

Confocal Spectrum Express 2004 02



FRAP with TCS SP2 (LCS 2.5 1347 a)

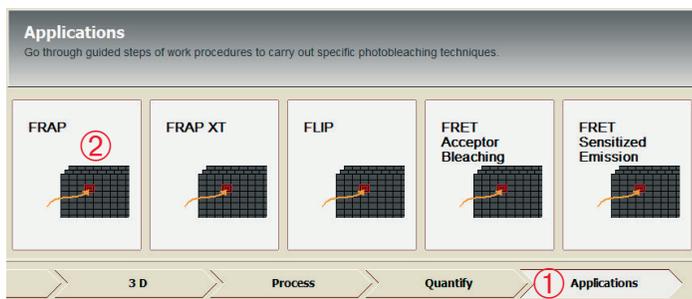
Part 1: FRAP wizard and FlyMode

Fluorescence recovery after photobleaching (FRAP) is a method for observing translational diffusion processes of macromolecules. With the LCS application wizard you can choose between different ways to carry out a FRAP experiment. You are able to adapt timing parameters for various experiments e.g. moderate, fast or multi step kinetics.

For very fast kinetics FlyMode is the best method. In this mode, reading out the signal during the x fly back of the scanner provides a time resolution "line by line" instead of "frame by frame" between bleach and postbleach images.

Depending on the necessary bleaching power you may choose ROI bleach or ROI-Zoom In bleach combined with one or multiple bleach steps. Free y format will reduce scanning time during bleaching.

Within the **Applications** menu ① there are two different wizards to perform FRAP experiments in various ways: FRAP and FRAP XT. This application note describes working with the FRAP wizard ②.

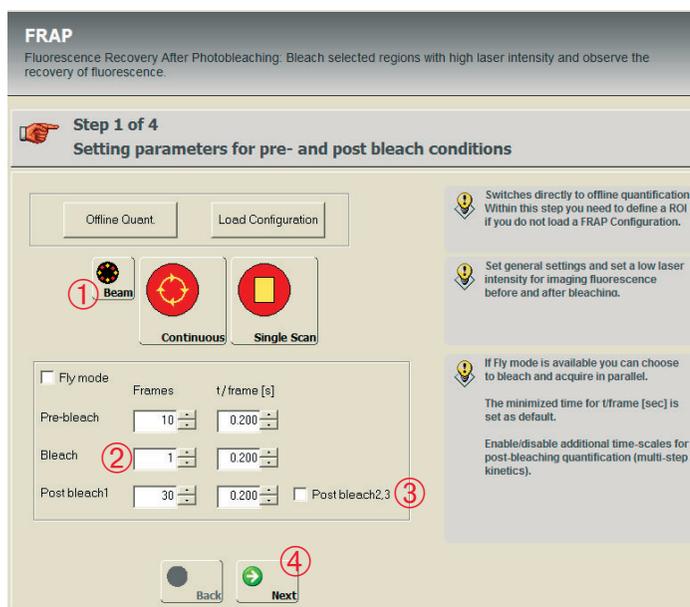


FRAP – step by step

■ Step 1: Setting parameters for pre- and postbleach conditions

First we will go through the FRAP routine without FlyMode. Define numbers of prebleach, bleach and postbleach intervals. A typical experiment could be the following: 10 frames prebleach, 1 frame bleach, 30 frames postbleach with 1000 Hz scan speed and 256 x 256 format. This results in a frame rate of 200 msec.

- Via the Beam Path Settings window ① adjust hardware parameters for pre- and postbleach imaging.
- Define the number of prebleach, bleach and postbleach steps ②.
- Click the checkbox **Postbleach 2, 3** ③ if you need additional different time scales
- Proceed with the next step ④.



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Note:

Argon laser: To achieve a high dynamic for bleaching turn the power control up to 75% and adjust low AOTF values for the pre- and postbleach intervals.

Step 2: Setting parameters for bleaching

- Now define the AOTF value(s) to tune the laser power ① for bleaching
- Define ROI for bleaching ②

Note:

If different laser lines for multiple ROIs are needed you can use the **Config** button by disabling the checkbox ③.

