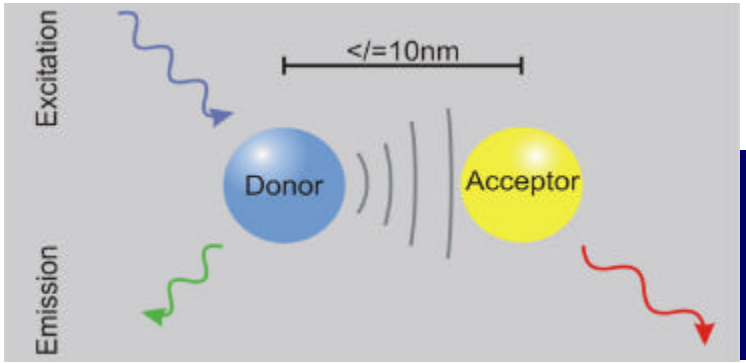
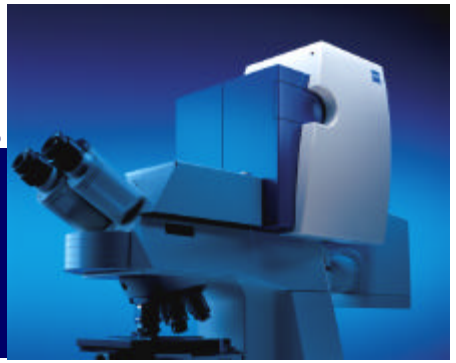
Quantitative FRET Analysis – FRET Macro Operation Guide (Rel. 3.2 Add On)



Carl Zeiss

LSM 5 Family

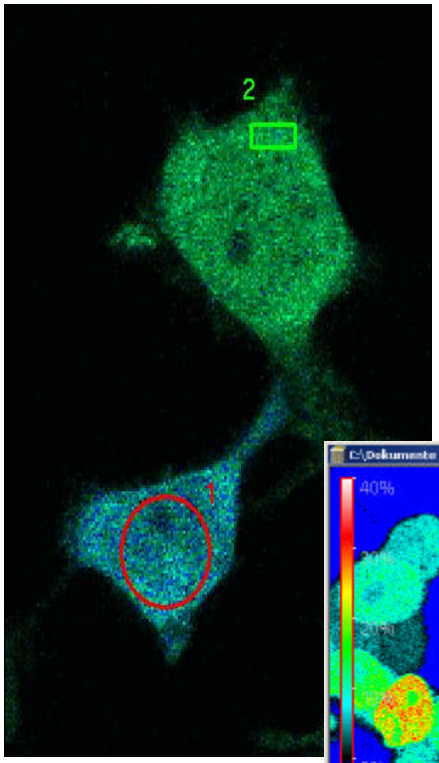


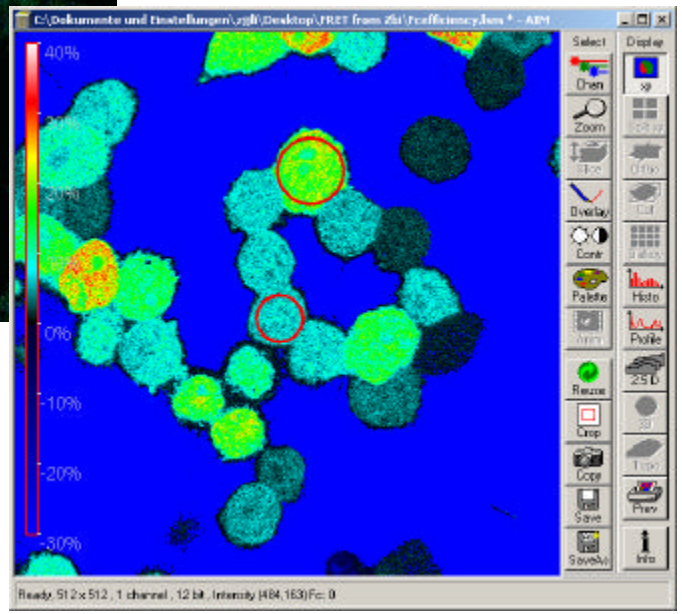
FRET Macro Guide

Aim / Content

This guide will help you to acquire FRET Data and to analyze this data with the FRET Macro (Rel 3.2 Add On) in order to get quantitative FRET efficiencies for defined ROIs. The data base on this CD contains FRET raw data which can be used instead online acquired data.

- Acquisition of Sensitized Emission FRET raw data
- Calculation of FRET values for Sensitized Emission via FRET Macro: Fc (FRET corrected), Fn (FRET net) and NF (normalized FRET)
- Acquisition of Acceptor Photobleaching data via META detector
- Calculation of FRET efficiency in Acceptor Pohtobleaching via FRET macro
- Interpretation of data





FRET Macro Guide

Acquisition of Sensitized Emission FRET Raw Data

Specimen used to acquire raw data on CD:

- HEK 293 cells culture expressing a CFP-YFP tandem as positive FRET control
- COS7 cell culture; single CFP or YFP expressing for acquisition of correction factors
- COS7 cell culture coexpressing CFP and YFP as negative FRET control

System used to acquire raw data on CD:

- LSM 510 META NLO @ Axiovert 200

Imaging settings

- *Multi Track* configuration with 3 tracks; donor excitation/donor emission detection, acceptor excitation/acceptor emission detection, donor excitation/acceptor emission detection
- Excitation 10 % 458 nm for CFP and 0,5% 514 nm for YFP excitation
- Emission detection; META detector in *Channel Mode:* 473-505 nm for CFP and 516-590nm for YFP detection

FRET Macro Guide

Acquisition of Sensitized Emission FRET Raw Data

Before Image Acquisition

- For correction of FRET data, images needs to be obtained from CFP and YFP only labeled specimen under the same conditions like the FRET specimen
- Check all three specimen to find cells of similar intensities for imaging
- The order of channels should be the same all three images (see *Set order of tracks* next page)

Acquire a 3 channel Multi Track image from the CFP only labeled specimen

(*Sens Em CFP Contr* in FERT Demo Database)

Acquire a 3 channel Multi Track image from the YFP only labeled specimen

(*Sens Em YFP Contr* in FERT Demo Database)

Acquire a 3 channel Multi Track image from the FRET specimen

(*Sens Em FERT* in FERT Demo Database)

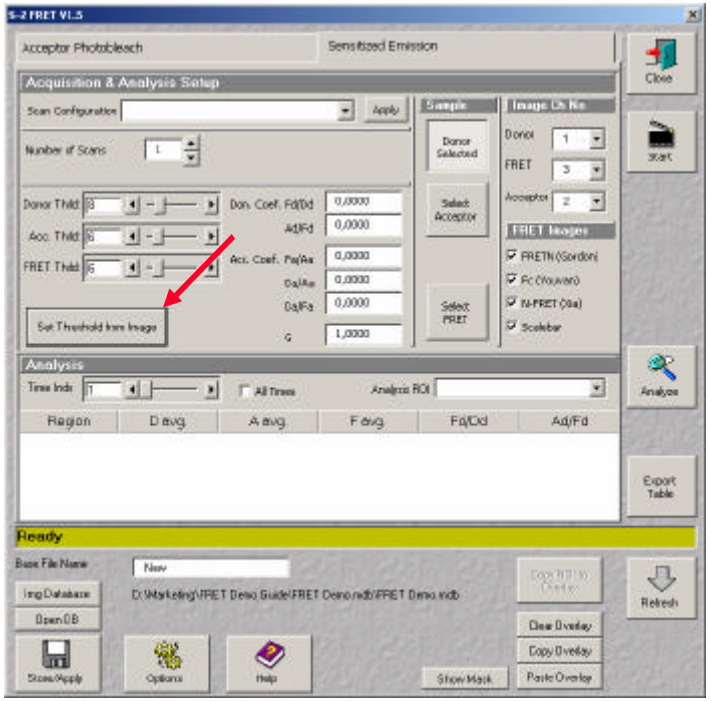
Note: Unmixed data can only be used for Acceptor Bleach analysis but not for Sensitized Emission!

