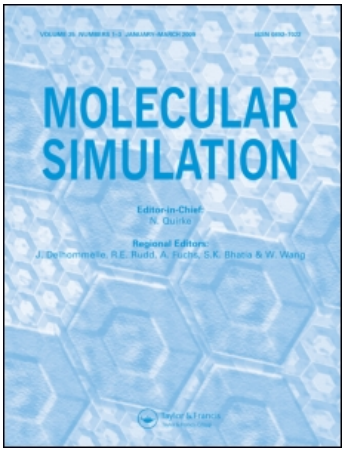

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Dimitris Glotsos[a]; Spiros Kostopoulos[a]; Kostas Ninos[a]; Dionisis Cavouras[a]
[a] Medical Image and Signal Processing Lab (MEDISP), Department of Medical Instruments Technology, Technological Educational Institute of Athens, Egaleo-Athens, Greece

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Interpretation of binding kinetics in fluorescence recovery after photobleaching experiments using a novel stochastic simulation strategy

Dimitris Glotsos*, Spiros Kostopoulos, Kostas Ninos and Dionisis Cavouras

Medical Image and Signal Processing Lab (MEDISP), Department of Medical Instruments Technology, Technological Educational Institute of Athens, Ag. Spyridonos Street, 122 10 Egaleo-Athens, Greece

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Fluorescence recovery after photobleaching (FRAP) has been extensively used for monitoring the binding kinetics of proteins with a goal to investigate the cellular processes, such as transcriptional regulation, cell membrane diffusion and signal transduction. In this study, a new approach for the interpretation of FRAP curves is presented based on the stochastic simulation of binding kinetics. The proposed method considers that proteins (a) randomly diffuse in a Brownian random-walk manner and (b) react with certain probability with compatible empty binding sites in a homogeneous well-stirred chemical environment. The proposed algorithm was compared with standard deterministic methods that are currently being used for analysis of FRAP curves. Predictions of recovery times of FRAP curves and sum of residuals revealed a good agreement. The stochastic simulation algorithm presents a firmer physical basis than its deterministic counterparts and it might be used to successfully model probabilistic events in the cell, deciphering information in FRAP experiments that cannot be computed using deterministic models.

Keywords: FRAP; reaction–diffusion; Gillespie; kinetics; binding

1. Introduction

Fluorescence recovery after photobleaching (FRAP) has been extensively used for monitoring binding kinetics of key biomolecules in order to investigate several important cellular process, such as transcriptional regulation, cell membrane diffusion and signal transduction [1,2]. FRAP is performed in three main stages: (a) molecules of interest are tagged with a fluorescent agent; (b) a small region within the cell is bleached; and (c) the redistribution of fluorescence within the bleached region, due to diffusion of fluorescent tagged molecules, is then recorded giving rise to the FRAP curve. Thus, the FRAP curve encodes information regarding the speed of migration of fluorescent-tagged molecules back into the bleached region. This 'migration speed' describes the diffusional mobility of the species involved and can be described by a simple diffusion equation $D = w^2/4t$, where D is the diffusion equation; w is the diameter of the bleached spot; and t is the time for recovery of 50% of fluorescence inside the bleached region. Apart from kinetics, FRAP can be used for describing binding properties of molecules (mostly proteins). In such studies, the examined protein is fused with a green fluorescent protein (GFP). If GFP-fused proteins bind to bio-affine cell receptors, then the recovery rate in the bleached spot is delayed by a factor related to the association and dissociation coefficients of binding. The latter comprises the basis for investigating the binding of proteins using FRAP. Probably the most popular model for analysis of FRAP curves has been proposed by Sprague and McNally [3]. The latter model predicts four main different scenarios. (a) Pure diffusion – fluorescent-tagged molecules diffuse freely (recoveries in less than 1 s). The FRAP curve, in this case, can be described by a simple diffusion equation [3] that can be used to compute the diffusion coefficient of involving species. (b) Effective diffusion – binding sites detain fluorescent-tagged molecules resulting in a slower FRAP recovery, which can be described by a new diffusion coefficient, the so-called effective diffusion coefficient [4,5]. (c) Reaction dominant – fluorescent-tagged molecules diffuse rapidly. Diffusion is so fast that it cannot be observed in the recovery; the resulting FRAP curve can be used to extract information regarding reaction rates. Recoveries, in this scenario, last from seconds to minutes [6,7]. (d) Full model – the full model covers any other regime than the above three.

