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A mathematical model to determine molecular kinetic rate constants under non-steady state conditions using fluorescence recovery after photobleaching (FRAP)

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Abstract

Fluorescence recovery after photobleaching (FRAP) analyses of binding and unbinding of molecules that interact with insoluble scaffolds, such as the cytoskeleton and nuclear matrix, in living cells commonly assume that this process is at equilibrium over the time scale of fluorescence recovery. This assumption breaks down for relatively fast intracellular processes like focal adhesion assembly at the leading edge of a migrating cell, or changes of transcriptional activation in the nucleus, that can occur in a matter of a few minutes. In this paper, we formulate a mathematical model that permits FRAP to be used to determine kinetic rate constants of molecules that interact with insoluble cellular structures under non-steady state conditions. We show that unlike steady state FRAP, fluorescence recovery time scales under these unsteady conditions are determined not only by unbinding rates, but also by the overall assembly and disassembly dynamics of the structural scaffold which supports these binding interactions. Experimental data from FRAP analysis and quantification of scaffold assembly dynamics may be combined and used with our mathematical model to estimate kinetic rate constants, as well as the apparent rate constant of scaffold assembly and disassembly.

Keywords: FRAP; Binding kinetics; Photobleaching; Unsteady; Adhesion

1. Introduction

Fluorescence recovery after photobleaching (FRAP) is a popular technique that has been used to measure mobilities of fluorescently tagged proteins inside living cells [1,12]. In this technique, a small spot in the cytoplasm, nucleus or plasma membrane of a living cell that expresses or is microinjected with a fluorescently tagged protein, is exposed to an intense laser beam at the excitation wavelength of the fluorophore. The intense irradiation causes photobleaching of the fluorescent protein in the spot making it optically invisible, although its binding functions are not altered. Because non-bleached fluorescent molecules present in surrounding areas diffuse into the irradiated region, fluorescence recovery occurs in the spot and this can be used to estimate the diffusion coefficient of the protein. If the photobleached spot contains a significant number of fluorescent molecules that are bound to insoluble scaffolds inside cells, then the recovery curve can be utilized to estimate binding (k_{ON}) and unbinding (k_{OFF}) constants of the proteins, in addition to the diffusion coefficients [7,8,10,14]. This requires the formulation of mathematical models that can be used to estimate kinetic rate constants for binding of proteins to scaffolds.

Mathematical models that estimate binding and unbinding constants for FRAP assume that the system is at steady-state [6,10,13,14]: the proteins bound to immobilized scaffolds are at equilibrium, so that the fluorescent intensity of bound molecules is constant over the time scale of the FRAP experiment. However, there has been no mathematical treatment of FRAP under non-steady state conditions. In particular, the steady state assumption may be inappropriate

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for focal adhesion proteins, such as integrins and talin, that recover over several minutes during FRAP analysis ([2] and unpublished observations), whereas whole focal adhesions remodel can remodel over time scales ranging from tens of seconds to several minutes in migrating cells [3,21]. A steady state treatment during FRAP experiments for such structures will be erroneous. The steady state assumption also might be inappropriate for nuclear proteins like Histone H1.5 that bind transiently to nuclear chromatin but exchange over several hundreds of seconds during FRAP analysis [19], i.e. on the same timescale of chromatin remodeling.

In this paper, we formulate a mathematical model that can be used to calculate binding constants for structures that are unsteady on time scales relative to the time scale of FRAP recovery. Unlike the steady state scenario, we show that FRAP recovery under non-steady state conditions is governed by k_{OFF} and k, the rate constant that describes overall remodeling of the structural scaffold of interest. With knowledge of k_{OFF} and the equilibrium constant K, it is possible to estimate k_{ON}.

