A New Method to Investigate RECA Therapeutic Effect

by

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Medical Physics Program

Duke Kunshan and Duke University

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James Bowsher

Thesis submitted in partial fulfillment of

the requirements for the degree of

Master of Science in the Department of

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ABSTRACT

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Abstract

Introduction: RECA (Radiotherapy Enhanced with Cherenkov photo-Activation) is a novel treatment that induces a synergistic therapeutic effect by combining conventional radiation therapy with phototherapy using the anti-cancer and potentially immunogenic drug, psoralen. This work presents a novel method to investigate the therapeutic effect of RECA using rat brain slices and the agarose-based tissue equivalent material.

Methods: 4T1 mCherry Firefly Luciferase mouse breast cancer cells are placed on the brain slice after exposed to psoralen solution. Taking fluorescent imaging of the brain slices every day after irradiation, an independent luciferase imaging was taken after the fifth fluorescence imaging. Using different imaging processing and analysis method to identify the cells.

Result: Four analyzing method give different result about the fluorescence signal or luminescence signal. The overall trend of the fluorescence signal is rising over day, reaches the lowest point at 48 hours after irradiation. Control group (no radiation and no Cherenkov lights) has the lowest signal compared with other groups. The signal of brain slices with 4T1 cells exposed to psoralen solution is lower than that of brain slices without psoralen exposition.

Conclusion: This work shows that rat brain slice can be used to simulate in vivo environment in exploring the therapeutic effect of RECA. Future work should focus on improving the image analyze method to better identify cells and noises.

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1. Introduction

Psoralen is a photosensitive drug which can absorb radiation and damage the function of DNA and other cell components. After been activated by the Ultravoilet A (UVA light, 320-400nm), psoralen molecules intercalate with DNA bases, forms photoadducts and cross-links. Psoralens also react with both proteins and lipids as well as other cell constituents, thus damage the cell function and inducing cell apoptosis [1]. In addition, studies showed that immunogenic responses have been observed in patients treated with psoralen, with proposed mechanisms including up-regulation of major histocompatibility complex I (MHC I), up-regulation of immunogenic transcription factors, and promotion of T-cell development, maturation, and proliferation [2]. 8-MOP is one kind of psoralen molecules which is normally used in clinic. Psoralens have been widely used for skin diseases, for example, vitiligo, psoriasis, and skin cancers. However, psoralen therapies are limited to treat superficial diseases because of the difficulty of generating UVA lights in deep-seated tissue, while UVA light is the required to activate psoralen. To solve this problem, a novel treatment, X-ray psoralen activated cancer therapy (X-PACT) [4] has been proposed.

X-PACT uses kilovoltage (kV) x-rays beam and phosphor to generate UVA lights in deep tissue. Phosphor particles absorb x-rays and emit UV lights which can activate the psoralen molecules. However, X-PACT also encountered some challenges in clinic application. The first one is that X-PACT requires to inject phosphor intermediaries into tissue, the other one is the high skin and bone dose due to requirement of kV x-rays.

To improve these two limitations of X-PACT, studies have been made to investigate another novel treatment based on the principle of psoralen, that is radiation therapy enhanced with Cherenkov photo-activation (RECA) [3]. RECA treatment utilizes clinical megavoltage (MV) x-rays and the psoralen to achieve the goal of combining the effect of conventional radiation therapy and the therapeutic effect of psoralen. The MV x-ray emits Cherenkov lights, which is a broad-spectrum UV-visible light product, in tissue while simultaneously delivers radiation dose to the target area. The Cherenkov lights emitted in tissue thus photo-activate psoralen.

In the recent in vitro RECA studies in B16 melanoma and 4T1 murine breast cancer cells, luminescence assays showed that cytotoxicity increased in RECA treatment, and flow cytometry showed that median MHC I expression was significantly higher in RECA treatment, also clonogenic assays showed decreases in tumor cell viability in RECA treatment. This in vitro RECA studies identifies the potential for Cherenkov lights activated psoralen treatment in deep seated tumor [3]. However, the solid water used in this study which mimics soft tissues has been proved having much lower UVA emission than bulk tissue sample in another study [5]. This study also established the soft tissue equivalent material have the similar response of UVA emission as soft tissue, which is made by agarose gel and India ink.

This work presents a novel method to investigate the therapeutic effect of RECA using rat brain slices and the agarose-based tissue equivalent material. The following chapters provide an overview on the theory of Cherenkov lights emission and the mechanism of psoralen, describe a series of experiments to explore RECA therapeutic effect and Cherenkov emission

from the agarose-based soft tissue equivalent material. Finally identified challenges for this work and improvements can be made for future work.

2. The Principle of Cherenkov Effect

Cherenkov radiation is electromagnetic radiation which is emitted when a charged particle, most commonly an electron, passing through a dielectric medium (soft tissue) with the speed higher than the phase velocity of light in this medium [6]. Cherenkov emission was first observed by the Soviet scientist Pavel Cherenkov and Sergei Wawilow [6]. As a charged particle moves inside a polarizable medium with molecules, it excites the molecules to the higher levels and excited states. After the molecules return back to the ground state, the molecules re-emit photons in the form of electromagnetic radiation, which moves out spherically at the phase velocity of the medium. If the speed of this charged particle is slow, the radiated waves bunch up slightly in the direction of motion, but do not cross. However, if the charged particle moves faster than the phase velocity of light, the emitted electromagnetic waves add up constructively leading to a coherent radiation at angle θ with respect to the particle direction, that is the Cherenkov radiation [7]. Figure 1 is a schematic of the Cherenkov radiation produced by the charged particle with the velocity of u in the angle of θ[8].

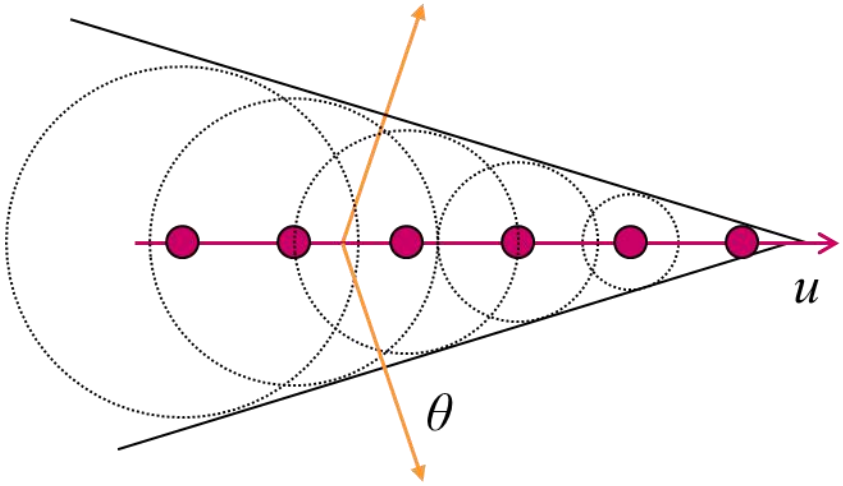

Figure 1: The schematic of Cherenkov radiation with charged particle velocity of u and angle of θ.

