Journal of Visualized Experiments

Irradiator commissioning and dosimetry for assessment of LQ α and β parameters, of radiation dosing schema, and of in vivo dose deposition.

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61692R1
Full Title:	Irradiator commissioning and dosimetry for assessment of LQ α and β parameters, of radiation dosing schema, and of in vivo dose deposition.
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Morgantown, West Virginia, United States

TITLE:

Irradiator Commissioning and Dosimetry for Assessment of LQ α and β Parameters, of radiation Dosing Schema, and of in vivo Dose Deposition

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KEYWORDS:

Radiation Dosimetry, Metastasis, Film, Cancer, Preclinical, Calibration

SUMMARY:

Radiation dosimetry provides a technique for enhancing the accuracy of preclinical experiments and ensuring that the radiation doses delivered are closely related to clinical parameters. This protocol describes steps to be taken at each phase during preclinical radiation experiments to ensure proper experimental design.

ABSTRACT:

Radiation dosimetry is critical in the accurate delivery and reproducibility of radiation schemes in preclinical models for high translational relevance. Prior to performing any in vitro or in vivo experiments, the specific dose output for the irradiator and individual experimental designs must be assessed. Using an ionization chamber, electrometer, and solid water setup, the dose output of wide fields at isocenter can be determined. Using a similar setup with radiochromic films in the place of the ionization chamber, dose rates for smaller fields at different depths can also be determined. In vitro clonogenic survival assays of cancer cells in response to radiation treatment are inexpensive experiments that provide a measure of inherent radio-sensitivity of cell lines by fitting these data with the traditional linear-quadratic model. Model parameters estimated from these assays, combined with the principles of biologic effective doses, allows one to develop varying fractionation schedules for radiation treatment that provide equivalent effective doses in tumor-bearing animal experiments. This is an important factor to consider and correct for in comparing in vivo radiation therapy schedules to eliminate potential confounding of results due to variance in the delivered effective doses. Taken together, this article provides a general method for dose output verification preclinical animal and cabinet irradiators, in vitro assessment of radio-sensitivity, and verification of radiation delivery in small living organisms.

INTRODUCTION:

Cancers collectively represent the second-leading cause of death in the U.S. and in many countries around the globe[1]. Radiation therapy is a cornerstone of treatment for many tumor subtypes and is administered to about half of all cancer patients[2,3]. Patient outcomes for nearly all cancers have improved over time as equipment used to deliver radiation doses has steadily advanced and some effective multimodal therapy approaches were developed[4-6], but recurrence and mortality rates for patients with certain types of tumors remain high[7-9]. Thus, radiotherapy for cancer continues to be an active area of basic and clinical research. Many pre-clinical radiotherapy studies employ the use of small-scale irradiators to deliver radiation doses to in vitro or animal models of cancers. With a multitude of potential experiments to conduct exploring mechanistic radiobiology details or novel treatments, common pitfalls may be encountered that lead to incorrect conclusions, poor reproducibility, and wasted resources. These pitfalls fall within three important areas: irradiator dosimetry, in vitro characterization of model cell lines, and in vivo irradiation dosing schedule and setup. Accurate and reproducible results from more advanced experiments are difficult to achieve without prior attention to these fundamental aspects of radiotherapy research.

The protocol detailed herein describes a generalized strategy for avoiding or mitigating these issues and draws upon several previously developed methodologies intended for independent use. These distinct methods have been merged so that a researcher interested in beginning or improving preclinical radiotherapy experiments could use this as a robust experimental layout. The suggested framework includes methodology for the commissioning of small-scale animal irradiators, for determining basic radiobiologic properties of model cancer cell lines, and for appropriately designing and administering a dosing and fractionation schedule for in vivo tumor models.

PROTOCOL:

Any steps of this protocol involving the use of laboratory animals, including handling and procedures, were approved by the Institutional Animal Care and Use Committee at West Virginia University in Morgantown, West Virginia (Protocol number: 1604001894).

1. Determination of dose output

1.1. Use this protocol, based on the "In-phantom Method" protocol of the American Association of Physicists in Medicine Task Group (AAPM TG) 61[10] and similar to the commissioning protocol set by Xstrahl, to determine beam output of the small animal irradiator with respect to particular geometry under the following setup conditions.

1.1.1. Set the irradiator to deliver radiation at 220 kVp and 13 mA, with an open field (17 cm by 17 cm) positioned at isocenter, or 35 cm from the source. Additionally, filter the beam with a 0.15 mm Cu filter with a broad focus. Some cellular irradiators contain only a radioactive source, this protocol can only be used for x-ray irradiators.

1.1.2. Align the solid water phantoms in the following order: 1 cm slab, 2 cm slab with ionization

chamber slot, 2 cm slab, 1 cm slab. Stacking the solid water phantoms in this order positions the ionization chamber at a depth of 2 cm, allowing 4 cm as well for backscatter. See **Figure 1** for a graphical portrayal of the dosimetry setup.

NOTE: To accommodate the large, fairly heavy stack of solid water, the authors recommend the acquisition of a custom 3D printed couch with variable support to ensure the phantom stack is level and at the correct distance from the source across the surface of the material, not just at the center.

1.2. Use the measurement equipment (i.e., ADCL calibrated ionization chamber, electrometer) and an explanation of correction factors used can be found in **Table of Materials** and **Table 1** respectively.

NOTE: That the ADCL provides values of N_k at a couple of points for different Half Value Layers (HVL, measure of beam quality). The value of N_k to be used in the protocol should be based on an interpolation of the ADCL values for the unit's measured HVL. The manufacturer measured the HVL of our unit and we used that in our dose rate output determination.

1.3. Set up the phantom stack and insert the ionization chamber into the phantom as specified in step 1.1.2.

1.3.1. Adjust the phantom stack such that the source to surface distance (SSD), or the distance from the radiation source to the first surface, is 33 cm when appropriately levelled.

NOTE: The authors suggest creating a custom, 3D printed couch, large enough to support the dimensions of the solid water slabs. Additionally the one, utilized in this protocol has an adjustable component for levelling the phantom stack.

1.4. Take the average of three separate x-ray exposures, one minute readings with the electrometer bias voltage set at 300 V. The result will be termed M^+.

NOTE: Irradiations are performed with the instrument set to deliver radiation at 220 kVp and 13 mA. This is the same for the next two steps (steps 1.5-1.6). For user safety, ensure the doors remain closed during treatments.

1.5. Perform another set of three separate x-ray exposures, 1 min readings with the electrometer bias voltage set at -150 V. The result will be termed M_L.

1.6. Perform another set of three separate x-ray exposures, 1 min readings with the electrometer bias voltage set at -300 V. The result will be termed M_H, or also M^-.

1.7. Calculate P_{pol} and P_{ion} using **Equation 1** and **Equation 2** respectively as described below:

$P_{pol} = (|M^+| + |M^-|)/2|M^-|$ (**Equation 1**)

$$P_{ion} = \left\{\frac{1-\left(\frac{V_H}{V_L}\right)^2}{\frac{M_H}{M_L}-\left(\frac{V_H}{V_L}\right)^2}\right\} = \left\{\frac{1-\left(\frac{-300}{-150}\right)^2}{\frac{M_H}{M_L}-\left(\frac{-300}{-150}\right)^2}\right\} = -3/(^{M_H}/_{M_L} - 4)$$ (**Equation 2**)

1.8. Measure the temperature, in Celsius, and the pressure, in kPa, inside the irradiator using a calibrated digital thermometer and barometer. Then, calculate P_{TP} as indicated below in **Equation 3**.

NOTE: This calculation assumes that the ADCL used standard temperature and pressure values of 22 °C and 101.33 kPa when stating their value for the air kerma calibration factor.

$$P_{TP} = \left(\frac{273.2+T}{295.2}\right)\left(\frac{101.33}{P}\right)$$ (**Equation 3**)

1.9. Calculate the corrected chamber reading, M, by multiplying the raw chamber reading, M_H, by P_{elec}, P_{pol}, P_{ion}, and P_{TP}. This equation can be found below in **Equation 4**.

NOTE: This calculation assumes that the ADCL performed their calibration with the bias voltage set to -300V, which is a fairly common practice.

$M = M_H P_{elec} P_{pol} P_{ion} P_{TP}$ (**Equation 4**).

1.10. Further multiply the corrected chamber reading by N_k, $[(\mu_{en}/p)^w_{air}]_{water}$, $P_{Q,\,cham}$, and P_{sheath}. P_{sheath} is only needed for measurements obtained in water. Therefore, for this protocol P_{sheath} is just 1.

NOTE: Using the conditions in this protocol, the latter three items give a value of 1.0731. This value depends on the beam quality, so the HVL must be known to determine it. The value of 1.0731 is specific to our unit and is given as an example. To determine the values of $P_{Q,cham}$ and $[(\mu_{en}/p)^w_{air}]_{water}$ specific to your unit, use the measured HVL and interpolate from **Table VII**, and **Table VIII**, and correct for the reference field size according to **Figure 3** and **Figure 4**. In our case, multiplying N_k by 1.0731 provides the dose to water, D_w, in Gy for a nominal time of 1 min, assuming the ADCL N_k value is given in Gy/Coulumbs.

