Development of a High Throughput Flow Cytometry Method to Analyze Changes in Epigenetic Histone Modifications in the Cell

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Development of a High Throughput Flow Cytometry Method to Analyze Changes in Epigenetic Histone Modifications in the Cell

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Abstract

Epigenetic studies commonly include assays such as Chromatin Immunoprecipitation (ChIP), western blotting, and immunofluorescence (IF), which are used to measure changes in chromatin modifications and in DNA methylation patterns associated with diseased states and resulting from drug treatments (Lo et al. 2004). Clinically, changes in epigenetic modifications are determined by intensive western blotting assays. However, other methods such as IF and flow cytometry are starting to become more prevalent, as these techniques are crucial to show that the drug response in a cell is specific before the drug enters clinical trials. The purpose of this study was to develop a high throughput flow assay to analyze a large number of histone modifications and to compare this assay to western blotting methods for ease of use and quantitative analysis.

First, I utilized antibodies against total Histone H3, H3K27Me2, H3K27Me3, H3K4Me3 and H3K9Me3 to develop a flow assay, to determine optimal drug treatment conditions, and compare to western blotting for assaying changes in histone modification in cells. Using this flow assay, I found that the EZH2-specific methyltransferase inhibitor, GSK343, most optimal drug treatment condition was 5µM for 96h in Jurkat cells, showing a 14–fold reduction in H3K27Me2 levels and a 21–fold reduction in H3K27Me3 levels. Additionally, I found that the general methyltransferase inhibitor, Adox, rapidly induced cell death and apoptosis without showing significant changes in
histone methylation. Finally, my results demonstrated that flow is a faster, more quantitative and less strenuous method compared to western blotting.

Second, I determined a medium-throughput flow protocol using a panel of thirteen monoclonal antibodies against key methylation and acetylation sites on total Histone H3. I determined one flow protocol, which used 0.3% Triton X-100 permeabilization and worked for all thirteen antibodies. Finally, I utilized this medium-throughput flow assay and antibody panel to interrogate the specificity of GSK343 and for that it specifically indicated methylation of H3K27, the known substrate of EZH2.

For this project, I was able to develop a medium-throughput flow protocol using a panel of thirteen monoclonal antibodies. I found this flow assay easier, more efficient and more quantitative than western blotting. Using the medium-throughput flow assay, I analyzed the specificity of GSK343 for EZH2. It is important to know of any off-target effects of epigenetic drugs before they are used on humans for drug therapies. Results from this project lay the groundwork so that I can expand upon and develop a high-throughout flow assay by introducing a larger panel of antibodies, potentially to every H2A, H2B, H3 and H4 modification site, for screening other epigenetic drugs for specificity.
Dedication

I dedicate my thesis to Kevin Kordosky, my loving husband. I began the Harvard Extension program back in September 2012; I did not attend my first class, Introduction to Biochemistry, as Kevin and I were on our honeymoon in Jamaica. I dedicated myself to the program upon our return and everyday thereafter for the next 5.5 years. I was very serious in my studies and doing well while learning. I found it difficult to relax and to be continuously happy during this time.

I discovered no matter how well I managed my time, working full time while attending classes with long commutes, I neglected many things. Kevin picked up my slack. He stepped up cooked our meals, grocery shopped and did our laundry. He agreed to help out when needed while I was pursuing my masters. This agreement turned into helping out quite frequently. Kevin took on all these added responsibilities with no complaints. I felt awkward relinquishing my chores for him to do alone. Kevin and I had always worked as team so this was difficult for me. I contemplated quitting many times when self-doubt and negativity became heavy.

I had difficult and painful ankle and back surgeries while attending school. Deaths in my family and stressful work situation added to my negative thoughts over this time period. Kevin motivated me and was my biggest supporter through all. He drove me to and from work everyday then to class and home while I was recovering from these surgeries. He drove me to my ethics class right after we attended my young cousins
funeral. He knew I was presenting that day for extra credit and wanted to give me moral supports after a difficult day.

As rewarding as the Harvard Extension Program has been, I am happy to move towards the next chapter of our lives. I plan on being less stressed and relaxed. I will feel more like my usual care-free self. My degree will offer additional career opportunities, which will enrich the life of my husband and I. Thank you, Kevin for your unwavering and continuous love and support.
Acknowledgements

Chris Fry, Ph.D. – Chris served as my thesis director on this project. I am very thankful for his time and support on this project. His motivation and love for science, especially epigenetics is inspiring. I have truly grown as a scientist; by enhancing my experimental designing and data interpretation skills and improving my writing skills because of his guidance.

Chris Manning, M.S. – Chris served as my second thesis director on this project. He specializes in flow cytometry. I am also very thankful for his time and support on this project. He encouraged and directed me beyond my comfort level towards interpreting data and conveying results at a more advance level.

Lori Teheen – Lori’s expertise in flow cytometry has helped me advance my skill set and career development. I am very thankful for her mentorship and friendship on a regular basis.

Angela Merluzzo and Mark Logan – These two colleagues have recently completed their Master’s at Harvard Extension. They helped me specifically with formatting and timeline questions. I confided in them during the entire thesis process, as they were supportive during my venting sessions.
Ryan Sinapius and Alicia Creesy – Ryan specifically shared all of his western blotting equipment with me. Additionally both Ryan and Alicia were very supportive to my emotional state during my thesis process, as they too soon will be performing their thesis with Harvard Extension, they helped keep me keep stable.

Flow Cytometry Group – Its make the workday so much tolerable when interacting with group members who are motivated, supportive and fun to be around. I would like to thank my flow group for their encouragement and continuous laugh out loud moments every day.

Cell Signaling Technology, Inc. – This is my employer where I performed all of my experiments. I am thankful as they funded the reagents for all of my experiments and have also reimbursed me for some of the tuition over the past five and a half years.

CST Colleagues – Numerous other colleagues were very willing to share supplies, reagents and expertise and I am very thankful.

My Family – I thank my family for being so understanding, this past year, as I have been more distant than ever allocating all my time into this thesis.
# Table of Contents

Dedication ........................................................................................................................................... v

Acknowledgements ............................................................................................................................ vii

List of Tables ....................................................................................................................................... xi

List of Figures ..................................................................................................................................... xii

I. Introduction ........................................................................................................................................ 1
   - Defining Epigenetics ...................................................................................................................... 1
   - Histone Methylation ....................................................................................................................... 4
   - EZH2 Histone Methyltransferase Complex .................................................................................. 5
   - EZH2 in Cancer ............................................................................................................................. 6
   - EZH2 and Drug Therapies ............................................................................................................. 8
   - Epigenetic Assays/Tools .............................................................................................................. 10
   - The Study of Epigenetics by Flow Cytometry ........................................................................... 11
   - Specific Aims ................................................................................................................................. 13

II. Material and Methods ...................................................................................................................... 15
   - Cell Culture ................................................................................................................................. 15
   - Cell Preparation ........................................................................................................................... 16
   - Cell Treatments ............................................................................................................................ 17
   - Western Blotting ........................................................................................................................... 18
   - Flow Cytometry ............................................................................................................................ 18
Antibodies.................................................................................................................. 19

III. Results........................................................................................................................................ 22

Specific Aim 1 - Compare western blotting versus flow cytometry methods and
determine optimal drug treatment conditions for assaying changes on histone
modifications.................................................................................................................. 22

Optimal GSK343 Incubation Time.................................................................................. 22

Optimal GSK343 Drug Dosage ...................................................................................... 25

Comparing Western Blotting to Flow Cytometry......................................................... 29

Adox Treatment Conditions......................................................................................... 31

Specific Aim 2 - Develop a flow protocol for use with a large panel of antibodies
against multiple key lysine methylation and acetylation sites on Histone H3 to
enable high-throughput flow cytometry........................................................................ 34

One Optimized Flow Cytometry Protocol ................................................................. 34

Epigenetics Monoclonal Antibody Histone Mark Panel Validation......................... 36

Specific Aim 3 - Demonstrate the functionality of the developed medium-
throughput flow cytometry assay by determining the specificity of the GSK343
EZH2-specific inhibitor and BIX-01294 G9a-specific inhibitor................................. 37

GSK343 Drug Screen .................................................................................................... 37

BIX-01294 Drug Screen .................................................................................................. 40

IV. Discussion................................................................................................................ 42

Bibliography .................................................................................................................... 49
List of Tables

Table 1. Cell lines with high expression for Wild-type EZH2 ........................................ 16

Table 2. Antibodies against histone mark used to compare western blotting versus flow cytometry .................................................................................................................. 20

Table 3. Flow epigenetics monoclonal antibody histone mark panel................................. 21

Table 4. Epigenetics monoclonal antibody histone mark panel validated by flow cytometry. ................................................................................................................................. 37