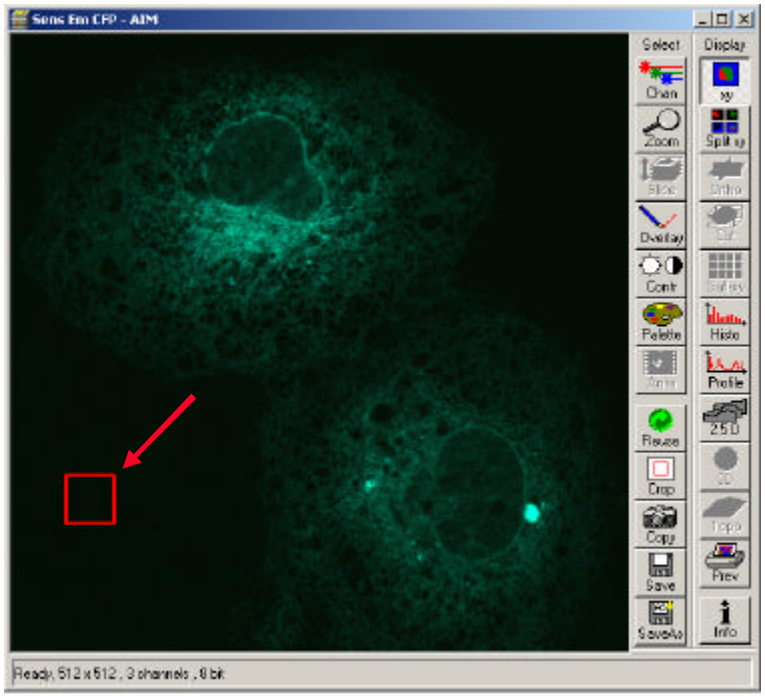
FRET Macro Guide

Analysis Sensitized Emission FRET, Donor Threshold Setting

- Open FRET Macro, select *Sensitized Emission*
- Open CFP only labeled control image (Sens Em CFP Contr, display image in X/Y (overlay) mode
- Adjust the number of channels in *Image Ch No* Window (arrow) to the order of channels in the image
- Click *Donor Selected* in *Sample* window
- Use one of two options to set threshold values
- A) Click Set Threshold from Image, draw a threshold measure area into the background of the CFP image (use x/y [overlay display] to apply the same ROI to all 3 channels)

Methode A

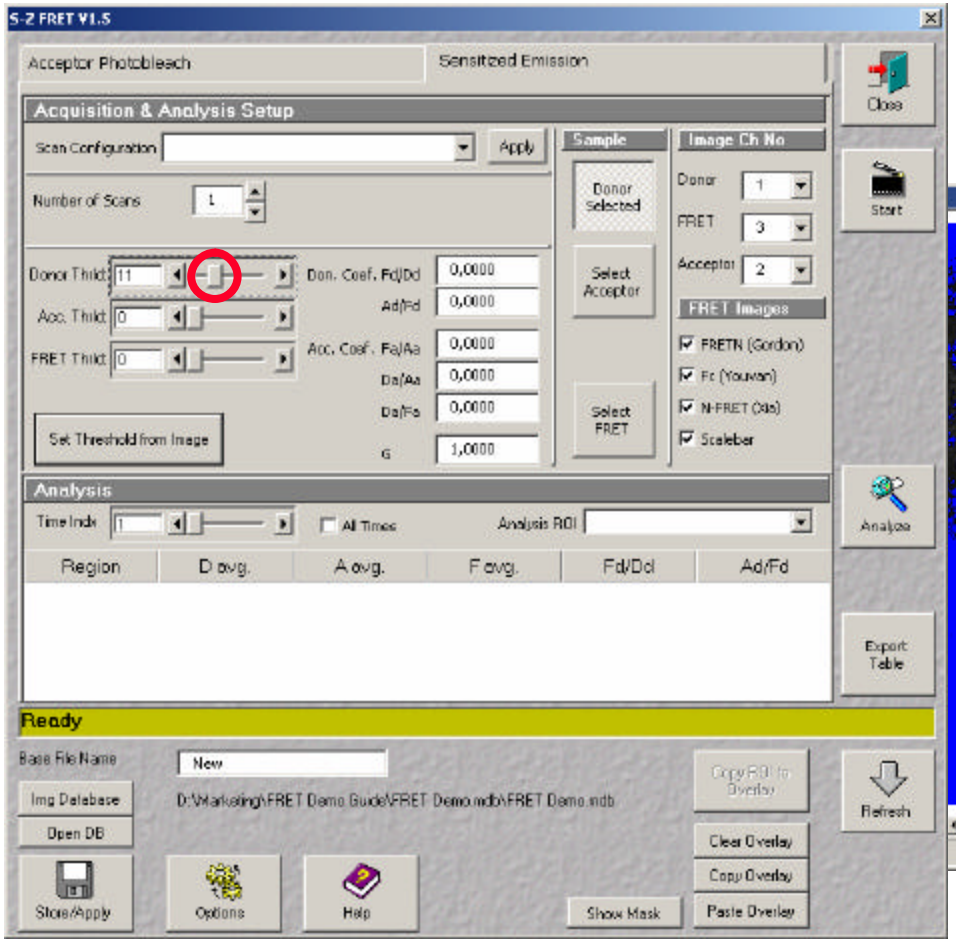




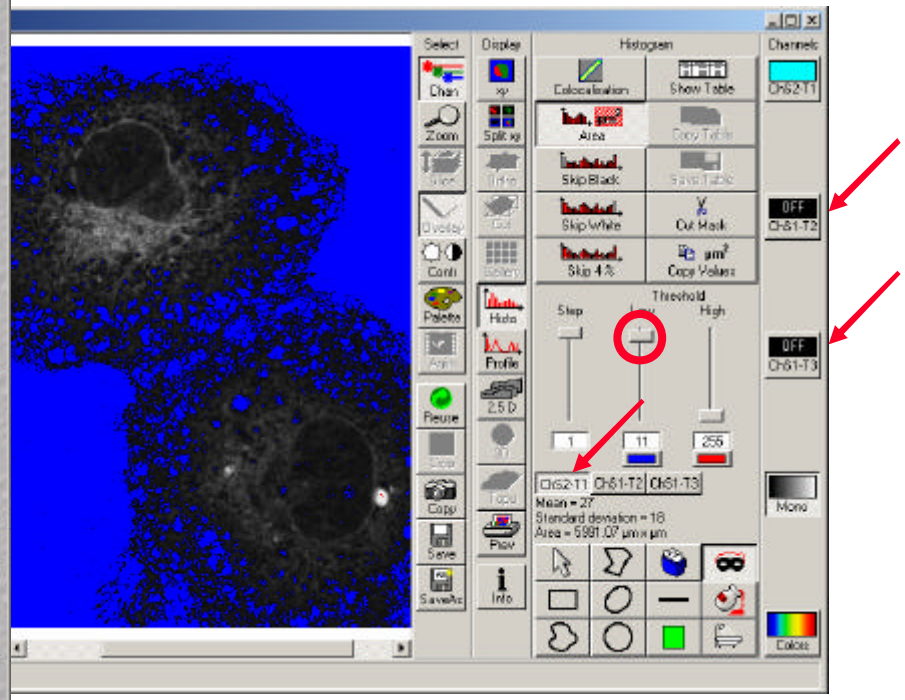
FRET Macro Guide

Analysis Sensitized Emission FRET, Donor Threshold Setting

- B) Click *Histo* in image window, activate donor channel only (display **and** channel selection in *Histo)*, adjust *Threshold Low* slider until you see only structures of interest, adjust donor threshold in the FRET macro accordingly, proceed in the same way for the other two channels



