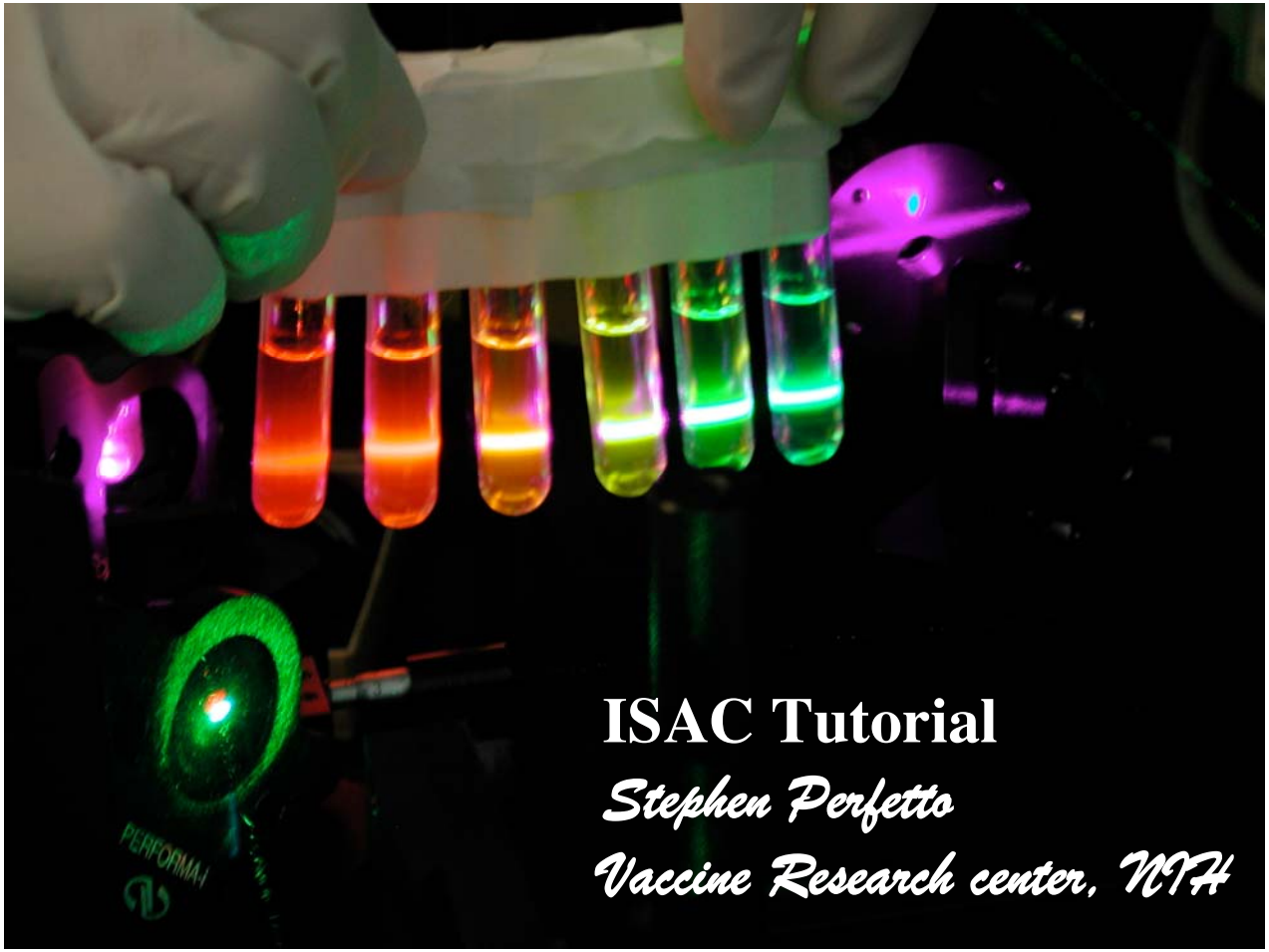
ISAC 2006 Tutorial #10

- Quality Assurance for Polychromatic Flow Cytometry and MAB Selection Criteria - *Stephen Perfetto*
- Critical Elements for a 12 Color Intracellular Cytokine Assay - *Laurie Lamoreaux*
- Emerging Fluorochromes for Flow Cytometry - *William Telford*

http://Home.NCIFCRF.gov/CCR/FlowCore/ISAC2006/ISAC2006.htm

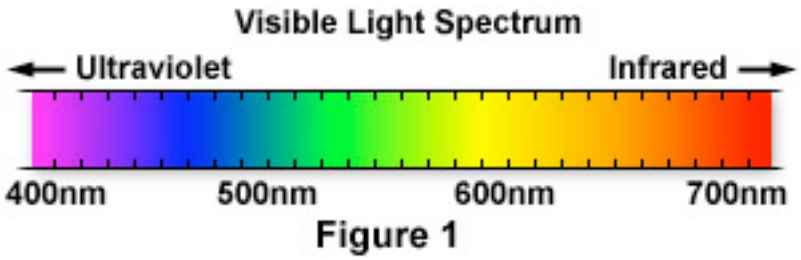
Quality Assurance in Polychromatic Flow Cytometry

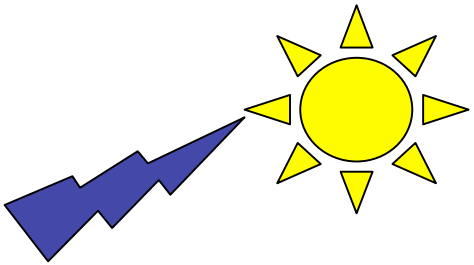


http://www.niaid.nih.gov/vrc/labs_flow.htm

Ideal FL detection and QFCM

One MESF (molecule of equivalent soluble fluorochrome)

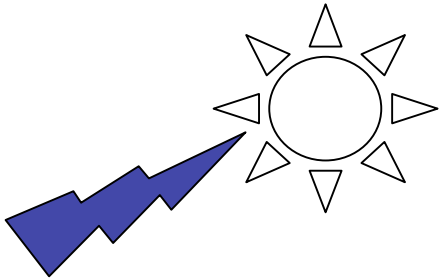




Photoelectrons

Q = photoelectrons per MESF

Unstained Particle



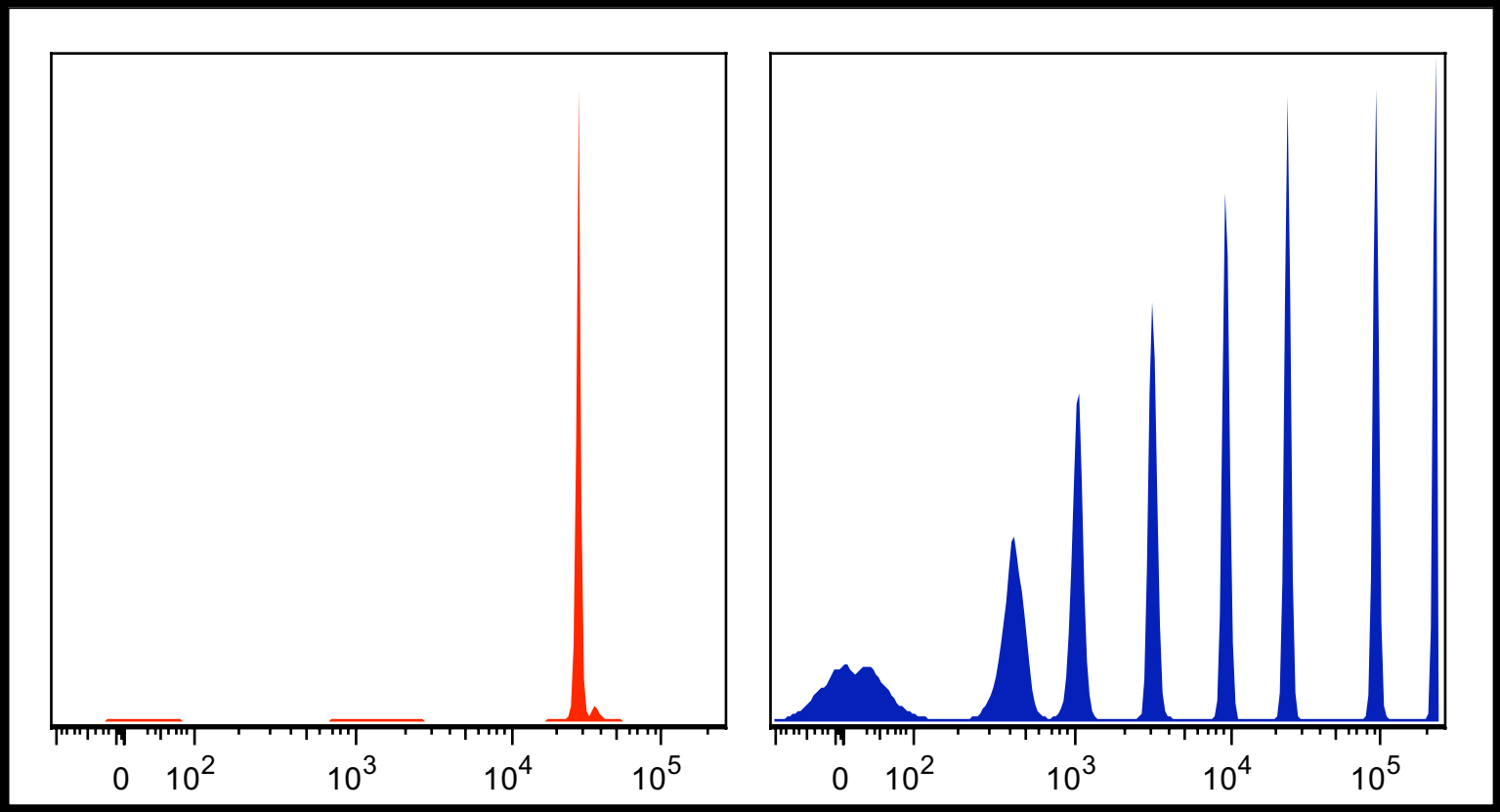
Instrument Background "B"

Particles Used for QA

Single and Eight Peak Rainbow Beads

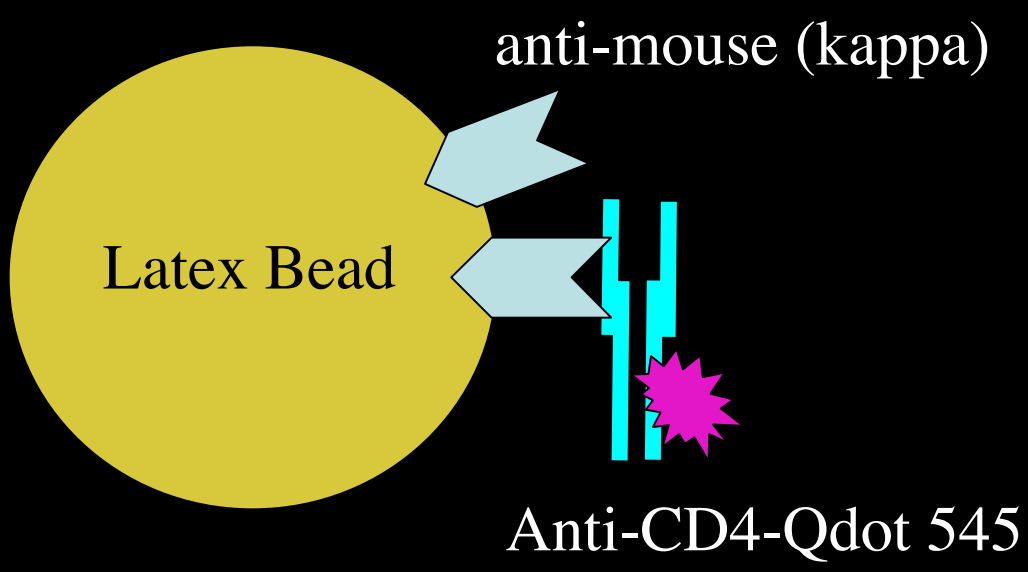
-Contains broad spectrum dyes

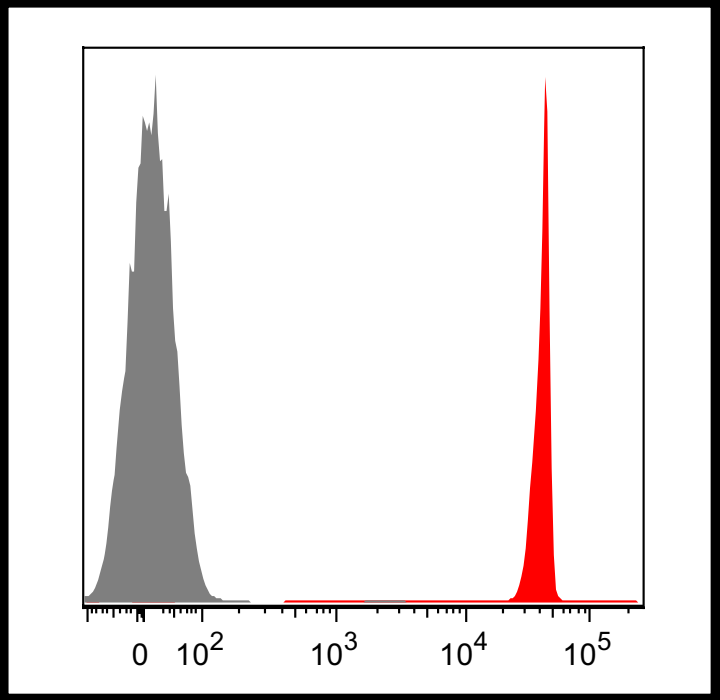
-NOT specific fluorescence



Particles Used for QA

Compensation Beads
-Stained with MAB-Conjugates
-Represents specific fluorescence
-Unstained bead represents background





