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“Broadbeam” irradiation of mammalian cells using a vertical microbeam facility

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Abstract A "broadbeam" facility is demonstrated for the vertical microbeam at Surrey's Ion Beam Centre, validating the new technique used by Barazzuol and coworkers recently (Rad. Res. 177 651–662, 2012). Here, droplets with a diameter of about 4 mm of 15,000 mammalian cells in suspension were pipetted onto defined locations on a 42 mm diameter cell dish with each droplet individually irradiated in "broadbeam" mode with 2 MeV protons and 4 MeV alpha particles, and assayed for clonogenicity. This method enables multiple experimental data points to be rapidly collected from the same cell dish. Initially the Surrey vertical beamline was designed for the targeted irradiation of single cells with single counted ions. Here, the benefits of both targeted single cell and broadbeam irradiations being available at the same facility are discussed: in particular, high throughput cell irradiation experiments can be conducted on the same system as time-intensive focused-beam experiments with the added benefits of fluorescent microscopy, cell recognition and time-lapse capabilities. The limitations of the system based on a 2 MV tandem accelerator are also discussed including the uncertainties associated with particle Poisson counting statistics, spread of linear energy transfer in the nucleus and a timed dose delivery . These are calculated with Monte Carlo methods. An analysis of how this uncertainty affects Relative Biological Effect measurements is made and discussed.

Introduction

The growth in the use of hadron therapy for the treatment of cancers (Levy *et al.*, 2009) gives impetus to the study of the radiobiological response of mammalian cells to ion irradiation. The energy and species available from current radiobiological facilities range from high energy cyclotrons and synchrotrons (e.g. National Institute of Radiobiological Sciences (NIRS) or Japan Atomic Energy Research Institute (JAERI), Japan, or Gesellschaft für Schwerionenforschung (GSI), Germany), to medium linear accelerators (e.g. the Superconducting Nanoprobe for Applied nuclear (Kern) physics Experiments (SNAKE), Tandem 14 MV, Germany) to lower energy accelerators (e.g. Radiobiological Research Accelerator Facility (RARAF), 5 MV, U.S.A). Many accelerators have horizontally orientated broadbeams, with beam diameters in the order of mm^2 or cm^2 (e.g. Schuff *et al.* 2002, Kosihi *et al.* 2005, Schmid *et al.* 2009). Fewer studies have been conducted using vertical beams with these dimensions (e.g. Besserer *et al.* 1999, Mortel *et al.* 2002). The experimental approaches adopted by these different facilities very much depends on the energy. Conventional plastic dishes are commonly used by high energy facilities, while dishes with micron-thin mylar or polypropylene windows are used by low energy facilities to minimise energy loss. Mylar or polypropylene has the disadvantage that cells have to be specially cultured on them, often using cell adhesive coatings such as fibronectin. Moreover, very careful consideration of the spread of Linear Energy Transfer (LET) has to be taken when using low energy particles that are approaching the Bragg peak in the nucleus of the cell. This is particularly relevant for ion species produced by low energy accelerators and radioactive sources (e.g. Goodhead *et al.* 1991 and more recently Beaton *et al.* 2011). Most cases in the literature have used a “volume average” (also known as track average) LET to summarise this spread. The merits and pitfalls of averaging the LET spread over the thickness of the nucleus will be discussed.

In parallel to broadbeams, microbeams are available at various institutes to irradiate individual cells with single ions. Microbeams have been instrumental in discovering a variety of phenomena such as very low dose and bystander effects (see Durante *et al.* 2011 for a review of recent microbeam advances). The Surrey Vertical Beam (VB) can operate as a microbeam (Merchant *et al.* 2012), but here we discuss how it can also operate in “broadbeam” mode. We

have a number of projects underway comparing broadbeam to microbeam mode. There are relatively few studies which have done such comparisons. One example is Miller *et al.* (1999), who used a microbeam to deliver single alpha particles to cell nuclei and compared the results to broadbeam irradiation and its associated Poisson uncertainty. They found that mutation rates were overestimated by broadbeam, as mutation was sensitive to multiple traversals. Another example is by Auer *et al.* (2011), who compared a continuous beam of 20 MeV protons, to a focused (100x100 μm) pulsed beam, and found there was little difference in survival curves from the ultra-high dose rate of the pulsed beam.