Table 5. GSK343 drug screen on panel of antibodies against Histone H3 marks............. 39

Table 6. BIX-01294 drug screen on panel of antibodies against Histone H3 marks....... 40
List of Figures

Figure 1. Nucleosome core particle ................................................................. 2
Figure 2. Mechanisms of Histone Modification and DNA ..................................... 3
Figure 3. Nucleosome Structure and Principal Modification Sites .......................... 5
Figure 4. The Composition of the Polycomb Repressive Complex 2 (PRC2) and Domain Organization of EZH2 ................................................................................. 6
Figure 5. Inhibitor Pathway of GSK343 .............................................................. 9
Figure 6. Flow cytometric analysis of histone modification levels determined that the most optimal time point for 5µM of GSK343 was 96 hours ......................... 24
Figure 7. Western blotting and flow cytometric analysis showing GSK343 has its greatest impact on histone modification sites at 5µM for 96 hours ................. 28
Figure 8. Adox cannot be used as a control because of significant cell toxicity ........ 32
Figure 9. Histogram overlays showing apoptosis of Jurkat cells untreated or treated with Adox measured by Cleaved Caspase-3 (Asp175) staining .......................... 34
Figure 10. Permeabilization by 0.3% Triton X-100 results in a slightly higher increase of antibody staining .......................................................... 36
Figure 11. GSK343 Histogram data generated from the developed medium-throughput flow cytometry assay ................................................................. 39
Figure 12. BIX-10294 Histogram data generated from the developed medium-throughput flow cytometry assay ................................................................. 41
Defining Epigenetics

Epigenetics is defined as heritable changes that occur on DNA and its surrounding chromatin that do not result from changes in DNA sequence. Epigenetic mechanisms include DNA methylation, replacement of canonical histones with histone variants, ATP-dependent nucleosome remodeling, RNA-based mechanisms, and post-translational histone modifications (Morera et al., 2016). The study of epigenetics involves the understanding of when and why a change in the modification of histones such as methylation and acetylation occurs. Observing changes in a genome-wide setting allows for an in-depth look into cancer development and progression, tumorigenesis and genetic disorders. Unlike DNA mutations, epigenetic modifications are reversible and their mechanisms that contribute to cancer are currently being discovered. Some efforts include understanding mechanisms involved in the silencing of tumor suppressor genes and activation of oncogenes (DeAngelis et al., 2008).

In the nucleus, DNA is highly compacted together with histone proteins forming the chromatin structure. The complex chromatin structure allows site-specific access and exposure to cellular machinery that regulates chromosome segregation, DNA transcription, replication, recombination and repair (Richmond and Davey, 2003). Different epigenetic mechanisms such as histone post-translational modifications, DNA
modifications, ATP-dependent nucleosome remodeling and non-coding RNA affect the accessibility to DNA (Watson et al., 2014).

The nucleosome is the basic subunit of chromatin, comprised of the nucleosome core, linker DNA and histone H1, all of which regulates DNA accessibility. The nucleosome core includes 146 base pairs of DNA wrapped around an octamer of two copies of each histone protein H2A, H2B, H3 and H4 (Figure 1). The nucleosome core can assemble into higher-order structures, which are stabilized by the linker histone H1 (Luger et al., 1997). Each individual nucleosome’s characteristics can also be influenced by the incorporated DNA sequence, in addition to surrounding sequences, which is significant because DNA sequences are specific for certain gene promoters. DNA sequences contribute to the variation in nucleosomes as they help to recruit transcription factors and co-factors (i.e. histone modification writer and eraser proteins) that modify the histone proteins, which allows for a wide range of post-translational modifications to occur.

Figure 1. Nucleosome core particle. 73-bp half of DNA phosphodiester backbones (brown and turquoise) and eight histone protein main chains (blue: H3; green: H4; yellow: H2A; red: H2B). Each super helix axis location label (1-7) represents one further DNA double helix turn from SHL0 (Luger et al., 1997).
Acetylation and methylation are the most frequent post-translational modifications on histones, localized abundantly at the histone N-terminal tails, in the core of the histones, and in the C-terminal tail regions. Enzymatic ‘writers’ are responsible for the addition of methyl or acetyl histone marks, the proteins that recognize and bind to these specific epigenetic marks are called ‘readers’, and since the epigenetic modifications are not permanent, ‘erasers’ remove the histone marks (Figure 2). Acetylated histones enable transcription of nearby genes, as they are less compact and more accessible to RNA polymerase and transcriptional machinery. Methylated histones however either repress or activate nearby genes depending on the site and amount of methylation (Arrowsmith et al., 2012).

Figure 2. Modification of histones and DNA are key mechanisms involved in epigenetic regulation of gene expression. DNA is wrapped around histone proteins and packed into chromatin to form a nucleosome. Nucleosomes are compacted into chromatin fibers by histone H1 and additional proteins which mediate histone post-translational modifications; writers, erasers and readers (Arrowsmith et al., 2012).
Histone Methylation

Lysines, arginines, serines, threonines, and tyrosines are all post-translationally modified in the N-terminal tail of the core histone proteins H2A, H2B, H3 and, H4. Lysine methyltransferases and arginine methyltransferases can transfer up to three methyl groups to each amino acid residue, resulting in mono-, di- or trimethylated lysine or mono- or di- methylated arginine (Shi et al., 2006). Methyl marks are written by histone methyltransferases, which require the coenzyme S-adenosylmethionine (SAM), and erased by histone demethylases of the amine oxidase lysine-specific demethylase (LSD) (Shi et al., 2004) and Jumonji C (JmJC) (Tsukada et al., 2006) families. Methylation of lysines 4, 36 and 79 of histone H3 (H3K4, H3K36 and H3K79) are associated with euchromatin and gene activation, while methylation of lysines 9 and 27 of histone H3 (H3K9 and H3K27) and lysine 20 of histone H4 (H4K20) are associated with heterochromatin and gene silencing (Morera et al., 2016).

The various methyl marks serve as specific signals for methyl-lysine binding domains that recognize the level of methylation and the surrounding amino acid sequence. For example, trimethylated lysine 4 of histone 3 (H3K4Me3), H3K9Me3 and H3K27Me3 each interact with a distinct set of reader, writer and eraser proteins (Figure 3). Proper expression and localization of these epigenetic marks results in proper regulation of gene expression and associated cellular processes. Altered levels or localization of these marks would result in deregulation of gene expression and would have the potential to drive diseases such as cancer (Arrowsmith et al., 2012).
Figure 3. Nucleosome structure and principal modification sites with reported writers, erasers and readers for the modifications depicted. Some histone lysines can be substrates of methylation as well as acetylation (Morera et al., 2016).

**EZH2 Histone Methyltransferase Complex**

As mentioned, lysine methyltransferases can transfer up to three methyl groups to each lysine residue (Shi et al., 2006). Polycomb repressive complex 2 (PRC2) is a gene silencer and major histone methyltransferase enzyme, which methylates H3K27 (Simon and Lange, 2008). The tri-methylated form of H3K27 is known to be associated with transcriptional repression. EZH2 and to a lesser extent, its homolog EZH1, members of the SET1 family of histone methyltransferases, are the catalytic subunits of PRC2. Shown in Figure 4, EZH2 requires the presence of the embryonic ectoderm development (EED) and the suppressor of zeste 12 (SUZ12) protein, as EZH2 can only methylate lysine residues when in complex with EED and SUZ12 (Cao and Zhang, 2004). The PHD
finger protein 1 (PHF1) is not a core subunit however it specifically stimulates the ability of EZH2 to catalyze H3K27 trimethylation (Sarma et al., 2008). Additionally the PRC2 complex binds to other subunits RbAP46/48 (histone chaperones), AEBP2 (regulates localization), Pcl proteins (interact with PCR2 through EZH2 and have crucial role in PRC2 functioning) and Jarid2 (regulates localization and increases activity on nucleosomal substrates) (Margueron and Reinberg, 2010).

Figure 4. The composition of the PRC2 and domain organization of EZH2. The four core subunits of PRC2 include EZH2, EED, SUZ12 and RbAp48. The five functional EZH2 domains are shown; the SET domain includes the histone methyltransferase active site and the CXC domain contributes to activity. Methyltransferase requires EZH2 assembly with both the EED and SUZ12, shown are the required domains for the subunits to bind (Simon and Lange, 2008).

**EZH2 in Cancer**

Vire et al. showed that EZH2 is required for DNA methylation of EZH2-target promoters, suggesting that EZH2 and/or H3K27 methylation recruits DNA methyltransferases (Vire et al., 2006). This study suggests that there is a link between the
polycomb group proteins and the DNA methylation machinery. EZH2 has been strongly implicated as an oncoprotein, as research has shown EZH2’s involvement in a broad spectrum of cancers including Large B-cell lymphoma (DLBCL), prostate, and breast cancer. Cancer-associated overexpression of EZH2 (Varambally et al., 2002, Simon et al., 2008, Kleer et al., 2002) and PRC2 components (Cao et al., 2008 and Sarma et al., 2008), in addition to loss of UTX H3K27 demethylase activity (van Haaften et al., 2009) have all been studied by researchers.

