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**Interpretation of binding kinetics in Fluorescence Recovery
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Interpretation of binding kinetics in Fluorescence Recovery After Photobleaching experiments using a novel stochastic simulation strategy

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Abstract: Fluorescence recovery after photobleaching (FRAP) has been extensively used for monitoring of binding kinetics of proteins with goal the investigation of 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*

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Introduction

Fluorescence Recovery After Photobleaching (FRAP) has been extensively used for motoring of binding kinetics of key biomolecules with goal the investigation of several important cellular process, such as transcriptional regulation, cell membrane diffusion, and signal transduction [1, 2]. FRAP is performed into two 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 relating with the association and dissociation coefficients of binding. The latter comprises the basis for investigating binding of proteins using FRAP. Probably the most popular model for analysis of FRAP curves has been proposed by Sprague et al [3]. The latter model predicts four main different scenarios: a/ pure diffusion – fluorescent tagged molecules diffuse freely (recoveries in less than 1 sec). The FRAP curve, in this case, can be described by a simple diffusion equation [3], which can be used to compute the diffusion coefficient of involving species.

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3 b/ Effective diffusion – binding sites detain fluorescent tagged molecules resulting in a
4 slower FRAP recovery, which can be described by a new diffusion coefficient, the so
5 called effective diffusion coefficient [4, 5]. c/ Reaction dominant – fluorescent tagged
6 molecules diffuse rapidly. Diffusion is so fast that it cannot be observed in the recovery;
7 the resulting FRAP curve can be used to extract information regarding reaction rates.
8 Recoveries, in this scenario, last from seconds to minutes [6, 7]. d/ Full model – the full
9 model covers any other regime than the above three.
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20 There have been proposed several mathematical models for extracting information
21 from FRAP curves [1, 5, 8-14]. These models suggest either numerical solutions based on
22 fitting, or Laplace transform, or analytical solutions. There have been proposed
23 mathematical treatments for 2-dimensional or 3-dimensional formulations for various
24 bleaching spot geometries; among the most popular are circular, strip, and Gaussian
25 profile geometries. In order to select the proper model for the analysis of a given FRAP
26 curve, several important factors need to be assessed, such as, the dimensionality and
27 shape of geometry, the number and type of binding sites, the presence or absence of
28 reactions, and the association and dissociation coefficients of suspected reactions. It has
29 been shown [14, 15] that selection of proper model plays an important role on both the
30 correctness and validity/accuracy of the analysis.
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46 Although the above models have been successfully used for analysis of FRAP curves,
47 they present important limitations [16-19]: a/ Proposed numerical and/or analytical
48 models are based on ordinary differential equations and rely on continuous
49 approximations. It is well known that such approaches are excellent for describing
50 phenomena of high density populations; however, most key biological molecules within
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3 the cell (i.e. proteins, transcription factors etc.) occur in very low densities (of the order
4 of $1-10^2 \mu\text{m}^{-3}$). Thus, using deterministic approximations, only average behaviors can be
5 assessed [19-24], and important cellular phenomena (such as polymerase binding, gene
6 expression), relying on the stochastic nature of low density populations, are smoothed out
7 [22, 25]. b/ Deterministic approximations assume that binding is a continuous process.
8 However, it is well known that binding occurs in discrete time intervals [22, 25]. For low
9 density populations, the discrete nature of binding plays important role when assessing
10 the 'preference' of a biomolecule to attach to specific cell sites. c/ Deterministic
11 approximations rely to a great extent on the geometry of the bleached spot (or the
12 bleaching laser profile –circular, Gaussian, etc-) [15, 26]. For complex geometries,
13 analytic solutions are difficult to compute. To the best of our knowledge, there is no
14 unified model treating any kind of geometry. d/ Most continuous approximation methods
15 have been based on the assumption that the cell environment is a homogeneous reaction
16 system. Although this assumption facilitates analytical solutions, it has been shown that
17 the reactant molecules of many, if not all, cellular biochemical pathways are highly
18 heterogeneously distributed within the cell compartments [15, 26]. e/ Regarding reactions
19 involving one diffusing molecule and one binding site, continuous approximation models
20 can only be used to estimate the ratio of association to dissociation rates. Such models
21 cannot be used for specifying unique values for reaction rate coefficients.

