University of Arkansas, Fayetteville
ScholarWorks@UARK

Biomedical Engineering Undergraduate Honors Theses

Biomedical Engineering

5-2017

Optimizing a Standard Fasting Time for 2-NBDG Uptake Studies in Murine Breast Cancers

Andrew C. Briley

Follow this and additional works at: http://scholarworks.uark.edu/bmeguht

Part of the Bioimaging and Biomedical Optics Commons, Cancer Biology Commons, and the Other Biomedical Engineering and Bioengineering Commons

Recommended Citation

Briley, Andrew C., "Optimizing a Standard Fasting Time for 2-NBDG Uptake Studies in Murine Breast Cancers" (2017). *Biomedical Engineering Undergraduate Honors Theses*. 41.
http://scholarworks.uark.edu/bmeguht/41

This Thesis is brought to you for free and open access by the Biomedical Engineering at ScholarWorks@UARK. It has been accepted for inclusion in Biomedical Engineering Undergraduate Honors Theses by an authorized administrator of ScholarWorks@UARK. For more information, please contact scholar@uark.edu, ccmiddle@uark.edu.

Optimizing a Standard Fasting Time for 2-NBDG Uptake Studies in Murine Breast Cancers

Senior Honors Thesis

By

Andrew Briley

Department of Biomedical Engineering

College of Engineering

University of Arkansas

May 2017

Abstract

Recently, there has been a larger use of 2-NBDG, a fluorescent glucose analog, to study glucose uptake in different cell types. These cell types have ranged anywhere from bacteria to human cancer cells. However, there has yet to be a standard procedure and practice for using 2-NBDG. In this study, our goal is to create a standard fasting time for the cells before introducing 2-NBDG to them. This study uses 4T07 cells, a murine breast cancer cell line, to help optimize this fasting time. These cells were fasted at different time points in order to find the ideal fasting time. This ideal fasting time ensures the highest uptake of 2-NBDG in the cells. Intensity measurements, resulting from 2-NBDG fluorescence, were calculated to determine the optimal fasting time. The following paper details the procedure used including cell culturing and passaging, microscope setup, fluorescent imaging, and image processing.

1. Introduction:

Through the years, research has shown that cancer cells exhibit increased amounts of glucose uptake when compared to normal cells [1]. Initially it was believed that cancers will always show an increase in aerobic glycolysis, which came to be known as the Warburg Effect [2]. However, recently the validity of the finding has been up for debate by researchers. Zu et al. concluded in their research that cancer cells might be glycolytic in vivo due to a hypoxic environment [3]. To this date, more research needs to be done to learn why cancers lean towards glycolysis or OXPHOS. Conclusive research is needed on all cancer cell lines to have concrete data on the metabolic properties of the different cancers.

To study increased glucose consumption in cancers, many researchers have used the positron emission tomography (PET) technique with the glucose analog tracer fluorodeoxy-glucose (FDG) [2]. However, Bingsheng Huang et al. studied the effects of using PET/CT scanners and concluded the scans are accompanied by increased radiation dosage and cancer risk [4]. Other difficulties with radiotracers, like FDG, include disposing of radioactive waste [5]. Researchers must evaluate the negative effects of using FDG-PET compared to the gain in the research results. More recently, an analog of FDG has been used in researching glucose uptake in cancer cell lines. 2 [N-(7-nitrobenz-2oxa- 1, 2-diaxol-r-7l) amino]-2-deoxyglucose (2-NBDG) has been a novel tool for imaging glucose uptake without having the side effects of radiation exposure. 2-NBDG has been studied in a variety of cell lines which shows the versatility of the glucose analog. Yoshioka et al. studied the application of 2-NBDG in E. coli [6]. O'Neil et al. researched the uptake of 2-NBDG in nonmalignant epithelial cells, breast

cancer cells, and liver cancer cells [7]. Zou et al. used 2-NBG as a fluorescent indicator to measure glucose uptake in human hepatocarcinoma cells and in rat skeletal muscle cells [5]. Langsner et al. studied 2-NBDG uptake in excised breast cancer tumors and normal breast tissue. From this research, it has been concluded that 2-NBDG uptake in cancerous breast tissue is inherently higher than non-cancerous breast tissue based on fluorescence signal intensity values [8].