According to Shrock's Monto Carlo simulation on modeling Cherenkov emission from medical linear accelerators (2016), the spectrum of Cherenkov lights which are generated by medical linac matches well with the psoralen absorbance spectrum between the wavelength range of 200nm to 400nm, as showed in Figure 2 [9]. This overlap in spectrums means that the photon generated Cherenkov lights works well for activating psoralen.

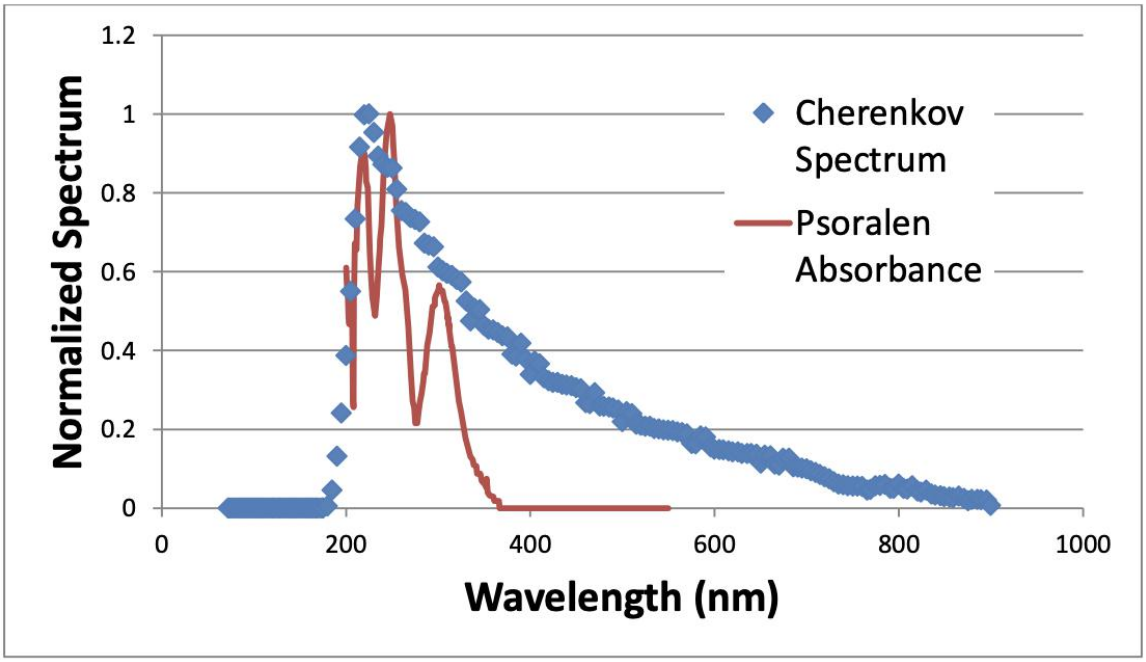

Figure 2: Cherenkov emission pectrum compared to psoralen absorbance spectrum [9].

3. Method

3.1 Experimental arms

In order to look at the effects of RECA on 4T1 cells expressing the mCherry red fluorescent protein (RFP) [6] in rat brain slices. The agarose phantom which has 250ppm (parts per million) India ink concentration created in Jain's work [5] was used to mimic tissue to produce the UVA Cherenkov lights, while the agarose phantom with 1000ppm was used to achieve same dose but blocked the UVA Cherenkov lights. Four arms are included in the study, with one 6-well plate corresponding to each arm. Every arm further included two categories (3 wells each), 8-MOP exposed and non 8-MOP exposed. The control arm received no irradiation. The second arm is kV arm which received 160keV irradiation to 4Gy and the third arm was exposed to MV irradiation, with a very dark agarose sample which has 1000ppm India ink concentration to block UVA lights. The final arm was exposed to 4Gy MV irradiation and UVA (RECA), by placing cell wells were on a tissue-simulating agarose phantom which has 250ppm India ink concentration.

Table 1: Experimental arms for RECA in vitro studies

Arms	Radiation	UVA lights
Control	No	No
kV (RT only)	kV	No

MV (RT only)	MV	No
MV (RECA)	MV	Yes

3.2 Cell culture and preparation

The rats used in this study are CD Sprague-Dawley rats from Charles River, which were sliced into 400μm thickness slices. The rat brain slices were plated in Corning 6-well plates with transwell inserts (one slice per well) as in Figure 3 and neurobasal media (Gibco) was used for the slice culture. Cell line used in this study is 4T1 mCherry Firefly Luciferase mouse breast cancer cells, which was infected with the mCherry Firefly Luciferase virus, with the Multiplicity of Infection ratio (MOI) of 50, using 8 μg/ml polybrene. After been plated on the 6-well plates, the 4T1 cells were exposed to the virus and been centrifuged at 32C for 30 minutes. A day after infection, flow cytometry can be used to sort the mCherry cells, then the cells were incubated at 37C, 5% CO2, and 90% humidity. The cell media for 4T1 cells consisted of RPMI1640 (Gibco), 10% FBS (Tissue Culture Biologicals), Pen/Strep (Gibco), HEPES 10mM (Gibco), Sodium Pyruvate 1mM (Gibco), D-Glucose 0.25% (Sigma-Aldrich).

Figure 3: Corning 6-well plates with transwell inserts [7].

On the day of irradiation, the 4T1 mCherry cells were trypsinized off of two 10cm dishes and counted using Nexelcom's Cellometer Auto 2000 in order to prepare two tubes with 1 million cells each. They were centrifuged again and re-suspended either in 100 µL of fresh media (no psoralen category), or media containing a pre-mixture of 99 µL media + 1 µL of 10mM 8-MOP in dimethyl sulfoxide (DMSO). The DMSO and 32 media were mixed in advance as the mixing is exothermic and could be detrimental to cell health. All the psoralen addition was performed in minimal room lighting to avoid UV contamination. 3 µL of this mixture (instead of 5µL in Jain's work [5]) was plated on each slice, ensuring roughly 10,000 cells (instead of 50,000 cells in Jain's work [5]) per slice. Four 6-well plates were hence prepared with three psoralen and non-psoralen containing ells each.

3.3 Irradiation and Imaging

In this study, we have both kV irradiation and MV irradiation. The MV irradiation was carried out in PA (posterior-anteriorly) position (as showed in Figure 4) using a single 15MV photon beam for these two MV irradiation arms. While the kV irradiation was carried out at the X-RAD 160 irradiator with 160kV X-rays and a tube current of 18mA (dose is roughly equal to 4Gy). The transwells which holds the rat brain slices and cells, were placed on top of 2cm-thick light (250ppm India ink concentration) and dark (1000ppm India ink concentration) 1% agarose slabs (which were placed at 100cm SSD) and were irradiated to 400MU (4Gy). The dark agarose slab ensured minimal UVA activation of the psoralen-exposed cells, whereas the light agarose mimicked tissue UVA emission and psoralen-activation. After irradiation, baseline fluorescent images were taken of each of the 24 slices using the Zeiss Lumar V12 Stereoscope with 17.6x magnification and +0.63mm focus. Rhodamine filter with an exposure time of 2500ms, using the Zen Blue Pro software were used to take fluorescence images. Images of each slice were acquired once every 24 hours for the next five days as showed in Table 2. After taken the fifth fluorescent images, the Luciferase imaging was taken for checking the Firefly Luciferase signal.