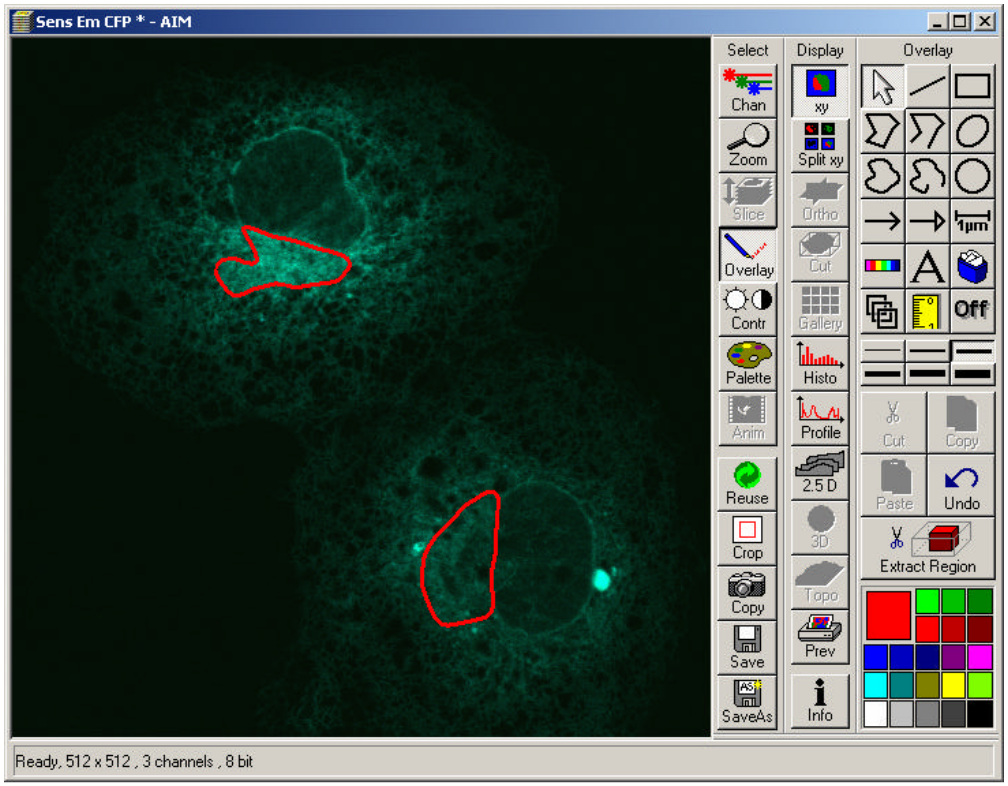
Methode B

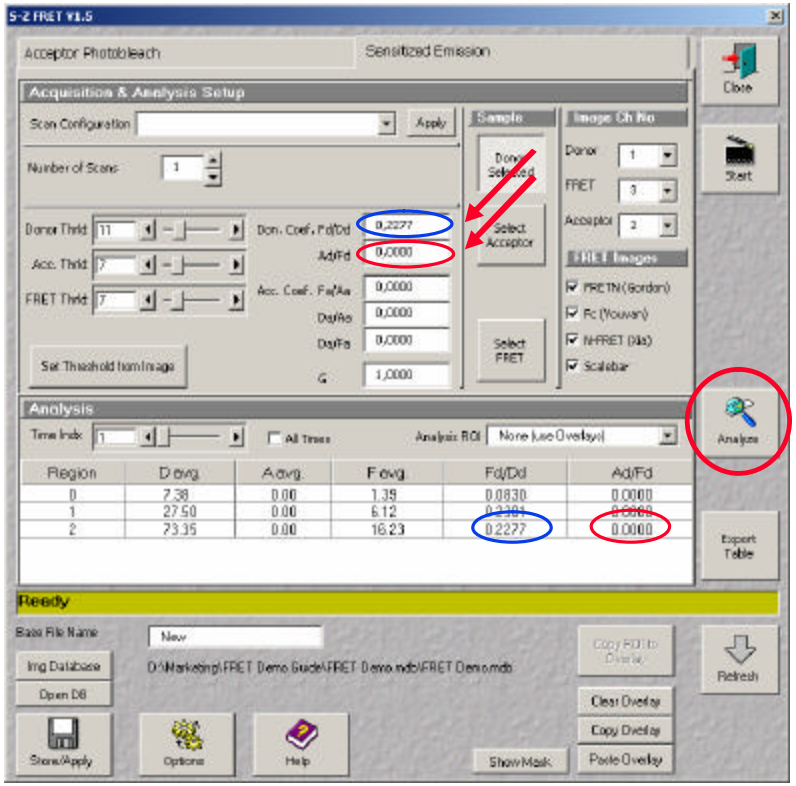


FRET Macro Guide

Analysis Sensitized Emission FRET, Analysis of Donor Coefficients

- Use *Overlay* function (image window) to draw a overlay region around a structure which correspond to those structures which you want to analyze for FRET
- When more then one overlay is drawn, only the last one will be considered for coefficient analyses
- Click Analysis in FRET macro, coefficient parameters will be calculated for each overlay region and the entire image as well (region 0), the coefficients from the region with the highest number will be copied in the corresponding windows (arrows)

