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Leica MICROSYSTEMS

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CONFOCAL APPLICATION LETTER

reSOLUTION

FRAP with TCS SP8

FRAP with TCS SP8 (LAS AF 3.1.0)

Introduction

Fluorescence recovery after photobleaching (FRAP) has been considered the most widely applied method for observing translational diffusion processes of macromolecules. The resulting information serves to determine kinetic properties such as the diffusion coefficient, mobile fraction, and transport rate of the fluorescently labeled molecules. FRAP employs irradiation of a fluorophore with a short laser pulse. State of the art laser scanning microscopes such as the Leica TCS SP8 have the advantage of using a high intensity laser for bleaching and a low intensity laser for image recording. The LAS AF application wizard offers different ways to carry out a FRAP experiment. Timing parameters can be adapted to various experiments, e.g. moderate, fast, or multi-step kinetics. Moreover, related experiments such as photoactivation and photoconversion or FLIP experiments are possible.

For very fast kinetics, FlyMode can be applied. Using FlyMode, the reading out of signals during the x fly back of the scanner provides a time resolution between lines – instead of between frames – for the FRAP experiment. Depending on the necessary bleaching power you may choose ROI bleach or ROI with Zoom In bleach combined with one or multiple bleach steps. A free y format reduces scanning time during bleaching when multiple bleach intervals are needed.

With the introduction of the Leica TCS SP8, the FRAP wizard offers improved functionality and additional features:

- z-stacking
- loop between bleach and post-bleach 1
- sequential scan between lines
- Zoom In ("FRAP Zoomer") for bleaching in RS mode.

FRAP – Step by Step

Choose the FRAP wizard

First, choose the FRAP wizard option under the TCS SP8 tab on top of the user interface (**Fig. 1**).

