

This is a repository copy of *Single-molecule imaging of DNA gyrase activity in living Escherichia coli*.

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/137893/>

Version: Accepted Version

---

**Article:**

Stracy, Mathew, Wollman, Adam [orcid.org/0000-0002-5501-8131](https://orcid.org/0000-0002-5501-8131), Kaja, Elzbieta et al. (9 more authors) (2019) Single-molecule imaging of DNA gyrase activity in living *Escherichia coli*. *Nucleic Acids Research*. pp. 210-220. ISSN 0305-1048

<https://doi.org/10.1093/nar/gky1143>

---

**Reuse**

Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

**Takedown**

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing [eprints@whiterose.ac.uk](mailto:eprints@whiterose.ac.uk) including the URL of the record and the reason for the withdrawal request.

1                   **Single-molecule imaging of DNA gyrase activity in living**  
2                   ***Escherichia coli***

3  
4 Mathew Stracy<sup>1</sup>, Adam J.M. Wollman<sup>2</sup>, Elzbieta Kaja<sup>5</sup>, Jacek Gapinski<sup>3</sup>, Ji-Eun Lee<sup>2</sup>,  
5 Victoria A. Leek<sup>4</sup>, Shannon J. McKie<sup>4</sup>, Lesley A. Mitchenall<sup>4</sup>, Anthony Maxwell<sup>4</sup>,  
6 David J. Sherratt<sup>1</sup>, Mark C. Leake<sup>2,\*</sup>, Pawel Zawadzki<sup>1,3,\*</sup>

7  
8 <sup>1</sup>Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1  
9 3QU, United Kingdom, <sup>2</sup>Biological Physical Sciences Institute (BPSI), Departments  
10 of Physics and Biology, University of York, York YO10 5DD, United Kingdom,  
11 <sup>3</sup>Molecular Biophysics Division, Faculty of Physics, A. Mickiewicz University,  
12 Umultowska 85, 61-614 Poznan, Poland, <sup>4</sup>Department of Biological Chemistry, John  
13 Innes Centre, Norwich Research Park, Norwich NR4 7UH, United Kingdom,  
14 <sup>5</sup>NanoBioMedical Centre, Adam Mickiewicz University, Umultowska 85, 61-614  
15 Poznan, Poland.

16  
17 \*To whom correspondence should be addressed. Email: [zawadzki@amu.edu.pl](mailto:zawadzki@amu.edu.pl) or  
18 [mark.leake@york.ac.uk](mailto:mark.leake@york.ac.uk)

19  
20 The authors wish it to be known that, in their opinion, the first two authors should be  
21 regarded as joint First Authors.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28

**ABSTRACT**

**Bacterial DNA gyrase introduces negative supercoils into chromosomal DNA and relaxes positive supercoils introduced by replication and transiently by transcription. Removal of these positive supercoils is essential for replication fork progression and for the overall unlinking of the two duplex DNA strands, as well as for ongoing transcription. To address how gyrase copes with these topological challenges, we used high-speed single-molecule fluorescence imaging in live *Escherichia coli* cells. We demonstrate that at least 300 gyrase molecules are stably bound to the chromosome at any time, with ~12 enzymes enriched near each replication fork. Trapping of reaction intermediates with ciprofloxacin revealed complexes undergoing catalysis. Dwell times of ~2 s were observed for the dispersed gyrase molecules, which we propose maintain steady-state levels of negative supercoiling of the chromosome. In contrast, the dwell time of replisome-proximal molecules was ~8 s, consistent with these catalyzing processive positive supercoil relaxation in front of the progressing replisome.**

## 1 INTRODUCTION

2 The double-helical structure of DNA leads to major topological problems during DNA  
3 replication and transcription. As DNA and RNA polymerases translocate along the  
4 chromosome they cause local over-winding of DNA ahead of them; if excessive  
5 positive (+) supercoiling accumulates it can inhibit the progress of the enzymes,  
6 leading to a shutdown of these essential cell processes. Furthermore, (+)  
7 supercoiling, which accumulates ahead of the replication fork, can diffuse backwards  
8 causing entanglement of daughter chromosomes, which must be unlinked before cell  
9 division can occur. In *Escherichia coli* these topological problems are resolved by  
10 two type II topoisomerases, DNA gyrase and DNA topoisomerase (topo) IV, which  
11 are essential enzymes that change topology by introducing transient double-  
12 stranded breaks into DNA and pass a second double-stranded DNA segment  
13 through the break before resealing it (1) (Figure 1A). Gyrase, the focus of this study,  
14 is formed from a dimer of GyrA, primarily responsible for DNA binding, and two GyrB  
15 subunits, which provide the ATPase activity.

16 In *E. coli*, the chromosome is maintained in a negatively (-) supercoiled state,  
17 and the appropriate level of supercoiling is important for regulation of almost all  
18 processes which take place on DNA, including transcription, replication, repair and  
19 recombination (2,3). For example, the expression level of many genes, including  
20 gyrase itself, is regulated by the level of supercoiling (4). Gyrase is unique in its  
21 ability to introduce (-) supercoils into DNA, and is therefore the central enzyme  
22 responsible for maintaining supercoiling homeostasis (5-8); however, local DNA  
23 supercoiling is constantly being altered by ongoing replication, transcription and  
24 repair. The activities of gyrase must therefore be responsive to these processes  
25 taking place in different regions of the chromosome (9,10).

26 The most acute topological problem arises during DNA replication, which is  
27 performed by two replisomes traveling in opposite directions around the circular  
28 chromosome at speeds of up to 1000 base pairs per second (bp/s) (11,12). Without  
29 the action of type II topoisomerases, replication of the 4.6 Mbp *E. coli* chromosome  
30 would result in two daughter chromosomes interlinked with a linking number of more  
31 than 440000 (given the DNA helical repeat of 10.4 base pairs). Type II  
32 topoisomerases change the linking number by 2 each catalytic cycle, and must  
33 therefore perform over 220 000 catalytic events before segregation can occur. When

1 the replisome is prevented from rotating around the DNA helix as it progresses, as  
2 originally suggested by Liu & Wang (13), (+) supercoils rapidly accumulate ahead of  
3 the replication fork. On the other hand, any rotation of the replication fork (14) allows  
4 (+) supercoils ahead of the fork to diffuse backwards forming precatenanes between  
5 the newly-replicated daughter chromosomes, which must be unlinked prior to  
6 chromosome segregation. Gyrase is inefficient in decatenation, and is believed to act  
7 ahead of the fork relaxing (+) supercoils, whereas topo IV acts preferentially behind  
8 the fork removing precatenanes (11,15,16).

9 To allow the replisome to maintain its incredibly high translocation rate, the  
10 two type II topoisomerases must relax up to 100 (+) supercoils per second for each  
11 fork (assuming a replisome translocation rate of 1000 bp/s, and DNA helical repeat  
12 of ~10bp) (Figure 1B). *In vitro*, the catalytic cycle for both gyrase and topo IV has  
13 been measured at ~2 s, with each cycle removing 2 supercoils (17-19), suggesting  
14 that up to 100 enzymes would be required per fork to keep up with the replication  
15 rate in live bacteria. Early studies of chromosome fragmentation in *E. coli* cells using  
16 the gyrase targeting drug, oxolinic acid (20), suggested that gyrase may be clustered  
17 near the replication fork. However, this raises the question of how so many gyrase  
18 enzymes can be acting ahead of the replication fork, while avoiding extremely toxic  
19 collisions with replication machinery. In single-molecule magnetic tweezers  
20 experiments *E. coli* gyrase was shown to act processively (18), confirming previous  
21 ensemble observations (1) and demonstrating that it is capable of performing  
22 multiple catalytic events without dissociating from DNA while relaxing (+) supercoils  
23 and introducing (-) supercoils (Figure 1A). More recent *in vitro* experiments on  
24 *Bacillus anthracis* gyrase suggests that gyrase 'bursting' activity might relax high  
25 levels of (+) supercoiling at faster rates (19). It remains to be established whether  
26 gyrase behaves processively or not *in vivo*, and whether its catalytic mode depends  
27 on the local supercoiling environment.

28 The action of gyrase is also essential for unperturbed transcription. Since  
29 coupling between RNA polymerase (RNAP) and (poly)ribosomes inhibits rotation of  
30 the transcription machinery, (+) supercoils accumulate ahead, and (-) supercoils  
31 behind, elongating RNAPs (Figure 1C) (7,9,21). While the rate of introduction of  
32 supercoils by a single RNAP is slow compared to replication (~60 bp/s, or ~6 (+)  
33 supercoils/s) (7-9), it is far more abundant. In a cell with 2 replisomes there are up to  
34 2000 RNAPs (22), introducing more (+) supercoiling overall than replication, but

1 distributed throughout the chromosome instead of accumulated in one region. The  
2 relative contribution of transcription and replication to gyrase activity is not clear.

3 We aimed to understand how gyrase acts in live *E. coli* cells and how  
4 topological problems arising during replication and transcription are resolved. Live  
5 cell epifluorescence showed that gyrase forms foci colocalized with active replication  
6 forks. However, single-molecule Slimfield (23,24) and photoactivated-localization  
7 microscopy (PALM) (25), showed that replication-dependent gyrase clusters  
8 comprise ~12 enzymes per replisome, while the remaining ~300 functional immobile  
9 enzymes interacted with the chromosome elsewhere to maintain steady-state levels  
10 of (-) supercoiling. An additional ~300 enzymes transiently interacted with dispersed  
11 regions of the chromosome. Measuring the dwell time of gyrase bound to DNA  
12 revealed that most gyrase remain immobile for ~2 s, whereas enzymes in the vicinity  
13 of the replisome had a ~8 s dwell time, suggesting that when an excessive (+)  
14 supercoiling is present due to the fast progression of the fork, gyrase performs  
15 multiple rounds of catalysis without dissociating from DNA.

16

## 17 **MATERIALS AND METHODS**

### 18 **Bacterial strains**

19 All strains were derivatives of *Escherichia coli* K-12 AB1157 (26). Replacement of  
20 endogenous genes with C-terminal fluorescent fusions was performed using  $\lambda$ -Red  
21 recombination with an *frt*-flanked kanamycin resistance (*kan*) cassette (27) using the  
22 primers listed in Table S1. The strains used in this study are: GyrApam  
23 (*gyrA::PAmCherry kan*); GyrBpam (*gyrB::PAmCherry kan*); PZ291 (*gyrA::mYPet*  
24 *kan*); PZ171 (*gyrA::PAmCherry kan, mYPet::DnaN frt*); PZ223 (*gyrA::mYPet kan,*  
25 *mCherry::DnaN frt*). See the Supplementary Materials and Methods for complete  
26 details of strain construction.

### 27 **Sample preparation**

28 Strains were streaked onto LB plates containing the appropriate antibiotics. Single  
29 colonies were inoculated into M9 media supplemented with 0.2% glycerol and grown  
30 overnight at 37°C to  $A_{600}$  0.4-0.6, diluted into fresh M9 glycerol and grown to  $A_{600}$

1 0.1. Cells were centrifuged and immobilized for imaging on 1% agarose (Bio-Rad)  
2 pads (made by mixing low-fluorescence 2% agarose in dH<sub>2</sub>O 1:1 with 2x growth  
3 medium) between 2 glass coverslips (Supplementary Methods).

#### 4 **Epifluorescence and colocalization microscopy**

5 Wide-field epifluorescence was performed using an Eclipse TE2000-U microscope  
6 (Nikon), equipped with 100x/NA1.4 oil objective and a Cool-Snap HQ<sup>2</sup> CCD. For  
7 colocalization analysis cell outlines were defined from phase contrast images using  
8 MicrobeTracker software (28). The positions of foci formed by mCherry-DnaN were  
9 established with Gaussian fitting (Supplementary Methods). Pairwise distances  
10 between the center of the brightest GyrA-mYPet pixel and the centroid of the nearest  
11 DnaN localization were calculated in MATLAB (MathWorks) from the square root of  
12 the squares of the summed coordinates in x and y. To determine the distribution of  
13 distances expected from chance GyrA localizations we calculated distances between  
14 a pixel randomly positioned within the cell and the centroid of the nearest DnaN  
15 focus. A threshold of 2 pixels (256 nm) was chosen to define colocalization.