Moreover, investigations into the possible synergistic effects of types of high-LET radiation and drug treatments are interesting. Indeed, Barazzuol *et al.* (2012), used the irradiation set-up described in the present paper, to conveniently assay the effects of temozolomide and high-LET radiation on glioblastoma derived cell lines. Temozolomide in conjunction with X-ray radiotherapy is routinely used to treat patients with this highly aggressive form of brain tumour. It was concluded that X-rays, protons and alpha particles have an additive rather than synergistic effect with temozolomide.

In the present paper, the broadbeam method of using the Surrey Vertical microbeam is presented, the limitations discussed and results compared to the literature.

Materials and methods

Vertical Beamline

A 2MV HVEE Tandetron accelerator (Simon *et al.* 2004) accelerates ions into the Surrey vertical beamline described by Merchant *et al.*(2012). A range of ions is available from either a duoplasmatron or sputter ion source. A 90° bending magnet (0.75 m radius) transports the beam from a horizontal to vertical orientation. The range of ion species is limited from protons to calcium due to the bending capability of the 90° magnet and the shorter range of heavier ions in

water. A table of ions with the associated range in water is presented by Merchant *et al.* (2012). A set of electrostatic plates are used as a “beam switch” to deflect the beam sufficiently to prevent particles from reaching the cells once the required dose has been achieved (Merchant *et al.* 2009). A range of apertures are used for beam transport and control of beam flux. The vertical section of the beamline ends on the fourth storey in a tissue culture laboratory, where the beam is extracted from vacuum into air through a 200 nm thick 2.25 mm² silicon nitride window (Norcada Inc, Edmonton, Canada). The beam is confined by this 2.25 mm² window in the beam nozzle and cannot be increased beyond this dimension. A computer controlled XY stage (Märzhäuser, Wetzlar, Germany) works together with a piezo-electric Z stage (Prior Scientific, UK) to position a cell dish with a thin polypropylene or mylar bottom over the beam nozzle, with an air-gap of 100 µm between the vacuum window and the base of the cell dish. A high performance, custom built optical microscope which uses software developed by the Gray Institute (Barber *et al.* 2001, Barber *et al.* 2007, Folkard *et al.* 1997) can be used to view the cell samples *in situ* during irradiation. Beam homogeneity is checked with a scintillator and beam flux is measured with a PIN diode (silicon *p-i-n* device) before the irradiation of cells.

Cell irradiation

In order to irradiate cells, a droplet-based method was developed, where cells are pipetted onto the polypropylene-bottomed dish in a 15 µl droplet. Two separate protocols were used depending on the range of the particles; for long range particles (i.e. 2 MeV protons) the cells were pipetted onto the polypropylene and were irradiated in suspension, while for short range particles (i.e. 4 MeV alphas) the cells were placed in droplets onto fibronectin so that they were flat and attached to the polypropylene. The overall schematic is shown in Fig. 1. Here, a 42 mm diameter dish contains a number of droplets each of which receives a different dose. The dish also contains a control droplet and a small piece of CR-39 to check the beam homogeneity. The diameter of each droplet was about 4 mm and so clearly larger than a single nozzle. The computer-controlled stage was arranged to move in a 3x3 patchwork configuration of nozzle areas, to allow irradiation of an area greater than the area of the droplet. The stage has an optical encoder and is capable of positioning successive irradiations side by side within micron accuracy. In this way, six different doses into six droplets could be delivered per dish (with a control droplet that is not

irradiated) with irradiations taking typically about 10-15 minutes per dish.