There have been several mathematical models proposed for extracting information from FRAP curves [1,5,8–14]. These models suggest numerical solutions based on fitting, Laplace transform or analytical solutions. There have been proposed mathematical treatments for 2D or 3D formulations for various

*Corresponding author. Email: dimglo@teiath.gr

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bleaching spot geometries; among the most popular are circular, strip and Gaussian profile geometries. In order to select the proper model for the analysis of a given FRAP curve, several important factors need to be assessed, such as the dimensionality and shape of geometry, the number and type of binding sites, the presence or absence of reactions, and the association and dissociation coefficients of suspected reactions. It has been shown [14,15] that the selection of a proper model plays an important role on both the correctness and validity/accuracy of the analysis.

Although the above models have been successfully used for the analysis of FRAP curves, they present important limitations that are as follows [16–19]: (a) proposed numerical and/or analytical models are based on ordinary differential equations and rely on continuous approximations. It is well known that such approaches are excellent for describing phenomena of high-density populations; however, most key biological molecules within the cell (i.e. proteins, transcription factors, etc.) occur in very low densities (of the order of $1-10^2\,\mu\mathrm{m}^{-3}$). Thus, using deterministic approximations, only average behaviours can be assessed [19–24], and important cellular phenomena (such as polymerase binding, gene expression), relying on the stochastic nature of low-density populations, are smoothed out [22,25]. (b) Deterministic approximations assume that binding is a continuous process. However, it is well known that binding occurs in discrete time intervals [22,25]. For low-density populations, the discrete nature of binding plays an important role when assessing the 'preference' of a biomolecule to attach to specific cell sites. (c) Deterministic approximations rely to a great extent on the geometry of the bleached spot (or the bleaching laser profile – circular, Gaussian, etc.) [15,26]. For complex geometries, analytic solutions are difficult to compute. To the best of our knowledge, there is no unified model treating any kind of geometry. (d) Most continuous approximation methods have been based on the assumption that the cell environment is a homogeneous reaction system. Although this assumption facilitates analytical solutions, it has been shown that the reactant molecules of many, if not all, cellular biochemical pathways are highly heterogeneously distributed within the cell compartments [15,26]. (e) Regarding reactions involving one diffusing molecule and one binding site, continuous approximation models can only be used to estimate the ratio of association to dissociation rates. Such models cannot be used for specifying unique values for reaction rate coefficients.

In this study, a new approach to the interpretation of FRAP curves is presented, based on stochastic simulation of binding kinetics. The proposed method considers that proteins (a) randomly diffuse in a Brownian random-walk manner and (b) react with certain probability with compatible empty binding sites in a homogeneous well-stirred chemical environment. There are certain advantages regarding stochastic interpretation of FRAP experiments, compared with deterministic models proposed in literature [1,5,8–14]. (a) Since it is well known that biological networks are characterised by discrete interactions, stochastic modelling of protein-binding kinetics is a more realistic interpretation of the behaviour of such processes than standard deterministic approaches [27]. To the best of our knowledge, such a stochastic approach has not been reported in literature for FRAP curve analysis. (b) Stochastic modelling requires no special formulation regarding geometry, size and/or the dimensionality of the bleached spot. (c) Stochastic simulation might be used to extract information that cannot be extracted from standard deterministic methods, such as distribution of time intervals to next reaction, determination of next event (types of reaction and diffusion), concentrations of each molecular species as a function of time and chemical systems behaviour towards equilibrium following any perturbation that alters molecular populations.

2. Materials and methods

Diffusion was simulated as a Brownian random-walk motion, with average displacement given by [28] the following:

$$P(r,t)=\frac{1}{(4\pi Dt)^{d/2}}\exp\left(\frac{-r^2}{4DT}\right), \tag{1}$$

where t is the time for next event of a random-direction displacement r of a molecule with diffusion constant D; d is the dimensionality of the geometry considered; and T is the temperature. In our experiments, we have considered $d=2$, and $T=36^{\circ}\mathrm{C}$.

