# Photons, Photoelectrons, & Q and B

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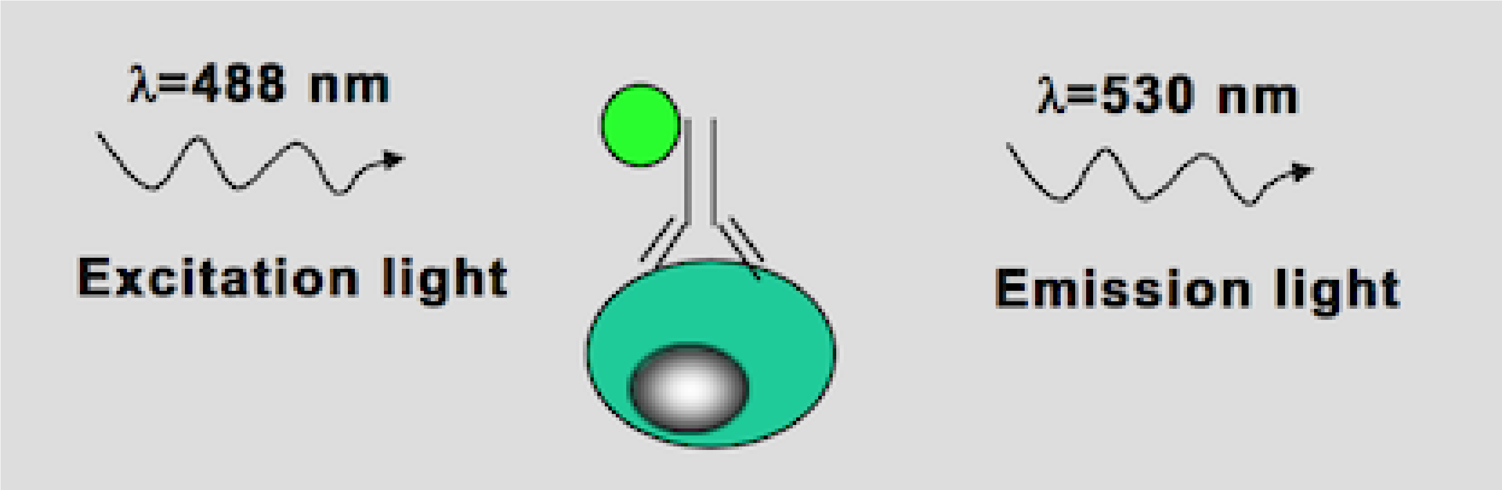
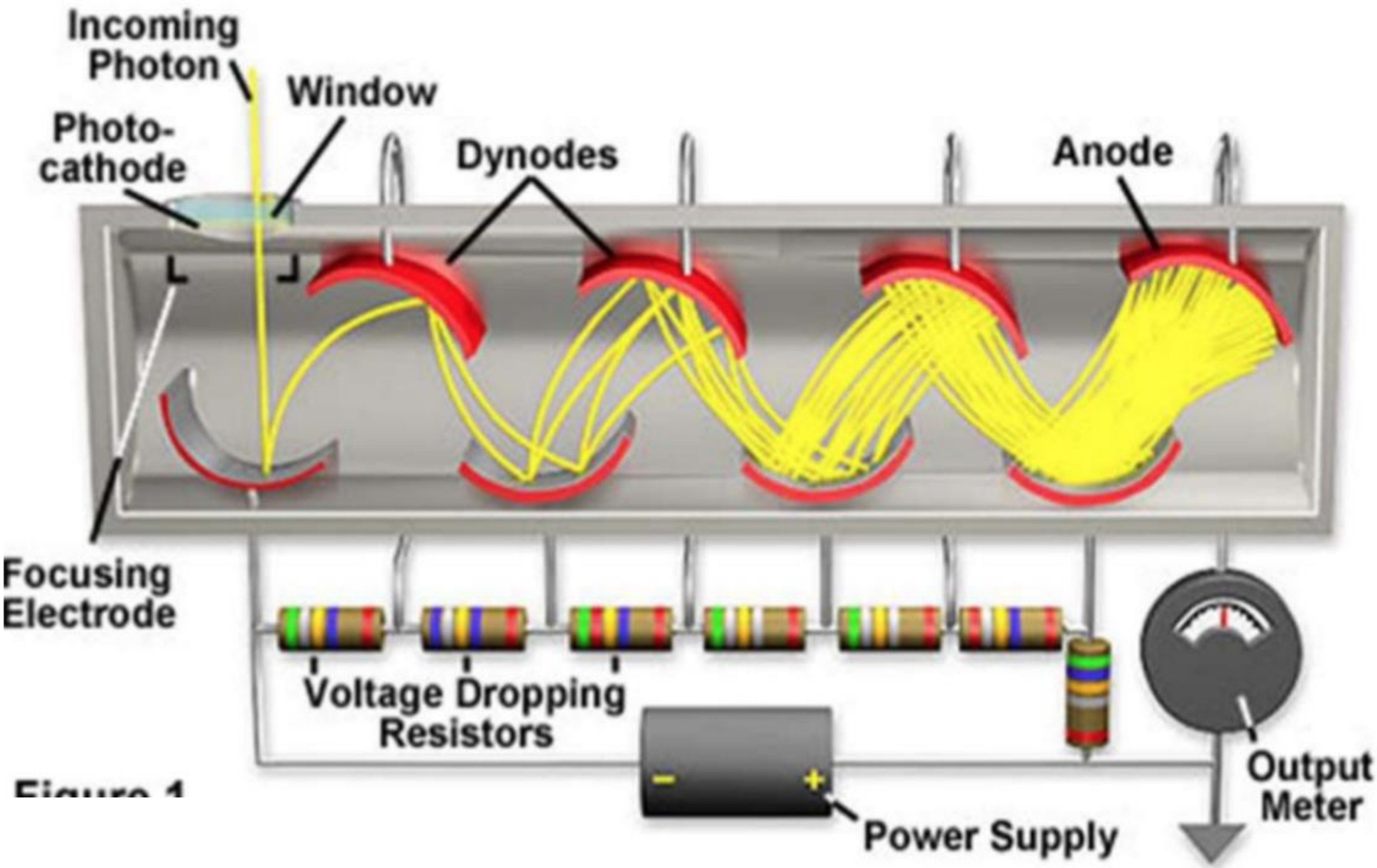
MetroFlow, October 12, 2017

# Photons are emitted from Dye Molecules on a Cell



# Photons generate photoelectrons



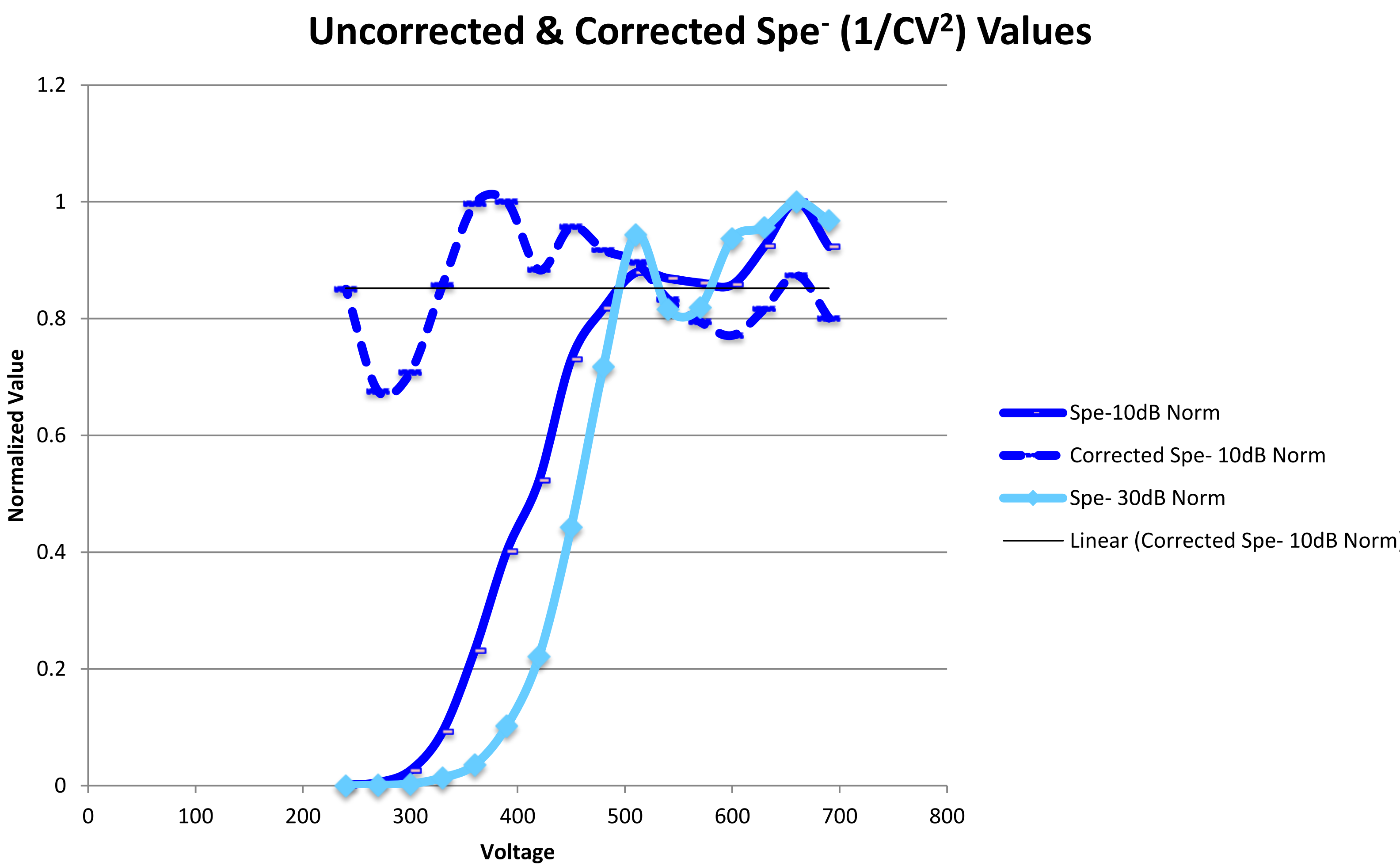
# Photoelectrons vs. Statistical photoelectrons ($Spe^-$)