2. Results and discussion

We assume that the bound population of a protein can be visualized clearly with fluorescence microscopy compared to the diffuse cytoplasmic protein concentration owing to a high concentration of binding sites on the insoluble cellular scaffold. This is a common occurrence for proteins that bind to localized binding sites on focal adhesions at the cell–substrate interface [4], junctional complexes at cell–cell adhesions [15], the cytoskeleton [11], and nuclear proteins, such as estrogen receptor [18], and RNA polymerase [6]. We model the concentration C (mol/mm^3) of freely diffusing protein; $\hat{C}$ (mol/mm^3), the concentration of protein bound to scaffolds; and S, the number of binding sites, (mol/mm^3). D (mm^2/s) denotes the diffusion coefficient of the freely diffusing protein, k_{ON} (s-mm^3/mol) and k_{OFF} (1/s) are the binding and unbinding rate constants of the protein, respectively. The model describing binding and unbinding of bound and free protein before the FRAP experiment can be written as

$$\frac{\partial C}{\partial t} = D\nabla^2 C - k_{ON}SC + k_{OFF}\hat{C} \tag{1}$$

$$\frac{\partial \hat{C}}{\partial t} = k_{ON}SC - k_{OFF}\hat{C}. \tag{2}$$

We assume that: 1) the experiment is designed such that fluorescently tagged diffusing proteins are negligibly bleached [9], 2) the diffusion time scale in the bleached spot is much smaller than the time scale of FRAP recovery, and 3) the contribution of the freely diffusing protein is negligible to the FRAP curve because $\hat{C} \gg C$. These assumptions imply that the recovery during FRAP is purely due to recovery in the bound concentration of protein which commonly holds for structures like focal adhesions [20] and nuclear scaffolds [16,17]. If $\hat{C}_F$ is the fluorescent bound protein and $\hat{C}_P$ is the photobleached bound protein, then under these assumptions the equations become:

$$\frac{d\hat{C}_F}{dt} = k_{ON}SC_0 - k_{OFF}\hat{C}_F \tag{3}$$

$$\frac{d\hat{C}_P}{dt} = k_{ON}SC_P - k_{OFF}\hat{C}_P \tag{4}$$

with initial conditions $\hat{C}_F = \alpha C_0$ and $\hat{C}_P = (1-\alpha)C_0$ where $\alpha < 1$ depends on the extent of photobleaching. $C = C_0$ is the (unchanged) concentration of freely diffusing protein that surrounds the structures. The implication is that the FRAP recovery curve is entirely due to recovery in the fluorescent bound protein concentration. Because the bound protein is not functionally destroyed, but only made optically invisible [13], these equations must obey the constraint $\hat{C}_F(t) + \hat{C}_P(t) = \hat{C}(t)$, where $d\hat{C}/dt = k_{ON}SC - k_{OFF}\hat{C}$.

In the FRAP literature, it is commonly assumed that throughout the FRAP experiment, $d\hat{C}/dt = k_{ON}SC - k_{OFF}\hat{C} = 0$ so that for all times, $\hat{C} = \hat{C}_0 = k_{ON}SC_0/k_{OFF}$ [10,14]. Then, letting $\hat{c}_F \equiv \hat{C}_F/\hat{C}_0$ and $C_0 = C_F$ (this latter equation follows from the assumption that the freely diffusing pool is negligibly bleached), Eq. (3) becomes $d\hat{c}_F/dt = k_{OFF}(1 - c_F)$ with the initial condition $c_F(0) = \alpha$ where $\alpha < 1$. The solution to this can be represented as $(\hat{c}_F - \alpha)/(\hat{c}_F(\infty) - \alpha) = 1 - e^{-k_{OFF}t}$ which is identical to the experimentally normalized FRAP recovery curve $(F(t) - F(0))/(F(\infty) - F(0))$. This means that FRAP uniquely yields k_{OFF} under steady state conditions when the diffusing protein pool is not bleached in FRAP [5,10].

