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The design and application of ion microbeams for irradiating living cells and tissues

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Abstract

The range of radiobiological experiments requiring microirradiation techniques continues to expand and diversify, creating ever-greater challenges for the designers of such systems. A versatile microbeam for radiation biology must excel in a number of areas. For studies of intracellular cell signalling where it may be of interest to target just the cytoplasm or nuclear membrane, targeting accuracies of micron or less are desirable. Other studies may use endpoints that are rare enough to require the irradiation of hundreds of thousands of cells in order to observe and quantify the effects. Inevitably, this means automating the cell finding, aligning and irradiation steps in order to achieve high cell throughputs. For investigations related to radiation risk, the effect of single particle traversals are paramount, therefore particle counting and single particle delivery are essential. A number of improvements have been implemented to the Gray Cancer Institute charged-particle microbeam, to extend its versatility and to meet these challenges. Specifically, improvements to the speed, alignment accuracy and environmental control have enabled investigations related to cell signalling, low-dose hypersensitivity, genomic instability and the visualisation of DNA repair to be successfully addressed.

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1. Introduction

Nuclear microprobe technology continues to be used in a wide range of applications. In the past decade, the use of particle microbeams as tool for inflicting highly localised damage to living cells and tissues has generated much interest in the field of radiation biology. Even so, relatively few fully-

developed microirradiation facilities exist for this purpose, although the number of institutes actively participating in their development grows year-by-year. Currently, at least 13 different institutes worldwide are committed to developing and applying particle microbeams for radiobiological purposes. This is roughly a two-fold increase in the past two years.

2. Factors influencing microbeam design

Two distinct approaches can now be identified for the development of radiobiological

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microbeams: those that have been developed for the express purpose of studying radiation damage to living cells and tissues and those that are an adaptation of an existing microirradiation facility, such as an analytical microprobe. The first type of microbeam tends to be vertically oriented and uses collimation to form the beam, while the second type tends to be horizontally configured and achieves a micron-sized beam by focusing. Examples of the first type are our own facility at the Gray Cancer Institute (GCI) [1–3], the facility at the Radiological Research Accelerator Facility (RARAF), Columbia University (New York) [4] and the heavy ion facility at the Japan Atomic Energy Research Institute (JAERI, Takasaki, Japan) [5]. Examples of the second type are those being developed at GSI Darmstadt [6], PTB Braunschweig and CENBG Bordeaux [7].

While it may seem that developing a radiobiological microbeam using an established microirradiation facility is advantageous, there are a number of reasons why this approach is problematic. This is because the criteria that define the design of a radiobiological microbeam are quite distinct from those required for an analytical microprobe. One key issue is that a radiobiological microbeam is used to irradiate cells in an environment compatible with life. Indeed, the cell or tissue environment should be as stress-free as possible, otherwise unwanted confounding factors could influence the results. Clearly, the exposure of the sample will have to take place outside the accelerator vacuum, which means that the beam must pass through a vacuum window at some point. For light ions, this will introduce scatter and will compromise the achievable beam spot size that would otherwise be the main advantage of a focussed system. Working with living cells also favours the use of vertically-aligned beams. This is because cells are typically attached to (and irradiated through) a thin membrane that forms the base of a cell dish, also containing a solution at a controlled temperature (although humid-gas arrangements can also be used [4]). In the case of a horizontal microbeam, the membrane will form one side of a container, which inevitably is a more complex and less flexible design.

Typical analytical microprobes are optimised to deliver high focussed beam currents and good *relative* spatial resolution (where sample, or beam scanning methods are employed). Radiobiological microbeams on the other hand, require single, or counted multiple particle delivery and good *absolute* spatial resolution such that individual particles can be aimed at pre-selected targets. This requires an arrangement for detecting single particles and shuttering the beam. For tissues and cells in solution, the detector must be located prior to the sample and be designed and sited such that the unwanted effects of scattering are minimised. In the case of the GCI microbeam, we use an 18 μm thick scintillator (Bicron BC400) mounted over the exit of the collimator. In order to minimise the effect of scattering, the collimator/scintillator assembly moves up about 0.1 mm prior to each exposure such that it just touches the base of the cell dish. After the exposure it moves down again so that the dish is free to move (to locate the next target). During irradiations, a photomultiplier tube above the dish is used to detect scintillator light, which in turn, triggers a fast electrostatic shutter.

The matter of achieving accurate targeting is one of the most challenging aspects of configuring a microbeam for a radiobiological application. The problem is confounded by the requirement, in some instances, for a large number of targets to be individually irradiated (in excess of 10^5 cells have been reported for some studies [8]). This requires a high sample throughput, which can only be achieved through automated procedures. The problem of achieving fast and accurate exposures is considered in the next section.