- We can't directly measure the photoelectrons at the photocathode.
- Detection efficiency (Q) is a property of the photocathode
- PMT amplification is nearly noiseless
- We estimate the statistical variation of the measured signal to be related to the number of photoelectrons generated by the pthotocathode
- Poisson statistics predicts $Spe^- = 1/CV^2$

# Measuring the $Spe^-$ generated

- Photosensitive surface generates electrons
- Independent of voltage
- $Spe^-$ definition: $Spe^- = 1/CV^2$
- Correct for background
- Correct for base line restorer
- Correct for offsets

# Voltage Ranging  to measure Spe$^-$




BV421A

# Benefits of an Spe$^-$ scale

- Standardize scale in Spe$^-$ units
- Instrument Independent scale
- HV used to adjust Spe$^-$ /channel
- Does not standardize Q (Spe$^-$ / ERF)
- Peak CVs reflect differences in Spe$^-$ s
- Higher CVs equate to lower Q values
- Not fluorochrome related

# $Spe^-$ vs Fluorochrome dye scales

- Target bead location does not uniquely define $Spe^-$ scale (nor FLU scale)
- $Spe^-$ value not guaranteed for a target channel
- Means alone do not imply a unique $Spe^-$ value
- Means and CVs must both match to standardize, implies Qs are the same
- CVs are influenced by alignment, Q, and $Spe^-$

# What is Q?

- Q is $Spe^-$ per fluorochrome
- Independent of voltage
- Measurement of Q at low levels affected by B

# What is not Q?

- $Spe^-$ / Channel is not Q
- $Spe^-$ is constant but channel depends on gain
- Gain dependent on voltage setting
- $Spe^-$ / Channel  decreases with increasing gain
- $Spe^-$ / Channel depends on dynode chain gain
- $Spe^-$ / Channel proportional (not equal) to photoelectrons generated at photocathode

# Relating $Spe^-$ value to Q

- $Spe^-$ per fluorochrome unit is Q
- Fluorochromes * Q = $Spe^-$
- Low Q will result in low Spe- values
- High Q will result in high Spe- values

# How to Estimate Q: SD$^2$ vs Intensity

$$Q\left(SD_{photonic}\right)^2 = f_{bead} + B$$

$$(SD_{photonic})^2 = \frac{f_{bead}}{Q} + \frac{B}{Q}$$

# Linear vs Quadratic Model

## Linear Model (CPCy 1.20)

$$(SD_{photonic})^2 = \frac{f_{bead}}{Q} + \frac{B}{Q}$$

$$(CV_{bead})^2 = (CV_{photonic})^2 + (CV_{intrinsic})^2 + (CV_{measurement})^2$$

## Quadratic Model

$$(SD_{bead})^2 = (f_{bead})^2 \times \{(CV_{intrinsic})^2 + (CV_{measurement})^2\} + \frac{f_{bead}}{Q} + \frac{B}{Q}$$

# Calculating Q

| | | | |
|---|---|---|---|
| Sample Name | V510-A | Copyright, 2015-2017; James C. S. Wood | Version 3.00 10/03/2017 |
| Date | 08-Oct-2017 | Evaluation only | |

| | Channels | | | | | ERFs | | | | | SPEs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | $SD^2$ | Predicted $SD^2$ (Channels) | Spe/channel | Mean | SD | $SD^2$ | Predicted $SD^2$ (ERFs) | Q Spe/ERF | Fluorescence (F) Spe |
| 1 | 11805.00 | 207.00 | 42849.00 | 43604.56 | 2.72E-01 | 13452.55 | 235.89 | 55643.97 | 56625.14 | 2.38E-01 | 3207.41 |
| 2 | 6054.00 | 151.00 | 22801.00 | 22252.46 | 2.72E-01 | 6898.92 | 172.07 | 29609.51 | 28897.18 | 2.38E-01 | 1644.87 |
| 3 | 2677.00 | 98.40 | 9682.56 | 9770.08 | 2.72E-01 | 3050.61 | 112.13 | 12573.83 | 12687.49 | 2.38E-01 | 727.34 |
| 4 | 1352.00 | 69.60 | 4844.16 | 4883.74 | 2.72E-01 | 1540.69 | 79.31 | 6290.66 | 6342.05 | 2.38E-01 | 367.34 |
| 5 | | | | | | | | | | | |
| 6 | | | | | | | | | | | |
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| 12 | | | | | | | | | | | |
| 13 | | | | | | | | | | | |
| 14 | | | | | | | | | | | |
| 15 | | | | | | | | | | | |
| 16 | | | | | | | | | | | |
| 100 Spe Channel | 368.05 | 35.49 | | 1259.24 | | 419.42 | | | | | 100.00 |
| 10 Spe Channel | 36.81 | 6.31 | | 39.82 | | 41.94 | | | | | 10.00 |
| 1 Spe Channel | 3.68 | | | -82.10 | | 4.19 | | | | | 1.00 |
| Marker Beads | 26046.00 | | | | | 29681.08 | | | | 0.238 | 7076.68 |
| Test Beads | 0.00 | | | | | 0.00 | | | | | 0.00 |
| B-Method Laser Off | 95.54 | 16.00 | 256.00 | | | | | | | | 25.96 |
| B-Method Laser On | 114.02 | 18.00 | 324.00 | | | | | | | | 30.98 |

CLEAR

PASTE

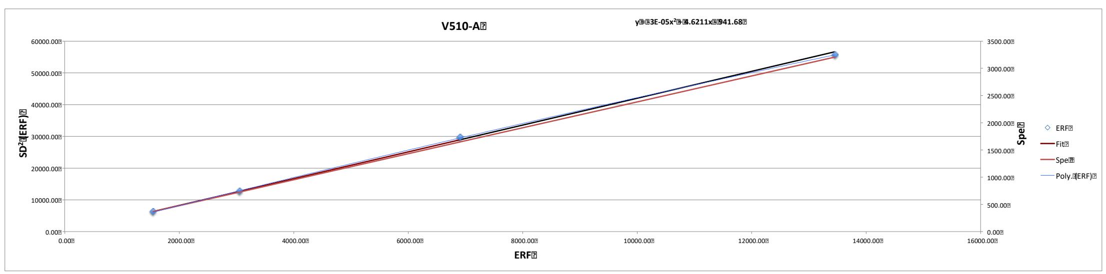
QB GRDLS

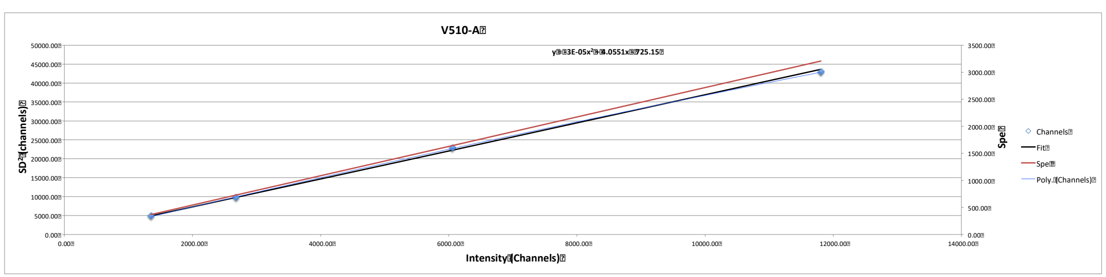
QBFIT+

QUAD_FIT

| | QBFIT+ | QBFIT+ |
|---|---|---|
| Intrinsic CV | 0.13% | 0.13% |
| Spe per Channel | 2.72E-01 | 2.72E-01 |
| Spe per ERF | 2.38E-01 | 2.38E-01 |
| Background Intensity Units | -2.60E+01 | -2.60E+01 |
| Spe | -7.06E+00 | -7.06E+00 |
| Background (ERF) | -2.28E+01 | -2.28E+01 |
| Goodness of Fit | 0.9741 | 0.9741 |
| Correlation | 0.9997 | 1.0000 |
| Q (ERF) | **0.238** | 0.238 |

| | | | |
|---|---|---|---|
| $B_{photonic}$ (Spe) | 5.02E+00 | $B_{electronic}$ (Spe) | -2.60E+01 |
| $B_{photonic}$ (IU) | 1.85E+01 | $B_{electronic}$ (IU) | -9.55E+01 |
| $B_{photonic}$ (ERF) | 2.11E+01 | $B_{electronic}$ (ERF) | -1.09E+02 |