Quality Assurance Procedures

- System Optimization
  - To assure proper signal measurement
- Calibration
  - To assure the best sensitivity and dynamic range of the detectors (PMT's)
  - To validate a voltage based on a specific fluorochrome and biological significance
  - To develop tolerance ranges
- Standardization
  - To monitoring tolerance ranges

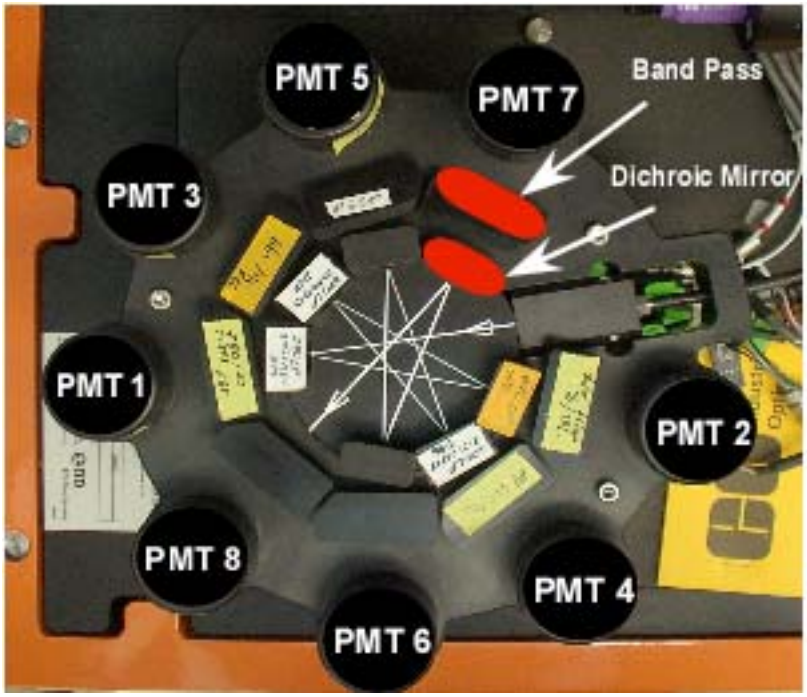
System Optimization

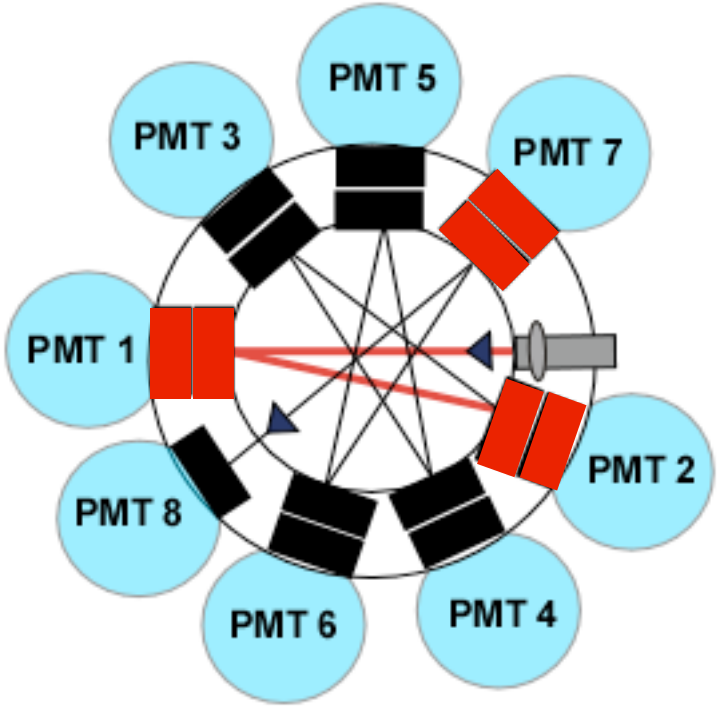
- Laser power and electronic pulse window (e.g. window extension)
- Laser Delay Settings (laser distances)
- Signal Synchronization (e.g. area scaling factor)
- Cascade test to determine photon efficiency of a given PMT (SPE)
- Filter transmission and reflection

LSR Configuration

Laser	Detector Name	Fluorochrome detected	Dichoric Filter (LP)	BP Filter
Blue 488nm	FSC	Forward Scatter		
Blue 488nm	B710	Cy55PerCP	685LP	710/50
Blue 488nm	B515	FITC, CFSE, GFP, Alexa 488, GrViD	505LP	515/20
Blue 488nm	SSC	Side scatter	488LP	
Green 532nm	G780	Cy7PE, Alexa 750PE	740LP	780/40
Green 532nm	G710	Cy55PE, Alexa 700PE	690LP	710/50
Green 532nm	G660	Cy5PE	640LP	660/40
Green 532nm	G610	TRPE, TR, OrViD	600LP	610/20
Green 532nm	G560	PE, Alexa 532	empty	575/25
Green 532nm	Empty	na		
Green 532nm	Empty	na		
Green 532nm	Empty	na		
Red 633nm	R780	Cy7APC,	740LP	780/60
Red 633nm	R710	Cy55APC, Alexa 680, 700	685LP	710/50
Red 633nm	R660	APC, Alexa 647	empty	660/20
Violet 407nm	V800	QD800	740LP	780/60
Violet 407nm	V705	QD705	670LP	705/70
Violet 407nm	V655	QD655	630LP	660/40
Violet 407nm	V605	QD605	595LP	605/40
Violet 407nm	V585	QD585	570LP	585/42
Violet 407nm	V565	QD565	557LP	560/40
Violet 407nm	V545	QD545, Pacific Orange	535 LP	550/40
Violet 407nm	V450	CBlue, ViViD, Pacific Blue	empty	450/50

Cascade Experiment



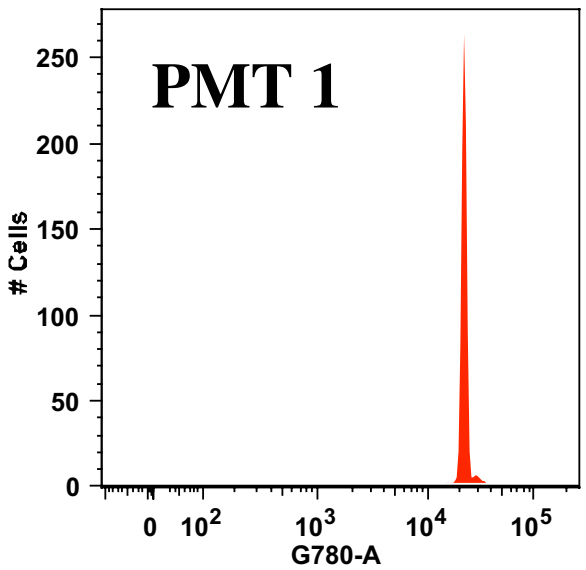


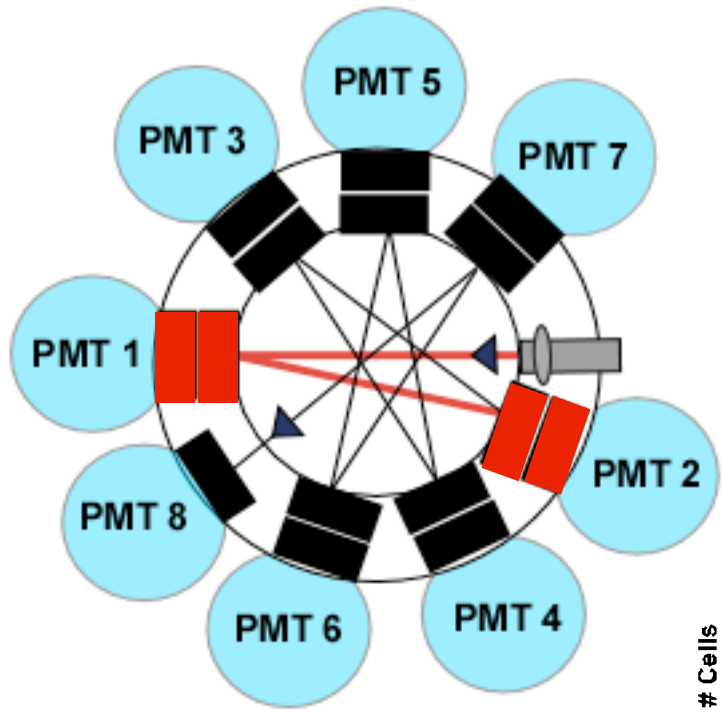
Measuring the SPE

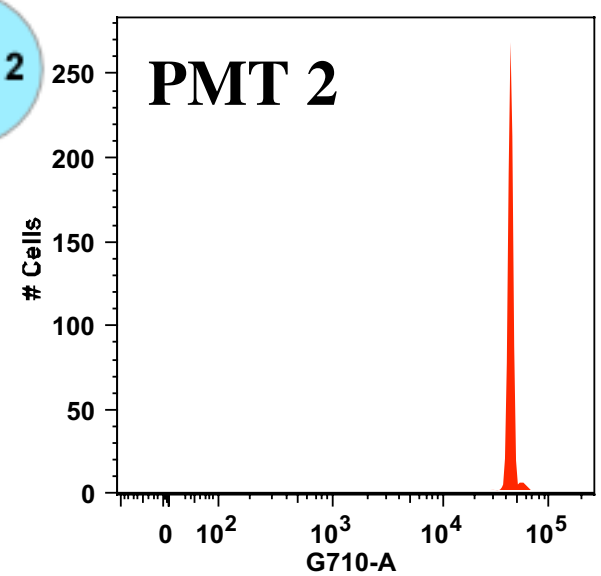
SPE (Statistical Photoelectron Estimate) = $1/(n)^2$

Range of SPE Values

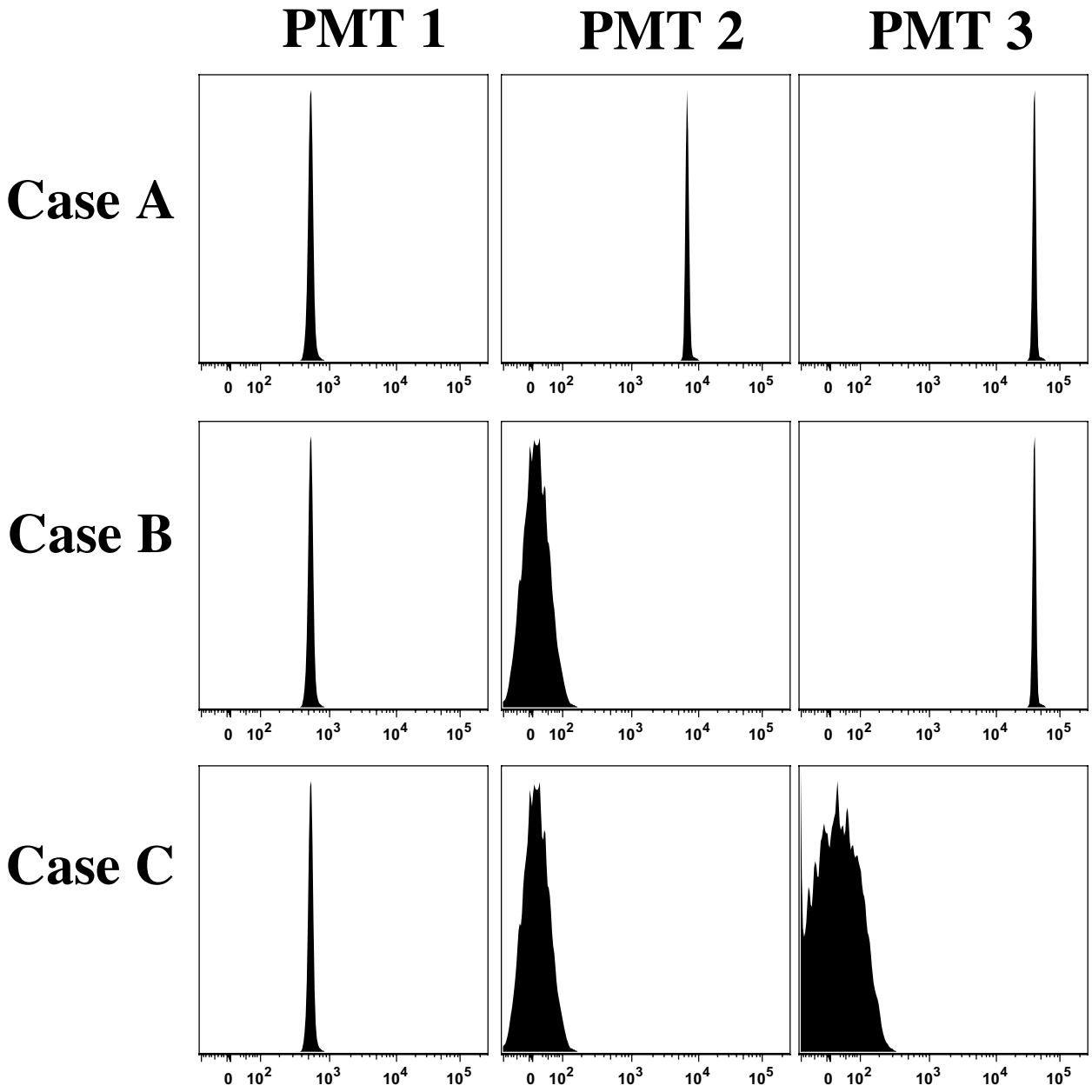
	699.87
	807.08
	726.53
	742.45
	1197.30
	1111.11
	802.51
	545.90
Mean	829.09
SD	217.53







