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Review

Fluorescence Recovery After Photobleaching: A Versatile Tool for Mobility and Interaction Measurements in Pharmaceutical Research

Tom K. L. Meyvis,[1] Stefaan C. De Smedt,[1,3] Patrick Van Oostveldt,[2] and Joseph Demeester[1]

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This review introduces the basics of fluorescence recovery after photobleaching (FRAP) from a theoretical and an instrumentational approach. The most interesting and innovative applications with a pharmaceutical point of view are briefly discussed and possible future applications are suggested. These future applications include research on the mobility of macromolecular drugs in macro- or microscopic pharmaceutical dosage forms, mobility, and binding of antitumor drugs in tumor tissue, intracellular trafficking of gene complexes and mobility of drugs in membranes prior to transmembrane penetration. The paper is also intended to be an introductory guideline to those who would like to get involved in FRAP related experimental techniques. Therefore, comprehensive details on different setups and data analysis are given, as well as a brief outline of the problems that may be encountered when performing FRAP. Overall, this review shows the great potential of FRAP in pharmaceutical research. This is complemented by our own results illustrating the possibility of performing FRAP in microscopic dosage forms (microspheres) using a high resolution variant of FRAP.

KEY WORDS: FRAP; mobility; interactions; diffusion; drug delivery.

INTRODUCTION

Fluorescence recovery after photobleaching (FRAP), also called (micro)photolysis, is a rather old, yet ever-evolving fluorescence technology. Basically, the translational diffusion coefficient of a fluorescent molecule is measured by bleaching fluorescent molecules that move in the focal area of a light beam. Immediately after the bleaching process, a highly attenuated light beam measures the recovery of the fluorescence in the bleached area due to the diffusion of fluorescent molecules from the surrounding unbleached areas into the bleached area (Fig. 1). The diffusion coefficient (D) of the fluorescent molecules can be derived from the recovery of the fluorescence in the bleached area. In pharmaceutical research, there are a vast amount of projects dealing with the delivery of macromolecular drugs. In many stages of drug delivery, starting at the release from a matrix, through the uptake of the drug at a specified location in the body, the mobility of the drug molecules is the driving force. Moreover, the dynamics of macromolecular drugs in solutions and networks are still very hard to predict theoretically and therefore need to be characterized experimentally. Microscopes and imaging tools are becoming standard equipment in biological, and more recently, pharmaceutical laboratories. Since well-established methods for fluorescently labeling macromolecular drugs exist, FRAP offers a chance to study the mobility of macromolecular drugs in both pharmaceutical matrices as well as in tissues and cells. This review introduces the basics of FRAP in theory and necessary instrumentation. A brief summary of new and innovative applications has been included while suggestions for future applications of FRAP are described. This paper is written not only as a comprehensive introduction but also an introductory guide for those who would like to get involved with FRAP technology.

[1] Laboratory of General Biochemistry and Physical Pharmacy, University of Gent, Harelbekestraat 72, 9000 Gent, Belgium.

[2] Laboratory of Biochemistry and Molecular Cytology, University of Gent, Coupure Links 653, 9000 Gent, Belgium.

[3] To whom correspondence should be addressed. (e-mail: tom.meyvis@rug.ac.be)

ABBREVIATIONS: FRAP, fluorescence recovery after photobleaching; AOM, acousto optical modulater; PMT, photomultiplier; TIR, total internal reflection; SCAMPER, scanning microphotolysis; CSLM, confocal scanning laser microscope; PSF, point spread function; FT, Fourrier transform; OTF, optical transfer function; GFP, green fluorescent protein; FITC, fluorescein isothiocyanate; dex-ma, dextran-methacrylate.

BASIC FRAP INSTRUMENTATION AND TECHNICAL VARIANTS

Basic Instrumentation

A wide variety of instrumentation has been created since FRAP was developed in the seventies. The scheme of an up-to-date FRAP setup is drawn in Fig. 2, illustrating two possible light intensity modulation setups. In whatever setup used, there has to be a light source with bleaching power and one to monitor

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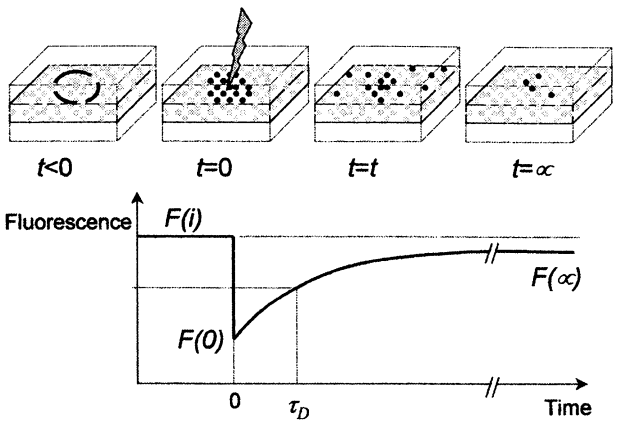

Fig. 1. Schematical representation of a FRAP experiment. Just before bleaching the monitoring light beam is focused within the sample and the fluorescence is measured in a specific area (circular in this example). The initial fluorescence before bleaching is recorded on the fluorescence recovery curve as $F(i)$. At $t = 0$ a high intensity light beam bleaches the molecules in the observed area causing a drop in fluorescence to $F(0)$. Due to random motion/diffusion the bleached molecules will exchange their position in the bleached area with non bleached fluorescent molecules from the surrounding. This results in a recovery of the observed fluorescence. The approximate characteristic diffusion time (τ_D) is indicated as the time at which half of the fluorescence has recovered. At the end of the experiment ($t = \infty$) the fluorescence has recovered to $F(\infty)$, that is equal to $F(i)$ if all fluorescent molecules in the observed area are mobile or less than $F(i)$ (as in this example) when a part of the fluorescent molecules is immobilized in the observed area during the time of the experiment.

