LSM FRET Tool Version 1.5
for Carl Zeiss AIM Software Release 3.5

(last update: May 17, 2005, zi)

Introduction:
The LSM FRET Tool is designed to provide efficient acquisition and analysis of FRET experiments.
Two different published methods are implemented:

a) Acceptor Photobleaching, and
b) Sensitized Emission, also known as Channel FRET (after Gordon, Youvan, Xia).

This manual is not meant as an introduction to FRET itself. Please refer to the literature references given at the end of the following pages to read more about the implemented methods. Do not hesitate to ask your Carl Zeiss LSM specialist if you have questions about how to use the macro. Also, if you have suggestions for improvements or additional functionality, you are most welcome to share these.
Soon there will be a Quick Start guide for the LSM FRET Tool available.

The LSM FRET Tool also allows for analysis of already acquired image sets stored in form of multi-channel LSM images.
The standard functions of the LSM software can be used to import image files (menu File - Import) and create LSM multi-channel images (menu Process - Copy).

Starting the FRET Tool for the first time will open the Acceptor Photobleaching tab. Also, the macro reopens with the same view and parameters used during the last session.

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Acceptor Photobleach | Sensitized Emission

Close

Acquisition & Analysis Setup

Scan Configuration FRETBleach Apply

Bleach Configuration: BleachAcceptor

Bleach ROI Bleach1ROI

Image Ch No

Donor 1

Acceptor 3

Start

Number of Scans 4 Bleach After # of Scans 2

Display Images

FRET

Donor Delta

Acceptor Delta

Scalebar

Donor Thrld: 6

Acceptor Thrld: 8

Set Threshold from Image

Avg PreBleach 2 Avg PostBleach 2 Analysis ROI None (use Overlays)

Analysis

Region	FRET(p) Eff.	FRET Eff.	D pre	D post	A pre	A post	DeltaD	DeltaA

Analyze

Export Table

Ready

Base File Name test

Img Database

Open DB

E:\FRET Demo.mdb\FRET Demo.mdb

Copy ROI to Overlay

Refresh

Clear Overlay

Copy Overlay

Paste Overlay

Store/Apply

Options

Help

Common dialog controls:

Close: Exits the LSM FRET Tool.

Refresh: Update changes of drop down list contents after changes through the main program.

Copy ROI to Overlay: Converts the ROIs defined in the **Analysis ROI** drop down list into overlay items.
This function is useful to copy the bleach ROI into the analysis image and then add more overlays for control/non-bleached areas and/or background.

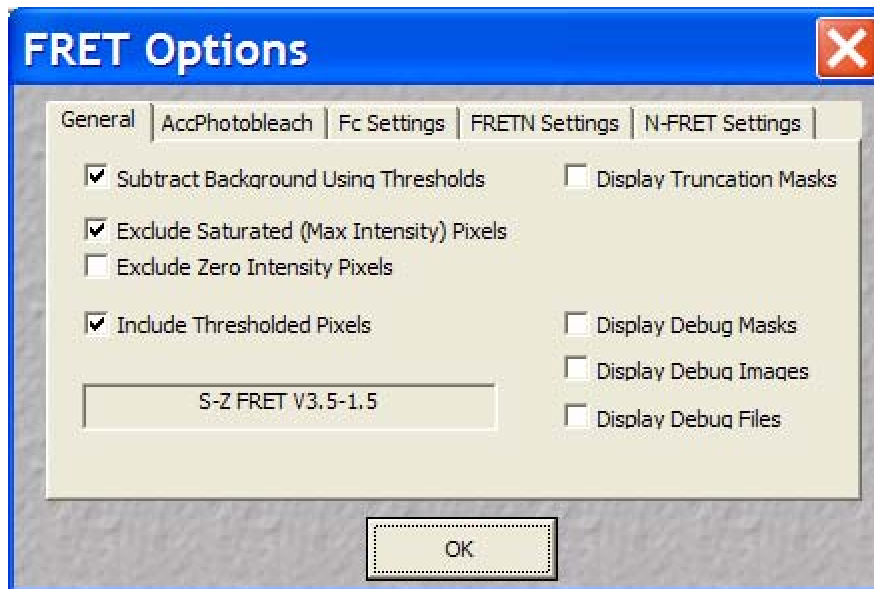
Clear Overlay: Delete all overlay items on current image.

Copy Overlay: Copy overlay items from current image into clipboard.

Paste Overlay: Paste overlay items from clipboard into current image.

Help: Displays this document.