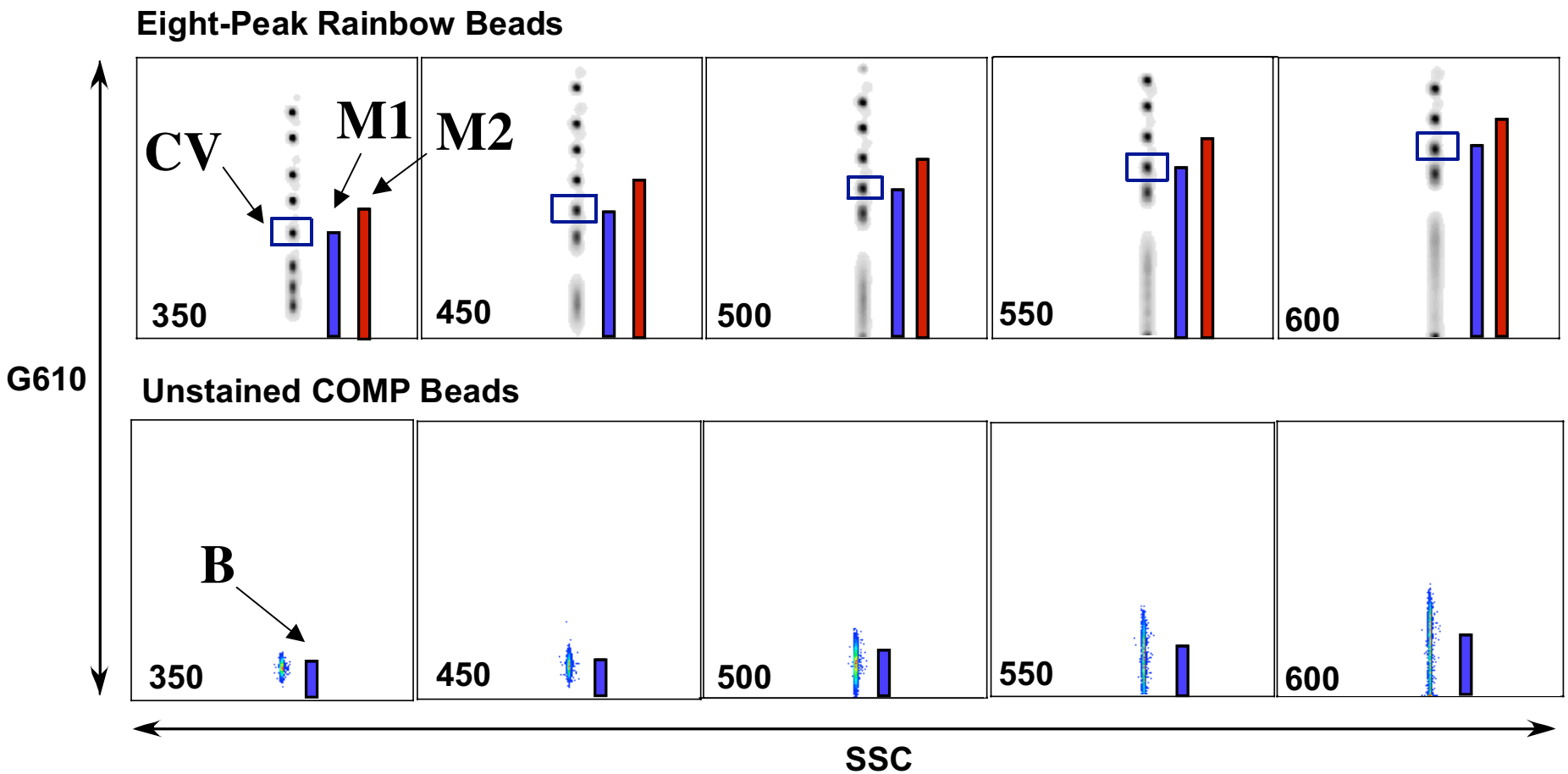
Reflection and Transmission



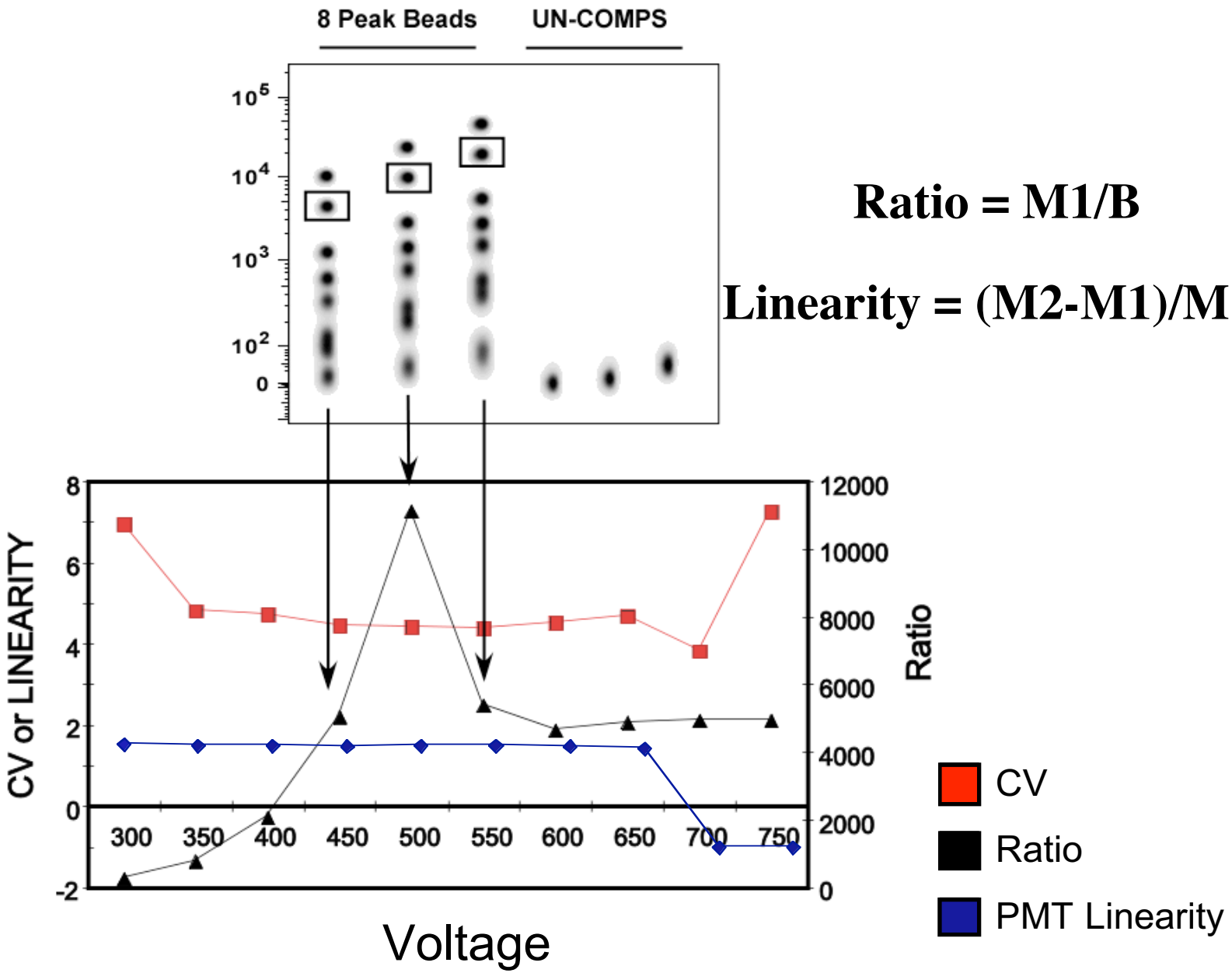
Calibration Procedures

- To assure the best sensitivity, dynamic range and linearity of the detectors (PMT's)
  - 8 Peak Rainbow Beads
  - Unstained COMP beads
- To validate a PMT voltage based on a specific fluorochrome and *biological significance*
  - COMP beads
  - Stained cells with linage and memory cell markers (ICS)
- To developing tolerance ranges
  - 1 Peak Rainbow beads

Bead Analysis Setting Voltages



Graphing Voltage Ranges



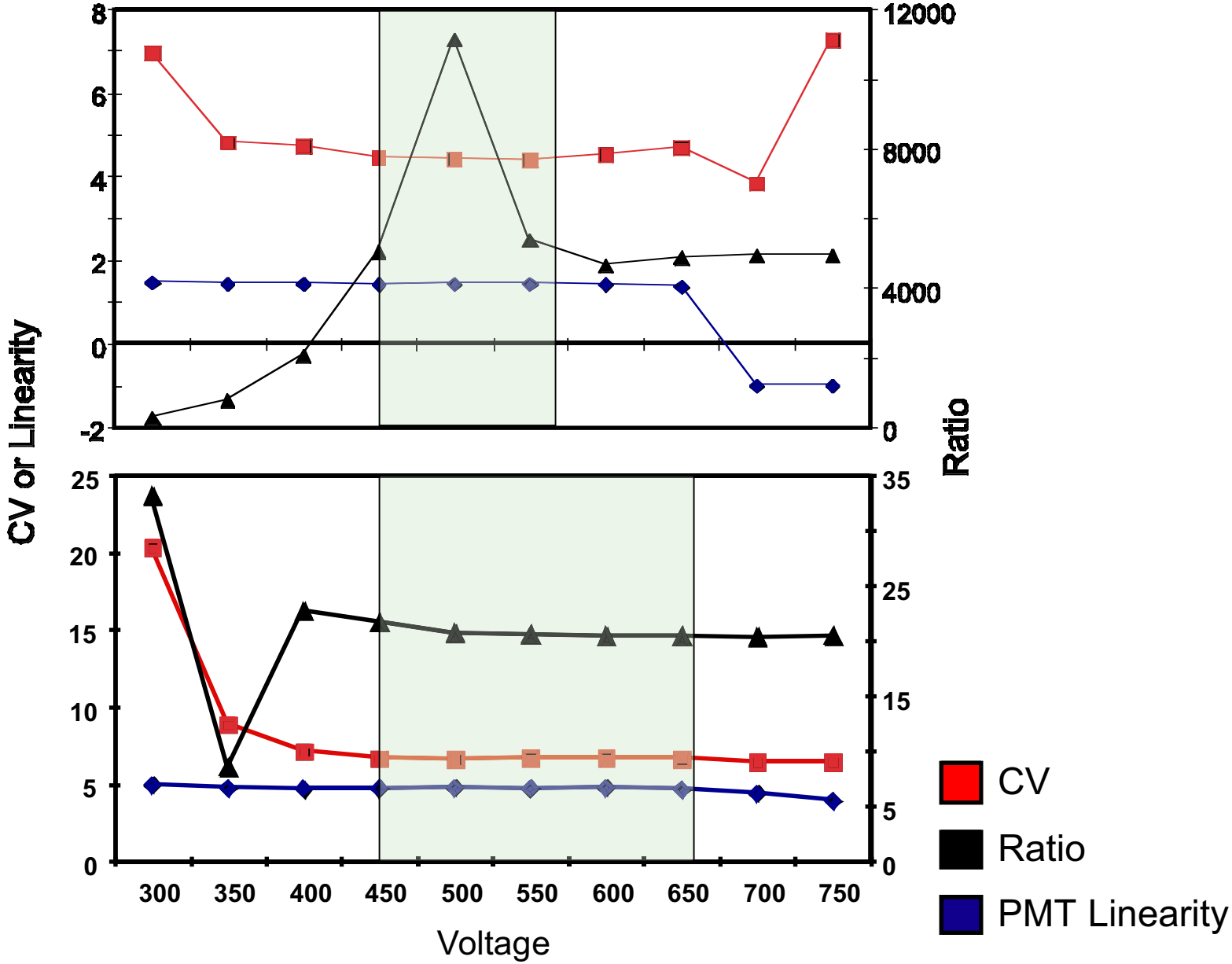
Ratio = M1/B

Linearity = (M2-M1)/M1

Criteria for the Selection of Voltage Ranges

- Lowest CV possible
- Lowest Voltage possible
- Highest MFI possible
- Lowest background possible (<200 MFI)

