SUPPLEMENTARY INFORMATION

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Supplementary Figures

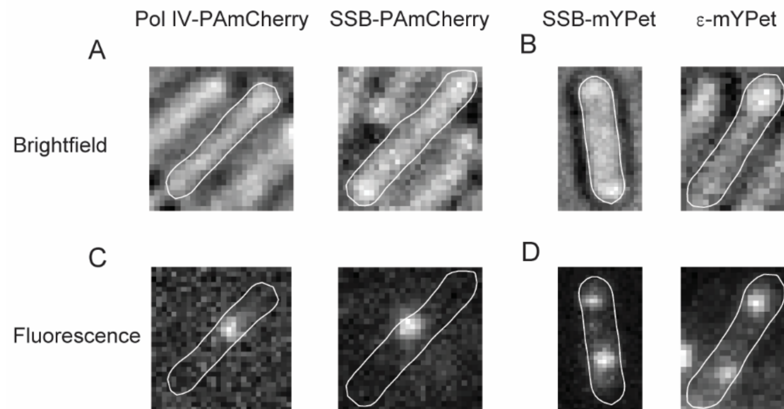

Fig. S1. Particle-tracking PALM imaging of Pol IV-PAmCherry and SSB-PAmCherry molecules and standard near-TIRF fluorescence imaging of SSB-mYPet and Pol III ε-mYPet foci. Representative brightfield micrographs of cells containing (*A*) PAmCherry (*pamcherry1*) fusions to the endogenous copy of the Pol IV gene *dinB* (left) or a second copy of the single-stranded DNA-binding protein gene *ssb* at the *lacZ* locus (right) and (*B*) mYPet (*mypet*) fusions to a second copy of *ssb* at the *lacZ* locus (left) or the endogenous copy of the ε subunit of Pol III *dnaQ* (right). Representative fluorescence micrographs of (*C*) single activated Pol IV-PAmCherry (left) or SSB-PAmCherry (right) molecules and (*D*) SSB-mYPet (left) and ε-mYPet (right) foci recorded with 250 ms integration times with overlays of the cell outlines.

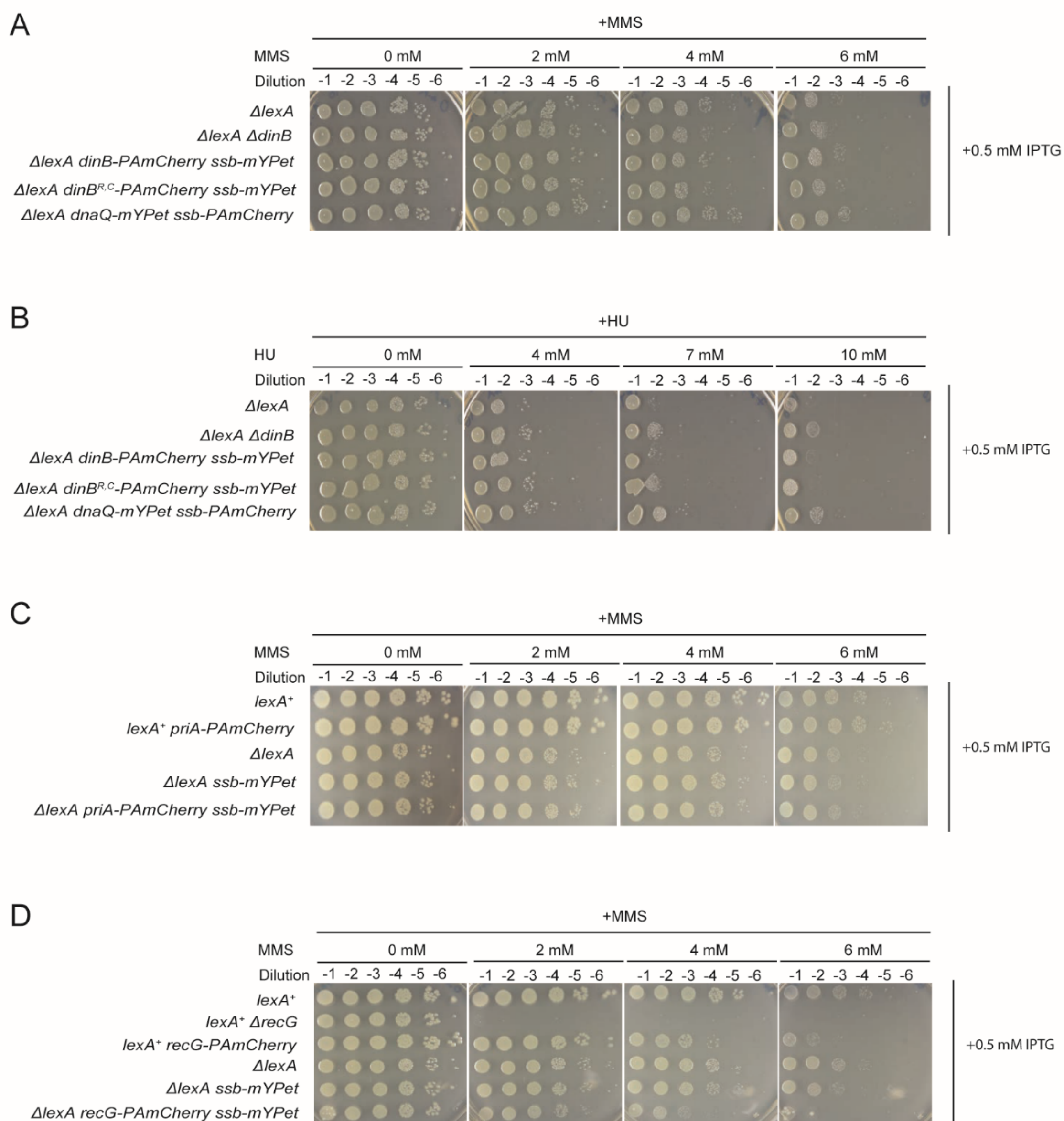

Fig. S2. (*A*) Serial 10-fold dilutions of *E. coli* strains with mutants and fusions to Pol IV (*dinB*) grown on LB agar plates with and without methyl methanesulfonate (MMS) added at 0, 2, 4, and 6 µM concentration. (*B*) Serial 10-fold dilutions of *E. coli* strains with mutants and fusions to Pol IV (*dinB*) grown on LB agar plates with and without hydroxyurea (HU) added at 0, 4, 7, and 10 µM concentration. Serial 10-fold dilutions of *E. coli* strains with fusions to (*C*) PriA (*priA*) or (*D*) RecG (*recG*) grown on LB agar plates with and without MMS added at 0, 2, 4, and 6 mM concentration.

