
Mark A. KuKuruga -- FDA CBER Flow Cytometry Core, Silver Spring, MD

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Disclaimer

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CBER Flow Cytometry Core: Laboratory Training

CFCC Core Laboratory – FDA White Oak Campus, Silver Spring MD
Main Lab – Bldg 52/ Rm 1151
BSL3 Lab – Bldg 52/ Rm 1151
Training for a Diverse Group of Users

- September 2012, CBER Flow Cytometry Core (CFCC) established at the FDA in Bethesda, MD

- A virtual Core -- BD FACSAnia II cell sorter, BD LSRII analyzer, BD LSRFortessa

- Spread across three buildings of the NIH (Bethesda MD)

- Diverse user-base:
  - Long-term career investigators, post-docs, post-bacs, grad students, etc.
Training for a Diverse Group of Users

The Core has recently relocated . . .

- FDA White Oak Campus -- Silver Spring MD
- Consolidated service in these newly constructed facilities.
- With consolidation, user-base also expands.
- (Large Number of Investigators)

The mission of the CCFC Core is to facilitate applications of advanced flow cytometric techniques towards mission-critical research on vaccines, blood products, cell and gene therapy products, and related biological products that fall under CBER's or CDER's regulatory purview.
What’s in the Core?

**BD FACS Aria IIu**
- Diva 8.0
- 4-5 lasers (UV thru Red)
- 17 color detectors
- FCS PMT
- Index Sort, ACDU

**BD FACS Aria II Fusion**
- Diva 8.0
- 4 lasers (Violet thru Red)
- 15 color detectors
- Index Sort, ACDU
- Integrated into a Baker Co. SteriGard-like BSC

**BSL3 Sorting -- BD FACS Aria IIu**
- Diva 8.0
- 4-5 lasers (UV thru Red)
- 17 color detectors
- Index Sort, ACDU
- Enclosed in a Baker Co. BioProtect IV BSC
What’s in the Core?

BD LSRII SORP Analyzer
- 5 lasers – UV thru Red
- 5 fluorescent parameters
- FSC PMT, HTS

BD LSRRFortessa SORP Analyzer
- 5 lasers -- UV thru Red
- 18 fluorescent parameters
- HTS

BD LSRFortessa X-20 Analyzer
- 4 lasers -- Violet thru Red
- 16 fluorescent parameters
- HTS

BD FACSCanto II Analyzer
- 3 lasers -- Violet thru Red
- 8 fluorescent parameters
- HTS
What’s in the Core?

- **Millipore EMD (Amnis)**
  Image Stream Mark II Analyzer
  - 5 lasers – UV (375nm), Violet (407nm), Blue (488nm), YellowGreen (561nm), Red (642nm)
  - 12 imaging parameters

- **Compucyte Laser Scanning Cytometer**
  (Thorlabs)
Flow Cytometry Training Outline

• Flow Cytometry Basics
  • Fluidics
  • Optics
    • Lasers
    • Collection optics
  • Electronics
  • Pulse generation
  • Analog vs Digital

• Signal Processing and Compensation

• Optimizing Instrument Settings for Multicolor Performance / QC
Training for a Diverse Group of Users

Support Documents

- Application Notes, Listing of Instruments and Parameters available, etc.
- Web links – spectrum viewers, commercial sites

CCFC Instrument / Diva SOP

This SOP covers the LSRII, Fortessa and X-20 Cytometers.

This document lists steps / specifications for starting Cytometers, general maintenance, QC, and experiment setup and execution.

Be sure to read Flow Core Information documents that display automatically on each computer desktop after startup. Herein you will find instructions for starting systems, as well as information for Cytometer Setup & Tracking (CS&T).

- Current Configuration(s)
- Current bead lot number
- Last Passing QC run
- Last cleaning cycle

Table of Contents:
1. Starting the System:
There are unique startup requirements for each instrument. Look the each section for specifications.
Training for a Diverse Group of Users

- Flow Cytometry Basics
  - Fluidics
  - Optics
  - Light Scatter
  - Lasers
- Panel Design
  - Collection optics
- QC and Controls
- Compensation
  - Optimization and Standardization
- Signal Processing and Compensation
- Optimizing Instrument Settings for Multicolor Performance / QC
Diagram of LSRFortessa / LSRII Fluidics

- Fluidics
Sample Flow -- Hydrodynamic Focusing

- Low Differential Pressure
- High Differential Pressure

- Lasers
- Low Sample Pressure 12µl/min
- Laminar Flow
- Sample Injection Needle
- Sheath Fluid
- Sample
- Sheath Fluid

- Fluidics
Many particles can be analyzed by Flow...

