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Detection of DNA damage by space radiation in human fibroblasts flown on the International Space Station

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ABSTRACT

Although charged particles in space have been detected with radiation detectors on board spacecraft since the discovery of the Van Allen Belts, reports on the effects of direct exposure to space radiation in biological systems have been limited. Measurement of biological effects of space radiation is challenging due to the low dose and low dose rate nature of the radiation environment, and due to the difficulty in distinguishing the radiation effects from microgravity and other space environmental factors. In astronauts, only a few changes, such as increased chromosome aberrations in their lymphocytes and early onset of cataracts, are attributed primarily to their exposure to space radiation. In this study, cultured human fibroblasts were flown on the International Space Station (ISS). Cells were kept at 37 °C in space for 14 days before being fixed for analysis of DNA damage with the γ-H2AX assay. The 3-dimensional γ-H2AX foci were captured with a laser confocal microscope. Quantitative analysis revealed several foci that were larger and displayed a track pattern only in the Day 14 flight samples. To confirm that the foci data from the flight study was actually induced from space radiation exposure, cultured human fibroblasts were exposed to low dose rate γ rays at 37 °C. Cells exposed to chronic γ rays showed similar foci size distribution in comparison to the non-exposed controls. The cells were also exposed to low- and high-LET protons, and high-LET Fe ions on the ground. Our results suggest that in G1 human fibroblasts under the normal culture condition, only a small fraction of large size foci can be attributed to high-LET radiation in space.

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1. Introduction

Space radiation consists of energetic charged particles of varying charges and energies (Dietze et al., 2013). Astronauts' exposure to space radiation on future long duration missions to Mars, or missions back to the Moon, is expected to result in deleterious consequences ranging from cancer to comprised central nervous system (CNS) function (Cucinotta and Durante, 2006). Space radiation can also cause mutations in microorganisms and potentially influence the evolution of life in space (Zhou et al., 2013). Measurement of the space radiation environment has been conducted since the very beginning of the space program, with radiation in a low earth orbit first detected with a Geiger counter on board the first US satellite Explorer 1 (Van Allen, 1983). The Van Allen Belts were later mapped by detectors on the satellites located in different orbits circling the Earth (Badhwar, 1997). Outside of the Earth's magnetosphere, the radiation environment around the Moon, including secondary neutrons emitted from the Lunar surface, has also been characterized (Mitrofanov et al., 2008). Recent measurements of cosmic radiation during the transit from Earth to Mars, as well as on the surface of Mars by the Curiosity rover, provided valuable

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information about the environment that astronauts will face in future exploration missions (Zeitlin et al., 2013; Hassler et al., 2014).

Compared to the quantification of the space radiation environment using physical detectors, reports on the direct measurement of biological consequences of space radiation exposure have been limited, due primarily to the low dose and low dose rate nature of the environment. Most biological assays have failed to detect the radiation effects at acute doses lower than 5 cSv (Tucker et al., 2013; Turner et al. 2015). In an attempt to detect mutations from space radiation exposure in repetitive microsatellite sequences, human tumor cells were flown on the Space Shuttle for 9 days. Even though these repetitive sequences are prone to alteration, no increase in mutation frequencies was reported (Ishizaki et al., 2001). Changes at the molecular level, e.g. in mRNA and proteins, can be detected for lower radiation doses, particularly in expressions of stress related genes (Amundson et al., 2003; Asaithamby and Chen 2009). However, multiple stress factors such as microgravity are present in space, and it is difficult to distinguish space radiation effects from effects induced by other stress factors that also contribute to the expression changes of these molecules (Paul and Ferl, 2002). Biological effects of radiation exposure also vary depending on the dose rate. For example, cytokine induction can be observed in human umbilical vein endothelial cells after γ irradiation at a dose rate of 4.1 mGy/h, but not at 1.4 mGy/h (Ebrahimian et al., 2015). Cellular or DNA damages by chronic radiation are particularly difficult to detect if the damages are allowed to repair during the exposure period. However, accumulated damages in frozen cells in space have been reported (Takahashi et al., 2008; Meyer et al., 2013; Yatagai et al., 2011).

Yet, space radiation exposure is the primary cause of some of the effects observed in astronauts. Although a number of astronauts who have flown in space have died of cancer, it is difficult to pinpoint space radiation exposure as the primary cause of these deaths due to the lack of statistical power or proper controls. One of the effects of space radiation exposures in humans is light flash, experienced first by astronauts on their trips to the Moon (Fazio et al., 1970) and in Skylab missions (Pinsky et al., 1975). On the ground, such light flashes were confirmed to be caused by exposure of the retina to charged particles (Budinger et al., 1972). Another effect of space radiation exposure is early onset of cataracts (Cucinotta et al., 2001). Although individuals in the general population are expected to develop cataracts at an older age, a group of astronauts that were exposed to higher levels of radiation in the eye were found to develop cataracts at earlier ages than a group receiving lower doses of radiation (Cucinotta et al., 2001). The third effect of space radiation exposure in astronauts is elevated chromosome aberrations in the lymphocytes detected after 3 to 6 month missions on the space station (Yang et al., 1997). However, the exposure after a typical two-week Space Shuttle mission was low, such that no changes in the chromosome aberration frequencies were significant (George et al., 2001).

