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Characterization of UVA biophoton emission and survival of α-irradiated HCT116+/+ cells via radium exposure

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Abstract

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Background: It's been hypothesized that biophoton production is a result of the generation of excited species followed by relaxation of these excited species to a stable state. This can occur as a result of many stressors including irradiation from radioactive materials. Cell exposure to radium (Ra-226) has not been thoroughly explored for biophoton production. Furthermore, biophoton production is often associated with oxidative stress and cell death which is concurrently also an area of exploration.

Objectives: The objective of this study was to characterize biophoton emission and additionally observe cell survival from α-irradiated HCT116+/+ cells via radium exposure. We aim to expand evidence that supports increased biophoton production and decreased cell survival as a result of oxidative stress and exposure to radioactive material.

Methods: HCT116+/+ cells were standardly cultured in this study. For characterization of biophotons, cell groups were irradiated with 10, 100, 1000 and 10,000mBq/ml of Ra-226 for 24 hours. A photon counter was used to quantify counts. For characterization of cell survival, standard clonogenic assay techniques were used. Cell groups were irradiated with the same concentrations for Ra-226 and colonies were counted 7-9 days later.

Results: No significant results were seen when observing biophoton counts from Ra-226 irradiated groups compared to a background count. Furthermore, there was a significant difference between Ra-226 irradiated groups when compared to a positive control known to produce biophotons. Some significant differences were seen in the surviving fraction of directly irradiated cells when looking at the different concentrations of Ra-226. However, overall there was a correlation that could be seen where an increase in concentration of Ra-226 resulted in a decreased surviving fraction of cells.

Conclusions: The hypothesis of this study was partially supported where increased exposure and sensitivity to Ra-226 showed decreased cell survival, and assumed to have implications on biophoton production at higher concentrations although was not observed here at environmentally relevant concentrations. It was also concluded that biophoton production and oxidative stress are independent events. The significance of biophoton production as a radiation-induced bystander effect was explored as an implication of this study.

Acknowledgements

I would like to dedicate this thesis to my late grandfathers MD Moazzem Hossain and MD Gholam Salmani who always believed in me; I miss them dearly.

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Background

Electromagnetic energy is energy that is manifests both properties of particles and waves. The emission of electromagnetic energy is represented by photons and phonons (Markov, 2015). Biophoton emission is a phenomenon that has been reported extensively previously in literature (Devaraj *et al.*, 1991; Niggli *et al.*, 2001; Ahmad *et al.*, 2013; Wijk *et al.*, 2013; Le *et al.*, 2015a; Le *et al.*, 2015b; Le *et al.*, 2017). It's been hypothesized that biophoton production is a result of the generation of excited species followed by relaxation of these excited species to a stable state. These excited species can arise from lipid peroxidation and oxidative stress in biological systems or as a result of excitation of organic molecules by ionising radiation. Part of the evidence is seen where single oxygen species and carbonyl compounds are identified as sources of biophoton emission and are also products of lipid peroxidation. As excitation decays, biophoton production is observed (Le, 2018). Photon emission can occur as a result of various stressors including ionization radiation. irradiation, visible light, UV light, and chemicals (Le *et al.*, 2017).

Radium is a highly reactive element that belongs to the alkaline earth metal group of the periodic table. All isotopes of radium are radioactive, however, Ra-226 is the most abundant radioisotope and of interest in this thesis. Ra-226 is a decay product of uranium. A notable source of radium and its radioactive decay products (such as bismuth-214 and lead-214) includes uranium mining (Virginia *et al.*, 2011). Canada is responsible for a quarter of the world's uranium mining, making us a leading producer of uranium (Goulet *et al.*, 2011). Although there have been improvements in uranium mining technology, low dose toxicity and indirect effects remains an explorable concern in regards to uranium mining effluent; including Ra-226.

The decay of Ra-226 results in primarily alpha particle emission and some beta particle emission. Alpha particles emitted in this decay have a mean energy of 4.78 MeV. This emission at elevated levels has a high possibility of causing adverse biological effects (Shi, 2016). Exposing Atlantic cod embryos to environmentally relevant concentrations of Ra-226 showed that there was oxidative stress and apoptosis in the cod embryonic cells (Olsvik *et al.*, 2012). Other articles have also looked at the effects of low-dose alpha particle emission via radium. Animals injected with Ra-226 saw that alpha-particles were able to cause skeletal lesions and osteosarcomas. Notably, this was similar to lesions found in radium-dial painting workers who had the practice of licking their paintbrushes (for finer points while painting) (International Atomic Energy Agency, 1969). Fathead minnows fed with environmentally relevant concentrations of Ra-226 experienced changes in DNA and RNA protein ratios and consequently, transient growth perturbations (Mothersill *et al.*, 2013). Similarly, in earth worms, Ra-226 exposure caused changes in growth and reproduction as a result of DNA damage (Lourenço *et al.*, 2012). Respectively, it is proposable that irradiation from alpha particles from radium would be responsible for some cases of ROS, oxidative stress and lipid peroxidation because of their ability to cause various biological complications that are often associated with oxidative stress and/or lipid peroxidation. Organisms contaminated with low-doses of Ra-226 can accumulate and absorb this radioactive element, which is why it is worthwhile to explore the irradiative effects (Hossain, 2021).

Previously in the Mothersill & Seymour Lab., electromagnetic UVA photon emission has been observed in cells that have been exposed to beta particles via tritium. These photons have been quantified and observed for relationships with cell lines, cell numbers and other parameters (Le *et al.*, 2015a; Le *et al.*, 2017). Gamma irradiation exposure via caesium has also shown the

production of UVA photon emission (Cohen *et al.*, 2020). There is evidence that other wavelengths of particles are also able to produce photons, but it has not been well explored. Thus, radium as an alpha particle emitter is appropriate to observe if biophotons are produced when cells are exposed; especially considering the biological effects that have been previously explored with Ra-226 exposure.

Furthermore, Ra-226 radioactivity and cell death has been previously explored and well-defined (Shi, 2016; Shi *et al.*, 2016; Fernando *et al.*, 2020). Exposure to UVA and UVB has also shown lethal mutations in HaCat cells (O'Reilly and Mothersill, 1997), as well as bystander effects in HaCat cells (Whiteside and McMillan, 2009). It is largely assumed due to increased ROS. It is also well known that increased ROS production and biophoton production both occur as a result of Ra-226 exposure; in many cases these processes seem simultaneous (Lyng *et al.*, 2011; Le *et al.*, 2017; Jella *et al.*, 2018). Both require high levels of excited species, thus share similarities in required conditions. Cell death (as an indicator of ROS production/oxidative stress) has not been measured alongside biophoton production, especially in the case of Ra-226 exposure. It is valuable to explore cell death in the context of biophoton production. This may have further implications on the correlation of ROS production and biophoton production when cells are exposed to radioactive materials.