The cell suspension was diluted to 1×10^6 /ml resulting in 15,000 cells per 15 μ l droplet, pipetted onto the polypropylene. It was observed with an inverted microscope (Axio, Zeiss, Germany) that after 10 minutes, the cells would sink to the bottom of the droplet (but would not attach if no fibronectin was present), with enough area for all the cells to be in contact with the bottom of the polypropylene. After irradiation, the droplet was pipetted off the polypropylene, and diluted to the appropriate concentration for a clonogenic assay. With a second careful washing of the area where the droplet had been, 100% of the cells could be retrieved. The polypropylene was examined by microscopy after the droplet had been removed to verify that no cells remained. For short-range particle irradiation, the cells were left to flatten onto fibronectin for an hour, washed to remove any unattached cells and then irradiated. Subsequently they were incubated in a droplet of trypsin and then plated out at an appropriate density. Again, the polypropylene was checked with a microscope to ensure that all the cells had detached. We found that this protocol did not affect the plating efficiency compared to cells cultured directly in tissue culture flasks. For each survival curve, at least three repeats were irradiated on the same day, with the experiment repeated at least once on a different day.

Dosimetry: Linear Energy Transfer calculations, beam homogeneity and beam stability

To calculate the dose rate given to cell nuclei, the following formula adapted from Belli *et al.* (1989) and Combs *et al.* (2009) was used:

$$\text{Dose Rate (Gy/s)} = 1.6 \times 10^{-9} \frac{L\phi}{\rho} \quad (1)$$

where $L(\text{keV } \mu\text{m}^{-1})$ is the LET, ϕ ($\text{particles cm}^{-2} \text{s}^{-1}$) is the particle flux, and ρ (g cm^{-3}) is the density of the medium.

A slab approximation is used for the cell nucleus even though the cell nucleus can be spherical. A more realistic model including spherical cell nuclei would be more representative. However, small variations in the thickness of the nucleus make very little difference to the “volume averaged LET”, as shown below. Hence, such a model makes very little difference to the

uncertainties presented in this paper.

The volume averaged LET of the particle will depend on the thickness of the nucleus. Concerning V79 cells that are attached to a substrate and lying flat, there is considerable literature on the thickness of the V79 cells nuclei (see Bettega *et al.* 1998 for experimental work and review). Here, the value of $6\text{ }\mu\text{m} \pm 2\text{ }\mu\text{m}$ for flattened nuclei was taken. To our knowledge, for V79 cells in suspension the nuclei dimensions have not been investigated, especially in the current “droplet” based experimental set-up. Therefore, confocal microscopy (Axiovert 2000, Zeiss, Germany) was used to analyse the central thickness of the cells, in $0.5\text{ }\mu\text{m}$ slices, lying on the polypropylene. The cells were fixed with 2% paraformaldehyde, permeabilised with 0.05% triton-X, treated with RNase, stained with To-Pro (Invitrogen, USA) and imaged through the polypropylene on a special jig constructed for the confocal microscope to accommodate custom dishes used in the present experiment. Sampling 100 cells, the average cell nucleus thickness was $10.1\text{ }\mu\text{m}$ with a range of about $6\text{ }\mu\text{m}$ to $17\text{ }\mu\text{m}$, which can be seen in the histogram shown in Fig. 2. Interestingly, it was observed that the nuclei were positioned at the bottom of the cell, next to the polypropylene. Moreover, it was found that cells did not move from their resting positions with stage movement (data not shown).

