

Macromolecule diffusion and confinement in prokaryotic cells

Jacek T Mika and Bert Poolman

We review recent observations on the mobility of macromolecules and their spatial organization in live bacterial cells. We outline the major fluorescence microscopy-based methods to determine the mobility and thus the diffusion coefficients (D) of molecules, which is not trivial in small cells. The extremely high macromolecule crowding of prokaryotes is used to rationalize the reported lower diffusion coefficients as compared to eukaryotes, and we speculate on the nature of the barriers for diffusion observed for proteins (and mRNAs) *in vivo*. Building on *in vitro* experiments and modeling studies, we evaluate the size dependence of diffusion coefficients for macromolecules *in vivo*, in case of both water-soluble and integral membrane proteins. We comment on the possibilities of anomalous diffusion and provide examples where the macromolecule mobility may be limiting biological processes.

Address

Department of Biochemistry, Groningen Biomolecular Science and Biotechnology Institute, Netherlands Proteomics Centre & Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

Corresponding author: Poolman, Bert (b.poolman@rug.nl)

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Introduction

The reductionist's approach in biochemistry is essential to elucidate biological mechanisms with molecular detail. However, to understand how a protein functions in a real cell and to capture all possible regulatory mechanisms, one needs to analyze molecules in their cellular environment and be able to perturb the system for obtaining mechanistic information. A major difference between *in vivo* and *in vitro* conditions is the crowdedness (and associated molecular complexity) of the cytoplasm and biological membranes [1]. For instance, the intracellular levels of proteins, RNA plus DNA in a *Escherichia coli* cell can reach up to 400 g/l (e.g. when cells are osmotically stressed), concentrations close to that in protein crystals. Figure 1 pictures the crowding of the *E. coli* cytoplasm at macromolecule concentration of 275 g/l [2•]. The con-

sequences of increased macromolecule crowding on the cellular constituents are most pronounced for the activity coefficients of the molecules, the oligomeric states of the proteins, reaction equilibria, and molecule diffusion coefficients (see [1,3,4] for excellent reviews). Diffusion, albeit passive, is the main process for transport and mixing of components in prokaryotic cells. A high crowding will lower the mobility and may thus slow down reactions and reduce the reorganization and dynamics of cellular components. On the other hand, a high crowding will favor (self)-association of molecules.

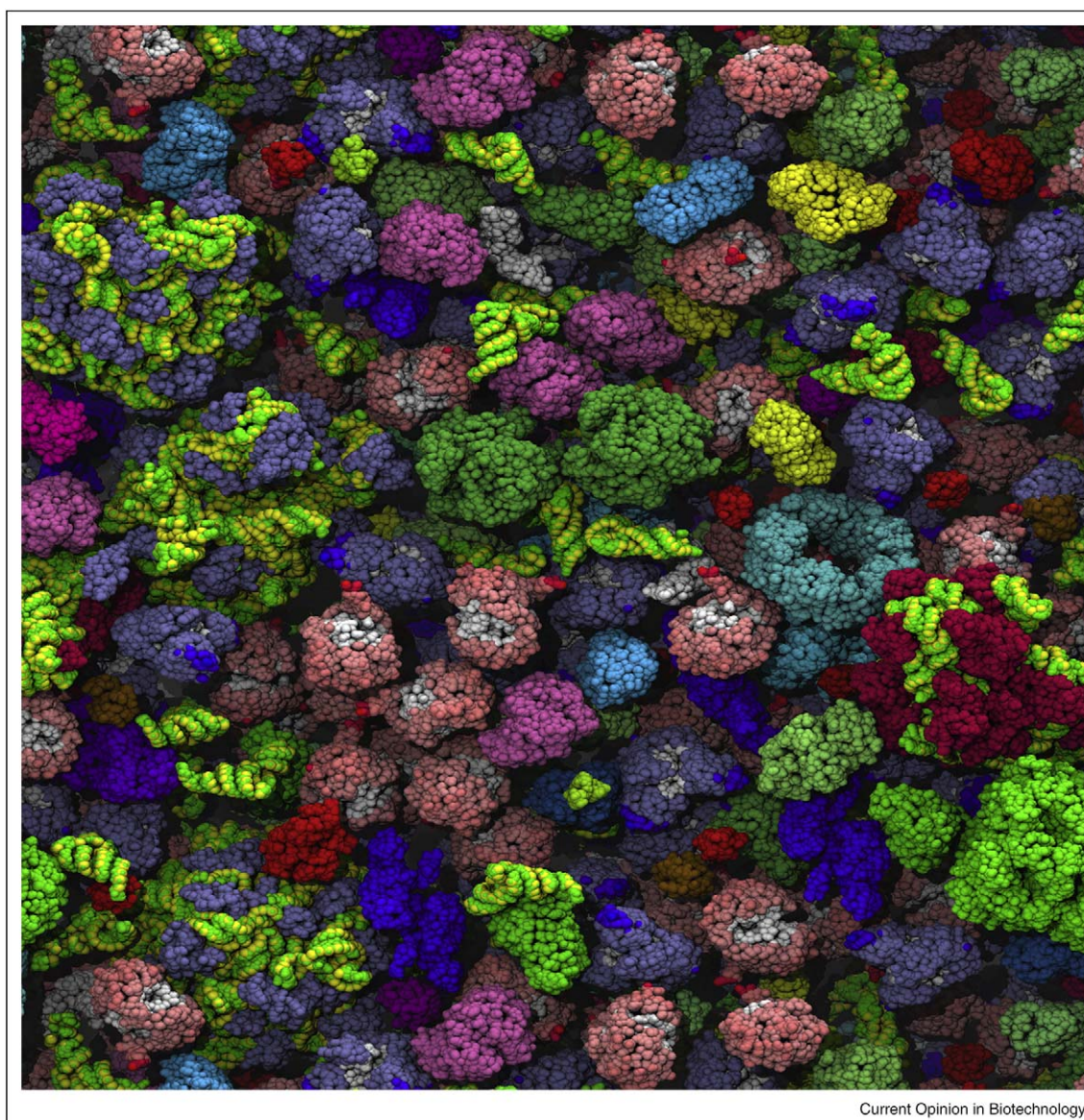
Although *E. coli* is probably the best-characterized organism in terms of genetics and physiology, until a few years ago surprisingly little was known about the translational diffusion and dynamics of macromolecule(s) (complexes) in this organism or any prokaryote. This is likely because of the small size of bacteria and archaea, which complicates dynamic studies by fluorescence microscopy. For instance, a typical *E. coli* cell is 3 μm long and 1 μm wide, which is only a few times more than the resolution of conventional light microscopy; for example, the diffraction limited spot is about 0.22 μm in the lateral direction for light at 550 nm [5].

