# A STOCHASTIC SIMULATION ALGORITHM FOR ANALYSIS OF FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING KINETICS

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## Abstract

**INTRODUCTION**: Fluorescence recovery after photobleaching (FRAP) is a confocal microscopy-based technique widely used for in vivo quantification of intracellular molecular movements and interactions. FRAP is very useful for elucidating several fundamental but complicated cellular activities, such as cell membrane diffusion and protein binding. **AIM**: The aim of this study was to investigate whether it is possible to develop stochastic simulation strategies for interpretation of FRAP kinetics. **METHODS**: A simulation algorithm based on a stochastic simulation of the time evolution of coupled reaction-diffusion biochemical systems was developed for investigating and interpreting FRAP experiments in terms of diffusion and binding. The proposed algorithm was compared with standard deterministic methods that are currently being used for analysis of FRAP curves. **RESULTS AND DISCUSSION**: Predictions of recovery times of FRAP curves and sum of residuals revealed a good agreement (Table I), at the level of both timescale and intensity, between the proposed model and the standard deterministic methods. The stochastic simulation algorithm presents a firmer physical basis that its deterministic counterparts and 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*

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# Introduction

Fluorescence recovery after photobleaching (FRAP) is a confocal microscopy-based technique widely used for in vivo quantification of intracellular molecular movements and interactions [1, 2]. FRAP is very useful for elucidating several fundamental but complicated cellular activities, such as cell membrane diffusion and protein binding. The basic idea of FRAP can be summarized into three steps: a/ the molecules within the observing specimen (usually in the cell for in vivo studies) are labeled with fluorescent probes, b/ a Region Of Interest (ROI) is bleached by exposure to proper exciting laser light; this process renders labeled molecules within the bleached ROI permanently non-fluorescent, c/ due to diffusion, the fluorescence is gradually restored within the bleached region; the rate of recovery of fluorescence is monitored giving rise to the FRAP curve, which contains information regarding both the diffusion potential and the binding interactions of the molecules of interest with the specimen's environment [3, 4]. In the absence of binding reactions, fluorescence is rapidly restored and the FRAP curve can be used to yield information regarding the diffusion rate of the molecules. On the other hand, longer FRAP recoveries usually imply that molecules' diffusion is delayed due to binding reactions [5, 6].

Deciphering the information from FRAP curves is not a straightforward task. There have been proposed several mathematical models [1, 3, 7-13], which have been formulated for proper interpretation of FRAP recoveries. Although these models differ in terms of mathematical basis, it is generally agreeable that three dominant scenarios might be used to describe any FRAP recovery involving a single species of molecules able to either freely diffuse or/and bind to a specific type of binding sites: pure diffusion dominant, effective diffusion, and reaction dominant scenarios. In the case of pure diffusion almost all fluorescent labeled molecules are assumed free to diffuse (in this case recovery for most biological molecules lasts less than 1 sec). The observed recovery can be described by a simple diffusion equation, which can be used to quantify the diffusion coefficient of the molecule into consideration [14]. When binding occurs faster than diffusion, then the recovery is slowed down due to the presence of binding sites that detain fluorescent labeled molecules from moving freely. Under the latter conditions, the recovery can be described by a new diffusion coefficient, the so called effective diffusion coefficient, which presents a reduced value compared to the free diffusion coefficient [3, 15]. The third scenario assumes that diffusion is faster than binding, so fast that it can be barely monitored; the recovery, then, depends mainly by the association and dissociation binding coefficients, which describe the association rate of a free molecule with a binding site and the dissociation rate of a bound complex to a free molecule and an empty binding site. In the reaction dominant and effective diffusion cases, recovery is of the order of seconds to minutes [16, 17]. There have also been presented models, such as in Sprague et al [7], that have been developed as an unified approach able to describe all possible FRAP recoveries in the presence of diffusion and single binding site using a single mathematical approach.

Although FRAP experiments involve confocal microscopy, thus, 3-D volumes are considered, due to the complexity of analytical solutions for the 3-D case, most of previous studies have been formulated for 1-D [9], or 2-D processes [1, 8, 18], assuming that such solutions approximate to a great extent the realistic 3-D solution. Only few studies have explored 3-D solutions [7, 19]. Moreover, most

studies have explored only circular bleached spots and have considered homogeneous distributions of diffusing molecules and binding sites [1, 3, 7-13]. There is no unified mathematical approach suitable for all geometries. The choice of the proper model depends on many aspects, among which, critical might be considered the geometry and size of the bleached region, the presence/absence of binding reactions, the number of different types of binding sites, and the dimensionality of the formulation. Improper model choice might lead to biased estimates of both diffusion and binding coefficients as it has been shown in recent literature [13, 20].

Mathematical models described above share a common reference characteristic: these models are based on continuous approximations and traditional ordinary differential equations, which on one hand have been successfully used for describing reaction-diffusion kinetics of biochemical systems, but on the other hand present four important limitations [21-24]: First, it is well known that living cells pose very low densities (of the order of $1-10^2\,\mu m^{-3}$) of most key biomolecules (including proteins, transcription factors, DNA, molecular regulators etc.) and due to these low molecular densities, many important cellular events, such as gene expression and polymerase binding, are governed by stochastic effects; such effects can be used to explain the variability of various phenomena at the molecular level for isogenic populations as, for example, the difference in expression of the same genes among cells sharing the same genetic material [25, 26]. Continuous approximation models are inappropriate for investigation of low density populations [24, 25, 27, 28], since only average behaviors are considered. Second, continuous approximation models treat binding, which involves association and dissociation of biomolecules with suitable binding sites, as a continuous process in time. The latter has been questioned in literature [25, 26], since it is well know that binding occurs at discrete time events. For high density populations discrete events become less prominent. However, for low density populations, such as at intracellular molecular level, the application of continuous approximation models 'smoothes out' these important discrete fluctuations [29, 30]. Third, 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 [24, 25]. Fourth, the mathematical formulation of continuous approximation methods changes significantly according to the shape of the geometry under investigation [20, 31]. For complex geometries, analytic solutions are difficult to compute. Thus, mathematical models based on stochastic time evolution appear as plausible, more realistic, than continuous approximations solutions to overcome the above limitations; stochastic simulation algorithms, properly formulated, might be a useful tool for describing and interpreting the information encoded in FRAP experiments regarding molecular populations that are distributed into the cell heterogeneously at low densities. To the best of our knowledge, such a stochastic model has not been presented in literature.