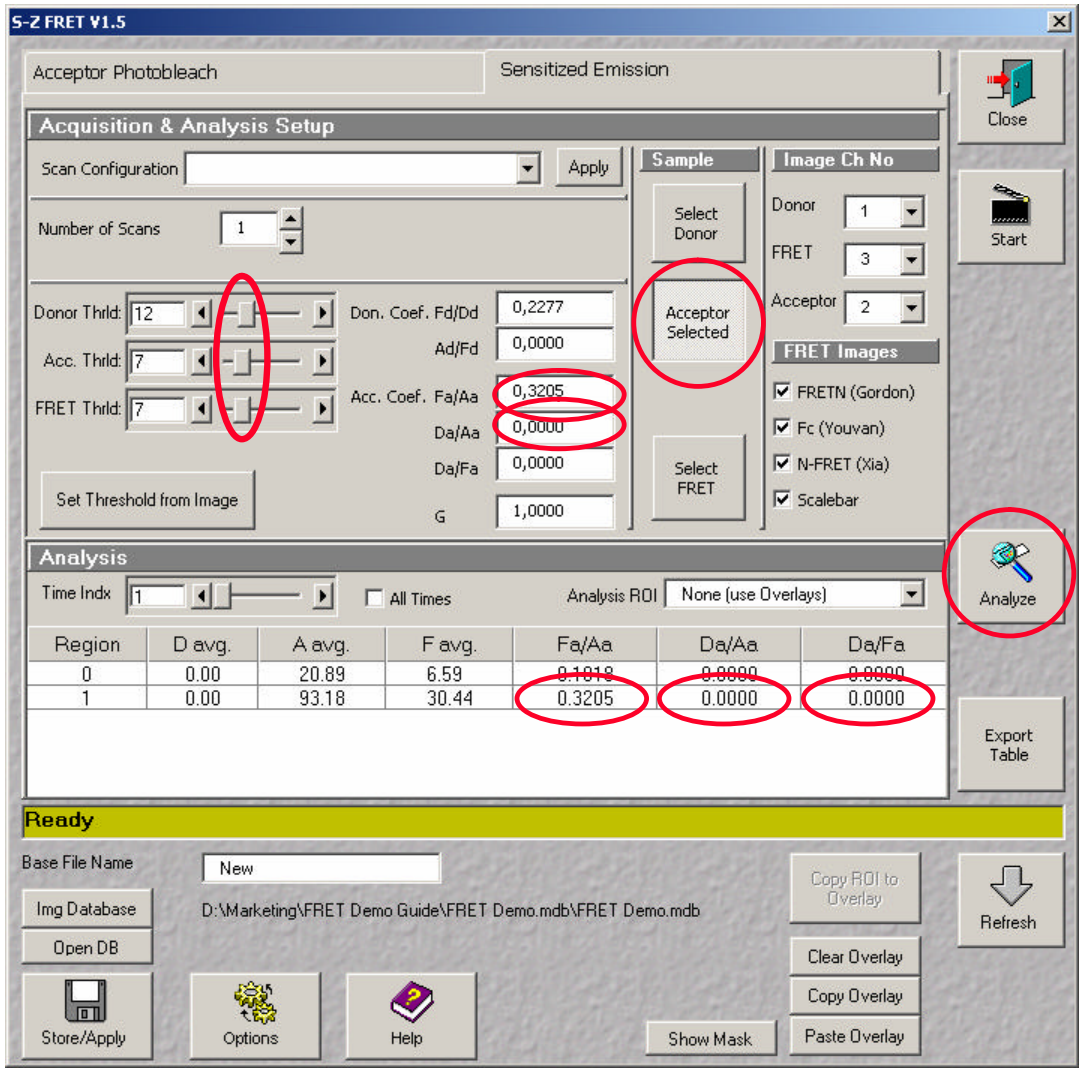




FRET Macro Guide

Analysis Sensitized Emission FRET, Acceptor Threshold and Coefficients Settings

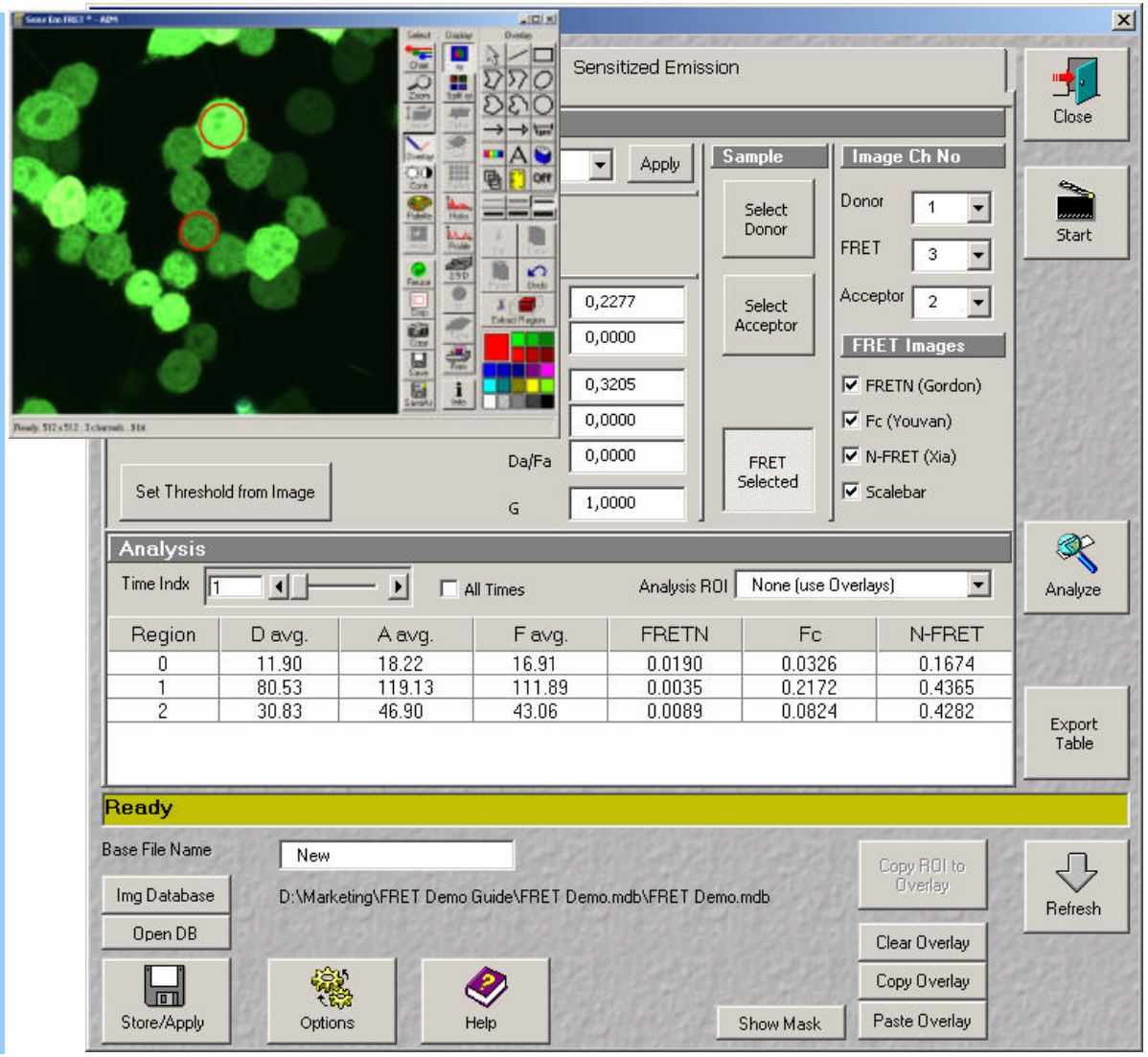
- Proceed as described before, now for the YFP only image (Sens Em YFP Contr in image database)
  - Define threshold setting for all three image channel
  - Calculate acceptor coefficients