#### 16 **Photoactivated Localization microscopy**

17 PALM microscopy was performed using a custom-built single-molecule microscope  
18 described in the Supplementary Materials and Methods. Photoactivatable mCherry  
19 activation was controlled with a 405 nm wavelength laser, and the photoactivated  
20 fluorophores were imaged with a 561 nm laser at 15.48 ms/frame for 30,000 frames.  
21 Data analysis was performed in MATLAB (MathWorks). Fluorescent signals from  
22 individual PAmCherry molecules in each frame were localized to ~40-nm precision  
23 by elliptical Gaussian fitting. Brightfield cell images were recorded from an LED  
24 source and condenser (ASI Imaging), and cell outlines were segmented with  
25 MicrobeTracker software (28). For colocalization analysis of super-resolved gyrase  
26 localizations with the replisome, snapshots of mYPet were taken with 488 nm  
27 excitation prior to PALM imaging of PAmCherry.

#### 28 **Single-particle tracking and diffusion analysis**

29 Localizations from PALM movies were linked together into trajectories using a  
30 MATLAB implementation of the algorithm described in ref (29). Positions were linked

1 to a track if they appeared in consecutive frames within a window of 5 pixels (0.48  
2  $\mu\text{m}$ ). In rare cases when multiple localizations fell within the tracking radius, tracks  
3 were linked such that the sum of step distances was minimized. We distinguished  
4 DNA-bound and diffusing proteins by calculating an apparent diffusion coefficient  
5  $D^* = \text{MSD} / (4\Delta t)$  from the mean-squared displacement (MSD) for each track with at  
6 least 4 steps at  $\Delta t = 15\text{ms}$  (30). Immobile molecules have a non-zero  $D^*$  value due to  
7 the localization uncertainty in each measurement,  $\sigma_{\text{loc}}$  (40nm), which manifests as a  
8 positive offset in  $D^*$  of  $\sim 0.1\mu\text{m}^2\text{s}^{-1}$ . Errors in  $D^*$  and fractions are SEM from fitting to  
9 at least 4 independent experimental repeats. Significance testing was performed  
10 using 2-sample  $t$ -tests of the fraction of immobile molecules extracted from these fits  
11 (Supplementary Materials and Methods).

12 **Dwell-time distributions using long exposure times** Long duration GyrA-  
13 PAmCherry binding was recorded at low continuous 561 nm excitation intensities  
14 using 1 s exposure times. The probability of observing a particular on-time is the  
15 product of the binding time and bleaching probabilities (30). The bleaching time  
16 distributions were measured independently using a control protein, MukB-  
17 PAmCherry, whose dwell time was previously shown to be  $\sim 1\text{ min} \gg$  bleaching time  
18 (31). MukB-PAmCherry was imaged with the same imaging conditions. On-time and  
19 bleaching time distributions were fitted with single-exponential functions to extract  
20 exponential time constants  $t_{\text{on}}$  and  $t_{\text{bleach}}$ , and the binding time constant calculated as  
21  $t_{\text{bound}} = t_{\text{on}} * t_{\text{bleach}} / (t_{\text{bleach}} - t_{\text{on}})$ . To determine binding times near the fork snapshots of  
22 mYPet-DnaN were taken prior to PALM imaging. DnaN foci were localized with  
23 Gaussian fitting and GyrA tracks within 200nm of a focus were used for binding time  
24 analysis. The bleaching time,  $t_{\text{bleach}} = 1.16 \pm 0.04$ . The uncorrected  $t_{\text{on}}$  time  
25 constants from 7 experimental repeats are shown in Table S2.

## 26 **Slimfield microscopy**

27 Slimfield microscopy was performed on a dual-color custom-made laser excitation  
28 single-molecule fluorescence microscope which utilized narrow epifluorescence  
29 excitation of 10  $\mu\text{m}$  full width at half maximum (FWHM) in the sample plane to  
30 generate Slimfield illumination from a 514 nm 20mW laser passed through a  $\sim 3\text{x}$   
31 Keplerian beam de-expander. Illumination was directed onto a sample mounted on  
32 an xyz nanostage (Mad City Labs, the Dane County, Wisconsin, USA). Imaging was



1 via a custom-made color splitter utilizing a dual-pass green/red dichroic mirror  
2 centered at long-pass wavelength 560 nm and emission filters with 25 nm  
3 bandwidths centered at 542 nm and 594 nm (Chroma Technology Corp.,  
4 Rockingham, Vermont, USA) onto an Andor iXon 128 emCCD camera, magnified to  
5 80 nm/pixel.

6 For dual color imaging we acquired 10 frames of brightfield, defocused to  
7 image the cell boundary, then acquired mCherry images by exciting with 1 mW 561  
8 nm laser until bleached after 500 frames. Then, the mYPet images were acquired,  
9 exciting with 10 mW of 514 nm laser for 500 frames. Brightfield imaging was  
10 performed with zero gain at 100 ms exposure time while single-molecule  
11 fluorescence was performed at maximum gain at 5ms/frame, with the addition of the  
12 561 nm laser for mCherry. Imaging of the single label mYPet-GyrA strain utilized  
13 only 514 nm laser excitation.

14 Stoichiometry was determined using a method which relies of step-wise  
15 photobleaching of fluorescent protein checked against surface immobilized purified  
16 mYPet using Chung-Kennedy filtration on single-molecule intensity bleach  
17 traces(24,32-38). Probability distributions for the relative displacement of GyrA-DnaN  
18 foci and for the stoichiometry of GyrA foci were rendered using kernel density  
19 estimation (KDE), a convolution of the data with a Gaussian kernel which has an  
20 advantage in objectifying the appearance of the distribution as opposed to using  
21 semi-arbitrary bin widths on a histogram plot. The kernel width was set to the  
22 appropriate experimental precision (0.7 molecules for the stoichiometry distribution  
23 and 40 nm for the distance estimates). See Supplementary Materials and Methods.

24

## 1 RESULTS

### 3 Gyrase foci colocalize with the replisome

4 To characterize gyrase activity in live cells we replaced the endogenous *gyrA* gene  
5 with a fusion to the fluorescent protein mYPet. Cells with *gyrA-mYPet* showed  
6 normal growth indicating the fusion is functional (Supplementary Figure S1A), and  
7 purified GyrA-mYPet showed normal supercoiling activity *in vitro* (Figure 1D). Using  
8 epifluorescence, gyrase formed foci in  $70 \pm 6$  % ( $\pm$ SD) of cells, with the remaining  
9 cells showing a diffuse fluorescent signal, consistent with gyrase localization  
10 throughout the chromosome (Figure 2).

11 Since gyrase is thought to remove (+) supercoils ahead of the replication fork,  
12 we constructed a strain expressing GyrA-mYPet and a replisome marker mCherry-  
13 DnaN (11). We find that the region with highest gyrase density is frequently  
14 colocalized with the replisome (Figure 2A), reflecting earlier findings from *B. subtilis*  
15 (39). To quantify colocalization we used Gaussian fitting to localize the replisome foci  
16 and examined the cumulative distributions of distances between the brightest pixel of  
17 gyrase signal and the nearest replisome focus within each cell (Figure 2B). To  
18 control for colocalization due to random coincidence we performed the same  
19 analysis with a simulated random gyrase focus position within the same cells,  
20 showing that  $80 \pm 4$ % of the brightest gyrase pixels were located within 2 pixels (256  
21 nm) from the replisome, compared to  $15 \pm 3$ % from random coincidence.

22 In the slow growth conditions used for our experiments, a single round of  
23 replication takes only  $\sim 2/3$  of the cell doubling time, leaving a population of young  
24 cells that have not initiated replication or cells approaching division that have  
25 completed replication (Figure 2A). Since the fraction of cells lacking replication foci  
26 ( $\sim 25$ % identified with spotFinder (28)) was similar to the fraction of cells lacking  
27 gyrase foci ( $\sim 30$ %), we asked whether the presence of gyrase foci was dependent  
28 on ongoing replication; in cells without a DnaN focus only  $30 \pm 10$ % of these non-  
29 replicating cells had a distinct gyrase focus. Taken together, this analysis suggests  
30 that distinct gyrase foci are largely associated with replication forks.

### 32 Slimfield microscopy reveals gyrase clusters of $\sim 12$ enzymes

1 Epifluorescence microscopy provides a description of the ensemble behavior of  
2 fluorescently labeled proteins inside cells, however it cannot provide a quantitative  
3 assessment of protein activity at the level of individual molecules. To enable single-  
4 molecule quantification of gyrase localization we used Slimfield microscopy on GyrA-  
5 mYPet in live cell (23,40), providing a ~40 nm spatial precision over a millisecond  
6 temporal resolution to enable blur-free analysis of individual proteins (SI Movie 1).  
7 Qualitatively, the patterns of GyrA localization with respect to DnaN (Figure 3A,  
8 Supplementary Figure S3) were similar to those observed earlier for epifluorescence  
9 (Figure 2A). Using analysis based on the integrated pixel intensity of Slimfield  
10 images (40) we quantified the GyrA copy number, giving 1300-3300 molecules per  
11 cell across all cells, which agrees broadly with earlier estimates based on immuno-  
12 gold electron microscopy of fixed *E. coli* cells (41).

13 To estimate the number of gyrase in localized clusters we used custom-  
14 written localization software to automatically track GyrA foci (42). We determined the  
15 stoichiometry of each as the initial focus brightness divided by the brightness of a  
16 single mYPet (32) (Material and Methods). Given the rate of relaxation of 2 positive  
17 supercoils per ~2 s previously reported for gyrase (18,43-45) and assuming minimal  
18 involvement of topo IV, we expected clusters to comprise of up to 100 gyrase (since  
19 100 enzymes are required to keep up with a replication rate of 1000 bp/s). However,  
20 the intensity of these foci indicated a mean of  $24 \pm 2$  ( $\pm$ SEM) GyrA molecules (i.e. just  
21  $12 \pm 1$  putative heterotetramer enzymes); note a key advantage of this single-  
22 molecule approach over ensemble methods is to render not just the mean value but  
23 also the full probability distribution, which we measure as having a broad range from  
24 a minimum of 2 molecules to over 100 per focus (Figure 3B). Using numerical  
25 integration of the overlap integral between green and red channel foci we observed  
26 that ~85% of all foci were colocalized with DnaN, comparable to epifluorescence.  
27 The relative separation between DnaN and GyrA foci centers was not peaked at zero  
28 but instead had a mean of  $135 \pm 14$  ( $\pm$ SEM) nm, exhibiting a unimodal distribution  
29 which extended up to ~400 nm (Figure 3C), larger than the ~50 nm replisome  
30 diameter, suggesting that gyrase does not act in tight proximity to the replisome. The  
31 hypothesis that gyrase acts at a distance from the fork might explain how collisions  
32 between the replisome and gyrase performing catalysis are prevented, however we  
33 note that while DnaN forms diffraction-limited foci, it has been shown that their

1 dissociation rate is slow and hence the focus centroid may be slightly behind the  
2 replication fork (46).

3

#### 4 **Photoactivated-localization microscopy and single-particle tracking of gyrase**

5 To explore the mobility of single gyrase we used photoactivated-localization  
6 microscopy (PALM), combined with single-particle tracking (sptPALM) (25), enabling  
7 localization and tracking of individual GyrA by controlling the photoactivation of a  
8 photoactivable fluorescent protein such that on average one fluorophore was active  
9 per cell at any given time. We labeled GyrA genomically with photoactivable  
10 mCherry (PAmCherry) (Figure 1D, Supplementary Figure S1A) and imaged cells  
11 with a PALM microscope at 15 ms intervals for 30,000 frames. Linking consecutive  
12 GyrA localizations from each frame into tracks allowed us to track gyrase movement  
13 until photobleaching (Figure 4B) (25,30).

