Original Research

Introducing simulated cellular architecture to the quantitative analysis of fluorescent microscopy

Mark A. DePristo a,b,1, Lynne Chang a,c,1, Ronald D. Vale a,d, Shahid M. Khan a,e,f, Karen Lipkow a,g,*

Contents

1. Introduction .................................................................................................................. 25
2. Materials and methods ................................................................................................. 26
   2.1. Bacterial strains and constructs .............................................................................. 26
   2.2. Sample preparation ................................................................................................. 27
   2.3. FRAP microscopy and analysis .............................................................................. 27
   2.4. Smoldyn simulations ............................................................................................... 27
3. Results ............................................................................................................................ 28
   3.1. Localization of fluorescent protein fusions ............................................................ 28
   3.2. Determination of diffusion coefficients ................................................................... 27
   3.3. Determination of binding coefficients ..................................................................... 27
4. Discussion ....................................................................................................................... 30
   4.1. Conclusion .............................................................................................................. 31
Acknowledgements ......................................................................................................... 32
Supplementary data ......................................................................................................... 32
References .......................................................................................................................... 32

1 These authors contributed equally to the work.

Abstract

Biological cells are complex and highly dynamic: many macromolecules are organized in loose assemblies, clusters or highly structured complexes, others exist most of the time as freely diffusing monomers. They move between regions and compartments through diffusion and enzyme-mediated transport, within a heavily crowded cytoplasm. To make sense of this complexity, computational models, and, in turn, quantitative in vivo data are needed. An array of fluorescent microscopy methods is available, but due to the inherent noise and complexity inside the cell, they are often hard to interpret. Using the example of fluorescence recovery after photobleaching (FRAP) and the bacterial chemotaxis system, we are here introducing detailed spatial simulations as a new approach in analysing such data.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Bottom-up Systems Biology takes the data produced in experimental approaches such as Molecular Biology and Genetics and builds them into computational models that simulate systems in their complexity. These aim to test whether the current concepts

* Corresponding author at: Department of Biochemistry, Cambridge Systems Biology Centre, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, UK. Tel.: +44 1223 760230; fax: +44 1223 760241.
E-mail address: KL280@cam.ac.uk (K. Lipkow).

0079-6107/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
and thought models can work, and to find new and emergent properties in the system. Benefits of this approach include that the models are easily manipulated, allowing a test of more, and more sophisticated, variations than would be feasible experimentally, and that details can be observed which are beyond the resolution of current experimental methods.

For the simulations to be meaningful, the data they are built from have to be as good as possible. In Cellular Systems Biology, it is not only important to know which molecules react, but the rates of their binding, unbinding, and reactions; how quickly they diffuse, move or are moved; how many there are, and where they are located at which time. Most of these values are significantly different in vitro than in the context of a living cell. Macromolecular crowding slows down diffusion, enhances association over dissociation, and allows reactions that would be impossible in an aqueous solution (Lipkow et al., 2004; Minton, 2001). Organisation in loose assemblies, clusters or highly structured complexes enhance effectiveness (Bray et al., 1998; Sourjik and Berg, 2004). Separation into compartments or regions keeps potentially volatile partners apart.

An array of new methods has been developed in the past few years to fulfill the need for quantitative in vivo data, most notably those based on fluorescent microscopy (Lippincott-Schwartz and Patterson, 2003; Phair and Misteli, 2001; Verveer and Bastiaens, 2004). Due to the complexity of biological systems, however, their interpretation is not always straightforward. We here present a novel approach in which computational simulations are not only used to model existing data, but to extract numerical values from quantitative microscopy.

The *Escherichia coli* chemotaxis system allows the bacteria to swim to their optimum environment (Fig. 1). It depends on two large multiprotein complexes: inputs are detected by a cluster of signalling components, clusters or highly structured complexes enhance effectiveness (Bray et al., 1998; Sourjik and Berg, 2004). Separation into compartments or regions keeps potentially volatile partners apart.

