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Establishment and validation of a method for multi-dose irradiation of cells in 96-well microplates

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ABSTRACT

Microplates are useful tools in chemistry, biotechnology and molecular biology. In radiobiology research, these can be also applied to assess the effect of a certain radiation dose delivered to the whole microplate, to test radio-sensitivity, radio-sensitization or radio-protection. Whether different radiation doses can be accurately applied to a single 96-well plate to further facilitate and accelerated research by one hand and spare funds on the other, is a question dealt in the current paper. Following repeated ion-chamber, TLD and radiotherapy planning dosimetry we established a method for multi-dose irradiation of cell cultures within a 96-well plate, which allows an accurate delivery of desired doses in sequential columns of the microplate. Up to eight different dose levels can be tested in one microplate. This method results in fast and reliable estimation of radiation dose–response curves.

1. Introduction

Microplates or microtiter plates are useful tools in chemistry, biotechnology and molecular biology. These were first designed by Gyula Takatsy, a Hungarian physician, during a serious influenza epidemic back in 1951, providing a fast and economic test method [1]. The side by side arrangement of six up to 1536 wells on a plate, allowed to fill simultaneously multiple sample tubes with defined volumes of liquid samples. The subsequent development of photometry with microplate readers that replaced titration provided a quick and accurate quantitative method of liquid sample analysis.

Apart from the diagnostic application in ELISA measurements of various proteins in patient serum, pharmaceutical screening of active compounds is also a major application of microplates in cancer research, allowing the rapid assessment of cell survival after exposure to various drugs and various drug concentrations. For cell cultures the surface of wells are modified using a plasma discharge to allow easy adhesion and growth of cells.

Multiple concentrations of a certain drug can be applied in sequential wells containing different cancer cell concentrations, facilitating the quick screening of cytotoxicity. Remnant cell can be stained and their density assessed in 96-well plate readers. Fluorescence or absorbance readers (according to the method applied) can plot survival/time curves for different dose levels. In radiobiology research, this can be also applied for a certain radiation dose level delivered to the whole microplate, to test radio-sensitivity, radio-sensitization or radio-protection.

Whether different radiation doses can be accurately applied to a single 96-well microplate to further facilitate and accelerate research, is a question dealt in the current paper.

2. Materials and methods

2.1. Radiation beam quality

Partial irradiation of a 96-well plate should be better performed with high energy X-rays so that the radiation field has sharp margins (more narrow penumbra than cobalt 60 or other isotope unit). The Linear Accelerator we use in the current study is the PRECISE (ELEKTA) with MultiLeaf Collimator and produces photons (energies 6 and 18 MV) and electrons (energies 6–18 MeV). The 6 MV photon energy produced has depth of maximum dose 16 mm water and $TPR_{20,10} = 0.680$.

2.2. Building a Plexiglas system

For this 6 MV X-ray beam to reach electronic equilibrium, the thickness of the irradiated material demanded is 16 mm (water equivalent). Thus, irradiation of the cell cultures within the wells, have to be performed with a posterior (upwards directed) beam and an adequate bolus material should be placed below the plate so that the beam could penetrate a material with at least 16 mm equivalent water before reaching the cells. Moreover, in order to reassure an equal radiation contribution to all wells from the back

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and side scatter radiation, a similar material has to be placed over and around the 96-well plate. We, therefore, used 30 × 30 cm plates of Plexiglas (PMMA polymethylmethacrylate) of 1.190 g/cm^3 density to construct a 'box' that embraces the 96-well plate well. 20 mm thick Plexiglas is equivalent to 22.39 mm of water so that a minimum thickness of 14.29 mm Plexiglas (equivalent 16 mm water) is demanded to allow electron equilibrium. A cross section of the Plexiglas box containing the 96-well plate, using a CT-scanner, is shown in Fig. 1(A). Using the chosen thickness of the Plexiglas, shown in Fig. 1, that are adapted to the dimensions of the 96-well plate, we can use a source surface distance SSD of 97 cm (gantry at 0°). When the gantry turns to 180° the center of the field is at the center of the cell culture in the well (98 cm from below). The isocenter is at the bottom of the well.

