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Payne-Dwyer, Alex L. orcid.org/0000-0001-8802-352X, Syeda, Aisha H. orcid.org/0000-0003-3201-4135, Shepherd, Jack W. et al. (2 more authors) (2022) RecA and RecB:probing complexes of DNA repair proteins with mitomycin C in live Escherichia coli with single-molecule sensitivity. *Interface*. ISSN 1742-5662

<https://doi.org/10.1098/rsif.2022.0437>

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1 **RecA and RecB: probing complexes of DNA repair proteins with mitomycin C in live *Escherichia coli*** 2 **with single-molecule sensitivity**

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10 11 **Abstract**

12 The RecA protein and RecBCD complex are key bacterial components for the maintenance and repair
13 of DNA. RecBCD is a helicase-nuclease that uses homologous recombination to resolve double-
14 stranded DNA breaks. It also facilitates coating of single-stranded DNA with RecA to form RecA
15 filaments, a vital step in the double-stranded break DNA repair pathway. However, questions remain
16 about the mechanistic roles of RecA and RecBCD in live cells. Here, we use millisecond super-resolved
17 fluorescence microscopy to pinpoint the spatial localization of fluorescent reporters of RecA or RecB
18 at physiological levels of expression in individual live *Escherichia coli* cells. By introducing the DNA
19 crosslinker mitomycin C, we induce DNA damage and quantify the resulting steady state changes in
20 stoichiometry, cellular protein copy number and molecular mobilities of RecA and RecB. We find that
21 both proteins accumulate in molecular hotspots to effect repair, resulting in RecA stoichiometries
22 equivalent to several hundred molecules that assemble largely in dimeric subunits before DNA
23 damage, but form periodic subunits of approximately 3-4 molecules within mature filaments of several
24 thousand molecules. Unexpectedly, we find that the physiologically predominant forms of RecB are
25 not only rapidly diffusing monomers, but slowly diffusing dimers.

26
27 **Keywords:** recombination; repair; DNA damage; mitomycin C; super-resolution microscopy; single-
28 molecule tracking; Slimfield.

29 30 **1. Introduction**

31 Accurate duplication of the genome is crucial in all organisms, accomplished by a sophisticated
32 molecular machine known as the replisome [1]. An inability to accurately replicate genetic material
33 can lead to cell death and/or cancers [2,3]. Mitomycin C (MMC) is a naturally occurring antibiotic that
34 can be used to controllably disrupt DNA replication, and thus a valuable reagent in studying DNA repair
35 processes. It is used as a chemotherapeutic in treating several cancers [4] and retinopathies [5], and
36 acts by targeting DNA deoxyguanosine (dG) residues [6], forming intrastrand or interstrand crosslinks
37 [7]. If unrepaired, these structures can interfere with cellular processes such as transcription and
38 replication, potentially leading to genome instability [8]. An encounter between a mitomycin C-
39 induced crosslink and an approaching replisome may result in replisome disassembly and eventually
40 a double strand break (DSB) [9]. RecBCD recognises DSBs in *E. coli* [10], processing the ends to
41 generate 3'-ended single-stranded DNA (ssDNA) as a landing pad for the principal recombination
42 protein, RecA [10]. Recombination of RecA-ssDNA complexes with the homologous DNA restores the
43 replication fork, on which the replisome can be reloaded. The replisome may resume replication if the
44 blocking adduct is repaired [11]. As a complex of individual RecB, RecC and RecD proteins, RecBCD is
45 a versatile helicase-nuclease and underpins two major pathways for homologous DNA recombination,
46 essential for DSB repair [10]. RecBCD activities involve several processes - it recognises and binds DSBs,
47 begins unwinding both DNA strands, and also degrades both [10]. This activity continues unhindered
48 until it encounters an octameric Chi site that induces a shift in enzyme activity to degrade only the 5'-

49 ended strand [12,13]. This activity shift results in a 3'-ended ssDNA overhang that facilitates RecA
50 loading. A key function of RecA is its ability to form nucleoprotein filaments on exposed ssDNA in
51 response to damage [14]. These filaments can infiltrate an intact duplex and, on finding homology,
52 recombine with the infiltrated duplex [15,16]. The extension of filaments along the cell accelerates
53 this homology search in a non-linear fashion [17]. Following further processing of the resulting
54 structure, primosome proteins establish an intact replisome thereby enabling replication to resume
55 [18]. Recombination proteins, such as RecBCD, need access to replication-transcription conflict sites
56 and collapsed forks, but if RecBCD is missing then double-stranded DNA (dsDNA) is degraded by
57 exonucleases [19,20], possibly resulting from replisome disassembly. However, how RecA stabilizes
58 blocked forks remains an open question.

59 The nucleoprotein filaments formed by RecA are both a requisite and a hallmark of the cell-wide SOS
60 response [21–24]. The SOS response is a regulatory shift that promotes cell survival in adverse
61 conditions associated with increased rates of interrupted replication and DNA damage [25]. The SOS
62 response to DNA damage induced by antimicrobials plays a major role in the emergence of persister
63 cells [26] and wider antimicrobial tolerance on a population level [27].

64 Given these far-reaching implications of RecA and RecB activity as studied comprehensively with
65 mutants [12,24,28–32], it is important to establish the number of molecules present in cells, how they
66 are spatially distributed and organized, and how these are affected by antimicrobials such as MMC.
67 Here, we use millisecond super-resolved Slimfield microscopy [33] in live *E. coli* containing
68 genomically-encoded fluorescent fusions RecA-mGFP [34] and RecB-sfGFP [35]. Since RecA fusion
69 constructs retain only partial function, our approach makes use of a merodiploid RecA fusion that
70 expresses from one copy of the native gene and one copy of the *recA4155* fusion construct [34]. This
71 strain rescues approximately wild-type sensitivity with mixed assemblies of the two RecA proteins
72 [34].

73 We use Slimfield microscopy to visualise the spatial distribution of RecA and RecB fluorescent proteins
74 in individual cells. From these quantitative images, we identify diffraction-limited local intensity
75 maxima (we denote these as *foci* – see Table 1 for a description of technical Slimfield microscopy
76 nomenclature used in this study) to a lateral spatial precision of 40 nm [36]. Slimfield uses ~millisecond
77 sampling that is sufficiently rapid to link the moving foci derived from the same emitter sources over
78 sequential image frames, following appropriate bespoke particle tracking analysis [33,37,38], into
79 *tracks*. Each of these tracks implies the presence of a particle containing one or more associated
80 molecules; typically more than one prior to photobleaching, so more generally, we term each a
81 *molecular assembly*. These tracks reveal the detailed diffusion of labelled RecA and RecB assemblies
82 in the cytoplasm of a living cell. By using the single-molecule sensitivity of Slimfield microscopy, we
83 are able to quantify single-molecule photobleaching steps in fluorescence intensity, to identify the
84 characteristic brightness of a single fluorescent protein [33]. Not only does this calibration apply to
85 the fraction of the fluorescence intensity for each tracked assembly, but also to the GFP fluorescence
86 in the whole, or part, of each cell. We use this to determine the number of GFP-labelled molecules
87 within each tracked assembly (the *stoichiometry*), and the total number of fluorescently-labelled
88 molecules within each cell (the *cellular protein copy number*), or intracellular segment (the *segment
89 protein copy number*). Those fluorescent molecules which contribute to the copy number above the
90 cell's autofluorescent background but are not detected as foci (typically due to high, uniform emitter
91 density and/or excessive mobility) are denoted the *pool*.

92 Slimfield has some similarities to single particle tracking photoactivation localization microscopy
93 (sptPALM) [39–41], however, our approach is simpler, requiring only constitutively expressed
94 fluorescent reporters such as GFP, and trades off the condition of observing exclusively single
95 molecules in order to measure the stoichiometry of dynamic assemblies far more accurately. This is
96 a deliberate advantage of our technique over other single-molecule microscopy techniques as
97 previously used to count RecB content in cells molecule-by-molecule [35].

98 Prior to MMC treatment, only point-like assemblies of RecA or RecB are detectable. RecA presents far
99 brighter fluorescence in a cell than RecB, indicating both a typical stoichiometry and a cellular protein
100 copy number that are 2-3 orders of magnitude greater. On treatment with MMC, we observe an
101 increase in the average cellular protein copy number of RecA, but not of RecB, in each cell, with up to
102 20% of cells devoid of RecB assemblies. MMC induces the formation of RecA assemblies larger than
103 can be captured in single foci, and we interpret these as RecA nucleoprotein filaments, or bundles of
104 filaments [24,30,42–47], typically associated with the SOS response.

105 Between cellular states of SOS readiness and MMC-induced response, the stoichiometries of RecA
106 assemblies increase, and the diffusion coefficients of assemblies decrease correspondingly. We also
107 discover surprisingly consistent intervals between the stoichiometries of different assemblies in each
108 condition. We interpret the average number of molecules in the intervals (the *periodicity*) as indicative
109 of an oligomeric structural repeat unit that comprises assemblies. The periodicity of RecA assemblies
110 changes from dimeric in character to groups of roughly 3-4 molecules in response to MMC, while the
111 periodicity of RecB assemblies is dimeric, and insensitive to MMC treatment.

112 Our results shed new light on the relations between structure and function for RecA and RecBCD in
113 mediating repair upon DNA damage.

114

Metric / Object	Definition
<i>Segment</i>	An area of the image defined by a contiguous subset of pixels in a binary mask. This area either corresponds to a whole cell (a <i>cell mask</i>), or more typically a region inside the cell (an <i>intracellular segment</i>) of high fluorescent intensity. The term “ <i>segment</i> ” refers to an intracellular segment unless otherwise stated.
<i>Cell mask</i>	A segment containing the outline of one cell. These are extracted using a machine learning protocol (Supplementary Methods).
<i>Intracellular segment</i>	A segment inside the cell. These are extracted from the set of foci localized in that cell by rendering a superresolved image, followed by local Otsu thresholding (Materials and Methods 4.3.5), with the intention of isolating RecA objects that resemble nucleoprotein filaments or bundles.
<i>Assembly</i>	A group of labeled molecules physically associated with one another, either directly or indirectly, such that their diffusive movement is strongly correlated, and therefore always detected in the same track.
<i>Focus (foci)</i>	A spot-like local intensity maximum in a single frame, which corresponds to a localized group of labeled molecules (Materials and Methods 4.3.1). Associated properties include centroid location, total intensity, and signal-to-noise ratio.
<i>Track</i>	A set of foci in adjacent frames that are spatially close enough to form a contiguous trajectory (Materials and Methods 4.3.1). Typically associated with a single molecular assembly, or a group of strongly colocalized assemblies.
<i>Diffusion coefficient</i>	Measure of the random microscopic motion of a specific track based on the increase in the mean square displacement of its intensity centroid over time (Materials and Methods 4.3.2).
<i>Characteristic single-molecule brightness</i>	The average sum of pixel values in foci associated with a single fluorescent reporter molecule (e.g. mGFP), under a fixed imaging condition (Materials and Methods 4.3.3). Equivalent to the modal step size in intensity for tracks in the final stage of photobleaching (Figure S1).
<i>Stoichiometry</i>	The number of fluorescently labeled molecules in a specific track. This is estimated by extracting the sequence of foci belonging to that track, then extrapolating the sum of pixel values in each focus backwards along that sequence to get an initial track intensity that is independent of photobleaching (Materials and Methods 4.3.4). The initial track intensity is then divided by the characteristic single-molecule brightness.
<i>Periodicity</i>	The population-averaged number of fluorescently labeled molecules in inferred repeat units within tracked objects. Estimated by averaging the consistent intervals between nearest-neighbor peaks in the population-level stoichiometry distribution (Materials and Methods 4.3.5).
<i>Integrated intensity</i>	The total fluorescence intensity of a segment in pixel counts, normalised by the characteristic single molecule brightness (Materials and Methods 4.3.6-7).
<i>Cellular (or segment) protein copy number</i>	The average number of molecules in a cell (or intracellular segment), as estimated from the increase in integrated intensity above negative control (i.e. subtracting the contribution from autofluorescence (Materials and Methods 4.3.6-7).
<i>Pool</i>	The intracellular fluorescence which is not detected in tracks.
<i>Pool stoichiometry</i>	The number of untracked, labeled molecules within an area of the pool equal to the size of one diffraction-limited focus (Materials and Methods 4.3.6).

