

Force Meets Chemistry: Analysis of Mechanochemical Conversion in Focal Adhesions Using Fluorescence Recovery After Photobleaching

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Abstract Mechanotransduction—the process by which mechanical forces are converted into changes of intracellular biochemistry—is critical for normal cell and tissue function. Integrins facilitate mechanochemical conversion by transferring physical forces from the extracellular matrix, across the cell surface, and to cytoskeletal-associated proteins within focal adhesions. It is likely that force alters biochemistry at these sites by altering molecular binding affinities of a subset of focal adhesion proteins, but this has been difficult to quantify within living cells. Here, we describe how the fluorescence recovery after photobleaching (FRAP) technique can be adapted and used in conjunction with mathematical models to directly measure force-dependent alterations in molecular binding and unbinding rate constants of individual focal adhesion proteins *in situ*. We review these recent findings, and discuss the strengths and limitations of this approach for analysis of mechanochemical signaling in focal adhesions and other cellular structures. The ability to quantify molecular binding rate constants in the physical context of the living cytoplasm should provide new insight into the molecular basis of cellular mechanotransduction. It also may facilitate future efforts to bridge biological experimentation and mathematical modeling in our quest for a systems biology level description of cell regulation. *J. Cell. Biochem.* 97: 1175–1183, 2006. © 2006 Wiley-Liss, Inc.

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Cells in tissues are constantly exposed to mechanical forces, including shear stresses on cells lining blood vessels and kidney collecting ducts, compression on bone, and stretching forces on skin and muscle. Conversion of these mechanical signals into intracellular biochemical changes, a process known as mechanotransduction, is critical for normal tissue growth and function. Mechanical forces exerted on whole organs and tissues result in physical distortion of the extracellular matrices (ECMs) that hold cells together. At a smaller size

scale, these mechanical forces are transferred across the cell surface by specialized transmembrane ECM receptors called integrins. The cytoplasmic tails of integrins are mechanically linked to actin microfilaments of the intracellular cytoskeleton through a large variety of proteins that co-assemble to form macromolecular anchoring complexes, known as focal adhesions. Stresses applied to integrin receptors can therefore be focused on distant parts of the cell, including the nucleus, by channeling over these microfilaments, as well as other cytoskeletal fibers (microtubules and intermediate filaments) to which they interconnect [Maniotis et al., 1997; Helmke et al., 2001; Hu et al., 2004]. The efficiency of force transfer through this network, and the resulting mechanotransduction response also can be altered by modulating the level of isometric tension (prestress) within the cytoskeleton [Hu et al., 2005].

When stress is applied to integrins with ligand-coated pipettes pulled with a micromanipulator [Riveline et al., 2001], ECM-coated

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microbeads tensed with an optical tweezer [Choquet et al., 1997], or ECM-coated magnetic microbeads displaced using applied magnetic fields [Wang et al., 1993; Matthews et al., 2004], cells mechanically adapt to these forces by becoming stiffer. Most of this response is mediated by an increase in focal adhesion size and density along with changes in their molecular composition that requires the presence of activated integrin receptors [Galbraith et al., 2002; Matthews et al., 2004, 2005]. Cell-substrate adhesion size correlates directly with the level of traction force that is exerted on the substrate at these sites in stationary cells [Balaban et al., 2001]. Moreover, when cytoskeletal prestress is dissipated by inhibiting actomyosin contractility, adhesions disassemble [Bershadsky et al., 2003]. In addition, focal adhesion disassembly at the cell base can be overcome by applying external mechanical loads to the apical surface of the cell [Riveline et al., 2001; Tzima et al., 2001]. This dependence of focal adhesion size, protein localization, and signaling on internal cytoskeletal prestress or external mechanical stress has led to the hypothesis that integrins act as mechanoreceptors, and that certain focal adhesion proteins function as mechanosensors by altering their conformation and binding kinetics in response to stress [Ingber, 1991, 1997; Khan and Sheetz, 1997; Bershadsky et al., 2003].

