Cellular homeostasis of Escherichia coli probed by super-resolution microscopy
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Chapter 1

Crowding homeostasis in microorganisms

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Abstract

Macromolecular crowding plays an important role in the functioning of cellular life. For example, crowding affects the mobility of biomolecules, protein folding and stability, and the equilibrium of association of molecules. The impact of macromolecular crowding not only influences individual proteins or complexes; it also has consequences for the structure of the cytoplasm of cells, which appears exquisitely organized. Local differences in crowding, arising from subcellular structures and supramolecular assemblies, contribute to the structural organization of the cytoplasm. In this chapter we describe the phenomenon of macromolecular crowding, how it can be measured, and how it affects cell physiology of microorganisms. Given the versatile effects of crowding, it is likely that cells try to maintain crowding conditions in a narrow window. Finally, we discuss some possibilities of how homeocrowding could be achieved.

JvdB, AB and BP formed the concept of the work and JvdB wrote the manuscript.
What is macromolecular crowding?

Cells are highly crowded

The biochemical processes of energy provision, gene expression and cell division that characterize living cells take place in a confined and highly crowded space (see Figure 1). High concentrations of macromolecules give rise to the phenomenon of macromolecular crowding. The chemical composition of *Escherichia coli* cells is given in Figure 2. Proteins make up the majority of the cell’s interior (~55% of the cell mass) and together with rRNA they are the most space-consuming molecules. The DNA is partly condensed to form the nucleoid, which makes up only about 2% of the cellular mass [1], although it does extend over 50-75% of the cytoplasmic volume [2]. mRNA and tRNA are less abundant, but are also considered molecular crowders, whereas small molecules like metabolites or ions do not contribute to macromolecular crowding. Overall, in *E. coli*, the macromolecular volume fraction has been reported to be in the range of 0.13-0.44 (v/v) [3-6]. Why has life evolved at high intracellular crowding and is crowding essential for life? We hypothesize that cells operate homeocrowding to control the reactions and interactions of molecules and maintain the dynamic structure of the cytoplasm.

![Figure 1: Artists impression of the crowded interior of an *E. coli* cell. A cross-section through the cell shows macromolecules at a magnification of 1,000,000x. The cell envelope is depicted in green colors, the cytoplasm in blue/purple, and the nucleoid in yellow/orange. Image from [7].](image)
Figure 2: Voronoi tree diagram of the composition of an *E. coli* cell growing with a doubling time of 40 minutes. The size of the polygons represents the fraction of each component relative to the total cell dry mass. Proteins and ribosomal RNA contribute most to macromolecular crowding. DNA, as well as tRNA and mRNA are less abundant, but are also molecular crowders, whereas metabolites are small molecules that do not contribute to macromolecular crowding. Lipids, lipopolysaccharides and peptidoglycan are components of the cell envelope. Image from [8].

**Theory behind macromolecular crowding**

Macromolecular crowding can be described as the volume that biomacromolecules occupy inside the cell, which is often referred to as macromolecular volume fraction ($\Phi$). Crowding can be quantified with different techniques, which are described in Text Box I. At high concentrations of biomacromolecules the volume fraction of water that is accessible to other macromolecules is significantly reduced, which is the excluded volume [9] (see Text Box I). The high excluded volume raises the effective concentration of a macromolecule, and therefore its chemical activity. In general, macromolecular crowding shifts the equilibrium towards higher order oligomeric states and favors the folded state of a molecule. Multiple theories have been developed to explain the effect of crowding on biomacromolecules: the Asakura-Oosawa depletion theory [10], solvent theory [11], Flory Huggins theory [12], scaled-particle theory [9], and other scaling arguments [13]. These theories consider the molecules as hard spheres that are chemically inert. This implies that the crowding effects are steric and purely entropic in nature. The magnitude of the steric crowding effects depends on the size and the shape of the molecules involved. However, the effects in cells of crowding are not just entropic but also include non-specific interactions (*vide infra*).
Text Box I: Crowding-related terms and methods

**Macromolecular volume fraction Φ:**
Macromolecular crowding is often described as the (macromolecular) volume fraction Φ. It is defined as the volume occupied by the macromolecules related to the whole cell volume and is expressed as (v/v) [3].

**Excluded volume:**
At high concentrations of macromolecules, the volume of water that is accessible to other macromolecules is reduced (excluded volume), which increases the chemical activity of macromolecules.

**Depletion force:**
Depletion force (first described by Asakura and Oosawa [10]) is an attractive force between macromolecules that arises when macromolecules and smaller solutes are suspended at high concentrations. When two macromolecules are close enough together, small particles are excluded from the space in between the large particles, leading to a local decrease in the concentration of the smaller solutes. This results in an osmotic pressure that pushes the macromolecules together.

**Buoyant density:**
Buoyant density refers to the cellular mass (biopolymers, salts and water) per cell volume, that is, the summation of the partial specific volumes times the concentration of all components of a cell. Buoyant densities are measured by applying cells to a concentrated medium (often polysaccharides like Ficoll, polysucrose, or dextrans) that form a linear density gradient upon ultracentrifugation. This method is useful to observe changes in densities during cell growth.

