

1    **Real-time dynamics of mutagenesis reveal the chronology of DNA repair and damage**  
2    **tolerance responses in single cells**

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12 **ABSTRACT**

13 Evolutionary processes are driven by diverse molecular mechanisms that act in the  
14 creation and prevention of mutations. It remains unclear how these mechanisms are  
15 regulated because limitations of existing mutation assays have precluded measuring  
16 how mutation rates vary over time in single cells. Towards this goal, I detected  
17 nascent DNA mismatches as a proxy for mutagenesis and simultaneously followed  
18 gene expression dynamics in single *Escherichia coli* cells using microfluidics. This  
19 general microscopy-based approach revealed the real-time dynamics of mutagenesis  
20 in response to DNA alkylation damage and antibiotic treatments. It also enabled  
21 relating the creation of DNA mismatches to the chronology of the underlying  
22 molecular processes. By avoiding population averaging, I discovered cell-to-cell  
23 variation in mutagenesis that correlated with heterogeneity in the expression of  
24 alternative responses to DNA damage. Pulses of mutagenesis are shown to arise from  
25 transient DNA repair deficiency. Constitutive expression of DNA repair pathways and  
26 induction of damage tolerance by the SOS response compensate for delays in the  
27 activation of inducible DNA repair mechanisms, together providing robustness  
28 against the toxic and mutagenic effects of DNA alkylation damage.

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30 **KEYWORDS**

31 mutagenesis / DNA repair / gene expression noise / antibiotics / single-cell microscopy

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33 **SIGNIFICANCE**

34 A central goal in genetics is to understand how mutation rates are regulated by the genome  
35 maintenance system in response to DNA damage or drug treatments. This has been

challenging because existing mutation assays only show time- and population-averages of mutation rates, and do not resolve the underlying molecular processes. Towards this goal, I utilized a microscopy-based method which enables relating the creation of DNA mismatches to single-cell gene expression dynamics in real-time. I show that DNA alkylation damage causes a distinct pulse of mutagenesis that is shaped by the chronology of constitutive and inducible DNA repair and damage tolerance pathways. Stochastic fluctuations in the expression of these pathways modulated the dynamics of mutagenesis in single *Escherichia coli* cells.

## INTRODUCTION

DNA damaging agents are widely used as antibiotics and cancer therapy drugs. These include DNA alkylating, oxidising, and crosslinking agents, and inhibitors of DNA transactions (1–3). However, besides the intended cytotoxicity, DNA damage also leads to heritable mutations that can accelerate disease progression and cause drug resistance in pathogenic bacteria and cancers (4–8). In addition, drug treatments trigger cellular stress responses that actively generate mutations (9). The molecular mechanisms of mutagenesis during normal cell growth and in response to DNA damage have been the focus of intense research and debate for decades. Owing to these efforts, many genes have been identified that affect mutation rates, as well as regulatory mechanisms that control their expression. However, we lack a clear understanding of how mutation rates are defined by the action of the replication and repair machinery as a whole. Which factors determine whether a mutagenic DNA lesion is accurately repaired or converted into a mutation? To address these unknowns, new experimental approaches are required that can measure the real-time dynamics of repair and mutagenesis in a way that individual mutation events can be linked to the underlying molecular processes in live cells.

Faithful completion of DNA replication is crucial for cell survival and genome stability. Therefore, multiple highly conserved mechanisms that deal with DNA damages exist in all domains of life from bacteria to humans (10). These mechanisms fall broadly into two categories: damage repair and damage tolerance. An abundant type of DNA damaging agents in the environment and inside cells are alkylating chemicals, which form base lesions that perturb the progression and fidelity of DNA synthesis (11). In *Escherichia coli* and many other diverged bacteria, the adaptive (Ada) response senses DNA alkylation damage and induces the expression of direct repair (DR) and base excision repair (BER) pathways to remove alkylation lesions (12, 13). Constitutively expressed DR and BER genes complement the inducible genes of the adaptive response. In contrast, DNA damage tolerance via Translesion Synthesis (TLS) or homologous recombination (HR) enables replication forks to bypass alkylation lesions without repair (14–16). Bacteria control DNA damage tolerance pathways through the SOS response, a large gene network that is induced by DNA breaks or stalled replication forks (17–19). Whereas Ada-regulated DR and BER pathways accurately restore the original DNA sequence, SOS-regulated TLS polymerases are intrinsically error-prone (16), but error-free lesion bypass and replication restart mechanisms are also activated by the SOS response (14, 15). DNA mismatch repair (MMR) corrects most misincorporated bases and short insertion/deletion loops before they turn into stable mutations (20, 21).

Despite extensive characterization of the individual DNA repair and damage tolerance pathways, it is still unclear how their overlapping and counteracting functions collectively control mutation rates. Furthermore, DNA damage responses change the expression and activity of the pathways. How do these dynamics influence mutation rates? These questions are unsolved due to limitations of existing methods to measure mutagenesis. Firstly, in vitro biochemical experiments or bulk genetics assays do not reveal the dynamics of DNA repair and mutagenesis in living wild-type cells. Secondly, DNA sequencing or fluctuation tests provide population- or time-averaged mutation rates from which the underlying molecular

mechanisms can only be inferred indirectly (20–22). Thirdly, it has become evident that the expression of DNA repair proteins is heterogeneous within cell populations (23–25), which may cause cell-to-cell variation in mutation rates (26–28, 24, 29). This demands experiments with single-cell resolution. Resolving the full distribution of mutation rates within a cell population is important for understanding evolutionary processes, which could be driven not by the population average but by a small fraction of individuals in the tail of a distribution of mutation rates.

