THE USE OF CONFIDENCE REGIONS TO DETERMINE OBJECTIVE CRITERIA FOR ANALYSIS OF SINGLE PARAMETER HISTOGRAMS OF MEAN FLUORESCENCE INTENSITY

by

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ABSTRACT

Several methods have been proposed for comparing fluorescence intensity curves obtained from flow cytometry analysis. The one comparison method that is most often used is the Kolmogorov-Smirnov (K-S) test, which is known to find statistically significant differences between curves even when clinical or scientific differences are doubtful. In the field of flow cytometry, the combination of the gating process and either Overton Subtraction or K-S test yields a histogram subtraction technique for analysis of flow cytometric frequency data. In order to help biologists assess the results of a K-S or other test for the difference between mean fluorescence intensity curves, we have proposed a method of producing bootstrap-based confidence bands around the difference in histograms to show the range of intensity channels for which a difference between histograms may be identified. As an illustration of the method, we obtain and display confidence bands for histogram differences between pumping cell subsets. The confidence bands are constructed using both pointwise and simultaneous confidence bands. We compare the results of these two approaches. Furthermore, for descriptive purposes, the confidence bands produced were found to provide a useful descriptive display to aid in the interpretation of results. Flow cytometry is a major research tool for immunology and, by

extension, infectious diseases and public health. Our statistical methodology is applicable to flow cytometry research in a variety of settings.

Key terms: Flow cytometry, Mean Fluorescent Intensity (MFI), Single parameter histogram, Bootstrapping, Difference, Pointwise confidence intervals, Simultaneous confidence bands

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PREFACE

I would like to thank my thesis advisor Dr. John W. Wilson for his insight, patience and editing skills in helping me to structure and write this thesis. I also would like to thank committee members: Dr. Andriy Bandos for his guidance to statistical methodology and constructing SAS programming code, and Dr. Donnenberg not only for allowing me to use her experimental data, also for her expert biological knowledge and countless support.

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Finally, I would like to thank my family and friends for their love, encouragement and support.

1.0 INTRODUCTION

1.1 BACKGROUND

P-glycoprotein, also known as Multi Drug Resistance (MDR) protein is a member of the ABC transporter protein family (Chaudhary and Roninson 1991, Chaudhary, Mechetner et al. 1992, Donnenberg, Burckart et al. 2001, Donnenberg, Burckart et al. 2004, Donnenberg, Wilson et al. 2004, Donnenberg, Landreneau et al. 2007). The multidrug resistance gene MDR1 encodes for P-glycoprotein (P-gp) (Thiebaut, Tsuruo et al. 1987, Chaudhary, Mechetner et al. 1992, Licht, Pastan et al. 1994, Petriz and Garcia-Lopez 1997). The adenosine triphosphate (ATP) binding cassette transporters extrude various molecules across membranes in a variety of cells including T cells (Schneider and Hunke 1998, Dean, Hamon et al. 2001). Further, an increased expression of the ABC transporters have been documented in pathologic states such as cancer (Izquierdo, Neefjes et al. 1996, Litman, Brangi et al. 2000) and immune rejection (Boisnard, Zickler et al. 2003, Parodi-Talice, Araujo et al. 2003), and results in pharmacological resistances to substrate drugs (Thiebaut, Tsuruo et al. 1987, Krishan, Sauerteig et al. 1991, Chaudhary, Mechetner et al. 1992, Leonce and Burbridge 1993, Licht, Pastan et al. 1994, Muller, Lennartz et al. 1994, Izquierdo, Neefjes et al. 1996, Petriz and Garcia-Lopez 1997, Litman, Brangi et al. 2000, Dean, Hamon et al. 2001, Thomas and Coley 2003, Donnenberg, Landreneau et al. 2007). Furthermore, the drug transporters that located in the plasma membrane play a major role in

acting as an efflux pump for the excretion of drugs and drug conjugates out of the cells (Chaudhary and Roninson 1991, Krishan, Sauerteig et al. 1991, Chaudhary, Mechetner et al. 1992, Muller, Lennartz et al. 1994, Petriz and Garcia-Lopez 1997, Wang, Casciano et al. 2000, Donnenberg, Burckart et al. 2001, Thomas and Coley 2003, Donnenberg, Burckart et al. 2004, Donnenberg, Wilson et al. 2004, Swerts, de Moerloose et al. 2004, Fletcher, Haber et al. 2010, Llaudo, Cassis et al. 2012, Pasquier, Rioult et al. 2013). Thus, P-gp activity in T cells is important for immunosuppression (Donnenberg, Burckart et al. 2004) or immunostimulatory drugs (Laupeze, Amiot et al. 2001).

P-gp activity could be described as having two components: 1) constitutive during LOAD and 2) substrate induced transport during EFFLUX (Fromm 2004). These activities are detected by exposing cells to a fluorescent substrate such as rhodamine 123 and measuring the degree of cell associated fluorescence. This is expressed as Load: how much dye entered a cell. Efflux is determined after loaded cells were further incubated during a specified time interval and R123 was allowed to be transported outside of the cell (Donnenberg, Wilson et al. 2004). This is expressed as substrate induced Efflux. Flow cytometry is the most widely used technique for measuring and analyzing multiple characteristics of cells in a suspension using monoclonal antibodies and fluorescent probes (Shapiro 2003). These fluorescently labeled cells are interrogated in a stream of a carrier fluid while passing through a beam of laser light (Shapiro, Glazer et al. 1983, Alberti, Parks et al. 1987). Relative fluorescent intensities are measured for each cell (Givan 2011). If p-glycoprotein is inactive, a fluorescent substrate probe will not be transported out of the cell, increasing the fluorescent intensity. In contrasts, when P-glycoprotein in active, the fluorescent dye is transported out of a cell, reducing or completely eliminating the fluorescent intensity of the cell. The change in fluorescence can be expressed as 1) change in

fluorescent intensity (MFI) and 2) percent of cells with low or no fluorescence (Pallis and Das-Gupta 2005, Solazzo, Fantappie et al. 2006). The MFI of each cell in a population of cells lead itself to evaluation as the statistical difference between two distributions by creating a virtual overlay of histograms generated for the control sample (no transporter activity, high fluorescence) and the test sample (high transporter activity, low fluorescence) by the subtraction of the control fluorescence intensity values from the test sample, for each channel (Solazzo, Fantappie et al. 2006, Gelderman and Simak 2008).

1.2 STATEMENT OF PROBLEM

Most flow cytometric data depend on the conversion of continuous biological measurements to discrete values. One of the methods for comparing two flow cytometric frequency distribution curves is the Kolmogorov-Smirnov (K-S) test (Massey 1951, Young 1977, Finch 1979, Cox, Reeder et al. 1988). This nonparametric statistical test was applied to flow cytometry data by Young et al (Young 1977). However, Young and Finch have warned that the test would be of limited value because this test usually concludes statistical significance due to the large sample size even when the difference is not biologically significant (Young 1977, Finch 1979). There have been additional efforts to compare flow cytometry histograms. In particular, Cox and Reeder developed a procedure that makes use of a normal approximation of frequencies in each channel (or binned adjacent channels) (Cox, Reeder et al. 1988). Despite of its endeavor to compensate hypersensitivity issue in K-S test, this alternative approach requires at least 20 events in channels (or binned adjacent channels) to utilize the normal approximation to the Poisson distribution. Lack of this number of counts will not allow confidence intervals for binned data in such channels. Besides statistical methods comparing frequency distributions, histogram subtraction techniques for analysis of flow cytometry data were developed (Overton 1988, Muller, Lennartz et al. 1994, Levin, van der Holt et al. 2004). In order to compare and evaluate histograms of fluorescence intensities, histogram subtraction methods are used to compare the numbers of cells with similar levels of fluorescent intensity (Schipper, Tilders et al. 1980, Mann, Hand et al. 1983, Overton 1988, Muller, Lennartz et al. 1994, Levin, van der Holt et

al. 2004). One of these methods, the Overton Subtraction method, is a cumulative subtraction method of determining percent positive cells accurately based on a comparison of two control curves (Overton 1988). It has an ability to enumerate difference between two samples even if their range is overlapping. However, this method does not address variability associated with estimated difference between histograms and is not primarily intended to aid in the decision of whether two histograms are significantly different.

1.3 PURPOSE OF STUDY

The purpose of this study was to provide a method for comparing flow cytometry histograms that showed at which fluorescence intensities difference occur, the direction of the differences, and the variability associated with the differences in the form of confidence bands. The method consists of obtaining pointwise and simultaneous confidence intervals around a difference curve. The display shows a region of plausible pointwise differences and confidence envelope which cover all true values simultaneously between the curves being compared. The displays are shown for two T-cell subsets, CD4 and CD8, and two different cell states, Load and Efflux, Inter- and intra-sample variability were also evaluated. The differences between these experimental conditions are both statistically and biologically significant. Taken together, the methods proposed provide objective criteria for analysis of single parameter histogram data of mean fluorescence intensity of R123 in T-cell subsets.

2.0 MATERIALS AND METHODS

2.1 BIOLOGICAL MATERIALS AND EXPERIMENTAL METHODS

2.1.1 HUMAN SUBJECTS

Peripheral blood (30 ml of venous blood, heparin from beef lung, 10 U/ml; Upjohn, Kalamazoo, MI) was obtained from one healthy control subject. Informed consent was obtained according to protocols approved by the University of Pittsburgh Institutional Review Board (Donnenberg, Wilson et al. 2004).

2.1.2 PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) PREPARATION

Thirty milliliters of heparinized blood from a control subject was diluted 1:2 with Dulbecco's $Ca2^{+}$ $Mg2^{+}$ -free phosphate buffered saline (PBS-A). PBMCs were obtained by centrifugation over Ficoll-Hypaque gradient (Sigma Diagnostics, St. Louis, MO) according to the manufacturer's instructions. Harvested cells were washed three times with 40 ml of PBS-A before surface staining. The cell pellet was re-suspended in a 15-ml polypropylene conical tube (Falcon Becton-Dickinson, Franklin Lakes, NJ) in 3 ml of staining buffer (PBS-A containing 4%, v/v, fetal calf serum and 0.1%, w/v, NaN3) (Donnenberg, Wilson et al. 2004).

2.1.3 BULK SURFACE STAINING OF PBMC

Before loading with R123, cells ($2–15 \times 10^6$) were centrifuged at 400*g* for 5 min at 4°C and decanted to a "dry" pellet. The cell pellet was first incubated for 20 min on ice with 2 µl each of conjugated monoclonal antibodies against surface epitopes for CD14 (phycoerythrin conjugated, BD-Pharmingen 347497, San Diego, CA), CD3 Energy Coupled Dye (ECD) (ECD conjugated, Beckman Coulter 6604701), CD8 (ECO conjugated, Beckman Coulter 6604728), and CD4 (phycoerythrin-Cy5 conjugated, Beckman Coulter IM2636; Beckman Coulter, Hialeah, FL). The resulting reaction volume was approximately 18 µl per tube (10 µl from the "dry pellet" and 8 µl from the added antibodies), and the final antibody concentration was consistent with the manufacturer's recommendations. After surface staining, the cells were resuspended to a concentration of 1×10^6 cells/ml in complete medium consisting of RPMI-1640 supplemented with 10% fetal calf serum, L-glutamine (200 µM), penicillin (100 U/ml), gentamicin (50 µg/ml), and HEPES buffer (10 mM) (Donnenberg, Wilson et al. 2004).

2.1.4 FLOW CYTOMETER SETUP

Samples were acquired on a four-color Beckman-Coulter EPICS XL flow cytometer (Beckman Coulter). Photomultiplier tubes (PMTs) were adjusted with FlowSet beads (Beckman Coulter) at a PMT setting that placed R123 bright cells in the third decade. The cytometer was checked on a daily basis by using FlowCheck fluorospheres to confirm laser alignment and fluidics and FlowSet fluorospheres to calibrate fluorescence measurement (Beckman Coulter). The latter was critical to these studies and ensures that cells of a given brightness appear in the

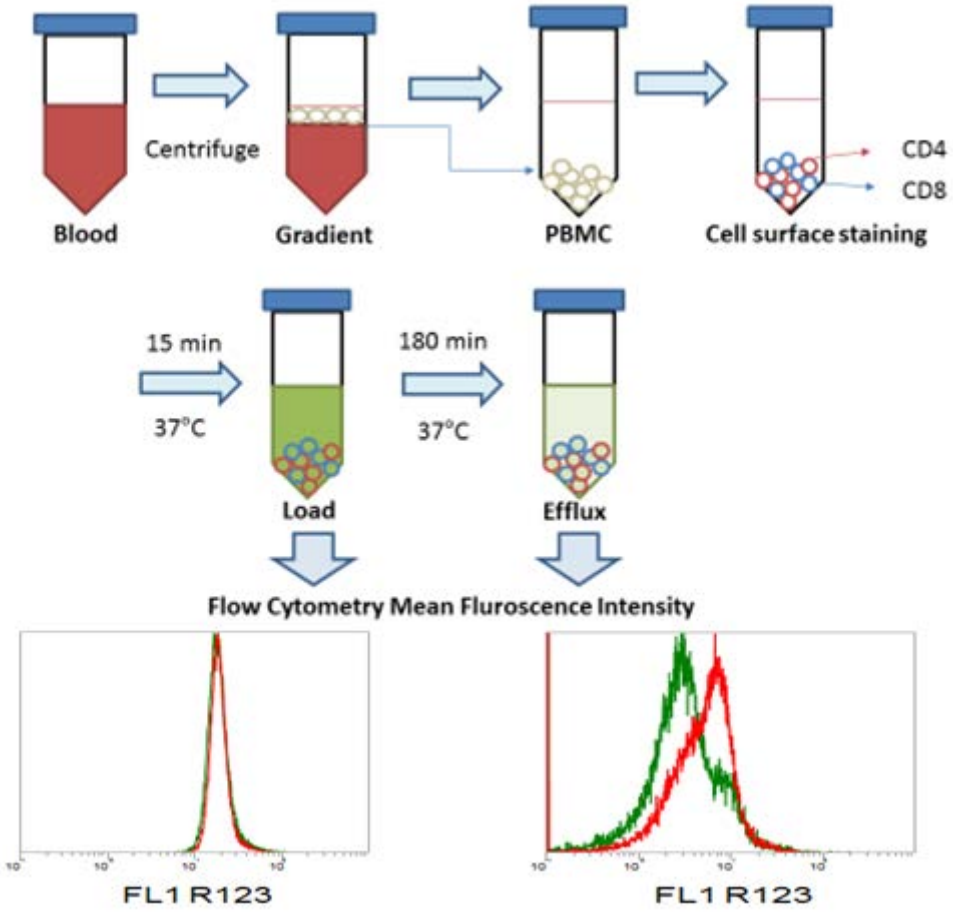
same fluorescence channel when assayed on different days. Compensation was performed with balanced PMTs using single-color–stained PBMCs and verified with a four-color stained sample. Linearity was confirmed during yearly maintenance by using six-peak Rainbow Calibration Particles (Spherotech, Libertyville, IL). Daily quality control validated our use of the same cytosettings throughout the study (Donnenberg, Wilson et al. 2004).

