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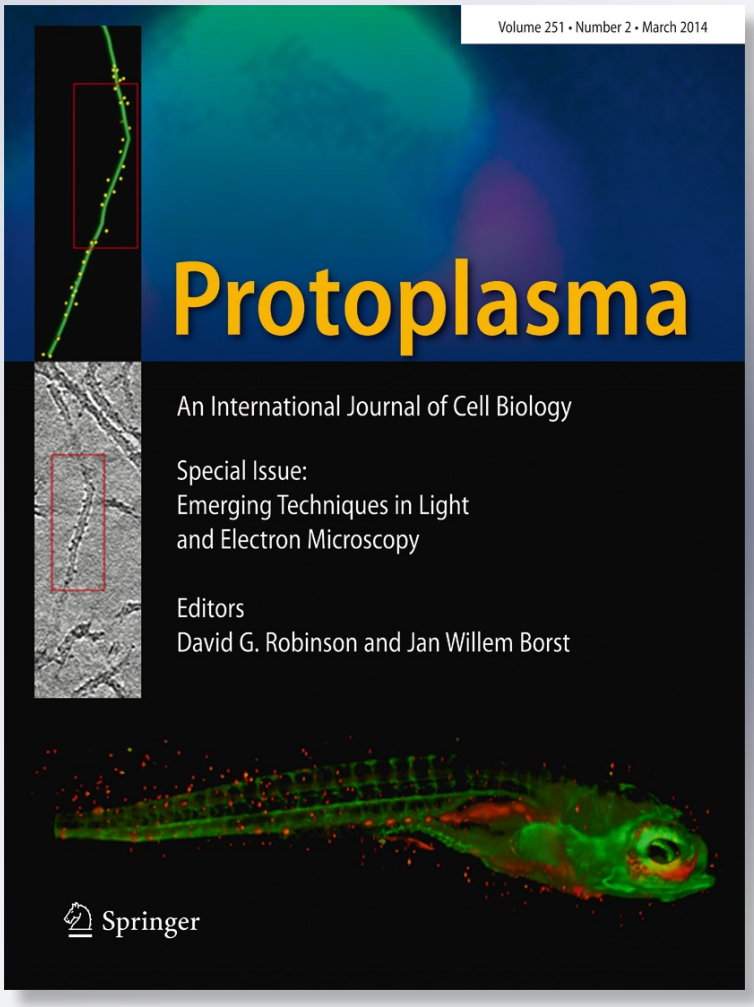

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SPECIAL ISSUE: NEW/EMERGING TECHNIQUES IN BIOLOGICAL MICROSCOPY

Molecular diffusion and binding analyzed with FRAP

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Abstract Intracellular molecular transport and localization are crucial for cells (plant cells as much as mammalian cells) to proliferate and to adapt to diverse environmental conditions. Here, some aspects of the microscopy-based method of fluorescence recovery after photobleaching (FRAP) are introduced. In the course of the last years, this has become a very powerful tool to study dynamic processes in living cells and tissue, and it is expected to experience further increasing demand because quantitative information on biological systems becomes more and more important. This review introduces the FRAP methodology, including some theoretical background, describes challenges and pitfalls, and presents some recent advanced applications.

Keywords Fluorescence recovery after photobleaching · Reaction–diffusion system · Fluorescence microscopy · Fluorescence correlation spectroscopy

Abbreviations

CLSM	Confocal laser scanning microscopy
CP	Continuous fluorescence photobleaching
ER	Endoplasmatic reticulum
FCS	Fluorescence correlation spectroscopy
FM	Fluorescence microphotolysis
FPR	Fluorescence photobleaching recovery
FRAP	Fluorescence recovery/redistribution after photobleaching
GFP	Green fluorescent protein
GR	Glucocorticoid receptor
HP1	Heterochromatin protein 1
mRNP	mRNA–protein complex
MSD	Mean squared displacement
NA	Numerical aperture
PSF	Point-spread function
RICS	Raster image correlation spectroscopy
ROI	Region of interest
SD	Spinning disk
TIRF	Total internal reflection fluorescence
YFP	Yellow fluorescent protein
3PEA	Pixel-wise photobleaching profile evolution analysis

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Introduction

Fluorescence recovery after photobleaching (FRAP; Axelrod et al. 1976; Koppel et al. 1976; Peters et al. 1974) was first introduced in the 1970s and is, today, probably the most widely used method to study transport and diffusion as well as interactions of biological molecules in living specimens. FRAP is also referred to as fluorescence redistribution after photobleaching, fluorescence photobleaching recovery (FPR), or fluorescence microphotolysis (FM) and represents a whole family of related methods all based on photoinduced bleaching (or activation) of marker molecules in selected areas of a cell. This imposes an unbalanced distribution on a large number of fluorescent molecules and allows observation of the relaxation back to equilibrium.

Originally, FRAP experiments could only be performed on custom-designed microscope systems and relied on the sometimes difficult introduction of chemically fluorescently labelled biomolecules into cells. FRAP became more and more popular in the second half of the 1990s because firstly,

commercially available confocal laser scanning microscopes (CLSMs) became very powerful with respect to resolution, speed, sensitivity, and flexibility, eventually enabling photobleaching experiments (Pawley 2006). Secondly, the emergence of the green fluorescent protein (GFP) and an ever-increasing number of other autofluorescent proteins (Miyawaki 2011) often derived from GFP has revolutionized fluorescent labelling of proteins in vivo.

