Flow Cytometry Workshop for the Department of Immunology

“New Approaches to Multicolor Compensation & Data Visualization In Flow Cytometry”

June 2, 2008
11am-12:30 pm
MSB 4279

Presented By:
Dr. Cynthia Guidos
Scientific Director,
SickKids/UHN Flow Cytometry Facility at TMDT
Senior Scientist
Program in Developmental & Stem Cell Biology
Hospital for Sick Children Research Institute
Toronto
Compensation

The single most common source of data error in multi-color flow cytometry experiments
The Importance of Proper Compensation!

Uncompensated

Proper

Improper (+3%)
The Importance of Proper Compensation!

Proper

Improper (-3%)
The Big Challenge: Signal / Background

• The most basic information to be derived from any flow cytometry experiment is
  – *Whether a cell of interest is positive for a given marker*

• To do this accurately, we must optimize resolution sensitivity without sacrificing specificity!
Sensitivity: Factors Impacting Resolution

Background
- Cell autofluorescence
- Unbound dye/fluorochrome
- Non-specific staining
- Ab titration
- Cell autofluorescence
- Unbound dye/fluorochrome

Spread
- Spectral overlap (spillover)
- Sample

Cytometer
- Q (Fluorescence detection efficiency)
- Non-optimized PMT gains

Experimental Design
- Optimize cytometer set-up
Resolution Sensitivity
The ability to resolve a dim population from background (noise)

- Negative population has low background
  - Populations well resolved

- Negative population has high background
  - Populations not resolved

- Negative population has low background and high CV
  - Populations not resolved

The ability to resolve populations is a function of both background and spread (variance!) of the negative population

Compensation has a huge effect on population variance
Fluorochromes emit in other channels

Spillover into other detectors contributes background to those detectors

Remember the basic assumption of flow analysis:

The signal in FL1 = the signal from FITC and only FITC and
the signal in FL2 = the signal from PE and only PE.

This is NOT TRUE for the raw data collected in each FLx parameter!
The process by which each fluorescence channel is “corrected” for this
spectral spillover is termed Fluorescence Compensation.
From fluorescent cell to histograms & dot plots
Analog vs Digital Instruments

In summary, the key differences between analog and digital systems include:

<table>
<thead>
<tr>
<th>implementation</th>
<th>performance</th>
<th>multicolor compensation</th>
</tr>
</thead>
<tbody>
<tr>
<td>at what point the</td>
<td>the digital system provides more</td>
<td>complete multicolor compensation can be performed in the</td>
</tr>
<tr>
<td>signals are digitized</td>
<td>accurate data</td>
<td>digital systems but not the analog systems</td>
</tr>
</tbody>
</table>

**Diagram**

**Analog**
- PMT → preamp → compensation circuit → amplifier → signal processing → ADC → sorting → computer

**Digital**
- PMT → preamp → ADC → thresholding and signal processing (compensation/ratio/log display/sorting) → computer
Why go digital?

**Current analog system**
- Small raw analog signal from the preamp
- Pulse height
- Log amp
- Maximal fluorescence
- ADC Gate open

**Digital system**
- A digital system with a 10MHz ADC will make 40 observations on a 4 µsec pulse
- The sum of the height values is the total fluorescence (pulse area).
- The total fluorescence value is approx. 16X the peak value.
Basic Principles of Compensation -

This subtraction is done electronically on analog instruments.

Following Compensation

\[
\begin{align*}
\text{FITC} &= \text{FL1} - x\% \text{ PE} \\
\text{PE} &= \text{FL2} - y\% \text{ FITC}
\end{align*}
\]

To get a true measure of the PE signal in FL2 you have to subtract a percentage of any PE signal present in the cell. This is compensating the signal.
## Analog vs Digital Compensation

<table>
<thead>
<tr>
<th></th>
<th>Analog</th>
<th>Digital</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Algorithm</strong></td>
<td>Subtractive: pulse matching</td>
<td>Corrective: matrix algebra</td>
</tr>
<tr>
<td><strong>PMTV</strong></td>
<td>Critical – narrow ranges for pulse matching</td>
<td>Above noise</td>
</tr>
<tr>
<td><strong>Comp Error</strong></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>Linearity</strong></td>
<td>Up to 6 – 10% error</td>
<td>Linear across dynamic range</td>
</tr>
</tbody>
</table>
Digital Compensation

Fluorescence Compensation

– We correct for fluorochrome spillover to align stained populations in fluorochrome space without bias from spectral overlap.
  • Analog system essentially subtracts pulses
  • Digital systems correct using a compensation matrix (inverted spillover matrix) using matrix algebra.

