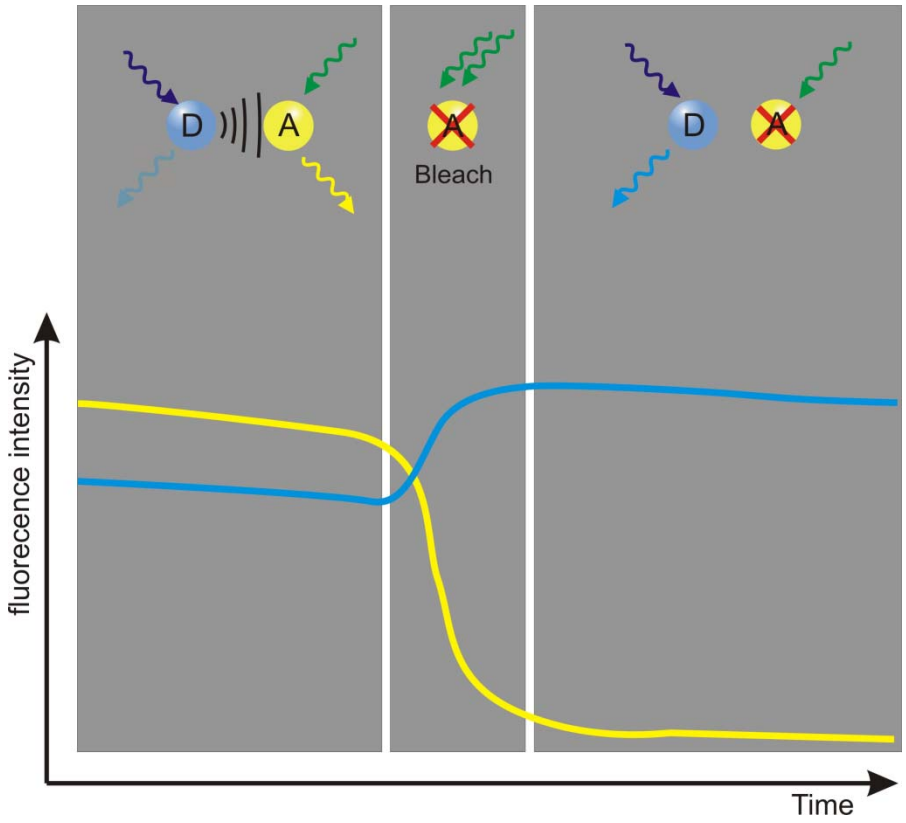
FRET Detection: Acceptor Photobleaching

Principle of Acceptor Photobleaching

Acceptor photobleaching:
a simple FRET detection method

De-quenching of the donor after selective photobleaching of the acceptor causes an increase in donor emission that can be readily quantified.



FRET Detection: Acceptor Photobleaching

ZEN 2010: Image acquisition

(1) Imaging Setup

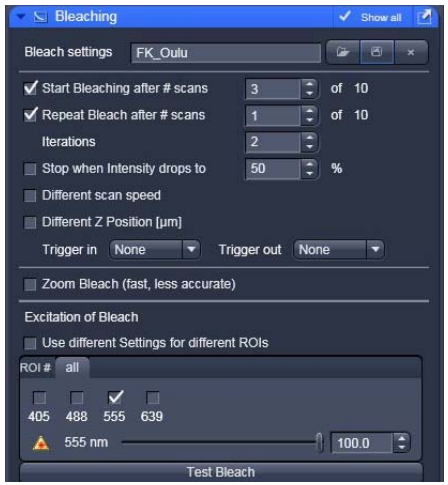
- Multitrack Channel Mode Linewise switch (fast)

1) Excite Donor and Detect Donor emission

2) Excite Acceptor and Detect Acceptor emission

(2) Regions

- Select the cells / structures to be analyzed by defining bleach ROIs (ROIs = Regions of Interest)