The LET of particles traversing an “average” nuclei (rounded to $10\text{ }\mu\text{m}$ thickness for unattached or $6\text{ }\mu\text{m}$ for attached cells) was calculated using the program “Stopping and Range of Ions in Matter” (SRIM) (Ziegler, 2004). Table 1 shows the incident energy of the particles, the entry, middle and exit LET of the particle in the cell (assuming it is 10 or $6\text{ }\mu\text{m}$), and the integrated LET through the cell, to give a volume averaged LET per cell nucleus. Figure 3 shows the energy loss of the particle through water, taking into account losses through the silicon nitride and polypropylene layers. An alternative calculation, which is more commonly used by researchers (e.g. Bettega *et al.* 1998), is to divide the difference between the entrance and exit energies ($E_{\text{in}}-E_{\text{out}}$) by the nuclear thickness giving an average LET. However, as can be seen in Fig. 3, there is a significant non-linear component to the stopping of the particles, especially with alpha particles. By integrating the LET from the entrance to the exit of the cell nucleus and dividing by the thickness of the nucleus, this non-linear element could be accounted for; however, the alternative calculation is sufficiently accurate when one considers the uncertainty

on the volume averaged LET. Importantly, a volume averaged LET does not take into account any possible non-linear biological effects. For instance, one could argue that the maximum LET reached for the particle (in our case 18.4 and 132.9 keV μm^{-1} for protons and alphas respectively), should be the LET assigned to the survival curve, depending on which LET is the most likely to have the most significant biological effect. With this caveat in mind, we assign the average volume LET to the survival curve and subsequent relative biological effectiveness (RBE) ratios.

The air gap between the nozzle and the polypropylene is about 100 μm which has a negligible effect on the energy of MeV protons and alpha particles (for 2 MeV protons it is 16 eV μm^{-1} and for 4 MeV alpha particles it is 103 eV μm^{-1}). From dish to dish, the air gap between the exit window and the sample does not change significantly. This is known as the depth of focus on a 40X objective is about 1 μm , and the image does not go significantly out of focus. The energy straggle for 4 MeV alpha particles after the polypropylene has a full width half maximum (FWHM) of 35.6 keV as calculated by SRIM. In terms of LET variation, this straggle has a negligible effect in comparison to variation in cell thicknesses. The variation in LET as a particle traverses an individual cell is small for 2 MeV protons (2 keV μm^{-1}), but much larger for 4 MeV alphas (16.9 keV μm^{-1}); this is why potential non-linear effects will be much more serious for alpha particles than for protons. However, if a population of cells is considered, with the large range of nuclear size described above, the volume-averaged LET has a relatively small uncertainty. For protons, the maximum percentage difference from cell to cell in volume averaged LET is less than 5 % (volume average LET range 17.2-17.9 keV μm^{-1}), when the average cell nuclear thickness is 10 μm (with a range of 4-17 μm). Similarly, for the alpha particles, the maximum percentage difference from cell to cell in volume averaged LET is less than 6% (volume average LET range 120-126.5 keV μm^{-1}), when the average cell nuclear thickness is 6 ± 2 μm . The LET variation is taken into account in the overall dose uncertainty by a Monte Carlo simulation (see below).

The beam homogeneity is observed on YAG:Ce (Yttrium Aluminium Garnet) scintillator (SPI supplies, West Chester, USA) using the endstation microscope, and the beam transport adjusted as necessary until uniform scintillation is achieved across the entire 2.25 mm² nozzle. Further

verifications of beam homogeneity were performed by irradiation of CR-39 track etch plastic (TASL Ltd, Bristol, UK). Figure 4a shows alpha particle hits on CR-39 where four irradiated 2.25 mm^2 areas are side by side. Each area shows a spread of particles with minimal overlap, which together irradiate an area of 20.25 mm^2 . An un-irradiated triangular area is visible in Fig. 4a where particles have been steered away from the top-right corner of the nozzle, to provide a marker of where each irradiated region starts and ends. Figure 4b shows that the homogeneity across the whole 20.25 mm^2 is consistent with Poisson counting statistics. Here, the count rate is about $6.5 \times 10^5 \text{ particles/cm}^2/\text{s}$.