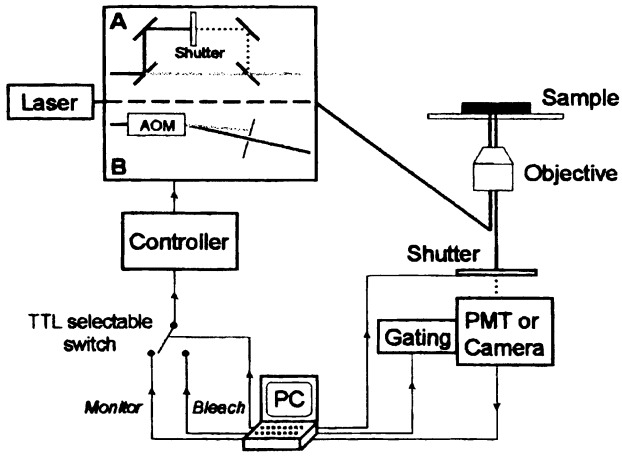

Fig. 2. Schematic representation of an up-to-date FRAP setup. This setup uses a single laser source as excitation and bleaching source. Two possible intensity modulation setups are represented. The dark and light lines represent respectively high and low intensity light. On top (A) is a dual beamsplitter in combination with a shutter (dashed line represents light path during bleaching when shutter is open). Underneath (B) is a setup using an AOM to diffract the light. According to the applied voltage either high or low intensity light is directed towards the microscope. Both systems are driven by a controller that translates the signals from a PC into either the appropriate voltage for the AOM or a signal controlling shutter movement. The laser beam is directed into the microscope (epi illumination in this example) and towards the sample. The fluorescence is detected by the photomultiplier. During bleaching the PMT is protected from high intensity light using either a shutter or a gating circuit both controlled and synchronized with the bleaching by the PC.

fluorescence before and during the fluorescence recovery process. Most often, intense laser light is used for bleaching. Monitoring is done either by laser light or by light from a mercury vapor lamp. When laser light is used, one single laser source is preferred for both bleaching and monitoring, instead of two separate laser sources. The easiest way to modulate the intensity of a single laser source is by inserting a neutral density filter in the beam path. A high intensity pulse is then generated upon brief withdrawal of this filter from the beam path (1). Other instruments use a dual beam splitter that splits the laser beam into a low and high intensity beam. A shutter interrupts the high intensity beam path and just before entering the microscope the two beams reunite. By controlling the shutter, pulses of high intensity light can be generated. Both systems are simple but their performance depends on the response time of the shutters and filter withdrawal mechanisms which is usually rather large (in terms of ms). Measuring the diffusion of low molecular weight components in watery environments is not possible with this kind of setup since the characteristic diffusion times are of the same magnitude as the response times. A faster system can be obtained by using an Acousto Optical Modulator (AOM) that has a typical response time of a few μs. Moreover by using an AOM, very high bleach/monitor power ratios (up to 1000) can be obtained. All these optical arrangements can be installed on conventional or confocal fluorescence microscopes. A confocal microscope however, allows fluorescence detection not only on the sample's surface but also at an arbitrary depth inside the sample, without any interference of fluorescence from out-of-focus levels of the sample (as encountered in a conventional microscope). An even deeper sample penetration can be obtained with a signal-to-noise confocal microscope. In this system only the fluorescent molecules located in the focal plane receive a photon density that is high enough for excitation and subsequent fluorescence emission (Fig. 3) (2).

The fluorescence intensity during recovery is recorded either directly by a photomultiplier (PMT) signal or by analysis of camera images taken during recovery. The use of cameras used to be limited to slow diffusion processes but with the ever-increasing 'speed' of cameras, very fast diffusion can now also be studied. During bleaching, PMT and camera should preferably be protected from the high light intensities during bleaching. Shutters can be used for this purpose but in a high-speed system an electronic gating circuit is preferred. The latter will put a counter voltage on the PMT during bleaching so that the high voltage created by the high intensity light is neutralized.

The main bleach geometries include circular spots with a Gaussian or a uniform intensity profile after bleaching, and stripes. The latter can be valuable in identifying anisotropic diffusion by changing the orientation of the stripe when repeating the experiment. A main disadvantage of bleaching spots with a Gaussian intensity profile is that the exact intensity profile after bleaching has to be determined in order to extract the 'half width' (ω) which is necessary for the calculation of the diffusion coefficient (see Eq. 2) (3). A recently developed technique called 'scanning microphotolysis' (SCAMPER) allows bleaching any geometry, as will be discussed in the next section.

Technical Variants

Several variants of the FRAP approach have been developed over the years. The most innovative developments will

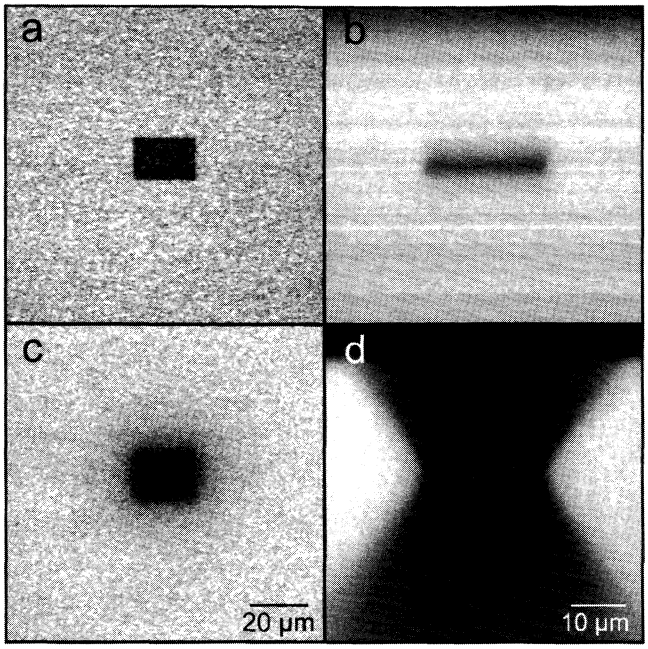

Fig. 3. Comparison of bleaching with a conventional and a two-photon confocal microscope. On the left are the x-y images of a rectangle bleached with a two-photon (a) and a conventional system (c). On the right are the images of the corresponding x-z planes through the rectangle just after bleaching. It clearly illustrates that in a conventional confocal microscope not only are the fluorescent molecules in the focal plane bleached, but that bleaching occurs throughout the whole light cone (d). Therefore the recovery in the bleached area will mainly arise from diffusion of non bleached fluorescent molecules in the focal plane. This is clearly not so for a two-photon confocal microscope (b). (from Kubitscheck *et al.*, 1995 (2)) Copyright 1996 the Royal Microscopical Society.