Quantification of compartmental volumes, volume fractions, macromolecular crowding and lateral diffusion:
- The water-accessible volumes of the cell, cytoplasm and periplasm can be obtained by comparing the volumes accessible to $^3$H$_2$O (all compartments) with that of $^{14}$C-inulin (probe excluded from the cell) or $^{14}$C-sucrose (probe excluded from cytoplasm but not from periplasm) [14].
  \[
  V_{cell} = V(^3H_2O) - V(^{14}C\text{-inulin}) \\
  V_{cytoplasm} = V(^3H_2O) - V(^{14}C\text{-sucrose}) \\
  V_{periplasm} = V_{cell} - V_{cytoplasm}
  \]
  The specific volume ($V_s$) is obtained by relating the volume to the total protein or dry weight of the suspension (expressed as volume per mg of protein or dry weight). For *E. coli* grown in a MOPS-based glucose minimal medium (MBM; analyzed at an external osmolarity of 0.28 Osm), the $V_{cytoplasm}$ and $V_{periplasm}$ are ~2.1 and ~0.4 mL/mg dry weight, respectively. The volume fraction is calculated as the fraction of the total volume occupied by macromolecules.

- Macromolecular crowding can be quantified with genetically encoded or synthetic crowding sensors [6, 15, 16]. The gene-based crowding sensor consists of two fluorescent proteins that make up a FRET pair, linked by two α-helices and three flexible linkers. In more crowded environments the sensor is compressed, which leads to an increase in FRET efficiency. By comparing intracellular FRET readouts with a calibration solution, i.e. Ficoll, the crowding in cells is estimated.

- Quantitative proteome analysis (e.g. by LC-MS quantification [17] or ribosomal profiling [18]) can be used to relate the protein mass to cell volume and obtain specific information about macromolecular volume fractions.
Lateral diffusion refers to the lateral movement of molecules. The lateral diffusion coefficient is related to the temperature \((T)\) and viscosity \((\mu)\) of the medium and the hydrodynamic radius \((R_s)\) of the particle as described by the Einstein-Stokes relationship:

\[
D = \frac{k_B T}{6 \pi R_s \mu}
\]

where \(k_B\) is the Boltzmann constant. Since \(D\) is also dependent of macromolecular crowding, diffusion measurements have been used to probe crowding in the cell [19-21]. Fluorescence recovery after photobleaching (FRAP) of fluorophore-tagged macromolecules is typically used when diffusion is fast; single-particle tracking can be used if diffusion is slow.

**Effects of macromolecular crowding \textit{ex vivo}\)**

The true effect of crowding is hard to measure \textit{in vivo}, because of the complexity of the cell. Synthetic crowders like Ficoll, dextran or polyethylene glycol (PEG) are often used to mimic \textit{in vivo} crowding effects. However, each crowder has a variety of non-desirable properties, e.g. PEG is a linear polymer that acts as a molten globule under dilute conditions, while at its critical concentration it will change its conformation and hence its volume. Furthermore, PEG can form coacervates at high concentrations and in the presence of inorganic salts. Ficoll is a sucrose-based polymer, roughly spherical in nature, but its branched structure makes it compressible and susceptible to changes in shape upon concentration due to self-crowding. Cell lysates can be used as biological components but are hard to concentrate to physiological levels of crowding without aggression of components, and solutions of purified proteins can become sticky when a single type of molecule is used at high concentration. Synthetic crowders have been shown to stabilize proteins [22, 23], promote aggregation [24], and influence protein-protein interactions [25]. Furthermore, crowding agents can stimulate transcription in cell-free expression systems. For more examples we refer to Zhou et al. [26].

In the highly crowded cytoplasm molecules not only experience excluded volume effects but also (non-)specific interactions such as electrostatic, H-bonding, van der Waals, and hydrophobic interactions. They can be either attractive or repulsive, depending on the surface chemistry of the molecules. This effect can oppose the steric crowding effects as was shown by a number of in-cell NMR experiments [27-29]. For example, the stability of some proteins \textit{in vivo} is lower than in solutions with synthetic crowders, highlighting the importance of (non-)specific interactions. Additionally, a FRET probe is compressed by synthetic crowders but is extended in a concentrated solution of cell lysates; an effect that was explained by a compensation of the depletion force (see Text Box I) by chemical interactions [30]. Besides these non-covalent chemical
interactions, macromolecular crowding agents also give rise to osmolyte effects, influencing protein hydration by changes in water polarity and activity [31]. Thus, synthetic crowding mimics excluded volume effects, but does not provide the same interactions as macromolecules in a cell; hence they do not answer all the questions pertinent to the effects of macromolecular crowding.