In the present study, we flew cultured confluent human fibroblasts, mostly in G1 phase of the cell cycle, to the International Space Station (ISS). The cells were fixed in space after being on the ISS for 14 days. The fixed cells were later returned to the ground and subsequently stained with the γ-H2AX antibodies that are commonly used as a marker for DNA damage, particularly DNA double strand breaks, induced by both low- and high-LET radiation (Rogakou et al., 1998; Takahashi et al., 2008; Jacob et al., 2009; Asaithamby et al., 2011; Meyer et al., 2013). In comparison to other methods, the γ-H2AX assay has been shown to detect DSB in cells at doses as low as 1 mGy, an improvement by several orders of magnitude over other methods (Rothkam and Lobrich 2003). This method has also been shown to be useful for detecting in vivo responses to radiation in normal tissue of radiation therapy patients (Ovarnstrom et al., 2004). Previously, we used the γ-H2AX assay to investigate DNA damages in the same cell type, as used in the present flight study, after low- and high-LET radiation exposures along the traversal of the charged particles (Desai et al., 2005a,b). The assay has also been applied to analyze DNA damage in cells that were kept frozen in space for 134 days (Ohnishi et al., 2009).

In this study, the γ-H2AX foci were captured with a laser confocal microscope. To confirm that some large track-like foci were from space radiation exposure, we also exposed, on the ground, the same type of cells to both low- and high-LET protons, and high-LET Fe ions. In addition, we exposed the cells to low dose rate γ rays in order to rule out the possibility that the large track-like foci can be induced by chronic low-LET radiation.

2. Materials and methods

2.1. Flight hardware

BioCells cell culture chambers manufactured by BioServe Space Technologies, University of Colorado, as shown in Fig. 1, were used to culture the cells in the flight experiments (Zhang et al., 2016). The top surface of the chamber is made of a gas permeable membrane allowing O_2 and CO_2 exchange in the medium. The bottom surface was cut from the bottom of a T-150 flask (Corning, Corning, NY) that was treated for tissue culture and mounted with seal gaskets. Adherent cells were grown on the bottom surface only. The chamber contains three ports that can be connected to syringes or bags containing washing or fixing solutions during the respective steps.

Prior to launch, the BioCells with confluent fibroblasts and fresh medium were sealed in a Cell Culture habitat (CHab) that contained 5% CO_2. The habitat was then placed into a Commercial Generic Bioprocessing Apparatus (CGBA, BioServe Space Technologies, University of Colorado, Boulder, CO) at a pre-set temperature. Once the cells reached the ISS, the habitat was transferred to another CGBA located in the US laboratory module of the ISS.

2.2. Cell culture

Normal human foreskin fibroblast cells (AG1522, National Institute of Aging) were grown in cultured dishes in a monolayer and the growth is contact-inhibited. Cells of less than 10 passages, routinely cultured at 37 °C, 95% humidity, and 5% CO_2 in α-MEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 50 mg/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA), were used as described in our previous study (Wu et al., 2006). The cell line has been used extensively in radiation research by investigators, including ourselves (Cornforth and Bedford 1983; Wu et al., 2003; Wu et al., 2006). Cells at passage 6 were frozen and shipped to Kennedy Space Center (KSC) months prior to the scheduled launch date. Two weeks prior to launch, the cells were thawed and plated in T-75 flasks. Seven days prior to the launch date, the cells were seeded in the BioCell cell culture chambers (10 flight and 10 ground control BioCells) at a density of 1×10^6 cells per BioCell. The cells reached confluence 2 days before launch, with 92% of the cells expected in the G1 phase of the cell cycle. One day prior to the launch, media in the BioCell was replaced with fresh media, and the cell culture chambers were inserted into sealed habitats (5 BioCells per habitat) that were each flushed with 5% CO_2/balance air .The samples were then transported to the launch pad and kept at 20 °C. Ground controls were prepared following the exact same procedure used for the flight samples.

