Instrument Characterization and Performance Tracking for Digital Flow Cytometers

BD Biosciences Cytometer Set-up & Tracking (CS&T) System

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Class I (1) Laser Products
23-9335-00

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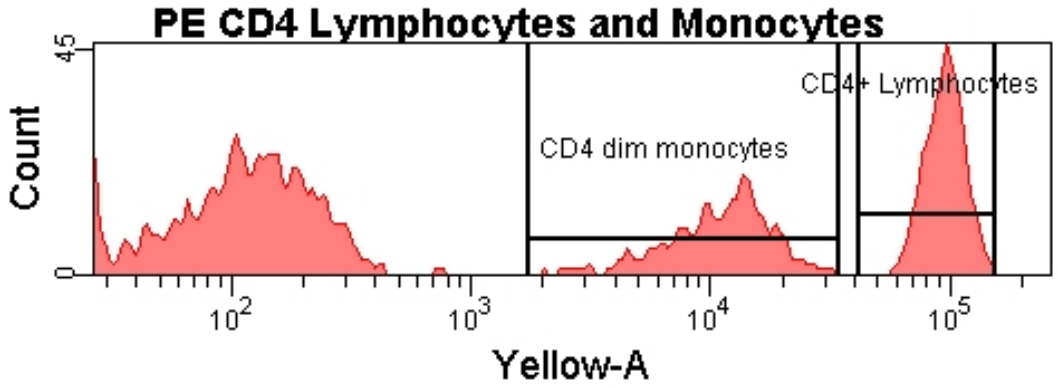
Key Performance Factors in High Quality Flow Cytometry Data



- Relative measured values of fluorescence
  - **Linearity and accuracy**
- Resolution of subpopulations, including dim subpopulations
  - **Sensitivity**
- Reproducibility of results and cytometer performance
  - **Tracking**
- Comparison of results across time and among laboratories
  - **Standardization**

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A Breakthrough for Flow Cytometry

- The Cytometer Setup & Tracking (CS&T) system is a fully automated software and reagent system for BD digital flow cytometers
  - **Functions of the CS&T system**
    - Defines and characterizes baseline performance
    - Optimizes and standardizes cytometer setup
    - Tracks cytometer performance
  - **Benefits of the CS&T system**
    - Provides consistent, reproducible data every day
    - Simplifies design of multi-color experiments
    - Yields higher quality data from multi-color experiments
    - Identifies any degradation of cytometer performance *early*

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Cytometer Setup & Tracking (CS&T) System: The Software

- BD FACSDiva™ 6.0 software
  - Has Unique Cytometer Setup & Tracking module
  - Compatible with BD FACSCanto™ , BD FACSCanto™ II, BD FACSAria™ , and BD™ LSR II digital cytometers
  - One tube–one click simplicity
- Newly developed BD CS&T beads
- Very flexible system using user-defined:
  - Cytometer configurations to support any number of lasers and parameters
  - Labeling - Fluorochrome parameters, filters, and mirrors including Q-dots and new fluorochromes
  - Alarm boundaries for tracking performance
  - Application settings – linked to CS&T setup

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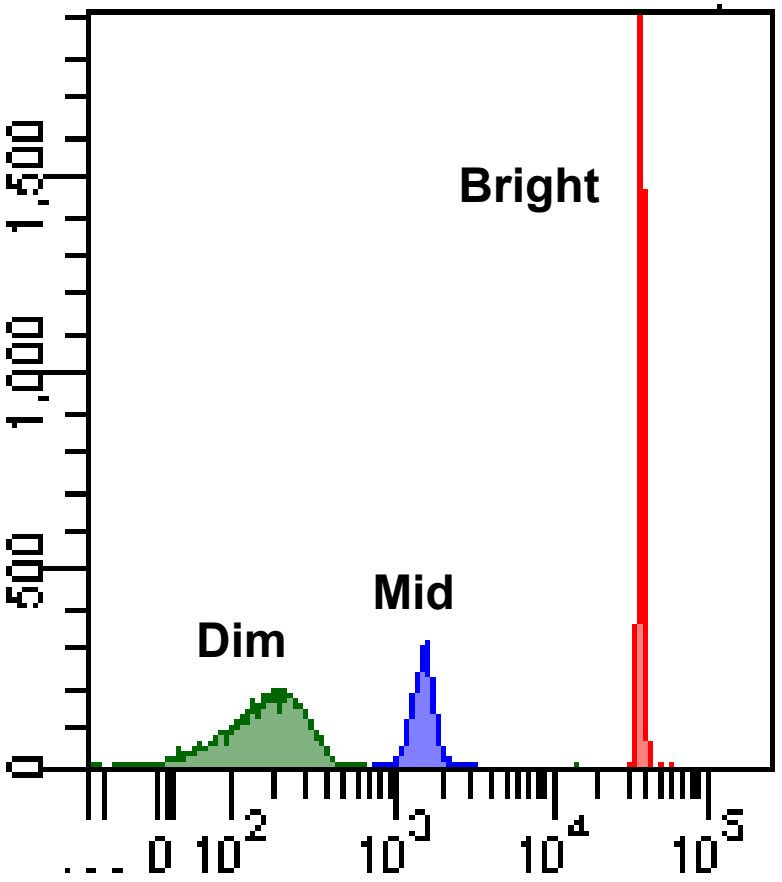
Cytometer Setup & Tracking (CS&T) System: The Beads

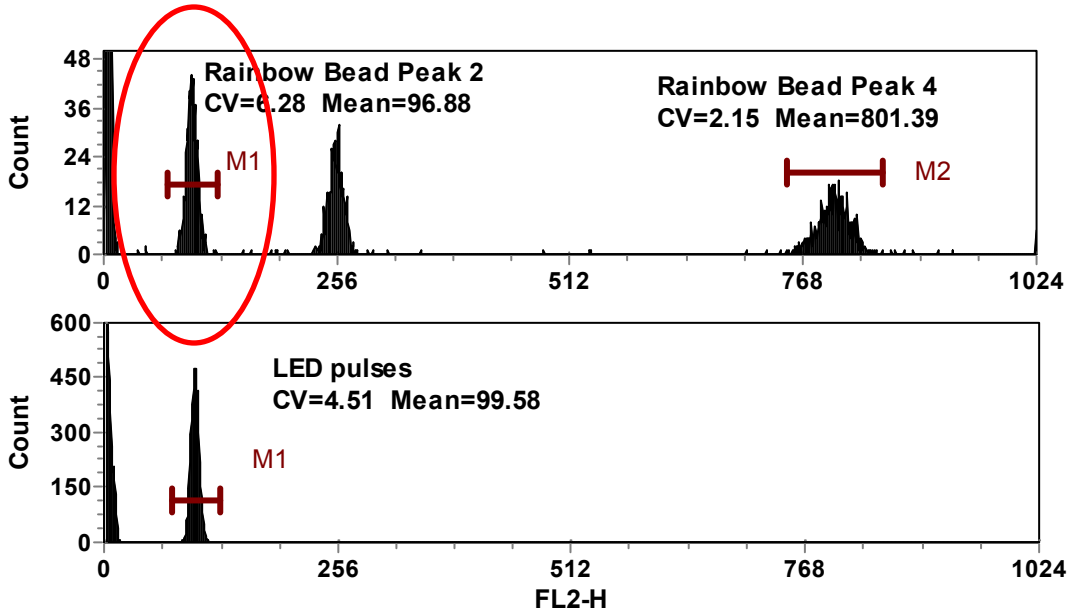
- The CS&T Bead set consists of uniform beads of 3 different intensities designed to fully characterize the flow cytometer

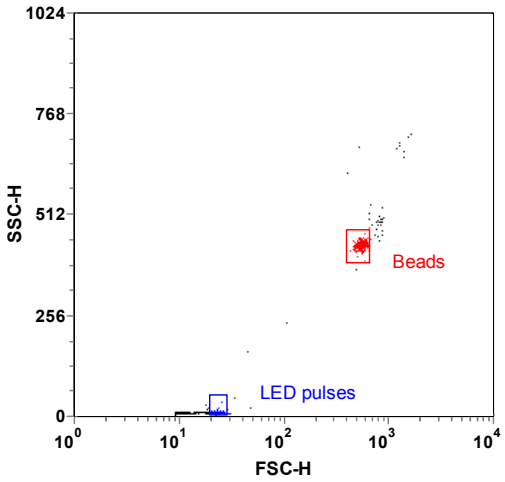
- **Bright Beads -** negligible photoelectron contribution to CV
- **Mid Beads -** large photoelectron contribution to CV
- **Dim Beads -** significant background contribution to CV



Determining the Intrinsic CV of Beads

In order to estimate bead photoelectron statistics we first need to know the intrinsic (non-photoelectron) CVs





RH000620.001
Rainbow Lin2.01PMT640
RGB FACScan 120 mW laser

Peak4 CV= 2.15%
LED CV at channel 800 predicted= 1.59%
Peak4 non-photoelectonCV= 1.48%

Peak3 CV= 3.24%,
LED CV at mean 255 predicted= 2.82%
Peak 3 non photoelectron CV= 1.59