(3) Bleaching

- Acquire a couple of pre-bleach images to enable the system to calculate average intensities for analysis (baseline)
- Instead of one-shot bleach define repetetive bleach events (maximum delta donor intensity)
- Start with a pre-experiment to test bleaching conditions: „Stop when intensity drops to XX %“
- Apply high laser power / iteration
- Bleach acceptor to ~ 10-20% of its initial value

(4) Time Series

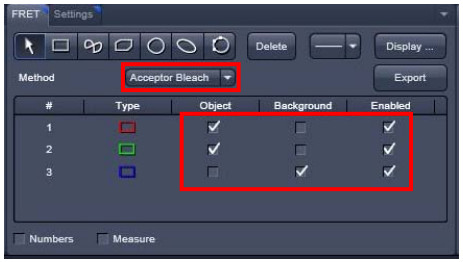
- Acquire a number of time points

FRET Detection: Acceptor Photobleaching

ZEN 2010: Data evaluation using FRET

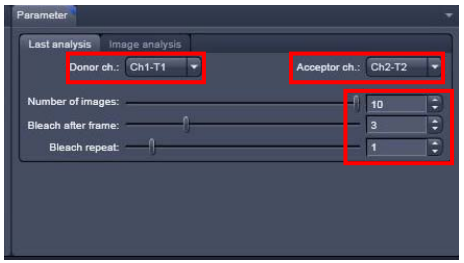
(1) FRET

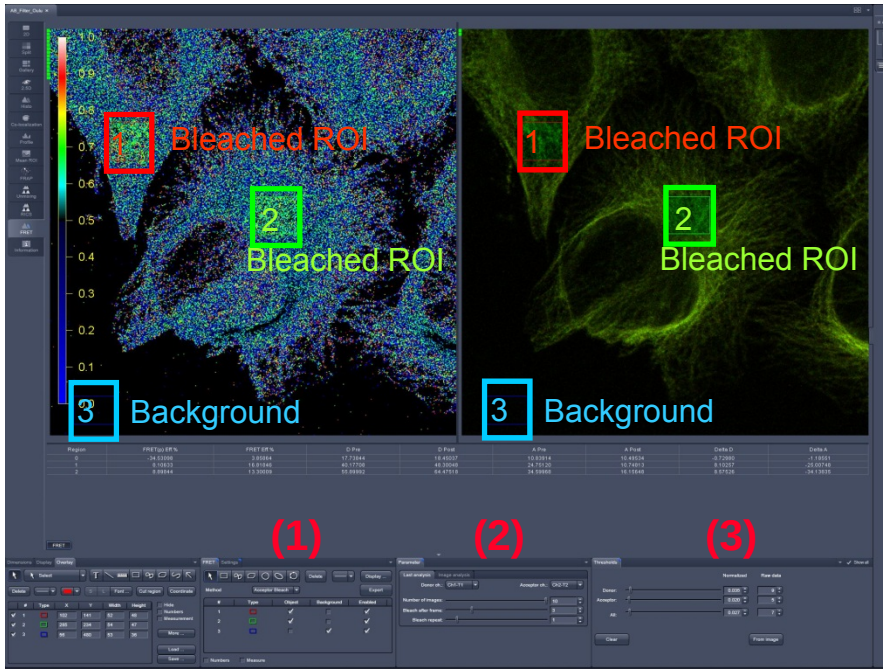
- Method: Acceptor Bleach
- Define ROIs: Enable Object(s) and Background
- NO reference possible



(2) Parameter

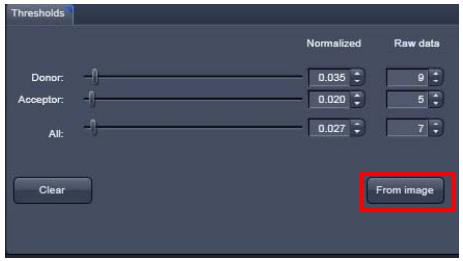
- Define acquisition and bleaching parameter





(3) Thresholds

- From image



(4) Data export

Region	FRET(p) Eff.%	FRET Eff.%	D Pre	D Post
0	-34.53098	3.85864	17.73844	18.45037
1	8.10633	16.81846	40.17708	48.30048
2	8.89844	13.30009	55.89992	64.47518