be discussed here. A first variant is the combination of FRAP with Total Internal Reflection microscopy ('TIR-FRAP') for measuring the mobility of molecules on or nearby surfaces. In a TIR microscope the incident light is directed onto the sample at a critical angle. Most of the incident light is reflected at the refractive index discontinuity formed by the interface between sample and coverslide. A part of the light enters the sample as an evanescent wave, meaning that the intensity will drop very rapidly to zero the further away (less than one wavelength) from the interface. Therefore fluorescence will only be observed in a thin layer close to the interface. By increasing the power of the incident light, bleaching of the surface molecules can be obtained (4). It can be used to study the mobility of drugs just underneath the plasma membrane.

A second FRAP variant called 'polarized FRAP' is used to monitor rotational instead of translational diffusion. The only difference from a basic FRAP setup is the use of polarized light for excitation and bleaching and the detection of polarized emission. Since 'polarized FRAP' analyzes rotational motion, a much faster data acquisition (µs range) than normally used in traditional FRAP is needed. Velez and Axelrod developed an appropriate theoretic analysis. The method is very sensitive to changes in the shape and size of the fluorescent molecules due to interactions or changing environment (5).

A third variant is 'continuous photobleaching'. In 'continuous photobleaching' experiments the excitation light does not switch between bleach and monitor intensity but illuminates the sample continuously at an intermediate intensity. This results in a continuous photobleaching of fluorescent molecules inside the illumination area together with a continuous influx of fluorescent molecules from the non-bleached surroundings. The diffusion coefficient of the fluorescent molecules can be calculated from the fluorescence decay in the illuminated area. Continuous photobleaching is very simple to perform but demands a more complex numerical analysis. A major drawback can be that the diffusion determined part of the decay is measured at low fluorescence intensities with a possibly worse signal-to-noise ratio.

A last variant called 'scanning microphotolysis' (SCAMPER) was recently developed and greatly enhanced the versatility and spatial resolution of photobleaching. The almost instant response of an AOM is used to bleach line segments during the scanning of a confocal scanning laser microscope (CSLM). In this way any figure can be bleached (e.g., an inhomogeneous region in a pharmaceutical formulation or a cell organelle). The combination of bleaching during scanning and the use of an AOM generating short bleaching pulses, prevents the broadening of the bleached area which occurs in conventional FRAP due to longer bleaching times and a stationary bleaching light beam. SCAMPER therefore allows bleaching spots of less than a micrometer at well defined locations in the sample. This may be very attractive when measuring inside cells or microscopic dosage forms (see Fig. 5) (6).

QUANTIFICATION OF MOLECULAR MOBILITY AND INTERACTIONS BY FRAP

Axelrod *et al.* developed the basic method of analysis for the quantification of molecular mobility and interactions from FRAP data. It is beyond the scope of this review to treat the theoretical analysis in full detail. However since nowadays most FRAP analysis are still based on the Axelrod equations, the outline of the theory will be briefly discussed. Another more recent approach based on Fourrier transforms will also be discussed, since it is becoming increasingly popular and is less stringent concerning the experimental conditions.

The Axelrod theory is based on some fundamental assumptions. First of all the fluorescence recovery must be the result of pure two-dimensional diffusion (no flow) in an infinite plane, which means that it has to arise from the diffusion of molecules within the same plane as the bleached area. This condition is approximately valid when high numerical aperture lenses are used, since a large cone of out-of-focus light bleaches the molecules above and below the focal plane (Fig. 3) so that only non-bleached molecules from the focal plane participate in the recovery. Secondly, no diffusion in or out of the bleached area may occur during bleaching. Under these conditions the fluorescence recovery in a circular bleached area, with a Gaussian fluorescence intensity profile, can be described by the normalized (fuorescence intensity before bleaching set to 1) fluorescence recovery curve ($f(t)$):

$$f(t) = \sum_{n=0}^{n=\infty} \frac{-\kappa^n}{n!} \cdot \frac{1}{1 + n\left[1 + \left(\frac{2t}{\tau_D}\right)\right]} \qquad (1)$$

were κ is the bleach constant, which is a measurement of the

amount of fluorescent molecules that are bleached, τ_D is the characteristic diffusion time that is related to the diffusion coefficient by:

$$\tau_D = \frac{\omega^2}{4D} \tag{2}$$

where ω is defined as half the width of the Gaussian intensity profile of the laser spot determined at e^{-2} times the height of the profile and D is the diffusion coefficient.

Due to physical and/or chemical interactions, all or a fraction of the fluorescent molecules may be immobile. This results in a partial recovery of the fluorescence after bleaching. The following fluorescence recovery equation takes the presence of immobile fluorescent molecules into account:

$$F(t) = F(i)[1 - R(1 - f(t))] \tag{3}$$

where $F(i)$ is the normalized fluorescence intensity of the bleach spot before bleaching and R is the mobile fraction of fluorescent molecules defined as:

$$R = \frac{F(\infty) - F(0)}{F(i) - F(0)} \tag{4}$$

where $F(\infty)$ is the normalized fluorescence intensity of the bleached spot at infinite time after bleaching and $F(0)$ is the normalized fluorescence intensity of the bleach spot just after bleaching (see Fig. 1). As will be discussed later, the immobile fraction observed in FRAP experiments can depend on the time window of the experiments.