The flux of the beam was measured using a silicon *p-i-n* (PIN) diode (model:S1223, Hamamatsu, Japan). The signal from the PIN diode was amplified and measured using a DAQ1000e multichannel analyser (Oxford Microbeams Ltd, Oxford, UK), giving a count rate. Since the pulse amplification electronics for the PIN diode signal are not accurate above 10,000 Hz, it is necessary to sample only a portion of the beam transmitted through the nozzle. To allow sampling of a portion of the beam, the PIN diode was capped with a $200 \mu\text{m}$ diameter aperture. A rate of 400 Hz transmitted through a $200 \mu\text{m}$ diameter aperture, corresponds to a flux of about $1.3 \times 10^4 \text{ particles/mm}^2/\text{s}$ over the 2.25 mm^2 nozzle. The beam stability was determined by measuring counts over time as can be seen in Fig. 4c. Here, the mean count rate is 402 Hz with a standard deviation of 15 Hz giving an uncertainty of about 5%, in agreement with Poisson counting statistics. This flux uncertainty is taken into account in the Monte Carlo simulation.

Monte Carlo Simulation

A Monte Carlo simulation was performed to calculate the uncertainty on the dose that cells received. The parameters included were a normally distributed variation of the volume-averaged LET corresponding to the thickness variation of the cells; a Poisson probability of ions hitting a cell; and a normally distributed flux uncertainty. The simulation was performed using the random number generators in OriginLab (Northampton, USA), and simulated over 15,000 virtual cells.

Tissue culture

V79-379A Chinese hamster cells (obtained from Mick Woodcock, Gray Institute for Radiation Oncology and Biology, UK) were grown in Eagle's essential media (EMEM) (Lonza, Wokingham, UK) with 10% fetal bovine serum (FBS), 2mM glutamine and 100 IU/ml penicillin & 1 µg/ml streptomycin incubated at 37°C with 5% CO₂. For clonogenic assays, appropriate densities of cells were prepared for the dose of the droplet. After allowing 5-7 days for colonies to develop, the colonies were stained with 10% crystal violet and counted.

Results

Table 2 shows the Monte Carlo results comparing the relative contribution of dose uncertainties. There are two types of uncertainty with three contributions: the “intrinsic uncertainties” (the Poisson distribution of hits to nuclei, and the LET variation based on cell thicknesses) are calculated, and the “physical uncertainty” (the flux variation of delivered particles) is measured (see Fig. 4b). The Poisson distribution of hits on nuclei is calculated from the dose per particle, and is shown in the second two columns, using a measured average cell nucleus area of 78 µm² (± 10 %). The uncertainty from the volume-averaged LET is calculated from the variation in cell thickness (17.2-17.9 or 120-126.5 keV µm⁻¹ for protons and alpha particles, respectively). Uncertainties are given at 1 standard deviation from the mean. All three parameters are part of a Monte Carlo simulation of 15,000 virtual cells.

Here, the percentage uncertainty in the Poisson statistics of hits on nuclei is the most significant, especially for the alpha particles. One would expect this as each single alpha particle imparts significant dose. The contribution of the uncertainty of the flux is much more important for the protons, as the contribution made from Poisson statistics is less. Least important in both cases is the uncertainty of the volume average LET. The overall dose uncertainty, is the sum of these three variables, and is plotted as a standard deviation from the mean (shown on the X-axis error bars in Fig. 5).

Figure 5 shows V79 Chinese hamster cells irradiated with 2 MeV protons and 4 MeV alpha

particles, and assayed through clonogenic growth survival. A reference curve for the same cell line irradiated with 300 kVp X-rays (Pantak, Royal Surrey County Hospital, U.K.) is also included. The results were first normalised to the control (with a plating efficiency of $80 \pm 10\%$), and then fitted with a linear-quadratic (LQ) model such that the survival fraction (SF) can be described as a function of the dose (D):

$$SF = \exp(-\alpha D - \beta D^2)$$

The alpha and beta values derived from the LQ fit are summarised in Table 3. The RBE is calculated as a ratio of the doses at a survival fraction of 10 % compared with X-rays. To calculate the uncertainty on the RBE value, the data is fitted using the upper and lower limit of the standard deviation on the dose (calculated by Monte Carlo), as well from the uncertainty of the LQ fit (see Table 3). Using these values, the RBE has about a 25 % uncertainty for the alpha particles and about a 15% uncertainty for the protons.

