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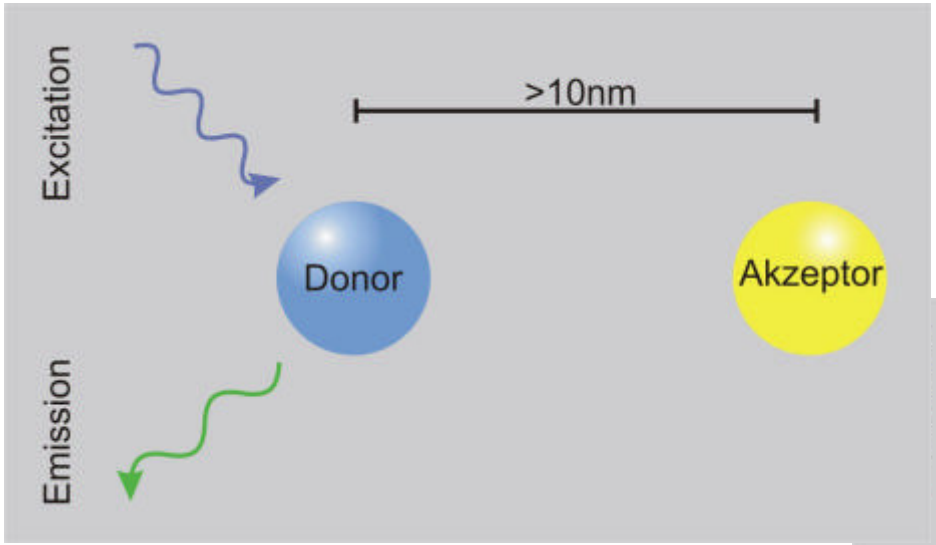
FRET Analysis in Laser Scanning Microscopy

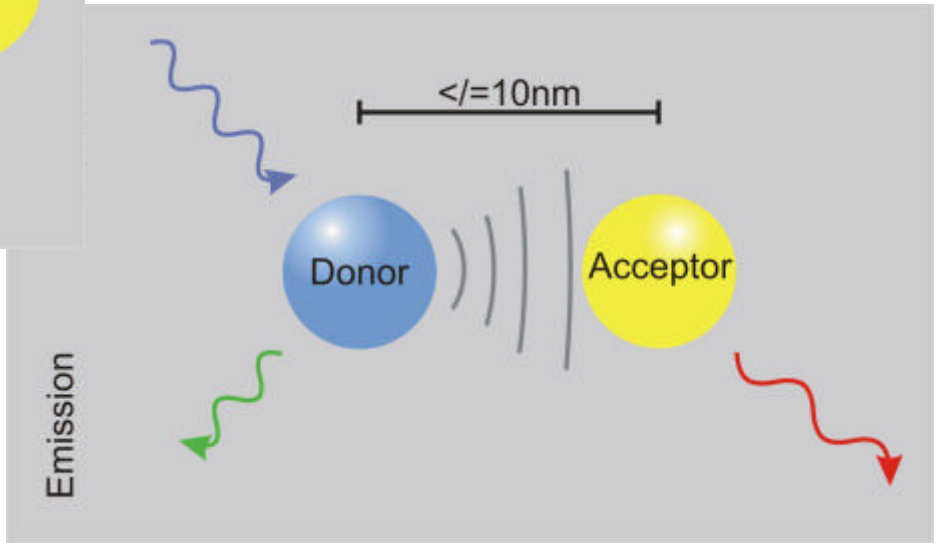
What is FRET ?

FRET (fluorescence resonance energy transfer) is the non-radiative transfer of photon energy from an excited fluorophore (the donor) to another fluorophore (the acceptor) when both are located within close proximity (1-10 nm).



FRET applications

- Protein/protein interactions
- Detection of conformational changes
- Specialized FRET tools like yellow Chameleon for Ca++ Imaging



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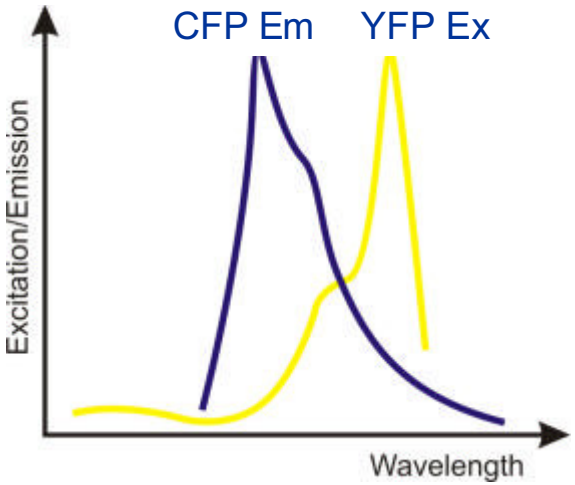
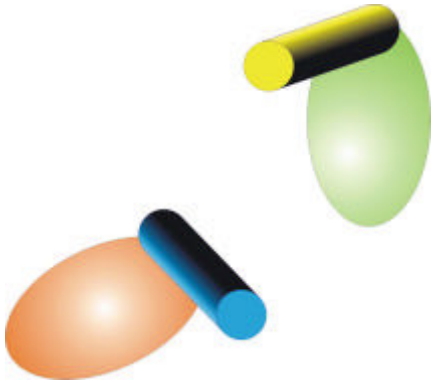
Preconditions for FRET Analysis

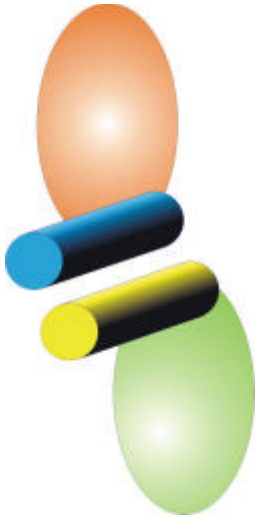
Appropriate FRET pair with overlap between donor emission and acceptor excitation

- EBFP & EGFP
- ECFP & EYFP
- EGFP & Rhodamine
- FITC & Rhodamine
- FITC & CY3

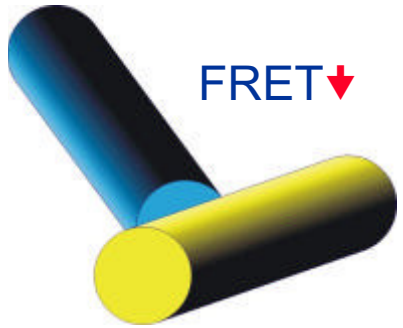


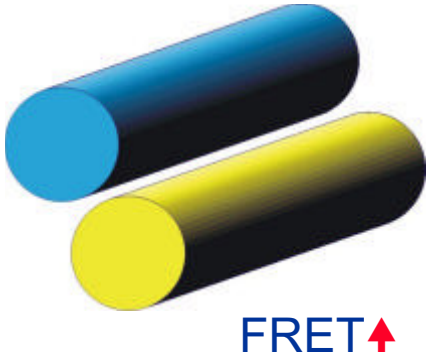
Specific staining of the molecule (protein) of interest