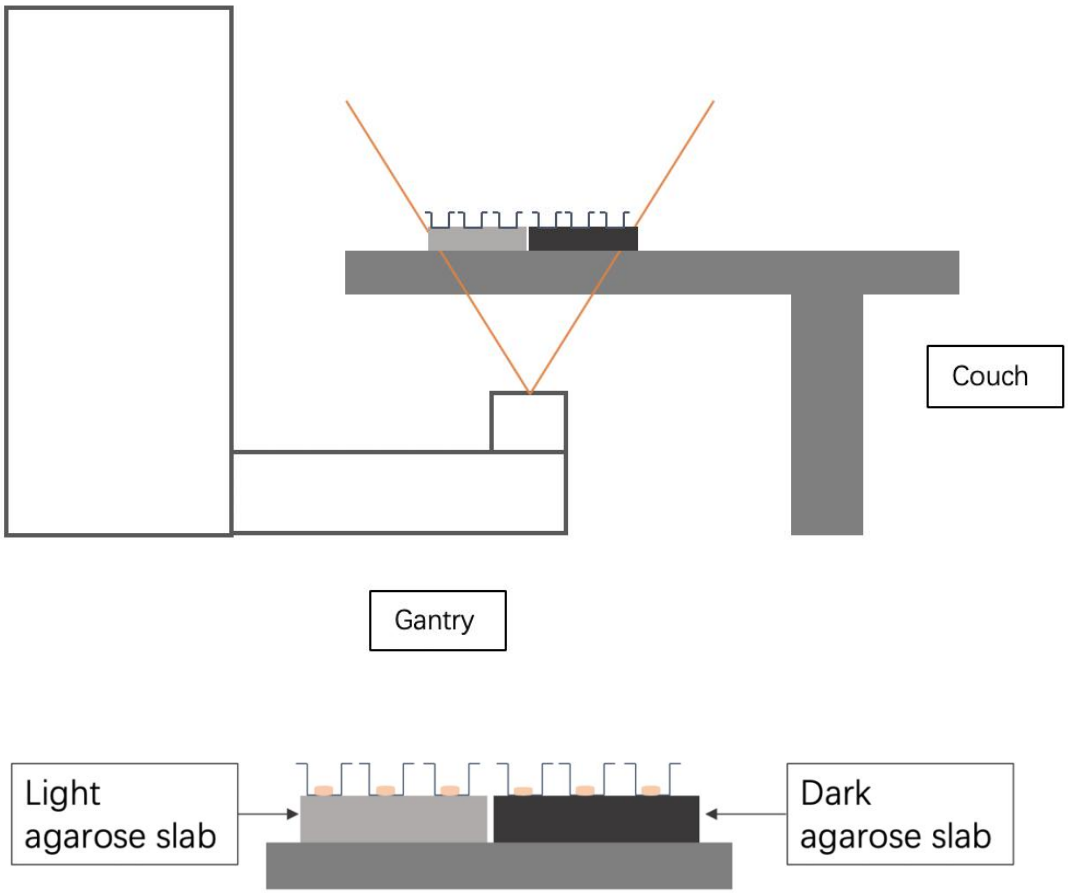

Figure 4: The geometry of MV irradiation

Table 2: Time of fluorescence images

	Day1	Day2	Day3	Day4	Day5
Time after irradiation	24 hours	48 hours	72 hours	96 hours	120 hours

3.3 Image analysis

Three analysis method were used for analyzing the Fluorescent images, single threshold analysis, multiple threshold analysis, and Cellprofiler analysis. The signal analyzed in this study is the fluorescent signals, which is bright in the images and reflects the survival rate of 4T1 mCherry Firefly Luciferase mouse breast cancer cells, thus we can explore the therapeutic effect of radiation therapy enhanced with Cherenkov photo-activation (MV radiation combined with Cherenkov light emission from the tissue equivalent agarose slab and 8-MOP). The single threshold analysis and multiple threshold analysis were taken with Fiji (ImageJ) [8] software, the Cellprofiler analysis was conducted with Cellprofiler software [9]. The first step of doing single threshold analysis and multiple threshold analysis is to convert the raw images into binary then counts the size of bright area (number of pixels) which is thought as cells emitting fluorescence signal. The single threshold analysis requires to set a threshold to separate the background noise and auto-fluorescence with the 4T1 cell fluorescence signal, then took an average over the fluorescent signals of the three brain slices in each group. The data from day2 to day5 was normalized based on day1's data. The multiple threshold analysis bears the same principle as the single threshold analysis, but applies two or three thresholds in order to better separate the background noise and auto-fluorescence with the fluorescence signals. At the center region of the brain slices, the fluorescence signals are higher than the signals at the edge of the brain slices as show in Figure 5, which makes it harder to identify the alive cells. Figure 6 shows two thresholds applied in one image. Theoretically, by applying multiple threshold, the background noise and auto-fluorescens can be

filtered, thus we can get the fluorescent signals which reflects the cancer cell survival rate. The Cellprofiler analysis utilizes Cellprofiler software, which is a free open-source software for measuring and analyzing cell images, to do the analysis. The first step of the Cellprofiler pipeline is to input the optical images, fluorescent images, and a mask compassing the brain slices. The optical images and the fluorescent images are directly derived from the microscope, while the mask is an ellipse generated by MATLAB. Then the pre-set modules are added to identify the brain slices, thresholds are set to filter cells, all these data and images are exported after all the analysis process finished. While single threshold analysis and multiple threshold analysis, which utilizes ImageJ software to conduct the image analysis, counts the pixel number of the area which is thought as have alive cancer cells. The Cellprofiler analysis measures the integral intensity of identified cells.