Table 1. Definitions of quantitative analysis metrics for Slimfield.

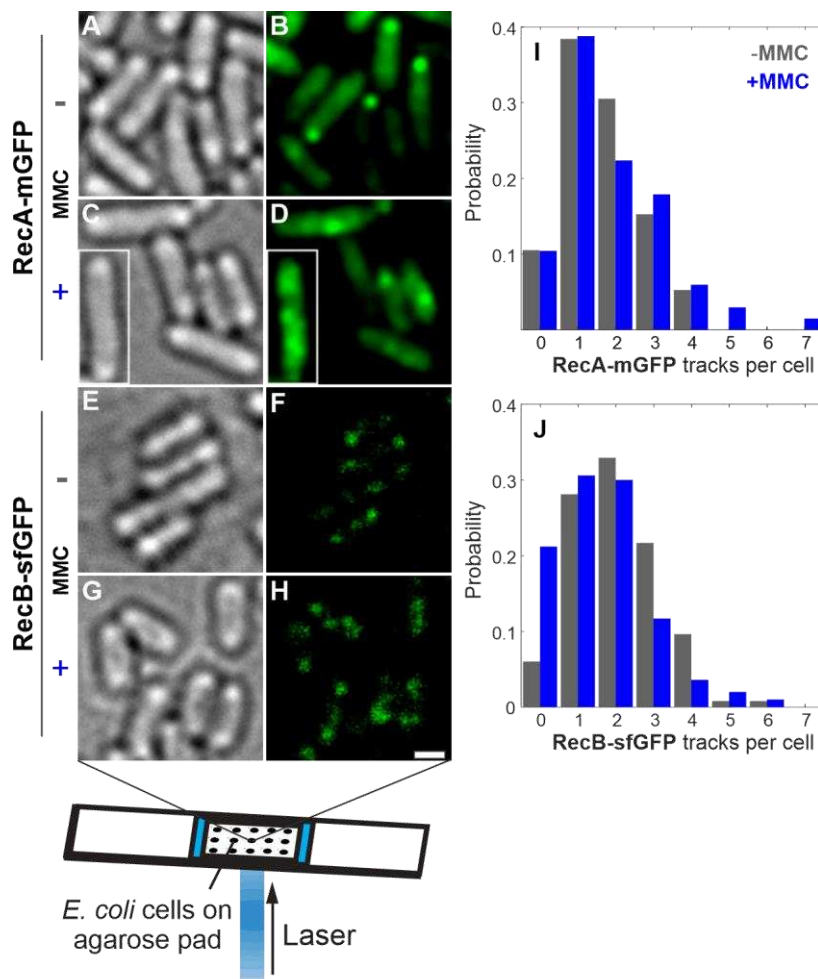
116

117 2. Results

118 2.1 Abundance of RecA, but not RecB, increases on MMC-induced DNA damage

119 We first optimised MMC treatment conditions so that they did not cause cellular filamentation in wild
120 type cells (Materials and Methods 4.1, Figure S1) but did induce the SOS response [48], since cells
121 would then be sensitised to MMC if the SOS response is blocked [48]. Filamentation and loss of viability
122 was also minimal for the labeled strains (Figure 1), hence we used the same MMC treatment for all
123 strains. Given that SOS induction in these and related strains typically takes <20 min [34,48], the
124 kinetics of initial SOS induction due to MMC will likely reach steady state within the 180 min MMC
125 exposure that we used. In light of the timescale of the initial SOS induction, RecA or RecB dynamics
126 were not in the scope of our study here, but rather the steady state effect of MMC on the distribution
127 and molecular organization of RecA and RecB.

128



129

130

131 **Figure 1.** Brightfield and Slimfield (mean average of 3 initial frames) of live *E. coli* in 56-salts minimal
132 media, labeled at RecA-mGFP or RecB-sfGFP before and after MMC treatment. Inset (C,D) is another
133 cell transplanted from the same acquisition outside the cropped field of view at the same scale.
134 Brightness of RecB-GFP Slimfield panels (F,H) scaled 100× vs. RecA-mGFP panels (B,D). Scale bar 1 μ m.
135 (I,J) Probability distributions for number of tracks detected per cell. Tracks are identified in post-
136 acquisition analysis (Materials and Methods 4.3.1) by first detecting foci as local fluorescent maxima,
137 then linking nearest-neighbour foci in subsequent frames.

138

139 We performed brightfield and Slimfield microscopy in each field of view (Materials and Methods 4.2).
140 Binary masks for each cell were extracted independently of any fluorescence signal, using a machine
141 learning segmentation protocol on brightfield images. We then applied these masks to fluorescence
142 images to eliminate extracellular background and facilitate statistics on a cell-by-cell level (Figure S5
143 and Supplementary Methods). Using home-written automated particle tracking and analysis software
144 ADEMScode [49], we identified fluorescent foci from local intensity maxima in each cell and linked
145 these into tracks (Materials and Methods 4.3.1). We determined the stoichiometry (the number of
146 molecules present) of each track, from the summed pixel intensity values corresponding to the start
147 of each track before any photobleaching has occurred (Materials and Methods 4.3.4), normalized by
148 brightness corresponding to a single molecule of GFP (Materials and Methods 4.3.3).

149 From the localizations of foci during photobleaching, we also reconstructed superresolved images of
150 fluorescent RecA structures. We extracted binary masks from highly fluorescent regions of interest in
151 these images (denoted *intracellular segments*, or simply *segments* for brevity) using a classical
152 segmentation method (Materials and Methods 4.3.7), which enabled statistics on an intracellular
153 segment level (Figure 3 and Figure S3).

154 Separately, from the cell masks (or intracellular segments) we also calculated the cellular (or segment)
155 protein copy number (Materials and Methods 4.3.6-7); first we summed the pixel values in each cell
156 or segment area and normalized these by the characteristic brightness of a single GFP to obtain the
157 total intensity within that region, expressed in molecules [50]. Taking the difference from an
158 equivalent area of the control strain that does not express GFP, then yields the cellular or segment
159 protein copy number corrected for any cellular autofluorescence.

160 Since the RecA-mGFP strain is merodiploid, both the *recA-mgfp* gene fusion construct and the
161 unlabeled endogenous *recA* gene are expressed simultaneously [34]. However, their expression levels
162 are not necessarily identical, nor equivalently inducible by MMC. From previous estimations of the
163 relative *lexA* suppression rates of the relevant *recA* promoters [51], reasonable expectations are that
164 a majority of the RecA present in the cell will be labelled with mGFP, and that RecA-mGFP is 2-3 fold
165 less inducible under the SOS response as endogenous RecA [52]. We estimated the different cellular
166 levels of unlabeled RecA vs. RecA-mGFP using Western blotting (Figure S4), which confirmed that the
167 RecA-mGFP was in excess compared to the endogenous protein before and after treatment. Both
168 qPCR and Western blots indicated that both endogenous RecA and RecA-mGFP are inducible by MMC
169 treatment (Materials and Methods 4.4), with the RecA-mGFP indeed about half as inducible (Figure
170 S4). Therefore, the total (i.e. labelled plus unlabelled) amount of RecA protein present, whether as
171 stoichiometry, periodicity or protein copy numbers, is higher than that reported for the RecA-mGFP
172 data directly, by an approximate correction factor of 1.3-fold in the presence of MMC. In the absence
173 of MMC the relative amount of RecA-mGFP to RecA is large enough that the correction factor is
174 effectively 1. As these corrections are indicative, we do not apply them in the early stages of the
175 Results, but present them later only where relevant to interpretations (Results 2.3 and Discussion).

176 We find that in the absence of MMC, RecA-mGFP has an an approximately uniform distribution in the
177 cytoplasm that is occasionally punctuated by bright fluorescent foci that can be linked into tracks
178 (Figure 1B). The cellular protein copy number of RecA-mGFP increases from $11,400 \pm 200$ molecules
179 (\pm SEM) in untreated cells to $19,500 \pm 300$ molecules in MMC treated cells (Figure S5A). MMC
180 treatment resulted in the subset of these RecA-mGFP molecules that are localized in tracks (i.e., the
181 mean summed stoichiometry of all tracks detected in the whole cell) approximately doubling from
182 510 ± 30 to $1,080 \pm 60$ molecules per cell (Table S1). We denote the fluorescently detected, but
183 untracked, molecules of RecA as residing in a *pool*. The pool typically comprises molecules that are
184 sufficiently dim, out-of-focus, and/or rapidly diffusing to evade direct particle-tracking-based
185 detection; here the RecA concentration is exceptionally high such that the stochastic fluctuations
186 corresponding to motion of discrete foci are partly averaged out. During photobleaching, the density
187 of foci decreases, overlap decreases and tracks become more evident. The proportion of RecA-mGFP
188 molecules in tracks is relatively low compared to the pool, but remains representative of the
189 population of assemblies containing RecA-mGFP.

190 RecB-sfGFP also exhibited fluorescent tracks against a relatively diffuse background, before and after
191 MMC treatment (Figure 1F,H). RecB-sfGFP foci were observed more commonly near the poles of the
192 cell regardless of MMC (Figure 1G-H). Since the RecB-sfGFP fluorescence signal is comparatively small,
193 estimates based on cellular protein copy number must account carefully for autofluorescence due to
194 native components other than GFP. We estimate that the contribution of autofluorescence from the
195 summed pixel intensity values from unlabeled MG1655 parental cells grown and imaged under
196 identical conditions. We find that the mean level of RecB-sfGFP fluorescence was almost three times
197 greater than the cellular autofluorescence (Figure S5B), therefore there is a comparatively large
198 population of the cellular RecB-sfGFP that evades direct particle-tracking-based detection (*c.f.*, slower
199 sampled images from commercial confocal/epifluorescence microscope systems) and thereby
200 comprise a RecB pool.

