Electronic Supplementary Material (ESI) for Chemical Communications.

Simultaneous detection of multiple DNA damage types by multi-colour fluorescent labelling

Dmitry Torchinsky, [a] Yael Michaeli, [a] Natalie R. Gassman [b] and Yuval Ebenstein [*a]

Supporting information:

Methods:

Cell culture: Human embryonic kidney (HEK 293) (a kind gift from Prof. Micha Fridman at Tel Aviv university) and immortalized human keratinocytes (HaCaT) cells (a kind gift from Prof. Carmit Levy at Tel Aviv university) were grown in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), at 37 ^{0}C in a 5% CO_2 atmosphere.

Exposure of HEK cells to oxidative stress: Cells were plated in 35 mm dishes at 10^6 cells/dish and grown until 70% confluent. Then, 30% hydrogen peroxide (H_2O_2) solution (Bio-Lab) was added directly to the cells' medium at the desired final concentration (0 to 200 µM), mixed gently and returned to the incubator for 15 min. The medium was then aspirated, cells were resuspended in 1 ml Dulbecco's phosphate-buffered saline (DPBS, Sigma) and centrifuged at 1000 g for 3 min at room temperature (~23 ^{0}C). DPBS was aspirated, and the cell pellet was washed again with DPBS followed by DNA extraction and purification (see below). For control samples, the same procedure was performed without adding H_2O_2.

Exposure of HEK cells to UV stress: Cells were plated in 35 mm dishes at 10^6 cells/dish and grown until 70% confluent. Cells were exposed to a range of UVA, UVB and UVC radiation (365 nm, 302 nm and 265 nm, respectively) doses from 0-60 J/m^2 using a UVP 3UV lamp with a power of 4 W/m^2 and controlled exposure times to achieve the indicated dose. For UV exposure, the growth medium was aspirated, and the lid of the plate was removed. Untreated controls were mock treated using the same procedure. Following exposure, cells were re-suspended in growth medium, centrifuged at 1000 g for 3 min and washed once with DPBS buffer (Sigma) prior to DNA extraction and purification (see below).

Exposure of HaCaT cells to UVB stress: Cells were plated in 35 mm at 10^6 cells/dish and grown until 70% confluent. Cells were exposed to UVB radiation (320 nm, Philips) with a power of 1 W/m^2 from 0-1800 J/m^2 or mock exposed as a control. For UVB exposure, the growth medium was aspirated, 100 µL of DPBS was added to the plate to prevent drying under long exposures, and the lid was removed. To monitor repair dyanmics, HaCaT cells were exposed to 1800 J/m^2 UVB, then allowed to repair or recover for the indicated times. For no repair/recovery time, the cells were resuspended in 1 ml of DPBS immediately after the exposure for harvesting. For indicated repair/recovery times, the growth medium was replaced, and cells were placed back into the incubator at 37 ^{0}C and 5% CO_2 for indicated times. All cells were harvested by scraping in 1 ml of DPBS, then centrifuged at 1000 g for 3 min and washed once with DPBS prior to DNA extraction and purification (see below).

DNA extraction and purification: For every experiment, DNA was extracted immediately after exposure or mock exposure. DNA was extracted using GenElute Mammalian Genomic DNA Miniprep kit (Sigma) following the manufacturer's protocol. Shortly, the cells were first lysed using the supplied reagents, then transferred to the provided purification column, washed twice with the washing buffer from the kit and

eluted using hot (65 ^{0}C) 10 mM Tris-HCl buffer pH=8.5. Pipetting steps were minimized to reduce shearing of DNA. All extracted samples were diluted to the same final concentration so that an equal volume is taken for each labelling reaction.

DNA PreCR labelling: 50 ng of extracted genomic DNA was mixed with 3 µL of Thermopol 10X buffer (NEB), 0.3 µL NAD+ 100X (NEB), 0.6 µL PreCR mix (NEB) and DI water up to 30 µL. The mixture was incubated at 37 ^{0}C for 30 min. Then, 0.2 µL of nucleotide mix containing dATP, dGTP, dCTP nucleotides (Sigma) and labelled dUTP-ATTO647N (Jena Bioscience) at a concentration of 10 µM each was added. The reaction was incubated for another 30 min at 37 ^{0}C followed by addition of 1 µL of 0.5 M EDTA pH=8 to stop the reaction. DNA was stored at -20 ^{0}C until analysed.