Here, I utilized a general microscopy-based method using microfluidics that complements existing mutation assays. It addresses the aforementioned challenges by simultaneously monitoring the occurrence of DNA mismatches, the expression of DNA damage responses, and the survival of single cells. The formation of MMR foci marks nascent DNA mismatches in real-time, thus providing a direct readout for the activity of mutagenic processes inside cells. This approach revealed a distinct pulse of mutagenesis in response to constant alkylation damage treatment. To determine which factors shape the dynamics, I performed a series of genetic perturbations combined with gene expression reporters. I discovered a temporal order in the activity of constitutive and inducible DNA repair and DNA damage tolerance pathways, a chronology that dictates the dynamics of mutagenesis and cell survival in response to DNA alkylation damage. In individual cells, mutagenesis was temporally correlated with variations in the expression of genome maintenance pathways. The generality of the approach is underlined by measurements of the real-time dynamics of mutagenesis in response to antibiotic treatments.

## Results

**Real-time measurements of DNA mismatch rates.** The action of a mutagenic process causes DNA mismatches or insertion/deletion loops which can be visualised as foci in live *E.*

*coli* cells using a fluorescent fusion of the MMR protein MutL-mYPet (Fig. 1A) (30). Because these foci contain multiple copies of MutL (31) they are sufficiently bright for detection in standard fluorescence microscopy snapshots (SI Appendix, Fig. S1). The frequency of MMR foci correlates with average genomic mutation rates, and can therefore be used as a reporter for mutagenesis (30, 32). Automated cell segmentation and tracking was facilitated by constitutive expression of a cytoplasmic mKate2 fluorescent protein. To image single cells in a controlled environment, I used a microfluidic device where cells grow inside channels with a generation time of 42 min under continuous growth medium supply (33) (Fig. 1B). Cell progeny are pushed out of the channels and flow into the waste stream while an individual “mother” cell remains at the end of each channel for long-term observation under constant conditions. Single-cell imaging also avoids any selection biases that complicate population-based mutagenesis assays. Furthermore, by measuring DNA mismatches instead of mutations, this approach directly shows the activity of mutagenic processes, irrespective of whether a mismatch is later converted into a fixed mutation. This strategy is related to the use of mismatch repair deficient strains in previous studies of mutagenesis (20, 34). Because DNA mismatches are rare and transient (30), each experiment typically followed 500–1000 mother cells at a temporal resolution of 3 min/frame for tens of generations.

The abundance of fluorescent MutL-mYPet was stable in cells over the course of the experiment (SI Appendix, Fig. S2), as expected when the photobleaching rate matches the synthesis rate of MutL-mYPet at equilibrium. During unperturbed growth, mismatches occurred randomly (Fig. 1C) with an average rate of  $0.15 \pm 0.015$  per generation (95% CI) (Fig. 1D). This value is in close agreement with independent measurements (32), and with the mutation rate in MutL-deficient cells of  $2.75 \times 10^{-8}$  per nucleotide (20), or 0.25 for the semiconservative synthesis of two  $4.6 \times 10^6$  bp genomes per generation. When completion of mismatch repair was prevented by deleting MutH, the rate of MutL-mYPet foci increased only slightly, but the foci persisted for a longer time, as expected (SI Appendix, Fig. S3). This demonstrates a high detection efficiency of mismatches and shows that unrepairable

mismatches persist until the next round of replication. Although these characterisations encourage quantitative interpretation of MutL foci rates, the following results are based solely on relative changes in the rates over time and between cells. The population-average mismatch rate was constant over time as judged by the flat slope of a linear fit (Fig. 1D). Mother cells continuously inherit the old cell pole, which has been linked to cell ageing effects (35, 33, 36). Interestingly, mutagenesis appeared unaffected by cell ageing at least over a period of 60 generations.

**Rapid increase in mutagenesis after DNA alkylation damage treatment.** Alkylating agents create a range of harmful DNA methylation products, including 7meG lesions that form toxic and mutagenic AP sites, mutagenic O6meG and O4meT lesions that mispair during replication, and cytotoxic 3meA lesions that stall DNA replication (13, 10, 11). The adaptive response increases resistance to DNA alkylation toxicity and mutagenesis (12, 37, 10). It is triggered when the DNA methyltransferase Ada repairs methylphosphotriester and O6meG lesions by directly transferring the aberrant methyl groups onto its Cys38 and Cys321 residues. Self-methylation at Cys38 turns Ada into a transcriptional activator of its own gene, and the genes encoding for AlkB (dioxygenase/demethylase), and AlkA (3meA DNA glycosylase II). The constitutively expressed repair proteins Ogt (O6meG DNA methyltransferase II) and Tag (3meA DNA glycosylase I) provide basal protection against alkylation damage.

To measure real-time dynamics of mutagenesis, I introduced the DNA alkylating agent methyl methanesulfonate (MMS) into the microfluidic chip by reversibly switching the growth medium supply during data acquisition (Fig. 1E). In single cells, mismatches frequently occurred during or shortly after the 1-hr period of MMS treatment (Fig. 1E). Strikingly, the population-average mismatch rate increased almost instantly (<0.5 generations) upon MMS addition (Fig. 1F, SI Appendix, Fig. S4-5). MMS enters cells quickly and the constitutively expressed BER proteins Pol I and Ligase localise at repair sites after a few minutes of

treatment (38). Despite this immediate repair activity, the rapid increase in the mismatch rate shows that some mutagenic lesions escape repair. Ongoing replication encounters these lesions and directly generates base-pair mismatches, such as O6meG-T or O4meT-G. After MMS removal, the mismatch rate decayed gradually and returned to the basal rate within ~2 generations (Fig. 1*F*).

