

# Crowding Effects on Diffusion in Solutions and Cells

James A. Dix<sup>1</sup> and A.S. Verkman<sup>2</sup>

<sup>1</sup>Department of Chemistry, State University of New York, Binghamton, New York 13902

<sup>2</sup>Departments of Medicine and Physiology, University of California, San Francisco, California 94143; email: Alan.Verkman@ucsf.edu

Annu. Rev. Biophys. 2008. 37:247–63

First published online as a Review in Advance on February 4, 2008

The *Annual Review of Biophysics* is online at [biophys.annualreviews.org](http://biophys.annualreviews.org)

This article's doi:  
10.1146/annurev.biophys.37.032807.125824

Copyright © 2008 by Annual Reviews.  
All rights reserved

1936-122X/08/0609-0247\$20.00

## Key Terms

Brownian diffusion, anomalous diffusion, molecular crowding, cell membrane, cytoplasm

## Abstract

We review the effects of molecular crowding on solute diffusion in solution and in cellular aqueous compartments and membranes. Anomalous diffusion, in which mean squared displacement does not increase linearly with time, is predicted in simulations of solute diffusion in media crowded with fixed or mobile obstacles, or when solute diffusion is restricted or accelerated by a variety of geometric or active transport processes. Experimental measurements of solute diffusion in solutions and cellular aqueous compartments, however, generally show Brownian diffusion. In cell membranes, there are examples of both Brownian and anomalous diffusion, with the latter likely produced by lipid-protein and protein-protein interactions. We conclude that the notion of universally anomalous diffusion in cells as a consequence of molecular crowding is not correct and that slowing of diffusion in cells is less marked than has been generally assumed.

## Contents

INTRODUCTION.....	248
PHYSICS OF DIFFUSION.....	248
Normal Diffusion .....	249
Anomalous Diffusion .....	249
Anomalous Diffusion in	
Membranes .....	250
Computational Approaches	
to Diffusion .....	252
EXPERIMENTAL	
MEASUREMENTS IN	
SOLUTIONS AND CELLS .....	254
Experimental Approaches	
and Limitations .....	254
Does Aqueous-Phase Molecular	
Crowding Always Produce	
Anomalous Diffusion? .....	255
Is Membrane Protein Diffusion	
Universally Anomalous? .....	256
Diffusion in Cellular Aqueous	
Compartments .....	257
CONCLUSIONS	
AND PERSPECTIVE .....	259

**Crowding:** the exclusion of solvent volume arising from the presence of a large number of solute particles

**Brownian diffusion:** diffusion in which the MSD of a particle increases linearly with time

**Anomalous diffusion:** diffusion in which the MSD of a particle does not increase linearly with time

## INTRODUCTION

The cytoplasm and the aqueous compartments of intracellular organelles such as mitochondria are crowded with small solutes, soluble macromolecules, skeletal proteins, and membranes. Cell membranes are crowded with lipids, some of which are organized into raft structures, and proteins, some of which are tethered to skeletal proteins and contain extensive external appendages. The consequences of this crowding remain a controversial and somewhat confused topic. Popular pictorial representations of the aqueous environment within cells (16) suggest that crowding would seriously hinder solute diffusion—a major determinant of metabolism, transport phenomena, signaling, and cell motility. One possible consequence of molecular crowding and hindered diffusion is the

need for compartmentalized metabolism to overcome diffusive barriers. A second predicted consequence of molecular crowding is that the physical chemistry of interactions in cells, such as protein-protein associations and enzyme reactions, is drastically altered.

This review focuses on the consequences of molecular crowding on translational diffusive phenomena in biological systems. Theoretical considerations and computational data regarding Brownian and non-Brownian diffusion are discussed, and experimental evidence on diffusion measurements in solutions and in cell aqueous compartments and membranes is reviewed. We conclude that crowding in cell aqueous compartments is largely Brownian with diffusion coefficients less than one order of magnitude slower than in water. We also conclude that protein diffusion in membranes can be Brownian if diffusion is slowed by crowding alone, but that specific interactions between proteins and lipids can produce anomalous diffusion.

## PHYSICS OF DIFFUSION

Translational diffusion is the movement of a substance from one region of space to another. In a homogeneous solvent where solute size is comparable to or greater than that of the solvent, solute movement is described well by phenomenological or statistical equations in which the primary determinants of diffusion are solute size and shape. We call this type of diffusion normal diffusion. In inhomogeneous environments, or where the solute is smaller than the solvent, or where a large fraction of the solution volume is occupied by another solute (a crowder), more complex equations may be necessary to describe solute movement. We call this type of diffusion in which solute movement is not described by the equations of normal diffusion as anomalous diffusion. In the context of this review, Brownian diffusion and normal diffusion are the same.

## Normal Diffusion

The flux of solute,  $J$ , through a planar area in space is proportional to the concentration gradient across the plane:

$$J = -D\nabla C, \quad 1.$$

where  $C$  is the concentration of solute and the operator  $\nabla$  represents the derivative of  $C$  with respect to spatial coordinates.  $C$  is generally a function of both time and space. Requiring mass balance, the diffusion equation becomes

$$\partial C / \partial t = \nabla \cdot (D\nabla C). \quad 2.$$

Equations 1 and 2 are Fick's first and second laws of diffusion, respectively. Solutions to Fick's laws are given by Crank (6); additional solutions are given by Hines & Madrox (20). Fick's laws are phenomenological laws that describe the spatial and temporal dissipation of a concentration gradient. The diffusion constant can be concentration dependent, although in practice  $D$  is assumed to be constant. For constant  $D$ , Fick's second law becomes  $\partial C / \partial t = D\nabla^2 C$ . Diffusion can also be anisotropic, in which case  $D$  becomes a tensor.

In the absence of a concentration gradient, normal diffusion is usually described by Einstein's equations of Brownian motion (11). For a solute comparable to or larger than the solvent, the diffusion coefficient is given by

$$D = kT/f, \quad 3.$$

where  $k$  is Boltzmann's constant,  $T$  is the absolute temperature, and  $f$  is the solvent friction coefficient. In normal diffusion, the solvent is usually thought of as a continuous hydrodynamic fluid in which the details of the solvent structure and the solvent-solute interaction are ignored. For a spherical particle in a hydrodynamic solvent of shear viscosity  $\eta$ , the solvent friction coefficient in Equation 3 is

$$f = 6\pi\eta r_b, \quad 4.$$

where  $r_b$  is the hydrodynamic radius of the particle. Equation 4 corresponds to stick boundary conditions between the solute and solvent in which the hydrodynamic solvent

is considered to stick to the solute at the solute-solvent boundary. For slip boundary conditions (no stickiness between the solvent and solute), the factor  $6\pi$  in Equation 4 is replaced by  $4\pi$ . For nonspherical shapes, the friction coefficient is multiplied by a shape factor that is greater than 1. From Fick's laws or from Einstein's relations, the mean squared displacement (MSD),  $\langle r^2 \rangle$ , of a solute particle in three dimensions is related to the  $D$  by

$$\langle r^2 \rangle = 6Dt. \quad 5.$$

For one and two dimensions, the factor 6 in Equation 5 is replaced by 2 and 4, respectively. The validity of Equation 5 for solute diffusion in fluid phases has been demonstrated many times over the past 100 years (19).

The equations of normal diffusion rest upon the central limit theorem: The average displacement of a particle is Gaussian-distributed if the displacements themselves have a finite second moment (i.e., a finite squared deviation from the origin) and are Markovian (i.e., the probability of a particular displacement is independent of previous displacements) (30). Displacements that do not follow this central limit theorem, such as displacement distributions having long tails that do not approach zero exponentially, or displacements that are correlated, will not in general lead to normal diffusion. These processes lead to anomalous diffusion.

