Single-ion microbeam as a tool for low-dose radiation effects investigations

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Abstract. Practical assessment of human radiation exposure risk deserves particular attention especially for low doses (and low dose rates), which concern environmental and occupational exposure. At these dose levels ionizing radiation exposures involve mainly isolated charged particle tracks, which strike individual cells at time intervals averaging from weeks to several years apart. Accelerator-based microbeam irradiation technique offers a unique tool to mimic such an exposure, allowing irradiating single cells individually with micrometer precision and with a preset number of charged particles down to one particle per cell. A horizontal single-ion microbeam facility for single-cell irradiations has been designed and set up at the INFN-LNL 7MV CN Van de Graaff accelerator. The light ion beam is collimated in air down to a section of 2-3\(\mu\)m in diameter by means of appropriate pinholes. Semi-automatic cell visualization and automatic cell positioning and revisiting system, based on an inverted phase contrast optical microscope and on X-Y translation stages with 0.1\(\mu\)m positioning precision, has been developed. An in-house-written software allows to control remotely the irradiation protocol. As a distinctive feature of the facility, cell recognition is performed without using fluorescent staining and UV light. Particle detection in air, behind the biological sample, is based on a silicon detector while in-air beam profile and precise hit position measurements are accomplished by a custom-made cooled-CCD camera and Solid State Nuclear Track detectors, respectively. A particle counting rate of less than 1 ion/sec can be reached.

1. Introduction

Common people are continuously exposed to low doses of ionizing radiation which comes from two major sources: natural (background) and man-made radiation. Natural sources of radiation, including terrestrial (above all radon gas), internal radiation sources and cosmic rays, constitute the major contribute of exposure of the population. Radiations are also routinely used in medicine for radio-diagnostic and radiotherapy [1].

Up to now no experimental and epidemiological data about low doses are available and so human cancer risk evaluation at low-doses derives from extrapolation of experimental and epidemiological data collected at high doses by means of \textit{in-vitro} and \textit{in-vivo} conventional cell irradiations as well as from Uranium miners, Japanese atomic bomb survivors, nuclear fallout accidents studies. In radiation protection a linear no-threshold (LNT) risk model is assumed in the low dose region in a precautionary way.

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Recent *in-vitro* radiobiological results following direct investigations of low dose effects seem to indicate a non-linear response to low dose exposure as a consequence of phenomena acting in this dose region: hypersensitivity, adaptive response and bystander effect.

At INFN - Laboratori Nazionali di Legnaro experiments with V79 cells irradiated with gamma-rays and helium-4 ion broad beams have shown a deviation at low doses from conventional linear-quadratic behaviour of survival curves: these data show a hyper-sensitivity in the low dose region followed by increased radio-resistance as the dose increases (the so called adaptive response), especially in the case of higher LET [2]. Analogue behaviour of survival curves has been found at GSI after irradiation of V79 cells with high LET carbon ions [3]. Considering that adaptive response could imply a sort of induction of repair processes triggered by increasing level of cellular damage, a sub-linear dose response curve could be taken into account.

A nowadays highly debated phenomenon (the so called bystander effect) seems to be present at low doses suggesting that the overall damage observed in an irradiated population could have a component from cells that are not directly hit by radiation. This effect implies that cell damage signals, induced by irradiation, might be transmitted from irradiated to neighbouring non irradiated bystander cells in the same population (by factors secreted in the medium or/and by direct cell-to-cell communication through gap junction). Considering this phenomenon, low dose effects might be higher than predicted by high dose extrapolation and cancer risk curve might be supra-linear at low doses. Anyway, it has to be considered that bystander effect (via apoptosis and differentiation) might be a protective mechanism, as adaptive response, causing overestimation of the low dose risk by the LNT model. So bystander effect and adaptive response may operate as in opposite as in the same direction to produce an overall biological effect; very few data on the role of these two phenomena are available [4].

Considering that ionising radiation exposures at protection dose levels involve mainly isolated charged particle tracks, which strike individual cells at time intervals averaging from weeks to several years apart, a “single-ion” microbeam facility is a powerful tool to be considered to irradiate single cells, one by one, with an exact and predefined number of particles down to one particle per cell. It allows overwhelming the limitation of Poisson distributed particle arrivals that are inherent to conventional broad-beam irradiation at low doses (either with accelerated broad beam or alpha sources). Moreover the high spatial resolution and targeting accuracy of these apparatuses allow selecting only few cells to be targeted inside a larger cell population offering unique opportunities for bystander studies. In particular an accelerator based microbeam facility is the more appropriate tool to perform systematic low-dose investigations in terms of radiation quality (type and energy of ions), defined target (selected cells inside a population) and defined and reproducible dose (number of counted particles per cell, down to one particle/cell).

