

University of Arkansas, Fayetteville
ScholarWorks@UARK

Biomedical Engineering Undergraduate Honors
Theses

Biomedical Engineering

5-2016

Optimized Protocol for Measuring 2-NBDG Uptake as a Cellular Marker of Glycolytic Demand

Drew Folgmann

University of Arkansas, Fayetteville

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

 Part of the [Bioimaging and Biomedical Optics Commons](#), and the [Other Biomedical Engineering and Bioengineering Commons](#)

Recommended Citation

Folgmann, Drew, "Optimized Protocol for Measuring 2-NBDG Uptake as a Cellular Marker of Glycolytic Demand" (2016).
Biomedical Engineering Undergraduate Honors Theses. 31.
<http://scholarworks.uark.edu/bmeguht/31>

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.

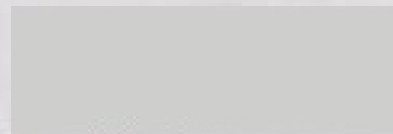
Optimized Protocol for Measuring 2-NBDG Uptake as a Cellular Marker of Glycolytic Demand

A thesis submitted in partial fulfillment
of the requirements for the degree of
Bachelor of Science in Biomedical Engineering

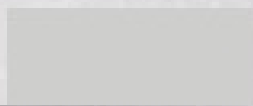
by

Drew Folgmann

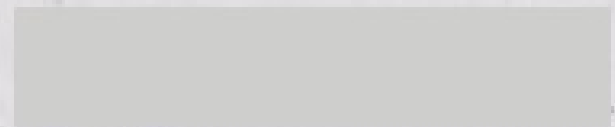
May 2016
University of Arkansas



Dr. Priya Puvanakrishnan
Thesis Director



Committee Member



Committee Member



Committee Member



Committee Member

Table of Contents

Abstract	2
Introdcution	3-5
Methods and Materials	5-8
Cell Viability Study	5 -6
2-NBDG Concentration vs. Cell Viability Study	6-7
2-NBDG Uptake Study	7-8
Image Analysis	8
Results	9-13
Cell Viability Study	9
2-NBDG Concentration vs. Cell Viability Study	10
2-NBDG Uptake Study	10-13
Discussion and Conclusions	14-15
Future Directions	15
Acknowledgements	16
References	17-18
Appendices	19-20
Appendix A: Supplementary Data	19-20

Abstract:

2[N-(7-nitrobenz-2-oxa-1,2-dioxol-4-yl)amino]-2-deoxyglucose (2-NBDG) a fluorescently tagged analog of FDG is ideal for *in vitro* studies and imaging. 2-NBDG has been proven to be an adequate marker for glucose uptake in many different types of cells [1]. However, across the spectrum of 2-NBDG use a lack of consensus is observed for the following questions. What is the ideal time to fast cells to optimize cellular uptake of 2-NBDG? Also, what is the ideal concentration of 2-NBDG to be used when quantifying glucose uptake? Finally, what is the effect of serum on the uptake of 2-NBDG? To answer these questions and understand glucose uptake, the Balb/cfC₃H 4T07 murine breast cancer cell line was fasted at varying time points between 0 and 150 minutes. Cell viability was evaluated for these time points using Promega's (Madison, WI) CellTiter-Glo® luminescent assay. Cells were also plated into 35mm glass bottom dishes, incubated for 24 hours, and fasted for varying times between 0 and 150 minutes. 400µM of 2-NBDG was introduced for 20 minutes and uptake was quantified using fluorescence microscopy. The peak of cell viability and glucose uptake was compared to find the optimal fasting time. Once fasting studies were complete, cells were fasted according to ideal conditions and concentration dependency of 2-NBDG was investigated. It was found that 4T07 cell viability is significantly decreased by 60 minutes of fasting cells in DMEM (-) glucose in the absence of 10% serum. The addition of 10% serum to the DMEM (-) glucose prolongs the fasting range to at least 150 minutes. 2-NBDG uptake is higher with the addition of 10% serum to DMEM (-) glucose in 20 minute fasting conditions. Also, 400µM 2-NBDG is the ideal concentration to optimize cell viability, cost effectiveness, and uptake.

Introduction:

Cancer cells have been shown to have an increased amount of glucose uptake relative to normal cells [1]. Currently Positron Emission Tomography (PET) is used to detect areas of high glucose tracer uptake [2]. The most commonly used tracer in the clinical diagnosis of cancer is radiolabeled fluorodeoxy-glucose (FDG). FDG-PET, however, is complicated to use *in vitro* due to its level of radiation exposure [3]. 2[N-(7-nitrobenz-2-oxa-1,2-dioxol-4-yl)amino]-2-deoxyglucose (2-NBDG) a fluorescently tagged analog of fluorodeoxy-glucose (FDG) is ideal for *in vitro* studies and imaging. The use of 2-NBDG allows researchers to quantify glucose uptake without using radioactive tracers such as FDG. It can be easily quantified through flow cytometry, fluorescence microscopy, or fluorescence plate readings. 2-NBDG has been proven to be an adequate marker for glucose uptake in many different types of cells [1]. Roger O'Neil et al. used 2-NBDG to look at glucose uptake of breast cancer and liver cancer cell lines [4]. Nathalie Rouach et al. used 2-NBDG to study the uptake of glucose in perivascular astrocytes [5]. These are just a few examples of the scope of 2-NBDG use. To uptake 2-NBDG effectively cells must be fasted, the time(s) for which to do so vary from study to study [1, 4-7]. Another interesting characteristic of 2-NBDG is that when added in high concentrations 2-NBDG 6-Phosphate can accumulate and kill the cell [6]. The point at which 2-NBDG concentration begins to inhibit the uptake of glucose is currently unknown. The effects of fetal bovine serum on 2-NBDG uptake will also be investigated. High concentrations of fetal bovine serum (FBS) in culture media has shown to significantly impair uptake of FDG [7]. The data obtained from these experiments will develop a standard for 2-NBDG uptake studies across the scientific community. 2-NBDG is a very important biomolecule that has potential applications in many areas of medical research.

Once the fasting times of murine Balb/cfC3H cell lines are optimized this knowledge will be applied to human breast cancer cells. These data sets will allow for direct applications in clinical imaging systems. *Ex Vivo* cancerous tissues can be fasted and imaged according to the findings of this study; this will give physicians a stronger diagnostic tool to identify breast cancer and develop treatment plans.