A Pre	A Post	Delta D	Delta A
10.83914	10.49534	-0.72980	-1.18551
24.75120	10.74813	8.10257	-25.00748
34.59968	16.15648	8.57526	-34.13835

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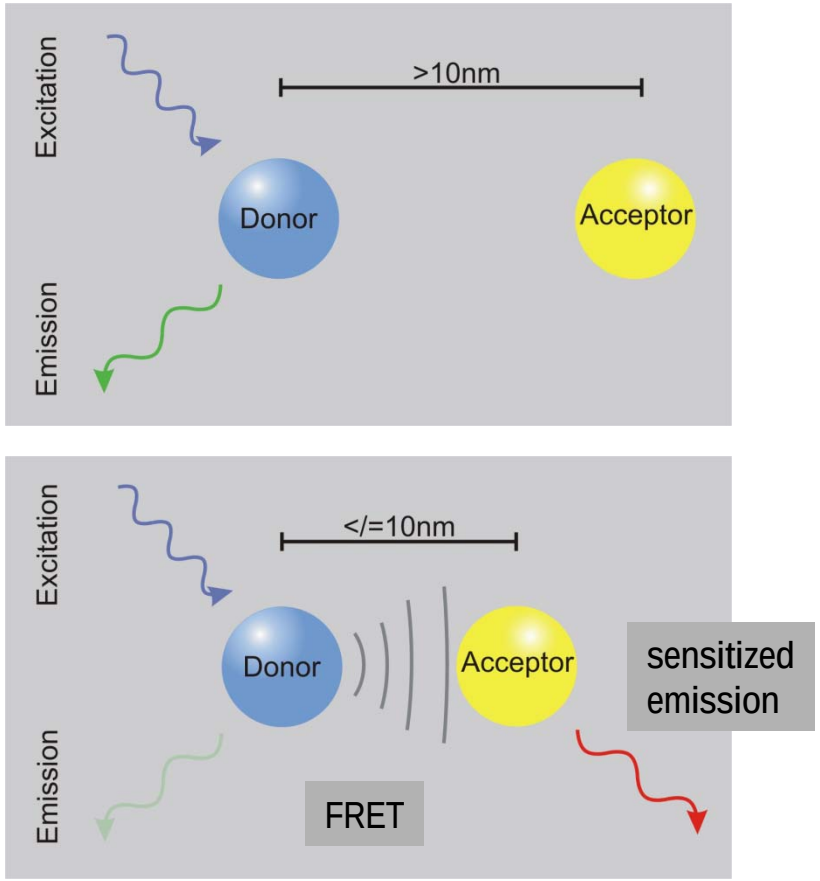
FRET Detection: Sensitized Emission
Principle of Sensitized Emission

Employing FRET (Fluorescence resonance energy transfer) as a molecular ruler in microscopy

FRET is a non-radiative transfer of an excited state from one fluorophore (donor) to another (acceptor).

FRET occurs if donor and acceptor are in close proximity (1-10 nm).

FRET permits microscopic proximity assays at the molecular level!



FRET Detection: Sensitized Emission

ZEN: Data evaluation in ZEN 2010 using FRET

(1) Imaging Setup

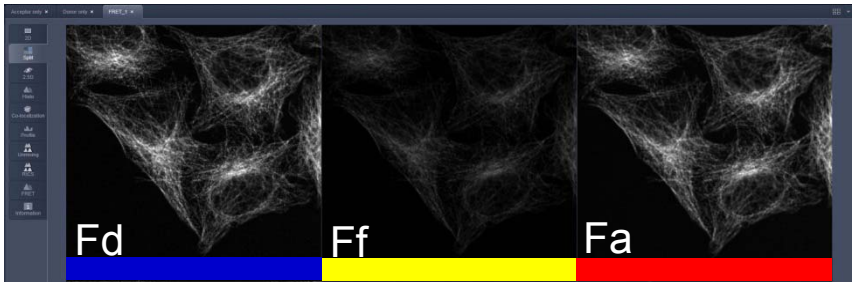
- Multitrack Channel Mode
- Linewise switch (fast)

