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TITLE:

Dosimetry for Cell Irradiation Using Orthovoltage (40–300 kV) X-Ray Facilities

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KEYWORDS:

dosimetry, low energy X-rays, radiobiology, irradiation protocol, cell irradiation, X-ray facility

SUMMARY:

This document describes a new dosimetry protocol for cell irradiations using low energy X-ray equipment. Measurements are performed in conditions simulating real cell irradiation conditions as much as possible.

ABSTRACT:

The importance of dosimetry protocols and standards for radiobiological studies is self-evident. Several protocols have been proposed for dose determination using low energy X-ray facilities, but depending on the irradiation configurations, samples, materials or beam quality, it is sometimes difficult to know which protocol is the most appropriate to employ. We, therefore, propose a dosimetry protocol for cell irradiations using low energy X-ray facility. The aim of this method is to perform the dose estimation at the level of the cell monolayer to make it as close as possible to real cell irradiation conditions. The different steps of the protocol are as follows: determination of the irradiation parameters (high voltage, intensity, cell container etc.), determination of the beam quality index (high voltage-half value layer couple), dose rate measurement with ionization chamber calibrated in air kerma conditions, quantification of the

attenuation and scattering of the cell culture medium with EBT3 radiochromic films, and determination of the dose rate at the cellular level. This methodology must be performed for each new cell irradiation configuration as the modification of only one parameter can strongly impact the real dose deposition at the level of the cell monolayer, particularly involving low energy X-rays.

INTRODUCTION:

The aim of radiobiology is to establish links between the delivered dose and the biological effects; dosimetry is a crucial aspect in the design of radiobiological experiments. For over 30 years, the importance of dosimetry standards and the harmonization of practices have been highlighted[1–5]. To establish a dose rate reference, several protocols exist[6–10]; however, as shown by Peixoto and Andreo[11], there can be differences of up to 7% depending on the dosimetric quantity used for the dose rate determination. Moreover, even if protocols exist, it is sometimes difficult to know which protocol is the most suitable for a particular application, if any, because the dose rate for the cells depends on parameters such as the cell container, quantity of cell culture media or beam quality, for example. The scattering and the backscattering for this type of irradiation is also a very important parameter to take into account. Indeed, for low and medium energy X-rays, in the AAPM TG-61 reference protocol[10], the absorbed dose in water is measured at the surface of a water phantom. Taking into account the very specific cell irradiation conditions, the small volume of cell culture media surrounded by air is closer to kerma conditions than those defined for an absorbed dose with a large water equivalent phantom as in the TG-61 protocol. Therefore, we have chosen to use the kerma in water as a dosimetric quantity for reference rather than the absorbed dose in water. Thus, we are proposing a new approach to provide a better determination of the actual dose delivered to cells.

Moreover, another crucial aspect for radiobiological studies is the complete reporting of the methods and protocols used for irradiation in order to be able to reproduce, interpret and compare experimental results. In 2016, Pedersen et al.[12] highlighted the inadequate reporting of dosimetry in preclinical radiobiological studies. A larger recent study from Draeger et al.[13] highlighted that even though some dosimetry parameters such as the dose, energy, or source type are reported, a large part of the physics and dosimetry parameters that are essential to properly replicate the irradiation conditions are missing. This large scale review, of more than 1,000 publications covering the past 20 years, shows a significant lack of the reporting of the physics and dosimetry conditions in radiobiological studies. Thus, a complete description of the protocol and the method utilized in radiobiological studies is mandatory in order to have robust and reproducible experiments.

Taking into account these different aspects, for the radiobiological experiments carried out at IRSN (Institute of Radiation Protection and Nuclear Safety), a stringent protocol was implemented for cell irradiations in an orthovoltage facility. This dosimetry protocol was designed in order to simulate the real cell irradiation conditions as much as possible and thus, to determine the actual dose delivered to cells. To this end, all the irradiation parameters are listed, and the beam quality index was evaluated by measuring the half value layer (HVL) for which some adaptations have been made as the standard recommendations from the AAPM protocol[10]

cannot be followed. The absolute dose rate measurement was then performed with the ionization chamber inside the cell container used for cell irradiations, and the attenuation and the scattering of the cell culture media was also quantified with EBT3 radiochromic films. As the modification of only one single parameter of the protocol can significantly impact the dose estimation, a dedicated dosimetry is performed for each cell irradiation configuration. Moreover, the HVL value must be calculated for each voltage-filter combination. In this present work, a voltage of 220 kV, an intensity of 3 mA, and an inherent and an additional filtration of 0.8 mm and 0.15 mm of beryllium and copper, respectively, are used. The cell irradiation configuration chosen is on a T25 flask, where cells were irradiated with 5 mL of cell culture media.

PROTOCOL:

1. Irradiation platform and determination of irradiation parameters

1.1. Use an irradiation platform delivering low to medium energy X-rays. Determine the parameters of the experiment to ensure the robustness and the reproducibility of the radiobiological experiment: High voltage, Intensity, Filtration (inherent and additional), Half Value Layer (HVL), Effective energy, Detector used for dosimetry measurements, Source Sample Distance (SSD), Irradiation field (shape, size, geometry), Dosimetry quantity, Dosimetry method, Dose rate, Cell container and Quantity of cell culture media. All parameters used in this protocol are given in **Table 1**.

2. Beam quality index: determination of the half value layer

NOTE: The HVL is defined as the thickness of an attenuator (usually copper or aluminum) to reduce the intensity of the beam by a factor of two compared with the original value.

2.1. Set up the equipment (support, collimator, diaphragm, ionization) inside the irradiation enclosure by following the instructions in **Figure 1**. No attenuator material is used at this step.

2.2. Ensure that all the distances reported in **Figure 1** are correct. Measure these with a tape measure.

2.3. Place that the ionization chamber in the horizontal position. For this work, we used a 31002 (equivalent to 31010) cylindrical ionization chamber calibrated in air kerma.

2.4. Pre-irradiate the ionization chamber for 5 min and measure the background (this step can be performed without a collimator).

2.5. Perform 10 measurements of 1 min each in charge collection mode corresponding to the M_{raw} value (in coulombs).

2.6. Take the temperature and pressure with suitable calibrated equipment placed inside the irradiation enclosure in our case (if it is not possible, place it near to the experiment). Correct the

M_{raw} reading on the electrometer by the temperature and pressure correction factor given as follows:

$$K_{T,P} = \left(\frac{T + 273.2}{T_{ref} + 273.2}\right) \times \left(\frac{P_{ref}}{P}\right)$$

where: T (°C) and P (hPa) are the actual temperature and pressure, respectively. T_{ref} and P_{ref} are the reference temperature and pressure when the ionization was calibrated by the standards laboratory. The pressure and temperature must be measured with calibrated instruments.
The obtained value in charge mode is the average reference value M (in coulombs).

NOTE: This step is not strictly necessary for HVL measurement, but it is recommended.

2.7. Place an attenuator of certain thickness above the diaphragm. The HVL set is composed of foils with different thicknesses (0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 and 10 mm of copper) with a dimension allowing to cover the entire beam (80 x 80 mm here).

