ANALYSIS OF SPATIO-TEMPORAL DYNAMICS BY FRAP

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ABSTRACT

The response of cells to contaminants is of great importance for human health. The project focus is to model the motion of a contaminants. Therefore, we use the well-known experiment Fluorescence Recovery After Photobleaching FRAP, which enables us to analyse binding and diffusion of fluorescent particles. Already published analytical solutions which describe the FRAP recovery for several cases only deal with diffusion of unbounded particles. First, we derived the laplace transformed solution for diffusion of all particles. Second, we fit different solutions to artificial data and try to identify both the strategy of acting and the parameters of binding and diffusion. Third, to give an example of use we fit the analytical solutions to real FRAP measurements.

1. INTRODUCTION

Whithin the last year the interest in noninvasive methods to observe and analyse cell mechanisms increased dramatic. Fluorescence After Photobleaching (FRAP) is one of this techniques[1]. The FRAP method is a well known and widely used experiment to investigate parameters of motion (diffusion) and interaction (reaction) in live cells. In order to extract these parameters out of the FRAP, researchers rely on models and their solutions describing those measurements. These models can be solved numerically or analytically. The drawback of numeric solutions are that it is time consuming to compute them and function values are only an approximation. The analytical solution by contrast is time expensive in calculation but exact function values are fast to compute. The disadvantage of analytical solutions is that not all models are solveable.
Up to now, there are different analytical solutions of simplified models which have one assumption in common: All models deal with one fraction of moving particles and all other fractions are immobile [2, 3, 4, 5]. Although, we can not assume that only one out of all fractions is mobile [6]. Other attemps to improve the model by substituting normal diffusion behavior for anomalous diffusion [7, 8] and by augmenting reaction with directed transport [9].
Our hypothesis is that some processes inside the cell can only described by a model which includes both reaction as well as diffusive motion of all particle fractions. We derive a so far missing (semi-) analytical solution for this model.
Additionaly, we will test a so far not used algorithm to fit FRAP data to model functions and apply the new solution to synthetic and real measurements.

2. MATERIALS AND METHODS

2.1. FRAP experiments

Fluorescence Recovery After Photobleaching (FRAP) is a widely known and applied experiment to get a deeper look inside processes of living cells. It enables us to answer qualitative questions, like: How many acting substances are present in a process? Is there a reaction between different substances? On the other hand, also quantitative informations like diffusion coefficients and binding parameters can be estimated by analysis of FRAP measurements. The method of FRAP experiments is described in Fig. 1. The resulting Recovery curve depends on particle properties.
To extract this properties out of the Recovery curve we describe the system by partial differential equations and solve them.

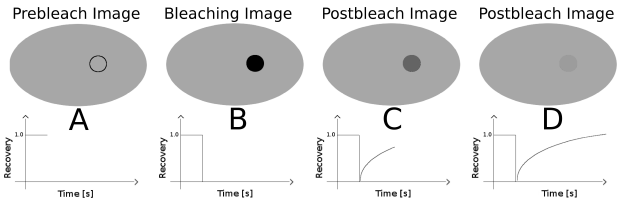

Figure 1. Concept of FRAP experiments: The gray oval represent a fluorescent area of the observed cell. (A) The Region of Interest (ROI) called Bleaching Spot is defined (black circle) and Prebleached Images are prepared. The fluorescence intensity inside the spot is set to 1. (B) The Bleaching Spot is treated by a laser pulse of high magnitude. Thus, particles inside the spot lost their fluorescence. The fluorescence intensity inside the spot sink to 0. (C-D) at intervals of fixed time steps Postbleach Images are taken to see the recovery of fluorescence inside the Bleaching Spot. The fluorescence intensity inside the bleaching Spot is increasing. The resulting curve is called Recovery.