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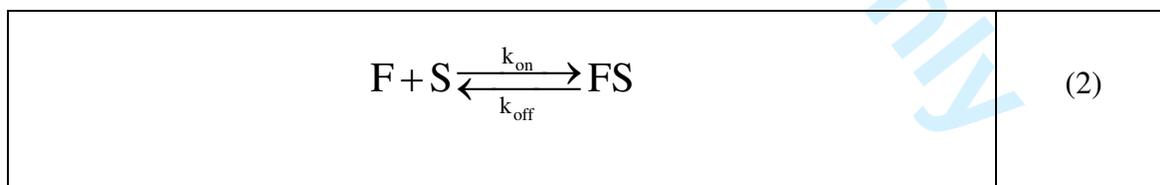
Material and Methods

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

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

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 $T=36^\circ\text{C}$.

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:



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

$\begin{bmatrix} [F] \\ [S] \\ [FS] \end{bmatrix} \Rightarrow \begin{bmatrix} -1 & 1 \\ -1 & 1 \\ 1 & -1 \end{bmatrix}$	(3)
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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 $P_0(t)$:

$P_0(t) = \exp\left(-\sum_{v=1}^M a_v t\right)$	(4)
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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:

$\text{FRAP}_{\text{stochastic}} = 1 - a_v \exp(-a_0 t)$	(5)
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where a_0 is the sum of propensities for both reactions.

The implementation of the proposed algorithm was realized as follows:

- (1) Initialization of molecular populations of participating species

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4 (2) Computation of each reaction propensity a_v
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7 (3) Computation of the sum of all reaction propensities a_0
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10 (4) Generation of two uniformly distributed random numbers r_1, r_2
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13 (5) Computation of time to next reaction as $\tau = \frac{\ln(r_1)}{a_0}$
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17 (6) Division of each reaction propensity with a_0 . Definition of the type of reaction
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20 that will occur next as follows:

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23 if $\left| \frac{a_1}{a_0} - r_2 \right| \leq \left| \frac{a_2}{a_0} - r_2 \right|$, then the forward reaction will occur: $F + S \rightarrow FS$
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27 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: $FS \rightarrow F + S$
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31 (7) Update of time t of simulation as $t = t + \tau$
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33 (8) Update of molecular populations according to stoichiometry of the system (see
34 equation 3). For example, if the forward reaction is selected, then one molecule of
35 unbound F and one molecule of binding site S will disappear, while one complex
36 of FS will appear. If the backward reaction will occur, one complex FS will
37 disappear, while one unbound F and one binding site S will appear.
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46 (9) Update of coordinates of F, S, and FS. Allow for unbound F to diffuse if $t \geq t_d$,
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49 where t_d is the time step to next diffusion.
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53 (10) If $t \geq t_f$, where t_f is the time where bleaching occurs, consider all F
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56 within the bleaching region as non fluorescent. Start recording fluorescence
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recovery within the bleached region as : $FRAP_{curve}(t) = \frac{F_{simulation-field}^0 F_{bleached-spot}(t)}{F_{bleached-spot}^0 F_{simulation-field}(t)}$

[29], where $F_{simulation-field}^0$ is the number of fluorescent F inside the simulation field

at $t=0$, $F_{bleached-spot}^0$ is the number of fluorescent F inside the bleached spot at $t=0$,

$F_{bleached-spot}(t)$ is the number of fluorescent F inside the bleached spot at t , and

$F_{simulation-field}(t)$ 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.

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]:

$FRAP_{sprague}(t) = \frac{1}{p} - \frac{F_{eq}}{p} (1 - 2K_1(qw)I_1(qw))x(1 + \frac{k_{on}}{p + k_{off}}) - \frac{C_{eq}}{p + k_{off}}$	(6)
<p>with $q^2 = (\frac{P}{D})(1 + \frac{k_{on}}{p + k_{off}})$</p>	

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 (sum of residuals R) as proposed in [30]:

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

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. $k_{on}=10^{-2} \text{ sec}^{-1}$ and $k_{off}=10^1 \text{ sec}^{-1}$) $R=0.84$, for effective diffusion (i.e. $k_{on}=10^{3.5} \text{ sec}^{-1}$ and $k_{off}=10^0 \text{ sec}^{-1}$) $R=0.94$, for reaction dominant (i.e. $k_{on}=10^{-0.5} \text{ sec}^{-1}$ and $k_{off}=10^{-1} \text{ sec}^{-1}$) $R=0.71$, and for full model (i.e. $k_{on}=10^2 \text{ sec}^{-1}$ and $k_{off}=10^{-1} \text{ sec}^{-1}$) $R=0.76$. The above results regarding 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 $t_{99}^{\text{diffusion_dominant}} = 0.082 \text{ sec}$, for effective diffusion $t_{99}^{\text{effective_diffusion}} = 25.5 \text{ sec}$ and for reaction dominant $t_{99}^{\text{reaction_dominant}} = 17.7 \text{ sec}$. Regarding the Sprague model, predictions were $t_{99}^{\text{diffusion_dominant}} = 0.083 \text{ sec}$,

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4 $t_{99}^{\text{effective_diffusion}} = 26.36 \text{ sec}$ and $t_{99}^{\text{reaction_dominant}} = 17.393 \text{ sec}$. According to t-test,

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7 there were no statistical differences between the proposed method and Sprague's formula
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9 in terms of predictions of 99% recovery.

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11 In the diffusion dominant case, each F was found to cover a mean distance of 0.18424
12 $\mu\text{m}/0.0005\text{sec}$, while 99% recovery was 0.0082 sec. In the effective diffusion case, that
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14 is, when unbound F are detained from binding reaction, thus, fluorescence is recovered
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16 slower, recovery to 99% occurred after 25.5 sec. Mean times to forward or backward
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18 reactions were found equal to 0.00023684 sec and 0.00017486 sec respectively with
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20 0.75768 reactions /sec. Moreover, 67.8% of the total molecular concentration of F was
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22 found unbound, whereas the remaining 32.2% was found in the form of binding complex
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24 FS. In the reaction dominant case, that is, when diffusion is a negligible process, time
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26 needed for 99% of recovery was 45.1 sec. Mean values of time distributions for next
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28 most probable forward or backward reaction, were found 0.00016634 sec and
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30 0.00013668 sec respectively. Regarding the forward reaction, 0.064319 F were reacting
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32 per sec, whereas regarding the backward reaction this ratio was 0.065025 /sec. Under
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34 these conditions, more than 50% of F was detained by binding sites S. Finally, for the full
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36 model case, which is used for fitting of FRAP curves that do not fall under the three
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38 above scenarios, mean time for forward reactions was found equal to 0.00018618 sec,
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40 while for backward reactions was equal to 0.00015898 sec. Ratios of forward and
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42 backward reactions to total number of F per unit of time were 0.064014 /sec and
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44 0.064696 /sec respectively, while the ratio of free to bound F was 0.53467.

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47 The proposed method presents several important advantages compared to other
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49 previous studies [3]: a/ It provides solutions for any kind of bleaching geometry, not only
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3 for uniform laser profiles and/or Gaussian laser profiles. b/ The coordinates of reactions
4 (forward and backward) can be monitored in space and time, enabling, thus, without any
5 special mathematical formulation, the interpretation of phenomena like anomalous
6 diffusion. c/ The propensities of reactions changes as a function of time based on the
7 exact molecular concentration of participating species, in contrast to standard
8 deterministic models, which assume reaction rates as constant, and forward-backward
9 reactions as independent processes. Thus, the physical basis of the algorithm is firmer. d/
10 Estimation of the time distribution to next forward and/or backward reaction, of next
11 event (diffusion, reaction, or no event), estimation of exact values for stochastic reaction
12 rates are based on distribution of propensities. Thus, the proposed method provides
13 information that cannot be extracted using standard deterministic models [3, 5, 10, 12,
14 31].

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

21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 **Acknowledgements**

47
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51 steps of this work.
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Figure legends

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

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

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

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ANSWERS TO QUESTIONS AND COMMENTS

“Interpretation of binding kinetics in Fluorescence Recovery After Photobleaching experiments using a novel stochastic simulation strategy”

By

Glotsos Dimitris, Ph.D.1, Kostopoulos Spiros, M.Sc., Ninos Kostas, M.Sc. and Cavouras Dionisis, Ph.D.

Dear Associate Editor,

Regarding the above paper we would like to thank you for the important comments and remarks. We have answered the comments made by the reviewers and we have made the appropriate changes in the manuscript as suggested.

LIST OF THE CHANGES MADE IN THE MANUSCRIPT AS RECOMMENDED BY THE REVIEWERS

REVIEWER 1

REVIEWER’S 1 COMMENT 1: The authors should discuss a bit more extensively the shortcomings of current, relevant methods.