In the following, I will give an overview of the concept and implementation of FRAP experiments, including the photo- and biophysical principles and the challenges of data analysis and how FRAP relates to other similar and complementary methods.

The typical FRAP experiment

In a typical FRAP experiment (Fig. 1a), the steady-state signal from fluorescent molecules in a region of interest (ROI), e.g., of a cell is recorded using low illumination conditions. Subsequently, the fluorescent molecules in the ROI are depleted rapidly by irreversible photo-induced bleaching with high-intensity laser light. Due to diffusion and other transport mechanisms, the bleached molecules exchange with fluorescent ones into the ROI. The replacement of bleached molecules with fluorescent ones at immobile binding sites results in an unbalanced distribution of fluorescent molecules, which gradually relaxes back to equilibrium. The redistribution is subsequently recorded with highly attenuated laser light, revealing a recovery of the fluorescence signal in the ROI (Fig. 1b) and yielding parameters such as the diffusion coefficient, the fraction of fully free, transiently bound fraction and fully immobilized molecules, or the residence time at the binding site responsible for immobilization.

Data analysis

To characterize the underlying diffusion and binding processes, a quantitative analysis is required. Typically, the averaged fluorescence intensities in the bleach ROI and other regions are plotted versus time, allowing extraction of the half time of recovery and an immobile, a slowly mobile/transiently bound, and a fully mobile/non-bleached fraction, as defined in Fig. 1b. However, averaging fluorescence intensities comes along with losing spatial information. Beyond the basic parametrization, spatially resolved analysis of the fluorescence intensity over time (Fig. 1c) can be performed with sets of partial differential equations, which describe coupled reaction-diffusion models and which are solved analytically or numerically. Like this, a more detailed biophysical interpretation is achieved. Depending on diffusion coefficients and reaction rates, most of the FRAP curves can be categorized as pure diffusion, effective diffusion, separated reaction and diffusion kinetics, or the case requiring full coupled reaction–diffusion modelling (Beaudouin et al. 2006; Carrero et al. 2003; Phair et al. 2004; Sprague et al. 2004). It is fundamentally challenging to dissect clearly the contributions of diffusion and binding, and the results depend intricately on experimental conditions, which may lead to misinterpretation and inconsistent data (Braga et al. 2004; Kang et al. 2009; McNally and Sullivan 2008; Mueller et al. 2008; Wachsmuth et al. 2008; Weiss 2004). Other sources of errors are neglecting diffusion during the bleach segment, undersampling data due to insufficient time resolution, or falsely estimating the three-dimensional shape of the bleach ROI.

Experimental setups for FRAP

Today, mostly confocal laser scanning microscopes are used in which a diffraction-limited laser beam is scanned across the sample and toggled appropriately between high and low intensities (Lippincott-Schwartz et al. 2001; Rabut and Ellenberg 2005; Sprague and McNally 2005; van Royen et al. 2009; Wedekind et al. 1994), termed imaging FRAP (Fig. 1d; Braeckmans et al. 2007; Calapez et al. 2002; Carrero et al. 2003; Kang and Kenworthy 2008; Müller et al. 2009; Phair and Misteli 2000). Initially, FRAP experiments were performed with a stationary bleach beam merged with a point or imaging fluorescence detector (Kao and Verkman 1996; Koppel et al. 1976; Lopez et al. 1988; Verkman 2003), referred to as point FRAP (Axelrod et al. 1976; Im et al. 2013; Simon et al. 1988; Soumpasis 1983; Swaminathan et al. 1997). Nowadays, many commercial and academic implementations combine a (scanned) bleach beam with other imaging modalities such as confocal SD or TIRF microscopy (Schneider et al. 2013; Skruzny et al. 2012).

Combining FRAP with fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS; Elson and Magde 1974; Magde et al. 1972, 1974) was also introduced in the 1970s. FCS is a relaxation technique, too. In contrast to FRAP, the focus of a confocal setup like a CLSM is fixed at a position of interest and the steady-state concentration fluctuations of small numbers of fluorescent molecules due to thermally induced Brownian motion are recorded. Applying a temporal correlation analysis of the fluorescence signal allows to determine concentrations and diffusion properties of free molecules and larger complexes (Gosch and Rigler 2005; Kim et al. 2007; Langowski 2008; Rigler and Elson 2001). Typical and in particular commercial FCS setups are based on a confocal laser illumination and fluorescence detection scheme and are often integrated into a CLSM (Pan et al. 2007; Wachsmuth et al. 2003).