Wild-type EZH2 proficiently catalyzes the mono-methylation of H3K27, with less processive di- and tri-methylation. Several somatic mutations in EZH2, resulting from a single amino-acid substitution at tyrosine 641 of EZH2 (Y641F, Y641N, Y641S, and Y641H) have been identified in DLBCL (Sneeringer et al., 2010 and Yap et al., 2011). These mutant EZH2 enzymes are highly proficient in catalyzing di- and tri-methylation of H3K27 suggesting a different transition state recognition compared to wild-type, thus expression of these mutant enzymes results in a higher catalytic efficiency and an increase in tri-methylated histone H3 lysine 27 (H3K27Me3). These DLBCLs are typically heterozygous, expressing both WT and mutant EZH2 alleles. The mutant EZH2 alleles lead to an increased H3K27Me3 in these cells, which alters gene expression and drives cancer.

Studies have shown that an increased level of EZH2 expression is directly correlated with the likelihood of developing cancers including prostate and breast (Varambally et al., 2002 and Kleer et al., 2003). In addition, somatic mutations in EZH2 have been identified in follicular lymphoma and the germinal-center B-cell like subtype of DLBCL (Morin et al. 2010 and McCabe et al. 2012). The dysregulation of H3K27
methylation and EZH2 is associated with tumor growth and survival. When EZH2 is abnormally unregulated by the over expression of EZH2 mutants the trimethylation activity increases.

EZH2 and Drug Therapies

The discovery of EZH2 as an oncoprotein and potential driver of cancer has made it an attractive target for drug discovery and therapy (Arrowsmith et al., 2012). For the case of the gain-of-function mutant EZH2 proteins, Sneeringer et al. suggest that inhibitors which target the transition state of the mutant EZH2 over the wild-type could be effective in blocking H3K27 methylation in cells containing the mutant protein (Sneeringer et al., 2010). However if an EZH2 inhibitor were to affect other key methylation or acetylation sites, i.e. H3K4Me3, H3K9Me3, H3K36Me2, H3K36Me3, H3K79Me3, H3K9Ac and H3K27Ac which are included in the antibody panel screened against EZH2 inhibitor GSK343 in aim 3 of this proposed study, then this would suggest that the inhibitor is not specific and could negatively impact gene expression and cell growth. Several inhibitors to EZH2 are in clinical trials but their off-target effects have not been well defined (Morera et al., 2016).

EZH2 inhibitors offer promising therapeutic approaches to cancer because they reduce the methylation levels of lysine 27. GlaxoSmithKline developed GSK343, a highly potent EZH2 inhibitor that competes with SAM binding, and is highly selective for EZH2 over EZH1 and other histone methyltransferases (Verma et al., 2012). A high-throughput screening of GSK compounds against EZH2 was performed. HCC1806 breast cancer cells were incubated with the EZH2 inhibitor for 72 hours, which led to a dose-dependent reduction in H3K27Me3. Liu et al. compared inhibitors 3-deazaneplanocin A
(DZNep) (known to deplete EZH2 protein expression through an indirect pathway) and GSK343 (Figure 5), and found that both inhibitors exerted differential effects on cancer cells. Most importantly they showed for the first time that SAM-competitive EZH2 inhibitors induce autophagy in cancer cells however whether this is a general effect of SAM competitors remains unanswered (Liu et al., 2015). According to Liu et al., advanced hepatocellular carcinoma has no reliable therapy, thus their data suggests GSK343 as a possible combinational therapy. It is important to note however, that the specificity for the inhibition of H3K27 has not been completely characterized. Therefore the off-site drug effects of the GSK343 EZH2-specific inhibitor are unclear. For instance if GSK343 reduced the methylation levels of other key lysine residues besides H3K27, then this could potentially have long term inheritable effects on gene expression leading to increased susceptibility to diseased states. It is important to determine if there are off-site drug effects before the GSK343 drug moves further into therapeutic studies and clinical trials where it severely could impact a patient.

Figure 5. Inhibitor pathway GSK343: Chemical structures of GSK343 (Lie et al., 2015).
Epigenetic Assays/Tools

Chromatin Immunoprecipitation (ChIP) is mainly used to identify and quantify the relative amount of a given histone modification or binding protein localized in a specific location in the genome, and does not provide a good assay for analyzing total histone modification levels in the cell (Obier et al., 2010). While, western blotting provides a good technique for measuring global changes in histone modifications, this method is not very quantitative, is low throughput, and also lacks the ability to detect changes at the single cell level. Additionally antibodies can be cross-reactive yet only show one clean specific band by western blotting, as the non-specific binding could be the same molecular size as the binding of the primary antibody (Watson et al., 2014).

Immunofluorescence (IF), is a great visualization technique for a specific target but can have restrictions in the number and types of cells (adherent vs. suspension) that can be analyzed as well as limitations in the ability to quantify fluorescent levels (Obier et al., 2010).

Flow cytometry would provide a faster, higher-throughput method that can analyze a large population of cells at a single cell level. With a flow rate of 10,000 cells/sec a flow cytometer can acquire 500,000-1,000,000 cell events (flow standard) in under a minute. The multiparametic measurements and the speed at which they are acquired generate a reliable quantitative statistical analysis of the cell populations. This ability to analyze a large population of cells at the single-cell level is a huge advantage compared to western blotting and IF. While western blotting measures modification of proteins that have been purified from a population of cells, thus only providing a readout in the change of histone modification of the population as a whole, flow cytometry can be
more dynamic by enabling the analysis of distinct cell types within a population at a single cell basis therefore providing the ability to look at heterogeneity in modification changes across the population of cells. In addition multi-color flow enables researchers to measure more variables per experiment, which allows for further scalability (Watson et al., 2014).

The Study of Epigenetics by Flow Cytometry

Flow cytometry allows for the characterization of epigenetic patterns in heterogeneous samples and can readout the function of enzymes that are regulating the epigenome. Therefore staining cells with antibodies against relevant readers, writers, erasers or relevant histone modifications that are epigenetically regulated will give valuable information. DNA methylation and histone acetylation were the first epigenetic target areas focused on for drug development (Arrowsmith, et al., 2012). Now histone methylation is a key area of focus for cancer, metabolic, and neurodegenerative diseases, among others.

Previous studies have demonstrated flow cytometry techniques to detect histone marks and study epigenetics. Zhou et al. depended on flow cytometry for a plant epigenetic study (the histone code is highly conserved across eukaryote species) (Zhou et al., 2015). Flow cytometry coupled with protoplast preparation detected \textit{in vivo} chromatin compaction from plant leaf cells. Obier and Muller implemented several Flow protocols that quantitatively displayed the levels of different histone modification on the single cell level (Obier and Muller, 2009). The authors took advantage of eleven available ChIP validated antibodies and adapted these antibodies to Flow protocols. They demonstrated how flow cytometry allows for the rapid and simultaneous analysis of the global levels of
chromatin marks; however, the flow protocols still need optimization to allow for simultaneous analysis of a large number of histone modifications by a cohort of antibodies. This includes varying the fixation and staining. Additionally a larger selection of antibodies against readers, writers, erasers and epigenetic modification sites need to be readily available to enable flow epigenetics as an application tool for clinical trials, cancer research and therapeutics.

Watson et al. also had a goal to implement flow epigenetics as a clinical tool. Given that histones are essential for chromatin stability and play a major role in transcriptional regulation, the authors selected antibodies against three clinically relevant histone marks, H3K27Me3, H3K9Ac and H3K9Me2 and studied their expression levels following treatments in the cell. With the testing of different flow protocols, the authors found as they increased the amount of formaldehyde concentration the staining intensities of their antibodies were reduced. This finding facilitated the authors to optimize a flow protocol, which was then used on donor blood from leukemia patients. Comparing flow cytometry to western blotting results showed comparable binding characteristics of the antibodies to the specific histone marks however western blotting was believed to be a more sensitive assay. This paper demonstrated the steps taken to develop an epigenetics flow assay and how the flow application can be a useful tool as an epigenetics assay.

However with only a small data set of three antibodies the authors were only able to find one modification site, H3K27Me3, as a potential therapeutic marker. The significance of this paper is that epigenetics by flow is possible and useful but optimized protocols and larger selections of antibodies need to be available.
Specific Aims

My goal is to develop a novel Flow Assay for analyzing changes in epigenetic modifications at the cellular level that can provide a valuable high-throughput method, quantitative and analytic tool for epigenetic studies. Aim 1 of this study compares flow cytometry and western blot methods for assaying histone modification. Antibodies against total Histone H3, H3K27Me2, H3K27Me3 and H3K4Me3 were used in the comparison of the two assays. Additionally a sub-aim for studying epigenetic modifications was to determine the optimal drug treatment conditions of GSK343 and Adox for assaying histone modifications in the potential novel flow assay.

