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Lighting Up Individual DNA Damage Sites by In Vitro Repair Synthesis

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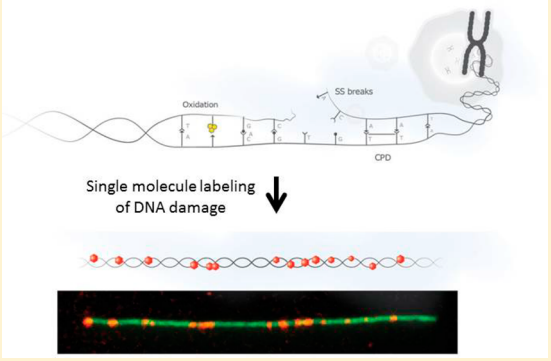
Supporting Information

ABSTRACT: DNA damage and repair are linked to fundamental biological processes such as metabolism, disease, and aging. Single-strand lesions are the most abundant form of DNA damage; however, methods for characterizing these damage lesions are lacking. To avoid double-strand breaks and genomic instability, DNA damage is constantly repaired by efficient enzymatic machinery. We take advantage of this natural process and harness the repair capacity of a bacterial enzymatic cocktail to repair damaged DNA in vitro and incorporate fluorescent nucleotides into damage sites as part of the repair process. We use single-molecule imaging to detect individual damage sites in genomic DNA samples. When the labeled DNA is extended on a microscope slide, damage sites are visualized as fluorescent spots along the DNA contour, and the extent of damage is easily quantified. We demonstrate the ability to quantitatively follow the damage dose response to different damaging agents as well as repair dynamics in response to UV irradiation in several cell types. Finally, we show the modularity of this single-molecule approach by labeling DNA damage in conjunction with 5-hydroxymethylcytosine in genomic DNA extracted from mouse brain tissue.



INTRODUCTION

The study of DNA damage and repair interfaces with most fields of biology and biomedical research. The extent of DNA damage and the capacity of cells to repair this damage are highly correlated with numerous metabolic and disease states.[1] The most pronounced damage, caused by double-strand (ds) DNA breaks, may lead to erroneous chromosomal translocations, deletions and loss of vital genomic information and, thus, to genomic instability and cancer. However, the majority of DNA lesions are manifested at the single-strand level, with a large array of different damage types caused by external stress, such as UV and ionizing radiation, exposure to ambient chemicals, as well as via normal extra- and intracellular metabolic processes. Exposure to UV radiation from the sun is one of the most common sources of DNA damage. UV radiation acts either by promotion of reactive oxygen species (ROS) that oxidize DNA or by directly inducing cyclobutane pyrimidine dimers (CPDs) or the more potent 6-4 photo products. These cross-linked bases can be misinterpreted during transcription and replication to cause mutations and, in severe cases, even complete pausing of replication that induces tension on the replication fork, rendering the DNA susceptible to double-strand breaks.[2,3]

ROS and other reactive agents are able to chemically modify DNA bases to create abnormal DNA lesions, with the most notable being 8-oxo-2′-deoxyguanosine (8-oxodG). This oxidized base can lead to mismatch pairing that in turn promotes conversion of G to T or C to A.[4] At any given time, the average human body sustains an approximated 0.1−100 RO- induced damage events per Mbp.[5] ROS are also produced extensively by the action of drug molecules, such as anticancer agents, by inhalation of reactive chemicals via smoking and during normal metabolic processes by the secretion of bioactive molecules in response to infection, tissue damage, and autoimmune response.[5,6] Quantifying the global levels of DNA damage lesions in the genomes of malignant cells has the potential to emerge as an informative biomarker for determining predisposition to disease, early diagnostics, and assessment of response to therapy. Modulations in the global levels of DNA damage lesions have been shown to correlate with various malignancies but are difficult to quantify in a cost-effective and timely manner.