**Effects of macromolecular crowding in living cells**
The interior of a cell is a complex network of proteins, nucleic acids and small molecules that act in concert to sustain all essential processes of life. It has been shown that high concentrations of polymers condense the DNA as a consequence of depletion interactions [32, 33]. A well-known effect of macromolecular crowding in living cells is the reduced mobility of biomacromolecules compared to dilute solutions. In crowded cells the encounter rate between all cellular molecules is increased, which leads to a higher number of interactions and consequently slower diffusion. Additionally, the viscosity of the cytoplasm has been reported ~3 times higher than that of water [34], which impacts diffusion as is evident from the Einstein-Stokes relationship (Text Box I). For example, it was shown that the diffusion of bulky tRNA complexes in the crowded cytoplasm imposes a physical limit on the speed of translation [35]. The lateral diffusion coefficient of GFP $D_{\text{GFP}}$ (27 kDa) in the *E. coli* cytoplasm ranges from 3-14 μm²/s, which is an order of magnitude lower than in aqueous media [4, 36-38]. The range of $D$ values reflect both biological variation and differences in experimental conditions [39].

Bacteria rely on diffusion for transport of molecules and cytoplasmic mixing. A crowded environment provides barriers for long-range solute diffusion, which can lead to apparent confined protein mobility [19]. For example, the majority of the ribosomes is confined to the endcaps of the cell and therefore excluded from the nucleoid, while free ribosomal subunits can reach the nucleoid and can bind to mRNA [40]. Because of the ribosome-nucleoid-segregation the mRNA (bound to a ribosomal subunit) first has to diffuse to the endcaps of the cell prior to translation [2].

Finally, macromolecular crowding and ionic strength change in parallel when the volume of a cell changes, and crowding has been shown to mitigate the effects of high salt. For instance, the interaction of lac repressor with the lac operator and of RNA polymerase with λPr promoter is strongly dependent on the salt concentration [41, 42]. However, the salt effect is not observed *in vivo* [43], which is attributed to the compensating increase in crowding. In another case, ionic strength and macromolecular crowding have been shown to act synergistically, i.e. the ion strength dependence of gating of the osmoregulatory
ABC transporter OpuA is diminished in the presence of synthetic crowders [44]. Thus, effects of macromolecular crowding are intimately linked to the electrostatic stabilization of cytoplasmic macromolecular surfaces. We note that other attractive physicochemical forces (hydrophobic, van der Waals and H-bonding) are undoubtedly also involved in the assembly of macromolecular complexes and structuring of the cytoplasm [45].

**Structure of the crowded cytoplasm**

Cells have subcellular structures [46, 47], known as metabolons [48] or hyperstructures [49]. The repulsive and attractive (non-covalent) forces, caused by the uneven distribution of hydrophilic, hydrophobic, and charged surface areas of macromolecules, favors the clustering of proteins and nucleic acids [45, 50]. As a consequence, the crowding can locally increase to levels above the average crowding, which goes along with the formation of ‘uncrowded’ areas in the cytoplasm. We recently proposed that the prokaryotic cytoplasm might form a multi-phase system of ‘supercrowded’ cytogel and ‘dilute’ cytosol [51], see Text Box II. In such a system the impact of molecule size on exploring the cytoplasmic space is different from that in a homogenous cytoplasm. Large particles get caged in the supercrowded areas and long-range motion is only possible if the pools rearrange. Consistent with this view, small molecule osmolytes explore a much larger volume of the cytoplasm of osmotically stressed cells than proteins [21]. This demonstrates that the effects of macromolecular crowding are diverse and might be locally amplified in regions of increased or decreased macromolecular densities.

A recent series of papers [52-54] supports the view of a dynamic structure of the cytoplasm as complex vectorial chemistry (Text Box II). In bacteria (*Escherichia coli* and *Caulobacter crescentus*) and lower eukaryotes (*Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Dictyostelium discoideum*) the cytoplasm changes from a fluid to a more solid-like (‘colloidal glassy’) state when the energy metabolism is shut down. In one study [53], it is proposed that the acidification of the cytoplasm causes reduced mobility of large particles, widespread macromolecular assembly and increased mechanical stability of the cell. Importantly, the lateral diffusion of a 54 kDa fluorescent protein was not affected by the energy depletion or acidification of the cytoplasm but strongly reduced in osmotically compressed cells. Munder and colleagues thus conclude that acid and osmotic stress result in different states of the cytoplasm; the transition to the solid-like state is required for cell
survival under conditions of energy starvation. The other study [54] proposes that the transition is due to the decrease in cell volume and the accompanying increase in macromolecular crowding and Joyner and colleagues equate energy starvation to osmotic stress.

Text Box II: Complex Vectorial Chemistry

The model of complex vectorial biochemistry combines the current understanding of bioenergetics and the vectorial character of the plasma membrane, including the chemiosmotic theory [55], with a semi-quantitative electrochemical model of a dynamically structured crowded cytoplasm [45, 50]. In this model, the cytoplasm has a dynamic architecture with a high volume fraction of reacting biomacromolecules, which divide the cytosol into networks of ion-conducting pathways and electrolyte pools (indicated as ATP/ADP pool in Figure 3). In a colloidal sense such a system is stable when the range of action of the stabilizing non-covalent forces remains commensurate during growth and cell division.

