Ionizing Radiation Microbeam Facilities for Radiobiological Studies in Europe

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A growing body of experimental evidence gathered in the last 10–15 years with regard to targeted and non-targeted effects of low doses of ionizing radiation (hyper-radiosensitivity, induced radio-resistance, adaptive response, genomic instability, bystander effects) has pushed the radiobiology research towards a better understanding of the mechanisms underlying these phenomena, the extent to which they are active in-vivo, and how they are inter-related. In such a way factors could be obtained and included in the estimation of potential cancer risk to the human population of exposure to low levels of ionizing radiation. Different experimental approaches have been developed and employed to study such effects in-vitro (medium transfer experiments; broad-field irradiation at low doses also with insert or shielding systems...). In this regard, important contributions came from ionizing radiation microbeam facilities that turn to be powerful tools to perform selective irradiations of individual cells inside a population with an exact, defined and reproducible dose (i.e. number of particles, in case of charged particle microbeams). Over the last 20 years the use of microbeams for radiobiological applications increased substantially and a continuously growing number of such facilities, providing X-rays, electrons, light and heavy ions, has been developing all over the world. Nowadays, just in Europe there are 12 microbeam facilities fully-operational or under-development, out of more than 30 worldwide. An overview of the European microbeam facilities for radiobiological studies is presented and discussed in this paper.

INTRODUCTION

Emerging radiobiological results in-vitro collected in the last 10–15 years with regard to radiation-related targeted and non-targeted effects of low doses (hyper-radiosensitivity, induced radio-resistance, adaptive response, genomic instability, bystander effects), suggest that the risk of low-level exposure to ionizing radiation is uncertain, and a simple extrapolation from high-dose effects may not be wholly justified in all instances. Epidemiological studies, which have demonstrated, through A-bomb survivor Life Span Study cohort, an excess risk of radiogenic cancer proportional to dose down to γ-ray doses of 50–100 mGy, have inadequate statistical power to detect and quantify the effects of doses much lower than such values, despite the large size of the irradiated population, detailed long term medical follow-up and reliable individual dose estimation. A better understanding of the mechanisms underlying low-dose phenomena, the extent to which they are active in-vivo, and how they are inter-related is needed before they can be evaluated as factors to be considered in the estimation of potential risk to the human population of exposure to low levels of ionizing radiation. Over the years, different experimental approaches have been developed and employed to study such effects in in-vitro systems (medium transfer experiments; broad-field irradiation at low doses also with insert or shielding devices...). In this regard, important contributions came from ionizing radiation microbeam facilities that turn to be powerful tools to perform selective irradiations of individual cells inside a population with an exact, defined and reproducible dose (i.e. number of particles, in case of charged particle microbeams).

Over the past 20 years the development and use of microbeams for radiobiological applications increased substantially and the number of such facilities, providing X-rays, electrons, light and heavy ions, is continuously growing worldwide. Some review papers have been published, reporting the features of the main facilities and the research opportunities offered by them. Taking advantage of new technological developments and innovations in particle delivery, focusing and detection, image processing and rec-

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ognition, computer control, and on the basis of the experiences acquired along the years, the features of microbeam facilities have been changing over the years with respect to first facilities born in the nineties.\textsuperscript{7,8}

At the European scale, after a pioneering development of a microbeam facility at GSI Darmstadt,\textsuperscript{9} later suspended for a number of years, the first operational microbeam facility was designed and realized in the early nineties in UK at the Gray Laboratory (now Gray Cancer Institute, GCI) by collimating beams of protons and helium-ions by a 1 \( \mu \)m-diameter glass capillary.\textsuperscript{10,11} Afterward, adapting some of the solutions adopted for this apparatus, a focussed X-ray microbeam facility was also developed by the same Group of GCI\textsuperscript{12}; very recently this latter apparatus moved to Queen’s University (Belfast, Northern Ireland, UK) with some further technical developments. Both the charged-particle and X-ray microbeams of GCI have been fully operational for long time producing a great body of data about low-dose effects and, in particular, on bystander effect\textsuperscript{13–17}; they have strongly contributed to the current knowledge in this field, giving in parallel high impulse to radiobiological research and promoting interesting debate on these issues in the world scientific community.

