FLUORESCENCE FOUNDATION

universiteit hasselt
BIOMEDISCH ONDERZOEKSINSTITUUT

Discovering Detail

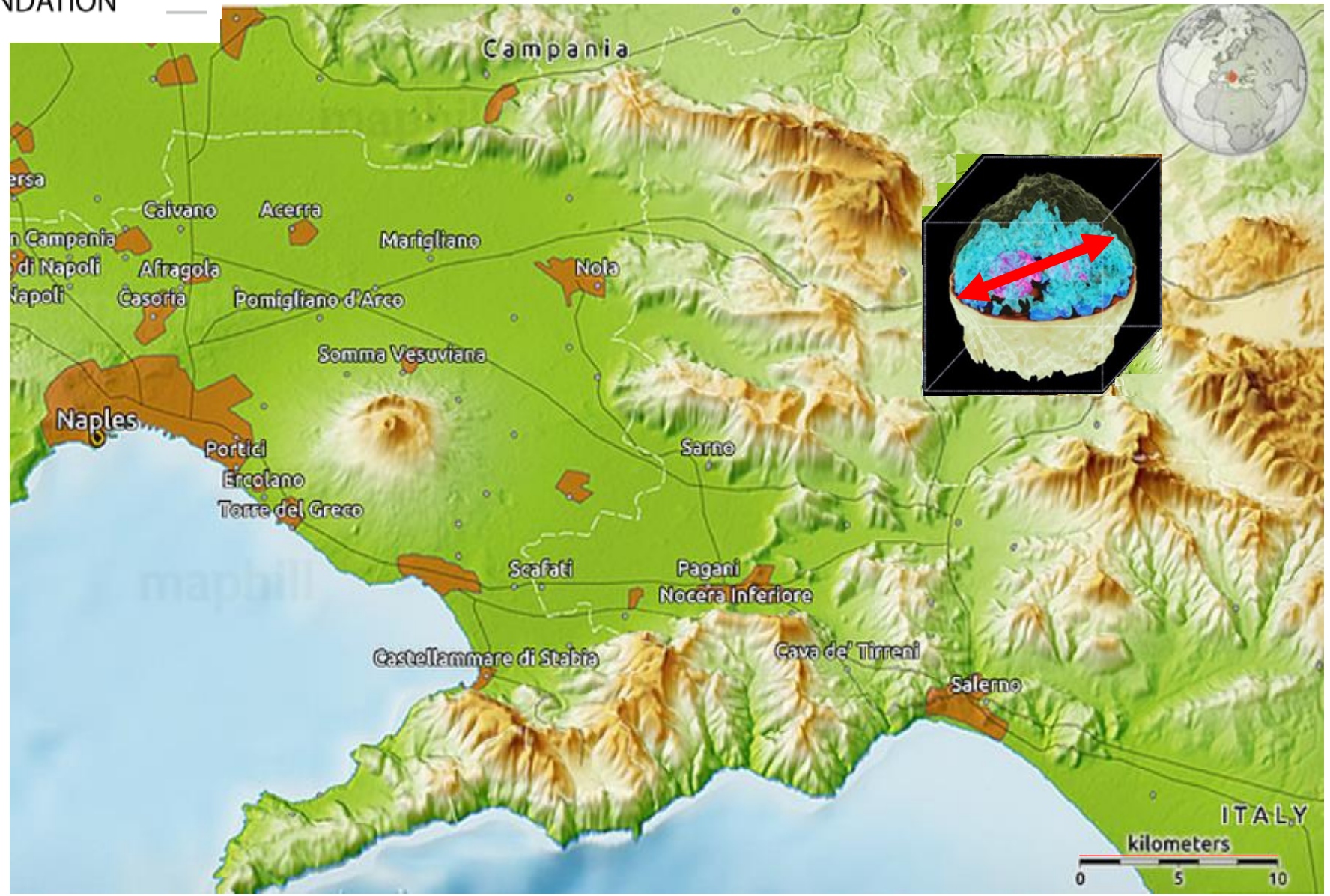
Nanoscopy

Spectroscopic Rulers Bridging Gaps
&
FRET

FRET – why it's useful, R^{-6} dependence; R_0 (3-7 nm), very convenient.

THE FLUORESCENCE FOUNDATION

Magnifying a 10 µm Cell One Billion Times



Sizing Proteins

Density of proteins ~ 1.37 gr/cm^3

Polyethylene terephthalate 1.37 gr/cm ^{3}

PVC 1.36 gr/cm ^{3}

Partial specific volume, v2, the reciprocal of the density.
v2 varies from 0.70 to 0.76 for different proteins. Average v2= 0.73 cm^3/gr

$$V(nm^3) = \frac{(0.73\ cm^3/g) \times (10^{21} nm^3/cm^3)}{6.023 \times 10^{23} Da/g} \times M(Da)$$

$$= 1.212 \times 10^{-3} (nm^3/Da) \times M(Da).$$

$$R_{min} = (3V/4\pi)^{1/3}$$

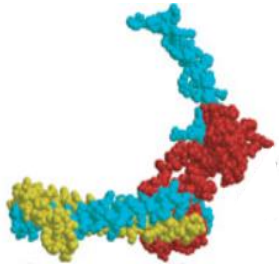
$$= 0.066 M^{1/3}$$ M(Da), R (nm)

Protein *M* (kDa)	5	10	20	50	100	200	500
R_{min} (nm)	1.1	1.42	1.78	2.4	3.05	3.84	5.21

Concentration	1 M	1 mM	1 µM	1 nM
Distance between molecules (nm)	1.18	11.8	118	1,180

Erickson, *Biol. Proc. Online*, 2009, 11 (1) 32-51

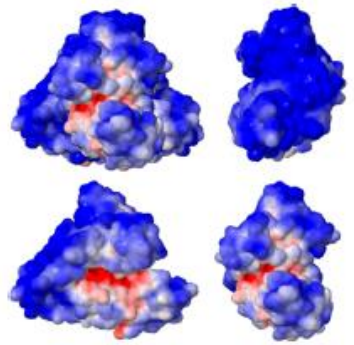
Proteins Space-Filling Shapes & Structure



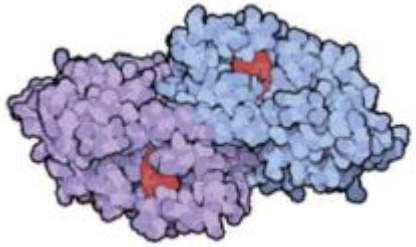
Troponin Core, 52 kDa

Trypsin, 23 kDa

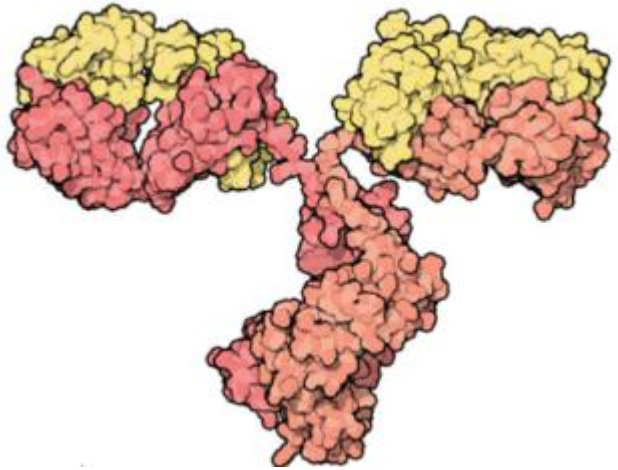
Insulin, 5.8 kDa



Human Serum Albumin, 66.5 kDa



Alcohol Dehydrogenase, ~ 150 kDa



Antibody, ~ 150 kDa

10 nm

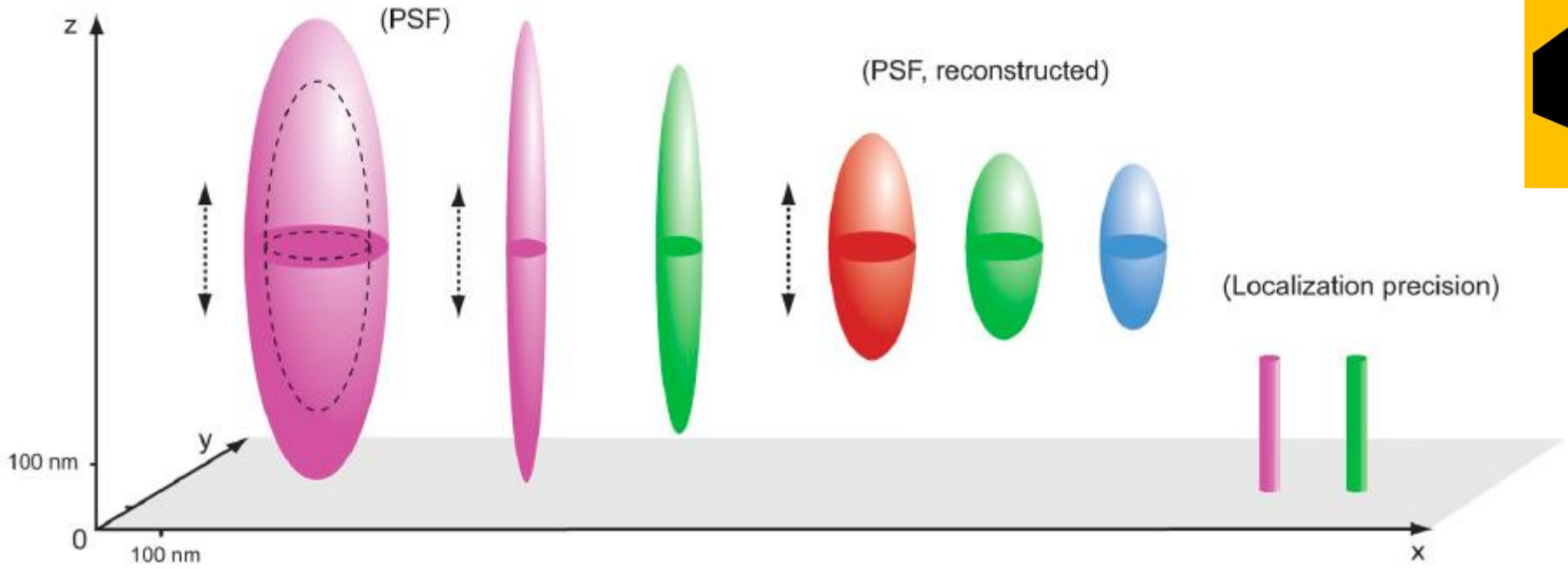
Erickson, *Biol. Proc. Online*, 2009, 11 (1) 32-51

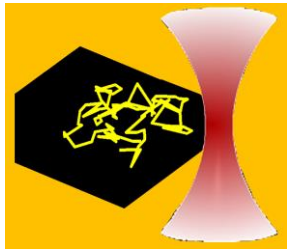
Takeda, *Nature*, 2003, 424 (6944):35-41

Treuel, *ACSNano*, 2014, 8 (1) 503–513

Super-Resolution Gap What can really be resolved ?

	CLSM	STED	CW-STED	3D-SIM			PALM/STORM	
~λem [nm]	460-670	670	520	620	520	460	670	520





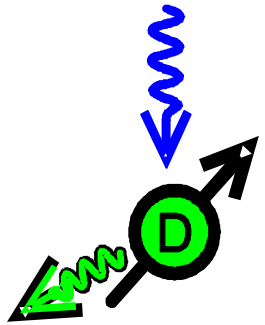
$\sim D_{x,y}$ [nm]	180-250	60	70	130	110	100	30
$\sim D_{z}$ [nm]	500-700	700	560	340	280	250	140 (TIRF-range)
$\sim V_{x,y,z}$ [$*10^{-3}$ µm^3]	10-23	1.3	1.5	3.0	1.8	1.3	0.1

CLSM Confocal Laser Scanning Microscopy
CW-STED Continuous Wave – Stimulated Emission Depletion

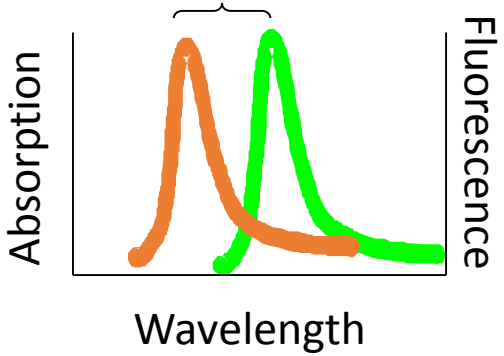
3D-SIM 3 Dimensional Structured Illumination Microscopy
(F)PALM Fluorescence Photo Activated Localization Microscopy
STORM STochastic Optical Reconstruction Microscopy

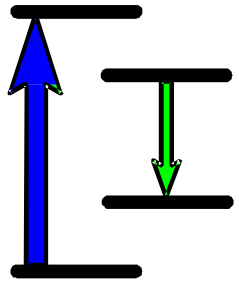
Schermelleh, *J. Cell Biol.*, 2010, 190 (2) 165–175

Refresher: the Perrin-Jablonski Energy Diagram

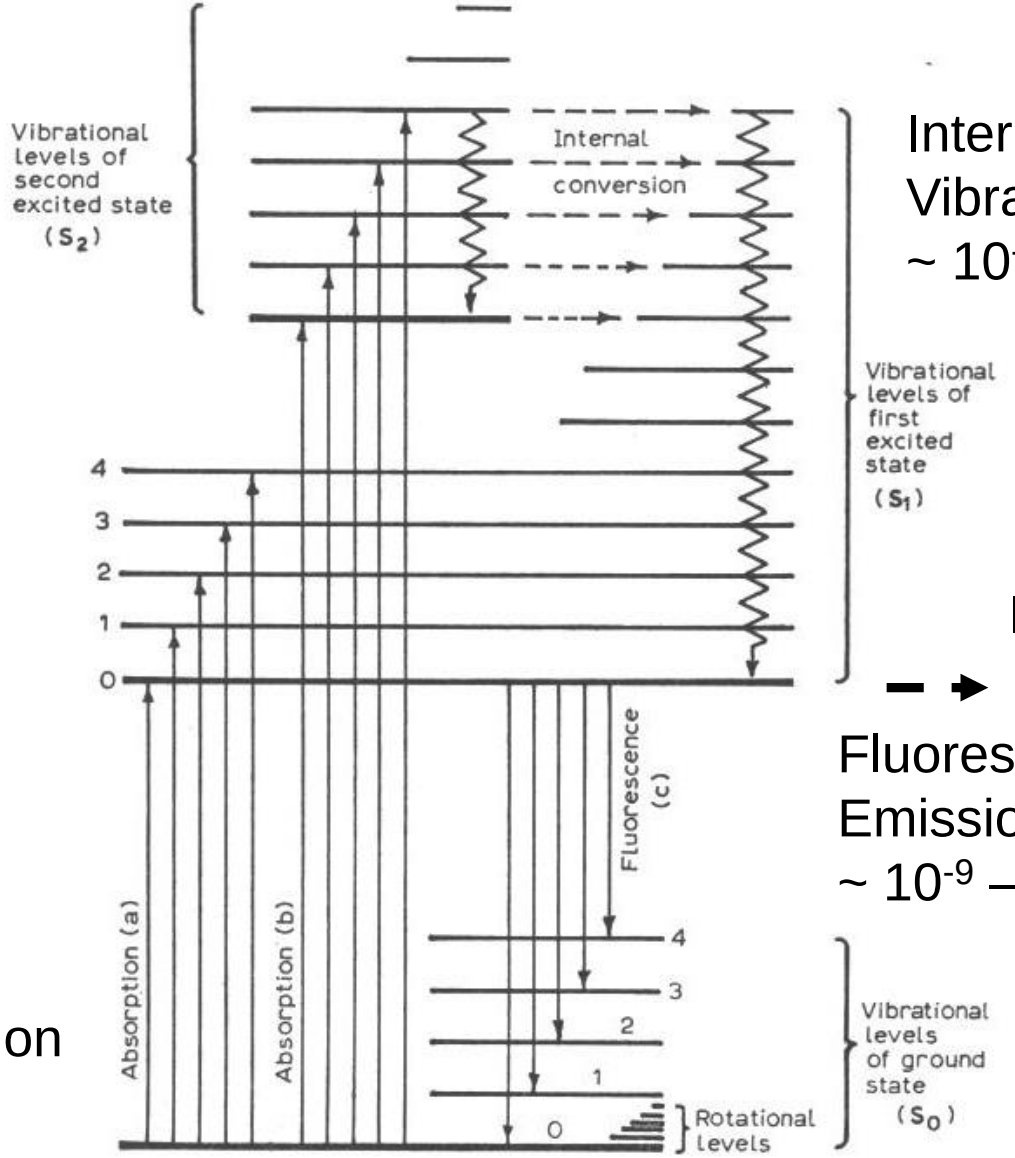


Stokes Shift
(10-100 nm)





Excitation / Absorption
~ 10^{-15} sec.



Internal Conversion &
Vibrational Relaxation
~ 10^{-14}- 10^{-11} sec.

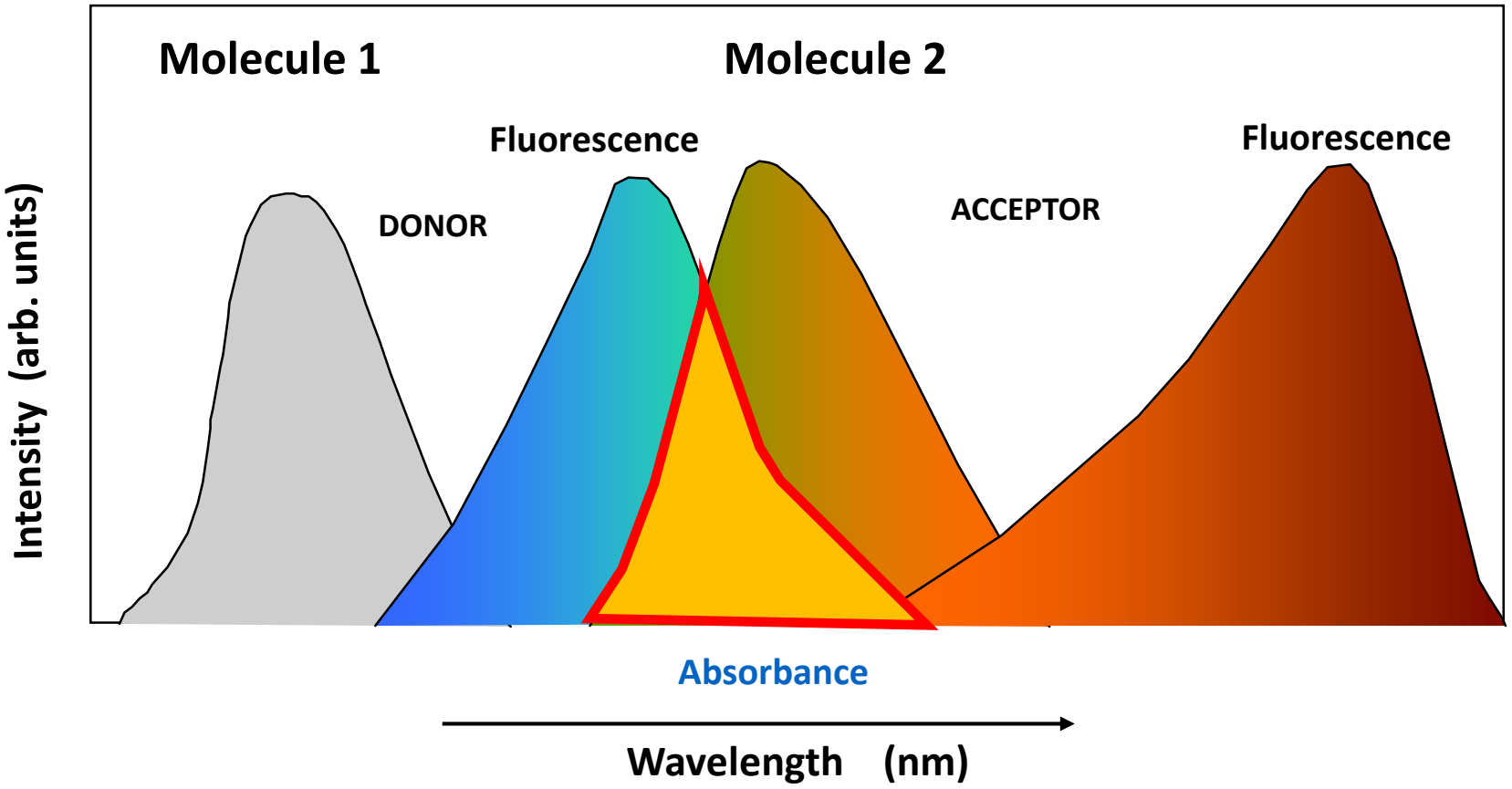
Intersystem Crossing
to Triplet excited state

Fluorescence
Emission
~ 10^{-9} – 10^{-7} sec.

Radiationless Decay
< 10^{-9} sec.

Fig. 1. Transitions giving rise to absorption and fluorescence emission spectra.

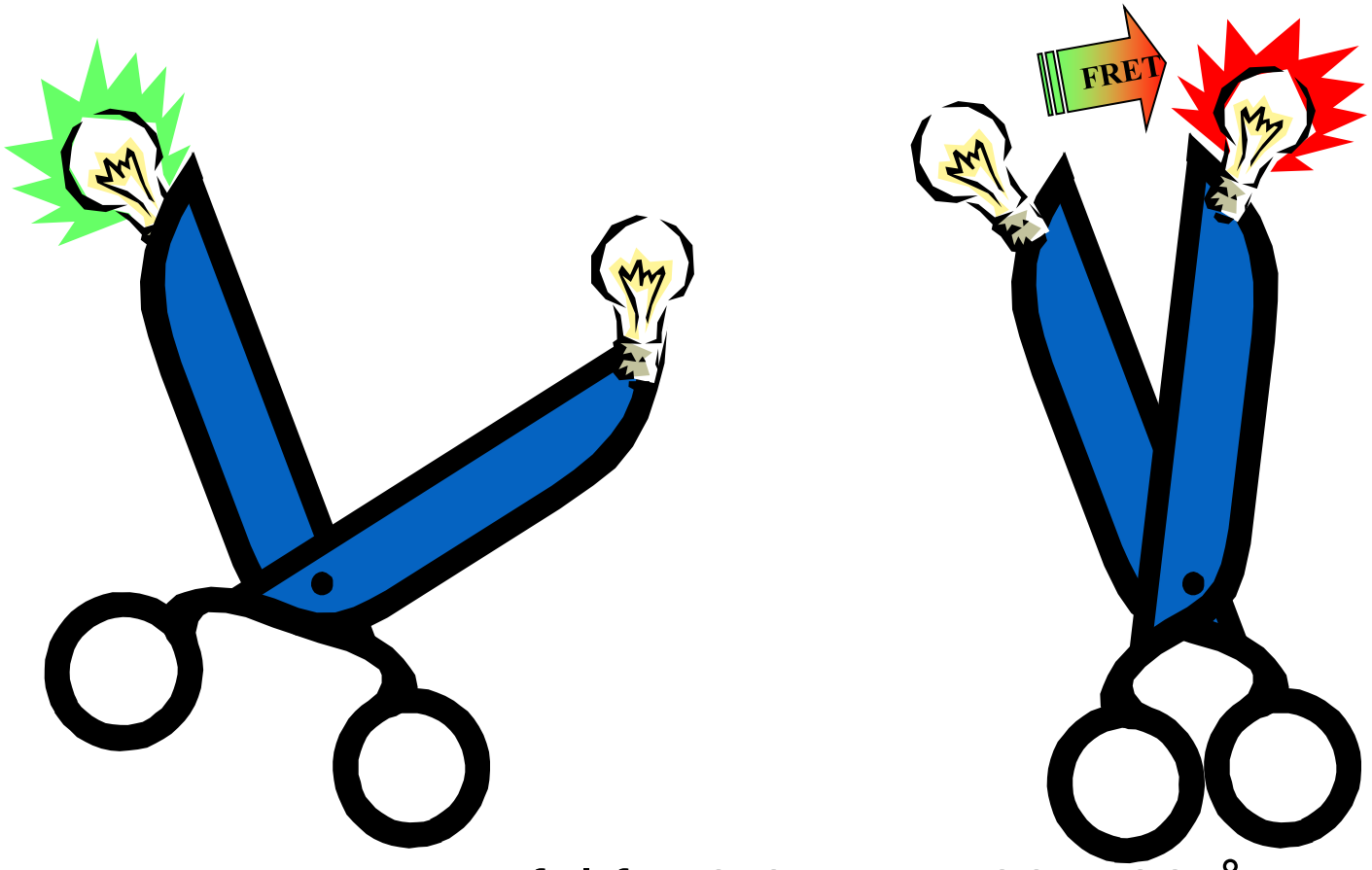
Förster Resonance Energy Transfer (FRET)



Spectral Overlap between Emission Spectrum of Donor Molecule 1 and absorption Spectrum of Acceptor Molecule 2

FRET: Quantifying Conformational Changes of (Single) (Bio)Molecules

Prof. Dr. R. Clegg



Prof. Dr. D. Jameson

FRET useful for 2-8 nm or 20 – 80 Å

Distance dependent interactions between green and red light bulbs can be used to deduce the shape of the scissors during the function.