Project Overview:

Some of the major questions we seek to answer are:

1. What are the ideal fasting time point(s) for optimal uptake of 2-NBDG?
2. Does 2-NBDG uptake depend on the addition of Fetal Bovine Serum metabolites?
3. After fasting, what concentration of 2-NBDG is optimal for uptake of cells with differing glycolytic demand?

To find the ideal fasting time point(s) of murine breast cancer cells, I first needed to determine the ideal cells for testing. Previous studies have shown good glucose uptake in select Balb/cfC3H lines (66cl4, 67NR, 4T07, 4T1, and 168FARN) [1]. To start the 4T07 cell line was deemed to be ideal because of its high glycolytic demand but non metastatic potential.

The 4T07 cells were seeded into 96- well plates for cell viability studies or 35mm glass bottom dishes for imaging. After 24 hours all media was removed and washed with Phosphate Buffer Solution (PBS) to ensure removal of all glucose media. Media with glucose was added into control wells/dishes and media without glucose (+/- serum) was added into respective test wells. The first set of test trials did not include fetal bovine serum in the glucose free fasting media. The second set of test trials did include fetal bovine serum in the glucose free fasting media. The cells were placed in the incubator for varying fasting times between 0 and 150 minutes. Once each plate's respective fasting time was up the cell viability was assessed or 2-NBDG was added for uptake quantification. The 2-NBDG uptake quantification plates/dishes

were then incubated for 20 minutes. Following, all 2-NBDG was removed and washed twice with PBS before quantification using fluorescence microscopy.

To study the effects of varying concentrations of 2-NBDG, cells were fasted according to the protocol above. A wide range of 2-NBDG concentrations were added for 20 minutes and glucose uptake was analyzed using a fluorescence plate reading.

Materials and Methods

Cell Viability Studies:

Cell Plating:

4T07 cells were obtained, thawed, and placed into a 75cm² cell flask with media containing glucose. Dulbecco's Modified Eagle Medium from Thermo Fisher Scientific (Waltham, MA). It was supplemented with L-Glutamine, Fetal Bovine Serum, Nonessential Amino Acids, and Penicillin Streptomycin. After three passages to remove Dimethyl sulfoxide (DMSO) freezing agent the cells were ready to be used for experimentation. The cells were passaged according to a standardized procedure handed down from Duke University. Cells were counted in a hemocytometer and centrifuged at 950 revolutions per minute for 4 minutes at 4 degrees Celsius. The cells were then re-suspended to achieve 10,000 cells in a volume between 100- 200 microliters. This is the recommended working volume of a standard 96-well plate. 10,000 cells was hypothesized to give a strong enough luminescent signal and proper confluency for cell viability assay readings. The cells were seeded onto a white walled, flat, clear bottom 96-well plate. The white walls prevent cross talk between wells and also provide a stronger luminescent signal for more accurate detection. Once the cells were seeded at the appropriate dilution with complete DMEM the plate was placed in an incubator at 37 degrees Celsius for 24 hours.

Fasting and Assay Protocol

After the 24 hour incubation period all DMEM was removed, and all wells were washed with PBS to ensure removal of glucose media. 100 microliters of DMEM (+) glucose or DMEM (-) glucose (+/-) 10% Fetal Bovine Serum (FBS) was added to the wells as indicated below.

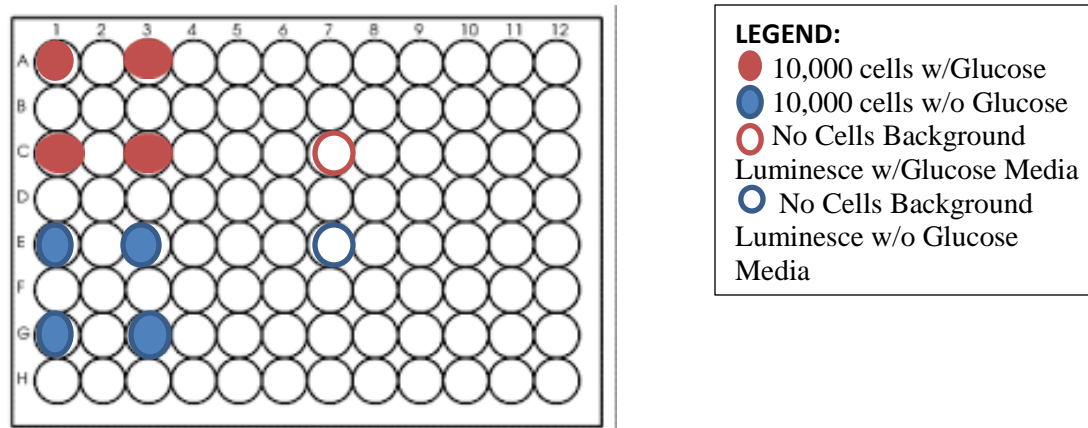


Figure 1: Indicating the locations of the test variables on the 96- well plate for the cell viability studies.

Source:<http://users.path.ox.ac.uk/~scobbold/tig/>

After all DMEM (+/-) glucose was added to appropriate wells the plates were placed in the incubator for 0, 30, 60, 90, 120, 150 minutes. While the plates were incubating the Promega Cell Titer-Glo® reagent was prepared. Once the fasting duration was complete for each respective plate 100 microliters of Cell Titer-Glo® reagent was added in addition to the 100 microliters of DMEM (+/-) glucose. Cell lysis was induced to allow the assay agents to attach to the cell ATP by placing the sample on an orbital shaker for 2 minutes prior to measuring luminescence.

Luminescence was measured using a BioTek Synergy HT plate reader.

2-NBDG Concentration vs. Cell Viability Study:

4T07 cells were plated on white 96-well plates. The ideal fasting time was determined from the cell viability study and was used to fast the cells according to the same protocol as above. Following the fast 2-NBDG was added according to the diagram below at concentrations between 50 and 400 μ M:

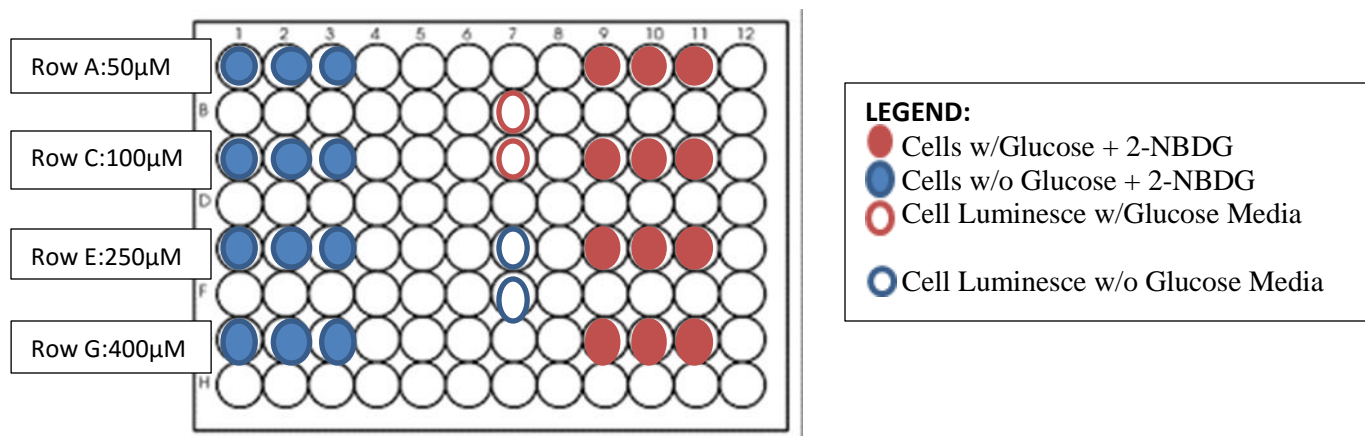


Figure 2: Indicating the locations of the test variables and their respective 2-NBDG concentrations by row on the 96- well plate. Row A contained 50µM of 2-NBDG in glucose free (columns 1-3) and glucose (columns 9-11) conditions. Row C contained 100µM of 2-NBDG in glucose free (columns 1-3) and glucose (columns 9-11) conditions. Row E contained 250µM of 2-NBDG in glucose free (columns 1-3) and glucose (columns 9-11) conditions. Row G contained 400µM of 2-NBDG in glucose free (columns 1-3) and glucose (columns 9-11) conditions. Column 7 contained cells without 2-NBDG in glucose free (rows E+F) and glucose (rows B+C) conditions.

Image source: <http://users.path.ox.ac.uk/~scobbold/tig/>

Once fasting was completed all media and 2-NBDG was removed. Each test well was then washed twice with Phosphate-Buffered Saline (PBS). Following, 100µL of the Cell Titer-Glo® reagent was added to each well. Cell lysis was induced to allow the assay agents to attach to the cell ATP by placing the sample on an orbital shaker for 2 minutes prior to measuring luminescence. Luminescence was measured using a BioTek Synergy HT plate reader.

2-NBDG Uptake:

Once the assessment of cell viability at all fasting time points and the ideal concentration was determined, glucose uptake was evaluated. 200,000 4T07 cells were plated on 35mm MaTek glass bottomed dishes. The cells were fasted using the same methods listed above in both (+/-) 10% FBS conditions. When each dish reached its respective fasting time 2-NBDG was introduced for 20 minutes at a concentration of 400µM. The concentration of 2-NBDG remained constant at 400µM for all samples, this was to rule out the effects of 2-NBDG concentration dependent uptake variability. Each dish was then removed from the incubator and washed twice

with PBS. To quantify 2-NBDG fluorescence a Nikon (Melville, NY) Eclipse ti-s inverted microscope fitted with a DS-fi2 camera was used for image collection. 2-NBDG has an excitation/emission spectra of 465/540nm respectively.

Image Analysis:

Fluorescence intensity images were analyzed using MATLAB. The background intensity from each image was subtracted out to yield the fluorescence of the cells. A background image was taken on every day cells were imaged. This was to account for day to day variability of the fluorescence signal. A histogram was constructed for the sum of each group of test sample images.

Results:

4T07 cell viability was evaluated in DMEM (-) glucose and DMEM (+) glucose both (+/-) serum as seen in **Figure 3**. Cell viability was significantly decreased at 60 minutes in the DMEM (-) glucose without serum. There wasn't a significant difference between cells fasted in DMEM (-) glucose or DMEM (+) glucose with the addition of 10% serum at all times between 0 – 150 minutes.

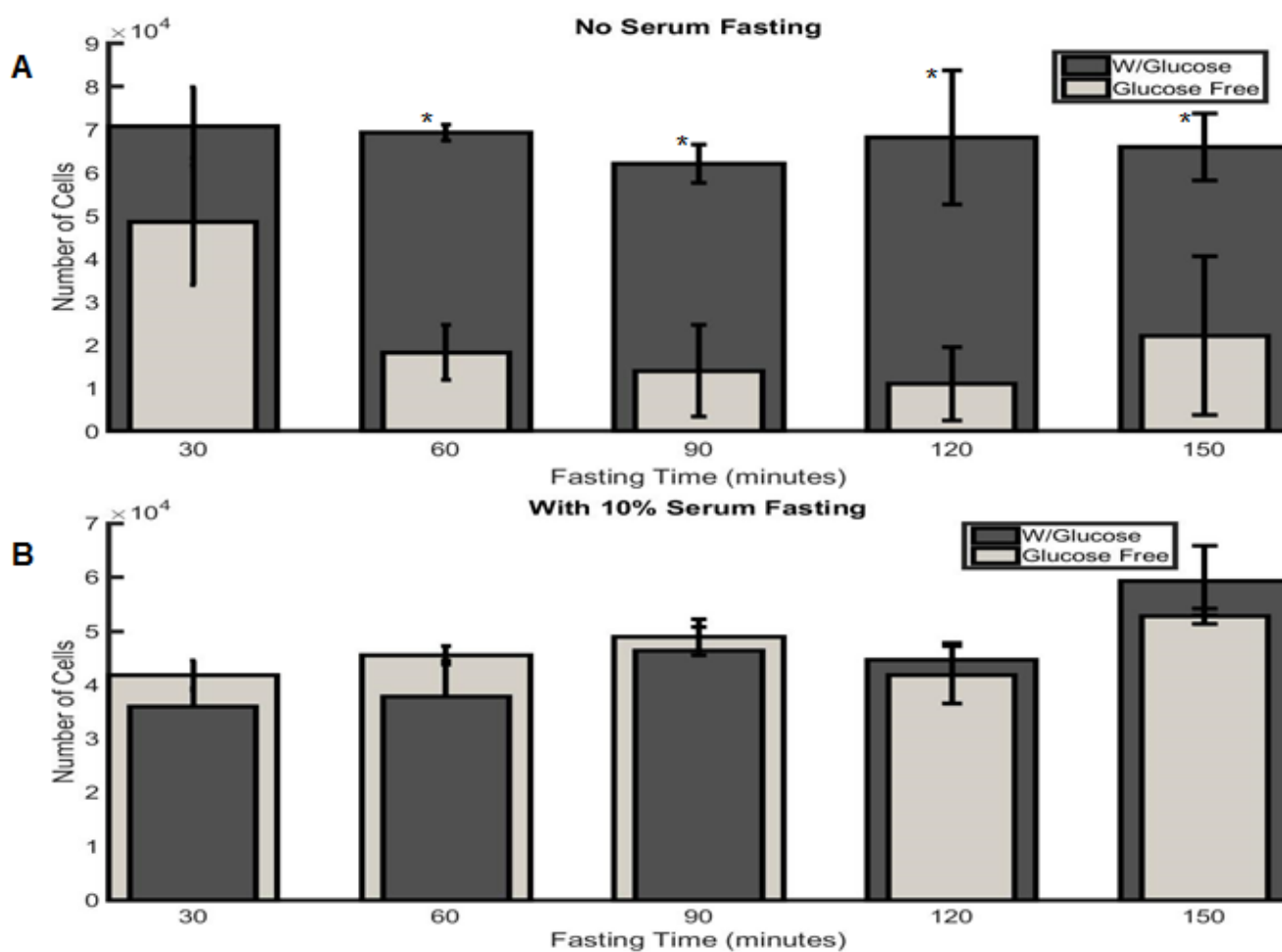
Cell Viability Studies:

Figure 3:A) In the absence of serum, viability of the cells fasted for 60 minutes in DMEM (-) glucose was significantly lower ($p < 0.001$) as well as for all time points following. **B)** 4T07 cell viability is comparable for all fasting times between 0-150 minutes in DMEM (+/-) glucose with the addition of 10% serum to the media. All data sets were collected on a white 96-well plate. Error bars indicate the standard deviation of the samples. * Indicates a significant difference with an alpha = 0.05.

2-NBDG Concentration vs. Cell Viability Study:

4T07 cell viability was evaluated for varying 2-NBDG concentrations (0 μ M, 50 μ M, 100 μ M, 250 μ M, 400 μ M) in the absence of serum. **Figure 4** shows the higher cell viability in DMEM (-) glucose as 2-NBDG concentration increased. There was no significant difference between the cell viability in DMEM (-) glucose and DMEM (+) glucose with 250 μ M and 400 μ M 2-NBDG.

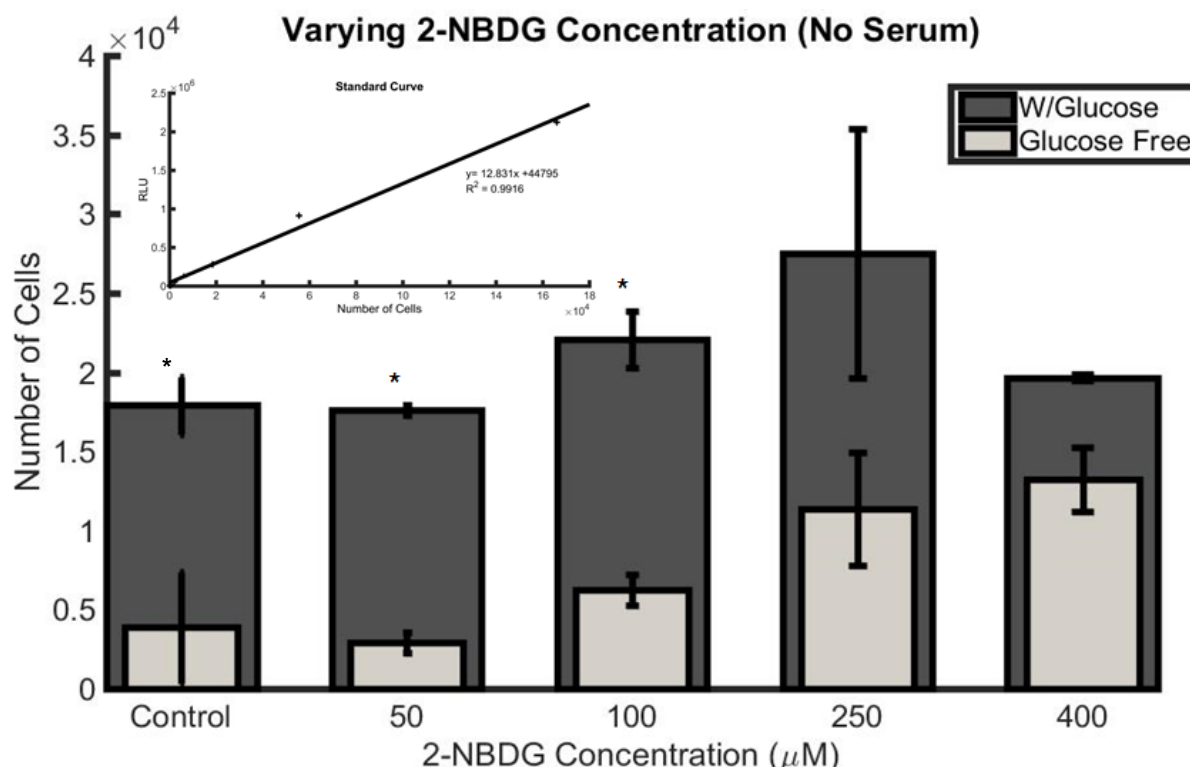


Figure 4: Cell viability is significantly higher ($p = 0.03$) in a 400 μ M 2-NBDG solution when compared to a 50 μ M solution. All samples were fasted for 10 minutes in DMEM (-) glucose then incubated with 2-NBDG with DMEM (-) glucose for 20 minutes. The control samples contained cells in their respective media. All samples were collected on a white 96-well plate. Error bars indicate the standard deviation of the samples. * Indicates a significant difference with an alpha = 0.05

2-NBDG Uptake:

4T07 cells were imaged after 20 minutes of fasting in DMEM (-) glucose (+/-) serum.

The addition of 10% serum to the DMEM (-) glucose fasting media increased 2-NBDG uptake as

indicated in **Figure 5**. The 2-NBDG uptake of 4T07 cells in both (+/-) serum for various fasting durations is shown in **Figure 6**.

