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Nucleocytoplasmic shuttling revealed by FRAP and FLIP technologies

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Protein mobility within cells is of key importance for many cellular functions. Although immunostaining can reveal protein locations in the steady-state, this might not represent the full picture and provides no information about protein movements. Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) are two techniques that enable the dynamics of intracellular protein mobility to be studied. These technologies have been successfully used to analyze the nucleocytoplasmic shuttling of STAT1, an intracellular signal transducer and activator of transcription, and can applied to the study of other proteins. Furthermore, FRAP and FLIP approaches have the added advantage of not affecting cell viability and might find application in the imaging of intracellular events in certain tissues and live animals.

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Abbreviations

EGFP	enhanced green fluorescent protein
FLIP	fluorescence loss in photobleaching
FRAP	fluorescence recovery after photobleaching
GFP	green fluorescent protein
IFN	interferon
STAT	signal transducer and activator of transcription

Introduction

The mobility of proteins within cells is an essential prerequisite for numerous cellular functions. Protein mobility is important for both the transfer of newly synthesized molecules towards their destination (e.g. to specific compartments) and for many other vital cellular activities, such as signaling events. The examination of different proteins reveals a great diversity in mobility, the size and shape of which have a minor role; determining factors are mainly the interaction with other proteins and macromolecular structures. In addition, intracellular membrane systems, like the nuclear envelope, function as barriers for their movement and regulate accessibility to distinct targets. The visualization of proteins by immunostaining reveals a steady-state picture with a defined distribution within the cell. This picture changes for certain signaling proteins when their activity is triggered by specific events. Steroid receptors, nuclear factor κB (NF-κB) and STATs move from the cytoplasm to the nucleus upon stimulation with steroid ligands, inflammation signals and cytokines, respectively. In the past few years it has become evident that the steady-state picture is misleading; it suggests the absence of some of these signaling proteins from the nucleus in the unstimulated state. In fact, it was shown for several proteins, including members of the families mentioned above, that they shuttle permanently between the cytoplasm and nucleus [1–5]. The discovery and engineering of green fluorescent protein (GFP) led to the availability of a widely applicable tool with few invasive properties that could be used to study protein dynamics and function. In particular, the combination of time-lapse imaging with photobleaching and photoactivation techniques has enabled analysis of the kinetic properties of a given protein in living cells. In this article, the application of fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) are discussed with particular reference to the study of STAT1 dynamics and its nucleocytoplasmic trafficking as an example.

STAT1: a model for differential protein mobility

Transcription factors and mediators of signal transduction execute their function through binding to certain substrates like DNA or phosphoproteins. Access to these specific sites within the cell is achieved by movement of the proteins. A well-studied example is the conditionally regulated nuclear trafficking of members of the STAT family [6]. The Jak-STAT pathway has a key role in cytokine-mediated signal transduction and in the reprogramming of gene expression. STAT proteins, named after their dual role as signal transducer and activator of transcription, have been described as latent cytoplasmic transcription factors until they are activated by tyrosine phosphorylation catalyzed by kinases of the Jak family [7]. It was further described that tyrosine phosphorylation induces dimerization of STAT molecules via recognition of the phosphorylated tyrosine residue by the C-terminal SH2 domain. These events lead to a mass translocation of dimeric STAT proteins to the nucleus where they direct the transcription of specific target genes after binding to

characteristic recognition elements. A prerequisite for their dual role is the ability of the protein to shuttle between nucleoplasm and cytoplasm. In this way the signal proteins STAT1 and STAT2 are responsible for interferon (IFN)-induced changes in cell function. To understand the regulation of STATs during IFN stimulation and the fine-tuning of subcellular transport, GFP fusion proteins were used. The analysis of several parameters of STAT1–GFP fusion protein activity revealed that it behaves indistinguishably from endogenous STAT1 [8,9]. Using different techniques of photobleaching a random walk model for the movement of STAT1 from the plasma membrane to the DNA was established [9].