The effect of crowding on protein diffusion in eukaryotic cells has been comprehensively reviewed by Dix and Verkman [6•]. Here, we focus on the challenges of determining translational diffusion in small cells (Box 1), and evaluate recent measurements on *in vivo* diffusion coefficients in prokaryotes. We feel that this kind of data and quantization of biology are important for systems biology approaches aimed at an understanding of the workings of a cell. We discuss the current literature on macromolecule mobility and barriers for diffusion inside living cells, with a focus on prokaryotes where crowding conditions seem most extreme. Some outstanding questions include: How much is the diffusion slowed down compared to aqueous media and what is the difference in mobility in prokaryotic and eukaryotic cells? What is the diffusion coefficient in different cellular environments such as the cytoplasm and the membrane? How much does the diffusion coefficient depend on the molecular weight (shape) of the macromolecules? Is the diffusion inside prokaryotes anomalous? Do proteins roam around in the cytoplasm freely and in a random fashion or is their mobility restricted to pools or sub-domains (spatial organization)? and What are the cases where diffusion is limiting a biochemical process?

Crowding and diffusion in the cytoplasm

Bacteria are described to be more crowded than eukaryotic cells, presumably because more functions need to

Figure 1



Crowding in the cytoplasm of bacteria. A snapshot of the *E. coli* cytoplasm at a macromolecule concentration (275 g/L) approximating *in vivo* conditions [2]. With permission from Adrian Elcock.

be concentrated in a small compartment (f.i. the volume of *E. coli* is 2 fL, which is 3–5 orders of magnitude less than that of mammalian cells). Macromolecule concentrations in *E. coli* grown under typical laboratory conditions (Luria–Bertani medium of osmolarity of ~ 0.44 Osm) are in the range of 300–370 g/L of protein plus RNA [7]. More specifically, Cayley *et al.* [8] have determined concentrations of 200 g/L of protein, 75 g/L of RNA and 10–20 g/L of DNA. By comparison, the slime mould *Dictyostelium discoideum* has a similar protein concentration (~ 220 g/L) but the levels of DNA (~ 1.13 g/L) and RNA (12.8 g/L) are much lower [9]. Studies on human brain

cells point toward protein concentrations in the range of 50–100 g/L protein [10,11], which is much lower than for bacteria. The high macromolecular crowding of bacteria (Figure 1) has clear impact on the mobility of molecules. For example, typical values for diffusion coefficients of GFP in the cytoplasm of *E. coli* are between 3 and 8 $\mu\text{m}^2/\text{s}$ (see Figure 3a; the wide range of values may reflect differences in physiological states of the cells and methodological differences). The mobility of GFP in live *E. coli* cells is an order of magnitude slower than that in diluted aqueous solutions (87 $\mu\text{m}^2/\text{s}$ [12]) and also slower than that in eukaryotic cytoplasm (e.g. 27 $\mu\text{m}^2/\text{s}$ in Swiss

Box 1 How to measure (macro)molecule mobility in live bacteria?

SMT. In Single Molecule Tracking (SMT) the localization of a fluorescent molecule is traced over time. The trajectory of movement can be plotted and analyzed as a function of time (Figure 2) to obtain a diffusion coefficient. If the molecule shows Brownian dynamics, the diffusion is quoted normal and the mean square displacement increases linearly with time. If the displacement of the molecule does not scale linearly over long(er) time scales, the diffusion is anomalous (see Figure 2a and Box 2). SMT is perhaps the method of choice to discriminate between normal and anomalous diffusion. While SMT is a powerful tool to study single molecule diffusion in dilute solutions, it encounters a number of experimental difficulties when performed in live cells. The main ones are background fluorescence of the cytoplasm, which renders it difficult to visualize single molecules *in vivo* (low signal from fluorescent proteins relative to autofluorescence), resulting in low signal-to-noise ratios. Although most observations on molecule diffusion in prokarya are based on *E. coli* [26,32,34,36,48,49], which is highly autofluorescent, it should be easier to perform SMT and other single-molecule measurements in fermentative bacteria lacking respiratory components. Moreover, progress in *in situ* single-molecule measurements is also facilitated by recent technical developments, such as total-internal reflection (TIR) microscopy, more sensitive EM-CCD cameras, stroboscopic illumination and new photoactivatable fluorescent proteins (e.g. Dronpa, mEos2).

