A New Method for the Simultaneous Detection of Mammalian Cells and Ion Tracks on a Surface of CR-39

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CR-39/UV exposure/Cell relief/Ion track/Heavy ion/γ-H2AX.

The geometric locations of ion traversals in mammalian cells constitute important information in the study of heavy ion-induced biological effects. We employed a contact microscopy technique, which was developed for boron imaging in boron neutron capture therapy to the irradiation mammalian cells by low-energy heavy ions. This method enables the simultaneous visualization of mammalian cells as a relief on a plastic track detector, CR-39, and the etch pits which indicate the positions of ion traversals. This technique provides visual geometric information about the cells and ion traversal, without any specially designed devices or microscopes. Only common laboratory equipment, such as a conventional optical microscope, a UV lamp, and commercially available CR-39 is required. To validate this method, CHO-K1 and HeLa cells were cultured on the CR-39 surface and then irradiated with low-energy Ar and Ne ions, respectively. The positions of induced DNA double strand breaks were detected as γ-H2AX fluorescent spots, which coincided with the positions of the etch pits in the cell relief image.

INTRODUCTION

Accelerated ions, which are known as high linear energy transfer (LET) radiation, dissipate their energy locally along their tracks, result in a non-random spatial dose deposition with a locally high distribution of energy.1,2) Thus, the induction of DNA double-strand breaks (DSBs) by heavy ions is more severe due to clustering along ion traversal compared to those of low-LET radiation, such as X- and γ-rays.2,3) Geometric information about where ions have traversed in a cell, especially the nucleus, has become important to understand the mechanisms of radiobiological effects.

A plastic track detector, CR-39, a polymer of allyl diglycol carbonate, produces pits at the positions of ion traversals after etching in an alkaline solution, and is commercially used as a personal neutron dosimeter and detectors for alpha particles emitted from a radon decay products. Recently, CR-39 is being applied to the field of radiation biology to detect the positions of ion tracks traversed in mammalian cells, and to compare with the positions of ion-induced clustered DNA damage, or with those DNA repair-related enzymes localized around DNA damage clusters.4-8) Scholz et al.9) used CR-39 to obtained direct evidence for a spatial correlation between individual ion traversals and localized CDKN1A (p21) binding to the sites of heavy ion-induced DNA damage. Funayama et al.6) developed a unique method, in which the positions of ion traversals can be detected during cell culturing by etching only the reverse side (opposite side from the cells) of the CR-39 at 37°C. Jakob et al. used 40 μm thick polycarbonate foils for the growth of cells, which made live cell imaging of heavy-ion-induced radiation responses by their beam-line microscopy possible.7) Recently, Gaillard et al.8) developed an extremely thin CR-39 with 10-μm thickness, which has expanded the possibilities in research for lower energy particle effects, such as 5.48 MeV α-particles emitted from 241Am.

In this paper, we report on a new and convenient method for validating the spatial correlation between individual ion traversal and DNA damaged positions, which was employed from a contact microscopy technique developed by Amemiya et al.9) It was introduced as an imaging technique for simultaneously detecting a relief image of sliced rat tis-
sue attached to the CR-39 surface and etch pits by neutron-induced α-particles. An application to the field of DNA repair using this technique by immuno-staining phosphorylated histone protein H2AX (γ-H2AX)\(^{10}\) is also demonstrated.

### MATERIALS AND METHODS

The experimental procedure of our method for the simultaneous detection of cell images and ion tracks on the CR-39 track detector (BARYOTRAK, Fukuvi Chemical Industry) surface is summarized briefly and stepwise; and the details will be described later. (1) Cells were cultured on the CR-39 one day before irradiation. (2) After the medium was removed and the dish was covered with a Mylar film; and the cells were then irradiated with ions in air through Mylar film. (3) After irradiation, the cells were immunostained with anti-γ-H2AX antibody, followed by the Alexa488 fluorescent secondary antibody to detect the positions of DSBs with a confocal laser scanning microscope. (4) The CR-39 was exposed to UV to make a latent image of the cells on the CR-39 surface. (5) After the cells were removed, the CR-39 was etched in \(7 \text{ M} \) NaOH at 70°C to develop embossment images of the cells as a relief and etch pits corresponding to ion tracks. (6) The images of the cell relief and the etch pits were captured by atomic force and optical microscopes, respectively.