Study Objectives

The overarching purpose of this study is to characterize UVA biophoton emission from α-irradiated cells via radium exposure. A particular aim is to determine if exposure of cells results in bio photon emission and if so, to what degree. We utilized multiple dosages of radium in order to achieve numerous possible outcomes of radium exposure. The HCT116+/+ cell line was used as it has shown positive results in the literature cited above, revolving biophoton production after exposure to tritium and caesium. There is a lack of information regarding biophoton production with alpha particle irradiation – thus this the purpose and motive behind this study. To further this study, we observed the effects of Ra-226 on cell survival. This is for a few reasons. Firstly, to verify the effects of Ra-226 on cell survival to compare to other literature. Secondly, to look at cell survival in the context of biophoton emission and ROS production after irradiation exposure. Both seem to occur simultaneously and require high energy levels; this study will be used to confirm. Previous studies have shown decreased cell survival with radium exposure (Shi, 2016; Shi *et al.*, 2016; Fernando *et al.*, 2020). Therefore, we should comprehensively see both a decline in cell survival and increase in biophoton production with exposure to α-irradiation via Ra-226. We hypothesize that the increased exposure and sensitivity to Ra-226 will reflect the biophoton production and cell survival of the HCT116+/+ cell line in accordance with expected adverse biological effects. Furthermore, we aim to use these results to discuss the capability of cells to produce bystander effects upon radium exposure as it is a relevant application.

Materials & Methods

Cell Culture

Human colon carcinoma 116 cells with wildtype p53 expression (HCT116+/+) cells (Bunz *et al.*, 1998) were cultured in T75 flasks using Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulphate. Cultures were incubated at 95% humidified air and 5% CO2 at 37°C. At 80-90% confluency, adherent monolayers were detached from flasks using 0.25% trypsin solution in 1 mM EDTA. Neutralization was achieved by supplying a greater volume of cell culture media to prevent cell lysing via trypsin. Cells were then seeded back into a new flask with RPMI solution at a cell density of approximately $1.0x10^6$ cells. Cells were cultured every 3-4 days as needed.

Characterization of UVA Biophotons

Irradiation

Cells were seeded into 100mm petri dishes at a density of 250,000 cells per 5 ml of cell culture media for the purpose of photon quantification from directly irradiated cells. For cell seeding, at 80-90% confluency, cells were trypsinized for cell detachment from large cell culture, and then neutralized with RPMI solution. This cell suspension was then used for cell counting to determine the number of viable cells. Cell counting was done using trypan blue and the BIO-RAD TC20 Automated Cell Counter (Bio-Rad Laboratories Inc., Berkeley, California, United States). Once counted, 250,000 cells were seeded into petri dishes respectively.

To prepare the radium stock solutions, a concentration of 100,000mBq/ml was made from stock concentration $6.98x10^4$Bq/ml; then, 1/10 serial dilutions were done to achieve concentrations of 100, 1000, 10,000, and 100,000mBq/ml. Final concentrations of 10, 100, 1000 and 10,000mBq/ml were achieved after adding radium stock solutions to cell petri dishes with RPMI. See Supplementary Equation 1.1 for details on radium dose calculation and administration. Tritium was also used as a positive control at a final concentration of 6.34MBq/ml. See Supplementary Equation 1.2 for details on tritium dose calculation. This was directly added from a stock concentration of $6.98x10^4$Bq/ml.

In total, there were 7 experimental groups (n=9) used to observe photon quantification. Four groups were treated with radium with respective concentrations of 10, 100, 1000, and 10,000mBq/ml. One group was treated with tritium at a concentration of 6.34MBq/ml as a positive control. One group remained untreated with only cells, to observe if there is any photon emission from the cells alone. And one more group served as a negative control with only the petri dish alone (no medium or cells) to ensure counts were not observed simply from the reflection of the dish.

Cells were seeded for 24 hours with an appropriate volume of radium or tritium for irradiation as outlined above. Once 24 hours has passed, medium was poured off and dishes were assessed for photon quantification within 5 minutes from pouring off medium.

Photon Quantification

Experimental groups were assessed for photon emission 24 hours after being exposed to irradiation.

Photon quantification was done using the photon counter, Hamamatsu Photonics (Bridgewater, NJ, United States) R7400P subminiature single-photon counting photomultiplier tube (PMT). The complete device was built by Dr. Bilal Ahmed who has passed on from the Mothersill & Seymour Laboratory (Ahmad, 2012). The PMT sat in a plastic container with room for a 25mm diameter convex lens and a 340 ± 5 nm optical filter. This unit was housed by a large sealable light-tight metal chamber which a petri dish can be placed for photon counting. In order to read UVA biophotons, the photon counter was fitted with a hard-coated interference type band pass optical filter centered at 340 ± 5 nm (Edmund Optics Inc., Barrington, NJ) (See Supplementary Information: Optical Filters in the appendix). The filter was 25mm in diameter and 10nm passband (>85% transmission); the blocking wavelengths include 200-1200nm excluding 340 ± 5 nm. In order to utilize the photon counter, the main power on the Nuclear Instrumentation Module (NIM) was switched on as well as the power for the high voltage supply which was then set to -800V. The machine was left on for thirty minutes before use to allow it to "warm up". The PMT unit had to be removed for each experiment to places the petri dish inside, but secured back into its original place afterwards. Petri dishes were places inside the photon counter without cell medium or 0.95mm lid, to avoid any obstacles interfering with photon quantification. The distance between the cell monolayer and the PMT device was approximately 5cm. The light-tight chamber was then sealed with a band and covered with a black cloak to prevent as much light from coming in and adding to background photon count for each experiment. The machine displayed photon

counts which were collected by manually operating the start stop and reset button on the scalar module. See Supplementary Figure 1.1 and Supplementary Figure 1.2 in the appendix for diagram of photon quantification apparatus.

Photon emission from each petri dish was observed for a total of 60 seconds per measurement and recorded. A background reading was taken to normalize counts. Three counts were taken from the photon counter for each experimental petri dish.

Characterization of Cell Survival

Irradiation

Cells were seeded into T25 cell flasks at a density of 300 cells per 5 ml of cell culture media, for the purpose of completing a clonogenic assay to assess survival of directly irradiated cells. For cell seeding, at 80-90% confluency, cells were trypsinized for cell detachment from large cell culture, and then neutralized with RPMI solution. This cell suspension was then used for cell counting to determine the number of viable cells. Cell counting was done using trypan blue and the BIO-RAD TC20 Automated Cell Counter (Bio-Rad Laboratories Inc., Berkeley, California, United States). Once counted, 300 cells were seeded into cell flasks respectively.