FCS. Another single molecule approach to determine diffusion coefficients is Fluorescence Correlation Spectroscopy (FCS; Figure 2b) [50]. While very powerful in determining *D* values in solution [51] and *in vitro* membrane systems like giant-unilamellar vesicles (GUVs) [21], FCS measurements of diffusion in small cells or cell organelles remain challenging. The reason is the small and confined volume of such structures. On the one hand, there are insufficient molecules to provide enough signal to reliably determine *D* before the photobleaching of the fluorophore occurs. On the other hand, the background fluorescence of cells makes it difficult to obtain satisfactory signal-to-noise ratios. Moreover, the presence of a bacterial cell (or a similar dense structure) in the observation volume can influence its geometry, which would hamper quantitative determination of *D* values. To date the number of studies reporting diffusion coefficients measurements by FCS in live bacteria is very limited [52].

FRAP. Despite lacking the single molecule sensitivity characteristic of SMT and FCS, Fluorescence Recovery After Photobleaching (FRAP) has proven to be very successful in determining the mobility of macromolecules both in prokaryotic [15,16,17*,18**,19,20,27,53] and eukaryotic cells [3,6**,13,54]. In a traditional FRAP experiment, a cell is first imaged with weak laser illumination. Subsequently a Region Of Interest (ROI) is photobleached with a strong laser pulse and the cell is then imaged over time with a weak laser illumination to record the recovery of fluorescence, which results from the diffusion of the non-bleached fluorophores into the ROI. Since bacterial cells are small, FRAP protocols need to be tailored to obtain satisfactory data (i.e. the laser beam needs to be small and weak enough to leave a significant portion of the cytoplasm unbleached to allow recovery). Below we summarize the main features of these methods.

Conventional-FRAP. In the initial pioneering studies to quantify protein diffusion in bacteria, Elowitz [19] used a FRAP protocol schematically depicted in Figure 2c, here referred to as 'conventional FRAP'. A region at the pole of the cell is bleached and subsequently the cell is imaged during recovery. By taking cross-sections through the longer cell axis at consecutive time points, one obtains a temporal plot of the fluorescence distribution throughout the cell (Figure 2c, right), which can be fitted to a one-dimensional diffusion model and yield *D* values. This approach was also employed by Konopka and co-workers to measure the changes of diffusion of GFP in the cytoplasm of *E. coli*, following osmotic upshift [15] and

osmoadaptation [53]. Some other groups [18**,20,27] have used a similar protocol since

Pulsed-FRAP. Van den Bogaart developed a version of FRAP termed pulsed-FRAP [16], schematically depicted in Figure 2d. In pulsed-FRAP a single, relatively weak laser beam is used both for bleaching and imaging. In brief, a cell is first imaged with a confocal microscope and a diffraction limited laser beam is positioned in the middle of the cell. Subsequently a series of weak, short pulses is applied, separated by time intervals to allow the fluorescence to recover. The fluorescence recorded during the pulses is influenced by the photobleaching of the GFP in the focal spot (decrease of fluorescence intensity) and by diffusion of the non-photobleached fluorophore into the focal spot (increase of fluorescence intensity). When a sufficient number of fluorescence decays have been acquired (on average 10–15), the data are fitted to a suitable diffusion model. Knowing the area (volume) of the probed cell one can calculate theoretical bleaching curves and fit the experimental data to obtain a diffusion coefficient and a bleaching constant. Pulsed-FRAP was used by van den Bogaart [16] to determine the diffusion of GFP in *E. coli* cells and later by Mika and colleagues [17*] to determine the diffusion coefficient of (macro)molecules of different molecular weight under various osmotic conditions.

CP-TIR. Slade and co-workers have combined Continuous Photobleaching (CP) with Total Internal Reflection (TIR) microscopy to determine diffusion coefficients of GFP under normal conditions [55] and in cells overexpressing proteins [56]. TIR illumination limits the laser light path in the axial direction and allows the photobleaching volume to be restricted to only a small subvolume of the cytoplasm, leaving enough non-photobleached GFP outside of the excitation region to observe fluorescence recovery with good signal-to-noise ratios (Figure 2e). In CP-TIR varying laser intensities are used to discriminate between the two competing processes: (i) photobleaching in the illuminated region and (ii) fluorescence recovery (resulting from diffusion of the non-photobleached fluorophore outside of that region). By comparing CP-TIR curves at different laser intensities, rate constants of diffusion and bleaching can be calculated

3T3 fibroblasts [13] or $24 \mu\text{m}^2/\text{s}$ [12] in *D. discoideum*). The faster diffusion coefficient in *D. discoideum* may reflect the much lower RNA concentration as the protein crowding seems similar to that of prokaryotic cells, whereas in higher eukaryotes both the protein and nucleic acid concentrations are lower than in bacteria.

