Visualization of Heavy Ion Tracks by Labeling 3’-OH Termi

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Heavy ion/Bragg peak/Ion track/DSBs/TUNEL/3’-OH termini.

African green monkey kidney cells, CV-1, were irradiated with Carbon ions (LET: 735 keV/μm Argon ions (LET: 3,000 keV/μm Argon ions to visualize ion tracks through the cell nucleus by labeling the 3’-OH termini result of DNA strand breaks. The 3’-OH termini of DNA were labeled with BrdU-triphosphate catalyzed by TdT. This method of TUNEL (TdT-mediated dUTP Nick End labeling) is based on the specific binding of TdT to 3’-OH termini of DNA. Subsequent immuno-fluorescent staining with the primary monoclonal antibody against BrdU, followed by a secondary antibody of Alexa Fluor 488, was performed to visualize the BrdU labeled DNA termini. Images of the cell nuclei were acquired by confocal laser microscopy. When cell monolayers were irradiated perpendicularly with argon ions, induced DSBs in cell nuclei were identifiable as fluorescent spots. In another irradiation setup, when cells were irradiated at a small angle with incident argon ions, DNA strand breaks were detected as fluorescent stripes across the cell nucleus. These results demonstrate the induction of 3’-OH termini at sites of DNA strand breaks along Argon ion tracks.

INTRODUCTION

Compared to other ionizing radiation such as electrons or photons, charged particles such as protons, helium ion, and heavy ions have a different depth distribution of the deposited dose, peaking at an energy-dependent depth at the end of the particle’s track, so called Bragg peak, and decreases drastically beyond this peak. In the case of heavy ions near the Bragg peak of several MeV/n, densely distributed secondary electrons along its ion path become sufficient. Resultantly under these conditions, the qualitative biological effects of the ion species itself are considered to be most the dominant.1,2) An investigation of heavy-ion track structures, and interactions at the atomic and molecular levels are essential for understanding the biological effects of radiation exposure. However, no direct measurement of energy deposition inside ion tracks has been measured with nanometer scale resolution. Instead, information on the track structure is largely based on models, or measurements performed in solid materials or gaseous targets, which are estimated according to the ratio of the density of targets and the density of biological materials, such as water.3,4)

Advances in laser-scanning microscopy and immuno-fluorescence staining techniques, based on antibodies against proteins involved in the DNA damage response, have detected fluorescent foci indicative of sites of DNA damage clearly accountable to ion traversal through a cell nucleus. For example, γ-phosphorylated histone H2AX is widely used as a marker for DNA double strand breaks (DSBs),5,6) and the visualization of fluorescently stained γ-H2AX has been frequently used for the detection of ion tracks in cell nuclei.8–13) Jakob. B et al. uniquely reported on the biological imaging of heavy charged ion tracks in human fibroblast cell nuclei, obtained using a unique irradiation geometry, characterized by a small angle (below 5°) between the beam incidence and the cell monolayer.14) This irradiation geometry corrected for both the poor resolution along the ion track due to the extreme flatness of the cell nucleus and the lower resolution of the confocal microscope in depth compared to the lateral direction. This method was successful in detecting the biological response distribution along the ion track by visualizing co-localization of DNA repair protein with H2AX phosphorylation in shapes of stripes traversing several micrometers in the cell nucleus.15) However, a few hundred histone H2AX phosphorylation events are due to single

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DSBs: the visualized fluorescent signals may appear to be much larger than the DSB region considered as the ion tracks. A similar diminished resolution occurs when visualizing ion tracks using DNA DSB repair related proteins.\(^8\)\(^{-12}\)\(^{-14}\)\(^{-15}\) As in these cases, the visualized fluorescent spots in the irradiated cell nucleus differ greatly from the actual size of the DSB region because of indirect visualization of ion tracks due to the biological response of the cell.