MECHANICAL CONTROL OF BIOCHEMICAL KINETICS

What is the molecular basis for the effect of mechanical force on focal adhesion size, protein localization, and remodeling? Force-dependent changes in the rate constants for binding and unbinding can give rise to net assembly or disassembly of molecules in adhesions. Mechanical stresses may alter molecular binding kinetics as a result of molecular distortion and changes in the three-dimensional (3D) conformation of critical mechanosensitive molecules [Ingber, 1997; Khan and Sheetz, 1997]. There have been numerous studies of the effect of mechanical force on the kinetics of protein-protein interactions. Bell [1978] first proposed that the unbinding rate constant, k_{OFF} , of a receptor that non-covalently binds to ligand could increase exponentially with an increase in the imposed mechanical force. This was shown to be experimentally valid for adhesive bonds

between neutrophils in shear flow that tether transiently to P-selectin-coated substrates [Chen and Springer, 2001]. Such ligand-receptor bonds that break more easily under increasing force are termed slip bonds. However, increasing force can also make bonds stronger; these are termed catch bonds [Dembo et al., 1988; Marshall et al., 2003; Konstantopoulos et al., 2003]. Direct experimental evidence for the existence of catch bonds has come from studies that used atomic force microscopy (AFM) to study bonds between purified P-selectin and P-selectin glycoprotein ligand-1 molecules [Marshall et al., 2003]. Thermodynamic models that incorporate force-dependence of binding kinetics also have been used to study force-dependent assembly of fibrillar adhesions [Nicolas et al., 2004] and microtubule polymerization against a resisting force [Hill, 1981].

Rate constants of binding and unbinding have been measured in studies analyzing interactions between cell-surface transmembrane receptors and extracellular ligands [Chesla et al., 1998; Hanley et al., 2004; Zhang et al., 2005]. These methods rely on contacting two apposing surfaces and allow the systematic quantification of the effect of a quick mechanical tug on properties such as the unbinding rate constant between interacting proteins. For example, studies that utilize AFM to exert force on individual isolated molecules have provided unprecedented insight into the unfolding of proteins under the influence of force and its potential effect on unbinding kinetics [Merkel et al., 1999; Marshall et al., 2005]. Molecular and Brownian dynamics simulations [Evans and Ritchie, 1997; Thomas et al., 2002; Bayas et al., 2003] also are powerful computational tools that have been used to explore the effect of mechanical force on protein conformation and interactions at the molecular level.

For focal adhesion proteins, evidence that mechanical force can alter molecular binding kinetics is rare. Steered molecular dynamics simulations suggest that the focal adhesion targeting domain (FAT) of focal adhesion kinase (FAK) may change conformation in response to mechanical force, and result in an increase in its binding affinity for paxillin [Kamm and Kazempour-Mofrad, 2004]. It is also possible that force-dependent conformational changes could cause unfolding of domains in the protein, exposing cryptic binding sites for partners. This

has been shown for force-induced fibronectin fibril assembly where cytoskeletal tension can cause fibronectin extension and unfolding in the ECM [Baneyx et al., 2002]. In fact, forces may alter focal adhesion remodeling using any one or all of these mechanisms. For example, stretching detergent-insoluble cytoskeletons causes alterations in the binding of several cytoplasmic proteins, including the focal adhesion molecules paxillin and FAK, to the cytoskeleton [Sawada and Sheetz, 2002]. This finding supports the concept that mechanical force is transduced directly into changes in molecular binding kinetics by physical distortion of critical focal adhesion proteins.

Importantly, with the exception of integrins, focal adhesion proteins are cytoplasmic. Hence, methods previously used to measure the binding properties of cell-surface receptors to extracellular ligands cannot be applied to study similar interactions between cytoskeletal-associated focal adhesion proteins *in situ*. Thus, there is a need for new experimental methods that can directly measure the binding rate constants of proteins in living cells in a spatially localized and tension-dependent manner. If effective, these methods may have value for analysis of mechanotransduction in other cellular structures, such as nuclei and microtubules that are at distant sites inside the cell.

FRAP APPLIED TO FOCAL ADHESIONS

Fluorescence Recovery after Photobleaching (FRAP) is an experimental technique that can be used to measure binding and unbinding rate constants of proteins in living cells [Phair and Misteli, 2001]. In FRAP, fluorescently-labeled molecules within a small region of the surface membrane, cytoplasm, or nucleus are exposed to a brief pulse of high intensity radiation from a laser beam at an excitation wavelength which bleaches the fluorescent tag, making it optically invisible without altering protein structure or function. Time-lapse fluorescence imaging of the bleached spot immediately after photobleaching allows the measurement of fluorescence recovery in this bleached spot. This recovery occurs because proteins diffuse in the cellular cytoplasm as well as bind and unbind from insoluble cellular scaffolds, such as focal adhesions.