1.11. Determine the end effect of the irradiator being used. When the x-rays are first generated, the output rises to its full value over some finite time. Similarly, when the x-ray source is turned off, the output decreases to zero over some finite time.

1.11.1. Account for the time for this transition, or the end effect. This can be done by taking the average of three readings with the electrometer bias voltage set at -300 V, for a variety of time settings. Do this for 6, 12, 18, 24, 30, and 60 seconds.

1.11.2. Plot the electrometer readings against time and find the best straight line. The total time, t, for a 1 minute treatment can be calculated by **equation** 5:

$t = 1 + \square t/60$ (**Equation 5**).

1.12. Calculate the dose rate for a given irradiator by **equation 6**:

$\dot{D} = D_W/(1 + \frac{\Delta t}{60})$ (**Equation 6**)

2. Creating a radiochromic film calibration curve

2.1. For a list of necessary materials, see **Table of Materials**.

2.2. Using a near identical set up as the previous protocol, place the film at a 2 cm depth in the solid water phantom stack. The order of solid water phantoms is insignificant so long as there are 2 cm of solid water above and 4 cm of solid water below for buildup and backscatter effects.

2.3. Using the determined dose commissioned in protocol 1, determine the treatment times for the doses listed in **Table 2** using equation 7

$T = \frac{D_X}{\dot{D}} * \frac{60s}{min}$(**Equation 7**)

2.4. Prepare several pieces of film ensuring that each film is of the same size and remains in the same orientation from treatment through scan acquisition. This can be done by placing a small diagonal cut in the lower left corner. Each film from this point forward must be from the same batch of film.

NOTE: Prepare 3 separate replicates for each dose point to be assessed.

2.5. Scan the cut pieces using a 48-bit color photo scanner with all corrections turned off. Ensure that each film is placed in the exact center of the scanning bed. The values obtained are the pre-exposure scans used for determining the unexposed optical density[11,12]. Save all images in the .Tiff file format to avoid compression of key data.

NOTE: The authors recommend scanning the films three times and using the obtained average as the single value for a given film.

2.6. Begin the irradiation of the films by placing a piece of film on top of 4 cm of solid water and positioned the remaining 2 cm of solid water above, as described previously in this section.

2.7. Adjust the phantom set so that the film is the same distance from the source as the ionization chamber was when determining the dose output. This is the isocentric point of the irradiator.

2.8. Program the treatment time calculated in step 2.3 above for one prescribed dose.

2.9. Repeat treatment for each of the doses listed in **Table 2**.

2.10. Allow films to rest for 24 hours protected from light.

2.11. Acquire the post-exposure film scans in the same manner as above.

2.12. Import images to ImageJ analysis software and perform all measurements on the red channel.

2.12.1. Drag the image in .Tiff file format into ImageJ.

2.12.2. Click the **Image** drop down menu. Select **Color** from **Image** drop down menu. Select split channels from **Color** option.

2.12.3. Using only the red image channel, draw a region of interest using the rectangle tool. Press **Ctrl+M**. Transcribe mean value from the results window.

2.12.4. Repeat steps 2.12.1-2.12.4 for all scanned films.

2.13. Obtain the pixel value in a centrally located 1 cm by 1 cm square for both the unexposed and exposed films. These values will be denoted as $PV_{U(D)}$ and $PV_{(D)}$ respectively, and can be used to calculate the net optical density as described in **Equation 8**.

$OD_{net} = \log(\frac{PV_{U(D)}}{PV_{(D)}})$ (**Equation 8**)[13]

2.14. Repeat step 2.13 for each pair of film images, both pre-exposure and post-exposure.

2.15. Plot a graph of the dose versus the net optical density and fit the curve to a cubic polynomial in the format of $y = ax^3 + bx^2 + cx + d$. An example can be found in **Figure 2B**.

3. Determination of α/β value for specific cancer cell lines via clonogenic assay

NOTE: The following protocol is a modified version of the methods described by Franken et al[14] and can be seen in **Figure 3**.

3.1. Grow cells to ~80% confluency. Avoid using over-confluent sources of cells for this experiment, as it is necessary that the cells are in the log-phase of cell growth. For the representative clonogenic assay results displayed in **Figure 3C**, brain-tropic MDA-MB-231 breast cancer cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin and were incubated at 37 °C and 5% carbon dioxide in a humidified incubator.

3.2. Seed the cells at the desired density for the colony assay. Accurate dilutions during the seeding are crucial to determining the assay's plating efficiency. Be sure to plate multiple replicates.

3.3. Proceed with this step if radiation treatment will precede cell plating (**Figure 3A**).

Alternatively, proceed to step 3.4 if cell plating will precede radiation treatment.

3.3.1. Perform desired radiation treatment on culture flasks. Any additional treatments (i.e. drug treatments) may be performed at any point before or after this. For the representative results in **Figure 3C**, radiation treatment occurred after plating cells, detailed in step 3.4.

3.3.2. Extract the cells using preferred trypsinization method and create a single cell suspension. Remove culture media and add recombinant enzyme (e.g., TrypLE Express) to detach cells from the flask. Incubate cells with the enzyme for approximately 3 minutes until cells were detached as detected using a light microscope. Neutralize the enzyme using an equal volume of cell culture media. Centrifuge cells at 300 x *g* for 10 min and resuspend to the desired concentration in culture medium.

3.3.3. Plate the cells at desired densities in multiple replicates.

3.3.4. Replace with fresh media after the first 24 h.

3.3.5. Continue to replace media every 2-3 days.

3.3.6. Continue culturing cells until control colonies exceed 50 cells per colony, ~9-14 days. Control colonies are those treatment groups that receive no radiation doses. For experiments using drug treatments as well, another control group with drug dosing but no radiation treatment will also be required.

3.4. Proceed with this step when seeding cells **before radiation treatment** (**Figure 3B**).

3.4.1. Extract cells using preferred trypsinization method and create a single cell suspension.

3.4.2. Place cells at desired densities in multiple replicates.

3.4.3. Allow cells to adhere to plate overnight.

3.4.4. Perform desired radiation doses. Additional treatments, such as drug dosing, may be performed at any point before or after this step, so long as cells have attached to their treatment plates. For the representative results in **Figure 3C**, 1250 brain-tropic MDA-MB-231 cells were plated prior to treatment (step 3.4). Then, cells were treated with 15 nM doxorubicin 3 hours prior to irradiation with 3 Gy of X-rays.

3.4.5. Replace the media after the initial 24 h.

3.4.6. Replace media every 2-3 days.

3.4.7. Culture the treated cells until control group colonies exceed 50 cells, ~9-14 days. Control colonies are those treatment groups that receive no radiation doses. For experiments using drug

treatments as well, another control group with drug dosing but no radiation treatment will also be required.

3.5. Remove culture media from wells or dishes, and wash with PBS.

3.6. Fix cells for 15 minutes in a 1:7 (v:v) solution of glacial acetic acid and methanol.

3.7. Remove the fixation solution.

3.8. After fixation, stain cells for 30 minutes, or 2 h if time is available, at room temperature with a 2.5-5.0 mg/mL in a 4:1 (v:v) solution of distilled water and methanol.

3.9. Remove staining solution and wash cells in a large, room-temperature water bath.

NOTE: Do not wash under running water.

3.10. Count the resultant number of colonies in each treatment group and calculate the survival fraction of each plate.

3.11. Plot the survival fraction against the corresponding dose delivered, and fit the curve with an exponential fit.

3.12. To estimate the α/β value, use an exponential fit of the above plot to estimate the values for each of the adjustable parameters in the linear-quadratic equation found below:

$SF = e^{-\alpha*D-\beta*D^2}$ (**Equation 9**)

NOTE: Irradiation of cells can typically be done at isocenter without any collimation provided the field size is large enough to accommodate well-plates or petri dishes. Potential pitfalls in this protocol may include yields such as no colony formation, significant cell migration with clear cell growth but no true colonies, or contamination due to treatment in a non-sterile irradiator chamber.

4. Determination of the specific dose output for variable experimental designs

4.1. Decide upon the desired field size and distance from source.

NOTE: Collimation will alter the dose rate no matter the size or distance of the collimator from the x-ray source.

4.2. Using solid water phantoms to provide buildup and backscatter, position a piece of film in the correct orientation that best portrays the experimental design.

NOTE: For any experimental setup solid water may not provide the most accurate representation of a given design. Instead we recommend using the actual experiment's vessels (i.e., Petri dish,

well-plates, small animal phantoms, etc.).

4.3. Irradiate films for 1 (N=3) and 2 (N=3) minutes.

4.4. Allow films to rest for 24 hours protected from light.

4.5. Determine the net optical density of each the films following the procedures from **Section 2**. Use the film calibration curve to determine dose from the net optical density.

