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Fluorescence Recovery after Photobleaching: Application to Nuclear Proteins

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Abstract Fluorescence redistribution after photobleaching (FRAP) has received increasing attention ever since it was first introduced into cell biological research. The method was developed in the 1970s, when its biological application mainly focused on the mobility of fluorescently labelled constituents of the cell membrane. The development of confocal scanning microscopy in the 1980s facilitated accurate investigation of the behaviour of molecules in the inside of cells without specialised equipment. However, FRAP did not yet become as popular as it is today, probably because of the dedicated and time-consuming methodology required to purify and label proteins or other compounds and, moreover, to inject them into cells. The revolution created by the development of GFP-technology finally lead to a tremendous boost of FRAP applications in studying the behaviour of proteins in the living cells. Finally, the ongoing increase of speed and memory of personal computers allows computer modelling of FRAP experiments for analysis of complex 3-D FRAP data, and for the development of new FRAP assays. Here we discuss several variants of FRAP on the basis of its application to the investigation of the behaviour of proteins in the living cell nucleus.

Keywords FRAP · Green fluorescent protein · Protein mobility · Cell nucleus · Protein-DNA interaction · DNA repair · Transcription · Replication

List of Abbreviations

AR Androgen receptor
ER Estrogen receptor
FLIP Fluorescence loss in photobleaching
FRAP Fluorescence recovery after photobleaching
FRET Fluorescence resonance energy transfer
GR Glucocorticoid receptor
NER Nucleotide excision repair
UV Ultra-violet

1 Introduction

In the past decade fluorescence recovery after photobleaching (FRAP) has received tremendously increased attention, especially in the field of live cell investigation of protein mobility. FRAP makes use of the property of fluorescent molecules that they not only can be visualised in fluorescence microscopes, but also can be photobleached, i.e. they can be made non-fluorescent by illuminating them at relatively high intensity of the excitation light. After selectively photobleaching a small volume inside a larger volume, the subsequent recovery of fluorescence in the illuminated region can be followed in time. The extent to which, and speed at which this recovery occurs are measures for the fraction of mobile molecules and the speed at which they move, respectively. Various novel findings in many fields of cell biology that came from the application of FRAP have been elaborately discussed in a large number of reviews (e.g. [1–7]). In this review we will focus on the utilisation of FRAP to study the mobility and behaviour of proteins in the inside of the cell nucleus. Specifically, we will focus on proteins and protein complexes that can interact with DNA, such as transcription and DNA repair factors. Live cell investigation of these DNA-transacting proteins provides an ideal model system to demonstrate the basic principles of FRAP, since they basically display two types of behaviour: they are either mobile, moving through the nucleus or they are (transiently) immobile when they interact with (usually immobile) DNA. As will be shown here, FRAP is a powerful method to study precisely this behaviour. In this chapter, we will first introduce the principles of FRAP in a stepwise manner, from determining overall protein mobility to measuring the immobile fraction. In addition, we will show how FRAP can be applied to determine duration of immobilisation when proteins are transiently immobilised. In each subsection, examples from biological research will be given to show how these qualitative and quantitative FRAP approaches contributed to understanding nuclear protein function. After this we will present a number of variations of FRAP, discuss their interpretation, and summarise some potential pitfalls in application and analysis of FRAP experiments. Throughout the review, examples of typical FRAP curves will be provided that were generated by computer

simulations. We will present a detailed description of the computer models that underlie these simulations.

2
Fluorescence Recovery After Photobleaching (FRAP)

2.1
Fluorescence

In biological research, fluorescent dyes are generally used to label molecules or cellular compartments. The labelled entities can be visualised by fluorescence microscopes, among which in current days the confocal microscope is one of the most frequently used. A large number of fluorescent dyes are available, in many cases as labels of specific biomolecules such as antibodies to detect and localise a specific constituent in a cell. In addition, a considerable number of proteins and other biologically relevant molecules have fluorescent properties themselves. The best known today is without doubt the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*. The unique property of this protein, and of its many colour variants, is that they require no specialised cellular machinery to reach their final fluorescent form. This allows researchers to express the protein in many different cell types, and to genetically label proteins with important cellular functions. The fact that labelling is encoded by the gene of interest avoids the elaborate purification, chemical labelling and injection into cells. This has lead to an impressive revolution in cell and molecular cell biology and it clearly is this revolution that has boosted the application of a relatively old fluorescence technique: fluorescence recovery after photobleaching [8].

2.2
Mobility

One potential problem in visualisation of fluorescent compounds is that after a certain period of excitation dependent on the intensity of the excitation light they loose their fluorescent capacity, a process known as photobleaching. Although in general photobleaching may hamper or even prevent extended time-lapse monitoring or imaging low dye concentrations, it can also be put to use to study the dynamic behaviour of labelled molecules. The technique to do so is named after the phenomenon that it makes use of: fluorescence recovery after photobleaching. The basic FRAP experiment is straightforward (Figs. 1 and 2): a region of limited dimensions within a larger volume is illuminated shortly at the excitation wavelength of the dye to be bleached, for instance with an intense laser beam. Immediately after illumination molecules in the exposed region will no longer be fluorescent. If the labelled molecules have a fixed position, the region will remain bleached and this situation will remain unchanged (Fig. 1A). However, if the fluorescent molecules are mobile, for instance because