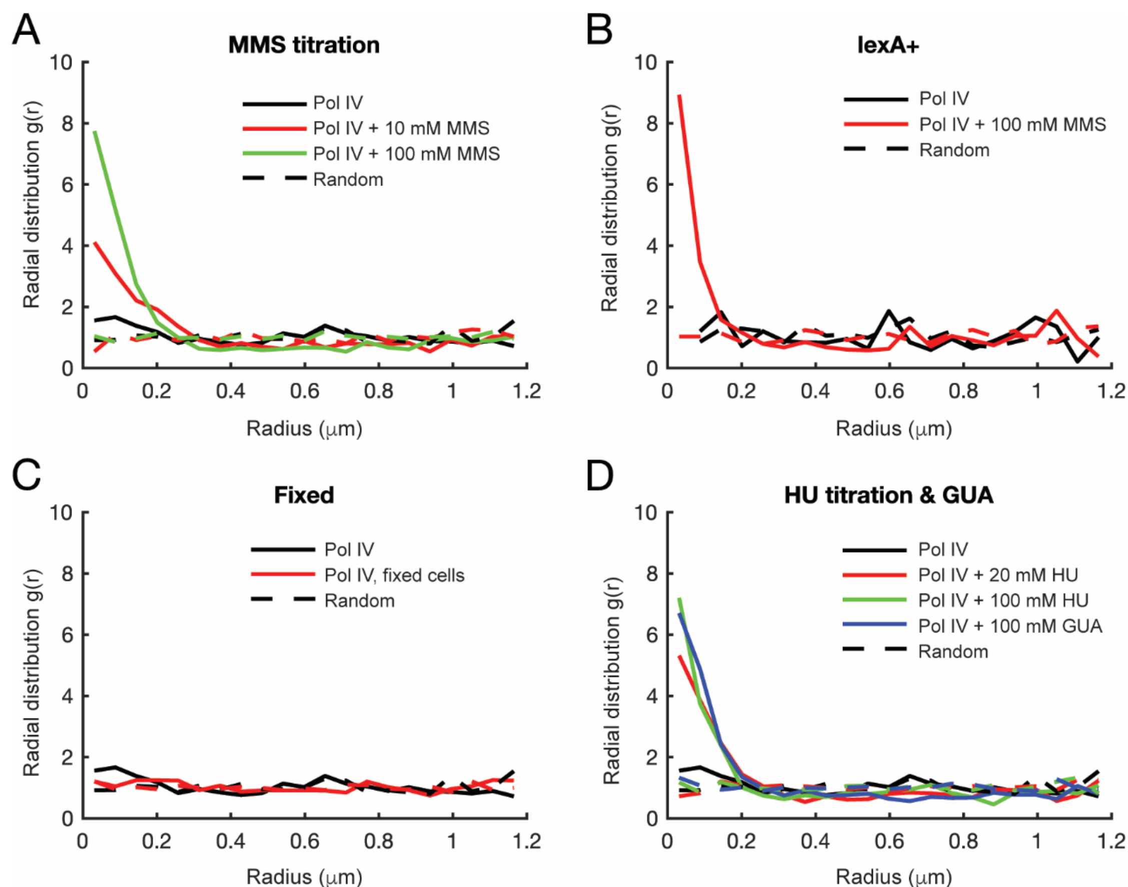

Fig. S3. Single-cell colocalization of Pol IV-PAmCherry and SSB-mYPet under different treatment conditions. (*A*) Radial distribution function *g(r)* between each static Pol IV-PAmCherry track and the nearest SSB-mYPet focus for untreated cells (black, *N* = 1,482) and cells treated with 10 mM (red, *N* = 1,273) or 100 mM (green, *N* = 2,754) MMS. (*B*) *g(r)* for untreated cells (black, *N* = 320) and cells treated with 100 mM MMS (red, *N* = 963) in the non-constitutive SOS-induced $lexA^+$ background. (*C*) *g(r)* for untreated cells (black, *N* = 1,482) and fixed cells (red, *N* = 5,596). (*D*) *g(r)* for untreated cells (black, *N* = 1,482) and cells treated with 100 mM HU (green, *N* = 1,539), 20 mM HU (red *N* = 2,267), and 100 mM guanazole (blue, *N* = 2,259. The untreated, 100 mM MMS, and 100 mM HU *g*(*r*) curves are replotted from Fig. 1 to enable comparison.

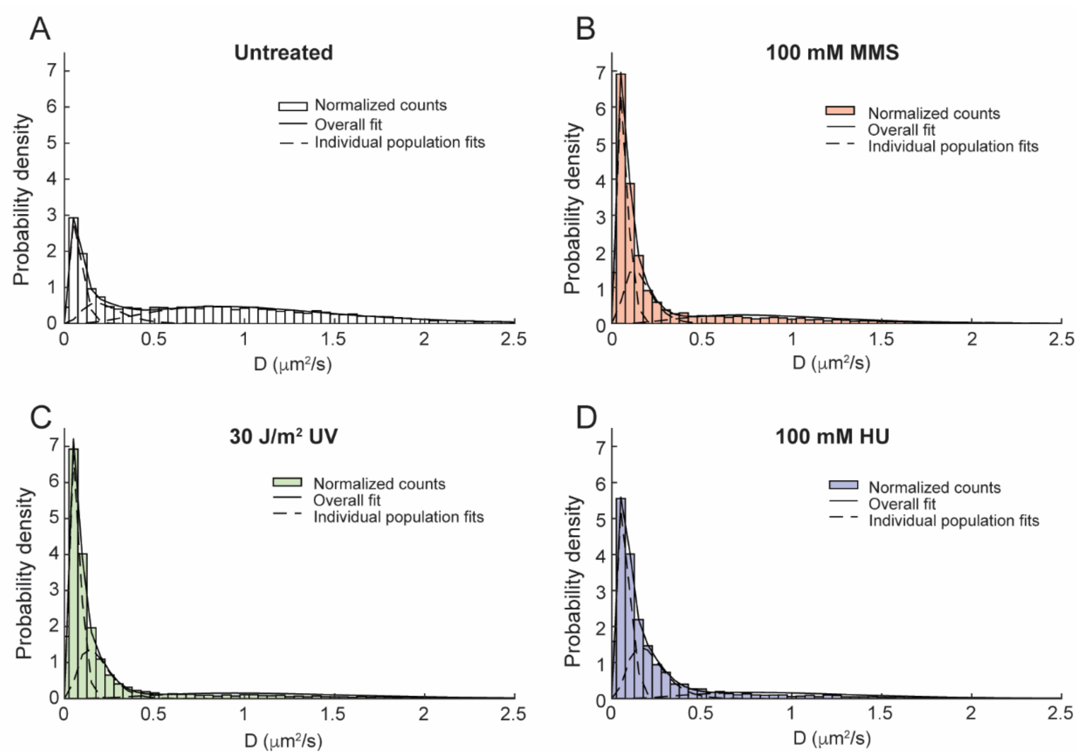

Fig. S4. Population fits to the distributions of the apparent diffusion coefficient (D*) for SSB-PAmCherry in (*A*) untreated cells (*N* = 9,251) and cells treated with (*B*) 100 mM MMS (red, *N* = 11,797), (*C*) 30 J/m^2 UV light (green, *N* = 16,542), and (*D*) 100 mM HU (blue, *N* = 5,690). See Methods and Table S6 for fit equations and parameters.

