

Direct measurement of the 3-dimensional DNA lesion distribution induced by energetic charged particles in a mouse model tissue

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Charged particles are increasingly used in cancer radiotherapy and contribute significantly to the natural radiation risk. The difference in the biological effects of high-energy charged particles compared with X-rays or γ -rays is determined largely by the spatial distribution of their energy deposition events. Part of the energy is deposited in a densely ionizing manner in the inner part of the track, with the remainder spread out more sparsely over the outer track region. Our knowledge about the dose distribution is derived solely from modeling approaches and physical measurements in inorganic material. Here we exploited the exceptional sensitivity of γ H2AX foci technology and quantified the spatial distribution of DNA lesions induced by charged particles in a mouse model tissue. We observed that charged particles damage tissue nonhomogeneously, with single cells receiving high doses and many other cells exposed to isolated damage resulting from high-energy secondary electrons. Using calibration experiments, we transformed the 3D lesion distribution into a dose distribution and compared it with predictions from modeling approaches. We obtained a radial dose distribution with sub-micrometer resolution that decreased with increasing distance to the particle path following a $1/r^2$ dependency. The analysis further revealed the existence of a background dose at larger distances from the particle path arising from overlapping dose deposition events from independent particles. Our study provides, to our knowledge, the first quantification of the spatial dose distribution of charged particles in biologically relevant material, and will serve as a benchmark for biophysical models that predict the biological effects of these particles.

charged particles | radial dose distribution | biodosimetry | γ H2AX foci | local effect model

Charged particles, including protons, α -particles, and heavy ions, are increasingly used in cancer radiotherapy and represent a significant component of the natural background irradiation on earth and in space (1–3). Their biological effect is often very different from that of photons (X- or γ -rays), largely because charged particles deposit their energy along a track, whereas photons produce a fairly homogeneous dose distribution. Linear energy transfer (LET; typically given in units of keV/ μ m) has been introduced as a parameter to describe the amount of energy that charged particles deposit along their track. Particles with high LET are densely ionizing and typically biologically more effective than photons or low-LET particles.

Energy deposition along a particle track is not restricted to the path itself (the so-called “track core”), but extends laterally into an area known as the penumbra of the particle, which can reach considerable distances for high-energy particles. Energy deposition in the penumbra arises from energetic secondary electrons, so-called δ -electrons, which are generated by ionization events of the charged particles and carry energy away from the immediate path into the penumbra. According to classical track structure theory, ~50% of the total energy is deposited in the penumbra,

where it spreads out over a much greater volume than the energy in the track core (4). Thus, the penumbra represents a sparsely ionized region within the track of high-LET particles. Thus, charged particles deposit their energy in a complex 3D manner and typically comprise a spectrum of high- and low-ionization densities. The total biological effect of a charged particle is a result of this complex energy deposition pattern.

Our knowledge about the energy deposition pattern of charged particles is based largely on theoretical predictions and physical measurements in inorganic material. Most information in this respect was gained from microdosimetric experiments with gas-filled detectors (5). In these devices, the dose distribution around an ion trajectory is measured and rescaled to a track structure profile in water by comparing the electron density of the gas with that of water. Such a density scaling approach is expected to fail, however, when the energies of the δ -electrons are in the same order of magnitude as the intermolecular binding energies (6, 7). These limitations call for alternative approaches for assessing the 3D energy deposition pattern of charged particles directly in biologically relevant material.

Computational models, mostly Monte Carlo codes, have been developed to describe the energy deposition patterns of charged particles. Results from such approaches are in reasonable agreement with physical measurements, although track structure modeling at a nanometer or micrometer scale remains a challenge.

Significance

Charged particles are applied in cancer radiotherapy because they are more efficient than X-rays or γ -rays in tumor cell killing. This efficiency results from the high dose deposition along the path of the particles. However, charged particles damage tissue inhomogeneously, such that many cells not directly hit by the particles receive a low dose and can survive with mutations. Because mutations can lead to secondary malignancies, this effect limits the applicability of charged particles in cancer radiotherapy. We provide the first direct measurement of the 3D DNA lesion distribution induced by energetic charged particles in a mouse model tissue. Our detailed analysis of the dose distribution will serve to benchmark biophysical models currently used for irradiation planning in cancer radiotherapy.

