

DNA double-strand breaks induced along the trajectory of particles

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ABSTRACT

It is well-known that the DNA damage caused by charged particles considerably differs from damage due to electromagnetic radiation. In the case of irradiation by charged particles the DNA lesions are more complex and clustered. Such clustered damage is presumed difficult to be repaired, and is potentially lethal. In this study, we utilize a 90°-scattering system and related imaging techniques to investigate the accumulation of γ -H2AX along the trajectory of charged particles. By immunostaining the γ -H2AX protein, optical images of corresponding double strand breaks were observed using a high resolution confocal microscope. We demonstrate the difference in the accumulation of γ -H2AX from irradiation by 1 MeV protons and that of 150 keV X-rays. The acquired images were arranged and reconstructed into a 3D image using ImageJ software. We discovered that the γ -H2AX foci, following irradiation by protons, have a tendency to extend in the beam direction, while those from X-ray irradiation tend to be smaller and more randomly distributed. These results can be explained by the physical model of energy deposition.

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1. Introduction

Radiobiological events such as cell death, mutagenesis, and carcinogenesis may be the result of DNA damage caused by ionizing radiation. Complex strand breaks are usually irreparable and lethal. Until now, the mechanisms behind radiation induced DNA damage and the ensuing repair process remained unclear. Charged particles can lead to complex and clustered damage to DNA, resulting in higher relative biological effects (RBE) than electromagnetic radiation [1]. It is known that the mechanisms governing energy loss in target cells is different when tissue is irradiated with charged particles or electromagnetic radiation. The transfer of energy and damage of DNA are related to cell response. In this work, we demonstrated a novel technique to directly observe DNA lesions resulting from irradiation by charged particles. We used a 90°-scattering system providing a uniform vertical beam of charged particles. The innovation of this approach was to replace the 90° magnet with a gold scattering foil [2]. In this manner, the high cost and complications involved in setting up an irradiation system could be reduced. Furthermore, a silicon nitride (Si_3N_4) membrane was applied to the system to eliminate energy loss and diffusion of the beam during propagation.

In the cell irradiation experiment, the damage to DNA was graded according to the expression of γ -H2AX protein. The H2AX protein is a member of the histone H2A family [3]. When double DNA strands break (DSB), the H2AX protein is extensively phosphorylated by ATM on serine 139 [4]. The phosphorylated H2AX protein is called γ -H2AX. The phosphorylation process is initiated in 1–3 min, reaching its maximum within 30 min [3]. The expression of γ -H2AX surrounding DSBs produces a docking site for many DNA repair proteins, such as 53BP1, MRE11-RAD50-NBS1 complex and BRCA1 [5–8]. For this reason, the γ -H2AX has been acknowledged as a damage marker in DNA [9]. In a previous study, we designed a proton scattering system to provide a convenient source of protons for radiobiological investigation [2]. In this study, we continue cell irradiation experiments using this system, and compare the results with those of X-rays. HeLa cells were incubated in a silicon nitride base culture dish and irradiated using both 1 MeV proton beam and 150 kVp X-ray. Following irradiation, a high resolution confocal microscope was used to acquire images of the γ -H2AX foci. The acquired images were arranged and reconstructed into 3D images using ImageJ software [10].

2. Materials and methods

2.1. Proton Irradiation system

A 90°-scattering cell irradiation system was installed at 3 MV HVEE (High Voltage Engineering Europe) Van de Graaff accelerator

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of the Accelerator Laboratory of National Tsing Hua University, Taiwan. A schematic diagram of this system is shown in Fig. 1(a). A 100 nm gold scattering foil was placed at the center of the scattering chamber, to replace the 90° bending magnet. When incident protons bombarded the scattering foil, they collided with target atoms to be scattered isotropically. An exit window for the beam was opened above the scattering foil and covered with a Si₃N₄ membrane to provide a vacuum seal. The particles scattered at right angle were able to pass through the 100 nm Si₃N₄ membrane and reach the cell sample. The design of a vertical beam was well suited to cell placement, preventing cell migration during irradiation. The details of the experimental setup are provided in a previous study [2].

We designed a cell culture dish to allow scattered protons to penetrate its base and reach the targeted cell. The dish consisted of three parts: the cover, the body and the open window bottom, (Fig. 1(b)). A 100 nm thin Si₃N₄ wafer was clipped between the body and bottom with an O-ring, and two open windows were used to divide the cells into the irradiation group and the control group. The dish had the advantages of being pervious to light and non-toxic to cells during the irradiation experiments. Because the protons only had to penetrate two layers of 100 nm Si₃N₄ membranes with a 3 mm air-gap before reaching the cell target, the energy loss and energy dispersion during propagation were minimized. A cell support chamber was installed at the nozzle of the beam to provide an appropriate environment for the cell incubation. During the irradiation, the environment inside the chamber was maintained at 37 °C with 95% humidity and a continuous supply of 5% CO₂ mixed air. In this manner, we were able to maintain the cells in a humid and stress free environment during the irradiation [11].

