Molecular characterisation of DNA double strand breaks with Tip Enhanced Raman Scattering (TERS)

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Individual Double Strand Breaks (DSBs) were first detected and located by AFM and then the molecular structure of this damage was characterized with Tip Enhanced Raman Scattering (TERS) using a top-down configuration and reflective substrate. Here we report the first experimental evidence proving that individual DSBs result from cleavage at the 3'– and 5'– bonds of deoxyribose upon exposure to UVC radiation based on the observation of P–O–H and methyl/methylene deformation modes enhanced in the TERS spectra. We hypothesise that strand fragments are hydrogen-terminated at the lesion, indicating the action of free radicals

during photon exposure.

Key words: Tip Enhanced Raman Scattering (TERS), DNA damage, Double Strand Break (DSB)

The depletion of stratospheric ozone and the accompanying increase in solar UV flux have led to concerns regarding increases in skin related cancers and decreases in global microbial productivity ^[1,2]. The main DNA lesions generated by UVB (315 – 280 nm) and UVC (280 – 100 nm) include the direct products of photochemical reactions within DNA, such as cyclobutane pyrimidine (CPDs) and 6–4 lesions ^[3,4]. Other forms of damage include single– and double–strand breaks (SSBs and DSBs, respectively) and the yield of DSBs/Gy are often used as a measure of biological effectiveness of different radiation types ^[5]. UVB and UVC exposure can also result in modified bases, such as 8–oxoguanine, thymine glycol, 5,6–dihydrothymine, and cytosine photohydrate ^[6]. These DNA alterations are chemically well characterised and have been accurately quantified for specific absorbed doses of UV. While UVC does not currently reach the Earth's atmosphere it does serve as an excellent source to generate high numbers of DNA DSBs for a proof of concept study.

DNA double strand breaks (DSBs) are deadly lesions that can lead to genetic defects and cell apoptosis^[5]. There are several molecular pathways that can cause DSBs following UVC exposure^[7]. Theoretically, 4.85 eV photons (the energy applied in this study) can damage DNA directly or indirectly, by the generation of delta electrons and free radicals, which react with the DNA molecule. The radiolysis products: free radicals ($\cdot\text{OH}$, $\cdot\text{H}$) can cause many kinds of DNA damage including SSBs, DSBs and cross–links ^[7]. Recently it has been demonstrated that even very low energy electrons 0.1 eV to 2 eV can interact with double stranded DNA and cause SSBs and DSBs ^[7–10]. A study on isolated DNA in water, demonstrated that 99.5 % of the energy from ionising radiation is absorbed directly by water and just 0.5 % by DNA ^[11].

Techniques that directly detect DNA DSBs include scanning electron microscopy^[12], Atomic Force Microscopy (AFM) ^[13–15] and fluorescence based approaches ^[16–20]. While these

techniques can be used to identify DSBs they provide no information on the molecular events occurring at the break. Tip Enhanced Raman Scattering (TERS) can provide molecular information from DNA at the nano-scale ^[21–23] and in combination with AFM provides a new way to visualise and characterise the molecular structure of DSBs. The primary use of AFM in this study is to identify potential sites of DSBs, which can then be targeted with TERS. TERS exploits the near-field enhancement generated by laser excitation of surface plasmons resulting in a strong electromagnetic field generated at the laser-irradiated apex of a metal or metalised scanning probe microscopy (SPM)-tip ^[24–26]. In this study DNA dissolved in buffer was irradiated then mixed with an untreated sample and the DNA was then deposited onto a mica surface. By mixing irradiated and treated samples one could perform AFM and TERS measurements using the same tip and under the same conditions. AFM imaging was performed to locate target spots (control and broken DNA) based on the DNA molecule conformation determined by AFM topographical maps and AFM phase images. TERS spectra were collected from these target spots and the spectra (control and irradiated DNA) were analysed with multivariate methods and compared with micro-Raman, SERS and calculated spectra. The hypothesis is that a combination of AFM and TERS can be used to determine the chemical structure of individual DSB following UV-C radiation exposure. It was found that DNA exposed to UVC resulted in 3 main types of chemical breaks the most important being cleavage at the 3'– and 5'– bonds of the deoxyribose unit resulting in terminal hydrogen bonding.

Experimental section

In this study pUC18 circular plasmid DNA was irradiated as a thin film in aqueous solution by a UVC lamp. The TERS methodology is described in the Supporting Information S1, S2 and includes an optical response image of the TERS tip (Supporting information S2) and a TERS image of DNA used to determine the absolute spatial resolution (Supporting

Information S3). A schematic diagram of the TERS upright configuration used to investigate irradiated DNA is shown in Fig. 1a. Mg^{2+} counter-ions are used to fix and orientate the DNA (Fig. 1b). To improve the enhancement and increase the percentage of functional TERS tips we coated one of the mica surfaces with a reflective silver coating and placed this underneath a second mica substrate (Fig. 1b). This increases the amount of laser light in the tip area through reflection from the silver surface increasing the number scattered photons interacting with the molecule and tip. The additional reflective surface leads to better enhancement and an increase in the percentage of TERS active tips (~90 %). This is because in the top-down configuration without a mirrored substrate the tip could cast a shadow on the sample at the measurement point and then the signal would not be enhanced. The mirrored surface illuminates more of the nanostructure at the tip apex, which is crucial in obtaining a higher percentage of active tips ^[27]. Fig. 1c and 1d shows Scanning Electron Microscopic (SEM) images from different orientations of the nano-droplet of silver deposited on the AFM tip.

