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Estimation of Diffusivity of Phycobilisomes on Thylakoid Membrane based on spatio-temporal FRAP images ¹

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Abstract:

The determination of phycobilisomes diffusivity (diffusion coefficient D) on thylakoid membrane from fluorescence recovery after photobleaching (FRAP) experiments is usually done by analytical models. However, analytical models need some unrealistic conditions to be supposed. This study describes the development of a method based on finite difference approximation of the process governed by the Fickian diffusion equation and on the minimization of an objective function representing the disparity between the experimental and simulated time-varying concentration profiles. Our method improves on other models by accounting for experimentally measured time-varying Dirichlet boundary conditions, and can include a reaction term as well. As a result we obtain both the overall (time averaged) diffusion coefficient D and the sequence of diffusivities D_j based on two successive fluorescence profiles in j -th time interval. Due to the noisy data, we cope with an inverse ill-posed problem and a regularization technique is mandatory.

Keywords:

Parameter estimation, FRAP, boundary value problem, optimization

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1 Introduction

The organization and dynamics of many photosynthetic pigment-protein complexes in the photosynthetic membrane is frequently studied using fluorescence confocal microscopy by means of Fluorescence Recovery After Photobleaching (FRAP) measuring technique [12]. FRAP has been used since 1970s to study lateral mobility on the cell surface. Later it has been extended to the investigation of protein dynamics within the living cells [10] including thylakoid membrane proteins [8, 6]. Based on spatio-temporal FRAP images, the mobility of photosynthetic complexes in a native intact membrane, i.e. the diffusivity or diffusion coefficient D ,³ is reconstructed using either a *closed form model* or *simulation based model* [9, 5]. The FRAP images are in general very noisy, with small signal to noise ratio (SNR), which requires an adequate technique assuring the reliable results.⁴

Our study describes the development of a method aiming to determine the phycobilisomes diffusivity on thylakoid membrane from FRAP experiments. As we know, this is usually done by experimental curve fitting to the analytical (closed form) models, see e.g. [1, 8, 6]. However, the closed form models need some unrealistic assumptions. For example, C. W. Moulineaux *et al.* [8] have exploited the rotational symmetry of the cells by bleaching a plane across the short axis of the cell. This approach allowed construction of bleaching profiles along the long axis. Supposing that: (i) $x \in \mathbb{R}$, i.e. the infinite domain, (ii) the initial bleaching profile is Gaussian: $y(x, t_0) = y_{0,0} \exp \frac{-2x^2}{r_0^2}$, where r_0 is the half-width of the bleach at time $t_0 = 0$, $y_{0,0}$ is the maximum depth at time t_0 , i.e. the depth ($y_{0,0} < 0$) of the first post-bleach signal at the center ($x = 0$), and (iii) boundary conditions correspond to the complete recovery: $y \rightarrow 0$ as $t \rightarrow \infty$, $y \rightarrow 0$ as $x \rightarrow \infty$; then the solution $y(x, t)$ of diffusion equation $\frac{\partial y}{\partial t} = D \frac{\partial^2 y}{\partial x^2}$ and the maximum depth at time t , i.e. $y(0, t)$ are as follows:

$$y(x, t) = \frac{y_{0,0}r_0}{\sqrt{r_0^2 + 8Dt}} \exp \frac{-2x^2}{r_0^2 + 8Dt}, \quad y(0, t) = \frac{y_{0,0}r_0}{\sqrt{r_0^2 + 8Dt}}.$$

The calculation of diffusion coefficient D then resides in the weighted linear regression: a plot of $(\frac{y_{0,0}}{y(0,t)})^2$ against time should give a straight line with the tangent $\frac{8D}{r_0^2}$.

As the analytical approach has several limitations (e.g. restriction to the cell geometry, full recovery is required, bleach profile must be gaussian-like, etc.) we model the process by the Fickian diffusion equation (with realistic initial and boundary conditions) instead. The estimation of diffusivity is further formulated as an optimization problem consisting in the minimization of an objective function representing the disparity between the experimental and simulated time-varying concentration profiles.

³I. F. Sbalzarini in [10] distinguishes between the molecular diffusion constant and the apparent diffusion constant; while the former is directly measured by single-molecule techniques, the latter is determined by coarse-grained methods such as FRAP, averaging over a certain observation volume.

⁴Let us mention that the fluorescence confocal microscope allows the selection of a thin cross-section of the sample by rejecting the information coming from the out-of-focus planes. However, the small energy level emitted by the fluorophore and the amplification performed by the photon detector introduces a measurement noise.