Based on this basic theory several other mathematical analyses of the fluorescence recovery curve were developed according to the shape and the intensity profile of the bleached area. Soumpasis, for example developed the analytical equations to analyze the fluorescence recovery when bleaching is obtained using a light source with a uniform rectangular intensity distribution instead of a Gaussian intensity distribution (7). Gordon *et al.* even extended the theory to two diffusing components, each with their own immobile fraction (8). The disadvantages of all the analytical approaches are that they strictly depend on the initial conditions and are not valid for the entire time span of the fluorescence recovery. This means that their accuracy depends on the precision of the data in a specific phase of the fluorescence recovery. The most significant part of the fluorescence recovery occurs directly after bleaching. In this time period the recovery curve is very steep and bad signal-to-noise ratios may exist due to the low fluorescence intensities in the bleached area (9).

Numerical approaches, based on a simulation of the Brownian motion of the molecules, were developed to analyze continuous photobleaching experiments, since no analytical solution was available for this illumination condition (10). Numerical methods allow more experimental flexibility concerning the size and shape of the bleached area, the probe concentration as well as the presence or absence of flow. Using numerical simulations the recovery of any bleached geometry can be modeled and studied. Wedekind *et al.* used numerical analysis to calculate the recovery kinetics of a linepart that was bleached during scanning. (11). The drawback of these numerical approaches is the need to transform the calculated fluorophore distributions to a fluorescence intensity image as seen by the microscope. This is done by a convolution with the point spread function (PSF) of the microscope that therefore needs to be defined exactly. In addition, a space independent relation between the concentration of fluorescent molecules and the fluorescence intensity must be valid. This is not always true for inhomogeneous samples such as thick tissue preparations or *in vivo* measurements. To resolve this problem FRAP, in combination with spatial Fourrier analysis, was developed by Tsay and Jacobsen. Fick's diffusion law extended in two dimensions describes the redistribution of the concentration profile of the bleached molecules (12). When this equation is subjected to a two dimensional Fourrier transform (FT) the solution of the transformed equation is a simple exponential decay:

$$C(u, v, t) = C(u, v, o) \exp(-4\pi(u^2 + v^2)Dt) \tag{5}$$

where $C(u, v, t)$ is the FT of the concentration distribution at time t, u and v are the spatial frequencies and D is the diffusion coefficient. The concentration distribution is related to the observed image by the PSF that becomes an Optical Transfer Function (OTF) after FT. So the FT of the intensity distribution $I(u, v, t)$ becomes:

$$I(u, v, t) = C(u, v, t)\ OTF(u, v) \tag{6}$$

Since the OTF is constant in time the FT of the image will obey the same exponential decay as the concentration distribution:

$$\frac{I(u, v, t)}{I(u, v, o)} = \frac{C(u, v, t)}{C(u, v, o)} = \exp(-4\pi(u^2 + v^2)Dt) \tag{7}$$

This means that D can be determined by measuring the decay of the Fourrier transform of the image without knowing the PSF of the microscope nor the true concentration distribution of the bleached molecules in the sample. By analyzing the x and y component separately, non-isotropic diffusion and flow can be detected and quantified (12). This approach offers great possibilities for FRAP measurements in strong light scattering media.

APPLICATIONS OF FRAP AND PERSPECTIVES FOR FUTURE RESEARCH

Mobility in Solutions, Gels, and Other Matrixes

Although FRAP was originally developed to study molecular mobility in biological samples it has become a valuable tool in studying diffusion in all kinds of environments, especially in the field of polymer solutions and gels. There has been considerable progress in describing diffusion of probes in such systems but no one has yet been able to come up with a uniform theory. Therefore experimental determination of diffusion coefficients remains a key issue in chromatography, membrane separation techniques, controlled delivery and the characterization of transport *in vivo*. The most widely used techniques for measuring diffusion in such matrixes are permeation through, or release from the matrix, the determination of concentration profiles inside the matrix, and dynamic light scattering. A review on these techniques from a pharmaceutical point of view was published by Westrin *et al.* (13). Dynamic light scattering measurements usually suffer from intense background scattering, caused by many biological (e.g., tissue) and non biological media (e.g., gels). The great advantage of FRAP in comparison to release and permeation experiments, which are mostly considered in drug delivery studies, is that samples don't have

to be placed between membranes or brought in contact with osmotically active solutions, that can change their solvent content. FRAP allows studying mobility and interactions in small (±100 μl), intact samples. This is especially important in biological gels like mucus and biofilms. These samples are very sensitive to mechanical treatment like spreading or mixing and usually limited volumes are available. By FRAP Saltzman *et al.* showed that the mobility of proteins like IgM are not significantly retarded in cervical mucus (14). As FRAP permits very localized diffusion measurements, Bryers *et al.* used it to elucidate a significant difference in molecular mobility in the bacterial clumps of biofilms and the water channels that run through the biofilms (15). FRAP has further been used to characterize diffusion in chromatographic gel beads (16,17) or to evaluate the several theories describing diffusion of macromolecules in polymer solutions and gels (18–20).

By FRAP we characterized the diffusion of macromolecules through dextran methacrylate (dex-ma) solutions and hydrogels, synthesized by radicalar cross-linking of the dextran methacrylate solutions (Fig. 4). FRAP allowed an analysis of the mobility of fluorescent polymers in the same sample both before (in dex-ma solutions) and after cross-linking (in dex-ma gels). The same sample could be analyzed before and after cross-linking allowing us to characterize the specific influence of the introduced cross-links on the diffusion (21). Since FRAP has been successfully used to measure molecular dynamics in gels and chromatographical beads it may be a versatile tool in studying drug mobility. Not only inside hydrogel based pharmaceutical dosage forms but also in conventional formulations like tablets or capsules that become a gel upon hydration. Understanding the mobility and interactions of drugs inside these matrixes may help to better understand the release and may expedite their development. This is especially the case with microscopic dosage forms like microspheres, were the most common way to characterize the matrix is by electron microscopy, giving only a qualitative image of a dehydrated matrix. Using the high spatial resolution of the SCAMPER we were able to do FRAP inside microspheres (Fig. 5).

