

# Obtaining cell survival curves in radiobiology: From the linear accelerator to the linear-quadratic fitting and alternatives

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**Abstract.** Radiobiology is the study of the biological effects of ionizing radiation on living organisms. A fundamental aspect is the use of cell survival curves (CSCs) to evaluate radio-sensitivity *in vitro*, with the clonogenic assay being the gold standard method for this purpose. Despite its widespread use, this assay has inherent limitations, such as variability in cell plating efficiency, and the need to adjust cell seeding density based on the expected cell kill at higher radiation doses. This issue is often overlooked in published protocols, potentially compromising reproducibility and robustness. Survival data from clonogenic assays can be fitted to the linear-quadratic (LQ) model to determine the  $\alpha/\beta$  ratio, a key metric for assessing tumor radiosensitivity. However, protocols for fitting curves to the LQ model and calculating  $\alpha/\beta$  values are poorly explained, lacking standardized protocols in the literature. Modern approaches, such as impedance-based real-time cell analyses (RTCAs) to monitor cell proliferation provide an alternative for obtaining CSCs, providing dynamic monitoring of cell proliferation and survival after irradiation. The present study demonstrates a fully described and reproducible protocol with step-by-step instructions for obtaining survival curves from clonogenic assays and fitting them to the LQ model. The present study also demonstrates a strong correlation ( $R^2 \geq 0.9222$ ) between RTCA results and clonogenic assay data, emphasizing potential of RTCA as a high-throughput, sensitive and more rapid alternative for assessing radiosensitivity *in vitro*.

## Introduction

Radiobiology, or radiation biology (RB), is the scientific field that explores the biological effects of ionizing radiation through the lenses of physics, chemistry, biology and medicine (1). Although fundamental in nature, RB studies underpin the development of radiotherapy (RT), providing conceptual frameworks, guiding innovative approaches and guiding treatment schedule recommendations in clinical RT (2). RT is grounded in the ‘7 Rs of radiobiology’, which explain the biological principles underlying tissue responses to ionizing radiation: repair of sublethal damage, reoxygenation, redistribution of cells through the cell cycle, regeneration (or repopulation), radiosensitivity, reactivation of antitumor immune response and reinforcement by the tumor microenvironment (3-6).

In RB, cell death is characterized by the loss of reproductive capacity, with cell survival linked to clonogenic potential. Consequently, RB studies often use cell survival curves (CSCs) to evaluate and quantify the effects of ionizing radiation *in vitro*. These curves illustrate the reduction in reproductive capacity that culminates in cell death or diminished proliferation post-irradiation (7). CSCs are typically represented as plots of survival fraction vs. radiation dose, traditionally generated using the clonogenic assay [or colony formation assay (CFA)], which has been the gold standard for *in vitro* analysis of radiosensitivity and cellular reproductive capacity since the 1950s (8). In this assay, cells that survive irradiation and can form colonies with  $\geq 50$  cells are referred to as clonogenic; this property is directly linked to radiosensitivity and repopulation, two of the 7 Rs of radiobiology (3,4). Based on the number of cells seeded and the number of colonies formed at the end of the experiment, the plating efficiency (PE) is calculated as follows (9,10):

$$PE = \frac{\text{number of colonies formed}}{\text{number of cells seeded}}$$

This value is subsequently used to determine survival fractions (SF) and plot the survival curve.

Despite the undeniable utility of the CFA in RB, cell seeding density remains a major issue in such experiments. A previous study that performed clonogenic assays with 50

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different cell lines demonstrated that the PE was not a constant value for each lineage, and its variability can significantly compromise the robustness of the assay (11). Furthermore, the necessity of increasing cell seeding density at higher radiation doses, to account for expected radiation-induced cell death and maintain countable colony numbers (10), is often overlooked. While this issue is addressed in classical radiobiology textbooks (10), it is rarely emphasized in scientific articles. Published protocols for clonogenic assays often neglect to discuss this factor (9,12), and only a minority of articles explicitly state the exact cell seeding density or whether it was adjusted at higher radiation doses, thereby compromising reproducibility. For instance, among 49 studies utilizing clonogenic assays to assess radiation effects (13–61), only 23 of these clearly mentioned that increased cell seeding densities were used for higher doses (13–35).

A critical aspect of constructing cell CSCs in RB is the application of the linear-quadratic (LQ) model, a mathematical framework describing the survival fraction as a function of radiation dose (D), using the following equation:

$$S = e^{-\alpha D - \beta D^2}$$

This model reflects the mechanisms of radiation-induced chromosomal aberrations, such as dicentric and rings, which require two chromosome breaks to form. If both breaks result from the same electron, the probability of an aberration is proportional to the dose (D). Conversely, if two separate electrons each cause one break, the probability becomes proportional to the square of the dose (D<sup>2</sup>) (62–64). Thus, the parameters  $\alpha$  and  $\beta$  represent intrinsic radiosensitivity, and their ratio ( $\alpha/\beta$ ) is used to assess the fractionation sensitivity of the cells (65). Although the biological interpretation of the  $\alpha/\beta$  ratio is not intuitive (66), it provides crucial insights into RT. A high  $\alpha/\beta$  ratio indicates a tumor primarily influenced by the linear component  $\alpha$ , rendering it less sensitive to fractionation; in contrast, a low  $\alpha/\beta$  ratio suggests greater sensitivity to fractionation (Fig. 1). The accurate estimation of  $\alpha$  and  $\beta$  parameters is essential for optimizing the therapeutic window and ensuring successful radiotherapeutic outcomes (65). Hence, the clonogenic assay and CSCs remain central to advancing clinical RT efficacy.

Despite the recognized importance and applicability of the clonogenic assay, its experimental limitations prompt the consideration of alternative methodologies to obtain critical radiobiological measurements and responses. In this context, impedance-based real-time cell analyses (RTCAs) that monitor proliferation have been employed to generate survival curves following radiation exposure (67–78), complementing CFA results. Notably, one study has proposed RTCA as a viable alternative to CFA (79). The principles of impedance-based assays have been detailed elsewhere (67,80–82). Briefly, micro-electronic electrodes located beneath each well of a cell culture plate serve as sensors to monitor bioimpedance. Variations in cell viability, number, morphology and adhesion alter impedance, which is detected by the sensors and quantified as the cell index, a unitless parameter. Although this assay does not directly measure clonogenic capacity, but rather cell proliferation, it provides a continuous curve, indicates real-time declines in cell growth, and quantifies proliferation in a growth curve. These features render it a valuable tool for analyzing radiosensitivity and repopulation following irradiation. Additionally,

RTCA does not require cell lines capable of colony formation or low cell seeding densities, as CFA does (83).

