Single-molecule DNA repair in live bacteria

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Cellular DNA damage is reversed by balanced repair pathways that avoid accumulation of toxic intermediates. Despite their importance, the organization of DNA repair pathways and the function of repair enzymes in vivo have remained unclear because of the inability to directly observe individual reactions in living cells. Here, we used photoactivation, localization, and tracking in live Escherichia coli to directly visualize single fluorescent labeled DNA polymerase I (Pol) and ligase (Lig) molecules searching for DNA gaps and nicks, performing transient reactions, and releasing their products. Our general approach provides enzymatic rates and copy numbers, substrate-search times, diffusion characteristics, and the spatial distribution of reaction sites, at the single-cell level, all in one measurement. Single repair events last 2.1 ± 0.5 s (Pol) and 2.5 ± 0.5 s (Lig), respectively. Pol and Lig activities increased fivefold over the basal level within minutes of DNA methylation damage; their rates were limited by upstream base excision repair pathway steps. Pol and Lig spent >80% of their time searching for free substrates, thereby minimizing both the number and lifetime of toxic repair intermediates. We integrated these single-molecule observations to generate a quantitative, systems-level description of a model repair pathway in vivo.

However, reaction pathways are not defined solely by protein concentrations, but by reaction rates that depend on protein and substrate concentrations and enzymatic rates in the cell. Consequently describing repair pathways quantitatively has proven difficult, because the rates are either unknown or have to be obtained from various in vitro experiments performed under different conditions (14, 15). Hence there is a need to quantify rates at the single-cell level and relate them to the concentrations and spatial distribution of substrates and repair proteins as well as to the timing of individual reactions and search processes.

To address these objectives, we developed a general single-molecule fluorescence method to directly visualize repair reactions in individual live Escherichia coli cells. We studied base excision repair (BER) of DNA-methylation damage by methyl methanesulfonate (MMS), which generates single-nucleotide gap intermediates (16). We focused on the final two BER steps, both conserved from bacteria to humans and across repair pathways: DNA synthesis by DNA polymerase I (Pol) and subsequent nick ligation by DNA ligase (Lig; Fig. 1 B) (11, 12); these reactions also link Okazaki fragments in lagging-strand DNA replication (17). Pol and Lig have been extensively characterized as model enzymes in vitro, but their actual cellular activities have not been directly observed and quantified. Fluorescence microscopy is well suited for this task (18), but is limited by its current spatial and temporal resolution. Fluorescence recovery after photobleaching experiments have revealed global DNA repair kinetics in eukaryotes (19, 20), but this model-dependent approach cannot easily resolve molecular subpopulations to extract binding and diffusion constants. Although single fluorescent proteins can be detected in live bacteria (21, 22), they typically report on only a few labeled molecules per cell. Attempts to visualize lagging-strand replication by fluorescent-labeled Pol molecules (~400 copies/cell) (1, 17) showed a broad diffusion-limited distribution in E. coli (Fig. 1 B) (22). To detect single reactions directly, we imaged molecules sequentially using photoactivated localization microscopy (PALM) (23–25) combined with single-molecule tracking (26–28) of photovactivatable mcHerry (PAmCherry) fusion proteins (29). This approach provided an unprecedented comprehensive dataset of Pol and Lig reaction rates, substrate search times, and diffusion coefficients as well as the number, localization, and lifetime of DNA damage sites in live E. coli. We quantified dynamic changes of these observables during the damage response and adaptation.

Results

PALM Tracking of Fluorescent Fusions. We replaced endogenous genes with C-terminal PAmCherry fusions (SI Appendix, Table S1). The growth rates and MMS sensitivity of the fusion strains were identical to WT (SI Appendix, Table S2), whereas a temperature-sensitive Pol mutant strain exhibited hypersensitivity to...
MMS (SI Appendix, Fig. S1). We measured live cells immobilized on agarose under continuous 561-nm excitation; >95% of cells remained viable after imaging (SI Appendix, Fig. S1). By controlling 405-nm photoactivation, we imaged less than one fluorophore per cell at any time (Fig. 1C, Movie S1), giving a clear fluorescent point-spread-function (PSF) in 15-ms exposures. We tracked consecutive localizations to follow the movement of single molecules until irreversible photobleaching (Fig. 1D and E).

**Pol and Lig in Undamaged Cells.** We first inspected Pol and Lig tracks in the absence of exogenous DNA damage. The spatial distribution of tracks reflected the structure of the nucleoid in replicating *E. coli* (30) (Fig. 1F and SI Appendix, Fig. S2). In short cells with a single chromosome, Pol tracks occupied the central area of the cell, avoiding the poles. Increasing cell length correlated with the progression of chromosome replication and segregation, as evident from the presence of two distinct areas of high Pol density in cells of intermediate length; these two areas separated entirely in the longest cells before the appearance of a constricting septum that marks cell division.

Mean-squared displacement (MSD) curves from tracks in live cells rise linearly but saturate at long lag times because of confinement within the nucleoid (Fig. 1G). We measured the small nucleoid-associated protein Fis (31) for comparison. The step lengths between consecutive localizations scaled with the expected molecular weights (Fig. 1H): 76-kDa Fis dimers showed the fastest diffusion, followed by 102-kDa Lig and 136-kDa Pol (molecular weights include 27 kDa per PAmCherry). These results confirmed integrity of the fusion proteins and show that Pol and Lig do not stably form larger replication or repair complexes in the cytoplasm. Pol displacements in fixed cells (red curve in Fig. 1H) represent the 40-nm localization precision.

We asked if subpopulations of Pol and Lig were active in lagging strands of replication and basal DNA repair. We hypothesized that molecules performing reactions while bound to DNA might have a significantly lower diffusion coefficient than free Pol and Lig, which are both expected to show only weak, nonspecific DNA-binding (SI Appendix), and considering that chromosomal DNA loci have a low diffusion coefficient of ∼10−5 μm²·s⁻¹ (32). Our single-molecule approach can reveal such molecular heterogeneity by measuring the apparent diffusion coefficient *D*⁺ of each track: *D*⁺ = MSD/(4 Δτ) − *σ*loc²/Δτ (correcting for the localization error *σ*loc). Undamaged cells showed primarily diffusing Pol and Lig with distributions centered at *D*⁺ ~0.8 μm²·s⁻¹ and ~1 μm²·s⁻¹, respectively (Fig. 2A). In contrast, fixed cells contained almost exclusively Pol molecules that appeared immobile, as expected (Fig. 2A). Using a threshold on *D*⁺ (based on the fixed cells control simulations), we identified populations of 2.7 ± 0.2% and 3.8 ± 0.4% bound Pol and Lig with *D*⁺ ~0 μm²·s⁻¹, respectively (red bars in Fig. 2A). As a positive control for our ability to identify single DNA-bound proteins against the pool of unbound molecules, we imaged DNA Pol III-PAmCherry (α subunit), exploiting the known positioning of replication forks in the cell (22). As expected, tracks of bound Pol III localized exclusively at midcell or at symmetric positions in each cell half; in contrast, tracks of diffusing molecules were distributed throughout the cell (SI Appendix, Fig. S3).