2 Problem formulation

2.1 Theory

FRAP (Fluorescence Recovery After Photobleaching) technique allows detection of diffusivity of autofluorescence compound like proteins (e.g. phycobiliproteins) and also other non-fluorescence compound that are fluorescently tagged (e.g. green fluorescence proteins - GFP). This method is based on application of short, intense laser irradiation (the so called bleach) to a small target region (Region Of Interest - ROI) of the cell that causes irreversible loss in fluorescence in this area without any damage in intracellular structures. After the "bleach" (or "bleaching"), the observed recovery in fluorescence in the "bleached area" reflects diffusion of fluorescence compounds from the area outside the bleach.

For an arbitrary bleach spot and assuming (i) local homogeneity, i.e. assuring that the concentration profile of fluorescent particles is smooth, (ii) isotropy, i.e. diffusion coefficient is space-invariant, (iii) an unrestricted supply of unbleached particles outside of the target region, i.e. assuring the complete recovery,⁵ the unbleached particle concentration C as a function of spatial coordinate \vec{r} and time t is modeled with the following diffusion-reaction equation on two-dimensional domain Ω :

$$\frac{\partial C}{\partial t} - \nabla \cdot (D \nabla C) = R(C) , \quad (1)$$

where D is the fluorescent particle diffusivity within the domain Ω and $R(C)$ is a reaction term.

The initial condition and time varying Dirichlet boundary conditions are:

$$C_0 = f(\vec{r}, t_0) \text{ in } \Omega, \quad C(t) = g(\vec{r}, t) \text{ in } \partial\Omega \times [t_0, T]. \quad (2)$$

The reaction term $R(C)$ is often viewed as negligible under assumptions that diffusion of fluorescence compounds (proteins) is not restricted (e.g. by some binding to the medium) and that photobleaching of these molecules during recovery is negligible. Consequently, if $R(C)$ is neglected, Eq. (1) becomes the Fickian diffusion equation. In contrast, under continual photobleaching during image acquisition, this reaction term could be described as a first order reaction:

$$R(C) = -k_S C , \quad (3)$$

where k_S is a rate constant describing bleaching during scanning [5].

It is of utmost importance to identify the relation between concentration of particles C and fluorescent signal ϕ . Although Eq. (1) and objective function J , cf. (10), works with concentrations, in fact we measure the fluorescence intensity level and not directly C . If the relation $C = k_F \phi$ holds, where k_F is a constant, than we can work with the measured signal without necessity of any recalculation. On the contrary, if k_F is space or time dependent, then we should design an experiment and estimate this dependence.

⁵The recovery is not always complete. It is usually modelled by introducing some correction term. More consistent method resides in the special time dependent Neumann boundary condition in form of a saturation curve.

Before bleaching, some number of so-called pre-bleach measurements are performed. Notice that the pre-bleach profile C_{pre} represents a steady-state constant concentration profile which has to be gradually recovered for $t \rightarrow \infty$. Thereafter, based on the pre-bleach data ϕ_{pre} (e.g. its average value), we reach the coefficient k_F as follows:

$$k_F = \frac{C_{pre}}{\phi_{pre}}.$$

Consequently, in order to have experimental values C_{exp} representing the concentration profiles after bleaching, we have to divide the post-bleach fluorescence signal by its pre-bleach value, as it is explained in the following.

2.2 One-dimensional model

For a linear bleach spot perpendicular to a longer axis (let this axis be denoted as r) and assuming local homogeneity and isotropy, the recovery of unbleached particle concentration as a function of spatial coordinate r and time t is modeled with a linear, diffusion-reaction equation

$$\frac{\partial C}{\partial t} - D \frac{\partial^2 C}{\partial r^2} = R(C). \quad (4)$$

If we adopt the form of reaction term according to (3), and introduce the dimensionless spatial coordinate x , the dimensionless diffusion coefficient p , the dimensionless time τ and the dimensionless concentration y by

$$r := xL, \quad D := p D_0, \quad t := \tau \frac{L^2}{D_0}, \quad y := \frac{C}{C_{pre}} = \frac{\phi}{\phi_{pre}}, \quad (5)$$

where L is the length of our specimen in direction perpendicular to bleach spot, D_0 is a constant with some characteristic value (unit: m^2s^{-1}), and C_{pre} is a pre-bleach concentration of C , we finally obtain the following form of dimensionless diffusion-reaction equation on one-dimensional domain, i.e. for $x \in [0, 1]$

$$\frac{\partial y}{\partial \tau} - p \frac{\partial^2 y}{\partial x^2} = -\frac{k_S L^2}{D_0} y. \quad (6)$$