Detector Voltage Examples

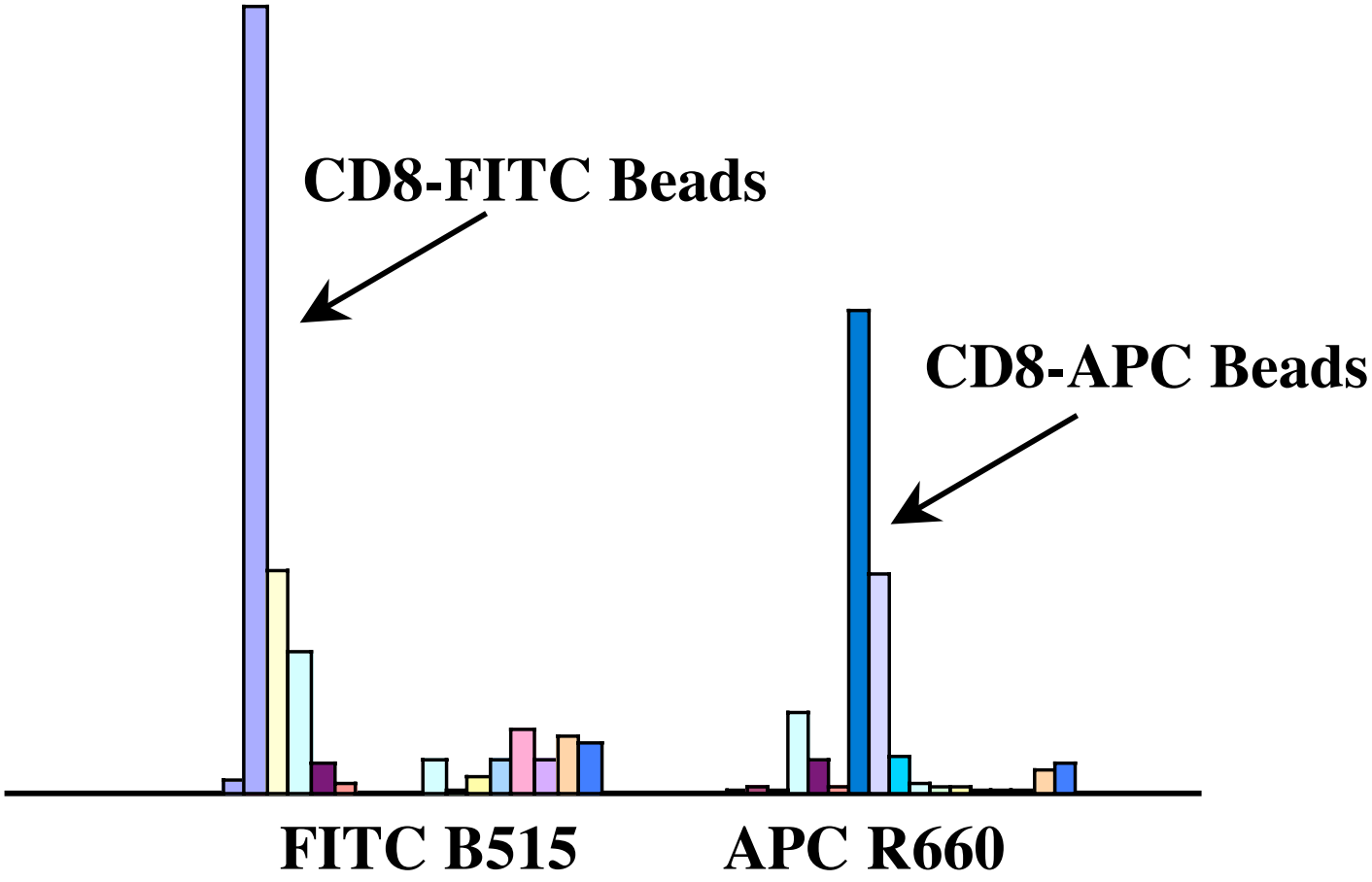


Specific FL COMP Beads

- Using the voltage ranges determine the "ideal" voltage for each PMT (*Tolerance Target Value*).

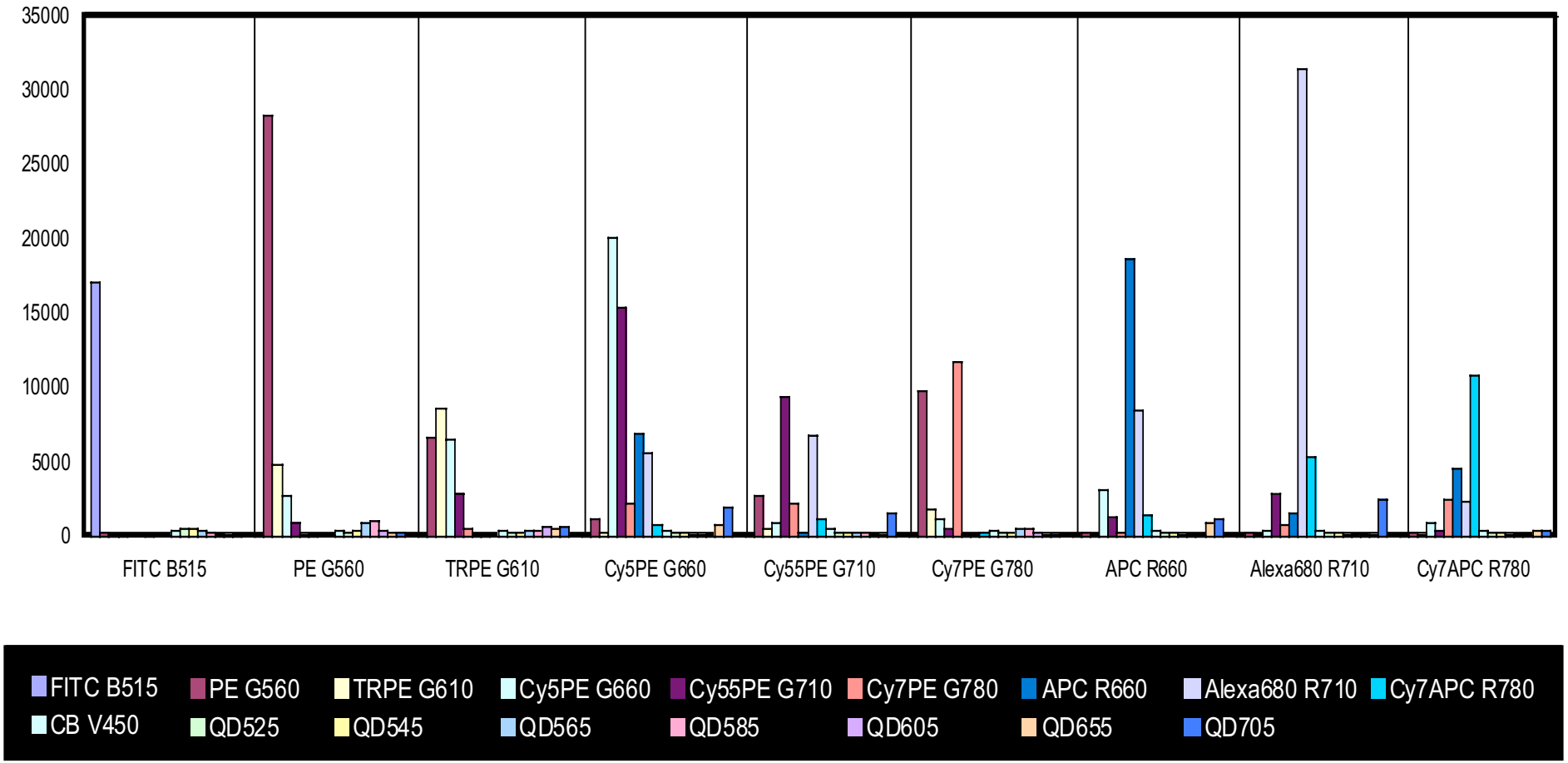
- The primary MFI should be the highest in each detector as compared to all secondary detectors.

Specific FL - COMP beads

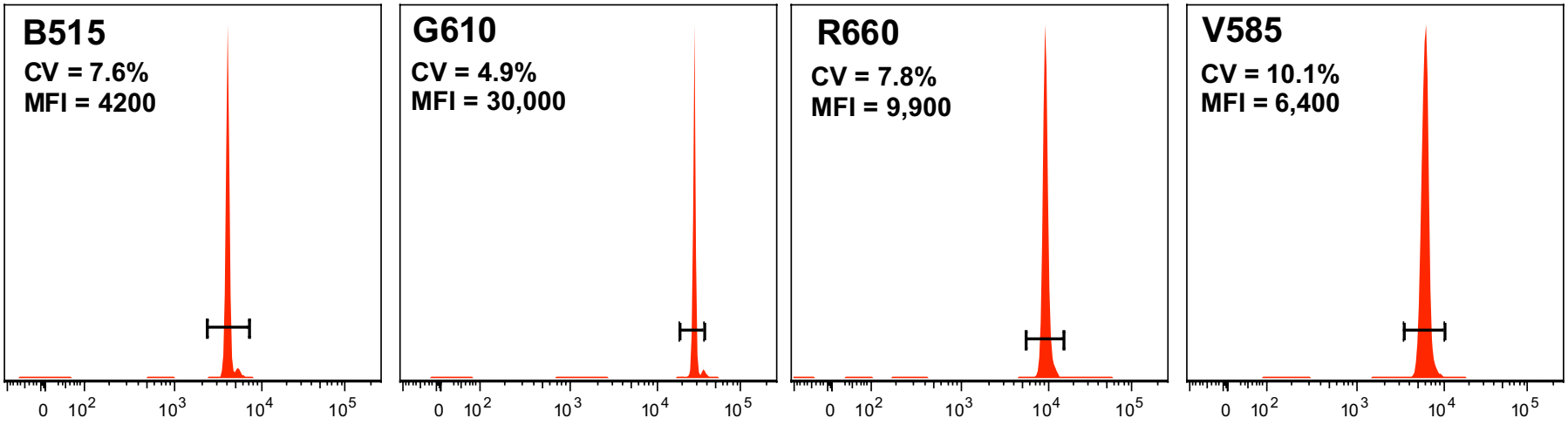


FITC B515 | PE G560 | TRPE G610 | Cy5PE G660 | Cy55PE G710 | Cy7PE G780 | APC R660 | Alexa680 R710 | Cy7APC R780
CB V450 | QD525 | QD545 | QD565 | QD585 | QD605 | QD655 | QD705

Specific FL - Primary Channels



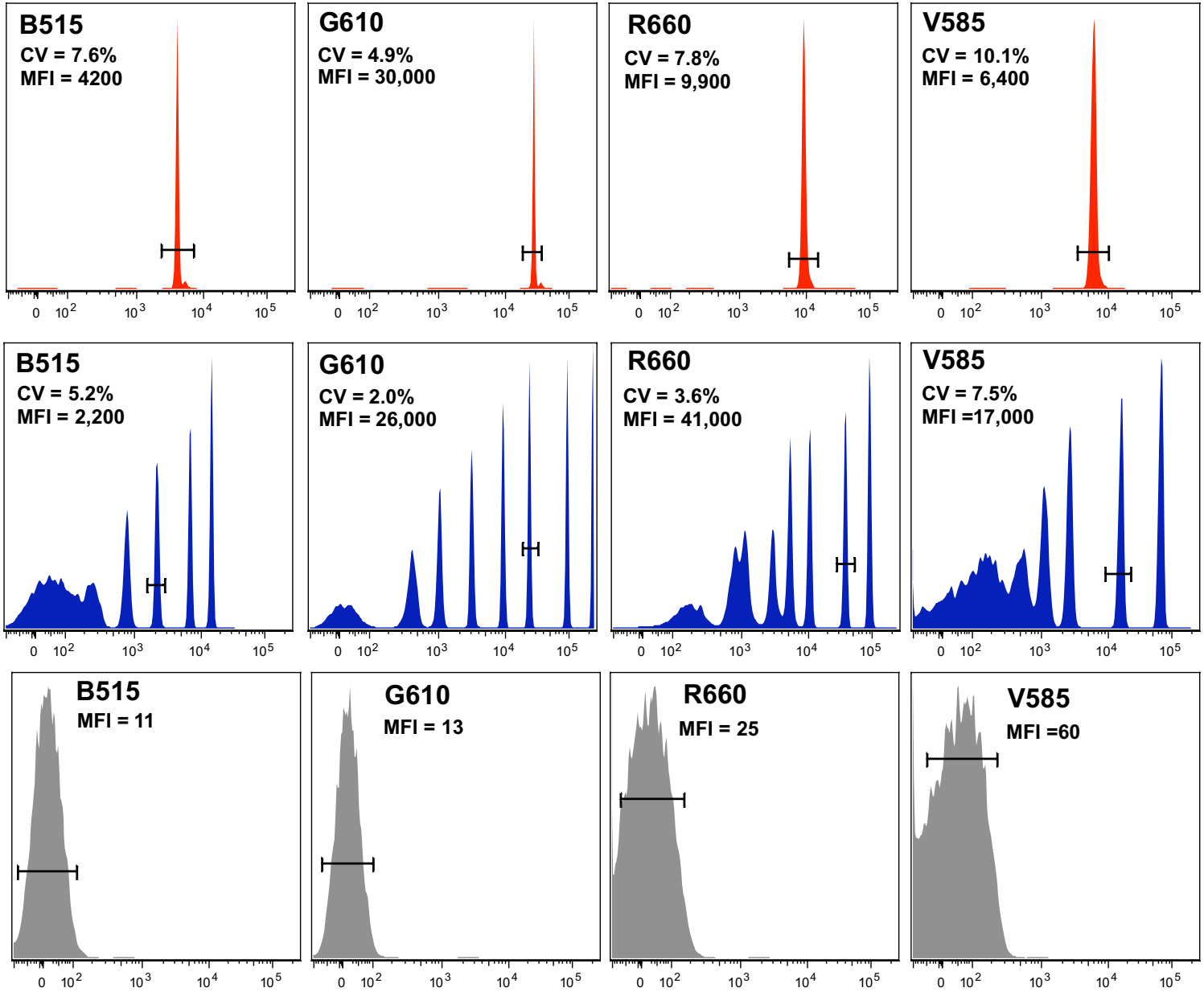
Tolerance Target Values
1 Peak Rainbow Beads



