Functional mapping of the *E. coli* translational machinery using single-molecule tracking

Sonisilpa Mohapatra and James C. Weisshaar*
Department of Chemistry, University of Wisconsin-Madison, Madison, WI 53706, USA.

Summary

The organization of the chromosomal DNA and ribosomes in living *Escherichia coli* is compared under two growth conditions: ‘fast’ (50 min doubling time) and ‘slow’ (147 min doubling time). Superresolution fluorescence microscopy reveals strong DNA-ribosome segregation in both cases. In both fast and slow growth, free ribosomal subunits evidently must circulate between the nucleoid (where they initiate co-transcriptional translation) and ribosome-rich regions (where most translation occurs). Single-molecule diffusive behavior dissects the ribosome copies into translating 70S polysomes and free 30S subunits, providing separate spatial distributions for each. In slow growth, ~21,000 total 30S copies/cell comprise ~65% translating 70S ribosomes and ~35% free 30S subunits. The ratio of 70S ribosomes to free 30S subunits is ~2.5 outside the nucleoid and ~0.50 inside the nucleoid. This new level of quantitative detail may motivate development of comprehensive, three-dimensional reaction-diffusion models of ribosome, DNA, mRNA and RNAP spatial distributions and dynamics within the *E. coli* cytoplasm.

Introduction

Within the cytoplasm of the bacterial species *E. coli* and *Bacillus subtilis*, the ribosomes exhibit strong segregation from the chromosomal DNA, despite the absence of membrane-bounded organelles (Lewis et al., 2000; Bakshi et al., 2012). The DNA occupies a region of space called the nucleoid, which is substantially smaller in volume than the complete cytoplasm (Kellenberger, 1991). Apparently most transcription of protein genes takes place separately from the ribosome-rich regions, where the bulk of translation must occur. In sharp contrast, in *Caulobacter crescentus* the chromosomal DNA occupies the entire volume of the cytoplasm and the DNA and ribosomes are thoroughly mixed (Montero Llopis et al., 2010). Thus far, strong DNA-ribosome segregation was observed in rapidly growing *E. coli* and *B. subtilis* (Lewis et al., 2000; Bakshi et al., 2012), which are relatively large cells containing multiple chromosome equivalents of DNA within their highly compacted nucleoids. Since *C. crescentus* is smaller and grows very slowly, we decided to extend our previous work to slowly growing *E. coli* cells that are comparable to *C. crescentus* in volume, doubling time and DNA content. We use superresolution fluorescence microscopy (Betzig et al., 2006; Hess et al., 2006; Rust et al., 2006) to locate and track 30S ribosomal subunits and the non-specific DNA binding protein HU, a proxy for the spatial distribution of DNA, in two growth conditions: ‘fast’ (50 min doubling time at 30°C) and ‘slow’ (147 min doubling time at 30°C).

In earlier work on fast growing *E. coli* (Bakshi et al., 2012), detailed single-molecule tracking experiments on ribosomal subunits and RNA polymerase (RNAP) supported a circulation model for the movement of translating 70S ribosomes and of free 30S and 50S ribosomal subunits between nucleoid and ribosome-rich regions (Fig. 1). Formation of poly-ribosomes (polysomes) begins within the nucleoid while a message is still being transcribed (co-transcriptional translation). Polysomes can diffuse to a ribosome-rich region in several seconds or less, a short timescale compared with the typical mRNA lifetime of several minutes (Bernstein et al., 2002). When each 70S ribosome completes a translation event within a ribosome-rich region, its 30S and 50S subunits are released. Many of these free subunits will find a new translation initiation site on an mRNA within the ribosome-rich region, but some must escape and return to the nucleoid where they can initiate further rounds of co-transcriptional translation (Sanamrad et al., 2014).

The new results extend the work of Elf and coworkers (Sanamrad et al., 2014) to provide a quantitative, comprehensive comparison of the organization of the *E. coli* cytoplasm in fast vs slow growth. We find that *E. coli* exhibits similarly strong DNA-ribosome segregation in fast and slow growth, as quantified by a modified version of the
Pearson Correlation Coefficient introduced here. We provide quantitative estimates of the peak DNA number density on a ~100 nm scale within the E. coli nucleoids of both fast and slow growing cells. The peak density is ~8-fold higher in fast growth. The slow-growth nucleoid is ‘fluffier’, perhaps because it is being driven by transcriptional and gyrase activity that is more widely distributed throughout the nucleoid (Stracy et al., 2015). In addition, we use single-molecule diffusive behavior to dissect the ribosome copies into slowly diffusing, translating 70S ribosomes (mostly polysomes) and more rapidly diffusing, free 30S subunits and provide separate spatial distributions for each species. Free 30S and 50S subunits can readily penetrate and 70S polysomes are strongly excluded from, both the high density nucleoid in fast growth and the low density nucleoid in slow growth. The circulation model evidently applies to E. coli at all growth rates. In slow growth, a simple model of the nucleoid as a spherocylinder enables us to provide quantitative estimates of the partitioning of 70S and 30S species between the nucleoid and the ribosome-rich regions. We hope this new body of information will stimulate development of a quantitative, fully 3D, comprehensive reaction-diffusion model of ribosome, DNA, mRNA and RNAP spatial distributions and circulation in slow growing E. coli (Castellana et al., 2016).

The underlying causes of the segregation of ribosomes from the chromosomal DNA in E. coli and B. subtilis have been the subject of intense speculation. As a bare, charged polymer in salt solution, the chromosomal DNA would occupy ~1000 times the volume of the E. coli cytoplasm (Vendeville et al., 2011), yet in fact it occupies only about half the cytoplasm in the living cell. This ‘compaction’ of the DNA into such a small volume involves both the physics of charged polymers confined to a small space and the complex biochemistry of the bacterial cytoplasm. Configurational and translational entropy (Mondal et al., 2011; Bakshi et al., 2014), excluded volume effects (Mondal et al., 2011; Castellana et al., 2016), crowding (Zimmerman and Murphy, 1996), repulsion between polyanionic DNA and polyanionic ribosomes (Joyeux, 2016), supercoiling of DNA (Woldringh et al., 1995) and binding of DNA to nucleoid-associated proteins (Dame Remus, 2005; Wang et al., 2011; Lin et al., 2012; Sato et al., 2013;) may all contribute to DNA compaction and DNA-ribosome segregation.

The same factors should be in play in the smaller, more slowly growing species Caulobacter crescentus. Our original hypothesis was that the C. crescentus chromosome occupies the entire cytoplasm because the cytoplasmic volume is so small. Perhaps the combined compaction forces are too weak to force the chromosome to be smaller still. Yet our slow-growing E. coli, comparable to C. crescentus in DNA content and cytoplasmic volume, exhibit strong DNA-ribosome segregation. Having eliminated one possible cause of extensive DNA-ribosome mixing in C. crescentus, we speculate on other possible explanations and suggest new studies.

**Results**

**Spatial distributions of ribosomes and DNA in fast and slow growth conditions**

To compare the spatial distributions of ribosomes and DNA in different growth conditions, we performed two-color superresolution fluorescence imaging of ribosomes (30S subunits via labeling of the S2 protein) and the non-specific DNA binding protein HU in live E. coli cells. In what we term fast growth, the cells are growing in EZRDM (EZ rich defined media) (Neidhardt et al., 1974) with glucose as carbon source at 30°C; the doubling time is 50 min. In what we term slow growth, the cells are grown in MBM minimal media with glycerol as carbon source at 30°C; the doubling time is 147 min. We began with an E. coli strain VH1000 in which the gene coding for the fluorescent protein YFP (observed in the green channel) (Nielsen et al., 2006) is fused to the C-terminus of the endogenous rpsB gene, which codes for the S2 ribosomal protein. On the timescale of YFP maturation (Nagai et al., 2002), S2 is efficiently incorporated into 30S ribosomal subunits (Lindahl, 1975). In effect we are imaging 30S subunits, which may themselves be freely diffusing (searching for translation initiation sites on mRNA) or incorporated into translating 70S ribosomes (primarily polysomes). Single 30S ribosome copies are imaged using the reversible photobleaching method described earlier (Biteen et al., 2008). An inducible plasmid that expresses HU labeled with the photoactivatable fluorescent protein PAmcherry (Subach et al., 2009) (observed in the red channel) was introduced into the same strain. HU binds non-specifically to the chromosomal DNA; we take its spatial distribution to be a proxy for that of the chromosomal DNA. In each growth
Fig. 2. A. Representative examples of single-molecule images of ribosome S2-YFP (Left) and HU–PAmcherry (Right) with cell outlines shown in red.

B. Left: Composite of 2D spatial distributions of 9839 ribosomes (Red) and 8762 HU copies (Black) imaged in 8 fast growing cells of length in the range 3.8-4.2 µm. Right: Composite of 2D spatial distributions of 10,251 ribosomes (Red) and 13,071 HU copies (Black) imaged in 27 slow growing cells of length in the range 2.1-2.5 µm. Note different scales.

C. Axial (x-axis) probability density distribution of ribosomes (Red) and HU (Black) in fast growing cells. Axial Distribution

D. Radial (y-axis) probability density distribution of ribosomes (Red) and HU (Black) in fast growing cells. Radial Distribution. Radial distribution for uniformly filled cylinder of radius $r_{cell} = 0.41$ µm is shown for comparison (Dashed line).

