Parameter Identification Problem Based on FRAP Images: From Data Processing to Optimal Design of Photobleaching Experiments

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Abstract. The aim of this study is to make a step towards optimal design of photobleaching experiments. The photobleaching techniques, mainly FRAP (Fluorescence Recovery After Photobleaching), are widely used since 1970's to determine the mobility of fluorescent molecules within the living cells. While many rather empirical recommendations for the experimental setup have been made in past decades, no rigorous mathematical study concerning optimal design of FRAP experiments exists. In this paper, we formulate and solve the inverse problem of data processing of FRAP images leading to the underlaying model parameter identification. The key concept relies on the analysis of sensitivity of the measured outputs on the model parameters. It permits to represent the resulting parameter estimate as random variable, i.e., we can provide both the mean value and standard error or corresponding confidence interval. Based on the same sensitivity-based approach we further optimize experimental design factors, e.g., the radius of bleach spot. The reliability of our new approach is shown on a numerical example.

Keywords: FRAP · Optimal experimental design · Sensitivity analysis · Parameter identification

1 Introduction

The image processing is certainly one of the fastest growing areas in informatics and applied mathematics. Many new applications, e.g., in biology and medicine, rise up every year. However, there is a gap between the level of sophistication of equipment for the data acquisition and the quality of further data processing. Particularly, discussion about the data noise propagation (from data to

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the resulting parameter estimates), i.e., the error or uncertainty analysis corresponding to respective methods, is rare and whole concept of parameter definition as random variable is often misunderstood by the biological community, cf. [9,10,15].

While in our previous papers we sought to elaborate reliable methods for the processing of spatio-temporal images acquired by the so-called FRAP (Fluorescence Recovery After Photobleaching) method [6,7,11–13], the aim of the present study is to make a step from the data processing to optimal design of photobleaching experiments. Further we show how to find a specific "optimal" experimental conditions maximizing a measure of sensitivity defined as the sum of squares of partial derivatives of the measured output on the estimated parameter, cf. (11).

Both FRAP & FLIP (Fluorescence Loss In Photobleaching) are based on the measuring the change in fluorescence intensity in a region of interest, e.g., in a finite 2D domain representing the part of a membrane, in response to an external stimulus (bleaching). A high-intensity laser pulse provided by the confocal laser scanning microscopy (CLSM) causes a presumably irreversible loss in fluorescence in the bleached area and the subsequent recovery in fluorescence reflects the mobility (related to diffusion) of fluorescent compounds from the area outside the bleach spot. CLSM allows to obtain high-resolution optical images with deep selectivity, however, the small energy level emitted by the fluorophore and the amplification performed by the photon detector introduces a measurement noise making the subsequent parameter identification problem highly unstable due to the ill-posedness in Hadamard's sense [3,4].

The rest of this paper is organized as follows: In Sect. 2 we describe the FRAP & FLIP data acquisition and structure. In Sect. 3 we formulate the inverse problem of parameter identification and introduce the sensitivity analysis. Then, in Sect. 4, we develop a new theoretical approach allowing the optimization of FRAP experimental factors and provide one numerical example. The novelty of our approach and outlooks for further research are discussed in the final Sect. 5.

2 Data Acquisition and Data Structure

The spatio-temporal FRAP data are graphically depicted in Fig. 1. Usually, the images are made with certain time period (in our case every 8 s) before and after the application of high-intensity laser pulse (so-called bleach). The prebleach image (see the top left image in Fig. 1) shows a typical distribution of phycobilisome fluorescence in a single cell. Application of high laser intensity across the vertical axis (red rectangle in the second image in top row in Fig. 1) reduced phycobilisome fluorescence to about 40 % of the initial value due to the destruction of a portion of the phycobilin pigments. The observed fluorescence recovery in the bleached zone is attributed to phycobilisome mobility in this red alga, see e.g., [5] and references therein.

