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Fluorescence time-lapse imaging of single cells targeted with a focused scanning charged-particle microbeam

Stéphane Bourret [a,b], François Vianna [a,b,c], Guillaume Devès [a,b], Vincent Atallah [a,b,d,e], Philippe Moretto [a,b], Hervé Seznec [a,b], Philippe Barberet [a,b,*]

[a] Univ. Bordeaux, CENBG, UMR 5797, F-33170 Gradignan, France
[b] CNRS, IN2P3, CENBG, UMR 5797, F-33170 Gradignan, France
[c] IRSN, BP 3, F-13115 Saint-Paul Lez Durance, France
[d] Department of Radiation Oncology, Institut Bergonié, Bordeaux, France
[e] Univ. Victor Segalen, Bordeaux, France

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ABSTRACT

Charged particle microbeams provide unique features to study targeted and non-targeted radiation response and have recently emerged as a powerful tool to investigate radiation-induced DNA damage and repair. We have developed a charged particle microbeam delivering protons and alpha particles in the MeV energy range equipped with online time-lapse imaging capabilities. The beam is focused to a sub-micrometer beam spot under vacuum by means of a triplet of magnetic quadrupoles and extracted in air through a 200 nm Si_3N_4 window. The end-station is equipped with an automated fluorescence microscope used for single cell targeting and online time-lapse imaging. Cells are kept in their medium during the irradiation procedure and the sample temperature is regulated to 37 °C. An overall targeting accuracy of 2.0 ± 0.7 μm has been measured by tracking the re-localization of the XRCC1 protein. First measurements of this re-localization shows the ability of our system to follow online the radiation-induced re-localization of proteins in the first minutes after irradiation.

1. Introduction

Charged particle microbeams are developed worldwide to perform targeted micro-irradiation of living cells. Although the first attempt to target living cells with charged particles has been performed by Zirkle and Bloom in the 50's [1], the routine use of automated systems really began in the mid 90's thanks to the developments in microscopy, motorized stages and computing [2]. The first automated charged particle microbeams, developed in the Gray Cancer Institute [3] and Columbia University [4], were initially based on collimated beams and have been extensively used to study the cellular response to low doses of ionizing radiation. More recently, microbeams using magnetic or electrostatic focusing have come to operation for radiobiology applications. They allow achieving a better lateral resolution and a higher irradiation throughput. Among the focused microbeams in operation today, several groups are routinely irradiating cells to investigate various endpoints: Microbeam II of the radiological research accelerator facility at Columbia University (New York, USA) [5], GSI (Gesellschaft für Schwerionenforschung, Darmstadt, Germany) [6], SNAKE (Bundeswehr University, Munich, Germany) [7], SPICE at NIRS (National Institute of Radiological Science, Chiba, Japan) [8], PTB (Physikalisch-Technische Bundesanstalt Braunschweig, Germany) [9], Surrey vertical nanobeam (Surrey university, UK) [10] and JAERI (Japan Atomic Energy Research Institute, Takasaki, Japan) [11]. Among them, several facilities provide sub-micrometer resolution [5–7] and even sub-micrometer targeting accuracy [12].

By their ability to target the radiation into specific subcellular regions in a highly controlled way, these charged particle microbeams are also increasingly used to study DNA damage and repair [13]. This is particularly true for heavy ion microbeams, which provide unique features to investigate DNA damage and repair following highly clustered damage. Moreover, by coupling these devices with fluorescent labeling and advanced fluorescence microscopy, it is possible to follow online the early response to radiation-induced DNA damage at the single cell scale [7,14,15]. Beside these applications, there is a growing interest in the use of microbeams for *in vivo* irradiation, either in tissues or small animals [16,17].

In the early 2000's, a charged particle microbeam has been commissioned at CENBG (Centre d'Etudes Nucléaires de

* Corresponding author at: Centre d'Etudes Nucléaires de Bordeaux Gradignan, Gradignan F-33175, France. Tel.: +33 5 57 12 08 64; fax: +33 5 57 12 08 01.
E-mail address: barberet@cenbg.in2p3.fr (P. Barberet).

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Bordeaux-Gradignan) for targeted irradiation of living cells using counted protons or alpha particles [18]. This prototype was initially developed for low dose studies and its ability to target living cells within a 10 μm-accuracy was demonstrated. However, a lack of targeting accuracy precluded any study requiring sub-nuclear irradiation. Therefore, we have re-designed our micro-irradiation set-up based on our light ion (protons or alpha particles) microbeam to improve the beam resolution and the targeting accuracy. In addition, this setup has been designed to follow the re-localization of GFP-tagged proteins by time-lapse fluorescence imaging. A great care has been put to provide optimal fluorescence microscopy quality and to minimize the possible cellular stress induced by the irradiation procedure (temperature control, cells kept in their growing medium, *etc.*). Currently, the following of the early cell response to radiation-induced DNA damage using fluorescence time-lapse imaging is conducted on a limited number of facilities. Most of these studies have been performed at SNAKE (Munich), GSI (Darmstadt) and PTB (Braunschweig). These facilities present the advantage of delivering a wide range of ions species, providing opportunities to investigate the DNA damage response as a function of LET (Linear Energy Transfer). There is a growing interest of using charged-particles to generate highly localized DNA damage since they offer the advantage of a well characterized dosimetry and a better understanding of the physical and biochemical processes of damage induction in comparison to the laser micro-irradiation commonly used in the DNA repair community [19]. Nevertheless, the use of charged particles generally suffers from the beam availability as well as low throughput and specific sample preparation. The evolution of the CENBG microbeam towards live-cell imaging online could therefore help to increase the number of facilities able to perform this type of research. The microbeam is installed on an ion beam platform opened to external users and can therefore meet the needs of the radiation biology community. This paper describes the performances of the CENBG microbeam as well as the first fluorescence time-lapse imaging acquisition showing the re-localization of the XRCC1-GFP protein at damaged sites in the first minutes after irradiation.