To prepare the radium stock solutions, the same procedure was utilized as outlined above in the Irradiation section. Final concentrations of 10, 100, 1000 and 10,000mBq/ml were achieved after adding radium stock solutions to cell flasks with RPMI. Tritium was also used as a positive control at a final concentration of 6.34MBq/ml.

In total, there were 6 experimental groups (n=9) used to observe cell survival. Four groups were treated with radium with respective concentrations of 10, 100, 1000, and 10,000mBq/ml. One group was treated with tritium at a concentration of 6.34MBq/ml as a positive control. And finally, one group remained untreated with only cells, as a negative control.

Clonogenic Assay

Cells were seeded into T25 flasks at a density of 300 cells in preparation for subsequent clonogenic assays to observe cell survival based on colony formation. Cell flasks were incubated for 7-9 days at 37°C and checked regularly for growth. Once colonies were observable to the naked eye in good formation, cells were ready for staining. Cells were stained with 10% carbol fuchsin solution in water for approximately 10 minutes. Subsequently after observing that colonies had been stained, flasks were rinsed and left to dry. Once dry, cell colonies were counted and recorded.

Cells counts were assessed as a surviving fraction in a percentage. The plating efficiency was determined from control cell counts with no irradiation. The plating efficiency was calculated by utilizing the average number of cells from each group of triplicates per each trial, then dividing this by the number of expected cells (300 cells seeded); it was multiplied by 100 to see as a percentage. Then, each individual cell count from each flask was corrected the plating efficiency per each trial. This was done by dividing each count by the number of cells expected (300) multiplied by the plating efficiency; it was multiplied by 100 to report as a percent. These calculations were done utilizing Microsoft Excel 2016.

Statistical Analysis

All experiments were conducted three times in triplicates tested for each trial, n=9. This data was inputted and analyzed in Microsoft Excel and Graphpad Prism 8. All data is represented as the mean, where error bars are represented as the mean ± standard error (SEM). For all data, a one-way ANOVA was performed followed by Tukey's post-hoc HSD test for significance. A p-value less than 0.05 was considered significant.

Results

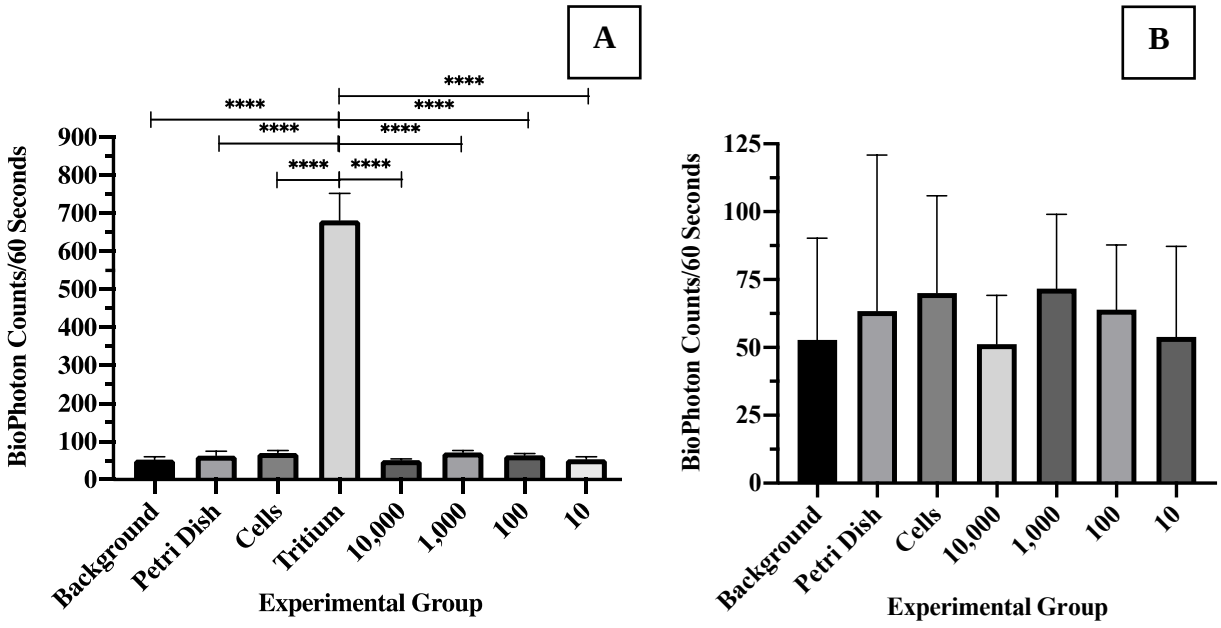

Figure 1. UVA photon counts from RA-226 directly irradiated HCT116+/+ cells. Counts were taken for 60 second intervals. **(A)** The first column on the left indicates photon counts with no experimental petri dish in the chamber (background count). The second column represents photon counts from the empty petri dish (no medium or cells) placed in the chamber. The third column indicates photon counts from the untreated group of cells. The Tritium column indicates photon counts from the group of cells treated with tritium at a concentration of 6.34MBq/ml, as a positive control. The last four columns represent photon counts from the experimental groups of cells treated with Ra-226 at the concentrations of 10, 100, 1000, and 10,000mBq/ml, respectively. **(B)** All columns are represented in the same manner as Figure (A), however, the Tritium column has been removed in order to visualize counts of all other experimental groups clearly. All experiments were conducted three times in triplicates tested for each trial, n=9. One-Way ANOVA - Tukey's post-hoc HSD test; A p-value less than 0.05 was considered significant; **** $p < 0.0001$. All data is represented as the mean, where error bars are represented as the mean ± SEM.

