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The use of microbeams to investigate radiation damage in living cells

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ABSTRACT

The micro-irradiation technique continues to be highly relevant to a number of radiobiological studies *in vitro*. In particular, studies of the bystander effect show that direct damage to cells is not the only trigger for radiation-induced effects, but that unirradiated cells can also respond to signals from irradiated neighbours. Furthermore, the bystander response can be initiated even when no energy is deposited in the genomic DNA of the irradiated cell (i.e. by targeting just the cytoplasm).

1. Introduction

Microbeams are being used to study a number of phenomena that challenge the classical view of how ionising radiation interacts with cells and tissues. One such phenomenon is the bystander effect, where unirradiated cells are damaged through signalling pathways initiated by a nearby irradiated cell (Morgan, 2003). This effect predominates at low doses and profoundly challenges our understanding of radiation risk. Microbeams have been used to show that the bystander response can be initiated even when no energy is deposited in the genomic DNA of the irradiated cell (i.e. by targeting just the cytoplasm). It is becoming evident therefore that other sub-cellular organelles may be initiating targets for radiobiological effects. In this regard, recent studies have shown an important role for mitochondria in the bystander effect (Tartier et al., 2007).

In order to study radiation damage using micro-irradiation techniques, a number of facilities have been developed worldwide based on charged particles (Gerardi, 2006), low-energy X-rays (Folkard et al., 2001), or electrons (Kim et al., 2006). For all three radiations, the radiation can be either focussed or collimated. The Gray Cancer Institute (GCI) has developed, or is developing a number of microbeam facilities for radiobiological applications. The GCI ion microbeam (Folkard et al., 1997a, b) was one of the first such microbeams to be operational in Europe (studies using this facility began in the mid-1990s) and at the time was of only two facilities routinely operational worldwide (the other was the RARAF facility based in New York, see Randers-Pehrson, 2001). The GCI facility has been used almost daily up to the present day, although it is scheduled to be decommissioned at the end of 2007, when the work at GCI relocates to a new institute at Oxford. In its place, a new focussed vertical ion nanobeam is being developed at the University of Surrey Ion Beam Centre. This is a collaborative project between GCI and Surrey. The new facility will be configurable both as an analytical and radiobiological nanoprobe and will have a number of highly desirable features not available on our current source, including a steerable, focussed beam allowing very fast cell throughputs (tens of cells per second), a versatile ion source (making a range of ions available) and state-of-the-art cell microscopy for both off-line and on-line target recognition.

In addition, it should be noted that GCI has also developed a number of tabletop focussed low-energy X-ray microprobe facilities, although these are not discussed in this report.

2. The role of microbeams in radiobiology

The micro-irradiation technique continues to be highly relevant to number of radiobiological studies, due to its ability to deliver precise doses of radiation to selected individual cells (or sub-cellular targets) *in vitro*. It is this exquisite control over dose delivery that makes the use of microbeams highly suitable to studies at low radiation doses. It has been known that ionising radiation can damage living cells and tissues for over 100 years. Despite this, the effects of radiations at low doses remain poorly understood. At high doses, above a few gray, it is likely that exposed cells will be sterilised, or die. At lower doses, most cells survive, but with the possibility of mis-repair, leading to carcinogenesis. Estimates of radiation risk have been based largely on studies of the survivors of the atomic weapons used against Japan in the Second World War (Morgan and Schwartz, 2007). However, below a few hundred mGy, risk estimates cannot be reliably derived from epidemiological data and a linear

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extrapolation to zero-effect at zero-dose is applied (the so-called 'linear no-threshold' model). For the vast majority of the population, the annual exposure to ionizing radiation is well below 100 mGy. Exposure arises through natural environmental sources (mainly radon, cosmic rays, and some foods) and through clinical diagnostic imaging procedures that use penetrating radiations. Some diagnostic CT procedures can impart doses of several tens of mGy. Of the environmental sources, radon accounts for over half of the effective dose on average, although large regional variations exist (high radon levels exist in granite-rich areas, such as south-west England, and parts of Scotland). Radon is an α-particle emitter that enters the body through inhalation, such that lung epithelial cells are exposed. However, at typical doses, only a tiny fraction of the cells are actually traversed by a particle and those that are hit are unlikely to see more than one traversal. Hence, we have a scenario where the bystander effect due to a single-particle traversal of a single cell could be significant. The bystander effect is an example of a 'non-targeted effect' where cells are seen to respond indirectly to ionizing radiation. This is in conflict with the conventional view of cellular radiation damage, which assumes that that direct damage to the DNA helix is necessary to induce critical effects. Such non-targeted effects tend to dominate at low-doses and could have a significant impact on the linear no-threshold model (Brenner and Sachs, 2006).

