Using BD FACSDiva™ CST To Evaluate Cytometer Performance, Create Custom Assay Settings and Implement Cross-Instrument and Cross-Site Standardization of Assays

PART 1

Alan M. Stall

Director, Advanced Cytometry Technologies
BD Biosciences

Agenda: Designing and Performing a Multi-color Assay Across Sites

Part 1

- **First principles**
  - **Resolution sensitivity**
    - **Qr: Fluorescence detection efficiency**
    - **Br: Background “noise”**
- **Choosing gain settings (MFI)**
  - **Taking into account differences among individual instruments**
    - **Electronic Noise (SDen)**
    - **Linearity**
  - **How does CST chose gain settings.**
    - **Setting Baseline Gains**
      - **Baseline MFI Target Values**
    - **Reset Target Values**

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Agenda: Designing and Performing a Multi-color Assay Across Sites

Part 2

- Insuring equivalent fluorescence intensities (MFI) across Multiple instrument
  - Using Application settings
- Choosing reagents
  - Taking into account differences among fluorochromes
- Optimizing for multiple cytometers-
  - Accounting for different instrument performance
  - Test assay by “detuning” an instrument
- A “real-world” example
  - The NIH ICS Assay Quality Assurance Project

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Instrument Sensitivity: Two definitions

- **Defining sensitivity**
  1. **Threshold**: Degree to which a flow cytometer can distinguish particles dimly stained from a particle-free background. Usually used to distinguish populations on the basis of Molecules of Equivalent Fluorochrome (MEF).
  2. **Resolution**: Degree to which a flow cytometer can distinguish unstained from dimly stained populations in a mixture.
- **How to measure instrument-dependent sensitivity?**
  - Resolution sensitivity is a function of three independent instrument factors: Q, B, and Electronic Noise (SDen) which are accurately assessed using BD™ Cytometer Setup and Tracking (CST) in BD FACSDiva v6 software.
    - ***This is the best measure of true assay sensitivity***
  - For flow cytometers that measure pulse area rather than pulse height, a blank bead MEF ***is not*** an effective measure of fluorescence sensitivity.
    - Based on concept that a “blank” bead is a measure of instrument noise

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Resolution Definition

Resolution – Degree to which a flow cytometer can distinguish unstained from dimly stained in a mixture.

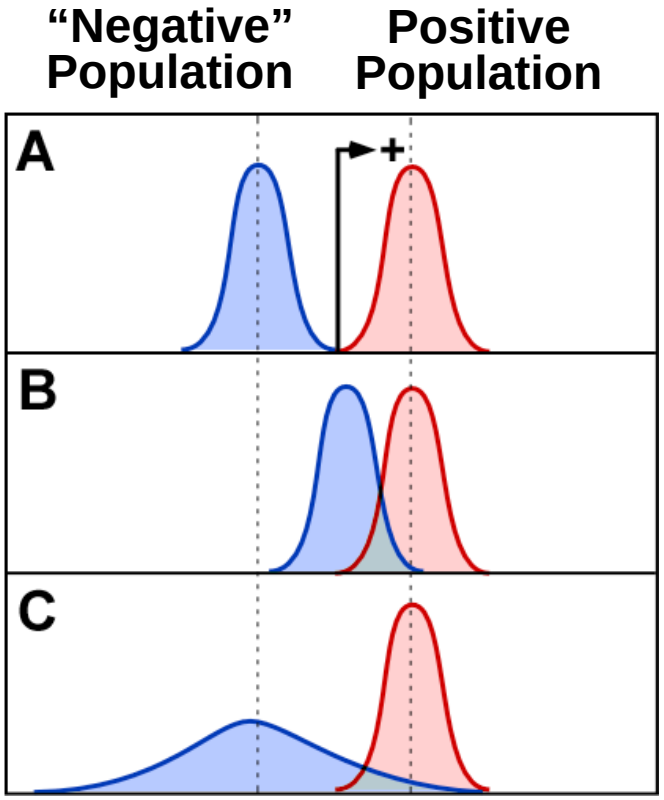
Can be very complicated in a polychromatic scenario.

Resolution vs Background

Negative population has
low background
Populations well resolved

Negative population has
high background
Populations not resolved

Negative population has
low background
high CV (Spread)
Populations not resolved

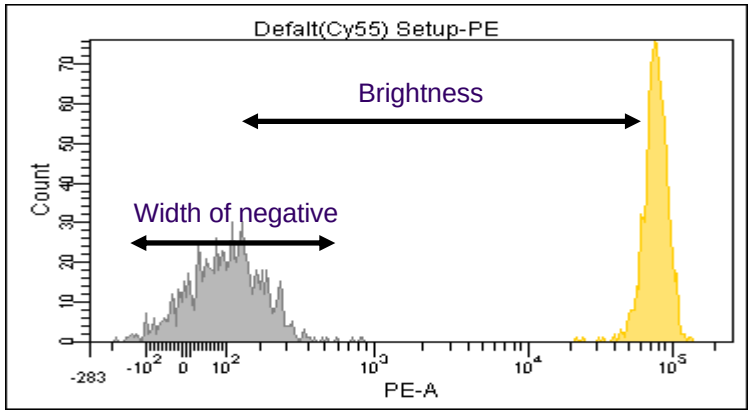


The ability to resolve populations is a function of both background *and* spread of the negative population.

Measuring Sensitivity: The Stain Index

- The **Stain Index** is a measure of reagent performance on a specific cytometer, a normalized signal over background metric.

$$\text{Stain Index} = \frac{\text{Brightness}}{\text{Width of Negative}} = \frac{\text{median}_{\text{positive}} - \text{median}_{\text{negative}}}{2 \times \text{rSD}_{\text{negative}}}$$



Experiment Name: CD4 Stain Intex (081304)
Tube Name: PE

Population	#Events	%Parent	PE-A Median	PE-A rSD
PE-	827	48.9	102	97
PE+	780	46.1	75,652	12,616

$$\text{Stain Index} = \frac{75852 - 102}{2 \times 97} = 390.5$$

- The brightness is a function of the ***assay*** (antigen density, fluorochrome used).
- The width of the negative is a function of
  - ***Instrument performance*** (Qr, Br, and SDen) [single color]
  - ***The assay***
    - (Fluorescence spillover / Compensation) [multicolor]
    - The cell population

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BD CS&T: Qr and Br – Relative Q and B

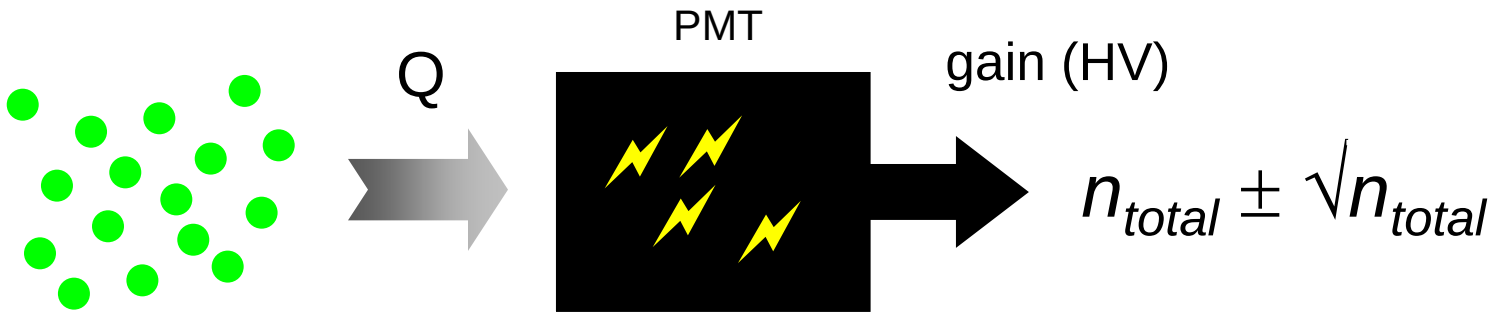
- **Qr** is *photoelectrons per fluorescence unit* and indicates how bright a reagent will appear on the sample when measured in a specific detector.
  - It is a function of
    - The instrument [laser power and alignment; optical design]
    - The reagent [quantum yield of the fluorochrome]
- **Br** is *measured optical background*, which helps indicate how easily (dim) signals may be resolved from unstained cells in that detector by providing a practical estimate of competing optical background.
- Qr and Br are independent variables, but both affect sensitivity.
- The relative detector sensitivity for a specific fluorochrome is proportional to Qr and Br:

$$\text{Sensitivity}_{\text{relative}} \propto \sqrt{\frac{\mathbf{Qr}}{\mathbf{Br}}}$$