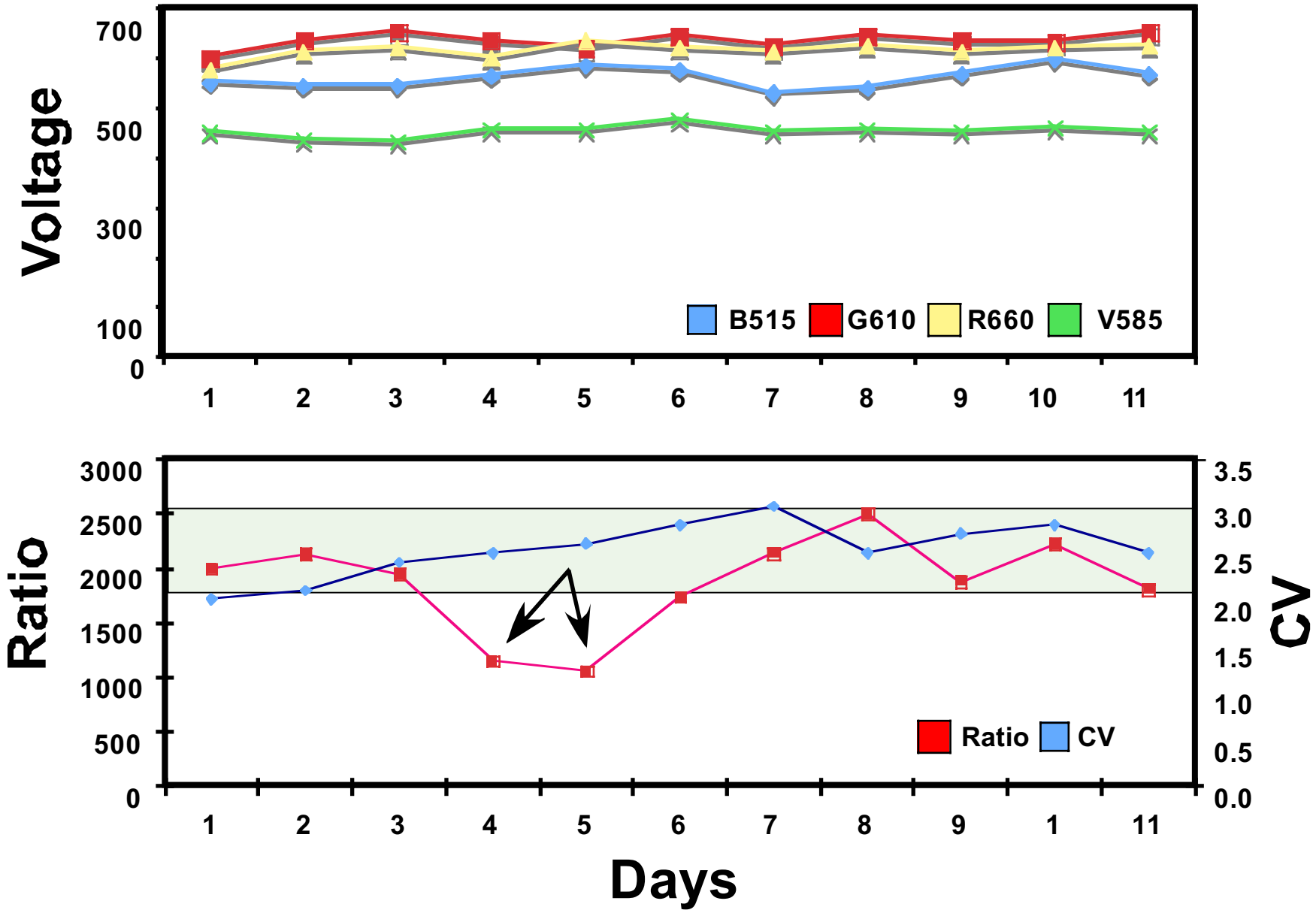
Standardization

- 1x Rainbow beads are used on a daily and adjust voltages to obtain the MFI within the established *Tolerance Target Range*.
- These voltages are used for all experiments throughout the day.
- *Tolerance Target Ranges* are check before each experimental run.
- Monitor voltage vs. time for each detector.
- Monitor CV and M1/B ratio of the 8x Rainbow bead.