We performed simulations to characterize the observed motion of Pol and Lig. Instead of simulating the full trajectories directly (27, 28), we fully modeled PALM movies of Brownian motion within the nucleoid, including the distinct populations of bound Pol and Lig molecules (SI Appendix, Fig. S4; SI Appendix). We found that simulations with diffusion coefficients *D*⁺ = 2.7 ± 0.4 μm²·s⁻¹ and *D*⁺ = 3.5 ± 0.3 μm²·s⁻¹ precisely matched our experimental results (SI Appendix, Fig. S4). Note that apparent diffusion coefficients (*D*⁺) are lower than these values because of the confinement and motion blurring (33). The close agreement between simulation and experiment suggests that Pol and Lig perform effective Brownian motion. Although the nucleoid association indicates nonspecific DNA interactions, the displacement between subsequent localizations limits transient DNA binding to <15 ms, excluding substrate search processes wherein Pol and Lig processively scan long chromosomal segments, as shown for other repair proteins in vitro (34, 35). The observed Brownian motion (i.e., Gaussian distribution of step lengths) likely results from a sum of multiple displacements and nonspecific binding events per frame.

**Direct Observation of DNA Repair.** To test whether the bound populations of Pol and Lig represent molecules performing DNA synthesis and ligation, we generated single-nucleotide gap substrates in vivo, using BER of exogenous DNA-methylation damage. MMS treatment of live cells for 20 min resulted in fivefold increased bound-molecule fractions: 13.0 ± 0.2% bound Pol and 17.9 ± 0.3% bound Lig (Fig. 2A and B). The DNA-binding...
protein Fis served as a control without DNA repair activity, showing no increase in binding with MMS (Fig. 2C and SI Appendix, Fig. S3). Because the MMS response was specific to the two DNA repair proteins, we interpret the tracks of single bound Pol and Lig molecules as direct reports of DNA synthesis and ligation reactions (red tracks in Fig. 2 D and E: Movie S2). Notably, the spatial organization and $D^*$ distributions of free Pol and Lig were similar with and without MMS, suggesting that their global diffusion and search processes did not change significantly within 20 min of DNA damage. By examining the locations of bound tracks, we found that the repair sites were spread throughout the cell, matching the spatial distribution of free Pol and Lig (SI Appendix, Fig. S5). This supports a distributive search model of individual Pol and Lig molecules for damage sites on the chromosome, instead of these sites being directed to spatially organized “repair factories” (7), as suggested for human cells and mitochondria (8, 9). Our results validate the common assumption of a uniform distribution of damage sites in computer models of DNA repair in E. coli (14, 15).

### DNA Repair Rates

To directly visualize complete substrate search and repair cycles, we identified individual tracks that showed free diffusion leading to a binding event, followed by dissociation and continued diffusion (Fig. 3A–C and SI Appendix, Fig. S6). Corresponding time-traces of the stochastic diffusion steps displayed characteristic binding intervals with $D^* \sim 0.8 \mu m^2 s^{-1}$. The bound population is hence in dynamic exchange with the pool of diffusing molecules. However, most molecules remained either in the diffusing or bound state for the entire track, limited by photobleaching. To minimize the influence of photobleaching on the apparent repair times, we used longer exposures with low excitation intensities and sparse photoactivation. Under these conditions, PSFs of diffusing molecules were dim and blurred (because of motion during exposure), but PSFs of bound molecules remained narrow (Fig. 3 D and E). Because the population of narrow PSFs increased with MMS (red bars in Fig. 3F and SI Appendix, Fig. S7), we interpret the exponentially distributed Pol and Lig binding times as repair times $t_{Pol} = 2.1 \pm 0.3$ s and $t_{Lig} = 2.5 \pm 0.4$ s (Fig. 3 G and H and SI Appendix, Fig. S7 and Table S3).

### Pol and Lig Activities Are Limited by Substrate Availability

Single-cell analysis uncovered significant variation in repair activities across cells, with distributions ranging from 0% to 40% bound Pol and Lig (Fig. 4B). We examined four factors that may influence the observed rates and their variation: Pol and Lig copy numbers, MMS concentration, upstream reactions in the BER pathway; and MMS treatment time. To test the influence of Pol and Lig copy numbers, we measured DNA-binding and protein copy number. PALM can be used to directly count molecules at the single-cell level, considering that each PAmCherry fluorophore is activated once (29). We counted 479 Pol, 226 Lig, and 1,560 Fis copies/cell (median values; Fig. 4A and SI Appendix, Fig. S8). These numbers are in agreement with previous reports of ~400 Pol (17), ~200 Lig (1), and thousands of Fis (31).

Using the median copy numbers, we estimated cellular reaction rates of ~350 min$^{-1}$ for Pol and ~210 min$^{-1}$ for Lig (Table 1). The basal activities match expectations: DNA replication accounts for 35–60% of the observed rates, considering two replication forks per cell-generating Okazaki fragments at a rate of ~200 min$^{-1}$ (17). Additionally, Pol and Lig are involved in the repair of replication errors and damage by reactive metabolites, including endogenous methylating agents (1, 16). Normal metabolism causes 3,000–5,000 lesions per E. coli generation by oxidative DNA damage alone, requiring a repair rate of 25–40 min$^{-1}$ (39). Under MMS treatment, the reaction rates increased to ~3.7 min$^{-1}$ and ~5.1 min$^{-1}$ per single Pol and Lig, leading to cellular repair rates of ~1,780 min$^{-1}$ and ~1,150 min$^{-1}$, respectively (Table 1); the 50% higher Pol activity over Lig likely reflects the wider range of Pol substrates and its nick-translation activity, which competes with ligation (40). As the final step in the pathway, the Lig activity corresponds to the overall BER rate.