2.8. Take a measurement of 1 min (M_{raw} corrected by the $K_{T,P}$ as described before).

2.8.1. If the dose rate is divided by a factor of 2 with respect to the starting value, the HVL value is found. Take 5 measurements of 1 min to estimate the average dose rate.

2.8.2. If the dose rate is not divided by a factor of 2 with respect to the starting value, increase or decrease the attenuator thickness and take another measurement. Adjust the thickness of the attenuator as necessary.

2.9. Once the thickness of the attenuator that decreases the intensity of the beam by a factor two is found, take 5 measurements of 1 min to confirm the HVL.

NOTE: In most cases, the exact thickness of the attenuator cannot be found from the foils available. In this case, proceed by bisection and interpolate the HVL.

3. Evaluation of the irradiation field (no dose estimation)

3.1. Place an EBT3 film on the support used for irradiation.

3.2. Irradiate this film to obtain a well-marked irradiation field (at least 2 Gy).

3.3. Scan the EBT3 film using a dedicated scanner.

3.4. Plot the dose profile using Image J using the **Analyze** and then **Plot Profile** option (**Figure 2**).

3.5. Determine the size of irradiation field usage for irradiation (homogeneous area, excluding penumbra regions, see **Figure 2**).

3.6. Make marks on the support used for irradiation to ensure that the cell container is in the right position.

NOTE: In this step, the size of the irradiation field is determined, and the dose is not estimated. The complete procedure for film reading and analysis is given in section 5. Also, take margins in order to avoid errors due to the cell container positioning.

4. Dose rate measurement with ionization chamber

4.1. Take the cell container and break a little part on the side or at the bottom (depending on the particular container and ionization chamber used) to be able to place the ionization chamber inside (**Figure 3**, upper section) or below (**Figure 3**, lower section) the container. The examples are given in **Figure 3** with different ionization chambers (cylindrical or plane parallel) and cell containers. In this case, a T25 flask was used (**Figure 3**, red box).

NOTE: a soldering iron or heated scalpel is a good alternative to make holes in plastic ware

4.2. Place the container inside the enclosure on the support used for irradiation (carbon plate here).

4.3. Place the ionization chamber in the container (**Figure 3**, red box), in the correct position and connect it to the electrometer.

4.4. Ensure that all irradiation parameters listed in section 1 are correct (high voltage, intensity, additional filtrations, source sample distance, etc.).

4.5. Pre-irradiate the ionization chamber for 5 min and perform the zeroing of the electrometer.

4.6. Take 10 measurements of 1 min to determine the average dose rate in air kerma ($Gy.min^{-1}$). Calculate the determination of the dose rate in K_{air} as follows:

$$K_{air} = M \times N_{Kair} \times K_Q$$

where M is the reading of the dosemeter corrected by temperature, pressure, polarity effect, ion recombination, and electrometer calibration. N_{Kair} and K_q are the calibration and correction factors for radiation quality, whose values are specific to each ionization chamber.

5. Measurement of cell culture media attenuation and scattering

NOTE: Handle EBT3 films with gloves throughout the procedure.

5.1. Preparation of the experiment

5.1.1. Cut small pieces of EBT3 films at least 24 h before irradiation.

5.1.2. Determine the size of the films as a function of the cell container used for radiobiology experiments (4 x 4 cm for a T25 flask, for example).

Cut two sets of radiochromic films: One set for the calibration curves composed of three pieces of EBT3 radiochromic film by dose or time point (nine points in total for this work) ; and one set for the quantification of the cell culture media attenuation, also three pieces per point.

5.1.3. Number all the films for identification (upper-right corner here) and scan them on the same position on the scanner.

5.1.4. Keep the films away from light.

5.1.5. Prepare the cell container used for the EBT3 film measurements and, if necessary, cut a part to put the film inside (an example with a T25 is given in **Figure 4**).

5.2. Dose rate estimation

5.2.1. Measure the dose rate for the configuration as described in the previous section.

5.2.2. Keep this configuration in place for the irradiation of the EBT3 radiochromic films and use the same type of cell container.

5.3. Construction of the calibration curve

5.3.1. Take the pre-cut EBT3 films for the calibration curve.

5.3.2. Do not irradiate three pieces (0 Gy).

5.3.3. Place the first film inside the cell container, in the same configuration as for cell irradiation.

5.3.4. Irradiate it to obtain the first dose points.

5.3.5. Repeat this operation to obtain three pieces of EBT3 films irradiated with the same dose.

5.3.6. Perform this for each dose point (nine dose points in this work (0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, and 3 Gy) as illustrated in **Figure 5**).

5.4. Evaluation of the attenuation of cell culture media and scattering.

5.4.1. Chose the same irradiation time for all irradiations (60 s, for example).

5.4.2. Irradiate three pieces of EBT3 films in the container without water.

5.4.3. Irradiate three pieces of EBT3 films in the container with water as follows.

5.4.3.1. Place the film inside the container.

5.4.3.2. Fill the container with the exact quantity of water to represent the cell culture media (5 mL here). Use small pieces of tape if the films do not remain submerged properly.

5.4.3.3. Place the cell container inside the enclosure and ensure that the film is correctly immersed.

5.4.3.4. When the irradiation is complete, take the EBT3 films, dry them with absorbent paper, and store them away from light.

6. Reading of EBT3 radiochromic films

6.1. Read EBT3 films at least 24 h after irradiation.

6.2. Scan the films on a dedicated scanner.

6.3. Set the scanner parameters as: 48 bit red-green-blue tiff format, 150 dpi in transmission mode, and no image correction.

6.4. Perform a warm up of the scanner as follows.

6.4.1. Place a non-irradiated film on the scanner.

6.4.2. Launch a preview of the scan.

6.4.3. Launch a timer and wait for 30 s.

6.4.4. Launch the scan.

6.4.5. At the end of the scan, launch a timer, and wait for 90 s.

6.4.6. At the same time, register the scan, open the image with ImageJ, trace a square ROI (always the same size and in the same position), and take a measurement of the average red pixel level of the area.

6.4.7. At the end of the 90 s, repeat the procedure from step 2 (without touching the film inside the scanner).

6.4.8. Repeat this at least 30 times to warm up and stabilize the scanner (no variations in the average red pixel level of the area selected on the non-irradiated films). If the scanner, i.e., the average red pixel value, is not stabilized, continue the procedure.

6.5. Scanning of the EBT3 films

6.5.1. Place the first film in the center of the scanner bed. Delimitate an area to always place the film in the same place and in the same orientation.

6.5.2. Launch a preview of the scan.

6.5.3. Launch a timer and wait for 30 s.

6.5.4. Launch the scan.

6.5.5. At the end of the scan, launch a timer, and wait for 90 s. During these 90 s change the EBT3 film.

NOTE: An analysis of the EBT3 radiochromic films was performed using a self-programmed C++ program. Different methods can be used for the EBT3 film analysis, such as the red channel method or the three channels method[14,15]. In this case, we have used the red channel method with no background subtraction, and the images were converted to optical densities and then to the dose using our program. As this method is already well defined, our C++ program was not included here. Moreover, dedicated software[16] can also be used for EBT3 film analysis.

