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FRAP analysis of binding: proper and fitting

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Dynamic molecular interactions are fundamental to all cellular processes. *In vivo* analyses of these interactions are frequently done using fluorescence recovery after photobleaching (FRAP). Proper interpretation of FRAP data yields information about the binding interactions of fluorescently tagged molecules, including the number of binding states and the binding strength of each state. This binding information can be gleaned from appropriate models of the process underlying a FRAP recovery. Continued application and development of these approaches promise to provide crucial information for a quantitative description of the molecular networks that regulate cellular function.

Introduction

A central challenge to cell biologists in the postgenomic era is to understand the extensive networks of protein–protein interactions that regulate cellular processes. Predicting the cellular responses that emerge from such networks requires identifying and ultimately quantifying the underlying interactions. Traditionally, binding interactions between cellular molecules have been measured by an assortment of *in vitro* techniques; however, live cell assays of binding are now possible owing to advances in protein labeling and fluorescence microscopy. Two relatively classic techniques, fluorescence recovery after photobleaching (FRAP) [1,2] and fluorescence correlation spectroscopy (FCS) [3,4], are experiencing a resurgence in interest, but as *in vivo* tools with which to probe cellular binding interactions. This renaissance is occurring because commercial microscopes and software are available for both techniques, and green fluorescence protein (GFP) fusion technology has made it possible to apply these methods readily to a range of biologically important proteins.

In FRAP, fluorescent molecules are permanently bleached at a region of interest in a specimen (Box 1). The rate of fluorescence recovery provides a measure of how quickly fluorescent molecules move into the bleached region. This 'mobility' is determined, in part, by the rates of diffusion and transport for the fluorescent molecule through the cellular milieu. Mobility is also influenced by binding interactions, which detain molecules that would otherwise diffuse freely.

In FCS, a diffraction-limited spot is illuminated in a specimen and the fluorescence intensity in the spot is measured (Box 2). The fluctuations in this intensity contain information on the mobility of molecules in the spot, essentially because the more mobile the molecule, the more frequent and short-lived are the fluctuations in the number of molecules in the spot. These effects

Box 1. Fluorescence recovery after photobleaching

FRAP was developed in the 1970s as a technique to study protein mobility in living cells by measuring the rate of fluorescence recovery at a bleached site [38,39] (Figure I). Several papers have discussed the technical considerations involved in a FRAP experiment [18,32,40,41]. The FRAP technique originally found success as a method to measure diffusion in cellular membranes [42,43]; however, the recent advent and availability of both fluorescent protein technology and confocal microscopy have led to a marked increase in the use of FRAP for studying protein mobility in the cell interior. The scope of these studies has expanded not only to address diffusion rates, but also to assess protein dynamics and interactions with other cellular components [9,33,41,44–46]. FRAP has now been adopted as a common technique for studying almost all aspects of cell biology, including chromatin structure [47], transcription [27], mRNA mobility [48], protein recycling [49], signal transduction [50], cytoskeletal dynamics [51], vesicle transport [52], cell adhesion [53] and mitosis [54].

Commonly, FRAP results are analyzed qualitatively to determine whether protein mobility is rapid or slow, whether binding interactions are present, whether an immobile fraction exists, or how a particular treatment (such as ATP depletion or a mutation in the protein of interest) affects these properties. Several mathematical models have been also developed to understand better the underlying processes, to ensure the accuracy of a qualitative interpretation, and to extract quantitative parameters from a FRAP curve.

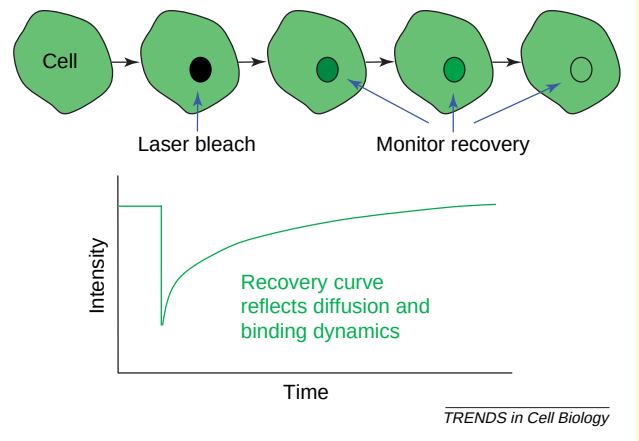

Figure I. Schematic illustrating the FRAP technique.