There are many parameters involved in using 2-NBDG to measure glucose uptake in different cell lines. Such parameters include fasting time of the cells, concentration of 2-NBDG, and incubation time with 2-NBDG. Zou et al. studied different concentrations of 2-NBDG as well as different incubation times with 2-NBDG present [5]. In order to effectively uptake 2-NBDG, cells must be fasted in media that does not contain d-Glucose. It has been shown by O'Neil et al. that media containing d-Glucose inhibits the uptake of 2-NBDG by competing for the glucose transporter (GLUT) to enter the cell [7]. When 2-NBDG is taken up into cells it becomes phosphorylated by hexokinase at the C-6 position which results in the fluorescent marker being trapped inside the cell [7]. Yoshioka et al. determined that the 2-NBDG metabolite was in fact 2-NBDG 6-phosphate by comparing molecular weights of the two [9]. By confirming the metabolite of 2-NBDG, it has been determined that 2-NBDG is an effective fluorescent marker for studying glucose uptake. Further research has shown that adding 10% fetal bovine serum (FBS) increases cell viability while fasting the cells in glucose free media [10]. With the addition of FBS to media lacking glucose allows researchers to keep cells viable with long fasting times, such as up to 150 minutes [10].

Since different studies have performed different fasting times [1,6-9], this paper's goals are to gain data from experiments on murine Balb/cfC3H cell lines to determine the ideal fasting time for cultured cancer cells before introducing 2NBDG to them. The fasting time can be defined as the amount of time cells are incubated without proper nutrients (glucose). To determine the ideal fasting time, multiple images were taken for each time point to observe the different intensities. We believed that by studying different fasting times we could find the most suitable amount of time for cells to be fasted prior to the addition of 2-NBDG to ensure the highest uptake of the fluorescent glucose analog. After finding this time, ex vivo tissue can be studied using the same fasting time found here to observe 2-NBDG uptake. It was decided to test 2-NBDG uptake at four different fasting times: 15 minutes, 30 minutes, 60 minutes, and 90 minutes. 2-NBDG uptake was also measured without fasting the cells prior to the addition of 2-NBDG (0 minutes).

In order to find the most appropriate fasting time for breast cancer tissues, it was decided to use the 4T07 cell line. The 4T07 cell line is one of the four tumor sublines that were isolated by Dr. Fred Miller from a spontaneously arisen tumor in a Balb/c mouse [11]. The 4T07 cell line is commonly used by researchers as a model for human mammary cancer because it is highly tumorigenic and has low metastatic potential [11]. As seen below, in Table 1, the different cell lines from this spontaneous tumor reach different levels of metastasis. 4T07 cells can be found in the blood and lungs, but no metastases ever form. Although the cancer cells do spread out from the primary location, they will not form tumors elsewhere. Using data found in this paper, along with other research [12], we can start to understand more about the metabolic processes

and glycolytic demand of different breast cancer cell lines. Since it has already been shown that 2-NBDG uptake is higher in cancerous breast tissue compared to non-cancerous tissue [8], we will be able to effectively differentiate between metastatic cancers from non-metastatic cancers. This will give physicians an advantage in diagnosing cancers. By knowing the specific metabolic properties and metastatic potential of cancer cell lines, physicians will be able to effectively treat patients with properly diagnosed cancer.

Table 1: Seen below is a table representing the metastatic capability of the sublines derived from a Balb/c mouse.

	Invasion	**Metastasis Location**
67NR	Primary Tumor only	N/A
168FARN	Local Invasion, Intravasation	Lymph Nodes (no nodules)
4T07	Extravasation	Blood, Lungs (no nodules)
4T1	Metastatic Growth	Lung, Liver, Bone

2.Methods:

2.1 Cell Culturing

As stated previously, this study focuses on studying the 4T07 cell line that was derived from a spontaneous breast tumor in a Balb/c mouse [10]. The cells were obtained from storage, thawed, and placed in a 75cm^2 flask with media. The media

used was Dulbecco's Modified Eagle's Medium (DMEM) (+) glucose with the addition of 10% (v/v) FBS, 2mM L-glutamine, 1% (v/v) nonessential amino acids, and 1% (v/v) penicillin-streptomycin. The cell culture flask was placed in a humidified incubator that was set to 5% CO_2 and 37 °C. Once the cells had reached approximately 80% confluency they were passaged into a new flask with DMEM. Cells were passaged based on standard cell culture techniques. After three passages, the cells were deemed ready to be used for the experiments.