2.3. Ion chamber dosimetry

The ion chamber 31003 flexible, with cavity volume 0.3 cm^3 (PTW, Germany) was used for simulated radiation dose distribution in the 96-well plate. Dosimetric recording was performed using the UNIDOS electrometer (PTW, Germany). The ion chamber provides dose assessment from irradiated point of less than 1 mm, so by moving the chamber by 3 mm steps we could assess differential dose distribution at these small distances. As the well's bottom is about 6 mm wide, three different measurements could be obtained within a well (middle area and two lateral areas in the well). With a specially constructed Plexiglas (same material as "The Plexiglas system") with an insertion to receive the ion chamber and with suitable table movements (electronically monitored by the LINAC table system), the ion chamber measure the radiation at the 3 points per well.

2.4. TLD dosimetry

To further measure the dose received by the wells, thermoluminescent dosimetry was applied using GR200 TLDs (LiF: Mg, Cu, P) (tablet form of 4.5 mm of diameter and 0.8 mm thick) and the LTM Reader (Fimel, France). TLDs were calibrated following irradiation at escalated doses with a 6 MV LINAC (ELECTA) and with a cobalt 60 unit (Theratron) (data not shown). Two TLDs were placed in each well. Tissue equivalent bolus was placed over the TLDs to simulate the height of the cell culture (total volume 200 μl).

3. Results and discussion

3.1. The width of the field

A 96-well plated was used to perform irradiation of two and three consecutive columns of the microplate, using a 6 MV X-ray beam. The microplate was scanned by a CT-scanner (Tomoscan EG, Philips) and, using a radiotherapy treatment planning system (Plato, Nucletron), a field was created to encompass two columns only leaving the adjacent ones outside the radiation portal. The center of the field was placed between the two wells. The width of this field was 1.8 cm. A significant variation of dose within one well was noted (data not shown). This field was subsequently applied to deliver 2 Gy of radiation to the ion chamber Plexiglas system, by moving the ion chamber by steps of 3 mm (three steps within one well). A significant drop of the percentage of dose within the same well was noted. From 100% measured at the center of the field (point between the two wells near the one edge of the wells) dropped to 81.5% to the center of the wells and to 59% to the distal edge of the wells. This shows that a field directed to two columns is unacceptable due to the up to 41% dose variation within the same well.

A field to cover 3 columns of wells (27 mm of width) was, thereafter, used to assess dose distribution in the irradiated wells. A central column was chosen to pace the center of the field and the dose distribution to the adjacent columns, within and outside the radiation portal, was assessed. Fig. 1(A) shows the isodose curves from the planning system. The dose distribution as shown by the ion chamber dosimetry showed that the central well receives 100% of the dose while the margins of the well received 99.3% of the dose (Fig. 1B). The intra-well dose distribution is, therefore, optimal. Further dosimetry performed using the ion chamber/Plexiglas system at distance corresponding to the consecutive wells confirmed the planning estimations. The percentage of dose to the adjacent well within the field drops to 84%. To the columns outside