There have been relatively few studies that have used FRAP to measure protein exchange

dynamics at cell-substrate adhesions, and all of these reported the half-time of fluorescence recovery ($t_{1/2}$). For example, focal adhesions that exhibit a high density of GFP-tagged $\beta 3$ integrins (as measured by fluorescence intensity) in melanoma cells were found to exhibit a lower binding affinity (faster recovery) relative to similar GFP-integrins in low density adhesions that displayed a higher binding affinity (slower recovery) [Ballestrem et al., 2001]. Disrupting the actin cytoskeleton with cytochalasin D (which dissipates cytoskeletal prestress) does not alter the half time of FRAP recovery of $\beta 3$ integrin in living endothelial cells; however, it changes the final steady state [Tsuruta et al., 2002]. FRAP analysis of the integrin binding protein α -actinin in fibroblasts reveals that the exchange rates of α -actinin are similar in adhesions and stress fibers with a $t_{1/2}$ of ~ 5 min [Edlund et al., 2001]. However, the fluorescence half time of recovery of α -actinin seems to be around 1 min in endothelial cells [Tsuruta et al., 2002] indicating the important contribution of the specific cellular microenvironment; similar cell type-specific effects have been observed for nuclear proteins [Kruhlak et al., 2000]. Separately, FRAP studies of GFP-vinculin and GFP-paxillin showed that the $t_{1/2}$ is dramatically lower in cells deficient in the protein tyrosine phosphatase Shp2, which was restored to normal levels by FAK inhibition [von Wichert et al., 2003]. Phosphorylation of FAK itself decreases the exchange rate between FAK and cytosol as measured by FRAP [Hamadi et al., 2005].

MATHEMATICAL MODELS FOR FRAP

What is the need for determining rate constants when $t_{1/2}$ appears to be adequate to report and compare FRAP data? The answer lies in the fact that $t_{1/2}$ represents a combination of diffusion (characterized by the protein's diffusion coefficient), binding kinetics (described by the rate constant k_{ON}), and unbinding kinetics (described by the rate constant k_{OFF}). Thus, measured changes in $t_{1/2}$ can arise from variations in one or more of these parameters. FRAP data have been extensively used to determine binding or unbinding constants of specific molecular binding interactions inside the nuclei of living cells [Kaufman and Jain, 1990, 1991; McGrath et al., 1998a,b; Lele et al., 2004; Sprague et al., 2004; Sprague and McNally,

2005]. This approach provides a significant advantage over biochemical methods that study protein function in solution (by destroying cells and isolating the protein of interest), because it permits the analysis of the influence of cellular microenvironments on molecular interactions.

FRAP may be an ideal technique to measure the effect of force on rate constants of focal adhesion protein interactions inside living cells, and recent work supports this hypothesis [Lele et al., 2005a]. But to accomplish this analysis, it is necessary to combine FRAP with use of mathematical models, and depending on the experimental design, solving the equations and estimating kinetic parameters may or may not be challenging (see Appendix). However, simplifications can be made if reaction or diffusion is rate-limiting [Phair et al., 2004; Lele et al., 2005a,b], and this can be leveraged to determine the unbinding rate constant k_{OFF} of focal adhesions using a relatively simple model. For example, the FRAP technique may be modified to measure only the exchange rate between bound and free protein [Lele et al., 2005a]. To ensure that the freely diffusing protein is not bleached, focal adhesions are bleached using a confocal laser spot size ($0.75\text{-}\mu\text{m}^2$ area) that is smaller than a single focal adhesion. This can be experimentally confirmed by demonstrating that when the laser beam is focused to bleach adjacent volumes in the cytoplasm in the same focal plane, there is negligible bleaching of freely diffusing GFP-focal adhesion proteins. This was shown to work for GFP-zyxin which diffuses very fast in the cytoplasm, and is not bleached during the very small times of laser exposure that were employed in this study (less than a millisecond for a $0.75\text{ }\mu\text{m}^2$ area) while the bound protein was ‘frozen’ in place over this time and therefore was bleached much more efficiently [Lele et al., 2005a].