Options: Calls the options dialog:



Subtract Background Using Thresholds: Threshold value will be subtracted from all pixels before FRET calculations (default: checked).

Exclude Saturated (Max Intensity) Pixels: Excludes pixels from calculation for which at least one of the 3 channels is at maximum intensity (default: checked).

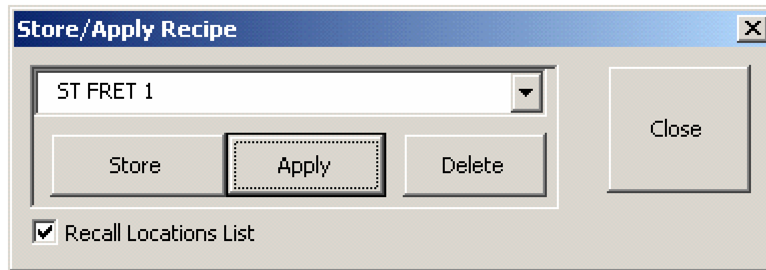
Exclude Zero Intensity Pixels: Excludes pixels from calculation for which at least one of the 3 channels is zero (default: unchecked).

Include Thresholded Pixels: Defines whether or not the pixels below threshold will contribute to calculating averaged FRET figures (default: checked).

Display Truncation Masks: Create images showing masks used for pixel exclusion/truncation.

Note: Explanation for other options settings can be found further down.

Store/Apply: Opens Store/Apply dialog box.



Store: Stores the entire FRET parameter set for both methods in the system registry with the user provided name.

Apply: Applies the settings stored in selected parameter set. Choose from drop down list.

Delete: Deletes selected parameter set.

Recall Locations List: Not applicable.

Close: Closes Store/Apply dialog box and returns to the FRET dialog.

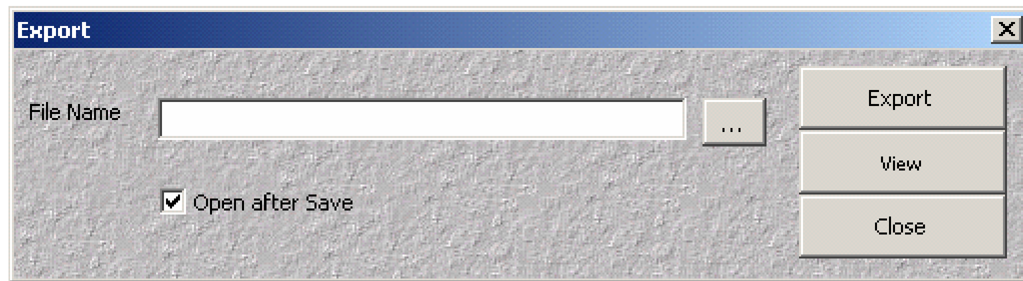
Status bar: Displays current state of the FRET Tool. Color is changed accordingly (Ready, Busy).

Base File Name: Acquired FRET images are automatically saved in the specified Image Database with file name prefix defined here.

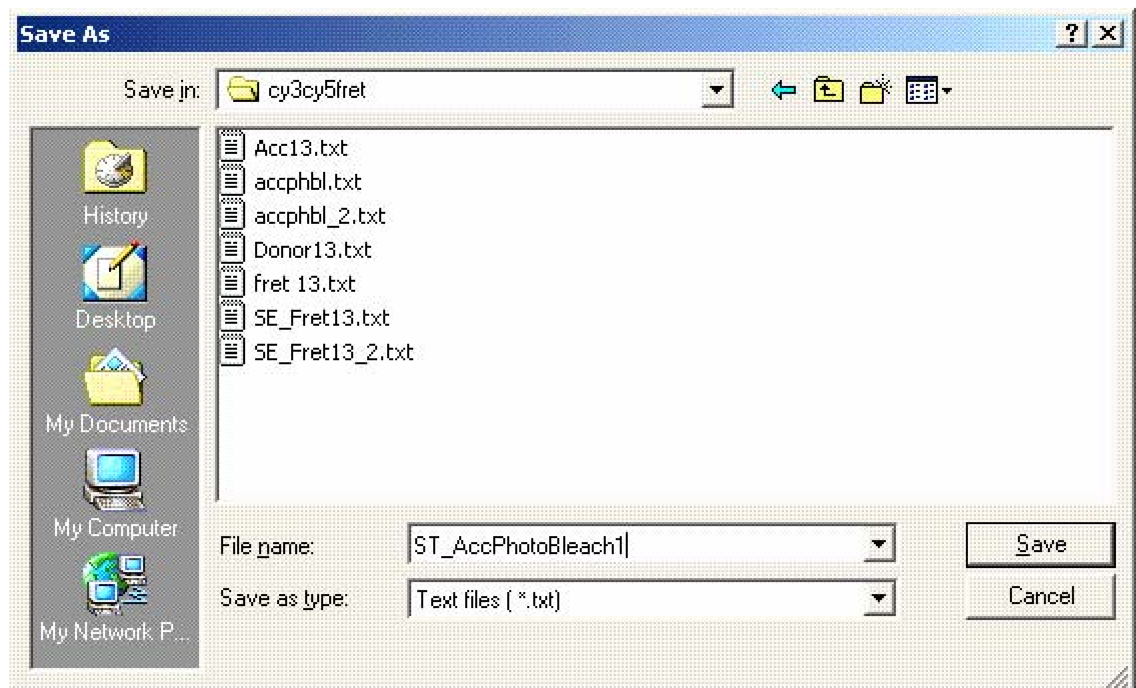
Img Database: Select the LSM image database where to acquisition images are stored and from where stored FRET images can be loaded.