2.1.5 MEASUREMENT OF P-GP FUNCTION BY FLOW CYTOMETRY

Cells were loaded with 0.13 μM R123 (Sigma Chemicals) for 15 min at 37°C. At the end of the "loading" incubation, cells were washed twice with 10 ml of ice-cold PBS-A. Washed cells were re-suspended in complete medium to a concentration of 1×10^6 cells/ml. At loading state, one half of the cell suspension was removed, chilled on ice, and acquired immediately on the flow cytometer. The remaining half of the cell suspension was allowed to efflux for 180min at 37°C. At the end of the efflux incubation, cells were chilled on ice to prevent further P-gp–mediated efflux and immediately acquired. We attempted to collect 10,000 events for both $CD4^+$ and $CD8^+$ T-cell subsets at a maximum of 1,000 events/s.

A viable acquisition list gate was established by using forward and side light scatter (FS, SS) parameters to exclude subcellular debris and multi-event clusters. Data were analyzed with WinList (Verity Software House, Topsham, ME). This technique allowed simultaneous measurement of the intracellular concentration of the fluorescent P-gp substrate R123 on $CD4^+$ and $CD8^+$ T cells (Donnenberg, Wilson et al. 2004).

The process is summarized in Figure 1.



CD4 (red circles) and CD8 (blue circles) T-cell subsets are incubated with R123 for 15 minutes to allow the fluorescent substrate to enter the cells. This is considered LOAD. R123 stained cells are washed and allowed to efflux for 180 minutes in R123 free medium. This is considered as EFFLUX.

Figure 1. The process of sample preparation

2.2 STATISTICAL METHODS

2.2.1 FREQUENCY

The flow cytometry data were analyzed by first converting the number of observations (events) into frequencies of observations in each fluorescence intensity channel. The sample size is different for each data set and represents the number of cells in the flow cytometry assay. In total, there are 3 data sets of CD4 at loading states, 3 data sets of CD8 at loading states, 3 data sets of CD4 at efflux states, and 3 data sets of CD8 at efflux states. In this study, each sample data of CD4 Load, CD4 Efflux, CD8 Load and CD8 Efflux is used to compute frequencies of observations in each data set. The observed cell counts are converted to relative frequencies by dividing each observed cell count by the total number of cells in the data set. Those relative frequencies are the basis of our procedure. To describe the proposed method in detail, suppose that a sample $\boldsymbol{z}$ is a data set of observations that correspond to channel numbers

$\boldsymbol{z}$: = $\{z_i\}_{i=1}^{m}$ = (z_1, …, z_m), where m = the number of observations, $z_i \in$ {0, …, 1,023}

In other words, each cell appears in the data set as a fluorescence intensity channel number.

Then, $\boldsymbol{y}$ specifies a set of relative frequencies by channel:

$\boldsymbol{y}$: $y_x = \frac{\sum_{i=1}^{m} I(z_i = x)}{m}$, x = (0, …, 1,023)

where $I(z_i = x)$ is an indicator variable for the condition $z_i = x$

Thus, $\boldsymbol{y}$ = (y_0, …,$y_{1,023}$)

Not every channel has events, which means that there are missing frequency values for a range of channels. Channel 0 and 1,023 represent channels in which fluorescence is undetectable (0) or saturated (1,023).

2.2.2 LINEAR INTERPOLATION

Since there are many missing values in a data set, linear interpolation has been applied to each data set to provide non-zero approximate frequencies for channels without cells representing them. The distribution of the relative frequencies of events is therefore smoother after linear interpolation {Meijering, 2002 #76}.

Suppose that there are two known data points given by the interval (j, y_j) and (l, y_l) from the X, Y axis where j and l values belong into channel numbers and $j < l$, and y_j and y_l values are observations from a $\boldsymbol{y}$ sample which correspond to j and l on X-axis.

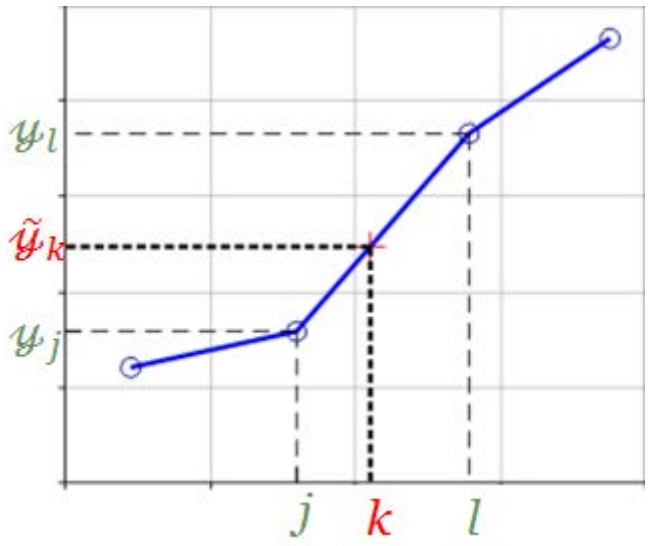
Then, linear interpolant is the straight line between these points. For any missing value *k* in the interval (j, l), the value $\tilde{y}_k$ along the straight line is given from the equation

$$\tilde{y}_k = y_j + (y_l - y_j) \times \frac{k-j}{l-j} \text{ for } \forall\, k \in [j, l],\ j < l$$

where *j* and *l* are observed channel numbers among 0 to 1,023

Thus, $\tilde{\boldsymbol{y}} = (\tilde{y}_0, \ldots, \tilde{y}_{1,023})$

Applying linear interpolation to all missing values would create all artificial observations into a sample B and those dummy values would be used to compute differences of each pair between two types of data sets and construct a continuous curve of differences.



A value $\tilde{y}_k$ at an unknown value k in the interval (j, l) is derived geometrically by linear interpolation.

Figure 2. Linear interpolation

2.2.3 BOOTSTRAPPING

Bootstrapping is a nonparametric approach to statistical inference that substitutes computation for more traditional distributional assumptions and asymptotic results (Efron 1979, Efron and Tibshirani 1994). To use this approach, a sample with the same size as the original sample is generated by simple random sampling with replacement (Efron 1979, Schreuder, Li et al. 1987, Williams 2013). That is, even though an observation has been selected once for the bootstrapping samples, it could be selected again. Thus, the population is to the sample as the sample is to the bootstrapped sample (Efron 1979, Freedman 1981, Efron and Tibshirani 1994, Davison and Hinkley 1997).

Note that a set of a bootstrapped sample $\mathcal{Z}_B^*$ is

$$\mathcal{Z}_B^* := \{z_{i\,b}^*\}_{i=1,\,b=1}^{m,\,B} = (z_{1\,b}^*, \ldots, z_{m\,b}^*),$$

where m = the number of observations in a sample $\mathcal{Z}$, $\mathcal{B}$ = the number of bootstrap replicates, and $z_{i\,b}^* \in \{z_1, \ldots, z_m\}$

Also, the set $\mathcal{Y}_B^*$ contains the relative frequencies of the bootstrap replicates by channel:

$$\mathcal{Y}_B^*: y_b^* = \frac{\sum_{i=1}^{m} I(z_{i\,b}^* = x)}{m},\ b = (1, \ldots, B)$$

where $I(\mathcal{Z}_{i\,b}^* = x)$ is an indicator variable for the condition $\mathcal{Z}_{i\,b}^* = x$

Thus, $\mathcal{Y}_B^* = (y_{0\,b}^*, \ldots, y_{1{,}023\,b}^*)$

In this study, bootstrap samples were taken from the original file of m cells/intensities. That is, a new file of m intensities is produced from each bootstrap sample. The process described above for producing a smoothed histogram was repeated 100,000 times. These histograms were used in the construction of the confidence bands described below.

After bootstrapping, linear interpolants are also applied to bootstrap replicates. Thus, we assumed that linear interpolant is the straight line between the two (j, $y^*_{j\,b}$), (l, $y^*_{l\,b}$), intervals from bootstrap samples, for any missing value k in the interval (j, l), the value $\tilde{y}^*_{k\,b}$ along the straight line is given from the equation

$$\tilde{y}^*_{k\,b} = y^*_{j\,b} + (y^*_{l\,b} - y^*_{j\,b}) \times \frac{k-j}{l-j} \text{ for } \forall\, k \in [j, l],\ j < l$$

where j and l are observed channel numbers among 0 to 1,023

Thus, $\tilde{\boldsymbol{y}}^*_B = (y^*_{0\,b}, \ldots, y^*_{1{,}023\,b})$

Therefore, a set $\tilde{\boldsymbol{y}}^*_b$ which include some imputed values within a bootstrapped replicate sample has been generated by a given equation above.

2.2.4 DIFFERENCE

By subtracting one flow cytometry histogram from the other, we obtained the difference in relative frequencies between two conditions, whether two T-cell subsets or two cell pump states, constructed on a channel by channel basis. Differences between histograms were obtained from the original and bootstrapped data sets.

Suppose that when there are two data sets named by “first” and “second”, a difference sample set $\mathcal{D}_B^*$ is

$$\mathcal{D}_B^*: = \{d_{x\,b}^*\}_{x\,=\,0',\,b\,=\,1}^{1,023\quad\ \mathcal{B}},\ d_{x\,b}^* = \tilde{\mathcal{G}}_{x\,b\,(1st)}^* - \tilde{\mathcal{G}}_{x\,b\,(2nd)}^*,$$

where $\mathcal{B}$ is the number of bootstrapped replicates

There are 100,000 bootstrapped samples in this study, each with 1,024 observations that indicate difference of the number of events between either two T-cell subsets or two cell pump states in each sample.

2.2.5 POINTWISE CONFIDENCE INTERVALS (PCI)

There are several ways to construct bootstrap confidence intervals (Efron 1979, Hall and Martin 1989, Aastveit 1990, Jöckel, Rothe et al. 1992, Efron and Tibshirani 1994, Davison and Hinkley 1997, Campbell and Torgerson 1999, Haukoos and Lewis 2005, Chernick and Wiley InterScience (Online service) 2008). The percentile method of constructing pointwise confidence intervals is a popular approach (Hall and Martin 1989, Efron and Tibshirani 1994, Polansky 1999, Imaizumi, Suzuki et al. 2006, Mandel and Betensky 2008). This method simply forms confidence intervals nonparametrically by using empirical quantiles of the bootstrap sampling distribution of the estimator.

For the PCI approach, we calculated differences between the two histograms being compared channel by channel. The set of 100,000 bootstrap samples thus yielded 100,000 differences at each fluorescence intensity channel. The 2.5th and 97.5th percentiles of sorted

differences were obtained as the 2,500th and 97,500th observations among the differences. These values provided a 95% confidence interval for the true difference at each channel.

