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# INVESTIGATING THE CELLULAR EFFECTS OF ISOLATED RADIATION TRACKS USING MICROBEAM TECHNIQUES

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## ABSTRACT

Studies of the effects of radiation at the cellular level have generally been carried out by exposing cells randomly to the charged-particle tracks of a radiation beam, Recently, a number of laboratories have developed techniques for microbeam irradiation of individual cells. These approaches are designed to remove much of the randomness of conventional methods and allow the nature of the targets and pathways involved in a range of radiation effects to be studied with greater selectivity. Another advantage is that the responses of individual cells can be followed in a time-lapse fashion and, for example, processes such as "bystander" effects can be studied clearly. The microbeam approach is of particular importance in mechanistic studies related to the risks associated with exposure to low fluences of charged particles. This is because it is now possible to determine the actions of strictly single particle tracks and thereby mimic, under *in vitro* conditions, exposures at low radiation dose that are significant for protection levels, especially those involving medium- to high-LET radiations. Overall, microbeam methods provide a new dimension in exploring mechanisms of radiation effect at the cellular level. Microbeam methods and their application to the study of the cellular effects of single charged-particle traversals are described.


## INTRODUCTION

There is a current resurgence in the use of microbeams in radiation biology. Recent technological developments, such as improved imaging techniques, microengineering and radiation detector technologies and the major advances in cell biology techniques, which allow one to measure effects at the single cell level, have generated new interest in this area. Three main research requirements have stimulated these developments: 1) There is a great need to understand radiation effects at the level of a single particle traversal of relevance to radiation risk. Low-dose studies with conventional particle beams are complicated by the Poisson distribution. The best that can be achieved with an average of one particle traversal, is an approximation wherein 37% of the population receives no-hits, 37% of the cells get 1-hit and 26% of the cells get 2 or more hits; 2) The importance of understanding the distribution of radiosensitivity across the cell nucleus and the cell in general, to determine where critical targets and processes are located; 3) The ability to target individual cells within a population and determine responses of relevance to the *in vivo* situation that can involve cell-cell communication.

Microbeams are one example of an approach designed to target radiation to regions of cells or tissues. Other approaches include: 1) The use of biostacks, which involves recording the positions of particle traversals using track-etch techniques and relating these to the positions of the cells growing on these detectors. This approach has already been used in several studies determining cell survival after exposure to low doses of α-particles, with a view to measuring the effects of a single particle traversal (Heimgartner et al. 1997; Pugliese et al. 1997; Søyland et al. 2000); 2) Targeting of radioisotopes into specific molecules within cells. For example, $^{125}$I dUrd has been used to target DNA and the cell nucleus and $^{125}$I concanavalin A has been used to target the cell membrane and cytoplasm (Warters et al. 1977). Other approaches include Boron Neutron Capture studies where epithermal neutrons are used to target boronated compounds in tumour cells leading to the production of short-range α-particles and $^{7}$Li particles. 3) Another approach in current use is to target groups of cells within a population using a grid which shields a known fraction of the culture being exposed (Lorimore et al. 1998; Nelms et al. 1998). This approach has the advantage of allowing large numbers of cells to be exposed quickly, but the disadvantage of not being able to specify the region within the irradiated cells.

## HISTORY OF MICROBEAMS

The first development of a microbeam for cell irradiation was in 1912 by Tchakhotine (reviewed by Zirkle, 1957). He described a technique for focusing, a 280nm UV beam down to around a few microns in diameter, using a quartz lens and objectives. These studies have evolved over the years to the sophisticated laser based UV microbeams in use today. For ionising radiations, Seed and colleagues in 1960 developed an approach for the focussing of an X-ray beam for biological studies (Seed, 1960). While Zirkle and others concentrated on the use of charged particles (reviewed in Zirkle, 1957). The latter utilised the limited range of charged particles as a means of titrating effects throughout a cell population (macrobeams) or the ability to precisely irradiate regions of an individual cell using, for example, polonium-tipped micro needles (Munro, 1970). Similar approaches have been used with partially penetrating low energy electrons (Cole et al. 1963). In recent times Braby (1992) developed collimation approaches which allowed the development of systems which could both target cells individually with counted particles and allow these to be delivered to within a few microns of the required target. The current generation of particle microbeams utilise this general principle of collimation coupled with particle counting (see Figure 1). (Braby, 1970; Geard et al. 1991; Folkard et al. 1997a; 1997b). Using this approach, current systems can achieve a 100% efficiency of particle detection, resolutions approaching 1 μm (Folkard et al. 1997a; 1997b) and a cell throughput approaching 3,000 cells per hour (Miller et al. 1999). For X-ray sources, a soft X-ray microprobe has been developed at the Gray Laboratory which generates a focus of $C_K$ X-rays with a radius less than 250 nm within a cell. This utilises zone plates -- devices used in soft X-ray microscopy -- which effectively behave as lenses and focus the X-rays (Schettino et al. 2000).