Prior to irradiation, the accumulated current of scattered particles at the scattering foil was measured and registered. A silicon detector mounted on the beam nozzle was used to perform measurements of energy and fluence. Meanwhile, the integrated beam current at the scattering foil was measured using a calibrated current meter. To consolidate these results, a conversion ratio of integrated scattering foil current to the exiting particle flux was retrieved. In this manner, the energy deposition in the target cell could be evaluated according to the linear energy transfer (LET) database [2]. Prior to the cultivation of cells, the culture dishes were cleaned using distilled water and sterilized for 1 h by UV irradiation. The cell line used in this study was HeLa cells, a human cervix carcinoma [12,13]. The cells were planted on the Si₃N₄ membrane at a density of approximately 100 cells per square millimeter, and incubated for 24 h, after which the culture dish was moved into the cell support chamber and aligned with the nozzle of the beam. The cells were irradiated using 1 MeV protons and subsequently moved back to the incubator for 30 min. During this

period, the number of phosphorylated H2AX proteins reached its maximum [12]. The proton intensity was maintained in the range of 6000–24,000 particles per square millimeter, or 8–32 particles in a cell nuclear region. Particle tracks were easily discriminated using this low intensity of protons.

2.2. The X-ray irradiation system

An XR200 portable X-ray tube (Golden Engineering) was used as a photon source. This tube was capable of providing a series of stable 150 kVp X-ray pulses. The throughput of this tube was 32 milli-Roentgens (mR) per 10 pulses at a point 30 cm in front of the window. During the cell irradiation experiment, the X-ray dose was controlled in the range of 8–32 mR. Following X-ray irradiation, the culture dish was incubated for 30 min to ensure the completion of the phosphorylation process. A dish of non-irradiated cells was used as control group.

2.3. The immunofluorescence staining

After incubation for 30 min, the cells were fixed and permeabilized using Methanol for 15 min at 4 °C. Following the fixation, the cell samples were blocked three times using PBS with 2% BSA solution at room temperature, each for 10 min [14]. Primary mouse monoclonal antibodies and secondary goat anti-mouse IgG antibodies conjugated with Alexa Fluor 488 dye (JBW301, Millipore) (A11001, Invitrogen) were used for immunofluorescence staining [15,16]. The phosphorylated H2AX proteins were labeled using primary antibodies and stored at 4 °C overnight. The following day, the cell samples were washed for 10 min using PBS with 2% BSA solution three times in order to remove the unbound antibodies. Subsequently, the cell samples were stained with secondary antibodies for 1 h at room temperature. Following the staining, the cells were washed four times with PBS, and mounted with antifade solution (AF3, Citifluor) [14].

2.4. The image acquisition

In this study, two types of images (wide field and confocal), were acquired using an inverted microscope (TE2000, Nikon) [17]. During the acquisition of the wide field images, the signals were excited using a UV lamp and collected with a Nikon NA1.2 60 × water immersion objective. For confocal microscopic applications, this microscope was equipped with a Nikon C1 scanning head. For the acquisition of confocal images, the objective lens was used to focus the excitation laser within the sample and collect the fluorescence signal. The collected fluorescence signal passed through a band-pass filter (Chroma Tech) and was detected using a photomultiplier [17]. Only the signal emitted from focal plane was acquired and registered, thereby enabling an improvement in image resolution. For the acquisition of confocal images, the field of view was set at 51.3 μm × 51.3 μm with a step size of 0.2 μm. The acquired image was reconstructed as a 3D image using ImageJ software with an ImageJ 3D viewer plug-in [10–18].

3. Results and discussion

Fig. 2(a) shows a γ-H2AX foci wide field image following irradiation with a 150 kVp X-ray. In the irradiation group, the foci signals were in high contrast with the background whereas in the control group, the image showed a dark blurred background (data not shown). When the wide field image was overlaid with a differential interference contrast image, Fig. 2(b), it became clear that all of the signals were located in the region of the nucleus. Moreover, the number of foci and signal brightness changed over the time (data

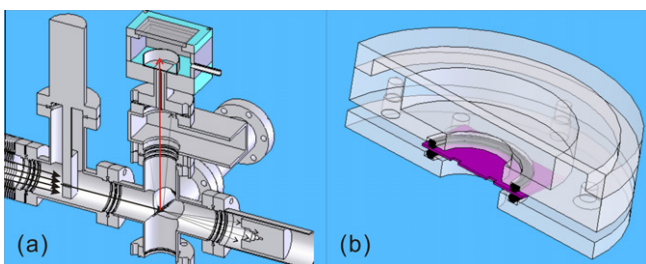


Fig. 1. A sketch view of the 90°-scattering irradiation system, and cell culture dish. (a) Irradiation system. Charged particles (black arrow) irradiate from the scattering foil in every direction. Only those particles scattered in specific solid angle (red arrow) are able to pass through the exit window, to irradiate the cell sample. (b) Cell dish. This dish comprised three plastic parts: cover, body, and open window bottom. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

