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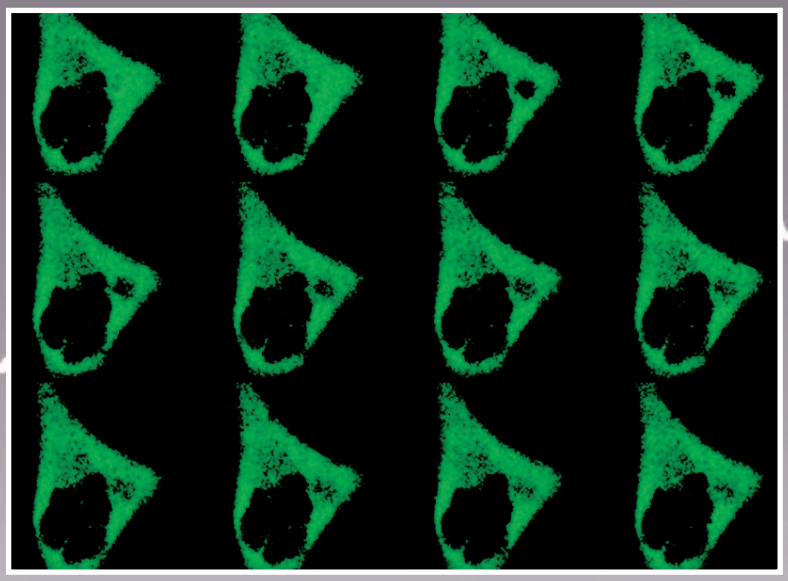
CONFOCAL APPLICATION LETTER

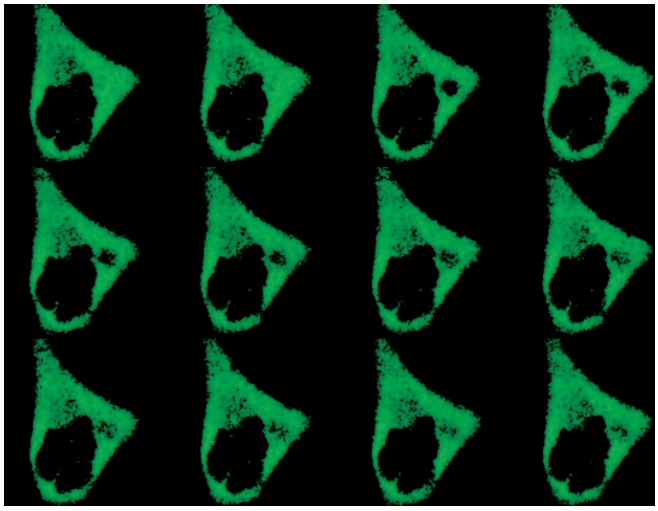
reSOLUTION



LAS AF APPLICATION WIZARD
FRAP WITH TCS SP5

Leica
MICROSYSTEMS

FRAP with TCS SP5 (LAS AF 1.5.1)



FRAP with Tubulin-GFP transformed HeLa cells.

Fluorescence recovery after photobleaching (FRAP) has been considered the most widely applied method for observing translational diffusion processes of macromolecules. The resulting information can be used to determine kinetic properties like the diffusion coefficient, mobile fraction and transport rate of the fluorescently labeled molecules. FRAP employs irradiation of a fluorophore in a living sample with a short laser pulse. Modern laser scanning microscopes like the Leica TCS SP5 have the advantage of using a high intensity laser pulse for bleaching and a low intensity laser pulse for image recording. With the LAS-AF 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 the FlyMode (pp. 9) is the best method. In this mode, reading out the signal 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. Free y-format will reduce scanning time during bleaching if multiple bleach intervals are needed.

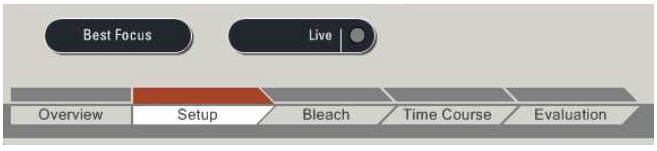
FRAP – step by step

Choose the FRAP-Wizard

First the FRAP routine without FlyMode is described. At the bottom of the wizard interface the working steps are displayed as buttons.

Step 1: Setup – Setting parameters for pre- and postbleach imaging

Click on the **Setup** button to adjust hardware parameters for pre- and postbleach imaging.



