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UNIVERSITY OF CALIFORNIA,  
IRVINE

Acute Alcohol Exposure Shifts Metabolism of Breast Cancer Cells

THESIS

submitted in partial satisfaction of the requirements  
for the degree of

MASTER OF SCIENCE

In Biomedical Engineering

by

Ivy Le

Thesis Committee:  
Assistant Professor Michelle Digman, Chair  
Assistant Professor Jered Haun  
Associate Professor Wendy Liu

2018



## **DEDICATION**

To

My parents and my husband.

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# **ABSTRACT OF THE THESIS**

Acute Alcohol Exposure Shifts Metabolism of Breast Cancer Cells

By

Ivy Le

Master of Science in Biomedical Engineering

University of California, Irvine, 2018

Associate Professor Michelle Digman, Chair

Alcohol consumption has been recognized as a risk factor for breast cancer. It is positively correlated with the progression of breast cancer. Ethanol is studied to shift the metabolism of breast cancer cells. According to the Warburg Effect, cancer cells predominantly produce their energy through a high rate of glycolysis. I hypothesized that with acute ethanol exposure the breast cancer cells will shift their metabolism from oxidative phosphorylation to glycolysis. The metabolic shift of cancer cells changes the ratio of free and protein bound NADH, which is an essential coenzyme in cellular metabolic pathway. As such, investigating the fluorescent lifetime of free and protein bound NADH of breast cancer cells through Fluorescence Lifetime Imaging Microscopy (FLIM), I report the preliminary results on the metabolic shifts of two breast cancer cell lines: MDA-MB-231 and MCF-7 due to acute alcohol exposure.

## **CHAPTER 1: INTRODUCTION**

Breast cancer is the most common type of cancer diagnosed among U.S. women with over 250,000 new cases of invasive breast cancer expected to be diagnosed in 2017. It also the second leading cause of cancer death among U.S. women after lung cancer. Approximately ~40,000 women and ~450 men are expected to die from this disease in 2017 [1]. Also, there are more than 3.5 million U.S. women with a history of breast cancer were alive as of January 1<sup>st</sup>, 2016 [1].

The development of breast cancer is associated with hereditary, hormonal, and behavioral factors. Alcohol consumption has been recognized as a risk factor that is linked to the progression of breast cancer [2]. Experimental studies demonstrate that acute alcohol exposure promotes the progression of existing mammary tumors [3][4]. The changes in cancer progression can be described via the shift in metabolism of cancer cells from oxidative phosphorylation to glycolysis [5][6].

Cancer cells have ability to modify their metabolism to meet the extremely high demand of energy to maintain their malignancy. They tend to undergo aerobic glycolysis (Warburg Effect), which produces APT rapidly, but less ATP per molecule of glucose than oxidative phosphorylation [7][8][9][10]. Lactic acid, byproducts from glycolysis pathway, accumulates creating acidic microenvironment, which has been shown to enhance cancer cells viability and invasiveness [11].

Nicotinamide adenine dinucleotide (NAD) and its reduced form NADH plays an important role in energy production and consumption within the cells [12][13]. NADH is an essential enzymatic cofactor of redox reaction, which strongly influences energy production, cell survival and proliferation. NADH is also an intrinsic fluorophore. Previous

studies have shown that the fluorescent lifetime measurement of two conformations of NADH: free and protein bound can be used to determine the cellular metabolic state of cancer cells [14][15][16][17][18].

Fluorescent lifetime imaging microscopy (FLIM) is a powerful technique to determine the metabolic state of living cells [14][19]. This optic technique provides a non-invasive method that able to real time capturing changes in metabolism in cancer cells due to acute exposure of alcohol. The phasor approach of FLIM (phasor FLIM) can characterize the fluorescent lifetime at pixel level. It provides the quantitative measure of the ratio of free and bound NADH, which reflects glycolysis and oxidative phosphorylation respectively [20][21].

In this study, I used phasor FLIM to measure the ratio of free and bound NADH in two different breast cancer cell lines (MDA-MB-231 and MCF-7) when treated with 0%, 0.3% and 0.6% ethanol. Both cell lines with acute alcohol exposure showed an immediate change in metabolism shifting toward glycolysis. This further confirm the relationship between alcohol consumption and the increase in cancer cell progression.

## **CHAPTER 2: BACKGROUND**

### **2.1 Breast Cancer and Alcohol Consumption**

The consumption of alcohol has been recognized as a major risk factor for breast cancer. Experimental studies showed that alcohol exposure promotes the progression of existent mammary tumors. Yet more studies are required to analyze the mechanism by which the alcohol affects women in breast cancer. Breast cancer is the most rampant malignant disease for females globally [22]. In the United States, it is the second cause of cancer-related death in the country. There are several risk factors for breast cancer among lifestyle factors, reproductive or hormonal factors and as well as factors related to genetics. However, epidemiological research showed that indicates the consumption of alcohol increases the risk associated with breast cancer in a dosage-dependent way. In addition, alcohol promotes the growth of existing breast tumors as well as enhancing metastasis [23].

Previous studies indicated that alcohol consumption increases women risk to hormone-receptor-positive breast cancer cells. Alcohol raises the level of estrogen and other hormones linked to hormone-receptor-positive breast cancer cells [24]. The other reason is that alcohol drinking increases breast cancer risks as it damages the DNA. In comparison to women who don't consume do not take alcohol at all, women those who take three alcoholic drinks per week are at 15% higher risk of contracting breast cancer [25]. Other studies suggested that the risk of breast cancer increases by 10% for every drink women takes in a day. Furthermore, individuals 9 to 15 years taking an average of 3 to 5 drinks per week are at three-time risk associated with benign breast lumps. Though the benign breast lumps are non-cancerous, the lumps are associated with the

development of cancer in years to come among women. Despite the fact there are few studies completed concerning the impact of alcohol to breast cancer; women who have been prior diagnosed with early-stage breast cancer are at a higher rate of breast cancer recurrence following consumption of alcohol. For reference, one drink is equal to 12 ounces of beer, 5 of wine and 1.5 ounces of hard liquor [2].

Metastasis and tumor growth are dependent on angiogenesis and vasculogenesis development. The role of vascular endothelial growth in alcohol promotes breast cancer development. The vascular endothelial growth factor (VEGF) plays a crucial role in tumor angiogenesis and cells as it enhances the endothelial cells proliferation, migration as well as their stabilization [26]. Studies have proved that VEGF expression in breast cancer is greater than any normal tissues. In a study involving a mouse xenograft model of mammal tumors and 3D endothelial tissue culture demonstrated that alcohol increased tumor angiogenesis and speed tumor development of MDA-MB-231 in 3D cell culture system with human vein endothelial cells [2]. Additionally, it appeared that alcohol-induced VEGF expression in breast cancer both *in vivo* and *in vitro*. The result indicated that alcohol promotes mammary tumor growth as it stimulates the VEGF dependable angiogenesis.