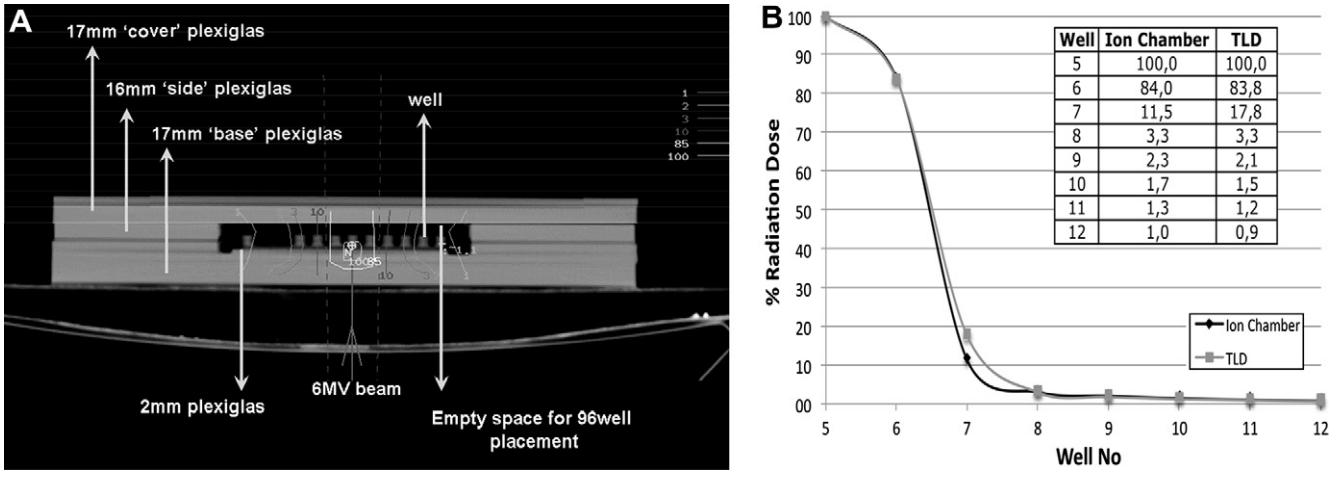

Fig. 1. (A) A cross section of the Plexiglas 96-well plate system, using CT-scan imaging. The dimensions of the 96-well plate are 12.8 × 8.6 × 1.6 cm. This is placed over a small 3 mm thick Plexiglas (2 mm additional plexi + 1 mm the bottom of the well) and this above a large 'base' of 17 mm thick Plexiglas; dimensions 30 × 30 cm). Subsequently, Plexiglas (30 × 30 × 1.6 cm) with a central empty rectangular space of 12.8 × 8.6 × 1.6 cm is place above the large base to encompass the 96-well plate. This is called the 'side' Plexiglas. Above this, a 17 mm thick 'cover' Plexiglas (dimensions 30 cm × 30 cm × 1.7 cm) is placed (total thickness of plexi 18 mm adding the 1 mm the plexi of the 96-well plate's cap). Using a source surface distance of 97 cm (at the surface of the cover Plexiglas) the gantry being at 0 degrees, the centre of the field is focused in the centre of the cell culture in the wells, as soon as the gantry turns to 180 degrees. In the central position of the cross section the 12 wells of the 96-well plate are evident (the 2nd and 3rd are empty, while the remaining are filled with culture medium). The isodose curves around a central well, using a three well covering 6 MV beam (27 mm width), as calculated by the radiotherapy planning system are displayed. (B) Dose distribution in subsequent wells, using a three well covering beam, starting from the center of the field (in this case placed at well number 5) and moving to the right to subsequent wells, using ion chamber and TLD dosimetry.

Table 1
The table shows the dose distribution in wells 4–11, using the calculations of dose distribution performed by ion chamber and TLD dosimetry. The table assumes that each column is irradiated with escalated doses a, . . .,h Gy, putting the central axis of a 3 column comprising field at the center axis of each well column.

4	5	6	7	8	9	10	11
a	0.84a	0.115a	0.033a	0.023a	0.017a	0.013a	0.01a
0.84b	b	0.84b	0.115b	0.033b	0.023b	0.017b	0.013b
0.115c	0.84c	c	0.84c	0.115c	0.033c	0.023c	0.017c
0.033d	0.115d	0.84d	d	0.84d	0.115d	0.033d	0.023d
0.023e	0.033e	0.115e	0.84e	e	0.84e	0.115e	0.033e
0.017f	0.023f	0.033f	0.115f	0.84f	f	0.84f	0.115f
0.013g	0.017g	0.023g	0.033g	0.115g	0.84g	g	0.84g
0.01h	0.013h	0.017h	0.023h	0.033h	0.115h	0.84h	h

the radiation filed the scattered dose gives 11.5%, 3,3%, 2.3%, 1.7%, 1.3% and 1.0% to the 3, 4, 5, 6, 7 and 8th columns, respectively. Using TLD dosimetry the above calculations were verified showing almost overlapping curves of the estimated dose distribution between the two methods (Fig. 1B).

3.2. The length of the field

An additional question raised was how long the field should be so that the upper and lower wells in the central and adjacent columns could receive an identical dose compared to the central wells. Thus, what should be the length of the field to apply. This is important as by moving farther from the centre of the field the dose distribution decreases. Using a 40 cm long field (the maximum of our LINAC), the best achievable homogeneity of dose distribution was obtained, with a variation of ±2% (data not shown).

3.3. The set-up of cultures in the 96-well plate

In order to acquire cell survival data at various dose increments that would allow the creation of dose response curves for a certain cell line, we proposed the following procedure.