# Baseline Restorer: SD vs CV

- SD is the preferred way to measure area peak width variations
- CV is only acceptable measure at high signal levels
- Electronic and photonic noise dominate lower decades
- In the lower decades the baseline restorer affects the mean measure, invalidates the CV values
- Correction for CV required

# What is B?

- Photonic component
- Electronic noise
- Quantizing noise from ADC
- Base line restorer

# Measuring the Modeled B

Sample Name: V510-A

Date: 08-Oct-2017



Evaluation only

Version 3.00 10/03/2017

| | Channels | | | | | ERFs | | | | | SPEs |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | $SD^2$ | Predicted $SD^2$ (Channels) | Spe/channel | Mean | SD | $SD^2$ | Predicted $SD^2$ (ERFs) | Q Spe/ERF | Fluorescence (F) Spe |
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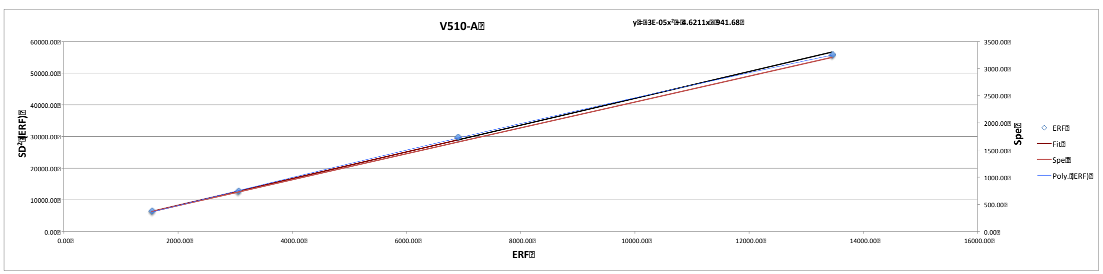
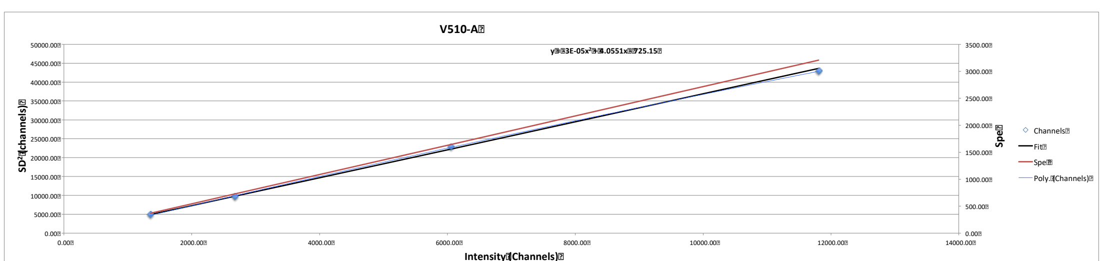
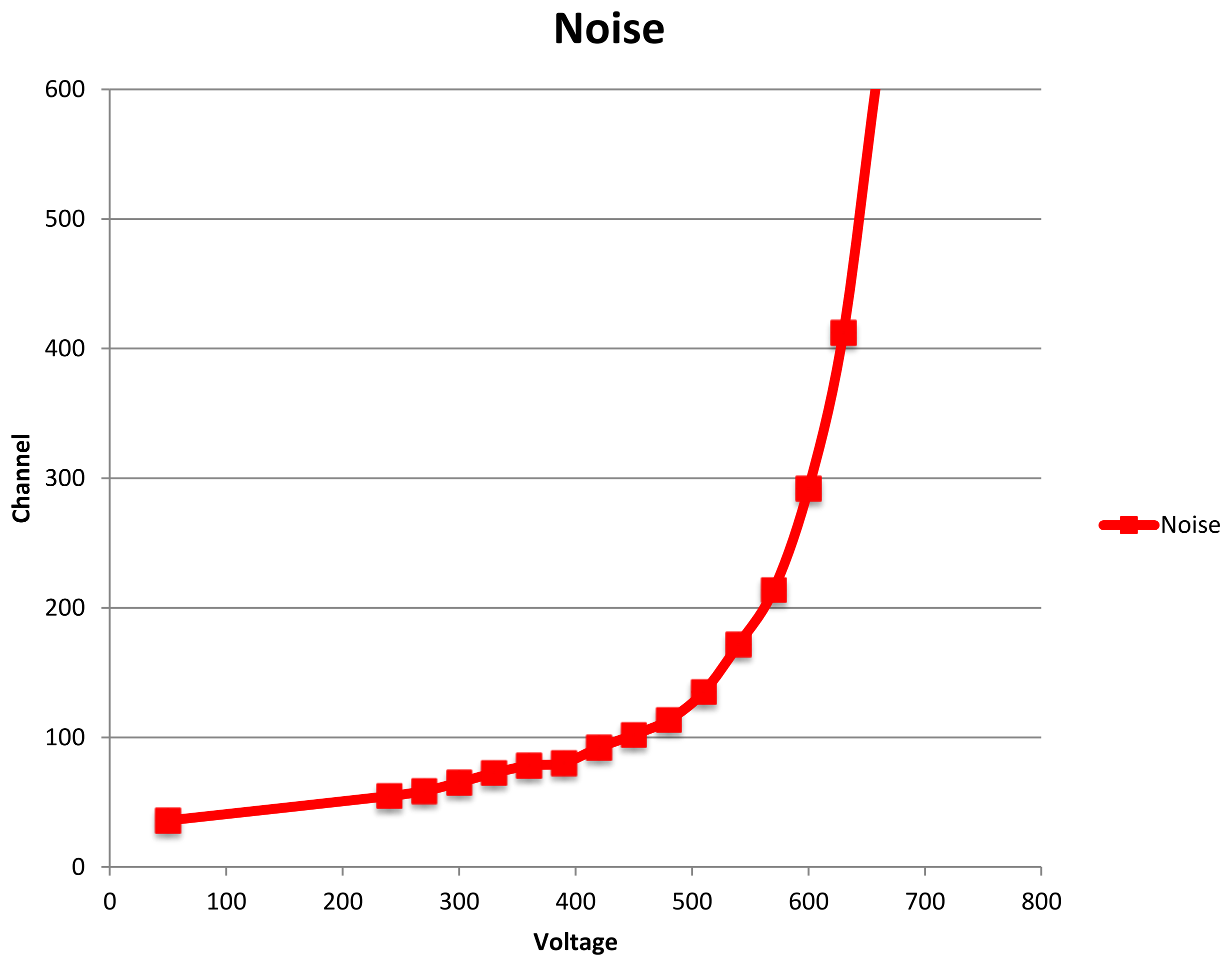