When bacterial cells are subjected to osmotic upshift (increase in the osmolality of external medium) a passive loss of cytoplasmatic water occurs. As a result the volume of the cytoplasm decreases. If the hyperosmotic shock is severe, cells can lose up to 70% of the cytoplasmatic water [14], and what is left are the hydration shells of the (macro)molecules. In *E. coli* grown at osmolarities of 1.02 Osm, the macromolecule concentration reaches values as high as 320 g/l of protein and 120 g/l of RNA [8]. Thus, by subjecting cells to osmotic upshift one can increase the cytoplasmatic biopolymer volume fraction. As shown by Konopka [15] and van den Bogaart [16], this increased crowding is reflected by lower molecule mobility; the drop in diffusion coefficients is proportional to the osmotic upshift applied [17*].

Barriers for diffusion in the cytoplasm

Konopka reports that in osmotically upshifted cells ($\Delta\text{Osm} = 0.7$, equivalent to a medium supplement of 400 mM NaCl) cytoplasmic diffusion of GFP is reduced to $0.014 \mu\text{m}^2/\text{s}$ [15], which is two orders of magnitude slower than in cells at typical osmotic conditions of $\sim 0.44 \text{ Osm}$. Under similar osmotic stress conditions van den Bogaart also reports a dramatic loss of GFP mobility, which coincides with the formation of cytoplasmic diffusion barriers [16]. The distribution of GFP is no longer equal throughout the cell, instead the cell appears compartmentalized with pools of GFP that do not exchange their contents. It was subsequently shown that the barriers for the mobility of GFP and bigger macromolecules are likely to be formed by the nucleoid (a kind of physical obstacle for diffusion), and the high macromolecular crowding of the cytoplasm [17]. Interestingly, no such barriers were observed for low molecular weight compounds (Figure 3b). Even in cells subjected to 2 M NaCl ($\Delta\text{Osm} = 4.5$), a fluorescent-labeled sugar molecule remained fairly mobile and distributed evenly over the cytoplasmic space. This result has two important consequences. Firstly, it implies that the cytoplasm acts as a molecular sieve. Under conditions of increased biopolymer volume fraction, when little 'free' cytoplasmic water is left and crowding is increased to an extent that proteins are on average less than 1 nm apart, proteins of only a few nm in diameter (e.g. 2 nm for the $\sim 27 \text{ kDa}$ GFP) are trapped by the constrictions or density of the macromolecular meshwork. Secondly, the relatively fast diffusion of small molecules (such as ions, sugars, signaling molecules and metabolites) will keep the cell biochemically active. The view of the cytoplasm as a molecular sieve is further supported by the recent work of Kumar [18], who observed a steep drop in diffusion coefficients with increasing molecular weight of the diffusing molecule.

Relationship between diffusion coefficient and macromolecule molecular weight

Over the last 10 years, diffusion coefficients have been determined for quite a number of (macro)molecules in live bacteria, in most cases using *E. coli* as a model organism. We have plotted the collected data and used the power law dependence as a fit (Figure 3a, Box 2). We find a value for x of -0.7 , which differs significantly from the Einstein–Stokes exponent of -0.33 . We note that most diffusion coefficients are in a limited range and that extrapolations to low molecular weight molecules and supramolecular complexes should be taken with care. There are a number of reasons to be cautious: Firstly, the spread in the actual data is large, which may have a biological origin (population heterogeneity, medium differences) and in part be technical (different types of measurement and data analysis). Secondly, by introducing a fluorescent tag, one not only renders the molecule amenable for fluorescence microscopy, but also influences

Box 2 Mean square displacement (MSD) and diffusion in 2D and 3D

The MSD (d^2) depends on the diffusion coefficient (D) and time (t), according Eq. (1):

$$[d^2] = bDt^\alpha \quad (1)$$

in which b is a dimension constant equal to 4 for 2D diffusion (e.g. in membranes) and $b = 6$ for 3D diffusion (in the cytoplasm); α is the anomalous diffusion coefficient. If $\alpha = 1$, we are dealing with normal (Brownian) diffusion, and MSD scales linearly with time. When $\alpha < 1$ the molecule's mobility decreases with time, which is referred to as sub-diffusion. If $\alpha > 1$, we are dealing with super-diffusion, see also Figure 2a.

The Einstein–Stokes relationship (3D diffusion):

Diffusion in dilute aqueous media is described by:

$$D = \frac{k_B T}{6\pi\eta R_s} \quad (2)$$

where D is the diffusion constant, k_B , the Boltzmann constant, T , temperature, η the viscosity of the medium and R_s , the Stokes radius of the particle. For globular proteins, R_s is related to the cubic root of the molecular weight:

$$R_s = \sqrt[3]{\frac{3M_w}{4\pi N_A \rho}} \quad (3)$$

where M_w is the molecular weight, N_A the Avogadro constant and ρ the density of a protein. This can be further simplified to a power law dependence:

$$D = aM_w^x, \quad (4)$$

where a is a scaling factor and x the exponent that describes the drop of D with increasing M_w . For proteins in infinitely dilute solutions (e.g. protein in buffer), the $D(M_w)$ dependence follows the Einstein–Stokes relationship and the exponent $x = -0.33$.