7. Determination of the dose rate at the level of the cell monolayer

7.1. Convert the average dose rate obtained with the ionization chamber corrected by the attenuation and scattering of the cell culture media (K) to the water kerma using the ratio of the mean mass energy absorption coefficient for water to air evaluated over the photon fluence spectrum (μ_{en}/ρ).

$$K_{water} = K \times \left[\left(\frac{\bar{\mu}_{en}}{\rho}\right)^{w}_{air}\right]_{air}$$

A dedicated software[17] was used to calculate the photon energy spectrum in air with no phantom, and we used the NIST table[18] to calculate the mean mass energy absorption coefficient.

REPRESENTATIVE RESULTS:

In this work, we used a platform dedicated to small animal irradiation[19]; however, this platform can be used to irradiate other types of samples such as cells. The irradiation source is a Varian X-ray tube (NDI-225-22) having an inherent filtration of 0.8 mm of beryllium, a large focal sport size of 3 mm, a high voltage range of about 30 to 225 kV and a maximal intensity of 30 mA.

The parameters used for this study are reported in **Table 1**. We have chosen to show an example

of the use of this protocol for cell irradiation in a T25 flask with 5 mL of cell culture media.

Half value layer

Table 2 reports the measurements performed to estimate the attenuator thickness needed to decrease the intensity of the beam by a factor of two. For this, 10 reference measurements were taken to estimate the average M_{raw} reading on the electrometer (in Coulombs), corrected by the temperature and pressure correction factor ($K_{T,P}$).

Different thickness of attenuators were then tested to find the thickness that decreased the beam intensity by a factor of two. When this thickness was found, five measurements were taken to evaluate the average M_{raw} value corrected by $K_{T,P}$.

For this configuration, a half value layer of 0.667 mm of copper was found. From the HVL measurement, we can calculate the effective energy of the beam, which is about 69 keV in our case.

Dose rate measurement

Before to these measurements, an EBT3 film was irradiated in order to determine the surface on which the irradiation field is homogeneous, allowing us to correctly place the cell container. This area is about 10 x 10 cm² excluding penumbra regions shown by dotted lines in **Figure 2**. Then, dose rate measurement was performed using a 31002 (equivalent to 31010) cylindrical ionization chamber calibrated in air kerma. For this configuration, with an open field irradiation field at 35 cm to the source in a T25 cell container placed on a carbon plate, the dose rate was about 0.626 $Gy.min^{-1}$ in K_{air}.

To determine the exact dose on cells, the measured K_{air} was converted in water kerma. **Figure 5** shows the X-rays energy spectrum obtained with dedicated software[17]. From this energy spectrum and the NIST table, we can convert the dose rate in K_{air} to K_{water}, which was 0.659 $Gy.min^{-1}$.

The overall uncertainty of the absolute dose rate measurement was about 3% at a 95% confidence level.

Cell culture media attenuation and scattering

For the quantification of cell culture media attenuation and scattering, dosimetry measurements with EBT3 radiochromic films were performed at room temperature. From the measurement with the ionization chamber, the dose rate was determined. Calibration films were then irradiated at the same position. EBT3 radiochromic films were calibrated between 0 and 3 Gy with 0.25 Gy steps between 0 and 1 Gy and 0.5 Gy steps between 1 and 3 Gy (nine dose points to construct the calibration curve) as shown in **Figure 6**. The dose points were fitted with a 4^{th}-degree polynomial curve. The EBT3 films were then irradiated with and without the exact quantity of cell culture media inside the cell container to evaluate the attenuation and the scattering due to the cell culture media. For this configuration, the attenuation of the cell culture media was about 1.5%.

The overall uncertainty of the EBT3 film measurements was about 4% at a 95% confidence level.

Routine measurements

Before performing the cell irradiations, the dose rate was measured each time in the same container used for irradiation. Thus, we used the daily dose rate to estimate the irradiation time. If we closely follow the protocol and do not change any parameters, the HVL measurement and the attenuation due to the cell culture media do not need to be repeated. As an example, the table used for the daily measurement is given in **Table 3**.

FIGURE AND TABLE LEGENDS:

Figure 1: Scheme of the configuration take in place on the SARRP enclosure for HVL measurements.

Figure 2: Evaluation of the irradiation field size. Dose profile obtained at 35 cm to the source without collimator. Dotted lines show the area considered for the irradiation.

Figure 3: Photographs of cell containers with the ionization chamber for dose rate measurement. Upper part: example for measurement with a 31002 cylindrical ionization chamber. Lower part: example for measurement with a TM23342 ionization chamber.

Figure 4: Photographs of the T25 used for the measurement of the cell culture media attenuation. The upper part of the T25 was cut out to be able to place the film inside the flask.

Figure 5: Simulated energy spectra for a 220 kV high voltage with 0.8 mm of Be and 0.15 mm of Cu filtrations[17].

Figure 6: EBT3 films irradiated to construct the calibration curve and the corresponding calibration curve.

Table1: A List of the configuration parameters.

Table 2: Measurement for the Half Value Layer determination.

Table 3: Daily dose rate measurements for cell irradiation.

DISCUSSION:

This work presents the protocol used and implemented for cell irradiations using low energy X-ray facility. Nowadays, many radiobiology experiments are performed with this type of irradiator as they are easy to use, cost effective and with very few radioprotection constraints, compared to cobalt source for example. Although these setups have many advantages, as they use a low X-ray energy source, a modification of only one irradiation parameter can significantly impact the dosimetry. Several studies have already highlighted the importance of dosimetry standards and

protocols for radiobiology studies[2,5,20,21]. Even though several protocols have already been well defined in the literature[1,5], we decided to develop a new protocol to perform dosimetry measurements to simulate real cell irradiation conditions as much as possible and take in account all the parameters that can influence the physical dose, especially for low energy X-rays[21,22]. Thus, we have chosen to implement a stringent protocol to minimize uncertainties. To this end, irradiation parameters were set (**Table 1**). The following three steps are then necessary: i) determination of the beam quality index, ii) measurement of the absolute dose rate with an ionization chamber and iii) measurement of the attenuation and scattering due to the cell culture medium with EBT3 radiochromic films.

The beam quality index corresponded to the voltage-half value layer (HVL) couple used to characterize low energy X-ray beams. The HVL is a practical indicator to describe poly energetic radiation and is defined as the thickness of an attenuator (usually copper or aluminum) to reduce the air kerma dose rate by a factor of two from the original value. HVL measurements were performed using the following recommendations of the AAPM protocol for a 40–300 kV X-ray beam[10]. However, some adaptations had to be made because in the irradiator enclosure it is not possible to achieve a distance of 1 meter between the source and the ionization chamber. Therefore, in the present work, we used a distance of 58 cm between the source and the detector for HVL measurements, as illustrated in **Figure 1**. We decided to let 25 cm after the ionization chamber because a lot of electronical material, support, and metallic elements are present at the bottom of the enclosure to limit the backscatter effect of these elements. Measurement of the HVL is one of the critical aspects of this protocol. Indeed, for many X-ray irradiators, the inside of enclosures is very restricted and these are not the optimal conditions to perform the measurements or it becomes impossible. Although experimental measurements are the best way to evaluate the HVL, when these measurements are too difficult or even impossible to perform, dedicated software[17] can be used to provide a good estimation for the HVL, or a Monte Carlo simulation can be used[23]. In the present work, we used a dedicated software to obtain the X-ray energy spectrum (**Figure 5**). We were also able to compare the measured and calculated HVL, which was the same, and to also compare the effective energy.