Here, we demonstrate the use of the TUNEL (TdT-mediated dUTP Nick End labeling) method, which is based on the catalysis of Terminal deoxynucleotidyl Transferase (TdT) incorporating BrdU to 3'-OH termini of DNA, for the visualization of ion tracks by detecting only a specific type of DSB that possesses 3'-OH termini. The TdT used for incorporating BrdU to 3'-OH termini does not need a template for its reaction, and catalyzes the incorporation of deoxynucleotides into the 3'-OH termini of single- or double-stranded DNA. Therefore, we show evidence for the existence of 3'-OH termini at the regions where DNA strand breaks were induced locally along the ion traversals.

**MATERIALS AND METHODS**

**Cell strains and culture media**

African green monkey kidney cells, CV-1, obtained from the Japanese Collection of Research Bioresources Cell Bank, were cultured at 37°C in 95% air, 5% CO\(_2\) in Eagle’s MEM (M4655, SIGMA) containing 2 mM MEM Non-Essential Amino Acids (NEAA) solution (11140-050, GIBCO), supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 15070-063, GIBCO).

**Irradiation setups**

Heavy ions were imported to the Medium energy beam (MEXP) course in Heavy Ion Medical Accelerator in Chiba (HIMAC) at National Institute of Radiological Sciences (NIRS). The irradiation system of the MEXP course has been described in detail elsewhere.\(^8\)\(^{17}\)\(^{18}\) Briefly, heavy ions of 6 MeV/nucleon were fed into air through a metal foil window (Havar foil: Hamilton Precision Metals Inc., PA, USA), and air was used as an energy absorber. For perpendicular irradiation (Fig. 1A), the ion energy and its LET in water of carbon ions and argon ions were calculated to be 0.96 MeV/nucleon and 735 keV/μm, and 1.04 MeV/nucleon and 3,000 keV/μm, respectively. The ion energy and LETs in water were calculated by the SRIM-2006 code software (available from http://www.srim.org/).\(^20\)

The ion fluence at the sample position was measured by a solid-state track detector, CR-39 (HarzLas TD-1, Fukui Chemical Industry Co., Ltd., Fukui, Japan) and the uniformity of the 20 mm diameter beam field was confirmed with an imaging plate and CR-39, and was ±10%.\(^{17}\)\(^{18}\)

CV-1 cells of 3 × 10^4 in a 0.5 ml culture medium were plated in a chamber of the Lab-Tek® Chamber Slide™ (177437, Nunc), and cultured for 24 hours, to reach the sub-confluent condition. Just before ion irradiation, 0.3 ml of the medium was removed, and the chamber was sealed with 5-μm thick Mylar film (Chempex Industries INC., FL, USA). The slides were set vertically and irradiated with the ion beam running horizontally ("perpendicular irradiation", see Fig. 1A). During irradiation, the remaining 0.2 ml medium kept the cells humidified, but did not disturb the beam.

Small angle irradiation (Fig. 1B) was performed using sub-confluent CV-1 cells, which were fixed with Streck Tissue Fixative (STF; 265138, Streck Laboratories, Inc., NE, USA) for 15 min at room temperature a few hours before irradiation, and kept in a Tris buffer saline (TBS, 10 mM Tris-HCl, 150 mM NaCl) solution at 4°C. Just before irradiation, the plastic and rubber frames of the chamber slides were removed, and the remaining solution on the surface of the slide, which would attenuate the ions from reaching the cells, was removed. The slides were set at a small angle of approximately 5° to the beam path, where the Ar ion energy and LET were calculated to be 3.0 MeV/nucleon and 2,400 keV/μm, respectively. The thin Mylar films, which were used in perpendicular irradiation setup to prevent dehydration, were inadequate for small-angle irradiation, since 5 μm thick Mylar film will be a layer of 5 μm/sin5 = 57 μm, and thus the range in water of 3.0 MeV/n Ar ion is only 46 μm. Therefore, pre-fixation of the cells was necessary to prevent artificial fluorescent signals, which were detected after a TUNEL assay due to damage from dehydration of the cells during the several minutes of irradiation procedure, which was the case for living cells. Hence, with the fixed cells, the duration between removals of the TBS buffer to ion irradiation was as short as possible, so as to prevent the cells from drying. Within minutes after irradiation, the slide was put back in the TBS solution and kept in 4°C.

