

ORIGINAL ARTICLE



Grid therapy using high definition multileaf collimators: realizing benefits of the bystander effect

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ABSTRACT

Background: In microbeam radiotherapy (MRT), parallel arrays of high-intensity synchrotron x-ray beams achieve normal tissue sparing without compromising tumor control. Grid-therapy using clinical linacs has spatial modulation on a larger scale and achieves promising results for palliative treatments of bulky tumors. The availability of high definition multileaf collimators (HDMLCs) with 2.5 mm leaves provides an opportunity for grid-therapy to more closely approach MRT. However, challenges to the wider implementation of grid-therapy remain because spatial modulation of the target volume runs counter to current radiotherapy practice and mechanisms for the beneficial effects of MRT are not fully understood. Without more knowledge of cell dose responses, a quantitative basis for planning treatments is difficult. The aim of this study is to determine if therapeutic benefits of MRT can be achieved using a linac with HDMLCs and if so, to develop a predictive model to support treatment planning.

Material and methods: HD120-MLCs of a Varian Novalis TXTM were used to generate grid patterns of 2.5 and 5.0 mm spacing, which were characterized dosimetrically using GafchromicTM EBT3 film. Clonogenic survival of normal (HUVCE) and cancer (NCI-H460, HCC-1954) cell lines following irradiation under the grid and open fields using a 6 MV photon beam were compared *in-vitro* for the same average dose.

Results and Conclusions: Relative to an open field, survival of normal cells in a 2.5 mm striped field was the same, while the survival of both cancer cell lines was significantly lower. A mathematical model was developed to incorporate dose gradients of the spatial modulation into the standard linear quadratic model. Our new bystander extended LQ model assumes spatial gradients drive the diffusion of soluble factors that influence survival through bystander effects, successfully predicting the experimental results that show an increased therapeutic ratio. Our results challenge conventional radiotherapy practice and propose that additional gain can be realized by prescribing spatially modulated treatments to harness the bystander effect.

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Background

The original motivation for using synchrotron generated kV x-ray beams in microbeam radiotherapy (MRT) was to treat pediatric brain tumors, where the high doses required for efficacy were limited by the radiosensitivity of the surrounding normal tissue. Animal studies have shown that irradiation using arrays of microbeams with spacings in the order of a 100 μm is effective in killing tumor cells and is well tolerated by normal tissue, despite the high-peak doses of hundreds of Gy [1]. The therapeutic benefit of MRT has been attributed to the narrowness and close spacing of the beamlets that allow rapid vasculature regeneration of damaged normal tissue [2,3]. MRT however, has not been widely translated into current clinical practice because of the poor availability of suitable medical synchrotron beamlines.

Spatial modulation of x-rays was first carried out in the clinic for the purpose of reducing skin reactions when delivering

high doses for the treatment of large bulky tumors. The method of irradiating through a collimating grid, commonly known as grid therapy, gives a non-uniform dose distribution with spatial periodicity in the range of 7.5–15 mm. Early studies of orthovoltage beams used with a grid collimator demonstrated a higher skin tolerance compared to an open field of the same dose [4]. Recent clinical studies using MV grid therapy have also yielded promising results in terms of beneficial palliative responses with reduced toxicity [5–7], supported by Monte Carlo and other treatment planning approaches [8,9], none of which incorporate bystander signaling. Despite these findings, periodic spatially modulated MV treatments are rarely prescribed for curative treatments in clinical practice for several reasons. Non-uniform treatments challenge traditional radiotherapy, which aims to deliver a uniform dose to the entire target volume, while avoiding the normal tissue. The lack of evidence for curative efficacy, combined with the lack

of any biological mechanism to support treatment planning, has prevented the widespread acceptance of intentionally modulated treatments. Existing studies of spatially modulated treatments have used dosimetry and patient response alone as endpoints, with little consideration of the underlying biological mechanisms. The ultimate potential benefits of periodic spatial modulation of treatment fields remain elusive to the patient. An understanding of the biological mechanisms would inform the design of spatial modulation in one and two dimensions to maximize the therapeutic ratio.

Spatial modulation of MV linac beams has usually been achieved using an attenuating grid made from a metal alloy, such as Cerrobend. Multileaf collimators provide a more convenient means of beam modulation, without the need for construction and mounting of a single purpose collimator. The advent of high definition multileaf collimators (HDMLCs) for stereotactic radiosurgery has provided modulated fields with fine collimation. Beamlets with an effective collimation width only 10 times larger than the several hundred micrometers typically used in MRT are now readily available. It is known from the literature that in synchrotron MRT, beam size affects response [10]. As the collimation width of a beamlet is increased to millimeter dimensions, it is not known at which scale size the observed benefits of MRT will disappear. For example, it is not known whether any benefits exist in a 2.5 mm field and if they do, whether they diminish in the commonly clinically available 5.0 mm field. Suggested biological mechanisms of spatially modulated fields include the volume effect [5], bystander effects [11] and differential normal to cancer cell responses [2], all of which could contribute to therapeutic effectiveness.

The biological mechanisms of spatially modulated treatments have been studied for MRT, but not for the larger beams of linac grid therapy. The data from palliative treatments of clinical grid therapy has not been sufficient to give the necessary support for widespread implementation in curative radiotherapy, nor has it been sufficient for the development of a mathematical predictive model. In this study, we investigate the *in-vitro* response of selected cancer and normal cells to a range of spatially modulated fields created using HDMLCs. An *in-vitro* study is performed for this work to include the effects related to cell-to-cell signaling, with the simplification to exclude effects on the vasculature. We will develop a mathematical model using experimental data with the aim of predicting survival in periodically modulated fields. The model is intended to support the planning of such treatments to realize the benefits of MRT in the clinic using readily available facilities.

Material and methods

Standard linear quadratic model

The linear quadratic model is widely used to predict the clonogenic survival S after a radiation dose D . Radiation creates a density of 'lesions' that cause cell death. The lesion density $N(D)$ is a function of dose with linear and quadratic terms:

$$N(D) = \alpha D + \beta D^2 \quad (1)$$

where α and β are cell specific parameters, determined from dose response experiments and G is the dose protraction factor that describes the effect of lesion repair during prolonged exposures. In this study, G is assumed to be 1 for the delivery of dose in a single fraction at a high-dose rate. The clonogenic survival S of cells that have a lesion density $N(D)$ is given by:

$$S = e^{-N(D)} \quad (2)$$

Local survival

In a spatially modulated field, the survival fraction at a position in the field is defined here as the local survival $S(\mathbf{r})$. The use of the LQ model to predict survival under a spatially modulated field has been described by Bromley et al. [12] assuming a local survival solely dependent on the local dose delivered. In this approach, the modulated dose profile was described by a series of elements, such that the dose within each element is constant to within 5%

$$S(\mathbf{r}) = e^{-\alpha D(\mathbf{r}) - \beta D(\mathbf{r})^2} \quad (3)$$

where $S(\mathbf{r})$ is the local survival and $D(\mathbf{r})$ is the local dose with the position co-ordinate $\mathbf{r}$. The LQ model was then applied to each dose element and the local survival in each element was summed to predict the overall survival. The prediction of average survival based on the mean dose was found to be inadequate using the LQ model. However, even when average survival was calculated from the LQ model using the local dose, the prediction was still inaccurate and it was concluded that there appeared to be 'some form of communication between neighbouring cells.' This communication has been well documented in the literature and is often referred to as the 'radiation-induced bystander effect'.