Parallel orientation of the axis of interacting dye molecules





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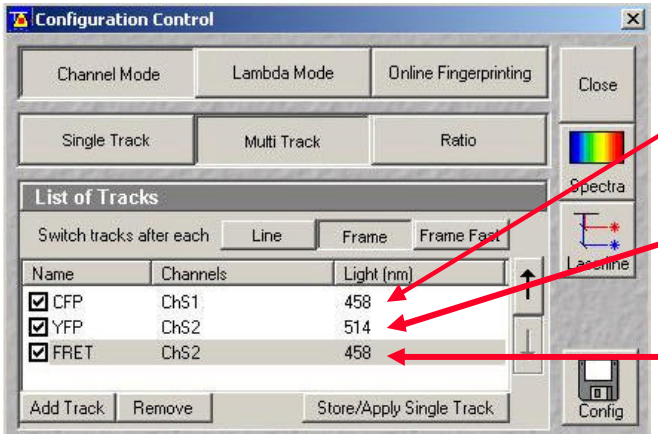
Quantitative Analysis using Filter FRET

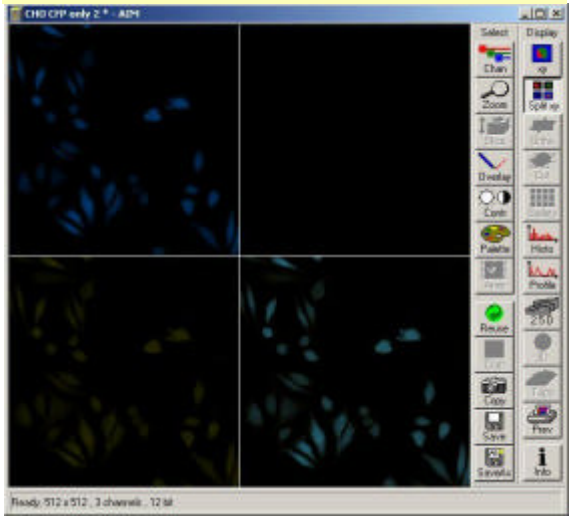


Excitation of CFP
Detection of CFP

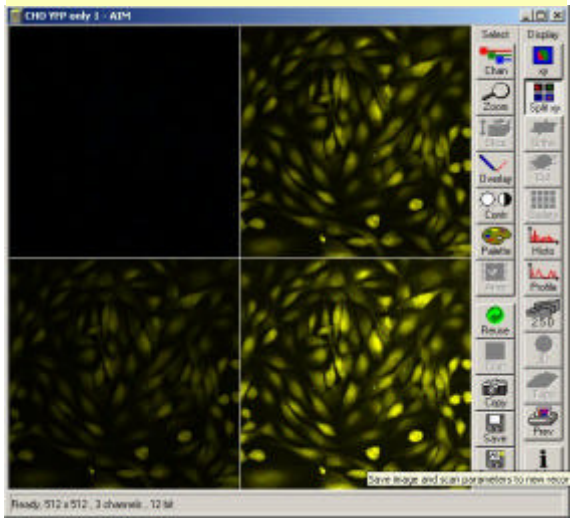
Excitation of YFP
Detection of YFP

Excitation of CFP
Detection of YFP ➢ FRET signal (not corrected)

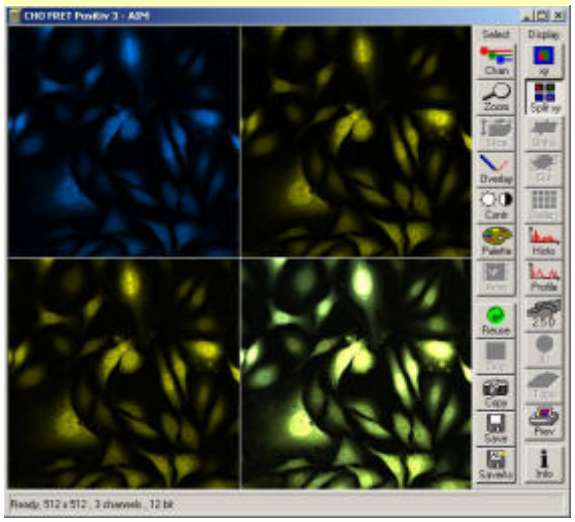
CFP only



YFP only



CFP and YFP doing FRET



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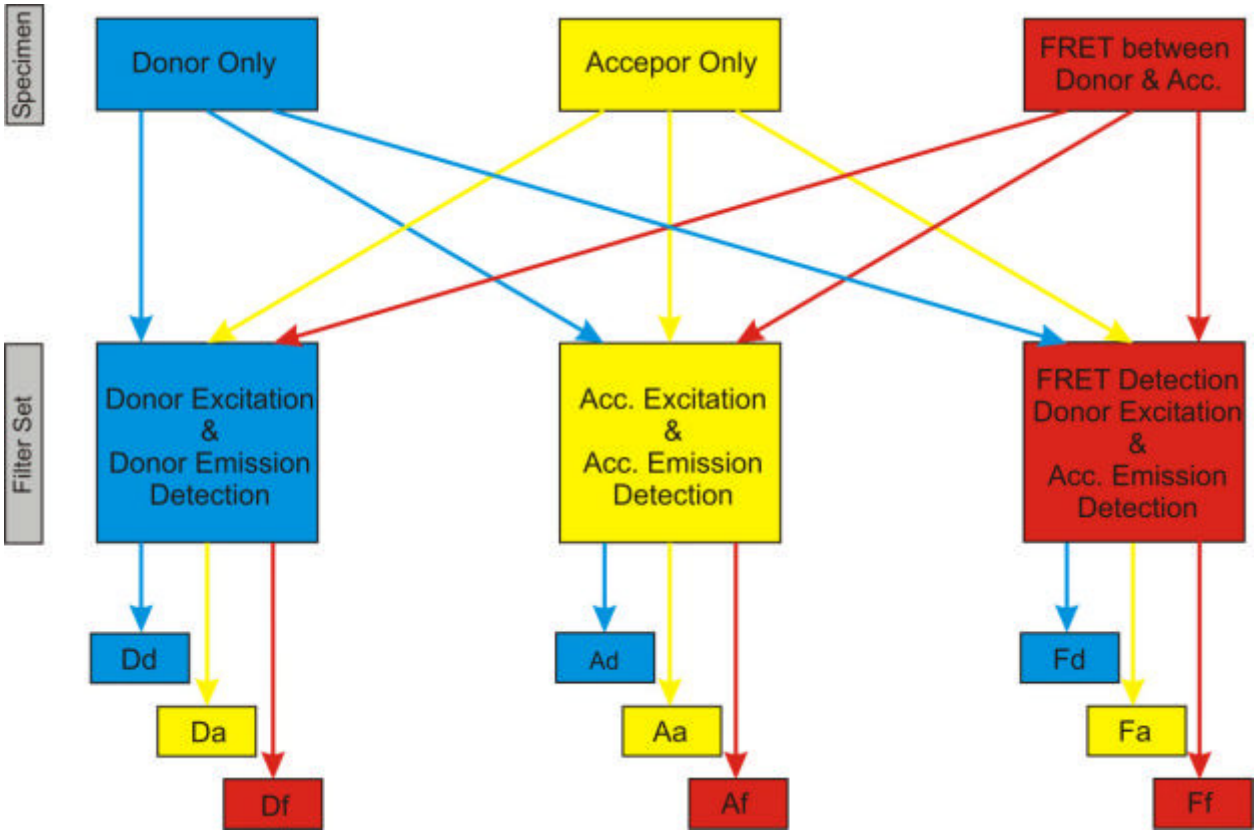
Sensitized Emission – Calculation of Fc