Quantitative FRAP techniques

Photobleaching occurs when a fluorophore permanently loses its ability to fluoresce due to photon-induced chemical damage and covalent modification (e.g. when exposed to repeated cycles of excitation and emission or to a pulse of high intensity light from a laser beam). Even though the exact mechanism of photobleaching is not fully understood, it is assumed to be linked to a transition from the excited singlet state to the excited triplet state. The excited triplet state is relatively long-lived and is chemically more reactive. Today, the photobleaching effect is utilized to obtain specific information in live-cell imaging that would not otherwise be available. The technique of fluorescence recovery after photobleaching (FRAP) has been used extensively to measure the mobility of molecules made visible by means of a fluorescent tag. Introduced in the mid-1970s [10] the method has seen a recent increase in popularity owing to advances in imaging technology and the ability to express fluorescent-tagged proteins such as GFP chimeras. GFP and its variants are exceptionally photostable, making them very reliable for imaging studies [11]. Fluorescent proteins are used as genetic labels that enable tagging and subsequent visualization of different types of molecules. For photobleaching experiments enhanced GFP (EGFP) appears to be one of the most suitable fluorophores, because it is bright and stable under low-intensity illumination but bleaches fast and irreversibly at high illumination power [11].

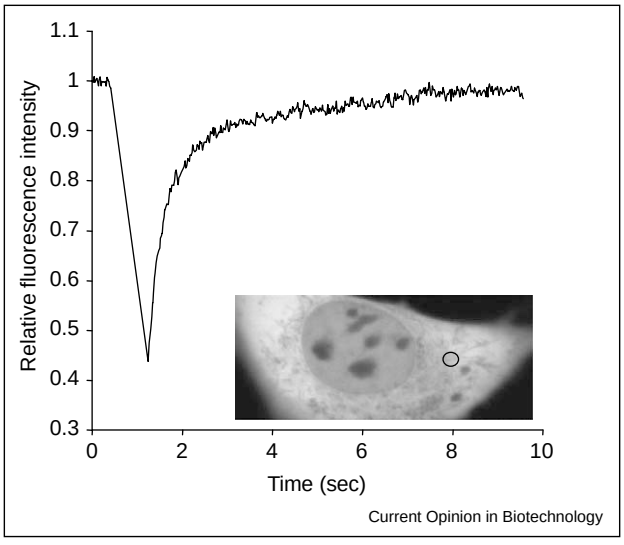
Standard confocal laser-scanning microscopes equipped with acousto-optical tunable filters (AOTF) are suitable for photobleaching experiments. The AOTF is needed to switch the laser power rapidly between the low-intensity imaging and high-intensity bleaching modes. A second important feature is that a laser-scanning microscope can limit laser illumination to selected regions of interest.

In typical FRAP experiments, GFP-labeled macromolecules within a small target region are subjected to intense illumination, usually with a short pulse of high-intensity laser light, which leads to complete photobleaching of fluorophores in this particular region. The result is a dramatic reduction or annihilation of fluorescence. As photobleaching is an irreversible process, fluorescence recovery is determined by the exchange of unbleached molecules with the bleached ones from the target region. Following the photobleaching pulse, the rate and extent of fluorescence intensity recovery in the bleached region is monitored as a function of time. Over time, the amount of fluorescence in the photobleached area increases owing to the re-population by unbleached molecules from regions outside the bleached spot. This replacement continues until a steady-state is reached. The course of recovery characterizes the nature of the targeted protein. Two key parameters of a protein discerned from quantitative FRAP studies comprise the mobile fraction, M_f, and the diffusion constant, D. D reflects the mean squared displacement explored by the protein through a random walk over time, whereas M_f is the fraction of fluorescent protein that can diffuse into the bleached region during the time course of the experiment. The mobile fraction can be determined by calculating the ratio of the final to the initial fluorescence intensity in the bleached area after a long recovery time, corrected for total fluorescence reduction by the bleach [12]. For FRAP applications, Houtsmuller *et al.* [13] developed a quantitative protocol, designated FRAP-FIM, to measure the immobile fraction of a particular molecule in living cells. Using this procedure, the fluorescence ratio of confocal sections before and after spot bleaching is plotted as a function of the distance to the bleached spot. Two recent articles detail the experimental design of FRAP and the mathematical data modeling [14•,15•].

