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THE STABILITY OF FLUORESCENCE-CONJUGATED ANTIBODIES UNDER TEMPERATURE AND TIME RELATED STRESSORS

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ABSTRACT

Fluorescence-activated Cell Sorting utilizes the natural mechanics of the antibody-target interactions to quantify and classify a sample of cells by way of their expressed proteins. Monoclonal antibodies, generated to target a singular cellular marker, are conjugated to a fluorescent molecule known as a fluorochrome. Because the fluorochrome is bound to the antibody, it will only emit light if the antibody is bound to the protein marker. Thus, it is imperative that the antibody binds with high affinity and the covalent bond between the fluorochrome and antibody remains stable.

We hypothesized the max fluorescence intensity as determined by flow cytometry would be decreased in fluorescence-conjugated antibodies as a result of temperature and time. We tested nine fluorescence-conjugated antibodies targeting cluster of differentiation 8 (CD8) against temperature stress by way of freeze-thaw cycles. A phenotyping master mix was also put through the freeze-thaw cycles to examine effects of temperature on a mixture of antibodies. A subset of antibodies was tested for effects of time, comparing results of four years of storage versus a few months. Experimental data revealed time had a pronounced impact on CD25-PE and CD8-APC. In terms of temperature stress, both individual antibody aliquots as well as the master mix experienced a decrease in overall intensity, but not to the severity predicted. PacBlue, PE-Cy7, PE-TxR, FITC, A700, and PerCP maintained viability over the duration of the freeze-thaw cycles. However, PacBlue, PE-Cy7, and PE-TxR demonstrated the least degree of destabilization with applied stress. As such, freezing aliquoted antibodies at -80˚ C may provide a useful method of prolonging shelf life and preventing the destabilization resulting from extended storage.

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Chapter 1

Introduction

1.1 The Mammalian Immune System

Whether in the air we breathe, the water we drink, or the surfaces we touch, the environment is riddled with pathogens. In order to combat the constant onslaught of these pathogens, the mammalian immune system has evolved many different mechanisms to prevent and fight off infection. The immune system takes on a multi-faceted approach, involving physical barriers, generalized molecular attacks, and specialized attacks that generate immunological memory. All such elements can be divided into two distinct branches – innate and adaptive immunity.

1.2 Innate Immunity

The innate immune system is the first line of defense for the host organism. It primarily operates under three main criteria: to identify pathogens, induce inflammation, and kill or contain the pathogen. But, all of these functions are only necessary if the pathogen is able to breach the barrier function of immunity. The skin is the outermost organ of mammalian species, serving as the first antimicrobial barrier. Unless the skin is compromised in some manner, only those species that are permeable to the skin layers can directly bypass this defense (Lee et. al, 2006). However, certain regions of the body lack the complete protection of the skin, despite

their exposure to the environment. The eyes, nose, mouth, throat, lungs, gastrointestinal tract, and vagina are all such regions of exposure. Yet, these regions are not without protection. All have a single-cell layer of epithelium, lined with mucus, making up the mucosal division of the innate immune system (McGhee and Fujihashi, 2012). The mucosal epithelium not only provides a physical barrier to trap pathogens, but also produces cytotoxic anti-microbial peptides known as defensins (Yang *et. al*, 2002).

If a pathogen breaches the epidermal and mucosal barriers, the innate immune system must identify and mark the pathogen for destruction in a non-specific manner. This process is known as opsonization, in which pathogens are covalently labeled with complement proteins to facilitate uptake and destruction by phagocytes. Most of these complement proteins are proteases, which become activated by the localized presence of a pathogen. One of the most common complement proteins, C3, is cleaved into C3a and C3b in the presence of a pathogen. The exposed thioester bond on C3b enables covalent binding to the surface of the pathogen (Wiesmann *et. al*, 2006). There are three main pathways of the complement system, including the classical, alternative, and lectin pathway. All result in amplified C3b opsonization. The C3b-pathogen complex binds to the CR1 receptor present on macrophages, initiating phagocytosis and subsequent digestion of the pathogen in the phagolysosome. Some types of complement lead to the formation of C5-9 membrane attack complexes, which create pores in the pathogen cell membrane to allow lysosomal enzymes to enter more directly (Rus *et. al*, 2005).

Unfortunately, many microbial species have the ability to overcome these non-cellular based attacks. In order for innate immune cells to act directly, they must be able to identify the pathogen through the use of pathogen recognition receptors (PRRs). PRRs recognize pathogen associated molecular patterns (PAMPs), allowing the host to distinguish the pathogen antigen

from self-antigen (Medzhitov and Janeway, Jr. 2002). These PRRs are constitutively expressed on most cells of the innate immune system, including macrophages, dendritic cells, and epithelial cells (Akira *et. al*, 2006). Most of the innate immune system is facilitated by macrophages and dendritic cells, which possess a specific class of PRRs known as Toll-like receptors (TLRs). TLRs are transmembrane complexes that, upon binding to a pathogen, initiate a signaling cascade. The protein kinase pathway results in the release of NF_KB – a transcription factor for the production of pro-inflammatory cytokines (Montero Vega and de Andrés Martín, 2008). These cytokines induce inflammation, increase vascular permeability, and serve to recruit effector cells, such as more macrophages, neutrophils, and natural killer cells. The response to the infection is magnified to reduce or eliminate the pathogen before the adaptive immune system is necessary (Akira *et al*, 2006).

1.3 Adaptive Immunity

While innate immunity provides a barrier against infection and rapid, short-term protection, the adaptive immune system enables the body to mount specific responses to specific pathogens and stimulate formation of immunological memory. This branch of the immune system is comprised of specialized effector cells known as B-lymphocytes (B cells) and T-lymphocytes (T cells). B cells participate in the antibody-mediated response, otherwise known as the humoral response; T cells dictate cytotoxic cell-mediated immunity. Both cell types carry out their respective functions in a pathogen-specific manner based on specific antigen binding to their receptors (Murphy *et. al*, 2008). The B cell receptor (BCR), also known as an immunoglobulin, recognizes a precise chemical moiety of the particular antigen. This is known

as the epitope and is different for each BCR (Sandel and Monroe, 1999). The T cell receptor (TCR) only recognizes protein antigens that are processed into peptide fragments and expressed on major histocompatibility class I (MHC I), present on all somatic cells, or MHC II, on the surface antigen-presenting cells (Turley *et. al,* 2000).

With the exception of red blood cells, all cells in the body possess MHC class I; but only antigen presenting cells, such as dendritic cells, macrophages, and B cells have MHC class II. The dendritic cells in the periphery engulf the invading pathogen, degrade the proteins into peptides, and present these pathogen-specific peptides on their surface in MHC class I and II. These cells migrate to the nearest secondary lymphoid organs where they stimulate naïve T cell differentiation into effector T cells (Fujii *et. al*, 2004).