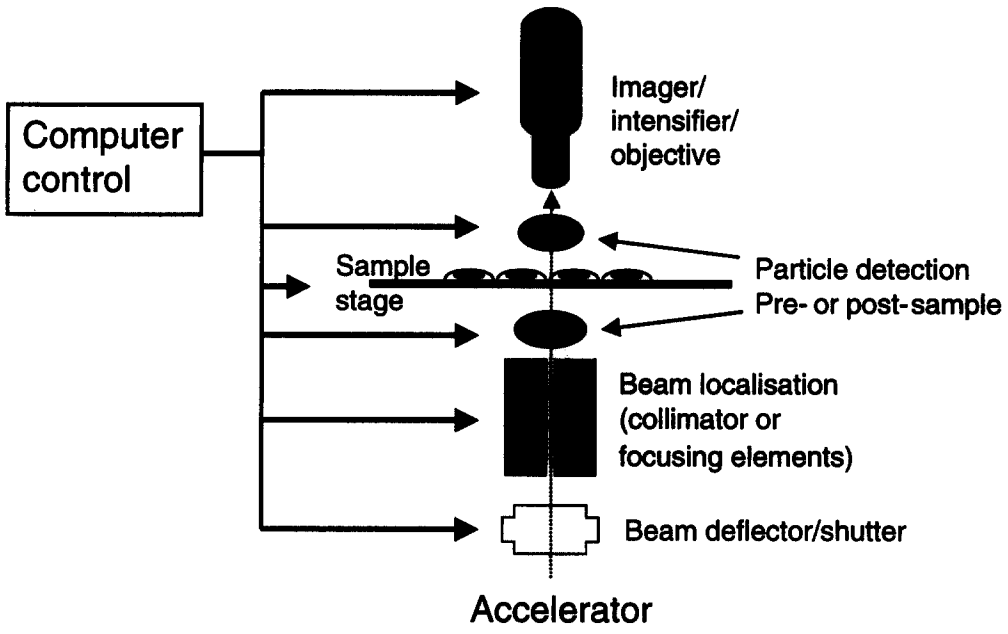


Fig. 1. Typical elements of a modern microbeam system.

## CURRENT APPLICATIONS

The types of experiments currently performed with microbeams fall into three general categories:- 1) experiments where every nucleus within a population is targeted, 2) experiments where a different subcellular region such as cytoplasm is targeted and 3) experiments where a single cell is targeted within a population. Microbeam techniques are unique in being able to perform all three of these types of experiments in contrast to the other approaches mentioned earlier. A range of biological endpoints and experiments have been used with the current generation of particle microbeams. The results are briefly summarised below along with some discussion of the experimental requirements for each type of procedure.

**Targeting every cell nucleus within a population**

A major consideration in these types of studies is the speed of finding and irradiating cells, particularly if one is measuring low frequency endpoints. Most of these studies to date have targeted cell nuclei with a view to comparing individual, versus average numbers of particle traversals. Targeting accuracy can be compromised to some extend providing the resolution available is sufficient to target the nucleus. Typical mammalian cell nuclei vary from about 7 μm to 20 μm in diameter, therefore a targeting accuracy of 5 μm for most cell systems is adequate. Imaging of cell nuclei is relatively straightforward using fluorescent approaches, upon which the current generation of microbeams rely on. Most commonly, low levels of the DNA binding dyes Hoechst 33258 or 33433 are used and the software systems only need to find and recognise ellipses of various forms. A more complex requirement is to differentiate cells from other fluorescent artefacts that may be present and to recognise newly divided cells properly so that each cell is targeted. A critical requirement is to count particles accurately so that exactly one particle is delivered to each cell. From studies comparing exact numbers of particles, both false positives (i.e. particle detected but cell not hit) and false negatives (cell hit but no particle detected) need to be avoided. The dose-rates also need to fall within a range in which individual particles can be adequately discriminated from each other by the detection and beam control system.