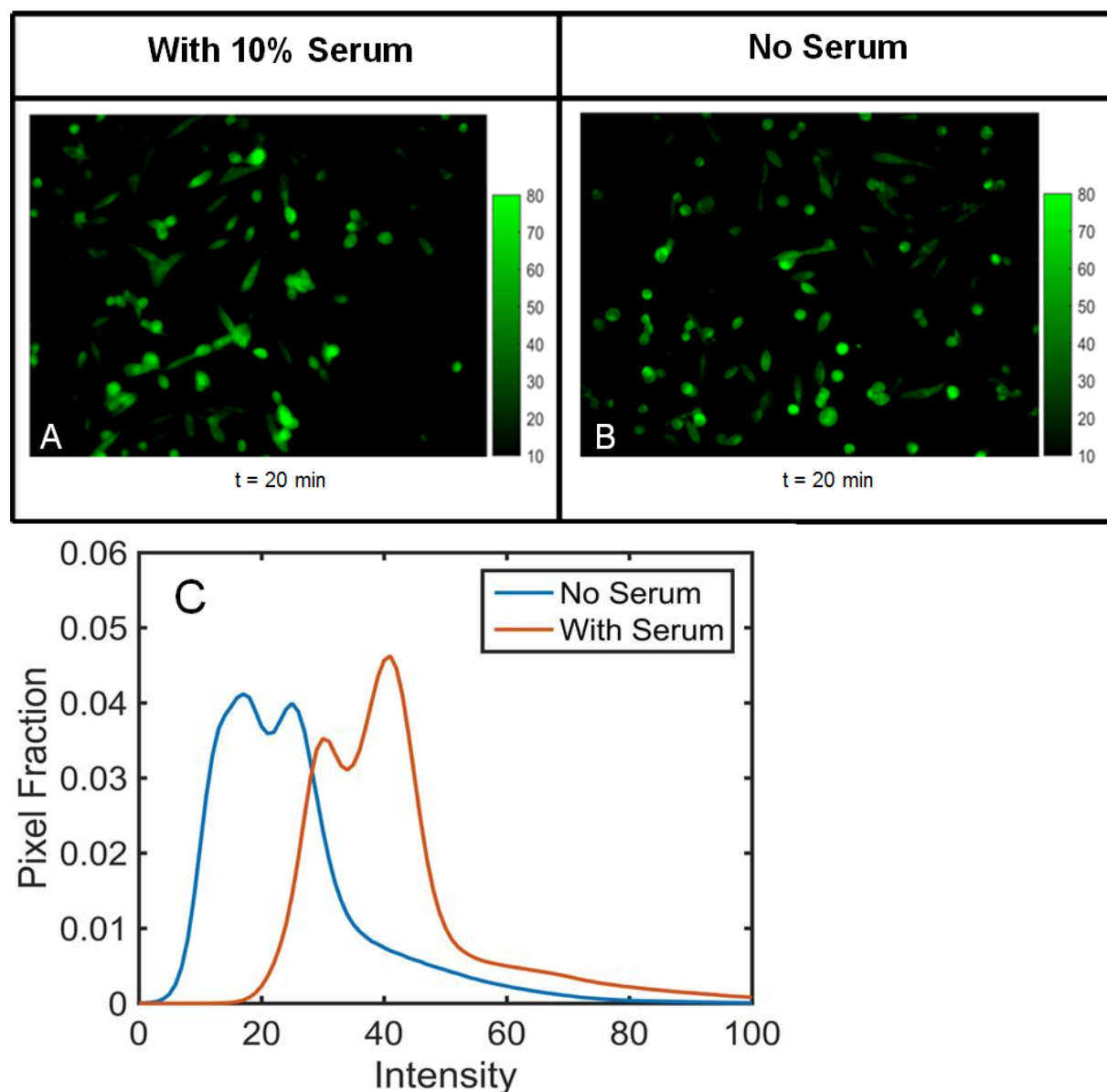


Figure 5: 200,000 4T07 cells were plated in 35mm glass bottom dishes and cultured for 24 hours prior to use. A Nikon (Melville, NY) Eclipse ti-s inverted microscope fitted with a DS-fi2 camera was used for image collection. 2-NBDG has an EX/EM of 465/540nm respectively. **A)** 4T07 cells incubated for 20 minutes with 400 μM 2-NBDG in DMEM (-) glucose with the addition of 10% serum. **B)** 4T07 cells incubated for 20 minutes with 400 μM 2-NBDG in DMEM (-) glucose. **C)** A histogram of fluorescence intensity in cells that were incubated for 20 minutes with 400 μM 2-NBDG and DMEM (-) glucose with and without the addition of serum.