FRET Macro Guide

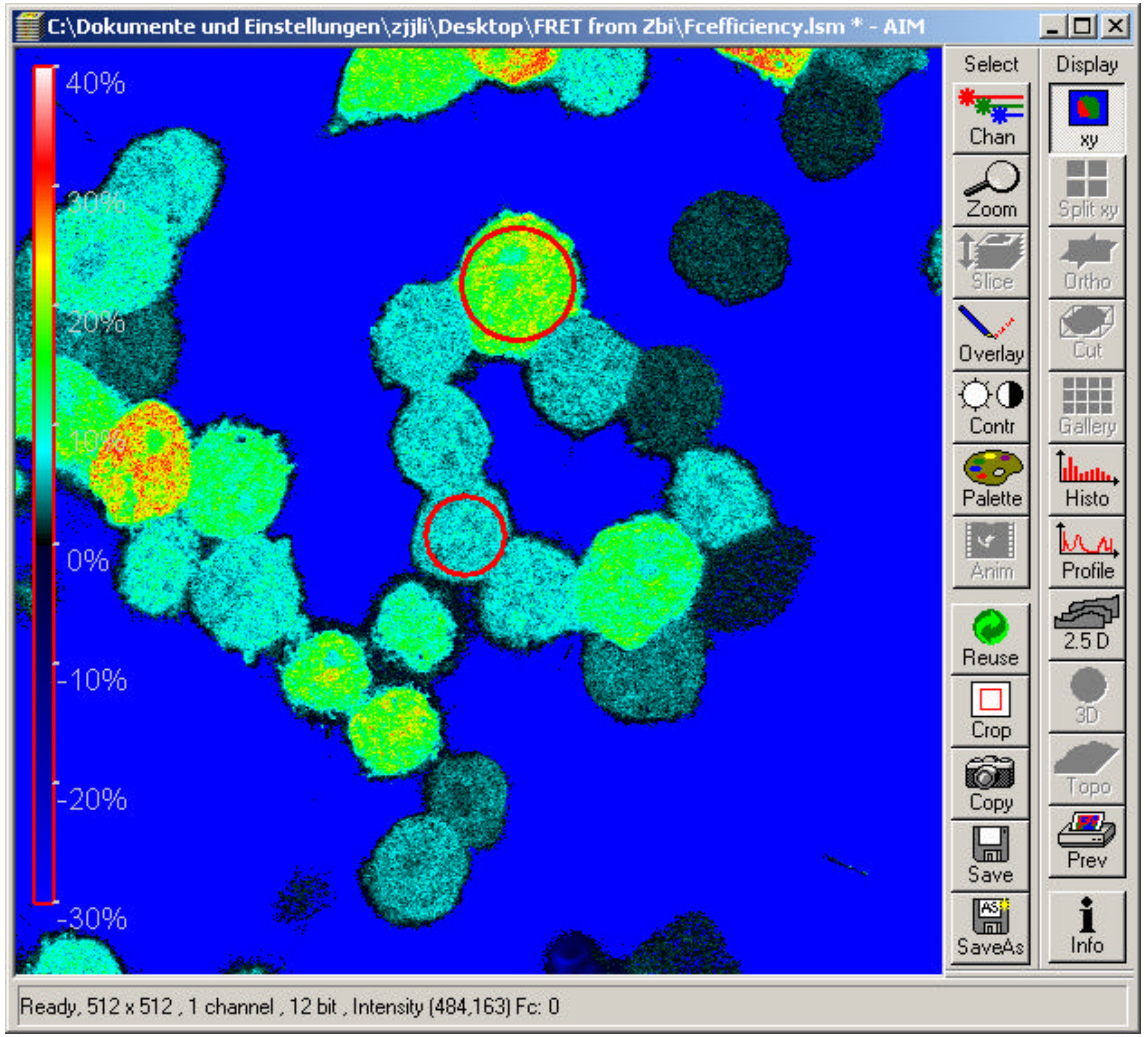
Analysis Sensitized Emission FRET Raw Data

- Select FRET image e.g. positive control in data base (Sens Em FERT)
- Define the threshold as described above
- Use the Overlay function of image tool bar to select structures for FRET analyses from the image
- Check the the different methods in the FRET macro window to apply for analysis (arrow)
- Press *Analyze*
- The system calculates a FRET image for each selected Method
- The system calculates FRET numbers for each selected Method and image region in the *Analysis* window of the FRET macro (region “0” is the whole image)



FRET Macro Guide

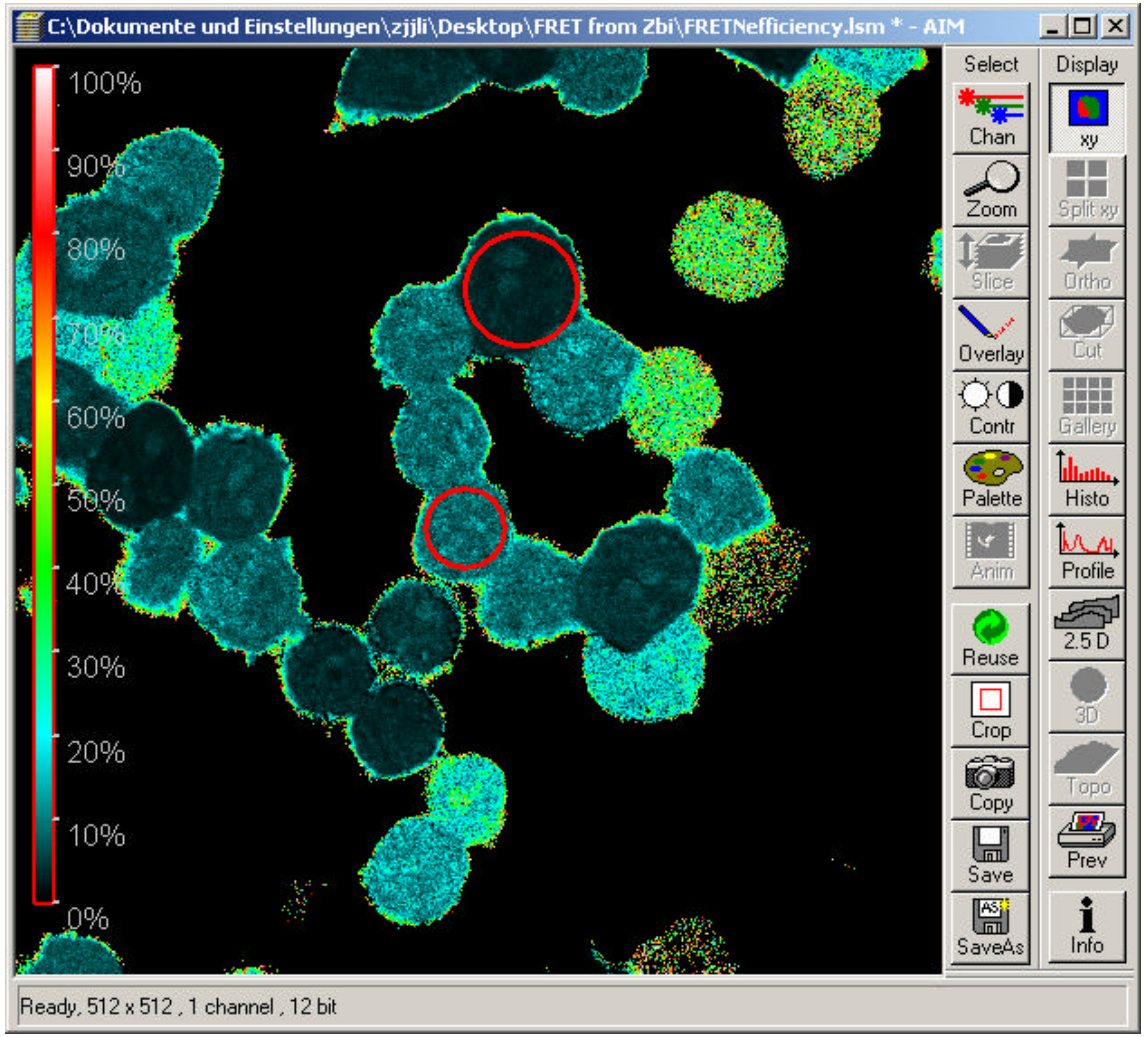
Interpretation of Sensitized Emission FRET Data



- The Fc (Youvan) FRET values are corrected for emission and excitation crosstalk, but not corrected for the concentration of donor and acceptor
- This results in FRET values proportional to the to the protein concentration (donor and acceptor)