Aim 2 of this study asks, can a larger panel of antibodies against multiple key lysine methylation and acetylation sites on Histone H3 be optimized with one single protocol to enable a high-throughput flow cytometry assay? Different protocols were explored by altering the staining and fixation conditions. The goal was to also implement one standard protocol in the assay and arrange the histone marks in a 96-well plate to enable the high-throughput application.

Flow cytometric analysis enables the study of drug effects on histone modification marks. Aim 3 of this study addresses the question; Can the functionality of a developed high-throughput flow cytometric assay be demonstrated by determining potential off-target effects of the GSK343 and BIX-01294 inhibitors? This novel flow assay will determine the specificity of the EZH2-specific GSK343 and G9a-specific BIX-01294 inhibitors. It is important to know of any off-target effects as these could have long-term inheritable effects on gene expression. It is useful to know this information before more time, money and resources are allocated towards drug discovery. The
developed flow cytometry assay tool has potential value for therapeutics if it can characterize epigenetics mechanisms and any off-site drug effects as it could track these mechanisms in cancer patients.
Chapter II
Material and Methods

The purpose of this study was to question whether a novel high-throughput flow cytometry assay could be developed to analyze epigenetic changes associated with diseased states and drug treatments on a large number of samples. This chapter describes the materials, techniques, and protocols used throughout the study, which enabled the development of a medium-throughput flow cytometry assay. Briefly, mammalian cells were cultured and different treatment dosages and incubation times were explored. Cells were then prepared for either western blotting or flow cytometry, and probed with antibodies from a selected histone mark panel. Analysis was performed and will be described in the following chapters.

Cell Culture

Cell lines with high expression of EZH2 (Table 1), as determined by mRNA levels with BioGPS (Wu at al., 2013 and 2016), were purchased from the American Type Culture Collection (ATCC®). Jurkat cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum. K562 cells were cultured in Iscove’s Modified Dulbecco’s medium containing 10% fetal bovine serum. Both cell lines were passaged with the addition of fresh medium every 2-3 days. Cell lines were treated with various histone methyltransferase inhibitors at varying concentrations and for varying times, as indicated.
Table 1. Cell lines with high expression for Wild-type EZH2 determined by BioGPS (Wu et al., 2013 and 2016) and acquired from American Type Culture Collection (ATCC®).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Tissue</th>
<th>Cell Type</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat</td>
<td>Peripheral Blood</td>
<td>Lymphocyte</td>
<td>Acute T Cell Leukemia</td>
</tr>
<tr>
<td>K562</td>
<td>Bone Marrow</td>
<td>Lymphocyte</td>
<td>Chromic Myelogenous Leukemia</td>
</tr>
</tbody>
</table>

**Cell Preparation**

Cells were cultured to ~80% confluency, then collected by centrifugation. The supernatant was aspirated and the cell pellet was resuspended in 1mL of 1x PBS. Cell counts were determined by the Countess™ II Automated Cell Counter (Cat# AMQAX1000, ThermoFisher Scientific). 25% of the cells were aliquoted to an eppendorf tube then pelleted by centrifugation. The supernatant was aspirated, and then the cell pellet was lysed with 1x SDS sample buffer (Red Loading Pack Cat# 7723, Cell Signaling Technology). The lysed cells were kept on ice then sonicated for 10-15 seconds to complete the lysis and DNA breakdown, boiled at 95°C for 5 minutes, then stored at -20°C. The remaining 75% of cells were stained with either Ghost Dye™ Violet 450 or 510 Viability Dye (Cat# 49826 or Cat# 59863, Cell Signaling Technology) then fixed with formaldehyde at a final concentration of 4%. These cells were fixed for 10 minutes at 37°C and then washed with 1x PBS before being pelleted by centrifugation. Two different permeabilization protocols were tested. For the first protocol, the cell pellet was slowly resuspended in 1x PBS, and then ice-cold 100% methanol was slowly added with gentle vortexing to a final concentration of 90% then placed at -20°C for storage. For the second protocol, the cell pellet was slowly resuspended in 1mL of 0.3% Triton X-100,
incubated at room temperature for 10 minutes, and then brought up in 0.1% Sodium Azide and placed at 4°C for storage.

**Cell Treatments**

Inhibition of H3K27 methylation was explored by using EZH2 and EZH1 histone-lysine- N-methyltransferase competitive inhibitor GSK343 (Cat# 66244, Cell Signaling Technology). GSK343 was received as a lyophilized powder and 1mg was reconstituted in 369.21µl of DMSO to generate a 5mM stock. Jurkat cells were first treated with 5µM of GSK343 for 48 hours, 72 hours and 96 hours and harvested for flow cytometric analysis. A 5-point, 3-fold titration of GSK343 from 0.5µM to 45µM at 96 hours in Jurkat cells was then prepared for flow cytometry and western blotting analysis. The inhibition of all Histone H3 methylation sites was explored by using the Adox general methyltransferase inhibitor (Cat# A7154-25MG, Sigma-Aldrich). 25mg of the lyophilized Adox was reconstituted in 943µl of DMSO to generate a 100mM stock. A 4-point, 4-fold titration of Adox from 1.25µM to 80µM at 48 hours in Jurkat cells was prepared for flow cytometry and western blotting analysis. Adox was also used at 20µM in Jurkat cells for 48 hours, 72 hours and 96 hours. Inhibition of H3K9 sites was explored by using the G9a-specific inhibitor, BIX-01294 (Cat# 13124-10mg, Cayman Chemical Company). 10mg of the lyophilized BIX-01294 was reconstituted in 166.66µl of DMSO to generate a 100mM stock. Jurkat cells were treated with BIX-01294 at 2µM for 24 hours, 48 hours and 72 hours in Jurkat cells by flow cytometry.
Western Blotting

3μl of lysate was loaded onto 4-20% SDS-PAGE gels along with 7μl of a biotinylated protein ladder (Cat# 7727, Cell Signaling Technology) to determine the molecular weights. Gels were run for 20 minutes at 120 V. Next the proteins were transferred from the gel to the Midi PVDF Membranes (Cat# 1704157, Bio-Rad) using a Trans-Blot® Turbo™ Transfer System (Cat# 1704150, Bio-Rad). The membranes were then incubated in blocking buffer (1x Tris Buffered Saline with Tween® 20 (TBST) (Cat# 9997, Cell Signaling Technology) with 5% nonfat dry milk (Cat# CU10109-00100, American Bioanalytical)) for 1 hour. Three, 5 minute washes in 1x TBST were performed then the membranes were incubated in 1x TBST overnight at 4°C with primary antibodies (dilution: 1:1,000 for H3K27Me3, H3K4Me3 and H3K9Me3, 1:3,000 for H3K27Me2 and 1:10,000 for Histone H3). The next day, three, 5 minute washes in 1x TBST were performed then the membranes were incubated for 1 hour at room temperature in blocking buffer with Anti-Rabbit and Anti-Biotin secondary antibodies (Cat# 7074 and 7075, Cell Signaling Technology) at 1:2,000. Three, 5 minute washes in 1x TBST were performed then the membranes were incubated with 1:1 solution of LumiGLO® and Peroxide (Cat# 7003, Cell Signaling Technology) for 1 minute. The membranes were imaged on the ChemiDoc™ XRS+ System (Cat# 1708265, Bio-Rad) and labeled with Image Lab™ Software (Version 5.2.1 build 11, Bio-Rad).

Flow Cytometry

Cells were washed by centrifugation in 1x PBS to remove the methanol or Sodium Azide. 200,000 cells were suspended in 50μl of Incubation Buffer (0.5g BSA in 100mL of 1x PBS) and incubated with 50μl of diluted antibody for 1 hour at room
temperature, covered and protected from light. These incubated samples were then washed by centrifugation in incubation buffer. When using a primary antibody, samples were resuspended in 100µl of Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 647 Conjugate) antibody (Cat# 4414, Cell Signaling Technology) used at 1:1,000 and incubated for 30 minutes at room temperature, covered and protected from light. These incubated samples were then washed by centrifugation in incubation buffer. Samples were then resuspended in 500µl and analyzed on the BD FACSCelesta™ flow cytometer.

Antibodies

Antibodies against five histone marks were chosen for the first aim (Table 2). H3K27Me2 and H3K27Me3 should detect histone methylation changes specific to the EZH2-specific GSK343 inhibitor. H3K4Me3 and H3K9Me3 should detect additional more general methylation changes induced by Adox. Total Histone H3 served as a positive control for both assays.
Table 2. Antibodies against histone mark used to compare western blotting versus flow cytometry.