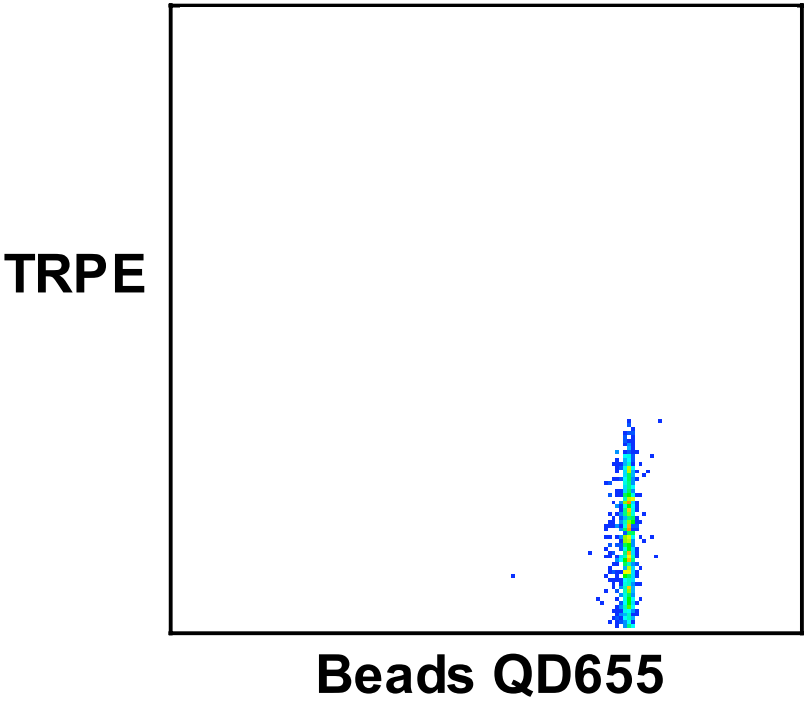
Standardization Checks

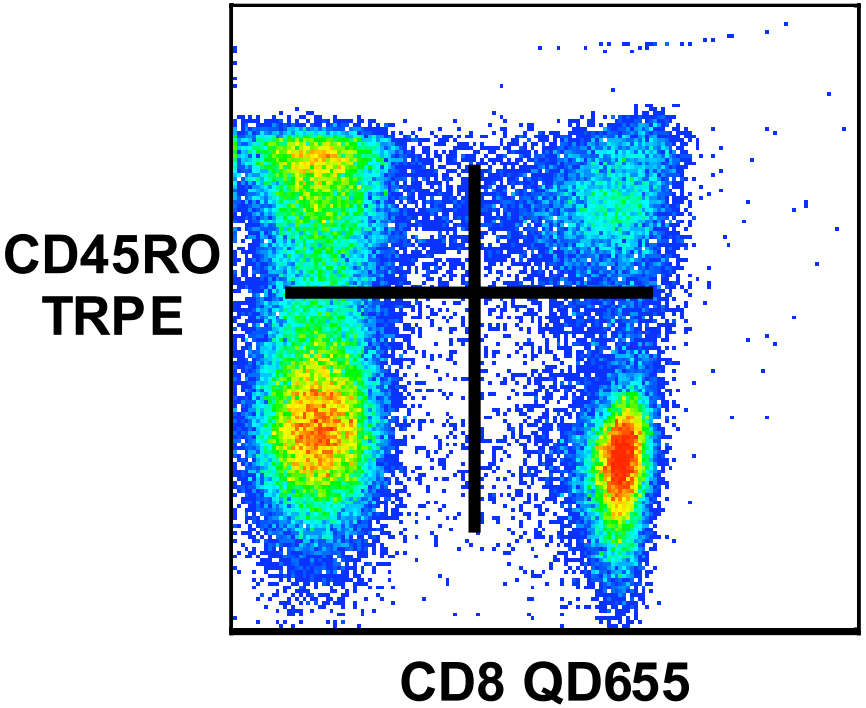


Ratio and Voltage Variation

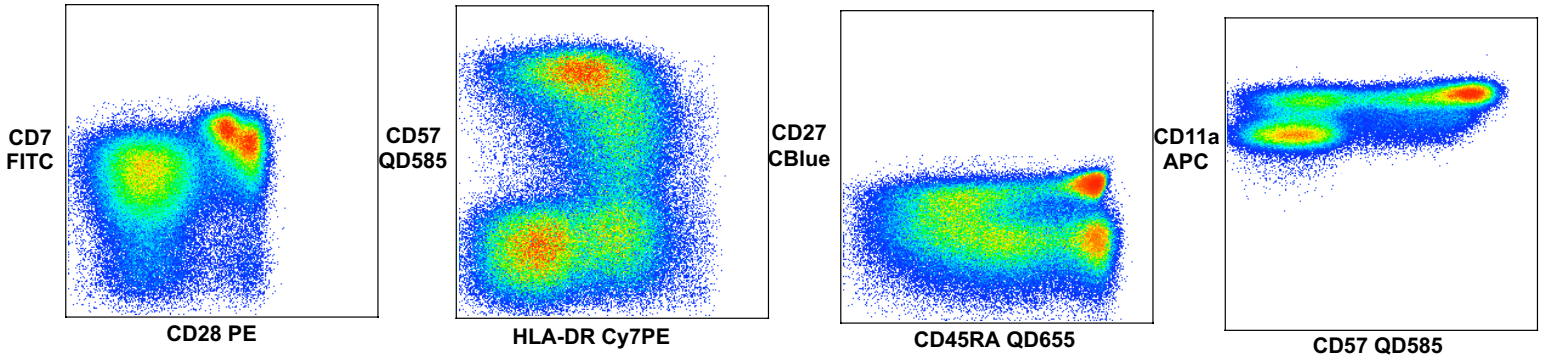


Biological Validation

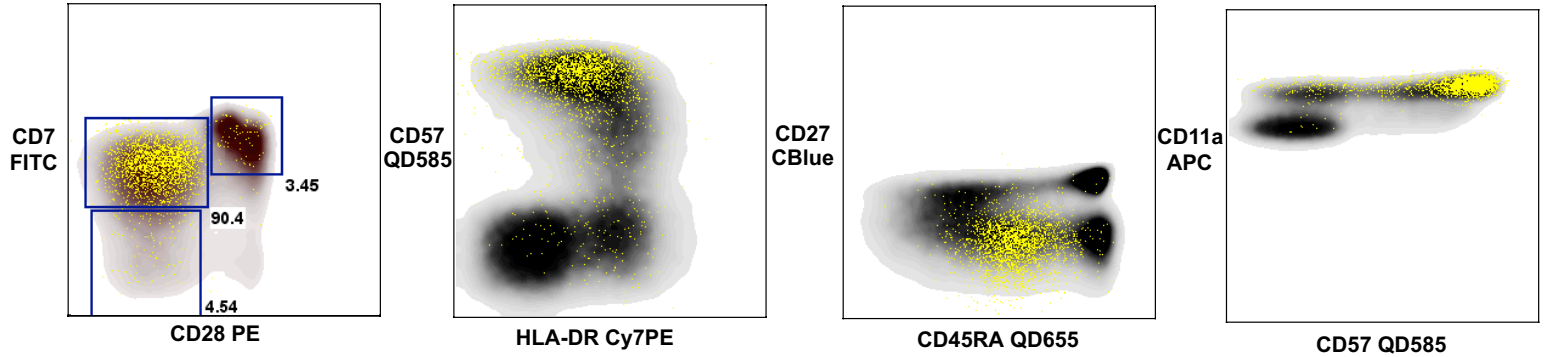




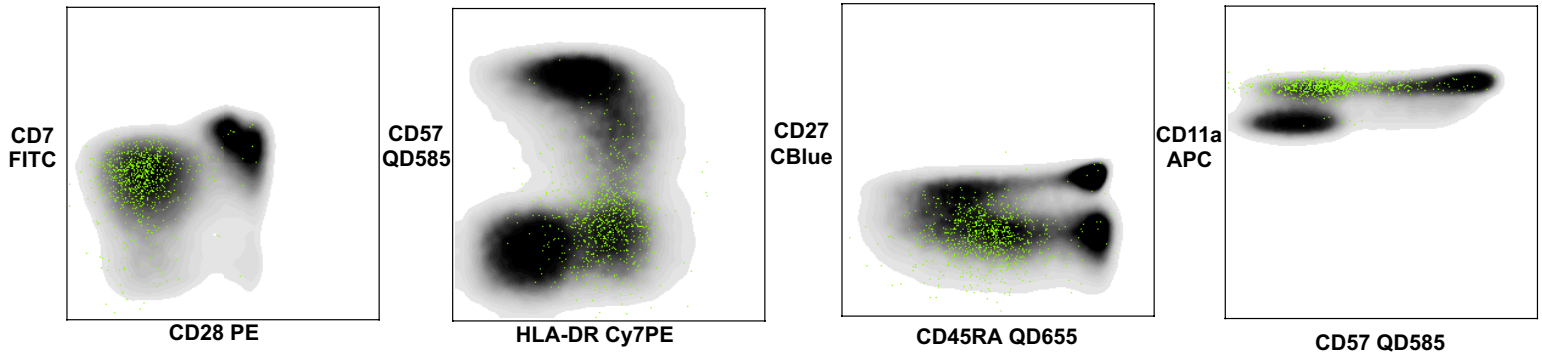
Qdot Tetramer Overlay



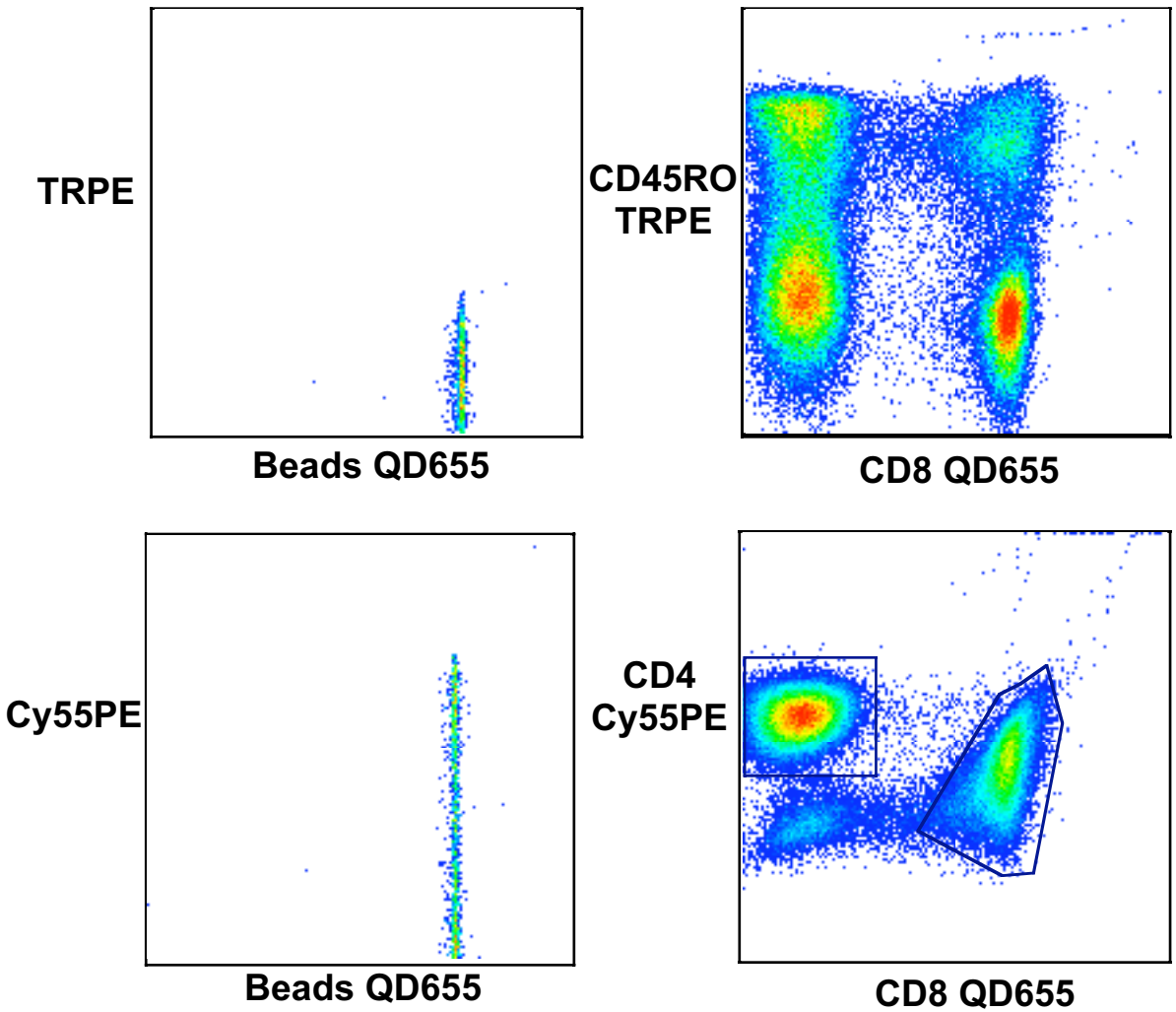
CMV Qdot Tetramer



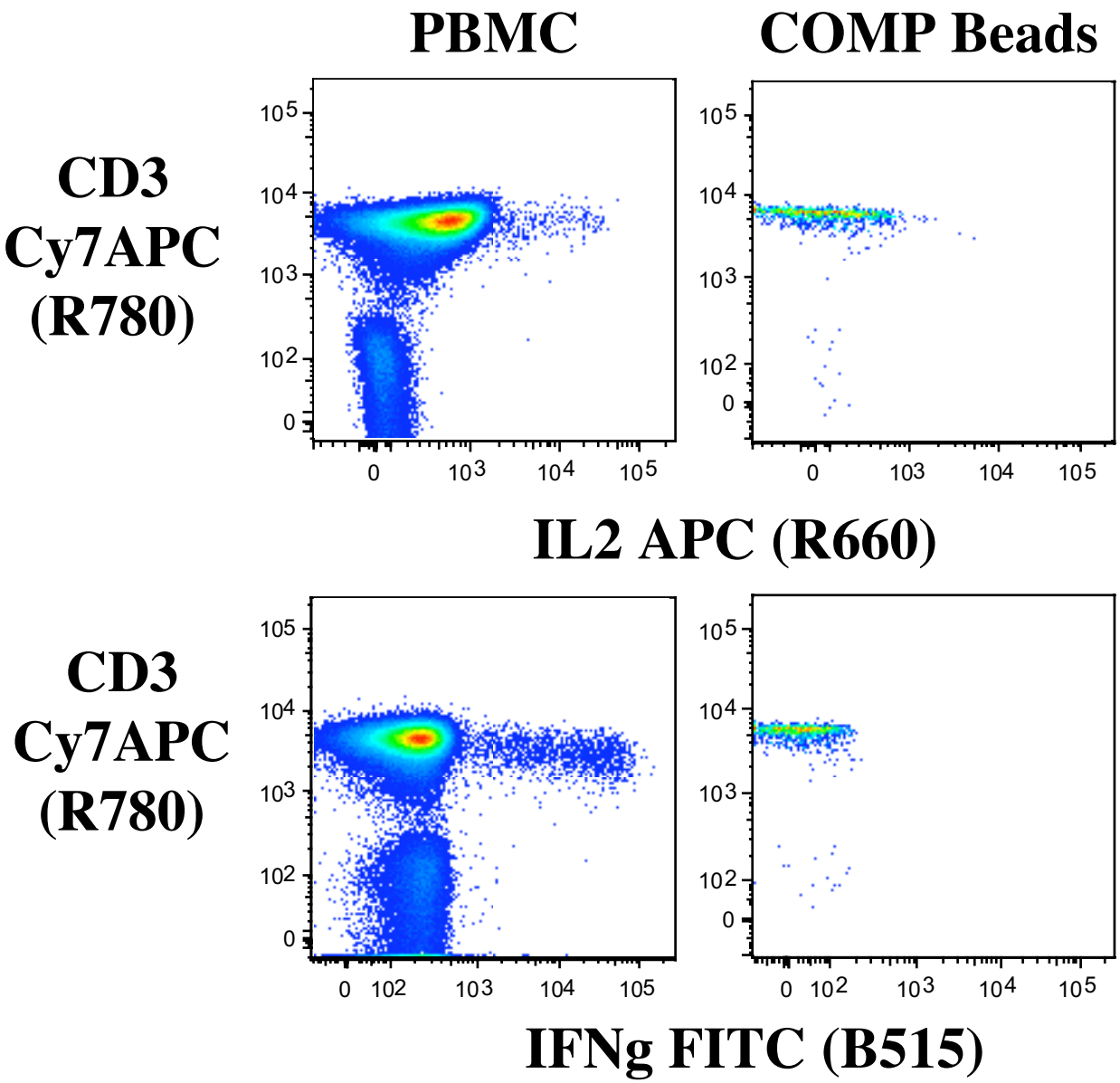
GAG Qdot Tetramer



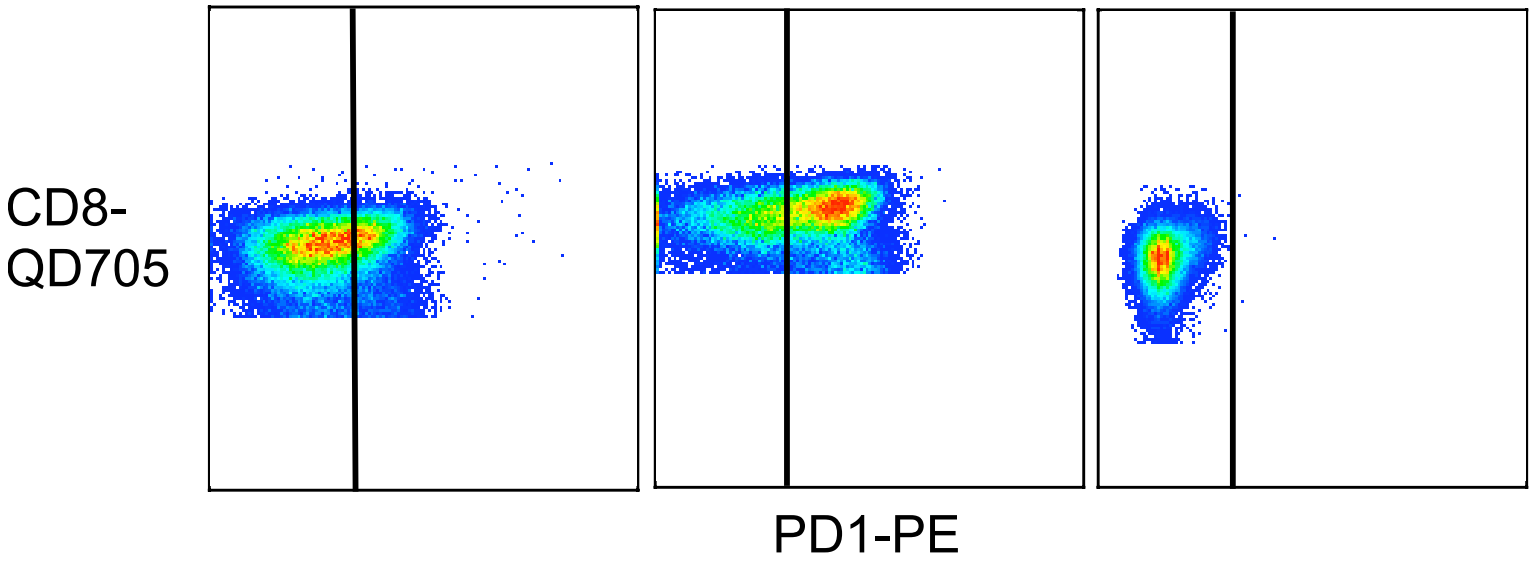
Spreading error (exp.1)



Spreading error (exp.2)



Spreading error (exp.3)