Peak2 CV= 6.28%
LED CV at mean 97= 4.58%
Peak2 non-photoelectron CV= 4.29

Measure the difference in CV

$$\text{Particle CV}_{\text{Intrinsic}} = \sqrt{\text{CV}^2_{\text{Particle}} - \text{CV}^2_{\text{LED}}}$$

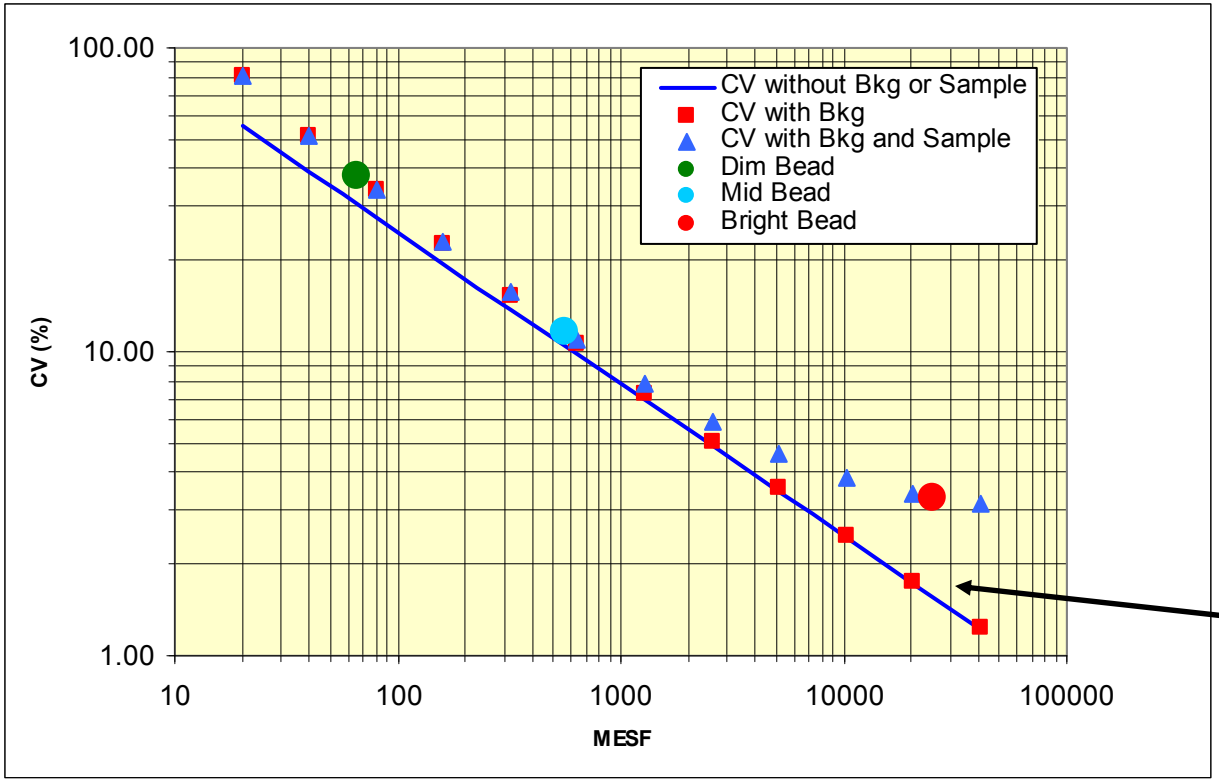
Of the beads against a "perfect particle", LED pulses

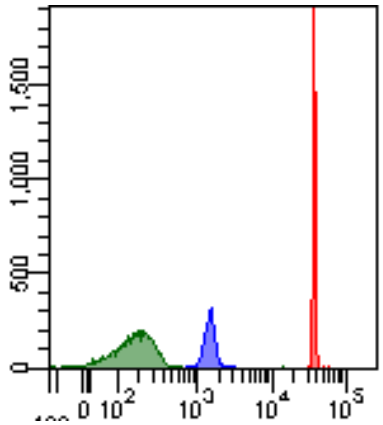
$$\text{Peak2 CV}_{\text{Intrinsic}} = \sqrt{6.28^2 - 4.58^2} = 4.29$$

Performance: Using the 3 bead set

By correcting for the bead intrinsic CVs, and for alignment and illumination effects at each step, we can calculate the photoelectron statistics.

If we know the variance we can determine performance.



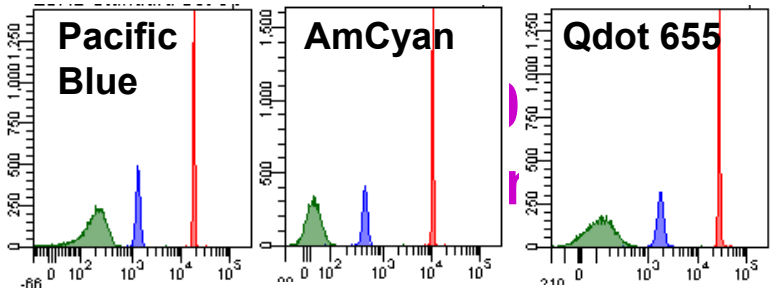


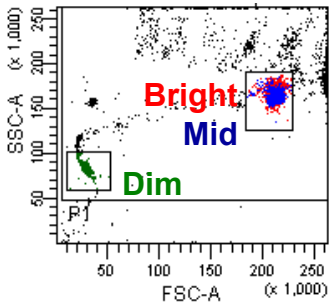
Photoelectron CV
Is proportional to

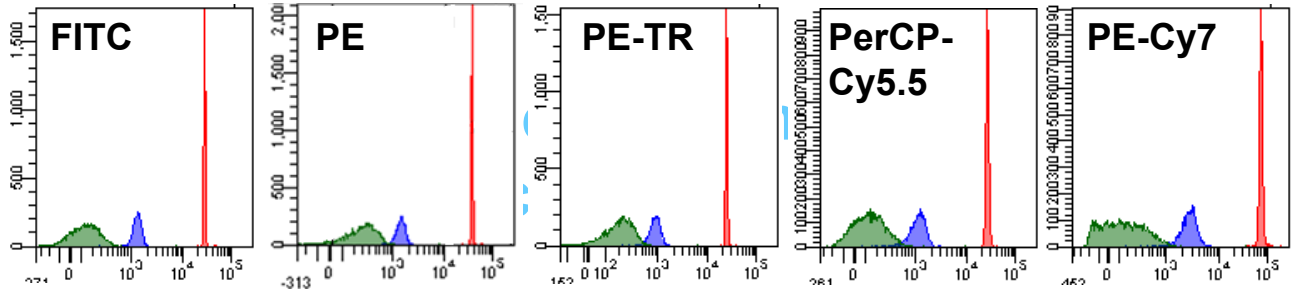
$$\frac{1}{\sqrt{Signal + Background}}.$$

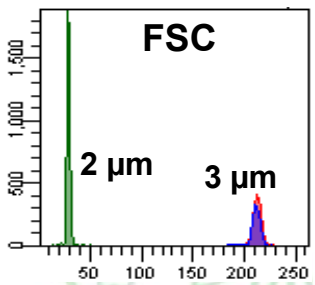
The Cytometer Setup & Tracking (CS&T) System: Beads Flexibility

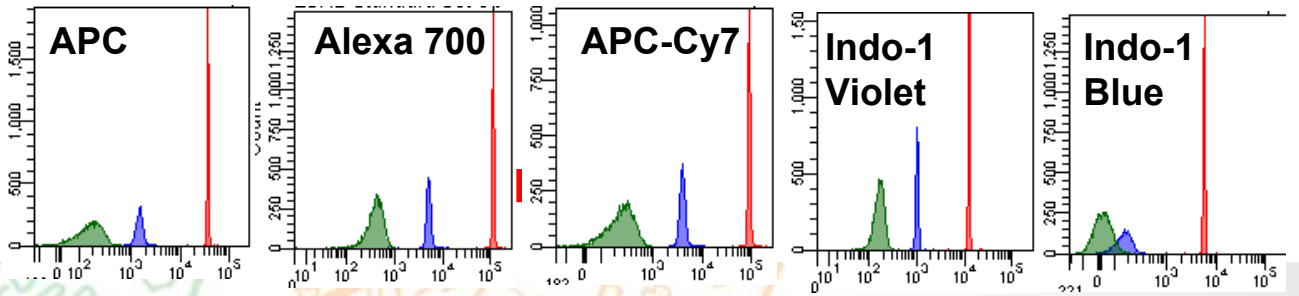
The beads are designed to be excited by all currently supplied lasers and emit in the respective detectors for virtually any fluorescent dye.