**Transient DNA repair deficiency causes a mutagenesis pulse.** Remarkably, during constant MMS treatment, the rate of DNA mismatches increased ~4-fold at first, but decreased again after ~2 generations (Fig. 2*A*). In fact, the mismatch rate returned close to the basal undamaged rate despite ongoing MMS treatment. The microscopy approach provides an opportunity to link mutagenesis with an individual cell's phenotype. In particular, it should be possible to correlate the occurrence of DNA mismatches with the expression of DNA repair pathways at that moment. To this end, I utilised a transcriptional reporter for the adaptive response, expressing a fast-maturing CFP fluorescent protein from the *Pada* promoter at an ectopic chromosomal locus (24, 39). *Pada*-CFP showed a ~1000-fold increase in response to continuous MMS treatment in the microfluidic chip (Fig. 2*B*). However, half-maximal induction was only reached after a delay of ~3 cell generations on average, with a broad distribution of response activation times across single cells (Fig. 2*B*). We recently discovered that stochastic delays in the activation of the *Ada* response are a consequence of gene expression noise (24). We found that the basal expression of the *ada* gene is so low that many cells do not contain a single *Ada* molecule when they first get exposed to DNA alkylation damage. These cells fail to sense the presence of damage until they produce at least one *Ada* molecule. Basal *Ada* expression follows memoryless Poissonian statistics, which means that some cells accumulate lesions for many generations before inducing the adaptive response.

The observation of a mutagenesis pulse during constant damage treatment is consistent with this conclusion, as the pulse duration of ~3 generations (Fig. 2*A*) matches the average



delay of the adaptive response (Fig. 2B). To further test this model, I measured mismatch rates in an Ada-deficient ( $\Delta ada$ ) strain and observed the same initial increase in mismatch rates as in wild-type cells but not the subsequent decrease (Fig. 2C). Instead, mismatch rates continued to rise gradually in  $\Delta ada$  cells. Therefore, accumulation of unrepaired lesions during a temporary repair deficiency leads to a spike in the creation of DNA mismatches. The spike terminates once the Ada response induces sufficient repair capacity.

**Linking the dynamics of mutagenesis and gene expression in single cells.** I had previously observed that mismatches were elevated in cells with low Ada expression in microscopy snapshots after a fixed time of MMS treatment (24). Here, using continuous imaging, I was able to relate the precise timing of mismatch events to the gene expression dynamics in single cells (Fig. 2D, SI Appendix, Fig. S6). Mismatches frequently occurred during the time between addition of MMS and induction of the Ada response (Fig. 2D, SI Appendix, Fig. S6). Quantification of this observation showed that mismatch rates were negatively correlated with the Pada-CFP level ( $R = -0.21$ ,  $p = 2 \times 10^{-6}$ ) and positively correlated with the delay time of the Ada response ( $R = 0.25$ ,  $p = 2 \times 10^{-8}$ ) in single cells (SI Appendix, Fig. S6). To measure how mutation dynamics differ before and after Ada activation, I categorized cells into two classes, those that had activated Ada expression (Ada-on) and those that had not yet activated (Ada-off) (Fig. 2D). After MMS addition, mismatch rates decreased in Ada-on cells, but continued to increase in Ada-off cells (Fig. 2E). The mismatch rate curves of Ada-off and  $\Delta ada$  cells were almost identical during the Ada delay period (Fig. 2E). In other words, cells have high mismatch rates until they activate the protective Ada response and lower mismatch rates afterwards. Random variation in the response activation time thus results in mutation rate heterogeneity across cells.

**Cell survival correlates with induction of repair.** Using single-cell microfluidics, it was possible to directly observe cell fates and their determinants in response to DNA damage. MMS exposure caused different types of cell death, including sudden growth arrest, gradual decay of the growth rate, lysis, or prolonged filamentation that lead to disappearance of the mother cell from the growth channel (SI Appendix, Fig. S7). The fraction of surviving wild-type cells gradually decayed during constant MMS treatment (Fig. 3A). Survival of  $\Delta ada$  cells begins to drop below the wild-type after ~2 generations of treatment (Fig. 3A), matching the time at which Ada expression first starts to increase (Fig. 2B). To test the influence of Ada response heterogeneity on cell fates, I categorized cells into Ada-On and Ada-Off subpopulations according to their P<sub>ada</sub>-CFP expression (as in Fig. 2D, SI Appendix, Fig. S8), and found that Ada-Off cells had lower survival than Ada-On cells (Fig. 3A). Therefore, random phenotypic variation in the abundance of Ada temporarily has the same consequences for MMS toxicity (Fig. 3A) and mutagenesis (Fig. 2E) as a genetic deletion of the Ada response.

**Constitutive and inducible repair shape the dynamics of mutagenesis.** These findings raise the question if cells rely on constitutively expressed DNA repair mechanisms when activation of the inducible repair genes is delayed. *E. coli* has a second methyltransferase, Ogt, which is expressed constitutively and reverts the same mutagenic O<sub>6</sub>meG lesions as Ada (Fig. 3B) (10, 13). In a  $\Delta ogt$  strain, Ada response activation was unaffected (SI Appendix, Fig. S9), but mismatch rates increased more rapidly after MMS addition and the peak of the mismatch pulse was elevated compared to wild-type (Fig. 3C). This deviation was temporary, as mismatch rates of  $\Delta ogt$  cells dropped to the wild-type level after ~1 generation and exactly matched the wild-type dynamics thereafter. Hence, the constitutive activity of Ogt prevents a sudden increase in mutagenesis after exposure to alkylation damage. But Ogt becomes dispensable as soon as Ada is induced.