Figure 1. Schematic of TERS configuration. a) AFM image of the pUC 18 DNA plasmid fixed onto a mica surface in the upright configuration showing the AFM tip and the direction of the green laser. b) method of DNA fixation and substrate configuration. c) and d) Scanning Electron Microscopy (SEM) images of the silver coated TERS tip.

Fig. 2 shows the AFM topography of control and irradiated samples. Untreated plasmid forms are dissolved in the solution and are supercoiled with some 6 to 10 nodes observed [28]. When the plasmid is damaged and providing there is at least one SSB it loses its supercoiled quaternary structure and goes into a relaxed state or a linear shape if there is a DSB [15–17, 27]. The corresponding histograms show the averaged number of supercoiled, relaxed and linear forms for control and irradiated DNA. For the actual TERS measurements, the control and irradiated solutions were mixed, enabling the collection of spectra from supercoiled – control DNA and at the end points of linear DNA where the DSBs occur based on height and phase AFM images.

Figure 2. AFM topographies of plasmid DNA. a) control and b) exposed to 85 kJ/m² UVC (256 nm) radiation of pUC18. Histograms summarise the averaged number of supercoiled, relaxed and linear plasmid forms corresponding to all control and irradiated samples. Three detectable plasmid forms were marked by arrows: supercoiled (blue), relaxed (cyan) and linear (pink).

At the nano-scale the DNA is not homogenous, so each spectrum is recorded from a different set of base combinations (*ca.* 54 base pairs including the terminal groups are located in the near field of the tip [Supporting information S3]) and so variation is expected. At the nano-scale the DNA is not homogenous, so each spectrum is recorded from a different set of base combinations (*ca.* 54 base pairs including the terminal groups are located in the near field of the tip [Supporting information S3]) and so variation is expected. The spatial resolution of our system (measured by mapping) was ~ 18.2 nm and the distance between two neighboring DNA base pairs (including one base pair) is 0.34 nm. The averaged number of base pairs located in the area of interest (in the near field of the tip) is calculated based on these approximations.

To assess the intrinsic variability associated with TERS measurements both the intra-sample and inter-sample variability were analysed by both visual inspection and Principal Component Analysis (PCA). Supporting Information S4 shows the variability observed from one tip for control and DSBs. TERS spectra from 350 different positions of control and DSBs in plasmid DNA were collected using 7 different tips and analysed with PCA. The results are presented in the Supporting Information S5. By visualising the data using a 3-Dimensional PCA Scores Plot (PC1 vs. PC2 vs. PC3) it became apparent that different tips yield different spectral profiles although general clustering of the control and DSBs could be observed.

Figure 3a–c show schematics of the potential cleavage sites and terminal hydrogen bonding sites based on detailed analysis of the TERS spectra. Fig. 3 (right) shows three general types of representative TERS spectra designated Type 1, 2 and 3 that were identified by a combination of PCA and visual inspection. Fig. 3 (left) shows the three corresponding mechanisms that are hypothesised to lead to DSB formation based on the spectral interpretation. Broken bonds are marked with yellow and blue arrows. Type 1 spectra (Fig. 3a) show strong bands corresponding to the CH₃ functional group. The schematic above the spectrum shows the position of the bond cleavage where the CH₃ group is bound. The appearance of CH₂/CH₃ (1368 cm⁻¹) scissoring modes along with the CH₂ (1439 cm⁻¹) deformation mode confirms that cleavage occurred at the 5'– bond position in a single DNA strand. Type 1 spectra do not exhibit the characteristic phosphodiester vibrations. This could be explained if the phosphodiester functional groups were removed upon the exposure to UVC radiation. If the absence of phosphodiester motions are indeed indicative of phosphate removal then this, suggests 3'– bond cleavage on the second DNA strand. Type 2 (Fig. 3c) spectra, collected from regions of DSBs, show the phosphodiester vibrations and a strong signal from P–O–H in-plane bending mode at 1160 cm⁻¹, which is coupled to the P–OH stretching mode at 1190 cm⁻¹ [29, 30] confirming that the phosphodiester fragment is hydrogen-terminated at the lesion site. The appearance of these bands indicates 3'– bond cleavage occurred in one strand and 5'– bond cleavage on the second strand (blue arrows). In the present case, there also exists a second possible cleavage site (yellow arrows).