Statistics of dimly fluorescent cells

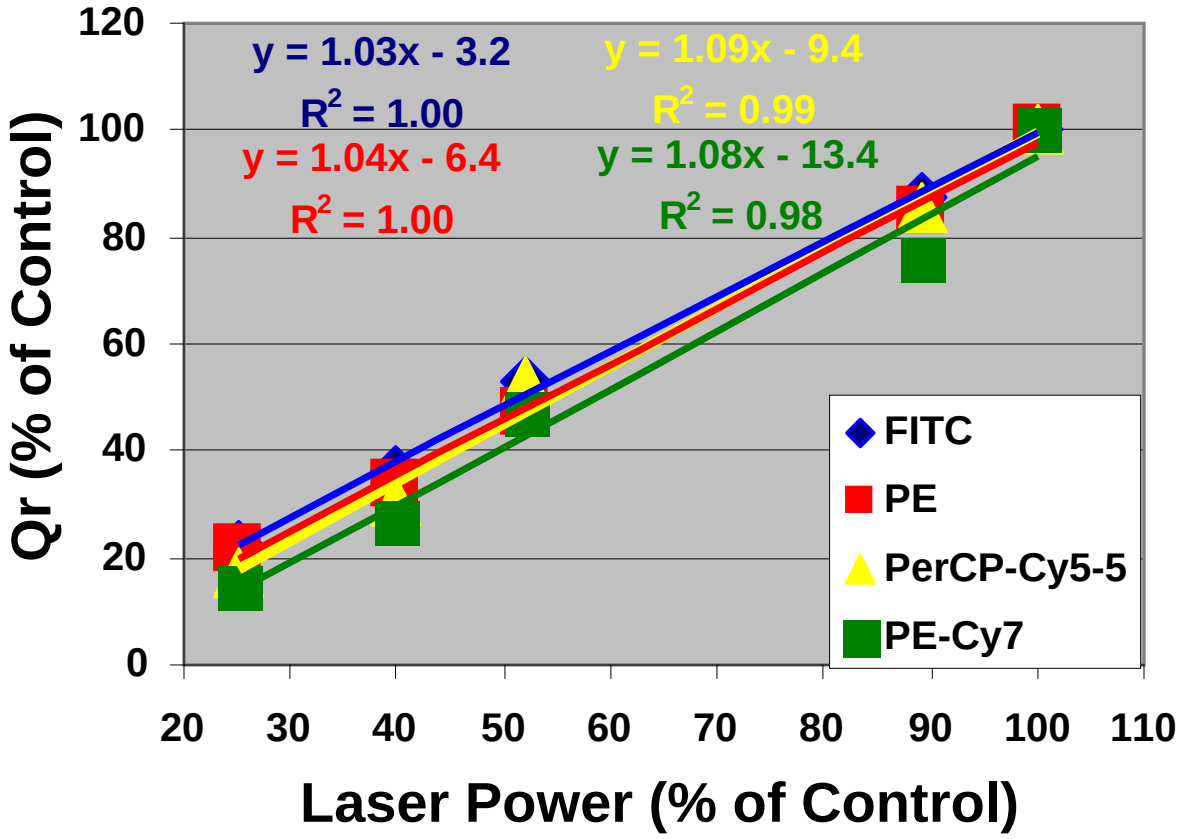
- **Fluorescence Sensitivity: *resolution* - ability to resolve dim cells from unstained cells**

- **Detection Efficiency (Q): a measure of the ability to excite and capture photons (S + B) of interest**
  - **The average number of photoelectrons *n* per molecule F**



  - **CV = SD/Signal = SD / (Gain x Q x F)**

Relationship Between Q and Resolution Sensitivity: Detuning- Laser Power



Lower laser power

Fewer photons per fluorochrome

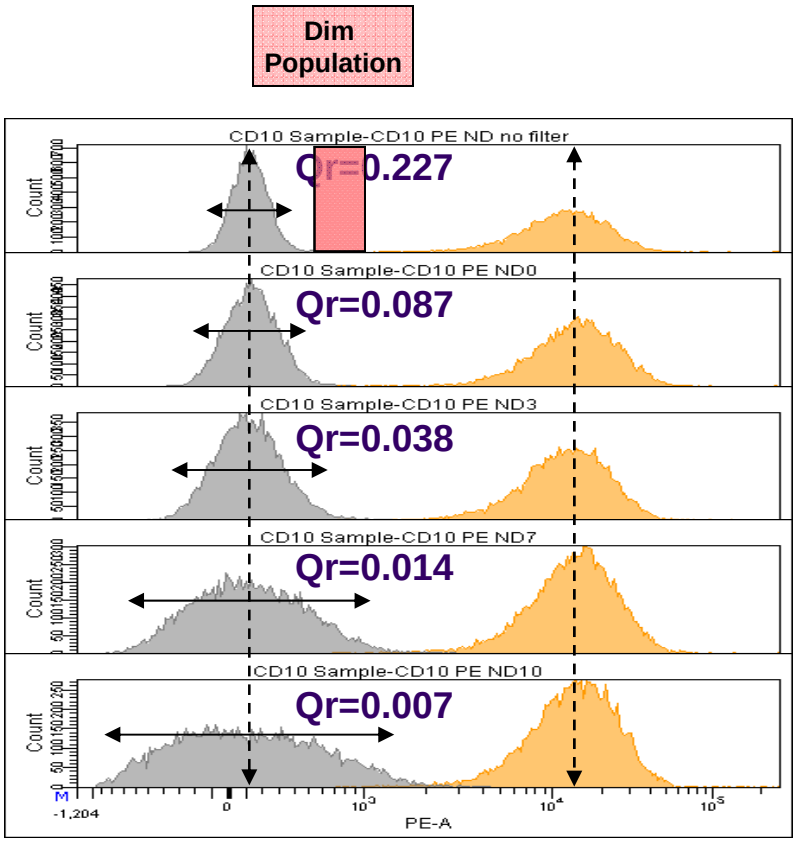
Lower Q

Decreased resolution sensitivity

Qr: Anti-CD10 PE Example (BD FACSCanto™)

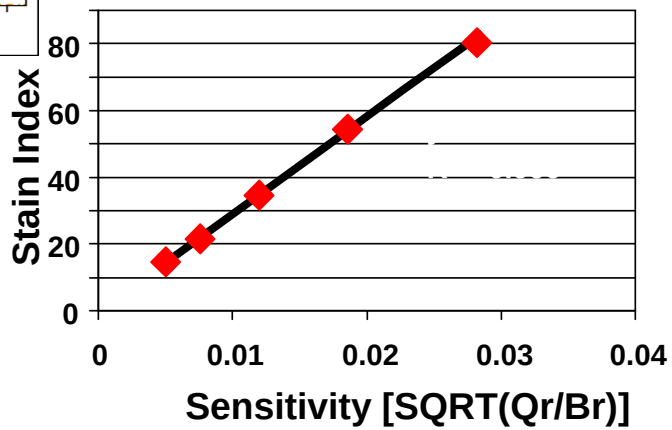
The laser and detectors were attenuated by ND filters over a 30-fold range to illustrate the effects of decreasing detector sensitivity on population resolution.

CS&T standardized the settings to place the positive at the same location.