Two alternative glycosylases can initiate the repair of replication-stalling 3meA lesions (Fig. 3B) (13, 10). Tag glycosylase is expressed constitutively whereas expression of AlkA glycosylase is initially low but induced by the adaptive response (13). Deletion of Tag and AlkA strongly increased mismatch rates (Fig. 3D-E), but the dynamics were very different for the two strains. For  $\Delta tag$  cells, mutagenesis occurred in a transient pulse that was 2-fold stronger than in the wild-type, but the mismatch rate was only slightly above the wild-type level when the pulse had terminated (Fig. 3D). In contrast, a distinct pulse was absent in  $\Delta alkA$  cells, for which mismatch rates continued to rise over time (Fig. 3E), similar to  $\Delta ada$  cells (Fig. 2C). The distinct chronology in the activity of Tag and AlkA thus resembles that of Ogt and Ada, reflecting a key function of the constitutive genes in maintaining genome stability until the delayed activation of the inducible genes.

Deletion of Ogt and Tag affected not only the height of the mutagenesis pulse, but also its timing. For  $\Delta ogt$ , the pulse peaked ~0.5 generations earlier than in wild-type cells, whereas the peak in  $\Delta tag$  cells occurred ~0.8 generations later than in the wild-type (Fig. 3C-D, SI Appendix, Fig. S10). This indicates that failure to revert O6meG lesions ( $\Delta ogt$ ) immediately results in DNA mismatches, whereas persistent 3meA lesions ( $\Delta tag$ ) lead to the formation of mismatches more slowly, potentially following replication stalling and mutagenic lesion bypass.

**Cell survival reflects the chronology of constitutive and inducible repair.** The dynamics of cell killing were also informative about the chronology of the constitutive and inducible repair pathways. There was little effect of Ogt deletion on cell survival, consistent with its role in repairing mutagenic, but not toxic lesions (Fig. 3F). This was not the case for Tag, deletion of which resulted in two distinct phases of cell killing (Fig. 3G). The first 3 generations of MMS treatment rapidly killed 25% of  $\Delta tag$  cells, but the remaining cells exhibited the same low mortality as wild-type cells. These  $\Delta tag$  survivors had elevated Ada expression, whereas cell death was associated with lack of Ada expression, as shown by categorising cells into

Ada-On and Ada-Off subpopulations (Fig. 3G). Therefore, the constitutive repair activity of Tag is crucial for the survival of the 25% of cells that fail to activate the Ada response quickly. In contrast, there was no difference in the survival of Ada-On and Ada-Off subpopulations for the  $\Delta alkA$  strain (Fig. 3H). Hence, it is the induction of AlkA that increases cell survival after activation of the adaptive response. In fact,  $\Delta alkA$  cells were more sensitive to MMS than  $\Delta ada$  cells (which cannot induce *alkA*), confirming that AlkA contributes to survival even at its low basal expression level, because it has a broad substrate specificity for removal toxic alkylation lesions that are not recognised by Tag (13, 10).

**Rapid induction of the SOS response is crucial for cell survival.** Replication stalling at unrepaired alkylation lesions triggers the induction of DNA damage tolerance pathways by the SOS response (10, 14, 18). Damage tolerance enables replication progression at the cost of replication fidelity, an important trade-off that has attracted much research attention (27, 16, 40, 9). Compared to wild-type cells, MMS rapidly killed the majority of cells in a *lexA3*(Ind<sup>-</sup>) mutant strain that cannot induce the SOS response (Fig. 4A), but the remaining ~10% of surviving cells after ~6 generations of MMS exposure exhibited lower death rates. This tail of long-lived survivors disappeared in a *lexA3*  $\Delta ada$  double mutant (Fig. 4A), showing that a functional Ada response is able to rescue a subpopulation of SOS-deficient cells. In fact, by measuring Pada-CFP in the *lexA3* strain, I found that Ada-On cells alone were responsible for the survivor subpopulation in the *lexA3* strain (Fig. 4B). Ada-Off *lexA3* cells showed the same high mortality as the *lexA3*  $\Delta ada$  double mutant (Fig. 4B).

To follow the dynamics of SOS gene expression, I constructed a fluorescent reporter plasmid expressing a fast-maturing CFP variant from the *PdinB* promoter. Here, DNA translesion synthesis polymerase Pol IV (*dinB*) serves as a general reporter for the whole SOS regulon, comprising numerous DNA repair and damage tolerance genes that are induced to promote survival and mutagenesis in response to replication stalling or DNA

double strand breaks (17, 41, 42). PdinB-CFP showed clear fluorescence signal in undamaged cells, and a rapid induction in response to MMS treatment that was almost uniform in all cells, in stark contrast to the delayed stochastic induction of Pada-CFP (Fig. 4C). PdinB-CFP reached steady-state induction levels within ~2 generations of MMS treatment (Fig 4C), which mirrors the sudden loss of viability in the *lexA3* strain. Therefore, whereas the adaptive response supports survival after prolonged damage exposure, induction of the SOS response is necessary for immediate survival of DNA alkylation damage.

The SOS and Ada responses are controlled by separate transcription factors, LexA and Ada, through independent biochemical mechanisms. However, considering that the amount of DNA damage determines the strength of both responses, it is possible that their expression levels are coordinated. On the one hand, any variation in the level of DNA damage per cell could produce a positive correlation between SOS and Ada expression. On the other hand, strong induction of one response might reduce damage levels so that the other response becomes less induced, causing a negative correlation. To test which of these scenarios is true, both responses were measured simultaneously in the same cells using the PdinB-CFP reporter plasmid and a functional endogenous Ada-mYPet fusion characterised previously (24). Surprisingly, there was no correlation between PdinB-CFP and Ada-mYPet expression after MMS treatment ( $R = -0.02$ ,  $p = 0.6$ ) (SI Appendix, Fig. S11). In agreement with this observation, SOS expression did not decrease after Ada activation (Fig. 4C). A possible explanation is the fact that overexpression of Ada and BER enzymes is toxic (11, 24). Although high repair rates will remove replication-blocking lesions efficiently, this can also cause SOS induction through the formation of DNA double strand breaks when replication forks encounter gapped excision repair intermediates (43).