Here, we relax this assumption by considering slow assembly and disassembly of the whole structural scaffold that results in a slow time-dependent change in the available binding sites. We let $S(t) = S_0 e^{-kt}$ so that $d\hat{C}/dt = k_{ON}SC - k_{OFF}\hat{C} \neq 0$, with the initial condition $\hat{C} = \hat{C}_0$. Depending on the sign of k, this structure either assembles or disassembles. In general, $|\frac{k_{OFF}}{k}| < 1$ so that assembly or disassembly occurs slower than fluorescence recovery during FRAP. On photobleaching, at time t_0, only some proteins become invisible, but this has no effect on the available binding sites so that $d\hat{C}_F/dt = k_{ON}S_0 e^{-kt}C_F - k_{OFF}\hat{C}_F$ and $d\hat{C}_P/dt = k_{ON}S_0 e^{-kt}C_P - k_{OFF}\hat{C}_P$. Adding these equations confirms that $\hat{C}_F(t) + \hat{C}_P(t) = \hat{C}(t)$.

Photobleaching can be performed at any time $t = t_0$; $t = 0$ is defined as the time when imaging starts. Throughout the experiment, we assume that C_F is constant. Thus, over the time $t = (0, t_0)$, there is no photobleaching and the dynamics is decided by the time scale $1/k$, while at $t = t_0$, the fluorescent protein is bleached causing the initial condition $\hat{C}_F(0) = \alpha\hat{C}_F$. Letting $\omega \equiv t - t_0$, the equations can be written as

$$\frac{d\hat{C}_F}{d\omega} = k_{ON}S_0 e^{-k(\omega+t_0)}C_F - k_{OFF}\hat{C}_F;\ t_0 \leq \omega < 0 \tag{5}$$

$$\frac{d\hat{C}_F}{d\omega} = k_{ON}S_0 e^{-k(\omega+t_0)}C_F - k_{OFF}\hat{C}_F;\ 0 \leq \omega \tag{6}$$

with $\hat{C}_F(-t_0)=\hat{C}_0$ and $\hat{C}_F(0)=\alpha\hat{C}_F(0-)$. This is a delay differential equation which can be solved analytically. Before that, we define $\hat{c}_F=\hat{C}_F/\hat{C}_0$. The equations become

$$\frac{d\hat{c}_F}{d\omega} = k_{ON}S_0 e^{-k(\omega+t_0)}\frac{C_0}{\hat{C}_0} - k_{OFF}\hat{c}_F; t_0\le\omega\le 0 \quad (7)$$

$$\frac{d\hat{c}_F}{d\omega} = k_{ON}S_0 e^{-k(\omega+t_0)}\frac{C_0}{\hat{C}_0} - k_{OFF}\hat{c}_F; 0\le\omega \quad (8)$$

where $\hat{c}_F(-t_0)=1\hat{c}_F(0)=\alpha\hat{c}_F(0-)$. The precise value of S_0 is not known, so we let $k^*_{ON}=k_{ON}S_0$ [14]. The Eqs. (7) and (8) become

$$\frac{d\hat{c}_F}{d\omega} = k^*_{ON}e^{-k(\omega+t_0)}\frac{C_0}{\hat{C}_0} - k_{OFF}\hat{c}_F; \quad \hat{c}_F(-t_0)=1,\ -t_0\le\omega<0 \quad (9)$$

$$\frac{d\hat{c}_F}{d\omega} = k^*_{ON}e^{-k(\omega+t_0)}\frac{C_0}{\hat{C}_0} - k_{OFF}\hat{c}_F; \hat{c}_F(0+)=\alpha\hat{c}_F(0-), 0\le\omega \quad (10)$$