1.4 B Cell Development

B cells arise by hematopoiesis in the bone marrow, governed by growth factors and cytokines produced by stromal cells (Sandel and Monroe, 1999). In order for these cells to reach maturity, they must be able to get past two key developmental checkpoints – the BCR heavy and light chain rearrangements (Gonzalez *et. al,* 2011). The checkpoints ensure the BCR is not reactive to self-antigen, but does result in a productive rearrangement of the variable (V), diversity (D), and joining (J) segments of the heavy and light chain genes. Collectively, the rearrangement of the VDJ regions is known as somatic recombination. This process enables exponential variations of the BCRs in order to ensure an immune response against the vast array of environmental pathogens (Murphy *et. al,* 2008). A single individual could have around 3.5 x 10^6 different antibody specificities from combinatorial diversity, but when combined with

junctional diversity and somatic hypermutation, the full antibody repertoire exceeds 10^{11} (Janeway *et al*, 2001). This incredible diversity enables the body to potentially respond to billions of environmental pathogens in a target specific manner.

Somatic recombination begins with the upregulation of recombinase genes, *RAG-1* and *RAG-2*, which catalyze the heavy chain rearrangement (Sandel and Monroe, 1999). The recombinase enzymes introduce a break in the DNA at specific recombination signal sequences (RSS), each of which have an associated intervening sequence of 12 or 23 base pairs. Recombination follows the 12/23 rule, only occurring between these 12-RSS and 23-RSS sequences. The first recombination involves bringing two of the RSS sequences together, forming a signal joint that is excised from the genome. The result is a coding joint that brings together a random D and J region. The D-J recombination occurs on both chromosomes of the early pro-B cell (Dudley *et. al*, 2005). The same process occurs to bring the V region to the D-J region, though this occurs on only one chromosome. If the recombination is nonproductive, VDJ recombination will occur on the second chromosome. If neither chromosome yields a productive rearrangement, the pro-B cell will undergo apoptosis (Murphy *et. al*, 2008).

If one of the chromosomes undergoes a productive VDJ rearrangement, the heavy chain will bind to a surrogate light chain, resulting in the pre-B receptor. The binding of a surrogate light chain sends a signal to the developing B cell that a successful rearrangement has occurred (Murphy *et. al*, 2008). The signal discontinues heavy chain recombination through the degradation of *RAG 1* and *RAG 2*, allowing the B cell past the initial checkpoint (Zhang *et. al*, 2004). This process enables only one functional heavy chain to be produced, known as allelic exclusion. Allelic exclusion ensures that each B cell expresses only a single type of BCR, which will later ensure uniform production of high avidity antibodies (Vettermann and Schlissel, 2010)

Following heavy chain formation, the developing B cell will divide multiple times – each cell expressing the pre-B receptor. Light chain recombination must also yield a productive rearrangement or the B cell will undergo apoptosis. Thus, the divisions maximize the likelihood that a productive BCR arrangement will occur without having to start from the beginning of the somatic recombination process. There are two classes of light chains, classified by the rearrangement of the κ and λ loci. Light chains lack the diversity region of the heavy chains, but V-J rearrangement occurs in an analogous manner. The κ locus is rearranged first, but only on one chromosome. The enzymatic rearrangement will proceed until all of the J segments have been tested on the first chromosome, but if nonproductive, the κ locus is rearranged on the second. The process will continue to the λ locus on the first and second chromosome until a functional BCR is formed (Murphy *et. al*, 2008).

During the original heavy chain rearrangement, the μ constant region is expressed, dictating the immunoglobulin class. The newly formed immature B cell now expresses IgM on its surface. The progressive rearrangements of the light chain enable the B cells to undergo negative selection (Murphy *et. al*, 2008). This selection can take the approach of deletion, anergy, or receptor editing, but the end goal is the same – to prevent autoreactive B cells from leaving the bone marrow (Sandel and Monroe, 1999). The B cell is presented with self-antigen from bone marrow stromal cells. If the BCR binds strongly to multivalent self-antigen, the crosslinking of the BCRs sends a strong signal that arrests the development (Murphy *et. al*, 2008). If early on in the light chain rearrangements, then receptor editing will occur, as there are many opportunities for rearrangement left. If it remains autoreactive after exhausting somatic recombination, the cell will be deleted. In rare cases, autoreactive cells may be rendered inactive, but not dead. These cells enter the periphery in a state of anergy (Sandel and Monroe,

1999). But, if the B cell is no longer reactive to self-antigen after receptor editing, it may leave the bone marrow and enter the periphery to continue maturing (Murphy *et. al*, 2008).

The immature B cells migrate to peripheral lymphoid organs in response to chemokine signaling. Peripheral immature B cells can be distinguished from bone marrow B cells in that they express IgD in conjunction with IgM on their surface (Sandel and Monroe, 1999). In the spleen and lymph nodes, the immature B cells may encounter self-antigen that was not expressed within the bone marrow. If autoreactivity occurs, clonal deletion will occur once more. Survival of this process classifies the B cell as naïve. Naïve B cells migrate through the circulatory and lymphatic systems, periodically stopping in primary lymphoid follicles to receive survival signals from follicular dendritic cells. This extends their life in order to increase the likelihood that they will encounter their specific antigen (Murphy *et. al*, 2008).

Antigen presentation and subsequent activation also occur in secondary lymphoid organs. As was previously mentioned, dendritic cells and macrophages express MHC II, which is used to present the antigen for T cell activation. A CD4+ T Cell (T_h) expressing a TCR specific to that antigen is activated by the TCR-MHC binding. But, B cells also have the ability to phagocytize and express antigens through MHC II. An activated T_h2 cell binds to the MHC II of a B cell expressing the specified antigen. Once again, the TCR-MHC binding results in activation of the B cell. The activated B cell will undergo proliferation, some of which will immediately differentiate into short-term plasma cells (Arpin *et. al*, 1995). Plasma cells no longer divide nor can they be further activated by antigen stimulation. Instead, they enter the periphery and secrete antibodies which will target the antigen and either enhance opsonization or neutralize the secreted toxins. Those that did not differentiate into plasma cells will join a primary follicle in the lymphoid organ, becoming part of a secondary follicle (Murphy *et. al*, 2008). The secondary

follicle contains a germinal center, consisting of growing B cells known as centroblasts that will divide into centrocytes after undergoing the germinal center reaction. Class switching and somatic hypermutation will alter the affinity for the antigen binding; those with the most optimal binding will be selected for further proliferation, known as affinity maturation. Some will migrate out into the periphery and become plasma cells, thus magnifying the B cell response (Arpin *et. al*, 1995). Others will become memory cells. Memory B cells possess high affinity BCRs that do not require T cell activation; they remain inactive in the body for years, but upon exposure to the antigen, can rapidly differentiate into plasma cells. Thus, the secondary response is faster and stronger than the primary response (Murphy *et. al*, 2008).