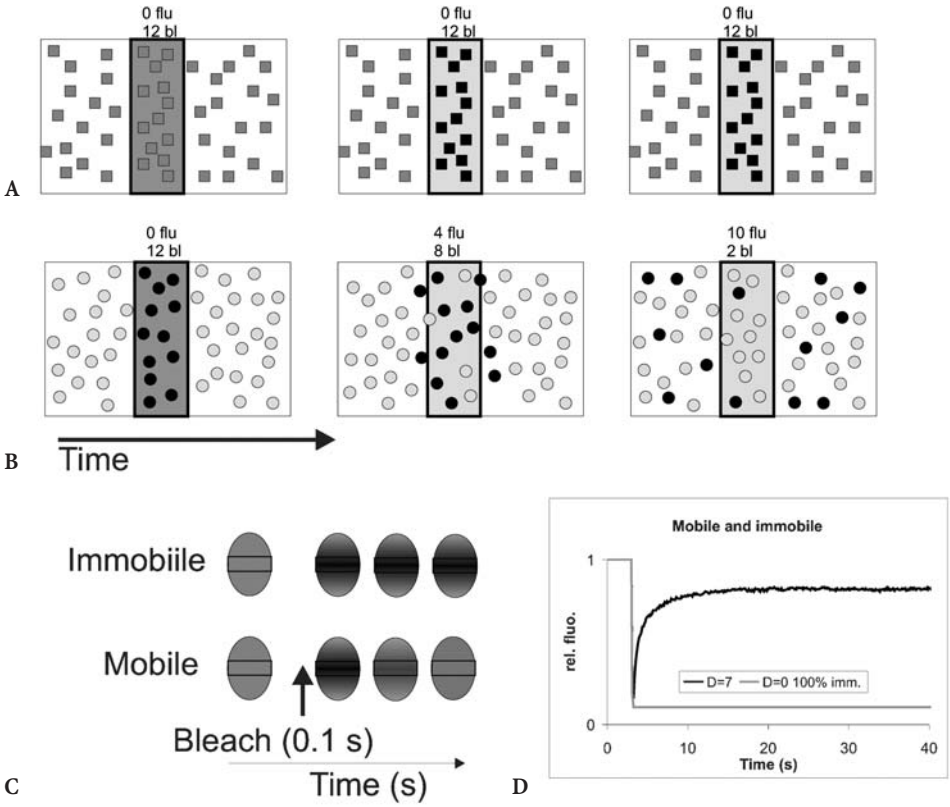


Fig. 1A–D The FRAP principle. When a part of a volume (*shaded rectangle*) containing fluorescent molecules is illuminated at high intensity excitation light, the molecules inside that volume lose their fluorescent property, a process also termed photobleaching: **A** when the molecules are immobile (*grey squares*), the bleached molecules in the strip and fluorescent molecules outside the strip will stay in place, so the bleached area will remain non-fluorescent after photobleaching; **B** when molecules are mobile (*grey spheres*), for instance because they diffuse, bleached molecules in the strip and fluorescent ones outside will redistribute throughout the nucleus after photobleaching, resulting in a gradual recovery of fluorescence after photobleaching. When the bleached area is not very small compared to the total volume (like in many biological cases) the final recovery will not reach the initial value; **C** schematic of strip-FRAP experiments on cell nuclei (*grey ellipses*) in the two cases in A and B. A narrow strip region in the centre of the nucleus is bleached with a short intense bleach pulse and the recovery of fluorescence in the strip is monitored at regular time intervals; **D** graph of the fluorescence recovery process in the two cases depicted in A and B. Relative fluorescence ($F(t)/F(0)$) is plotted against time. The curves are based on computer simulated FRAP experiments (see text). The molecules in case B, where they were all mobile, had a diffusion coefficient of 7 $\mu m^2/s$ in the simulated experiment

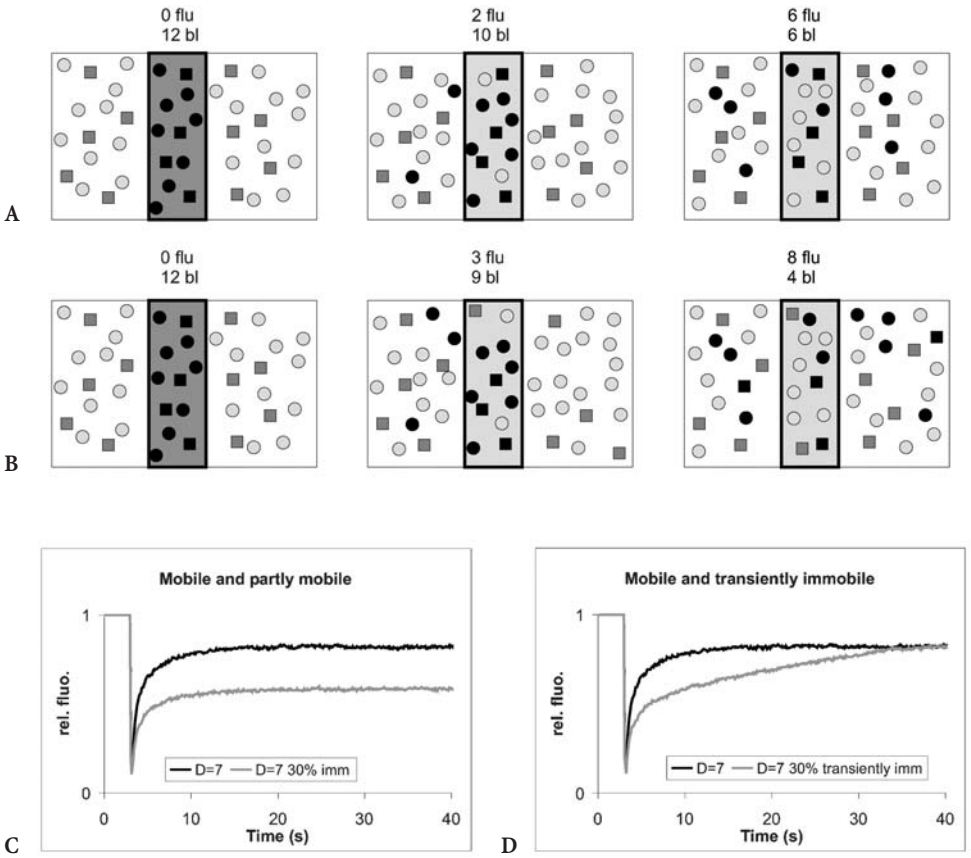


Fig. 2A–D the FRAP principle in two, more complicated cases than the ones in Fig. 1: **A** a situation where only a part of the molecules is immobilised (*grey squares*). In this case only the mobile fraction of molecules (*grey spheres*) causes recovery of fluorescence. Since the immobile do no longer contribute to the signal after photobleaching, fluorescence after complete redistribution will be considerable lower than before bleaching. After correction for the fraction of bleached mobile molecules, the size of the immobile fraction can be calculated from the FRAP curves; **B** a situation where a fraction of the molecules are transiently immobile, i.e. they bind for a while and release. In this case the recovery of fluorescence will initially be due to redistribution of mobile molecules. A secondary recovery of fluorescence will occur due to fluorescent immobile molecules outside the strip becoming mobile and redistribute. The graphs at C and D represent the recovery of fluorescence in the cases in A and B plotted against time; **C** *black curve* represents free molecules in Fig. 1B. The *grey curve* represents the situation in A where a 30% fraction of the molecules is permanently immobilised, resulting incomplete recovery of fluorescence; **D** *black curve* is the same as in C. The *grey curve* now represents a situation where a steady state exists in which always a 30% fraction of the molecules is immobilised, but individual ones are immobile for 45 s, resulting in an initial recovery due to diffusion of free molecules and a secondary (linear) recovery of fluorescence due to release of fluorescent immobile molecules. The time point where the recovery reaches a plateau is a good estimate of the average binding time of individual molecules. The curves are based on computer simulated FRAP experiments (see text)

they diffuse, after the illumination the non-fluorescent and fluorescent molecules inside and outside the area will remix (Fig. 1B), leading to an increase of fluorescence in the bleached region which continues until bleached and fluorescent molecules have completely redistributed over the entire volume. If the bleached area is relatively large compared to the volume to which the investigated molecules are restricted, the final recovery of fluorescence will not be to the pre-bleach level (Fig. 1B). The process can be followed by monitoring fluorescence either in the bleached area or in the total volume in which the investigated molecules reside (Fig. 1C).