Following the GCI successful experience, other microbeam facilities have been developed and installed in Europe in the last 10 years, also (but not exclusively) in the framework of the CELLION Marie Curie European project (MRTN-CT-2003-503923; 2004–2008), which was focused on the development of single-ion microbeam facilities for radiobiological studies by promoting the exchange of knowledge among the Partners.\textsuperscript{18}

Nowadays in Europe there are 12 microbeam facilities fully-operational or under-development, out of more than 30 worldwide.

**SINGLE-ION MICROBEAM FACILITIES IN EUROPE: THE SECOND GENERATION**

The single-ion microbeam facilities can be roughly divided into two generations. The first generation includes few facilities which were realized in the early-1990s and have been operational till recent years, producing a lot of very interesting data about low-dose effects. Worldwide the light-ion microbeams at Columbia University,\textsuperscript{19} in USA, and at Gray Cancer Institute,\textsuperscript{10,11} in Europe, can be inserted in this category. These were vertical facilities installed at low-energy electrostatic accelerator and based on collimation of light-ion beams down to micrometer size, where mammalian cell recognition was automated and performed by means of fluorescence microscopy. A second generation of microbeams spread all over the world and, in particular, also in Europe. In Table 1 an updated list of the European facilities either operational or under development is reported.

These second-generation microbeam facilities differ substantially from the first ones, differences concerning most of their key-elements and in particular:

- microbeam formation;
- beam extraction into air;
- radiation detection and dose delivery;
- cell visualization and recognition system;
- cell holder.

In the following paragraphs a comparative overview of the European microbeam facilities will be given with respect to the distinctive key-elements mentioned here above. This review is intended to be an updating of the previous one, published in 2006,\textsuperscript{3} with the focus on the European scale. For a review on microbeam facilities in America and in Asia refer to the companion papers in this issue by Bigelow et al. and Kobayashi et al., respectively.

**Microbeam formation**

Regarding charged particle microbeams, in recent years, a general move can be observed from microcollimator-based facilities to focused microbeam facilities, in Europe as well as all over the world.\textsuperscript{4,20} Up to few years ago single-ion experiments have been performed mainly by collimating ion beams by small aperture of micrometer dimensions. Currently in Europe the mechanical collimation is used at INFN-LNL and CEA-LPS facilities. At INFN-LNL a tantalum pinhole, 100 \( \mu \)-thickness and 2 or 5 \( \mu \) aperture diameter, is placed in air downstream of the exit window and beam spot size is reduced down to 10 \( \mu \)-diameter for proton and H\textsuperscript{4+} ions in an energy range of 5–10 MeV.\textsuperscript{21–23} At CEA-LPS a 2 mm-long 5 \( \mu \)-inner diameter fused silica capillary is mounted just below the exit window; a 10 \( \mu \)-diameter beam spot is measured with this set-up for 3 MeV H\textsuperscript{4+} ions.\textsuperscript{24–26}

Notwithstanding the higher cost of focusing elements than collimators, the majority of European facilities (see Table 1) are currently based on electrostatic/magnetic lenses which allow the beam spot to be easily reduced down to sub-micrometer size, at least in vacuum. Three main reasons can be asserted to explain this general trend:

1. Most of the recently developed facilities come from re-adaptation of already existing focused nuclear microprobes, originally intended for elemental analysis, ion microlithography etc. and then modified for single-ion single-cell irradiation, mainly in terms of a re-design of the irradiation chamber.
2. Particle scattering on the inside walls of the aperture limits the minimum beam spot size of microcollimator-based facilities to a few micrometers and increases the energy spread of the microbeam. Focused ion microbeams have a substantially reduced number of scattered ions, therefore offer the advantage of better-defined energy and smaller beam spots (below 1 \( \mu \)m).
3. Focused microbeams allow beam spot-scanning irradiation and therefore can speed-up and make easier indi-

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individual cell irradiation: cells are in fixed position and beam is moved from one cell to another. This feature has the potential of higher cell throughput.