DNA oxidative damage labelling: 50 ng of extracted genomic DNA was mixed with 1 µL of NEB2 10X buffer (NEB), 0.5 µL hOGG1 (0.8U, NEB), supplemented with bovine serum albumin (BSA, NEB) to a final concentration of 100 ng/µL and DI water up to 10 µL. The mixture was incubated at 37 ^{0}C for 1 h. Then, 0.5 µL Endonuclease IV (5U, NEB) was added, and the reaction was incubated for another hour at 37 ^{0}C. Next, the following were added: 4 µL NEB2 10x buffer, 0.5 µL of NAD+ 100X (NEB), 0.2 µL nucleotide mix containing dATP, dGTP, dCTP nucleotides (Sigma) and labelled dUTP-ATTO647N (Jena Bioscience) at 10 µM each, 0.2 µL Taq DNA Ligase (8U, NEB), 0.2 µL Large Fragment Bst DNA Polymerase (1.6U, NEB), supplemented with BSA to a final concentration of 100 ng/µL and DI water up to 50 µL. The mixture was incubated at 65 ^{0}C for 30 min followed by addition of 3 µL of 0.5 M EDTA pH=8 to stop the reaction. DNA was stored at -20 ^{0}C until analysed.

DNA UV damage labelling: 50 ng of extracted genomic DNA was mixed with 1.5 µL NEB4 x10 buffer (NEB), 0.5 µL T4 PDG (5U, NEB), supplemented with BSA (NEB) to a final concentration of 100 ng/µL and DI water up to 15 µL. The mixture was incubated at 37 ^{0}C for 1 h, followed by addition of 0.5 µL Endonuclease IV (5U, NEB), and the reaction was incubated for another hour at 37 ^{0}C. Next, the following were added: 3.5 µL NEB4 x10 buffer, 0.5 µL of NAD+ 100X (NEB), 0.2 µL of nucleotide mix containing dATP, dGTP, dCTP nucleotides (Sigma) and labelled dUTP-ATTO647N (Jena Bioscience) at 10 µM each, 0.2 µL Taq DNA Ligase (8U, NEB), 0.2 µL Large Fragment Bst DNA Polymerase (2U, NEB), supplemented with BSA to a final concentration of 100 ng/µL and DI water up to 50 µL. The mixture was incubated at 65 ^{0}C for 30 min followed by addition of 3 µL of 0.5 M EDTA pH=8 to stop the reaction. DNA was stored at -20 ^{0}C until analysed.

Labelling of oxidative and UV DNA damage lesions with two distinct colours: For labelling of oxidative damage, 50 ng DNA was mixed with 1 µL NEB4 x10 buffer (NEB), 0.5 µL of hOGG1 (0.8U, NEB), supplemented with BSA (NEB) to a final concentration of 100 ng/µL and DI water up to 10 µL. The mixture was incubated at 37 ^{0}C for 1 h followed by addition of 0.5 µL of Endonuclease IV (5U, NEB) and another hour incubation at 37 ^{0}C. Next, the following were added: 1 µL NEB4 x10 buffer, 0.2 µL NAD+ 100X (NEB), 0.2 µL of nucleotide mix containing dATP, dGTP, dCTP nucleotides (Jena Bioscience) and labelled dUTP-ATTO550 (Jena Bioscience) at 10 µM each, 0.2 µL Taq DNA Ligase (8U, NEB), 0.2 µL Large Fragment Bst DNA Polymerase (1.6U, NEB), supplemented with BSA to a final concentration of 100 ng/µL and DI water up to 20 µL. After incubation at 65 ^{0}C for 30 min, 0.2 µL of Shrimp Alkaline Phosphatase (rSAP, 0.2U, NEB) were added to the sample to remove the phosphate group from the remaining free nucleotides. The mixture was incubated at 37 ^{0}C for 10 min and then at 65 ^{0}C for 5 min.

Next, for labelling UV DNA damage, 0.5 µL of NEB4 x10 buffer, 0.5 µL of T4 PDG (5U, NEB) were added to the mixture, supplemented with BSA to a final concentration of 100 ng/µL and DI water up to 25 µL. The reaction was incubated at 37 ^{0}C for 1 h followed by addition of 0.5 µL of Endonuclease IV (5U, NEB) and another hour incubation at 37 ^{0}C. Next, the following were added: 2.5 µL of NEB4 x10 buffer, 0.5 µL of NAD+ 100X, 0.2 µL of nucleotide mix containing dATP, dGTP, dCTP nucleotides (Sigma) and labelled dUTP-ATTO647N (Jena Bioscience) at 10 µM each, 0.2 µL Taq DNA Ligase (8U), 0.2 µL Large Fragment Bst DNA Polymerase (2U), supplemented with BSA to a final concentration of 100 ng/µL and DI water up to 50 µL. The mixture was incubated at 65 ^{0}C for 30 min followed by addition of 3 µL of 0.5 M EDTA pH=8 to stop the reaction. DNA was stored at -20 ^{0}C until analysed.

