

# FRET GUIDE

This guide is written for acceptor photobleaching FRET on the Zeiss 510 Meta confocals.

## Channel mode (CFP/YFP example)

### Control experiments

When carrying out a FRET experiment, if possible, have a positive control using the same FRET pair as your experiment. Otherwise it is difficult to be confident about a negative FRET result.

Before starting the FRET experiment, there are two control experiments that you should carry out to aid in the FRET design.

#### 1) Background bleaching

This involves a simple time-lapse to discover the extent of background bleaching during imaging. You want to minimise background bleaching so that you do not complicate the analysis of FRET.

#### 2) Find the bleaching conditions for the acceptor

Again a simple time-lapse with multiple bleaching events to establish the number of bleach iterations needed to create an appropriate bleaching curve for the acceptor.

### 1) Background bleaching

- Set up a two channel configuration to image the donor and the acceptor.
- Choose a cell/object as similar as possible to the cell/object you wish to image during FRET.
- Centre the cell/object in the field of view and adjust scan settings to achieve the fastest scan rate possible (for example, reduce the Y value of the box size [Scan Control Panel and Mode tab] to minimise the number of lines in a scan).
- Set up a time-series to scan continually for as many scans as you estimate will cover your FRET experiment [Time Series Control panel].
- Image the entire cell/object to avoid any FRAP effects from non-imaged regions.
- Collect the time series [Start T].
- To analyse the time series data in the LSM software, go to Mean ROI in the image window. Draw an ROI **within** the cell/object and generate a graph over time for each channel.
- If excessive bleaching is occurring, then adjust the scan settings (for example lower the laser power) and repeat as above.

## 2) Find the bleaching conditions for the acceptor

- Choose a new cell/object as similar as possible to the cell/object you wish to image during FRET.
- Set up a configuration to image the acceptor (in this example, YFP).
- Centre the cell/object in the field of view and adjust scan settings to achieve the fastest scan rate possible (for example, reduce the Y value of the box size [Scan Control Panel and Mode tab] to minimise the number of lines in a scan).
- Set up bleach parameters [Edit Bleach - Bleach control panel]. Set the required number of pre-bleach scans [Bleach after number of scans (tick) Scan number e.g. 3]. Set to scan once after each bleach event [Bleach repeat after number of scans (tick) Scan number 1]. Set the number of bleach iterations for each bleach event [Bleach parameter - Iterations e.g. 2] see Figure 1.
- Set the appropriate laser to 100% power for the bleaching events (for YFP, the 514 line) [Excitation of bleach track].
- Define a bleach area [in Define Region] to include the entire cell/object. Bleaching the entire cell/object avoids any FRAP effects from non-bleached regions. At this point use a rectangle ROI if you are scanning at rates of 10 frames per second or faster.
- Set up a time-series to include the pre bleach scans and then a reasonable number of bleach events (see Figure 2). The more data points for the bleaching curve the better.
- Collect the bleaching time series [Start B].
- To analyse the time series data in the Zeiss software, go to Mean ROI in the image window. Draw an ROI **within** the cell/object and generate a graph over time.
- Adjust the number of bleach iterations in each bleach event to achieve a steady bleaching curve (see Figure 3 green curve, for an example of a bleaching curve).