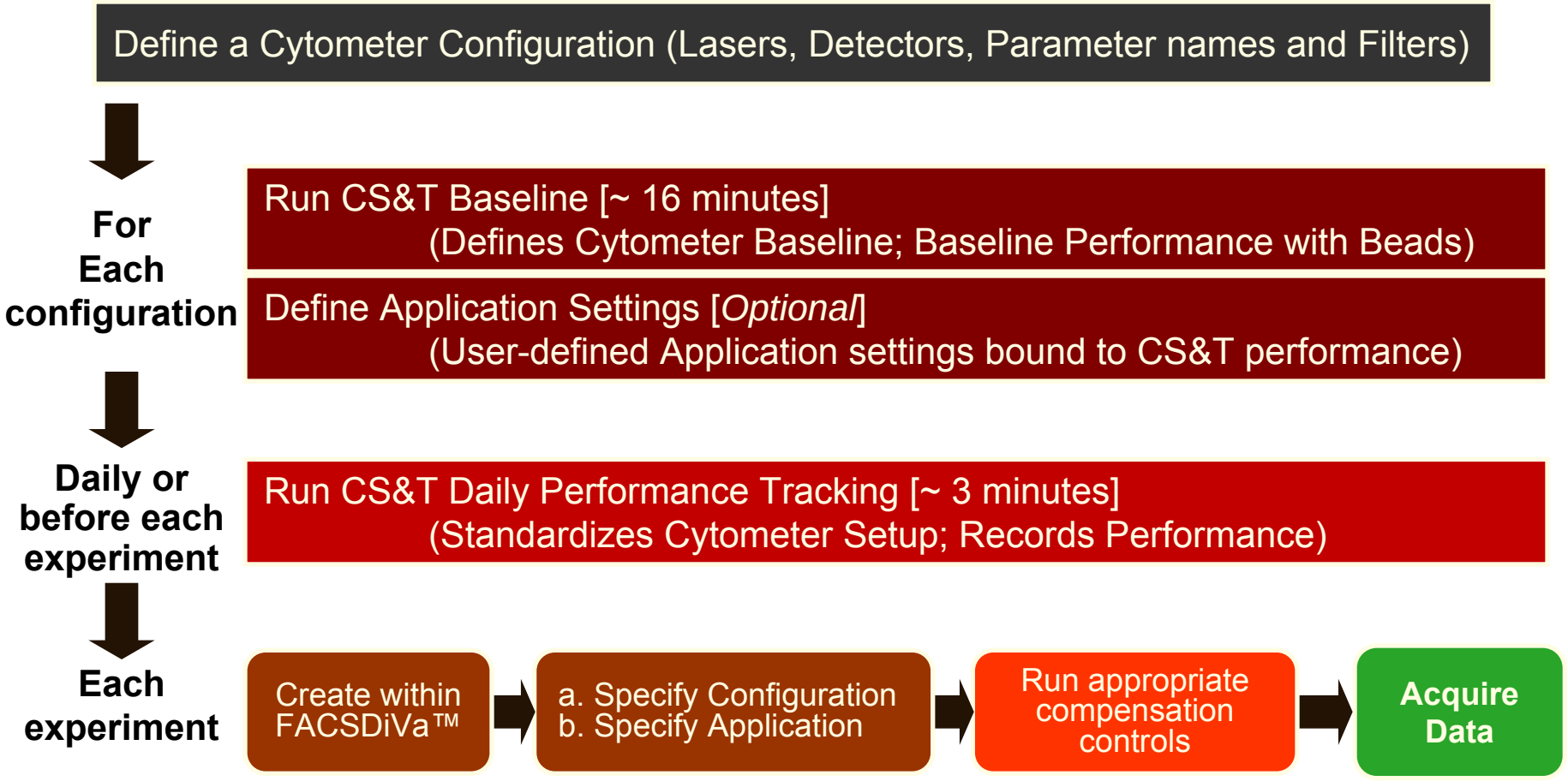








The Cytometer Setup & Tracking (CS&T) System: Multicolor work flow

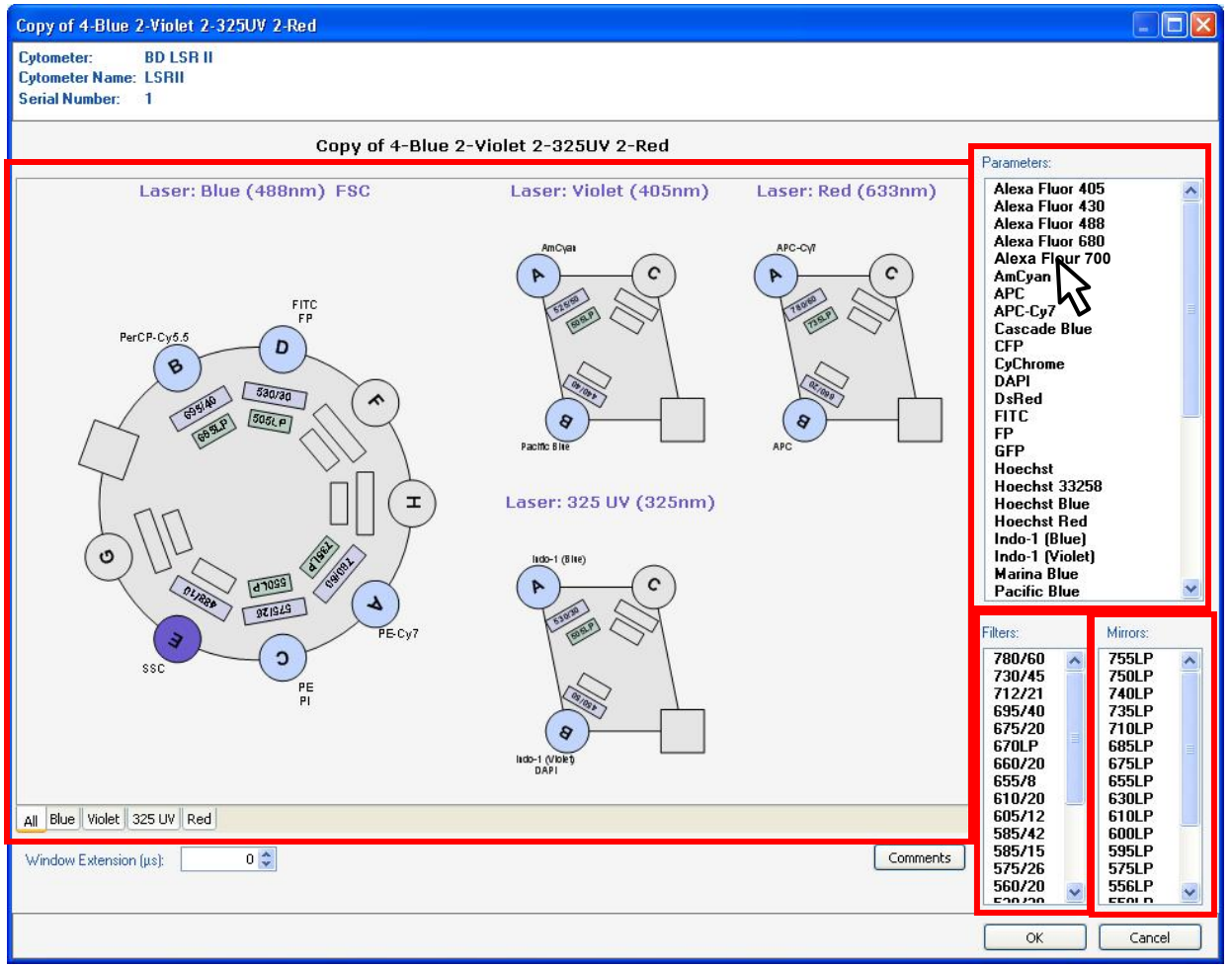


Defining Cytometer Configurations

Total User Flexibility

- Any number of lasers
- Octagon and trigon optical benches
- Any number of fluorochrome parameters
- Any number of bandpass filters
- Any number of dichroic mirrors

Simply drag parameters, filters, and mirrors onto octagon and trigon optical benches



Characterizing the Cytometer Baseline

- Perform ONCE for every new cytometer configuration or if you change or add a configuration (lasers, PMTs or filters)
- For each fluorescence detector, BD FACSDiva™ 6.0 software's CS&T system automatically determines the following performance parameters
    - Laser Delays (and adjust) – uses bright bead
    - Linear Range (±2%) – uses mid and bright beads
    - Fluorescence Detector Efficiency (Qr) - Mid Bead (with adjustments from bright and dim bead performance)
    - Optical Background (Br) - dim bead (with adjustments from mid bead performance)
    - Electronic Noise (SD_{EN}) – uses dim bead performance
    - Baseline PMT Voltage Settings: Minimal Effects from Electronic Noise – based on dim bead performance (using SD_{EN})
    - Creates Target Values for Ongoing Standardization - bright bead

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Performance Parameter: Linearity

- Define Linearity as proportionality of output to input (*signal : number of photons*)
- Important for accurate fluorescence compensation
- Important for quantitative measurements
  - DNA measurements
  - Antigen - Antibody binding
- The BD FACSDiva™ 6.0 CS&T module uses a robust and reliable method (dual signal ratio)