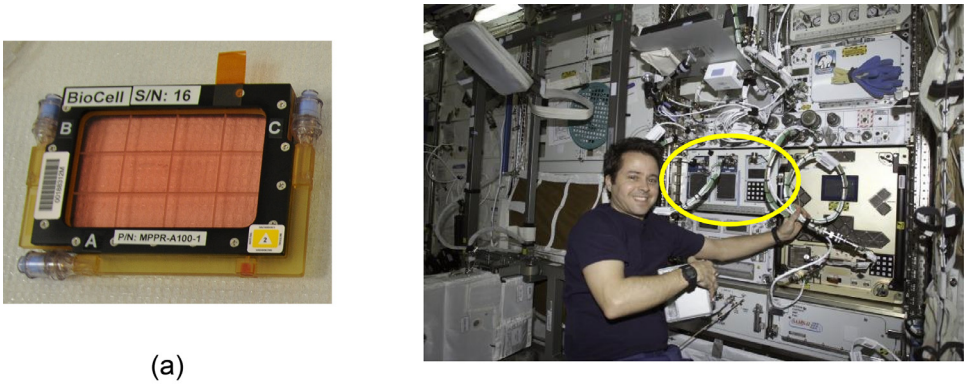

Fig. 1. (a) Human fibroblasts were cultured in a BioCells cell culture chamber used for the flight experiment. Cells were grown on only the bottom surface. (b) The cells were kept in a CGBA (BioServe Space Technologies, circled in the photo) incubator inside the US module of the ISS at 37 C.

2.3. Flight experimental timeline

On April 18, 2014, our experimental payload was launched from NASA Kennedy Space Center (KSC) in Florida. The cells were kept at 20 °C until they reached the ISS. On April 22, the two habitats containing the cells were then transferred to another CGBA preset at 37 °C. On May 6, cells in the BioCell were washed with PBS and fixed with a final concentration of 2% paraformaldehyde (PFA) for 30 min at ambient temperature before being washed with PBS. By this time, the cells had been cultured at 37 °C for 14 days. No medium change or subculture of the cells were performed during the 14 day period. Immediately after fixation, the samples were transferred to a 4 °C refrigerator onboard the ISS. On May 18, 2014, the samples were returned to the Earth. The samples were kept at 4 °C until they arrived at NASA Johnson Space Center (JSC) in Houston, Texas.

Processing of the samples on the ISS was performed by Astronaut Thomas Marshburn in the Microgravity Science Glovebox. The ground control experiment was performed in the same manner at KSC, but with a 6 h offset from the flight schedule.

2.4. Ground experiment with low dose rate γ rays

Confluent AG1522 cells in chamber slides were exposed to a low activity Cs-137 γ source at NASA Johnson Space Center, Houston, Texas for 14 days continuously in an incubator that was maintained at 37 °C during the exposure period. The dose rate at the sample was 0.045 cGy/hr and the accumulate dose over the period of 2 weeks was 16.3 cGy. At the end of exposure period, the cells were washed with PBS and fixed with 2% paraformaldehyde for 15 minutes at room temperature. Non-exposed controls were kept in an incubator for the same period.

2.5. Ground experiment with charged particles

AG1522 cells cultured in the chamber slide were maintained in the same culture solution as in the flight experiment in an incubator at 37 °C. Cells at confluence were exposed to 150 MeV protons at entrance and near the Bragg peak, as well as 600 MeV/u Fe ions, at the NASA Space Radiation Laboratory located in Brookhaven National Laboratory, New York. The particles traversed in parallel to the monolayer cells along the length of the slide, following the exposure scenario as in our previous study (Desai et al., 2005a; Wu et al., 2006), as shown in Fig. 2. Protons of 150 MeV have a residual range of 16.4 cm in water, and an LET value of 0.54 keV/µm. To achieve high-LET values for protons, polyethylene blocks were placed in front of the chamber slide so that the primary protons stop in the middle of the slide. The entrance dose of protons was 0.2 at a dose rate of 0.5 Gy/min. Confluent cells were also exposed to 600 MeV/u Fe ions in the same exposure scenario without polyethylene shielding. The LET value of Fe ions at entrance is 172 keV/µm and the residual range in water 12.6 cm. The entrance dose of Fe ions was 0.1 at a dose rate of 0.5 Gy/min. At 30 min and 24 h after irradiation, the cells were fixed with 2% paraformaldehyde for 15 min at room temperature.

2.6. Immunohistochemistry analysis

Cells fixed with paraformaldehyde from space flight and ground experiments were rinsed with 1 x PBS, and permeabilized with 0.25% Triton-X-100 in 1x PBS for 15 min at room temperature. After blocking with 3% BSA in PBST (1 x PBS + 0.5% Tween-20) overnight at 4 °C, the samples were incubated with anti-γH2AX (1:500, Novus Biologicals, NB100-79,967; 1:400, EMD Millipore, 05-636) and appropriate fluorescence-conjugated secondary antibodies. The stained samples, counter stained with DAPI, were mounted with ProLong® Gold Antifade Mountant medium (Life Technologies).

