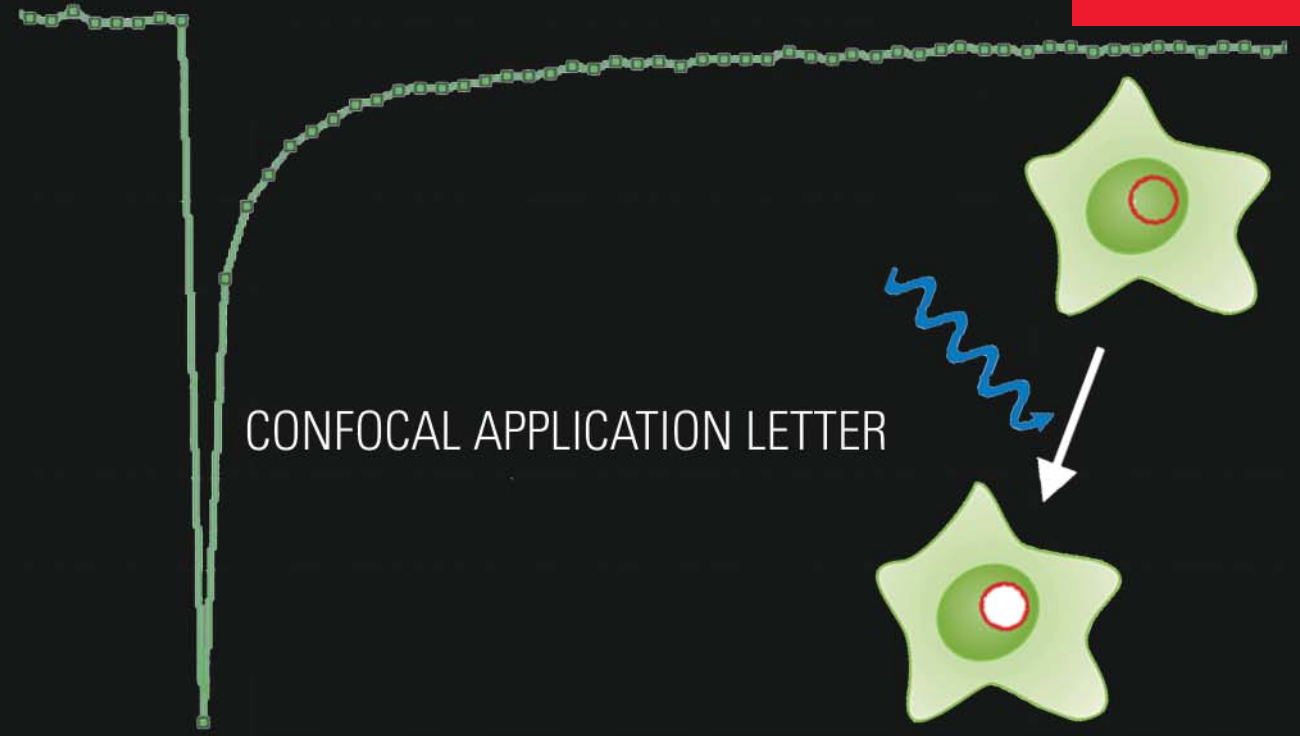
Aug.
2004
No. 18



CONFOCAL APPLICATION LETTER

reSOLUTION

Fluorescence recovery after photobleaching with the Leica TCS SP2

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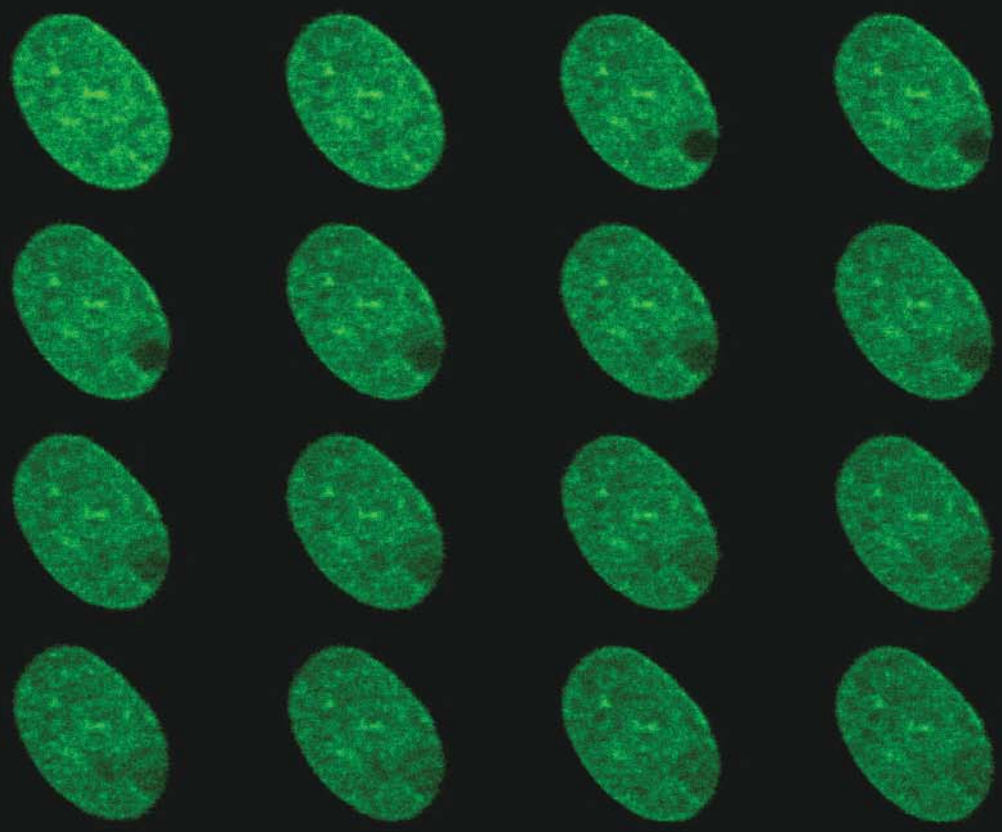

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Fluorescence recovery after photobleaching with the Leica TCS SP2

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Introduction

Among all photobleaching experiments which have been described, fluorescence recovery after photobleaching (FRAP) is the most popular. It employs irradiation of a fluorophore in a living sample with a short laser pulse to degrade it and thereby abolish fluorescence followed by time-resolved image recording of the sample. If the fluorophore (or a so-called mobile fraction of it, see below) is able to freely diffuse through the sample a recovery of fluorescence can be observed. Photobleaching experiments can be conducted with modern laser scanning microscopes, where the laser is used at high intensity for bleaching and low intensity for image recording. The LCS software provided by Leica contains a FRAP application wizard that guides the user through the steps of a FRAP experiment. This wizard will be used in the examples below.