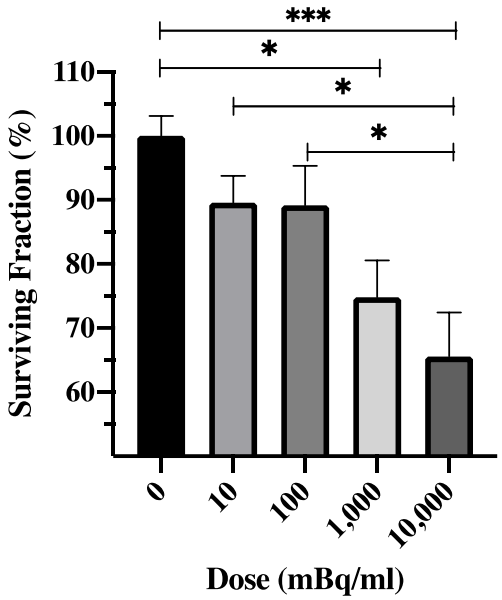

Figure 2. Surviving fraction of RA-226 directly irradiated HCT116+/+ cells. Cell survival was assessed within 7-9 days of exposure with 300 cells originally seeded. Cell survival was assessed as a surviving fraction (%) with respect to colony counts with no irradiation observed. The first column indicates cell flask with no irradiation (control). The plating efficiency was average ~47%. The last four columns represent photon counts from the experimental groups of cells treated with Ra-226 at the concentrations of 10, 100, 1000, and 10,000mBq/ml, respectively. All experiments were conducted three times in triplicates tested for each trial, n=9. One-Way ANOVA - Tukey's post-hoc HSD test; A p-value less than 0.05 was considered significant; * $p < 0.05$, *** $p < 0.001$. All data is represented as the mean, where error bars are represented as the mean ± SEM.

UVA photon counts from RA-226 directly irradiated HCT116+/+ cells

Figure 1. shows the UVA photon counts from Ra-226 directly irradiated HCT116+/+ cells. No significant results were observed between any radium groups, or background, petri dish and cell controls (≤100 counts/60 seconds) (Figure 1B). Therefore, radium produced no significant photon emissions above the background count. However, all groups showed a significant difference when compared to the 6.34MBq/ml tritium positive control ($p < 0.0001$) (Figure 1A).

Surviving fraction of RA-226 directly irradiated HCT116+/+ cells

Figure 2. shows the surviving fraction of RA-226 directly irradiated HCT116+/+ cells. Here, it is observable, that as the dose of Ra-226 was increased, the survival fraction was seen to decrease. This is further indicated, as the greatest significant difference is seen between dosages 0mBq/ml and 10,000mBq/ml ($p = 0.0005$). There are also significant differences existing between 0mBq/ml versus 1,000mBq/ml ($p = 0.0164$), 10mBq/ml versus 10,000mBq/ml ($p = 0.0246$) and 100mBq/ml versus 10,000mBq/ml ($p = 0.0282$). Although significant differences are not observed with each and every column, they are observably different as dose increases, with some concentrations being significantly different. Non-significant results were concluded when comparing 0mBq/ml versus 10mBq/ml, 0mBq/ml versus 100mBq/ml, 10mBq/ml versus 100mBq/ml, 10mBq/ml versus 1,000mBq/ml, 100mBq/ml versus 1,000mBq/ml and 1,000mBq/ml versus 10,000mBq/ml ($p > 0.05$).

Cell survival was assessed as a surviving fraction (%) with respect to colony counts with no irradiation observed. The plating efficiency was average ~47%. For trial 1, the surviving fraction was determined from a plating efficiency of 42.56%, followed by 56.44% and 42.11% for trial 2 and 3 respectively. Correspondingly, all three flask counts for each trial were normalized to each plating efficiency. The plating efficiency was as expected and reasonably stable during the experiment. Since cell survival was observed for short-term effects, only one passage was observed at the at a time interval of 7-9 days.

Discussion

After observing the results, the hypothesis where it was assumed that the increased exposure and sensitivity to Ra-226 will reflect the biophoton production and cell survival of the HCT116+/+ cell line in accordance with expected adverse biological effects was mostly supported. Radioactive material such as Ra-226 may still be the determinant source of causing biophoton production, although it was not observed here. In regards to cell survival, it was expected that Ra-226 is responsible for increased ROS, oxidative stress and perturbations in cell survival – this was supported in this study. It is also confirmed that cell death and consequently ROS production is an independent process and not always accompanied with biophoton production, as seen in this study.

UVA biophoton production from RA-226 directly irradiated HCT116+/+ cells

As seen in Figure 1., no significant results were observed from any radium groups when comparing to a background count, indicating that radium does not lead to biophoton production after exposure to HCT116+/+ cells. This is justified, as tritium has been previously explored and was used as a positive control in this experiment. Tritium has been previously seen to cause biophoton emission in multiple scenarios (Le, 2018; Rusin, 2021). Thus, it can be concluded that within this study, cells show no significant biophoton production when exposed to low doses of Ra-226.

This was an unexpected result, as it had been assumed that the root cause of biophoton production stems from high energy excited species, thus ROS induced by radiation exposure. As mentioned, biophoton production is spontaneous emission of ultra-weak light that comes from living organisms and hypothetically produced as a response to the generation of excited species followed by relaxation of excited species to a stable state (Wijk and Wijk, 2005; Cifra *et al.*, 2011).

It would be appropriate to assume increased ROS would be able to generate excited species that would then need to be brought back to a stable state by the organism system. Therefore, ROS production and biophoton production would be seen simultaneously. It is known that radioactive materials such as radium are able to induce oxidative stress. In a study observing the impact of exposure to relevant concentrations of Ra-226 in Atlantic cod embryonic cells, it was indicated that radium exposure is able to motivate oxidative stress and apoptotic trigger factors because a significant upregulation in transcription of associated genes was seen (Olsvik *et al.*, 2012). DNA damage as a result of oxidative stress was also seen in earthworms (Lourenço *et al.*, 2012), and changes in DNA and RNA were seen in cultured fish cells when exposed to Ra-226 (Shi *et al.*, 2016) . Thus, it had originally been hypothesized that exposure to Ra-226 (at low, environmentally relevant concentrations), would initiate highly energetic species from ROS and biophoton production, and these phenomenon are simultaneous and connected.

Interestingly, the effects of radium on cells had been explored previously, however there is a lack of discourse when discussing the production of biophotons via radium exposure. However, an explanation of the results achieved may be because increased ROS or oxidative stress alone does not produce a physical bystander signal i.e. photon production as reported by Rusin who had looked at the characterization of biophotons emitted from cells exposed to tritium (2021). It is said that irradiation is required to cause biophoton production because the energy of the incident radiation is absorbed by biological molecules. This causes electrons to occupy higher energy states and as we mentioned, the relaxation results in biophoton production (Rusin, 2021). Now, as we will explore later, we have definitely observed ROS and oxidative stress from this study overall. However, it is unsure that radium exposure was able to be absorbed due to the low