Acquisition speed

For freely diffusing molecules 1400 Hz line frequency scan speed with bidirectional scan should be used. In combination with an image format of 256 x 256 pixels one can record one image every 118 ms.

Excitation light

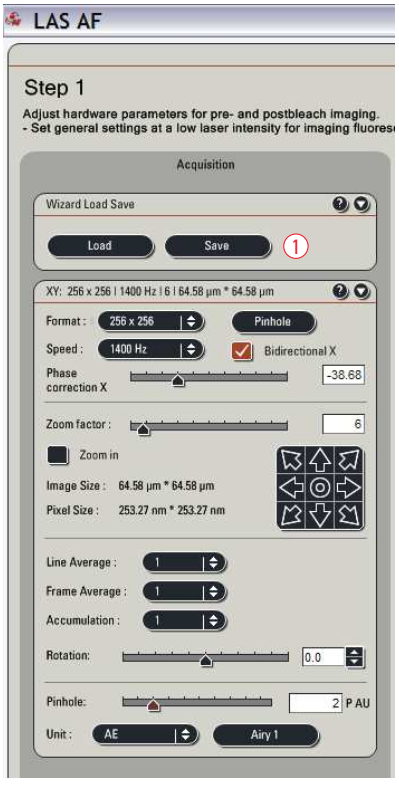
To allow highest dynamic range between monitoring and bleaching adjust Argon laser power to 100% with the configuration/laser menu. For imaging set the AOTF values at low percentage.

Pinhole size

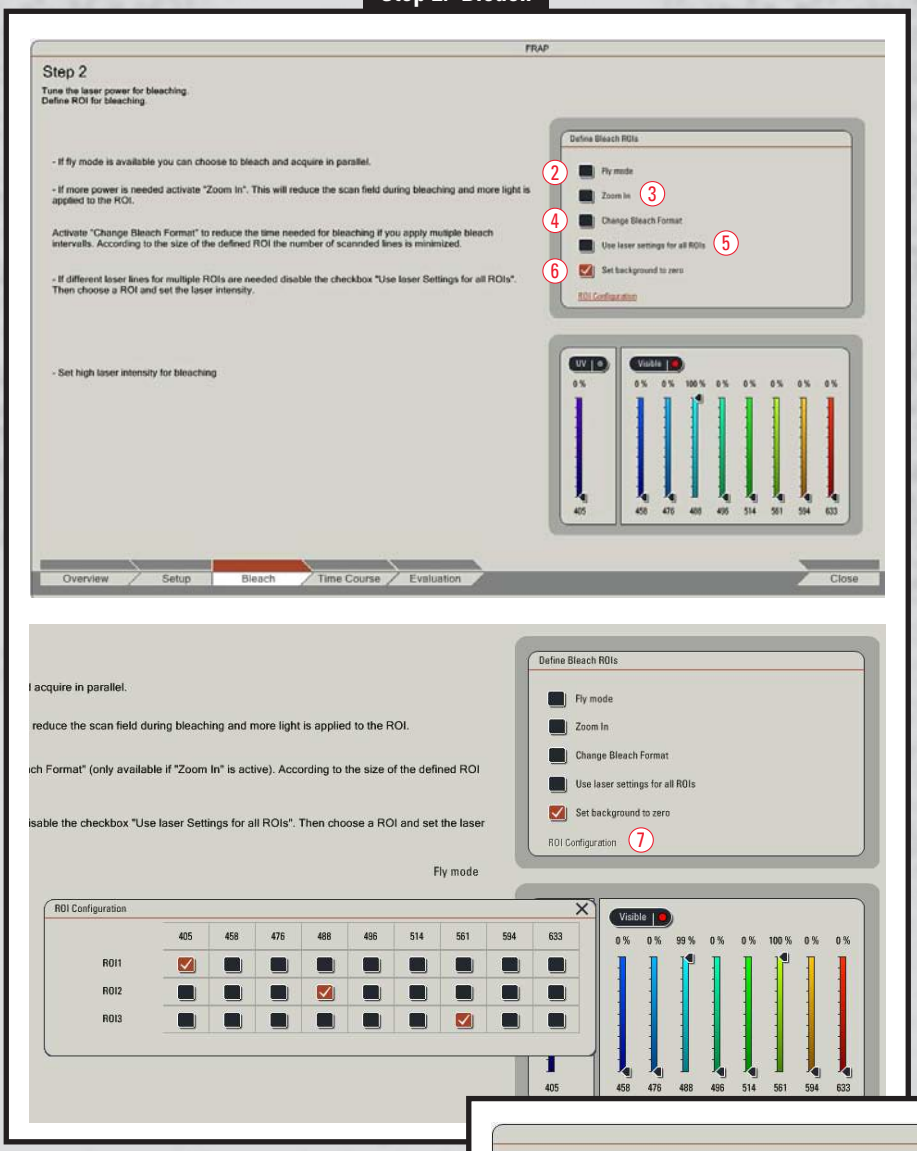
You may set the pinhole sizes to 2 airy units if you work with thin cell layers. You will collect more information about kinetics in the depth and improve the signal-to-noise ratio.