201 The cellular protein copy number of RecB-sfGFP does not decrease significantly following MMC-
202 induced DNA damage, comprising 126 ± 11 molecules per cell before treatment and 101 ± 14
203 molecules following MMC treatment (Figure S5B, Brunner-Munzel (BM) test, $n=246$, $p=0.0216$ | NS,
204 not significant at Bonferroni-adjusted $\alpha = 0.01$). However, the mean number of RecB-sfGFP localized
205 into tracks does decrease with MMC; just 13.6 ± 0.5 molecules per cell in all tracks, decreasing to 9.3
206 ± 0.3 on MMC treatment (BM test, $n=246$, $p<0.001$). This is clearly much smaller absolute number of
207 tracked molecules per cell compared to RecA-mGFP, but a similar proportion of the cellular protein
208 copy numbers (ranging from 6-10% in each case). The complementary fractions of the total RecA-
209 mGFP and RecB-sfGFP molecules assigned to their respective pools are thus consistently high (89-
210 95%). In the respective strains, the total concentration of RecB-sfGFP is much lower than that of RecA-
211 mGFP, and this likely indicates the correspondingly more rapid diffusion of RecB-sfGFP species within
212 the pool.

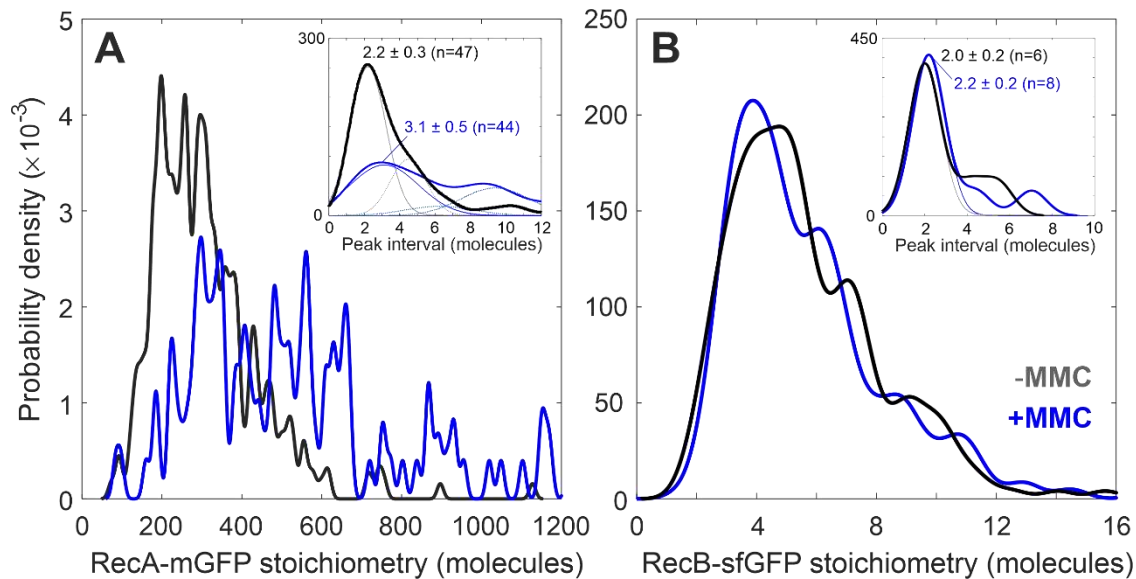
213

214 *2.2 RecB forms characteristic puncta which are partially lost on MMC exposure*

215 We detected typically 1-3 tracks of RecA-mGFP or RecB-sfGFP in each cell above the local background
216 fluorescence (Figure 1I,J). However, each showed strongly opposing trends in the number of tracks
217 observed upon MMC treatment. While RecA-mGFP showed no significant change in the mean number
218 of tracks on MMC treatment (from 1.66 ± 0.06 to 1.86 ± 0.16 tracks per cell, BM test, $n=60$, $p=0.50$
219 | NS), MMC reduced the population average number of RecB-sfGFP tracks significantly, from $2.06 \pm$
220 0.09 to 1.56 ± 0.06 per cell.

221 If, however, we set aside the fraction of cells with no detected RecB-sfGFP tracks, the change in the
222 mean number of RecB-sfGFP tracks is marginal, from 2.20 ± 0.07 to 1.98 ± 0.07 tracks (BM test, $n=234$,
223 $p=0.006$). We see that the cells which continue to harbor RecB tracks are relatively unchanged by
224 MMC, each containing an average of 12.1 ± 0.3 molecules per cell. The unexpected subset of cells
225 that are devoid of RecB-sfGFP tracks increases from 6% to 21% of the population on MMC treatment.
226 These otherwise resemble the other treated cells; rather than filamenting, they retain $92 \pm 3\%$ of the
227 population averaged cell length and retain the same pool level of untracked RecB-sfGFP molecules.

228 The increase in the fraction of cells lacking RecB-sfGFP foci agrees with a model of random,
229 independent survival of assemblies (Figure 1J, the MMC+ condition is consistent with Poisson
230 distribution with same mean; Pearson χ^2 test, $dof=6$, $n=234$, $p=0.004$).



231
232

233 **Figure 2.** Stoichiometry distributions of detected foci of A) RecA-mGFP and B) RecB-sfGFP with (blue)
 234 or without MMC treatment (black), shown as kernel density estimates [53]. The statistics used for
 235 MMC- (MMC+) conditions include N=190 (67) whole RecA-mGFP cells containing n=316 (125) tracks,
 236 or whole N=249 (307) whole RecB cells containing n=514 (478) tracks within the cell masks. The use
 237 of ‘probability density’ reflects the fact that each distribution is continuous with a total area equal to
 238 1, such that areas under the curve correspond to the probability that the stoichiometry of a given
 239 assembly falls within a range. The kernel width (the width for smoothing the discrete stoichiometry
 240 of each track) is 0.7 molecules following the known detection sensitivity to single GFP (A, inset and B,
 241 both panels), or 8 molecules for clarity (main panel A). Insets are the distributions of intervals between
 242 nearest neighbor stoichiometry peaks (solid curves) whose modal position, or periodicity, indicates
 243 the number of GFP-labeled molecules in a repeating subunit within molecular assemblies (Table 1).
 244 Overlaid are heuristic Gaussian fits that minimize a reduced χ^2 metric, with components of equal width
 245 and whose centers are fixed at integer multiples to account for the detected optical overlap of an
 246 integer number of subunit repeats of tracked foci. The resulting fits comprise three components for
 247 RecA-mGFP with MMC treatment (blue, Pearson’s $R^2 = 0.979$, 5 degrees of freedom (dof)) and two
 248 components for RecA-mGFP without MMC (grey, Pearson’s $R^2 = 0.961$, 4 dof). The mode of the peak
 249 interval is indicated $\pm 95\%$ confidence interval, alongside the number of contributing peak pairs in the
 250 original stoichiometry distribution.

251

252 RecA-mGFP foci were approximately two orders of magnitude brighter than those of RecB-sfGFP,
 253 corresponding to a greater apparent stoichiometry. A subset of polar assemblies in untreated cells are
 254 especially bright (Figure 2B); we defined this subset quantitatively by thresholding at $2\times$ the mean
 255 stoichiometry of all assemblies. The mean stoichiometry of these bright assemblies is itself as high as
 256 760 ± 40 molecules (Figure S3A).

257 On treating with MMC, the RecA-mGFP mean stoichiometry almost doubled from 310 ± 8 to 580 ± 30
 258 molecules per focus, reflecting further local accumulation of RecA-mGFP protein (Figure 2A). We find
 259 that most RecA-mGFP molecules comprise an untracked, diffusive pool, in which there are ~ 30 RecA-
 260 mGFP molecules in an area corresponding to that of a typical diffraction-limited focus (which we
 261 denote as the *pool stoichiometry*, Table 1). The fact that the relative increase in pool stoichiometry
 262 with MMC treatment to ~ 50 RecA molecules (Figure S5C) is smaller than the fractional increase in the
 263 amount of RecA in tracks (Figure 2), indicates that the MMC-driven upregulation of RecA
 264 disproportionately affects tracked assemblies. As such, either i) new assemblies are formed which
 265 contain much more RecA than those before MMC treatment, or ii) those assemblies that already

266 contain local concentrations of RecA accumulate more RecA. Under our treatment protocol, these
267 changes do not deplete the reservoir of RecA in the cytoplasm. This observation of localized
268 accumulation of RecA-mGFP is consistent with prior reports of long nucleoprotein filament formation
269 on single stranded DNA [34]. The increased number of RecA tracks we observe upon MMC treatment
270 may therefore indicate greater occurrence of processed ssDNA.

271

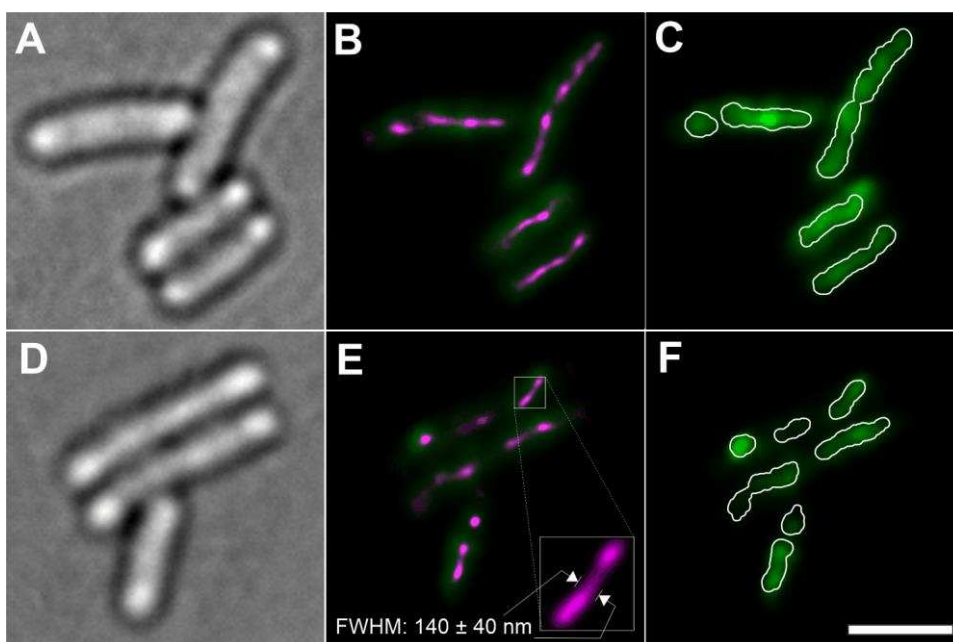
272 The RecB-sfGFP mean stoichiometry decreases very slightly from 6.6 ± 0.1 to 6.1 ± 0.2 molecules per
273 focus (BM, $n=478$, $p < 10^{-6}$) (Figure 2B). A mean of approximately 6 RecB-sfGFP molecules in each case
274 can be explained if the assembly contains 3 identical subunits whose periodicity is 2 molecules (Figure
275 2B inset). A pool stoichiometry of ~ 1 molecule of RecB-sfGFP (Figure S5B,D) suggests that the
276 untracked RecB-sfGFP are likely to be monomers irrespective of MMC treatment (BM test, $n=243$,
277 $p=0.27$ | NS). We find that the untracked pool of RecB-sfGFP comprises $90 \pm 1\%$ of the total RecB-
278 sfGFP molecules in the cell.