2.7. Image analysis

Three dimensional images of γ-H2AX foci were captured with a Leica laser confocal microscope with a 60 X objective lens at a step of 0.3 µm. Images were analyzed with Imaris version 7.6.3 (Bitplane Technologies, Zurich Switzerland). Nuclei were identified via their DAPI signal and a rectangular region of interest was constructed around each nucleus. To analyze foci, a standardized set of parameters to maximize object detection and minimize inclusion of background objects were developed and employed on each region of interest. Specifically, thresholding was done using a local contrast approach. In this method, background subtraction is used to estimate and remove the background intensity at each voxel. This was done through a Gaussian filter employing a user selected 4 pixel width kernel which generates resultant intensities by weighing central voxel intensities more than neighboring voxels. The filter size was selected based on approximate sizes of the signal objects in order to reduce noise and improve later thresholding without biasing the intensity value obtained. Following this processing, a user selected threshold, which captures signal objects without including non-specific extraneous signal, was applied to all the regions of interest. Finally, every created surface was checked to verify there were no artifacts such as merging of neighboring objects or inclusion of small extra-nuclear objects. If such objects were present, they were deleted. Data was generated automatically by Imaris, which calculates statistics such as volume and sphericity for detected objects.

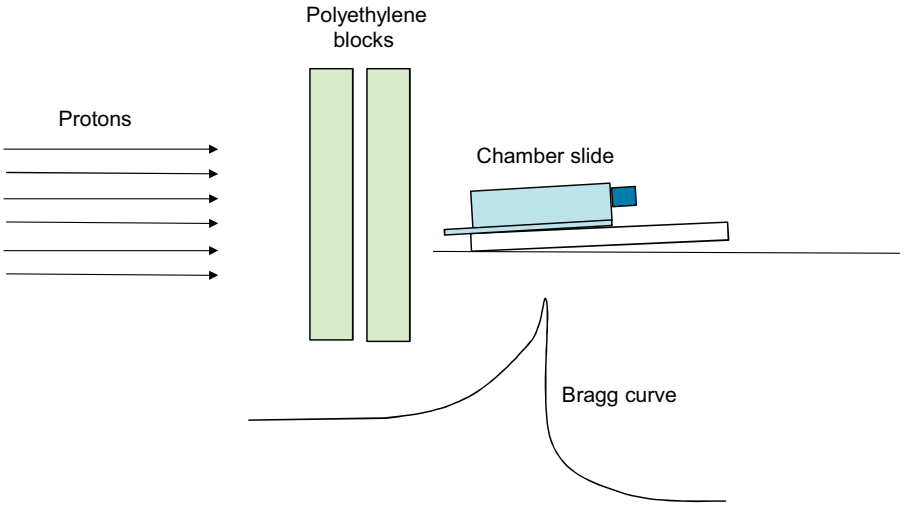

Fig. 2. Human fibroblasts exposed to energetic particles. Polyethylene blocks were placed in front of the chamber slides to achieve high-LET protons at the location of the Bragg peak in the middle of the slide. For Fe ions and low-LET protons, no polyethylene blocks were used.

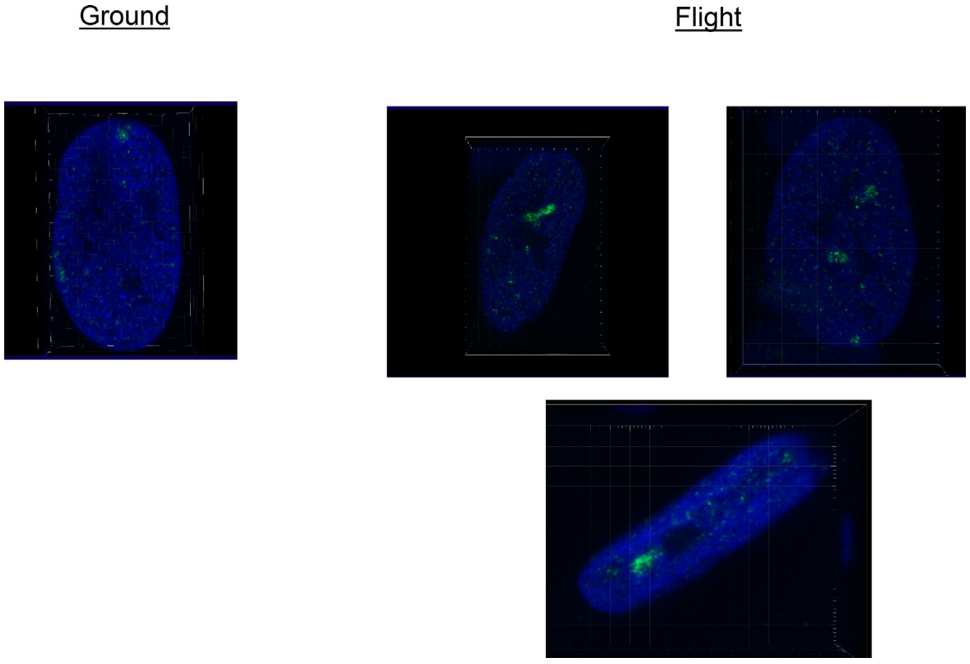

Fig. 3. Images of γ-H2AX foci in human fibroblasts that had been flown in space for 14 days and on the ground.