Bystander effect

The bystander effect was first observed by in 1992 by Nagasawa and Little, when a low dose of alpha particles was delivered to a cell culture [13]. The biological damage, as measured by sister chromatid exchanges, in cells that were not directly irradiated by the alpha particles, was greater than expected. Subsequent studies of communicated responses have identified several mechanisms of intercellular communication, such as those mediated by gap-junctions [14] and those mediated by medium that has been conditioned by the exposed cells [15]. Gap-junction mediated bystander signaling requires cells to be in contact so that irradiated cells are able to transmit signals to unirradiated cells. Medium mediated signaling involves the release of soluble factors that diffuse through the medium, inducing biological effects in unirradiated cells. Studies of bystander effects have reported both protective and damaging bystander responses, which encourage cell proliferation and cell death respectively [16,17], depending on the dose, dose rate, cell type [16,18–20] and spatial and temporal modulation [21]. The extent of bystander effects on survival is known to be cell specific and proportional to the dose received, saturating at higher doses where direct effects of radiation dominate [18,19]. Protective bystander effects have been attributed to the production of

growth factors and the inhibition of toxic factors while damaging bystander effects have been attributed to the release of factors that are either directly or indirectly cytotoxic through the generation of reactive oxygen species [11,22].

The influence of bystander effects in spatially modulated fields has been well established. The same effects are likely to operate in uniform fields where they are likely to be responsible for a substantial part of cell death [23] and have been identified as a potential mechanism for the different radiosensitivity of various cell types. In low-dose regions, survival is more dependent on intercellular signaling rather than direct radiation damage, which dominates in high-dose regions [18,24]. The dose gradients generated from spatial modulation result in a different survival pattern compared to a uniform field of the same dose. Diffusion of bystander factors driven by dose gradients redistributes the bystander factors (Figure 1). Since the cell specific parameters of the standard LQ model are evaluated in uniform fields, therefore, it is not surprising that predictions of the model are inadequate in modulated fields. Experimental studies show survival in modulated fields differs significantly from open field LQ predictions [12,16,25,26]. Methods of incorporating bystander effects into the prediction of survival have been explored in various studies. Using the standard LQ model to predict survival, two sets of α , β parameters were determined to separate the bystander effects from the direct effects of radiation [23]. Similarly, direct radiation and indirect bystander induced effects were incorporated into Monte Carlo modeling of cellular damage by McMahon et al. [24]. The computational model incorporates the release of bystander signals from irradiated cells, which rapidly diffuse throughout the medium until an equilibrium concentration is reached. The computational model demonstrated a more accurate survival prediction in exposed and blocked regions of a cell culture.

Extended LQ model with bystander signaling

We now make the following assumptions concerning bystander effects, to allow us to develop an extended LQ model that includes them.

1. Bystander signaling affects survival outcomes in modulated fields.
2. The generation of the bystander signals is proportional to the local dose.
3. The spreading of bystander signals is proportional to the average dose gradient.

Our extended LQ model for the local survival at position $\mathbf{r}$ with bystander signaling follows from assumptions 1–3:

$$S(\mathbf{r}) = \exp - [(\alpha + A\bar{D})D(\mathbf{r}) + \beta D^2(\mathbf{r})] \quad (4)$$

where A is the bystander parameter, $D(\mathbf{r})$ is the local dose at the position with co-ordinate $\mathbf{r}$ and $\bar{D} = \langle |\frac{\partial D}{\partial \mathbf{r}}| \rangle$ is the average of the magnitude of the dose gradient. The bystander parameter is cell specific and depends on the ability of exposed cells to emit bystander-signaling factors, the ability of the factors to diffuse and the ability of cells receiving the factors to respond. The local dose is responsible for generating the bystander signals that are distributed to an extent determined by the average dose gradient. The additional bystander term is therefore equal to the product of the bystander parameter A , the average dose gradient $\bar{D}$ and the local dose $D(\mathbf{r})$.

Considering a field where dose depends on a single spatial variable x , overall survival is then the average of the local survival:

$$\bar{S}(x) = \frac{1}{x_2 - x_1} \int_{x_1}^{x_2} e^{-\alpha D(x)} e^{-(A\bar{D})D(x)} e^{-\beta D(x)^2} dx \quad (5)$$

where x_1 and x_2 specify the field limits.

Creation of grid fields

A Varian Novalis TxTM linear accelerator (Varian Medical Systems, Palo Alto, CA, USA) was used to deliver the radiation fields through its integrated HD120 MLC, using a 6 MV photon beam, at a dose rate of 600 MU/min. The open field size as defined by the secondary collimators was set to 20×17 cm. The HDMLC fields were generated using only the inner 32 leaf pairs, which have a projected leaf width of

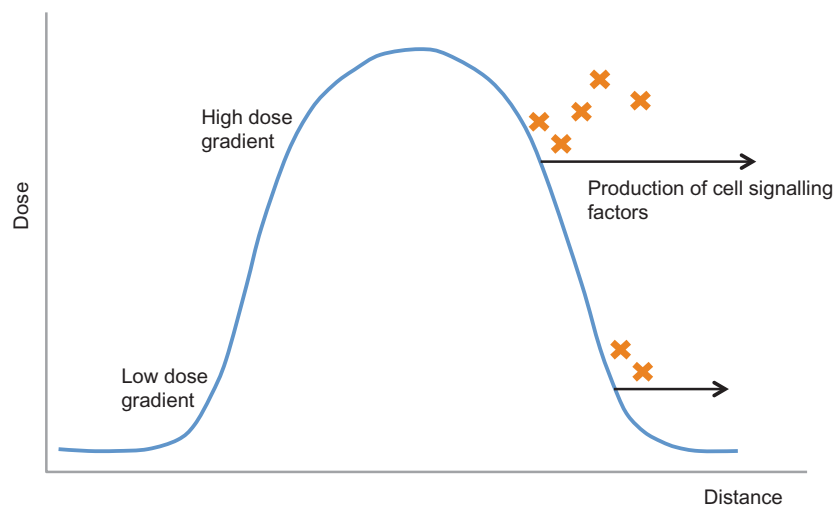


Figure 1. Illustration of the production and diffusion of soluble cell signalling factors.