14 We calculated an apparent diffusion coefficient ( $D^*$ ) for each GyrA from the  
15 mean squared displacement of its track (Materials and Methods). We fitted an  
16 analytical expression (22,47) to the distribution of  $D^*$  values from all 85529  
17 measured tracks. We found that the distribution of  $D^*$  values was best described by  
18 a three-species model: immobile ( $46 \pm 5\%$ ;  $D_{\text{imm}}=0.1 \mu\text{m}^2\text{s}^{-1}$  set by the localization  
19 precision), slow-diffusing ( $42 \pm 4\%$ ;  $D_{\text{slow}}=0.25 \pm 0.01 \mu\text{m}^2\text{s}^{-1}$ ) and fast-diffusing ( $12 \pm$   
20  $4\%$ ;  $D_{\text{fast}}=0.82 \pm 0.10 \mu\text{m}^2\text{s}^{-1}$ ) populations (Figure 4C). Fitting one or two species to  
21 the  $D^*$  distribution provided a poor description of the data (Supplementary Figure  
22 S1B,C).

23 We interpret immobile tracks as DNA-bound gyrase and fast-diffusing tracks  
24 as gyrase undergoing free 3D diffusion, possibly GyrA molecules not incorporated  
25 into functional gyrase heterotetramers with GyrB. Slow-diffusing gyrases have lower  
26 mobility than expected for free 3D diffusion, consistent with transient interactions  
27 with DNA without engaging in stable binding required for catalysis.

28 To assess gyrase expression, we photoactivated and tracked all GyrA-  
29 PAmCherry molecules present in each cell, indicating a mean of  $\sim 1450 \pm 550$  (SD)  
30 GyrA per cell (Figure 4A). We note that the copy number measured using PALM may  
31 underestimate the true copy number due to a population of PAmCherry which do not  
32 become fully photoactivatable (although this has never been characterized in  
33 bacteria) (48). Nevertheless, this estimate falls within the range estimated earlier

1 from Slimfield microscopy, which does not use photoactivatable fluorescent proteins  
2 and hence does not suffer the same technical issue. For simplicity we have based all  
3 calculations derived from PALM experiments on the unmodified mean copy number  
4 of 1450 GyrA, but we acknowledge that the true copy number could potentially be up  
5 to two-fold larger.

6 To estimate the proportion of GyrA able to form functional heterotetramers,  
7 we treated GyrA-PAmCherry cells with ciprofloxacin, which traps gyrase on DNA by  
8 stabilizing the covalently linked DNA-gyrase complex formed during catalysis (49).  
9 We find that  $80 \pm 3\%$  of GyrA are immobile after drug treatment (Figure 4D), a  
10 significant increase ( $p = 6 \times 10^{-5}$ ) from unperturbed cells and more than twenty-fold  
11 higher than early estimates of  $\sim 45$  stabilized gyrase based on chromosome  
12 fragmentation with the much less potent quinolone, oxolinic acid (50). Since  
13 ciprofloxacin is not known to be able to capture gyrase subunits not incorporated into  
14 heterotetramers, and only stabilizes enzymes during catalysis, this demonstrates  
15 that the GyrA-PAmCherry stabilized on DNA after ciprofloxacin treatment were  
16 incorporated into functional enzymes that underwent catalysis. Assuming a copy  
17 number of 1450 GyrA subunits, of which 12% are fast-diffusing putative  
18 unincorporated subunits, our findings show that in an average cell there is enough  
19 GyrA to form  $\sim 600$  functional enzymes, of which  $\sim 300$  are DNA-bound and likely  
20 performing catalysis.

21

## 22 **Gyrase activity in cells not undergoing replication or transcription**

23 Epifluorescence microscopy indicates that gyrase foci are less common in cells not  
24 undergoing replication (Figure 2C). These cells show only a minimal reduction in the  
25 fraction of DNA-bound, immobile GyrA compared to replicating cells (Figure 4C and  
26 5A) from  $46 \pm 5\%$  immobile GyrA to  $44 \pm 5\%$ , within statistical error, equating to a  
27 difference of just  $\sim 15$  additional gyrase enzymes per cell (with 2 replisomes), broadly  
28 consistent with Slimfield observations suggesting an average of  $\sim 12$  gyrase  
29 associated with each replisome.

30 We constructed a mYPet-DnaN, GyrA-PAmCherry strain to determine  
31 positions of replisomes relative to PALM-tracked gyrase (Figure 5B). The fraction of  
32 immobile gyrase 'proximal' (within 200 nm) to the replisome is  $16 \pm 12\%$  which, when  
33 corrected by a fraction of simulated randomly distributed gyrase in the same region

1 (8 ± 0.5%), equates to ~25 more gyrase located next to both replisomes than  
2 expected from a random distribution, consistent with the small reduction of immobile  
3 gyrase observed in non-replicating cells (Figure 5A). In summary, on average only 8-  
4 12 gyrase are involved in relaxation of (+) supercoiling introduced by each replisome,  
5 and most of the remaining ~300 DNA-bound gyrases are immobile throughout the  
6 rest of the chromosome. To test where immobile gyrase is catalytically active we  
7 treated cells with ciprofloxacin and analyzed the distribution of immobile molecules  
8 within the cells. We found immobile gyrase throughout the chromosome  
9 (Supplementary Figure S2B), suggesting that molecules close to and far from the  
10 replisome perform catalysis.

11 Gyrase not associated with the replisome could be relaxing (+) supercoils  
12 introduced by RNAP or be involved in maintaining steady-state levels of  
13 chromosomal (-) supercoiling. To distinguish these possibilities, we treated cells with  
14 the transcription initiation inhibitor rifampicin, resulting in a moderate reduction (by  
15 11%) in the fraction of immobile gyrase (Figure 5C), consistent with earlier  
16 experiments which showed that rifampicin reduces plasmid supercoiling (51)  
17 Nevertheless, since 33% of gyrase remain immobile after rifampicin treatment, this  
18 suggests that gyrase performs its activity even when no (+) supercoils are being  
19 introduced due to transcription. We conclude that the role of the majority of gyrase in  
20 the cell is not directed towards relaxing (+) supercoiling introduced by replication, but  
21 rather towards maintaining steady-state chromosomal supercoiling.

22

### 23 **Different modes of gyrase**

24 To address the conundrum of how a low number of gyrase in the vicinity of the  
25 replisome can relax up to 100 supercoils per second, we aimed to determine  
26 whether the catalytic mode depended on proximity to the replisome. To do this we  
27 measured the binding time of gyrase inside live cells using sparse photoactivation  
28 with a low excitation intensity and long (1s) exposure time. Under these conditions  
29 mobile gyrases are motion blurred, whereas immobile molecules appear as distinct  
30 diffraction-limited foci (30,52) (Figure 6A).

31 The observed dwell time for gyrase was corrected for photobleaching as  
32 described previously (30), giving a mean binding time of 2.4 ± 0.5s (Figure 6B,  
33 Figure S4). As a control we also measured the binding time of topo IV using a ParC-

1 PAmCherry fusion strain from our previous study, described in reference 16. For  
2 topo IV we measured a similar binding time ( $1.7 \pm 0.2$  s), consistent with the rate of  
3 ATP hydrolysis estimated *in vitro* for both enzymes (18,53). Ciprofloxacin resulted in  
4 a drastic increase in the fraction of immobile molecules (Figure 4D) as well as  
5 increasing the binding time (to at least 30 s, the upper limit of our assay), indicative  
6 of gyrase trapped during its catalytic cycle (Figure 6B). We suggest that bound  
7 gyrase exhibiting binding times of  $\sim 2.5$  s are undergoing single rounds of catalytic  
8 activity, however we cannot exclude the possibility that some gyrase bind DNA  
9 without performing catalysis.

10 While the observed binding time for gyrase is consistent with rates measured  
11 *in vitro* (17,18), it does not resolve the puzzle of how gyrase foci comprised of only  
12  $\sim 10$  molecules relax (+) supercoils at a rate sufficient for replication fork progression  
13 at up to 1000 bp/s. By taking a snapshot of replication foci prior to measuring binding  
14 times, we categorized binding events taking place within ('proximal') or beyond 200  
15 nm ('distal') from a mYPet-DnaN replisome marker. The binding time of distal gyrase  
16 ( $2.5 \pm 0.4$  s) shows no significant difference from  $2.4 \pm 0.5$  s measured for the entire  
17 population (Figure 6C); however, proximal gyrase has a significantly longer binding  
18 time ( $7.7 \pm 1.5$  s). We propose that the longer binding time close to the replisome  
19 results from gyrase performing multiple rounds of catalytic activity without  
20 dissociating, which is facilitated by the high level of (+) supercoiling ahead of the  
21 fork.

22

## 23 **DISCUSSION**

24 DNA gyrase has been the subject of many biochemical and structural studies  
25 since its discovery in 1976 (1,43), however, many questions remain regarding how it  
26 acts in living cells. For example, *in vitro* gyrase can relax (+) supercoils, and also  
27 introduce (-) supercoils into relaxed DNA. Yet, little is known about what proportion  
28 of gyrase activity is directed towards different DNA substrates in the cell: removing  
29 (+) supercoiling introduced by replication, removing (+) supercoiling introduced by  
30 transcription, and maintaining steady-state (-) supercoiling of the chromosome. The  
31 relative activities of gyrase and topo IV during replication also remains a mystery.  
32 Furthermore, while *in vitro* studies have observed different modes of gyrase  
33 catalysis, it remains to be established if the catalytic mode depends on the substrate

1 *in vivo*. In this work we have used a combination of live-cell fluorescence microscopy  
2 techniques, with the aim of bridging the gap between our understanding of how  
3 gyrase acts in the test tube, to how it behaves in the native environment inside living  
4 cells. While the super-resolution techniques used in this study cannot rival the  
5 atomic-level precision of structural biology studies, placing limitations on the extent  
6 of what we can really know about the activity of any individual gyrase enzyme, they  
7 offer an order of magnitude better spatial resolution than the standard optical  
8 resolution limit, and come with the substantive advantage that it is performed in living  
9 cells and thus allows us to answer questions which are impossible to answer with  
10 structural biology or *in vitro* biochemical techniques alone, such as ‘how many  
11 gyrase act in proximity to the replication fork?’

12         Based on PALM and Slimfield analysis we estimate that an average of ~600  
13 gyrase per cell are present of which ~300 are tightly DNA-bound and presumably  
14 performing catalysis. We find that gyrase forms foci which colocalize with replisomes  
15 and comprise on average of ~10 gyrase enzymes. In agreement with this, the  
16 fraction of DNA-bound gyrase is reduced by only a few % in cells that had either not  
17 yet initiated replication or had terminated replication but not divided. Despite the  
18 regions with the highest gyrase occupancy being close to the replisome, the vast  
19 majority of gyrase are immobile elsewhere on the chromosome. In a cell containing  
20 two replisomes there are at least ~1000 transcribing RNAPs, introducing (+)  
21 supercoils with an overall rate up to 30-fold higher than replication (~6000 compared  
22 to ~200 supercoils/s) (6,22). Since we find only ~20 out of 300 immobile gyrase are  
23 involved in relaxation of (+) supercoils introduced by replication, we expected the  
24 ~280 remaining to participate in relaxation of (+) supercoils introduced by  
25 transcription. We find that the fraction of immobile gyrase is reduced only modestly  
26 after transcription is blocked with rifampicin, indicating that the primary activity of  
27 gyrase is instead directed towards maintaining a steady-state level of (-)  
28 supercoiling, with a caveat that rifampicin itself has a major effect on nucleoid  
29 organization through decompaction (22), which may influence gyrase activities in an  
30 unknown way. Since the time taken to transcribe an average gene is short, it is  
31 inevitable that some of the (+) and (-) supercoiling created during transcription is  
32 cancelled out after RNAP dissociation. Similarly, on highly-expressed genes (+)  
33 supercoils produced ahead of multiple RNAPs will be neutralized by (-) supercoils  
34 introduced behind. Our results show that gyrase activity should not be considered as



1 merely removing (+) supercoils to ensure unimpeded progression of transcription  
2 and replication, but contributes to multiple interdependent processes affecting global  
3 chromosome organization and segregation.