The Saffman–Delbrück relationship (2D diffusion):

Saffman–Delbrück's continuum hydrodynamic model describes lateral and rotational diffusion of objects moving in a 2D fluid, for example, a lipid membrane [22]. The protein is considered a cylindrical entity moving in a continuous viscous fluid of defined height (h), which is separated by fluids of lower viscosity (aqueous environment). The lateral diffusion coefficient (D) can be expressed as:

$$D = \frac{k_B T}{4\pi\mu h} \left(\ln \left(\frac{\mu h}{\mu' R} \right) - \gamma \right), \quad (5)$$

where k_B is the Boltzmann constant, T the absolute temperature, h the thickness of the bilayer, μ the viscosity of the membrane, μ' the viscosity of the outer liquid, R the radius of the diffusing object, and γ the Euler's constant.

its size, shape and stability. Kumar *et al.* [18] report that their YFP fusions were subject to degradation and it cannot be excluded that heterogeneous populations of macromolecules have been probed in some studies, rather than one type of species with a well defined M_w . Similarly, upon overexpression part of the protein may be present in aggregates, for example, inclusion bodies, which may explain the discrepancy in diffusion coefficient for the same large complex as reported by Elowitz *et al.* [19] and Mika *et al.* [17]. Thirdly, molecules may non-specifically

(electrostatically) interact, which would lead to an underestimation of diffusion coefficients. Indeed, in a comprehensive Brownian dynamics simulation of the bacterial cytoplasm [2[•]], where some 50 most abundant *E. coli* proteins were studied at a concentration that resembles the *in vivo* situation (275 g/L), proteins of similar mass (M_w) yielded different diffusion coefficients (D). The simulations are supported by recent experimental data [18,20]. Nonetheless, a general decrease of mobility with increasing macromolecule size is consistently observed [2[•],17[•],18^{••}] and the decay of D with M_w in bacterial cells is steeper than what is predicted by the Einstein–Stokes relationship (see Box 2).

Diffusion in bacterial membranes

Diffusion of membrane proteins and peptides has been well characterized in artificial membrane systems like Giant Unilamellar Vesicles (GUVs) [21[•]]. In contrast to soluble (cytosolic) proteins, the overall M_w of membrane proteins seems to have little influence on their diffusion coefficients. What matters is their mass (radius or number of transmembrane segments (TMS)) in the membrane [18^{••},21[•],22], which can be explained by the much higher viscosity of the lipid bilayer than that of the aqueous media in which the soluble domains reside. Different models have been proposed to describe the lateral diffusion of membrane proteins in lipid bilayers. In the Saffman–Delbrück (SD) model (Box 2), D is logarithmically dependent on the radius (R) of the diffusing object [$D \sim \ln(1/R)$] and inversely proportional to the thickness of the bilayer and viscosity of the lipid membrane. An alternative heuristic model proposes the diffusion of membrane proteins to be more strongly dependent on their radii than suggested by Saffman–Delbrück, that is, D scales with $1/R$. However, a recent experimental study, using peptides and proteins with lateral radii ranging from 0.5 nm to 4 nm, is most consistent with the SD model. In agreement with these observations, coarse-grained simulations by Guigas and Weiss suggest that the SD model holds for diffusion of membrane proteins with radii smaller than 7 nm, but fails for objects with larger dimensions [23]. Thus, different diffusional regimes may have to be considered when comparing relatively small proteins (e.g. channels, transporters, and redox enzymes) and supramolecular systems (respiratory complexes, flagellar motor). The D values measured *in vivo* are at least an order of magnitude lower than those measured *in vitro*, which can be rationalized by the higher crowding of biological membranes as compared to the artificial membrane systems. In fact, similar to the cytoplasm, biological membranes are highly crowded and lipid-to-protein ratios on weight basis range from ~ 0.35 (inner mitochondrial membrane) to ~ 1 (plasma membrane) to >1 secretory vesicles [24]. The membrane area fraction occupied by these proteins ranges from 15% to 35% [25]. This implies that a typical membrane protein with a perimeter of 15 nm is surrounded on average by a shell of lipids of only a few