DNA backbone staining: Total genomic DNA was stained with YOYO-1 (Invitrogen) at a 4:1 ratio of DNA base-pairs to YOYO-1 molecules with a final concentration of DNA in the staining solution of 150 pg/µL. For staining, 3 ng of DNA from the damage labelling reaction, was mixed with 4 µL of aqueous 2 M DTT solution (Sigma), 0.2 µL of 5 µM YOYO-1 solution in DMSO (Invitrogen) and TE buffer (10 mM Tris, 1 mM EDTA, pH=8.5) in a total reaction volume of 20 µL. The sample was incubated at 45 ^{0}C for 30 min prior to imaging.

Glass coverslips activation: 22x22 mm glass coverslips (Merienfeld) were submerged in freshly prepared piranha solution (1:3 hydrogen peroxide 30% to concentrated sulphuric acid 98%) and incubated for 1.5 h. Next, the coverslips were transferred to a DI water bath and mechanically washed for a couple of minutes before being transferred to a second DI water bath. The coverslips were again washed mechanically for several minutes and then transferred to a third DI water bath and warmed to 85 ^{0}C for 30 min. Then, coverslips were transferred to a technical ethanol 96% (Bio-Lab) bath and mechanically washed for several minutes before being dried with nitrogen. The dried coverslips were immediately submerged in a freshly prepared silanization bath containing a solution of (volume to volume ratios) 10% sec-butyl alcohol (Frutarom), 10% 2-propanol (EMSURE), 10% ethanol (Bio-Lab), 1% v/v N-trimethoxysilylpropyl-N,N,N-trimethylammonium chloride (Gelest), 0.33% v/v vinyltrimethoxysilane (Gelest) in deionized water. The silanization bath was placed in a 65 ^{0}C incubator (Cleaver Hybrid, Scientific ltd) for 1.5 h with circular mixing of 25 rpm and a reverse of direction after every 10 cycles. After silanization, the slides were transferred into DI water bath and mechanically washed as before three times. Finally, the coverslips were transferred to technical ethanol 96% bath, washed mechanically for several minutes and transferred into another technical ethanol 96% bath for storage at 4 ^{0}C. We note that in our hands the improved protocol for glass coverslip activation reduces the total preparation time from three days to around seven hours, improving the homogeneity of the stretching of DNA molecules and the stability of the substrates during storage(43).

DNA sample deposition for imaging: A microscope slide (Color Frosted, Bar Naor) was washed with technical grade acetone, technical ethanol 96% and DI water, then dried with nitrogen. Silanized glass coverslip was washed with technical ethanol 96% and deionized water then dried with nitrogen and placed on top of the cleaned slide. 8 µL of 150 pg/µL labelled DNA was slowly deposited at the edge of the coverslip and drawn between the coverslip and glass slide by capillary forces.

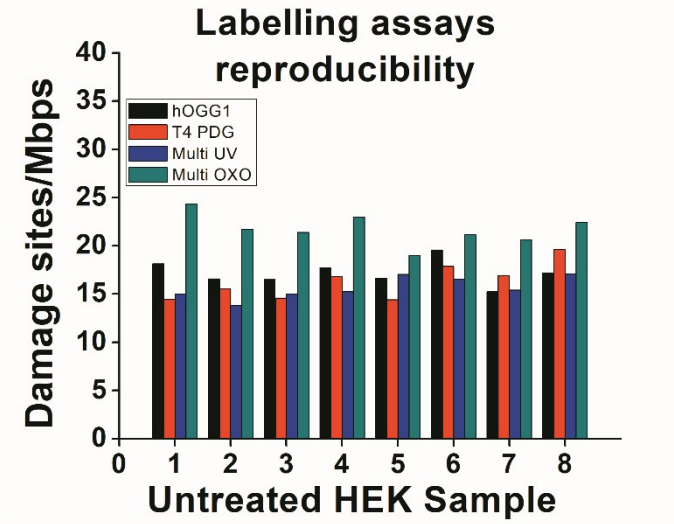
DNA imaging: The slide with the DNA sample was placed in an epi-fluorescence microscope (TILL Photonics) with the coverslip facing the objective. We used 100x oil objective (UPlanSApo, ∞/0.17/FN

26.5/ N.A. 1.4, Olympus) and an EMCCD camera (Ixon3, Andor) for imaging. Multiple fields of views (FOVs) were imaged using an autofocus feature based on infrared reflection from the surface of the sample. In each FOV sequential images were taken to visualize the backbone of the DNA (YOYO-1 ex485/20, em525/30) and the labelled damage sites (ATTO550 ex563/9, em578/16, ATTO647N ex640/14, em684/24).