The initial condition and time varying Dirichlet boundary conditions are:

$$y(x, \tau_0) = f(x), \quad x \in [0, 1], \quad (7)$$

$$y(0, \tau) = g_0(\tau), \quad y(1, \tau) = g_1(\tau), \quad \tau \geq \tau_0. \quad (8)$$

2.3 Experimental data

Based on FRAP experiments, see Fig. 1 [6], we have a 2D matrix of dimension $(N+1, M+m+1)$ with pre-bleach and post-bleach experimental values

$$y_{exp}(x_i, \tau_j), \quad i = 0 \dots N, \quad j = -m \dots M, \quad (9)$$

which can be read by columns as the concentration profiles (along x axis) in $M + m + 1$ discrete time points (m corresponds to the number of columns with pre-bleach data). Recall that τ_0 corresponds to the first post-bleach measurement, and $x_0 = 0$, $x_N = 1$.

Consequently $y_{exp}(x_i, \tau_0)$, $i = 0 \dots N$, represents the initial condition, and $y_{exp}(0, \tau_j)$, resp. $y_{exp}(1, \tau_j)$, $j = 0 \dots M$, the left and right Dirichlet boundary conditions, respectively.

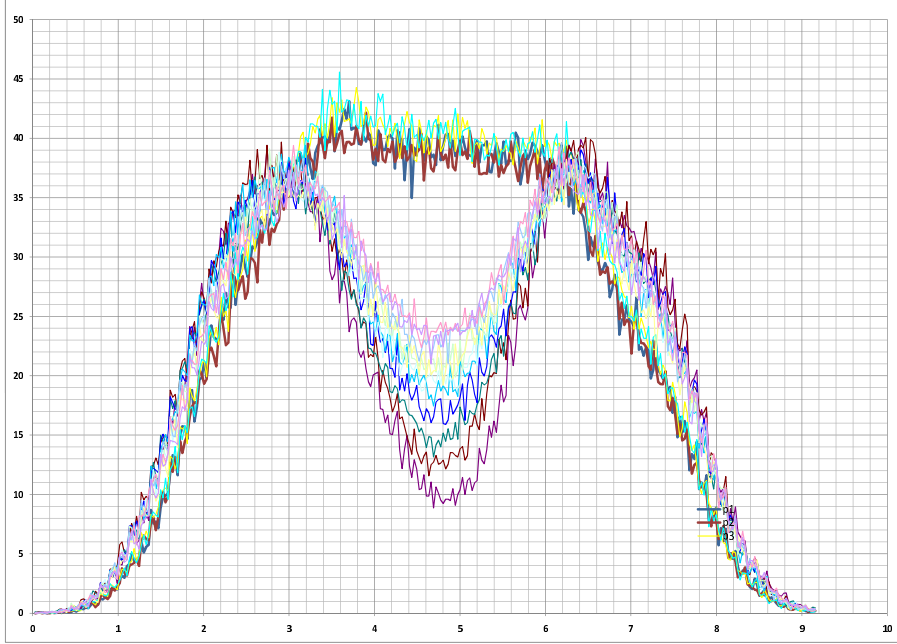


Figure 1: Fluorescence intensity (in arbitrary units) vs. Distance [μm]. Experimental data from FRAP experiment with red algae *Porphyridium cruentum* describing the phycobilisomes mobility on thylakoid membrane [6].

2.4 Determination of diffusivity as a parameter estimation problem

The problem of autofluorescence compound (e.g. phycobilisomes) diffusivity determination based on time series of FRAP experimental data will be further formulated as a parameter estimation problem. We construct an objective function J representing the disparity between the experimental and simulated time-varying concentration profiles, and then within a suitable method we look for such a value p minimizing J . The usual form of an objective function is the sum of squared differences between the experimentally measured and numerically simulated time-varying concentration profiles:

$$J = \sum_{i=0}^N \sum_{j=0}^M [y_{exp}(x_i, \tau_j) - y_{sim}(x_i, \tau_j)]^2, \quad (10)$$

where $y_{sim}(x_i, \tau_j)$ are simulated values resulting from the solution of PDE (6) with the initial and boundary conditions (7)-(8) for the known parameter p .

Taking into account the biological reality residing in possible time dependence of phycobilisomes diffusivity, we further consider two cases. First, we can take both sums for i and j in (10) together. In this case, the scalar p is a result of a minimization problem for J . Secondly, we can consider each j -th time row separately. In this case, the M solutions p_1, \dots, p_M with values J_1, \dots, J_M correspond to each minimization problem for fixed j in sum (10) and we have a 'dynamics' of diffusivity p evolution.