The aim of this study was to investigate whether it is possible to develop stochastic simulation strategies for interpretation of FRAP kinetics. The add-on value of such an approach is threefold: a/ stochastic simulation can be used for reliable estimation of reaction-diffusion biochemical systems involving molecular species occurring at low densities, b/ in the presence of binding, reaction rates are not considered constant as in continuous approximation models. Stochastic reaction rates are combined

into a single probability density function taking into account the inherent fluctuations of the binding process in space and time and are described on a unified framework; the latter scenario is closer to realistic conditions [29], in contrast to continuous approximation models that consider association and dissociation as independent processes occurring in a continuous manner in space and time, c/ the stochastic model is independent on the geometry and size of the bleached spot, in contrast to standard models that require special formulations for different geometries (i.e. circular, stripe etc.) [14].

## Material and Methods

***Diffusion model***

Diffusion was seen as a Brownian motion process. The Brownian motion is due to collisions with molecules (i.e. water molecules), which makes the particles undergo random-walk motion with no preferred direction. The average displacement of a molecule depends on the diffusion coefficient and, additionally, on the time diffusion step. The probability of a random motion is given by [32]:

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

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$^{o}$C.

**Reaction model**

The binding reaction process was seen as a numerical Monte Carlo procedure based on the pioneer work of Gillespie [27]. The basic idea of the Gillespie's algorithm is to track the exact molecular population of chemical species, which are able to interact via multiple reaction channels in a fixed volume. The Gillespie's algorithm was originally proposed to simulate coupled chemical reaction systems, while later efforts have extended this algorithm to coupled reaction-diffusion biochemical systems [24]. In this study we have suitably modified the Gillespie's algorithm for simulating FRAP recoveries as follows:

In a FRAP experiment, considering well-stirred distribution of the fluorescent labeled molecule $F$ at fixed temperature $T$, in a fixed volume $V$ inside the cell, which contains homogeneously distributed binding sites $S$, every free molecule $F$ may react with any vacant $S$ to create the complex $FS$ according to:

$$F + S \underset{koff}{\overset{kon}{\rightarrow}} FS \quad (2)$$

This can be broken down into two elementary reactions:

$$\text{Reaction 1:} \quad F + S \xrightarrow{kon} FS \tag{3}$$

$$\text{Reaction 2:} \quad FS \xrightarrow[koff]{} F + S \tag{4}$$

where $kon$ and $koff$ are the association and dissociation coefficients respectively. The stoichiometry of such system will be

$$\begin{bmatrix} [F] \\ [S] \\ [FS] \end{bmatrix} \Rightarrow \begin{bmatrix} -1 & 1 \\ -1 & 1 \\ 1 & -1 \end{bmatrix} \tag{5}$$

Equation 5 gives the net flow of populations during reactions. For example, if Reaction 1 occurs, then the populations of *F* and *S* are reduced, whereas the population of *FS* is increased. For such a system the time evolution of the populations would depend from starting concentrations and reaction coefficients. The time *t* to next reaction or next release is assumed to be exponentially distributed according to $P_0(t)$:

$$P_0(t) = \exp(-\sum_{\nu=1}^{M} a_\nu t) \tag{6}$$

where *M* denotes the type reaction (Reaction 1 or Reaction 2, equations 2 and 3 respectively), and *a* is the propensity for a particular reaction. The term propensity reflects the ability of the system to restore its equilibrium and it depends on the current state of molecular populations and the association and dissociation coefficients. Following any perturbation that changes the molecular population distribution over time -in our case this perturbation is diffusion- the system tries to restore its steady state by performing vibrations around its equilibrium state. Equation 6 basically gives the probability that a particular reaction will happen in the next infinitesimal time interval *t*. The expectation or mean value of such distribution is given by $mean = 1/sum(a)$. A plot of such probability from the simulation is given in Figure 8.

***Reaction-diffusion simulation algorithm***

The simulation starts by placing *F (free), S (binding sites)* randomly to the full extent of the simulation field (we have tested circular and strip-like spots with radius and width of 0.5 $\mu m$ respectively. Such settings are common in FRAP experiments [7]). *F* are allowed to diffuse. *S* are considered immobile. Every *F* is allowed to react with an empty *S* forming and *FS* complex. Then as

the simulation proceeds, it gives some time to the system to reach a steady state-equilibrium. This is needed because the initial choice of the concentrations is user defined and does not guarantee steady state conditions (either for diffusion or reaction). After the steady state has been reached, the region of interest (either circular or strip-like) is bleached (after this point, all *F* inside the bleached region are considered as non-fluorescent) and the FRAP curve is measured. The simulation filed is significantly bigger than the bleached spot. More specifically, the steps that are required for implementation of the proposing stochastic simulation are:

INITIALIZATION

1. Set time $t$ =0
2. Set initial numbers for molecular populations $N_F$, $N_S$, $N_{FS}$
3. Calculate concentrations according to $[C_i]=\frac{N_i * 1e9}{V * A * 1e-9}(nM)$, where $N$ denotes the number of molecules at time $t$=0, $i$ denotes the different chemical species, $V$ is the volume and $A$ the Avogadro number
4. Set initial values for association ( $kon$ ) and dissociation ( $koff$ ) coefficients
5. Set initial value for the diffusion coefficient $D$
6. Set the geometry and size of the bleached spot and of the simulation field. The simulation field should be at least 50 times bigger than the bleached spot [24, 27]
7. Place within the simulation field randomly, inside and outside the bleached spot, molecules *F*, and binding sites *S*. There are no initial *FS*. Store coordinates of initial positioning
8. Set time sapling step $t$, time $t_{bleach}$ (time that bleaching occurs; from this time and on, recording of the recovery inside the simulated bleached spot is initiated), and time $t_{stop}$ (time for terminating the simulation). $t_{bleach}$ should be greater than the time that the system needs to restore equilibrium. $t_{stop}$ should be greater than the time needed to restore 99% of initial fluorescence inside the bleached spot