1.5 Antibodies

Antibodies are the primary component of the humoral response, released by plasma cells into the blood and lymphatic fluid (Murphy *et. al*, 2008). Antibodies are merely secreted forms of the initial B cell receptor. Thus, they retain the structure of the four polypeptide chains – two identical heavy chains and two identical light chains. As displayed in Figure 1-1, the two sets of chains are held together via a disulfide bond known as the hinge region. The hinge region provides valuable flexibility to accommodate multivalent binding interactions (Janeway *et. al*, 2001). This generates a 'Y-shaped' molecule with two antigen-binding regions at the tips of the Y and one constant region at the base. Both the light and heavy chains have repeated sequences known as the immunoglobulin domains. These domains interact to provide the structural stability of the molecule; the constant regions of the heavy chains interact, the constant region of the light chains interact with the heavy chains, and the variable regions interact all through these

Ig domains (Murphy *et. al*, 2008). The overall framework of the protein is comprised of multiple β sheets, but in the variable region, the loops, known as hypervariable regions, represent the site of antigenic variation. Subtle modifications in the hypervariable loops enable selection and proliferation of the antibody that binds a given antigen with the highest affinity (Janeway *et. al*, 2001).

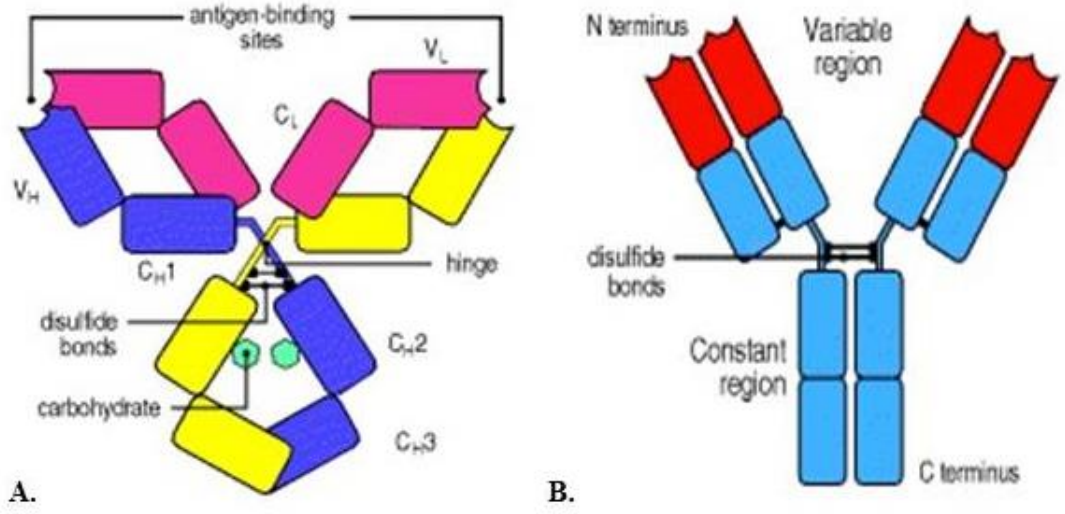

Figure 1-1. Structure of an Antibody Molecule (Janeway et. al, 2001). A. The antibody molecule is made up of two identical light chains (pink) and two identical heavy chains (yellow and dark blue). **B.** The disulfide bonds make up the hinge region to give the Y shape and flexibility. Image c displays the constant regions of the heavy and light chains (light blue) and the variable regions (red), which serve as the antigen binding sites.

The first form of antibody diversification was dictated by somatic recombination; but secondary diversification arises from somatic hypermutation, gene conversion, and class switching. These alterations generate the five classes of antibodies – IgM, IgD, IgG, IgE, and IgA. IgM is the class present on every immature B cell, the μ region is the first constant region brought to the VDJ region on the chromosome. Maturation results in concurrent expression of IgD on the surface of the B cell after it leaves the bone marrow. Antigen-based affinity

maturation results in the expression of IgG. Unlike IgM, which binds as a pentamer, IgG binds as a monomer; thus, the secondary immune response is characterized by much stronger, higher affinity signaling (Murphy *et. al,* 2008). IgA and IgE are unique immunoglobulin classes. IgA functions as a secretory dimer to aid in mucosal immunity (Mazanec *et. al,* 1993). IgE binds to mast cells in the periphery to mediate responses to allergens (Galli and Tsai, 2012). IgM, IgD, IgG, IgA, and IgE are derived from the recombination events that bring the VDJ region of the chromosome close to the μ, δ, λ, α, and ε constant regions, respectively. All such aspects provide an individual with a selective, diversified antibody repertoire that is essential for proper immune functionality (Murphy *et. al,* 2008).

1.6 Research Applications

The nearly unlimited diversity in antibodies generated by the humoral response provides us not only with a powerful tool to adapt to and neutralize any invading pathogen, but has also been targeted by scientists as a powerful research tool in protein quantification and function analysis. Antibodies that are naturally produced in the body can be manipulated to suit a variety of research applications. But in order to accomplish large-scale target-specific binding, a population of identical antibodies must be made that all bind the same epitope – known as monoclonal antibodies (Jiskoot *et. al,* 1991).

Monoclonal antibodies are generated by injecting a mouse or other test animal with the antigen being targeted. This immunization is conducted over time until the desired antibody titer is attained. The spleen is then harvested and the B cells are extracted and placed in a culture with a myeloma cell line. Though these myeloma cells do not produce antibodies, they have the

capacity to divide exponentially, which can be exploited by induced cell fusion into B cell-myeloma hybrids or hybridomas. The fusion is triggered by the addition of polyethylene glycol (PEG). The short lifespan of the unfused B cells quickly eliminates their presence in the culture. The remaining cells are then placed on a salvage nucleotide synthesis pathways. Only cells containing the enzymes for the salvage pathway are enabled to survive. The myelomas do not contain these enzymes, and thus cannot survive in this culture. In this way, the culture promotes selection of the hybridomas, which retain the B cell capacity of antibody production, but the immortality of tumor cells. The hybridomas are isolated and cultured individually, resulting in the production of clonal populations. The individual populations are screened for the production of the desired antibody based on antigen binding tests. Those that test positive for binding can be grown in mass culture for high production of monoclonal antibodies (Davis et. al, 1982). Formation of monoclonal antibodies is essential for any antibody-targeted analysis (Jiskoot et. al, 1991).

Technological advancements have made it possible to take a multivariate approach to cellular analysis to greater depths through the use of fluorescence-conjugated antibodies. High specificity antibodies generated to a specific protein are then covalently bonded to a fluorochrome. To actually visualize the degree of binding interactions, laser excitation via flow cytometry is the common approach (Murphy *et. al*, 2008). Flow cytometry enables segregated analysis for a large number of cells in a rapid, precise manner. Using fluorescence-conjugated antibodies, each cell will bind the number of antibodies in proportion to how much of the given protein it expresses. The cell can then be specifically analyzed based on measuring the degree of fluorescence of the antibody (Rieseberg *et. al*, 2001). This process, known as immuno-

phenotyping or Fluorescence Activated Cell Sorting (FACS) allows for classification of cells based on proteins expressed on the cellular surface (Baumgarth and Roderer, 2000).