2.2 Cell Plating

Once the cells were deemed ready to be passaged based on confluency, cells were plated at the same time. Three sample plates and one control plate was used for each fasting time point. For an adequate signal, it was decided to suspend 300,000 cells with 2 mL of DMEM in 35mm MatTek glass bottom dishes. Glass bottom dishes were used to warrant a true fluorescent signal from the cells without any interference from plastic. The total volume of cells used was dependent on the number of living cells per milliliter. Cell viability was measured using the Countess II Automated Cell Counter. The plated dishes were placed in the humidified incubator at 5% CO_2 and 37 °C for 24 hours. 4T07 cells were plated in four plates for each of the four fasting times plus the baseline. This gives a total of 20 plates for the 4T07 cell line.

2.3 Fasting and Fluorescence Imaging

Once the 24-hour incubation time was completed, plates were taken out of the incubator individually to undergo fasting. The DMEM media was removed from the plates and each was washed with PBS to ensure there was no glucose left in the plate. After washing, 0.5mL of DMEM (-) glucose was added to each plate. With the addition

of the glucose free media, plates were put into the incubator to “fast” per their respective times (0, 15, 30, 60, 90 minutes). After the respective fasting times were completed, plates were removed from the incubator to allow the addition of 2-NBDG. Each plate received 2-NBDG at a concentration of 400μM and was incubated for 20 minutes. This concentration was chosen based on prior studies [9]. After the 20-minute incubation time was completed, plates were removed and washed twice with PBS to ensure proper removal of 2-NBDG. 1mL of DMEM (-) glucose was added to each plate prior to imaging.

Fluorescent imaging was done using an Olympus IX81 microscope, shown in Figure 1. The optical setup includes an Olympus IX2-UCB controller, 75-Watt Xenon Burner, and a Hamamatsu Digital Camera. Images were taken using a 10X objective with a 2x2 binning. Based on the excitation/emission profile of 2-NBDG, an excitation filter of 465nm was used. The microscope was initially set up to view the cells using bright field microscopy. Once cells were in focus, the shutter was opened to allow the fluorescent excitation light reach the sample. From here, an image was captured, and then the stage was moved until the next FOV was chosen. A total of 5 images were taken per plate.