Image analysis and calculations: Custom developed software (CVI NI, C language), described in our previous paper (43), was used to analyse the DNA images. The software detects DNA molecules and co-localized labels in each of the different channels, finding the number of labels for each channel for every detected DNA molecule. The software uses custom enhancement filtering algorithms and enables restriction on the detected molecules physical properties such as size, intensity, length, width and linearity. The co-localization is based on the geometrical overlap of the shapes from the different channels and can be set by the user to account for only tightly overlapping shapes or loosely overlapping ones as well. The software outputs the physical properties of every detected molecule, as well as the number of its labels in each channel. For each sample, the total number of labels was divided by the entire length of detected DNA molecules to receive a ratio that represents the average labels per pixel for the sample. To convert the length of detected DNA to bps we used a conversion factor of 240 bps/pixel calculated from the experimental magnification (80 nm/pixel) and the theoretical value of 3 bps/nm. To check for convergence of every data set, we calculated the standard deviation of the last 200 points of the cumulative ratio of damage sites to sampled DNA length and verified it is less than 1%. Samples with results that did not converge, due to an insufficient amount of sampled data, were reimaged. To evaluate our experimental error we performed sets of identical experiments (N=8) for each assay and derived the percent error for the DNA damage estimation (see Fig. S1).

Error calculation for various labelling assays

For every labelling assay described in the manuscript, we calculated the error in DNA damage estimation by conducting multiple reactions on native DNA extracted from untreated cells. These multiple measurements of the basal DNA damage level allowed us to calculate the variability between reactions representing the same biological condition. As we expect to get the same damage levels for all the reactions, we calculated the average damage levels for these reactions and the standard deviation. Using the ratio between these two values, we derive the estimation error percentage for each assay. The percent error for each assay was used to calculate the error for each data point in the following experiments. Figure S1 summarizes the results received for the T4 PDG assay, hOGG1 assay and the multi-labelling assay.



	H2O2	T4 PDG	Multi UV	Multi OXO
Sample 1	18.1	14.4	15.0	24.4
Sample 2	16.6	15.5	13.8	21.7
Sample 3	16.5	14.6	15.0	21.4
Sample 4	17.7	16.8	15.2	23.0
Sample 5	16.6	14.4	17.0	19.0
Sample 6	19.5	17.9	16.5	21.1
Sample 7	15.2	16.9	15.4	20.6
Sample 8	17.2	19.6	17.1	22.4
Avg	**17.2**	**16.3**	**15.6**	**21.7**
Std	**1.2**	**1.8**	**1.1**	**1.5**
RE	**7.0%**	**10.8%**	**6.8%**	**6.9%**

Figure S1. Labelling assays reproducibility. Comparison of the native Damage levels as detected by the individual reactions for labelling oxidative lesions and photoproducts and the multi-labelling reaction. The data represents the native levels detected in eight untreated HEK samples where black data represents hOGG1 results, red data represents the results of the T4 PDG reaction and blue and green data represents the photoproducts and oxidative damage detected by the multi-labelling reaction respectively. The table summarizes the average labelling efficiency (Avg) of the eight samples, the standard deviation (Std) of the eight samples and the relative estimation error (RE) in percentage.

Specific labelling of photoproducts and oxidative DNA damage

To facilitate the distinction between damage types via labelling with different colours we first optimized two individual reactions for labelling oxidative damage and photoproducts. As a model for photoproducts, we used HEK cells exposed to either UVA, UVB or UVC radiation for increasing periods of time, resulting in an increasing dose of UV radiation. Oxidative damage was induced by exposing HEK cells to increasing concentrations of hydrogen peroxide. We used the PreCR repair mix (NEB), a broad spectrum lesion processing cocktail formulated to repair damaged template DNA, to label both oxidative and photoproduct damage by incorporating fluorescent nucleotides during the *in-vitro* repair process[1]. We developed optimized enzyme cocktails for labelling specific damage types. Labelling of photoproducts was performed with pyrimidine dimer glycosylase (T4 PDG), an enzyme that recognizes cis-syn-cyclobutane pyrimidine dimers caused by UV irradiation[2]. Oxidative damage was specifically labelled using human 8-oxoguanine DNA glycosylase (hOGG1), an enzyme that releases damaged purines from double stranded DNA[3].

For every experiment, a control DNA sample extracted from untreated cells served to evaluate the endogenous DNA damage baseline. After damage labelling, without further cleaning, the backbone of the DNA was stained and then stretched on modified glass slides for imaging. Data were analysed using

custom software. Damage labelling for either oxidative lesions or photoproducts for each individual DNA sample was measured and compared to the overall damage levels detected using the PreCR mix. Figure S2 shows the response of HEK cells to increasing doses of various types of UV radiation.

