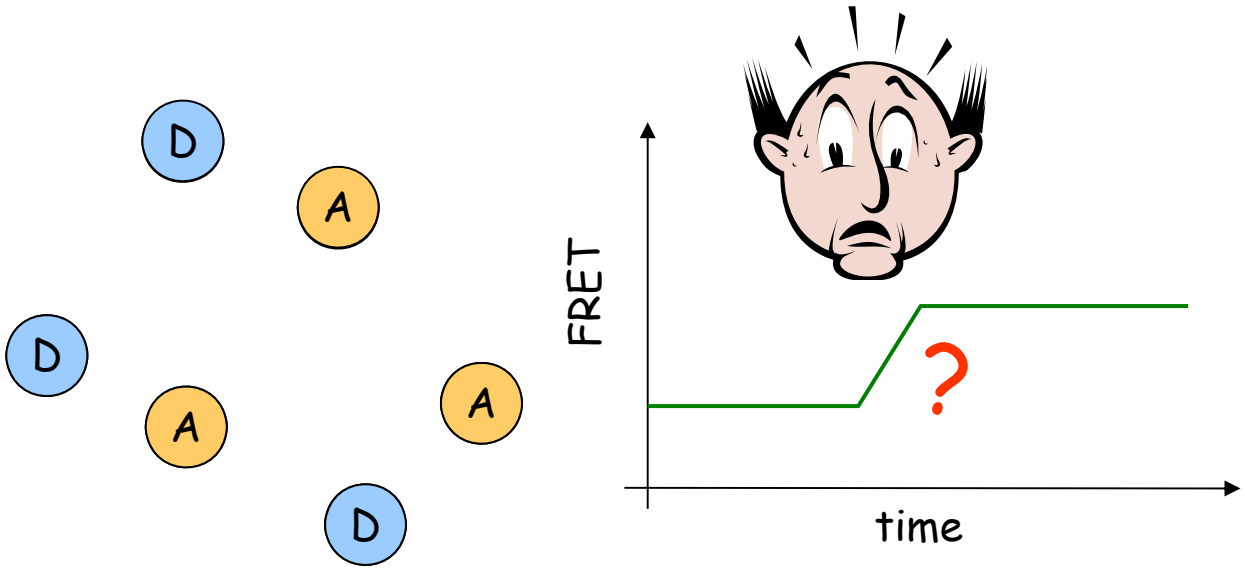




Applications of FRET



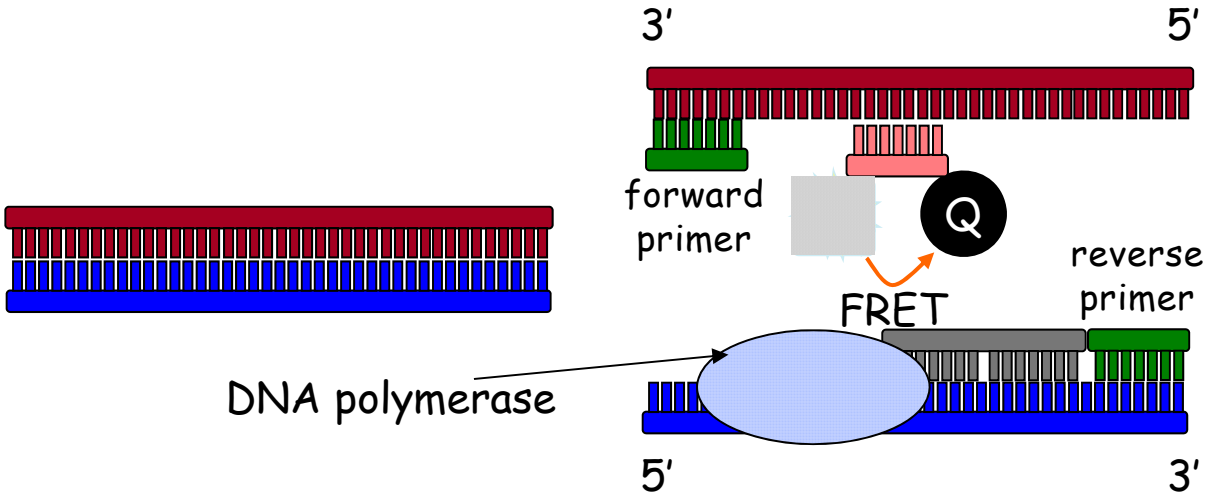
Nat. Rev. Mol. Cell Biol. 3, 906.