DNA damage is addressed by diverse and highly efficient enzymatic repair machineries. In the normal state, these repair mechanisms ensure that single-strand damage is not further catalyzed into a double-strand break, and maintain the normal function of the replication and transcription machinery that uses DNA as its template. Single-strand damage is repaired in a process termed repair synthesis, mainly by the nucleotide excision repair (NER) or the base excision repair mechanisms. In both pathways, the damaged DNA is excised, leaving a single

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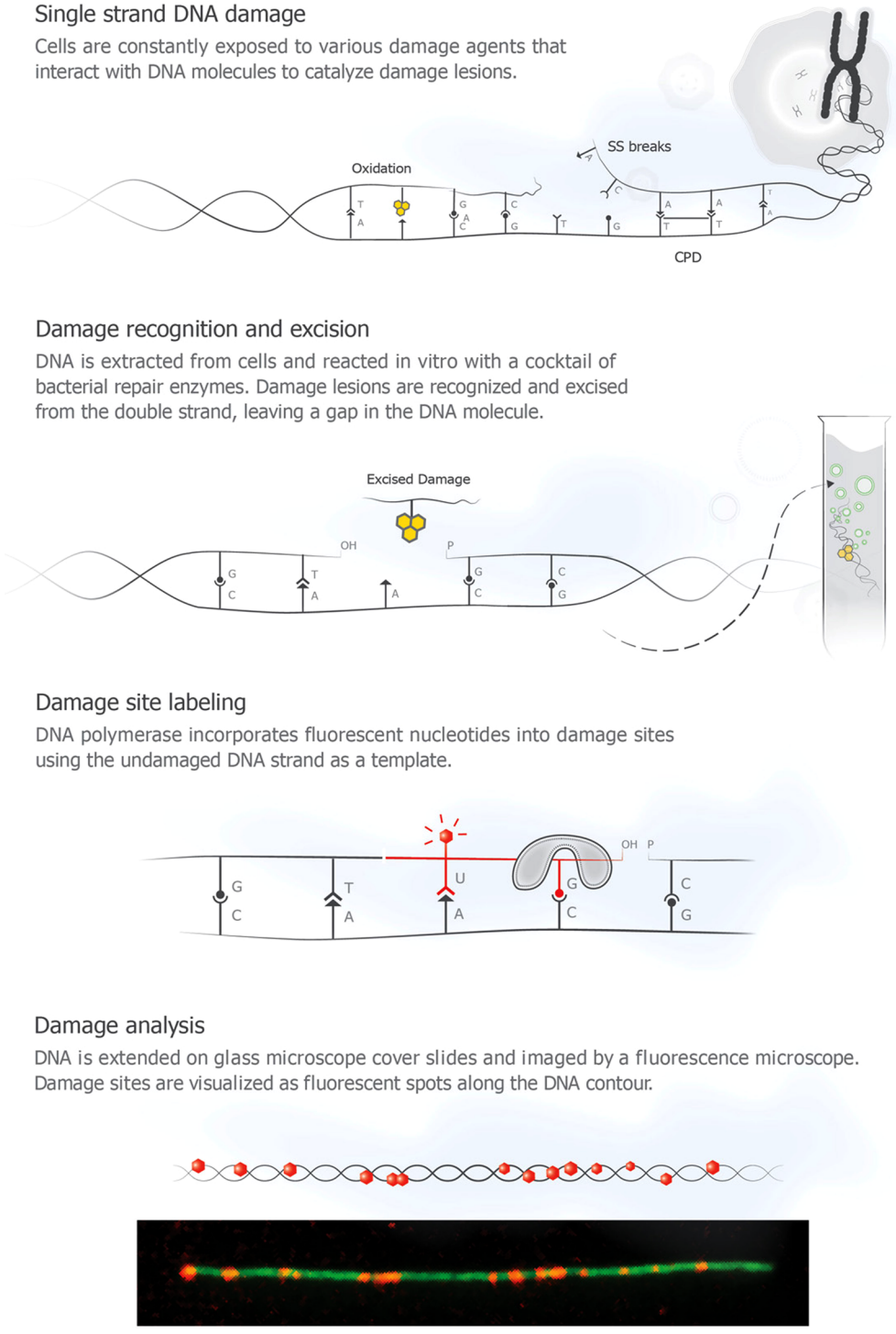