FOR THE REACTION SYSTEM

9. According to *P(t)* (equation 6) calculate the most probable next event (Reaction 1 or Reaction 2 – see equations 3 and 4 respectively).
10. Based on the selected event, update the current molecular populations of $N_F$, $N_S$, and $N_{FS}$ according to stoichiometry of the system (see equation 5)
11. Update the status at each coordinate (initialized at step 8) according to the following three scenarios: a/ If at a specific coordinate a new *FS* complex has been created, then label this particular coordinate as *FS*, which means that at the next event after time $t$ only a dissociation of *FS* to *F* and *S* may occur at this coordinate. b/ If an *FS* complex dissociates to an *F* and *S*, then label this particular coordinate as *F*; this *F* will be able to diffuse or react at next event after time $t$. Moreover, label this particular coordinate as *S*, which means that an empty binding site exists, which can react with a free diffusing *F* at next event after time t.

FOR THE DIFFUSION SYSTEM

12. Allow free *F* to diffuse randomly and cover distance r based on to *P(r, t)* (equation 1).
13. Update the status at each coordinate as in step 11.

TIME EVOLUTION

14. Progress time step *t*.
    a. If $t < t_{bleach}$, repeat steps 11-14
    b. If $t >= t_{bleach}$, then label each *F* inside the bleached spot as non-fluorescent as and all remaining *F* outside the spot as fluorescent. Generate a point at the FRAP curve for time *t* as follows: $FRAP_{curve}(t) = \frac{F^0_{simulation-field} F_{bleached-spot}(t)}{F^0_{bleached-spot} F_{simulation-field}(t)}$ [33], where $F^0_{simulation-field}$ is the number of fluorescent *F* inside the simulation field at *t=0*, $F^0_{bleached-spot}$ is the number of fluorescent *F* inside the spot of interest at *t=0*, $F_{bleached-spot}(t)$ is the number of fluorescent *F* inside the bleached spot of interest at *t*, and $F_{simulation-field}(t)$ is the number of fluorescent *F* inside the simulation field at *t*. Repeat steps 11-14.
    c. If $t < t_{stop}$, then terminate simulation

**Comparison with other methods**

The performance of the proposed stochastic simulation algorithm was compared with the model proposed by Sprague et al [7], which can be used to describe all possible scenarios of FRAP recoveries. According to the above model, the recovery of any FRAP curve involving circular bleached spot and a single type of binding sites is given by:

$$FRAP_{curve}(t) = \frac{1}{p} - \frac{F_{eq}}{p}(1 - 2K_1(qw)I_1(qw))x(1 + \frac{k_{on}}{p + k_{off}}) - \frac{C_{eq}}{p + k_{off}} \quad (7)$$

with

$$q^2 = (\frac{P}{D})(1 + \frac{k_{on}}{p + k_{off}}) \quad (8)$$

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 1st and 2nd 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 and predictions of 99% of recovery.

# Results

***Simulation initializations***

Simulations were performed with starting molecular conditions [F] = 0.041513 nM, [F] = 3.3626 nM, and [FS] = 0.041513 nM in a reaction volume of 10 pl considering steady temperature conditions, homogeneous molecular distributions and a single binding site, circular bleached spot, and diffusion coefficient D=30 $\mu m^2 / \sec$ .

***Steady state conditions of the reaction system***

For the pure diffusion case (for $k_{on}$=$10^{-2}$ $sec^{-1}$ and $k_{off}$=$10^{1}$ $sec^{-1}$), the system reached equilibrium at 0.4 sec, for the effective diffusion case (for $k_{on}$= $10^{3.5}$ $sec^{-1}$ and $k_{off}$ = $10^{0}$ $sec^{-1}$) at 0.02 sec, and for the reaction dominant case (for $k_{on}$= $10^{-0.5}$ $sec^{-1}$ and $k_{off}$=$10^{-1}$ $sec^{-1}$) 45.1 sec and for the full model case (for $k_{on}$= $10^{2}$ $sec^{-1}$ and $k_{off}$= $10^{-1}$ $sec^{-1}$) 5 sec. Results are given in Figure 1.

*Comparison with deterministic models*

In the case of pure diffusion (Figure 2) the time needed for 99% recovery of fluorescence inside the spot was 0.0082 sec, for effective diffusion (Figure 3) 25.5 sec, and for reaction dominant (Figure 4) 17 sec. Relative predictions for the above scenarios were obtained using deterministic model as described in [7] (equation 7). According to this model, recovery times were 0.083 sec, 26.36 sec, and 17.393 sec respectively for the pure diffusion, effective diffusion and reaction dominant scenarios. Additional simulations were performed for the full model case (Figure 5). Figure 6 illustrates the resulting probability density function for the reaction system (equation 6).

The goodness of fit between the proposed stochastic model and the existing model of equation 7 [7] is presented at table I. The goodness of fit was quantified by the sum of residuals ($R$) of the two recovery curves, for each scenario (see Figures 2-5), as proposed in [34] according to the following expression:

$$R = \sum_{t=1}^{m} \left| frap_{stochastic}(t) - frap_{deterministic}(t) \right| \quad (9)$$

**Table I: Comparison of the proposed stochastic simulation algorithm with the deterministic model [7] of equation 7, in terms of Sum of residuals for different FRAP scenarios**

| **Scenario** | **Sum of residuals (*R*)** |
|---|---|
| Diffusion dominant | 0.84 |
| Effective diffusion | 0.94 |
| Reaction Dominant | 0.71 |
| Full Model | 0.76 |

After having confirmed that the proposed stochastic model gives reasonable results and agrees with other standard methods, experiments were performed to investigate the effect of the bleach spot geometry to the prediction of the recovery. We have simulated circular spots of different radius and we

have compared the recoveries prediction for a circular and strip spot of the same area for the pure diffusion case. Results are presented in Figures 7 and 8. Finally, table II gives a full report of information that can be extracted using the proposed algorithm for the effective diffusion case.