Our problem is ill-posed in the sense that the solution, i.e. the diffusion coefficients $D_j = p_j D_0$, $j = 1, \dots, M$, does not depend continuously on the data. This led us to the necessity of some stabilizing procedure and the formulation of another cost function, see e.g. [3, 4, 11]

$$J = \sum_{i=0}^N \sum_{j=0}^M [y_{exp}(x_i, \tau_j) - y_{sim}(x_i, \tau_j)]^2 + \alpha (p - p_{est})^2, \quad (11)$$

where $\alpha \geq 0$ is a regularization parameter, and p_{est} is an estimation of our result. Note that taking $\alpha = 0$, function (11) turns to (10), so in next sections we will consider only expression (11) for function J .

3 Implementation

In this section we describe how we have implemented both the direct problem, i.e. solution of problem (6)-(8), and the parameter estimation problem, i.e. minimization of J in (11). For the sake of clarity we further neglect the bleaching during scanning, i.e. we put $k_S = 0$.

Minimization of the objective function J with respect to p represents a one-dimensional optimization problem. We use a suitable optimization method from the so-called UFO system [7] to solve the problem

$$\min J(p), \quad \text{subject to } p > 0.$$

After choosing initial $p^{(0)}$, the algorithm generates a sequence of iterates $\{p^{(l)}, l > 0\}$ leading to a value p^* which minimizes J . For our experimental results in Section 4 we assume that $p^{(0)} = p_{est}$. The computed value p^* is an approximation of the solution p in case of the scalar or p_j in case of considering each j -th time row separately, $j = 1 \dots M$, respectively.

Taking into account the above mentioned problem residing in a measurement noise, we try to cope with by two methods:

1. "removing" noise from all experimental values (both pre- and post-bleach data) by their smoothing using the Fourier transformation,
2. applying the regularization technique based on minimization of functional (11) instead of (10), i.e. taking $\alpha > 0$.

In order to compute a function value $J(p^{(l)})$ in (11) for a given $p^{(l)}$ in the l -th iteration, we need to know both the experimental values $y_{exp}(x_i, \tau_j)$, and the simulated values $y_{sim}(x_i, \tau_j)$, $i = 0 \dots N$, $j = 0 \dots M$. It means that in each l -th iteration we need to solve the problem (let put further $y_{sim} \equiv y$ for simplicity)

$$\frac{\partial y}{\partial \tau} - p^{(l)} \frac{\partial^2 y}{\partial x^2} = 0, \quad (12)$$

with the initial and boundary conditions defined by the experimental data

$$y(x, \tau_0) = y_{exp}(x, \tau_0) \quad \text{for } x \in [0, 1], \quad (13)$$

$$y(0, \tau) = y_{exp}(0, \tau), \quad y(1, \tau) = y_{exp}(1, \tau) \quad \text{for } \tau \geq \tau_0. \quad (14)$$

Problem (12)-(14) for simulated data $y(x_i, \tau_j)$ was solved numerically using the following two finite difference schemes for uniformly distributed nodes with the space steplength Δh and the variable time steplength $\Delta \tau$ [2]: (i) the explicit scheme of order $\Delta \tau + \Delta h^2$

$$y_{i,j} = \beta y_{i-1,j-1} + (1 - 2\beta) y_{i,j-1} + \beta y_{i+1,j-1}$$

and (ii) the Crank-Nicholson (CN) implicit scheme of order $\Delta \tau^2 + \Delta h^2$

$$-\frac{\beta}{2} y_{i-1,j} + (1 + \beta) y_{i,j} - \frac{\beta}{2} y_{i+1,j} = \frac{\beta}{2} y_{i-1,j-1} + (1 - \beta) y_{i,j-1} + \frac{\beta}{2} y_{i+1,j-1}.$$

Here $\beta = \frac{\Delta \tau}{\Delta h^2} p$ and $y_{i,j} \equiv y(x_i, \tau_j)$ are the computed values in nodes, which enter Eq. (11) as $y_{sim}(x_i, \tau_j)$. Recall that for the explicit scheme the condition $\beta \leq 1/2$ must hold.

Concerning the steplengths used in the numerical schemes, we set the space steplength to be $\Delta h = 1/N$ (smaller splitting $\Delta h = 1/(\kappa_s N)$ with $\kappa_s \in \mathbb{N}$ can also be considered). The time steplength $\Delta \tau$ is variable but should be ideally of the same order as Δh^2 (or Δh in the CN scheme) and in the explicit scheme has to fulfill the relation $\Delta \tau \leq \frac{\Delta h^2}{2p}$. In order to get from the $(j-1)$ -th time row to the j -th, we need to perform $\kappa_t = \lceil \frac{TD_0}{L^2 \Delta \tau} \rceil$ substeps of the above chosen scheme, where $\kappa_t \in \mathbb{N}$ is the smallest integer that is not less than $\frac{TD_0}{L^2 \Delta \tau}$.