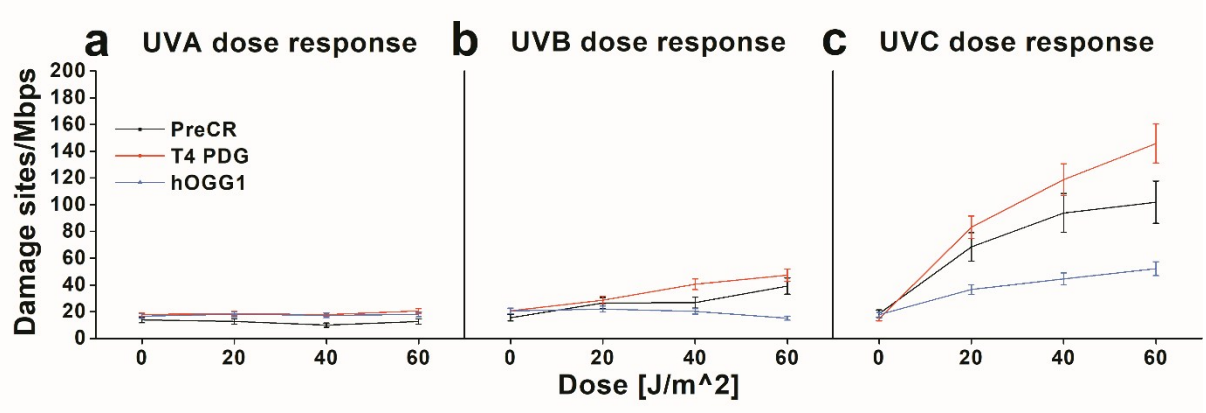


Figure S2. Quantification of DNA damage in HEK cells exposed to increasing doses of various types of UV radiation. (a) Dose response of HEK cells exposed to UVA radiation. (b) Dose response of HEK cells exposed to UVB radiation. (c) Dose response of HEK cells exposed to UVC radiation. In each graph, red data represents the amount of photoproducts as detected using the T4 PDG reaction, blue data the amount of oxidative damage as detected with the hOGG1 reaction, and black data the overall damage levels received using the PreCR reaction.

As expected, more DNA lesions were induced by shorter wavelength, higher energy UV. Both increased photoproducts and oxidative damage were observed with UVC in a dose-dependent manner (Figure S2c). UVB radiation showed only a slight increase in photoproducts detected by the T4 PDG reaction, while no increase in oxidative damage detected by the hOGG1 reaction, which is in correlation with a slight increase in overall damage levels observed by PreCR. The UVA radiation produced no detectable increase in any of the damage types measured over the dose range tested and is in agreement with the PreCR cocktail.

To validate our individual labelling reactions for oxidative lesions, we analysed HEK cells exposed to increasing doses of H_2O_2 and compared our results to the PreCR reaction. Figure S3 summarizes the results.

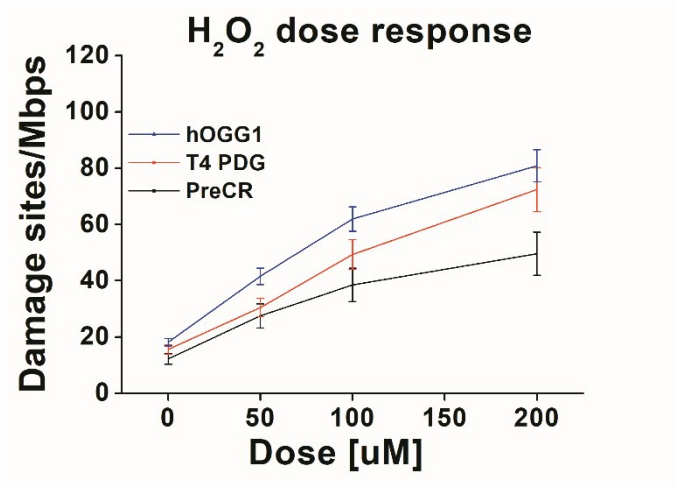
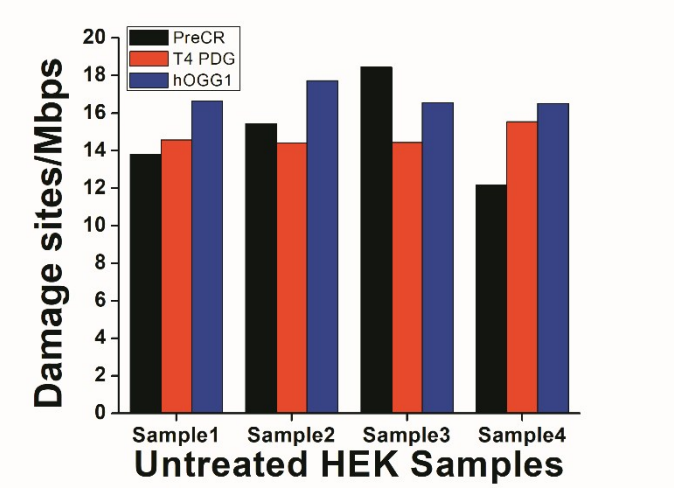


Figure S3. Quantification of DNA damage lesions in HEK cells exposed to increasing doses of hydrogen peroxide. Red data represent the results of the individual reaction for labelling photoproducts, blue data the results of the individual reaction for oxidative lesions and black data the PreCR reaction.