4.6. Determine the dose at 1 minute, D_1, as the output dose rate, Ḋ, for this experimental setup defined by **Equation 10** as follows:

$$D_{exp1} = {}^{D_1}/_{1\ min} \text{ (Equation 10)}$$

4.7. Similarly, caluclate the dose at 2 minutes by **Equation 11** as follows:

$$D_{exp2} = {}^{D_2}/_{2\ min} \text{ (Equation 11)}$$

4.8. Due to the end effect, the dose rate for the above calculations may be slightly different. For this reason to calculate D_{exp} for the desired experimental design, use an average of the individual D_{exp} as indicated in **Equation 12**:

$$D_{exp}^{avg} = ({}^{D_{1exp}}/_{1\ min} + {}^{D_{exp2}}/_{2\ min})/2 \text{ (Equation 12)}$$

4.9. Using this average, define the time for treating to any desired dose for this particular setup in **Equation 13**:

$$T = D_{exp}^{avg}/D \text{ (Equation 13)}$$

5. Treating mice bearing tumors in anatomical location of interest

5.1. Anesthetize mouse with safe and humane anesthesia techniques approved by the institution's IACUC.

5.2. Place anesthetized animal in restraint as indicated in the desired experimental design.

5.3. This step is **optional, if not available proceed to step 5.6.** Obtain a radiogram, using an onboard portal camera, of the mouse without collimation using an aluminum filter.

5.4. Obtain a second radiogram with collimation in place.

5.5. Overlay radiograms in ImageJ to demonstrate beam positioning.

5.6. Using the predetermined α/β value, determine the dose scheme that provides the most reasonable approach to answer a research question (i.e., if wanting to model the effects of a dose of 30Gy delivered in 10 fractions of 3 Gy, but only wish to give four fractions). Using **equation 2,**

with an assumed α/β value of 10 (this value can be determined for individual cancer cell lines in **protocol 3**) and a BED similar to that of 30 Gy/10 F, treat with 24 Gy in 4 fractions of 6 Gy.

5.7. Treat animal for the prescribed time given for the desired dose.

6. Histological confirmation of dose deposition in vivo

6.1. Following **protocol 5**, collect tissue of interest within 1 hour of treatment[15,16]. Following tissue harvest, proceed with preferred immunohistochemistry protocol. An example is given below.

6.2. Perfuse animal with ice cold 4% paraformaldehyde (PFA).

6.3. Post-fix in PFA at 4 °C.

6.4. Following fixation, fix tissue sequentially in 10%, 20%, and 30% sucrose for 24 hours each at room temperature.

6.5. Embed tissue in gelatin and sequentially fix in 4% PFA and again in 10-30% sucrose for 24 hours each at room temperature.

6.6. Trim block and place at -80 °C for 30 minutes.

6.7. Slice tissue into 20-30 µm sections.

6.8. Immunostain slides as free floating sections in a six well-plate[17,18].

6.8.1. Wash three times and permeabilize for 30 minutes on a shaker with 1.83% lysine in 1% Triton, and 4% heat-inactivated goat serum.

6.8.2. Incubate sections with anti-γH2AX antibody for 24 hours, followed by a 2 hour incubation with desired secondary antibody.

6.8.3. Coverslip slides with glass coverslips using preferred mounting media.

6.9. Image on a fluorescent microscope.

REPRESENTATIVE RESULTS:

Following protocol 1 will provide a dose rate in Gy/min, which is specific to the irradiator being used. However, regardless of the type of irradiator, with a known dose rate a calibration curve can be generated using protocol 2 yielding similar films and a similar calibration curve to that in **Figure 2A-B**. A successful assay from protocol 3 will yield distinct, well-demarcated colonies of cells that stain homogenously violet. The estimate of α/β can be compared to literature values or other treatment groups to interpret the radio-sensitivity of the given cell line. Utilizing the

calibration curve developed following protocol 2 and displayed in **Figure 2B**, protocol 4 will yield two film samples resembling **Figure 2A** that can be used to estimate required experimental irradiation times. If an on-board portal imaging camera is available for the irradiator being used, radiograms of small animals can be obtained with and without collimation. Overlaying these images will demonstrate the exact positioning of the collimated radiation beam relative to the small animal being treated as depicted in **Figure 4A**. Successful dose-deposition in protocol 5 can be confirmed following protocol 6. One indication that radiation is being deposited in an in vivo or in vitro systems is through the detection of double stranded DNA breaks. Illustrated in **Figure 4B**, the same mouse treated solely through the right hemisphere in **Figure 4A**, demonstrates positive γH2AX staining only in the treated hemisphere. In this figure, the nuclei are stained with DAPI to show two things; 1) the whole are of the brain which the anti γH2AX antibody was applied to during histological analysis, and 2) the untreated hemisphere of the brain remains unstained.

FIGURE AND TABLE LEGENDS:

Figure 1: Rough set up of ionization chamber and water phantom set up for determination of dose output. The pictogram illustrates a basic setup utilizing the various components required for dosimetry using an ionization chamber and solid water phantoms within the cabinet of the irradiator.

Figure 2: Generation of a calibration curve using radiochromic film. (**A**) Representative color change of radiochromic film with increasing dose. Top left (0 cGy); bottom right (2000 cGy). (**B**) Potential radiochromic film calibration curve comparing net optical density and dose.

Figure 3: Clonogenic Assay of Cancer Cells. Radiation treatment of cells can be done prior to plating in six well plates/petri dishes (**A**), or after (**B**). In panel (**C**), a representative image is displayed of a successful clonogenic assay with MDA-MB-231 breast cancer cells after following Protocol Section 3.

Figure 4: Use of dual overlayed radiograms for positioning (if available) and positive γH2AX immunohistochemical staining for confirmation of dose deposition. (**A**) Representative overlayed radiograms depicting placement of radiation beam. (**B**) Representative results indicating dose deposition to the right hemisphere as demonstrated by increased γH2AX intensity.

Table 1: Correction factors needed for determination of dose rate in Protocol 1.

Table 2: Doses to be used in generation of radiochromic film calibration curve.

DISCUSSION:

The above protocol describes a user-friendly approach for radiation dosimetry, determination of α/β values in cancer cell lines, and a brief example of an approach for irradiation in a preclinical model of breast cancer brain metastasis. These methods can be used to study any model of cancer and are not just limited to brain metastasis of breast cancer. In this section we will discuss the relevant intricacies underlying preclinical radiotherapy experiments.

Dosimetry involves two parts: 1) calibrate the output with a farmer chamber, so that the dose rate of the x-ray unit is established, and 2) prepare a practical dosimetry measurement system using radiochromic film. With regards to output calibration, TG-61 provides a reproducible method in water. The protocol here uses Gammex RMI 457 solid water, as recommended by XStrahl, the manufacturer of the irradiator. Although relative dosimetry (profiles or depth dose curves normalized to maximum dose) analysis with solid water, agrees to better than 1% with that of water, there is a difference of about 3 to 4% in absolute dose due to a higher mass energy absorption coefficient for solid water compared to water. However, as all installations of the XStrahl system use the solid water protocol for output calibration, we did not correct for these differences. Knowing the output allows the calculation of the exposure time required to deliver a desired dose. Placing film in the same setup as the farmer chamber allows us to deliver known doses to the film. Scanning the film then provides optical densities. The dose to the film can then be graphed against the corresponding net optical density (difference in optical density after and before exposure). This produces a film calibration curve. When we change experimental setups, the dose rate in that situation could change, since dose rate depends on field size, depth and the material being irradiated. Exposing film with the experimental setup provides us with a net optical density, and using the film calibration curve, we can then determine the corresponding dose. Dividing this dose by the time the film was irradiated, we get the dose rate. This dose rate can then be used to calculate the exposure time to deliver a desired dose for the given experimental setup. The protocol described above handles several nuances associated with film dosimetry. For example, after exposure, the film requires approximately 24 hours for the chemical reactions in the film's active layer to be virtually complete. Not waiting for this amount of time will lead to a lower optical density.

For any study to have reproducible dosimetry it is important to know and understand several of the key elements of a given irradiator. In particular, it is crucial to know and detail to other researchers the make and model of the irradiator used, the source type (x-ray, radioactive, etc.), energy, half-value layer, field size, source to surface and source to isocenter distances, size of material irradiated, attenuation before and backscatter after the irradiated material, experiment-specific dose rate, fractionation schema, exact dosimetry equipment utilized, and the dosimetry protocol used. All of these points of information are what cohesively describe the beam quality of a given irradiator prior to delivering a dose to any animal or cell[19]. Another pertinent point of information from this protocol and others is that the dose rate achieved in **Protocol 1** is simply the output of the irradiator being used. For any given experiment it is important to define the dose rate for that particular setup (**Protocol 4**) by comparison with a generated radiochromic film calibration curve (**Protocol 2**).

