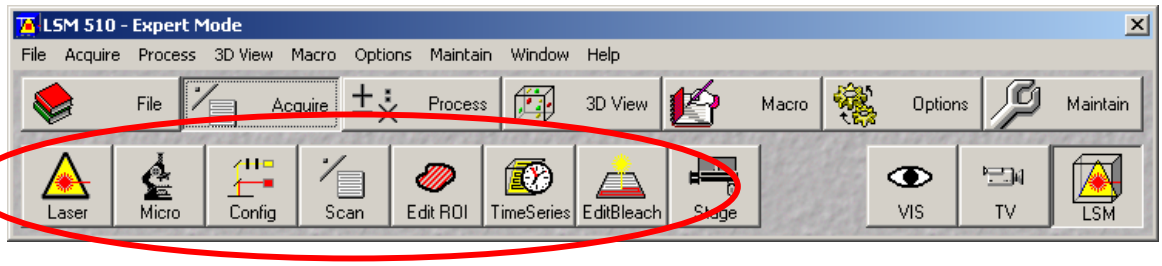
Protocol: "Acceptor Photobleaching FRET (CFP/YFP)"

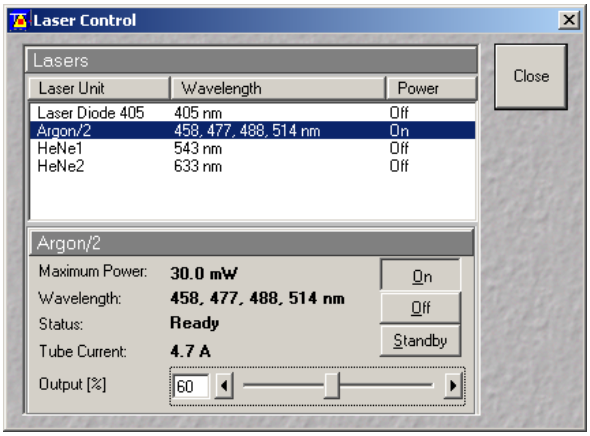
Version 221104

Settings:

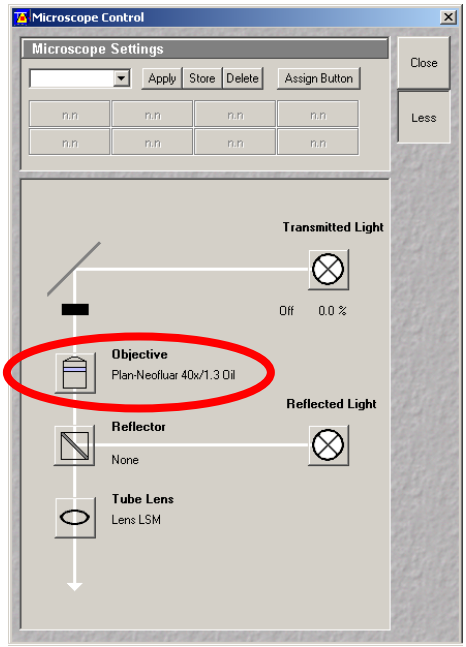
In the "acquire" toolbar in the main menu several settings can be adjusted for the measurements:



LASER (Laser control):
- Argon **458**/477/488/**514** nm ON
If the Laser is off, first "standbye" until laser can turned on.
- Tube current of 6.1A (Output[%] ~ 60%). *If the current is lower, slowly increase the Output [%]*