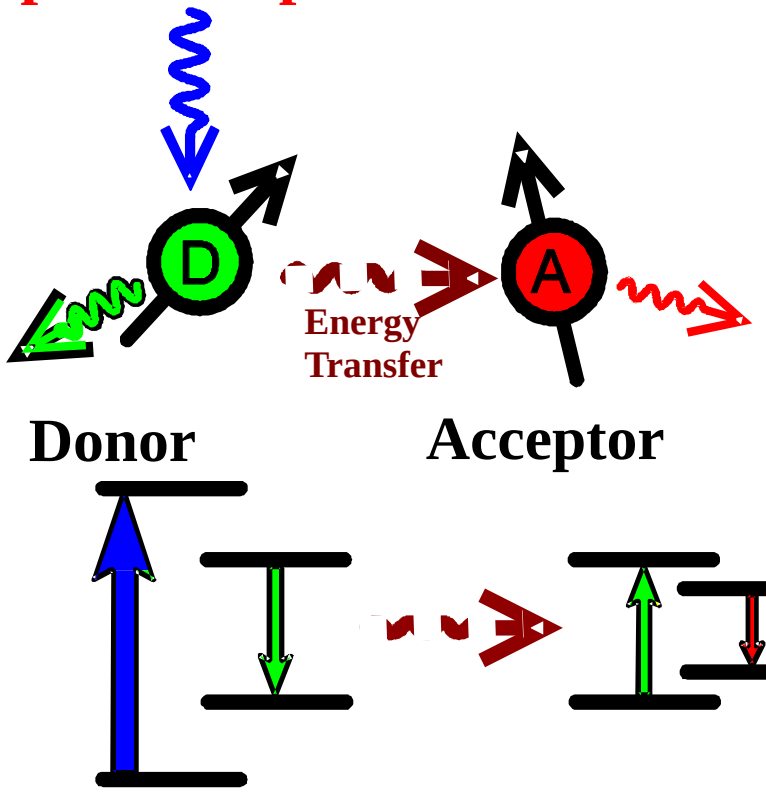
Förster Resonance Energy Transfer (FRET)

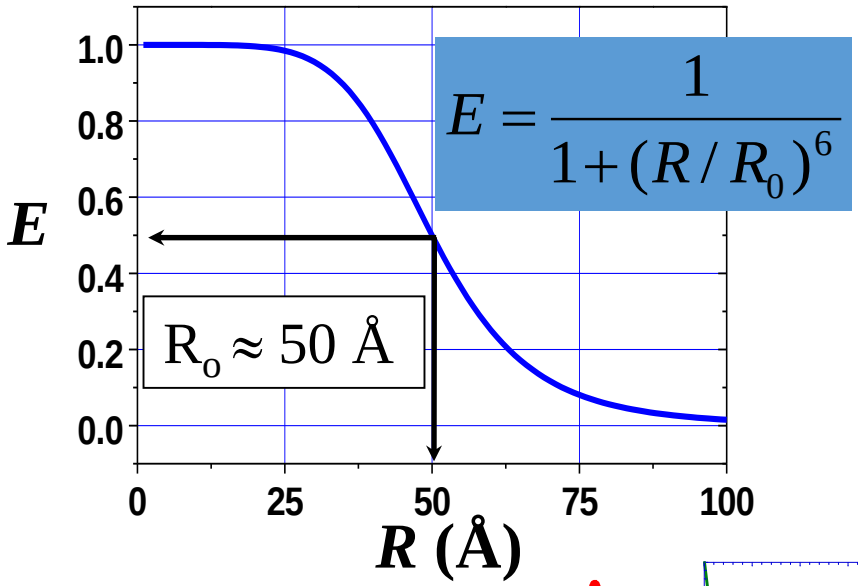
Spectroscopic Ruler for measuring nm-scale distances, binding

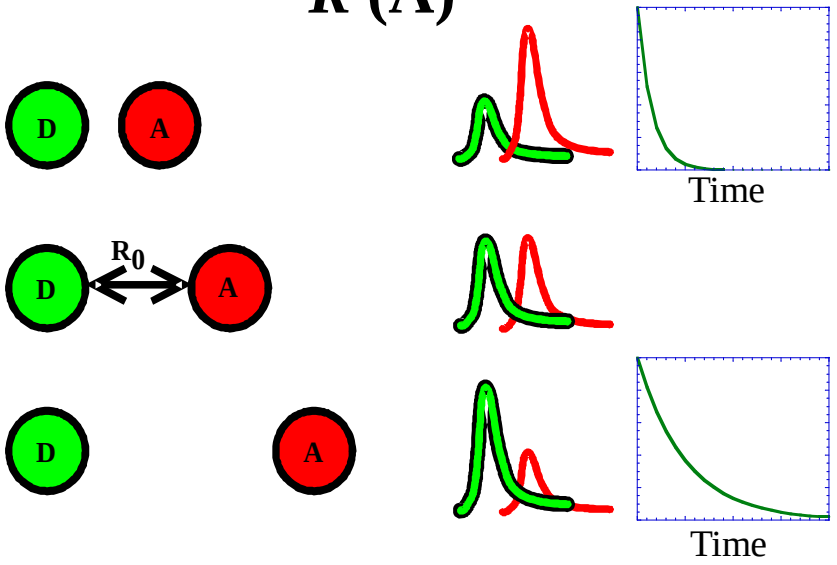
Radiationless

Energy Transfer

NO Photons involved



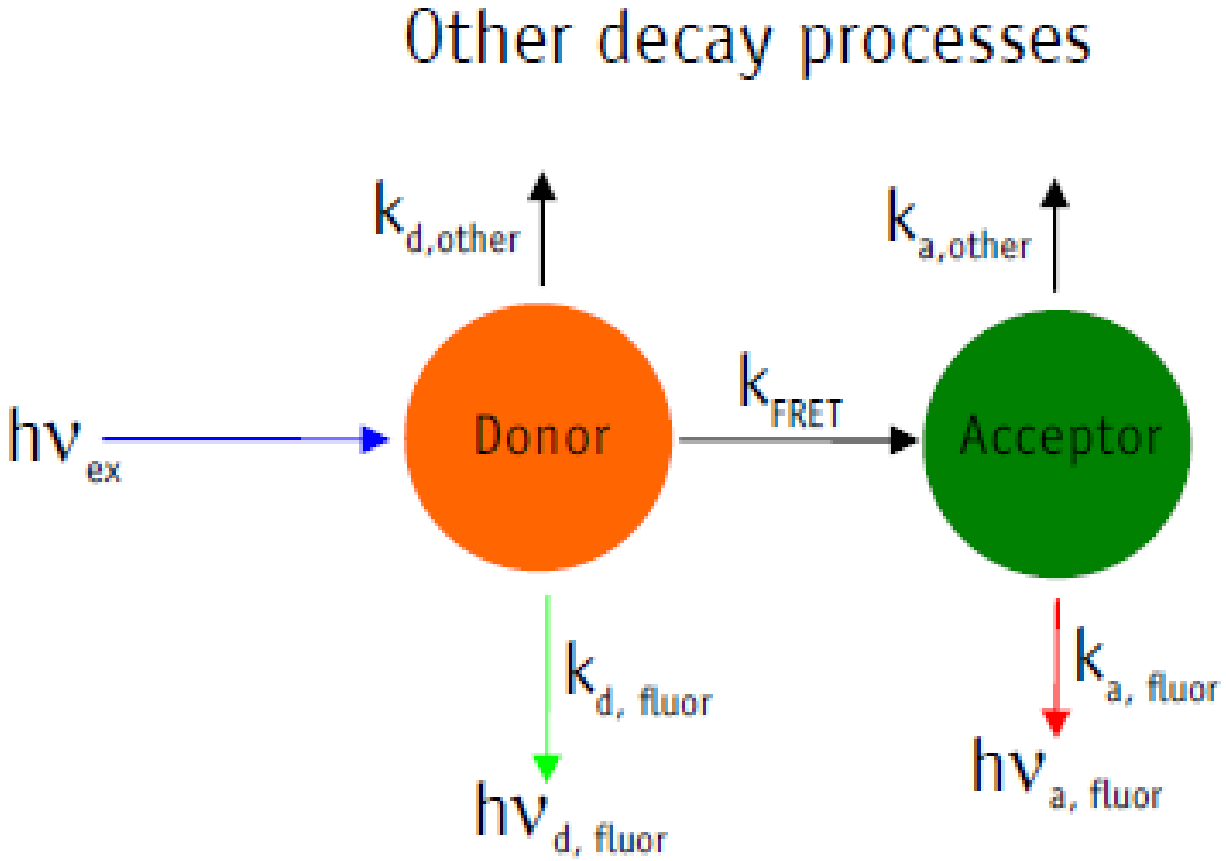




Dipole-dipole Distant-dependent energy transfer

Look at relative amounts of green & red

Fluorescence Quantum Yield, QY & FRET Efficiency, E



$$QY_d = \frac{k_{\text{d, fluor}}}{k_{\text{d, fluor}} + k_{\text{d, other}}}$$

$$QY_{da} = \frac{k_{\text{d, fluor}}}{k_{\text{d, fluor}} + k_{\text{d, other}} + k_{\text{FRET}}}$$

$$E = 1 - \frac{QY_{da}}{QY_d}$$

$$E = \frac{k_{\text{FRET}}}{k_{\text{FRET}} + k_{\text{d, other}} + k_{\text{d, fluor}}}$$

$$E = 1 - \frac{I_{da}}{I_d} \qquad E = 1 - \frac{\tau_{da}}{\tau_d}$$

Daenen, *Thesis Hasselt U.*, 2003

Terms in R_o

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

Förster distance

$$R_0 = 0.021084\ (\ J(\lambda)\ q_D\ n^{-4}\ \kappa^2\ )^{1/6} \quad \text{(nm)}$$

• J (in $M^{-1}\ cm^{-1}\ nm^4$) is the normalized spectral overlap of the donor emission (f_D) and acceptor absorption (ε_A); **Wavelength λ (nm)**

• q_D is the quantum efficiency (or quantum yield) for donor emission in the absence of acceptor (q_D = number of photons emitted divided by number of photons absorbed).

How do you measure this? Compare to known standard.

pronounced Kappa squared

• n is the index of refraction (1.33 for water).(~ 1.35 for cytoplasm)

• κ^2 is a geometric factor related to the relative orientation of the transition dipoles of the donor and acceptor and their relative orientation in space. Varies from 0 to 4; usually = 2/3.

R_0 and The Overlap Integral, J

$$J = \int_0^\infty \epsilon_A(\lambda) I_F^D(\lambda) \lambda^4 d\lambda = \int_0^\infty \frac{\epsilon_A(\bar{\nu}) I_F^D(\bar{\nu})}{\bar{\nu}^4} d(\bar{\nu})$$

with I^D_F normalized.

Note: $I_F^D(\bar{\nu}) = \lambda^2 I_F^D(\lambda)$

Note: wavenumber $(\bar{\nu})$ equals $1/\lambda$

$$R_0^6 = \frac{9000 \kappa^2 \ln 10 \, q_D}{128 \pi^5 n^4 N_A} \int \epsilon_A(\lambda) I_F^D(\lambda) \lambda^4 d\lambda$$

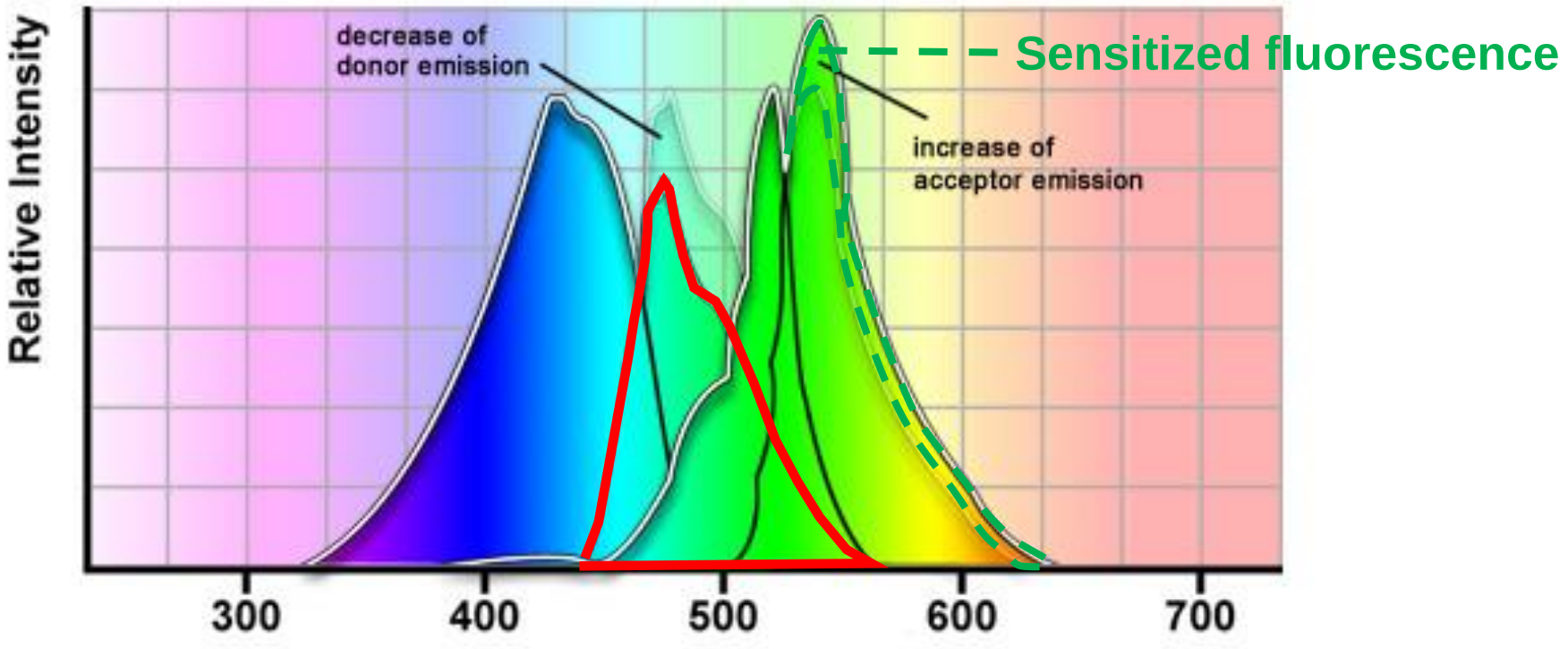
$R_0 = 0.021084 \, (J(\lambda) \, q_D \, n^{-4} \, \kappa^2)^{1/6}$ (nm) valid **for λ in nm units**

$R_0 = 978.6438 \, (J(\lambda) \, q_D \, n^{-4} \, \kappa^2)^{1/6}$ (nm) valid **for λ in cm units**

$q_D = 1$, n= 1.37 cytosolic refr. index, $\kappa^2 = 2/3$ leads to $R_0 = 0.016 \, \varepsilon^{1/6} \, \lambda^{2/3}$ (nm)

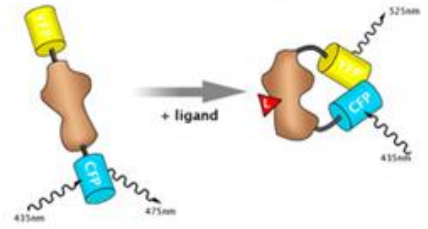
Lakowicz, *Principles of Fluorescence Spectroscopy*, Springer 3rd ed., 2006

E.T. leads to decrease in Donor Emission & Increase in Acceptor Emission



FRET between CFP and YFP

When FRET occurs this is hallmarked by a decreased donor (CFP) emission and increased acceptor (YFP) emissior

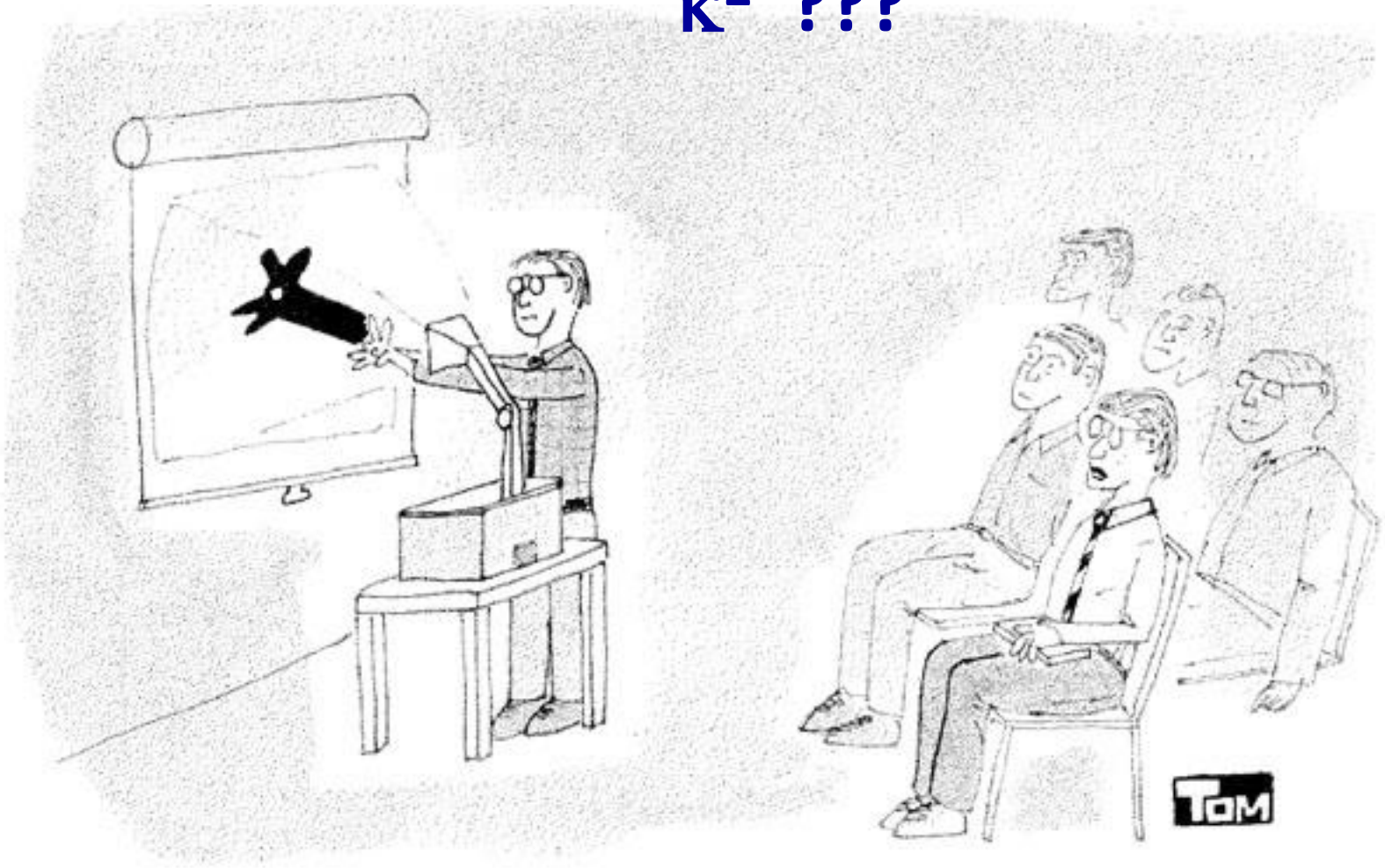


$R_o \approx$ 4.9-5.2 nm

Sp13Lec20FRETIIPhysics475-Clegg.ppt

http://mekentosj.com/science/fret/

κ^2 ???

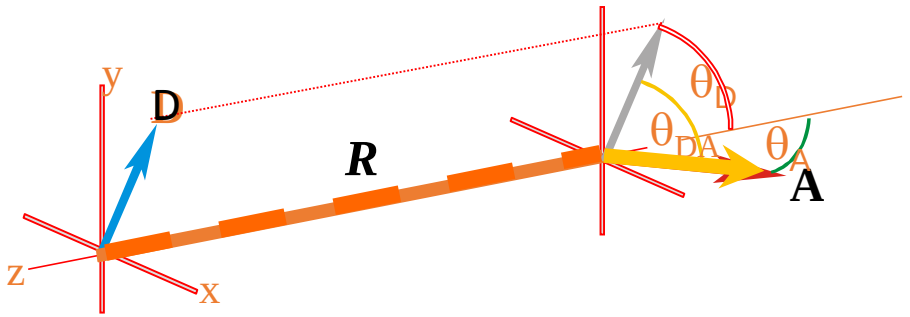


"I WAS HOPING TO SEE MORE EVIDENCE on this kappa square"

κ^2 Orientation Factor

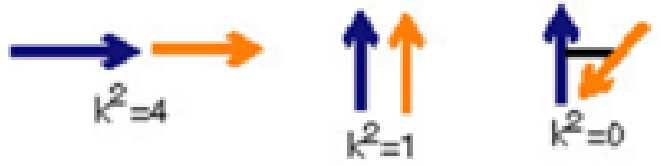
The spatial relationship between the DONOR emission dipole moment and the ACCEPTOR absorption dipole moment

$$\kappa^2 = (\cos\theta_{DA} - 3\cos\theta_D \cos\theta_A)^2$$



$(0 \le \kappa^2 \le 4)$

κ^2 often = 2/3



where θ_{DA} is the angle between the donor and acceptor transition dipole moments, θ_D (θ_A) is the angle between the donor (acceptor) transition dipole moment and the R distance vector joining the two dyes.

✓ **κ^2 is usually not known and is assumed to have a value of 2/3 (Randomized distribution) where** D and A probes exhibit a high degree of rotational motion

Dale, Biophys. J., 1979, 26, 161-193

κ^2 Orientation Factor Best Practice

Except in very rare case, κ^2 can not be uniquely determined in solution. What value of κ^2 should be used ?

1. We can **assume** isotropic motions of the probes and a value of $\kappa^2 = 2/3$, and verify experimentally that it is indeed the case.

***By swapping probes*:** The environment of the probe will be different and if κ^2 is not equal to 2/3, because orientations of the probes are not dynamically average (during the lifetime of the probe) due to restricted motions of the fluorophores, then the distance measured by FRET will be different.

By using different probes: If the distance measured using different probe pairs are similar (taking into account the size of the probes) then the assumption that κ^2 is equal to 2/3 is probably valid.

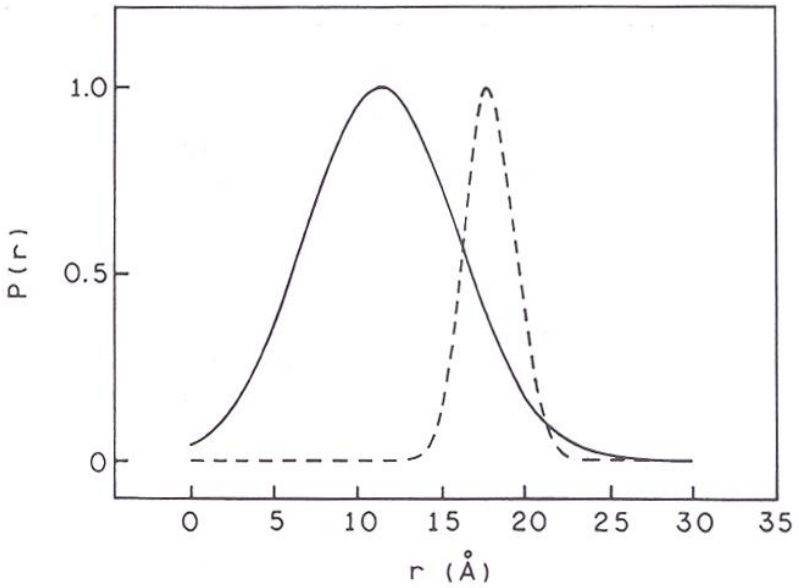
2. We can **calculate** the lower and upper limit of κ^2 using polarization data (Dale, Eisinger and Blumberg: 1979 *Biophys. J.* 26:161-93).

Distance Distribution Analysis

For a flexible biomolecule, the distance between two "target" points on the molecule, appropriately labelled with donor and acceptor groups, will not be fixed but experience a distribution of separation distances which reflect the solution dynamics. The observed efficiency of energy transfer will directly be related to this distribution of distances.

This distribution cannot be determined by steady-state methodologies in a single donor/acceptor experiment.
Methodologies based on lifetime procedures permit recovery of a distribution and the applicability of these methods, using both time and frequency domain techniques, has been demonstrated in a number of model and unknown systems.

First suggested by Haas, et al., (1975) *Proc Natl Acad Sci USA* **72**, 1807, an example of this analysis is shown here, from the work of She et al. 1998 *J Mol Biol.* **281:**445-52.

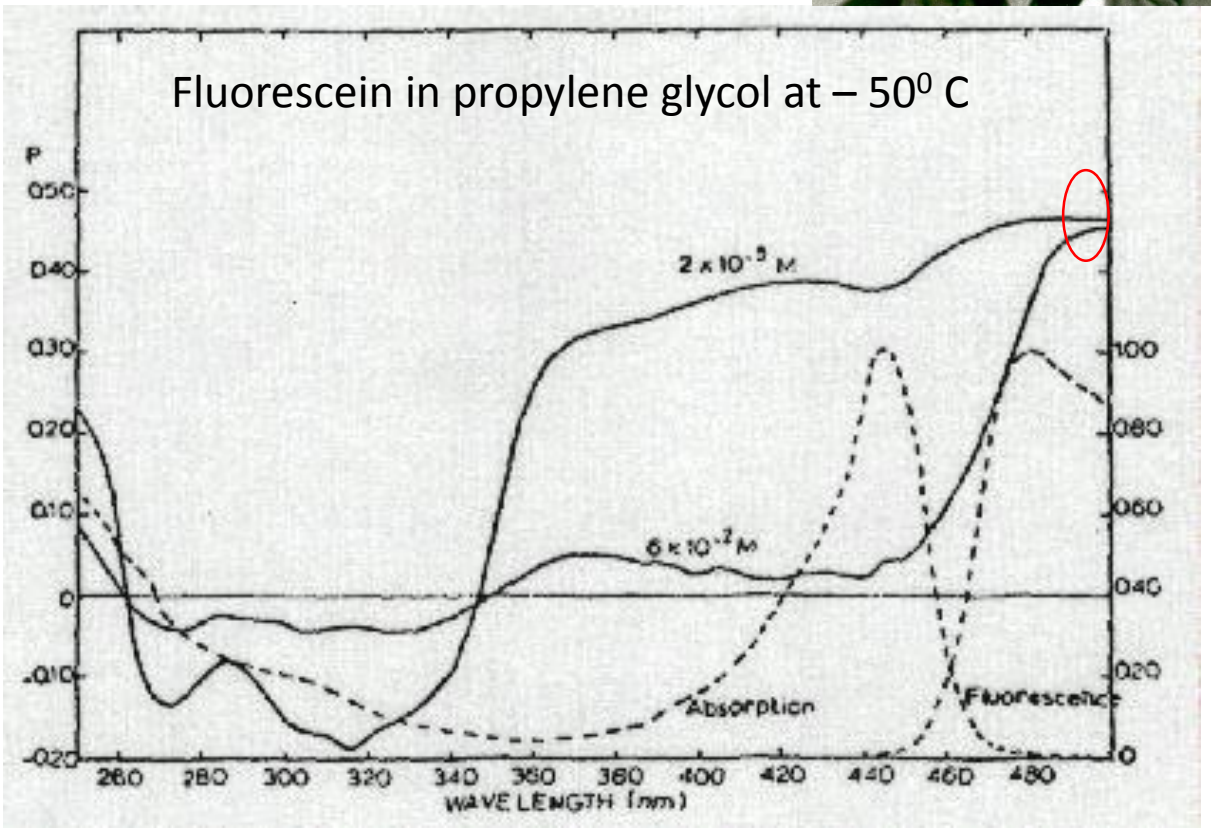


Distance distribution functions between tryptophan 22 and AEDANS-Cys52 in **troponin** in the presence (dashed line) and absence (solid line) of calcium.

Weber's Red-Edge Effect

Electronic energy transfer between identical fluorophores was originally observed by Gaviola and Pringsheim in 1924. In 1960 Weber was the first to report that **homotransfer** among indole molecules disappeared upon excitation at the red-edge of the absorption band - this phenomenon is now known as the "Weber red-edge effect".

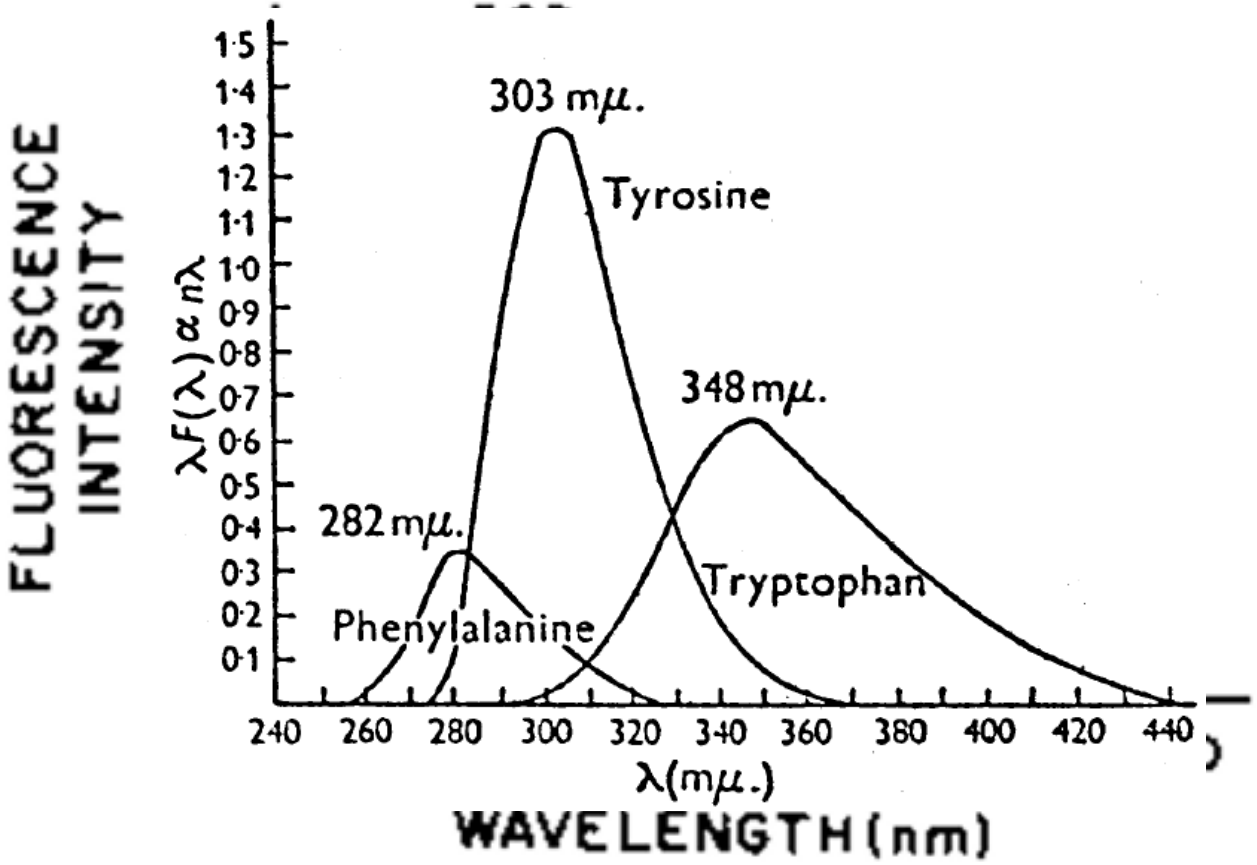
In 1970 Weber and Shinitzky published a more detailed examination of this phenomenon. They reported that in the many aromatic residues examined, transfer is much decreased or undetectable on excitation at the red edge of the absorption spectrum .