**Table II: Analytic presentation of information that can be obtained using the proposed stochastic simulation algorithm for the effective diffusion scenario**

| | | |
|---|---|---|
| Reaction coefficients | $k_{on} = 10^{3.5}$ sec$^{-1}$<br>$k_{off} = 10^{0}$ sec$^{-1}$ | |
| Sampling step | 0.5 sec | (time step that the FRAP curve was sampled) |
| Starting Concentrations for each compartment | [F] = 0.041513 nM<br>[S] = 0.033211 nM<br>[FS] = 0.041513 nM | In a reaction volume of V=10pl |
| Total starting concentrations | [F] = 3.3626 nM<br>[S] = 2.6901 nM<br>[FS] = 3.3626 nM | $concentration = \frac{CountOfMolecules_i * 1e9}{V * Avogadro * 1e-9}(nM)$, *i* denotes the different chemical species |
| Reaction Volume | V = 10 pl | |
| Recovery prediction | *Radius=0.5 μm*: 25.5 sec<br>*Radius=1.1 μm*: 130.5 sec | (time needed for 99% recovery) |
| Reaction equilibrium | ≈0.02 sec | Time that Reaction Equilibrium is reached for any possible starting population considering volume V is fixed |
| $F + S \xrightarrow{kon} FS$<br>If a reaction will happen, then it will happen at a mean time after the previous event | After equilibrium:<br>0.00023684 sec | |
| $F + S \xrightarrow{kon} FS$<br>If a release will happen, then it will happen at a mean time after the previous event | After equilibrium:<br>0.00017486 sec | |
| $\frac{Number\_of\_reactions}{Feq + FSeq}$ | 0.75768 /sec | In equilibrium state for a fixed Volume V |
| $\frac{Number\_of\_releashes}{Feq + FSeq}$ | 0.75768 /sec | In equilibrium state for a fixed Volume V |
| Ratio of free to bound molecules | 0.32223 | In equilibrium state for a fixed Volume V |

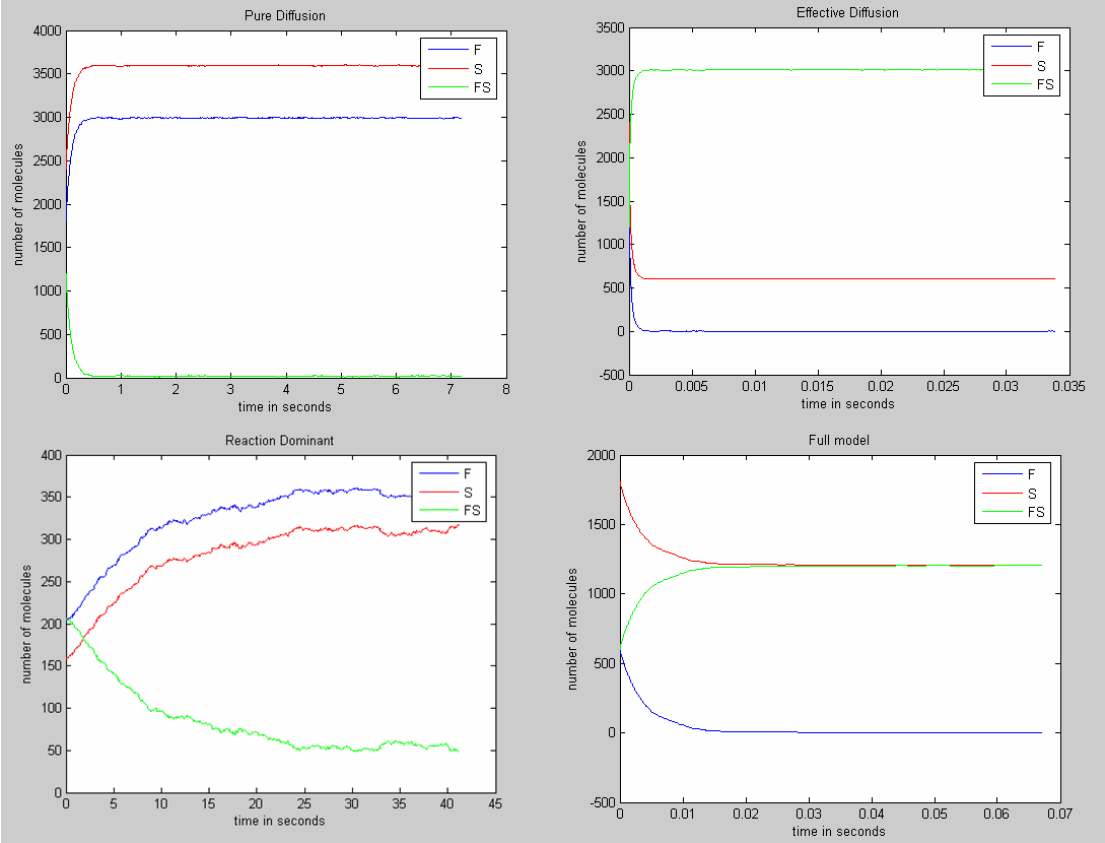


**Figure 1:** Convergence to equilibrium for the pure diffusion, effective diffusion, reaction dominant and full model scenarios.