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Computational models are also used to predict the biological effects of charged particles, such as the induction of DNA damage (8–11). Unlike Monte Carlo calculations, amorphous track structure models exploit the radial dose distribution to predict the biological response (12, 13). One of these approaches, known as the local effect model (LEM), is currently applied in particle cancer therapy to predict the biological effects of heavy ions (14, 15). The LEM assumes that the same local dose, independent of the radiation type that deposits this dose, leads to the same biological effect. Essentially, LEM derives the biological effects of charged particles from the response of cells or tissues to photon radiation.

Double-strand breaks (DSBs) are among the most hazardous DNA lesions induced by ionizing radiation (IR) because they can give rise to more complex lesions when occurring in clusters or associated with other lesions (16). On DSB induction, the histone variant H2AX is phosphorylated at the break site to γ H2AX (17). This histone modification can be visualized by microscopy and forms so-called γ H2AX foci, which arise within minutes after IR in a 1:1 ratio to isolated DSBs and are lost with time due to DSB repair (18, 19). Because this technology detects single isolated DSBs in single cells, it has exceptional sensitivity and can monitor the effect of IR doses of a few mGy (20, 21). Not surprisingly, γ H2AX foci analysis has been applied to assess radiation doses encountered by humans during diagnostic medical procedures, such as computed tomography scanning (22–25). Moreover, this technology can be applied to different tissues (26, 27) and is becoming the gold standard for various biodosimetric applications (28, 29).

In the present study, we combined the particular sensitivity of the γ H2AX foci technology with its second major advantage, the ability to precisely determine the position of the DNA lesions, to investigate the spatial distribution of γ H2AX foci induced by charged particles. Calibration experiments allowed transformation

of the lesion distribution into a 3D dose distribution, which could be compared with predictions from the amorphous track structure model, the LEM. Using this procedure, we were able to quantify the lateral (radial) dose profile of titanium (Ti) ions with sub-micrometer resolution and verify the theoretically predicted $1/r^2$ dose decline using a biological model system.

Results

Charged particles deposit high doses in the core (i.e., the inner part) of their track and low doses in the surrounding penumbra (i.e., the outer part). Because the physical dose deposited by δ -electrons in the outer part of the track is predicted to decrease strongly with the distance to the particle path (5, 30), we aimed to biologically quantify the dose in a 3D geometry around the path. We used mouse tissue as a model system and quantified DSBs by counting γ H2AX foci, which arise linearly with radiation dose (18, 19, 31). To further enhance the sensitivity of this biodosimetric approach, we investigated the mouse retina with its densely packed and uniformly arranged photoreceptors (32). Lamin B staining showed that mouse photoreceptors contained a low amount of cytoplasm and little intercellular space (Fig. 1A). The nuclei of photoreceptors contained a central heterochromatic chromocenter, which is surrounded by euchromatic DNA (33) (Fig. 1B). This specialized chromatin structure represents a unique adaptation of photoreceptors to optimal vision in low-light conditions (33). Collectively, the various special features of the mouse retina make it an ideal model system for analyzing the spatial dose distribution of charged particles.

γ H2AX Foci in Mouse Photoreceptors After Low X-Ray Doses. To characterize the formation of γ H2AX foci in mouse photoreceptors, we quantified the number of foci per cell at different time