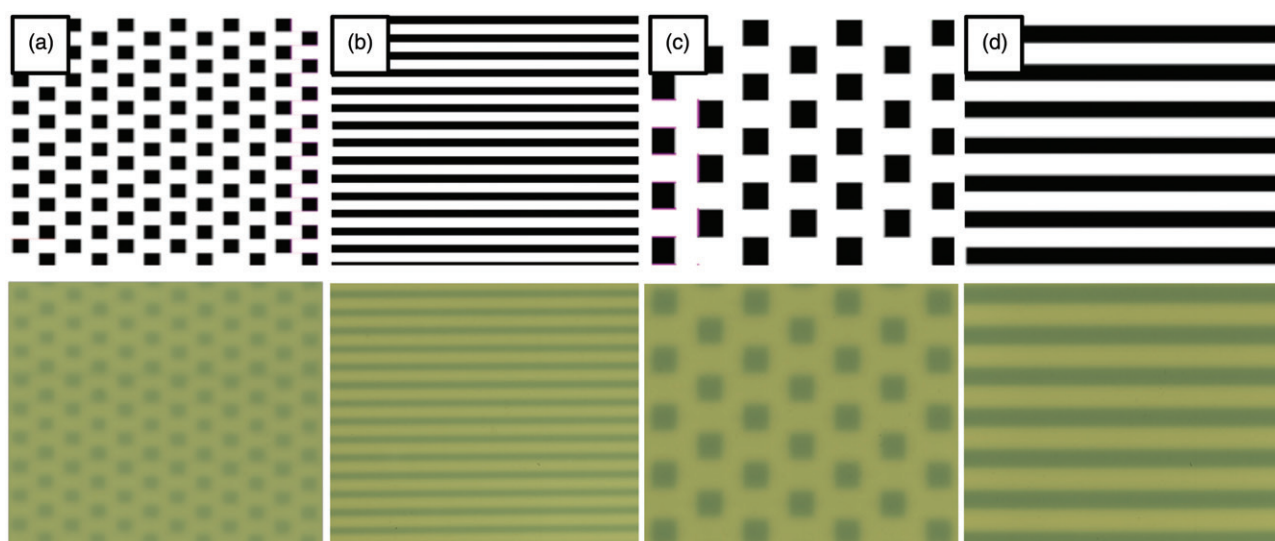


Figure 2. Top: schematic of HD-MLC fields generated by Shaper Software (Varian Medical Systems, Inc., Palo Alto, CA, USA). Bottom: Gafchromic EBT3 film exposed to the HDMLC modulated fields. Black and darkened areas represent exposed regions of the field, where dose is delivered.

2.5 mm at the isocenter, while the outer leaves were kept, closed and offset to minimize interleaf end leakage. Using only the inner leaves, the fields were limited to a maximum of 8 cm in one dimension, sufficient to cover the dimensions of the cell culture flasks to be irradiated.

Four HDMLC defined grid fields (2.5 and 5.0 mm, dots and stripes) were created as text files and then saved using the ShaperTM software (Varian Medical System, Palo Alto, CA, USA). Grid patterns of striped fields of open–close exposures were delivered in a single static field, while alternating holes were delivered through a multisegment step-and-shoot method. The dot and stripe patterns are similar to the collimation used in grid therapy and MRT, respectively.

Dosimetry

To verify the dosimetric accuracy and the placement of the cells relative to the fields, GafChromicTM EBT3 (lot No. 04211502) film was positioned under the cell flask in the same experimental set-up and irradiated under each modulated field (Figure 2). The films were processed according to the procedures specified for radiochromic film dosimetry in the AAPM Task Group 55 report.

Dose calibration of the films was carried out immediately after the grid field exposures. Squares of the same batch of EBT3 film were cut and marked on the upper right corner to indicate their orientation for scanning. They were then exposed to a range of absorbed doses from 0 to 330 cGy where 1 Gy = 100 MU under calibration conditions. A standard calibration configuration was used: 100 cm SSD, 10 × 10 cm field size, 1.5 cm depth. The calibration films were placed on 15 cm of solid water and positioned perpendicular to the central axis of the beam. An additional 1.5 cm of solid water was placed above the films before irradiation with the gantry at 0°.

Three films were used for each exposure to minimize errors. All films were read out 72 h after irradiation, using an Epson 10000XL Flatbed scanner (Epson America, Inc., Long Beach, CA, USA) to digitize the films. The films were scanned

though the Epson scan driver in transmission mode, without any color correction setting to obtain RGB positive images at 16 bits per color channel and a spatial resolution of 300 dots per inch (dpi). A cardboard template was used to ensure that all films were scanned in the same orientation, at a reproducible central position on the scanner, as EBT3 film is known to be sensitive to its scanning orientation and position on the scanner. The calibration films were positioned along the central axis of the scanner in the same orientation, as previously marked and scanned all together. The scanned images were then saved as (TIFF) files and imported to ImageJTM version 1.50e (National Institutes of Health, Bethesda, MD, USA), using only the red channel data for analysis as EBT film has maximum absorption in the red region of the visible spectrum.

An area was selected from the center of each calibration film to measure the mean pixel value, given as a 16-bit number (0–65,535) to establish its relationship with the known absorbed dose. Using ImageJ, an appropriate calibration function was chosen to convert pixel value to dose for all films.

Cell culture and survival assays

In this work, the clonogenic survival is the chosen end point to evaluate the effects of radiation. Clonogenic survival is defined as the ability of a single cell to maintain its reproductive viability to form colonies of greater than 50 cells. The normal human umbilical vein endothelial cell line (HUVEC) (unmodified) and two human cancer cell lines: non-small cell lung (NCI-H460) and a HER2+ breast (HCC-1954), from the American Type Culture Collection (ATCC) breast were used. The cancer cell lines were maintained in RPMI-1640 medium, supplemented with 10% v/v fetal bovine serum (FBS) (Gibco Life Technologies, Australia) as recommended by ATCC. The normal (HUVEC) cells were kept in Medium M199 and supplemented with 20% v/v FBS, 13.4 mM sodium bicarbonate, 20 mM HEPES, 1 × non-essential amino acid, 1 mM sodium pyruvate, 1 × Glutamax (all from Gibco Life Technologies, Australia), 3 µL mL^{−1} heparin (Sigma-Aldrich, St Louis, MO,

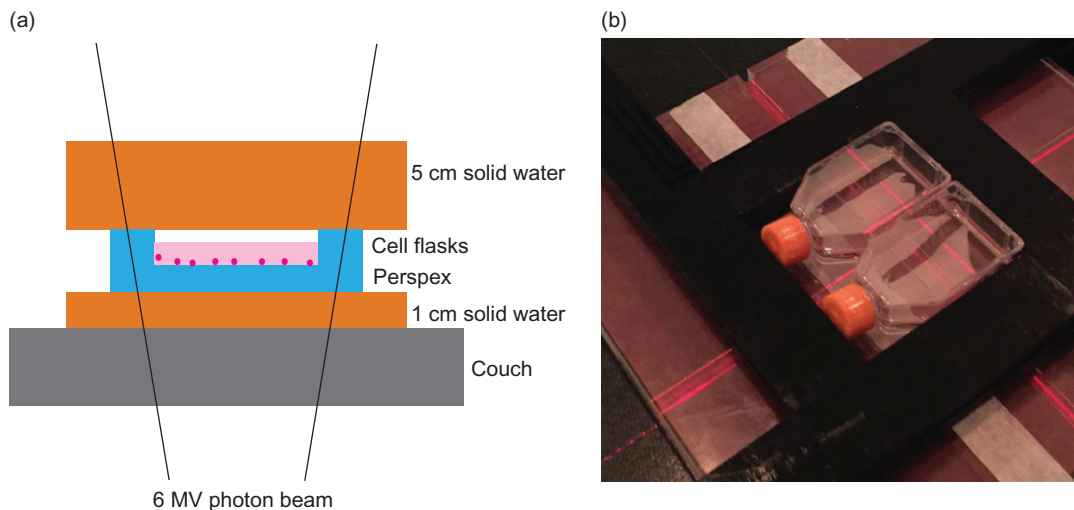


Figure 3. Schematic of cell irradiation set-up (a) and the alignment of the cell flasks (b).

