Co-visualization of DNA damage and ion traversals in live mammalian cells using a fluorescent nuclear track detector

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The geometric locations of ion traversals in mammalian cells constitute important information in the study of heavy ion-induced biological effect. Single ion traversal through a cellular nucleus produces complex and massive DNA damage at a nanometer level, leading to cell inactivation, mutations and transformation. We present a novel approach that uses a fluorescent nuclear track detector (FNTD) for the simultaneous detection of the geometrical images of ion traversals and DNA damage in single cells using confocal microscopy. HT1080 or HT1080–53BP1-GFP cells were cultured on the surface of a FNTD and exposed to 5.1-MeV/n neon ions. The positions of the ion traversals were obtained as fluorescent images of a FNTD. Localized DNA damage in cells was identified as fluorescent spots of γ-H2AX or 53BP1-GFP. These track images and images of damaged DNA were obtained in a short time using a confocal laser scanning microscope. The geometrical distribution of DNA damage indicated by fluorescent γ-H2AX spots in fixed cells or fluorescent 53BP1-GFP spots in living cells was found to correlate well with the distribution of the ion traversals. This method will be useful for evaluating the number of ion hits on individual cells, not only for micro-beam but also for random-beam experiments.

Keywords: fluorescent nuclear track detector; ion beam tracks; DNA damage; localization

INTRODUCTION

Information obtained from the geometric location of ion traversals through cells is important for radiobiological studies. Energy deposition from radiation, especially from ion beams, is strictly localized in tracks. Clustered DNA damage is also known to be induced along the ion tracks. This leads to severe biological effects such as cell killing, mutation or transformation of cells, even with a few traversals through the nuclei [1–3]. Several methods have been employed to obtain microscopic information on ion traversal within cells. The microbeam irradiation system [4–6] is a popular technique that enables researchers to precisely deliver the desired number of ions to cell nuclei and other subcellular organelles. Other approaches based on the usage of plastic nuclear track detectors [5, 7–9], poly-allyl-diglycol-carbonate (PADC) or CR-39 as substrate for cell adhesion, have facilitated the microscopic resolution of the intranuclear location of ion traversals through cells. The two images, cellular and ion, have to be taken separately, and the correlations have to be analysed by taking into account many geometric parameters, such as the resolution, distance and rotation [8, 9]. However, removal of cells before etching of the detector is essential, and this poses a technical barrier to biological applications. Although live cell imaging with track detection using CR-39 has been reported [5], the time gap affects the later biological analysis due to cellular mobility and metabolism. Moreover, most of the biological analysis is limited to the retrospective
detection of ion traversals through the cells enabled by the etching process of the track detector.

Recently, novel passive integrated fluorescent nuclear track detectors (FNTDs, Landauer Inc.) have been developed with Al₂O₃:C,Mg; and have demonstrated high performance in the dosimetry of neutrons, protons and heavy ions [10–12]. FNTDs have aggregate oxygen vacancy defects denoted as F₂²⁺(2Mg) centres. They produce radiation-induced colour-centres that have a light-absorption band at around 620 nm and emit fluorescence at 750 nm with a high quantum yield and a short lifetime of 75 ns [10]. For radiobiological applications, FNTDs are advantageous over CR-39 by virtue of (i) their non-destructive readout, that can be performed using a commercialized confocal fluorescence microscope, (ii) a lower LET sensitivity threshold of 0.4 keV/μm compared with that of CR-39 (5 keV/μm), and (iii) no saturation to high-LET charged particle radiations, even at 1800keV/μm in water [11, 12].

In this paper, we describe an approach that uses FNTDs for the detection of the geometric location of ion traversals and cell images immediately after irradiation at a submicron scale resolution using confocal laser scanning microscopy. To demonstrate the suitability of FNTD for biological studies, the geometrical correlation between the ion traversals and the DNA damage sites was observed as fluorescent images of ion tracks from FNTDs and the localization of γ-H2AX [13] or 53BP1 [14, 15], respectively.

MATERIALS AND METHODS

Cell lines and cell culture
HT1080 cells (ATCC CCL-121) were maintained in Dulbecco’s Modified Eagle Medium (05919, Nissui, Japan) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin at 37°C in a humidified 5% CO₂ incubator. HT1080-53BP1-GFP cells were constructed by transfecting pDC3.1Hyg-F2GFP-53BP1 (12.24 kb, a kind gift from Dr Burong Hu of the Institute of Modern Physics, Lanzhou, China) [14] into the HT1080 cells by electroporation and culturing for 3 weeks in selection medium containing 100 μg/ml hygromycin B (10687-010, Life Technologies, CA). Subsequently, the GFP⁺ cells were sorted into 96-well plates with a single cell per well using the FACSAria cell-sorter (BD Biosciences, NJ), following which each clone was independently expanded. About six clones from a total of 500 GFP⁺ clones were obtained. A single clone was finally chosen after verifying its morphology, growth curves, and radiation sensitivity, such as 53BP1-GFP fluorescent spot formation and survival curves.

