Compensation: An Instrumental Perspective

Why Digital? Visualization Issues

Joe Trotter
Joe_Trotter@bd.com
Review: Instrument Sensitivity

- Measuring Sensitivity - 2 definitions
  - **Threshold** - Degree to which a flow cytometer can distinguish dimly stained from particle free background. Usually used to distinguish means/medians.
  - **Resolution** – Degree to which a flow cytometer can distinguish unstained and dimly stained in a mixture. Can be very complicated in a polychromatic scenario.

- Good Sensitivity?
  - We have generally improved instrument sensitivity – threshold definition (50 – 200 MESF FITC, for example).
  - Good threshold sensitivity does not necessarily guarantee good resolution.
Things that impact sensitivity

Instrument

Q

Optical design efficiency
Laser power intensity
PMT quantum efficiency
Sheath flow rate

B

Component autofluorescence
Component scatter
Raman scatter
Dark current

Sample

Cell autofluorescence

Laser power intensity

Unbound dye/fluorochrome

Laser power intensity
Sample flow rate

Spectral overlap

Laser power intensity
Filter design
Fluorescence Sensitivity is a Function of Detection Efficiency - Q

Fluorescence sensitivity:
a measure of the ability to resolve dimly stained cells from noise

Detection Efficiency (Q):
a measure of the ability of the instrument to excite and capture photons of interest

10 fold increase in laser power

30 mW
Q = 0.030

15 mW
Q = 0.018

7.5 mW
Q = 0.0076

3.75 mW
Q = 0.0040
Sources of Background Light That Reduce Fluorescence Sensitivity - B

- Unbound antibody or fluorochrome
- Scatter from flow cell, capillary, or fluid jet
- Raman Scatter
- Spectral overlap on multiple-stained cell
- Cell Autofluorescence
Fluorochromes emit in other channels

Spillover into other detectors increases background

Double click over graph to unzoom, or over a filter to view spillover estimates.
Without compensation, the amount of PE MESF background contributed by bright CD45-FITC staining can be determined (8700 PE MESF).

**Analog Data:**

**CD45 FITC only**

**Dim CD4 PE double stained cells not visible**
Effect of Spillover on Double Stained Cells

Compensated analog data:
CD45 FITC makes dim CD4 difficult to measure due to FITC spillover into PE and resultant “spread”

Compensated analog data:
CD45 PerCP allows same dim CD4 cells to be separated from bkg. – little spillover into PE
Analog Cytometers

- They have set the standard from the 1970s to now.

  - 2 stage linear display 0 – 10V:
    1. Preamp
    2. Amplifier/integrator

  - 3 stage logarithmic Display:
    1. Preamp (current to pulse)
    2. Differential amp(s) (compensation by pulse subtraction)
    3. Logarithmic amp
      a. Response error ±0.5dB from true log
      b. Linear error 6% to 10% over range
      c. Largest errors are from offsets
Simple 4 detector analog example

- HV Power Supplies 0 – 999 V
- Linear Differential Amplifiers (Compensation)
  - Preamps
  - BLR
- Amplifiers
  - linear
  - log
- Signal Processing
  - Trigger circuit
  - ADCs
  - DACs
- Sorting Electronics
  - Drop Charging
  - Gate circuits

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Simple 4 detector digital example

Channel DAQs
ADCs
FPGAs BLR

Preamps

DSP Chips
Embedded CPU
Sort control

FSC

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Base Line Restore - Analog vs Digital

1: PMT current

2: Pre-amp

3a: Analog BLR

3b: Digital BLR

4: Digitized parameter

Events

Common

Bipolar current to pulse amplifier

Circuit is constantly averaged - duty cycle dependent

Negative is clipped at 0

Running average is subtracted outside window ext. (w)

Virtual zero

+50 mV bias

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A Continuously Digitizing Cytometer

FACSAria 10 MHz ADC: 5.8 μsec pulse has ~ 58 observations

Sum 14 bit height measurements into area as IEEE 32 bit floating point
Pulse area is a measurement of total fluorescence (18 bit resolution)
We live in an imperfect world

Fluorescence Compensation

- We correct for dye spillover to align stained populations in dye space without bias from spectral overlap.
  - Analog system essentially subtracts pulses
  - Digital systems correct using a compensation matrix (inverted spillover matrix) and 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 $\sqrt{\text{number of photons}}$
  - Analog systems dampen spread due to errors in compensation circuits and logamp nonlinearity

**Analog Compensation Errors**

- **High Low**
  - Ideal: No significant pulse distortion
  - Mild Mismatch: Mild Pulse Distortion
  - Medium Low Mismatch: Significant Pulse Distortion and Compensation Error