– Compensated parameters exhibit spread.
  • Nonlinear error from photon counting statistics\(^1\)
  • Worse in red and far red (fewer photons)
    – Sampling error is function of SQRT(number of photons)
  • Analog systems dampen spread due to errors in compensation circuits and logamp nonlinearity

Key Factors for Successful Compensation

1) Optimize Filter Configuration
2) Choose the Correct Comp Controls
3) Use Software (Digital), rather than electronic (Analog) compensation
4) Use Extreme caution when manually adjusting compensation matrix
5) Validate that compensation was performed correctly
6) Understand how & why compensation “spreads” distributions
7) Use FMO staining controls and proper gating strategy
8) Use Bi-exponential transformations for proper visualization
<table>
<thead>
<tr>
<th>PMT</th>
<th>LP</th>
<th>BP</th>
<th>Fluorochromes</th>
<th>Peak Em</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>735</td>
<td>780/60</td>
<td>PE-CY7</td>
<td>776</td>
</tr>
<tr>
<td>B</td>
<td>685</td>
<td>695/40</td>
<td>PE-CY5.5 OR PerCP-Cy5.5</td>
<td>689</td>
</tr>
<tr>
<td>C</td>
<td>635</td>
<td>660/20</td>
<td>PE-CY5</td>
<td>667</td>
</tr>
<tr>
<td>D</td>
<td>600</td>
<td>610/20</td>
<td>PE-Texas Red OR PI</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>550</td>
<td>575/26</td>
<td>PE</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>505</td>
<td>530/30</td>
<td>FITC</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>-</td>
<td>488/10</td>
<td>SSC</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>-</td>
<td>-</td>
<td>empty</td>
<td></td>
</tr>
<tr>
<td>PMT</td>
<td>LP</td>
<td>BP</td>
<td>Fluorochromes</td>
<td>Peak Em</td>
</tr>
<tr>
<td>-----</td>
<td>----</td>
<td>----------</td>
<td>----------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>A</td>
<td>735</td>
<td>780/60</td>
<td>PE-CY7</td>
<td>776</td>
</tr>
<tr>
<td>B</td>
<td>685</td>
<td>695/40</td>
<td>PE-CY5.5 OR PerCP-Cy5.5</td>
<td>689</td>
</tr>
<tr>
<td>C</td>
<td>635</td>
<td>660/20</td>
<td>PE-CY5</td>
<td>667</td>
</tr>
<tr>
<td>D</td>
<td>600</td>
<td>610/20</td>
<td>PE-Texas Red OR PI</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>550</td>
<td>575/26</td>
<td>PE</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>505</td>
<td>530/30</td>
<td>FITC</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>-</td>
<td>488/10</td>
<td>SSC</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>-</td>
<td>-</td>
<td>empty</td>
<td></td>
</tr>
</tbody>
</table>
How to determine if Compensation is correct?
Compensation Quiz!
Does Improper Compensation Matter?

Matters Most for:
Accurate Ag Density Measurements (MFI)
Distinguishing Dim from Negative Populations (Resolution Sensitivity)
Multi-color Applications
Why should the amount of compensation by minimized & optimized?

Compensation “spreads” the data (increases variance) and therefore decreases resolution sensitivity (big problem for detecting dim markers).

Under-compensation can cause false positives (ie, calling a negative cell positive).

Over-compensation will cause underestimation of the population’s MFI in the compensated channel.
Spillover Increases “Spread”

Resolution for a given fluorescence parameter is decreased by increased spread due to spillover from other fluorochromes.

This spread is NOT eliminated by Compensation.

