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FRAP: Analysis of Protein Dynamics with a Non-fluorescent Protein

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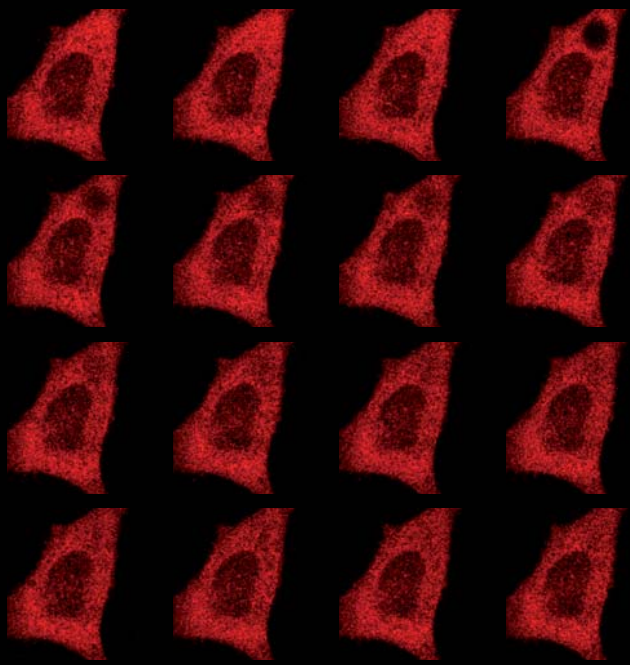

HaloTag, Promega – TCS SP5, Leica Microsystems

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FRAP: Analysis of Protein Dynamics with a Non-fluorescent Protein*

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Fluorescence Recovery after Photobleaching (FRAP) is often employed to analyze protein dynamics[1,2]. All FRAP experiments discussed below were performed with a Leica TCS SP5 confocal microscope. Instead of fluorescent proteins (FPs) the HaloTag Interchangeable Labelling Technology, which is based on non-fluorescent HaloTag protein, was used. Signals were detected by incubation with various fluorescent ligands, which are able to covalently bind to the HaloTag protein.

In FRAP experiments fluorescent molecules are bleached by a laser pulse in a selected region of a specimen and the subsequent increase of fluorescence is measured in the same region. If fluorescent molecules diffuse through the specimen, kinetic parameters of a protein can be determined by the increase of fluorescence. For example, the diffusion constant, mobile fraction, transport rate or binding and dissociation rate of other proteins can be determined[3]. To investigate protein dynamics in living cells, genetic fusions of the examined proteins with fluorescent proteins (FPs) are mostly used[4]. In recent years, new imaging technologies have been developed which are based on the genetic mutation of non-fluorescent protein motives[5] or modified enzymes[6,7]. They can be detected either by membrane permeable ligands attached to fluorescent dyes bearing a high affinity to the protein motive or ligands which undergo a covalent binding with the modified enzyme. The HaloTag Interchangeable Technology (Promega, Mannheim, Germany) is based on the genetic mutation of a monomeric prokaryotic hydrolase enzyme expressed

Table 1: Hardware settings of Leica TCS SP5 laserscan microscope

	HaloTag® diAcFAM	HaloTag® TMR
Objective	HCX PL APO 63.0x1.20 WATER UV	HCX PL APO 63.0x1.20 WATER UV
Scan speed (Line frequency)	1400 Hz bidirectional scan	1400 Hz bidirectional scan
Excitation wavelength	488 nm	561 nm
Emission range	495–600 nm	568–672 nm
Format	256 x 256 pixels	256 x 256 pixels
Zoom	~ 6	~ 6
Laser power (Argon laser)	~100 %	–
AOTF (imaging) 488 nm	5 %	0 %
AOTF (imaging) 561 nm	–	10 %
AOTF (bleaching) 488 nm	100 %	0%
AOTF (bleaching) 561 nm	–	100 %
ROI geometry	circle (diameter 3 µm)	circle (diameter 3 µm)
Prebleach	10 x 118 ms	10 x 118 ms
Bleach	1 x 118 ms	1 x 118 ms
Postbleach 1	50 x 118 ms	50 x 118 ms
Postbleach 2 30 x	1 s	1 s

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in the cells as a monomer. Due to its prokaryotic origin, endogenous activities in mammalian cell systems can almost be excluded. The HaloTag protein and the HaloTag ligands do not show any detectable cellular toxicity or morphological side effects with the applied experimental conditions[7]. To perform FRAP experiments with the Leica TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany), two HaloTag fusion proteins stained with HaloTag-diAcFAM or TMR ligands were used. Both are easy to apply and are specific for FRAP.