E. Axial (x-axis) probability density distribution of ribosomes (Red) and HU (Black) in slow growing cells. Axial Distribution

F. Radial (y-axis) probability density distribution of ribosomes (Red) and HU (Black) in slow growing cells. Radial Distribution. Radial distribution for uniformly filled cylinder of radius $r_{cell} = 0.29$ µm is shown for comparison (Dashed line). Error bars added at highest points of each experimental curves are based on $\pm \sqrt{N}$, where $N$ is the number of counts in a bin. [Colour figure can be viewed at wileyonlinelibrary.com]
condition, cells were grown at 30°C, plated on a glass coverslip and imaged with 30 ms/frame exposure time. For 30S-YFP, the dynamic localization error estimated from MSD plots (Fig. S1) is $\sigma = 36$ and 27 nm in fast and slow growth respectively. For the more rapidly diffusing HU-PAmcherry, $\sigma = 60$ nm and 55 nm in fast and slow growth respectively (Fig. S1). Details of strain construction, growth conditions and imaging conditions are provided in Methods.

Typical single-molecule images are shown for 30S-YFP and for HU-PAmcherry in Fig. 2A. The imaged cells were sorted by tip-to-tip length based on phase contrast images in order to minimize broadening of the spatial distributions due to the range of cell lengths. We combine all locations of red HU and green 30S molecules that lasted for four frames or more, imaged in cells of essentially the same length. For the fast growth condition, we chose cells whose length ranges from 3.8 to 4.2 µm, the bin with the highest number of imaged cells. For fast growing cells, the resulting composite 2D distributions of 9839 ribosomal S2-YFP copies and 8762 HU–PAmcherry copies from 8 cells are shown in Fig. 2B (Left). In fast growth, the projected 1D axial distributions of DNA and ribosomes are strongly anti-correlated with each other (Fig. 2C), in agreement with earlier work by Bakshi et al using wide-field imaging of ribosomes S2-YFP and DRAQ5 staining the DNA (Bakshi et al., 2012). The axial distribution for all 30S copies shows a peak-to-trough ratio of 3:1 between the ribosome-rich regions and the densest part of the nucleoids. In the chosen length bin, the DNA has segregated into two nucleoid lobes (Fig. 2C). The axial distribution of HU–PAmcherry molecules shows two peaks with a peak-to-trough ratio of 7:1. Evidently there are some ribosomes within the nucleoids, but little or no DNA in the three ribosome-rich regions (Bakshi et al., 2012).

The projected 1D radial (short, y-axis) distributions of ribosomes and DNA in fast growing cells are shown in Fig. 2D. These radial profiles exclude the end cap regions as well as the ribosome-rich region in the central part of the cell in order to get a clearer picture of the degree of radial DNA-ribosome segregation. To minimize noise in this smaller sample, we symmetrized the radial ribosomal profile about the x-axis (long cell axis). The ribosome distribution extends outward to the cytoplasmic membrane. The dashed line shows a projected radial distribution for a model of a uniform distribution in a cylinder with radius $r_{cell} = 0.41$ µm, chosen from modeling of the spatial distribution of Kaede (Fig. S2). In comparison with the uniform distribution, the ribosome concentration is slightly depleted near the x-axis (where $y = 0$). The symmetrized radial distribution of the DNA marker HU is much narrower and strongly depleted in the annular regions near the cytoplasmic membrane.

For the slow growing cells, the length bin with the highest number of imaged cells is 2.1 to 2.5 µm. This length bin primarily samples cells before DNA segregation (Jin et al., 2013); there is a single nucleoid lobe. For 10,251 S2-YFP copies and 13,071 HU-PAmcherry copies imaged in 27 slow growing cells with trajectories that lasted for four frames or more, the corresponding 2D scatter plot of locations is shown in Fig. 2B (Right). The projected axial distributions are shown in Fig. 2E. The peak-to-trough ratio in the axial distribution of ribosomes in slow growth conditions is only ~1:3:1. The axial distribution of HU molecules shows a single broad peak; DNA is depleted near the endcaps. The symmetrized radial distributions excluding the two endcap regions (Fig. 2F) show that the DNA radial distribution is narrower than that of ribosomes. Comparison with the model radial distribution for a uniform distribution (cylinder with $r_{cell} = 0.29$ µm; see Methods and Fig. S2) shows that ribosomes are somewhat depleted near the central x-axis, i.e. within the nucleoid region. DNA is completely excluded from the annular region near the cytoplasmic membrane.

**Degree of segregation of ribosomes and DNA in fast and slow growth conditions**

Superficially, the 1D projections suggest that the ribosome-DNA segregation is stronger in fast growth than in slow growth. Next we return to the measured 2D projected locations (Fig. 2B and C) seeking to quantify the degree of segregation of ribosomes and DNA. Our metric is a modification of the commonly used Pearson Correlation Coefficient (PCC) (Pearson, 1896; Manders et al., 1992), adapted for 2D projections of 3D distributions from the *E. coli* spherocylindrical geometry. A number of different procedures for assessing co-localization between two images are described in detail in a recent review (Aaron et al., 2018). The standard PCC measures the degree of linear correlation between two pixelated image matrices $R$ (red channel, here HU) and $G$ (green channel, here ribosomes), on a scale from $-1$ (perfect anti-correlation) to $+1$ (perfect positive correlation) (Pearson, 1896; Manders et al., 1992). Standard PCC compares every matrix element of $R$ and $G$ with the constant mean value $\overline{R}$ or $\overline{G}$ and calculates the normalized correlation coefficient between these positive and negative deviations. However, the standard PCC procedure fails both quantitatively and qualitatively for spatial distributions of molecules imaged in spherocylindrical cells, such as *E. coli* (Mohapatra and Weisshaar, 2018). The projection of molecules randomly distributed in a 3D spherocylindrical volume is non-uniform in 2D, owing to strong curvature at the two endcaps and in the cylindrical region. As a result, standard PCC returns the same $+1$ value for 2D projections of distributions that are either perfectly correlated in 3D or completely uncorrelated in 3D. The standard PCC also systematically underestimates the
Fig. 3. Calculation of modified pearson correlation coefficient (MPCC) between ribosome and DNA (HU) spatial distributions in fast growing cells.
A. 2D localization probability density maps of 9839 ribosomes (G, Left) and 8762 HU (R, Right) molecules imaged in fast growing cells. Color scale indicates number of molecules in each 50 nm pixel.
B. Reference distributions \( \hat{U}^G \) (Left) and \( \hat{U}^R \) (Right), which are 2D projections of 3D random distributions of 100,000 molecules within the spherocylinder (\( l_{cell} = 3.82 \mu m, r_{cell} = 0.41 \mu m \)) and normalized to have same total number of molecules as G and R respectively. \( \hat{U}^G \) and \( \hat{U}^R \) are subtracted from images G and R respectively.
C. Normalized difference matrices \( \hat{\Delta}^G \) (Left) and \( \hat{\Delta}^R \) (Right) obtained after subtraction. The Frobenius inner product of these two difference matrices gives the value MPCC\(_{\text{fast}} = -0.30 \) for fast growth conditions. [Colour figure can be viewed at wileyonlinelibrary.com]

The degree of anti-correlation between the projections of two perfectly anti-correlated 3D distributions. The reasons for these failures of standard PCC are described in detail elsewhere (Mohapatra and Weisshaar, 2018).

We therefore applied a modified PCC procedure (called MPCC) that compares individual elements of the image matrices R and G with the matrix elements of the 2D projection of a large set of molecules distributed randomly within a 3D spherocylinder (Eq. 1 in Methods). For the composite images from trajectories three steps or longer in fast growing cells (Fig. 2B, Left), we pixelated the spatial distribution of \( N_G = 9839 \) ribosomes and \( N_R = 8762 \) HU molecules into 50 nm × 50 nm bins to yield the experimental image matrices R and G (Fig. 3A). The mean number of molecules per pixel is 8.8 and 7.9 for the ribosome and HU channels respectively, which is greater than the recommended mean occupancy of at least 7 molecules per pixel for accurate estimation of MPCC values (Mohapatra and Weisshaar, 2018). To form the MPCC reference distributions (Fig. 3B), we simulated two random 3D distributions of 100,000 molecules each filling a spherocylinder, corresponding to the ribosome (green) and HU (red) channels. The dimensions of the randomly filled spherocylinder are the same as those of the imaged cells, length \( l_{cell} = 3.82 \mu m \) and diameter \( 2r_{cell} = 0.82 \mu m \). The resulting reference images are normalized to have the same number of molecules as were imaged for ribosomes and HU, generating the matrices \( \hat{U}^G \) and \( \hat{U}^R \) shown in Fig. 3B. We then subtracted \( \hat{U}^R \) and \( \hat{U}^G \) from the corresponding image matrices R and G and normalized the difference matrices to generate \( \hat{\Delta}^R \) and \( \hat{\Delta}^G \) (Fig. 3C), so that the sum of the squares of individual pixel values in each difference matrix is 1. The Frobenius inner product of the two normalized matrices \( \hat{\Delta}^R \) and \( \hat{\Delta}^G \) (Eq. 1) gives the result MPCC\(_{\text{fast}} = -0.30 \), indicating spatial anti-correlation.