<table>
<thead>
<tr>
<th>Histone Mark</th>
<th>Function</th>
<th>CST Cat. #</th>
<th>Western Dilution</th>
<th>Flow Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone H3</td>
<td>Primary building block of chromatin. Acetylated at Lys9/14/18/23/27/36 and 56. Methylated at Lys4/9/27/36 and 79. Phosphorylated at Ser10/28, Thr3 and 11.</td>
<td>4499 L9 (10 ug/ml)</td>
<td>1:10,000</td>
<td>1:50</td>
</tr>
<tr>
<td>H3K4Me3</td>
<td>Associated with euchromatin and gene activation</td>
<td>9751 L10 (190 ug/ml)</td>
<td>1:1,000</td>
<td>1:200</td>
</tr>
<tr>
<td>H3K9Me3</td>
<td>Associated with heterochromatin and gene silencing</td>
<td>13969 L3 (165 ug/ml)</td>
<td>1:1,000</td>
<td>1:50</td>
</tr>
<tr>
<td>H3K27Me2</td>
<td>Associated with heterochromatin and gene silencing</td>
<td>9728 L15 (452 ug/ml)</td>
<td>1:2,000</td>
<td>1:400</td>
</tr>
<tr>
<td>H3K27Me3</td>
<td></td>
<td>9733 L8 (67 ug/ml)</td>
<td>1:1,000</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Note: There is not a flow cytometry antibody against H3K27Me1 commercially available.

For Aims 2 and 3, thirteen antibodies against Histone H3 methylation and acetylation sites were chosen for the two assays (Table 3). Primary monoclonal antibodies were used for western blotting and Alexa Fluor® 647 conjugated monoclonal antibodies of the same clone were used for flow cytometry. Please note that five Alexa Fluor® 647 conjugated monoclonal antibodies were not available therefore primary antibodies were used instead. Twelve of the thirteen chosen antibodies are all products of Cell Signaling Technology. Antibody H3K79Me3 is a product of Epigenetek.
**Table 3. Flow epigenetics monoclonal antibody histone mark panel.**

<table>
<thead>
<tr>
<th>Histone Mark</th>
<th>Function</th>
<th>CST Cat. #</th>
<th>Flow Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone H3</td>
<td>Primary building block of chromatin. Acetylated at Lys9/14/18/23/27/36 and 56. Methylated at Lys4/9/27/36 and 79. Phosphorylated at Ser10/28, Thr3 and 11.</td>
<td>12230 L8 (100 ug/ml)</td>
<td>1:50</td>
</tr>
<tr>
<td>H3K4Me3</td>
<td>Assoicated with euchromatin and gene activation</td>
<td>12064 L5 (50 ug/ml)</td>
<td>1:50</td>
</tr>
<tr>
<td>H3K9Me1</td>
<td>Associated with heterochromatin and gene silencing</td>
<td>14186 L1 (230 ug/ml)</td>
<td>1:3200</td>
</tr>
<tr>
<td>H3K9Me2</td>
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<td>66070 L2 (200 ug/ml)</td>
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<tr>
<td>H3K9Me3</td>
<td></td>
<td>13969 L3 (165 ug/ml)</td>
<td>1:50</td>
</tr>
<tr>
<td>H3K27Me2</td>
<td>Associated with heterochromatin and gene silencing</td>
<td>12244 L3 (100 ug/ml)</td>
<td>1:50</td>
</tr>
<tr>
<td>H3K27Me3</td>
<td></td>
<td>12158 L9 (100 ug/ml)</td>
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</tr>
<tr>
<td>H3K36Me2</td>
<td>Assoicated with euchromatin and gene activation</td>
<td>15090 L2 (50 ug/ml)</td>
<td>1:50</td>
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<tr>
<td>H3K36Me3</td>
<td></td>
<td>4909 L2 (142 ug/ml)</td>
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<td>H3K79Me3</td>
<td>Assoicated with euchromatin and gene activation</td>
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<td>1:400</td>
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<tr>
<td>H3K9Ac</td>
<td>Associated with transcriptional activation</td>
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<tr>
<td>H3K27Ac</td>
<td>Associated with transcriptional activation</td>
<td>39030 L1 (200 ug/ml)</td>
<td>1:50</td>
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<tr>
<td>H3K36Ac</td>
<td>Associated with transcriptional activation</td>
<td>27683 L1 (230 ug/ml)</td>
<td>1:50</td>
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</table>

Note: There is not a flow cytometry antibody against H3K27Me1 commercially available.
Chapter III

Results

This chapter shows the results of the experiments performed in the development of a high-throughput flow cytometry assay. First, data from Aim 1 suggest that flow cytometry was easier to use and more capable of high-throughput and quantification than western blotting. Additionally, the optimal drug treatment conditions for GSK343 and Adox were determined. Next, results from Aims 2 and 3 demonstrate how the proposed flow epigenetics monoclonal antibody histone mark panel was validated and applied as a medium-throughput flow cytometry assay to determine if an EZH2-specific GSK343 inhibitor and G9a-specific BIX-01294 inhibitor had off-target effects in the methylation and acetylation of histones.

Specific Aim 1 - Compare western blotting versus flow cytometry methods and determine optimal drug treatment conditions for assaying changes on histone modifications

Optimal GSK343 Incubation Time

Throughout this study, Jurkat cells were the chosen model system. Cell passage number, availability, ease of use, doubling time and growth medium were all contributing factors for this decision. To determine the most optimal treatment conditions for the EZH2-specific GSK343 inhibitor, a time-course study was first performed. For the time-

22
course study I chose to use GSK343 at the 5µM concentration based on previous studies (Agarwal et al., 2016, Tripathy et al., 2015 and Xiong et al., 2016). Because I was looking at relatively stable histone modifications, which are known to have a long half-life, I chose longer treatment conditions. Therefore I treated Jurkat cells with 5µM of GSK343 then harvested the cells for flow cytometric analysis at 48 hours, 72 hours and 96 hours. Antibodies against total Histone H3 (control), H3K27Me2, H3K27Me3 (two Histone H3 sites regulated by EZH2), H3K4Me3 and H3K9Me3 (Histone H3 sites that are not be regulated by EZH2, controls) were chosen to not only validate the treatments but also to compare the western blotting and flow cytometry assays. Shown in Figure 6, the GSK343 5µM drug treatment reduced the levels for H3K27Me2 and H3K27Me3 while the levels for total Histone H3 and H3K4Me3 did not change. These results suggested that the most optimal time for the 5µM drug dose was 96 hours. Figure 6B illustrates with histogram overlays, the significant inhibition of GSK343 on H3K27Me2 and H3K27Me3. Jurkat cells treated with 5µM of GSK343 for 96 hours had a 2-fold reduction in levels of H3K27Me2 and a 4-fold reduction in levels for H3K27Me3 when compared to untreated Jurkat cells.
Figure 6. Flow cytometric analysis of histone modification levels determined that the most optimal time point for 5µM of GSK343 was 96 hours. (A) Histone modification levels in Jurkat cells untreated (light gray) or GSK343 treated (5µM) (dark gray) measured by mean fluorescence intensity (MFI) at three time points (hours). (B) Histogram overlays showing the MFI of histone modifications versus the number of Jurkat cells in untreated (solid line, white) or GSK343 treated (5µM) (dotted line, gray) conditions at the most optimal time point, 96 hours.
Optimal GSK343 Drug Dosage

To determine the most optimal dosage of GSK343, I performed a 5-point, 3-fold drug titration at the optimal time point of 96 hours. The five antibodies from Table 2 were used to validate the treatment conditions by western blotting and flow cytometry. Shown in Figure 7, both methods agreed that the most optimal concentration for GSK343 was 5µM. Western blotting data in Figure 7A, showed that H3K27Me2 levels began to decrease at 1.5µM and reached a maximum reduction at 5µM. At 0.5µM, H3K27Me3 levels were very weak and appeared to be completely reduced for all higher dosages 1.5µM thru 45µM. Interestingly a significant reduction in H3K4Me3 levels and some reduction in H3K9Me3 levels began at 5µM. Additionally, I observed reduced levels at 45µM for all four methylation sites. However when comparing these methylation sites to total Histone H3 and beta-actin, two loading controls, 45µM appeared to have less total protein. Therefore all four methylation sites were likely to have a weaker band at 45µM because of the less protein content.

A scatter plot graph of flow cytometric analysis displayed in Figure 7C, showed that GSK343 only inhibits H3K27Me2 and H3K27Me3. As I increased the drug dosage, both sites reached optimal inhibition at 5µM. H3K4Me3, H3K9Me3 and total Histone H3 levels appeared to be consistent without any change therefore not exhibiting any sign of EZH2-specific GSK343 inhibition. These sites served as controls since GSK343 should only effect H3K27 specific methylation sites.
D.