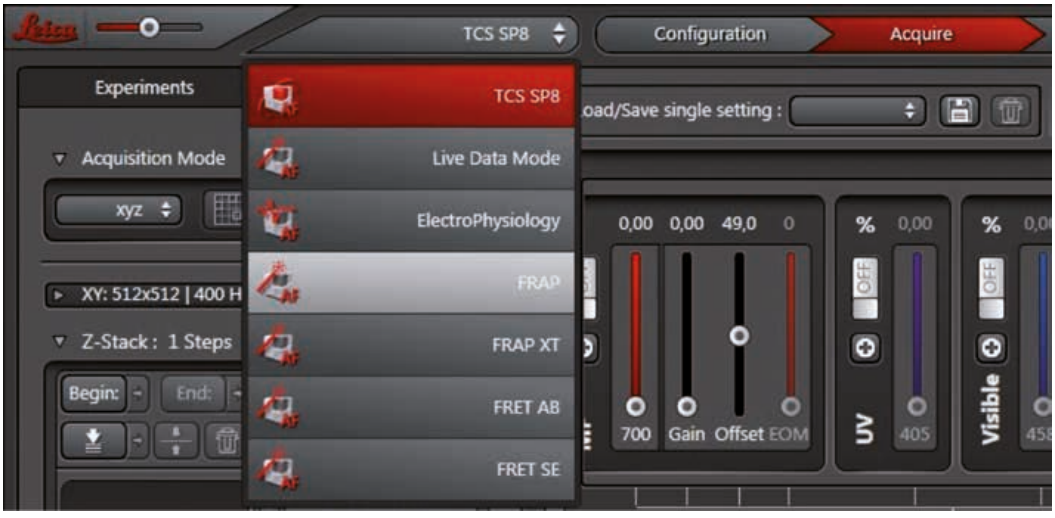

Fig. 1

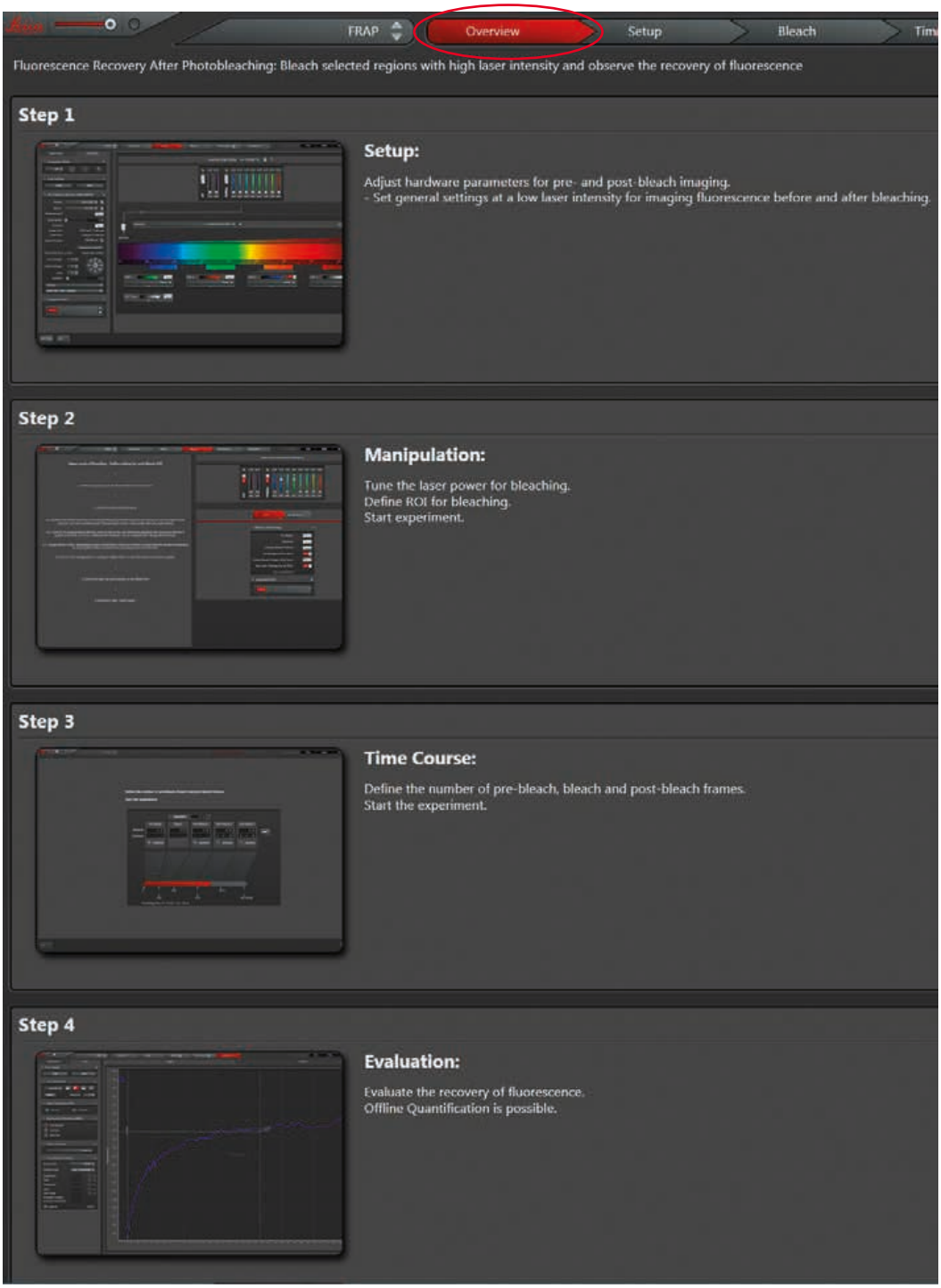

Fig. 2

Wizard overview

Clicking on **Overview** shows a description of steps 1 to 4 (**Fig. 2**).

Step 1:

Setup – Setting parameters for pre- and postbleach imaging

Click on **Setup** (see **Fig. 3** to adjust hardware parameters for pre- and postbleach imaging.

Note:
For FRAP experiments on TCS SP8 systems with Hybrid Detectors (HyDs), it is recommended to use a PMT as detector. During the bleach sequence, high intensity levels are usually reached inside the Region of Interest (ROI). This may cause the HyD detectors to switch off to protect them from photon overload. As the detector has to be actively switched on afterwards, the post-bleach sequence will be lost for quantification.

Fig. 3

Scan Mode

For analysis of a 3D stack, the scan mode can be changed from xyt to xyzt (**Fig. 4**). Then, pre- and postbleach series are scanned in xyzt mode. A single plane within the stack can be defined for bleaching.

