Available online at www.sciencedirect.com
ScienceDirect
J. Biochem. Biophys. Methods 70 (2008) 1224–1231
JOURNAL OF biochemical and biophysical methods
www.elsevier.com/locate/jbbm

Improving parameter estimation for cell surface FRAP data

Omer Dushek, Daniel Coombs *

Department of Mathematics and Institute of Applied Mathematics, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z2

Received 25 August 2006; received in revised form 3 July 2007; accepted 8 July 2007

Abstract

Fluorescence Recovery After Photobleaching (FRAP) using the confocal laser scanning microscope has become a standard method used to determine the diffusion coefficient and mobile fraction of cell surface proteins. A common experimental approach is to bleach a stripe on the cell surface and fit the ensuing FRAP curve to a 1D diffusion model. This model is derived from the time course of recovery to an infinitely long stripe bleached on an infinite flat plane. This choice of model dictates the use of a long bleach stripe. We demonstrate that, in the case of a long bleach stripe, the finite extent of the cell leads to significant errors in parameter estimation. We further show that these errors are reduced when a relatively small stripe is bleached. Unfortunately, diffusion to such a region is fundamentally two dimensional and therefore applying the 1D model of diffusion leads to significant errors. We derive an equation suitable for fitting to FRAP data acquired from small bleach regions and analyze its accuracy using simulated data. We propose that the use of a small bleach region along with a two dimensional diffusion model is the ideal protocol for cell surface FRAP.

Keywords: Fluorescence recovery after photobleaching; Membrane diffusion; Confocal microscopy; Mathematical modeling

1. Introduction

Fluorescence Recovery After Photobleaching (FRAP) using the confocal laser scanning microscope (CLSM) is routinely used to determine the diffusion coefficient and mobile fraction of cell surface proteins (e.g. [1–3]). The method relies on the ability to conjugate a fluorescent probe, known as a fluorophore, to the protein of interest. In a specific region of the cell surface, high laser intensity denatures/bleaches the fluorophores reducing the fluorescence in that region to background levels. Motion (thermal or otherwise) of unbleached proteins from outside the bleached region causes fluorescence in the bleached region to recover over time. The exact form of the recovery is governed by the mobility properties of the labeled protein itself, modulated by the details of the experimental protocol (i.e. size and shape of the bleached region, amount of bleaching, etc.). Excellent reviews of the technique are given in [4–7]. In particular, the book chapter by Rabut and Ellenberg [7] provides a complete general description of the technique, including numerous experimental practicalities.

As described in the review of Goodwin and Kenworthy [6], a common method to obtain the diffusion coefficient (D) of the labeled protein and the fraction of these proteins that are mobile (M_f) is to fit an equation to the fluorescence recovery in the bleached region. The equation is derived from a model that captures the experimental protocol and the underlying physical processes. Commonly used models are described by Ellenberg et al. [8] and Axelrod et al. [9]. We will show here that for the space and time-scales of FRAP experiments for cell surface anchored proteins, certain key assumptions of the common models are violated resulting in significant errors in D and M_f. We will then present a refined experimental protocol and fitting procedure for use in obtaining the diffusion coefficient and mobile fraction of cell surface molecules.

2. Materials and methods

2.1. Experimental protocols for cell surface FRAP

In what follows, we assume, as is usually the case for a CLSM, that the bleaching beam and monitoring beam are parallel to each other and to the z-axis.

There are two common protocols for performing confocal FRAP for cell surface molecules using a CLSM. Protocol 1, the

* Corresponding author.
E-mail address: coombs@math.ubc.ca (D. Coombs).

0165-022X/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbbm.2007.07.002

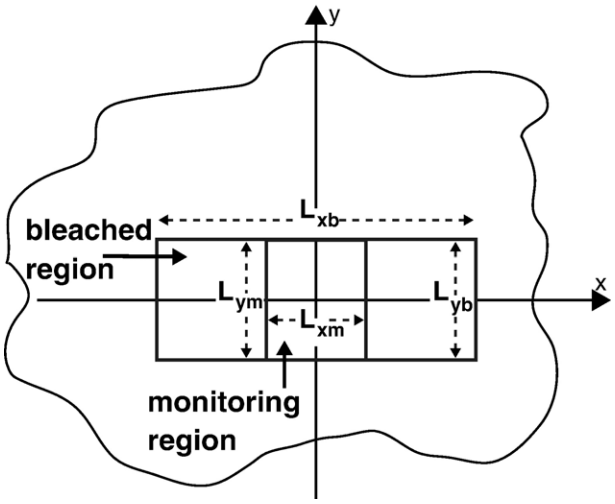

Fig. 1. Experimental geometry for protocol 1 (not to scale). Shown here is a schematic of a cell that has spread on a substrate. The focal plane of the CSLM is adjusted to obtain fluorescence from membrane anchored proteins proximal to the substrate. The bleached region is a rectangle measuring L_{xb} by L_{yb} and the monitoring region is a subset of this region measuring L_{xm} by L_{ym}.