### Pol and Lig Activities Are Limited by Substrate Availability

Single-cell analysis uncovered significant variation in repair activities across cells, with distributions ranging from 0% to 40% bound Pol and Lig (Fig. 4B). We examined four factors that may influence the observed rates and their variation: Pol and Lig copy numbers, MMS concentration, upstream reactions in the BER pathway; and MMS treatment time. To test the influence of Pol and Lig copy numbers, we measured DNA-binding and protein copy number. PALM can be used to directly count molecules at the single-cell level. By linking the natural copy number variation between cells with the bound-fraction distribution across cells, we found that the percentage of bound Pol and Lig was lower in cells with higher copy numbers but similar size (Fig. 4C). In those cells, the excess copies joined the diffusing pool of molecules and the total number of bound...
Pol and Lig molecules saturated at \( \sim 60 \) and \( \sim 40 \) bound molecules/cell, respectively (Fig. 4D). This demonstrates that Pol and Lig activities are limited by substrate sites available for binding. Dose–response curves showed that Pol and Lig binding saturated with increasing MMS concentration (at \( \sim 13\% \) and \( \sim 18\% \) bound fraction, Fig. 4E). The Pol activity is therefore not limited by the initial damage, but instead by upstream BER steps, such as the glycosylase and nuclease reactions. Below saturating MMS concentrations, the steady-state ligation rate is expected to equal the rate at which lesions are generated. Based on previous in vitro and in vivo reports (41, 42), we estimated the damage rate by 25 mM MMS to \( \sim 430-460 \) lesions \( \cdot \) chromosome \( \cdot \) min \(^{-1} \), in agreement with our measured Lig rate of \( 469 \pm 136 \) reactions-cell \(^{-1} \) min \(^{-1} \) at 25 mM MMS (SI Appendix). We measured the same trend of saturated Pol activity during the MMS response over time, with the bound Pol fraction increasing significantly in 5 min and reaching a plateau at the limiting activity of \( \sim 13\% \) binding after 15 min of treatment (red curve in Fig. 5A). We also explored the Pol response to brief MMS treatment followed by MMS removal, showing rapid reduction of Pol binding after 5 min and return close to basal activity within \( \sim 15 \) min of recovery (Fig. 5B); Pol catalyzed \( \sim 18,000 \) repair events per cell during the damage and recovery experiment (orange shaded area in Fig. 5B). Residual activity after the initial fast recovery may reflect slower repair of complicated lesions such as double-strand breaks (39, 43).

Pretreatment of \( E. \) coli with a low MMS dose induces the adaptive response, which up-regulates 3-methyladenine DNA glycosylase II, rendering cells more resistant to subsequent higher doses (1, 16). Adapted cells showed the same timing of Pol activity during the initial MMS response as nonadapted cells, but saturation occurred at a higher value of \( \sim 19\% \) binding (cellular repair rate \( \sim 2,500 \) min \(^{-1} \); magenta curve in Fig. 5A). Increased repair

Fig. 3. Visualizing and timing individual repair events. (A) Single Pol track in an undamaged cell. (Scale bars, 0.5 \( \mu \)m.) Time trace of the corresponding diffusion steps from the displacements between localizations; dotted blue and red lines are the average \( D^* \) of free and bound Pol, respectively. (B) Two example Pol tracks in MMS damaged cells showing the substrate search path (light blue) that leads to a repair event (red), and continued search (dark blue). Corresponding time traces show \( D^* \sim 0 \) \( \mu \)m\(^2\) s \(^{-1} \) during repair events. (C) Lig search (magenta), repair (red), and continued diffusion (black), with \( D^* \) time trace. (D) Accumulated Pol-PAmCherry fluorescence of a whole movie. (Scale bars, 1 \( \mu \)m.) (E) Example frame at 750-ms exposure time with PSFs of bound and diffusing Pol (red and blue outlines). (F) Fitted PSF width in fixed cells, undamaged cells, and with 100-mM MMS at 750-ms exposure time. Bound populations with narrow PSFs are in red. (G) On-time distributions for bound Pol and Lig with exponential fits (solid lines) and photobleaching-corrected binding time distributions (dashed circled lines). (H) Mean Pol and Lig binding times in undamaged cells and with MMS (\( \pm \) SEM; from three exposure and excitation conditions).

Fig. 4. Single-cell analysis shows that Pol (Upper row) and Lig (Lower row) saturate repair substrates. (A) Protein copy number distributions. (B) Distributions of the percentage of bound Pol and Lig across cells in undamaged cells and with 100-mM MMS. (C) Percentages of bound Pol and Lig with MMS as a function of their copy numbers per cell (\( \pm \) SEM). (D) Number of diffusing and bound Pol and Lig with MMS as a function of their copy numbers per cell. Black lines: hypothetical case of unlimited substrates in which the bound percentage was independent of the copy number. (E) MMS dose–response curves show the average percentage of bound Pol and Lig for different MMS concentrations (\( \pm \) SEM; \( n = 4 \)).
initiation in adapted cells hence propagates through the pathway to enhance the overall BER rate and modulate the Pol activity that is limited by upstream pathway steps. We noticed that adaptation slowed the diffusion of free Pol and confined tracks to a smaller area in the cell (SI Appendix, Fig. S9); this observation indicates a global nucleoid compaction that might facilitate repair and physically protect the chromosome against damage (44).

**Substrate Search Times.** Binding times and reaction rates also inform about the search time \( t_{\text{search}} \), between subsequent reactions. Dictated by the availability of free substrates, \( t_{\text{search}} \) decreased from \(~85\) s for Pol in undamaged cells to \(~14\) s with saturating MMS damage, and increased again during recovery (Fig. 5C and D). Even at maximum BER activity, Pol and Lig spent \( >80\% \) of time searching for free substrates. Using the relation between the diffusion-limited association rate (45) and search times, we estimated four to five free gaps or nicks with lifetimes of \(~140\) ms and \(~90\) ms, respectively, to be present at any time with saturating MMS; this contrasts to 62 Pol-bound and 40 Lig-bound molecules. The short lifetime of free gaps and nicks, a consequence of the distributive search by excess numbers of Pol and Lig, minimizes accumulation of these repair intermediates and safeguards genomic stability.

**Discussion**

DNA synthesis and ligation are essential steps in base excision, nucleotide excision, and mismatch repair pathways of all cellular organisms. Here, we presented a direct view on DNA repair and generated a quantitative systems-level account of a model reaction pathway in vivo (Table 1 and Fig. 5E and F). Individual Pol and Lig molecules diffuse with little affinity for undamaged DNA but become transiently immobilized for repair on randomly distributed damage sites. Although Pol and Lig mutants are hypersensitive to DNA damage (11, 12), only a small percentage of WT activity appears to be required for normal growth (1, 17).

### Table 1. Pol and Lig activities in undamaged cells and under saturating MMS damage

<table>
<thead>
<tr>
<th>Experiment</th>
<th>% bound</th>
<th>Rate, molecule(^{-1}).min(^{-1})</th>
<th>Rate, cell(^{-1}).min(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pol</td>
<td>2.7 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>329 ± 76</td>
</tr>
<tr>
<td>Lig</td>
<td>3.8 ± 0.4</td>
<td>0.0 ± 0.2</td>
<td>210 ± 54</td>
</tr>
<tr>
<td>Pol MMS</td>
<td>13.0 ± 0.2</td>
<td>3.7 ± 0.5</td>
<td>1,782 ± 248</td>
</tr>
<tr>
<td>Lig MMS</td>
<td>17.9 ± 0.3</td>
<td>5.1 ± 0.7</td>
<td>1,152 ± 167</td>
</tr>
</tbody>
</table>

Bound molecules and rates per cell are stated for exact copy numbers of 479 Pol and 226 Lig (median values in Fig. 4A). Errors are ± SEM for % bound and binding times; other errors were estimated by error propagation. Variation in copy numbers will introduce additional variation in the number of bound molecules and rates per cell.