Figure 1. Schematic representation of the reported method. DNA damage lesions are recognized and repaired in vitro. During this process, damage lesions are excised and replaced with fluorescent nucleotides by DNA polymerase. The labeled DNA is extended on glass slides and imaged by a fluorescence microscope to reveal repaired damage sites as fluorescent spots along the DNA contour.

strand gap. The latter is further filled by DNA polymerase, using the opposite strand as a template, followed by ligation by DNA ligase.[7,8]

Malfunction of either of these repair mechanisms is a major cause of several severe disease states. One example is xeroderma pigmentosum (XP), a deficiency in one of the NER repair proteins that leads to inability of cells to repair UV-induced DNA damage.[9] As a result, XP patients must refrain from exposure to sunlight, and in many cases develop melanoma and carcinoma. This disorder affects approximately 1:250,000, and is 6-fold more frequent in the Japanese population.[10] Recent studies have also linked DNA damage and repair levels with the susceptibility and progression of lung cancer and coronary artery disease, pointing out DNA damage as a possible disease marker for diagnostics.[11−14]

Given all of the above, it is clear that information regarding the level of DNA damage, as well as the state of the DNA repair process, is essential for both basic research and clinical

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applications. Although several methods for double-strand breaks detection exist, the ability to assess and quantify the relative abundance of single-strand (ss) damage is very limited, mostly because of a lack of comprehensive techniques that can detect multiple damage types with high sensitivity. In the past decade, several approaches have been developed for in situ optical detection of DNA damage. These include indirect detection by immunostaining of histone gamaH2X (mainly detects ds-breaks) or directly labeling DNA damage with fluorescent antibodies against specific damage lesions, such as 8-Oxo-dG.[15,16]

In addition, several less commonly used approaches have utilized enzymatically assisted labeling of damage sites. These include the detection of DNA nicks in fixed cells via incorporation of biotinylated nucleotides by DNA polymerase or labeling CPDs in lambda phage DNA by the use of pyrimidine dimer glycosylase (PDG).[17−19] ELISA assays have recently emerged as convenient tools to measure ss-DNA damage, with the limitation of being dependent on antibodies against the various damage types, antibodies that in many cases do not exist or exhibit low sensitivity due to inefficient antibody−antigen interaction.[20,21]

All of the above assays address a specific damage type and do not report on the overall damage state of the cell. Other techniques, such as the comet or alkaline unwinding flow cytometry assays, are based on the unwinding of DNA as a result of single- and double-strand breaks.[22−24] These techniques use reagents that induce DNA unwinding proximal to double strand breaks and nicks. As a result, only physical ds- or ss-DNA breaks are detected, and sensitivity to DNA damage adducts that do not directly interfere with DNA integrity is low. Moreover, damage such as CPDs is detected only for sites under active repair in which a ss-DNA gap is enzymatically exposed, resulting in poor sensitivity to such damage types.

In this report, we present a single-molecule approach that takes advantage of natural repair processes performed in vitro to directly label damage sites with fluorescent nucleotides. The labeled DNA is stretched on microscope slides and imaged so that damage sites are detected as fluorescent spots along the DNA backbone. These images are analyzed by measuring the length of DNA molecules and counting the number of spots along the DNA, finally reporting the number of damage sites per Mbp. By harnessing the repair capabilities of a combination of bacterial and viral repair enzymes, our method is capable of detecting a broad range of damage types, limited only by the composition of the repair cocktail. This modular approach also allows tailoring of the assay for specific damage-type recognition. In addition, the ability to count individual damage sites enabled us to quantify the basal level of DNA damage in different cell types. Such low damage levels are not accessible by other techniques and will be required if DNA damage-based diagnostics are envisioned. We used the technique to analyze the extent of damage and the dynamics of DNA repair in human total genomic DNA.

RESULTS

Bacteria have evolved highly efficient DNA repair enzymes compatible with in vitro repair of damaged DNA. Such enzymes are commonly used as reagents for repairing ancient and forensic DNA samples for downstream applications, such as PCR amplification and sequencing. We use such enzymes in combination with DNA polymerase and DNA ligase to incorporate fluorescent nucleotides as part of the repair process (Figure 1).