Note:

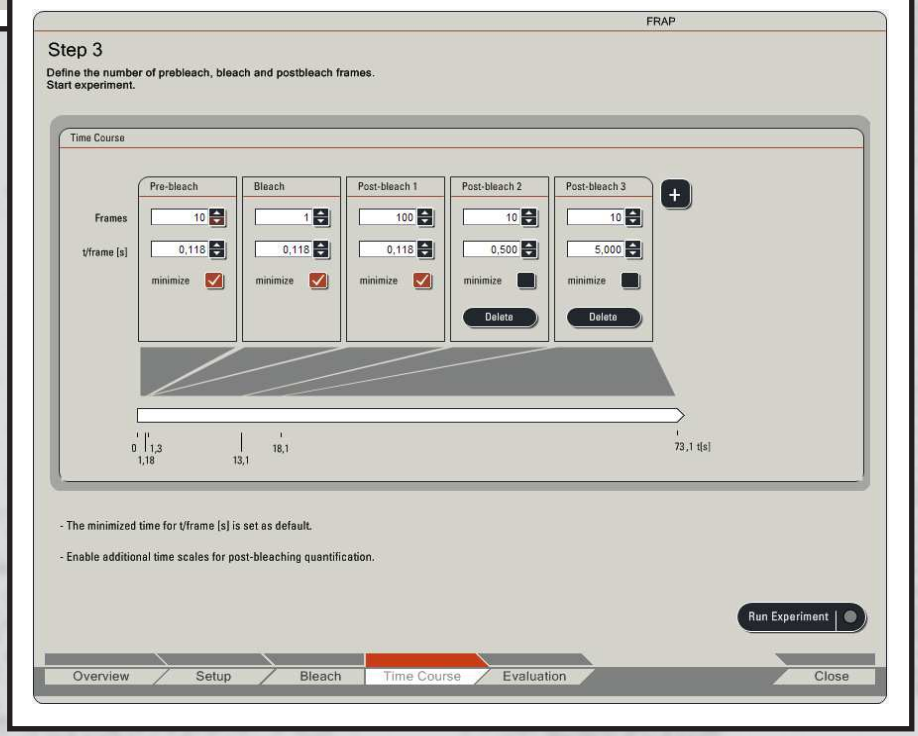
Set the intensity below saturation and slightly above zero as setting to zero can interfere with data analysis. An appropriate lookup table (glow over/glow under) can help to adjust gain/offset. Make sure to use the same gain settings for all experiments. For reproducibility it is recommended to save ① the settings that include all settings from the **Setup, Bleach** and **Time Course** Tabs.



Step 2: Bleach



Step 3: Time Course



Step 2: Bleach – Define parameters for bleaching
Click on the **Bleach** button to set the parameters for bleaching.

You can choose any of the following options:

FlyMode ②
Allows faster time resolution for the whole FRAP series

Zoom In ③
For most bleaching applications we recommend the **Zoom In** option. This reduces the scan field during bleaching and more light is applied to the ROI.

Set background to zero ⑥
This option is recommended when **Zoom In** is active. Thus the background outside the exposed ROI is not bleached

Change Bleach Format ④
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, e.g. 10 or more, are needed. This option can be combined with **Zoom In**.

Use laser settings for all ROIs ⑤
When several ROIs should be exposed with the same laser lines one can choose this option.

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 in the Define **Bleach ROIs** box when individual laser lines should be active for several ROIs.

Photo-activation
You can use the FRAP wizard for photo-activation as well. Open the UV-shutter and use the 405 laser line instead of the 488 laser line.