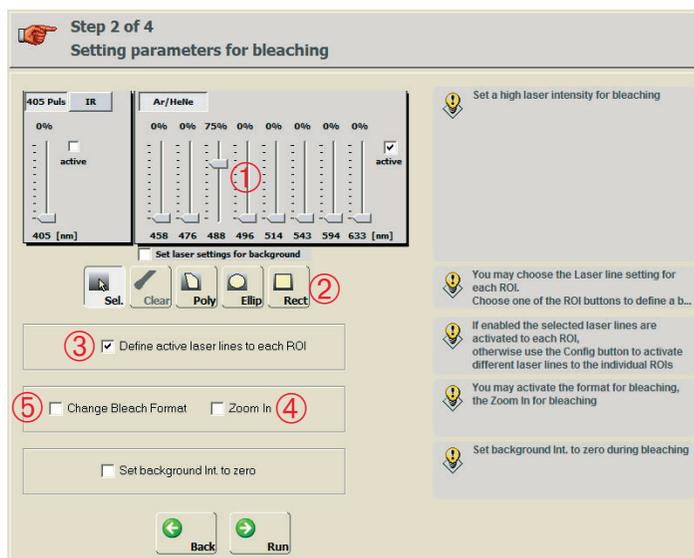
There are two ways to increase the bleach power by combining ROI with Zoom In:

1) ROI-Zoom In

If more power is needed activate the **Zoom In** check box ④. This will reduce the scan field during bleaching and more light is applied to the ROI.

2) ROI-Zoom In with Change Bleach Format

For even more power you may define multiple bleach iterations in step 1. Activate the check boxes **Zoom In** ④ and **Change Bleach Format** ⑤ simultaneously. According to the size of the defined ROI the number of scanned lines is reduced. This will reduce the bleaching time.

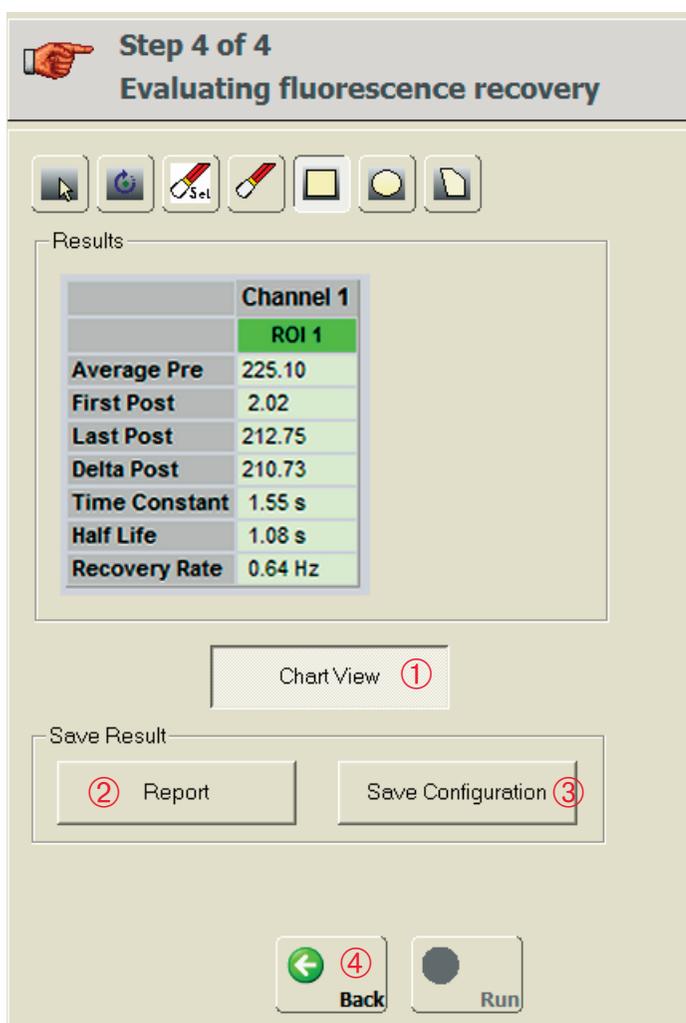


Click **Run** to start the experiment (step 3).

Step 3: Experiment runs automatically, leads to step 4

Step 4: Evaluation of the fluorescence recovery

- ① opens the recovery chart
- ② generates the xml-report
- ③ saves the whole experiment procedure
- ④ leads again to step 1 and keeps the hardware settings



Displayed values:

The table shows the grey values averaged over the ROI(s).