Figure 3. Potential cleavage sites and corresponding assigned TERS spectra. a) Type 1, b) Type 3 (mixture of Type 1 and Type 2) and c) Type 2 spectra. The blue highlight shows bands common to Type 1 and Type 3 spectra, while the pink shadow highlights bands common to Type 2 and Type 3 spectra. The arrows indicate the potential cleavage sites that become hydrogen terminated. c) second derivative of Type 3 spectrum zoomed in spectral range: 1250 – 1050 cm⁻¹. d) the number of Type 1, Type 2 and Type 3 spectra collected in this experiment.

Close inspection of the asymmetric and symmetric phosphodiester bands ($1240\text{--}1225\text{ cm}^{-1}$ and $1090\text{--}1070\text{ cm}^{-1}$) of DNA reveals distinct shoulder features not observed in spectra recorded with the micro-Raman system. By applying a second derivative to the Type 3 spectrum two sets of doublet bands are clearly resolved (Fig. 3d). The appearance of these doublets is consistent with the phosphodiester groups being in slightly different molecular environments following the breakage and demonstrates the extremely high sensitivity that can be achieved with TERS. Type 3 spectra (Fig. 3b) appear to be the sum of Type 1 and Type 2 spectra. Bands from the phosphodiester vibrations and the CH_3 group are observed, along with the bands at 1160 cm^{-1} and 1190 cm^{-1} , indicating radicals resulting from UVC irradiation preferentially attack O–C bonds. In Fig. 3e a histogram showing the percentage of the three described types of TERS spectra are presented. Type 3 spectra indicating O–C ($5'$ –) cleavage were collected more often than the others (56.8 % (Type 3) : 23.9 % (Type 1) : 19.3 % (Type 2)). This result suggests that the O–C bond in the DNA backbone is the most sensitive to UVC radiation and its cleavage causes DSBs the majority of the time. The spectroscopic evidence for cleavage at the O–C band includes the identification of intense bands associated with the CH_2/CH_3 and P–O–H bending modes. To correlate the TERS measurements we compared the results with those obtained with Surface Enhanced Raman (SERS) and conventional Raman spectroscopy. UVC exposed DNA showed higher intensity bands in both SERS and micro-Raman compared to the controls. The SERS spectra resemble the TERS Type 3 spectra showing enhanced phosphodiester and CH_2 deformation modes. These results are presented and explained in detail in Supporting Information S6.

Additionally computations of Raman activity for control (undamaged) and damaged (Type 3: $5'$ – C–O cleavage) DNA structures have been carried out in the framework of hybrid QM/MM (quantum mechanics/molecular mechanics) method (ONIOM model). The technical details including a comparison of experimental and calculated spectra are described in

Supporting Information S7. The calculated spectra show good agreement for the band positions of the hydrogen terminated functional groups following 5'– C–O bond cleavage including the P–O–H bending, P–OH stretching along with methyl/methylene deformation modes and the DNA phosphodiester modes.

We have shown that the combination of AFM–TERS provides a new way to visualize DNA and investigate the molecular structure of terminal groups at high spatial resolution and with extreme sensitivity. Moreover, we have demonstrated the potential of TERS to investigate the susceptibility of bonds to radiation damage and in particular have shown that O–C cleavage is the most likely form of DNA damage as this accounted for more than half of the cleavages detected.

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ASSOCIATED CONTENT

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Supporting Information for the paper:

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S1. Methods

Tips and sample preparation. The tip size and shape are crucial in obtaining high quality TERS spectra. Silicon-based AFM tips from ATEC-NC (Nanosensors) were coated with 20 nm of silver (Aldrich 99.99 % pure) using a CE. 12/14 (Dynavac Engineering Pty Ltd) evaporation system, under air pressure (5×10^{-5} Torr), Probes were angled at 30°C to evaporate the silver exactly on the tip. During the evaporation the deposition ratio was 0.05 nm per second. The sample-source distance was approximately 20 cm. Double-stranded pUC 18 DNA plasmid (Sigma Aldrich, Australia) was used as the source for control and UV damaged DNA. The DNA, in a radioprotective aqueous buffer (20 mM HEPES 100mM NaCl solution), was homogeneously irradiated as a thin film from the 40 μ L solution on Petri dishes. A UVC (256 nm, 4.85 eV) lamp was used as the radiation source. The exposure time was set to 30 mins and the distance between the lamp and the sample was ~ 2.5 cm. The deposited energy at the sample was 85 kJ/m². After irradiation, the cationic solution (10 mM MgCl₂) was used to dilute the DNA samples (1:1). 10 μ l final solution (final concentrations: 1–3 μ g/ml DNA, 10 mM HEPES, 50 mM NaCl, 5 mM MgCl₂) was deposited on freshly cleaved mica (~ 0.1 mm thickness). After 5 mins incubation samples were washed 10 times in deionised water and dried in nitrogen. The physiological solution mimics the natural osmolarity of the DNA environment. In this physiological solution the plasmid DNA is mainly supercoiled showing between 6 – 10 nodes^[S1] with AFM microscopy if the plasmid breaks, it is no longer supercoiled and the DNA becomes relaxed when at least one SSB occurs or linear if a double strand is broken^[S1]. The prepared samples were placed on silver coated (160 nm) mica (Fig. 1B) to increase the reflectivity from the bottom surface during TERS acquisition.