Fluorescence recovery after photobleaching (FRAP) is a powerful method for studying the kinetic properties of a molecule including its mobility and binding interactions as well as the viscous properties of its surrounding environment (reviewed in Lippincott-Schwartz and Patterson, 2003). The method involves rapidly and irreversibly photobleaching fluorescently labelled molecules within a select region of the cell. This photobleach event effectively creates two spatially distinct populations: the photobleached and unbleached, fluorescent molecules in the neighbouring regions. Mobility of the molecules can then be visualized by monitoring the recovery of fluorescence in the photobleached region by time-lapse microscopy. The fluorescence recovery resulting from the influx of unbleached molecules can be analyzed using simple measurements to determine half-times for recovery and more complex mathematical modelling to estimate more biologically meaningful values such as the effective diffusion coefficient and the size of mobile fractions.

The analysis of FRAP data obtained on bacteria poses special challenges due to the small cell size (Elowitz et al., 1999). The low copy number of proteins results in a very noisy signal. Analysis is furthermore complicated by changing properties of molecules such as CheY and CheZ, which shuttle between a cytoplasmic and membrane-localized state (reviewed in Sourjik and Berg, 2004). We pioneered a spatial model of the *E. coli* chemotaxis system using the Brownian dynamics algorithm Smoldyn (Lipkow, 2006; Lipkow et al., 2005; Lipkow and Odde, 2008), which models the movements and reactions of each relevant molecule at high spatial and temporal resolution (Andrews and Bray, 2004; http://www.smoldyn.org). Building these models from published data, not much information was available on 3D protein dynamics. We therefore set out to determine the diffusion coefficients and cluster interaction dynamics of the signalling molecule and its phosphatase, CheY and CheZ.

The bacterial chemotaxis system is arguably the best-studied signal transduction system in biology. All components are known, as well as their structures, copy numbers, localisations, and much of the reaction kinetics (Li and Hazelbauer, 2004; Wadhams and Armitage, 2004; http://www.pdn.cam.ac.uk/groups/comp-cell/Data.html). This wealth of knowledge has made it a favoured subject of mathematical and computational models (Barkai and Leibler, 1997; Bray and Bourret, 1995; Bray et al., 1993, 2007; Mello and Tu, 2003; Shimizu et al., 2003). Most treat the bacterium as a well-stirred solution. We pioneered a spatial model of the *E. coli* cell and its chemotaxis pathway using the Brownian dynamics algorithm Smoldyn (Lipkow, 2006; Lipkow et al., 2005; Lipkow and Odde, 2008), which models the movements and reactions of each relevant molecule at high spatial and temporal resolution (Andrews and Bray, 2004; http://www.smoldyn.org). Building these models from published data, not much information was available on 3D protein dynamics. We therefore set out to determine the diffusion coefficients and cluster interaction dynamics of the signalling molecule and its phosphatase, CheY and CheZ.

Fluorescence recovery after photobleaching (FRAP) is a powerful method for studying the kinetic properties of a molecule including its mobility and binding interactions as well as the viscous properties of its surrounding environment (reviewed in Lippincott-Schwartz and Patterson, 2003). The method involves rapidly and irreversibly photobleaching fluorescently labelled molecules within a select region of the cell. This photobleach event effectively creates two spatially distinct populations: the photobleached and unbleached, fluorescent molecules in the neighbouring regions. Mobility of the molecules can then be visualized by monitoring the recovery of fluorescence in the photobleached region by time-lapse microscopy. The fluorescence recovery resulting from the influx of unbleached molecules can be analyzed using simple measurements to determine half-times for recovery and more complex mathematical modelling to estimate more biologically meaningful values such as the effective diffusion coefficient and the size of mobile fractions.

The analysis of FRAP data obtained on bacteria poses special challenges due to the small cell size (Elowitz et al., 1999). The low copy number of proteins results in a very noisy signal. Analysis is furthermore complicated by changing properties of molecules such as CheY and CheZ, which shuttle between a cytoplasmic and a clustered localization. Here, we describe the use of Smoldyn simulations that deal with these problems of analysis by replicating all relevant aspects of the system. By building a cell and chemotaxis system with known parameters and varying those numbers which we were aiming to find out, we were able to deduce the missing parameters from those simulated curves which best matched the experimental results.