# Measuring Trigger B

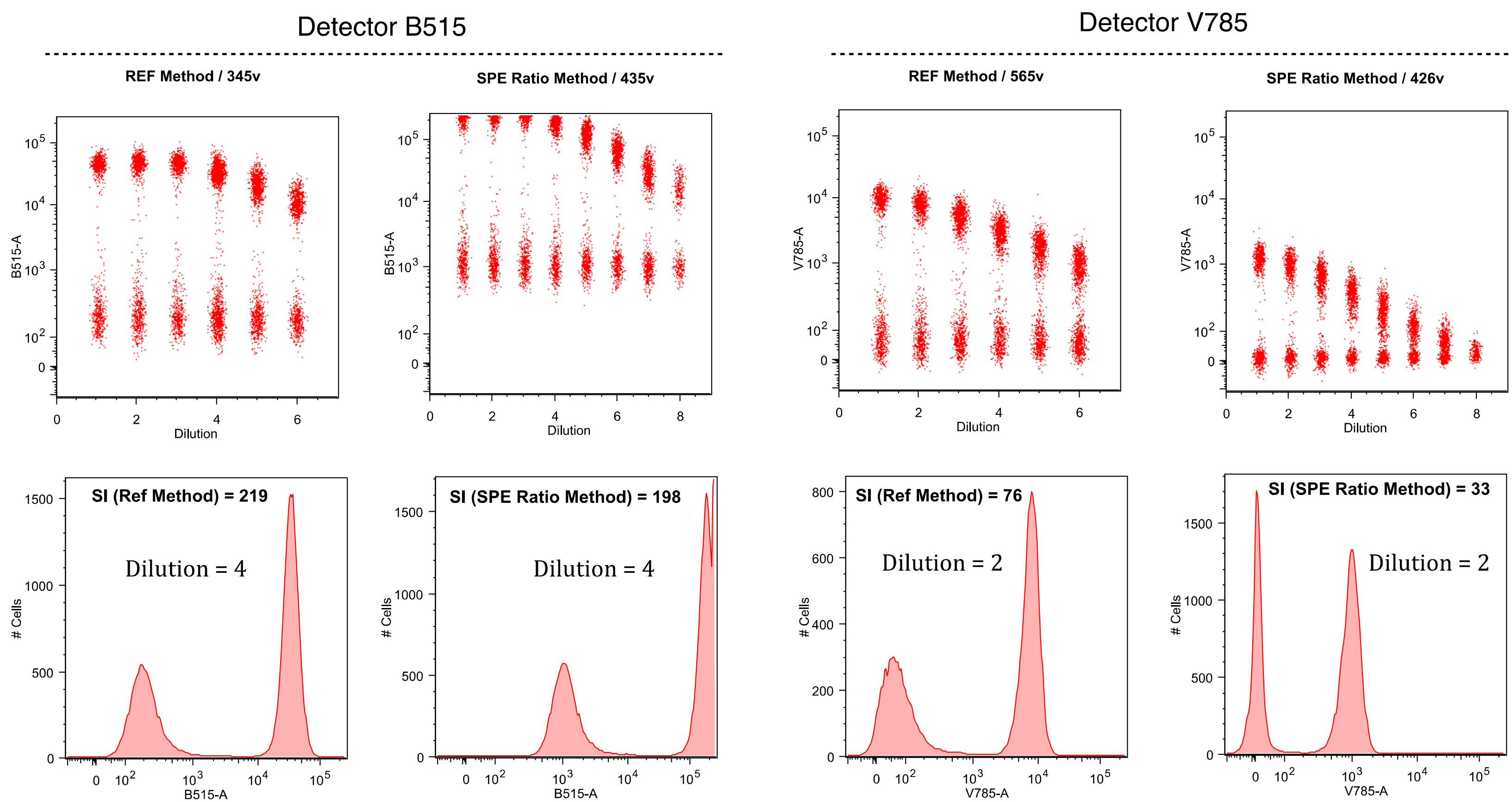
- Use quantiFlash to trigger flow cytometer
  - FSC,SSC or any other unused channel
- Lasers should be on
- No other signals should used
- Measure area signal mean and rSD for all tested channels