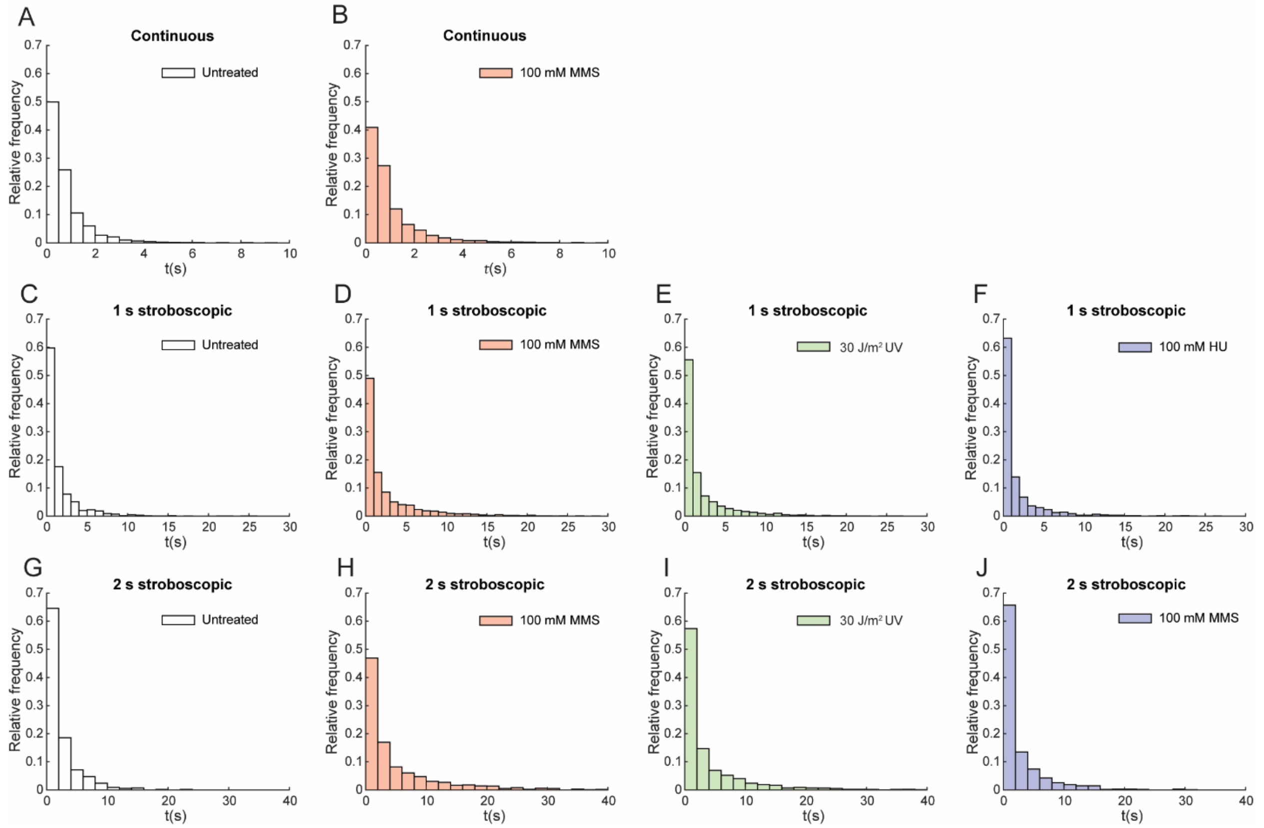

Fig. S5. Lifetime distributions of static SSB-PAmCherry tracks. Continuous imaging in (*A*) untreated cells (white, $N = 2{,}965$) and (*B*) cells treated with 100 mM MMS (red, $N = 6{,}924$). Stroboscopic imaging with a 1-s interval in (*C*) untreated cells (white, $N = 1{,}403$) and cells treated with (*D*) 100 mM MMS (red, $N = 3{,}837$), (*E*) 30 J/m^2 UV light (green, $N = 2{,}922$), and (*F*) 100 mM HU (blue, $N = 1{,}659$). Stroboscopic imaging with a 2-s interval in (*G*) untreated cells (white, $N = 545$) and cells treated with (*H*) 100 mM MMS (red, $N = 1{,}171$), (*I*) 30 J/m^2 UV light (green, $N = 1{,}755$), and (*J*) 100 mM HU (blue, $N = 956$).

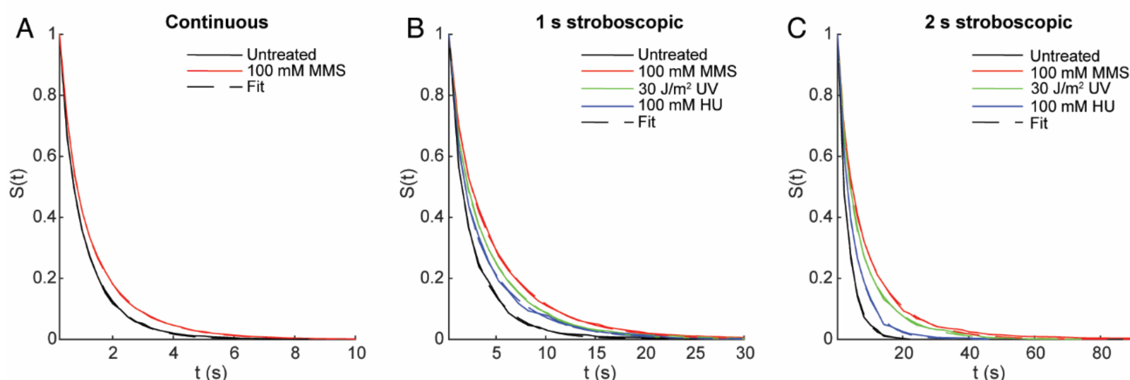

Fig. S6. Survival curves of static SSB-PAmCherry tracks fit to double-exponential decay functions. (*A*) Continuous imaging in untreated cells (white, $N = 2{,}965$) and cells treated with 100 mM MMS for 20 min (red, $N = 6{,}924$). (*B*) Stroboscopic imaging with a 1-s interval in untreated (white, $N = 1{,}403$) cells and cells treated with 100 mM MMS (red, $N = 3{,}837$), 30 J/m^2 UV light (green, $N = 2{,}922$), and 100 mM HU (blue, $N = 1{,}659$). (*C*) Stroboscopic imaging with a 2-s interval in untreated cells (white, $N = 545$) and cells treated with 100 mM MMS (red, $N = 1{,}171$), 30 J/m^2 UV light (green, $N = 1{,}755$), and 100 mM HU (blue, $N = 956$).