Radiotherapy (RT) remains one of the key strategies for treating many cancers and exploits the ability of ionizing radiations to kill cells at high doses. Although the basic methodology underpinning RT has remained unchanged for decades, evermore precise dose delivery strategies are being developed and applied (such as intensity-modulated RT and particle RT). As cure rates improve, the occurrence of secondary cancers arising from unwanted exposure of the normal tissue becomes increasingly significant. Indeed, it has been pointed out that the use of intensity-modulated RT may actually increase the risk of secondary cancers, due to stray fields and through the use of more fields (Hall, 2006). These observations underline the importance of understanding radiobiological effects at low doses for RT as well as environmental exposures.

3. Strategies for micro-irradiating cells

Most particle microbeams make use of light ions, either protons or helium ions. Protons are the most penetrating for given accelerator: they cover a useful LET range (10–40 keV μm^{-1}), and they are relevant to proton RT, but are easily scattered. Helium ions are radiobiologically relevant to risk (i.e. exposure to radon); they have good penetration at modest energies and are less readily scattered than protons. Some microbeams use heavy ions for studies requiring very high LET particles (i.e. see Heiss et al., 2006; Hauptner et al., 2004). They can be used in studies pertinent to particle RT that use particles other than protons (i.e. carbon ions) and for radiation fields encountered during space travel. Heavy ions are advantageous in that they are less readily scattered, and particle detection is less problematic.

To study the much lower doses that occur when a photon or electron traverses a cell requires the use of an X-ray or electron microbeam. Also, low-energy X-rays are the source of choice for achieving the finest probes. This is because unlike particles and electrons, they interact almost entirely through the photoelectric effect and are therefore are not scattered. The 'fineness' of an X-ray probe is ultimately limited by the range of the secondary electrons it sets in motion, but can be well below 1 μm.

In the first wave of microbeam development, all facilities (including the GCI microbeam) used a vertically up beam orientation. This is the preferred direction for ease of sample manipulation. Typically, cells to be irradiated are attached to thin membranes that form the base of a dish and cells are irradiated through the membrane. If the dish is mounted above a vertical beamline then it is straightforward to add cell culture medium to the dish. Later, microbeams tended to be developed around existing analytical microprobes which were invariably oriented horizontally (i.e. Hauptner et al., 2004). In this case, a more complex dish design is required to retain a media layer over the cells. Typically, another membrane is added beyond the cell layer, with only a thin gap to retain media. Alternatively, the cells are bathed in a humidified gas, but this is not ideal for studies of media-borne cell signalling.

With regard to ion microbeams, two methods of probe formation are possible; collimation or focussing. The GCI facility uses a 1 mm by 0.25 mm glass capillary with a 1 μm bore. This is mounted on gimbal at the end of the beamline. The advantage of collimation is that it is relatively straightforward to implement and (in our experience) easily aligned. Ultimately, however, the finest probes will be achieved using focussing systems. Although sub-micrometre probes are readily achieved by focussing, this will be compromised by scattering in the vacuum window and (if used) the transmission detector. Of the focussing systems, both magnetic and electrostatic quadrupole lenses have been used.

One aspect of ion microbeam design that has proved problematic is the issue of particle detection. Clearly, reliable particle detection is needed to deliver single particles to cells, but a solid detector placed before the cell will inevitably scatter the beam. This is particularly problematic for light ions. It is not possible to place the detector after the cells unless the cell media is removed, or a very thin 'sandwich' is constructed with cells between two thin windows. As mentioned, some horizontal microbeams use just such a dish design and can therefore adopt this method of detection. One possibility for detecting the particle without introducing scattering is to detect the charge by capacitive pickup. This technique is currently being explored by the group developing the RARAF microbeam.

4. The GCI charged-particle microbeam

A schematic arrangement of the GCI charged-particle microbeam is shown in Fig. 1. There are four key aspects that underpin the GCI microbeam specification:

1. *Accuracy*: Need to reliably target the cell nucleus, or cytoplasm. Therefore about 1–2 μm targeting accuracy is required.

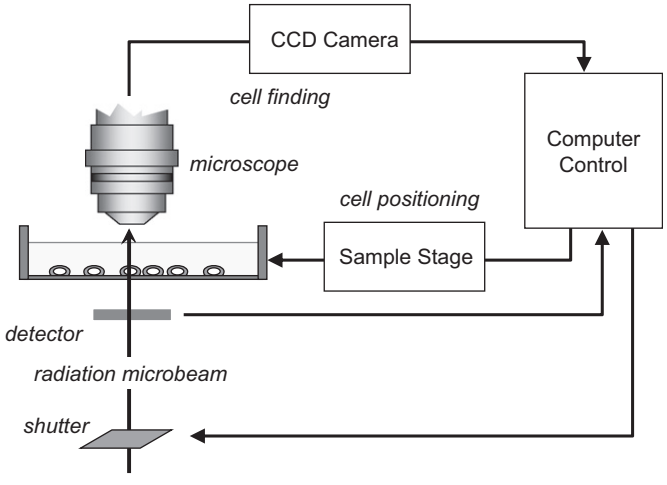

Fig. 1. Schematic arrangement of the GCI charged-particle microbeam.