Built-in Intrinsic FRET Pairs

Aromatic amino acid residues: Trp, Tyr, Phe

Possible FRET pairs: Phe – Tyr, Tyr - Trp



Jameson, *MAF6 Paris*, 1999, 1-25

Förster Distances for Donor – Acceptor Dye Pairs

Donor	Acceptor	R_0 (Å)
Fluorescein	Tetramethylrhodamine	55
IAEDANS	Fluorescein	46
EDANS	Dabcyl	33
Fluorescein	Fluorescein	44
BODIPY FL	BODIPY FL	57
Fluorescein	QSY 7 and QSY 9 dyes	61
Alexa fluor 555	Alexa fluor 647	51
Cy3	Cy5	51

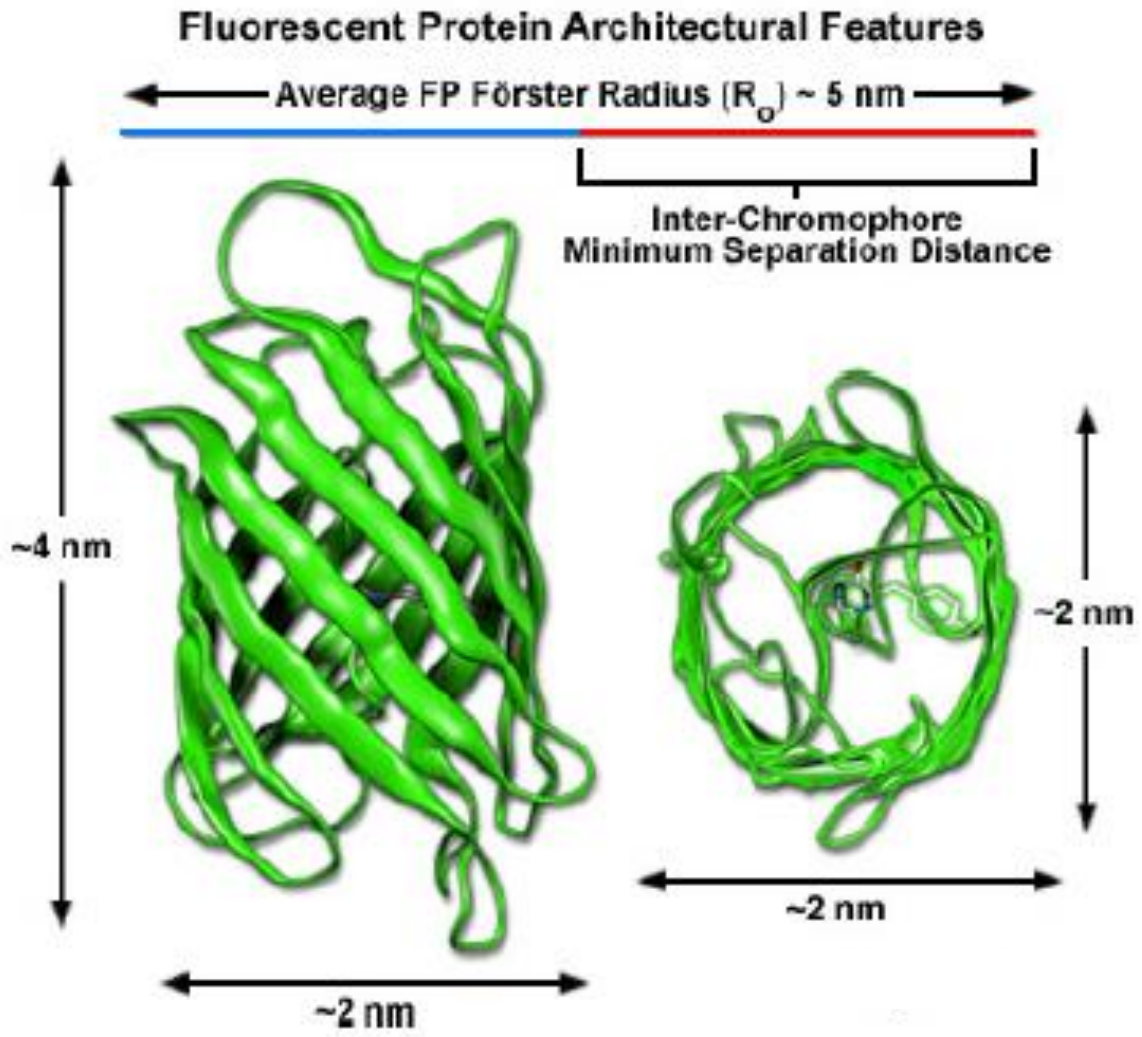
Donor	Acceptor	Förster distance (R_0, nm)
Naphthalene	Dansyl	2.2
LY	TNP-ATP	3.5
Dansyl	ODR	4.3
LY	EM	5.3
FITC	EM	6.0
BPE	CY5	7.2

Abbreviations: **BPE**, B-phycoerythrin; **CY5**, carboxymethylindocyanine; **Dansyl**, just dansyl group; **EM**, eosin maleimide; **FITC**, fluorscein-5-isothiocyanate; **LY**, Lucifer yellow;**ODR**, octadecylrhodamine; **TNP-ATP**, trinitrophenyl-ATP.

http://chemwiki.ucdavis.edu/Core/Theoretical_Chemistry/Fundamentals/Fluorescence_Resonance_Energy_Transfer

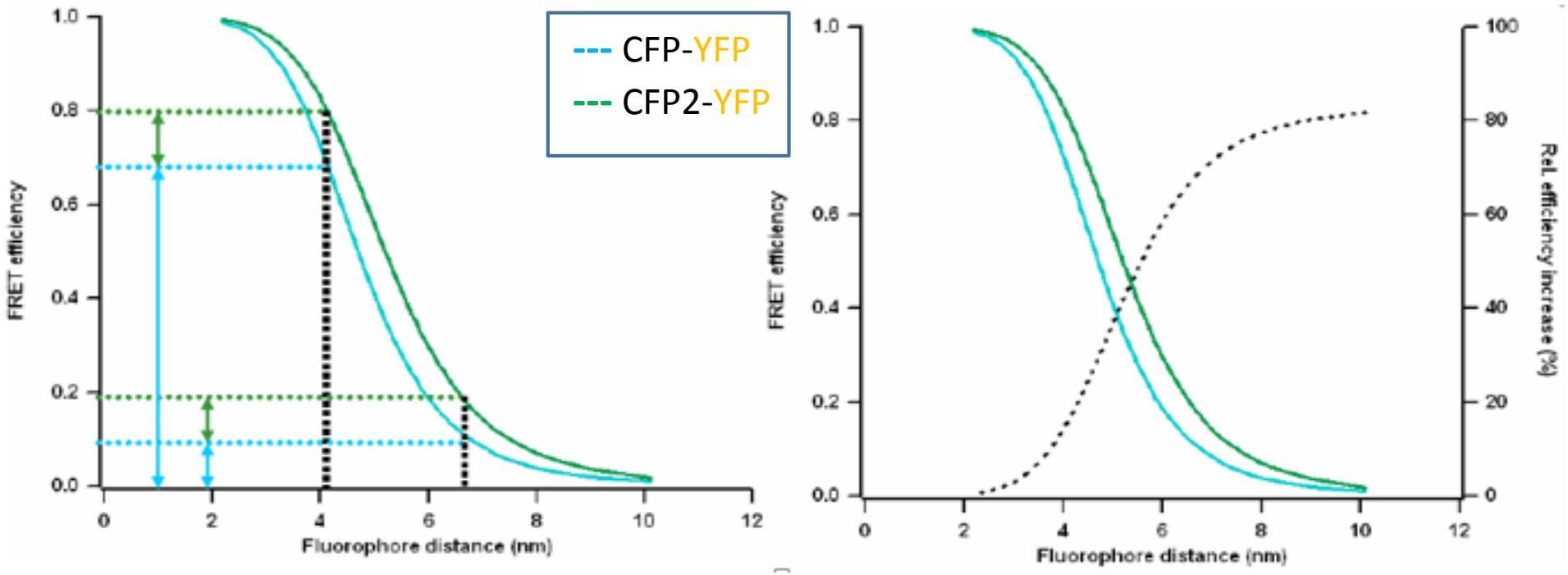
Daenen, *Thesis Hasselt U.*, 2003

Fluorescent Proteins as FRET Probes

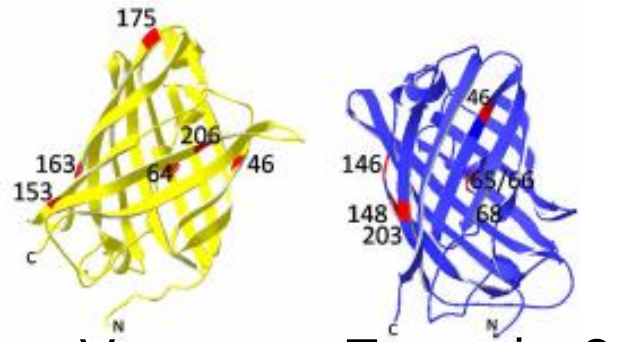


Yildiz, PHYS250 Berkeley, 2012

Higher FRET Efficiency Enhances Detection Range



R_0 mTurquise2 – mVenus 5.8 nm
R_0 ECFP -- EYFP 4.9 nm



mVenus

mTurquoise2

Mueller, *Frontiers in Plant Sc.*, 2013, 4 (413) 1-20
FRET_teaching_module.pdf , EMBL, Zimmermann

Förster-Radii for Fluorescent Protein FRET-Pairs

Fluorophores	Förster radius R_0	Dynamic range	References
BLUE DONOR			
EBFP/ECFP	3.8 nm	1.9–5.7 nm	Patterson et al., 2000
EBFP/EGFP	4.1 nm	2.1–6.2 nm	Patterson et al., 2000
EBFP/EYFP	3.8 nm	1.9–5.7 nm	Patterson et al., 2000
EBFP/DsRed	3.2 nm	1.6–4.8 nm	Patterson et al., 2000
CYAN DONOR			
ECFP/EGFP	4.8 nm	2.4–7.2 nm	Patterson et al., 2000
ECFP/EYFP	4.9 nm	2.5–7.3 nm	Patterson et al., 2000
ECFP/mVenus	5.0 nm	2.5–7.5 nm	Rizzo et al., 2006
mCerulean/EYFP	5.4 nm	2.7–8.1 nm	Rizzo et al., 2006
mCerulean/mVenus	5.4 (5.2) nm	2.7–8.1 nm	Rizzo et al., 2006; Markwardt et al., 2011
mCerulean/mCitrine	5.4 nm	2.7–8.1 nm	Rizzo et al., 2006
mCerulean3/mVenus	5.7 nm	2.9–8.6 nm	Markwardt et al., 2011
SCFP3/SYFP2	5.4 nm	2.7–8.1 nm	Goedhart et al., 2007
mTurquoise/mVenus	5.7 nm	2.9–8.6 nm	Markwardt et al., 2011
mTurquoise2/mVenus	5.8 nm	2.9–8.7 nm	Goedhart et al., 2012
ECFP/DsRed	4.2 (5.1) nm	2.1–6.3 nm (2.6–7.7 nm)	Patterson et al., 2000; Erickson et al., 2003
ECFP/mCherry	3.5 nm	1.8–5.3 nm	He et al., 2005
GREEN DONOR			
EGFP/EYFP	5.6 nm	2.8–8.4 nm	Patterson et al., 2000
EGFP/DsRed	4.7 (5.8) nm	2.4–7.1 nm (2.9–8.7 nm)	Erickson et al., 2003
EGFP/mRFP1	4.7 nm	2.4–7.1 nm	Peter et al., 2005
Clover/mRuby2	6.3 nm	3.2–9.5 nm	Lam et al., 2012
Kaede/Kaede	5.8 nm	2.9–8.7 nm	Wolf et al., 2013a
Dronpa/mCherry	5.6 nm	2.8–8.4 nm	This work
YELLOW/ORANGE DONOR			
EYFP/DsRed	4.9 nm	2.5–7.4 nm	Patterson et al., 2000
EYFP/mCherry	5.7 nm	2.9–8.6 nm	Akrap et al., 2010
SYFP2/mStrawberry	6.3 nm	3.2–9.5 nm	Goedhart et al., 2007
mKo/mCherry	6.4 nm	3.2–9.6 nm	Goedhart et al., 2007

Mueller, *Frontiers in Plant Sc.*, 2013, 4 (413) 1-20

Spectral Properties for Donor – Acceptor FRET Pairs

Donor (Emission)	Acceptor (Excitation)
FITC (520 nm)	TRITC (550 nm)
Cy3 (566 nm)	Cy5 (649 nm)
EGFP(508 nm)	Cy3 (554 nm)
CFP (477 nm)	YFP (514 nm)
EGFP (508 nm)	YFP (514 nm)

Quantum Yield & Extinction Coef. Of FRET Proteins

Protein (acronym)	Ex (nm)	Em (nm)	EC × 10^{-3}/M/cm	QY	Relative brightness (% of EGFP)[a]	Use as FRET probe	Reference
Aequorea-based FPs							
EBFP2	383	448	32.0	0.56	53	Donor to GFP/YFP	[9]
mCerulean3	433	475	40.0	0.87	103	Donor to YFP	[53]
mTurquoise	435	477	35.0	0.51	53	Donor to YFP	[52]
EGFP	488	507	56.0	0.60	100	Donor to OFP, RFP	[5]
mVenus	515	528	92.2	0.57	156	Acceptor for CFP, donor to RFP	[7]
mCitrine	516	529	77.0	0.76	174	Acceptor for CFP	[51]
T-Sapphire	399	511	44.0	0.60	79	Long Stokes shift donor	[55]
mAmetrine	406	526	45.0	0.58	78	Long Stokes shift donor	[56]
REACh	515	528	92.2	0.04	1	Strong absorber, weak emitter, acceptor for FLIM studies	[77, 78]
Coral FPs							
Midoriishi Cyan	472	495	27.3	0.90	73	Donor to mKO	[15]
mTFP1	462	492	64.0	0.85	162	Donor to YFP, OFP, RFP	[57]
Kusabira Orange2	551	565	63.8	0.62	118	Acceptor for CFP	[71]
mCherry	587	610	72.0	0.22	47	Acceptor for GFP	[65]
TagRFP-T	555	584	81.0	0.41	99	Acceptor for GFP	[70]
mRuby	558	605	112.0	0.35	117	Acceptor for GFP	[76]

Large Quantum Yield, QY good donor
Large Extinction Coef., EC good acceptor

Day, *Bioessays* 2012, 34 341–350

Photostability of FRET Proteins

	Ex λ(nm)	Em λ(nm)	ε($mM^{-1}cm^{-1}$)	Q	Photostability (s)	Ref
mCerulean	433	475	43	0.62	36	36
mTurqoise2	434	474	30	0.93	90	37
CyPet	435	477	35	0.51	59	29
mTFP1	462	488	64	0.85	110	38
mCitrine	516	530	77	0.76	49	39
YPet	517	530	104	0.77	49	29
TagRFP	555	584	100	0.48	37	40
TagRFP-t	555	584	81	0.41	337	41
mRuby2	559	600	113	0.38	123	42
mCherry	587	610	72	0.22	96	43

Scott, *Scientific Reports*, 2015, 5 10270 1-13

Reporting on Recent Additions to FRET Proteins

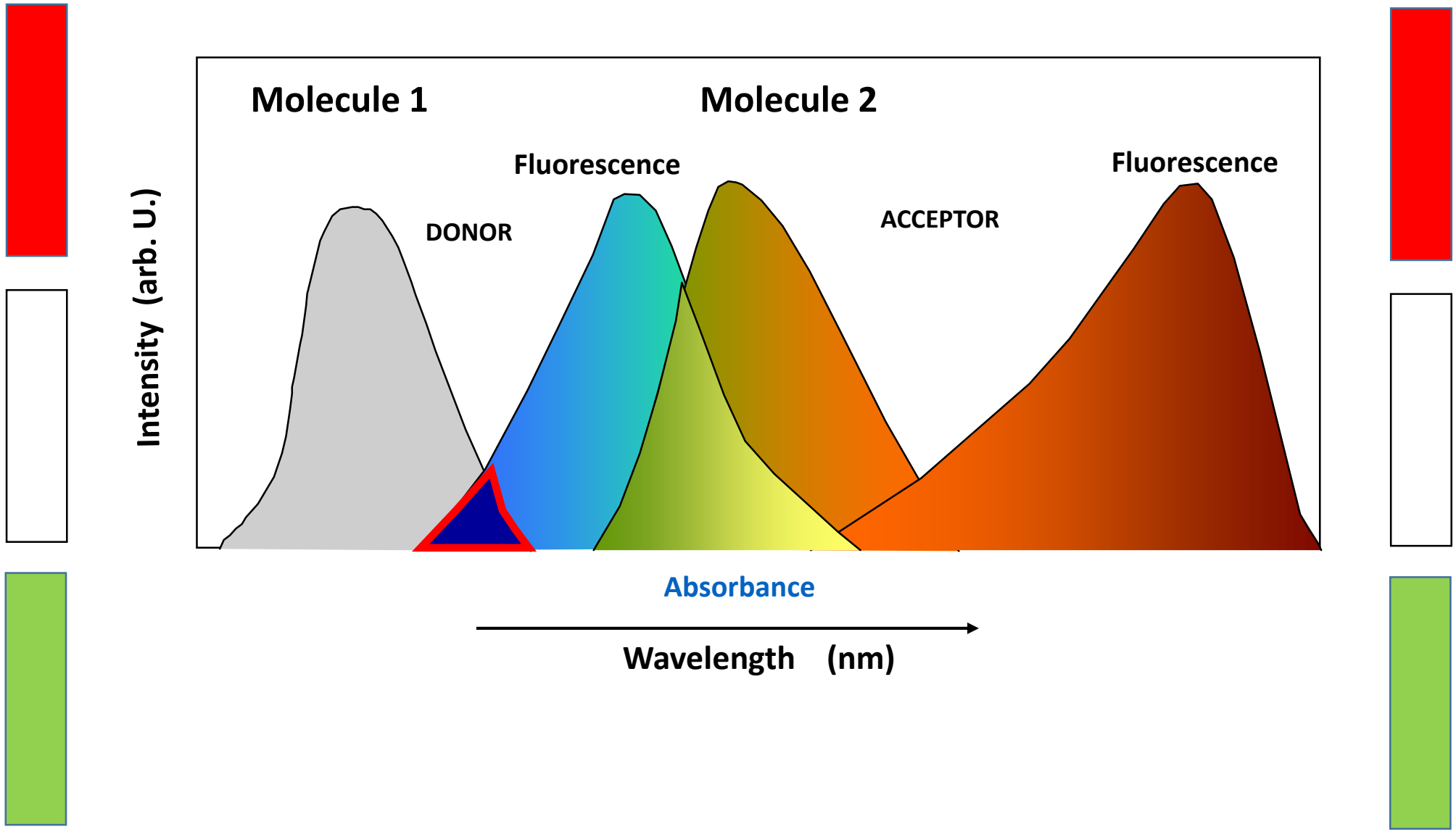
Fluorescent protein	λ_{Abs} (nm)	λ_{Emiss} (nm)	ε (M^{-1} cm^{-1})	QY	J (λ)$M^{-1}cm^{-1}nm^4$*	R_0 (Å)*
NowGFP	494	502	56700	0.76	-	
mOrange	548	562	71000†	0.69†	2.48×10^{15}	57.63
mRuby2	559	600	113000‡	0.38‡	3.74×10^{15}	61.72
TagRFP	555	584	100000§	0.48§	2.91×10^{15}	59.17
tdTomato	554	581	138000†	0.69†	5.43×10^{15}	65.67

Note: * NowGFP is donor

Desirable Design: Photo-switching FPs

Abraham, *PLOS ONE*, 2015, August 3, 1-15

Energy Migration Between Like Fluorophores

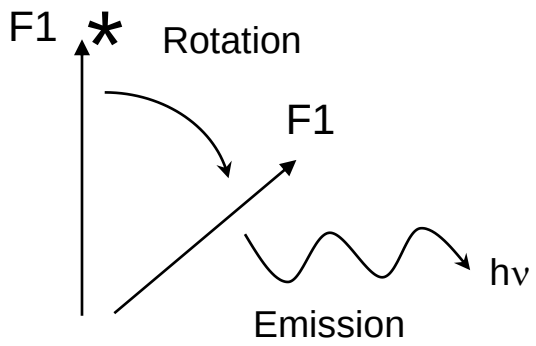


Shrestha, *Int. J. Mol. Sci.* **2015**, *16*, 6718-6756

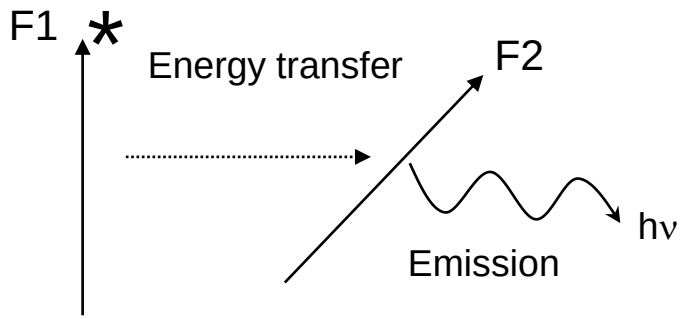
Energy Migration ie. HOMO-FRET

"…Excitation transfer between alike molecules can occur in repeated steps. So the excitation may *migrate* from the absorbing molecule over a considerable number of other ones before deactivation occurs by fluorescence or other process. Though this kind of transfer cannot be recognized from fluorescence spectra, it may be observed by the decrease of fluorescence polarization…" (Förster, 1959)

A.



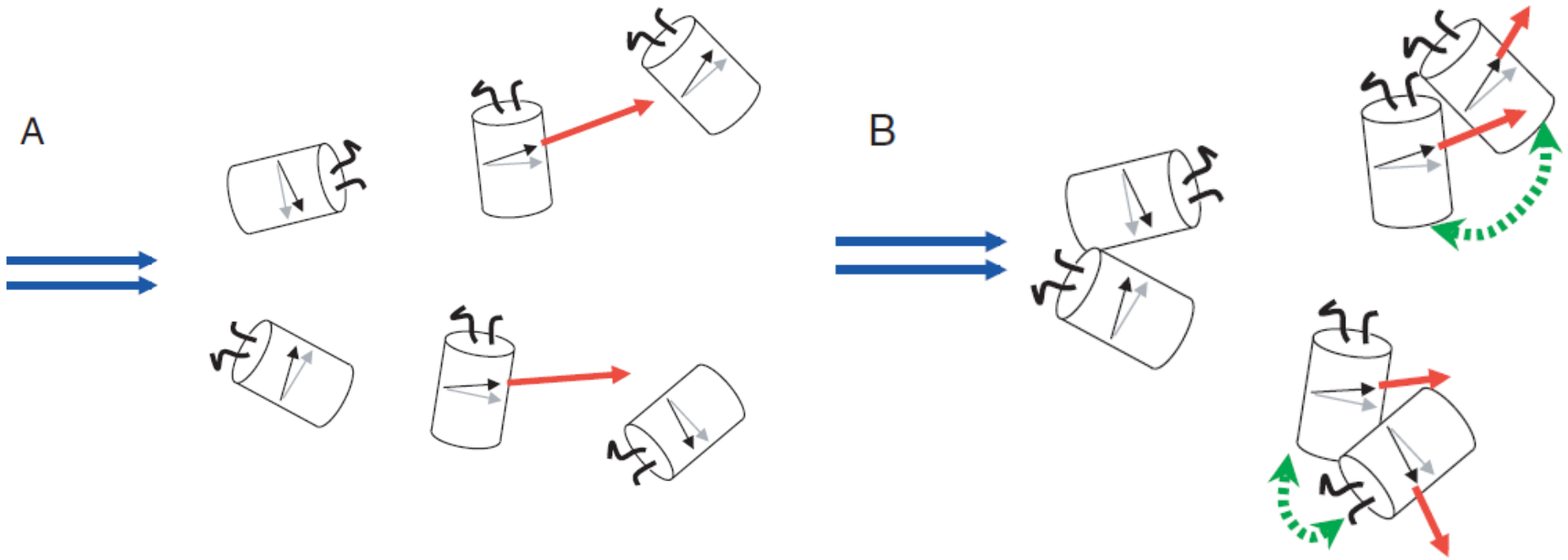
B.



A. Depolarization resulting from rotational diffusion of the fluorophore. The excited fluorophore (F1*) rotates then emits light.

B. The excited fluorophore (F1*) transfer energy to another fluorophore F2 which in turn emits light.

HOMO-FRET utilizing Fluorescence Anisotropy



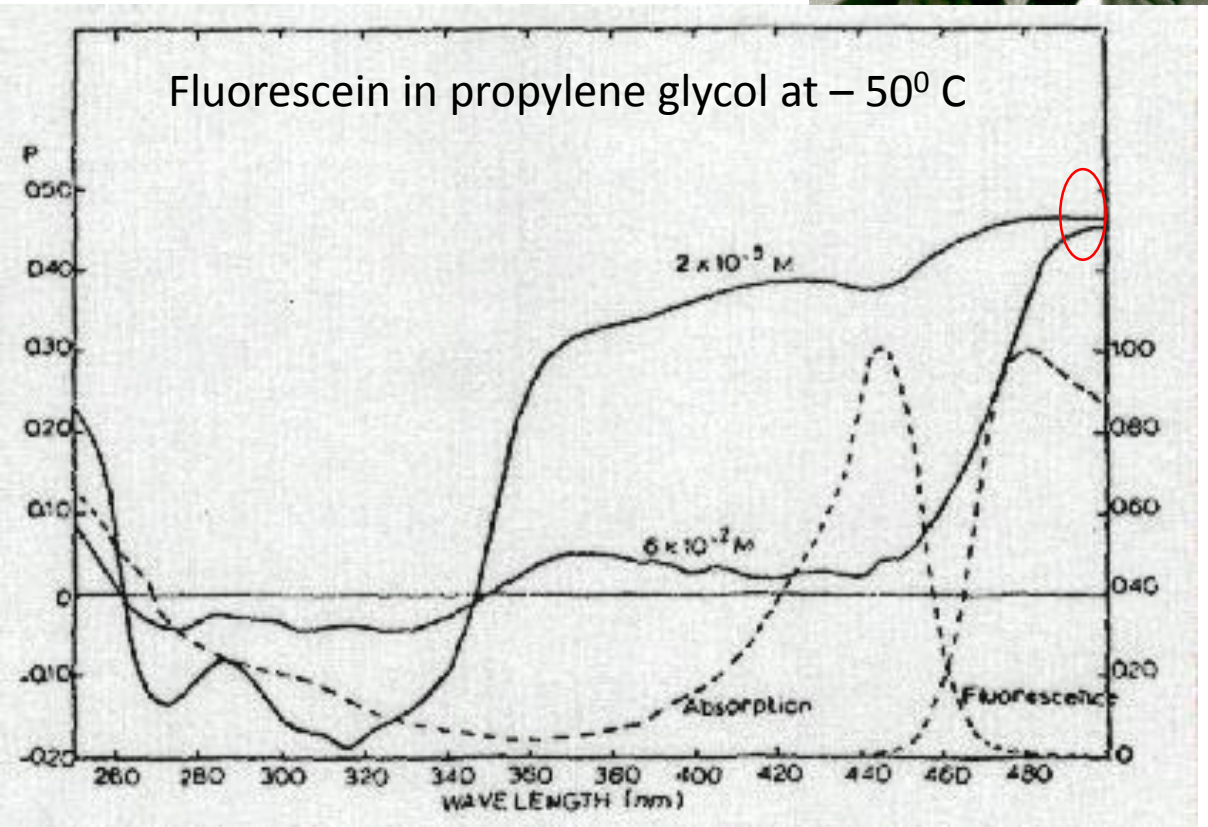
Higher sensitivity as compared with hetero-FRET
No dual labelling
No curtailing of different expression levels
Whole donor emission spectrum can be used
(Varma & Mayor, 1998)

Pietraszewska-Bogiel, *J.Microsc., 2011, 241(2) 111–118*

Weber's Red-Edge Effect

Electronic energy transfer between identical fluorophores was originally observed by Gaviola and Pringsheim in 1924. In 1960 Weber was the first to report that homotransfer among indole molecules disappeared upon excitation at the red-edge of the absorption band - this phenomenon is now known as the "Weber red-edge effect".

In 1970 Weber and Shinitzky published a more detailed examination of this phenomenon. They reported that in the many aromatic residues examined, transfer is much decreased or undetectable on excitation at the red edge of the absorption spectrum .