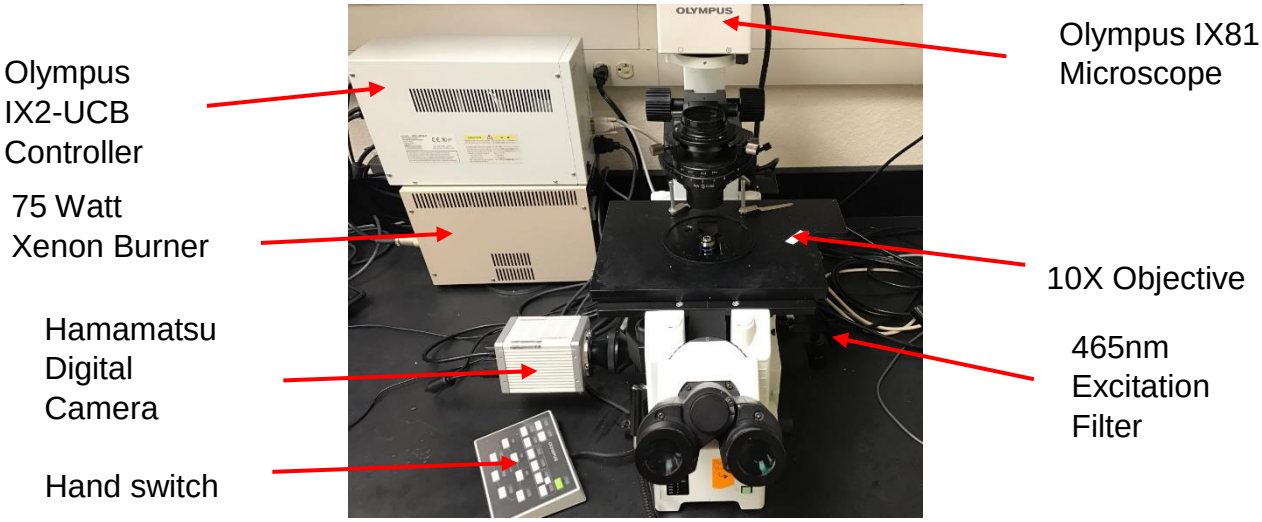

Figure 1: Above is the microscope setup used for imaging 4T07 cells.

2.4 Image Analysis

Using MATLAB, fluorescence intensity values were analyzed and calculated. Each image was run through a program to select a background region of interest. The average intensity of the ROI was measured and subtracted from each individual image. The average intensity of each image was calculated after background subtraction. This final intensity value is used for results.

2.5 Statistical Analysis

A nested, one-way analysis of variance test (ANOVA) was performed for intensity values of 2-NBDG 4T07 cells. The breast cancer cell lines used and the fasting times were considered fixed effects. The different plates and fields of view for each plate were nested in each group and considered to be random effects.

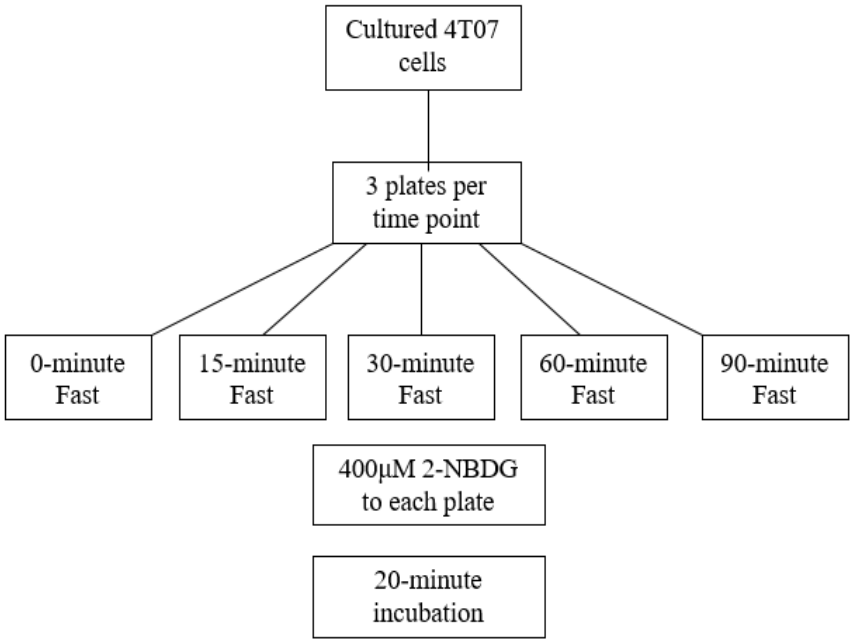

Figure 2: Shown above is brief flow chart that overviews the methods performed. represents the procedure for the 4T07 cell line. One additional plate was used for each fasting time, but did not receive any 2-NBDG.

3. Results:

4T07 Studies:

Images taken from each fasting time are shown in **Figure 3** for 4T07 cells. After a nested, one-way analysis of variance test, there was shown to be no significant difference in 2-NBDG uptake intensity values for 4T07 cells.