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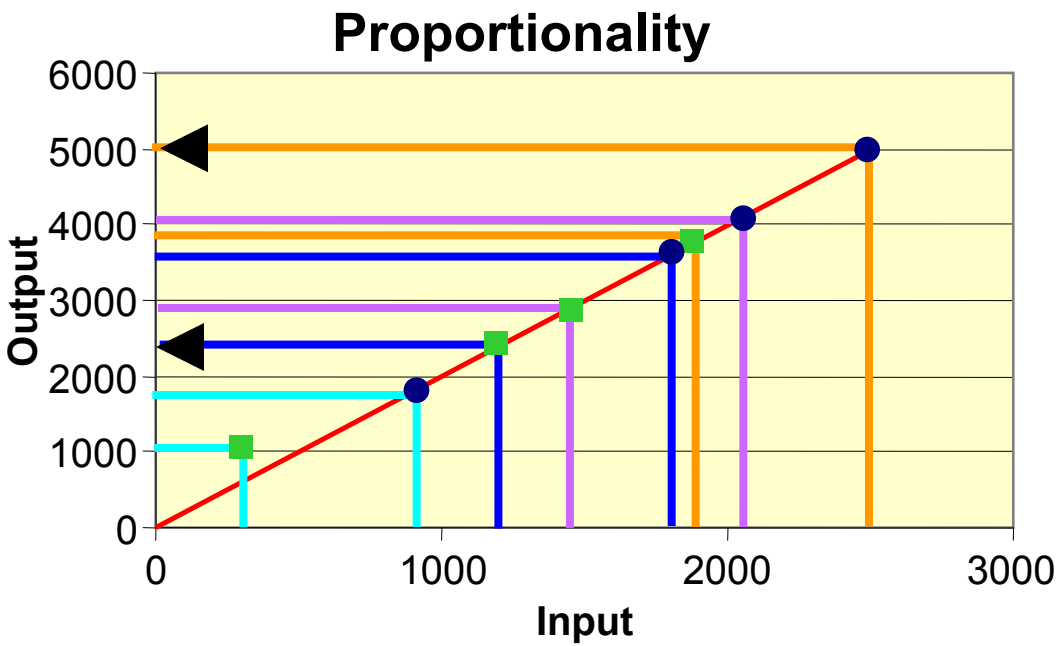
Linearity: Using Ratios

Linearity means proportionality

$R_1 = R_2 = R_3 \neq R_4$



$$\frac{Y_2}{X_2} = \frac{Y_1}{X_1}$$

Or equivalently

$$\frac{Y_2}{Y_1} = \frac{X_2}{X_1}$$

The detector is linear from 2500 to 5000 units

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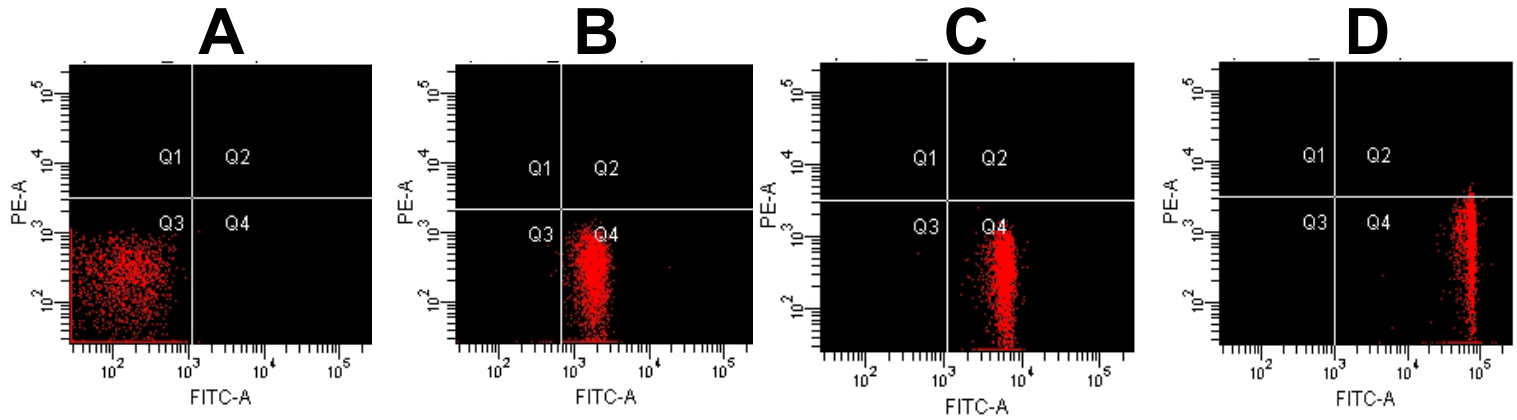
Linearity: Effect on Compensation

- Compensation of data in the last decade involves subtraction of large numbers
- Errors (non-linearity) in one or both large numbers can cause a large absolute error in the result

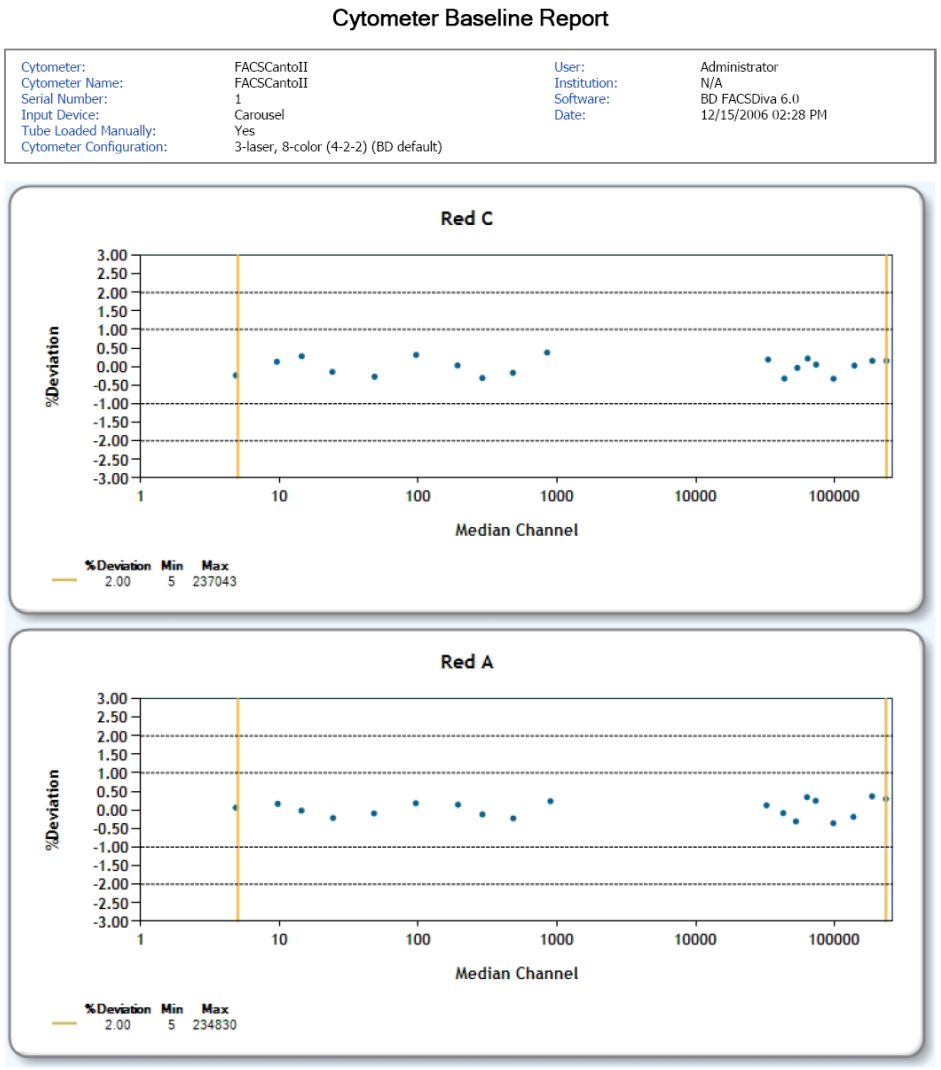


Detector	Median Fluorescence Intensity (MFI)			
FITC	68	1796	5921	73,000
PE	80	75	79	365

BD CompBeads stained with varying levels of FITC-Ab.
Compensation was set using samples A and C.
This cytometer had a 2% deviation from linearity above 50,000 units.

Linearity: CS&T Baseline Report

- BD FACSDiva™ 6.0 CS&T module automatically generates a Cytometer Baseline Report
  - Linearity range charted for every fluorescence detector
  - Default tracking boundary of ± 2.0% deviation
  - Entire reports or charts can be printed



Performance Parameter: Sensitivity

- Defining sensitivity
  - **Threshold -** Degree to which a flow cytometer can distinguish dimly stained particles from particle free background. Usually used to distinguish populations on the basis of Molecules Equivalent Soluble Fluorochrome (MESF).
  - **Resolution -** Degree to which a flow cytometer can distinguish unstained and dimly stained particles in a mixture. Can be very complicated in a polychromatic scenario.
- What is good sensitivity?
  - Generally cytometer threshold sensitivity is high – threshold definition (50–200 MESF FITC, for example)
  - Good threshold sensitivity does not necessarily guarantee good resolution of dim cells from unstained cells
- Detector efficiency (Q)
  - Contributes to sensitivity (both kinds) and is predetermined by factors such as laser power and optical design
- Goal: Optimize cytometer setup for the best resolution sensitivity by using enough detector gain to place measured populations above noise