Binding was simulated on the context of the Gillespie [20] stochastic simulation algorithm, which can be used to monitor the exact concentrations of reactants in a homogeneous, well-stirred environment. In this study, we have suitably modified the Gillespie's algorithm for simulating FRAP recoveries as follows: considering a protein species F and binding sites S, unbound F may react with vacant S to create bound complexes FS according to:

$$\mathrm{F}+\mathrm{S}\underset{k_{\mathrm{off}}}{\overset{k_{\mathrm{on}}}{\rightleftarrows}}\mathrm{FS}, \tag{2}$$

where k_{on} and k_{off} are the association and dissociation coefficients, respectively. The stoichiometry matrix for this system can be written as follows:

$$\begin{bmatrix}[\mathrm{F}]\\ [\mathrm{S}]\\ [\mathrm{FS}]\end{bmatrix}\Rightarrow\begin{bmatrix}-1 & 1\\ -1 & 1\\ 1 & -1\end{bmatrix}. \tag{3}$$

According to the Gillespie algorithm, molecular concentrations of participating species change as a

function of time, based on the stoichiometry of the system (Equation (3)) according to $P_0(t)$:

$$P_0(t) = \exp\left(-\sum_{\nu=1}^{M} a_\nu t\right), \tag{4}$$

where M denotes the type of reaction, and a is the propensity for a particular reaction (this parameter expresses the tendency of the system to reaction equilibrium for the forward or backward reaction; it depends on exact molecular concentrations at each time t and the reaction coefficients). According to Equation (4), we may derive the following formula for simulating FRAP recoveries in the context of the proposed binding diffusion model:

$$\mathrm{FRAP}_{\mathrm{stochastic}} = 1 - a_\nu \exp(-a_0 t), \tag{5}$$

where a_0 is the sum of propensities for both reactions.

The implementation of the proposed algorithm was realised as follows:

(1) Initialisation of molecular populations of participating species.
(2) Computation of each reaction propensity a_ν.
(3) Computation of the sum of all reaction propensities a_0.
(4) Generation of two uniformly distributed random numbers r_1 and r_2.
(5) Computation of time to next reaction as $\tau = \ln(r1)/a_0$.
(6) Division of each reaction propensity with a_0. Definition of the type of reaction that will occur next is as follows:

$$\text{if } \left|\frac{a_1}{a_0} - r_2\right| \le \left|\frac{a_2}{a_0} - r_2\right|,$$

then the forward reaction will occur

: F + S → FS

$$\text{if } \left|\frac{a_1}{a_0} - r_2\right| > \left|\frac{a_2}{a_0} - r_2\right|,$$

then the backward reaction will occur

: FS → F + S

(7) Update of time t of simulation as $t = t + \tau$.
(8) Update of molecular populations according to stoichiometry of the system (see Equation (3)). For example, if the forward reaction is selected, then one molecule of unbound F and one molecule of binding site S will disappear, while one complex of FS will appear. If the backward reaction occurs, one complex FS will disappear, while one unbound F and one binding site S will appear.