AFM imaging. AFM images were recorded using a NT-MDT Integra AFM Raman system in the tapping mode. The NT-MDT NSG10 probes were used for imaging in air. The spring constant of the AFM cantilevers was ~ 11.8 N/m and their resonance frequency was ~ 240 kHz. All images were collected at a scan rate of 0.2–0.3 Hz, a scan resolution of 512–512 or 256–256 pixels, and scan sizes of 1000–3000 nm. Additionally (in order to improve the statistics) two further instruments namely Cypher and JPK nano Wizard AFM were used for AFM imaging. The imaging was faster with the scan rate of 0.8–1.5 Hz but the other parameters and tips were the same.

AFM imaging using TERS tips. The silver coated top visual silicon-based AFM cantilevers of ATEC-NC (Nanosensors) were used for imaging in air. The spring constant of the AFM cantilevers was ~ 50 N/m and their resonance frequency was ~ 300 kHz. All images were collected at a scan rate of 0.2–0.3 Hz, a scan resolution of 512×512 or 256×256 pixels, and scan sizes of 1000–3000 nm.

TERS spectra collection.

Based on AFM topographies and phase images we have selected our targets – the places where the spectra were collected (from two groups of points (1) at the end of linear plasmid: broken, (2) supercoiled form with 6-10 nodes: control.

TERS spectra were collected using an “Andor” CCD camera (cooled to -30°C) in the spectral range of $1725\text{--}580\text{ cm}^{-1}$. The green (532 nm) Nd:YAG laser power measured at the sample level was 2.5 mW. The laser exposure time for a single spectrum acquisition was 5 seconds.

S2. Focusing the laser on the tip

To focus the laser light exactly at the tip apex an area of $10\text{ }\mu\text{m} \times 10\text{ }\mu\text{m}$ was scanned with the TERS tip in 30×30 incremental steps. The sum of Raman intensity in the spectral range: $1700\text{--}1320\text{ cm}^{-1}$ is presented in 3-dimensions in Fig. S1. In the selected spectral range there are many DNA peaks and the maximum counts shows the spot where the tip and laser were situated on the DNA during the measurement.

Figure S1. The optical response of a focused laser scanned through the TERS tip.

S3. Spatial resolution

The spatial resolution of the TERS method was measured by mapping the plasmid DNA using the integrated area of some important DNA bands. The DNA strand was placed at the normal angle to the scanning direction. The results are presented in Fig. S2. The AFM topography of the sample is shown in Fig. S2a and the area, that was mapped using the TERS technique is marked by a green rectangle. Fig. S2b shows the TERS mapping of ν_{asym} (O-P-O) band based on the sum of TERS intensities in the spectral range: $1255 - 1225 \text{ cm}^{-1}$ along with the background (mica peaks). The pixel size is $2.6 \times 2.6 \text{ nm}$ for all TERS maps. A high intensity Raman signal is found in the central part of the map where the DNA strand is located. Fig. S2c shows TERS mapping of the A/C/G/T pyridine/purine ring-breathing modes in the spectral range ($1580 - 1465 \text{ cm}^{-1}$) along with the background (mica peaks). The corresponding AFM images of height and phase are presented in Fig. S2d and Fig. S2e, respectively. Fig. S2f and Fig. S2g show the TERS maps from Fig. S2b and Fig. S2c after background subtraction. Fig. 2Sh presents the spectra extracted from the pixels: blue: the DNA TERS spectrum with the background (mica peaks), black: the background spectrum (mica peaks), grey: the DNA TERS spectrum after background subtraction. The DNA double stranded diameter is approximately 2 nm based on the AFM images. For TERS maps utilizing the TERS tip and integrating the area under the DNA bands (ν_{asym} (O-P-O) at $1255 - 1225 \text{ cm}^{-1}$ and pyridine/purine ring-breathing modes at $1580 - 1465 \text{ cm}^{-1}$) a 7 pixel resolution could be achieved equivalent to $\sim 18,2 \text{ nm}$. The distance between two neighboring DNA base pairs (including one base pair) is 0.34 nm . The number of base pairs located in the area of our interest (in the near field of the tip) calculated based on our spatial resolution and the distance between two neighboring DNA base pairs is 54.

Figure S2. The TERS spatial resolution. a) The AFM image (height) of DNA double strands, the images in panels b-g images were collected from the area marked with a green rectangle. b) TERS mapping of ν_{asym} (O-P-O) – the sum of TERS intensities in spectral range: 1255 – 1225 cm^{-1} along with the background (mica peaks), the pixel size: 2.6x2.6 nm. c) TERS mapping of A/C/G/T pyrimidine/purine ring stretching vibration using the sum of TERS intensities in spectral range: 1580 – 1465 cm^{-1} along with the background (mica peaks). d) The AFM image (height) of DNA double strands. e) The AFM image (phase) of DNA double strands. f) TERS mapping of ν_{asym} (O-P-O) – the sum of TERS intensities in spectral range: 1255 – 1225 cm^{-1} after background (mica peaks) subtraction. g) The TERS mapping of A/C/G/T pyrimidine/purine ring stretching vibration using the sum of TERS intensities in the spectral range: 1580 – 1465 cm^{-1} after the background (mica peaks) were subtracted. h) The spectra extracted from the pixels: blue: The DNA TERS spectrum with the background (mica peaks), black: The background spectrum (mica peaks), grey: The DNA TERS spectrum after the background subtraction.