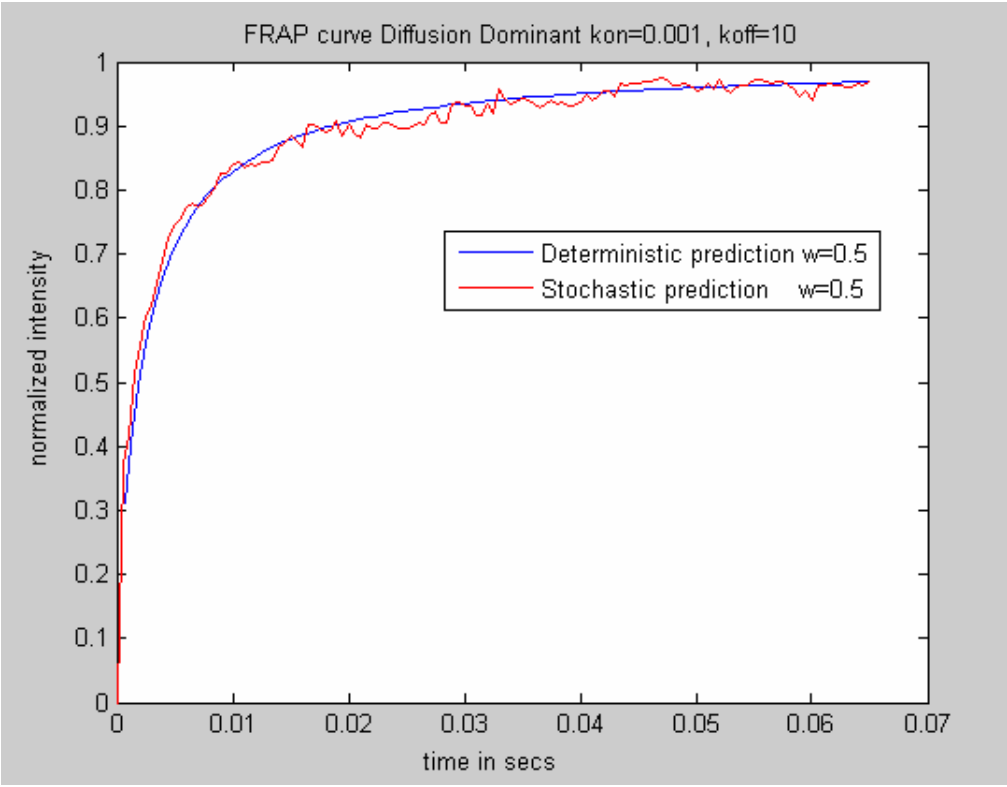


**Figure 2:** Comparison of the proposed stochastic simulation algorithm with the deterministic model of equation 7 for the pure diffusion scenario

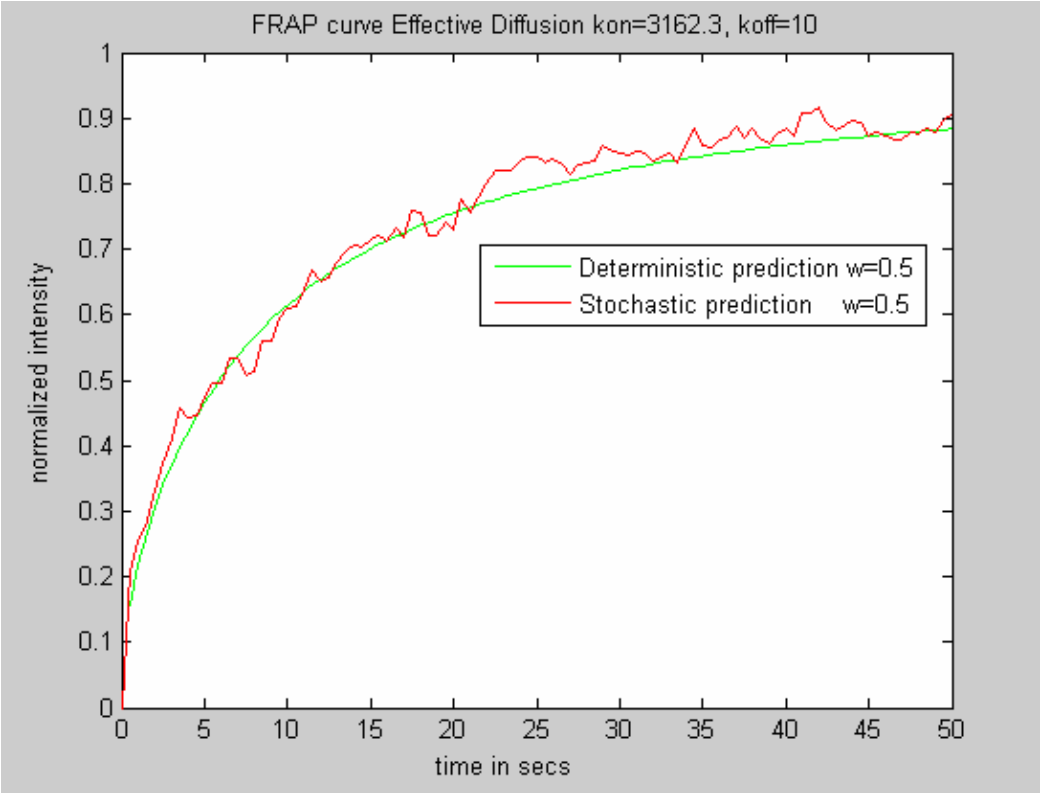


**Figure 3:** Comparison of the proposed stochastic simulation algorithm with the deterministic model of equation 7 for the effective diffusion scenario

