Chapter 5

Nucleoid organization under increased crowding and confinement in vivo

Macromolecular crowding is important for many cellular processes such as cytoplasmic transport and metabolic reactions, but its effects on essential DNA transactions in vivo remains largely unexplored. Here we ask whether and to what extent crowding of the cytoplasm affects the spatial organization of the E. coli nucleoid. Although crowding has been shown to drive compaction of isolated nucleoids in vitro, demonstrating its role in vivo has been challenging, in part because numerous other specific factors influence nucleoid configuration, but more fundamentally because tools for directly quantifying crowding in live cells has been lacking. Here we present experiments in which a recently developed Förster resonance energy transfer (FRET) sensor of cytoplasmic crowding was combined with high-throughput live-cell imaging to dissect the role of crowding on nucleoid spatial organization in vivo. We followed the temporal evolution of both crowding and nucleoid morphology simultaneously in thousands of exponentially growing cells. To probe the response of the nucleoid to crowding perturbations, we subjected cells to osmotic shocks. Nucleoids collapsed in size upon increased crowding and expanded upon decreased crowding, suggesting that crowding plays an important role in determining nucleoid size during steady growth. In agreement with previous observations, we find that nucleoids adopt different sizes and morphologies at different growth rates, but the relationship between nucleoid size and crowding was highly stereotyped, suggesting that physical parameters relevant to crowding are largely invariant. Our data also provide clear evidence that nucleoid size and morphology is not determined by crowding alone. Changes in both crowding and nucleoid size demonstrated hysteresis, likely reflecting the role of active processes during osmoadaptation. Treatment with the drug 2,4-dinitrophenol (DNP), which inhibits active processes in the cell, led to dramatic nucleoid expansion while crowding remained invariant, suggesting that the net force exerted on the
nucleoid by active processes during steady growth is compressive. Taken together our data confirm the importance of crowding in the compaction of chromosomal DNA in vivo, and provide new insights on the role of active processes in the homeostatic regulation of nucleoid size in bacteria.

5.1 Introduction

In its native cellular environment, the bacterial chromosome is a highly compacted polymer, a DNA molecule of length $\sim 1\text{ mm}$ confined within a cytoplasmic volume of $\sim 1\text{ µm}^3$. Unlike eukaryotic chromosomes, which reside in a membrane-bound nuclear compartment, it resides within the cytosol but its density nevertheless occupies a distinct subvolume known as the nucleoid. Recent advances in optical microscopy and sequencing-based technologies have revealed that chromosomal DNA in the nucleoid are by no means random polymers but possess a complex multi-scale spatio-temporal organization ([126, 129, 83, 270–272, 69, 273]). Despite very high degrees of compaction, nucleoids are visibly dynamic, demonstrating a rich repertoire of morphologies depending on growth conditions [84], cell cycle [129], and various physiological perturbations [274, 275]. The demand for sophisticated spatial organization of the nucleoid is tangible if one considers the degree of compaction on the one hand, and the plethora of vital functions that require access to chromosomal DNA on the other. Such essential DNA transactions include chromosome replication, segregation and gene expression in response to external stimuli [276, 277, 21], and to support these functions the nucleoid volume includes, in addition to chromosomal DNA, polymerases, nascent messenger RNAs, transcription factors, as well as a variety of other DNA-associated proteins. Yet, how bacterial cells achieve, maintain, and dynamically modulate the spatial organization of their genetic material in the crowded cytoplasmic environment remain largely open questions, and the underlying mechanisms and design principles have become subjects of an increasingly active area of research.

A variety of specific biochemical processes have been proposed to modulate nucleoid configuration in vivo, with varying degrees of experimental and theoretical support. These processes include transcription by RNA polymerases (RNAPs) [69, 83], cotranscriptional translation and bilayer insertion of membrane proteins (transertion) [278], DNA supercoiling [279], nucleoid associated proteins (NAPs) and structural maintenance of chromosome proteins (SMCs) [58, 273]. Indeed, the panel of proposed mechanisms are nearly as diverse as the repertoire of chromosomal DNA transactions, and although some proposed effects have been studied/confirmed in more detail than others, most proposals are not mutually exclusive, and it is likely that in reality a combination of many (if not all) of these specific
mechanisms play some role in the complex overall organization of bacterial nucleoids across diverse environmental and physiological conditions.

In this chapter, we focus on the effects of macromolecular crowding, an ubiquitous physical phenomenon in living cells, on the spatial organization of the E. coli nucleoid in vivo. Unlike the specific biochemical processes postulated to affect nucleoid size and morphology discussed above, macromolecular crowding does not depend on the activity of specific molecular species, but rather reflects generic features of the physical environment such as the cell’s volume and the size distribution of its molecular constituents. In recent years, the effects of crowding have gained increased attention not only in mechanistic studies of nucleoid compaction but also in those focused on the relationship between gene expression and DNA spatial organization [39, 52, 280, 281]. By slowing diffusion, affecting spatial proximity of interaction partners and rates in biochemical reactions, crowding appears indeed to be relevant for many important cellular processes like metabolic activity, initiation of DNA replication, and transcription [52, 282, 283].

Recent in vitro experiments on nucleoids isolated by cell lysis have unambiguously demonstrated that the addition of artificial crowding agents can compact the E. coli nucleoid, with the compaction degree increasing with the magnitude of the crowding perturbation (change in volume fraction of synthetic polymers) [43, 44]. Other in vitro experiments and theoretical works have shown that isolated nucleoids can be compacted by increased protein or polymer concentrations (e.g. lysozyme and polyethylene glycol), that crowding could account for the formation of the two-arms chromosome configuration, and that the nucleoid can undergo a continuous collapse mediated by depletion interactions [60, 44, 284, 285, 66, 45]. All of these results point towards a fundamental role of crowding in shaping the chromosome’s configuration in live bacteria, but in lieu of direct experimental test in vivo, exactly how and to what extent crowding impacts nucleoid morphology in its native cellular environment remains a subject of active debate.

We have developed a novel live-cell imaging approach that allows parallel measurement of nucleoid morphology and the crowding state of the cell across time as bacteria experience environmental changes that affect cytoplasmic crowding and other physiological parameters. We made use of a genetically encoded protein FRET sensor developed by Boersma and coworkers [54] to quantify crowding in growing E. coli cells, and perturb crowding by osmotic challenges under different growth and metabolic conditions. By employing microfluidic growth chambers of the ’mother machine’ type ’ [98], we achieve continuous imaging at single-cell resolution and high throughput during exponential growth, with rapid medium exchange to implement step-like environmental changes. Imaging through multiple spectral
channels allowed us to follow changes in the nucleoid and cell morphologies together with the crowding level of the cytoplasm upon applied osmotic shocks of varying magnitude.

Our ability to simultaneously follow nucleoid and crowding dynamics in a large number of cells (\(\sim 2000\) cells per experiment) enables data-rich inquiries of crowding effects on the nucleoid \textit{in vivo}. Here, we exploited this capability to address the following questions:

- \textit{What is the role of crowding in setting the nucleoid size in living cells?} Given the variety of important cellular processes that require a certain degree of crowding and the scaling of cell size with growth rate, the idea that crowding might be actively controlled by the cell has recently introduced \cite{52}. We sought to determine whether and to what extent crowding shapes the steady state chromosome configuration by perturbing the steady-state level of crowding by osmotic shocks.

- \textit{Does crowding play a role in the distinct nucleoid sizes observed at different growth rates?} The shape and size of the nucleoid is different under fast and slow growth \cite{84} but the underlying causes of such differences remain unknown. It is well established that cytoplasmic composition varies when cells are growing at different rates and in particular ribosomal protein fraction of the proteome increases linearly with growth rate \cite{286, 287}. Thus crowding might act differently in cells growing at different rates, and we sought to compare the response of the nucleoid to crowding perturbations in cells under contrasting growth conditions.

- \textit{What is the relative importance of active and passive processes in determining the state of the nucleoid?} It has been shown that shutting down metabolism with 2,4-dinitrophenol (DNP) can drastically affect the physical state of the cytoplasm, as evidenced by a strong attenuation in the mobility of large particles \cite{70}. We reasoned that monitoring the state of the nucleoid and cytoplasmic crowding upon metabolic shutdown could provide new insights regarding the balance between active and passive forces acting on the nucleoid.

By simultaneous monitoring of cell size and morphology, in addition to the state of the nucleoid and crowding, our approach also allowed us to further dissect the expected causal chain of events upon osmotic shock, namely the change in crowding upon osmotic perturbations to the cell volume, and the change in the nucleoid state in response to crowding.
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5.2.1 Hyperosmotic shock in growing cells leads to transient crowding increase and nucleoid compaction

To achieve parallel measurement of nucleoid and cell morphologies as well as intracellular crowding at high throughput, we developed a multi-channel imaging system based on a microfluidic device of the ‘mother machine’ type [98] (Fig. 5.2.1). This device consists of two linear arrays of parallel slit-like growth chambers, of width comparable to that of the cells and of length several times that of cells. These growth-chamber arrays flank a larger central channel which provides a constant nutrient feed by steady flow of growth medium (Fig. 5.2.1A). Because of the short length of the slit-like growth chambers (~10µm), the cells experience a rapid change in their environment upon a switch in the incubation medium, controlled by a fluidic valve near the device’s inlet. Throughout the experiment, 30 fields of view (FOV), each containing 100-200 cells are imaged in rapid succession at regular intervals in four spectral channels to monitor three observables (Fig. 5.2.1B): (i) phase contrast channel for cell morphology, (ii) FRET donor (CFP) and acceptor (YFP) fluorescence channels for intracellular crowding, and (iii) red (mRuby2) fluorescence channel for nucleoid morphology.