FRET Macro Guide

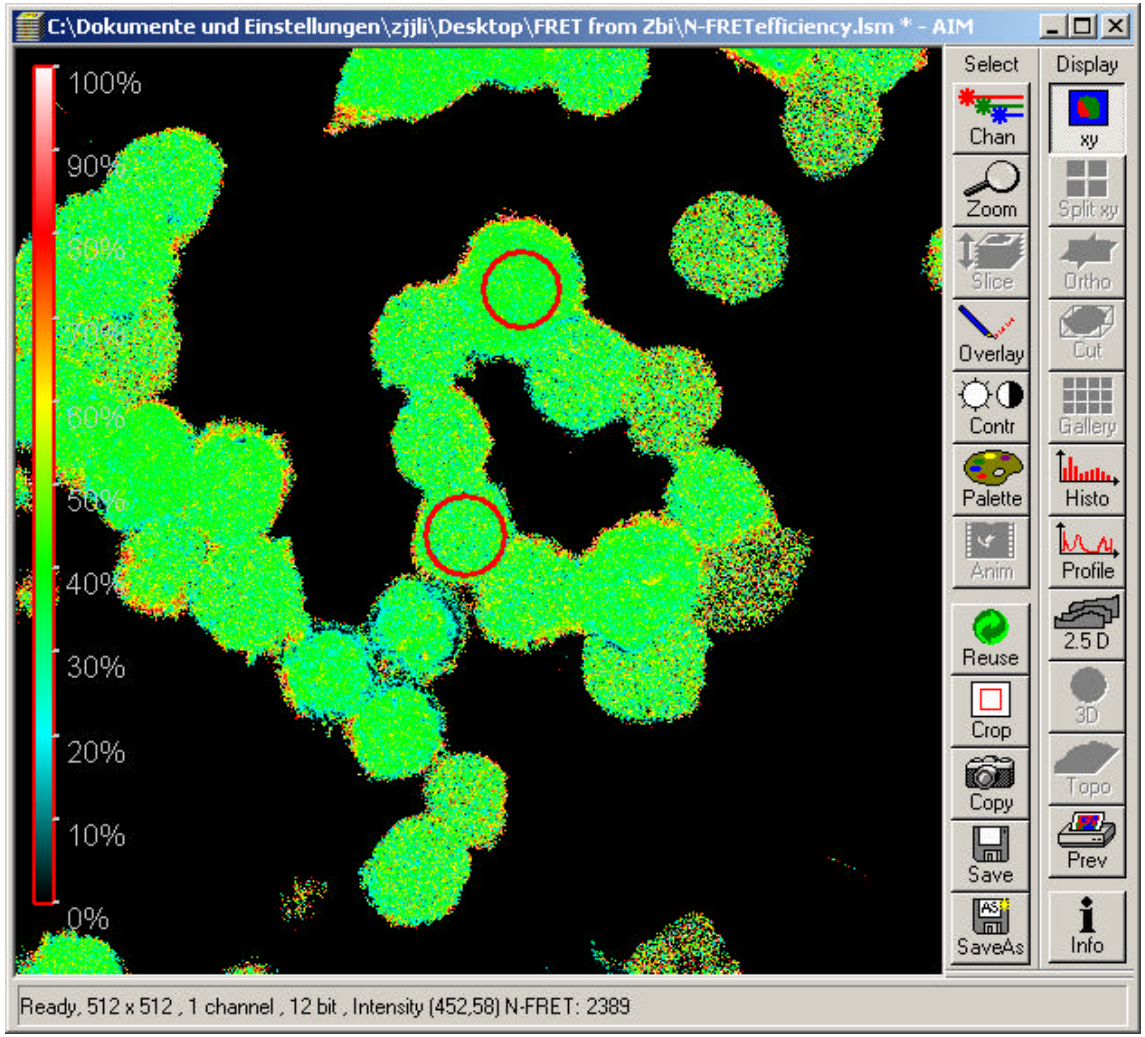
Interpretation of Sensitized Emission FRET Data



- The FRETN (FRET net, Gordon) FRET values are corrected for emission and excitation crosstalk.
- In addition this method over-compensates for protein concentration, resulting in FRET values inverse proportional to protein concentration (donor and acceptor)

FRET Macro Guide

Interpretation of Sensitized Emission FRET Data



- N-FRET (normalized FRET, Xia) values are corrected for emission and excitation crosstalk and are normalized for the concentration of donor and acceptor
- Since the donor and acceptor are connected via a molecular linker the FRET efficiency in the whole image should be constant independently from the local concentration of the donor-acceptor construct
- The result in the image looks like expected: a homogeneous distribution of N-FRET values

FRET Macro Guide

Sensitized Emission FRET – Add On

Faster Acquisition of Sensitized Emission raw data

Instead of acquiring the raw data with a 3 track Multitrack configuration one can also use a 2 track configuration were one of the tracks acquires the donor and the FRET Ch simultaneously:

- Track 1
  - Excitation 458 or 405nm (if available)
  - Emission detection; META detector in *Channel Mode*: 473-505 for CFP (Ch 1; donor), 516-590nm (Ch2; FRET)
- Track 2
  - Excitation 514nm
  - Emission detection; META detector in *Channel Mode*: 516-590nm (Ch3; acceptor)

FRET Macro Guide

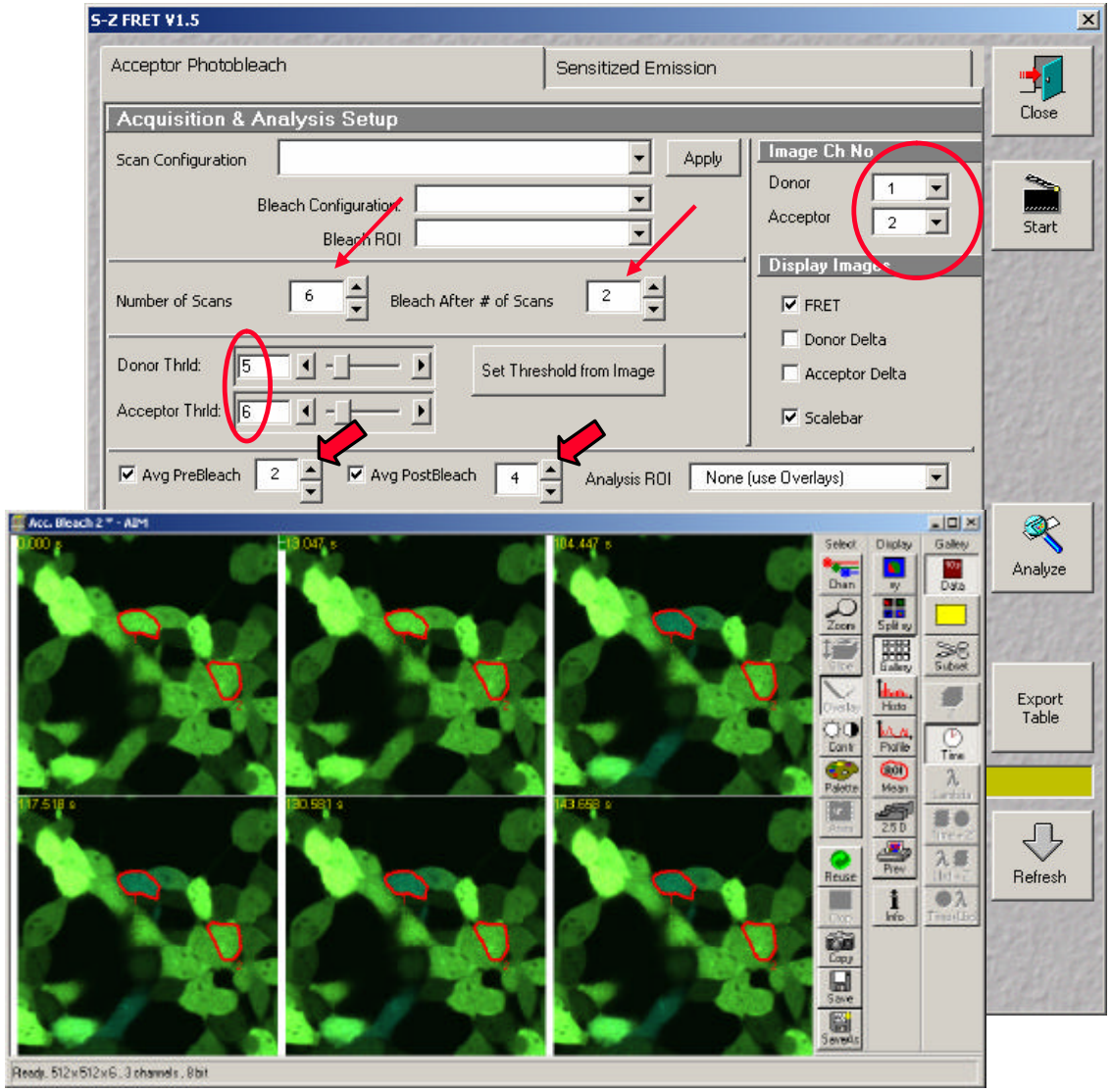
Acquisition of Acceptor Photobleaching Data for FRET Macro Analysis