The solution to Eq. (9) is

$$\hat{c}_F = \frac{k^*_{ON}}{k-k_{OFF}}\frac{C_0}{\hat{C}_0}\left(-e^{-k(\omega+t_0)}+e^{-k_{OFF}(\omega+t_0)}\right) + e^{-k_{OFF}(\omega+t_0)} \text{ for } -t_0\le\omega<0 \quad (11)$$

while that to Eq. (10) for $0\le\omega$ is

$$\hat{c}_F = -\frac{k^*_{ON}}{k-k_{OFF}}\frac{C_0}{\hat{C}_0}e^{-k(w+t_0)} + e^{-k_{OFF}w-kt_0}\frac{k_{ON}}{k-k_{OFF}}\frac{C_0}{\hat{C}_0} - e^{-k_{OFF}w-2kt_0}\frac{k_{ON}}{k-k_{OFF}}\frac{C_0}{\hat{C}_0} + e^{-k_{OFF}w-2k_{OFF}t_0}\alpha\left(\frac{k_{ON}}{k-k_{OFF}}\frac{C_0}{\hat{C}_0}+1\right) \quad (12)$$

We recast the solution to FRAP recovery as

$$\hat{c}_F = e^{-k_{OFF}(\omega+t_0)}\left(1-\frac{1}{K(1-\varphi)}\frac{C_0}{\hat{C}_0}\left(1-e^{(1-\varphi)k_{OFF}(\omega+t_0)}\right)\right) \text{ for } -t_0\le\omega<0 \quad (13)$$

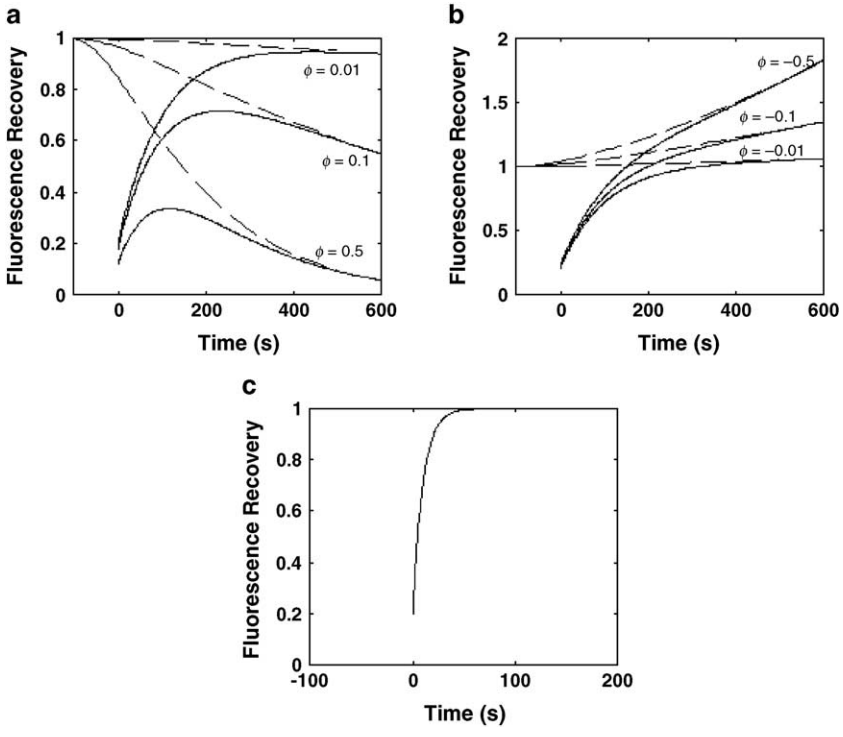

Fig. 1. Model calculations of FRAP recovery curves for molecular binding to structural scaffolds under non-steady state conditions (Eqs. (13) and (14)). (a) Predicted FRAP recovery curve for disassembling structures with $K=0.01$, $\alpha=0.2$, $k_{OFF}=0.01$, $t_0=100$ s and different values of ϕ. As ϕ increases, unsteady dynamics of disassembly of the structural scaffold have an increasing effect on the nature of the recovery curve (b) FRAP recovery curve for assembling structures with $K=0.01$, $\alpha=0.2$, $k_{OFF}=0.01$, $t_0=100$ s and different values of ϕ. (c) FRAP recovery curve for structures that are under steady state, i.e. they do not assemble or disassemble during the time scale of the FRAP experiment ($k=0$), $\alpha=0.2$, $k_{OFF}=0.01$, $t_0=100$ s.