concentrations used, given time frames, or other factors. Other studies have looked at ionizing radiation that can cause oxidative stress, however were observed at much higher concentrations (Thornalley and Vašák, 1985; Satoh *et al.*, 1989; Cohen *et al.*, 2020). One study observing Ra-226 and its effects on the ragworm *H. diversicolor* at environmentally-relevant concentrations, found that Ra-226 had no effect on the oxy-radical scavenging capacity of the organisms compared to control. The study suggested that the dose given and the dose absorbed of Ra-226 are different and therefore, low concentrations may have no effect in regards to excitation from irradiation. They concluded that it is unlikely that environmental exposure to Ra-226 causes high levels of oxidative stress (Grung *et al.*, 2009). This was also supported by another study that explores given external dosages of radioactive materials such as uranium, protactinium and polonium are different than internal absorbed amounts in benthic invertebrates (Thomas and Liber, 2001). The conclusion of this study is slightly different, where it is believed that environmentally relevant concentrations of Ra-226 are able to produce oxidative stress (which will be further discussed in the cell survival section below); per contra, these concentrations are not potent enough to be well absorbed by the cells and have enough energy to excite electrons to higher energy states (and consequent relaxation) where photon production would occur. Oxidative stress or ROS alone is not enough to cause biophoton production, but instead high irradiation to reach specific high energy states that would allow biophoton production. This would unequivocally need to be confirmed with additional experimentation, yet the limiting factor seems to be dosage and possibly time of irradiation. From this study, increased exposure and sensitivity to Ra-226 at environmentally relevant concentrations did not affect the biophoton production of the HCT116+/+ cell line. Though, it is predicted that higher concentrations will yield better results considering abundant

excitation states. It is also determined that biophoton production is independent of oxidative stress and more so reliant on irradiation exposure.

Cell Survival of Ra-226 directly irradiated HCT116+/+ cells

Observing Figure 2., it seems that Ra-226 is able to affect cell survival and colony formation. In general, as the dose of Ra-226 increased, the survival fraction of the cells decreased. There were significant differences between many groups going across the graph, including 0mBq/ml versus 1,000mBq/ml, 10mBq/ml versus 10,000mBq/ml and 100mBq/ml versus however the most significant was between the dosages 0mBq/ml and 10,000mBq/ml, as expected. It can be concluded that Ra-226 has an impact on the cell survival of directly irradiated HCT116+/+ cells since as dose was increased, the survival fraction was seen to overall decrease.

This was an expected result. It is assumed that oxidative stress causing lethal mutations in cells is the reason for a decreased cell survival and colony formation. Oxidative stress is a result of an imbalance (usually increase) in reactive oxygen species (ROS) due to abnormal production and accumulation within a system (Pizzino *et al.*, 2017). The production of ROS is a normal part of cellular homeostasis; however, it becomes concerning when it manifests as an imbalance - oxidative stress. Oxidative stress can cause damage to major biological macromolecules including DNA, proteins and membrane phospholipids. Severe damage to these macromolecules can lead to cell death (Schieber and Chandel, 2014). It has been reported that accumulation of heavy metals and isotopes such as uranium, radium etc. can cause an abnormal increase in ROS including superoxide radical, hydroxyl radical, and hydrogen peroxide leading to oxidative stress. This is because of the radioactive and unstable nature of these elements (Rosemond *et al.*, 2005; Bessa *et*

al., 2016). The effects of Ra-226 on cell culture survival has been previously observed in many studies, explored below.

Fernando *et al*. (2020), found that HaCaT progeny cells showed reduced survival after being exposed to Ra-226. Significant decreases in cloning efficiency were seen in secondary progeny cells. Some findings were comparable; the study used 100mBq/ml, 1,000mBq/ml and 10,000mBq/ml to directly irradiate cells, similarly to this study. It is important to note that this was the lower end of the concentrations of Ra-226 used by Fernando et al. At 100mBq radium, directly exposed cells had a survival fraction of 69±8%. At 100mBq/ml in our study, the surviving fraction was 89%±6%. The surviving fraction was lower by approximately 20% in the study observed by Fernando et al. At 1,000 mBq/ml, directly irradiated cells saw 71%±13% survival rate compared to 74±6% survival rate in our study. This is extremely comparable, as both rates fall within deviation of each other. Similarly, at 10,000 mBq/ml, our study saw 66±7% survival while Fernando et al., saw 70±7 survival rate. Again, these findings are comparable considering both fall within error. These similar findings indicate that similar survival rates are seen in cells exposed to the same concentrations of Ra-226. The differences may be due to different experimental conditions (such as different cell lines) but overall, many findings were similar and comparable suggesting that it is justified in concluding that Ra-22 has negative effects on cell survival when exposed. The authors related this to hyper-radiosensitivity followed by increased radioresistance which allows for some cell survival. The authors noted however, that at low doses alpha rays should cause non-targeted effects like genomic instability that arises in distant progeny with mortality seen later. This was because further cell death was seen with progeny cells in that study.

However, it was conclusive in both cases that increased Ra-226 exposure reduces cell survival even at the directly-irradiated level.

Another study also observed the effects of Ra-226 on HaCaT cells and saw reduced survival with exposure. Shi (2016) saw significantly decreased clonogenic survival in cultures continuously cultured with Ra-226 for 20 days. The differences became greater overtime. However, even with directly irradiated cells, there was a general pattern of a decreased cell count with increased dosage of Ra-226 similarly seen with the current study. The percentages were not reported by Shi, however, taking a look at the results, it is visible that the cell count was approximately 15% lower in cells directly irradiated by 10,000mBq/ml radium compared to the control of 0, five days into irradiation. This is higher than our findings, but it is worthwhile to consider that both studies saw a decrease in colony formation with increased dosing of Ra-226 within cells. It is also substantial that this is again a different cell line with other experimental differences (such as incubation periods). This again confirms our hypothesis in which HCT+/+ cell survival is affected when exposed to Ra-226, specifically increased exposure leads to decreased cell survival.

Considering this, it is conclusive that exposure and sensitivity to Ra-226 at environmentally relevant concentrations does effect the cell survival and colony formation of the HCT116+/+ cell line. It would be important to consider that higher concentrations than this may have alternate effects. As well, it is suggested that longer incubation and exposure periods such as that seen in the studies by Shi (2016) and Fernando et al. (2020), would show further significant differences in cell survival in both directly exposed and progeny cells. Thus, it is justified that Ra-226 comprehensively decreases cell survival overall, likely due to increased ROS and oxidative stress.

Limitations

Initial attempts to achieve photon counts were quite unsuccessful. Petri dishes were seeded with cell densities of 20,000 to 200,000 cells per 5ml of medium for testing. Originally, cells were observed for photon counts directly after exposure to Ra-226 or tritium irradiation. Later cells were tested for photon counts 6 hours after exposure to irradiation. None of the conditions listed above were able to produce results, and no photon counts were observed greater than the background count (data not shown). Photon counts were partially observed after 24 hours after irradiation (data not shown); however, it was determined that the optimal photon count could be achieved by pouring off the medium. The reason for this, was so that no photons were absorbed by the medium and thus undetected while photon counting. The optimal conditions as addressed in the material and methods section was accomplished after 5-6 rounds of preliminary testing. It is very likely that higher concentrations of Ra-226 may have seen more valuable results and greater thresholds, however it was important to test environmentally relevant concentrations. It is also possible that there are better optimal conditions, however due to financial and time restrictions and limitations, this method was chosen as results were able to be seen.