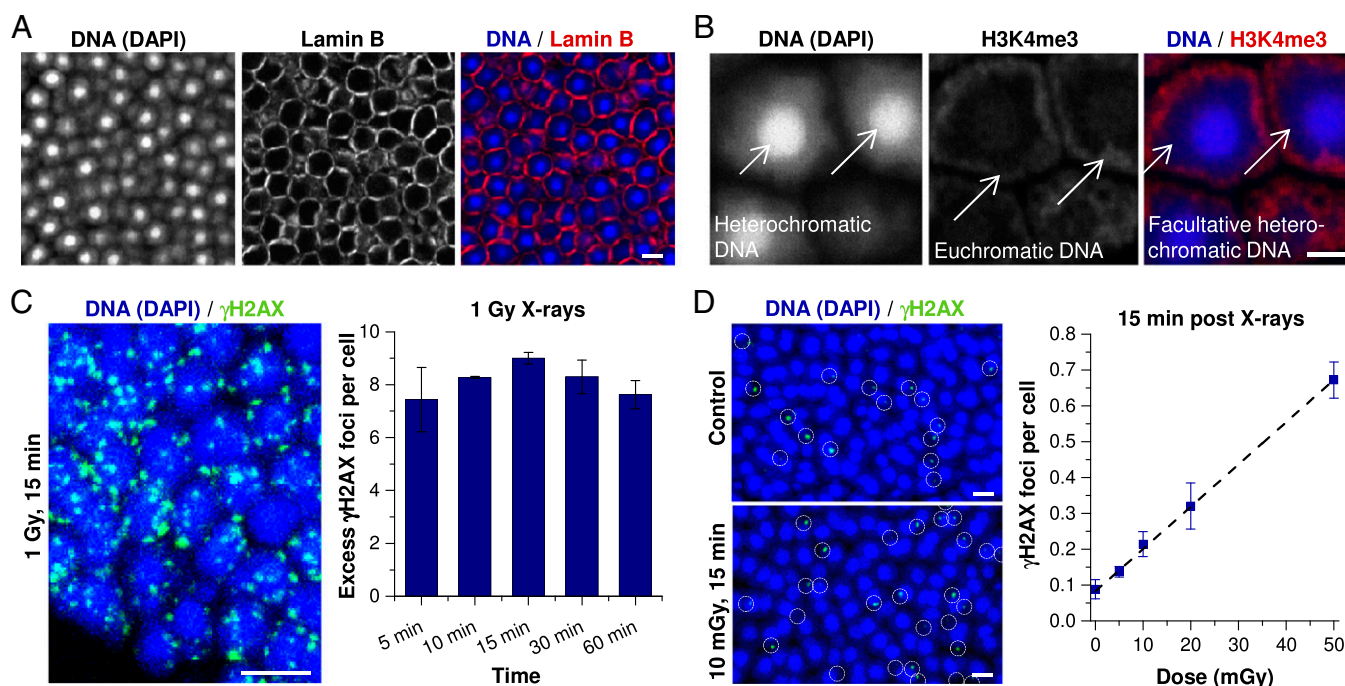


Fig. 1. γ H2AX foci in mouse photoreceptors after low X-ray doses. (A) IF image of Lamin B in mouse photoreceptors. (Scale bar: 5 μ m.) (B) IF image of H3K4me3 in mouse photoreceptors. Different chromatin regions in the nucleus are indicated by white arrows. (Scale bar: 2 μ m.) (C) Time-dependent quantification of γ H2AX foci per cell after receipt of 1 Gy X-irradiation. Retinae were left unirradiated or were analyzed at 5–60 min postirradiation. (Left) IF image (maximum intensity projection, MIP) of γ H2AX in irradiated photoreceptors. (Scale bar: 5 μ m.) (Right) Spontaneous γ H2AX foci (fewer than 0.1 per cell) were subtracted. Error bars represent the SD between two retinae with 40 cells each. (D) Quantification and regression analysis of γ H2AX foci per cell after various X-ray doses. Retinae were left unirradiated or were analyzed at 15 min after receipt of 5–50 mGy. (Left) IF images (MIP) of γ H2AX in unirradiated or irradiated photoreceptors. γ H2AX foci were framed with white circles for better visualization. (Scale bars: 5 μ m.) (Right) Error bars representing the SD among three retinae with 1,500 cells each ($R^2 = 0.994$).

could be readily visualized by a chain of relatively big and bright γ H2AX foci over numerous cell nuclei (Fig. 24; Fig. S24 shows the geometry of an imaged tissue section relative to the ion beam). In addition, smaller and less intense γ H2AX foci were observed in the cells surrounding the particle trajectory, representing isolated DSBs induced by δ -electrons (Fig. 24). The average number of ion traversals visible on a single microscopic image of approximately $100 \times 100 \times 12 \mu\text{m}^3$ was less than one after irradiation with 7.5×10^3 and 7.5×10^4 ions/cm² and increased proportionally with higher fluences (Fig. S2B).