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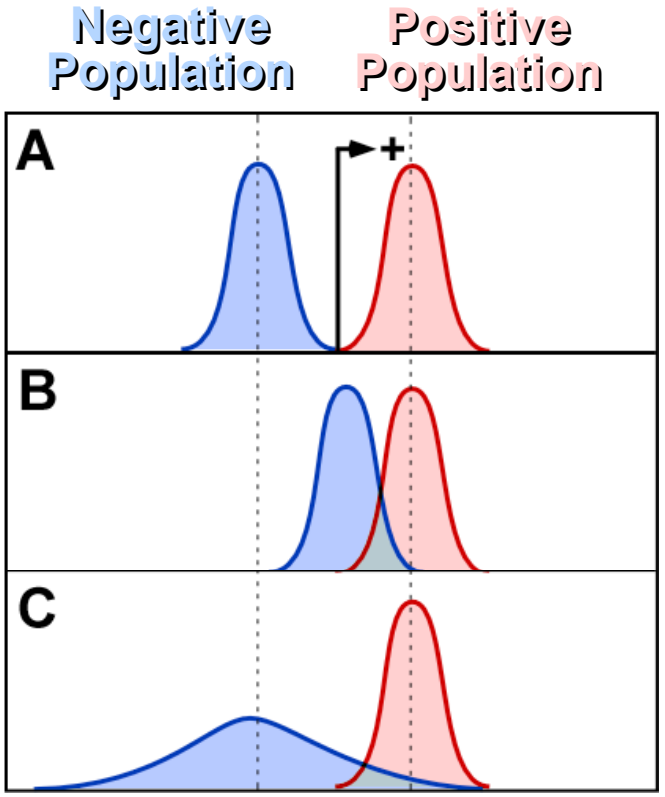
Sensitivity: Resolution vs. Background

Negative population has low signal and low CV
Populations well resolved

Negative population has high signal
Populations not resolved

Negative population has low signal and high CV
Populations not resolved



The ability to resolve populations is a function of both the relative signal levels and spread of the populations

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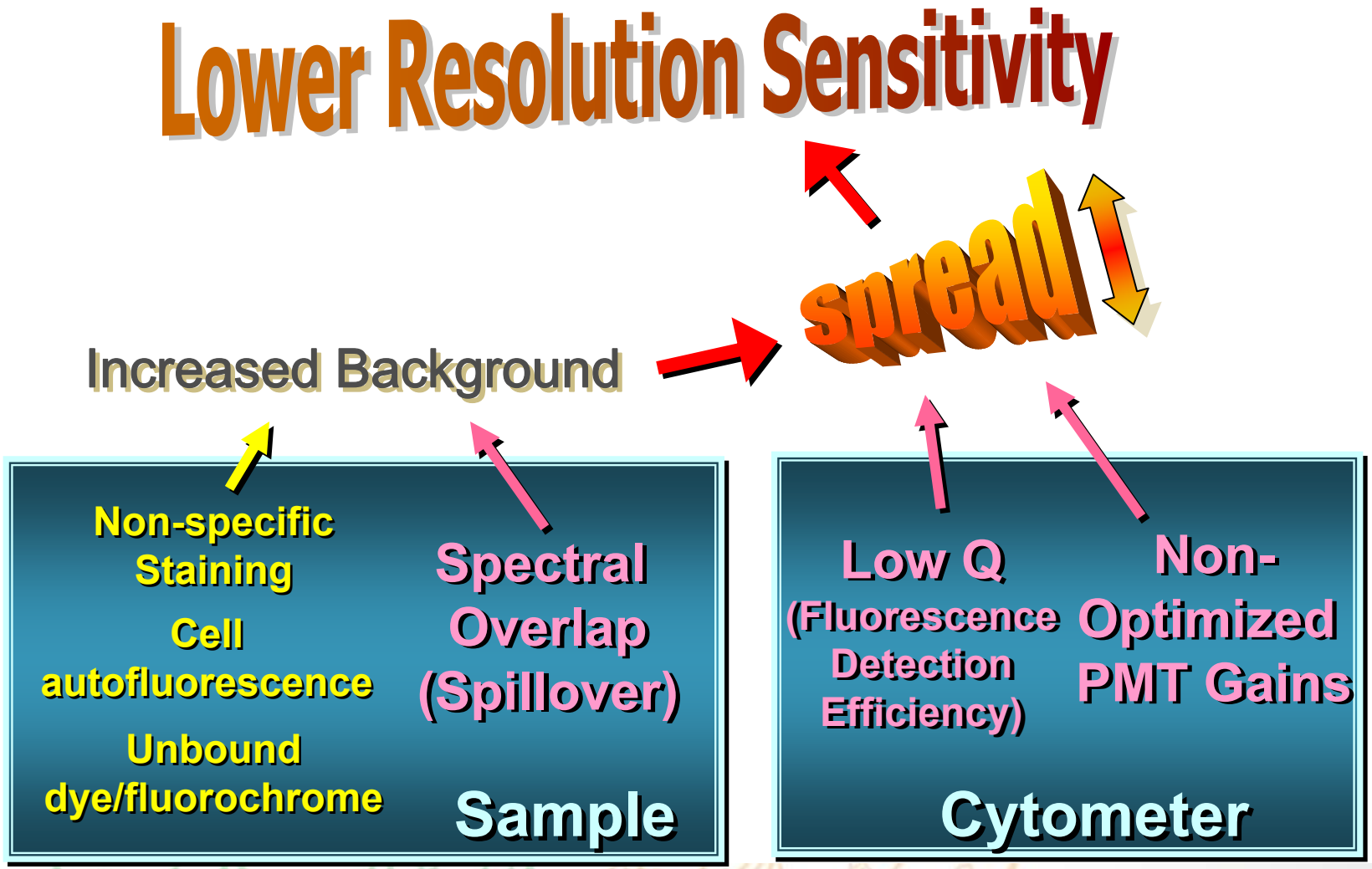
Sensitivity: Factors Impacting Resolution



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Performance Parameter: Q

- Measures the relative efficiency of a given detector to measure fluorescence
  - Photoelectrons detected per equivalent fluorochrome molecule
    - Fewer photons detected per molecule = lower Q = higher CV = increased spread = lower resolution sensitivity
  - Accepted standard in the flow cytometry community
- BD FACSDiva™ 6.0 CS&T module calculates a relative Q (Qr)
  - All fluorescence detectors
  - Based upon the median and robust CV (rCV) values for each of the CS&T fluorescent bead types
- The following affect Q:
  - Laser power and laser alignment
  - Cleanliness of optics (cuvette, lenses, mirrors, etc…)
  - PMT spectral sensitivity: PMTs become less sensitive towards the red; therefore, Q is lower for these detectors (e.g. PE-Cy7 lower than FITC)
  - Optical Design: number of dichroic mirrors, N.A. of collection lens, etc…

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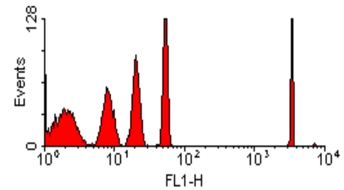
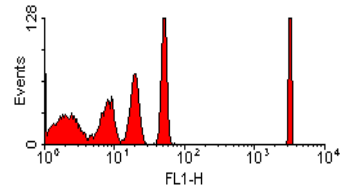
Q: Relationship to Resolution Sensitivity

Sample of multi-intensity beads analyzed on the BD FACSCalibur™ cytometer

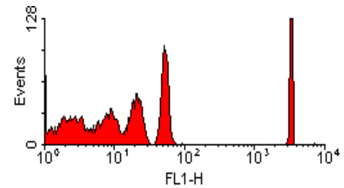


Vary laser power which affects Q

PMT voltages adjusted to keep bright bead median constant



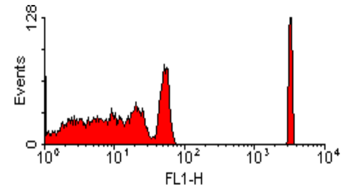
Resolution of dim populations depends more on the width than the mean of the distributions



Lower laser power →

Fewer photons per fluorochrome →

Lower Q →



Laser Power	Q
30 mW	**.030**
15 mW	**.018**
7.5 mW	**.008**
3.75 mW	**.004**

Decreased Resolution Sensitivity

Performance Parameter: B

- Optical background
  - B is a measure of optical background signal in the detector
  - Baseline restoration electronics subtract constant backgrounds during current to pulse conversion
  - Baseline restoration electronics subtract background signal levels, but cannot remove measurement error when their levels are high (works like compensation)

    Increased optical background broadens the distribution of all populations and results in decreased resolution sensitivity, most evident when measuring dim populations.
  - Background Contributing Factors
    - Free fluorescent dye in sample
    - Raman scattering
    - Ambient light
    - Damaged optics

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Performance Parameter: Electronic Noise (SD_{EN})

- Effects due to 9 electronics signal connections, PMT noise, ADC noise, digital error, etc…
- Broadens the distribution of all populations, most noticeable at the low end of the scale: dim populations
- Increases in electronic noise results in decreased resolution sensitivity (*most noticeable at the low end*)
- Used by the BD FACSDiva™ 6.0 CS&T module to establish baseline targets that minimize the spread of negative and dim populations
  - Baseline Target value of dim bead ≈ 10 x SD_{EN}
  - Set application voltage in DiVa so that MFI of negative cells remains at CS&T baseline target or above