In comparison to other articles that have observed tritium, photon counts from this experimentation were significantly lower in magnitude (Ahmad *et al.*, 2013; Le, 2018). This may be indicative of various experimental methods, although we did not have success utilizing the methods outlined in these articles. Rusin (2021) had utilized the same photon counter one year prior and reported various technical complications. This includes the suspicion that the photon counter has become less sensitive over time as it is an older hardware. Rusin reported a possible "lossy signal transmission between the PMT and scalar card" (2021). Additionally, possible

deterioration of the PMT itself or changes in settings that are not present on the immediate switchboard. A combination of these mechanical issues may be the culprit for an overall lower photon count achieved in this study.

Furthermore, it would have been worthwhile to explore progeny cell survival in HCT+/+ cells with exposure to Ra-226. This could be achieved with a greater number of clonogenic assays overtime, especially observing cells "born from" directly irradiated cells in an unirradiated healthy environment. This would allow further conclusions regarding non-targeted effects and radiation-induced bystander effects (RIBE). Nonetheless, due to time limitations, it was decided not to pursue this avenue within this study.

Applications

The important application of biophoton production stems from the ability for them to potentially cause radiation-induced bystander effects (RIBE). A bystander effect is considered to be an effect on a unirradiated cell or population of cells where they respond similarly to cells that have been directly irradiated. Rather this includes progeny cells of irradiated cells, cells physically close to irradiated cells, or cells in a similar environment as irradiated cells (Mothersill and Seymour, 2004; Marín *et al.*, 2014). A radiation-induced bystander effect is a type of non-targeted effect that occurs due to the release of different types of signals from irradiated cells. This includes multiple types of non-targeted effects including activation of the MAPK pathway, calcium signaling, mitochondrial effects etc. (Rusin, 2021). This can also include the emission of light, such as biophotons as a form of unsoluable factor signal which can then act as a cautionary signal to unirradiated cells to "warn" them regarding potential harm from irradiation. This is a very new

phenomenon that is currently being explored, and is not yet fully understood. It is unclear exactly what the purpose of biophoton production is, in the sense that the signaling system is not yet understood. Even the way that biophotons are produced is hypothesized and not yet completely clarified as we explored above. Additionally, it has always been assumed that biophoton production and production of reactive oxygen species are associated, as we have also explored above (Marín *et al.*, 2014; Mothersill *et al.*, 2019). This is also especially observed in bystander cells and their production of ROS (Lyng *et al.*, 2011). This is justified again, since it is thought that biophoton signaling is done comprehensively with other soluble signaling factors (Le *et al.*, 2017; Jella *et al.*, 2018). All-in-all non-targeted biophoton production independently and in correlation with other factors, as a source of radiation-induced bystander effects, is a new and explorable avenue in radiobiology at the moment.

At the same time, the reason for the imperative explorations of biophotons as a radiation-induced bystander effect is because of its relevant applications in the field of radiation sciences. Main considerations with non-targeted effects include cancer induction and micro environmental plasticity (Mothersill *et al.*, 2019).

When looking at the manifestation of cancer and bystander effects, there is an increased likelihood for cancer to re-establish because existing cells that were once effected have bestowed bystander effects on “recipient” cells. These bystander cells are then able to act in a cancerous fashion once again, without ever being triggered by the same cancerous stimulus – this includes progeny cells and nearby healthy cells (Wang *et al.*, 2018; Mothersill *et al.*, 2019). Many studies have shown that after low-dose exposure to cancer inducing triggers, there has been persistent

expression of clastogenic factors, micronuclei or microsatellite instability as a result of bystander effects seen in distant progeny cells of those exposed previously or in the blood of people who were exposed several years prior (Dubrova *et al.,* 1996, 1997; Emerit *et al.,* 1997; Marozik *et al.,* 2007; Mothersill *et al.*, 2019). Adverse effects of radiotherapy are also another consideration when observing cancer and non-targeted effects. Bystander effects may exacerbate carcinogenic damage from these treatments. Reducing non-targeted bystander effects in normal tissues is a potential solution and novel target for improving radiotherapy treatments for cancer. Yet, this does not consider the implication that non-targeted effects are important for adaptive and protective responses to harmful stimuli (such as ionizing radiation – radium) (International Atomic Energy Agency, 1969; Mothersill *et al.*, 2019; State Institution «National Research Center for Radiation Medicine of the National Academy of Medical Sciences of Ukraine *et al.*, 2019). What does consider this, is the idea of stimulating bystander effects from healthy normal cells to tumor cell populations. It is hypothesized that UVA/biophoton exposure concomitant with radiotherapy could be a possible avenue to conduct this potential therapy (Mothersill *et al.*, 2019). Nonetheless, fully understanding the mechanisms of non-targeted effects, in all cases, including biophoton production will allow for the development of particular, and pristine treatments in cancer therapy. It can also provide insight into the prevention, diagnosis and treatment of cancer.

Another relevant application involves the possibility of micro environmental plasticity with the occurrence of radiation-induced bystander effects. Essentially, this refers to the micro environmental changes in a cell or organ in response to changes in the system (Wisdom *et al.*, 2018). This is similar to interests in cancer research, as here we can also discuss impacts on bystander cells and how they are influenced by non-targeted effects. But, here we are particularly

looking at cross-talk and plasticity between functional units such as organs, endothelium, fibrous tissue, and components of the endocrine system; instead of cancerous cells (Gandhi and Chandna, 2017; Mothersill *et al.*, 2019). For example, patients who have experience low-dose medical diagnostic exposure would be pertinent to this application. Even more so, in the case of radium, it is very much pertinent to look at bystander effects in the natural environment where organisms may be exposed; this could include ecosystems surrounding radium deposits, organisms living in uranium milling effluent environments, even offspring of organisms briefly exposed etc. Minor micro environmental changes could potentially lead to large organism level changes, which is relevant to consider. Overall, it is important to understand the non-targeted bystander effects such as RIBE and biophoton production on micro environmental plasticity and the greater implications of it, as seen here.

Positively, it is important to consider the applications of the effects of α-irradiation/Ra-226 exposure on cell biophoton production and cell survival. This particularly involves the implications of non-targeted RIBE effects, which in this case encompasses cancer research and environmental ramifications.

