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The application of charged-particle microbeams in radiobiology

Melvyn Folkard*, Boris Vojnovic, Kevin M. Prise, Barry D. Michael

Gray Cancer Institute, Mount Vernon Hospital, P.O. Box 100, Northwood, Middlesex HA6 2JR, UK

Abstract

For radiobiological applications, the strength of the microirradiation technique lies in its ability to deliver precise doses of radiation to selected individual cells (or sub-cellular targets) in vitro. There is particular interest in studying the risks associated with environmental exposures to α-particle emitting isotopes (which are predominantly due to single-particle effects) and for investigating the so-called 'bystander effect' where non-irradiated cells are seen to respond to signals from nearby irradiated cells. The Gray Cancer Institute charged particle microbeam is one of only two facilities currently in routine use for radiobiology; although a number other facilities are at various stages of development. To be useful in a radiobiological study, a microbeam facility is required to reliably deliver an exact number of particles to a pre-selected sub-cellular target. Furthermore, the low incidence of some biological endpoints means that a large number of cells may have to be individually irradiated (>100,000 cells), therefore some form of automation is essential. Our microbeam uses a 1 μm diameter bore glass capillary to vertically collimate protons, or helium ions accelerated by a 4 MV Van de Graaff. Using $^3He^{2+}$ ions, 99% of cells are targeted with an accuracy of ±2 μm, and with a particle counting accuracy >99%. Using automated cell finding and irradiation procedures, up to 10,000 cells per hour can be individually irradiated.

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1. Introduction

The ion microbeam is now a recognised analytical technique in materials science. The use of ion beams in biological applications is less established, but in one area at least is having a significant impact. The field of radiation biology seeks to understand the mechanisms by which ionising radiations interact with living tissues and in so doing, develop improved strategies for cancer treatment by radiotherapy and develop better estimates for the risks associated with occupational and environmental exposures to ionising radiations. The microbeam is proving to be an exquisite tool for probing the biological effects of radiations at the cellular and sub-cellular level. By targeting cells individually with counted particles, it is possible to address a number of important radiobiological questions. For example, microbeams can be used to study the risks associated with environmental exposures to α-particle emitting isotopes.

* Corresponding author. Tel.: +44-1923-828611; fax: +44-1923-835210.
E-mail address: folkard@gci.ac.uk (M. Folkard).

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Naturally occurring radon gas is one of the primary sources of environmental exposure to ionising radiation. Radon (and its daughters) produces α-particles that can damage epithelial cells in the lung. It is extremely unlikely however, that a cell will be traversed by more than one α-particle, therefore to study the risks associated with exposure to radon, it is important to understand the biological effects of a single α-particle. The microbeam provides an elegant method for delivering single particles to cellular and sub-cellular targets.

There is increasing interest in the role of so-called 'non-targeted' effects where cells respond *indirectly* to energy deposited by radiations. One such effect is the occurrence of damage arising from radiation induced cell signalling, both intracellular (i.e. from the cytoplasm to the nucleus) and inter-cellular (from cell to cell). The interest in inter-cellular signalling followed a report by Nagasawa and Little [1], who observed chromosome damage in 30% of cells following exposure to a broad field of α-particles such that only 1% of cell nuclei are actually hit. Subsequently, Deshpande et al. [2] reported a similar finding in primary human fibroblasts, while Hickman et al. [3] showed greater than expected levels of p53 (a tumor suppressor protein) in α-particle irradiated epithelial cells. The implication of these findings is that damage is occurring in non-irradiated cells in response to signals from neighbouring irradiated cells. This phenomenon has been termed the 'bystander-effect' and microbeam techniques that allow the selective irradiation of cells (or even just a single cell) within a population are clearly advantageous for this type of study. There is also evidence that DNA damage can result from irradiating just the cell cytoplasm. Historically, experiments to study this effect have used very short-range α-particles that only partially penetrate the cell [4]. A microbeam with micron or submicron targeting capability is well suited to this type of investigation.