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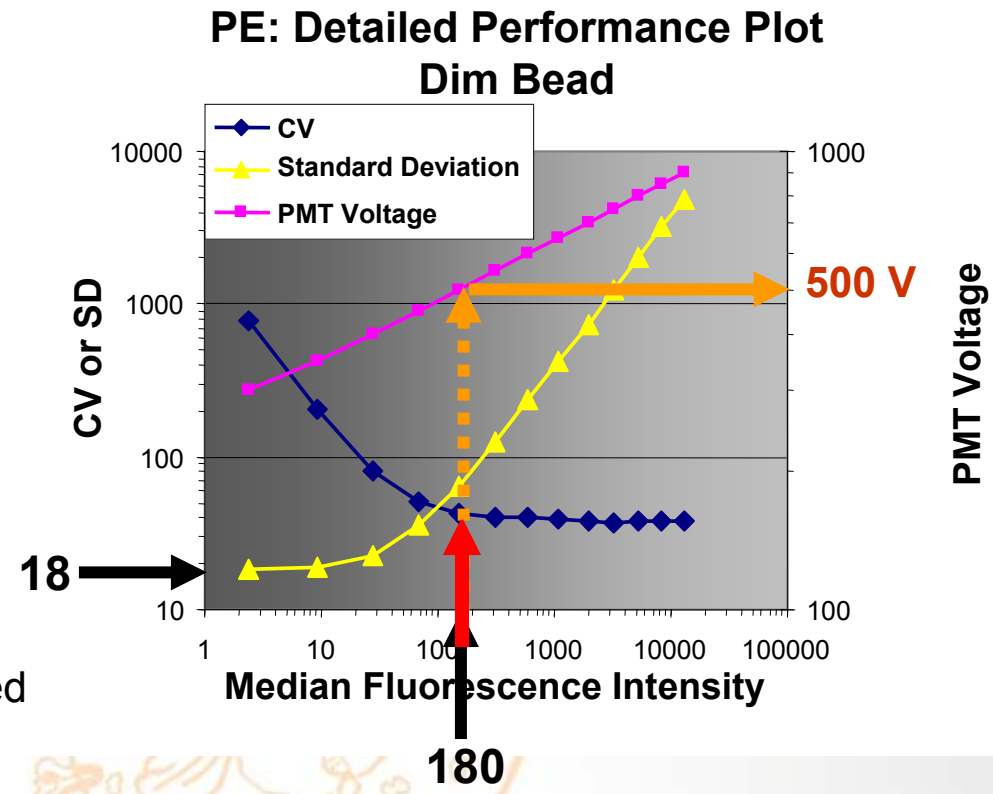
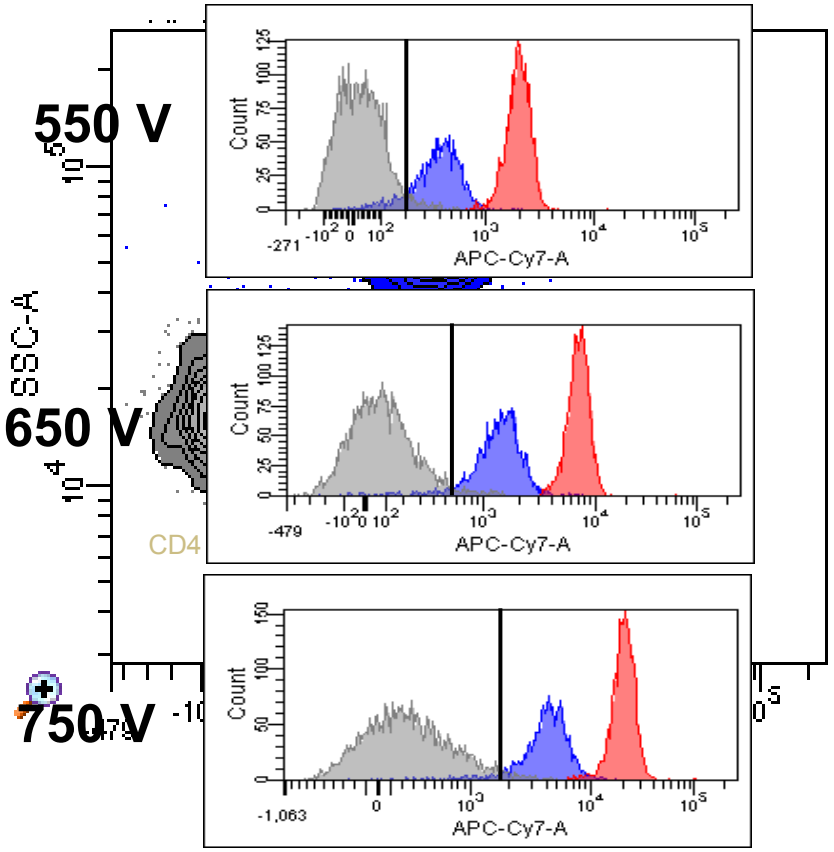
Electronic Noise (SD_{EN}): Determining Baseline PMT Voltages

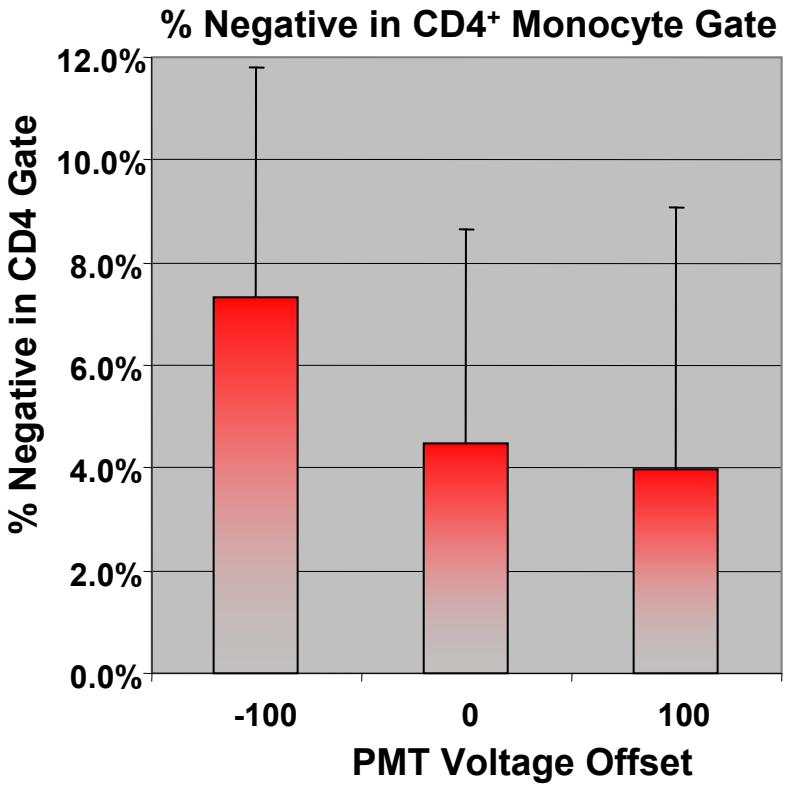
The BD FACSDiva™ 6.0 CS&T module analyzes dim particles, which are similar to dim cells' brightness, allowing relevant detector baselines to be visualized by plotting fluorescence intensity vs (PMT gain, CV, and SD)

- For this detector, the SD_{EN} = 18
- Fluorescence intensity of dim bead = 10 x SD_{EN} = 180
- Determine PMT voltage required to set the dim beads at 180 = 500 volts = baseline voltage
- As PMT voltage is lowered, CV increases → resolution decreases
- As PMT voltage is increased CV unchanged → resolution unchanged



PMT Voltages: Optimal Gains Can Reduce Classification Errors





Performance Parameters: Summary

- Linearity
  - Lets you know where potential errors in compensation may occur
  - Visually inspect linear range (± 2%, ± 1% or ± 0.5% error)
  - Avoid reagents with medians outside the linearity range
- Detector Efficiency (Q)
- Optical Background (B)
- Electronic Noise (SD_{EN})
- All performance parameters affect resolution sensitivity
- Important dim fluorescent markers should be measured in detectors with high Q values, low B, and low SD_{EN}
- Changes in these parameters can indicate cytometer problems
  - Increases in SD_{EN} → bad PMT connections or other electronic problem
  - Decreases in Q → low laser power, dirty flowcell, alignment or filter issue
  - Increases in B → fluorescent contaminant, failing laser or filter problems

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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**

Cytometer Baseline Report

Cytometer:	FACSCanto	User:	BDService
Cytometer Name:	FACSCanto	Institution:	N/A
Serial Number:	DEMOV0056	Software:	BD FACSDiva 6.0
Input Device:	Carousel	Date:	12/13/2006 03:39 PM
Tube Loaded Manually:	Yes		
Cytometer Configuration:	2-laser, 6-color (4-2) (BD default)		

Setup Beads

Bead Product: CST Setup Beads, Part #: 345678
Lot ID: 54321, Expiration Date: 10/13/2007