**Mutagenesis is correlated with SOS expression level.** Considering that SOS induction was essential for immediate survival of MMS damage, the observed pulse of mutagenesis

(Fig. 2A) could be the combined effect of transient DNA repair deficiency together with SOS induction of mutagenic damage tolerance pathways. Although PdinB induction occurred almost simultaneously in all cells within ~1 generation of MMS treatment, the magnitude of the SOS response varied randomly between cells and fluctuated over time (Fig. 4C-D). A small fraction of cells (~3%) showed elevated SOS expression even before MMS treatment. These observations are consistent with previous single-cell studies that showed cellular heterogeneity in the expression of SOS-controlled genes (23), spontaneous triggering of the SOS response in a subpopulation of cells (44), and SOS expression pulses after UV irradiation (45). These studies left open the important question whether heterogeneity in the SOS response impacts mutation rates in single cells, which can now be addressed using my time-resolved measurements. Indeed, mismatches often coincided with peaks of SOS expression during continuous MMS treatment (Fig. 4D). Overall, cells with higher SOS expression showed more mismatches than cells with lower SOS expression (Fig. 4E). There was a highly significant positive correlation between mismatch rates and SOS expression during the first 5 generations after MMS addition ( $R = 0.30$ ,  $p = 1.4 \times 10^{-11}$ ; Fig. 4E), in agreement with the long-standing model that the SOS response is associated with DNA damage-induced mutagenesis. Furthermore, phenotypic heterogeneity in the magnitude of the SOS response correlates with variation in the rates of mutagenesis across cells.

In contrast to the positive correlation between SOS expression and mismatch rates during the early response (Fig. 4E), there was little correlation later (>5 generations) after MMS addition ( $R = 0.12$ ,  $p = 0.02$ ; SI Appendix, Fig. S12). Evidently, fluctuations in SOS expression do not significantly affect mutagenesis once the Ada response is activated. Interestingly, the mismatch rate drops to the basal rate while SOS expression stays constant after Ada induction (Fig. 2A, Fig. 4C). Indeed, elevated SOS expression only causes mutagenesis in the presence of DNA lesions (40), and several layers of regulation appear to control the access of error-prone TLS Pols to replication forks (16, 46–48).

**Pol IV and Pol V promote cell survival but role in mutagenesis is undetected.** DNA translesion synthesis polymerases Pol IV (*dinB*) and Pol V (*umuDC*) have been shown to function in error-free and error-prone bypass of alkylation lesions, respectively (15, 49). As expected, cell survival during MMS treatment was mildly impaired in strains where either or both polymerases were deleted (Fig. 5A). Combining these deletions with a Tag deletion strongly decreased viability, confirming that TLS is essential when replication-blocking lesions persist without repair (Fig. 5A). The viability of  $\Delta tag$  strains with  $\Delta dinB$  and  $\Delta umuD$  deletions was rescued after ~2.5 generations (Fig. 5A), because AlkA induction by the Ada response compensates for Tag deficiency. Despite the clear impact of Pol IV and Pol V on cell survival, no effect of  $\Delta dinB$  and  $\Delta umuD$  deletions on mismatch rate dynamics could be detected (Fig. 5B). This was also the case for strains carrying additional  $\Delta ada$  (Fig. 5C) or  $\Delta tag$  deletions (Fig. 5D). These experiments are at odds with several genetics studies showing that Pol V deletion lowers MMS mutagenesis, especially when BER or error-free TLS by Pol IV are impaired (41, 15, 49, 50). A possible explanation is that MutL-mYPet foci do not efficiently mark mismatches generated by TLS Pols because they are poorly recognised by MMR. A recent report came to a similar conclusion following an observation that the frequency of MMR foci was lower than expected for a mutator strain that frequently incorporates oxidatively-damaged nucleotides (32).

**Real-time mutagenesis and damage response during antibiotic treatment.** Inflicting DNA damage and blocking DNA replication are successful therapeutic strategies for curing bacterial infections and cancers (1–3). The important class of fluoroquinolone antibiotics inhibit the bacterial type II DNA topoisomerases, which leads to DNA double strand breaks, accumulation of positive DNA supercoiling, and replication stalling at crosslinked topoisomerase-DNA adducts. During therapy, bacteria can evolve de-novo resistance to fluoroquinolone antibiotics by acquiring chromosomal point mutations (51). The SOS response promotes antibiotic-induced mutagenesis leading to resistance (4, 21, 52), and is

responsible for a range of other complications during bacterial infections, such as the spreading of antibiotic resistance genes by horizontal gene transfer and the activation of virulence and persistence (19). The generation of reactive oxygen species by sublethal concentrations of various antibiotics also contributes to mutagenesis (5, 53).

Simultaneous imaging of DNA mismatches, SOS dynamics, and cell survival could provide direct insight into the regulation of mutagenesis in response to antibiotics. After several generations of unperturbed growth in the microfluidic chip, I treated cells with the minimum inhibitory concentration (MIC, 60 ng/μl) of the fluoroquinolone norfloxacin for one hour, which rapidly killed 90% of cells (Fig. 6A). Death rates of the surviving cells after removal of norfloxacin were as low as before the treatment, showing that DNA damages had been efficiently repaired in these cells. Prolonged norfloxacin exposure at a sublethal concentration of ¼ MIC (15 ng/μl) gradually killed all observed cells with a constant death rate of 0.25 per generation (Fig. 6A). Using the PdinB-CFP reporter, I observed the dynamics of SOS response induction during norfloxacin treatment. A transient 5-fold increase in PdinB expression followed a 1-hr pulse of MIC treatment, and a gradual 2-fold increase in expression occurred during constant ¼ MIC treatment (Fig. 6B).