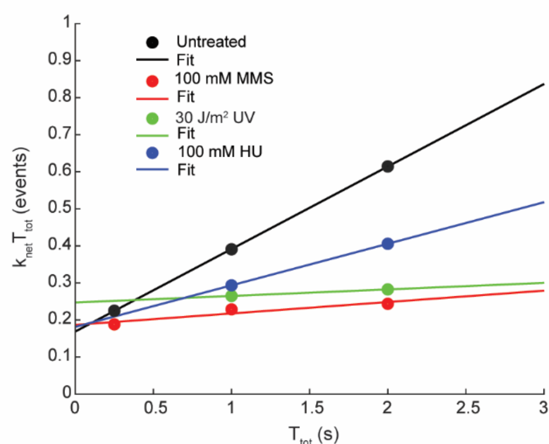

Fig. S7. Photobleaching correction to estimated SSB-PAmCherry binding lifetimes. Measured apparent dissociation rate constants k_{net} from exponential fits to survival curves (see Figure S6) multiplied by the stroboscopic interval T_{tot} plotted against T_{tot} for untreated cells (black circles) and cells treated with 100 mM MMS (red circles), 30 J/m^2 UV light (green circles), and 100 mM HU (blue circles). Linear fits (solid lines) for each condition give the photobleaching-corrected SSB-PAmCherry off rate as the slope and the PAmCherry photobleaching rate as the intercept (see Methods).

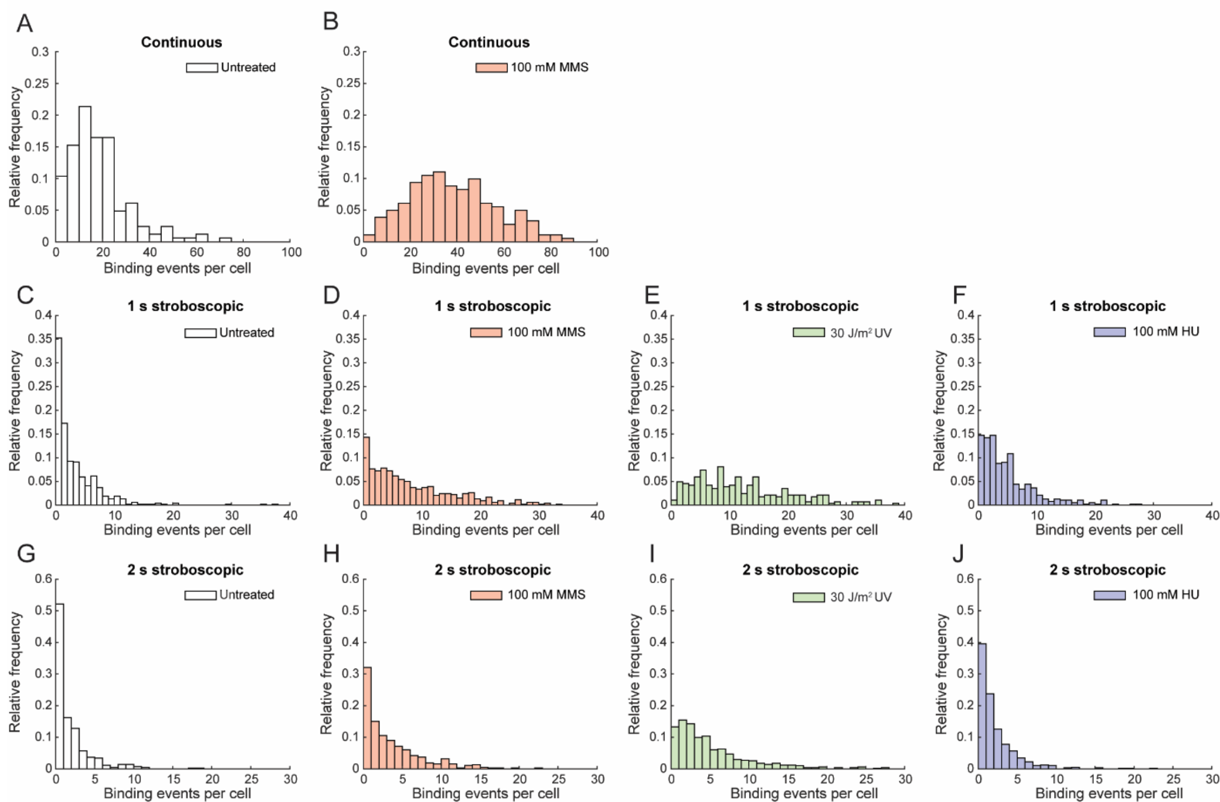

Fig. S8. Binding number distributions of static SSB-PAmCherry molecules for continuous imaging in (*A*) untreated cells (white, $N = 164$) and (*B*) cells treated with 100 mM MMS (red, $N = 181$). Stroboscopic imaging with a 1-s interval in (*C*) untreated cells (white, $N = 539$) and cells treated with (*D*) 100 mM MMS (red, $N = 538$), (*E*) 30 J/m^2 UV light (green, $N = 285$), and (*F*) 100 mM HU (blue, $N = 387$). Stroboscopic imaging with a 2-s interval in (*G*) untreated cells (white, $N = 437$) and cells treated with (*H*) 100 mM MMS (red, $N = 380$), (*I*) 30 J/m^2 UV light (green, $N = 512$), and (*J*) 100 mM HU (blue, $N = 581$).

Supplementary Tables

Table S1: Value of the mean radial distribution function *g*(*r*) for Pol IV, PriA, or RecG colocalization with SSB at the smallest value of *r*, and the standard error of the mean (S.E.M.) at that *r* value for the 100 calculated *g*(*r*) curves

Fig.	Protein	Condition	*g*(*r*) ± S.E.M.
1A	Pol IV^{WT}	Untreated	1.46 ± 0.04
	Pol IV^{R,C}		0.68 ± 0.03
	Pol IV^{T120P}		0.73 ± 0.04
1B	Pol IV^{WT}	100 mM MMS	7.8 ± 0.2
	Pol IV^{R,C}		5.4 ± 0.1
	Pol IV^{T120P}		3.04 ± 0.07
1C	Pol IV^{WT}	30 J/m^2 UV	5.1 ± 0.1
	Pol IV^{R,C}		2.79 ± 0.05
	Pol IV^{T120P}		1.7 ± 0.1
1D	Pol IV^{WT}	100 mM HU	7.7 ± 0.2
	Pol IV^{R,C}		4.4 ± 0.1
	Pol IV^{T120P}		1.02 ± 0.08
4A	PriA	Untreated	1.4 ± 0.1
		100 mM MMS	3.24 ± 0.08
4B	RecG	Untreated	1.60 ± 0.06
		100 mM MMS	10.1 ± 0.3

Fig.	Protein	Condition	*g*(*r*) ± S.E.M.
S3A	Pol IV^{WT}	Untreated	1.46 ± 0.04
		10 mM MMS	4.1 ± 0.2
		100 mM MMS	7.8 ± 0.2
S3B	Pol IV^{WT} (*lexA*$^+$)	Untreated	See note
		100 mM MMS	8.9 ± 0.3
S3C	Pol IV^{WT}	Untreated	1.46 ± 0.04
		Fixed	1.21 ± 0.02
S4C	Pol IV^{WT}	Untreated	1.46 ± 0.04
		100 mM HU	7.7 ± 0.2
		20 mM HU	5.2 ± 0.1
		100 mM GUA	6.3 ± 0.1

Note: The first *g*(*r*) bin is not populated for this dataset due to the smaller number of trajectories.

