Nanoscale spatial induction of ultraviolet photoproducts in cellular DNA by three-photon near-infrared absorption

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The high-resolution spatial induction of ultraviolet (UV) photoproducts in mammalian cellular DNA is a goal of many scientists who study UV damage and repair. Here we describe how UV photoproducts can be induced in cellular DNA within nanometre dimensions by near-diffraction-limited 750 nm infrared laser radiation. The use of multiphoton excitation to induce highly localized DNA damage in an individual cell nucleus or mitochondrion will provide much greater resolution for studies of DNA repair dynamics and intracellular localization as well as intracellular signalling processes and cell–cell communication. The technique offers an advantage over the masking method for localized irradiation of cells, as the laser radiation can specifically target a single cell and subnuclear structures such as nucleoli, nuclear membranes or any structure that can be labelled and visualized by a fluorescent tag. It also increases the time resolution with which migration of DNA repair proteins to damage sites can be monitored. We define the characteristics of localized DNA damage induction by near-infrared radiation and suggest how it may be used for new biological investigations.

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INTRODUCTION

Although known as physical phenomena for many years, two- and three-photon excitation processes have recently generated interest because localization of the photonic interaction is possible within a femtolitre volume (Kawata *et al.*, 2001; He *et al.*, 2002). In biological studies, two- and three-photon phenomena have been applied mainly to imaging microscopy, where fluorescence excitation by multiphoton absorption can provide unique three-

dimensional detail with submicrometre resolution. Near-infrared (NIR) multiphoton microscopy is becoming the tool of choice for biological imaging as it allows nondestructive multiphoton fluorescence excitation in biological materials (König, 2000).

The main photoproducts induced in DNA by one-photon absorption around 260 nm, the peak absorption of DNA, are cyclobutane pyrimidine dimers (CPDs). Localized induction of these and other ultraviolet (UV) photoproducts in cellular DNA can be achieved by irradiating cells with far-UV light through micrometre-sized holes in a polycarbonate filter (Volker *et al.*, 2001). However, resolution of less than a few micrometres would be impossible in this approach because of the diffraction limitations of the light. Here we demonstrate that three-photon NIR absorption may be used to induce CPDs in the nuclear DNA of cells (in this case MH1C1 rat liver epithelial cells). The damage can be generated in a precise and distinct pattern with nanometre three-dimensional resolution. Evidence for a low level of three-photon NIR induction of CPDs in isolated plasmid DNA has been reported (Shafirovich *et al.*, 1999), but there has been no previous demonstration of three-photon NIR induction of UV photoproducts in cellular DNA. The maximum irradiance from an 810 nm pulsed laser beam used in experiments with plasmid DNA was less than 100 GW cm⁻², which is about half the irradiance intensity required to induce clearly detectable CPDs in our experiments. NIR laser light at 710 nm has been used to create three-photon excitation of fluorescence in the endogenous chromophores tryptophan, serotonin and dopamine in live cells (Maita *et al.*, 1997). Gross damage to the cells was not observed and they survived for several hours following the imaging radiation. However, three-photon excitation by the incident 710 nm infrared laser beam would be expected to inflict DNA damage.

Laser-induced multiphoton processes have been detected before in living cells (Calmettes & Berns, 1983) and the spatial resolution of two-photon absorption has been used to induce DNA damage in sensitized DNA (Berns *et al.*, 2000), but this is the first clear visual demonstration of specific photoproducts produced by a laser microbeam in nonsensitized cellular DNA.

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RESULTS AND DISCUSSION

Fig. 1A shows how a focused 750 nm laser beam can generate a defined pattern of DNA damage in a live cell nucleus as revealed by staining of CPDs with a specific antibody (Mori *et al.*, 1991). The photoproducts may also be induced by a range of wavelengths above and below 750 nm in the tunable range of the Tsunami laser used here (710–850 nm).