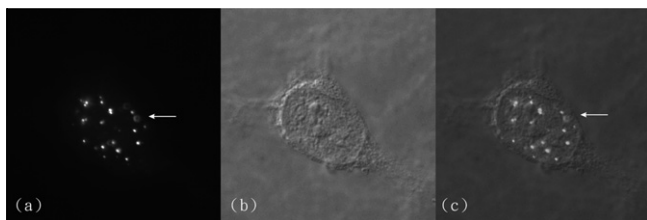


Fig. 2. A wide field image of γ -H2AX staining, (a) wide field image, (b) differential interference contrast image, and (c) the wide field image overlaid with the morphology image. The γ -H2AX foci, which were not formed near the focal plane of the objective (white arrow), appear blurred and unclear.

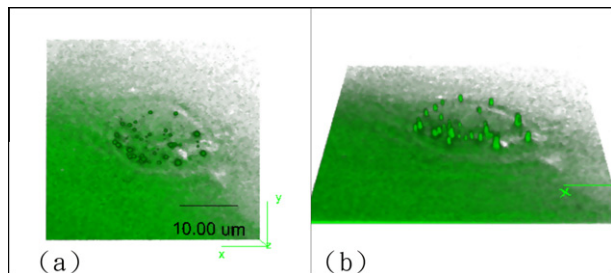


Fig. 3. A reconstructed 3D image of charged particle induced γ -H2AX foci, (a) top view and (b) oblique view. A portion of the γ -H2AX foci formed from the surface of the Si_3N_4 membrane and extending in the direction of the beam. The remainders, even not formed from the surface, still show a tendency to extend in the direction of the beam.

not shown). To consolidate these results, the foci signals were related to γ -H2AX protein, in which the γ -H2AX foci in the nucleus were randomly distributed throughout the 3D image, and only the γ -H2AX foci near the focal plane were focused and distinguishable. Nonetheless, the wide field images are still widely used in preliminary testing of the immunofluorescence staining.

Fig. 3 shows a reconstructed 3D image of proton irradiation of the γ -H2AX foci. The Si_3N_4 membrane and γ -H2AX foci are easily identified with a portion of the γ -H2AX foci formed from the surface of Si_3N_4 membrane extending in the beam direction. The rest, even those not formed from the surface, still had a tendency to extend in the beam direction. Using the image measurement tool, we estimated the size of proton induced γ -H2AX foci to be approximately 2–2.5 μm .

A reconstructed 3D image of γ -H2AX foci following 150 kVp X-ray irradiation is shown in Fig. 4. Compared to proton irradiation, the γ -H2AX foci appear much smaller and more randomly distributed. These findings are in good agreement with the physical model of energy deposition: charged particles are believed to release energy in matter through serial collisions with target atoms to form secondary electron clusters along the path of the particle. As a result, high density ionization events along the track of the particle may directly interact and damage DNA. In contrast to protons, photons release their energies in random events, such as Compton scattering and photoelectric effect, causing less direct interaction with DNA. Thus, in the nucleus of the cell irradiated with X-rays, the γ -H2AX foci appear smaller and more randomly distributed.

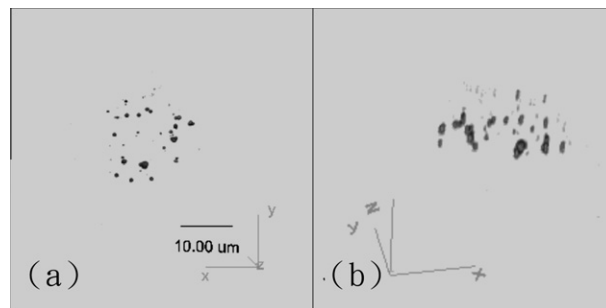


Fig. 4. Reconstructed 3D image of X-ray induced γ -H2AX foci, (a) top view and (b) oblique view. The γ -H2AX foci in the X-ray case were much smaller and randomly distributed.

4. Conclusions

In this study, we compared DNA lesions caused by proton- and X-ray irradiation. We discovered that the DNA double strand breaks measured by γ -H2AX foci have the tendency to extend in the direction of the proton beam, while those from X-ray irradiation to be more randomly distributed. The DNA damage due to proton- and X-ray irradiation can be discriminated in the 3-D image reconstructed from γ -H2AX foci. This is a useful technique for investigating biological expressions like apoptosis and oncogenesis. Moreover, the 90°-scattering system proved to be a reliable source of charged particles and an appropriate setup for radiobiological research.

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