Discussion

Table 3 summarises the results and compares them to those obtained by Folkard *et al.* (1989). Their experiments are similar to the present ones but differ in some aspects. They used a 4 MV Van der Graaff accelerator to irradiate cells in a horizontal orientation which were temporarily fixed in a moist filter membrane, and they irradiated the cells on a rotating gantry, varying the speed of the rotation to control the dose given to the cells. They also used layers of polypropylene to vary the energy of the beam, whereas we changed the terminal voltage. It should be noted that the He energy was limited to 4 MeV here specifically to match their experiment.

Comparing the two sets of data in Table 3, the 10% RBE of X-rays, protons and alpha particles are very similar, and well within the range of the uncertainty on such RBE measurements. Folkard and coworkers measured an RBE at 10 % survival of 1.61 and 1.91 with volume-average LET of $17 \text{ keV } \mu\text{m}^{-1}$ and $24 \text{ keV } \mu\text{m}^{-1}$ protons respectively. The uncertainty on the RBE was given as a “worst case” fit according to their dose uncertainty of about 18% in total.

Folkard's RBE uncertainty is about 10% and 20% for protons and alpha particles respectively. These values are comparable to the present data (15% and 25% respectively). Both the experiment described in the present paper and that of Folkard and coworkers investigate particles which impart significant dose, and are therefore dominated by unavoidable Poisson uncertainty. Indeed, one of the driving motivations to develop systems which could deliver single particles to single nuclei, was to overcome Poisson uncertainties in the very low dose regions (Miller *et al.* 1999). Similarly using a microbeam Schettino *et al.* (2001), found low dose hypersensitivity with 3.2 MeV protons below 1 Gray, a result which cannot be observed with survival curves obtained with equivalent broadbeams. However, it was clear that a comparison above 1 Gy gave very similar results for both 1 and 3.2 MeV protons. With a broadbeam, many thousands of cells can be irradiated so that the uncertainty in the dose is well averaged giving results which are comparable to more accurate microbeam methods. In this way, despite the intrinsic uncertainties, broadbeam irradiation with low energies is a valid method of irradiating cells, particularly at higher doses.

More generally, the uncertainty of RBE measurements has been the focus of a recent paper by Friedrich *et al.* (2012) who created a database containing more than 800 survival curves from laboratories all over the world. They found that there was a large variation (frequently 20% or more) in reported RBE values between laboratories with the same cell line and a similar LET. For example, Belli *et al.* (1989) and Folkard *et al.* (1989) measured an RBE value at $24 \text{ keV } \mu\text{m}^{-1}$ for protons at 1.9 and 2.4, respectively. Interestingly, at lower energies where the range of LET is much larger, the difference in measurement was also much larger. At $32 \text{ keV } \mu\text{m}^{-1}$, Folkard measured an RBE of about 3.3, while for Belli it was about 2. Folkard *et al.* (1996) later revised the RBE to about 2 in accordance with Belli's data, proposing that a better experimental set-up excluded very low energy protons which were probably present in the original measurements. Thus, a large spread of LET can complicate the RBE measurement, as the uncertainties in the experiment are larger.