Almost all current application of FRAP in the field of nuclear research are performed using modern confocal microscopes. An advantage of the confocal microscope is that any region can be selected for bleaching. Usually a small area is selected somewhere inside the nucleus and the area is scanned prior to bleaching at a low laser intensity to determine a prebleach fluorescence value. After that a short bleach pulse is given at a high laser intensity. Subsequently the recovery of fluorescence inside the area is monitored at low intensity, preferably until complete recovery of the fluorescent signal is reached. Usually the selected area is kept small, but if cells are studied that contain fluorescently tagged molecules at a physiologically relevant concentration, the obtained signal may be quite low. In such a situation it is advisable to select a larger area to increase the signal to noise ratio, for instance a narrow strip spanning the entire nucleus. With the newest versions of the confocal microscope it is possible to scan an area of 20×1 μm (200×10 pixels at optimal resolution) in only 20 ms. After a statistically relevant number of cells has been sampled, the average mobility of the fluorescent molecules can be determined by averaging the normalised fluorescent data of individual cells. There are several ways of normalising FRAP data. The most straight forward way is to express the data relative to the prebleach value: $I_{norm,t}=(I_t-I_{background})/(I_{prebleach}-I_{background})$, where $I_{prebleach}$ is the measurement before the bleach (or the average of a number of recordings before bleaching), and $I_{background}$ is the signal level when no fluorescence is present. A second method of normalisation is used to fit data to analytically derived descriptions of diffusion and is expressed as: $I_{norm,t}=(I_t-I_0)/(I_{final}-I_0)$, where I_{final} is the final value when no more fluorescence recovery occurs. This way of normalising the data yields a curve that starts at zero right after bleaching and reaches one at recovery, allowing to fit the data (e.g. using least squares methods) to any equation that represents the diffusion process. Since many different approaches have yielded equations that were described in abundant literature we will not address the topic in this review but refer to the following citations. Axelrod and coworkers were the first to develop a detailed analytical solution for the basic FRAP principle, which was later refined by Soumpasis [9]. They were able to do so by using a single laser beam to both bleach a small region, i.e. the spot created by a focused laser beam (at high laser intensity), and detect the recovery of fluorescence (at low laser intensity) inside that same region. This approach enhanced the analysis of the data, since the bleached region exactly overlapped the region monitored afterwards. More-

over, the authors provided ways to determine whether observed mobility was due to flow or to diffusion. However, these analytical solutions were limited to membrane studies. Therefore, other mathematical approximations have been used to fit data obtained with confocal microscopy. A one-dimensional model of diffusion into a strip was originally used to study diffusion in nuclear and ER membranes [10] and later extended to investigate nuclear protein diffusion [11]. Various other equations representing 2-D diffusion models can be found in Carrero et al. and the literature cited therein [12]. A very thorough 3-D approach was presented by Blonk et al. [13]. In addition Monte Carlo simulation may be used to generate FRAP curves to fit experimental data [11]. The curves presented in the figures of this review are generated using this approach. This will be discussed in more detail later.

After the advent of GFP technology, the first FRAP experiments in research of protein behaviour in the cell nucleus were applied to determine the mobility of proteins or other biomolecules in the living cell nucleus. It had long been a matter of great concern whether DNA-transacting processes like transcription, replication and repair and also RNA-splicing were governed by highly organised and compartmentalised holo-enzymes, (sometimes termed factories), comprised of many different factors. This hypothesised high degree of organisation and formation of stable protein structures suggested a relatively low mobility of nuclear constituents. The development of GFP tagging made it possible to thoroughly investigate the issue in its most relevant context: the living cell. One of the first investigations in which FRAP provided information on the mobility of nuclear proteins were focused on constituents of the nucleotide excision repair (NER) machinery, a repair system that removes single strand DNA damage caused by ultra violet (UV) light. The authors investigated cell lines that stably expressed functional GFP-tagged NER-factors at physiological levels. The latter was considered important since over-expression of the factors under investigation may result in a large fraction of the molecules being mobile simply because they are redundant. In another bad-case-scenario, over-expression may lead to precipitation of the excess of protein, for instance caused by misfolding due to insufficient availability of chaperones. In this type of situations mobility studies may not be very informative.

By application of the confocal FRAP experiments described above to two repair factors (ERCC1, XPA) and a repair/transcription factor (TFIIH) it was shown that nuclear proteins have a much higher mobility than previously anticipated [11]. In the absence of DNA damage the NER factors ERCC1/XPF and XPA appeared to be freely mobile and diffuse through the nucleus as separate entities. After this discovery, the surprisingly high mobility of DNA repair factors was also found for other nuclear proteins using a similar FRAP approach. In a study of three proteins involved in different nuclear processes, Misteli and coworkers showed a surprisingly high mobility of HMG-17, SF2/ASF and fibrillarin, which are involved in transcription, pre-mRNA splicing and rRNA processing respectively [14]. Others also found, using FRAP that molecules can be highly mobile in the nucleus. Various studies revealed that dextrans up to a

molecular weight of 500 kD and also fluorescently labelled oligonucleotides diffuse freely through the nucleus [15–17] and have access to most regions of the nucleus [18].

Complementary to the investigation of DNA metabolising factors, the mobility of their template, chromatin, was also studied using FRAP. In a non-confocal microscope set-up, using an aperture diaphragm to minimise out-of-focus-light detection, FRAP was applied to cells of which the DNA was stained with an ethidium bromide derivate [19]. After bleaching small spots in the stained chromatin the spots remained non-fluorescent for periods of more than one hour, indicating that interphase chromatin is largely immobile, at least at the spatio-temporal resolution of the described experiment.

These examples show that already the very straightforward, largely qualitative application of FRAP to determine whether biomolecules are mobile or not has provided important conceptual implications for the way nuclear processes are organised in the living cell.

2.3 Immobilisation

As already pointed out above, FRAP is not only able to determine whether molecules are mobile or not, but can also be applied to quantitatively determine a second mobility parameter, i.e. immobile fraction. If only a fraction of the investigated fluorescently tagged molecules is immobile, those outside the bleached area will never enter it, similar to the situation in Fig. 1A, whereas those inside the area will never leave (Fig. 2A). Immobile molecules that were inside the area contributed to the fluorescent signal before bleaching, but will no longer do so after bleaching. Therefore, recovery of fluorescence in the bleached area will reach a lower final level compared to when no immobile fraction would be present (Fig. 2C). To estimate the immobile fraction the data is usually normalised in a different way than described above: $I_{norm,t}=(I_t-I_0)/(I_{prebleach}-I_0)$, where I_0 is the intensity immediately after bleaching. Using this way of normalising the data yields a curve of which the prebleach value is one and the fluorescence level immediately after bleaching is zero. Provided that the fraction of mobile bleached molecules is negligible compared to the total amount of fluorescence molecules, i.e. if the FRAP curve would return to prebleach levels when no immobile fraction was present, the immobile fraction can be estimated as $1-I_{norm,final}$ (Fig. 2C). However, in most biological applications the volume in which the fluorescent molecules are present is very small, for instance in the cell nucleus. As stated above, a substantial portion of the fluorescent molecules may then be bleached, giving rise to an incomplete recovery for which the signal should be corrected if one wants to determine immobile fraction. There are several ways to deal with the problem. First, and probably best is to study the same proteins in a situation where they are freely mobile, in case of gene transcription or DNA repair factors, when they are inactive. For DNA repair this is not a great problem, since one can study cells without DNA damage. However,

in the case of essential transcription factors, it is more problematic to do so. In that case the signal can be divided by the total nuclear fluorescence outside the bleached region. However, this method may have a potential drawback when cells are studied under condition where photobleaching due to monitoring occurs, a problem that will be discussed in detail later. An alternative way to estimate the fraction of fluorescent mobile molecules removed by the bleach could be obtained from experiments on cells expressing free GFP. In fact, it would be of great value if such control experiments were always presented allowing a better comparison of data obtained in different labs using different microscopes and FRAP settings.