- Bacteria
- Blood cells
- Cell lines - reporter genes
- Protozoa
- Primary tissue cells - Stem cells
- Microparticles/Coated Bead Capture Array

• Light Scatter
• Light Scatter
• Light Scatter

Lysed Whole Blood

- Granulocytes
  - Basophils
  - Neutrophils
  - Eosinophils
- Monocytes
- Lymphocytes
- Debris, red cells, platelet aggregates
Principles of Panel Design

• Screen clones for efficacy
• Match fluorochrome conjugates to antigens:
  • by brightness (as estimated by stain index)
  • Antigen density and distribution (molecules per cell, smeared vs discrete distribution).
• Minimize spectral overlap.
• Use tandem dyes with consideration of their technical limitations.
• Use appropriate controls.
Panel Design

Fluors and Filters

http://wwwbdbiosciencescomresearchmulticolorspectrum_viewersonpageindexjsp#search=(spectrum viewer)

Panel Design

**Match Fluorochromes by Brightness**

- Know the Stain Index for each detector of the instrument for which you are designing a panel.
  - Specific to a set of lasers and filters
- Refer to the ranking of fluorochromes by stain index for a platform.
Measuring Resolution

Signal/Background vs. Stain Index\(^1\)

Stain Index (SI) = \( \frac{D}{W} \)

\( D = S - B \)

Goal: Normalize the signal to the spread of background where background may be autofluorescence, unstained cells, or compensated cells from another dye dimension.

\[
\begin{align*}
SI &= \frac{1.645 \times (\text{positive} - \text{background})}{\text{background}_{95\%} - \text{background}_{5\%}} = \frac{\text{positive} - \text{background}}{2 \times \text{StdDev}_{\text{background}}} \\
\end{align*}
\]

\(^1\)Stain Index: Dave Parks, Stanford
• Panel Design

LSR II Optics

- Blue 488nm Laser
- Red 640nm Laser
- Green 532nm Laser
- UV 355nm Laser
- Violet 405nm Laser
- Flow Cell
• Panel Design

LSRII Optics

- Red trigon
- Blue trigon
- UV trigon
- Violet trigon
- Green Octagon
- Flow Cell
• Panel Design

Match Fluorochromes by Brightness

• Know your instrument!
Panel Design

Use tandem dyes with consideration of their technical limitations.

- Compensation requirements for tandem dye conjugates can vary, even between two experiments with the same antibody
  - Require compensation that is: lot-specific, experiment-specific and label-specific.

- Certain tandem dye conjugates (APC-Cy7, PE-Cy7) can degrade with exposure to light, elevated temperature, and fixation, etc.
  - Minimize exposure to these conditions
  - Keeping tandems cold improves stability
  - Add tandems to cell preps not into empty tubes
False positives due to tandem degradation

A. With CD8 APC-Cy7 and CD4 PE-Cy7

- CD8 APC-Cy7+ cells
- CD4 PE-Cy7+ cells

B. Without CD8 APC-Cy7

- False positives in APC channel reduced in absence of APC-Cy7
- False positives in PE channel remain
Panel Design

**Compensation: Tandems**

- Panel Design

<table>
<thead>
<tr>
<th>CD8 PE-Cy7</th>
<th>CD3 PE-Cy5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time Sample Left in Light</td>
<td></td>
</tr>
<tr>
<td>0 hours</td>
<td></td>
</tr>
<tr>
<td>2 hours</td>
<td></td>
</tr>
<tr>
<td>22.5 hours</td>
<td></td>
</tr>
</tbody>
</table>

- PE
How is Compensation Done?

- Analog Circuits (FACScan, FACSort, Calibur)
- Post Acquisition Software Compensation
  - Software – FlowJo, WinList, FCS Express . . .
- Diva Acquisition Software -- LSRII, LSRFortessa, FACS Aria
  - Run Single Color Controls
  - In Diva – create compensation tubes, spillover matrix is automatically applied
Why Fluorescence Compensation is necessary

- Compensation
• Compensation

Error in measurement

Log scale: negative or zero values are set to > zero (arbitrarily?)
• Compensation

**Compensation Spread**

**Uncompensated**

**Compensated**
Display of Multicolor Data – digital data display issues

- 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.