We see that there is an increase in damage levels detected by both the individual reaction for oxidative lesions and the PreCR reaction as expected. An interesting finding was that T4 PDG reaction optimized for photoproduct repair was efficient at repairing oxidative damage induced by hydrogen peroxide. This implies that the T4 PDG reaction is not specific for photoproducts but rather labels oxidative damage as well. Oxidative labelling by T4 PDG is likely due to the presence of Endonuclease IV in the reaction, which is known to detect and excise apurinic/apyrimidinic sites (AP sites) and 5,6 dihydrothymine base paired with an adenine (DHT:A)[4,5]. Moreover, excluding the Endonuclease IV from the T4 PDG reaction drastically decreases its efficiency, despite its known AP-lyase activity (see Fig. S7).

Baseline damage levels of various labelling reactions

We compared the native damage levels of four untreated HEK samples with the two individual reactions for labelling oxidative lesions and photoproducts and the PreCR reaction. Figure S4 summarises these results.



	PreCR	T4 PDG	hOGG1
Sample1	13.8	14.6	16.6
Sample2	15.4	14.4	17.7
Sample3	18.4	14.4	16.6
Sample4	12.2	15.5	16.5
Avg	15.0	14.7	16.9
Std	2.3	0.5	0.5
RE	15.5%	3.2%	3.0%

Figure S4. Comparison of the native Damage levels as detected by the individual reactions for labelling oxidative lesions and photoproducts and the PreCR reaction. The data represents the native levels detected in four untreated HEK samples where black data represents PreCR results, red data represents the results of the individual reaction for labelling photoproducts and blue data represents the results of the individual reaction for labelling oxidative lesions. The table summarizes the average labelling efficiency (Avg) of the four samples, the standard deviation (Std) of the four samples and the relative estimation error (RE) in percentage.

The results show that the reproducibility of the two individual reaction is higher than the PreCR. Moreover, judging by the levels detected by the individual reactions as compared to those of the PreCR, we see that the efficiency of the individual reactions is higher. This difference in labelling efficiency is likely due to the fact that the PreCR mix has various enzymes at unknown ratios and concentrations, making it difficult to optimize the reaction conditions, while in the case of our assays we optimized each and every step to get the highest labelling efficiency possible.

Fpg non-specific labelling

We analysed HEK cells exposed to UVB and UVC irradiation as well as to H_2O_2 using the Fpg enzyme from the commercial PreCR mix. For all analysed samples we got high non-specific labelling even for the untreated samples as can be seen in Figure S5.

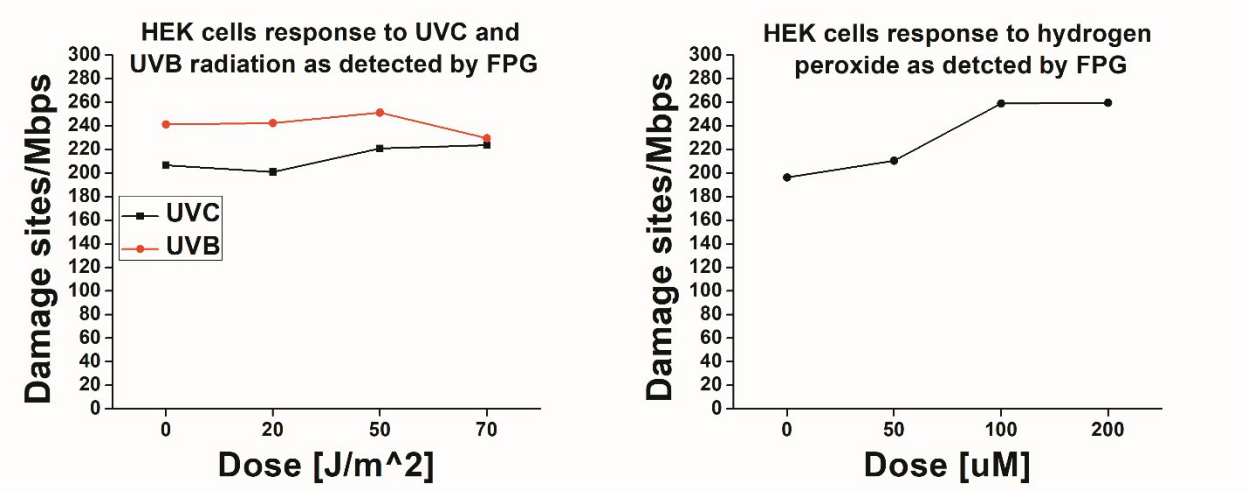


Figure S5. HEK cells response to UVB and UVC irradiation as well as to hydrogen peroxide as detected by the Fpg enzyme.