Qualitative vs. Quantitative FRAP

FRAP experiments have been applied to study the dynamics within and between subcellular compartments (Phair and Misteli, 2000) as well as changes in their morphology (Beaudouin et al. 2002).

In this way qualitative information about intracellular transport processes can be rendered in live cells. If a time series is recorded immediately after the bleaching pulse at high rate (> 5 s^{-1}) a quantitative evaluation becomes possible. Parameters that can be obtained are the diffusion coefficient D, and the mobile fraction M_f. Alternatively, instead of D the characteristic half-time of the recovery $t_{1/2}$, can be retrieved from the data. In fact, $t_{1/2}$ is reciprocally proportional to D with the assumptions made by Axelrod et al. (1976). See also “data processing” in this paper. For review, see Phair and Misteli (2001), Snapp et al. (2003) or Lippincott-Schwartz et al. (2003).

Preparation

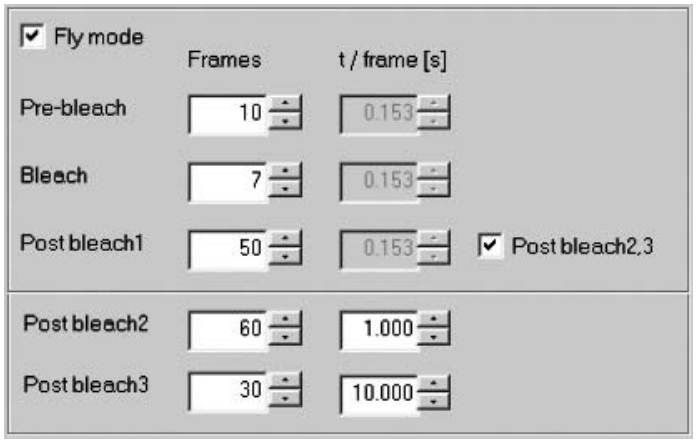
The most common way to fluorescently tag proteins in living cells is the GFP technology (green fluorescent protein cloned from the jelly fish *A. victoria*). Of the different spectral GFP mutants EGFP is the best, because of its high quantum yield, its low tendency of photobleaching as well as its relative photostability during post-bleach image acquisition. Cells both stably or transiently expressing an EGFP-tagged version of your protein of interest (ypi) are suitable for FRAP experiments. Transiently transfected cells are often used 16 – 48 hours post transfection. Here the optimal time for the experiment has to be determined empirically depending on the construct and the cell type or culture conditions. To maintain appropriate culture conditions for most cells it is necessary to have at least a heatable stage, ideally contained within a moisturized and CO_2-controlled box. Prerequisite for FRAP experiments is a confocal laser scanning microscope capable of modulating the beam intensity during the scan. The Leica TCS SP2 capability of switching between two laser intensities enables even more sophisticated experiments.

Establish experimental conditions

1. Prepare the cells in an imaging chamber 16 – 48 hours prior to the FRAP experiment.
2. Set up microscope and prewarm the stage. Let the UV-lamp (for optical control) and the lasers warm up until steady.

3. Identify a cell of interest and focus.

Note: It can be helpful to use a fluorescent dye to counter stain for the subcellular compartment under study (s.a. DAPI (4',6-Diamidino-2-phenylindole) for the nucleus). With transiently transfected cells, also check that the cell is not overexpressing ypi as this will interfere with data analysis. Overexpression can disturb the endogenous protein distribution. Furthermore, this may also make it harder to define the subcellular compartment under study.



4. Adjust laser intensity, detector gain, pinhole, format and scan speed, as well as line averaging or bidirectional scanning if appropriate. For freely diffusing (i.e. non-binding) molecules the fastest scan speed setting and a small image format e.g. 256x256 pixels should be used. Save settings for reproducibility.

Note: Make sure to set the intensity below saturation and slightly above zero as this can interfere with data analysis. An appropriate lookup table (glow over/under) can help. Make sure to use the same gain settings for all cells in a given series.

Initial experiments

For a new protein one has to decide which laser lines to use for the bleaching, which bleaching intensity and length of the bleach pulse is necessary. Furthermore, depending on the size and environment of ypi, the recovery can be very rapid. Also the number of post-bleach images has to be optimized in order to acquire enough information for analysis and to avoid photobleaching during post-bleach acquisition by taking too many images.

5. Now you can start the FRAP application. Double-check or reload the imaging settings determined before. To start with, using the FlyMode is a good idea, because it will help to find the optimum length for the bleach pulse. 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 micro-seconds). 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.

Take 10 – 20 prebleach images. They determine the initial intensity and will be important for normalization of the data series later on. For EGFP a bleach pulse between 500 – 1000 ms with maximum laser power often will be enough (unpublished result). This time frame may be obtained by adequate choice of bleach iterations (i.e. if your recording interval is 300 ms, setting the number of bleach iterations to 3 will result in a 900 ms bleach pulse).The most important information about the fluorescence recovery is contained within the data immediately after the bleach pulse. Therefore the first post-bleach step (Fig. 1) should be recorded at the highest possible rate. For proteins which interact with other structures or are membrane-bound the recovery process can take several minutes or even more, so further post-bleach steps at slower frame rates may be necessary. Initially, to set Post 2 to 60 images at 2 s intervals and Post 3 to 30 images at 10 s intervals will cover more than 6 minutes and will be long enough for most diffusion processes.