279

280 2.3. RecA reorganises into filaments with 3-4-mer subunits in response to MMC

281 RecA-mGFP and RecB-sfGFP stoichiometry distributions show clear and reproducible peaks (Figure 2A
282 and 2B). One explanation is that each detected fluorescent focus has a diffracted-limited width of
283 ~ 250 nm that may potentially contain more than one 'subunit' of RecA-mGFP or RecB-sfGFP, bound
284 sufficiently to co-track, such that the measured focus stoichiometry may appear as an integer multiple
285 of that subunit, manifest as periodic peaks on the focus stoichiometry distribution. The expected
286 difference between pairs of values on the stoichiometry distribution is thus either zero or an integer
287 multiple of the periodicity within measurement error. The magnitude of the most likely non-zero
288 pairwise difference value corresponds to the periodicity, with less likely values corresponding to
289 harmonic peaks. Our approach uses a modal estimate of the nearest-neighbor peak intervals
290 (Materials and Methods 4.3.5), and therefore produces a continuous, heuristic estimate for the
291 periodicity. We then compare this periodicity metric to realistic models with integer numbers of
292 molecules. RecA-mGFP tracks have a periodicity of 2.2 ± 0.3 molecules before addition of MMC (Fig
293 2A inset). This is clearly most consistent with a dimeric subunit of RecA in structures before MMC
294 treatment. After MMC treatment, the most likely interval value is 3.1 ± 0.5 RecA-mGFP molecules,
295 and estimating the additional unlabeled RecA content indicates a likely overall periodicity range of 3-
296 4 RecA molecules (see Discussion and Figure S4).

297



298

300 **Figure 3.** Bundles of RecA-mGFP filaments in MMC treated cells as observed in A,D) brightfield and
301 B,E) The initial Slimfield fluorescent frame (green) overlaid with all super-resolved single-molecule
302 tracks from the acquisition (*ca.* 40 nm spatial precision, with point localizations from foci visualized as
303 a Normalised Gaussian rendering in ThunderSTORM, Materials and Methods 4.3.7), revealing
304 filaments with high spatial precision (magenta); note that the contrast for the green Slimfield channel
305 is set to half to aid the visibility of the superresolution rendering. C,F) Slimfield at full contrast, overlaid
306 with segments derived from each super-resolved bundle by Otsu thresholding and expanding the
307 resulting image masks by the point spread function width of 180 nm, so as to match the diffracted-
308 limited widefield image optical resolution (white overlay); these segments were then used to calculate
309 the segment protein copy number. Scale bar 2 μm .

310

311 In MMC-treated cultures we observe strikingly bright, elongated structures (Figures 3B-E). These
312 resemble parallel or intertwined RecA-mGFP nucleoprotein filaments, that we denote as *bundles*
313 following similar observations by others [21,28,34,42,54]. The bundles were identified in a pointillistic
314 manner by overlaying the tracked foci with our measured localization precision of 40 nm. Though it is
315 unclear whether this segmentation is able to distinguish individual filaments or bundles of RecA from
316 one another, the segments reproduce the contiguous morphology of the bright structures at a
317 diffraction-limited optical resolution (Figure 3C,F).

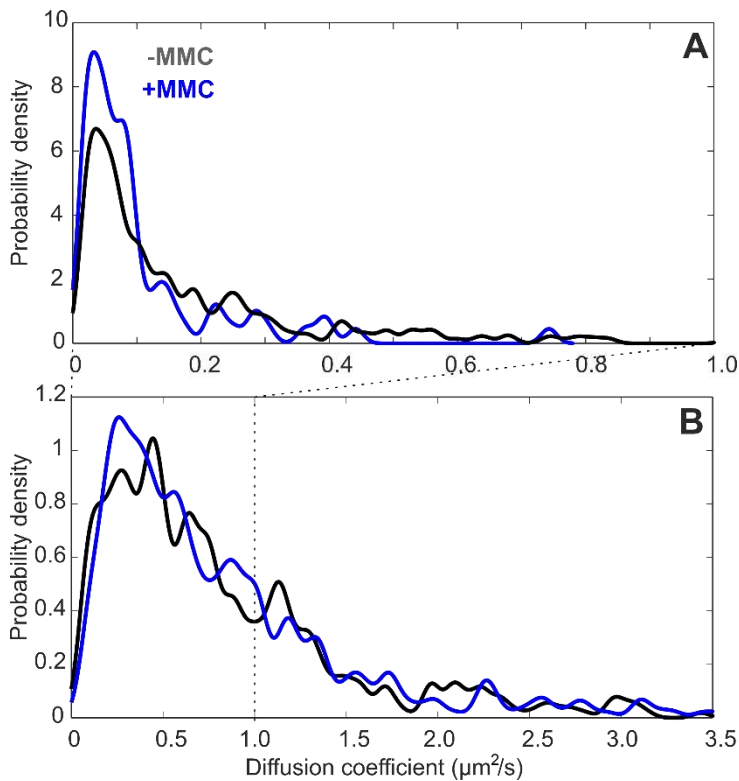
318 Though single contiguous segments are evident along the full length of some cells (Figure 3C), the
319 mean number of segments is 1.8 ± 0.4 per cell (Figure S3B). We occasionally observed several small
320 segments per cell, in quantitative agreement (Figure S3B) with our expectation that segments occur
321 at random under of a Poisson distribution, albeit conditioned on the presence of at least one segment
322 persistently occurring per cell. Assuming that DNA damage also occurs randomly but under
323 unconditional Poisson statistics, the number of unaffected cells can only be small when there is
324 significantly more than one segment-inducing damage site per cell at any time. It is not clear how
325 many DSBs per cell cycle an *E. coli* culture can sustain without loss of viability, but repeated stalling
326 and collapse of the replisome is common, and cells with single chronic DSBs are known to replicate
327 almost normally within the confines of an elevated SOS response [55]. Under the relatively mild MMC
328 treatment conditions of our study, we observe a comparatively small fraction of the available RecA-
329 mGFP in bundle-associated segments (here the sum of the segment protein copy numbers is <40% of
330 the cell protein copy number, in contrast to 70% [34]). These observations favour an explanation that
331 much of the RecA in elongated MMC-induced structures is bound to a relatively large number of ssDNA
332 nicks as well as a small number of DSBs per cell at any one time.

333 The high estimated amount of RecA (Figure 2), and the substantial super-resolved breadth of these
334 objects (Figure 3, Figure S3D) above the ~ 40 nm width of individual filaments [17] suggests that these
335 are bundles comprised of either multiple RecA filaments, and/or multiple windings thereof. We
336 calculate the segment protein copy number within each of these segments in a similar manner to each
337 whole cell. We find the segment protein copy number is $2,800 \pm 200$ RecA-mGFP molecules (Figure
338 S3A). That means each segment typically includes about three times as much RecA in total than the
339 brightest polar assemblies detected in untreated cells (Figure S3A). Greater than 95% of these
340 segments contain a track whose stoichiometry exceeds twice the mean stoichiometry of untreated
341 cells. The RecA structures observed after MMC treatment cannot therefore be produced solely from
342 the large RecA assemblies prior to MMC treatment, but most likely recruit additional RecA from the
343 cytoplasmic pool. We cannot measure the ratio of RecA to available ssDNA directly, however, the high
344 measured amount of RecA provides some indication that it occurs in high enough excess to form RecA-
345 rich bundles rather than simple nucleoprotein filaments. The binding site density on each helical
346 filament containing ssDNA was found in previous studies to be 1.5 nm per RecA in the presence of ATP
347 [56,57]. As the individual filaments are known to be undersaturated with RecA under physiological
348 conditions [58], one would expect a longer filament per molecule. In contrast, we find that each

349 bundle-associated segment typically measures $900 \pm 400 \mu\text{m}$ (mean \pm s.d.) in length, $140 \pm 40 \mu\text{m}$ wide
350 (Figure S3C-E) and no greater than $\sim 0.4 \mu\text{m}$ deep (based on depth of focus constraints), but contains
351 a quantity of RecA we estimate sufficient to produce $>7 \mu\text{m}$ total length of individual helical filament
352 based on known structures [59]. The longest segments have a more efficient packing density of RecA-
353 mGFP (Figure S3F) which approaches the binding site saturation limit of $1.5 \text{ nm} / \text{molecule}$. This link
354 between length and efficiency could result from the functional alignment and elongation of the
355 filament along the cell axis, meaning fewer re-entrant windings of any bundles, and exposure of vacant
356 binding sites to free RecA in cytoplasm.

357 In contrast, the brightest RecA assemblies in untreated cells occur in isolation, and are never elongated
358 but reside within diffraction-limited foci (Figure 1B). Defining these as containing RecA exceeding
359 twice the mean labeled stoichiometry, these occur in $10 \pm 3\%$ of untreated cells and have a typical
360 content of 800 ± 100 RecA molecules (Figure S3). This relatively high density is equivalent to $>2 \mu\text{m}$ of
361 filament packing inside a sphere $<0.4 \mu\text{m}$ in diameter. While these assemblies resemble RecA storage
362 bodies, as suggested previously [21], the *recA4155* R28A mutation has been shown to inhibit the
363 formation of true DNA-independent storage bodies [24]. Despite the presence of wild type RecA, it is
364 likely that our observations before MMC treatment indicate DNA-bound RecA bodies that are not
365 filamentous.

366



367

368

369 **Figure 4.** Distributions of instantaneous microscopic diffusion coefficient for tracks of A) RecA-mGFP
370 and B) RecB-sfGFP obtained from Slimfield. Kernel density estimates were generated with a kernel
371 width of $0.008 \mu\text{m}^2/\text{s}$ corresponding to the lower bound uncertainty in diffusion coefficient, estimated
372 as the localization precision / (timestep)². Statistics are as shown for Figure 2.

373

374 The diffusive dynamics of RecA assemblies are also indicative of their state of condensation into
375 filaments. Returning to the tracked foci of RecA-mGFP, we noticed that the mean diffusion coefficient
376 decreases sharply from $0.17 \pm 0.02 \mu\text{m}^2/\text{s}$ to $0.07 \pm 0.01 \mu\text{m}^2/\text{s}$ following MMC treatment (Figure 4A).
377 This initially low diffusivity, and the further drop in diffusivity, likely reflect the proportion of RecA

378 condensed onto ssDNA. MMC induces formation of filaments and these are relatively static on the
379 *ca.* 10 s timescale of the Slimfield acquisition. In contrast, we find that the mean diffusion coefficient
380 of tracked RecB is not significantly affected by MMC treatment (Figure 4B), with untreated and treated
381 values of $0.82 \pm 0.03 \mu\text{m}^2/\text{s}$ and $0.79 \pm 0.03 \mu\text{m}^2/\text{s}$ respectively (BM test, $n=478$, $p=0.48$ | NS). The
382 diffusivity of RecB-sfGFP in tracks is still lower than expected for a single molecule freely diffusing in
383 bacterial cytoplasm of $\sim 10 \mu\text{m}^2/\text{s}$, based on simplistic assumptions of a hydrodynamic diameter of
384 ~ 10 nm, and contrasts with the large amount of pool RecB-sfGFP that diffuse too quickly to be tracked.
385 This observation hints at the tracked subset of RecB forming larger complexes with other partners not
386 detected here, such as RecC and RecD.

387

388 **3. Discussion**

389 We used Slimfield to investigate the stoichiometry and spatial location of fluorescently tagged RecA
390 and RecB proteins in live *E. coli* upon treatment with the DNA cross-linking and alkylating agent MMC.
391 RecA and RecB are repair proteins whose involvement in MMC-specific damage repair pathways, as
392 part of the SOS response or otherwise, is unclear. We probed the steady state effect of MMC on RecA
393 and RecB at the minimum inhibitory concentration, which is relevant to sub-lethal antimicrobial
394 exposure. Our results show that the sensitivity and dynamic range of Slimfield is sufficient to quantify
395 counts, either by stepwise photobleaching of multi-molecular complexes or by direct detection of
396 single molecules using millisecond sampling.