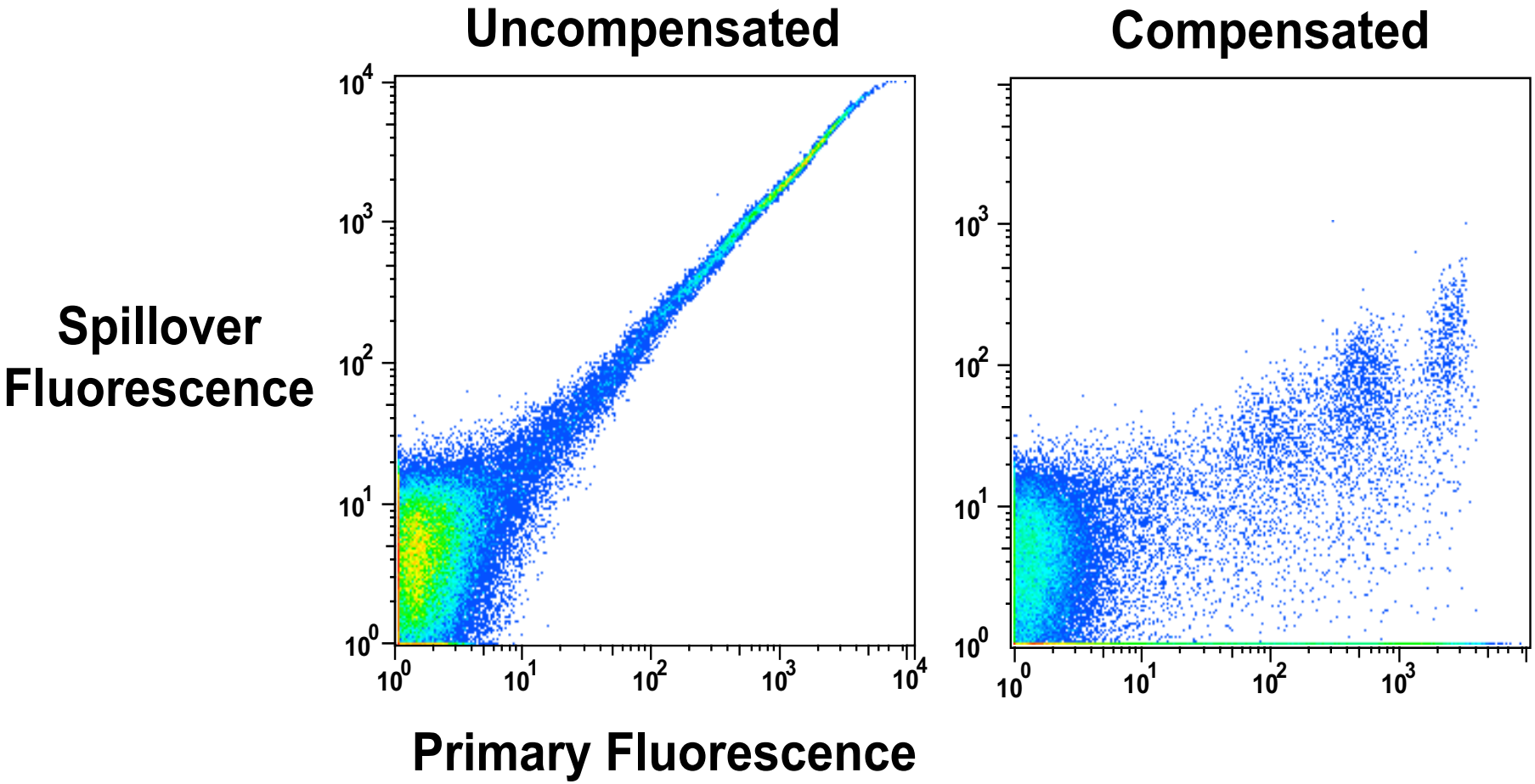


CD4 Cy55PE
PD1 PE
CD45RO TRPE
CD27 Cy5PE
CD8 Qd705
CD3Cy7APC
CD14, CD19 Cas Blue; Vivid

PD1 PE
CD8 Qd705
CD3Cy7APC

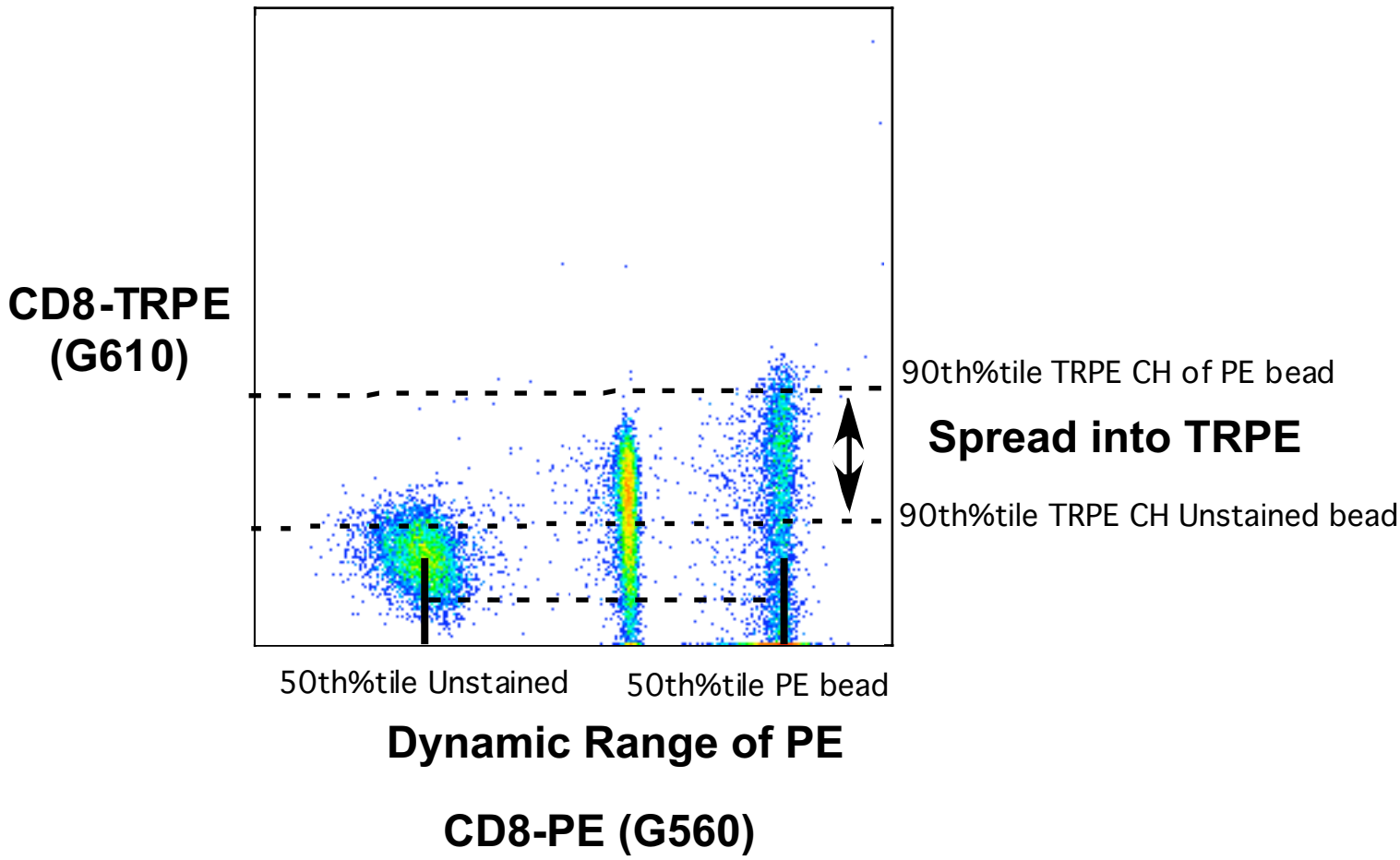
CD8 Qd705
CD3Cy7APC

Panel Development
Spreading Error in Compensated Data



Why is there a 400-unit spread? Photon counting statistics.

Compensated PE labeled Beads



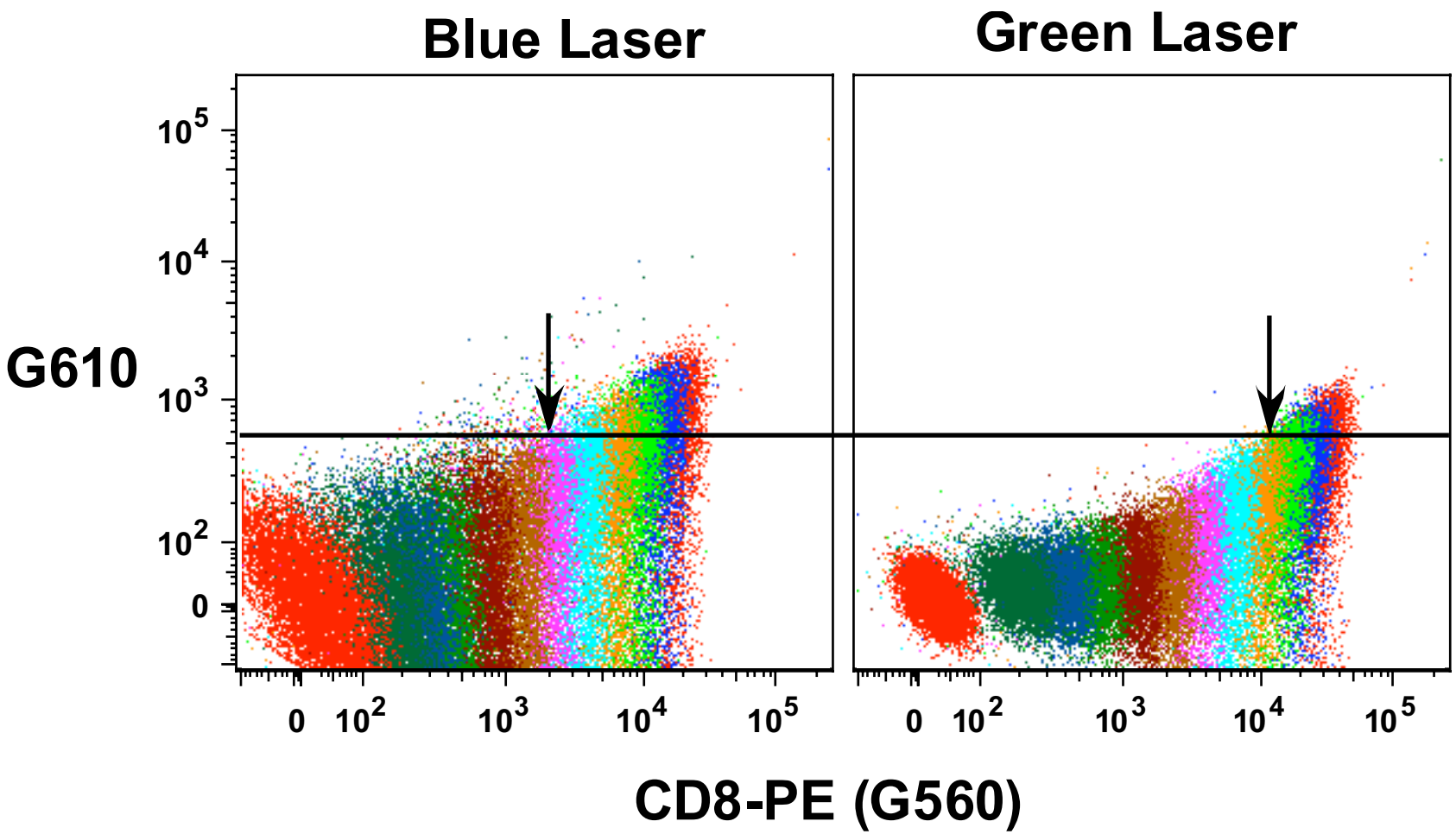
$$\text{Spreading Error} = \frac{\text{Spread into the TRPE detector}}{\text{SQRT Dynamic Range of PE detector}}$$

How to reduce SE?