- Average of the prebleach intensities
- Intensity of the first postbleach image
- Intensity of the last postbleach image
- Delta Post (Last Post minus First Post)

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All following values are calculated on base of a single exponential fit: Time Constant, Half Life and Recovery Rate (reciprocal of Time Constant) according to the following algorithms:

$$\text{Intensity}(t) = \text{FirstPost} + \text{RecoveryAmplitude} \left[1 - \exp\left(-\frac{t}{\text{TimeConstant}}\right) \right]$$

For full recovery: $\text{RecoveryAmplitude} \approx \text{LastPost} - \text{FirstPost}$

Half Life = Time Constant • ln2

Recovery Rate = 1/Time Constant

Graph (Chart View)

The graph shows the grey values averaged over the ROI(s) for every frame.

Note:

During bleaching signal is in oversaturation. Therefore these intensities are not shown. The only exception is the FlyMode (see below).

Save Configuration

Here FRAP conditions, e.g. AOTF values and ROIs are saved.

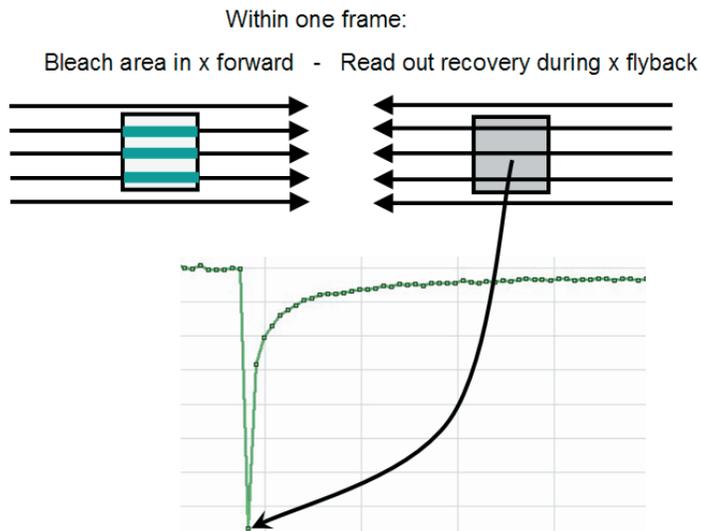
Note:

To recall your hardware settings for a new experiment proceed as follows: In step 1, open your last FRAP experiment and use the **Apply** function to call up the hardware settings. Then click on **Load Configuration**.

FRAP – FlyMode:

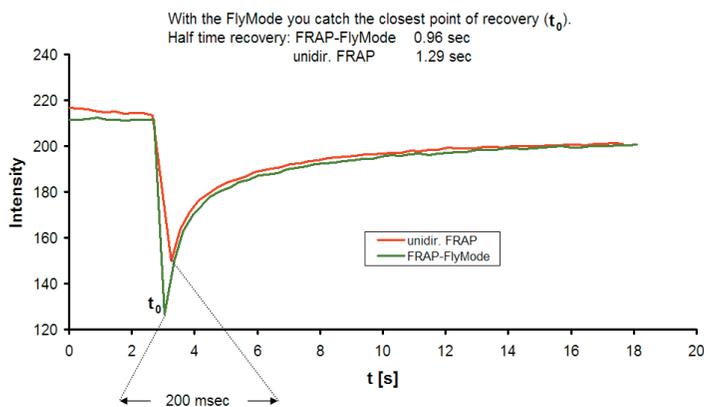
With FlyMode you may reduce the time resolution down to 0.35 msec since the readout of recovery is done between lines. This means, your recovery readout is closest possible to the real zero time (t_0) of the postbleach intensity.

The FlyMode combines both, the bleach scan and first image scan after bleaching. Bleaching is performed during fly forward using ROI Scan features and high laser power. During fly back, the laser intensity is set to imaging values (AOTF switching within microseconds). Thus, the first image is acquired simultaneously with the bleaching frame. And consequently, the delay time between bleaching and data acquisition is half the time needed to scan a single line.