Fig. 4

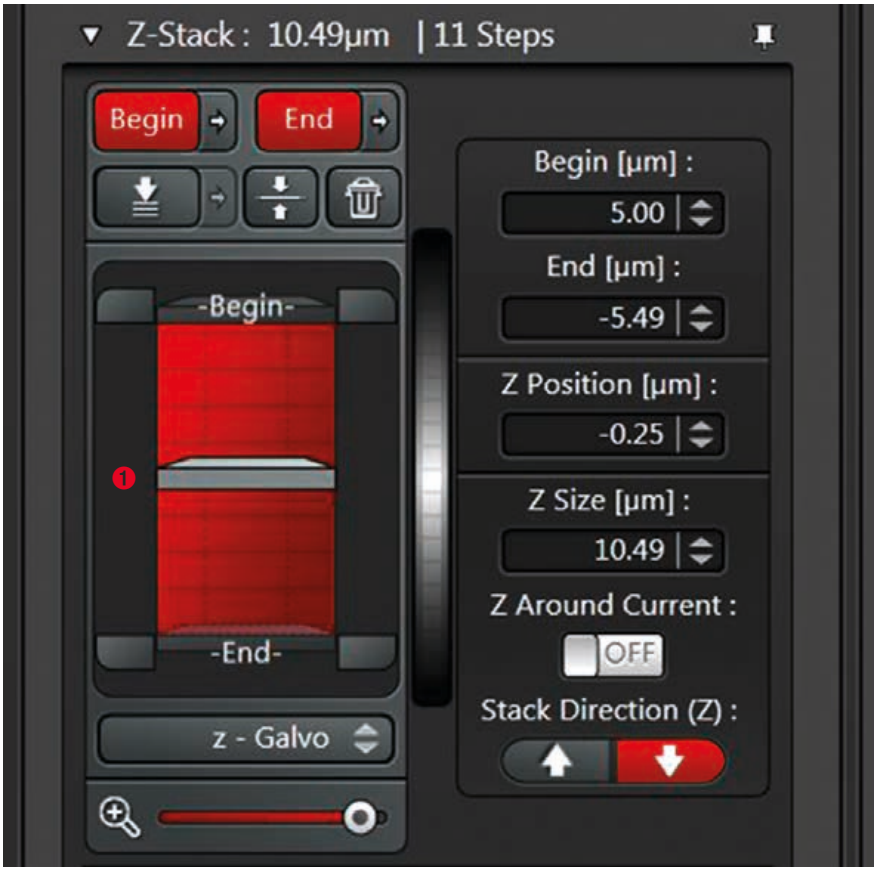

Fig. 5

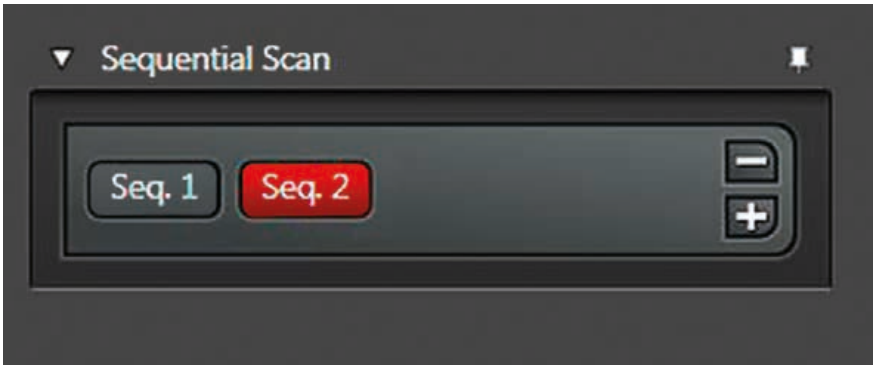

Fig. 6

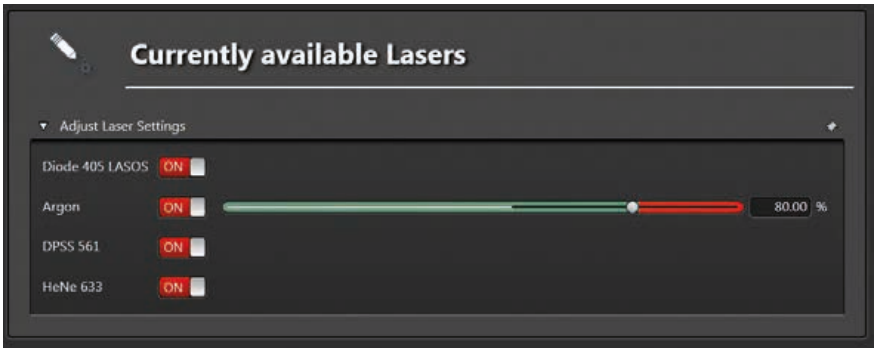

Fig. 7 shows the increasing tube current of the Argon laser.

Any plane within the stack can be defined for bleaching: The position of the actual z-plane ① (**Fig. 5**) is automatically saved and displayed at the bottom within the UI. The bleach pulse is only executed in this plane. There is no acquisition of a z-stack during the bleaching process.

Acquisition speed

Depending on the expected mobility of the molecules, the appropriate acquisition speed can be adjusted. Thus, for freely diffusing molecules 1,800 Hz line frequency, a scan speed with bidirectional scan should be used. In combination with an image format of 256 x 256 pixels, image recording at every 79 ms is possible.

Sequential Scan

Sequential (**Fig. 6**) scan prevents crosstalk in case of a multiple channel setup: This is useful for photoconversion – experiments with photoswitchable proteins like KAEDE or DENDRA2.

Light Dose

To allow the highest dynamic range between monitoring and bleaching, adjust the Argon laser power to 80–100 % (**Fig. 7**) within the configuration/laser menu.

For imaging, set the AOTF values to a low percentage.

FRAP Booster

In case the amount of excitation light is not sufficient, e.g. during fast acquisition with resonant scanning, FRAP Booster (**Fig. 8**) is recommended if the system is configured with the FRAP Booster option. After selection, the FRAP Booster option is active for the whole experiment.

If the FRAP Booster is active, the beam expander is retracted from the beam path. As a result, the back aperture of the objective is not completely filled with light any more (see **Fig. 9A**): The amount of light remains but is concentrated to a spot in the center. This results in about 2 to 5 times more light, depending on the objective used (see **Fig. 9B**).

Fig. 8

Pinhole size

You may set the pinhole size to two or more Airy units if you work with thin cell layers. Opening of the pinhole improves the signal-to-noise ratio and allows more information to be collected about kinetics within the depth.

Note:
Set the intensity below saturation and slightly above zero as setting to zero can interfere with data analysis. An appropriate lookup table (glow over/glow under) can help to adjust gain/offset. Make sure to use the same gain settings for all experiments. For reproducibility, it is recommended to save (**Fig. 10**) the settings that include all settings from the tabs **Setup, Bleach** and **Time Course** either in this step or in the very last one. This will save all hardware settings including the ROI shape and position.