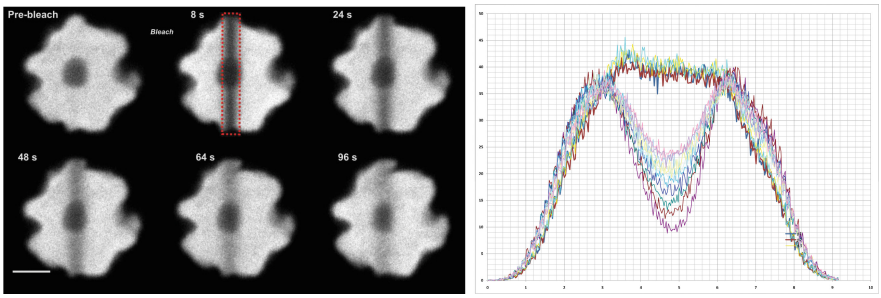

Fig. 1. Left: representative FRAP image sequence for a single cell of red algae *Porphyridium cruentum* for phycobilisome fluorescence. First, a fluorescence image before bleaching was detected (pre-bleach), then the phycobilisome fluorescence was bleached out across the middle of the cell in the vertical direction (red dashed rectangle). The sequence of five post-bleach images is shown. The length of the scale bar is 3 μm. Right: experimental (noisy) data in form of one-dimensional bleach profiles for different time instants after the bleach. The abscissa represents the position along the axis perpendicular to the bleach stripe. In the ordinate there is the corresponding average fluorescence (in arbitrary units) along the axis parallel to bleach. In the central region we see the step-wise recovery of the signal: from the lowest value (first post-bleach) to the highest pre-bleach (steady-state) values on the top (Color figure online).

A FRAP data structure usually consists of a time sequence of rectangular matrices, where each entry quantifies the fluorescence intensity u at a particular spatial point in a finite 2D domain (e.g., by a number between 0 and 255):

$$u(x_{kl}, t_j)_{j=0}^{N_t}, \quad k = 1 \dots N_x, \; l = 1 \dots N_y,$$

where k, l are the spatial indexes uniquely identifying the pixel position where the signal u is measured, and j is the time index (the initial condition corresponds to $j = 0$), cf. [6,7,11]. Usually, the data points are uniformly distributed both in time (the time interval Δt between two consecutive measurements is constant) and space, i.e., on an equidistant 1D or 2D mesh. Let see the right part of Fig. 1, where we observe an example of 1D fluorescence intensity profiles (in arbitrary units) for different time instants $t_0 \dots t_{N_t}$.

Further, in sake of simplicity, we shall infer about the parameter D by using direct measurements of discrete data in a space-time domain when only one index is employed, i.e., we use the following form of data

$$u(x_i, t_i)_{i=1}^{N_{\text{data}}} \in \mathbb{R}^{N_{\text{data}}}.$$

3 Problem Formulation

Let us consider the isotropic diffusion process characterized by one single scalar parameter: a diffusion coefficient D (constant in space). Right now we assume that D is time-dependent, i.e., an anomalous diffusion is allowed. The governing

equation for the spatio-temporal fluorescence signal $u(x,t)$, proportional to the fluorescent particles concentration, is Fick's diffusion equation as follows

$$\frac{\partial}{\partial t}u(x,t) = D\Delta u(x,t) \qquad x \in \Omega,\ t \in [0,T] \tag{1}$$

$$u(x,0) = u_0(x) \qquad x \in \Omega \tag{2}$$

$$\text{boundary conditions} \qquad \partial\Omega \times [0,T]. \tag{3}$$

Boundary conditions could be, e.g.,

$$u(x,t) = 0 \quad \text{or} \quad \frac{\partial}{\partial n}u(x,t) = 0 \quad \text{on} \quad \partial\Omega \times [0,T].$$

We also consider the simplest case of unbounded domains $\Omega = \mathbb{R}^n$, in which case we set appropriate decay conditions at $|x| \to \infty$, $t \in [0,T]$. The above formulation (1)–(3) (and variants) is the basis for all the further analysis.

In the case of constant coefficient D, the solution to this problem can be expressed by means of the Green function $G(x,t;y)$ for the heat equation

$$\frac{\partial}{\partial t}G(x,t;y) = \Delta G(x,t;y) \qquad x \in \Omega,\ t \in [0,T]$$

$$G(x,0;y) = \delta(x-y) \qquad x \in \Omega$$

$$\text{boundary conditions for } G(x,t;y) \qquad \partial\Omega \times [0,T].$$

Some frequently used cases are that of a diffusion in free space, e.g., in the one-dimensional domain $\mathbb{R}$ without boundary conditions, the Green function is the heat kernel

$$G(x,t;y) = \frac{1}{\sqrt{4\pi t}}\ \exp\left[-\frac{(x-y)^2}{4t}\right] \qquad x, y \in \mathbb{R}.$$