Table S2: Estimated SSB-PAmCherry diffusion coefficients (± S.E.M.)

Treatment condition	**Mean ± S.E.M. (µm^2/s)**	**Median (µm^2/s)**	***N* (events)**	***N* per cell**	**Wilcoxon rank-sum *P*-value relative to untreated**
Untreated	0.743 ± 0.007	0.638	9,251	5.1	-
100 mM MMS	0.266 ± 0.004	0.095	11,797	12.9	0.00000
30 J/m^2 UV	0.245 ± 0.003	0.094	16,542	23.2	0.00000
100 mM HU	0.246 ± 0.005	0.118	5,690	6.8	0.00000

Table S3: Estimated SSB-PAmCherry diffusion coefficients sorted by distance from the replisome

Treatment condition	*N* (D^* < 0.25 µm²/s, static)	*N* (D^* > 0.25 µm²/s, mobile)
Untreated (all)	2,947	5,753
Untreated (< 200 nm replisome)	1,014	262
Untreated (> 200 nm replisome)	1,933	5,491
100 mM MMS (all)	8,763	2,681
100 mM MMS (< 200 nm replisome)	4,405	371
100 mM MMS (> 200 nm replisome)	4,358	2,310
30 J/m² UV (all)	12,268	3,077
30 J/m² UV (< 200 nm replisome)	2,771	232
30 J/m² UV (> 200 nm replisome)	9,497	2,845
100 mM HU (all)	3,854	1,201
100 mM HU (< 200 nm replisome)	511	84
100 mM HU (> 200 nm replisome)	3,343	1,117

Table S4: SSB-PAmCherry diffusion coefficient distribution fit parameters (± uncertainties from 95% fit confidence intervals)

Treatment condition	D_1 (µm²/s)	A_1	D_2 (µm²/s)	A_2	D_3 (µm²/s)	A_3
Untreated	0.0752 ± 0.004	0.242 ± 0.021	0.236 ± 0.038	0.149 ± 0.023	1.150 ± 0.06	0.609 ± 0.044
100 mM MMS	0.0664 ± 0.0094	0.470 ± 0.116	0.163 ± 0.051	0.274 ± 0.11	0.972 ± 0.308	0.256 ± 0.226
30 J/m² UV	0.0689 ± 0.0086	0.501 ± 0.113	0.184 ± 0.056	0.287 ± 0.101	1.256 ± 0.644	0.212 ± 0.214
100 mM HU	0.0756 ± 0.008	0.452 ± 0.083	0.213 ± 0.051	0.346 ± 0.082	1.069 ± 0.52	0.202 ± 0.165

Table S5: Integrated SSB-mYPet focus intensity (± S.E.M.)

Treatment condition	Mean ± S.E.M. (AUe-5)	Median (AUe-5)	*N* (foci)	Wilcoxon rank-sum *P*-value relative to untreated
Untreated	2.40 ± 0.05	1.61	2,138	-
100 mM MMS	3.16 ± 0.07	2.53	1,309	0.00000
30 J/m² UV	6.6 ± 0.2	3.57	2,015	0.00000
100 mM HU	2.87 ± 0.08	2.04	1,207	0.00000

Table S6: Number of SSB-mYPet foci per cell (± S.E.M.)

Treatment condition	Mean ± S.E.M.	Median	*N* (cells)	Wilcoxon rank-sum *P*-value relative to untreated
Untreated	1.06 ± 0.02	1	2,021	-
100 mM MMS	1.11 ± 0.02	1	1,182	0.00311
30 J/m^2 UV	1.22 ± 0.02	1	1,646	0.00000
100 mM HU	1.29 ± 0.03	1	938	0.00000

Table S7: SSB-PAmCherry binding lifetimes (± S.E.M.)

Treatment condition (Stroboscopic interval)	Mean ± S.E.M. (s)	*N* (events)	*N* (cells)	Wilcoxon rank-sum *P*-value relative to untreated
Untreated (2 s)	1.7 ± 0.1	545	437	-
100 mM MMS (2 s)	5.2 ± 0.3	1,171	380	0.00000
30 J/m^2 UV (2 s)	3.6 ± 0.2	1,755	512	0.00001
100 mM HU (2 s)	2.3 ± 0.1	956	581	0.69151
Untreated (1 s)	1.41 ± 0.06	1,403	539	-
100 mM MMS (1 s)	2.66 ± 0.07	3,837	538	0.00000
30 J/m^2 UV (1 s)	2.08 ± 0.07	2,922	285	0.00003
100 mM HU (1 s)	1.62 ± 0.08	1,659	387	0.29163
Untreated (continuous)	0.72 ± 0.02	2,965	164	-
100 mM MMS (continuous)	0.94 ± 0.01	6,924	181	0.00000

Table S8: SSB-PAmCherry survival fit parameters (± uncertainties from 95% fit confidence intervals)

	2 s interval				**1 s interval**				**Continuous**			
Treatment condition	A	T_1 (s)	B	T_2 (s)	A	T_1 (s)	B	T_2 (s)	A	T_1 (s)	B	T_2 (s)
Untreated	0.1±0.6	0±4	0.9±0.2	3.4±0.4	0.40±0.07	0.9±0.2	0.60±0.09	3.3±0.3	0.30±0.02	0.27±0.05	0.70±0.07	1.01±0.05
100 mM MMS	0.38±0.06	2.7±0.5	0.61±0.07	11.3±0.8	0.31±0.03	1.1±0.2	0.68±0.03	5.5±0.2	0.44±0.02	0.44±0.03	0.56±0.03	1.49±0.04
30 J/m^2 UV	0.47±0.08	3.0±0.5	0.52±0.09	10±1	0.32±0.02	0.9±0.1	0.68±0.03	4.7±0.2	-	-	-	-
100 mM HU	0.3±0.1	1.7±0.8	0.7±0.1	5.7±0.5	0.45±0.05	1.1±0.2	0.55±0.06	4.8±0.4	-	-	-	-

Table S9: Photobleaching-corrected SSB-PAmCherry survival times (± uncertainties from 95% fit confidence intervals)

Treatment condition	k_{off} (s^{-1})	T_{off} (s)	k_{bleach} (s^{-1})
Untreated	0.22±0.07	4±1	0.7±0.2
100 mM MMS	0.031±0.009	32±9	0.7±0.2
30 J/m^2 UV	0.018±0.04	60±10	1.0±0.2
100 mM HU	0.11±0.06	9±5	0.7±0.4
Mean k_{bleach}			0.8±0.6

Table S10: Number of SSB-PAmCherry binding events (± S.E.M.)