**Sample preparation**

A human cervix carcinoma cell line, HeLa, and a Chinese hamster ovary cell line, CHO-K1, were used. The cell lines and culture conditions were described previously.\(^{11}\) These two cell lines were chosen for their differences in geometrical configuration, which may produce different contrasts on the cell image embossed on the CR-39 surface after the etching procedures mentioned below. For example, the nuclear area of HeLa cells was 1.7-fold larger than CHO-K1 cells, as mentioned elsewhere,\(^{11}\) and the total cell area was roughly estimated to be 2.5-fold larger than for CHO-K1 cells, (data not shown). These geometrical differences affect the embossment image produced on the surface of CR-39.

A CR-39 of \(2 \text{ cm} \times 2 \text{ cm} \) size was scratched crosswise at the center of the back side by a knife as a landmark, which was later used to match the positions of the cell images observed with an optical microscope with the position of relief images of the cells. The CR-39 was sterilized with 70% ethanol, dried in clean air, and then glued with nail varnish onto the bottom surface of a 3.5 cm plastic dish. A cell suspension of \(5 \times 10^4 \) cells in 2.5 ml was seeded in a dish 24 hours before ion irradiation. Just before irradiation, the culture medium was removed and the dish was covered with a 5 μm Mylar film to prevent drying and contamination during irradiation.

### Irradiation

All irradiation was performed at the Medium Energy Beam (MEXP) course at the Heavy Ion Medical Accelerator in Chiba (HIMAC), the National Institute of Radiological Sciences (NIRS). The irradiation system built in the MEXP course provides a variety of ion species of H to Xe, which are accelerated to 6 MeV/nucleon, and leads ions out into the air through a beam exit window. The ion energy was controlled by changing the distance of the sample position from the beam exit, where the air functioned as an absorber to control the ion energy; thus, the ion energy was continuously selectable. The beam field was spread out to 20 mm in diameter with a uniformity of ±10% for the irradiation of mammalian cells and other biological samples.\(^{12}\) Details of the irradiation set up are described elsewhere.\(^{13}\) The HeLa cells were irradiated with Ne ions with an energy of 3.1 MeV/nucleon and a LET of 970 keV/μm; CHO-K1 cells were irradiated with Ar ions with 2.0 MeV/nucleon and 2,650 keV/μm. The LET and ion energy at the sample position were calculated using SRIM-2003.\(^{14}\)

### Detection of DNA double-strand break positions in the cell nucleus

After irradiation, the cells were incubated at 37°C in 95% air, 5% CO\(_2\) for 30 minutes for the phosphorylation of H2AX to reach its maximum. The cells were then fixed with a fixative solution (modified Streck Tissue Fixative (S.T.F., Streck Inc.) supplemented with 50 mM EDTA-2Na) for 15 minutes at room temperature. The cells were permeabilized with a solution of 100 mM Tris pH 7.5, 50 mM EDTA and 1% Triton-X-100 for 20 minutes at 56°C, and blocked for 60 minutes in PBS at 37°C. The DSBs were visualized by immunostaining the phosphorylated histone protein, γ-H2AX,\(^{10}\) with an anti-γ-H2AX-antibody (Trevigen), followed by Alexa 488 secondary antibody. The cell nucleus was counterstained by propidium iodide of 1 g/ml in PBS. An antifade solution (1.5mM 1,4-diaxabicyclo[2,2,2]octane, 100 mM Tris-HCl, 90% Glycerol) was loaded, and a cover slip was placed on the surface before an observation with a confocal laser scanning microscope (LSM 510, Carl Zeiss; hereafter abbreviated LSM). The immunostaining assay was held exactly the same way as mentioned elsewhere.\(^{11}\)

### Cell image transferring to the CR-39 surface and detection of the ion traversal position

The principle of cell image transferring to the CR-39 surface is shown in Fig. 1. UV exposure accelerates the etching speed of CR-39. Therefore, the amount of UV transmission through the cells depends on the cellular materials attached to the CR-39 surface, which results in an embossment of the cell relief on the surface of the CR-39 after etching in an alkaline solution. The enhanced bulk etching speed had been measured to estimate the etching time needed to produce a
clear relief of the cell image and its structure. Details are described later.