## Anomalous Diffusion

A central assumption in describing normal solute diffusion is that the solute diffuses in a continuous hydrodynamic fluid. This assumption is clearly not valid in most biological systems. For example, cell cytoplasm contains many different solutes with a large distribution of sizes (26), so that diffusion of any one type of solute in the cytoplasm would not occur in a hydrodynamic fluid. Also, there are boundary effects as the solute encounters spectrin networks or organellar membranes. For these and other reasons, one would not anticipate a priori that solute diffusion in

### Mean squared displacement (MSD):

the square of the displacement of a particle at some time relative to the position of the particle at zero time, averaged over many particles

biological systems would be described by the equations of normal diffusion. Despite this, solutes in many biological systems do follow the equations of normal diffusion (see Experimental Measurements, below). In other cases, solutes do not follow normal diffusion.

Following Bouchaud & Georges (4), we define solute diffusion that cannot be described by Einstein's equations for Brownian motion (Equations 3 and 5) as anomalous diffusion. The non-Brownian behavior is usually described by the equation:

$$\langle r^2 \rangle = 6Dt^\alpha. \quad 6.$$

Although strictly true if the solute hops from one potential trap to another potential trap under restricted conditions (4), Equation 6 has nonetheless been used extensively as a semiempirical description of anomalous diffusion. If  $\alpha < 1$ , the diffusion is called anomalous subdiffusion, and if  $\alpha > 1$ , the diffusion is called anomalous superdiffusion.

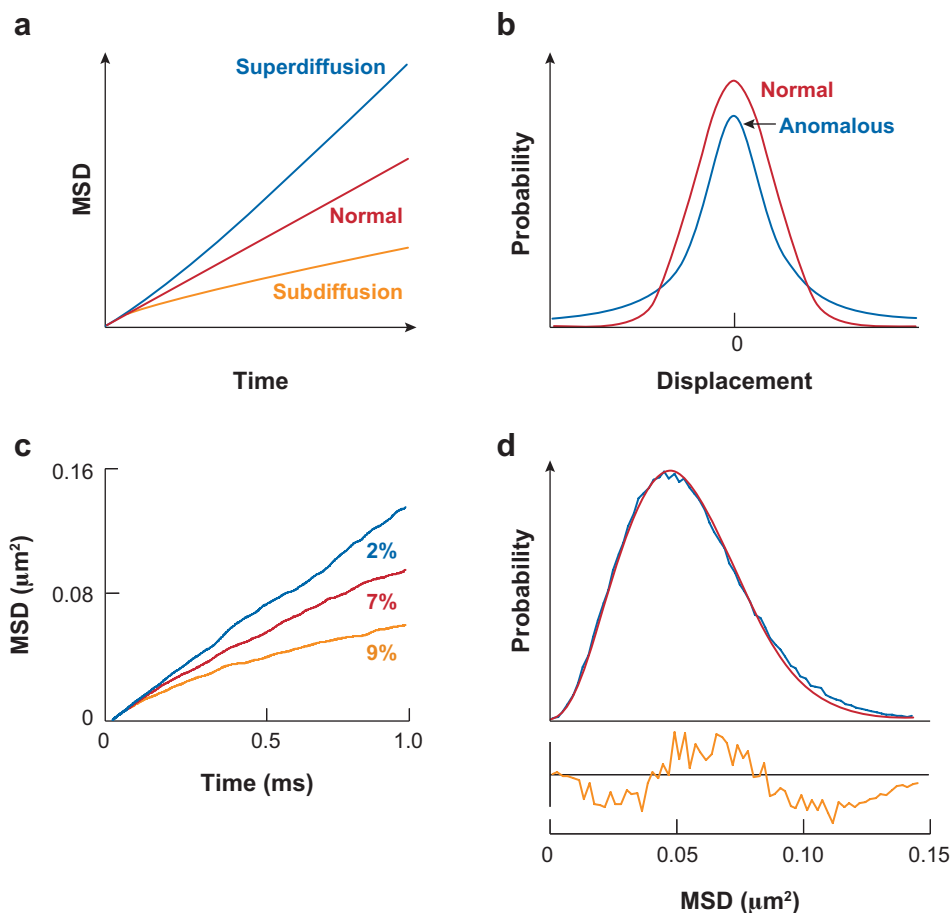
In noncrowded systems, the solute has almost the entire hydrodynamic fluid (the solvent) in which to diffuse. Crowding is the reduction of the available solvent volume by a crowder (volume exclusion). The crowder can be either mobile, as in intracellular globular proteins, or fixed, as in a spectrin network. A consequence of volume exclusion is that the effective solute concentration increases, thus increasing the chemical potential of the solute. This thermodynamic consequence is discussed by Minton and colleagues (56) in this volume. In terms of diffusion, the crowder provides barriers to solute movement. If crowding gives rise to anomalous diffusion, it will always be manifest as anomalous subdiffusion ( $\alpha < 1$  in Equation 6) at long enough times and distances. **Figures 1a,b** define normal and anomalous diffusion in terms of MSD plots and displacement distributions.

Even if a particle undergoes normal diffusion, the MSDs may not be linear with time (Equation 5) for all time and distances. As discussed by Berne & Pecora (3), at very short times ( $\langle r^2 \rangle$  of a Brownian solute is proportional to  $t^2$ , corresponding to free

motion of a particle under the force of nearby solvent molecules (ballistic diffusion). At longer times, the particle experiences the frictional force of the hydrodynamic solvent. As formalized by the Langevin equation,  $\langle r^2 \rangle$  increases linearly with time in this regime. The crossover from ballistic to Brownian diffusion occurs on a time characteristic of the correlation time of the velocity autocorrelation function. For the case of distance-limited diffusion, in which the particle is constrained to move in a limited two- or three-dimensional area or volume (e.g., solutes constrained to move within an organelle or a corral on the surface of a membrane), there is a limit to the distance a solute can diffuse, resulting in a plateau in  $\langle r^2 \rangle$  at long times. Thus, the interpretation of  $\langle r^2 \rangle$  that varies linearly or nonlinearly with time must be tempered with the characteristic time over which the measurement is made. An observation of anomalous diffusion for a solute can therefore be compatible with normal solute diffusion over biologically relevant timescales, but anomalous diffusion over the measurement timescale. Further, as discussed below, there are experimental artifacts that can give rise to apparent anomalous diffusion. Because diffusion time and distance are related (Equations 5 and 6), similar considerations apply to biologically relevant and experimentally measured distances.

## Anomalous Diffusion in Membranes

The Singer-Nicolson model of biological membranes (43) posits a two-dimensional lipid sheet into which proteins are embedded. In a sense, diffusion of lipids and proteins in a membrane is a process occurring at nearly maximal crowding, because the biological solvent, water, is at very low concentration. Alternately, the membrane lipids can be thought of as the solvent into which proteins are dissolved. For pure lipid bilayers (no protein or other membrane constituents), diffusion of individual lipids appears to be Brownian (28, 38, 45). However, diffusion of



**Figure 1**

Characteristics and simulations of anomalous diffusion. (a) MSD curves defining normal (Brownian) diffusion and anomalous subdiffusion (downward curvature) and superdiffusion (upward curvature). (b) Distribution of displacements for normal and anomalous diffusion. Initial particle position is at the origin. For normal diffusion, the distribution is Gaussian and gives rise to Brownian motion. The curve labeled anomalous has long tails and an infinite second moment, resulting in nonlinear MSD plots and anomalous diffusion. (c) MSD plots for simulations of crowding in an aqueous phase. Simulations done for 75-nm-radius spherical particles in a  $3 \times 3 \times 9 \mu\text{m}$  box for 1 ms using the method of Dix et al. (10). (d) (Top) MSD distributions for simulation of 75-nm-radius particles for 10 ms at 9% volume exclusion. The smooth curve is fitted using the expected distribution for normal diffusion. (Bottom) Difference between fitted and observed MSD distributions.

lipids and proteins in plasma membranes of cells can show anomalous diffusion (14, 24, 44) as well as normal diffusion (54). The variation in experimentally measured diffusion properties in the plasma membrane may relate, in part, to the timescales over which the measurements are made (24). The finding of

anomalous diffusion of membrane proteins has led to an update to the Singer-Nicolson model (52), in which clusters of proteins or lipids, such as rafts, contribute to biological function.