For dosimetry measurements, we then chose to simulate real cell irradiation conditions as much as possible. For this, we directly performed the absolute dose rate measurements with the ionization chamber inside the cell container used for cells irradiation (**Figure 3**). However, as we used a cylindrical ionization chamber calibrated for beams over 100 kV, we were not exactly at the same position as the cells because of the thickness of the ionization chamber. For lower beams (15–70 kV), where plane parallel chamber can be used, we can be even closer to the real cell irradiation conditions. Then, relative dosimetry measurements were performed to evaluate the attenuation and the scattering due to the cell culture medium. The results presented on this work do not highlight a significant variation in the dose deposited with or without the exact quantity of cell culture media as we used a voltage of 220 kV, an additional filtration of 0.15 mm of Cu and we only had 5 mL of cell culture medium. However, in a previous study[21] carried out at 80 kV, we pointed out that a variation of the cell culture media and filtration significantly impacts the physical dose, up to 40% compared to the reference configuration when we used a 1 mm aluminum filtration. This impact was also demonstrated in terms of biological effects by

measuring the surviving cell fraction using a clonogenic assay[21,24]. Thus, depending on the voltage, additional filtration, the container and quantity of cell culture media, the dose deposited on the cells can be different if the protocol is not closely followed for all irradiations.

Consequently, a dedicated dosimetry should be set up for all cell irradiation configurations. Although this is restrictive and the modification of only a single parameter requires the implementation of a new configuration, we have decided to make this choice to be as close as possible to the real cell irradiation conditions. This requires a close collaboration between the physicists and the radiobiologist in order to set up the best design for the configuration. At our institute, a dozen protocols were established on our platform for a voltage range of 40 to 220 kV for which T25, T75, 6- to 96-plate wells or Petri dishes can be irradiated.

Although this protocol seems quite long to implement, once the configuration is established, the only measurement to be taken on the day of irradiation is the measurement of the dose rate with the ionization chamber inside the cell container. This measurement is also a quality control that enables us to ensure that the dose rate is as expected.

To ensure the reproducibility of radiobiological studies, and to be able to compare and interpret experiments, it is important to rigorously follow established protocols and report all the dosimetry and configuration aspects, particularly for facilities using low or medium energy X-rays. The new protocol proposed here is for cell irradiations, applicable to many X-ray facilities, and takes into account all the parameters influencing the dosimetry and provides a better estimation of the actual dose delivered to the cells.

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None

DISCLOSURES:
The authors have nothing to disclose.

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569 computation in digital mammography. *Radiologia Brasileira*. **48** (6), 363–367 (2015).
570