Fig. 5.2.1 Mother machine setup, experimental approach and observables (A) Schematic of the "mother machine" PDMS device used in this study. The fluidic switch valve at the inlet is used to rapidly exchange the medium to apply osmotic shocks and other perturbations to cellular physiology. (B) Representative images of one field of view within the mother machine device, in the four spectral channels used for parallel monitoring of three observables: (i) phase contrast channel for cell morphology, (ii) FRET donor (CFP) and acceptor (YFP) fluorescence channels for intracellular crowding, and (iii) red (mRuby2) fluorescence channel for nucleoid morphology.
To test the effect of osmotic upshifts on cell size, nucleoid size, and crowding, we exchanged the nutrient medium during exponential growth with an identical one supplemented with an osmotic agent (sucrose) and followed the state of the cell and nucleoid in the four imaging channels of our setup. Fig. 5.2.2 shows images and extracted observables from a typical experiment in which cells were challenged with a 0.6M osmotic upshift during fast growth (in a chemically defined rich medium; see Methods). Both the cytoplasm and nucleoid were visibly attenuated in size just after addition of sucrose (Fig. 5.2.2A), which permeates *E. coli*’s outer membrane but not the inner membrane [288]. Volume reduction of the cytoplasm was often accompanied by noticeable deformations due to plasmolysis (indicated by red arrow in Fig. 5.2.2A), at a frequency that increased with the magnitude of applied osmotic upshifts. Nucleoid compaction was also far from isometric, with initially elongated density profiles often collapsing into globular density peaks.

The average cell area and nucleoid area, as well as the acceptor/donor fluorescence ratio (YFP/CFP) of the FRET crowding sensor (hereafter referred to as "FRET ratio") were stationary during exponential growth in the mother machine, but upon the application of hyper-osmotic shock, all of these observables were significantly perturbed (Fig. 5.2.2B). The fractional change $\Delta A_c / A_{c0} = (A_c - A_{c0}) / A_{c0}$ of the cytoplasmic area $A_c$ from its steady-state value $A_{c0}$ peaked immediately after the 0.6M sucrose stimulus with an average magnitude $|\Delta A_c / A_{c0}| \approx 15\%$, in good agreement with the higher time-resolution measurements of Pilizota and Shaevitz [288] who found that cytoplasmic volume change reaches a maximum amplitude $\Delta V_c / V_{c0} (= (1 + \Delta A_c / A_{c0})^{3/2} - 1)$ of $|\Delta V_c / V_{c0}| \approx 20\%$ within seconds after a sucrose stimulus of comparable magnitude for cells grown in another rich medium (LB).

Strikingly, initial changes in nucleoid size and crowding following the shock were antiparallel, with the change in the FRET ratio indicating an increase in crowding concomitant with osmotic compression of the cytoplasm, while nucleoid area decreased during the same interval, as expected if increased crowding were driving the observed nucleoid collapse. By contrast, changes in cell and nucleoid areas were approximately parallel, both decreasing rapidly just after osmotic upshift, followed by a slower recovery. Interestingly, however, the overall degree of compression/compaction was greater for the nucleoid than that for the cell, as could be seen in the clear decrease of the nucleoid area fraction (defined as nucleoid area divided by cell area) just after the osmotic shock Fig. 5.2.2B). The fact that the nucleoid is compacted by a factor greater than that for the cytoplasmic volume strongly suggests that the observed nucleoid compaction is not due to increased confinement of the nucleoid polymer by the cytoplasmic membrane.
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Fig. 5.2.2 Image sequences and time series of extracted observables in a typical hyperosmotic shock experiment (A) Image sequences of mRuby (top) and phase contrast (bottom) channels showing the time evolution of the nucleoid and cytoplasm morphologies upon hyperosmotic shock (addition of 0.6M sucrose) during fast growth, for a representative cell lineage (image sequence aligned at the midpoint of progenitor cell). Red arrow indicates visible plasmolysis event. Time scale is as indicated in panel B. (B) Time traces from a 0.6M sucrose osmotic upshift experiment, from top to bottom: cell area from phase contrast image segmentation, FRET signal from crowding sensor, nucleoid area from mRuby2 fluorescence images segmentation, nucleoid area fraction defined as the ratio of nucleoid area to cell area. For all observables per cent changes with respect to steady state values, normalized to the steady state, are shown (for an observable $x$, traces represent $\frac{\langle \Delta x \rangle}{\langle x \rangle_0} \times 100$). Red dashed line indicates the time points at which the sucrose-growth medium solution arrived in the microscope field of view, grey dashed lines indicate the steady-state average level prior to shock. Colored curves represent averages over single growth chambers, and black curve and points represents the ensemble average over all growth channels.
Taken together, the decrease in nucleoid area fraction combined with the antiparallel trajectories of the nucleoid area and FRET crowding signal implicate increased crowding, and not confinement, as the driver of nucleoid compaction upon osmotic upshift.

Over longer times after osmotic upshift, the initial rapid compression is followed by a slower recovery of both the nucleoid and cytoplasm, with their respective sizes slowly increasing, while the crowding FRET signal concomitantly decreased, toward pre-shock values. Interestingly, recovery of both the nucleoid and cytoplasm eventually overshoots the pre-shock level, but the overshoot amplitude is greater for the nucleoid which thus occupies a larger fraction of the cytoplasm at long times after osmotic upshift (Fig. 5.2.2B, lower two panels).

In summary, we showed that the nucleoid in fast-growing *E. coli* cells can be further compacted by osmotic shock, that the collapse upon shock is likely not caused by increased cellular confinement, and that increased crowding correlates with compaction.

### 5.2.2 Hypoosmotic shock reduces crowding and increases nucleoid size

The results of the previous section suggested that crowding might play a significant role in determining the steady-state size of the nucleoid within growing cells. We reasoned if that were true, decreasing intracellular crowding could also affect nucleoid size. We thus tested whether an opposite osmotic stimulus (a hypoosmotic shock) could induce a crowding perturbation of the opposite sign by challenging growing cells with a sudden downshift in environmental osmolarity.

By contrast to the hyperosmotic shock response, upon a hypoosmotic shock applied by rapid exchange of the growth medium with distilled water (dH$_2$O), no rapid changes in the observables were detected (Fig 5.2.3). We note that these data are not inconsistent with previous work [289] in which the *E. coli* cytoplasm was found to transiently expand upon hypoosmotic shock, because the duration of the volume peaks reported in that study were shorter than the image acquisition interval of our experiment (2 min).

Over longer times, our data indicate a mild decrease in the cytoplasmic volume while the FRET crowding signal slowly decreased over the same interval (Fig 5.2.3A). A decrease in crowding implies a reduction of the macromolecular volume fraction $\phi = V_m/V$, which could result from a change in the cytoplasmic volume, $V$, the volume occupied by macromolecules $V_m$, or both. Given that the population averaged cytoplasmic volume $\langle V \rangle$ is decreasing, $\langle V_m \rangle$ must also be decreasing and at a faster relative rate, *i.e.* $d \ln \langle V_m \rangle / dt < d \ln \langle V \rangle / dt < 0$. During steady exponential growth, the net rate of cytoplasmic volume production $\alpha$ is balanced by the rate of cell division $\mu$, so that the average cytoplasmic volume is stationary, $d \ln \langle V \rangle / dt = \alpha - \mu \ln 2 = 0$. The net rate of macromolecular volume production $\alpha_m$ must also
balance the cell division rate, so that \( d\ln\langle V_m \rangle / dt = \alpha_m - \mu \ln 2 \). Thus, \( \alpha_m = \alpha = \mu \ln 2 \) during steady growth, and the decrease in both cytoplasmic volume (\( d\langle V \rangle / dt < 0 \)) and FRET crowding signal (\( d\langle \phi \rangle / dt < 0 \)) in our data implies a hierarchy among the rates \( \alpha_m < \alpha < \mu \ln 2 \) during the prolonged hypoosmotic challenge.

Visual inspection of the image sequences indeed confirmed an altered balance between cell elongation and division under the hypoosmotic condition in our experiment. After exchange of the growth medium with dH\(_2\)O, both cell elongation and division gradually slowed and eventually halted. But decay of the elongation rate (which is proportional to \( \alpha \)) proceeded more rapidly than that of the division rate (\( \mu \)), resulting in a reduction of the average cell size (i.e., \( \alpha < \mu \ln 2 \)). Changes in the cytoplasmic growth rate \( \alpha \) could in principle reflect in addition to bona fide changes in growth, also the inflation/deflation of the cell upon the osmotic shock and subsequent osmo-adaptation. In fact, it has been shown that certain hypoosmotic stimuli can trigger a transient decrease of the cytoplasmic volume (after the aforementioned transient increase immediately following the shock) that can last for tens of minutes [289]. However such osmo-adaptive volume reductions typically play out much more rapidly (within a few minutes after the shock) compared to the gradual decay of cytoplasmic volume we observed here (Fig 5.2.3A). We therefore think it more likely that the decrease in cell volume observed here is due to cumulative effects of the dH\(_2\)O environment on biosynthetic processes that support growth. Our experiment does not provide a direct means of monitoring the net rate of macromolecular volume production \( \alpha_m \), but the hierarchy noted above suggests that it slows down even more rapidly than the production rate of cytoplasmic volume, such that \( \alpha_m < \alpha \) under the sustained hypoosmotic stimulus.