Slow recovery indicates low mobility, for example, owing to a more viscous cell environment or limited lateral mobility within membrane compartments. Diffusion barriers or discontinuities within the target structure are additional parameters that influence protein mobility [16]. Under ideal conditions the recovery kinetics are only dependent on the mobility of the protein of interest; however, the formation of protein complexes of higher order or binding to complex structures can affect the mobility of the molecule under observation. Thus, the time course of FRAP reflects several different parameters that influence the mobility of the protein.

In typical FRAP experiments the average intensities are measured as a function of time (illustrated in Figure 1). In the given example, STAT1–EGFP rapidly recovers within the bleached area. If the whole population of STAT1–EGFP is freely mobile the fluorescence intensity recovery curve should reach a plateau close to 100% of the initial fluorescence of the pre-bleach. Binding of a fraction of this protein to immobile structures would result in a reduction of fluorescence recovery. Using the FRAP technique it was possible to demonstrate reduced intranuclear STAT1 mobility when a mutant

Figure 1



Fluorescence recovery after photobleaching (FRAP). Live NIH3T3 cells expressing STAT1–EGFP were subjected to quantitative FRAP analysis. FRAP analysis was performed with a Zeiss 510 META inverted confocal laser-scanning microscope equipped with an on-stage heating chamber using a Plan-Apochromat 100× oil immersion lense (1.3 numeric aperture). Cells were excited with an argon laser at 488 nm and emission was collected using a 505–550 nm bandpass filter. An area within the cytoplasm (indicated by a black circle) was bleached with maximum laser power for approximately 0.8 s. Fluorescence recovery in the bleached area and intensities in different cytoplasmic and nuclear regions as well as the background signal were quantified with minimal laser power. The raw data obtained for recovery were corrected for the background intensity, loss of total fluorescence removed by bleaching a cellular subregion, and for any loss of fluorescence that occurs during post-bleach signal collection. The panel shows a normalized plot of fluorescence intensity versus time.

with enhanced DNA-binding activity was introduced [17].

FRAP has been used to examine protein dynamics and mobility in different cellular compartments. These include the plasma membrane, the nucleus, mitochondria, the Golgi apparatus and the endoplasmic reticulum [18]. Today, confocal laser-scanning microscopy is the prevalent tool for studies of the diffusional and kinetic properties of proteins in living cells by FRAP.

Application of FRAP to nucleocytoplasmic shuttling

Quantitative FRAP analysis, as described above, can be used to measure the mobility of molecules in a given compartment where in an ideal situation the boundaries (e.g. intracellular membrane systems) do not influence recovery. By contrast, the undirected and directed exchange of molecules between the cytoplasm and nucleus is strongly influenced by the nuclear membrane [19]. To study the process of nucleocytoplasmic exchange the application of 'selective FRAP' is appropriate. The nucleus or a segment of the cytoplasm is photobleached until a dramatic reduction or extinction of fluorescence in the complete compartment is observed. Subsequently, fluorescence recovery due to the movement of unbleached GFP fusion proteins is recorded by sequential imaging scans.