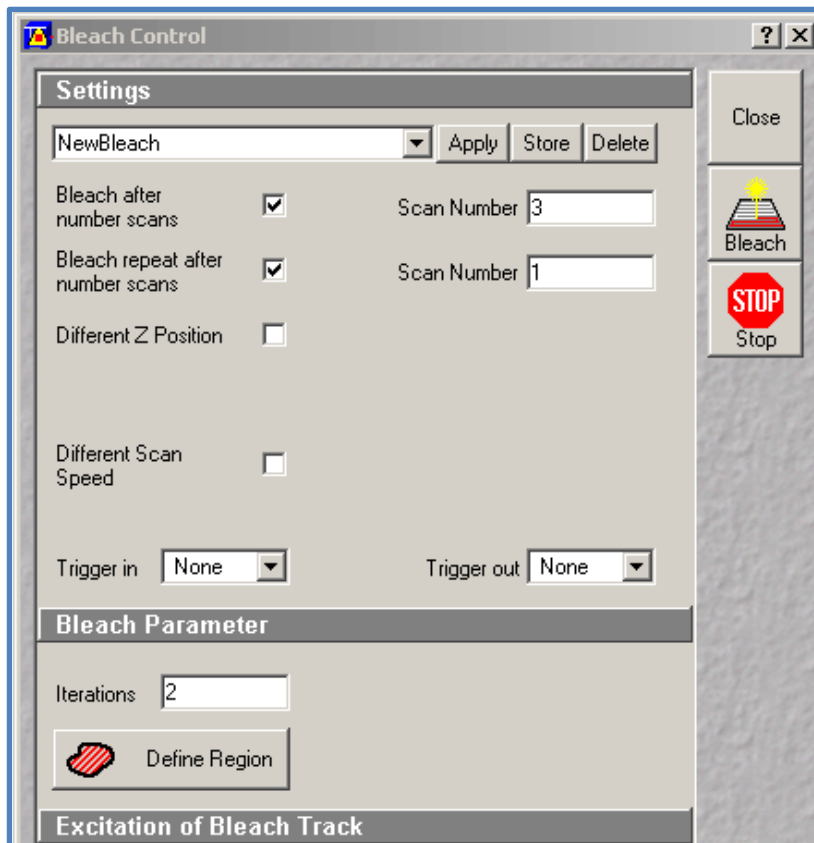


Figure 1.

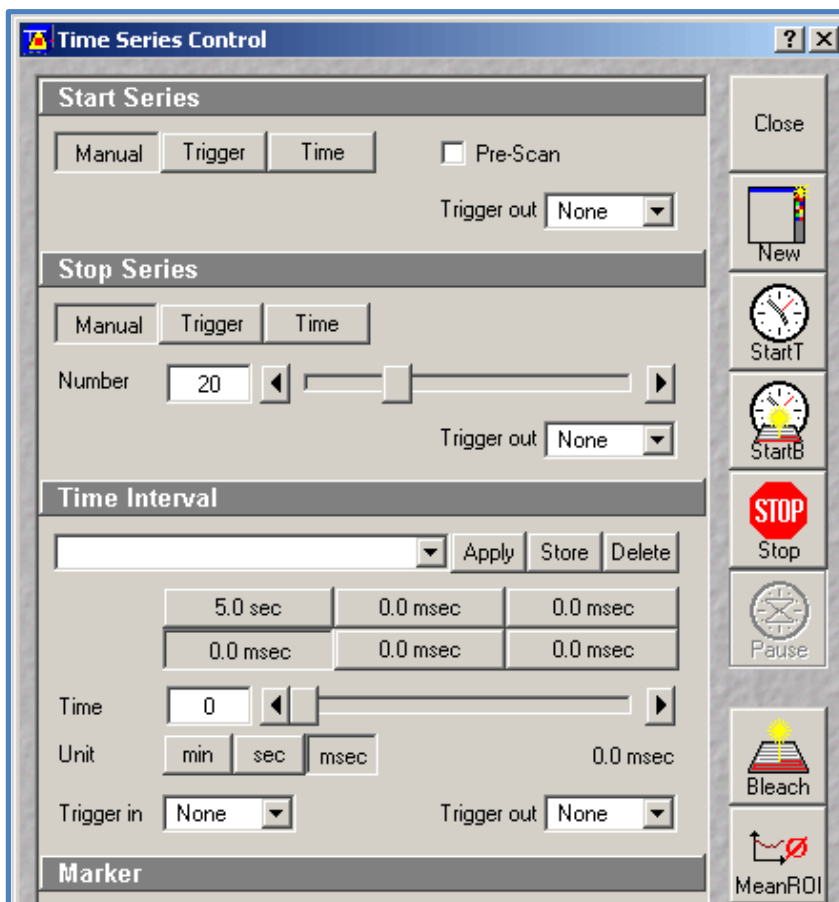


Figure 2.