Fa ← capital letter : Filter Set

Fa ← small letter : Specimen

$$Fc = Ff - \left[\frac{Fd}{Dd} \cdot Df\right] - \left[\frac{Fa}{Aa} \cdot Af\right]$$

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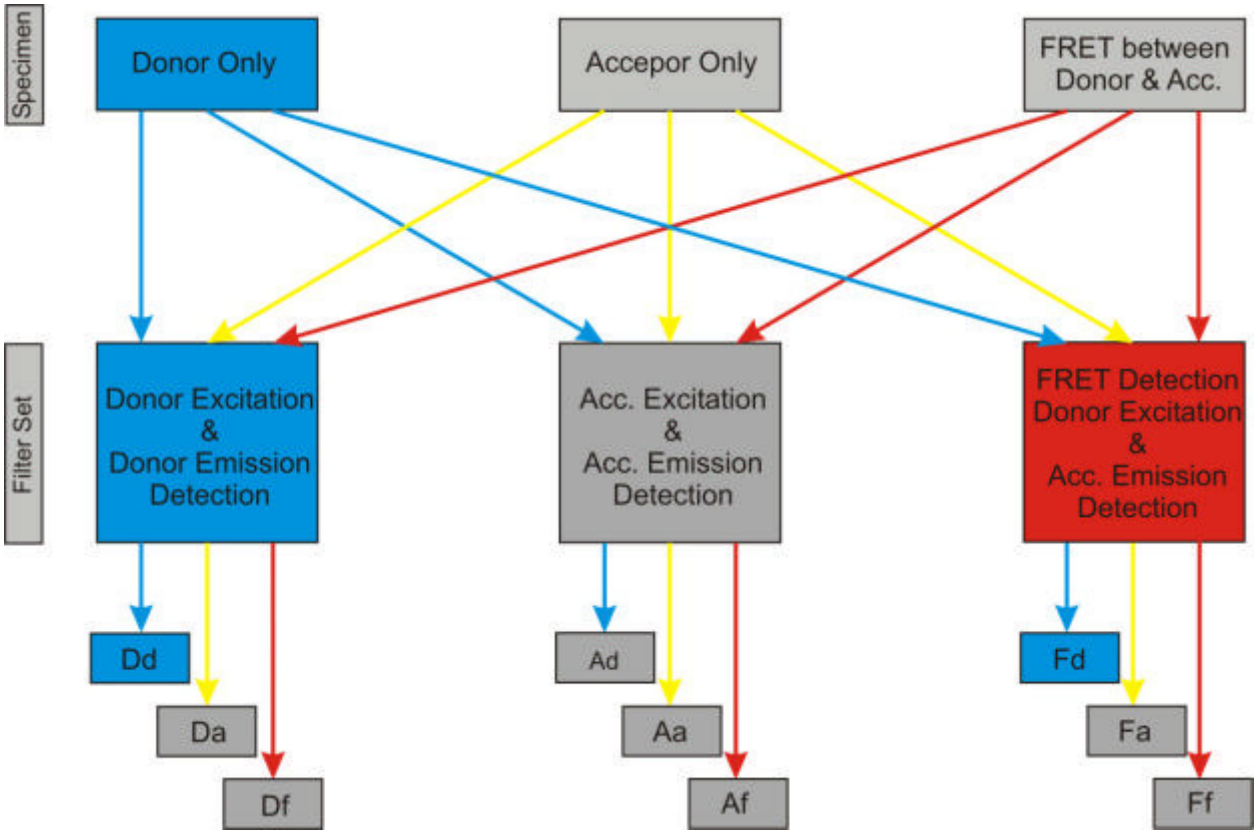
Sensitized Emission – Calculation of Fc



Fd/Dd is a measure for the ratio of donor signal detected in the donor channel and emission crosstalk of donor signal detected in the FRET channel.

Once determined for an experiment this value remains constant.

Fa
- capital letter : Filter Set
- small letter : Specimen

$$Fc = Ff - \left[\frac{Fd}{Dd} \cdot Df\right] - \left[\frac{Fa}{Aa} \cdot Af\right]$$

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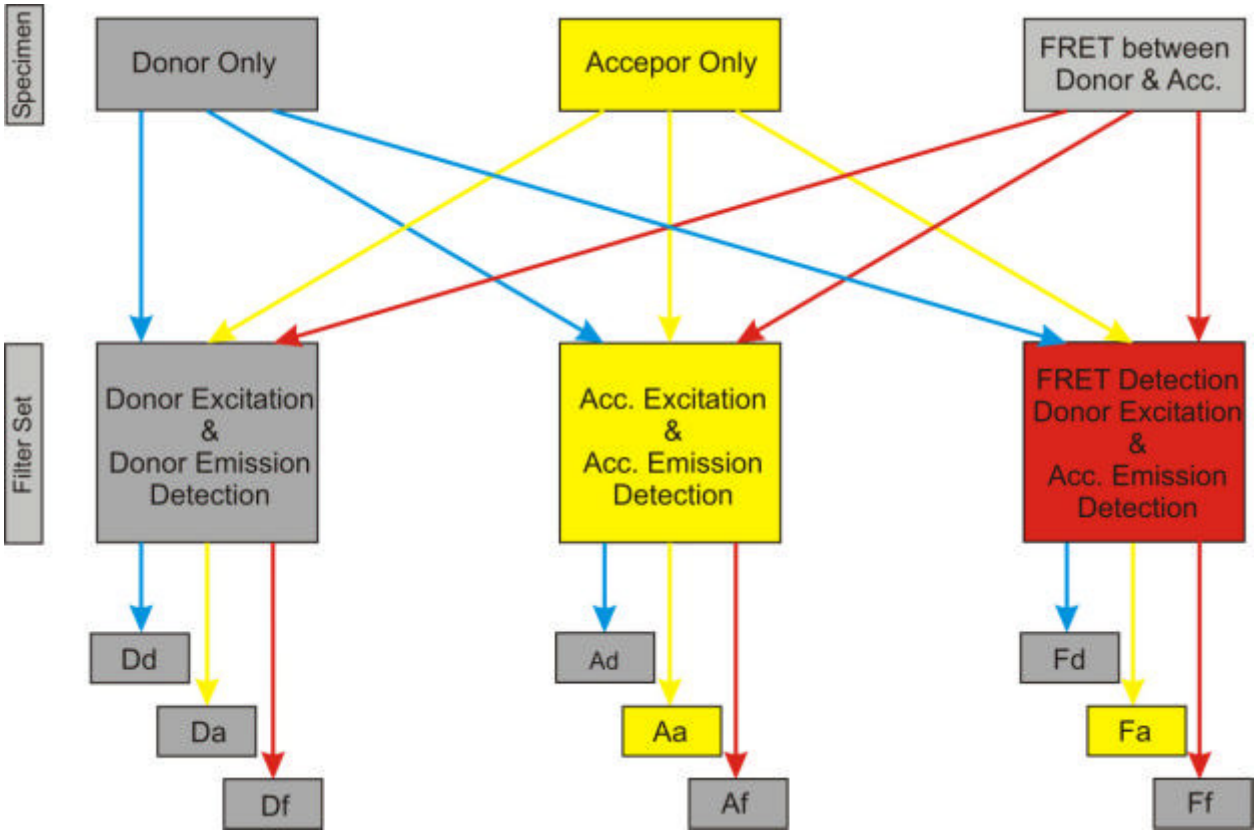
Sensitized Emission – Calculation of Fc



Fa/Aa is a measure for the ratio of acceptor signal detected in the acceptor channel and excitation crosstalk of acceptor signal detected in the FRET channel.