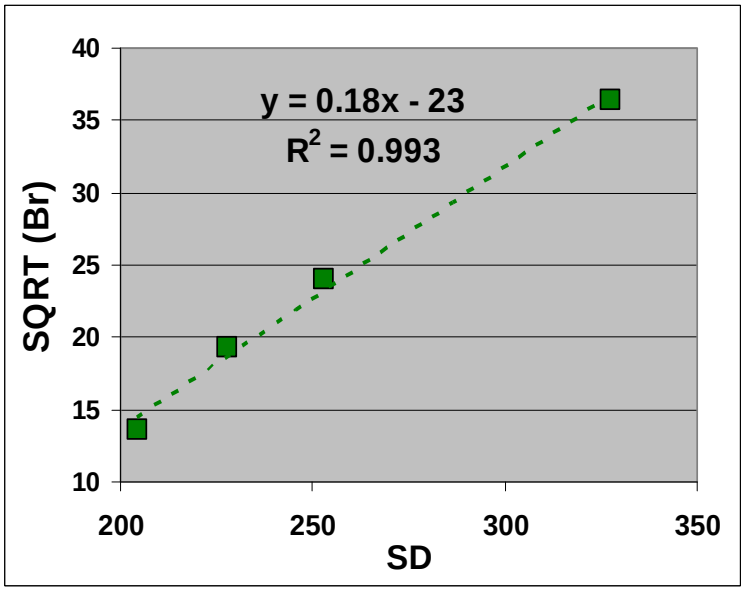
Trans-mission	SI Corrected
100%	83
35.5%	55
17.8%	35
7.1%	20
3.5%	14

Qr	Br
0.2274	277
0.0867	251
0.0379	255
0.0135	254
0.0071	266



Br: Optical Background (Detuning – Free Dye)

- **Example: APC-IgG was added in increasing amounts to the buffer containing CS&T beads, and Qr and Br estimated by the CS&T baseline procedure:**

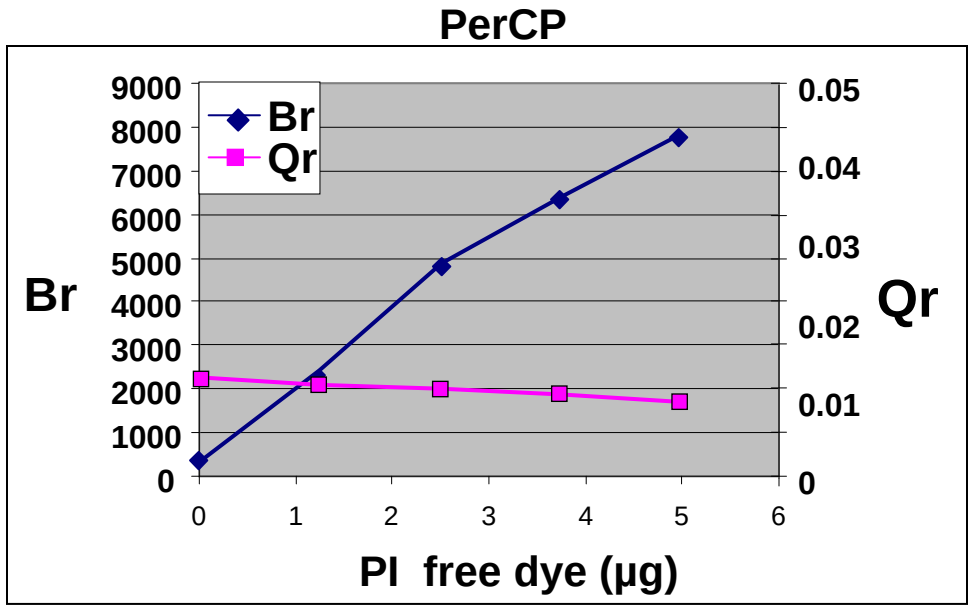


			Dim Population		
IgG1-APC, ng/ml	Br	Qr	MFI	%CV	SD
3.1	64	0.0171	513	36.8	189
6.3	120	0.0175	516	37.8	195
12.5	185	0.0174	513	39.9	205
25.0	372	0.0175	512	44.5	228
50.0	577	0.0175	514	49.2	253
100.0	1325	0.0173	515	63.6	328
200.0	2552	0.0159	528	83.7	442

- **Note that as Br increases, Qr remains constant.**
- **Although the Dim bead MFI remains constant (via baseline restore), the spread (SD and %CV) increases.**

Br: Optical Background from Propidium Iodide

- **Example: It is common to use propidium iodide (PI) to distinguish live from dead cells. Propidium iodide was added in increasing amounts to the buffer containing CS&T beads, and Qr and Br estimated by CS&T baseline procedure:**



- **Residual PI in your sample tube will increase Br, which will reduce sensitivity.**

Summary: Instrument Performance and Sensitivity

CS&T Baseline Report

Laser	Detector	Parameter	Linearity Min Channel	Linearity Max Channel	Slope	Intercept	Electronic Noise Robust SD	Qr	Br
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	A	PE-Cy7	146	153291	7.4885	-16.42	19.21	0.0078	6.98

- Instrument performance can have a significant impact on the performance of an assay, especially for the farther red channels.
- Instrument sensitivity is a function of Qr, Br, and SDen.
  - Increases in Br or decreases in Qr can reduce sensitivity and the ability to resolve dim populations.
  - On digital instruments, BD FACSDiva software v6 and CS&T provides the capability to track performance data for all of these metrics, allowing users to compare performance between instruments.

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Designing Multicolor Experiments for Use Across Multiple Instruments

1. Choosing Gain Settings (MFI)
    a. Optimizing for a Single Instrument

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Factors to Consider for an Optimal Gain Setup

- **Things to consider when *optimizing* the cytometer setup for the immunofluorescence application**

  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.**

  2. **Dynamic range assessment for each fluorescence parameter**
     - a) **Are the brightest populations within the *linear range* of the detector?**
       - **Leave room for ~ 2-fold increase in expression levels and ensure the cells are in the linear range of the detector.**
     - b) **Are the compensation controls within the linear range of the detector?**
       - **If positive cells are out side of the linear range compensation may be inaccurate**
     - c) **Are the negatives (in a stained sample) too high?**
       - **This is a matter of taste**

  3. **An optimal cytometer gain setting is one for which both conditions are met.**

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Electronic noise (SDen)

- Background signal due to electronics
  - Contributed by
    - PMT connections / PMT Noise
    - Cables too near power sources
    - Digital error
- Broadens the distribution of unstained or dim particles
  - Removed by baseline restoration electronics
  - However, the broadness or noise of the distribution (SDen) cannot be removed by baseline restore
    - **Therefore, increases in electronic noise results in <u>decreased resolution sensitivity</u>**
  - Most important for channels with low cellular autofluorescence
    - APC-Cy7, PE-Cy7, PerCP-Cy5.5
- Diva 6/CST software uses the SDen to set PMT voltages to minimize CV (spread) of negative / dim populations