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Box 2. Fluorescence correlation spectroscopy

FCS was created by Elson, Magde and Webb [55,56] in the early 1970s as a technique to measure reaction kinetics and diffusion coefficients by measuring the fluctuations in fluorescence intensity in a small volume (Figure I). FCS was used initially for *in vitro* experiments, but advances in fluorescence signal detection have enabled it to become increasingly useful for measurements in living cells. More-sensitive photon detectors have enabled smaller illumination volumes, leading to larger fluctuations and a shorter time required for observation.

Because FCS requires many measurements to have proper statistical power, it is most commonly used to study extremely fast processes such as diffusion [3]; however, FCS can be also used to study the kinetics of chemical reaction systems and, in fact, the underlying equations describing mobility in the presence of binding are the same in FCS and FRAP [57].

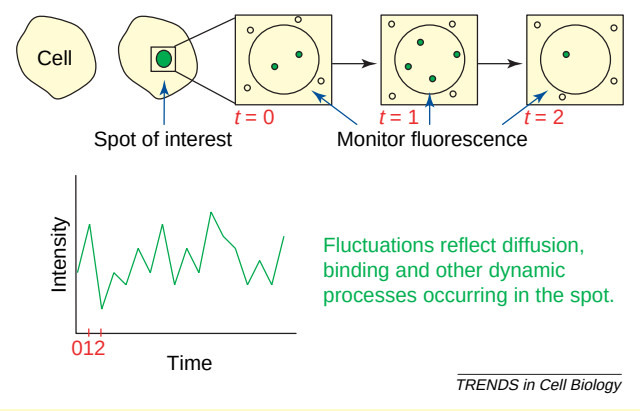

Figure I. Schematic illustrating the FCS technique.

can be quantified to obtain information from FCS data about various processes, including the rate of diffusion and the strength of binding interactions in the illuminated spot [3,5,6].

At present, FRAP is used much more widely than FCS [7–10], mainly because it can be accomplished on any standard confocal microscope, whereas FCS requires special instrumentation. The two approaches are, however, complementary [11,12]. FCS is well suited to fast processes on a scale of microseconds, whereas FRAP is better suited to slower processes on a scale of seconds. Continued progress in the field will come from the application and development of both of these techniques.

In this review, we focus on FRAP as a method for the *in vivo* measurement of cellular binding interactions. Our goal here is to provide an intuitive understanding of what happens in a FRAP recovery when binding interactions are present and, in the process, to offer guidelines that will help in selecting and evaluating a proper approach to obtain information about the binding interactions.

FRAP in the presence of diffusion and binding

When binding interactions are present, they retard a FRAP recovery in relation to what would be observed if only diffusion occurred. To assess whether binding interactions are involved in a FRAP recovery requires a comparison between the FRAP of an inert, non-binding molecule and that of a GFP fusion of the protein of interest. When the FRAP recovery of the fusion protein is slower than that of its non-binding counterpart, binding is implicated and the degree of slowdown in the FRAP recovery is a measure of the strength of binding. Typically, this binding has been evaluated by comparing the FRAP recovery of the fusion protein to that of unconjugated GFP. Under typical FRAP conditions, GFP alone recovers in less than a second, whereas some GFP fusion proteins require many seconds or longer for full recovery. Such large differences in recovery time are not due to the increased mass of the fusion protein as compared with GFP, because both the diffusion constant (see Glossary) and the recovery time for FRAP change extremely slowly with mass (Table 1).

Although a significantly slower recovery time suggests that binding is retarding the FRAP recovery of the fusion protein, there is an important caveat. A better comparison would be made by using molecules of the same size, because fusion proteins (being larger in diameter than unconjugated GFP) can be more sensitive to 'molecular sieving' effects. These size-dependent effects arise from cellular structures, such as a microtubule network, that temporarily trap the random movements of a freely

Glossary

Association rate: the product of the on rate and the concentration of available binding sites when the fusion protein is at equilibrium (also known as the 'effective first order association rate constant' [20] or the 'pseudo on rate' [19]). The inverse of the association rate is the time required for a free molecule to become bound.

Diffusion constant: a parameter that characterizes the rate at which a molecule diffuses in a specific environment. A cellular diffusion constant can be determined by using FRAP to measure the mobility of an inert, non-binding molecule in the cellular compartment under study.