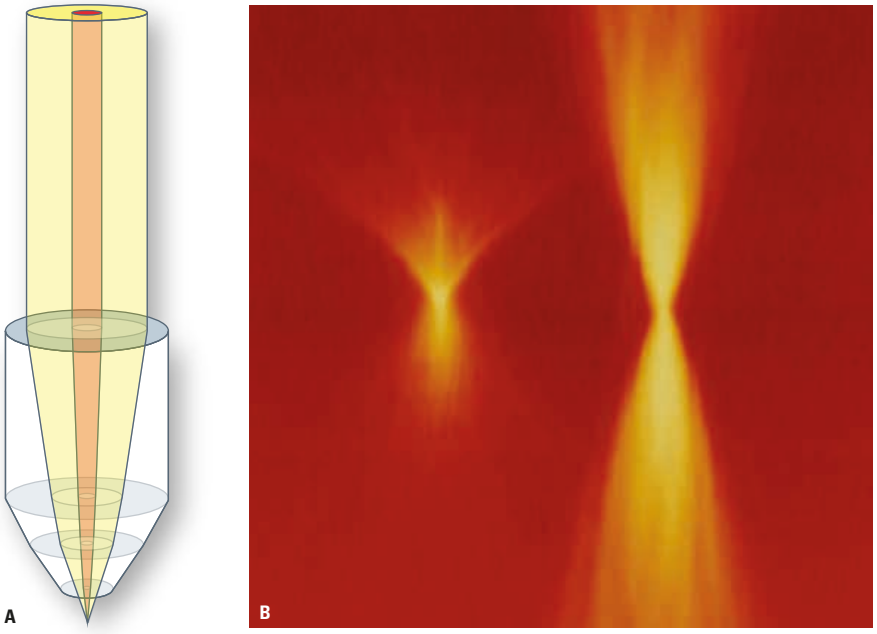

Fig. 9A: The sketch illustrates how the pupil of the objective is filled in yellow, beam expander in position. The red color shows the more concentrated light caused by the retracted beam expander (FRAP Booster active).
Fig. 9B: Beampark experiment with a fluorescent slide. Much more light was applied to the sample using the FRAP Booster option. After beam-park, a xzy scan was done and processed to a maximum projection. Left: without FRAP Booster. Right: with FRAP Booster.

Fig. 10

Step 2:
Bleach –
define parameters for bleaching

Click on the **Bleach** button (**Fig. 11**) to set the parameters for bleaching. On the left side you see a description of all options.

First choose whether to apply ROI ① or Bleach Point ② (**Fig. 11**).