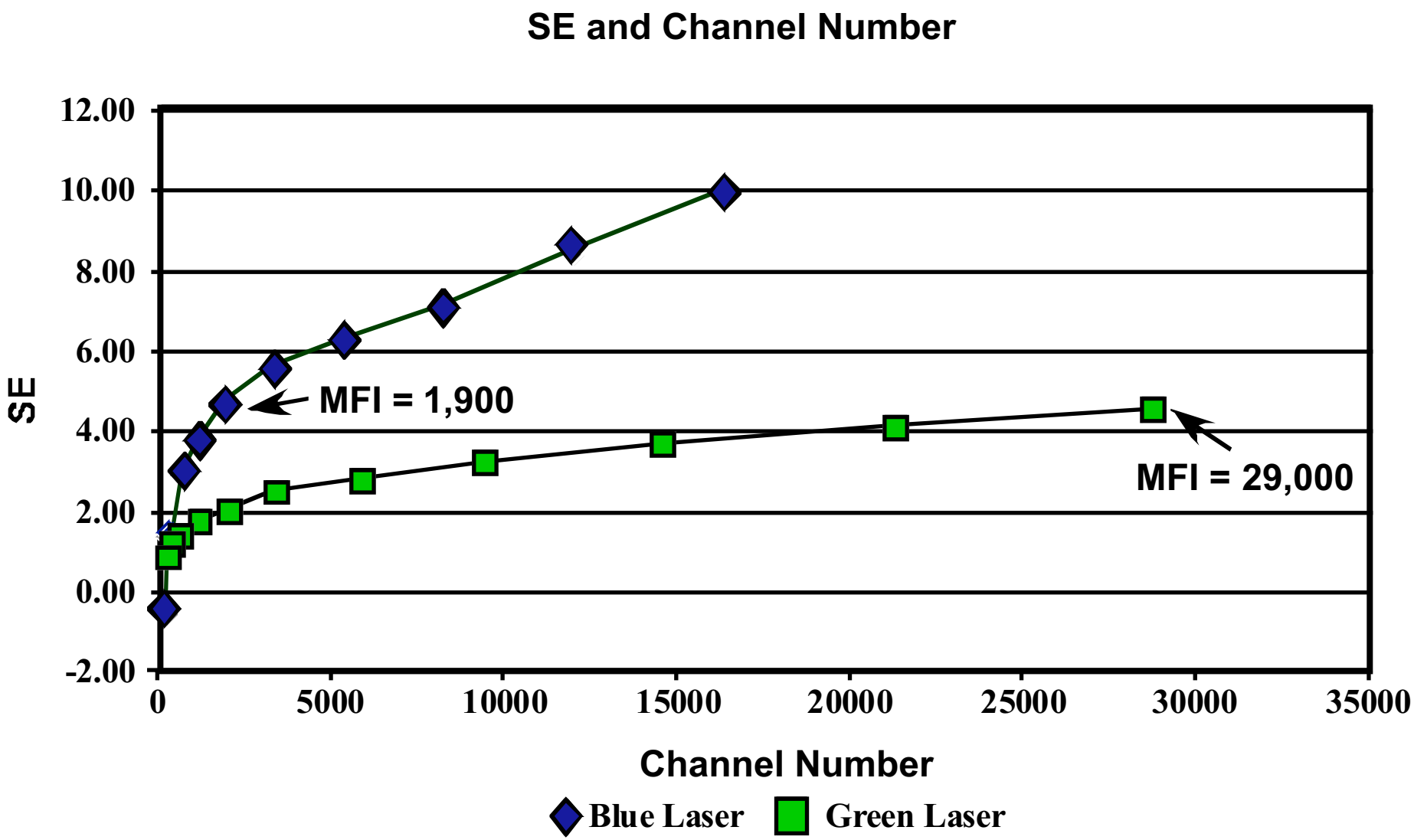
- Efficiency of PMT: Higher efficiency detectors can improve photoelectron detection, esp. at the far-red spectrum detection.

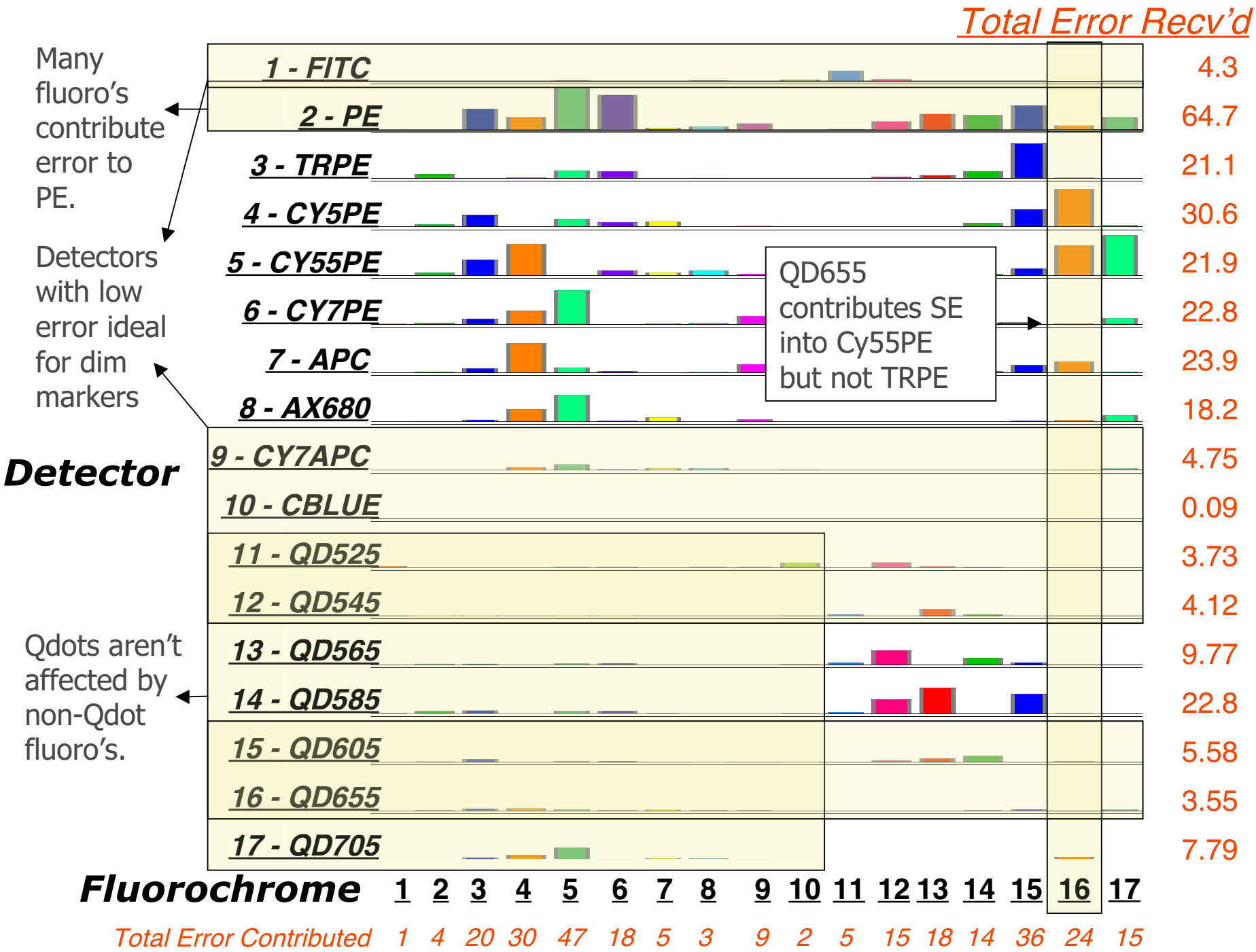
- Difference in lasers: The green laser excites the PE molecule to emit *MORE* photoelectrons as compared to the blue laser.

Titration of cells



Reduced SE of the Green Laser

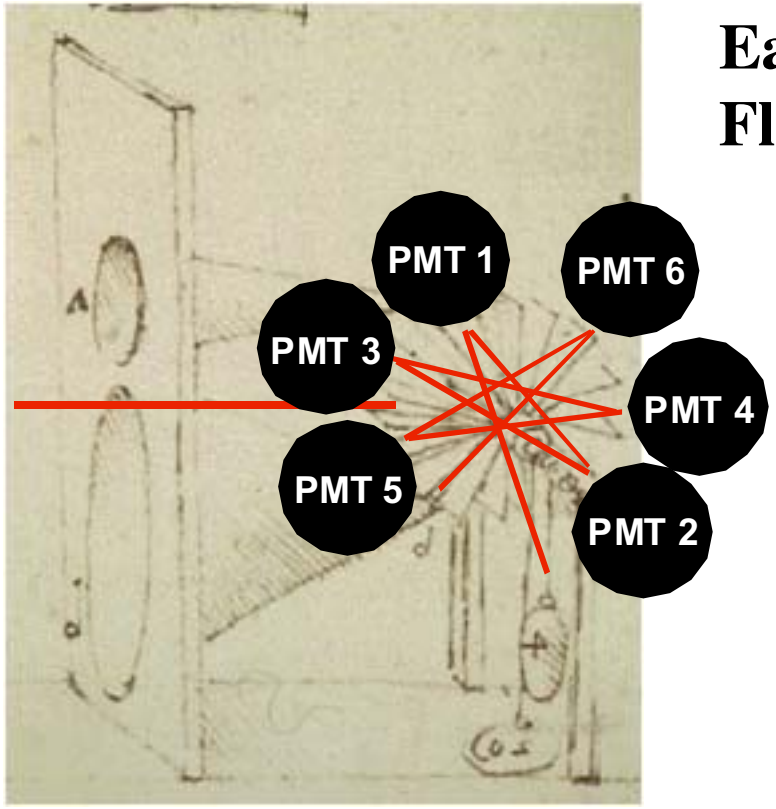



Total Error Recv'd
Many fluoro's contribute error to PE.
Detectors with low error ideal for dim markers
Qdots aren't affected by non-Qdot fluoro's.
Detector
1 - FITC
2 - PE
3 - TRPE
4 - CY5PE
5 - CY55PE
6 - CY7PE
7 - APC
8 - AX680
9 - CY7APC
10 - CBLUE
11 - QD525
12 - QD545
13 - QD565
14 - QD585
15 - QD605
16 - QD655
17 - QD705
QD655 contributes SE into Cy55PE but not TRPE
4.3
64.7
21.1
30.6
21.9
22.8
23.9
18.2
4.75
0.09
3.73
4.12
9.77
22.8
5.58
3.55
7.79
Fluorochrome
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Total Error Contributed
1 4 20 30 47 18 5 3 9 2 5 15 18 14 36 24 15

Conclusions on panel Development

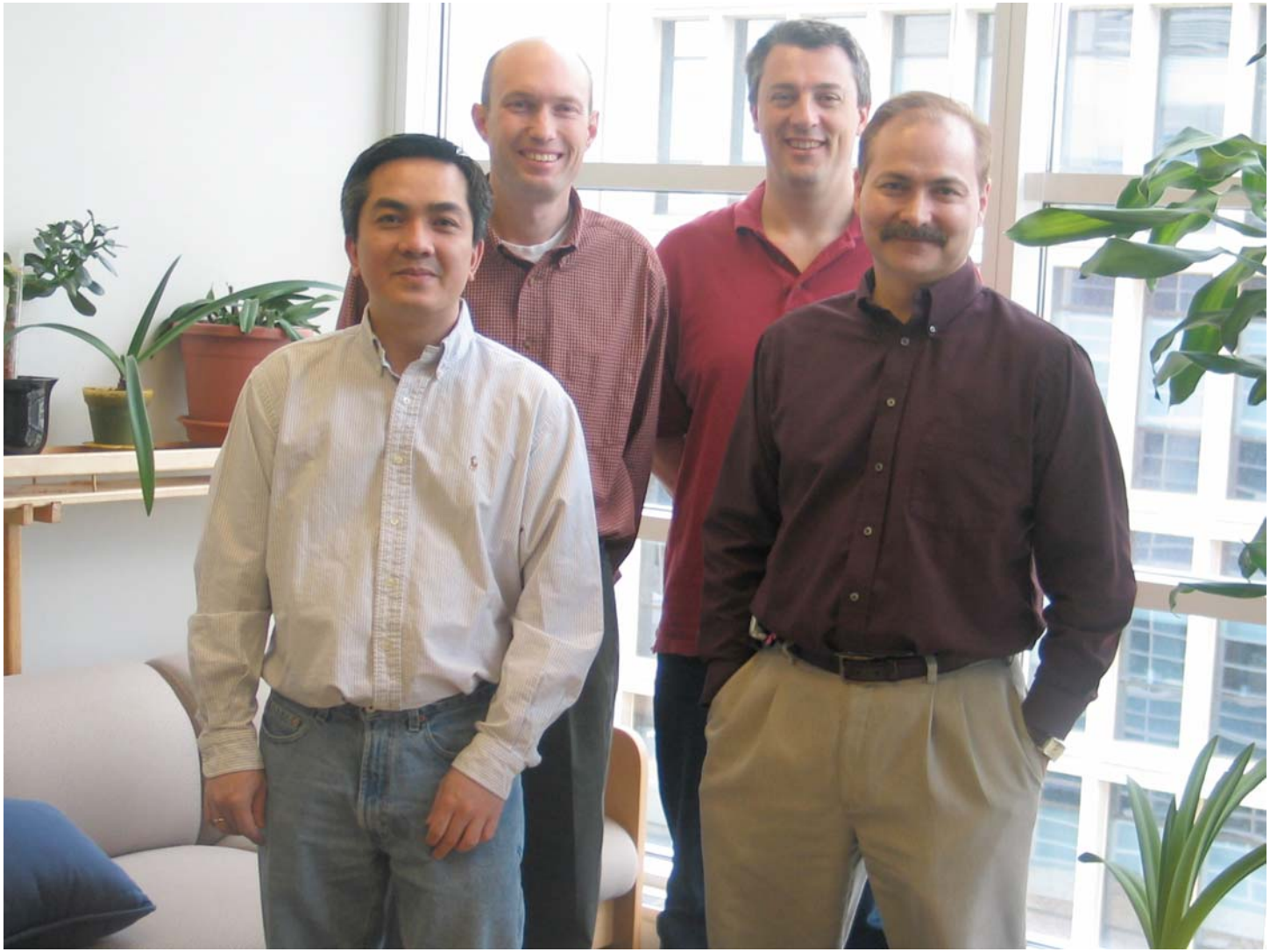
- Use an FMO control to define Pos/Neg.
- Titration of ALL reagents to within the dynamic range of the detector.
- Develop small panels (5-9 clone-conjugate).
- COMP the COMP beads.
- Reduce SE: Spreading error chart, PMT efficiency and type of lasers.
- Panels must be biologically significant.

Leonardo De Vinci's Mystery Machines



Early Design of a Flow Cytometer

FCC at the VRC



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