(1) Track1: Donor (blue) + FRET (yellow)
(2) Track2: Acceptor (red)

(2) Acquire images of FRET sample and references with the same settings

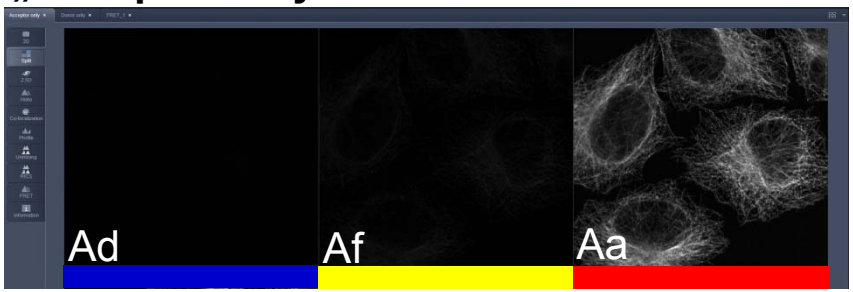
- Avoid noisy images (→Ratios)
- Adjust settings for FRET sample
- Acquire references with the same acquisition parameters

FRET



„Donor only“

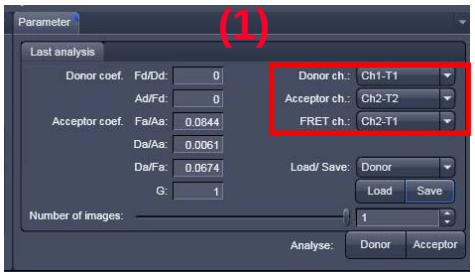
„Acceptor only

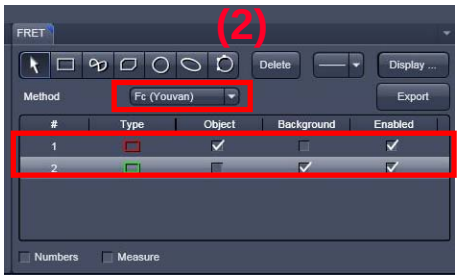


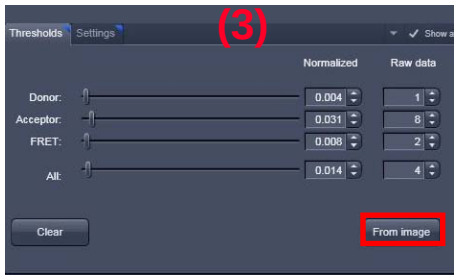
FRET Detection: Sensitized Emission

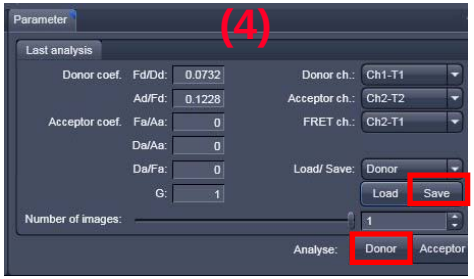
ZEN: Data evaluation in ZEN 2010 using FRET