4 During replication of the chromosome over 220,000 catalytic events by the  
5 combined action of topo IV and gyrase must be performed, with gyrase removing (+)  
6 supercoils ahead of the replication fork and topo IV decatenating interlinked daughter  
7 chromosomes caused by diffusion of (+) supercoils behind the fork. These processes  
8 can occur simultaneously, yet the division of catalytic events between gyrase and  
9 topo IV during replication remains to be determined. Unlike gyrase, topo IV does not  
10 form foci in the proximity of the replisome (16,39,54). Nevertheless, blocking of topo  
11 IV prevents decatenation-segregation of all loci tested (11,16), demonstrating that  
12 the replisome can rotate and introduce precatenanes. Indeed, recent findings that  
13 most components of the replisome turnover every few seconds (55), suggest that the  
14 replisome is unlikely to be a barrier to replication fork rotation. The copy number of  
15 topo IV is much lower than gyrase; our previous measurements of topo IV under the  
16 same growth conditions as this study, showed that ~30 DNA-bound enzymes are  
17 present per cell, and the action of 1/3 of these are dependent on ongoing replication,  
18 indicating that during replication ~10 topo IVs are performing decatenation per cell  
19 (~5 per replication fork), most of which will be distal from the progressing forks since  
20 decatenation takes ~12 min (16).

21 The combined action of ~5 topo IV and ~10 gyrase enzymes per replication  
22 fork is nearly 10-fold lower than the number theoretically needed to keep up with  
23 replication rate, given the catalytic rate for both enzymes, which has been measured  
24 at ~1 supercoil/s. Importantly, topo IV is unlikely to decatenate processively, since *in*  
25 *vitro* topo IV acts distributively on (-) supercoils (with the same local topology as  
26 right-handed replicative catenanes) (56,57), confirmed by our previous  
27 measurements of topo IV dwell times (16). In contrast, gyrase can remove (+)  
28 supercoils processively *in vitro* (17-19), consistent with our observations that its dwell  
29 time significantly increases close to the replisome. Previous *in vitro* measurements of  
30 the processive catalytic rate were the same as for distributive catalysis (1  
31 supercoil/s), and thus remains insufficient to account for the rate of supercoils  
32 introduced by each replisome (up to 100 supercoil/s). Intriguingly, a recent single-  
33 molecule *in vitro* study suggests that processive relaxation of (+) supercoils by *B.*

1 *anthracis* gyrase may be faster than previously measured for *E. coli* gyrase (18), with  
2 mean of ~6 supercoils/s (19), though with individual bursts of catalysis measured as  
3 high as  $107 \pm 23$  supercoils/s. Therefore, we suggest that the acute topological  
4 problem introduced by replication is primarily dealt with by gyrase enzymes  
5 performing processive catalysis to remove (+) supercoils ahead of replication,  
6 possibly at a higher rate than 1 supercoils/s, and we speculate that when gyrase fails  
7 to remove sufficient (+) supercoiling, replisome rotation is induced forming a  
8 substrate for topo IV behind the fork. However, it remains to be established whether  
9 *E. coli* gyrase *in vivo* can perform bursts of processive catalysis at higher rates than  
10 1 supercoil/s.

11 The *E. coli* chromosome is organized into looped topological domains  
12 (8,21,58), within which supercoils can rapidly diffuse (5) and thus may delimit gyrase  
13 activity. Since the global net supercoiling of the chromosome is (-), most DNA loops  
14 will be relaxed or (-) supercoiled, and gyrase binding to these regions will perform a  
15 single round of catalysis. Our data suggest that local supercoiling may strongly  
16 influence gyrase off-rate, as we find with replication proximal gyrase remaining  
17 immobile for >8s. Since the fork progresses at a rate of up to 1000 bp/s this would  
18 require initially binding ~10 kbp ahead of the fork to avoid collisions rather than  
19 directly ahead of it. This predicts a displacement of gyrase foci in relation to  
20 replisome position. Indeed, Slimfield analysis (Figure 3C) showed that gyrase and  
21 replisome foci are displaced by ~100 nm. Therefore, diffusing (+) supercoils may  
22 promote processive catalysis of gyrase bound many kbp away from replication,  
23 which could help to protect against detrimental gyrase-fork collisions.

24 Together, our results show that *in vivo* a small number of gyrase acting  
25 processively ensures unimpeded progression of the replisome, while a majority of  
26 gyrase is involved in maintaining steady-state levels of chromosome supercoiling.

27  
28  
29

1 **Data availability.** Data included in full in the main text and supplementary files. Raw  
2 data available from the authors.

3  
4 **Author contributions:** M.S., D.J.S., M.C.L., and P.Z., designed research. M.S.,  
5 A.J.M.W., E.K., J.G., J.-E.L., V.A.L., S.J.M., L.A.M., P.Z., performed experiments  
6 and analyzed data. M.S., D.J.S., M.C.L., A.M., and P.Z., wrote the paper.

## 7 8 **FUNDING**

9 This work was supported by: the National Science Centre Poland  
10 [2015/19/P/NZ1/03859 to P.Z.] and FNP [First TEAM/2016-1/9 to P.Z.]; the Medical  
11 Research Council [MR/K01580X/1 to M.L.]; the Biotechnology and Biological  
12 Sciences Research Council [BB/N006453/1 to M.L., BB/R001235/1 to M.L. A.M., J.-  
13 E.L., BB/J004561/1, BB/P012523/1 to A.M.]; The Wellcome Trust through the Centre  
14 for Future Health at University of York [204829 to A.J.M.W.]; The Wellcome Trust  
15 [099204/Z/12Z to D.J.S.] and via a Sir Henry Wellcome Fellowship [204684/Z/16/Z to  
16 M.S.]; the Leverhulme Trust [RP2013-K-017 to A.M.]; and a Junior Research  
17 Fellowship at Trinity College Oxford [to M.S].

## 18 19 **REFERENCES**

- 20 1. Bush, N.G., Evans-Roberts, K. and Maxwell, A. (2015) DNA Topoisomerases. *EcoSal Plus*, **6**.
- 21 2. Lal, A., Dhar, A., Trostel, A., Kouzine, F., Seshasayee, A.S. and Adhya, S. (2016) Genome scale  
22 patterns of supercoiling in a bacterial chromosome. *Nature communications*, **7**, 11055.
- 23 3. Dorman, C.J. and Dorman, M.J. (2016) DNA supercoiling is a fundamental regulatory  
24 principle in the control of bacterial gene expression. *Biophysical reviews*, **8**, 89-100.
- 25 4. Peter, B.J., Arsuaga, J., Breier, A.M., Khodursky, A.B., Brown, P.O. and Cozzarelli, N.R. (2004)  
26 Genomic transcriptional response to loss of chromosomal supercoiling in Escherichia coli.  
27 *Genome biology*, **5**, R87.
- 28 5. Koster, D.A., Crut, A., Shuman, S., Bjornsti, M.A. and Dekker, N.H. (2010) Cellular strategies  
29 for regulating DNA supercoiling: a single-molecule perspective. *Cell*, **142**, 519-530.
- 30 6. Vos, S.M., Tretter, E.M., Schmidt, B.H. and Berger, J.M. (2011) All tangled up: how cells  
31 direct, manage and exploit topoisomerase function. *Nature reviews. Molecular cell biology*,  
32 **12**, 827-841.
- 33 7. Rovinskiy, N., Agbleke, A.A., Chesnokova, O., Pang, Z. and Higgins, N.P. (2012) Rates of  
34 gyrase supercoiling and transcription elongation control supercoil density in a bacterial  
35 chromosome. *PLoS genetics*, **8**, e1002845.

- 1 8. Postow, L., Hardy, C.D., Arsuaga, J. and Cozzarelli, N.R. (2004) Topological domain structure  
2 of the Escherichia coli chromosome. *Genes & development*, **18**, 1766-1779.
- 3 9. Menzel, R. and Gellert, M. (1983) Regulation of the genes for E. coli DNA gyrase:  
4 homeostatic control of DNA supercoiling. *Cell*, **34**, 105-113.
- 5 10. Zechiedrich, E.L., Khodursky, A.B., Bachellier, S., Schneider, R., Chen, D., Lilley, D.M. and  
6 Cozzarelli, N.R. (2000) Roles of topoisomerases in maintaining steady-state DNA supercoiling  
7 in Escherichia coli. *The Journal of biological chemistry*, **275**, 8103-8113.
- 8 11. Wang, X., Reyes-Lamothe, R. and Sherratt, D.J. (2008) Modulation of Escherichia coli sister  
9 chromosome cohesion by topoisomerase IV. *Genes & development*, **22**, 2426-2433.
- 10 12. Reyes-Lamothe, R., Possoz, C., Danilova, O. and Sherratt, D.J. (2008) Independent positioning  
11 and action of Escherichia coli replisomes in live cells. *Cell*, **133**, 90-102.
- 12 13. Liu, L.F. and Wang, J.C. (1987) Supercoiling of the DNA template during transcription.  
13 *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 7024-  
14 7027.
- 15 14. Schalbetter, S.A., Mansoubi, S., Chambers, A.L., Downs, J.A. and Baxter, J. (2015) Fork  
16 rotation and DNA precatenation are restricted during DNA replication to prevent  
17 chromosomal instability. *Proceedings of the National Academy of Sciences of the United  
18 States of America*, **112**, E4565-4570.
- 19 15. Joshi, M.C., Magnan, D., Montminy, T.P., Lies, M., Stepankiw, N. and Bates, D. (2013)  
20 Regulation of sister chromosome cohesion by the replication fork tracking protein SeqA.  
21 *PLoS genetics*, **9**, e1003673.
- 22 16. Zawadzki, P., Stracy, M., Ginda, K., Zawadzka, K., Lesterlin, C., Kapanidis, A.N. and Sherratt,  
23 D.J. (2015) The Localization and Action of Topoisomerase IV in Escherichia coli Chromosome  
24 Segregation Is Coordinated by the SMC Complex, MukBEF. *Cell Rep*, **13**, 2587-2596.
- 25 17. Nollmann, M., Crisona, N.J. and Arimondo, P.B. (2007) Thirty years of Escherichia coli DNA  
26 gyrase: from in vivo function to single-molecule mechanism. *Biochimie*, **89**, 490-499.
- 27 18. Nollmann, M., Stone, M.D., Bryant, Z., Gore, J., Crisona, N.J., Hong, S.C., Mitelheiser, S.,  
28 Maxwell, A., Bustamante, C. and Cozzarelli, N.R. (2007) Multiple modes of Escherichia coli  
29 DNA gyrase activity revealed by force and torque. *Nature structural & molecular biology*, **14**,  
30 264-271.
- 31 19. Ashley, R.E., Dittmore, A., McPherson, S.A., Turnbough, C.L., Jr., Neuman, K.C. and Osheroff,  
32 N. (2017) Activities of gyrase and topoisomerase IV on positively supercoiled DNA. *Nucleic  
33 acids research*, **45**, 9611-9624.
- 34 20. Drlica, K., Engle, E.C. and Manes, S.H. (1980) DNA gyrase on the bacterial chromosome:  
35 possibility of two levels of action. *Proceedings of the National Academy of Sciences of the  
36 United States of America*, **77**, 6879-6883.
- 37 21. Chong, S., Chen, C., Ge, H. and Xie, X.S. (2014) Mechanism of transcriptional bursting in  
38 bacteria. *Cell*, **158**, 314-326.
- 39 22. Stracy, M., Lesterlin, C., Garza de Leon, F., Uphoff, S., Zawadzki, P. and Kapanidis, A.N. (2015)  
40 Live-cell superresolution microscopy reveals the organization of RNA polymerase in the  
41 bacterial nucleoid. *Proceedings of the National Academy of Sciences of the United States of  
42 America*.
- 43 23. Plank, M., Wadhams, G.H. and Leake, M.C. (2009) Millisecond timescale slimfield imaging  
44 and automated quantification of single fluorescent protein molecules for use in probing