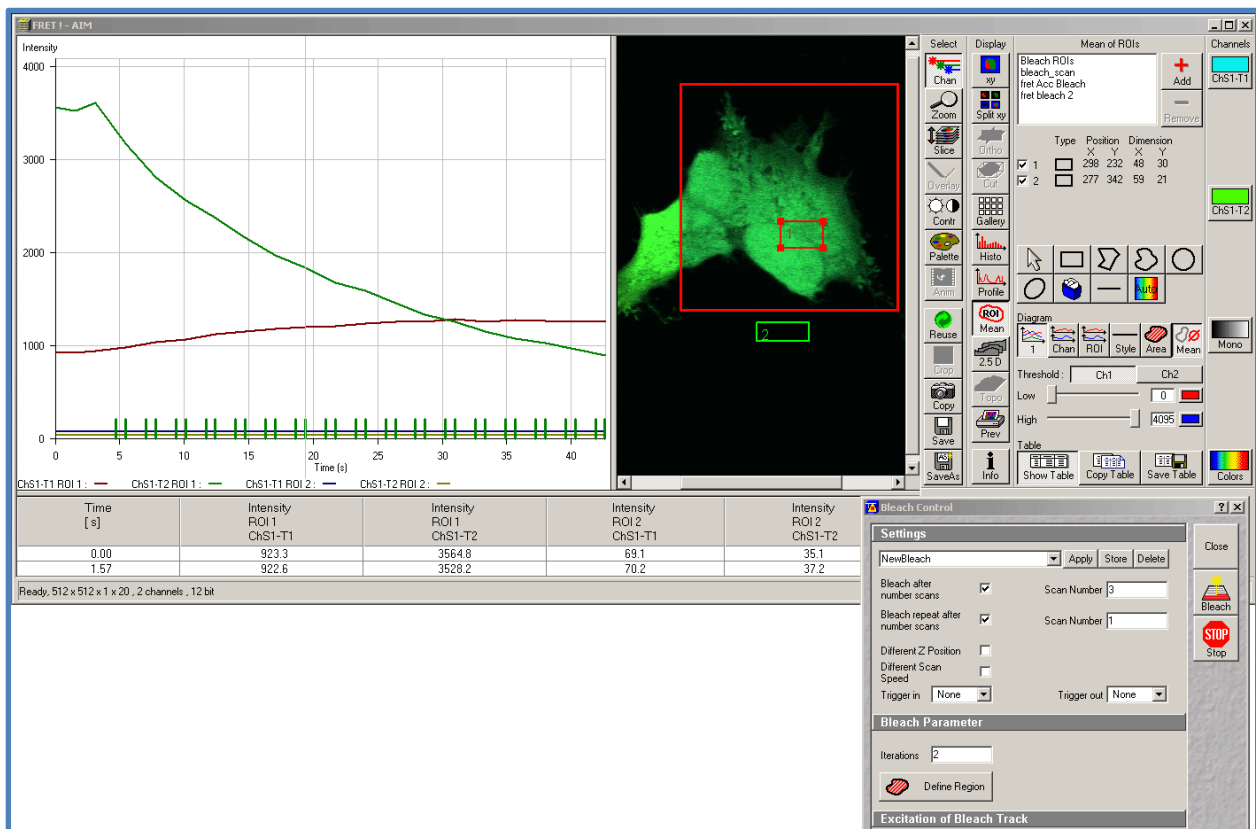


Figure 3.

The region shown in the large red box has been acceptor bleached using the settings shown in the bleach control panel (bottom right). The average intensities of the donor and acceptor from ROI 1 have been plotted. The green line shows the bleaching curve of the acceptor. The small green bars along the X-axis show the start and end of the bleaching events. The decrease in acceptor intensity (green line) is accompanied by an increase in donor intensity (red line) illustrating that FRET is occurring.

## The FRET experiment

- Set up a two channel configuration to image the donor and the acceptor (in this case, CFP and YFP). Minimise cross-talk - if the donor detection band is too wide then you might see an effect of bleaching the acceptor in the donor channel. NB Using the meta detector for both channels gives the best emission settings for CFP and YFP without having to change any mirrors or filters (this allows you to use line by line multi-tracking).
- Set up the bleach parameters to bleach the acceptor as described above (in this case YFP) [Edit Bleach - Bleach control panel].
- Set the appropriate laser (514 line for YFP) to 100% power for the bleaching events [Excitation of bleach track].
- Define a bleach area [in Define Region] to include the entire cell/object. Ensure you are bleaching the entire cell/object to avoid any FRAP effects from non-bleached regions. At this point a rectangle ROI is a good idea if possible - especially if you are scanning at rates of 10 frames per second or faster.
- Set up a time-series to include the pre bleach scans and the bleach events.
- Collect the bleaching time series [Start B].
- To analyse the time series data in the Zeiss software, go to Mean ROI in the image window. Draw an ROI **within** the cell/object and generate a graph over time for the two channels. If FRET is occurring then a decrease in acceptor signal should be accompanied by an increase in donor signal (see Figure 3, Figure 4 and Figure 5 ).
- Export the intensity values [use the 'Show Table' button, then copy and paste] to an appropriate software package such as Excel.
- Plot a graph of % initial YFP intensity {100 x intensity value / average pre-bleach value} against % change in CFP intensity {(100 x intensity value / average pre-bleach value) - 100} see Figure 6.
- Apply linear regression back to the X-axis to give values for donor intensity without acceptor (CFPmax). In the example shown in Figure 6, the % change in CFP intensity when YFP intensity is zero is 62%. Therefore CFPmax is 162%.
- FRET efficiency (E) can then be calculated as follows:

$$\frac{\text{CFPmax}-\text{CFPmin}}{\text{CFPmax}} = \frac{\text{Max \% change in CFP}}{\text{CFPmax\%}} = \frac{62}{162} = 38\%$$

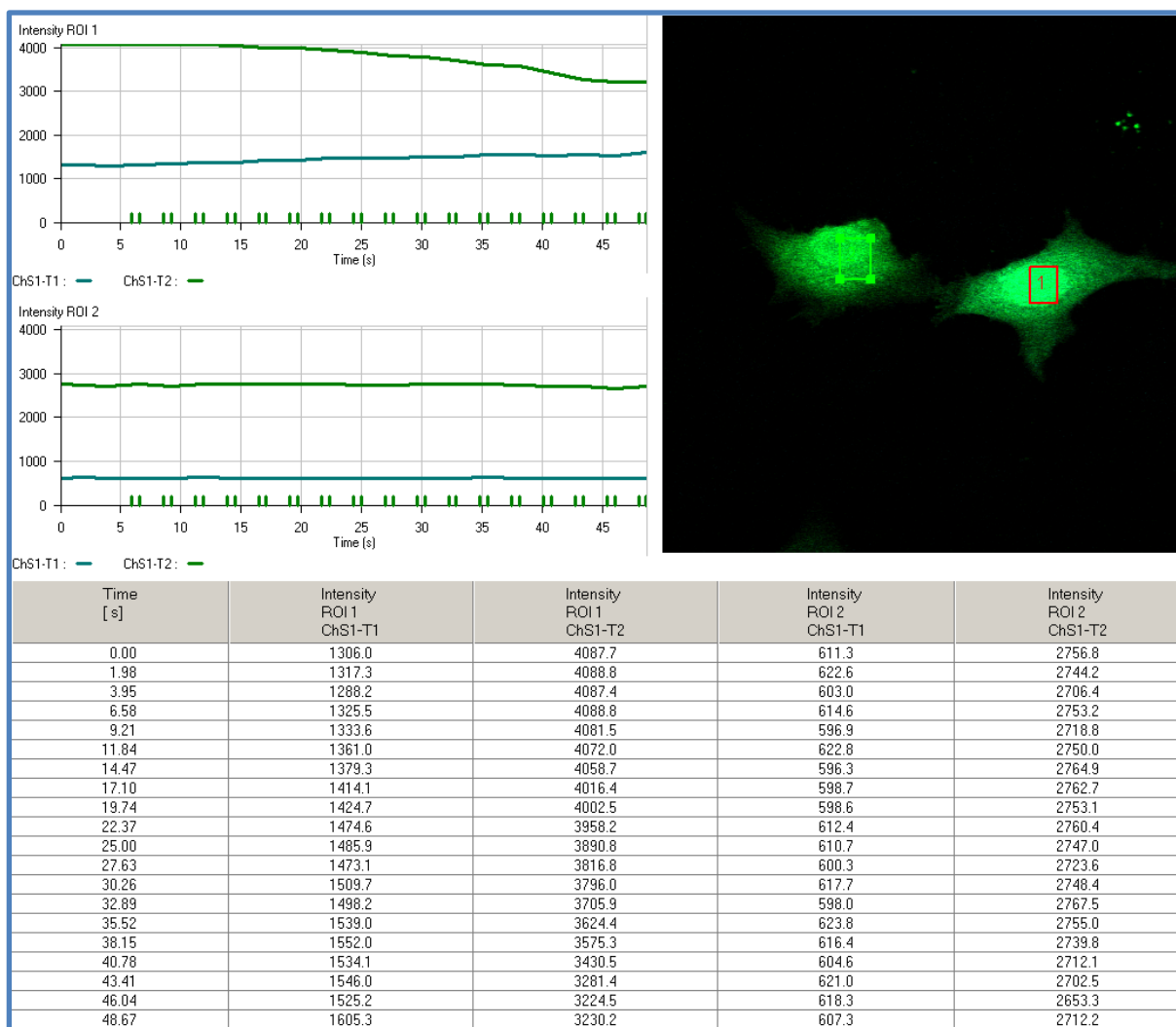


Figure 4.