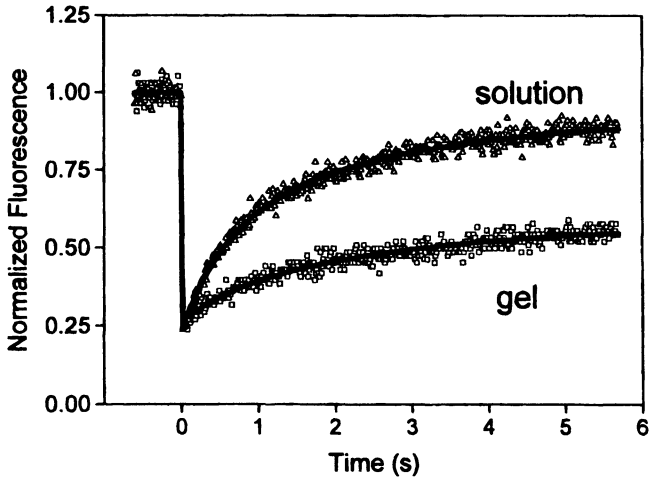

Fig. 4. Fluorescence recovery profiles as measured for FITC conjugated dextran (487 000 g/mol) in a dex-ma solution and a dex-ma gel (100 mg/ml). The introduction of the cross-links clearly slows down the diffusion and partially immobilizes the FITC conjugated dextran chains.

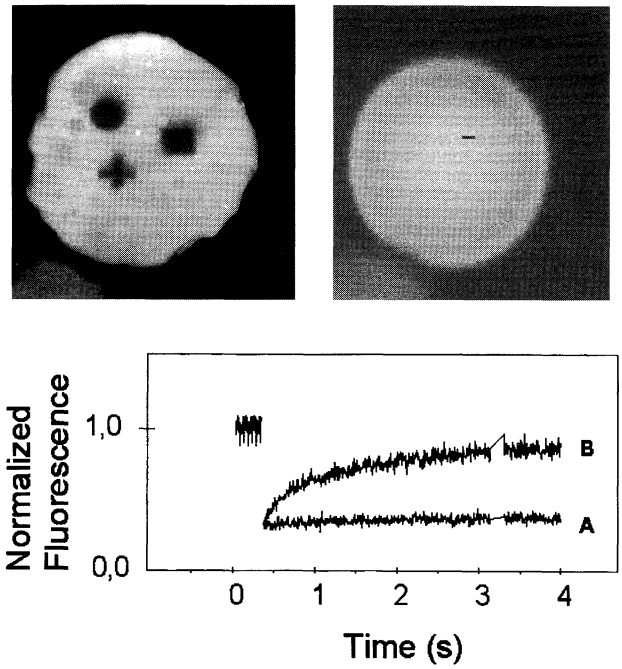

Fig. 5. On the left an illustration of FRAP in a dextran methacrylate microsphere (diameter = 100 μm) using the SCAMPER setup to bleach different geometries. On the right an illustration of FRAP on a dextran-methacrylate microsphere using SCAMPER technology (Line-SCAMP). The bleached area is a line of about 3 μm. At the bottom are the normalized fluorescence recovery curves of FITC labeled dextran (148 kDa) in dextran methacrylate microspheres, for curve A the fluorescent molecules were present during gelation of the microspheres, for curve B the microspheres were loaded with fluorescent molecules after preparation.

Mobility in Cellular Compartments and Tissues

For a long time FRAP has been successfully used to assess the translational mobility of all kinds of solutes in cytoplasm. For molecules with a molecular weight between 2 and 500 kD it has been shown by FRAP that the mobility in the cytoplasm is only 3 to 4 times slower than in aqueous solution. FRAP experiments have also shown that osmotic swelling or shrinkage respectively increased or decreased the mobility by a similar magnitude which indicated that the diffusion is influenced by the concentration of obstacles (probably the cytoskeleton) present in the cytoplasm. Recent developments in molecular biology have allowed the intracellular synthesis of green fluorescent protein (GFP) derivatives. This opened the way for FRAP in cells without the need to inject fluorescent molecules, risking a disruption of the cytoskeleton or a defective cell volume regulation (22,23). Recently Wedekind *et al.* used the high resolution SCAMPER technology to study the mobility of molecules (fluorescein isothiocyanate labeled dextrans) inside the cell nucleus (Fig. 6) (11). Since nowadays FRAP allows bleaching with a high spatial resolution it may be a worthwhile method for studying the intracellular trafficking of gene complexes in research on gene therapy.

Besides its cellular applications FRAP also allows studying the mobility of molecules in interstitial spaces of tissues, which is very important for the delivery of blood-born materials to cells. Chary and Jain used FRAP to evaluate the antitumor