USA), 0.048 gL⁻¹ endothelial cell growth factor (ECGS) (In Vitro Technologies, Australia). All flasks containing HUVEC were pre-coated with 0.1% gelatin overnight at 4 °C. All cell cultures were maintained in a humidified incubator containing 5% CO₂ at 37 °C.

The cells were left for exponential growth as monolayers until 80% confluent before discarding the growth medium and washing the adherent cells with phosphate buffered saline (Gibco Life Technologies, Australia) twice. Using 0.05% trypsin-EDTA solution, the cells were detached for 4–6 min at 37 °C and then centrifuged at 252 × g for 5 min at room temperature.

The cells were plated in T25 cm² flasks (Corning, MA) in 5 mL of their respective growth media at densities of 600, 3000 and 1500 cells for the NCI-H460, HCC-1954 and HUVEC cell lines, respectively. The plating efficiencies observed in our cell lines are 54% for NCI-H460, 11% for HCC1954 and 14% for HUVEC, which are typical for these cell lines. Flasks containing normal cells were pre-coated with gelatin. All flasks were incubated overnight for 24 h, allowing cells to adhere. Prior to irradiation, flasks were topped up to 10 mL of medium to create constant electronic scatter conditions.

Following irradiation, the cells were incubated for 6, 10 and 10 d for the NCI-H460, HCC-1954 and HUVEC cell lines, respectively, to allow colonies greater than 50 cells to form. The doubling times are 1.24 d for the NCI-H460 and 1.95 d for the HCC1954 and HUVEC cell lines. Unmodified HUVEC cells were used at passage 6 or less. Colonies of the cancer cell lines were fixed and stained using 0.3% methylene blue in 50% ethanol (Sigma-Aldrich, St Louis, MO, USA). HUVEC cells were fixed and stained using 4% crystal violet, which provides a high absorbency per cell thus increasing the contrast for the disperse colonies, characteristic of this cell line.

Using the ColCountTM colony counter (Oxford Optronix Ltd., Abingdon, UK) with predefined parameters for the individual cell lines, each flask was scanned and colonies of more than 50 cells were counted at a resolution of 205 dpi. The counts for irradiated flasks were normalized to the unirradiated controls to determine the survival fraction.

A higher plating density of 6000 cells per flask was used for the NCI-H460 cell line to give a clearer representation of

the spatial distribution of survival in response to the spatially modulated (2.5 and 5.0 mm) striped fields. Treatment and irradiation of the higher plating density assay were the same as for the NCI-H460 clonogenic assay, for which a plating density of 600 cells per flask was used. Clonogenic survival was not calculated for the high-plating density assay. Its purpose was for the determination of the optical density and colony distribution.

Irradiation with MLC fields

To achieve full scatter conditions, the cell monolayers in the culture flasks were positioned on 1.2 cm of Perspex, and completely surrounded by additional Perspex plates. The experimental set up was sandwiched between slabs of solid water: 1 cm beneath and 5 cm above (Figure 3). The experimental set-up was placed on the treatment couch and irradiated with an isocentric set up to the cell monolayer, with the gantry at 180°, thereby avoiding any air above the medium filled cell culture flasks. The dimensions of the grid fields were sufficient to cover two T25 cm² flasks. All cells were kept in a thermally insulated container except when being irradiated and returned to the incubator after exposure. Sham exposed flasks were prepared for each experiment and used as controls for the determination of survival fraction.

Clonogenic cell survival was determined after exposure to uniform and HDMLC modulated fields. Dose response experiments were performed with a uniform field covering the full range of doses used in this work, to obtain the α , β values of each cell line by fitting survival data with the LQ model. For the HDMLC modulated fields, an average dose of 3.5 Gy was prescribed for the NCI-H460, 2 Gy for the HCC-1954 and 4 Gy for the HUVEC cell lines. Taking into consideration the different radiosensitivities of each cell line, these different dose prescriptions were selected such that the cell lines would result in a similar survival fraction after irradiation. For each cell line the flasks were grouped into exposure types: 2.5, 5.0 mm dots, 2.5, 5.0 mm stripes, open and unirradiated controls, with n for each group being between 8 and 14.

Statistical analysis

All experiments were repeated with data presented as the mean $\pm$ standard error in all cases. The one-tailed, equal variance Student's *t*-test was used to assess the significance of differences in survival fraction. The one-tailed *t*-test was used to test the hypothesis that 'the survival of cells in a modulated field is lower than in an open field of the same average dose.' A one-tailed *t*-test is used because the direction is specified. Statistically, significant differences were defined at a level of $p < .05$ (*).

Prediction of survival in spatially modulated fields using the LQ and extended LQ models

The predictions of average survival fractions using the LQ models (standard and extended to include bystander signaling) were made for striped fields, where dose varies along one axis. As described above, the dose profiles obtained from film dosimetry were binned into 256 dose bins. Average survival as predicted by both the standard LQ model using Equation (3) and the extended LQ model using Equation (4) was calculated by averaging the predicted local survival of all 256 dose bins.

Local survival

For the striped fields, the theoretical local survival (from film dosimetry) as predicted by the extended LQ model with bystander signaling and compared to the experimental local survival (from cell staining) to further validate the model. Flasks with a high-plating density (NCI-H460) were used to provide sufficient statistics in the determination of a local survival. Using the ColCount™ colony counter, both the processed image (outlines of colonies) and unprocessed image (raw image) were obtained and analyzed using ImageJ™ to give two representations of local survival. From the unprocessed images, optical density is a measure of the total amount of nucleic acid, which in turn is a measure of the density of living cells in the cell culture flasks, including cells that are capable of forming colonies and cells that are not. This provides a different measure of cell survival to the clonogenic assays, but shows a smaller statistical fluctuation in the spatial distribution. Colony counts from the processed images are representative of the local clonogenic survival. Using Excel, spatial data was binned at the same scanning resolution of 205 dpi to obtain a histogram of colony counts, in the vertical direction across the field. Potentially, there is a small under-reporting of colony density in areas of high colony density, as the higher plating density may give rise to joined colonies, which are identified as a single colony.

Results

Dose response

The values of α and β in Table 1 are determined in this study by fitting the LQ model to the survival data for an open field

Table 1. α , β parameters and R^2 value of the least square fit determined for each cell line from experimental survival curves.