FRET with Multiple Acceptors

Assume n independent acceptors

$$E_n = \frac{nk_{transfer}}{nk_{transfer} + k_{other}}$$

$$= \frac{nk_{transfer}}{k_{transfer} + k_{other}} \Big/ \frac{k_{transfer} + k_{other} + (n-1)k_{transfer}}{k_{transfer} + k_{other}}$$

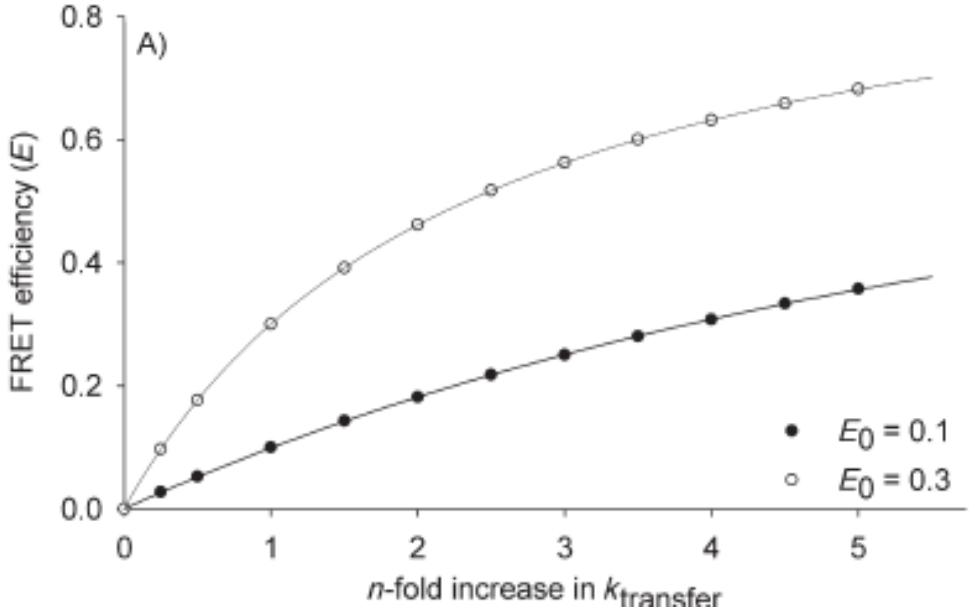
$$= \frac{nE_0}{1 + (n-1)E_0}$$

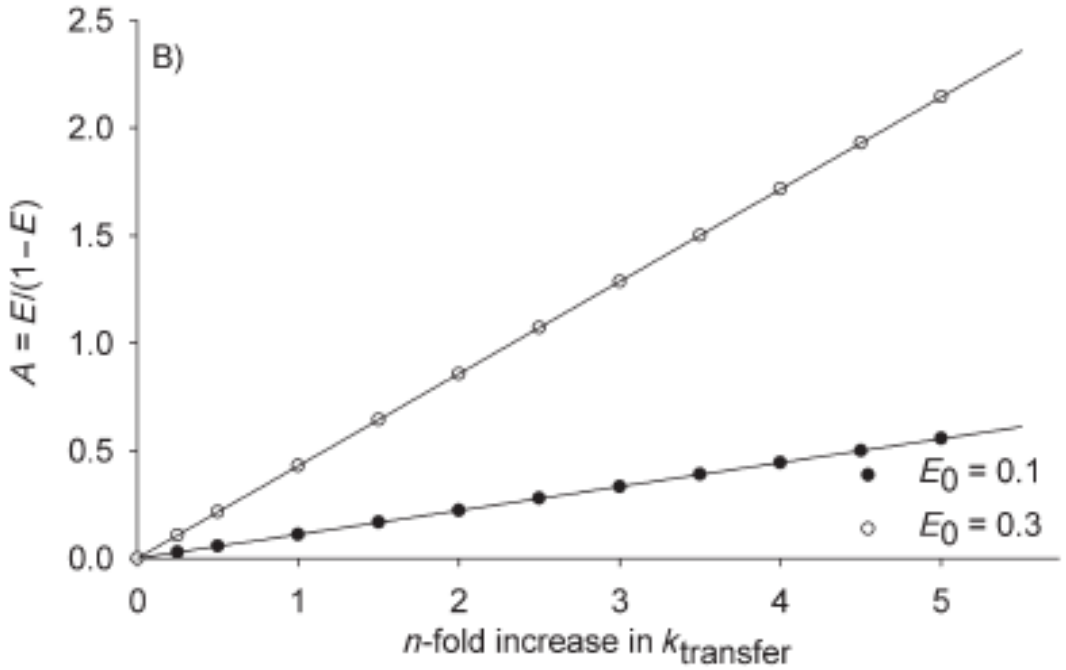
$$A_0 = \frac{E_0}{1 - E_0}$$

$$A_n = \frac{E_n}{1 - E_n} = n\frac{E_0}{1 - E_0} = nA_0$$

Mueller, *Frontiers in Plant Sc*., 2013, 4 (413) 1-20 Bojarski, *J. Phys. Chem. B* 2011, 115, 10120–10125

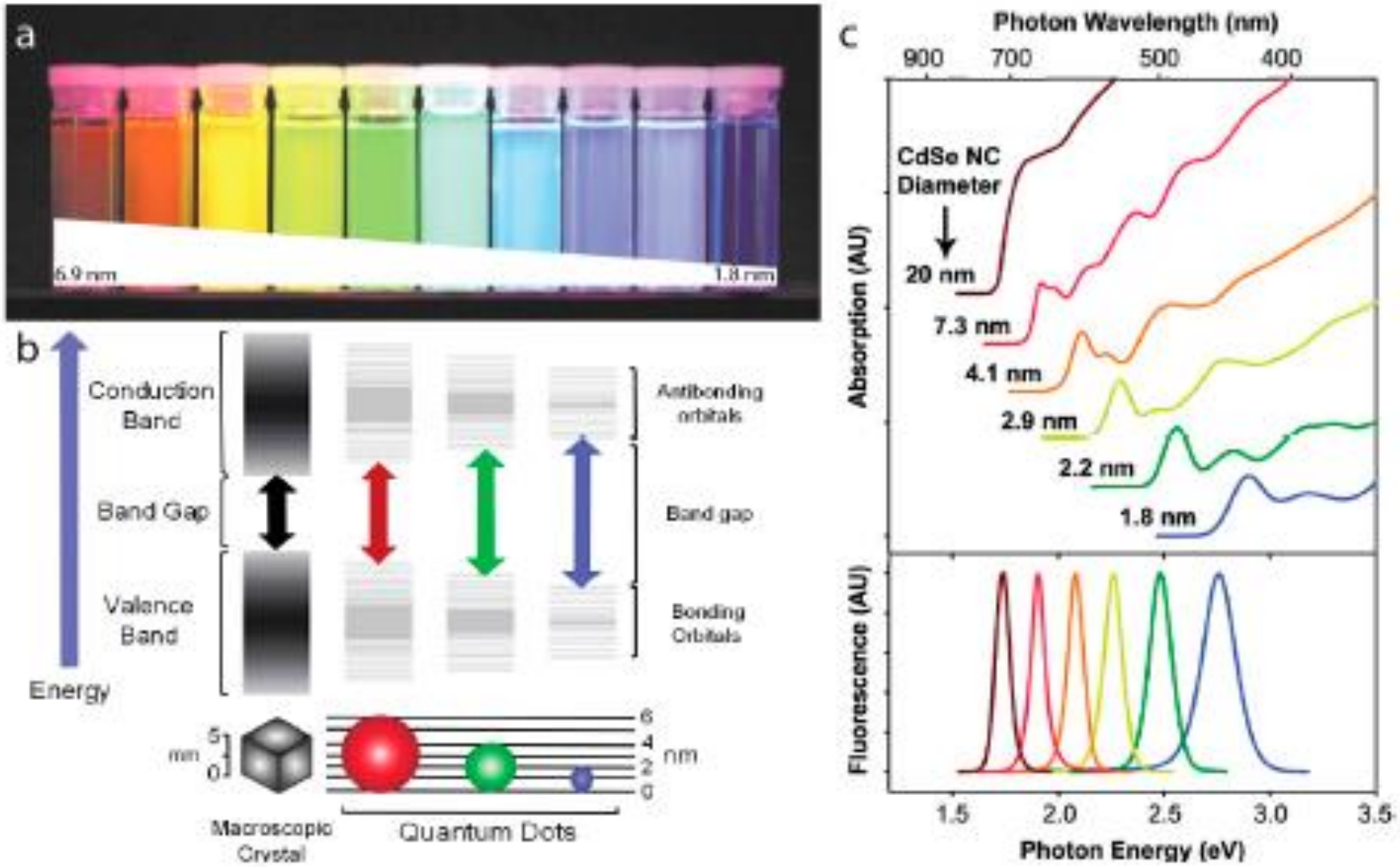
Modeling FRET with Multiple Acceptors





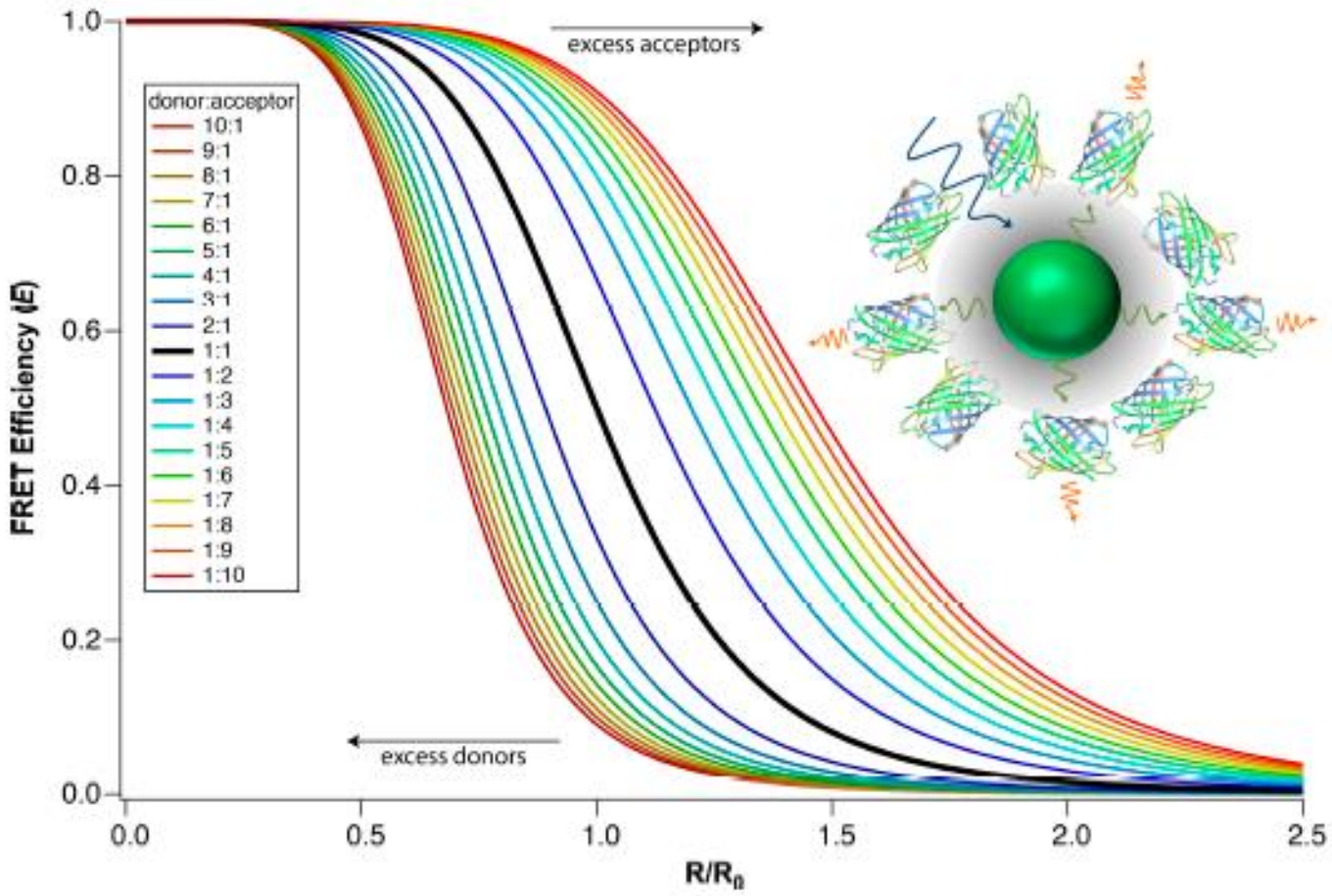
Mueller, *Frontiers in Plant Sc*., 2013, 4 (413) 1-20

Nanoparticle Based FRET Sensing



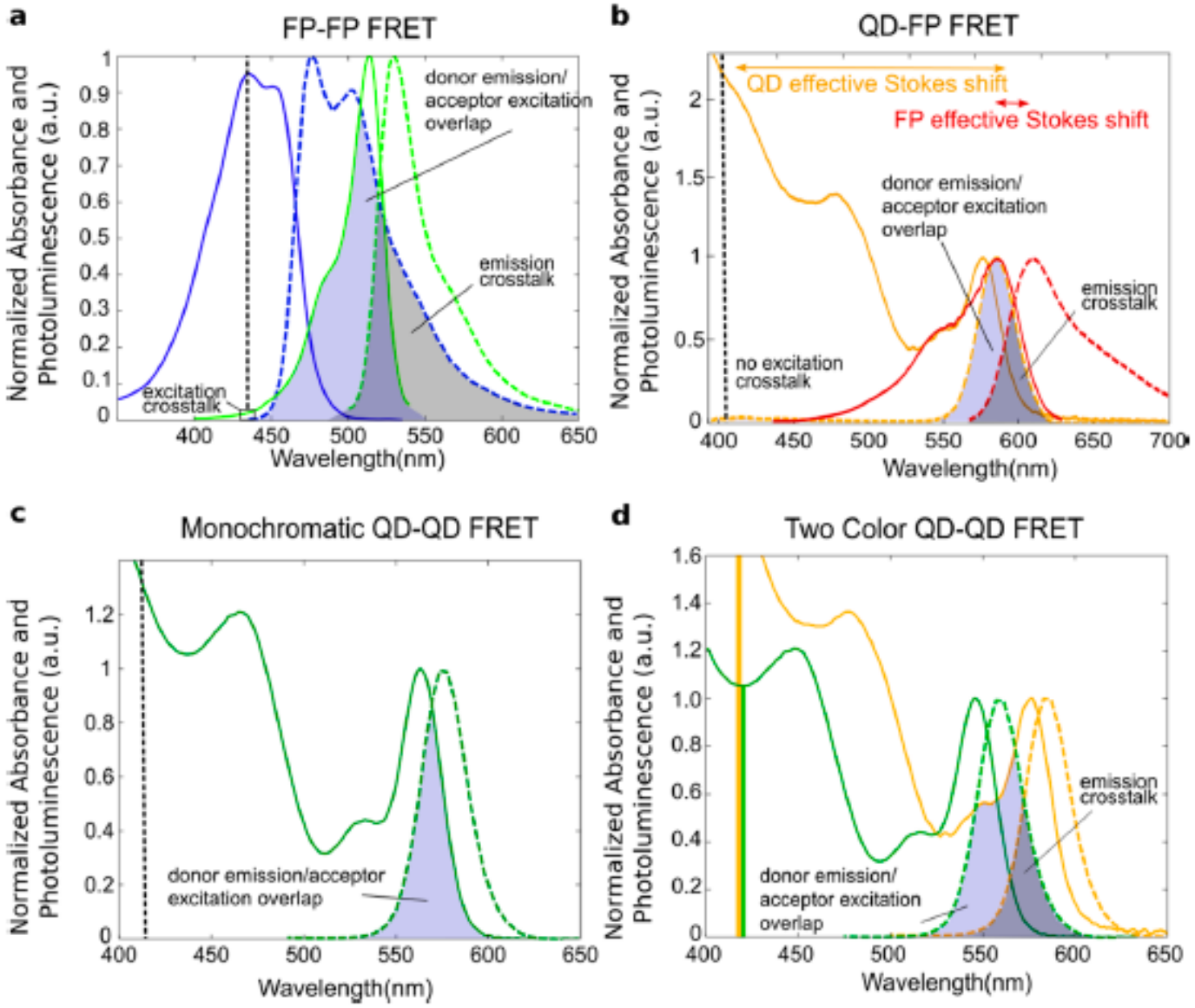
Chou, *Sensors* **2015**, *15*, 13288-13325

Predicting FRET Sensor Range



Chou, *Sensors* **2015**, *15*, 13288-13325

Energy Transfer Modalities



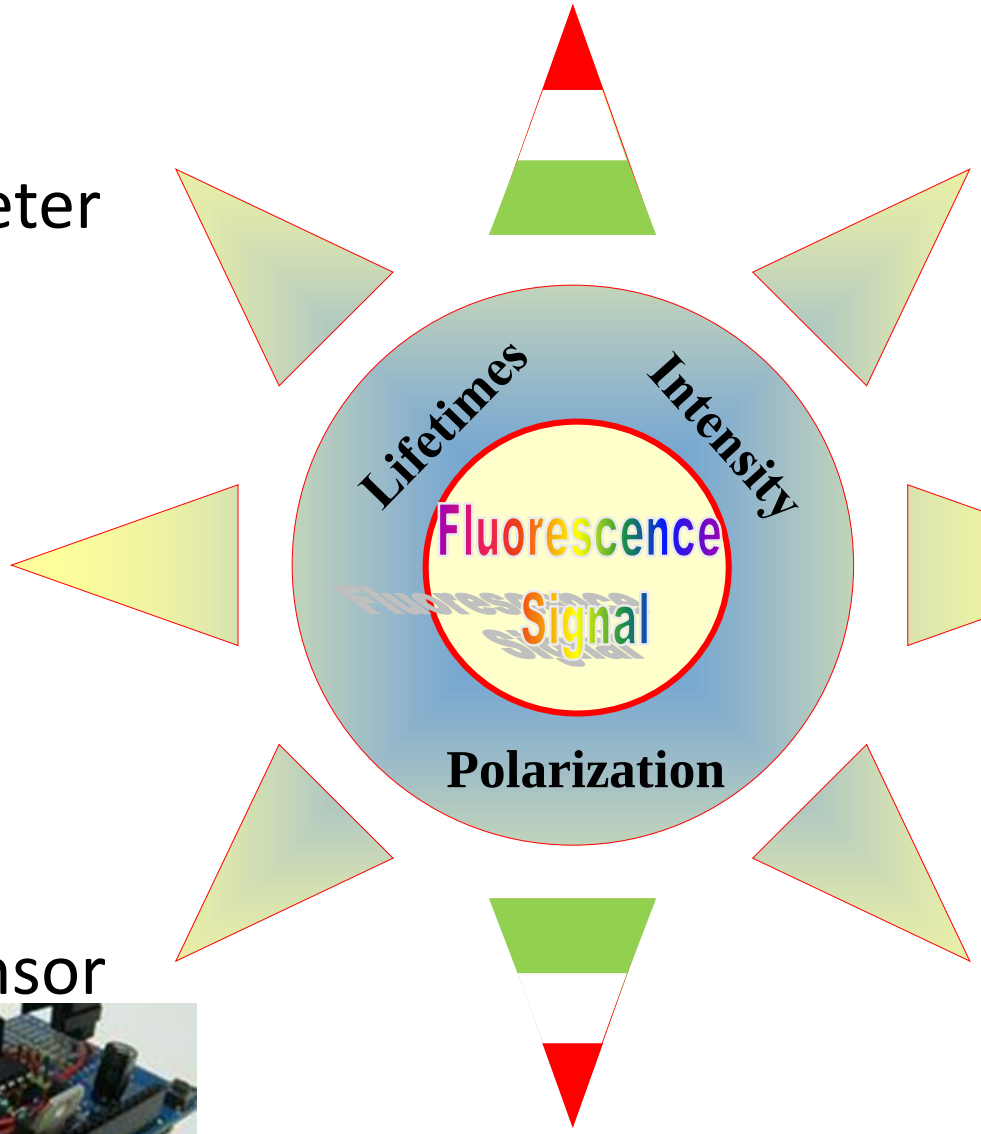
Chou, *Sensors* **2015**, *15*, 13288-13325

Example: Quantum Dot – Fluorescent Protein FRET

FRET Pair	Donor Molar Extinction Coefficient (ε_D; $M^{-1}\cdot cm^{-1}$)	Acceptor Molar Extinction Coefficient (ε_A; $M^{-1}\cdot cm^{-1}$)	Donor Quantum Yield (Φ_D)	Overlap Integral (J; $M^{-1}\cdot cm^{-1}\cdot nm^4$)	Förster Distance (R_0; nm)
FP-FP	32,500 [a] (ECFP)	83,400 (EYFP)	0.40	1.99×10^{15}	4.53
QD-FP	190,860 (λ_{1S}); [b] 389,700 (λ_e) [c]	72,000 (mCherry)	0.60 [d]	6.20×10^{15}	5.86
QD-QD (Homo-FRET)	142,220 (λ_{1S}); 208,800 (λ_e)	142,220 (λ_{1S}); 208,800 (λ_e)	0.60	8.52×10^{15}	6.18
QD-QD (Hetero-FRET)	102,370 (λ_{1S}); 116,200 (λ_e)	190,860 (λ_{1S}); 387,900 (λ_e);	0.60	1.29×10^{16}	6.63

Chou, *Sensors* **2015**, *15*, 13288-13325

FRET Photophysical Parameters

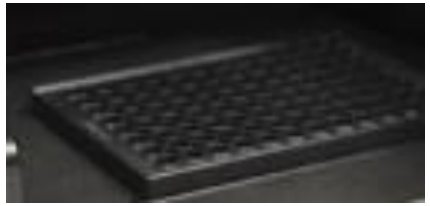


Flow cytometer

Microscope

Microfluidic device

Plate reader



HTS

Biosensor

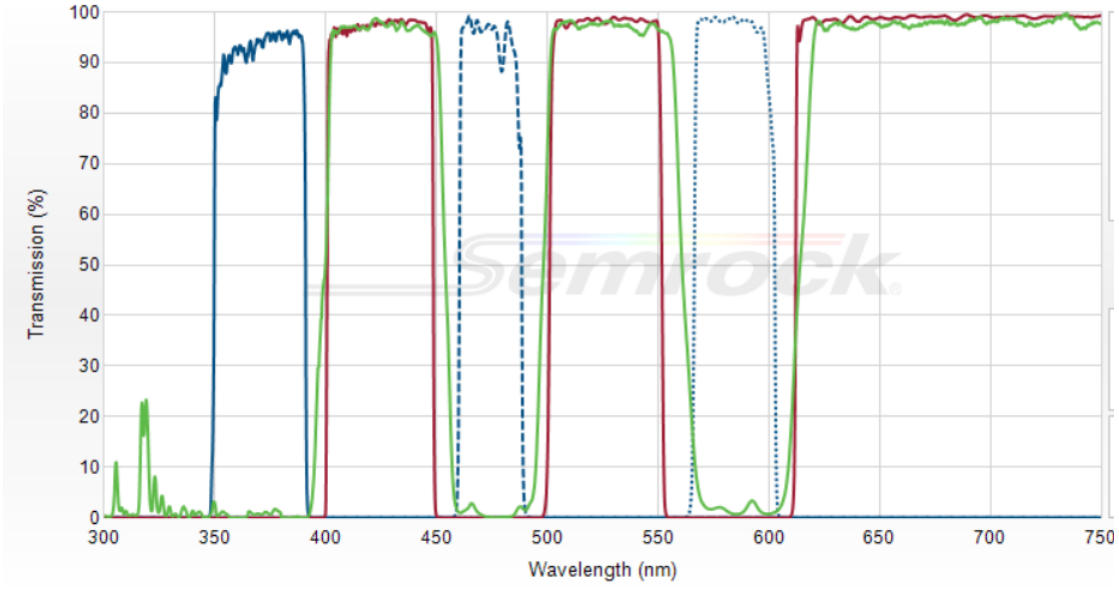
Spectrofluorimeter

FRET Measurement Protocols

Matching FRET protocol strengths and weaknesses with sample characteristics

Static differences: Acceptor photobleaching Donor photobleaching	Fixed samples
Dynamic information: Ratio imaging Sensitized emission	Dynamics, in vivo samples
Fluorescence lifetimes collection	

Optical Filter Choices & Scatter from Water Buffer



Exc1 Exc2 Exc3

BFP GFP HcRed

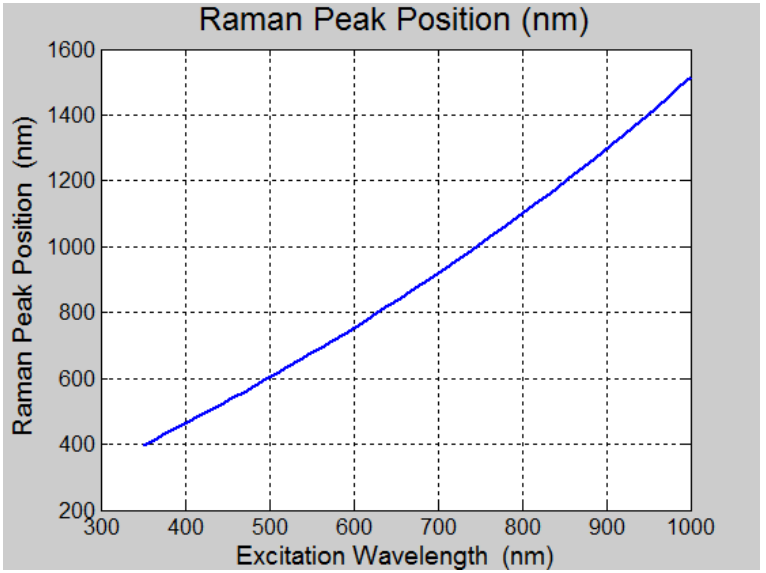
emitter interference filter

dicroic beamsplitter

Light Scattering

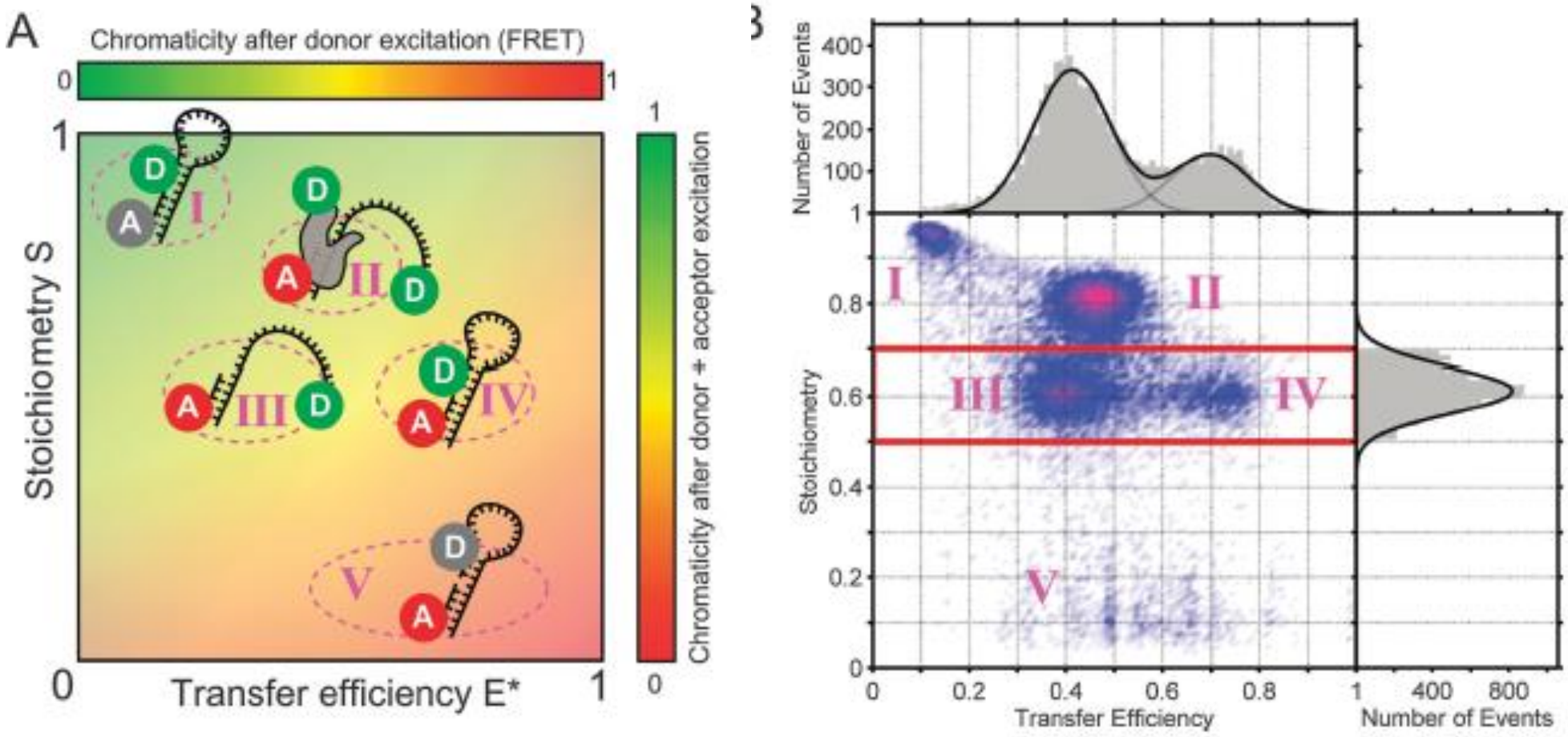
Rayleigh (Elastic) scatter

Raman (Inelastic) scatter



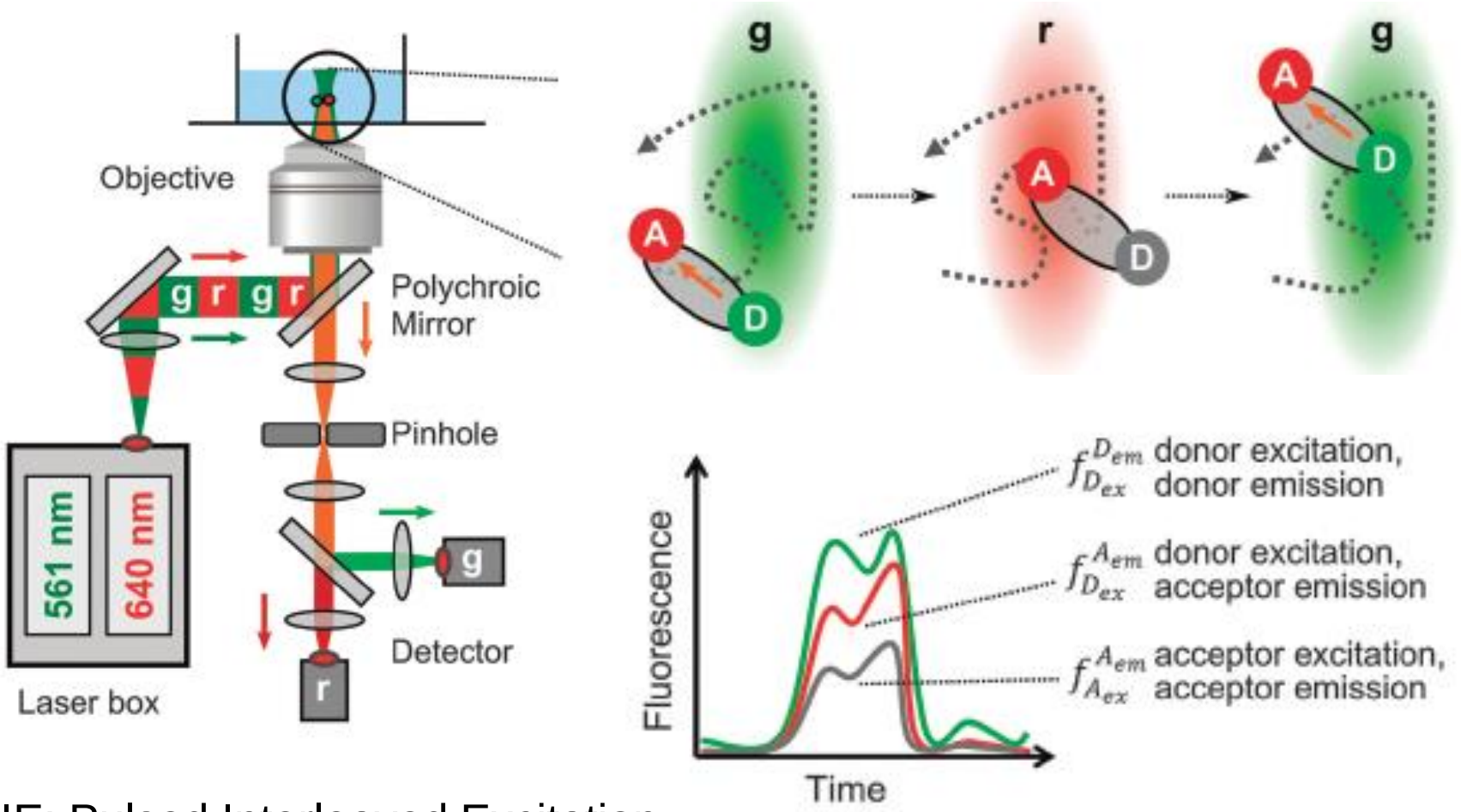
https://www.semrock.com/SetDetails.aspx?id=2716