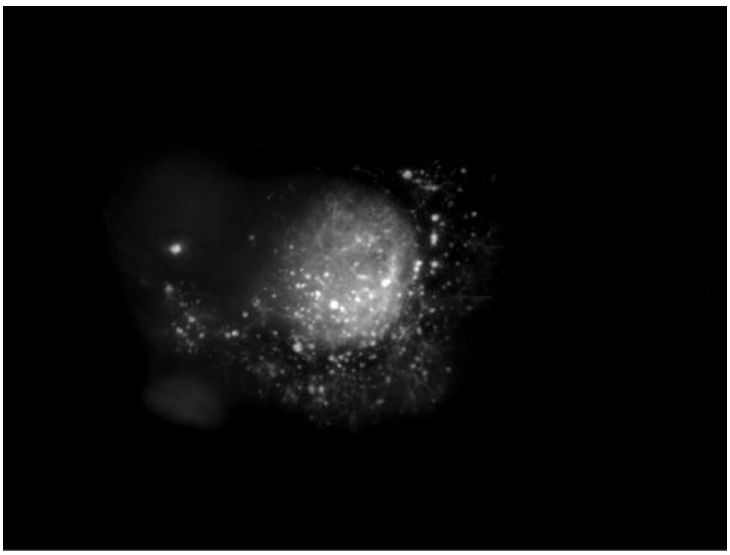

Figure 5: A fluorescent image of the brain slices.

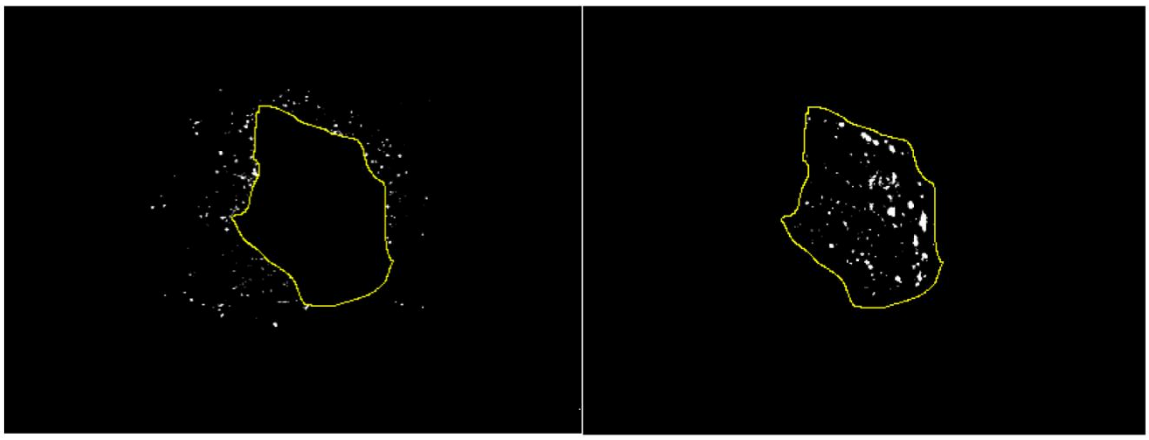

Figure 6: Two threshold applied in one image.

3.4 UVA imaging of agarose slabs

In order to check the accuracy of the results and determine whether agarose has the same Cherenkov emission as soft tissue, an UVA imaging was performed to the dark agarose slab (1000ppm) and light agarose slab (250ppm). The set-up is showed in Figure 7. The camera is CCD camera [10] equipped with the visible light lens, cooled to -85℃. The camera and agarose slabs were placed into the dark chamber on the LINAC couch. These slabs were irradiated by an MV photon beam at 100cm SAD with a $4 \times 4cm^2$ field size. While the camera was angled at 45° towards the slabs. The MV photon beam was delivered at 6 and 15 MV with 600MUs with the dose rate of 600MU/min. The slabs is placed in a 3D printed cuboid mold to stand them up.

Imaging analysis of the UVA images also took with ImageJ. 30-pixel-radius median filter was used to remove noise, which was produced by scattered and leakage radiation hitting the CCD detector and dark current in the camera electronics. Region of interest (ROI) was taken in the agarose

slabs and background. The background signal was subtracted from the average counts in the agarose slabs.

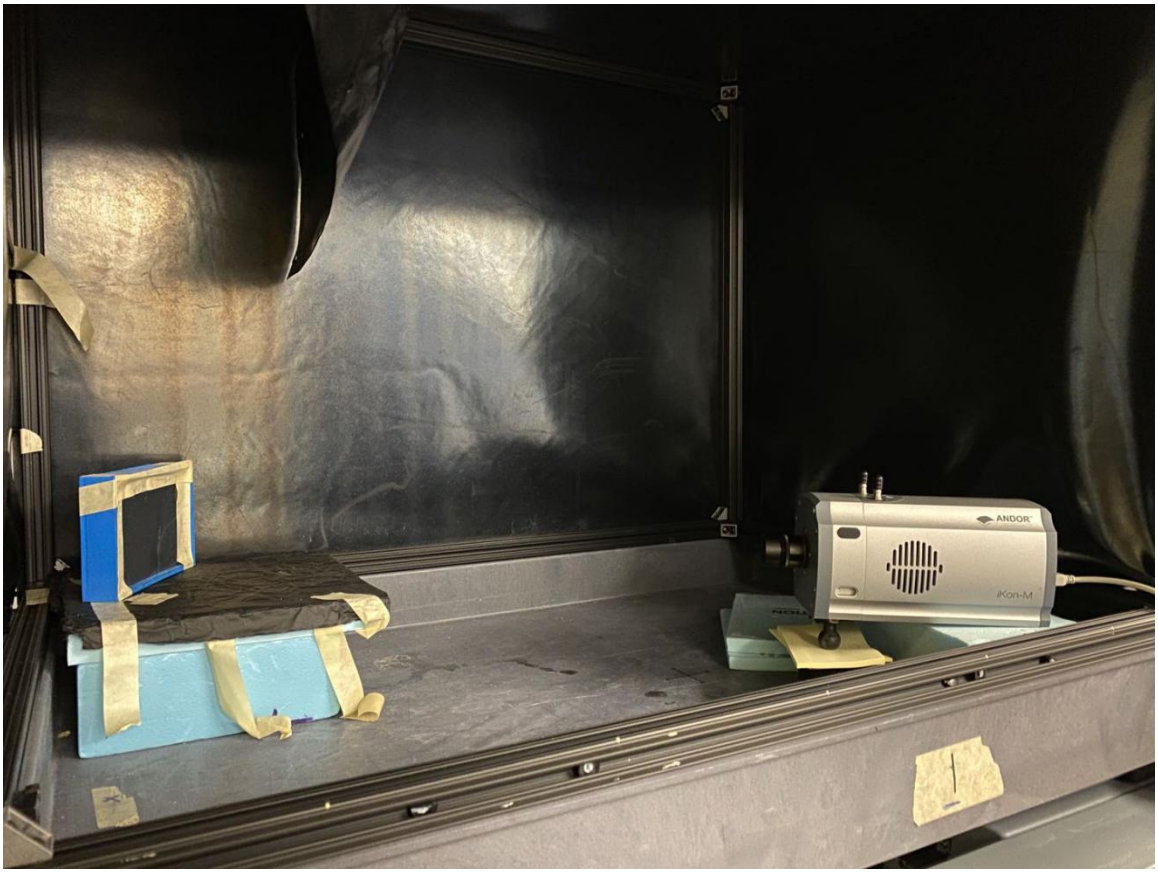

Figure 7: Set-up of UVA imaging of the agarose slabs.