From the difference sample set $\mathcal{D}_B^*$, observations are arranged in an ascending way in every channel:

$$\tilde{\mathcal{D}}_B^* := \{\tilde{d}_{x\,b}^*\}_{x=0,\,b=1}^{1,023,\;\mathcal{B}},$$

$\tilde{d}_{x\,1}^*$ = the minimum observation value among 100,000 observations at given channel *x*,

where *x* = (0, …, 1,023)

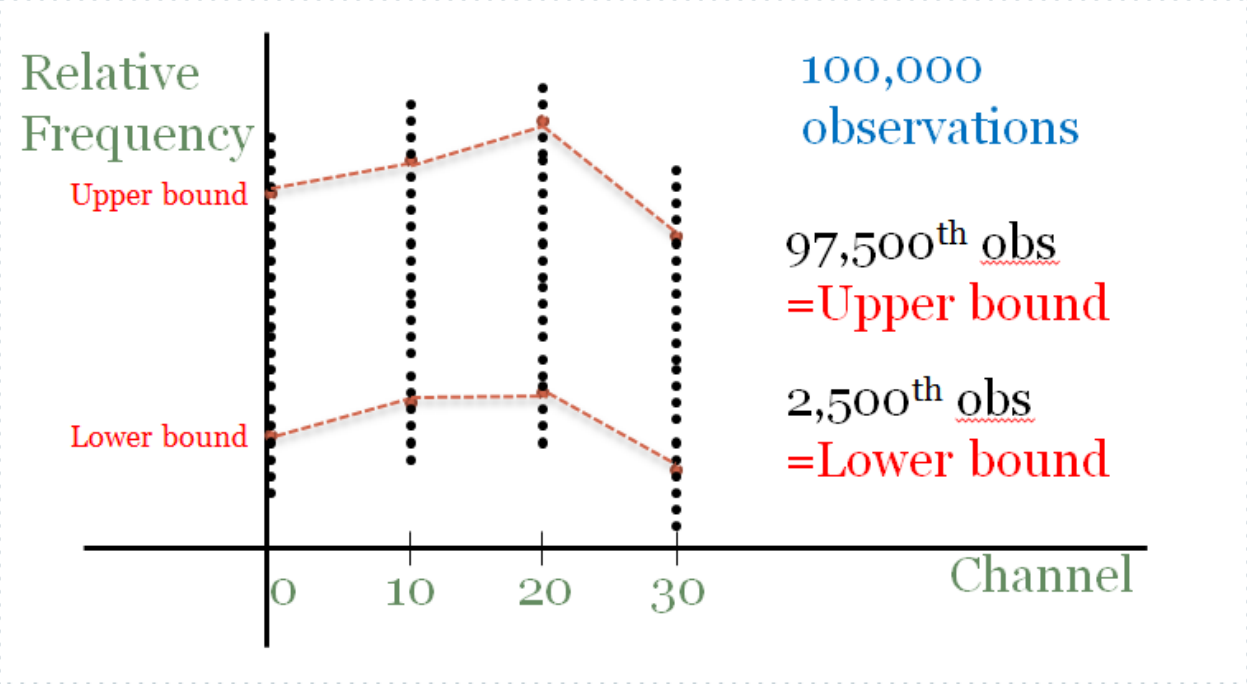
$\tilde{d}_{x\,B}^*$ = the maximum observation value among 100,000 observations at given channel *x*,

where *x* = (0, …, 1,023)

Then, The 100 × (1 - α) % percentile interval for differences is

$[\tilde{d}_{x\,(lower)}^*\,,\ \tilde{d}_{x\,(upper)}^*]$; lower = $\lfloor \mathcal{B} \times {}^{\alpha}/_{2} \rfloor$ and upper = $\lceil \mathcal{B} \times (1 - \{{}^{\alpha}/_{2}\}) \rceil$

In this study 95% percentile confidence intervals were established, as shown in Figure 3.



Pointwise confidence intervals are constructed in channels by percentile method.

Figure 3. Pointwise confidence intervals

2.2.6 SIMULTANEOUS CONFIDENCE BANDS (SCB)

Although pointwise confidence intervals (PCI) delineate confidence boundaries for each channel, they do not provide a confidence band with the property that the true histogram falls completely within the band. That is, even though the percentile confidence intervals are constructed, the simultaneous coverage probability would not be fulfilled with pointwise confidence intervals. Thus, to cover all true values which fall inside of the confidence regions, collection of the confidence intervals for all values should be obtained simultaneously. In nearly all cases, simultaneous confidence bands constitute wider contour than pointwise confidence intervals for all values. Davison and Hinkley (1997) provide an algorithm to a related simpler problem of calculating the overall coverage of simultaneous confidence intervals. Their

algorithm counts the number of bootstrap samples that fall outside the confidence region. In order to calculate a simultaneous confidence band of a pre-specified level 1 − α, one can utilize their algorithm for obtaining the target coverage.

Suppose that for all curves, indicator functions $I(\tilde{d}^*_{x\mathscr{b}})$ are defined as

$$I(\tilde{d}^*_{x\mathscr{b}})_{x=0,\mathscr{b}=1}^{1,023\quad\mathcal{B}} := \begin{cases} 1, & \tilde{d}^*_{x\ (lower)} > \tilde{d}^*_{x\mathscr{b}} \ \ or \ \ \tilde{d}^*_{x\ (upper)} < \tilde{d}^*_{x\mathscr{b}} \\ 0, & \tilde{d}^*_{x\ (lower)} < \tilde{d}^*_{x\mathscr{b}} < \tilde{d}^*_{x\ (upper)} \end{cases},$$

where lower = $\lfloor \mathcal{B} \times {}^{\alpha}/_{2} \rfloor$ and upper = $\lceil \mathcal{B} \times (1 - \{{}^{\alpha}/_{2}\}) \rceil$

The indicator variables represent the condition that a difference is outside a pointwise confidence interval. Otherwise, they take a value of 0, which means that all observed differences are located within the pointwise confidence limits.

And then, the sum of the indicator variables is

$$\sum_{x=0}^{1,023} I(\tilde{d}^*_{x\mathscr{b}})_{\mathscr{b}=1}^{\mathcal{B}} = \begin{cases} \sum_{x=0}^{1,023} I(\tilde{d}^*_{x\mathscr{b}})_{\mathscr{b}=1}^{\mathcal{B}}, & \tilde{d}^*_{x\mathscr{b}\ (lower)} > \tilde{d}^*_{x\mathscr{b}} \ \ or \ \ \tilde{d}^*_{x\mathscr{b}\ (upper)} < \tilde{d}^*_{x\mathscr{b}} \\ 0, & \tilde{d}^*_{x\mathscr{b}\ (lower)} < \tilde{d}^*_{x\mathscr{b}} < \tilde{d}^*_{x\mathscr{b}\ (upper)} \end{cases},$$

for each replicate $\mathscr{b}$, where $\mathscr{b}$ = (1, …, *B*) and $\mathcal{B}$ is the number of bootstrapped replicates

The sum of the indicator variable values within each replicate represents frequencies: how often a curve is falling outside of confidence boundaries over all channel numbers under a fixed bootstrapped replicate.

Given b bootstrap replicates, for $\forall\, x \in \{0, \ldots, 1{,}023\}$

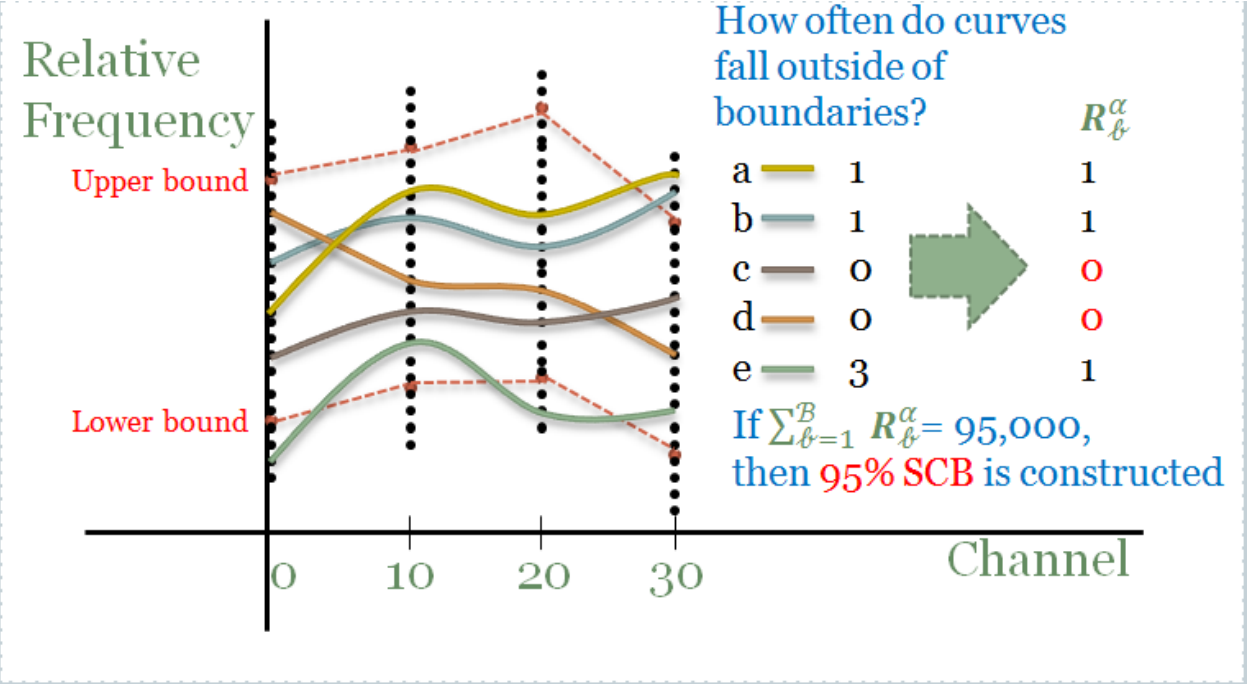
$$R_b^{\alpha} = I\left[\sum_{x=0}^{1,023} I(\tilde{d}^*_{x\,b})_{b=1}^{B} = 0\right],$$

where $I\left[\sum_{x=0}^{1,023} I(\tilde{d}^*_{x\,b})_{b=1}^{B} = 0\right]$ is an indicator function of condition that $\sum_{x=0}^{1,023} I(\tilde{d}^*_{x\,b})_{b=1}^{B} = 0$

Lastly, the error rate ε_{α} is

$$\varepsilon_{\alpha} = 1 - \frac{\sum_{b=1}^{B} R_b^{\alpha}}{B}$$

The band itself is constructed by adjusting the pointwise confidence interval alpha level until 95% of the bootstrapped histograms fall entirely within the band, as shown in Figure 4.



Simultaneous confidence bands are constructed by three steps: 1) See if there are curves falling outside of confidence intervals, 2) Sum the number of counts how many times curves fall outside of confidence region, 3) Summarize frequency of curves falling outside of CI. The number of counts of 0 specifies the number of curves stays in the confidence limits.

Figure 4. Simultaneous confidence bands

2.3 PROGRAM ALGORITHM

2.3.1 CHANNELS

The CHANNELS data set has been created as the first step of the SAS program. This process is designed to be merged with other data sets for both original and bootstrap samples. There is a CHANNEL variable which takes a range from 0 to 1023. Moreover, a CHANNELSBOOT data set is created to fit into bootstrap sample size.

2.3.2 MACRO

All data steps are processed using macros to make the code simpler to read and understand. We generated a comprehensive macro named FC_Band containing five subordinate macros: ReadFile, Bootstrap, Difference, Confidence_intervals, and Plot.

2.3.3 INPUT PARAMETER

There are five input parameters in FC_Band macro, which are ‘filename1’, ‘filename2’, ‘bootstrap’, ‘alpha1’ and ‘alpha2'. The two ‘filename’ parameters command SAS to read two different data files into the program in order to compute a difference between the two data sets. A ‘bootstrap' variable in the macro is used for specifying the size of bootstrap replicates in the SAS Bootstrap macro. Lastly, there are two alpha level parameters named by ‘alpha1’ and

‘alpha2’. Those parameters specify the level of significance when SAS constructs pointwise and simultaneous confidence intervals.

2.3.4 OUTPUT RESULTS

Using the input parameters above, SAS creates datasets to draw totally four plots of difference curves with both pointwise (Red) and simultaneous (Blue) confidence intervals. In each plot, a difference curve has been delineated with the two confidence intervals.

2.3.5 BRIEF OUTLINE

PROC IMPORT imports data files into SAS, PROC FREQ is applied for generating relative frequency of data sets, PROC EXPAND specifies linear interpolation, PROC SURVEYSELECT does bootstrapping, PROC UNIVARIATE is used for construction of confidence intervals, and PROC GPLOT draws plots in SAS output.

The SAS program is given in the Appendix.

3.0 RESULTS

Two different tests for MDR transporter states and two T-cell subsets have been compared. In this study, loading and efflux assays using R123 fluorescence dye are used to evaluate P-glycoprotein activity. It is known that frequency intensity is higher at load, while lower fluorescent intensity of the fluorescent dye is detected at efflux. Each test result from Load and Efflux in T-cell subsets consists of a difference histogram with both pointwise and simultaneous confidence intervals. Each figure is delineated with one black trace with two colored regions. The black trace shows the channel-specific difference between the histograms being compared. The red region represents pointwise confidence intervals between histograms and the blue region specifies simultaneous confidence bands. Histograms of data sets which were analyzed were given in Figures 5 and 8.

3.1 COMPARISON OF LOAD AND EFFLUX IN CD4 AND CD8 T-CELL SUBSETS

Difference histograms with pointwise confidence intervals and simultaneous confidence bands for load and efflux in CD4 and CD8 T-cell subsets are shown in Figures 6 and 9, respectively. Figure 6 conveys three messages regarding the comparison of CD4 and CD8 cells at load. First, the observed differences are in the negative direction just below channel 600 and are in the positive direction near channel 600, when the direction that differences are is in a way of subtracting CD8 from CD4. Second, the pointwise confidence intervals include 0 difference for almost all channels. Third, the simultaneous confidence bands include 0 for all channels. Although there are observed differences between the two histograms, it appears that these differences are not statistically significant. That is, we do not have evidence that the histograms are truly different in the population of cells from which these cells were drawn. In Figure 9, which shows the difference curve between CD8+ cells in LOAD and EFFLUX states, it is clear that the pointwise confidence intervals and simultaneous confidence bands indicate that there are truly differences between the histograms being compared. The difference is shown to be negative between channels 200 and 550 and positive between channels 550 and 650, based on subtracting Load to Efflux data. The fact that the simultaneous confidence band departs from 0 over a wide range of channels suggests that the observed difference is outside the realm of chance. In Table 3, we compare our results to results of other test statistics. The K-S test and Cox et al.'s method concluded that two T-cell subsets during LOAD incubation are statistically significantly different. However, this conclusion is opposite to an expectation where the

difference is not detected in biology. In Figure 7 and 10, PCI shows that there are some channel points not including zero within it, whereas SCB takes zero value into overall channels. Table 1 and 2 are provided as numerical reference with corresponding to channel area in Figure 7 and 10.