Fluorochromes are small organic dyes that have been optimized based on their wavelength range of excitation and emission, brightness levels, photostability, and reduction of self-quenching (Giepmans *et. al*, 2006). Self-quenching results from intermolecular interactions and subsequent energy transfer between fluorochromes, most notably when the fluorochrome-to-protein ratio is too high. The strength of the dipole-dipole interactions increases with proximity between the molecules, causing enhanced overlap between the emission spectrum of one fluorochrome and the absorption spectrum of its neighbor. Quantum yield, the number of photons emitted divided by the number of photons absorbed, describes the overall efficiency of a given flurochrome. But, the conflicting signals decrease the overall quantum yield of the fluorescence. This process alters the results to appear as though cells are expressing a fewer number of a target protein (Deka *et. al*, 1996). Self-quenching can also be a problem when utilizing multiple fluorochromes in a single solution, known as master mixes. In order to distinguish multiple fluorochromes, they must have little spectral overlap in their fluorescent emissions (Baumgarth and Roederer, 2000).

Because fluorescence conjugated antibodies can be utilized in both *in vitro* and *in vivo* applications, fluorochromes must be biologically inert such that they do not interfere with cellular elements. In addition, they must be readily conjugated to monoclonal antibodies in a process known as immunolabeling (Baumgarth and Roederer, 2000). There are two approaches to immunolabeling – through the use of either primary or secondary antibodies. Both methods are displayed in Figure 1-2. One method is to utilize a primary antibody (shown in yellow) to label the cellular protein of interest followed by a fluorescence-conjugated secondary antibody

(shown in red) that binds to the primary antibody. This method, known as indirect immunolabeling, is contingent upon the affinity of two separate interactions; the primary antibody must bind specifically to the cellular protein and the secondary antibody to the primary antibody. Immunohistochemistry, western blotting, and enzyme-linked immunosorbent assays (ELISA) all utilize this technique (Kim *et. al*, 2008). The direct approach is the use of fluorescence-conjugated primary antibodies (yellow). In this process, the primary antibody binds to the target protein and, as it is conjugated to the fluorochrome, provides a direct fluorescent signal upon excitation with a laser. This method is more efficient when working with live cells or when targeting for multiple proteins (Giepmans *et. al*, 2006). In both cases, the degree of fluorescence corresponds to the degree of antigen binding that has occurred; more binding leads to an increase in measured fluorescence intensity (Deka *et. al*, 1996).

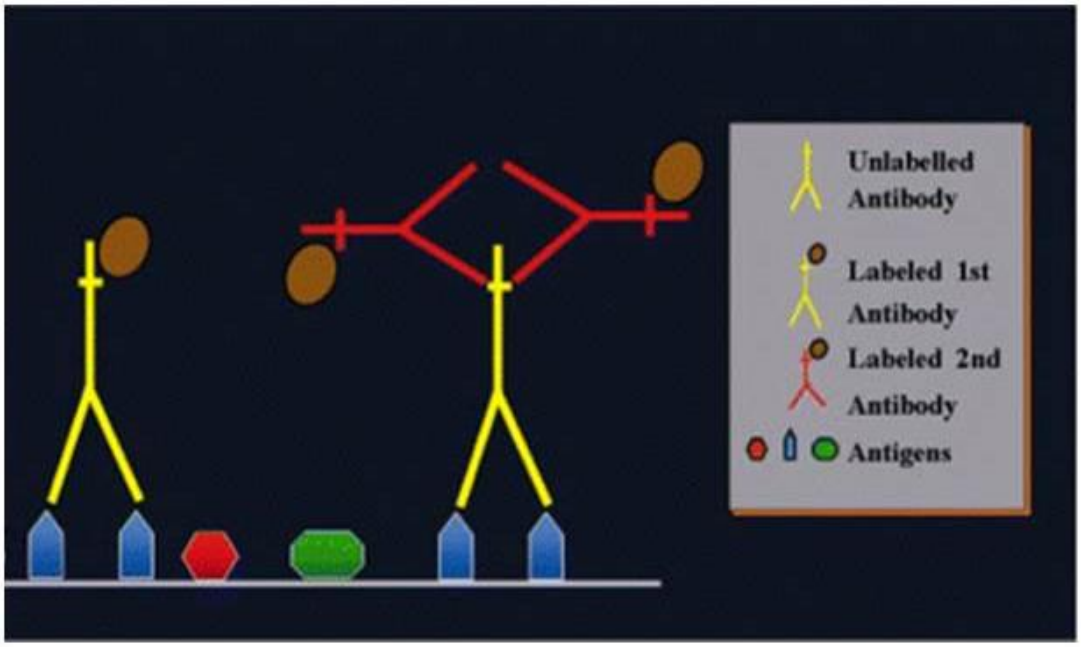

Figure 1-2. Direct and Indirect Methods of Immunofluorescence (Ramos-Vera, 2005). In direct immunofluorescence staining, a primary antibody (yellow) is conjugated to a fluorochrome (brown) and binds the antigen. This is shown on the left. In indirect staining, a primary antibody (yellow) binds the antigen. A secondary antibody (red) labeled with the fluorochrome binds the constant region of the primary antibody. This is shown on the right.

Immunophenotyping can provide a highly detailed analysis of each cell within a population, yet its accuracy relies on several crucial factors. The intensity of fluorescence is dependent upon the concentration of antibodies, concentration of cells, expression of the target protein on the cell, and affinity of the antibody for the designated protein (Petit *et. al*, 1993). The concentration of cells present in a given suspension requires a specific concentration of antibodies for optimal binding. This interaction is based on the specificity to the target antigen, temperature, pH, and components of the buffer (Jiskoot et. al, 1991.) When all such interactions are optimized, the final component in cellular analysis is stability of the bond between antibody and conjugated fluorochrome. Even with high affinity antibody-target binding, results cannot be accurately quantified without a stable fluorochrome.

1.7 Experimental Purpose

The purpose of this research is to determine the stability of fluorescence-conjugated antibodies based on mean fluorescence intensity as determined by flow cytometry. The maximal fluorescence intensity will be determined by titration of nine different fluorescence-conjugated antibodies, thereby providing a broad-spectrum analysis of how antibody concentration affects fluorescence of a fixed number of CD8+ T cells. This experiment will utilize antibodies that are specifically staining for cluster of differentiation 8 (CD8), a protein expressed on the surface of cytotoxic T cells. Using the lowest concentration of maximal fluorescence, we will be analyzing effects of temperature and time-related stressors on the mean fluorescence intensity. The antibodies will be exposed to a series of freezing and thawing cycles to evaluate effects of temperature, providing a better understanding of how current storage methods affect antibody stability. In addition, an older stock of antibodies will be used to stain for CD8 and CD25, detailing the effects of the length of storage on mean fluorescence intensity. The goal of this research is to discern how the stability of fluorescence-conjugated antibodies is affected by temperature and time. Study results will provide insight into how rigorously antibodies must be managed in order to maintain optimal fluorescent staining. We hypothesize that the max fluorescence intensity will be decreased in all of the antibodies as a result of temperature and time. However, antibodies will not all respond to the same degree. The following experiment will reveal the behavior of fluorescent-conjugated antibodies and their stability after external stress has been applied.