With the experimental method above, the concentration of freely diffusing molecules may be assumed to effectively be constant (in time and space) during the course of the FRAP experiment. In this ‘well-mixed’ condition, where the diffusing molecular concentration is spatially homogeneous and temporally constant, the normalized recovery curve depends only on the unbinding constant k_{OFF} , and it is independent of the binding constant k_{ON} . Although this relationship seems counterintuitive, it follows from mathematical arguments

and is well-established in the literature [Bulinski et al., 2001; Lele et al., 2004; Sprague et al., 2004]. The rate constant k_{OFF} is therefore easily determined by fitting normalized FRAP recovery data to the expression $1 - e^{-k_{\text{OFF}}t}$. Additionally, the $t_{1/2}$ (time for 50 % recovery) under these conditions is directly related to k_{OFF} by the expression $t_{1/2} = -\ln(0.5)/k_{\text{OFF}}$.

FRAP was recently used to show that mechanical force affects the binding kinetics of the focal adhesion molecule, zyxin [Lele et al., 2005a]. Three independent experimental approaches were used to dissipate cytoskeletal tension in this study—Rho kinase inhibition with Y27632, femtosecond laser ablation of individual stress fibers, and culturing cells on compliant substrates. Interestingly, these experiments revealed that relaxing tension on focal adhesions increases the k_{OFF} of zyxin (suggesting catch-bond like behavior), but not vinculin. These findings suggest that force-dependent changes in zyxin kinetics may result either from direct conformational changes in zyxin or in one or more of its binding partners. This hypothesis is supported by recent findings that suggest zyxin to be a mechanosensitive protein [Yoshigi et al., 2005; Lele et al., 2005a].

One assumption that is made when formulating mathematical models for FRAP is that the system is at steady state before and during the time scale of fluorescence recovery. This assumption was valid in past studies [Lele et al., 2005a] that were carried out using stationary cells which form focal adhesions that are stable over time scales of FRAP recovery. However, the steady state assumption may be inappropriate for proteins, such as integrins and talin, that recover over several minutes during FRAP analysis [Ballestrem et al., 2001]; whole focal adhesions also remodel over time scales ranging from tens of seconds to several minutes in migrating cells [Beningo et al., 2001; Zaidel-Bar et al., 2003]. A steady state treatment during FRAP experiments for such structures will be erroneous; however, a mathematical model has been developed that can be used to interpret FRAP data under unsteady conditions that can address this issue [Lele and Ingber, 2005].

SIGNIFICANCE OF RATE CONSTANTS

Measuring binding affinities in the form of rate constants using FRAP may offer new

insight into basic biological mechanisms of force-dependent focal adhesion remodeling and associated mechanical activation of signal transduction. For example, force-dependent interactions between actin filament associated protein (AFAP) and the protein tyrosine kinase, Src, results in conformational change in the kinase that leads to its activation [Han et al., 2004]. This is critical because Src is a key kinase that mediates tyrosine phosphorylation of multiple focal adhesion proteins and associated signaling molecules. Because FRAP yields information about protein interactions at the level of binding affinities, it might offer direct insight into the biophysical mechanism by which stresses applied to surface integrins alter specific rate constants that govern AFAP-c-Src interactions in the future.

Mechanical strain and shear stress both induce tyrosine phosphorylation of FAK [Yano et al., 1996; Li et al., 1997; Sai et al., 1999] and this may cause changes in its affinity to substrates. The association of the signaling protein Shc with integrins in response to fluid shear stress [Jalali et al., 2001] may also be explained by changes in Shc-integrin binding kinetics. Integrin receptors are directly activated by shear stress in endothelial cells [Tzima et al., 2001] and by mechanical stretch in fibroblasts [Katsumi et al., 2005]. This activation is believed to result from conformational changes in the protein. While such conformational changes can be detected by binding to conformation-specific antibodies [Katsumi et al., 2005], determining binding kinetics of the protein in adhesions as well as its diffusion coefficient in the membrane may provide an alternative method to detect and characterize these force-dependent changes in molecular shape and function. This is particularly attractive because unlike immunolabeling, FRAP can be performed in living cells and therefore can be interpreted in the context of dynamic processes that are critical for tissue development, such as cell migration.