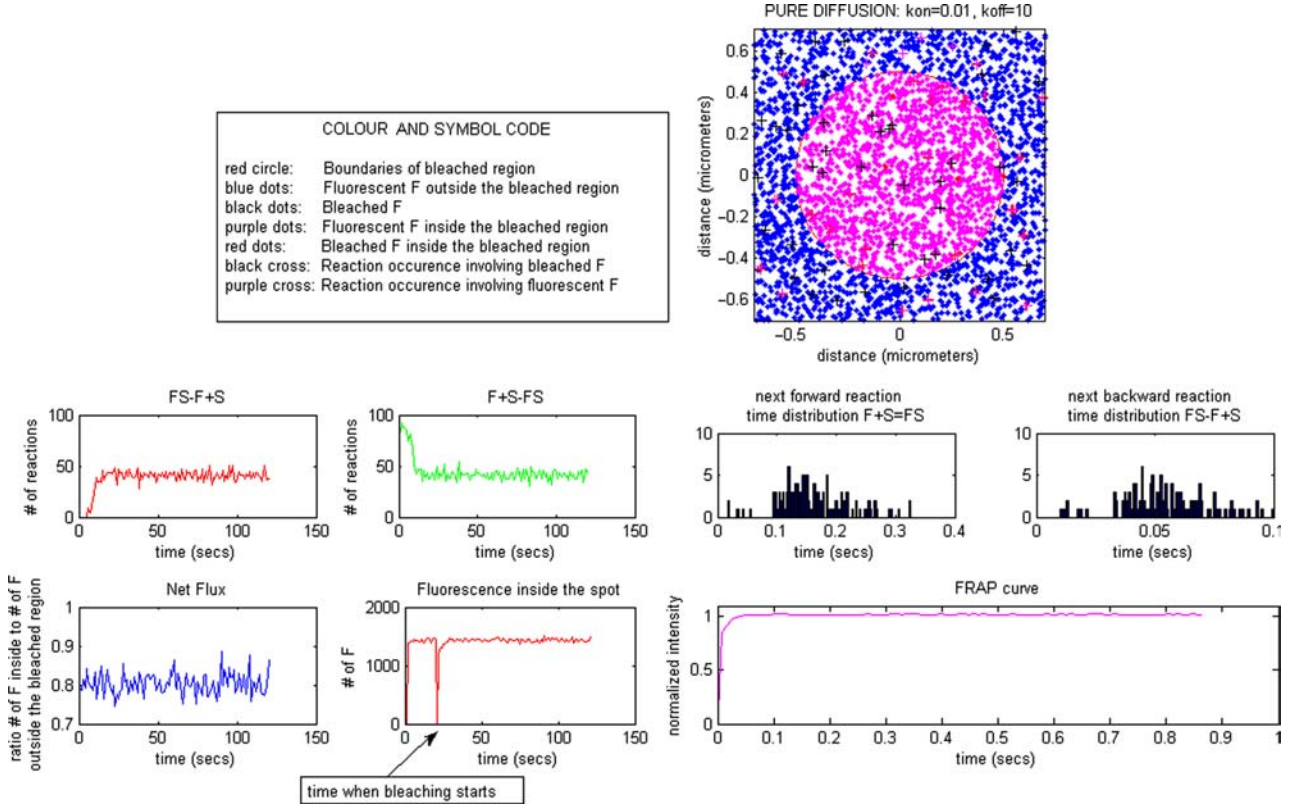

Figure 1. The proposed stochastic simulation algorithm for the diffusion-dominant scenario.

(9) Update of coordinates of F, S and FS. Allow for unbound F to diffuse if $t \geq t_d$, where t_d is the time step to next diffusion.

(10) If $t \geq t_f$, where t_f is the time, where bleaching occurs, consider all F within the bleaching region as non-fluorescent. Start recording fluorescence recovery within the bleached region as:

$$\mathrm{FRAP}_{\mathrm{curve}}(t) = \frac{\mathrm{F}^0_{\text{simulation-field}}\mathrm{F}_{\text{bleached-spot}}(t)}{\mathrm{F}^0_{\text{bleached-spot}}\mathrm{F}_{\text{simulation-field}}(t)}$$

[29], where $\mathrm{F}^0_{\text{simulation-field}}$ is the number of fluorescent F inside the simulation field at $t = 0$; $\mathrm{F}^0_{\text{bleached-spot}}$ is the number of fluorescent F inside the bleached spot at $t = 0$; $\mathrm{F}_{\text{bleached-spot}}(t)$ is the number of fluorescent F inside the bleached spot at t; and $\mathrm{F}_{\text{simulation-field}}(t)$ is the number of fluorescent F inside the simulation field at t.

(11) Update of coordinates of F, S and FS. Allow for unbound F to diffuse if $t \geq t_d$, where t_d is the time step to next diffusion. t_d is user defined.

(12) Go to step 2, repeat all steps until $t \geq t_t$, where t_t is the simulation's termination time.