4. Result

The optical images and fluorescent images obtained by the microscope are showed in Figure 8. By comparing these images over five days, some general observations were got. (1) The fluorescence signal increases over time but reaches the lowest point at day2. (2) The background noise increases over time.

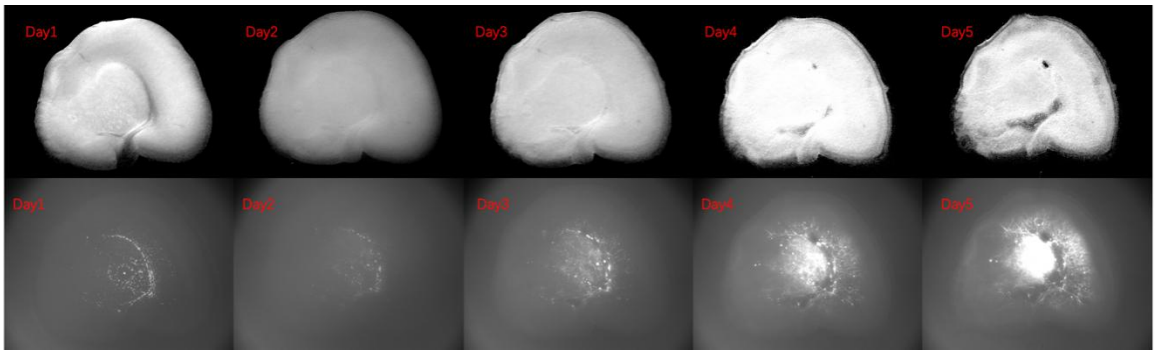

Figure 8: The optical images (upper row) and fluorescent images (lower row) of the brain slices from day1 to day5.

4.1 Single Threshold method

The average changes of fluorescence signal over day 1 is shows in Figure 9. The overall trend of the fluorescence signal goes up, but it reaches the lowest value at day2. The control groups, both with 8-MOP and without 8-MOP, have the highest signal after day3, which is corresponding with what we excepted. The data of MV (RECA) with psoralen drug group also matches the expectation, which goes lowest from day3 to day5. In addition, the groups with 8-MOP have lower signal than those from groups without 8-MOP, which means that psoralen drug works in proliferating 4T1 cancer cells. However, during day1 to day2, the fluorescence signal of MV (RT

only) without 8-MOP has the highest signal. This high signal means most 4T1 mCherry Firefly Luciferase mouse breast cancer cells survive, which is contradicts the expectations because MV radiation is applied in this group.

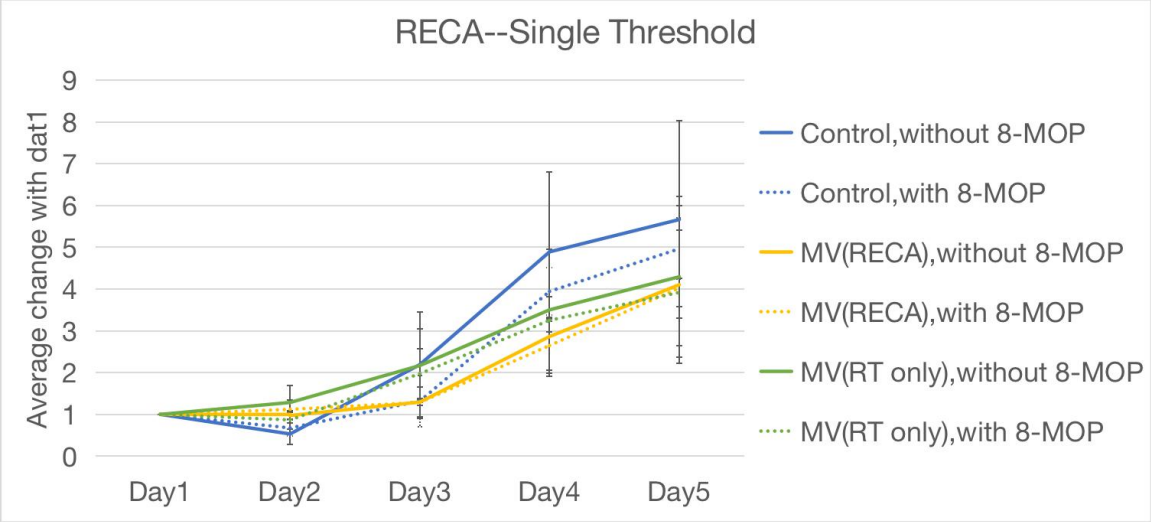

Figure 9: The average change of fluorescence signal normalized over day1 using single threshold method.

4.2 Multiple Threshold method

The average signal change over day1 of each group is showed in Figure 10. Same as what shows in 4.2, the fluorescence signals of Control groups, MV (RECA) without 8-MOP group, and MV (RT only) without 8-MOP group reached the lowest point at day2, while the fluorescence signals of MV (RECA) with 8-MOP group and MV (RT only) without 8-MOP group reached the lowest point at day3. Considering excessive suppression of the fluorescence signals due to multiple thresholding, the overall trend after day 2 is still rising.