Fig. 1B shows a plane parallel to the laser beam axis giving an image commonly known as the z-plane section. The image, which is of the same sample as in Fig. 1A, consists of consecutive confocal scans taken at steps of 500 nm through the depth of the cell. The true profile of the image of the DNA damage distribution in this plane is compressed because the fixation flattens the cell. However, it illustrates that the focus of the damage is confined to the central section of the nucleus. The distribution of CPD damage by the focusing of 750 nm light is likely to differ from that induced by 250/260 nm UV light. Light from a UV source will be absorbed more strongly on the side of the nucleus where the beam first impinges and will tail off as a result of absorption by RNA and DNA as the light passes into the depth of the nucleus. Single-photon UV irradiation of cells through micropores in polycarbonate filters will produce a column of damage through the depth of the cell nucleus, but precise localization of the damage will also be compromised by the additional problem of light scattering. In the localized area of three-photon NIR processes, absorption will cause small changes in the intensity of the 750 nm beam but disproportionately large changes in the three-photon processes on

account of the cubic relationship between these factors; however, light scattering will not affect the focus of the damage spot.

Pattern projection through different objectives

Fig. 2 shows that the three-photon absorption can be obtained even when the focus and refractive index are changed. An identical pattern was traced through a $\times 40$ (numerical aperture (NA)=0.85) (Fig. 2A) and a $\times 100$ (NA=1.3) (Fig. 2B) objective. The reduction in the size of the pattern written through the $\times 100$ objective is clearly illustrated.

As the NAs of the two objectives are different, this might be expected to give rise to different intensities of three-photon absorption in an individual pixel area. For instance, the 1.3 NA of the $\times 100$ objective should give rise to spots of greater density of induced damage, because the laser power was increased to give 10 mW at the stage of the microscope. The higher NA also alters the shape of the focal volume. This means that the intensity of the NIR irradiation, which induced the damage, was about 300 GJ cm^{-2} . The $\times 100$ objective was used in oil immersion, whereas the $\times 40$ objective was used dry. The settings of laser power and pixel dwell times used here represent an intuitive and experimental compromise, but clearly the parameters can be optimized with further experimentation to obtain the lowest level of damage detectable within the minimal focal volume.

Collateral two-photon NIR damage in cells

Predictions about biological effects of collateral one- and two-photon radiation can be made from mathematical modelling of the volumes affected by single- and multiphoton 750 nm laser excitation. Numerical calculations (see supplementary data online) were carried out based on Gaussian beams focused using a microscope objective with an NA of 1.2 with an arbitrary choice of beam size at the objective. These assumptions are only approximate to the conditions used here, but the calculations provide sufficient information for the assessment of the biological

Fig. 1 Focused induction of ultraviolet photoproducts. (A) Pattern of cyclobutane pyrimidine dimers (CPDs) generated in a cell nucleus by three-photon near-infrared absorption. This image shows the letters DNA outlined by antibody-stained CPDs. The nuclei of the cells are visible as the DNA is counterstained red with propidium iodide. The cell cytoplasm is not visible. The minimum diameter of these nuclei is 10–15 μm . The pattern is made up of 200 pixel points, but in principle the damage could be induced by irradiation of only one pixel area, which has a diameter of 500 nm. The minimum width measured by fluorescence intensity across a line of the pattern is 450 nm. (B) Image of the vertical or z-plane of (A). The two central intense spots correspond to the intense limbs of the letters DNA where the laser beam has retraced pixel areas while writing the pattern. The projections of (a) and (b) onto the areas demarcating the induced photoproducts correspond approximately to the same projection in image (A). The depth of the fixed cell was measured as 3 μm . Although the counterstaining by propidium iodide was present in image (B), the dye has been photobleached by the confocal scanning procedure; thus, the rest of the cell nucleus is not clearly visible as it is in image (A).