2.1. *Comparison with other methods*

The proposed method was compared against the binding diffusion model of Sprague et al. [8], which is suitable for uniform circular bleaching profiles and has been used as a benchmark model in recent studies [3,6,12]:

$$\mathrm{FRAP}_{\mathrm{sprague}}(t) = \frac{1}{p} - \frac{\mathrm{F}_{\mathrm{eq}}}{p}(1 - 2K_1(qw)I_1(qw))x \times \left(1 + \frac{k_{\mathrm{on}}}{p + k_{\mathrm{off}}}\right) - \frac{C_{\mathrm{eq}}}{p + k_{\mathrm{off}}} \quad (6)$$

$$\text{with } q^2 = \left(\frac{P}{D}\right)\left(1 + \frac{k_{\mathrm{on}}}{p + k_{\mathrm{off}}}\right),$$

where w is the radius of the bleached spot; D is the diffusion coefficient; I_1 and K_1 are the modified Bessel functions of the first and second kind; F_{eq} and S_{eq} are the concentrations of F and S at equilibrium; and p is the Laplace variable. Comparison was performed in terms of goodness of fit (sum of residuals R) as proposed in Ref. [30]:

$$R = \sum_{t=1}^{m} \left|\mathrm{FRAP}_{\mathrm{stochastic}}(t) - \mathrm{FRAP}_{\mathrm{sprague}}(t)\right|. \quad (7)$$

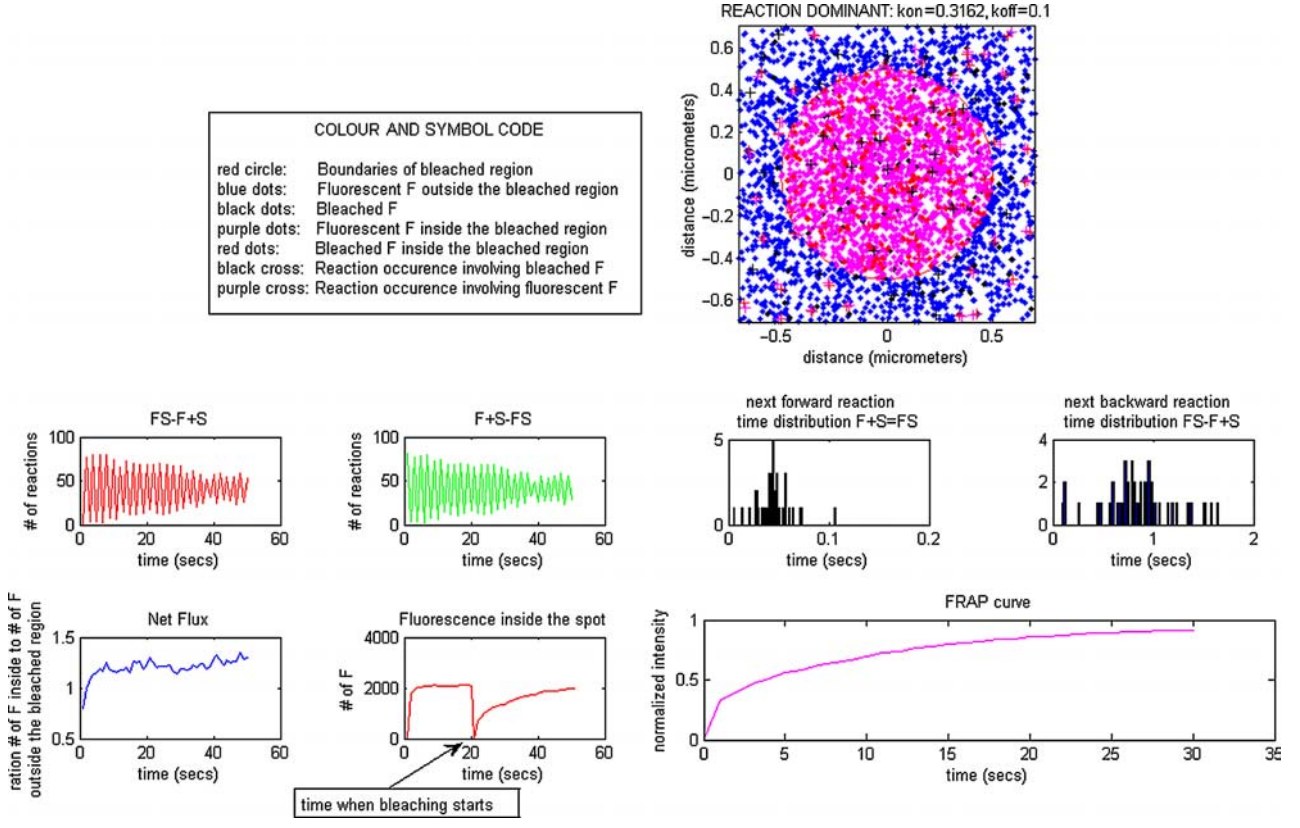

Figure 2. The proposed stochastic simulation algorithm for the reaction-dominant scenario.