In another study, scientists were seeking to understand the risks factors that are involved in the development of breast cancers to provide effective preventive measures as well as help patients identify the effective therapeutic measure. Epidemiological studies indicated that breast cancer is positively related not only alcohol or also the amount alcohol consumption. Furthermore, alcohol consumption positively correlates with increased risk in breast cancer as they are concealed by dense parenchymal tissue

as well as reducing carotene circulation and these parameters are leading causes of breast cancer [27].

In addition, studies showed that for the given popularity of alcohol in the US, alcohol is a known modifiable factor in the development of breast cancer [28]. Candelaria *et al.* examined the molecular mechanism of actions of alcohol through the application of molecular, genomic and genetic approaches are the characterization of estrogen receptor-positive breast cancer cells. Their treatment with alcohol promotes the cells proliferation increased growth while regulated the transcription of estrogen receptor target gene *GREB1* but not canonical targets *TFF1/pS2*. In their microarray analysis using alcohol treatment found a large number of alcohol-responsive genes including those with cell proliferation pathways as well as those that function in apoptosis. Expression profiles of responsive genes provided clinical outcomes in a patient using endocrine therapy. Moreover, alcohol treatment diminished the anti-proliferation impacts of endocrine therapy drug tamoxifen in estrogen receptive cell. In order to determine the contribution of and response of genes, variation in expression was assessed between outcome groups. Using the proto-oncogene *BRAF* was the novel estrogen and alcohol gene which showed higher expression in patients with poor cells. The knock-down of *BRAF*, despite preventing the proliferation of breast cancer cells and increased risk for diseases reoccurring and incidents [29].

Narayanan *et al.*'s study investigated the molecular mechanism involved in ethanol mediated proliferation of breast cancer cells [4]. MDA-231 and MCF-7 breast cancer cells were used. The study used immunoblotting and flow cytometry to evaluate the gene expression related to the increased proliferation caused ethanol exposure. The

mechanism of ethanol-mediated proliferation was determined using reverse transcription polymerase chain reaction (PCR), and immunoblotting. The qualitative real-time polymerase chain reaction (qPCR) results showed the involvement of Signal transducer and activator of transcription 3 (STAT3) activation. This study not only demonstrated that exposure to ethanol caused an increase in cell proliferation, but also the accumulation of cells in S-phase of both MDA MB-231 and MCF-7 cell lines. In addition, an increase in reactive oxygen species (ROS) release confirmed to enhance proliferation due to inflammatory response in breast cancer cells. The pro-inflammatory response followed the phosphorylation of STAT3. The importance of STAT3 activation in ethanol-mediated proliferation was confirmed via silencing of STAT3 [30].

Ethanol is known as a tumor initiator and tumor promoter. The correlation between alcohol consumption and breast cancer risk has been intensively study for the past decades [31]. Hence, it is important to investigate how alcohol affects cells to bring awareness for people, especially women.

## **2.2 Metabolism and Oxidative Stress**

Cell metabolism has been extensively studied in order to understand cellular physiology, functions and fate. Metabolism plays an important role in cell growth, proliferation, differentiation and viability [32]. Metabolism is a very complicated process involving multiple biochemical pathways catalyzed by specific enzymes, which results in energy consumption and production.

There are three important processes of cellular respiratory: oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect) [33][34][35]. Cells depend on glucose for energy to function normally. Cellular respiratory

generates adenosine triphosphate (ATP), the main energy source of the organism. Glycolysis is the major metabolic pathway to break down glucose to pyruvate in cytosol in all cells, which results in production of nicotinamide adenine dinucleotide (NADH). It can occur aerobically or anaerobically depending on the availability of oxygen. In the presence of oxygen, pyruvate is decarboxylated into acetyl-coA in the mitochondrial matrix, which then enters the Krebs cycle. The oxidative phosphorylation process produces energy due to the electrons acceptance of the electron transport chain from NADH and reduced flavin adenine dinucleotide (FADH<sub>2</sub>). Oxygen is an essential metabolite since it is the final electron acceptor at the end of this pathway. This process generates 30-36 ATP. The oxidative phosphorylation pathway also creates reactive oxygen species. In addition, in the absence of oxygen, the cell metabolism shifts to anaerobic glycolysis, where NADH is reoxidized instead by reducing pyruvate to lactate. Thus, the energy released from anaerobic condition is severely limited in comparison to aerobic glycolysis. Studies showed that cancer cells tend to perform aerobic glycolysis even in the presence of oxygen, which results in lactate accumulation. This phenomenon is called “Warburg effect”; and it also generates less ATP than oxidative phosphorylation pathway [33].

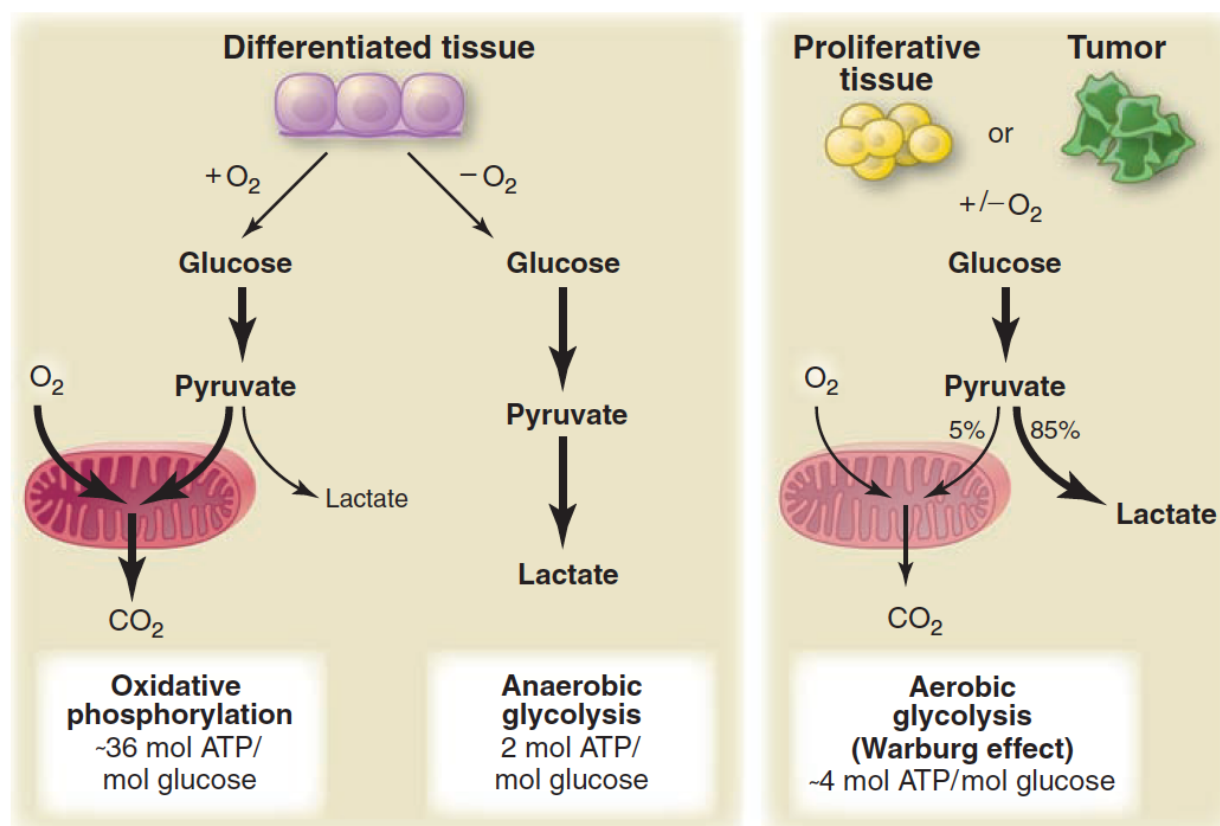


Figure 1: A simplified schematic of glucose metabolism. There are three main cellular respiratory processes to metabolize glucose: oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (from left to right) (Warburg Effect) [36].