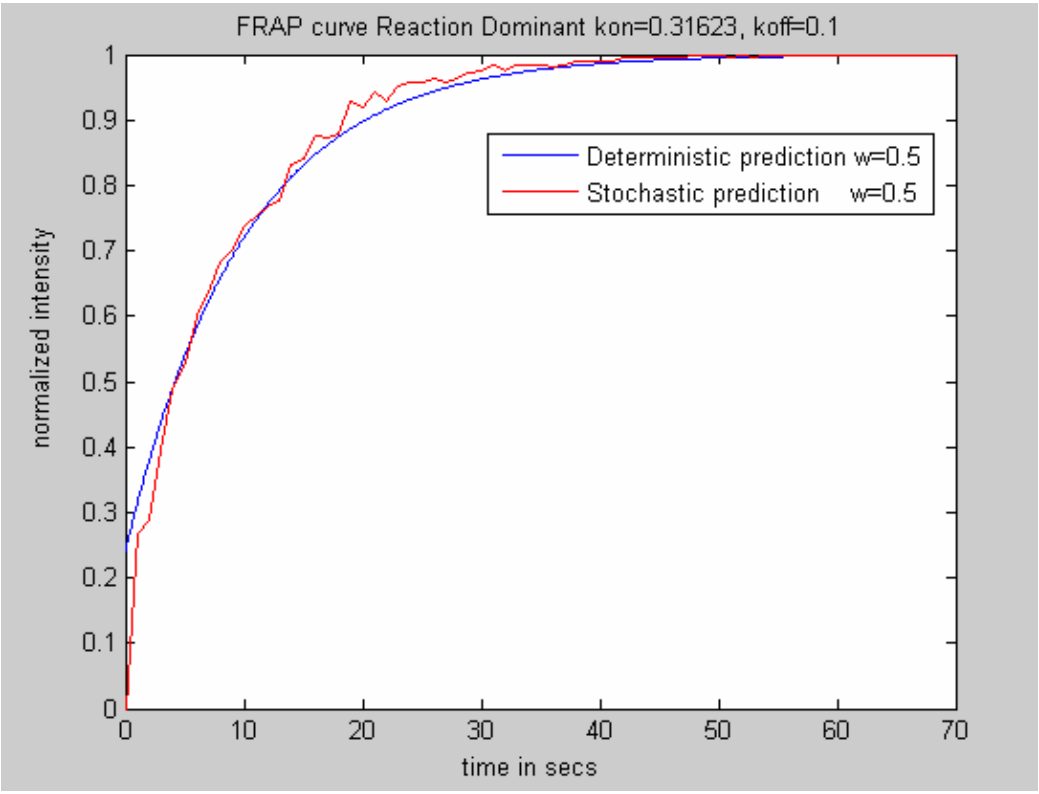


**Figure 4:** Comparison of the proposed stochastic simulation algorithm with the deterministic model of equation 7 for the reaction dominant scenario

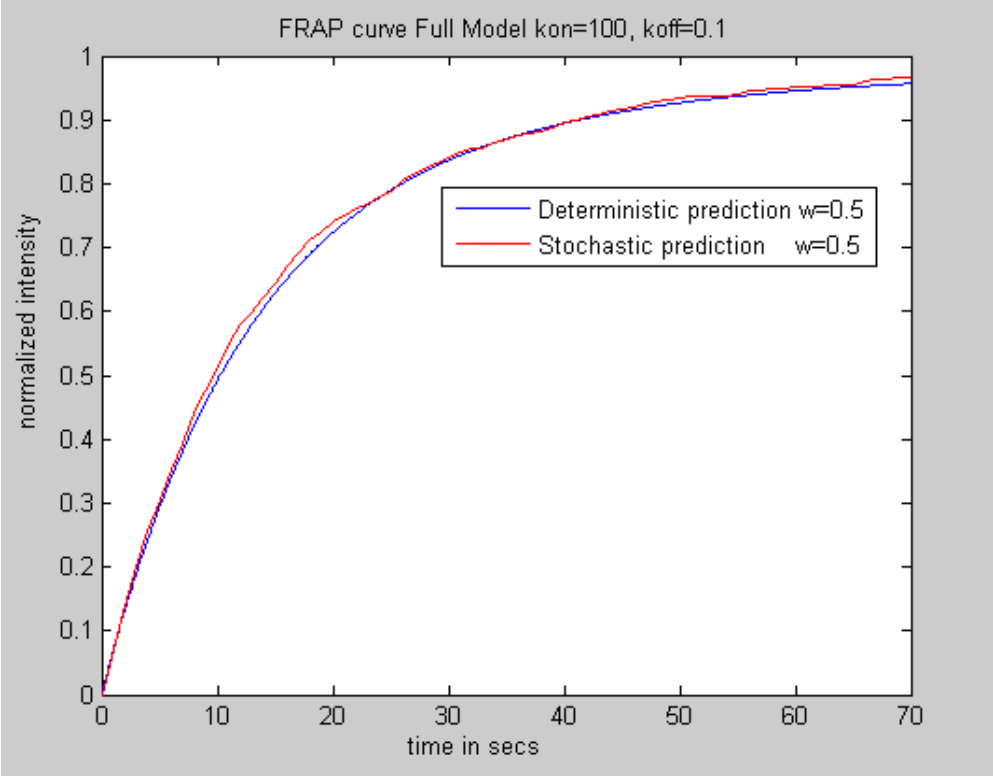


**Figure 5:** Comparison of the proposed stochastic simulation algorithm with the deterministic model of equation 7 for the full model scenario

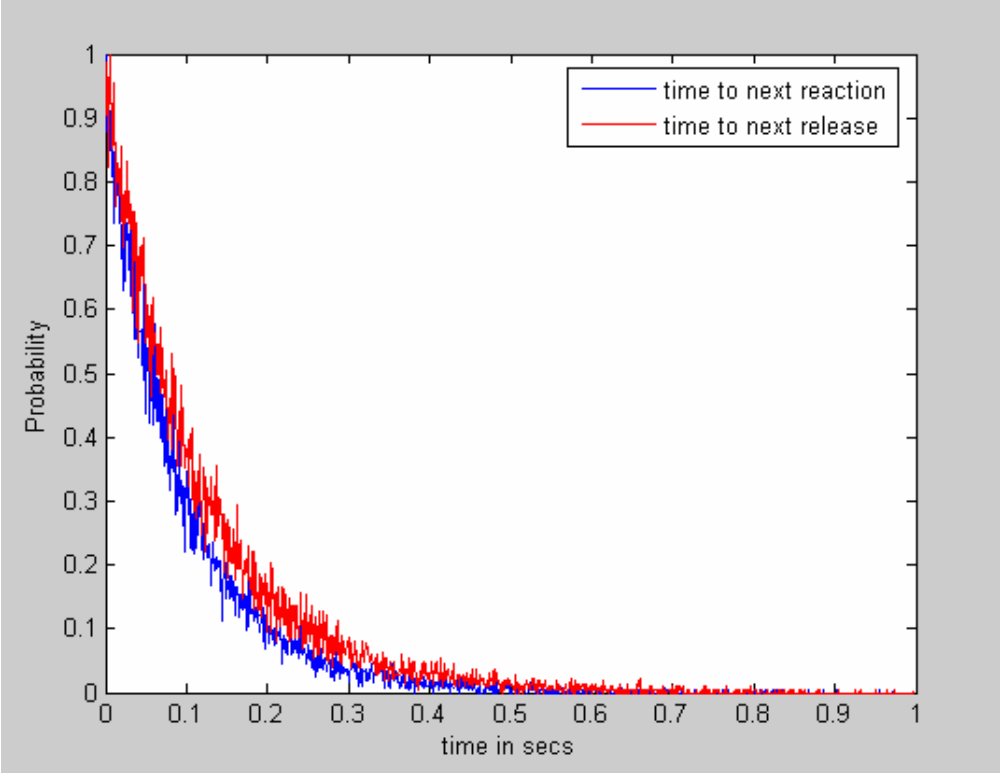


**Figure 6:** Probability density function of the Gillespie algorithm (equation 6). The red line gives the probability of occurrence of a reaction of the type $F + S \xrightarrow{kon} FS$, whereas the blue line gives the probability of occurrence of a release of the type $FS \xrightarrow[koff]{} F + S$