I simultaneously imaged MutL-mYPet foci in the same cells, and found that mismatch rates also increased ~5-fold in response to the MIC treatment pulse, and ~2-fold during constant ¼ MIC treatment, respectively (Fig. 6C). The fold-changes in mutagenesis are in excellent agreement with population- or time-averages of norfloxacin-induced mutagenesis obtained from fluctuation tests or DNA sequencing (5, 21, 54). Strikingly, the dynamics of the mismatch rates precisely matched the induction curves of the SOS reporter for both the MIC pulse and constant ¼ MIC treatment (Fig. 6C). This nearly perfect correlation implicates the SOS response as the key driver of mutagenesis during fluoroquinolone treatment. The observation that mismatch rates increase multiple-fold in less than an hour suggests that SOS-induced mutagenesis may play an important role in the evolution of fluoroquinolone resistance even during brief or low-level treatments.



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## 410 **Discussion**

411 Our current knowledge of mutational mechanisms in cells largely relies on observations that  
412 deletion and over-expression of certain genes affect average mutation rates or mutation  
413 spectra. However, overlapping and pleiotropic functions of DNA repair and replication factors  
414 hinder the interpretation of genetics studies. Single-cell imaging can help to solve the  
415 dilemma by detecting nascent DNA mismatches in relation to the expression levels of  
416 replication and repair factors in wild-type cells. Combining correlation analysis with genetic  
417 and environmental perturbations can then establish causal mechanisms of mutagenesis.  
418 Following the proof-of-principle with alkylation damage and a topoisomerase inhibitor, the  
419 generality of this method opens many opportunities study the mutational mechanisms  
420 underlying other important mutagens, such as UV light and diverse antibiotics.

421 However, my analysis also points at an important limitation of the approach. Not all  
422 mismatches are recognised by the MMR system with the same efficiency (55), and thus may  
423 not be detected as foci using microscopy. The observation that mismatch rates were  
424 unaffected by Pol IV and Pol V deletions is likely a consequence of this detection bias.  
425 Popular mutation reporter assays suffer from a similar constraint, which by design only  
426 detect the small subset of all genome-wide mutations that occur at a defined marker  
427 sequence and that produce a selectable phenotype (22). As a result, mutation rate estimates  
428 using different markers or methods of selection can differ by several orders of magnitude  
429 (49).

430 Although the mismatch detection constraint remains, the microscopy approach circumvents  
431 several other important limitations which make it a useful complementation to existing  
432 mutation assays. Firstly, gene reporter assays and whole genome sequencing both suffer  
433 from selection biases due to the potential fitness effects of deleterious or beneficial  
434 mutations. Secondly, these methods can in principle measure dynamic changes in mutation

rates by sampling from cell populations multiple times. However, in practice, this is limited to relatively few discrete time points and cannot follow real-time mutation dynamics. Thirdly, although single-cell sequencing is under development for resolving mutational heterogeneity, it does not allow repeated analysis of the same cells and accurate DNA amplification remains challenging. By contrast, the microfluidic imaging setup achieves real-time measurements of mutagenesis in individual wild-type cells without selection biases in a high-throughput manner. Further characterisation of the method by direct comparison to established mutation assays will help to resolve current discrepancies and enable conversion of the observed mismatch rates to mutation rates.

With this method, I was able to link the chronology of DNA damage responses with the dynamics of mutagenesis and survival of individual cells (Fig. 7). Although the DNA repair system is tightly regulated, it is also subject to stochastic variations in protein abundances due to gene expression noise. This study demonstrates that robustness against the mutagenic and toxic effects of noise in DNA repair is achieved through the combined activity of constitutive and inducible repair pathways together with damage tolerance. This redundancy ensures a basal damage resistance to compensate for noise and delays in the inducible repair system, whereas loss of constitutive repair or damage tolerance causes severe sensitivity to noise. In the specific case of DNA alkylation damage, constitutive DR prevents a sudden increase in mutagenesis, while constitutive BER and rapid induction of the SOS response are necessary for immediate cell survival after damage exposure. These mechanisms become mostly dispensable when the adaptive response induces additional BER and DR capacity after prolonged damage treatment. However, if constitutive repair or damage tolerance are unavailable, only the subpopulation of cells that is able to activate the adaptive response rapidly will survive. Despite the basal resistance, sudden damage exposure can saturate constitutive repair and cause a distinct pulse of mutagenesis, the duration of which is determined by the stochastic activation time of the adaptive response. Cell-to-cell variation in the number of mutations after DNA alkylation damage treatment was

hypothesised by John Cairns in 1980 (56), and has indeed been observed by whole-genome sequencing (28).

DNA damage tolerance has been described as a strategy of last resort for cells (15), being used only when the repair system becomes overwhelmed after prolonged exposure to DNA damaging agents. For instance, within the SOS regulon, transcription of accurate nucleotide excision repair genes is induced 10 – 20 min earlier than genes encoding for TLS polymerases (17). Using long-term imaging, I found that cells follow the opposite strategy to cope with exogenous DNA alkylation damage, prioritising the continuation of DNA replication over the potentially detrimental consequences of mutagenesis. In fact, the time difference between SOS and Ada induction of >100 min is an order of magnitude larger than differences in the induction of accurate and error-prone pathways within the SOS regulon (17).