Nelson and colleagues measured the ability of a single α-particle to produce chromosomal damage in hamster CHO-K1 cells when each cell was exposed (Nelson et al. 1996). A yield of 0.13 micronuclei per binucleated cell per particle was observed for 3.2 MeV helium ions. In their system they relied on non-fluorescent epi-illumination of their cells, which reduced the confidence in targeting the nucleus (< 90%). Similar studies, using staining with Hoechst 33258 (Prise et al. 2000) were able to measure the chromosomal damage induced by a single 3.2 MeV proton traversal delivering less than 0.02 Gy per cell nucleus. Hei et al. (1997) measured the production of specific mutations at the *CD59 (S1)* locus in human-hamster hybrid cells ($A_L$) cells exposed to counted α-particles targeted through the nucleus. A significant yield of mutants was observed after a single particle traversal. The mutation frequency increased from 45 per $10^5$ survivors to 155 per $10^5$ survivors, while the surviving fraction was reduced to 0.82, suggesting that a single α-particle is highly mutagenic. The risk associated with a single α-particle in terms of the probability of it inducing a transformed cell within a cell population has also been measured and compared with average numbers of particle traversals (Miller et al. 1999). Individual mouse C3H10T1/2 cells were exposed to exactly 0, 1, 2, 4, or 8 α-particles and the transformation frequency compared with average numbers (Poisson-distributed) of particles delivered by a broad-beam α-source. Important differences in the low dose region were observed, namely that the transformation frequency was 2-3 fold lower when a single particle was delivered in comparison to an average of 1 particle. This suggests that in this system, multiple traversals of a cell are required for significant risk of transformation, in contrast to the mutation system where a single particle is highly mutagenic. Methods have also been developed to target non-attached cells such as human lymphocytes (Kadhim et al. 2000). These studies have shown evidence for the induction of chromosomal instability after only a single α-particle traversal in the progeny of single cells many generations later.

**Targeting subcellular regions**

A major advantage of the microbeam approach is the ability to localise the radiation to regions of interest within the target cells or tissues. Many questions of radiation effects at the cellular level revolve around targets and pathways required for various biological responses. For example, the processes leading to apoptosis can be triggered by either external stimuli or direct damage to cellular DNA with different signalling pathways involved. For nuclear DNA, damage responses may be heterogeneous across the nucleus leading to differential damage expression. The major limiting factor in spatial studies is resolution of the beam relative to the target(s) of interest. In most attached mammalian cell systems studies can be easily performed by targeting either nucleus or cytoplasm. An important aspect is target recognition. For cytoplasmic irradiation, it is essential to avoid hitting the nucleus, therefore studies to date normally use a dual labelling system whereby the nucleus is stained with Hoechst and the cytoplasm is recognised either using a membrane dye such as Nile Red or a mitochondrial probe such as rhodamine 123. With the current generation of microbeams, where targeting accuracy is around 1-5 μm, probing within the nucleus and then targeting the cytoplasm is possible. However, good validation of the target finding and accuracy is required (Folkard et al. 2000). To localise damage to subcellular organelles such as mitochondria spatial resolution below 1 μm is required. At present soft X-ray microbeams are the only method available which gives this resolution with the current generation easily achieving 250 nm (Schettino et al. 2000). The future generation of these facilities will be capable of providing focal spots approaching 30 nm. They also have inherent advantages over particle microbeams in that scattering is not an issue.

Several studies, using microbeams have shown evidence for the cellular cytoplasm being an important target for biological effects. Wu and colleagues (1999) targeted the cytoplasm of $A_L$ cells with α-particles and monitored mutation expression at the CD59 locus. An increased production of mutations was observed after 4 – 16 particle traversals with a concomitant reduction in cell survival to around 80%. Importantly, the molecular spectra of these mutations was similar to spontaneous mutations found in the background rather than radiation-induced mutations. Studies with radical scavengers and inhibitors of cellular glutathione synthesis suggested that the induction of these mutations was dependent on the production of reactive oxygen species (ROS).

In our own studies, with primary human fibroblasts, we have shown that cytoplasmic targeting with 5 helium ions leads to ROS production and chromosomal damage in the form of micronucleus induction (Ozols et al. 2000). These studies have been performed using dual staining with Hoechst and rhodamine 123. Evidence for chromosomal instability at delayed times after cytoplasmic irradiation is also observed although the relationship between this and ROS production is not clear.

Microbeams have also been used in spatial studies of DNA damage and repair. The track of a particle through a cell nucleus was observed by post-labelling of the DNA ends that were present. This approach probably does not measure the initial damage produced, but breaks associated with the repair processes, caused by repair enzymes which are recruited to process the damage sites (Metting and Braby, 1997). These studies were an extension of previous reports with partial irradiation using synchrotron generated soft X-rays and promised to be a useful approach to follow the functional association of repair proteins with damaged DNA sites *in situ* (Nelms et al. 1998).

### Targeting individual cells within a population

Another major area of research has been to target individual cells within a population. These studies have directly confirmed earlier work (Nagasawa and Little, 1992) showing the production of bystander effects (Prise et al. 1998; Belyakov et al. 2001). These findings have important implications for measurements of radiation risk, which may not be evident in experiments where every cell has been targeted. There is an expanding interest in so called "non-targeted" effects of radiation. To some extent these are the most straightforward of microbeam experiments to perform if cell monolayers are being used. Experiments can be done with minimal image processing requirements provided that the position of the beam is accurately known. This approach is particularly suitable for time-lapse studies to follow individual cell responses in real time.