The efforts towards sub-micrometric beams derive from the interest in the investigation on the sensitivity of subcellular targets and spatial aspects of cell stress response, mechanisms and pathways. Recent data, for instance, show that Reactive Oxygen Species (ROS) are candidate to be among the principal mediators of bystander effects, the source of ROS being also mitochondria, i.e. a subcellular site.27)

Focused beam spot size of 100–500 nm can be easily achieved in vacuum in STIM mode (i.e. high beam current) at CENBG,28) GSI,29,30) Lund NMP,31,32) PTB.33–36) At LIPSION nanoprobe the smallest beam spot has been obtained in vacuum, down to 40 nm in the low current mode, while a beam spot size of 100 nm can be regularly achieved in STIM mode.37) Anyway the extraction of the beam from vacuum into air, required for living cell irradiation, produce significant deterioration in the beam quality, in terms of particle scattering and energy straggling due to the crossing of the exit window and air gap. Moreover a high beam current, which is the optimal working condition for elemental

| Microbeam Facility | Radiation type | Energy/LET | Beam formation | Beam size on cell | Cell recognition |
|--------------------|----------------|------------|----------------|-------------------|-----------------|
| CENBG Bordeaux, France | p, α | Up to 3.5 MeV | focusing | 10 μm | Fluorescence-based system, Automated |
| GSI Darmstadt, Germany | From α to U-ions | Up to 11.4 MeV/n | focusing | 0.5 μm | Fluorescence-based system, Automated |
| IFJ Cracow, Poland | p | Up to 2.5 MeV | focusing | 12 μm | Fluorescence-based system, Automated |
| INFN-LNL Legnaro, Italy | p, ³He⁺⁺⁺, ⁴He⁺⁺⁺ | 7–150 keV/μm | microcollimation | 10 μm | Unstained-cell recognition system, Semi-automated, in development |
| LIPSION Leipzig, Germany | p, ⁴He⁺⁺⁺ | Up to 3 MeV | focusing | 0.5 μm | Unstained-cell recognition system, Automated |
| Lund NMP Lund, Sweden | p | Up to 3 MeV | focusing | 5 μm | Unstained-cell recognition system, Automated, in development |
| CEA-LPS Saclay, France | p, ³He⁺ | Up to 3.75 MeV | microcollimation | 10 μm | Fluorescence-based system, Automated |
| PTB Braunschweig, Germany | p, α | 3–200 keV/μm | focusing | <1 μm | Fluorescence-based system, Automated |
| Queen’s Univ. Belfast, Northern Ireland UK | X-ray | 0.3–4.5 keV | zone plate | <1 μm | Fluorescence-based system, Automated |
| SNAKE Munchen, Germany | From p to HI | 2–1000 keV/μm | focusing | 0.5 μm | Unstained-cell recognition system |
| Univ. of Surrey Guilford, UK | p, α, HI | <1 μm (in vacuum) | focusing | 0.01 μm | Fluorescence-based system, Automated |

Table 1. Ionizing radiation microbeams for radiobiological studies in Europe
analysis microprobes, instead a critical condition for single-ion cell irradiation experiments where a counted number of particles has to be delivered in a highly reproducible way. Therefore the focused-microbeam current has to be strongly reduced in its intensity before reaching the biological target. In this respect most of the work carried out so far at focused-microbeam facilities was aimed at achieving a small/sub-micron beam spot with low particle flux and a high beam quality in air on cells. Good performances have been achieved: for example, at PTB beam diameters from 0.2 to 2 μm (fwhm) can be obtained at the position of the cells while at LIPSION the beam spot size in air in the low current mode is around 350–500 nm in diameter.

However, it should be pointed out that a sub-micrometric beam spot size is not enough to guarantee a sub-micrometric targeting accuracy required for sub-cellular irradiations. The various factors influencing targeting accuracy will be discussed in more details later in this paper.