More colors = more spillover.
Visualization Artifacts from Compensation-Induced Spread

8 modeled populations – 2 of which are double positive

Difficult with low autofluorescence and compensation because of high spillover (22%) of X into Y, low spillover (3%) of Y into X causes “high background” of X into Y on single positive bright X population, which inflicts significant data spread after compensation.
Blue/Green, Red and Violet laser dyes

- Current BD Biosciences Immunofluorescent Dyes

![Graph showing fluorescence emission spectra for Blue/Green Laser, Violet Laser, and Red Laser with different dyes and their wavelengths.](Image)
Compensation-Induced Spreading is Worse in the Far Red Channels

APC vs APC-Cy7
Compensation

Setting up Contols for Compensation
Setting Compensation

• **Myths about setting compensation**
  – You should set your compensation on the same tissue you are going to analyze
  – Compensation controls should be the same intensity as the reagent to be used
    • Bright reagents require more compensation than dull reagents
  – **Compensation can be set by eye**
  – Compensation settings can be saved and used from day-to-day
  – Improper compensation doesn’t affect the data very much
4 Simple Rules for Setting Compensation

1. The fluorescence spectrum (% Spillover) of the compensation control reagent should be identical to the reagent used in the experiment
   a. Critical for Tandem reagents where different reagents can have different spillovers
   b. Even similar fluorochromes like FITC / Alexa 488 or APC/ Cy5 should be compensated for separately

2. The negative and positive populations must have the same autofluorescence
   a. Critical when using stained cells as compensation controls
      i. i.e. compare CD3⁺ lymphocytes and CD3⁻ lymphocytes
      ii. don’t use CD3⁺ lymphocytes and CD3⁻ monocytes

3. The positive population should be as bright as possible
   a. Insures values in spillover channel are above background
   b. Provides greater accuracy for calculating low (but real) spillovers
      i. FITC -> PE-Cy7 (1-2%) or APC -> PE-Cy7

4. Take enough events to get statically accurate numbers
   a. 10,000 events gives a 1% SD
   b. Provides greater accuracy for calculating low (but real) spillovers
Tandem Dye Reagents

- **Tandems** - PE-Cy5, PE-Cy7, PerCP-Cy5.5

Typically, the chemical coupling of a protein fluorochrome (e.g. PE) which acts as the donor and a small organic fluorochrome (e.g. TR) which acts as the acceptor.
Tandem Compensation: Different lots have different spillovers

Comparison of FL2- %FL3 Compensation required for 4 different PE-TR conjugates
4 Simple Rules for Setting Compensation

1. The fluorescence spectrum (% Spillover) of the compensation control reagent should be identical to the reagent used in the experiment
   a. Critical for Tandem reagents where different reagents can have different spillovers
   b. Even similar fluorochromes like FITC / Alexa 488 or APC/ Cy5 should be compensated for separately

2. The negative and positive populations must have the same autofluorescence
   a. Critical when using stained cells as compensation controls
      i. i.e. compare CD3\(^+\) lymphocytes and CD3\(^-\) lymphocytes
      ii. Don’t use CD3\(^+\) lymphocytes and CD3\(^-\) monocytes
      iii. Don’t mix comp beads and cells to compensate individual parameters
      iv. OK to compensate some parameters with beads and others with cells
      v. It is crucial to accurately measure the autofluorescence of the “neg” pop
         MUST use optimal PMT voltages!!

3. The positive population should be as bright as possible
   a. Insures values in spillover channel are above background
   b. Provides greater accuracy for calculating low (but real) spillovers
      i. FITC -> PE-Cy7 (1-2\%) or APC -> PE-Cy7

4. Take enough events to get statically accurate numbers
   a. 10,000 events gives a 1\% SD
   b. Provides greater accuracy for calculating low (but real) spillovers
Effect of Suboptimal PMT Voltage on Compensation

As PE voltage decreases, PE values <0.1 are not accurately reported (limitation of log scaling). The values are “pinned” at 0.1. Therefore, the difference in PE fluorescence of FITC pos and FITC negs will be underestimated (data will be undercompensated).
4 Simple Rules for Setting Compensation

1. The fluorescence spectrum (% Spillover) of the compensation control reagent should be identical to the reagent used in the experiment
   a. Critical for Tandem reagents where different reagents can have different spillovers
   b. Even similar fluorochromes like FITC / Alexa 488 or APC/ Cy5 should be compensated for separately

2. The negative and positive populations must have the same autofluorescence
   a. Critical when using stained cells as compensation controls
      i. i.e. compare CD3+ lymphocytes and CD3- lymphocytes
      ii. don’t use CD3+ lymphocytes and CD3- monocytes

3. The positive population should be as bright as possible
   a. Insures values in spillover channel are above background
   b. Provides greater accuracy for calculating low (but real) spillovers
      i. FITC -> PE-Cy7 (1-2%) or APC -> PE-Cy7

4. Take enough events to get statically accurate numbers
   a. 10,000 events gives a 1% SD
   b. Provides greater accuracy for calculating low (but real) spillovers
Why Bright Compensation Controls

Estimating a low spillover fluorescence accurately is impossible if the level of fluorescence into the spillover channel is near or at background levels in that channel.