Figure1

Click here to access/download;Figure;Figure_1.TIF

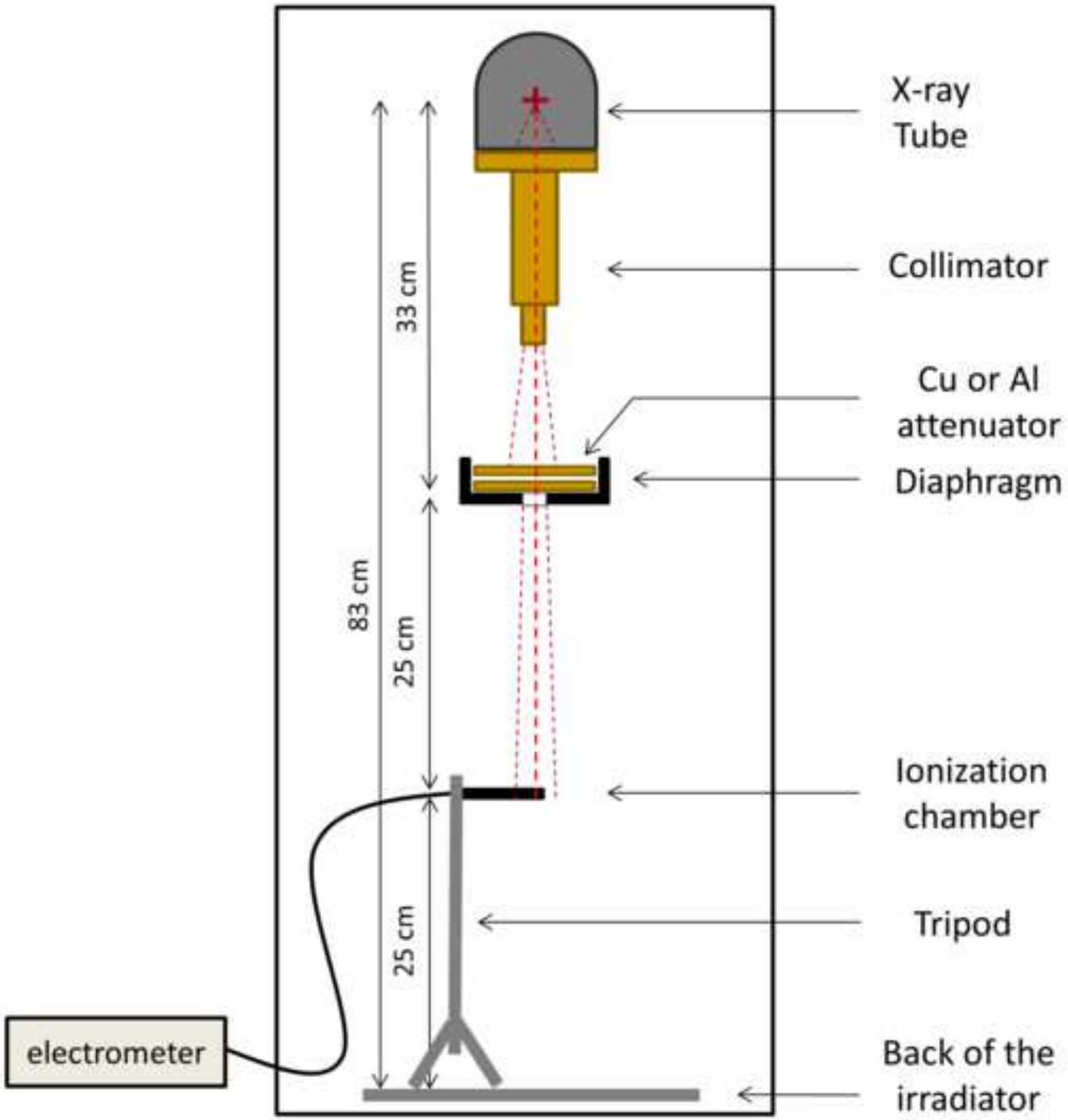


Figure2

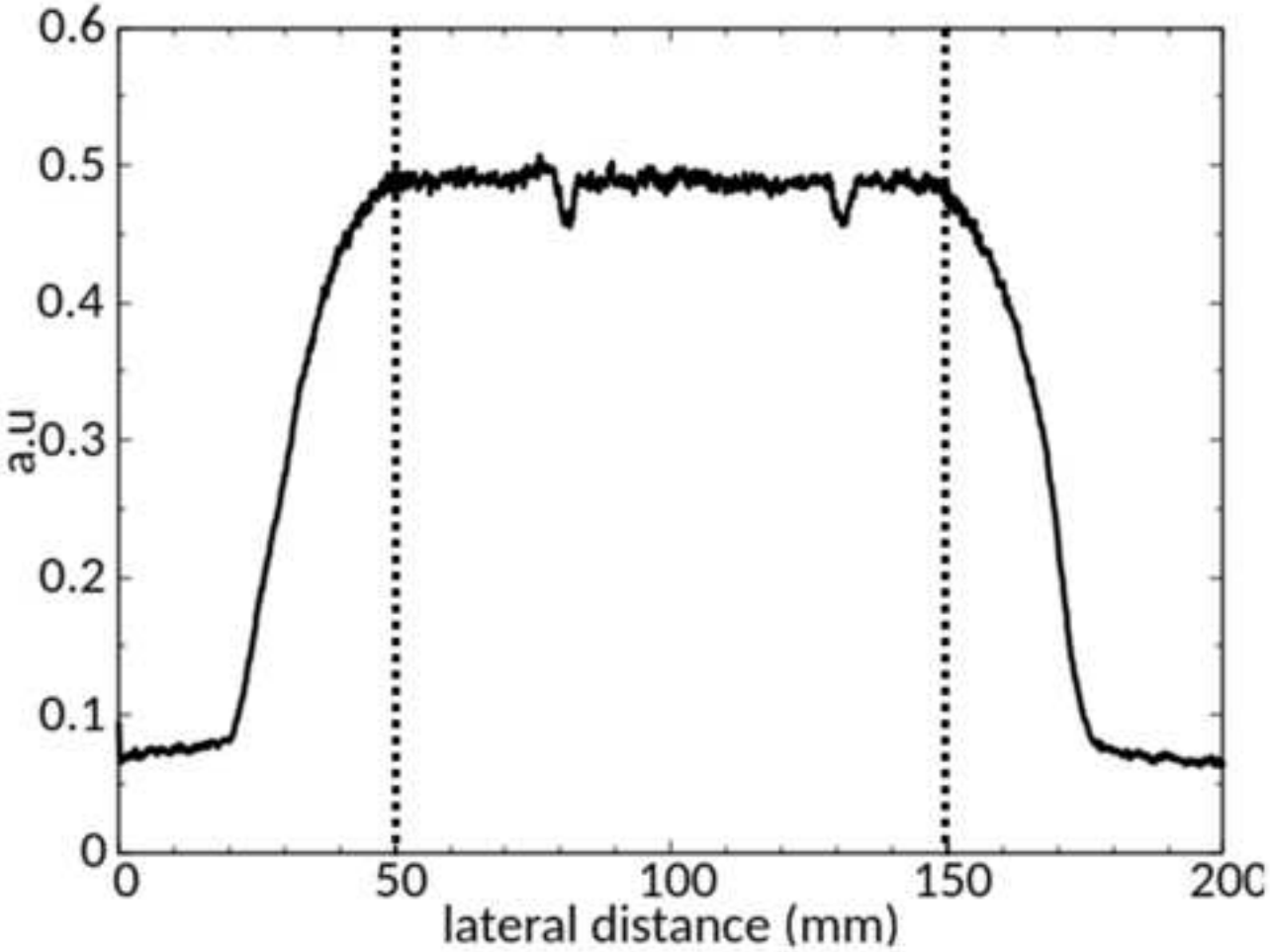


Figure3