1.8 Experimental Results

All fluorochromes demonstrated a decrease in maximal fluorescence intensity as a result of the temperature stress from the freeze-thaw cycle; however, it was not to the degree predicted. With the exception of a select few, most of the fluorescence-conjugated antibodies maintained the necessary stability to continue to function in an optimal way. The time of storage, reflecting the age of the antibodies, had a much more consistent impact on the stability of the antibodies. This study suggests that freezing aliquots of fluorescence-conjugated antibodies may in fact provide a valuable tactic in prolonging their respective shelf lives. This method can be further carried over into freezing aliquoted master mixes, which retained their efficacy in multivariate analysis after temperature-related stress.

Chapter 2

Materials and Methods

2.1 Mice

For these experiments B6 mice (C57BL6) were utilized, purchased from the Jackson Laboratory. These wild type mice are bred for the sake of studying immunological functions and interactions in a natural setting. Mice were bred and maintained in facilities provided by the Pennsylvania State University. Two mice were used for each experiment, giving a total of ten mice, all within eight to ten weeks of age. All animals were used in accordance with IACUC guidelines.

2.2 Euthanasia and Splenocyte Processing

The target cells chosen for the assays were CD8+ T cells, which can be isolated, analyzed, and quantified from the spleen. Gaseous carbon dioxide was administered at a rate of 2 L per minute to euthanize the mice, followed by cervical dislocation. The spleens were excised, cut in half, and added to a solution of 4 mL RPMI with 1% fetal bovine serum (FBS) in a 15 mL tube. The tube was immediately placed on ice. The spleen, still in the RPMI solution, was emptied into a small petri dish. The cells were extracted by disrupting the spleen matrix between two frosted glass slides. A 5 mL syringe filled with 1% FBS supplemented RPMI was used to wash the cells off the slides and into the petri dish. The same syringe was used to extract the

cells and return them to the 15 mL tube. The tube was centrifuged at 1,200 rpm and 4°C for 10 minutes, forming a splenocyte pellet at the bottom of the tube. The excess media was carefully poured off, retaining the pellet at the bottom. The pellet was disrupted and cells went back into solution, followed by the addition of 0.83% ammonium chloride to lyse the splenic red blood cells. The cells were resuspended and centrifuged at 1,200 rpm and 4°C for another 10 minutes to was off the residual ammonium chloride. The excess media was emptied and cells were resuspended in RPMI supplemented with 10% FBS and placed on ice.

2.3 Antibody Labeling

Throughout the experiment the following antibodies were utilized. For the freeze-thaw experiment: CD8-FITC, CD8-PerCP, CD8-PE, CD8-PE-TxR, CD8-PE-Cy7, CD8-APC, CD8-A700, CD8-APC-Cy7, and CD8-PacBlue; for the old-new experiment: CD25-PE and CD8-APC; for the master mix freeze-thaw: CD8-FITC, GP33-APC, Thy1.1-PerCP, Thy1.2-PE, and CD44-APC-Cy7.

The splenocytes were counted and resuspended to ensure $20x10^6$ cells/mL. The cells were distributed in a 96-well U-bottom plate in FACS buffer and the plate was placed on ice. The plate was centrifuged for 2 minutes at 1,800 rpm and 4°C to form pellets. The plate was rapidly inverted and flicked to discard the excess media into a biohazard container, then lightly blotted on a paper towel before re-inverting to prevent cross-well contamination. The plate was tapped gently along the side in order to resuspend the cells. For each subset of the experiment, only a surface stain was required. Fluorescent-conjugated antibodies were added to each well in 50 μL aliquots of FACS buffer. Cells were incubated for 45 minutes on ice in the dark. Following this,

cells were washed with 150 μL of FACS buffer and centrifuged for at 1,800 rpm and 4°C for 2 minutes. The plate was inverted and flicked as before to remove the media and the cells were resuspended by tapping on the side of the plate. The cells were washed two additional times with 200 μL of FACS. Cells were fixed using 100 μL of 4% paraformaldehyde dissolved in PBS and 100 μL of FACS, then incubated for 30 minutes on ice in a darkened state.

2.3a. Titration

A serial dilution was conducted to evaluate the lowest concentration of maximum fluorescence intensity for each of the nine CD8-targeting antibodies. A 1:50 dilution was made using 2 μL of antibody and 98 μL of FACS buffer. This dilution served as the stock solution for the proceeding dilutions. This was followed by a 1:250, 1:500, 1:1000, 1:5000, 1:10,000, 1:50,000, and 1:100,000 dilution from the original stock solution. Once this concentration was determined by flow cytometry, it was used as the optimal concentration for the freeze-thaw portion of the experiment.

2.3b. Freeze-Thaw

Two sets of each fluorochrome were aliquoted into 1.5 mL eppendorf tubes, each containing 2 μL. Both sets were frozen at -80°C. The second set was removed from the freezer after 24 hours, completely thawed, then placed back in at -80°C. After 24 hours, both sets were removed, completely thawed and diluted into FACS buffer, then applied to $1x10^6$ splenocytes for staining at the lowest concentration of maximal fluorescence intensity as determined by titration.

2.3c. Master Mix Freeze-Thaw

Three equivalent mouse phenotyping master mixes were aliquoted, each consisting of CD8-FITC, D^bGP33 Tetramer-APC, Thy1.1-PerCP, Thy1.2-PE, and CD44-APC-Cy7. While the control mix remained unfrozen (made right before staining), the other two mixes were frozen at -80°C. The third mix was removed from the freezer after 24 hours, completely thawed, then placed back in at -80°C. After 24 hours, both mixes were removed, completely thawed, then applied to $1x10^6$ splenocytes for staining.

2.3d. Old-New

Both an old (4 years) and new (~2-5 months) set of CD25-PE and CD8-APC from the antibody stock were aliquoted and applied to $1x10^6$ splenocytes for staining to observe the effects of time on the maximum fluorescence intensity.

2.4 Flow Cytometry

Each of the former experiments concluded with cellular analysis by flow cytometry. The 96-well U-bottom plate containing the samples was placed into the cytometer. The cells labeled by the fluorescence-conjugated antibodies, were drawn up by the probe into the flow chamber. The cells were directed through the chamber by way of laminar flow. The cells passed one at a time out of the chamber and through a laser. The energy was absorbed by the fluorochromes tagged to the cell surface; the resulting emission spectrum scattered the light, which was detected by the light detector. This data was transmitted by the computer which identifies the

fluorochrome present based on the wavelength of light emitted. The flow cytometer was also able to identify the size and granularity of the cell based on the scatter induced by passing through the laser. All of this data was analyzed using FlowJo Single Cell Analysis software. Cells were gated based on the expression of the targeted surface proteins and plotted based on the concentration and mean fluorescence intensity of the fluorochromes.