Preconditions for effective bleaching in resonant scanning mode
If very fast scan modes are needed (e.g. measurement of diffusion in aqueous media) you may scan bidirectional in 512 x 128 format which results in a very short time per frame, e.g. 18 ms. Here it is recommended to apply multiple bleach frames, e.g. three or four to apply sufficient light for bleaching.

Furthermore the resonant scanner needs some time for settling if the zoom value is changed. Therefore the zoom option that is applied only for bleaching is not available in step two for the resonant scanning mode. However, one can define a higher zoom value for the complete FRAP series in step one.

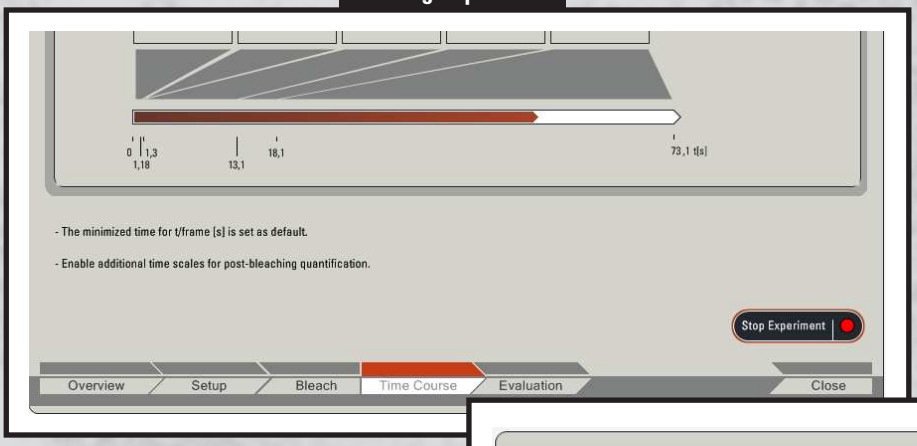
Step 3: Time Course – Define number of prebleach, bleach and postbleach intervals
Next, choose **Time Course** to define number of prebleach, bleach and postbleach intervals.

A typical experiment with 1400 Hz scan speed (bidirectional scan) and 256 x 256 format can be defined as follows:

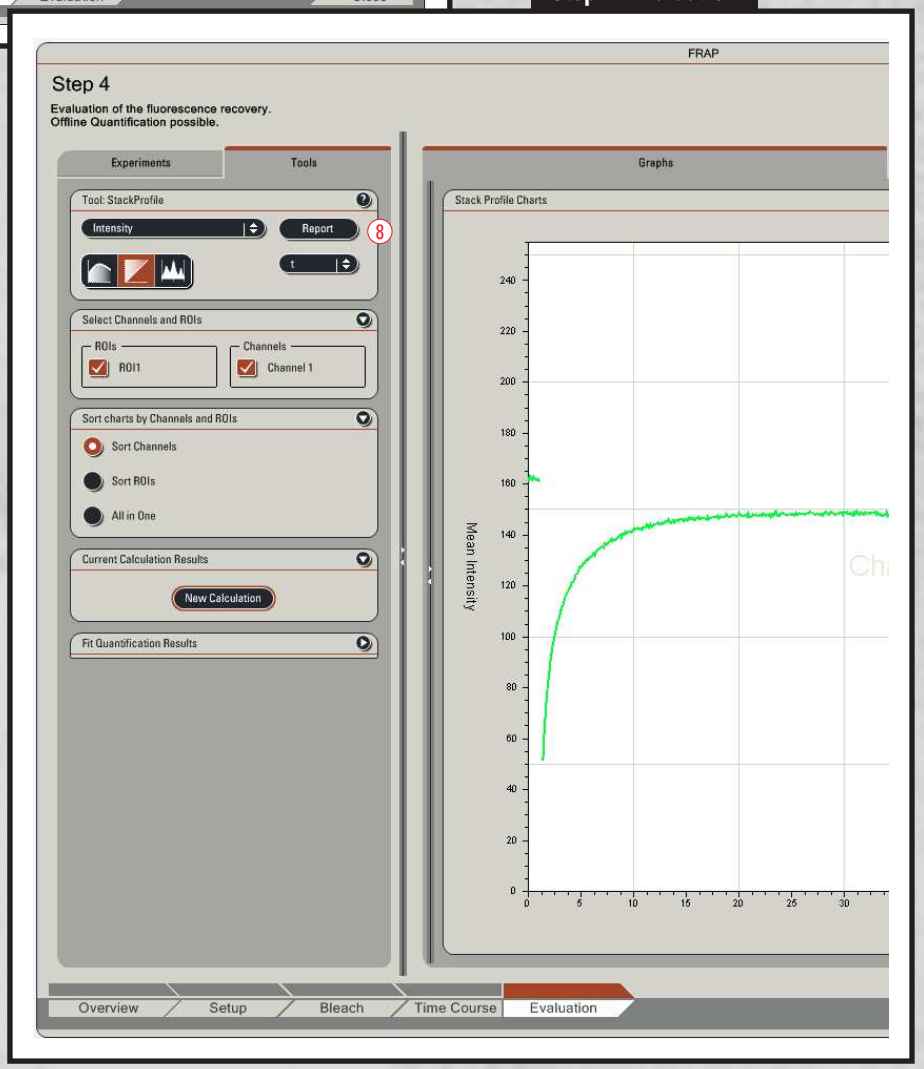
Prebleach	10 frames	minimized time frame → 118 ms each interval
Bleach	1 frame	minimized time frame → 118 ms each interval
Postbleach 1	100 frames	minimized time frame → 118 ms each interval
Postbleach 2	10 frames	→ 1 sec each interval
Postbleach 3	10 frames	→ 5 sec each interval