Fig. 5. Timing of the Pol response to DNA damage and recovery and the influence of the adaptive response on Pol repair rates. (A) Percentage of bound Pol (left axis), repair rates per Pol molecule and per cell (right axes), measured over time during constant 100-mM MMS treatment (red) and control (blue). The adaptive response was induced with 3-mM MMS for 1 h before measuring adapted cells under constant 100-mM MMS treatment (magenta) (± SEM; \( n = 3 \)) for nonadapted cells, \( n = 5 \) for adapted cells). (B) Pol DNA-damage response during 100-mM MMS treatment for 15 min, followed by recovery after removing MMS (blue background) (± SEM; \( n = 3 \)). The integrated Pol repair activity above the basal level is highlighted in orange. (C and D) Pol substrate search times based on data in A and B. (E) Model for Pol and Lig activities in undamaged E. coli, and under saturating MMS damage repair (F).
MMS (2). Abundance of high-fidelity Pol also favors competition against ~200 copies per cell of error-prone DNA Pol IV to lower mutagenesis associated with DNA repair (7, 43). Finally, gene expression noise and damage responses challenge the pathway balance and overall repair rates; excess amounts of Pol and Lig ensure sufficient repair capacities even in cells that contain relatively fewer copies.

Our approach can be used to elucidate a wide range of molecular processes in vivo (46). In principle, it allows quantification of reaction rates for any enzyme that changes diffusion characteristics upon substrate binding and release. Our results provide evidence for the existence of a balance and overall repair rates; excess amounts of Pol and Lig ensure sufficient repair capacities even in cells that contain relatively fewer copies.

Materials and Methods

Details on materials and methods are available in the SI Appendix. In brief, E.coli AB1157 endogenous PAM-Cherry fusions were created by J-Red recombination. Cells were grown in M9 minimal medium to OD ~0.1 and immobilized for imaging on 1% agarose pads, containing 100 mM MMS (Sigma) for DNA damage experiments (different concentrations in Fig. 4E).

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Supporting Information

Uphoff et al. Single-molecule DNA repair in live bacteria

1. Construction and characterization of photoactivatable mCherry (PAmCherry) fusion strains.
PAmCherry (1) fusion strains of DNA polymerase I (Pol), DNA ligase (Lig), and Fis protein, expressed from their native promoter in E.coli AB1157, were generated by replacing the polA, ligA, and fis genes by their PAmCherry fusion versions as described (2). Genes coding for PAmCherry1 and Kanamycin resistance cassette were amplified from the plasmid pROD85 (carrying an 11aa linker preceding PAmCherry and followed by frt flanked kan), using primers with 40-50 nt overhangs homologous to the insertion site at the chromosome (Supplementary Table 1). DNA fragments were treated with DpnI and ~1 μg was used for electroporation of AB1157 cells overexpressing lambda-Red proteins from pKD46 (3). Correct insertion of the fragment into the chromosome was evaluated by PCR using primers flanking the insertion site (Table S1). The growth rates in LB and M9 glycerol medium as well as methyl methanesulfonate (MMS, Sigma) sensitivity were the same for AB1157 wild-type and fusion strains (Table S2 and Fig. S1).

2. Cell preparation for microscopy. Strains were streaked onto LB plates with 25 μg ml⁻¹ kanamycin. Single colonies were inoculated into LB and grown at 37°C for 4-5 h, then diluted 1:10,000 into M9 medium (M9 salts, MEM amino acids + proline, MEM vitamins, 0.2% glycerol) and grown overnight at 37°C to OD 0.4-0.6. The following morning, cultures were diluted to OD of 0.025 in M9 medium and grown for 2 h at 37°C to early exponential phase (OD ~ 0.1). Cells were centrifuged and immobilized on agarose pads between two glass coverslips (1.5 thickness, burned to remove any fluorescent background particles). We prepared 1% agarose pads by mixing low-fluorescence 2% agarose in dH₂O 1:1 with 2x M9 medium. For DNA damage experiments, MMS was diluted into M9 medium before mixing with agarose solution. We used 100 mM MMS in all experiments; the lowest concentration that resulted in saturated Pol and Lig binding. For Fig. 4E we used 25 - 200 mM MMS. The adaptive response was induced by 3 mM MMS treatment in liquid M9 culture at 37°C for 1 h (Fig. S1 shows that this MMS concentration is non-lethal); adapted cells were subsequently prepared for imaging on 100 mM MMS agarose pads identical to the damage response experiments with non-adapted cells in Fig. 5A. For DNA damage recovery experiments, cells were immobilized using 1% polyethylenimine on glass coverslips with silicon gaskets. This allowed imaging in M9 medium first, then incubating with M9 medium + 100 mM MMS for 15 min, followed by removal of MMS and washing with M9 medium to allow recovery. For fixation, centrifuged cells prepared as above were resuspended into 2.5% paraformaldehyde in PBS buffer and fixed for 45 min shaking at 22°C. Fixed cells were washed with PBS and immobilized on agarose pads as above.

3. Microscope setup. PAmCherry fusion proteins were imaged on a custom-built total internal reflection fluorescence (TIRF) microscope with a 561 nm laser (SLIM-561 200 mW, Oxxius) and photoactivated with a 405-nm laser (MLL-III-405 100 mW, CNI). The 561-nm and 405-nm laser beams were coupled into the same single-mode optical fiber. At the fiber output, the laser beams were collimated and focused (100x oil immersion objective, NA 1.4, Olympus) onto the sample under an angle allowing for highly inclined thin illumination (4). Fluorescence emission was filtered by a dichroic mirror and notch filter (ZT405/473/561rpc & ZET405/473/561NF, Chroma). PAmCherry emission was projected onto the central region of an EMCCD camera (iXon 897, 512x512 pixels, Andor) using a custom-built triple view component (T540lpxr & T640lpxr, Chroma). The pixel size was measured as 114.5 nm. Cell outlines were recorded with an LED source (pE-100, coolLED) in an Olympus condenser (IX-2, Olympus). Sample position and focus were controlled with a motorized stage and z-motor (MS-2000 and LS-50, ASI Imaging).