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## Cytometer Quantitative Characteristics

### Quantitative Characteristics Over a 4 Decade Range

<table>
<thead>
<tr>
<th>Decade</th>
<th>Log amp output</th>
<th>Log amp input</th>
<th>Cathode photons/10 μsec</th>
<th>CV (%)</th>
<th>MESF</th>
<th>% error 1.0 mV offset</th>
<th>ADC channel (10 bit)</th>
<th>ADC channel (18 bit)</th>
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<tbody>
<tr>
<td>1</td>
<td>10.0 V</td>
<td>10.0 V</td>
<td>4430000</td>
<td>0.13</td>
<td>1000000</td>
<td>0.01</td>
<td>1023</td>
<td>262143</td>
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<td>10000</td>
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<tr>
<td>1</td>
<td>0.0 V</td>
<td>1 mV</td>
<td>443</td>
<td>12.9</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>26</td>
</tr>
</tbody>
</table>

Analog vs Digital Electronics

High Low
Mild Mismatch

Pulse Distortion
No negative values
(Clipped at 0)

Compensation

Nonlinearity from
6% to 10% over range

PMT → preamp → differential amps → logamp → ADC

BLR and Compensation

PMT → preamp → ADC → FPGA and DSP
Digital Cytometer Numerical Range

• BLR zero is really brought to “0.0” numerically
  • An asymptotic logarithmic scale can never get there…
    • 100,000 - 10,000 - 1,000 - 100 -10 - 1 - 0.1 - 0.01…
• Range is from 1% below 0 to maximum value
  • 18 bits: That means from -2621 to 262144 in uncompensated data, more negative values appear after compensation due to spread
  • Typical range is from -300 to 200000 before compensation
• BD digital 4 decade range plots from 26.0 - 262143.0
  • Raw values < 26.0 are visualized at 26.0
  • Statistics use the actual raw IEEE 32 bit FP values from less than zero up to 18 bit range of 262143.0
Uncompensated CD20 FITC on capture beads

Spillover calculation
AutoCompensation method

Compensation coefficient = slope

1. \( k_{12} = \frac{(2010 - 5)}{(7981 - 10)} \)

2. \( k = \begin{bmatrix} 1 & k_{12} \\ k_{21} & 1 \end{bmatrix} \)

3. \( k^{-1} \)

4. \( PE_{comp} = PE \times k^{-1} \)
CD20 FITC Capture Beads Compensated

Populations are aligned in dye space

\[ PE_c = PE + FITC \times -0.25 \]

using matrix algebra

Not a subtraction, rather a correction because we use compensation coefficients instead of spillover coefficients.
Compensation spread

The linear plot below shows the alignment of compensated CD20 FITC positive and negative capture beads in the PE dimension.

The CD20+ standard deviation decreases from 192 to 33.4 after compensation – yet the CV* goes to 1500% because the mean drops from 2010 all the way to 2.2.

*The Coefficient of Variation (CV) is inappropriate with compensated data. CV = 100 x Std/Mean, so as the mean approaches 0, the CV approaches ∞
Generally accepted assumptions among the Asilomar 2002 flow community attendees

- Analog compensation error is responsible for the observed differences between analog- and digitally-compensated data
- Pure linear matrix compensation gives the best available estimates for the dye signals from each cell
- Statistical results should be computed on the matrix compensation output without further manipulations
- Logarithmic display of compensated data interferes with proper interpretation of samples with populations that include low and negative data values
- Data display transformations that are not simply linear or simply log would provide better and more interpretable visualizations

1 October 2002 - Courtesy Dave Parks
Synthetic data example – log scale

8 modeled populations – 2 of which are double positive

Difficult with low autofluorescence and compensation because high cross-over (22%) of X into Y, low cross-over (3%) of Y into X causes “high background” of X into Y on single positive bright X population, which inflicts significant data spread around zero after compensation
Full Log Display – Visualization Artifacts

Standard Log Scale

Blended Scale
95% Log with zero shown
More Linearization minimizes bifurcation

20% Linear

50% Linear

Vanishing linear populations
Logicle: Compensated Biexponential Display

Upper decades are log, becomes linear near zero. Biexponential transform where data zero is shown by the crosshairs in the plot.

- This example shows the value of combining the blend with a mostly logarithmic scale on the upper such that the linear portion occupies a significant plot area over that in blend alone.
  - Compensated single pos are continuous
  - All populations are visible
Data dependant display capability

Input variables may be adjusted based on sampled population variance to optimize the display for any particular data set.