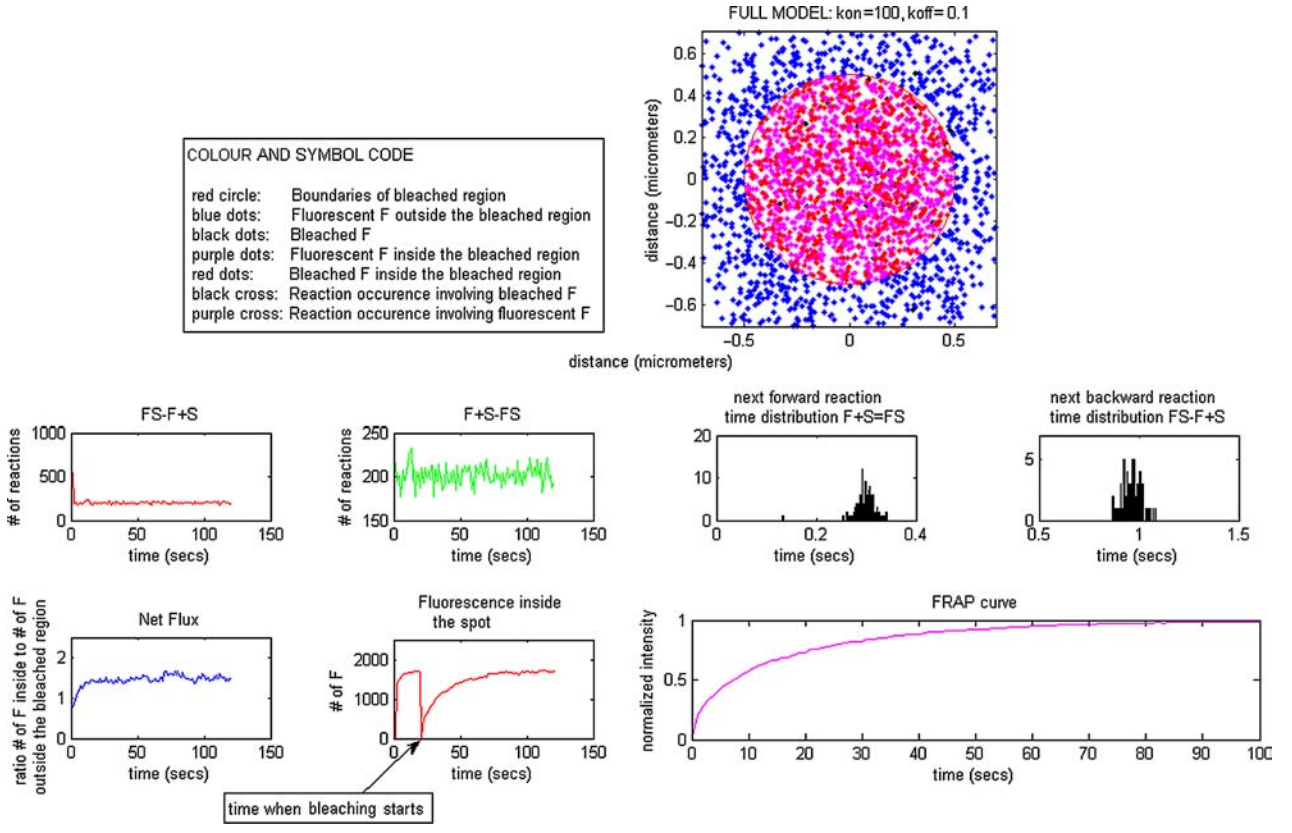

Figure 3. The proposed stochastic simulation algorithm for the full-model scenario.