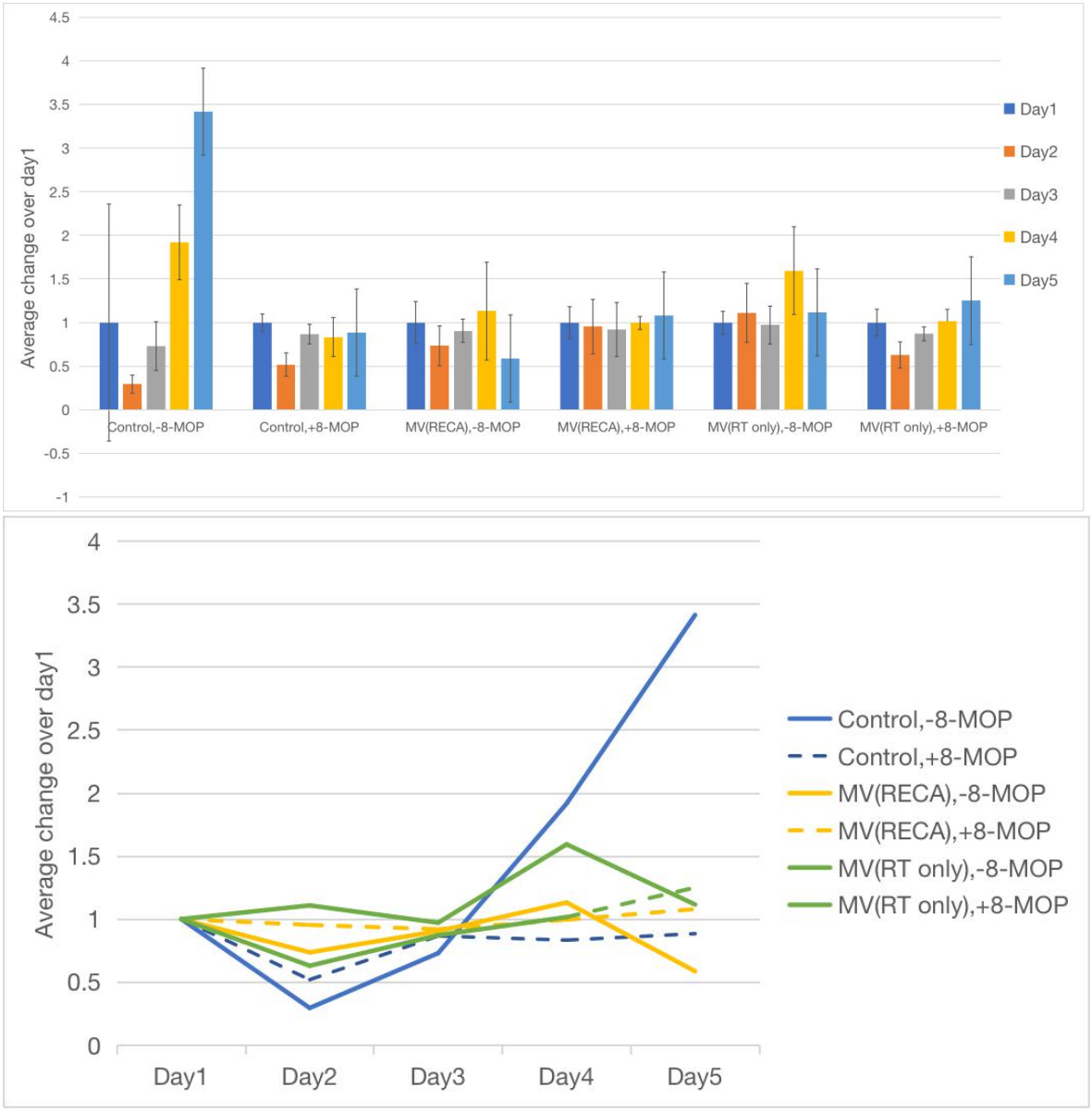

Figure 10: The average change of fluorescence signal normalized over day1 using multiple threshold method.

The fluorescence signal of Control without 8-MOP group at day5 is 3.41 times as that at day1, while the lowest change of 0.59 of MV (RECA) without 8-MOP group. The number of 4T1 cells at 120 hours after

irradiation was 3.5 times as that at 24 hours after irradiation. The Control without 8-MOP group has the most survival cells, which matches the expectation. Another observation is that the fluorescence signals of MV (RECA) without 8-MOP and MV (RT only) without 8-MOP at day 5 decrease compared with day 4. This decrease might due to excessive suppression of the signal by multiple thresholds, another possibility is that the brain slices is not healthy enough for cancer cells to survive.

4.3 Cellprofiler analysis

The key step in the Cellprofiler analysis is to identify cells and filter cells, so that the background noise and auto-fluorescence can be filtered. There are two thresholds need to be clarified in the Cellprofiler pipeline, the minimal integrated intensity and the minimal mean intensity. In this study, the minimal integrated intensity was set to 1 and the mean integrated intensity was set to 0.05.

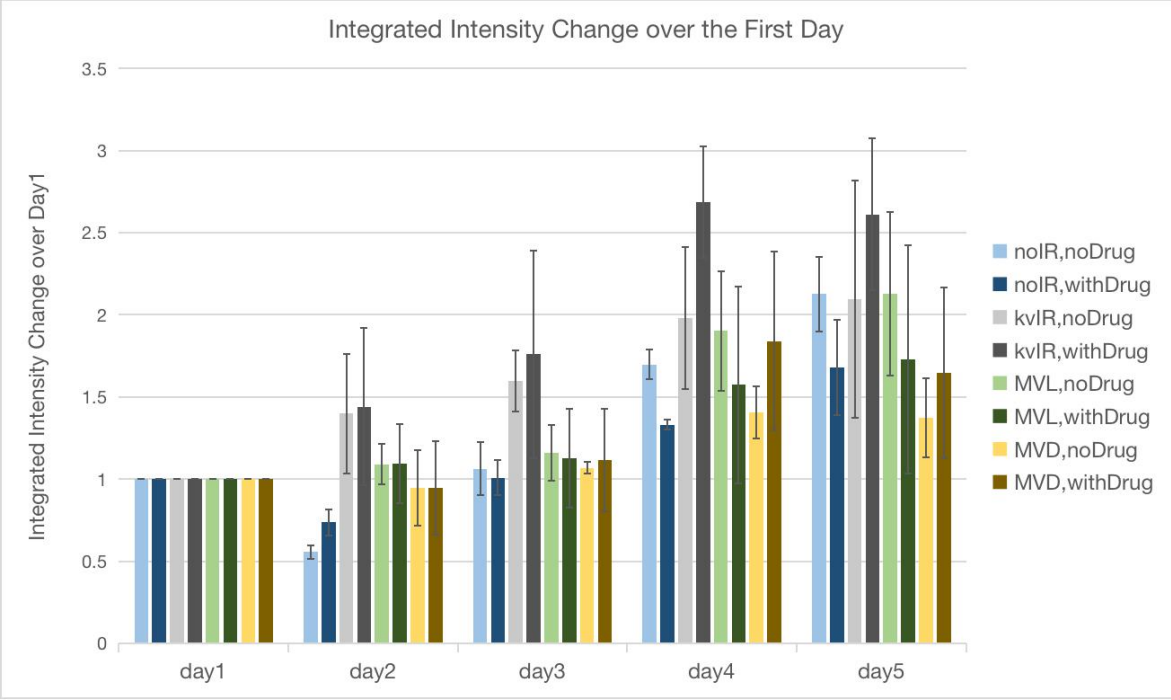

Figure 11: Integrated intensity change over day1.

As shown in Figure 11, the overall trend of the integrated intensity is still rising. All the groups reached the lowest point at day2, which is consistent with what shows in single threshold analysis. The kV groups have the highest integral intensity, and it is higher with psoralen presents than without psoralen. While the data of day3 and day2 is close, the integrated intensity changes a lot from day4 to day3. In addition, the Control group and MV (RECA) group show the inhabitation of cell proliferation by psoralen. However, in contrast, the integrated intensity in kV groups and MV (RT only) groups have higher survival rates.

4.4 Luciferase analysis

The 4T1 firefly luciferase reading as shown in Figure 12, indicates that No IR (Control) group has the highest luminescence signal, which means that the Control group have the highest cell survival. While in the kV groups, the luminescence is lowest. Additionally, in the kV groups and MV groups, the luminescence signal is higher with psoralen presents than that without psoralen, which is contradicts with expectation.