In FRAP experiments, the initial condition, i.e., the first post-bleach profile (with the background or pre-bleach signal subtracted) is often modeled as a Gaussian, cf. Fig. 1, which leads in the one-dimensional case to initial condition of the form

$$u_0(x) = u_{0,0}\ \exp\left(-\frac{2x^2}{r_0^2}\right), \tag{4}$$

where $u_{0,0} \geq 0$ is the maximum depth at time t_0 for $x = 0$, $r_0 > 0$ is the half-width of the bleach at normalized height (depth) $\exp(-2)$, i.e., $\frac{u_0(r_0)}{u_{0,0}} = \exp(-2)$, cf. [11]. An explicit solution for u in the one-dimensional free space case is then given by

$$u(x,t) = u_{0,0}\frac{r_0}{\sqrt{r_0^2 + 8Dt}}\ \exp\left(-\frac{2x^2}{r_0^2 + 8Dt}\right). \tag{5}$$

Parameter Identification Problem Based on FRAP data

Define a forward map (also called a parameter-to-data map)

$$F : \mathbb{R} \to \mathbb{R}^{N_{\text{data}}} \tag{6}$$

$$(D) \to u(x_i, t_i)_{i=1}^{N_{\text{data}}}. \tag{7}$$

Our regression model is now

$$F(D) = \text{data}, \tag{8}$$

where the data are modeled as contaminated with additive white noise

$$\text{data} = F(D_T) + e = u(x_i, t_i)_{i=1}^{N_{\text{data}}} + (e_i)_{i=1}^{N_{\text{data}}}. \tag{9}$$

Here D_T denotes the true coefficient and e is a data error vector which we assume to be normally distributed with variance σ^2

$$(e_i)_{i=1}^{N_{\text{data}}} \in \mathbb{R}^{N_{\text{data}}}, \quad e_j = \mathcal{N}(0, \sigma^2), \quad j = 0, \ldots, N_t.$$

Given some data, the aim of the parameter identification problem is to find D such that (8) is satisfied in some appropriate sense. Since (8) usually consists of an overdetermined system (there are more data points than unknowns), it cannot be expected that (8) holds with equality, but instead an appropriate notion of solution (which we adopt for the rest of the paper) is that of a least-squares solution D_c (with $\|.\|$ denoting the Euclidean norm on $\mathbb{R}^{N_{\text{data}}}$):

$$\|F(D_c) - \text{data}\|^2 = \min_{D>0} \|F(D) - \text{data}\|^2. \tag{10}$$

The above defined parameter identification problem is usually ill-posed for nonconstant coefficients, so that regularization has to be employed; see, e.g., [4]. A solution of practical example based on FRAP data was presented in [11].

Sensitivity Analysis and Confidence Intervals

For the sensitivity analysis, cf. [2,7], we require the Fréchet-derivative $F'(D) \in \mathbb{R}^{N_{\text{data}} \times 1}$ of the forward map F, that is

$$F'(D) = \frac{\partial}{\partial D} F(D) = \begin{pmatrix} \frac{\partial}{\partial D} u(x_1, t_1) \\ \cdots \\ \cdots \\ \frac{\partial}{\partial D} u(x_{N_{\text{data}}}, t_{N_{\text{data}}}) \end{pmatrix}.$$

A corresponding quantity used further as our key sensitivity measure is a number

$$M(D) = F'(D)^T F'(D) \quad \in \mathbb{R}. \tag{11}$$

Based on the book of Bates and Watts [1], we can estimate confidence intervals. Suppose we have computed D_c as least-squares solutions in the sense of (10). Let us define the residual as

$$res^2(D_c) = \|F(D_c) - \text{data}\|^2 = \sum_{i=1}^{N_{\text{data}}} \left[\text{data}_i - u_{D_c}(x_i, t_i)\right]^2, \tag{12}$$

where u_{D_c} is computed from (1)–(3) for the parameter value D_c. Then according to [1], it is possible to quantify the error between computed parameter D_c and true parameter D_T. In fact, we have an approximate $1-\alpha$ confidence interval

$$(D_c - D_T)^2 \sum_{i=1}^{N_{\text{data}}} \left[\frac{\partial}{\partial D} u(x_i, t_i)\right]^2 \leq \frac{res^2(D_c)}{N_{\text{data}} - 1} f_{1,N_{\text{data}}-1}(\alpha). \tag{13}$$

In equation (13), several simplifications are possible. Note that according to our noise model, the residual term $\frac{res^2(D_c)}{N_{\text{data}}-1}$ is an estimator of error variance such that an approximation

$$\frac{res^2(D_c)}{N_{\text{data}} - 1} \sim \sigma^2 \tag{14}$$

holds for N_{data} being large [1]. The term $\frac{res^2(D_c)}{N_{\text{data}}-1}$ in (13) can be viewed as rather independent of D_c or N_{data}. Moreover, we remember the reader that the Fisher distribution with 1 and $N_{\text{data}} - 1$ degrees of freedom converges to the χ^2-distribution as $N_{\text{data}} \to \infty$. Hence, the term $f_{1,N_{\text{data}}-1}(\alpha)$ can approximately be viewed as independent of N_{data} as well and of moderate size.