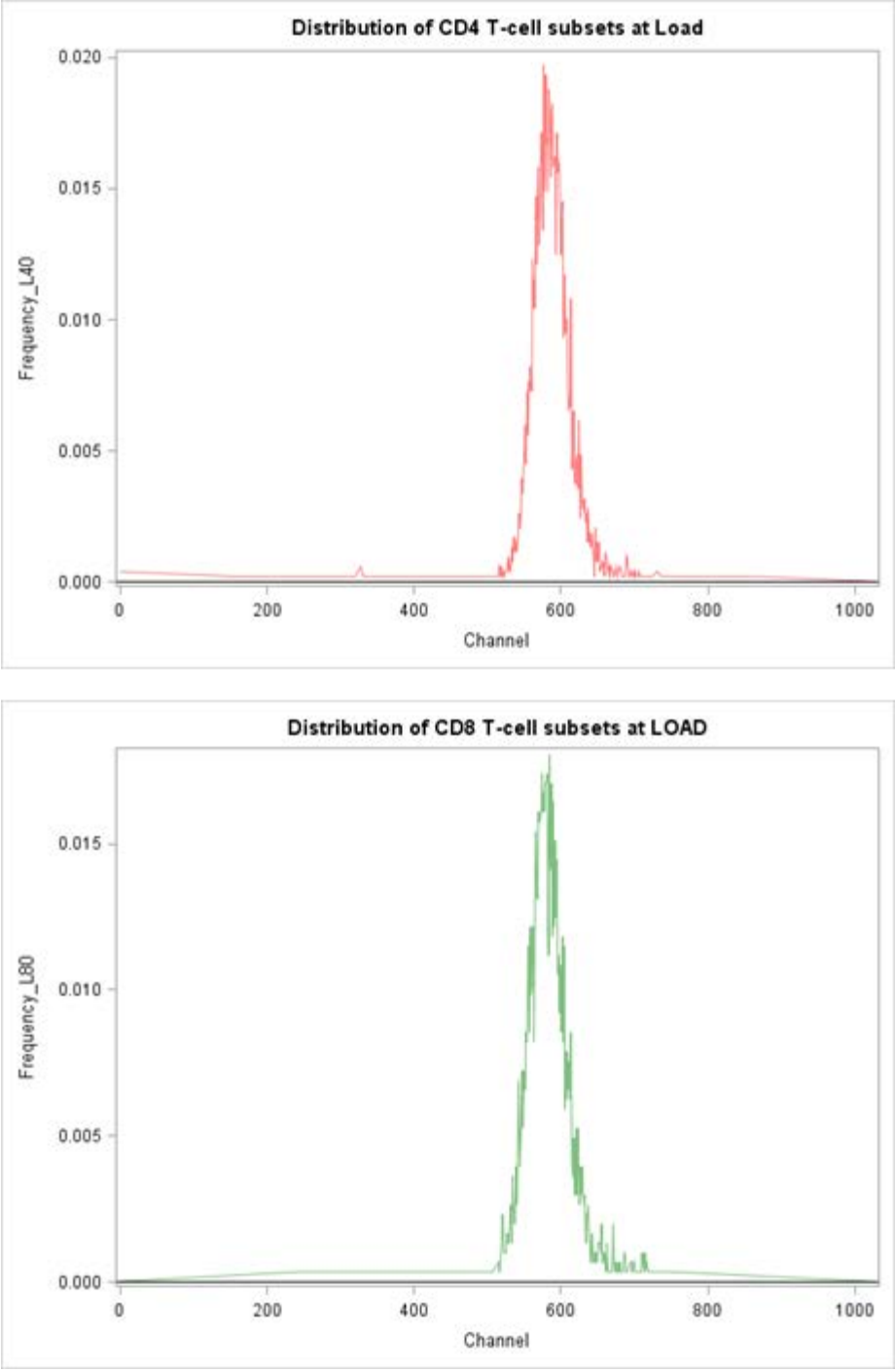
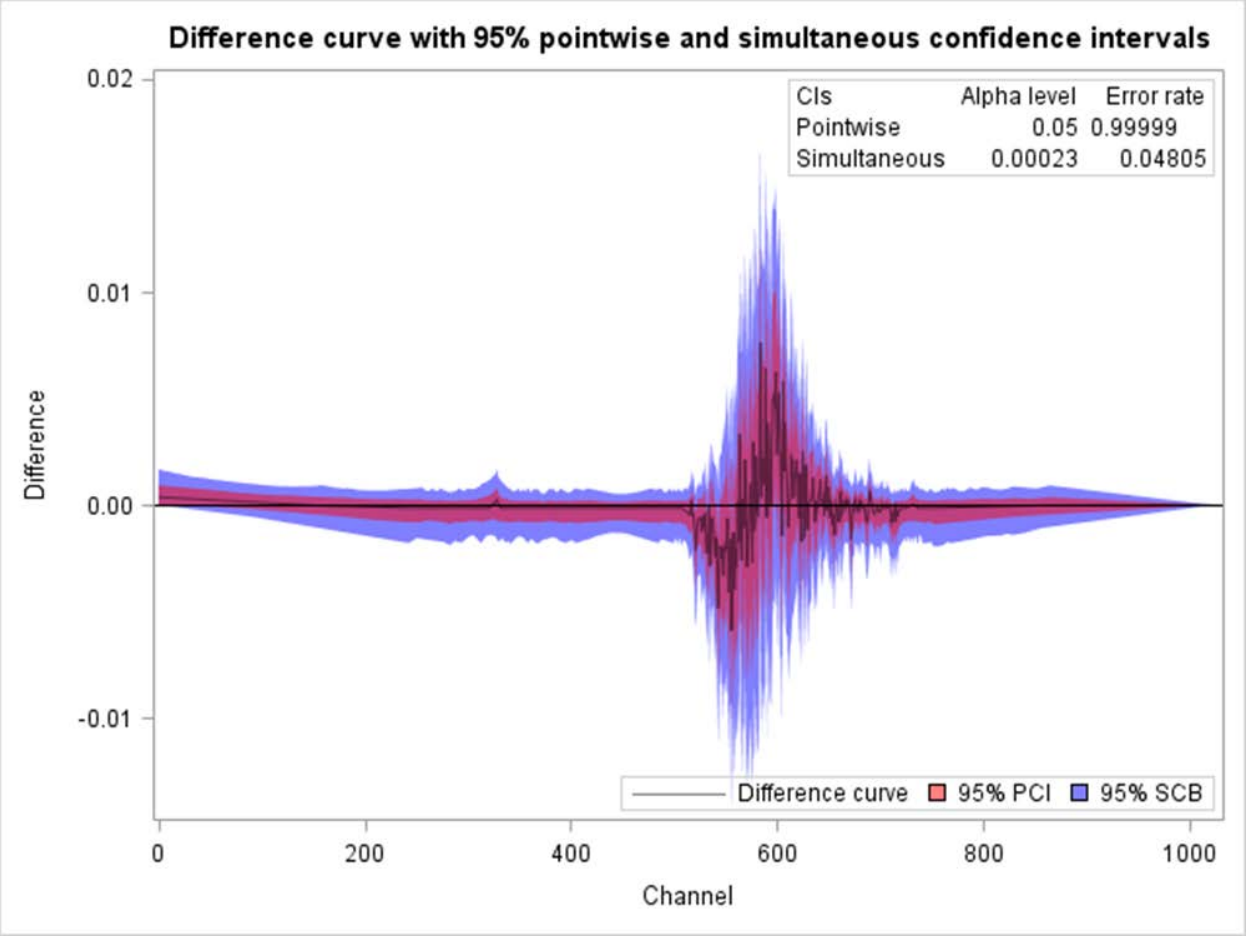
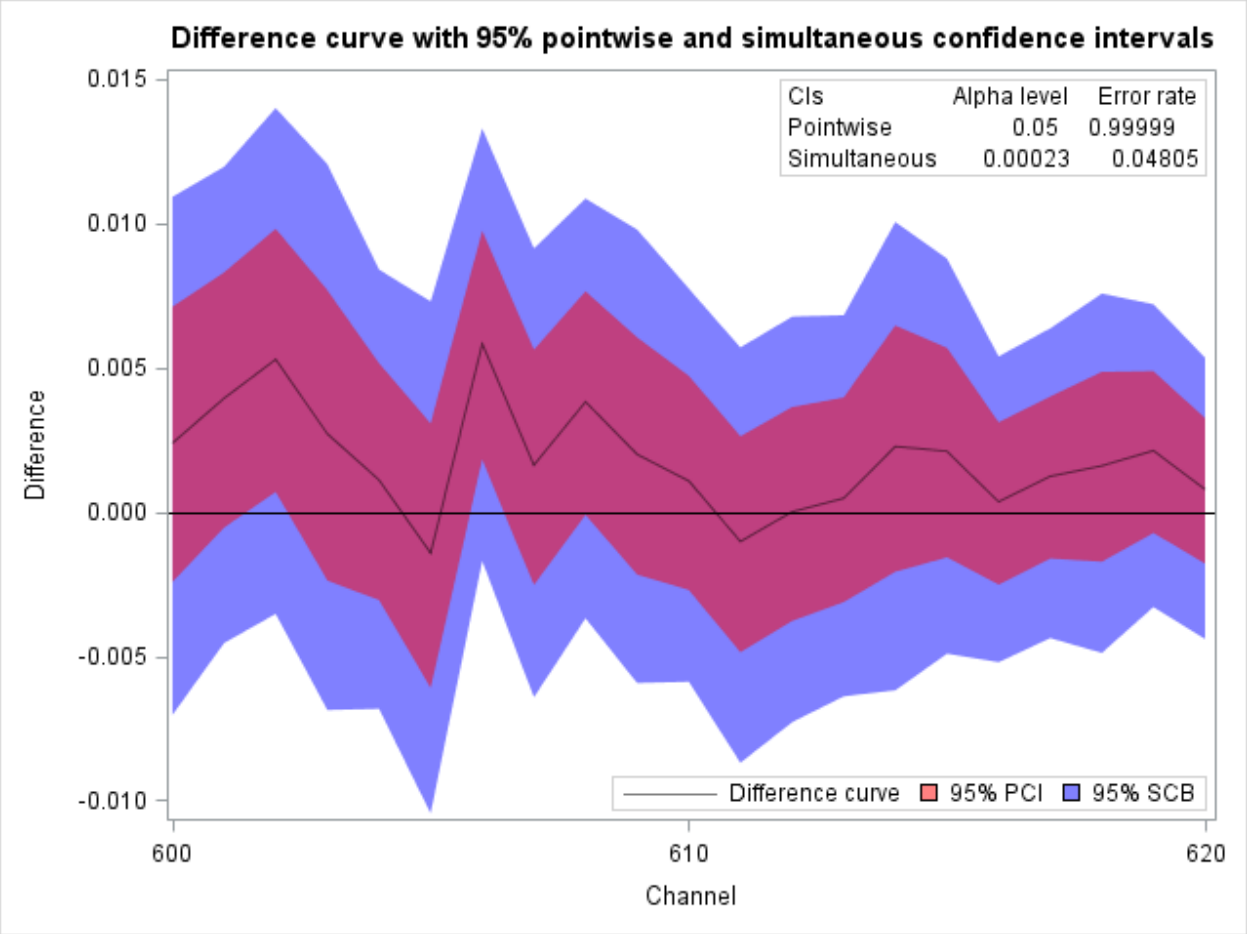


Figure 5. R123 MFI in CD4+ and CD8+ T-cell subsets at LOAD



95% pointwised and simultaneous Confidence Bands of differences between the LOAD in CD4 and CD8 T cells. The black line shows a difference curve and pointwised and simultaneous confidence intervals are drawn as red and blue lines, respectively.

Figure 6. Comparison between CD4+ and CD8+ T-cell subsets at LOAD



95% pointwised and simultaneous Confidence Bands of differences between the LOAD in CD4 and CD8 T cells from channel 600 to 620. The black line shows a difference curve and pointwised and simultaneous confidence intervals are drawn as red and blue lines, respectively.

Figure 7. Highlighted area of Figure 6 to compare test results between PCI and SCB