Although the observation alone that proteins are highly mobile in densely compacted compartments such as the cell nucleus has attributed to understanding of how biomolecules function, it is the fact that FRAP can also be used to determine the percentage of immobilised molecules that makes it a strong tool for investigating protein behaviour in living cells. This becomes strikingly apparent in the case of the investigation of nuclear proteins that have a function in regulating DNA metabolism (transcription, replication and repair). It is obvious that in order to perform their function, these factors have to bind to DNA, either directly or indirectly through other chromatin constituents. Since DNA is essentially immobile, at least during a typical FRAP experiment, the activity of DNA-transacting enzymes and supportive factors leads to their immobilisation. The first reports on the application of FRAP to study DNA binding in vivo were from the investigation of repair of UV-lesions of DNA. By application of a FRAP procedure (see also below) specifically developed to study immobilisation in small compartments (like the nucleus) it was shown that NER factors ERCC1/XPF, XPA, and the transcription/repair factor TFIIH bind to DNA damage (and are thus immobilised) in UV dose dependent amounts [11, 20, 21], reaching a plateau at an immobile fraction of approximately 30–40%, at a UV-dose of 8–16 J/m^2 (UV-C irradiation was used). In addition it was shown that the apparent diffusion coefficient of the freely mobile fraction was not affected by the induction of DNA damage. Note that this can be judged already by visual inspection of the FRAP curves from simple strip-FRAP experiments (Fig. 2C): the (incomplete) recovery of fluorescence in the situation where there is an immobile fraction (grey line) reaches a plateau at the same time after photobleaching as in the case where there is no immobile fraction, the only difference being that the final level is lower.

2.4 Transient Immobilisation

In the FRAP experiments described above to determine immobile fraction of repair proteins, the immobile fraction is constant at the time scale of the FRAP experiment. However, it is also possible that individual molecules immobilise for a shorter period, leading to a turn-over of immobile and mobile molecules during the FRAP experiment. If the system studied is in steady state, i.e. if an

equal amount of molecules bind and release per unit time, this scenario will result in a secondary recovery of fluorescence. FRAP analysis can be applied to determine the third 'mobility parameter': average duration of transient immobilisation of individual molecules. In Fig. 2B such a situation is depicted. It can be seen that the release of molecules that were immobile at the time of bleaching will give rise to a secondary recovery of fluorescence in the bleached area (Fig. 2D). Such a scenario may well be expected in the case of transcription, replication or DNA repair since binding to DNA by these factors in many cases may be limited to the time needed for their action. For instance a DNA repair factor like the endonuclease heterodimer ERCC1/XPF can be expected to bind to repair complexes at the DNA for at least the time required to cut the 5′ end of the damaged DNA strand. After the DNA lesion has been repaired successfully it is well possible that the repair complex releases, since the DNA is now repaired. Indeed, it was shown that individual NER repair factors are immobilised to damage for approximately three to five minutes [11, 20, 21]. In addition, it was recently reported for the androgen receptor (AR) that the wild type AR shows a reduced mobility compared to a non-DNA-binding mutant, and that this drop is caused by the transient binding of ARs to DNA in the order of 45–90 s [22]. For this, the authors used two complementary FRAP assays, a strip-FRAP and a combined FLIP and FRAP assay, which are discussed in detail below; see Fig. 4.

Although it is possible to determine the average duration of immobilisation in cases where the immobilisation is relatively long compared to the time the mobile fraction redistributes completely within the nuclear volume, often transient immobilisation will be too short to distinguish from (slow) diffusion (see also below). Nevertheless, in many cases conclusions can be drawn from the qualitative observation that a reduced mobility compared to a situation where the protein is inactive is probably due to interaction with immobile elements in the nucleus. For estrogen receptors (ER), it was reported that binding to ligand slowed down the previously freely mobile ER. In addition, the authors report a distinct slow down induced by antagonists as well as by proteasome inhibitors, which they contribute to binding to the (operationally defined) nuclear matrix [23]. Moreover, their data suggest that in the presence of ligand, the tagged cofactor SRC-1 shows dynamics similar to the ER, suggesting interaction of these molecules prior to DNA binding. Similarly, the RNA splicing factor ASF was reported to move considerably slower through the nucleus than expected for free diffusion. The authors argue that frequent but transient interactions with relatively immobile nuclear binding sites causes the observed slow nucleoplasmic mobility [24].

2.5 Residence Time

The previous sections discussed the application of FRAP to determine the overall nucleoplasmic mobility of nuclear proteins. FRAP has another, more read-

ily appreciated application in the investigation of residence time of nuclear factors that accumulate in small nuclear substructures such as nucleoli, nuclear bodies, telomeres or DNA double strand break related foci. To determine residence time of proteins at foci, it is possible to conduct two complementary photobleaching assays, FRAP and FLIP (fluorescence loss in photobleaching). Again, the FRAP experiment is straight forward: the accumulation is bleached and fluorescence recovery monitored at regular intervals after bleaching. The time it takes to fully recover is a measure for the residence time of proteins in the accumulation. In the complementary FLIP experiment, a region at a distance from the accumulation is bleached and the loss of fluorescence in the distant accumulation is monitored (see below).

Similar to the results of measurement of overall nuclear mobility, FRAP and FLIP revealed in many cases a surprisingly high exchange of many factors with nuclear bodies. One of the most elaborate studies was conducted by Misteli and coworkers who combined what was termed kinetic modelling with FRAP of factors that regulate transcription of rRNA genes in the nucleolus, providing a kinetic framework for RNA polymerase I transcription [25]. The authors show a high turnover of transcription initiation factors at rRNA genes. In addition, it was shown by others that also transcription factor TFIIH is associated with nucleoli in a highly dynamic fashion [20]. Similarly, nuclear bodies such as Cajal bodies, and also DNA double strand break foci and telomeres were investigated by FRAP and FLIP. In these studies the authors show that different factors with different functions associate to foci in a differential manner [26–30]. Replication factors also interact with replication foci in a differential way. Cardoso and coworkers have reported that PCNA, but not RPA34, is stably associated with replication foci [31].

Apart from studying naturally occurring nuclear substructures, researchers have also used various methods to introduce artificial accumulations into cell nuclei. One of the first reports comes from steroid receptor research. Hager and co-workers investigated the activity of GFP-tagged glucocorticoid receptors in cells containing long tandem arrays of the MMTV promoter. It was shown that liganded GR-GFP accumulated to these arrays in such amounts that they could be readily visualised by fluorescence microscopy as bright fluorescent spots [32]. In addition, FRAP and FLIP measurements suggested a transient interactions between MMTV-promoters and GRs in the order of seconds to a minute, suggesting a 'hit-and-run' mechanism for GR transcription initiation. In a similar way, nuclear factors involved in various types of DNA repair were investigated, by inducing DNA damage in a subregion of the nucleus [20, 33]. Interestingly, these studies show that DNA repair factors of UV-damage tend to bind considerable longer to damage than transcription factors like steroid receptors and general transcription factors [11, 20, 34]. An obvious exception to this is RNA polymerase II which was reported from FRAP studies to be immobilised for more than 10 min [35].