1. All wells at the margins of the 96-well plate are simply filled with culture medium to prevent evaporation of the inner (cell containing) wells during the time the 96-well plate is incubated for measurements. So there are 10 columns left, each one having 6 wells available for cell culture placement.
2. The left 2nd and 3rd columns are used to place cultured cells after the irradiation of the plate, so that this well columns function as control un-irradiated (0 Gy) cultures. The upper, middle and lower 4 wells are loaded with three different known cell concentrations, respectively, to allow creation of a curve translating the scoring of the photometric reader to actual cell concentration. The middle concentration was the one used in wells used for irradiation.
3. The remaining 8 columns (6 wells each) are used for irradiation aiming to deliver escalated doses starting from the 4th left column and moving consecutively to the 11th right column.

3.4. Dose to give and column to irradiate

Taking into account the dose distribution in the central and adjacent left and right columns following irradiation with a three-column field, but also the scattered dose distributed to the well columns outside the main radiation portal we created the Table 1. This shows the dose that each columns receives if columns 4, 5, ...,11 are consecutively used as central columns to give a, b, . . .,h Gy, respectively. In this way column 4, 5, . . .,11 receive S1, S2, . . .,S8 Gy, respectively, as the sum of the dose received from the contribution of the 8 consecutive fields. Assuming that the dose desired to give to column 4, thus S1, is 1/2 S2, 1/3 S3, . . .,1/8 S8 we can create a series of doses (e.g., 1 Gy up to 8 Gy) that would be convenient to build a dose response curve. In this way we create a system of equations with 8 variables to solve (Table 1). This system, however, has negative values for some columns (data not shown) so it is not feasible to apply.

In order to overcome this mathematical dead-end we proposed a practical solution that gives dose increments to sequential columns convenient to create a dose–response curve. We simplified the problem by proposing the irradiation of only 4 columns (5, 7,

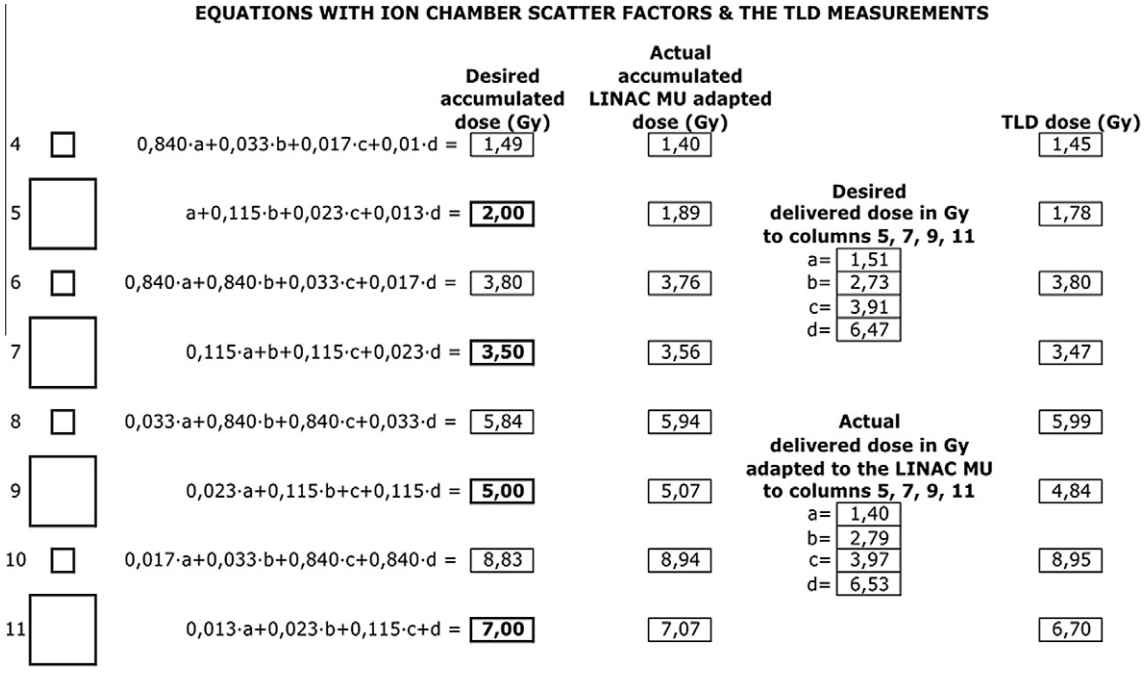

Fig. 2. Dose a, b, c and d Gy is given in columns 5, 7, 9 and 11, respectively. The dose received by columns 4–11 is given by the equations shown. In the figure, an example with a, b, c, d and values equal to 1.40, 2.79, 3.97 and 6.53, respectively is shown. TLD values are also shown, verifying an actual dose similar to the theoretically calculated.