The ion fluence for small-angle irradiation was calculated as follows. The mean nucleus of the cells was estimated to
be a spheroid with a long axis of \(x\) μm, which was calculated from the mean geometrical cross section, \(A\) μm\(^2\), and with a short axis (nucleus thickness) of \(d\) μm. When the nucleus was orientated laterally (with an angle of 0°), the cross section of the nucleus could be calculated as, \(x^2 \times d^2 \times \pi\). In the case of an angle of 5°, the elliptical height axis was increased by \(d \times \sin(5°)\), leading to a mean cross section of \(x \times (1 + \sin(5°))2 \times d^2 \times \pi\). The expected numbers of traversals are given in the Fig. legends based on the actual geometric parameter (nuclear thickness, \(d\), and the nucleus area, \(A\)) and fluence.

For γ-ray irradiation, \(^{60}\)Co γ-rays with a dose rate of 0.54 Gy/min were used. Cells were irradiated with a culture medium of 5 mm thick, which were fully filled in the chamber slides to ensure buildup of secondary electrons.

**Labeling of 3′-OH termini**

After irradiation by the conventional setup shown in Fig. 1A, cells were rinsed twice with ice-cold TBS (100 mM Tris-HCl (pH 7.5) containing 150 mM NaCl), and fixed with STF supplemented with 50 mM EDTA-2Na for 15 min at room temperature, followed by 15 min at 56°C in a permeabilization buffer consisting of 100 mM Tris (pH7.4), 50 mM EDTA-2Na and 1% Triton X-100. The cells were briefly washed in double-distilled water and incubated for 1 h at 37°C in a reaction buffer (M1893, Promega Corp.) consisted of 100 mM sodium cacodylate (pH7.6), 1 mM CoCl\(_2\), 0.2 mM dithiothreitol, 0.05% tritonX-100, 45 nmol/ml 5-bromo-2′-deoxyuridine 5′-triphosphate (BrdU) (B0631, Sigma-Aldrich Co.), and 400 U of Terminal deoxynucleotidyl Transferase (TdT) (M1875, Promega Corp.). The labeling reaction was stopped by rinsing with TBS, and the cells were incubated for 20 min at 37°C in a blocking solution consisting of 2% Bovine Serum Albumin (A2153, Sigma) and 0.3% TritonX-100 in TBS. The cells were then rinsed with TBS twice, followed by being incubated with 40 μg/ml anti-BrdU antibody (anti-bromodeoxyuridine, mouse IgG(H+L), 11017, Molecular Probes Inc.) for 1 hr, and rinsed with TBS twice again. Secondary-mouse Alexa 488 antibodies (Alexa Fluor 488 F(ab′)\(_2\) fragment of goat antimouse IgG(H+L), 11017, Molecular Probes Inc.) were diluted to 30 μg/ml in the blocking solution incubated for 1 hr at 37°C and rinsed twice with TBS, followed by counterstaining the nucleus with PI, and mounted in antifade solution (2.3% diazabicyclo [2.2.2] octane, 90% (V/V) glycerol. 10 mM Tris-HCl (pH 7.5)).

Cells irradiated with the small-angle setup as shown in Fig. 1B, were fixed with STF prior to irradiation, as mentioned above. Following irradiation, the immuno-fluorescent assay was performed exactly as mentioned above.

**Microscopy**

The control and irradiated cells immuno-stained as mentioned above were observed with a confocal laser scanning system (LSM 510, Carl Zeiss) equipped with an Axiovert 200 M microscope, 40x lens (C-Apochromat 40×/1.2 W, Carl Zeiss). Images of the cells were obtained with a 3×−6× digital zoom at a in resolution of 1.024 × 1.024 pixels with the Multi Track mode. The areas of the cell nuclei stained with PI were obtained by surrounding the girth of the PI fluorescent area using the software LSM Ver. 3.0. Cross-sectional view images of a single cell were reconstructed with the software using the 20 sliced images taken with an interval of 0.54 μm over 10.45 μm for the CV-1 cell.