Once determined for an experiment this value remains constant.

Fa ← capital letter : Filter Set

Fa ← small letter : Specimen

$$Fc = Ff - \left[\frac{Fd}{Dd} \cdot Df\right] - \left[\frac{Fa}{Aa} \cdot Af\right]$$

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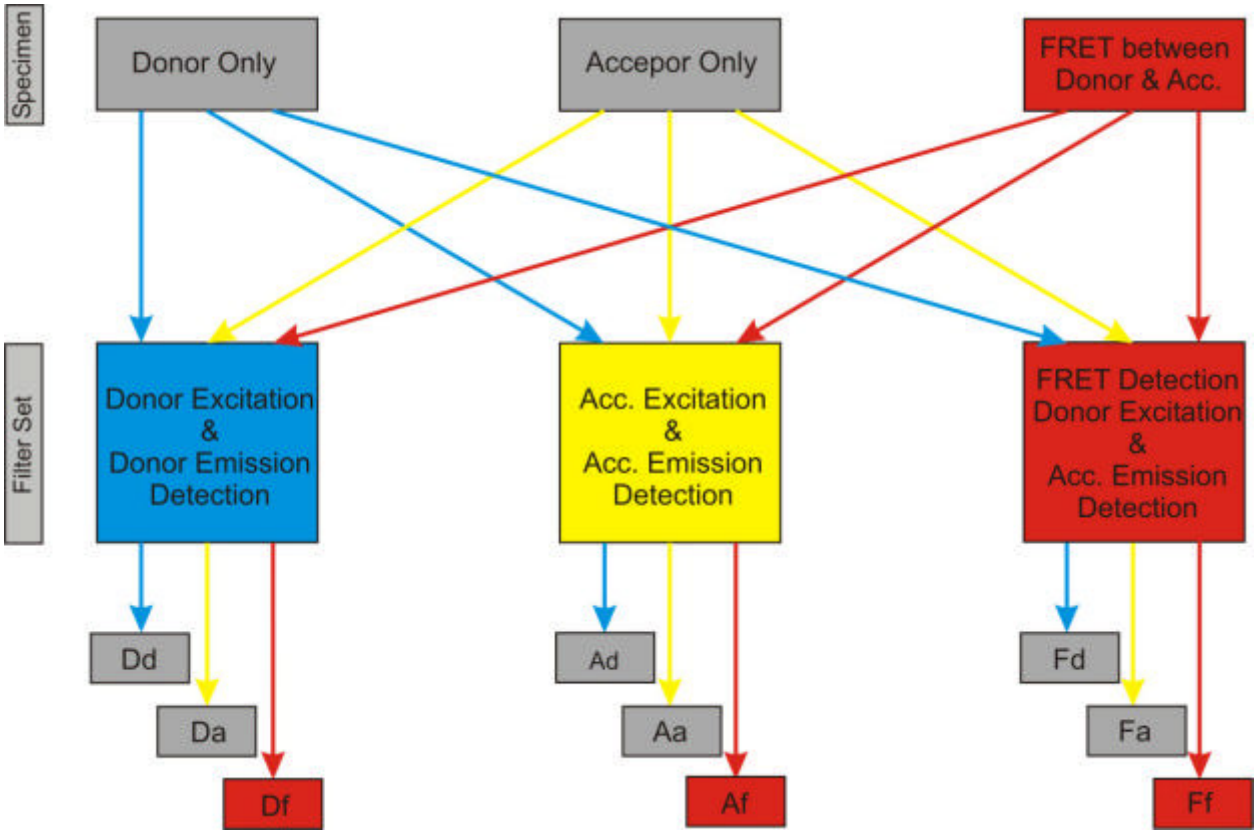
Sensitized Emission – Calculation of Fc



In a FRET experiment the values Df and Af correlate with donor and acceptor concentration respectively.

Multiplied with the previously determined ratios Fd/Dd and Fa/Aa, the FRET value Ff can be corrected to get Fc.

Fa
- capital letter : Filter Set
- small letter : Specimen

$$Fc = Ff - \left[\frac{Fd}{Dd} \cdot Df\right] - \left[\frac{Fa}{Aa} \cdot Af\right]$$

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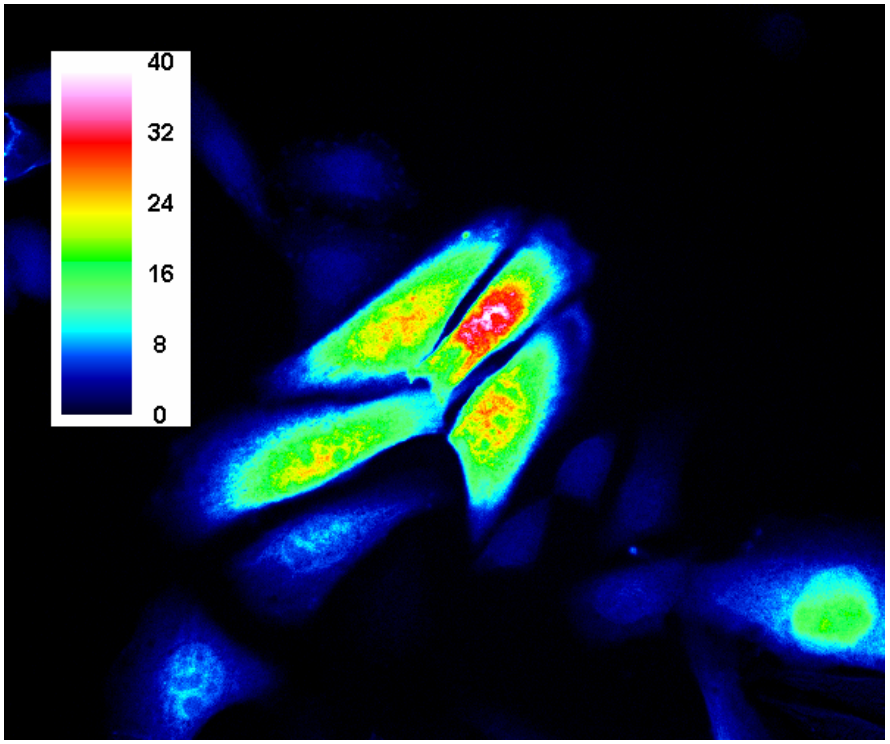
Sensitized Emission – The 3 Methods

Method 1: Fc (FRETcorrected) D.C. Youvan et al. 1997



Fc is corrected for donor and acceptor contribution to the signal measured with the FRET filter set. Fc is not normalized for the donor acceptor concentration. High Fc numbers occur were high concentration of donor and acceptor are present.