Our own studies with primary human fibroblasts have shown that targeting a single cell within a population of 600 – 800 cells with a single helium-ion (~110 keV/μm) leads to damaged cells being produced up to 5 mm away from the targeted cell. Typically an additional 80 – 120 damaged cells are observed containing micronuclei. The number of damaged cells produced was found to be independent of the number of helium ions targeted through the cell nucleus and was independent of the number of cells targeted, up to 25% of the total number of cells seeded. Pre-treatment of the population with a low dose of X-rays (0.1 Gy) did not remove the bystander response suggesting that it is not only a consequence of interactions between hit and non-hit cells. Studies by other workers in $A_L$ cells have also shown a bystander response measured as additional mutated cells. When 20% of the cells within a population were targeted through their nuclei, the mutant fraction was 3-fold higher than that predicted on the basis of the number of cells targeted. The radical scavenger DMSO had no effect on the bystander response but cell-cell communication appeared to be involved (Zhou et al. 2000).

These cellular studies have now been extended to tissue models to start to understand the role of cell-to-cell communication and tissue architecture on radiation response. Our preliminary work has been performed with sections of human or porcine ureter where a 4 – 5 cell layer of uroepithelium surrounds the lumen of the ureter. Individual protons or helium ions targeted into the epithelial layers of the tissue or into specific uroepithelial cells within explants induced. A significant bystander response, in contrast to the cellular studies, leads to several thousand additional damaged cells being produced (Belyakov et al. 2000).

## FUTURE DEVELOPMENTS

With several microbeams now fully operational worldwide and several more under construction or planned, there is a potential for a sea change in radiation biology. There is now the possibility to follow biological processes on an individual cell basis and this will provide more detailed information than could be obtained. This has coincided with a need to determine the effects of very low doses of radiation, of particular relevance to radiation risk, and to be able to precisely control where the radiation is deposited within a cell. In the future, the main effort will be to increase the accuracy of targeting down to 1μm and below with particle microbeams. Several

laboratories in Europe, Japan and the USA are currently developing new microbeams around existing microprobe facilities used for elemental PIXE analysis. Many of these will use focusing systems with an electrostatic or magnetic lens (e.g. Dymnikov et al., 2000). Electrostatic focusing will be used instead of collimation. For particles, the resolution is ultimately limited by the inherent scattering of the tracks, which takes place within the sample of interest. For soft X-ray microbeams, resolutions of less than 1 μm have been achieved and the emphasis will be to push the limit of resolution down toward a few tens of nanometers. This goal will require new developments in zone-plate technology. Further advancements in imaging, micropositioning control, radiation detection and the use of off-line cell identification systems will allow much faster processing of cells, allowing irradiation of up to 50,000 cells per hour. Current microbeam systems utilise fluorescent detection of targets, as they have to work in epi-illumination mode. The explosion in the availability of fluorescent markers for cellular targets and processes will impact directly on the ability of future experiments. With the high throughput of cells which will be possible, studies utilising micro-array technologies to monitor changes at the gene expression level will be possible. *In situ* PCR will allow changes on an individual cell basis to be monitored coupled with the use of microinjection techniques for anti-sense oligonucleotides and expression vectors. The use of focusing techniques allows radiation traversals to be controlled in 3 dimensions and the use of particles, which stop within the region of interest, will ultimately be possible. Combining two-photon confocal imaging techniques with microbeams will lead to powerful 3-D probing of cells and tissue models. To date, no studies have been reported using microbeams for determining dose-rate effects. Given that cell locations are logged on the current systems, the ability to revisit and give additional particle traversals to cells is possible allowing precise monitoring of dose-rate effects. Spatial separation of particle traversals at the sub-cellular level will allow questions related to the interactions between target molecules (e.g. chromosomal domains) to be answered. Time-lapse approaches, well utilised in other areas of biology, will be an invaluable approach not just for dose-rate type experiments in single cells but for following other biological processes (e.g. apoptosis).

In the future, we may also see a general move, from experiments concentrating on the use of microbeams to understand radiation responses to the use of microbeams as probes of other biological processes, for example, spatial aspects of stress responses, mechanisms and pathways involved in apoptosis, DNA damage and repair, gene activation and protein expression. Increasingly, microbeams will be used in cell surgery techniques particularly as related to understanding developmental systems (Kobayashi et al. 1997). In conclusion, microbeams have an immense potential to answer important questions in radiation biology, but ultimately they may also impact on many other areas of biology.

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