Treatment condition (Stroboscopic interval)	Mean ± S.E.M. (events)	Median (events)	*N* (cells)	Wilcoxon rank-sum *P*-value relative to untreated
Untreated (2 s)	1.4 ± 0.1	0	437	-
100 mM MMS (2 s)	3.1 ± 0.2	2	380	0.00000
30 J/m^2 UV (2 s)	4.7 ± 0.2	3	512	0.00000
100 mM HU (2 s)	1.8 ± 0.1	1	581	0.00086
Untreated (1 s)	3.0 ± 0.2	1	539	-
100 mM MMS (1 s)	7.6 ± 0.3	5	538	0.00000
30 J/m^2 UV (1 s)	11.8 ± 0.5	10	285	0.00000
100 mM HU (1 s)	4.6 ± 0.2	3	387	0.00000
Untreated (continuous)	18 ± 1	15	164	-
100 mM MMS (continuous)	38 ± 1	37	181	0.00000

Table S11: Oligonucleotides and Gene Blocks used in this study

Number	Designation	Sequence (5′-3′)
oET110	recG-G4S-PAmC-KI-for	CTGATAGAACGCTGGATGCCGGAGACGGAACGTTACTCGAATG CGGGTGGTGGTGGTTCTGGTGG
oET111	recG-PAmC-KI-rev	GGAAGGTAGGGTAACCTGAAATGGCGGTCTTCTCACTGCCGCC TTTCTTATGAATATCCTCCTTAGTTCC
oET114	priA-G4S-PAmC-KI-for	TCCCGTAAGGTGAAATGGGTGCTGGATGTTGATCCGATTGAGG GTGGTGGTGGTGGTTCTGGTGG
oET115	priA-PAmC-KI-rev	GTGATGAATATTGAATTTTTCGATCCGCCTCGCATCGTGAGCG GTTCTTATGAATATCCTCCTTAGTTCC
oJK022	Lambda red P1	GTGTAGGCTGGAGCTGCTTC
oJK197	lacZ-SSB-mYpet-KI_for	TGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCT ATGGCCAGCAGAGGCGTAAA
oJK198	lacZ-SSB-mYpet-KI_rev	TCATCATATTTAATCAGCGACTGATCCACCCAGTCCCAGACGA AGATGAATATCCTCCTTAGTTCCTA
oJK277	rc_pKD4_9_30	AATCGCTCAAGACGTGTAATGC
SO572	pKD3/4-recG-KO-for	ACTGGTGGGCTACTATGCAGGCTGCAGGGTAAGTGCCATGGTG TAGGCTGGAGCTGCTT
SO573	pKD3/4-recG-KO-rev	GTCTTCTCACTGCCGCCTTTTACGCATTCGAGTAACGTTCTAT GAATATCCTCCTTAGT
gbS282	Gblock-pKD4-ssb-linker-Pamcherry	GCTTGCATGCAGATTGCAGCATTACACGTCTTGAGCGATTATG GCCAGCAGAGGCGTAAACAAGGTTATTCTCGTTGGTAATCTGG GTCAGGACCCGGAAGTACGCTACATGCCAAATGGTGGCGCAGT TGCCAACATTACGCTGGCTACTTCCGAATCCTGGCGTGATAAA GCGACCGGCGAGATGAAAGAACAGACTGAATGGCACCGCGTTG TGCTGTTCGGCAAACTGGCAGAAGTGGCGAGCGAATATCTGCG TAAAGGTTCTCAGGTTTATATCGAAGGTCAGCTGCGTACCCGT AAATGGACCGATCAATCCGGTCAGGATCGCTACACCACAGAAG TCGTGGTGAACGTTGGCGGCACCATGCAGATGCTGGGTGGTCG TCAGGGTGGTGGCGCTCCGGCAGGTGGCAATATCGGTGGTGGT CAGCCGCAGGGCGGTTGGGGTCAGCCTCAGCAGCCGCAGGGTG GCAATCAGTTCAGCGGCGGCGCGCAGTCTCGCCCGCAGCAGTC CGCTCCGGCAGCGCCGTCTAACGAGCCGCCGATGGACTTTGAT GATGACATTCCGTTCAGCTCGGCTGGCTCCGCTGCTGGTTCTG GCGAATTCATGGTTAGCAAGGGCGAGGAGGATAACATGGCCAT CATTAAGGAGTTCATGCGCTTCAAGGTGCACATGGAGGGGTCC GTGAACGGCCACGTGTTCGAGATCGAGGGCGAGGGCGAGGGCC GCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAA GGGTGGCCCCCTGCCCTTCACCTGGGACATCCTGTCCCCTCAA TTCATGTACGGCTCCAATGCCTACGTGAAGCACCCCGCCGACA TCCCCGACTACTTTAAGCTGTCCTTCCCCGAGGGCTTCAAGTG GGAGCGCGTGATGAAATTCGAGGACGGCGGCGTGGTGACCGTG ACCCAGGACTCCTCCCTGCAGGACGGTGAGTTCATCTACAAGG TGAAGCTGCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAAT GCAGAAGAAGACCATGGGCTGGGAGGCCCTCTCCGAGCGGATG TACCCCGAGGACGGCGCCCTGAAGGGCGAGGTCAAGCCGAGAG TGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGAC CACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTAC

		AACGTCAACCGCAAGTTGGACATCACCTCACACAACGAGGACT ACACCATCGTGGAACAGTACGAACGTGCCGAGGGCCGCCACTC CACCGGCGGCATGGACGAGCTGTACAAGTAAGTGTAGGCTGGA GCTGCTTCGAAGTTCCTATACTTTCTAG

Table S12: Plasmids used in this study

Designation	Containing Strain	Source or Reference
pKD3	JEK42	(1)
pKD4	JEK45	(1)
pSIM5	JEK168	(2)
pCP20	JEK169	(3)
pKD4-G4S-PAmCherry	JEK573	(4)
pKD3-ssb-linker-PAmCherry	SE99	This study
pSIM6	S235	(2)

Table S13: *Escherichia coli* bacterial strains used in this study

Number	Designation or description	Relevant genotype	Construction or source strain designation	Reference
ET227	Δ*lexA* Pol IV^{T120P}-PAmCherry SSB-mYPet	RW542 *dinB*^{T120P}-*PAmCherry-frt-kan-frt lacZ::ssb-mypet-frt*	Same	(5)
ET240	Δ*lexA* SSB-PAmCherry	RW542 *lacZ::ssb-PAmCherry-frt-cat-frt*	P1*vir*: S146 → JEK418	This study
ET340	Δ*lexA* SSB-PAmCherry	RW542 *lacZ::ssb-PAmCherry-frt*	ET240 pCP20 Flp-FRT recombination	This study
ET350	Δ*lexA* SSB-PAmCherry ε-mYPet	RW542 *lacZ::ssb-PAmCherry-frt dnaQ-mypet-frt-cat-frt*	P1*vir*: JEK466 → ET340	This study
ET352	Δ*lexA* Δ*dinB*	RW542 Δ*dinB::frt-kan-frt*	P1*vir*: JEK337 → JEK418	This study
JEK5	*E.coli* K-12 type strain	MG1655	—	—
JEK168	*E.coli* K-12 type strain with Cm^R recombineering plasmid	pSIM5 in MG1655	Same	(2, 4)
JEK170	*E.coli* K-12 type strain with Amp^R recombineering plasmid	pKD46 in MG1655	Same	(1, 4)
JEK337	Δ*dinB*	MG1655 Δ*dinB::frt-kan-frt*	Same	(4)
JEK418	Δ*lexA*	*lexA51(Def) rpsL31 xyl-5 mtl-1 galK2 lacY1 tsx-33 supE44 thi-1 hisG4[Oc]*	RW542	(6)