(A) Workflow „Donor only“

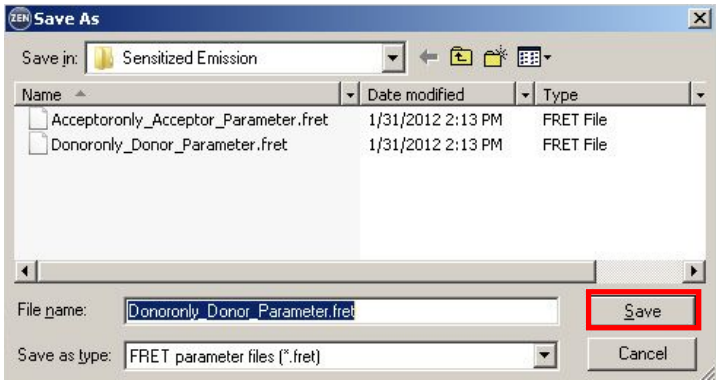








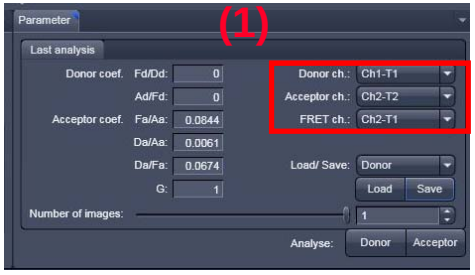
(1)	**Parameter**	Assign channels (→ Donor, → Acceptor, → FRET)
(2)	**FRET**	Method Fc Youvan
		Define ROIs / Enable Object(s) and Background
(3)	**Thresholds**	„From image“
(4)	**Parameter**	Analyse „Donor“
		Save „Donor_Parameter.fret“

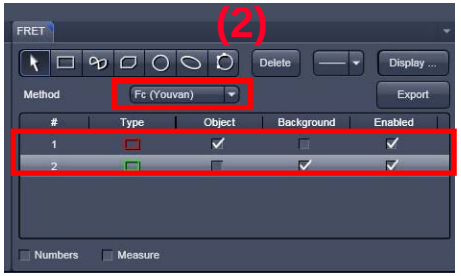


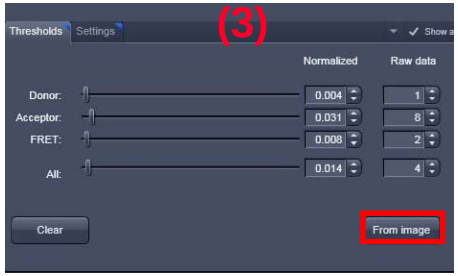
FRET Detection: Sensitized Emission

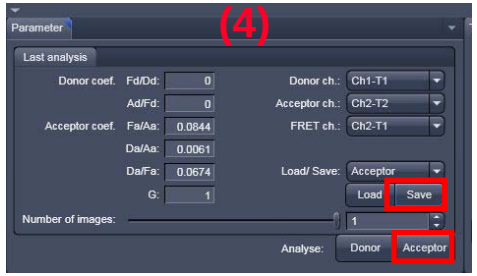
ZEN: Data evaluation in ZEN 2010 using FRET

(B) Workflow „Acceptor only“

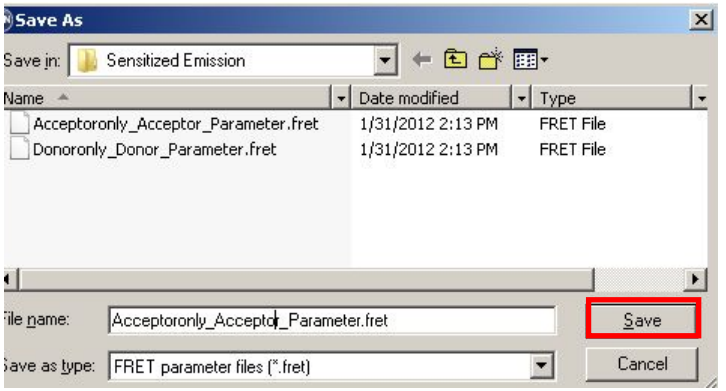








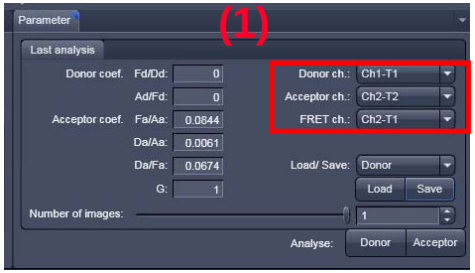
(1)	**Parameter**	Assign channels (→ Donor, → Acceptor, → FRET)
(2)	**FRET**	Method Fc Youvan
		Define ROIs / Enable Object(s) and Background
(3)	**Thresholds**	„From image“
(4)	**Parameter**	Analyse „Acceptor“
		Save „Acceptor_Parameter.fret“