397 RecA assembly formation is not solely correlated with induced DNA damage. Before treatment with
398 MMC, we find that a portion of RecA appears in foci at an average incidence of approximately 2 foci
399 per cell. In 10% of cells, at least one of these foci is especially bright, circular and localized to one of
400 the cell poles. A previous study reports that a minority of cells (4-9%) exhibit spontaneous RecA foci
401 near the poles prior to DSB induction [34]. It has been suggested that wild type RecA foci at the cell
402 membrane might act as nucleation points for later filament formation across DSBs [60], or that these
403 are storage bodies outside the nucleoid [21]. However, the RecA-mGFP strain used here (and in [34])
404 is a *recA4155* (R28A) genotype which abolishes DNA-independent aggregation of RecA [52,61]. In this
405 strain, we cannot eliminate the possibilities that wild-type RecA forms native storage bodies that are
406 undetected due to exclusion of RecA-GFP, or indeed visible storage structures which do recruit the
407 mutant RecA-mGFP (RecA4155), which would account for the resemblance of detected foci to
408 previous observations of these bodies outside the nucleoid [21,34,61]. In the case where RecA-mGFP
409 cannot participate in storage bodies and can only aggregate in the presence of DNA, there is an
410 alternative explanation for the subset of RecA-mGFP foci we observe, distinct from membrane
411 anchors and storage bodies. These foci do not appear to require RecB for spontaneous assembly [34]
412 indicating that they are independent of DSBs and instead assembled at incidental sites of ssDNA. The
413 foci lie consistently at the periphery of the cell, which indicates they are not likely to be associated
414 with ssDNA within replication forks. These occasionally bright foci may instead simply reflect
415 stochastic ssDNA nicks in a small proportion of cells of an otherwise healthy culture.

416 Our findings show that the RecA-mGFP copy number increases upon treatment with MMC. We
417 observed a modest increase in the number of tracks, but whose stoichiometry per focus is almost
418 twice those of untreated cultures. This observation of spatially localized RecA and is consistent with
419 significant assembly formation ultimately leading to formation of long nucleoprotein filaments on
420 ssDNA as nucleated from polar locations [34]. These filaments are known to accumulate into bundles
421 as posited by Story et al. [42]. We observed filamentous bundles in MMC treated cultures, possibly
422 due to increased availability of processed ssDNA from DNA damage sites. RecA-assisted homologous
423 recombination and RecA* disassembly occur on a timespan between 15 min [17] and 2 hours [34]. We
424 detect a large increase in RecA stoichiometry (Figure 2) and cellular protein copy number (Figure S5A)
425 and decrease in diffusivity (Figure 4) even after 3 hours' treatment, indicating that RecA bundles
426 continue to form in response to constantly accumulating DNA damage.

427 Our observation of an about 2 intracellular segments per cell (Figure S3B) is consistent with
428 approximately 2 MMC-induced RecA bundles each extending along opposite halves of a cell (Figure
429 3F) at any one time in the steady state. This observation may indicate the presence of a double-strand
430 break (DSB) with nearly-bridged loci. However, according to the schemes in previous work [17,34], the
431 development and breakdown of filaments [17] and bundles [34] takes typically <20 min, while for
432 bundles only, recombination is the rate-limiting step, taking up to 90 min [34]. It follows that labeled
433 bundles associated with DSBs would be expected to be bridged for most of their visible lifetime. It is
434 therefore possible that either i) multiple DSBs are present and the segments correspond to different
435 simultaneously bridged DSBs, or that ii) one bridged DSB is present alongside other defects which
436 support RecA filament binding, such as ssDNA nicks.

437 Intracellular segments were typically aligned along the cell axis (Figure 3B,E) in agreement with the
438 observations of filaments and bundles by other authors [17,34]. While some degree of alignment is
439 expected for all segments much longer than the cell diameter ($0.78 \pm 0.05 \mu\text{m}$), we note that more
440 than half of the detected segments are shorter than this (Figure S3E), which may suggest an alignment
441 mechanism that is not solely due to segment length. Moreover, segments appeared to follow the
442 central axis of the cell, rather than the cell outline (Figure 3B,E), which suggests they fall mostly within
443 the nucleoid rather than residing at the cell membrane, in keeping with the known DNA repair function
444 of the filaments. Filament extension along the cell axis is not predicated on the presence of sister
445 homology [34] but inherently reduces the dimensionality of the search for any homology to one across
446 the cross-section of the cell, independent of cell length or DNA content [17]. Thus, extension vastly
447 accelerates the search time [17]. However, the cause of the extension is unclear. It may reflect simple
448 polymeric elongation under spatial confinement inside the cell, but extension is entropically
449 unfavourable for a flexible polymer. Stiffening and/or thickening of filaments into bundles [34] would
450 therefore facilitate extension. The bundle model in [34] suggested a thickened central backbone
451 flanked by thin filament ends. The bundles observed in our study appear to be thickened with a typical
452 cross-sectional full-width half-maximum of $140 \pm 40 \text{ nm}$ (Figure S3C) in agreement with previous
453 observation, $160 \pm 30 \text{ nm}$ [34]. Rather than a monolithic central section, our observations resemble
454 beads on a chain, or a sequence of thick and narrow sections (Figure 3B,E). We find the median width
455 increases rapidly with segment length (Figure S3D), which in this binary framework, suggests the bulk
456 of the increase in bundle length is taken up by the thickened portions and that the thin sections are
457 relatively short. Yet, individual $\sim 40 \text{ nm}$ -wide filaments without thickened portions have also been
458 observed previously to extend dynamically on the scale of minutes or less along the length of the cell
459 [17]. We speculate that this suggests an active process of pole-to-pole translocation of thin filament
460 ends (for example, as proposed in [59]), to facilitate the reduced search time.

461 The observable periodicity of RecA structures could indicate a difference in their macromolecular
462 organization in response to MMC treatment. We observe a change in the periodicity of RecA
463 stoichiometry from ~ 2 molecules in foci in untreated cells, to a ~ 3 -4-mer within spatially extended
464 filaments following treatment with MMC, after accounting for the unlabelled RecA content per cell
465 with a correction factor of 1.3 ± 0.1 (Results 2.3 and Figure S4). Previous *in vitro* and *in vivo* studies
466 indicate that RecA undergoes linear polymerization in a head-to-tail fashion, with dimeric nucleation
467 points on ssDNA mediated by SSB [62] consistent with our finding of dimeric periodicity prior to
468 treatment. These also provide evidence for stable trimeric, tetrameric, hexameric and the filamentous
469 forms when ssDNA is present [63], consistent with our findings post-treatment. Our snapshot
470 observation of filament stoichiometry cannot shed light directly on models of dynamic nucleation or
471 stepwise growth, as explored in [64–66]. Rather, it explores molecular details of the characteristic
472 protein subunits within the mature filament at steady state. The helical geometry of the filament,
473 with a pitch of 6 RecA molecules per turn, implies that each group of 6 RecA forms a split-ring structure
474 related to the intact hexameric ring of DNA helicases, but distorted axially such that rings each
475 complete a single helical turn around ssDNA [67]. Such ring-shaped hexamers have been identified *in*
476 *vitro* for both the wild type RecA protein, and the RecA (R28A) mutant [61] that is fused with GFP in
477 our experiment. Even if isolated oligomers were somehow unstable *in vivo*, a polymeric filament could

478 conceivably still result from a small, periodic barrier to polymerization corresponding to this split-ring
479 distortion. This points to the hypothesis that the fundamental building block of RecA filaments is a
480 factor of 6, if not a hexamer. However, our stoichiometry analysis suggests variability in the total size
481 of assemblies, with our periodicity results indicating a range of 3-4 molecules per subunit. This could
482 reflect trimers which form half-turns in the filament, or perhaps tetramers as an intermediate
483 between preexisting dimers and hexameric rings. Although these data cannot directly establish
484 whether independent oligomers of wild type RecA occur *in vivo* either on DNA or in the cytosol, it is
485 conceivable that assembly and rearrangement of RecA subunits on DNA could generate the canonical
486 ATP-inactive and ATP-active DNA-binding filaments [68,69]. In light of a recent study highlighting the
487 role of RecN in RecA filament formation and activity [59], it is interesting to pose whether RecA
488 assemblies with the dimeric subunit may be devoid of RecN and are ATP-inactive, and if these might
489 then change to a higher oligomeric form upon DNA damage via the involvement of RecN and its
490 associated ATP-activity.

491 Our measurements confirm that RecA has a very high concentration in the cytosol of live cells. We
492 observe that untreated cultures comprise approximately 11,000 molecules of RecA-mGFP per cell,
493 which increases to 20,000 RecA-mGFP molecules in cells treated with MMC. Of the latter, $28 \pm 7\%$
494 resides in filamentous bundles large enough to be resolved in millisecond widefield fluorescence
495 images. Applying the approximate merodiploid correction factors that we estimated of 1.0 ± 0.1 and
496 1.3 ± 0.1 respectively (Results 2.3 and Figure S4), the total copy number is approximately $11,400 \pm 200$
497 RecA molecules in untreated cells, increasing to $25,300 \pm 400$ molecules in treated cells. Though less
498 than the 4-5-fold transcriptional increase suggested by qPCR (Figure S4), the more than two-fold
499 increase of total RecA with MMC resembles the increase detected in western blots (Figure S4). While
500 the RecA copy number we estimate in untreated cells exceeds the *ca.* 5,000 molecules reported
501 previously by Lesterlin et al [34], our more direct estimations are of similar order and correlate with
502 previous work indicating 2,900-10,400 molecules, with the high end of this range obtained from cells
503 in EZ rich medium using a ribosome profiling method [70]. Approximately 15,000 RecA molecules per
504 cell in rich medium were reported previously, using semi-quantitative immunoblotting [71]; the same
505 study found that the RecA copy number increased to 100,000 molecules upon MMC treatment. Large
506 discrepancies between studies in the increase in RecA due to MMC treatment are not only due to
507 treatment dose [72] but also arise from differences in *recA* genotype, culture media and growth
508 conditions, as noted by others [21]. In particular, our study uses a minimally inhibitory treatment with
509 MMC (Figure S1).

