

Fluorescence recovery after photobleaching

This protocol describes the FRAP Wizard set-up and is an extension on the <u>SP5 Quick</u> start Manual found on the website. FRAP is only possible on SP5-604 and SP5-1315.

### General Image set-up (also in SP5 Quick start manual)

Turn on the temperature,  $CO_2$  and humidity. Start LAS-AF

- 1. Use as configuration: Machine with manual AFC
  - a. Initialize stage
- 2. Start the lasers in the Configure tab and turn the Argon laser to 50%



3. Go back to the Acquisition tab and enable the FRAP booster.



- 4. Place your samples on the microscope and focus.
- Setup the desired channel(s) in sequential mode, like you would do for live cell imaging experiments e.g. low laser powers and high gain.
  Note: PMT's are preferred above Hyd detectors. Hyd detectors can turn off during the high bleaching laser powers and need to reset in between. This takes time and there for not optimal for the short time intervals we want to measure during recovery.
- 6. Take a first picture to store your settings.

### **Experiment setup**

Imaging setup:

- Pixels: 512\*512
- Average: line, 2 times
- Laser: 1-2%
- Speed: 400Hz
- Gain: 800-1000V (no saturation)
- Pinhole: 1AU

Fluorescence recovery after photobleaching

### **FRAP WIZARD**

1. Go to the left top of the screen and click the small arrow directing down. A menu will appear, select FRAP Wizard.

#### Setup tab

- Press live and search for a cell of interest. By using a target (press Ctrl+t) move the cell to the middle of the screen. Press Ctrl+t again to hide the target.
- 3. Rotate the view until the longest axis of the cell is vertical
- 4. Zoom in to the cell in such a way that it is completely filling the screen (e.g. for nucleus about 8x zoom)
- 5. Stop live imaging.
- 6. Press "Capture Image" to have the basic initial image of your cell.
- 7. Change you field of view to perform strip-FRAP
  - a. Go to the XY menu and click the double arrow (4a).
  - b. In the drop down menu, that appears there will be the option to choose "More".
  - c. A second window will appear where you can change the Y to 16 pixels and click OK.
- 8. Change the speed to 1400Hz (5)
- 9. Enable the Pinhole in the XY menu and set to 1-2 AU
- 10. Press "Live" again to see the result of changing the settings.



Leica Microsystems LAS AF - TCS SP5	File Help
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FRAP Wizard 1	0
FRAP XT Wizard	



Adjust hardware parameters for pre- and post-bleach imaging. - Set general settings at a low laser intensity for imaging fluoresc







Fluorescence recovery after photobleaching

#### **Bleach tab**

	FRAP
Bleach	
Workflow      Select mode of bleaching - Define setting for each Bleach-ROI      1. Define the positions of the Bleach-ROI in the Viewer Tool      2. Define method of bleaching      2. Define method of bleaching      2. Define method of bleaching      2.1: "FlyMode" (for fastest recording of recovery by bleaching and data readout in one frame), can not be combined with "Change Bleach Format".      2.2: "Change Bleach Format" (advantageous when several Bleach-Frames are defined; it reduces the time needed for bleaching by reducing the number of scanned lines according to the size of the ROI.      2.3: Click on "ROI-Configuration" to configure multiple ROIs. For each ROI a laser line should be applied.	NOI    Black Point      Image: Black house    Image: Black house      Image: Black house    Im
3. Define the laser line and intensity of the Bleach-ROI	405 488 496 514 561 504 600

- 11. Draw a square ROI in the cell (ROI 1; covering the whole nucleus)
  - a. Place lines of ROI half inside image, half outside
- 12. Draw a second ROI in the background (ROI 2)



Click in ROI Configuration and make sure the correct lasers are selected for the ROI's (8)

	405	458	476	488	496	514	561	594	633
R0[1									
R012									

14. Make sure the shutter of the bleaching laser is open and that the laser has a power of 100%.

#### Method of bleaching

- Fly mode Off
- Change Bleach format: Off
- Set background to zero: off



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### **Time Course Tab**

### Time Course Define the number of pre-bleach, bleach and post-bleach frames. Start the experiment. Time Course Pre-bleach Bleach Post-bleach 1 Frames 100 ≑ 5 🜲 2000 🖨 0.020 ≑ 0.022 🖨 0.022 🖨 t/frame [s] minimize minimize 0 2.3 22 46.3007142857143 t(s) - The minimized time for t/frame [s] is set as default. - Enable additional time scales for post-bleaching quantification.

#### Interval settings

- a. Pre-bleach
- b. Bleach

100 frames, 22ms interval (uncheck minimize) 9 frames or ~100ms,

c. Post-Bleach 1

2000 frames, 22ms interval (uncheck minimize)

15. Check settings and press start experiment



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### **Evaluation tab**



- 16. Enable a graph in the left window and scale accordingly by clicking right mouse button and go to scaling. A new window will open and you can change the scaling.
- 17. Check the curves created by the bleaching.
  - a. Is the pre-bleach signal stable or is it declining; if declining this means that the Imaging laser is to strong and bleaching your fluorophore. Change the settings for the general image set-up.
  - b. Is the Bleach "drop" almost as low as the background? No, then more Iterations are needed to bleach the fluorophore in the Time-Course settings.
  - c. Is the signal coming back in the post-bleach? No, use more Iterations to see the signal coming back.
- 18. When finished, export the data by clicking the right mouse button and export the data to a csv file.



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