As a consequence of the increasing interest in microbeams, a number of groups are now actively engaged in developing microbeams for radiobiological applications, or in adapting existing analytical microprobes for radiobiological use. Nevertheless, relatively few facilities are in routine operation in this application. Our own microbeam facility at the Gray Cancer Institute is fully operational, and has been in routine use for some years [5,6]. Similarly, the Radiological Research Accelerator Facility (RARAF) at Columbia University (New York) has been routinely operating a fully-automated microbeam for irradiating cells since the 1990s [7]. The Japan Atomic Energy Research Institute (JAERI, Takasaki, Japan) has developed a heavy ion microbeam to microirradiate living organisms with 10 MeV/u ions from a cyclotron [8]. The system is operational but is available for radiobiological experiments infrequently. Partly developed facilities exist at Texas A&M [9,10], CENBG Bordeaux (based on an existing light ion microprobe [11]), GSI Darmstadt (using heavy ions [12]), PTB Braunschweig and MIT Boston.

One reason that radiobiological microbeams are not more widespread is that the full potential of such a facility can only be realised if versatile cell finding and alignment facilities are available. Many radiobiological effects occur infrequently with dose. In some cases, in excess of 100,000 cells must be individually irradiated to obtain statistically significant results [13], therefore the procedures used for finding, aligning and irradiating cells must be both fast and automated.

Finally, it should be noted that microbeams of ionising radiation other than charged-particles are in use, or being developed. In fact, the Gray Cancer Institute has developed a unique facility that uses X-ray diffraction optics to focus 278 eV X-rays to a sub-micron spot [14,15]. Low-energy X-rays have the advantage that they interact almost entirely through photoelectric absorption, such that scattering by the vacuum window and other intervening materials does not degrade the resolution.

2. Requirement for a radiobiological microbeam

While existing analytical microirradiation facilities can be developed (and indeed, are being developed) for use in a radiobiological application, the requirements differ from traditional uses of particle microbeams in a number of ways. Most notably, materials analysis applications usually

require a bright source of focused ions to irradiate a single sample, or a small batch of samples that are often mounted in vacuum. By contrast, to study damage to living cells requires just a few particles (or even a single particle) delivered with micron accuracy to many thousands of cells in solution (or humidified) and at atmospheric pressure. This means that computer controlled micropositioning, imaging and image analysis techniques must be used to find and align cells automatically, and that particles crossing the cells can be reliably detected and controlled without compromising the targeting accuracy. There is also a considerable practical advantage in using a vertically-oriented beam, rather than the horizontal configuration common to microprobes used in analytical applications, although existing (horizontal) microbeam facilities being adapted for radiobiological use [11,12].

As only small particle numbers are required, it is possible to use achieve micron-sized beams by collimation as well as focusing. Indeed, the three established radiobiological facilities (at the Gray Cancer Institute, at RARAF and at JAERI) all currently use collimation. While focusing is ultimately capable of the producing the finest beams, the benefits are compromised by the requirement for a particle detector and for a vacuum window, both of which can scatter the beam. Despite this, an electrostatic focusing system is being developed for use with the RARAF facility. A potential advantage of focusing the beam is that allows for the possibility of targeting many cells by steering the beam to each cell, rather than moving the cell dish. Such an arrangement has the potential to be very fast.

3. Methods

The Gray Cancer Institute charged-particle microbeam makes use of a purpose-built beamline from our 4MV VdG accelerator to transport particles, either protons, or $^{3}He^{2+}$ ions, vertically upward through the floor of the laboratory above to the cell irradiation apparatus, mounted on an optical table at bench height. Note that $^{3}He^{2+}$ ions are radiobiologically equivalent to $^{4}He^{2+}$ ions

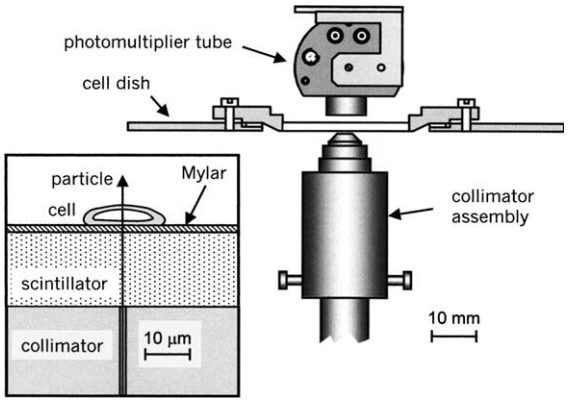

Fig. 1. The arrangement for micro-collimation and particle detection. The inset shows (to scale) the position of the collimator, the scintillator and the cell, aligned for irradiation.