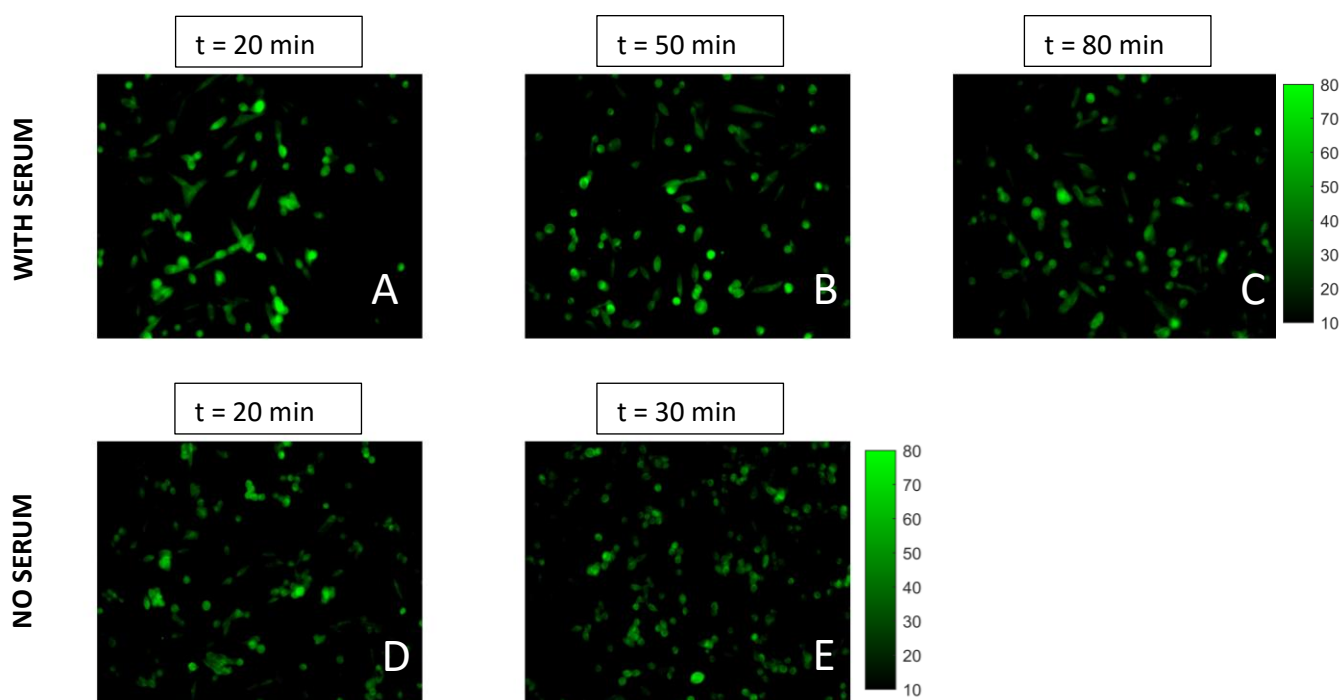


Figure 6: 200,000 4T07 cells were plated in 35mm glass bottom dishes and cultured for 24 hours prior to use. A Nikon (Melville, NY) Eclipse ti-s inverted microscope fitted with a DS-fi2 camera was used for image collection. 2-NBDG has an EX/EM of 465/540nm respectively. **A)** 4T07 cells incubated for 20 minutes with 400 μ M 2-NBDG in DMEM (-) glucose with the addition of 10% serum. **B)** 4T07 cells fasted for 50 minutes with 400 μ M 2-NBDG in DMEM (-) glucose with the addition of 10% serum. **C)** 4T07 cells fasted for 80 minutes with 400 μ M 2-NBDG in DMEM (-) glucose with the addition of 10% serum. **D)** 4T07 cells incubated for 20 minutes with 400 μ M 2-NBDG in DMEM (-) glucose in absence of serum. **E)** 4T07 cells fasted for 30 minutes with 400 μ M 2-NBDG in DMEM (-) glucose in absence of serum.

Between images A, B, and C in **Figure 6** there wasn't a significant difference in 2-NBDG uptake and similarly between images D and E in the absence of serum. This is shown by the fluorescence intensity histograms in **Figure 7** and **Figure 8**. In **Figure 7**, the 2-NBDG uptake by the 4T07 cells was highest with 20 min fasting in DMEM (-) glucose in the presence of 10% serum. In **Figure 8**, there wasn't a significant difference between the 4T07 cells fasted for 20 minutes or 30 minutes in the absence of serum. The cells were not fasted past 30 minutes due to the cell viability issues in the absence of serum indicated in **Figure 3**.

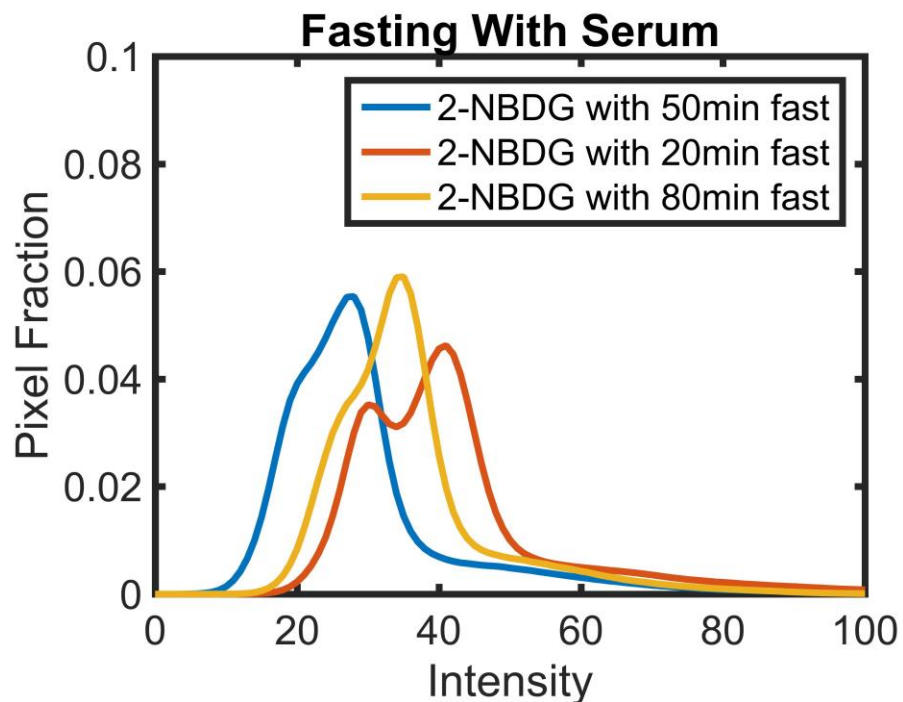


Figure 7: Histogram of 2-NBDG uptake of 4T07 cells fasted for 20, 50, and 80 minutes with 10% serum.

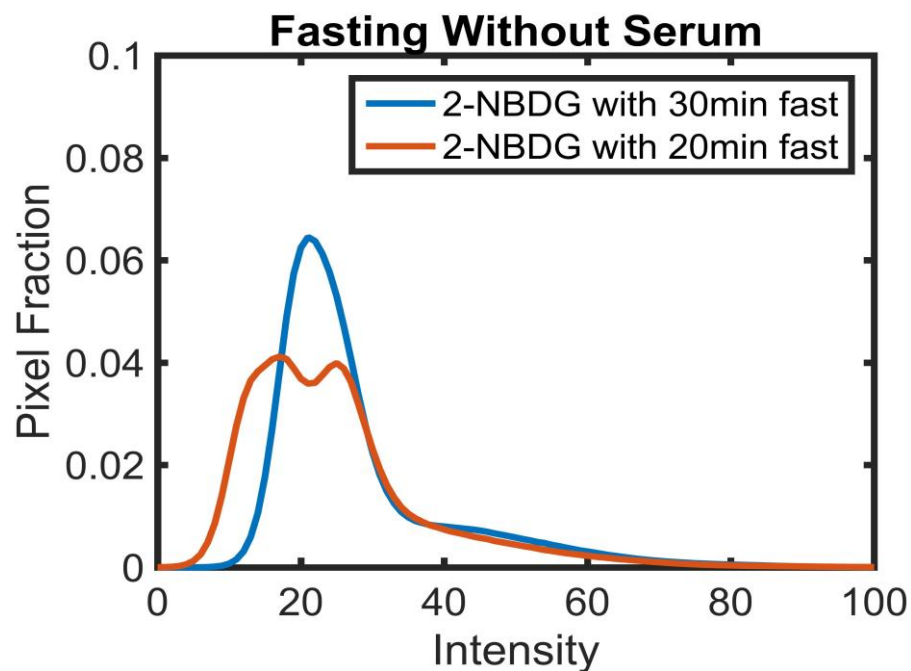


Figure 8: Histogram of 2-NBDG uptake of 4T07 cells fasted for 20 and 30 minutes in absence of serum.