To the best of our knowledge, no published protocol to date compiles radiobiology-based instructions for performing assays to obtain CSCs and  $\alpha/\beta$  values. The present study aimed to provide a clear and reproducible protocol with step-by-step instructions for obtaining survival curves from clonogenic assays, including curve fitting with the linear-quadratic model through a user-friendly software. Furthermore, the CFA results were correlated with data obtained from the real-time proliferation assay, demonstrating the robustness of the latter as a radiobiology assay. The protocol described herein includes fully described plating and irradiation conditions, with a setup that utilizes equipment commonly used in quality control and dosimetry of linear accelerators and in clinical radiotherapy routine, thereby avoiding additional costs.

## Materials and methods

**Cells and cell culture.** The immortalized head and neck cancer cell line, 93-VU-147T (floor of mouth squamous cell carcinoma), was kindly provided by the Cell Bank of Barretos Cancer Hospital, Barretos, São Paulo, Brazil and had been originally deposited by Dr Lidia Maria Rebolho Batista Arantes (Barretos Cancer Hospital, Barretos, Brazil). The cells were cultured in DMEM high-glucose medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Cytiva) and maintained in a humidified incubator at 37°C and 5% CO<sub>2</sub>. For subcultures, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and detached with trypsin (Cytiva), with subsequent trypsin inactivation with DMEM 10% FBS and 1% penicillin/streptomycin. Cell counting and cell viability were assessed using an automatic counter (Invitrogen Countess Automated Cell Counter, Thermo Fisher Scientific, Inc.) with Trypan blue 0.4% solution (Gibco, Thermo Fisher Scientific, Inc.) in a 1:1 dilution. The cell line was authenticated by short tandem repeat (STR) profiling at the beginning and at the end of the study work and was regularly monitored every 2 weeks for the detection of potential mycoplasma contamination, using the Mycoalert kit (Lonza Group, Ltd.) or conventional PCR, maintaining a mycoplasma-free cell culture environment.

**Clonogenic assay.** This assay was conducted following the protocol described in the study by Franken *et al* (9). The cells (5x10<sup>2</sup> cells/well) were plated in 6-well plates in a final volume of 2 ml/well of DMEM with 10% FBS and 1% P/S and incubated at 37°C and 5% CO<sub>2</sub>. A separate plate was prepared for each dose, plus the non-irradiated control. Following cell adhesion (24 h), the plates were sealed with parafilm and irradiated with 1, 2, 4, 6 and 8 Gy, apart from the control, which was mock-irradiated. The plates were then returned to the incubator and, following the appropriate time (mean incubation time, 6.6 days), when colonies were larger but not touching, the medium was removed, and the colonies were washed with DPBS. They were then fixed and stained with 5% crystal violet (Merck KGaA) dye in 50% methanol for 15 min under gentle agitation at room temperature. The dye was removed, and the wells were extensively washed three times with distilled water, followed by a final rinse with tap water. The plates were left

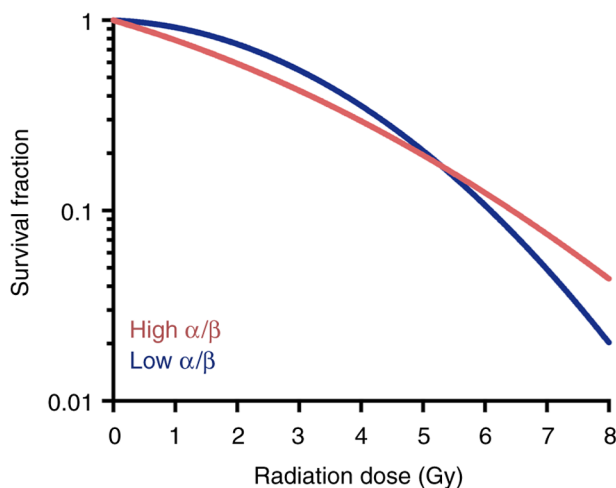


Figure 1. A cell survival curve illustrating a high  $\alpha/\beta$  ratio compared to another curve with a low  $\alpha/\beta$  ratio. The blue curve exhibits greater curvature, with a characteristic shoulder, reflecting a larger contribution from the  $\beta$  component and a low  $\alpha/\beta$  ratio. By contrast, the red curve appears more linear, consistent with a high  $\alpha/\beta$ .

open, protected from light, and allowed to dry overnight at room temperature. Subsequently, all wells were photographed using a stereoscopic microscope (Olympus SZX7, Olympus Corporation) and colonies were counted using ImageJ software (Fiji, version 2.14.0; National Institutes of Health) with a custom macro (Data S1). In the non-irradiated control, a colony containing 50 cells was manually identified under the microscope. Using ImageJ software, the area of this colony (in pixels<sup>2</sup>) was used to calibrate the macro as the minimum size of particles to be counted. Therefore, only colonies  $\geq 50$  cells were included in the count.

Note 1: Extensive optimization of cell seeding number should be performed before starting this experiment. First, the mean plating efficiency (PE) for each cell line should be determined by calculating the ratio of counted colonies to seeded cells in a non-irradiated test. This PE value should be used to estimate the maximum number of colonies expected for each condition (non-irradiated control and irradiated samples) by multiplying the number of seeded cells by the plating efficiency. Subsequently, pilot tests should be conducted with the desired radiation doses to assess the magnitude of cell killing (e.g., 30, 40 and 70%, etc.). Finally, the seeding densities for each condition should be adjusted to ensure that a sufficient amount of colonies (one colony should contain at least 50 cells) remain to be counted at the end of the assay, considering both intrinsic plating efficiency and expected cell kill. The specific used densities should be clearly stated when describing methodology and results.

Note 2: When using ImageJ (Fiji) to automatically count colonies, it should always be verified that the output is reliable and consistent with the number of colonies observed in each well. If necessary, the colonies can be counted manually to validate automated analysis and ensure accuracy, as the number of colonies counted directly affects the survival fractions and the fitting to the LQ model.