- Set up time series of two channel images (donor excitation, donor emission detection / acceptor excitation , acceptor emission detection needs to be acquired
- Include a bleach routine into the Time Series, acquire a couple (5-10) of pre and after bleach images to enable the system to calculate average intensities for analysis
- Select the cells / structures to be analyzed by defining bleach ROIs
- Apply a high laser dosage (multiple iterations) to bleach the acceptor to app. 20% of its initial intensity at one shot
- In contrast to the alternative method on this CD (Acceptor Photobleaching – Manual Data Evaluation) here, the only need is the acquisition of pre and post bleach images from donor and acceptor (no intermediate data are necessary)
- Alternative to a channel image a time/bleach series of a lambda stack can be also acquired. For further processing the lambda stack series needs to be unmixed in to a series of two channel images
- In the following example a time/bleach series of three channel image was acquired. Only the donor and the acceptor channel, but not the FRET have been used for analyses (*Acc. Bleach 2* in FRET Demo Database)

FRET Macro Guide

Analysis of Acceptor Photobleaching Data via FRET Macro

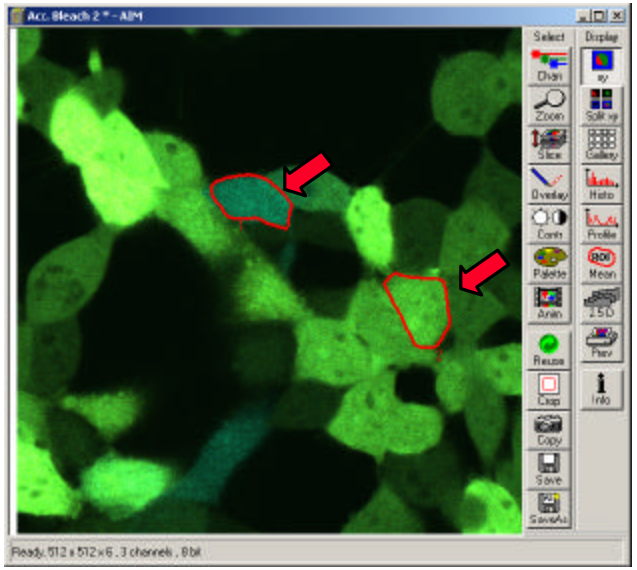
- Open FRET macro, select *Acceptor Photobleaching*
- Adjust the number of channels in *Image Ch No* Window to the order of channels in the image (circle)
- Define threshold values in XY overlay display as described for Senstized Emission analyses
- Indicate fill in number of scans and Bleach after # of Scans (arrows)
- Indicate the number of images for pre and after bleach averaging (total number of images was 6, bleaching was applied after the second image, thick arrows)

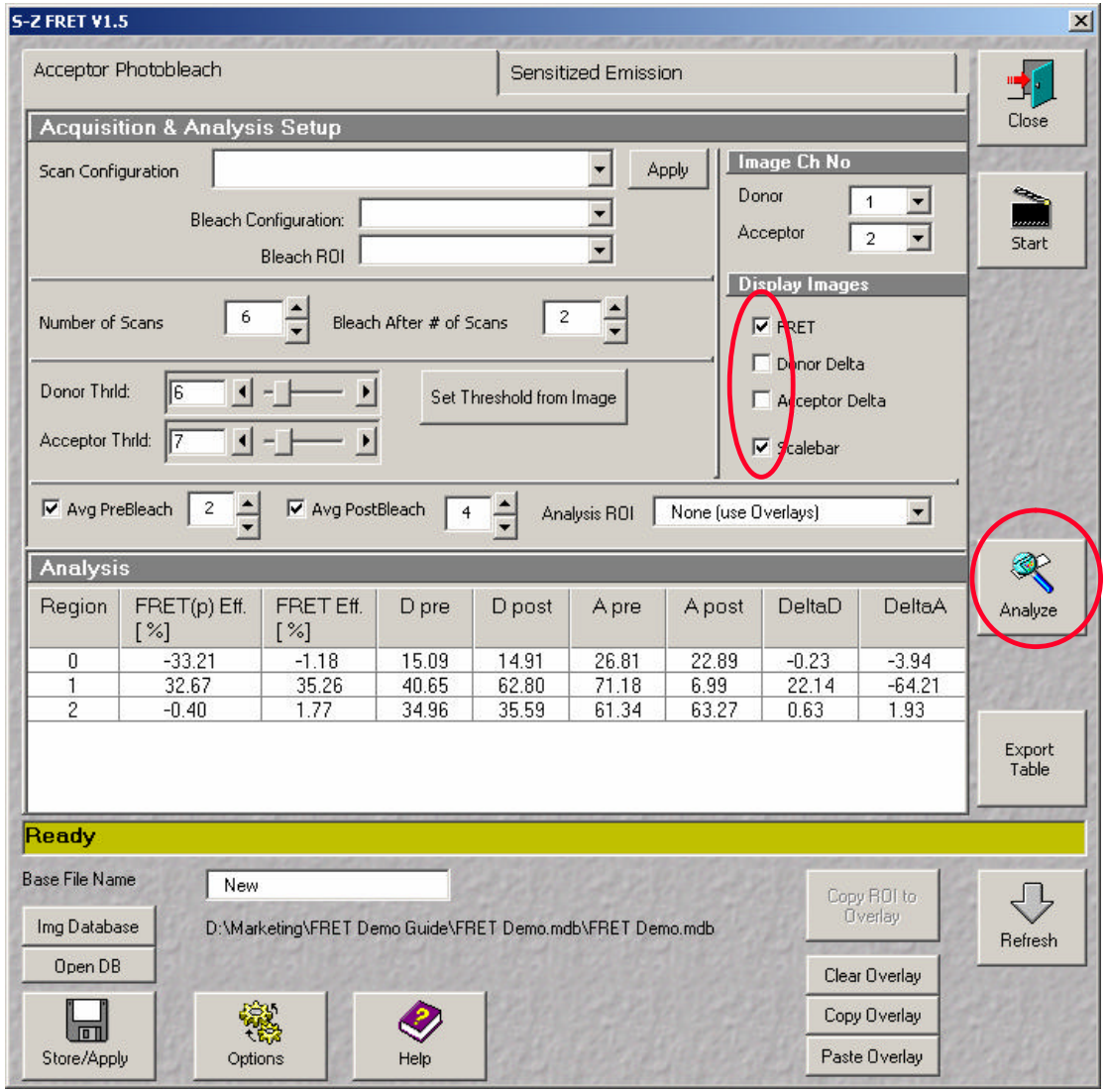


FRET Macro Guide

Analysis of Acceptor Photobleaching Data via FRET Macro

- Select the areas to be analyzed via ROI list or use the Overlay function in image tool bar to draw overlays
- Check FRET and Scalebar box in Image Display dialogue of FRET macro
- Press Analyze