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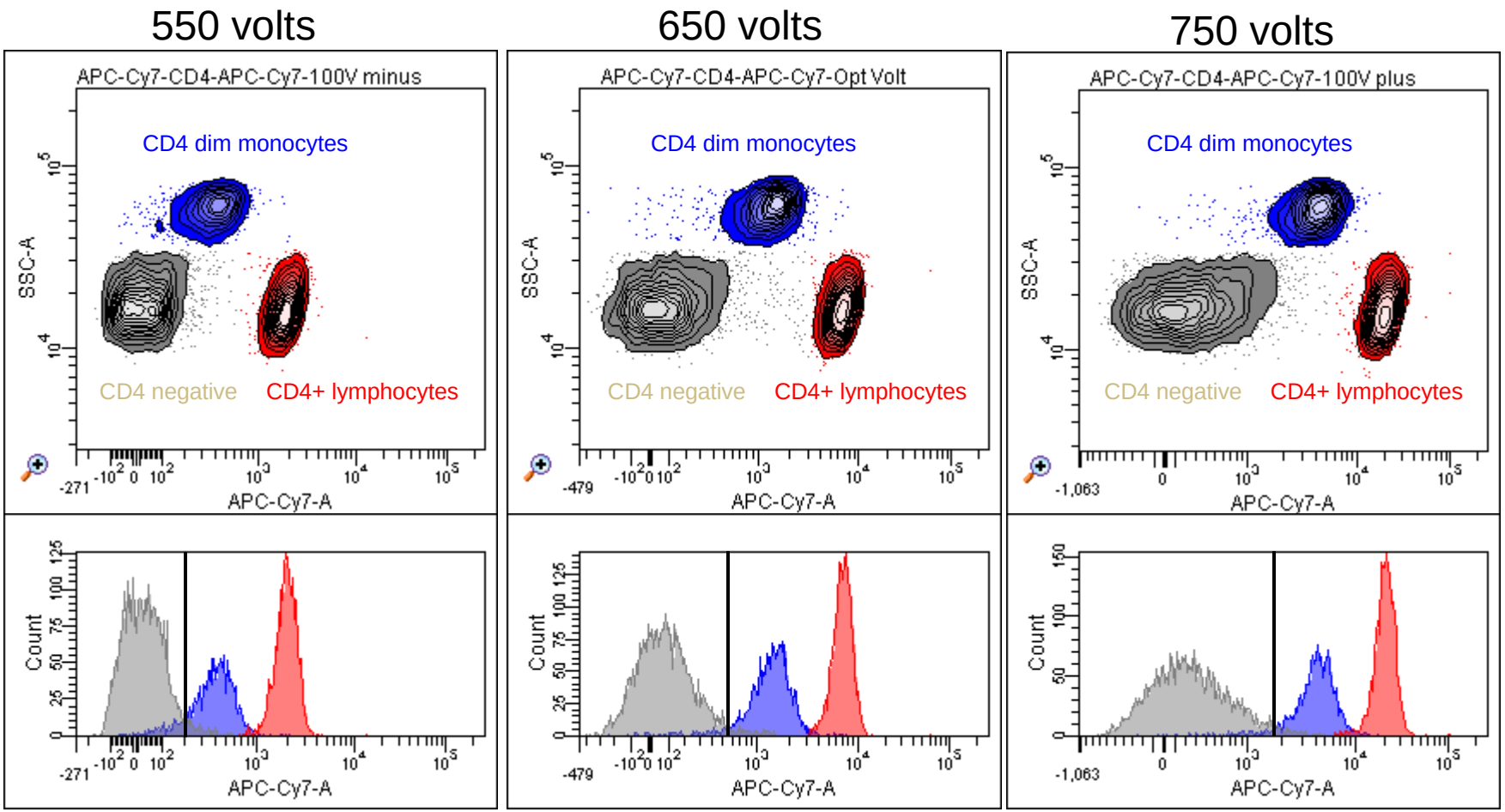
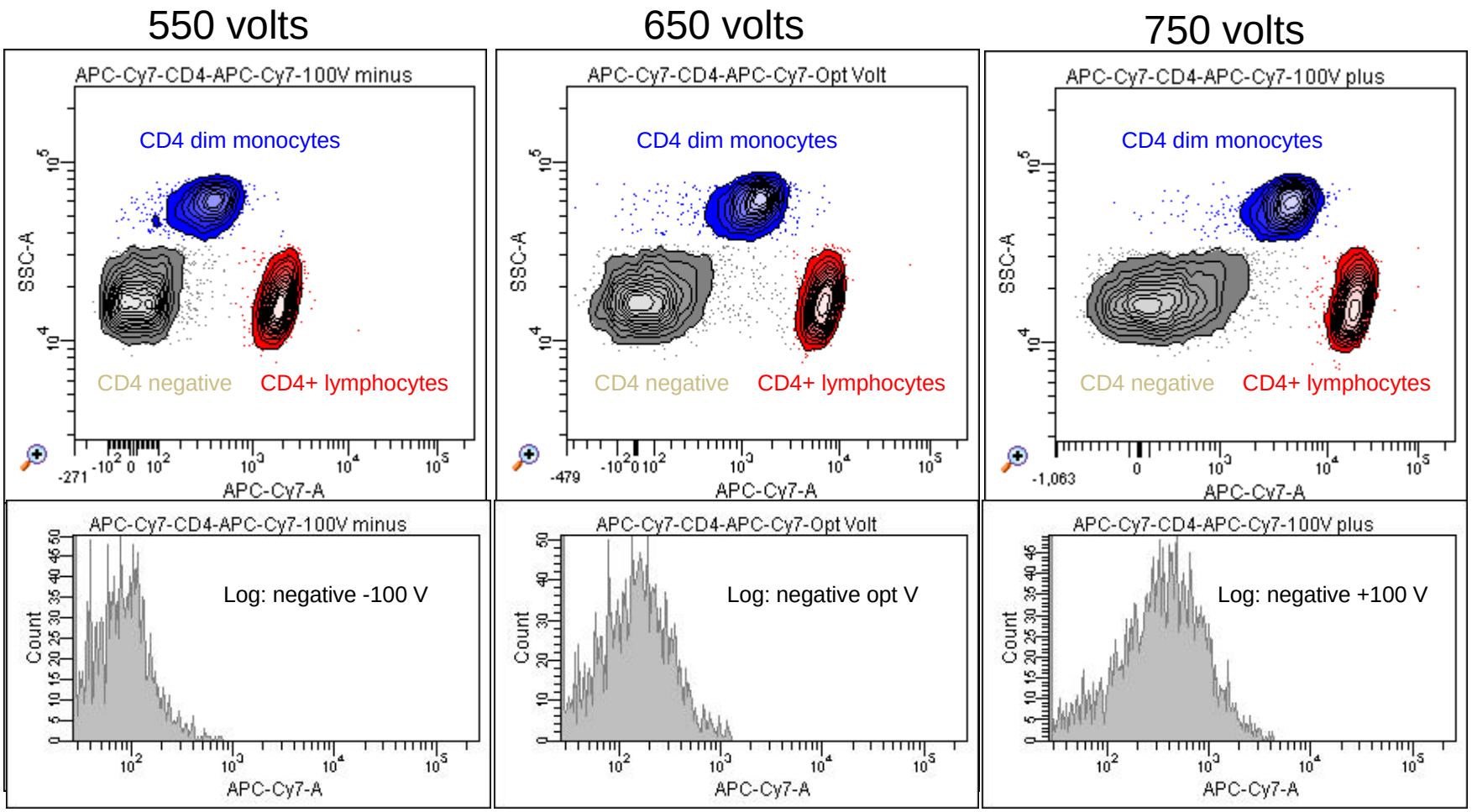
Correctly Setting PMT Voltage Gain Improves Resolution



Correctly Setting PMT Voltage Gain Improves Resolution



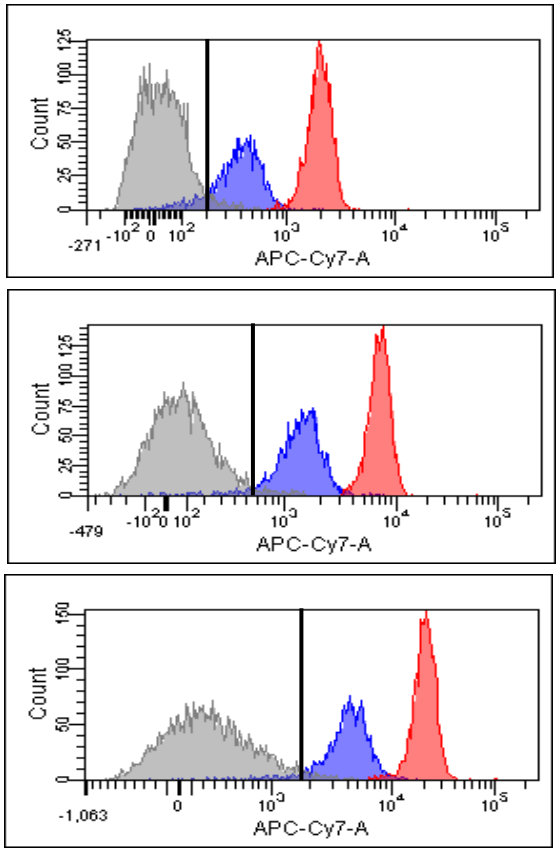
Optimal Gains Can Reduce Classification Errors