The calculation for sphericity (Ψ) (Wadell, 1935) is the ratio of the surface area of a sphere with the same volume as the measured particle to the surface area of the particle.

3. Results

3.1. Spaceflight experiment

A total of 200 cell nuclei from flown samples and 200 from ground controls were scanned with a laser confocal microscope, and the number of γ-H2AX foci per cell nucleus, as well the volume of each of the foci, was analyzed. Fig. 3 shows several images of the cell nuclei in the flight and ground samples. Several flown cells contained γ-H2AX foci that have apparent track shapes. These large track shaped foci were found only in the flown cells, but not in the ground controls. Fig. 4a shows the number of foci per cell nucleus as a function of the foci volume cutoff. Such numbers would increase when small size foci are included. Several of the flown cells had large size foci in comparison to the ground control, as shown in the distribution for sizes above 0.5 μm^3. To quantify the shape of these large size foci, we present in Fig. 4b the sphericity as a function of the volume. It is shown that several foci that are greater than 6 μm^3 had low sphericity below 0.6, indicating that these foci had a track shape, potentially induced by high-LET particles.

3.2. Experiments with low dose rate γ rays

To verify whether large size foci are caused by space radiation, we exposed human fibroblasts to low dose rate γ rays at a similar cell culture condition as in the flight experiment. The cells were exposed continuously at 37 °C for 14 days. At the end of the irradiation, the cells received an accumulated dose of 16.3 cGy. Fig. 5a shows the comparison of the size distribution as a function of the cut-off foci volume. At such a low dose and low dose rate, there were no significant differences in foci number between irradiated

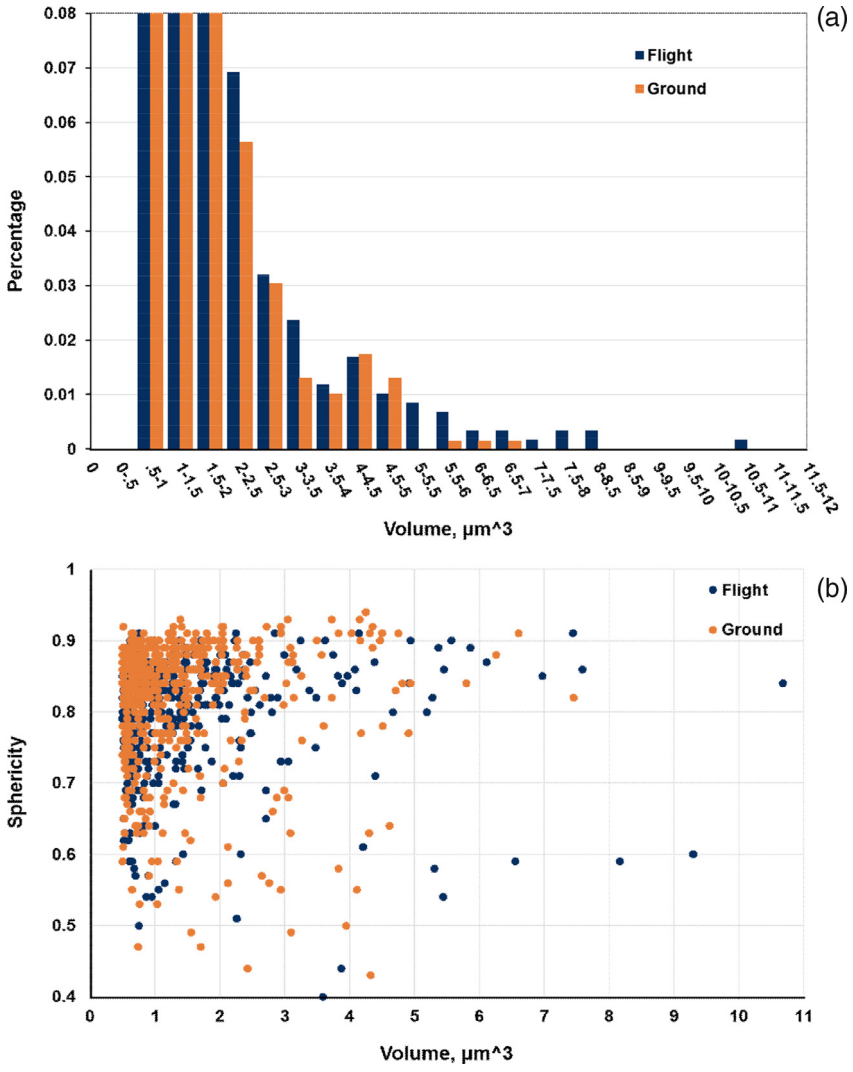

Fig. 4. (a) Volume distribution of the γ-H2AX foci in flown and ground cells fixed on Day 14.Several large size foci were observed in the flown cells. (b) Sphericity of foci in cells fixed on d14 between ground and space. Several large size foci with low sphericity were found in only the flown samples.