The presence of fixed barriers to diffusion can in effect produce volume exclusion

on the two-dimensional membrane surface (41). These effects are often framed in percolation theory (46). In percolation theory, as the concentration of obstacles increases, solutes encounter increasing difficulty in finding their way around obstacles until a percolation threshold is reached. At the percolation threshold, the fraction of excluded volume is such that the solute becomes trapped. Simulations of these kinds of barriers reveal initial anomalous diffusion, followed by normal diffusion (41). The crossover from anomalous diffusion to normal diffusion occurs at longer times as the percolation threshold is reached. For mobile membrane proteins, crowding can have a significant effect on the mobility of membrane constituents in model membrane systems (36, 40).

The Singer-Nicolson membrane model has recently been recast to take into account new experimental data (12, 24, 52). The updated model views the plasma membrane as a dynamic mosaic composed of clumps consisting of lipids, proteins, and lipid/protein complexes. These clumps are dynamic, rearranging often in response to the biological needs of the cell. Given this updated membrane model, it is not clear whether crowding (as defined by simple volume exclusion) is a useful model to explain mobility in cell plasma membranes. Two biologically relevant models of membrane mobility are protein binding and unbinding to fixed anchors on the membrane (47), and diffusion within and between membrane microdomains such as lipid rafts (21). Binding must be transient to allow measurable mobility. The protein is thought to remain fixed at its binding site for some length of time and then hop to another binding site. This model contrasts with the normal diffusion model discussed above, in which the particle incrementally moves a distance taken from a Gaussian distribution. In the binding model, for sufficiently small time increments, there is a less than unity probability that a particle will move. With a non-Gaussian distribution of unbinding times, diffusion is anomalous (4).

Microdomains, corral proteins, or lipids within the membrane can exclude proteins and lipids located outside the corral. Proteins or lipids may diffuse normally within the corral as well as hop between inside and outside of the corral. The diffusion times in the two environments may be different. As is the case for transient binding, such movement can produce anomalous subdiffusion.

As pointed out by Kusumi et al. (24), the small reduction in protein diffusion coefficients upon aggregation suggests that protein aggregation by itself probably does not play a significant role in anomalous diffusion in plasma membranes. For example, using the membrane hydrodynamic model of Saffman & Delbrück (39), the diffusion coefficient for a cylinder of radius  $r$  in a two-dimensional membrane varies only logarithmically with  $1/r$ . A doubling of the radius of the cylinder decreases the diffusion coefficient by only 10%. In contrast, for example, the diffusion coefficient of bradykinin receptors decreases by a factor of 10 when the receptor is coupled with G proteins (37). These considerations indicate that specific protein-membrane interactions can dominate the translational mobility of proteins on the plasma membrane.

## Computational Approaches to Diffusion

Given the complexity of the models used to explain anomalous diffusion, many papers reporting experimental studies of anomalous diffusion also report ancillary computations to interpret the experimental data. The goal of simulations of diffusion is to tabulate the spatial position of particles as a function of time (the trajectory). The trajectory is then processed to simulate experimental data (10).

As reviewed by Takahashi et al. (49), there are several methods to obtain the trajectory. The most detailed trajectory is given by an all-atom molecular dynamics simulation in which solute-solute and solute-solvent interactions are specified on an atomic level. Unfortunately, both the size (femtoliter) and time



( $\mu\text{s}$ ) of cytoplasmic or membrane systems to be simulated are too large to simulate with current computer hardware and software.

A common alternative approach to simulate crowding is stochastic dynamics simulation, in which the particle's displacement is calculated from forces from nearby particles as well as from a random displacement (10, 13). This method assumes that particles have no inertia; they are stationary in one position and then jump instantaneously to another stationary position. The solvent is considered a continuous fluid without structure. Typically, intermolecular interactions are represented by Lennard-Jones and Coulomb types of potentials. With this method, time steps of adequate size can be used so that areas and volumes applicable to crowding experiments can be simulated (29).

**Figure 1c,d** give examples of molecular dynamics simulation with Lennard-Jones repulsive potentials (J. Dix & K. Hiranuka, unpublished observations). **Figure 1c** shows anomalous subdiffusion, seen as downward curvature in MSD plots, in simulations with large particles at 7 and 9 volume% exclusion. **Figure 1d** gives the distribution of MSDs for the simulation at 9 volume% exclusion, compared with what would be expected for Brownian diffusion. The difference suggests that the anomalous diffusion at 9 volume% exclusion arises because of a shift in the MSDs toward higher values. The shift may occur as the large particles, trapped in a cage by other large particles, suddenly break free and jump to another cage, mimicking some aspects of a Lévy walk and giving rise to anomalous diffusion (4). Smaller particles diffusing in solutions containing large crowders find their way around the crowder particles, resulting in slowed but Brownian diffusion (10).

The simulation time and space regimes needed for crowding computations are barely accessible with common laboratory computers, even at fairly low crowder concentrations. For example, simulation of  $1\ \mu\text{m}^3$  volume with 25 volume% mobile crowders 10 nm in diameter for 1 ms (values typical for simulating

experimental data) requires keeping track of  $6 \times 10^4$  molecules for  $1 \times 10^6$  time steps; at each step, costly interaction potentials have to be calculated. This type of simulation would require several weeks on a typical fast laboratory computer. As the volume percent occupied by the crowder increases, the number of particles increases and the time step decreases (to avoid a particle straying into the excluded volume of another particle), greatly increasing computational time.

A common variation of the stochastic dynamics simulation method is to specify a lattice within the simulation area or volume and to restrict jumps to vertices of the lattice (32, 40), thereby increasing the computational speed by eliminating the computation of interaction potentials. The lattice jumps are governed by a set of rules appropriate to the simulation; typically, jumps are not allowed to vertices already occupied by a molecule. Another approach is to allow jumps to any region of space subject to constraints but to ignore intermolecular interactions (1). Other methods of trajectory simulation have been developed, such as dissipative particle dynamics, in which intermolecular forces are computed for particles surrounded by blobs of solvent of variable volume (15), and Green's function reaction dynamics, in which particles are moved at variable time steps and the pair-wise interactions are solved exactly by Green's functions (51). These other methods have not been applied extensively to crowding simulations.

Stochastic simulations of crowding in cytoplasm reveal both normal and anomalous diffusion. Dix et al. (10) found that for diffusion of 0.7-nm-radius particles, 150-nm crowders, and a repulsive potential, diffusion was normal but slowed by a factor of 2.5 at 50–60 volume% crowder. Weiss et al. (55) found that in simulations with 3.6- to 5.4-nm particles, a Poisson distribution of large crowders, and a repulsive potential, diffusion was anomalous and slowed at 13 volume% crowder. Dix and Kazushi (unpublished observations) found slowed and anomalous diffusion at 25 volume% crowders with 150-nm

**Single-particle tracking (SPT):** an experimental method to follow a single labeled particle over time

**GFP:** green fluorescent protein

**Fluorescence recovery after photobleaching (FRAP):** an experimental method in which a sample volume containing fluorophore is bleached and the subsequent recovery of fluorescence is followed

particles, 150-nm crowders, and a repulsive potential, as well as smaller-sized particles (**Figure 1c,d**). Lipkow et al. (25) found a twofold decrease in diffusion of an 8-nm-radius particle at 41 volume% with infinite step potentials. These simulations indicate that in all cases crowders reduce particle diffusion and that under some conditions diffusion can become anomalous.