Finally, we estimated the probability \( \rho \) that a value of MPCC\(_{\text{fast}} = -0.30 \) or larger in magnitude would be obtained from two random 3D distributions in spherocylinders of the same dimensions as the imaged fast growing cells, with the same number of imaged molecules and the same pixel size used for the experimental data. In Fig. S3A, we show a histogram of the outcomes of 200 such simulations. The best-fit Gaussian distribution has a mean value \( \langle \text{MPCC} \rangle = -0.0017 \) and standard error \( \sigma_{\text{MPCC}} = 0.0309 \).
The measured MPCC\textsubscript{fast} value lies 9.6 $\sigma_{MPCC}$ away from zero. Under the assumption that the statistics of the simulated MPCC trials are Gaussian, the probability that two random 3D distributions would produce an MPCC value of magnitude 0.30 or larger on either side of the Gaussian curve is $p \sim 10^{-21}$. Thus we reject the null hypothesis that MPCC\textsubscript{fast} = −0.30 arose from two random, uncorrelated 3D distributions and assert significant negative correlation between the ribosome and HU spatial distributions in fast growth with high confidence.

In the same way, we used the MPCC procedure to quantify the degree of segregation between ribosomes and DNA in slow growing cells. The composite spatial distribution of $N_\text{G} = 10,251$ ribosome and $N_\text{R} = 13,071$ HU molecules from trajectories three steps or longer in 27 slow growing cells is pixelated into 50 nm × 50 nm bins to obtain $\mathbf{R}$ and $\mathbf{G}$ (Fig. 4A). The mean number of molecules per pixel is 16.2 and 20.7 for the ribosome and HU channels. $\mathbf{U}_\text{G}$ and $\mathbf{U}_\text{R}$ shown in Fig. 4B are generated by normalizing two simulated random distributions of 100,000 molecules within the spherocylinder ($L_\text{cell} = 2.14 \mu m$, $r_\text{cell} = 0.29 \mu m$) and normalized to have same total number of molecules as $\mathbf{G}$ and $\mathbf{R}$ respectively. $\mathbf{U}_\text{G}$ and $\mathbf{U}_\text{R}$ are subtracted from images $\mathbf{G}$ and $\mathbf{R}$ respectively.

The estimated values MPCC\textsubscript{fast} = −0.30 and MPCC\textsubscript{slow} = −0.23 for ribosome and HU spatial distributions in the two growth conditions are both negative and significantly different from the value MPCC = 0 for random, uncorrelated distributions. In both growth conditions, the ribosomes and DNA are segregated from each other. The apparent degree of segregation of ribosomes from DNA in slow growth conditions is only slightly smaller than that for fast growing cells. These 2D MPCC values surely underestimate the degree of segregation in the actual 3D $E. \text{coli}$ cells. Ribosomes at the top and bottom of the 3D annular region near the cytoplasmic membrane are projected into the nucleoid region, making a strong positive contribution to the 2D MPCC even though they are segregated from the DNA in 3D.

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Classification of translating 70S ribosomes and free 30S subunits in fast and slow growth

We also carried out single-30S tracking experiments in both fast and slow growth conditions in an attempt to distinguish free 30S subunits from 30S subunits that are incorporated into translating 70S ribosomes. The smaller free 30S subunits should diffuse more rapidly than the larger translating 70S copies, most of which are incorporated into polysomes. By modeling the distribution of single-step displacements as arising from two populations that do not exchange on the 30-ms imaging timescale, we can provide an estimate of the fraction of translating 70S ribosomes in both fast and slow growth conditions. The two-state analysis will also enable estimation of separate spatial distributions for free 30S subunits and for translating 70S ribosomes in both growth conditions. The photoswitchable fluorescent protein mEos2 (McKinney et al., 2009) gives us better control over the average number of fluorescent copies in each camera frame than the YFP label. For the ribosome tracking experiments, we fused the gene encoding mEos2 to the C-terminus of the endogenous rpsB gene (which encodes S2). Details regarding the construction of this strain were given previously (Bakshi et al., 2014). The imaging conditions and tracking analysis are described in Methods. Only 0, 1 or 2 30S-mEos2 molecules per cell were activated in each frame to enable accurate tracking of each copy over successive frames. A typical S2-mEos2 movie is included in Supporting Movies S1.

We selected trajectories that last at least seven frames; longer trajectories were truncated to seven frames. Examples are shown in Fig. S4. The chosen trajectories were divided into individual steps and single-step displacements were calculated and binned to form a distribution $P_{\text{rib}}(r)$ in both fast and slow growth (Fig. 5).

The static two-state model assumes that all translating 70S/polysomes have diffusion coefficient $D_{\text{slow}}$ and all non-translating, free 30S subunits have diffusion coefficient $D_{\text{fast}}$. Each distribution of experimental single-step displacements $P_{\text{rib}}(r)$ was fit in a least-squares sense to a numerical model function $P_{\text{model}}(r)$, which includes two populations diffusing under confinement within a spherocylinder of appropriate length and diameter. The three fitting parameters are $D_{\text{slow}}, D_{\text{fast}},$ and the fractional population of slow, 70S ribosomes $f_{\text{slow}}$, which in turn fixes the fractional population of fast, 30S copies, $f_{\text{fast}} = (1 - f_{\text{slow}})$. The fitting procedure (Methods) includes confinement effects and was described in detail earlier (Mohapatra et al., 2017).

For fast growth (Fig. 5A), the experimental $P_{\text{rib}}(r)$ distribution comprises 88,506 individual steps from 14,751 trajectories truncated to six steps, if they lasted longer.

We collected these trajectories from 271 different cells of varying lengths. Since the ribosomes are slowly diffusing molecules that seldom suffer from confinement by the cell boundary, we include trajectories from all imaged cells. The best fit $P_{\text{model}}(r)$ shown in Fig. 5A used parameters $f_{\text{slow}} = 0.80 \pm 0.07, D_{\text{slow}} = 0.020 \pm 0.007 \, \mu m^2/s$ and $D_{\text{fast}} = 0.20 \pm 0.03 \, \mu m^2/s$ ($\chi^2 = 1.46$). For slow growth (Fig. 5B), the experimental $P_{\text{rib}}(r)$ distribution comprises 112,146 individual steps from 378 different cells. The best fit $P_{\text{model}}(r)$ shown in Fig. 5B has parameters $f_{\text{slow}} = 0.65 \pm 0.05,$
Fast Growth
50 nm pixels

A
Translating 70S

B
Free 30S

C

70S
Free 30S

D

70S
Free 30S
Uniform dist.

\[ r_{\text{cell}} = 0.41 \, \mu \text{m} \]

\[ D_{\text{slow}} = 0.009 \pm 0.003 \, \mu \text{m}^2/\text{s} \] and \[ D_{\text{fast}} = 0.13 \pm 0.02 \, \mu \text{m}^2/\text{s} \]

\( (r_{x} = 1.55) \)

Bootstrapping the data enabled us to obtain error estimates (±1 standard deviation) for each of the fitted parameters.

We infer a moderate decrease in the fraction of 30S subunits incorporated into translating 70S ribosomes from 0.80 ± 0.07 in fast growth to 0.65 ± 0.05 in slow growth conditions. These results are quantitatively consistent.

Fig. 6. Spatial organization of translating 70S and free 30S ribosomes in fast growing cells.
A. 2D localization probability density maps of translating 70S ribosomes (blue swath in Fig. 5A). Color scale indicates number of molecules in each 50 nm pixel.
B. 2D localization probability density maps of free 30S ribosomes (red swath in Fig. 5A). Color scale indicates number of molecules in each 50 nm pixel.
C. Axial probability density distribution of translating 70S (Blue) and free 30S (Red) ribosomes.
D. Radial probability density distribution of translating 70S (Blue) and free 30S (Red) ribosomes. Radial distribution of uniformly filled cylinder \( (r_{\text{cell}} = 0.41 \, \mu \text{m}) \) is shown for comparison (Dashed Line). Error bars added at highest points of each experimental curves are based on \( \sqrt{N} \), where \( N \) is the number of counts in a bin. [Colour figure can be viewed at wileyonlinelibrary.com]
with recent biochemical measurements of the fraction of translating ribosomes vs growth rate by Hwa and co-workers (Dai et al., 2016). They obtained 87% translating ribosomes for a doubling time of 50 min and 66% translating ribosomes for a doubling time of 147 min. According to the best-fit values of $D_{slow}$ in both growth conditions the root-mean-square single-step displacement of a translating ribosome is comparable to the localization error ($\sigma \sim 30$ nm). The difference in the fitted values of $D_{slow}$ between the two growth conditions may not be significant.