Click here to access/download;Figure;Figure_3.TIF

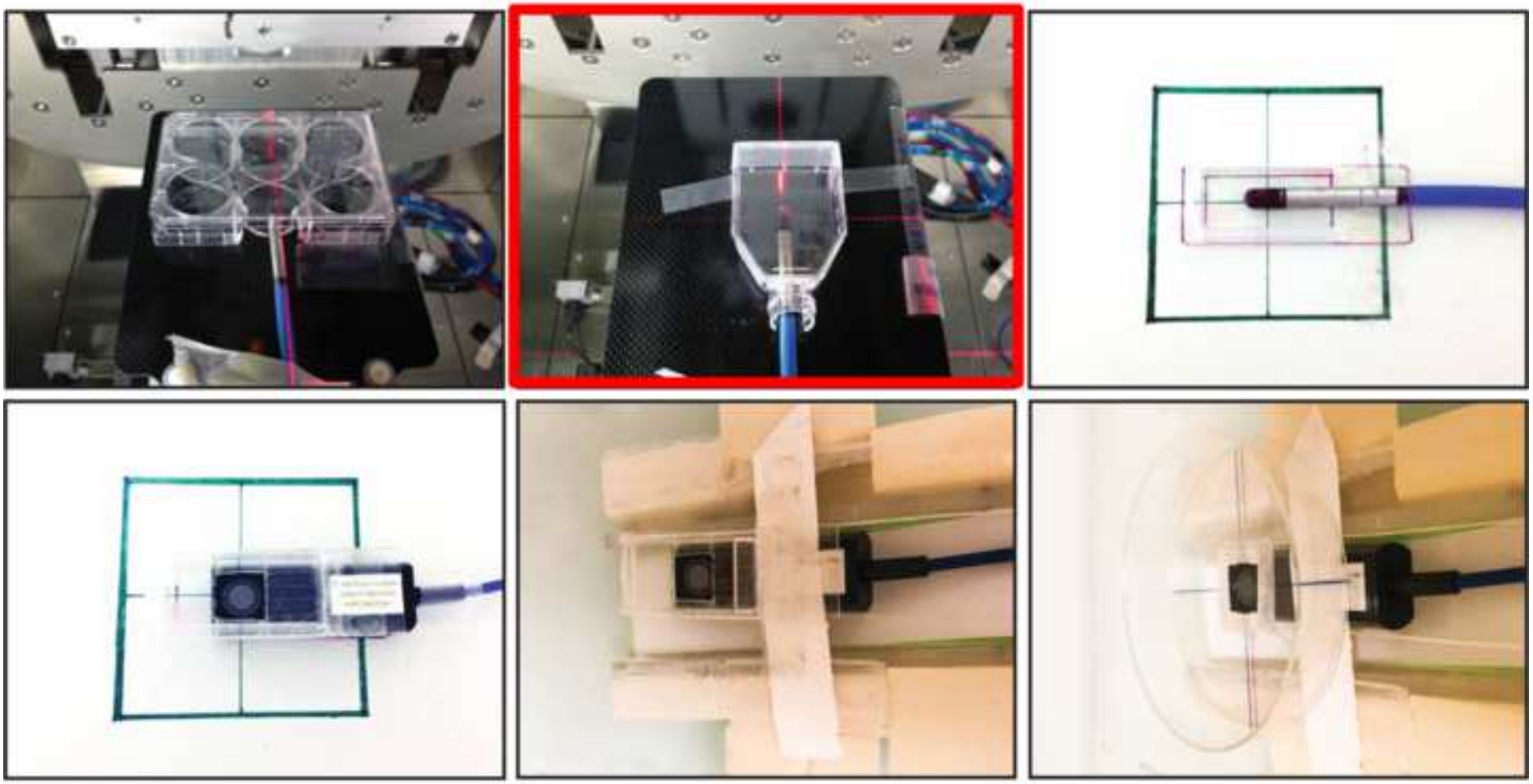
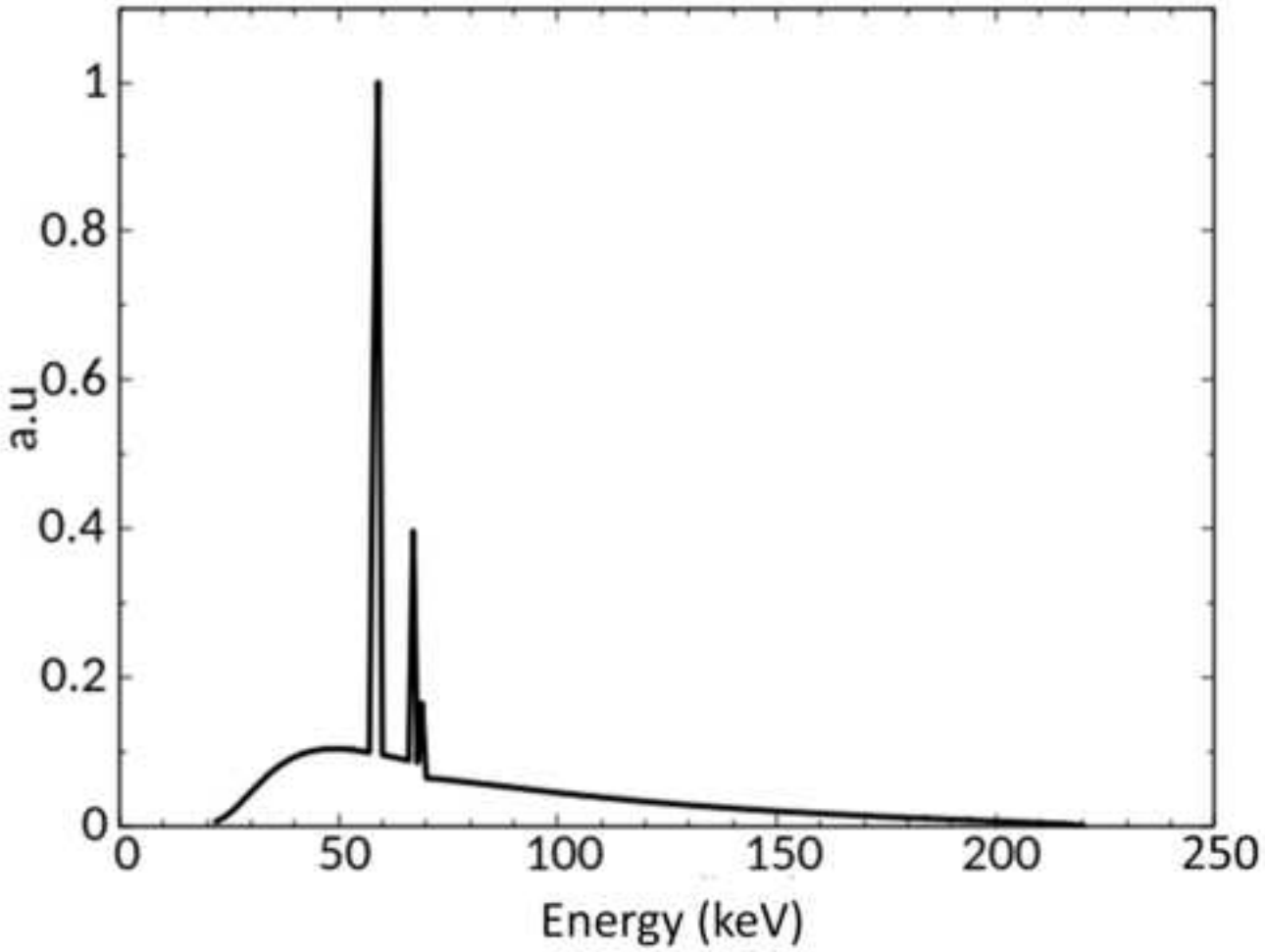
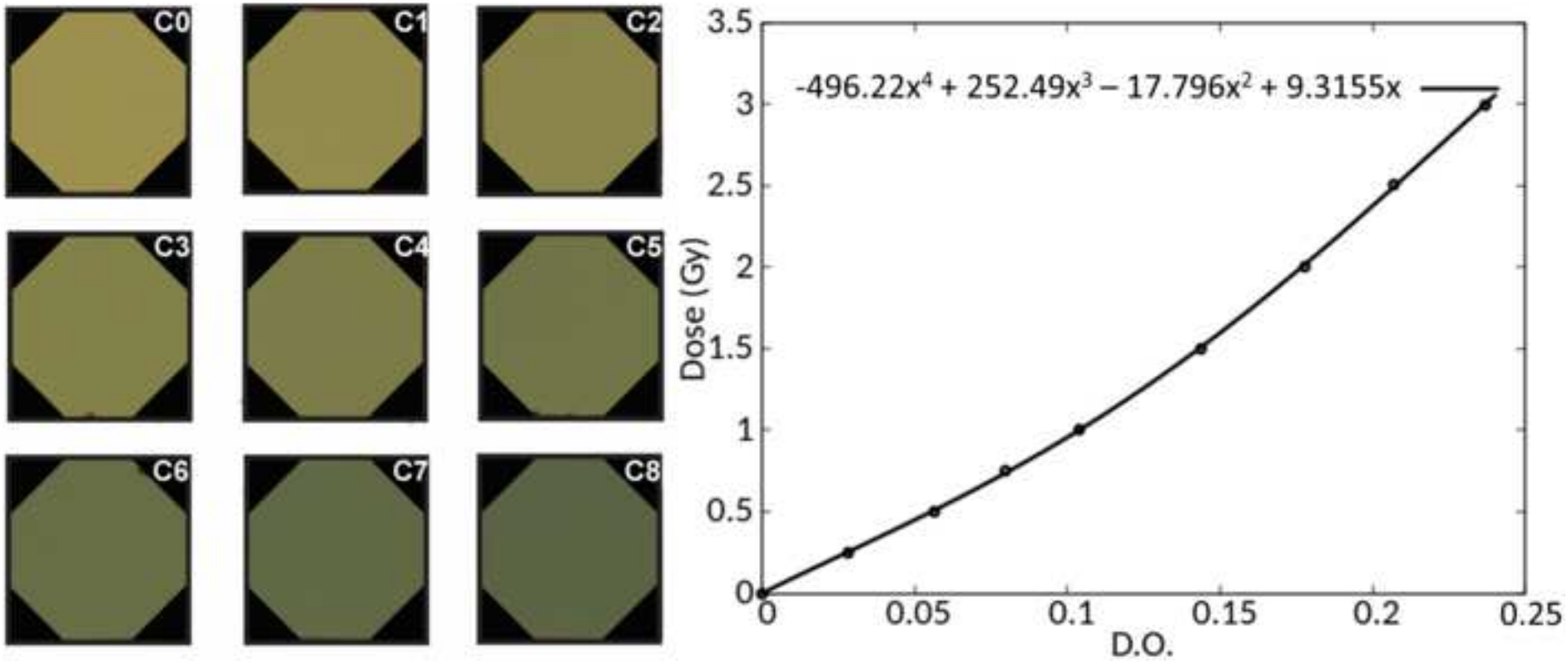