Open DB: Opens the specified LSM Image Database.

Export Table:



(Button) ...: Opens the file selection dialog:



Specify the location for the file containing the FRET analysis data:
Type in the **File Name** and choose the proper location by browsing to the folder of your choice.
Click **Save** to take over these settings and go back to the Export dialog (does not export the data).

Export: Stores the analysis data set.

Open after Save: If this option is checked then the text file will be opened in Notepad after data is exported.

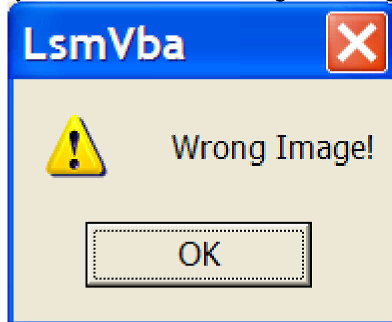
View: Opens the export file specified before.

Close: Close the Export dialog.

Note: See Table Content explanation below.

Analyze: Starts the analysis of the FRET imaged opened and in focus. The analysis can be stopped by pressing this button again (button caption reads **Stop** during analysis).

In case you see the following message box:



check the settings for **Number of Scans** and **Image Channel No** under **Acquisition & Analysis Setup**.

Start: Commence the acquisition process using the parameters set in the **Acquisition & Analysis Setup**. The acquisition can be stopped by pressing the button again (button caption reads **Stop** during acquisition).

Acceptor Photobleach mode:

Acceptor Photobleaching analyses FRET by observing donor channel intensity changes upon bleaching of the acceptor. If there was FRET in the bleached region than donor intensity should go up since fluorescence energy transfer is inhibited.
The analysis requires an image set consisting of a time lapse recording of donor and acceptor channels with acceptor photobleaching carried out in between. Optionally the FRET and other channels can be contained in the image set as well, though will be disregarded by the FRET Tool.

Acquisition & Analysis Setup:

Scan Configuration: Select acquisition settings from drop down list. Use **Refresh** if previously changed in main program. Only needed for acquisition.

Apply: Apply (Reuse) the selected configuration.

Bleach Configuration: Select bleach settings from drop down list. Use **Refresh** if previously changed in main program. Only needed for acquisition.

Bleach ROI: Select bleach ROI from drop down list. Use **Refresh** if previously changed in main program. Only needed for acquisition.

Number of Scans: Defines number of images of the bleach series.

Bleach After # of Scans: Defines the number of images taken before acceptor photobleach occurs.

Donor Thrld: Threshold value for background in donor channel. Edit field or slider can be used to change value.

Acceptor Thrld: Threshold value for background in acceptor channel. Edit field or slider can be used to change value.

Set Threshold from Image: Click on this button and then click into FRET image to determine threshold levels for donor and acceptor channels automatically.
If split view is enabled then this function can also be used to set acceptor and donor channel separately by clicking into corresponding image channel.
Threshold is determined by averaging the intensities inside the chosen area plus adding 2 times the noise level (standard deviation).
The function creates an overlay of a size determined by **Area Size**. This overlay can also be used for later analysis.

Area Size: Choose the threshold area size from 2x2 up to 80x80 pixels.

Image Channel No: Select the image channels for both **Donor** and **Acceptor** in the FRET image. Image channels are numbered from 1 through 8 (drop down list). Use the Channels button (top button in first row next to image window) to determine channel allocation.