4 Optimizing Experimental Design Variables

There are many rather *empirical recommendations* related to the design of a photobleaching experiment, e.g., the bleach spot shape and size (design factor r_0), the region of interest location and size (design factor L), total time of measurement (T), see [7,15] and references therein. However, we should have a *more rigorous tool* for the choice of experimental design factors. Based on the process model (1)–(3) and just introduced sensitivity analysis, we can define an optimization problem residing in the maximization of the sensitivity measure (11).

The key parameter in FRAP measurements is the size (and shape) of bleach spot, e.g., the characteristic radius r_0 in case of a circular bleach. If the size of bleach spot can be varied (at the same time keeping the bleach depth $u_{0,0}$ fixed), we should ask the question if there is an optimal bleach size that can be used. Thus, we can try to look for such a bleach radius r_0 which leads to maximal sensitivity since this corresponds to minimal confidence intervals (for comparable experiments).

More precisely, in the one-dimensional case of the Fick diffusion on a line, having the set of observations on a space-time cylinder $Q = [-L, L] \times [0, T]$, we try to infer about the optimal bleach radius r_{opt} yielding maximal sensitivity.

We introduce a function

$$S(r_0) = \sum_{i=1}^{N_{\text{data}}} \left[\frac{\partial}{\partial D} u(x_i, t_i) \right]^2 = \sum_{k=1}^{N_x} \sum_{j=1}^{N_t} \left[\frac{\partial}{\partial D} u(x_k, t_j) \right]^2, \tag{15}$$

where $N_x = \frac{2L}{\Delta x} + 1$ and $N_t = \frac{T}{\Delta t}$, and we try to find out a maximal value

$$S(r_{opt}) = \max_{r_0 > 0} S(r_0).$$

Note that $S(r_0)$ is equal to M from (11).

Numerical Example

In the following example we compute a least-squares estimate D_c and the sensitivity $S(r_0)$, cf. (15). We consider a rectangular spatio-temporal data grid with space interval $x_i \in [-6, 6]$, i.e., $L = 6$, and time interval $t_i \in [0, T]$ for various T. For our test purposes we used various grid sizes Δx and Δt and also various exact diffusion coefficient D_T. We simulated data by assuming D_T with different bleach radii r_0 and computed the data for the 1D case by (5). Based on these data we computed a least-squares estimate D_c of the diffusion coefficient using a procedure described in [11]. It is a one-dimensional minimization problem (10) for D. To obtain a solution, we used variable metric method implemented in our optimization system [8]. The values $M = S(r_0)$ were then computed numerically using central differences.

To see what may influence a value of optimal bleach radius r_{opt}, we considered different values of $D_T, T, \Delta x, \Delta t$ defined in Table 1.

Table 1. Input values for numerical experiments.

Data set	D_T	Δx	N_x	Δt	N_t	T
Data 1	1	0.1	121	0.1	40	4
Data 2	1	0.1	121	0.01	400	4
Data 3	1	0.01	1201	0.1	40	4
Data 4	2	0.1	121	0.01	200	2
Data 5	1	0.1	121	0.01	200	2
Data 6	2	0.1	121	0.01	100	1

Typical behaviors of dependence of $M = S(r_0)$ on r_0 and computed values D_c on r_0 are shown in Fig. 2. For this purpose we used the results for data set *Data 1*. One can see that there exists a unique maximum of function $S(r_0)$ which is marked with a black circle. There exists an optimal bleach radius r_{opt} leading to maximal sensitivity.