S4. Spectral reproducibility for control and irradiated plasmid DNA

Fig. S3 shows an AFM images: topography and phase recorded with the TERS tip along with the locations where spectra were acquired of control (black) and double strand breaks (blue). The AFM image, in this case, is recorded with a TERS tip and a drop of silver (~ 20 nm) at its apex hence the AFM image is not as resolved as those AFM images presented in Figure 1 and 2 of the main article, which were recorded with a standard AFM tip. The AFM phase image of the same sample is presented in Fig. S3b. The typical bands for the DNA are the symmetric phosphate stretch at ~ 1083 cm^{-1} and the asymmetric stretch ~ 1237 cm^{-1} . These are not readily observed in control DNA TERS spectra (Fig. S3, black numbers : 1, 2, 3, 4, 5). This is because the DNA is fixed onto the mica surface by coulomb interaction between negatively charged phosphate groups and positively charged magnesium ions, which results in different

orientations of the phosphate groups relative to the surface (Fig. 1) Consequently these vibrational motions are observable in some spectra of damaged DNA (Fig. S3, blue numbers: 1, 2, 3, 4, 5) but not all. Alternatively the terminal phosphate groups in some cases could have been completely removed by exposure to the radiation.

As shown in Fig. 3 of the main text there are 3 types of possible DSBs: one generates OH-terminated phosphates (DSB Type 2), another results in a CH₃ group terminal on the ribose ring (DSB Type 1), and the third is a combination of both (Type 3). The variability observed in TERS spectra originates primarily from these bond differences and their relative orientation to the tip. Here, the 1440 cm⁻¹ and 1360 cm⁻¹ bands dominate Type 1 DSB spectra, which are assigned to CH₂ bending motions. These bands are absent in Type 2 DSB spectra. Similarly, bands absent in Type 1 spectra include the P-O-C symmetric stretching (1115 cm⁻¹), phosphate stretching (1079, 1237 cm⁻¹) and P-OH bending (1160 cm⁻¹), which are all highly intensified in the type 2 spectra. These observations support the proposed sites of DSB of DNA under ionising radiation.

Figure S3. The intra-sample variability of TERS spectra recorded with a single tip. a) The AFM topography of plasmid DNA recorded with a TERS active tip showing the spots where the tip was located during spectral acquisition, (control DNA – black numbers and DSBs DNA – blue numbers). b) The AFM phase image corresponding to a). c) Representative TERS spectra of control DNA, corresponding to tip positions marked on a. d) Representative TERS spectra (Type 1) of DSBs DNA, corresponding to tip positions marked on a. e) Representative TERS spectra (Type 2) of DSBs DNA, corresponding to tip positions marked on a the band assignments are presented in Table 1 (Supporting information Section 6).

S5. Principal Component Analysis

The evaporation method employed to coat the tips is random at the nano-scale, and hence the nano-structures at the end of the tip vary in size and shape, which can lead to variation in the spectral profiles. Additionally during the experiment the TERS tip properties can change. For instance these structures can be damaged during an image measurement in semi-contact mode or alternatively the nano-tip can become oxidised. The variability of spectra collected from one tip in both the control and damaged DNA are presented in Figure S3. The sample structure can also have an influence on the variability. At the nano-scale the DNA is not homogenous, so every time a spectrum from different base combination (ca 54 base pairs) is collected, some variation is expected. Nevertheless consistent differences can be observed between TERS spectra of control and DSBs.

To further examine the spectral variability induced by the tip Principal Component Analysis (PCA), a multivariate clustering technique, was applied to analyse the spectra. In the first case the intra-tip variability was investigated by using a single TERS tip for recording multiple TERS spectra from control and irradiated DNA. The PC1 versus PC2 Scores Plot is shown in Fig. S4a. Two distinct clusters, corresponding to the two groups of spectra (control and DSB) were identified. The corresponding loading factors are presented in Fig. 4Sb. The PCA results show that PC1 contributed to the 23 % of the total variance. The important positive loadings show a positive correlation with wavenumber values attributed to DNA base-sugar bonds ($\sim 690\text{ cm}^{-1}$), C-O furanose stretching vibration (970 cm^{-1} and 1030 cm^{-1}), $\nu_{\text{asym}}\text{ O-P-O}$ ($\sim 1240\text{ cm}^{-1}$), $\nu\text{ P-(OH)}$ (1159 cm^{-1}) and $\delta\text{ P-O-H}$ (1194 cm^{-1}). P-O-H motions are not typically observed in control DNA and are only observed in spectra of damaged DNA. PC2 explains 18 % of the total variance and shows strong negative loadings for CH_2 , CH_3 scissoring vibrations (1368 and 1439 cm^{-1}). These modes are also only observed in damaged DNA.