To reconstruct the trajectory of the particle in the tissue, we approximated the location of each focus by its center and performed a 3D regression analysis of the chain of foci representing the inner part of the track (hereinafter termed γ H2AX_I; indicated in red in Fig. 24, *Right*, and displayed in 3D in Fig. S2C). We then calculated the distance of all γ H2AX foci in the outer region of the track (γ H2AX_O) to the approximated ion trajectory. Because irradiation occurred in living tissue, whereas the analysis of foci position was performed in tissue after fixation, cutting, and staining, we performed control experiments to verify that cell dimensions were not altered by this procedure (Fig. S2D).

The first step of our analysis involved distinguishing the γ H2AX_I foci in the inner part of the track from the γ H2AX_O foci in the surrounding area. For this analysis, we separated γ H2AX foci located $<2 \mu\text{m}$ from the regression line from foci located farther away. Approximately 99% of the γ H2AX_I foci that were identified by eye to represent the inner part were positioned within $2 \mu\text{m}$ (Fig. S2E). We then measured the size and intensity of each focus and confirmed our visual impression that γ H2AX_I foci along the trajectory were on average larger (Fig. 2B) and brighter (Fig. S2F) than γ H2AX_O foci in the surrounding region (diameter, $\sim 0.5 \mu\text{m}$ vs. $\sim 0.3 \mu\text{m}$; mean signal intensity, 142 vs. 99). Interestingly, the γ H2AX_O foci were similar in size to X-ray-induced foci, consistent with the notion that foci after X-irradiation as well as foci in the outer part of the particle track are induced by sparsely ionizing δ -electrons.

We next determined the number of foci per cell below and above the $2 \mu\text{m}$ limit. For this, we approximated the location of each cell by its center. Thus, a cell with its center inside the $2 \mu\text{m}$ border was considered to fall within this region even though it partly protruded beyond the $2 \mu\text{m}$ limit, whereas a cell with its center outside the $2 \mu\text{m}$ border was not considered for the 0 – $2 \mu\text{m}$ category even if it protruded inside this area. We obtained an average of 5.4γ H2AX foci/cell inside the 2 – μm border and 0.12γ H2AX foci/cell at 2 – $10 \mu\text{m}$ (Fig. 2C).

Because both γ H2AX_O and X-ray-induced γ H2AX foci originate from secondary electrons, we converted the number of 0.12γ H2AX foci/cell into a dose of 10 mGy by using the induction rate of $11.8 \text{ foci/cell per Gy}$ obtained in Fig. 1D. This dose of 10 mGy , which was evaluated based solely on biological measurements, is in excellent agreement with a theoretical track structure model that predicts an average dose of approximately 8 mGy at 2 – $10 \mu\text{m}$ around Ti ion trajectories (14). These results provide proof of principle that our biological dosimetry approach yields meaningful dose estimates in the low-dose range.

In the second step of our analysis, we investigated the radial decline in dose with increasing lateral distance from the particle path. We extended the analysis up to a distance of $20 \mu\text{m}$ and assessed the number of γ H2AX foci per cell in 10 categories with a size of $2 \mu\text{m}$ each. We observed a decrease in γ H2AX foci per cell in the first four categories up to $8 \mu\text{m}$, which is in reasonable agreement with the theoretically predicted decline with increasing distance from the approximated trajectory (Fig. 2D). However, the agreement between γ H2AX foci and predicted X-ray dose is less convincing in the first category (0 – $2 \mu\text{m}$) where the measured foci arise from the densely ionized inner part of the particle tracks. Moreover, γ H2AX foci numbers per cell varied considerably for categories larger than $8 \mu\text{m}$, precluding a reliable analysis of the

dose profile above this distance. Thus, despite the apparently good correlation between measured foci and predicted doses, this agreement was essentially limited to three size categories.

Radial and Longitudinal Distance Analysis of γ H2AX Foci. To further refine the observed decline of γ H2AX foci with increasing distance from the particle trajectory, we enhanced the statistical power in the categories further away from the trajectory where low γ H2AX foci numbers resulted in high variations. The enhancement was achieved by defining categories that increase in width with increasing distance from the trajectory. We applied a logarithmic scale in which the distance between 0.1 and $20 \mu\text{m}$ is divided into 36 categories, with the width of each category increasing proportionally to the distance from the trajectory. This procedure reduces the data scatter in the larger categories, but has limitations for the smaller categories close to the particle trajectory (below approximately $0.5 \mu\text{m}$), because the number of cells in these categories is very small. Thus, we introduced a further step in the analysis and reconstructed the analyzed tissue volume using small voxels with a dimension of $0.8 \times 0.8 \times 0.645 \mu\text{m}^3$ (Fig. S3A).