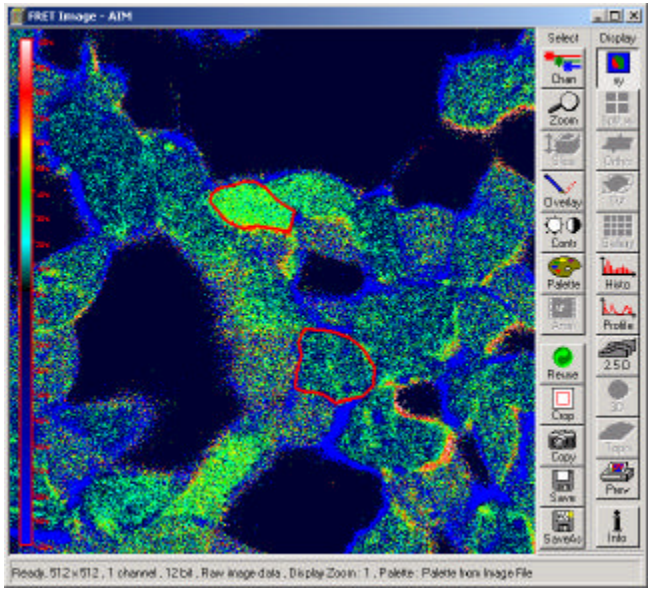


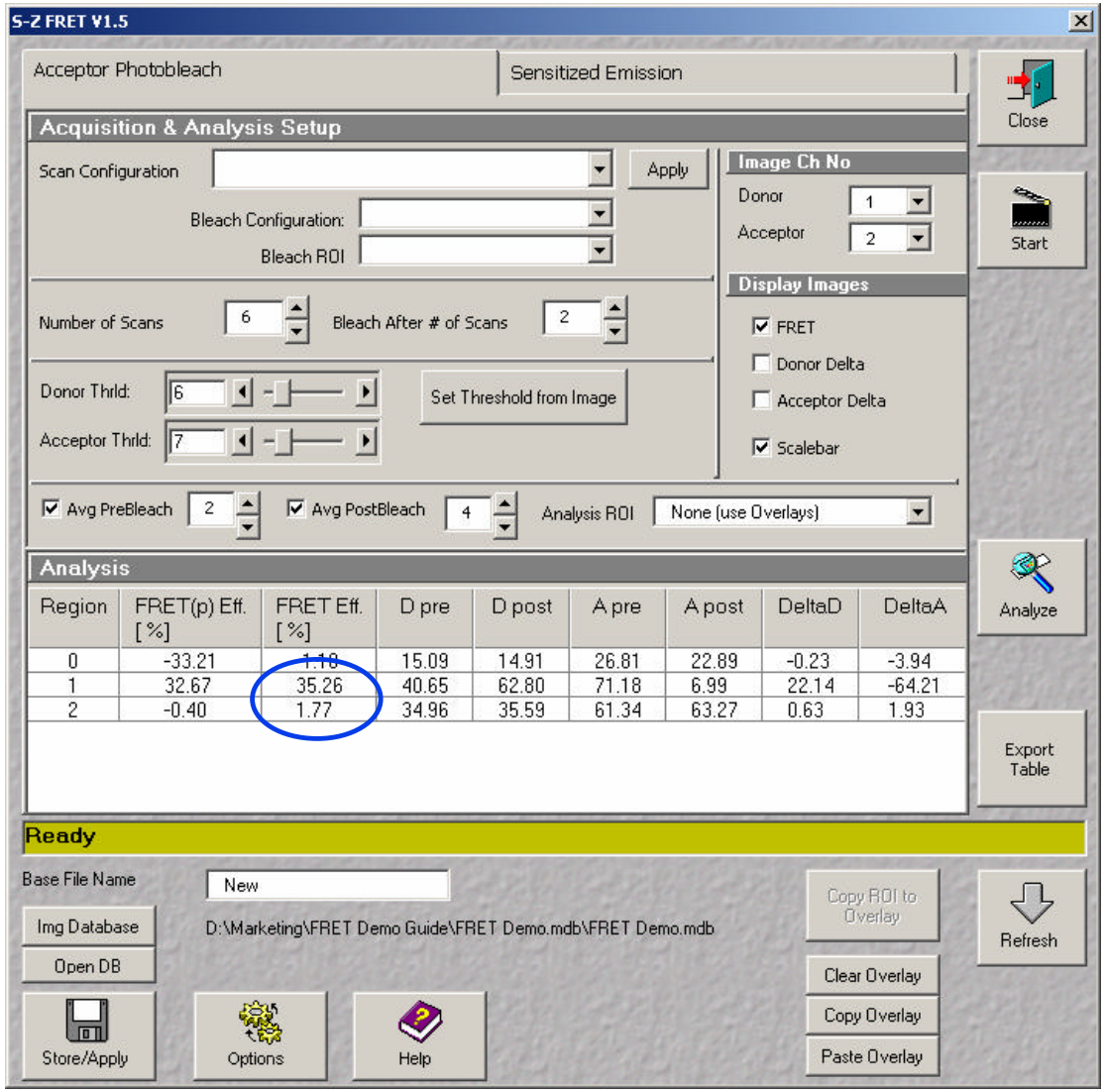


FRET Macro Guide

Results of Acceptor Photobleaching Analysis via FRET Macro

- The FRET efficiencies of the bleached cell (upper overlay) is 35.2%
- For the unbleached cell the system calculated an efficiency of 1.7%





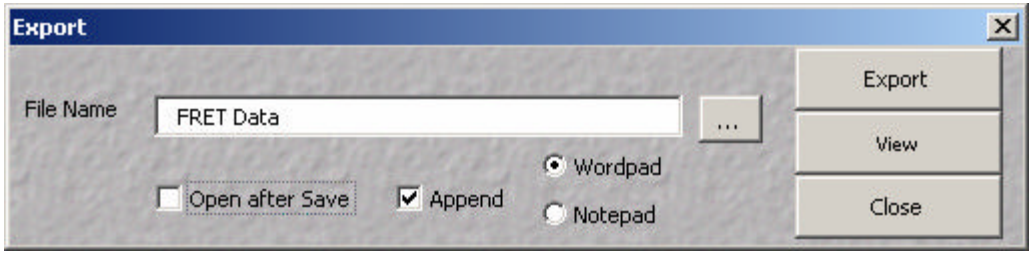
FRET Macro Guide

Interpretation of Acceptor Photobleaching Results

- The macro calculated a FRET efficiency 35%
- If compared to the value determined by manual calculation (38%) the macro calculated a little lower FRET efficiency for the same specimen (the estimated FRET was app. 40%)
- This is due to two reasons:
  1. In this example the macro used raw date not proceeded to Emission Fingerprinting – depending from the impact of crosstalk the dynamic range is lower then after unmixing
  2. The macro uses the donor value after bleaching for the calculation (even if the acceptor is not totally bleached). The manual method extrapolates the donor intensity increase to the estimated total bleaching of the acceptor.
- For further reading about the manual calculation see: H.Amiri et al. in Cell Calcium (2003)
- Please find also the Talk “Live cell Imaging” on this CD for some introduction into FRET

FRET Macro Guide

Data Export



- Numerical date calculated from the macro can be exported to either Wordpad or Notepad
- The option *Append* in the *Export* window allows to add data from a new analysis session to the previous file. This function might be helpful while analyzing the progression of FRET in a time series e.g.