Display Images: Check/uncheck to see the following analysis images:

FRET: Display FRET image with intensities converted from the FRET index calculated for each pixel.

FRET = (Donor after - Donor before) / Donor after.

Donor Delta: Display the difference image for the donor channel after and before the bleach:

Donor delta = Donor after - Donor before.

Acceptor Delta: Display the difference image for the acceptor channel before and after the bleach:

Acceptor delta = Acceptor before - Acceptor after.

Note: The Donor Delta and Acceptor Delta images are useful for judging the bleaching inside the bleach area (intended) as well as outside the bleach area (not intended). In case photobleaching outside the bleach area occurs the corresponding numbers in the table can be used to correct the FRET index results.

Scalebar: Check, if a scalebar shall be applied to the FRET image.

Note: A special color look-up table (LUT) is used to indicate the value of the corresponding pixels. Blue shades are negative with bright blue representing most negative numbers (only visible when corresponding option setting is not checked), black are zero intensity pixels, and positive numbers are shown with increasing value in the following colors: cyan, green, yellow, red, white.

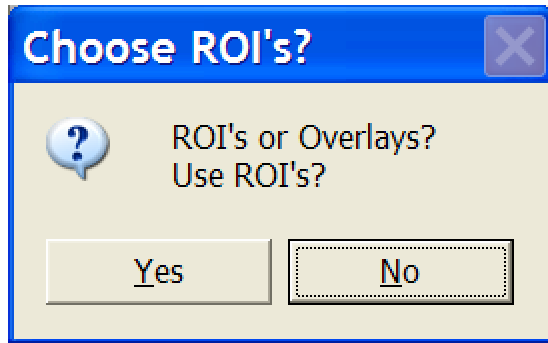
Avg PreBleach: Determines the number of consecutive images before the bleach to be averaged.

Avg PostBleach: Determines the number of consecutive images after the bleach to be averaged.

Note: Averaging of images can be used to improve signal to noise ratio and increase calculation reliability.

Analysis ROI: Select the ROI set from drop down list. Use Refresh to update list if previously changed in main program.
Select **None (use Overlays)** when overlays shall be used for selecting the analysis regions (recommended).
This option is also selected automatically after the function Copy **ROI to Overlay** is executed.

In case there are overlays drawn on the FRET image and a ROI list is selected there will be a dialog asking for which of the two region types to choose for analysis purposes:



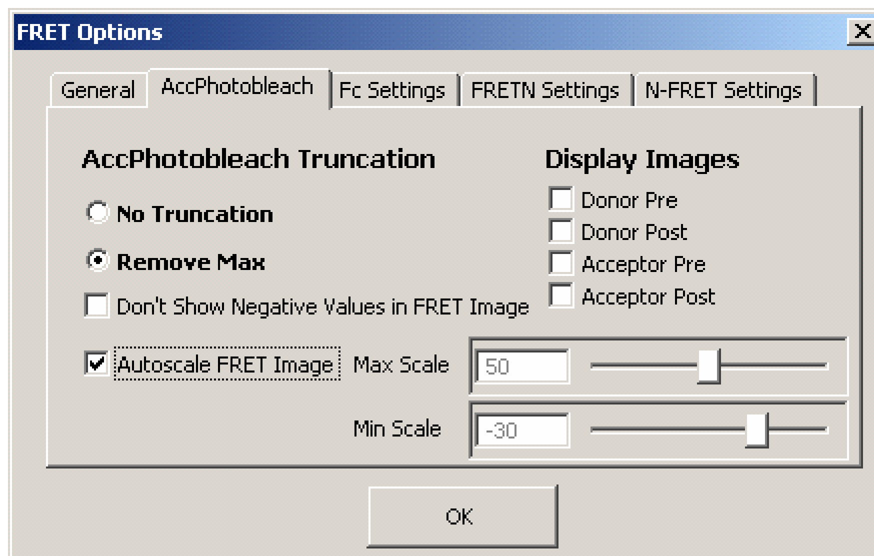
Click **No** if you want to evaluate FRET using overlays. Click **Yes** if you want to evaluate ROIs defined under the selected ROI list.

Note: Use the drawing tools of the **Overlay** function - opened by clicking the 4th button from top in the **Select** column next to the image.