2.2. Analysis of FRAP: A new analytical solution

We describe a binding reaction of a substance F with BS binding sites S_i to form bound particles B_i. Our nomenclature for reactions is

$$F + S_\mathrm{i} \underset{k_{\mathrm{off_i}}}{\overset{k_{\mathrm{on_i}}}{\rightleftharpoons}} B_\mathrm{i} \quad , \quad \mathrm{i} = 1 \ldots \mathrm{BS} \tag{1}$$

where F represents the unbound (free) fraction, S_i the vacant binding sites and B_i the bound fraction. $k_{\mathrm{on_i}}$ and $k_{\mathrm{off_i}}$ are the association rate and dissociation rate in $[\mathrm{mol} \cdot s^{-1}]$ and $[s^{-1}]$ respectively.
Let us assume that free particle fraction F is a fluorescenct one. Since fluorescent particles F are part of the bound ones B_i the bound particles are also fluorescent. The FRAP experiment observe all fluorescent particles. Thus, the recovery curve describe both free particles F and bound particles B_i. In case both of these particles are mobile, we have to solve the following set of partial differential equations:

$$\frac{\partial c_\mathrm{F}}{\partial t} = D_\mathrm{F} \nabla^2 c_\mathrm{F} - \sum_{\mathrm{i=1}}^{\mathrm{BS}} \left(k_{\mathrm{on_i^*}} c_\mathrm{F} - k_{\mathrm{off_i}} c_{\mathrm{B_i}} \right) \tag{2a}$$

$$\frac{\partial c_{\mathrm{B_i}}}{\partial t} = D_{\mathrm{B_i}} \nabla^2 c_{\mathrm{B_i}} + k_{\mathrm{on_i^*}} c_\mathrm{F} - k_{\mathrm{off_i}} c_{\mathrm{B_i}} \quad \text{with } \mathrm{i} = 1 \ldots \mathrm{BS} \tag{2b}$$

where ∇^2 is the Laplacian Operator, c represents the concentration and D is the diffusion coefficient. The indices F and B_i represents the different kinds of free and bound particle fractions. $k_{\mathrm{on_i^*}}$ is a pseudo-on rate and is defined as $k_{\mathrm{on_i^*}} = k_{\mathrm{on_i}} \cdot c_{\mathrm{S_i}}$ [5]. Index i denotes the reaction like it is described in Eq. 1.
We are interested in the recovery curve $\mathrm{frap}(t)$. This curve describes the average fluorescence intensity inside a circular Bleaching Spot with radius R:

$$\mathrm{frap}(t) = \frac{1}{\pi R^2} \cdot \int_0^{2\pi} \int_0^{R} (c_\mathrm{F} + \sum_{i=1}^{BS} c_{\mathrm{B_i}}) \cdot r \; dr \; d\varphi \tag{3}$$

Thus, we need a solution of the set of differential equations (Eq. 2). Since there is so far no analytical solution, we derived it. Therefore, we use known theoretical results [10, 11] and apply them to analysis of FRAP experiments. This yields for case of one binding site ($BS = 1$)

$$\begin{aligned} \overline{\mathrm{frap}}(s^*) = {} & 2\mathbf{I_1}\left(R^*\sqrt{p_1}\right)\mathbf{K_1}\left(R^*\sqrt{p_1}\right) \times \\ & \frac{(K - s^* + p_1 - 1)(s^* - p_2)}{(K-1)s^*(p_1 - p_2)} \\ & - 2\mathbf{I_1}\left(R^*\sqrt{p_2}\right)\mathbf{K_1}\left(R^*\sqrt{p_2}\right) \times \\ & \frac{(K - s^* + p_2 - 1)(s^* - p_1)}{(K-1)s^*(p_1 - p_2)} \end{aligned} \tag{4a}$$

$$p_{1/2} = \frac{D(s^*+1) - K + s^*}{2D} \pm \frac{\sqrt{(D(s^*+1) + K - s^*)^2 - 4DK}}{2D} \tag{4b}$$

where $\overline{\mathrm{frap}}(s^*)$ defines the averaged fluorescence intensity within the bleaching spot and is given as the Laplace transform solution [12]. The original time variable t changed to the Laplace variable s caused by a Laplace transformation during the derivation. D and K are ratios of diffusion coefficients D_B/D_F and binding rates $k_{\mathrm{off}}/k^*_{\mathrm{on}}$ respectively. $\mathbf{I_1}$ and $\mathbf{K_1}$ are modified Bessel functions of the first and second kind. R represents the radius of the circular bleaching spot. The superscribed stars denote nondimensional variables

$$R^* = R \cdot \sqrt{\frac{k^*_{on}}{D_F}} \tag{5a}$$

$$t^* = k^*_{on} \cdot t \tag{5b}$$

We denote this model and solution as **Reaction Diffusion Model with Full Diffusion** (M_3). Since the solution is Laplace transformed, we have to retransform it to $\mathrm{frap}(t^*)$. This transformation require a numerical algorithm. We apply the Stehfest algorithm [13].