Simulation results on crowding are subject to several caveats. A major assumption in most simulations is that interparticle interactions can be approximated by pair-wise additive potentials. At high crowder concentrations, this assumption becomes less tenable as three-body and higher-order interactions become increasingly important. Apparent anomalous behavior seen with two-body interactions may become averaged out when higher-order interactions are taken into account. Simulations based on lattice models are subject to lattice artifacts when distances are analyzed on the order of the lattice spacing. For crowding at high volume percent, these distances are the predominant distances to be analyzed. Most crowding simulations neglect hydrodynamic interactions and weak attractive and long-range potentials. Consideration of these interactions may reduce the tails on non-Gaussian displacement distributions, changing the diffusion type from anomalous to normal.

## EXPERIMENTAL MEASUREMENTS IN SOLUTIONS AND CELLS

### Experimental Approaches and Limitations

The continuous, high-resolution tracking of the motion of individual molecules in three dimensions is the benchmark in describing diffusive phenomena. Single-particle tracking (SPT) involves the selective labeling of proteins or lipids with fluorophores, such as quantum dots, green fluorescent protein (GFP) or organic dyes (e.g., cyanine dyes), or probes

visible with transmitted light (gold or latex beads), such that particle position can be measured with as low as nanometer spatial and submillisecond temporal resolution using suitable camera detectors. Unlike ensemble-averaged methods to measure diffusion, SPT provides information about individual particles and so can identify heterogeneous and complex diffusive behaviors such as transient confinement or barriers (22). SPT has become the method of choice for studying the two-dimensional diffusion of membrane proteins; however, it is not yet suitable for measurement of diffusion of aqueous-phase solutes in three dimensions because of their generally rapid diffusion as well as limitations in determination of particle *z* (axial) position.

Fluorescence recovery after photobleaching (FRAP) has been used extensively for diffusion measurements. Fluorescently labeled molecules are introduced into cells by microinjection or incubation or by targeted expression of fluorescent proteins. In spot photobleaching, fluorophores in a defined volume of a fluorescent sample are irreversibly bleached by a brief intense laser pulse. Using an attenuated probe beam, the diffusion of unbleached fluorophores into the bleached volume is measured. A variety of optical configurations, detection strategies and analysis methods have been used to quantify diffusive phenomena in photobleaching measurements (reviewed in Reference 53). Recently, we developed a microfiberoptic epifluorescence photobleaching method in which photobleaching is done at the tip of a micron-sized fiber that can be introduced deep into solid tissues such as tumors and brain (50).

Besides being an ensemble-averaged method describing the averaged diffusive properties of many fluorescent particles, FRAP studies are generally limited in measurement time, such that long-tail phenomena expected in anomalous subdiffusion are easily overlooked and misinterpreted as incomplete recovery arising from diffusion-inaccessible compartments (14). As discussed by Periasamy & Verkman (35), the



determination of solute diffusion coefficients from fluorescence recovery curve shape is challenging when multiple diffusing species are present, or when diffusion is anomalous or geometrically restricted. Quantitative comparison of recovery curve shapes measured in cells to that in standards (fluorophore in thin layer of saline) is useful in the experimental determination of diffusion coefficients (23). Another potential problem in the interpretation of FRAP data is the presence of reversible photobleaching processes, such as triplet-state and flicker phenomenon, which produce recovery signals unrelated to fluorophore diffusion (34).

Fluorescence correlation spectroscopy (FCS) relies on the analysis of fluctuations in the number of fluorescent particles in a femtoliter volume defined by a focused laser spot. Increased diffusion results in more rapid fluctuations and a smaller probability that a particle found in the beam initially will be found in the beam at a later time. This probability is quantified by the autocorrelation function. Although FCS methods have been used to study molecular diffusion in cells (reviewed in Reference 2), it remains uncertain given available SPT, FRAP, and direct imaging methods whether FCS methods can provide clear-cut quantitative information in the complex cellular environment. FCS methods require very low fluorophore concentrations and are confounded by reversible photophysical processes, cell autofluorescence, and complexities in beam and cell geometry. Subtle differences in shape of autocorrelation functions due to such effects are easily mistaken for anomalous diffusion when fitted to an equation that assumes single-component isotropic diffusion with ideal Gaussian beam geometry and absence of photophysical phenomena.

From these considerations we conclude that where experimentally possible, as in the case of diffusion in membranes, SPT is preferred because it provides direct information about trajectories of individual particles. When SPT is not feasible because of

limitations in labeling or rapid diffusion, as in the case of diffusion in aqueous cellular compartments, FRAP provides useful ensemble-averaged information about diffusion. FCS is of particular utility in artificial solutions because of its wide dynamic time range, though its utility in complex cellular environments may be limited as discussed above.

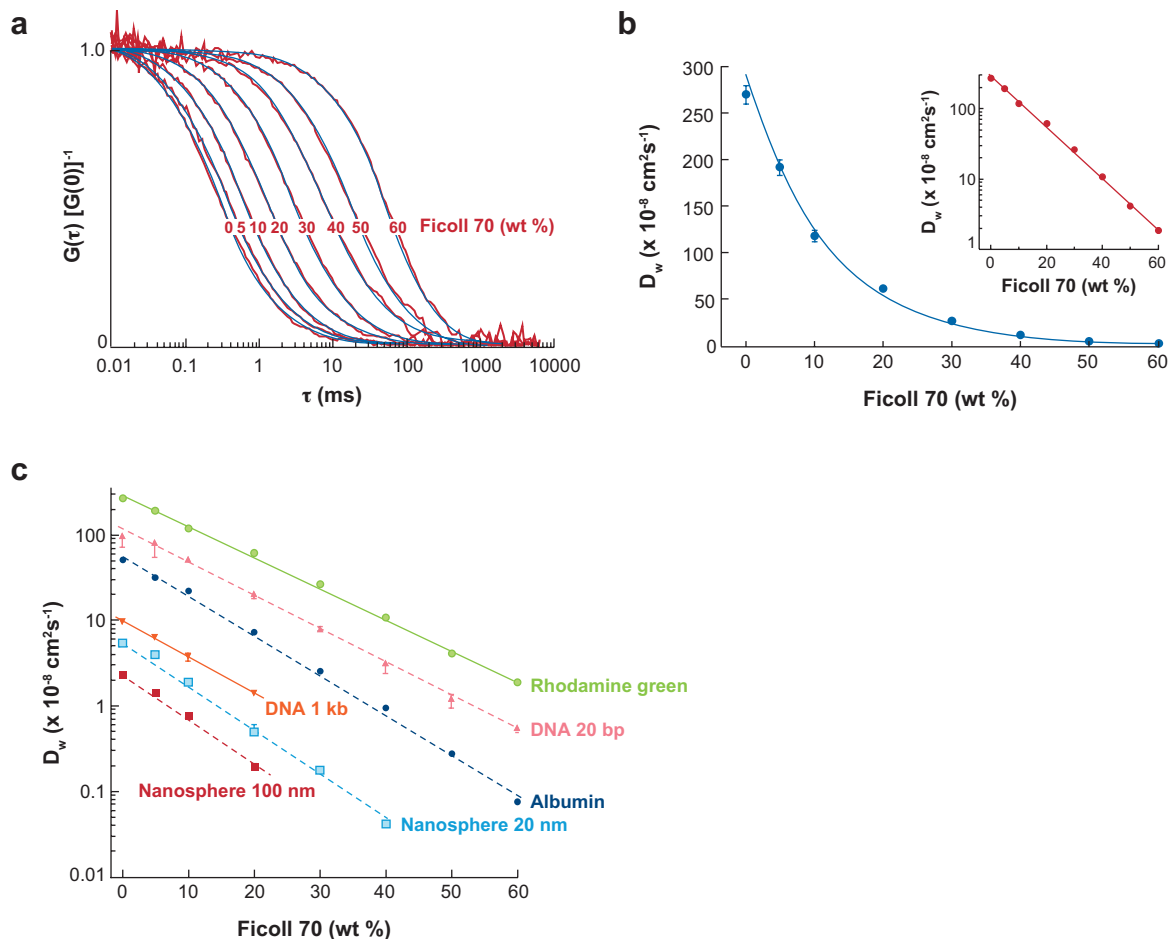
### Does Aqueous-Phase Molecular Crowding Always Produce Anomalous Diffusion?