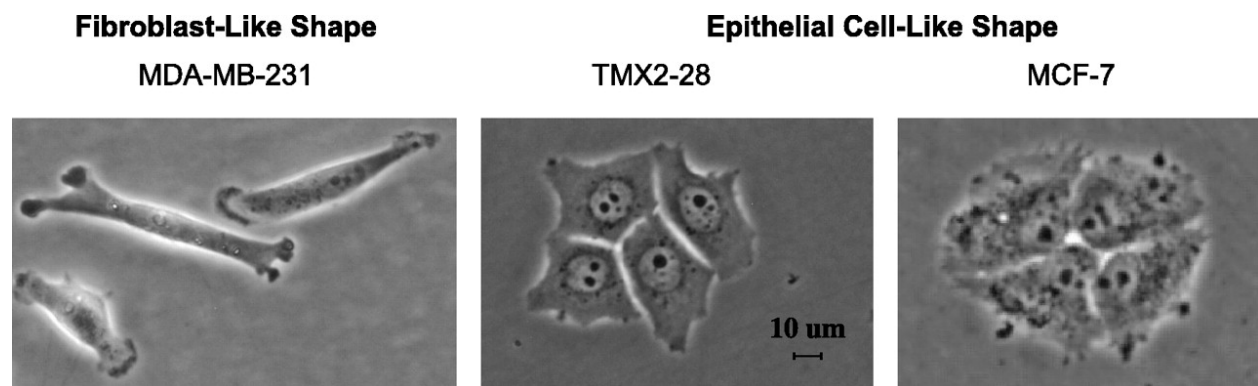
Metabolism shift is one of the hallmarks to differentiate normal and cancer cells along with other key factors including abnormal morphology, increased in rate of growth, resisted to cell death, increased mobility and invasiveness. Therefore, study of cancer metabolism can provide powerful insights into the development of cancer cells.

## 2.3 Breast Cancer Cell Lines

In this study, I used MDA-MB-231 and MCF-7 cell lines, which are commonly used breast cancer cell lines in medical research laboratories. MDA-MB-231 is an epithelial, human breast cancer cell derived from a 51-year-old Caucasian female, who had a metastatic mammary adenocarcinoma [43]. MDA-MB-231 is highly invasive and poorly

differentiated. It is triple-negative cell line, which lacks Estrogen receptor (ER), progesterone receptor (PR) expressions, and human epidermal growth factor receptor 2 (HER2) amplification [44]. MDA-MB-231 is known for its endothelial-like morphology [Fig. 2]. The triple negative breast cancer is highly aggressive with limited treatment options. Thus, it can serve as an excellent model for breast cancer study.

MCF-7 cell line is isolated from a 69-year-old Caucasian female [45]. This cell line is less aggressive in comparison to MDA-MB-231 since it still retains several characteristics of differentiated mammary epithelium. MCF-7 is ER and PR positive, and HER2 negative, which means it still have hormone response ability including process estrogen in cell cytoplasm [46][47][48]. The morphology of MCF-7 is significant different from MDA-MB-231 [Fig. 2]. MCF-7 cells are smaller with a rounder cell body; and they tend to clump together as they grow. MCF-7, MDA-MB-231 and T-47D contributes up to two-thirds of reporting studies of breast cancer according to a Medline-based survey [49].



*Figure 2: Morphologies of three types of breast cancer cells: MDA-MB-231, TMX2-28, and MCF-7. MDA-MB-231 has fibroblast-like shape, while TMX2-28 and MCF-7 has epithelial cell-like shape [37].*

## **CHAPTER 3: RESEARCH DESIGN AND METHODS**

### **3.1 NADH, a Label-free Biomarker for Metabolic Imaging**

Cells, tissues along with other biological systems consist of numerous endogenous fluorophores that have been intensively study to explore and access the physiological functions like metabolism. Table 1 listed several intrinsic fluorophores along with there one photon excitation and emission wavelengths. The properties of emission light from intrinsic fluorophores are affected by their morphology, microenvironment, and importantly, their metabolic state. Hence, intrinsic biomolecules remain powerful biomarkers in study of cancer.

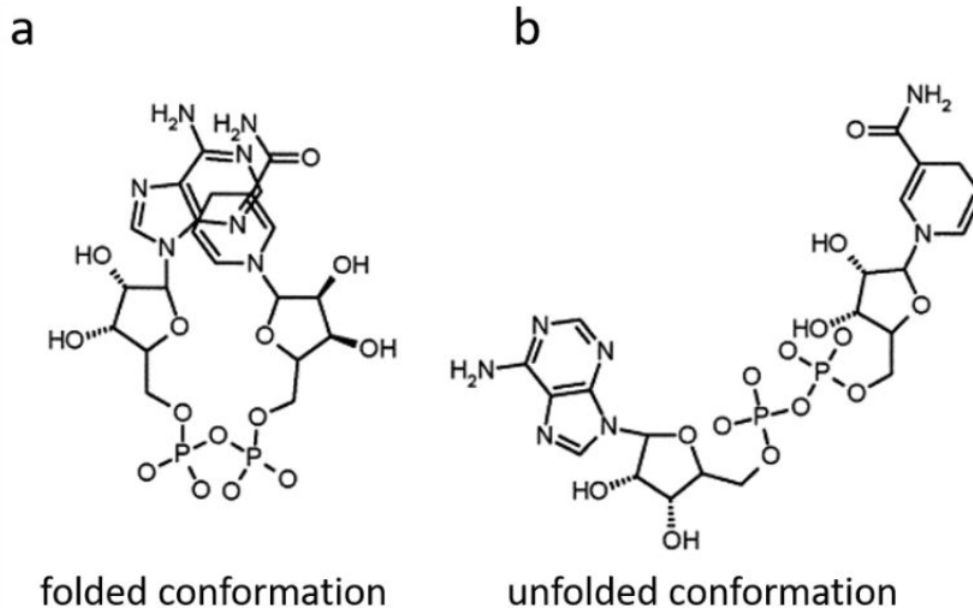
*Table 1: Excitation and Emission Wavelength of intrinsic fluorophores [38][39][40][41][42]*