Future Direction

In future studies, it would be beneficial to observe the effects radium exposure to a greater degree - in a fashion that is more comprehensive. This would include observing the effects of Ra-226 in higher concentration, as this study only observed low-dose effects. It would be worthwhile to observe effects on cell survival and biophoton production in a similar manner as this study with high doses. Similarly, it would be interesting to observe long term effects of Ra-226. The thesis written by Le et al., is an excellent starting point for looking at long-term effects of Ra-226, however there is no exploration on biophoton production overtime. Additionally, observing the cell survival of progeny cells of exposure, or cells in continuous exposure would be an interesting take. Furthermore, utilizing medium run-off from exposed cells with "new" cells with no irradiation may test the ability of the bystander effects via biophotons. Another important adaptation of this study would be to measure and quantify oxidative stress and ROS production alongside observing biophoton production, and consequently clarifying the independence of these events. There are absolutely many possibilities. Due to the financial and time-related limitations of this study, only preliminary data regarding biophoton production as a result of radium exposure was observed; although it is still a relevant area of study that has not been well explored.

Otherwise, it is also valuable to explore other forms of irradiation in order to comprehensively understand the effect and correlation between biophoton production and radioactive material exposure. This information is very valuable in understanding the bystander effects of biophotons, as explored above.

Conclusion

To conclude, the hypothesis of this study was partially supported, in which an increased exposure and sensitivity to Ra-226 showed decreased cell survival, and assumed to have implications on biophoton production at high concentrations. However, at environmentally significant low doses studied in this report, Ra-226 did not affect biophoton production via α-irradiation. We did expand evidence that biophoton production results from exposure to radioactive material, although not substantially. It was also contended from the results that increased ROS/oxidative stress and biophoton production are independent events that effect cells, but can both arise when cells are irradiated and both observe highly energetic species. This is confirmed when looking at cell survival after Ra-226 exposure in this study, where it was concluded that higher concentrations of Ra-226 will consequently lead to decreased cell survival. It was also suggested that longer exposure periods would further support this idea. We effectively explored the application of Ra-226 and biophoton production, as a means of non-targeted radiation induced bystander effect and this relevance in environmental impact and cancer research. Finally, it is acknowledgeable that biophoton production is an important area of study in radiobiology and radiation sciences at the moment, exceptionally because it is a novel phenomenon in the peak of its discovery.

Abbreviations

ANOVA	Analysis of variance
CO2	Carbon dioxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
HCT116+/+	Human colon carcinoma cell line 116 with wildtype protein 53 expression
MAPK	Mitogen-activated protein kinase
NTE	Non-targeted effect
NIM	Nuclear Instrumentation Module
p53	Protein 53
PMT	Photomultiplier tube
Ra-226	Radium-226
RIBE	Radiation-induced bystander effect
RNA	Ribonucleic acid
ROS	Reactive oxygen Species
RPMI	Roswell Park Memorial Institute
SEM	Standard error of the mean
Tukey's HSD	Tukey's honestly significant difference
UV	Ultraviolet
UVA	Ultraviolet A

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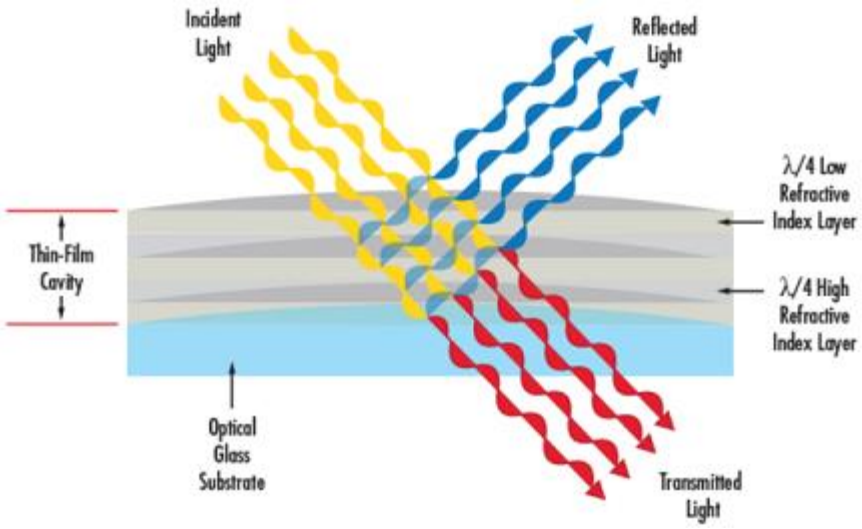
Appendix:

Supplementary Information: Optical Filters

In this study, only a 340nm optical bandpass interference filter was used to exclusively look at UVA biophoton emission.

Optical filters are used to block out other emissions of light except for the wavelength specified (Ahmad, 2012). In this case, UVA has a wavelength of 320-400nm. All other biophotons were blocked out to ensure we were only observing biophotons – alpha particles in the UVA range that would be expected with radium decay and exposure in the cells.



This diagram was created by Edmund Optics (Optical Filters | Edmund Optics, 2021). Incident particles (incident light) hit the filter which is made up of multiple layers of material of varying indexes of refraction. This exploits the interference of light waves. The filter is made so that the desired wavelength of light constructively interferes with the incident light to pass through the filter (transmitted light), while the light of all other wavelengths is reflected (reflected light).

Supplementary Equation 1.1 Radium Dose Calculation and Administration

The activity of radium was assumed to be the same over the course of the experiment in a four-month span. This is because the half-life of radium is approximately 1600 years and any amount of decay occurring within the experimentation time frame would be considered negligible (Radium-226 Decay Chain, n.d.). The activity of the stock Ra-226 is 9.436 μCi in 5 ml.

Converting this into mBq/ml:

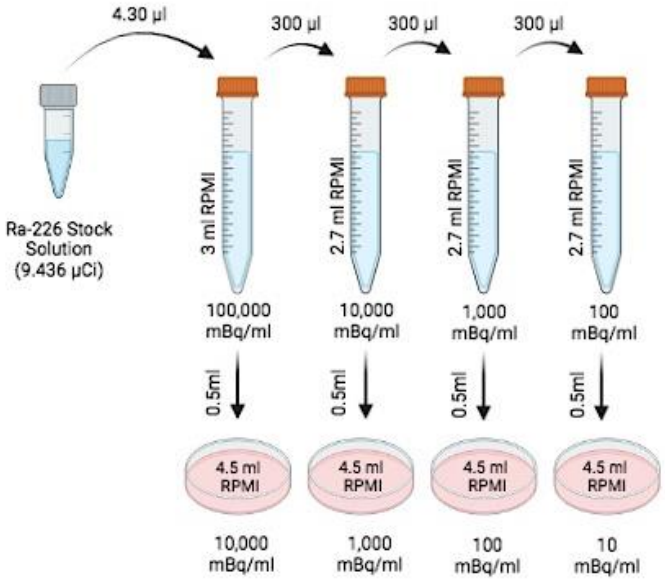
9.436 μCi = 349 132 Bq
349 132 Bq ÷ 5 ml = 69 826 400 mBq/ml or 6.98×10^4 Bq/ml