2. Materials and methods

2.1. Bacterial strains and constructs

*E. coli* strain VS100 (∆cheY) containing plasmid pVS18 (amp⁸) was obtained from Dr. H.C. Berg. pVS18 encodes CheY with a carboxy terminal fusion of EYFP separated by a three glycine linker (CheY-YFP), under control of the pTrc promoter (Sourjik and Berg, 2002b). Strain BC200 (∆cheZ) was obtained from Dr. M.D. Manson. Its chromosome encodes amp⁸ and CheZ with a carboxy terminal fusion of EGFP separated by a flexible seven amino acid linker (CheZ-GFP), under control of the tac promoter (Cantwell et al., 2003). VS100/pVS18 and BC200 are wild-type for chemotaxis when induced with 0.05 mM and 1 mM isopropyl-β-D-thio-galactopyranoside (IPTG) respectively (Cantwell et al., 2003; Sourjik and Berg, 2002b).
2.2. Sample preparation

Motile cells were grown and prepared as described (Sourjik and Berg, 2004). Briefly, overnight cultures were grown while shaking in tryptone broth supplemented with 25 μg/ml of ampicillin (TB/amp) at 30 °C. Overnight cultures were diluted 1:100 into TB/amp containing IPTG (0.05 mM for VS100/pVS18 and 1 mM for BC200) and allowed to grow for 4 h in a rotary shaker at 30 °C. Slides were prepared by dispensing 100 μl of poly-l-lysine to each well, incubated for 20 min, and washed three times with 100 μl of buffer (67 mM NaCl, 10 mM potassium phosphate (pH 7.0), 100 μM EDTA, 1 mM L-methionine, 10 mM sodium lactate (pH 7.0)) (Khan et al., 2004).

2.3. FRAP microscopy and analysis

Cell populations were screened for the presence of polar clusters on a Zeiss Axiosplan microscope with a Plan Apochromat 100×1.0 NA immersion objective at room temperature (~22 °C). Cells exhibited a pronounced cluster of fluorescence at one or both poles or were uniformly fluorescent.

Confocal microscopy and FRAP were performed at room temperature (~22 °C) on an LSM 510 confocal microscope (Carl Zeiss, Inc., Thornwood, NY) with a Plan Apochromat 100×1.4 NA oil immersion objective and an Argon ion laser (30 mW power, 6.5 A tube current).

For FRAP, individual cells were selected from phase-contrast and fluorescence images, and the scan region was limited to include only the subject and one or two other cells. For cells with clusters, the bleach zone was selected to include only the pole. For FRAP of cytosolic molecules, the zone was positioned over an arbitrary end of a diffuse cell so as to include approximately one third of the cell body. For each cell, three pre-bleach fluorescence images were captured followed by photobleaching at 488 nm for CheZ-GFP and 514 nm for CheY-YFP (100% laser power; average photobleach duration = 186 ms, standard deviation = 87 ms). Recovery of fluorescence was monitored (3% laser power) using the time-series function with no cycle delay and frame scan rates ranging from 41 to 319 ms. Parallel measurements were taken from a region of similar size in a neighbouring unbleached cell. The integrated intensities of the bleached \( I_{\text{bleached}} \) and unbleached \( I_{\text{unbleached}} \) regions for the FRAP experiments were calculated using the Zeiss LSM 510 analysis software. Frames were normalized so that the bleach event occurred at time \( t = 0.0 \) s. To account for the photobleaching we expressed the recovery as a ratio of \( I_{\text{bleached}} / I_{\text{unbleached}} \).