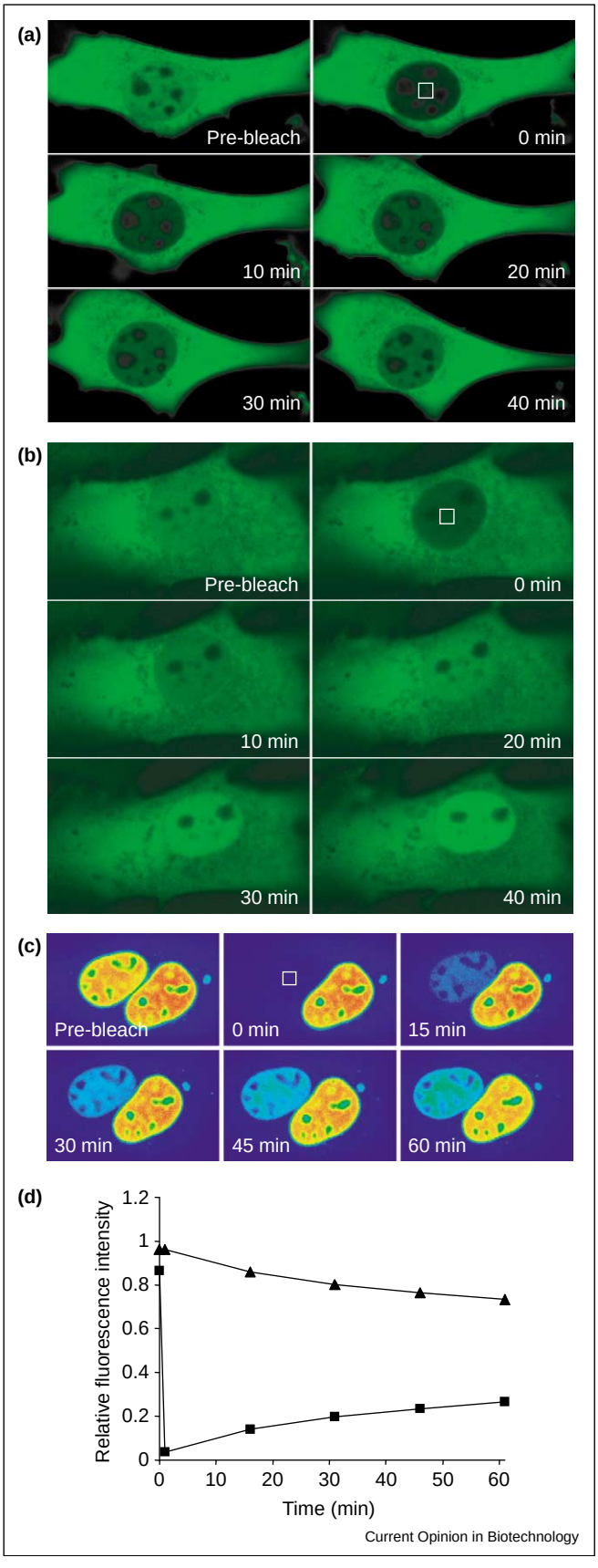
STATs are large molecules that need specific transport systems to enter the nucleus, and their steady-state levels are strictly controlled by extracellular signals. Using the selective FRAP technique the rate of nuclear import of STAT1–EGFP was determined (Figure 2a,b). Steady-state pictures show that in the absence of activating stimuli STAT1–EGFP localizes predominantly in the cytoplasm, with some protein in the nucleus. Extended photobleaching of a small area within the nucleus eliminates nuclear fluorescence. Recovery of GFP-competent molecules within the nucleus was determined by low-power imaging scans of the whole cell at different time points. This example and other experiments [17,20] demonstrate that in unstimulated cells STAT1 shuttles between the cytoplasm and nucleus, whereas IFN-γ-induced activation leads to a higher fraction of STAT1 molecules within the nucleus. Measuring the recovery of fluorescence in the nuclear and cytoplasmic compartment over time further indicate that the rate of nuclear import for the IFN-γ-activated form of STAT1–EGFP is much higher than that of the non-activated protein.

Upon IFN-γ treatment STAT1 participates in an activation–inactivation cycle, which includes nuclear import and subsequent export into the cytoplasm [21]. This process was uncovered by visualization of these repeated activation cycles using selective FRAP in multinucleated cells in accordance with the procedure described by Howell and Truant [22]. After complete bleaching of STAT1–EGFP in one nucleus of a binuclear IFN-γ-stimulated cell, the rate of re-entry from the non-bleached nucleus was measured (Figure 2c,d). The increase in relative fluorescence intensity in the bleached nucleus corresponded to the decrease of STAT1–EGFP in the non-bleached nucleus. This indicates a reactivation of STAT1–EGFP during IFN-γ stimulation in living cells.