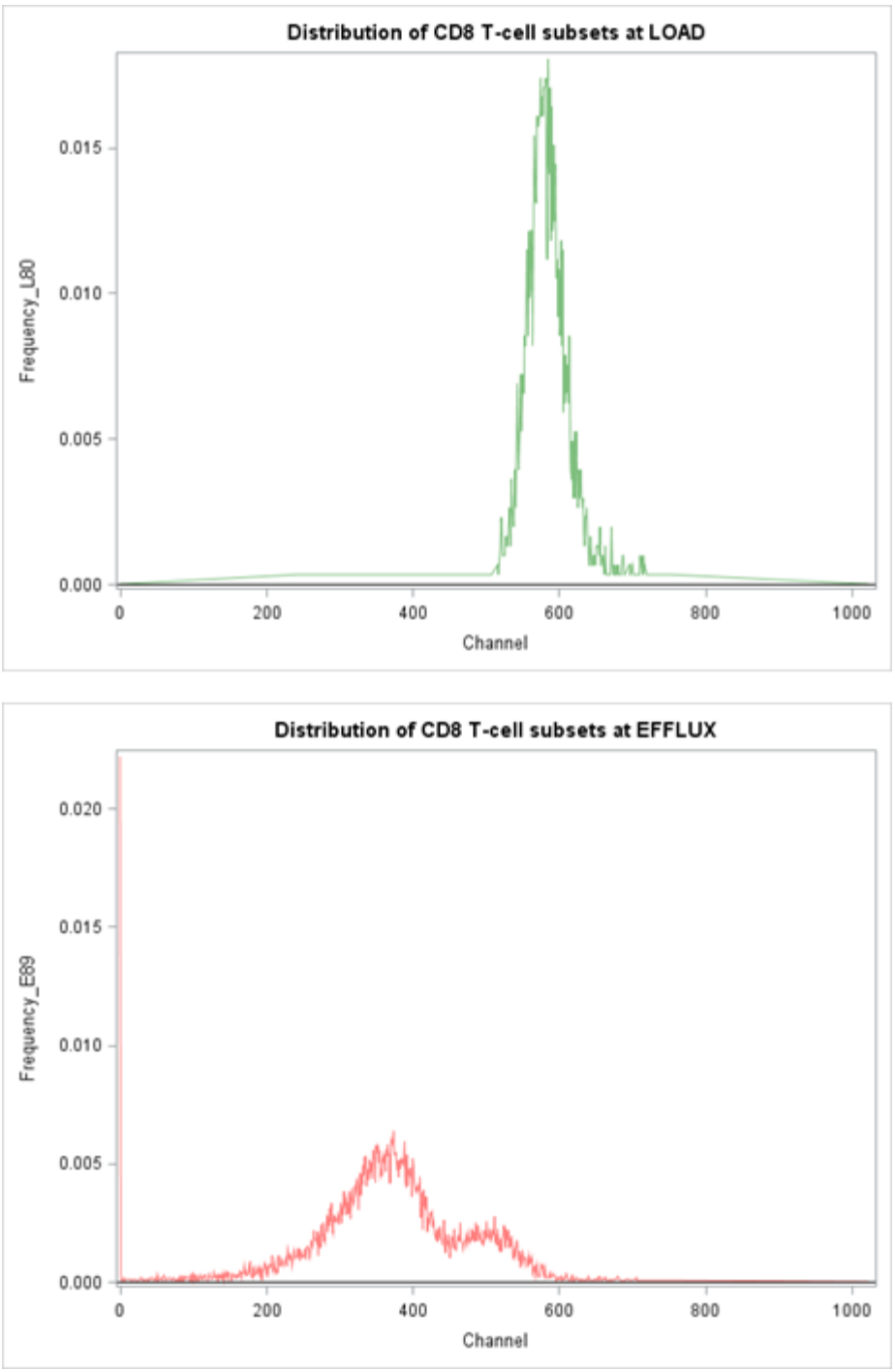
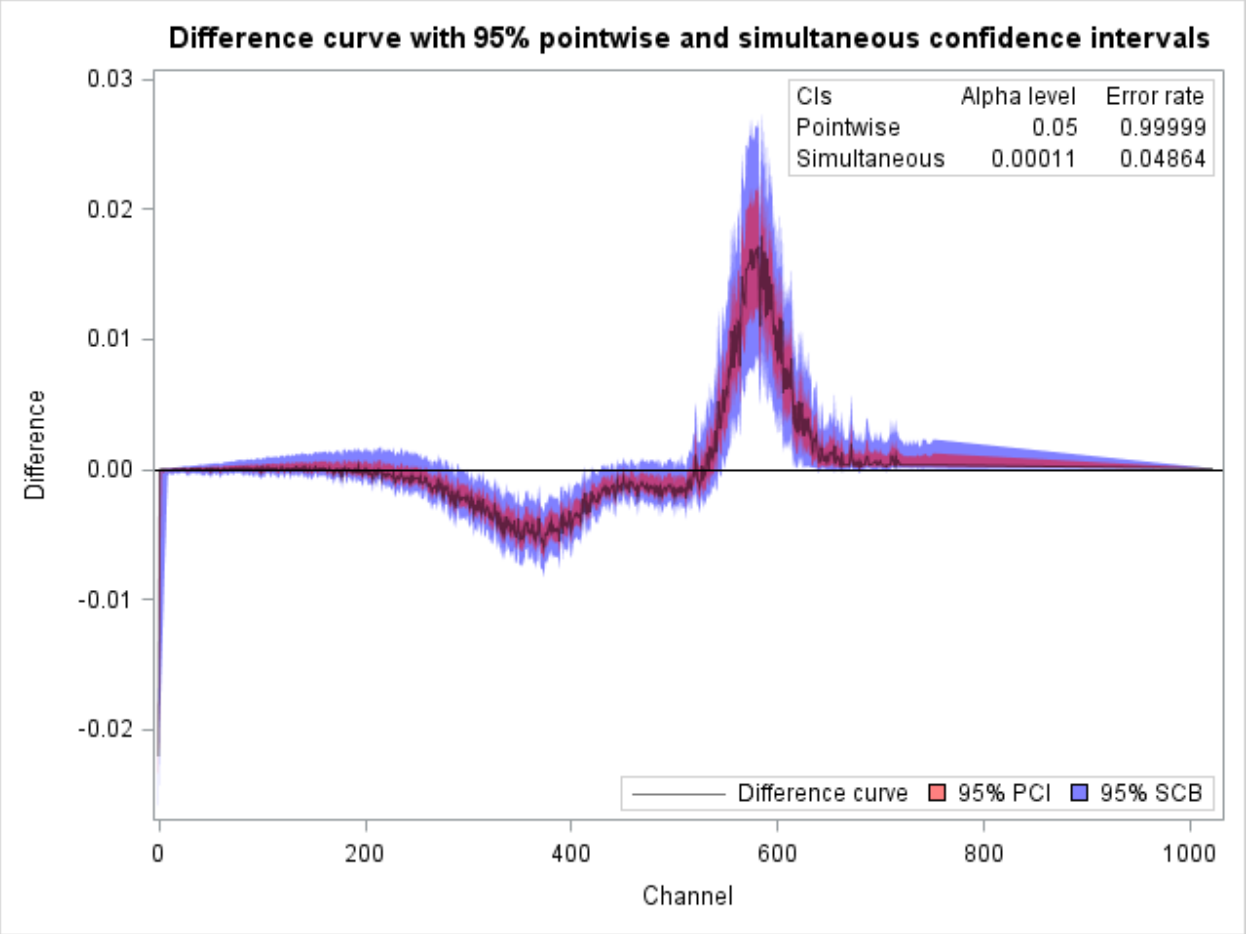
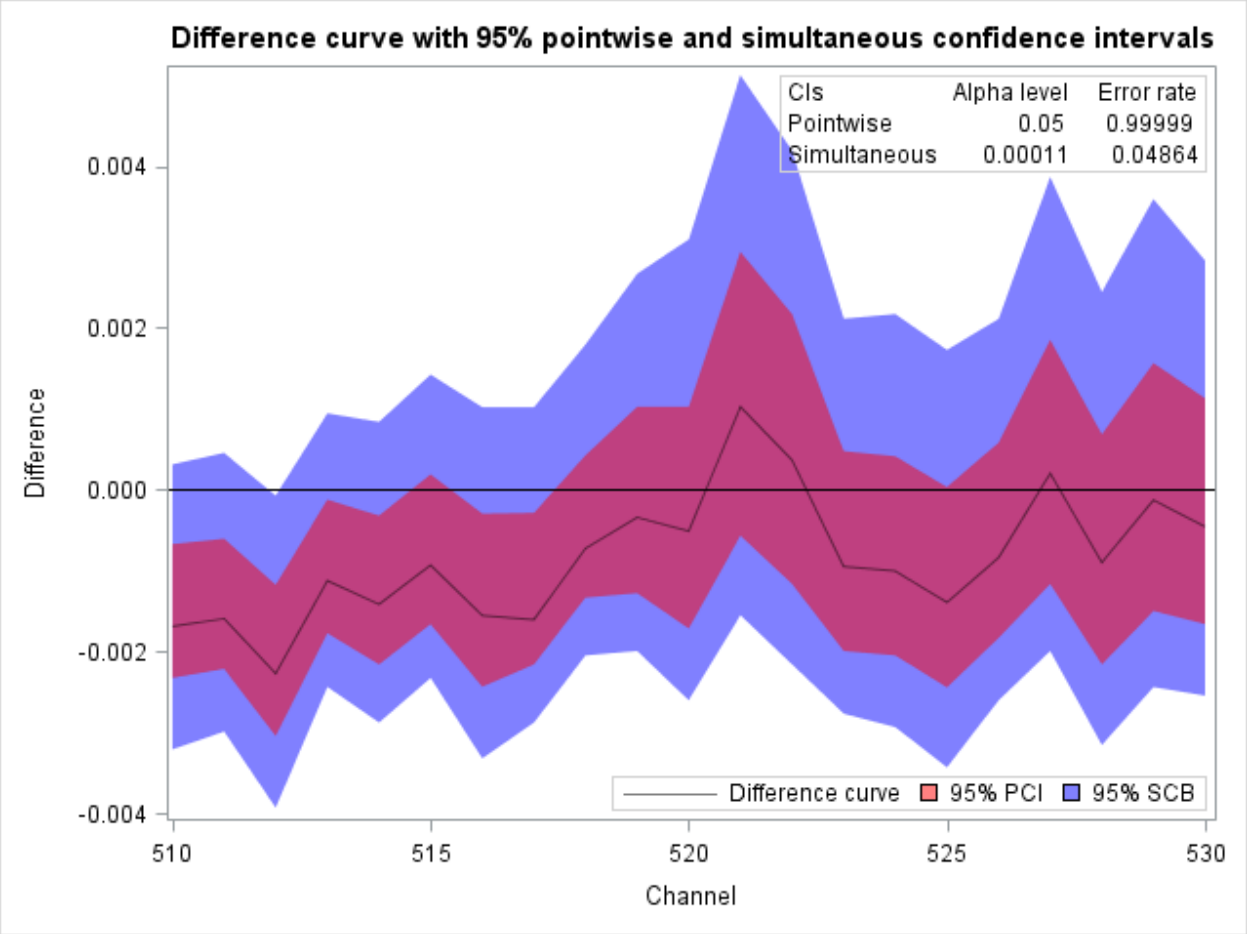


Figure 8. R123 MFI at LOAD and EFFLUX states in CD8+ T-cell subsets



95% pointwised and simultaneous Confidence Bands of differences between CD8 T-cell subsets in LOAD and EFFLUX states. The black line shows a difference curve and pointwised and simultaneous confidence intervals are drawn as red and blue lines, respectively.

Figure 9. Comparison between Load and Efflux states in CD8+ T-cell subsets



95% pointwised and simultaneous Confidence Bands of differences between the LOAD in CD4 and CD8 T cells from channel 600 to 620. The black line shows a difference curve and pointwised and simultaneous confidence intervals are drawn as red and blue lines, respectively.

Figure 10. Highlighted area of Figure 9 to compare test results between PCI and SCB

Table 1. Comparison of confidence intervals by Cox et al.'s approach to PCI and SCB limits between CD4+ and CD8+ T-cell subsets at LOAD

Channel	Difference	Cox et al		Percentile		Simultaneous	
		Lower	Upper	Lower	Upper	Lower	Upper
600	2.38E-03	-2.42E-03	7.18E-03	-2.44E-03	7.10E-03	-7.04E-03	1.09E-02
601	3.93E-03	-5.00E-04	8.36E-03	-5.51E-04	8.30E-03	-4.55E-03	1.20E-02
602	5.28E-03	6.68E-04	9.89E-03	6.86E-04	9.80E-03	-3.55E-03	1.40E-02
603	2.70E-03	-2.32E-03	7.72E-03	-2.39E-03	7.67E-03	-6.88E-03	1.20E-02
604	1.10E-03	-3.02E-03	5.22E-03	-3.07E-03	5.14E-03	-6.84E-03	8.39E-03
605	-1.44E-03	-6.09E-03	3.21E-03	-6.10E-03	3.06E-03	-1.05E-02	7.29E-03
606	5.81E-03	1.84E-03	9.79E-03	1.81E-03	9.73E-03	-1.71E-03	1.33E-02
607	1.61E-03	-2.47E-03	5.70E-03	-2.54E-03	5.61E-03	-6.43E-03	9.12E-03
608	3.81E-03	-6.55E-05	7.68E-03	-1.15E-04	7.64E-03	-3.70E-03	1.09E-02
609	1.98E-03	-2.13E-03	6.10E-03	-2.18E-03	6.04E-03	-5.94E-03	9.78E-03
610	1.06E-03	-2.64E-03	4.77E-03	-2.72E-03	4.71E-03	-5.90E-03	7.73E-03
611	-1.03E-03	-4.80E-03	2.73E-03	-4.87E-03	2.62E-03	-8.71E-03	5.69E-03
612	-6.64E-06	-3.69E-03	3.68E-03	-3.79E-03	3.62E-03	-7.30E-03	6.76E-03
613	4.63E-04	-3.09E-03	4.02E-03	-3.13E-03	3.96E-03	-6.40E-03	6.80E-03
614	2.26E-03	-2.04E-03	6.55E-03	-2.08E-03	6.46E-03	-6.19E-03	1.00E-02
615	2.09E-03	-1.53E-03	5.72E-03	-1.58E-03	5.68E-03	-4.92E-03	8.77E-03
616	3.41E-04	-2.49E-03	3.17E-03	-2.53E-03	3.10E-03	-5.22E-03	5.38E-03
617	1.23E-03	-1.60E-03	4.05E-03	-1.63E-03	3.99E-03	-4.38E-03	6.35E-03
618	1.59E-03	-1.70E-03	4.88E-03	-1.73E-03	4.85E-03	-4.89E-03	7.56E-03
619	2.11E-03	-7.12E-04	4.94E-03	-7.40E-04	4.87E-03	-3.31E-03	7.19E-03
620	7.67E-04	-1.76E-03	3.29E-03	-1.80E-03	3.24E-03	-4.41E-03	5.34E-03

95% confidence intervals from Cox et al.'s approach were compared with pointwise confidence intervals and simultaneous confidence bands for differences between two T-cell subsets at LOAD states. Channel numbers were selected (600 to 620) with corresponding to a range in Figure 7.