In this experiment, the cell on the right (including ROI 1) has been subjected to acceptor bleaching whereas the cell on the left (including ROI 2) has not. The average intensities of the donor (CFP) and acceptor (YFP) have been plotted over time. In ROI 1, the decrease in acceptor intensity (green line - upper graph) is accompanied by an increase in donor intensity (blue line - upper graph) illustrating that FRET is occurring. The fluorescence intensities in ROI 2 remain reasonably constant over time (lower graph) showing that background bleaching of both the donor and acceptor is minimal. The average intensity values can be displayed in table form [use the 'Show Table' button in the Zeiss software] and exported into other analysis software packages.

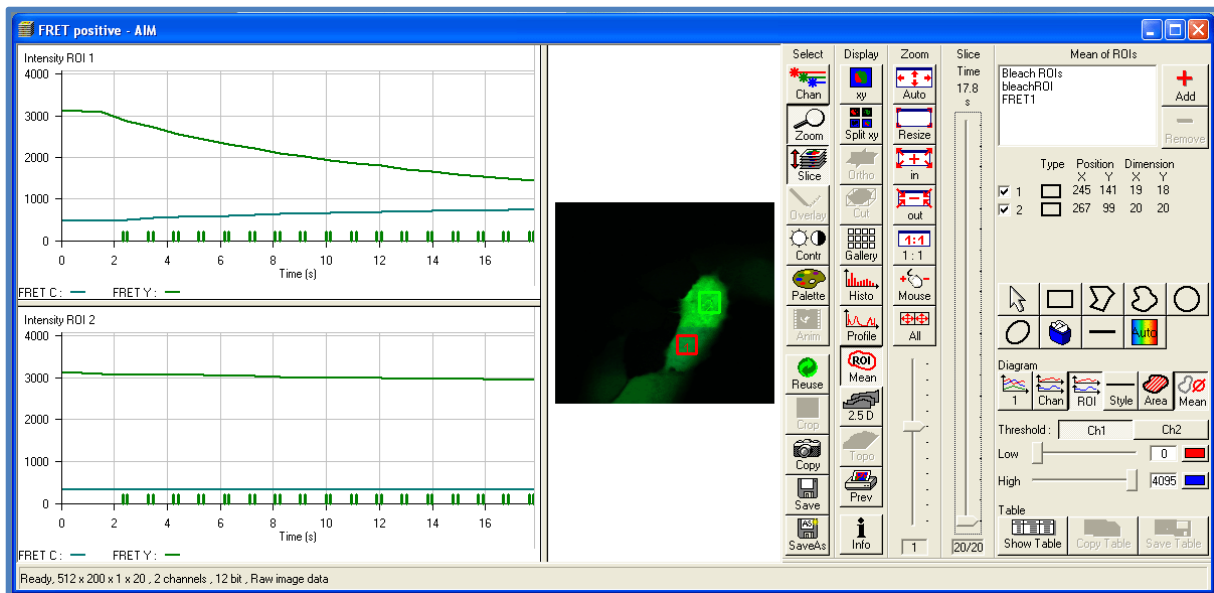


Figure 5.

In this example, the lower half of the cell has been acceptor bleached (ROI 1) and the upper half has not (ROI 2). The intensity decrease of acceptor fluorescence in ROI 2 is probably due to FRAP as fluorescent molecules move from the non-bleached to the bleached region. This illustrates the requirement to bleach the entire cell to avoid fluorescence recovery from non-bleached regions.