It is widely believed that crowding produces anomalous diffusion. However, we have reported FCS measurements in aqueous solutions containing diffusing solutes and crowders (7), showing that this is not the case at least to a crowder concentration of 60 volume%. Ficoll 70 was used as the crowder because it is noninteracting and of intermediate size, such that smaller and larger diffusing solutes can be studied. **Figure 2a** shows FCS data for diffusion of the small solute rhodamine green in saline solutions containing the crowder. The data fitted well to a simple Brownian diffusion model for a single species described by a single correlation time,  $\tau_c$ . In FCS,  $\tau_c$  is inversely proportional to the diffusion coefficient. As expected,  $\tau_c$  increased greatly with Ficoll 70 concentration. Anomalous diffusion was not seen even at the highest crowder concentration. **Figure 2b** summarizes deduced diffusion coefficients, showing an ideal, exponential dependence of  $D_w$  on Ficoll 70 concentration. Diffusion was also measured for a series of rhodamine green-labeled macromolecules, including albumin, linear double-stranded DNAs, dextrans, and nanospheres. Remarkably, although the diffusing substances differed greatly in size, physical properties, and absolute diffusion coefficients, being both smaller and larger than the crowding agent Ficoll 70, FCS data fitted well to a simple Brownian diffusion model with similar exponential dependences of diffusion coefficient on

---

**Fluorescence correlation spectroscopy (FCS):** an experimental method in which fluctuations in fluorescence intensity are correlated

---



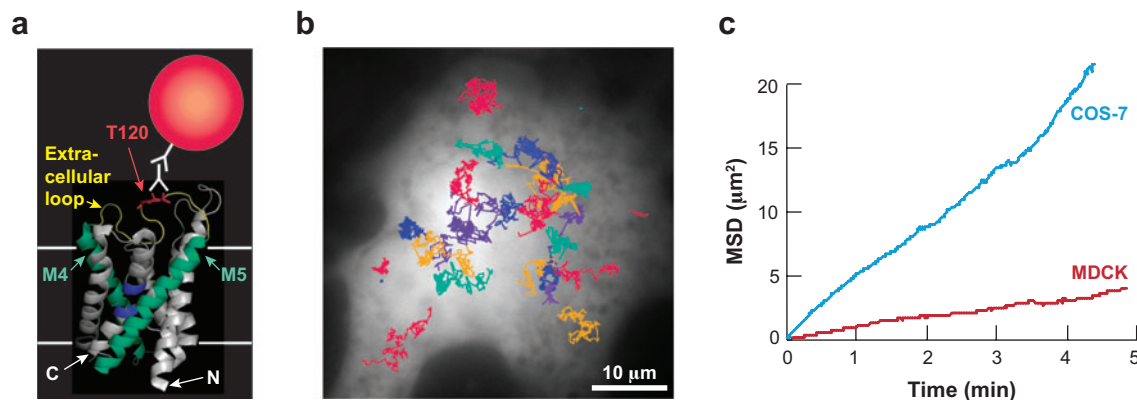
**Figure 2**

Nonanomalous solute diffusion in saline solutions crowded with Ficoll 70. (a) Normalized autocorrelation data  $G(\tau)/G(0)$  for fluorescence correlation measurements of rhodamine green diffusion for increasing Ficoll 70 concentrations. Fitted curves shown for a single-component Brownian diffusion model. (b) Deduced diffusion coefficients as a function of Ficoll 70 concentration shown on linear and log (*inset*) scales (mean  $\pm$  SE, 10–20 measurements). (c) Diffusion of indicated small solutes, macromolecules, and nanospheres in saline solutions crowded Ficoll 70. Adapted from Reference 7.

crowder concentration (Figure 2c). Of note, hard-sphere models of diffusion in crowded media (18, 31) predict significant deviations from exponentials in the range of Ficoll 70 concentrations studied here, a prediction that was not confirmed in FCS. Further work is needed to resolve the apparent discrepancy between results predicted by simulation and those obtained experimentally.

## Is Membrane Protein Diffusion Universally Anomalous?

Diffusion in membranes is remarkably slower than in aqueous compartments and can be complex because of membrane crowding with proteins, the presence of distinct lipid domains, and membrane-cytoskeletal interactions. As mentioned above, there are many examples of nonsimple diffusion of



**Figure 3**

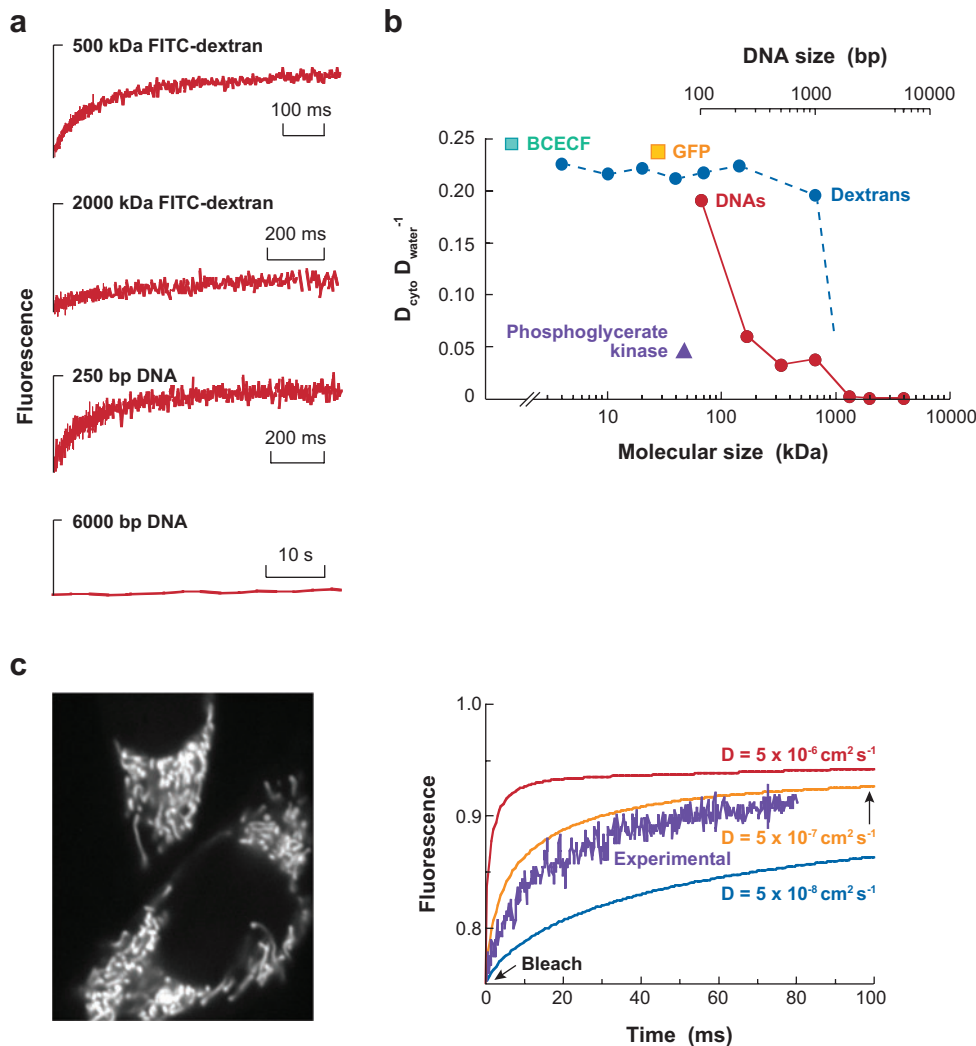
Long-range nonanomalous diffusion of aquaporin-1 (AQP1) water channels in cell plasma membranes. (a) Schematic of a quantum dot (Qdot)-labeled AQP1 monomer. Labeling was done with an engineered human c-myc epitope inserted between residues T120 and G121, which reside in the second extracellular loop between transmembrane helices M4 and M5. (b) AQP1 trajectories shown over ~5 min in the plasma membrane COS-7 cell. Each trajectory is shown in a different color. (c) MSD versus time curves for AQP1 diffusion in COS-7 and MDCK cells (~300 individual trajectories averaged for each cell type). Adapted from Reference 5.