**Survival curve analysis.** After counting the number of colonies formed in the clonogenic assays, the methodology described

by Bright *et al* (18) was used to obtain survival curves fitted to the linear-quadratic model of radiobiology.

An XY table was created in GraphPad Prism (Dotmatics), with the X column representing the radiation doses and Y being replicate values in side-by-side columns (the number of technical replicates performed in each plate). Radiation doses were placed on the X column, and the number of colonies counted in ImageJ (Fiji) were placed on the Y column. Each biological replicate (independent experiments) should be entered in separate Y column groups. This initial table was named '147T-number of colonies'. In the *Analyze* menu, *Grouped analyses* → *Row Statistics* → *Compute the mean for each data set column* and *Calculate row means with SD*, *N* → *OK* were selected to obtain the mean values of counted colonies.

These results were copied into a new XY table (named 'Row Statistics-147T-number of colonies'), where the X column represents the radiation doses, and the Y column is *Mean, SD and N values calculated elsewhere*, where the results were pasted. Subsequently, the survival quotient (SQ) was calculated for each condition by selecting *Analyze* → *Transform* → *Transform Y values using  $Y=Y/K$*  → *Same k for all datasets*. The number of K should be the number of cells that were seeded in each condition (*note that this value varies according to the cell lineage and experimental design*). The results of SQ were copied into a new XY table (named 'SQ-147T').

Thereafter, to determine the plating efficiency, *Analyze* → *Nonlinear fit* were selected to use an unnormalized version of the linear-quadratic model. In the non-linear regression parameters, 'explicit equation' was selected as the equation type and the equation was defined as follows:

$$Y = PE \times \exp(-\alpha X - \beta X^2).$$

A complete guide for this step is available in Data S2.

Using the PE value of each independent experiment, in the table 'SQ-147T', SQ values were transformed again by selecting *Analyze* → *Transform* → *Transform Y values using  $Y=Y/K$*  → *Different k for each dataset*. For each condition (e.g., experiment 1, experiment 2...), the appropriate PE was selected for K and *OK* was then selected, to obtain SF values.

The results were copied into a new XY table (named 'SF-147T'), in which X is each radiation dose, and Y is *Mean, SD and N values calculated elsewhere*, where the results will be pasted. In column A (named 'All Data'), all survival fractions calculated in the previous step were pasted, with an empty line between each independent experiment. In columns B, C, and so on, the survival fractions corresponding to experiments 1, 2, etc. were pasted. To fit the SF results with the LQ model, once again *Analyze* → *Nonlinear fit* were selected and the following equation was used:  $Y = \exp(-\alpha X - \beta X^2)$  (Data S2).

To create a graph of the results fit to the LQ model, a *Row Statistics* was performed in columns B, C and D of the 'SF-147T' table by selecting *Analyze* → *Row Statistics* → *Compute one mean for the entire row* → *Calculate row means with SD, N*. After creating a new graph (XY type), the datasets to be displayed are the last *Row Statistics* and the non-linear regression of SF that determined the  $\alpha/\beta$  values. The Y-axis should be on a logarithmic scale (log 10).

**Optimization of the cell seeding density for the real-time proliferation assay with MTS.** Before conducting the real-time proliferation assay, optimal cell seeding densities were standardized using the tetrazolium compound [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] (MTS; CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega Corporation). Cells were plated in 96-well plates at seeding densities of  $5 \times 10^2$ ,  $1 \times 10^3$ ,  $2 \times 10^3$  and  $4 \times 10^3$  cells/well. Cell viability was monitored at 24-, 48-, 72- and 96-h post-plating by the addition of 20  $\mu$ l MTS reagent to each well. Following a 4-h incubation period with the reagent at 37°C, cells were gently shaken, and the absorbance was measured at 490 nm using a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific, Inc.).

**Real-time proliferation assay.** The real-time proliferation assay was performed using the xCELLigence Real-Time Cell Analysis system (Agilent), which was calibrated following the manufacturer's instructions prior the experiment. The system was placed inside a humidified incubator at 37°C and 5% CO<sub>2</sub>. A volume of 50  $\mu$ l of DMEM with 10% FBS and 1% P/S was added to each of the 16 wells in the E-plate (Agilent Technologies, Inc.), while the space surrounding the wells was filled with DPBS to minimize evaporation. The plate was then incubated at 37°C for 30 min to 1 h before being inserted into the xCELLigence station and scanned. The schedule for reading was set as follows: step 1, a single 1-minute reading for background measurement; step 2, six readings every 20 min; and a sub-step of step 2, 999 readings every hour. Each well was labeled according to the cell line and cell number in the layout session. After the background impedance was measured, cells were added at a seeding density of  $2 \times 10^3$  cells/well, completing a final volume of 150  $\mu$ l/well, and the analysis continued. Following cell adhesion (24 h), the plates were irradiated with doses of 2 and 8 Gy (and the non-irradiated control was mock-irradiated), then returned to the station for incubation. The equipment generated real-time proliferation curves for each condition based on periodic impedance measurements, expressed as the cell index. The experiment was concluded after 12 days (288 h). A troubleshooting guide for common issues in the real-time cell analysis has been previously described (81), along with the manufacturer's manual for further consultation if required.

Note: A detailed description of each tab in the xCELLigence software has been published elsewhere (84).

**Irradiation conditions.** The adhered cells in parafilm-sealed 6-well plates or 16-well E-plates were irradiated with X-rays at a dose rate of 600 monitor units per minute (MU/min), delivered by a 6-MV clinical linear accelerator (LINAC) (Synergy, Elekta Medical Systems), following a methodology adapted from Tesei *et al* (85) and Hao *et al* (86). The LINAC, which is part of Barretos Cancer Hospital's clinical routine, undergoes a quality assurance program that aligns with Task Group 142 standards from the American Association of Physicists in Medicine (AAPM) (87).

To ensure reproducible positioning of the cell culture plates, the following setup was used: i) 30x30x1 cm (height x width x thickness) solid water plates with water-equivalent density;

ii) a 30x30x1 cm gel buildup bolus as a human tissue compensator; and iii) a custom-built support measuring 30x30x2 cm, comprised of thermoplastic material from immobilization masks used in head and neck tumors treatments. This support includes a central template matching the cell culture plate layout, providing secure and consistent positioning for the plates and solid water layers. The same support was used for both 6-well plates and RTCA E-plates (Agilent Technologies, Inc.), as it was compatible despite the difference in plate sizes.