Material and Methods

HeLa cells were plated on 8-well chambered cover glass (Nunc, Wiesbaden) and incubated under conditions for standard cell cultures. The next day cells were transiently transfected with TransIT® LT1 reagent with or without HaloTag expression plasmids (Mirus, Madison, USA). The plasmids were fused with human α-Tubulin or with the human p65 subunit of the transcription factor nuclear factor-κB (NF-κB). 16 hours after transfection, cells transfected with HaloTag α-Tubulin were stained with HaloTag-diAcFAM and p65-HaloTag cells were stained with TMR ligands according to the manufacturer's protocol (Promega). FRAP experiments and data analysis were done with the Leica FRAP application wizard. The experimental settings of the confocal microscope are displayed in table 1.

Results

For the FRAP experiments, cytoplasmic regions of stained cells were chosen and selectively bleached. The loss of fluorescence caused by image acquisition was corrected in the data and normalized with respect to the prebleach intensity. Transmitted light images were recorded in parallel to demonstrate the cell shape (Fig. 1). Cells stained with the HaloTag-diAcFAM ligand showed a decrease in fluorescence by 35% of the original intensity after the bleaching pulse. Approx. 30 seconds later, the original intensity was reached (Fig. 2). This experiment represents other experiments with HaloTag-diAcFAM stained cells. In all experiments, the half-time recovery of 1.14 +/- 0.38 seconds was observed. Cells stained with HaloTag TMR ligand were inves-tigated under the same conditions recovered with a half-time of 1.24 +/- 0.15 seconds, a slightly slower kinetic (Fig. 3). For image acquisition before and after bleaching, the DPSS 561nm laser was used. To achieve more effective bleaching, the 488 nm Argon laser was additionally applied only for

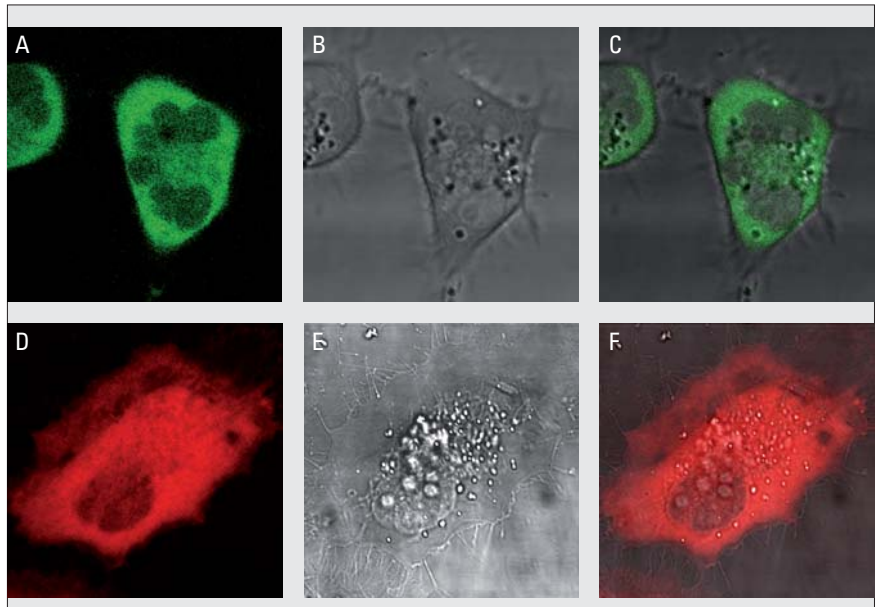

Figure 1: A–C, α-Tubulin-HaloTag fusion protein stained with HaloTag-diAcFAM ligand. **D-F**, p65-HaloTag fusion protein stained with HaloTag-TMR ligand. **(A, D)** fluorescence, **(B, E)** transmitted light, **(C, F)** Overlay of fluorescence- and transmitted light image.