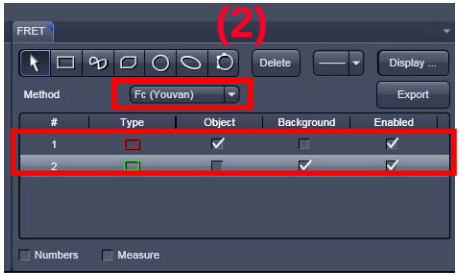


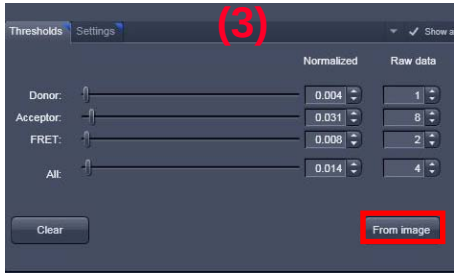
FRET Detection: Sensitized Emission

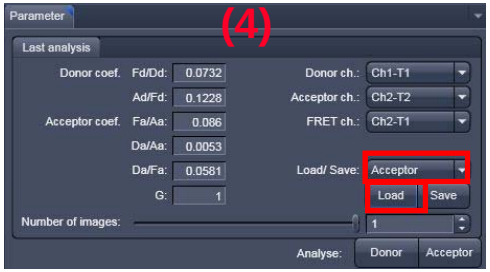
ZEN: Data evaluation in ZEN 2010 using FRET

(C) Workflow „FRET“

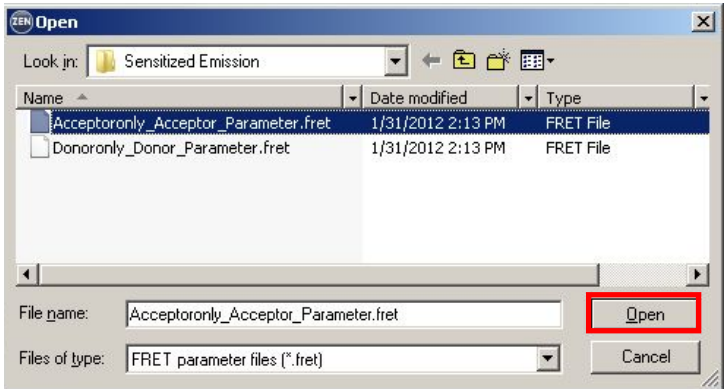








(1)	**Parameter**	Assign channels (→ Donor, → Acceptor, → FRET)
(2)	**FRET**	Method Fc Youvan
		Define ROIs / Enable Object(s) and Background
(3)	**Thresholds**	„From image“
(4)	**Parameter**	Load „Donor“
		Load „Acceptor“ (shown)