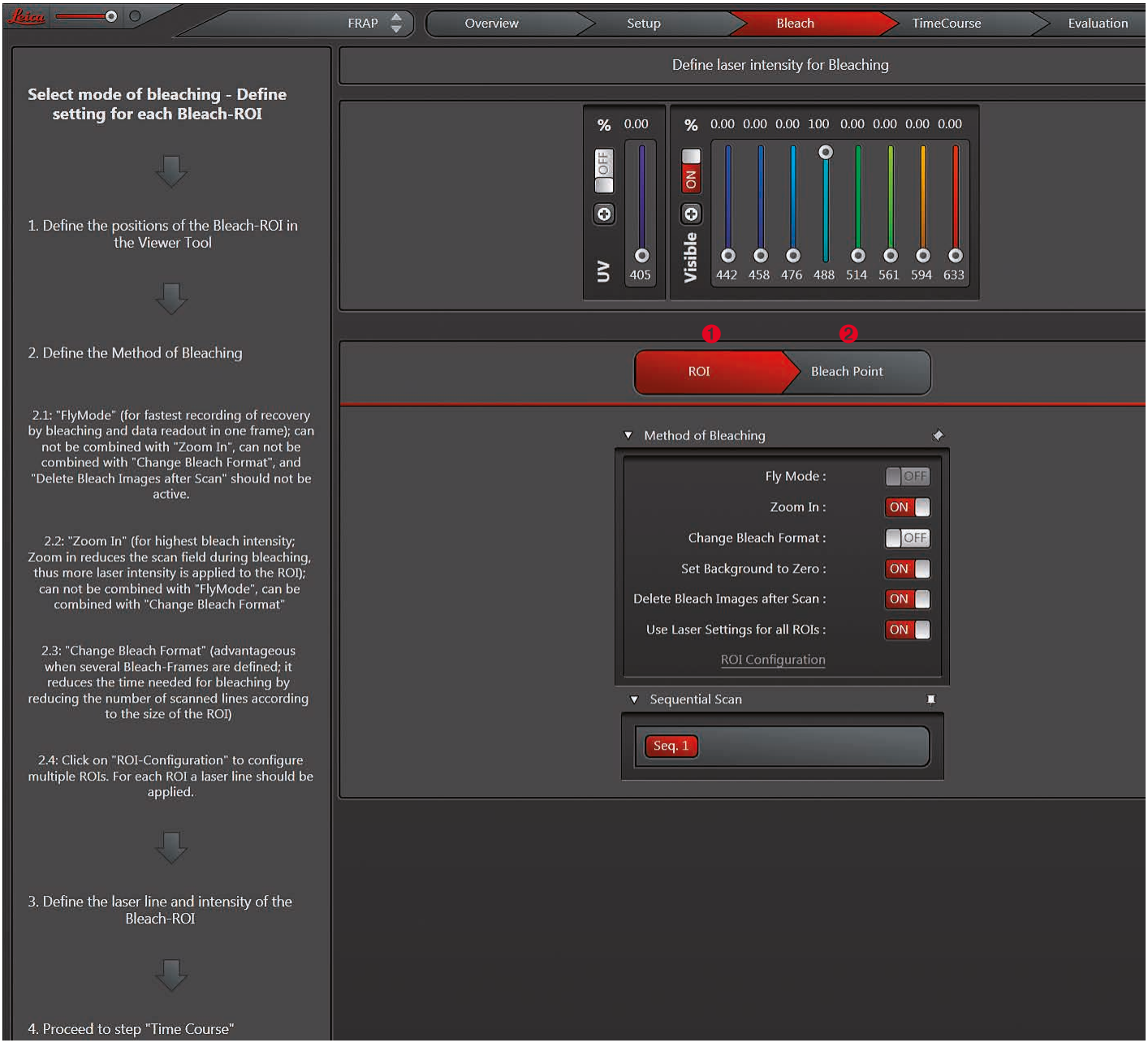

Fig. 11

You can choose any of the options displayed in **Fig. 12**.

FlyMode ①

FlyMode provides faster time resolution for the whole FRAP series. Instead of frame-wise time resolution, line-wise time resolution can be obtained. You may reduce the time resolution down to 0.35 ms since the measurement of recovery is already done between lines instead of between frames. This means the measurement of recovery starts the closest possible to the zero time (t_0).

The FlyMode combines both the bleach scan and the first image scan after bleaching (**Fig. 13**). Bleaching is performed during the forward motion using ROI scan features together with high laser power. During flyback, the laser intensity is set to imaging values (AOTF switching works within microseconds). Thus, the first image is acquired simultaneously with the bleaching frame. Consequently, the delay time between bleaching and data acquisition is less than half the time needed to scan a single line.

Zoom In ②

For most bleaching applications we recommend the **Zoom In** option. It minimizes the scan field during bleaching so that more light is applied to the ROI.

New: Using Leica TCS SP8, Zoom In is possible with the resonant scanner, too.