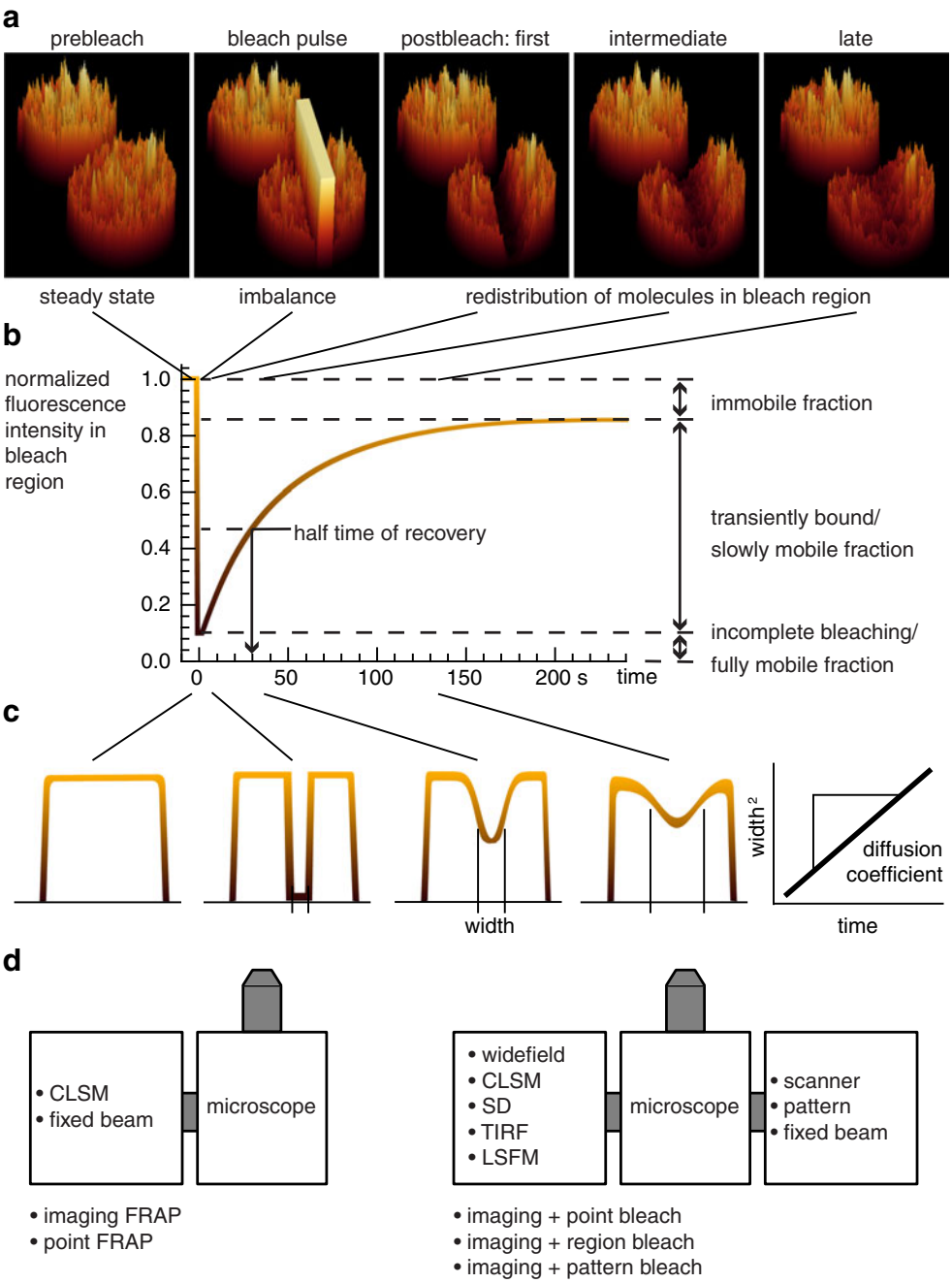

Fig. 1 A typical FRAP experiment. **a** Sequence of prebleach images, bleach pulse, and post-bleach images in height-encoded intensity representation. **b** Average fluorescence intensity obtained from the bleach ROI over time, showing the fundamental parameters to be extracted from the FRAP data. **c** Example for a spatial analysis of FRAP data: the profile of the bleach ROI perpendicular to and averaged along its main axis for different time points. The squared width plotted against the post-bleach time yields the diffusion coefficient. **d** Schematic of different microscope setups and configurations that can be used for FRAP in different implementations

FCS features quite standardized experimental conditions so that a theoretical description renders it possible to determine quantitatively the diffusion coefficients and absolute concentrations and to distinguish different modes of diffusion (Schwille et al. 1999; Wachsmuth et al. 2000) by simply fitting analytical model functions to experimental data. As opposed to FRAP, FCS is insensitive to immobilized molecules. The correlation time from an FCS experiment typically characterizes diffusion whereas a major readout of a FRAP experiment, the half-time of recovery, is often determined by convoluted diffusion and binding, making FCS and FRAP nicely complementary methods.