After an observation with the LSM, the CR-39 with the irradiated cells was immersed into distilled water for 20 minutes for the cover slip to come off, and was immersed again into fresh distilled water for an additional 10 minutes to remove any residual antifade solution. CR-39 was dried in air for at least one day, and then UV was exposed for 8 hours with a total dose of $2.6 \times 10^{5}$ J/m$^2$ at 2.5 cm away from the center of a 10W low-pressure mercury lamp (GL-10, Toshiba). The cells were removed from the CR-39 by shaking in a neutral detergent for a few hours and rinsed in distilled water repeatedly. CR-39 was dried in air for more than 24 hrs, and then etched in 7 M NaOH at 70°C for 9 minutes to measure with an atomic force microscope. For observation with an optical microscope, CR-39 was etched for an additional 3 minutes. The CR-39 surfaces were scanned with an atomic-force microscope (Nanoscope IV, Digital Instruments; hereafter abbreviated as AFM) with a cantilever (NCH-W, Digital Instruments) in a tapping mode at a scan rate of 0.2 Hz over 100 μm × 100 μm. The cantilever of the AFM was set near to the relief of the cell that had been measured with the LSM, using an optical microscope of 100× magnification equipped to the AFM with the aid of a scratched cross on the back side of the CR-39. The CR-39s were also observed with a conventional optical microscope (DM IRB, Leica).

**Measurement of the enhanced bulk etching speed by with and without UV exposure**

The UV sensitivity of CR-39 was measured using a method introduced by Yasuda et al. A schematic drawing of the procedure is shown in Fig. 2. The CR-39 surface was partly masked by an epoxy adhesive resin (Araldite, Huntsman Advanced Materials) that protected the surface from etching in 7 M NaOH at 70°C; about one day was necessary for the resin to harden and to protect against etching. After etching, the mask was easily peeled off from the surface. The height of the step that was visible along the mask boundary on the CR-39 surface was measured by tapping-mode AFM. This height corresponded to the amount of bulk etching, B.
**RESULTS AND DISCUSSION**

The method mentioned here is based on the fact that the etching speed of CR-39 is enhanced by UV exposure, and applied to the contact UV microscopy technique proposed by Amemiya et al., in which a relief image of a slice of rat tissue attached to the CR-39 surface and etch pits by neutron-induced α-particles were simultaneously recorded on the CR-39 surface. We extended this technique to the simultaneous recording of etch pits by heavy ions and cell relief images (principle shown in Fig. 1). We first measured the amount of bulk etch using the partial masking method to estimate the appropriate etching time to obtain a cell relief images. Fig. 2A shows a schematic drawing of the principle and Fig. 2B shows a typical AFM image of CR-39 after etching for 9 min. The difference in the heights between the masked and unmasked surfaces indicates the amount of bulk etching. The amount of bulk etching with (UV+) and without UV exposure (UV–) is plotted as a function of the etching time (Fig. 3). The amount of bulk etch increased linearly with the etching time. The slopes of the regression lines were the bulk etching speeds: 25 ± 1.0 nm/minute for UV– and 48 ± 1.2 nm/minute for UV+ (1.9 times larger than that for UV–).

Since almost all of the organic molecules constituting a cell, such as protein and nucleic acid, absorbs UV light strongly, the amount of UV light reaching to the CR-39 surface depends on the amount of UV absorbing materials of the cell.

**Fig. 3.** Amount of bulk etching of the CR-39 surface plotted as a function of the etching time. The solid and open circles represent the results with and without UV exposure, UV+ and UV–. The regression lines represent the etching speed; the speed (with the standard error) for UV+ was 48 ± 1.2 nm/minute, which was two-fold larger than that of UV–, 25 ± 1.0 nm/minute.