Diffusion-coupled FRAP: a FRAP recovery in which diffusion across the bleach spot is slower or similar to the time required for a free molecule to bind. After the bleach, the diffusing molecules do not rapidly re-equilibrate throughout the bleach spot, but instead are quickly bound, then released for more diffusion, then quickly bound again, and so on. The resultant FRAP recovery is governed by diffusion and binding throughout the recovery period.

Diffusion time: the time required for the fusion protein to diffuse across the bleach spot in the absence of any binding sites. This is roughly proportional to the area of the bleach spot divided by the diffusion constant in that cellular compartment.

Diffusion-uncoupled FRAP: a FRAP recovery in which diffusion across the bleach spot is much faster than the time required for a free molecule to bind. The diffusing molecules rapidly re-equilibrate after the bleach, leaving binding interactions as the determining factor governing the remainder of the FRAP recovery.

Effective diffusion: a simplified form of diffusion-coupled FRAP in which the recovery exactly mimics diffusion, but at a rate that is retarded by binding interactions. Our use of this term is based on what we consider to be its original definition [29]. Note, however, that some FRAP studies have used 'effective diffusion' as a general term to describe diffusion in the cellular milieu. Thus, it is important to determine exactly what is meant when this term is used.

Equilibrium: a state in which the fusion protein has been expressed long enough to reach a constant concentration because production is matched by degradation and steady-state levels of free and bound protein have been established.

Homogeneous distribution: the average concentration of fluorescently tagged molecules and their binding sites remains constant over a region that is much larger than the bleach spot, yielding a fluorescent distribution that is relatively uniform. For example, a homogeneous distribution might arise in the case of a nuclear protein that binds generically to chromatin. By contrast, a non-homogeneous distribution would arise, for example, with a centromere-associated protein that accumulates specifically at these chromosomal sites.

Off rate: the rate of the reverse unbinding reaction in which a protein is released from its binding site.

On rate: the rate of the forward binding reaction in which a protein binds to a binding site to form a bound complex.

Recovery time: the length of time after bleaching required for the fluorescence intensity to reach a constant value.

Table 1. FRAP recovery rate due to diffusion is weakly dependent on protein mass

Mass (kDa)	Half-time for FRAP recovery (s)[a]	
	Cytoplasmic or nuclear	Transmembrane
27	0.011	0.020
100	0.017	0.023
500	0.030	0.028
1000	0.037	0.031
100 000	0.173	0.096

[a]The recovery half-time is the time required for the fluorescence intensity to reach 50% of its pre-bleach intensity. Half-times here are calculated on the basis of a circular bleach spot with a radius of 1.0 μm by using a model for simple diffusion [36] and standard formulas relating the diffusion constant and mass for either transmembrane [37] or cytoplasmic and nuclear proteins [9]. GFP (27 kDa) diffusion constants were estimated to be 20 $\mu m^2/s$ for cytoplasmic or nuclear proteins, and 11 $\mu m^2/s$ for transmembrane proteins.

diffusing molecule, leading to a reduction in mobility that is not caused by binding interactions. Such sieving effects are well established in membranes [13,14], but there is still debate about whether, and at what size limit, they become significant in the cytoplasm or nucleus [15–17]. Although GFP remains the standard marker of mobility in the absence of binding, the development and use of 'size-matched' markers would facilitate the definitive identification of binding effects.

When diffusion has been properly characterized and binding interactions are deemed to be present, the next step in FRAP analysis is to ascertain the respective contributions of diffusion and binding to the recovery curve. Sometimes diffusion is so fast relative to binding that it can be ignored, but at other times it cannot [18,19]. These 'diffusion-uncoupled FRAP' and 'diffusion-coupled FRAP' regimes yield distinct behaviors that require completely different qualitative and quantitative interpretations of the FRAP curve.

Whether diffusion can be ignored depends on the relative magnitude of two parameters: the diffusion time and the association rate. The diffusion time is the time required for the molecule, in the absence of any binding, to diffuse across the bleach spot. The association rate is determined by the on rate of binding multiplied by the concentration of available binding sites at equilibrium. Its inverse is a measure of the time required to begin binding. The relative magnitude of these two parameters therefore reflects the potential interplay between diffusion and binding, and thus determines whether a FRAP recovery is diffusion coupled or uncoupled.

Below we first describe the features of these two behaviors and then provide guidelines for determining which type of model is appropriate for a given FRAP experiment.