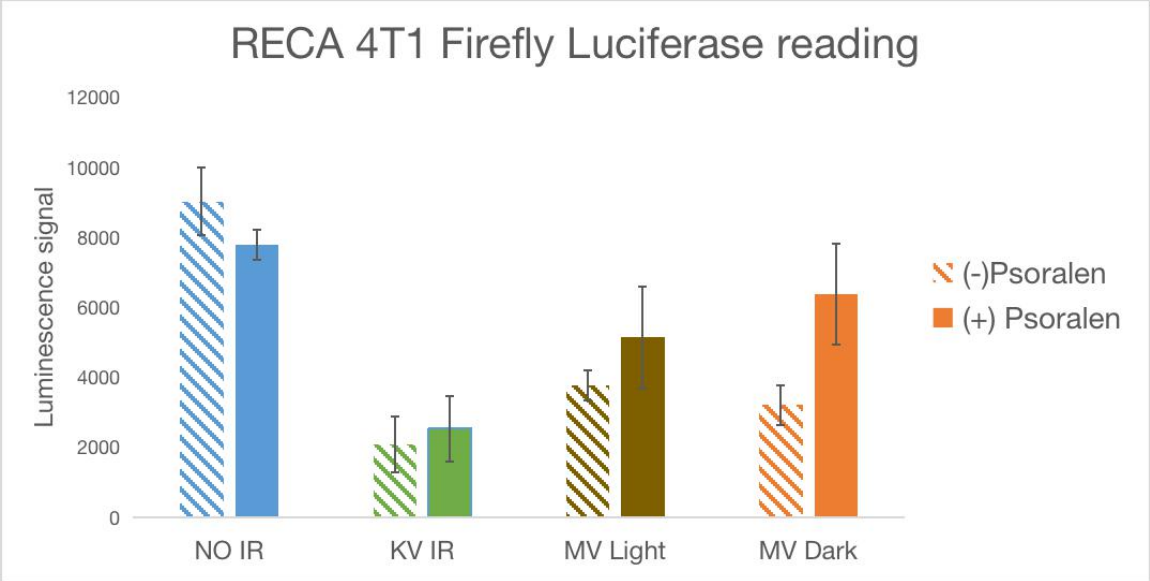

Figure 12: 4T1 Firefly luciferase data.

4.5 UVA imaging of agarose slabs

The UVA images filtered by 30-pixel-radius median filter are shown in Figure13 and Figure 14 shows the average counts per unit area after subtracts background. The Cherenkov emission of light agarose slab is higher than that of dark agarose slab, while under 15MV radiation, the Cherenkov emission is higher than that under 6MV radiation. The dark agarose slab emits Cherenkov light with 6MV and 15MV photon beam radiation.

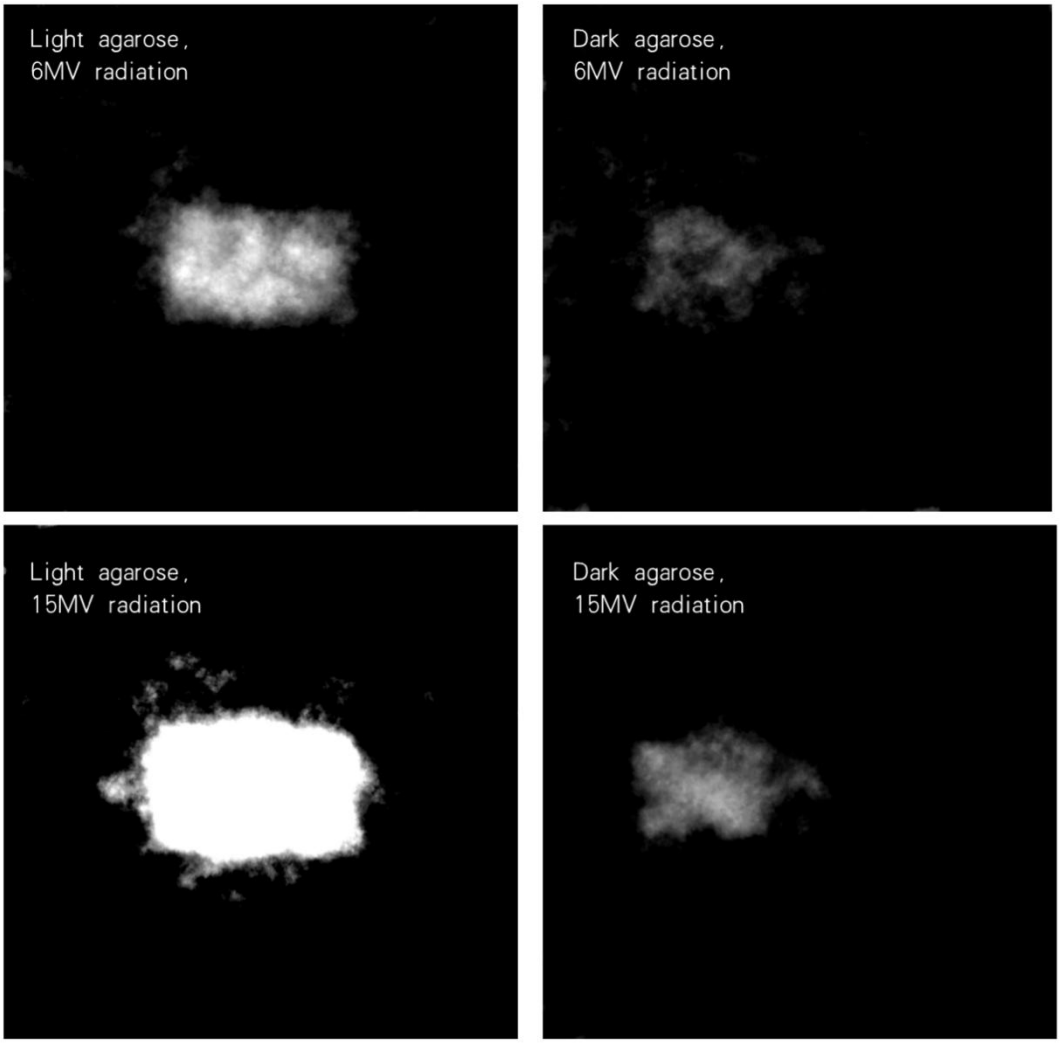

Figure 13: UVA images of agarose slabs after filtered with proper window and leveling.