Fig. 7. Spatial organization of translating 70S and free 30S ribosomes in slow growing cells.
A. 2D localization probability density maps of translating 70S ribosomes (blue swath in Fig. 5B). Color scale indicates number of molecules in each 50 nm pixel.
B. 2D localization probability density maps of free 30S ribosomes (red swath in Fig. 5B). Color scale indicates number of molecules in each 50 nm pixel.
C. Axial probability density distribution of translating 70S (Blue) and free 30S (Red) ribosomes.
D. Radial probability density distribution of translating 70S (Blue) and free 30S (Red) ribosomes. Radial distribution of uniformly filled cylinder ($r_{cell} = 0.29 \mu m$) is shown for comparison (Dashed Line). Error bars added at highest points of each experimental curves are based on $\sqrt{N}$, where $N$ is the number of counts in a bin. [Colour figure can be viewed at wileyonlinelibrary.com]
Spatial distributions of free 30S subunits and translating 70S ribosomes

As shown in Fig. 5, the slow and fast components of the experimental distribution of single-step displacements $P_{\text{ribo}}(r)$ from the 30S-mEos2 tracking experiments show significant overlap for both growth conditions. This makes it difficult to determine whether any particular copy is fast or slow. To characterize the spatial distributions of slow and fast copies, we defined two cutoff values for each distribution $P_{\text{ribo}}(r)$. Steps shorter than the lower cutoff value $r_{\text{slow}}$ are likely to come from the translating, 70S population. Steps longer than the higher cutoff value $r_{\text{fast}}$ are likely to come from the searching, free 30S population. For fast growing cells, we chose $r_{\text{slow}} = 0.075 \mu m$ and $r_{\text{fast}} = 0.175 \mu m$ (Fig. 5A). For slow growing cells we chose $r_{\text{slow}} = 0.05 \mu m$ and $r_{\text{fast}} = 0.15 \mu m$ (Fig. 5B). The blue and red shaded regions show the range of single-step displacements that are classified as translating 70S and free 30S copies and suggest in each case the purity of the assignments, according to the two-state model. The
chosen thresholds for fast growth conditions indicate that the fast copies are 95% pure and the slow copies are 91% pure. For slow growth, the model indicates 95% purity for fast copies and 82% percent purity for slow copies.

For fast growth conditions, we analyzed 13,716 displacements from 52 cells whose tip-to-tip length varied from 3.8 to 4.2 μm based on phase contrast images. Trajectories that lasted longer than seven frames (six single steps) were selected. We calculated single-step displacements r for all individual steps of the trajectories. The midpoint of the two endpoints \((x_1, y_1)\) and \((x_2, y_2)\) for each single step was defined as the location of that step. Each step was classified as coming from translating 70S or free 30S according to the cutoff prescription described above. In Fig. 6A, B, we show 2D probability density heat maps of the spatial locations of short steps (translating 70S) and of long steps (free 30S). Both translating 70S and free 30S are concentrated at the endcap regions and in the central region between the two nucleoid lobes, as seen in the axial distribution symmetrized about y-axis (Fig. 6C). The radial distributions are obtained only from molecules imaged in the two nucleoid lobes; they exclude the endcap regions and the central ribosome–rich region and are symmetrized about the x-axis (Fig. 6D). The radial distributions of translating 70S and free 30S are very different from each other. The translating 70S copies concentrate in the annular regions near the cell membrane and avoid the nucleoids. The free 30S copies readily access the nucleoids but are sparse near the cell membrane.

For slow growing cells (Fig. 7), 40,854 displacements from 87 cells in the length bin 2.1–2.5 μm were classified as translating 70S or free 30S based on the cutoffs \(r_{\text{slow}}\) and \(r_{\text{fast}}\) (Fig. 5B) and located at the midpoint of each step. The location heat map suggests that slowly diffusing ribosomes concentrate in the endcaps (Fig. 7A). The symmetrized axial distribution of translating 70S ribosomes is indeed concentrated in the endcaps and somewhat depleted in the central part of the cell where the DNA resides (Fig. 7C). In contrast, the symmetrized axial distribution of free 30S shows low concentration in the endcap tips and higher concentration in the nucleoid region. The radial distributions (Fig. 7D) exclude molecules in the endcap regions and are symmetrized about x axis. The radial distribution of the translating 70S ribosomes peaks away from the central cell axis and extends all the way to the cytoplasmic membrane. In contrast, the radial distribution of the free 30S subunits is essentially uniform (Fig. 7D), as shown by comparison with a uniformly filled model cylinder of radius \(r_{\text{cell}} = 0.29 \mu m\).

**Slow growth: partitioning of 70S and free 30S species inside and outside of nucleoid**

In our slow growth conditions, we can use the measured axial and radial spatial distributions of fast and slow diffusing copies (free 30S subunits and translating 70S ribosomes respectively) and a simple geometric model of the nucleoid region to estimate how many free 30S and translating 70S copies reside inside and outside of the nucleoid. We have used the 30S-YFP construct to estimate the mean total 30S copy number to be ~21,000 per cell for slow growing cells in the 2.1–2.5 μm length bin (Methods, Fig. S5). According to the analysis of Fig. 5B, this includes ~7350 free 30S subunits (35±5%) and ~13,650 70S copies having 30S incorporated into translating 70S ribosomes (65±5%).

In the simplified 3D geometric model depicted at the top of Fig. 8, the full cell volume is represented by a spherocylinder of tip-to-tip length \(L_{\text{cell}} = 2.14 \mu m\), radius \(r_{\text{cell}} = 0.29 \mu m\) and volume \(V_{\text{cell}} = 0.51 \mu m^3\). These dimensions are based on model fits to the measured distribution of Kaede copies in slow growing cells in the 2.1–2.5 μm length bin, as estimated by phase contrast (Methods, Fig. S2). Slow growing cells have a single nucleoid lobe and a fairly smooth distribution of DNA density, as shown in Fig. S6A. The nucleoid is represented by a shorter, narrower spherocylinder of dimensions \(L_{\text{nucleoid}} = 1.55 \mu m\), \(r_{\text{nucleoid}} = 0.19 \mu m\) and \(V_{\text{nucleoid}} = 0.16 \mu m^3 = 0.31V_{\text{cell}}\). These were determined from spatial distributions of HU copies in slow growing cells within the same length bin, as detailed in Methods (Fig. S6).

Then we determined the fraction of translating 70S copies residing inside the nucleoid that best fits the experimental axial and radial distributions of the slowly diffusing copies (Fig. 7C and D). We randomly filled the model cell volume with 100,000 mathematical points. This places 31,353 points inside the nucleoid and 68,647 points outside
than the estimate from Kaede localizations) and radius $r_{cell} = 0.29 \mu m$ (matching the Kaede estimate, Fig. S2).

The need to shorten the model cell length by 70 nm at each tip is an artifact of our choice of very long steps (50 > 150 nm in length; Fig. 5B) to obtain a fairly pure set of rapidly diffusing 30S copies. The midpoints of such long steps are necessarily depleted at the very tips of the cell. Detailed comparison of the axial distributions of static molecules and molecules with single step displacements longer than 150 nm (Fig. S8) shows that indeed the chosen long steps are depleted from the tips of the cell. This effect is less pronounced for the radial distribution. Since a uniform distribution of points within a spherocylinder of tip-to-tip length $L_{cell} = 2.00 \mu m$ (140 nm shorter than the estimate from Kaede localizations) and radius $r_{cell} = 0.29 \mu m$ (matching the Kaede estimate, Fig. S2).

For the free 30S copies, we found that the experimental axial and radial distributions are well matched by a uniform distribution of points within a spherocylinder of tip-to-tip length $L_{cell} = 2.00 \mu m$ (140 nm shorter than the estimate from Kaede localizations) and radius $r_{cell} = 0.29 \mu m$ (matching the Kaede estimate, Fig. S2). The need to shorten the model cell length by 70 nm at each tip is an artifact of our choice of very long steps ($r > 150$ nm in length; Fig. 5B) to obtain a fairly pure set of rapidly diffusing 30S copies. The midpoints of such long steps are necessarily depleted at the very tips of the cell. Detailed comparison of the axial distributions of static molecules and molecules with single step displacements longer than 150 nm (Fig. S8) shows that indeed the chosen long steps are depleted from the tips of the cell. This effect is less pronounced for the radial distribution. Since a uniform distribution of points within a spherocylinder of tip-to-tip length $L_{cell} = 2.00 \mu m$ (140 nm shorter than the estimate from Kaede localizations) and radius $r_{cell} = 0.29 \mu m$ (matching the Kaede estimate, Fig. S2).

We refrained from attempting similar calculations for fast growing cells due to increased cell to cell heterogeneity in nucleoid size, shape, location and density. This renders the composite HU distribution both wider and longer than typical single-cell distributions (Fig. S10C, D). A spherocylindrical model of the nucleoid volume is inappropriate in fast growth.

**Discussion**

**Comparison of E. coli and C. crescentus at similar total DNA content and cell volume**

Our original motivation for the present work was the observation of very different spatial organization within the cytoplasm of fast growing *E. coli* and *B. subtilis* compared with relatively slow growing *C. crescentus*. Strong segregation of the chromosomal DNA (the nucleoid) from the ribosomes was first observed in rapidly growing *B. subtilis* (Lewis et al., 2000). A similar degree of axial and radial segregation has now been observed for *E. coli* in fast (Bakshi et al., 2012), moderate (Sanamrad et al., 2014) and slow growth (present work). Evidently in those two species, most transcription of protein genes occurs in different regions of space than the bulk of translation. In sharp contrast, in the slow-growing species *C. crescentus*, whose doubling time lies in the range ~120–150
min (Campos et al., 2014), the chromosomal DNA and the ribosomes are apparently thoroughly mixed and occupy the entire cytoplasmic volume (Montero Llopis et al., 2010).