2.4. Smoldyn simulations

Simulations were performed with version 1.58 of the Brownian Dynamics simulator Smoldyn (Andrews and Bray, 2004; http://www.smoldyn.org). The simulations were set up by modelling a single bacterium as a rectangular box of 2.4 μm × 0.88 μm × 0.88 μm, having the same volume and proportion as an average E. coli cell. For FRAP experiments on cells lacking polar clusters, the cell volume was partitioned into a “bleached zone” occupying 1/3 of the cell with the remaining being the “unbleached zone”. The cell was populated with randomly positioned protein molecules, based on published estimates: we chose 16,000 Che-YFP monomers, and for our induction conditions (Sourjik and Berg, 2002a), and 1600 CheZ dimers, as measured in wild-type cells in rich medium (Li and Hazelbauer, 2004). For modelling FRAP experiments on cells with polar clusters, the bleached volume was taken to be approximately diffraction limited (0.3 μm × 0.88 μm × 0.88 μm). The bleached zones in both cases mimicked the standards used in the experiments. The cluster was modelled as a 0.72 μm × 0.72 μm patch adjacent to the membrane in the bleached zone. We worked with the assumption that there are 3000 and 1000 available binding sites for CheY and CheZ respectively, derived from the molecule numbers measured in wt cells, 4500 CheA and 2200 CheA monomers (Li and Hazelbauer, 2004). CheY binds to the CheA P2 domain (Swanson et al., 1993), which is present in both translational variants; one or two CheY molecules each will therefore be able to bind to most of the 3350 dimers of any composition. CheZ binds selectively to the truncated P1 domain of CheA (Cantwell et al., 2003; Wang and Matsumura, 1996); assuming unbiased dimerisation efficiency, CheZ will therefore have on average 391 A_2 and possibly up to 1478 A_2 binding targets. No attempt was made to distinguish between CheY and CheYp.

Changing cytoplasmic positions of the free CheY and CheZ molecules and their patch occupancies were simulated at time steps of 0.1 ms. Local concentration effects were simulated by changing patch size. The molecules were colour-coded. After a time delay, to allow the pre-bleach steady state to be attained, “bleaching” was effected by changing the colour-code of the cytoplasmic molecule. As diffusion and unimolecular reactions, the bleaching reaction was stochastic: each molecule in the relevant zone was converted with a probability of \( P \) (e.g. \( P = 0.315 \) for Fig. 5B). Lists of the molecule positions with time, read out at 0.2 s intervals, were processed using Matlab version 7.0 (Mathworks Inc., Natick, MA) to generate FRAP recovery trajectories for comparison with the experimental data. For Fig. 5 and Supplementary movies 3 and 4, TIFFs of the simulation were generated from within Smoldyn.

3. Results

3.1. Localization of fluorescent protein fusions

Im mobilized cells of E. coli strains VS100/pVS18, expressing CheY-YFP, and BC200, expressing CheZ-GFP, exhibited pronounced localization of their fluorescence, in accordance with previous studies (Cantwell et al., 2003; Sourjik and Berg, 2000). While we did not attempt to subdivide the observed clusters into polar clusters versus the more diffuse patches (Skidmore et al., 2000; Sourjik and Berg, 2000), our impression was that the large majority of the clusters were tight rather than diffuse. The distribution of the clusters in the cell population was heterogeneous in both cases. Within one population, bacteria with uniform, mono-polar or bi-polar clustered fusion protein distributions were all found. Cell-to-cell variation in expression level may account, in part, for this heterogeneity.

The population fraction for the three distribution types for CheZ-GFP was found to depend on the level of induction. At a physiological expression level, i.e. the IPTG concentration needed to obtain wild-type chemotactic behaviour (1 mM), c. 90% of the cells had at least one clear cluster (Fig. 2). At reducing the inducer concentration, only c. 25% of cells displayed any clustering, but at increasing it 10 fold, no significant change was observed. This implies that the distribution obtained at physiological levels was close to saturation and that the lack of clusters in a proportion of the cells has to be due to other reasons than expression levels, such as dynamic protein localization of CheZ in response to signalling state (Lipkow, 2006). IPTG levels needed to obtain physiological levels of the fusion proteins were used for the FRAP studies.