Interestingly, the decrease in the FRET crowding signal was accompanied by a concomitant enlargement of the nucleoid (Fig 5.2.3A). The nucleoid area increased by \( \sim 10 \) per cent over \( \sim 50 \) mins, despite the \( \sim 10 \) per cent reduction in the cytoplasmic volume during the same interval. As a result, the fraction of the cytoplasmic area occupied by the nucleoid increased substantially (by \( \sim 20 \) per cent; Fig 5.2.3A), as could also be confirmed by visual inspection (Fig 5.2.3B).

In summary, under sustained hypoosmotic conditions we observed a gradual decrease of cytoplasmic crowding, and a concomitant expansion of the nucleoid. The changes in the observables were smaller in amplitude and slower compared to those upon hyperosmotic shock, but nevertheless are statistically significant (Fig. 5.2.3A). The decrease in crowding occurs despite a reduction in cytoplasmic volume during the same interval, and likely reflects a shift in the balance between growth of the cytoplasm and that of macromolecules that act as crowders.
Fig. 5.2.3 **Response of the cell and nucleoid to hypoosmotic shock.** (A) Time series of average cell area, FRET, nucleoid area and area fraction (nucleoid area divided by cell area) upon a sustained hypo-osmotic stimulus (exchange of growth medium by dH$_2$O). The shock arrives at time 0 (red dashed vertical line). Shaded regions correspond to mean ± s.e.m. For comparison, the average single-cell time series for the hypoosmotic shock experiment of Fig. 5.2.2 are also shown (in lighter shades of the same color). For all observables changes with respect to steady state mean, normalized by the steady state mean, are shown (*i.e.* we plot $\langle \Delta x \rangle / \langle x \rangle_0$ for an observable $x$ with steady-state mean $\langle x \rangle_0$). Asterisks indicate time points from which the values for Figure 5.2.4B were taken. (B) Images of representative cells before and after sustained hypo-osmotic stimulation showing (from top to bottom) background-subtracted phase contrast, YFP channel from the FRET sensor, HU::mRuby2 signal from the nucleoid and the latter overlaid to the cell body image at the top.
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5.2.3 Crowding plays an important role in setting the steady-state nucleoid size in vivo

We summarize in Figure 5.2.4 our observations on the relationship between crowding and nucleoid size in the hyper- and hypo-osmotic shock experiments of Figs. 5.2.2 and 5.2.3, using data from time points at which we observed the maximum change relative to the pre-stimulus steady state (the time of the peak response in Fig. 5.2.2 and the time indicated by asterisks in 5.2.3). Although both hyper- and hypo-osmotic shocks induced a reduction in the cytoplasmic volume (Fig. 5.2.4A, upper left panel), the fraction of that volume occupied by the nucleoid decreases upon hyperosmotic shock and increases during sustained hypooosmotic stimulation (Fig. 5.2.4A, lower right panel). Furthermore, hyper- and hypo-osmotic stimulation exerted opposite effects on crowding (Fig. 5.2.4A, upper right panel) and nucleoid size (Fig. 5.2.4A, lower left panel).

A

Fig. 5.2.4 Crowding and nucleoid size can be modulated by both hyper- and hypo-osmotic shock. (A) Distributions of cell area, FRET ratio, nucleoid area and nucleoid area fraction are shown for different osmotic conditions: unperturbed (blue) peak response after the 0.6 M sucrose hyper-osmotic shock of the experiment of Fig. 5.2.2 (purple) and at under sustained hypo-osmotic shock (magenta). Data for the hypo-osmotic case correspond to the time point marked by the asterisks in Fig. 5.2.3A. For cell area, FRET ratio and nucleoid area, single-cell statistics were used. For nucleoid area fraction, growth-channel-level averages were used. (B) Average changes in FRET (green) and nucleoid area (dark red) for hyper- and hypo-osmotic shock, normalized to the unperturbed values \( \frac{\Delta x}{x_0} \). Upper panel: representative images show typical cells in each condition over which the average was performed to compute the values reported in the plot. Lower panel: Error bars represent standard error of the mean computed over the single-cell statistics.
Although these histograms demonstrate that there exists considerable variability in each of these observables at the single-cell level, the changes of opposing sign in both nucleoid size and crowding upon hypo- and hyper-osmotic stimulation are clearly statistically significant (Fig. 5.2.4B).

The fact that the cytoplasmic volume fraction occupied by the nucleoid can increase or decrease while the cytoplasmic volume itself decreases indicates that changes in confinement by the cytoplasmic membrane are not the primary driver of nucleoid size changes upon osmotic shocks. By contrast, nucleoid size is clearly anticorrelated with the crowding FRET signal, upon both hyperosmotic and hypoosmotic shock. The fact that both upward and downward perturbations to crowding affect nucleoid size supports the idea that crowding plays a significant role in shaping nucleoid configuration in living cells during steady-state growth.

5.2.4 Magnitude of nucleoid-size responses to hyperosmotic shock are comparable at different growth rates

The morphology and cellular volume fraction of the *E. coli* nucleoid are known to depend on growth conditions [84]. To examine the role of crowding in the contrasting nucleoid organization under different growth conditions, we measured the response of the cell and nucleoid to osmotic shocks of varying magnitude in under fast- and slow-growth conditions (with doubling time $\sim 20$ min and $\sim 45$ min, respectively; see Methods for media composition).

Prior to osmotic stimulation, the cytoplasmic area was significantly larger under fast growth compared to the slow growth condition (mean±s.e.m. = 2.47±0.01 vs. 1.93±0.01 $\mu$m$^2$; Fig. 5.2.5A). The nucleoid area, however, was very similar (1.01±0.003 vs. 1.03±0.01 $\mu$m$^2$; Fig. 5.2.5B), thus yielding a similar fold-difference in the nucleoid area fraction but of opposing sign (0.55±0.002 vs. 0.62±0.002; Fig. 5.2.5C) comparable to that of the cytoplasm alone. The observed distributions of the FRET crowding signal (Fig. 5.2.5C) indicates an apparent difference between the two growth conditions, but interpreting this absolute difference between growth conditions requires care as a rigorous comparison would require additional calibration of the FRET sensor within our experimental setup to make the absolute level of the signal interpretable. We thus focus hereafter on the relative change in FRET signal within each growth condition, and defer discussion of this apparent difference in the steady-state FRET level to the Discussion section.

We tested responses to osmotic shocks of varying magnitude under both fast and slow growth conditions, applying the same protocol as that of the experiment in Fig. 5.2.2,
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Fig. 5.2.5 **Steady-state distribution of observables under fast and slow growth conditions.** Distributions of (A) cell area, (B) nucleoid area, and (C) the fraction of cell area occupied by the nucleoid, and (D) the crowding FRET signal (YFP/CFP fluorescence ratio). In all panels, blue corresponds to the fast growth condition, and light blue to the slow growth condition.

but with varying sucrose concentrations. The qualitative features of the time series were similar across all tested shock magnitudes (Fig.5.2.6A), with the cell and nucleoid areas initially decreasing while the crowding FRET signal increased, before reaching a peak value from which each observable recovered towards its steady-state value. The magnitude of fractional response at the response peak, which is likely determined by the balance between the passive response to the shock and the active processes that account for the subsequent recovery, increased monotonically with the applied shock magnitude, and the dependence on the shock magnitude was nearly linear in all three observables (Fig.5.2.6B), indicating that the shocks we applied are far from saturating the response.

Interestingly, the peak fractional change in nucleoid size at each tested sucrose shock magnitude (Fig.5.2.6B, lower panel) was very similar between the two growth conditions, despite the steady-state differences in cell size and nucleoid fraction noted above. The dependence of the peak change in crowding FRET (Fig.5.2.6B, middle panel) on the shock magnitude was also similar within experimental error, although as noted above, quantitative comparisons of the FRET signal across different growth conditions requires caution.
By contrast, the peak cytoplasmic volume response demonstrated significant differences in magnitude between the two growth conditions, with the fast-growing cells demonstrating a greater change in size compared to slow-growing cells.

5.2.5 Both crowding and nucleoid dynamics demonstrate hysteresis across hyperosmotic shock response and recovery

Another notable feature of the time series data of Fig. 5.2.6 is that the times at which the change in the observables reach their peak value generally do not coincide. In particular, at larger shock magnitudes, the peak time of the nucleoid-size response tends to be delayed relative to those for the cytoplasmic volume and the crowding FRET signal. The fact that the nucleoid can continue to decrease in size even after cytoplasmic volume in crowding begin...
their recovery indicates that the nucleoid, though affected by it, is not in equilibrium with the crowding state of the cell.