of the same ionisation density, but have greater penetration. Fig. 1 shows the principle of the microirradiation method. A fine radiation beam is formed using a 1 μm diameter bore fused silica capillary collimator, mounted at the end of the beamline. Cells to be irradiated are attached to a thin plastic membrane that forms the base of a cell dish containing cell culture medium. The dish is located on a three-axis micropositioning stage above the collimator. During irradiation, each cell (or sub-cellular target) is located, in turn, above the collimator and exposed to an exact, predefined number of particles. The particles incident on the cell are counted using a photo-multiplier (PM) tube mounted just above the cell dish. The PM tube detects the pulse of light (due to the passage of a particle) from a thin scintillator 'sandwiched' between the collimator exit and the cell dish. This detector arrangement is close to 100% efficient. A fast electrostatic shutter terminates the irradiation of each cell once the preset number of particles has been delivered. The targeting accuracy is limited primarily by particle scattering from the vacuum window and scintillator. The effect of scattering is minimised by arranging for the collimator to be as close as possible to the cell. In fact, the collimator just touches the base of the cell dish prior to each exposure. In this way, we can achieve a targeting accuracy (for 99% of cells) of ± 2 μm when $^{3}He^{2+}$ ions are used (validated using etch-track methods).

One of the strengths of the Gray Laboratory microbeam is that it uses fast, automated procedures for cell-finding, alignment and irradiation. This is important, as many biological assays require a large number of cells to be irradiated. The control features of facility are shown schematically in Fig. 2. Cell finding is achieved using an epifluorescent microscope to view cells in the dish in situ. The microscope objective and the PM tube can be readily exchanged depending on whether cell finding, or irradiations are occurring. An intensifier-coupled electronic camera can capture live, snapshot, or integrated images through the microscope and in conjunction with computer image processing, is used to identify and store the co-ordinates of cells on the dish. Typically, it takes about 10 minutes to identify around 1000–2000 cells in a 1 cm^2 area. The intensifier greatly reduces the dye concentration and UV exposure required to view stained cells. The irradiation step takes place once all the cells have been found, and their co-ordinates stored. Again, this step is entirely automated, such that it is possible to individually irradiate up to 10,000 cells per hour. After irradiation, the cell dishes are stored in an incubator prior to scoring. After an appropriate interval, the dish can be returned to the stage and the stored cell co-ordinates used to revisit each cell individually with the microscope, such that the induced damage can be assessed on a cell-by-cell basis.

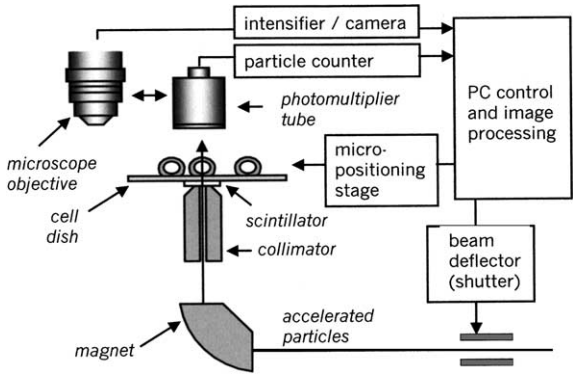

Fig. 2. The arrangement for controlling the microbeam experiment. All aspects of the experiment are automated and controlled using a PC.

4. Biological experiments

Three main types of biological experiment are being performed using our facility:

(a) Uniformly target every cell through the centre of the nucleus: These are standard experiments for studies aimed at understanding risks associated with low dose effects. A single particle can be individually delivered to many thousands of cell nuclei. More complex distributions, such as delivering an average of one particle (to a cell population) can also be performed automatically. Normally low levels of non-toxic DNA binding dyes are used for identification of the nucleus and for subsequent alignment and targeting.