In vitro experimentation provides important details about the radiobiologic behavior of cancer cell lines. In vitro clonogenic cell survival assays accurately estimate and quantify the inherent radio-sensitivity of a cell line[20], aiding in the design of fractionation schedules in subsequent cellular or small animal experiments[21]. Specifically, these assays approximate values for the parameters α and β that are used in the linear-quadratic model to predict cell death in response to radiotherapy according to the equation:

$$SF = e^{-\alpha * D - B * D^2}$$ (**Equation 9**)

where SF is the surviving fraction of clonogenically viable cells, D is radiation dose in Gy, and α and β are fitted parameters[22]. The ratio α/β provides an inherent measure of cellular radio-sensitivity, with higher values correlating with increased sensitivity of a cell line[22]. Because this functional relationship is non-linear with respect to dose, the biologic effects of a radiotherapy fractionation scheme are not only related to the total delivered dose but also the number and size of fractions[23]. The biologic effective dose (BED) is a measure of the true biological dose delivered to a tissue and permits direct comparison of different fractionations schemes[24,25]. The BED equation only requires an estimate of a/b, and is displayed below:

$$BED = n * D * \left[1 + \frac{D}{\alpha/\beta}\right]$$ (**Equation 14**)

where n is the number of fractions of dose D. Clonogenic cell survival assays estimate α/β and facilitate the direct comparison of radiotherapy fractionation schemes via the BED equation. Incorrect conclusions may be drawn regarding a tissue or organ response to radiotherapy (or combinations of radiotherapy with other modalities) if the BED in the treatment groups is not equitable within or between experiments. For example, 2 fractions of 10 Gy compared with 4 fractions of 5 Gy do not yield the same BED, and thus these dosing schemes cannot be directly compared in terms of biologic response. The BED equation, while imperfect due to inherent limitations in the linear-quadratic model, reliably estimates equitable effects for a wide range of experimental treatment conditions[24,25].

Clonogenic cell survival assays clearly play an important role in studying radiotherapy effects in cancer models, but in vitro experimentation offers a number of additional options to further explore mechanistic details of cancer cell radiobiology. Simple modifications of the clonogenic cell survival assay were used to determine the modes of action for some radio-sensitizing chemotherapies, such as paclitaxel or etoposide[26,27]. Further in vitro experimental options include immunocytochemistry studies to examine specific cellular repair pathways, such as γ-H2AX foci and/or 53BP1 staining for double-stranded DNA break repair[28]. These experiments may be of particular interest when comparing radiotherapy as a single modality with combination therapies, especially when probing mechanistic details for a given cell line. Other experimental options include cytokine measurements to examine the innate role of a cell's inflammatory response to irradiation or analyses of the mode of cell death (i.e., apoptosis, necrosis, mitotic catastrophe, etc.) under different therapeutic conditions[29-31]. This type of experimentation can complement or replace animal experimentation and provide a more complete understanding of a cancer cell line's radiobiology. Regardless of the choice of additional experiments to conduct, a standard clonogenic cell survival assay as described in protocol 3 is an important initial radiobiologic assessment of a cell line.

Clonogenic assays and radiation dosimetry provide the researcher with a means to precisely plan

experiments to more directly resemble clinical scenarios. With the addition of preclinical cancer small rodent models, it is possible to study the response to radiation alone or in the context of a treatment plan in vivo. Prior to using animals, it is important to determine the relative dose output of the specific setup if it differs from the setup used for determination of dose output[32,33]. When it comes to determining a dose rate for field sizes of <10 mm, use of an ionization chamber becomes less accurate due to alignment within a small field and partial volume averaging effects[33]. The use of radiochromic film to determine output in combination with in vivo immunohistochemical experiments has been used to determine output and dose deposition in the past[16,34-38].

ACKNOWLEDGMENTS:

The authors would like to thank the Microscope and Animal Models Imaging Facilites at WVU for the use of their equipment supporte by grant number P20GM103434. Additionally, this work was supported by grant number P20GM121322 from the National Institue of General Medical Sciences and the Mylan Chair Endowment Fund.

DISCLOSURES:

The authors have no disclosures to make.

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Figure 1

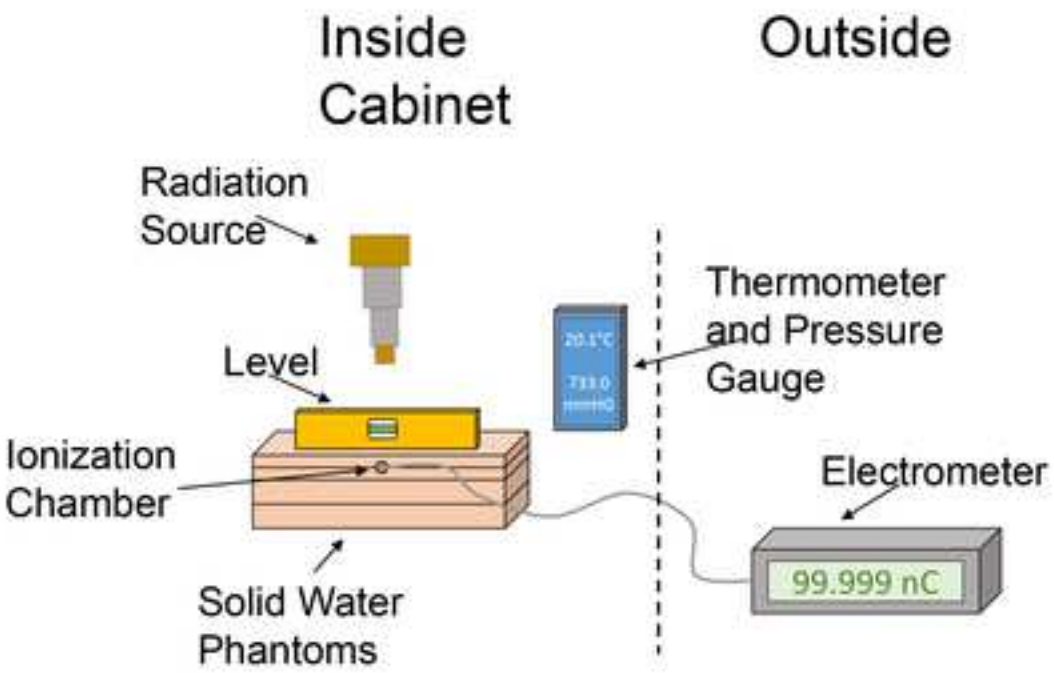


Figure 2

Click here to access/download;Figure;Figure.2_Calibration.Curve.psd

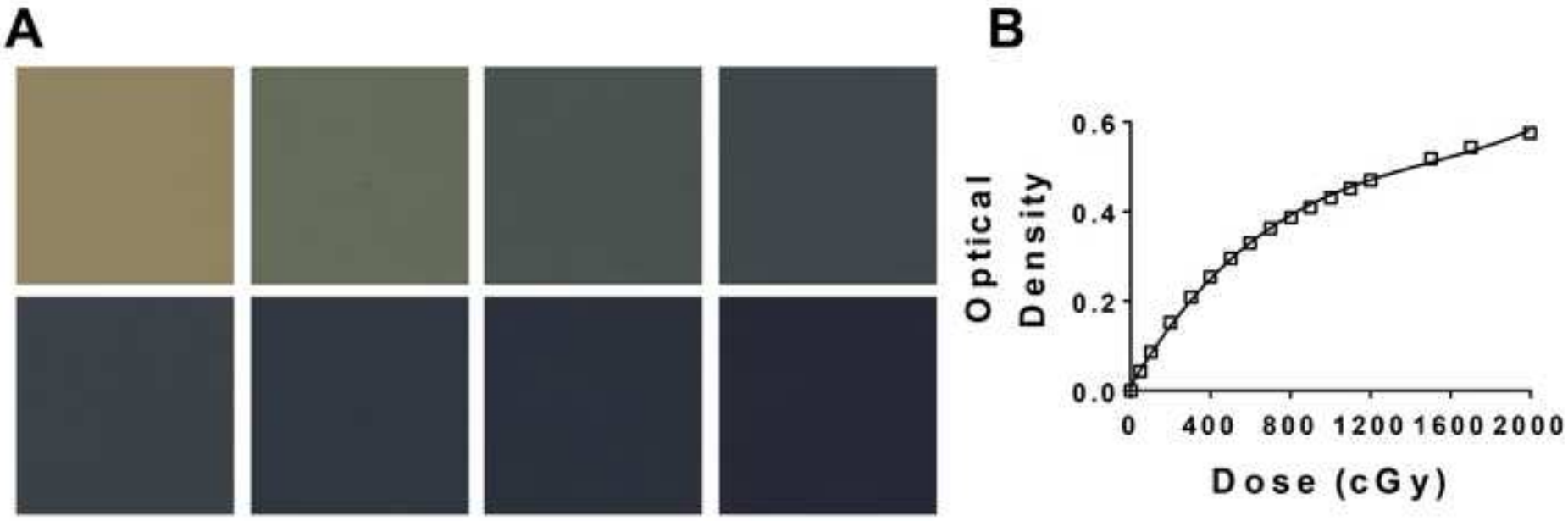
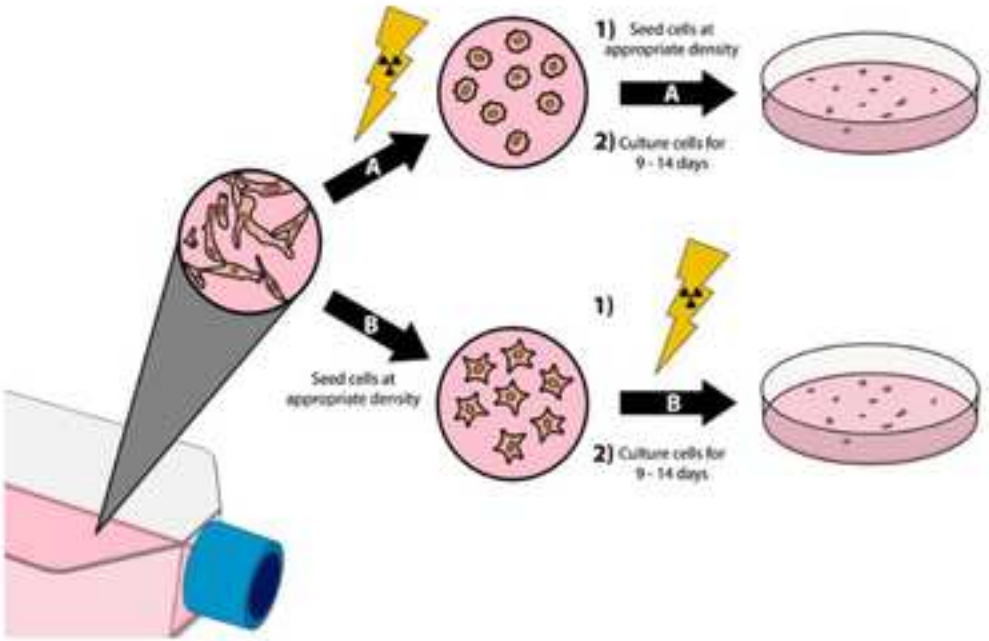