H3K27Me2

H3K27Me3

H3K4Me3

H3K9Me3

E.

0.5 μM GSK343

GSK343 5 μM

GSK343 Untreated

H3K27Me2

FSC-A

SSC-A

Gated Cells

Gated Cells

Ghost Dye™ Violet 510

Ghost Dye™ Violet 510

Gated Cells

Gated Cells

F.

1.5 μM GSK343

15 μM GSK343

5 μM GSK343

45 μM GSK343

H3K27Me2

H3K27Me2

H3K27Me2

H3K27Me2

G.

45μM GSK343

H3K27Me2

Violet 510 Ghost Dye

Violet 510 Ghost Dye

Live Cells

Live Cells

Violet 510 Ghost Dye

Violet 510 Ghost Dye
Figure 7. (A and B) Western blotting and (C-J) flow cytometric analysis showing GSK343 has its greatest impact on histone modification sites at 5µM for 96 hours. (A) Western blots showing histone modification protein levels compared to total Histone H3 and beta-actin protein levels (loading controls). (B) Graphs showing relative protein quantity levels obtained from western blotting after being quantified and normalized to total Histone H3 using Image Lab™ Software (Version 5.2.1 build 11, Bio-Rad). (C) Scatter plot graph comparing MFI values (Y-axis) of histone modifications versus GSK343 (µM) drug concentrations (X-axis). The MFI for each was then normalized to total Histone H3 and graphed (D) separately to quantitatively compare with the graphed western blotting relative protein quantity levels (B). Histogram overlays showing the MFI values of H3K27Me2 (E and F) and H3K27Me3 (H and I) versus the number of Jurkat cells in untreated (solid line, white) or GSK343 (µM) treated (dotted line, gray) conditions at 96 hours. (G and J) Side-Scatter (SSC) versus Forward Scatter (FSC) plots with a gate drawn on the viable Jurkat cells, which was then applied to the SSC versus the Ghost Dye™ Violet 510 plot. A gate was then drawn on the viable cells and applied to the histogram measuring the MFI values of H3K27Me2 and H3K27Me3 (X-axis) versus the number of untreated or treated Jurkat cells collected.
Comparing Western Blotting to Flow Cytometry

Attempts to observe protein expression by eye using western blotting is informative but can be subjective and allows for human error. Therefore, I tried to quantify the western blot results using Image Lab™ Software (Version 5.2.1 build 11, Bio-Rad). Band intensities were normalized to the total Histone H3 control and then the relative protein quantity for that tested modification site was graphed against the amount of GSK343 concentrations (Figure 7B). Western blot data showed a significant reduction in H3K4Me3 levels (similar to H3K27Me2) and some reduction in H3K9Me3 levels, suggesting that GSK343 was not EZH2-specific. Notably I observed the H3K4Me3 signal to be quite low in my baseline, untreated cells, which may have impacted the quantification. Similar to western blotting, for flow cytometric analysis I normalized the histone modification levels to total Histone H3 then graphed these against the amount of GSK343 concentrations (Figure 7D). Unlike western blotting, I had not observed a reduction in H3K4Me3 or H3K9Me3 levels, suggesting that GSK3 may be EZH2-specific. In comparison, Jurkat cells treated with 5µM GSK343 for 96 hours had a ~10-fold reduction in H3K27Me2 levels and ~20-fold reduction in H3K27Me3 levels compared to untreated Jurkat cells. These results suggested that my flow cytometry data was very sensitive and potentially more quantitative than my western blot data.

With flow cytometry I could specifically gate and see which portion of cells were dead and which portion survived after being induced with the GSK343 drug treatment, as opposed to western blotting where I collected every cell population including debris into my lysate, measured the total protein content, consequently not having it known how much protein was a result of cells that outlasted or died upon treatment. Examples of healthy viable cells are shown in Figure 7E and 7H. Jurkat cells were collected and
displayed according to size and complexity on a dot plot of side-scatter (SSC) versus forward scatter (FSC). Dead cells typically appear on the lower left side of these plots because they are small and less complex. Therefore a gate was drawn around the suspected viable Jurkat cells of interest which was then applied to a second dot plot with SSC versus Ghost Dye™ Violet 510. The purpose of using this viability dye was to confirm my gating strategy. The SSC versus FSC plots in Figure 7G and 7J showed a larger amount of dead cells in the lower left corner. A gate was drawn around the suspected viable Jurkat cells of interest. Contrasting from Figure 7E and 7H, two populations appeared on the SSC versus Ghost Dye™ Violet 510 plot. The second population was considered dead because of the higher MFI values. Therefore I could apply another viable gate around the first population (lower MFI values), which did not incorporate the Ghost Dye™ Violet 510. This data suggests that the viability dye was necessary to confirm that I was analyzing cells that survived upon treatment, as I could not fully distinguish between live and dead cells with only a SSC versus FSC plot.

These plots allowed me to discriminate between live and dead cells at the time of the treatment. A gate could then be drawn on the viable cells and applied to histograms measuring the MFI values of H3K27Me2 and H3K27Me3 (Figure 7E, 7F, 7H and 7I). These histograms indicated that as the GSK343 dosage increased from 0.5µM to 15µM, a significant separation in MFI values between the untreated and treated Jurkat cells could be observed, which revealed that GSK343 only reduced methylation levels for H3K27Me2 and H3K27Me3. Notably as the GSK343 dosage reached 45µM, I did not observe a reduction in methylation levels. This high of a dosage induced cell death
(Figure 7G and 7I) as shown in the scatter plots, therefore the only levels I could measure were from cells that were not yet inhibited by the treatment.

Adox Treatment Conditions

Adenosine-2’,3’-dialdehyde (Adox) was supposed to serve as a positive control for optimizing the western blotting and flow cytometry protocols for the entire antibody panel, as it is a general methylation inhibitor that inhibits methylation of most lysine and arginine residues. H3K27Me2, H3K27Me3, H3K4Me3, H3K9Me3 levels should all be reduced with Adox treatment, since it is a general inhibitor of SAM production in the cell, while total Histone H3 should show normal levels and facilitate the normalization of histone methylation levels.

First, I performed a 4-point, 4-fold drug titration from 1.25µM to 80µM in Jurkat cells at 48 hours. For both western blotting and flow cytometry no significant change in methylation levels was observed (Figure 8A and 8B). Histogram overlays were gathered to illustrate this observation, for example, H3K27Me3 (Figure 8C) and H3K4Me3 (Figure 8D) in the untreated and treated conditions of Jurkat cells at 48 hours exhibited no significant change in MFI values for every concentration.
Figure 8. Adox cannot be used as a control because of significant cell toxicity. Western blotting (A) and flow cytometric analysis (B, C, and D) showing histone modification levels in Jurkat cells untreated and treated with Adox at the labeled concentrations for 48 hours. (A) Western blot images showing histone modification levels compared to total Histone H3 and beta-actin protein levels (loading controls). (B) Scatter plot graph comparing MFI values (Y-axis) of histone modifications versus Adox drug concentration (µM, X-axis). Histogram overlays showing the MFI of H3K27Me3 (C) and H3K4Me3 (D) versus the number of untreated (solid line, white) or Adox treated (dotted line, gray) Jurkat cells collected.
Second, I performed a 48 hour, 72 hour and 96 hour time course at 20µM. Unfortunately these results also exhibited no change in methylation levels, again suggesting that Adox was not inhibiting methylation of histone proteins. However, given that Adox is a general inhibitor of SAM production, it is possible that Adox treatment was highly toxic to the Jurkat cells. During cell harvest, before fixing or lysing cells, I stained the cells with trypan blue and observed ~50% cell death in the Adox-treated Jurkat cells. To determine the level of Adox-induced toxicity in Jurkat cells in my experiments, I performed flow cytometric analysis of untreated and Adox-treated Jurkat cells using an antibody against Cleaved Caspase-3, a marker of apoptotic cell death, and Ghost Dye™ Violet 450, a marker of live/dead cells. This analysis showed that although treatment of cells with 20µM Adox did not reduce H3K27Me2, H3K27Me3, H3K4Me3 levels, it did induce high levels of Cleaved Caspase-3 (Asp175) (Figure 9B). Using the Ghost Dye™ Violet 450, which stained live/dead cells, I was able to omit the dead cells induced by the Adox treatment (Figure 9A) then apply the viable cell gate to histograms, which then showed that Cleaved Caspase-3 (Asp175) had similar low levels at all three time-points in both the Adox treated Jurkat cells (dotted line, gray) and untreated Jurkat cells (solid line, white). However increased levels of Cleaved Caspase-3 (Asp175) only appeared in the treated Jurkat cells as indicated by arrows. This data suggested that at the time of cell harvest, Adox was inducing apoptosis. Perhaps as the cells responded to the Adox treatment they were dying as histone methylation was decreased, thus being removed from analysis. Overall this data suggested that Adox was toxic to the cells.
Specific Aim 2-Develop a flow protocol for use with a large panel of antibodies against multiple key lysine methylation and acetylation sites on Histone H3 to enable high-throughput flow cytometry