'Apparent' rate constants of focal adhesion proteins have been calculated by fitting an exponential curve to the changes in the total fluorescence intensity of molecules in the focal adhesion measured over several minutes in response to various perturbations [Webb et al., 2004], such as inhibition of cytoskeletal tension generation. However, there is an important distinction between the k_{OFF} measured by

FRAP and the apparent k_{OFF} determined by measuring changes in overall protein concentration in adhesions. This is because unlike rate constants measured by FRAP that contain information about molecular affinity to binding partners in the adhesion complex, apparent rate constants may represent global remodeling changes where the imaged protein merely serves as a marker for this response. For example, slow changes in focal adhesion assembly may result from removal and addition of molecular components due to protease degradation, enzymatic phosphorylation-dephosphorylation, or alterations in the number of available binding sites for a given molecule, even if intrinsic molecular kinetic parameters do not change. Thus, while the time course of focal adhesion formation or disappearance measured over minutes may be a useful metric to compare relative changes of focal adhesion assembly or disassembly, apparent rate constants measured using these data only represent gross properties; they are not interpretable in terms of changes in the native binding affinities of a given protein at the biophysical level.

The measurement of kinetic rate constants may also help in systems biology models of signaling pathways. For example, rate constants and changes in these properties in relation to mechanical force can be incorporated into mathematical models that can be used to predict biological phenomena, as well as generate and test new hypotheses. This cannot be done by simply measuring $t_{1/2}$ because it represents a combination of distinct terms in the mathematical model, such as the diffusion coefficient, and binding and unbinding constants. In fact, we recently showed the predictive potential of rate constant measurements by using force-dependent changes in the unbinding rate constant of zyxin to mathematically predict the overall force-dependent disassembly of this molecule from focal adhesions in living cells [Lele et al., 2005a]. In other words, this combined experimental and modeling technique can explain phenomena that take place over several minutes (overall adhesion remodeling) by measuring protein exchange rates that occur in seconds, and quantifying this information in terms of k_{OFF} . Thus, rate constants of binding and unbinding are an essential link between the world of mathematical modeling and biological experimentation.

POTENTIAL LIMITATIONS AND FUTURE DIRECTIONS

A typical protein in focal adhesions is bound to several binding partners, and the rate constants measured by FRAP represent an average value of rate constants that represent each interaction. FRAP measurements combined with techniques based on siRNA silencing or knockdowns along with more sophisticated mathematical models and parameter estimation techniques will be needed to address this question of molecular specificity in the future. Additionally, more reliable quantification of the dependence of k_{OFF} on force requires new methods that allow the simultaneous measurement of k_{OFF} and traction force in the same focal adhesions [Munevar et al., 2001; Tan et al., 2003]. Estimating the precise level of force on a given adhesion protein may be difficult, because mechanical force loaded on the integrins or the internal face of the focal adhesion may not be distributed equally to all adhesion proteins. This is important because while individual proteins may unfold peptide domains when mechanically loaded in vitro using techniques, such as AFM, this may not happen in cells under physiological conditions. For example, models of red blood cell membrane deformation suggest that physiological distortion of cell surfaces does not result in stress-dependent unfolding of spectrin molecules [Vera et al., 2005], even though this has clearly been demonstrated in studies with purified spectrin [Rief et al., 1999].

An important unexplored potential limitation of the FRAP technique itself is that the photobleaching process might alter the binding properties of the protein. Alternative methods to measure the kinetics need to be explored; one possibility is to measure these properties in vitro with isolated focal adhesion binding partners using AFM to allow comparisons. Other potential methods include performing FRAP using different markers like fluorescein or rhodamine for the same protein or using photoactivable GFP to activate rather than photobleach GFP-proteins and following changes in their binding interactions with focal adhesions in whole cells. It is also important to unequivocally confirm that FRAP does not functionally destroy proteins using methods, such as immunodetection after photobleaching.