In the second model the inter-tip variability was investigated. The PCA analysis was applied to more than 350 TERS spectra collected with 7 different tips (Fig. S4c, d). The 3D (PC1 vs. PC2 vs. PC3) Scores Plot (Fig. 4c) shows general clustering of the data. Clusters corresponding to control and DSBs DNA spectra are observed; however, the 3D-Scores Plot confirms that the tip has an influence on the spectral clustering. The corresponding loadings plots show that the peaks at the wavenumber values 970 cm^{-1} , 1160 cm^{-1} , 1236 cm^{-1} , 1335 cm^{-1} , 1440 cm^{-1} are responsible for the general clustering between control and irradiated DNA as was the case for the single tip in Fig. 3b.

Figure S4. PCA analysis of TERS spectra. a) Scores Plot of TERS spectra collected by using one tip, control spectra – black stars, spectra of DSBs DNA – blue stars. b) Loadings Plot corresponding to a. c) Scores Plot of 350 TERS spectra collected by 7 different tips (marked by 7 various colours). c – control DNA (filled spheres), d – damaged DNA, (hollow spheres). d) Loadings Plot corresponding to c.

S6. Micro-Raman, SERS and TERS

To correlate the TERS measurements we compared the results with those obtained with SERS (Surface Enhanced Raman Scattering) and micro-Raman spectroscopy. Single channel Micro-Raman and SERS spectra of irradiated and untreated pUC-18 DNA plasmid were collected using a Confocal Raman Microscope alpha300 R equipped with Nd-Yag 532 nm (green) laser with a CCD camera cooled to -60°C. The 50x objective was used. For micro-Raman measurements the sample, which is a drop of 385 µg/ml DNA solution, was placed on aluminum coated Petri dishes and left to dry. The spectra were recorded in the range of 3700 cm⁻¹ – 200 cm⁻¹. The acquisition time was 20 seconds per spectrum, the beam power was set to 3.6 mW at the sample. The spectral resolution was 2 cm⁻¹. For SERS measurements the 3.9 µg/ml DNA solution was placed on a purpose-built SERS substrate which incorporated a silver nanostructure on silicon surface providing SERS enhancement when using a green laser line^[S2]. The SERS spectra were collected in the range of 3700 cm⁻¹ – 200 cm⁻¹. The acquisition time was 2.5 seconds per spectrum and the laser power was

1.63 μW on the sample with a spectral resolution of 2 cm^{-1} . The spectra were processed using Opus 6.5 software (Bruker Optik), which entailed spectral smoothing (9 smoothing points) and baseline correction (rubberband correction with 16 baseline points and 4 iterations).

The spectra collected using all three methods are shown in Figure S5 and the detailed band assignments based on previous studies and theoretical calculations are reported in the Table (Table S1). The comparison of TERS, SERS and micro-Raman spectra of control and irradiated pUC18 DNA plasmid is presented in Fig. S5a and Fig. S5b, respectively. SERS and micro-Raman spectra are averaged from more than 10 spectra. Averaging of TERS spectra results in a loss of spectral information from the sample because DNA is not homogenous at the nanoscale and as discussed in sections 3 and 4 above spectral variability is expected. For comparison of the damaged DNA spectra, a representative type 3 spectrum was chosen because it shows peaks from all the characteristic terminal hydrogen functional groups including CH_2 , CH_3 and P-O-H groups. The TERS peaks observed in SERS or micro-Raman are marked using yellow highlight. The spectra, achieved by using three various techniques: micro-Raman, SERS and TERS, of control in comparison with damaged DNA are presented in Figure S5c, S5d, S5e respectively, where differences in peak positions are marked by using grey highlight.

Figure S5. A comparison of TERS, SERS and micro-Raman spectra. a) spectra of untreated pUC18 DNA plasmid, the TERS peaks observable by other methods are marked by using yellow highlight. b) spectra of irradiated pUC18 DNA plasmid, the TERS peaks observable by other methods are marked by using yellow highlight. c) Raman spectra of control and irradiated pUC18 DNA plasmid, differences marked by using grey highlight. d) SERS spectra of control and irradiated pUC18 DNA plasmid, differences are marked by using grey highlight. e) TERS spectra of control and irradiated pUC18 DNA plasmid, differences marked by using grey highlight.

Raman bands arising from the DNA phosphodiester and sugar groups are comparatively less intense compared to other biomolecules because of the low Raman scattering cross-section of the phosphate moieties. In SERS and TERS, the functional groups closest to the metal surface are subject to the greater enhancements (EM enhancements scale as r^{-10} from the surface). DSBs cause the phosphate terminals and nucleic bases to become exposed to the metal surface leading to the greater enhancement of these modes.