To investigate further this disequilibrium between crowding and nucleoid size, we examined the time series of Fig.5.2.6 as trajectories in the phase space of observables. Projections of these trajectories in the nucleoid size-crowding plane (Fig.5.2.7, upper two rows) demonstrate considerable hysteresis; within each trajectory, segments corresponding to the response phase (orange curve) and recovery phase (gray curve) do not overlap. Furthermore, characteristic loops were observed around the response peak for experiments with the largest shock magnitudes (0.6 and 0.8 M sucrose). This looped profile reflects both the delay in the onset of recovery for the nucleoid relative to crowding at early times, as well as the faster recovery of the nucleoid at later times.

By contrast, projections in the cytoplasmic volume-crowding plane (Fig.5.2.7, lower two rows) demonstrate that the peak response of these two observables do coincide in time across all tested shock magnitudes. Nevertheless, hysteresis was observed in the relationship between cytoplasmic volume and crowding at larger shock magnitudes (0.6 and 0.8 M sucrose), with the crowding FRET signal recovering more slowly than the cytoplasmic volume. The trajectories in this plane did not form loops, however, reflecting that the recovery of the FRET signal remains slower than that of the cytoplasmic volume throughout the recovery trajectory. We mention in passing that this threshold for hysteresis in the cytoplasmic volume-crowding relationship (between 0.4 and 0.6 M sucrose) approximately coincides with that above which plasmolysis events become noticeable. Although we have not undertaken a systematic analysis of plasmolysis frequency here, one can speculate that the recovery from plasmolysis could involve additional active mechanisms not deployed upon the recovery from milder osmotic shocks and hence contribute to the observed disequilibrium at large osmotic shocks.

Finally, cytoplasmic volume-crowding trajectories (Fig.5.2.7, lower two rows) also indicate that the cytoplasmic volume typically did not overshoot its initial level by large margins, if at all, and their endpoints tended to coincide with their origin. A notable exception is the experiment with 0.8M sucrose under fast growth (Fig.5.2.7, third row, rightmost panel), where the trajectory does give the impression of a significant overshoot. However, inspection of the time series revealed that this could be due to incomplete relaxation at the end of the finite duration of the experiment (see Discussion). By contrast, nucleoid size-crowding trajectories (Fig.5.2.7, upper two rows) typically overshoot by a considerable margin, and in most cases did not recover to their origin. Thus, changes in cytoplasmic volume alone can not explain the dynamics of crowding, and changes in crowding alone can not explain the dynamics of the nucleoid. These apparently irreversible/hysteretic trajectories demonstrate...
that both the nucleoid and cytoplasmic volume are not rapidly equilibrated with the crowding state of the cell, and indicate that their dynamics involve active processes.

Fig. 5.2.7 Phase-space trajectories following hyperosmotic shock reveal disequilibrium between cytoplasmic volume, crowding, and nucleoid size. Projections of the phase space trajectories on to the nucleoid area-crowding plane (upper two rows) and cytoplasmic area-crowding plane (lower two rows). Each panel corresponds to a representative experiment for the indicated shock size (columns) and growth condition (rows). The coordinates correspond to fractional changes from the pre-shock steady-state mean. Segments of the trajectory preceding the crowding (FRET) response peak (collapse phase) are indicated by orange curves, and segments thereafter (recovery phase) by gray curves. Shaded regions indicate the 70% contours of single-cell distributions, in orange for the collapse phase, and blue (fast growth) or light blue (slow growth) for the recovery phase.
5.2.6  **Nucleoid collapse, but not recovery, upon hyperosmotic shock follows a stereotyped trajectory**

Overlaying the phase space trajectories of Fig. 5.2.7 in the nucleoid size-crowding plane revealed that trajectory segments corresponding to the initial collapse phase (orange), but not the subsequent recovery phase (gray), follow a simple pattern (Fig. 5.2.8). The collapse-phase paths for all shock magnitudes fall on top of one another, and are well approximated by a straight line. This is consistent with the approximate linearity of the dose-response relations of both the FRET crowding signal and nucleoid size against osmotic stimuli observed in Fig. 5.2.6B. Because those dose response relations address the peak response at the onset of osmo-adaptive recovery, the (approximate) proportionality of their amplitude with respect to the applied shock size indicates that recovery begins within the linear regime of the initial collapse response upon hyperosmotic shock.

![Image](image.png)

**Fig. 5.2.8** **Relationship between crowding and nucleoid area following hyperosmotic shock is stereotyped during the collapse phase, but not during recovery phase.** Overlays of the nucleoid area-crowding trajectories (Fig. 5.2.7) for all shock magnitudes under fast (A) and slow (B) growth conditions. Color scheme of curves and shaded regions are as in Fig. 5.2.7. Symbols mark the beginning and of the collapse phase (red) and the final time point of the experiment (gray). Shape of symbols indicate the shock magnitude of the experiment, as in Fig. 5.2.9.

Remarkably, the slopes of these linear trajectory segments in the nucleoid size-crowding plane (Fig. 5.2.8, orange) are very similar not only across all tested shock magnitudes, but also between the fast and slow growth conditions (-3.75 ± 1.79 and -3.78 ± 0.97 for the fast and slow growth conditions, respectively). A parsimonious explanation for this stereotypy is
that crowding drives nucleoid collapse, and the manner in which it does so is similar under these contrasting growth conditions.

By contrast, trajectory segments corresponding to the recovery phase are far more diverse, following tortuous nonlinear paths that vary with both the applied shock magnitude and the growth conditions under which the cells experience the shock. The stark contrast between the recovery- and collapse-phase trajectories suggest that the mechanisms driving nucleoid compaction and recovery are different from one another.

5.2.7 Metabolic shutdown dilates the nucleoid independently of crowding and inhibits recovery from collapse

Whereas the highly stereotyped relationship between nucleoid size and crowding during the collapse phase upon hyperosmotic shock (Fig. 5.2.8) suggests that crowding is a key determinant of the compaction magnitude during the collapse phase, the distinct phase space trajectories during osmoadaptive recovery indicate the involvement of additional mechanisms other than crowding in regulating nucleoid size. Furthermore, given the hysteretic nature of the collapse-recovery cycle, one or more active processes must be involved in either the collapse phase, the recovery phase, or both. To directly test the role of active processes in nucleoid organization, we subjected cells to the drug 2,4-dinitrophenol (DNP), a membrane permeable protonophore that de-energizes cellular metabolism by uncoupling oxidative phosphorylation.

Treatment of fast-growing cells with DNP led to a dramatic decondensation of the nucleoid, which expanded by $\sim 35$ per cent in $\sim 10$ mins while cytoplasmic growth was arrested more rapidly, within $\sim 5$ mins (Fig. 5.2.9 A). Interestingly, the FRET crowding signal and the average cytoplasmic volume, remained nearly invariant throughout the course of this nucleoid dilation (Fig. 5.2.9 B), during which the fraction of cytoplasmic area occupied by the nucleoid increased from $\sim 51$ per cent to $\sim 67$ per cent. This observation confirms that crowding, despite playing an important role in determining the steady-state nucleoid organization (Fig. 5.2.4), is not the sole player in determining nucleoid configuration in living cells.

Subsequent application of a hyperosmotic shock (0.6 M sucrose) led to a large decrease in both the cytoplasmic volume and the nucleoid size, with a concomitant large increase in the FRET crowding signal. Given the shutdown of metabolic activity by DNP, these changes in cytoplasmic volume and nucleoid size represent a response to osmotic shock in the absence of active processes that require dissipation of metabolic energy.
5.2 Results

Fig. 5.2.9 Effect of metabolic shutdown on nucleoid size and hyperosmotic shock response. (A) Image sequences of the nucleoid (upper row, mRuby channel) and cytoplasm (lower row, phase-contrast channel) across time during DNP treatment and subsequent hyperosmotic shock. Top panel shows the medium exchange protocol of the experiment, in which 2mM DNP and 0.6mM sucrose were added at the indicated times. Time axis is shared with panel B. (B) FRET, normalized nucleoid and cell area time traces. Color code is indicated in legend. Shaded areas represent mean ± s.e.m. (C) Comparison of trajectories in the nucleoid size-crowding phase space for the same osmotic shock magnitude (0.6M sucrose) in the presence (dark green) and absence (blue) of 2mM DNP. Color code and shaded regions are as in Figs. 5.2.7 and 5.2.8, except for the color of shaded regions for DNP experiment, which is given in legend. Orange curves indicate the collapse phase of the shock, black curves indicate the recovery phase. Green curve corresponds to the expansion phase of nucleoid under the effect of 2mM DNP, prior to shock. The dashed line is a fit to the collapse phase of the no-DNP experiment, showing that the collapsed phase in the presence of DNP lies very close to the extension of the collapse-phase dynamics in the absence of DNP.
These passive responses in the cytoplasmic and nucleoid areas led to decreases of $\sim 34$ per cent and $\sim 84$ per cent, respectively, corresponding to a $\sim 15$ per cent decrease in the area fraction occupied by the nucleoid. Upon sustained shock, we did not observe recovery of any of our observables (cytoplasm area, FRET crowding signal, and nucleoid size), confirming that recovery from osmotic shock is an active process.