Cell line	α (Gy^{-1})	β (Gy^{-2})	R^2
NCI-H460	0.043 ± 0.023	0.076 ± 0.007	0.99824
HCC-1954	0.32 ± 0.14	0.009 ± 0.009	0.86900
HUVEC	0.249 ± 0.043	0.002 ± 0.007	0.98872

in two ways. The first was to use a linear regression fit of the log of survival in Excel and the data is shown in Table 1. The second was using the software package, CFAssay for R [27], which had consistent α , β values and uncertainties with Excel.

Because of the small values of the β parameters for the HCC1954 and HUVEC cell lines, their uncertainties are comparable to the numerical value.

Dose distributions

The dose profiles obtained from film dosimetry are shown in Figure 4. The dot fields have different profiles in different orientations. Given the complexity of the dose gradients of the dot fields, only the striped fields were modeled using Equation 4. Profile 1 of the dot fields (Figure 4(a)) shows a large region of low-constant dose while the striped fields (Figure 4(b)) show a smaller region of low dose. The dose-area histograms (Figure 4(c)) reveal this difference. The striped fields show almost equal high- and low-dose regions while the dot fields show a small area of high dose and a relatively large area of constant low dose. These differences in the dose distribution lead to differences in the dose gradient distribution. Slow diffusing factors will show different behavior in the dot and striped fields as a result of the different dose gradient distributions. In the dot fields, the large region of constant low dose, associated with low-dose gradients would prevent slow diffusing factors from spreading across the field. In the striped fields, more of the field has high-dose gradients, enabling the spreading of slow diffusing factors. Fast diffusing factors would be able to spread in both dot and striped fields. Therefore, the two types of factors are likely to respond differently to a change in the distribution of the dose gradients across the field.

Survival after spatially modulated irradiation

The clonogenic survival fractions for the modulated fields are shown in Figure 6 and Table 4 for the cell lines NCI-H460, HCC-1954 and HUVEC, with the associated uncertainties. The *t*-test indicates if the responses to the modulated fields and to the open field are significantly different for the same average dose. For the cancer cell lines, a significant difference in the survival fraction is found following irradiation to 2.5 mm modulated fields of stripes and dots compared to an open field. However, the significance is lost for the larger spatial modulation of 5.0 mm. In contrast, the survival fraction of normal cell line (HUVEC) for the 2.5 mm striped field is the same as that of the open field, while the survival fraction for the dot field is significantly less.

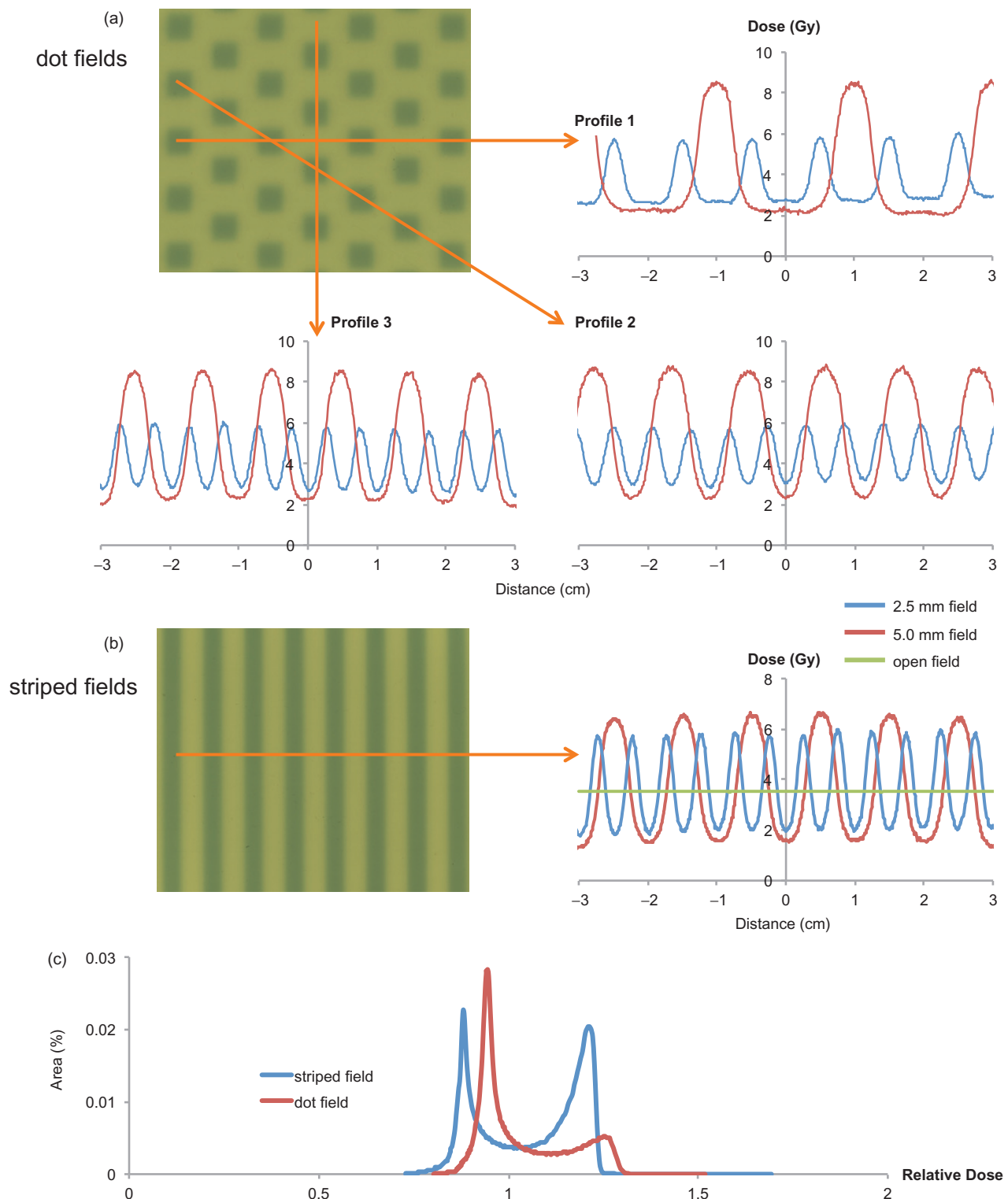


Figure 4. Analysis of dose profiles for an average dose of 3.5 Gy for dot fields (a) and striped fields (b) measured by Gafchromic film dosimetry. Blue curves represent 2.5 mm fields, red curves represent 5.0 mm fields and the green curve represents an open field (a, b). Measurement of the dot fields was carried out in three directions as specified in (a), with the corresponding dose profiles. Dose profiles of the striped fields and open field are shown in (b). Comparison of dose-area histograms (analogous to a DVH) for striped and dot fields (c). The blue curve represents striped fields while the red curve represents the dot fields (c).