Table 2. Comparison of confidence intervals by Cox et al.'s approach to PCI and SCB limits between LOAD and EFFLUX states in CD8+ T-cell subsets

Channel	Difference	Cox et al		Pointwise		Simultaneous	
		Lower	Upper	Lower	Upper	Lower	Upper
510	-1.69E-03	-2.70E-03	-8.47E-04	-2.33E-03	-6.75E-04	-3.21E-03	3.13E-04
511	-1.60E-03	-2.64E-03	-7.98E-04	-2.22E-03	-6.07E-04	-2.99E-03	4.55E-04
512	-2.27E-03	-3.30E-03	-9.20E-04	-3.05E-03	-1.18E-03	-3.93E-03	-7.40E-05
513	-1.13E-03	-2.09E-03	8.23E-05	-1.77E-03	-1.24E-04	-2.44E-03	9.43E-04
514	-1.42E-03	-2.45E-03	-2.18E-04	-2.16E-03	-3.21E-04	-2.88E-03	8.37E-04
515	-9.34E-04	-1.97E-03	1.82E-04	-1.66E-03	1.88E-04	-2.33E-03	1.42E-03
516	-1.56E-03	-2.69E-03	-4.18E-04	-2.44E-03	-2.96E-04	-3.32E-03	1.02E-03
517	-1.61E-03	-2.52E-03	-7.00E-04	-2.16E-03	-2.88E-04	-2.88E-03	1.02E-03
518	-7.27E-04	-1.78E-03	3.31E-04	-1.33E-03	4.23E-04	-2.05E-03	1.79E-03
519	-3.44E-04	-1.58E-03	8.90E-04	-1.28E-03	1.02E-03	-1.99E-03	2.67E-03
520	-5.13E-04	-1.94E-03	9.15E-04	-1.72E-03	1.02E-03	-2.60E-03	3.09E-03
521	1.02E-03	-7.55E-04	2.80E-03	-5.69E-04	2.94E-03	-1.55E-03	5.12E-03
522	3.63E-04	-1.32E-03	2.04E-03	-1.17E-03	2.17E-03	-2.16E-03	4.19E-03
523	-9.52E-04	-2.24E-03	3.33E-04	-1.99E-03	4.74E-04	-2.77E-03	2.11E-03
524	-1.01E-03	-2.30E-03	2.82E-04	-2.05E-03	4.11E-04	-2.93E-03	2.17E-03
525	-1.39E-03	-2.71E-03	-7.37E-05	-2.45E-03	3.17E-05	-3.43E-03	1.73E-03
526	-8.41E-04	-2.12E-03	4.34E-04	-1.83E-03	5.81E-04	-2.60E-03	2.11E-03
527	2.02E-04	-1.34E-03	1.74E-03	-1.17E-03	1.85E-03	-1.99E-03	3.86E-03
528	-9.01E-04	-2.36E-03	5.56E-04	-2.16E-03	6.83E-04	-3.16E-03	2.44E-03
529	-1.30E-04	-1.69E-03	1.43E-03	-1.50E-03	1.56E-03	-2.44E-03	3.59E-03
530	-4.58E-04	-1.88E-03	9.66E-04	-1.66E-03	1.13E-03	-2.55E-03	2.83E-03

95% confidence intervals from Cox et al.'s approach were compared with pointwise confidence intervals and simultaneous confidence bands for differences between two T-cell subsets at LOAD states. Channel numbers were selected (510 to 530) with corresponding to a range in Figure 10.

Table 3. Test of significance by K-S test and Cox et al.'s approach for CD4+ and CD8+ T cells in LOAD and EFFLUX states

Test of significance by K-S test and Cox et al.'s approach

CD4 vs CD8 in LOAD

Kolmogorov-Smirnov Two-Sample Test (Asymptotic)			
KS	0.186523	D	0.373047
KSa	8.441087	Pr > KSa	**<.0001**

Chi-Square Test for Equal Proportions	
Chi-Square	186.9673
DF	117
Pr > ChiSq	**<.0001**

LOAD vs EFFLUX

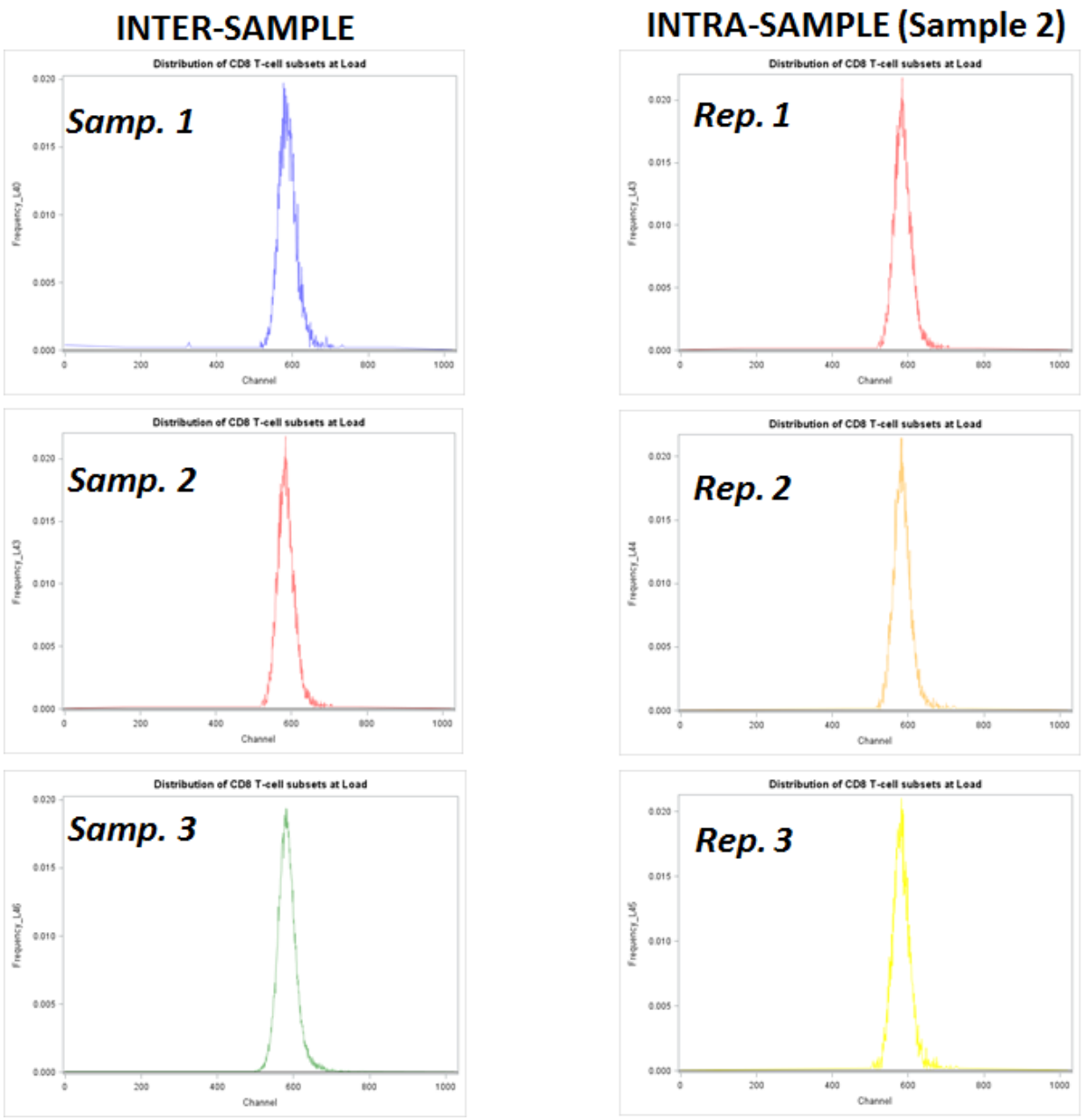
Kolmogorov-Smirnov Two-Sample Test (Asymptotic)			
KS	0.154297	D	0.308594
KSa	6.982679	Pr > KSa	**<.0001**

Chi-Square Test for Equal Proportions	
Chi-Square	6909.084
DF	117
Pr > ChiSq	**<.0001**

The overall test of significance from K-S test and Cox et al.'s approach were processed and shown to having different results against SCB. Both test statistics conclude that the two cases of comparison turns out to be statistically significant.

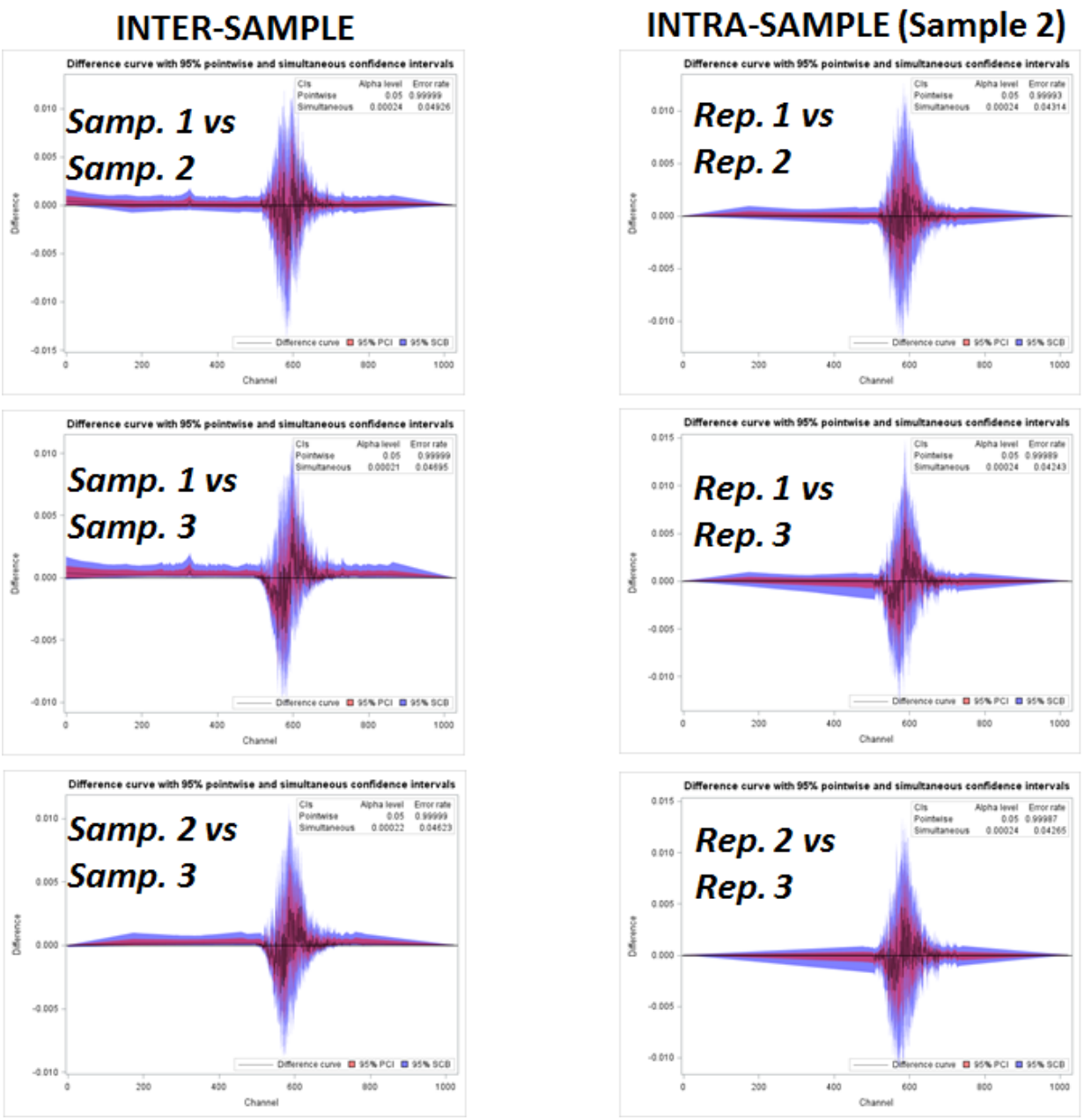
3.2 COMPARISON OF REPLICATE R123 LOADING IN CD4 T-CELL SUBSESTS

Assay variability was examined by creating difference histograms for inter-sample (left column) and intra-sample (right column) determinations. Inter-sample determinations were made on 3 separately prepared blood samples from the same individual, prepared and acquired on the flow cytometer on the same day. Histograms of inter- and intra-sample are shown in Figure 11. For intra-sample comparisons (right column), the second sample was divided into 3 replicates after sample preparation, and analyzed individually on the flow cytometer. Difference histograms with pointwise confidence intervals (red) and simultaneous confidence bands (blue) are shown in Figure 12. No significant differences were observed between samples or replicates. In Table 6, we compare test results from the K-S test and Cox et al.'s approach for inter- and intra-sample cases. Both procedures conclude that the two T-cell subsets during LOAD incubation are statistically significantly different, which is not expected because the difference is not biologically significant. In Figure 12, simultaneous confidence bands include zero for all inter- and intra-sample cases. We highlighted some channels for which pointwise confidence intervals do not include 0 in Figure 13. Tables 4 and 5 are provided as numerical references for corresponding channels in Figure 13.