2.3. Analysis of FRAP: Fitting

In addition to this new analytical solution which include diffusion of all involved particles are solutions of special cases already published.
The Recovery curve of a system where you assume that the diffusion of free particle fraction is very fast compared to reaction is called **Reaction Dominant Model** (M_1) and derived by Sprague et al. [5].
The analytical solution of a system where only free particle fraction is mobil and all bound fractions are immobile is also given by Sprague et al. [5] and is named as **Reaction Diffusion Model with Single Diffusion** (M_2).
In order to identify which behavior is occuring during the FRAP experiment we fit all model functions to the measurements and take the model which yield the least error. Afterwards, we set the fitted parameters of this best model to be the properties of particle fractions. We use a Simulated Annealing (SA) strategy for fitting analytical solutions to measurements. We take the Mean Absolute Error function (sum of absolute distance between measured values and values of fitted function) as our objective function.

2.4. Artificial FRAP measurements

For the purpose of testing this strategy to identify the correct model and parameters, we applied the method to artificial dataset. Therefore, we set the Bleaching Spot to 15, fix the different parameter values (parameter values not shown)and discretize the analytical solutions to get 100 data points. In order to actualize measurements, we add gaussian distributed errors e ($e \sim N[0, \sigma]$, $\sigma_1 = 0.01$, $\sigma_2 = 0.03$) to the discrete analytical dataset (Fig. 2). Afterwards, we apply SA to fit every artificial dataset by all of the three analytical solutions. Since, SA is a heuristic fitting algorithm, we repeat the fitting procedure 500 times for each solution and measurement.

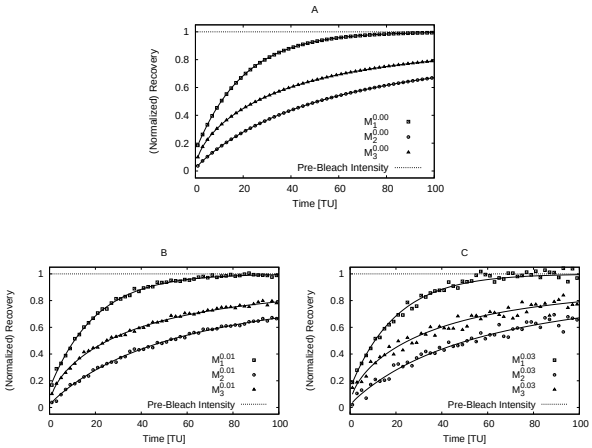

Figure 2. Artificial Datasets: Calculation of the model function values with fixed parameter settings and addition of Gaussian distributed noise where the mean of noise is zero and the standard deviation σ is (A) $\sigma = 0.00$ - no noise signal, (B) $\sigma = 0.01$ - low noise signal and (C) $\sigma = 0.03$ - high noise signal

2.5. Real FRAP measurements

The murine Hepa1c1c7 clone Tao BpRc1 deficient in endogenous Ah-receptor was used for all experiments. Cells were transfected with a YFP labelled AhR construct (generously provided by A. Puga) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) as previously described [14]. All experiments were carried out 18 hours after transfection.

The FRAP experiments were performed on a Zeiss 510 confocal microscope (Carl Zeiss, Thornwood, NY, USA) with a 100X/1.4 NA oil immersion objective. Cells were kept at 37°C using an air stream stage incubator (Nevtek, Burnsville, VA, USA). Bleaching was performed with a circular spot (radius 1.12305 μm) using the 488- and 514-nm lines from an argon laser operating at 74% laser power. A single iteration was used for the bleach pulse. Five pre-bleach images were taken and the fluorescence recovery was monitored at 78.2 ms intervals.

We treated the cells for 1 hour with 50 nM Benzo[a]pyrene (BaP) to induce translocation, and performed 51 separate FRAP experiments in the nucleus.

The raw image data were used to extract the fluorescence recovery curves. Afterwards each recovery curve was double normalized using the pre-bleach images as well as two reference areas as described by Phair et al. [3]. We take the mean of the sample recovery values to create the average recovery shown as dots in Fig. 3.