4. Data acquisition. PALM movies were recorded under continuous 400 W·cm⁻² 561-nm excitation at 15.26 ms/frame for 7,500 or 10,000 frames; transmitted light microscopy images of the cell outlines were acquired before PALM imaging. Photoactivating less than one PAmCherry fluorophore per cell at any time was controlled by adjusting the 405-nm excitation (from 0 - 1 W·cm⁻²) over the duration of the movie. We confirmed cell viability after PALM imaging (Fig. S1). Using cells immobilized on agarose pads with rich defined medium (Teknova), we followed normal cell growth and division on the microscope slide at room temperature for 45 min. We subsequently imaged Pol-PAmCherry with standard acquisition and excitation conditions. After PALM imaging, cells continued normal growth, but we noticed a 15-20 min time lag before growth continued. 67 out of 70 imaged cells (95.7%) recovered growth, showing no further sign of light-induced DNA damage.
5. Data processing. Localization analysis was performed using custom-written MATLAB software (MathWorks), adapted from Refs. 5 and 6. PSFs were identified for localization by band-pass filtering and applying an intensity threshold to each frame of a super-resolution movie. Candidate positions were used as initial guesses in a two-dimensional elliptical Gaussian fit for high-precision localization. Free fit parameters were x-position, y-position, x-width, y-width, elliptical rotation angle, intensity, background. Single-particle tracking analysis was performed by adapting the MATLAB implementation of the algorithm described in Ref. 7. Positions were linked to a track if they appeared in consecutive frames within a window of 5 pixels (0.57 μm) for Pol and Lig; this window size ensures 98% of steps are correctly linked for an apparent diffusion coefficient of 1 μm²·s⁻¹. The tracking window for Fis was 8 pixels (0.92 μm). In rare cases when multiple localizations fell within the tracking radius, tracks were linked such that the sum of step distances was minimized. We used a memory parameter of 1 frame to allow for transient disappearance of the PSF within a track due to blinking or missed localization.

6. Diffusion analysis of single-molecule tracks. We distinguished bound and diffusing proteins by calculating an apparent diffusion coefficient \( D^* = \text{MSD}/(4 \Delta t) - \sigma_{\text{inc}}^2/\Delta t \) from the mean-squared displacement (MSD) for each track with a minimum of 4 steps, correcting for the localization standard deviation of 40 nm. Shorter tracks were discarded for this analysis because the statistical error prohibits clear separation of bound and diffusing proteins. Histograms of \( D^* \) show a peak at \( D^* \sim 0 \) μm²·s⁻¹ for the bound molecules and a separate distribution for the diffusing molecules, the width of which matched simulated data (Fig. S4). We note that \( D^* \) is an apparent diffusion coefficient, used to identify bound and diffusing molecules, and does not equal an accurate diffusion coefficient because of cell confinement and motion blurring (8); instead, we simulated Brownian motion and modelled PALM movies to find the input diffusion coefficient that reproduced observed experimental data (see below). Guided by the \( D^* \) distributions in fixed cells, we set two thresholds \( D^* < 0.15 \) μm²·s⁻¹ for MSD(Δt=15.26ms) and \( D^* < 0.075 \) μm²·s⁻¹ for MSD(Δt=30.52ms) to identify bound tracks. The percentage of bound molecules was calculated from the number of bound tracks divided by the total number of tracks with 4 or more steps. Binding distributions across cells were measured by identifying the fraction of bound molecules within manually segmented cells.

Using simulated data of a single diffusing species matching experimental data (see below), we estimated false positive identification of bound tracks (erroneous bound identification of diffusing molecules) to 0.7%. Using simulations of a mixed population of bound and diffusing tracks (containing 15% of tracks with \( D = 0 \) μm²·s⁻¹) we also estimated a false negative identification (missed identification of a bound track) of 1.1%.

Complete repair events in our experimental data (Fig. 3A-C, Fig. S6) were identified in tracks that showed diffusion flanking a full binding event by calculating a time trace of \( D^* \) over all steps of a track. Only tracks with 12 or more steps were included in this analysis. 1.1% of 3700 analyzed Pol traces showed complete binding events in MMS damaged cells; whereas 0.2% of ~2200 Pol traces in undamaged cells, respectively. This reflects the ~5 fold increase of Pol binding with MMS.

7. Simulations. We simulated three-dimensional Brownian motion with diffusion coefficient \( D \) by creating random steps in x, y, and z directions. The step length in each direction was drawn from a Gaussian distribution with zero mean and variance \( 2D \cdot dt \), with a simulation time step \( dt = 0.1526 \) ms (100 time steps per 15.26-ms frame). The population of immobile tracks was generated by randomly generating individual tracks with \( D = 0 \) μm²·s⁻¹ for the whole duration of the track, such that the average percentage of immobile tracks matched experimental results of 2.7% bound Pol and 3.8% bound Lig in undamaged cells. The rod-shaped three-dimensional confinement was approximated by a cylinder with spherical caps (2 μm length, 0.65 μm diameter); this area accounts for confinement to the nucleoid rather than the whole cytoplasm, as can be seen in Fig. 1F and Fig. S2. Initial molecule positions were uniform within the confinement. Steps that would lead outside the confinement were rejected. For stochastic photoactivation and bleaching, single tracks started at random times uniformly distributed over the total number of time steps and each track length was drawn from an exponential distribution with an experimentally determined mean duration of 54 ms. We simulated 400 tracks per cell in 152 s long movies (10^6 time steps) such that the density of activated particles was similar to experiments.

To accurately model the raw experimental fluorescence data including motion blurring, PSFs were centred on each position of the simulated Brownian motion tracks that were projected onto the x-y plane. Matching our experimental frame rate, every 100 simulation time steps were integrated to form a frame of a movie.
with 10,000 frames in total. Simulation parameters for the confinement volume, PSF intensity, PSF width, background noise per pixel, number of frames per movie, number of molecules per cell, and mean number of frames per track were chosen to match experimental values. Simulated movies were saved for localization, tracking, and further data analysis (MSD curves, cumulative distribution curves, $D^*$ distributions), performed exactly the same as for experimental data.

To evaluate whether simple Brownian motion with a bound population matches experimental observations and to extract accurate diffusion coefficients, we performed simulations for a range of diffusion coefficients $D = 1.5 - 5.5 \mu m^2 s^{-1}$ with increments of 0.1 $\mu m^2 s^{-1}$. Simulation results were evaluated by calculating the squared deviation between simulated and experimental data based on MSD curves, cumulative distribution curves, and $D^*$ distributions. The best parameter estimate for the accurate diffusion coefficient $D$ was obtained from the minimum of the squared deviation values (least squares estimate). We found diffusion coefficients $D_{Pol} = 2.7 \pm 0.4 \mu m^2 s^{-1}$ and $D_{Lig} = 3.5 \pm 0.3 \mu m^2 s^{-1}$ from simulations that precisely matched experimental data (Fig. S4). These results are consistent with previous reports for proteins of similar size (9, 10). Because of confined diffusion and motion blurring, $D^*$ distributions peak at lower values $D^*_{Pol} \sim 0.8 \mu m^2 s^{-1}$ and $D^*_{Lig} \sim 1 \mu m^2 s^{-1}$, as for experimental data.

The nucleoid-association indicates non-specific DNA-binding of Pol and Lig in vivo. On the other hand, their diffusion in undamaged cells is consistent with ordinary Brownian motion. Taken together, these two findings suggest that Pol and Lig interact transiently with DNA, on a time-scale shorter than our exposure time (15 ms/frame). Multiple cycles of binding and diffusion per frame result in a Gaussian distribution of displacements characteristic of Brownian motion. The combination of three-dimensional diffusion and one-dimensional sliding on DNA can facilitate the search for specific DNA sites (11); however, the relative non-specific binding and diffusion times in vivo remain unknown for most proteins, including Pol and Lig. Our observation of weak non-specific binding is in agreement with the requirement of a primer-template DNA structure for tight binding of Klenow fragment in vitro (12-14). Similarly, the affinity of DNA ligases for dsDNA appears to be much weaker than binding to a nicked DNA substrate (15, 16).