The major bands observed in Type 1,2 and 3 TERS spectra of damaged DNA are all shown in the SERS spectrum. This can be explained based on the spectral acquisition area. In TERS, the enhancement occurs in the vicinity of the metallised tip and the spectrum is collected from this localised area, while in SERS, the sampling area is largely determined by the laser spot size, which is *ca.* 100 times larger than the average TERS tip. Thus, in the SERS measurement multiple DNA strands are sampled with various surface orientations, while DSBs are probed one at a time in TERS. The P-O-H and CH₃/CH₃ motions associated to DSBs can not be observed by micro-Raman because of the sensitivity, the number of broken bonds relative to the amount of non-damaged helical DNA is below the detection limit. SERS is more sensitive than conventional micro-Raman because of the close proximity of the DSB to the surface resulting in the enhancement of terminal hydrogen bonded and methylated functional groups at the site of the break. In the case of micro-Raman spectroscopy an averaged spectrum of packed DNA is recorded dominated mainly by normal DNA and there is no localized enhancement of the terminal hydrogen groups.

Table 1. Bands assignments for Raman, SERS and TERS spectra of pUC18 DNA plasmid control and irradiated with UV-C

Control DNA, Raman band	Irradiated DNA, Raman band	Control DNA, SERS band	Irradiated DNA, SERS band	Control DNA, TERS band	Irradiated DNA, TERS band	Assignment
1694	-	1684	-	1702	-	$\nu(\text{C=O})$, $\delta(\text{NH}_2)$ of

						T/G/C, base stacking vibration ^[S3]
1665	1657	1660	1667	1651	1653	δ (NH ₂) sci. ^[S4]
1570	1571	1573	1581	1578	1571	in plane ring vibration A/G, NH ₂ sci. ^[S4, S5]
-	-	1519	1507	1512	1515	Ring ^[S5]
1482	1468	1484	1478	1483	1472	ν (C=N) Pirymidine ^[S4] , C ₂ H ₂ def. ^[S5]
-	-	-	1440	-	1440	ρ_w (CH ₂) ^[S6]
1418	1412	1402	1400	1406	1403	ν (C ₆ -N ₁) Pirymidine ^[S4] , ring ^[S5]
-	-	-	1370	-	1350-1365	ρ_w , scissoring(CH ₂ , CH ₃) ^[S6]
1332	1336	1324	1313	1328	1317	(C-N) ^[S5] , ring ^[S3]
1303	1293	1324	1311	1296	1296-1313	ring ^[S5]
1234	1240	1245	1254	1234	1237	ν_{asym} (O-P-O) ^[S5]
1183	1192	1202	1198	-	1190	ν P-(OH) ^[S7, S8]
-	1140	-	-	-	1160	δ (P-O-H) ^[S7, S6]
-	-	-	-	-	1115	ν_{sym} (P-O-C) ^[S9] , ν_{sym} (O-P-O) ^[S10, S11]
1089	1086	1128	1090	1085	1079	ν_{sym} (O-P-O) ^[S10]
1060	1062	1068	1070	1067	1061	ν (N-sugar) A ^[S4]
1020	1019	1030	-	-	1034	ν (C8-N9, N9-H, C8-H) A ^[S12]
963	982	980	980	-	980	ν (CC, CO) T, ribose ^[S11] ρ_w (NH ₂) T ^{S11}
916	913	-	930	-	930	ρ_r (NH ₂) A/C/G ^[S4, S12] , deoxyribose ^[S13]
890		-	880	-	870	deoxyribose ring ^[S14]
-	852	841	850	-	830	deoxyribose ring ^[S14]
784	767	786	780	-	-	T ring breathing ^[S12, S15]
680	630, 644	658	648	-	655	G ring breathing ^[S16, S17]

s strong, *w* weak, ν stretching, δ bending, ρ rocking.

S7. Comparison of the TERS results with DFT calculations

According to Placzek's polarizability theory the non-resonant Raman intensity associated with vibrational mode Q_i is defined by the Raman scattering activity:

$$S_i = g_i [45(\tilde{\alpha}'_i)^2 + 7(\tilde{\gamma}'_i)^2] \quad (1)$$

and the depolarization ratio:

$$\rho_i = \frac{3(\tilde{\gamma}'_i)^2}{45(\tilde{\alpha}'_i)^2 + 4(\tilde{\gamma}'_i)^2}, \quad (2)$$

where g_i is degeneracy of normal mode Q_i , and $\tilde{\alpha}'_i$ and $\tilde{\gamma}'_i$ are the derivatives of isotropic

and anisotropic invariants of Raman tensor defined respectively by:

$$\tilde{\alpha} = \frac{1}{3}(\alpha_{xx} + \alpha_{yy} + \alpha_{zz}) \quad (3)$$

and

$$\tilde{\gamma}^2 = \frac{1}{2} \left[(\alpha_{xx} - \alpha_{yy})^2 + (\alpha_{yy} - \alpha_{zz})^2 + (\alpha_{zz} - \alpha_{xx})^2 \right] + 3(\alpha_{xy}^2 + \alpha_{xz}^2 + \alpha_{yz}^2) \quad (4).$$

In equation (3) and (4) α_{ij} are components of the Raman tensor.