4 Experimental results

In this section we illustrate the difficulties caused by the ill-posedness of our problem. We have performed numerical experiments with the real experimental data for $N = 286$ and $M = 59$ described in [6], set initial $p^{(0)} = 1$, and consider each j -th time row separately, i.e. j is fixed in sum (11). We report the results using the CN scheme (they are independent of the used scheme).

In Fig. 2 we can see big jumps in computed approximated values p_j , $j = 1, \dots, M$ when using no smoothing of experimental data neither using regularization. No significant

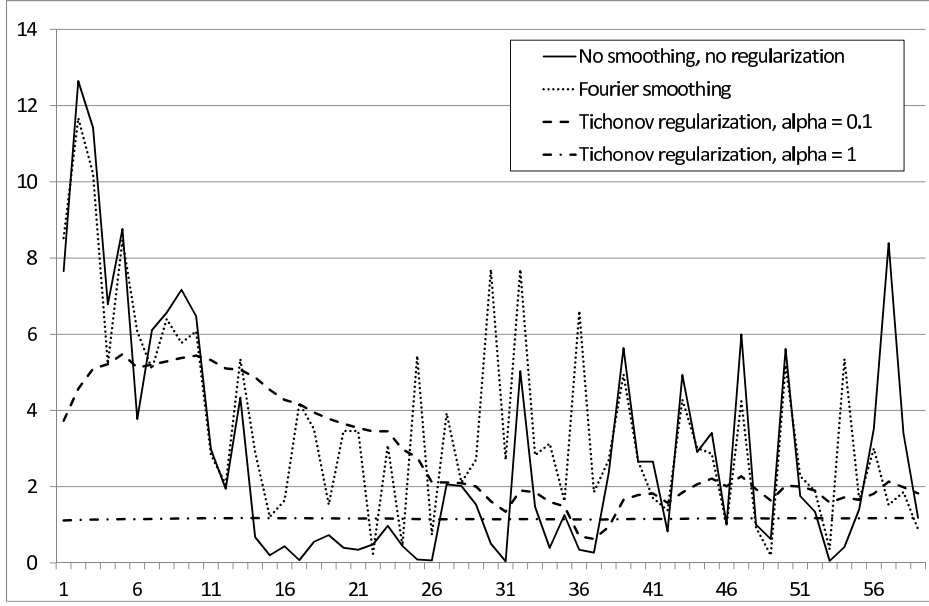


Figure 2: Dimensionless diffusivities $p_j = \frac{D_j}{D_0}$: Values p_1, \dots, p_{59} .

changes are depicted after smoothing the experimental data which is in agreement with theory (smoothing techniques usually do not improve the solution of ill-posed problems). In contrast, regularization technique seems to cope with ill-posedness quite well. However, when using this approach we would like to know more about the noise. In (11) it corresponds to a regularization parameter α or estimated expected value p_{est} . As already mentioned, we set $p_{est} = p^{(0)} = 1$ and for our experimental results, we took $\alpha = 0.1$ and $\alpha = 1$. The solution then becomes smoother and tends to the estimated value p_{est} for larger α (larger weight of the regularization term).

5 Conclusions

The purpose of this paper was to present the real problem residing in the estimation of diffusivity of phycobilisomes on thylakoid membrane based on spatio-temporal FRAP images. While the state-of-the-art methods in FRAP measurement of photosynthetic complexes mobility are usually based on the curve fitting to an analytical (closed form) models, which need some unrealistic conditions to be supposed, our method is based on finite difference approximation of diffusion process and on the minimization of an objective function evaluating both the disparity between the experimental and simulated time-varying concentration profiles and the smoothness of the time evolution of diffusivity. This approach naturally takes into account the time-dependent Dirichlet boundary conditions and can include also a reaction term (e.g. modeling the low level bleaching during scanning) and the time varying fluorescence signal as well.

Our program is actually under testing, however, for the previously known diffusion coefficient and the synthetic data corrupted by the Gaussian noise it computes correct results. Afterward, we determined the diffusivities for the real data of FRAP measurements (with the red algae *Porphyridium cruentum*). The range of result $10^{-15}\text{m}^2\text{s}^{-1}$ ($10^{-3}\mu\text{m}^2\text{s}^{-1}$) is in agreement with reference values.

In the near future, we would like to improve our method by an adequate assessment of the measurement noise and by an implementation of a more rigorous regularization algorithm.

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