Example 1:

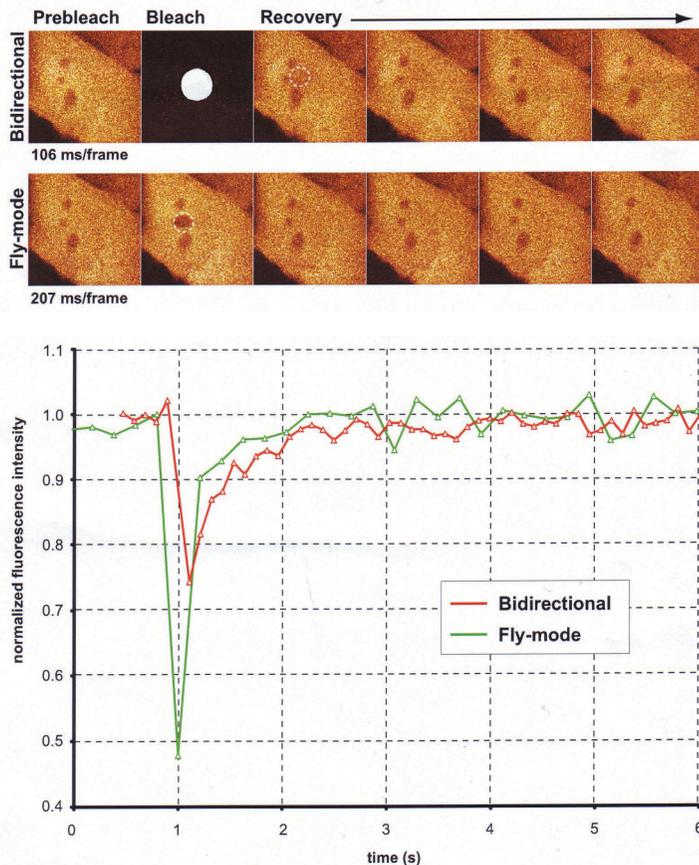
Free diffusion of FITC in glycerol: With FRAP-FlyMode time resolution is improved about 200 msec compared to unidirectional FRAP. Conditions: Objective: HCX PL APO CS 63.0 x 1.32 OIL UV, Zoom 6, ROI area = 90 μm^2 , 1000 Hz scan speed, 256 x 256 format.



Example 2:

Living cells, diffusion of GFP tagged proteins. 1400 Hz scan speed, 128 x 128 format. The experiment was performed in bidirectional scan mode and FlyMode.

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FlyMode operation is fully automated, just activate the check box **FlyMode** ① in step 1 and proceed as already shown above.

The screenshot shows the 'FRAP' software interface. The title is 'FRAP' with a subtitle 'Fluorescence Recovery After Photobleaching; Bleach selected regions with high laser intensity and observe the recovery of fluorescence.' The main window is titled 'Step 1 of 4: Setting parameters for pre- and post bleach conditions'. It features several control elements: 'Offline Quant' and 'Load Configuration' buttons; 'Beam' icon; 'Continuous' and 'Single Scan' radio buttons; a checked 'Fly mode' checkbox with a circled '1' next to it; and input fields for 'Frames' and 't/frame [s]' for 'Pre-bleach', 'Bleach', and 'Post bleach1'. There are also 'Back' and 'Next' buttons at the bottom. On the right side, there are three informational messages with warning icons.

Fly forward and fly back channels are displayed during the experiment. Since readout is done via fly back channel only the data of the fly back channel is saved after experiment.

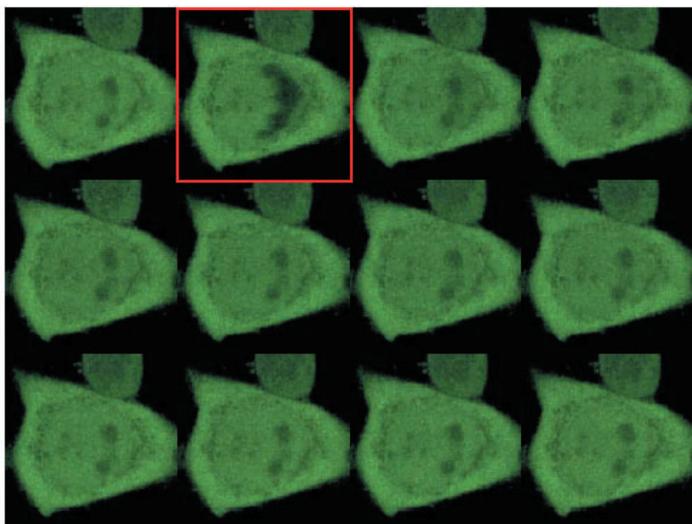
Note:

FlyMode is only possible with systems equipped with the Cambridge Galvo 6210. You may check this easily: If your system supports bidirectional ROI scan you can do the FlyMode, too.