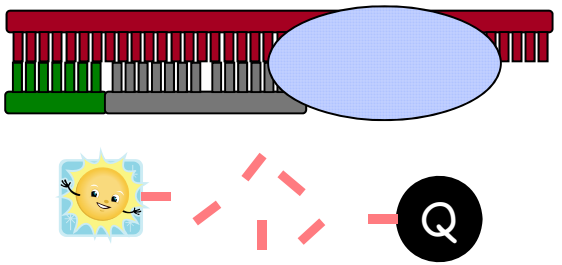
1. every donor-acceptor pair gets a little closer to each other
2. some donor-acceptor pairs get much closer to each other, while the distance between the others do not change

Applications of FRET

TaqMan PCR:

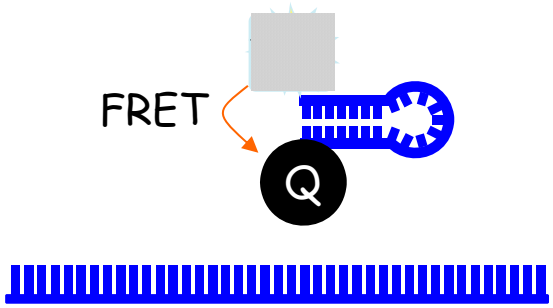


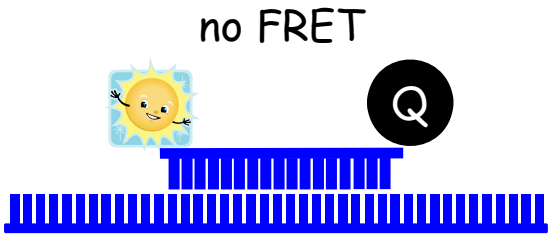
the 5′-3′ exonuclease activity of the polymerase degrades the TaqMan probe



no FRET

Molecular beacons:

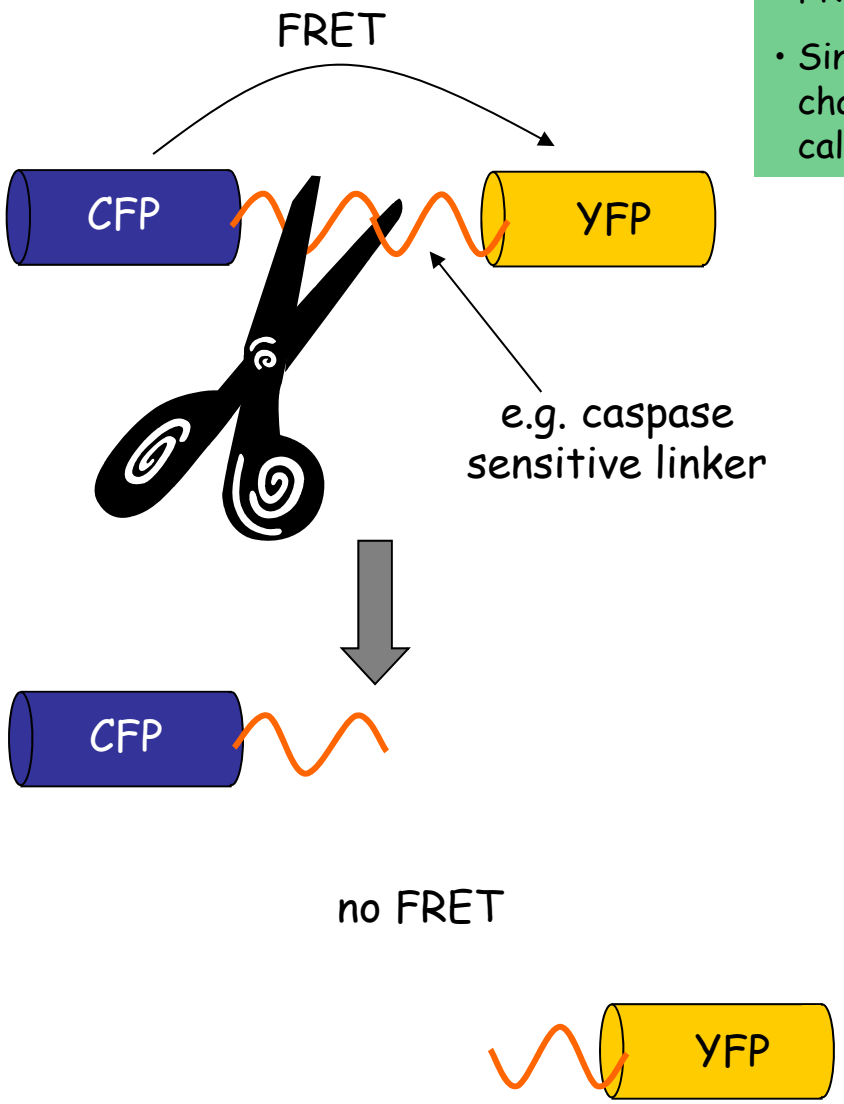




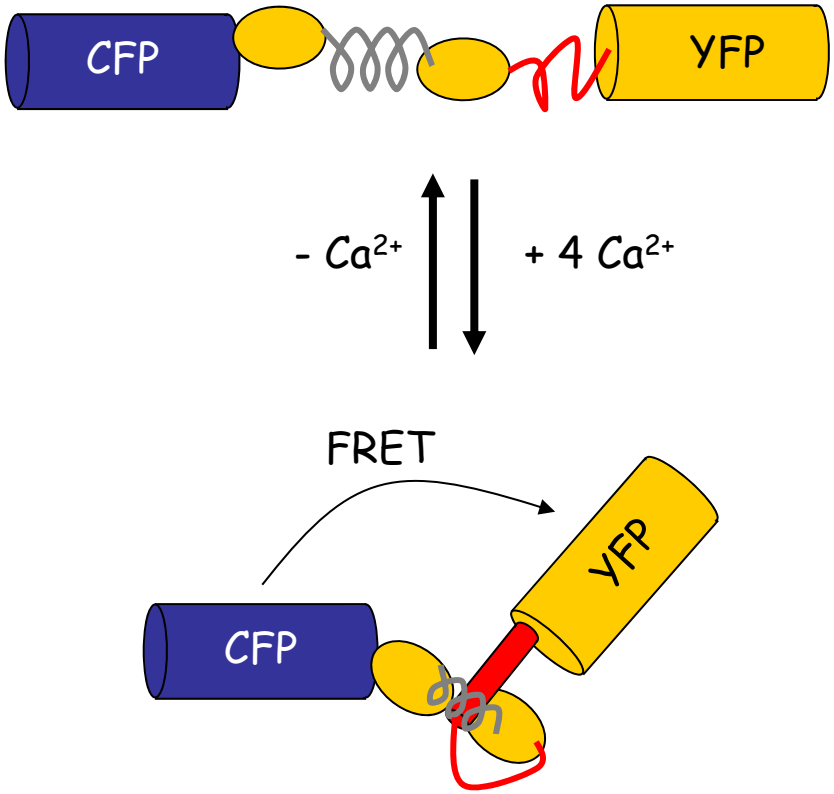
Applications of FRET

- In principle, any biological parameter leading to a change in the donor-acceptor distance, can be measured by FRET.
- Since FRET is a normalized parameter (the relative change in donor fluorescence intensity), it can be calibrated reliably and reproducibly.