Using the above-described procedures, we determined the number of γ H2AX foci per voxel as a function of the logarithm of the distance to the approximated particle trajectory. We also used a logarithmic scale for the vertical axis, given that a double-logarithmic plot allows visualization of the expected $1/r^2$ dependency as a linear function. For the three independent experiments from Fig. 2D, we obtained a plateau for γ H2AX foci per voxel in the inner region up to a distance of approximately $0.4 \mu\text{m}$, followed by a decrease slightly steeper than a $1/r^2$ dependency between 0.4 and $4 \mu\text{m}$ and by another plateau region above $4 \mu\text{m}$ (Fig. 3A). Although the decreasing foci numbers in the middle region roughly follow the theoretical prediction, the plateaus in the smaller and larger categories reflect expected experimental restrictions. The resolution of the focus structure with a typical size of roughly $0.5 \mu\text{m}$ and slight foci movement processes (37, 38) (Fig. S2E) might contribute to the deviation from the $1/r^2$ dependency below $0.4 \mu\text{m}$, and also might give rise to a slightly steeper decrease than expected above $0.4 \mu\text{m}$. Moreover, dose depositions from neighboring particles below or above the analyzed tissue slices represent a background dose and contribute to foci numbers in larger categories, which can explain the deviation from the $1/r^2$ dependency above $4 \mu\text{m}$.

We next compared our experimental data with a theoretical model for high-LET radiation that is based on the dose deposition within particle tracks and takes the restrictions of the experimental system into account. We exploited the LEM (14) and considered the track structure description of the local doses deposited around a particle trajectory, as well as the enhanced DSB production for high doses (i.e., close to the trajectory) from the induction of two single-strand breaks from two different δ -electrons. We found that the latter effect played only a marginal role in the particle energies used, however, and had only a slight effect on the simulated focus yields in the inner part of the track. Fig. 3A shows that the simulation reflects the experimental foci distributions. In particular, the dose decrease for intermediate distances was well reproduced and slightly steeper than the expected $1/r^2$ dependency. For small distances ($< \sim 0.4 \mu\text{m}$), the number of foci was slightly higher in the simulation compared with the experiments. For large distances ($> \sim 4 \mu\text{m}$), the data from the three different experiments lie within the predictions for the two different ion fluences used in these experiments.

To evaluate the fluence dependency of the background dose with better statistics, we also analyzed images from irradiated retinæ without visible particle tracks. Of note, the two high fluences provided a substantially higher background dose than the low fluence (Fig. S3B).

part of the particle track, which is consistent with the notion that several nearby DSBs in this high-dose region can form a single, microscopically detectable focus. Such DSB clustering was previously observed by others (40, 43–46) and is also evident from our analysis of foci sizes and intensities showing that foci in the inner part of the particle track were on average larger and brighter than foci in the outer part of the track.

Cells hit directly by the track core can have a low probability of survival, but the tissue exposed in the penumbra will almost certainly survive with little damage. Because the extension of the penumbra increases with particle velocity, tissues exposed to very high-energy ions, such as those in space or used in therapy, will experience hotspots of high doses and large volumes with low dose exposure. Controversy surrounding the effects of low doses persists (2, 47, 48), but our results support the view that every exposure to charged particles should be considered a 3D entanglement of high-dose and low-dose DNA damage induction in tissues.

Materials and Methods

All animal experiments were approved by the Regional Board of Darmstadt. Details of the preparation, irradiation, and immunostaining of retina explants; image acquisition and evaluation; and modeling analysis are provided in *SI Materials and Methods*.

Preparation of Retina Explants. After the mice were killed, the eyes were removed, and the retinæ together with lenses and vitreous bodies were isolated and cultured in medium before irradiation.

Irradiation. Low-LET irradiation was performed with an X-ray machine at two different settings. High-LET Ti ion irradiation was performed at a synchrotron accelerator of the GSI.

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