# Voltage Ranging to Measure B



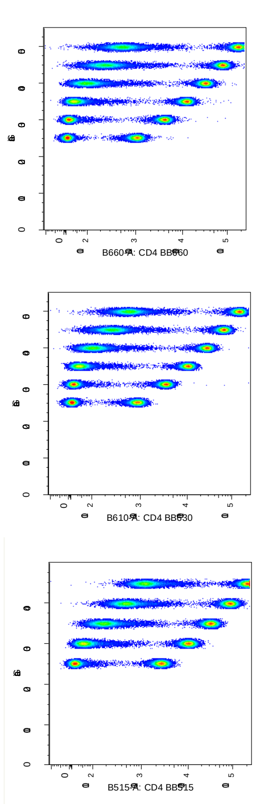

BV421A rSD

# Importance of Voltage Calibration

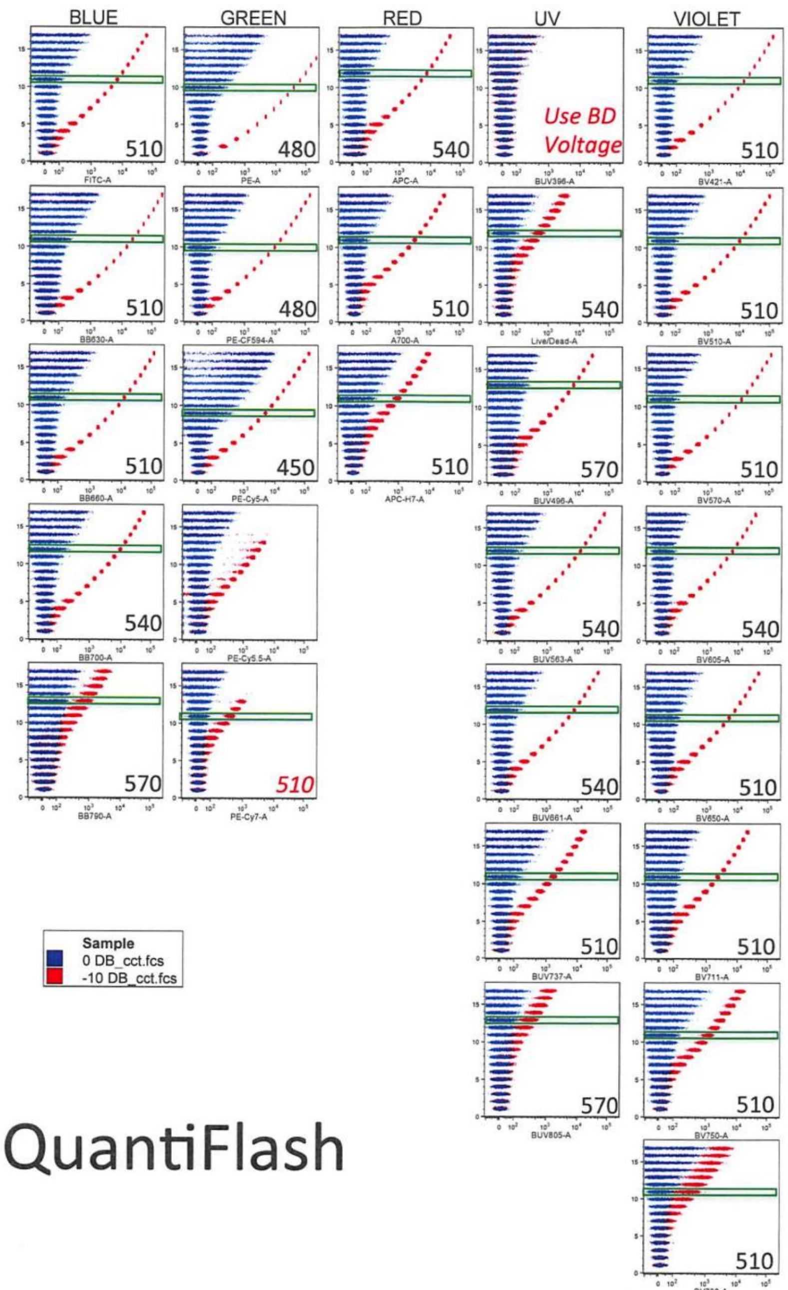



REF: 170721_XF_PBMC and Bead Data.jo

# Optimizing operating Voltages




CD4 Cells

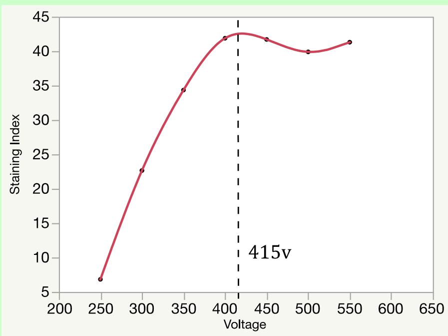



QuantiFlash

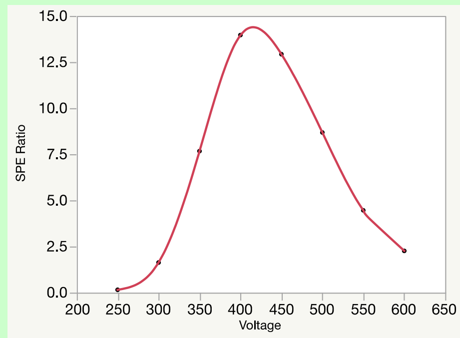
# Overview

## Optimization, Calibration and Monitoring

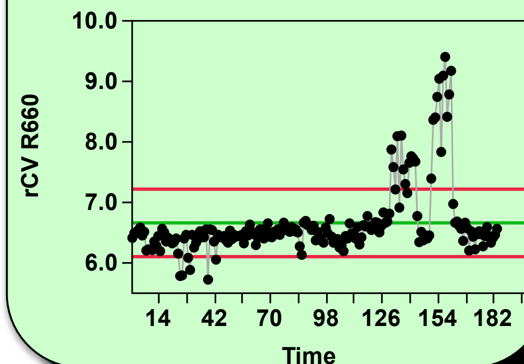
### SI Volt. Ranging




### SPE Volt. Ranging

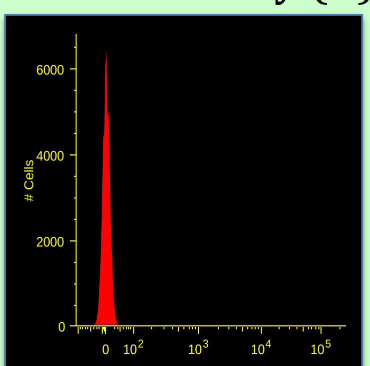



### Monitoring

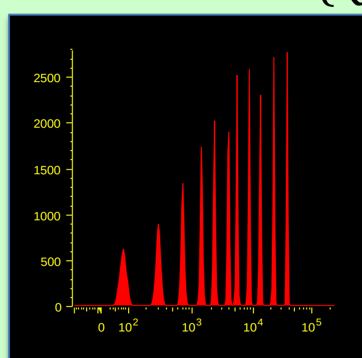



## Standardization

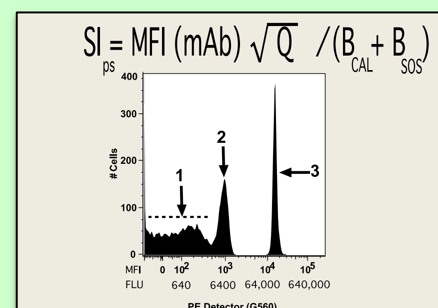
### PMT sensitivity (B)




### PMT resolution (Q)



### Panel Development



# quantiflash Measurements

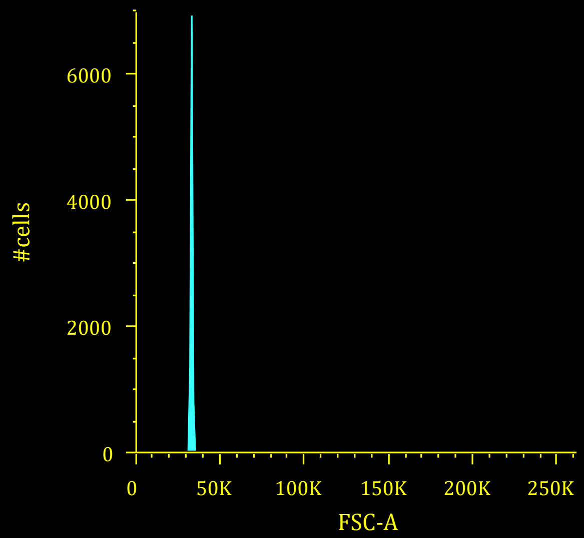
*Trigger B value (tB)* – measured with pulsed LED light into the FSC detector, simulates a particle in the laser target with only background light. Measured with either laser off or laser on.

- Baseline noise in either the laser or to electronics
- Used in the calculation for voltage ranging
- Used in calculations for panel development

*Q value or detector efficiency* - measured with LED light pulsed through the flow cell to all detectors while the trigger is on and can be collected at multiple voltages or a single voltage.

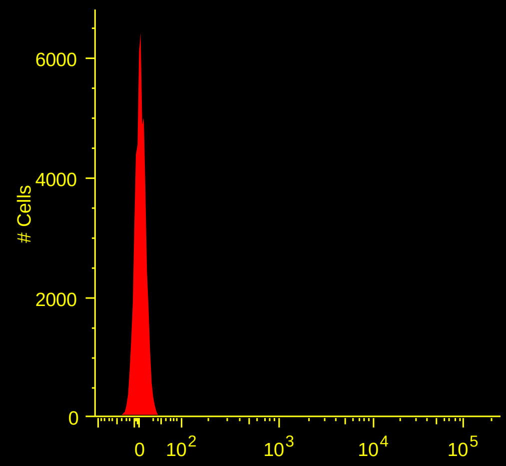
- Used in calculations for voltage ranging
- Used in calculations for panel development
- Standardizes all PMTs on all instruments ("holy grail" of flow cytometry)

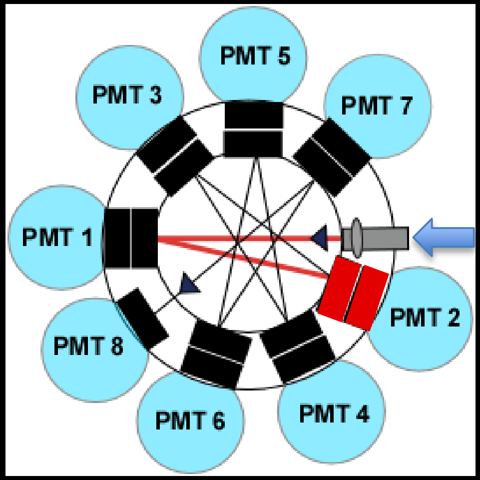
# quantiFlash Used to Measure Background in Detectors (Trigger B)





FSC - trigger

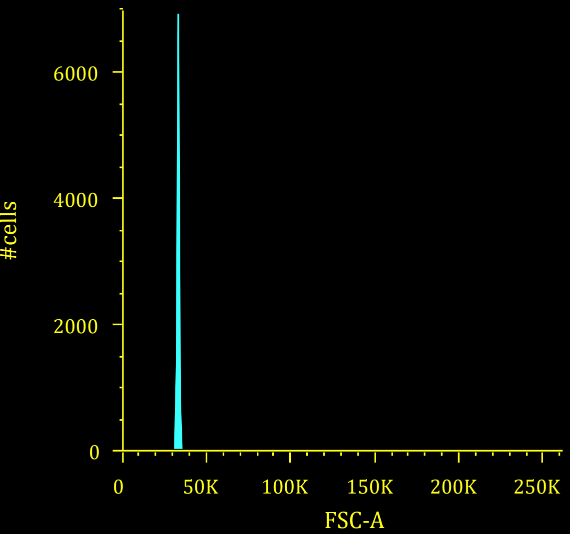


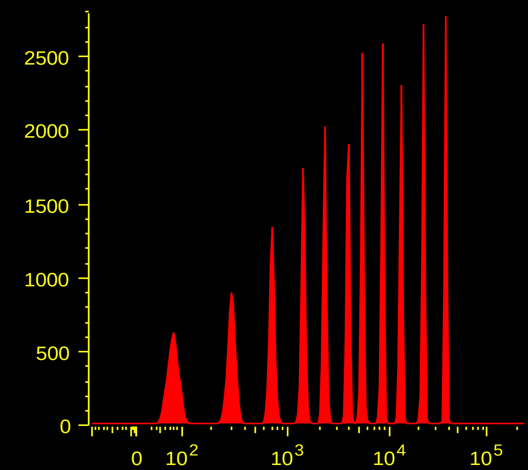

Photons from Lasers

Measurement = rSD

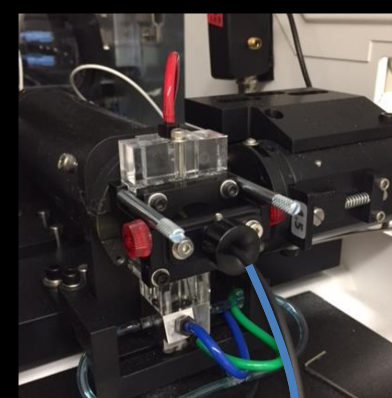
# quantiFlash Used to Measure LED Pulses in the Detector (Q-Mode)