End labelling of various polymerases

A ~12.5 kbp pNCS plasmid was linearized by cutting the dsDNA at a single location using the XhoI restriction enzyme. Then four different DNA polymerases [Bst. Full Length (NEB), Bst. Large Fragment (NEB), Polymerase I - Large (Klenow) Fragment (NEB), Vent exo- (NEB)] and T4 DNA ligase were used to perform a labelling reaction with only a mixture of dATP, dGTP and dCTP nucleotides (Sigma) and labelled dUTP-ATTO647N nucleotides (Jena Bioscience). Because the plasmid DNA is untreated and hence is intact, as well as we don't use any DNA damage repair enzymes in this labelling, we expect no labelling to occur. In practice, labelling does occur at the ends of the DNA molecules and its amount is dependent on the type of the polymerase used. For each reaction, we analysed the non-specific labelling ratio and the results are summarized in Figure S6.

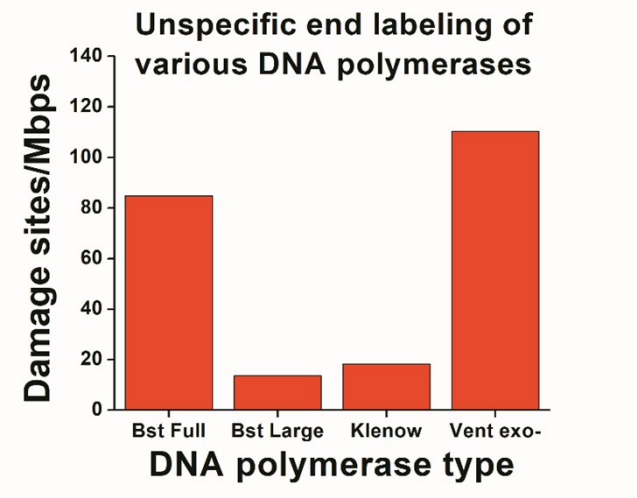


Figure S6. A 12 kbp plasmid labelled using a mixture of dATP, dGTP, dCTP and dUTP-ATTO647N nucleotides and different DNA polymerases in the presence of DNA ligase.

These results show that the lowest non-specific labelling is achieved using the Bst. Large Fragment DNA Polymerase. Moreover, in our hands the vast majority of the non-specific labelling occurred at the ends of the molecules, with negligible amount of labels being detected at the centre of the molecules. To reduce the non-specific labelling to the minimum, we chose the Bst. Large Fragment DNA Polymerase for conducting our DNA damage labelling reactions.

T4 PDG reaction efficiency

HEK cells exposed to UVC irradiation were analysed using the T4 PDG enzyme with and without the use of Endonuclease IV. Figure S7 summarizes results.

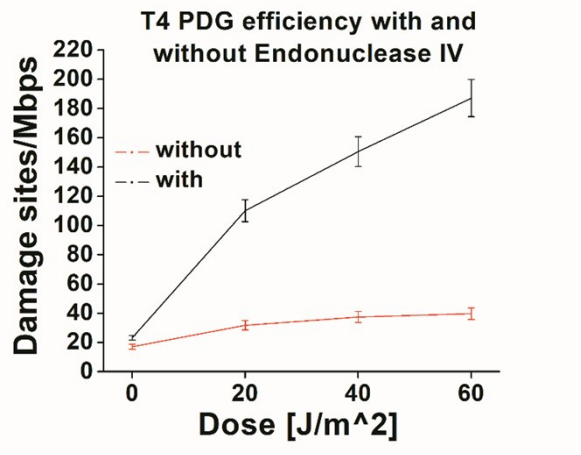


Figure S7. Quantification of DNA damage lesions in HEK cells exposed to UVC irradiation as detected by the T4 PDG enzyme in the presence and absence of Endonuclease IV. Black data represents the damage levels received with the Endonuclease IV enzyme and the red data without its use.

It is clearly visible from the results that in the absence of Endonuclease IV the labelling efficiency of the T4 PDG reaction drastically drops.