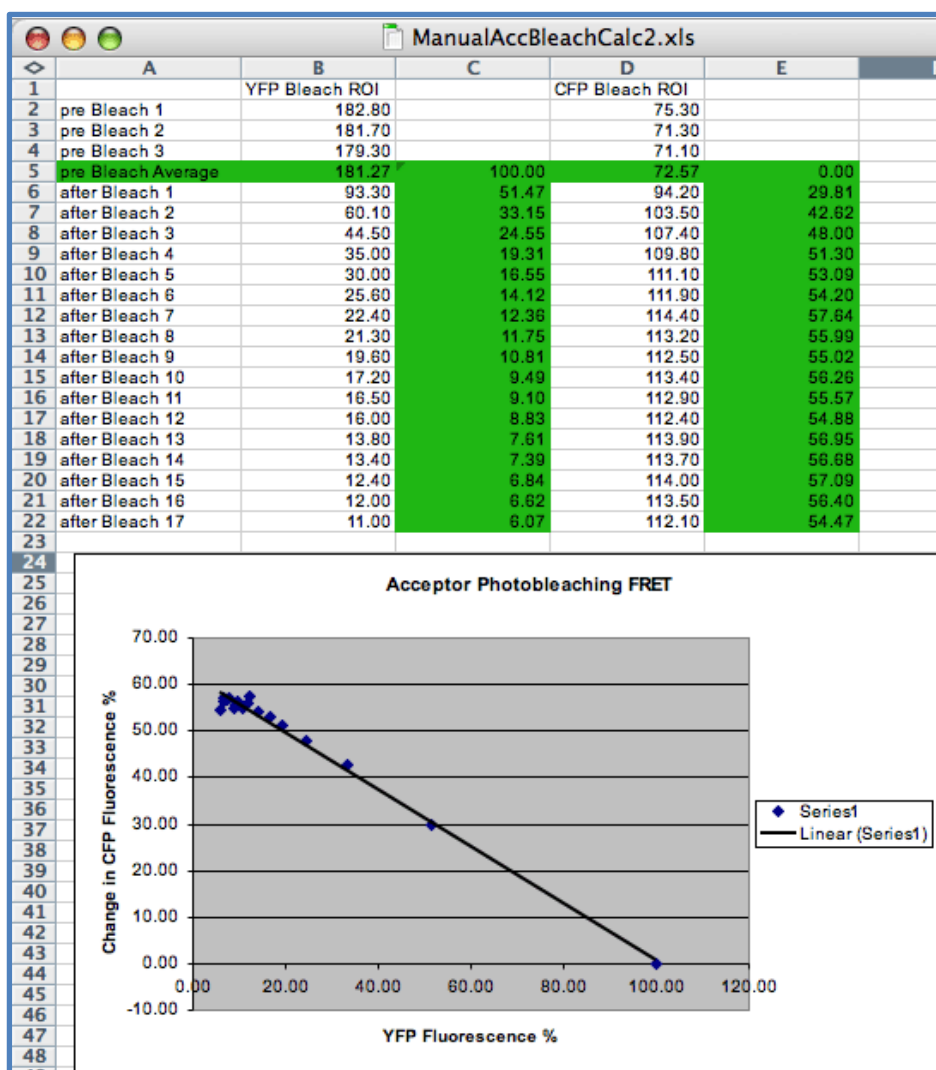


Figure 6

The average intensity values of the acceptor YFP (column B) and donor CFP (column D) were imported into Microsoft Excel. An average pre bleach value as calculated (row 5). The percentage of acceptor YFP fluorescence (column C) was then plotted against the percentage change in donor CFP fluorescence (column E).



## **Lambda mode (CFP/YFP example)**

### **Control experiments**

#### **1) Background bleaching**

As above using a lambda configuration rather than a channel configuration to image.

#### **2) Find the bleaching conditions for the acceptor**

As above using a lambda configuration rather than a channel configuration to image.

#### **3) Reference Spectra**

- Prepare samples containing donor only and acceptor only.
- Take reference spectra of the donor and acceptor using the same dichroic filters and active laser lines as you intend to use for the final experiment.
- Use 'Sum' if the signal is weak. Make sure you have a low noise image as follows: Turn the laser off and bring down the gain until the channel looks black. Use this gain setting for the imaging, compensating with 'Sum' or opening the pinhole slightly.
- Save the reference spectra.

### **The FRET experiment**

- Set a configuration in lambda mode to image the donor and acceptor.
- Set bleaching parameters as described above.
- Set up a time-series as described above.
- Collect the bleaching time series [Start B].
- Play through time and you should see the donor peaks increasing and the acceptor peaks decreasing.
- 'Process' -> 'Unmix' -> 'Channels'
- Choose the reference spectral curves that you saved earlier for the donor and acceptor.
- You now have two channels that should be separated for bleedthrough.
- Do the FRET analysis as described above for channel mode.