Fundamentals

Photobleaching

Photobleaching is the photo-induced irreversible transition of a fluorophore into a non-fluorescent chemical conformation. The rate of photobleaching is usually assumed to be proportional to the illumination intensity (Axelrod et al. 1976; Lanni and Ware 1981). However, many experimental observations indicate a deviation from linearity (Harms et al. 2001; Song et al. 1996), which can be explained based on a Jablonski diagram (Lakowicz 1999), see Fig. 2a: Upon continuous

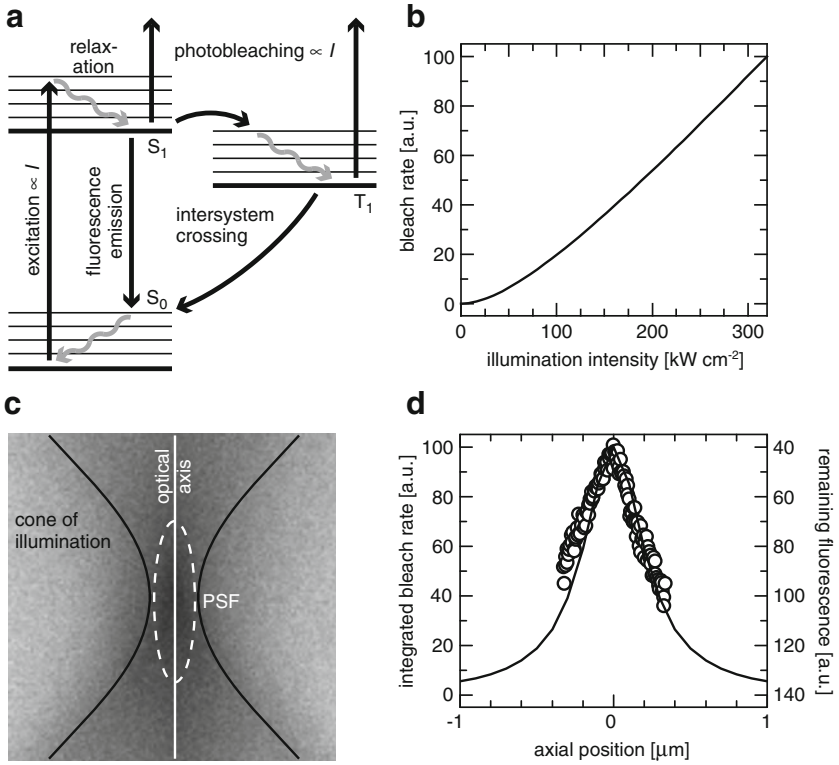

Fig. 2 Photobleaching. **a** Jablonski diagram of electronic states of a typical fluorophore, showing the transitions between states involved in laser excitation, fluorescence emission, triplet state occupation, and photobleaching. **b** Nonlinear excitation intensity dependence of the bleach rate based on (**a**). **c** 2D fluorescence image along and perpendicular to the optical axis of an objective lens of a spot bleached into an immobile fluorescent layer, highlighting the point-spread function (PSF) and the cone of illumination. **d** Laterally averaging the remaining fluorescence in (**c**) in the vicinity of the optical axis yields an axial intensity profile (*circles*), which agrees well with the laterally integrated bleach rate from (**b**; see also Im et al. (2013) for details)

illumination, molecules are raised from singlet ground state S_0 to the excited singlet state S_1 and, after a certain delay, the fluorescence lifetime, decay back upon emission of a photon. Thus, the steady-state occupation of S_1 and of an excited triplet state T_1 depends on the illumination intensity I (Im et al. 2013). Then, assuming that the photobleaching process results from the further photo-induced excitation of fluorophores already in S_1 or T_1, respectively (Song et al. 1995), the photobleaching rate depends more than linearly on the illumination intensity, as shown in Fig. 2b for a fluorophore with fluorescein-like properties (Im et al. 2013). Therefore, using a CLSM for photobleaching a single spot into a fluorescent layer (Fig. 2c) creates a 3D profile whose laterally integrated axial profile agrees quantitatively with the predictions assuming non-linear photobleaching processes (Fig. 2d).

This is beneficial for FRAP experiments: Firstly, increasing the laser power for the bleach pulse of a FRAP experiment is more useful than increasing the length of the bleach pulse, which is also desirable to reduce the effect of diffusion during the bleach pulse. Secondly, single-photon excitation generally results in significant unwanted out-of-focus bleaching of the sample. The non-linear intensity dependence of the photobleaching rate results in a desirable axial confinement of the bleach region even for single photon fluorescence excitation (Braeckmans et al. 2007; van Royen et al. 2009), not only for two-photon excitation (Brown et al. 1999; Mazza et al. 2008).

Diffusion and binding

The thermally induced stochastic movement, e.g., of fluorescently labelled molecules in a solution or inside a cell is called diffusion or Brownian motion. This concept can be generalized to all thermally driven stochastic processes such as fluctuations in molecular structures. On the macroscopic scale of a whole cell where large numbers of molecules are involved, diffusion aims at equilibrating macroscopic concentration gradients, like those generated by depleting the fluorescent molecules from a certain area of a cell—the very process observed in a FRAP experiment. The diffusion coefficient characterizes the relation between the gradient and the resulting flux. From the point of view of single molecules, which randomly roam in their environment, concentration fluctuations are generated when they enter and leave a small observation volume—the very process observed in an FCS experiment. The area covered by roaming or the mean squared displacement (MSD), respectively, increases linearly in time, thus, the corresponding slope of a plot of MSD against time yields the diffusion coefficient, often given in micrometers per second.