and non-irradiated samples. Comparison of sphericity between the exposed cells and non-exposed controls (Fig. 5b) showed some large size foci, but none of large ones had a low sphericity as found in the flown cells. Since γ rays are expected to produce similar foci yield as high energy protons, we conclude that high energy protons (low-LET) at the low dose rate in space are unlikely to produce observable foci in the flight samples.

3.3. Experiments with charged particles

When charged particles traverse along the plane of monolayer cells, γ-H2AX tracks can be visualized. Fig. 6 shows several images of AG1522 cells after exposure to low-LET protons, high-LET protons and high-LET Fe ions. Distinct foci that were scattered in the cell nucleus can be seen at 30 min after high energy proton exposure (LET ~0.54 keV/µm), and these foci would disappear at 24 h after irradiation. Protons at the Bragg peak region induced γ-H2AX foci that formed apparent tracks. At 24 h post irradiation, a significantly higher yield of foci were still present, although these foci may not form a straight line, as the tracks visibly seen immediately after irradiation.

High-LET Fe ions of 600 MeV/u (LET = 172 keV/µm) produced dense tracks in the cell nucleus immediately post irradiation (Fig. 6). Some of the foci size were apparently greater than the size found in the unirradiated controls. At 24 h post irradiation, some of the large size foci still remained. This study was intended to confirm that high-LET radiation induces γ-H2AX tracks. Detailed kinetics of high-LET radiation-induced foci has been reported previously (Desai et al., 2005a; Asaithamby et al., 2011).

4. Discussion

Detection of radiation effects directly in biological systems is challenging due primarily to the low dose and low dose rate nature of the radiation environment. Most of the biological damages that have been detected were accumulated after several months in space with a total exposure reaches above 5 cSv. In cell culture experiments, the accumulation was achieved by keeping the cells frozen in space so that no repair of the damages occurs until the cells are thawed after returning to the ground (Takahashi et al., 2008; Yatagai et al. 2011). In astronauts, chromosome aberrations that were detected post 3–6 month missions were mostly of a stable type that can be accumulated in the blood (George et al., 2001). When the cells in the present study were cultured on the ISS for 14 days, a thermoluminescent radiation dosimeter was placed in a locker adjacent to the samples. The dosimeter detected an absorbed dose rate of 0.21 mGy/day, resulting in an accumulated dose of 2.9 mGy over a period of two weeks