Acquisition speed
The acquisition speed should be adjusted to resolve the dynamic range of the recovery with good temporal resolution. Thus ideally acquire at least 10 data points during the time required for half of the recovery.

Running Experiment



Step 4: Evaluation



Duration of the FRAP experiment
Initial experiments should be conducted until no noticeable further increase in fluorescence intensity is detected.

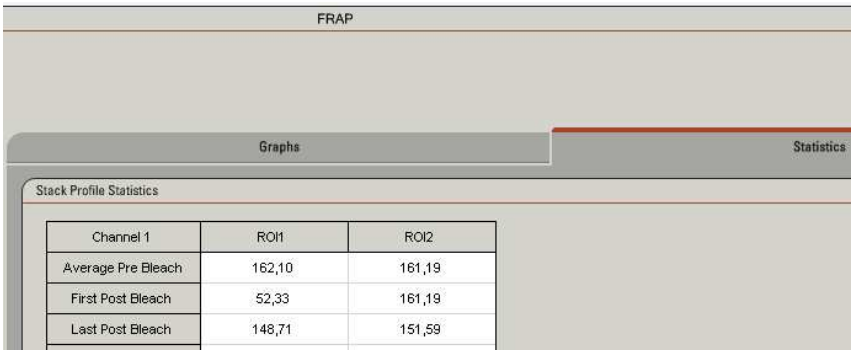
If you want your ROIs & Time Course included in your saved settings you can do this in Step1. Proceed again to **Time Course** and click **Run** to start the experiment. The experiment runs automatically and leads to the evaluation step.

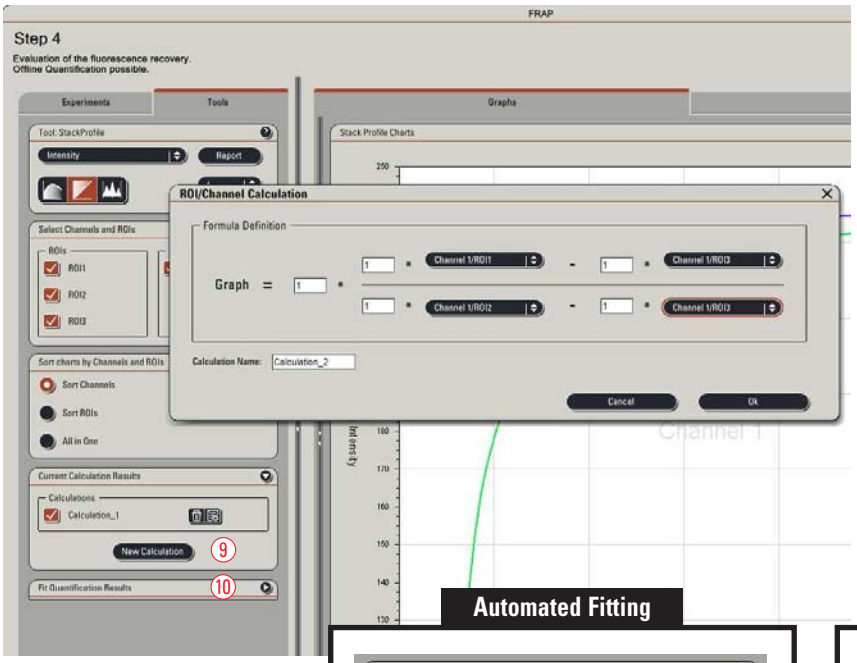
Note:
If you use fluorescent proteins usage of postbleach 2 & 3 may lead to intensity changes during transition between the different time scales. Altering the imaging frequency during the experiment can alter the fraction of fluorescent protein driven into dark states (see Weber et al., 1999).