Full field of view image analysis for photoproducts and oxidative DNA damage

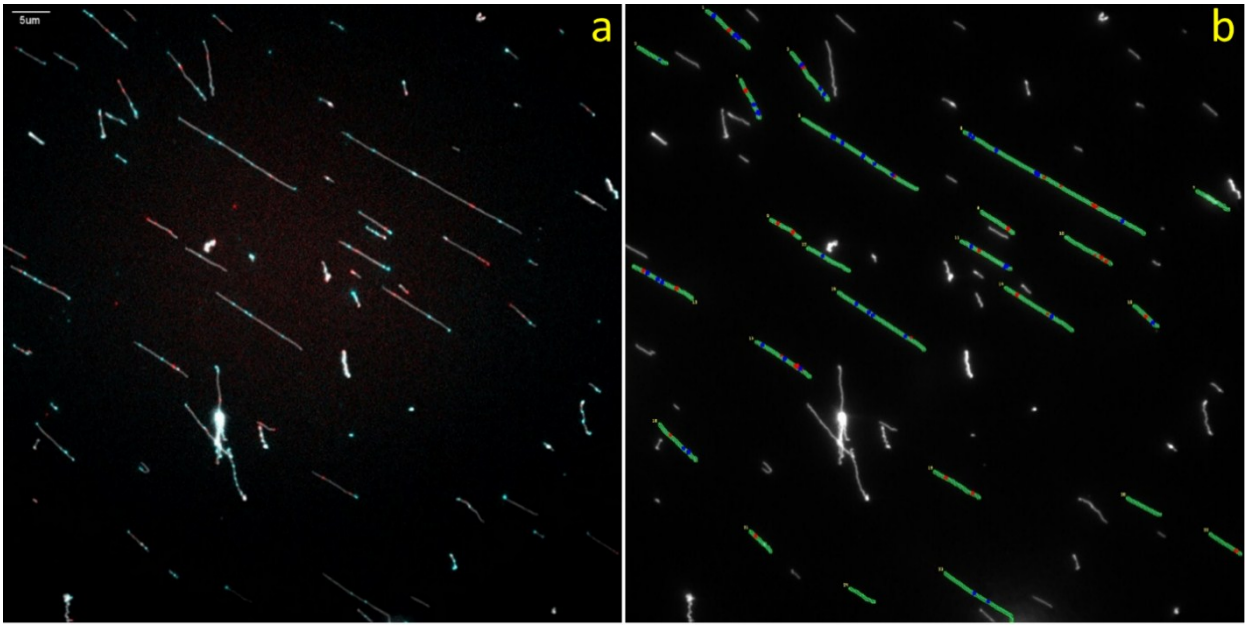


Figure S8. A full representative field of view of imaged DNA molecules in three colours and its analysis. (a) raw image overlay as detected in our optical system. Each grey structure is a DNA molecule, with most of them being stretched and some randomly coiled on the surface. The red dots represent repaired photoproduct sites while blue dots represent the repaired oxidative damage sites. (b) analysis results as detected by our image processing software. Green structures represent detected DNA molecules used for downstream analysis while red dots are the detected photoproduct labels and blue dots the detected oxidative damage sites.

Reverse multi-labelling reaction

In order to validate our simultaneous labelling reaction, we performed a reverse multi-labelling reaction of HEK cells exposed to UVC irradiation, where photoproducts are labelled first and oxidative lesions second. Figure S9 shows the levels of detected damage types.

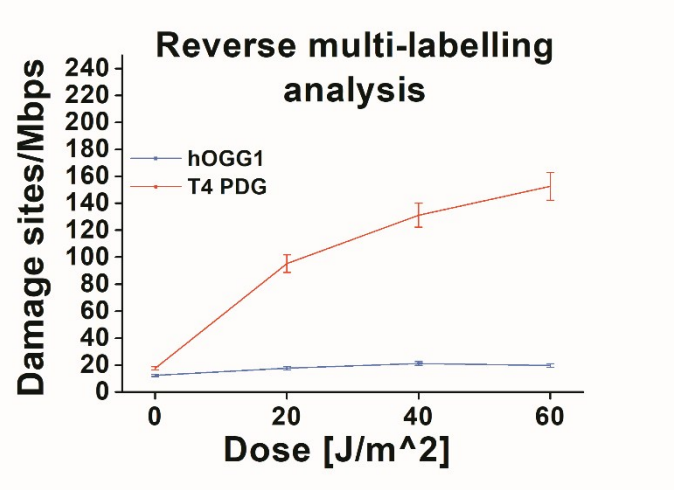


Figure S9. Reverse multi-labelling analysis, where photoproducts are labelled first and oxidative lesions second, of HEK cell exposed to UVC irradiation. Red data represent the levels of detected photoproducts and blue data the levels of oxidative damage.

Carrying out the reaction in a reverse order, labelling the photoproducts firsts and the oxidative lesions second, produced no detectable oxidative damage. This lack of labelling is likely due the Endonuclease IV enzyme which detects and excises oxidative damage lesions. Labelling the oxidative damage first eliminates this enzymatic overlap, leaving only photoproducts to be detected and labelled in the second step. This emphasizes the importance of carrying the multi-labelling reaction in a specific order.

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