In contrast to these low-energy experiments, the RBE value associated with higher energy beams has significantly less uncertainty, as the dose is inherently more accurate. This is because each high energy particle carries relatively little dose meaning that Poisson uncertainties are

insignificant and there is very little uncertainty in the LET. This is important in medical treatment planning, where the RBE values have to be known with at least 10% accuracy. Generally, a standard RBE is applied to the treatment plan, as most of the beam energy deposited in the tumour has the same RBE value. For example, protons above 3 MeV (below about 10 keV μm^{-1}) have an RBE of about 1.1 and only below this energy does the RBE change significantly. However, as this region is only a small fraction of the total energy deposited it makes little practical difference clinically. That said, there has been a concerted effort to model the effect of using a variable RBE in treatment plans. For example, Frese *et al.* found through simulation that caution had to be used for radioresistant normal tissue as it was very sensitive to LET distributions.

Conclusion

In this paper, a novel method of irradiating cells *in vitro* with charged particles on a vertical microbeam line has been explained, demonstrated and validated. The purpose was to increase the versatility of the microbeam line. The limitations of this system include a 2.25 mm² exit nozzle, so that large regions can only be irradiated when this area is stitched together to form larger irradiated regions. Moreover, the relatively low energy of the accelerator means that most ion species have a large spread of LET through the nucleus of the cell requiring a volume-average to be LET assigned to any particular survival curve; care must therefore be taken when interpreting results in relation to RBE calculations. Survival curves were obtained of V79 cells irradiated in an about 4 mm² droplet of media; these correspond well to the literature and indicate RBE values which are well within the range of the uncertainties on such type of measurements. The dosimetry measurements and their associated uncertainties show that for 4 MeV alpha particles and 2 MeV protons, Poisson uncertainty is the most significant factor in the overall dose uncertainty. In contrast, the contribution due to flux and volume average LET uncertainty is less significant. This broadbeam irradiation setup has already proved itself to be a convenient method of assessing the effect of high-LET radiation and, overall, has allowed pertinent biological questions to be addressed.

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Figure captions

Fig 1 Schematic of the droplet irradiation set-up. The stainless steel dish holds a 4 μm sheet of polypropylene, onto which seven droplets containing about 15,000 cells are pipetted. Six different doses can be given (i.e d1-d6), including a unirradiated control (C). The beam exit nozzle is 1.5mm². A larger area (cm²) is irradiated by moving the dish on an XY stage over the nozzle.

Fig 2 Histogram of the thicknesses of randomly selected V79 cells lying on polypropylene. Measurement was performed by confocal microscopy under the very similar conditions as carried out during irradiation (although the cells were fixed in paraformaldehyde and treated with RNase; for details see materials and methods section).

Fig 3 LET (keV μm^{-1}) of protons (o) and alpha particles (■) as a function of distance in water (μm) calculated in SRIM (Ziegler, 2004), with an incident beam of 1.93 and 3.53 MeV for protons and alpha particles respectively, which takes into account the energy lost through the 0.1 nm silicon nitride window and 4 μm polypropylene film. Ten and six μm was assumed here to be the approximate thickness of the nucleus of a cell, for cells irradiated with protons and alphas respectively. Table 1 shows the integrated LET value and the volume average LET (keV μm^{-1}) through the nucleus.

Fig 4 (a) CR-39 irradiated with 4 MeV alpha particles. The image shows the middle of four irradiated regions. The triangular section in the middle of the image was created by steering the beam from the corner of the nozzle, to give an indication of how the irradiated regions line up in respect to each other. The scale bar corresponds to 200 μm ; (b) Number of particles on CR-39 plastic sampled in 100 μm^2 in nine fields of irradiation. The error bars show the standard deviation in each of the nine fields. The average over the entire irradiated region is 66 ± 6 particles, consistent with counting statistics; (c) Beam stability over time sampled every minute on a PIN diode. The mean count rate was 402 Hz with a standard deviation of 12 Hz, consistent with Poisson counting statistics.

Fig 5 Broadbeam results from irradiating V79 cells with alpha particles and protons. (■) 300 kVp X-rays, (□) 2 MeV protons, (Δ) 4 MeV alpha particles. The data were fitted with the linear-quadratic model (solid lines). Standard errors on the survival fraction values are shown. The dashed lines are the LQ fitted data from Folkard *et al.* (1989) included for comparison. The incident beam energy, LET and RBE are summarised in Table 1. The error bars in the X-axis are the standard deviation from the mean dose (see Table 2).