A variety of mechanisms have been proposed to explain why the E. coli chromosome occupies only a fraction of the total cytoplasmic volume and why the DNA and ribosomes are strongly segregated from each other. Joyeux provides an excellent summary of possible physical and biochemical contributions to the compaction of the chromosomal DNA (Jayeux, 2016). These include segregative phase separation due to repulsive forces between polyanionic DNA and the highly negatively charged 70S ribosomes (~4500 each); DNA condensation arising from binding to small cations; macromolecular crowding ('depletion forces'); supercoiling; and binding to nucleoid associated proteins. He argues that the strongest forces arise from segregative phase separation. On the other hand, a minimalist model of DNA as a hyperbranched chain of beads and polysomes as freely jointed chains of beads of appropriate size found that the DNA polymer when confined by itself within a spherocylindrical volume already avoids the walls to enhance its configurational entropy (Mondal et al., 2011). Entropic and hard-sphere excluded volume effects alone were sufficient to cause strong DNA-polysome segregation in that very simple model. No electrostatic repulsion was necessary.

Regardless of the underlying causes of segregation in E. coli, we wondered if the C. crescentus chromosome fills the entire cytoplasm because the cell volume is so small that the combined nucleoid compaction forces are too weak to cause the DNA to occupy a still smaller volume. Ribosomes and DNA would then necessarily mix. An estimate of the C. crescentus cytoplasmic volume as that of two truncated cones of radius 250 nm and height 1.5 µm each yields a lower bound on V_{crescentus} of 0.20 µm³; a spherocylindrical model of radius 250 nm and length 3 µm yields an upper bound of 0.55 µm³. We take V_{crescentus} = 0.4 µm³ as a sensible estimate. Our slow growth condition yields an E. coli doubling time of 147 min and median cell volume V_{cell} = 0.51 µm³, both comparable to those of C. crescentus. This is nearly four times less volume than the median V_{cell} = 1.88 µm³ for E. coli in our fast growth conditions (doubling time 50 min). Both the slow growing E. coli and C. crescentus harbor ~1 chromosome per cell (Jensen, 2006; Kuhlman and Cox, 2012) and the size of the chromosome is similar, 4.63 Mb and 4.01 Mb (Nierman et al., 2001) respectively. The comparison in terms of cell volume and amount of DNA seems apt; nevertheless, we find substantial nucleoid-ribosome segregation in slow growing E. coli. The MPCC metric shows a comparable degree of anti-correlation between 30S ribosomes and the DNA marker HU for E. coli in our fast and slow growth conditions. The degree of radial segregation is strong in both growth conditions, and the endcaps concentrate ribosomes and exclude DNA in both cases.

Thus the underlying cause of the different spatial distributions in E. coli and C. crescentus remains unclear. Perhaps the compacting forces are somehow weaker in C. crescentus. There may be significant morphological differences between the E. coli chromosome and the C. crescentus chromosome. HiC analysis of the latter revealed a 'bottlebrush' structure (Le et al., 2013); a study of the E. coli chromosomal structure at comparable resolution might prove informative. Finally, a recent 3D superresolution study in C. crescentus discovered spatial clustering of ribosomes, suggesting the possibility of localized pockets of segregation of ribosomes from DNA with the pockets widely dispersed throughout the cytoplasm (Bayas et al., 2018). A similar study investigating the spatial distribution of HU in C. crescentus suggested clustering of HU in different stages of the cell cycle (Lee et al., 2011). It might prove informative to extend that work to 3D superresolution imaging of HU and ribosomes in the same cell to test for 'patchy' segregation. 3D experiments on E. coli would also be informative; with enough data, they would yield 3D correlation coefficients undistorted by the 'squashing' of the image from 3D to 2D inherent in the present study.

**Degree of nucleoid compaction in fast and slow growth**

With one exception (Kuhlman and Cox, 2012), there is widespread agreement in the literature that the E. coli chromosome is denser ('more compacted') in fast growth than in slow growth (Hadizadeh Yazdi et al., 2012; Jin et al., 2013; Stracy et al., 2015; Castellana et al., 2016; Joyeux, 2016). That conclusion is sometimes reached based on visual inspection of fluorescent images of nucleoids in various growth conditions (Hadizadeh Yazdi et al., 2012; Jin et al., 2013). Our new data corroborate this idea. In addition, we now attempt quantitative estimates of the peak DNA density on the ~100 nm length scale in our slow and fast growth conditions. In our slow growth condition (147 min doubling time), there is one nucleoid lobe containing ~1 chromosome = 4.6 Mb of DNA (Kuhlman and Cox, 2012). The volume of our cylindrical model nucleoid is V_{nucleoid} = 0.16 µm³, yielding an estimated mean DNA density within the nucleoid of ~2.9 × 10^7 bp/µm³. The DNA spatial distribution appears smooth on the 100 nm length scale, so the peak and average density are similar. In our fast growth condition (50 min doubling time), there are two nucleoid lobes containing a total of ~2.2 chromosome equivalents = 10.1 Mb of DNA (Bremer and Dennis, 2008). The HU distribution within each nucleoid lobe is typically crescent shaped, with much higher density near the center of the crescent
A spherocylindrical model of the composite HU distribution averaged across cells (Fig. S10C) yields $V_{\text{nucleoid}} = 0.21 \ \mu m^3$ for the total volume of the two lobes, providing a lower bound on the mean DNA density within each lobe of $\sim 4.8 \times 10^7$ bp/µm$^3$. However, as shown in Fig. S10D, the nucleoid volume in a single cell is perhaps two-fold smaller than the estimate derived from the multicell average distribution. In addition, the density in the center of each lobe is perhaps 2.5 times higher than the average (Fig. S10A). A rough estimate of the peak DNA density on a ~100 nm scale in our fast growth conditions is thus $\sim 2.4 \times 10^8$ bp/µm$^3$, some 8 times larger than in slow growth.

Why is the nucleoid ‘fluffier’ in slow growth than in fast growth? One possible explanation derives from the very different spatial distributions of actively transcribing RNA polymerase copies in the two conditions. In fast growth, most transcription occurs on the seven $rrn$ operons, six of which have been shown to cluster with each other (Gaal et al., 2016). These ‘transcription foci’ (Jin et al., 2013) tend to locate at the periphery of the nucleoid (Jin et al., 2013; Stracy et al., 2015), perhaps due to excluded volume effects (Mondal et al., 2011). In slow growth, transcription of $rrn$ operons is less important, many more protein genes are expressed (Tao et al., 1999), and transcription of protein genes is broadly distributed throughout the nucleoid (Stracy et al., 2015). Perhaps the widely distributed activity of transcription, which causes local supercoiling and necessitates DNA gyrase activity, somehow causes expansion of the overall DNA volume. The detailed mechanism remains unclear. Perhaps the nucleoid should be considered a form of ‘active matter’, whose overall volume is controlled by the density and distribution of energy dissipation by RNAP and other force-producing proteins (Needleman and Dogic, 2017).

Circulation model of movement of 70S and 30S ribosomal species

Co-transcriptional translation has been shown to be important to prevent backtracking of RNAP, premature termination of transcription, and premature degradation of mRNA by ribonucleases (Burmann et al., 2010; Proshkin et al., 2010; McGary and Nudler, 2013). Indeed we find clear evidence of translating 70S ribosomes in the core of the nucleoid in both fast and slow growth (Figs. 6 and 7). The strong segregation of most translating 70S ribosomes outside the nucleoid combined with the occurrence of co-transcriptional translation within the nucleoid requires that 30S and 50S ribosomal subunits be able to circulate throughout the cell (Fig. 1). When a transcript is complete, it is already presumably decorated with 70S ribosomes and diffuses as a polysome, with $D_{\text{slow}} \sim 0.01$ F06Dm$^2$/s. For $r_{\text{nucleoid}} \sim 0.19$ µm, the typical nascent mRNA will escape the nucleoid and find the annular ribosome-rich region surrounding the nucleoid very quickly, in ~0.1 s (SI, S1). Polysomes in the ribosome-rich annular region will exchange with polysomes in the ribosome-rich endcaps on a timescale of ~10 s (SI, S2). The same forces that exclude polysomes from the nucleoid will tend to prevent their re-entry into the nucleoid after escape. As each 70S ribosome within the ribosome-rich regions completes translation of a protein, 30S and 50S dissociate. These free ribosomal subunits will either find a new mRNA within the ribosome-rich regions or return to the nucleoid to initiate another co-transcriptional translation event.

This means that it is functionally important that 30S and 50S subunits be able to penetrate the nucleoid meshwork rapidly to enable co-transcriptional translation within the nucleoid. One might worry that in fast growth, the nucleoid is so dense that it precludes re-entry of ribosomal subunits into the nucleoid. This in fact occurs for the highly condensed nucleoids of erythromycin-treated cells (Sanamrad et al., 2014). Elf and co-workers earlier showed that for E. coli in ‘medium growth’ conditions (doubling time = 120 min at 25°C), free 50S and 30S subunits distribute uniformly across the cytoplasm, indicating ready access of subunits to the bulk of the nucleoid (Sanamrad et al., 2014). The present work shows (Figs. 6 and 7) that free 30S subunits distribute uniformly across the cytoplasm for both our fast and slow growth conditions (doubling times of 50 min and 147 min). Although the peak DNA density is ~8 times greater in fast growth than in slow growth, it is evidently not so high as to exclude ribosomal subunits. A model of ribosomal subunit circulation including co-transcriptional translation (Fig. 1) appears to be applicable over the entire range of E. coli growth rates studied in this fashion.