3.2. Determination of diffusion coefficients

To measure cytoplasmic dynamics for the two proteins independently from their association with the polar cluster, we chose...
cells lacking polar clusters from the experimental population. We laser photobleached part (c. 1/3) of the cells (Fig. 3A). Ten or more cells were bleached for each of the CheY-YFP and CheZ-GFP cell populations. From each set, 5-6 example traces are shown in Fig. 4A and B. The recovery trajectories are very noisy, and only those for CheY-YFP were suitable to be fitted by single exponentials as expected (Elowitz et al., 1999). The decrease in fluorescence intensity of CheZ-GFP in response to photobleaching was less relative to the decrease observed with CheY-YFP, consistent with the three-fold higher photostability of EGFP versus EYFP (Shaner et al., 2007).

The FRAP recovery data in cells lacking clusters were modelled based on the assumption that the molecules were homogenous (had a single diffusion coefficient) in an isotropic environment. We created in silico cells of the volume and proportions of E. coli cells and populated them randomly with our best estimation of CheY-YFP or CheZ-GFP molecule numbers (16,000 and 1600 respectively, see Materials and methods). As above, we simulated the photobleaching event by simultaneously converting a fraction of the molecules in one third of the cell to a differently coloured species (Fig. 5A). Both the volume and the fraction of molecules bleached was based on our FRAP experiments. Coloured and bleached molecules were allowed to continue diffusing with the same diffusion coefficient, and their distribution throughout the cell was recorded.

Simulated recovery curves show the same level of noise as the experiments (Fig. 6). We ran the simulations with a series of diffusion coefficients, in five repetitions each. For CheY-YFP, these could be fitted by single exponentials. Both the exponential fits and the shape of the recovery traces were closest to the experimental measurements at a diffusion coefficient of 2 $\mu$m$^2$ s$^{-1}$ (Fig. 6A and B, compare with Fig. 4A).

Due to the 10$^5$ lower molecule numbers, the CheZ-GFP curves are a lot noisier. The mean standard deviation for the CheZ-GFP traces is about 5%, while that for the CheY-YFP traces should be $(10^{0.5}) = 3.3$ times smaller, consistent with our observations. The best match between simulated and experimentally measured curves was achieved with a diffusion coefficient of 2 $\mu$m$^2$ s$^{-1}$ (Fig. 6C, compare with Fig. 4B).

### 3.3. Determination of binding coefficients

Ten cells in each strain with a polar cluster were chosen for FRAP study of the interaction dynamics of the proteins with the cluster. The cluster was more prominent in CheZ-GFP containing cells due...
to a lower cytosolic background, compared to cells expressing CheY-YFP when the proteins were expressed at physiological concentrations. When a cytosolic region was photobleached in CheY-YFP localized cells, the entire cell’s fluorescence rapidly diminished, implying that the clustered CheY-YFP exchanged rapidly with the cytosolic pool. Most FRAP-curves of clustered CheY-YFP exhibited near-complete recovery, but at a slower rate than the cytosolic CheY-YFP in diffuse cells (Fig. 4C).

Polarly localized CheZ-GFP exhibited a radically different behaviour than diffuse CheZ-GFP or polarly localized CheY-YFP (Fig. 4D). The laser photobleaching pulse created a dark pole that lasted for at least 20 s without appreciable recovery (Fig. 3B). An initial, slight recovery was presumably due to exchange of CheZ-GFP molecules between the bleached and unbleached cytoplasmic zones. Observations of the photobleached cells beyond 20 s were difficult due to gradual bleaching of the entire cell fluorescence by the monitoring laser beam. In the reverse experiment (photobleaching of a central cytosolic region), the polar patch was left as a bright spot against a dark intracellular background over tens of seconds (data not shown). The fact that under the same experimental conditions, cells containing CheY-YFP or uniformly distributed CheZ-GFP completely recovered their fluorescence profiles ruled out cell damage as responsible for the lack of recovery seen in cells with polarly localized CheZ-GFP.