Note: The frame rate can be maximized by using the highest scan speed, small image formats and bidirectional scanning. Acquisition speed is proportional to the number of lines scanned (y-format). The smaller the number of pixels in x (x-format) the brighter the image will be.

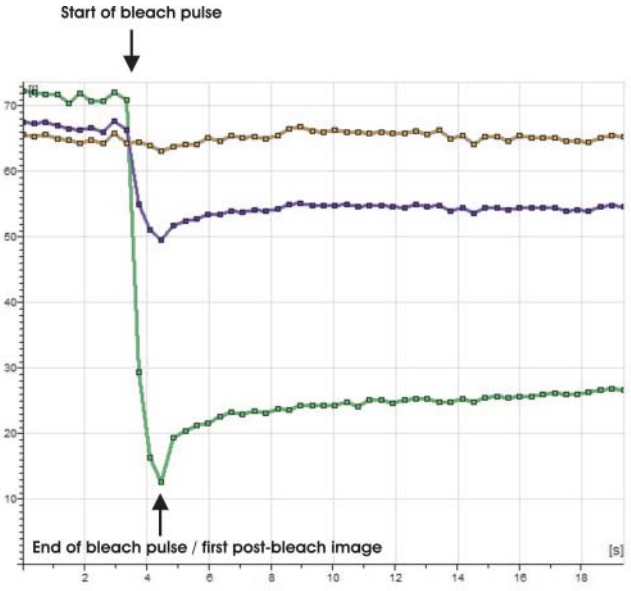


Figure 1
Live cell: Fluorescence recovery plotted versus time in 8-bit format (i.e. max = 255). Time integrated grey values (intensities) of bleach ROI (green), whole cell ROI (purple), unbleached part of cell (orange, more information, see text). Three iterations with bleach intensity were applied (between arrows). The background intensity was not taken into consideration in this example . Note: Only in the Fly-Mode you are able to monitor the fluorescence decay during bleaching.

6. Press next. Now the laser intensity for bleaching has to be set. EGFP or FITC have an absorbance maximum near the 488 nm line of an Ar-Ion laser. Additionally activating the 458 and 476 nm laser lines can increase the bleaching depth. The bleaching depth is the ratio between the post-bleach intensity F_0 and the prebleach intensity within the bleached region. F_0 should be close or equal to background intensity, otherwise the curvature of the recovery curve will be small which can be detrimental to quantitative data analysis (see below).

Note: It can be difficult to bleach rapidly diffusing species to background intensity. In this case establishing the FRAP procedure using a fixed sample can help (Snapp et al. 2003).

7. Define a region of interest (ROI) for bleaching. It is always a good idea to save the ROI for quantification later on (right-click in the image window/ROI/ save).

Note: The larger the ROI the longer the recovery will take. Proportionally, the total fluorescence intensity of the whole cell is reduced which aggravates the problem of photobleaching during post-bleach acquisition. As a rule of thumb use a larger ROI for rapidly diffusing proteins and a smaller one for more slowly diffusing proteins.

Note: There are different geometries for bleach ROIs available in LCS. The most widely used ones are spot photobleaching (circular ROI) and strip photobleaching (rectangular ROI extending beyond the borders of the labelled organelle). Strip photobleaching has been described by Snapp et al. (2003), Ellenberg et al. (1997) and Siggia et al. (2000), while spot photobleaching was used in the landmark paper by Axelrod et al. (1976) and for FRAP experiments in the nucleus (Phair and Misteli, 2000).

8. Run experiment. After the experiment has finished, a window will open plotting the integrated intensity for each ROI (Figure 1, Figure 5, raw). The software offers a simple exponential fit to the FRAP data giving an estimate of the time constant, characteristic half time and rate of the recovery. However this fit result has to be considered preliminary, because at this point no background subtraction, bleaching correction and normalization has been carried out. For data processing export the ROI data as a text file. You can then import it into your favourite data processing or statistics software (i.e. Excel, Mathematica, Matlab or others).

Note: In order to be able to repeat the experiment with exactly the same settings (i.e. for repeated photobleaching of the same cells as a test for phototoxicity and reproducibility) it is recommended to save the settings.

9. Repeat the FRAP experiment with other cells in the sample (about a dozen cells for some basic statistics).

FRAP experiments

To provide an example demonstrating the FRAP procedure we will use a 464 kD dextran labelled with fluorescein-isothiocyanate (FITC-dextran, FD464), which is commercially available from most laboratory suppliers. The dextrans can serve as a system to practice the steps of a real FRAP experiment without having to use live cells. A FRAP experiment using live cells will also be given.

Photobleaching a fluorescein-labelled polymer in solution

FITC-dextran (Sigma-Aldrich) of MW 464,000 was prepared in PBS buffer pH 7.4 at 1.5 mg/ml with 30% (w/w) glucose added. The solution was filled into microslides with ground cavity (Karl Hecht KG, Sondheim, Germany), covered with a coplanar coverslip and sealed with acetone-free nail polish.

Experimental conditions were:	
Ojective	63x NA 1.4 Oil
Scan mode	xyt
Scan speed	1400 Hz
Excitation wavelength	488 nm
Emission range	498-600 nm
Format	256 x 256
Zoom	8
Laser power/potentiometer (Ar/KrAr)	~75 %
AOTF (imaging) 488 nm	1.5 %
AOTF (bleaching) 488 nm	100%
ROI geometry	Circle
ROI diameter	9.2 µm
Prebleach	20 x 294 ms
Bleach	3 x 294 ms
Post 1	15 x 294 ms
Post 2	60 x 1 s
Post 3	50 x 5 s

After having established experimental conditions we now have to optimize the bleach pulse as well as the number and rate of post-bleach images. For this purpose the FlyMode is activated. It allows us to examine the bleaching process in detail. For the first trial a bleach pulse of 4 x 294 ms = 1,2 s was set. Figure 1 shows the first 40 Post-bleach images. From the plot it becomes evident that the last bleach iteration only results in a ~5% decrease of fluorescence intensity. Therefore, for the following experiments only 3 iterations will be used. This way we can optimize the length of the bleach pulse and can now monitor the early onset of the fluorescence recovery.