Table entries:

Region: Number of analysis area. 0 represents the entire image. Numbers 1, … are assigned according to the order the overlays are created or ROIs are listed (check ROI dialog of main program).
FRET Eff.: FRET efficiency calculated using area averages for Donor pre and post.
FRET Eff. (p).: FRET efficiency calculated pixel by pixel, then averaged for entire region.
D pre: Intensity of donor channel before the bleach.
D post: Intensity of donor channel after the bleach.
A pre: Intensity of acceptor channel before the bleach.
A post: Intensity of acceptor channel after the bleach.
DeltaD: Difference of donor intensities after and before the bleach.
DeltaA: Difference of acceptor intensities before and after the bleach.
Only in the exported file:
dD pre: Averaged intensity difference of consecutive donor images before the bleach.
dD post: Averaged intensity difference of consecutive donor images after the bleach.

Acceptor Photobleaching options settings:



No Truncation: Turns truncation of calculated pixels off.

Remove Max: Exclude extreme/maximum pixel intensity from FRET images (default).

Don’t Show Negative Values in FRET Image: (default: unchecked). Negative values mean that the donor intensity went down after bleach - which usually takes place outside the acceptor bleached region.

Autoscale FRET Image: Defines whether or not FRET image will be scaled according to result of analysis (default: checked).

Max Scale: Set the upper limit for FRET percentages. Not available if **Autoscale** is checked.

Min Scale: Set the lower limit for FRET percentages (can be negative if **Don't Show Negative Values in FRET Image** is not checked).
Not available if **Autoscale** is checked.

Sensitized Emission mode:

Sensitized Emission is based on 3 channel images:

a) Donor channel - using donor excitation and donor emission filter
b) Acceptor channel - using acceptor excitation and acceptor emission filter
c) FRET channel - acquired using donor excitation and FRET emission filter (can be identical to acceptor emission filter)

The analysis requires three image sets taken from the donor only, acceptor only and the FRET sample.
The first two are used to evaluate the cross talk of signals caused by imaging settings and fluorophore properties (see below).

Scan Configuration:
It is recommended to set up a Multitracking configuration that acquires the Donor and FRET channel in one track using only donor excitation. The other track should collect the Acceptor signal using only acceptor excitation.

The detection bandwidth for the FRET channel and the Acceptor channel can be identical.
In some cases it is useful to push the FRET detection band a little towards longer wavelength in order to minimize the donor emission bleed trough - even though this will be corrected by the sensitized emission method.

Switching between tracks should be line wise - especially when imaging living cells.
Please note that when using the same detector for the FRET and acceptor channel then the PMT voltage cannot be changed on a line by line basis.

Imperative is to use the exact same acquisition parameters for all three samples.
This may require some switching between the reference donor only and acceptor only and the FRET sample to find the ideal configuration and fit all signals into the available dynamic range.

Please note: Saturated and zero intensity pixels will be excluded from calculation.

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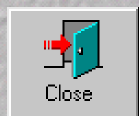
Acceptor Photobleach | Sensitized Emission


Close

Acquisition & Analysis Setup

Scan Configuration: FRETSensitized | Apply

Number of Scans: 1

Donor Thrld: 5

Acc. Thrld: 6

FRET Thrld: 6

Set Threshold from Image

Don. Coef. Fd/Dd: 0.2430

Ad/Fd: 0.0000

Acc. Coef. Fa/Aa: 0.3692

Da/Aa: 0.0036

Da/Fa: 0.0100

G: 1.0000

Sample

Select Donor

Select Acceptor

FRET Selected

Image Ch No

Donor: 1

FRET: 3

Acceptor: 2

FRET Images

- [x] FRETN (Gordon)
- [x] Fc (Youvan)
- [x] N-FRET (Xia)
- [x] Scalebar

Start

Analysis

Time Indx: 1 | All Times | Analysis ROI: None (use Overlays)

Analyze

Region	D avg.	A avg.	F avg.	FRETN	Fc	N-FRET
0	12.32	18.22	16.91	0.0153	0.0281	0.1529
1	79.26	115.27	107.95	0.0034	0.1809	0.3874
2	57.92	85.36	79.09	0.0045	0.1314	0.3830
3	34.78	52.67	48.28	0.0074	0.0799	0.3821
4	31.35	45.61	42.13	0.0083	0.0693	0.3787

Export Table

Ready

Base File Name: test

Img Database

Open DB

E:\FRET Demo.mdb\FRET Demo.mdb

Copy ROI to Overlay

Refresh

Clear Overlay

Copy Overlay

Paste Overlay

Store/Apply | Options | Help | Show Mask

Acquisition & Analysis Setup:

Scan Configuration: Select acquisition settings from drop down list. Use **Refresh** if previously changed in main program. Only needed for acquisition.

Apply: Apply (Reuse) the selected configuration.

Number of Scans: Defines number of one or more images for the FRET time series.