membrane proteins, which are generally related to confinement due to protein-protein interactions. However, it has not been clear whether anomalous diffusion of membrane proteins, as a consequence of protein/lipid crowding, is a universal phenomenon. We recently used SPT to demonstrate long-range, nonanomalous diffusion of aquaporin-1 (AQP1) (5), which is an integral membrane protein that facilitates osmotic water transport across cell plasma membranes in epithelia and endothelia. AQP1 is present in membranes as a tetrameric association of monomers, each of molecular mass of ~30 kDa. AQP1 has no known interactions with cytoplasmic or membrane proteins. We tracked the membrane diffusion of AQP1 molecules labeled with quantum dots bound to an engineered external epitope (Figure 3a) at frame rates up to 91 Hz for more than 5 min (trajectories shown in Figure 3b). In several cell types, nearly linear MSD plots were obtained (Figure 3c) over long and short times. From several different methods of single trajectory analysis, it was determined that the majority of AQP1 molecules diffused in a Brownian

manner. Thus, anomalous diffusion of membrane proteins is not a universal phenomenon, and when anomalous diffusion occurs, there should be an identifiable cause(s) other than simple crowding by volume exclusion.

### Diffusion in Cellular Aqueous Compartments

Diffusion in cellular aqueous compartments is determined by solute properties and the composition, organization, and geometry of the cellular compartment. We have used spot photobleaching to measure the diffusion of small solutes and a series of macromolecules in cytoplasm, including GFP (48) and fluorescently labeled dextrans (42) and DNAs (27). Examples of photobleaching recovery curves are given in Figure 4a and the results are summarized in Figure 4b as relative diffusion of in cytoplasm versus water ( $D_{\text{cyto}}/D_{\text{water}}$ ). The general observation is that diffusion in cytoplasm is slowed only a few-fold compared with that in water, except for linear DNAs and large macromolecules (dextrans >500 kDa), where diffusion is greatly slowed because of limited movement through the actin



**Figure 4**

Diffusion of macromolecules in cytoplasm and mitochondria. (a) Spot photobleaching ( $60\times$  objective, short bleach time) of indicated fluorescein-labeled dextrans and linear double-stranded DNA fragments in cytoplasm. (b) Ratio of diffusion coefficients in cytoplasm versus saline ( $D_{\text{cyto}}/D_{\text{water}}$ ) for indicated solutes and macromolecules. Data taken from References 27, 42, and 48. (c) Diffusion of green fluorescent protein (GFP) in the mitochondrial matrix. (Left) Fluorescence micrograph of matrix-targeted GFP in transfected CHO cells. (Right) Spot photobleaching ( $100\times$  lens), with Brownian diffusion model predictions for indicated GFP diffusion coefficients. Adapted from Reference 33.

cytoskeleton. An FCS study of the diffusion of dextrans in cytoplasm reported evidence for anomalous diffusion even for relatively small dextrans (55). The DNA size-dependent reduction in  $D_{\text{cyto}}/D_{\text{water}}$  could be reproduced in artificial solutions containing actin net-

works (8) and was ascribed to the barrier properties of actin networks for diffusion of DNAs greater than their persistence length. Measured  $D_{\text{cyto}}/D_{\text{water}}$  is reduced not only by steric factors (molecular crowding), but also by the intrinsic fluid-phase viscosity of the

aqueous environment and by binding interactions with mobile and fixed obstacles in cells. When these three independent factors reducing diffusion of a small molecule were measured independently, it was found that fluid-phase viscosity and binding accounted for less than 40% of total slowing, with molecular crowding being the major determinant (23). Also, notwithstanding limitations in the application of FRAP to detect anomalous diffusion, fluorescence recovery curves were generally monophasic and fitted well to Brownian diffusion models.

We have also found unexpectedly minor slowing of diffusion in aqueous compartments of intracellular organelles. The mitochondrial matrix, a major site of metabolic processes, is the aqueous compartment enclosed by the inner mitochondrial membrane. Theoretical considerations have suggested that the diffusion of metabolite- and enzyme-sized solutes might be severely restricted, by more than 1000-fold, in the mitochondrial matrix because of its high density of proteins. However, the diffusion of GFP was only slowed about threefold compared with its diffusion in water (33). **Figure 4c** shows mitochondrial specific GFP targeting. Spot photobleaching of GFP with a  $100\times$  objective (0.8- $\mu\text{m}$  spot diameter) gave a half-time for fluorescence recovery of 15–19 ms with greater than 90% of the GFP mobile (**Figure 4c**). Predicted recovery curves for different diffusion coefficients are also shown; the best fitted value was  $2\text{--}3 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ , only  $\sim$ threefold less than that for GFP diffusion in water. We proposed that clustering of matrix enzymes in membrane-associated complexes might serve to establish a relatively noncrowded aqueous space in which solutes can freely diffuse. Subsequent measurement of diffusion of various GFP-tricarboxylic enzyme chimeras provided experimental evidence for such a multi-enzyme macromolecular complex (17). A similar analysis of GFP diffusion in the lumen of the endoplasmic reticulum showed only  $\sim$ threefold slowing compared with GFP diffusion in water (9). Together, these data suggest relatively

minor effects of molecular crowding on diffusion in cellular aqueous compartments (at least for small solutes and relatively small macromolecules such as GFP). These results are consistent with those obtained from simulations of small solutes diffusing in the presence of large crowders (10).

## CONCLUSIONS AND PERSPECTIVE

Molecular crowding and complexity in and around cells can in principle produce marked slowing of diffusion as well as anomalous and complex diffusive behaviors, such as strongly size-dependent diffusion. Although there are examples of restricted and anomalous diffusion in cellular aqueous and membrane compartments, greatly slowed or anomalous diffusion in cells is not a universal phenomenon. Experimental evidence supports the possibility of simple Brownian diffusion even in highly crowded aqueous media and when the diffusing species is as large as or larger than the crowding agent. Experimental data also support the possibility of long-range nonanomalous diffusion of integral membrane proteins. Thus, the observation of anomalous or greatly slowed diffusion in cells indicates the presence of significant interactions, fixed obstacles that impede diffusion, and/or high-order supermolecular organization such as membrane rafts. Further, the possibility of factitious anomalous diffusion mandates consideration as discussed in this review with regard to experimental measurement methods and limitations. Experimental data in cells indicate relatively minor consequences of molecular crowding at least for the diffusion of fairly small solutes, such that diffusion is slowed only a few-fold (compared with diffusion in water) in cytoplasm and intracellular organelles including mitochondria. Further refinement of the ideas presented here is likely to follow technological advances for single-molecule tracking and computational advances for simulations of diffusion in highly crowded media.