$$Fc = Ff - \left[\frac{Fd}{Dd} \cdot Df\right] - \left[\frac{Fa}{Aa} \cdot Af\right]$$

$$Fc = Ff - [Donor\ corr.] - [Acc.\ corr.]$$

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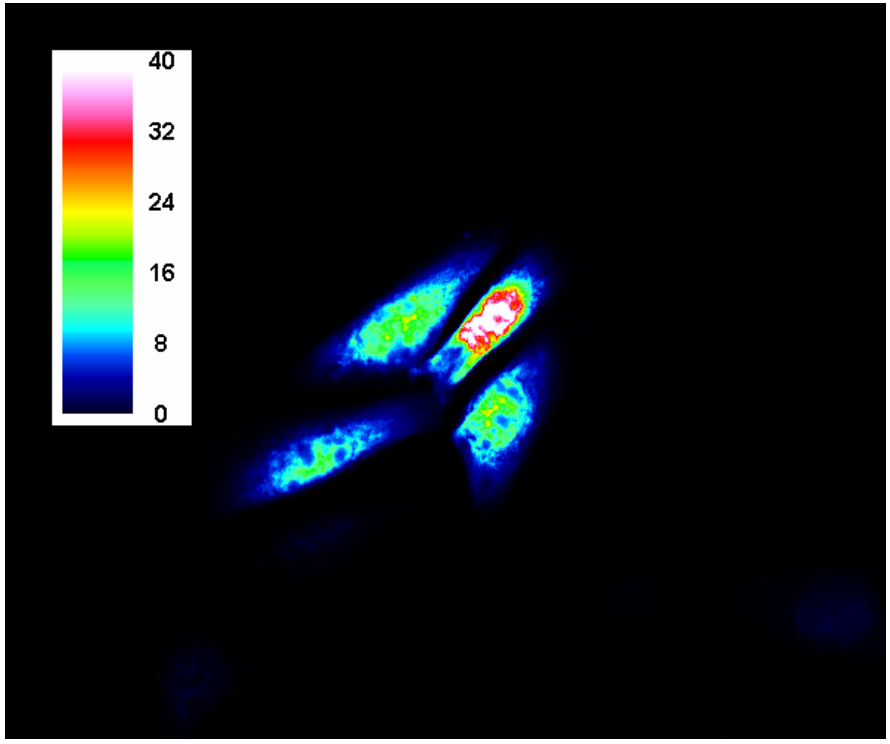
Sensitized Emission – The 3 Methods

Method 2: Fn (FRET net) G.W. Gordon et al. 1998



Fn is corrected for donor and acceptor contribution to the signal measured with the FRET filter set as Fc.
Fn is given as Fc divided by the multiplied concentrations of donor and acceptor. This emphasize FRET occurring at low concentrations of donor and acceptor.

$$Fn = \frac{Ff - [Donor\ corr.] - [Acc.\ corr.]}{G \cdot Df \cdot Af}$$

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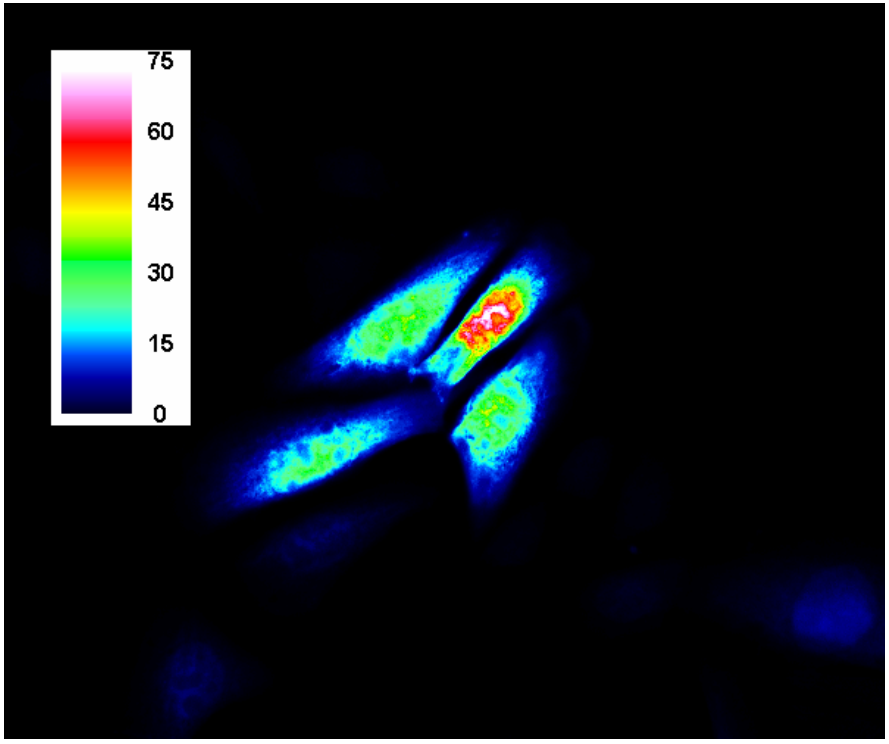
Sensitized Emission – The 3 Methods

Method 3: NF (normalized FRET) X. Xia et al. 2001



NF is corrected for donor and acceptor contribution to the signal measured with the FRET filter set as Fc.
NF is given as Fc divided by the square root of the multiplied concentrations of donor and acceptor. This results in FRET values normalized for donor and acceptor concentration.