The labeling procedure relies on the ability of specialized enzymes to recognize specific damage lesions and remove the damaged bases, rendering these sites available for nucleotide incorporation by DNA polymerase.[25] To cover a wide range of damage types that may reflect reliably the damage state of the genome, we use a broad acting enzymatic cocktail composed of bacterial and bacteriophage enzymes (Table 1). These enzymes catalyze the repair of various oxidative DNA damage, such as 8-Oxo-dG,[26−28] CPDs, and 6-4 photoproducts incurred by UV radiation[29] and removal of uracil from DNA.[30] The enzymes are all commercially available and exist also as a premixed cocktail known as PreCR repair mix (New England Biolabs).

Table 1. Composition and Function of the Enzymatic Repair Cocktail Used for in Vitro Repair[a]

enzyme	function
formamidopyrimidine DNA glycosylase (FPG, 8-oxoguanine DNA glycosylase)	recognizes and removes various types of oxidized purines, such as 8-oxoguanine
endonuclease VIII	repairs various types of damaged pyrimidines, including oxidized pyrimidines
endonuclease IV	apurinic/apyrimidinic (AP) endonuclease, repairs oxidative DNA damage
uracil-DNA glycosylase (UDG)	catalyzes the removal of uracil from DNA, such as those caused by deamination of cytosine to uracil
pyrimidine dimer glycosylase (PDG, endonuclease V)	recognizes and removes CPDs and 6-4 photoproducts incurred by UV radiation

[a]These specialized enzymes recognize specific damage lesions and catalyze the formation of apurinic/apyrimidinic (AP) sites (also known as abasic sites) by hydrolysis of the N-glycosydic bond. These AP sites are converted to nicks that leave behind a 3′-OH end and a 5′-phosphate, making them readily available for nucleotide incorporation by DNA polymerase.

We first tested our ability to detect UV damage in total genomic DNA extracts from U2OS cells. We exposed cell culture plates to increasing intensities of UV radiation (20−60 J/m^2) and extracted genomic DNA immediately following irradiation. The purified genomic DNA was treated by the repair cocktail spiked with ATTO-550-dUTP fluorescent nucleotides. DNA was repaired in vitro, stained with the intercalating dye YOYO-1, and then extended on chemically modified glass coverslips and imaged by a fluorescence microscope as previously reported.[31] Typical images are shown in Figure 2a. Images from each data set were analyzed by measuring the length of the DNA molecules and the number of labeled damage sites. Clearly, damage distribution is nonhomogeneous along the genome, and adequate coverage is needed to ensure reliable representation of global genomic damage. We found that depending on damage levels, between 10 Mbp and 30 Mbp of DNA was sufficient for genomic representation. In Figure 2b, we plotted the number of damage events detected for the various doses of UV irradiation, obtaining a linear dose response curve, as expected. Similarly, we incubated cells with physiologically relevant concentrations of hydrogen peroxide to simulate oxidative damage. Again, the response to increasing concentration of H_2O_2 was readily detected as an increase in the number of damage spots on the DNA (Figure 2c).

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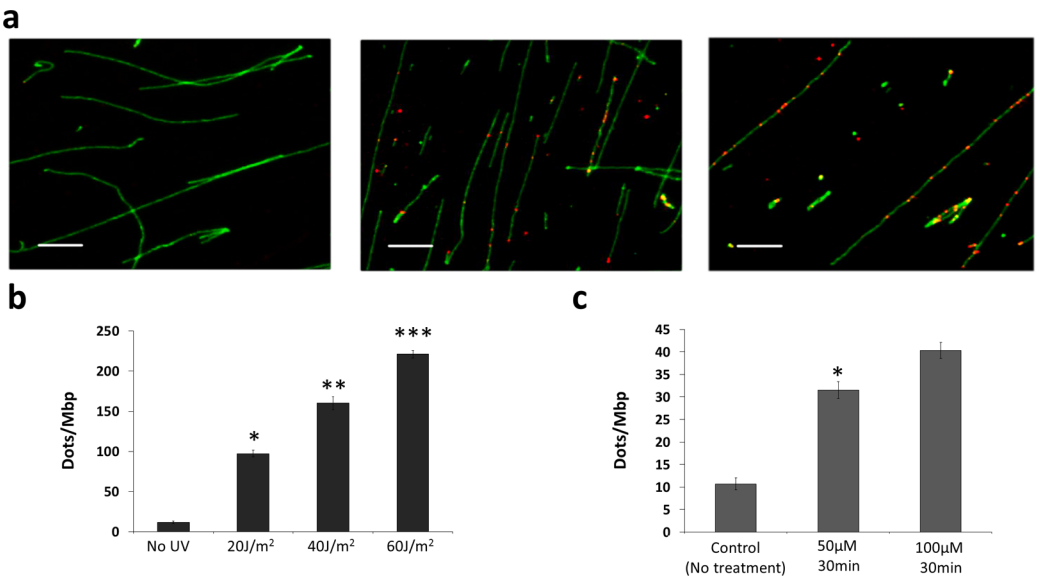