FRET Biochemical & Photophysical Caveats



Hohlbein, *Chem. Soc. Rev.*, 2014, 43, 1156-1171

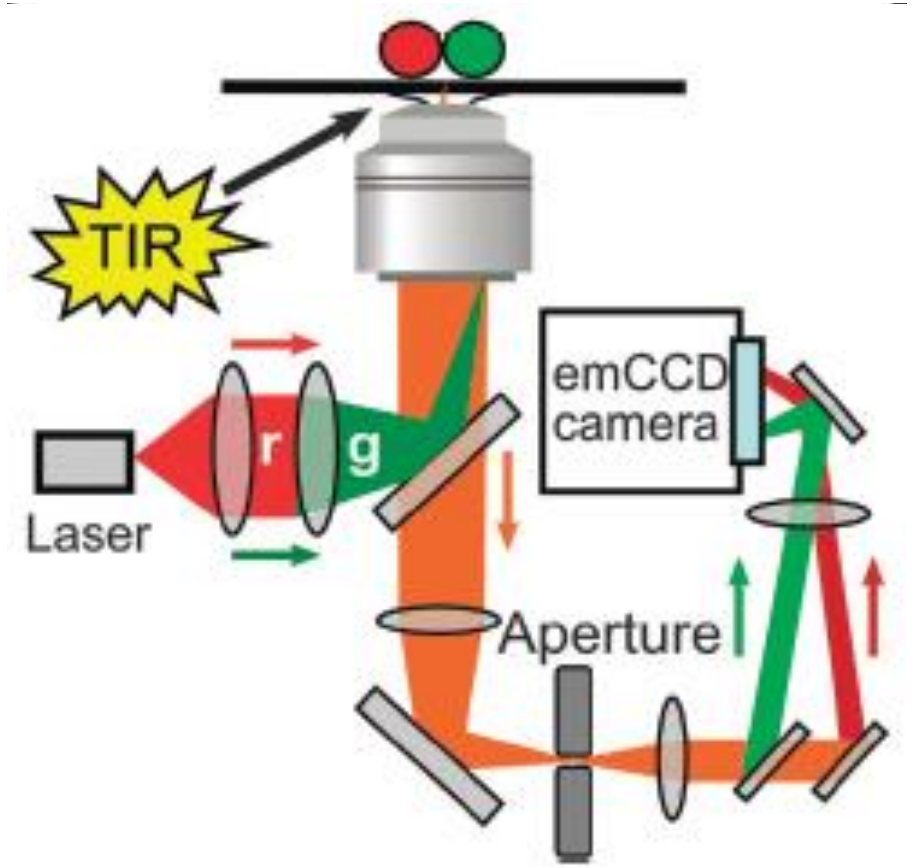
Single Molecule FRET, smFRET



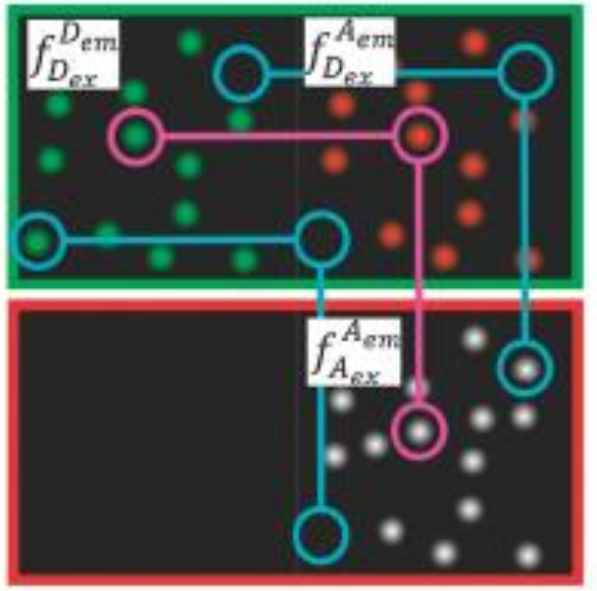
PIE: Pulsed Interleaved Excitation
ALEX: Alternating Laser Excitation

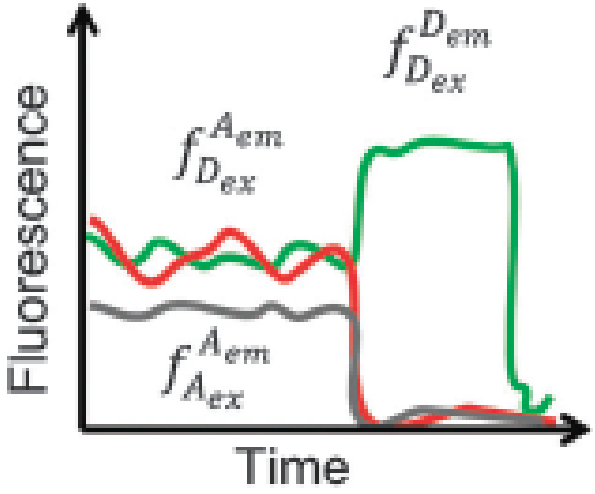
Hohlbein, *Chem. Soc. Rev.*, 2014, 43, 1156-1171

Single Molecule FRET, smFRET



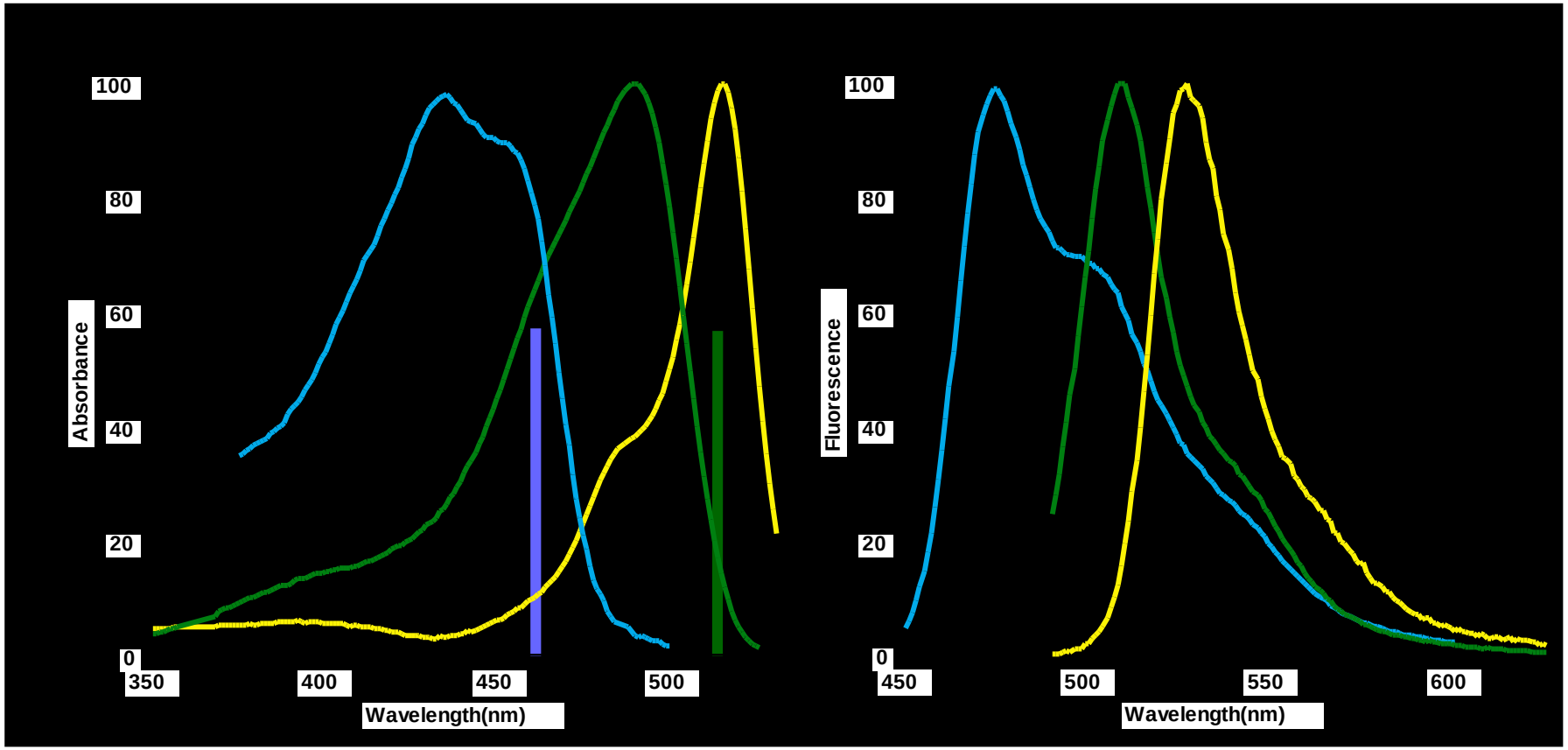
TIR: Total Internal Reflection setup





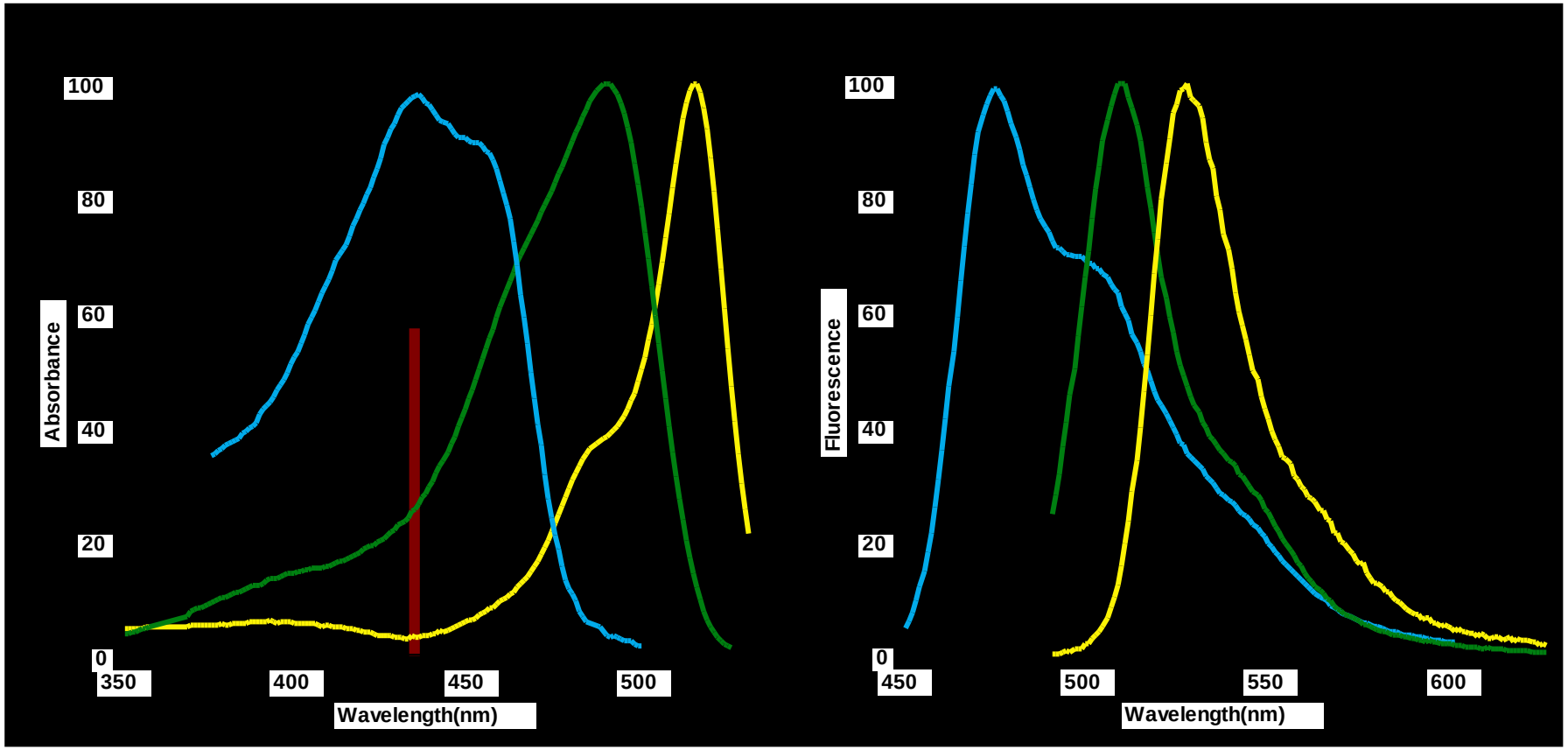
Hohlbein, *Chem. Soc. Rev.*, 2014, 43, 1156-1171

FRET with GFP and One-Photon Confocal Microscopy



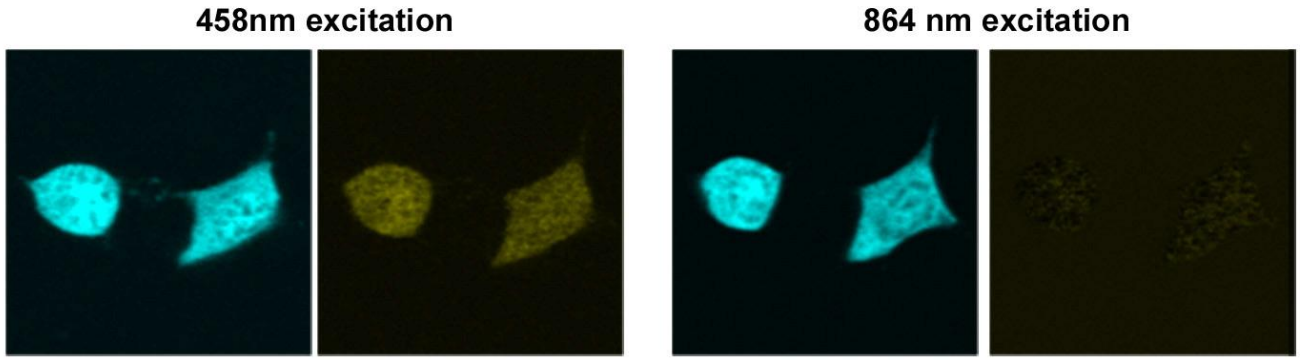
Argon laser line excites **CFP** at 70% efficiency, and **YFP** at 10% efficiency **YFP** is ~5 fold brighter than **CFP**. This causes the **YFP** signal to be ~half as bright as that from the **CFP**.

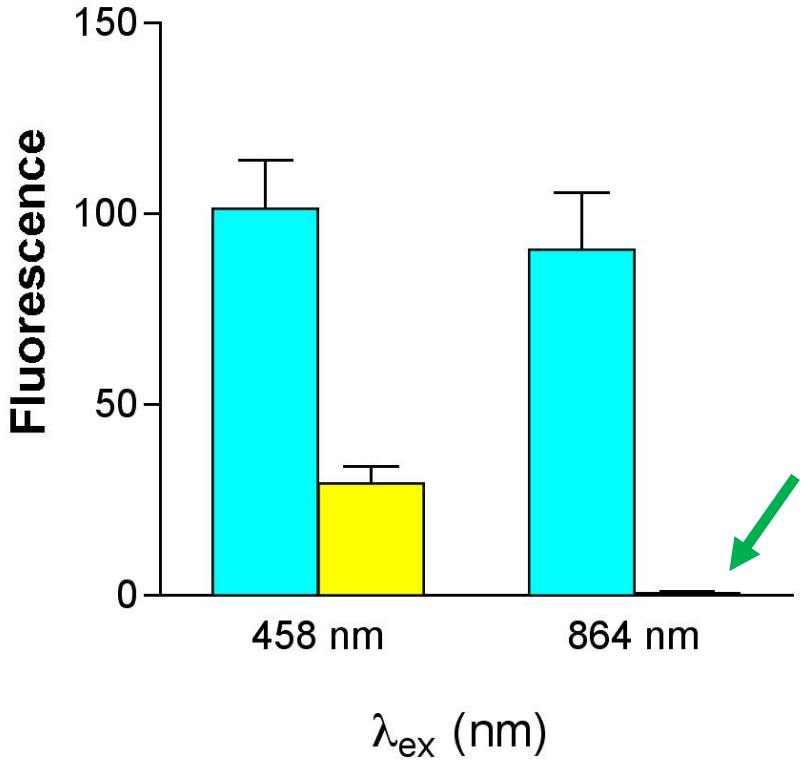
Better FRET with Two-Photon Excitation



Two-photon excitation can be tuned to optimally excite **CFP** and minimize direct absorption of **YFP**. This reduces the **YFP** signal to less than 3% of that from the **CFP**.

Better FRET with Two-Photon Excitation





Lifetime Imaging is Not Well-Suited for *CFP-YFP* FRET

CFP photophysics is complicated:

- **Two non-interacting states, that appear to be fixed at initial folding**

- **Two lifetimes that can exhibit homotransfer, thus greatly complicating data analysis and interpretation**

Optimization Challenges

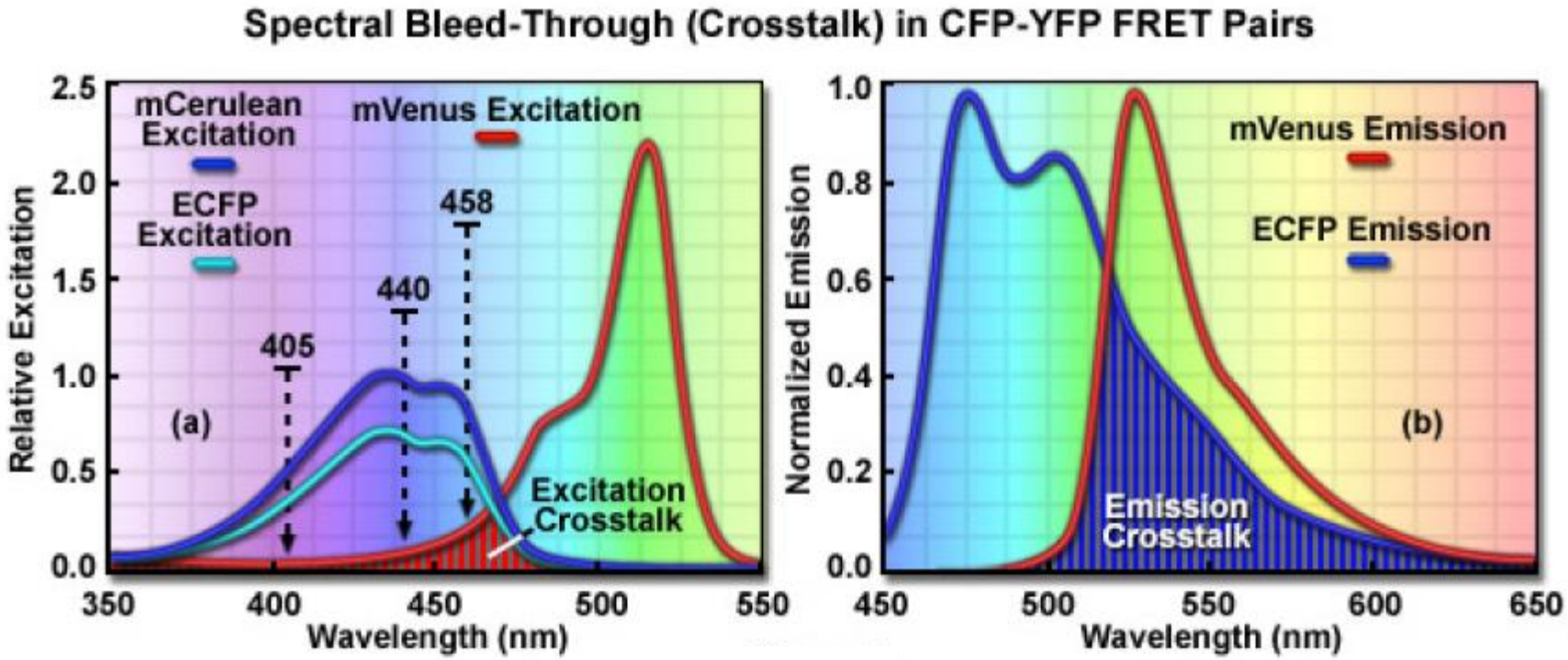
Acceptor excitation by Donor excitation
Donor emission contributes to FRET em.
Lightsource & detector gain fluctuation

Concentration <> Quantum Yield variation
Autofluorescence & Scatter contributions
Photobleaching

Linker length for free rotation, κ^2
Cell medium
Image registration & Co-localization

Setup & Sample related attention points

Spectral Bleed-Through (Crosstalk) …

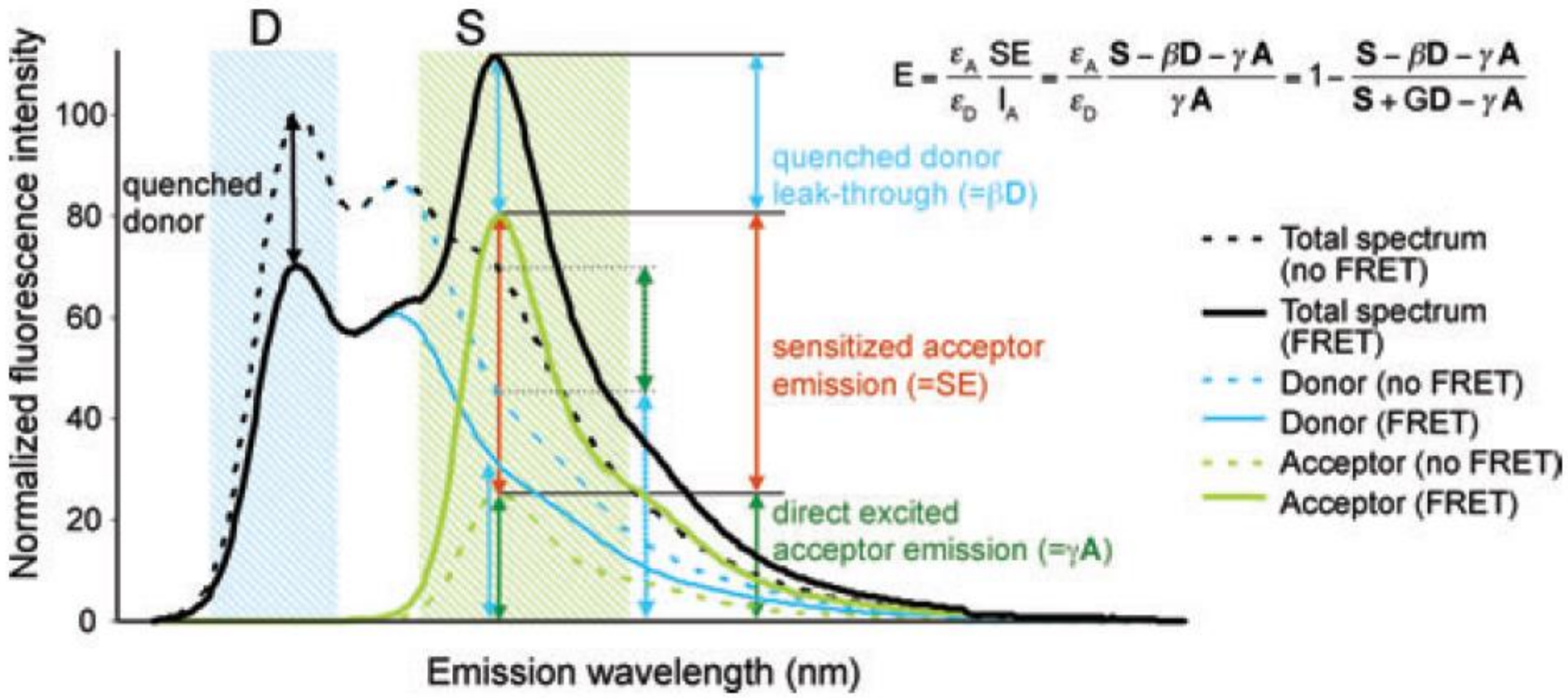


Acceptor Excitation

Donor Emission

Yildiz, PHYS250 Berkeley, 2012

Sensitized Emission, SE-FRET



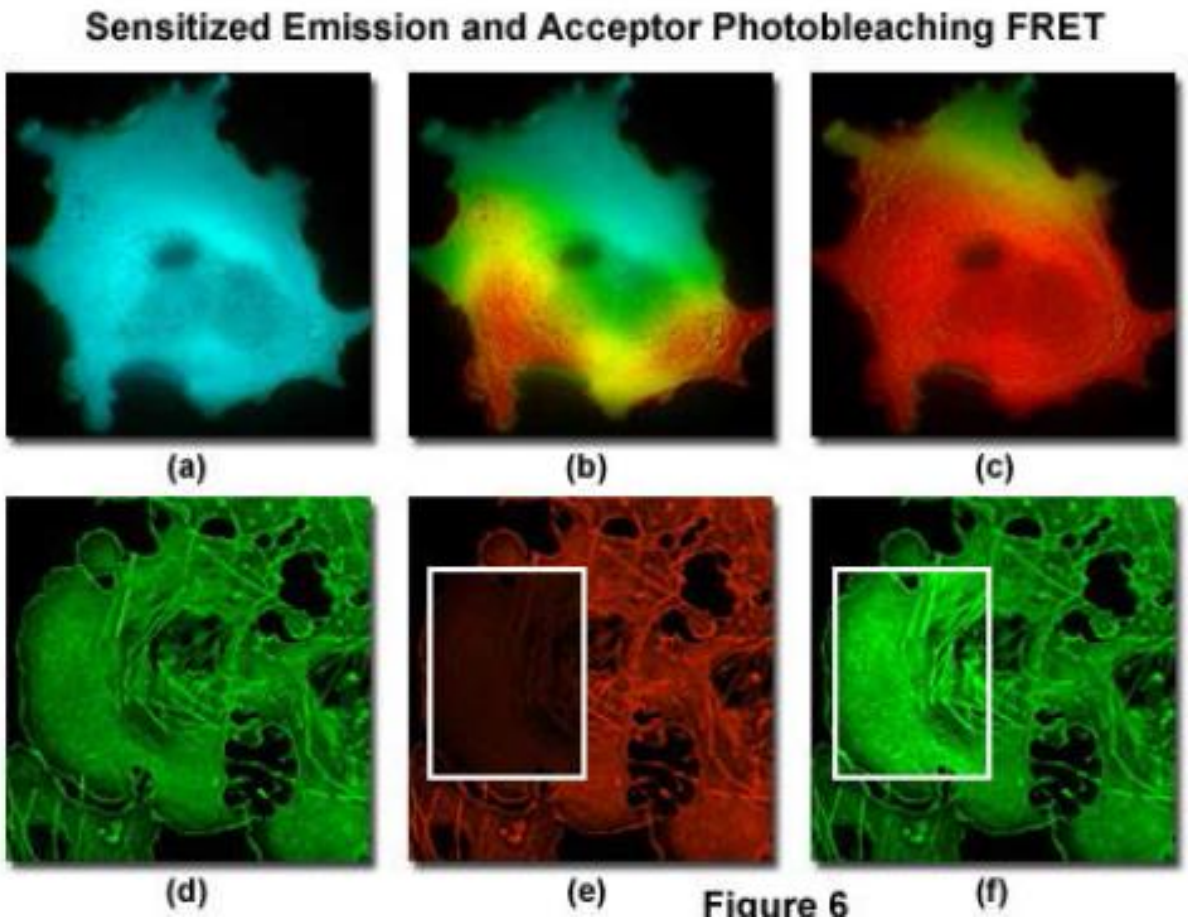
$$E = \frac{\varepsilon_A}{\varepsilon_D}\frac{SE}{I_A} = \frac{\varepsilon_A}{\varepsilon_D}\frac{S-\beta D-\gamma A}{\gamma A} = 1-\frac{S-\beta D-\gamma A}{S+GD-\gamma A}$$

Pietraszewska-Bogiel, *J.Microsc., 2011, 241(2) 111–118*

Accpb-FRET Acceptor PhotoBleaching FRET

HeLa cells. Effect of Calcium in Calmodulin binding peptide association.