510 While the RecA-mGFP protein is not identical to native RecA in its enzymatic activity [32,43,52], the
511 merodiploid strain used in our study also includes a full complement of native RecA from a single
512 allele. This wild-type protein is expected to mitigate the partial loss of sensitivity in RecA-mGFP in any
513 given mixed assembly, as shown on a cellular level by the similar SOS response profile [34] and lack of
514 filamentation under treatment with MMC (Figure 1, Figure S1). The *recA* wild type allele is expressed
515 under control of the native operator, while the *recA-mGFP* allele is expressed under the *recAo1403*
516 operator. In the absence of treatment with MMC, this operator is known to result in an increased
517 transcription rate of the *recA-gfp* allele relative to the wild type *recA* gene under its native promoter
518 by a factor of 2-3, while both alleles are upregulated to the same level under induction of SOS [52].
519 Using quantitative Western blotting we estimate that prior to MMC treatment, RecA-mGFP is actually
520 present at several tenfold more than the unlabeled protein (Figure S4), and that in the presence of
521 MMC the ratio of RecA-mGFP to RecA is lower, at approximately 3-4 to 1. From these ratios, we
522 derived our approximate correction factors of 1.0 or 1.3-fold for the total amount of RecA protein, in
523 the absence or presence of MMC respectively. While RecA-mGFP is known to label RecA assemblies
524 [34], it cannot form DNA-independent assemblies by itself [52], and is therefore reasonable to
525 conclude that all labelled sites here represent occupied DNA on which wild type RecA and RecA-GFP
526 are interchangeable. Even if the binding partition of wild type RecA were higher, for example reflecting
527 the relative sensitivity (Figure S4A and [52]), the high relative concentration of RecA-mGFP (Figure
528 S4B) would conceivably result in the majority of RecA sites on DNA being occupied by RecA-mGFP.

529 Lesterlin *et al.* showed that RecA immunostaining of filaments (agnostic as to GFP labelling) correlates
530 with the fluorescent distribution of RecA-GFP [34], proving that dark filaments exclusively of wild-type
531 RecA cannot be present. Though this result could potentially be interpreted that the structure is
532 entirely RecA-GFP and that the highly sensitive wild-type RecA is excluded, this wild-type RecA would
533 have to somehow rescue DNA repair function in the cytoplasm rather than in filaments, which has no
534 known basis. We therefore assume the presence of hybrid filaments. In any case, the effect of
535 excluding wild-type RecA from filaments would simply mitigate our periodicity correction factor
536 toward unity, and narrow our estimate of the periodicity within filaments toward a value of 3 RecA
537 molecules.

538 Unlike RecA-mGFP, we detected only modest quantities of RecB-sfGFP in untreated cells grown in
539 minimal medium: 13.6 ± 0.5 molecules in tracks, and 126 ± 11 molecules in total per cell based on
540 integrated GFP fluorescence corrected for cellular autofluorescence. Several previous reports also
541 indicate that RecB is very scarce – typically less than 20 molecules per cell [35,73]. One of these studies
542 estimated that there are just 4.9 ± 0.3 RecB molecules per cell using a HaloTag fusion allele labeled
543 with HTL-TMR, and 4.5 ± 0.4 molecules per cell using magnetic activated cell sorting of the same RecB-
544 sfGFP strain that we use here, albeit in M9 medium and restricted to nascent cells for which the
545 average copy number is approximately halved [35]. An earlier mass spectrometry study used intensity
546 based absolute quantification to estimate 9-20 RecB molecules per cell across different stages of
547 growth in M9 minimal media [73]. Ribosome profiling estimated the RecB copy number to be 33-93
548 molecules per cell in different growth media [70]. However, these techniques are either *ex vivo* or
549 necessitate significantly perturbed intracellular crowding that may conceivably result in potentially
550 non-physiological molecular assemblies.

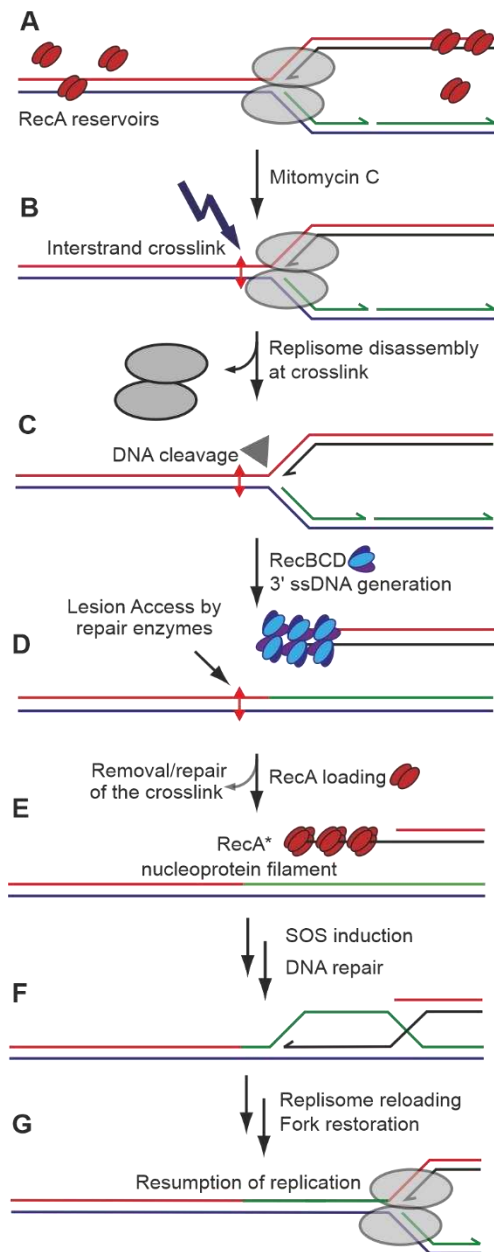
551 Comparing the number of RecB in tracks in our present study with the number of RecB in distinct foci
552 per cell reported previously, we find a similar albeit slightly higher estimate, possibly because our
553 approach is based on fluorescent fusions with a high labelling efficiency in unsynchronized cultures,
554 as opposed to selecting nascent cells. However, our measurement of RecB copy number exceeds
555 previous estimates. The large remainder in summed pixel fluorescence intensity may represent two
556 possible contributions. The first is from RecB that diffuses faster than Slimfield can track. The highest
557 diffusion coefficients of tracked RecB assemblies approach $3 \mu\text{m}^2/\text{s}$ (95% quantiles, Figure 4). We
558 estimate the limit of measurement as approximately $5 \mu\text{m}^2/\text{s}$, though it is conceivable that free
559 monomeric RecB-sfGFP could exceed this, given that it has been estimated to reach diffusion
560 coefficients equivalent to approximately $8 \mu\text{m}^2/\text{s}$ in *E. coli* cytosol [74,75]. A second possible source is
561 an increase in net autofluorescence relative to the parental strain when the real RecB are labeled; it
562 is unlikely that could account for the discrepancy, since this would require a 3-fold increase in
563 autofluorescence based on our measurements, and such a drastic increase lacks precedence (for
564 example, upon treatment with MMC at a high level sufficient to induce widespread RecA
565 filamentation, our estimation suggests only an increase in autofluorescence of no more than 20%).
566 Furthermore, the measured rate of photobleaching of the diffuse RecB-sfGFP signal matches that of
567 RecB-sfGFP tracks and is roughly half the rate of the autofluorescent parental cells (Figure S6, Table
568 S2). The implication is that untracked RecB-sfGFP is the major contributor to mean cellular
569 fluorescence, which is then a more accurate reflection of total copy of RecB than simply the number
570 of molecules in tracks.

571 The cellular protein copy number of RecB does not change significantly with MMC (Table S1, Figure
572 S5B,D), suggesting that there may only be a modest regulatory response to DNA damage. Although
573 MMC is known to induce the SOS response and cell cycle arrest [4,48], *recB* expression is itself not
574 induced directly as part of the SOS response. RecB-sfGFP foci increase neither in number (Figure 1J)
575 nor stoichiometry (Figure 2B), which compares with earlier observations that treatment with MMC
576 under similar concentrations to those used in our study do not significantly change RecB expression
577 [22]. In fact, the number of observed tracks per cell *dropped* considerably after MMC treatment, due
578 to a sharp increase in the proportion of cells in which RecB assemblies were absent, from 6% to 21%.
579 This reduction in RecB assemblies was at odds with our expectation that MMC would eventually

580 increase the recruitment of RecB in response to damage, if not increase the cellular production of
581 RecB. MMC treatment is known to increase the occurrence of DSBs and thereby drive demand for
582 DSB processing [4] that is typically mediated by RecB. Yet, rather than initiating cellular upregulation
583 of RecB, treatment with MMC acts to partially deplete localized assemblies. Given that DSBs are likely
584 to occur in the majority of cells under our MMC treatment, as indicated by the ubiquitous induction
585 of RecA filaments (Figure 3), the fate of RecB assemblies cannot simply reflect the presence or absence
586 of DSBs. The increase in the fraction of cells lacking RecB-sfGFP tracks is consistent with random,
587 independent survival or breakdown of assemblies (Figure 1J). This result may indicate a situation
588 where pre-existing RecB (hetero)complexes at foci are occasionally disassembled while interacting
589 with sites of MMC-induced DNA damage, such as DSBs. This instability of (presumably heteromeric)
590 RecB assemblies might result from successfully bridged pairs of RecA filaments, however, we did not
591 detect any correlated loss of pairs of RecB foci, as might be expected for recombination events.
592 Notably, the number of tracked foci detected per cell is approximately 2 for both RecA-mGFP and
593 RecB-sfGFP. Future colocalization studies of RecA and RecB assemblies may offer more direct insight
594 into the functional interaction and turnover of these repair proteins in regards to whether the average
595 of 2 be related to the number of replication sites, or perhaps simply reflects a small average number
596 of severe DNA damage sites per cell.

597 Independent of MMC treatment, we observed a dimeric periodicity for RecB-sfGFP. This suggests that
598 RecBCD heterotrimers occur in pairs *in vivo*. Indeed, earlier *in vitro* studies identified the occurrence
599 of (RecBCD)₂ complexes, possibly held together by the nuclease domains of the two RecBCD
600 monomers [12]. However, the authors concluded that the monomeric form is functional while the
601 dimeric form is nonfunctional [13]. Furthermore, crystallization of the RecBCD complex for structural
602 studies contained two RecBCD-DNA complexes in the asymmetric unit [76]. Our observations cannot
603 determine covalent interactions directly between RecB molecules, but their cotracking is very strongly
604 correlated. We can infer two details: first, that the dimeric form of the complex, (RecBCD)₂, occurs in
605 live cells, and second, that previous *in vitro* observations of dimers are carried over from their
606 physiological state. Our findings suggest a hypothesis that assemblies with multiple pairs of RecB have
607 a greater activity on DSBs than isolated RecB in the pool. Making the distinction between monomeric
608 RecBCD in tracks and monomeric RecB in the untracked pool suggests that RecB monomers in the pool
609 could potentially act as a reservoir. One may consider the alternative situation, where the monomeric
610 pool are the functional RecB elements and the assemblies are reservoirs that disassemble in response
611 to damage, but this makes less sense, since those monomers would already be in excess. A mean
612 stoichiometry of ~6 molecules indicates that RecB foci may occur as colocalized assemblies that
613 comprise roughly three pairs of RecBCD heterotrimers (Figure 5). It would be interesting to estimate
614 the stoichiometries of RecC and RecD in future studies to understand their association in processing
615 DSBs in greater detail.