Fig. 2 Focus of three-photon near-infrared induction of cyclobutane pyrimidine dimers using objectives of different magnification and numerical aperture. (A) An envelope pattern was drawn by the 750 nm laser beam focused through a $\times 40$ objective. The pattern is estimated to be 8 μm at its widest extremities. The six lines forming the pattern are clearly discernible. (B) The same pattern has been drawn through a $\times 100$ objective, through which its maximum width is estimated to be 5 μm . The individual lines are not discernible in the image, but the general shape can be seen to be the same as that drawn through the $\times 40$ objective.

effects of collateral one- and two-photon radiation. The models of the volumes affected by one-, two- and three-photon excitation are shown in Fig. 3. These represent the volumes that the beam would affect if only one pixel area was irradiated. The one-, two- and three-photon volumes (Fig. 3B,C,D, respectively) are illustrated so that they are directly comparable in relative size to each other. The relative volumes within a live cell nucleus are demonstrated by the projection in Fig. 3A. The calculations neglected phase aberrations caused by refractive index changes and intensity changes due to absorption that may have some effect in the case of three-photon, 750 nm absorption. The volume affected by three-photon absorption is $\sqrt{3}$ times smaller than one-photon absorption, and two-photon absorption is $\sqrt{2}$ times smaller than one-photon absorption. The shapes of the one- and two-photon excitation profiles also differ slightly from the three-photon excitation volume. The length (axis parallel to the laser beam) and the equatorial diameter are given for each volume at the contours where the beam is 50% (Table 1) and 10% (Table 2) of the peak intensity. It can be seen from these dimensions that no collateral radiation will spread outside the cell nucleus, which is 10–20 μm in size. Measurement of the fluorescent intensity across a line of CPDs created by the focused 750 nm laser beam indicates that 50% of the peak value of intensity of the beam is about the minimum intensity at which three-photon absorption gives rise to CPDs. The width of this cross-section of fluorescent-stained CPDs is 450 nm (data not shown). The distribution of the point spread function in the axial plane (z-axis) obtained by confocal imaging does not allow an accurate estimate of the extent of CPD formation through the depth of the cell.

Two-photon excitation from 750 nm radiation will excite molecules that absorb at 375 nm. At this wavelength, flavins ($\lambda_{\text{max}} = 370 \text{ nm}$) and NADH ($\lambda_{\text{max}} = 340 \text{ nm}$) are potential en-

dogenous cellular absorbers. The major localization site of these coenzymes is the mitochondria, which are situated in the cytoplasm. The method that we have described here confines two-photon excitations within the cell nucleus; thus, it is unlikely that significant absorption will occur at 375 nm and collateral effects from this radiation will be minimal.

Despite the intense NIR radiation required to produce three-photon absorption in the cellular DNA, effects from heating do not occur. The absorption of NIR by DNA and RNA is known to be almost negligible. To estimate the possibility of heating, the absorption by water must be considered the most relevant factor. At a similar pulse energy to that used here (10 mW at 82 MHz), a

Table 1 | Dimensions of the volume affected in the cell up to 50% of the peak intensity of the beam

	Equatorial diameter (nm)	Length (nm)
One-photon	366	814
Two-photon	259	542
Three-photon	211	415

Table 2 | Dimensions of the volume affected in the cell up to 10% of the peak intensity of the beam

	Equatorial diameter (nm)	Length (nm)
One-photon	667	2,441
Two-photon	472	1,197
Three-photon	385	847

Fig. 3 | Numerically evaluated contour plots of the affected volume of one-, two- and three-photon near-infrared intensities when the beam dwells on one individual pixel area. Table 1 gives the dimensions of the volumes extending from the central peak intensity of the beam to 50% of this intensity. Table 2 gives the dimensions of the volumes extending from the central peak intensity to 10% of this intensity. The legend depicts which colours represent the various fractions of the peak intensity of the laser beam. The projection of these onto a diagram of a cell drawn to scale (A) illustrates the relative volumes in the cell nucleus. The vertical axis of this plot represents a plane lying parallel to the axis of the laser beam and therefore the shape of the affected volume in the cell nucleus if it is viewed from the side (z-plane).

temperature rise of about 10^{-5} K was noted (Langford *et al.*, 2001). The most important factor is whether the rate of cooling during the 82 MHz pulse train is sufficiently fast when compared to the rate of heating. Integral solutions for heating by absorption at the focus of an objective lens estimate that for a focused beam of 750 nm, a mean laser power of 100 mW, and through an objective of 1.2 NA, the temperature rise due to absorption by water would be 0.11 K in 1 ms (Schönle & Hell, 1998). For a dwell time of 25 ms per pixel and a mean power of 10 mW, the maximum possible temperature rise would be 0.25 K. As the interval between pulses used to create the three-photon NIR absorption in cellular DNA is 12 ns, there is sufficient time to allow diffusion of heat out of the irradiated volume and thus the actual temperature rise will be considerably less than 0.25 K.