Extended LQ model with bystander signaling

The parameters of the extended LQ model are summarized in Tables 2 and 3. The bystander parameter A was determined by minimizing the root mean square deviation (RMSD) when fitting the extended model to experimental data for

striped fields. The normal cell line had a value of A much smaller than for the cancer cell lines (Table 3). This suggests that either the effect of bystander signaling is less in the normal cell line compared to both of the cancer cell lines, or there are two compensating contributions of opposite sign, one a cytotoxic effect and the other a beneficial effect. Using

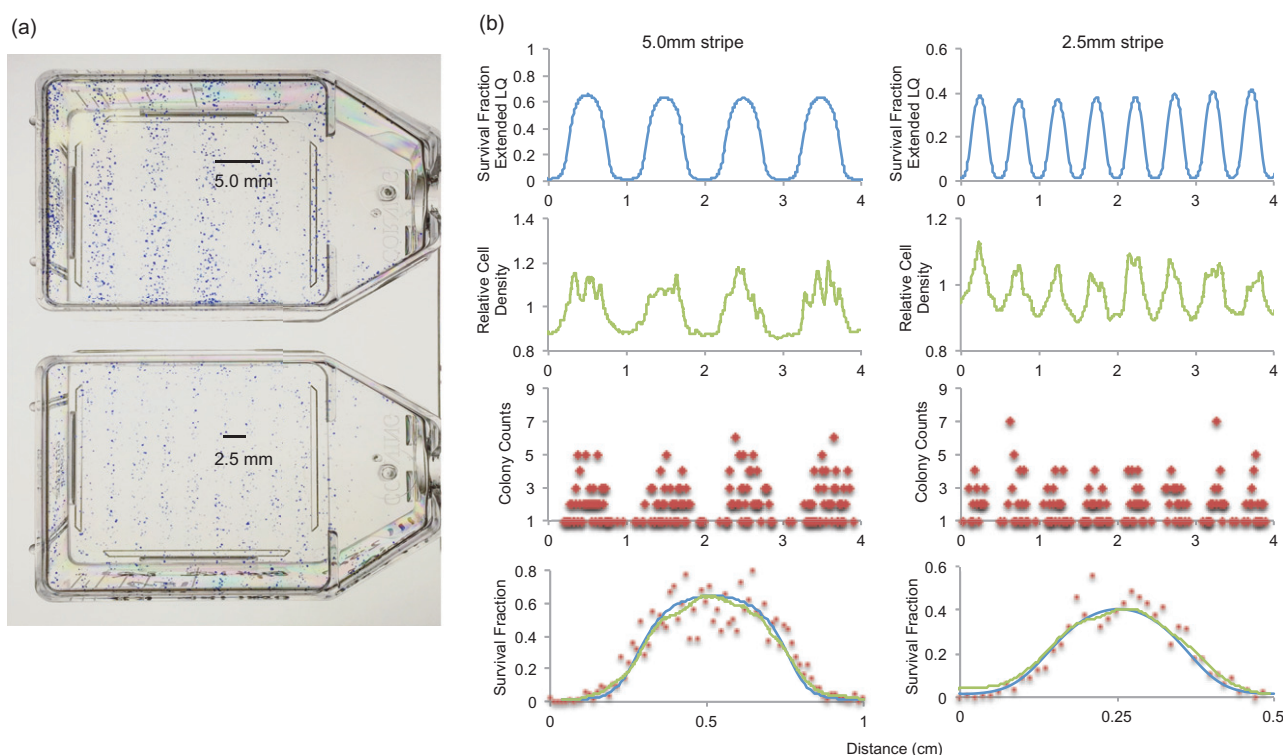


Figure 5. (a) Image of cell culture flasks for the higher plating density assay, showing the surviving colonies following irradiation to modulated fields of 5.0 mm periodicity (above) and 2.5 mm periodicity (below). (b) The local survival fraction as predicted by the extended model is shown in the blue curve (top); the relative cell density determined using the optical density across the flasks is shown in green (second from top); the total number of colonies in each stripe as a function of position is shown in red (third from top); a composite representation of all data ($n = 6$), averaged to give the result for a single period (bottom).

the t -test to test for an assumed population mean, survival predictions made by the extended LQ model (Figure 7) were not significantly different to experimental results for the cancer cells. For the normal cells, the RMSD for the standard and extended model were similar.

Comparison of local survival with the predictions of the extended LQ model

The experimental local survivals measured using colony counts and optical density values show agreement with each other as shown in Figure 5. The local survival predictions of the extended LQ model are in good agreement with the measurements and give a similar shape for the local survival curves.

Discussion

In the past, two different approaches have been taken in the study of periodic modulation of radiation beams for therapeutic applications. The first approach is grid therapy, where the period of the modulation is between 7.5 and 10 mm [6,7] and the main reported benefits arise from normal tissue sparing by the volume effect. The second approach is MRT using a synchrotron, where the period of modulation is smaller than 400 μm and the beam width is typically 25–50 μm [2,3]. The bystander effect has been suggested as an important mechanism for the cellular response in the highly modulated fields in MRT [20]. With the introduction of more sophisticated MLCs in clinical linacs, a period of modulation

smaller than 7.5 mm has become available [28], enabling us to explore opportunities for modulation with periods between the two approaches traditionally taken and classified above. Our results, using modulation periods of 5.0 and 2.5 mm, show that the biological response is sensitive to the period of modulation in this region.

Positive and negative bystander effects have been described in the literature, with various signaling mechanisms suggested [17,29]. The extent and complexity of non-targeted bystander effects highlight the involvement of multiple factors, including the system under study (cell type). The experimental survival data of the different cell lines in our study show that the response of normal cells differs markedly from that of cancer cells. Under our modulated fields, the same trend was observed for both cancer cell lines (NCI-H460 and HCC-1954), but is not displayed by normal cells. The NCI-H460 and HUVEC cells both have wild type TP53 status, while the HCC1954 have mutated p53 (IARC TP35 database). Mothersill et al. [30] discuss the correlation of TP53 status with bystander responses and conclude the evidence as described in the literature is inconsistent. Some report that wild type p53 is associated with radiosensitivity, while Zhang et al. [31] find that the response to or production of bystander signals was not dependent on the TP53 status.

Since mechanisms of action for bystander effects are diverse, there is potential for more parameters in our present simple extended LQ model. However, the success of our extended LQ model for both normal and cancer cells is significant, given that there is only a single bystander (A) parameter for each cell line.