		argE3[Oc] araD139 thr-1 Δ[gpt-proA]62 sulA211		
JEK466	ε-mYPet	CH1358 *dnaQ-mypet-frt-cat-frt*	Same	(4)
JEK625	Pol IV-PAmCherry	MG1655 *dinB-PAmCherry-frt-kan-frt*	Same	(4)
JEK726	Pol $IV^{R,C}$-PAmCherry	MG1655 *dinB*^{R,C}-*PAmCherry-frt-kan-frt*	Same	(4)
JEK762	Δ*lexA* SSB-mYPet	RW542 *lacZ::ssb-mypet-frt*	Same	(4)
JEK766	Δ*lexA* Pol IV-PAmCherry SSB-mYPet	RW542 *dinB-PAmCherry-frt-kan-frt lacZ::ssb-mypet-frt*	Same	(4)
JEK784	*lexA*$_{+}$ Pol IV-PAmCherry SSB-mYPet	RW118 *dinB-PAmCherry-frt-kan-frt lacZ::ssb-mypet-frt*	Same	(4)
JEK790	Δ*lexA* Pol $IV^{R,C}$-PAmCherry SSB-mYPet	RW542 *dinB*^{R,C}-*PAmCherry-frt-kan-frt lacZ::ssb-mypet-frt*	Same	(4)
S146	SSB-PAmCherry	MG1655 *lacZ::ssb-PAmCherry-frt-cat-frt*	λ Red: SSB-PAmCherry-FRT-Cat-FRT → MG1655 pSIM5	This study
S235	*E.coli* K-12 type strain with Amp^R recombineering plasmid	pSIM6 in MG1655	Transformation: pSIM6 → JEK5	(2)
S473	Δ*recG*	MG1655 Δ*recG::frt-kan-frt*	λ Red: ΔrecG::FRT-Kan-FRT → MG1655 pSIM5	This study
SCP004	RecG-PAmCherry	MG1655 *recG-PAmCherry-frt-kan-frt*	λ Red: RecG-PAmCherry-FRT-Kan-FRT → MG1655 pSIM5	This study
SCP007	PriA-PAmCherry	MG1655 *priA-PAmCherry-frt-kan-frt*	λ Red: PriA-PAmCherry-FRT-Kan-FRT → MG1655 pSIM5	This study
SCP008	Δ*lexA* PriA-PAmCherry SSB-mYPet	RW542 *priA-PAmCherry-frt-kan-frt lacZ::ssb-mypet-frt*	P1*vir*: SCP007 → JEK762	This study
SCP012	Δ*lexA* RecG-PAmCherry SSB-mYPet	RW542 *recG-PAmCherry-frt-kan-frt lacZ::ssb-mypet-frt*	P1*vir*: SCP004 → JEK762	This study

Table S14: Imaging dataset size

Dataset	Fig.	Number of Days	Number of Replicates	Number of Cells	Number of Tracks or Foci
Pol IV^{WT} untreated *g*(*r*)	1A, S3A, S3C – D	2	2	343	1,482
Pol IV^{R,C} untreated *g*(*r*)	1A	3	4	539	1,090
Pol IV^{T120P} untreated *g*(*r*)		2	2	269	878
Pol IV^{WT} 100 mM MMS *g*(*r*)	1B, S3A	2	2	360	2,754
Pol IV^{R,C} 100 mM MMS *g*(*r*)	1B	4	5	562	2,894
Pol IV^{T120P} 100 mM MMS *g*(*r*)		3	3	690	2,331
Pol IV^{WT} 30 J/m^2 UV *g*(*r*)	1C	2	3	630	2,681
Pol IV^{R,C} 30 J/m^2 UV *g*(*r*)		4	4	899	3,261
Pol IV^{T120P} 30 J/m^2 UV *g*(*r*)		2	3	581	1,142
Pol IV^{WT} 100 mM HU *g*(*r*)	1D, S3D	2	3	439	1,539
Pol IV^{R,C} 100 mM HU *g*(*r*)	1D	2	4	630	1,479
Pol IV^{T120P} 100 mM HU *g*(*r*)		2	4	866	1,207
SSB untreated *D** (only cells with an ε-mYPet focus)	2A – C	3	3	631	8,772
SSB untreated *D**	2C – F, S4A	3	3	631	9,251
SSB 100 mM MMS *D**	2D, S4B	3	3	705	11,797
SSB 30 J/m^2 UV *D**	2E, S4C	3	3	581	16,542
SSB 100 mM HU *D**	2F, S4D	3	4	653	5,690
SSB untreated focus intensity	3A – C	6	6	2021	2,138
SSB 100 mM MMS focus intensity	3A	3	3	1182	1,309
SSB 30 J/m^2 UV focus intensity	3B	2	3	1646	2,015
SSB 100 mM HU focus intensity	3C	2	3	938	1,207
SSB untreated survival and binding events (2-s stroboscopic)	3D – F, S4G, S6C, S7, S8G	4	4	437	545
SSB 100 mM MMS survival and binding events (2-s stroboscopic)	3D, S4H, S6C, S7, S8H	3	3	380	1,171
SSB 30 J/m^2 UV survival and binding events (2-s stroboscopic)	3E, S4I, S6C, S7, S8I	2	3	512	1,755
SSB 100 mM HU survival and binding events (2-s stroboscopic)	3F, S4J, S6C, S7, S8J	5	5	581	956
PriA untreated *g*(*r*)	4A	2	3	755	781
PriA 100 mM MMS *g*(*r*)		3	3	1105	2,412
RecG untreated *g*(*r*)	4B	2	3	577	1,189
RecG 100 mM MMS *g*(*r*)		2	3	492	1,694
Pol IV^{WT} 10 mM MMS *g*(*r*)	S3A	1	2	402	1,273
Pol IV^{WT} *lexA*$^+$ untreated *g*(*r*)	S3B	4	6	536	320
Pol IV^{WT} *lexA*$^+$ 100 mM MMS *g*(*r*)		4	6	647	963
Pol IV^{WT} untreated fixed *g*(*r*)	S3C	4	6	168	5,596
Pol IV^{WT} 20 mM HU *g*(*r*)	S3D	2	3	587	2,267
Pol IV^{WT} 100 mM GUA *g*(*r*)		3	4	800	2,259
SSB untreated survival and binding events (continuous)	S5A, S6A, S7, S8A	1	1	164	2,965
SSB 100 mM MMS survival and binding events (continuous)	S5B, S6A, S7, S8B	1	1	181	6,924
SSB untreated survival and binding events (1-s stroboscopic)	S5C, S6B, S7, S8C	4	4	539	1,403