Region -2 to 3 "Dec"

Display function "Decades"

PE Capture Beads

BiExponential

Log

Log Plot

Input variables may be adjusted based on sampled population variance to optimize the display for any particular data set.
PacBlu-CD4 FACSARia example

5 decade pulse area

Transformed plot

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PacBlu-CD4 FACSARia with lymph gate

5 decade pulse area

Transformed plot
Summary: Current data display issues

- As pointed out by Mario Roederer, Dave Parks\(^1\), Randy Hardy and others, what often looks like properly compensated analog data tends to be overcompensated, "leading to systematically biased dye level estimates".
- Compensated digital data does not systematically bias dye level estimates.
- Logarithmic display of flow cytometry immunofluorescence data can be misleading and often difficult to interpret.
- Digital immunofluorescence data, with its virtual zero and floating point database, is more vulnerable to log distortion than analog, and many events cannot be visualized on a log scale even before compensation.

\(^1\)R Hardy data - Cytometry email thread 2002, DR Parks - Asilomar Workshop 2002
Can Compensated B Cells Correctly Align at Different Dye Concentrations?¹

10⁷ Murine Spleen Cells

Spleen

Stain B cells with B220 FITC at 1/40 saturating concentration

Split each half into 4 samples and stain with saturating CD19 Cy7-PE at:

Recombine so 50% of each Cy7-PE CD19 concentration is B220 Cy5-PE+

1X  1/10X  1/100X  none

Wash ½

Wash ½ & Stain with Saturating B220 Cy5-PE

¹ Randy Hardy – Fox Chase Cancer Center
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Analog Compensation Behavior

• “Y” reagent stained at high, medium, low and blank levels (CD19 Cy7-PE).
• Half are stained with the “X” reagent (B220 Cy5-PE) and half with mock stain (B220 FITC 1/40) only.
• Recombine the corresponding pairs and analyze at various compensation settings.
• No compensation value satisfies all tubes of the same stain.
Digital & Analog: Software Compensation

Digital

1X

0.1X

0.01X

Blank

Analog

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Correct Population Alignment

1X 0.1X 0.01X Blank

Log\(_{10}\)

B220 Cy5 PE-A

BiExp

CD19 CY7 PE-A

26283 24911

16916 16120

2229 2484

77 10

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Hyper-Log: Another Solution

Four Fluorescent Proteins: ECFP, EGFP, EYFP, DsRed

¹ Courtesy Bruce Bagwell - Verity Software House

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Optimized BiExponential

Algorithm self-adjusts and is automatically scaled
CD20 FITC Capture Beads Revisited

BiExponential display reveals proper compensation visually

2% over

Correct

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### 8-color antigen-specific immunophenotyping

<table>
<thead>
<tr>
<th>Ab Conjugate</th>
<th>Laser $\lambda$</th>
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<tbody>
<tr>
<td>CD28 PerCP-Cy5.5</td>
<td>488</td>
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<tr>
<td>CD45RA PE-Cy7</td>
<td>488</td>
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<tr>
<td>CD27 APC</td>
<td>633</td>
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<tr>
<td>CD8 APC-Cy7</td>
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<tr>
<td>CD3 Pacific Blue</td>
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<tr>
<td>CD4 AmCyan</td>
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<tr>
<td>Anti-IFN$\gamma$ FITC</td>
<td>488</td>
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<tr>
<td>Anti-IL-2 PE</td>
<td>488</td>
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</tbody>
</table>

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8 Color Compensation (LSR II)

Single-stained controls:

- **CD4 FITC**
- **CD4 PE**
- **CD28 PerCP-Cy5.5**
- **CD45RA PE-Cy7**
- **CD27 APC**
- **CD8 APC-Cy7**
- **CD3 Pacific Blue**
- **CD4 AmCyan**

**Auto-comp**

<table>
<thead>
<tr>
<th></th>
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<th>PE</th>
<th>PerCP-Cy5.5</th>
<th>PE-Cy7</th>
<th>APC</th>
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<td>0.6</td>
<td>0.1</td>
<td>100.0</td>
<td>0.0</td>
<td>3.6</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td><strong>APC</strong></td>
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<td>0.0</td>
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<td>0.2</td>
<td>100.0</td>
<td>2.7</td>
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<td>19.9</td>
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<td>0.6</td>
<td>1.5</td>
<td>0.0</td>
<td>17.1</td>
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Phenotype of CMV-responsive CD8 T cells

IFN$\gamma$ Response

CD45RA

CD27

IL-2 Response

CD45RA

CD27

CD28

CD27

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Reading: Compensation/Digital

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