| <b>Fluorophores</b>        |               | <b>Excitation (nm)</b> | <b>Emission (nm)</b> |
|----------------------------|---------------|------------------------|----------------------|
| <b>Metabolic Coenzymes</b> | NADH          | 340 (max)              | 470 (max)            |
|                            | NADPH         | 336 (max)              | 464 (max)            |
|                            | FAD           | 450 (max)              | 535 (max)            |
| <b>Vitamins</b>            | Retinol       | 327 (max)              | 510 (max)            |
|                            | Vitamin K     | 335 (max)              | 480 (max)            |
|                            | Vitamin D     | 390 (max)              | 480 (max)            |
|                            | Vitamin B12   | 275 (max)              | 305 (max)            |
| <b>Pigments</b>            | Melanin       | 300 - 800              | 440, 520, 575        |
| <b>Amino Acids</b>         | Tyrosine      | 275 (max)              | 300 (max)            |
|                            | Tryptophan    | 280 (max)              | 250 – 310            |
|                            | Phenylalanine | 258 (max)              | 280 (max)            |
| <b>Structural Proteins</b> | Collagen      | 325, 360 (max)         | 400, 405 (max)       |
|                            | Elastin       | 290, 325 (max)         | 340, 400 (max)       |

Nicotinamide adenine dinucleotide (NAD) is an essential metabolic coenzyme, it involves in numerous cellular processes such as mitochondrial function, gene expression, oxidative stress, and apoptosis. In oxidative phosphorylation pathway, NADH molecules produced Krebs cycle are oxidized to NAD<sup>+</sup> by donating electrons to the electron

transport chain. However, in anaerobic glycolysis, the oxidative phosphorylation is diminished leading in the reduce in oxidation of NADH molecules, which results in the increase in free NADH. The ratio of reduction-oxidation (redox) pair NADH/NAD<sup>+</sup> indicates the balance between oxidative phosphorylation and glycolysis. Studies showed that there is a correlation between the redox ratio of NADH/NAD<sup>+</sup> and the ratio of free/protein-bound NADH. One of the important note is that NAD<sup>+</sup> is not fluorescent. However, NADH has been extensively studied as a tool to investigate the metabolism both *in vitro* and *in vivo* [50][51]. The autofluorescence NADH has absorption maximum at 340nm and emission maximum around 470nm. The fluorescent is influenced by local microenvironment and metabolism state.

NADH-FLIM takes the advantages of NADH lifetime properties for metabolic imaging. NADH exists in two different configurations: folded and extended. The reduced nicotinamide is the fluorescent group in NADH. Free NADH has a folded configuration (Fig. 3a); and the reduced nicotinamide is quenched by the adenine group. On the other hand, the protein-bound NADH exists in extended configuration (Fig. 3b), which rescue the quenching. Hence, the fluorescent lifetime of free NADH is significant lower in comparison to bound NADH due to its self-quenching property. The fluorescent lifetime of free and protein-bound NADH is 0.4ns and 3.4ns respectively [53][54]. The significant difference between the fluorescent lifetime of two species provides a strong quantitative measure of the ratio of free/bound NADH.



*Figure 3: NADH Conformations. Folded conformation (free NADH) (a), and extended conformation (protein-bound NADH) of NADH [52].*

### 3.2 Fluorescence Lifetime Imaging Microscopy

Fluorescence Lifetime Imaging Microscopy (FLIM) is a powerful biomedical optics tool to measure the rate of the fluorescence exponential decay from a sample post-excitation of a photon [55]. A fluorophore is excited by a short pulse of light, its fluorescence lifetime ( $\tau$ ) is determined by the time it takes for the intensity,  $I(t)$ , to decrease to  $1/e$  of the original value. The intensity at time  $t$  is given by the formula below:

$$I(t) = \alpha e^{-t/\tau}$$

where,  $\alpha$  is the pre-exponential factor.

FLIM can obtain high sensitivity result of fluorescence lifetime of various biological molecules due to the differences in fluorescence lifetime of each specific molecular. The setup of FLIM is differed based on multiple factors such as light source, optics, filters, and

analyzing software. In this study, I used 740nm two-photon microscopy to excite free and bound NADH molecules.

There are several approaches to analyze the fluorescence lifetime data including Laplace transform method, global fitting algorithm, Laguerre deconvolution technique [56][57][58]. However, in this study, I used phasor approach to interpret the raw data. The advantage of this method is that the decay information can be obtained at each pixel of the image. It can identify more than one fluorescent molecular species at a single pixel [55]. In addition, the phasor approach provides instantaneous visualization of special location of the specific molecular species. In this method, the fluorescence exponential decay information of each pixel is Fourier transformed into a point in the phasor plot. A phasor distribution of an image can be created using plotted phasors at each pixel. Considering time-domain FLIM measurement, the coordinates in the phasor plot (g,s) is given by [59]:

$$g_{i,j}(\omega) = \frac{\int_0^T I_{i,j}(t) \cos(\omega t) dt}{\int_0^T I_{i,j}(t) dt}$$

$$s_{i,j}(\omega) = \frac{\int_0^T I_{i,j}(t) \sin(\omega t) dt}{\int_0^T I_{i,j}(t) dt}$$

where, P(i,j) is a pixel in the FLIM image with the coordinates (i,j);  $I_{i,j}$  is the fluorescence intensity decay at pixel (i,j);  $\omega = 2\pi f$ , and  $f = 1/T$  is the laser frequency. In this study,  $f = 80\text{MHz}$  is used.

The phasor coordinates are also expressed in term of lifetime and angular laser frequency. The coordinates of single pixel on the FLIM image can be determined using the equations below:

$$g_{i,j}(\omega) = \frac{1}{1 + (\omega\tau)^2}$$

$$s_{i,j}(\omega) = \frac{\omega\tau}{1 + (\omega\tau)^2}$$

Equation (6) can be derived from equation (4) and (5):

$$s_{i,j}^2 + (g_{i,j} - 1/2)^2 = 1/4$$

From equation (6), we can create a *Universal Circle* (Fig. 3) since all the single exponential lifetimes will fall on a semi-circle of radius  $1/2$  and center at  $(1/2, 0)$ . Hence, a short lifetime will fall to the right of the *Universal Circle* toward the point  $(1,0)$ ; while a long lifetime will lie on the left on the circle toward the point  $(0,0)$ . However, the FLIM image is usually very complex with multiple single lifetimes components. Especially, a biological system such as cells and tissue contains multiple fluorescence molecules. Thus, the overall coordinates will be given by:

$$G(\omega) = \sum_n f_n g_n(\omega)$$

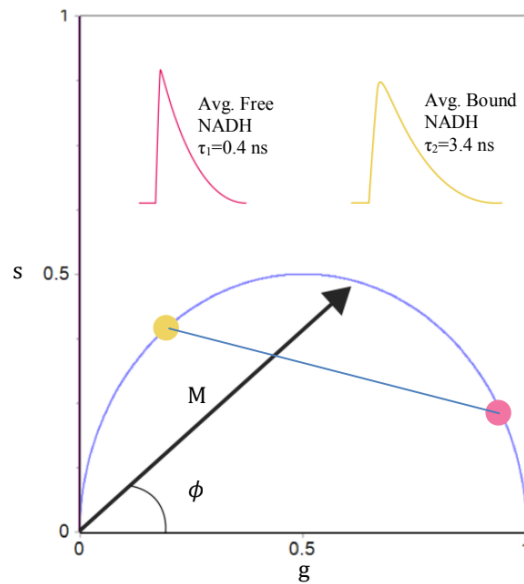
$$S(\omega) = \sum_n f_n s_n(\omega)$$

The phasor of the mixture of two pure single exponential molecules will fall on a linear line connecting the phasor location of each individual molecule on the *Universal Circle* [55][60]. Therefore, the relative fractional distribution of each component can be calculated based on the position of the phasor.