3. Achieving fast and accurate sample throughputs

The factors that influence the accuracy of a microbeam for targeting cells fall into two categories: ‘beam geometry’ and ‘aiming accuracy’. ‘Beam geometry’ covers effects that arise from the initial size and shape of the particle beam, the effect of scattering sources (such as the vacuum window and detector) and the geometrical arrangement of the various elements. ‘Aiming

accuracy' addresses factors such as the accuracy with which targets can be identified (and assigned coordinates), the accuracy with which the collimator position can be established and the ability to accurately align the target over the collimator. A number of studies characterising the GCI microbeam have already been reported [1,2,9] these findings show that using a 1 μm diameter collimator, a 3 μm thick Mylar window and an 18 μm thick scintillator, beam geometry factors contribute $\pm 2 \mu\text{m}$ uncertainty to the overall accuracy of our microbeam. Note that much of this uncertainty is caused by scattering and could not be improved significantly by switching from a collimated system to a focussed source.

More recently, the factors influencing the aiming accuracy of the GCI facility have been closely scrutinised. The arrangement for finding and positioning cells has been reported in detail previously [1,10] and is described only briefly below. Cells are found in situ using a modified epifluorescent Olympus BX microscope that views stained cells from above using typically, a $\times 20$ water-immersion objective. The microscope objective and the PM tube can be readily exchanged depending on whether cell finding, or irradiations are occurring. A 3-axis micropositioning stage (Märzhäuser, Germany) is used to support and align the cell dish. 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 coordinates of cells on the dish. The intensifier greatly reduces the dye concentration and UV exposure required to view stained cells. Typically, it takes about 10 min to scan a 1 cm^2 area of a dish and identify (and assign coordinates to) around 1000–2000 cells. The same imaging system can be used to locate the collimator position. This is undertaken at the beginning of each experimental day by operating at a high beam current (typically >1000 particles/s through the collimator) and imaging the light from the scintillator (with a dummy dish filled with water in place). Note that since the collimator/scintillator assembly just touches the base of the dish, the scintillator will be at virtually the same height as the cells, and therefore in focus. Once the collimator position is established, the scintillator is

viewed under white light and structural features within the scintillator are used as fiducial marks that serve to instantly indicate the collimator position in subsequent exposures. A check of the collimator position by viewing scintillator light is made at the end of each day, and is normally within 1 μm of the expected position.

The accuracy for finding and positioning cells has been studied using a purpose-built dish that supports a light-emitting diode, masked by a 10 μm diameter pinhole. The dish is mounted on the cell positioning stage and when viewed by the microscope, the imaging software normally used to identify stained cell nuclei will locate the back-illuminated pinhole. This light source serves a number of purposes. One function is to map the optical distortions in the image. This is achieved by successively imaging the pinhole in 50 μm steps until a square array is formed over the entire image field. The image is processed and analysed to produce a look-up table that is used to correct all subsequent images. Without this distortion correction, errors up to 15 μm in the assigned coordinates of objects will occur. The distortions are due mostly to the use of an image intensifier, which in many ways is a poor optical device (but necessary in our current system to reduce UV exposure to acceptable levels). However, CCD cameras with much improved light-sensitivity are becoming available at reasonable cost, and we propose to replace our intensifier-coupled camera with such a device, thereby removing the image-degrading qualities of an intensified system.

Even with the distortion correction implemented, it was evident that cell-positioning errors still occurred. By using the automated procedures to find the pinhole at various points in the image field, then moving it to the collimator, these errors can be mapped. Errors up to 3 μm were observed for objects found in some parts of the image field. This error is tolerable for experiments that target mammalian cell nuclei, but is too large for some other types of experiment. Where increased accuracy is required, a 'two-pass' system is used. This involves automatically finding each cell, moving it over the collimator and then finding it a second time. Any positioning error from the first imaging

step will then be evident, and can be corrected for. Using this method 90% of the cells can be positioned within 1 μm . Note however, that the two-pass method introduces a two- to three-fold increase in the overall time to find cells and doubles the UV dose to each cell. The error is due to the mechanical properties of the stage, which uses a stepper-motor and lead-screw arrangement in an open-loop configuration (i.e. no position feedback). To overcome this limitation, we have designed and built a new stage to meet our current and future requirements. The decision to develop the stage ourselves was necessary as commercial stages with the required accuracy and geometry (i.e. an 'open-frame' type, with access through the centre) were not available. The new stage uses two voice-coil DC motors (BEI Technologies Ltd., USA) and two linear encoders with ± 100 nm position sensitivity (Jena Numerik, Germany) to move, and provide closed-loop feedback control of the stage over a 25 mm square area. Improved accuracy has been achieved partly by making the stage compact (the moving platform is 100 mm square), which improves rigidity and places the encoders as close as possible to the point of interest. Initial tests of the stage indicate that a positioning accuracy and reproducibility significantly less than 1 μm has been realized.