## SUMMARY POINTS

1. Crowding can slow the diffusion of solutes in aqueous-phase compartments and in membranes without leading to anomalous diffusion.
2. Large reductions in solute diffusion and/or anomalous diffusion are probably indicators of interactions between the solute and cellular or membrane components, or of fixed barriers to diffusion.
3. Crowding reduces the diffusion of small solutes and many macromolecules in cytoplasm by only a few-fold compared to their diffusion in water.
4. Discrepancies between simulations and experiments on crowding effects on solute diffusion require further investigation.

## DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

## ACKNOWLEDGMENTS

This work was supported NIH grant EB000415 and by the Research Foundation of SUNY.

## LITERATURE CITED

1. Andrews SS, Bray D. 2004. Stochastic simulation of chemical reactions with spatial resolution and single molecule detail. *Phys. Biol.* 1:137–51
2. Bacia K, Kim SA, Schwille P. 2006. Fluorescence cross-correlation spectroscopy in living cells. *Nat. Methods* 3:83–89
3. Berne BJ, Pecora R. 2000. *Dynamic Light Scattering with Applications to Chemistry, Biology and Physics*. Mineola, NY: Dover
4. Bouchaud JP, Georges A. 1990. Anomalous diffusion in disordered media—statistical mechanisms, models and physical applications. *Phys. Rep.-Rev. Sec. Phys. Lett.* 195:127–293
5. Crane J, Verkman AS. 2008. Long-range nonanomalous diffusion of quantum dot-labeled aquaporin-1 water channels in the cell plasma membrane. *Biophys. J.* 94:702–13
6. Crank J. 1975. *The Mathematics of Diffusion*. Oxford: Oxford Univ. Press
7. Dauty E, Verkman AS. 2004. Molecular crowding reduces to a similar extent the diffusion of small solutes and macromolecules: measurement by fluorescence correlation spectroscopy. *J. Mol. Recognit.* 17:441–47
8. Dauty E, Verkman AS. 2005. Actin cytoskeleton as the principal determinant of size-dependent DNA mobility in cytoplasm. *J. Biol. Chem.* 280:7823–28
9. Dayel MJ, Hom EFY, Verkman AS. 1999. Diffusion of green fluorescent protein in the aqueous-phase lumen of endoplasmic reticulum. *Biophys. J.* 76:2843–51
10. Dix JA, Hom EFY, Verkman AS. 2006. Fluorescence correlation spectroscopy simulations of photophysical phenomena and molecular interactions: a molecular dynamics/Monte Carlo approach. *J. Phys. Chem. B* 110:1896–906

---

4. A thorough physical and mathematical description of anomalous diffusion.

---

5. SPT of an integral membrane protein that moves rapidly over large distances without anomalous diffusion.

---



11. Einstein A. 1955. *Investigations on the Theory of the Brownian Movement*. Mineola, NY: Dover
12. Engelman DM. 2005. Membranes are more mosaic than fluid. *Nature* 438:578–80
13. Ermak DJ, McCammon J. 1978. Brownian dynamics with hydrodynamic interactions. *J. Chem. Phys.* 69:1352–60
14. Feder TJ, BrustMascher I, Slattery JP, Baird B, Webb WW. 1996. Constrained diffusion or immobile fraction on cell surfaces: a new interpretation. *Biophys. J.* 70:2767–73
15. Flekkoy EG, Coveney PV, De Fabritiis G. 2000. Foundations of dissipative particle dynamics. *Phys. Rev. E* 62:2140–57
16. Goodsell DS. 2005. Visual methods from atoms to cells. *Structure* 13:347–54
17. Haggie PM, Verkman AS. 2002. Diffusion of tricarboxylic acid cycle enzymes in the mitochondrial matrix in vivo. Evidence for restricted mobility of a multienzyme complex. *J. Biol. Chem.* 277:40782–88
18. Han JN, Herzfeld J. 1993. Macromolecular diffusion in crowded solutions. *Biophys. J.* 65:1155–61
19. Haw MD. 2002. Colloidal suspensions, Brownian motion, molecular reality: a short history. *J. Phys. Condens. Matter* 14:7769–79
20. Hines AL, Maddox RN. 1984. *Mass Transfer: Fundamentals and Applications*. Englewood Cliffs, NJ: Prentice Hall
21. Jacobson K, Mouritsen OG, Anderson RGW. 2007. Lipid rafts: at a crossroad between cell biology and physics. *Nat. Cell Biol.* 9:7–14
22. Jin S, Verkman AS. 2007. Single particle tracking of complex diffusion in membranes: simulation and detection of barrier, raft, and interaction phenomena. *J. Phys. Chem. B* 111:3625–32
23. Kao HP, Abney JR, Verkman AS. 1993. Determinants of the translational mobility of a small solute in cell cytoplasm. *J. Cell Biol.* 120:175–84
24. Kusumi A, Nakada C, Ritchie K, Murase K, Suzuki K, et al. 2005. Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules. *Annu. Rev. Biophys. Biomol. Struct.* 34:351–54
25. Lipkow K, Andrews SS, Bray D. 2005. Simulated diffusion of phosphorylated CheY through the cytoplasm of *Escherichia coli*. *J. Bacteriol.* 187:45–53
26. Luby-Phelps K. 2000. Cytoarchitecture and physical properties of cytoplasm: volume, viscosity, diffusion, intracellular surface area. *Int. Rev. Cytol.* 192:189–221
27. Lukacs GL, Haggie P, Seksek O, Lechardeur D, Freedman N, Verkman AS. 2000. Size-dependent DNA mobility in cytoplasm and nucleus. *J. Biol. Chem.* 275:1625–29
28. Martin DS, Forstner MB, Kas JA. 2002. Apparent subdiffusion inherent to single particle tracking. *Biophys. J.* 83:2109–17
29. McGuffee SR, Elcock AH. 2006. Atomically detailed simulations of concentrated protein solutions: the effects of salt, pH, point mutations, and protein concentration in simulations of 1000-molecule systems. *J. Am. Chem. Soc.* 128:12098–110
30. Metzler R, Klafter J. 2000. The random walk's guide to anomalous diffusion: a fractional dynamics approach. *Phys. Rep.-Rev. Sec. Phys. Lett.* 339:1–77
31. Muramatsu N, Minton AP. 1988. Tracer diffusion of globular-proteins in concentrated protein solutions. *Proc. Natl. Acad. Sci. USA* 85:2984–88