FLIP technology

A complementary technique called fluorescence loss in photobleaching (FLIP) is used when the continuity of a cell compartment or the mobility of a molecule within the whole compartment is studied. In FLIP experiments, a region of fluorescence within the cell is subjected to repeated photobleaching, thereby preventing recovery of fluorescence in that region. Over time, this leads to a loss of the fluorescent signal of certain cellular compartments and eventually throughout the cell, given that the fluorescent molecules are mobile and are able to enter the

Figure 2



bleached region. The rate at which fluorescence is lost within the entire cell is monitored. The observation that molecules do not become bleached suggests that they are isolated (immobilized) in distinct cellular compartments. For this reason FLIP is ideal for studying the exchange of molecules between two compartments (e.g. compartments that are separated by lipid bilayers). The continuity of cellular structures including the Golgi apparatus, the endoplasmic reticulum, the nucleolus and splicing factor compartments have been verified using FLIP [23–25].

Application of FLIP to nucleocytoplasmic shuttling

The rate of nucleocytoplasmic shuttling of STAT1–EGFP was investigated using FLIP. The repeated bleaching of STAT1–EGFP in a small cytoplasmic region results in complete loss of detectable fluorescence in the whole cytoplasm (Figure 3a). The movement of STAT1–EGFP molecules from distant sites of the cytoplasm leads to a certain delay until fluorescence is completely lost. Nevertheless, the fluorescent population within the nucleus is not affected. Thus, during this short time period, no significant export of STAT1–EGFP from the nuclear compartment takes place, indicating that the nucleocytoplasmic shuttling rate is low [9]. To calculate the exact rate of fluorescence loss due to unspecific bleaching in the non-bleached compartment the imaging scan should include an unbleached neighboring cell.

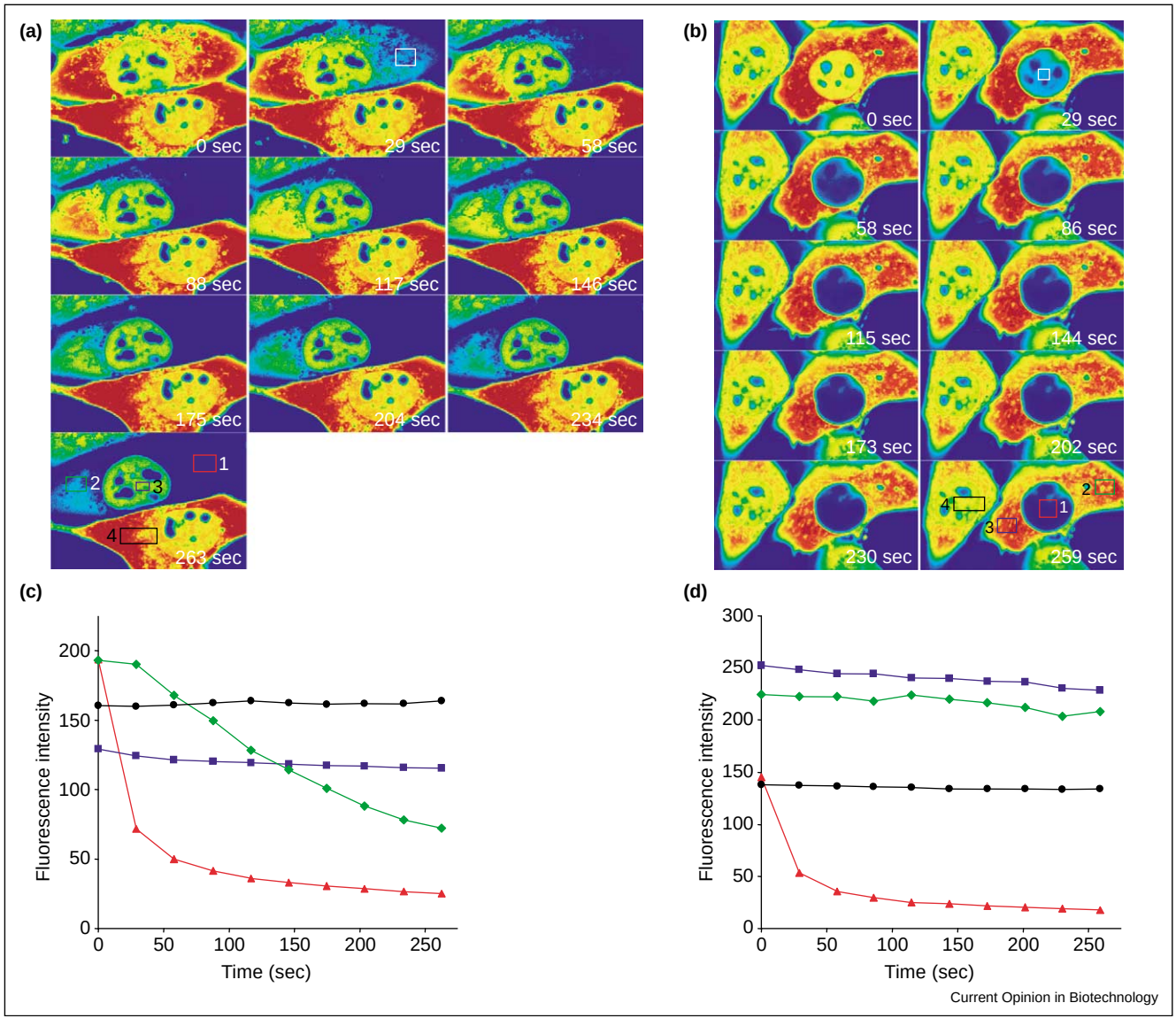
For certain proteins the steady-state localization within the nucleus is very low, owing to rapid nuclear export. To analyze nucleocytoplasmic exchange of cytoplasmically localized proteins bleaching should be carried out in a