FRET Detection: Sensitized Emission
Quantifying FRET: Sensitized Emission

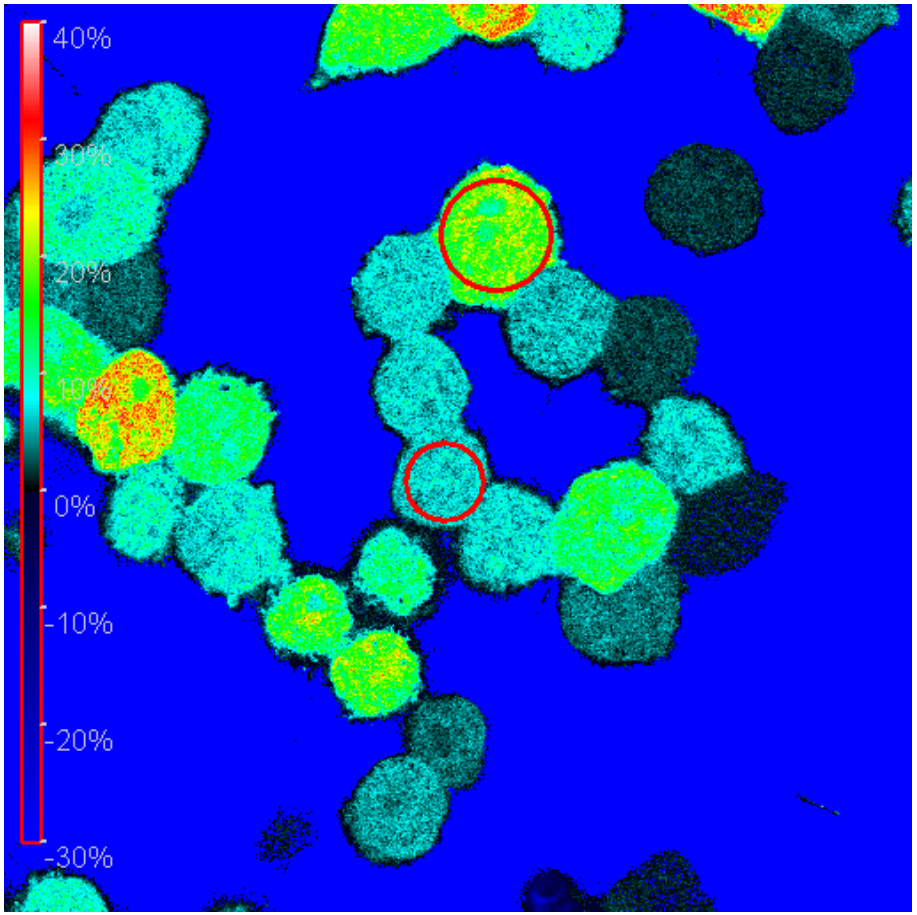
Method 1

Fc (FRETcorrected)

D.C. Youvan et al. 1997

This method calculates the absolute intensity of sensitized emission in the FRET channel as Fc. Therefore, high Fc values occur at high molecular concentrations.

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



FRET Microscopy with LSM Systems
Quantifying FRET: Sensitized Emission

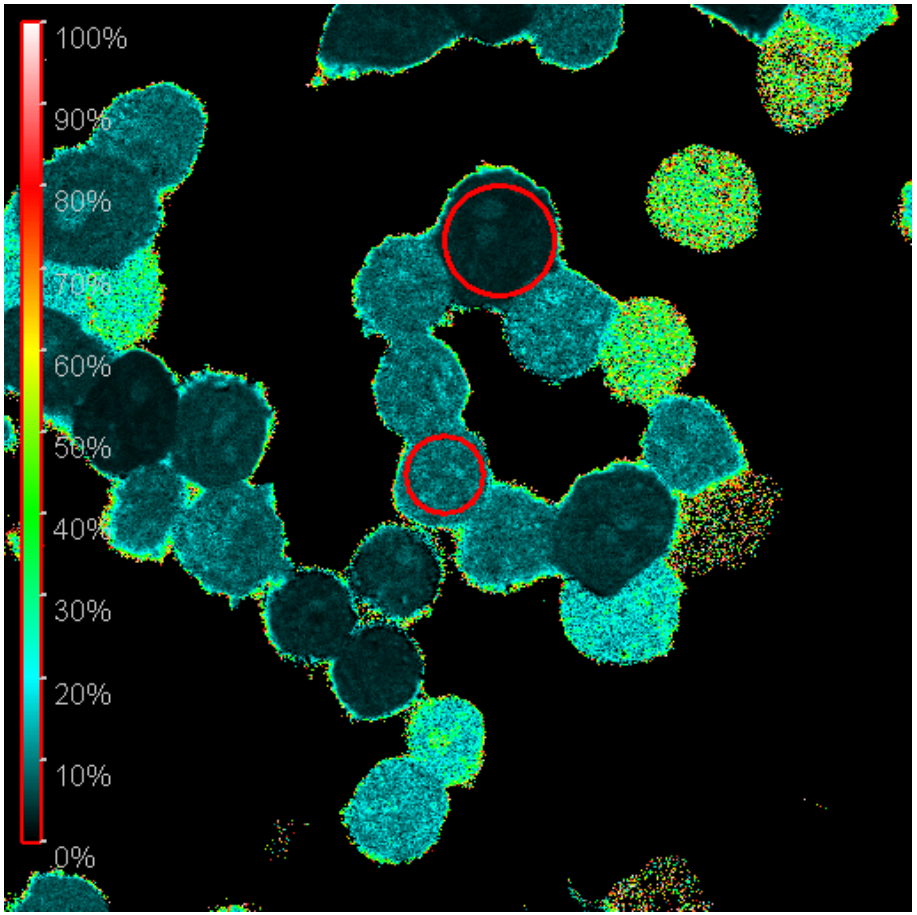
Method 2

Fn (FRET net)

G.W. Gordon et al. 1998

This method emphasizes FRET occurring at low concentrations of FRET pairs.