The diffusive behavior of fluorescently labeled biomolecules can change as a result of different processes: Firstly, in complex with intracellular compartments, the MSD often shows a less than linear time dependence owing to collisions to and non-specific binding with immobile structures (Saxton and Jacobson 1997; Schwille et al. 2000; Wachsmuth et al. 2000). Secondly, if molecules bind to larger yet mobile

complexes, the movement is slowed down, showing a reduced actual diffusion coefficient. Thirdly, specific binding to immobile cellular structures also leads to a reduced mobility. Here, it is useful to distinguish three cases: If the binding reaction is diffusion-limited, a certain fraction of binding sites is not occupied at any given time, and if a molecule is released from a binding site, it is rapidly recaptured by another one, and the molecules appear as one fraction with a reduced effective diffusion coefficient. In the presence of a detectable fraction of free molecules, it depends on the ratio of association/dissociation rates and diffusional "escape rate" from an observation area whether both a free and a bound fraction can be identified or whether a coupled description of reaction and diffusion is required to describe that behavior (Carrero et al. 2003; Müller et al. 2009; Phair et al. 2004; Sprague and McNally 2005; Sprague et al. 2004; Stasevich et al. 2010a, b). Here, experimental settings such as the spatial and temporal sampling or the size and geometry of the bleach ROI in a FRAP experiment play a crucial role so that this is described in more detail below.

Imaging FRAP data analysis

For the data analysis of an imaging FRAP experiment, several correction and normalization steps must or should be applied independent of the underlying molecular process resulting in redistribution and recovery. After that, the spatio-temporal fluorescence distribution can be analyzed analytically or numerically to extract binding, diffusion, and other transport parameters.

Correction and normalization of intensity values

It is useful to define not only the bleach ROI ("bl" in Fig. 3a), but also a ROI, which allows extraction of the background signal ("bg" in Fig. 3a), a ROI covering the overall (total) fluorescence intensity, e.g., of the bleached cell that is available for the recovery ("tot" in Fig. 3a) and/or a reference ROI comprising a non-bleached and topologically separated area, e.g., a neighboring cell ("ref" in Fig. 3a). Subsequently, for every time step, the "bg"-averaged background signal is subtracted from each pixel of the other ROIs (Fig. 3c, d). Next, for every time step again, the pixel values of the bleach ROI are divided by the "ref"- or "tot"-averaged pixel values to account for slow fluctuations such as unwanted yet unavoidable photobleaching during the post-bleach sequence (scan bleaching) (Fig. 3e). Finally, the resulting values are normalized to the prebleach values (Fig. 3f) in order to have full recovery correspond to unity. These steps can be performed either for each pixel or for the average of the "bl" ROI. The resulting time traces are subject to further analysis.

Extraction of diffusion and reaction parameters

The simplest model for binding of diffusive molecules to immobile structures is determined by the diffusion coefficient D of free molecules and their association rate k_{on} to and the dissociation rate k_{off} from binding sites (Fig. 3g). As mentioned above, in a FRAP experiment, different cases must be distinguished (Beaudouin et al. 2006; Carrero et al. 2003; Phair et al. 2004; Sprague et al. 2004): If the diffusional escape rate D/A, which depends crucially on the experiment because A is the area of the bleach ROI, is significantly bigger than the binding rates (k_{on}, k_{on}), the diffusional recovery is much faster than the binding-related one (Fig. 3h), and they can easily be distinguished and evaluated independently. The binding-related contribution is an exponential recovery whereas the description of the diffusional one depends crucially on the geometry of the bleach ROI (Im et al. 2013). On the other hand (Fig. 3j), if the diffusional rate is much smaller than the binding rates, one observes an effectively diffusional behavior with a reduced apparent diffusion coefficient (Wachsmuth et al. 2003). In the intermediate regime (Fig. 3i), molecules are captured by a binding site where they dwell for some time until getting released again, resulting in repeated hopping between free and bound state before they leave the observation volume. Thus, a complete coupled reaction–diffusion scheme must be employed to fit the data by solving the coupled differential equations analytically (and mostly approximately), using appropriate coordinate transforms or numerically. Alternatively, molecular dynamics simulations allow to model more complex modes of binding and transport such as broad distributions of affinities, inhomogeneous distributions of binding sites or facilitated/1D and obstructed diffusion, e.g., of transcription factors along and within chromatin (van Royen et al. 2011).