From Mario Roederer - NIH
Types of Compensation Controls

- **Beads stained with dyes “similar” to a known fluorochrome**
  - Very Bad- small differences in emission spectra can result in large compensation errors (Rule #1)

- **Beads coated with a known fluorochrome**
  - CaliBrite / 7-color Beads coated with FITC, PE, APC, APC-Cy7, etc.
    - **Advantages**
    - Easy to use; automated set-up and compensation
    - **Limitations**-
      - Spectra (especially tandems) may be slightly different than a conjugated mAb
      - Best used with pre-defined clinical cocktails with pre-tested reagents
      - Limited number of fluorochrome-conjugated beads
Types of Compensation Controls

• *Cells stained with a fluorochrome-conjugated mAb*
  – Advantages
    • Best match of spectra
    • Can be used for any fluorochrome
  – Disadvantage
    • Have to stain cells
    • Assume identical autofluorescence of + and - pop. (Rule #2)
    • Positive stained cells may be dull or few in number (Rules #3 & 4)

• *Beads which capture a defined amount of conjugated mAb*
  – *BD™ Anti-mouse/Rat IgG CompBeads*
    • Advantages
      – All of the above
    • Disadvantages
      – None of the above
Setting-up a Flow Cytometer - Compensation

BD Anti-Mouse CompBeads

<table>
<thead>
<tr>
<th>mAb</th>
<th>FITC</th>
<th>PE</th>
<th>FL3</th>
<th>APC</th>
<th>APC-Cy7</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td></td>
<td>PE-Cy5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#2</td>
<td>PerCP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#3</td>
<td>PerCP-Cy5.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Choosing the Best Compensation Controls

Wrong controls

Right controls

FMO (APC)  APC-CD117  FMO (APC)  APC-CD117

Other stains: FITC, PE, Sytox Blue
Summary

- Spillover from other fluorescence channels will increase the background and spread (CV) of a given fluorescence parameter.
- Compensation can remove the background component but NOT the increased spread.
- Software (digital) compensation is more accurate than electronic (analog) compensation.
  - Where possible compensate analog data off-line with software.
- Correct Compensation Controls are the key to a good Multi-color experiment.
  - Run Compensation controls for EVERY parameter in an experiment.
  - Run individual compensation controls for EACH tandem reagent.
  - In general Ig-capture beads are the best controls.
8 modeled populations – 2 of which are double positive

With highly compensated data with increased spread from spillover, quadrant gates set using isotype controls are not valid and will lead to false positives.
8 modeled populations – 2 of which are double positive

Bent quadrant gates available in Diva 4.1 provide a much more accurate discrimination of positives and negatives. Gates are best set using FMO controls.
8 modeled populations – 2 of which are double positive

Individual gates are always the safest way to identify separate populations.
Gates are best set using FMO controls
FMO (Fluorescence Minus One)

- Compensated data exhibits spread
- Bright single positives may change threshold levels between dim and background in other dimensions
- Unstained and/or isotype controls are NOT useful for determining threshold over background
- The best control is one stained with all reagents except the one of interest
Use Fluorescence Minus One (FMO) Controls to Identify Positives

PBMC were stained as shown in a 4-color experiment. Compensation was properly set for all spillovers.

<table>
<thead>
<tr>
<th>Unstained Control</th>
<th>FMO Control</th>
<th>Fully Stained</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC None</td>
<td>CD3</td>
<td>CD3</td>
</tr>
<tr>
<td>PE None</td>
<td>None</td>
<td>CD4</td>
</tr>
<tr>
<td>Cy5PE None</td>
<td>CD8</td>
<td>CD8</td>
</tr>
<tr>
<td>Cy7PE None</td>
<td>CD45RO</td>
<td>CD45RO</td>
</tr>
</tbody>
</table>

Courtesey Mario Roederer
Isotype Controls are (Mostly) Useless!!
Visualizing Compensated Data
**Visualizing Data on Log vs Linear Scales**

**Best for parameters with small range of intensities (0-10)**
- (DNA, FSC, SSC)

**Best for parameters with large range of intensities (0-100,000)**
- (most surface markers)

**Linear Scale**
- Compresses values at low end
- Spreads high end
- Lower range of values can be effectively visualized
- Can display negative values!