3 FRAP Variants

3.1 Applications

In this section we will discuss various FRAP variants that have been used to study the behaviour of nuclear proteins. The development of confocal microscopy in the 1980s made it possible to extend the straightforward methods provided by early researchers. The scanning capacity of this type of microscopes made it possible to bleach specific areas and measure the redistribution of fluorescence in other regions, or even scan the entire cell. This enabled various more dedicated approaches that will be discussed below: FRAP-FIM (FRAP for immobilisation measurement) (Fig. 3A) [11], combined strip-FRAP (Fig. 3B) and FLIP-FRAP (Fig. 3C,E) [22], FLIP (Fig. 3D) [28–30, 32–34] and iFRAP (Fig. 3F) [25].

A FRAP variation termed FRAP-FIM was developed to study the immobilisation of nuclear repair factors. The method differs from the 'conventional' FRAP applications, in that the spot is illuminated at a relatively low intensity for a relatively long time [11], typically 4–12 s. If all molecules are immobile, only those in the beam will get bleached, resulting in a dark hole in the nucleus when a new image is taken at the confocal plane, even if this image is taken after a long period of time. However, if all molecules are mobile, a significant part (depending on diffusion rate) will pass through the beam in this period, get bleached and diffuse through the nucleus. If a period of redistribution of the bleached and unbleached mobile molecules is allowed after the bleaching, this will result in a homogeneously distributed decrease of fluorescence in the entire nucleus. In an intermediate situation, when a part of the molecules is mobile, the resulting image will be an intermediate between the two extreme situations. To obtain images of these extremes, cells expressing GFP can be used that are either paraformaldehyde-fixed or alive, representing the 100% immobile and 100% mobile scenarios respectively. Next, the fluorescence intensity ratio in images taken after and before bleaching are calculated as a function of the distance to the bleach spot. To do so the image is divided into concentric rings around the bleach spot. Finally, the weighted sum of the fluorescence ratio profiles (FRPs) of the extremes, $a(\mathrm{FRP}_{\mathrm{immobile}})+b(\mathrm{FRP}_{\mathrm{mobile}})$, is fitted to the curves obtained in cells under investigation and the weight factors a and b represent the immobile and mobile fraction respectively.

Of special interest is the combination of the strip-FRAP (Figs. 3B and 4A) and FLIP-FRAP method (Figs. 3C and 4B) that can be used to study transient immobilisation [22]. In the combined FLIP-FRAP experiment a region distant from the bleach region is monitored simultaneously with the FRAP region. In such an experiment (Figs. 3C and 4B), a strip at one pole of the nucleus is bleached for a relatively long period (typically 4–8 s) at a moderate excitation intensity. Subsequently the fluorescence is monitored in that region (FRAP), but

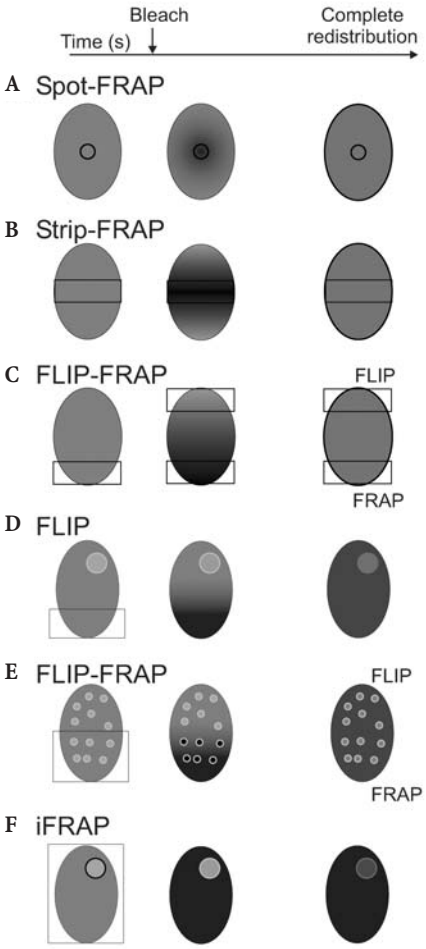
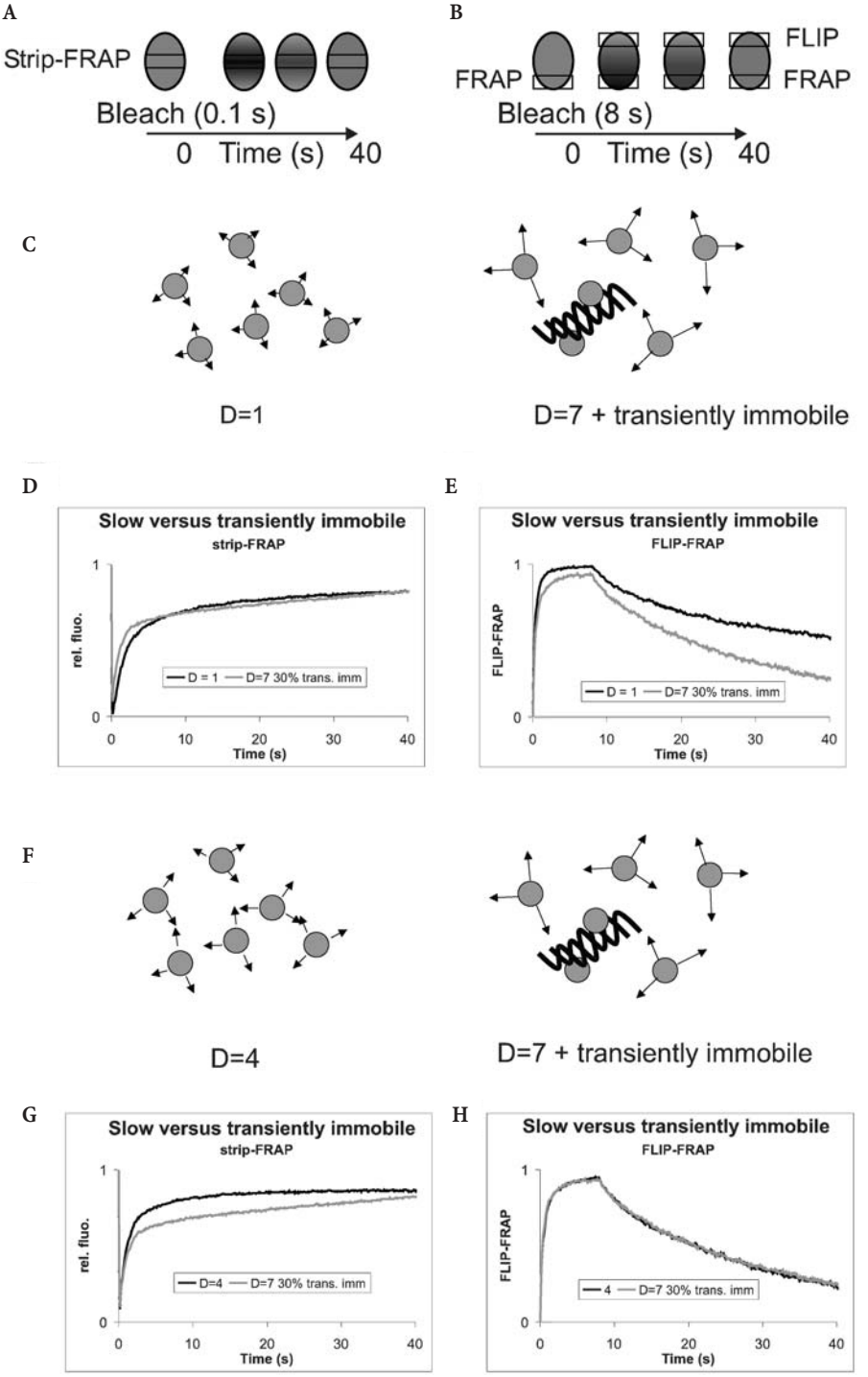