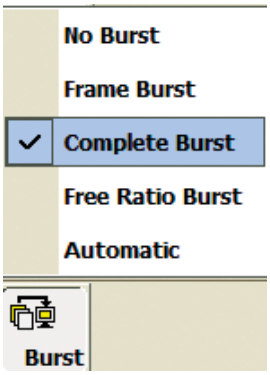
In much the same way the graphical view helps us to optimize Post 1 step. After 15 iterations most of the recovery has taken place, so that for Post 2 and 3 a slower rate can be used. If the intensity has not altered for about 1 min while observing the recovery, it can be assumed to have reached its asymptotic (plateau) value.

Note: One can be tricked into believing the plateau is reached at this point, because photobleaching during post-bleach acquisition decreases the signal and can mimic an equilibrium state (this will become apparent after bleaching correction during data analysis). See data processing.

As useful as the FlyMode is, sometimes an even higher bleach depth is needed. For this purpose one can activate the Zoom-In option (cannot be combined with FlyMode). With this option checked, the aspect ratio of the image will be adjusted to accommodate the bleach ROI resulting in a net zoom in during the bleach. As a result every pixel will be irradiated more often during the scan which leads to a larger fluorescence loss.

Note: During the bleach with ZoomIn no images will be recorded, so the recovery curve will have a 'hole' (e.g. will not be steady).

Note: If really maximum recording speed is essential, the time lag between the acquisition of two subsequent images can be minimized by switching on "complete burst" mode. With this setting image acquisition is at maximum speed. Therefore the screen will not show any live images during recording.



FRAP of a nuclear protein in live cells

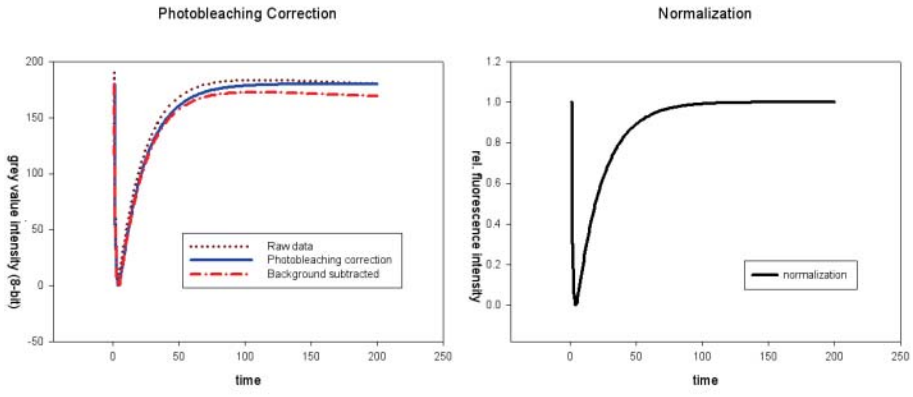
Histone H1 – tagged with EGFP (contruct kindly provided by T. Misteli) was transfected into 3T3 NIH mouse fibroblast cells. A FRAP experiment was conducted with the following conditions:

Experimental conditions were:	
Ojective	63x NA 1.4 Oil
Scan mode	xyt
Scan speed	1400 Hz
Excitation wavelength	488 nm
Emission range	498-600 nm
Format	256x256
Zoom	~ 8
Laser power/potentiometer (Ar/KrAr)	~75 %
AOTF (imaging) 488 nm	1.2 %
AOTF (bleaching) 488 nm	100 %
AOTF (bleaching) 476 nm	100 %
AOTF (bleaching) 456 nm	100 %
ROI geometry	Polygon
ROI size (enclosing rectangle)	15.3 µm x 4.1 µm
Prebleach	20 x 294 ms
Bleach	3 x 294 ms
Post 1	40 x 294 ms
Post 2	30 x 2 s
Post 3	24 x 10 s

Figure 5 (raw, Pre) shows a prebleach image of a mouse fibroblast nucleus transfected with histone H1 fused to GFP with bleach ROI (green), whole cell ROI (red) and the unbleached part of the cell (orange) overlaid. Figure 5 also shows the first postbleach (Post1) and the last image (Post3) of a FRAP series with the same cell.

Data processing

As mentioned above the values for the characteristic relaxation time τ, the recovery half-time $t_{1/2}$ and the recovery rate are only rough estimates, because the data has not been corrected. There are three necessary corrections: Background subtraction, correction for (unintended) photobleaching during acquisition of post-bleach images and finally normalization (Figure 2). All three will be explained in the following.



$$F_b = F(t) - background$$

$$F_{b,corr}(t) = F_b(t)\frac{F_{precell}}{F_{infcell}}$$

$$F_{b,corr,normAxelrod}(t) = \frac{F_{b,corr}(t) - F_{b,corr}(0)}{F_{b,corr}(inf) - F_{b,corr}(0)}$$

Figure 2
Necessary corrections represented schematically. Raw data given in dark red (dots), after background subtraction (red dash-dots) and correction for photobleaching artifacts (solid blue). Note the intensity decrease after time 100 caused by photobleaching during acquisition. After normalization relative fluorescence values are plotted over time (right figure, solid black line).

Figure 3 illustrates the meaning and positions of the different regions of interest (ROIs) used. For clarity ROIs are not drawn to scale and the bleach ROI is depicted in red, but will be depicted in green with actual data (see below).