2. *Detection*: Single particle delivery requires efficient real-time detection and shuttering.
3. *Imaging and alignment*: Requirement to image target, log position, and to precisely align target to microbeam. Need ability to relocate dish and revisit cells to score damage after extended periods.
4. *Speed*: There can be up to 5000 cells per dish, therefore rapid, automated cell finding and irradiation is essential.

4.1. Accuracy

To achieve the desired accuracy, a fine radiation beam is formed using a 1 μm diameter bore fused silica capillary collimator, mounted at the end of the beamline and covered with a 3 μm thick Mylar vacuum window. During irradiation, each cell, or sub-cellular target, is located in turn above the collimator and irradiated. The effect of scattering of the beam by the exit window and detector (described below) is minimised by positioning the collimator as close as possible to the cell. For each exposure, the collimator is raised until it just touches the base of the cell dish and then lowered about 0.5 mm after the exposure. We have determined the targeting accuracy and particle counting efficiency of our collimated facility using CR-39 track-etch plastic (Peng et al., 2001). Our measurements show that for protons, we can hit 90% of targets with an accuracy of ± 2 μm, or 96% of cells with an accuracy of ± 5 μm. Using $^{3}He^{2+}$ ions (which are less easily scattered), 99% of cells are targeted with an accuracy of ± 2 μm. Calculations using SRIM2003 show that as little as 20 μm of air gap between the end of the collimator and the cell dish can decrease the targeting accuracy by $> \pm 1$ μm for protons (see Fig. 2).

4.2. Particle detection

The particles incident on the cells are counted using a photomultiplier (PM) tube mounted just above the cell dish. The PM tube detects the pulse of light (due to the passage of a particle) from an 18 μm think scintillator (Bicron BC400) mounted on the end of the collimator. A fast electrostatic shutter terminates the irradiation of each cell once the preset number of particles has been delivered. CR-39 has been used to determine the efficiency of the detection system. Fig. 3 shows a typical experiment, where exactly three ions have been delivered in a triangle pattern to 25 locations. With one exception (circled), the correct number of ions has been delivered. Using this method, we estimate that for single particle delivery, the detection efficiency is greater than 99% with no missed particles and less than 1% false positives (see Fig. 4).

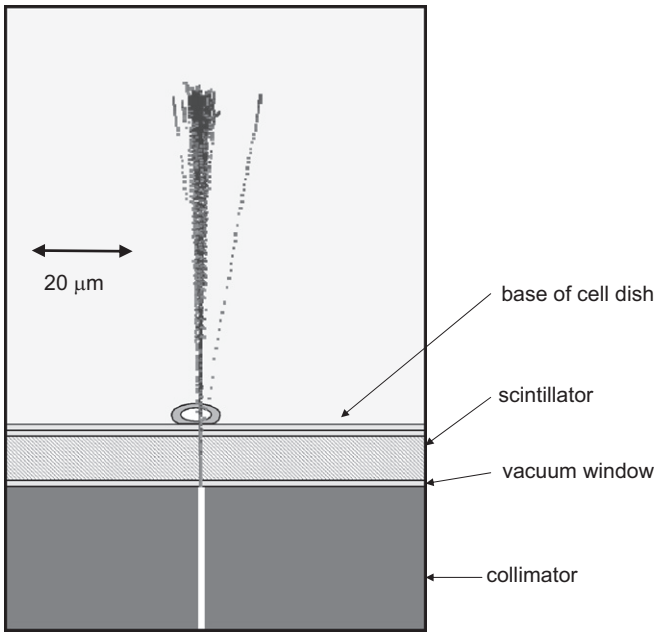

Fig. 2. SRIM2003 calculations of the scattering of a 2.5 MeV proton as it exits the collimator.

4.3. Imaging and alignment

To image and align cells, the dish is located on a three-axis micro-positioning stage (Marzhauser, Germany) above the collimator. Cells are found *in situ* using a modified epi-fluorescent Olympus BX microscope that views stained cells from above using typically a 20 × water-immersion objective. 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 imaging step is completed prior to the irradiation of the cells, as the objective lens is replaced with the PM tube during the cell exposures. During irradiation, the stored cell coordinates are used to align the cell to the beam. The same stored coordinates can be used to relocate cells and score damage after extended periods, for example, after incubation of the cells as part of a cell survival assay. The imaging system can also be used to establish the collimator position by imaging the light from the scintillator in part of the dish with no cells.