Chapter 3

Results

3.1 Titration

The serial dilution of the nine antibodies resulted in the titration curves in Figure 3-1. The general trend displays that the mean fluorescence intensity increased with the concentration, though not in a linear fashion. Upon reaching an inflection point, the titration curves for CD8-FITC, CD8-PerCP, and CD8-PacBlue leveled off, an indication of saturating. The histograms provide a more clear indication of the changing fluorescence with the decreasing concentration, as the intensity and number of protein binding interactions diminished. CD8-PE-TxR, CD8-PE-Cy7, and CD8-A700 reached a point of inflection, but a higher concentration would need to be tested in order to determine full saturation. CD8-PE, CD8-APC, and CD8-APC-Cy7 demonstrated no significant quenching even at the highest concentration being tested. Regardless of the behavior of these fluorochromes, the titration provided a starting point for the following experiments, as the lowest concentration of maximum fluorescence had been determined.

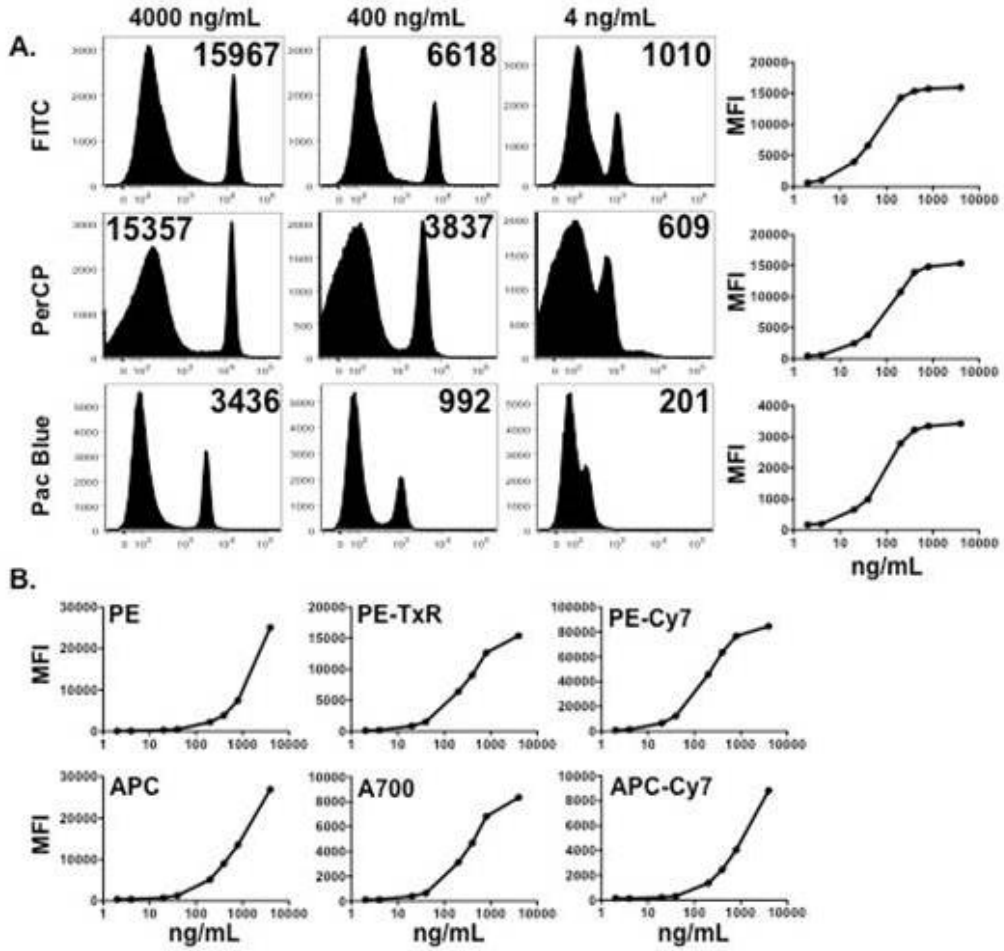

Figure 3-1. Titration of Fluorochromes: Mean Fluorescence Intensity Affected by Concentration. Nine fluorescence-conjugated antibodies, all targeting CD8, were titrated to determine the lowest concentration required to elicit the highest degree of fluorescence intensity. **A.** CD8+T cells were gated to produce the histograms for CD8-FITC, CD8-PerCP, and CD8-PacBlue, indicating the decrease in MFI with the serial dilution. The titration curves for these three fluorochromes are positioned to the right of the respective histograms. **B.** The titration curves for the remaining six fluorochromes were produced to display the behavior of each antibody over the duration of the dilution. The curves have been reversed to indicate that an increase in concentration (ng/mL) results in a steady increase in MFI until the antibody-CD8 binding has been quenched.

3.2 Freeze-Thaw

All nine fluorescence-conjugated antibodies were tested for stability against cold extremes. In all cases, the control group maintained the highest overall fluorescence, as was

expected. For CD8-PE (B), CD8-APC (C), and CD8-APC-Cy7 (I), the intensity of fluorescence experienced a significant decrease with the freeze cycles (Figure 3-2). CD8-FITC (A), CD8-PerCP (D), and CD8-A700 (F) demonstrated a slight decrease in overall fluoresce intensity with the freeze cycles. However, CD8-PacBlue (G), CD8-PE-TxR (E), and CD8-PE-Cy7 (H) did not experience any significant decrease as a result of the freeze cycles. This is a good clarification because it also shows that binding affinity of the CD8 antibody is not affected by freeze-thaw