Obviously, averaging intensities over the bleach ROI improves the signal-to-noise ratio, however, at the expense of spatial resolution. Since it is always difficult to dissect diffusion and binding and even more challenging to decide whether the above-mentioned simple binding model or a more intricate one is required to describe the data, a refined spatial sampling (as indicated by dashed lines in Fig. 3h, ranging from a few partitions of the ROI to individual pixels) combined with numerically solving the differential equations may help (Beaudouin et al. 2006; Im et al. 2013; Soumpasis 1983). Often, simple geometries such as squares, rectangular strips, or circles are chosen that allow to extract averaged intensity profiles (see above) as a compromise between averaging over ROIs and spatially resolved analysis (Braeckmans et al. 2003; Carrero et al. 2003; Müller et al. 2009; Stasevich et al. 2010a; van Royen et al. 2011). As mentioned above, not only the lateral geometry, but also the axial profile of the bleach ROI, which depends on the NA of the objective lens and on the process of bleaching itself, must be taken into consideration

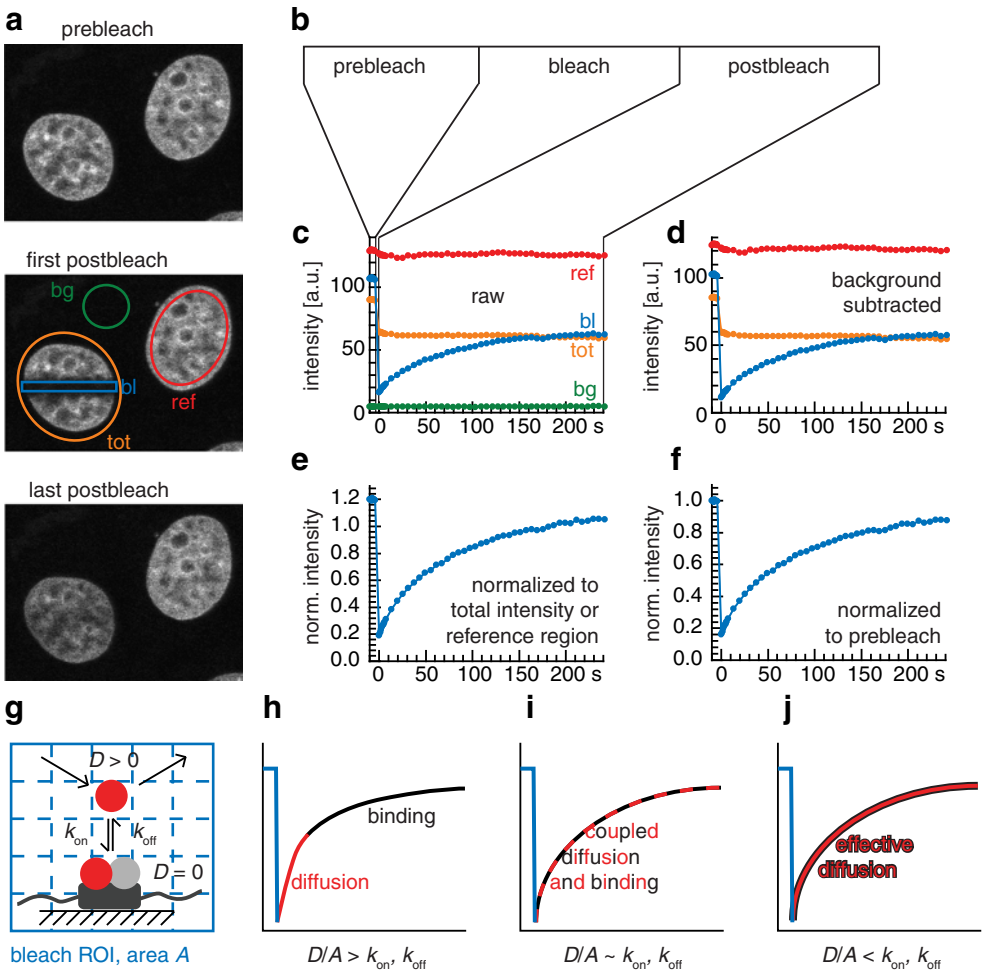

Fig. 3 Evaluating a FRAP experiment. **a** Prebleach and post-bleach images of a FRAP experiment of a nuclear protein with the bleach ROI ("bl", *blue*), a ROI for background signal ("bg", *green*), a ROI for the total signal from the bleached cell ("tot", *yellow*), and a ROI for signal from a reference cell ("ref", *red*). **b** Typical relative durations of prebleach, bleach pulse, and post-bleach sequence. **c** The averaged fluorescence intensities from the ROIs shown in (**b**) versus time; time zero corresponds to the first post-bleach image. **d** Intensities "bl," "ref," and "tot" from (**c**) after background correction. **e** Intensity "bl" from (**d**) normalized to "tot" or "ref" from (**d**). **f** Intensity "bl" from (**e**) normalized to the prebleach intensity. **g** Bleach ROI of area *A* and simple model of diffusive molecules that associate to and dissociate from immobile binding sites. Finer spatial sampling (*dashed blue lines*) can help to better assess and distinguish diffusional and binding contribution. Depending on the ratio of diffusion coefficient *D*, area *A*, and the reaction rates k_{on} and k_{off}, binding events occur at different spatial and temporal densities, such that different cases must be distinguished: **h** fast diffusion when compared with binding, such that the recovery curve is biphasic with a diffusive and a binding-related contribution. **i** Binding and diffusive hopping events are sufficiently intermingled, such that the recovery curve is best described by a coupled reaction-diffusion scheme. **j** Binding is very short-lived whereby it just slows down effectively the diffusion process, which determines the recovery

carefully (Braeckmans et al. 2007; Mazza et al. 2008; van Royen et al. 2009).