The main elements necessary in the design of an accelerator based microbeam apparatus for single cell irradiation are:
- a mono-energetic ion beam of appropriate energy, reduced at micrometer size, comparable to cell dimension;
- a low intensity beam current;
- a cell visualisation and localisation system based on a microscope coupled to a software for logging cell co-ordinates and image analysis;
- a cell micro-positioning and revisiting system for cell examination under microscope, to place cells individually in front of the beam with high precision and repeatability and to revisit them after irradiation under the microscope;
- a particle detection system to count particles hitting (or crossing) cells (placed before or behind the cell holder);
- a fast beam deflection system to switch on/off the beam to deliver the pre-set number of particles per cell with high reliability;
- an appropriate cell holder to keep cells in sterile and humid conditions during irradiation.
In the present paper the solution adopted for each of these elements in setting up the “single-ion microbeam facility at INFN - Laboratori Nazionali di Legnaro will be described [5]. The facility overall features and performances will be also reported.

2. Experimental set-up
The single ion microbeam apparatus has been installed at the horizontal broad-beam Radiobiology facility set up at the -45° beam pipe of the 7MV Van de Graaff accelerator in order to allow alternatively as single-cell single-ion irradiation experiments as broad beam irradiation experiments. The Radiobiology facility delivers beams of protons, deuterons, He-3 and He-4 ions covering a LET range from 7 to 150 keV/µm (in tissue). The facility is based on two scattering chambers housing gold foils which broad the beam over 1 cm diameter area and reduce its intensity current [6].

2.1. Micro-collimation device
Beam is extracted in air through a bi-aluminized mylar window and then collimated at micrometer scale. As a distinctive element of the LNL microbeam facility, the ion beam is micro-collimated in air (and not in vacuum) by means of a tantalum pinhole of 200 µm thickness with a central hole of 2-3 µm or, alternatively, 5 µm diameter. A tilting flange is used for the alignment of the pinhole aperture with respect to the beam axis.

2.2. Cell holder
An especially designed stainless steel Petri dish (Ext. Dia.: 100 mm; Int. Dia.: 70 mm) with a 20 µm thick cell chamber has been realized in order to keep cells in humid and sterile conditions during irradiation and in vertical position in front of the beam. The bottom and the cover of the cell chamber are 7 µm thick mylar foils, allowing particles to cross the cell chamber (containing cells and culture medium) and impinge on the detector placed downstream of it. Cells are grown attached to the mylar foil base, which faces the beam.

In order to have a fixed reference system to both Petri dish and seeded cells in such a way that each cell (to be irradiated or not) can be univocally identified under microscope and after Petri dish is removed from irradiation platform, a gold grid pattern has been devised to be printed on the mylar foil. Very recently first samples of patterned mylar foils have been produced through microlithography technique by the Engineering Sciences Dept. of Uppsala University, Sweden. The grid step is 100 µm and the grid line is 7 µm wide. Every row and every column of the grid are labelled by a number and so every square and every cell can be uniquely identified. Preliminary tests are underway.

2.3. Particle detection system
A high resolution and high sensitivity custom made cooled-CCD camera (DTA, Pisa, Italy) is used as particle detector to monitor the spatial particle distribution in air. It has been developed without optics nor shutter, but only with a 3 µm thick havar foil as window before the pixel matrix. The centroid of the particle distribution is taken as beam position.

A silicon detector is placed downstream of the Petri dish and is used to perform counting and energy measurement of the particles crossing the cell. Furthermore, it generates a trigger signal for electrostatic deflector to switch off the beam when the pre-set number of particles has been delivered. The electrostatic deflector is installed 5 m upstream of the mylar extraction window and has a response time of 150 ns [7].

As a post-irradiation off-line check of every particle impact points and individual hit cells, a very thin Solid State Nuclear Track (SSNT) detector foil could be placed before the cell sample during single-ion single-cell irradiation. The overlap of the etched particle tracks with the recorded cell positions image allows verifying the correctness of cell targeting. With this aim LR115, 13 µm thick, SSNT detector foils (courtesy by DOSIRAD, France) have been recently tested (see par. 3). Taking into account its manufacturing characteristics, LR115 is intended to be also used as basic substrate for cells, in place of the presently used mylar foil.
2.4. Cell visualization and recognition system

Cells visualization and recognition is performed by using an inverted phase contrast optical microscope (Olympus) coupled to a CCD and an in-house-written dedicated software for cell image acquisition and coordinates logging. As a distinctive feature of the INFN-LNL facility, cell recognition is performed without using fluorescent staining and UV light. However, this implies that the cell recognition procedure cannot be fully automatic but expert operator assisted, requiring time for cell identification. A specific developed software routine drives the micro-positioning translator stages (Physik Instrumente, Germany) for an automatic scanning of the cell sample under the microscope allowing cell identification and cell coordinates’ logging. The automatic scanning procedure can be stopped by the operator when a cell is visualized and identified; the fine localization of the cell can be executed, the cell coordinates can be logged and the scanning can be restarted from the point where it was stopped to complete the cell search.