### 3.3 NADH Phasor FLIM

In this study, I focused on the application of FLIM and phasor approach on the analysis of NADH. Lakowicz *et al* first proved the possibility to obtain the lifetime images of Free and bound NADH in 1992. Figure shows the phasor position of free and bound

NADH. The mixture of free and bound NADH will fall on the straight line joining two phasors. Thus, the analysis of relative abundance of free and bound NADH can be easily calculated using the relative fractional distribution of the phasor on the plot. The connected line between the position of pure free NADH and pure bound NADH was referred as the metabolic trajectory by Stringari et al. The ratio of free/bound NADH is different depending on the location inside the cell and the metabolism state of the cell. For instance, the ratio will vary between nucleus and cytoplasm; between glycolysis and oxidative phosphorylation. Free and bound NADH display a lifetime of 0.4 ns and 3.2 ns respectively (Fig. 4). Thus, the ratio of free/bound NADH can deduce the dominant metabolic pathway; which is a goal of this study.



*Figure 4: The transformation of free and Bound NADH onto a phasor plot. The average fluorescent lifetime of free, and bound NADH are 0.4ns and 3.4ns respectively.*

### 3.4 NADH Absolute Concentration

The accurate concentration of NADH is very important to understand cellular metabolism. Normally, the measurement of a fluorophore can be calculated using a

calibration of the signal of known concentration of that fluorophore in the cell. This calibration method works when the intensity of the fluorophore is greater than any signal of background fluorescent molecules. As mentioned in previous sections, NADH exists in two different conformations which has two different fluorescent lifetime. The measurement of autofluorescent NADH is achieved by a calibration procedure on known concentration of homogeneous solution of free NADH. This approach uses the changes in phasor positions at each pixel due to the addition of a given amount of unmodulated light to determine the absolute concentration of NADH [61]. Because the fluorescent lifetime is an independent measurement. The addition of an unmodulated light at the origin will not change the fluorescent exponential decay measured in either frequency or time domain. The phasor of free NADH will move towards the origin as the constant increases. The amount of the shift depends on the relative intensity of free NADH and the amount of unmodulated added. In this study, I added a constant which is a fluorescent decay of a known amount of free NADH instead of an external light. For a given amount of light, the measurement of the shift can be measured by comparison with the calibrated solution of homogeneous free NADH. The distance (M) of the phasor position to the origin can be determined using the formula below [61]:

$$M = \frac{L}{L + F}$$

where L is external light intensity; F is the amount of fluorescence emitted by a known concentration of free NADH.

In a FLIM image, a pixel which contains a smaller amount of NADH than the calibration solution will move further toward the origin; and the shift is larger than pixels

where the concentration of NADH is higher. Hence, the absolute concentration of NADH in each pixel can be determined.

### **3.5 Cell Culture and Alcohol Treatment**

MDA-MB-231 and MCF-7 cells were cultured in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) (ThermoFisher Scientific, San Diego, CA), and 1% penicillin-streptomycin (Genesee Scientific, San Diego, CA). 5000 cells of MDA-MB-231 were counted and plated in 8-well plates 48 hours before the alcohol treatment.

Before the experiment, the media in each well is taken out. Ethanol is added to the concentration of 0%, 0.3%, and 0.6%. The highest concentration is 0.6%, which mimics the highest biological reference of ethanol inside human cells before the lethal concentration. After the additional of ethanol, the media is immediately transfer back to the corresponding well. I used the old culture media for imaging to eliminate the addition of nutrients that can greatly affect the cell metabolism. Cells were incubated at 37°C with 5% CO<sub>2</sub> for 15 minutes before the imaging.

### **3.6 FLIM Setup and Data Acquisition**

FLIM images of MDA-MB-231 were imaged on an inverted confocal Zeiss LSM 710 (Carl Zeiss, Jena, Germany). Olympus 40X/1.2NA water objective is used. The images had consistent size of 256 x 256 pixels with 50 frames per view; and were taken at a pixel with a scan speed of 25.21 $\mu$ s. The LSM 710 is coupled with a two-photon Ti:Sapphire laser (Spetra-Physics Maitai, Mountain View, CA). FLIM data of NADH is acquired with 740nm two-photon excitation by an ISS A320 FastFLIM box (ISS, Champaign, IL). The emissions were separated at 690nm followed by a blue emission filter of 420-500nm to collect signals of NADH. The signals were collected by a

photomultiplier tubes (H7422P-40, Hamamatsu, Japan). Coumarin-6 in ethanol, which has a known single exponential lifetime of 2.5ns, was used as reference to calibrate all the FLIM measurements.

FLIM analysis was performed using Globals for Images software (SimFCS), which was developed at Laboratory of Fluorescence Dynamics, University of California, Irvine. The absolute concentration of NADH is calculation based on the calibration of known concentration of free NADH.

### **3.7 Statistical Analysis of Data**

Statistical significance was calculated using Student's t-test in Microsoft Excel.

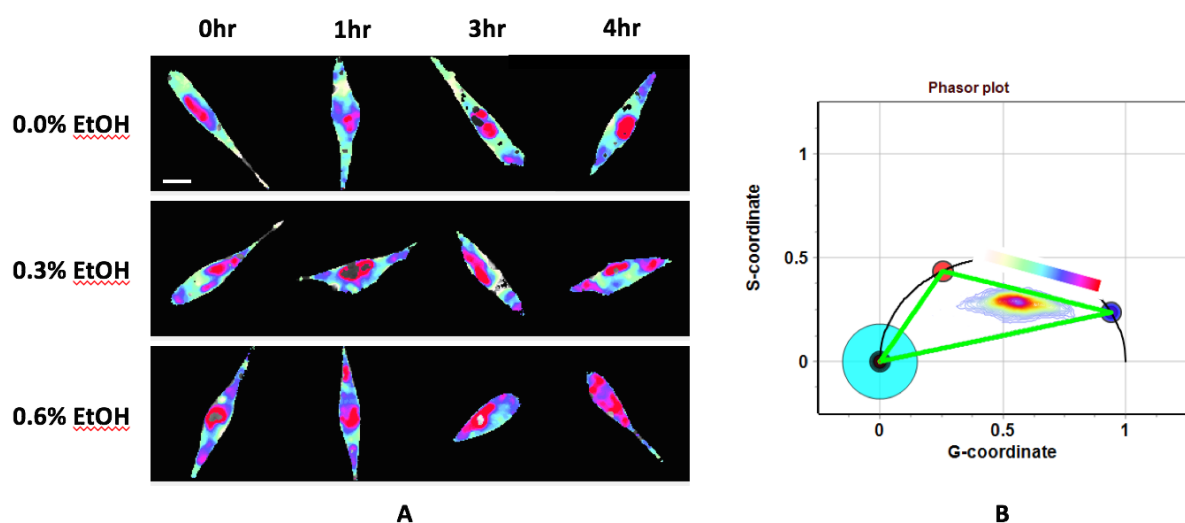
## **CHAPTER 4: RESULTS AND DISCUSSION**