**RESULTS**

CV-1 cells were cultured to sub-confluency and irradiated with γ-rays at a dose rate of 0.54 Gy/minute. Figure 2 shows a representative image of control, 210 Gy, 500 Gy, 1,000 Gy irradiated CV-1 cell nucleus using DNA 3′-OH labeling. As mentioned, the 3′-OH termini of fragmented DNA of DSBs were labeled with BrdU enzymatically by TdT, and then an immuno-fluorescent assay was performed against BrdU. The green fluorescence visible in the cell nucleus images of panels C and D in Fig. 2 is the fluorescence of the Alexa fluor 488 secondary antibody against the anti-BrdU antibody. The green fluorescence was detected in 500 Gy and
Carbon-ion irradiation was carried out in the beam direction perpendicular to the cell monolayer, as shown in Fig. 1A. Representative images of the C ion (LET in water: 735 keV/μm) irradiated cell are shown in Fig. 3. The cells shown in panel B–F were irradiated at ion fluences of $1.2 \times 10^2$, $6.0 \times 10^2$, $2.6 \times 10^3$, $4.54 \times 10^3$, and $1.30 \times 10^4$, per nucleus, respectively. The fluorescence indicating the 3’OH termini was detected as dabble-like fluorescence in the cells irradiated with massive ion fluence of $4.54 \times 10^3$ and $1.30 \times 10^4$ ions traversal per nucleus (panel E and F). However, fluorescence was not detected with the cells irradiated with $1.2 \times 10^2$, $6.0 \times 10^2$, and $2.6 \times 10^3$ per nucleus.

Argon-ion irradiation was also carried out in the beam direction perpendicular to the cell monolayer, as shown in Fig. 1A; a spot-like fluorescence was visible in the CV-1 cell nucleus. Figure 4 shows a typical image of an CV-1 cell nucleus, irradiated with Ar ions (1.0 MeV/nucleon, LET: 3,000 keV/μm) at a fluence of $4.0 \times 10^{-2}$ ions/μm², calculated to be 13.5 ion traversals per nucleus. Panel A in Fig. 3 is constructed of 16 sliced images obtained from the top to bottom at a 0.4 μm interval of the nucleus. Panels B and C are the cross-sectional images along the longitudinal lines, B and C in panel A, respectively. The fluorescence indicating the position of induced 3’-OH termini can be observed as a fluorescent spot in the top view (panel A) and fluorescent stripes in the cross-sectional view of the nucleus (panel B, C).

The nucleus area of the cell in Fig. 4 is $S = 337 \, \mu m^2$ and the fluence of Ar ion irradiated was $F = 4.0 \times 10^{-2} \, ions/\mu m^2$. 

1,000 Gy irradiated cells, but not in control cells and a 210 Gy irradiated cell nucleus.

Carbon-ion irradiation was carried out in the beam direction perpendicular to the cell monolayer, as shown in Fig. 1A. Representative images of the C ion (LET in water: 735 keV/μm) irradiated cell are shown in Fig. 3. The cells shown in panel B–F were irradiated at ion fluences of $1.2 \times 10^2$, $6.0 \times 10^2$, $2.6 \times 10^3$, $4.54 \times 10^3$, 1.30×10⁴, respectively. Panel E and F shows the green fluorescence in the cell nucleus, but panel B to D, for those cells irradiated with the lower fluences, show no detectable fluorescence.
The expected number of ion traversals in the individual cell nucleus can be calculated as \( n = S \times F \), where \( S \) is the cross-sectional area of the individual nucleus and \( F \) is the ion fluence. To support that the 3’-OH termini observed as fluorescent spots in the cell nucleus are in fact due to ion traversal, the correlation between the observed number of fluorescent spots and the expected number of ion traversals is plotted in Fig. 5. The regression line is shown as a solid line, and the slope of the line is 0.99 ± 0.02, showing nearly 1.0, indicating that single Ar ion traversals through the cell nucleus can be successfully detected with this method.