The stabilizing non-covalent forces are excluded volume, screened electrostatic repulsions, hydrogen bonding and charge-dipole forces (hydration). The range of the interaction is ~1 nm for excluded volume (assuming a macromolecular volume fraction \( \Phi \) of 0.3), 0.2-1 nm for the electrostatic force (for ionic strength, \( I \), from 2-0.1 M), and ~0.6 nm for the hydration force. Thus, at \( \Phi > 0.3 \) the average surface-to-surface distance is commensurate with the screened electrostatic and hydration interactions.

Although an average \( \Phi = 0.3 \) is on the high end for (osmotically) non-compressed cells (see sections below), it is likely that the cytoplasm dynamically segregates into ‘supercrowded’ and ‘uncrowded’ areas. A non-random clustering of biomolecules is supported by the recent findings of microscopically visible structures in energy-starved yeast cells [53, 57, 58]. In an extension of the complex vectorial chemistry model, the biomolecular crowding within the cytoplasm may constitute a multiphase system of supercrowded cytogel and dilute cytosol [51]. Importantly, the supercrowded regions would extend the vectorial nature of reactions at the plasma membrane deeper into the cytoplasm, and, like in a ‘microfluidic device’, they would direct reactants and products without getting mixed in the cytosol.
In living cells biopolymers are always exposed to (non-)specific interactions with the surrounding molecules (like people in a bustling city). The short distances between the biomolecules are in the range over which screened electric forces are operational (Text Box II), which stabilizes the cytoplasm against random collapse but allows unequal crowding [45]. The idea of a dynamically structured crowded cytoplasm is consistent with the observation that cytosolic acidification triggers the assembly of many proteins into microscopically visible structures [53, 58], because at low internal pH the electrostatic screening is affected and the solubility of the ‘acidic proteins’ is diminished. The dynamic reorganization of proteins into intracellular bodies can involve filament or foci formation [57] a highly cooperative process that is strongly dependent on macromolecular crowding. Thus, even though the molecular mechanism that underlies the dynamic heterogeneity of the cell is still elusive, it is evident that the cytoplasm is more than a bag with randomly organized enzymes and the formation of phase-separated macromolecular assemblies is at the heart of localized biochemistry.

What is the optimal level of crowding?

From an evolutionary standpoint the optimal performance of biochemical reactions is achieved in its natural crowded environment, because proteins have coevolved in the presence of each other. An unanswered question is how the magnitude of macromolecular crowding affects the performance of biomacromolecules. We first review how biochemical reactions perform in (diluted) cell-free environments and then describe how cells react to an increase in macromolecular crowding to find out what is the optimal level of crowding. We then report levels of crowding in bacteria under different conditions.

Biochemical reactions in cell-free systems

It was in 1897, when Buchner provided the first evidence that a complex biochemical process can be separated from a living cell [59]. He observed that a yeast cell-free extract could still ferment sugars to produce alcohol. The rate of Büchner’s cell-free fermentation was later found to be less than 5% of that in living yeast cells [60], which could be a consequence of the more dilute environment compared to in vivo conditions.

Macromolecular crowding increases the efficiency of some biochemical reactions, although the effect in most cases is moderate. For example, Vöpel et al. compared the kinetic parameters of several enzymatic reactions, including
oxidotransferases and phosphatases, carried out either with or without addition of Ficoll [61]. Most of the reactions tested showed a small change in the Michaelis constant (K_M) of the enzyme for the substrate (less than 2-fold change in K_M). However, even small changes of the kinetic parameters of individual enzymes might result in large changes for synthesis or breakdown of molecules, since the metabolism is an elaborate network of interconnected enzymes and metabolites. For an in vitro replication system, a plasmid, bearing the origin of the E. coli chromosome (OriC), could only be replicated in the presence of high concentrations (~6%) of polyethylene glycerol (PEG) or polyvinyl alcohol (PVA) [62]. In a cell-free protein expression system the mRNA production was enhanced in the presence of Ficoll, while translation was inhibited by the synthetic crowder, which was attributed to (non-)specific binding events [63]. Binding of RNA polymerase to DNA is enhanced when adding dextran [64]. Similarly, the rate constant for transcription is five times higher when the environment is crowded rather than dilute and the values under crowded conditions are strikingly similar to what is observed in vivo [65]. On the other hand, the crowding agent negatively affected the translation. In conclusion, enzymes are typically only moderately affected by macromolecular crowding, but there are several indications that in particular transcription is enhanced under crowded conditions.