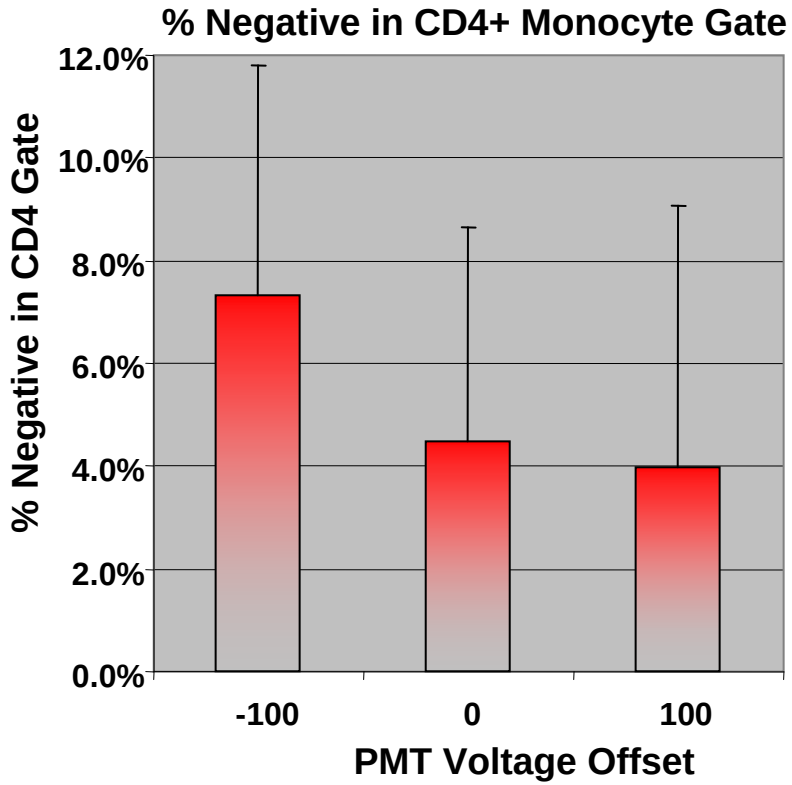
GAIN

550 V

650 V

750 V





Increasing the gain pulls dim populations out of the electronic noise.
Increases accurate resolution / identification of the dim population

Further increases in gain does not improve resolution.
Can cause potential problems in bright populations going off-scale

Linearity

- **Defined as proportionality of output (MFI) to input (Fluorescence/ # of photons)**

- **Important for fluorescence compensation**
  - Compensation of data in the last decade involves subtraction of large numbers
  - Small errors (non-linearity) in one or both large numbers can cause a large absolute error in the result

Actual	**82000**
Measure	**80000**
Spillover	**0.2**
Error =	**2000 X 0.2 = 400**

- **Important for quantitative measurements**
  - **DNA Measurements**
  - **Antigen / Antibody binding**

- **CST uses a robust reliable method for assessing fluorescence detector linearity**
  - **Dual signal ratio method**

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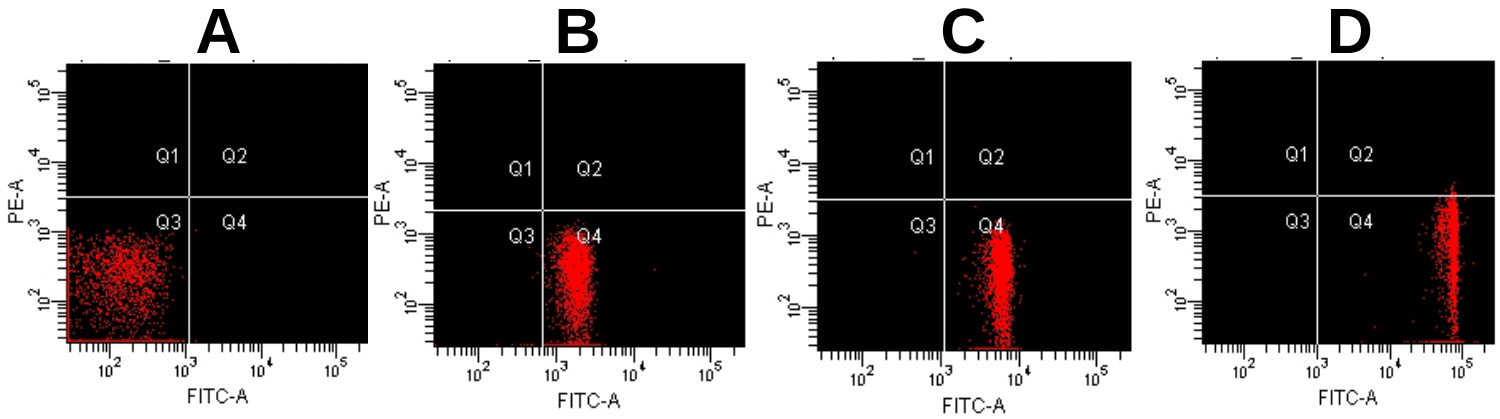
Effect of non-linearity on compensation

- 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

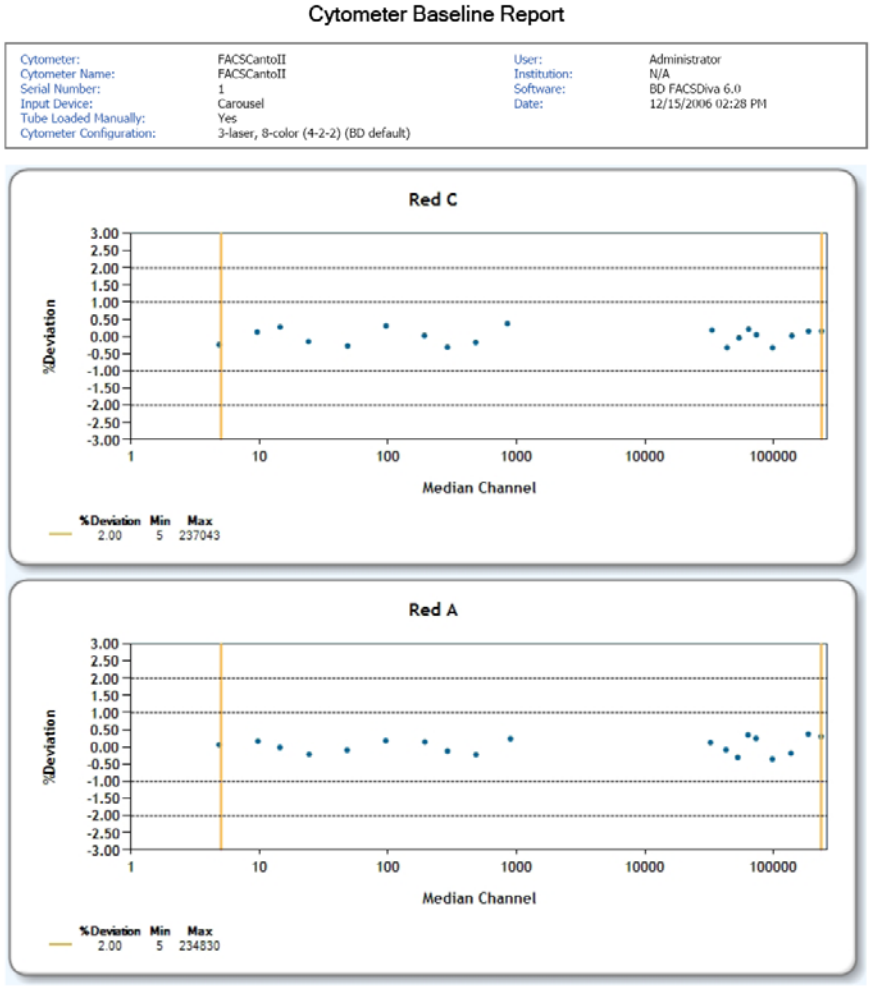


Channel	Median Fluorescence Intensity			
FITC	68	1796	5921	73,000
PE	80	75	79	365

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

CST Baseline Report- Linearity

- CST reports the linearity range for every fluorescence detector (± 2.0% deviation) in the Cytometer Baseline Report
- Users can print out data plots for any detector



How Does Diva 6 / CST Determine Gain Setting (PMTV)?