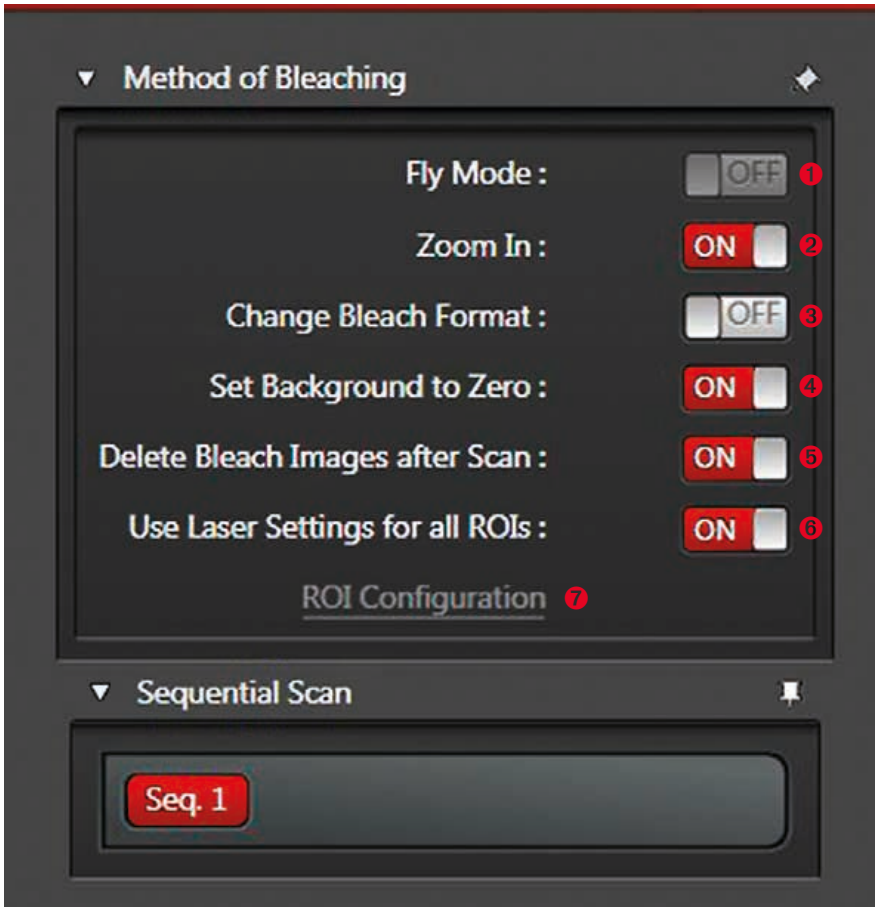

Fig. 12

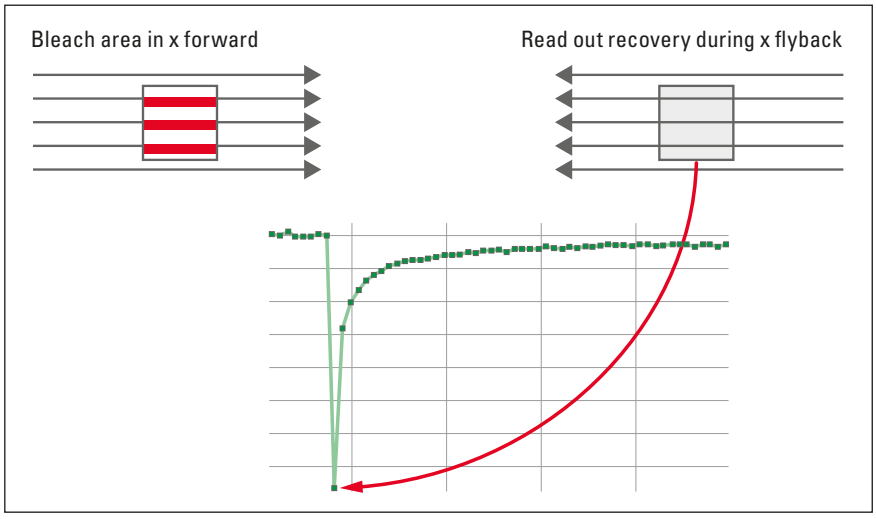

Fig. 13

Change bleach format ③

According to the size of the defined ROIs, the number of scanned lines is reduced during bleaching (strip scan). You may use this option to speed up the bleaching when multiple bleach intervals are needed, e.g., 10 or more. This option can be combined with **Zoom In**.

Set background to zero ④

This option is recommended when **Zoom In** or **FlyMode** is active.

Zoom In: Normally the area outside a ROI will be exposed with the background intensity. This background intensity can lead to bleaching if the image is zoomed in. Using **Set background to zero**, the area outside the exposed ROI will get no light.

When the FlyMode is active, a forward channel and a fly back channel is enabled (see above). Using **Set background to zero**, the forward channel collects light during prebleach and postbleach. Only during the bleach step, the forward (left) channel illuminates the ROI.

Delete bleach images after scan ⑤

Usually, the bleached ROI is very bright and in many experiments Zoom In for bleaching is used which hampers meaningful quantification. The same is valid for the option **Change Bleach Format**. In these cases, the option **Delete Bleach Images** after Scan avoids accumulation of waste data.

Use laser settings for all ROIs ⑥

Activate the **Use laser settings for all ROIs** option when several ROIs should be exposed with the same laser lines. Now, draw the ROI for bleaching and define the AOTF value(s) to tune the laser power for bleaching.

Bleaching with several laser lines and several ROIs

Click on **ROI Configuration** ⑦ when individual laser lines should be active for several ROIs. In advance you have to uncheck **Use laser settings for all ROIs**.

Preconditions for effective bleaching in resonant scanning mode

If very fast scan modes are required for measurement of diffusion in aqueous media, you may bi-directionally scan in 512 × 128 format. This will result in a very short time per frame, e.g. 12 ms. Here, it is recommended to apply multiple bleach frames, e.g. three or four to supply sufficient light for bleaching.

To compensate for too little light due to short illumination time, you may use

- Zoom In during bleaching. Using Leica TCS SP8, Zoom In is available for the resonant scanner, too (FRAP Zoomer).
- the FRAP Booster option within Set Up.

Photoactivation

The FRAP wizard for photoactivation, e.g. pa-GFP is an additional option. Open the UV shutter and use the 405 laser line instead of the 488 laser line for bleaching.

Step 3:

Time Course – Define number of prebleach, bleach and postbleach intervals

Select **TimeCourse** in the upper menu to define the number of prebleach, bleach and postbleach intervals (**Fig. 14**). A typical experiment with 1,800 Hz scan speed (bidirectional scan) and 256 × 256 format can be defined as shown in the table below: You may add different additional timescales as well by clicking the + symbol.

If required, the running experiment can be stopped, e.g., during postbleach. The user will then be guided to the evaluation step. This is particularly useful if the total time for full recovery is unknown as it allows the experiment to be ended during postbleach once the full recovery (i.e. no more increase in intensity) is reached. There is no need to wait until the predicted number of frames has been acquired.

	Frames	Minimized time frame	Time per frame [ms]
Prebleach	10	yes	79
Bleach	1	yes	79
Postbleach 1	100	yes	79
Postbleach 2	10	no	1,000
Postbleach 3	10	no	5,000