Net fractional changes in the nucleoid size and crowding level reached upon sustained shock in the presence of DNP exceeded the peak changes observed upon the same 0.6M shock applied in the absence of DNP (Fig. 5.2.9C), with the nucleoid area decreasing by $\sim 35$ percent and the crowding FRET signal increasing by $\sim 10$ per cent. Interestingly, this terminal phase-space coordinate of the passive response of DNP-treated cells lies very close to the extension of the phase-space trajectory of the collapse phase of DNP-untreated cells (Fig. 5.2.9C, dashed line). This observation lends further support to the idea that nucleoid dynamics during the collapse phase of hyperosmotic responses (Figs. 5.2.7, 5.2.8) largely reflect the passive response to changes in crowding.

In summary, examining nucleoid dynamics upon DNP-induced metabolic shutdown provided clear evidence that not only crowding, but also additional active processes determine the steady-state nucleoid size in growing \textit{E. coli} cells. Furthermore, neither cytoplasmic volume nor nucleoid size recovered following hyperosmotic compression/condensation in these metabolically arrested cells, thus demonstrating that osmoadaptive recovery requires dissipation of metabolic energy. The observed extent of nucleoid size and crowding changes are compatible with the hypothesis that nucleoid collapse upon hyperosmotic shock is dominated by the passive response to crowding changes in the cytoplasm.

### 5.3 Discussion

By combining fluorescence imaging, microfluidics, and a FRET sensor of macromolecular crowding, we have carried out a quantitative experimental investigation of the effect of crowding on nucleoid spatial organization within growing \textit{E. coli} cells. Although the effects of crowding on nucleoid organization has been studied both theoretically and through \textit{in vitro} experiments, to our knowledge this is the first systematic investigation of crowding effects on the bacterial nucleoid \textit{in vivo}.

**Crowding plays a significant role in nucleoid spatial organization \textit{in vivo}**

From a polymer physics point of view, it is non-trivial that the nucleoid occupies only a fraction of the available cellular volume. The most entropically favorable configuration of an ideal confined polymer with a radius of gyration larger than the confining region is one that
fills the available volume, and based on *in vitro* experiments, it has been estimated that the *in vivo* compacted state of the *E. coli* nucleoid stores about $10^5 k_B T$ of mechanical energy [43]. Theoretical and experimental results of previous studies have identified four main classes of mechanisms that could contribute to this remarkable degree of compaction within living cells: (1) confinement by the plasma membrane [290, 291, 43], (2) macromolecular crowding in the cytoplasm [44, 292, 43], (3) the activity of DNA associated proteins (RNA polymerase, ribosomes, NAPs and SMCs) [69, 293–295, 273], and (4) supercoiling of chromosomal DNA [44, 292].

Confinement was suggested to be an important factor for nucleoid organization, by limiting the number of possible configurations the chromosomal DNA polymer can assume. It has been proposed by means of theoretical arguments and numerical simulations that confinement could promote segregation and that a confined polymer can assume a nucleoid-like configuration with appropriate choice of parameters [290, 296]. We found that during nucleoid collapse, both confinement and crowding increase but that the ratio of nucleoid size over cell size decreases significantly. This suggests that it is crowding rather than confinement that induces the collapse of the nucleoid. The possibility that crowding can induce nucleoid collapse is also supported by previous *in vitro* results [44, 43, 295] where the size of isolated nucleoids were found to be modulated by tuning the concentration of artificial crowders.

Our results show a clear negative correlation between the crowding FRET signal and nucleoid size, both when crowding is increased rapidly by hyperosmotic shock, and when it is decreased more gradually under sustained hypoosmotic conditions. For the case of hyperosmotic shock, the causal relationships connecting the osmotic upshift and the increase in the crowding FRET signal is straightforward: the cell volume $V$ decreases as water flows out to balance the increased extracellular osmolarity, leading to an increase in the macromolecular volume fraction \( \phi = \frac{V_m}{V} \) which in turn reduces the average distance between the donor and acceptor domains of the FRET sensor (and hence increases FRET [54]). The causal sequence of events is less obvious for the hypoosmotic perturbation, as we observed a slow decrease, rather than increase, in the average cell size upon the reduction in extracellular osmolarity, yet still observed a decrease in the crowding FRET signal. These hypoosmotic results are counterintuitive at first, but could be explained by considering the net balance between the rates of cytoplasmic growth, cell division, and macromolecule production. The decrease in the crowding FRET signal under the hypoosmotic (dH$_2$O) environment implies a decrease in the average macromolecular volume fraction \( (d\langle \phi \rangle/dt = d\langle V_m/V \rangle/dt < 0) \). Given that the cytoplasmic volume also decreased during the same interval \( (d\ln\langle V \rangle/dt < 0) \), the average volume occupied by macromolecules must also have decreased, and at a faster rate than that of the cytoplasmic volume \( (d\ln\langle V_m \rangle/dt < d\ln\langle V \rangle/dt < 0) \). Thus, the negative
correlation between crowding and nucleoid size holds both for the hypo- and hyper-osmotic conditions, strongly suggesting the importance of crowding in the steady-state compaction of the nucleoid in vivo.

The role of active processes in maintaining the nucleoid’s steady state and osmoadaptive recovery

In addition to macromolecular crowding — a set of passive (equilibrium) effects that manifest at high macromolecular volume fraction $\phi$ — our data also demonstrate the importance of active (non-equilibrium) processes in shaping nucleoid spatial organization and dynamics in vivo. Depression of metabolic activity in fast-growing cells via the membrane potential uncoupler DNP led to a dramatic increase in the nucleoid volume while the cytoplasmic volume and crowding FRET signal remained invariant (Fig. 5.2.9). These observations clearly demonstrate that active processes dependent on the availability of metabolic energy contribute to the steady-state nucleoid compaction in growing cells. The fact that the crowding FRET signal remained invariant during this shutdown-induced nucleoid expansion further suggests that the manner in which these active processes contribute to the steady-state nucleoid compaction is orthogonal to that of crowding. Interestingly, Joyner et al. recently reported a small, but significant volume reduction in E. coli cells treated with DNP[297] and speculated that crowding might thus be affected by metabolic shutdown. This contrasts with our observation (Fig. 5.2.9B) that the cell size remains invariant under DNP treatment. The reason for this apparent discrepancy remains unclear at this time, but we note that the experiment of Joyner et al. differs in many ways from our own (strain, growth conditions, measurement techniques), including the duration of the DNP exposure (30 minutes), which in that work was more than twice as long as that in our experiment. Thus, it remains possible that a similar reduction would be observable under our experimental setup upon longer exposure to DNP, but the results presented here nevertheless indicate that a change in nucleoid size can be induced by DNP treatment in the absence of changes in cell size or crowding. Decondensation of the nucleoid upon DNP treatment had been noted in a previous study [151], in which the authors’ focus was the effects of metabolic shutdown not on the nucleoid but rather the physical state of the cytoplasm — the mobility of large particles (protein aggregates of length $\sim$ 900 nm) diffusing in the cytoplasm was found to be strongly diminished upon DNP treatment. The invariance of the FRET signal in our experiments upon DNP treatment suggests that the DNP-induced changes in the cytoplasm reported in ref. [151] do not affect macromolecular crowding effects felt by molecules of more moderate size (the FRET sensor used here measures $< 10$ nm in length).
Active cellular processes reported to affect the nucleoid configuration include transcription by RNA polymerase (RNAP) [298, 69, 83], cotranscriptional translation and membrane insertion of transmembrane proteins (transertion) [293, 278], as well as ATP-hydrolyzing structural maintenance of chromosome (SMC) proteins, such as the MukBEF complex [63, 273]. Interestingly, while some of these active processes (RNAPs and SMC proteins) contribute to compaction of the nucleoid, others (transertion) contribute to its expansion. The increased nucleoid volume we observed upon DNP treatment during fast growth (Fig. 5.2.9) thus indicates that the net balance among forces exerted by these active processes during steady growth favors nucleoid compaction, at least under the experimental conditions tested here.

Effects of active processes were also apparent in the nucleoid dynamics following hyperosmotic shock (Figs. 5.2.7, 5.2.8). The relationship between the crowding FRET signal and nucleoid size contrasted strongly between the initial collapse phase of the response and the subsequent recovery phase, demonstrating considerable hysteresis (Fig. 5.2.7). Although hysteresis could in principle arise from the involvement of active processes in the collapse phase, recovery phase, or both, the highly stereotyped trajectories of the collapse dynamics (Fig. 5.2.8), and the absence of the recovery phase upon metabolic shutdown (Fig. 5.2.9) motivate the interpretation that active processes contribute primarily to osmoadaptive recovery, and not to the initial collapse response.