In the absence of a physical method of measuring direct heating effects from the intense NIR irradiation, the cellular responses reflect the extent of physical and chemical processes. The viability of cells is maintained even when femtosecond laser microscopy of living cells has been performed for several minutes with a peak intensity of about 200 GW cm^{-2} (König, 2000). High intensities in the TW cm^{-2} range are required to produce intracellular plasma formation (König *et al.*, 1999a), and this is confined to the central part of the illumination spot. In contrast to nanosecond laser pulses, femtosecond laser pulses do not generate thermal damage in the surrounding structures (König *et al.*, 1999a). We detected no significant effects on cell viability in our experiments, and we thus conclude that the only significant excitation arising from the irradiation applied in our experiments is confined to the DNA damage illustrated. Any RNA lying in the focus of the three-photon NIR absorption would also suffer damage in the form of CPDs, but RNA was removed from these samples by RNase treatment before antibody staining.

The estimates of the affected volumes presented here are approximations, but calculations that take into account beam aberrations created by absorption and refractive index changes will estimate the volume of the damage spot more accurately. The details of the media (oil, coverslip and cells) affect the intensity distribution, but provided an objective is used with the correct combination of media it should produce an optimized focus characterized by the system NA.

Repair and movement of photoproducts

Cells were irradiated using the pattern seen in Fig. 1 and then replaced in a CO_2 incubator at 37°C . Fig. 4A shows the loss in intensity and definition of the staining of CPDs when the cells were incubated for 30 min before staining. In this case, the loss of intensity suggests that significant repair of the lesions has occurred. Movement of the lesions also takes place as can be seen in Fig. 4B, which shows dramatic changes from the inscribed pattern DNA. Compaction and migration of the remaining lesions to the periphery of the nucleus are clearly evident in some cells. The damage induced here is relatively sparse compared to that inflicted under whole or partial cell irradiation by one-photon UV light from a mercury lamp. Initial estimations suggest that a maximum of 30 lesions are produced per pixel area of 500 nm^2 at the intensity of NIR used here (see supplementary data online). A pattern covering 100 pixels would induce about 3,000 lesions. This means that the loss of movement during the repair of relatively small numbers of lesions can be discerned especially

Fig. 4 | Repair and movement of three-photon near-infrared (NIR)-induced photoproducts. The image seen here is that obtained by the immunostaining of cyclobutane pyrimidine dimers (CPDs) after the cells were replaced in a 37°C CO_2 incubator for 30 min following the same focused NIR radiation. The reduction in the intensity and the blurring of the pattern is evidence of repair of CPDs in the cells in (A). (B) Distinct movement and compaction of the lesions are seen since the inscribed pattern of radiation on the cell was in the form of the letters DNA.

when the damage is generated in a distinctive pattern. If this is carried out on repair-inhibited cells or repair-deficient cells, the movement of lesions due to only DNA diffusion or chromatin movement should be distinguishable.

The incidence and repair of 6–4 photoproducts and oxidative DNA damage such as 8-hydroxyguanine lesions are the focus of ongoing studies.

Localization of a repair protein to the photoproducts

Localization of proliferating cell nuclear antigen (PCNA) to the DNA damage induced by the focused infrared laser beam was seen to occur in a dose-dependent and time-dependent manner. Fig. 5 shows the protein accumulation on a pattern of damage induced in a W shape. The incident radiation was adjusted to give the approximate equivalent of damage levels induced by single-photon UV at 250 nm at 1, 5 and 10 J m^{-2} (see supplementary material online for calculations). The minimum time at which protein localization is shown here is 5 min, but at the highest dose the PCNA was seen to localize at the damage within 1 min. As DNA repair proteins can be imaged almost immediately after radiation of the cells, the method increases the time resolution with which the evolution of repair of DNA photoproducts may be followed.