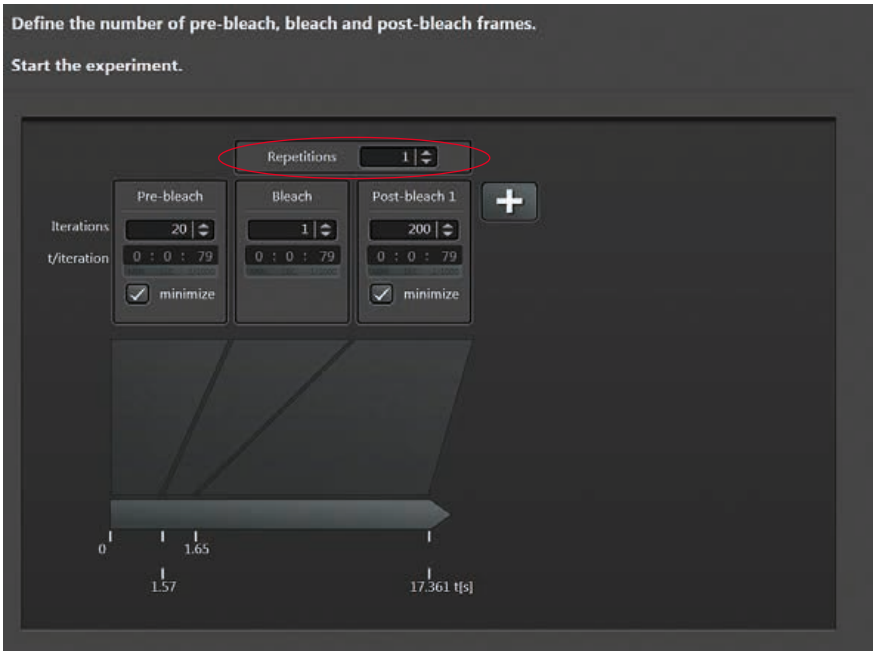

Fig. 14

Acquisition speed

The acquisition speed should be adjusted to resolve the dynamic range of the recovery with good temporal resolution. At least 10 data points halfway through the recovery are required.

FRAP with Loop

To perform FLIP experiments, you can define the number of repetitions (**Fig. 14**) between bleach and first postbleach.

Duration of the FRAP experiment

Initial experiments should be continued as long as noticeable further increase in fluorescence intensity is detected. If you want your ROIs & **TimeCourse** included in your saved settings you can do this in the first or the last step. Click **Run Experiment** to start the experiment. The experiment automatically runs and leads to the evaluation step.

Note:
If you perform experiments with fluorescent proteins, the use of postbleach sequences with different time scaling may lead to intensity decrease during transition between the different time scales. Altering the imaging frequency during the experiment can alter the fraction of fluorescent protein driven into dark states (see Weber et al., 1999). Another strategy is defining 40 or 50 prebleach intervals to achieve a steady state intensity after a first decrease of intensity within the first 10 to 20 intervals of the prebleach series.

Fig. 15:
① Experimental conditions and procedure can be saved
② Report generates a data sheet in xml format
③ Background can be subtracted
④ Fitting can be applied to the data

Step 4:
Evaluation

Now, the displayed recovery (**Fig. 15**) shows all intensity values averaged over the ROIs for all frames. This chart can be exported to Excel via a right mouse click.