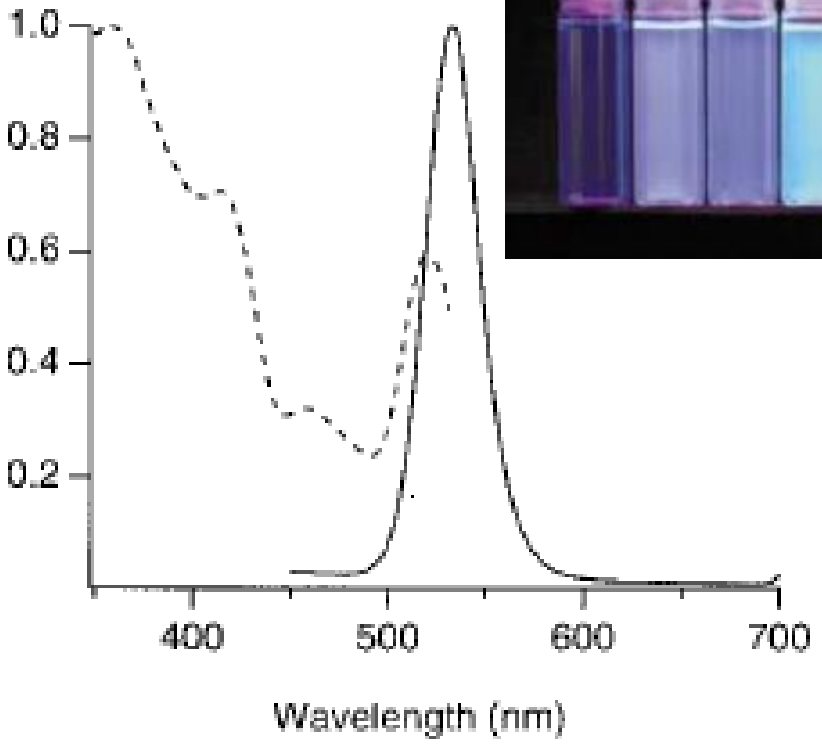
Kidney cells. Cholera toxin B-subunit and targeting the plasma membrane



Yildiz, PHYS250 Berkeley, 2012

A Solution to Spectral Bleed-Through

Use Quantum Dots as Donors



Advantages:

-Broad absorption

-Narrow Emission

-Size tunability

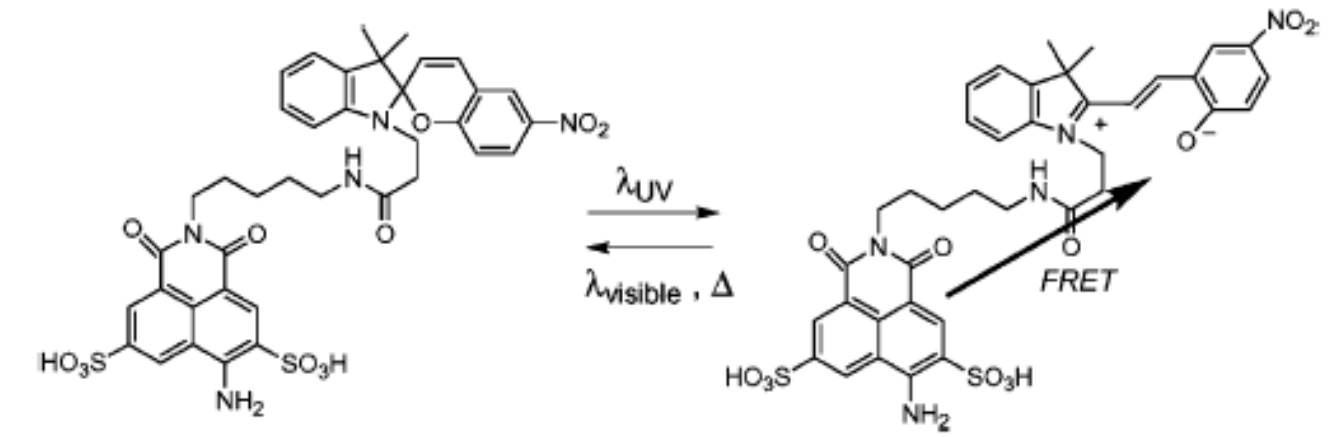
-Surface functionalisation

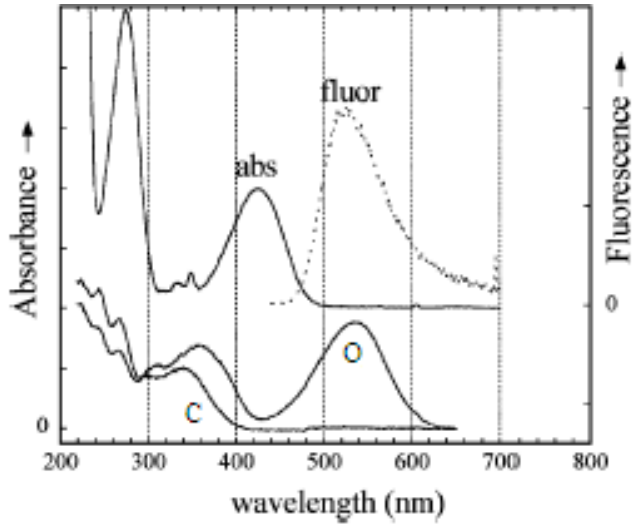
Kronemeijer, Nanoscience Symposium 2005

Maliwal, *J. Biomed. Optics* 17(1), 011006 1-8

Solutions to Spectral Bleed-Through

Photochromic FRET

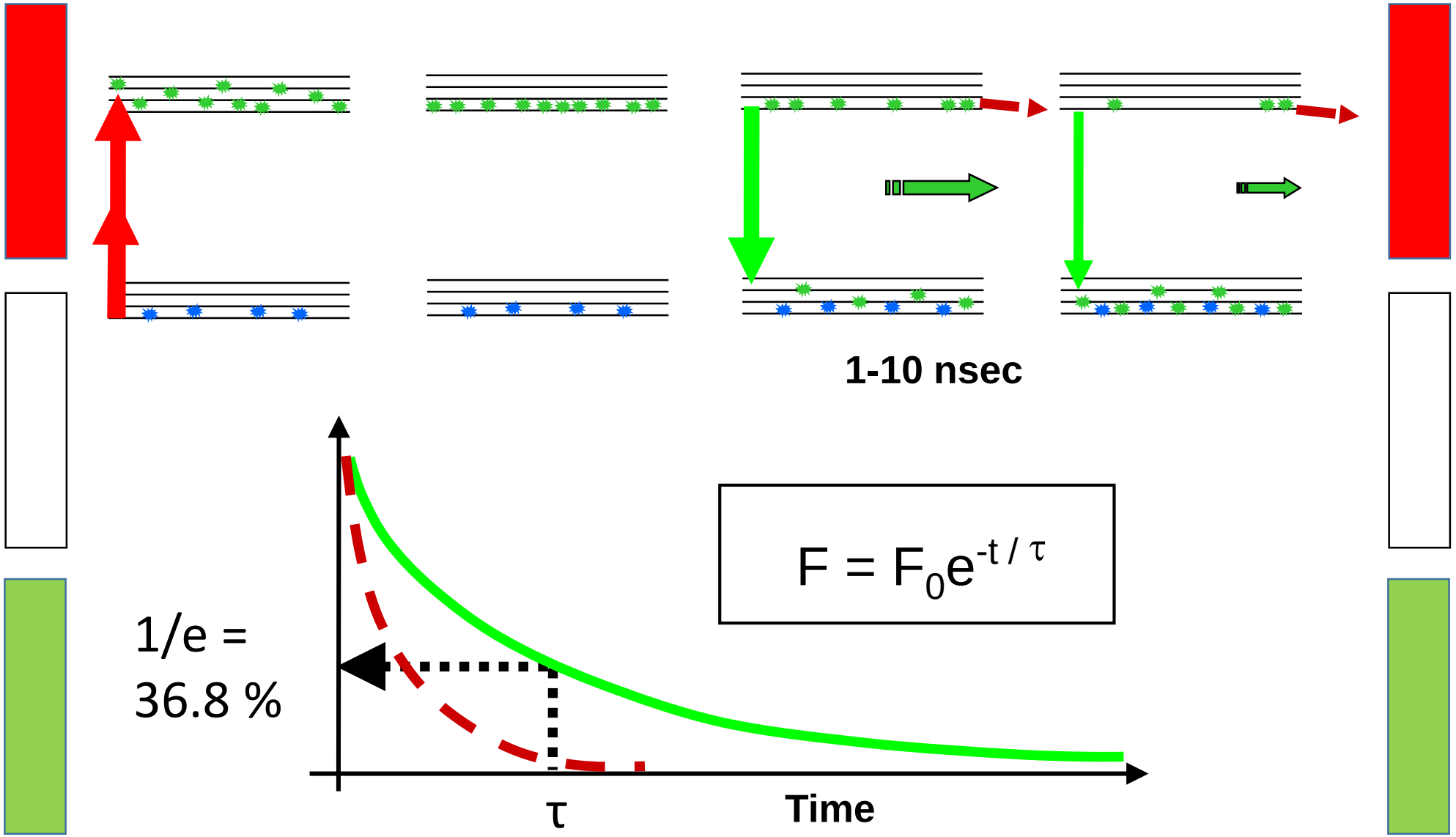




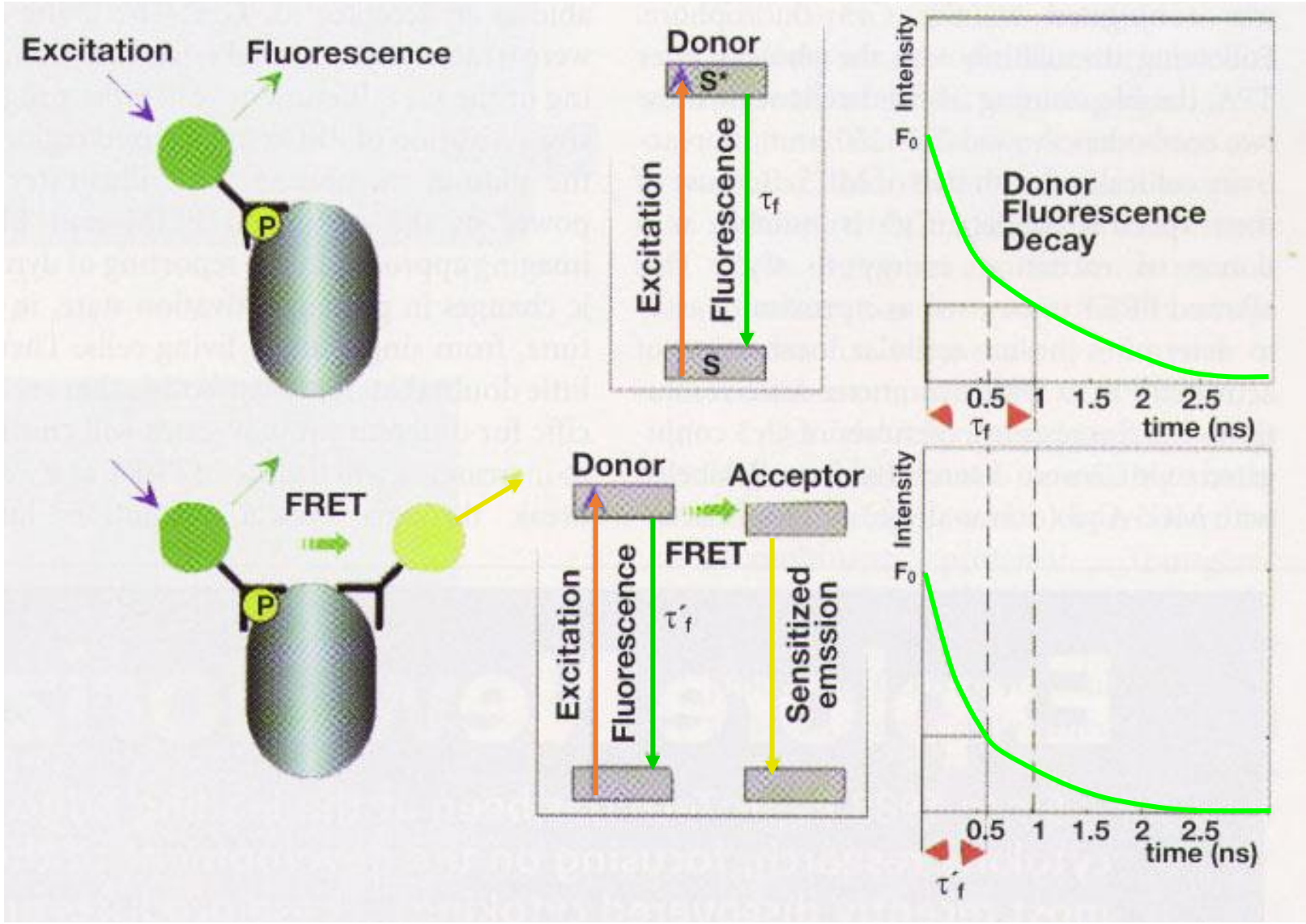
"Switching on and off FRET mechanism to determine FRET contribution to total emission"

Kronemeijer, *Nanoscience Symposium* 2005

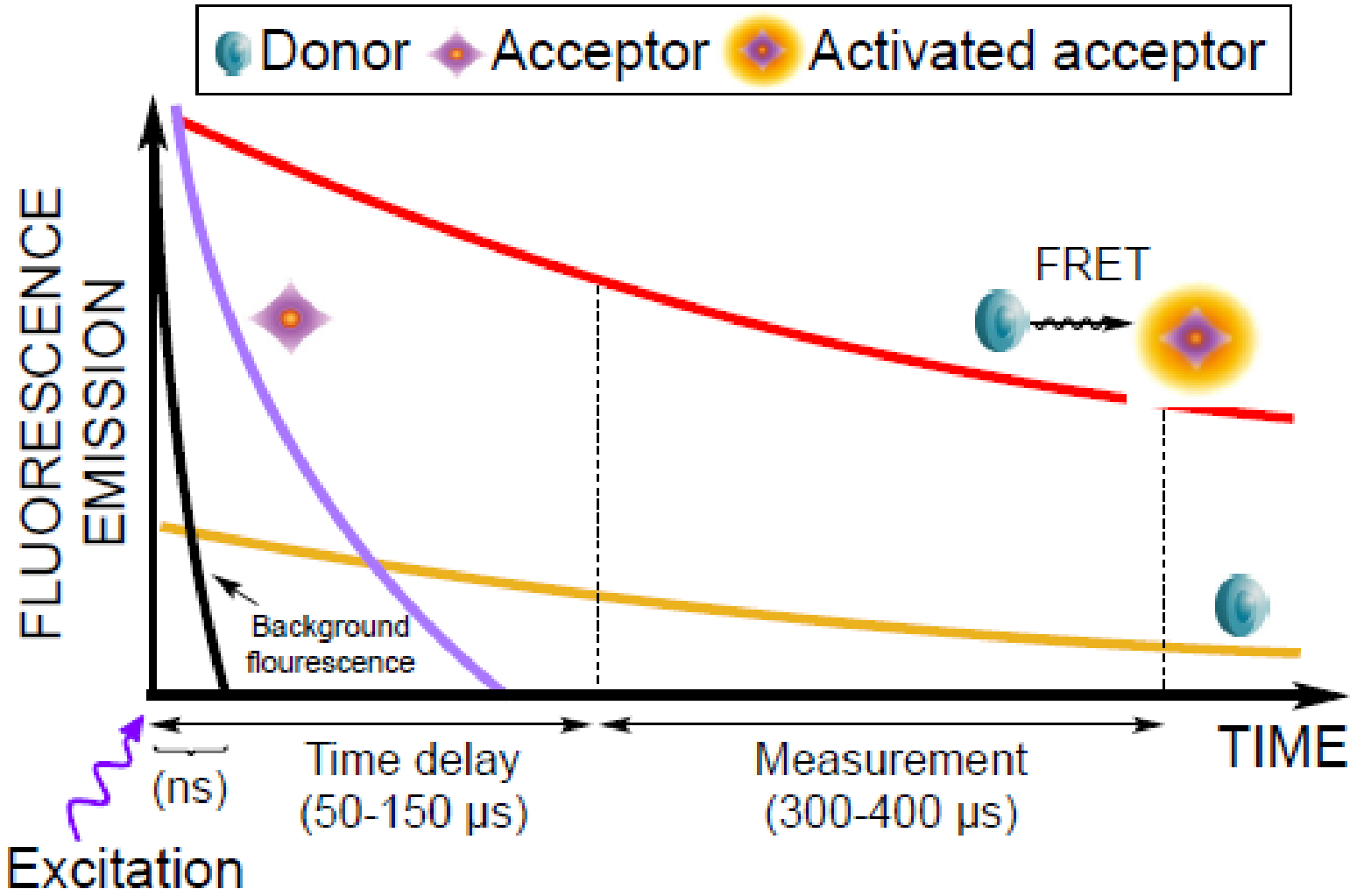
Fluorescence is an Exponential Decay Process



FRET Assayed by Fluorescence Lifetime



HTS Related FRET for Ratiometric I_A/I_D



Initially Eu – Allophycocyanin (105 kDa)
later improved: Tb - Fluorescein FRET pair

Noerskov-Lauritsen, *Int. J. Mol. Sci.* **2014**, *15*, 2554-2572

How to measure Energy Transfer ?

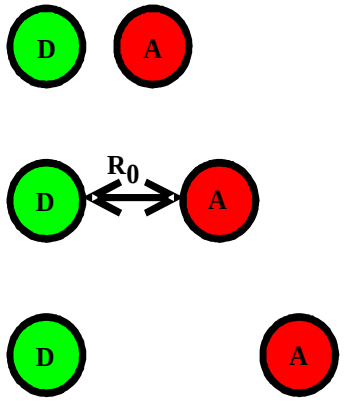
Donor intensity decrease, donor lifetime decrease, acceptor increase.

E.T. by decreases in donor emission.

Need to compare two samples, d-only, and D-A.

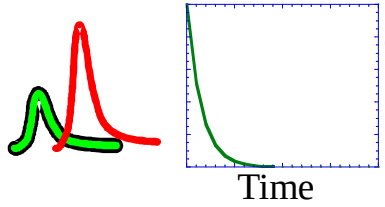
$$E = \left(1 - \frac{I_{D_A}}{I_D}\right) = 1 - \frac{\tau_{D_A}}{\tau_D}$$

Where I_{D_A}, τ_{D_A} are the donor's intensity, and excited state lifetime in the presence of acceptor, and I_D, τ_D are the same but without the acceptor.

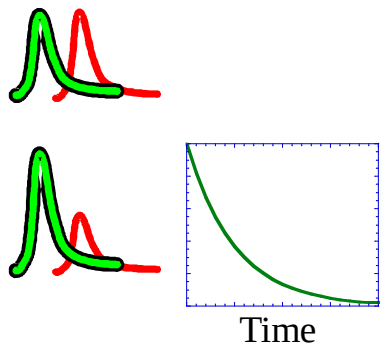


E.T. by increase in acceptor fluorescence and compare it to residual donor emission.

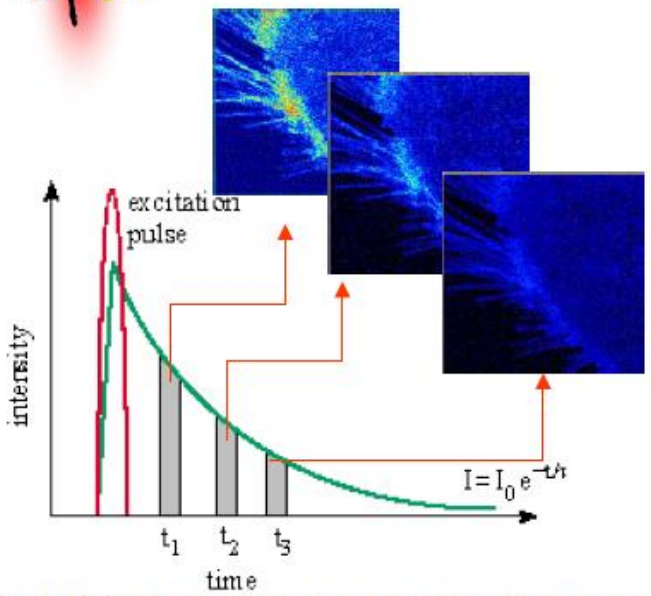
Need to compare one sample at two λ and also measure their quantum yields.



$$E = \frac{\frac{I_{A_D}}{q_A}}{\frac{I_{D_A}}{q_D} + \frac{I_{A_D}}{q_A}}$$

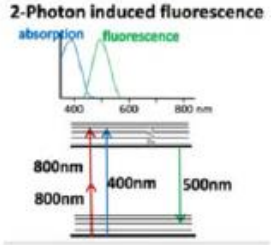


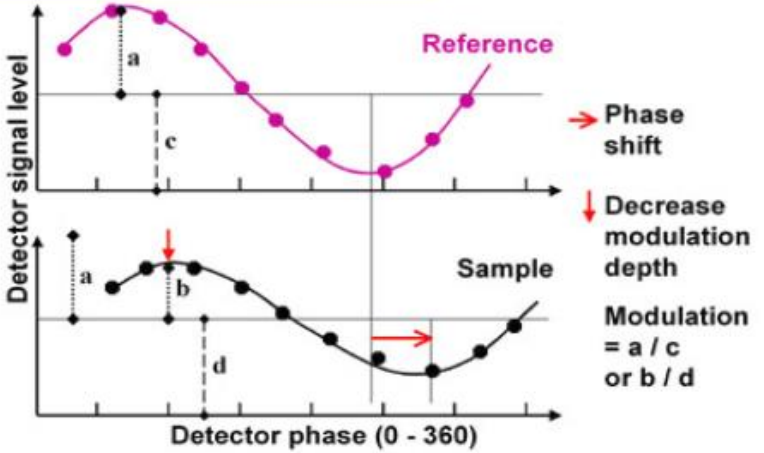
Fluorescence Life Time Measured with the Time Domain and Frequency Domain



A sample is flashed many times by a short duration laser source

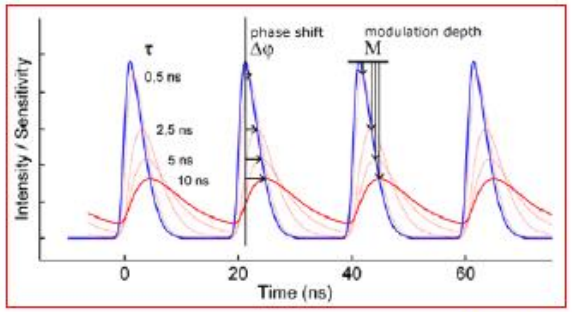
The histogram of the time intervals between the excitation flash, and 1st emitted photon is measured





A sample is excited by a modulated light source

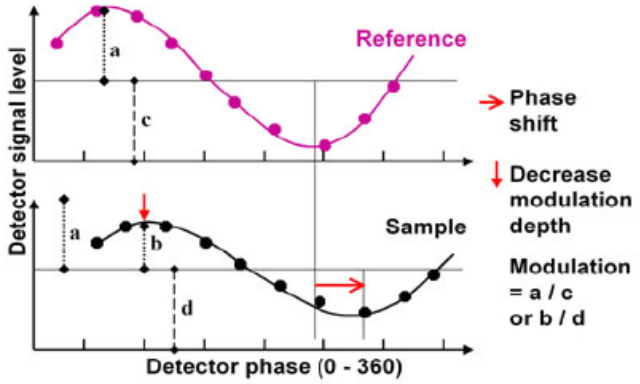
The fluorescence emission has the same frequency but is modulated and phase-shifted from the excitation source

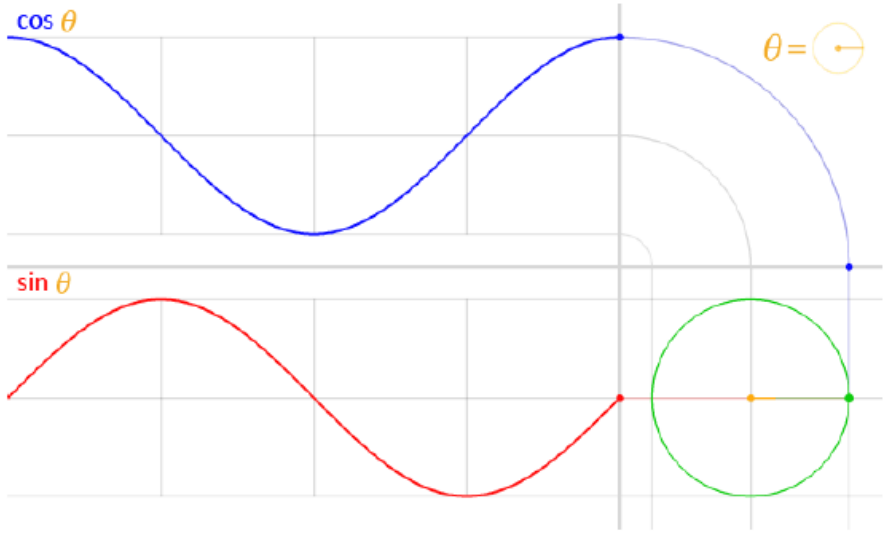


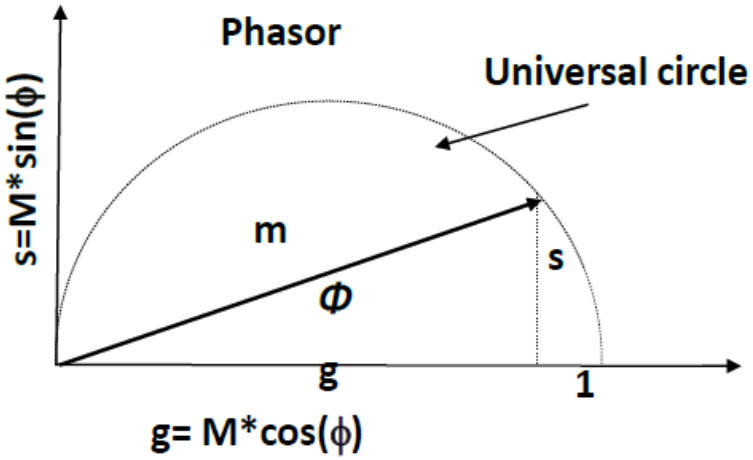
(Michelle Digman, LFD UCI)

Phasor Plot Construction

The Phasor Plot (Universal circle)







How to calculate the components g and s of a phasor from the time decay?