Detector Settings

Laser	Detector	Parameter	PMTV	New Target Value	Old Target Value	Bright Bead % Robust CV	Mid Bead Median Channel	Mid Bead %Robust CV	Dim Bead Median Channel	Dim Bead %Robust CV
Blue	FSC	FSC	359	119758	N/A	N/A	119690	N/A	15963	N/A
Blue	E	SSC	362	125902	N/A	N/A	125321	N/A	67856	N/A
Blue	D	FITC	530	32769	N/A	2.38	1498	9.36	170	34.42
Blue	C	PE	441	23152	N/A	1.72	871	9.61	192	29.42
Blue	B	PerCP-Cy5.5	531	36093	N/A	2.95	1581	11.52	186	34.03
Blue	A	PE-Cy7	707	82540	N/A	4.88	3133	20.51	184	85.77
Red	B	APC	546	43003	N/A	2.63	1557	13.26	183	40.21
Red	A	APC-Cy7	524	60540	N/A	3.11	2617	10.22	200	39.08

Detector Settings (Continued)

Laser	Detector	Parameter	Linearity Min Channel	Linearity Max Channel	Slope	Intercept	Electronic Noise Robust SD	Qr	Br
Blue	FSC	FSC	N/A	N/A	0.0038	3.69	N/A	N/A	N/A
Blue	E	SSC	N/A	N/A	7.5987	-14.30	N/A	N/A	N/A
Blue	D	FITC	197	174901	7.5100	-15.94	18.11	0.0842	92.32
Blue	C	PE	177	157054	7.4772	-15.40	20.38	0.1802	309.13
Blue	B	PerCP-Cy5.5	355	155736	7.4947	-15.86	19.36	0.0491	0.00
Blue	A	PE-Cy7	146	153291	7.4885	-16.42	19.21	0.0078	6.98
Red	B	APC	91	188536	7.5379	-16.00	18.43	0.0371	5.83
Red	A	APC-Cy7	204	167284	7.6259	-15.96	20.76	0.0391	22.26

Cytometer Baseline Report: Qr, Br, and SD_{EN}

Data from a BD FACSCanto cytometer

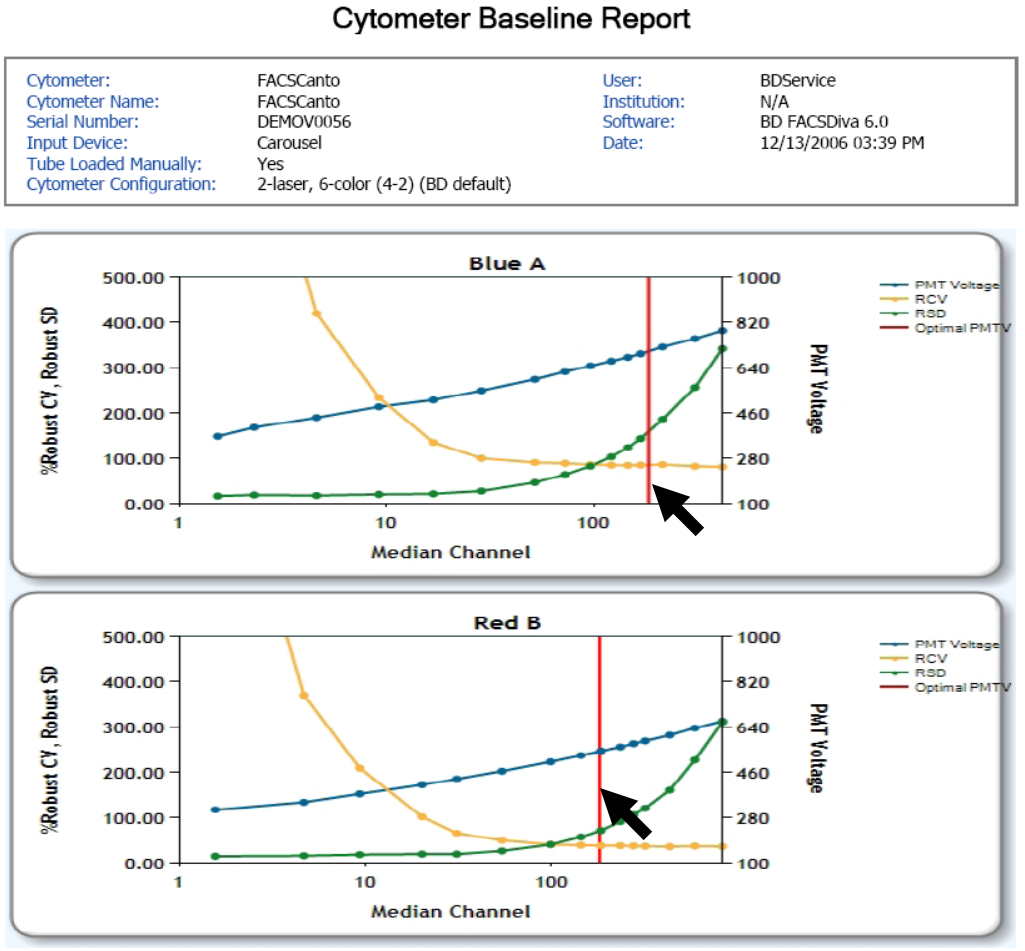
Laser	Detector	Parameter	Linearity Min Channel	Linearity Max Channel	Slope	Intercept	Electronic Noise Robust SD	Qr	Br
Blue	FSC	FSC	N/A	N/A	0.0038	3.69	N/A	N/A	N/A
Blue	E	SSC	N/A	N/A	7.5987	-14.30	N/A	N/A	N/A
Blue	D	FITC	197	174901	7.5100	-15.94	18.11	0.0842	92.32
Blue	C	PE	177	157054	7.4772	-15.40	20.38	0.1802	309.13
Blue	B	PerCP-Cy5.5	355	155736	7.4947	-15.86	19.36	0.0491	0.00
Blue	A	PE-Cy7	146	153291	7.4885	-16.42	19.21	0.0078	6.98
Red	B	APC	91	188536	7.5379	-16.00	18.43	0.0371	5.83
Red	A	APC-Cy7	204	167284	7.6259	-15.96	20.76	0.0391	22.26

Background from Raman Scattering; PE and FITC

Lower detector efficiency in far red; PE-Cy7

Cytometer Baseline Report: Performance Plots

- Report provides performance plots for every detector
  - Shows data for dim beads
- Shows baseline target and PMT voltage
  - Recommended as a starting PMT voltage based on bead performance, can be overridden by the operator



Daily Performance Checks

Perform at least once a day or before each experiment

For each fluorescence detector, BD FACSDiva™ 6.0 CS&T module will automatically:

- Check and adjust laser delays
- Check and adjust area scaling factors
- Adjust detector PMT voltages to place bright beads at their target values in each detector (linked to application settings)
- Measure performance parameters
  - Detector efficiency (Qr), background (Br), rCVs, and PMT voltages
- Record and track performance parameters with Levey-Jennings Graphs

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Daily Performance Check: Report

Detectors

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

Detector Settings

Laser	Detector	Parameter	Target Value	Actual Target Value	% Difference Target Value	Bright Bead %Robust CV	Mid Bead Median Channel	Mid Bead % Robust CV
Blue	FSC	FSC	119348	118976	-1	1.78	118827	1.50
Blue	E	SSC	126468	126453	-1	2.53	125646	1.90
Blue	D	FITC	37118	36603	-2	2.06	1670	8.95
Blue	C	PE	23292	23262	-1	1.56	874	9.33
Blue	B	PerCP-Cy5.5	36831	36335	-2	2.90	1587	11.84
Blue	A	PE-Cy7	94428	94346	-1	5.03	3546	20.06
Red	B	APC	42714	42226	-2	2.48	1540	12.93
Red	A	APC-Cy7	61839	61044	-2	3.09	2659	10.02

Detector Settings (Continued)

Laser	Detector	Parameter	Dim Bead Median Channel	Dim Bead %Robust CV	PMTV	Δ PMTV	Qr	Br	P/F
Blue	FSC	FSC	16156	7.12	359	N/A	N/A	N/A	Pass
Blue	E	SSC	68067	1.44	362	N/A	N/A	N/A	Pass
Blue	D	FITC	191	33.21	538	-1	0.0818	103.86	Pass
Blue	C	PE	194	29.19	442	0	0.1986	360.52	Pass
Blue	B	PerCP-Cy5.5	186	35.24	532	-1	0.0460	0.00	Pass
Blue	A	PE-Cy7	210	85.95	721	0	0.0073	24.00	Pass
Red	B	APC	181	40.90	545	-1	0.0398	23.92	Pass
Red	A	APC-Cy7	200	39.38	525	-1	0.0404	32.20	Pass

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Performance Tracking: Levey-Jennings Graphs

- All measured performance parameters are tracked
  - Laser Alignment (CVs), detection efficiency (Qr), optical background (Br)
  - PMT voltages
    - Fluorescence levels (target values) are held constant, changes in PMT voltages can reflect changes in laser output, cleanliness, laser alignment and optical alignment
  - Data can be analyzed in Levey-Jennings graphs
    - All data points are directly linked to their respective daily performance reports