Diffusion-uncoupled FRAP recoveries

Qualitative features of diffusion-uncoupled recovery curves

In a diffusion-uncoupled FRAP recovery (Figure 1a), the diffusion time is much shorter than the time to begin binding. Consequently, recovery due to diffusion occurs first and recovery due to binding follows later, resulting in a FRAP curve that can be separated into two phases. The initial diffusive phase, in which the fluorescence due to free molecules recovers completely, lasts less than 1 s in most biological FRAP experiments because diffusion by itself is rapid. After this rapid diffusive phase, exchange at binding sites occurs over a slower period of seconds to minutes (or longer), leading to the recovery of fluorescence due to bound molecules.

Thus, in the diffusion-uncoupled mode, the FRAP curve contains an extremely short initial diffusive phase, followed by a much longer binding phase. Consequently, almost all of the FRAP curve reflects the binding interactions, and much can be gleaned about binding by a simple inspection of the shape of the recovery curve. The rate of recovery reflects the strength of binding, whereby slower recoveries correspond to tighter binding. If the curve has a 'shoulder', then it probably reflects the presence of two different binding states with distinctive binding affinities. For example, 'fast' and 'slow' components correspond to 'weak' and 'tight' binding states, respectively. The fraction of molecules in each of these two states is determined by the location of the shoulder, which partitions the curve into two segments, the relative heights of which are proportional to the fraction of molecules that occupy each binding state. As an example, fast and slow components have been found in FRAP recoveries for several DNA-binding proteins, leading to the suggestion that there are two binding states: non-specific and specific DNA binding [20].

Quantitative interpretation of diffusion-uncoupled recovery curves

These qualitative interpretations can be made more quantitative by fitting a mathematical model to the FRAP recovery. At present, several options of varying complexity are available for diffusion-uncoupled FRAP. In the simplest case of a single binding state, the predicted FRAP recovery curve is the inverse of an exponential decay, namely $1-Ae^{-k_{off}t}$ [19,21,22]. By fitting this equation to the FRAP data, a direct estimate can be obtained for both the off rate of binding (k_{off}) and the parameter A, which can be used to calculate the association rate [19]. Fits with one exponential term have been reported in the FRAP literature [21–23], although it has not always been appreciated that both the off rate and the association rate can be extracted from these fits.

The next simplest case for curve fitting occurs when two independent binding states are present. For example, the same GFP fusion protein might bind to microfilaments and microtubules. In such examples, the predicted FRAP recovery curve contains two exponential terms from which an association rate and an off rate for each binding state can be determined [19]. Fits with two exponential terms have been also reported in the literature [24,25], but an extra degree of caution is required here because a sum of exponentials can inappropriately fit many diffusion-coupled FRAP recoveries, leading to erroneous conclusions about the number and strength of binding interactions.

A more complicated case for curve fitting arises when multiple, dependent binding states are present. This situation can occur, for example, when the first binding