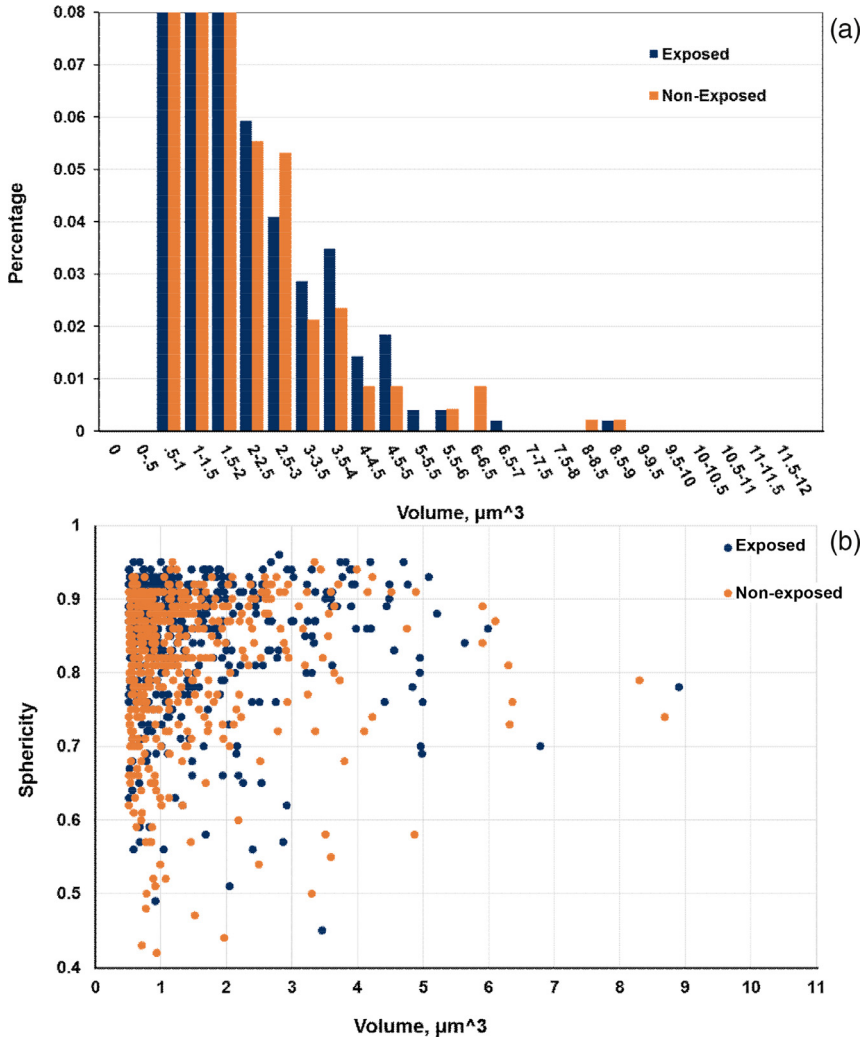

Fig. 5. (a) Volume distribution of the γ-H2AX foci in the cells exposed to low dose rate γ rays for 14 days on the ground in comparison to the unexposed controls. (b) Sphericity of foci in the cells exposed to low dose rate γ rays on the ground in comparison to the unexposed cells.

(Gaza et al., 2014; Ruedas et al., 2015). After 1 Gy X irradiation acutely, the average number of radiation-induced γ-H2AX foci per cell nucleus was estimated to be 25 in primary human fibroblasts, and the average number per Gy of α-particles was slightly less (Staaf et al., 2012), without consideration of small size foci detected with laser confocal microscope as in the present study. Estimated with 25 foci/Gy, the number of increased foci due to the exposure to 2.9 mGy space radiation would be 0.07/cell nucleus, even if the damage is accumulated. Therefore, exposure of live cells at 37 °C to space radiation is not expected to yield changes of the average number of γ-H2AX foci in comparison to the ground controls. In the present study, we did not detect a significant difference in the number of foci between ground and flown cells. However, we found that several γ-H2AX foci in the flown cells were large, and some of the large ones had low sphericity with a clear track shape (Fig. 3). These foci had a distinct shape of high-LET signature and we concluded that they were induced by direct exposure to space radiation. No such track shaped foci were found in the ground controls.

To confirm that the distinct foci were induced from high-LET particles in space, we exposed AG1522 cells to low- and high-LET protons, and high-LET Fe ions using a ground-based accelerator. Low-LET protons are known to produce similar biological effects of γ rays or X-rays (George et al., 2002). Low-LET induced foci of similar patterns as gamma rays initially (Fig. 6). Most of the low-LET proton-induced foci disappeared at 24 h after irradiation. High-LET protons near the Bragg peak induced foci that form a clear track at 30 min after irradiation. At 24 h post irradiation, some of the high-LET proton-induced foci can still be found in the cell nuclei, with some still forming a clear track in the cell nucleus (Fig. 6). High-LET heavy ions induced dense, large size foci. Some large size foci still remained at 24 h post irradiation. Previously, we have reported the initial and residual γ-H2AX foci after high-LET charged particle irradiation of AG1522 human fibroblasts (Desai et al., 2005a,b). Repair kinetics of radiation-induced foci has also been reported for other cell types and for different radiation qualities (Asaithamby et al., 2011; Antonelli et al., 2015). The track shaped foci found in cells fixed on Day 14 in our present study matched the shapes of the high-LET radiation induced foci on the ground. Although the cells were fixed on Day 14, our ground data indicated that these foci may have been induced within days of the fixation (Desai et al. 2005a).

To further confirm that the large size foci in the flown cells were from exposure to high-LET radiation, we exposed the same cell type to γ rays continuously for two weeks. Although the accumulated dose of 16.3 cSv over two weeks was higher than in the flight experiment, the dose rate of $\sim$ 1 cSv/day was low enough to be considered chronic. Analysis of the volume and sphericity of the