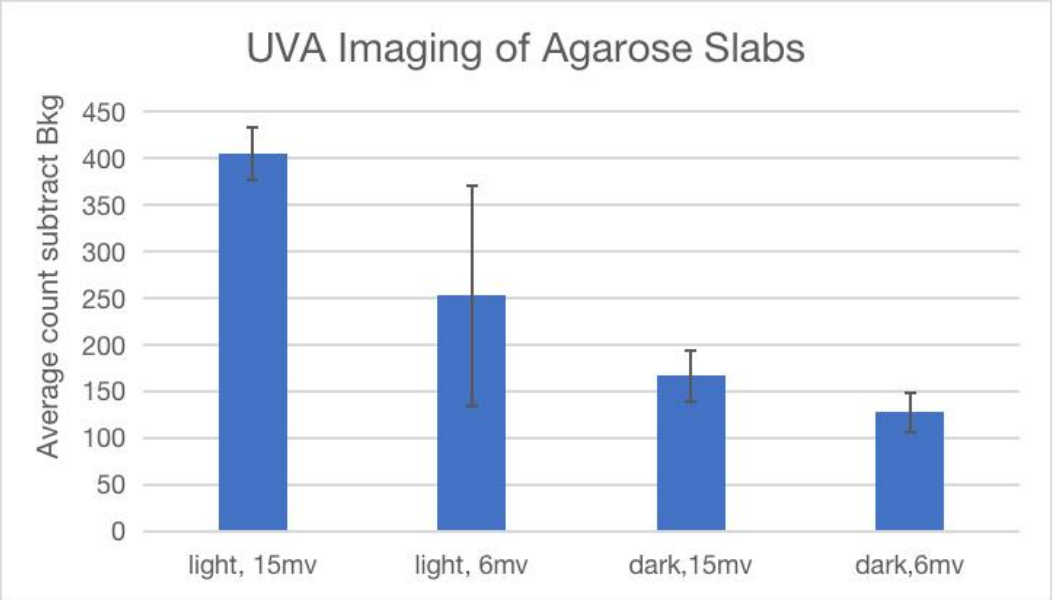

Figure 14: Average counts after subtracts background.

5. Discussion

The fluorescence signal analysis method in this study, including single threshold analysis, multiple threshold analysis, and Cellprofiler analysis. Single threshold analysis and multiple threshold analysis, which utilizes ImageJ software to conduct the image analysis, counts the pixel number of the area which is thought as have alive cancer cells. While the Cellprofiler analysis measures the integral intensity of identified cells.

As expected, the Control group demonstrated the highest cell survival since it does not have any treatment to the 4T1 mouse breast cancer cells. While the survival cells in groups with kV radiation and MV radiation should less than that in Control group, and the MV (RECA) group should have even lower cell survival rate. In this work, four methods are used for analyzing the fluorescence signal and luminescence signal, including single threshold analysis and multiple threshold analysis taken with ImageJ, Cellprofiler analysis using Cellprofiler software, and an independent analysis based on Luciferase signal 120 hours after irradiation. The overall trend of the fluorescence signal and luminescence signal is rising due to the proliferation of the 4T1 mCherry Firefly Luceferase breast cancer cells. The lowest point was achieved around 48 hours or 72 hours after irradiation. However, different image analysis method have different ways to identify cells and filter the background noise and auto-fluorescence, so that different analysis result would also appear. For example, as the result of the single threshold analysis, the fluorescence signal is lower in the groups with psoralen than without psoralen, which indicates that psoralen has some effect on inhibit cell proliferation. While the luciferase analysis result shows

that the groups with psoralen have higher signal than without psoralen. Same brain slices gave different result under different analysis method. Comparing the Luciferase signal and the fluorescence signal at 120 hours after irradiation, the 4T1 Firefly Luciferase data shows that kV group has the lowest luminescence signal at day5 while the Cellprofiler analysis shows that the MV (RT only) group has the lowest signal at day5, also the single threshold and multiple threshold analysis taken with ImageJ shows that MV (RECA) group has the lowest signal at day5. In addition, the data from Cellprofiler analysis shows that the kV group has the highest fluorescence signal or in another expression, has the highest cell survival, while the result of single threshold analysis, multiple threshold analysis, and the luminescence data indicates that the Control group has the highest cell survival at day5, which is consistent with the expectation. From all these data, it can be identified that radiation and psoralen have some effect on inhibiting cell proliferation. However, the therapeutic effect of radiation combined with Cherenkov photo-activation has yet to be demonstrated, and requires further experimental investigation.

According to the result of UVA imaging, which measures the UVA emission from the agarose-based phantom, the generation of UVA in dark agarose phantom could be one of the reason that the fluorescence signal in MV (radiation only) arm, using dark agarose phantom, is close to that in RECA arm, which uses light agarose phantom. The difference between light agarose phantom and dark agarose phantom is that light agarose phantom mimics the Cherenkov light emission as soft tissue, which the dark agarose phantom should absorb all the UVA light. The UVA generated by agarose phantom could photo-activate the psoralen, thus psoralen damages cell

functions. However, the UVA imaging shows that dark agarose phantom also emits UVA, which means that psoralen in MV (radiation only) arm had been activated.

The next set of experiment should focus on reducing noises on the experiment set-up and imaging procession as well as removing noises and auto-fluorescence in image analysis procession. A calibration image before every imaging session is an efficient way to keep all the imaging in consistent. Emphases should also be put on checking psoralen loading, which can be achieved by exposing the 4T1 cancer cell exposed to the psoralen directly to known UVA source. A set of rat brain slices without placing 4T1 cancer cells can help check the background of the brain slice itself. In addition, considering the dark agarose slab, which is expected to have minimal Cherenkov emission, generate around one-third to half UVA light as the tissue equivalent agarose slab, placing a light block on the dark agarose slab is necessary. Besides, an improved analysis method capable of better identifying cells and filtering background and auto-fluorescence will be performed in future work. For higher statistical power, we could either use multiple ROIs in the same image set, or expand the experiment to include more wells per arm.

6. Conclusion

In this study, we used a new method to explore therapeutic effect of Radiotherapy Enhanced with Cherenkov photo-Activation, which is using brain slices and 4T1 mCherry Firefly Luciferase mouse breast cancer cells to simulate in vivo environment. Fluorescence images and Luciferase reading are taken for analyze the cell survival in different arms, including 4T1 cells with or without being exposed to radiation (which can produce Cherenkov lights in soft tissue) and psoralen (which can be activated by UVA lights). Three methods are used in analyzing the fluorescence images, which is single threshold analyze, multiple threshold analyze, and analyze using Cellprofiler software. The results from both imaging analysis and Luciferase reading showed that the overall trend of the fluorescence signal is rising over day, and reaches the lowest point at 48 hours (the second day of fluorescence imaging) after irradiation. While control group which has no radiation and no Cherenkov lights has the lowest signal compared with other groups. The signal of brain slices with 4T1 cells exposed to psoralen solution is lower than that of brain slices without psoralen exposition. All these observations indicate that brain slices with agarose slab (tissue equivalent material) can be used to simulate an in vivo environment for exploring the therapeutic effect of RECA, but the experiment need further improvement.

To get a better understanding of the usage of brain slice as a simulation of in vivo environment, there are some point can be applied in future experiment. Before each of the fluorescence imaging session, a calibration image can be taken to keep the images in consistent and remove some of the

background noise. The backgound noise and auto-fluorescence is an critical problem in analyzing the fluorescence images, a better analyze process to remove these noises can help in explore the application of brain slices in vivo environment and RECA. Also as the result of the Cherenkov imaging of the tissue equivalent slab used in this study, although we expect that dark slab should have no UVA emission, it actually emits, which means that a light block is necessary.

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