Qualitative FRAP analysis. **(a)** Selective FRAP was used to determine the kinetics of constitutive nucleocytoplasmic transport of STAT1–EGFP in NIH3T3 cells. The indicated nuclear area was bleached for 40 s. Subsequently, recovery of unbleached STAT1–EGFP into the nucleus was monitored with a low-power image every 10 min. Over a 40 min period, approximately 50% of nuclear STAT1–EGFP was recovered in unstimulated cells. **(b)** The kinetics of STAT1–EGFP nuclear import increases after IFN-γ stimulation. In contrast to unstimulated cells, IFN-γ-activated STAT1–EGFP recovered to 100% within 10 to 15 min. Ongoing IFN-γ stimulation resulted in increased accumulation of STAT1–EGFP in the nucleus. **(c)** Selective FRAP was used to demonstrate repeated nucleocytoplasmic shuttling of activated STAT1–EGFP. NIH3T3 cells expressing STAT1–EGFP were pre-treated with cytochalasin D to induce multinuclear cells. IFN-γ treatment results in complete accumulation of STAT1–EGFP in both nuclei. To monitor the repeated use of existing STAT1–EGFP molecules new protein synthesis was inhibited with cycloheximide. After bleaching of one nucleus the recovery of STAT1–EGFP during ongoing signaling was monitored for 60 min. Fluorescence intensities of the entire unbleached and bleached nucleus were measured and corrected for background intensity. Fluorescence of a single nucleus was normalized to the total fluorescence of both nuclei at each time point to correct for non-visible cytoplasmic STAT1–EGFP molecules becoming activated after the bleach pulse. **(d)** The increase of STAT1–EGFP in the bleached nucleus (squares) correlates with the loss of EGFP fluorescence in the unbleached nucleus (triangles).

nuclear area. Subjecting such proteins to nuclear FLIP analysis, the decrease of fluorescence in the cytoplasm monitors the exchange of molecules between both compartments. Additionally, the collected raw data must be corrected for background bleaching of the cytoplasmic pool of fluorescent molecules. This is particularly important in rounded cells, because here the high-intensity bleach pulse touches not only the nuclear region of interest, but also the cytoplasmic fraction above and below the nucleus.