Calculating for initial concentration of 100,000 mBq/ml:

$C_1V_1 = C_2V_2$
V_2 chosen to be 3ml

(69 826 400 mBq/ml) V_1 = 100,000 mBq/ml (3ml)
$V_1 = 4.296 \times 10^{-3}$ ml = 4.30 μl

The following serial dilution was done followed by administration to petri dishes to achieve final concentrations of 10,000 mBq/ml, 1,000 mBq/ml, 100 mBq/ml and 10 mBq/ml in respective petri dishes and/or flask (in the same fashion):



Supplementary Equation 1.2 Tritium Dose Calculation

Since tritium was used as a positive control, dose delivered to directly irradiated cells was done using the same methods as described in previous publications. 0.5 Gy tritium is considered to be an adequate dose to allow for biophoton production when exposing HCT+/+ cells (Ahmad, 2012; Rusin, 2021).

The dose 0.5 Gy tritium was solved for using this equation:

$$D = \frac{N_0 \lambda_R \bar{E}_\beta t}{m}$$

This equation represents the dose of ß-radiation emitted by tritium and absorbed by cell culture in Gray (Gy). Where D represents dose (Gy); $N_0\lambda_R$ represents activity of tritium in decays per second or Becquerel (Bq); $\bar{E}_\beta$ represents the average energy of tritium beta particles in Joules (J); t is the duration of irradiation in seconds; and m represents the mass of the irradiated object in kilograms (kg).

The activity of tritium was assumed to be the same over the course of the experiment in a four-month span. This is because the half-life of tritium is approximately 12.3 years and any amount of decay occurring within the experimentation time frame would be considered negligible (PubChem, n.d.). Therefore, the activity of the tritium ($N_0\lambda_R$) was 31,727,500 Bq (as provided by the supplier).

The average energy of tritium beta particles ($\bar{E}_\beta$) is 9.7KeV or 9.13×10^{-16} J (Radioactivity : Tritium, n.d.).

The time (t) or durations of irradiation used in this experiment was 24 hours or 86400 seconds.

The mass (m) was considered to be the mass of the cell culture medium. The density of the cell culture medium was approximated to the density of water at ~1g/ml under normal atmospheric conditions. Because 5ml cell culture medium was used, the mass (m) was assumed to be 5g or 0.005 kg.

Solving for this equation gave a dose of 0.5 Gy. This was then converted back to Becquerel to remain consistent with radium dosing.

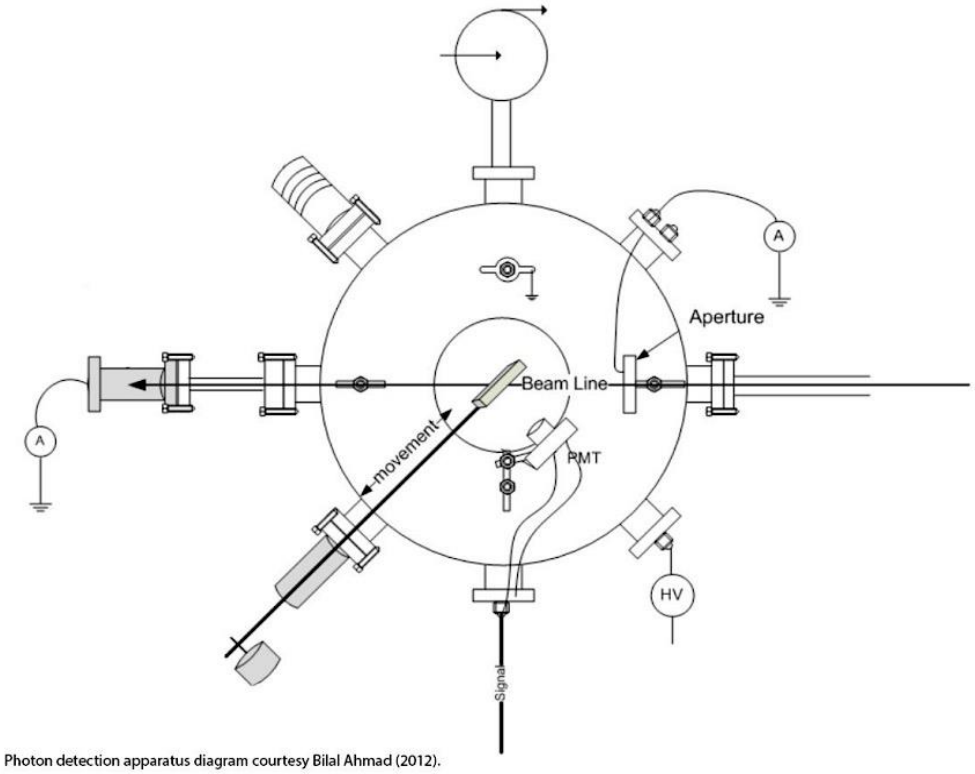
0.5 Gy = 31,727,500 Bq

31,727,500 Bq ÷ 5 ml = 6,345,500 Bq/ml = 6.34 MBq/ml

It was then divided by 5 to account for dilution in 5ml cell medium and consistent units. Thus, dose for tritium was determined to be **6.34 MBq/ml.**

31,727,500 Bq = 0.0008575 Ci = 857.5 μCi

1 μCi = 1 μl tritium, thus 857.5 μl was administered to control tritium cell groups.



Photon detection apparatus diagram courtesy Bilal Ahmad (2012).

Supplementary Figure 1.1 Aerial top view of photon quantification apparatus. In this view the top lid is open; while counting, it was to be closed and completely light tight with blanket covering. The PMT is the photomultiplier tube, which is the primary part used for photon detection. The inner circle represents the area where the petri dish was placed for quantification. This diagram was illustrated by Bilal Ahmed (2012).

Supplementary Figure 1.2 Nuclear Instrumentation Module (NIM) of Photon Counter. The panels used for operating the photon counter is seen here. The power to the NIM is found on the right side. The power was turned on and voltage on the HV Power panel was turned on and dialed to -800V for 30 minutes before use, to allow for the apparatus to "warm up". The Stop, Start and Reset buttons on the Scaler panel were used to respectively start, stop and reset photon counts during detection. All other dials and setting remained the same, as set up by previous students in the laboratory. This image was taken by and courtesy of Andrej Rusin (2021).