We thank the reviewer for this comment. We have tried to elaborate the discussion regarding shortcomings of existing methods in the revised manuscript (please see third paragraph of the introduction section).

REVIEWER’S 1 COMMENT 2: The authors should more extensively explain how binding kinetics are extracted from FRAP curves.

We thank the reviewer for this comment. We have tried to elaborate the discussion regarding binding kinetics extraction from FRAP curves in the revised manuscript (please see first paragraph of the introduction section).

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5 **REVIEWER'S 1 COMMENT 3:** When discussing the need for stochastic modeling
6 over standard deterministic approaches, the authors should also note the following
7 relevant papers: Blake et al. Nature 422: 633 (2003) and Kaern et al. Nature Reviews
8 Genetics 6: 451 (2005).
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11 **Suggested references have been included in the revised manuscript.**
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REVIEWER 2

REVIEWER'S 2 COMMENT 1: Already in the introduction the authors state several limitations of existing methods, but the manuscript does not contain examples that their method achieves better results in these cases; that is, I would like to see a "real" analysis/evaluation of some experimental data, and not only a comparison of simulations.

We thank the reviewer for the constructive comment. To the best of our knowledge, the proposed method is the first stochastic approach to FRAP experiments. As a first step to establish our method, we have selected to compare our method with other well known methods in terms of justifying that our results are reasonable. Second, we have indicated the advantages of our method compared to others (such as firmer physical basis due to stochasticity of cellular events, one model for all geometries etc). Third, we have analyzed what kind of additional information can be extracted using our method (such as mean diffusing distance, distribution of times to next reaction or release). Application to further experimental data will only involve fitting of generated curves and experimental curves, something that we have done indirectly by comparing our method with Sprague's method [1. B. L. Sprague, Pego, R. L., Stavreva, D. A. and McNally, J. G., Analysis of binding reactions by fluorescence recovery after photobleaching, Biophys J, Vol (2004), 3473-95.]. Sprague's method has been shown to apply well in analysis of binding of a transcription factor, the glucocorticoid receptor. These issues are now clarified in the revised manuscript in the discussion section.

REVIEWER'S 2 COMMENT 2: The manuscript does not mention CPU and memory effort of the method, nor is mentioned anything about availability of the program.

For low density starting molecular population, for the full model case, the algorithm converges at 1500 sec on PC with Pentium V 300MHz processor and 512MB RAM, in contrast to the deterministic model, which gives instantaneous estimations. Algorithms have been developed in custom MATLAB® code and are available upon request. These issues are now clarified in the revised manuscript in last paragraph of the discussion section.

REVIEWER'S 2 COMMENT 3: Most equations (both those on separate lines as in the text) need some polishing; the ugly appearance, however, might be due to WORD(?) usage.

We have tried to improve the appearance of equations.

REVIEWER'S 2 COMMENT 4 The three figures are too small (it's even difficult to read the axis labels) and seem to have a bad resolution (line art as JPGs).

We have tried to increase quality of figures.

REVIEWER'S 2 COMMENT 5 Chemical substances have not to be given in italics (or math mode); for example, F, S, and FS in equation (2) should be set in normal font.

We have made appropriate corrections throughout the manuscript.

REVIEWER'S 2 COMMENT 6 Units have not to be given in italics (for example "microm" on page 3, 2nd par., line 7).

We have made appropriate corrections throughout the manuscript.

REVIEWER'S 2 COMMENT 7 - Page 7, item 5: The equation contains a " $\ln(r1)$ "; should this be " $\ln(r^{-1})$ "?

Equation is $\tau = \frac{\ln(r1)}{a_0}$ where $r1$ is a uniformly distributed random number as described in [J. H. Gillespie, Exact stochastic simulation of coupled chemical reactions, Journal of Physical Chemistry, Vol (1972), 2340-61.]

REVIEWER'S 2 COMMENT 8 Page 7, item 6: Both equations contain some text that has not to be given in italics.