$$NF = \frac{Ff - [Donor\ corr.] - [Acc.corr.]}{\sqrt{G \cdot Df \cdot Af}}$$

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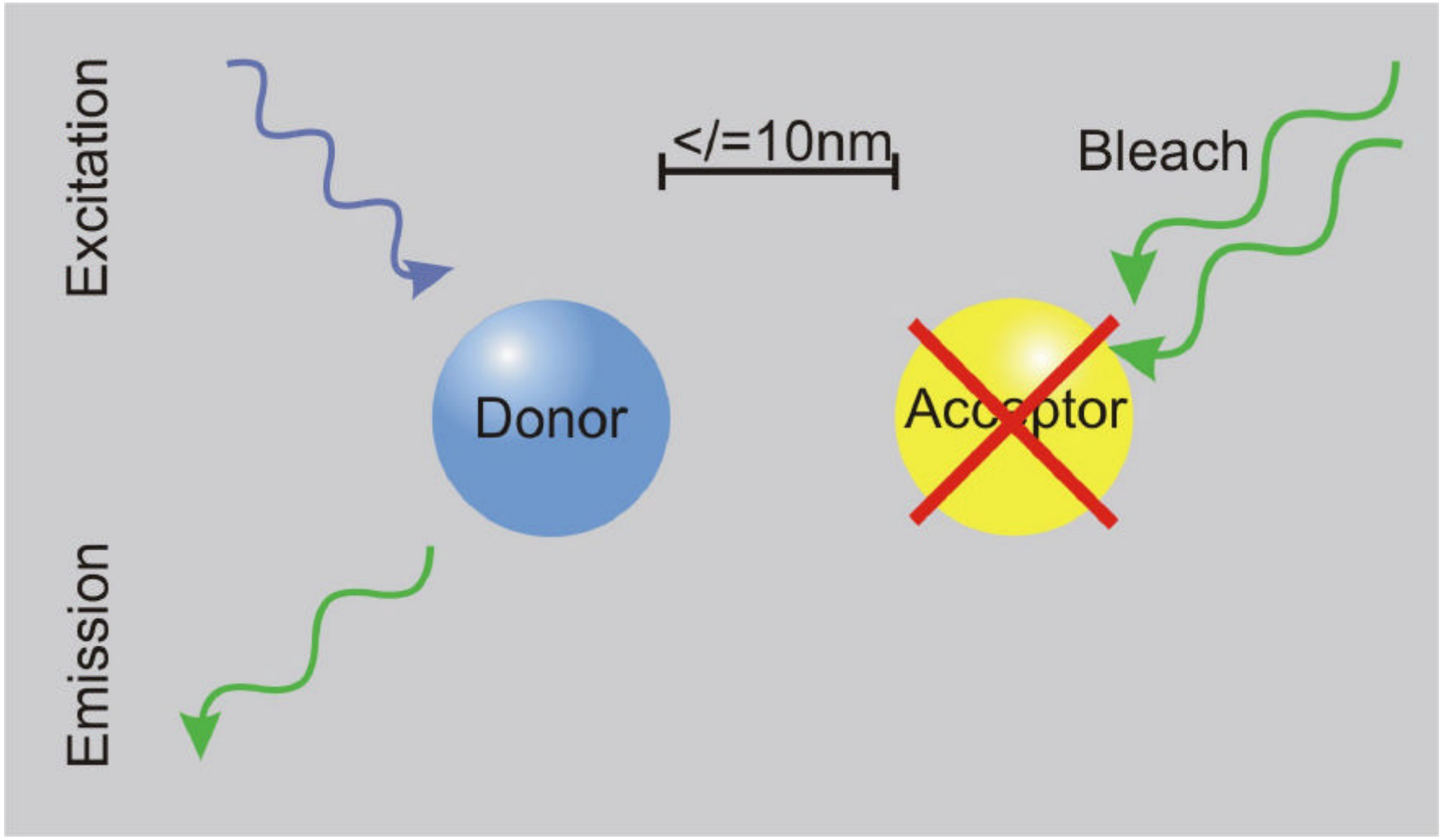
Quantitative FRET Analysis using Acceptor Bleach



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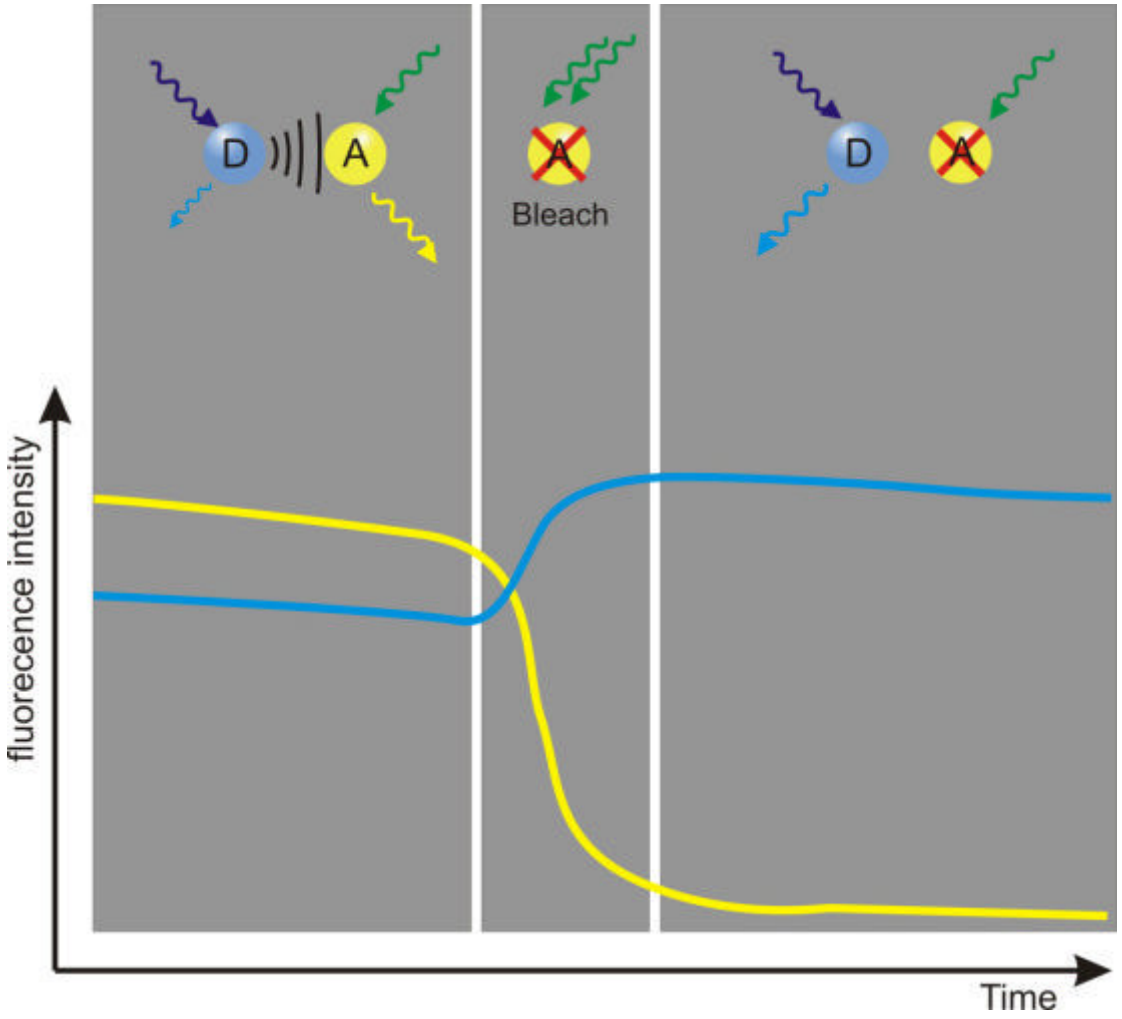
Quantitative FRET Analysis using Acceptor Bleach



Principle

- Some donor (CFP) signal is transferred (FRET) to the acceptor (YFP)
- The acceptor is bleached (chemically destroyed)
- The donor signal increases (up to 30%) since no energy transfer to the acceptor is possible.