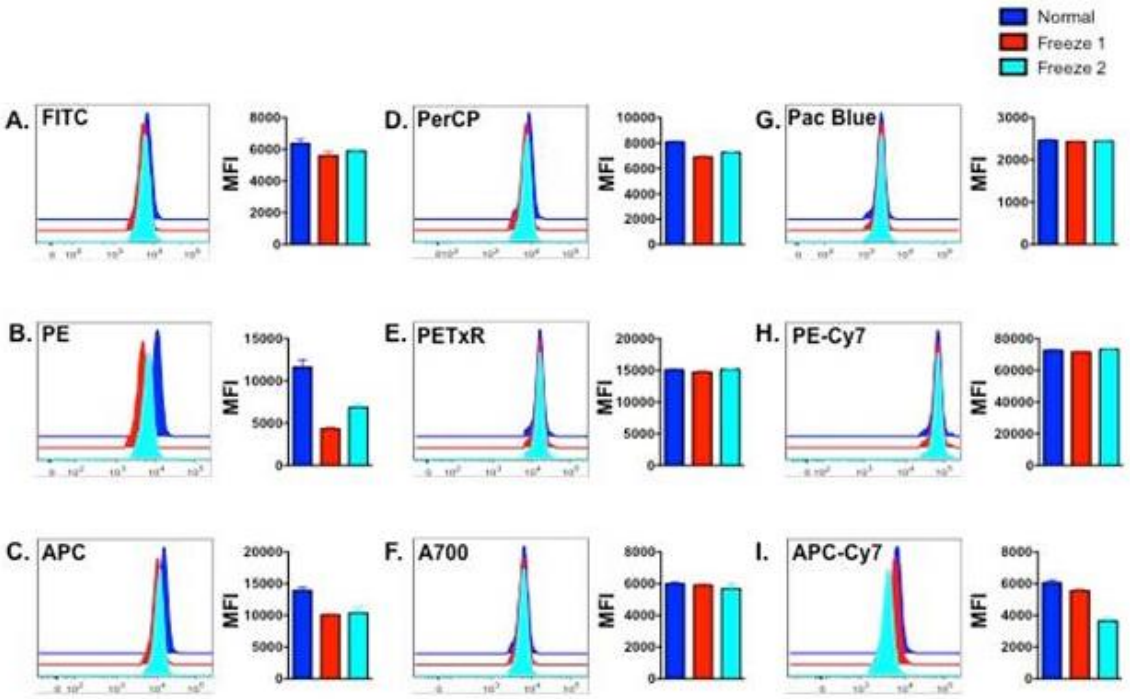

Figure 3-2. The Effects of Temperature on Aliquoted Fluorescence-Conjugated Antibodies. Each of the nine fluorescence-conjugated antibodies was aliquoted and applied at the lowest concentration of maximal fluorescence intensity as determined by titration. Three sets of each antibody were applied: an unfrozen control, Freeze 1, and Freeze 2. Freeze 1 was placed in the freezer at -80° C for 24 hours then applied to splenocytes for cell staining. Freeze 2 was placed in the freezer at -80° C for 24 hours, completely thawed, then placed back into the freezer for 24 hours. Freeze 2 was then removed and applied to splenocytes staining for CD8. Histograms were made using gated CD8+ T cells and offset to indicate the change in the fluorescence depending on the conditions. The bar graphs were made using the average MFI values for each group, as each set was duplicated using two mice.

3.3 Master Mix Freeze-Thaw

The phenotyping master mix containing CD8-FITC, GP33-APC and Thy1.1-PerCP displayed a similar trend as the individual freeze-thaw experiment (Figure 3-4). Thy1.2-PE and CD44-APC-Cy7 are not displayed in this figure, despite their inclusion in the master mix because they did not display any noticeable decrease in fluorescence. The MFI values are directly presented in each of the histograms; however, the values were normalized in the bar graphs to better demonstrate the slight degree of variance. Thy1.1-PerCP showed the greatest decrease in maximal fluorescence similar to what we observed during the individual freeze-thaw results. CD8-FITC maintained its nature of overall stability and even the change in APC was not as pronounced as it had been on an individual basis.

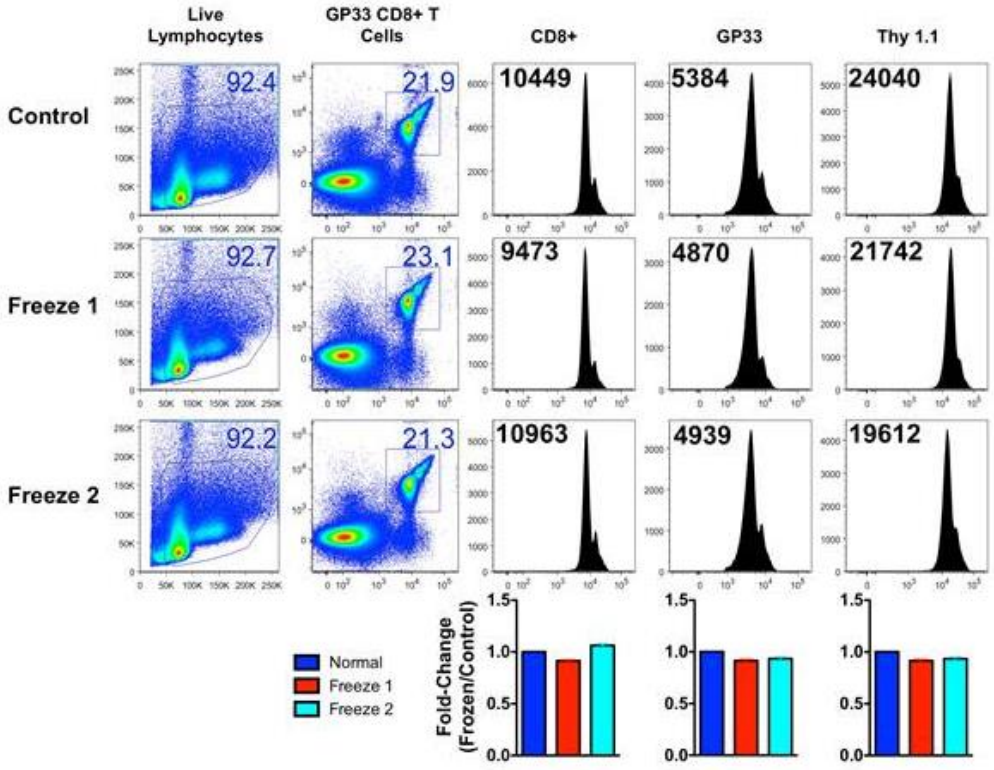

Figure 3-3. The Effects of Temperature on a p14 Master Mix. A p14 master mix was aliquoted using CD8-FITC, GP33-APC, Thy1.1-PE, Thy1.2-PerCP, and CD44-APC-Cy7. The mix was divided into three sets: an unfrozen control, Freeze 1, and Freeze 2. Freeze 1 was placed in the freezer at -80° C for 24 hours then applied to splenocytes for cell staining. Freeze 2 was placed in the freezer at -80° C for 24 hours, completely thawed, then placed back into the freezer for 24 hours. Freeze 2 was then removed and applied to splenocytes. Live lymphocytes were gated for GP33 CD8+ T cells. Histograms of CD8-FITC, GP33-APC, and Thy1.1-PE were made to indicate the change in MFI as a result of the freeze-thaw cycles. Bar graphs were made using fold-change of Frozen/Control to standardize the MFI values.

3.4 Old-New

The Old and New antibodies were applied and cells were gated based on the expression CD8 for APC and CD25 for PE. The MFI for New APC matched what was determined in the control for the Freeze-Thaw (Figure 3-2), reinforcing the behavior of APC without applied stress. However, the MFI of the Old APC experienced a dramatic decrease, being almost half of the control value. The affects for PE were not quite as pronounced, but a decrease was still

noted. However, laboratory stock did not contain a 4 year-old stock of CD8-PE; thus, we could not compare the values of the PE fluorochrome due to the different target proteins. Regardless, the storage time represented a marked decrease in fluorescence.