Donor Thrld: Threshold value for background in donor channel. Edit field or slider can be used to change value.

Acc. Thrld: Threshold value for background in acceptor channel. Edit field or slider can be used to change value.

FRET Thrld: Threshold value for background in FRET channel. Edit field or slider can be used to change value.

Set Threshold from Image: Click on this button and then click into FRET image to determine threshold levels for donor, acceptor, and FRET channels automatically.
If split view is enabled then this function can also be used to set acceptor and donor channel separately by clicking into corresponding image channel.
Threshold is determined by averaging the intensities inside the chosen area plus adding w times the noise level (standard deviation).
The function creates and overlay of a size determined by **Area Size**. This overlay can also be used for following analysis.

Area Size: Choose the threshold area size from 2x2 up to 80x80 pixels.

Sensitized Emission ***blead through*** *(crosstalk)* ***coefficients****:*

Don. Coef. **Fd/Dd**: Determines the amount of crosstalk of donor signal into the FRET channel.

Ad/Fd: Determines the amount of crosstalk of FRET signal into the Acceptor channel.

Acc. Coef. **Fa/Aa**: Determines the amount of crosstalk of the acceptor signal into the FRET channel.
Da/Aa: Determines the amount of crosstalk of the acceptor signal into the donor channel.
Da/Fa: Determines the amount of crosstalk of the FRET signal into the donor channel.

G: System constant. Usually set to 1 (default).

Sample *Selector:*

Select Donor: Click to select to acquire/analyze donor only sample (changes to **Donor Selected** when clicked).

Select Acceptor: Click to select to acquire/analyze acceptor only sample (changes to **Acceptor Selected** when clicked).

Select FRET: Click to select to acquire/analyze FRET sample (changes to **FRET Selected** when clicked).

Image Channel No: Select the image channels for **Donor, FRET** and **Acceptor** in the FRET image. Image channels are numbered from 1 through 8 (drop down list). Use the Channels button (top button in first row next to image window) to determine channel allocation.

FRET Images: Check/uncheck to see the following analysis images:

Fc: Display Fc image with intensities converted from the FRET index calculated for each pixel using the Youvan method. This method assumes that the signal recorded in the FRET channel is the sum of real FRET signal overlaid by donor crosstalk and acceptor signal induced by direct (donor) excitation. There is no correction for donor and acceptor concentration levels.

$$Fc = Ff - Df\,(Fd/Dd) - Af\,(Fa/Aa)$$

FRETN: Display FRETN image with intensities converted from the FRET index calculated for each pixel using the Gordon method. This method calculates a corrected FRET value and divides by concentration values for donor and acceptor.

$$FRETN = \frac{FRET1}{Dfd * Afa} \infty \frac{[bound]}{[total\ d] * [total\ a]}$$

N-FRET: Display N-FRET image with intensities converted from the FRET index calculated for each pixel using the Xia method. This method is similar to the Gordon method with the difference that for concentration compensation the square root of donor and acceptor concentration is used.

$$N_{FRET} = \frac{FRETN}{Dfd*Afa} = \frac{FRET1}{(Dfd * Afa)^{1/2}} \infty \frac{[bound]}{[total\ d] * [total\ a]}.$$

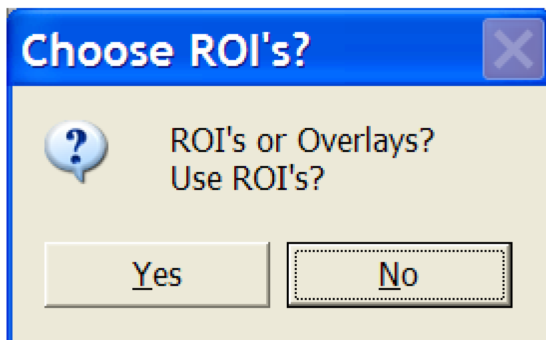
Note: For description of mathematical equations please check the papers by Gordon et al. and Xia et al. (see references listed at the end of this document) - which give a good overview of all three implemented formulas.

Scalebar: Check, if a scale bar shall be applied to the FRET images.

Note: A special color look-up table (LUT) is used to indicate the value of the corresponding pixels. Blue shades are negative with bright blue representing most negative numbers (only visible when corresponding option setting is not checked; not available for FRETN and N-FRET images), black are zero intensity pixels, and positive numbers are shown with increasing value in the following colors: cyan, green, yellow, red, white.