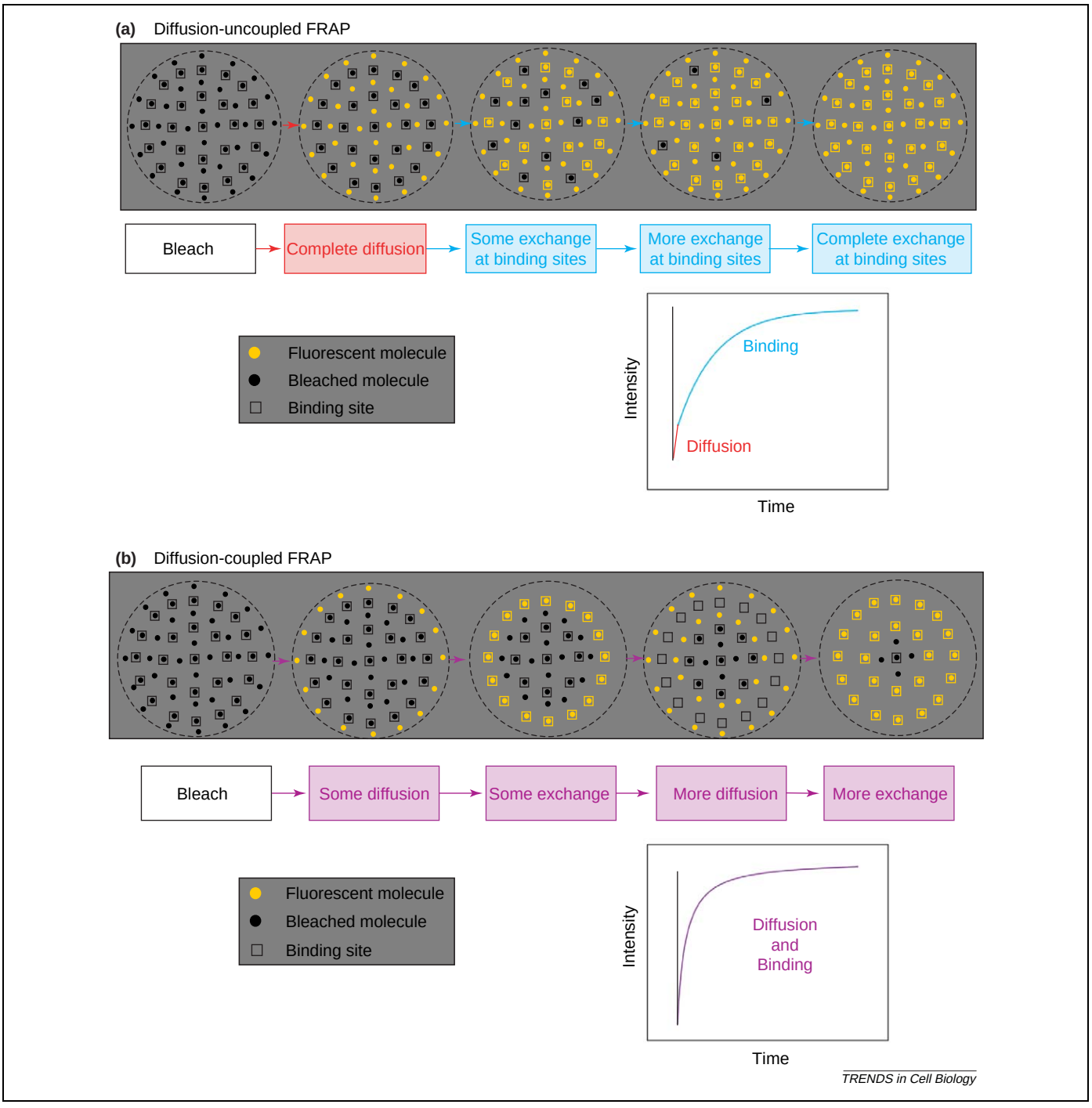

Figure 1. FRAP behaviors. **(a)** Diffusion-uncoupled behavior. After the bleach, fluorescent molecules rapidly diffuse throughout the bleach spot. Only bound bleached molecules remain in the spot. Gradually, these bleached molecules dissociate from their binding site and rapidly leave the spot. Fluorescent molecules then replace the bleached molecules at the binding sites as they become vacant. The diffusion-uncoupled FRAP recovery curve consists of two separable components: the early recovery due to diffusion (red) and the slower recovery due to exchange at binding sites (blue). **(b)** Diffusion-coupled behavior. Fluorescent molecules diffusing into the spot rapidly associate with the first binding sites that they encounter. Dissociation from these sites leads to more diffusion, but fluorescent molecules bind again rapidly. In this way, fluorescent molecules gradually make their way into the interior of the bleach spot. A diffusion-coupled FRAP curve cannot be separated into distinct components but, instead, reflects both diffusion and binding (red+blue=purple) throughout the recovery. Note that, in both (a) and (b), certain simplifications have been made for clarity.

state is a weakly bound state that induces a conformational change that enables a second reaction to occur that, in turn, yields a more tightly bound state. Simple formulas describing the FRAP recovery for such sequential binding reactions have not been described as yet. Instead, an alternative approach has been used to predict the FRAP recovery in which a set of ordinary differential equations describing the binding interactions is solved numerically [26,27] and fitted to the FRAP data by various techniques [18,28]. Although in this approach an explicit equation is not available for the FRAP recovery curve, estimates of the on and off rates of binding can still be obtained.

In summary, diffusion-uncoupled FRAP occurs when recovery due to binding can be separated from recovery due to diffusion. In this situation, the FRAP curve can be

described either by simple equations involving exponential decays or by numerical solution of ordinary differential equations. These fits enable a direct estimation of the association and off rates of binding.

Diffusion-coupled FRAP recoveries

Qualitative features of diffusion-coupled recovery curves

In a diffusion-coupled FRAP recovery, the time for the molecule to diffuse across the bleach spot is either slower than, or comparable to, the time for the molecule to associate with a binding site. Therefore, a molecule is quickly bound before it can diffuse far, it remains bound for a time determined by the off rate and then unbinds to repeat this cycle (Figure 1b). Because diffusion and binding are intermixed throughout the recovery, the FRAP curve cannot be separated into a diffusive phase and a binding phase. Thus, although diffusion-coupled FRAP curves might appear biphasic, the different 'phases' do not reflect fast and slow components that correspond to weak and tight binding, respectively, as might be presumed.