- Diva 6/CST software uses the SDen determined at Baseline to set PMT voltages high enough to minimize CV (spread) of negative / dim populations
  - Set PMTV so that SDen is less than 10% the MFI of neg / dim cells
    - Dim CST MFI are normalize to autofluorescence of human lymphocytes
  - Normalized MFI of Dim bead = 10 x SDen
- Advantages
  - No cells required
  - Automatic
- Disadvantages
  - Does not account for differences in autofluorescence or inherent SD of negative populations
    - Can result in higher gain settings than needed to minimize SDen

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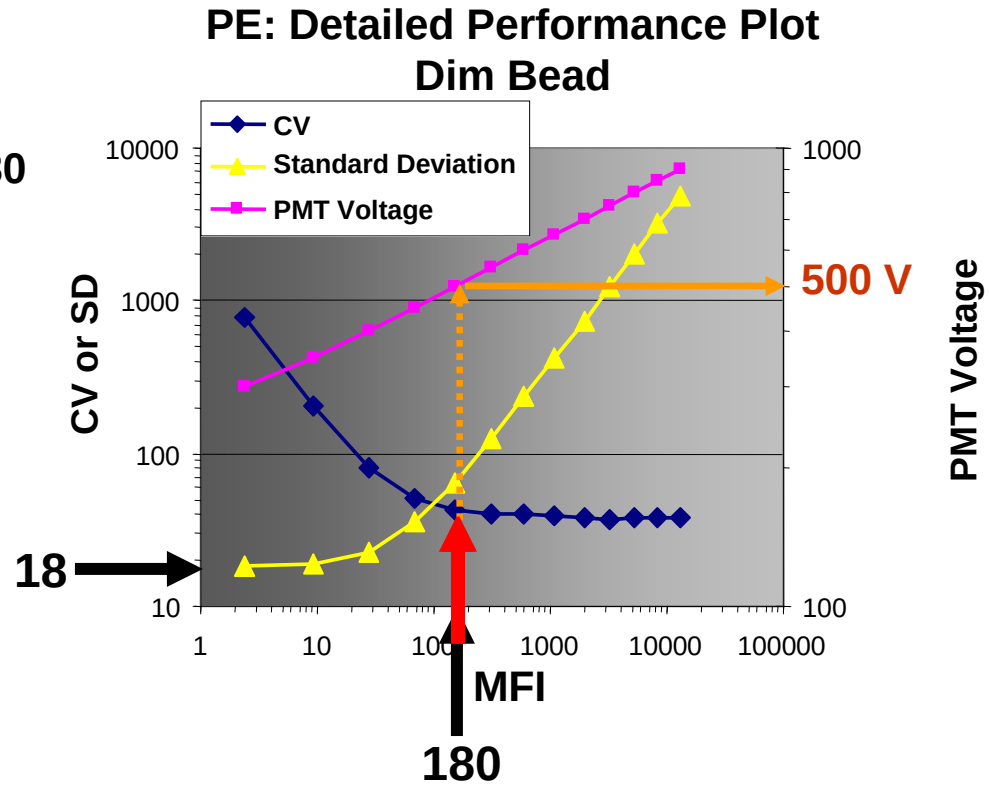
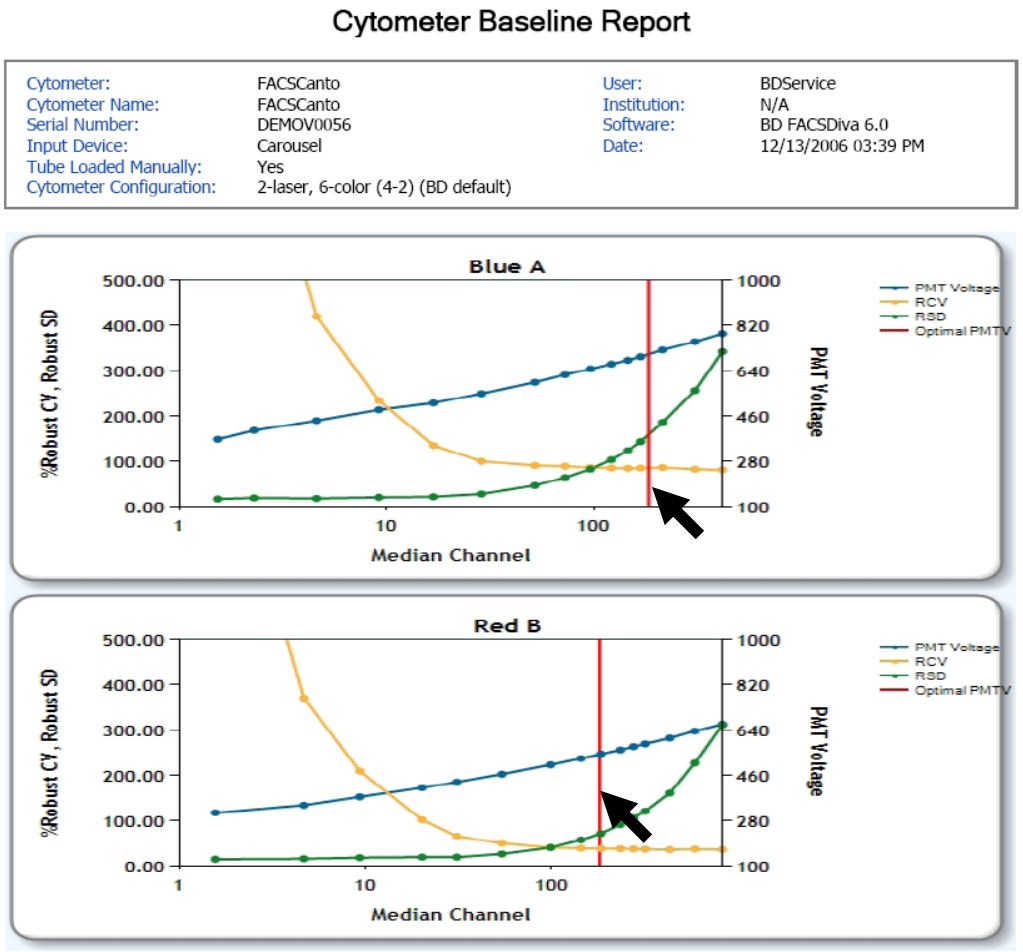
Determining Baseline PMT Voltages Using SD_{EN}

- CST analyzes dim particle MFI which is normalized to dim cell brightness allowing relevant detector baselines to be visualized by plotting MFI vs gain and CV
  - For this detector the SD_{EN} = 18
  - MFI of Dim bead = 10 x SD_{EN} = 180
  - Determine PMT Voltage required to achieve MFI of 180
    = 500 Volts = Baseline voltage
  - As PMT Voltage is lowered the CV increases → resolution decreases
  - As PMT Voltage is increased the CV remains unchanged → resolution unchanged



Cytometer Baseline Report-
Detailed Detector Performance Plots

- **CST provides Performance Plots for every detector**
  - **Data shown for Dim Beads**
- Shows Baseline PMT voltage
- This is a recommended starting PMT Voltage based on bead performance, but can be overridden by the operator



An Alternative Approach to Settings Gains

- One of the criteria for setting gain is to ensure that electronic noise does not impact low end sensitivity
  - The goal is to have the dimmest cells (unstained) where electronic noise is no more than 10% to 20% of the total variance.
- CST uses Dim particle MFI which is normalized to dim cell brightness to set the MFI gain
  - While this is a good general approach (for a single instrument) it does not take into account differences in cells or assay conditions
    - Cellular autofluorescence
    - Autofluorescence due to fixation
    - Intrinsic variance (standard deviation) of the negative cells
- An alternative approach is for a given assay to measure the rSD of the negative cells at different gain (PMTV) settings.
  - A good rule of thumb is to set the gain so that the rSD of the negative cell is greater than 2.5 times the SD of the electronic noise

$$\mathrm{rSD}_{\mathrm{Neg\,Cells}} > 2.5 \times SD_{\mathrm{EN}}$$

  - Adjusted Gain settings can then be applied through the use of Application Settings (explained in detail in the next Part)