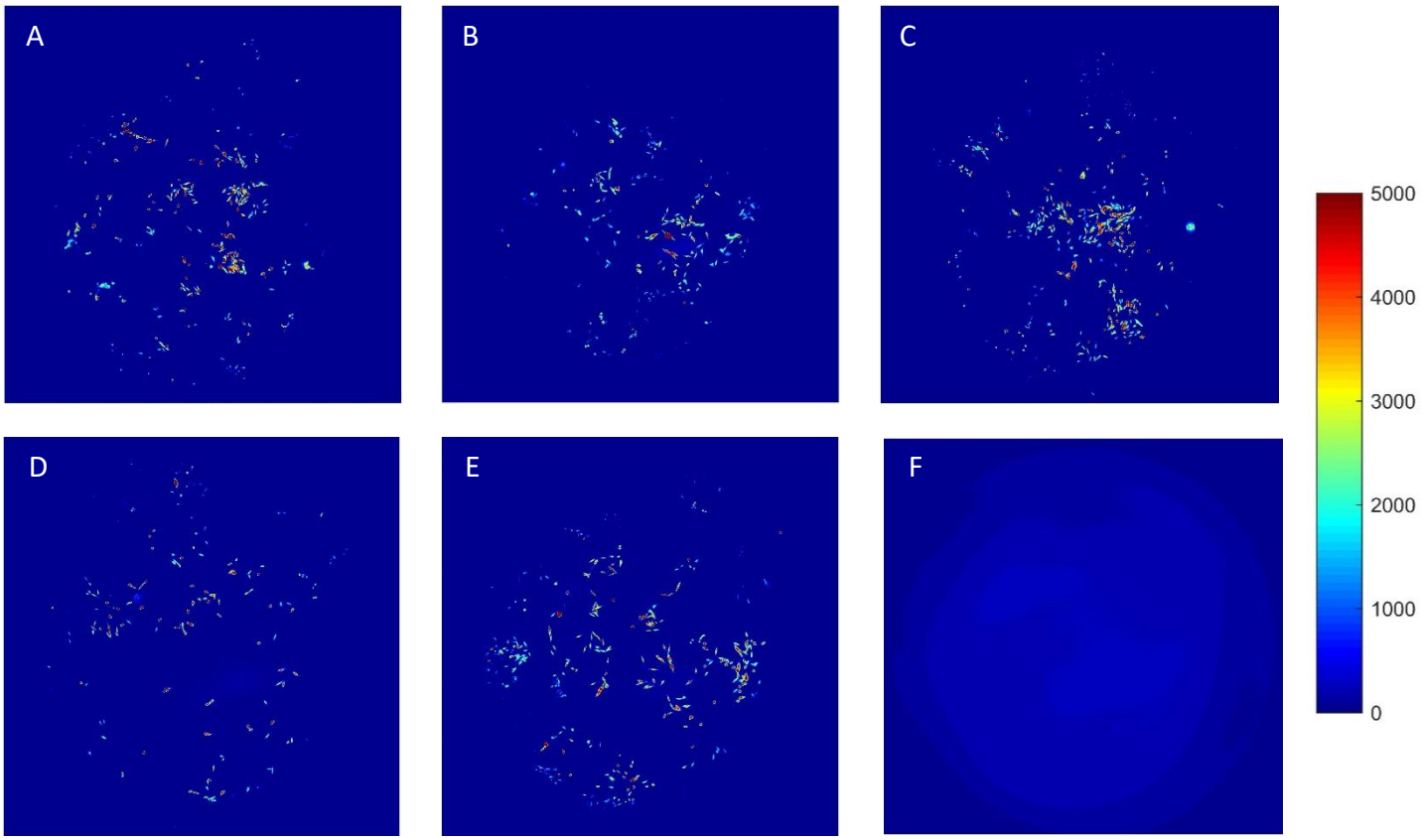

Figure 3: A. 2-NBDG uptake in 4T07 cells with a 0-minute fasting time. B. 2-NBDG uptake with a 15-minute fasting time. C. 2-NBDG uptake with a 30-minute fasting time. D. 2-NBDG uptake with a 60-minute fasting time. E. 2-NBDG uptake with a 90-minute fasting time. F. Control image of 4T07 cells without the addition of 2-NBDG. As seen in all 5 images, there is little variation in intensity between the different fasting times. Images shown above were processed using MATLAB and background-subtracted. The calculated background intensity is then subtracted from the total image to give the final images seen above. Images A-F were used to calculate the average intensity of each time point. The color bar represents intensity values as it relates to 2-NBDG uptake. Each image is 1024x1024 pixels and 1.3x1.3 mm.