1R Hardy data - Cytometry email thread 2002, DR Parks - Asilomar Workshop 2002
• Compensation

Synthetic data example – log scale

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.
• Compensation

Logicle: Compensated Biexponential Display

Log at the upper end, linear at the low, and symmetrical about zero. Biexponential transform where data zero is shown by the crosshairs in the plot

• This FlowJo example shows the value of a mostly logarithmic scale on the upper end, and a lower linear region occupies a reasonable plot area compared to that in the blended scale.
  – Compensated single pos are continuous
  – All populations are visible
Use Appropriate Controls

<table>
<thead>
<tr>
<th>What</th>
<th>Why</th>
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<tbody>
<tr>
<td><strong>Instrument setup controls</strong></td>
<td>Ensure consistent setup and</td>
</tr>
<tr>
<td>• CS&amp;T beads</td>
<td>compensation</td>
</tr>
<tr>
<td>• CompBeads</td>
<td></td>
</tr>
<tr>
<td>• Unstained and single color</td>
<td></td>
</tr>
<tr>
<td>stained cells</td>
<td></td>
</tr>
<tr>
<td><strong>Gating controls</strong></td>
<td>Obtain reliable gates for</td>
</tr>
<tr>
<td>• FMO</td>
<td>problematic markers</td>
</tr>
<tr>
<td>• Unstained / Isotype control</td>
<td></td>
</tr>
<tr>
<td>• Live / Dead stains</td>
<td></td>
</tr>
<tr>
<td><strong>Biological controls</strong></td>
<td>Make appropriate biological</td>
</tr>
<tr>
<td>• Unstimulated samples</td>
<td>comparisons and conclusions</td>
</tr>
<tr>
<td>• Healthy donors</td>
<td></td>
</tr>
</tbody>
</table>
Use FMO Controls for Accurate Data Analysis

- Fluorescence Minus One (FMO) Controls contain all the lineage markers except the one of interest.
- For low density or smeared populations (e.g., activation markers) FMOs allow accurate delineation of positively vs negatively stained cells.
  - T Regs are a good example.
• QC and Controls
• QC and Controls
Optimizing for performance . . .

- Manual Method
- Vary voltage incrementally for each parameter
- Determine interface – signal vs. “noise”
Why is PMT Optimization Important?

Finding PMT settings that maximize resolution sensitivity for each experiment:

Move fluorescent populations out of electronic noise

550 volts
650 volts
750 volts

CD4 dim monocytes
CD4+ lymphocytes
CD4 negative
Find the "best" voltage . . .

**Figure 3.** Analyzing the instrument response curves and determining the voltage required for optimal resolution sensitivity.
CS&T . . . the basics

- CS&T beads and software (in Diva 6.0) will automatically derive and store baseline and application-specific settings
- They will also determine and track:
  - Background (Br)
  - Sensitivity (Qr)
  - Linearity
  - Laser delay
  - Area scaling
- SDen
Evaluating Fluorescence Sensitivity on Flow Cytometers: An Overview

James C.S. Wood¹ and Robert A. Hoffman²

¹Beckman Coulter, Inc., Miami, Florida
²Becton Dickinson Immunocytometry Systems, San Jose, California

Received 9 June 1998; Accepted 17 June 1998

The current paradigms for assessing fluorescence sensitivity on flow cytometers do not provide an adequate assessment of an instrument’s ability to detect and measure weak fluorescence on stained particles. The capability to resolve dimly stained populations depends on two factors: the background noise (B), and the efficiency (Q) with which the fluorescence from the fluorochrome molecules are converted to photoelectrons. Any single statistical measure of fluorescence histogram distributions will be unable to uniquely characterize an instrument. Therefore, neither of the routinely used methods (detection threshold and delta channel) measure sensitivity completely and unambiguously. We show the limitations of these methods and propose that instrument sensitivity be characterized in terms of both background noise and detection efficiency in order to determine better the capability to detect and resolve weakly fluorescent particles. Cytometry 33: 256–259, 1998. © 1998 Wiley-Liss, Inc.