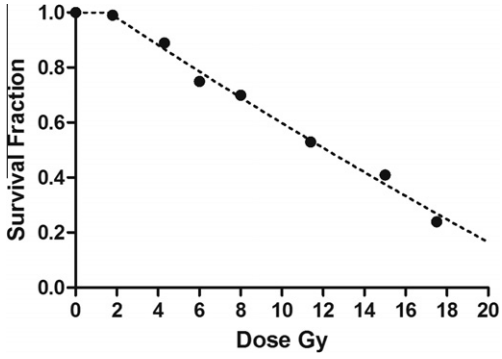

Fig. 3. Dose response curve of T98G glioblastoma cells obtained following two 96-well microplate irradiation experiments and the AlamarBlue® assay applied 96 h post-irradiation.

9, 11) creating an equation system with 4 variables and demanding to give values around 2, 3.5, 5 and 7 Gy to these columns (Fig. 2). By delivering 1.40, 2.79, 3.97 and 6.53 Gy to columns 5, 7, 9 and 11, respectively, the dose received by columns 4, 6, 8 and 10 is 1.40, 3.76, 5.94, and 8.94 Gy respectively (Fig. 2). The 4 parameter equation system produced by dosimetric analysis and shown in Fig. 2 can be used to adapt the dose values to columns at desired levels.

In order to verify that the above proposed irradiation method delivers indeed the theoretically calculated radiation doses to each column, TLDs were implanted in the wells and irradiation was performed using the method described. Indeed, values similar to the theoretically calculated were obtained, as shown in Fig. 2.

3.5. Application of the method

The T98G glioblastoma radio-resistant cell line [2] was chosen for experiments. Cells were grown in plates in appropriate culture medium, in CO_2 incubators at 37 °C and 18% oxygen ambient. For cancer cell susceptibility testing we used clear-bottomed, 96-well plates in order to minimize background fluorescence. Outer perimeter wells were filled with culture medium to prevent dehydration in inner used wells. Following initial experiments to determine the optimal cell concentration, the 250 cells/well was chosen. This concentration allowed prolonged monitoring without culture medium replacement.

Two 96-well plates were used to assess multi-dose radiation survival curves at 96 h. The first well received the above described dose schedule. The second received a three-fold higher dose, given the fact that this cell line is radio-resistant and higher than 9 Gy dose is demanded to achieve death effect above 50%.

The AlamarBlue® assay was chosen to assess cell viability (Serotec, Oxon, UK). This test is based on the reduction of a weakly fluorescent resazurin sodium salt to a strongly fluorescent resorufin sodium salt [3]. This reaction is only performed intracellularly by viable cells. Following 8 h incubation of cells in wells at a concentration of 10% AlamarBlue®, fluorescence (excitation 530 nm, emission 590 nm) was measured by a microplate reader (FLUOstar Omega). The percentage of viable cells in each well was calculated as the ratio: florescence signal (relative fluorescence units; RFU) obtained from the irradiated well minus the signal obtained from three negative control wells (containing cell free culture medium and AlamarBlue® 10%), divided by the mean signal recorded from non-irradiated wells (same initial cell concentration as the irradiated ones) minus the signal from negative control wells. Using these calculations the survival curve shown in Fig. 3 was obtained.

It is concluded that multi-dose irradiation of cell cultures within a 96-well plate is feasible and the provided methodology allows an accurate delivery of desired doses in sequential columns of the microplate. This method results in fast and reliable estimation of radiation dose–response curves at the same time sparing resources.

References

[1] G. Takatsy, The use of spiral loops in serological and virological micro-methods, Acta Microbiol. Acad. Sci. Hung. 3 (1955) 191–202.
[2] A. Karim, K. McCarthy, A. Jawahar, D. Smith, B. Willis, A. Nanda, Differential cyclooxygenase-2 enzyme expression in radiosensitive versus radioresistant glioblastoma multiforme cell lines, Anticancer Res. 25 (2005) 675–679.
[3] S. Anoopkumar-Dukie, J.B. Carey, T. Conere, E. O'sullivan, F.N. van Pelt, A. Allshire, Resazurin assay of radiation response in cultured cells, Br. J. Radiol. 78 (2005) 945–947.