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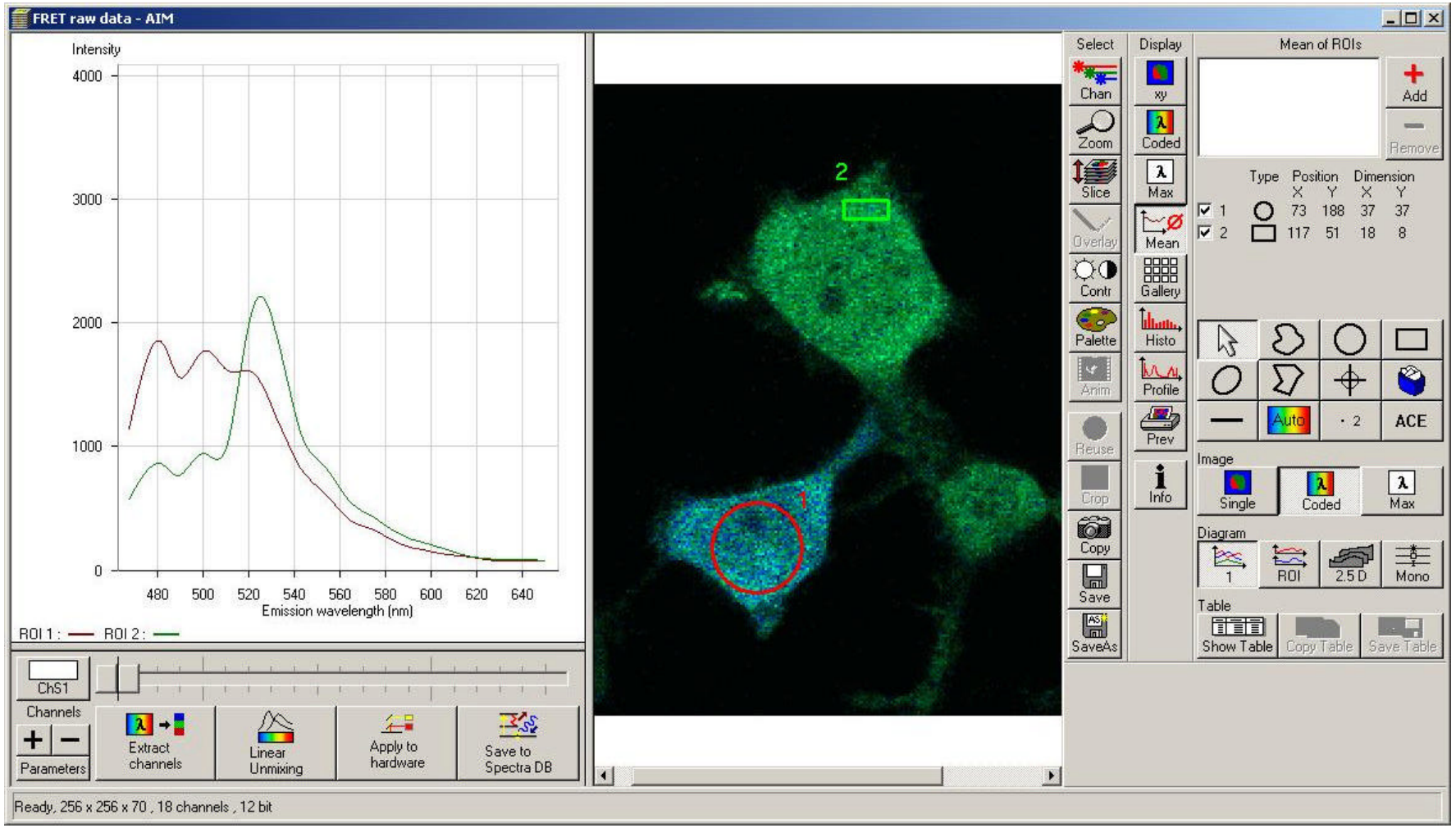
Quantitative FRET Analysis using Acceptor Bleach



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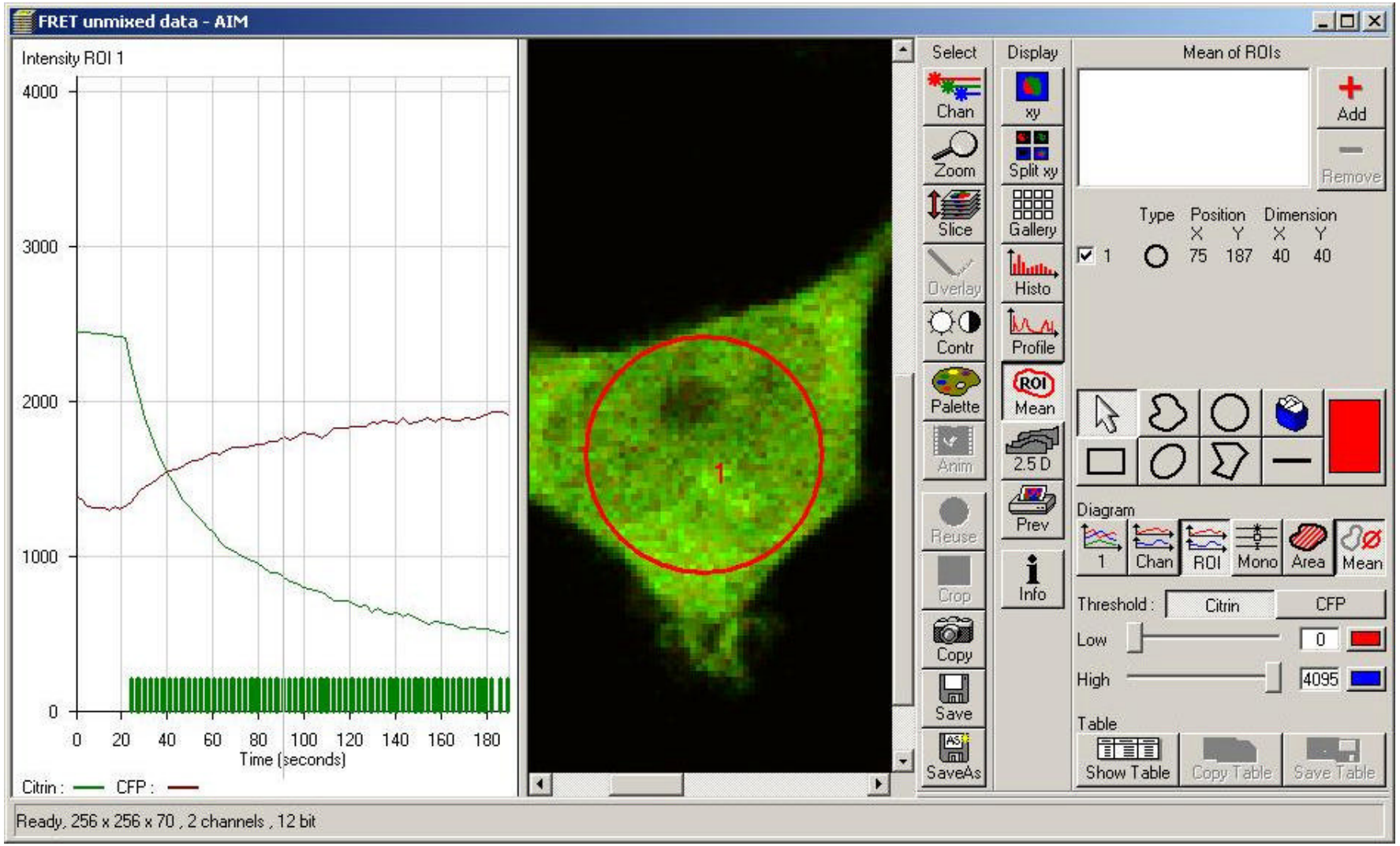
Quantitative FRET Analysis using Acceptor Bleach