(b) Target different locations within cells: With the accuracy of the particle microbeam, sub-cellular targeting is possible. Many studies involve comparison of nuclear versus cytoplasmic targeting. Targeting is either fully automated, or can be experimenter-operated using an on-screen point-and-click system. Various fluorescent probes can be used to highlight sub-cellular organelles for targeting. For example, this type of study could involve targeting regions of cells with mitochondria present.

(c) Target individual cells within a population: The system is also used for many studies where only one or a few cells need to be targeted within a population. These can be individual cells randomly plated on a cell dish, individual cells within pre-grown colonies or tissue-like samples such as explants, or cell layers within a tissue fragment. For tissue fragment studies, different layers within a tissue section can be reached by altering the energy of the beam, giving three-dimensional information.

The microbeam is ideally suited to investigating the survival of cells at low doses (because particles are counted individually). The Gray Cancer Institute facility has been used to measure the survival of V79 mammalian cells following exposure to 3.2 MeV protons at doses below 1 Gy (between 5 and 50 proton traversals per cell). At the lowest doses, the survival curve is very steep, indicating that the cells are very sensitive. Beyond about 10 protons per cell, the curve becomes less steep as the cells exhibit increased resistance to the radia-

tion. This phenomenon, known as 'low dose hypersensitivity' [16] has been shown previously for other radiations. It has been proposed that the onset of reduced radio-sensitivity may indicate that an inducible repair mechanism has been triggered.

The oncogenic potential of a single α-particle has been measured by Miller et al. [13] using the RARAF microbeam. They irradiated C3H10T1/2 mouse fibroblast cells with either an *exact*, or an *average* number of α-particles and measured the transformation frequency (per surviving cell). Their results showed that the risk associated with exposure to a single particle is not significantly higher than that for zero dose, suggesting that extrapolating to low doses from multiple traversal data will significantly overestimate the risk of radon exposure at domestic levels.

The ability to select and irradiate sub-cellular targets (such as the cell cytoplasm) makes it possible to study the biological effect of radiation other than by direct damage to the DNA by ionisation (or through free radical production in the water layer close to the DNA). Historically, direct damage to the DNA is thought to be a requirement for critical biological damage. Several studies, using microbeams have shown evidence for the cell cytoplasm being an important target for biological effects. Studies at the Gray Cancer Institute using primary human fibroblasts have shown that targeting the cell cytoplasm with 5 helium ions, leads to the production of reactive oxygen species (ROS) and micronucleus induction (a form of chromosomal damage [17]). Hei and colleagues [18] have used the RARAF facility to target the cytoplasm of human-hamster hybrid A_L cells with α-particles and monitor mutation expression. An increased production of mutations was observed after 4–16 particle traversals, and with a reduction in cell survival to around 80%. They also observed that the molecular spectra of these mutations were similar to spontaneous mutations that occur in un-irradiated cells.

Effects that arise in the nucleus following irradiation of the cytoplasm indicate that intra-cellular signalling processes are occurring. It is now known that cell-to-cell communication also takes place (the 'bystander effect'). Studies at the Gray Cancer Institute using primary human fibroblasts have shown that targeting a single cell within a population of 600–800 cells with a single helium-ion leads to an additional 80–120 damaged cells (scored as cells containing micronuclei) being produced uniformly across the population [19]. Increasing the number of helium ions through the cell nucleus, or the number of cells targeted does not appear to increase the effect. A similar approach using the RARAF microbeam has shown a radiation-induced response for the production of mutations in the A_L cell line with a 30% higher mutation frequency than that assumed from the fraction of cells hit [20]. An important advance in the study of the bystander response is to understand the role of cell-to-cell communication in tissue systems. We have performed preliminary work using sections of human or porcine ureter where a 4–5 cell layer of uroepithelium surrounds the lumen of the ureter. Single protons or helium ions have been used to target the epithelial layers of the tissue, or specific uroepithelial cells within explants. Such an exposure leads to several thousand additional damaged cells being produced indicating a significant bystander response.

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