Different techniques are used to produce X-ray micro-probes. To date the finest probes have been obtained in the field of X-ray microscopy by using circular diffraction gratings, known as “zone plates”. This is a very established technique that allows to obtain device that can focus X-rays to diameters of 50 nm or less at energies of few hundred eV. This technique was implemented for radiobiological studies first at GCI X-ray facility, and now at Queen’s University, where an X-ray beam size of less than 1 μm is available in an energy range between 0.3 and 4.5 keV. As an alternative technique, X-rays in this energy range can be focused to micrometric size by means of reflecting mirrors. These are used at IFJ-Cracow facility, where a beam spot size of 7 μm has been achieved so far.

Beam extraction into air

In order to irradiate living cells, as stated here above, the beam has to be extracted into ambient air, at atmospheric pressure, by means of an appropriate vacuum-air window, whose characteristics have to be chosen on the basis of the type and energy of the radiation beams.

All particles undergo multiple scattering as they traverse whatever material. Small angle scattering in the vacuum window and in the air gap between the exit window and the cell sample leads the beam to be spatially broadened at the position of the cell target, affecting the targeting accuracy and reproducibility. This effect depends on particle type and energy as well as on material and thickness of traversed layers, i.e. the vacuum window and air gap before cell sample, if any. In addition, the diameter of the vacuum window has to be considered: depending on its dimension, the air pressure produces an inward bending curve in the window foil and therefore an increase of the air gap between the vacuum window and the cell sample.

To reduce the particle scattering at the vacuum exit window as much as possible, a common choice at low-energy ion accelerators is the use of 0.1–0.2 μm thick Si3N4 windows, which are commercially available also in small diameter (1–3 mm). This choice has been adopted at CEA-LPS, GSI, IFJ, LIPSION, Lund NMF, facilities while at INFN-LNL, PTB and SNAKE thin aluminized mylar, mylar and kapton foils are routinely used respectively.

On the other hand, an ambient air gap between the vacuum window and the cell sample is necessary to allow the sequential positioning of cells one-by-one in front of the vacuum window, in correspondence of the beam position. As a sort of compromise to have a free air gap for sample-beam movement and a reduced particle scattering in air, a distance of 100–200 μm is usually adopted at the various facilities.

Radiation detection and dose delivery

Many different options have been adopted for radiation beam detection and low-dose delivery (i.e. counted particles, for charged particle microbeams). Even if it is difficult to find two microbeam facilities using the same detection system, however two main approaches can be distinguished, in placing the detector upstream or downstream of the cell holder.

In particular, when the ion beam does not have enough energy to cross the cell holder, a transmission detector is adopted, usually a very thin scintillator, coupled to a photomultiplier as at CENBG and PTB. At CENBG, as an option for low-energy alpha-particle beams, a low-pressure transmission detector (5 mbar, isobutene), equipped with a 100 nm-thick Si3N4 window, has been also developed and mounted as final part of the beam line. At Lund NMP an ultra-thin silicon semiconductor transmission detector is used as a pre-cell hit detector.

When the beam has enough energy to cross the cell sample, the general trend is placing the detector downstream of the cell holder in order to avoid further scattering component on the beam due to crossing of the detector. A silicon detector placed under vacuum and a silicon detector behind cell sample, as a double-counting system, to improve the overall detection efficiency for low-energy light particles (i.e. protons).

Cell visualization and recognition system

A crucial task in microbeam irradiation experiments is a...
reliable recognition and localization of each living cell to be irradiated. A variety of approaches has been applied in the different laboratories. Some of the cell recognition systems are in continuous development, all taking profit of the more advanced technology in microscopy and software.

The most commonly used method for cell imaging is based on fluorescence microscopy and cell staining. One of the first systems of this kind was developed at GCI, and soon was employed at many other microbeam facilities thanks to its performances: cell recognition and coordinates logging can be performed automatically on-line (often without the need of moving the cell sample), in a short time, allowing the irradiation of a high number of cells per hour. This method is currently used at CENBG, CEA-LPS, GSI, IFJ, Queen’s University, and University of Surrey. In brief, to identify the cell nuclei, cells are stained with a UV-fluorescent dye, such as the DNA-binding dye Hoechst 33342 or 33258 which are used at extremely low concentrations (50–200 nM) to avoid the known radio-modifying effects at typical working concentrations. In addition, to reduce the induction of any unwanted phototoxic effects, during the scoring of the cell holder, the UV light exposure time is minimized as much as possible by using appropriate low-power objectives and high sensitivity CCD cameras. It should be noted that the cell recognition efficiency of this system is very high reaching 90–95% of the cells detected.