As mentioned above, the irradiation setup perpendicular to the beam direction to the cell nucleus resulted in fluorescent spots when observed from the top, and a fluorescent stripes with the cross-sectional view. The low resolution of a confocal laser microscope in depth and the extreme flatness of a cell nucleus decreases the resolution of induced DNA damage along the ion traversals in cell nuclei. We therefore adopted the unique irradiation setup introduced by Jakob et al.,\(^{14}\) where Ar ions (3.0 MeV/nucleon, LET in water: 2,400 keV/\( \mu \)m) were irradiated to the cell monolayer with a small angle (approximately 5°), as drawn in Fig. 1B. A representative image of an irradiated CV-1 cell nucleus, traversed by an Ar ion from left to right, is shown in Fig. 6. This cell nucleus was irradiated at a fluence of 1.2 \( \times 10^{-1} \) ions/\( \mu \)m\(^2\), and showed approximately 6 independent green fluorescent stripes indicating the existence of 3’-OH termini. The expected number of ion traversal calculated from the cross-sectional area mentioned in the materials and methods was 12.7. The difference in the number may be due to the accuracy of the low-angle, and also to the removal of TBS solution from the cell monolayer, which may have inhibited the Ar ion to traverse the cells. However, this image clearly ensures that the visualization of DSBs induced by Ar ion traversal was successful with the method of labeling the 3’-OH termini of fragmented DNA.

**DISCUSSION**

The method presented here was developed from one described in Kodym et al.,\(^{16}\) which avoids DNA unwinding, and will only leave 3’-OH termini of DSBs as optimal priming sites for nucleotide incorporation, while the action of TdT on 3’-OH termini of SSBs will be hindered by the remaining strand downstream of the breaks.\(^{24,25}\) They demonstrated that cell nuclei which that irradiated X-ray with showed an increased terminal transferase catalyzed incorporation of tritiated nucleotides when compared to unirradiated cells.\(^{25}\) Nakamura et al. (1976) did not find an increased labeling of nuclei in mouse brain immediately after doses of up to 200 Gy,\(^{26}\) yet Kodym et al. reported that this may be due to the fixation procedures performed by methanol:acetone (1:1), which could not prohibit
the activation of endogenous cellular nucleases, leading to excessive background signals in unirradiated cells.\textsuperscript{10} Although we applied the fixation method mentioned by Kodym \textit{et al.} performed with modified STF consisting of 2-bromomo-2-nitro-1, 3-promanediol, diazidilindyl urea, sodium citrate, and EDTA, our results also could not detect the fluorescence of 3’-OH termini up to 500 Gy of γ-ray irradiation, as shown in Fig. 2C. It is interesting that Henner \textit{et al.} irradiated pure DNA in water with γ-rays, and concluded that 3’-phosphoryl and 3’-phosphoglycolate, but no 3’-OH termini, are produced.\textsuperscript{5,29} From reports of Belli \textit{et al.}, γ-rays induce 6 DSBs/Gbp/Gy, and 500 Gy γ-rays induce 1.8 × 10\textsuperscript{4} DSBs/nucleus. In other words, the density of DSBs per nucleus of Fig. 2C, the area of which was 391 μm\textsuperscript{2}, can be calculated to be 46 DSBs/μm\textsuperscript{2}.\textsuperscript{29} Therefore, the cell nucleus with 210 Gy shown in Fig. 2B does not indicate a lack of DSBs, but the fraction of 3’-OH termini per DSBs was extremely low, which made 500 Gy necessary for significant detection of the 3’-OH termini. Also with longer incubation up to three hours of TdT reaction for 200 Gy and 500 Gy irradiated cells were tested, however the significant difference in fluorescent signal were not detected.