For planning, a computed tomography [CT580 RT (GE Healthcare Systems)] scan of the proposed setup was performed (Fig. 2). Images were imported into the treatment planning system Eclipse v15.6 (Varian Medical Systems), where calculations determined the monitor units required to deliver the prescribed dose at a 5 cm depth in an isocentric technique. Field sizes beams of 30x30 cm were delivered at anteroposterior (AP=0°) and posteroanterior (PA=180°) gantry angles with equal weighting at a source-to-surface distance (SSD) of 95 cm. Dose calculations were performed with the AAA v15.6 algorithm at the following doses: 1; 2; 3; 4; 5; 6; 7; 8; 9; 10; and 15 Gy. The LINAC table was included in the calculations to account for its attenuation effects.

Note: In the present study, the cells were irradiated 24 h after plating and adhesion. Alternatively, cells can be treated prior to plating, depending on the objectives of the study, as previously described by Franken *et al* (9).

**Statistical analyses.** Data were collected with two or three independent biological replicates, each including samples with triplicate, quadruplicate, or sextuplicate technical replicates. Statistical analyses were conducted using GraphPad Prism 9.2.0. Normality tests (Shapiro-Wilk test for  $n < 9$ ) were applied. Parametric data were analyzed using one-way ANOVA with Tukey's multiple comparison test, while non-parametric data were evaluated using the Kruskal-Wallis test of variance followed by Dunn's multiple comparisons test. For correlation analyses, only cell index values obtained over the same period as the clonogenic assay were used. Cell index values of the irradiated cells in each independent experiment were normalized and expressed relative to the highest cell index value of the non-irradiated control, set as 1-fold, yielding cell index data on a 0-1 scale (88), referred to as the 'normalized cell index'. Surviving fractions and normalized cell index values at 2 and 8 Gy were then correlated with Spearman's Rho (non-parametric data), with a simple linear regression to fit the points to a linear trend. A value of  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

The complete setup was carefully positioned on the LINAC table, with alignment meticulously adjusted for each experiment using reference lasers (Fig. 3).

The mean incubation time for the clonogenic assay was 6.6 days, after which the cells were fixed, stained and counted (Fig. 4A and B). The plating efficiency was determined based on the number of counted colonies and subsequently used for further calculations (Fig. 4C). Survival fractions were plotted on a logarithmic scale as a function of radiation dose (Fig. 4D).

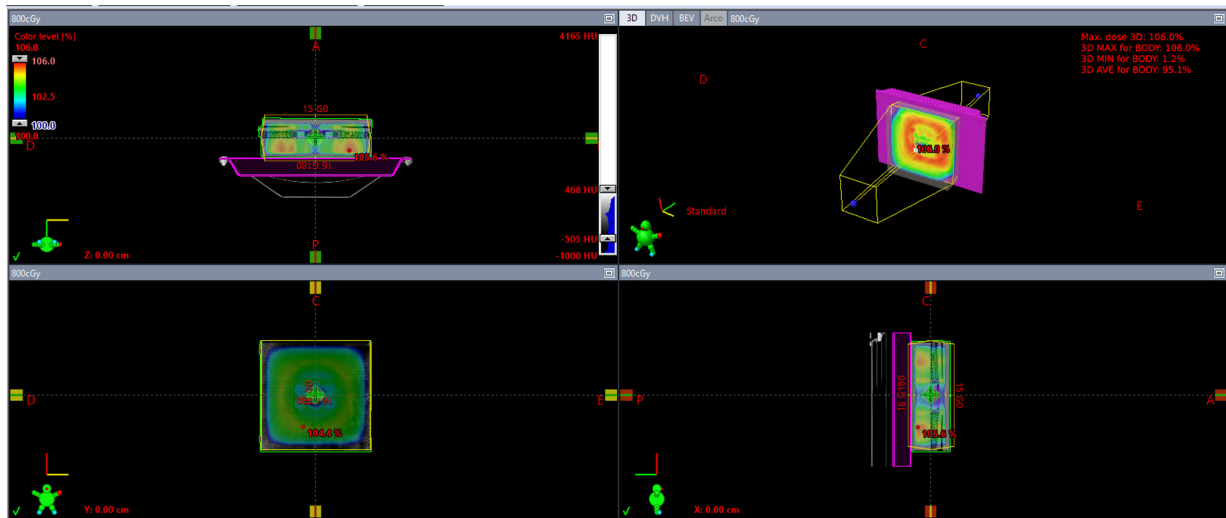


Figure 2. Computed tomography images of the complete setup that were imported to the treatment planning system.