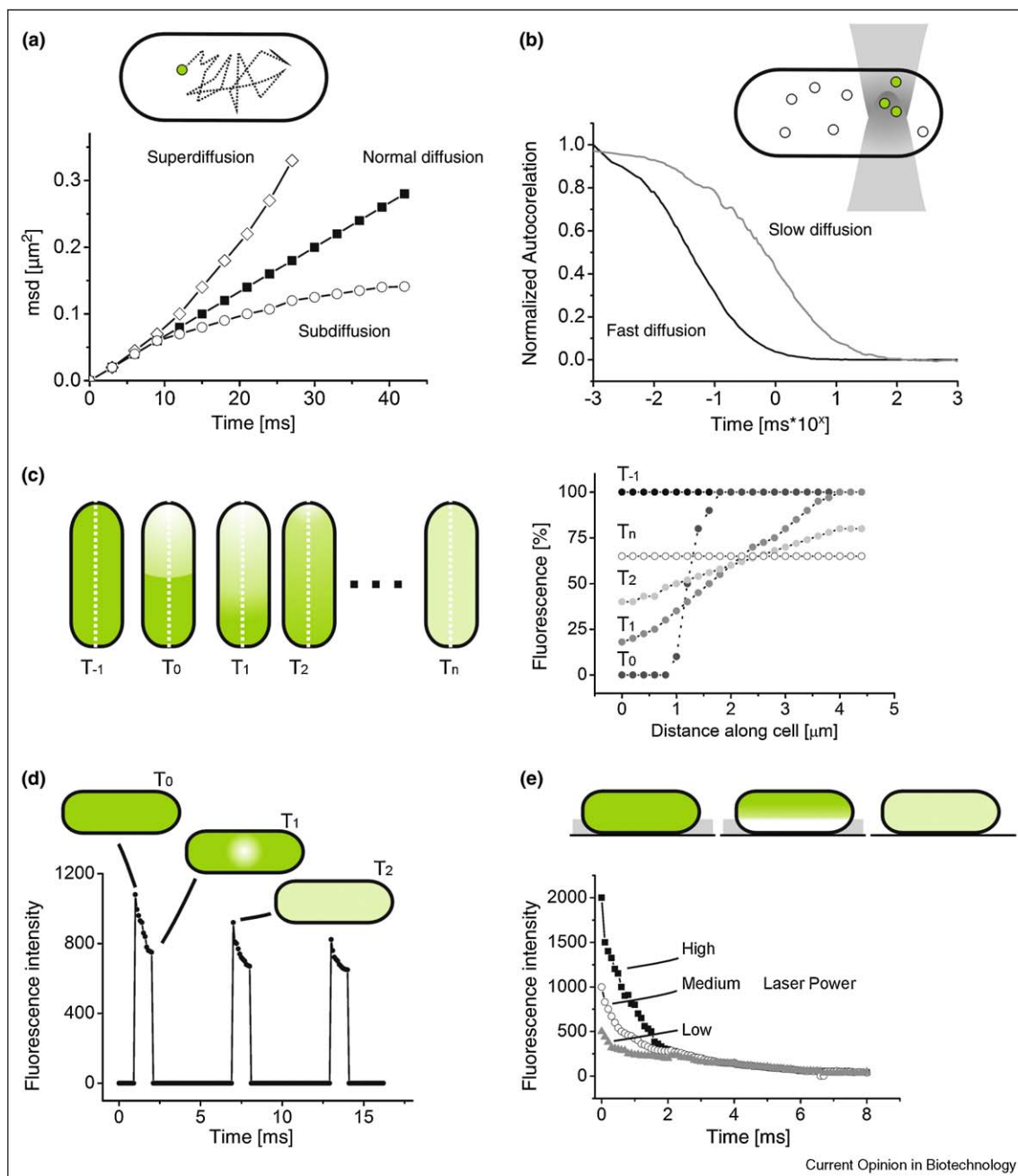
layers thick. Consequently, diffusing objects will be hindered in their mobility. Ramadurai *et al.* [21[•]] has shown that in GUVs the membrane protein diffusion coefficients drop linearly with increasing protein-to-lipid ratio in the range of 3–3000 proteins per μm^2 . However, the highest protein density exploited is still 8-fold lower than that of a typical biological membrane, which has $\sim 25,000$ proteins per μm^2 (i.e. an area occupancy of 30% [25]).

Provided proteins are not linked to the cytoskeleton or other cellular structure, diffusion rates obtained in bacterial membranes [18^{••},26,27] are similar to rates obtained for eukaryotic membranes [28,29]. What matters is the membrane crowdedness; however, we emphasize that to date relatively few systematic studies are available and specific lipids effects (degree of saturation, the presence of sterols) have not been investigated *in situ* (see Ramadurai *et al.* [30,31] for the effects on lateral diffusion in membranes of protein crowding, hydrophobic thickness of the lipids, and lipid headgroup composition). In general, the diffusion of proteins in membranes of live bacterial cells is slower than that of proteins in the cytosol (Figure 3a); typical D values for a 30 kDa soluble protein are 3–6 $\mu\text{m}^2/\text{s}$, while for a 30 kDa membrane protein with a radius in the membrane of 2 nm D is in the range of 0.1–0.2 $\mu\text{m}^2/\text{s}$. Even though diffusion is slow, very large complexes are still mobile [32,33] with D values of 0.005 $\mu\text{m}^2/\text{s}$ for the 3.2 MDa *E. coli* flagellar motor FliG-GFP [32].

Is the diffusion normal or anomalous?

The question of whether diffusion in crowded cells is normal or anomalous is still under debate, and depending on the experimental system researchers have come to opposing views. The high excluded volume fraction of the bacterial cytoplasm and membrane is expected to lead to anomalous diffusion (see Box 1), that is, the effective mean square displacement (msd) of a diffusing species would not scale linearly with time (Figure 2a) [6^{••}]. Moreover, the heterogeneity of the environment (microdomains in cytoplasm and in membranes) would result in the formation of zones of different apparent viscosity and crowding and thus different mobility. While the diffusion within these microdomains might be normal (Brownian), the overall mobility for a molecule traveling a long distance would be affected by hopping between these domains and result in anomalous diffusion. Also the presence of obstacles such as the cell membrane, the nucleoid or the cytoskeleton will introduce boundary conditions to diffusion [6^{••}]. On the basis of measurements of macromolecule mobility in *in vitro* crowded solutions and in eukaryotic cells, Dix and Verkman indicate that the notion of anomalous diffusion as a consequence of crowding alone is not correct. They argue that molecules display anomalous diffusion behavior as a result of specific macromolecular interactions (protein–protein or protein–lipid) or in the