Figure4

Click here to access/download;Figure;Figure_4.TIF

Figure5





High voltage (kV)	220
Intensity (mA)	3
Filtrations (inherent and additional)	0.8 mm of Be + 0.15 mm Cu
Half value layer (mm Cu)	Determined below
Effective energy (keV)	Determined below
Detector used	Cylindrical ionization chamber + EBT3 radiochromic films
Source sample distance	35 cm
Irradiation field (shape, size, geometry)	Open field (no collimator), square, 20 x 20 cm
Dosimetry quantity	Kair and Kwater
Dosimetry method	As described on the protocol section
Cell container	T25
Quantity of cell culture media	5 ml
Dose rate (Gy/min)	Determined below

Table2 Click here to access/download;Table;Table2.xlsx

	Attenuator (mm Cu)	IC measure (nC)	Temperature (°C)	Pressure (hPa)
reference measurements (Mref)	0	10.480	21.6	993.2
		10.480	21.6	993.1
		10.490	21.6	993.1
		10.490	21.6	993.1
		10.490	21.6	993.2
		10.490	21.6	993.2
		10.490	21.6	993.1
		10.490	21.6	993.2
		10.490	21.6	993.2
		10.490	21.6	993.1
Finding of attenuatior thickness (M)	0.514	5.840	21.7	993.2
	0.564	5.651	21.7	993.2
	0.584	5.569	21.7	993.2
	0.604	5.491	21.7	993.2
	0.615	5.441	21.7	993.2
	0.627	5.380	21.7	993.2
	0.647	5.307	21.7	993.2
	0.667	**5.240**	**21.8**	**993.2**
Measurments with the right attenuator (M)	0.667	5.231	21.8	993.4
	0.667	5.236	21.8	993.1
	0.667	5.235	21.8	993.2
	0.667	5.236	21.8	993.2
	0.667	5.235	21.8	993.3

$k_{T.P}$	IC measure corrected by $k_{T.P}$ (nC)	Corrected Mean value (nC)	ST deviation
1.026	10.752	10.761	0.005
1.026	10.752		
1.026	10.763		
1.026	10.763		
1.026	10.763		
1.026	10.763		
1.026	10.763		
1.026	10.763		
1.026	10.763		
1.026	10.763		
1.026	5.992	-	-
1.026	5.798	-	-
1.026	5.714	-	-
1.026	5.634	-	-
1.026	5.582	-	-
1.026	5.520	-	-
1.026	5.445	-	-
1.026	5.376	-	-
1.026	5.368	5.373	0.003
1.026	5.375		
1.026	5.373		
1.026	5.374		
1.026	5.373		

Attenuation estimation (M / Mref)
-
0.557
0.539
0.531
0.524
0.519
0.513
0.506
0.500
0.499

Table3 Click here to access/download;Table;Table3.xlsx

IC measure (nC)	Temperature (°C)	Pressure (hPa)	$k_{T,P}$
2.495	22.3	1001	1.020
2.496	22.3	1001	1.020
2.497	22.3	1001	1.020
2.498	22.3	1001	1.020
2.496	22.3	1001	1.020
2.495	22.3	1000.9	1.020
2.494	22.3	1000.9	1.020
2.495	22.3	1000.9	1.020
2.496	22.3	1000.9	1.020
2.496	22.3	1000.9	1.020

IC measure corrected by $k_{T.P}$ (nC)	Corrected Mean value by $k_{T.P}$ (nC)	ST deviation	Corrected mean value by all correction factors	Dose rate in air kerm (Gy/min)
2.545	2.546	0.001	2.536	0.626
2.546				
2.547				
2.548				
2.546				
2.545				
2.544				
2.545				
2.546				
2.546				

Dose rate at cell level in K_{water} (Gy/min)
0.659

Name of Reagent/ Equipment	Company	Catalog Number
31010 ionization chamber	PTW	ionization Radiation, Detectors including code of practice, catalog 2019/2020, page 14
EBT3 radiochromic films	Meditest	quote request
electrometer UNIDOSEwebline	PTW	online catalog, quote request
HVL material (filter, diaphragm)	PTW	online catalog, page 70, quote request
scanner for radiochromic films	Epson	quote request
temperature and pressure measurements, Lufft OPUS20	lufft	quote request

Comments/Description
https://www.ptwdosimetry.com/fileadmin/user_upload/DETECTORS_Cat_en_16522900_12/blaetterkatalog/index.html?startpage=1#page_14
https://www.meditest.fr/produit/ebt3-8x10/
https://www.ptwdosimetry.com/en/products/unidos-webline/?type=3451&downloadfile=1593&cHash=6096ddc2949f8bafe5d556e931e6c865
thickness foils: 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 and 10 mm of copper, https://www.ptwdosimetry.com/fileadmin/user_upload/Online_Catalog/Radiation_Medicine_Cat_en_58721100_11/blaetterkatalog/index.html#page_70
Epson V700, seiko Epson corporation, Suwa, Japan
https://www.lufft.com/products/in-room-measurements-291/opus-20-thip-1983/

Response letter

Manuscript: JoVE61645_R2

We would like to thank the Editor and referees for their constructive comments that have help us to improve the quality of our manuscript. We hope that this new version is now suitable for publication. Our responses to your comments can be found hereafter and the modifications in the manuscript are in blue.

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached file for revision.

Reply: We have used the attached file for revision

2. Please address all the specific comments marked in the manuscript.

Reply: All specific comments in the manuscript have been taken into account. See below for details:

- Line 2, title reformulation: We agree with the modification
- Manuscript need a thorough proofreading: The manuscript was proof-read by a professional English reader before to submit our first revision. For this new revision, all comments by the editor and the reviewers were taken into account.
- Line 64, please describe the kerma condition or provide a citation: the kerma conditions correspond to an irradiation in air, this is now specified this point in the manuscript (page 2, line 63).
- Line 101 to 107, Please note that we need a specific experiment with specific experimental values/parameters in order to film. We cannot film a generalized protocol. Can you include a specific example film used for this protocol?: We now included a specific example with the specific parameters given in Table 1. For the film, we will use these specific parameters. We added the following sentence at the end of this paragraph: "*All the parameters used in this protocol are given in table 1*."
- Line 117, How do you check this?: We check this with a tape measure. We specified this point in the manuscript page 3, Line 118).
- Line 121, What are the settings for the ionization chamber used?: We can use different type of ionization chamber and each ionization chamber has its specific calibration. In the results section (page9, line 373), we specified the ionization chamber use for this work, a 31002 (equivalent to 31010) ionization chamber calibrated in air kerma. In the section 2.3, we also specified the ionization chamber used for this work (page 3, line 122).
- Line 127, Where is the weather station? Presumably inside near the experiement?: In our case, the weather station is located inside the irradiation casemate so very close to the experiment. This is now specified it in the manuscript page 4 line 130.
- Line 160, How is this done? What is the irradiation dose?: For this example we irradiated the film during 120 seconds. We don't need to measure the dose for this verification we only need to have a well-marked film to determine the size of the irradiation field and the area where we have a homogeneous distribution of the dose. It is the reason that we specified in the title of this section that it is not a dose estimation. To have a well-marked

film, a dose of 2 Gy is widely sufficient, this is now specified in the manuscript page 4 line 166.