Another reason for developing the new stage has been to improve the cell throughput. With the old system, it takes on average 400 ms to irradiate each cell (~ 9000 cells/h), of which 320 ms is accounted for by stage movement. The new stage is compact, and therefore light and capable of much faster accelerations. Also, by switching to DC motors, the software communication step with the stage is significantly shortened. Over a ten-fold reduction in the time required for stage movement has now been achieved, resulting in an average cell throughput of about 100 ms/cell ($\sim 36,000$ cells/h) without compromising positioning accuracy. This is a substantial improvement and will enable a range of new experiments to be considered, particularly certain studies concerned with low-dose radiation risk (malignant transformation and mutation) where radiobiological effects occur only rarely.

4. Biological applications

Microbeams continue to be a powerful research tool in many areas of radiobiology, particularly in studies of effects at low-doses. This is because microbeams can be used to deliver exact low-doses of radiation to individual cells and the subsequent damage can be analysed on a cell-by-cell basis. Microbeams are also well suited to studies of 'non-targeted effects' such as the bystander effect (where unirradiated cells are affected by damage to nearby irradiated cells), low-dose hypersensitivity and genomic instability. There is increasing evidence that such effects may predominate at low-doses [11] and could cause deviations from the assumed 'Linear, No-Threshold model' currently used to estimate low-dose radiation risk. This model is based on a linear extrapolation of the risk at high-doses into the important low-dose region where few data exist. Our studies of the bystander effect include investigations using a tissue explant model. The move from irradiating individual cultured cells to organised tissues systems is seen as an important step towards understanding these effect *in vivo*.

A question of great interest is whether the bystander effect *increases* or *decreases* the risk associated with low-dose exposures. We 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 [12]. This observation suggests an increased risk at low-doses due to the bystander effect. A similar conclusion can be drawn from a study using the RARAF microbeam to irradiate defined fractions of A_L cells on a dish with just one α -particle per cell [13]. Their data show that irradiating just 20% of the cells on the dish produces a frequency of mutations almost as great as that seen when every cell on the dish is irradiated. Other studies suggest a decreased risk however. In a recent series of experiments using the GCI microbeam, a single cell (out of ~ 150 cells) was targeted with between 1 and 50 protons. This resulted in a loss of clonogenic potential in cells other than just the targeted cell. The level of

cell killing in the population reached 7% after five 3 MeV proton traversals. Increasing the dose to the targeted cell further did not produce more cell killing. This process could be viewed as the removal of potentially unstable cells that would otherwise pose a risk to the organism. In another study, our tissue explant model was used to investigate the influence of bystander processes on growing and differentiating tissues [14]. When a single location in a section of intact tissue was targeted with 10, 5 MeV He³ ions, a significant increase in the number of differentiated cells is observed in the explant outgrowth. This response involves up to 20% of the cells present and could be indicative of a protective mechanism whereby cells that may be potentially damaged (leading to instability) are removed by premature differentiation, thus losing their ability to proliferate.

5. Summary

The number of institutes involved in developing and applying microbeam in a radiobiological application has grown dramatically in the past few years. Many of these groups have recognised the potential that microbeams provide to this research field and at the same time, have come to terms with a unique set of technological challenges that must be addressed if the microbeam is to fulfil its potential as a major radiobiological tool. To be truly versatile, the microbeam must excel in three areas; it must offer a controlled, stress-free environment to the cells, it must be fast (i.e. high cell throughput) and it must be accurate. Recent developments to the GCI microbeam have yielded improvements in all three areas. In particular, the development and implementation of a new micropositioning stage has vastly increased the potential cell throughput (up to 36,000 cell/h) without compromising overall targeting accuracy ($\pm 2 \mu\text{m}$). The facility continues to be used for a number of studies, particularly those that address non-targeted effects such as the bystander effect. Both cell and tissue models are being used to understand the underlying mechanisms and possible role of the bystander effect. The experimental evidence to date

indicates that the bystander effect may cause deviations from the Linear No-Threshold model in the low-dose region, especially with high-LET radiations. However, further research is needed to resolve whether the overall effect is to increase, or to decrease, low-dose risk.

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