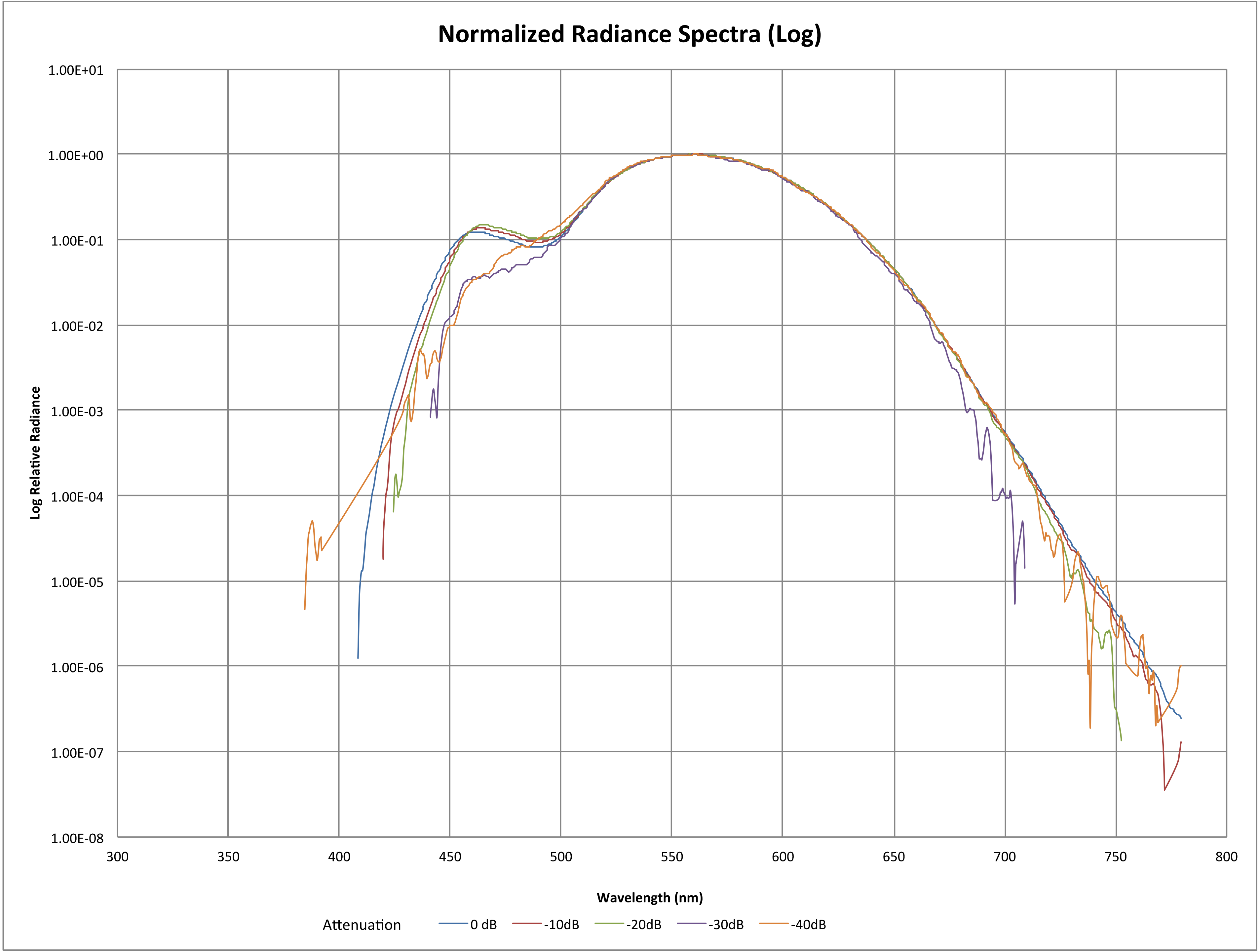

FSC - trigger



LED Pulse

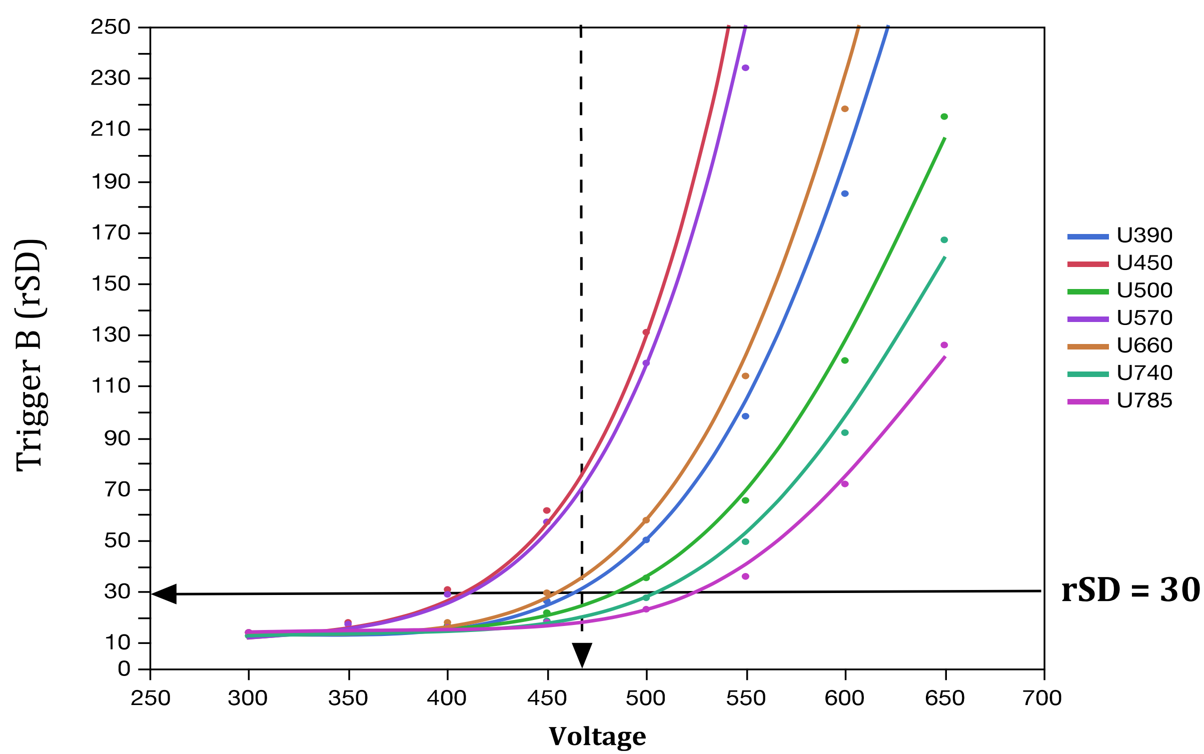
Measurement = MFI, rCV and rSD

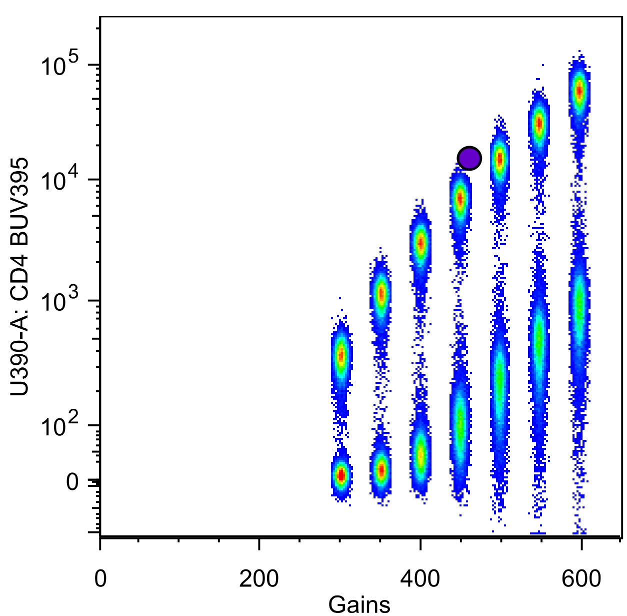
quantiFlash®: A.P.E.

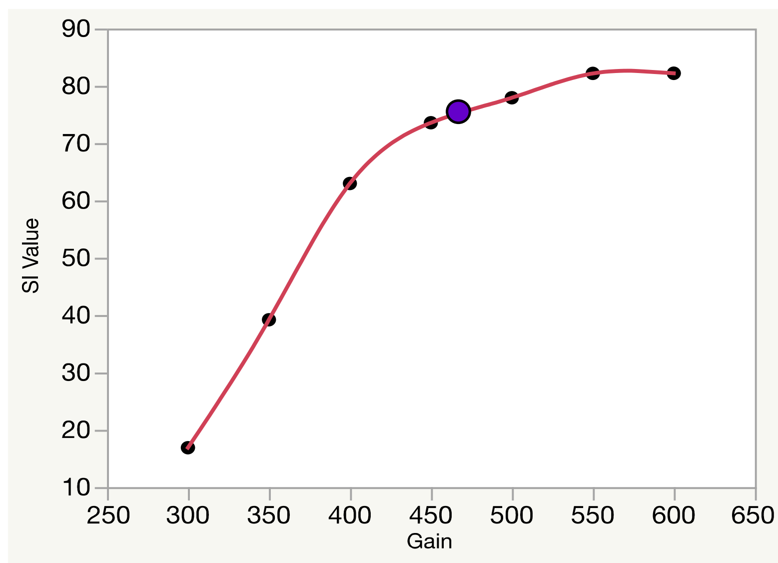


Normalized Radiance Spectra (Log)
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
1.00E-07
1.00E-08
Log Relative Radiance
300
350
400
450
500
550
600
650
700
750
800
Wavelength (nm)
Attenuation
0 dB
-10dB
-20dB
-30dB
-40dB