616



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618

619 **Figure 5.** A model of DNA damage caused by treatment with MMC and subsequent repair at the
620 replication site by RecA and RecBCD. A) Intact replication fork; occasional binding of multiple RecA
621 dimers to DNA away from the fork as well as RecA dimers as DNA-free storage bodies in the cytoplasm;
622 B) exposure to MMC and induction of an interstrand crosslink that acts as a barrier to an approaching
623 replication fork; C) replisome dissociates if unable to overcome barrier; dissociated fork is recognised
624 by branched DNA specific endonucleases (filled triangle) that can eventually cause DSBs leading to
625 replication fork collapse; replication fork collapse allows access to repair enzymes to recognise the
626 lesion; D) a newly generated DSB is recognised by RecBCD and processed to generate a 3' single strand
627 end; E) RecA dimers identify the newly generated ssDNA and assemble in groups of 3-4-mers into
628 RecA* filaments; RecA is shown as a short stretch for illustrative purposes but may extend for many
629 thousands of molecules over several hundreds of nm of ssDNA, and these filaments may be twisted
630 and/or grouped into bundles. F) Strand exchange followed by processing of the double strand break,
631 then recombination sufficiently upstream of the lesion and subsequent G) reloading of the replisome.
632 This process allows sufficient time for the repair enzymes to repair the lesion on the template strand,

633 so that replication may resume. For a detailed overview of the possible pathways to fork restoration,
634 refer to [11].

635 While MMC-induced damage constitutes a range of chemical moieties [77], the canonical mechanism
636 of MMC toxicity is of interstrand crosslinks at dG sites [6,7]. The specific repair of interstrand crosslinks
637 (implied in Figure 5) can involve several repair pathways, primarily nucleotide excision repair (NER),
638 which converts the crosslinks into dsDNA breaks [78]. Although NER enzymes such as UvrD typically
639 degrade RecA filaments, NER is involved in the cleavage of damaged replication forks into suitable
640 substrates for downstream processing, including RecA-mediated recombination [79]. Repair of the
641 fork is then completed, for example by PriA-, Rep- and PriC-dependent pathways [11,18,80] on sets of
642 ssDNA and a dsDNA end (Figure 5). The observation of a greater increase in RecA-mGFP copy numbers
643 and foci compared to RecB-sfGFP could indicate a significant proportion of single-strand breaks and
644 single-strand gaps at sites of crosslinks. While two previous studies reported that NER action on
645 crosslinks also produces ssDNA nicks [78,81], we do not know if this applies strictly to MMC-induced
646 NER, as our present work does not pertain to genes that *process* ss-gaps. Future analysis of proteins
647 that process ssDNA breaks may potentially shed light on the relative occurrence of the two types of
648 breaks by MMC and their relation to repair of replication forks.

649 While others have shown that *recB* deletion abolishes UV-induced filaments of RecA [34], we do not
650 know the effect of *recB* deletion and MMC treatment on RecA dynamics. To avoid RecA interference
651 in 'normal' ssDNA processes such as replication, the cell maintains strict control over filament
652 nucleation, based on RecA and associated cofactor concentrations. It is therefore likely that the
653 observed filamentation upon treatment with MMC is dependent on RecBCD, indirectly pointing
654 towards increased occurrences of DSBs in these cells. Alternatively, if RecA nucleation is independent
655 of RecBCD, one might anticipate little change in RecA dynamics upon *recB* deletion. However, further
656 analysis of MMC-dependent RecA stoichiometry and copy number in a strain devoid of RecBCD activity
657 - and with a controlled RecFOR pathway [19] - is needed to differentiate between these models.

658 In conclusion, RecA occurs as assemblies located near poles of wild type cells in a dimeric periodicity
659 consistent with nucleation models. Upon mild treatment with MMC, RecA is upregulated at least two-
660 fold, and assembles into long filamentous bundles on newly generated ssDNA in effectively all cells
661 without exhausting the cytoplasmic reservoir. These mature bundles have a much lower diffusivity,
662 reflecting their aggregation of a few thousand molecules each, with a structural periodicity in the
663 range of 3-4 RecA molecules. The bundles are typically wider than single filaments, but both forms are
664 known to facilitate homology search for homologous base-pairing with an intact duplex. Generation
665 of ssDNA is known to occur at a DSB induced by processing of disassembled forks upon recognition by
666 RecBCD. We observed RecB as a moderately diffusive set of three associated dimers at two locations
667 in the cell, providing further evidence that RecBCD predominantly occurs as pairs of heterotrimers
668 inside the cell at either end of DSBs. Our work implies the existence of a separate, significant reservoir
669 of highly diffusive RecB monomers. Neither of these forms of RecB are upregulated upon MMC
670 exposure, nor do they change their mobility. Accordingly, RecB is not a part of the SOS regulon.
671 Instead, MMC-induced DNA damage impacts the formation - or induces a higher turnover - of these
672 periodic RecB assemblies potentially associated with further DSB repair.

673

674 **4. Materials and Methods**

675 *4.1. Strains, culture and MMC protocol*

676 Three strains of *E. coli* were used in this study without alteration:

677 **Control:**

678 *MG1655*

679 **RecA-mGFP** [34]:

680 *MG1655 rpsL (Str^R, lac+) ygaD1::kan recAo1403 recA4155,4136-gfp901, fhuB::recAwt-cm*

681 **RecB-sfGFP** = MEK706 [35]:

682 *MG1655 recB::sfGFP*

683
684 The RecA-mGFP strain used here is the same as in [34]. It is a merodiploid, natively promoted
685 derivative of the SS3085 strain [52,82,83]. It expresses both i) the wild type unlabelled RecA protein
686 from a single, ectopic wild-type *recA* allele, under a wild-type operator, and ii) a labeled mutant
687 protein at the native *recA* site with a mutant operator *recAo1403*, which has a higher transcription
688 rate than wild type [52]. The labelled *recA* is a *recA4155* (R28A) mutant, which complements the wild
689 type recombination function *in vitro* [34,61] but has important differences in self-assembly. Unlike
690 wild type RecA, the *recA4155* forms of RecA cannot alone form assemblies independently of DNA as
691 proven by comparisons *in vitro* [61] and competitive binding studies *in vivo* [52]. The *gfp-901* label is
692 the same as *mut2* (A206T) which corresponds to a monomeric GFP (mGFP) [84]. The notation
693 *recA4136* refers to the insertion of a linker, as well as the *gfp-901* gene, between the penultimate and
694 ultimate stop codons of *recA*. The fusion with mGFP impairs the recombinant sensitivity of the labeled
695 RecA protein; were the strain to include only the fused allele, it would be fully as SOS inducible as wild
696 type MG1655, but only approximately two-thirds as UV resistant, and would be compromised up to
697 *ca.* 10-fold for recombination activity [21,52]. Induction of the SOS response would also take 30 min,
698 or roughly twice as long as wild type (the R28A mutation prevents this from being an additional 2×
699 slower) [52]. The merodiploid strain rescues both these functions and their kinetics: functional RecA
700 filaments labeled with 70% of the total available mutant fusion protein form within just 15 min of DNA
701 damage and reach steady state within 90 min, similar to wild type MG1655 [34].
702 The RecB-sfGFP fusion was constructed in [35] by wild-type *recB* replacement under plasmid-mediated
703 recombination. N-terminal fusions were shown in [35] to be functional using growth curves and tests
704 of DNA repair, in contrast to C-terminal fusions which may disrupt RecBCD complexation [35].
705 *E. coli* strains were grown overnight in 56-salts minimal media at 30°C to mid-log phase in an Innova
706 44 shaker incubator (New Brunswick). The mid-log phase cultures were concentrated to ~100 cells/ml
707 (OD₆₀₀ ~0.3) and split into two equal fractions. Aliquots were adjusted to either nil (MMC-) or the
708 minimum wt inhibitory concentration of 0.5 µg/ml MMC (MMC+) (Figure S6 and [48]) and incubated
709 at 30°C for a further 3 h (Figure S6). Cells were harvested for microscopy on 1% w/v agarose pads
710 suffused with the same liquid media and imaged within 1 h.

711

712 4.2. Slimfield

713 A custom-built Slimfield microscope was used for single colour, single-molecule-sensitive imaging with
714 a bespoke GFP/mCherry emission channel splitter as described previously [11,37]. The GFP channel
715 was recorded, while the mCherry channel was used only as a negative control. The setup included a
716 high-magnification objective (NA 1.49 Apo TIRF 100× oil immersion, Nikon) and the detector was a
717 Prime95B sCMOS camera (Photometrics) operating in 12-bit gain at 180 Hz and 3 ms exposure/frame,
718 for a total magnification of 53 nm/pixel. The samples were illuminated either in brightfield, or for
719 Slimfield fluorescence in camera-triggered frames by a collimated 488 nm wavelength continuous
720 wave OPAL laser (Coherent, Obis LS) in Gaussian TEM₀₀ mode at a power density of 5 kW/cm². The
721 number of frames per acquisition was 2,000 for RecA and 300 for RecB strains.

722

723 4.3 Quantitative tracking and protein copy number analysis

724 4.3.1 Identification of Slimfield foci and assignment into tracks

725 Slimfield image sequences were processed by custom ADEMscode software in MATLAB (Mathworks)
726 [33,80,85–87]. This pipeline identified foci from local maxima in pixel values within individual frames.
727 An iterative Gaussian mask algorithm was used to detect the centroids of foci, using a circular region
728 of interest of radius 5 pixels within a sliding window of 17 pixels. The intensity of each focus was
729 calculated as the sum of the circular region corrected for the average background in the surrounding
730 annular region. The prospective foci were accepted if their intensity was >0.4× the standard deviation
731 in the background region. The nearest neighboring foci in adjacent frames within 8 pixels of each other
732 were assigned to the same track, with a minimum of 4 foci per track. The typical track duration was

733 limited by diffusion and/or photobleaching to a mean of >13 foci per track over ~75 ms real time, or
734 ~40 ms cumulative exposure (Table S1).

735 4.3.2. Diffusion coefficient

736 The centroids of the foci within each track, as generated from the ADEMScode tracking analysis above,
737 were used to calculate displacements over the length of each track in chronological sequence. From
738 these, the mean square displacements (MSDs) of each track were calculated by averaging the square
739 of the displacements across equal lag times, corresponding to all possible intervals between frames
740 up to the length of the track. For each track, the MSDs at the four lowest lag times were linearly
741 interpolated (with a constraint on the fit of passing through a specified intercept on the lag time axis,
742 equal to the square of the measured localization precision of 40 nm divided by the frame interval of
743 5.7 ms). The initial slope of this fit (and corresponding error) was then divided by a factor of 4
744 according to the 2D diffusion equation [88] to yield a diffusion coefficient (and error) for that track.

745 4.3.3 Characteristic single-molecule brightness

746 The intensity of each focus was estimated by integrating the local pixel values with a local sliding
747 window background subtraction. After photobleaching sufficiently to show single photoactive GFP
748 molecules, the characteristic single-molecule brightness of a single GFP molecule was estimated from
749 the modal brightness of these foci. These were confirmed to be broadly consistent with estimates of
750 the signal per GFP in each dataset were determined from the monomeric intervals in total number of
751 counts due to stepwise photobleaching, as identified by a Chung-Kennedy edge-preserving filter
752 (15 ms window, 50% weighting, Figure S7) [89]. This integrated intensity is characteristic for each
753 fluorescent protein under fixed imaging conditions, although mGFP and sfGFP were found to be
754 indistinguishable in this respect, and hereafter referred to collectively as GFP. To ensure consistent
755 counts per single-molecule probe, analysis was restricted to the uniformly illuminated area lying
756 within half of the $1/e^2$ beamwaist of the excitation laser in the sample plane. The integrated intensity
757 of GFP *in vivo* was found to be within 14% and 9% respective errors in RecA and RecB (88 ± 18 and 177
758 ± 16 pixel grey values per GFP for the respective gain modes). The combined equivalent is 88 ± 7
759 photoelectrons per GFP per frame, which is precise enough to unequivocally identify groups or steps
760 of up to 12 GFP molecules.