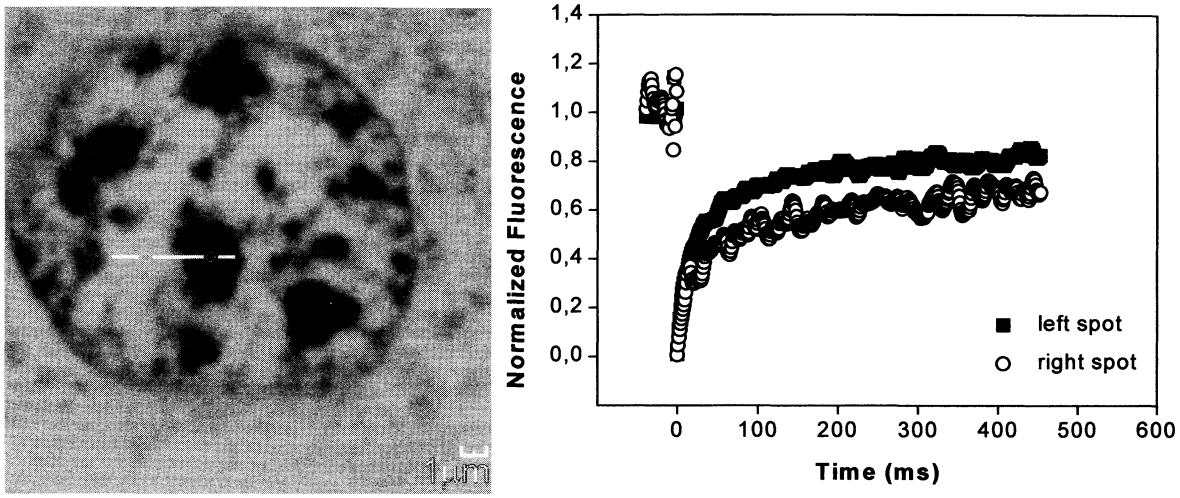

Fig. 6. FRAP in the cell nucleus using SCAMPER technology (LineSCAMP). The spots on the image indicate where the mobility of FITC labeled dextran (150 kDa) was measured. The graph on the right shows the normalized fluorescence recovery curves showing a spatial dependence of the mobility of the labeled dextran in the nucleus.(from Wedekind *et al.*, 1995 (2)) Copyright 1996 the Biophysical Society.

efficiency of high molecular weight therapeutics. (24). The main barrier to drug targeting to tumors is formed by the vascular endothelium (25). FRAP could become a useful technique to quantify the extent of binding with the vascular endothelium and the subsequent penetration towards the tumor tissue. In combination with two-photon microscopy FRAP will offer a chance to investigate the performance of drug complexes in the core of undisturbed tumor tissue *in vivo*.

Mobility in Cell Membranes

Researchers have made extensive use of FRAP in studying the lateral diffusion in cell membranes. It has proven to be a very versatile and sensitive method for this purpose. The early research (until 1990) on these topics was recently reviewed by Henis (26). An often-mentioned observation in these measurements is the occurrence of large immobile fractions as well as great variations in the diffusion coefficients. The interpretation of FRAP results, when measuring mobilities in strongly heterogeneous media, should be taken with precaution. Feder *et al.* showed by single particle tracking that the same protein can move in different ways within a cell membrane including random Brownian motion (which is assumed in FRAP analysis), directed motion due to active transport and constrained random motion due to interactions. When several of these motions occur simultaneously, the diffusion coefficient and the immobile fraction as measured by FRAP will become time and distance dependent. As a rule, FRAP can only be used to compare the mobility of proteins in cell membranes when fixed observation times and length scales are considered (27).

In the framework of transdermal delivery, Johnson *et al.* used FRAP to evaluate the molecular weight dependence of lateral diffusion of lipid compounds in human stratum corneum and found a very strong dependence in the lowest molecular weight range (200–500 Da) (28).

Binding Studies and Immunological Assays

In addition to the characterization of mobility, FRAP also allows estimating the fraction of molecules that are immobile during the experimental time which allows calculating binding parameters. This aspect of FRAP may be used to study drug-receptor interactions and this *in vitro* as well as *in vivo*. Very recently FRAP has been used to detect binding in cell membranes, cytoplasm and various other preparations (29).

When reversible binding occurs the rate of fluorescence recovery is influenced by both translational diffusion and binding kinetics. 'Diffusion limited' and 'reaction limited' cases can be considered. When the fluorescence recovery is 'diffusion limited', bound and unbound molecules rapidly exchange (i.e., in a time frame much faster than the duration of the experiment) and a full fluorescence recovery is obtained. The binding slows down the diffusion which results in a larger τ_D. An idea of the binding kinetics can be obtained by comparing the measured τ_D with the τ_D of the fluorescent molecules in a similar medium without interactions. When the fluorescence recovery is 'reaction limited', a given molecule exists either bound or unbound and will stay in this state for the time span of the experiment. In this case an immobile fraction will be observed, as fluorescence only partially recovers, and kinetic data can be obtained by studying the relation between the immobile fraction and the concentration of fluorescent molecules.

A theoretical framework for the characterization of binding from FRAP experiments was published by Berk *et al.* and Kaufman & Jain. The latter applied FRAP to the *in vitro* screening of monoclonal antibodies. The great advantage of FRAP over traditional immunoassays is that once the inactive fraction of the labeled antibody is known, no separation of antibody and antigen is needed (29,30). *In vitro* all variables can be controlled, preset and easily varied. This is a limiting step towards *in vivo* measurements. Berk *et al.* solved part of this problem by applying a pharmacokinetic model to calculate parameters like antibody concentration in a certain tissue compartment. These concentrations are hard to measure non-invasively due to the diverging optical properties within a tissue. In this way they were able to determine binding parameters *in vivo* within a human tumor xenograft.

Specific receptor-ligand binding is crucial to the function of many biologically active molecules and is the basis for a

wide range of novel therapeutics and diagnostics. A great deal of controversy regarding the performance of targeting agents has arisen from the possible discrepancies between *in vitro* and *in vivo* results. These may differ substantially due to differences in receptor density, presentation and/or accessibility and due to microenvironment related changes in binding kinetics. FRAP can be a powerful tool to study these discrepancies.

Finally it is important to stress that fluorescent labels can be coupled to many molecules of interest (e.g., antibodies) so that virtually any binding study can be done using FRAP. Coupling a fluorophore to the molecule of interest may change it's binding affinity. The influence can, however, be easily quantified using traditional *in vitro* binding tests like immunoassays. It was shown for antibodies that the binding of the fluorophore either hardly influences the affinity or completely destroys it (31).