Then again, not all mutations are deleterious and increased mutation rates can be beneficial to accelerate genetic adaptation in unfavourable environments (27, 57). In fact, bacterial isolates from infections are frequently found to be hypermutators, for example due to genetic inactivation of mismatch repair (58). Contrary to fixed hypermutation, transient induction of mutagenic pathways allows cells to dynamically regulate mutation rates, which appears to contribute to the evolution of antibiotic resistance (4, 53, 52) and drug resistance in cancers (7, 9). Here, I reported real-time hypermutation dynamics during fluoroquinolone treatment. Even low drug concentrations or brief treatment caused multiple-fold increased mutagenesis within less than an hour of drug exposure. Considering that the mismatch rate dynamics precisely matched the timing of SOS expression, targeting the DNA damage response may indeed be a promising strategy to combat the evolution of antibiotic resistance. Time-resolved data of mutagenesis in single cells will be highly valuable for modelling such evolutionary processes (59) and could guide efforts to devise improved antibiotic treatment regimens that achieve efficient cell killing without inducing mutagenesis.

This is also an important aim for cancer therapy. For example, increased expression of MGMT, the human homologue of Ada/Ogt, is associated with cancer resistance to the alkylating chemotherapy drug temozolomide (TMZ) (60). Intra-tumour genetic variation and phenotypic heterogeneity in MGMT expression are both hallmarks of TMZ-treated tumours and a cause of therapeutic failures (8, 61). These features share similarities with the mutation rate variation and heterogeneous expression of Ada observed in bacteria.

## Materials and Methods

Detailed information of all experimental procedures is provided in SI Appendix. Cell strains and plasmids are available upon request.

**Sample preparation.** Plasmids and *Escherichia coli* AB1157 derived strains were created using standard molecular biology and genetics techniques. Cultures were grown at 37°C in M9 minimal medium containing 0.2% glucose, and supplemented with amino acids, thiamine, and Pluronic F127. The microfluidic single-cell imaging device (“mother machine”) was based on the original design (33), and fabricated according to published procedures (62). Polydimethylsiloxane (PDMS) chips were prepared in two steps, using a negative PDMS mold of the silicon master as an intermediate. PDMS devices were bound to microscope cover slips using air plasma and cells were inserted into the growth channels by centrifugation of the chip in a benchtop centrifuge. Growth medium was continuously flowed into the devices using motorized infusion pumps. For drug treatments, a second syringe loaded with identical growth medium and containing additional MMS or norfloxacin was attached to the same microfluidic channel with a Y-junction.

**Data acquisition.** Time-lapse movies were acquired on a Nikon Ti Eclipse inverted fluorescence microscope equipped with perfect focus system, 100x NA1.4 oil immersion objective, sCMOS camera (Hamamatsu Flash 4), motorized stage, and 37°C temperature chamber (Okolabs). Fluorescence images were automatically collected using NIS-Elements software (Nikon) and an LED excitation source (Lumencor SpectraX) at 3 min intervals. Exposure times were 300 ms for mYPet, 100 ms for mKate2, 75 ms for CFP using 50% LED intensity (SI Appendix, Fig. S1). Typical acquisitions had 50 fields of view containing ~20 growth channels with cells, giving a total of ~1000 cells per experiment.

**Data analysis.** Automated data analysis was performed in MATLAB (Mathworks). Cell outlines were segmented based on the cytoplasmic signal of mKate2. Only mother cells at the end of each channel were included in the analysis. Cell deaths were manually detected when growth ceased, or when time traces terminated abruptly because cell filamentation led to the disappearance of the cell from the growth channel. CFP reporter intensities were calculated from the average pixel intensities inside the segmented cell area and subtracting the background signal outside of cells. MutL-mYPet foci were detected using a spot-finding algorithm (63). When foci persisted for several frames, only the first frame was counted as a mismatch event. Mismatch rates were calculated by dividing the number of observed mismatch events by the observation time interval. I generated cell-average time traces of mismatch rates by dividing the number of mismatch events by the number of observed cells in each frame. Pearson correlation coefficients and p-values were calculated using the corrcoef function. Further data analysis, statistics, and plotting were also performed in MATLAB.

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## Figure Legends

**Fig. 1.** Real-time imaging of DNA mismatch rate dynamics. (A) Nucleotide misincorporation by a mutagenic process results in a DNA mismatch that is bound by fluorescent MutL-mYPet. The mismatch can either be successfully repaired by MMR or turn into a stable mutation during the next round of replication. (B) Single-cell imaging of DNA mismatches using microfluidics. Individual mother cells (black outline) grow and divide at the bottoms of many parallel fluidic channels. Example frame from a time-lapse movie shows MutL-mYPet foci marking DNA mismatches (red circles; thick circle indicates mismatch in a mother cell). Automated cell tracking and foci detection generates a time trace of mismatch events in mother cells. (C) Example time trace showing continuous growth and division of a single

mother cell. The occurrence of DNA mismatch events is indicated by vertical red markers. (D) Mismatch rate time trace, showing the number of mismatch events divided by number of cells observed in each frame, scaled to units of mismatches per generation (average rate of  $N = 526$  cells). Linear fit (black line)  $\pm$  95% CI (shaded background). (E) Cells are treated with DNA alkylation damage by introducing MMS into the microfluidic chip. Two example cell time traces showing cell growth and mismatch events with 10 mM MMS added at time 0 for 1 hr (shaded background). (F) Cell-average mismatch rate with MMS treatment as in panel E ( $N = 591$  cells).