Fig. 3A–F Examples of different FRAP applications that have been developed to study the dynamics of nuclear proteins. The curves are based on computer simulated FRAP experiments (see text): **A** spot bleaching – the most straightforward FRAP experiment based on bleaching only a diffraction limited spot. A specific application of the method was developed to study the immobilisation of nuclear repair factors (see text and [9]); **B** strip bleaching – when signals are relatively low, e.g. due to low expression of the tagged protein, a larger area, for instance a strip spanning the entire nucleus can be bleached, and the fluorescence monitored at regular intervals; **C** combined FLIP and FRAP – the FLIP-FRAP method differs from the strip-FRAP in that two areas are monitored after bleaching. D–F represent methods to determine the residence times of proteins associated with nuclear accumulation such as nucleoli, nuclear bodies, telomeres locally damaged nuclei or DNA double-strand break associated foci; **D** fluorescence loss in photo bleaching (FLIP). An area at a distance of nuclear accumulation (*bright circle*) is bleached, either repetitively, or with one prolonged bleach pulse at low intensity. The velocity at which fluorescence is lost in the distant accumulation is a measure for the residence time at the accumulation, the point in time where a new steady state is reached being a good measure for residence time; **E** combined FLIP and FRAP. One half of the nucleus is bleached, including foci (*bright circles*) in that half; **F** iFRAP (inverse FRAP). iFRAP was developed to estimate the rate of dissociation of molecules from the nucleolus (see text)


A
Strip-FRAP
Bleach (0.1 s)
0 Time (s) 40
B
FRAP
FLIP
FRAP
Bleach (8 s)
0 Time (s) 40
C
D=1
D=7 + transiently immobile
D
Slow versus transiently immobile
strip-FRAP
rel. fluo.
D = 1
D=7 30% trans. imm
Time (s)
E
Slow versus transiently immobile
FLIP-FRAP
FLIP-FRAP
D = 1
D=7 30% trans. imm
Time (s)
F
D=4
D=7 + transiently immobile
G
Slow versus transiently immobile
strip-FRAP
rel. fluo.
D=4
D=7 30% trans. imm
Time (s)
H
Slow versus transiently immobile
FLIP-FRAP
FLIP-FRAP
4
D=7 30% trans. imm
Time (s)

also in the area at the other side of the nucleus (FLIP). The power of combining two different FRAP protocols (strip-FRAP and FLIP-FRAP) is exemplified in Fig. 4. In a scenario where molecules transiently bind to immobile elements in the nucleus (Fig. 4C, left panel), it will often, if not always, be possible to define another scenario where the entire population of molecules is mobile (but moves slower) (Fig. 4C, right panel) which results in similar FRAP curves (Fig. 4D). For instance, using simple strip-FRAP methods in a situation where molecules diffuse at a D of 7 $\mu m^2/s$ of which 30% is immobile, individual molecules being on average immobilised for 45 s, it will be difficult to distinguish this from a situation where the entire population diffuses at a D close to 1 $\mu m^2/s$ (Fig. 4D). However, a way to cope with this problem is to perform a complementary FLIP-FRAP experiment. The two situations described above yield similar curves in the strip-FRAP experiments but are clearly separated in the accompanying FLIP-FRAP experiment (Fig. 4E). From this, one may erroneously conclude that the FLIP-FRAP experiment is apparently more suited to identify the presence of secondary fluorescence recovery. However, scenarios also exist that give similar curves in the FLIP-FRAP experiment, but not in the strip-FRAP (Fig. 4F): if D=4 $\mu m^2/s$ the FLIP-FRAP curve are similar to the faster but transiently immobile molecules whereas the strip-FRAP curves are now different (Fig. 4G,H). In addition to the clear advantage of using different FRAP approaches, it also allows one to apply global analysis of the data, a statistical method to enhance measurement accuracy often used in fluorescence methodology.

When binding times are very short, typically less than approximately 5 s, the combined strip-FRAP/FLIP-FRAP method, or any other FRAP-method is not capable to distinguish this scenario from a situation where all molecules are freely mobile (but moving slower). However, several approaches to cope with this problem have been developed. In the investigation of nuclear factors, it can

Fig. 4A–H Comparison of two different variants of FRAP: strip-FRAP and FLIP-FRAP (see also Fig. 3B, C). The combined use of the two protocols may allow to discriminate between transient binding and slow diffusion. The curves are based on computer simulated FRAP experiments (see the text): **A**, **B** schematic drawings of the strip-FRAP (see also Fig. 1C) and FLIP-FRAP methods. The FLIP-FRAP method differs from the strip-FRAP in that two areas are monitored after bleaching. Briefly, a strip at one pole of the nucleus is bleached for a relatively long period at a moderate excitation intensity. Subsequently the fluorescence is monitored in that region (FRAP), but also in the area at the other side of the nucleus (FLIP). Subsequently the difference between the two (normalised) fluorescence levels is plotted against time; **C** schematic drawing of two scenarios where molecules are either free, but relatively slow (D=4 $\mu m^2/s$, top panel), or relatively fast (D=7 $\mu m^2/s$), but transiently immobilised such that 30% is immobile in steady state and individual molecules are immobilised for 45 s (*bottom panel*); **D**, **E** strip-FRAP and FLIP-FRAP curves of the scenarios depicted in C. In this case strip-FRAP can discriminate between the two cases, whereas the FLIP-FRAP curves are nearly identical; **F** schematic drawing of a situation where freely mobile molecules are slower (D=1 $\mu m^2/s$, *top panel*) than in C; **G, H** strip-FRAP curves are identical whereas the FLIP-FRAP method can now discriminate between the two scenarios

be expected that if immobilisation is observed, this is due to binding to DNA or other immobile structures in the nucleus. If the DNA binding domain of the molecule is known, and mutations are known that specifically disable the DNA-binding capacity, one may compare the mobility of mutants that lack the ability to bind to DNA with wild type mobility [22, 36]. If functional mutants are not available, it is possible to inhibit the process in which the protein under surveillance is involved. For instance the mobility of general transcription factors can be compared in untreated and transcription-inhibited cell exposed to agents like DRB or actinomycin D [20, 35]. Finally, a powerful set of experiments may be to study protein mobility at different temperatures [14, 17, 20]. Since diffusion changes linearly with temperature a limited effect is expected when temperature is dropped from 310 K to 300 K (assuming that the effect on viscosity on the cellular interior is also limited, since diffusion is also linearly dependent on the reciprocal of viscosity). However, if the molecules are transiently immobilised in a temperature dependent fashion, e.g. in an enzymatic reactions, their binding time is expected to increase considerably resulting in a notable drop in mobility.