3. Results and discussion

Figures 1–3 illustrate FRAP curves generated using the proposed stochastic simulation algorithm for the pure diffusion (Figure 1), reaction-dominant (Figure 2) and full-model (Figure 3) regimes. For pure diffusion conditions (i.e. $k_{on} = 10^{-2}\,s^{-1}$ and $k_{off} = 10^{1}\,s^{-1}$) $R = 0.84$, for effective diffusion (i.e. $k_{on} = 10^{3.5}\,s^{-1}$ and $k_{off} = 10^{0}\,s^{-1}$) $R = 0.94$, for reaction dominant (i.e. $k_{on} = 10^{-0.5}\,s^{-1}$ and $k_{off} = 10^{-1}\,s^{-1}$) $R = 0.71$ and for full model (i.e. $k_{on} = 10^{2}\,s^{-1}$ and $k_{off} = 10^{-1}\,s^{-1}$) $R = 0.76$. The above results regarding the sum of residuals indicate that FRAP curves, generated using the proposed method (see Figures 1–3), match those of the well-established Sprague binding-diffusion model. Additionally, comparison was performed in terms of time needed for 99% (t_{99}) recovery of fluorescence inside the spot for each scenario. Regarding the proposed method, for pure diffusion $t_{99}^{\text{diffusion\_dominant}} = 0.082\,s$, for effective diffusion and for reaction dominant $t_{99}^{\text{reaction\_dominant}} = 17.7\,s$. Regarding the Sprague model, predictions were $t_{99}^{\text{diffusion\_dominant}} = 0.083\,s$, $t_{99}^{\text{effective\_diffusion}} = 26.36\,s$ and $t_{99}^{\text{reaction\_dominant}} = 17.393\,s$. According to t-test, there were no statistical differences between the proposed method and Sprague's formula in terms of predictions of 99% recovery.

In the diffusion-dominant case, each F was found to cover a mean distance of 0.18424 μm/0.0005 s, while 99% recovery was 0.0082 s. In the effective diffusion case, i.e. when unbound F are detained from binding reaction, thus, fluorescence is recovered more slowly, recovery to 99% occurred after 25.5 s. Mean times for forward or backward reactions were found equal to 0.00023684 and 0.00017486 s, respectively, with 0.75768 reactions/s. Moreover, 67.8% of the total molecular concentration of F was found unbound, whereas the remaining 32.2% was found in the form of binding complex FS. In the reaction-dominant case, i.e. when diffusion is a negligible process, time needed for 99% of recovery was 45.1 s. Mean values of time distributions for the next most probable forward or backward reaction were found 0.00016634 and 0.00013668 s, respectively. Regarding the forward reaction, 0.064319 F were reacting per seconds, whereas regarding the backward reaction this ratio was $0.065025\,s^{-1}$. Under these conditions, more than 50% of F was detained by the binding sites S. Finally, for the full-model case, which is used for fitting of FRAP curves that do not fall under the three above scenarios, mean time for forward reactions was found equal to 0.00018618 s, while for backward reactions was equal to 0.00015898 s. Ratios of forward and backward reactions to total number of F per unit of time were 0.064014 and $0.064696\,s^{-1}$, respectively, while the ratio of free to bound F was 0.53467.

The proposed method presents several important advantages compared with other previous studies [3]: (a) it provides solutions for any kind of bleaching geometry, not only for uniform laser profiles and/or Gaussian laser profiles. (b) The coordinates of reactions (forward and backward) can be monitored in space and time, enabling, thus, without any special mathematical formulation, the interpretation of phenomena like anomalous diffusion. (c) The propensities of reactions change as a function of time based on the exact molecular concentration of participating species, in contrast to standard deterministic models, which assume reaction rates as constant, and forward–backward reactions as independent processes. Thus, the physical basis of the algorithm is firmer. (d) Estimation of the time distribution to next forward and/or backward reaction, of next event (diffusion, reaction or no event), estimation of exact values for stochastic reaction rates are based on distribution of propensities. Thus, the proposed method provides information that cannot be extracted using standard deterministic models [3,5,10,12,31].

In terms of computational burden, for low density starting molecular population, for the full-model case, the algorithm converges at 1500 s on PC with Pentium V 300 MHz processor and 512 MB RAM, in contrast to the deterministic model [3], which gives instantaneous estimations. Algorithms have been developed in custom MATLAB® code and are available upon request.

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