The time for complete recovery in a diffusion-coupled scheme is determined by both the association rate and the off rate at each binding step. A slow off rate can yield an extremely slow recovery that still depends on diffusion throughout, even though the association rate and diffusion are fast. Therefore, contrary to the intuitive expectation, a long recovery time does not rule out the diffusion-coupled mode. In fact, on the basis of a rough estimate of typical cellular binding and diffusion rates, diffusion-coupled FRAP is likely to be more common than is currently appreciated [19].

Considerations for quantitative models of diffusion-coupled recovery curves

In all cases of diffusion-coupled behavior, a FRAP analysis of an inert, non-binding molecule of comparable size should, ideally, be used first to define the diffusive behavior. In practice, this analysis is more commonly done by fitting the FRAP recovery of unconjugated GFP with one of the diffusion models described in Box 3. This diffusive behavior then forms the baseline on which the binding interactions are superimposed.

For example, if the FRAP recovery of GFP is fitted well by a simple model of diffusion [19], then a simple diffusion equation with binding terms should be sufficient to model both diffusion and binding. If a more complicated diffusion model is required, however, then that model for diffusion must be incorporated into the more general model describing diffusion and binding. As outlined in Box 3, these more complicated models might be required in complex cellular geometries such as the Golgi, or when molecular sieving effects are important such as for transmembrane proteins and potentially some cytoplasmic or nuclear proteins, especially those that are part of large complexes.

Because the underlying diffusive behavior must be included, the equations describing a diffusion-coupled FRAP are, in most cases, more complex than those used to describe diffusion-uncoupled FRAP. Furthermore, several factors that influence the diffusion of molecules into the bleached zone become important in diffusion-coupled FRAP. Most current models for the analysis of binding in diffusion-coupled FRAP require several assumptions to deal with these factors: first, an instantaneous bleach that yields a cylindrical bleach pattern; second, a homogeneous distribution of binding sites that are immobile during the fluorescence recovery; and third, a bleach at a reasonable distance from cellular boundaries. Where possible, experiments should be designed to approximate these conditions. If they are not, then the simple equations described below for fitting FRAP curves will be inaccurate and some of the computational approaches described further on will be required to account for the complexities of the problem.

Although the diffusion-coupled mode is generally complex, an important simplification can arise that enables straightforward curve fitting to estimate the ratio of the association rate to the off rate of binding. We discuss it first before considering more-complicated situations.

Effective diffusion

When the time to associate with a binding site is much faster than the time required to diffuse across the bleach spot, then a diffusion-coupled FRAP recovery exactly mimics diffusion, but at a slower rate determined by the strength of binding [19,29–31]. In this case, the recovery can be fitted by just the diffusion equation and the solutions described in Box 3 for pure diffusion in one, two or three dimensions apply. When fitted to the FRAP

Box 3. Modeling fluorescence recovery after photobleaching in the absence of binding

When binding and transport are absent, then only a term for diffusion is included in the equation for FRAP recovery. The diffusion equations have been solved for FRAP with a rectangular, circular or Gaussian-shaped bleach profile, including one-dimensional [58,59], two-dimensional [36,38], and three-dimensional geometries [60,61].

The above FRAP models presume that diffusion occurs in the absence of obstacles or complicated cellular structures that might impede a random walk and temporarily trap an otherwise freely diffusing molecule. To account for the possible complications of this 'molecular sieving', more-elaborate models of 'anomalous' diffusion have been developed [59,62–65]. Several recent reviews [1,2,9] describe in detail the use of these models, the necessary precautions in their application, and the special circumstances under which more-complex models are necessary.