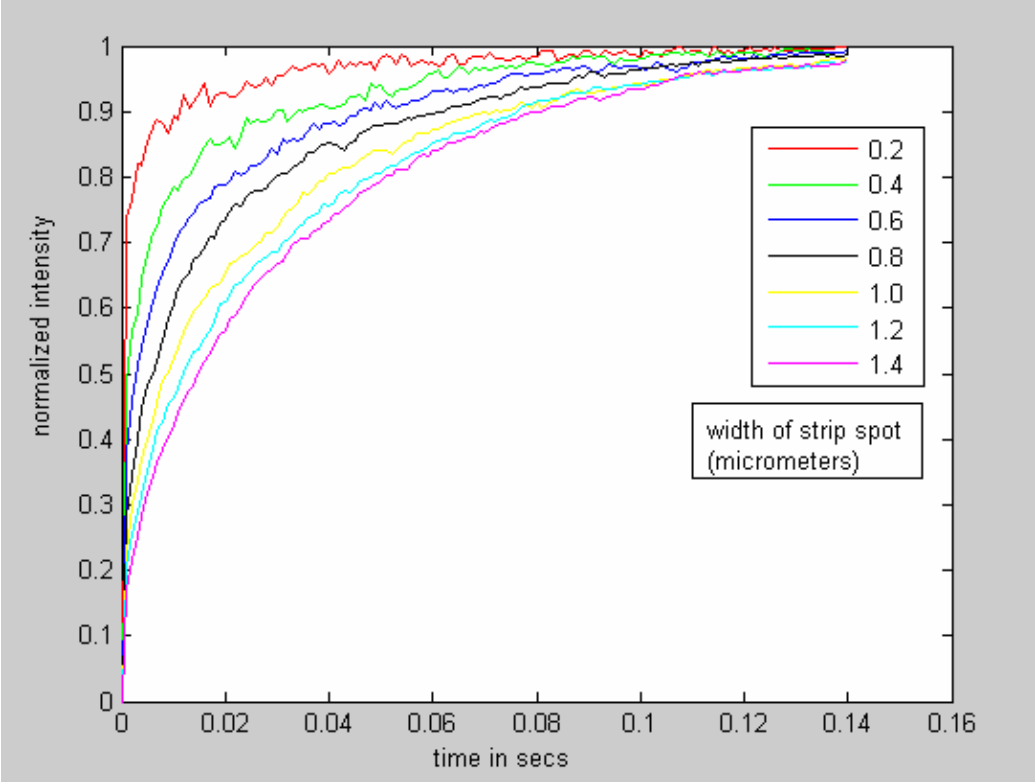


**Figure 7:** Effect of the size (radius) of circular bleached spots to the recovery of fluorescence in the bleached region