Figure 3

Click here to access/download;Figure;Figure.3_Clonogenic.Assay.psd



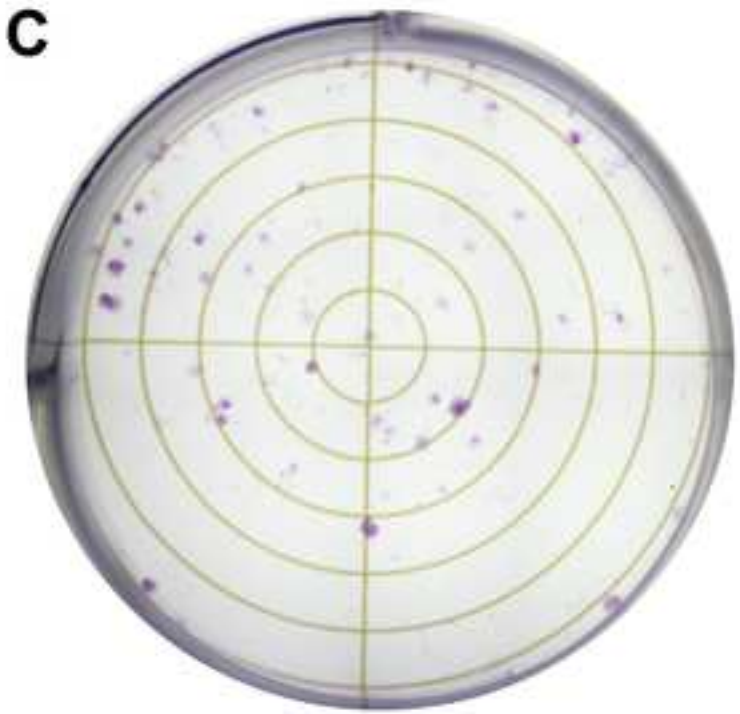


Figure 4

Click here to access/download;Figure;Figure.4_H2AX.psd

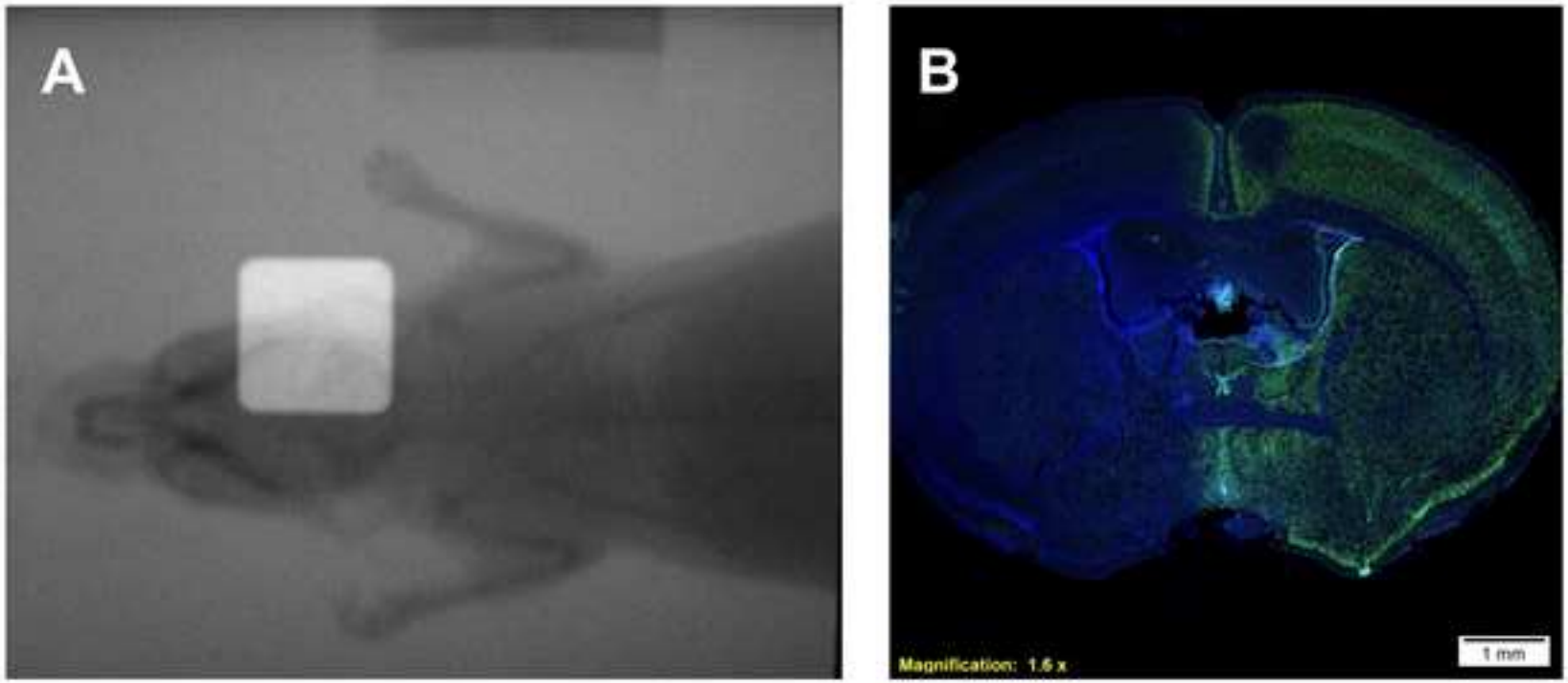


Table 1: Correction factors needed for determination of dose rate in Protocol 1.

Correction Factor	Explanation
N_K	Air kerma calibration factor
$[(\mu_{en}/\rho)^{W}_{air}]_{water}$	Ration of mass energy absorption coefficients of wat
$P_{q,Cham}$	Correction accounting for chamber stem affecting ph
P_{sheath}	Correction accounting for sheath protecting ionizatio
P_{pol}	Correction factor accounting for polarity; determined
P_{ion}	Correction factor accounting for ion recombination; c
P_{Tp}	Correction factor acocunting for temerpature and pre

er to air; approximately 1.05

oton fluence perterbation by chamber; approximately 1.022

n chamber; value of 1, as chamber is waterproof

l in Protocol 1

determined in Protocol 1

essure on day of experiment; determined in Protocol 1

Table 2: Doses to be used in generation of Gafchromic film calibration curve.

Dose	N
0.5	3
1	3
2	3
3	3
4	3
6	3
8	3
10	3
12*	3
15*	3
20*	3

*Only necessary for doses exceeding 10 for individual experiments.

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Material/ Equipment	Company
Acetic acid, glacial	Sigma-Aldrich
Crystal Violet	Sigma-Aldrich
Digital Baraometer	Fisher Scientific
Electrometer	Standard Imaging
Film	Gafchromic
Ionization Chamber	Farmer
Methanol	Sigma-Aldrich
Photo Scanner	Epson
XenX	Xstrahl

Catalog Number

A6283

C6158

14-650-118

CDX 2000B

EBT3 Film

PTW TN30013

34860

Perfection V700

NA

Comments/Description

This or comparable glacial acetic acid products are acceptable.

This or comparable crystal violet products are acceptable.

For pressure and temperature measurements.

Calibrated by an ADCL; Need correction factor, P_{elec}

Comes in sheets of 25; calibration films and experimental films must come from same set

Calibrated by an ADCL @ two calibration points

This or comparable methanol products are acceptable.

Equivalent scanners are V800, V10000, V11000, V12000

Irradiator used.

Dear Dr. Sprowls,

Your manuscript, JoVE61692 "Radiation dosimetry and its use in preclinical in-vitro and in-vivo metastatic models.," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Your revision is due by **Jul 20, 2020**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

Vineeta Bajaj, Ph.D.
Review Editor
JoVE
617.674.1888
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Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Editorial comments:

NOTE: Please read this entire email before making edits to your manuscript. Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Introduction:** Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the

technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.
We have revised the introduction and feel that the text describes the technique and its importance in the context of radiobiological research adequately.

• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) Examples NOT in the imperative: 1.1, 1.2, 1.11 etc.
These grammatical errors have been corrected throughout the manuscript.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:
1) Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.
2) 1.4-1.6: It is unclear how the readings are performed. Is the irradiator on during these steps? Briefly mention precautions for experimenter safety.
3) 1.8: What is the measurement cite? How is pressure measured?Is this atmospheric pressure in the room? Mention all instruments used.
4) 1.9, 1.10: Define all variables and mention their values.
5) 1.11: Unclear what is being done here. Check missing variable symbols.
6) 2.2: Avoid personal pronouns "you" and "your".
7) 2.8: Specify the step where this was calculated.
8) 2.12: Mention all software steps including button clicks and menu selections.
9) 3.2: Mention cell line, growth media and culturing environmental conditions.
10) 3.4..A: When was the treatment performed?
11) 3.4.A.1: Specify treatment parameters
12) 3.4.A.2: Describe the steps for trypsinization in greater detail including neutralization.
13) 3.4.A.6: define the control conditions.
14) Your manuscript title to too broad. Please focus it on the protocol and results presented.
We thank the editor for pointing out these issues. We fixed them to the best of our knowledge. Additionally, the variables referred to in item 4 among others are listed and described in table 2.

• **Protocol Numbering:**
1) Please adjust the numbering of your protocol **section 3.4.>/b>; 1. should be followed by 1.1. and then 1.1.1. if necessary.**
The numbering in the protocol now aligns appropriately.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

• **Results:**
1) We require results (figures/tables) that demonstrate the success of your technique, this can be an application of your method to a specific study or general results that validate the technique. These must be fully discussed in the Representative results. The current results do not sufficiently support and validate the technique you present.
This protocol describes a method for getting to the point of doing radiobiological experiments. The Results are the calibration curve, the *in vivo* response in figure 4, as well as the outcome of the clonogenic assay described in protocol 3. We have tried to redefine this in our results section to be clearer.

2) Results in Fig 4 must be discussed in greater detail. Currently it is unclear what was achieved and whether or not this is a successful outcome.
Figure 4 has been explained in greater detail regarding what was achieved and how it is a successful result.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

• **Figures:**
1) Fig 4A: This figure is quite blurry. Is a better figure available?
This is an image of an entire brain to demonstrate a large area of targeted irradiation. A better image has been updated.

• **References:** Please spell out journal names.
The references should now be in the preferred format.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Gafchromic®, solid water,
All commercial sounding language has been removed. As an additional note to this point, the reviewers mentioned the use of our irradiator out right. We are told not to include the manufacturer or instrument name per JoVE.

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by

"(see table of materials)" to draw the readers' attention to specific commercial names.
2) Please check figures as well.

• **Table of Materials:**
1) Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include all items used such as phantoms, ionization chambers, thermometers, pressure gauge, irradiator, cell lines, antibodies, animal strains etc.
2) Sort the list alphabetically.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The purpose of this submission is to describe dosimetry methodology for a preclinical irradiator. Proper dosimetry is an essential experimental component in radiation biology that is too often underappreciated. Many manuscripts in our field unfortunately gloss over these important details that can have a major impact on our results. In that sense, this manuscript provides fundamental knowledge and I can see my lab using this as training materials and to ensure proper calibration. The protocol provides basic instructions for a complete radiobiology assessment starting with dosimetry all the way to animal irradiations. A big challenge is balancing specificity versus general use. Clearly the authors have a specific use in mind though the procedures are widely applicable. Overall, this is a good manuscript that would be quite useful to the radiobiology field but which may need a little bit of re-framing in a more generalized way.

Major Concerns:
1. The introduction seems out of place. Why the focus on CNS tumors when the methods described have much broader application. Roughly 50% of all cancer patients receive radiotherapy. The methodology described is applicable to virtually every cancer model. This is in fact mentioned be the authors in the discussion.
We thank the reviewer for pointing out this comment. The introduction has been reworked to specifically include all cancer types and not solely pertain to brain tumors.

2. At times, it feels like the authors have covered too much: the discussion of clonogenic survival and fractionation are not really directed to dosimetry. They are still important components of radiobiological

experiments. Maybe a title that better reflects all of the elements of this protocol in a general sense would be better
We kindly thank and agree with the reviewer on this point. The title has been reworded to better reflect the scope of the manuscript.

Minor Concerns:
1. Looking at section Protocol 1.1.1, no information is given for which irradiator is being used (X-RAD, SAARP, etc.). Could this section include a mention of how this can be generalizable to any irradiator? That would make this protocol more broadly useful.
Per JoVE we are instructed not to mentioned manufacturer specific details.

2. Looking at section Protocol 1.1.9, I believe you meant equation 4 and not 3.
This mistake has been corrected.

3. I have minor concerns about the histological method.
a. Blocking usually refers to the addition of serum or some other protein to prevent non-specific binding. Instead that is included under permeabilization.
b. The hydrogen peroxide is only relevant if the secondary is conjugated to horseradish peroxidase (HRP).
c. For incubations, usually temperature is also included. A 24 hour incubation with the primary, for example, would usually be done at 4º C
We thank the reviewer for pointing out this issue. The protocol was incorrect as it was written, but has been fixed to accurately shown the method used.

Reviewer #2:
Manuscript Summary:
The aim of this work is to provide dosimetry techniques to establish accurate delivery of radiation dose in preclinical experiments. This is accomplished by providing step by step directions on how to obtain dose output and film calibrations for preclinical irradiators to construct experiment-specific treatment times derived from dose rate calculations. Determination of alpha/beta values through clonogenic assays for mouse tumour irradiations and histological confirmation protocols are also described.

This is an important work as there is a critical need for establishing proper dosimetry in preclinical radiobiological research, as a large proportion of these studies have poor dosimetric accuracy and reproducibly [4]. I believe a video format for carrying out preclinical dosimetry would help alleviate this deficiency in the field. However, there are a number of comments and concerns I have made below that must be properly addressed before I can recommend this work for publication.

In General, the protocols are sparse and most of the steps could benefit from a higher level of detail and explanation. It appears that the authors have written dosimetry protocols for a very specific preclinical irradiator, but the abstract and protocols feel vague enough as if it they may have wanted the dosimetry to be used for any preclinical irradiator. Any details are not brought to light until the Discussion section at the end of the document. I would recommend that the authors establish a clear focus on what preclinical irradiators this protocol is meant for; if it's written for an Xstrahl SARRP, or if it's written for only commercial image-guided small animal irradiators, or if it's written for any preclinical irradiator, make it known from the beginning and tailor the step by step dosimetry protocols to that. The

dosimetry protocols needed between different preclinical irradiators require different steps and details in their setup and dosimetry due to their different geometry and components.
We thank the reviewer for their thorough and meticulous approach in reviewing our manuscript. We hope the comments below are sufficient to answer any concerns, major or minor.

Major Concerns:
1) Dose output for kilovoltage beams is only half of the story regarding accurate and reproducible dosimetry. This submission is missing HVL beam quality measurements as included in the AAPM TG-61 and must be included in this protocol to fully describe the dosimetry of a kilovoltage irradiator [1,2]. An HVL measurement is needed to properly obtain the mass energy-absorption coefficient of Table VII in the AAPM TG-61 for use in the dose calculations following Equation 4 in the AAPM TG-61, as using a nominal HVL is not appropriate. Even with a specific dose output for kilovoltage beams, a change in beam quality (beam quality is defined by both kVp and HVL together for kilovoltage beams) may affect the biological response even when using the same dose rate [3]. Beam quality measurements are also key in establishing reproducibility for preclinical studies [4].
The HVL was not measured in our laboratory. HVL was measured and provided to us by the manufacturer. Including this measurement in the protocol would only add to the intense detail. It seems more than likely that this information can be provided by most manufacturers.

2) I recommend creating an introduction to this protocol that addresses the following comments regarding sections 1.1 through 1.2. The journal requires a "sufficient introduction for the protocol" which this submission is missing. The authors present some information in the Discussions section but need an introduction. It is also unclear to the reader (until much later on) that the dosimetry in Section 1 (Equation 6) is used for calibrating film and not used for experiment-specific irradiations. This should also be addressed in an introduction.
The introduction has been rewritten entirely to encompass both the suggested details as well as a larger focus on all cancer types, rather than those of solely the CNS.

3) 1.1.1: Define 35 cm from the source as "isocenter." Also, is your protocol intended to be used for any preclinical irradiator or written for a specific preclinical irradiator? (Based on 1.1.1 it looks like you're using the Xstrahl SARRP for your protocol). If so pleases state this, if not, state which types of preclinical irradiators this can be used for.
We have defined isocenter in the text as 35cm from the x-ray source.

4) 1.1.1 What about preclinical irradiators that cannot change the field size at isocenter, or do not use a 0.15 mm Cu external filtration, or do not operate at 220 kVp and 13 mA? All of the different types of standard non-image-guided and image-guided preclinical irradiators use different kVp, mA, filtration, treatment isocenter, field sizes, etc. Thus the parameters you propose in 1.1.1 are only possible with a certain preclinical irradiator and are vastly different for other preclinical irradiators (i.e., PXi SmART uses 0.30 mm of external filtration, PXi XRAD 320 operates at 320 kVp, ect.). In order to establish this protocol, you either need to state that these methods of 1.1.1 are only for a specific irradiator, or it must be changed to recommend using the beam energy, filtration, collimation, isocenter, etc. that is actually used for a user's specific irradiator.
We are unable to state explicitly in the text that we used the Xstrahl XenX. However, in the discussion we point out that many different factors affect dose output and therefore experimental dosing parameters will vary from instrument to instrument.