Connection with 1D global reaction-diffusion model of transcription and translation

Recently Wingreen and co-workers developed a quantitative numerical model of the E. coli transcription-translation machinery based on available experimental input for cells growing with doubling time of 60 min (Castellana et al., 2016). Their steady-state reaction-diffusion model includes many details of the dynamics and spatial distributions of 50S ribosomal subunits, 70S ribosomes, mRNA and chromosomal DNA. For numerical simplicity, the model is one-dimensional, with all concentrations expressed as molecules/length. The single nucleoid lobe is represented as a distribution of DNA density centered at mid-cell with FWHM of ~60% of the total $L_{\text{cell}} = 3$ µm, or ~1.8 µm. In the 1D model, the ribosome-rich endcaps map onto line segments at either end. An important prediction of the model is that ~90% of mRNAs are
segregated within the endcaps, where most translation occurs.

Next we attempt to compare our estimated free 30S and translating 70S partition fractions (Fig. 9) with the 1D model results. We use our slow growth data (147 min doubling time), for which we were able to estimate free 30S and translating 70S copy numbers and percentages both inside and outside the nucleoid. The comparison is admittedly imperfect. The 1D model is built for a 60 min doubling time, but like our slow growth condition (147 min), it includes only one nucleoid lobe. The FWHM of 1.8 μm in the 1D nucleoid is similar to the length of our 3D nucleoid model in slow growth, $L_{\text{nucleoid}} \approx 1.55 \text{μm}$. Of necessity, there is no ribosome-rich annular region surrounding the nucleoid in the 1D model.

Within the dense part of the nucleoid region of the 1D model (Castellana et al., 2016), only ~34% of ribosomal subunits are carrying out co-transcriptional translation; ~29% are free subunits and the remaining ~37% are in short, free polysomes whose message was previously transcribed. The only way a free polysome born in the dense nucleoid region can escape to a ribosome-rich endcap is by 1D axial diffusion over an average distance of ~450 nm (SI, S3). In our 3D model of the experimental spatial distributions in slow growth (Fig. 8), most free polysomes born in the nucleoid region will escape by 2D radial diffusion to the annular shell of translating ribosomes surrounding the nucleoid. In SI, S3 and Fig. S12, we show that ~90% of free polysomes initially uniformly distributed within our 3D model nucleoid volume would escape radially by 2D diffusion rather than axially by 1D diffusion. The average distance traveled to escape is only ~60 nm (SI, S1), ~7 times less than in the 1D model. Since the root-mean square distance diffusively traveled in time $t$ is $\text{MSD} = (2Dt)^{1/2}$ in 1D (linear diffusion) and $\text{MSD} = (4Dt)^{1/2}$ in 2D (radial diffusion), for the same diffusion coefficient the free polysomes in the 1D model will remain in the dense nucleoid region ~100 times longer than they would in our 3D model.

Therefore, we feel justified in ‘re-assigning’ the ~37% free polysomes in the 1D nucleoid as the free polysomes in the annular region surrounding the nucleoid in our 3D model. This means that 34% + 37% = 71% of ribosomes within the nucleoid of the 1D model are slow-moving and would be assigned as translating, 70S ribosomes in our experiments. Stated the other way around, for comparison with the 1D model we discard the two endcaps and calculate the fraction of free 30S and translating 70S ribosomes in the remaining volume, which includes both the nucleoid spherocylinder and the surrounding tube of ribosomes. In that volume, our 3D partitioning model finds 63% 70S translating ribosomes and 37% free 30S subunits, in remarkably good agreement with the re-assigned 1D model results. This agreement is encouraging but it may be fortuitous, given the many assumptions involved in both models. We hope our new data provide impetus for development of a full, three-dimensional reaction-diffusion model of the transcription/translation machinery in slow-growing E. coli.

**Experimental procedures**

**Strain construction**

All experiments were performed on modified E. coli strains with a VH1000 background. For superresolution co-imaging of ribosomes and DNA binding protein HU in the same cell, we constructed a strain SM6 in which the S2 ribosomal protein was labeled with eYFP as before (Bakshi et al., 2012) and HU was labeled with photoactivatable fluorescent protein PAmcherry (Subach et al., 2009). The HU encoding gene (hupA) was amplified from MG1655 using the primers 5′-ATCGGAATTCGCGCCCGCTTAACCTCGTC-3′ and 5′-GATCGAATTCTAGGAGGTATTCATACGAAACAGACTC-3′ and then subcloned into the plasmid pASK-IBA3plus between EcoRI and BamHI sites to generate plasmid pSM5. The PAmcherry encoding gene was amplified with forward primer 5′-GATCGGATCCATGGTGAGCAAGGGCGAGG-3′ and reverse primer 5′-GATCTAAGCTTTTTACTTGTACAGCTCGTCGTC CATGCC-3′ and then subcloned into the above mentioned plasmid pSM5 expressing HU between the BamHI and HindIII sites. This resulted in plasmid pSM6, which expresses HU–PAmcherry upon induction with tetracycline. The construct MSG192 described in the work of Bakshi et al (2012) already contained a translational fusion of yfp to the C-terminus of rpsB (the gene encoding the S2 ribosomal protein). We transformed MSG192 with the plasmid pSM6, which expresses HU–PAmcherry. The transformants were selected on an ampicillin containing plate. A successful transformant was named strain SM6 (S2-YFP, HU–PAmcherry). The doubling time of this strain was measured to be 50 ± 7 min in our fast growth conditions (EZRDM media with glucose at 30°C) and 147 ± 18 min without induction in our slow growth conditions (MBM minimal media with glycerol at 30°C).

For single-molecule tracking of trajectories of diffusing ribosomes, we imaged S2 ribosomal proteins labeled with mEos2 (McKinney et al., 2009), a photoconvertible fluorescent protein in the strain MSG196. The construction of this strain is described in an earlier work (Bakshi et al., 2014).

**Cell growth and preparation for imaging**

For imaging cells under fast and slow growth conditions, 2 mL cultures from frozen glycerol stock were grown overnight with continuous shaking in EZRDM or MBM media respectively, in a 30°C water bath. We subsequently made subcultures by diluting the stationary phase culture at least 1:100 into 2 mL of fresh EZRDM or MBM as required. EZRDM is a morpholinepropanesulfonic acid (MOPS)-buffered solution with supplemental metal ions (M2130; Teknova), glucose (2 mg/ml), supplemental amino acids and vitamins (M2104; Teknova), nitogenous bases (M2103; Teknova), 1.32 mM $K_2HPO_4$ and 76 mM NaCl. Slow growth media MBM is also a MOPS-based medium with supplemental metal ions.
The strain SM6 that endogenously expresses S2–YFP and includes the plasmid expressing HU–PAmcherry was grown with addition of 100 µg/mL ampicillin. When the cells reach mid-log phase, anhydroteracycline was added to a final concentration of 45 nM to induce the expression of HU–PAmcherry. After 10 min of induction, the cells were centrifuged and resuspended in fresh growth media with 100 µg/mL ampicillin to remove the inducer. The cells were then incubated again in growth media for 15 min at 30°C to enable maturation of PAmcherry (Subach et al., 2009) prior to imaging.

After the subcultures had grown to midlog phase (OD600 = 0.3 – 0.6), ~150 µL of culture was placed in a CoverWell perfusion chamber (70326-56, Electron Microscopy Sciences, Radnor, PA) on a polylysine-coated clean coverslip to fill the entire chamber volume. We allowed ~2 min for the cells to adhere to the coverslip. The plated cells were then rinsed with fresh, warm, aerated growth media to wash away the non-adhered cells. The cells are maintained at 30°C throughout the imaging and imaged for no longer than 30 min after plating.

**Superresolution fluorescence imaging of ribosomes and DNA**

The cells were imaged using an Eclipse Ti inverted microscope (Nikon, Melville, NY) equipped with an oil immersion objective (CFI Plan Apo Lambda DM 100X oil, 1.45 N.A; Nikon Instruments), a 1.5X intermediate magnification lens and the Perfect Focus system (Nikon, Melville, NY). Fast shutters (Uniblitz LS2; Vincent Associates, NY) were used to synchronize illumination and image acquisition. Images were recorded by a back-illuminated EMCCD camera with 16 µm × 16 µm pixels (iXon DV – 887; Andor Technology, CT, USA). Each pixel corresponds to 105 × 105 nm2 at the sample with an overall magnification of 150X. All images were collected at a frame rate of 31.2 Hz, with an exposure time within each frame of 30 ns.

For superresolution co-imaging of ribosomes and HU in the strain SM6, first the S2-YFP molecules were imaged with the 514 nm laser with a power density of 2 kW/cm². Images were acquired only when the rate of return of molecules to the fluorescent state became small enough that at most 3-4 copies per camera frame are fluorescent in each cell, enabling single molecules to be distinguished from each other. Immediately after imaging of single S2-YFP copies at 514 nm, the HU–PAmcherry molecules were photoactivated with 405 nm diode laser (CrystaLaser, Reno, NV) and subsequently imaged with the 561 nm laser. The 405 nm power density at the sample was 1 to 10 W/cm² to ensure only 0–2 molecules of S2–mEos2 are fluorescent per camera frame per cell. The power density of the 561 nm laser was ~2 kW/cm². Emission was collected through a 617/73 band pass filter (bright line 617/73, Semrock, Rochester, NY).

In all imaging modes, the required lasers illuminated the sample for the entire duration of image acquisition. To minimize the phototoxic effects of the lasers, we collected data for <35 s per cell for each channel. This necessitates formation of composite, multi-cell distributions for both HU and S2.