Bidirectional Scan, Zoom In and Change Format for bleaching are not available in the FlyMode.

Very important for exact timing with the FRAP wizard: Always work with active "complete burst mode" to assure a proper timing.

FlyMode: The green graph shows the improved time resolution. NRK cells expressing GFP: maltose binding protein. Courtesy of Peter Lenart (Ellenberg group), EMBL, Heidelberg.



Sequence of images of a FRAP experiment using FlyMode
 HeLa Cells expressing YFP
 1st frame: prebleach image
 2nd frame (red): bleach and first postbleach image in one frame
 Following frames: postbleach images

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Part 2: Sequential acquisition between lines to exclude x-talk

This application note describes how to set up a FRAP experiment with two fluorescent dyes. For example one dye is used to study FRAP and the second dye is used as a reference. Very frequently the excitation and/or emission bands of two dyes are overlapping and this will cause crosstalk (x-talk or bleed through). You can avoid this phenomenon by doing the FRAP experiment in sequential scan mode between lines.

Sequential scan between lines may be useful for the following dye combinations:

- Hoechst/GFP
- CFP/YFP
- GFP/RFP

In the FRAP wizard you may adjust all needed sequential settings.

■ Step 1: Setting parameters for pre- and postbleach conditions

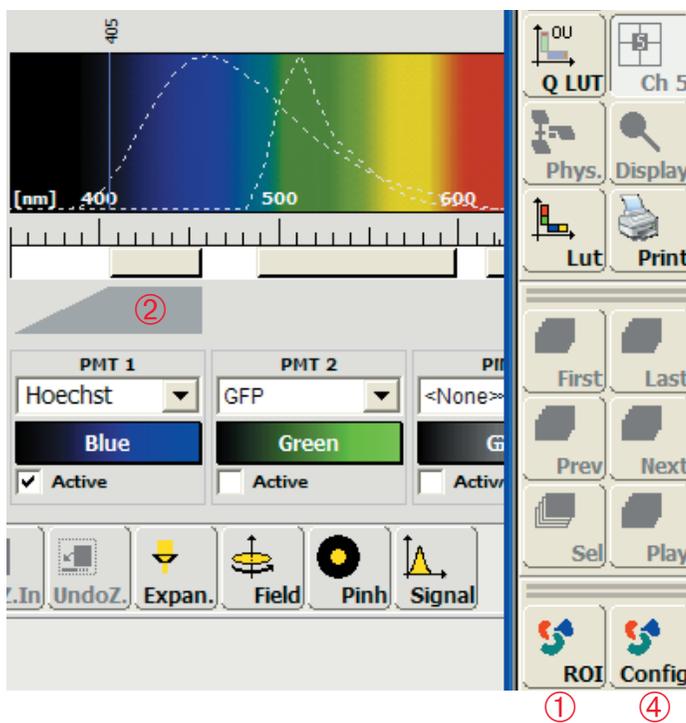
We assume a sample is labeled with Hoechst and GFP, whereas GFP is the marker to be bleached and Hoechst serves as a reference. Adjust the sequential scan settings between lines as described in the LCS help and the Confocal Application Letter Nr. 15 "Sequential Image Recording" (Art.-No: 1593104002).

- The major difference to conventional sequential image recording is that the sequential setting for each dye (reference-IPS for Hoechst ②, bleach-IPS for GFP ③) is saved while the ROI scan button is active ①. Use the AOTF values needed for pre- and postbleaching. The AOTF values for ROI- and background scan must be the same. Click on „Config“ ④ and assure for reference- and bleach IPSs that both, the 405 nm and 488 nm laser lines are enabled. Save each setting separately.