What cellular processes could be responsible for this active osmoadaptation of nucleoid size? Osmoadaptation of the cytoplasmic volume involves both passive and active processes, with an initial rapid volume recovery triggered by passive potassium ion (K\(^+\)) influx through constitutively expressed potassium channels, and subsequent recovery requiring active synthesis of additional transporters and biosynthetic enzymes that eventually replace the increased K\(^+\) with organic osmoprotectants such as glycine betaine, proline, and trehalose [299–301]. More specifically for the nucleoid, Cagliero et al. [69] found by imaging cells chemically fixed at various times after osmotic shock that the initial collapse of the nucleoid upon hyperosmotic shock is accompanied by dissociation of RNAPs from the nucleoid (possibly as a result of the transient increase in cytoplasmic K\(^+\) concentration), and that the dissociated RNAPs return to the nucleoid during the osmoadaptive recovery phase. Although RNAP activity is generally considered to contribute to nucleoid condensation [69, 83], it is also involved in transertion, and thus could contribute to nucleoid expansion [293, 278], especially if expression of transmembrane proteins were augmented during the recovery phase. In the future, it will be interesting to further dissect the interplay between these active processes and crowding effects on nucleoid compaction, by extending the experiments presented here to include perturbations to the osmoadaptation pathways (e.g. by removing environmental...
K\(^+\), or deleting genes involved in the transport/synthesis of organic osmoprotectants), as well as other active processes such as transcription and translation using drugs that specifically target each process (e.g. rifampicin for transcription, chloramphenicol for translation).

**The dependence of crowding and nucleoid configuration on growth conditions**

In agreement with previous reports \[84\], we found that the fraction of cytoplasmic space occupied by the nucleoid differs between cells growing at fast and slow rates, with fast growing cells exhibiting a lower area fraction occupied by the nucleoid (Fig. 5.2.5C). The absolute area occupied by the nucleoid was remarkably similar between the two growth conditions (Fig. 5.2.5B), but considering also that faster growing cells contain more chromosomal DNA [302], we conclude that the degree of nucleoid compaction is higher in fast growing cells. The mechanistic determinants of this difference remain unknown, but likely involve the activity of both active (RNAPs, ribosomes, SMCs) and passive (NAPs, crowding) molecular machinery. Interestingly, we observed a higher level of the crowding FRET signal in slow-growing cells (Fig. 5.2.5D); the mean (±s.e.m.) values for this ratiometric signal (YFP/CFP) were 0.70±0.001 and. 0.88±0.002 for the fast and slow growth conditions, respectively. As noted in Results, interpretation of the absolute level of the FRET signal in our experiments requires caution as the sensor has not been calibrated within our experimental setup. It will be of interest to confirm with future experiments with calibration (using appropriate controls of cellular background fluorescence and purified FRET sensor) whether this represents a true difference in the degree of cytoplasmic crowding, but we note here that the trends we observe in the fractional changes to the various observables upon osmotic shock (Fig. 5.2.6B) could in principle be explained by a higher level of crowding in slow growing cells (though other interpretations are also possible).

Consider a bacterium immersed in a medium of osmotic pressure \(p\). The cytoplasm is turgid, that is, the internal osmotic pressure of the cytoplasm \(p^*\) is greater than that of the environment \((p^* > p)\), leading to a positive turgor pressure \(\Delta p = p^* - p\). When the external osmotic pressure exceeds that of the cell interior \((p > p^*)\), the cell loses turgidity \((\Delta p \to 0)\) and the cytoplasmic volume \(V\) decreases according to a conservation law for osmotic work \(p(V - V_m) = p^*(V^* - V_m)\). Here the difference \(V - V_m\) is the osmotically active volume. Rearranging, and writing in terms of the macromolecular volume fraction \(\phi = V_m/V\), we have

\[
\frac{V}{V^*} = \frac{1 - \phi^*}{\phi^*} \frac{p^*}{p} + \phi^*,
\]

where \(V^*\) and \(\phi^*\) are the cytoplasmic volume and the macromolecular volume fraction, respectively, at the isoosmotic point \(p = p^*\). Eq. 5.1 (or close variants thereof) has been
found to fit well the cytoplasmic volume response to osmotic shock for \( p > p^* \) in a number of previous studies [303, 304], and implies that the dependence of cytoplasmic volume on external osmotic pressure

\[
\frac{\partial \ln V}{\partial p} \bigg|_{p=p^*} = -(1 - \phi^*)/p^*
\]  

(5.2)

will become steeper if either the initial level of crowding \( \phi^* \), the initial internal osmotic pressure \( p^* \), or both are reduced. The data of (Fig. 5.2.6B, upper panel) does indeed indicate a steeper dependence of the fractional change in cytoplasmic volume on the change in external osmolarity \((\Delta V/V)/\Delta p \approx \partial \ln V/\partial p)\) for the fast growth condition, for which the data of Fig. 5.2.5D indicate a lower degree of crowding. Thus, the observed difference in the slope of the cytoplasmic volume response \((\Delta V/V)/\Delta p\) is at least qualitatively consistent with a difference in the crowding parameter \( \phi \) although we can not exclude the possibility that it is instead due to differences in the internal osmotic pressure \( p^* \).

On the other hand, the observed response in nucleoid size to jumps in external osmolarity (Fig. 5.2.6B, lower panel) was very similar between the fast and slow growth conditions. Combined with the observation that the cytoplasmic volume decreases more rapidly with external osmolarity for the fast growth condition (Fig. 5.2.6B, upper panel), this implies a greater fractional change in nucleoid size per fractional change in cytoplasmic volume for the slow growth condition. The latter trend could also be explained, in principle, by a higher steady-state level of crowding \( \phi \) for the slow growth condition, because the strength \( f_{\text{dep}} \) of the depletion interaction driving nucleoid compaction is, to leading order, proportional to the crowder volume fraction \( \phi = V_m/V: f_{\text{dep}}/k_B T \approx (V_{\text{excl}}/v_m)(V_m/V) \) where \( V_{\text{excl}} \) is the excluded volume around the DNA (modeled as a cylinder) and \( v_m \) is the volume of individual crowder molecules [37, 292]. This means the dependence of the depletion interaction on fractional changes to the cytoplasmic volume,

\[
\frac{\partial f_{\text{dep}}}{\partial \ln V} \bigg|_{V=V^*} \approx -\phi^*V_{\text{excl}}/v_m,
\]  

(5.3)

is proportional to the steady-state crowder volume fraction \( \phi^* \). Thus, the slow-growth condition’s steeper nucleoid-size response to changes in cytoplasmic volume (Fig. 5.2.6B, upper panel) is again compatible with its higher crowding FRET signal level (Fig. 5.2.5D), although we can not rule out the possibility that the steeper response rather reflects a difference in the typical size of crowder molecules \( v_m \) between the two growth conditions, which could arise from differences in proteome composition at different growth rates.
Outlook and future directions

By high-throughput imaging at the single-cell level under a variety of environmental perturbations, we investigated the role of macromolecular crowding in nucleoid spatial organization \textit{in vivo}. Our results strongly suggest a significant role of crowding in determining the steady-state level of nucleoid compaction under steady growth conditions, and open the way to a variety of future studies on the complex interplay between nucleoid spatial organization and cellular physiology. In the future, it will be exciting to extend the experimental approach presented here to further dissect both the causes and consequences of nucleoid spatial organization. We conclude by highlighting a few particularly promising directions for future work.

NAPs are expected to drastically affect nucleoid spatial organization through multiple effects. On the one hand, they can enhance the response to crowding changes by increasing the effective cross-sectional area of the DNA polymer. On the other hand, NAPs can also counter crowding effects by crosslinking chromosomal DNA segments at multiple sites. Although we used a fluorescently labeled NAP (HU::mRuby2) to image the nucleoid, our experiments did not address the role of NAPs in determining the \textit{in vivo} configuration of the nucleoid. By carrying out experiments similar to those presented here in mutants of specific NAPs, such as H-NS, HU or Fis. By comparing steady-state and osmotically perturbed values of nucleoid size and measuring the spatial distribution of such proteins in mutant and wild type strains, future experiments could clarify whether and how NAPs contribute to nucleoid organization under both physiological and perturbed conditions.

Similarly, extending the approach of our experiment to mutants of active proteins that contribute to nucleoid organization, such as SMCs, RNAPs and ribosomes, would be both exciting and informative in dissecting the role of active processes in steady-state nucleoid compaction, as well as in the adaptation following osmotic shocks.

Another important set of experiments that could be carried out in this context are osmotic upshifts delivered in a potassium-free medium. It is well established that potassium (K$^+$) import is, together with the slower timescale glycine import, the main process responsible for the initial cell size recovery upon sustained osmotic shocks [288, 299, 304, 305]. When osmotic shock is applied through a potassium-free medium, cells are not able to recover from plasmolysis, and a previous study has indicated that the absence of potassium in the medium also reduces of the effects of osmotic shock on the nucleoid [305]. By comparing the degree of nucleoid compression after osmotic shock between potassium-free and potassium-containing media, one could quantify the effects of the transient K$^+$ influx at the onset of osmoadaptation. This would help disentangle the effect of the crowding perturbation from
those associated with the active nucleoid expansion during sustained osmotic upshifts in metabolically active cells.

To better understand the dependence of nucleoid organization on growth physiology, it will be instrumental to calibrate within our experimental setup the FRET sensor against in vitro measurements of crowding (using e.g. Ficoll) and taking into account the growth-rate dependent cellular background fluorescence. This will allow more meaningful comparisons of the absolute level of the crowding FRET signal between different growth conditions, and help resolve whether growth-condition dependent differences in cytoplasmic and nucleoid osmotic responses are due to crowding or other factors (see discussion in above).