**Log Scale**
- Spreads values at low end
- Compresses high end
- Can’t display negative values!
Cells with "negative" fluorescence - How can this be!

Measurements of dim cells/particles often near zero (esp for far/far red PMT’s)

Both analog & digital cytometers carry out "invisible" background corrections (subtract the background (estimated) from stray light and electronic noise)

Log scaling in analog system can't display negative numbers - pile up in Channel "0"

Digital instruments (& Flow-Jo) provide "bi-exponential" scaling to avoid this artifact

<table>
<thead>
<tr>
<th>PE-Cy7 PMT Voltage:</th>
<th>baseline</th>
<th>+50 V</th>
<th>+100 V</th>
<th>+150 V</th>
<th>+200 V</th>
</tr>
</thead>
<tbody>
<tr>
<td>99.9</td>
<td>92.4</td>
<td>84</td>
<td></td>
<td>79.1</td>
<td>79.2</td>
</tr>
<tr>
<td>+50 V</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+100 V</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+150 V</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+200 V</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The Biexponential Scale

This example shows the value of the Biexponential Scale— a mostly logarithmic scale on the upper end, linear at the low end and symmetrical about zero.

- Compensated single positives are continuous
- All populations are visible
The Biexponential Scale

The Biexponential transformed display shows aligned populations in the APC-Cy7 dimension

Antibody capture beads stained with 3 levels of an APC reagent
Bi-exponential Displays Improve Visualization of Dim/Negative Populations

Log$_{10}$

BiExp

1X 0.1X 0.01X Blank

26283 24911 16916 16120 2229 2484 77 10
Bi-exponential Displays Improve Visualization Population Centers

Dot Plots are Evil!

Figure 1: ‘Logicle’ displays provide improved representation of cells with minimal fluorescence. Cells with minimal fluorescence can be visualized with ‘logicle’ displays (right) but are ‘piled up’ on the axis with logarithmic displays (left). The true center of each gated population (median fluorescence value in each dimension; dark red crosses) matches the visual peak for that population in ‘logicle’ displays (right, top and middle) but does not match the visual peak in the logarithmic displays (left, top and middle). Because logarithmic scales cannot display cells with zero or negative values, these cells are ‘piled up’ on the axis in the logarithmic displays. However, they are properly visualized in the ‘logicle’ display (bottom right, red shaded region). Data provided by E. Ghoen (Stanford University, Stanford, California).
Optimizing Bi-exponential Scaling Parameters

Effects of Bi-exponential Scaling:
- Allows proper visualization of dim/neg cells at axes origins (reduces “pile-up”) esp in far red/red channels
- Makes it easier to see if data is compensated correctly
- Ensures that visual population centers correlate better with statistical centers (medians)

Applying Bi-exponential Scaling:
- First, apply minor transformation to uncompensated data as it is imported into the Workspace (Set preferences for importing Diva files)
- During this step, a single automatic transformation is applied to all fluorescence parameters
- When data is compensated in FlowJo, the compensated parameters will get the same minor transformation by default
- Often this is adequate to optimally view compensated data
- When it is not, you can manually create a custom transformation to better view your compensated data
- Ensures that visual population centers correlate better with statistical centers (medians)

Setting Bi-exponential Scaling Parameters (Uncompensated Data):
- Total number of decades: decrease if dim/neg subset is split or too wide (start @ 3.5-4.5) increase if dim/negs are too compressed
- Additional negative display size:
  - adds extra space below 0, thus decreasing positive space
  - increase in increments of 0.5 or less if negs offscale below 0
- Width Basis: negative number representing the total # channels compressed to linear scale around 0
  - usually need to increase (default=-10) to accommodate neg values in far red
  - increase if neg/dims are “split”
  - decrease if negs are spread over too much of the scale

Customizing Bi-exponential Scaling Parameters (Compensated Data):
- Compensation will increase spread and data points below zero (esp far red)
- to improve visualization, add neg space (to see points<0 better) and/or change #positive decades displayed
  - decrease if negs split or spread over too much of scale
  - increase if negs too compressed