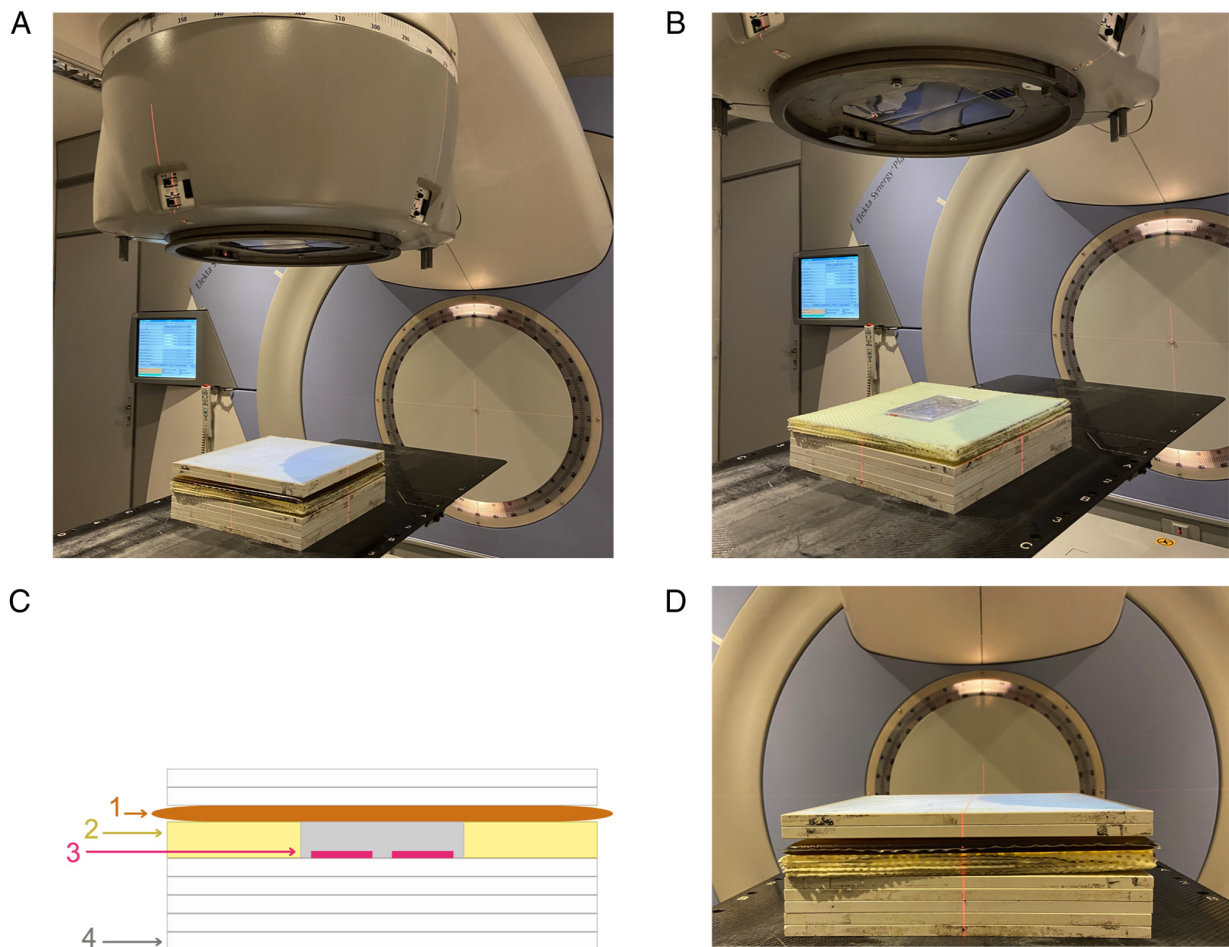


Figure 3. The setup used for irradiations. (A) Complete setup from top to bottom, consisting of two 1 cm-thick solid water plates, a 1 cm-thick gel bolus, a 2 cm-thick custom thermoplastic support holding a 6-well culture plate, and five additional 1 cm-thick solid water plates. The total setup height was 10 cm, with an SSD of 95 cm, so that the cells are at the isocenter, at a depth of 5 cm in both directions (anteroposterior/posteroanterior). (B) Open view of the setup, highlighting the cell culture plate positioned at the isocenter, and the opening of the radiation field. (C and D) Schematic cross-sectional illustration of (C) the setup. 1, gel bolus; 2, custom thermoplastic support; 3, culture plate; 4, solid water plates. (D) Image alongside the actual setup.

The mean plating efficiency of the non-irradiated control, considering the standard deviation across three independent experiments, was  $0.5828 \pm 0.0455$ . The survival curve showed

minimal evidence of a shoulder, consistent with a calculated  $\alpha/\beta$  ratio of 20.23 Gy, with  $\alpha=0.2259$  (0.1611-0.2941) and  $\beta=0.0111$  (undefined 0.0232).