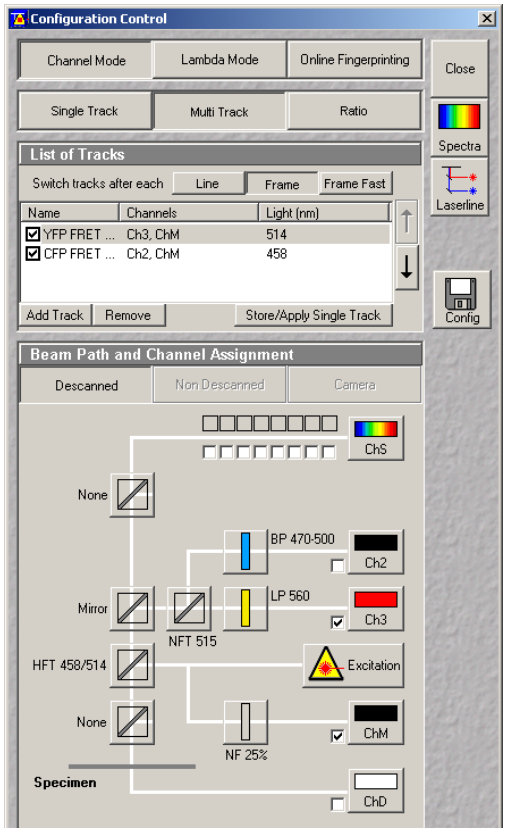


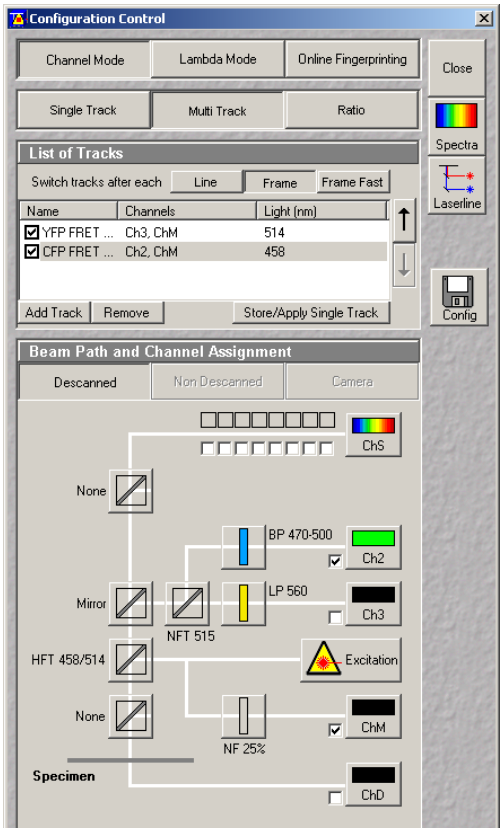
MICRO (Microscope control): -
Objective: Plan Neofluar 40x/1.3 Oil



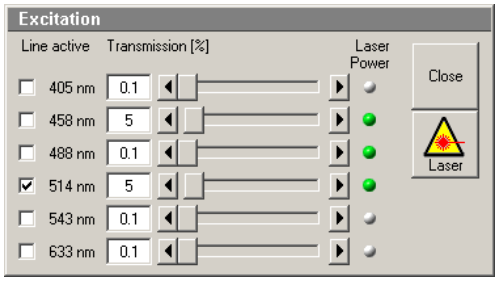
CONFIG (Configuration control):

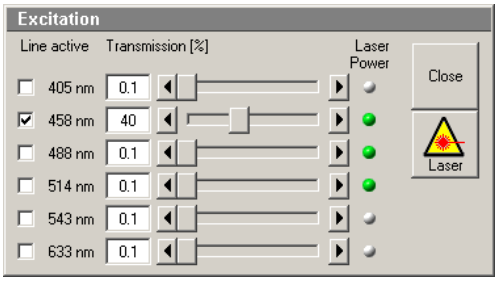
CFP/YFP FRET will be imaged in "Multi Track" mode. The two tracks will be used to image the CFP and YFP signals separately:





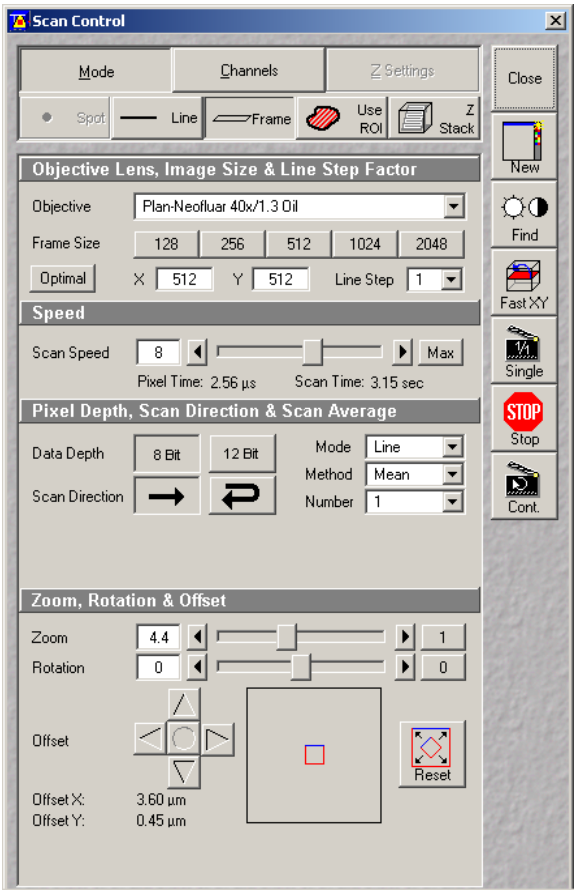
With each track a specific excitation, where the transmissions should be set resulting an a 5 µW 514nm excitation of YFP (~5%) and a 10 µW 458nm excitation of CFP (~40%):





Scan (Scan Control - Mode):

- Scan in Frame mode
- Image size: 512x512 pixels
- Scan Speed 8
- Pixel Depth 8 Bit, unidirectional
- No averaging
- For searching cells zoom 1, for imaging Acceptor bleaching FRET Zoom 4.4



Scan (Scan Control - Channels):

YFP channel (Ch3-T1)

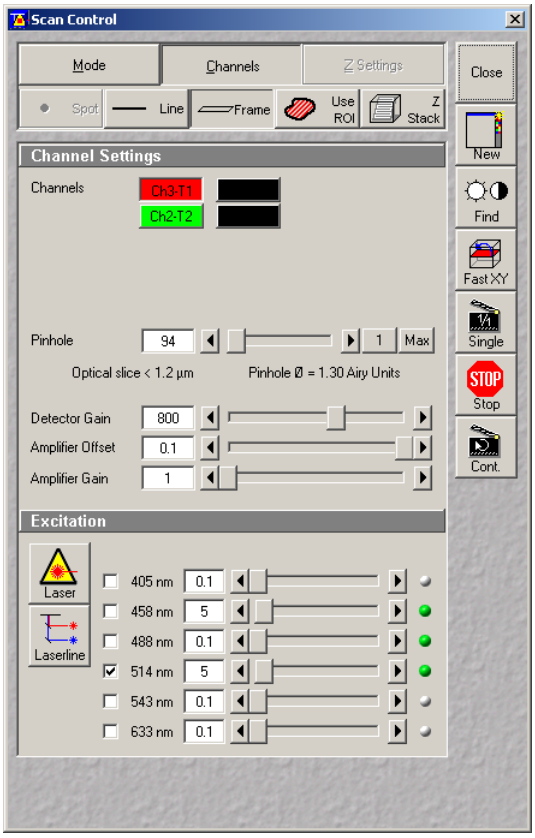
- Pinhole: 94 (Optical slice <1.2 µm)
- Detector Gain: 800
- Amplifier Offset 0.1
- Amplifier Gain 1