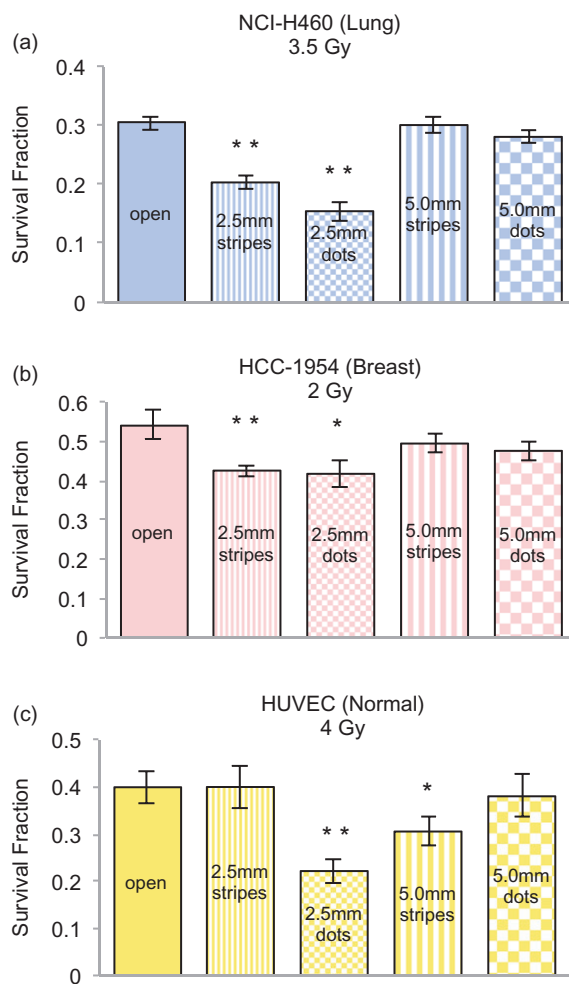


Figure 6. Comparison of percent survival after irradiation to four different spatially modulated fields: 2.5, 5.0 mm stripes, 2.5 and 5.0 mm dots, for (a) NCI-H460 lung, (b) HCC-1954 breast and (c) HUVEC normal cell lines. Survival fraction data is also shown in Table 4. Statistically significant difference in survival from the open field irradiation is shown as * if $p < 0.05$ and ** if $p < 0.01$. For the cancer cell lines, none of the 5.0 mm dot or striped fields showed significantly different survival to the open field.

Table 2. Average dose gradients for striped fields with average doses of 2 Gy, 3.5 Gy and 4 Gy. The uncertainty is derived from the average of three films scanned at 300 dpi.

Field	$\bar{D}$ (Gy/cm)		
	2 Gy (HCC-1954)	3.5 Gy (NCI-H460)	4 Gy (HUVEC)
5.0 mm stripe	5.84 ± 0.05	10.2 ± 0.1	11.7 ± 0.1
2.5 mm stripe	8.76 ± 0.15	15.3 ± 0.3	17.5 ± 0.3

Table 3. Bystander parameters of each cell line obtained by minimizing the RMSD.

Cell line	A (cm/Gy ²)	RMSD (cm/Gy ²)
NCI-H460	0.0177	0.00678
HCC-1954	0.0130	0.00714
HUVEC	0.00200	0.0734

For cancer cells, the fields with a 2.5 mm spatial modulation exhibit a significantly lower survival compared to the open field, which we attribute to a cytotoxic bystander effect, consistent with previous work [12,16,24,25]. However, the

5.0 mm spatially modulated fields were not effective in engaging the cytotoxic bystander effect, giving no significant difference compared to the open field. Therefore, our finding is that HDMLCs capable of 2.5 mm modulation are necessary to obtain the benefits of periodically modulated treatment fields. A mechanism based on bystander signaling to explain this response is schematically illustrated in Figure 8. The dose gradients of the 2.5 mm modulated fields are higher than for the 5.0 mm fields and the resulting concentration gradients of bystander signals drive them more effectively from the high-dose regions to the low-dose regions. This results in the cytotoxic bystander signals, assumed fast diffusing, becoming more evenly distributed across the field and cause greater cancer cell kill in the 2.5 mm fields.

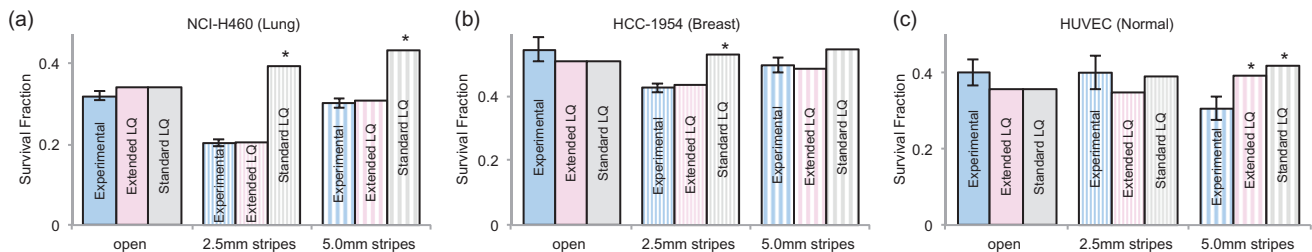
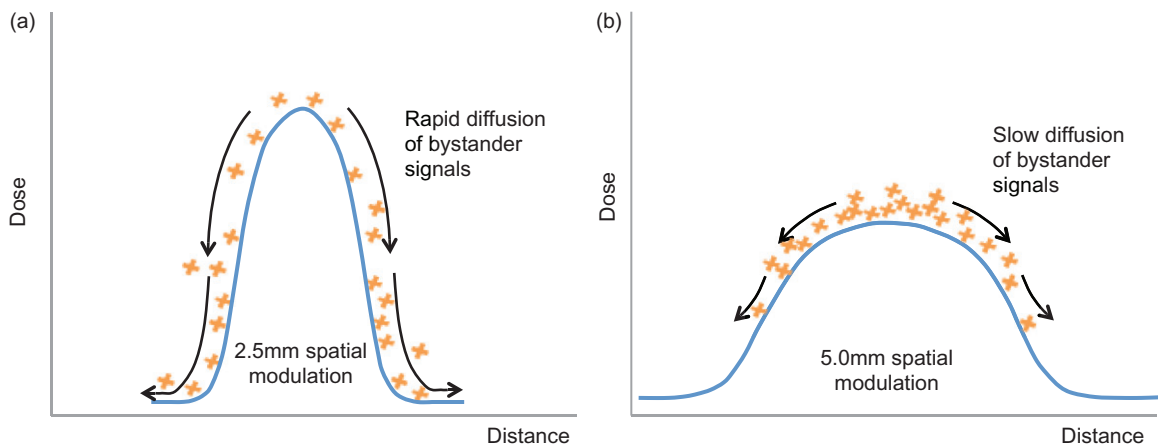
We now consider the response of normal cells to modulated fields. Our results (Figure 6 and Table 4) for exposure of normal cells to 2.5 mm modulated fields, showed a response different to that of cancer cells. For the 2.5 mm dot field, there was a decrease in survival, relative to an open field, while for the 2.5 mm striped field, there was a similar survival, relative to an open field. The finding for the 2.5 mm striped field is consistent with synchrotron MRT studies for normal tissue response [20]. Although bystander effects have been reported to have cytotoxic effects, there are also reports of bystander effects contributing to normal tissue sparing, therefore, supporting cell survival and proliferation [15,16,20,22]. The response of the normal cells to a 2.5 mm striped field is consistent with the involvement of a component of a beneficial bystander effect, where tissue regeneration is encouraged by the release of growth factors.