Key terms: fluorescence; sensitivity; resolution; flow cytometry
Electronic Noise: \( SD_{\text{EN}} \) is the constant low level background noise contributed by the electronics system.

<table>
<thead>
<tr>
<th>Laser</th>
<th>Detector</th>
<th>Parameter</th>
<th>Electronic Noise Robust SD</th>
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</thead>
<tbody>
<tr>
<td>Blue</td>
<td>FSC</td>
<td>FSC</td>
<td>N/A</td>
</tr>
<tr>
<td>Blue</td>
<td>F</td>
<td>SSC</td>
<td>N/A</td>
</tr>
<tr>
<td>Blue</td>
<td>E</td>
<td>FITC</td>
<td>23.19</td>
</tr>
<tr>
<td>Blue</td>
<td>D</td>
<td>PE</td>
<td>23.24</td>
</tr>
<tr>
<td>Blue</td>
<td>B</td>
<td>PE-Cy5</td>
<td>24.17</td>
</tr>
<tr>
<td>Blue</td>
<td>A</td>
<td>PE-Cy7</td>
<td>25.07</td>
</tr>
<tr>
<td>Red</td>
<td>C</td>
<td>APC</td>
<td>21.02</td>
</tr>
<tr>
<td>Red</td>
<td>A</td>
<td>APC-Cy7</td>
<td>22.73</td>
</tr>
<tr>
<td>Violet</td>
<td>B</td>
<td>Pacific Blue</td>
<td>22.90</td>
</tr>
<tr>
<td>Violet</td>
<td>A</td>
<td>AmCyan</td>
<td>28.99</td>
</tr>
</tbody>
</table>
Factors to Consider for an Optimal Gain Setup

Optimizing the cytometer setup for flow applications

1. Electronic Noise can affect Resolution Sensitivity
   ✓ A good *minimal* application PMT voltage would place the dimmest cells (unstained) where electronic noise is no more than 10% to 20% of the total variance

\[
\frac{rSD_{EN}}{\sqrt{0.10}} < rSD_{Neg Cells} < \frac{rSD_{EN}}{\sqrt{0.20}} \quad \text{and} \quad rSD_{Neg Cells} > \frac{2.5 \times rSD_{EN}}{
\]

2. Dynamic Range Assessment
   a) Are the positives too high? Insuring the cells are in the linear range of the detector is essential for accurate data
   b) Are the Negatives (in a stained sample) too high?
   c) Are the compensation controls within the linear range of the detector?

3. An Optimal cytometer setting is one where all conditions are met
Standardization across instruments – similar / different configurations

Determine the Virtual Predicate (Worst Instrument)

Run Cells to Generate Application Settings with 2.5X rSD_{EN} targets and bright cells are below Max linearity

Using the Application Settings, run CS&T beads to record bright bead target values

Adjust all other instruments to hit bright bead target values and save as Application Settings
1 Assay – 4 Platforms

- CD4 V450
- CD8 PE
- CD3 FITC
- CD20 APC
Standardizing application setup can ensure consistency of results over time across multiple systems. To achieve this consistency, each cytometer must be properly and efficiently characterized and its performance levels maintained. BD™ Cytometer Setup and Tracking (CS&T) software and BD™ Cytometer Setup and Tracking beads (dim, mid, and bright) were developed to deliver this characterization and performance maintenance capability.

In this technical bulletin, we describe how users can create and save the optimal settings for specific applications (called application settings in BD FACSDiva™ software) using BD CS&T bright bead target values for each fluorescence detector, to obtain consistent and reproducible results over time. As needed, these CS&T bright bead target values can be used to reproduce application settings on other cytometers with identical optical configurations, or to create new application settings. Multiple cytometers with different optical components such as lasers, laser power, and filters also can be standardized. This technical bulletin explains the principles underlying application settings and the standardization process, and provides details on how to:

- Create optimal application settings
- Save the settings using CS&T bright bead target values
- Standardize multiple cytometers
Sort – TFH and GC from C57BL mice inoculated w/ SRBC, Day10
- B220 BV605
- CD4 PerCP-Cy5.5
- FAS Alexa647
- GL-7 FITC
- CD44 Alexa 700
- PD-1 PE
- CXCR5 BV421