Conclusions:

The 4T07 cell viability was significantly decreased by 60 minutes of fasting cells in DMEM (-) glucose in the absence of serum as indicated in **Figure 3**. The addition of 10% serum to the DMEM (-) glucose prolongs the fasting range to at least 150 minutes. The addition of 10% serum allows for an adequate fasting window to optimize 2-NBDG uptake.

400 μ M 2-NBDG was determined to be the ideal concentration to optimize cell viability. **Figure 4** shows that for a concentration of 400 μ M of 2-NBDG there wasn't a significant difference in cell viability of the cells in both DMEM (-) glucose and DMEM (+) glucose in the absence of serum. It is believed this is because the cells metabolize the high concentration of 2-NBDG into 2-NBDG-6-Phosphate and in doing so it preserves the cell viability.

2-NBDG uptake is higher with the addition of serum to DMEM (-) glucose in 20 minute fasting conditions (**Figure 5**). **Figure 7** and **Figure 8** show no significant evidence to suggest that cells should be fasted beyond 20 minutes. Fasting beyond 20 minutes showed decreased 2-NBDG uptake in the presence of 10% serum and no difference in the absence of serum.

It is recommended that before any study is begun a cell viability study during fasting conditions be conducted. This will give an idea of the glucose dependency of the cell line which helps to gauge the appropriate fasting duration. In the absence of serum, the 4T07 cell viability was significantly decreased after 30 minutes of fasting. It is believed this is why the maximum uptake was observed in both conditions ((+/-) serum) at 20 minutes of fasting (**Figure 7** and **Figure 8**). 400 μ M of 2-NBDG is the recommended concentration of 2-NBDG to maximize cell viability and thus 2-NBDG uptake for the reasons discussed prior. It is also recommended that serum be added to the fasting media to increase cell viability and 2-NBDG uptake (**Figure 5**).

The facts discussed above answer the proposed questions of the study. These guidelines will hopefully provide clarity to the scientific community interested in using 2-NBDG for *in vitro* studies.

Future Directions:

2-NBDG concentration vs. cell viability in 10% serum conditions will need to be evaluated. This is to validate the belief that there isn't a significant difference in cell viability for all concentrations of 2-NBDG with the addition of serum. Microscopy images for increasing concentration of 2-NBDG in both (+/-) serum conditions will need to be evaluated to confirm that 400 μ M is the ideal concentration for maximum uptake.

To further validate that the addition of 10% serum to the fasting media increases 2-NBDG uptake, images will need to be collected for all fasting time points (30 minutes, 60 minutes, 90 minutes, etc.) with and without serum.

The 4T07 cell line is very glucose dependent; this may impact the 2-NBDG uptake. To confirm that the above findings are true for all cell types other cell lines will need to be evaluated. A549 lung cancer cells would be ideal because of the OXPHOS leaning metabolism. The direct comparison of these cells lines of varying glycolytic demand will provide a basis for researchers to maximize 2-NBDG uptake in all types of cells based on their bioenergetic profile.

Upon completion of *In Vitro* murine studies In/Ex Vivo 2-NBDG uptake should be investigated. Balb/c/c₃H cells will be injected into mice to induce tumor formation. The tumors will then be harvested as an *Ex Vivo* tissue and fasted according to the determined optimal *In Vitro* protocol. 2-NBDG will then be introduced to the sample for 20 minutes. The *Ex Vivo* tissue will then be imaged using fluorescence microscopy.

Acknowledgements

I would like to thank Dr. Priya Puvanakrishnan and Dr. Narasimhan Rajaram for their support and guidance of this project. I would also like to thank the Department of Biomedical Engineering at the University of Arkansas at Fayetteville for their funding of this research. I would also like to acknowledge the University of Arkansas Honors College for their funding of this research through the Honors College Research Grant.

References:

1. Rajaram, Narasimhan, Amy E. Frees, Andrew N. Fontanella, Jim Zhong, Katherine Hansen, Mark W. Dewhirst, and Nirmala Ramanujam. "Delivery Rate Affects Uptake of a Fluorescent Glucose Analog in Murine Metastatic Breast Cancer." *PLoS ONE* 8.10 (2013): n. pag. Web. 28 Sept. 2015.
2. Walsh, Alex, Rebecca S. Cook, Brent Rexer, Carlos L. Arteaga, and Melissa C. Skala. "Optical Imaging of Metabolism in HER2 Overexpressing Breast Cancer Cells." *Biomedical Optics Express Biomed. Opt. Express* 3.1 (2011): 75. Web. 28 Sept. 2015.
3. Huang, Bingsheng, Martin Wai-Ming Law, and Pek-Lan Khong. "Whole-Body PET/CT Scanning: Estimation of Radiation Dose and Cancer Risk 1." *Radiology* 251.1 (2009): 166-74. Web. 5 Oct. 2015.
4. O'Neil, Roger G., Ling Wu, and Nizar Mullani. "Uptake of a Fluorescent Deoxyglucose Analog (2-NBDG) in Tumor Cells." *Mol Imaging Biol Molecular Imaging and Biology* 7.6 (2005): 388-92. Web. 5 Oct. 2015.
5. Rouach, N., A. Koulakoff, V. Abudara, K. Willecke, and C. Giaume. "Astroglial Metabolic Networks Sustain Hippocampal Synaptic Transmission." *Science* 322.5907 (2008): 1551-555. Web. 5 Oct. 2015.
6. Yoshioka, Kazuaki, Mikako Saito, Ki-Bong Oh, Yasuyuki Nemoto, Hideaki Matsuoka, Masahiro Natsume, and Hiroshi Abe. "Intracellular Fate of 2-NBDG, a Fluorescent Probe for Glucose Uptake Activity, in Escherichia Coli Cells." *Bioscience, Biotechnology and Biochemistry* 60.11 (1996): 1899-901. Web. 7 Oct. 2015.