The two opposing bystander effects may be carried by soluble factors of different molecular weights, giving different degrees of diffusion in the same field, depending on the configurations of dose gradients. The response to bystander signaling would then be a balance between signals promoting cytotoxicity and signals promoting cell survival. In dot fields, the high doses are confined to localized regions and, therefore, the high-dose gradients are also localized. Poorly diffusing bystander factors will be driven away from the high-dose regions and then stop diffusing. If the bystander signals are protective, this effect reduces their activity in the high-dose region. The factors accumulate just outside the high-dose regions but do not reach much of the low-dose regions. The net effect is a reduction in protective bystander effects and an increase in cell death.

In this study, the HUVEC cells are a normal cell line, which has not been modified to give immortality, as is often the case in cell biology studies of 'normal' cells. As a consequence, the cell line can only be reliably used for less than six passages and even then they are significantly more difficult to grow than the cancer cell lines. Therefore, it is not surprising that the uncertainty in the data for the normal cell line is greater than that achieved for the more robust cancer cell lines. Despite this, with $n = 10$, statistically significant differences between the open and some modulated fields were found, confirming the presence of bystander signaling. Further investigation of positive and negative bystander

Table 4. Comparison of experimental survival fraction with predictions made by the standard and extended LQ models.

Field	NCI-H460 (3.5 Gy)			HCC-1954 (2 Gy)			HUVCEC (4 Gy)		
	Standard LQ	Extended LQ	Experimental	Standard LQ	Extended LQ	Experimental	Standard LQ	Extended LQ	Experimental
2.5 mm dots	0.363	–	0.155 ± 0.017	0.518	–	0.418 ± 0.036	0.371	–	0.221 ± 0.025
5.0 mm dots	0.440	–	0.281 ± 0.010	0.542	–	0.476 ± 0.024	0.410	–	0.380 ± 0.048
2.5 mm stripes	0.392	0.204	0.202 ± 0.007	0.528	0.434	0.426 ± 0.015	0.390	0.348	0.400 ± 0.045
5.0 mm stripes	0.431	0.307	0.301 ± 0.013	0.544	0.484	0.495 ± 0.022	0.418	0.392	0.305 ± 0.029
Open	0.339	0.339	0.319 ± 0.011	0.510	0.510	0.543 ± 0.037	0.359	0.359	0.400 ± 0.035


Figure 7. Comparison of the extended and standard model with experimental survival fraction for striped fields (data from Table 4). The prediction of the extended model for both cancer cell lines is not significantly different from the experimental survival. For the normal cell line, the prediction of the extended model is superior to that of the standard model but is significantly different from the experimental survival for the 5.0 mm striped field. Significant departures of models from the experimental survival for each field are indicated by (*).

Figure 8. Schematic illustration of the diffusion of bystander signals in spatially modulated fields. The higher dose gradient in the 2.5 mm fields (a) drives the bystander signals more effectively into the lower dose regions than the lower dose gradient of the 5.0 mm fields (b). Therefore, for cancer cells having a cytotoxic bystander response, more killing will occur in (a) than (b).

signaling in normal cell lines will require more experiments on a greater range of normal cell lines.

The standard LQ model is inferior to our extended LQ model in its predictions for the response of cancer cells in modulated fields. From Table 4 and Figure 7, it can be seen that the standard LQ model over-predicts clonogenic survival following modulated exposures for the cancer cell lines, especially for the NCI-H460. Although Butterworth et al. [26] did not use highly modulated exposures, they also report out-of-field clonogenic survival of cancer cells to be significantly lower than that predicted by the standard LQ model. Therefore, it is not recommended that the standard LQ model be used to predict survival in fields containing high dose gradients, conditions that induce additional biological effects influencing the overall survival.

The extended LQ model with bystander signaling we developed with input from the experimental data contains an additional term that defines the contribution of the bystander signal. This term contains the product of a cell specific bystander parameter A , the local dose $D(r)$ and the

average dose gradient $\bar{D}$ of the modulated field. For uniform exposures, the standard and extended LQ models predict the same survival since the additional gradient term in the extended model vanishes. For the majority of cases, in individual predictions, the standard LQ model gives significant deviations (t test) from the experimental values (Figure 7). Two components are likely to contribute to the value of the parameter A : the strength of the bystander signal released and the ability of these signals to diffuse. Therefore, there is a balance between diffusion effects and strength effects of the signals represented by the parameter A . Diffusion effects will be dependent on the signal itself and on the microenvironment.

This work provides evidence, although preliminary, of how radiation field modulation can be used to influence cell survival for the same average dose. We find that a modulation of periodicity of 2.5 mm but not 5.0 mm is able to engage bystander effects, significantly changing the survival fraction of both cancer and normal cells, but in different ways. Overall, for the same average dose, the best therapeutic

results would be given by delivering the 2.5 mm striped field, since it has enhanced cancer cell toxicity compared to an open field, without enhanced toxicity for normal cells. For low-frequency spatial modulation, for example, at the common grid spacing in the order of 2 cm, calculation of the equivalent uniform dose (EUD) and therapeutic ratio (TR) using the standard LQ model may be acceptable in some circumstances [8,9]. However, when the frequency of spatial modulation is high, with spacing in the order of 2.5 mm, our findings show that the bystander effect must be included in the predictive models of survival.

Using modern MLC technology, we currently have the capability to deliver fields modulated with 2.5 mm periodicity. Our treatment planning platforms are able to describe the dose distribution created by such technology. In this work, we provide an extended LQ model to translate the dose distribution into a biological response. The next step to progress our findings to the clinic is to validate them *in vivo*.

Conclusions

Some of the beneficial therapeutic effects reported for synchrotron MRT treatments were reproduced *in-vitro* using a 6 MV photon linac beam and modulated fields with spatial periodicity of 2.5 mm. When the periodicity was increased to 5.0 mm, the enhanced toxicity for cancer cells was no longer statistically significant. For normal cells, striped fields of 2.5 mm period resulted in less toxicity than the dot fields.

The standard LQ model was not able to predict the survival in a modulated field. We have developed an extended LQ model based on experimentally derived parameters that includes an additional bystander signaling term, dependent on the product of the average dose gradient, local dose and a cell specific parameter describing the cell signaling. The extended LQ model was able to predict the survival for highly modulated fields with better accuracy than the standard LQ model.

The significance of this work is in establishing that radio-sensitivity is not solely dependent on dose and that high-frequency spatial modulation of the radiation beam is a powerful factor in the ultimate radiobiological response. Of significance to current clinical radiotherapy, is that a 2.5 mm striped spatial modulation of a 6 MV linac beam appears sufficient to decrease the tumor cell survival and preserve the normal cell survival, relative to the same dose in an open field. This study establishes a proof of principle only and the next stage is to validate these *in-vitro* findings in animal studies, before they can be clinically exploited. Further *in-vitro* studies to identify the bystander mechanisms that drive the response to modulated fields would give further confidence in their clinical application.

Modulation of the radiation beam is easily achievable with current clinical linac technology and is an option for customizing treatments to an individual patient's tumor type and normal tissue response. The extended LQ model, with further validation, would be a natural partner in customizing a clinical treatment.

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