The experiment can be stopped while it is already running 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 not known, since it allows to end the experiment during postbleach as soon as 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.

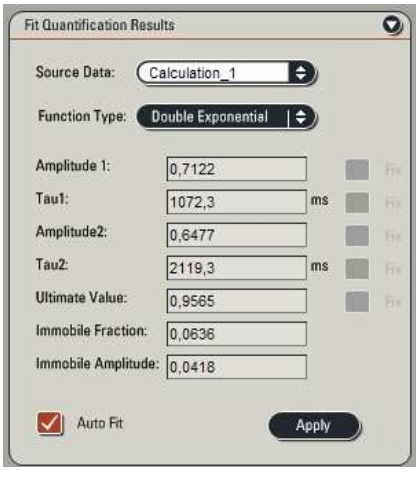
Step 4: Evaluation
Now the recovery chart is displayed. The chart shows all intensity values averaged over the ROIs for all frames. This chart can be exported to Excel via right mouse click. The **Report** button ⑧ generates a data sheet in xml format. To compare different experiments it is essential to use bleaching regions with the same size, relative position in the cell and scanning parameters. You can save the experiment procedure in the **Setup** step (p. 3).

Step 4: Evaluation

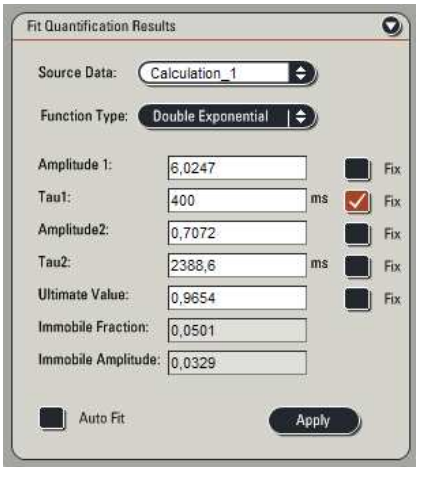




Automated Fitting



User Defined Fitting



You can display the relevant FRAP data such as last prebleach or first postbleach value in the statistics tab.

Usually the chart has to be corrected for background bleaching which is caused during imaging. Define a second ROI, e.g. enclosing the whole cell, and click on **New Calculation** ⑨ on the **Current Calculation Results** box. This opens a window where you can enter the desired formula using the values of all ROIs as well as arbitrary constants which can be used to subtract offset values. The result appears in a second chart at the bottom.

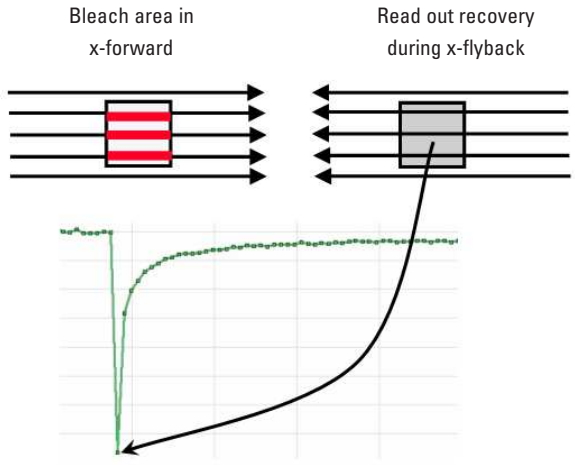
Fitting of FRAP data

Quantities like the relaxation time (τ) or amount of immobile fraction can be determined by single or double exponential fitting: After correction the calculated data is ready to be processed by LAS AF according to Levenberg Marquardt (see Press et al., 1993, pp 681-687). Click on Fit Quantification Results ⑩ and choose which ROI should be applied with which channel or which calculation should be used as source data, e.g. Calculation_1. Apply either an automated fit (see figure "Automated Fitting") or a user defined fit using fixed values for certain parameters e.g. for tau1 (see figure "User Defined Fitting"). The resulting fit is then overlaid with the calculated graph.