**Fig. 4.** Microscopic images of a HeLa cell and etch pits of CR-39 after irradiation with Ne ions of 3.05 MeV/nucleon at a fluence of $3.6 \times 10^2$ ions/μm². Panel A: a differential interference image of a cell. Panel B: an AFM image of the CR-39 surface after etching, corresponding to the cell in panel A, clearly showing the cell relief. Panel C: phase contrast image taken by a conventional optical microscope of the same cell of panel B after additional etching for three minutes. The bar size is 10 μm.
the cell attached to the surface. Thus, cell relief images on the CR-39 surface were developed as an embossment after etching. Fig. 4A shows a differential interference image of a fixed cell obtained with the LSM. Fig. 4B is an AFM image of etch pits originating from ion tracks, and the relief corresponding to the cell is shown in Fig. 4A, demonstrating intracellular structures. The AFM may not be a convenient tool for observing cell relief and etch pits because of its high cost, laborious set up and long measuring time. We therefore used the AFM just to show how clearly the relief can be seen in detail. After additional etching for 3 minutes, the cell-relief image can also be observed with a conventional optical microscope, as shown in Fig. 4C.

As an example, our new method was applied to determine whether the positions of fluorescent spots of γ-H2AX, which is an indicator of DSBs, agree with the positions of the ion tracks. Typical fluorescent images were superimposed with differential interference images obtained with the LSM (Fig. 5A and C) and relief images with the AFM of a CHO-K1 cell irradiated with Ar ions (Fig. 5 B) and a HeLa cell irradiated with Ne ions (Fig. 5 D). In the fluorescent images, the green fluorescent spots indicate the positions of DSB clusters, which had been produced along ion tracks and immunostained with the anti-γ-H2AX antibody. The result demonstrated that the bright-green fluorescent spots in the cell nuclei co-localized with the etch pits in the relief images of the cell. However, the contour of cell nuclei is difficult to identify from the cell relieves in image Fig. 5B. This is considered to be due to the similar UV absorption of the nuclei and the cytoplasm. It did not make any significant difference concerning the amount of UV light reaching the CR-39 surface, resulting in a similar etching velocity. However, clearer embossments of the relief of the cells may be achieved with different etching times and UV exposures.

The etch pit, indicated by the arrow at the center of the nucleus in Fig. 5B, is hardly visible. This is may be due to...
an uneven enhancement of the etching velocity by UV exposure during the cell image-transferring procedure. For example, the etch pit size at the area where the cells do not exist is larger than those in the area of cell relieves, which reflects the UV absorption of CR-39. Therefore, the difference in the size of the etch pit can be identified in Figs. 5B and 5C, which is due to the uneven thickness of the cell. Three γ-H2AX spots can be seen in Fig. 5C, where the etch pit indicated by the arrow in Fig. 5D is outside of the nucleus, but the geometrical position of the spots and tracks can be considered to be agreeable. Therefore, this is may be due to the cell displacements during the incubation between irradiation and fixation of cells.

The most valuable point of the method described in this paper, compared to the other introduced techniques, is the simultaneous detection of the ion traversal positions as etch pits and the cell reliefs on the same surface of CR-39. Another point is that no specially designed devices and special CR-39 are necessary, but only a conventional optical microscope, a UV lamp, and a commercially available CR-39. For example, Scholz et al. used CR-39 to obtained direct evidence for a spatial correlation between individual ion traversals and localized CDKN1A (p21) binding to the sites of heavy ion-induced DNA damage. They cultured cells on the CR-39 surface, which were previously irradiated and etched to produce etch pits; these etch pits were used as landmarks when comparing the position of the fluorescent CDKN1A foci with the etch pits of ion traversal through the nuclei of cells. This method required many procedural steps. A specially designed microscope system was used to define the correlation of the fluorescent foci and ion traversal through a cell. Also Funayama et al. cultured cells on CR-39, and developed a unique method, in which the positions of ion traversals can be detected by etching only the reverse side (opposite side from the cells) of CR-39 at 37°C. This etching technique enabled them to detect etch pits during cell culturing, and it may be most effective method for analyzing the cellular responses of irradiated living cells. However, this is applicable only to experiments with ion energies high enough to penetrate through CR-39 or, alternatively, when thinner polycarbonate foils are used for growth and etching, as demonstrated by Jakob et al., with live cell imaging of heavy-ion-induced radiation responses by their beamline microscopy. Recently, Gaillard et al. developed extremely thin CR-39 with 10-μm thickness, which enable them to detect 5.48 MeV α-particles emitted from 241Am. However their technique of producing very thin CR-39 may demands skills of accomplished technical assistance. The method introduced in this paper may be the most convenient way to detect the ion traversal position in cells, and an effective method for irradiation experiments with low-energy heavy ions.

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