Cell preparation on FNTD
FNTDs (8 × 4 mm², Crystal Growth Division, Landauer Inc., OK) were glued with an epoxy adhesive onto a 35-mm petri dish with a 13-mm glass bottom (D111505, Matsunami Glass Ind. Ltd, Osaka). The dish with FNTD was sterilized with 70% ethanol and UV light. Approximately 24 h before irradiation, the FNTD was coated with fibronectin (063-00591, Wako Chemical, Tokyo) and the cells were seeded. An aliquot of 50 μl (100 ng/ml fibronectin in PBS) was dropped onto the surface of the FNTD, incubated at 37°C for 1 h, and rinsed with the culture media. Cell suspensions of 50 μl (containing 1500 cells) were seeded onto the surface of the FNTD and incubated for 3 h to allow for cell attachment. Then, 2 ml of media was added to the dish to completely cover the FNTD. Immediately before irradiation, the dishes were covered by a Mylar film (2.5 μm thickness, Cat# 100, Chempex Industries Inc., FL) to prevent drying and contamination of the cells during the irradiation procedure.

Irradiation setup
Irradiation was performed at the Medium Energy Beam (MEXP) course [16] at the Heavy-Ion Medical Accelerator in Chiba (HIMAC) [17] in the National Institute of Radiological Sciences (NIRS), Chiba, Japan. The cells were irradiated with a horizontal beam and set in perpendicular to the beam direction. For each beam time experiment, uniformity of the beam profile was reproduced in the range of ± 5% within a 10-mm diameter area by defocusing the beam with a quadrupole magnet and scattering with a secondary electron monitor (SEM) installed for dosimetry in the accelerator tube and beam extraction window (6 μm Ni alloy foil). The energy and LET of the neon ion beam at the sample position were calculated to be 5.1 MeV/n and 736 keV/μm in water, respectively, using the SRIM-2013 code software [18].

The cells on the FNTD were irradiated with an ion fluence of 1.5 × 10⁻² ions/μm², which dose was calculated to be 1.7 Gy. The irradiated cells were kept on ice for further radiobiological experiments. The total irradiation procedure was accomplished in 20 min, and the cells were kept on ice immediately after the irradiation to prevent DNA repair until the later procedure.

Immunostaining with γ-H2AX for visualization of DNA double-strand breaks
The culture media was replaced with pre-warmed media. The cells were kept in a CO₂ incubator at 37°C for 15 min and then fixed with 4% paraformaldehyde for 15 min at room temperature. Subsequently, the samples were immunostained [6] with anti-phospho-histone H2AX (Ser139) clone JBW301 antibody (05-636, Merck Millipore, MA) and Alexa Fluor®488 secondary antibody (A-11001, Molecular Probes, OR).

Confocal laser microscopy
Images were obtained using a confocal laser scanning microscope (LSM; FV-1000, Olympus Co., Tokyo) equipped with three lasers at 473, 559 and 635 nm, and captured using a × 40 objective lens (LUMPLFLN 40XW, Olympus Co., Tokyo) for a scan area of 317.2 × 317.2 μm² (2,048 × 2,048 pixels) with a scan speed of 20 μm/s and at 45% laser light transmissivity. A CO₂ incubation chamber (INUG2F-UK,
Tokai-Hit, Shizuoka) with a gas mixer (GM-5000, Tokai-Hit, Shizuoka) was installed on a motorized X-Y stage (MPT-AS04FV, SIGMAKOKI, Tokyo) of the LSM. For the FNTD read-out [10], a dichroic mirror split at 670 nm was used and scanned at 10 µm below the surface. For cell imaging with AlexaFlour®488 or 53BP1-GFP, a 505-nm dichroic mirror was used. The image resolution and size were set to be the same as the cell images. For all the image acquisitions, the FNTDs were first scanned with a 635-nm laser to image ion traversals and then scanned with a blue laser for cell imaging to avoid ‘recording’ of fluorescence in the FNTD crystal with the focused blue laser light. The image acquisition started 20 min after irradiation, and live images were captured for up to 6 h.