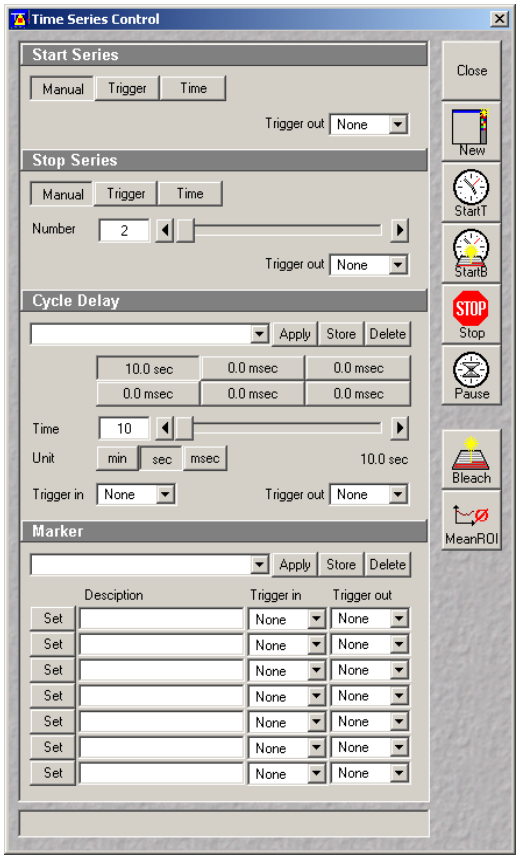
(Excitations are already set in "config")

CFP channel (Ch2-T2)

- Pinhole: 94 (Optical slice <1.2 µm)
- Detector Gain: 900
- Amplifier Offset 0.1
- Amplifier Gain 1

(Excitations are already set in "config")

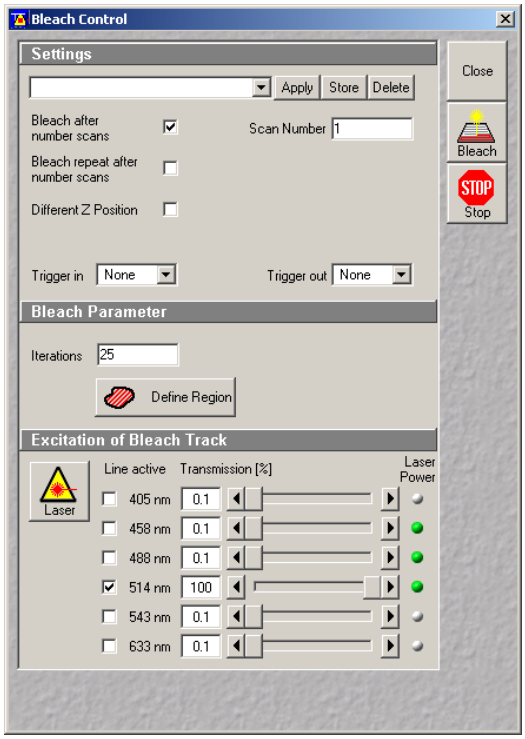




TimeSeries

We scan both signals (YFP and CFP) twice, before and after bleaching YFP. Therefore the procedure will be: Image YFP, Image CFP, Bleach YFP, Image YFP, and Image CFP. This procedure can be programmed in a timeserie.

- Start series: manual
- Stop series: after two scans (of both channels)
- Cycle delay: set to 10 sec, but the procedure will take longer resulting in a continuous scan
- A timeserie can be started using StartB (a timeserie with bleaching)

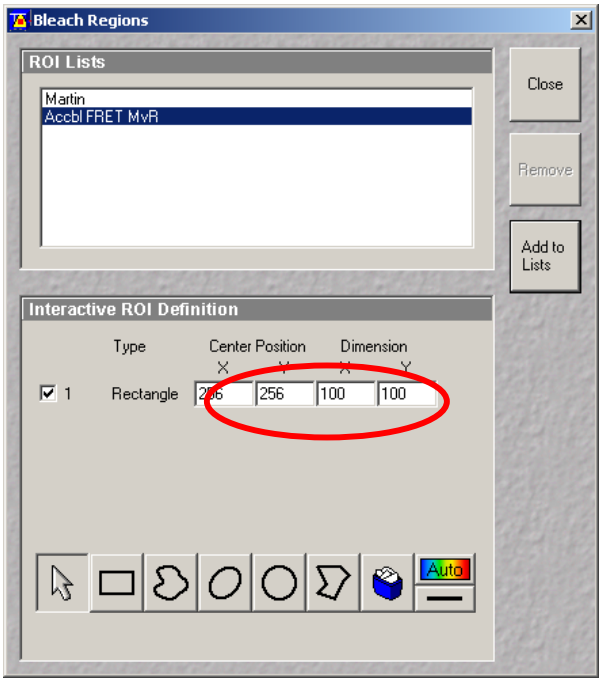


Bleach Control

We bleach YFP after one scan of both channels and only in a region of interest (ROI). To bleach a large fraction of YFP the ROI will be scanned 25 times using the YFP excitation wavelength (514nm) at a high laser power (100%).