Figure 2. U2OS cells were UV irradiated or exposed to H_2O_2, followed by DNA extraction, damage labeling, DNA extension and fluorescence imaging. Damage was analyzed by counting the number of spots along the DNA molecules. (a) Typical images of labeled DNA from cells exposed to no UV radiation (left), 20 J/m^2 (middle), and 60 J/m^2 (right). DNA stained with YOYO-1 (green) and damage sites labeled with ATTO 550-dUTP fluorescent nucleotide (red). Scale bar = 5 μm. A clear increase in DNA damage levels can be seen as the dose of UV radiation increases. (b) Analysis of UV damage 5 min post radiation of 20, 40, 60 J/m^2. Control cells were not radiated. (*p < 0.01 comparing no UV with 20 J/m^2, **p < 0.01 comparing 20 J/m^2 with 40 J/m^2, and ***comparing 40 J/m^2 with 60 J/m^2). (c) Analysis of H_2O_2 damage. The cells were exposed to 50 or 100 μM H_2O_2 for 30 min; control cells were not exposed to H_2O_2 treatment (*p < 0.01 comparing the control with 50 μM H_2O_2 treatment).

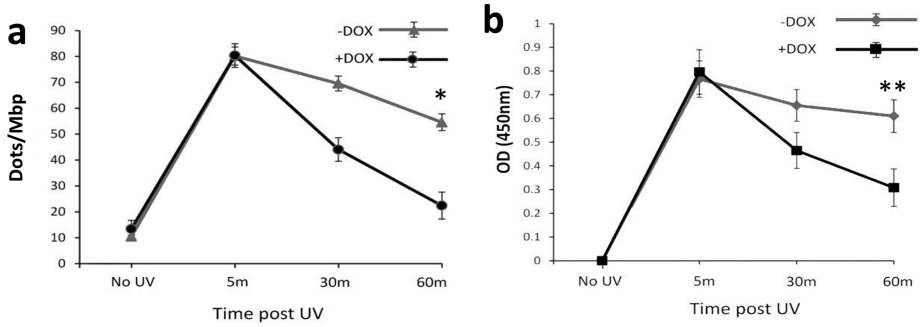

Figure 3. Monitoring DNA repair in PIM-2 overexpressing cells (+DOX) versus control cells (−DOX). The cells were exposed to UV radiation (30 J/m^2), and DNA was extracted at various time points post irradiation (allowing cells to repair the UV induced damage). DNA was further analyzed using our method (a) or a commercial ELISA for CPD detection (b) (*p < 0.01, **p < 0.05).

Next, we validated the utility of our approach to probe DNA repair dynamics. Overexpression of the PIM-2 protein was shown to increase the rate of DNA repair following UV-induced DNA damage.[32] We used a doxycycline-dependent PIM-2 inducible U2OS cell line[33] to follow the rate of UV-damage repair under normal vs overexpression conditions. DNA was extracted at different time points post UV irradiation, and the number of damage spots per Mbp was plotted as a function of time (Figure 3a). As expected, uninduced cells exhibited a decreasing number of damage sites over time, indicating that their repair mechanism was triggered and active. However, cells induced to overexpress PIM-2 showed significantly more efficient repair, visualized as a rapid decline in the number of damage spots detected over time and reaching levels of damage close to nonirradiated control cells after 1 h.