5) 1.1.1 What about preclinical irradiators that have a different isocenter distance? (i.e., Xstrahl SARRP and PXI SmART have 35 and 30 cm isocenter distances, respectively, and the PXi XRAD 320 has a much farther isocenter), would you recommend all units measure at a 35 cm distance regardless of their treatment isocenter, or would you recommend obtaining the dose rate at the treatment isocenter of the respective preclinical irradiator?
We do not recommend using 35cm for all irradiators, but rather suggest that the readers use the isocenter for their individual units.

6) 1.1.1 You need to state that you're using the "In-phantom Method" as there is also the in-air method in the AAPM TG 61. The AAPM TG 61 in-phantom method states that absorbed dose to water should be measured at 100 cm SSD, 2 cm depth, and a 10 x 10cm2 field size, so you need to mention that your protocol is a modified version of the AAPM TG-61 due to geometry constraints.
This detail has been added to section 1.1.

7) 1.1.2 Do you have a recommendation on the size of the solid water slabs other than the depths, i.e., 20 x 20 cm2 vs 6 x 6 cm2. What about the types of solid water, for kilovoltage dosimetry non-commercial pieces of solid water vary wildly in their dosimetry properties. [5] Do you recommend using a commercial solid water phantom such as GAMMEX RMI-457? How do you place the solid water in the irradiator? i.e., it may be challenging to setup and position on the Xstrahl SARRP couch (couch bows) for large solid water slabs, but easier in the PXi XRAD 320 with its large platform.
We recommend the larger (20 x 20 cm^2) solid water slabs, as those are what we are accustomed to working with. Yes commercial phantoms, in this protocol we used GAMMEX RMI-457, are recommended to be used. The solid water was placed upon a custom 3D printed couch to prevent bowing and has a variable design to allow for proper levelling and SSD determination across the surface of the phantom stack. This information has been added to the protocol.

8) 1.2. Tables 1, 2, 3, and 4 are missing from the submission and so I cannot review them.
Tables were provided for the first submission. It is possible that they were somehow lost when the PDF was compiled. The tables will again be provided.

9) 1.10 The statements here are incorrect. The mass energy-absorption coefficient ([(μen/p)wair]water) can only be accurately obtained my measuring the half-value layer (HVL). Using a non-measured nominal value is inappropriate. HVL describes the beam quality for the respective mass energy-absorption coefficients for the specific irradiator, and different irradiators will have different values. Even two Xstrahl SARRPs will have different HVLs (and thus different mass energy-absorption coefficients) with a common HVL being around 0.67 mm Cu but some Xstrahl SARRPS have as low as 0.59 mm Cu HVL. The HVL of a PXi SmART irradiator is around 1.0 mm Cu.
The value used in our experiments is not a nominal value. The HVL was provided by the manufacturer as stated previously and in the manuscript we have suggested the reader to either measure this themselves, or contact the manufacturer.

Minor Concerns:
We thank the reviewer for the following minor concerns and have made all of the suggested linguistic changes. Where necessary, the minor comment response was elaborated.

10) Line 25: I would recommend adding "…accurate delivery and reproducibility…" as reproducibility has been seen as a problem that proper dosimetry can address in preclinical irradiations.

11) Line 27: Would replace recommend replacing "instrument" with "irradiator" with regards to dose output.

12) Line 29: You can just state "similar setup" instead of "very similar setup."

13) Line 70: Define AAPM TG 61 (American Association of Physics in Medicine Task Group 61). Also state that the AAPM TG 61 determines beam quality for kilovoltage beams between 40 and 300 kVp.

14) 1.1.1 In addition, some cell irradiator use a radioactive source and these protocol are not appropriate for radioactive sources, and thus you must mention that this dosimetry protocols can only be used by x-ray irradiators.

15) 1.2 Need to state that the Farmer ionization chamber must be calibrated or by or traceable to a calibrated ionization chamber from an Accredited Dosimetry Calibration Laboratory (ADCL).

16) 1.3 With setting up the phantom stack, it does not mention here or in 1.1.2 what the source to surface distance (SSD) is of the solid water. There is also no discussion describing leveling the solid water (only shows a level in Figure 1), and does not mention how to center the solid water/ionization chamber/film. For the SSD do you trust the lasers for positioning (may not be accurate)? Do you have a tool such as an SSD-pointer to verify the z-axis depth position of the solid water?

17) 1.8 The nomenclature is Celsius instead of Centigrade.

18) 1.8. Where do you recommend taking the temperature, inside the cabinet of the irradiator our outside the cabinet? Where do you recommend obtaining the pressure from, a calibrated barometer inside the irradiator cabinet, outside the irradiator cabinet but inside the room? Most pressure is recorded in mmHg, so it might be easier for the reader to have your Equation 3 converted to mmHg and presented as PTP = ((273.2 + °C) / 295.2) *(760/P). This conversion to mmHg is presented in the Appendix C.2 in the AAPM TG-61.

19) 1.9 The equation referenced should be Equation 4 (not Equation 3). You also don't mention how to obtain Pelec.

20) 1.10 PSheath is only needed if measurements are obtained in water, so since you recommending solid water measurements the factor is just 1.

21) 1.10 You need to define what Nk is and how to obtain it.

22) Line 115: Consistency is important when using an established protocol such as the AAPM TG-61. I recommend writing down Equation 4 of the AAPM TG-61 (the in-phantom method) and asking the reader to input their values into that equation to obtain dose to water Dw. This equation and converting Dw into dose rate is also presented in Appendix C.2 in Equations 11 and 12 in the AAPM TG-61.

23) 2.2. How do you define "near identical?" Is using film not the same identical setup? I would still describe the film setup here even if you did write a similar setup in section 1.1.2.

24) 2.2. Do you recommend any type of Gafchromic film or just EBT 3? Is the process different if you use EBT 3 vs EBT or EBT2?
We recommend using EBT3 film as it is appropriate for dose deposition to film for up to 1000cGy, and reaches a saturation point near 2000cGy. For the purposes of our work and many other preclinical radiation experiments, it is unlikely that the dose delivered will fall outside of this range.

25) 2.2.1. The thickness of the individual solid water slabs underneath the film is insignificant as long as you just state that you just need a total of 4 cm of solid water under the film for backscatter.

26) 2.3. Be specific. State that the time obtained from the dose rate calculation using Equation 6 is what determines the treatment times.

27) 2.4 Why 2 cm by 2.5 cm film? Would it not be simpler to cut squares? Label them with a sharpie or a pen? The pressure of using a pen can damage the integrity of the film for analysis. You can also maintain the same orientation by cutting a small corner off of the film on the bottom left.

28) 2.4 Be specific, how many pieces do you need for the zero-dose scans? Also mention that all films, including the zero-dose and irradiated films, all need to be from the same batch of film to be calibrated.

29) 2.5. 48 bit color photo scanner? Why this? Do you recommend a professional scanner such as an Epson 10000 XL? Do you only use the Red channel or all three (red, blue, and green). Do you save in the .Tiff file format? (you should save in an uncompressed .Tiff). Please elaborate in more detail and reference any film techniques from published papers to validate your choice of film protocol.

30) 2.5 - 2.6. Your transition from the pre-scanning setup of the film in 2.5 to the irradiation setup of the film 2.6 is abrupt, and it is unclear to the reader that the transition is happening. Consider better organizing the pre-irradiation, irradiation, and post-irradiation film sections.

31) 2.7 State that the film depth has to be positioned specifically at the center of the ionization chamber, aka, isocenter.

32) 2.10 Many papers recommend specifically scanning 24 hours after irradiation before scanning.

33) 2.11 Do you scan one film at a time? Nine at a time? Do you scan each film only once or three times and take an average reading? (You should scan multiple times and take an average to reduce noise). Do you only irradiate a single film per dose point or do you irradiated three films per dose point? (You should irradiate multiple films per dose point). This is because optical densities in Gafchromic film for kilovoltage energies can vary to a large degree for the same dose exposure, and it is recommended that each dose point be irradiated three times and then averaged [6, 7]. Please cite a paper here describing a more detailed film scanning protocol.

34) 2.14 It is unclear what "each pair of film images" means. Does this mean every single irradiated film also have an unirradiated film that pairs with it? Or does it mean that each dose point had a pair of films irradiated?

35) 4.2. The determination of setup-specific dose is very important and a fantastic addition to this work. You want to simulate approximately the same amount of attenuation and backscatter as you would get from your experimental setup using a petri dish, well chamber, etc. The dose rate from having too much attenuation and/or backscatter from solid water could be greatly different compared to the dose rate given to cells with minimal attenuating and backscattering materials present in the actual experimental setup.

36) 4.3 Three films should be irradiated and averaged as film has been shown to vary between exposures.