Previously, we obtained images of HeLa cells irradiated with 1.1 MeV/n Fe ions (LET in water: 4.760 keV/μm) near the Bragg peak, which showed an equal number of γ-H2AX fluorescent spots per expected number of ion traversals to the cell nucleus, and also verified that the geometric location of γ-H2AX fluorescent spots that appeared in the cell nucleus agreed with the position of etch pits, indicating the position of ion traversal of the 3.1 MeV/n Ne ion (LET in water: 970 keV/μm) in HeLa cells and 3.1 MeV/n Ne ions (LET in water: 970 keV/μm) in CHO-K1 cells using the contact microscopy technique.\textsuperscript{10} Contrary to γ-ray irradiated cells, Ar ion traversals in the nuclei of cells were detectable as fluorescent spots using the TUNEL method, as shown in Fig. 4 and fluorescent stripes in Fig. 6, demonstrating the induction DSBs along the Ar-ion traversal. This indicates that DSBs were not only induced locally along the Ar ion traversal, but 3’-OH termini were produced efficiently along the ion tracks.

The possibilities of the conversion of other radiation-induced 3’ moieties to 3’-OH termini by cellular enzymes, as mentioned by Kodym \textit{et al.},\textsuperscript{10} cannot be excluded, although the cell nucleus shown in Figs. 3, 4 and 6 was kept on ice, and fixed immediately after irradiation, and the irradiation was completed in no longer than 5 minutes; thus, such conversion is considered to be low in extent. The cell nucleus shown in Fig. 6 was fixed with STF prior to irradiation; therefore, this possibility of a biological process of cellular enzyme can be considered as low extent. However, the production of 3’OH termini due to the chemical conversion of other 3’moieties to 3’OH termini by the reaction of radicals cannot be excluded.

It is known that the induction of a DNA double strand break will induce several hundred phosphorylation of H2AX proteins.\textsuperscript{5,6} This indicates that the cell image of immunostaining with the anti-γ-H2AX antibody will result an amplified image of the DNA double strand break region. In addition, it has been reported by others\textsuperscript{5,7,11} that the percentage of H2AX of all histone protein is different with each cell line. Therefore a comparison with different cell lines will be inadequate for analyzing the size of the DNA double strand break region using γ-H2AX as its marker; therefore, 3’OH labeling may be a more direct imaging of the DNA stand-break region. Simultaneous fluorescent staining of γ-H2AX and TUNEL assay would be most significant for a comparison of the size of the fluorescent region induced by ion traversal, although we were not successful in obtaining the cell images with our standard protocol. As for the fluorescent spot detected in Fig. 4, the average area was 0.36 ± 0.08 μm\textsuperscript{2}. At total of 30 irradiated cells were analyzed and the fluorescent spot size was 0.41 ± 0.12 μm\textsuperscript{2}. As for a comparison, CV-1 cells irradiated with Argon ion of 1.0 MeV/n were immunostained against γ-H2AX (data not shown) and the size of fluorescent spots were measured to have an average size of 2.04 ± 0.34 μm\textsuperscript{2}, which was 5-fold the size of the TUNEL assay.

The 3’OH termini were not detected with the cells irradiated with C ions at a fluence below 2.6 × 10\textsuperscript{3} per nucleus, as shown in Fig. 3, although with higher fluence double like fluorescence spots were detected, which may be due to an over lapping of the C ion tracks. Contrary to the C ion, individual Ar ion tracks were detected as spot like fluorescence with perpendicular irradiation (Fig. 4), and as stripes along the nucleus with low angle irradiation (Fig. 6). The production of 3’-OH termini may be due to the difference in the ion track structure. Figure 7 shows the local dose distribution in the ion tracks for C ions and Ar ions with their incident ener-

![Fig. 7. Radial dose distribution of energy deposited around the carbon and argon ion in water.](image)
3′OH-termini Production along the Ion Tracks

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