In addition to the above described method to determine overall nucleoplasmic mobility, methods have also been developed to determine the residence times of proteins associated with nuclear accumulations such as nucleoli, nuclear bodies, telomeres, locally damaged nuclei or DNA double-strand break associated foci. These accumulation are frequently studied using the most popular variant of the basic FRAP experiment discussed already above: FLIP (Fig. 3D). The FLIP approach to study accumulations or small compartments, is to bleach an area at a distance of a nuclear accumulation, either repetitively, or with one prolonged bleach pulse at low intensity. If molecules in the accumulation are permanently bound, fluorescence will not go down in the accumulation, since bleached molecules do not exchange with the unbleached in the accumulation. In contrast, fluorescence in the accumulation is expected to decrease if molecules in the accumulation have limited residence times. The velocity at which fluorescence is lost is a measure for the turn-over at the accumulation, the point in time where a new steady state is reached being a good measure for the average residence time in the accumulation. A variant of FLIP for measuring residence times in multiple foci was introduced in the investigation of foci associated with DNA double strand break [26] and of telomeric proteins [27] (Fig. 3E). Briefly, one half of the nucleus is bleached, including the foci present in that half. Subsequently the entire image is monitored at regular time intervals. Image analysis is then used to determine the changes in fluorescence level in each of the bleached and unbleached foci. Another sophisticated method, termed iFRAP (inverse FRAP) was introduced by Misteli and coworkers (Fig. 3F) [25]. It was developed to estimate the rate at which molecules associated with the nucleolus exchange with the surrounding nucleoplasm. The method optimally makes use of the advanced possibilities provided by the operating software of modern confocal microscopes to indicate bleach regions with complicated shape. In iFRAP the entire nuclear volume is bleached with

exception of the investigated accumulation (nucleolus). Immediately after bleaching, the loss of fluorescence in the accumulation fully represents the releasing molecules, whereas in normal FLIP the loss is the result of unbalance of dissociation and association of fluorescent molecules. Therefore determination of the rate of exchange does not require further complicated analytical methods.

4 Pitfalls

In the interpretation and analysis of FRAP experiments there are some unexpected pitfalls that we will discuss below. A first potential difficulty may arise when fluorophore levels are low requiring monitoring at relatively high excitation intensity. Correction for the resulting monitor bleaching may lead to erroneous results when an immobile fraction is present. The second potential pitfall concerns the influence of exchange of bleached and fluorescent molecules with compartments distant from the bleach region. This potential problem specifically may lead to erroneous interpretation of FRAP results when the protein under surveillance accumulate in nuclear subcompartments like the nucleolus, in replication or other foci, or in locally applied DNA damage. Third, the phenomenon of reversible bleaching or blinking may affect FRAP analysis. In the next three paragraphs we will discuss these items.

When the concentration of the tagged protein is low, which is frequently observed when factors are stably expressed at physiological levels, for instance in transfected cells or knock-in mice, fluorescence can only be detected using a laser intensity at which a certain degree of acquisition bleaching will occur. Specifically when the investigator is interested to quantify an observed or expected secondary recovery, this may cause a substantial problem: the secondary recovery may be cancelled out or even exceeded by monitor bleaching during monitoring fluorescence after bleaching (Fig. 5A,B). The obvious method to deal with this problem is to monitor a region similar to the one subjected to FRAP, without applying the bleach pulse, derive the bleach rate and use that to subsequently correct the FRAP curves. Although this works in the case of a freely mobile population of molecules, the correction method is not applicable when a (transiently) immobile fraction is present (Fig. 5C). This is due to the fact that in the control experiment performed to derive bleach rate, the immobile fraction will contribute more to the loss of fluorescence than the mobile one since immobile molecules are constantly in the monitored area, whereas mobile bleached are replaced by mobile fluorescent. This immobile fraction, however, does not contribute to monitor bleaching observed after photobleaching, since it is then largely bleached by the bleach pulse. The result is an overcompensation of the FRAP curve (Fig. 5C). A frequently used alternative correction for acquisition bleaching is to monitor the entire nucleus and normalise the fluorescence intensity in the bleached area to the entire fluorescence level in the nucleus (minus the bleached region). However, when a confocal

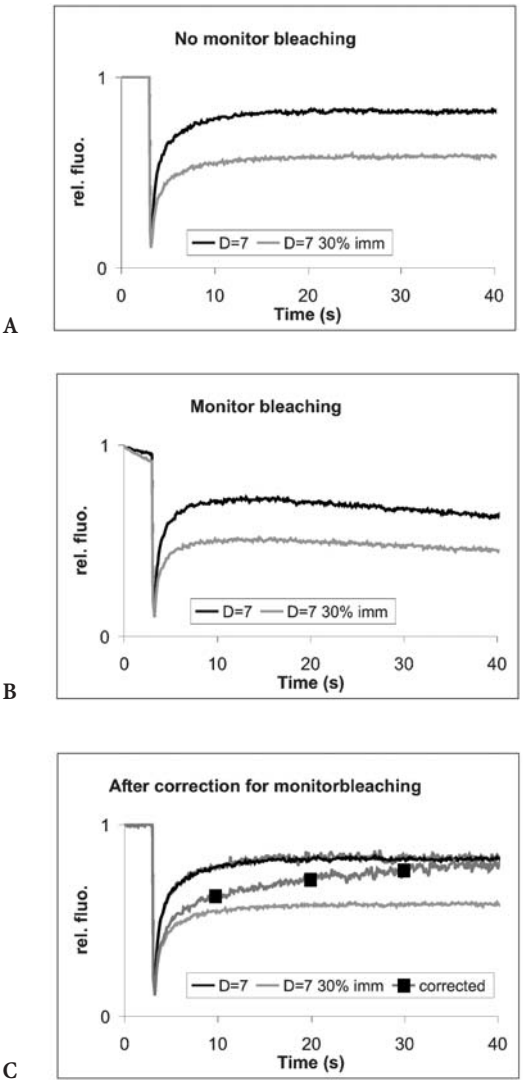


Fig. 5A–C The effect of correction for monitor bleaching in FRAP experiments. The curves are based on computer simulated FRAP experiments (see text): **A** strip-FRAP curves of freely mobile (D=7 $\mu m^2/s$) molecules and the same of which 30% is permanently immobilised; **B** strip-FRAP of the same situations as in A with the difference that now a considerable monitor bleaching is simulated. This may in practice often occur when molecules are investigated at low concentration; **C** to correct for monitor bleaching the experiments were repeated without the bleach pulse. The corrected curve fits well in a situation where the molecules are all freely mobile. However, when an immobile fraction is present, the correction overcompensates the monitor bleaching that actually occurred. This is due to the fact that during the control experiment the immobile fraction bleaches with different kinetics than the free fraction. In the FRAP experiment, after the bleach pulse the immobile fraction is bleached and the signal will have different bleaching characteristics

microscope is used, this method is also hampered by the same phenomenon as in the above example. If an immobile fraction is present, it will contribute in a different way to acquisition bleaching (outside the bleach area) than the mobile fraction which is constantly diffusing to and from the confocal plane. In cases where considerable acquisition bleaching occurs this way of normalising the data will lead to considerable underestimation of the immobile fraction. The longer the experiment is continued, the smaller the fraction will appear, and eventually, he immobile fraction will be fully obscured, when it is completely bleached by the monitoring beam.