Suggested background reading

1. Phair, R.D., Misteli, T.: High mobility of proteins in the mammalian cell nucleus. Nature 404: 604 (2000).
2. Beaudouin, J., Gerlich, D., Daigle, N., Eils R., Ellenberg, J.: Nuclear Envelope Breakdown Proceeds by Microtubule-Induced Tearing of the Lamina Cell 108: 83–96 (2002).
3. Axelrod, D., Koppel, D.E., Schlessinger, J., Elson, E., Webb, W.W.: Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. Biophys. J. 16: 1055–1069 (1976).
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5. Snapp, E.L., Altan, N., Lippincott-Schwartz, J.: Measuring protein mobility by photobleaching GFP chimeras in living cells. Curr. Prot. Cell. Biol. 21.1–21.1.24 (2003).
6. Ellenberg., J., Siggia, E.D., Moreira, J.E., Smith, C.F., Presley, J.F., Worman, H.J., Lippincott-Schwartz, J.: Nuclear membrane dynamics and reassembly in living cells: Targetting of an inner nuclear membrane protein in interphase and mitosis. J. Cell. Biol. 138: 1193–1206 (1997).
7. Siggia, E.D., Lippincott-Schwartz, J., Bekiranov, S.: Diffusion in inhomogenous media: Theory and simulations applied to a whole cell photobleach recovery. Biophys. J. 79: 1761–1770 (2000).
8. Lippincott-Schwartz, J., Altan-Bonnet, N., Patterson, G.H.: Photobleaching and photoactivation: following protein dynamics in living cells. Nature Cell Biology Suppl. S7–S14 (2003).
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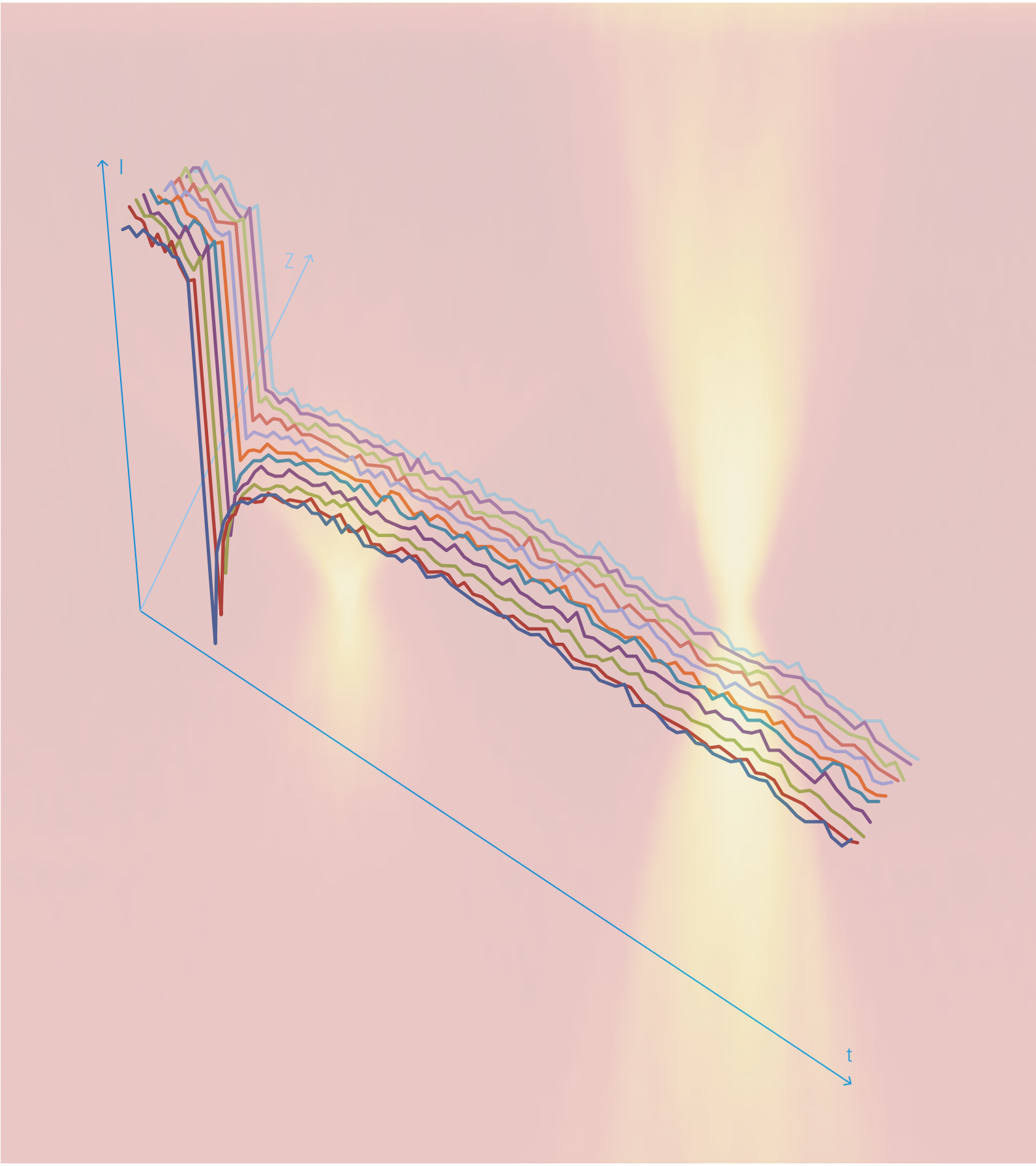
10. Misteli, T., Gunjan, A., Hock, R., Bustink, M., Brown, D.T.: Dynamic binding of histone H1 to chromatin in living cells. Nature 408: 877–880 (2000).
11. Beaudouin, J., D. Gerlich, N. Daigle, R. Eils, J. Ellenberg: Nuclear Envelope Breakdown Proceeds by Microtubule-Induced Tearing of the Lamina. Cell 108: 83–96 (2002).
12. Lippincott-Schwartz, J., E. Snapp, A. Kenworthy: Studying protein dynamics in living cells. Nature 2: 444–456 (2001).
13. Press, W.H., B.P. Flannery, S.A. Teukolsky, W.T. Vetterling: Numerical Recipes in C: The Art of Scientific Computing, 2nd edition, Cambridge University Press (1993).
14. Rabut, G., J. Ellenberg: Photobleaching techniques to study mobility and molecular dynamics of proteins in live cells: FRAP, iFRAP, and FLIP, in Live Cell Imaging: A Laboratory Manual. Goldman, R.D. and Spector, D.L. (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York: 101–126 (2005).
15. Snapp, E.L., N. Altan, J. Lippincott-Schwartz: Measuring protein mobility by photobleaching GFP chimeras in living cells. Curr. Prot. Cell. Biol., chapter 21.1 (2003).
16. Weber, W., V. Helms, J.A. McCammon, P.W. Langhoff: Shedding light on the dark and weakly fluorescent states of green fluorescent proteins. Proc. Natl. Acad. Sci. USA 96 (11): 6177–6182 (1999).

Useful links

- Molecular dynamics: http://www.embl.de/eamnet/html/molecular_dynamics_2005.html
- Analysis of FRAP Curves: Kota Miura: www.embl.de/eamnet/frap/FRAP6.html
- Introduction to FRAP, Timo Zimmermann: www.embl.de/eamnet/downloads/courses/FRAP2005/tzimmermann_frap.pdf
- Video on FRAP Technique from Essential Cell Biology, 3rd Edition Alberts, Bray, Hopkin, Johnson, Lewis, Raff, Roberts, & Walter, ISBN: 978-0-8153-4129-1:
  www.youtube.com/watch?v=LicQb_SnCSI
- Lecture of Jennifer Lippincott-Schwartz Part 2: Photobleaching and Photoactivation: www.youtube.com/watch?v=bhjP9PqfJRE
- Online Manual for the MBF-ImagsJ: Collection: www.macbiophotonics.ca/imagej/intensity_vs_time_ana.htm
- Leica Science Lab: www.leica-microsystems.com/science-lab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