- Line 164, How is this done? Please include button clicks in the software: Done. Following a comment by the fourth referee, we also added a step in this section to make this section clearer
- Line 166, How do you determine this?: From the EBT3 film irradiated in the section 3 of the protocol we can know determine the size of the irradiation field and the area where we have a homogeneous dose. As we recorded the position of the support inside the irradiation enclosure and the position of the EBT3 film on it we can locate and mark the area with a homogeneous distribution of dose on the support used for irradiations and always place the cell containers inside this area.
- Line 248: Please provide the dose points here as well for greater experimental fidelity: done
- Line 421: Please expand on the limitations of the protocol: the main limitations of this protocol are explained and discussed on the discussion section. This protocol was developed in order to perform dosimetry measurement as close as possible to the real cell irradiations conditions on orthovoltage facilities. The difficulty or even the impossibility to perform beam quality index measurements have been highlighted on the second paragraph of the protocol (page 11, line 452). As the enclosure of this type of installation are small, it is not possible to follow the AAPM recommendations and some adjustments have to be made. Then the dosimetry measurements with the ionization chamber were discussed especially the influence of the shape of the ionization chamber used to be as close as possible to the cell irradiation condition. In this third paragraph we also highlight the influence of a lot of parameters on the dosimetry (filtrations, quantity of cell culture media, cell container…). Thus, dedicated dosimetry has to be set up multiplying the number of configurations. The protocol developed here may seem long and very restrictive, but once the measurements have been taken, only the daily dose rate measurements remain to be carried out with the ionization chamber. To insist in these two last critical points, we have added some comments in the fourth paragraph of the discussion. We believe that all the limitations of the protocol are highlighted.

3. Once done please ensure that the highlight is no more than 3 pages including headings and spacings.

Reply: done

4. Please address all the reviewers comments as well.

Reply: done

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript is greatly improved. Having an English reader has increased the readability greatly. It is now an acceptable manuscript. I have a few comments that should be addressed but I do not need to review again. Minor revisions with points made below.

1. Page 2 line 58: dosimetry does not appear to be - dosimetry is crucial. The attitude of the past has not worried about dosimetry. Let's no longer work with this attitude.

Reply: done

2. Page 4 line 134 I object to implying that a weather station is equivalent to a measurement using a thermometer and barometer. Change this to state that temperature and pressure needs to be measured with calibrated equipment.

Reply: done. The sentence has been modified as follow: *"Take the temperature and pressure with suitable calibrated equipment placed inside the irradiation enclosure in our case (if it is not possible place it near to the experiment)."*

3. It is implied in your Figure 1 but should be stated clearly in line 126 on page 4 that the filters need to cover the entire beam.

Reply: we added this information in the new version of the manuscript in the section 2.7: Place an attenuator of certain thickness above the diaphragm. The HVL set is composed of foils with different thicknesses (0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 and 10 mm of copper) with a dimension allowing to cover the entire beam (here 80 x 80 mm).

Reviewer #2:

The authors have sufficiently addressed all my concerns. There remain a few small comments, mostly wording errors, likely due to translation from french:

in many places:

"tension" is used in some places instead of "voltage"

Reply: done

"casemate" should be "casement" or better: "enclosure"

Reply: done

page 4 line 130 "make the zero" should be "measure background"

Reply: done

Page 4 line 132: "charge measurment" should be "charge collection"

Reply: done

page 5 line 142: does the obtained value refer to the average value? if so please note.

Reply: done

steps 2.8.1 and 2.8.2 are not worded clearly

Reply: We added some information and reworded these two sections. We hope that these steps are clear now.

step 4.1: a note to the authors unrelated to this manuscript: a soldering iron or heated scalpel is a good alternative to make holes in plasticware,

Reply: We thank the reviewer for the tip. We find appropriate to add this suggestion in the manuscript (page 5 line 191).

page 5 line 155: "dichotomy" should be "bisection"

Reply: done

page 6 line 187: again mention that you are averaging multiple readings

Reply: done

page 8 line 161: "can" should probably be replaced by "should". it is good practice to always measure the film in the same position, as recommended by Ashland.

Reply: done

Fig 1 caption: "schema" should be "scheme" or "drawing"

Reply: done

table of materials,

"quote request" is not really useful. can you provide the model instead? e.g. for the electrometer: "unidos E"

Reply: we added some information in the Jove materials table.

for the ion chamber 30010 would be the catalog number

Reply: It is an online catalog, we don't have the catalog number but in the Jove Materials table we added the year of the catalog (2019-2020)

Reviewer #4:

Manuscript Summary:

The manuscript improved after the last revision, most of the recommendations were followed. There are couple of sentences in the manuscript which are not clear and would benefit from proofreading by a native speaker (figure legends). After these improvements I recommend the manuscript for publication.

Major Concerns:

What is still missing is the background reading for EBT3 films. Since the background of each film and even within a film varies, it is common to perform a film background scan prior to irradiation which is then subtracted from the irradiated film scan. This step is missing in the procedures. Could you please comment on this?

Reply: effectively, we have not specified this step in the protocol because we have not formally measured the background in this work. In order to reduce as much as possible the influence of the background noise on our measurements, all the EBT3 films used for a precise experiment come from the same sheet. Moreover, all the films used for the experiment are randomly distributed on the sheet (see figure). On the note at the end of the section 6, we now specified that we used the red channel method with no background subtraction.

0 Gy	0,25 Gy	0,5 Gy	0,75 Gy	1 Gy	1,5 Gy
2 Gy	2,5 Gy	3 Gy	No_water_1	water_1	0 Gy
0,25 Gy	0,5 Gy	0,75 Gy	1 Gy	1,5 Gy	2 Gy
2,5 Gy	3 Gy	No_water_1	water_1	0 Gy	

Minor Concerns:

Line 130, Section 2.4: perform zeroing

Reply: we replaced “make the zero” by measure background as recommended by the second referee.

Line 165-166: it is not clear how big the margins should be please be more specific

Reply: For our irradiations we consider only the area where we have the maximal and homogeneous distribution of dose. So, we exclude the penumbra regions to avoid heterogeneous irradiation. In this case we consider a square of about 10 x 10 cm. We added a step in this section to better explain the determination of the area used for irradiation:

3.4 Plot the dose profile using Image J by using the Analyze and then plot Profile option (Figure 2)
3.5 Determine the size of irradiation field usage for irradiation (homogeneous area, excluding penumbra regions, see figure 2)

Line 251: be more specific about the ROI size

Reply: We did not specified the size of the ROI because it depends on the size of the film used at this step.

Line 332: one digit after comma is more than sufficient 1.5%

Reply: done

Lines 343-355: English in figure legends is not clear. Often not possible to get the meaning.

Reply: The manuscript has already been proof-read by a professional English reader for the first review.