Flow Velocity Measurements

Next to diffusion the transport of molecules may occur by flow. When characterizing diffusion, flow should be avoided. When flow is significantly faster than diffusion the fluorescence recovery in a FRAP experiment can be related to the flow velocity. The theoretical analysis for flow measurements using FRAP was developed by Flamion *et al.* and calibrated using glass capillaries and high precision pumps that create a hydrostatic pressure over the observed part of the capillaries. A part of the tubules were uniformly illuminated and fluorescence was measured. After a short bleaching pulse, bleached molecules were transported out of the illuminated area and the fluorescence recovery was observed. When flow is the sole mechanism of molecular transport the fluorescence recovery is linear within time until $L/2v_m$, where L is the length of the illuminated region and v_m is the mean axial velocity. This linearity is almost independent of the flow regime (e.g., poiseuille versus plug flow). When both flow and diffusion are present their relative contribution in the fluorescence recovery can be assessed by calculating the Péclet number (*Pé*):

$$Pé = \frac{v_m d}{D} \tag{8}$$

where d is the diameter of the tube and D is the diffusion coefficient of the fluorescent molecules in the stationary medium. For high *Pé* numbers diffusion has no significant contribution and the above-mentioned linearity can be obtained (32). Berk *et al.* studied the natural flow occurring *in vivo* in the lymphatic vessels of mouse tails using a variant of the Fourrier transform FRAP described earlier (Fig. 7). The displacement of the bleached spot was measured by examining the relative changes in the real and imaginary parts of a given Fourrier component. The flow was then calculated from the phase shift in the Fourrier transform of the images caused by the bleached area leaving the observed image frame (33). Chary and Jain measured diffusion and flow in the interstitial space of tumors in a rabbit's ear chamber. The diffusion of FITC labeled albumin was characterized by measuring the dissipation of the Gaussian fluorescence intensity distribution in the bleached area, while the flow was characterized by tracing the movement of the center of the dissipating bleached area. This complex approach demands a uniform flow within the bleached area to maintain the Gaussian distribution of the fluorescence intensity (24).

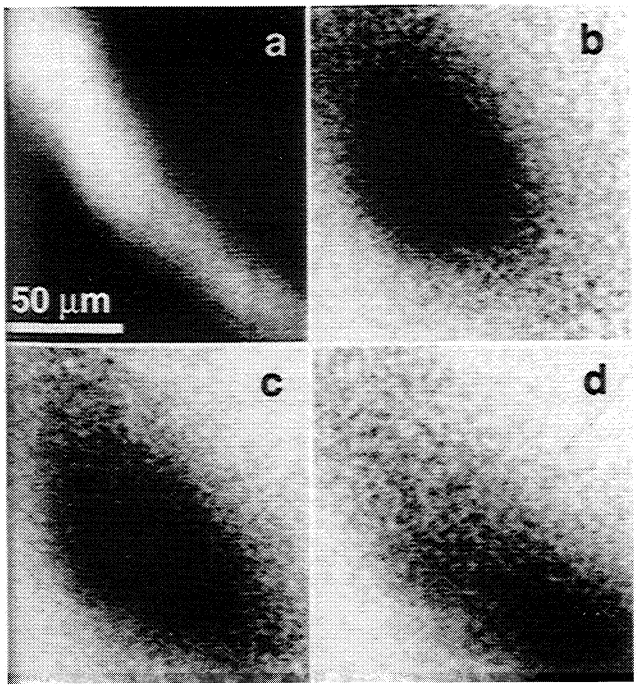

Fig. 7. Illustration of FRAP for the measurement of flow velocities in lymphatic capillaries. a: a lymphatic capillary loaded with fluorescein isothiocyanate conjugated dextran; b. differential image (image obtained after subtraction of the first image) just after bleaching; c and d are differential images at respectively 4 and 9 s after bleaching showing the displacement of the bleached area towards the bottom right corner of the images as well as the dissipation of the bleached area (from Berk *et al.*, 1996(33)) Copyright 1996 the American Physiological Society.

PRACTICAL GUIDELINES FOR FRAP RESEARCH

As mentioned in the introduction, this paper not only aims to review the most important developments in FRAP, but is also intended as a guide to those who would like to get involved with FRAP technology. The following sections include information on the fluorophores which are suited for FRAP and the 'fluorescence related phenomena' one has to be aware of when doing FRAP. The section on 'experimental guidelines' outlines basic rules for performing good FRAP experiments. While the final section focuses on some artifacts that can be induced in biological samples due to illumination with high bleach intensities.

Fluorophores for FRAP

Which fluorophore to choose for FRAP experiments depends on the available excitation source, the hydrophilic or lipophilic properties of the medium in which the fluorophore has to dissolve, and the chemical ways available to attach the fluorophore to the molecule of interest. Unfortunately the choice of fluorophore is also a trade off between its photostability or instability. An easily bleachable fluorophore can be bleached at low bleach intensities, which is a clear advantage. However it will also be more sensitive to bleaching during the recovery process which should be avoided as much as possible (see 'experimental guidelines'). The most commonly used hydrophilic fluorophore is fluorescein isothiocyanate (FITC). It can be easily attached to proteins and polysaccharides and has a

good balance between photostability and instability. Another fluorophore suitable for FRAP is β-phycoeritrine, a fluorescent protein. The recent development of GFP offers great possibilities for FRAP in undisturbed cells as already shown by Swaminathan *et al.* (23). On top of that, the bleaching efficiency of GFP is insensitive to radical quenchers or singlet oxygen scavengers because the chromophore part of the molecule is completely shielded from bulk solvent (34).

The most frequently used lipophylic fluorophores include Diphenylhexatriene derivatives and Nitrobenzoxadiazole derivatives. These are mostly incorporated in structural analogues of membrane constituents such as phospholipids, where they can be bound to either the polar head group or one of the fatty alcyl chains. Other lipophylic fluorophores that are non polar and widely used unbound are dialkylcarbocyanine derivatives.

Fluorescence Phenomena

FRAP is entirely based on fluorescence. Therefore it is necessary to briefly outline some fluorescence-related phenomena that demand attention when performing or analyzing FRAP experiments.

The bleaching of fluorophores is caused by oxidation of the excited molecule. Therefore the bleach rate is dependent on the collision rate of molecular oxygen with the excited fluorophore. This implies that the bleach rate will, among other things, depend on temperature, viscosity and oxygen content of the surrounding medium (35).