**Increased crowding leads to growth arrest**

Relatively modest osmotic upshifts (e.g. addition of 100 mM sorbitol to E. coli cells in LB medium) do not affect cell functioning even though the volume is decreased by ~5% [66] and the crowding may increase accordingly. The diffusion coefficient of GFP in mildly upshocked cells (up to ~0.3 Osm) is hardly changed [19]. However, large osmotic upshifts severely disturb cell functioning and lead to growth arrest. The efflux of water upon hyperosmotic stress leads to an instantaneous cell shrinkage and the cytoplasmic volume can decrease up to 40% [67, 68], which leads to plasmolysis and a dramatic increase in macromolecular crowding (see Figures 4A and B). The diffusion coefficient of GFP in plasmolyzing cells decreases proportionally with the magnitude of the osmotic upshift and under extreme conditions the macromolecules are essentially frozen [19, 21]. In plasmolyzing E. coli cells barriers for diffusion are observed for molecules as small as GFP but not for metabolites [69]. Thus, the cytoplasm of osmotically stressed cells forms a meshwork of biopolymers, allowing the free passage of small molecules while restricting the diffusion of bigger ones. Additionally, the cytoplasm shows molecular sieving properties that are likely caused by invaginations of the cytoplasmic membrane pushing against the nucleoid in a way that proteins get trapped [21].
Osmolytes are osmotically active compounds. Changes in medium osmolarity lead to release (osmotic downshift) or accumulation (osmotic upshift, hyperosmotic stress) of osmoprotectants to counterbalance the differences in extracellular and intracellular osmolarity. The accumulation of K⁺ is used by *E. coli* to quickly respond to hyperosmotic stress. Over longer periods of time the ionic osmoprotectant(s) are replaced by neutral (e.g. trehalose) or zwitterionic osmoprotectants like glycine betaine, proline, carnitine or trimethylamine N-oxide (TMAO). These molecules are also referred to as compatible solutes and can be accumulated by uptake or biosynthesis. They are usually kosmotropes, compounds that structure water and stabilize biological macromolecules.

Osmolarity is a measure of the concentration of solutes (osmolytes), which contribute to the osmotic pressure of a solution, defined as osmoles per liter.

Turgor pressure is the hydrostatic pressure difference (∆π) that balances the difference in internal and external osmolyte concentration. In equation: ∆π = RT([c]_{in} - [c]_{out}), in which ∆π is the osmotic pressure difference, *R* is the gas constant, *T* the absolute temperature, and [c] the osmolyte concentration. The turgor (cytoplasmic volume) increases upon osmotic downshift and decreases upon osmotic upshift of the external medium.

Cells adapt to osmotic upshift by accumulating compatible solutes, often referred to as osmoprotectants (see Text Box III), either through import or synthesis [37, 70, 71], which allows restoration of the cytoplasmic volume (Figure 4C). This response takes place over timescales from minutes to hours.
The compatible solute glycine betaine is accumulated by osmotically stressed cells and has also been shown to mitigate the destabilizing effect of a high protein crowding [72] and to counter protein aggregation in vivo [73]. When cells face an osmotic downshift, they release solutes in a non-specific manner enabling a rapid decrease in osmolyte concentration and, consequently, in the osmotic driving force for water entry [74]. We are not aware of studies that have addressed the structure of the cytoplasm immediately after an osmotic downshift, but the assembly of biomolecules or the formation of supramolecular complexes is likely to be affected after the majority of electrolytes and metabolites have left the cell.

**Modeling the optimal macromolecular crowding**

So far, we have seen that in dilute, cell-free environments biochemical reactions may perform suboptimal, whereas an increase of macromolecular crowding above a certain threshold leads to growth arrest. Thus, cells need to regulate their intracellular crowding, because deviations from the optimal crowding have a negative effect on their growth. Here, we report two models that address the question of what is the optimal crowding for cells.

Under the assumption that nutrient uptake is fast compared to the metabolic reactions inside the cell, a flux balance model was developed that simulates the highest possible macromolecular density where cells still benefit from increased enzyme concentrations (to speed up reaction rates) without diffusion of reactants becoming too slow and therefore a limiting factor [75]. According to Vazquez, it is the interplay of these opposing factors that likely determines the optimal crowding of cells. This depends on the number of reactions that are diffusion limited. The authors assume that reactions are diffusion limited if the substrate concentration is low compared to the \( K_M \) (\( S < K_M \)), whereas at high substrate concentrations all enzymes are saturated (\( S > K_M \)) and the rate is limited by the chemical steps in the formation of the product, which is assumed to be fast. The biochemical reactions were therefore divided into two groups: diffusion-limited reactions (\( R_L \)) and reactions at saturation (\( R_S \)). The optimal macromolecular volume fraction \( \Phi \) was simulated as a function of \( R_S/R_L \), ranging from \( \Phi = 0.22 \) (all reactions are diffusion limited) to \( \Phi = 0.8 \) (maximum density and all reactions saturated with substrate). To deduct the number of diffusion-limited reactions, Vasquez took advantage of a study from Bennett et al., who determined the concentration of more than 100 metabolites in *E. coli* [76] and found that three times more reactions are in the saturated regime, i.e. with \( S > K_M \). Vasquez concludes that the most likely ratio is \( R_S/R_L = 3 \), which would correspond to an optimal volume fraction \( \Phi = 0.37 \).
Another modeling approach is based on the idea that cells have an optimal protein density that maximizes the reaction rates [77]. Dill at al. assumes that a cell with a fixed amount of proteins can regulate its density (and consequently the crowding) to an optimal level by adjusting the cell volume. Big cells would therefore be less crowded and have a density below the optimum, which slows down the reaction rates. On the other hand, in small cells with a high crowding, the slow diffusion of macromolecules would be a limiting factor. The model assumes that the rate of diffusion-limited reactions is proportional to the concentration of reactants and the lateral diffusion constant $D$. Using the volume fraction $\Phi$ instead of concentration, they find $\Phi = 0.19$ as the optimal density for maximal reaction rates.