2.4.1. Software routine for semi-automatic sample scanning. The software routine allows scanning the whole Petri dish area, step-by-step, by means a zigzag shaped pattern composed by long paths along the Y axis (in the ascending and descending directions) and short ones along the X axis. The minimum step of the scanning procedure can be set by the operator through the user dialog window panel as well as the number of steps that are necessary to cover the Petri dish diameter. Considering the dimensions of the especially designed Petri dish, a good choice is 100μm for the minimum step; 700 steps are set for the long paths to cover the diameter. During the scanning procedure the biological sample image is continuously acquired through the CCD camera coupled to the microscope and transferred to the PC by means of the IMAQ PCI-1409 (National Instruments) analog frame-grabber. The routine for the automatic scanning is based on three cycle indexes: n⁺, n⁻, n̂. The n⁺ and n⁻ indexes range from 0 to 700 and are related to the ascending and the descending scanning paths; n̂ ranges from 0 to 350 and, during the scanning, counts the numbers of performed single cycle (ascending path / short path /
descending path / short path). The indexes are visualized and continuously updated on the user dialog window panel and give the scanning status (at rest; active; in pause). Every time the operator stops the automatic scanning in order to log cell coordinates the three indexes are recorded by the software. This allows to restart the scanning from the point where it was stopped.

2.5. Cell positioning system

By means of a helicoidal guide the cell sample is moved from the horizontal position under the microscope to the vertical one in front of the ion beam. The custom-made cooled CCD camera is used to monitor beam particle distribution and measure beam position. The fine positioning of the cell dish in the X-Y plane under the microscope during the cell recognition step/phase and in front of the collimator for single cell irradiation is achieved by means of two precision translation stages with 0.1 µm positioning reproducibility.

Under the microscope, a fiducial marker placed near the cell holder is localized and its coordinates logged. This marker is taken as the origin of the reference system for each single cell coordinates. A CCD camera, equipped with a 40x objective is mounted close to the micro-collimator assembly and is used to re-localize the fiducial marker after the sample platform is moved in beam-position.

The beam position is measured relatively to the fiducial marker with the custom-made cooled-CCD camera, used as beam monitor. An appropriate software has been developed to analyze the image acquired by the cooled-CCD and calculate the centroid of particle spatial distribution. Every cell’s X-Y coordinates logged under microscope are corrected considering the new coordinates of the reference marker and (centroid) beam position. By recalling the corrected coordinate values every cell is placed in turn in front of beam and irradiated.

After irradiation, sample platform is moved again under microscope for cells revisiting and image acquisition to check the accuracy of cells locations.

3. Results and discussion

The optimization of the micro-collimated ion beam quality is achieved in terms of energy spectrum and particle counts. A measurement of the energy spectrum provides a reliable method for checking the collimator alignment and is always performed before each irradiation experiment. Different micro-collimator geometries and materials have been compared on the basis of the energy spectrum in air, that is on percentage of full-energy particles and peak full width at half maximum (FWHM). By using a tantalum pinhole collimator, for 10 MeV ⁴He²⁺ ion beam, at cell position, a full-energy peak percentage of 75% is obtained with a FWHM of 5% in energy (see figure 2, 5.6 MeV spectrum). Similar results have been also obtained with 8.9 MeV ³He²⁺ and 3 MeV ¹H⁺ ion beams.

The double scattering system and the micro-collimator in air allow achieving a very low counting rate, down to less than 1 particle/ second on the cell sample.

Test on beam has given good results in terms of the micro-collimated beam quality when the LR115 foil is inserted before the Petri dish (figure 2, 2.4 MeV spectrum). Energy spectra relative to different LR115-Petri dish set-up conditions are reported in figure 2. Preliminary tests of biocompatibility between LR115 detector and cells and of maintaining its registration properties against special treatments (as, for instance, UV sterilization necessary before cell plating) have been performed. Further experiments are in progress. Etching conditions (solution molarity, temperature of the etching, etching time…) are now under optimization in order to obtain a reliable protocol.

The microbeam facility described here shows attractive features capabilities that make it suitable for single-cell irradiations with counted particles allowing to perform systematic investigations on the low-dose effects of ionizing radiations in cultured mammalian cells.
Figure 2. Energy spectra of 10MeV $^4$He$^{2+}$ ion beam micro-collimated by 5μm tantalum pinhole: after the air gap between collimator and silicon detector ($E=5.6$MeV); with a 7μm mylar foil before the silicon detector ($E=4.9$MeV); with two 7μm mylar foils before the silicon detector ($E=4.2$MeV), representative of the present Petri dish configuration; with a 7μm mylar foil and a 13μm LR115 foil before the silicon detector ($E=3.4$MeV), when LR115 is used as bottom of the Petri dish; with two 7μm mylar foils and a 13μm LR115 foil ($E=2.4$MeV) when LR115 is placed before the Petri dish in the present configuration.

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