Recent methodological developments

Consideration of diffusion during bleaching

A considerable source of error may be diffusion during bleaching, which is usually not taken into consideration. Especially in the presence of a fast diffusive fraction of molecules and if a CLSM is used for measuring FRAP, the bleach segment is often long enough to allow a significant amount of molecules to diffuse across the bleach ROI at the same time. This results in mis-estimation of diffusive, transiently, and permanently immobilized fractions and wrong assumptions for the post-bleach concentration distribution (Weiss 2004). Recently, both for imaging and for point FRAP, this has been taken into account explicitly (Im et al. 2013; Kang et al. 2009, 2010), which has made the analysis much more consistent, also with FCS data.

Cross-validation and combination with FCS

A crucial step of FRAP data analysis is a proper separation of diffusion and binding contributions. However, while FRAP is

very well suited to study slower processes like association to and dissociation from immobile binding sites, the often much faster diffusion is difficult to assess. On the other hand, FCS is conceptually "blind" for immobilized or slowly binding molecules. Therefore, a combination of FRAP and FCS is most promising to extract more reliably both diffusion and binding properties, as implied by the conceptual proximity and suggested already 30 years ago (Icenogle and Elson 1983a, b; Petersen and Elson 1986).

More recently, this was applied in different ways for monitoring protein dynamics in living cells: Both methods were used separately to study the binding properties of