One Optimized Flow Cytometry Protocol

My results suggested that flow cytometry was a good method for measuring changes in histone modifications associated with drug treatment. To develop a high-throughput flow cytometry assay, I needed one optimized flow cytometry protocol. When
studying epigenetic marks it is important to keep in mind that chromatin is highly compact which can make antigen accessibility more difficult. Therefore I explored both methanol and Triton X-100 permeabilization, as these are two different methods to provide access for antigens after fixation with formaldehyde cross-linking. Methanol is an organic solvent that can dissolve lipids from the cell membrane, while Triton X-100 is a detergent, which is slightly less invasive leaving holes in the membrane. Antibodies against H3K27Me2, H3K27Me3, H3K4Me3 and Histone H3 (Figure 10A) were used to stain untreated Jurkat cells versus concentration matched isotype controls, fixed with 4% formaldehyde and either permeabilized with 90% MeOH or 0.3% Triton X-100. Results suggested that permeabilization by 0.3% Triton X-100 enabled a higher binding of antibody as a brighter signal was observed with all four antibodies, implying that this was the best protocol. Additionally histogram overlays (Figure 10B) suggested that the coefficient of variation (cv) was tighter with 0.3% Triton X-100, which produced an increase in sensitivity and brighter signal.
Figure 10. Permeabilization by 0.3% Triton X-100 results in a slightly higher increase of antibody staining. (A) Graph showing signal to noise ratios (Antibody MFI versus Isotype MFI) of histone modification levels in Jurkat cells fixed with 4% formaldehyde then permeabilized with 90% MeOH (dark gray) or Triton X-100 (light gray). (B) Histogram overlays showing antibody staining for H3K27Me3 and Histone H3 (solid line, white) versus a concentration matched-isotype control (dotted line, gray) using two different permeabilization methods; 90% MeOH (top row) or 0.3% Triton X-100 (bottom row).

Epigenetics Monoclonal Antibody Histone Mark Panel Validation

A panel of antibodies against multiple key lysine methylation and acetylation sites on Histone H3 was compiled (Table 3). The purpose of this panel was to enable flow cytometric analysis to analyze epigenetic changes associated with diseased states and drug treatments on a large number of samples. Displayed in Table 4 are the signal to noise ratios (antibody MFI/concentration matched Isotype control MFI) for antibodies stained in untreated Jurkat cells using 4% formaldehyde fixation with 0.3% Triton X-100 permeabilization. Every antibody gave at least a 2-fold shift, which was the passing criteria, supporting my antibody panel validation. Because my panel only consisted of 13
antibodies, I changed my aim of developing a high-throughput flow assay to a medium-throughput flow assay.

Table 4. Epigenetics monoclonal antibody histone mark panel validated by Flow Cytometry.

<table>
<thead>
<tr>
<th>CST Ab#</th>
<th>Dilution</th>
<th>Histone Mark</th>
<th>S / N</th>
</tr>
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<tbody>
<tr>
<td>12230</td>
<td>1:50</td>
<td>Histone H3</td>
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<tr>
<td>12064</td>
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Specific Aim 3 - Demonstrate the functionality of the developed medium-throughput flow cytometry assay by determining the specificity of the GSK343 EZH2-specific inhibitor and BIX-01294 G9a-specific inhibitor

GSK343 Drug Screen

A 5-point, 3-fold titration of GSK343 concentration was screened using the developed medium-throughput flow cytometry assay (Table 5). Screening of this GSK343 drug titration using the validated antibody panel demonstrated the specificity of
this EZH2-specific inhibitor as only H3K27Me2 and H3K27Me3 levels were reduced. Additionally these results agreed with previous data that the optimal treatment dosage for GSK343 was 5µM. As the dosage of GSK343 was increased, the fold inhibition (histone modification level in untreated/treated cells) increased for H3K27Me2 and H3K27Me3. Both these sites exhibited the greatest inhibition with GSK343 at 5µM; ~14-fold reduction in levels of H2K27Me2 and ~21-fold reduction in levels of H3K27Me3. I did not observe significant reduction of any other histone modifications in the panel, which suggested that there are not any off-site drug effects for GSK343. Histogram overlays in Figure 11 illustrated GSK343 inhibition. H3K27Me2 and H3K27Me3 levels in untreated (solid line, white) Jurkat cells were significantly higher than in GSK343 (5µM, 96 hours) treated (dotted line, gray) Jurkat cells, suggesting that I was observing GSK343 specifically inhibiting the H3K27Me2 and H3K27Me3 levels. Interestingly, I also observed an increase in H3K27Ac levels in GSK343-treated Jurkat cells, which can be expected, as acetylation and methylation of H3K27 are opposing modifications.
Table 5. GSK343 drug screen on panel of antibodies against Histone H3 marks.

<table>
<thead>
<tr>
<th>Histone Mark</th>
<th>CST Aby#</th>
<th>Dilution</th>
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</tr>
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<td>1.05</td>
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Figure 11. Histogram data generated from the developed medium-throughput flow cytometry assay. Histogram overlays showing the MFI of histone modifications in Jurkat cells untreated (solid line, white) or treated (dotted line, gray) with the most optimal GSK343 treatment condition 5µM at 96 hours.
BIX-01294 Drug Screen

BIX-01294 is a specific inhibitor of euchromatic histone-lysine N-methyltransferase 2 (EHMT2, also known as G9a), which does not compete with the cofactor SAM. G9a is the key enzyme responsible for H3K9Me1 and H3K9Me2. The purpose of including the BIX-01294 inhibitor in this study was to have an additional example of an epigenetics-specific drug, screened with the medium-throughput flow assay. BIX-01294 treatment should inhibit methylation of H3K9; however, I observed unexpected, off-target effects when using this drug (Table 6). H3K27Me2, H3K36Me3 and H3K36Ac levels all showed significant reduction in levels, while all the other sites including H3K9Me1, H3K9Me2 and H3K9Me3 did not show significant changes. Histograms overlays (Figure 12) illustrated the observed reduced levels of H3K27Me2, H3K36Me3 and H3K36Ac compared to total Histone H3, H3K9Me1 and H3K9Me2 sites. These results suggest that the BIX-01294 may have some off-target effects.

Table 6. BIX-01294 drug screen on panel of antibodies against Histone H3 marks.

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<th>Dilution</th>
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<th>Jurkat BIX-48hr 2uM</th>
<th>Jurkat BIX-72hr 2uM</th>
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<td>H3K9Ac</td>
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Figure 12. Histogram data generated from the developed medium-throughput flow cytometry assay. Histogram overlays showing the MFI of histone modifications in Jurkat cells untreated (solid line, white) or treated (dotted line, gray) with the most optimal BIX-01294 treatment condition 2µM at 72 hours.
Chapter IV
Discussion

The overall goal of this project was to determine if flow cytometry would give comparable results to western blotting for the analysis of epigenetics modifications, and if so, to develop a high-throughput flow cytometry assay for analyzing a large number of histone modifications which could then be leveraged to determine potential off-target effects of an EZH2-specific methyltransferase inhibitor. Additionally, demonstrating the functionality of this high-throughput flow cytometry assay could demonstrate the value of having this tool to look at on-target and off-target effects for histone modification-specific drug discovery/validation. Historically, western blotting has been used (Agarwal et al., 2016, Tripathy et al., 2015 and Xiong et al., 2016) as a low-throughout method to show target specificity of epigenetics drugs with a small number of antibodies. Previous studies have used flow cytometry (Obier and Muller, 2009, Watson et al., 2014 and Zhou et al., 2015); however, currently there are no publications exhibiting flow cytometric assays with a large number of antibodies against histone modification sites to study off-target effects of epigenetic inhibitors.

I compared flow cytometry and western blotting methods by observing changes at major histone lysine methylation and acetylation sites upon drug treatment in Jurkat cells. I found flow cytometry to be more quantitative as every cell that passed through the cytometer was detected as a single event then plotted individually versus its signal of light intensity. I was able to more accurately measure the difference in signals between
untreated and treated cells populations. Analysis could then extract numeric values as mean fluorescent intensities. These numeric values were effortlessly graphed in Figures 7C and 7D, for example, which gave me the ability to measure levels of modifications at the single-cell level.