- 1 complex biological processes. *Integrative biology : quantitative biosciences from nano to*  
2 *macro*, **1**, 602-612.
- 3 24. Reyes-Lamothe, R., Sherratt, D.J. and Leake, M.C. (2010) Stoichiometry and architecture of  
4 active DNA replication machinery in Escherichia coli. *Science*, **328**, 498-501.
- 5 25. Manley, S., Gillette, J.M., Patterson, G.H., Shroff, H., Hess, H.F., Betzig, E. and Lippincott-  
6 Schwartz, J. (2008) High-density mapping of single-molecule trajectories with photoactivated  
7 localization microscopy. *Nature methods*, **5**, 155-157.
- 8 26. Bachmann, B.J. (1972) Pedigrees of some mutant strains of Escherichia coli K-12.  
9 *Bacteriological reviews*, **36**, 525-557.
- 10 27. Datsenko, K.A. and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in  
11 Escherichia coli K-12 using PCR products. *Proceedings of the National Academy of Sciences of*  
12 *the United States of America*, **97**, 6640-6645.
- 13 28. Sliusarenko, O., Heinritz, J., Emonet, T. and Jacobs-Wagner, C. (2011) High-throughput,  
14 subpixel precision analysis of bacterial morphogenesis and intracellular spatio-temporal  
15 dynamics. *Molecular microbiology*, **80**, 612-627.
- 16 29. Crocker, J.C. and Grier, D.G. (1996) When Like Charges Attract: The Effects of Geometrical  
17 Confinement on Long-Range Colloidal Interactions. *Physical review letters*, **77**, 1897-1900.
- 18 30. Uphoff, S., Reyes-Lamothe, R., Garza de Leon, F., Sherratt, D.J. and Kapanidis, A.N. (2013)  
19 Single-molecule DNA repair in live bacteria. *Proceedings of the National Academy of Sciences*  
20 *of the United States of America*, **110**, 8063-8068.
- 21 31. Badrinarayanan, A., Reyes-Lamothe, R., Uphoff, S., Leake, M.C. and Sherratt, D.J. (2012) In  
22 vivo architecture and action of bacterial structural maintenance of chromosome proteins.  
23 *Science*, **338**, 528-531.
- 24 32. Leake, M.C., Chandler, J.H., Wadhams, G.H., Bai, F., Berry, R.M. and Armitage, J.P. (2006)  
25 Stoichiometry and turnover in single, functioning membrane protein complexes. *Nature*,  
26 **443**, 355-358.
- 27 33. Leake, M.C., Greene, N.P., Godun, R.M., Granjon, T., Buchanan, G., Chen, S., Berry, R.M.,  
28 Palmer, T. and Berks, B.C. (2008) Variable stoichiometry of the TatA component of the twin-  
29 arginine protein transport system observed by in vivo single-molecule imaging. *Proceedings*  
30 *of the National Academy of Sciences of the United States of America*, **105**, 15376-15381.
- 31 34. Delalez, N.J., Wadhams, G.H., Rosser, G., Xue, Q., Brown, M.T., Dobbie, I.M., Berry, R.M.,  
32 Leake, M.C. and Armitage, J.P. (2010) Signal-dependent turnover of the bacterial flagellar  
33 switch protein FliM. *Proceedings of the National Academy of Sciences of the United States of*  
34 *America*, **107**, 11347-11351.
- 35 35. Wollman, A.J., Shashkova, S., Hedlund, E.G., Friemann, R., Hohmann, S. and Leake, M.C.  
36 (2017) Transcription factor clusters regulate genes in eukaryotic cells. *eLife*, **6**.
- 37 36. Miller, H., Cosgrove, J., Wollman, A.J.M., Taylor, E., Zhou, Z., O'Toole, P.J., Coles, M.C. and  
38 Leake, M.C. (2018) High-Speed Single-Molecule Tracking of CXCL13 in the B-Follicle. *Frontiers*  
39 *in immunology*, **9**, 1073.
- 40 37. Leake, M.C., Wilson, D., Bullard, B. and Simmons, R.M. (2003) The elasticity of single kettin  
41 molecules using a two-bead laser-tweezers assay. *FEBS letters*, **535**, 55-60.
- 42 38. Lenn, T. and Leake, M.C. (2012) Experimental approaches for addressing fundamental  
43 biological questions in living, functioning cells with single molecule precision. *Open biology*,  
44 **2**, 120090.

- 1 39. Tadesse, S. and Graumann, P.L. (2006) Differential and dynamic localization of  
2 topoisomerases in *Bacillus subtilis*. *Journal of bacteriology*, **188**, 3002-3011.
- 3 40. Wollman, A.J. and Leake, M.C. (2015) Millisecond single-molecule localization microscopy  
4 combined with convolution analysis and automated image segmentation to determine  
5 protein concentrations in complexly structured, functional cells, one cell at a time. *Faraday*  
6 *discussions*, **184**, 401-424.
- 7 41. Thornton, M., Armitage, M., Maxwell, A., Dosanjh, B., Howells, A.J., Norris, V. and Sigeo, D.C.  
8 (1994) Immunogold localization of GyrA and GyrB proteins in *Escherichia coli*. *Microbiology*,  
9 **140 ( Pt 9)**, 2371-2382.
- 10 42. Miller, H., Zhou, Z., Wollman, A.J. and Leake, M.C. (2015) Superresolution imaging of single  
11 DNA molecules using stochastic photoblinking of minor groove and intercalating dyes.  
12 *Methods*, **88**, 81-88.
- 13 43. Gellert, M., Mizuuchi, K., O'Dea, M.H. and Nash, H.A. (1976) DNA gyrase: an enzyme that  
14 introduces superhelical turns into DNA. *Proceedings of the National Academy of Sciences of*  
15 *the United States of America*, **73**, 3872-3876.
- 16 44. Higgins, N.P., Peebles, C.L., Sugino, A. and Cozzarelli, N.R. (1978) Purification of subunits of  
17 *Escherichia coli* DNA gyrase and reconstitution of enzymatic activity. *Proceedings of the*  
18 *National Academy of Sciences of the United States of America*, **75**, 1773-1777.
- 19 45. Reece, R.J. and Maxwell, A. (1991) DNA gyrase: structure and function. *Critical reviews in*  
20 *biochemistry and molecular biology*, **26**, 335-375.
- 21 46. Moolman, M.C., Krishnan, S.T., Kerssemakers, J.W., van den Berg, A., Tulinski, P., Depken,  
22 M., Reyes-Lamothe, R., Sherratt, D.J. and Dekker, N.H. (2014) Slow unloading leads to DNA-  
23 bound beta2-sliding clamp accumulation in live *Escherichia coli* cells. *Nature*  
24 *communications*, **5**, 5820.
- 25 47. Vrljic, M., Nishimura, S.Y., Brasselet, S., Moerner, W.E. and McConnell, H.M. (2002)  
26 Translational diffusion of individual class II MHC membrane proteins in cells. *Biophysical*  
27 *journal*, **83**, 2681-2692.
- 28 48. Durisic, N., Laparra-Cuervo, L., Sandoval-Alvarez, A., Borbely, J.S. and Lakadamyali, M. (2014)  
29 Single-molecule evaluation of fluorescent protein photoactivation efficiency using an in vivo  
30 nanotemplate. *Nature methods*, **11**, 156-162.
- 31 49. Collin, F., Karkare, S. and Maxwell, A. (2011) Exploiting bacterial DNA gyrase as a drug target:  
32 current state and perspectives. *Applied microbiology and biotechnology*, **92**, 479-497.
- 33 50. Snyder, M. and Drlica, K. (1979) DNA gyrase on the bacterial chromosome: DNA cleavage  
34 induced by oxolinic acid. *Journal of molecular biology*, **131**, 287-302.
- 35 51. Drlica, K., Franco, R.J. and Steck, T.R. (1988) Rifampin and rpoB mutations can alter DNA  
36 supercoiling in *Escherichia coli*. *Journal of bacteriology*, **170**, 4983-4985.
- 37 52. Stracy, M., Uphoff, S., Garza de Leon, F. and Kapanidis, A.N. (2014) In vivo single-molecule  
38 imaging of bacterial DNA replication, transcription, and repair. *FEBS letters*, **588**, 3585-3594.
- 39 53. Charvin, G., Bensimon, D. and Croquette, V. (2003) Single-molecule study of DNA unlinking  
40 by eukaryotic and prokaryotic type-II topoisomerases. *Proceedings of the National Academy*  
41 *of Sciences of the United States of America*, **100**, 9820-9825.
- 42 54. Nicolas, E., Upton, A.L., Uphoff, S., Henry, O., Badrinarayanan, A. and Sherratt, D. (2014) The  
43 SMC complex MukBEF recruits topoisomerase IV to the origin of replication region in live  
44 *Escherichia coli*. *mBio*, **5**, e01001-01013.

- 1 55. Beattie, T.R., Kapadia, N., Nicolas, E., Uphoff, S., Wollman, A.J., Leake, M.C. and Reyes-  
2 Lamothe, R. (2017) Frequent exchange of the DNA polymerase during bacterial chromosome  
3 replication. *eLife*, **6**.
- 4 56. Crisona, N.J., Strick, T.R., Bensimon, D., Croquette, V. and Cozzarelli, N.R. (2000) Preferential  
5 relaxation of positively supercoiled DNA by E. coli topoisomerase IV in single-molecule and  
6 ensemble measurements. *Genes & development*, **14**, 2881-2892.
- 7 57. Stone, M.D., Bryant, Z., Crisona, N.J., Smith, S.B., Vologodskii, A., Bustamante, C. and  
8 Cozzarelli, N.R. (2003) Chirality sensing by Escherichia coli topoisomerase IV and the  
9 mechanism of type II topoisomerases. *Proceedings of the National Academy of Sciences of  
10 the United States of America*, **100**, 8654-8659.
- 11 58. Lioy, V.S., Cournac, A., Marbouty, M., Duigou, S., Mozziconacci, J., Espeli, O., Boccard, F. and  
12 Koszul, R. (2018) Multiscale Structuring of the E. coli Chromosome by Nucleoid-Associated  
13 and Condensin Proteins. *Cell*, **172**, 771-783 e718.

14

## 15 **Figure legends**

16 **Figure 1. The activity of gyrase.** A) DNA gyrase catalytic cycle. B) Replication  
17 introduces (+) supercoils ahead and precatenated DNA behind. Gyrase acts ahead  
18 of the fork while topo IV removes precatenanes behind. C) Gyrase removes (+)  
19 supercoiling from ahead of RNAP to ensure unperturbed transcription. D) Time  
20 course supercoiling assays presenting the activity of GyrA fusion proteins against the  
21 wild-type GyrA after different incubation periods at 37°C. Gyrase was incubated with  
22 relaxed pBR322 DNA in standard supercoiling assays. Samples were taken at the  
23 intervals indicated and loaded onto a 1% agarose gel and analysed by  
24 electrophoresis.

25

26 **Figure 2. Epifluorescence of E. coli gyrase.** A) Example cells with gyrase, fork  
27 marked with mCherry-DnaN, and overlay of signal from both channels; scale bar 1  
28  $\mu\text{m}$ . B) Cumulative distributions of distances between centroids of fork foci and  
29 brightest gyrase pixels in each cell (red), or a randomly simulated position (black).  
30 Colocalization (gray shaded rectangle) defined as when the fork centroid is  $\leq 2$  pixels  
31 (256 nm) from the brightest gyrase pixel. C) % of cells from population containing  
32 fork or gyrase foci plotted as a histogram. SD error bars from N=3 experiments.