4.4. Speed

The process of identifying and aligning cells is automated. Typically, it takes about 6–8 min to scan a 5×5 mm^2 area of a dish and identify and assign coordinates to around 1000–2000 cells.

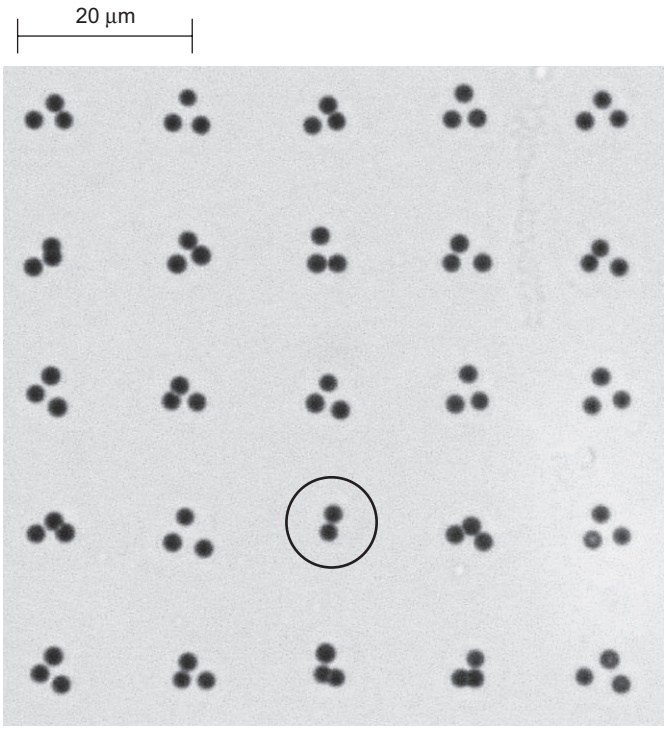

Fig. 3. CR-39 track-etch plastic targeted by exactly three helium ions in a triangle pattern to 25 separate locations. The observed deviations from an equilateral triangle are due to scattering. In one instance a 'false-positive' count has resulted in only two ions being delivered.

No. particles Delivered \ Preset No. particles	1	2	3	4	5
0	0.8	-	-	-	-
1	99.2	0.9	-	-	-
2	-	99.1	2.5	-	-
3	-	-	97.5	2.2	0.3
4	-	-	-	97.2	3.1
5	-	-	-	0.6	96.3
6	-	-	-	-	0.3
7	-	-	-	-	-

Fig. 4. The measured detection efficiency of the GCI scintillation detector arrangement for helium ions. The data reveal that deviations from the required number of particles tend to be 'false positives'.

The irradiation of each cell is a four-step process: Firstly, the cell is positioned above the collimator. Secondly, the collimator is raised. Third is the actual irradiation of the cell, and finally, the collimator is lowered to allow the next cell to be aligned. The overall time to irradiate each cell is about 450 ms, of which about 350 ms is due to the first step (cell positioning by the stage). Overall, the cell throughput is about 8000 cells per hour during the irradiation step.

5. The vertical scanning nanoprobe

The GCI charged-particle microbeam has been in routine use for over a decade, but work using this facility will cease towards the end of 2007. Some of the experience gained on the GCI facility will be applied to a new micro-irradiation facility under development at the University of Surrey. The new facility will exploit an existing 2 MV tandetron accelerator, equipped to supply a range of light and heavy ions. The new nanobeam will be vertically oriented and be focussed and steered using electromagnetic fields. It is anticipated that the beam will be capable of targeting areas of <10 nm with single ions *in vacuo* (but with reduced accuracy for the externalised beam). The ion focussing system will be based on a compact triplet of magnetic quadrupoles using a proven design. A great strength of the new facility is that rapid beam steering will be used to target cells. This will largely eliminate the time-consuming cell alignment step of our current arrangement; although some cell dish repositioning will be necessary if the cells are plated over an area larger than can be accessed through beam steering. It is estimated that eventually the facility will be capable of irradiating ~100,000 cells per hour. The development of the scanning nanoprobe is reported elsewhere in these proceedings.

6. Conclusions

Without doubt, the use of microbeams in radiobiology has advanced our understanding of how ionizing radiations interact with cells and tissues. This is particularly true at low doses, where ability to precisely target sub-cellular features with an exact number of particles has been used identify and quantify the some of the processes involved in non-targeted effects, such as the bystander effect.

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