As I increased the GSK343 drug dosage from 15µM to 45µM it was difficult to determine if changes in intensity of the lighter bands in the western blots, as measured with Image Lab™ Software (Figure 7A and 7B) were due to actual GSK343-mediated inhibition or due to signal intensities outside the optimal range for western blotting. However, flow cytometry allowed for measurement of histone modification levels in single cells, in addition to providing the ability to identify and select live cell populations using cell viability dyes and gating strategies, providing the ability to measure changes in histone modification occurring in live cells. In addition, flow cytometry provided for a more quantitative measurement of changes in histone modification resulting from drug treatment, as shown in Figure 7. Flow cytometry analysis demonstrated that GSK343 specifically inhibited H3K27Me2 and Me3, while having only minimal effects on H3K4Me3 and H3K9Me3. In addition, staining with Ghost Dye™ Violet 510 viability dye allowed for identification of live vs. dead cells and confirmed that only a small population of cells was viable after treatment with 45µM GSK343, suggesting that this concentration of drug was toxic to cells. Cell toxicity leading to cell death and low overall protein concentration may have led to the observed decrease in H3K4Me3 and H3K9Me3 levels by western blot.

Watson et al., had developed a flow cytometry protocol for epigenetic studies by exploring different concentrations of formaldehyde fixation (0.5%, 1%, 2% or 4%) with
0.1% Triton X-100 permeabilization, and determined 4% formaldehyde at room
temperature for 10 minutes and permeabilization with 0.1% Triton X-100 for 15 minutes
at 37°C to be the best flow cytometry protocol for blood samples. With this protocol they
found three histone marks to be successful and one, H3K9Me2, to be problematic. Loss
of staining intensity with increasing formaldehyde concentrations forced the authors to
remove H3K9Me2 from their studies. To study multiple histone modifications with a
high-throughput flow assay I would need to improve and expand upon these authors’
fixation conditions and flow protocols.

To address these concerns I developed one optimized fixation and staining
method using 4% formaldehyde fixation at 37°C and permeabilization with 0.3% Triton
X-100 at room temperature for 10 minutes (Figure 10). All four antibodies against
H3K27Me2, H3K27Me3, H3K4Me3 and Histone H3 were observed to have an increase
in sensitivity and brighter signal as they produced a tighter cv with 0.3% Triton X-100
and broader cv with 90% MeOH permeabilization. Additionally I applied this optimal
method to a larger panel of antibodies, against key methyl and acetyl lysine residues in
Histone H3 (Table 4). I found that in untreated Jurkat cells versus concentration-matched
isotype controls all of these antibodies produced at least a 2-fold induction in signal.

Interestingly the H3K9Me2, H3K36Me2, H3K36Me3 and H3K36Ac antibodies
showed a very low signal versus concentration matched isotype controls, as compared to
the nine other marks in the panel (Table 4). It is possible that these antibodies bind more
weakly to their modifications or that these modified lysine residues are less accessible to
antibodies in their local chromatin environment, resulting in lower sensitivity to changes
in modification levels induced by drug treatment. This would have a negative implication
for my assay, as it would hinder the ability to accurately measure changes in these modifications. Possible solutions would include further investigation of other formaldehyde fixation and permeabilization techniques and possible addition of mild denaturing conditions, and/or the use of other antibodies against these histone modifications. These modifications to the protocol may increase the sensitivity of my flow assay for these specific modification sites.

My results demonstrated that not only can I use flow cytometry as an assay to study Histone H3 modification sites, but also specifically demonstrate the advantages of using flow cytometry, as it allows for simple and accurate detection, quantification, and normalization of histone modification levels at the single-cell level, in addition to measuring cell viability upon treatment of cells with the various drugs. Because flow cytometry has these advantages, I chose this method to look at numerous histone modification sites at once in a medium-throughout assay to determine potential on- and off-target effects of EZH2-specific and G9a-specific methyltransferase inhibitors.

I selected 13 Histone H3 modification antibodies for the panel and first screened the EZH2-specific GSK343 inhibitor. My flow cytometry data suggests that this inhibitor is specific to EZH2 and does not have any off-target effects in the modifications of histones, as I only observed inhibition of H3K27Me2 and H3K27Me3 across a range of drug concentrations (Table 5). Additionally I observed increased levels of H327Ac, which is expected as H3K27Me and H3K27Ac are opposing regulatory marks.

Adox, an indirect inhibitor, inactivates S-adenosylhomocysteine hydrolase producing an accumulation of adenosine homocysteine levels resulting in the feedback inhibition of most methylation reactions (Chen et al., 2004 and Fujji et al., 2016). This
global SAM-dependent methyltransferase inhibitor was supposed to serve as a positive control for development of my flow protocol and validation of my histone modification antibody panel. However I could not determine the optimal treatment conditions for Adox. At time-points higher than 24h and dosages higher than 20µM I observed a significant amount of cell death. Cell death was confirmed by trypan blue at the time of cell harvest and additionally by the Ghost Dye™ viability dye during flow cytometric analysis (Figure 9A).

Upon further investigation of the literature, I found other studies demonstrating that global methylation plays a role in cell survival and when inhibited by Adox, cell death occurs (Ghemrawi et al. 2013 and Dasgupta et la. 2008). Low concentrations of Adox were found to cause normal apoptosis while higher concentrations happened to cause a caspase-independent form of cell death (Schwerk and Schulze-Osthoff, 2005). I observed significant levels of cleaved caspase-3 (Asp175) in Adox treated Jurkat cells (Figure 9 B), indicating induction of apoptosis. Since Adox should inhibit methylation of most of the lysine and arginine residues, it is possible that upon treatment methyltransferase inhibition is induced but it is so potent that simultaneously apoptosis is induced and ultimately cell death. During flow cytometric analysis I gated and omitted the dead cells using the Ghost Dye™; however, my results suggest that cells entering apoptosis did not absorb this dye. Therefore apoptotic cells remained in my viable gate. Consequently I could not use the Adox inhibitor as a control for the histone methyltransferase in the antibody panel because of significant cell toxicity.

Because Adox induced cell toxicity I chose the G9a-specific BIX-01294 inhibitor as a second drug to be used with the medium-throughput flow assay. BIX-01294 has been
shown to reduce the levels of H3K9Me2 (Kubicek et al., 2007). Additional studies have shown BIX-01294 to reduce levels of H3K9Me1 and H3K9Me2 but not H3K9Me3 (Huang et al., 2017). However, my experiments show that BIX-01294 decreases H3K27Me2/3, but not H3K9Me2/3. Interestingly, in addition to methylating H3K9, G9a is suggested to also methylate H3K27 and potentially coordinate the cross-talk between H3K9, H3K27 and H3K36 methylation (Wu et al., 2011). This study agrees with my observation that BIX-01294 decreases levels of H3K27Me2, H3K36Me3 and H3K36Ac (Figure 12). Perhaps these modifications are specifically inhibited by the G9a inhibitor and are not off-target effects.

Even though signal to noise ratios, determined during antibody validation, suggested low antibody binding levels for H3K9Me2, H3K36Me2, H3K36Me3 and H3K36Ac, levels in both H3K36Me2 and H3K36Ac were reduced with BIX-01294 treatment. So why did I not detect changes in H3K9Me2 upon treatment of cells with BIX-01294? It is possible that the H3K9Me2 antibody was less sensitive or that the H3K9Me2 modification was less accessible to antibody binding, as previously described. Further investigation of BIX-01294 drug effects needs to be examined.

To fully leverage my flow cytometry assay, expanding to a larger selection of antibodies for the panel will make it more applicable for epigenetics studies. I developed the assay to screen GSK343, an inhibitor specific to EZH2, a writer for methyl lysines on H3K27, and selected antibodies against H3K27Me2, H3K27Me3 and Histone H3. From there I selected other Histone H3 methylation and acetylation sites for my panel. Future experiments could develop a larger antibody panel which could include sites on other histones: H2A, H2B, and H4. Many inhibitors of deacetylases and methyltransferases are
in drug development and in clinical trials. Providing a tool to address the specificity of these inhibitory drugs and any off-site drug effects would offer an advantage to researchers.

In future experiments, screening additional drugs with my panel would demonstrate the scope of knowledge that this analytic tool can bring. For instance further EZH2 inhibitors in clinical trials could be screened. If one happened to show weak or moderate inhibition of H3K4Me3, H3K9Me3, H3K36Me3 or H3K79Me3 then this would suggest that the EZH2 inhibitor is not specific and the inhibition/change of these off target epigenetics marks could negatively affect gene expression and cell growth.

Having this knowledge in the early stages of clinical trials is powerful. Additionally many other relevant inhibitors with potential use for cancer therapies could be screened. Relevant disease-associated targets for inhibitors include the methyltransferases DOT1L (H3K79Me), G9a (H3K9Me), and SET8 (H4K20Me), and demethylases LSD1 (H3K4Me and H3K9Me).