8. Binding-time distributions using long exposure times. PALM movies to measure binding times with reduced photobleaching were recorded at low continuous 561-nm excitation intensities (40 W·cm$^{-2}$, 60 W·cm$^{-2}$, and 80 W·cm$^{-2}$) using long exposure times (500.26 ms/frame, 750.26 ms/frame, and 1000.26 ms/frame). Bound and diffusing molecules were distinguished by the mean PSF width over each track, with thresholds x-width < 160 nm and y-width < 200 nm to identify bound molecules (Fig. 3D-F, Fig. S7A-G). Note that the percentage of bound molecules under these conditions appears larger than for short exposure times since this approach focuses mainly on the bound molecules and their binding-time distribution, which is defined by the duration of tracks that passed the width thresholds. The probability of observing a particular on-time is the product of the underlying binding-time probability and the bleaching probability. The bleaching-time distributions were measured independently using Pol-PAmCherry in fixed cells with the same acquisition and excitation conditions (Table S3). On-time and bleaching-time distributions were fitted with single-exponential functions to extract exponential-time constants $t_{on}$ and $t_{bleach}$, and the binding-time constant was calculated by $t_{bound} = t_{on} \cdot t_{bleach} / (t_{bleach} - t_{on})$.

Stochastic photoactivation of molecules before or during binding events does not influence our measurement, because the observed binding times follow an exponential distribution and are therefore memoryless. We confirmed that the bleaching rates in fixed cells and live cells were the same by using a PAmCherry fusion protein that unbinds DNA much slower than photobleaching, such that the observed on-time distribution in live cells equals the bleaching-time distribution. The Structural Maintenance of Chromosomes complex in E.coli, MukBEF, binds DNA in 1-3 large clusters per cell with a turnover rate of ~50 s (17). Localizations of bound MukE-PAmCherry were at the expected positions and the on-time distribution at 1-s exposure and 40 W·cm$^{-2}$ excitation was essentially identical to the bleaching-time distribution from Pol-PAmCherry in fixed cells under the same exposure and excitation conditions (Fig. S7H).

9. Protein copy number measurements. To obtain protein copy numbers per cell (Fig. 4A, Fig. S8), we ensured activation of essentially all PAmCherry molecules by acquiring long movies (up to 20,000 frames; lasting for 5 min) with a constant photoactivation rate by increasing 405-nm excitation gradually (18). We used highly inclined thin illumination (4) to image the majority of molecules within ~0.8 $\mu m$ thick cells. Any
residual excitation gradient will not affect molecule counts in live cells because all diffusing molecules will get activated and imaged when they move into the excitation/activation field. We manually segmented cell outlines in transmitted light microscopy images to determine the number of tracks within each cell. Cells were split if a septum was clearly visible in the transmitted light microscopy image. On the basis of the PAmCherry maturation time of 23 min (1) and a steady-state cell generation time of ~120 min in M9 glycerol at room temperature, we estimated a molecule detection efficiency of ~80%. In fixed cells, Pol counts were 20% lower than in live cells.

10. Estimating repair rates, search times, and substrate numbers. Using the measured binding times, we calculated the search time from the fraction of bound molecules:

$$N_{\text{bound}}/(N_{\text{bound}} + N_{\text{free}}) = t_{\text{bound}}/(t_{\text{bound}} + t_{\text{search}}).$$

The repair rate per molecule is given by

$$r = 1 / (t_{\text{bound}} + t_{\text{search}}).$$

The total repair rate per cell for a given copy number $N_{\text{copies}}$ is

$$r_{\text{cell}} = N_{\text{copies}} \cdot r.$$

The diffusion-limited search time of a single enzyme with diffusion coefficient, $D$, for free substrates with concentration, $C_{\text{substrate}}$, and association radius, $a$, is given by Ref 11:

$$t_{\text{search}} = 1 / (4 \pi \cdot D \cdot a \cdot C_{\text{substrate}}).$$

Using $C_{\text{substrate}} = N_{\text{substrate}}/V_{e.coli}$ we calculate the number of free substrates:

$$N_{\text{substrate}} = V_{e.coli} / (4 \pi \cdot D \cdot a \cdot N_{\text{enzymes}}).$$

We use parameter values $V_{e.coli} = 10^{-18} \text{ m}^3$; $D_{\text{pol}} = 2.7 \mu\text{m}^2\text{ s}^{-1}$ and $D_{\text{lig}} = 3.5 \mu\text{m}^2\text{ s}^{-1}$ (see simulations); $a = 0.5 \text{ nm}$; $t_{\text{search}} \approx 14 \text{ s}$ for Pol and $t_{\text{search}} = 9 \text{ s}$ for Lig (with saturating MMS). This gives $N_{\text{substrate}} = 4.2$ for Pol, and $N_{\text{substrate}} = 5.1$ for Lig.

We further calculate the lifetime of a free substrate before enzyme binding:

$$t_{\text{free}} = \frac{V_{e.coli}}{(4 \pi \cdot D \cdot a \cdot N_{\text{enzymes}})}.$$

$N_{\text{enzymes}}$ refers to the number of free enzymes that are available for substrate binding, accounting for the fraction of enzymes already bound. We obtained this fraction from our measurements at saturating MMS (Table 1): $N_{\text{enzymes}} = 479 - 62 = 417$ for Pol, and $N_{\text{enzymes}} = 226 - 40 = 186$ for Lig.

The lifetime of gapped substrates, considering only Pol binding, gives $t_{\text{free}} = 140 \text{ ms}$. With Pol and Lig both competing for nicked substrates, we find $t_{\text{free}} = 90 \text{ ms}$. Alternatively, we can use the fact that the turnover rate of free substrates equals the enzyme repair rate $r_{\text{cell}}$ (Table 1) at equilibrium:

$$r_{\text{cell}} = N_{\text{substrate}} / t_{\text{free}}.$$

Using $r_{\text{cell}} = 29.7 \text{ s}^{-1}$ for Pol, and $r_{\text{cell}} = 19.2 \text{ s}^{-1}$ for Lig gives the same results for $t_{\text{free}}$.

11. Estimating MMS damage rates from published data. An in vitro method for quantification of DNA methylation damage has been reported (19). The authors measured 47 7meG lesions per 10^6 bases after 25 mM MMS treatment for 60 min. Considering that 7meG accounts for 83% of all MMS lesions, this gives a total damage rate of 430 lesions-chromosome$^{-1}$·min$^{-1}$. Consistent with this, early in vivo work showed that 60 min treatment with 25 mM radioactively labelled MMS causes methylation of 0.6% of DNA bases in E.coli (20). This gives an estimate for the damage rate of 460 lesions-chromosome$^{-1}$·min$^{-1}$. These numbers can be compared to our measured ligation rate, which is expected to equal the rate at which lesions are generated under non-saturating MMS concentrations in steady state. Using the Lig rate at 25 mM MMS (Fig. 4E) and subtracting the basal reaction rate gives a repair rate of 469 ± 136 reactions-cell$^{-1}$·min$^{-1}$.