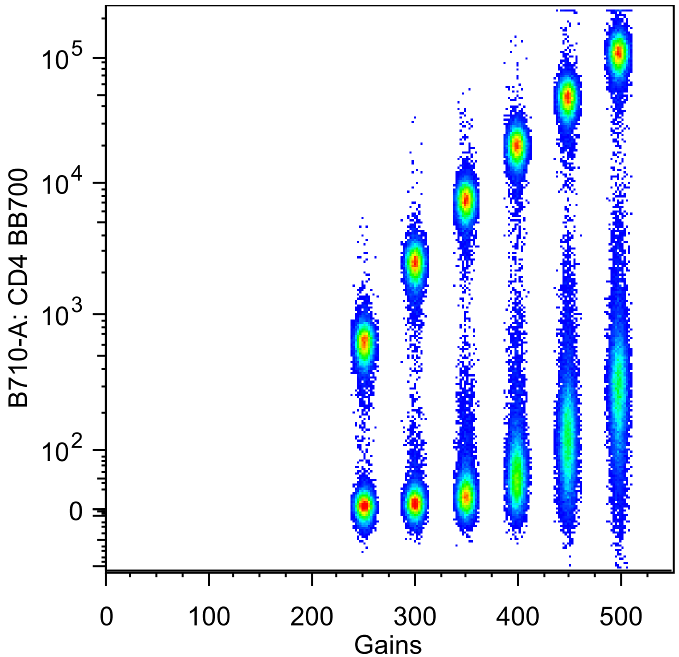
**UV Laser Detectors vs. Trigger B Value (lasers on)**