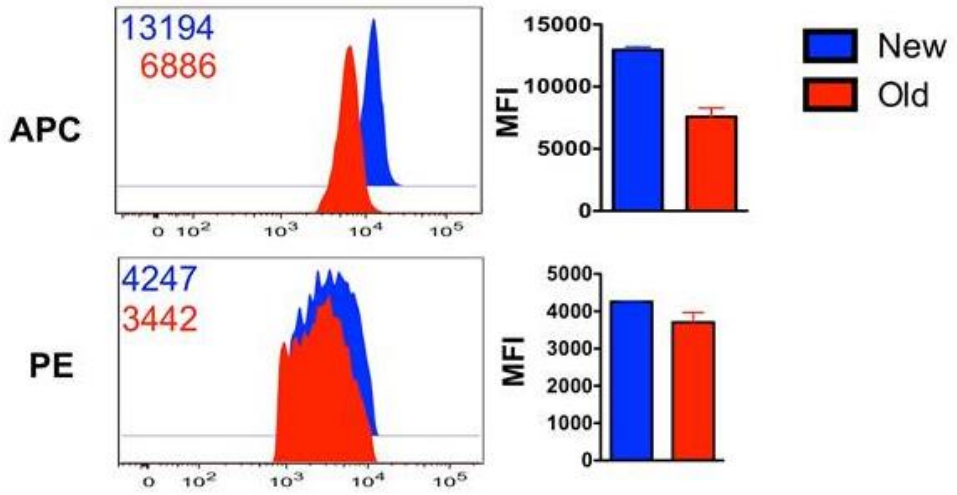

Figure 3-4. The Effects of Time on Fluorescence-Conjugated Antibodies. CD8-APC and CD25-PE were aliquoted and applied to splenocytes at the optimal concentration as determined by titration. The New (~2-5 months) group serves as a control, while the Old group represents fluorescence-conjugated antibodies that have been in storage for four years. The cells were gated based on expression of CD8 for APC and CD25 for PE. Histograms were made and offset to indicate the change in mean fluorescence. The MFI values were placed in the upper left hand corner for clarification. As each set was duplicated using two mice, the bar graphs reflect the average MFI values.

Chapter 4

Discussion

4.1 Effects of Temperature Stress

The freeze-thaw cycles placed the fluorescence-conjugated antibodies under significant temperature stress, which was thought to have a drastic effect on the covalent bond between fluorochrome and antibody. However, the behavior of each individual antibody remained fairly consistent amongst the different tests. All of the antibodies were titrated using the same initial concentrations, yet only CD8-FITC, CD8-PerCP, and CD8-PacBlue reached peak levels of fluorescence that were maintained for multiple dilutions. Others, such as CD8-PE, CD8-APC, or CD8-APC-Cy7 have a much higher peak fluorescence that was not achieved by the experimental concentrations. Thus, a much greater concentration is required of these antibodies in order to saturate fluorescence. Considering that all of the antibodies are targeting CD8, the inflection point for antibodies should be the same regardless of the fluorochrome, as this aspect is controlled by the binding affinity. Variations in the binding affinity may be attributed to the addition of the fluorochromes. It is possible that the additional molecule impairs the binding interaction, thus preventing maximal binding efficiency. Further research is required in order to ascertain the variations in the binding affinity.

In the individual freeze-thaw cycles (Figure 3-2.) many of the fluorochromes maintained similar behaviors. CD8-PacBlue (G), CD8-PE-Cy7 (H), CD8-PE-TxR (E), CD8-FITC (A), and CD8-A700 (F) showed only minor decreases in fluorescence intensity; yet, as indicated by the titration graphs (Figure 3-1.), these fluorochromes had reached saturation levels at lower concentrations and thus were more resistant to change. On the other hand, CD8-PE (B), CD8-

APC (C), and CD8-APC-Cy7 (I), which had failed to reach saturation levels in the serial dilution, experienced a marked decrease in mean fluorescence intensity when put through the freeze-thaw cycles.

The phenotyping master mix (Figure 3-3.) comprised of CD8-FITC, D^bGP33 Tetramer-APC, and Thy1.1-PerCP remained consistent with the individual fluorochrome behavior as well. The behavior of CD8-FITC fluctuated faintly, but remained fairly close to the control value. D^bGP33 Tetramer--APC and Thy1.1-PerCP decreased slightly more noticeably, as was consistent (Figure 3-2), but the entire mix retained viability. The decrease could have changed in a uniform manner due to some degree of self-quenching, but this is unlikely as the fluorochromes are selected for the master mix due to their limited overlap. Given that an antibody cocktail must be used within one or two days on a normal basis, these results bear significant implications about antibody management.

4.2 Effects of Time-Related Stress

One of the major hurdles in the experiment was the lack of fluorescence-conjugated antibodies that had been in storage for four years or longer. Though this reflects the need of the laboratory to utilize only the optimal stock to produce the most accurate results, a more concrete fluorochrome analysis could have been completed. However, even with limited availability, the impact of age on fluorochrome-antibody stability was far greater than the influence of temperature, as there was almost a 50 percent reduction seen in CD8-APC (Figure 3-4).

When an object is frozen, the energy of the system is dramatically reduced; however, once placed in a warmer environment, the energy is reapplied. This could explain why after the

first freeze-thaw cycle, the subsequent change was not significant. But, with the response to time, there is no re-application of energy. Instead, over time the energy binding the fluorochrome to the antibody was merely lost. Though both molecules may individually be intact, the fluorescence cannot be detected unless the complex is bound. It is likely that the covalent bond adjoining APC to the antibody was weaker than that of PE to CD25, as the decrease was much more evident (Figure 3.4). Although we recommend that this experiment be repeated with a greater number of fluorochromes, it still bears significance in evaluating the potential detriment of long-term storage.

Chapter 5

Conclusion

Overall, the effects of temperature and time resulted in a reduction of maximal fluorescence intensity for all nine fluorochromes. However, based on the behavior of those tested against time, the length of storage had a far more profound impact on stability than the freeze-thaw cycles. This would suggest that freezing aliquoted antibodies, whether individually or in a master mix, could in fact be a manner of preserving their efficacy rather than depleting it. Furthermore, certain fluorochromes demonstrated superior stability over the duration of the experiment, namely PacBlue, PE-Cy7, and PE-TxR. FITC, A700, and PerCP lacked the stability of the former fluorochromes, but maintained viability such that freezing would not significantly detract from immunolabeling. Fluorescence-conjugated antibodies are highly expensive, so it is important to try and maximize their usage. Since not every cellular analysis will require all antibodies within the laboratory stock, freezing may be highly useful in prolonging those with less frequent use. All in all, this experiment detailed that fluorescence-conjugated antibodies are quite stable in response to temperature stress, providing a new perspective on laboratory storage methods.

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