Background subtraction

All images contain a background signal. Some of it is due to noise of the photomultiplier tubes (PMTs) and the electronics, some is background fluorescence due to residual staining or overexpression or mislocation of the GFP-tagged protein. The former kind of background originates from the hardware and can only be reduced by setting a lower PMT voltage at the expense of sensitivity. It can be treated by recording an image with all lasers turned off and the respective PMT turned on. This "noise image" can later on be subtracted from the image series.

Note: Make sure that there are no pixels with zero intensity. You can check for zero values with the "O/U LUT (glow over/under)" (they are green). If there are zero values increase the offset of the PMT. The histogram can also help to find the background value. The simulation software by Siggia et al. (2000) uses the histogram peak near the low end of the histogram for background subtraction.

If the image contains parts of a cell which is not stained in the respective channel, background due to fluorescence can be corrected for by subtracting the averaged intensity of unstained parts from the FRAP series. In the example Figure 5 (upper row, right) the averaged intensity of ROI 4 (cyan) would be subtracted.

Correct for fluorescence loss due to photobleaching

We have to distinguish two kinds of photobleaching: Firstly, the intended photobleach with full laser intensity during the bleach pulse, secondly, photobleaching during post-bleach acquisition. While the former photobleaching is intended, the latter is inherent to the imaging process and can lead to some considerable loss of total fluorescence. Therefore, the fluorescence intensity can never recover to 100%, even if all fluorophore molecules were mobile (see "Calculation of mobile fraction"). It is possible to correct for this amount of photobleaching by multiplying every element

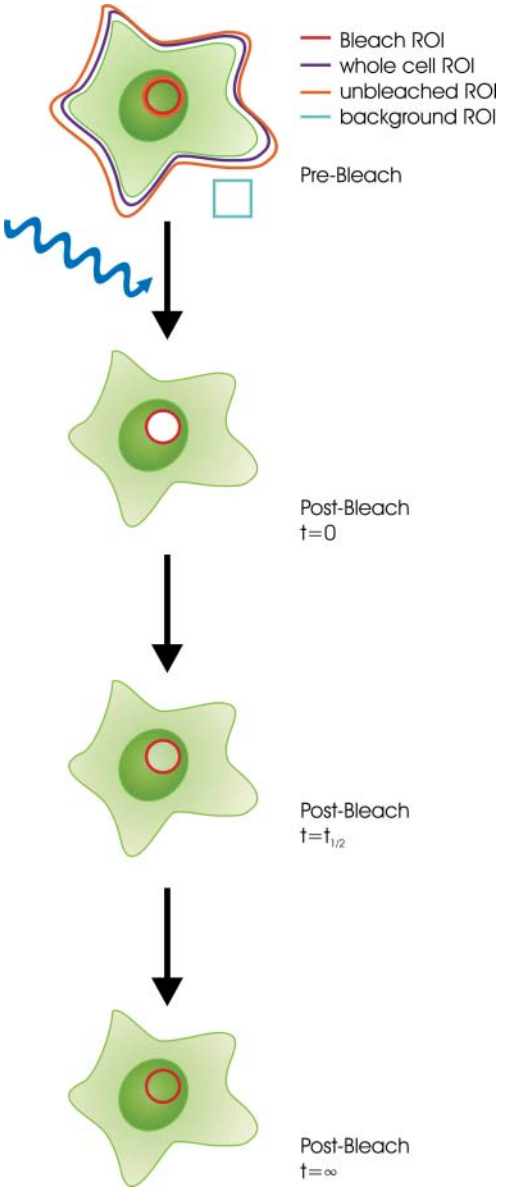


Figure 3
Schematic cell expressing GFP mainly in the nucleus. A region is bleached in the nucleus (red circle). After the bleach pulse the cell is imaged at low intesity to follow the fluorescence recovery. Pre-Bleach shown with Bleach ROI (region of interest) as well as further quantification ROIs (not drawn to scale).

of the data set by ($F_{precell}/F_{infcell}$), where $F_{precell}$ means prebleach intensity within the whole cell, $F_{infcell}$ stands for postbleach intensity (whole cell minus Bleach ROI) at any given time point. This correction should be done after subtraction (see above). In any case, it is preferable to optimize the experimental parameters in order to avoid strong photobleaching. An acceptable range would be between 5-15% fluorescence loss.

Note: Alternatively, a second cell, which does not take part in the FRAP experiment, can be used as an independent control. In this case the ratio $F_{precell}/F_{infcell}$ would be determined for this cell.

Note: $F_{precell}/F_{infcell}$ can only be accurately determined for a closed compartment without exchange with the surroundings within the time-scale of the experiment (s.a. the nucleus in our live cell example). In spite of this limitation the data from the FITC-dextran experiment was used here, for illustration purposes only.

Normalization

Normalization means to rescale the images in a way that represents plateau fluorescence as 100% (fractional fluoresence intensity) in order to make several image series mutually comparable as well as to enable us to readily estimate the relaxation half-time $t_{1/2}$.

The most commonly employed normalization used was introduced by Siggia et al. (2000). Here every frame (time point) is divided by the first frame (time point). This way Pre-Bleach intensities will be normalized to 100%. This type of normalization is suitable to graphically determine the mobile fraction from normalized data, while the relaxation half-time can not. This method can also be applied to incomplete recoveries (i.e. fluorescence intensity has not reached a plateau value.).

An alternative method was published by Axelrod et al. (1976).

$$Fn(t) = (F(t) - F(0))/(F(inf) - F(0)) \quad (1)$$

With
F(t)..fluorescence intensity at time t inside bleach ROI
F(0)..fluorescence intensity at time 0
(immediately after bleaching)
F(inf)..fluorescence intensity at equilibrium

Using the “Axelrod method” the plateau value is set to 100%. Therefore, the relaxation half-time can be graphically determined from a normalized plot, while the mobile fraction cannot be determined.

The following sections introduce methods for formal estimation of mobile fraction and relaxation half-times.