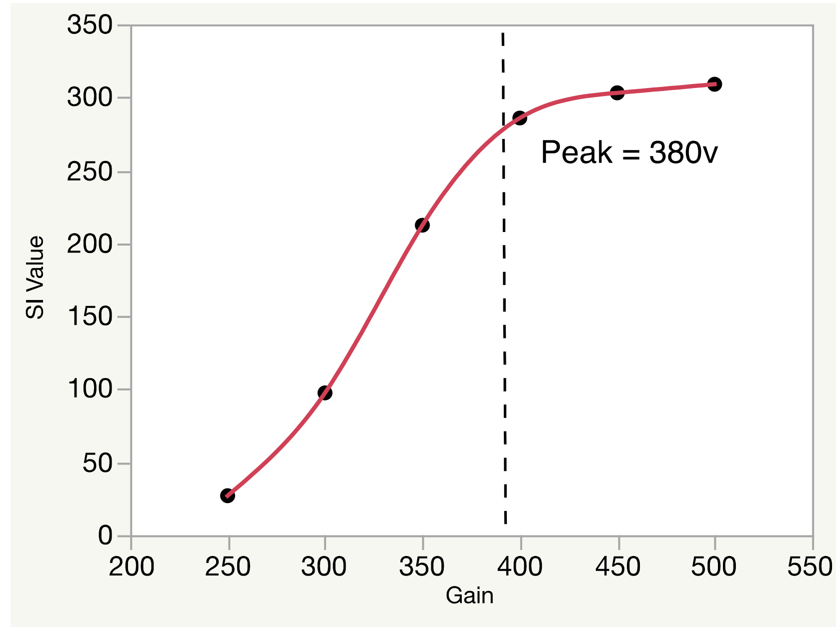


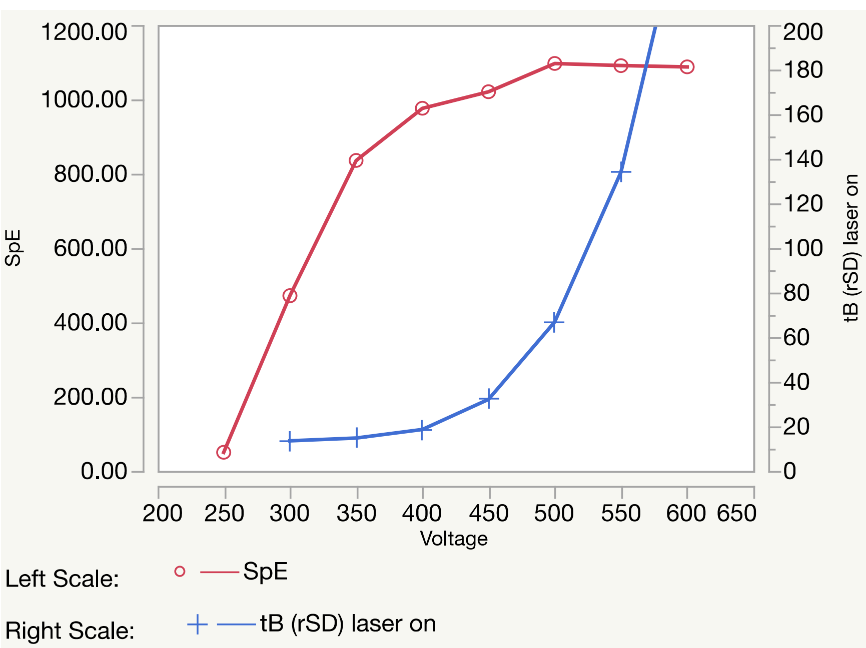



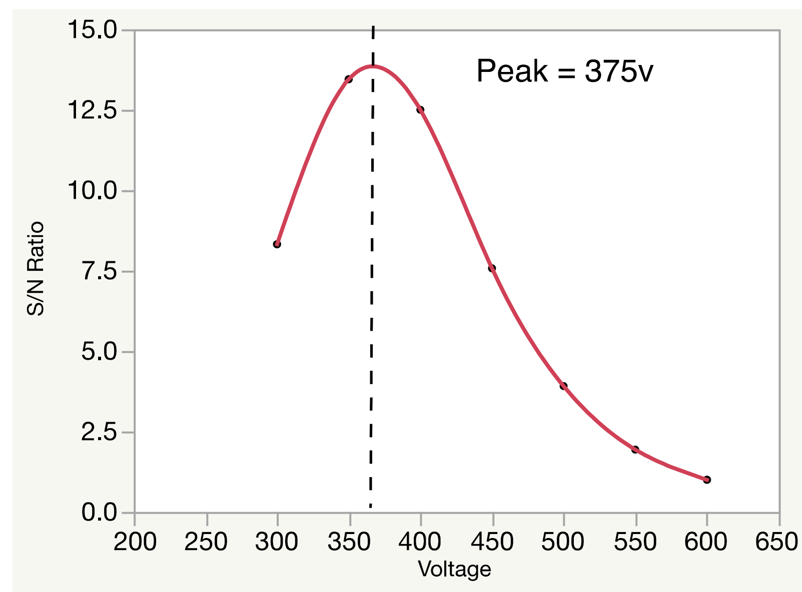
**PBMC Data**



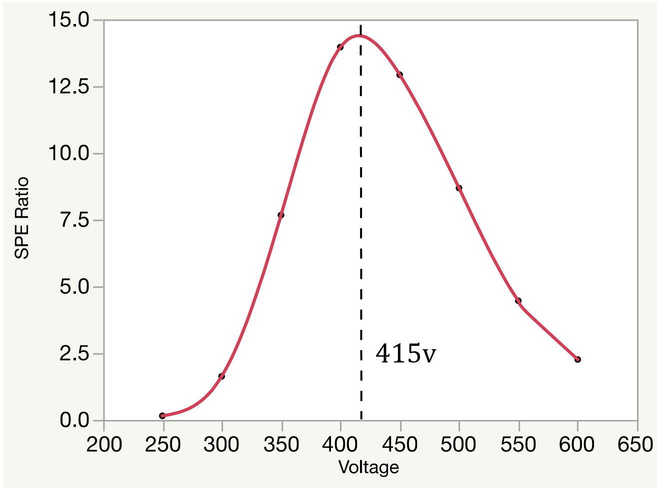

**quantiFlash Data**



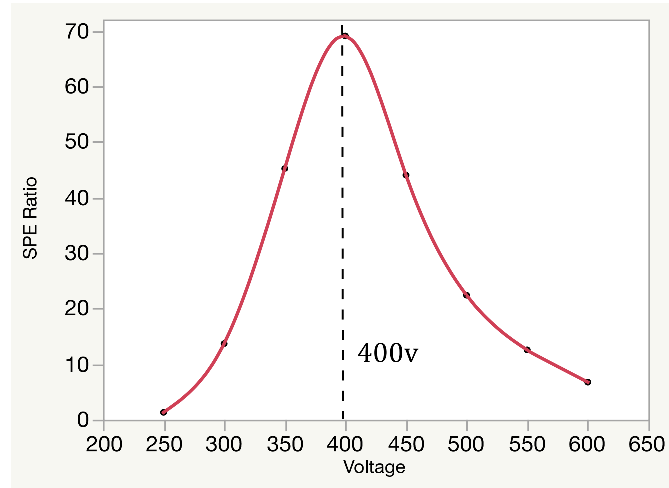



# SpE Ratio vs. SI Voltage Ranging

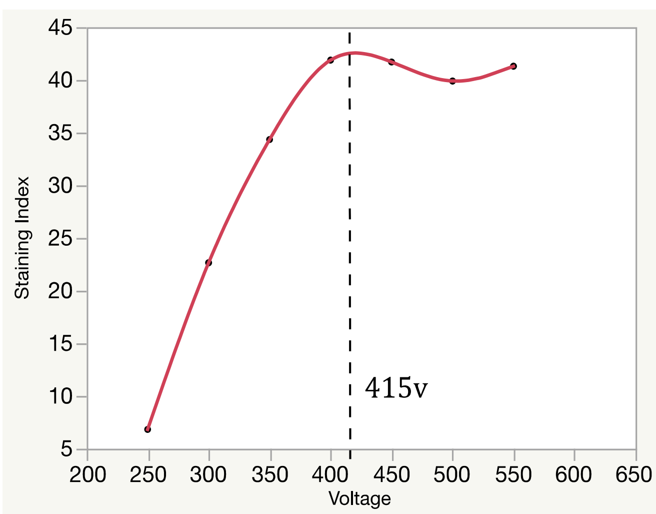
## G575 Detector




## R780 Detector




## G575 Detector

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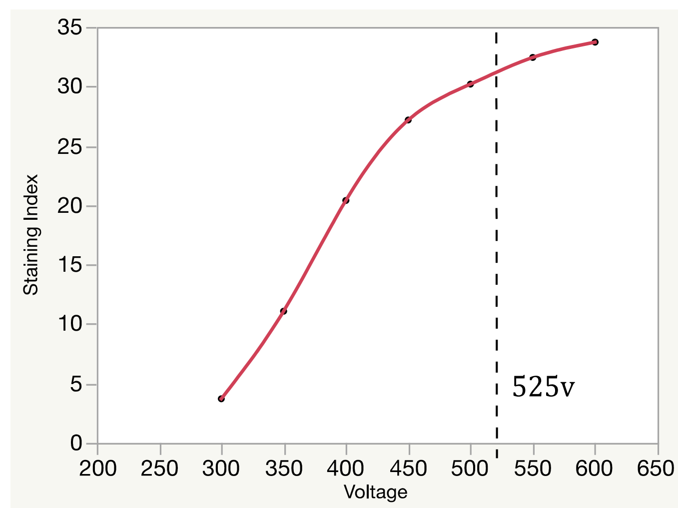


# Data Input Form

| File name | Voltage | tB ON (rSD) | Detector | LED Pulser (MFI) | LED Pulser (rCV) | LED Pulser (rSD) | LED Pulser SPE | SPE Ratio | Calibration Bead Type | Calibration Bead MFI | Calibration Bead FLU | Calibration Bead SPE | Q value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 170830_SPE and Q_Workshop.jo | 250 | 32.9 | G575-A | 76.7 | 45.3 | 34.8 | 4.86 | 0.15 | | • | • | • | • |
| 170830_SPE and Q_Workshop.jo | 300 | 37.8 | G575-A | 316 | 12.7 | 40.3 | 61.48 | 1.63 | | • | • | • | • |
| 170830_SPE and Q_Workshop.jo | 350 | 42.9 | G575-A | 945 | 5.51 | 52.1 | 328.99 | 7.67 | | • | • | • | • |
| 170830_SPE and Q_Workshop.jo | 400 | 53.3 | G575-A | 2638 | 3.67 | 96.7 | 744.21 | 13.96 | | • | • | • | • |
| 170830_SPE and Q_Workshop.jo | 450 | 73.8 | G575-A | 6332 | 3.24 | 205 | 954.06 | 12.93 | | • | • | • | • |
| 170830_SPE and Q_Workshop.jo | 500 | 127 | G575-A | 14144 | 3.02 | 426 | 1102.36 | 8.68 | COMP Besds | 33207 | 13989 | 2588.11 | 0.185 |
| 170830_SPE and Q_Workshop.jo | 550 | 245 | G575-A | 29645 | 3.03 | 897 | 1092.24 | 4.46 | | • | • | • | • |
| 170830_SPE and Q_Workshop.jo | 600 | 445 | G575-A | 58444 | 3.15 | 1843 | 1005.61 | 2.26 | | • | • | • | • |
| 170830_SPE and Q_Workshop.jo | 250 | 18.8 | R780-A | 105 | 20.2 | 21.2 | 24.53 | 1.30 | | • | • | • | • |
| 170830_SPE and Q_Workshop.jo | 300 | 21.7 | R780-A | 405 | 5.81 | 23.5 | 297.01 | 13.69 | | • | • | • | • |
| 170830_SPE and Q_Workshop.jo | 350 | 25.1 | R780-A | 1226 | 2.97 | 36.4 | 1134.43 | 45.20 | | • | • | • | • |
| 170830_SPE and Q_Workshop.jo | 400 | 33.1 | R780-A | 3275 | 2.09 | 68.5 | 2285.82 | 69.06 | FC (Dried Beads) | 10867 | 3387 | 7584.72 | 2.239 |
| 170830_SPE and Q_Workshop.jo | 450 | 57.1 | R780-A | 7770 | 2 | 155 | 2512.92 | 44.01 | | • | • | • | • |
| 170830_SPE and Q_Workshop.jo | 500 | 110 | R780-A | 17121 | 2.01 | 345 | 2462.75 | 22.39 | | • | • | • | • |
| 170830_SPE and Q_Workshop.jo | 550 | 222 | R780-A | 34749 | 1.89 | 658 | 2788.90 | 12.56 | | • | • | • | • |
| 170830_SPE and Q_Workshop.jo | 600 | 414 | R780-A | 66814 | 1.89 | 1264 | 2794.09 | 6.75 | | • | • | • | • |

Photoelectrons (Spe)= $1/n^2$

Where, n= rSD/MFI

e.g. G575 at 500v; rSD = 426 and a MFI =14,144 would yield an SpE = 1,102

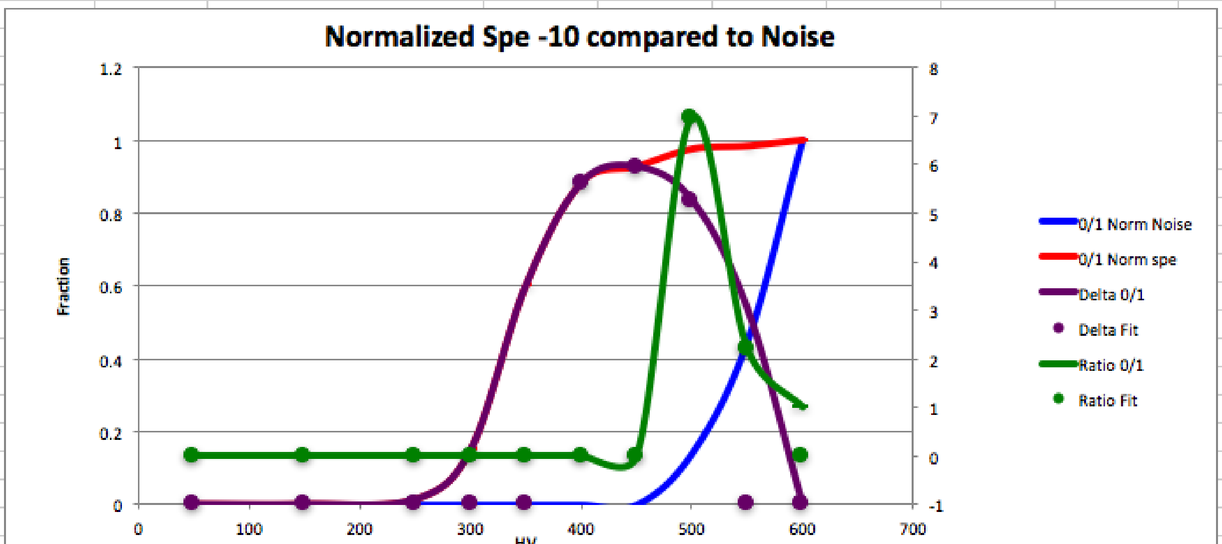
Convert Calibration Bead MFI to SpE = Calibration bead MFI * (LED pulser SpE / LED pulser MFI)

Q value (G575) = Calibration Bead SpE / Calibration Bead FLU

Appendix: I

# Normalize SpE Ratio Curves and Q

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| -2.73946E-05 | 0.02418 | -4.406244 | 0 | | | | | | | |
| HV | 441 | DELTA | FITC-A | | 0.92741382 | 10 | | Spe | 8708.7 | |
| | 8 | $A$8 | $A$10 | $A$8:$A$10 | HV_Values | 50 | | Q | 8.4 | Spe/ERF |
| | 10 | $Z$8 | $Z$10 | $Z$8:$Z$10 | Delta_Values | | | | | |
| -0.002342441 | 2.364477 | -589.67039 | | | | | | | | |
| HV | 505 | RATIO | FITC-A | | 6.95790167 | | | | | |
| | 9 | $A$9 | $A$11 | $A$9:$A$11 | HVR_Values | | | | | |
| | 11 | $AB$9 | $AB$11 | $AB$9:$AB$ | Ratio_Values | | | | | |



# Summary

- Spe$^-$ is independent of PMT voltage
- Q is independent of PMT voltage
- Spe$^-$/channel is <u>dependent</u> on PMT voltage
- Reference measurements to fluorochromes
- Maximize signal/noise for optimized channel
- Noise must be factored into measurement
- Trigger B gives good estimate of noise floor
- Pulsed LED (quantiFlash) for easy interpretation