RESULTS AND DISCUSSION

Cytotoxicity of FNTD and laser light
We have introduced an approach that uses FNTDs and a confocal LSM for the detection of the geometric positions of ion traversals and DNA damage. A–D: magnification × 40 (scalebar = 50 µm): (A) phase contrast, (B) γ-H2AX, (C) FNTD and (D) merged. Digitally enlarged images E, F and G (scalebar = 10 µm) correspond to e, f and g, respectively, in panel D. Arrows indicate γ-H2AX spots that correspond to the ion traversals.
traversals and cells immediately after radiation at a submicron scale resolution. To validate the suitability of FNTD, HT1080 cells were cultured on the surface of fibronectin-coated FNTD crystals, as depicted by the phase contrast microscopic image in Fig. 1A. Fibronectin is one of the most commonly used primary cell adhesion molecules for coating dishes for cell culture. Fig. 1A shows that the cells are well stretched and attached to the surface of the crystal and grow as well as on fibronectin-coated CR-39. The applicability of fibronectin-coated FNTD as a substrate for cell culture growth has also been demonstrated previously [19].

Another concern is the phototoxicity of the 635-nm laser light, since a highly transmitted and focused laser is necessary for the detection of ion tracks in FNTD. Our preliminary investigation showed that any significant increase in the γ-H2AX signals can be seen for up to 10 repeated scans, suggesting that the toxicity caused by the 635-nm laser scans of the LSM is negligible.

**Correlation patterns of ion traversals and DNA damaged signal**

Representative images of the Correlation patterns of ion traversals and γ-H2AX spots are shown in Fig. 1. The green spots in Fig. 1B denote fluorescent γ-H2AX spots that correspond to sites of cellular DNA damage. On the other hand, the red spots in Fig. 1C demonstrate the locations of ion traversals on the FNTD crystal. These spots are randomly distributed all over the observed field. The ion traversals and regions of DNA damage in cells showed a strong geometrical correlation. However, a systematic parallel shift of several micrometres was observed between the tracks and the fluorescent damage spots (Fig. 1E–G). These shifts are not caused by instrumentation displacement between the 635- and 435-nm laser positions during the two sequential scans. This was confirmed prior to the measurements and calibrated based on the scanning of fluorescent microbeads with the two lasers (confirmed by Olympus Co., data not shown). Nonetheless, the systematic shift of the DNA damage images was observed in the upper right positions relative to the FNTD spots (Fig. 1E–G). A similar shift between the ion traversals and 53BP1-GFP localization can be observed in Fig. 2. The FNTD spots stay fixed without moving from the position of ion traversals, and they must be observed at their fixed position (Fig. 2A). However, the HT1080 cells rotate their nucleus while moving several micrometres within 15 min (Fig. 2A and 2B), and it has also been reported by others that this is common to living cell behaviour in similar conditions [5, 8, 19]. Ideally, if the irradiated cells were to be set on the LSM system immediately after irradiation, such as in an on-site imaging system, it would facilitate the improvement of resolution for determining the precise correlation between the ion tracks and cell images. However, our experimental setup was unable to achieve this resolution.

**Live cell imaging**

As seen in Fig. 2, the time-course of the 53BP1 localization in cells can be observed. Figure 2B depicts five fluorescent 53BP1-GFP spots in the nucleus (Fig. 2B). The distribution pattern of 53BP1-GFP and the ion traversals showed good agreement with ion traversals, as shown by the red arrows, demonstrating that 53BP1 is localized at the ion traversals. However, a discrepancy was seen in the distribution of the ion traversals and 53BP1 localization, possibly due to the mobility of the cells within the period between irradiation...
Advantages of FNTD and fluorescent stain

FNTD provides nearly 100% efficiency of detection of the ion traversals with an LET range of 0.5–61 000 keV/µm, when imaged by LSM [10–12]. In comparison with CR-39 track detectors, with which the detection of low-LET ions (below 5 keV/µm) is impossible [20], FNTD provides prompt detection of ion traversals without any etching procedures. This property of FNTD makes it most advantageous for continuous imaging of living mammalian cells.

In addition, the read-out signals of FNTDs can be translated into an LET value [12]; mostly practically, this is within the clinically relevant LET range [21]. These characteristics and the applicability of FNTDs for live cell imaging microscopy are expected to further expand the field of radiobiology. For example, the FNTD technology can be applied to determine the biological response in a mix-beam radiation field, where cells targeted with radiation at different LETs demonstrate synergistic biological effects [21, 22]. Radiation exposures in space and also during clinical therapy are representative examples of the mix-radiation environment [21–23].

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