The cluster was modelled as a polar patch as detailed in Materials and methods (Fig. 5B). We examined the effect on FRAP kinetics of local binding site concentration by changing patch size from 1.3 μm²/receptor to 10³ nm²/receptor. There was a modest effect (c. 25%) on steady-state patch occupancies, but not on FRAP kinetics. Thus, local concentration effects are not significant over this range of receptor densities.

Next, we estimated the affinities needed to make clusters visible over the cytoplasmic background. Brownian dynamics simulations by Northrup and Erickson (1992) revealed that due to the stereospecificity of such interactions, the typical diffusion-limited on-rate \((k_{on})\) for protein–protein associations is c. \((2 – 6) \times 10^{6} \text{ M}^{-1} \text{s}^{-1}\). This was fixed at \((6 \times 10^{6} \text{ M}^{-1} \text{s}^{-1})\) and the dissociation constant, \(K_D\), changed by changing \(k_{off}\). We found that visible clusters will only be observed if the \(K_D\) for CheY and CheZ is < 10 μM (\(k_{off} < 10^2 \text{ s}^{-1}\)). At saturation, a CheZ-GFP cluster will be much brighter than the CheY-YFP cluster relative to the cytosolic background; due to the fact that the pool of cytosolic CheZ is much smaller than that for CheY while saturation patch occupancies are similar (Fig. 7A).

Comparing the simulated recovery curves with the experimentally obtained ones revealed a good match for the following values: CheY-YFP: \(k_{off} = 1 \text{ s}^{-1}\), giving a \(K_D\) in the order of 0.1 μM (Fig. 7B, compare with Fig. 4C). CheZ-GFP: \(k_{off} = 0.01 \text{ s}^{-1}\), giving a \(K_D\) in the order of 1 nM (Fig. 7C, compare with Fig. 4D). With these

![Fig. 4. Individual FRAP experiment traces for diffuse CheY-YFP (A), diffuse CheZ-GFP (B), clustered CheY-YFP (C), and clustered CheZ-GFP (D). The bleach event occurred at 0 s in all cases. In (A), continuous lines denote single exponential fits \((1 – Ae^{-bt})\) to the data, colour-coded to match the data points \((b = 2.8 \pm 0.8; n = 6)\).](image-url)
values, and the diffusion coefficient of $2 \, \mu m^2 \, s^{-1}$, the measured and simulated curves are similar in overall shape, recovery level, recovery time and noise level.

4. Discussion

Our strategy shows the application of simulations as a powerful new technique to extract quantitative information from complex experimental data. Demonstrating the detail of our method, the level of noise we observed was comparable in experiments and simulations. This confirms the published numbers (Cantwell et al., 2003; Li and Hazelbauer, 2004; Sourjik and Berg, 2002a) and means also that the noise was due to the low number of molecules and inherent stochasticity, and not due to potential limitations of the equipment.

Conducted within the time-constraints of a summer course, we only managed a limited number of repetitions and used unconfirmed estimates for some of the numbers that went into our model. Nevertheless, our results are within the same order of magnitude as a more thorough study that was carried out in the meantime (Schulmeister et al., 2008), and our diffusion coefficient for CheY-YFP is even within the margin of error of their best estimate. They also chose to use numerical methods to quantify their complex FRAP data.

Our estimated apparent diffusion coefficient for CheY-YFP is lower than that determined for GFP-CheY ($4.6 \, \mu m^2 \, s^{-1}$) in *Salmonella typhimurium* (Cluzel et al., 2000) and than the normally assumed value of $10 \, \mu m^2 \, s^{-1}$ (Sourjik and Berg, 2002a). The difference between the experimentally determined and calculated values could be due to a number of factors such as concentration effects (Elowitz et al., 1999), macromolecular crowding (Hall and Minton, 2003) and interactions with other cytoplasmic proteins, including CheY binding partners CheA, CheZ and FliM. In line with its role as a diffusible messenger molecule, CheY-YFP was found to have a lower affinity to the receptor cluster than CheZ-GFP. It is important to point out that we have investigated the behaviour of CheY-EYFP and CheZ-EGFP, not of CheY and CheZ on their own. At a molecular weight of c. 28 kDa, GFP and its variants are heavier than both CheY (14 kDa) and CheZ (24 kDa), and might well dominate the diffusion properties of the fusion proteins.