and for $0 \leq \omega$

$$\hat{c}_F = -\frac{1}{K(\varphi-1)}\frac{C_0}{\hat{C}_0}e^{-k_{OFF}\varphi}(w+t_0) + e^{-k_{OFF}w-k_{OFF}\varphi t_0}\frac{1}{K(\varphi-1)}\frac{C_0}{\hat{C}_0} - e^{-k_{OFF}w-2k_{OFF}\varphi t_0}\frac{\alpha}{K(\varphi-1)}\frac{C_0}{\hat{C}_0} + e^{-k_{OFF}w-2k_{OFF}t_0}\alpha\left(\frac{1}{K(\varphi-1)}\frac{C_0}{\hat{C}_0}+1\right) \quad (14)$$

where $\varphi = k/k_{OFF}$ and $K = k_{OFF}/k^*_{ON}$, the equilibrium constant. Note that the time scale during FRAP recovery is governed not only by k_{OFF} but also by the overall assembly/disassembly dynamics determined by k.

Sample calculations which directly resemble the type of experimental data (normalized to the intensity at $t=0$) that would be necessary for fitting to Eqs. (13) and (14) are shown in Fig. 1. As the separation between $1/k$ (overall assembly/disassembly time scale) and $1/k_{OFF}$ (unbinding time scale) becomes small (i.e. φ approaches 1 or -1), the FRAP recovery curve becomes more and more influenced by the overall unsteady state behavior (Fig. 1 a and b).

If the structure (e.g., a focal adhesion) is at equilibrium initially, then at $\omega = -t_0$, $C_0/(K\hat{C}_0)=1$. Otherwise, for estimating rate constants, it is required to measure $C_0/(K\hat{C}_0)$. This can be done by measuring the constant K in a separate experiment under steady state conditions (assuming they exist) by measuring the ratio $K = C_{0,S}/\hat{C}_{0,S}$ where the subscript denotes steady state; this measurement also allows the estimation of $k_{ON} = k_{OFF}/K$. For example, for focal adhesions in resting (steady) cells, this ratio would correspond to the ratio of fluorescent intensity in the cytoplasm to the intensity at the adhesion which can be easily measured. At $\omega = -t_0$, when the measurement starts, the ratio $C_0/\hat{C}_0$ can be measured similarly. Experimental data can be fit to the solution in Eqs. (13) and (14) to yield φ and k_{OFF}, which together can yield the value of k since $\varphi = \frac{k}{k_{OFF}}$. From Fig. 1, it is clear that ignoring the unsteady situation may result in large errors in the estimation of the unbinding rate constant particularly as $\varphi \to 1$.

In conclusion, we have developed a mathematical model that can be used to interpret FRAP experiments performed under unsteady state conditions. This model may be used for studying the kinetic properties of proteins that interact at insoluble structural scaffolds. It is hoped that this work will help to further our understanding of protein function underlying complex dynamics of the cytoskeleton, nuclear matrix and adhesions within the microenvironment of living cells.