33

34 **Figure 3. GyrA form foci of a few tens of molecules.** A) Dual-color Slimfield  
35 enables single-molecule tracking in two separate color channels with millisecond

1 sampling, for the strain GyrA-mYPet:DnaN-mCherry, cell outline indicated (white  
2 dash). B) Stoichiometry distribution rendered as a kernel density estimate(38) for all  
3 detected GyrA-mYPet foci, mean ( $\pm$ SEM) indicated for all GyrA, kernel width 0.7  
4 molecules. C) Distribution of displacements between foci centers for colocalized  
5 DnaN and GyrA rendered as a kernel density estimate, kernel width 40 nm. Data  
6 acquired from 72 foci using N=35 cells.

7

8 **Figure 4. Intracellular characterization of *E. coli* gyrase.** A) Copy number of GyrA  
9 in exponentially growing culture. B) Selected tracks colored according to apparent  
10 diffusion coefficient ( $D^*$ ) of individual GyrA. C) Distribution of  $D^*$  for 85529 tracked  
11 GyrA. D) Distribution of  $D^*$  for 30813 GyrA treated for 10 min with 10  $\mu$ g/ml  
12 ciprofloxacin.

13

14 **Figure 5. Effect of replication and transcription on gyrase mobility.** A)  
15 Distribution of  $D^*$  for 16597 tracks in cells without fork foci. B) Cell (i - brightfield) with  
16 mean position of immobile molecules (ii) and position of fork marker mYPet-DnaN  
17 (iii). iv) Superimposed images of ii and iii. C) Distribution of  $D^*$  for 41632 GyrA in  
18 cells treated for 30 min with 50  $\mu$ g/ml rifampicin.

19

20 **Figure 6. Binding times of gyrase inside live cells.** A) PALM images of an  
21 example cell imaged with 1 s exposure times; only immobile GyrA-PAmCherry  
22 produce distinct foci, while mobile GyrA are blurred and produce signal below the  
23 detection threshold. B) Photobleaching-corrected binding times extracted from 1s  
24 exposures of GyrA-PAmCherry, topo IV subunit (ParC-PAmCherry) and GyrA after  
25 10 min treatment with 10  $\mu$ g/ml of ciprofloxacin. C) Photobleaching-corrected binding  
26 times for GyrA, dependent on the distance from fork, categorized as proximal (<200  
27 nm), distal ( $\geq$ 200 nm) or all binding events.

28

29

30 **Movie 1. Legend** Example Slimfield GyrA-mYpet (yellow) fluorescence  
31 photobleaching. Time in ms shown, scale bar 1  $\mu$ m.

32



# Supplementary Material & Methods and Supplementary Figures

## Increased activity of DNA gyrase near replication forks revealed by *in vivo* single-molecule imaging

### Authors:

Mathew Stracy<sup>a,1</sup>, Adam J.M. Wollman<sup>b,1</sup>, Elzbieta Kaja<sup>e</sup>, Jacek Gapinski<sup>c</sup>, Ji-Eun Lee<sup>b</sup>, Victoria A Leek<sup>d</sup>, Shannon J. McKie<sup>d</sup>, Lesley A. Mitchenall<sup>d</sup>, Anthony Maxwell<sup>d</sup>, David J. Sherratt<sup>a</sup>, Mark C. Leake<sup>b,2</sup>, Pawel Zawadzki<sup>a,c,2</sup>

### Affiliations:

<sup>a</sup>Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, United Kingdom.

<sup>b</sup>Biological Physical Sciences Institute (BPSI), Departments of Physics and Biology, University of York, York YO10 5DD, United Kingdom

<sup>c</sup>Molecular Biophysics Division, Faculty of Physics, A. Mickiewicz University,

Umultowska 85, 61-614 Poznan, Poland

<sup>d</sup>Department of Biological Chemistry, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, United Kingdom

<sup>e</sup>NanoBioMedical Centre, Adam Mickiewicz University, Umultowska 85, 61-614 Poznan, Poland.

<sup>1</sup>These authors contributed equally

<sup>2</sup>Co-corresponding authors. Email: [zawadzki@amu.edu.pl](mailto:zawadzki@amu.edu.pl) or [mark.leake@york.ac.uk](mailto:mark.leake@york.ac.uk)

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18

**Bacterial strains**

All strains were derivatives of *Escherichia coli* K-12 AB1157 (1). The oligonucleotides used for replacement of genes with C-terminal mYPet fusions by  $\lambda$ -Red recombination (2) are shown in Table S1. PCRs were performed with the template plasmid pROD10, containing the sequence for the monomeric YPet fluorescent protein preceded by a flexible 11 amino acid linker (SAGSAAGSGEF), and followed by an *frt*-flanked kanamycin resistance gene (*kan<sup>r</sup>*). For PAmCherry fusions the same oligo sets were used with the template plasmid pROD85 containing PAmCherry instead of mYPet. For multiple insertions of modified genes, the *kan<sup>r</sup>* gene was removed using site-specific recombination through expression of the Fip recombinase from plasmid pCP20 (2). Correct insertion of the fragment into the chromosome was evaluated by PCR using primers flanking the insertion site.

Oligonucleotides	
Name	Sequence
gyrApamcherryfor	GGACGATGAAATCGCTCCGGAAGTGGACGTTGACGACGAG CCAGAAGAAGAATCG GCT GGC TCC GCT GCT GGT TC
gyrApamcherryrev	TCAATTCAAACAAGGGAGATAGCTCCCTTTTGGCATGAAGA AGTAAAATTAGAGGATCCCATATGAATATCCTCC
gyrBpamcherryrev	GCCGTGCGTTTTATTGAAGAGAACGCCCTGAAAGCGGCGAA TATCGATATTTTCG GCT GGC TCC GCT GCT GGT TC

gyrBpamcherryfor	GCCTGATAAGCGTAGCGCATCAGGCACGCTCGCATGGTTA GCGCCATTAGAGGATCCCATATGAATATCCTCC
------------------	---

1 **Table S1.**

2

3 **Sample preparation**

4 Strains were streaked onto LB plates containing appropriate antibiotics. Single colonies were  
5 inoculated into M9 growth media with a glycerol carbon source (0.2%) and grown overnight  
6 at 37°C to  $A_{600}$  0.4-0.6, then diluted into fresh M9 and grown to  $A_{600}$  0.1. Cells were  
7 centrifuged and immobilized on agarose pads between two glass coverslips. For PALM  
8 microscopy 0.17 mm thickness coverslips were cleaned of any background fluorescent  
9 particles before use by heating in an oven to 500°C for 1 h. For Slimfield microscopy BK7  
10 coverslip were plasma-cleaned before use. 1% agarose pads were prepared by mixing 2%  
11 low-fluorescence agarose (Bio-Rad) in dH<sub>2</sub>O 1:1 with 2x M9 growth medium. Where  
12 indicated cells were incubated with, 1 µg/ml ciprofloxacin for 10 minutes prior to imaging, or  
13 50 µg/ml rifampicin for 30 minutes prior to imaging.

14

15 **Epifluorescence microscopy and colocalization analysis**

16 Wide-field epifluorescence microscopy was performed using an Eclipse TE2000-U  
17 microscope (Nikon), equipped with a 100x/NA1.4 oil PlanApo objective and a Cool-Snap  
18 HQ<sup>2</sup> CCD, and using Nikon NIS-Elements software for image acquisition. A chromosomally  
19 encoded mCherry-DnaN fusion protein was used as a marker for the replisome (3,4).

20 For colocalization analysis cell outlines were first delineated from a phase image  
21 using the MicrobeTracker software, creating a “mesh” for each cell, within which each pixel  
22 is characterized by a specific x,y coordinate. The positions of foci formed by mCherry-DnaN  
23 were established with Gaussian fitting described in the section titled ‘ Localization and  
24 tracking’. Since GyrA-mYPet did not form well-defined diffraction-limited foci, we determined  
25 the brightest pixel of GyrA-mYPet signal within each cell, as described in ref (6). It should be  
26 noted that the Gaussian localization analysis for mCherry-DnaN can identify multiple  
27 fluorescent foci within one cell or none at all, but the brightest pixel analysis finds exactly one  
28 pixel with the highest intensity for GyrA.

1 The pairwise distances between the brightest GyrA pixel and the nearest DnaN  
2 localization was calculated in Matlab (Mathworks) as described in ref (5). To determine the  
3 distribution of distances expected from an entirely random localization of GyrA, we also  
4 calculated distances between a pixel randomly positioned within the cell and the nearest  
5 DnaN focus. A threshold of 2 pixels (258 nm) was chosen to define colocalization.

## 6 7 **Photoactivated Localization Microscopy**

8 Live cell single-molecule-tracking PhotoActivated Localization Microscopy (PALM) was  
9 performed on a custom-built total internal reflection fluorescence (TIRF) microscope built  
10 around the Rapid Automated Modular Microscope (RAMM) System (ASI Imaging).  
11 Photoactivatable mCherry activation was controlled by a 405 nm laser and excitation with  
12 561 nm. All lasers were provided by a multi-laser engine (iChrome MLE, Toptica). At the  
13 fibre output, the laser beams were collimated and focused (100x oil immersion objective, NA  
14 1.4, Olympus) onto the sample under an angle allowing for highly inclined thin illumination  
15 (6). Fluorescence emission was filtered by a dichroic mirror and notch filter  
16 (ZT405/488/561rpc & ZET405/488/561NF, Chroma). PAmCherry emission was projected  
17 onto an EMCCD camera (iXon Ultra, 512x512 pixels, Andor). The pixel size was 96 nm.  
18 Transmission illumination was provided by an LED source and condenser (ASI Imaging).  
19 Sample position and focus were controlled with a motorized piezo stage, a z-motor objective  
20 mount, and autofocus system (MS-2000, PZ-2000FT, CRISP, ASI Imaging). PALM movies  
21 were aquired with a frame time of 15.48 ms. For colocalization analysis snapshots with 488  
22 nm excitation were performed prior to PALM imaging.

## 23 24 **Localization and tracking**

25 PALM data for single-molecule-tracking analysis was localized using custom-written  
26 MATLAB software (MathWorks): fluorophore images were identified for localization by band-  
27 pass filtering and applying an intensity threshold to each frame of the super-resolution  
28 movie. Candidate positions were used as initial guesses in a two-dimensional elliptical  
29 Gaussian fit for high-precision localisation. Free fit parameters were x-position, y-position, x-  
30 width, y-width, elliptical rotation angle, intensity, background. Single-particle tracking  
31 analysis was performed by adapting the MATLAB implementation of the algorithm described  
32 in ref (7). Positions were linked to a track if they appeared in consecutive frames within a  
33 window of 5 pixels (0.48  $\mu$ m). In rare cases when multiple localizations fell within the tracking

1 radius, tracks were linked such that the sum of step distances was minimized. We used a  
2 'memory' parameter of 1 frame to allow for transient (1 frame) disappearance of the  
3 fluorophore image within a track due to blinking or missed localisation.

4

## 5 **Molecule counting**

6 We counted the total number of GyrA or GyrB molecules by recording long movies (50000  
7 frames), until no further activation was observed. Cells were segmented from transmission  
8 images using MicrobeTracker (8). Localizations within cell boundaries were tracked and the  
9 number of tracked molecules per cell established. We note that the copy numbers  
10 presented here may be underestimates of the true copy numbers, since only 49% of  
11 PAmCherry were shown to be photoactivatable in studies in eukaryotic cells (9).

12

## 13 **Measuring the diffusion of PAmCherry labeled proteins.**

14 We determined the mobility of each molecule by calculating an apparent (or nominal)  
15 diffusion coefficient,  $D^*$ , from the one-step mean-squared displacement (MSD) of the track  
16 using:

17

$$18 \quad D^* = \frac{1}{4n\Delta t} \sum_{i=1}^n [x(i\Delta t) - x(i\Delta t + \Delta t)]^2 + [y(i\Delta t) - y(i\Delta t + \Delta t)]^2$$

19

20 Where  $x(t)$  and  $y(t)$  are the coordinates of the molecule at time  $t$ , the frame time of the  
21 camera is  $\Delta t$ , and  $n$  is the number of steps in the trajectory. Tracks shorter than  $n = 4$  steps  
22 long were discarded for this analysis because the higher uncertainty in  $D^*$  value.