FRAP of cytosolic and clustered proteins is not the only technique that can be enhanced by Smoldyn simulations. We already used it to quantify the clearance of antibody-labelled membrane proteins in auditory hair cells (Grati et al., 2006). Other suitable kinetic microscopy techniques include photoactivation or photoconversion, FRET (Förster resonance energy transfer), FLIP (fluorescence loss in photobleaching), and FCS (fluorescence correlation spectroscopy). Photoactivation and photoconversion are alternate methods to photobleaching for visualizing a subpopulation of molecules by selectively initiating or altering fluorescence emission profiles (Lukyanov et al., 2005). FRET is a distance-dependent transfer of energy between an excited donor molecule and an acceptor molecule and a widely used technique for determining protein–protein interactions as well as conformational changes.

---

**Fig. 5.** Graphical output from Smoldyn simulations. The subsequent bleach zone is marked in red. (A) Diffuse CheY-YFP, as in Fig. 3A. CheY-YFP molecules (yellow) are distributed randomly in the cell. At $t = 0.0$, c. 31.5% of fluorescent molecules in the bleach zone are replaced by bleached molecules (grey). (B) Clustered CheZ-GFP, as in Fig. 3B. The binding sites (CheA, red) are randomly positioned and immobilized within the square patch at the bottom. After 2–5 s to allow saturated binding of CheZ-GFP (green) to CheA, c. 55% of both free and clustered fluorescent molecules were bleached in the marked zone by replacing them with dark green ones.
within a protein (Piston and Kremers, 2007). In FLIP, a small region of the cell is repeatedly photobleached while the loss in fluorescence in the surrounding area is monitored to analyze the mobility of the fluorescently tagged molecule (Lippincott-Schwartz et al., 2001). In FCS, a small, defined volume in the femtoliter range is monitored for fluctuations in the fluorescence signal, over a short period of time (Van Craenenbroeck and Engelborghs, 2000). These fluctuations can be analyzed to determine the concentration of the proteins in the sample volume as well as diffusion coefficients and binding constants.

Smoldyn employs the Smoluchowski level of detail, generally representing molecules as individual points in continuous space. Development is ongoing; its current version (2.03) can model internal surfaces and molecules with excluded volume. This allows simulation of very complex cellular structures, including eukaryotic cells with their membrane-bound compartments. Great care has been employed in its development, and at the level relevant for cell biology, it is exceptionally accurate. This makes it an excellent analysis tool: the user can trust the results to be biophysically correct, and does not need to develop the equations on his own every time.

4.1. Conclusion

Fluorescent microscopy has contributed tremendously toward our understanding of the inner workings of a cell and brought to light much of the inherent intricate structural complexity. It is this aspect that now makes accurate quantification of microscopy data hard to do by conventional methods. We show a first instance of how to add back some of that structural complexity into the analysis process.
Acknowledgements

This project was carried out as part of the 2004 Physiology course at the Marine Biological Laboratory (MBL). We thank the course instructors and students, particularly Drs. Tim Mitchison and Dennis Bray, for discussions, and Dr Steven Andrews for assistance with Smoldyn. We are indebted to MBL and Rudi Rotenfusser (Carl Zeiss, Inc.) for loan and help with the microscopy equipment and thank Sven Sewitz for valuable suggestions on the manuscript. We received additional support from MBL post-course research fellowships (MAD and LC), the Bauer Center for Genomic Research, a National Science Foundation graduate research fellowship (MAD), a MERIT Award (LC), grants RO1-GM64713 (Dennis Bray) and RO1-GM49319 (SMK) from the National Institutes of Health, and a Royal Society University Research Fellowship (KL). We wish Dennis Bray many happy returns.

Appendix. Supplementary data


References