Core client -- Shafiuddin “Shafi” Siddiqui, PhD.
Training for a Diverse Group of Users

• Fluidics
• Light Scatter
• Panel Design
• Compensation
• QC and Controls
• Optimization and Standardization
• Practice flow for a long, long time . . .
Questions?
Know the Stain Index of Your Reagents

- The Stain Index of a reagent is a function of:
  - Relative fluorescence intensity of the fluorochrome
  - Density of the antigen on the cell
  - Autofluorescence of the cell in the detector

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Stain Index</th>
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<tbody>
<tr>
<td></td>
<td>CD19</td>
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<tr>
<td>FITC</td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td></td>
</tr>
<tr>
<td>PerCP</td>
<td></td>
</tr>
<tr>
<td>PerCP-Cy5.5</td>
<td></td>
</tr>
<tr>
<td>PE-Cy7</td>
<td></td>
</tr>
<tr>
<td>APC</td>
<td></td>
</tr>
<tr>
<td>APC-Cy7</td>
<td></td>
</tr>
<tr>
<td>APC-H7</td>
<td></td>
</tr>
<tr>
<td>Horizon V450</td>
<td></td>
</tr>
<tr>
<td>AmCyan</td>
<td></td>
</tr>
<tr>
<td>Horizon V500</td>
<td></td>
</tr>
</tbody>
</table>
Spread Antigens Across Lasers

If multiple antigens are present on a cell, spread them across as many lasers as possible to minimize spillover.

Example:
CD3 “bright”          APC-Cy7 (s.i. = 42.2)
CD7 “less bright”   PE (s.i. = 356.3)

Both antigens expressed on same cell, low spillover of CD3 into CD7 and vice versa.

CD3 = 124K molecules/cell
CD7 = 20K molecules/cell
- Compensation

**Biexponential Display**

Log Display

- Log at the high end of the scale

Biexponential

- Linear at the lowest end of the scale
- Symmetrical around zero
Digital & Analog -- Software Compensation

Digitally compensated analog data bears a striking resemblance to digital data

Courtesy of Randy Hardy, Fox Chase Cancer Ctr
• Compensation

Correct Population Alignment

\[ \text{Log}_{10} \]

\[ \begin{align*}
\text{BiExp} & \quad 26283 & 24911 \\
& \quad 16916 & 16120 \\
& \quad 2229 & 2484 \\
& \quad 77 & 10
\end{align*} \]
Optical Filters

- For a given laser pathway, fluorescence is combined – colored filters limit fluorescent wavelengths detected
- Filters typically used
  - Dichroic Mirrors – long or short pass w/ reflective surface
  - Band Pass – combines long and short pass limits
  - ND Filters – defined in log-units of signal diminishment

• Compensation
- Compensation

**Optimized BiExponential**

*Diva -- algorithm is automatically scaled*
Comparison of controls

FMO vs. Isotype
Cytometer Baseline: Report

Cytometer Information

Bead Information

Detectors

- **Bead Medians and rCVs**
- **Linearity**
- **Qr and Br**
- **SD Electronic Noise**
- **Baseline PMT Voltage**
- **Bright Bead Target Values**
  - **Median Fluorescence**
### Detectors

- **Bead Medians and rCVs**
- **PMT Voltage -- Δ from Baseline**
- **Qr and Br**
- **Detector Pass/Fail**

<table>
<thead>
<tr>
<th>Laser</th>
<th>Detector</th>
<th>Parameter</th>
<th>Target Value</th>
<th>Actual Target Value</th>
<th>% Difference Target Value</th>
<th>Bright Bead %Robust CV</th>
<th>Mid Bead Median Channel</th>
<th>Mid Bead %Robust CV</th>
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<tbody>
<tr>
<td>Blue</td>
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<td>FSC</td>
<td>119348</td>
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<td>1.78</td>
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<td>36603</td>
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**Detector Settings (Continued)**

<table>
<thead>
<tr>
<th>Laser</th>
<th>Detector</th>
<th>Parameter</th>
<th>Dm Bead Median Channel</th>
<th>Dm Bead %Robust CV</th>
<th>PMTV</th>
<th>Δ PMTV</th>
<th>Qr</th>
<th>Br</th>
<th>P/F</th>
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<tr>
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