23

24 For a molecule with apparent diffusion coefficient  $D$ , the probability distribution of obtaining a  
25 single-molecule  $D^*$  value,  $x$ , is given by (10) :

26

27

$$f(x; D, n) = \frac{(n/D)^n x^{n-1} e^{-nx/D}}{(n-1)!}$$

Where  $n$  is the number of steps in the trajectory. In order to determine the apparent diffusion coefficient,  $D$ , from the population of individual single-molecule  $D^*$  values, longer tracks were truncated after 5<sup>th</sup> localization (i.e.  $n = 4$ ). The  $D^*$  distribution,  $x$ , was then fitted to the  $n = 4$  analytical expression equation:

$$f(x; D) = \frac{(4/D)^4 x^3 e^{-4x/D}}{6}$$

Fits were performed using maximum likelihood estimation in MATLAB, and errors were estimated as the SD in each estimated parameter using bootstrap resampling with 100 resamples, rounded up to the nearest  $0.01 \mu\text{m}^2\text{s}^{-1}$ . A single species model fits poorly to the data (Supplementary Fig. 1c). We reasoned that at least two species with different mobilities are present: mobile molecules diffusing and binding only transiently to DNA, and immobile molecules bound to DNA for the entire trajectory. We therefore introduced a second species:

$$f(x; D_1, D_2, A) = \frac{A(4/D_1)^4 x^3 e^{-4x/D_1}}{6} + \frac{(1-A)(4/D_2)^4 x^3 e^{-4x/D_2}}{6}$$

Where  $D_1$  and  $D_2$  are the diffusion coefficients of the two different species, and  $A$  and  $1 - A$  are the fraction of molecules found in each state.

The localisation uncertainty in each measurement,  $\sigma_{\text{loc}}$ , manifests itself as a positive offset in the  $D^*$  value of  $\sigma_{\text{loc}}^2/\Delta t$  (11). Based on the estimated localisation uncertainty of  $\sim 40$  nm for our measurements, we expected a positive shift in the mean  $D^*$  value of immobile molecules to  $\sim 0.1 \mu\text{m}^2\text{s}^{-1}$ .

### Estimating colocalization with the replisome

The replisome position was established using a mYPet-DnaN fusion. Snapshots of mYPet-DnaN were taken prior to PALM imaging, and the exact position estimated using gaussian fitting as described in the section 'Localization and tracking'. Cells were segmented based on transmission images using MicrobeTracker, and the number of PALM localization within each cell outline was determined. The pairwise distances between centroid positions of

1 DnaN and all GyrA PALM localizations within each cell was determined using the `pdist2`  
2 function in Matlab, and the fraction located within 200 nm was determined. The mean  
3 colocalized fraction was determined from all cells from the data set (containing at least 100  
4 cells), and the SEM established from the means of five experimental data sets.

5

## 6 **Measuring long-lasting binding events**

7 PALM movies to measure long duration binding events were recorded at low  
8 continuous 561 nm excitation intensities using 1 s exposure times(12,13). At this  
9 exposure times mobile GyrA-PAmCherry molecules are motion blurred over a large  
10 fraction of the cell, whereas immobile GyrA-PAmCherry molecules still appear as  
11 point sources, producing a diffraction limited spot. Elliptical Gaussian fitting was used  
12 as described in the 'Localization and tracking' section. Bound and mobile molecules  
13 were distinguished by the width of the elliptical fits, with thresholds short axis-width <  
14 160 nm and long axis-width < 200 nm to identify bound molecules. The probability of  
15 observing a particular on-time is the product of the underlying binding-time  
16 probability and the bleaching probability. The bleaching-time distributions were  
17 measured independently using MukB-PAmCherry which has a binding time >>  
18 bleaching time. On-time and bleaching-time distributions were fitted with single-  
19 exponential functions to extract exponential-time constants  $t_{on}$  and  $t_{bleach}$ , and the  
20 binding-time constant was calculated by  $t_{bound} = t_{on} \cdot t_{bleach} / (t_{bleach} - t_{on})$ . Stochastic  
21 photoactivation of GyrA-PAmCherry molecules before or during binding events does  
22 not influence our measurement, because the observed binding times follow an  
23 exponential distribution and are therefore memoryless. The MukB-PAmCherry  
24 bleaching time constant,  $t_{bleach} = 1.16 \pm 0.04$ . The uncorrected  $t_{on}$  time constants from  
25 7 experimental repeats are shown in Table S2.

26 To determine binding times near the replisome, snapshots of mYPet-DnaN were  
27 taken prior to PALM imaging. DnaN foci were localized with Gaussian fitting and GyrA  
28 trajectories within 200 nm of a foci were used for binding time analysis. As a control, the  
29 binding times within 200 nm of mid-cell (where the replisome is expected to  
30 assemble/diassemble) were determined in cells lacking DnaN foci. Mid-cell position was  
31 determined from segmenting the transmission image.

32

Uncorrected on-time measurements in seconds
---

All tracks	Tracks >200nm from the replisome	Tracks <200nm from the replisome
0.781	0.740	1.087
0.790	0.758	1.168
0.879	0.861	1.004
0.823	0.802	1.046
0.819	0.791	1.029
0.894	0.871	1.113
0.909	0.887	1.132

1

2 **In vitro DNA supercoiling assay.**

3 Wildtype and fluorescently tagged GyrA and GyrB subunits were purified according to  
4 standard protocols (14). Supercoiling assays were carried out as before (14). Briefly, a 1.5  
5  $\mu\text{L}$  aliquot of the 0.1  $\mu\text{M}$  respective GyrA sample was added to 17  $\mu\text{L}$  of  $\text{H}_2\text{O}$ , 4  $\mu\text{L}$  of dilution  
6 buffer, 6  $\mu\text{L}$  of assay buffer, 0.5  $\mu\text{L}$  of relaxed DNA and 1  $\mu\text{L}$  of GyrB (0.75  $\mu\text{M}$ ). This  
7 resulted in a final concentration of GyrA and GyrB of 5 nM and 25 nM, respectively. Full  
8 supercoiling activity was observed after 5-10 minutes for the GyrA wildtype, which remained  
9 consistent across the repeats. However, the activities of the two fusions were minimally  
10 lower but still comparable to the wild type.

11

12

13

14 **Slimfield image analysis**

15 Foci from Slimfield images were automatically detected and tracked using custom-written  
16 Matlab software discussed previously (15). In brief, bright foci were identified by image  
17 transformation and thresholding. The centroid of candidate foci were determined using  
18 iterative Gaussian masking and accepted if their intensity was greater than a signal to noise  
19 (SNR) of 0.4. Intensity was defined as the summed pixel intensity inside a 5 pixel circular  
20 region of interest (ROI) corrected for the background in an outer square ROI of 17x17 pixels.  
21 SNR was defined as the mean BG corrected pixel intensity in the circular ROI divided by the  
22 standard deviation in the square ROI. Foci were linked together into trajectories between  
23 frames if they were within 5 pixels of each other.



1           Stoichiometry was determined by fitting the first 3 intensity values of a foci to a  
2 straight line, using the intercept as the initial intensity and dividing this by the characteristic  
3 intensity of mYPet or mCherry. This characteristic intensity was determined from the  
4 distribution of foci intensity values towards the end of the photobleach confirmed by  
5 overtracking foci beyond their bleaching to generate individual photobleach steps of the  
6 characteristic intensity (Fig S2). Red and green images were aligned based on the peak of  
7 the 2D cross correlation between brightfield images using individual green channel image  
8 frame cross correlated against 10 frame average images from the red channel.  
9 Colocalisation between green and red foci and the probability of random colocalisation was  
10 determined as described previously(16).

11           Copy numbers were determined using the first excited mYPet image frame. The  
12 image was segmented and background corrected using the mean intensity from images of  
13 the wild type *E. coli* without mYPet but imaged using identical conditions. A model 'sausage  
14 function' *E. coli* shape was fitted to the segmented area using the minor and major radii. A  
15 model 3D point spread function was integrated over this volume and the molecular  
16 concentration determined by solving a set of linear equations for each pixel in the real,  
17 background corrected image and model convolved image (17).

18

19

20

## 21 **Supplementary References**

22

- 23 1.     Bachmann, B.J. (1972) Pedigrees of some mutant strains of Escherichia coli K-12.  
24     *Bacteriological reviews*, **36**, 525-557.
- 25 2.     Datsenko, K.A. and Wanner, B.L. (2000) One-step inactivation of chromosomal  
26     genes in Escherichia coli K-12 using PCR products. *Proceedings of the National*  
27     *Academy of Sciences of the United States of America*, **97**, 6640-6645.
- 28 3.     Moolman, M.C., Krishnan, S.T., Kerssemakers, J.W., van den Berg, A., Tulinski, P.,  
29     Depken, M., Reyes-Lamothe, R., Sherratt, D.J. and Dekker, N.H. (2014) Slow  
30     unloading leads to DNA-bound beta2-sliding clamp accumulation in live Escherichia  
31     coli cells. *Nature communications*, **5**, 5820.
- 32 4.     Reyes-Lamothe, R., Sherratt, D.J. and Leake, M.C. (2010) Stoichiometry and  
33     architecture of active DNA replication machinery in Escherichia coli. *Science*, **328**,  
34     498-501.

- 1 5. Nicolas, E., Upton, A.L., Uphoff, S., Henry, O., Badrinarayanan, A. and Sherratt, D.  
2 (2014) The SMC complex MukBEF recruits topoisomerase IV to the origin of  
3 replication region in live *Escherichia coli*. *mBio*, **5**, e01001-01013.
- 4 6. Tokunaga, M., Imamoto, N. and Sakata-Sogawa, K. (2008) Highly inclined thin  
5 illumination enables clear single-molecule imaging in cells. *Nature methods*, **5**, 159-  
6 161.
- 7 7. Crocker, J.C. and Grier, D.G. (1996) When Like Charges Attract: The Effects of  
8 Geometrical Confinement on Long-Range Colloidal Interactions. *Physical review*  
9 *letters*, **77**, 1897-1900.
- 10 8. Sliusarenko, O., Heinritz, J., Emonet, T. and Jacobs-Wagner, C. (2011) High-  
11 throughput, subpixel precision analysis of bacterial morphogenesis and intracellular  
12 spatio-temporal dynamics. *Molecular microbiology*, **80**, 612-627.
- 13 9. Durisic, N., Laparra-Cuervo, L., Sandoval-Alvarez, A., Borbely, J.S. and  
14 Lakadamyali, M. (2014) Single-molecule evaluation of fluorescent protein  
15 photoactivation efficiency using an in vivo nanotemplate. *Nature methods*, **11**, 156-  
16 162.
- 17 10. Vrljic, M., Nishimura, S.Y., Brasselet, S., Moerner, W.E. and McConnell, H.M. (2002)  
18 Translational diffusion of individual class II MHC membrane proteins in cells.  
19 *Biophysical journal*, **83**, 2681-2692.
- 20 11. Michalet, X. and Berglund, A.J. (2012) Optimal diffusion coefficient estimation in  
21 single-particle tracking. *Physical review. E, Statistical, nonlinear, and soft matter*  
22 *physics*, **85**, 061916.
- 23 12. Uphoff, S., Reyes-Lamothe, R., Garza de Leon, F., Sherratt, D.J. and Kapanidis,  
24 A.N. (2013) Single-molecule DNA repair in live bacteria. *Proceedings of the National*  
25 *Academy of Sciences of the United States of America*, **110**, 8063-8068.
- 26 13. Zawadzki, P., Stracy, M., Ginda, K., Zawadzka, K., Lesterlin, C., Kapanidis, A.N. and  
27 Sherratt, D.J. (2015) The Localization and Action of Topoisomerase IV in *Escherichia*  
28 *coli* Chromosome Segregation Is Coordinated by the SMC Complex, MukBEF. *Cell*  
29 *Rep*, **13**, 2587-2596.
- 30 14. Reece, R.J. and Maxwell, A. (1989) Tryptic fragments of the *Escherichia coli* DNA  
31 gyrase A protein. *The Journal of biological chemistry*, **264**, 19648-19653.
- 32 15. Miller, H., Zhou, Z., Shepherd, J., Wollman, A.J.M. and Leake, M.C. (2018) Single-  
33 molecule techniques in biophysics: a review of the progress in methods and  
34 applications. *Reports on progress in physics. Physical Society*, **81**, 024601.
- 35 16. Llorente-Garcia, I., Lenn, T., Erhardt, H., Harriman, O.L., Liu, L.N., Robson, A., Chiu,  
36 S.W., Matthews, S., Willis, N.J., Bray, C.D. *et al.* (2014) Single-molecule in vivo  
37 imaging of bacterial respiratory complexes indicates delocalized oxidative  
38 phosphorylation. *Biochimica et biophysica acta*, **1837**, 811-824.
- 39 17. Wollman, A.J. and Leake, M.C. (2015) Millisecond single-molecule localization  
40 microscopy combined with convolution analysis and automated image segmentation  
41 to determine protein concentrations in complexly structured, functional cells, one cell  
42 at a time. *Faraday discussions*, **184**, 401-424.