### **4.1 FLIM Results**

NADH fluorescent lifetime measurement from FLIM provided a non-invasive determination of the spatial shifts in metabolism of breast cancer cell lines: MDA-MB-231 and MCF-7. For this study, the fluorescent lifetime of free NADH (0.4ns) and lactate dehydrogenase bound (LDH) NADH (3.4ns) were when quantifying the fraction of free NADH at each pixel on the FLIM image [54]. The fraction of free and bound NADH can be calculated based on the position of the phasor on the linear line connecting 100% free NADH and 100% bound NADH.

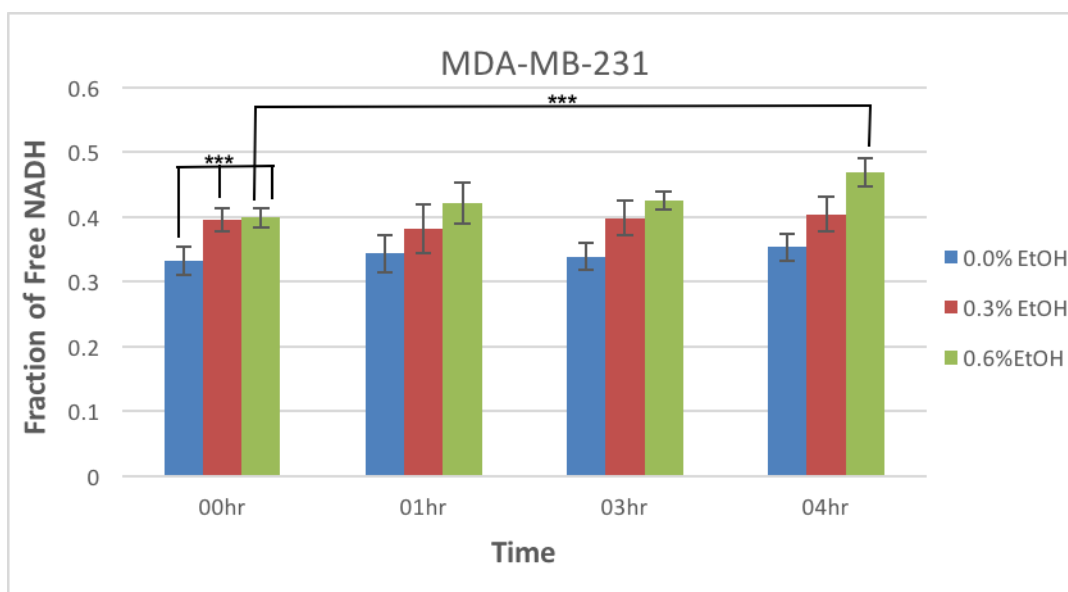
Before the FLIM imaging, the cells were incubated with different concentration of ethanol for 20 minutes (0.0%, 0.3% and 0.6% ethanol); 20-minutes mark was considered as time point 0 hours. I took image of 5 cells for each condition at 4 different time points: 00 hours, 01 hours, 03 hours, and 04 hours. The experiment was repeated twice for each cell lines (n =10).

The fluorescent lifetimes were collected for a whole cell in an effort to characterize the total concentration of NADH inside the cell. Figure 5b depicted the fluorescent lifetime of the whole population. It lied on the linear line connected the pure free NADH (blue circle) and 100% protein bound NADH (red circle). The colored image of FLIM confirmed the hypothesis that alcohol shifts the metabolism of cancer cells. Figure 5a indicated that as the percentage of alcohol, and the time increased, the MDA-MB-231 cells shifted from oxidative phosphorylation (OXPHOS) (white/cyan) to glycolysis (GLY) (pink/red).



*Figure 5: Metabolic indexes of MDA-MB-231 cells on various ethanol concentration in 4 hours. (A) Colored FLIM images of MDA-MB-231 with 3 different alcohol treatments: 0.0%, 0.3%, and 0.6% ethanol. Images were taken at 0, 1, 3 and 4 hours mark. (B) Fluorescent lifetime of the whole MDA-MB-231 population. Blue dot is 100% free NADH (0.4ns), while red dot is 100% protein bound NADH (3.4ns). An increase in free NADH indicates the shift toward GLY (pink/red), while an increase in bound NADH shows the shift toward OXPHOS (white/cyan). Scale bar: 5um.*

The effect of alcohol on MDA-MB-231 breast cancer cells was immediate. The fraction of free NADH changed significantly by 19.2% and 20.2% in the alcohol treatment groups 0.3% and 0.6% ethanol in comparison with no alcohol group at time 0 hours respectively [Fig. 6]. Throughout the course of 4 hours, the fraction of free NADH did not change in 0.0%, and 0.3% ethanol treatment groups; they fluctuated around 0.342 and 0.394 respectively. Only the 0.6% ethanol group displayed a significant increase in free NADH by 17.6% at 4-hour mark [Fig. 6], which indicated that 0.6% alcohol exposure caused a shift in metabolism of MDA-MB-231 cancer cells immediately, and the effect continued to increase with time.



*Figure 6: Fraction of free NADH of MDA-MB-231 breast cancer cells with alcohol treatment. MDA-MB-231 were treated with 0.0%, 0.3% and 0.6% ethanol. Images were taken at 0, 1, 3, and 4-hour marks (n=10). \* $p < 0.05$ , \*\* $p < 0.001$  by Student's *t*-test.*

MCF-7 also showed a shift in metabolism from OXPHOS to GLY as the concentration of alcohol increased [Fig. 7]. However, the shift in MCF-7 was more dramatic in comparison to MDA-MB-231 cells. At time 0hr, MCF-7 cells with alcohol treatment strongly shifted toward GLY, which was proved by the increase in fraction of free NADH of 26.0% and 35.4% in 0.3% and 0.6% ethanol treatment groups respectively in comparison with no alcohol group [Fig. 8]. As expected, MCF-7 cells with no ethanol maintained their fraction of free NADH throughout the course of experiment (average 0.298). Similar to MDA-MB-231, the fraction of free NADH of MCF-7 cells in 0.6% alcohol treatment group continued to increase as the time increased. 0.6% ethanol MCF-7 at 4hr mark displayed an increase in free NADH by 11.1% in comparison with 0hr mark. However, the remarkable raise in free NADH of 0.3% ethanol group at 4hr in comparison with they were at 0hr indicating that alcohol the effect of ethanol in MCF-7 cells was greater than it was in MDA-MB-231.