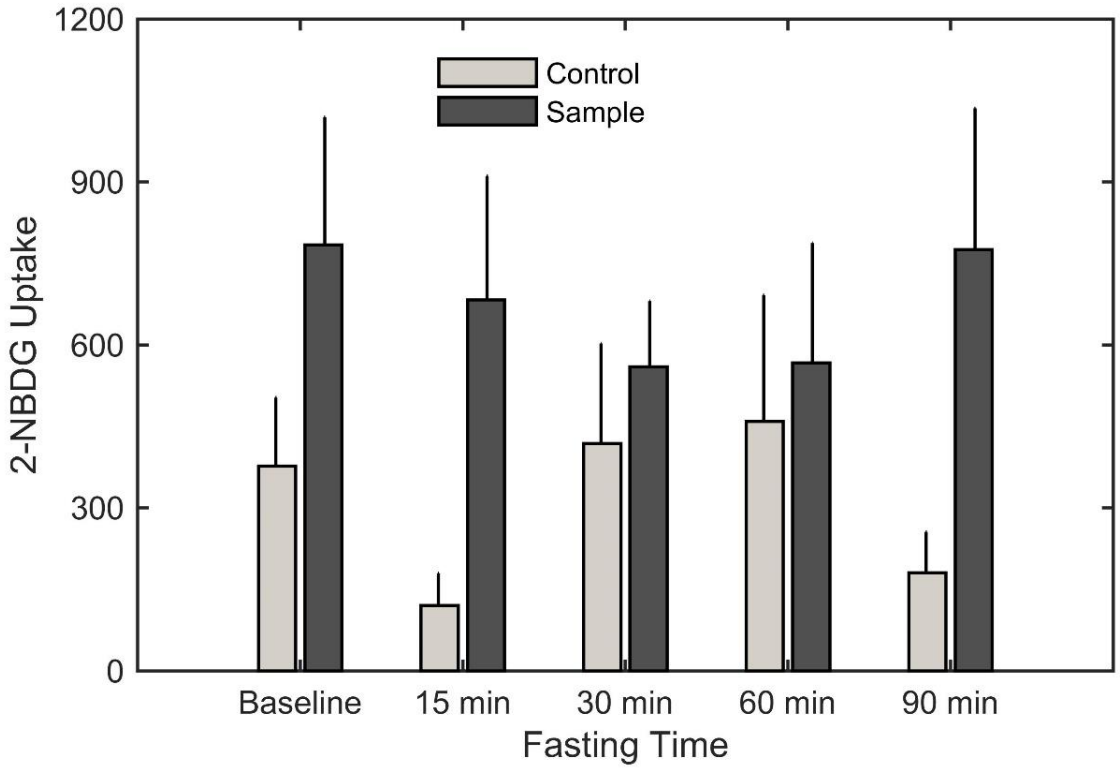

Figure 4: Represented above is the average intensity measured from 2-NBDG uptake at each time point. Baseline corresponds to no fasting time. Intensities from each plate were averaged from 5 different fields of view. No significant difference was found between time points from the nested, one-way ANOVA. Error bars show standard deviation. Due to the discrepancies in the fasting time's control values, we cannot successfully determine if one fasting time is preferred over others.

Conclusion:

After obtaining high quality images of 4T07 cells post 2-NBDG introduction, we have shown the potential and need of a standard practice for 2-NBDG uptake. While going through the imaging process, we figured the best optical setup for our microscope and camera. Originally, we used an LCTF to control the input of light into the camera. However, the resultant images were not as clear as needed. It was decided to take off the LCTF to allow all wavelengths of light reach the camera. This allowed us to get the best quality of image possible without overexposing the CCD. It also reduced the complexity of the imaging process by not having to make the decision of what wavelengths would be passed through the LCTF.

Looking at the data from the 4T07 studies we can see discrepancies in intensity values. These discrepancies will not allow us to reach a reasonable conclusion on which fasting time should be preferred. The issue arises not in the data from the samples but from the control plates. We have seen too high of deviations (Figure 4) in intensity values from each time point's control plate. This makes the data flawed. The control plates must all have similar intensity values for accurate analysis of the sample data. Since the control plates have such different intensity values, this might mean the output of the fluorescent burner was not constant on a day to day basis. Potentially, the burner had a different output hourly as well. Even with looking at the sample values, the nested, one way ANOVA test concluded there was no significant difference in fasting times. However, we can still take away the optimal protocol for introducing 2-NBDG and imaging the cells. Based on other work in our lab, we have previously determined that FBS is necessary when fasting cells and that 400μm is an adequate concentration of 2-

NBDG [9]. Along with this previous information and the information gained from these studies, we have shown the great possibilities of 2-NBDG being a novel tool for studying glucose consumption in cancer cells.