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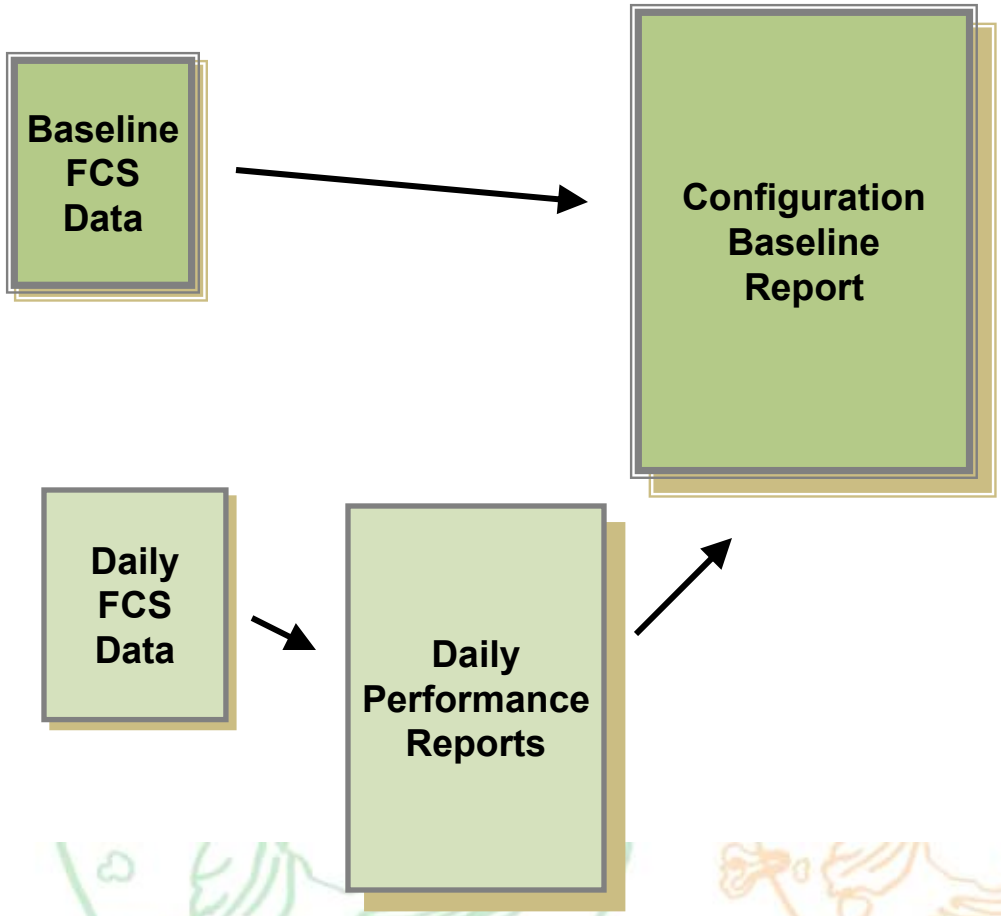
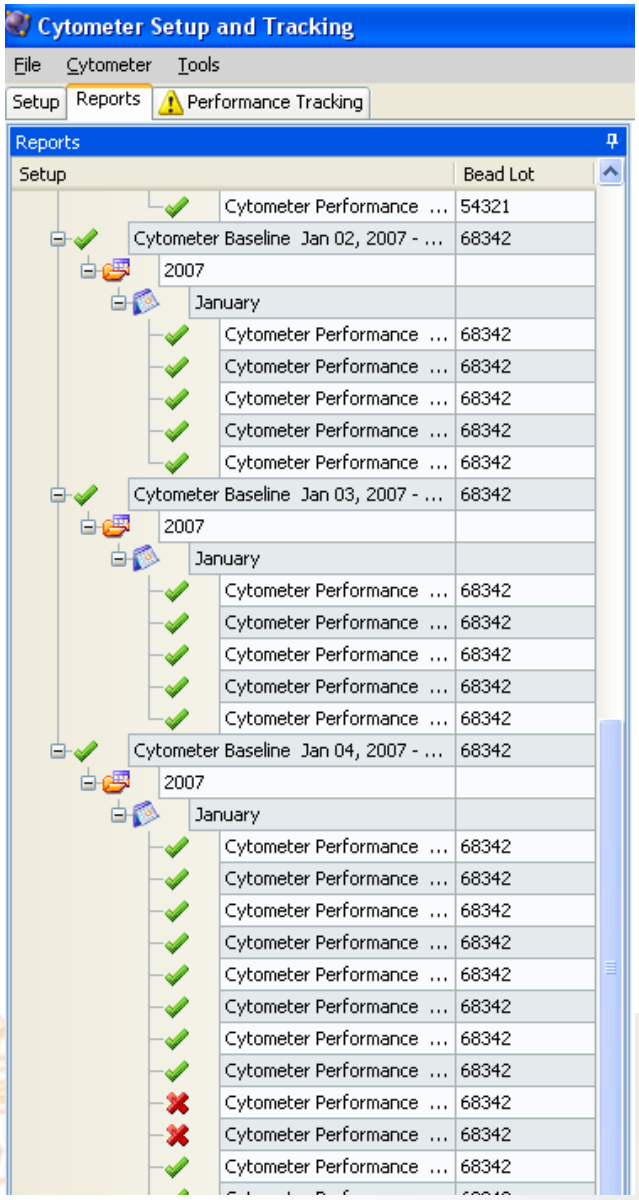
Performance Data: Reports

All data is directly traceable back to the original Baseline Report





Applications: Cytometer Settings

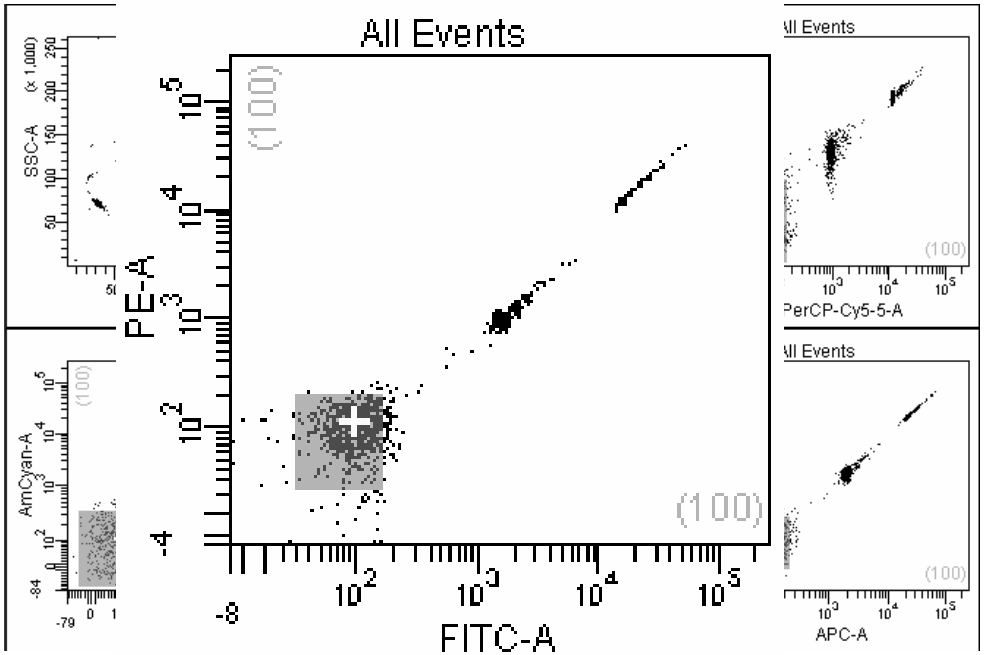
- Automated flow cytometer setup software has offered users two basic application settings
  - HuPBL – Lyse/Wash
  - HuPBL – Lyse/No Wash
- However, some applications may have reproducible high-fluorescence backgrounds
  - Cell lines, activated cells, etc…
  - Intra-cellular staining (e.g. cytokine or BD Phosflow)
  - In these cases, PMT voltages can be decreased without adversely affecting resolution sensitivity

Application Settings: User Defined

- The BD FACSDiva™ 6.0 software provides customers a means to define proper application settings and link to CS&T performance data
- A pre-defined application settings Worksheet provides visual guides to setting PMT voltages for the low end of the scale for stained samples

- Crosshair indicates the minimum recommended target value for the negative population = 10 x SD_{EN}
- The gray box indicates the 97th percentile of the dim beads

- For maximum low end resolution sensitivity, PMT voltages should be adjusted to place the negative population within or above the gray box
- Actual target center is at the crosshair – note asymmetry due to log scale



Summary

- BD Cytometer Setup & Tracking (CS&T) beads work with BD FACSDiva™ 6.0 software on the BD FACSCanto™, BD FACSCanto™ II, BD FACSAria™, and BD™ LSR II platforms
  - Any number of lasers or fluorescence parameters
  - Compatible with novel fluorochromes
- The BD FACSDiva CS&T module will:
  - Fully characterize the cytometer's performance
    - Linearity, detector efficiency (Qr); background fluorescence (Br); electronic noise (SD_{EN}); and laser alignment (rCV)
  - Optimize cytometer settings
    - Laser delays; Area scaling factors; PMT voltages
  - Set up your cytometer with reproducible performance
    - Obtain consistent, reproducible experimental data from day to day
    - Define application settings in FACSDiVa™ and link to automated setup
  - Track cytometer performance
    - Detect component failures and alterations
    - Provide graphical representations of performance trends over time

Conclusion

Designing and performing high-level multi-color experiments is no longer so limited by the complexity of cytometer setup

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