FRAP – FlyMode:

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 closest possible to the 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 the forward motion using ROI scan features together with high laser power. During fly back, the laser intensity is set to imaging values (AOTF switching works within microseconds). Thus, the first image is acquired simultaneously with the bleaching frame. And consequently, the delay time between bleaching and data acquisition is less than half the time needed to scan a single line.

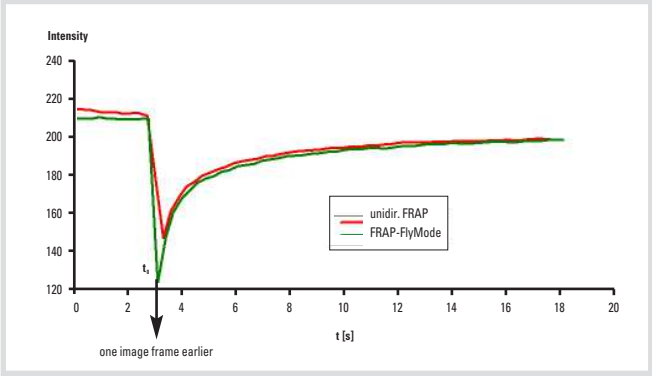


Free diffusion of FITC in glycerol, 1400 Hz scan speed and 256x256 format. With FRAP-FlyMode readout starts one frame earlier compared to unidirectional FRAP.

FlyMode operation is fully automated; just activate bidirectional scan in step 1 (setup), activate FlyMode in step 2 and proceed as already shown above.

Forward and fly back scan are displayed as separate channels during the experiment. The viewer shows on the left side the forward scan and on the right side the fly back scan.

Note:
Zoom In and **Change Format** for bleaching is not available in the FlyMode.



With the FlyMode you can catch the closest point of recovery (t_0) because it can already be acquired within the bleach frame.

Suggested background reading:

- Axelrod, D., D.E. Koppel, J. Schlessinger, E. Elson, W.W. Webb. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. Biophys. J. 16: 1055-1069 (1976)
- Braeckmans, K., L. Peeters, N.N. Sanders, S.C. De Smedt, J. Demeester. Three-Dimensional Fluorescence Recovery after Photobleaching with the Confocal Scanning Laser Microscope. Biophys. J. 85: 2240-2252 (2003)
- Beaudouin, J., D. Gerlich, N. Daigle, R. Eils, J. Ellenberg. Nuclear Envelope Breakdown Proceeds by Microtubule-Induced Tearing of the Lamina. Cell 108, 2002: 83-96
- Ellenberg, J., E.D. Siggia, J.E. Moreira, C.F. Smith, J.F. Presley, H.J. Worman, J. Lippincott-Schwartz. (1997) 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)
- Lippincott-Schwartz, J., E. Snapp, A. Kenworthy. Studying protein dynamics in living cells. Nature 2: 444-456 (2001)
- Lippincott-Schwartz, J., N. Altan-Bonnet, G.H. Patterson. (2003) Photobleaching and photoactivation:following protein dynamics in living cells. Nature Cell Biology Suppl: 7-14 (2003)
- Phair, R.D., T. Misteli. High mobility of proteins in the mammalian cell nucleus. Nature 404 (6778): 604-609 (2000)
- Phair, R.D., T. Misteli. Kinetic modelling approaches to in vivo imaging. Nat Rev Mol Cell Biol. 2: 898-907 (2001)
- Misteli, T., A. Gunjan, R. Hock, M. Bustink, D.T. Brown. Dynamic binding of histone H1 to chromatin in living cells. Nature 408: 877-880 (2000)
- 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)
- 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)
- Schröder, J., T.N. Bui. Analyse der Proteindynamik mit einem nicht-fluoreszierenden Protein. Biospektrum 12 (5): 515-517 (2006)
- Siggia, E.D., J. Lippincott-Schwartz, S. Bekiranov. Diffusion in inhomogenous media: Theory and simulations applied to a whole cell photobleach recovery. Biophys. J. 79: 1761-1770 (2000)
- 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)
- 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)

Internet resources:

http://www.embl.de/eamnet/html/molecular_dynamics_2005.html
http://www.dkfz.de/tbi/projects/imagingAndFunctionalScreening/diffusionalDynamicsNucleus.jsp

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