simpler of the two, is more suitable if the cell is able to spread considerably on a substrate. In this case, the focal plane of the microscope (depicted as the *xy* plane in Fig. 1) is adjusted to obtain fluorescence from membrane proteins that are directly above the substrate. A region of the cell membrane, generally a rectangle measuring L_{xb} by L_{yb}, is selected for bleaching and in a subset of this region, which we call the monitoring region, the fluorescence is integrated to obtain the iconographic FRAP recovery curve. The dimensions of the monitoring region are L_{xm} by L_{ym}. In this case, it is quite easy to visualize both the bleached and monitoring regions. Studies where this protocol is employed are [6,10–13]. This protocol cannot always be used because many cells do not spread sufficiently and further, light scattering can cause problems when the microscope is focused close to the substrate.

The geometry is more complicated in protocol 2 because the focal plane of the microscope is set to the equatorial plane of the cell. We assume the cell is spherical and centered at the origin of a Cartesian coordinate system. A ring of fluorescence, represented as a circle in the *xy* plane, is observed from the equatorial plane of the cell (Fig. 2a). The thickness of this ring is dependent on the confocal pinhole, which controls the depth, W_z, about the focal plane where fluorescence is collected as shown in Fig. 2b. The box (W_x by W_y) in Fig. 2a represents a typical region selected for bleaching. Unlike protocol 1, here one cannot directly observe the bleached region on the cell surface. However, since we do know that the bleaching beam penetrates the entire depth of the cell [7], we can safely assume that the bleached region is a projection of the box of Fig. 2a onto the cell surface. Therefore the bleached region is a stripe, shown in Fig. 2c, measuring L_{xb} by L_{yb}. The monitoring region in this case is the intersection of the focal plane and the bleached stripe. Its dimensions are L_{xm} by L_{ym} as indicated in Fig. 2c. The fluorescence in the monitoring region is integrated to obtain the FRAP recovery curve. Examples where this protocol is employed are [1–3]. Note that, in describing protocol 2, we label experimentally adjustable parameters with a *W* and the quantities describing the actual shape of the bleached and monitoring regions by *L*.

The most common equation used to fit FRAP recovery curves generated by protocol 1 or 2 is the empirical equation presented by Ellenberg et al. [8]. We restate their 1D approximation as

$$\frac{F(t)}{F_p} = \left(1 - \frac{F_o}{F_p}\right) M_f \left(1 - \left(\frac{4\pi D t}{(L_{yb})^2} + 1\right)^{-\frac{1}{2}}\right) + \frac{F_o}{F_p} \quad (1)$$

where $F(t)$ is the time-dependent fluorescence intensity during the recovery phase, F_p the fluorescence prior to bleaching, F_o the fluorescence immediately after bleaching, D the diffusion coefficient, and L_{yb} the width of the stripe. The mobile fraction, M_f, is defined as

$$M_f = \frac{F_\infty - F_o}{F_p - F_o} \quad (2)$$

where F_8 is the asymptotic fluorescence (reached after a long recovery period). The exact analog of the empirical formula given in Eq. (1) can be derived by modeling the cell surface as an infinite (flat) plane of diffusing labeled proteins. The initial fluorescence distribution models the experimental geometry, i.e. a stripe of width L_{yb} and infinite length ($L_{xb}=\infty$) with zero intensity and a constant intensity everywhere else. This initial condition reduces the problem from 2D to 1D. The solution obtained from the diffusion equation is then integrated over the width of the stripe and is normalized. This solution agrees well with the empirical Eq. (1) [8].

Henceforth we will refer to Eq. (1) as the 1D model. To achieve an effective 1D diffusion on a finite cell, an effectively infinite stripe must be bleached on the cell surface. This can be achieved by bleaching a stripe that wraps around the cell. In protocol 1 this is accomplished by extending the bleached rectangle, shown in Fig. 1, through the entire length of the cell and in protocol 2 it is accomplished by picking $W_x=2R$. In both protocols, a symmetric 1D recovery is ensured. However, as we discuss in the results, several sources of error exist when a large stripe is bleached.

2.2. An improved protocol for cell surface FRAP

As we will show in the Results section, an improvement to the common experimental protocols for cell surface FRAP can be made by bleaching a sufficiently small region and using a 2D model of diffusion. Below we derive the necessary equations and describe the protocol. We begin by deriving the 2D equation to use when a finite rectangular region is bleached, as shown in Figs. 1 and 2.

We begin by solving the 2D infinite plane diffusion equation,

$$\frac{\partial f(x,y,t)}{\partial t} = D\left(\frac{\partial^2 f(x,y,t)}{\partial x^2} + \frac{\partial^2 f(x,y,t)}{\partial y^2}\right) \quad (3)$$