A second problem may occur when proteins under surveillance accumulate in one or multiple foci (see above) or when they exchange with neighbouring compartments (shuttling between nucleus and cytoplasm. In many cases, residence times can very well be determined by FLIP, FRAP or iFRAP (Fig. 3). However, a potential problem may arise if the overall nucleoplasmic mobility of the protein is to be determined. Even if the area that is bleached contains no foci, nucleoli, or local damage, a secondary recovery may be observed due to the exchange of bleached and fluorescent molecules with the distant accumulation(s). In the case the focal residence time is in the range of the redistribution time of the protein, this secondary recovery will partly overlap the initial recovery, leading to an apparent slow down of the proteins mobility. A similar effect will take place if the protein shuttles between nucleus and cytoplasm.

A third potential threat to proper FRAP analysis is the 'blinking' behaviour of GFP. It has been shown that many fluorescent proteins rapidly switch between a dark non-fluorescent state and a fluorescent state. The period GFPs are in the dark state (off-time) is not dependent on laser whereas the on-times are [37, 38]. Since the bleach pulse is at a much higher intensity as the monitoring after bleaching, it can be expected that a part of the recovery of fluorescence is due to decrease of the pool of molecules in the off-state. This is not a great problem if all conditions are kept constant, since it will only introduce a constant bias in the results However, comparison between different variants of GFP that have different blinking properties is hampered.

5
Monte Carlo Simulation of FRAP Experiments

From the above it may have become clear that the application of FRAP to constituents of the cell nucleus has dramatically changed the view of the way nuclear processes are organised. This is in spite of the fact that much of the early work, with some exceptions, has been based on semi-quantitative and qualitative interpretation of the FRAP data. It is expected that to push further the limits of FRAP application to processes in the cell nucleus, more sophisticated quantification will be required for future work.

Most of the analytical techniques that have been developed for FRAP quantification are based on mathematical analytical models of diffusion of the

labelled molecules, requiring the solution of known diffusion equations of transport. In these types of analyses, photobleaching is considered as an irreversible process and often simplified into an initial condition for the concentration of chromophores, since the duration of the applied bleach pulse is supposed to be very small compared to the characteristic diffusion time of the studied molecules. Further simplifications are applied to these models by using a reduced set of spatial dimensions or neglecting boundary effects, or by replacing the full point spread function of the focused laser beam with some geometrical approximation. Clearly, these methods capture the essence of FRAP experiments [8, 9, 12, 13], a very thorough 3-D approach being the one developed by Blonk and coworkers [13]. However, when considering experiments in cellular compartments such as the nucleus, some of the approximations may sometimes be oversimplifications, as the system is three-dimensional, finite and bounded, and molecules may be immobile for longer or shorter periods of time. Therefore, it may also be useful to apply another approach to the analysis of FRAP data, i.e. computer modelling of the FRAP procedures and the behaviour of molecules inside small volumes [39]. We have applied in most of our studies a straightforward Monte Carlo method [11, 20, 22], and the examples in this review are also based on this approach. Simulations were performed using experimentally obtained parameters, describing the properties of the microscope lens (beam shape and 3-D intensity distribution, during monitoring and during bleach pulse), the fluorescent label (quantum yield, susceptibility to bleaching, 'blinking' behaviour) and properties of the cell nucleus (size and shape). All these parameters were kept constant in the simulations. Simulations were then performed varying the three protein mobility parameters described above, diffusion coefficient, immobile fraction and duration of binding of individual molecules. Diffusion was simulated by randomly picking a direction to step into with a step size derived from the equation: D=stepsize^2/(6·cycletime), where cycletime in our simulations ranges from 20 to 100 ms. An immobile fraction was established by giving molecules a chance to bind at each cycle, derived from the equation: $P_{\text{bind,1 cycle}}$=(immobile fraction)·(mobile fraction)$^{-1}$·cycletime^{-1}. This software was used to generate the curves presented in this chapter.

6 Discussion

FRAP is a powerful tool for investigating protein behaviour in nuclei of living cells. FRAP experiments are most informative when an inducible system can be used, allowing to compare protein behaviour in their active and inactive state. Alternatively, comparison of the mobility of functional mutants can be compared with wild type mobility. The possibility to estimate immobile fraction and, moreover, the average duration of immobilisation of individual proteins is very useful in studying the dynamics of nuclear proteins, and specifically those that are involved in DNA transacting processes, transcription, repair

and replication. In addition, accumulation in subnuclear regions allows direct visualisation of the exchange (or the absence of it) of proteins with the subnuclear structure. Such accumulations may be either naturally occurring foci or artificial accumulations on for instance stably integrated large promotergene arrays [32] or locally inflicted DNA damage [20, 33]. FRAP probably is less effective when applied to proteins of which the function is not known.

Due to the power of FRAP in the study of nuclear processes, an impressive amount of data has accumulated indicating that many nuclear factors are highly mobile. However, assuming free Brownian diffusion may very likely be an oversimplification of the actual situation. Since many nuclear factors have DNA binding properties and since the nucleus is packed with proteins and DNA it may be expected that the overall mobility of such factors reflects not only (relatively) free movement but also very transient interactions with chromatin or other nuclear structures. In addition, diffusion of factors that do not show interaction may very well be anomalous rather than completely free, due to the crowded nature of the nucleus. The presence of relatively dense chromatin territories may cause diffusing proteins to temporarily get trapped inside such areas giving rise to anomalities in their mobility. However, in many cases FRAP data fit quite well to diffusion models [22] suggesting that at the spatial and temporal resolution of confocal FRAP experiments the effects of anomalous or restricted diffusion may be too weak to be detected by FRAP. Determining the effective diffusion coefficient at which a free fraction moves may then be of less functional relevance than determining their immobilisation characteristics. At least in the case of DNA-metabolising proteins, these immobilisation parameters are likely to represent the timing of the activity of the proteins when they are associated with immobile DNA-protein complexes. Nevertheless, effective diffusion coefficients may, in well controlled experiments, provide an indication of the molecular size of complexes in which a tagged factor resides, although future work is required to provide decisive evidence.

Concluding, FRAP has clearly shown its applicability in nuclear research and is expected to contribute largely to further unravelling and quantifying nuclear processes such as transcription, replication, RNA splicing and DNA repair. In addition, the combined application of FRAP with other quantitative techniques, like FRET (fluorescence resonance energy transfer) and FCS (fluorescence correlation spectroscopy) will be instrumental for future research of the functional organisation of the cell nucleus.

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