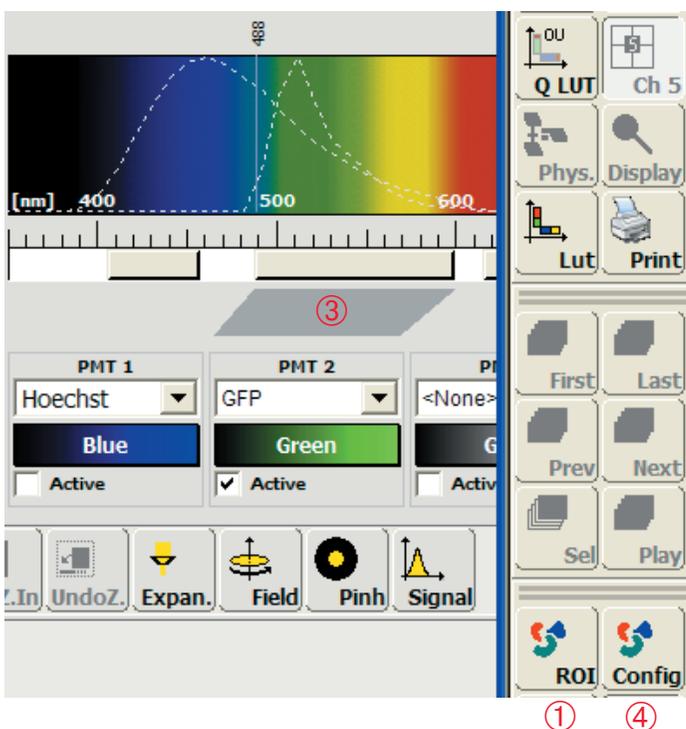
Note:

After having saved the first setting, deactivate ROI scan before adjusting the second setting. Then press again the ROI scan button.

Reference: IPS 405/Hoechst

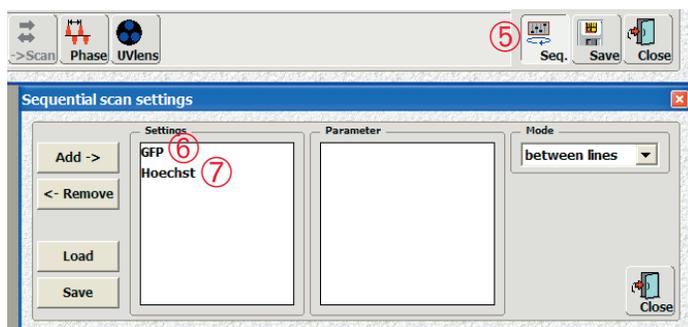


Bleaching: IPS 488/GFP



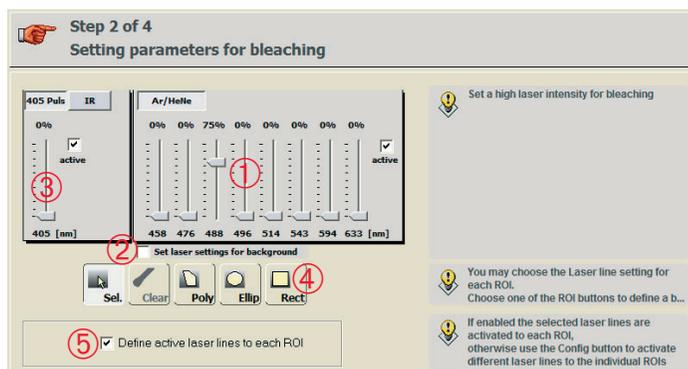
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- Activate sequential scan ⑤ and add the predefined IPs. The correct sequence of the IPs is crucial! The bleach setting (e.g. GFP) ⑥ has to be first, followed by the setting for the reference (e.g. Hoechst) ⑦. Leave the Sequential Scan Settings window open and continue with step 2.



■ Step 2: Setting parameters for bleaching

- Bleach procedure: Define the AOTF values for the laser intensity ① during bleaching (e.g. 75 % for the 488 nm laser line).
- Click the checkbox ② and raise the laser intensity (e.g. 10 %) ③ for the reference channel. Adopt the AOTF values from the IPS you already defined in step 1.
- Define ROI for bleaching ④.
- Check that both laser lines are active via **Config** by clicking ⑤.



Now, you may run the experiment.

Note:

When repeating the same experiment, the laser settings for the reference channel must be set again in step 2 ②.

During sequential scan between lines, FlyMode is not available.

The frame rate decreases by a factor of 2 because of sequential scan.

The detection ranges of the two channels have to remain in the same positions and must not be altered. The operation of sequential scan is described in the LCS help and in the Confocal Application Letter Nr. 15 "Sequential Image Recording" (Art.-No: 1593104002).