Both models achieve optimal volume fractions that are within the range of experimentally obtained values. A volume fraction of $\Phi = 0.37$ is in accordance with Zimmerman et al. [3] and a volume fraction of $\Phi = 0.19$ is closer to more recent observations from Boersma et al. [6]. However, the assumptions made are not necessarily correct. Both values for the optimal volume fraction were obtained by assuming that a large fraction of the biochemical reactions is diffusion-limited, but there is little experimental evidence for this assumption. In fact, the lateral diffusion of most molecules inside cells is fast and even for a large enzyme like β-galactosidase it takes less than 1 s to travel by Brownian motion from one end of the *E. coli* cell to the other [68]. Additionally, the maximization of reaction rates, not only depends on the concentration of reactants, but also for example pH, ionic strength, and temperature and in many cases the availability of ATP. These effects are not addressed in both models, however the fact that volume fractions can be simulated close to the experimentally observed ones makes it tempting to assume that the general idea of two opposing effects on reaction rates is valid.

**Experimentally determined crowding in *E. coli***
The macromolecular volume fraction of *E. coli* has been reported to be in the range of 0.13-0.44 (v/v) [3-6]. The spread is large and likely caused by differences in experimental approaches and data analysis. Accurate determination of volume fractions requires quantification both of the biomass that contributes to the macromolecular crowding and of the cytoplasmic volume (Text Box I). The biomass is usually approximated as arising solely from proteins and RNA. Different experimental techniques are available to determine cytoplasmic water and are based on the exclusion of a solute from the cell or the cytoplasm (Text Box I). Zimmerman et al. obtained the highest cytoplasmic volume fractions of $\Phi = 0.33$ to 0.44 (v/v) for cells grown in LB.
medium (~0.4 Osm) [3], while Konopka et al. reported $\Phi = 0.16$ for cells grown in MBM (~0.28 Osm) [36, 78]. The differences are likely not due to growth conditions, but are arising from the determination of the cytoplasmic volume, and the subsequent conversion of volumes to the volume fraction using the partial specific volumes of the macromolecules.

The macromolecular volume fraction can also be obtained by using a FRET-based crowding sensor, which showed that the macromolecular crowding of *E. coli* cells grown in minimal medium is equivalent to ~19% (w/w) Ficoll [6], which corresponds to a volume fraction of $\Phi = 0.13$; a value close to the volume fraction determined by Konopka et al. [36]. The advantage of the FRET-based sensor is that macromolecular crowding can be determined with high temporal and spatial (diffraction-limited) resolution. Another method to estimate the crowding is based on determining the dry weight of a cell pellet in relation to its wet weight. Usually, only 60% of the water in the cell pellet is intracellular water [79]. This method will not lead to a value for the volume fraction excluded by macromolecules, because the dry mass also includes metabolites and compatible solutes. Additionally, the weight of the wet pellet before drying also contains water adhering to the surface of cells, which leads to an underestimation of the dry mass per cell. Yet, this method allows rapid analysis of many organisms or screening of a wide range of conditions; for example, Norland et al. examined more than 300 bacterial cells and found a linear relationship between dry mass and volume [80].

**Is the macromolecular volume fraction dependent on medium osmolarity?**

The cytoplasmic volume fraction was reported to increase from $\Phi = 0.15$ in cells growing in low osmolarity medium (0.1 Osm) to $\Phi = 0.36$ in cells adapted to high osmolarity medium (1.45 Osm) [36, 78]. At the same time the lateral diffusion coefficient of GFP in cells growing in either low or high osmolarity medium is similar [36]. The authors conclude that the protein mobility is only weakly dependent on the macromolecular volume fraction, which is contradictory to what one would expect for the diffusivity of proteins in upshocked cells. By increasing the medium osmolarity by 1 Osm the ‘free’ water fraction of the *E. coli* cytoplasm decreases 75% [5]. After adaptation of *E. coli* to the high osmolarity medium (1 Osm), the ‘free’ water only increased to 50% of its initial value. At the same time the cell now contains large amounts of compatible solutes. The ‘bound’ water is part of the hydration shell of proteins and makes up ~20% of the total amount of water in non-stressed cells and cannot be removed, even at very high medium osmolarities.
Unpublished data from our lab indicates that the diffusion coefficients found by Konopka et al. are correct but that the macromolecular crowding of adapted cells is overestimated [36]. Using the crowding sensor we find that the crowding of cells adapted to \( \sim 0.9 \) Osm is similar to that of cells grown at 0.32 Osm, which is more in line with the observations on the lateral diffusion of proteins. Thus, cells seem to operate crowding homeostasis when adapting to high osmolarity medium.