In parallel, we analyzed the same DNA samples using a commercial ELISA kit for detection of CPDs (Figure 3b). The ELISA results closely resembled the data acquired by our single-molecule approach with several noticeable differences. The standard deviation (STD) calculated from the ELISA data was ∼3-fold larger than the STD of the single molecule measurement. In addition, the single-molecule experiments were able to detect the basal levels of damage in nonirradiated cell populations, whereas these were nondetectable by the ELISA kit. This degree of sensitivity and low noise open up possibilities for comparative studies of DNA damage in native tissue and blood samples. It is important to note that the ELISA kit specifically measures the abundance of CPDs and our single molecule technique is also sensitive to 6-4 photo products as well as to the indirect damage caused by UV-induced ROS.

Xeroderma pigmentosum patients are deficient in one of the NER proteins, resulting in the inability of cells to recover from UV-induced DNA damage. We tested the utility of our method to characterize the repair process in a XP patient-derived cell line that lacks the xeroderma pigmentosum (XPA) protein (XPA-). This protein is essential for the normal function of NER, and therefore, UV-induced DNA damage is not expected to be repaired efficiently. To assess the crucial role of XPA in the DNA repair process in this cell line, a gene encoding the recombinant full-length XPA protein was introduced by

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transfection to these cells (XPA+), and the repair dynamics of the XPA- and XPA+ cells in response to a moderate UV dose (20 J/m^2) was compared.

Figure 4a depicts a Western blot, confirming expression of the recombinant XPA in the transfected XPA− cells.

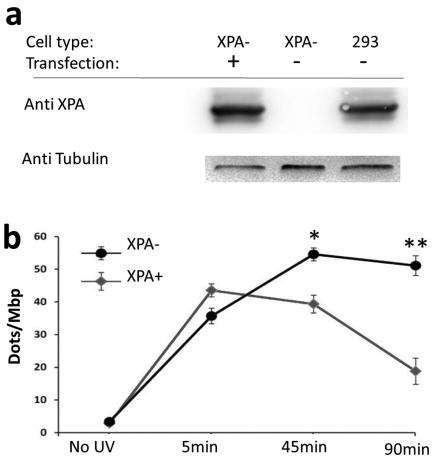

Figure 4. Following DNA repair in xeroderma pigmentosum-derived cells. (a) A Western blot assessing the XPA protein level in a XP120 cell line, which lacks functioning XPA protein (XPA−) compared with XP120 cells, which were transfected with the XPA gene (XPA+) or a HEK-293 cell line. (b) Monitoring of DNA repair in XPA− cells compared with XPA+ cells. The cells were exposed to UV radiation (20 J/m^2), and DNA was extracted at various time points post irradiation. The number of damage signals per Mbp of DNA is plotted as a function of time post irradiation (*p < 0.02, **p < 0.01).

We extracted DNA from the XPA− and XPA+ cell populations, and damage sites were quantified at regular intervals after UV irradiation. The amount of damage was plotted as a function of time post UV irradiation (Figure 4b). At 5 min post irradiation, both cell types exhibited comparable amounts of damage; however, the XPA complemented cells exhibited clear evidence for DNA repair at 45 min post irradiation, whereas the XPA− cells showed no evidence for repair, even 90 min post irradiation. Interestingly, the XPA− cells continued to accumulate damage and showed increased DNA damage from 5 to 45 min post irradiation. We postulate that this damage accumulation may be due to indirect damage promoted, for example, by UV-induced ROS. Such damage would not have been detected by traditional methods, such as the previously used ELISA assay for CPD detection.

Finally, we demonstrate the utility of this method to analyze multiple genomic observables on the same DNA molecule. Genomic DNA was extracted from mouse brain tissue, and the epigenetic mark 5-hydroxymethylcytosine (5hmC) was fluorescently labeled using our recently reported technique.[31] In the next step, the DNA repair cocktail was added to the sample, and damage sites were labeled with a second color using fluorescent nucleotides. DNA images show that it is highly hydroxymethylated, as expected. In addition, damage sites are observed in conjunction with the 5hmC marks and display higher damage levels than the basal damage levels observed in the previous experiments with cultured cells. This preliminary observation may suggest a possible correlation between the levels of 5hmC and ss-DNA damage and highlights the utility of this single-molecule approach for multiplexing (Figure 5).