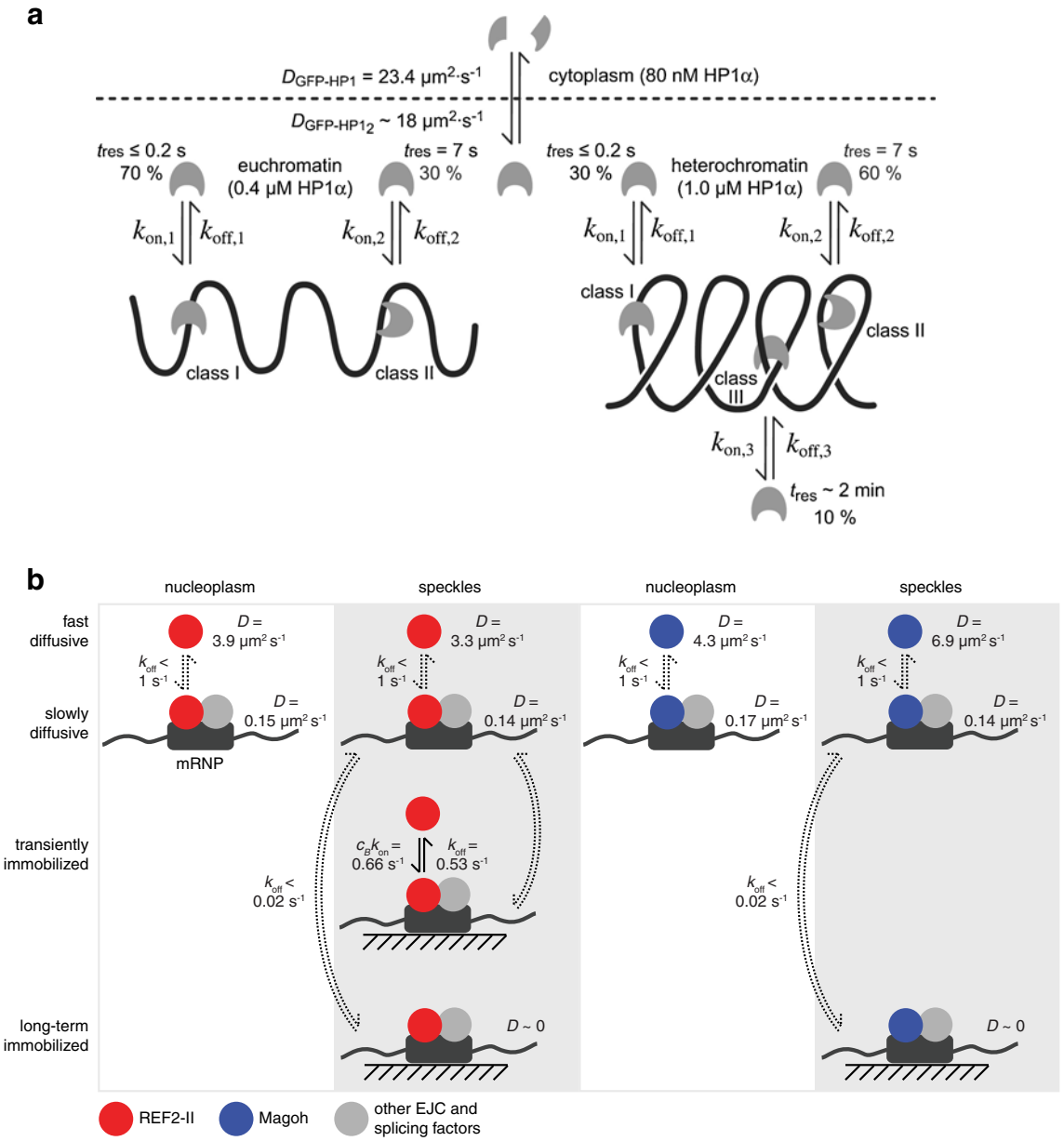

Fig. 4 Kinetic models extracted from combined FRAP and FCS studies. **a** Kinetic model of HP1 mobility and interactions in the cell. A highly diffusive, non-interacting species was found in the cytoplasm. Different fractions of HP1 molecules were detected within the nucleus. A highly mobile fraction diffuses throughout the whole nucleus, showing unspecific binding interactions (class I); some HP1 molecules bind transiently but specifically to euchromatin or heterochromatin (class II); and a third fraction is stably incorporated into chromatin, probably via interaction with various binding partners (class III). Values for diffusion coefficients and residence times are given for HP1a. Taken from Müller et al. (2009) with permission. **b** Model for the nuclear mobility of REF2-II and Magoh. Apparent diffusion coefficients were calculated and averaged from FRAP and FCS data (REF2-II) or taken from FRAP data (Magoh). The reaction rates of transient binding of REF2-II to immobile sites in speckles were determined from FRAP. The exchange between the fast and the slowly diffusive fraction was not observed in FCS and FRAP, so that an upper limit for the rate was estimated (*dashed straight reaction arrows*). The exchange between the diffusive and the immobile pool of the mRNP complex was not observed in FRAP, so an upper limit from the duration of the post-bleach segments could be estimated (*dashed curved reaction arrows*). Taken from Im et al. (2013) with permission

heterochromatin protein 1 (HP1) to chromatin (Müller et al. 2009). Imaging FRAP in combination with analytical and numerical fitting of the data, continuous fluorescence photobleaching (CP; another photobleaching method to extract especially reaction rates; Arkhipov et al. 2007; Delon et al. 2006; Im et al. 2013; Wachsmuth et al. 2003), and FCS allow the identification of different classes of HP1 binding sites on chromatin and quantifying of diffusion coefficients and reaction rates in a spatially differentiated way, i.e., by distinguishing transcriptionally more active and more open euchromatin from transcriptionally less active and more condensed heterochromatin (Fig. 4a; Müller et al. 2009).

In another systematic study on the combination of FRAP and FCS (Stasevich et al. 2010b), the authors compared very carefully the different regimes for binding rates and diffusion coefficients that can be assessed with either of the methods. By studying the chromatin binding properties of the glucocorticoid receptor (GR), a transcription factor, only the combination helped to estimate accuracies and to rule out respective systematic errors.

An even more explicit combination of FRAP and FCS even on the instrument level was carried out recently (Im et al. 2013): A CLSM equipped for FCS was modified for allowing point FRAP resulting in simultaneous FRAP and FCS measurements. Like that, point FRAP benefits from the microsecond time resolution of FCS equipment, making diffusion measurements even of free yellow fluorescent protein (YFP) more accurate. This approach was then applied to characterize the mobility of the exon–exon junction complex proteins REF2-II and Magoh, suggesting that they bind to a maturating mRNA protein complex (mRNP) in a protein-specific and spatially differential way (Fig. 4b; Im et al. 2013).

Combination with other CLSM-based approaches

The time resolution of CLSM-based imaging FRAP is inherently limited by the scanning velocity and frame rate of the microscope such that diffusion (see above) and very short-lived interactions with immobilized binding sites are difficult or impossible to resolve. However, the raster-scanning process of image formation of the CLSM provides a well-defined spatio-temporal relationship of pixel intensity values, which was used in a recent study (Erdel and Rippe 2012). In this study, the redistribution after photobleaching of a circular area of ISWI chromatin remodelers was analyzed. Pixel-wise photobleaching profile evolution analysis (3PEA) relates to FRAP in the same way as raster image correlation spectroscopy (RICS; Digman et al. 2005) does to FCS: This allows extraction of both the free diffusion coefficient and the diverse dissociation rates of Snf2H and Snf2L from chromatin with very high accuracy.

Automated high-content microscopy was developed to study the impact of large numbers of proteins or genes, e.g., on cellular pathways. In addition, it is useful to overcome the sometimes-anecdotal character of experimental observations by simply scaling up the number of replicates. A FRAP experiment is a complex sequence of observations and decisions, such as finding an appropriate bleach ROI in a preview image and configuring and starting the FRAP imaging. However, these steps could also be delegated to a computer such that imaging FRAP experiments could be carried out in an automated way (Conrad et al. 2011), revealing the cell-cycle dependence of HP1 chromatin binding.

Conclusion

In summary, FRAP is a powerful fluorescence-microscopy-based method that assesses quantitatively the transport, diffusion, and binding processes in living cells and tissue. An ever-increasing number of applications range from a comparison of more basic and descriptive parameters, e.g., to describe the response to a certain treatment, to a full biophysical characterization of intracellular pathways towards systems biology. En route from its invention in the 1970s, limitations and pitfalls became obvious. Therefore, nowadays, especially the combination with other advanced fluorescence microscopy and computational methods, the commercial availability and the accessibility of equipment, e.g., in imaging facilities, it has come of age and it is straightforward to use for a variety of cell biology applications.

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Conflict of interests The author declares that he has no conflict of interest.

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