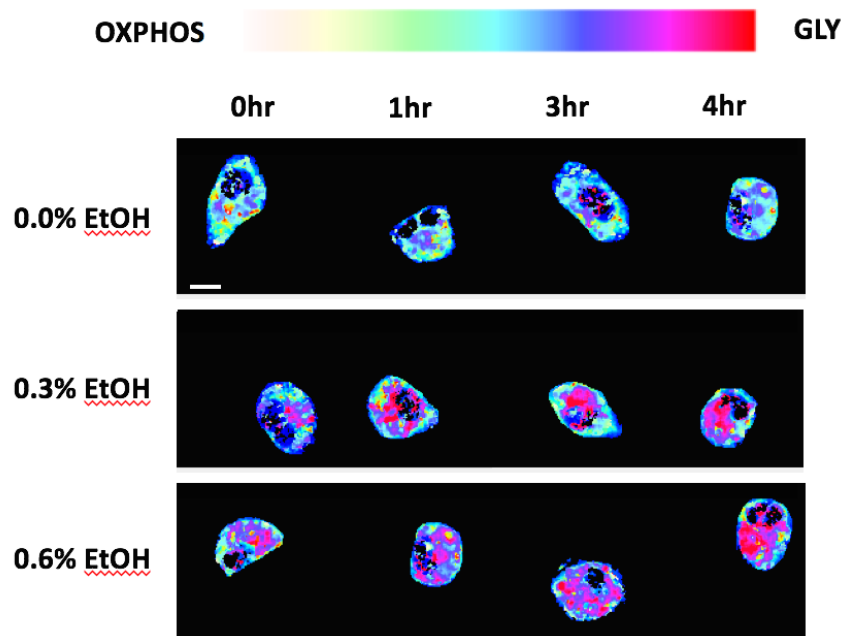


Figure 7: Metabolic indexes of MCF-7 cells through colored FLIM images after alcohol treatment. MCF-7 cells were treated with 0.0%, 0.3%, and 0.6% ethanol. Images were taken at 0, 1, 3 and 4-hour mark. An increase in free NADH indicates the shift toward GLY (pink/red), while an increase in bound NADH shows the shift toward OXPHOS (white/cyan). Scale bar: 5 $\mu$ m.

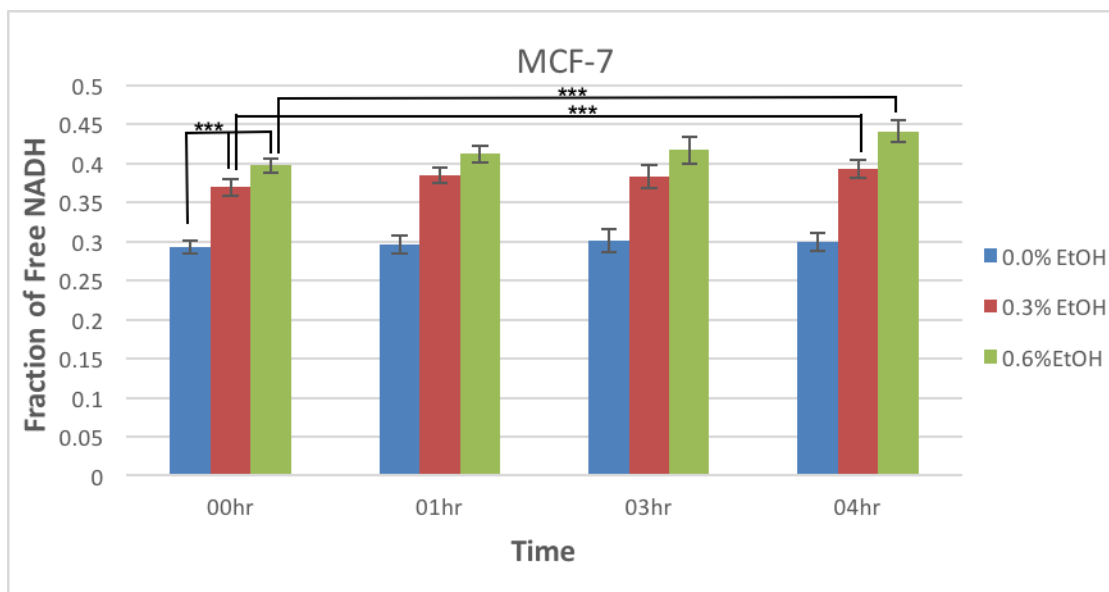


Figure 8: Fraction of free NADH of MCF-7 breast cancer cells with alcohol treatment. MDA-MB-231 were treated with 0.0%, 0.3% and 0.6% ethanol. Images were taken at 0, 1, 3, and 4-hour marks ( $n=10$ ). \* $p<0.05$ , \*\* $p<0.001$  by Student's  $t$ -test.

The stronger effect of alcohol in MCF-7 cell lines might due to the nature that MCF-7 cells still have ER receptor to response to the changes in hormones. Study showed that alcohol is linked to the increase in breast cancer risk, and also is associated with hormone-dependent development of breast cancer [28]. Alcohol believed to promote cancer cell proliferation via up-regulating the transcription of *GREB1*, which is an ER target gene. The study from Lu *et al* also confirmed through flow cytometry analysis that ethanol enhances cell proliferation in MCF-7 significantly more than MDA-MB-231 [3].

## **4.2 NADH Concentration Results**

As expected, the total NADH concentration of MDA-MB-231 and MCF-7 were matching with the fraction of free NADH. The total concentration of NADH increased immediately at 0hr in alcohol treatments group in comparison with no alcohol group (average of 22% in MDA-MB-231, and 12% in MCF-7) [Fig. 9] [Fig. 10]. The abundance in NADH concentration can be explained by the shift toward glycolysis of cancer cells. The accumulation of NADH occurs when oxidative phosphorylation is dismissed.

Also, the increase in NADH concentration of both MDA-MB-231 and MCF-7 cells have a prolonged effect after 4 hours. This indicated that the influence of alcohol is both dose- and time-dependent. The NADH concentration also confirmed the observation that alcohol effect on MCF-7 was stronger than it was on MDA-MB-231 since the lower concentration of ethanol (0.3%) could continue to shift the cell metabolism after 4 hours in MCF-7 cells, but not in MDA-MB-231.