To determine the unwanted experimental background bleaching in the cytoplasm a nuclear FLIP experiment with static cytoplasmic fluorescent protein should be

Figure 3



Fluorescence loss in photobleaching (FLIP). Live NIH3T3 cells expressing STAT1–EGFP were subjected to FLIP analysis. Defined areas of **(a)** the cytoplasm and **(b)** the nucleoplasm were bleached by scanning for nine consecutive periods of approximately 30 s with maximum laser intensity. To correct the acquired fluorescence intensities for a generalized bleaching effect, which results from the imaging scan, an unbleached neighboring cell in the same window was monitored. The total fluorescence of the bleached cell and of a neighboring cell was monitored between the times of bleaching. The bleached regions are indicated with white rectangles in the first post-bleach image. Each image series shows the fluorescence intensity in false color code (intensity increases from blue to red) before bleaching (0 s) and after the consecutive bleaching periods. **(c,d)** Fluorescence intensities of different cytoplasmic areas and the nucleoplasm were measured, corrected for the background signal and plotted over time. (c) Fluorescence intensity of the bleached area (1, red triangles); a distant cytoplasmic region (2, green diamonds); the nuclear area (3, blue squares); and a non-bleached cell (4, black circles). (d) Fluorescence intensity of the bleached area (1, red triangles); two regions in the cytoplasm (2, green diamonds; 3, blue squares) and a non-bleached cell (4, black circles).

carried out. In the experimental set-up shown in Figure 3b, repeated bleaching of the nuclear STAT1–EGFP was used to determine the extent of cytoplasmic background bleaching. The result shows that loss of fluorescence intensity in the cytoplasmic fraction due to bleaching through the vertical axis is negligible, most probably because of the flat cell morphology. The results obtained with both FLIP techniques confirm the status of a slow shuttling rate of the STAT1–EGFP protein.

Taken together, these data indicate that STAT1 permanently shuttles between the cytoplasm and the nucleus; IFN-γ stimulation increases nuclear import, but shuttling continues. This behavior leads to steady-state pictures that show cytoplasmic dominance in the absence of IFN-γ and movement to the nucleus after activation. These findings are quite important if one wants to understand the mechanisms of direct protein transport.

Conclusions

The experiments described demonstrate the value of FLIP and FRAP for studying the dynamics of signaling proteins. It is clear that these technologies can be applied to many types of proteins, including cell-cycle regulators. Furthermore, studies on membrane trafficking and on nuclear architecture and function have also been carried out using these techniques. As individual cells are studied, these methods allow differences between cells of a population or resulting from the state of the cell within the cell cycle to be determined. This is of fundamental interest. By contrast, measurements on many individual cells are required before general conclusions for the whole population can be drawn.

A major obstacle to the wide application of FLIP and FRAP is the possible influence of the fluorescent tag on protein features. In specific cases the fusion of the fluorophore can perturb the properties of the target protein including dynamics, function and destination. The use of low molecular weight fluorophores bound to specific sites might help to overcome this problem, but this has to be carefully demonstrated in each case. Several direct and indirect controls are needed to compare the native and labeled target proteins before final conclusions can be drawn.

An alternative to photobleaching concerns the gain of fluorescence (photoactivation) or a shift of the emission spectra (photoconversion) by illumination with ultraviolet or violet light. Recently, photoactivatable GFP (PA-GFP) and Kaede, a tetrameric fluorescent protein from corals, have been introduced [26,27]. Initially, both proteins show no or only marginal fluorescence under the imaging wavelength. Once excited using a brief pulse of high-intensity irradiation, their fluorescence at the imaging wavelength becomes activated. This approach allows the selective labeling of pre-existing proteins in defined subdomains of cells or of individual cells (e.g. during development). The technique enables the identification and tracking of the movement of molecules emanating from certain regions of the cell. It is expected that the availability of this type of fluorescent tag will significantly advance the field.

Lastly, it should be noted that FLIP and FRAP do not significantly interfere with the viability of the cells under examination. Thus, they could be used to image intracellular events in certain tissues of living animals.

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- •• of outstanding interest

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