**Fig. 2.** Transient lack of DNA repair capacity causes a pulse of mutagenesis. (A) Mismatch rate dynamics (cell-average) during constant 1.5 mM MMS treatment added at time 0 (shaded background) ( $N = 3529$  cells). (B) Pada-CFP expression with constant 1.5 mM MMS treatment added at time 0. Example time traces of single cells and cell-average (dashed line,  $N = 519$  cells). (C) Mismatch rate dynamics for  $\Delta ada$  strain during constant 1.5 mM MMS treatment added at time 0 (average of  $N = 514$  cells) compared with wild-type. (D) Example time traces showing mismatch events in cells with a rapid and a delayed Ada response with 1.5 mM MMS (Pada-CFP expression: blue curves, mismatch events: red vertical markers). Cells are classified “Ada-Off” and “Ada-On” before and after Pada-CFP induction, respectively. (E) Mismatch rate dynamics with 1.5 mM MMS for wild-type cells in the Ada-Off state (orange) or Ada-On state (green) (classified as indicated in panel D), and  $\Delta ada$  strain (purple). Mismatch rate traces were smoothed using a moving average of 0.7 generations. Below: Number of cells in each category. Initially, all cells are in Ada-Off state and gradually convert into Ada-On state after MMS addition at time 0.

**Fig. 3.** Constitutive and inducible DNA repair shape the dynamics of mutagenesis and cell killing. (A) Distribution of cell survival times during time-lapse imaging in microfluidics without

treatment (wild-type, N = 549 cells) and with constant 1.5 mM MMS treatment (wild-type: N = 529,  $\Delta ada$  N = 547). Wild-type cells were classified as Ada-On or Ada-Off according to Pada-CFP intensity after 2 generations of MMS treatment (see Fig. 2D, and SI Appendix, Fig. S8). (B) Constitutive (Ogt, Tag) and inducible (Ada, AlkA) repair of DNA alkylation lesions. (C-E) Mismatch rate dynamics (cell-average) during constant 1.5 mM MMS treatment added at time 0 for  $\Delta ogt$  (panel C, N = 636),  $\Delta tag$  (panel D, N = 622),  $\Delta alkA$  (panel E, N = 600), compared to wild-type. (F-H) Distribution of cell survival times during constant 1.5 mM MMS treatment for  $\Delta ogt$  (panel F),  $\Delta tag$  (panel G),  $\Delta alkA$  (panel H), with Ada-On and Ada-Off cells classified as in panel A.

**Fig. 4.** SOS induction is crucial for initial survival and correlates with mutagenesis in single cells. (A) Distribution of cell survival times during time-lapse imaging in microfluidics with constant 1.5 mM MMS treatment for wild-type (N = 529), *lexA3* (N = 560), double mutant *lexA3*  $\Delta ada$  (N = 342). (B) Survival time distributions for the boxed area in panel A with *lexA3* cells classified as Ada-On or Ada-Off according to Pada-CFP intensity after 4 generations of MMS treatment (see Fig. 2D, and SI Appendix, Fig. S8). (C) Time traces of PdinB-CFP expression with constant 1.5 mM MMS added at time 0. Overlay of 569 single-cell traces and cell average (dashed line). (D) Example time traces of PdinB-CFP expression (blue) and mismatch events (red vertical markers) with 1.5 mM MMS added at time 0. (E) Positive correlation between mismatch events and SOS response across cells. Each dot shows the mismatch rate and average PdinB-CFP fluorescence per cell during the first 5 generations after MMS addition (N = 410 cells). Open circles: moving average ( $\pm$ SEM).

**Fig. 5** Deletion of Pol IV and Pol V impairs survival but no effect on mutagenesis is detected. (A) Distribution of cell survival times during time-lapse imaging in microfluidics with constant 1.5 mM MMS treatment for wild-type (N = 529 cells),  $\Delta umuD$  (N = 632),  $\Delta dinB$  (N = 635),

double mutant  $\Delta umuD \Delta dinB$  (N = 595),  $\Delta tag$  (N = 622), double mutant  $\Delta tag \Delta umuD$  (N = 593), triple mutant  $\Delta tag \Delta umuD \Delta dinB$  (N = 582). (B) Mismatch rate dynamics (cell-average) during constant 1.5 mM MMS treatment added at time 0 for strains  $\Delta umuD$ ,  $\Delta dinB$ , double mutant  $\Delta umuD \Delta dinB$ , compared to wild-type. (C) Mismatch rate as in panel (B) with double mutant  $\Delta ada \Delta dinB$  (N = 655), and  $\Delta ada \Delta umuD$  (N = 429), compared to  $\Delta ada$ . (D) Mismatch rate as in panel (B) with double mutant  $\Delta tag \Delta umuD$ , triple mutant  $\Delta tag \Delta umuD \Delta dinB$ , compared to  $\Delta tag$ .

**Fig. 6.** Mismatch rates during fluoroquinolone antibiotic treatment match SOS response dynamics. (A) Distribution of cell survival times with norfloxacin (Nor, shaded background) treatment added at time 0 at MIC concentration (60 ng/μl) for 1 hr (left), or constant ¼ MIC (15 ng/μl) (right). (B) PdinB-CFP expression traces in single cells and cell average (dashed line); same cells as in panels A-C. (C) Mismatch rate (raw data: light red, moving average: red) measured together with PdinB-CFP (dashed line, from panel B); same cells as in panels A-C.

**Fig. 7.** Exposure to DNA alkylation damage causes a pulse of mutagenesis that is shaped by the distinct chronology of constitutive and inducible DNA repair and DNA damage tolerance. Mutagenesis increases immediately after damage exposure when unrepaired lesions lead to replication errors and stalling. Constitutive BER and DR mechanisms prevent accumulation of lesions, thereby limiting mutagenesis and cell death. Subsequent activation of the SOS response induces DNA damage tolerance, which promotes continuation of DNA replication and cell survival, but comes at the cost of increased mutagenesis. After several generations of alkylation damage exposure, the adaptive response is activated. Ada increases the expression of accurate repair pathways, leading to a decrease in mutagenesis. As the timing of Ada induction is stochastic, the duration of the mutagenesis pulse varies

779 randomly between cells. Ada activation also rescues survival of cells that lack constitutive  
780 repair or damage tolerance.