**Macromolecular crowding remains similar under different growth conditions**

Schaechter was the first to report that cell size is determined by the nutrient availability [81]. In *E. coli*, the cell mass, the amount of DNA and RNA and the average number of chromosomes per cell are exponential functions of the growth rate (\( \mu \)). In a recent ribosome profiling study, Li et al. find that the mass of proteins (240 fg per cell) in cells grown at \( \mu = 0.74 \) h\(^{-1}\) is \( \sim 3 \) times less than that of cells grown at \( \mu = 1.9 \) h\(^{-1}\) (680 fg per cell) [18]. Since the volume of the cell increases with growth, the overall crowding may be relatively constant. Furthermore, the buoyant density of *E. coli* cells grown at different growth rates is constant [82]; the partial specific volume of proteins and nucleic acids are \( \sim 0.73 \) and \( \sim 0.58 \) mL/g, respectively, and significantly increased macromolecular crowding would have increased the buoyant density.

By quantifying the majority of *E. coli* proteins and determining their location in the cell, one finds that with increasing growth rate the mass of proteins in the cytoplasm increases and the mass of protein in the periplasm decreases [17]. Because parallel measurements of the cytoplasmic and periplasmic volume show the same trend, this implies that the crowding of the cytoplasm and periplasm is constant under these different growth conditions (see Chapter 4). The lateral diffusion coefficient of GFP in the periplasm is reported to be two times slower than in the cytoplasm [83], but in terms of crowding both compartments may not be compared directly. Cytoplasmic biomacromolecules vary in molecular weight from 10 kDa to \( \sim 2,500 \) kDa (ribosomes), whereas the weight of periplasmic proteins are in the range of 25-80 kDa [17]. The periplasm does not contain nucleic acids, whereas the peptidoglycan structure is not present in the cytoplasm. Also, interactions of the fluorescent probe with the cytoplasmic or outer membrane might affect protein diffusion. Thus, while lateral diffusion is a measure of crowding within one compartment, the biomolecular composition and structure of the cytoplasm and periplasm are so different that a change in lateral diffusion may not simply be translated to a change in crowding.
In summary: too high or too low macromolecular crowding is expected to hamper the functioning of the cell. Abrupt changes in external osmolarity immediately affect cell volume and crowding (as well as pH and ionic strength) but cells are capable to adapt quickly to osmotic stress and ultimately may bring the volume and physicochemical parameters back to optimal values. Given the remarkable robustness and repeatability of the bacterial cell cycle, it is likely that cells maintain crowding conditions within narrow limits, suggesting a mechanism of crowding homeostasis that could be related to cell volume regulation.

**How do cells achieve homeocrowding?**

**Carbon Catabolite Repression as control mechanism**

Carbon catabolite repression (CCR) is a mechanism that allows bacteria to selectively take up substrates from a complex medium with mixed carbon sources. If glucose is present, CCR is activated and the uptake of substrates other than glucose is prevented. This process is mediated through underlying transcriptional regulation or by inhibition of synthesis of enzymes that are involved in the catabolism of carbon sources other than the preferred one. Zhou et al. found that slow growing *E. coli* cells at very low concentrations of the carbon source do not display CCR in a mixed carbon medium [84]. With increasing growth rate, CCR is gradually switched on along with higher glycolytic fluxes. Under such conditions *E. coli* cells do not fully metabolize the carbohydrate to obtain maximal ATP yield, but instead they produce ethanol, acetate, formate and lactate as fermentation products in a seemingly ‘wasteful’ process called overflow metabolism.

Using a flux balance analysis that includes the spatial constraints that occur in a densely packed environment, called flux balance analysis with macromolecular crowding (FBAwMC), the *E. coli* growth rate, nutrient uptake, and the ‘wasteful’ metabolite excretion were successfully predicted [84]. This led to the idea that CCR could be a means to maintain constant crowding conditions at high glycolytic flux. Slow growing cells (at low glucose) oxidize the carbohydrate to carbon dioxide. Faster growing cells (at higher glucose) require more enzymes to keep up with all the biosynthetic needs, consequently cells are bigger in size to accommodate the increase in biomass, but the biomass per unit of volume may stay constant (see Figure 5, transition A). By further increasing the growth rate, the amount of enzymes that the cell would have to synthesize in order to fully metabolize glucose exceeds the available space.
Instead of increasing their macromolecular crowding (transition C, which would lead to suboptimal growth), they increase their glycolytic flux and divert part of their metabolism towards fermentation products (Figure 5, transition B).

The CCR mechanism controls the transcription of the key steps in the metabolic switch in *E. coli*. Zhou et al. show that substrate uptake and growth rate correlate to macromolecular crowding (measured as buoyancy), and they propose that CCR could also be a regulatory mechanism to keep the cell density unchanged. Notably, transient perturbation of CCR and hence the concomitant perturbation of crowding resulted in delayed cell growth with altered cell volume and density, indicative of the importance of homeocrowding.

**Figure 4**: Model of metabolic adaptation and crowding homeostasis via carbon catabolite repression (CCR). Slow growing cells oxidize glucose to carbon dioxide. At increasing growth rate cells expand their volume to accommodate the increased biomass (transition A). At faster growth rate cells turn on CCR and the flux of glucose via the glycolytic pathway increases relative to the TCA cycle and respiration (transition B), which changes the composition of the whole proteome and possibly fine-tunes the macromolecular crowding. A corollary of this hypothesis is that without CCR the macromolecular crowding would become be too high and slow down growth (transition C). Figure adapted from [84].