SSB 100 mM MMS survival and binding events (1-s stroboscopic)	S5D, S6B, S7, S8D	5	5	538	3,837
SSB 30 J/m^2 UV survival and binding events (1-s stroboscopic)	S5E, S6B, S7, S8E	2	3	285	2,922
SSB 100 mM HU survival and binding events (1-s stroboscopic)	S5F, S6B, S7, S8F	5	5	387	1,659

Supplementary Methods

Overview of strain construction strategy:

Bacterial strains containing fluorescent protein fusions were constructed using Lambda Red recombineering(1) and P1*vir* transduction as previously described.(4) In brief, the *E. coli* K-12 type strain, MG1655, was transformed with the temperature-sensitive recombineering plasmids pSIM5 (chloramphenicol) or pSIM6 (ampicillin).(2) Recombineering fragments bearing approximately 50 bp length homology arms were generated by PCR amplification (Q5 polymerase, New England Biolabs) of pKD3- or pKD4-based plasmids and transformed into MG1655 pSIM5 or pSIM6 by electroporation. Recombinants were selected on LB agar plates containing the appropriate antibiotic (chloramphenicol for pKD3, kanamycin for pKD4) and the recombineering plasmids were cured by growth at 37 °C. The modified alleles were then moved into the appropriate strain background for imaging using P1*vir* transduction. When required for further strain construction steps, FRT-flanked antibiotic cassettes were removed using Flp-FRT recombination with the temperature-sensitive plasmid pCP20, leaving a scar of approximately 80 bp containing a single FRT site.(3) Fusions were validated at each step by PCR amplification of genomic DNA and Sanger DNA sequencing. All oligonucleotides, plasmids, and bacterial strains used in this study are listed in Tables S11 – S13 and construction details for individual strains are given below.

The photoactivatable fluorescent protein PAmCherry1(7) was used to make Pol IV, SSB, PriA, and RecG fusions for PALM imaging, and the monomeric YFP variant mYPet(8) was used to make SSB and ε replisome marker fusions. All fusions contained a linker between the C-terminus of the protein and the N-terminus of the fluorescent protein: $(GGGS)_4$ for PamCherry fusions to Pol IV, PriA, and RecG; SSAGSAAGSGEF for the mYPet and PAmCherry fusions to SSB; and SAGSAAGSGEF for the mYPet fusion to ε.

Detailed strain construction information:

ET240/ET340: Δ*lexA* SSB-PAmCherry. P1*vir* transduction was used to transfer the *ssb-PAmCherry* allele from strain S146 to strain JEK418. Plasmid pCP20 was then transformed into strain ET240 and Flp-FRT recombination was used to remove the Cm cassette, giving strain ET340.

ET350: Δ*lexA* SSB-PAmCherry ε-mYPet. P1*vir* transduction was used to transfer the *dnaQ-mYPet* allele from strain JEK466 to strain ET340.

ET352: Δ*lexA* Δ*dinB*. P1*vir* transduction was used to transfer the *ΔdinB::frt-kan-frt* allele from strain JEK337 to strain JEK418.

S146: MG1655 SSB-PAmCherry. In this construct, nucleotides nt1-1697 of *lacZ* were replaced with *ssb-PAmCherry-frt-cat-frt*. A backbone fragment of the plasmid pKD3 was amplified using the oligonucleotides Lambda red P1 and rc_pKD4_9_30 and combined with a Gene Block containing the SSAGSAAGSGEF linker and PAmCherry (Gblock-pKD4-ssb-linker-Pamcherry) by Gibson assembly, yielding plasmid pKD3-ssb-linker-PAmCherry. A recombineering fragment was then generated by amplifying this plasmid with primers lacZ-SSB-mYpet-KI_for and lacZ-SSB-mYpet-KI_rev and transformed into strain MG1655 pKD46 (JEK170), then pKD46 was cured to give strain S146.

S473: MG1655 Δ*recG*. In this construct, *recG* was replaced with *frt-kan-frt*, leaving only the first amino acid and the last 6 amino acids of *recG*. A recombineering fragment was generated by amplifying pKD4 with primers pKD3/4-recG-KO-for and pKD3/4-recG-KO-rev and transformed into S235 (MG1655 pSIM6), then pSIM6 was cured to give strain S473.

SCP004: MG1655 RecG-PAmCherry. A recombineering fragment was generated by amplifying pKD4-G4S-PAmCherry with primers recG-G4S-PAmC-KI-for and recG-PAmC-KI-rev and transformed into JEK168 (MG1655 pSIM5), then pSIM5 was cured to give strain SCP004.

SCP007: MG1655 PriA-PAmCherry. A recombineering fragment was generated by amplifying pKD4-G4S-PAmCherry with primers priA-G4S-PAmC-KI-for and priA-PAmC-KI-rev and transformed into JEK168 (MG1655 pSIM5), then pSIM5 was cured to give strain SCP007.

SCP008: Δ*lexA* PriA-PAmCherry SSB-mYPet. P1*vir* transduction was used to transfer the *priA-PAmCherry* allele from strain SCP007 to strain JEK762.

SCP012: Δ*lexA* RecG-PAmCherry SSB-mYPet. P1*vir* transduction was used to transfer the *recG-PAmCherry* allele from strain SCP004 to strain JEK762.

Supplementary References

1. K. A. Datsenko, B. L. Wanner, One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6640–6645 (2000).

2. S. K. Sharan, L. C. Thomason, S. G. Kuznetsov, D. L. Court, Recombineering: a homologous recombination-based method of genetic engineering. *Nat. Protoc.* **4**, 206–223 (2009).

3. P. P. Cherepanov, W. Wackernagel, Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158**, 9–14 (1995).

4. E. S. Thrall, J. E. Kath, S. Chang, J. J. Loparo, Single-molecule imaging reveals multiple pathways for the recruitment of translesion polymerases after DNA damage. *Nat. Commun.* **8**, 2170 (2017).

5. S. Chang, E. S. Thrall, L. Laureti, V. Pagès, J. J. Loparo, "Compartmentalization of the replication fork by single-stranded DNA binding protein regulates translesion synthesis" (bioRxiv, 2020) https:/doi.org/10.1101/2020.03.03.975086 (April 29, 2021).

6. A. R. Fernández de Henestrosa, *et al.*, Identification of additional genes belonging to the LexA regulon in Escherichia coli: Novel LexA-regulated genes in E. coli. *Mol. Microbiol.* **35**, 1560–1572 (2002).

7. F. V. Subach, *et al.*, Photoactivatable mCherry for high-resolution two-color fluorescence microscopy. *Nat. Methods* **6**, 153–159 (2009).

8. A. W. Nguyen, P. S. Daugherty, Evolutionary optimization of fluorescent proteins for intracellular FRET. *Nat. Biotechnol.* **23**, 355–360 (2005).