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Maintaining Consistent Fluorescence Measurements Over Time

Configurations, Baselines,
Bead Lots & MFI Target Values

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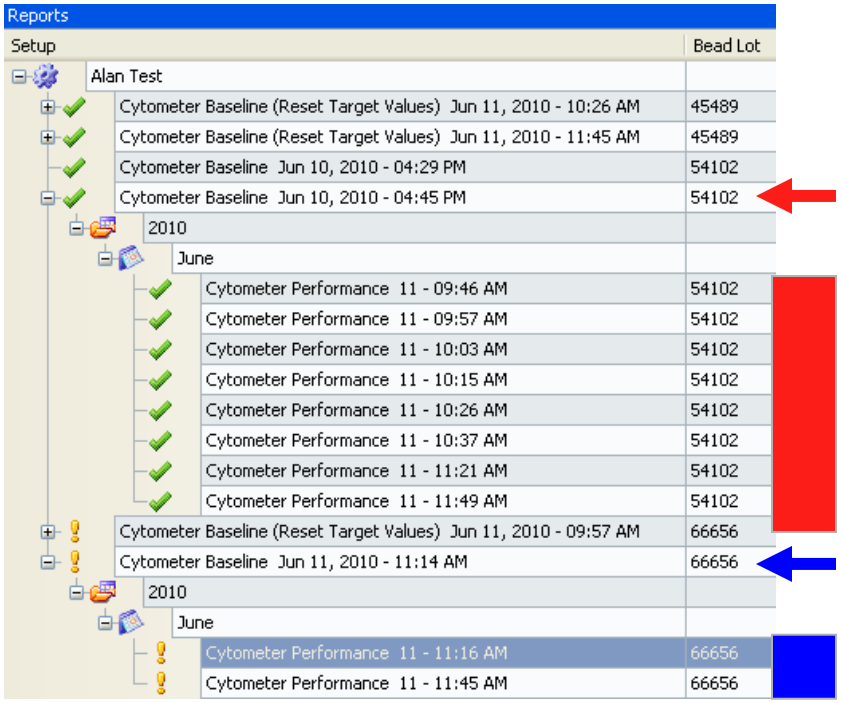
Diva 6 / CST Maintains Consistent MFI Over Time - 1

- **One of the main features of Diva 6 / CST is that it ensures consistent fluorescence measurements over time**
  - "Define Baseline" determines MFI target values for every channel
    - Advantage- Instrumental is always optimized
    - Disadvantage- MFI target values can change depending upon SDen
      - If the SDen changes the MFI Target values will change when the Baseline is run
  - The MFI Target values are based upon the fluorescence of the CS&T Bright beads used for that Baseline
    - ***Thus the target values for that Baseline are linked to a specific bead Lot***



Diva 6 / CST Maintains Consistent MFI Over Time - 2

- **Every time CST "Check Performance" is run PMTV are adjusted so that the MFI Target Values are hit**
  - **Thus equivalent fluorescence measurements will be made even if instrument performance changes**
    - **Decrease Laser power; misalignment**

Cytometer Performance Report

Cytometer:	FACSCantoII	User:	Administrator
Cytometer Name:	FACSCantoII	Institution:	N/A
Serial Number:	1	Software:	BD FACSDiva 6.1.3
Input Device:	Manual	Date:	06/11/2010 10:37 AM
Cytometer Configuration:	Alan Test	Cytometer Baseline:	06/10/2010 04:45 PM
		P/F:	Pass

Setup Beads

Bead Product: CST Setup Beads, Part #: 910723
Lot ID: 54102, Expiration Date: 09/30/2011
Bead Lot Information: Available

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	125000	123767	-1	3.91	123838	3.44
Blue	F	SSC	125000	122662	-2	5.96	123516	5.16
Blue	E	FITC	10069	9921	-2	5.17	376	22.21
Blue	D	PE	12267	12067	-2	3.11	559	10.57
Blue	B	PerCP-Cy5-5	21998	22001	0	2.59	811	10.07
Blue	A	PE-Cy7	19031	18461	-3	5.93	467	29.6
Red	C	APC	30227	29776	-2	2.95	1530	11.98
Red	A	APC-Cy7	26046	25682	-2	3.45	971	10.35
Violet	B	Pacific Blue	10205	10208	0	2.52	463	7.35
Violet	A	AmCyan	37615	37647	0	2.73	2010	9.67

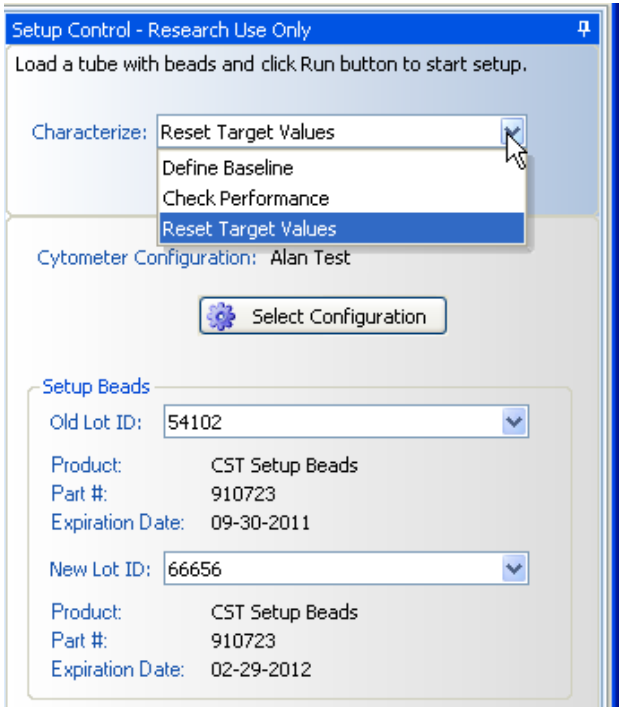
Using Two (or More) Bead Lots With One Baseline

- **Different Lots of CS&T beads have differences in MFI**

	MFI of CS&T Bright Bead						
	Channel						
Bead Lot	FITC	PE	PerCP-Cy5.5	PE-Cy7	Pacific Blue	APC	APC-Cy7
#1	11258	17540	35750	29664	9598	48293	38006
#2	11236	18418	33400	22457	10644	47810	30395
#3	11130	18174	32576	27907	11164	48793	40622

- **Diva 6 / CST allows you to use more than one Bead Lot with the same Baseline**

- **"Reset Target Values" transfers the MFI target values from one Bead Lot to another**
  - **Both Bead Lots are linked to the same Baseline**



Reset Target Values - 1

- **"Reset Target Values" transfers the MFI target values from one Bead Lot to another**
  - **The software maintains a unique set of Target values for each Bead Lot**
  - **Thus the two lots of Beads will give the exact same Gain settings**

Cytometer Baseline Report (Reset Target Values)

Cytometer:	FACSCantoII	User:	Administrator
Cytometer Name:	FACSCantoII	Institution:	N/A
Serial Number:	1	Software:	BD FACSDiva 6.1.3
Input Device:	Manual	Date:	06/11/2010 11:45 AM
Cytometer Configuration:	Alan Test	Cytometer Baseline:	06/11/2010 11:14 AM

Old Setup Beads

Bead Product: CST Setup Beads, Part #: 910723
Lot ID: 66656, Expiration Date: 02/29/2012
Bead Lot Information: Available