12. Discussion on potential aggregation and localization artifacts of fluorescent fusion proteins. It has been shown that fluorescent protein fusions can aggregate in cells (21) and may create artifacts in protein localization (22). We closely inspected our data in this respect and found that all characteristics of the tracking data exclude localization artifacts or aggregation of the PAmCherry fusion proteins in live E.coli: (i) The majority (>95%) of Pol, Lig, and Fis showed rapid diffusion in undamaged live cells; (ii) The MSD and cumulative distribution curves scaled with the expected individual molecular weights of the Pol, Lig, and Fis fusion proteins; (iii) Protein localizations were distributed over a large area in the cell corresponding to the nucleoid without any sign of clustering; (iv) Simulations showed that the observed diffusion is consistent with ordinary Brownian motion and the extracted diffusion coefficients for Pol and Lig matched those of other proteins with similar molecular weight; (v) We did not observe any change in MMS sensitivity of strains carrying Pol or Lig PAmCherry fusions compared to wild type as would be caused by aggregation or localization artifacts; (vi) Bound tracks of Pol III-PAmCherry (ε subunit) were localized exclusively at the
expected positions of the replication fork in live *E. coli*, while diffusing Pol III were distributed throughout cells; there was no sign of binding, sticking, or aggregation at other positions in the cell; (vi) Tracking MukBEF-PAmCherry fusions in live *E. coli* also showed bound molecules only at positions in the cell that were previously observed using different fluorophores (17).

References
Fig. S1. MMS sensitivity and viability after imaging. (A) Serial dilutions of strains were grown overnight on LB agar at 42°C (top), and 37°C (bottom) without MMS (left), 3 mM MMS (middle), 6 mM MMS (right). Wild type AB1157 showed the same MMS sensitivity and growth rates as fusion strains polA-PAmCherry and ligA-PAmCherry. The temperature sensitive DNA polymerase I mutant strain polA<sub>ts</sub> was hyper-sensitive to MMS. (B) E.coli cells are viable after imaging. 0 - 45 min: Cells grow on the slide. 50 - 53 min: PALM imaging of Pol-PAmCherry under standard illumination conditions. 53 - 75 min: Cells halt growth for ~20 min. 75 - 200 min: Cells continue normal growth. 67 out of 70 cells (95.7%) recovered growth, with no sign of light-induced damage.
Fig. S2. The spatial distributions of Lig and Pol tracks reflect the organization of the nucleoid in replicating E.coli. Tracks are shown in blue/grey, histograms of localizations projected onto the normalized long cell axis are shown underneath together with the cell length. Like the nucleoid, Pol and Lig tracks occupied the central area of the cell, avoiding the cell poles. Chromosome separation is evident from discrete areas of high track density in longer cells. (A) Short cells with a single chromosome. (B) Medium length cells during replication in the process of chromosome separation. (C) Long cells with two separate chromosomes. (D) Lig localizations from 20 cells and Pol localizations from 47 cells projected onto the short cell axis, showing the ~0.6 μm width of the nucleoid. (E) Based on Pol localization distributions, cells were manually sorted by the number of chromosomes and counted (134 cells). (F) Average cell length versus the number of chromosomes (±SEM, 134 cells).
Fig. S3. Single-molecule tracking controls. Scale bars: 1 μm. (A) Tracking the ε subunit of Pol III in live *E. coli* as a positive control for DNA-binding. Tracks of molecules that were classified as bound are shown in random colours; all other tracks, corresponding to the diffusing molecules, are shown in blue. The yellow track in the lower quarter of the second panel also displays the search path of a single Pol III molecule. (B) Tracking the nucleoid associated protein Fis as control without DNA repair activity. Fis tracks are shown in undamaged cells, 20 min 100 mM MMS-treated cells, and 120 min 100 mM MMS-treated cells. Tracks of diffusing and bound molecules are plotted in green and red, respectively. (C) Apparent diffusion coefficient ($D^*$) distributions corresponding to Fis tracking data in panel B. N > 10,000 tracks; populations of bound molecules are shown in red.
Fig. S4. Pol and Lig diffusion match simulations of Brownian motion with diffusion coefficients $D_{\text{Pol}} = 2.7 \pm 0.4 \, \mu m^2 \cdot s^{-1}$ and $D_{\text{Lig}} = 3.5 \pm 0.3 \, \mu m^2 \cdot s^{-1}$. (A) Simulated 3D Brownian motion tracks. (B) Simulated tracks were used to model PALM movies (example frame shown) for localization and tracking analysis that was performed identical to analysis of experimental data. (C) Example frame from an experimental PALM movie and resulting tracks. (D-I) Comparison between simulation (orange) and experiment for Pol (blue, Upper) and Lig (gray, Lower) in undamaged cells: (D-E) Mean-squared displacement (MSD) versus lag time (± SD). (F-G) Cumulative distributions of the diffusion step length (curve width: ±SD.). (H-I) Apparent diffusion coefficient ($D^*$) distributions (normalized to maximum values).
Fig. S5. The spatial distribution of Pol and Lig repair sites with 100 mM MMS is uniform across the nucleoid. (A) Projection of the distribution of mobile Pol localizations (top) and bound Pol localizations (bottom) onto the normalized long cell axis (combined data from 95 cells). (B-F) Top: Example cells with diffusing Pol (blue tracks) and mean positions of bound Pol (red dots). Bottom: Projection of mobile Pol localizations onto the normalized long cell axis for the cell shown above. Mean localizations of bound Pol are shown in red. (G-L) Spatial distribution of Lig DNA repair sites (data displayed as for Pol).
Fig. S6. Further examples of Pol and Lig binding events in undamaged cells and under 100 mM MMS treatment. (A-B) Example tracks (starting at green dot, finishing at black dot) and time traces of the diffusion steps showing Pol and Lig movement in undamaged cells. Dotted blue and red lines indicate the average $D^*$ for mobile and bound molecules. (C-D) Example Pol and Lig tracks and time traces showing full binding events (highlighted as red arrows) flanked by diffusion in undamaged cells. (E-F) Example Pol and Lig tracks and time traces showing full binding events (red arrows), flanked by diffusion in MMS-damaged cells. Scale bars: 0.5 μm.
Fig. S7. Measuring binding times. (A-C) Fitted elliptical Gaussian PSF width at 750-ms exposures (fit parameters: x width, y width, and rotation angle; x width was assigned to the shorter elliptical axis). (A) Pol in fixed cells showed exclusively narrow PSFs. (B) Pol in undamaged cells showed blurred PSFs of diffusing molecules. 100 mM MMS treatment increased the population of narrow PSFs. (C) Lig in undamaged cells and with 100 mM MMS measured at different exposure times and excitation intensities. Solid lines are exponential fits to the on-time distributions for bound molecules (t_{on} ± SD). Dashed lines with circles are the on-time distributions (t_{bound} ± SD), recovered by correcting t_{on} for photobleaching t_{bleach} of PAmCherry (Table S3). Curves were normalized by maximum value. (D) 500 ms exposure time, 80 W·cm^(-2) excitation intensity. (E) 750 ms, 60 W·cm^(-2). (F) 1000 ms, 40 W·cm^(-2). (G) 750 ms, 60 W·cm^(-2); undamaged cells. (H) Independent measure of the photobleaching rate in live cells using MukE-PAmCherry. Clusters of bound MukE (green dots; as reported before: Ref. 17) are shown on a transmitted light microscopy image. (I) t_{on} distribution for bound MukE-PAmCherry (1000 ms exposure, 40 W·cm^(-2) excitation; same conditions as for Pol and Lig in panel F). Because MukE unbinding is slow (~50 s; Ref. 17) compared to photobleaching, t_{on} matches the photobleaching time t_{bleach} = 2.15 ± 0.15 s measured for Pol-PAmCherry in fixed cells (Table S3). This confirms that the PAmCherry photobleaching rates are similar in live and fixed cells.
**Fig. S8.** Protein copy numbers per cell. (A) Pol-PAmCherry in live *E. coli* (median value 479). (B) Pol-PAmCherry in fixed *E. coli* (median value 421). (C) Fis-PAmCherry in live *E. coli* (median value 1560). (D) Lig-PAmCherry in live *E. coli* (median value 226). Insets show copy numbers as a function of the cell length.