**Single molecule image analysis**

Images were analyzed using a MATLAB graphical user interface (GUI) developed in our lab (Bakshi et al., 2011). Images were smoothed and filtered to obtain ero-based image. Bright spots were located with pixel-level accuracy by a peak finding algorithm that detects the local intensity maxima within an image. A user defined intensity threshold was used as the minimum brightness of a pixel arising from a single molecule. The threshold is carefully set by the user so that it will not be so high as to reject a real single molecule in the raw images or so low as to include background noise.

A modified MATLAB version of the tracking program written by Crocker and Grier (1996) was used. A centroid algorithm was used to locate the identified particles with subpixel resolution. Centroids of the bright spots were calculated from a 7 × 7 pixel square containing the entire bright spot, centered on the local maximum determined by the peak finding algorithm. The centroid positions from successive frames were connected to form a trajectory. For ribosomal and HU trajectories, the centroid positions are connected only if they lie within 3 px = 315 and 6 px = 630 nm of each other respectively.

To generate the spatial distribution of molecules from several cells such as the one shown in Fig. 2C–F, the camera based coordinates are reoriented so that the x axis and y axis correspond to the long and short cell axis and y axis respectively. For identifying spatial localizations within the cell of choice, cell outlines generated by Microbe Tracker (Sliusarenko et al., 2011) from phase contrast images are used.

**Calculation of modified pearson correlation coefficient (MPCC)**

The MPCC of two images R and G is evaluated as follows:

\[
\text{MPCC} = \frac{\sum_{i=1}^{m} \sum_{j=1}^{n} (R_{ij} - \bar{R}) (G_{ij} - \bar{G})}{\sqrt{\sum_{i=1}^{m} \sum_{j=1}^{n} (R_{ij} - \bar{R})^2} \sqrt{\sum_{i=1}^{m} \sum_{j=1}^{n} (G_{ij} - \bar{G})^2}}
\]
Here \( m \) and \( n \) are the number of rows and columns in the image matrices \( \mathbf{R} \) and \( \mathbf{G} \); there are \( m \times n \) total pixels in each image. The \( R_{ij} \) and \( G_{ij} \) are the corresponding intensities of pixel \( i,j \) in \( \mathbf{R} \) and \( \mathbf{G} \). \( U_R^{ij} \) and \( U_G^{ij} \) denote the intensity of pixel \( i,j \) in the 2D projection of a large set of molecules distributed randomly in a 3D spherocylinder. The total number of molecules in \( U_R \) and \( U_G \) has been scaled to be the same as the total number of molecules in \( R \) and \( G \) respectively.

\( R \) and \( G \) are generated by pixelating the composite distribution of spatial localizations of HU-PAmcherry and S2-YFP molecules from cells in the chosen length bin to 50 nm. We simulated two random distributions of 100,000 molecules each, corresponding to the ribosome (green) and HU (red) channels, using a spherocylinder whose dimensions match those of the chosen cells. The resulting reference images are normalized to give \( U_R \) and \( U_G \), which have same number of molecules as imaged ribosomes and HU. The two simulated 3D random distributions, \( U_R \) and \( U_G \) incorporated localization errors \( \sigma_{R,\text{nu}} \) and \( \sigma_{R,\text{rib}} \) determined by the intercepts of MSD plots for respective growth condition (Fig. S1). The detailed procedure for generating \( U_R \) and \( U_G \) is described elsewhere (Mohapatra and Weisshaar, 2018).

### Classifying the ribosome single-step displacements into two diffusive states

To investigate the diffusive behavior of ribosomes in live cells under different growth conditions, we chose trajectories that lasted at least seven frames. The chosen trajectories were divided into individual steps (two locations) with \( \Delta t = 30 \) ms. The resulting distribution \( P_{\text{rib}}(t) \) of experimental single-step displacements is modeled as a sum of two static (non-exchanging) populations: \( P_{\text{model}}(t) = f_{\text{slow}} P(r;D_{\text{slow}}) + (1 - f_{\text{slow}}) P(r;D_{\text{fast}}) \). First a series of numerical basis functions \( P(r;D) \) is generated for a series of diffusion coefficients \( D \) by simulating a large number of random walk trajectories that incorporate localization error \( \sigma \) and confinement within a spherocylinder that mimics the dimensions of an \( E. \) coli cell. The static localization error \( \sigma = 35 \) and 27 nm for fast and slow growth conditions respectively, is estimated from the intercept of the MSD(\( \tau \)) plots for ribosome S2-mEos2 molecules shown in Fig. S1. The experimental \( P_{\text{rib}}(t) \) is fit to a weighted average of two static populations, \( P_{\text{model}}(t) \), in a least-squares sense. The least-squares fitting procedure involved a numerical search for the lowest value of \( \chi^2 \) on a 3D grid of combinations of the three independent adjustable parameters \( (D_{\text{fast}}, D_{\text{slow}}, f_{\text{slow}}) \). We varied \( D_{\text{slow}} \) from 0.001 \( \mu \)m\(^2\)/s to 0.05 \( \mu \)m\(^2\)/s in increments of 0.001 \( \mu \)m\(^2\)/s. Similarly, \( D_{\text{fast}} \) was varied from 0.1 \( \mu \)m\(^2\)/s to 1 \( \mu \)m\(^2\)/s in increments of 0.01 \( \mu \)m\(^2\)/s. The parameter \( f_{\text{slow}} \) was scanned from 0 to 1 in intervals of 0.05. Additional details of generation of \( P_{\text{model}}(t) \) and fitting procedure are described in a previous work (Mohapatra et al., 2017). Bootstrapping the data into 10 subsets enabled us to obtain error estimates for each of the fitted parameters.

### Localization error of imaged molecules

The mean-square displacement MSD as a function of lag time \( \tau \) is given by \( \text{MSD}(\tau) = \langle r(t+\tau) - r(t) \rangle^2 \), where \( r(t) \) is the two-dimensional location of the particle at time \( t \), \( \tau \) is the lag time, and the average is taken over all times \( t \) and over many trajectories. We chose trajectories that lasted at least seven frames (six steps) for analysis. Tracks longer than seven frames were truncated to seven frames. Suppose the best fit to the first two experimental points of a two-dimensional mean-square displacement plot is given by the equation \( \text{MSD}(\tau) = a + b \tau \), with \( b \) the slope and \( a \) the extrapolated intercept at lag time \( \tau = 0 \). Then the best estimate of the diffusion coefficient is \( D = b/4 \) and the best estimate of the dynamic localization error is \( \sigma = \frac{\tau}{4} (a + 4D_{\text{ex}}/3)^{1/2} \), where \( \tau_{\text{ex}} \) is the exposure time per camera frame (Michalet, 2010).

In fast growing cells, we used 2117 trajectories to determine the mean diffusion coefficient of ribosome S2-YFP to be \( \langle D \rangle = 0.05 \pm 0.001 \mu \)m\(^2\)/s and the dynamic localization error to be \( \sigma = 36 \) nm (Fig. S1A, Red). For HU-PAmcherry, from 1180 trajectories we determined the mean diffusion coefficient \( \langle D \rangle = 0.25 \pm 0.04 \mu \)m\(^2\)/s and \( \sigma = 60 \) nm (Fig. S1A, Black). In slow growing cells, from 2692 trajectories we determined the mean diffusion coefficient of ribosome S2-YFP to be \( \langle D \rangle = 0.05 \pm 0.0017 \mu \)m\(^2\)/s and \( \sigma = 27 \) nm (Fig. S1B, Red). For HU-PAmcherry, using 9922 trajectories we determined the diffusion coefficient to be \( \langle D \rangle = 0.17 \pm 0.01 \mu \)m\(^2\)/s and \( \sigma = 55 \) nm (Fig. S1B, Black).

### Cell radius and length in fast and slow growth

We model the \( E. \) coli cytoplasm as a spherocylinder. For a given growth condition, the cytoplasmic radius varies little from cell to cell. To determine the radius \( r_{\text{cell}} \), we imaged photoactivatable Kaede molecules under the assumption that they distribute homogenously within the cytoplasmic volume (Bakshi et al., 2011). The exposure time was 2 ms/frame. For fast growth, we used data from 15 cells whose length based on phase contrast images falls within the bin 3.8-4.2 \( \mu \)m, the same range used for detailed analysis in Figs. 2, 3 and 6. For slow growth, we chose 15 cells of length 2.1-2.5 \( \mu \)m, matching Figs. 2, 4 and 7.

In each case, we chose Kaede trajectories that lasted for at least seven frames. Tracks longer than seven frames were truncated to seven frames. The dynamic localization error \( \sigma_{\text{kaede}} \) was estimated using the mean diffusion coefficient \( D_{\text{kaede}} \) and intercept of the MSD(\( \tau \)) plot. To estimate the cytoplasmic radius \( r_{\text{cell}} \), we then simulated Kaede diffusion using \( D_{\text{kaede}} \) and \( \sigma_{\text{kaede}} \) in a series of uniformly filled spherocylinders of varying radii, seeking a match to the experimental transverse spatial distribution. At \( t = 0 \), 15,000 particles were randomly distributed within the cell volume. Each particle undergoes an independent random walk. To model each 2 ms camera image, three-dimensional microtrajectories (1000 steps of 2 \( \mu \)s each) were generated. At each time step, each particle chooses a displacement in each of three Cartesian directions. These displacements are chosen from a Gaussian distribution whose standard deviation corresponds to the three-dimensional diffusion coefficient \( D_{\text{kaede}} \). In the rare event that a particle attempts to step outside of the cell boundaries, the displacement for that microstep is taken to be zero. The location of each particle during each camera frame is obtained as the centroid of the model microtrajectories in order to mimic the analysis procedure used for the experimental images. The appropriate dynamic localization
error was then applied to each centroid location in both x and y coordinates by sampling a Gaussian distribution with standard deviation $\sigma_{\text{Kaede}}$. By adding the error vector to the centroid position we obtain the model 'measured' location for each 2 ms camera frame. The x and y coordinates of each location are stored for further analysis. For the next model camera frame, each particle continues to make microsteps in 3D starting from the exact endpoint of the previous camera frame. Only the particles in the central cylindrical region are used to generate the simulated radial distribution; the endcaps are deleted.