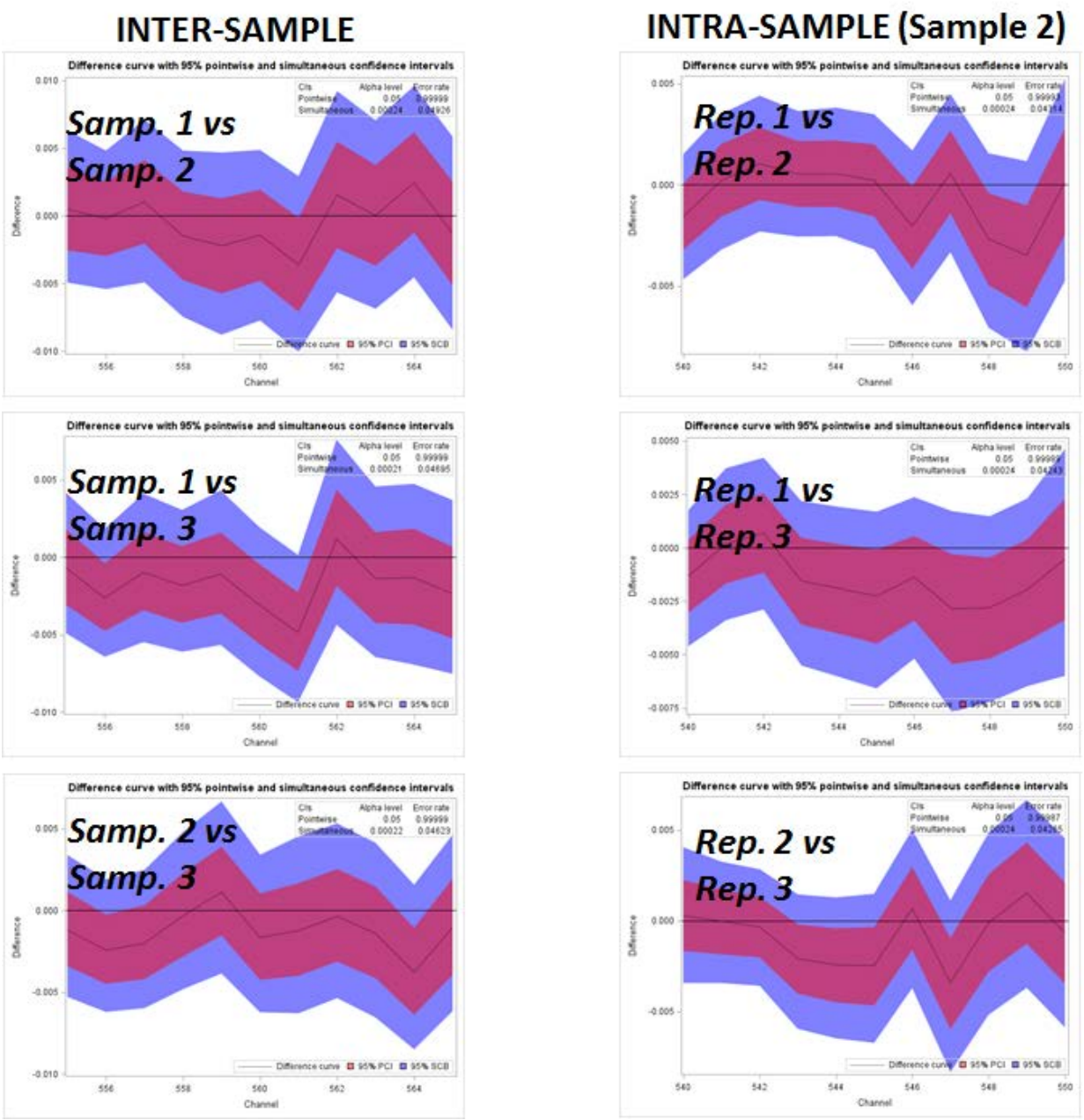
R123 frequency distributions are provided with 1) inter-sample: blue – sample 1; red – sample 2; green – sample 3, and 2) intra-sample: red – replicate 1; orange – replicate 2; yellow – replicate 3

Figure 11. Distribution histograms of inter- and intra-sample



95% pointwised and simultaneous Confidence Bands of differences between inter-sample and inter-sample of R123 Loading. In all case of comparisons,no diifferences between two R123 Loading samples are observed.

Figure 12. Intra-sample and inter-sample variability



95% pointwised and simultaneous Confidence Bands of differences between the LOAD in CD4 and CD8 T cells for inter-sample (channel 555 to 565) and intra-sample (channel 540 to 550). The black line shows a difference curve and pointwised and simultaneous confidence intervals are drawn as red and blue lines, respectively.

Figure 13. Highlighted area of Figure 12 to compare test results between PCI and SCB

Table 4. Comparison of confidence intervals by Cox et al.'s approach to PCI and SCB limits for inter-sample

Inter-sample

Sample Comparison	Channel	Difference	Cox et al		Pointwise		Simultaneous	
			Lower	Upper	Lower	Upper	Lower	Upper
Sample 1 vs Sample 2	555	4.89E-04	-2.59E-03	3.57E-03	-2.57E-03	3.57E-03	-4.94E-03	6.32E-03
	556	-1.95E-04	-2.96E-03	2.57E-03	-2.96E-03	2.55E-03	-5.43E-03	4.83E-03
	557	1.03E-03	-2.08E-03	4.13E-03	-2.05E-03	4.12E-03	-4.93E-03	7.12E-03
	558	-1.49E-03	-4.76E-03	1.78E-03	-4.75E-03	1.79E-03	-7.47E-03	4.84E-03
	559	-2.21E-03	-5.73E-03	1.32E-03	-5.74E-03	1.30E-03	-8.80E-03	4.69E-03
	560	-1.43E-03	-4.81E-03	1.95E-03	-4.81E-03	1.93E-03	-7.75E-03	4.88E-03
	561	-3.63E-03	-7.11E-03	-1.59E-04	-7.10E-03	-1.46E-04	-1.01E-02	2.94E-03
	562	1.55E-03	-2.39E-03	5.49E-03	-2.39E-03	5.49E-03	-5.66E-03	9.24E-03
	563	2.17E-05	-3.72E-03	3.77E-03	-3.69E-03	3.76E-03	-6.88E-03	7.07E-03
	564	2.46E-03	-1.28E-03	6.20E-03	-1.24E-03	6.19E-03	-4.55E-03	9.62E-03
	565	-1.30E-03	-5.15E-03	2.56E-03	-5.14E-03	2.52E-03	-8.45E-03	5.86E-03
Sample 1 vs Sample 3	555	-7.16E-04	-3.17E-03	1.74E-03	-3.08E-03	1.79E-03	-4.93E-03	4.16E-03
	556	-2.64E-03	-4.85E-03	-4.35E-04	-4.77E-03	-4.00E-04	-6.44E-03	1.84E-03
	557	-9.87E-04	-3.51E-03	1.54E-03	-3.43E-03	1.62E-03	-5.49E-03	4.09E-03
	558	-1.83E-03	-4.31E-03	6.47E-04	-4.24E-03	7.14E-04	-6.11E-03	3.05E-03
	559	-1.10E-03	-3.72E-03	1.51E-03	-3.64E-03	1.58E-03	-5.67E-03	4.30E-03
	560	-3.09E-03	-5.69E-03	-4.96E-04	-5.60E-03	-4.54E-04	-7.70E-03	1.94E-03
	561	-4.88E-03	-7.43E-03	-2.34E-03	-7.35E-03	-2.28E-03	-9.37E-03	1.41E-04
	562	1.17E-03	-1.98E-03	4.33E-03	-1.87E-03	4.35E-03	-4.38E-03	7.59E-03
	563	-1.39E-03	-4.33E-03	1.56E-03	-4.24E-03	1.61E-03	-6.45E-03	4.56E-03
	564	-1.33E-03	-4.43E-03	1.77E-03	-4.36E-03	1.82E-03	-6.95E-03	4.73E-03
	565	-2.36E-03	-5.33E-03	6.04E-04	-5.24E-03	6.67E-04	-7.53E-03	3.68E-03
Sample 2 vs Sample 3	555	-1.21E-03	-3.47E-03	1.06E-03	-3.41E-03	1.11E-03	-5.30E-03	3.38E-03
	556	-2.45E-03	-4.58E-03	-3.16E-04	-4.50E-03	-2.59E-04	-6.22E-03	1.90E-03
	557	-2.01E-03	-4.27E-03	2.46E-04	-4.20E-03	2.98E-04	-5.98E-03	2.47E-03
	558	-3.44E-04	-2.90E-03	2.21E-03	-2.82E-03	2.26E-03	-4.83E-03	4.79E-03
	559	1.11E-03	-1.64E-03	3.86E-03	-1.54E-03	3.88E-03	-3.86E-03	6.66E-03
	560	-1.67E-03	-4.32E-03	9.83E-04	-4.25E-03	1.03E-03	-6.24E-03	3.39E-03
	561	-1.25E-03	-4.11E-03	1.62E-03	-4.00E-03	1.65E-03	-6.30E-03	4.52E-03
	562	-3.78E-04	-3.20E-03	2.45E-03	-3.14E-03	2.51E-03	-5.37E-03	5.31E-03
	563	-1.41E-03	-4.21E-03	1.39E-03	-4.14E-03	1.45E-03	-6.55E-03	4.12E-03
	564	-3.79E-03	-6.46E-03	-1.12E-03	-6.39E-03	-1.07E-03	-8.51E-03	1.56E-03
	565	-1.07E-03	-4.03E-03	1.90E-03	-3.94E-03	1.94E-03	-6.17E-03	4.62E-03

Table 5. Comparison of confidence intervals by Cox et al.'s approach to PCI and SCB limits for intra-sample

Intra-sample

Sample Comparison	Channel	Difference	Cox et al		Pointwise		Simultaneous	
			Lower	Upper	Lower	Upper	Lower	Upper
Replicate 1 vs Replicate 2	540	-1.58E-03	-3.26E-03	1.05E-04	-3.22E-03	5.47E-05	-4.67E-03	1.52E-03
	541	2.20E-04	-1.56E-03	2.00E-03	-1.57E-03	2.02E-03	-3.20E-03	3.51E-03
	542	1.03E-03	-7.22E-04	2.79E-03	-7.52E-04	2.83E-03	-2.31E-03	4.40E-03
	543	5.35E-04	-1.07E-03	2.14E-03	-1.09E-03	2.17E-03	-2.56E-03	3.65E-03
	544	5.38E-04	-1.12E-03	2.20E-03	-1.10E-03	2.18E-03	-2.54E-03	3.81E-03
	545	2.16E-04	-1.51E-03	1.94E-03	-1.56E-03	2.00E-03	-3.19E-03	3.48E-03
	546	-2.05E-03	-4.09E-03	-5.81E-06	-4.15E-03	-8.11E-05	-5.95E-03	1.70E-03
	547	5.64E-04	-1.48E-03	2.61E-03	-1.41E-03	2.67E-03	-3.34E-03	4.49E-03
	548	-2.68E-03	-4.95E-03	-4.05E-04	-4.96E-03	-4.11E-04	-7.06E-03	1.55E-03
	549	-3.47E-03	-5.98E-03	-9.68E-04	-6.05E-03	-1.02E-03	-8.25E-03	1.17E-03
	550	1.28E-04	-2.55E-03	2.80E-03	-2.49E-03	2.76E-03	-4.77E-03	5.21E-03
Replicate 1 vs Replicate 3	540	-1.32E-03	-3.00E-03	3.62E-04	-3.03E-03	3.60E-04	-4.61E-03	1.77E-03
	541	1.85E-04	-1.64E-03	2.01E-03	-1.67E-03	2.03E-03	-3.39E-03	3.73E-03
	542	6.90E-04	-1.16E-03	2.54E-03	-1.17E-03	2.55E-03	-2.89E-03	4.22E-03
	543	-1.55E-03	-3.56E-03	4.68E-04	-3.60E-03	4.63E-04	-5.51E-03	2.19E-03
	544	-1.91E-03	-4.03E-03	2.18E-04	-4.02E-03	1.64E-04	-6.04E-03	1.92E-03
	545	-2.26E-03	-4.44E-03	-7.82E-05	-4.48E-03	-8.34E-05	-6.58E-03	1.69E-03
	546	-1.37E-03	-3.36E-03	6.13E-04	-3.40E-03	5.46E-04	-5.19E-03	2.37E-03
	547	-2.86E-03	-5.44E-03	-2.75E-04	-5.44E-03	-3.00E-04	-7.69E-03	1.72E-03
	548	-2.80E-03	-5.16E-03	-4.48E-04	-5.20E-03	-4.55E-04	-7.22E-03	1.48E-03
	549	-1.96E-03	-4.33E-03	4.13E-04	-4.36E-03	3.91E-04	-6.48E-03	2.30E-03
	550	-5.37E-04	-3.35E-03	2.27E-03	-3.38E-03	2.29E-03	-6.00E-03	4.62E-03
Replicate 2 vs Replicate 3	540	2.60E-04	-1.69E-03	2.21E-03	-1.67E-03	2.22E-03	-3.43E-03	4.05E-03
	541	-3.46E-05	-1.81E-03	1.74E-03	-1.85E-03	1.71E-03	-3.44E-03	3.25E-03
	542	-3.43E-04	-2.00E-03	1.31E-03	-2.03E-03	1.31E-03	-3.59E-03	2.83E-03
	543	-2.08E-03	-4.00E-03	-1.60E-04	-4.02E-03	-2.10E-04	-5.96E-03	1.44E-03
	544	-2.45E-03	-4.62E-03	-5.95E-04	-4.51E-03	-4.27E-04	-6.48E-03	1.28E-03
	545	-2.47E-03	-4.62E-03	-3.33E-04	-4.67E-03	-3.64E-04	-6.73E-03	1.48E-03
	546	6.74E-04	-1.88E-03	2.58E-03	-1.61E-03	2.96E-03	-3.72E-03	5.02E-03
	547	-3.42E-03	-6.06E-03	-1.10E-03	-5.97E-03	-9.59E-04	-8.35E-03	1.12E-03
	548	-1.25E-04	-3.80E-03	1.29E-03	-2.80E-03	2.55E-03	-5.19E-03	4.84E-03
	549	1.52E-03	-1.70E-03	3.76E-03	-1.26E-03	4.30E-03	-3.70E-03	6.65E-03
	550	-6.65E-04	-3.45E-03	2.12E-03	-3.45E-03	2.10E-03	-5.88E-03	4.53E-03

Table 6. Test of significance by K-S test and Cox et al.'s approach for inter- and intra-sample