Another imaging method that can be very effective for measuring the effect of mechanical

force on molecular conformation is the Fluorescence Resonance Energy Transfer technique (FRET). FRET measures the efficiency of energy transfer between fluorescent moieties and can yield dynamic information about the nanometer-range proximity between molecules. It has been used to measure nanoscale clustering of adhesion ligands and their relationship to substrate mechanics [Kong et al., 2005], the direct effect of mechanical force on the activation and spatio-temporal dynamics of Src protein kinase that phosphorylates focal adhesion proteins [Wang et al., 2005], cytoskeletal tension-mediated unfolding of fibronectin [Baneyx et al., 2002], and the locally heterogeneous patterns of vinculin activation in focal adhesions in living cells [Chen et al., 2005]. Combining FRET with FRAP may yield simultaneous information on protein conformation changes coupled with binding kinetics; this may be invaluable in studying direct force-mediated changes on protein conformation in the physical context of living cells. Such measurements together with advances in quantitative microscopy that can measure protein concentrations based on fluorescence [Iyer et al., 2005] may be used as direct inputs into predictive mathematical models. Such interdisciplinary methods may prove very useful in our quest for understanding cell regulation in the era of quantitative systems biology.

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APPENDIX

To model molecular binding kinetics within focal adhesions in living cells, let the concentration of the fluorescent tagged diffusing protein be denoted by C_F (moles/m³) and that of bound protein at the adhesion scaffold be \hat{C}_F (moles/m²). Equations describing exchange between bound and free protein are given by $\frac{\partial C_F}{\partial t} = D\nabla^2 C_F + k_{\text{ON}} C_F S - k_{\text{OFF}} \hat{C}_F$ over Ω (the cytoplasmic/membrane domain) and $\frac{\partial \hat{C}_F}{\partial t} = -k_{\text{ON}} C_F S + k_{\text{OFF}} \hat{C}_F$ over $\partial\Omega$ (the adhesion scaffold is treated as a surface and diffusional transport of bound protein is assumed negligible). D (m²/s) is the diffusion coefficient of freely

diffusing cytoplasmic or membrane protein, S is the concentration of available binding sites (moles/m³), k_{ON} (s·m³/mole) is the binding constant and k_{OFF} (1/s) is the unbinding constant; $\nabla C_F \cdot n = 0$ at all cellular boundaries. Photobleaching may be performed on a small sub-domain such that Ω_b is the bleached spot in the cytoplasm and $\partial\Omega_b$ is the bleached spot at the adhesion. The initial condition is $C_F = \alpha(r)C_0$ in Ω_b and $\hat{C}_F = \beta(r_S)\hat{C}_0$, where C_0 and \hat{C}_0 are the initial unbleached concentration of freely diffusing and bound protein respectively, r is the position vector, r_s is the surface position vector on $\partial\Omega_b$ and $\alpha < 1$, $\beta < 1$ describe the positional dependence of the bleached spot intensity for a Gaussian beam. The fluorescence recovery is $F(t) = \int_{\partial\Omega_F} \hat{C}_F dA + \int_{\Omega_F} C_F dV$. This normalized to the initial intensity gives

$$\frac{F(t)}{F_0} = \left(\int_{\partial\Omega_F} \hat{C}_F dA + \int_{\Omega_F} C_F dV \right) / \left(\int_{\partial\Omega_F} \hat{C}_0 dA + \int_{\Omega_F} C_0 dA \right)$$

that can be determined by solving the differential equations and fit to normalized FRAP data for determining rate constants and diffusion coefficients. Detailed procedures for solving the full set of equations and determining kinetic parameters from FRAP data have been published and extensively reviewed elsewhere ([Sprague et al., 2004; Sprague and McNally, 2005]).

Photobleaching does not disturb local equilibrium but merely makes a fraction of fluorescent protein invisible. Thus, assuming that structures have equilibrated before the FRAP experiment; an equilibrium constraint exists such that the concentration of bleached and fluorescent protein add up to the total initial steady concentration prior to photobleaching. This constraint causes equations for FRAP in the mathematical model to be linear [Lele et al., 2004]. If the photobleached spot is chosen in such a manner that $\frac{l}{w} \gg 1$ where l is the characteristic length scale of the cell and w the length scale of the photobleached spot, cellular boundaries are no longer relevant and the equations can be solved using simple methods of Laplace transforms [Axelrod et al., 1976; Sprague et al., 2004]. However, if the bleach spot is very close to membrane boundaries (for example bleaching adhesions at the cell periphery) numerical methods are needed to solve these equations because cellular boundaries are asymmetrical and irregular in 3D. However, simplifications may result if reaction or diffu-

sion is rate-limiting [Phair et al., 2004; Lele et al., 2005a,b].

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