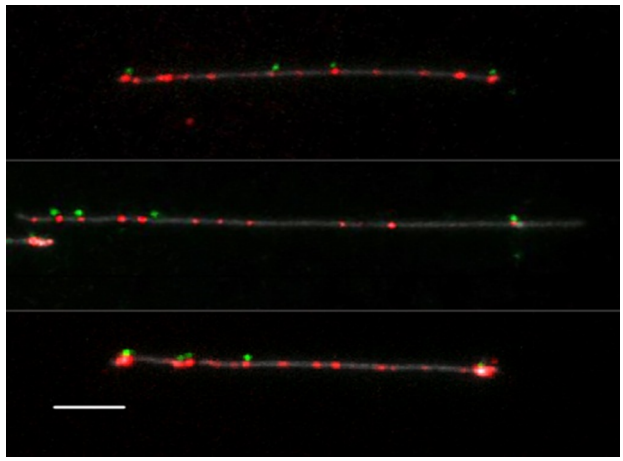

Figure 5. Double labeling of 5hmC and DNA damage sites in mouse brain tissue. DNA stained with YOYO-1 (gray) 5hmC was labeled with cy5 (red), and damage sites were labeled with ATTO 550-dUTP fluorescent nucleotide (green). Green spots were shifted upward for clarity. Scale bar = 5 μm.

DISCUSSION

Single-strand DNA damage is the most common form of damage incurred in DNA and is composed of an array of different damage types. If not repaired rapidly, this damage can extend to double-strand breaks with fatal consequences to the cell. The characterization of single-strand damage is instrumental to our full understanding of DNA damage and repair processes and may have implications in biomedical research and clinical settings. Techniques for analyzing DNA damage are inadequate, and this holds especially true for the analysis of single-strand damage. Current methods for the analysis of such damage are tedious, address only specific damage types, are not sufficiently quantitative, and suffer from low sensitivity. We demonstrate herein a method for direct quantification of single-strand DNA damage by single-molecule analysis. Our in vitro repair assay addresses a broad spectrum of damage types by utilizing the repair abilities of bacterial repair enzymes. Total genomic DNA extracted from cells is repaired with fluorescent nucleotides that are incorporated into damage sites. This labeled DNA is imaged, and damage sites are quantified by image analysis. The method is easy to implement, and results are readily available within several hours. Although our results show that relative amounts of damage can be compared between samples with high sensitivity, the exact efficiency of the labeling procedure relies on the efficiency of the repair enzymes used. This should be further calibrated by use of appropriate control samples to produce absolute quantitative results. Nevertheless, our results indicate that when comparing multiple samples of a similar nature, such as experiments with dose-dependent exposure to specific damaging agents, or dynamics of DNA repair on the same cell type, the relative damage levels detected are highly reliable.

One of the most exciting features of our technique is the ability to resolve multiple observables on the same DNA molecule. For example, damage sites may be correlated with other genomic features, such as epigenetic DNA modifications. In Figure 5, we demonstrate the colocalization of 5hmC (red) and ss-DNA damage (green) on DNA extracted from mouse brain tissue. The relatively high basal levels of DNA damage observed may be related to the high consumption of oxygen by the brain (20%),[34] a fact that may render the DNA more

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susceptible to oxidative damage.[35] In addition, the conversion of methyl cytosine (mC) to 5hmC is in itself an oxidative process that may influence native DNA, especially in 5hmC-rich tissues, such as the brain.[36] Yet another option is that higher 5hmC levels also imply higher activity of thymine DNA glycosylase, which performs excision of demethylated bases and leaves behind an AP site that is detected and repaired with our procedure.

Several optical DNA mapping studies in recent years have demonstrated the ability to define the genetic identity of an observed DNA molecule by fluorescent barcoding. The combination of our technique with such mapping approaches may provide the sequence-specific locus of damage sites to correlate DNA damage (as well as other observables) with the underlying sequence.[37−39] These possible extensions may open up new research avenues for the characterization of DNA damage in the context of genomic information and may find use in clinical diagnostics and personalized medicine.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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