2. Materials and methods

2.1. Micro-irradiation facility

2.1.1. Beam characteristics

The micro-irradiation setup is installed on the AIFIRA ion accelerator facility (Applications Interdisciplinaires des Faisceaux d'Ions en Région Aquitaine). This accelerator (Singletron™, High Voltage Engineering Europa, The Netherlands) delivers protons and helium ions with energies up to 3.5 MeV. It presents an excellent beam brightness and energy stability, which makes it ideal to produce microbeams and even sub-micrometer ion beams [20]. In order to target single living cells, the beam is strongly collimated to reduce the particle flux to a few thousand ions per second on target and focused using a triplet of magnetic quadrupoles to achieve a sub-micron resolution under vacuum. A typical beam spot size under vacuum below 0.5 μm is routinely achieved when a 5-μm object collimator is used.

The exposure of the target cells to the charged particles is controlled using a fast electrostatic beam deflector allowing to open and close the beam within 1 μs (DEI PVM-4210). The delivered dose can be either controlled by counting the particles with a detector or by adjusting the opening time. In the latter case, the mean number of particles (N) hitting the cells is linearly related to the opening time and the relative statistical fluctuation in the number of traversals delivered to the cell decreases as N increases.

The micro-irradiation setup allows the exposure of cells to 3 MeV protons and alpha particles presenting a linear energy transfer of 12 and 128 keV μm^{-1}, leading to a maximum range in liquid water of 148 and 18 μm, respectively. As reported previously [21] a 3 MeV incoming alpha particle deposits from 1 to 2 MeV when passing through a cell nucleus, the fluctuation coming from the variety of cell geometries. In comparison, the traversal of a 3 MeV proton leads to an energy deposit ten times smaller. The typical delivered dose for one incoming 3 MeV alpha particle being thus between 0.1 and 0.2 Gy and between 0.01 and 0.02 Gy for one incoming 3 MeV proton.

2.1.2. End-station

In comparison to the system reported previously [18], the beamline and microscopy end-station have been fully re-designed to improve the ion microbeam performances as well as the microscopy features available online. An overview of the micro-irradiation apparatus is shown on Fig. 1. The ion beam is extracted in air through a 200 nm thick Si_3N_4 window (Silson Ltd., Northampton, England) and enters the sample through a 4-μm thick polypropylene (Goodfellow) foil used as a cell support. The traversal of the exit window, residual layer of air and polypropylene leads to a loss of resolution due to particle scattering in these materials. The thicknesses of the different materials used have been reduced as much as possible to preserve the micrometer resolution at the position of the cell monolayer. The ion beam is positioned on target by means of electrostatic scanning plates situated downstream of the last quadrupole. The cells present in a microscope field of view can be irradiated by moving the beam spot cell by cell. Larger displacements are done by the mechanical sample positioning stage (Newport™ M436 linear stages moved by Newport™ LTA-HS actuators). The full irradiation stage is included in an incubation cage regulated at 37 °C by a temperature control unit (model H201-T1, OKOLAB™).

The fast deflector, beam scanning and particle counting are performed by a stand-alone system called CRionScan as described by Daudin et al. [22]. Briefly, it consists in a real-time scanning and imaging instrument based on a Compact Reconfigurable Input/Output (Compact RIO™, National Instrument). This system is equipped with analog and digital Input/Output modules (NI 9401, NI 9263) used to steer the beam on target, to open or close the beam and to count pulses provided by a particle detector. It is based on a real-time controller and a Field Programmable Gate Array (FPGA), providing a fast handling of digital and analog signals. It is addressed by the irradiation software using TCP/IP messages to achieve automated calibration and irradiation procedures.

2.1.3. Dose control

In order to monitor the beam and to count the incoming particles, a PIN diode has been installed on the microscope objective wheel. This diode is used to measure precisely the incoming particles counting rate prior to the irradiation of a sample. In the case of light particles of a few MeV, it has to be noted that the range in matter is very limited (a few tens of micrometers) and that the only way to detect the particles downstream the cells during the irradiation is thus to remove the nutrient medium. This approach is performed on other systems [4,8] but this is not desirable when the cells are observed online on rather long periods during and after irradiation (a few tens of minutes). The use of a pre-cell detector becomes then mandatory. To achieve this goal, several developments are in progress to detect the particles upstream the cells. Two detectors have been developed and tested for the counting of the different types of particles: a secondary electron detector for alpha particles and a thin plastic scintillator for protons. At