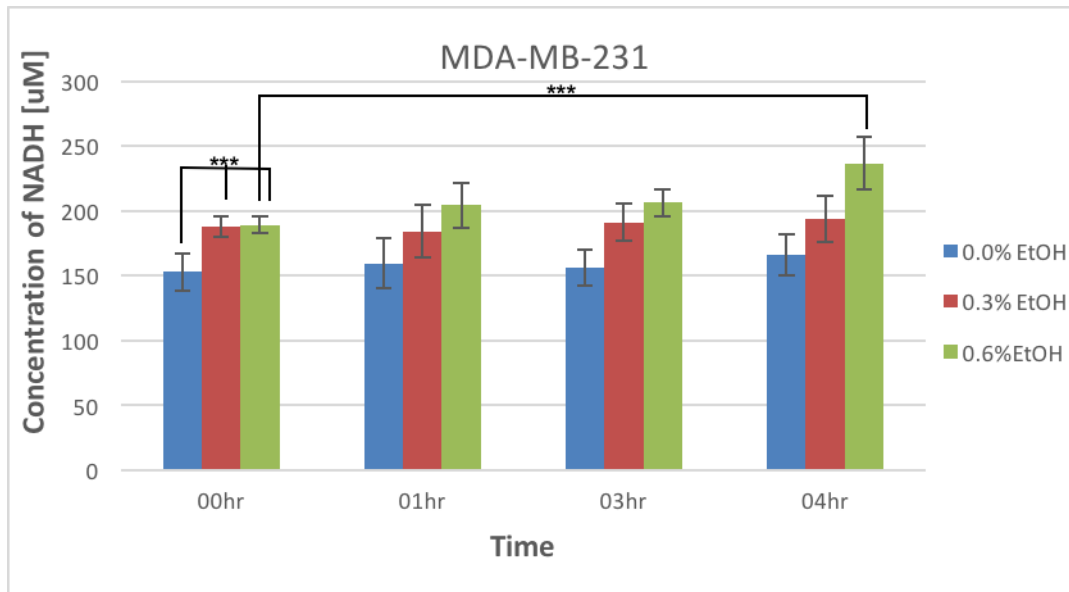


Figure 9: Absolute Concentration of NADH in MDA-MB-231 cells after 4 hours of alcohol treatment. MDA-MB-231 cells were treated with 0.0%, 0.3% and 0.6% ethanol. Data were taken at 0, 1, 3, and 4-hour marks (n=10). \* $p < 0.05$ , \*\* $p < 0.001$  by Student's *t*-test.

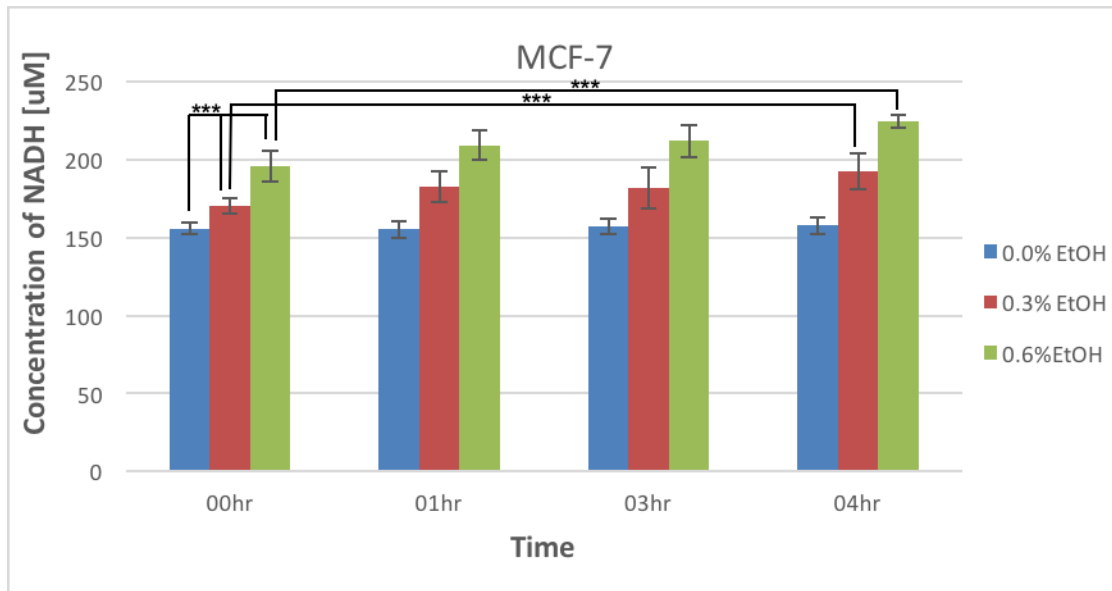


Figure 10: Absolute Concentration of NADH in MCF-7 cells after 4 hours of alcohol treatment. MCF-7 cells were treated with 0.0%, 0.3% and 0.6% ethanol. Data were taken at 0, 1, 3, and 4-hour marks (n=10). \* $p < 0.05$ , \*\* $p < 0.001$  by Student's *t*-test.

## **CHAPTER 5: CONCLUSIONS**

### **5.1 Conclusion Remarks**

The Warburg effect is the hallmark of cancer cell metabolism. Cancer cells prefer fermentation pathway (glycolysis) as their energy source rather than undergo the more efficient pathway of oxidative phosphorylation. This is due the high energy demand of cancer cells. The changes in metabolic pathway play an important role in regulating cancer invasiveness and proliferation. Importantly, phasor FLIM is a powerful, non-invasive imaging tool to investigate the metabolic shift of cancer cells due to changes in cellular microenvironment. I used FLIM of the autofluorescence NADH to measure the metabolic changes in breast cancer cell line MDA-MB-231 and MCF-7. The cells were treated with 0.0%, 0.3%, and 0.6% ethanol.

This study has shown that acute alcohol exposure can immediately shift the metabolism of breast cancer cells MDA-MB-231 and MCF-7 from OXPHOS to GLY. In addition, the effect of ethanol on MCF-7 was stronger than it was on MDA-MB-231; which was confirmed by the increase in fraction of free NADH and total NADH concentration. I observed a prolonged effect of shifting to GLY of MDA-MB-231 in high concentration of alcohol (0.6% ethanol) and of MCF-7 in medium and high concentration of alcohol (both 0.3% and 0.6% ethanol) after 4 hours.

This study provides insight for breast cancer research in an effort to understand the link between cancer cells proliferation/invasiveness and diet. It also characterizes the relationship between alcohol exposure and ER activation. Moreover, it brings awareness to people with high alcohol consumption about the risk of breast cancer.

## **5.2 Limitation of the study**

The study demonstrated the shift in metabolism of both types of breast cancer cells: MDA-MB-231 and MCF-7 due to acute alcohol exposure. However, due to the time constraints, there was no non-tumorigenic breast cells as a negative control. To further confirm that the changes in the ratio of free and bound NADH were due to the shift in metabolism, more studies should be done including several inhibition experiments.

## **5.3 Future Work**

As mentioned above, to further confirm the effect of acute alcohol exposure on breast cancer cells metabolism. There are a set of follow up experiment including FLIM imaging on non-cancerous cells such as MCF-10A. Also, inhibition experiments including dichloroacetate and 2-deoxyglucose (glycolysis inhibitors), rotenone and antimycin A (oxidative phosphorylation inhibitors) should be done. Also, extending the experiment time can provide the maximum fraction of free NADH, and the maximum effective time of alcohol on two cell lines.

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