Finally, simply expanding the range of osmotic shock magnitudes tested in the experiment will be highly informative. Due to the relatively mild osmotic shocks applied here, the nucleoid responses observed in the present study appear to be within (or at least not far from) the linear regime of the response (Fig. 5.2.6B). The full dynamics of disordered polymers are nearly always characterized by nonlinear functions (often power laws) [68], and in the case of the nucleoid, it remains an open question whether the collapse we observed in vivo upon osmotic shock represents a continuous [44] or discontinuous [43, 306] transition. Testing the osmotic response over a broader range of shock magnitudes would also help estimate the steady-state osmotic pressure of the cytoplasm (by fitting Eq. 5.1 to changes in the cytoplasmic volume, $V$). Future studies that combine a larger range of osmotic shock magnitudes with FRET calibration and the expanded repertoire of mutant strains described above would undoubtedly help to dissect further the roles of the multiple factors (passive and active DNA-binding proteins, osmoadaptation, growth physiology, and crowding) in shaping nucleoid spatial organization and dynamics in vivo.

### 5.4 Materials and methods

#### 5.4.1 Bacterial strains and plasmids.

The strains and plasmids used in this study are listed in Table 5.1 and 5.2.
### Table 5.1 Plasmids used in this study

<table>
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<tr>
<th>Name</th>
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<th>Gene(s)</th>
<th>Resistance</th>
<th>Induction</th>
<th>Source</th>
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<tbody>
<tr>
<td>n/a</td>
<td>pRSET-A</td>
<td>FRET crowding sensor</td>
<td>Amp</td>
<td>IPTG</td>
<td>B. Poolman [54]</td>
</tr>
<tr>
<td>pSJAB159</td>
<td>pTrc99Aα*</td>
<td>FRET crowding sensor</td>
<td>Amp</td>
<td>IPTG</td>
<td>This study</td>
</tr>
</tbody>
</table>

*pTrc99Aα* is a modified version of pTrc99A. In this version the plasmid-borne start codon is deliberately mutated to ATG for cloning the native RBS of the protein of interest. This way no unintended translation occurs from the RBS that comes with the vector.

### Table 5.2 Strains used in this study

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Genotype</th>
<th>Parent</th>
<th>Source</th>
<th>Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJ540</td>
<td>hupA::mRuby2, (native promoter),</td>
<td>MG1655</td>
<td>Suckjoon Jun</td>
<td>-</td>
</tr>
<tr>
<td>TSS1961</td>
<td>SJ540 transformed with crowding sensor plasmid</td>
<td>MG1655</td>
<td>this study</td>
<td>pSJAB159</td>
</tr>
</tbody>
</table>

### 5.4.2 Growth conditions

In this study we used two types of growth media which were both based on the AB medium described for the first time in [307] (solution A: 2.0 g \((NH_4)_2SO_4\), 6.0 g \(Na_2HPO_4\), 3.0 g \(KH_2PO_4\), 3.0 g \(NaCl\), 0.011 g \(Na_2SO_4\), dissolved in 200 ml. of water. Solution B: 0.2 g
**5.4 Materials and methods**

*MgCl*$_2$, 0.010 g *CaCl*$_2$ and 0.0005 g *FeCl*$_3$, 7*H*$_2$O dissolved in 800 mL water. A and B are mixed after autoclaving by pouring A into B. No precipitate is formed. To achieve fast growth conditions with a doubling time of about 25 minutes at 37°C, we supplemented the AB medium with EZ (teknova 5X Supplement EZ), all 4 nucleotides (10X ACGU solution, teknova) to a final concentration of 0.02mM and 0.5% glycerol (which does not interfere with our IPTG inducing system). For achieving slow growth with an average doubling time of 45 minutes we supplemented AB with 0.5% glycerol and 6 amino acids (methionine, histidine, arginine, proline, threonine and tryptophan, final concentration is 0.5mg/ml for each amino acid).

For both slow and fast conditions, cells were grown overnight in the respective medium. The morning after, the saturated culture was diluted 1:10 into fresh medium and cultured for about 4hrs. Cells were then harvested and concentrated ∼10 times via centrifugation at 10000rpm for 1 minute (Eppendorf Centrifuge 5424) prior to loading them in a mother machine device (see Sample Preparation section below).

### 5.4.3 Overview of experimental setup and measurement strategy.

Our study required simultaneous monitoring of nucleoid size, intracellular crowding and cell size and a means to perturb intracellular crowding in growing *E. coli* cells. To implement step-like perturbations in time, we also needed a way to rapidly exchange the medium in the environment of growing cells. To meet these requirements, we made use of a PDMS-based "mother machine" microfluidic device, which enables microscopic imaging of thousands of individual cells during steady-state growth [98].

For nucleoid-size readout we employed an *in situ* DNA label based on the nucleoid associated protein HU, which has been used widely for nucleoid labeling purposes and has been shown to colocalize with DNA-intercalating dyes such as DAPI [130, 129, 43, 60]. We made use of an MG1655 derivative strain bearing a genomic mRuby2 fusion to hupA (SJ540, see materials and methods section). In this strain the nucleoid is labeled with good contrast by the HU::mRuby2 fusion upon imaging live cells growing within the mother machine, as shown in Fig. 5.2.1.

To measure intracellular crowding, we made use of a FRET sensor recently developed by Boersma et al. [54]. The sensor consists of a clamp-like protein composed by two arms with a donor CFP (mCerulean) and acceptor YFP (mCitrine) fluorescent protein respectively on the tip of each of its arms. CFP acts as the donor for FRET while YFP is the acceptor. Under constant environments, the FRET signal (acceptor-to-donor fluorescence ratio; $I_{YFP}/I_{CFP}$) is stationary and its amplitude determined by the average distance between the CFP and the YFP domain. Increased crowding forces the two domains into closer spatial proximity,
resulting in an increased FRET signal. The sensor has been shown to robustly measure crowding changes *in vitro* under variation of different environmental conditions relevant for *in vivo* measurements (temperature, pH, NaCl and DNA) for a number of different crowders (Ficoll, PEG, sucrose), crowder sizes and sensor concentrations. The FRET signal increase is nearly proportional to the volume fraction of crowders \( \phi = V_m/V \) across the physiologically relevant range \( 0 < \phi < 0.3 \) and the sensor has been shown to be insensitive to osmoprotectants such as potassium glutamate and glycine betaine. Tests *in vivo* on bacterial cells under osmotic shock induced by increasing concentrations of sucrose, showed that the FRET signal increases nearly proportionally with the magnitude of the shock (for details see ref. [54]).

To monitor in parallel the size of the cells’ cytoplasm, the nucleoid as well as crowding, we developed a multichannel imaging system capable of phase contrast imaging in addition to fluorescence imaging in the relevant wavelength bands for mRuby2, CFP and YFP fluorophores. Excitation filters and dichroic mirrors were mounted on the motorized turret of an inverted fluorescence microscope (Nikon Ti-E), and emission filters on a motorized filter wheel (Nikon TI-FLBW-E) attached at the microscope’s emission output port. The motorized turret and filter wheel enable rapid switching between imaging channels, so that each field of view (FOV) could be imaged in all four channels (phase contrast, mRuby2, CFP and YFP) in \(< 2 \text{ s}\).

The microscope was placed in a temperature-controlled room (37°C) and a fast-switching (< 200 ms) emission filter wheel was positioned just before the camera for spectral separation of our imaging channels. Actual imaging time of our four channels sum up to about 0.7 s (fluorescence excitation is carried out with a multi-wavelength LED, Lumencor Spectra X) but switching both the excitation filter turret and the emission filter wheel takes on average about 0.2s per imaging channel so the time needed to image a field of view is about \(1.5\text{ s}\). Image acquisition (including excitation light pulses, filter switching, and translation of the motorized microscope stage) was automated using custom-written scripts in NIS-Elements software (Nikon Instruments, NIS-Elements Advanced Research). The script repeats the 4-channel imaging across a set of FOVs pre-selected by the user (in a typical experiment, ~ 30 FOVs) at regular time intervals. We observed minimal sample drift under these conditions when a time series are constructed from image sequences of each FOV. Since we image around 30 FOVs per experiment, our frame rate is limited by acquisition time. Moving the stage from one FOV to another takes about 1.5s which added to the acquisition time sum up to about 3 seconds per FOV. Thus to acquire images at one time point for 30 FOVs requires at minimum \( 3 \times 30 \text{ s} = 90 \text{ s} \). To leave some margin and prevent errors in the timing of the image acquisition, we set our inter-frame interval to \(120 \text{ s} \).
We chose to implement perturbations to crowding via sucrose hyper-osmotic shock which has been shown to perturb crowding in live E. coli cells [54]. As discussed in Chapter 3, the mother machine microfluidic device enables continuous imaging during steady state exponential growth at high-throughput (here 3000 cells per experiment), as well as rapid exchange of media for step-like environmental perturbations [98, 43].

A typical experiment comprises the following steps: (i) load cells in mother machine and incubate under constant temperature and nutrient flow to achieve steady exponential growth, (ii) image cell body morphology in phase contrast channel, (iii) image nucleoid morphology in red fluorescence emission channel, (iv) image FRET donor (cyan) and acceptor (yellow) fluorescence emission channels for crowding analysis, (v) repeat steps (ii)-(iv) to obtain statistics of observables during steady-state growth, (vi) exchange medium to apply osmotic shock or other perturbations to cellular parameters, (vii) repeat steps (ii)-(iv) to obtain statistics of dynamical response to the perturbation applied in (vi).