A complication in nomenclature is that in much of the FRAP literature, particularly that on membranes, anomalous diffusion is attributed not only to obstacles but also to binding interactions. Indeed, either of these effects can give rise to FRAP recoveries that cannot be explained by simple diffusion models. With the recent interest in specifically extracting binding information from FRAP experiments, however, it has become important to distinguish between the contribution of obstructed diffusion and the contribution of binding to a FRAP recovery. Ideally, distinction should be made by measuring the FRAP recovery of an inert molecule of the same size as the fusion protein, so that the size-dependent effects of molecular sieving can be calibrated in the absence of binding interactions. These effects define the appropriate diffusion behavior, either simple or anomalous, which serves as the baseline on which binding interactions must be added to explain the FRAP recovery of the GFP fusion protein.

curve, these solutions yield an effective diffusion constant, D_{eff}. This constant will be smaller than the cellular diffusion constant, D, determined, for example, by FRAP of unconjugated GFP. The binding parameters – namely, the ratio of the association rate to the off rate – can then be determined by simply computing the value of $D/D_{eff}-1$.

This effective diffusion mode is estimated to occur in about a third of biological FRAP experiments where binding interactions are present [19]. It can be distinguished from pure diffusion because the FRAP recovery curve will be noticeably slower than that for unconjugated GFP, but will still be fitted by a pure diffusion model. Unfortunately, this type of behavior is not commonly considered. Several FRAP studies describe diffusion fits to GFP-tagged proteins, often presuming that this reflects a larger molecule or an unusually high local viscosity, but without discussing the possibility that these fits could reflect effective diffusion, whereby binding information could be directly extracted from the fit of the diffusion model to the FRAP curve.

There are two important constraints when extracting binding information in the effective diffusion mode. First, effective diffusion does not permit independent estimates of the association rates and off rates, but only their ratio. Second, it is impossible to determine the number of binding states present because, when effective diffusion holds, the same FRAP curve can arise from one, two, three or more binding states, as long as the sum of the ratios of individual association rates to off rates is the same. Coupled with the first constraint, this means that, in the effective diffusion regime, the FRAP fit yields a predicted ratio of association rates to off rates that might reflect either a single binding state or the sum of several states. It is crucial to appreciate this constraint in numerical or computational analysis of FRAP, where independent estimates of on and off rates can yield a good fit to an effective diffusion curve, even though the data determine only a single ratio.

More-complex diffusion-coupled models

A more complicated diffusion-coupled scheme arises when the diffusion time is comparable to the association time. In this case, the recovery curve is not described by effective diffusion but instead requires a more complex model. Predicted FRAP recoveries for this type of situation have been calculated for circular [19] and rectangular [32] bleach spots. Practically speaking, these equations can be used to fit a diffusion-coupled FRAP recovery curve when the effective diffusion model fails.

The above-mentioned diffusion-coupled approaches presume that the fluorescent molecule is uniformly distributed and that an instantaneous bleach is performed at some distance from a cell boundary. When these conditions are violated, the solutions described above become inaccurate [33], and a numerical analysis that can account for a more complicated scheme is needed. This analysis can be done either by software such as the 'Virtual Cell' [28], which solves the appropriate differential equations, or by using a computer program that directly simulates the diffusion and binding of the fluorescent protein in the bleach spot [34].

In summary, it is more difficult to extract binding information from diffusion-coupled FRAP because both diffusion and binding contribute to the recovery curve. Nevertheless, solutions for the simplest situations are available and computer software has been described for more-complex situations and, in some cases, is freely available [28].

Distinguishing diffusion-coupled from diffusion-uncoupled FRAP

As the above discussion conveys, completely different behaviors arise depending on whether the FRAP recovery is diffusion coupled or uncoupled. To extract binding information correctly from FRAP data, therefore, it is essential to assess which of these regimes is appropriate. Common sense might suggest that FRAP recoveries that last significantly longer than the time for free diffusion in a cell ($\sim$1 s) must reflect much slower binding compared with diffusion and, therefore, must be diffusion uncoupled. As described above, however, diffusion-coupled FRAP can also last an arbitrarily long time even when diffusion is extremely fast. Thus, the distinction between diffusion-coupled and diffusion-uncoupled FRAP cannot be made on the basis of the time for complete recovery.

Instead, an independent assessment is required. Phair *et al.* [18] have suggested an approach in which the fluorescence recovery is measured in various areas of the bleached zone. If the time for diffusion throughout the bleached zone is much faster than the association rate, then the recovery will be independent of position in the bleached zones and diffusion-uncoupled behavior is indicated.

An alternative strategy is to use different spot sizes for bleaching and to determine whether the FRAP recovery changes [19]. This strategy is also based on the fact that diffusion, but not the reaction kinetics, depends on the spatial scale. Thus, in the diffusion-uncoupled mode, there is almost no change in the recovery with bleach spot size because diffusion occurs instantaneously and occupies a miniscule initial portion of the curve that is often not even recorded. By contrast, in the diffusion-coupled mode, there is a detectable change in recovery with bleach spot size, because diffusion and binding are intertwined throughout the measured recovery phase. Thus, simple tests can and should be performed to determine whether the FRAP recovery is diffusion coupled or uncoupled.