Table 1 Low-energy particle beam ranges calculated with SRIM (Ziegler 2004). The volume-averaged LET is calculated for rounded, unattached V79 cells for protons (average thickness of 10 μm) or for attached, flat V79 cells for alpha particles (average thickness of 6 μm). The LET is shown as it enters the nucleus at the entrance (0 μm), the middle of the cell (5 μm or 3 μm , for protons or alphas respectively) and as it leaves the nucleus (at 10 μm or 6 μm) (see also Fig. 3). Calculations were for an incident energy of 1.93 MeV and 3.53 MeV for protons and alpha particles respectively, which takes into account energy lost through the 0.1 μm silicon nitride window, the 4 μm polypropylene film, and the air gap (using SRIM energy losses). The energy spread through the nucleus is integrated to give a total LET over the nucleus, and divided by 10 or 6 for the average LET ($\text{keV } \mu\text{m}^{-1}$) in the nucleus.

Particle	Incident Energy MeV	Max. range in water microns	LET at cell entrance $\text{keV}/\mu\text{m}$	LET at cell middle $\text{keV}/\mu\text{m}$	LET at cell exit $\text{keV}/\mu\text{m}$	Volume averaged LET $\text{keV}/\mu\text{m}$
H^+	2	77.7	16.4	17.7	18.4	17.6
He^+	4	37.3	116	122.6	132.9	123

Table 2 Contributions to the dose uncertainty.

	Average number of particles per cell		Standard deviation of particles assuming Poisson distribution		Dose uncertainty contribution due to Poisson statistics		Dose uncertainty contribution due to volume average LET variation		Dose Uncertainty due to Flux variation (5%)	
unit	Particles		Particles		Gy		Gy		Gy	
Average dose (Gy)	Protons	Alphas	Protons	Alphas	Protons	Alphas	Protons	Alphas	Protons	Alphas
0.5		2		1.41		0.35		0.004		0.03
1	28	4	5.52	1.85	0.20	0.46	0.003	0.008	0.05	0.05
2	56	8	7.43	2.87	0.27	0.72	0.006	0.016	0.10	0.10
3	84	12	8.98	3.45	0.32	0.87	0.008	0.024	0.15	0.15
4	112	16	10.26	3.99	0.37	1.00	0.011	0.032	0.20	0.20
5	138	20	11.52	4.47	0.41	1.12	0.014	0.040	0.25	0.25
6	166		12.73		0.46		0.017		0.30	

Table 3 Comparison of Linear Quadratic fits and RBE values calculated at 10% surviving fraction (SF).

Radiation	Incident Energy (MeV)	volume-averaged LET (keV/ μ m)	α (Gy ⁻¹)	β (Gy ⁻²)	RBE (10% SF)
X-rays (Folkard <i>et al.</i> 1989)	250kVp	~ 2	0.11 ± 0.027	0.027 ± 0.003	1
X-rays (present work)	300kVp	~ 2	0.12 ± 0.027	0.023 ± 0.005	1
Proton (Folkard <i>et al.</i> 1989)	1.90	17.0 ± 5	0.13 ± 0.038	0.078 ± 0.007	1.6 ± 0.2
Proton (present work)	2.00	17.6 ± 1.2	0.12 ± 0.062	0.067 ± 0.006	1.5 ± 0.2
He ⁺² (Folkard <i>et al.</i> 1989)	5.09	105 ± 10	1.31 ± 0.023	-	4.2 ± 0.75
He ⁺² (present work)	4.00	123 ± 9.6	1.5 ± 0.151	-	3.9 ± 1

Figure 1

Figure 2

Figure 3

Fig. 4a

Fig. 4b

Fig. 4c

Figure 5