---

29. State-of-the-art in stochastic dynamics simulations.

---

32. Palo K, Metz U, Jager S, Kask P, Gall K. 2000. Fluorescence intensity multiple distributions analysis: concurrent determination of diffusion times and molecular brightness. *Biophys. J.* 79:2858–66
33. Partikian A, Ölviczky B, Swaminathan R, Li YX, Verkman AS. 1998. Rapid diffusion of green fluorescent protein in the mitochondrial matrix. *J. Cell Biol.* 140:821–29
34. Periasamy N, Bicknese S, Verkman AS. 1996. Reversible photobleaching of fluorescein conjugates in air-saturated viscous solutions: singlet and triplet state quenching by tryptophan. *Photochem. Photobiol.* 63:265–71
35. Periasamy N, Verkman AS. 1998. Analysis of fluorophore diffusion by continuous distributions of diffusion coefficients: application to photobleaching measurements of multicomponent and anomalous diffusion. *Biophys. J.* 75:557–67
36. Peters R, Cherry RJ. 1982. Lateral and rotational diffusion of bacteriorhodopsin in lipid bilayers: experimental test of the Saffman-Delbrück equations. *Proc. Natl. Acad. Sci. USA* 82:4317–21
37. Philip F, Sengupta P, Scarlata S. 2007. Signaling through a G protein-coupled receptor and its corresponding G protein follows a stoichiometrically limited model. *J. Biol. Chem.* 282:19203–16
38. Przybylo M, Sykora J, Humpolickova J, Benda A, Zan A, Hof M. 2006. Lipid diffusion in giant unilamellar vesicles is more than two times faster than in supported phospholipid bilayers under identical conditions. *Langmuir* 22:9096–99
39. Saffman P, Delbrück M. 1975. Brownian motion in biological membranes. *Proc. Natl. Acad. Sci. USA* 72:3111–13
40. Saxton MJ. 1987. Lateral diffusion in an archipelago. The effect of mobile obstacles. *Biophys. J.* 52:989–97
41. Saxton MJ. 1994. Anomalous diffusion due to obstacles: a Monte-Carlo study. *Biophys. J.* 66:394–401
42. Seksek O, Biwersi J, Verkman AS. 1997. Translational diffusion of macromolecule-sized solutes in cytoplasm and nucleus. *J. Cell Biol.* 138:131–42
43. Singer SJ, Nicolson GL. 1972. The fluid mosaic model of the structure of the cell membrane. *Science* 175:720–31
44. Smith PR, Morrison IEG, Wilson KM, Fernandez N, Cherry RJ. 1999. Anomalous diffusion of major histocompatibility complex class I molecules on HeLa cells determined by single particle tracking. *Biophys. J.* 76:3331–44
45. Sonnleitner A, Schutz GJ, Schmidt T. 1999. Free Brownian motion of individual lipid molecules in biomembranes. *Biophys. J.* 77:2638–42
46. Sung BJ, Yethiraj A. 2006. Lateral diffusion and percolation in membranes. *Phys. Rev. Lett.* 96:4
47. Suzuki K, Sheetz MP. 2001. Binding of cross-linked glycosylphosphatidylinositol-anchored proteins to discrete actin-associated sites and cholesterol-dependent domains. *Biophys. J.* 81:2181–89
48. Swaminathan R, Hoang CP, Verkman AS. 1997. Photobleaching recovery and anisotropy decay of green fluorescent protein GFP-S65T in solution and cells: cytoplasmic viscosity probed by green fluorescent protein translational and rotational diffusion. *Biophys. J.* 72:1900–7
49. Takahashi K, Arjunan SN, Tomita M. 2005. Space in systems biology of signaling pathways—towards intracellular molecular crowding in silico. *FEBS Lett.* 579:1783–88
50. Thiagarajah JR, Kim JK, Magzoub M, Verkman AS. 2006. Slowed diffusion in tumors revealed by microfiber optic epifluorescence photobleaching. *Nat. Methods* 3:275–80

51. van Zon JS, ten Wolde PR. 2005. Green's-function reaction dynamics: a particle-based approach for simulating biochemical networks in time and space. *J. Chem. Phys.* 123:16
52. Vereb G, Szollosi J, Matko J, Nagy P, Farkas T, et al. 2003. Dynamic, yet structured: the cell membrane three decades after the Singer-Nicolson model. *Proc. Natl. Acad. Sci. USA* 100:8053–58
53. Verkman AS. 2003. Diffusion in cells measured by fluorescence recovery after photobleaching. *Methods Enzymol.* 360:635–48
54. Vrljic M, Nishimura SY, Brasselet S, Moerner WE, McConnell HM. 2002. Translational diffusion of individual class II MHC membrane proteins in cells. *Biophys. J.* 83:2681–92
55. Weiss M, Elsner M, Kartberg F, Nilsson T. 2004. Anomalous subdiffusion is a measure for cytoplasmic crowding in living cells. *Biophys. J.* 87:3518–24
56. Zhou H-X, Rivas G, Minton AP. 2008. Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences. *Annu. Rev. Biophys.* 37:375–97

# Contents



Annual Review of  
Biophysics

Volume 37, 2008

Frontispiece	
<i>Robert L. Baldwin</i> .....	xiv
The Search for Folding Intermediates and the Mechanism of Protein Folding	
<i>Robert L. Baldwin</i> .....	1
How Translocons Select Transmembrane Helices	
<i>Stephen H. White and Gunnar von Heijne</i> .....	23
Unique Rotary ATP Synthase and Its Biological Diversity	
<i>Christoph von Ballmoos, Gregory M. Cook, and Peter Dimroth</i> .....	43
Mediation, Modulation, and Consequences of Membrane-Cytoskeleton Interactions	
<i>Gary J. Doberty and Harvey T. McMahon</i> .....	65
Metal Binding Affinity and Selectivity in Metalloproteins: Insights from Computational Studies	
<i>Todor Dudev and Carmay Lim</i> .....	97
Riboswitches: Emerging Themes in RNA Structure and Function	
<i>Rebecca K. Montange and Robert T. Batey</i> .....	117
Calorimetry and Thermodynamics in Drug Design	
<i>Jonathan B. Chaires</i> .....	135
Protein Design by Directed Evolution	
<i>Christian Jücker, Peter Kast, and Donald Hilvert</i> .....	153
PIP <sub>2</sub> Is A Necessary Cofactor for Ion Channel Function: How and Why?	
<i>Byung-Chang Sub and Bertil Hille</i> .....	175
RNA Folding: Conformational Statistics, Folding Kinetics, and Ion Electrostatics	
<i>Shi-Jie Chen</i> .....	197
Intrinsically Disordered Proteins in Human Diseases: Introducing the D <sup>2</sup> Concept	
<i>Vladimir N. Uversky, Christopher J. Oldfield, and A. Keith Dunker</i> .....	215
Crowding Effects on Diffusion in Solutions and Cells	
<i>James A. Dix and A.S. Verkman</i> .....	247

Nanobiotechnology and Cell Biology: Micro- and Nanofabricated Surfaces to Investigate Receptor-Mediated Signaling <i>Alexis J. Torres, Min Wu, David Holowka, and Barbara Baird</i> .....	265
The Protein Folding Problem <i>Ken A. Dill, S. Banu Ozkan, M. Scott Shell, and Thomas R. Weikl</i> .....	289
Translocation and Unwinding Mechanisms of RNA and DNA Helicases <i>Anna Marie Pyle</i> .....	317
Structure of Eukaryotic RNA Polymerases <i>P. Cramer, K.-J. Armache, S. Baumli, S. Benkert, F. Brueckner, C. Buchen, G.E. Damsma, S. Dengl, S.R. Geiger, A.J. Jasiak, A. Jawhari, S. Jennebach, T. Kamenski, H. Kettenberger, C.-D. Kubn, E. Lehmann, K. Leike, J.F. Sydow, and A. Vannini</i> .....	337
Structure-Based View of Epidermal Growth Factor Receptor Regulation <i>Kathryn M. Ferguson</i> .....	353
Macromolecular Crowding and Confinement: Biochemical, Biophysical, and Potential Physiological Consequences <i>Huan-Xiang Zhou, Germán Rivas, and Allen P. Minton</i> .....	375
Biophysics of Catch Bonds <i>Wendy E. Thomas, Viola Vogel, and Evgeni Sokurenko</i> .....	399
Single-Molecule Approach to Molecular Biology in Living Bacterial Cells <i>X. Sunney Xie, Paul J. Choi, Gene-Wei Li, Nam Ki Lee, and Giuseppe Lia</i> .....	417
Structural Principles from Large RNAs <i>Stephen R. Holbrook</i> .....	445
Bimolecular Fluorescence Complementation (BiFC) Analysis as a Probe of Protein Interactions in Living Cells <i>Tom K. Kerppola</i> .....	465
Multiple Routes and Structural Heterogeneity in Protein Folding <i>Jayant B. Udgaonkar</i> .....	489
<b>Index</b>	
Cumulative Index of Contributing Authors, Volumes 33–37 .....	511

## Errata

An online log of corrections to *Annual Review of Biophysics* articles may be found at  
<http://biophys.annualreviews.org/errata.shtml>