Analysis ROI: Select the ROI set from drop down list. Use Refresh to update list if previously changed in main program.
Select **None (use Overlays)** when overlays shall be used for selecting the analysis regions (recommended).
This option is also selected automatically after the function **Copy ROI to Overlay** is executed.

In case there are overlays drawn on the FRET image and a ROI list is selected there will be a dialog popping up during analysis asking for which of the two region types to choose for analysis:



Click **No** if you want to evaluate FRET using overlays.
If you want ROIs from the selected list only, click **Yes**.

Note: Use the drawing tools of the **Overlay** function - opened by clicking the 4th button from top in the **Select** column next to the image.

Analysis *frame:*

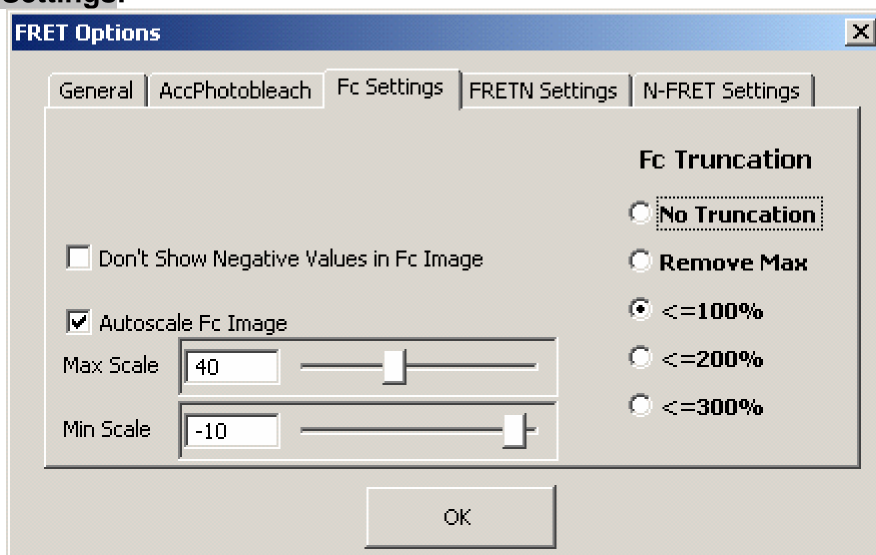
Time Indx: Select time index (i. e. number of image in the series) to view corresponding analysis results.

All Times: Show complete table using additional row with time indices.

Table entries:

Region: Number of analysis area. 0 represents the entire image. Numbers 1, … are assigned according to the order the overlays are created or ROIs are listed (check ROI dialog of main program).
Time: Time index according to number of image scanned in the series (only visible when **All Times** is checked).
D avg.: Average donor channel intensity for corresponding region.
A avg.: Average acceptor channel intensity for corresponding region.
F avg.: Average FRET channel intensity for corresponding region.
FRETN: The average FRET efficiency calculated using the formula described above.
Fc: The average FRET efficiency calculated using the formula described above.
N-FRET: The average FRET efficiency calculated using the formula described above.

Fc Settings:



Don’t Show Negative Values in FRET Image: (default: unchecked). Negative values mean that the donor intensity went down after bleach.
Note: This can occur due to movements within the sample as well as of the entire sample itself. It also can hint to photobleaching of the donor signal due to too high laser power or questionable bleaching settings.

Autoscale Fc Image: Defines whether or not FRET image will be scaled according to result of analysis (default: checked).

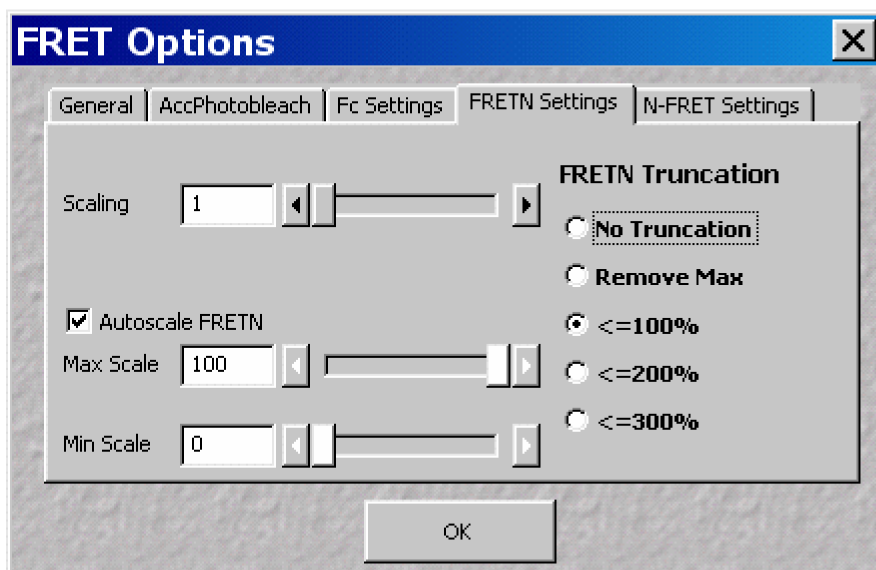
Max Scale: Set the upper limit for FRET percentages. Not available if **Autoscale** is checked.