Computations of Raman activity for untreated and radiation-induced damaged (double strand break within O-C bond region) DNA structures have been carried out using GAUSSIAN 09 software package in the framework of hybrid QM/MM method (ONIOM model) with Density Functional Theory formalism with B3LYP hybrid exchange-correlation functional using 6-311++G(d,p) basis functions set for a guanine-cytosine pair and Universal Force Field model for the rest of the system (additional 6 pairs treated classically). The molecular system is schematically shown in Fig. S6b. Initial positions of atoms were taken from Nucleic Acid Database [<http://ndbserver.rutgers.edu>]. The system has been optimized using default integration grid size (i.e. 75, 302), default integral cutoffs and EDIIS+CDIIS convergence algorithm without damping. Phosphate groups atoms positions were fixed during optimization due to interaction with divalent cations Mg^{2+} present on the mica surface. Then, Raman activities were calculated based on the optimized geometries. Frequencies obtained from vibrational analysis have been scaled by 0.967 factor [<http://cccbdb.nist.gov/vibscalejust.asp>] because the DFT/B3LYP method tends to overestimate vibrational frequencies because of anharmonicity effects in the theoretical treatment. Errors also arise due to the incomplete incorporation of electron correlation and the use of finite basis sets.

After frequency correction Raman spectra intensities have been calculated according to^[S18].

$$I_i = C(\nu_0 - \nu_i)^4 \nu_i^{-1} B_i^{-1} S_i \quad (5)$$

where C is a constant, ν_i is the frequency of normal mode, ν_0 is the excitation frequency (in this work the Nd:YAG laser operating at 532 nm was used which corresponds to a wavelength of 18797 cm^{-1}), S_i is the Raman scattering activity of the normal mode Q_i and B_i is the Boltzmann distribution factor for temperature T (we assumed $T = 300K$):

$$B_i = 1 - \exp\left(-\frac{h\nu_i}{kT}\right) \quad (6)$$

where h and k are the Planck and Boltzmann constants, respectively and c is speed of light.

Fig. S6a (green) shows the result of the calculation for the untreated DNA along with the corresponding TERS spectrum of control DNA. Fig. S6b shows the calculated damaged DNA spectrum with induced cleavage at the O-C bond (5'-) along with a representative Type 3 TERS spectrum of damaged DNA. Fig.6b presents a computed model of DNA with induced cleavage at the O-C bond (5'-). The calculated spectra confirm our experimental results and the major band assignments for a strand break between the O-C bond are detailed in Table S2 for both calculated and experimental wavenumber values.

Table S2 The differences between the positions of the key bands from our point of view (correlated with particle DNA damage)

Observed effect	Experiment	Theory
Deoxiribose-phosphate motion peaks	852 cm ⁻¹	827 cm ⁻¹
	908 cm ⁻¹	920 cm ⁻¹
	956 cm ⁻¹	947 cm ⁻¹
	1019 cm ⁻¹	1014 cm ⁻¹
		1054 cm ⁻¹
δ P-O-H	1180 cm ⁻¹	1182 cm ⁻¹
ν P-(OH) stretching	1160 cm ⁻¹	1171 cm ⁻¹
ρ _w sci. CH ₂ , CH ₃	1350 cm ⁻¹	1376 cm ⁻¹
ρ _w CH ₂	1440 cm ⁻¹	1443 cm ⁻¹
ν P-O-C stretching in DNA backbone		
ν C-O Ribose - DNA backbone	1112 cm ⁻¹	-
ν _{asym} O-P-O	1244 cm ⁻¹	1255 cm ⁻¹
ν _{sym} O-P-O	1079 cm ⁻¹	1085 cm ⁻¹

s strong, *w* weak, *ν* stretching, *δ* bending, *ρ* rocking.

Figure S6. The comparison of experimental and calculated spectra. a) Raman spectrum calculation for untreated control DNA (green top) along with the experimental TERS spectrum of control DNA (green bottom) compared to the computed spectrum of DNA with an induced DSB at the C-O bond (red top) and the experimental TERS spectrum (Type 3: -5' cleavage (O-C) in both DNA strands). b) schematic model showing the theoretical molecular system where the A-C pair was treated quantum mechanically and the additional six base pairs classically treated.

At first glance one can see a correlation between the spectra of control and damaged DNA in both cases (experimental and theoretical spectra). The intensity of DNA backbone bands are higher in damaged DNA spectra compared to the control

All differences in the peak positions are presented in detail in Table 1. The appearance of O-H bending and CH₂, CH₃ bending and wagging bands in the calculated spectrum of damaged DNA confirms the experimental results

One of the major differences between the experimental and calculated spectra is that first; not all bands are enhanced in the TERS spectrum, whereas the calculated spectrum provides an intensity value for every band. Secondly, the QM/MM model is a simplification of the real system because only one nucleic base pair is quantum-mechanically treated as opposed to a TERS measurement where approximately ~54 bases and the terminal group are interrogated. Minor effects on the variability could also result from neglecting anharmonicity effects in the computations (even after scaling with an empirically determined factor) along with an

incomplete description of electron correlations in DFT calculations and the use of a finite basis set.

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