Figure 2



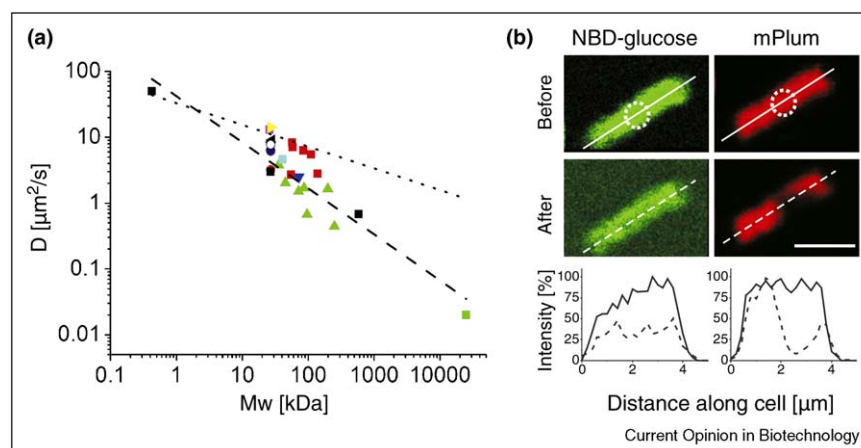
Fluorescence microscopy methods for determining the lateral diffusion of (macro)molecules. (a) Single molecule tracking; (b) fluorescence correlation spectroscopy; (c) conventional FRAP; (d) pulsed-FRAP; and (e) continuous photobleaching with total internal reflection microscopy.

presence of a barrier [6^{••}]. Below we summarize the major experimental observations.

By tracking single molecules of mRNA coated with on average 96 bacteriophage MS2-GFP fusion proteins (~ 3 kb long, $M_w > 12$ MDa), Golding and Cox [34] report that the diffusion of this ~ 100 nm long molecule inside the *E. coli* cytoplasm is anomalous on the scale of seconds to

minutes with $\alpha = 0.7$. The observed anomalous diffusion (see Figure 2a and Box 1) is ascribed to the high macromolecular crowding of the cytoplasm and is not affected by the bacterial cytoskeleton. In a computational study of protein diffusion at concentrations reminiscent of those *in vivo*, McGuffe and Elcock [2[•]] report for proteins of 72–84 kDa a transient deviation from normal diffusion with α as low as 0.7–0.8. The diffusion of the molecules, however,

Figure 3



Molecule diffusion in live cells. Panel (a) Molecule diffusion *in vivo* as a function of molecular weight (M_w). The data were fitted with a power-law dependence: $D = aM_w^x$ (see Box 1 for details). Dashed and dotted lines represent the fits; dashed: $x = -0.7$ (best fit) and dotted: $x = -0.33$ (Einstein–Stokes dependence). Symbols: (■) NBD-glucose, GFP and (β -galactosidase-GFP) $_4$ [17*]; (●) GFP [16]; (▲) YFP, PtsH-YFP, Crr-YFP, CFP-CheR-YFP, DnaK-YFP, HtpG-YFP and CFP-CheA-YFP [18**]; (▼) GFP and MBP-GFP [19]; (■) CheY-GFP [52]; (■) mEos2 [40]; (●) GFP [15]; (●) GFP [53]; (●) GFP [55]; (●) TorA-GFP [27]; (○) GFP [56]; (■) NlpA_(No LB)-GFP, TorA-GFP2, AmiA_(no SP)-GFP, TorA-GFP3, TorA-GFP4 and TorA-GFP5 [20]; (■) plasmid–protein complex [57]. Panel (b) The cytoplasm of osmotically upshifted cells forms mobility barriers for proteins (mPlum) but not for small molecules (NBD-glucose). Pseudo-colored images of a mPlum expressing *E. coli* single cell loaded with NBD-glucose and treated with 0.5 M NaCl (Δ LB = 0.85 Osm). Left panels, NBD-glucose (green) and right panels, mPlum (red). The dotted circle indicates the photo-bleached spot. Upper panels show cells before, and lower panels 2 min after, photo-bleaching. The graphs indicate the normalized fluorescence intensities of the cell along the longer cell axis (indicated by the white lines). Scale bar 2 μ m. From Mika *et al.* [17*].

returned to normal with α values between 0.8 and 1 on longer time scales. Anomalous diffusion has also been reported in a study of the *E. coli* chemotaxis pathway proteins [35], but here it is questionable whether all the molecules are freely diffusing. The cytoskeletal protein FtsZ, fused to the photoswitchable Denra2, showed two populations, one being stationary and forming cell division rings and another being mobile but diffusing anomalously (with $\alpha = 0.74$) [37]. The anomalous diffusion of FtsZ was rationalized by the transient binding of a fraction of free protein to the cytoskeletal rings. Surprisingly, single-molecule tracking of another bacterial cytoskeleton protein MreB (and fused to YFP) in *Caulobacter crescentus* showed normal, Brownian diffusion [38]. Normal diffusion was also reported for the membrane proteins PleC-YFP (a histidine kinase involved in cell division in *C. crescentus*; [39]) and for the TatA-YFP (a component of the twin-arginine protein translocation system of *E. coli*; [26]). Similarly, a recent study of mEos2, a GFP variant, in the cytoplasm of *E. coli* points toward normal diffusion in the bacterial cytoplasm [40]. The information on the diffusion of membrane proteins is scarce. CydB-GFP [36] (from the respiratory cytochrome *bd-I* complex) and FliG-GFP [32] (part of the bacterial flagellar motor) have been described to obey both Brownian and anomalous diffusion. Thus, despite convincing theoretical considerations in favor of anomalous diffusion, the lateral mobility in the cytoplasm and biological membranes is not generally observed as non-Brownian. At least in some cases this may relate to the length-scale over which the diffusion has been probed.