**Homeocrowding mediated by a threshold protein**

When overexpressing large amounts of a ‘useless’ LacZ protein in nutrient-limited medium, Basan et al. observed that cell size is 6-8 times larger than what would be expected from the growth law (the cell volume was almost 8 µm³ at a growth rate of µ = 0.3 h⁻¹) [85]. Under these conditions, the amount of RNA, DNA and cellular proteins paralleled the cell volume in a way that dry mass per µm³ – and therefore crowding – was constant. To explain their observations, they introduced the threshold initiation model that requires the
existence of a division protein $X$. This protein has to reach a threshold amount $P_X^*$ to initiate division (Figure 6, Cell I). With the overexpression of LacZ (Figure 6, Cell II) the amount of $X$ would be lower at the same cell size (compare Figure 6, Cell I and II), and the cell would continue to elongate until the threshold amount of $X$ is reached (Figure 6, Cell III). The model explains why cell division is delayed when (foreign) proteins are (over)expressed, which could potentially prohibit overcrowding. A prerequisite is that cell division is sensitive to very small changes in $X$. The protein FtsZ, that forms a septal ring structure at the mid-cell during cell division, is a potential candidate for $X$. However, other molecules could be involved, for example the small metabolite UDP-glucose, which was shown to indirectly inhibit the FtsZ ring formation [86]. Additionally, the alarmone ppGpp contributes to the regulation of various cellular processes that are related to nutrient starvation and other environmental stress [87].

**Figure 5:** The threshold initiation model of cell size control assumes that a cell division protein $X$ has to reach a certain threshold amount $P_X^*$ per cell to trigger cell division. When the abundance of the protein $X$ reaches a threshold level (represented by the dashed line), the cell divides at this size (Cell I). Overexpression (OE) of useless protein (LacZ) reduces the proteome fractions of $X$ and other growth rate-dependent proteins $G$, while a certain fraction of the proteome $Q$ remains constant and cannot be reduced (Cell II). Hence, the cell continues to grow until protein $X$ reaches the threshold level $P_X^*$ and then divides (Cell III). Figure from [85].

**Crowding control via sensing of ionic strength and membrane stretch**

Osmotically stressed cells control their volume via the gating mechanisms of osmoregulatory transporters and mechanosensitive (MS) channels [88, 89]. Upon osmotic upshift, the cell volume decreases and next to macromolecular crowding, the internal ionic strength ($K^+$-concentration) increases, which gates osmoregulatory transporters (shown for *E. coli*, *L. lactis* and *Corynebacterium glutamicum* [90-92]), and thereby restores the volume of the cell in due time. Upon osmotic downshift, cells rapidly release osmolytes via membrane-stretch activated mechanosensitive channels and thereby mitigate the cell expansion due to water influx and prevent cells from lysing [93]. MS channels are universal in prokaryotes and eukaryotes, *E. coli* has even seven genes coding for MS channels and the function of several is still enigmatic. We consider it likely that the osmoregulatory transporters and some of the (more sensitive)
MS channels play a role not only in osmoregulation but also in cell volume regulation and thus crowding homeostasis in growing cells. We note the activity of the osmoregulatory ABC transporter OpuA, which is gated by ionic strength, is tuned by macromolecular crowding, at least in vitro using synthetic crowders [44]. Also, it has not escaped our attention that E. coli has six MS channels belonging to the MscS family that may have roles in addition to osmoregulation, e.g. YjeP has a role lipid biosynthesis [94] and thus control the cytoplasmic volume.

**Conclusions**

A high macromolecular crowding is a universal property of all living cells. Crowding influences the properties of macromolecules and increases the effective concentration, as well as the encounter rate and (non-)specific interactions between cellular components. The use of synthetic crowders revealed that crowding can modulate the activity of enzymes, but in general the effects are small. The strongest effect of crowding on reactions is observed for transcription and the assembly of oligomeric structures. The excluded volume effects on reactions can be balanced by (non-)specific chemical interactions between the cellular components. Thus, the effects of crowding are diverse and depend on the nature and the size of the biomolecule. At a more macroscopic level, crowding impacts the physiology of the cell by organizing the cytoplasm into dynamic compartments [50] and may induce phase transitions [52]. Because of the versatile effects of macromolecular crowding, not only on individual molecules, but also on the interactions between molecules and the ability to organize the cytoplasm, crowding can be considered essential for life.

Considering its importance, cells likely maintain crowding within narrow limits for which evidence is emerging. Possible mechanisms for homeocrowding are based on (i) transcriptional regulation of metabolism in order not to ‘overcrowd’ the cell; (ii) sensing of a signaling molecule to control cell division; and (iii) sensing of ionic strength or/and membrane stretch to control the cytoplasmic volume. Whatever the mechanism or combinations thereof crowding homeostasis has to include some flexibility, as otherwise even slight changes in cytoplasmic volume would result in immediate growth arrest.
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