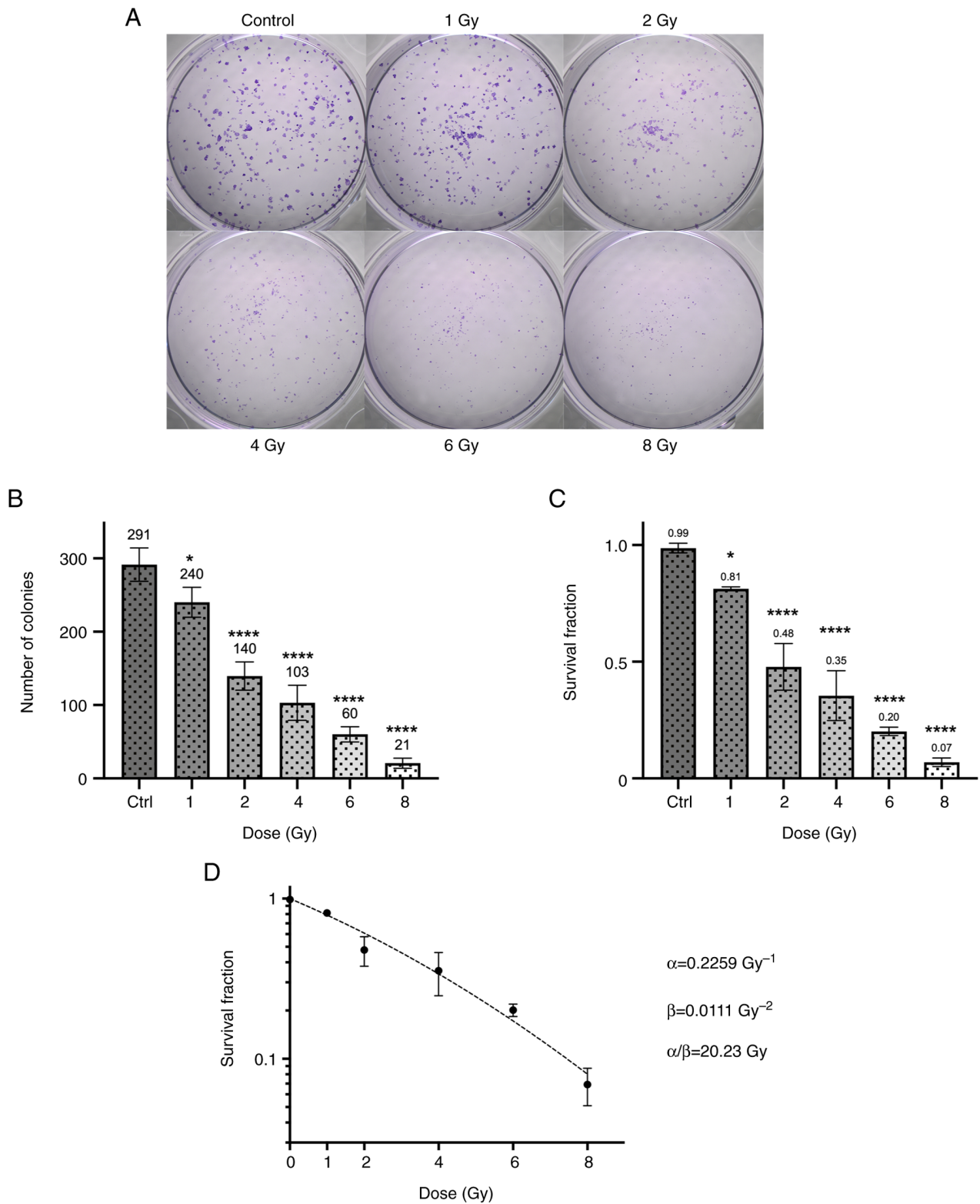


Figure 4. Clonogenic assay using single-fraction radiation doses. (A) Representative images of the assay in 6-well plates following a mean incubation time of 6.6 days. (B) Graph depicting the number of colonies counted in each dose. (C) Survival fractions at different radiation doses. (D) Survival curve fit to the linear-quadratic model equation (dashed line), with the Y-axis shown on a logarithmic scale. Plotted values of colony counts, and survival fractions are displayed above each bar and error bar. Data represent the mean of sextuplicates ( $\pm$  standard deviation) from three independent experiments. The parameters  $\alpha$ ,  $\beta$  and  $\alpha/\beta$  were calculated using the linear-quadratic model with a weighted least squares nonlinear regression. Statistics are relative to the non-irradiated control. \* $P \leq 0.05$  and \*\*\*\* $P \leq 0.0001$ . Ctrl, non-irradiated control.

To support the results of clonogenic assay, a real-time proliferation assay was conducted. An MTS cell viability assay was used to standardize cell seeding density, minimizing the

influence of intrinsic growth biases and ensuring that observed effects were not due to excessive proliferation-induced cell death. Various cell densities ( $5 \times 10^2$ ,  $1 \times 10^3$ ,  $2 \times 10^3$  and

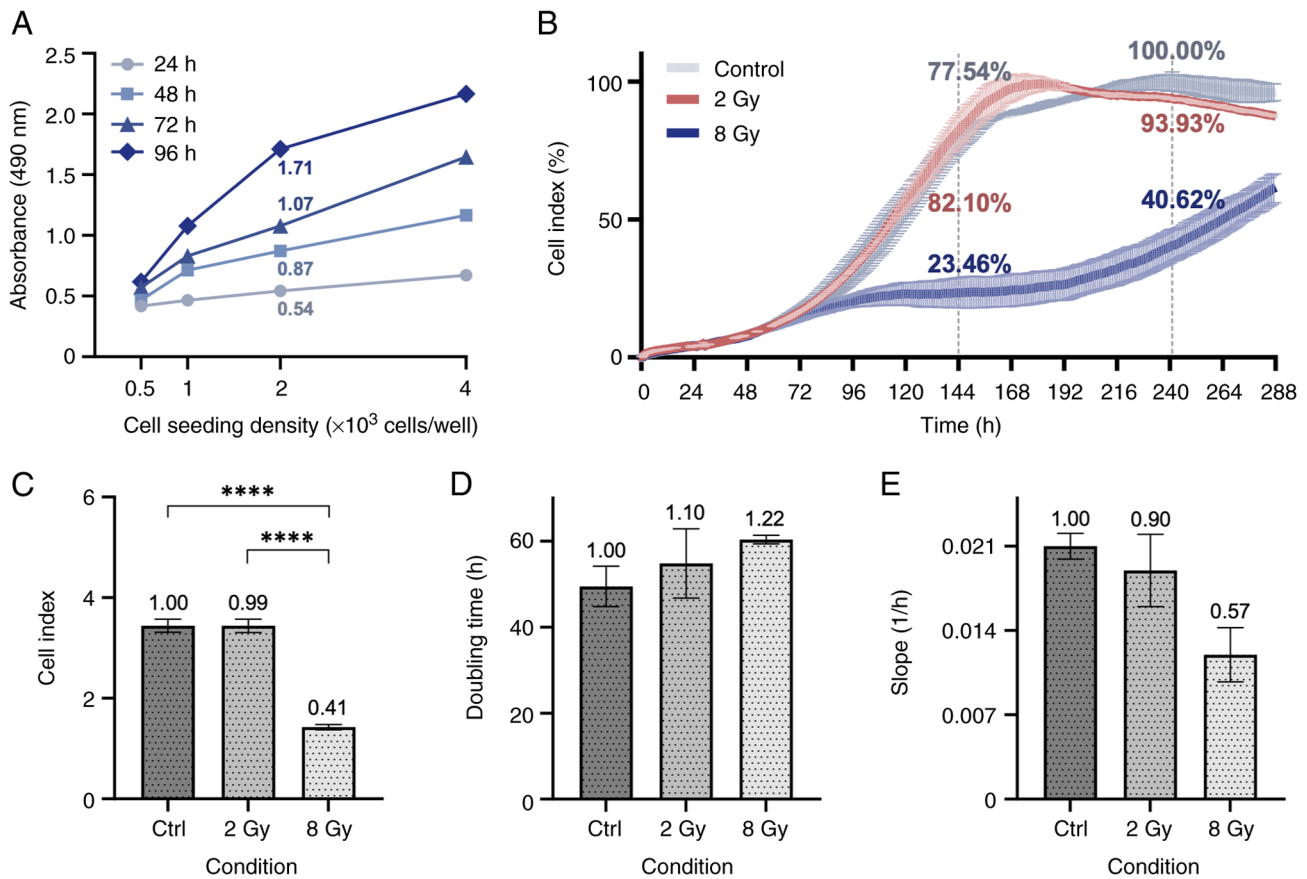


Figure 5. Real-time proliferation assay using single-fraction radiation doses. (A) Optimization of cell seeding density with an MTS cell viability assay, including four cell densities across time points indicated by different symbols: 24 h (circles), 48 h (squares), 72 h (triangles) and 96 h (diamonds) post-plating. (B) Real-time proliferation curve following irradiation with 2 Gy (blue) and 8 Gy (red) at 24 h post-plating. Cell index values were normalized to the highest value observed in the non-irradiated control, which occurred at 240 h, and expressed as percentages. (C) Cell index, (D) doubling time, and (E) slope values for the three conditions. Bars represent the mean values  $\pm$  standard error of the mean of quadruplicates from three independent experiments. Within bars, mean values of cell index, doubling time, and slope were expressed relative to the non-irradiated control (1-fold). \*\*\*\* $P \leq 0.0001$ . Ctrl, non-irradiated control.