- Bleach after Scan Number 1
- Iterations 25
- Excitation of bleach track: 514nm 100%
- Define Bleach Region (ROI)

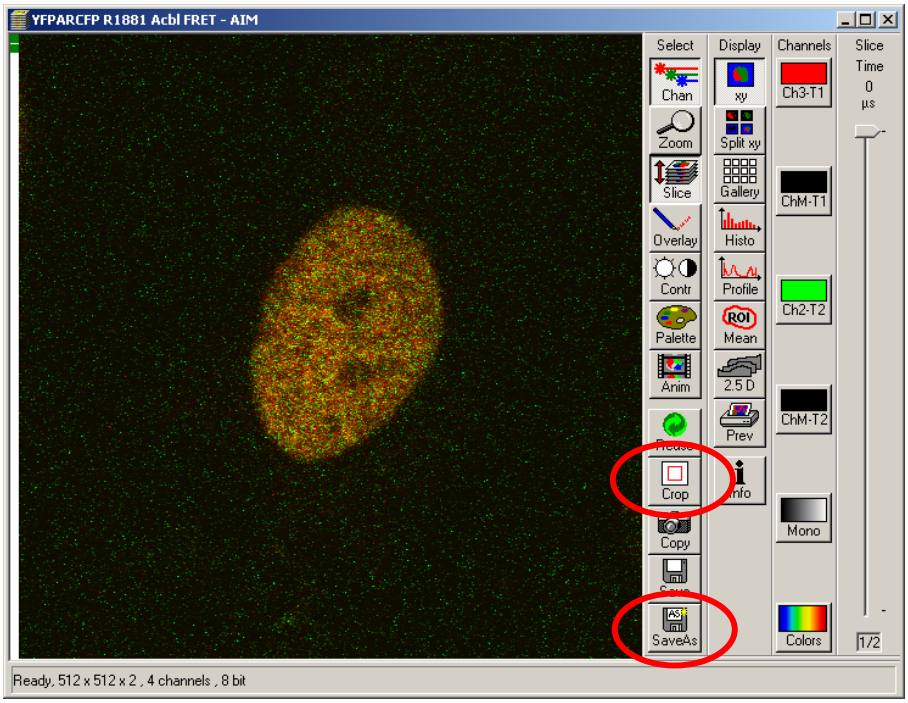
Bleach region (define bleach region)
- Any shape of ROI is possible, but we bleach a square of 100x100 pixels in the middle of the image.



Procedure of FRET measurement:

Because we work with living cells the cells should be kept on 37°C. Allow the cells (and coverglas) to get to this temperature to avoid focal plane shifts because of temperature adjustments.

- -Use normal light ("**VIS**" in the main menu) to get the cells in focus. Switch back to "**LSM**" and adjust the focal plane to the cells in zoom 1.
- -Select a cell and put this cell in the middle of the image using the "**Gert Center**" – macro in the "Macro" menu. Close the "Gert Center" macro.
- **Crop** the image with the cell of interest in the middle of the square.
- Adjust **zoom** to 4.4.
- **Single** scan in the scan menu.



- If the cell of interest is still in focus and in the middle of the image define bleach region (already preset in bleach menu), activate by clicking "**Define Region**". A square (ROI) will be visible in the middle of the image.
- Start FRET measurement by "**StartB**" in the Time Series menu.
- Use "**Save as**" to save the time serie after the scan.
- Reset to zoom 1 and select next cell.