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References

[1] D. Axelrod, D. Koppel, J. Schlessinger, E. Elson, W. Webb, Mobility measurement by analysis of fluorescence photobleaching recovery kinetics, Biophys. J. 16 (1976) 1055–1069.
[2] C. Ballestrem, B. Hinz, B.A. Imhof, B. Wehrle-Haller, Marching at the front and dragging behind: differential alphaVbeta3-integrin turnover regulates focal adhesion behavior, J. Cell Biol. 155 (2001) 1319–1332.
[3] K.A. Beningo, M. Dembo, I. Kaverina, J.V. Small, Y.L. Wang, Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts, J. Cell Biol. 153 (2001) 881–888.
[4] A.D. Bershadsky, N.Q. Balaban, B. Geiger, Adhesion-dependent cell mechanosensitivity, Annu. Rev. Cell Dev. Biol. 19 (2003) 677–695.
[5] J.C. Bulinski, D.J. Odde, B.J. Howell, T.D. Salmon, C.M. Waterman-Storer, Rapid dynamics of the microtubule binding of ensconsin in vivo, J. Cell Sci. 114 (2001) 3885–3897.
[6] M. Dundr, U. Hoffmann-Rohrer, Q. Hu, I. Grummt, L. Rothblum, R. Phair, T. Misteli, A kinetic framework for a mammalian RNA polymerase in vivo, Science 298 (2002) 1538.
[7] E.N. Kaufman, R.K. Jain, Quantification of transport and binding parameters using fluorescence recovery after photobleaching, Biophys. J. 58 (1990) 873–885.
[8] E.N. Kaufman, R.K. Jain, Measurement of mass transport and reaction parameters in bulk solution using photobleaching, Biophys. J. 60 (1991) 596–608.
[9] T. Lele, J. Pendse, S. Kumar, M. Salanga, J. Karavitis, D. Ingber, Mechanical forces alter zyxin unbinding kinetics within focal adhesions of living cells. J. Cellular Physiology (in press).
[10] T.P. Lele, P. Oh, J. Nickerson, D. Ingber, An improved mathematical approach for determination of molecular kinetics in living cells with FRAP, Mech. Chem. Biosyst. 1 (2004) 181–190.
[11] N.M. McKenna, J.B. Meigs, Y.L. Wang, Exchangeability of alpha-actinin in living cardiac fibroblasts and muscle cells, J. Cell Biol. 101 (1985) 2223–2232.
[12] R. Phair, T. Misteli, High mobility of proteins in the mammalian cell nucleus, Nature 404 (2000) 604–609.
[13] R. Phair, T. Misteli, Kinetic modelling approaches to in vivo imaging, Nat. Rev., Mol. Cell Biol. 2 (2001) 898–907.
[14] B.L. Sprague, R.L. Pego, D.A. Stavreva, J.G. McNally, Analysis of binding reactions by fluorescence recovery after photobleaching, Biophys. J. 86 (2004) 3473–3495.
[15] M.S. Steinberg, P.M. McNutt, Cadherins and their connections: adhesion junctions have broader functions, Curr. Opin. Cell Biol. 11 (1999) 554–560.
[16] D.L. Stenoien, M. Mielke, M.A. Mancini, Intranuclear ataxin1 inclusions contain both fast- and slow-exchanging components, Nat. Cell Biol. 4 (2002) 806–810.
[17] D.L. Stenoien, A.C. Nye, M.G. Mancini, K. Patel, M. Dutertre, B.W. O'Malley, C.L. Smith, A.S. Belmont, M.A. Mancini, Ligand-mediated assembly and real-time cellular dynamics of estrogen receptor alpha-coactivator complexes in living cells, Mol. Cell Biol. 21 (2001) 4404–4412.
[18] D.L. Stenoien, K. Patel, M.G. Mancini, M. Dutertre, C.L. Smith, B.W. O'Malley, M.A. Mancini, FRAP reveals that mobility of oestrogen receptor-alpha is ligand- and proteasome-dependent, Nat. Cell Biol. 3 (2001) 15–23.
[19] J.P. Th'ng, R. Sung, M. Ye, M.J. Hendzel, H1 family histones in the nucleus. Control of binding and localization by the C-terminal domain, J. Biol. Chem. 280 (2005) 27809–27814.
[20] G. von Wichert, B. Haimovich, G.S. Feng, M.P. Sheetz, Force-dependent integrin-cytoskeleton linkage formation requires downregulation of focal complex dynamics by Shp2, EMBO J. 22 (2003) 5023–5035.
[21] R. Zaidel-Bar, C. Ballestrem, Z. Kam, B. Geiger, Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells, J. Cell Sci. 116 (2003) 4605–4613.