Evaluating a good fit

So, an appropriate model has been implemented and a good fit has been obtained, but is the fit correct? An affirmative answer is never guaranteed, but some checks for consistency can be applied to help to confirm or to reject fits that look good.

One simple consistency test is to analyze the parameter estimates produced by the model fit. When an association rate is predicted, for example, it can be used to compare the characteristic time for binding (the inverse of the association rate) to the characteristic diffusion time. The characteristic diffusion time reflects the time required for diffusion across the bleach spot. In general, this time is proportional to w^2/D, where D is the diffusion coefficient

and w is the length scale of the bleach spot (e.g. the radius of a circular spot). A diffusion-uncoupled model is appropriate only when the characteristic diffusion time is much faster than the time for binding. A diffusion-uncoupled fit that estimates a time for binding that is faster or on the same order of magnitude as the time for diffusion should be rejected or at least treated with skepticism. Similarly, diffusion-coupled models should predict binding times that are faster or similar to the time for diffusion.

Another consistency check is to shift the FRAP behavior between different regimes by bleaching with different spot sizes. In general, as the bleached area gets smaller, the time to diffuse across the spot gets shorter as compared with the time to begin binding. This relationship between the times for diffusion and binding determines whether the FRAP recovery is diffusion coupled or uncoupled and whether the equations describing the recovery can be simplified or not. Thus, by using different spot sizes, the resultant FRAP curve might be fitted with completely different equations, thereby yielding independent estimates of the binding parameters.

These checks for consistency provide some reassurance that a good fit to a FRAP curve is not fortuitous and, thus, that the binding parameters predicted are likely to be accurate.

Concluding remarks

The ability of FRAP to investigate *in vivo* molecular dynamics has stimulated a resurgence in interest in this approach. At the forefront of this research are attempts to extract quantitative binding information from FRAP recovery curves. In this article, we have summarized the key principles underlying FRAP recovery in the presence of binding and have provided guidelines for selecting appropriate mathematical models to determine binding parameters. These quantitative approaches have shown that the analysis of FRAP data no longer needs to be limited to a mere qualitative appraisal of the shape of the recovery curve. In fact, in many cases the shape of the curve can be misleading. As discussed, only in specific cases (diffusion-uncoupled) can the biphasic or multiphasic shape of the curve be interpreted to represent distinct phases of recovery due to one or more binding states separated from diffusion. In many situations (diffusion-coupled), diffusion is intertwined with one or more binding states throughout the whole curve. Thus, a qualitative interpretation of a change in shape caused by a particular treatment or mutant condition cannot always be easily accomplished.

Despite the intense, recent interest in methods to extract binding information from FRAP data, considerable work remains to be done to validate and to disseminate these developing approaches. Among the intriguing predictions of such analyses are the residence times of transcription factors on chromatin [19,20], the elongation rate of RNA polymerase I [27] and RNA polymerase II [35], and the dissociation rate of microtubule-associated proteins [22]. How accurate are these estimates? This question will be answered in due course as different groups tackle the same or related issues by applying different methods for FRAP analysis. Will the results obtained from different approaches agree or will they be highly dependent on the model? In this regard, it would be particularly valuable if *in vitro* or *in vivo* systems could be identified or devised as positive controls – in essence, to provide a gold standard by which a FRAP analysis procedure can be validated.

Also important for advancing this field will be the development of more-detailed and accurate models, necessarily involving the numerical analysis of a FRAP recovery, that account for the complexity of cellular architecture, bleach spot profiles and finite cellular volumes with boundaries. Such detailed models would serve as '*in silico*' positive controls by which to evaluate how well the current more simplified models perform, thereby defining the limitations of our present approaches. Finally, means of making these new analysis methods available to biologists via 'easy-to-use' software will be necessary to realize in full the potential of quantitative FRAP analysis. Proper guidelines for using these models will be equally essential so that crucial assumptions and limitations are not lost in the dispersal of these methods.

In the coming years, we can look forward to the increasing accuracy and widespread use of FRAP analysis methods. The benefit will be an improved knowledge of binding interactions in live cells, thereby bringing us a step closer to understanding the complex protein networks that regulate cellular behavior.

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