Conclusions

The images presented here show that three-photon absorption is a versatile tool for the introduction of UV photoproducts in DNA in a geometrically defined fashion. The resolution obtained is considerably higher than that obtained using polycarbonate micropore filters to mask single-photon UV light at 260 nm. In addition to giving much higher resolution, the damage can be focused on a specific cellular structure that can be visualized and positioned microscopically to introduce a controlled level of damage in a spatial volume, unlike the methods that use masking of UV light to introduce localized damage in cells in a random manner. The choice of 750 nm NIR facilitates induction of UV photoproducts by three-photon absorption with minimal collateral

effects of two- and one-photon radiation, and as the infrared wavelength is below $1\ \mu\text{m}$, heating effects are avoided.

In relation to the application of multiphoton imaging techniques to biological experimentation and medical diagnostics, the data presented here allow evaluation of the likelihood that DNA damage may be coincidentally induced.

Fig. 5 | Localization of proliferating cell nuclear antigen (PCNA) to three-photon near-infrared (NIR)-induced DNA photoproducts. The notation on the left side shows the estimated equivalent levels of ultraviolet intensity that the incident NIR beam would give rise to when focused on CHO GFP-PCNA cells. The pattern consisted of 150 pixels; therefore, the number of cyclobutane pyrimidine dimers induced at 1, 5 and $10\ \text{J m}^{-2}$ is 4,500, 22,500 and 45,000, respectively. The notation along the bottom shows the time after irradiation at which the image was collected.

Fig. 6 | Schematic outline of the experimental set-up.

MATERIALS AND METHODS

Laser irradiation of cells. A 750 nm laser beam from a Spectra-Physics Tsunami titanium–sapphire laser (82 MHz, pumped by an argon-ion laser) of pulse length 120 fs was focused through an Olympus Planapo $\times 40$ objective with a focal length of 2 mm and an NA of 0.85. The pulse length of 120 fs was chosen since shorter pulse lengths cause damage to cells (König *et al.*, 1999b). Live cells growing on a glass coverslip were brought into focus under white light and positioned using a micromanipulator stage so that the beam would centre on a cell nucleus. A schematic illustration of the experimental set-up is shown in Fig. 6. The power output of the laser was attenuated with neutral density filters to give a final power of 10 mW at the stage. The peak fluence of the IR laser beam that induced UV photoproducts detected was calculated to be about $190\ \text{GJ cm}^{-2}$. DNA damage was induced and detected at a minimum laser power of 10 mW when the dwell time at each pixel position ($0.25\ \mu\text{m}^2$) was 25 ms. When the power was reduced to 5 mW, no CPDs were detected. This reflects the cubic relation of the three-photon absorption to the peak intensity of the NIR radiation, and that the induction of the lesions is a nonlinear process. The peak fluence of the IR laser beam that induced UV photoproducts detected was calculated to be approximately $190\ \text{GJ cm}^{-2}$. The laser beam was guided by a galvanometric scanning mirror moving at a rate of about 1 kHz and was interfaced with computer software that generated a chosen pattern.

Cell culture and antibody staining. MH1C1 rat liver epithelial cells were grown to confluence in Dulbecco's modified Eagle's medium supplemented with antibiotics. The choice of MH1C1 rat liver epithelial cells was arbitrary.

Chinese hamster ovary (CHO) green fluorescent protein (GFP)-tagged PCNA cells were supplied by Jeroen Essers. Antibody staining was carried out as has been described previously (Mori *et al.*, 1991).

Images of fixed and antibody-stained cells were recorded using a Biorad Radiance 2000 laser scanning system and a Plan Apo $\times 60$, 1.4 NA oil immersion lens with a working distance of 0.21 mm. z-Plane scans were taken at steps of 500 nm. Confocal images of the GFP-PCNA cells were recorded by an MRC600 laser scanning system coupled to a Nikon TE 2000 microscope.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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