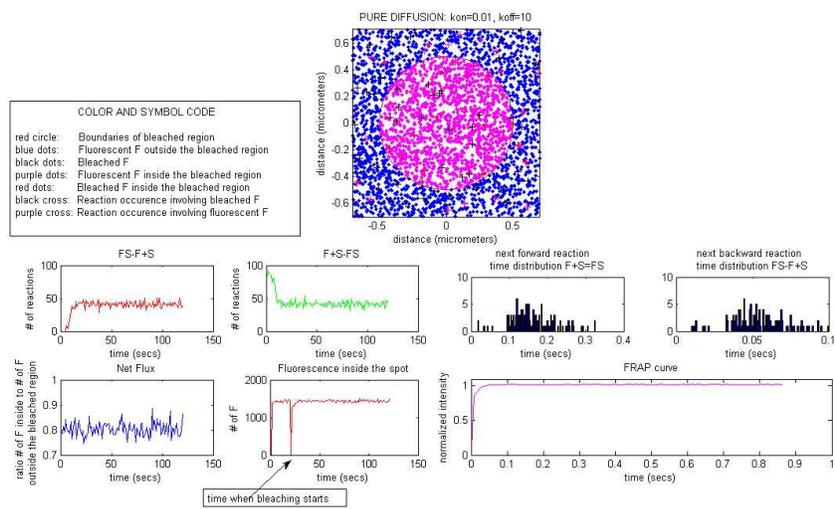
We have made the appropriate correction.

REVIEWER'S 2 COMMENT 9 Page 9, bottom:

The superscripts ``diffusion_dominant" and ``reaction_dominant" should not be given in italics. Similar, equation (5) and equations in item 10 (page 7/8) contain text in subscripts that should not be given in italics.

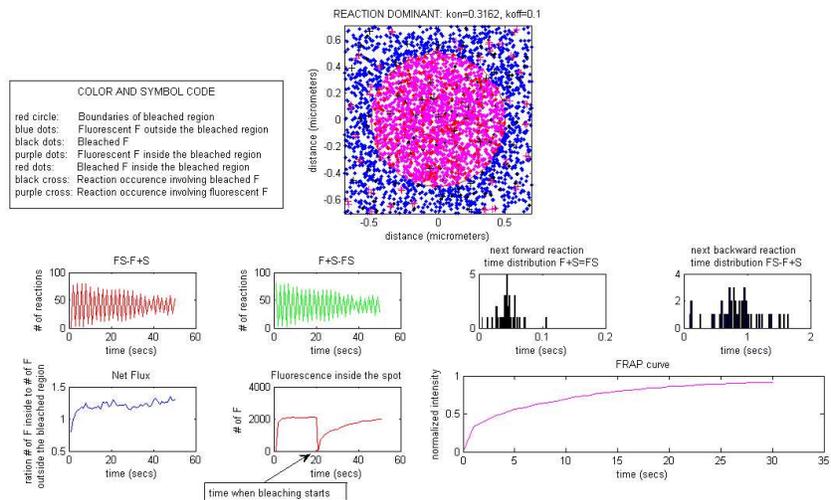
We have made the appropriate correction.

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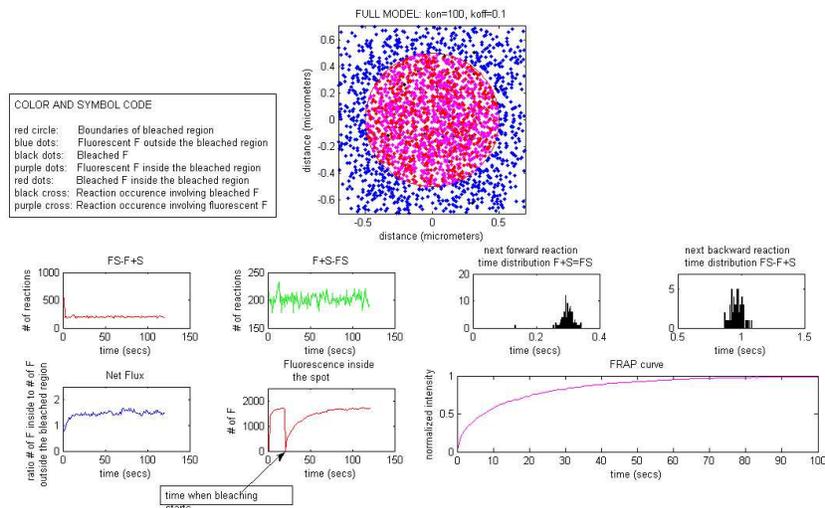
The proposed stochastic simulation algorithm for the diffusion dominant scenario
336x176mm (96 x 96 DPI)

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The proposed stochastic simulation algorithm for the reaction dominant scenario
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The proposed stochastic simulation algorithm for the full model scenario
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