761 4.3.4 Stoichiometry

762 Each track is associated with an assembly that contains a certain number of molecules, or
763 stoichiometry, at the initial point of acquisition. To estimate this stoichiometry for a given track, the
764 intensities of the constituent foci were linearly extrapolated using the first 4 datapoints in the track
765 back to the timepoint of initial laser exposure. This initial intensity of this fit was divided by the
766 characteristic single-molecule brightness signal associated with one fluorescent protein under a fixed
767 excitation-detection protocol. The result is a stoichiometry expressed as a number of molecules. The
768 standard error associated with a stoichiometry value of 1 molecule is approximately 0.7 molecules. To
769 avoid undercounting bias due to photobleaching, only tracks in the first 10 frames after laser exposure
770 were considered for stoichiometry estimates.

771 4.3.5 Periodicity

772 The distributions of track stoichiometry may show periodic peaks, whose smallest reproducible
773 interval can be interpreted as a physical repeat unit or *periodicity* within assemblies. To calculate
774 periodicity, first the stoichiometries of all tracks within each acquisition were represented as a kernel
775 density distribution. The kernel width used was the empirical standard deviation on the characteristic
776 single molecule brightness of 0.7 molecules [41]. Peaks in this distribution were detected using the
777 MATLAB *findpeaks* function, and the intervals between nearest neighbor peaks were calculated. These
778 sets of nearest neighbor intervals for each acquisition were then aggregated across the relevant
779 population of cells. A second kernel density estimate was calculated over the intervals for a
780 population, with a kernel width of 0.7 molecules multiplied by the square root of the mean
781 stoichiometry, divided by the square root of the number of interpolated intervals. The fundamental
782 value of this interval distribution (corresponding to the center of the leftmost peak in Figure 2 insets)

783 was refined by fitting the curve with a sum of Gaussian terms centered at multiples of the fundamental
784 value. To accommodate the uncertainty in the single molecule characteristic brightness, the
785 fundamental value of the fit was not constrained to an exact integer value but represents a heuristic
786 model for the periodicity. The number of terms in the fit was set to minimize the reduced χ^2 metric in
787 the fit. This modal value was reported with 95% confidence interval as the periodicity of assemblies in
788 each population. This method of estimating periodicity was verified as independent of the mean
789 stoichiometry using simulated data drawn from noisy Poisson-distributed multiples of an oligomeric
790 ground truth (artificial input value). This analysis reproduced the expectation that the minimum
791 number of tracks required for sufficient peak sampling, and therefore the limit of periodicity
792 detection, scales with the square root of the mean stoichiometry.

793 4.3.6 Cellular protein copy numbers and pool stoichiometry

794 The cellular protein copy numbers as reported in the Results Section 2.1, Table S1, Figure S5 and
795 Discussion correspond to whole cell masks, as identified using the manual annotated machine learning
796 segmentation output from brightfield images (Figure S2 and Supplementary Methods). Integrated
797 intensities of cells (uncorrected cellular protein copy numbers) and pool stoichiometries, were
798 determined not from tracked foci, but directly from the raw image sequences using the CoPro package
799 in ADEMscode software following [37] with the characteristic single-molecule brightness of GFP (as
800 described in 4.3.2), the cell masks, and the camera's dark pixel bias as input. The procedure effectively
801 adds up all of the pixel values within the mask in question in an initial frame, and accounts for the
802 convolution of the 3D cell volume with the widefield point spread function, followed by projection
803 onto a 2D image. To obtain the cellular protein copy number in the labeled strains, and account for
804 the contribution of autofluorescence, we calculated the difference in mean integrated intensity per
805 segment between the labeled and parent strains under the corresponding MMC \pm condition, adjusted
806 by the ratio of mean segment area. The pool stoichiometry in each cell is a measure of its untracked
807 molecular concentration. It is calculated in CoPro as the cell's integrated intensity, less the mean
808 integrated intensity of the parental cells, less the total stoichiometry of tracked foci in the cell, divided
809 by the area of the cell mask relative to the area within one diffraction limited focus.

810 4.3.7 Super-resolved images and segment protein copy numbers of RecA-mGFP

811 The segment protein copy numbers as reported in Results Section 2.3 and Figure S3, were calculated
812 with ImageJ, using as input the segments corresponding to bundles or foci, instead of whole cells.
813 These segments were obtained starting from the coordinates of localized, tracked foci in Slimfield
814 analysis, from the latter stages of photobleaching below a threshold stoichiometry of 2 molecules;
815 these were imported into ThunderSTORM software [90]. The Visualization module to build a
816 pointillistic super-resolved image at 40 nm lateral spatial precision (as shown in Figures 3B,E) at 5 \times
817 upscaling (11 nm pixel size), which was then smoothed with a Gaussian filter of 4 pixels' width, and
818 automatically Otsu thresholded to generate a superresolved binary mask. The masks were then
819 expanded by a distance equal to the widefield resolution of 17 pixels (~180 nm) to match the features
820 in the Slimfield images. The integrated intensities were extracted, as in [91], from the sum of
821 fluorescent pixel counts in the Slimfield images (Analyze Particles > Multi-Measure function in ImageJ)
822 less the area multiplied by the camera pixel dark value. To yield segment protein copy numbers (Figure
823 S3A), the resulting integrated intensities were corrected for the relative autofluorescence, by
824 subtracting the integrated intensity of parental cells adjusted by the ratio of mean segment area.
825 The Multi-Measure output also included the Feret diameter of each segment which was used as an
826 estimate of its end-to-end length (Figure 3D-F).

827 4.3.8 Photobleaching rates

828 Photobleaching rates were estimated by fitting the decrease in background-subtracted cellular
829 protein copy number or mean track stoichiometry over the exposure time using MATLAB *cftool*. The
830 fit consisted of a monoexponential decay to the first 10 frames with variable initial intensity and decay
831 constant, but with a baseline fixed to the average intensity after 50 frames. Fits were then refined to
832 include only data within the initial $1/e$ decay time (Table S2). RecA-mGFP and RecB-sfGFP photobleach

833 decay times were consistently dissimilar at 13 ± 2 and 6 ± 1 frames respectively; sfGFP is typically
834 several-fold less photostable than comparable enhanced GFPs under high intensity illumination [92].

835 4.3.9 Statistical tests

836 We performed multiple statistical comparisons on each set of tracked data (typically ~5: number of
837 tracks, stoichiometry, periodicity, diffusivity, copy number), which we account for using the standard
838 Bonferroni correction; the significance level is adjusted downwards by a factor of the number of
839 comparisons, $\alpha = 0.05/5 = 0.01$).

840

841 4.4 Gene expression assays

842 4.4.1 Quantitative PCR (qPCR)

843 Treated and untreated cultures were grown as in section 4.1. Total RNA was then isolated using
844 Monarch Total RNA Miniprep Kit (New England Biolabs). cDNA was synthesised from 350 ng of total
845 RNA from each sample using Superscript IV reverse transcriptase (Invitrogen) according to the
846 manufacturer's instructions using random hexamer primer (ThermoScientific).

847 The cDNA was then subjected to qPCR using Fast SYBR Green Master Mix (ThermoFisher) in a
848 QuantStudio 3 Real-Time PCR System. The *recA* primer pair amplified *recA* cDNA in the wild type and
849 both *recA* and *recA-GFP* mRNA in the labeled strain. *recA-GFP* alone in the labeled strain was amplified
850 using *GFP* primer pair. 16s rRNA was used as a housekeeping control.

851 Data obtained was analysed using the standard curve method [51]. Standard curves were generated
852 from serial dilutions of PCR products with known concentrations derived from genomic DNA. Fold
853 increase in mRNA levels was calculated by dividing the values obtained for treated mRNA with the
854 untreated. Results are shown in Figure S4A.

855

Primer	Sequence 5'-3'	Complementary region
oAS216	GCAGGCACTGGAAATCTGTG	<i>recA</i> (forward)
oAS217	GCCGATTTTCGCTTCGATTTTC	<i>recA</i> (reverse)
oAS220	CTACAAGACACGTGCTGAAGTC	<i>GFP</i> (forward)
oAS221	AGTTGTATTCCAATTTGTGTCCAAGAATG	<i>GFP</i> (reverse)
oAS23	GTAGAATTCCAGGTGTAGCGGTG	16s rRNA (forward)
oAS24	CATCGTTTACGGCGTGACTACCAG	16s rRNA (reverse)

856 **Table 2. Primers used for qPCR to quantify mRNA of *recA*, *recA-gfp* and housekeeping gene *rrsA*.**

857

858 4.4.2 Western blots

859 Six samples of normalised *E. coli* cell cultures were prepared as above (section 4.1) in 1 ml aliquots at
860 $OD_{600} \sim 0.2$. The cells were isolated using centrifugation at $10,000 \times g$ for 2 min in a microfuge to
861 prepare them for SDS-PAGE / immuno-detection. The cell pellets were resuspended in 75 μ l of SDS
862 loading buffer and boiled for 5 min at 95°C before application of 15 μ l onto a 4-20 % gradient gel. The
863 gel was subsequently transferred to nitrocellulose and the membrane was placed in blocking solution
864 (PBS-T, 5% (w/v) non-fat milk) for 3 h. Primary antibody (anti-RecA) was incubated at 1/500 overnight
865 in blocking solution before the membrane was washed (4 x 5 min) in blocking solution. Secondary
866 antibody (goat anti rabbit-HRP) was incubated at 1/2,000 dilution for 4 h in blocking solution before
867 the membrane was again washed (4 x 5 min) in blocking solution. A final wash in PBS was performed
868 before development using ECL and image acquisition (iBRIGHT). Results are shown in Figure S4B.

869

870 Author Contributions:

871 A.P-D, A.S, and M.L. designed the research; A.S. cultured and treated cells; A.P-D performed
872 microscopy and data analysis, visualization and curation, J.S. and L.F. wrote and validated
873 segmentation software; A.P-D and A.S drafted the paper; A.P-D, A.S, J.S. and M.L. edited the paper;

874 M.L. supervised and administered the project. This research was funded by BBSRC, grant numbers
875 BB/P000746/1 and BB/N006453/1, and EPSRC, grant number EP/T002166/1.

876

877 Data Availability Statement: The raw imaging data is available on reasonable from
878 <https://doi.org/10.5281/zenodo.6639101>; the MATLAB tracking analysis code can be found at
879 <https://github.com/alex-payne-dwyer/single-molecule-tools-ald>. The U-Net image segmentation
880 architecture originated from code obtained from the NEUBIAS Academy workshop
881 (<http://eubias.org/NEUBIAS/training-schools/neubias-academy-home/>).

882

883 **Acknowledgments:**

884 The authors thank Dr. Christian Lesterlin for the gift of the RecA-mGFP strain and Prof. Meriem El
885 Karoui for the RecB-sfGFP strain. We thank the Biosciences Technology Facility for technical assistance
886 with gene expression assays.

887 **Conflicts of Interest:**

888 The authors declare no conflict of interest. The funders had no role in the design of the study; in the
889 collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to
890 publish the results.

891

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