Relevance of diffusion for biological processes

Although diffusion in the crowded cytoplasm of prokaryotes is slow compared to that in aqueous media, it is still fast on the time-scale of most cellular processes. One can calculate how long it would take for a molecule to travel from one end of the cell to another, using Eq. (1) (Box 1). If we assume an *E. coli* cell to be 3 μ m long, we obtain diffusion times of 30 ms for NBD-glucose ($M_w \sim 0.3$ kDa; $D = 50 \mu\text{m}^2/\text{s}$), 0.5 s for GFP ($M_w \sim 27$ kDa; $D = 3 \mu\text{m}^2/\text{s}$), 2 s for β -galactosidase-GFP $_4$ ($M_w \sim 580$ kDa; $D = 0.8 \mu\text{m}^2/\text{s}$) and 75 s for 25–50 MDa ribosome-loaded mRNAs ($D = 0.02 \mu\text{m}^2/\text{s}$). Assuming a typical doubling time of *E. coli* of 30 min even very large complexes can travel forward and backwards several times during the life-span of the cell. The situation, however, becomes very different in osmotically stressed cells and here the diffusion of macromolecules most likely limits various biochemical processes. A similar estimation can be made for membrane proteins and D values of $\sim 0.2 \mu\text{m}^2/\text{s}$, for example, as observed for the Tar aspartate chemoreceptor [18**]), translate to a diffusion time of about 11 s to travel between the poles of the cell. On the other hand, for the flagellar rotor (FliG; $D = 0.005 \mu\text{m}^2/\text{s}$) it would take 7.5 min and here the traveling time gets close to the cells' doubling time.

Although the majority of protein(s) and protein complexes will be able to traverse the cytoplasmic and membrane space multiple times during the life-span of a cell,

machineries like those involved in transcription and translation are localized in the cell. In a recent paper, Jacobs-Wagner and coworkers even propose that bacteria spatially organize translation by using the chromosome layout as a template [41^{••}]. At these places, newly synthesized proteins may thus be concentrated as well. We speculate that the slow diffusion in crowded environments contributes to the formation of functional compartments, for instance by promoting rapid interactions and formation of supramolecular complexes. Moreover, the crowding and composition of a cell are not homogenous and proteins can be excluded or enriched at positions like the nucleoid [17[•]] or cell pole [42], which creates different regimes for molecular interactions. In fact, as previously emphasized the 'structure' of the cytoplasm is transient [43]; it changes over time and is unlikely to persist throughout the volume of the whole cell.

What are other documented cases of how macromolecule diffusion relates to function? One example comes from studies of the interaction of the Lac repressor with the nucleoid DNA [44[•]]. This DNA-binding protein displays 1D diffusion ($D_{1D} = 0.046 \mu\text{m}^2/\text{s}$) as it slides along the DNA in search of its sequence of recognition. This movement would be too slow for the protein to scan the entire chromosome. If the molecule does not find its target sequence soon enough, it dissociates from the DNA and exhibits fast 3D diffusion throughout the cytoplasm ($D_{3D} \sim 3 \mu\text{m}^2/\text{s}$). It can then bind non-specifically to another DNA segment where it can start the slow search again. Overall, the molecule is estimated to spend 90% of its time nonspecifically bound to and diffusing along the DNA, and this translates to an apparent diffusion coefficient D_{eff} of $0.4 \mu\text{m}^2/\text{s}$ [44[•]].

Another example comes from a FRAP study of the mobility of lipid probes in the membrane of *Bacillus* spores [45]. In dormant spores only part of the phospholipids is mobile with D values of $0.11\text{--}0.13 \mu\text{m}^2/\text{s}$, which can be rationalized by the tight packing of lipids to keep the membrane impermeable and to protect the spore. As soon as the spores germinate, yielding vegetative cells, the lateral mobility increases to $1.7\text{--}1.8 \mu\text{m}^2/\text{s}$ with most of the phospholipids being mobile. The increase in lipid mobility coincides with increased membrane fluidity (and permeability) to allow full biochemical activity of the cell. Other cases where a limitation in free diffusion may be a determining factor are in signal transduction (e.g. chemotaxis [46] and cell division [47]).

Conclusion

Prokaryotes are generally devoid of cellular organelles and as such they are less compartmentalized than eukaryotic cells. However, the bacterial cytoplasm is not a random-organized soup of macromolecules, and proteins and nucleic acids seem spatially organized. Despite the high crowding of the cytoplasm and the membrane(s), the

mobility of molecules is relatively high provided they are not interacting with other cell components. But, even for freely diffusing species, their mobility can limit physiological processes. There is increasing evidence that the (high) macromolecular crowding is used as a means of confining molecules (proteins) to a given location, where they need to perform their function. Also, there is a wealth of theoretical and *in vitro* data to show that at high concentrations macromolecules non-specifically enhance protein association rates or binding to surfaces. As a consequence, slow processes ('transition-state limited') are sped up, whereas the fast ones ('diffusion-limited') are slowed down. To precisely measure such phenomena *in vivo* remains a challenge but we expect more experimental data of protein diffusion, molecular association, and enzyme activity in live cells, owing to fantastic developments in *in situ* labeling and optical microscopy.

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