Estimation of parameters

Calculation of the mobile fraction

In an "artificial" *in vitro* experiment, such as the diffusion of FD 464 in aqueous solution all particles will be subject to Brownian motion and therefore spread by diffusion. However, in real cells the situation can be more complicated, because biomolecules/proteins interact (bind to) each other. This can lead to a substantial fraction of the labelled molecule, which is bound and therefore does not diffuse, the so-called immobile fraction ($1 - M_f$, see below). Only the mobile fraction M_f can move. Figure 4 illustrates the effect of an immobile fraction on the FRAP curve.

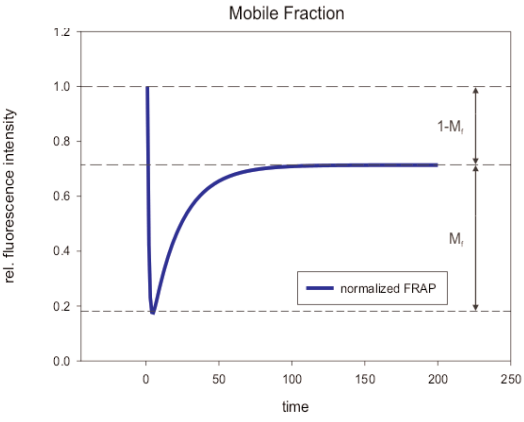


Figure 4
Only the mobile fraction can diffuse. As a result the fluorescence level becomes lower than 1 (100%).

The mobile fraction M_f can be calculated directly from the integrated intensities within the bleach ROI (ROI 1 in Figure 5, raw) and a ROI including the whole cell (ROI 2 in Figure 5, raw) according to:

$$M_f = 100 * \frac{F_{precell}}{F_{infcell}} * \frac{F_{infcell} - F(0)}{F_{pre} - F(0)} \qquad (2)$$

With $F_{precell}$ = prebleach intensity within the whole cell
$F_{infcell}$ = postbleach intensity (whole cell) at equilibrium
F_{pre} = bleach ROI prebleach intensity
$F(0)$ = bleach ROI intensity at time 0

Note: The first term in eqn. (2) contains a correction for photobleaching during the acquisition of the postbleach images. The latter leads to a fluorescence loss over time, so the equilibrium intensity would be underestimated. Without this correction the recovery could not reach 100 % even with M_f = 100%. If a "correction for fluorescence loss due to photobleaching" was performed already (see above), the first term eqn. (2) has to be omitted.

Calculation of $t_{1/2}$

The time it takes for the fluorescence to recover to 50% of the asymptote (plateau) intensity is called recovery half-time, $t_{1/2}$. It can be used to compare relative recovery rates, if it is not possible to estimate D by fitting a diffusion equation to the data. $t_{1/2}$ can be either determined visually from a graph plotting fractional fluorescence vs. time or by solving (comp. Snapp et al. 2003):

$$F(t) = 100 \times (F_0 + F_{inf}(t/t_{1/2})) / (1+(t/t_{1/2})) \qquad (3)$$

Note: The recovery half-time strongly depends on the bleach geometry as well as numerous other experimental parameters (see Table "Experimental Conditions"). In order to compare experiments with each other they should be conducted with identical conditions, bleach ROI geometry and ROI size.

Estimation of the diffusion coefficient D

Instead of using equation (3) one could fit an exponential function as empirical approach to the data set:

$$y = a^*(1-\exp(-t/\tau))^+c \qquad (4)$$

This is implemented in the Leica FRAP wizard and yields the time constant t of the recovery as well as the recovery half-time $t_{1/2}$:

$$t_{1/2} = \tau^* \ln 2 \qquad (5)$$

In the original publication by Axelrod et al. (1976) a somewhat complicated expression is given for the temporal behaviour of fluorescence recovery. In the case the nature of the transport is pure diffusion they suggest to use the following simplified equation to estimate the diffusion coefficient:

$$D = 0.88^* w^2/(4\, t_{1/2}) \qquad (6)$$

With w ... radius of bleached area

However, this equation makes the assumptions that the bleached area is a spot (disc) and that diffusion occurs only laterally (in 2D as in membranes). In some experiments these assumptions may not apply. In such a case calculations will only serve as a rough estimation.

Note: For fitting eqn 4 to the data it must be normalized and plotted with the time point of the first postbleach image $t_0 = 0$ s.

Modeling

More accurate estimates of D can be achieved by fitting a mathematical model numerically to the image series recorded (In this case not only the integrated ROI intensities, but the whole image data are used). One such simulation for recovery of a strip bleach ROI has been published by Siggia et al. (2000). Other simulation packages dealing with diffusion or reactive flows can be suitable, too, but usually require a considerable amount of work to implement a simulation tailored for live cell image series. The authors plan to publish soon a web-based tool for quantitative FRAP analysis on their homepage (http://www.dkfz.de/ibios/index.jsp).

Evaluation of demo experiments

In this section our showcase examples, namely FD 464 and the H1-GFP cell, will be examplarily evaluated.

FD 464

Figure 5, upper row, contains integrated fluorescence intensities for the bleach ROI (green) and a ROI comprising the whole cell (purple). The "whole cell" ROI (purple) covers the complete field of view. In the case of FD464 it was not possible to define a ROI for background intensity, like in H1-GFP (cyan). For background subtraction an image was taken using the respective photomultiplier with lasers turned to 0%.

Table 1 summarizes the results.
In the left column the experimental results are given. $FD464_{theor}$ are the expected values for M_f and D. Since all FD464 molecules are free to diffuse we expect mobile fraction to be 100%. The experiment shows that nicely. The 3 % deviation could be attributed to noise or, more generally, errors during estimation of $F_{infcell}$. The theoretical D value was estimated using the Stokes-Einstein equation,

$$D = k^*T/(6\pi\eta r_H) \quad (7)$$

With k..Boltzmann constant
T..absolute temperature
η..Viscosity of solvent
r_H..hydrodynamic radius of solute molecules

where r_H can be estimated using the empirical equation (Braeckmans et al. 2003):

$$r_H = 0.015^*M_w^{0.53} \quad (8)$$

With M_w..molecular weight

Note: This is only the case for dextrans

From the D obtained the characteristic relaxation time τ and the recovery half-time $t_{1/2}$ were estimated using $D = w^2/(4\tau)$. Since two approximations have been made to obtain these parameters, they will be rather inaccurate and are therefore spelled in parentheses.