**Fig. S9.** Influence of the adaptive response to MMS damage on Pol diffusion and binding. The adaptive response was induced with 3 mM MMS for 1 h and adapted cells were measured subsequently under constant 100 mM MMS treatment identical to non-adapted cells. (A) Apparent diffusion coefficient ($D^*$) distributions for Pol in non-adapted cells (top, blue) and in adapted cells (bottom, magenta). Adaptation leads to increased Pol binding under saturating MMS treatment (18.6 ± 0.2 % in adapted cells vs. 13.0 ± 0.2 % in non-adapted cells). The shift of the unbound Pol population to lower $D^*$ values in adapted cells (shown by vertical dashed lines) indicates compaction and increased viscosity of the nucleoid upon adaptation. N > 10,000 tracks; populations of bound molecules are shown in red. (B) Pol tracks in non-adapted cells and (C) in adapted cells with 100 mM MMS. Diffusing tracks in blue/magenta, bound tracks in red. The spatial distribution of the tracks reflects the size and position of the nucleoid, which appears more compacted in adapted cells. Scale bars: 1 μm.
Lambda polA-F: TGCCGTGCGTCGCTGGGAGTGGGGAGTGGCGAAAACTGGGATCAGGC
Lambda polA-R: GTGACAGCTTGTTCTTACTTACGAAAGGCATGTTCCGGCGAACTTATGAAATTATCCTCTTTAG
Lambda ligA-F: TGGGCATTGAAGTCATCGACGAAGCGGAAATGCTGCGTTTGCTGGGTAGCTGGCTGGCTGCTGGCTGCTGC
Lambda ligA-R: CAAACGGCATTATCGCTATTTCAATCAATGGCCTACGGCTACGGCATGCACCGAATACGCATGTAACATTGAAAAATGC
Lambda fis-F: gcattaatgctgccgacctgtggcaaataggccagtggcctggccagagccag
Lambda fis-R: ATCACTTCAAACAGCCAGTCGC
polA seqF: GAAGCGCAAGTACAGTGA
polA seqR: TGCTTGCCGGATGTGGC
ligA seqF: TTGGGACACGTGAAAGCGG
ligA seqR: AGACCGAAGTCAGGG
fis seqF: ATCTGGACACTGGGGAGTTG
fis seqR: ATCACTTCAAACAGCCAGTGC

Table S1. Primers used in the construction and characterization of strains carrying PAmCherry fusions.

<table>
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<th>AB1157 wild type</th>
<th>polA-PAmCherry</th>
<th>ligA-PAmCherry</th>
<th>fis-PAmCherry</th>
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<tr>
<td>LB (min)</td>
<td>28.7</td>
<td>29.7</td>
<td>28.8</td>
<td>28.4</td>
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<tr>
<td>M9 glycerol (min)</td>
<td>86.6</td>
<td>87.7</td>
<td>88.8</td>
<td>84.5</td>
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Table S2. Growth rates at 37°C.

<table>
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<tr>
<th></th>
<th>500 ms, 80 W·cm⁻² + MMS</th>
<th>750 ms, 60 W·cm⁻² + MMS</th>
<th>1000 ms, 40 W·cm⁻² + MMS</th>
<th>750 ms, 60 W·cm⁻² no MMS</th>
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</thead>
<tbody>
<tr>
<td>t_{bleach} [s]</td>
<td>0.97 ± 0.04</td>
<td>1.33 ± 0.06</td>
<td>2.15 ± 0.15</td>
<td>1.33 ± 0.06</td>
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<tr>
<td>Pol t_{on} [s]</td>
<td>0.66 ± 0.05</td>
<td>0.82 ± 0.05</td>
<td>1.05 ± 0.03</td>
<td>0.85 ± 0.03</td>
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<tr>
<td>Pol t_{bound} [s]</td>
<td>2.07 ± 0.57</td>
<td>2.16 ± 0.56</td>
<td>2.06 ± 0.59</td>
<td>2.36 ± 0.53</td>
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<tr>
<td>Lig t_{on} [s]</td>
<td>0.70 ± 0.02</td>
<td>0.87 ± 0.04</td>
<td>1.16 ± 0.04</td>
<td>0.86 ± 0.04</td>
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<tr>
<td>Lig t_{bound} [s]</td>
<td>2.44 ± 0.56</td>
<td>2.53 ± 0.62</td>
<td>2.52 ± 0.74</td>
<td>2.48 ± 0.61</td>
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Table S3. Exponential time constants for PAmCherry photobleaching t_{bleach}, measured on-times t_{on}, and corrected binding times t_{bound} for Pol and Lig with 100 mM MMS treatment (columns 1-3) and without MMS treatment (column 4) (±SD).
Movie S1. Part of a photoactivated localization microscopy (PALM) movie of an undamaged cell showing diffusion of a single DNA polymerase I (Pol) protein labeled with photoactivatable mCherry (cyan), superimposed onto transmitted light microscopy image (gray).
Movie S2. Part of a PALM movie showing binding of individual Pol-PAmCherry proteins (red) in MMS-treated cells, superimposed onto transmitted light image (gray).

Movie S2

Other Supporting Information Files

SI Appendix (PDF)