For fast growing cells, we obtained $D_{\text{Kaede}} = 4.77 \pm 0.21$ $\mu$m$^2$ s$^{-1}$ and $\sigma_{\text{Kaede}} = 97$ nm using 709 trajectories. In slow growing cells, $D_{\text{Kaede}} = 5.23 \pm 0.16$ $\mu$m$^2$ s$^{-1}$ and $\sigma_{\text{Kaede}} = 95$ nm using 2126 trajectories. The measured $D_{\text{Kaede}}$ and $\sigma_{\text{Kaede}}$ in each growth condition were used to generate simulated transverse distributions in spherocylinders of varying radii.

For each of 15 cells in both fast and slow growth conditions, we found the best fit of the experimental distribution to the spherocylindrical model in a least-squares sense by varying the model radius in increments of 10 nm. Single cell experimental radial distribution of Kaede locations, excluding the endcap regions of the cell, along with best fits are shown in Figs. S2A (Black) and S2B (Black) for fast and slow growth conditions. Averaged over 15 cells, the mean cytoplasmic radius for fast growing cells is $r_{\text{cell}} = 412 \pm 22$ nm ($\pm 1$ SE). The mean cytoplasmic radius for slow growing cells is $r_{\text{cell}} = 290 \pm 14$ nm ($\pm 1$ SE).

For both fast and slow growing cells, a 2D composite scatter plot of Kaede localizations obtained from cells in the chosen length bin is used to estimate the tip-to-tip cell length $L_{\text{cell}}$, as illustrated in Fig. S2C. The points at which the scattered distributions begin to curve mark the beginnings of the hemispherical endcap regions and the ends of the cylindrical region. The error in determination of this boundary is $\pm 50$ nm, much smaller than the total cell length. The distance between these two boundary points becomes the length of the cylindrical region of the cell ($L_{\text{cyl}}$). The measured tip-to-tip cell length is then $L_{\text{cell}} = L_{\text{cyl}} + 2r_{\text{cell}}$. The result is $L_{\text{cell}} = 3.82$ $\mu$m for fast growing cells in the nominal length bin of 3.8–4.2 $\mu$m (based on rougher estimates from phase contrast). For slow growing cells of nominal length 2.1–2.5 $\mu$m, the result is $L_{\text{cell}} = 2.14$ $\mu$m.

**Nucleoid radius and length**

We also model the composite HU distributions in slow and fast growth conditions as spherocylinders. To determine the nucleoid radius and length in each growth condition, we followed a procedure similar to that used for determining cell width and length.

For fast growing cells, a 2D composite scatter plot of HU molecules in the two nucleoid lobes of 8 cells with nominal length in the range 3.8–4.2 $\mu$m is shown in Fig. S10C. The composite HU spatial distribution in each nucleoid lobe roughly resembles a 2D projection of a spherocylinder with hemispherical end caps. The experimental radial distribution of all HU localizations in the cylindrical region of each nucleoid lobe, symmetrized about the long axis of the cylinder, is generated (Fig. S10B, Black). To estimate the nucleoid radius $r_{\text{nucleoid}}$, we simulated projected 2D distributions from uniformly filled cylinders of varying radii in increments of 20 nm and broadened the distribution with $\sigma = 60$ nm, determined for HU molecules imaged in fast growing cells. The best-fit simulated radial distribution had $r_{\text{nucleoid}} = 190$ nm (Fig. S10B, dashed line). The simulated radial distribution does not fit the tails on either end of the experimental composite radial distribution. This is likely due to cell-to-cell heterogeneity in nucleoid shape, size and location relative to the cell body.

For measuring the nucleoid length $L_{\text{nucleoid}}$, the points at which the 2D composite scatter plot of individual nucleoids (Fig. S10C) begin to curve mark the beginnings of the hemispherical endcap regions and the ends of the cylindrical region. The distance between these two boundary points is the length of the cylindrical region of the nucleoid ($L_{\text{cyl}}$). The measured tip-to-tip cell length for each nucleoid lobe is then $L_{\text{nucleoid}} = L_{\text{cyl}} + 2r_{\text{nucleoid}}$. The reported $V_{\text{nucleoid}}$ for fast growing cells is the total volume of the two lobes.

For slow growing cells, we combined localizations of HU molecules imaged in cells with length in the length bin 2.1–2.5 $\mu$m. An experimental radial distribution of the HU molecules in the cylindrical region, symmetrized about the $x$-axis is generated (Fig. S6B, black). Similar to the method adopted for determining the nucleoid radius in fast growing cells, we generated a family of radial distributions of uniformly filled cylinders of varying radii broadened with $\sigma = 55$ nm, determined for HU molecules imaged in slow growing cells. The best-fit simulated radial distribution for slow growing cells had $r_{\text{nucleoid}} = 190$ nm (Fig. S6B, dashed line). $L_{\text{nucleoid}}$ was determined to be 1.55 $\mu$m following similar procedure.

For slow growing cells, the fit of the composite radial HU distribution to the spherocylinder model is very good. Single-cell HU distributions in slow growth are smooth and there is little cell-to-cell heterogeneity. The composite distribution is much more representative of the single-cell distribution in slow growth than in fast growth.

**Total number of ribosomes in fast and slow growth**

A detailed description of the procedure employed for counting ribosomes in the cells is provided in a previous work by Bakshi et al. (2012). We determined the copy number of ribosomes (S2-YFP) in the two different growth conditions by dividing the mean total ribosome S2-YFP intensity per cell belonging to the chosen length bin by the mean intensity of a single ribosome S2-YFP molecule. Intensities of fluorescent ribosome S2-YFP single molecules imaged over multiple cells are used to determine the mean intensity of a single ribosome S2-YFP molecule. The total intensity of YFP in individual cells is measured by averaging over the first 10 frames of imaging using a much smaller laser intensity to avoid photobleaching. A correction to the mean total YFP intensity per cell is applied to account for the difference in laser intensity used for imaging single molecule YFP fluorescence and total YFP fluorescence per cell. All other parameters of image acquisition for single molecule and wide field fluorescence imaging were kept constant. An additional small correction is applied to the total YFP intensity to account for the presence of immature YFP copies that do not contribute to the total cell fluorescence intensity, as per Eq. S8 of Ref. (Bakshi et al., 2012).
The mean copy number of ribosomes per cell was measured to be 56,000 in fast growing cells with length in the range of 3.8–4.2 µm and 21,000 in slow growing cells with length in the range of 2.1–2.5 µm. The number of ribosomes per cell correlates with the cell volume across the different growth conditions, as shown in Fig. S5.

Additional controls

To test whether fluorescently labeled HU faithfully stains the chromosomal DNA, we carried out experiments in which the same set of cells was imaged by both HU-YFP and Sytox Orange labeling. We previously showed that weak staining with Sytox dyes is a non-perturbative way to image the nucleoid (Bakshi et al., 2014). Two-color imaging enabled us to compare widefield images from both methods in the same cell. The two methods yield nearly identical results (Fig. S13).

To test for possible effects of fluorescently labeled HU expression levels on the DNA spatial distribution, we carried out experiments comparing the DNA spatial distribution obtained from the non-perturbative DNA stain Sytox Orange for cells which express HU-YFP and for wild type cells that do not express HU-YFP. We needed to change labels here because HU-PAmCherry fluorescence overlaps Sytox Orange fluorescence. The HU-YFP gene was encoded into the same type of plasmid that encodes HU-PAmCherry, so expression levels should be similar. In Fig. S14, for the slow growth condition we compare composite (averaged over 5 cells in the length bin 2.1–2.5 µm) widefield axial and radial distributions of Sytox Orange fluorescence from the same strain with and without induction of HU-YFP. The results are quite similar. The difference in FWHM of the composite radial distributions is <40 nm, insignificant compared with the differences between distributions across growth conditions.

As an additional test for possible growth defects caused by labeling of the ribosomal protein S2, we measured doubling times for all the label strains at 37°C. In addition, at 30°C we measured cell length distributions for the same strains to test for any phenotypic variation. The results are shown in Table S1 and Fig. S15. Growth defects in the ribosome-labeled strains are minor if any.

The ribosomal P(r) distributions in Fig. 5 were formed from all localizations from trajectories of at least six steps, truncated to six steps. To test for possible effects of the choice of the minimum trajectory length on the distribution, we also formed P(r) from trajectories of at least four steps, truncated to four steps. Results are shown in Fig. S16. The two distributions are essentially identical.

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Author contributions

JCW conceptualized the study. JCW and SM designed the experiments. SM was involved in data acquisition and analysis. JCW and SM interpreted the data. JCW and SM wrote the paper.

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**Supporting information**

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