5.4.4 Microfluidic experiments in the mother machine

PDMS (SYLGARD 184, 10 g clip-pack) was mixed with cross-linker to a 10:1 ratio in weight for a total weight of 70g. The solution was stirred for about 5 minutes and poured onto the epoxy mold (SJ110 model from Prof. Suckjoon Jun’s lab) where the devices (12 per mold) were impressed. The mold with the PDMS was then baked for 8 hours at 60C. After that, the PDMS, which has a thickness of about 0.8cm at this stage, was detached from the mold via a metal cutter, single devices cut apart and holes were punched to create inlet and outlet ports at the two ends of the main channel (see Fig. 5.2.1). Single devices were then immersed in pure pentane and stirred continuously for 4 hours with a magnetic stirrer. After that, the devices were transferred into pure acetone and washed twice for 2 hours with continuous stirring. Subsequently the devices were left to dry in air and stored in plastic containers. We used the devices within two weeks after drying.

After devices were washed and dried, they were plasma treated (30W Harrick Plasma system) for 1 minute together with the Willco dish (WillcoWells, GWST-5040, 50X7mm, glass thickness 170 µm), promptly sticked to the dish and incubated at 60C for about 15 minutes to stimulate adhesion. To prepare the sample for microscopy, a concentrated bacterial solution (O.D. ∼ 2) from a day culture (see Growth Conditions section) was injected into the main channel by pipetting. When the bacterial suspension had filled the main channel, the Willco dish was secured by means of a custom holder into a custom centrifuge (based on a commercial fan rotor) taking care to place it such as to make the main microfluidic channel and the radius of the centrifuge perpendicular to each other. This way bacteria were pushed into the growth channels upon centrifugation. The centrifuge was run for 10 minutes at about
800rpm and in the meanwhile a microfluidic tubing (PTFE 24AWG .55mmID .30mm Wall 100ft, Zeus, SC, USA of about 50cm length was filled with fresh medium via a syringe with needle. After centrifugation, one of the holes punched earlier on the device was connected to the tubing filled with fresh medium (inlet) and the other to a piece (∼ 20 cm) for the waste (outlet). The inlet was then connected to a syringe driven by a pump (PHD ULTRA, Harvard Apparatus, MA, USA), which was loaded with fresh growth medium. The pump was turned on, set to a flow rate of 1.5ml/h and the bacteria were left to grow at 37°C while fresh medium was flowing in the main channel. When exponential growth was achieved (4 hours for fast growth condition and 6 for slow growth condition) about 10 frames for each imaging channel were acquired at 2-minutes interval under unperturbed conditions. After that the medium flowing in the mother machine was switched to apply the environmental perturbation (hyper-/hypo-osmotic shock, and/or DNP) by connecting the pump to a different syringe filled with the relevant medium. Phase contrast, YFP, CFP and mRuby images were then recorded at 2 minute s inter-frame interval in the presence of the environmental perturbation.

5.4.5 Microscopy

For our experiments with the mother machine, which were mainly carried out in San Diego at UCSD, we make use of a Nikon Ti-E inverted microscope, a 60X objective suitable for phase contrast imaging (Nikon CFI Plan Apochromat DM60x oil), a sCMOS camera (Andor Neo sCMOS 5.5), a LED (Lumencor Spectra X) for fluorescence excitation, multiband dichroic (Chroma 69008bs), multiband excitation filters (Chroma 69008x) and multiband emission filter (Chroma 69008m). LED illumination was coupled into the microscope via a fluorescence illumination attachment (Nikon Ti built-in LED or lamp coupling arm) to produce a homogeneous illumination pattern on the sample plane. Emission filters were mounted on a motorized filter wheel (Nikon TI-FLBW-E) placed in the emission path just before the camera while excitation filters and dichroics were mounted into standard filter cubes for Nikon Ti microscopes. The sample holder is mounted on a motorized stage (Nikon Ti built-in motorized stage). The exposure times for imaging in each imaging channel were 30ms for phase contrast, 50ms for YFP, 400ms for CFP and 200ms for mRuby. Camera exposure and LED illumination times were the same for all imaging channels and the LED power was kept to its maximum value. For achieving high throughput, the 4-channel image sequences were acquired in about 30 FOVs in each experiment. With the specifications of the current setup, each cycle of 30-FOV acquisitions could be completed within 2 minutes (see above).
5.4.6 Quantitative data analysis and image processing.

As discussed briefly in chapter 3, for all analyses described in this chapter we utilized Python code that was initially developed in the laboratory of Prof. Suckjoon Jun (UCSD), but expanded and adapted here for the specific needs of the present study. The code was already optimized for channel identification, channel slicing, empty channel averaging and background subtraction while we added the module that compute FRET ratios of CFP and YFP images, the nucleoid segmentation and the morphological quantification modules. The code comprises the following modules (explained more in detail in Chapter 3):

1. Growth-channel identification and slicing from phase contrast images:
   Starting from time series of images similar to those presented in Fig. 5.2.1, we compute the projection of phase contrast images on the horizontal axis. We thus obtain a peaked profile where peaks correspond to the middle axes of single growth channels. The position of the peak is used for channel identification and slicing. Each growth channel in each FOV is sliced and stacked at each time point.

2. Empty-channel identification:
   This module of the analysis involves a manual selection of identified channels that are empty, as well as channels that are to be discarded from the analysis because of imperfections in the PDMS or other defects. Via a GUI it is possible to select which channels to discard and mark empty channels for background subtraction.

3. Background subtraction of empty channels from filled channels:
   Here images of empty sliced channels are first averaged together to obtain a more general estimate of the background spatial profile. The averaged empty channel image is aligned with images of filled channels by means of cross correlation maximization between two images. After alignment the empty channel image is subtracted from the filled one.

4. Phase contrast subtracted image segmentation:
   The resulted growth channel subtracted images are passed through a segmentation algorithm based on watershed transform [308]. To identify seeds for the watershed we cast a straight Otsu algorithm to threshold the subtracted image. The thresholded image is then passed through a distance transform and the transformed image is thresholded to retain only those parts of binary regions that have a distance greater than 1.5 pixels (159nm) from the edge of the segmented region(s). The resulting image is used as the seed image for watershed transform. After watershed, the final image is filtered to remove segmented cells touching the boundary of an image (most likely coming from a segmentation artifact), cells smaller than 0.75µm$^2$ in area and cells exceeding 8µm$^2$. 
A challenge of mother machine image segmentation is indeed to properly separate cells with automatic segmentation as they often are found in very close spatial proximity and sometimes segmentation identifies multiple cells as a single binary region. Each resulting image from each segmented growth channel time series in each FOV is stacked together.

5. mRuby-labeled nucleoid image segmentation:
A very similar approach is used for segmenting nucleoid images. Here the challenge deriving from cells being close to each other is less drastic but it can be present when segmenting nucleoids in slow growing cells where nucleoid area fraction is higher. As before, the seeds are identified by distance thresholding a rough binary mask obtained via Otsu’s algorithm mRuby from which the (fluorescence) background was also subtracted employing empty channels images as described above for phase contrast images. For slow growth the threshold value obtained from Otsu’s algorithm is rescaled by a factor of 1.25 to attenuate the aforementioned proximity issue. Since upon collapse a replicating nucleoid can split into two distinct blobs for some osmotic shock magnitudes, our segmentation algorithm could assign two different binary regions to the two blobs even though before shock they were part of the same nucleoid in the segmented image. As a consequence our measured nucleoid size change would be biased at the time point of sucrose arrival since the number of identified nucleoids would increase due to nucleoid splitting upon osmotic shock and we would thus underestimate the average nucleoid size at the time of the shock. To resolve this issue, we always force segmented nucleoids whose centroids are closer than 250nm to belong to the same binary region. Filtering is applied as for segmented phase contrast images but we set the size threshold to \(4 \mu m\) this time.

6. FRET ratio computation from CFP and YFP images:
To measure crowding levels using the crowding sensor from [54] we need to compute the ratio of the acceptor fluorophore (YFP) to the donor (CFP) from the two different images. We use the segmented phase contrast image to identify rectangular boxes around each segmented cell from phase contrast. We then crop regions corresponding to the boxes from both the YFP and the CFP images, and take the sum of the cropped YFP image and divide it by the sum of the corresponding cropped CFP image. We note that we used the same phase contrast image to define the boxes, such as the YFP and CFP images are clipped with an identical box. We repeat this procedure for each cropped image of single growth channels and store Acceptor/Donor values at each time point.
7. Cell and nucleoid morphological quantification:
   All the morphological parameters in this work are extracted from segmented images. Each segmented image is assigned a unique label. We make use of the function "regionprops" of the morphology module of the scikit-image python package (freely available online http://scikit-image.org/). The function returns (among other properties) area, centroid position, major and minor axes of an input binary region. As input, we pass to the function segmented images of isolated single cells which are possible to isolate thanks to our segmented images being labeled: each region (cell) identified by the watershed segmentation has a different numerical label.

5.5 Acknowledgments

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