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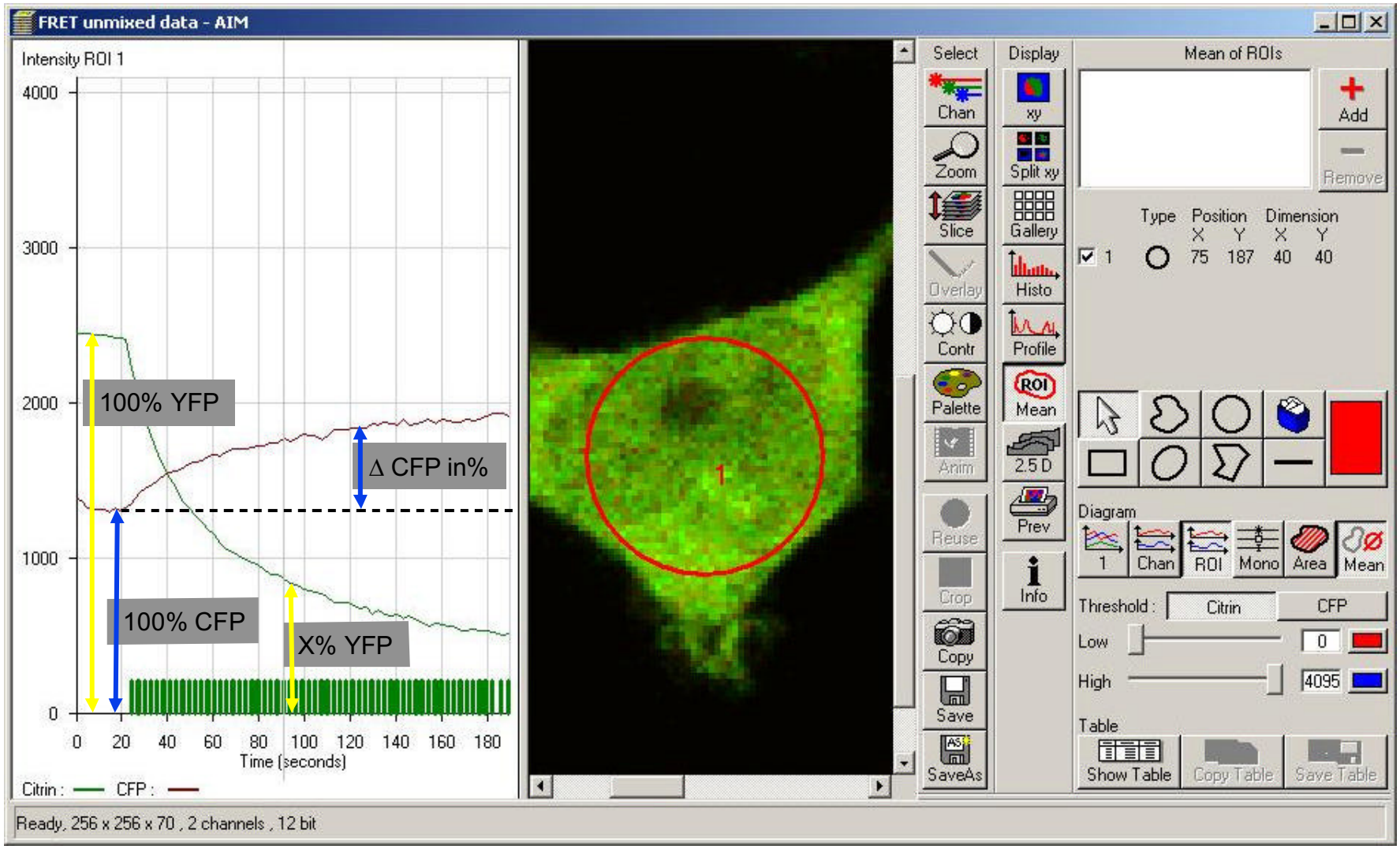
Quantitative FRET Analysis using Acceptor Bleach



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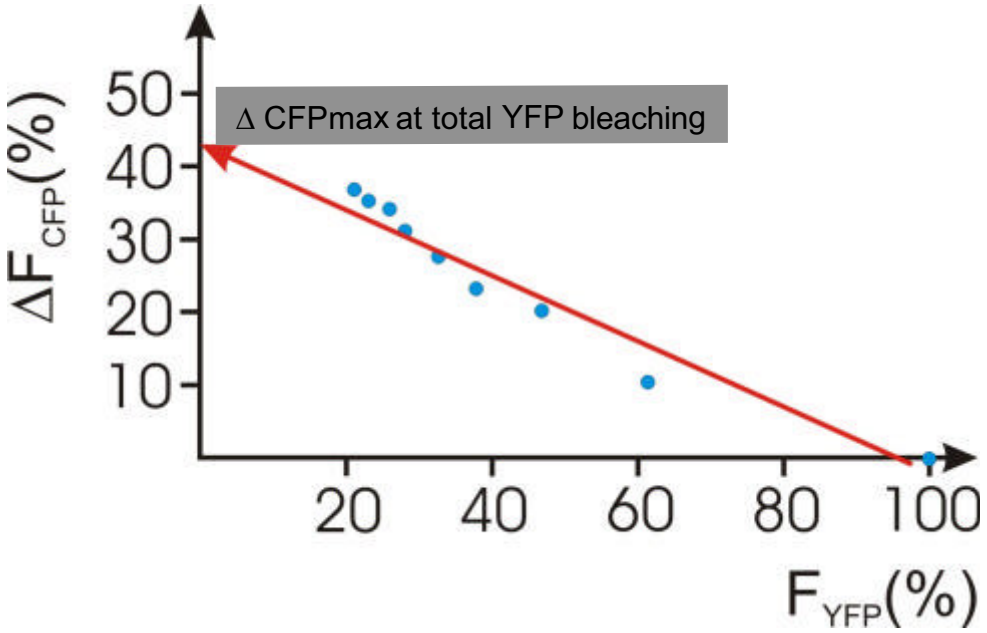
Quantitative FRET Analysis using Acceptor Bleach



$$E = \frac{F_{CFP-\max} - F_{CFP-\min}}{F_{CFP-\max}} = 30\,\%$$

Experimental Conditions

- Use non bleaching laser intensities of 458 and 514nm for CFP and YFP imaging
- Bleach YFP from 100 to 10% with 100% power of 514nm laser line
- Apply linear regression analyses to yield values for CFP intensities without acceptor ($F_{CFP\text{-}max}$ at YFP = 0)
- Lit.: H. Amiri et al. in Cell Calcium (2003)

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Methods and Systems for Quantitative FRET Analysis in LSM

		System Configuration				
		LSM 5 PASCAL/510 plus AxioCam	LSM 5 PASCAL	LSM 510	LSM 510 META	LSM 510 with FLIM Module
FRET Analysis Method	Sensitized Emission (Filter FERT) via FRET Macro	X/X	X	X	X	-
	Acceptor Photobleaching via FERT Macro	(X)/X	(X)	X	X	-
	Acceptor Photobleaching via manual calculation	-/-	-	-	X	-
	Fluorescence Lifetime FRET	-/-	-	-	-	X

- Calculation via FRET Macro requires Rel. 3.2 Add On
- With the LSM 5 PASCAL no real regions of interest can be applied for bleaching
- Calculation of Lifetime FRET requires external Hard and Software