1

2

3

4

5

6

7

8

9

10

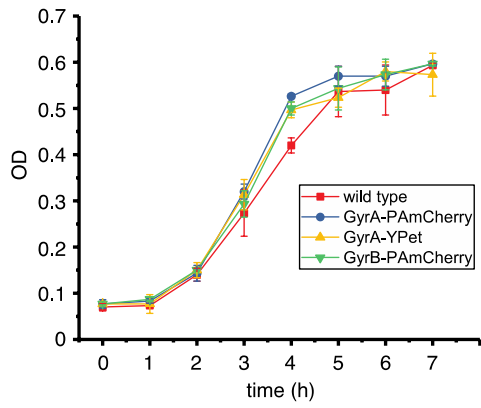
11

12 **Supplementary Figures**

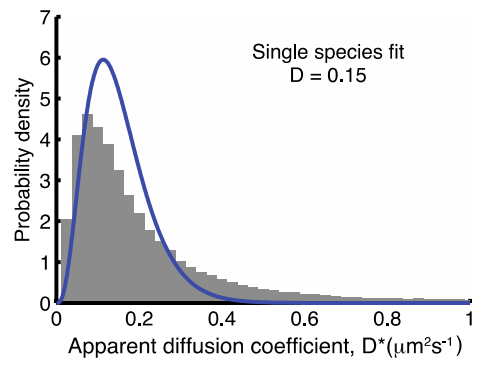
13

14

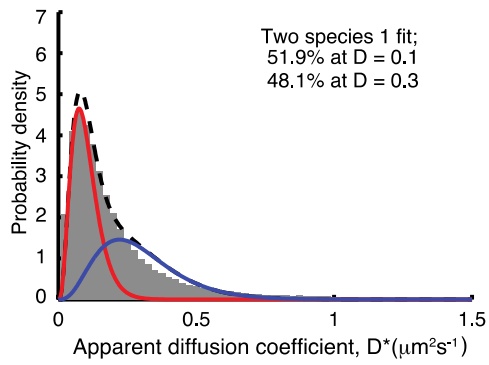
A



B



C



1

2

3 **Figure S1.** A) Growth curves of indicated strains in LB media at 37°C. B) Single-species fit  
4 to GyrA data. C) Double-species fit to GyrA data.

5

6

7

8

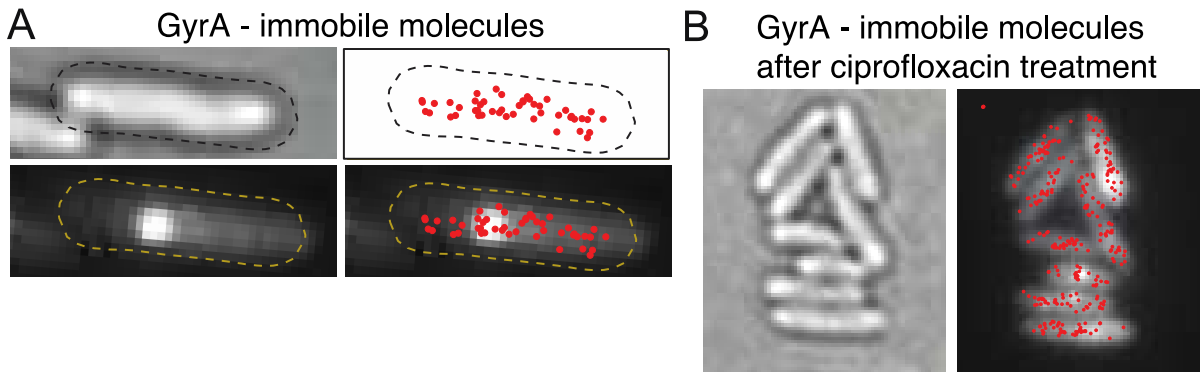
9

10

11

12

13



1

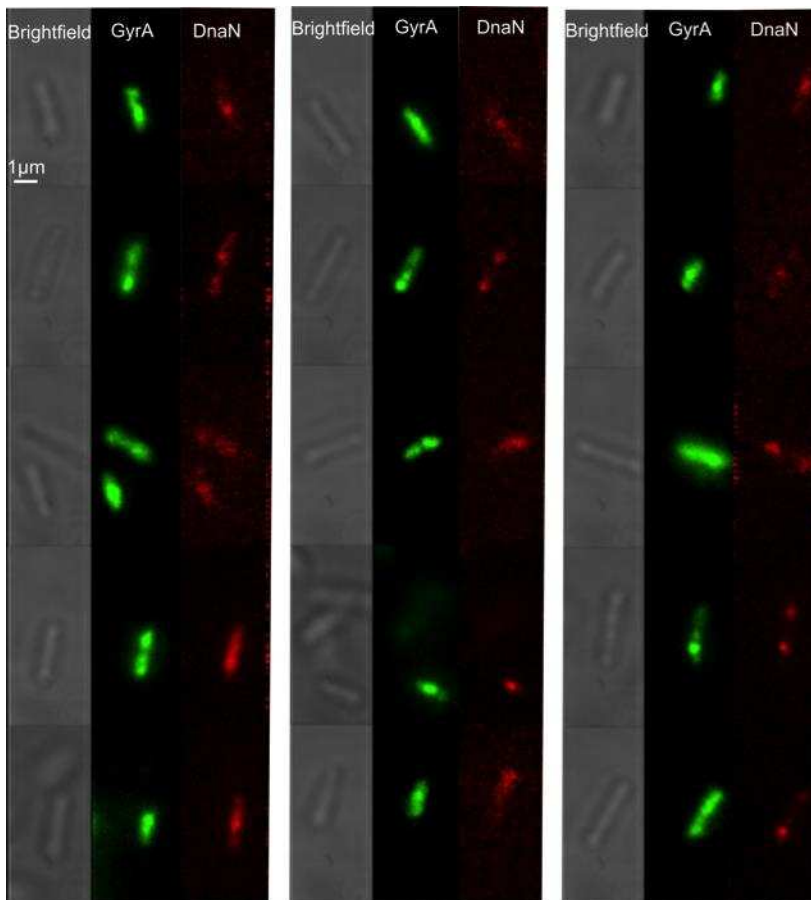
2 **Figure S2.** A) An example of the cell where no clear enrichment of GyrA close to the  
 3 replisome was observed. Red dots represent mean position of immobile molecule. B) Group  
 4 of cells after ciprofloxacin treatment (molecules treated for 10 min with 10  $\mu\text{g/ml}$   
 5 ciprofloxacin) demonstrating that catalytically active gyrase are distributed throughout the  
 6 chromosome.

7

8

9

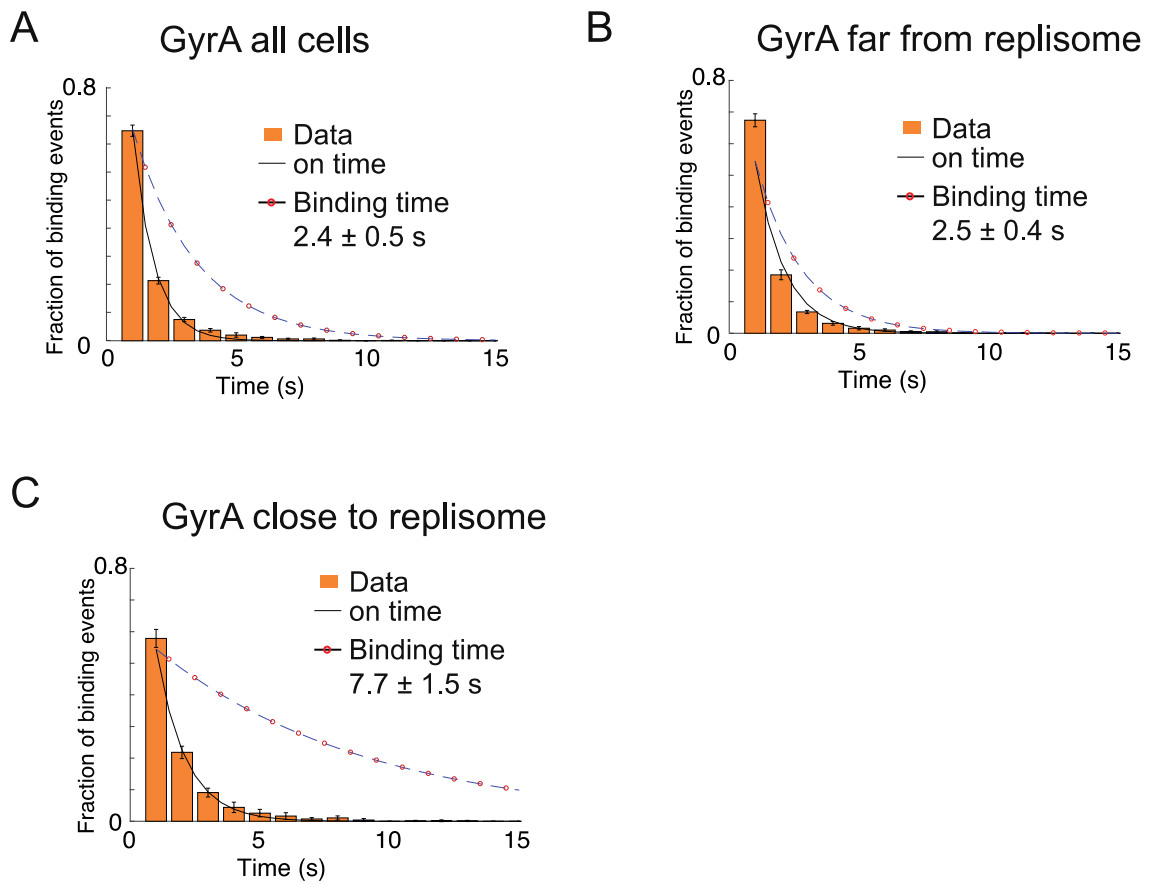
10



11

1 **Figure S3.** Brightfield and equivalent GyrA-mYPet and DnaN-mCherry dual-color Slimfield  
2 images (frame averages, from first five frames).

3  
4  
5



6  
7

8 **Figure S4. Dwell times of GyrA.** On-time distributions for immobile GyrA-PAmCherry  
9 imaged with 1 s exposure times. Single exponential fits (solid lines) and photobleaching-  
10 corrected on time distributions (dashed circled lines). Photobleaching times were estimated  
11 by imaging, under the same conditions, cells with MukB-PAmCherry fusion, which has been  
12 shown to have a dwell time of  $\sim 50$  s. Error bars shows S.E.M. of three experimental repeats.

13  
14  
15