However in order to avoid completely the potentially phototoxic effect of fluorescent dye and UV illumination, systems for unstained cell imaging have been developed. In this case cell recognition is not automated and is performed by eye by the operator, and the microscope has to be mounted off-line. A system of this type is in use at INFN-LNL, based on a phase-contrast optical microscope: a home-developed software drives an automated scanning of the cell holder under the microscope; when the operator recognizes a cell, the scanning is stopped for a while and cell coordinates are logged. Of course this is a time consuming approach which prevents from the achievement of a high cell throughput.

In recent years some laboratories have undertaken several attempts to develop automated recognition systems for unstained cells based on bright field optical microscopy. A system based on differential interference contrast method was developed at GSI, and a Qpm-based system at IFJ. Both systems did not give satisfactory results and were abandoned. More recently a neural network based system has been conceived and is currently under development and test at INFN-LNL, in collaboration with the Bialystok Technical University: the automated recognition system is based on texture segmentation, using texture discrimination technique (by the classifier) and an algorithm for joining over-segmented objects into one cell-object with the help of the classifier. Cell coordinates to be used for irradiation are calculated from inscribed circle into each cell-object. To gain adequate speed calculations are spread over the parallel cluster machine.

A different cell visualization and recognition system is the one developed at SNAKE facility. It makes use of both phase-contrast and epifluorescence microscopy on-line by means of a Zeiss Axiovert 200M microscope installed at the beam-line exit: the beam-line exit nozzle contains an LED illumination for phase-contrast microscopy, that is used for cell imaging before irradiation and cell positioning on beam; after irradiation the protein of interest tagged with GFP is observed in the living cells by switching on the light (λ = 470 nm). With this approach cellular dynamics or kinetics can be observed on-line, within 0.5 sec after irradiation.

Cell recognition without staining via dark field microscopy (correctness higher than 95%) is under development at LIPSION. Cell detection is performed off-line, in cell laboratory, by using a home-made software (CellCognition) in no-staining condition. The projection of cell coordinates from off-line to on-line reference system is obtained via fiducial markers in mylar (realized by high current beam painting); the projection of on-line cell coordinates to beam coordinates is obtained by visualizing beam spots on glass substrate or scintillator material.

**Cell holder**

The need to visualize, recognize and localize individually the cells to be irradiated, prompted the different laboratories to design specific cell holders (Petri-dishes). As common features, optically transparent and thin membranes have to be mounted on the Petri dishes as cell substrate, in order to allow cell visualization under microscope and ion crossing during irradiation; moreover cell holders have to guarantee humid, stress-free (and sterile, for some specific investigations) culture conditions during all phases of microbeam irradiations. Considering the variety of accelerator and ion beam characteristics as well as cell visualization systems, each microbeam facility has developed a specific cell holder. Therefore different experimental solutions in terms of cell substrate material, and cell holder geometry have been adopted to fit the (vertical or horizontal) beam-line set up and reduce stress to cells as much as possible.

One of the first examples of Petri dish for microbeam experiments was realized at GCI. The cell substrate was made of few μm thick mylar or polypropylene foils and mounted in such a way that cells could be kept with culture medium during irradiation and access from both sides of the holder was possible for the beam, the detector and for viewing. A similar approach is now adopted at Queen’s University.

A standard approach is realizing a cell chamber where the entrance foil is usually a thin film, like mylar and polypropylene, where cells are seeded, and the exit window is a cover glass. The use of a cover glass, being transparent and flat, optimizes the cell visualization under microscope. The
cell chamber is filled with appropriate amount of medium to maintain cells in culture conditions during irradiation. However this solution requires that particle detection is performed upstream of the Petri dish or that medium is removed during irradiation. This experimental approach is adopted at PTB, GSI, and CENBG where different materials are chosen as cell substrate (biofoil at PTB, polypropylene at CENBG and GSI).