7. Wahl, R. L., C. A. Henry, and S. P. Ethier. "Serum Glucose: Effects on Tumor and Normal Tissue Accumulation of 2-[F-18]-fluoro-2-deoxy-D-glucose in Rodents with Mammary Carcinoma." *Radiology* 183.3 (1992): 643-47. Web. 12 Oct. 2015.
8. Alonso-Castro, Angel Josabad, Ana Cristina Miranda-Torres, Marco Martín González-Chávez, and Luis A. Salazar-Olivo. "Cecropia Obtusifolia Bertol and Its Active Compound, Chlorogenic Acid, Stimulate 2-NBDglucose Uptake in Both Insulin-sensitive and Insulin-resistant 3T3 Adipocytes." *Journal of Ethnopharmacology* 120.3 (2008): 458-64. Web. 28 Sept. 2015.

Appendix A
Supporting Data:

Cell Viability

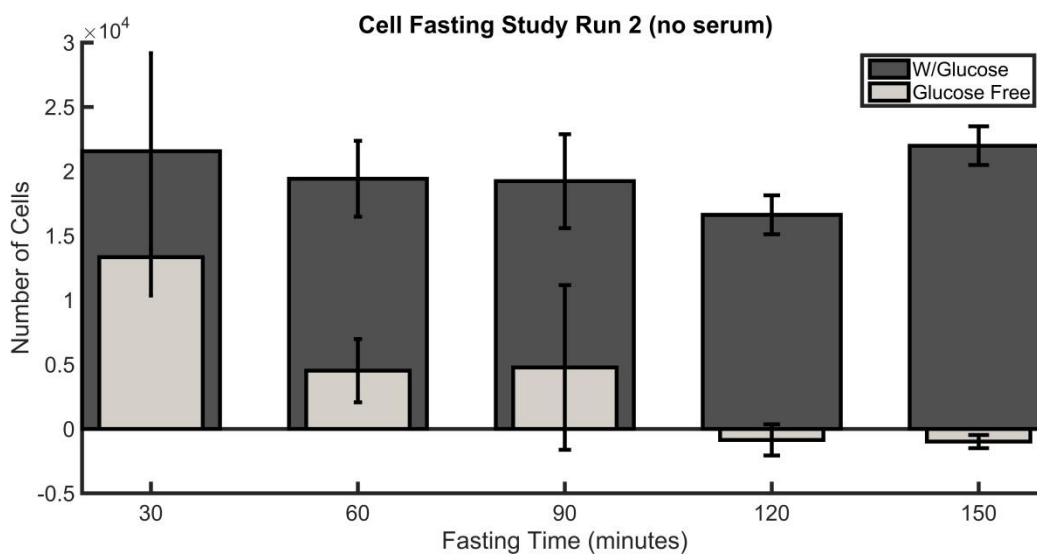


Figure 9: 4T07 cell viability for varying fasting durations in the absence of serum

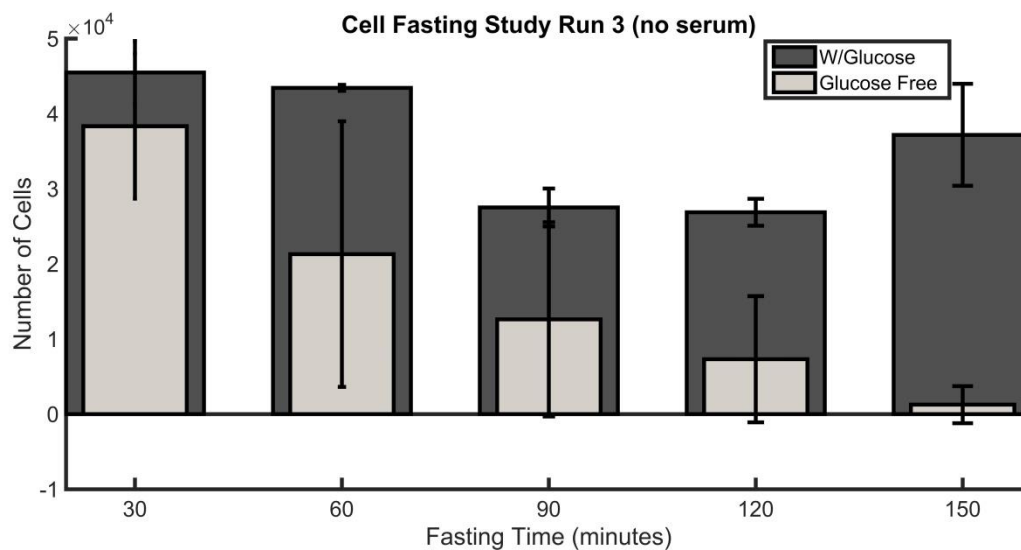


Figure 10: 4T07 cell viability for varying fasting durations in the absence of serum

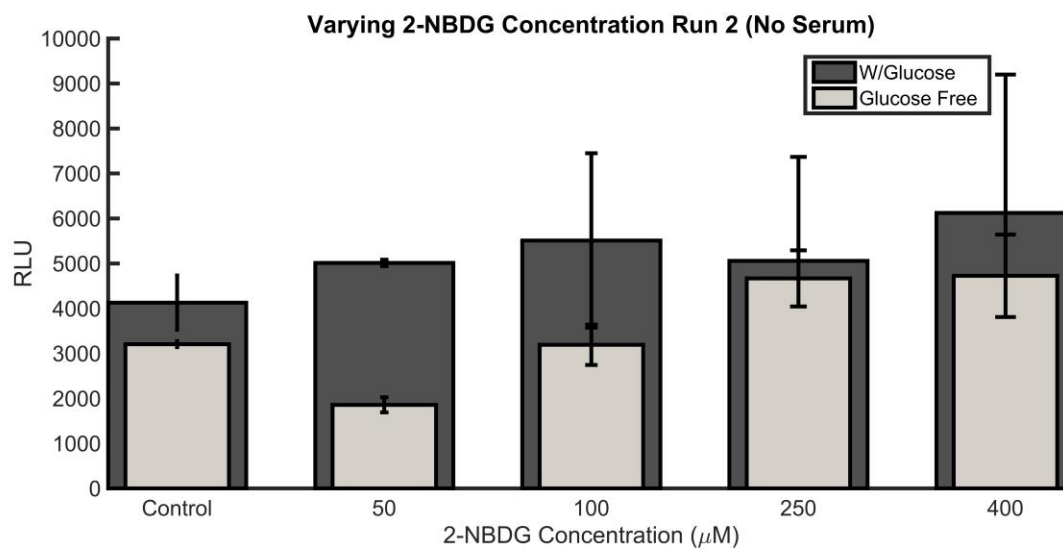
2-NBDG Concentration vs. Cell Viability:

Figure 11: 4T07 cell viability for varying 2-NBDG concentrations at 30 minute fast in absence of serum.