H1-GFP

The green curves in the right column graphs of Figure 5 show clearly a much slower recovery rate for the H1-GFP construct under *in vivo* conditions. Such a slow recovery can be indicative of molecular interactions (between histone H1 and chromatin in this case). ROI2 in H1-GFP stays relatively constant, but the intensity increases by a few percent towards the end of the experiment. In general, such an increase could indicate specimen movement or flux from image planes above or below the one observed. Such effects can sometimes complicate the quantitative analysis of a FRAP experiment. The corrected curves in the middle row of Figure 5 demonstrate the correction for fluorescence loss during acquisition (needed for calculation of M_f) using the whole cell ROI. The bottom row contains the normalized bleach ROI data together with a single exponential fit, similar to the fit used by LCS to calculate the time constant of the recovery. Table 1 summarizes the results. Note, that the time constant depends (among other parameters) on the bleach ROI geometry, as shown by comparing an arbitraryly shaped ROI in Figure 5 and a circular ROI in Figure 6. The time constants determined here lie within about 10% deviation of previously published results for H1-GFP (Misteli et al. 2000). If more than a rough estimate is desired, an appropriate model should be chosen (comp. Braeckmans et al. 2003). The same holds true for the time constants (D_{eff}) estimated for the FITC-dextran solution.

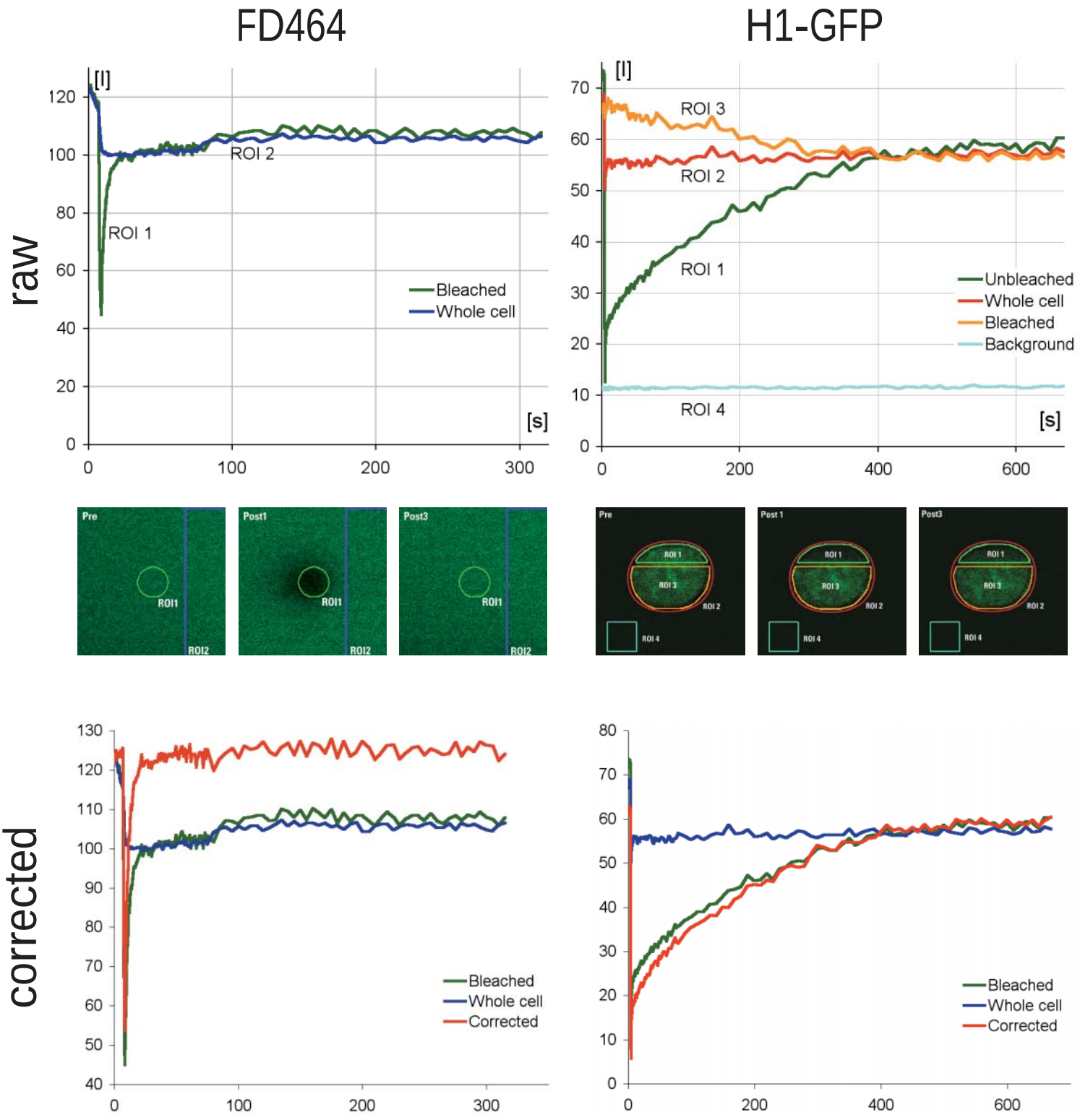


Figure 5: to be continued on page 11

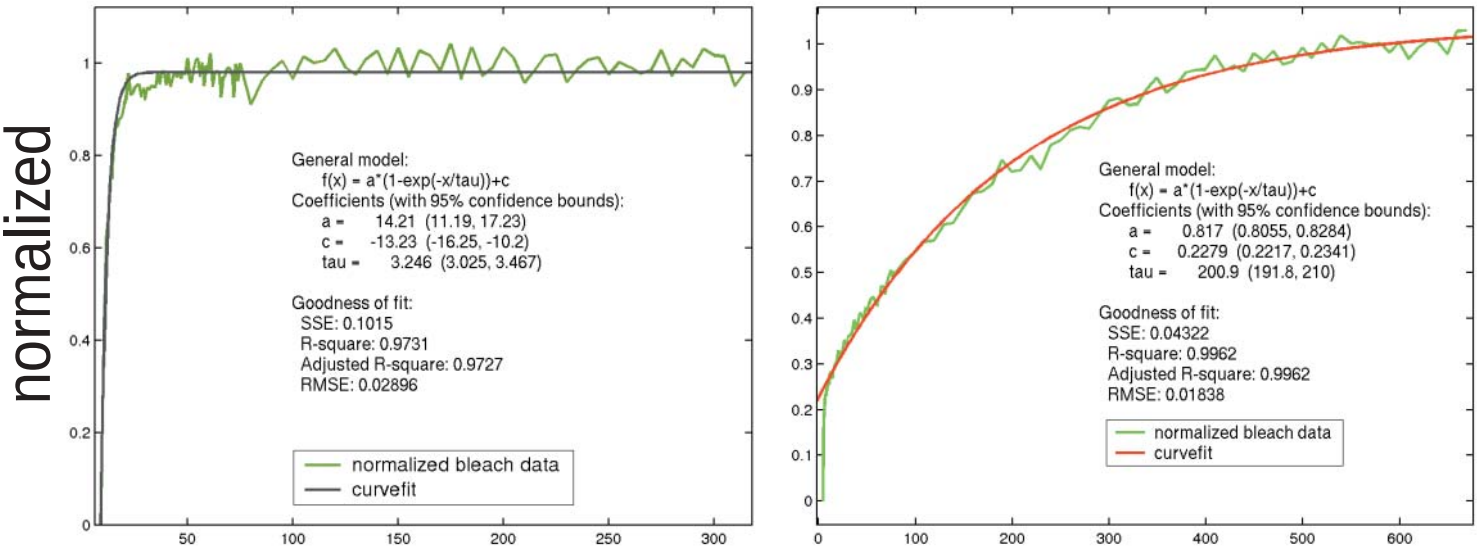


Figure 5
Recovery curves with intensity plotted versus time [s]. FITC-dextran (FD464) and live cells (H1-GFP). Upper row with time integrated ROI intensities (raw) and prebleach (Pre), immediate post-bleach (Post1) and equilibrium images (Post3). Bleach ROI shown in green, whole cell: ROI 2, unbleached part of the cell in orange and background intensity in cyan. Middle row compares data corrected for fluorescence loss (red) with raw data. Bottom row shows normalized data with single exponential fit.