Protease sensor:

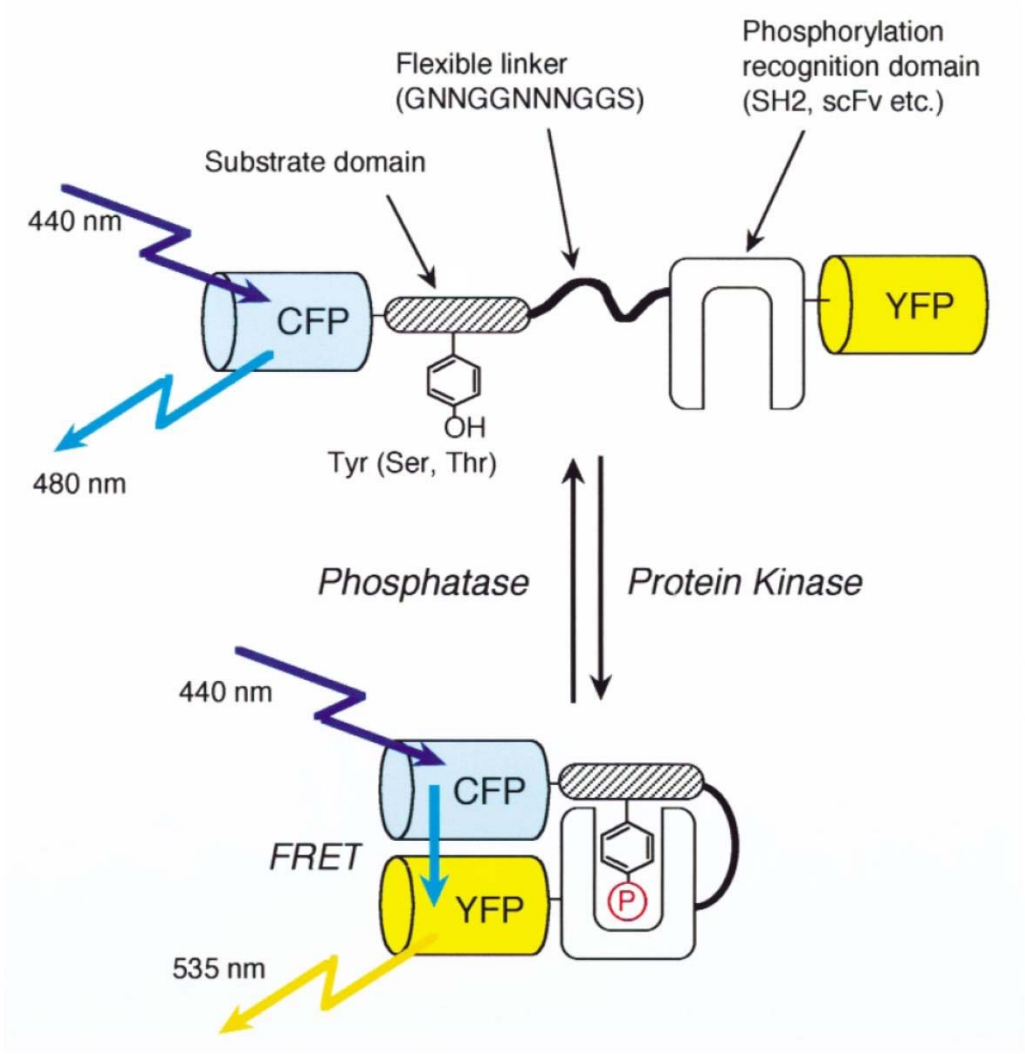


Calcium sensor:



Applications of FRET

Tyrosine phosphorylation sensors:



Conclusions

- FRET is usually relatively easy to implement and cheap (conventional instrumentation is needed).
- FRET has several manifestations. Careful consideration is needed to choose the optimal one.
- FRET is quantitative. Make use of its quantitative nature → It is not only insufficient, but misleading to say only if FRET is present or not.
- Calculate FRET efficiency, do not just show uncalibrated FRET-related parameters.
- Careful controls are needed to prevent misinterpretation and to achieve the desired level of quantitativity.