$4 \times 10^3$  cells/well) were seeded in a 96-well plate and analyzed 24-, 48-, 72- and 96-h post-plating (Fig. 5A). These time points were selected to capture radiation-induced effects on proliferation, typically observed 48-72 h post-irradiation (corresponding to 72-96 h post-plating in this setup), while also ensuring that cell viability was not compromised by nutrient depletion over the course of the experiment. A density of  $2 \times 10^3$  cells/well yielded absorbance values within the response range of the MTS assay across all time points, making it an optimal choice for the real-time proliferation assay.

Following seeding, the cells were allowed to adhere in the xCELLigence Real-Time Cell Analysis system for 24 h. Subsequently, the adhered cells received single radiation doses of 2 and 8 Gy, followed by incubation and monitoring for an additional 264 h, completing the experiment at 288 h (12 days in total). In addition to cell index values (Fig. 5B), the system generated a cell index vs. time graph (Fig. 5C). After 240 h, the non-irradiated control reached its maximum cell index value; at this time point, the 2 and 8 Gy curves exhibited reductions of  $\sim 7$  and 60%, respectively. By contrast, at 144 h (the same duration as the clonogenic assay), the cell index of the non-irradiated control was still at 77.54% of its maximum. The 2 Gy curve had even exceeded the control, reaching 82.10% of proliferation, while the 8 Gy was significantly reduced, with a decrease of  $\sim 77\%$ . The parameter

doubling time (Fig. 5D) and slope (Fig. 5E) were analyzed at 288 h to characterize the time required for cell index doubling and the angular coefficient of the cell index curve in the entire experiment, respectively.

The correlation between surviving fractions and cell index values was determined at 144 h (6 days) and 120 h (5 days) (Fig. 6). The results revealed a strong linear correlation at both times ( $R^2=0.9838$ ,  $r=0.9919$ ,  $P=0.0081$  for cell index at 6 days; and  $R^2=0.9222$ ,  $r=0.9603$ ,  $P=0.0397$  for cell index at 5 days). Cell index values at 96 (4 days) and 72 h (3 days) were also analyzed, but did not correlate with surviving fractions (Fig. S1).

## Discussion

Survival curves are essential tools in radiation biology used to determine tumor radiosensitivity and  $\alpha/\beta$  ratios, which significantly influence clinical decisions in radiotherapy, as this ratio reflects cell sensitivity to fractionation (65). The clonogenic assay is the gold standard for obtaining CSCs; however, the lack of detailed protocols for assessing them leads to low reproducibility of results. To address this gap, the present study demonstrates a step-by-step protocol using an immortalized floor-of-mouth squamous cell carcinoma cell line, revisiting all parameters, from cell plating to curve construction, necessary

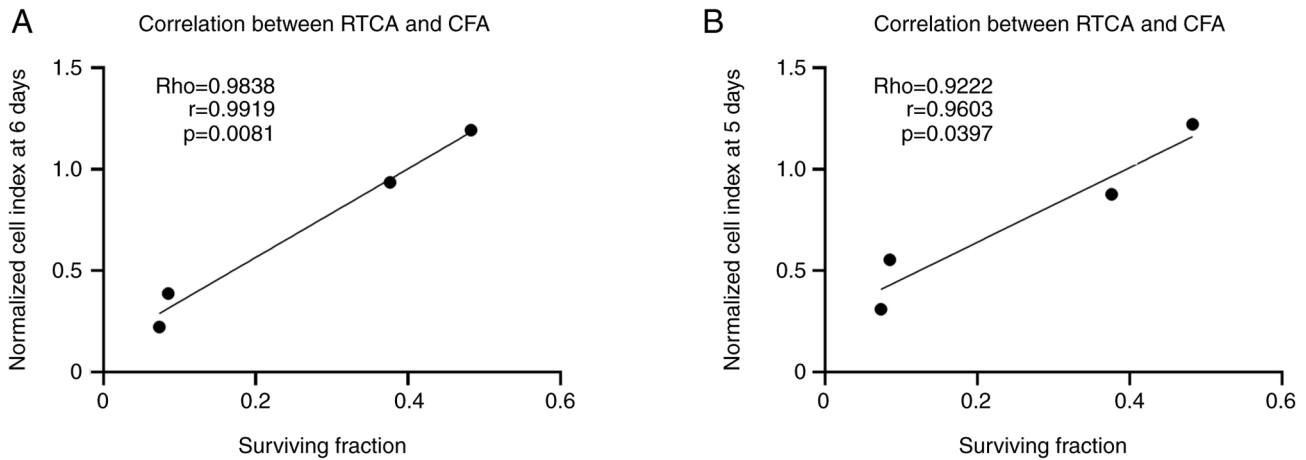


Figure 6. Correlation between surviving fractions and cell index values in irradiated assays. (A) Cell index values 6 days after plating and surviving fractions at the end of the experiment (6 days post-plating). (B) Cell index values 5 days after plating and surviving fractions at the end of the experiment (6 days post-plating). RTCA, real-time proliferation assay. CFA, colony formation assay.

for extracting  $\alpha/\beta$  values, with an adaptable and easy-to-follow guideline.

An  $\alpha/\beta$  ratio of 20.23 Gy was calculated after fitting the CSC to the LQ model, using radiation doses of 1, 2, 4, 6 and 8 Gy. Typically,  $\alpha/\beta$  values of  $\geq 10$  Gy are considered high and correspond to early-response tumors, while  $\alpha/\beta$  values of  $\sim 2$  Gy or lower are associated with late-responding tumors. These values reflect the sensitivity of the tumor to fractionation. The findings of the present study are consistent with those presented in the literature (89-91), which suggests that head and neck tumors are clinically treated as early-responding tumors. Although the typical  $\alpha/\beta$  ratio for these tumors is  $\sim 10$  Gy, accepted values range from 10 to 30 Gy for squamous cancer cells (92,93). In addition, a 2018 review of 149  $\alpha/\beta$  estimates across tumor sites revealed significant heterogeneity ( $I^2 > 75\%$ ), primarily due to variability among studies: for example, in head and neck cancer,  $\alpha/\beta$  values varied from -83.6 to 30 Gy ( $I^2 = 87\%$ ), and from -0.1 to 29.9 Gy ( $I^2 = 94\%$ ) in prostate tumors (65). *In vitro*  $\alpha/\beta$  ratios also appear to vary across different fractionation regimens (94-96). Herein, the calculated ratio represents a high  $\alpha/\beta$ , exceeding 10 Gy, suggesting a tumor largely influenced by the linear  $\alpha$  component.