Min Scale: Set the lower limit for FRET percentages (can be negative when **Don’t Show Negative Values in FRET Image** is not checked). Not available if **Autoscale** is checked.

Fc Truncation: Selects the method for excluding extreme values from both, the FRET image as well as the data table calculation. These extreme values represent artifacts that can stem from movement within the sample or of the entire sample itself.

> **No Truncation:** Turns truncation of calculated pixels off.
>
> **Remove Max:** Exclude extreme/maximum pixel intensity from FRET images (default).
>
> **<= 100%**, **<=200%**, **<=300%**: Further truncation levels for extreme pixel values.

FRETN Settings:



Scaling: Arbitrary factor (1-4095) to scale the Gordon formula results

Autoscale FRET Image: Defines whether or not FRET image will be scaled according to result of analysis (default: checked).

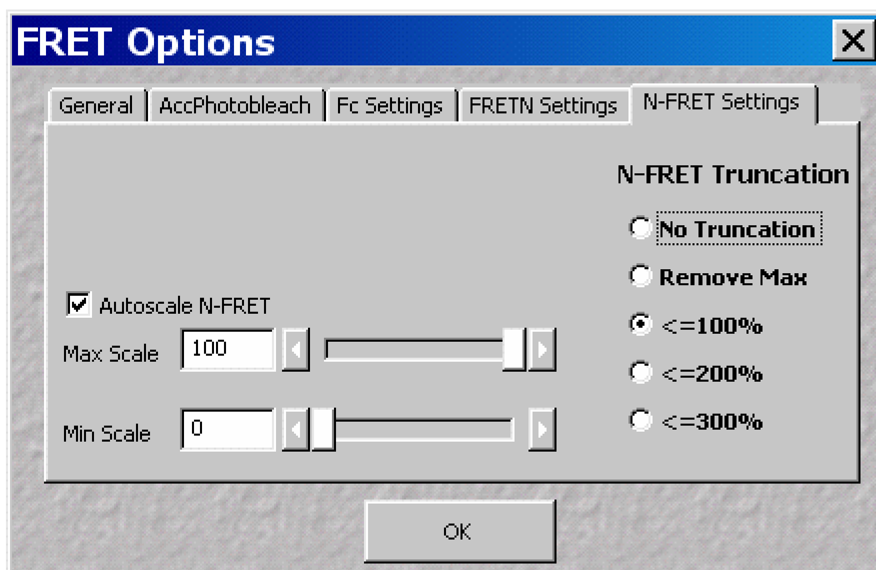
Max Scale: Set the upper limit for FRET percentages. Not available if **Autoscale** is checked.

Min Scale: Set the lower limit for FRET percentages (>=0). Not available if **Autoscale** is checked.

FRETN Truncation: Selects the method for excluding extreme values from both, the FRET image as well as the data table calculation. These extreme values represent artifacts that can stem from movement within the sample or of the entire sample itself.

> **No Truncation:** Turns truncation of calculated pixels off.
>
> **Remove Max:** Exclude extreme/maximum pixel intensity from FRET images (default).
>
> **<= 100%**, **<=200%**, **<=300%**: Further truncation levels for extreme pixel values.

N-FRET Settings:



Autoscale FRET Image: Defines whether or not FRET image will be scaled according to result of analysis (default: checked).

Max Scale: Set the upper limit for FRET percentages. Not available if **Autoscale** is checked.

Min Scale: Set the lower limit for FRET percentages. Not available if **Autoscale** is checked.

(there are no negative values for this method).

N-FRET Truncation: Selects the method for excluding extreme values from both, the FRET image as well as the data table calculation. These extreme values represent artifacts that can stem from movement in or of the sample.

> **No Truncation:** Turns truncation of calculated pixels off.
>
> **Remove Max:** Exclude extreme/maximum pixel intensity from FRET images (default).
>
> **<= 100%**, **<=200%**, **<=300%**: Further truncation levels for extreme pixel values.

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