FRAP generally assumes that the fluorescence is directly proportional to the concentration of fluorescent molecules. However when a high concentration of fluorescent molecules is present 'concentration quenching', which is essentially characterized by a concentration dependency of the quantum yield of the fluorescent molecules, can occur. Since the recovery kinetics are extracted from intensity data the fluorescent molecules should have a constant, concentration independent quantum yield or erroneous recovery curves will be recorded (36). The easiest way to avoid such errors is to examine the linearity between the concentration of the fluorescent molecules and the measured fluorescence intensity under conditions as close as possible to those in the FRAP experiments. Reliable FRAP experiments should only be done using concentrations of the fluorescent molecules within the linear region. Unfortunately there is always a trade off between concentration of the fluorescent molecules and the signal-to-noise ratio. In turbid or high light scattering samples it may be necessary to use high concentrations of fluorescent molecules to observe fluorescence. In these circumstances it is possible, though not advisable, to model the concentration dependence of the quantum yield, using the curve obtained in the linearity tests, and include this relation in the recovery analysis model.

In all analyses of fluorescence recovery curves, irreversible bleaching is assumed so that the recovery is fully explained by the translational movement of fluorophores. However, Periasamy *et al.* observed that the fluorescence recovery of fluorescein in air saturated viscous solutions partially originates from reversible bleaching. It occurred within 1 ms after bleaching and was independent of the translational diffusion or the concentration of the fluorescent molecules. The reversible bleaching did depend on the solution viscosity, the oxygen content and the nature of the macromolecule to which the fluorescein was conjugated. It was sugggested that the reversible bleaching is probably caused by a triplet state relaxation. Since the fluorescence recovery due to reversible bleaching occurs in a very short time range it only has to be considered when studying very fast diffusion with characteristic diffusion times in the ms range (37).

Experimental Guidelines

The amount of bleaching and the way it is experimentally obtained are essential features for performing a reliable FRAP experiment. Generally the amount of bleaching should be between 30–70% ($0.3 < F(0)/F(i) < 0.7$). If the amount of bleaching is too low, the shape of the recovery curves is not 'typical enough' to be analyzed properly. If the amount of bleaching is too high, errors may arise from the low signal-to-noise ratio in the very weakly fluorescent bleached area. Experimentally the amount of bleaching can be influenced by changing the bleach time or the intensity of the bleaching light. Increasing the bleach time will increase the amount of bleaching but since FRAP theory assumes no significant diffusion occurs during the bleaching it should be restricted to a minimum. As a rule of thumb a maximal ratio of 1/15 between bleach time and characteristic diffusion time is usually respected. If no sufficient bleaching is obtained within the allowable time range the intensity of the bleaching light should be increased. Since most of the fluorescence recovery occurs in the initial part of the recovery curve it is very important to start measuring the fluorescence as soon as possible after bleaching. This is the only way to get accurate and reproducible values of the characteristic diffusion time.

When studying slow diffusion the fluorescence recovery can take a very long time. Although the monitoring light has only a low intensity, the extended illumination during long fluorescence recovery periods may cause substantial bleaching of the fluorescent molecules which interferes with the recovery kinetics. A correction has to be made for this 'bleaching during recovery' phenomenon. This can be done by normalizing the measured fluorescence recovery with the fluorescence intensity measured simultaneously in an area well outside the bleached area. This correction is only allowed to a limited extent, as in extreme cases the fluorescence recovery profile will become more determined by the 'bleaching during recovery' than by the fluorescence recovery due to diffusion. This may introduce serious errors especially towards the end of the recovery. If a strong 'bleaching during recovery' is observed it may be preferable to record the fluorescence recovery intermittantly so that the total illumination time is reduced.

Finally, in all FRAP experiments for the determination of diffusion coefficients, flow should be avoided. The two most important causes of flow are temperature gradients and movement. Therefore enough time should be taken between the application of the sample and the actual measurement to allow an equilibration of the sample. Flow can mostly be detected by a fast and atypical (thereby hard to fit) fluorescence recovery.

Radiation Induced Artifacts

When working with biological specimens one could wonder weither no damage is done by the high illumination intensities during bleaching. Known processes caused by high intensity

ight include cross-linking of membrane proteins, loss of enzymatic activity and cell lysis. Most of these originate from photooxidation and may be reduced by adding singlet oxygen quenchers. It has been shown that under the typical illumination conditions of FRAP, where high intensities are only used for very short periods, no damage occurs. Another question often heard is whether bleaching can cause a local temperature increase, which may influence the mobility of the molecules. Based on theoretical considerations of the heat flow, it was calculated, that for a basic FRAP setup, the temperature increase caused by the illumination rarely exceeds 1°C (38).

CONCLUSIONS

As shown in this review FRAP is a very versatile technique. It offers the possibility of microscopically examining a sample and getting information on molecular motion and interactions in a specific part of that sample. The three main advantages are the speed of the experiments, their resolution (both spatial (μm) and time (μs)) and the possibility of measuring intact samples both *in vivo* and *in vitro*. The main disadvantage is the need for fluorescent molecules. Labeling a molecule of interest can change its properties and, whenever possible, this should be controlled using appropriate techniques like immuno assays, partition experiments or photon correlation spectroscopy. However, given its great versatility and the ever increasing penetration of microscopic instrumentation in research laboratories, the possibilities of FRAP have only barely been exploited in pharmaceutical research. Some possible future applications include research on the mobility of macromolecular drugs in macro- or microscopic pharmaceutical dosage forms, mobility and binding of antitumor drugs in tumor tissue, intracellular trafficking of gene complexes and mobility of drugs in membranes prior to transmembrane penetration. The future developments in FRAP go together with the technological developments in microscopy, image processing and mathematics. The most promising evolution is probably FRAP in combination with two-photon microscopy and the appropriate model for three dimensional diffusion.

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