3. RESULTS & DISCUSSION

3.1. Artificial FRAP measurements

The least error function values (sum of absolute difference between fitted function and dataset) of 500 runs of SA for every artificial dataset and model function are shown in Tab. 1. This tabular shows that for every dataset the correct model type is predicted (bold values are always in correct column). We also recognize that small differences between the error function values of two different fitted models are relevant and meaningful in order to decide for the model type. In oder to emphasize that also small distances between error function values are relevant, we also analyse the distribution of all 500 error values. To describe these method and results here would go beyond the scope of this paper. We illustrate this in Mai et al. [12].

Furthermore, the parameter values of no-noise datasets ($M_1^{0.00}$, $M_2^{0.00}$, $M_3^{0.00}$) are accurately estimated by fitted functions (data not shown). The estimated parameters of noise datasets ($\sigma_1 = 0.01$, $\sigma_1 = 0.03$) are more spreaded, but also close to simulated settings (data not shown).

This leads us to be convienced that SA technique is applicable to fit FRAP measurements and to estimate both the correct model and the correct model parameters. It is obvious that noisy datasets result in more spreaded parameters.

	Model 1	Model 2	Model 3
$M_1^{0.00}$	**0.00000**	1.92217	2.07131
$M_1^{0.01}$	**0.78581**	2.09649	2.24620
$M_1^{0.03}$	**2.11161**	3.03402	3.16262
$M_2^{0.00}$	1.50488	**0.00885**	0.06056
$M_2^{0.01}$	1.70500	**0.78243**	0.78933
$M_2^{0.03}$	2.87615	**2.23449**	2.35603
$M_3^{0.00}$	2.17175	0.03437	**0.00092**
$M_3^{0.01}$	2.26306	0.77783	**0.77635**
$M_3^{0.03}$	2.97956	2.48055	**2.45850**

Table 1. Error function values of fitting artificial datasets: The artificial datasets with different noise intensity are fitted by the three model functions. The bold printed values represents the best result out of 500 Simulated Annealing fitting runs.

3.2. Real FRAP measurements

In order to fit the real FRAP measurements, we extend our bunch of model functions which we take into account for fitting by increasing the number of binding sites BS. Within Tab. 2 the fitted model functions are listed as well as the least error function value for each model after 500 SA runs. The least error function value of all fitted model functions is given by *Reaction Diffusion Model with Full Diffusion (2BS)*. This means there is a free particle fraction F and two bound particle fractions B_i. All fractions are moving diffusively and coupled by two reactions .

This result shows us that our new model is required to describe processes inside a cell. Here, we see that there have to be 2 partners inside the cell which interact with the BaP-AhR complex inside the nucleus. This fits to our so far knowledge. We still know that the BaP-AhR complex have to bind to a special nuclear translocator (ARNT) (which we assume to be BS 1) before it can bind to DNA (which we assume to be BS 2).

	Error
Reaction Dominant (1BS)	4.41299
Reaction Dominant (2BS)	2.86201
Reaction Dominant (3BS)	2.78075
Reaction Diffusion with Single Diffusion (1BS)	2.90925
Reaction Diffusion with Single Diffusion (2BS)	2.52231
Reaction Diffusion with Single Diffusion (3BS)	2.51706
Reaction Diffusion with Full Diffusion (1BS)	2.42556
Reaction Diffusion with Full Diffusion (2BS)	2.40706

Table 2. Results of fitting real FRAP measurement: *Full Reaction Diffusion with Full Diffusion (2BS)* identified as the model with least value of error function value of all fitted models.

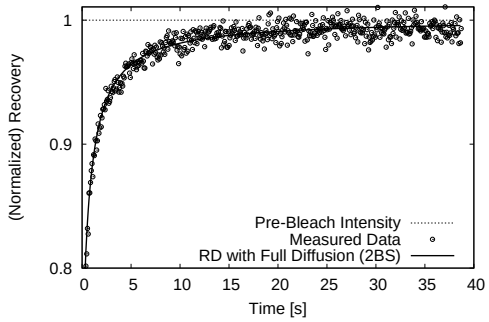

Figure 3. Best results of fitting real measurements: FRAP measurement within nucleus of treated (1h; 50 nM BaP) cells (51 samples) represented as dots and model function of Reaction Diffusion with Full Diffusion (2BS) using fitted parameters. Fluorescence intensity measured during experiment is caused by aryl hydrocarbon receptor (AhR) which is labelled with yellow fluorescent proteins (YFP).

4. CONCLUSION

We showed that a Simulated Annealing technique is applicable to fit model function to measurements in order to identify the model structure as well as certain parameters. Further, we provide a new model function and show their relevance to describe processes inside a cell.

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