At LIPSION a commercial plastic Petri dish (35 mm in diameter) is used with cut-out bottom, where a 2.5 μm-thick mylar foil is mounted as base and fixed with a special ring; medium is removed during vertical irradiation.

At INFN-LNL a specially designed stainless steel Petri dish (external diameter: 100 mm; internal diameter: 70 mm) with a 20 μm-thick cell chamber has been realized to keep cells in humid and sterile conditions during irradiation and in a vertical position in front of the beam. The base and the cover of the cell chamber are 7 μm-thick mylar foils, allowing ions 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 during irradiation.

At CEA-LPS cell sample is based on standard 50 mm-diameter polystyrene Petri dishes, with a central hole where a 2.25 mm² area, 100 nm-thick Si₃N₄ membrane is glued using low fusion temperature wax. A similar approach, with Si₃N₄ membranes as cell substrate, is adopted at IFJ. Moreover at CEA-LPS a heated cage incubator, integrating microscope objectives and sample micropositioning stages, has been mounted as a microenvironment chamber that guarantees standard culture conditions (37°C, controlled humidity, 5% CO₂) and therefore permits long-term experiments, in-situ observations and irradiation cycles.

At SNAKE facility an appropriate Petri dish has been developed for their live cell image system that foresees on-line cell observation and also long observation periods after irradiation (up to 10–12 h). The cell holder is in stainless steel with a cavity to be filled with culture medium. The entrance window is a mylar foil, the exit window is a 170 μm-thick plastic scintillator (BC418), used as cell substrate and for beam localization. These two windows define a cell chamber that is 4 mm-thick and guarantees cells to be kept vital for 24 h in the cell holder. During irradiation however the beam-line exit nozzle is pushed towards the entrance mylar foil in such a way that it is positioned close to the cells with a reduced medium layer (just 30 μm-thick) to be crossed by the ions. The microscope objective is in contact to the cell chamber via immersion oil and is heated to 37°C to keep cells at this temperature during irradiation and observation.

CONCLUDING DISCUSSION

The overall performances of a single-ion microbeam facility for single-cell irradiation are generally evaluated in terms of targeting accuracy and cell throughput, which are the results of the combination of all the elements of the facility, discussed here above.

A sub-micron targeting accuracy is required to study the radiation sensitivity of sub-cellular sites and the influence of cytoplasmatic signal transduction or investigate on bystander effect mediators. The first requirement to obtain a sub-micron targeting accuracy is achieving a sub-micron beam spot size. With focusing technique a beam spot size in the range between 0.5 and 1 μm in air can be obtained quite easily, as already discussed. However targeting accuracy depends also on other parameters like accuracy to which beam position is identified, accuracy to which targets are identified, stage positioning accuracy or reproducibility in the raster scanning system. Although many microbeam facilities have now a sub-micron beam size, till today no microbeam facility has reached an overall targeting accuracy, including all these contributions, below 1 μm suggesting that also all other elements have to be optimized. In particular, PTB, LIPSION, GSI facilities are featured by a target accuracy of 2–3 μm in spite of their submicrometric beam spot sizes.

As regards cell throughput, this is mostly influenced by irradiation and cell recognition speed. To increase irradiation speed, micropositioning stage velocity (as at CEA-LPS) or raster scanning speed (as at PTB) has to be increased, when cells are placed one-by-one on beam or beam is moved from one cell to another, respectively. However cell recognition speed is the most significant parameter for cell throughput. Currently fluorescence-based recognition systems allow cell throughput up to 50000 cell per hour (as at CEA-LPS, PTB, Queen’s University), and therefore also studies of mutagenic and oncogenic endpoints where yields are ~10⁻⁴. Moreover high speed in the irradiation protocol allows to minimize potentially stressing conditions to cells in the cell holder, which might influence cellular response. The underway automated no-staining recognition systems could turn as useful tools to achieve high speed in cell imaging and then in the irradiation protocol, avoiding the use of cell staining and UV light that can potentially influence cell response as well. The development of such a kind of cell recognition systems together with the development of cell holder guaranteeing stress-free conditions represent the key-elements for “clean” single-ion single-cell experiments, where cellular response can be directly correlated to radiation quality and cell communication mechanisms, without any confounding factors.

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