New Setup Beads

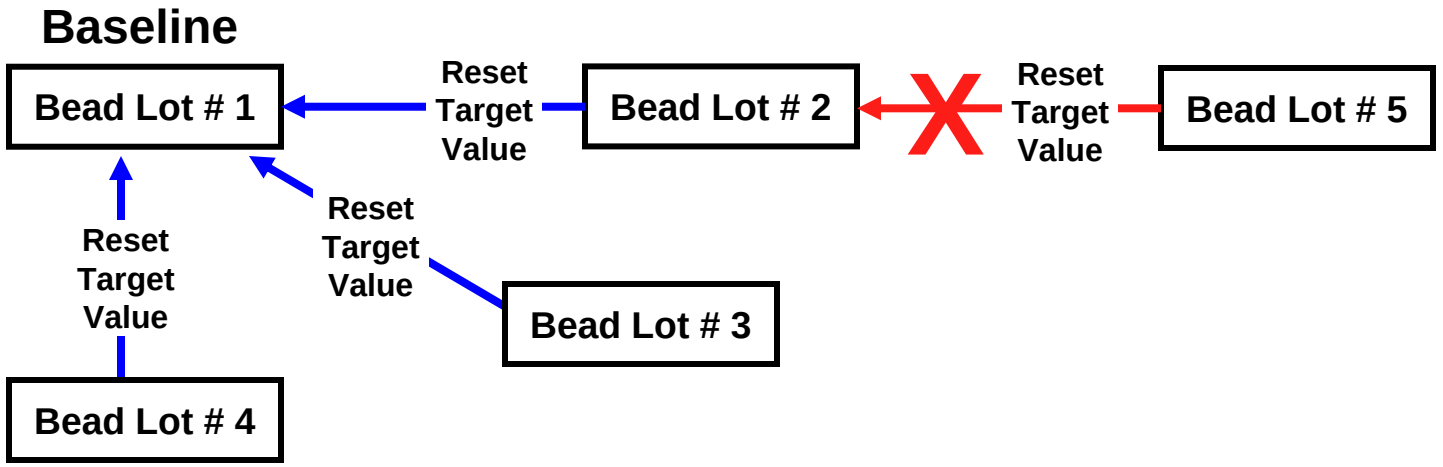
Bead Product: CST Setup Beads, Part #: 910723
Lot ID: 45489, Expiration Date: 05/31/2011
Bead Lot Information: Available

Detector Settings

Laser	Detector	Parameter	PMTV	Target Value Old Lot	Target Value New Lot	Bright Bead % Robust CV
Blue	FSC	FSC	520	125000	125000	3.53
Blue	F	SSC	415	125000	125000	5.71
Blue	E	FITC	516	16481	11868	5.37
Blue	D	PE	459	12547	12283	3.01
Blue	B	PerCP-Cy5-5	413	20253	20116	2.49
Blue	A	PE-Cy7	512	18016	21929	6.58
Red	C	APC	473	28241	29345	3.07
Red	A	APC-Cy7	490	24860	29458	4.04
Violet	B	Pacific Blue	355	10253	10077	2.64
Violet	A	AmCyan	408	38214	38266	2.80

Resetting Target Values For Multiple Lots

- Diva 6 / CST allows you to Reset Target Values for multiple lots to the same Baseline Lot
  - For a given Baseline all Resets must be against the original Bead Lot



- For long-term studies where it is important to have consistent MFI
  - Only create and use one Baseline
  - Save a bottle of the Bead Lot used to create that Baseline (Target MFI)
  - When first using a new lot of Beads, DO NOT create a new Baseline (unless you specifically want to)
    - Reset the Target Values of the new Bead lot to the original (old) Bead lot

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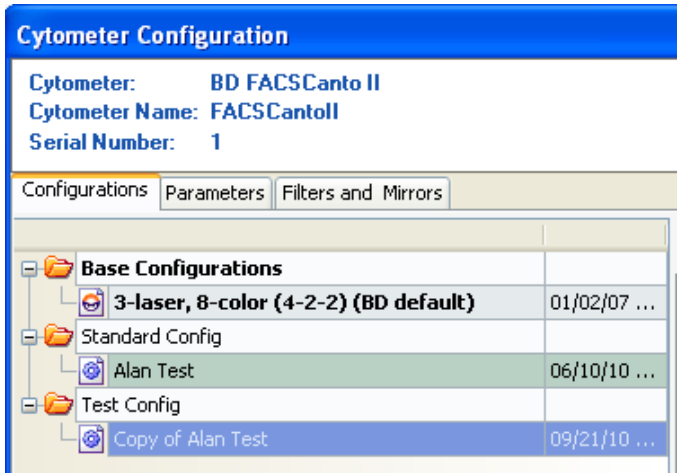
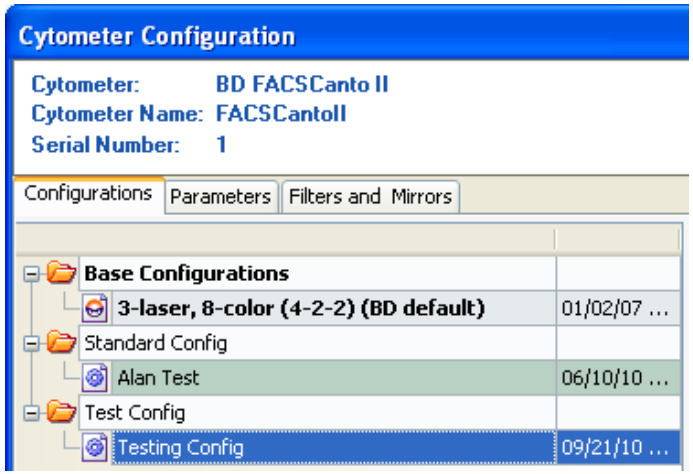
Running a Baseline Without Changing MFI Target Value

- Performing a “Define Baseline” is the only way to evaluate SDen and Linearity
  - However, re-running the Baseline can results in new MFI target values which could change the fluorescence measurements.

- The solution is to create a “Test Configuration” which you can use anytime you want to check the SDen and Linearity without affecting the MFI Target Values.
  1. Under Cytometer Configuration create a new folder “Test Config”.
  2. Copy the original configuration and paste into the new folder.
  3. Rename the new configuration,
     - e.g Testing Configuration
  4. When you want to just check the instrument
     a) chose the configuration “Testing Configuration”
     b) Perform “Define Baseline”
     c) Change configuration back to the standard configuration “Alan Test”
     d) Run experiments





Designing Multicolor Experiments for Use Across Multiple Instruments

1. Choosing Gain Settings (MFI)
   b. Taking into Account Differences Among **Multiple Instruments**

Settings Gain: One Instrument vs. Many Instruments

- Finding the best gain/PMTV for any given channel is a compromise among many parameters
  - Instrument: Electronic noise (SDen) and Linearity
  - Assay: Brightness of reagent / antigen expression
- How one assesses the compromises and the resulting settings depends upon whether you are looking at one instrument or many
- One Instrument
  - The gain settings are a function of the SDen and Linearity of that cytometer
- Multiple Instruments / Multiple Sites
  - To give equivalent fluorescence measurements and equivalent assay performance, the gain must work for the poorest performing instrument
    - the gain settings are a function of the highest SDen and lowest Linearity range among all of the cytometers

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Setting Gains for Use Across Multiple Instruments

- When determining gain settings for assays to work across multiple instruments, the gains need to be set according to the limitations of the poorest performing instrument.

- Example*:

Inst. No.	SDen	Qr	Br	Sens	Upper End of Linearity
1	26	0.015	976	3.9	230,000
2	17	0.042	92	21.4	200,000
3	23	0.01	298	5.8	180,000
4	22	0.01	613	4.0	200,000
5	9	0.007	2322	1.7	230,000
6	20	0.018	2768	2.6	190,000

- Instrument 3 has the lowest upper end of linearity: 180,000.
  - The gain should be low enough so the brightest population in the assays is lower than 180,000 on any instrument (150,000 would give some room for variability).
- Instrument 1 has the highest electronic noise: 26.
  - The gain should be high enough so the SD of negative cells is >2.5 x 26 = 65.
    - This is critical only if you are measuring dim events in this channel.
- If both conditions can't be met, then you must choose which is more important for this channel: identification of bright populations or resolution of dim populations.

* This variation in instruments is outside that expected for properly maintained instruments.

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