Frequency-domain components of a phasor. m and ϕ is what is measured

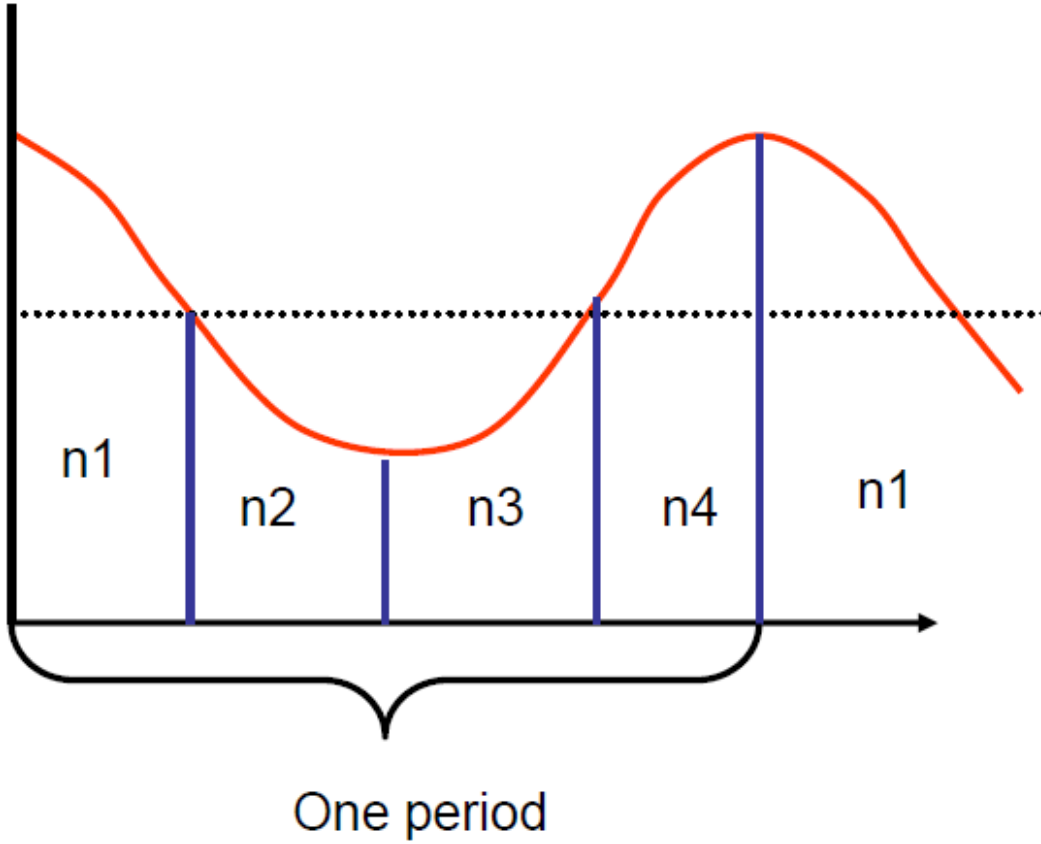
$$g_i(\omega) = m_i \cos(\varphi_i)$$
$$s_i(\omega) = m_i \sin(\varphi_i)$$

Time-domain components of a phasor. I(t) is what is measured

$$g_i(\omega) = \int_0^\infty I(t)\cos(\omega t)dt \,/\, \int_0^\infty I(t)dt$$
$$s_i(\omega) = \int_0^\infty I(t)\sin(\omega t)dt \,/\, \int_0^\infty I(t)dt$$

Note that I(t) is not resolved in components!!

Frequency Domain
Calculation of Phase Shift and Demodulation



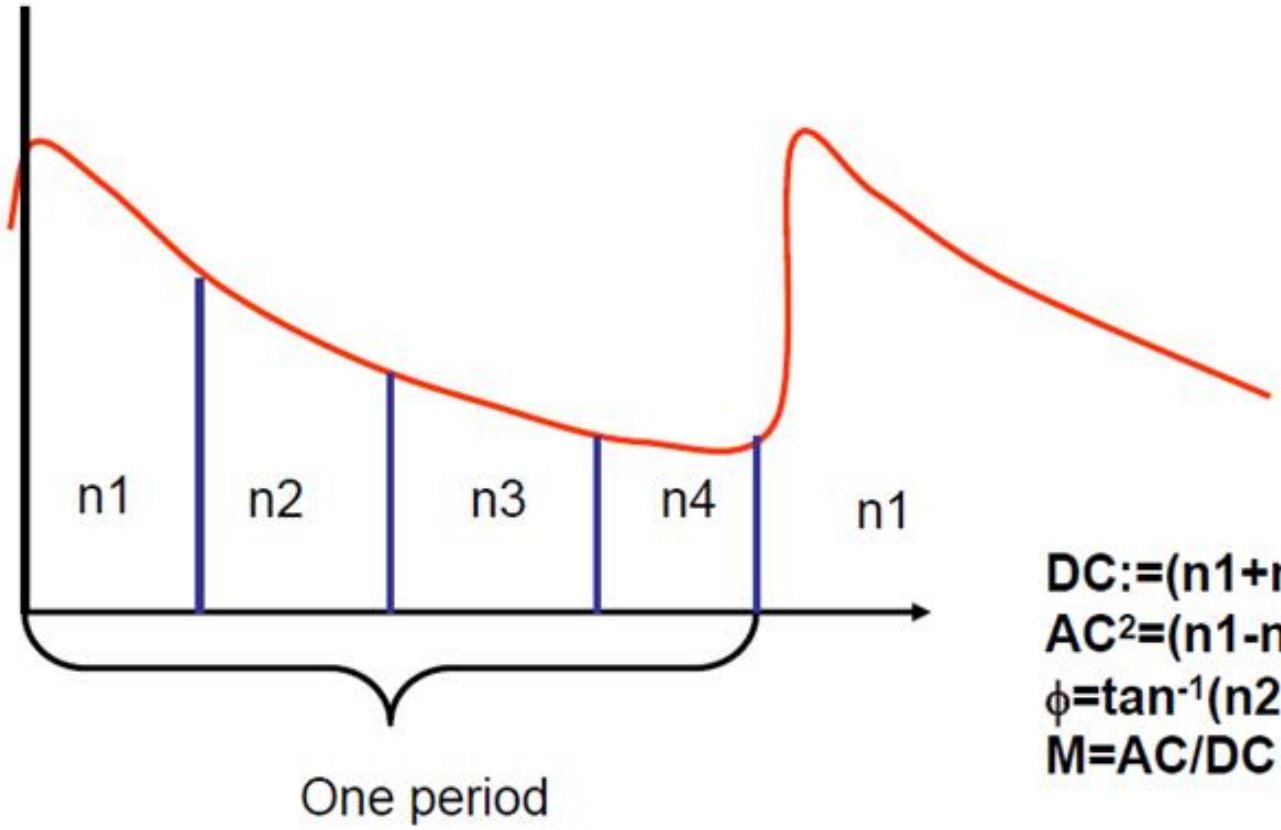
$DC:=(n1+n2+n3+n4)/4$

$AC^2=(n1-n3)^2+(n2-n4)^2$

$\phi=\tan^{-1}(n2-n4)/(n1-n3)$

$M=AC/DC$

Time Domain
Calculation of Phase Shift and Demodulation



$DC:=(n1+n2+n3+n4)/4$

$AC^2=(n1-n3)^2+(n2-n4)^2$

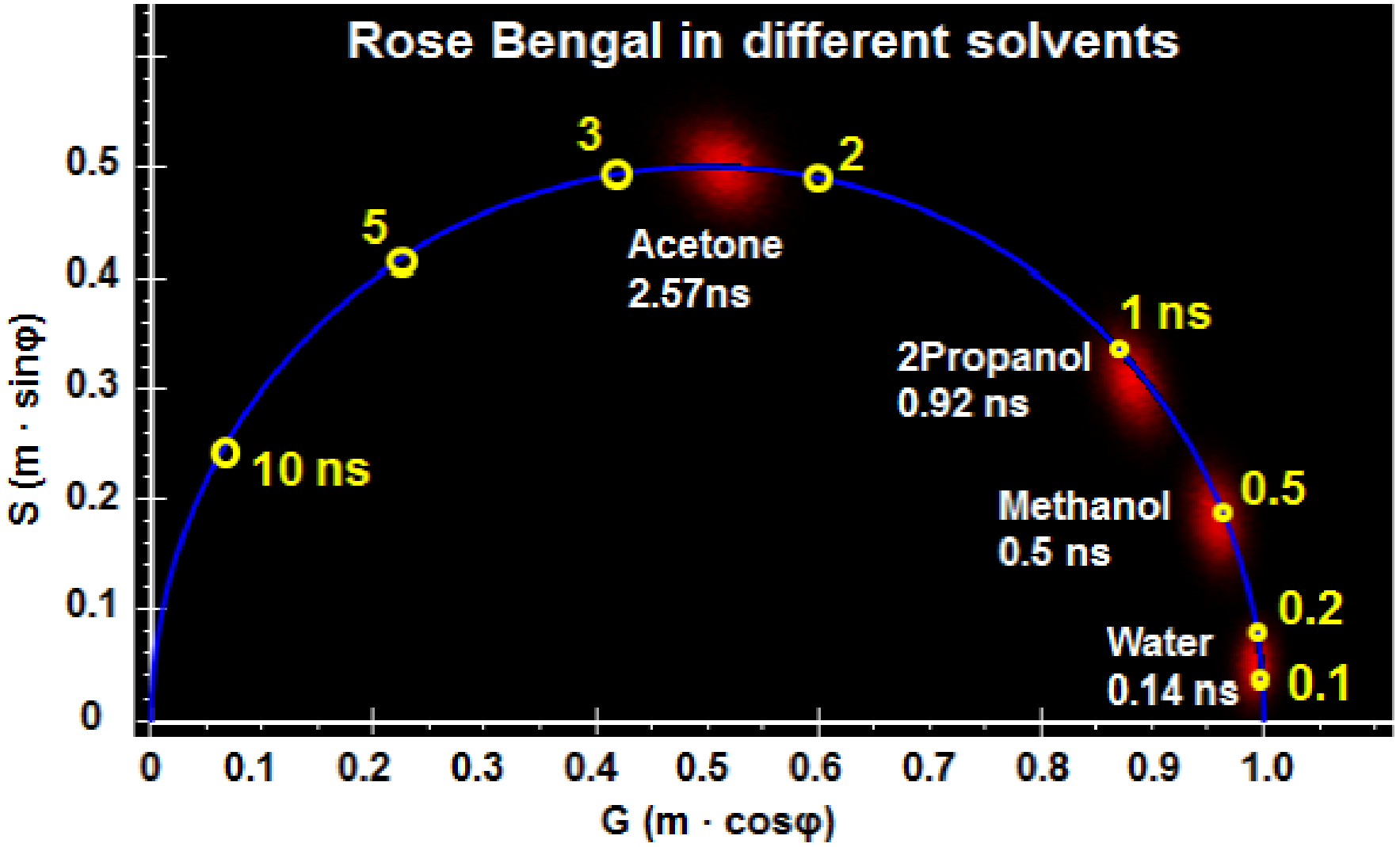
$\phi=\tan^{-1}(n2-n4)/(n1-n3)$

$M=AC/DC$

We use identical formulas!!!

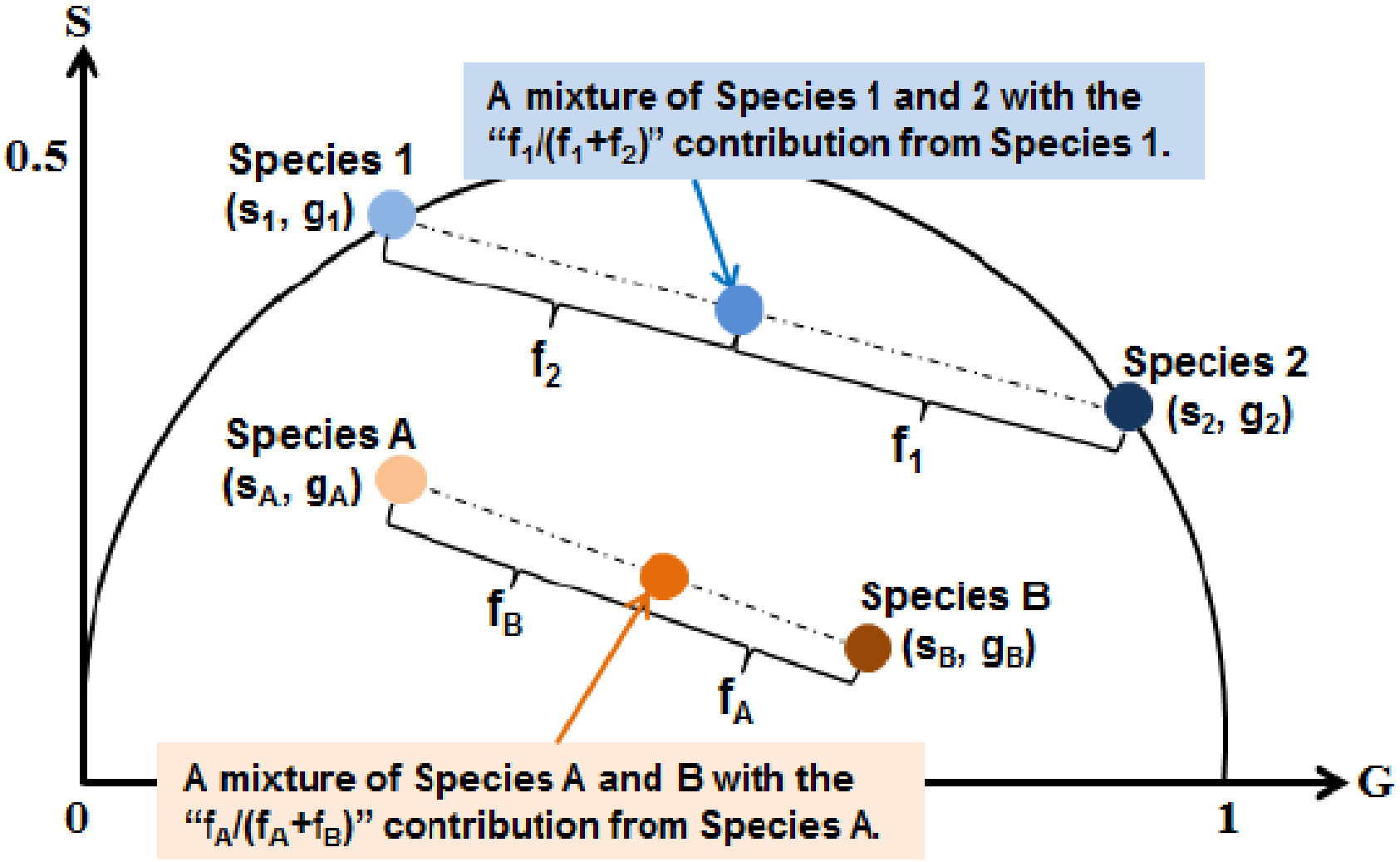
Stefl, *Anal Biochem*., 2011, 410 (1) 62–69

Single Fluorescence Intensity Decay



Sun, *ISS Applic. Note*, 2014, 1-9

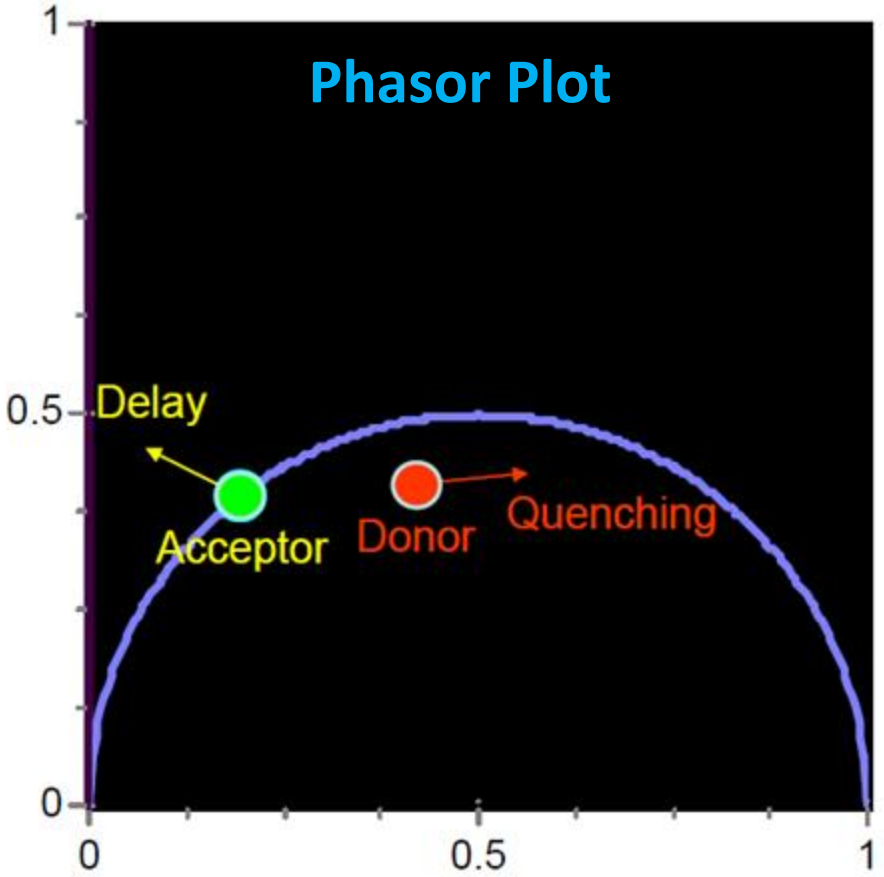
A Mixture of Two Chromophores



Sun, *ISS Applic. Note*, 2014, 1-9

How to Identify Processes ?

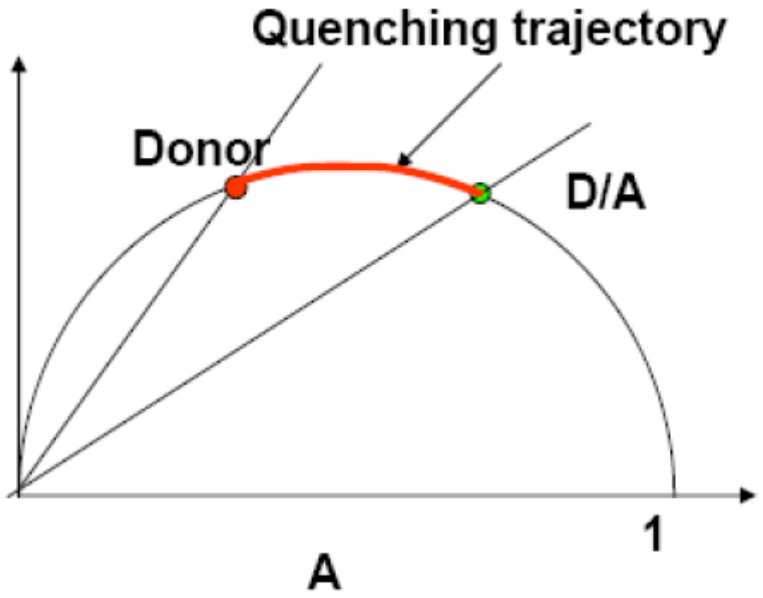
Prof. Dr. I

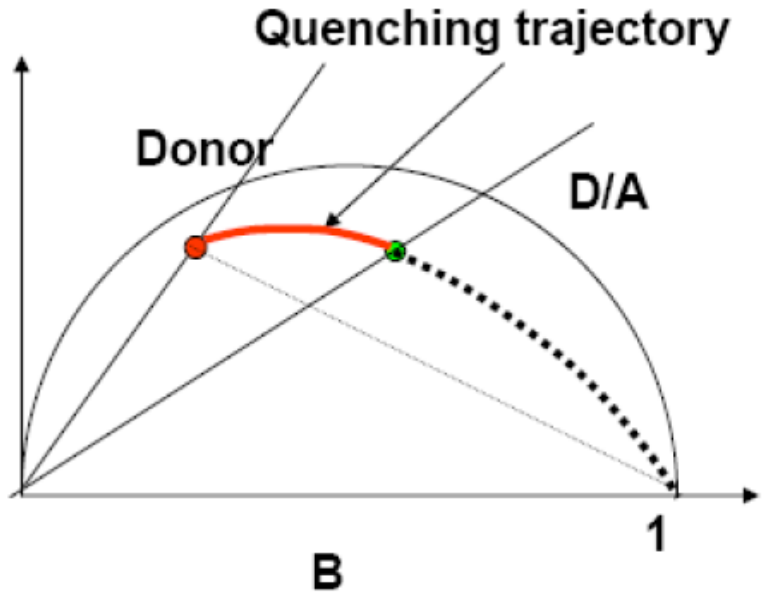


Delay of the excitation of the acceptor due to FRET moves the acceptor phasor to the left (yellow arrow). If the delay is sufficiently long, the phasor could fall outside the semicircle. The donor phasor moves to the right (red arrow) due to quenching (shorter lifetime).

The FRET calculator

If we have a donor with a single exponential decay that is quenched by the presence of a acceptor. What should we expect?



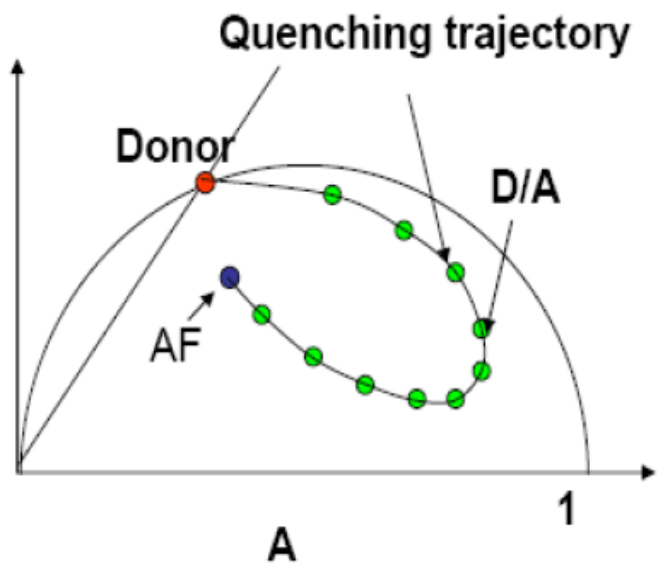


The lifetime of the donor is quenched
The FRET efficiency can be calculated by the ratio of the two lifetimes

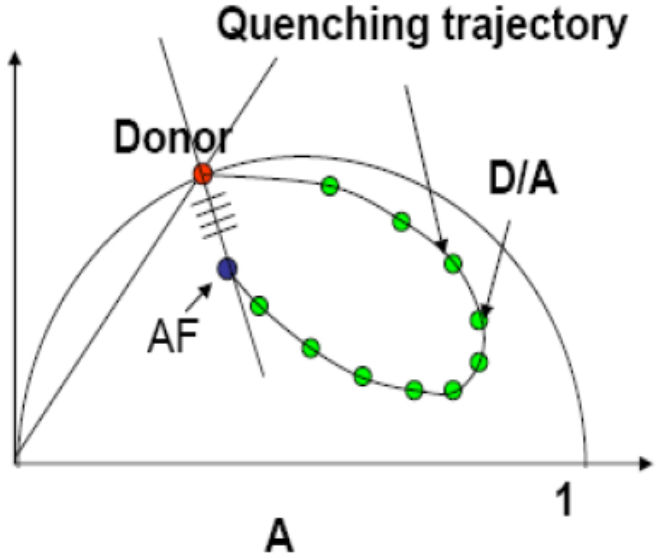
The lifetime of the donor is along a different "trajectory", Why is the trajectory an arc rath than a line to the (1,0) point?

The FRET Calculator

After all the Donor is quenched, what is left? The cell autofluorescence!!



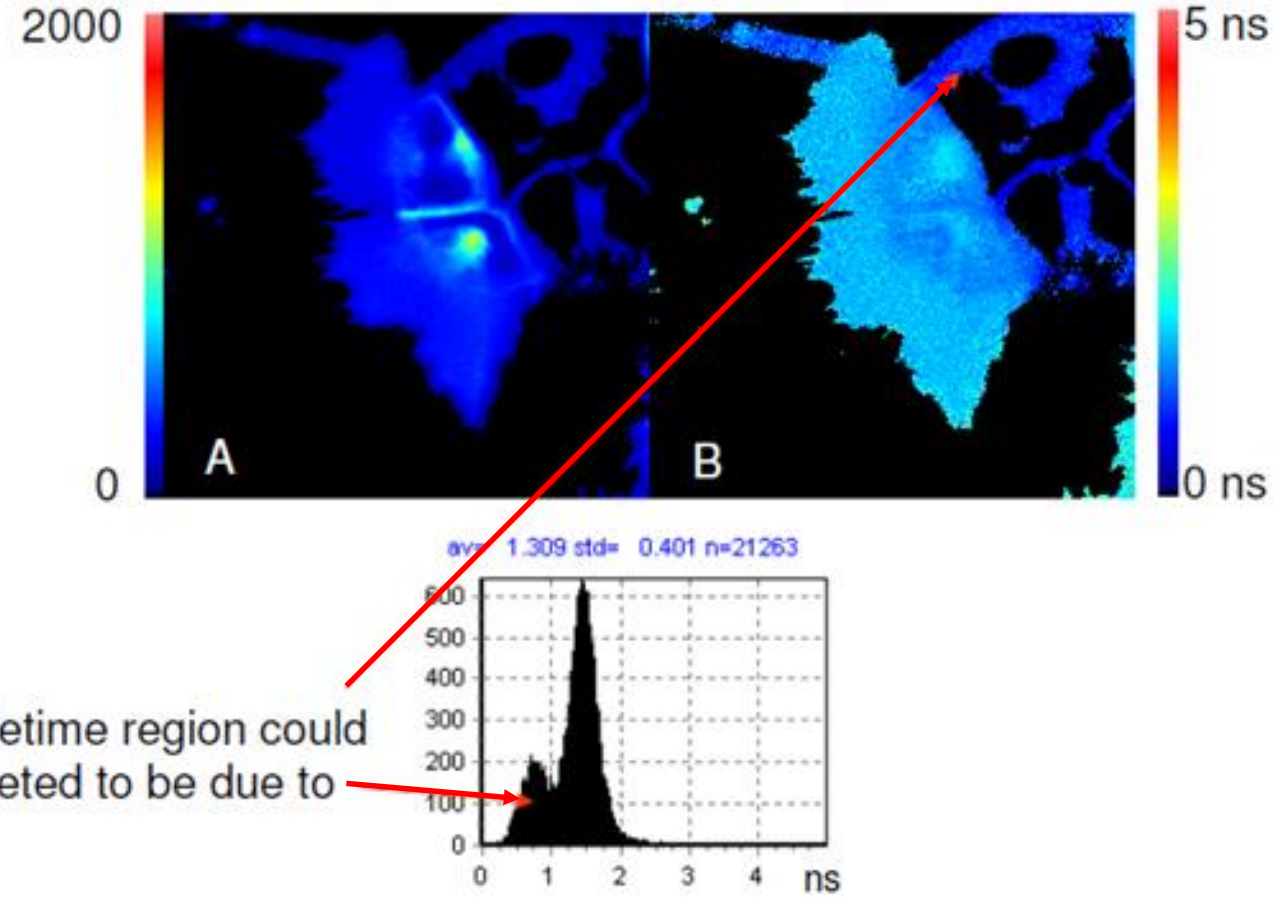
As the lifetime of the Donor is quenched, the phasor of the quenched Donor is added to the phasor of the autofluorescence



If there is a fraction of Donor that cannot be quenched, the final point will be along the line joining the Donor with the autofluorescence phasor