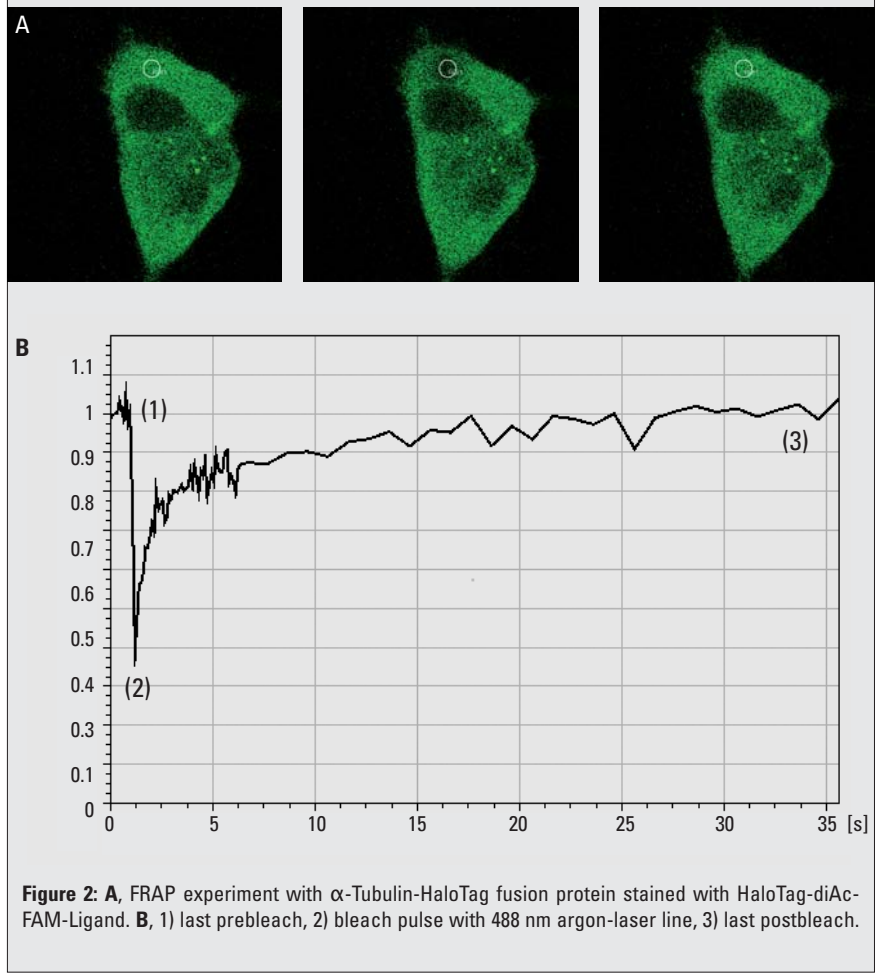

Figure 2: A, FRAP experiment with α-Tubulin-HaloTag fusion protein stained with HaloTag-diAc-FAM-Ligand. **B**, 1) last prebleach, 2) bleach pulse with 488 nm argon-laser line, 3) last postbleach.

bleaching. Here the initial fluorescence intensity decreased more than 80% and after 30 seconds the fluorescence reached 85 % of the initial fluorescence (Fig. 3). Non-transfected cells were as control of the specificity of the staining with both ligands. With the excitation wavelengths mentioned above the cells did not show any detectable fluorescence (data not shown) either for diAcFAM or for TMR.

Discussion

The use of new specific fluorescence ligands[5-7] with different spectral characteristics allows for more flexibility to detect non-fluorescent proteins. Time-consuming and cumbersome sub-cloning work can be avoided with the described methods. However, potential toxicity and specificity need always to be taken into account when applying these technologies. Since the HaloTag Interchangeable Technology is based on a prokaryotic protein, non-specific staining in mammalian cells is significantly reduced. Additionally, toxicity could not be observed during long-term investigations[7].