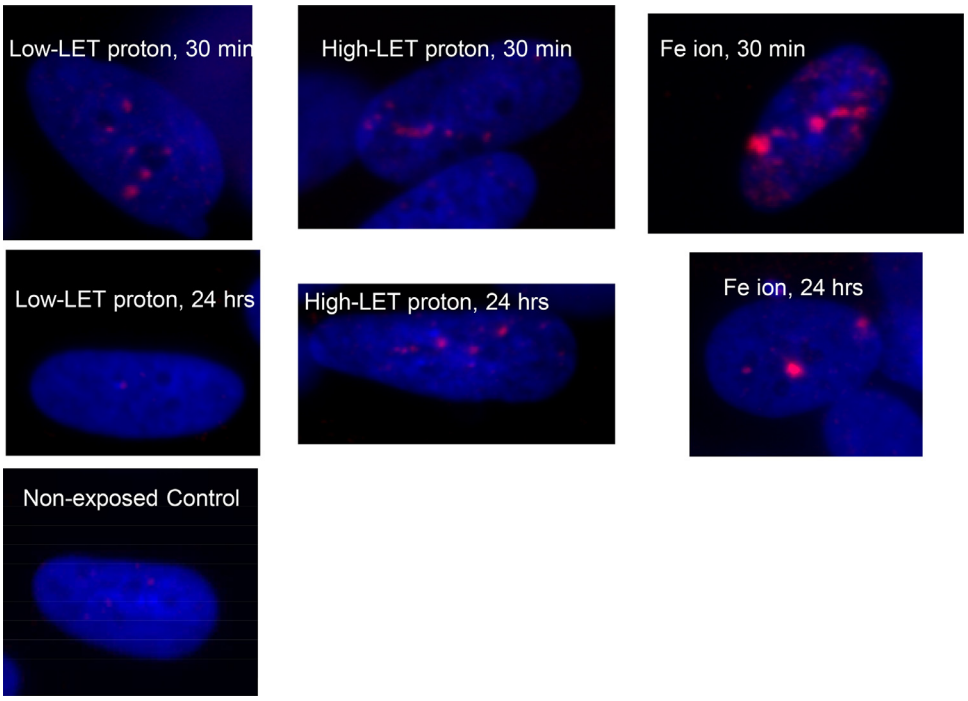

Fig. 6. γ-H2AX foci in human fibroblasts exposed to low- and high-LET protons and high-LET Fe ions acutely. Cells were fixed at 30 min or 24 h after exposure.

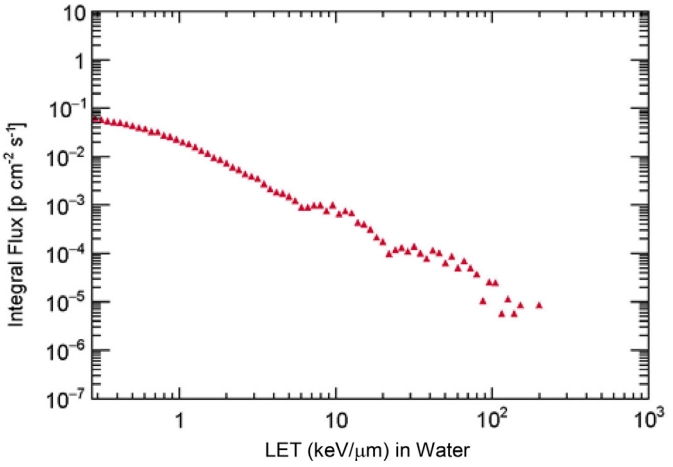

Fig. 7. Integral LET spectrum measured inside the US module of the ISS at the time of the spaceflight experiment.

foci indicated that chronic exposure to low-LET radiation resulted in similar distribution of the volume and sphericity as the non-exposed controls (Fig. 5). It has been reported previously that the number of foci in human fibroblasts exposed to 10 cGy/h γ rays for a total dose of 1 Gy was similar to the unexposed control (Kato et al., 2006), in agreement with our current finding. Differences in the mean number of foci after low-dose rate γ irradiation were found in ATM$^{-/-}$ and ATM$^{+/-}$ cells, however (Kato et al., 2006).

In the orbit of the International Space Station, the majority of radiation that the cells were exposed to is protons, with a small fraction of heavy ions (O' Sullivan et al., 2004). Fig. 7 shows the integral LET spectrum of the radiation field measured inside the US module of the ISS by the Radiation Environmental Monitor (REM) active instrument for approximately one week around the time when the cells were flown in space (Stoffle, 2015). The fluence in the LET spectrum is for particles from all directions as measured by a planar detector, which is similar to the shape of the cell nuclei in AG1522 cells that are flat. Assuming that large size tracks were produced with traversal of particles with LET > 10 keV/μm, and an average cross section area of the cell nuclei of 140 μm^2 (~280 μm^2 from both sides) (Azzam et al., 1998), only 1 in 300 cells will be traversed by a high-LET particle for over a period of 14 days. Some of the high-LET radiation induced tracks are known to persist for days, and therefore, it is difficult to estimate the number of high-LET tracks observed in the cells. Of 200 cells analyzed in the present study, only several cells were expected to be traversed by high-LET particles, which was in agreement with the estimation from the measurement of the LET spectrum.

Our present study was the first to use the γ-H2AX assay to detect DNA damage in live cells from direct traversal of high-LET particles in space. Quantification of such damages will require more cells to be analyzed to achieve a greater statistical significance. Future studies will also be performed with co-staining with other DNA damage response markers.

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