In addition to the CFA, the present study performed real-time proliferation assay as a reliable complement for determining the radiosensitivity of tumor cells. Radiation-induced effects were observable after 72 h of experimentation. After 6 days (144 h), the 2 and 8 Gy curves exhibited decreases of  $\sim 18$  and 77%, respectively, while in the CFA, the reductions were  $\sim 52$  and 99%. This difference may be attributed to several factors related to the experimental models. Low seeding densities, as required in the CFA, can influence the duration of the lag phase and the initiation of the log phase of growth. By contrast, intermediate and high densities facilitate entry into the log phase and reduce adaptation time to the environment (97,98). Therefore, with a lower cell density than the RTCA, the clonogenic assay may misestimate survival calculations (11), which also impairs sensitivity evaluation (99).

Furthermore, the survival fractions in the CFA are directly linked to the number of colonies counted. However, this

measurement can be biased by intra-individual variability, as size-based counts can often be imprecise (100,101), even when made manual or automatically. Colonies can be difficult to distinguish, and the process is both time-consuming and labor-intensive; they may also be lost during washing steps (101). Moreover, the incubation time for the CFA is not predetermined and depends on the preferences of the researcher, as colonies should be sufficiently large to count, but not touching each other. This not only significantly affects the survival fractions obtained (11) but also limits the possibility of extended observations. Conversely, the RTCA continued monitoring cells for an additional 6 days, totaling 12 days of observation. At the end of experiment, the cell index in the 2 Gy condition did not significantly differ from the non-irradiated control, with a decrease of only 7% (in contrast to the 18% reduction at 6 days). However, the 8 Gy condition exhibited a significant difference compared to the control (adjusted P-value  $\leq 0.0001$ ), with a reduction of  $\sim 60\%$  and a marked alteration in the curve's angular coefficient (slope). Cells were less proliferative than the non-irradiated control, but more proliferative compared to the 77% cell index at 6 days after 8 Gy. Thus, real-time cell analysis provides a more comprehensive view of cell behavior, including repopulation, which was not observed in the CFA, likely due to the assay's limited duration.

The RTCA outcomes were correlated with the results of clonogenic assay using linear regression, as it was considered the most appropriated method for the dataset. Other analyses, such as Bland-Altman plots, are designed to assess agreement between methods that measure the same continuous variable, whereas RTCA and CFA evaluate distinct biological endpoints (cell index and survival fraction, respectively). Nonetheless, the two methods exhibited a strong correlation, with a linear correlation ( $R^2 = 0.9222$ ) after 5 days. This is consistent with the findings of previous studies (79,88,102), highlighting role of RTCA in radiosensitivity evaluation even in a shorter time frame than the clonogenic assay.

RTCA is thus a robust, sensitive, reproducible and high-throughput complement to the colony formation assay, overcoming a significant limitation of the clonogenic assay,



its discontinuous analysis model. On the other hand, despite advantages, such as dynamic monitoring of parameters beyond reproductive capacity (such as viability, morphology and adhesion), a notable limitation of the RTCA is the current lack of available studies demonstrating whether its data can be fitted to the linear-quadratic model to obtain  $\alpha/\beta$  values. To the best of our knowledge, no studies to date have investigated how to address this specific limitation, reinforcing the continued need for the clonogenic assay for this purpose. Additionally, RTCA can be considered more costly than the clonogenic assay; however, its plates can be reused (103), which may help reduce costs over time, and it requires less culture medium.

The representative results of the present study can be adapted to other cell lineages. A crucial step in this process is optimizing the cell seeding number based on plating efficiency and the expected cell kill, ensuring that a sufficient amount of colonies remain to be counted at the end of the assay, e.g., 20 to 50 colonies surviving colonies following irradiation. Additionally, when other radiation doses and fractionation schemes are applied, the monitoring units must be recalculated according to the desired dose. The present study has certain limitations, which should be mentioned, including the use of a single cell line derived from one tumor type, which restricts the generalizability of our findings. It is necessary to expand the models to include additional cell lines and radiation doses. In addition, the authors recognize the inherent limitations of 2D cell cultures, particularly their inability to replicate the true tumor microenvironment. Lastly, the present study did not use that same cell seeding densities in the CFA and RTCA assays; as a result, RTCA did not exactly measure growth from a single cell, as CFA does. This is a key consideration when assessing tumor regrowth after subcurative treatments (2).

Establishing and sharing reproducible models to explore radiation effects *in vitro* can improve the ability of researchers to obtain results that can be translated to clinical practice. Radiation biology had long been surpassed by technological advances in radiotherapy until very recently; however, it is currently regaining traction to elucidate outcomes from modern RT, underscoring the need for uniform, reproducible protocols that reflect clinically relevant experimental conditions.

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## Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

## Authors' contributions

JOD was involved in data curation, formal analysis, in data investigation and validation, in visualization, as well as in the writing of original draft of the manuscript, and in writing, reviewing and editing the manuscript. AVDC was involved in data investigation, methodology, in the provision of resources for the irradiation setup, and in the writing, reviewing and editing of the manuscript. DDCSADS was involved in data investigation and methodology, in the provision of resources for the irradiation setup, and in the writing, reviewing and editing of the manuscript. ISF was involved in data investigation, and in the writing, reviewing and editing of the manuscript. LBDS was involved in data investigation, methodology, the provision of resources for the irradiation setup, and in the writing, reviewing and editing of the manuscript. MDCB was involved in data investigation, and in the writing, reviewing and editing of the manuscript. MG was involved in data investigation, methodology, in the provision of resources for the irradiation setup, and in the writing, reviewing and editing of the manuscript. RG was involved in data investigation, and in the writing, reviewing and editing of the manuscript. AAJ was involved in the conceptualization of the study, in funding acquisition, in the provision of resources for experimental supplies, in study supervision, and in the writing, reviewing and editing of the manuscript. WFA was involved in the conceptualization of the study, in funding acquisition, data investigation, project administration, in the provision of resources, for experimental supplies, in study supervision, and in the writing, reviewing and editing of the manuscript. JOD and WFA confirm the authenticity of all the raw data.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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