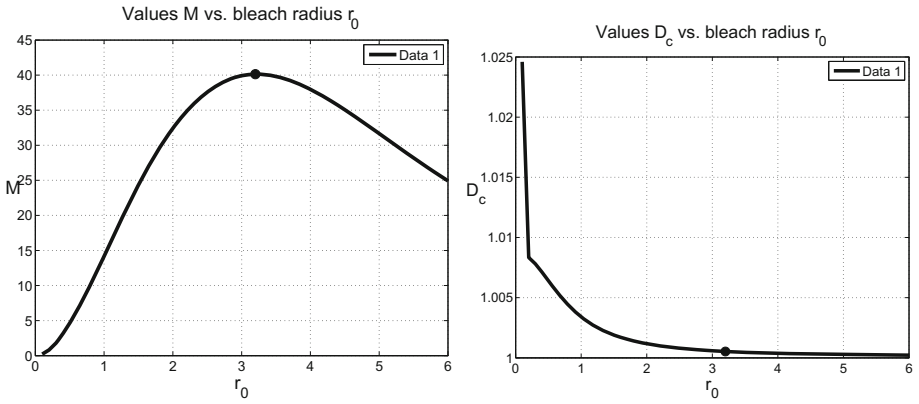

Fig. 2. Values $M = S(r_0)$ and D_c vs. bleach radius r_0 for data set *Data 1*.

Optimal bleach radii for all data sets together with computed values D_c are presented in Table 2. We found out that the optimal bleach radius is the same for data sets *Data 1 – Data 4* and for data sets *Data 5 – Data 6*. The value of r_{opt} is influenced by exact diffusion coefficient D_T and time interval of measurement T. The function value $S(r_{opt})$ depends on the number of spatio-temporal points. For example, this value is approximately 10 times larger for data sets *Data 2* and *Data 3* in comparison with data set *Data 1* because the number of points is 10 times larger (10 times larger number of $N_x N_t$), see the sums in (15).

Table 2. Results of numerical experiments.

Data set	r_{opt}	$S(r_{opt})$	D_c	$\lvert D_c - D_T \rvert$
Data 1	3.2	40.13	1.000528	5.28E–4
Data 2	3.2	395.36	1.000343	3.43E–4
Data 3	3.2	399.77	0.999999	1.00E–6
Data 4	3.2	198.08	2.001091	1.09E–3
Data 5	2.4	149.83	1.000810	8.10E–4
Data 6	2.4	75.21	2.001912	1.91E–3

The obtained results correspond quite well with our theoretical findings published in [14], where we argue that the value r_{opt} depends on the square root of the product of time interval of measurement T and exact diffusion coefficient D_T. Indeed, the optimal value r_{opt} is the same for the same product TD_T.

5 Conclusion

In this paper, we propose the interconnection of two important activities in performing experiments: (i) experimental design, i.e., optimal or near-optimal setting of experimental factors, and (ii) data processing based on a mathematical model containing the specific experimental conditions as parameters. Although our idea is illustrated only on a widely used case of photobleaching experiment, our approach is more general. We formulate the problem of parameter identification in precise terms of parameter-to-data map, parameter estimates and their confidence intervals. Then, we introduce the key concept of sensitivity of measured data on estimated parameters.

Despite the fact that some recommendations and findings concerning the FRAP experimental protocol exist, cf. [10], their applicability is limited because they are based on very specific experimental conditions. Our approach is more general and accurate (always when the process model is reliable).

In order to validate our idea of the model-based optimization of experimental conditions, we provide one numerical example. We prove that one of the most important experimental design factors in photobleaching experiments, the bleach size r_0, can be actually optimized, i.e., there exists a value r_{opt} for which the sensitivity measure $S(r_{opt})$ reaches the maximal value, hence assuring the shortest confidence interval, cf. (13).

Our findings are expected to be incorporated into a process of FRAP experimental protocol development – it is not computationally expensive and the enhancement of the parameter estimation process can be substantial, e.g., a four times higher $S(r)$ assures half upper bound for the standard error of the estimated parameter, cf. Fig. 2.

Certainly, the more realistic model formulation should be conceived in order to get reliable results, e.g., taking into account the anisotropic diffusion on finite 2-dimensional domain, binding reaction, bleaching during scanning, more general bleaching shapes and topologies. All these issues are only some extension of the presented study and do not question neither the governing Fick diffusion PDE nor the nature of the computation domain Ω (if it is a Euclidean domain or a fractal set modelling the molecular crowding). This is the subject of our ongoing research together with an ambitious goal consisting of the computationally effective *on-line* model-based sensitivity analysis. The appealing idea is to suggest the optimal values of experimental design variables *on-line*, i.e., to perform the experimental protocol modification (or tuning) during FRAP measurements. The main drawback of this very last idea is neither mathematical nor technical difficulty but the complicated communication between the members of mathematical and biological community.

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