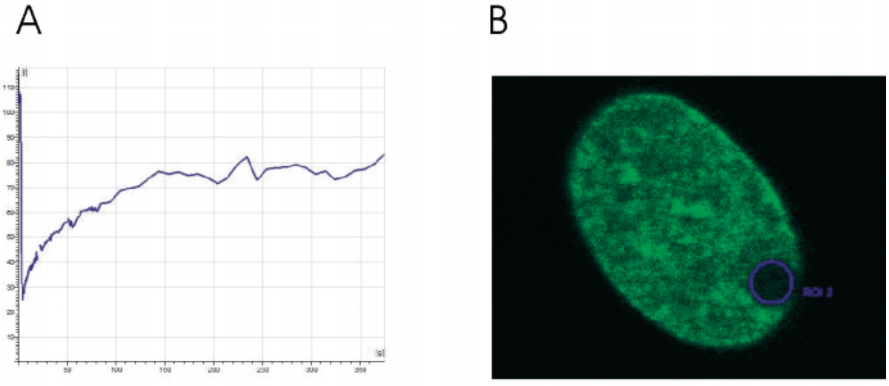


Figure 6
(A) Recovery curve for bleaching of heterochromatin-rich region of H1::GFP transfected 3T3 nucleus. (B) First postbleach image with ROI (circle).

	FD464	$FD464_{theor}$	$H1\text{-}GFP_{arbitrary\ ROI}$	$H1\text{-}GFP_{circular\ ROI}$	H1-GFPliterature
M_f [%]	103	100	91	–	~ 90
$t_{1/2}$ [s]	2.3	(1.0)	138.6	59.9	~ 55
τ [s]	3.3	(1.4)	200.9	41.5	–
D_{eff} [$\mu m^2/s$]	1.6	3.7	–	0.01	–

τ: Time constant of recovery (calculated by LCS, circular ROI)

$t_{1/2}$: Half-life of recovery (calculated by LCS, circular ROI)
D_{eff}: Effective diffusion coefficient (Axelrod et al. 1976)

Table 1
Time constants obtained from exponential fit. $FD464_{theor}$ calculated using an estimation of the hydrodynamic radius and viscosity as determined in Breackmans et al. (2003). H1-GFP literature value from Misteli et al. (2000). Results for our running live cell example ($H1\text{-}GFP_{arbitrary}$) as well as another cell bleached with a circular ROI ($H1\text{-}GFP_{circular}$) are given.

Final Remarks

We would like to recommend beginners, who want to practice FRAP experiments, to use a system similar to the FITC-dextrans as demonstrated here before trying live cells. Keep in mind that in particular the bleach area size, the laser intensities, FRAP modes, bleach format, scan speeds as well as the iteration numbers and intervals for bleach and post-bleach acquisition have to be optimized for each cell type and protein of interest. The results given here were obtained by evaluating just one data set. For meaningful results the experiment should be repeated at least 10-20 times to get an estimate for the standard deviation. Repeating the FRAP experiment using the same cell and bleach ROI can also reveal potential phototoxicity, if the results are not reproducible. In such a case the FRAP protocol has to be further optimized (s.a. decreasing the laser power).

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