Test of significance by K-S test and Cox et al.'s approach

Inter-sample				Intra-sample			
Sample 1 vs Sample 2				Replicate 1 vs Replicate 2			
Kolmogorov-Smirnov Two-Sample Test (Asymptotic)				Kolmogorov-Smirnov Two-Sample Test (Asymptotic)			
KS	0.349609	D	0.699219	KS	0.206055	D	0.412109
KSa	15.821514	Pr > KSa	**<.0001**	KSa	9.324971	Pr > KSa	**<.0001**
Chi-Square Test for Equal Proportions				Chi-Square Test for Equal Proportions			
Chi-Square			202.7839107	Chi-Square			129.3706191
DF			90	DF			90
Pr > ChiSq			**<.0001**	Pr > ChiSq			**<.0001**
Sample 1 vs Sample 3				Replicate 1 vs Replicate 3			
Kolmogorov-Smirnov Two-Sample Test (Asymptotic)				Kolmogorov-Smirnov Two-Sample Test (Asymptotic)			
KS	0.375488	D	0.750977	KS	0.151367	D	0.302734
KSa	16.99266	Pr > KSa	**<.0001**	KSa	6.850097	Pr > KSa	**<.0001**
Chi-Square Test for Equal Proportions				Chi-Square Test for Equal Proportions			
Chi-Square			421.8090844	Chi-Square			199.5725173
DF			90	DF			90
Pr > ChiSq			**<.0001**	Pr > ChiSq			**<.0001**
Sample 2 vs Sample 3				Replicate 2 vs Replicate 3			
Kolmogorov-Smirnov Two-Sample Test (Asymptotic)				Kolmogorov-Smirnov Two-Sample Test (Asymptotic)			
KS	0.339844	D	0.679688	KS	0.058594	D	0.117188
KSa	15.379572	Pr > KSa	**<.0001**	KSa	2.651650	Pr > KSa	**<.0001**
Chi-Square Test for Equal Proportions				Chi-Square Test for Equal Proportions			
Chi-Square			358.041764	Chi-Square			133.6344351
DF			90	DF			90
Pr > ChiSq			**<.0001**	Pr > ChiSq			**<.0001**

4.0 CONCLUSION

Comparing changes in MFI of R123, which represents P-glycoprotein (P-gp) activity in T-cell subsets by flow cytometry can be carried out using a number of statistical methods. The Kolmogorov-Smirnov (K-S) test is one of the most popular and widely used methods for comparing cumulative distributions generated by flow cytometry. However, this statistical method of comparing empirical distribution function of a sample to cumulative distribution function of a reference distribution has been criticized as being too sensitive for showing significance for differences on distributions. In particular, the K-S test may detect statistical differences in flow cytometric measurements that may not be biologically significant. An alternative analytical method for comparing flow cytometric single parameter histogram is the Overton histogram subtraction (Overton Subtraction), which can be used to estimate the percent positive cells from immunofluorescence histograms. Despite its convenience and simplicity, this method does not assess the variability in the difference observed between histograms. Also, Cox et al. proposed a method of comparing frequency distributions in flow cytometry which assumes Poisson distributions for channel-wise cell counts and uses a normal approximation to assess the variability in the difference. However, when the Poisson parameter estimate is small, as it often is for the observed counts, the normal approximation is not useful. Here, we provide an alternative, correct and more robust graphical procedure to quantitatively assess the differences in flow cytometry data. The procedures presented show pointwise confidence intervals for each

channel number and simultaneous confidence bands and may be used regardless of the probability distribution of frequencies at each channel number.

The data used for illustration made use of two different flow cytometric files, load and efflux, in two T-cell subsets, CD4 and CD8. First, the differences between histograms for CD4 and CD8 T-cell subsets at load were compared. Then, histograms in Load and Efflux states from CD8 T-cell subsets were compared to see the differences.

The statistical method described here is based on counting frequencies in each channel. Zero counts were approximated using linear interpolation between any observed values. Using bootstrapping, we obtained pointwise confidence intervals and simultaneous confidence bands for differences between flow cytometry histograms. The goal of this study was to determine whether confidence bands include zero difference for each comparison. Because the study handles differences data sets, the null hypothesis of test is that difference between two samples is equal to zero for all channels. We hypothesize that the result should show no difference between two T-cell subsets at load states and verify this as confidence bands established including a zero line are observed. The other dataset compared P-gp activity of two flow cytometry intensities in CD8 T-cell subsets. The second test results show differences clearly with large space departing from zero values. Finally, our investigation of intra- and inter-sample variability, where by definition, no biological variation exists, did not reveal differences where they were not expected. We compared both pointwise confidence intervals and simultaneous confidence bands around a difference curve of R123 mean fluorescence intensity in CD4 and CD8 T cells to other pointwise confidence intervals generated by Cox et al.'s approach. The confidence boundaries constructed using the normal approximation turned out to be very similar to those of pointwise confidence intervals. Also, hypothesis test result from K-S test and Cox et al.'s chi-square

statistic derives completely different conclusion comparing to test result from simultaneous confidence bands. Therefore, in our analysis of these samples, our confidence region procedure is more robust to detect differences with wider confidence range with simultaneous confidence bands, while K-S and Cox et al.'s method shows significant difference where it should not be biologically significant.

The limitations of this study include the fact that the original data sets of flow cytometry analysis have many variables which should be considered together. In this study, the channel numbers are only taken into account of as one independent variable but, the other types of data sets may have many independent variables to be considered. Also, this study doesn't consider assay-to-assay variability. This is an additional level of variability not accounted for by methods that have been proposed. In principle, it would be possible to generate confidence intervals and bands using results from multiple assays. However, we have not proposed such a procedure in this manuscript.

For the next step of this study, quantification of differences would be processed to see how much the two sample are different quantitatively. This step can be worked out by calculating area under the difference histogram.

APPENDIX: SAS PROGRAMMING CODES

```
/* Dongha Kim */
/* Master of Science */
/* Department of Biostatistics */
/* Graduate School of Public Health */
/* University of Pittsburgh */

/* Master Thesis Data Coding */

%macro fc_band(filename1=, filename2=, bootstrap=, alpha1=, alpha2= );

/* 1st step                          */;
/* Read data files into SAS */;
data Channels;
      do Channel = 0 to 1023;
            output;
      end;
run;

%macro ReadFile(filename= );

proc import file="F:\Cytometry\&filename..txt" out=Flow_&filename
dbms=dlm ;
run;

proc freq data = Flow_&filename noprint;
      table FL1 / nocum out = out1_&filename;
run;

data out2_&filename;
  set out1_&filename;
      PERCENT_&filename = PERCENT / 100;
      rename FL1=Channel;
      drop Count Percent;
run;

data out3_&filename;
      merge out2_&filename Channels;
      by Channel;
      if Channel = 0 then do; if PERCENT_&filename = .  then
PERCENT_&filename = 0; end;
      if Channel = 1023 then do; if PERCENT_&filename = .  then
PERCENT_&filename = 0; end;
```

```
run;

proc expand data=out3_&filename out=Total_&filename;
      convert PERCENT_&filename=Frequency_&filename / method=join;
      id Channel;
run;

%mend ReadFile;

%ReadFile(filename= &filename1);
%ReadFile(filename= &filename2);


%macro Bootstrap(filename=, b= );

/* 2nd step                                    */;
/* Bootstrap to generate samples */;
data Channelsboot;
      do Replicate = 1 to &b by 1;
            do Channel = 0 to 1023;
                  output;
            end;
      end;
run;

proc surveyselect data=Flow_&filename out=outboot_&filename
 seed=0
 method=urs
 samprate=1
 outhits
 rep=&b;
 run;

proc freq data = outboot_&filename noprint;
      table FL1 / nocum out = outboot1_&filename;
      by Replicate;
run;

data outboot2_&filename;
  set outboot1_&filename;
      PERCENTB_&filename = PERCENT / 100;
      rename FL1=Channel;
      drop COUNT PERCENT;
run;

data outboot3_&filename;
      merge outboot2_&filename Channelsboot;
      by Replicate Channel;
      if Channel = 0 then do; if PERCENTB_&filename = . then
PERCENTB_&filename = 0; end;
      if Channel = 1023 then do; if PERCENTB_&filename = . then
PERCENTB_&filename = 0; end;
run;

proc expand data=outboot3_&filename out=Totalboot_&filename;
      convert PERCENTB_&filename=FrequencyB_&filename / method=join;
      id Channel;
```

```
      by Replicate;
run;

%mend Bootstrap;

%bootstrap(filename= &filename1, b=&bootstrap);
%bootstrap(filename= &filename2, b=&bootstrap);


%macro Difference(filename1=, filename2= )

/* Merge L4 with L8 */ ;
data Diffboot;
      merge Totalboot_&filename1 Totalboot_&filename2;
      by Replicate Channel;
      DiffB_&filename1._&filename2. = FrequencyB_&filename1 -
FrequencyB_&filename2;
run;

proc sort data = Diffboot;
      by Channel;
run;

%mend Difference;

%Difference(filename1 = &filename1, filename2= &filename2);


%macro Confidence_intervals(error=, ci=);

/* 3rd step                                    */;
/* Confidencen Bands */;

/* Percentile Confidence Intervals */;
%let alpha = &error;
%let lb = %sysevalf(&alpha/2*100);
%let ub = %sysevalf((1 - &alpha/2)*100);
* creating confidence interval, percentile method;
proc univariate data = Diffboot alpha = &error noprint;
  var DiffB_&filename1._&filename2.;
  by Channel;
  output out=CIboot pctlpts=&lb &ub pctlpre = CI pctlname = _lb _ub;
run;

/* Simultaneous Confidence Bands */;
data Final_CI;
       merge Diffboot CIboot ;
       by channel;
           outofbound =  (DiffB_&filename1._&filename2. < CI_lb) +
(DiffB_&filename1._&filename2. > CI_ub);
run;

proc means data = Final_CI noprint;
           class replicate;
           var outofbound;
           output out=Final_CI_bound
           sum(outofbound) = sumofcount;
```

```
run;

proc freq data = Final_CI_bound;
      table sumofcount / out= Final_CI_count;
run;

data Final_&ci;
      set FInal_CI_count;
      where sumofcount = 0;
      Error_rate = (100 - Percent) / 100 ;
      Alpha = &error;
run;

%mend Confidence_intervals;


%macro plot(alpha1=, alpha2=);

%Confidence_intervals(error=&alpha1, ci=PCI);
data CIboot_Per_&filename1._&filename2.;
            set CIboot;
            rename CI_lb = CI_lb_Per_&filename1._&filename2.;
            rename CI_ub = CI_ub_Per_&filename1._&filename2.;
run;

%Confidence_intervals(error=&alpha2, ci=SCB);
data CIboot_Sim_&filename1._&filename2.;
            set CIboot;
            rename CI_lb = CI_lb_Sim_&filename1._&filename2.;
            rename CI_ub = CI_ub_Sim_&filename1._&filename2.;
run;


* 95% Confidence intervals plots around a difference curve *;
data Final;
            merge Total_&filename1. Total_&filename2.
CIboot_Per_&filename1._&filename2. CIboot_Sim_&filename1._&filename2.;
            Difference = Frequency_&filename1. - Frequency_&filename2.;
            by channel;
run;

data _null_;
   set Final Final_PCI Final_SCB;
   if sumofcount = 0 and alpha = &alpha1 then call symput('Error1',
put(error_rate, best7.));
   else if sumofcount = 0 and alpha = &alpha2 then call symput('Error2',
put(error_rate, best7.));
   else if missing(sumofcount) then call symput('Error1', 0.99999);
run;

proc sgplot data=Final;
      title 'Difference curve with 95% pointwise and simultaneous confidence
intervals';
      band x= Channel lower= CI_lb_Sim_&filename1._&filename2. upper=
CI_ub_Sim_&filename1._&filename2. /
            fillattrs= (color= blue) transparency= .5
            legendlabel= "95% SCB" name="band2";
```

```
      band x= Channel lower= CI_lb_Per_&filename1._&filename2. upper=
CI_ub_Per_&filename1._&filename2. /
            fillattrs= (color= red) transparency= .5
            legendlabel= "95% PCI" name="band1";

      series x= Channel y= Difference /
      lineattrs= (color= black) transparency= .5
      legendlabel= "Difference curve" name="series";

      inset ("CIs" = "Alpha level     Error rate"
               "Pointwise" = "            &alpha1  &error1"
          "Simultaneous" = "     &alpha2       &error2" ) /
      border  valuealign=left position=topright
    titleattrs=GraphLabelText;

      refline 0 / lineattrs= (color= black);
      keylegend "series" "band1" "band2" / location=inside
position=bottomright;

run;

%mend plot;

%plot (alpha1=&alpha1, alpha2=&alpha2);


%mend fc_band;

* Comparison of CD4 and CD8 T-cell subsets in R123 LOAD *;
%fc_band (filename1=L40, filename2=L80, bootstrap=100000, alpha1=0.05,
alpha2=0.00024) ;
* Comparison of R123 LOAD and EFFLUX in CD8 T-cell subsets *;
%fc_band (filename1=L80, filename2=E89, bootstrap=100000, alpha1=0.05,
alpha2=0.00011) ;

* Comparison of Replicate R123 LOAD in inter-sample *;
%fc_band (filename1=L40, filename2=L43, bootstrap=100000, alpha1=0.05,
alpha2=0.00024)
%fc_band (filename1=L40, filename2=L46, bootstrap=100000, alpha1=0.05,
alpha2=0.00021)
%fc_band (filename1=L43, filename2=L46, bootstrap=100000, alpha1=0.05,
alpha2=0.00022) ;

* Comparison of Replicate R123 LOAD in intra-sample *;
%fc_band (filename1=L43, filename2=L44, bootstrap=100000, alpha1=0.05,
alpha2=0.00024)
%fc_band (filename1=L43, filename2=L45, bootstrap=100000, alpha1=0.05,
alpha2=0.00024)
%fc_band (filename1=L44, filename2=L45, bootstrap=100000, alpha1=0.05,
alpha2=0.00024) ;
```

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