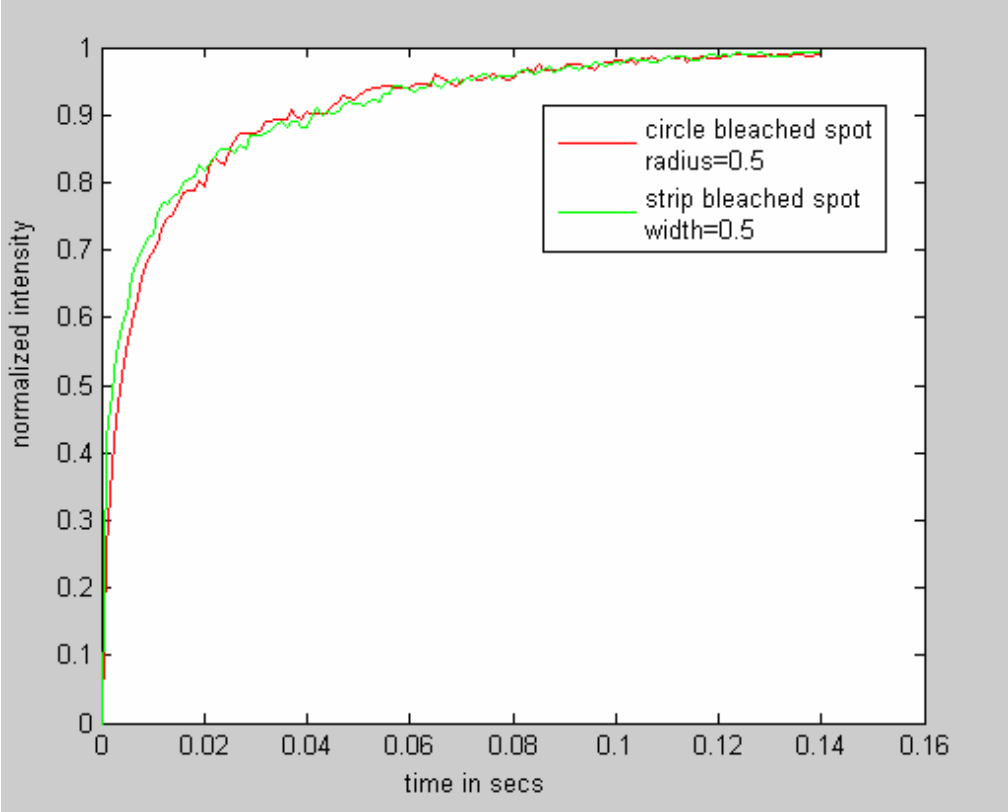


**Figure 8:** Comparison of recoveriesw resulted from circular and strip-like bleached spots of the same area

## Discussion

In this study, a stochastic model was presented for numerical simulation of FRAP experiments. The model was compared with standard methods presented in literature [1, 7, 34], with promising results. Predictions of recovery times and sum of residuals revealed a good agreement (Table I), at the level of both timescale and intensity. Figure 2 illustrates the diffusion dominant case, which agrees with the solution of equation 7 with sum of residuals 0.84. Reaction equilibrium was reached at about 0.4 sec for any possible starting molecular concentrations for a fixed volume of V=10pl. Diffusion dominates, with each diffusing protein covering a mean distance of 0.18424 μm/0.0005sec. Time for 99% recovery was 0.0082 sec. In the effective diffusion case (Figure 3), agreement with the solution of equation 7 resulted in 0.94 sum of residuals. Recovery to 99% of fluorescence occurred after 25.5 sec, while steady state was restored in 0.02 sec. In this case, bleached molecules *F* find it hard to enter or escape the bleached spot since, almost after a every single dissociation, a fast association follows. If an association will occur, then it will occur at 0.00023684 sec after the previous event. On the other hand, if a dissociation will occur, then it will happen after 0.00017486 sec. The number of associations and dissociations divided by the molecular population of *F* is 0.75768 reactions /sec. Moreover, the ratio of free *F* to bound *F* (in the form of *FS*) was 0.32223. In Figure 4 the reaction dominant scenario is investigated. The agreement with the model of equation 7 in terms of sum of residuals was 0.71. Equilibrium was accomplished at 4 sec, whereas time needed for 99% of recovery was 45.1 sec. The slower recovery is due to binding reactions that delay *F* from diffusing. Under these conditions, diffusion can be considered negligible. The next most probable event (association, dissociation or no event) was determined based on a probability density function (equation 6), which depended on both association and dissociation coefficients and the current number of molecular populations. This probability is exponentially distributed as shown at Figure 8. Based on this probability, most probable times to next association and dissociation were determined 0.00016634 sec and 0.00013668 sec respectively. The ratio of associations to total population of *F* in equilibrium per unit of time was 0.064319 /sec, whereas the ratio of dissociations to total population of *F* per unit of time was 0.065025 /sec. The ratio of free to bound *F* in equilibrium was found 0.56734. The latter means that more than 50% of *F* are detained by binding sites *S*. Finally, for the full model case (Figure 5), a good fit was found (sum of residuals 0.76) with the model of equation 7. The full model case is used for describing FRAP recoveries that cannot be explained by any of the three main scenarios. For the full model, reaction equilibrium was achieved after 5 sec. Next association will occur after 0.00018618 sec and next dissociation after 0.00015898 sec. The total number of reactions per equilibrium concentration of free and bound F per unit of time was 0.064014 /sec and the respective ratio of dissociations per unit of time was 0.064696 /sec. Finally, the ratio of free to bound F was 0.53467. It worth noticing that the algorithm makes no assumptions for equilibrium concentrations, but converge to equilibrium concentrations independently on the initial molecular populations, which are user defined. The deterministic models, such that of equation 7 require assumptions for estimating equilibrium concentrations.

Figure 7 illustrates the effect of the size of circular bleached spot to recovery. The larger the diameter of the spot the longer fluorescence recovers. Similar predictions are obtained by standard deterministic models [1, 7, 34]. In figure 8 a comparison is performed for circular and strip-like bleached regions. The recovery for strip-like regions depends on the width of the strip and we get the same results with a circular bleach spot only if the width of the strip equals to the radius of the circular spot. It is worth noticing that the proposed stochastic simulation algorithm needs no special formulation for strip-like or any other shape bleached regions, in contrast to standard deterministic methods [3, 7, 34, 35].

The above results might be regarded as an indication that the proposed stochastic simulation may be used for proper interpretation of FRAP. To the best of our knowledge, this effort comprises the first investigation towards stochastic simulation of FRAP experiments. The add-on value of the proposed model lies on two major issues: a/ firmer physical basis, and most importantly, b/ extraction of additional information that cannot be estimated using the deterministic methods presented in literature.

The firmer physical basis is due to the following: i/ Stochastic reaction rates change dynamically according to the current state of the population of reactant species and are not assumed constant, ii/ Association and dissociation are not considered as independently occurring processes, but are combined into a single probability density function, accounting in this way for inherent fluctuations and correlations of binding in time. The probabilistic manner of the proposed method enables the description of the reaction system under simulation by a unique probability density function, according to which the next most probable event is decided: will it be an association of the type $F + S \xrightarrow{kon} FS$, between a free diffusing *F* with an empty binding site *S*, will it be a dissociation of a binding complex of the type $FS \xrightarrow[koff]{} F + S$ to a free diffusing *F* and to an empty binding site *S*, or a random diffusion will occur?

Moreover, information that can be extracted using the proposed stochastic simulation algorithm but cannot be extracted by standard deterministic methods are: i/ an exact estimation of molecular concentrations at equilibrium conditions, ii/ the coordinates of associations and dissociations, which make possible, without any special mathematical formulation, to investigate phenomena like anomalous diffusion (i.e. the diffusing proteins form aggregates that exhibit different diffusion coefficients), iii/ the recovery of the system to equilibrium following any perturbation (such as diffusion in our case) can be investigated in space and time, iv/ Dynamic estimation of time interval-distributions regarding next association or dissociation, as well as the ratio of free to bound *F* in equilibrium and the number of associations and dissociations per unit of time (see table II), v/ it is possible to quantify and interpret the fluctuations of molecular populations.

The computational burden of the algorithm depends on the starting molecular populations and the time sampling step. For low density concentrations of the order of 1000 molecules, for the full model scenario, the algorithm converges at 1500 sec, in contrast to the deterministic model of equation 7, which gives instantaneous estimations.

## Future work

The proposed stochastic simulation algorithm can be used to interpret FRAP experiments considering geometries of various sizes, well-stirred conditions, fixed volume, steady temperature and single binding sites. Future efforts should concentrate on testing this algorithm on real and simulated FRAP curves, expand the algorithm to spatially inhomogeneous systems, modify the algorithm for application in realistic 3-D scenarios and consider more than one binding sites.

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