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



FRET Microscopy with LSM Systems
Quantifying FRET: Sensitized Emission

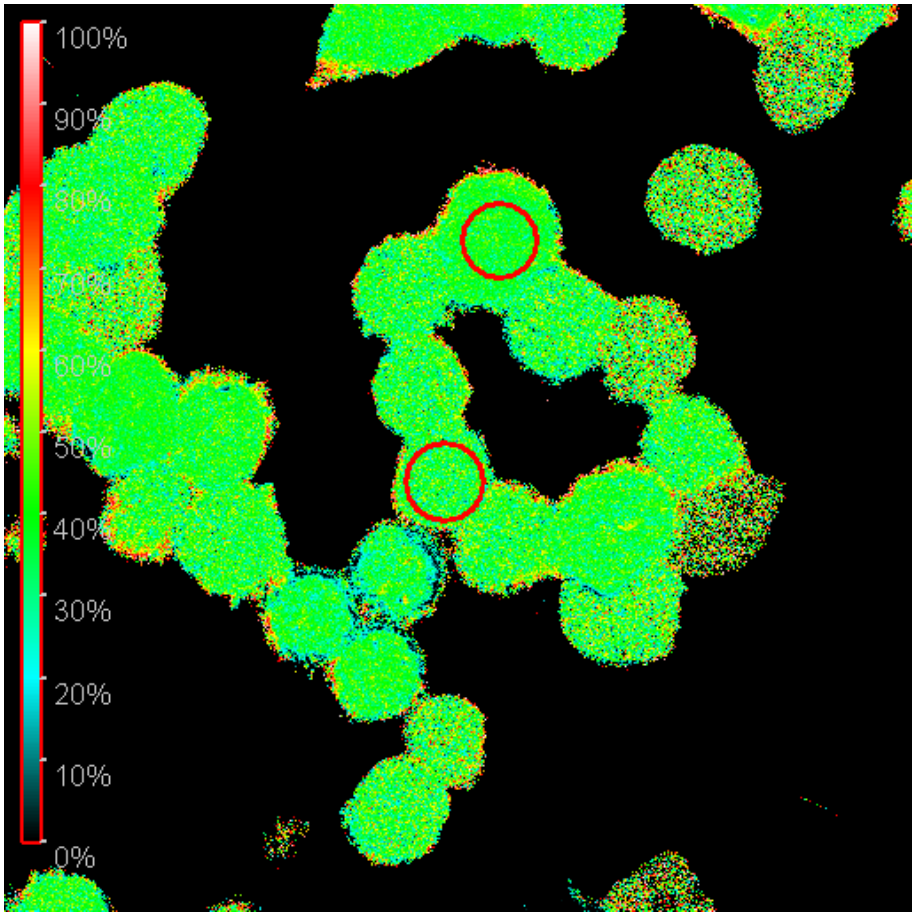
Method 3

NF (normalized FRET)

X. Xia et al. 2001

NF provides FRET values normalized for concentrations of FRET pairs.

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



Sensitized Emission – The 3 (main) Methods

Method 1: Fc (FRET corrected) D.C. **Youvan** et al. 1997
Dependend on Donor / Acceptor concentration. If its less → no FRET Signal

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

Method 2: Fn (FRET net) G.W. **Gordon** et al. 1998
Facilitates getting FRET Signals also if Donor / Acceptor signals are weak

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

Method 3: NF (normalized FRET) X. **Xia** et al. 2001
Independent of Donor und Akzeptor concentration

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