Future Directions

More work needs to be done in order to establish a uniform protocol for 2-NBDG studies. The experiments conducted here provide good preliminary data for optimizing a protocol. In order to ensure there is no discrepancies in the data, all samples should be tested in one day, after the microscope, camera, and burner are all calibrated. Tests should be run to ensure a uniform output of power from the burner before and after imaging is conducted. This will ensure the controls for each time point must have similar intensity values with low standard deviation. This allows the researcher to properly observe and analyze the differences in intensities seen in different fasting times. In the future, more experiments should be performed on the different breast cancer cell lines as well as expanding to different cancer types. Once the 4T1 family of cell lines has been thoroughly studied with 2-NBDG, the information learned can be tied with redox ration and oxygen consumption. This will help further the knowledge on metabolic properties of the breast cancer cell lines. By finding the optimal fasting time for other cancer types, we can begin to compare the similarities and differences in cancerous cells' other metabolic tendencies. Finding this optimal fasting time will let future researchers have a standard fasting time for in vivo studies. From there, researchers can effectively compare glucose uptake in tumors in vivo. This allows researchers to understand the kinetics behind glucose uptake in vivo. Along with glucose uptake, the

breast cancer cell line family can be further studied to learn about redox ratio and oxygen consumption. Using redox ration, oxygen consumption, and glucose uptake, researchers will have a detailed knowledge on the metabolic properties of the cancer cell lines. This will allow physicians to properly diagnose and treat patients with select cancers.

References:

1. Rajaram N, Frees A, et al. (2013). Delivery rates affects uptake of a fluorescent glucose analog in murine metastatic breast cancer. PLoS ONE. 8 (10): e76524
2. Gatenby R.A., Gillies R.J. (2004). Why do cancers have high aerobic glycolysis? Nature Reviews Cancer. 4, 891-899.
3. Zu XL, Guppy M (2004). Cancer metabolism: facts, fantasy, and fiction. Biochem and Biophys Res Commun. 313 (3): 459-465.
4. Huang B, Wai-Ming Law M, Khong P.L. (2009). Whole-Body PET/CT Scanning: Estimation of Radiation Dose and Cancer Risk. Radiology. 251 (1).
5. Zou C, Wang Y, Shen Z. (2005). 2-NBDG as a fluorescent indicator for direct glucose uptake measurement. J. Biochemical and Biophysical Methods. 64, 207-215.
6. Yoshioka K, Takahashi H, Homma T, et al. (1995). A novel fluorescent derivative of glucose applicable to the assessment of glucose uptake activity of Eserichia coli. Biochemica et Biophysica Acta. 1289, 5-9.
7. O'Neil R.G., Wu L, Mullani N. (2005). Uptake of a fluorescent deoxy-glucose analog in tumor cells. Molecular Imaging and Biology. 7, 388-392.
8. Langsner R.J., Middleton L.P., Sun J., et al. (2011). Wide-field imaging of fluorescent deoxy-glucose in ex vivo malignant and normal breast tissue. Biomedical Optics Express. 2 (6): 1514-1523.
9. Yoshioka K, Saito M, Oh KB, et al. (1996). Intracellular fate of 2-NBDG, a fluorescent probe for glucose uptake activity in E. coli cells. Biosci Biotechnol Biochem. 60 (11): 1899-1901.
10. Folgmann D. (2016) Optimized protocol for measuring 2-NBDG uptake as a cellular marker of glycolytic demand. Biomedical Undergraduate Honors Theses. Paper 31.
11. Heppner G.H., Miller F.R., Malathay Shekhar P.V. (2000) Nontransgenic Models of Breast Cancer. Breast Cancer Research. 2 (5): 331-334.
12. Alhallak K, Rebello L.G., Muldoon T.J., Quinn K.P., Rajaram N. (2016). Optical redox ratio identifies metastatic potential-dependent changes in breast cancer cell metabolism. Biomedical Optics Express. 7 (11).