We used the Leica TCS SP5 confocal microscope for the FRAP analysis of human α-Tubulin and p65-HaloTag fusion proteins. Both HaloTag-diAcFAM and TMR ligand bound specifically to the HaloTag fusion protein and could not be detected in control cells. The HaloTag α-Tubulin experiments showed nearly complete fluorescence recovery which implies that nearly all molecules are mobile. Thus, the immobile fraction is negligible. The kinetic of p65-HaloTag showed a higher immobile fraction which is likely due to the effect of its interaction with other NF-κB sub-units and the cytoplasmatic inhibitor protein IκB[8]. Furthermore, it could be shown that FRAP experiments can also be successfully conducted with the excitation of the HaloTag-TMR ligand by 561 nm and with a high bleach efficiency. This enables the use of the "red" channel as an alternative for FRAP analysis in case the "green" channel may be occupied.

These examples demonstrate that the HaloTag Interchangeable Technology (Promega) together with modern confocal microscopes like the Leica TCS SP5 is a new flexible and specific approach to analyze protein dynamics with FRAP experiments.

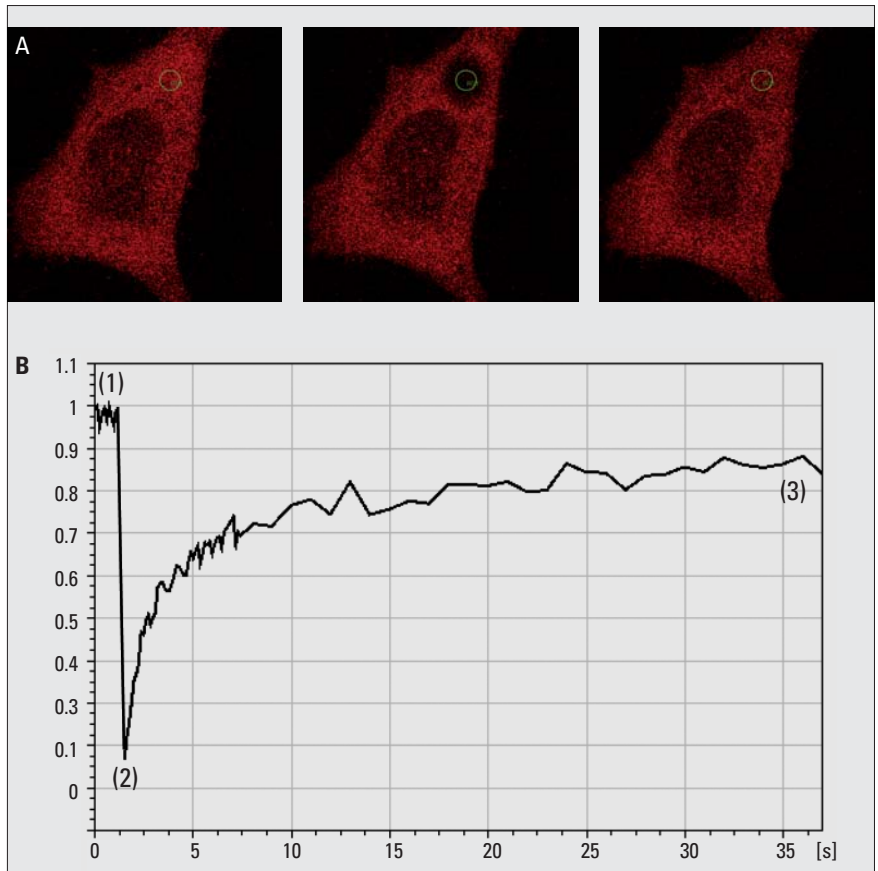

Figure 3: A, FRAP experiment with p65-HaloTag fusion protein stained with HaloTag-TMR-digand, excitation with 561 nm DPSS laser. **B**, 1) last prebleach, 2) bleach pulse with 488 nm argon laser line together with 561 nm DPSS-laser, 3) last postbleach.

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