37) 4.6. The equation you are referencing is Equation 9 (not Equation 3)

38) 4.9 The equation reference number is wrong again. Define that "D" is any desired dose. Also, shouldn't Equation 12 be, T = DAvg / D?

39) 5.3 By radiogram do you mean obtaining an electronic image using an electronic portal imaging device (EPID)? If so please specify.

40) 5.4 and 5.5 Please include more detail on how exactly you are using the radiogram/EPID for positioning.

41) Line 312-314: In addition to field size, depth, and material being irradiated, other factors that can change the dose rate are changes to the HVL, SSD, attenuation before the material, backscatter after the material, and any changes to the geometry of the setup.

42) Discussion Section: In order for a study to have reproducible dosimetry, all of the following factors should be clearly stated and documented in every radiobiology study: The irradiator make and model, source type, energy, HVL, field size, SSD, material being irradiated, size of material being irradiated, attenuation before the material, backscatter after the material, the depth of the material, the experiment-specific dose rate, fractionation, make and model of all dosimetry equipment used, and the dosimetry protocol used. [4] This information is extremely important for dosimetry and should be stated in the Discussions section.

43) Discussion Section: In the Discussions section it should be made clear that the dose rate achieved in this protocol for a 2 cm depth in water (the in-phantom method of the AAPM TG-61) is for film calibration, and should not be the dose rate used for delivering cell irradiations, and that Section 4 should be used to acquire study specific dose rates for cell irradiations. The attenuation, scatter, and backscatter of a 220 kVp beam will change the dose rate at a depth of 2 cm by over 30% compared to the dose rate at the surface of a solid water phantom [7].

44) Equation 1: The AAPM TG-61 has Ppol = |(M+raw - M-raw) / 2Mraw|, your equation is not consistent to this.

45) Equation 2: The AAPM TG-61 has Pion = 1-(VH/ VL)2 / MHraw/ MLraw - (VH/ VL)2. If you are simplifying the AAPM TG-61 equation, I would recommend including the full equation and then simplifying.

46) Equation 7: Please cite a reference for the OD equation here.

47) Equation 9: For the Dose Rate D, I recommend inserting a subscript such as DExp to not confuse the reader between the dose rate of Equation 6 and the experimental dose rate.

48) Equation 12: Shouldn't this be T = DAvg / D?

49) Figure 1: The ionization chamber/film are not labeled. The Radiation Source is stated but is not shown where in the figure. State "Inside Cabinet" and "Outside Cabinet."

50) Figure 2: Figure 2A shows 8 film exposures but Figure 2B shows 17 dose points. It would be nice to have the Dose delivered to the films in Figure 2A labeled, including the 0 Gy film, and then seeing the same dose points labeled in Figure 2B

51) Tables: Line 274 states that there are Table Legends but they are not present in the PDF document. The actual tables themselves are also missing and thus I cannot review them.

References:
[1] C. Ma et al. AAPM Protocol for 40-300 kV x-ray beam dosimetry in radiotherapy and radiobiology. Medical Physics 2001.
[2] Y. Poirier et al. A simplified approach to characterizing a kilovoltage source spectrum for accurate dose computation. Medical Physics 2012.
[3] Y. Poirier et al. The Potential Impact of Ultrathin Filter Design on Dosimetry and Relative Biological Effectiveness in Modern Image-Guided Small Animal Irradiators. British Journal of Radiology 2018.
[4] E. Draeger et al. A Dose of Reality: How 20 Years of Incomplete Physics and Dosimetry Reporting in Radiobiology Studies May Have Contributed to the Reproducibility Crisis. International Journal of Radiation Oncology Biology Physics 2020.
[5] R. Hill et al. The water equivalence of solid phantoms for low energy photon beams. Medical Physics 2010.
[6] L. Wack et al. High throughput film dosimetry in homogeneous and heterogeneous media for a small animal irradiator. Physica Medica 2014.
[7] C. Johnstone et al. MicroCT imaging dose to mouse organs using a validated Monte Carlo model of the small animal radiation research platform (SARRP).Physics in Medicine and Biology 2018.

Reviewer #3:
Manuscript Summary:
This manuscript details a dosimetry protocol for biological irradiations for the Xstrahl SARRP irradiator. Though the SARRP is never named explicitly, it is definitely the SARRP - it includes the SARRP phantom included in its calibration kit, its calibration geometry, large-field calibration protocol, etc.. The 0.15 mm Cu filter, for instance, is only used for this particular irradiator, and therefore the protocol cannot be adapted to many other irradiators. The manufacturer is only mentioned in the discussion and then, not the model - which is crucial, as several Xstrahl irradiators exist, but this particular protocol is specific to the SARRP.

The protocol is sound, except for the missing crucial step of HVL measurement to obtain the values detailed in L102-105. The protocol is not particularly novel and should be fairly obvious for experts in the field, but this is not a publication criteria for JOVE. It is based on the use of TG-61 to calibrate EBT3 Gafchromic film which is then used to measure dose in the experimental position. Nothing in this

protocol is particularly ground-breaking, though it does not exist in its present form to serve as a resource for physicists who have not yet performed it and as such is of interest to new physicists approaching the SARRP for the first time.

The biological protocol is similarly not ground-breaking, as it details a fairly standard cell-curvival curve methodology, but is nicely laid out and explained and would be of use to people newly entering the field. It is better supported than the dosimetric portion of the protocol. Many biological experiments are detailed, which makes the protocol .

Since the protocol is specific to this irradiator, and this irradiator also contains many moving parts (small collimators, rotating gantry, moveable couch, etc...) that make it non-ideal for cell irradiations (one of the experiments covered in the protocol), greater discussion should be had in its limitations and in which parameters should be considered if an other irradiator was to be calibrated (hint - different HVL, different uen/rho, Bw factors, different Nk for the chamber from the ACDL, field size considerations, distance considerations, etc...)

The major concerns with the protocol follow in the next section.

Major Concerns:
The protocol is essentially identical to the Xstrahl SARRP's manufacturer's protocol, but at no point is the SARRP mentioned and the authors are presenting this work as though it was entirely of their own design. At the very least, manufacturer's protocol should be cited and acknowledged.

1) Measurement of the Half-Value Layer, which determines the correction factors from TG-61. It is unclear why the protocol details the minutia of the Ptp, Pion, Ppol correction factors, without mentioning the HVL and the important correction factors it details - Backscatter factor, and mass-energy attenuation coefficient ratio. Instead, these values are given verbatim (L103-105), though they can change irradiator to irradiator. Thus, the most crucial step of the entire operation is missing.
The measurement of Half-value layer for our particular irradiator (Xstrahl, XenX) was not measured in our laboratory, but was provided and measured by the manufacturer. Adding this information into the protocol would make this manuscript cover an even larger swath of detail than currently provided.

2) Nk is never discussed. The determination of Nk is not trivial, as ACDL are typically incapable of producing a beam identical to the Xtrahl SARRP (220 kVp + 0.15 mm Cu filtration), and therefore is never entirely correct. A lot of thought is put into determining which beam qualities best approximate the SARRP HVL=0.65 mm Cu/220 kVp spectrum, and this is missing from the protocol.
Determination of Nk was not measured in our lab. Our ionization chamber was sent out to an accredited ACDL laboratory for proper measurements. While we agree this measurement is definitely not trivial, the total error produced from the combination of all corrective factors in this protocol is less than 2%.

3) The figures are of low quality, being of very low resolution. Worst offenders are Figures 1, 2
Higher qualities figures have been provided.

4) The text refers to Tables 1-4, but no such tables are in the text. There is an additional material (page 20), but this leads to an empty table with only three headings: Company, Catalog Number, and Comments/Description
Tables were provided for the first submission. It is possible that they were somehow lost when the PDF was compiled. The tables will again be provided.

5) L200- The choice of "sufficient buildup and backscatter" are not trivial - see for instance Chen et al, Impact of backscatter material thickness on the depth dose of orthovoltage irradiators for radiobiology research, Phys Med Biol 64(5) 055001 (2019) or Subiel et al., The influence of lack of reference conditions on dosimetry in pre-clinical radiotherapy with medium energy x-ray beams, Phys Med Biol 65(8) 2020. This *must* be better described, as use of insufficient backscatter (up to 10 cm in some circumstances) will lead to incorrect dosimetry.

We thank the reviewer for acknowledging this deficit. It is hard to provide an accurate measure of buildup and backscatter for a given experiment when writing a broad protocol. A medical physicist and the researcher, while paying specific attention to their experimental design, should decide upon that particular piece of information. There would not be much use for the authors to recommend using a particular amount of buildup and backscatter material without consulting about a given experiment first.

We have also added a note to this section describing a scenario in which the most accurate way to measure dose of a particular design is to implement the use of film within a well-plate or dish when applicable.

Minor Concerns:

L72-74: This is the maximum field size when no collimation is used, but the text implies that the field should be set to 17x17 intentionally.

We have fixed this contextual error to make it more clear that this size is the maximum field size and cannot be set.

L102: Spell out the equation, as in TG-61 - this is critical

All equations are written to best reflect the calculations performed.

L147: The equation should be spelled out, like the others the equation of best fit should be shown with the figure when it is produced

The general format of a cubic equation has been detailed in the text.