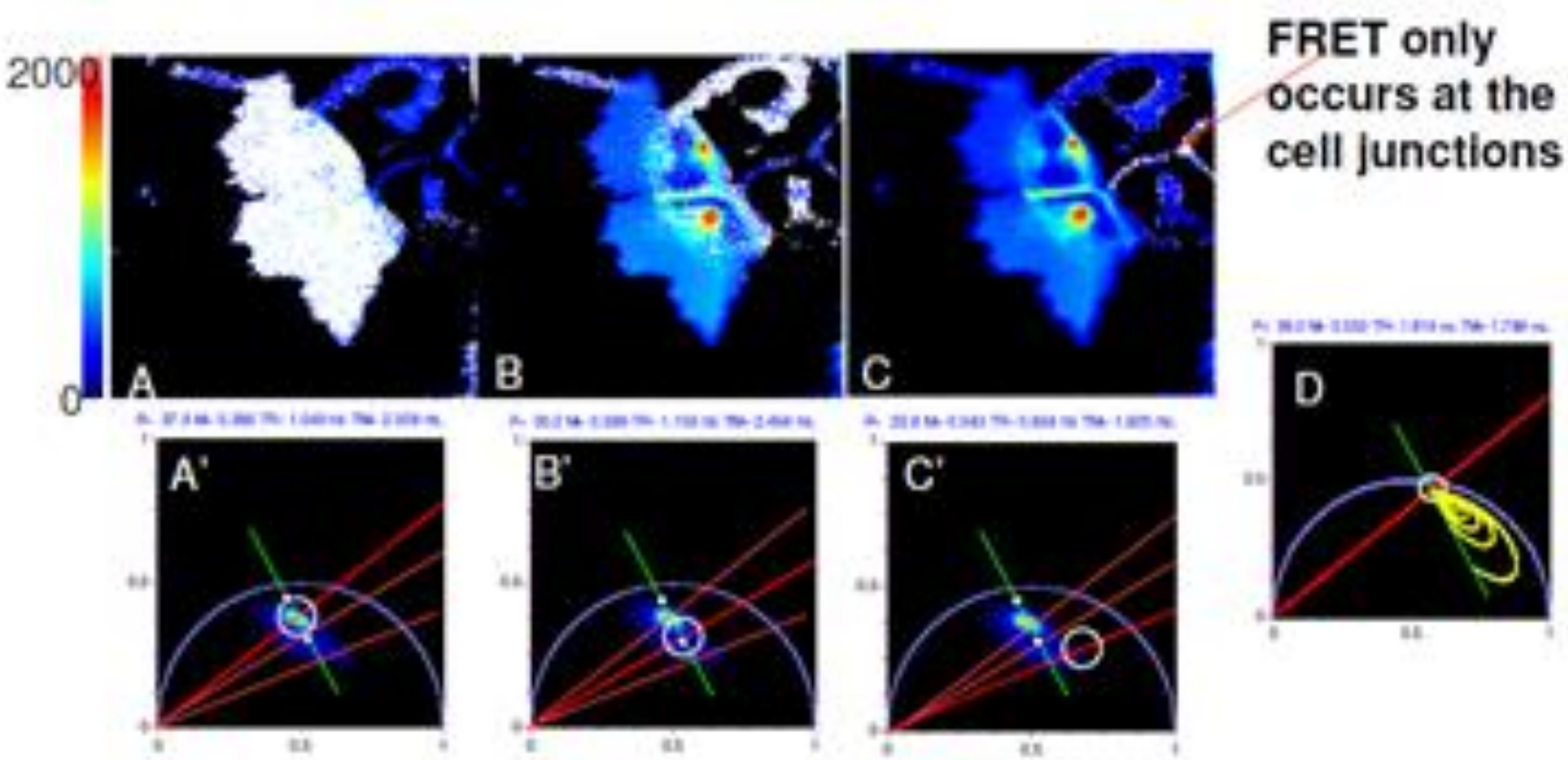
The Pitfalls of 'Conventional' FLIM Analysis

Image obtained using B&H 830 in our 2-photon microscope



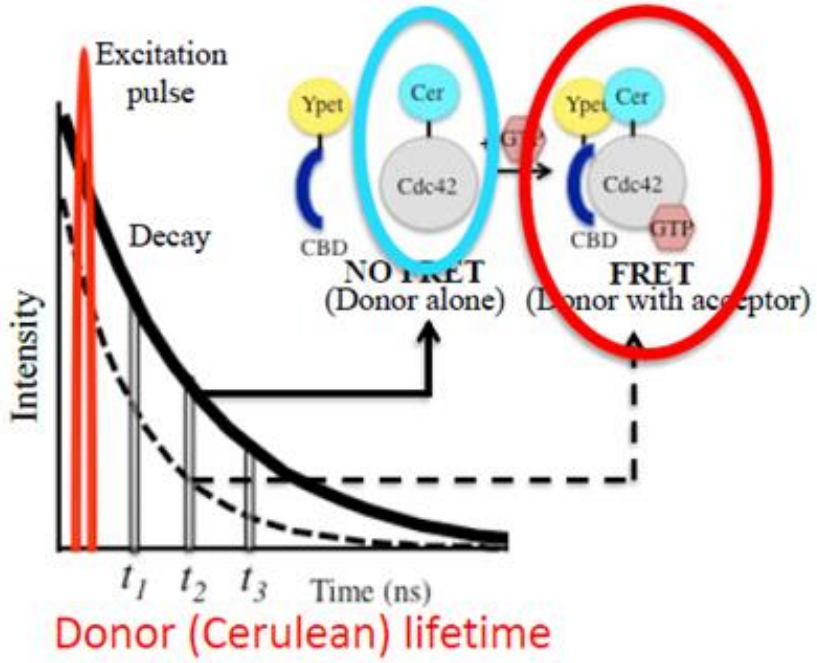
Donor+acceptor+ligand. A) intensity image after background subtraction, B) τ_p image

Identification of FRET Using The Phasor Plot

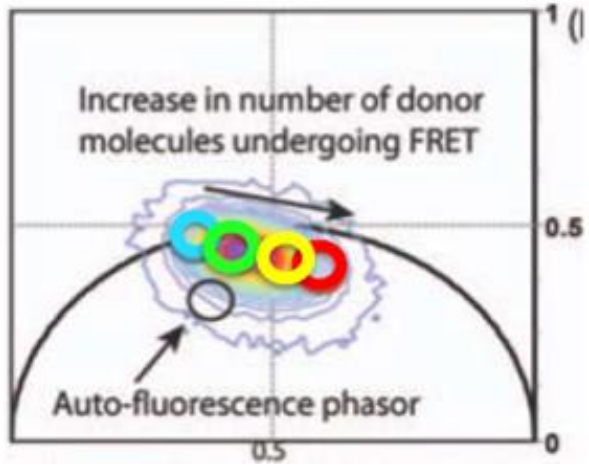


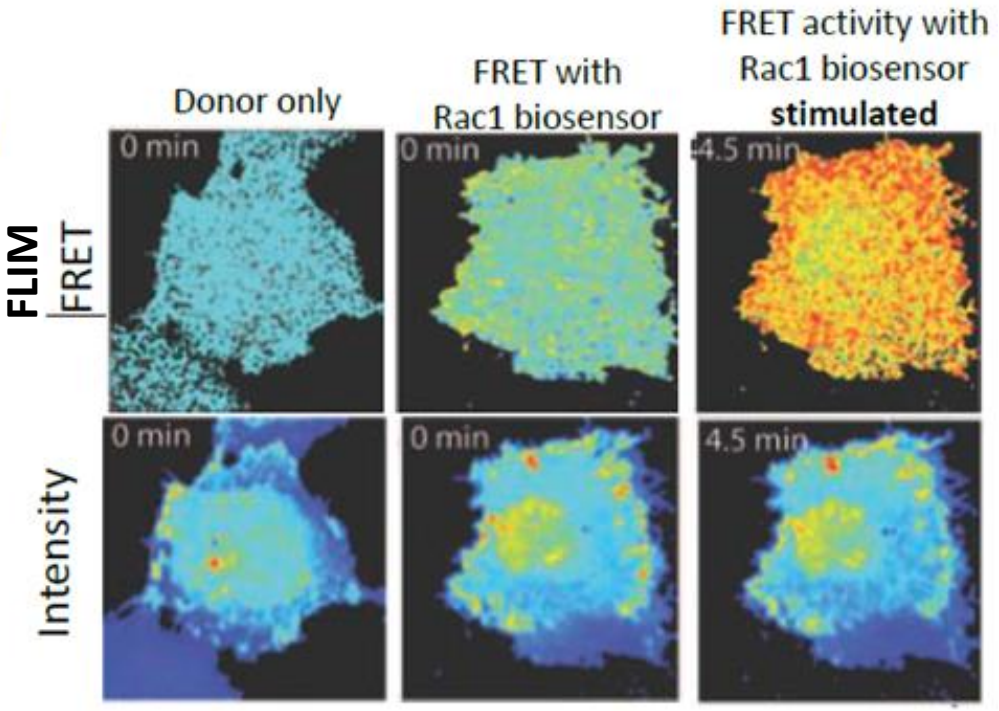
Selecting regions of the phasor diagram. Selecting the region in **A'** (donor +acceptor) the part in white lights up (**A**). Selecting the region in **B'** (autofluorescence) the part in white in lights up (**B**). The color scale in **B'** has been changed to better show the region of the autofluorescence. Selecting the region in **C'** (along the donor quenching line as shown in **D**) the part in white in at the cell junction lights up in **C**.

FRET Signal Is Independent Of Intensity



Donor (Cerulean) lifetime





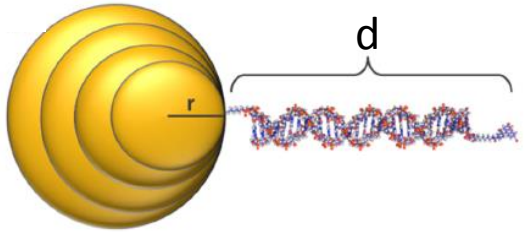
Nanometal Surface Energy Transfer

Energy transfer from a dipole to a metallic surface

Interaction of the electromagnetic field of the donor dipole with the nearly free conduction electrons (plasmons) of the accepting metal

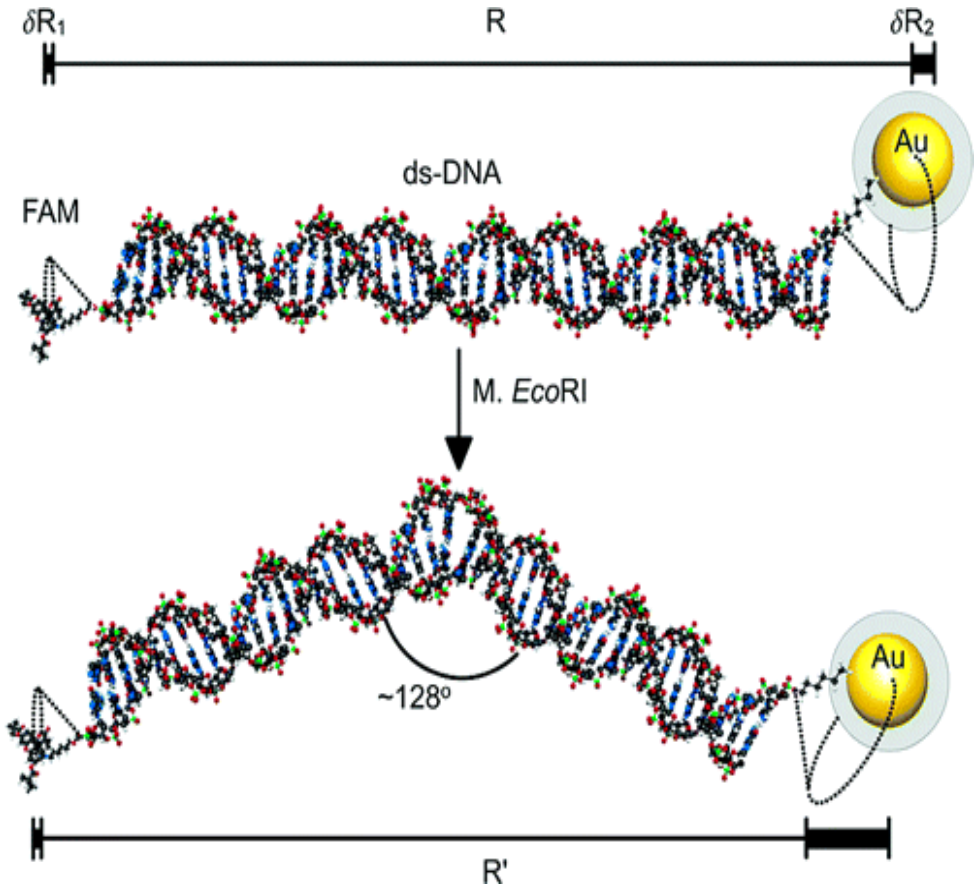
Surface energy transfer efficiency :

$$K_{SET} = (1/\tau_D)\ (d_o/d)^4$$



Breshike, *J. Phys. Chem. C* 2013, 117, 23942−23949

FRET Spectroscopic Rulers



6-Carboxy Fluorescein (FAM) double stranded ds-DNA with length *R* (varying from 15 to 60bp) Au nanoparticle (*d* = 1.4 nm) construct

*C*6 linkers for both moieties.

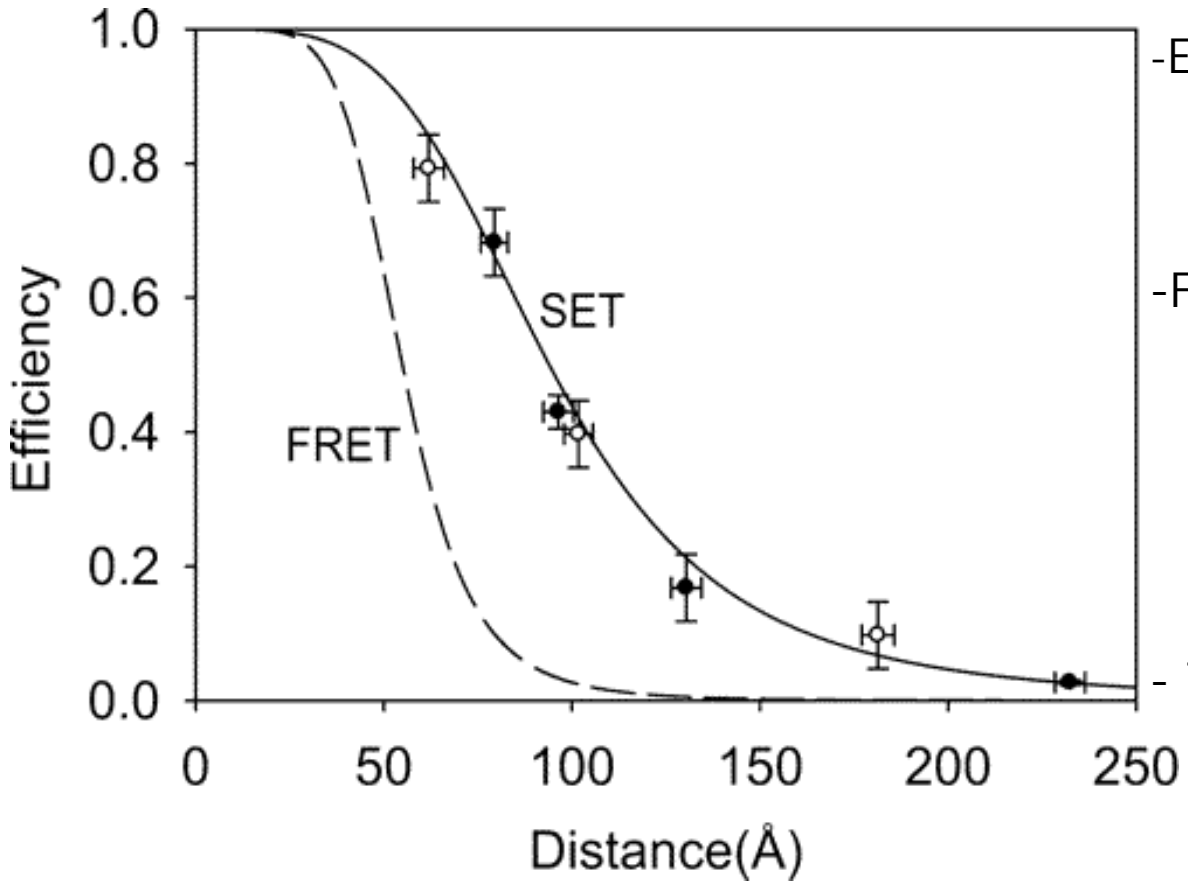
Addition of *EcoRI* (methyltransferase) bends the ds-DNA at the GAATTC site by 128°

15 bp ; 62 A
20 bp ; 96.4 A
30 bp ; 130.4 A
60 bp ; 232.4 A

10 bases per turn
3.4 Å per base

Breshike, *J. Phys. Chem. C* 2013, 117, 23942−23949

SET Efficiency vs distance



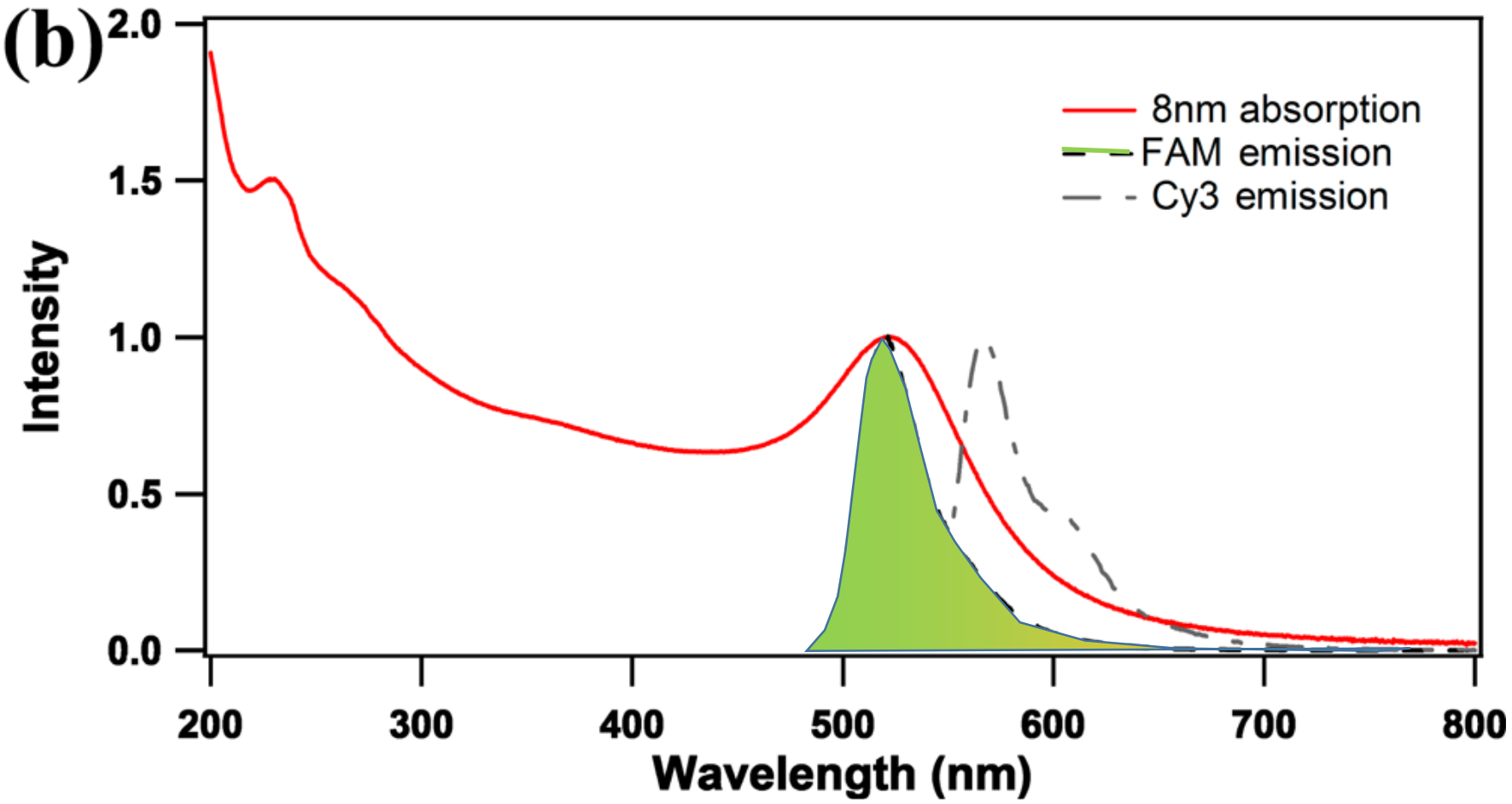
-Energy transfer efficiency plotted versus separation distance between FAM and Au(NM).

-Filled circles (·) represent DNA lengths of 15bp, 20bp, 30bp, and 60bp. The measured efficiencies of these strands with the addition of *M.EcoRI* are represented by the open circles.

- The dashed line is the theoretical FRET efficiency, while the solid line the

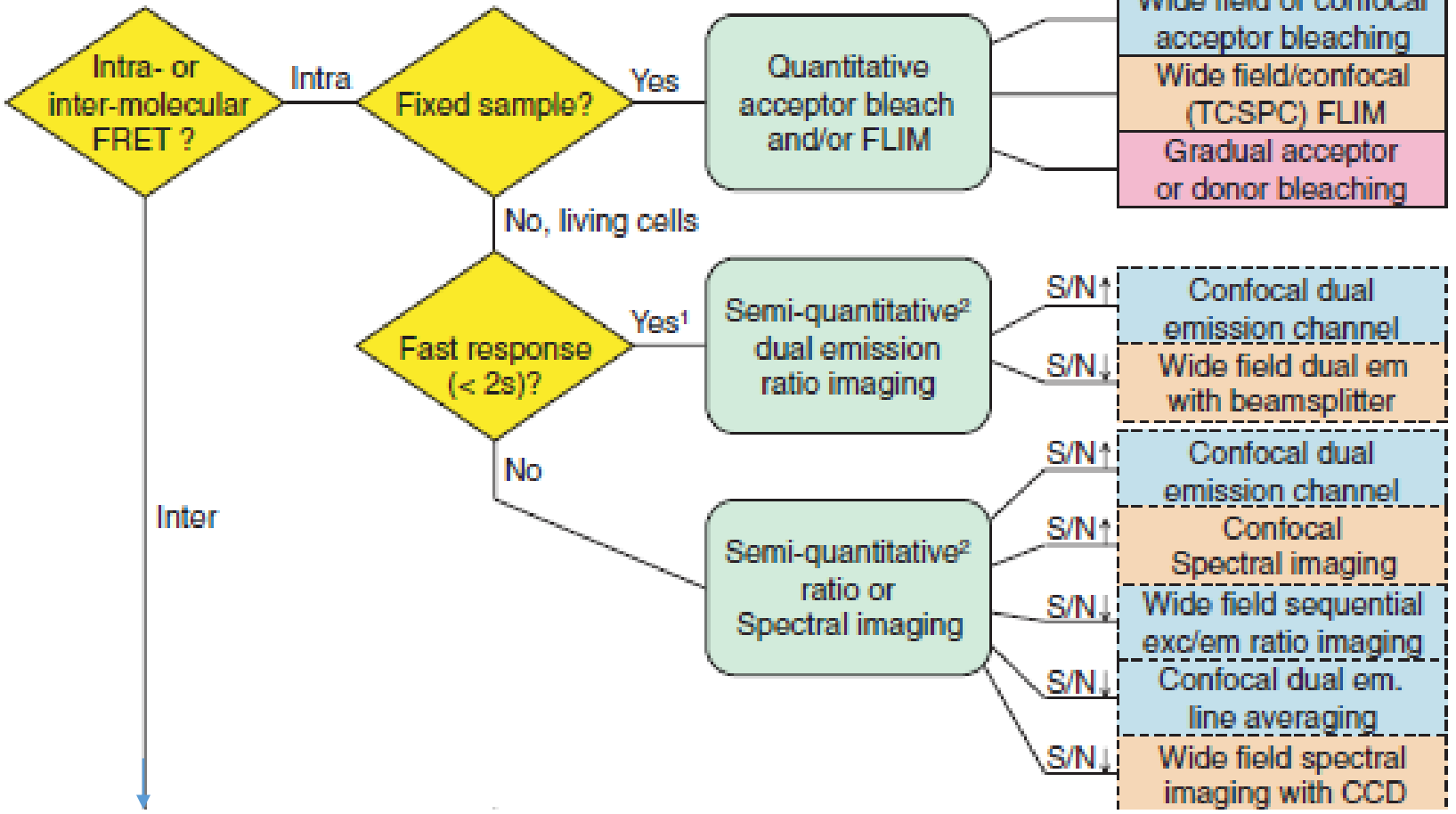
theoretical SET efficiency

Au Nanoparticle, Donor & Acceptor Spectra



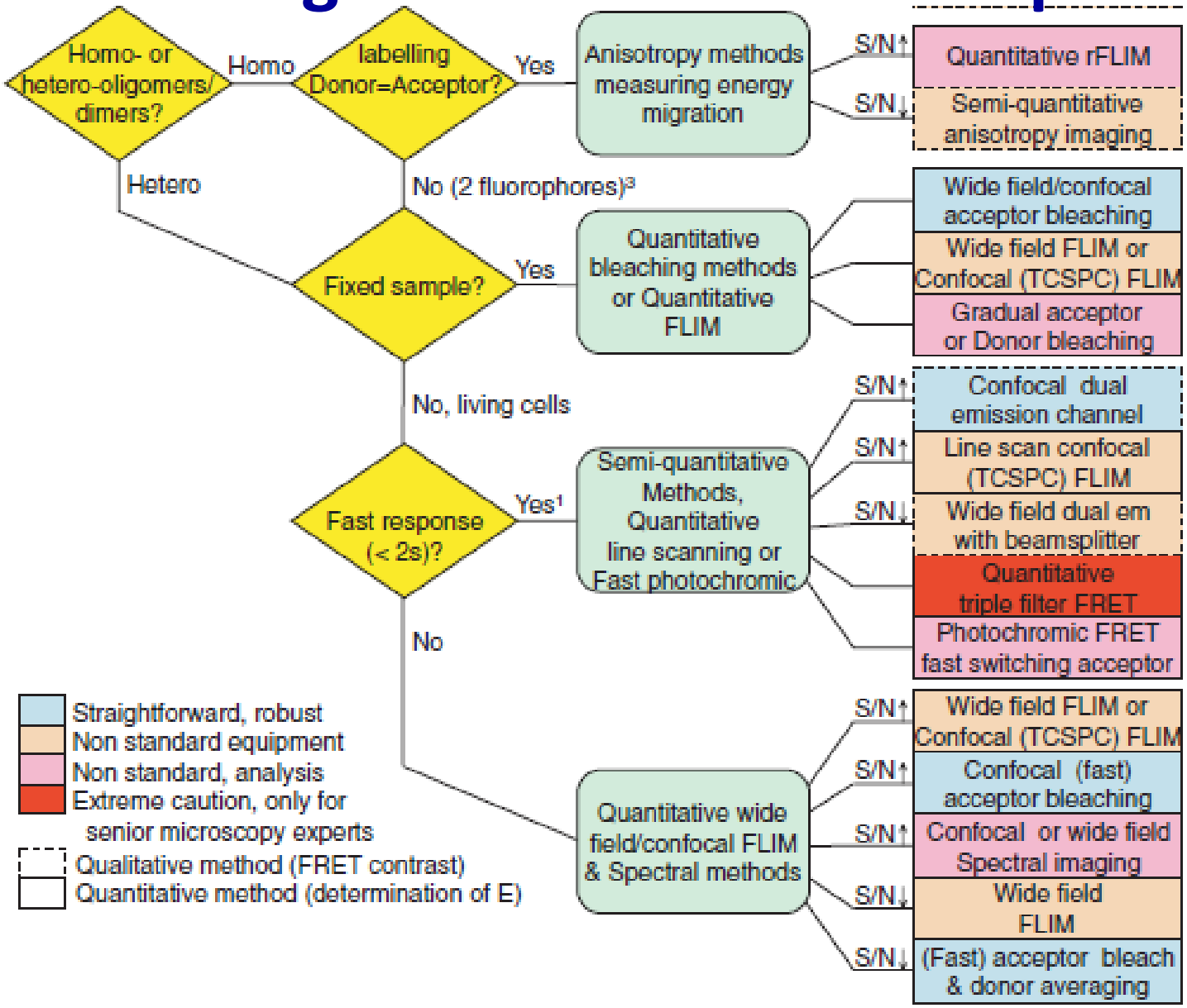
Breshike, *J. Phys. Chem. C* 2013, 117, 23942−23949

Selecting a 'Best' FRET Technique



Pietraszewska-Bogiel, *J.Microsc., 2011, 241(2) 111–118*

Selecting a 'Best' FRET Technique



Pietraszewska-Bogiel, *J.Microsc., 2011, 241(2) 111–118*

Acknowledgements

The presenter (MV) greatly appreciates and acknowledges the use of several excellent FRET training & instruction presentations as made available by
Profs. R. Clegg (ł, UIUC) , E. Gratton (LFD, UCI), D. Jameson (U. of Hawaii) , J. Mueller (UMN), A. Periasamy (Keck, UVA), D. Piston (Vanderbilt U.), Zimmermann (EAMNet, EMBL) etc.

Resources

Apart from information available on the web a number of excellent BOOKs have been written by Profs. R. Clegg, R. Day, D. Jameson, J. Lakowicz, A. Periasamy, B. Valeur and several others for further consultation.

FRET data processing SOFTWARE is available ao. from the LFD, UIUC; Keck, UVA and several other sources like ImageJ plugins, McNamara2005 Excel worksheet, Python and Matlab scripts

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Grazie mille