



Using Fluorescence Spillover to Advantage

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Compensation: Why and When is it Necessary?

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Summary

- C6 design and manufacturing process is unique
 - Scale for fluorescence measurement is standardized (calibrated)
 - Alignment of lasers, flow cell and optics performed during final phase of manufacture
 - Voltage on each detector is set to deliver specific bead performance requirements
- Results in predictable fluorescence spillover
- Useful tool



Pre-optimized fluorescence detection and locked-down optic table

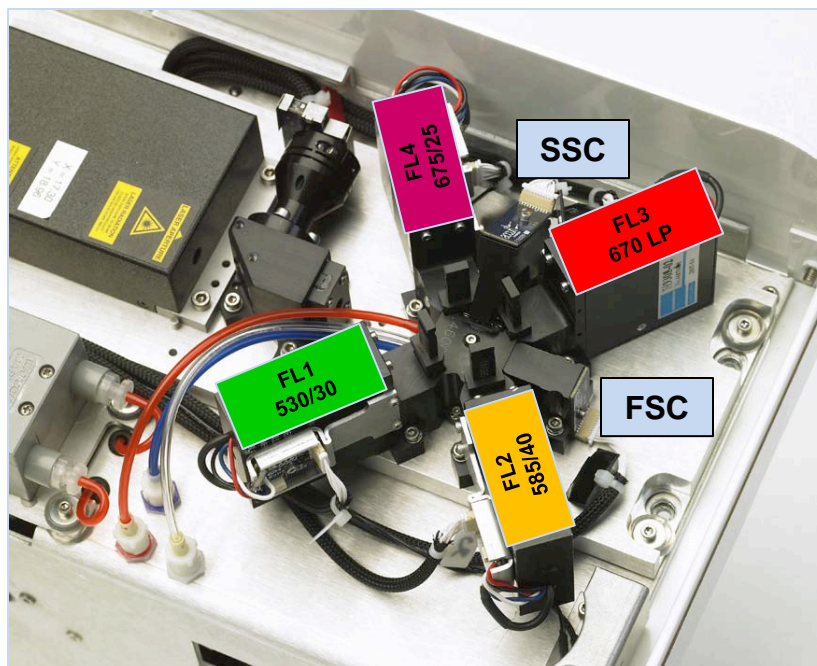
Compact optical system design reduces cost and eliminates alignment issues

488 nm solid state laser

640 nm diode laser

PMTs for fluorescence Detection

Diodes for scatter detection



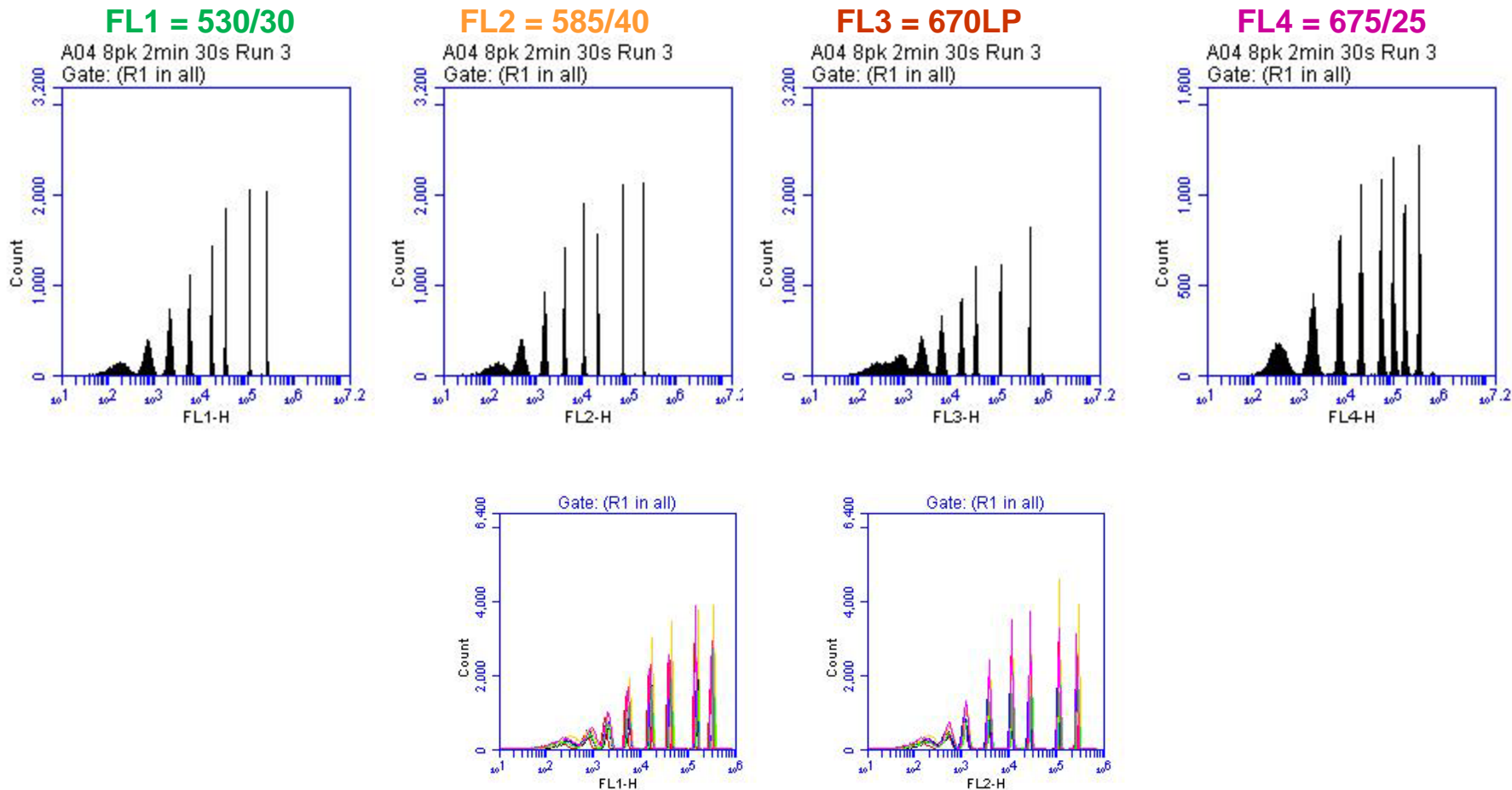
User changeable optical filters

510/15
540/20
565/20
610/20
780/60

Selectable Lasers

3 blue 1 red
2 blue 2 red
4 blue

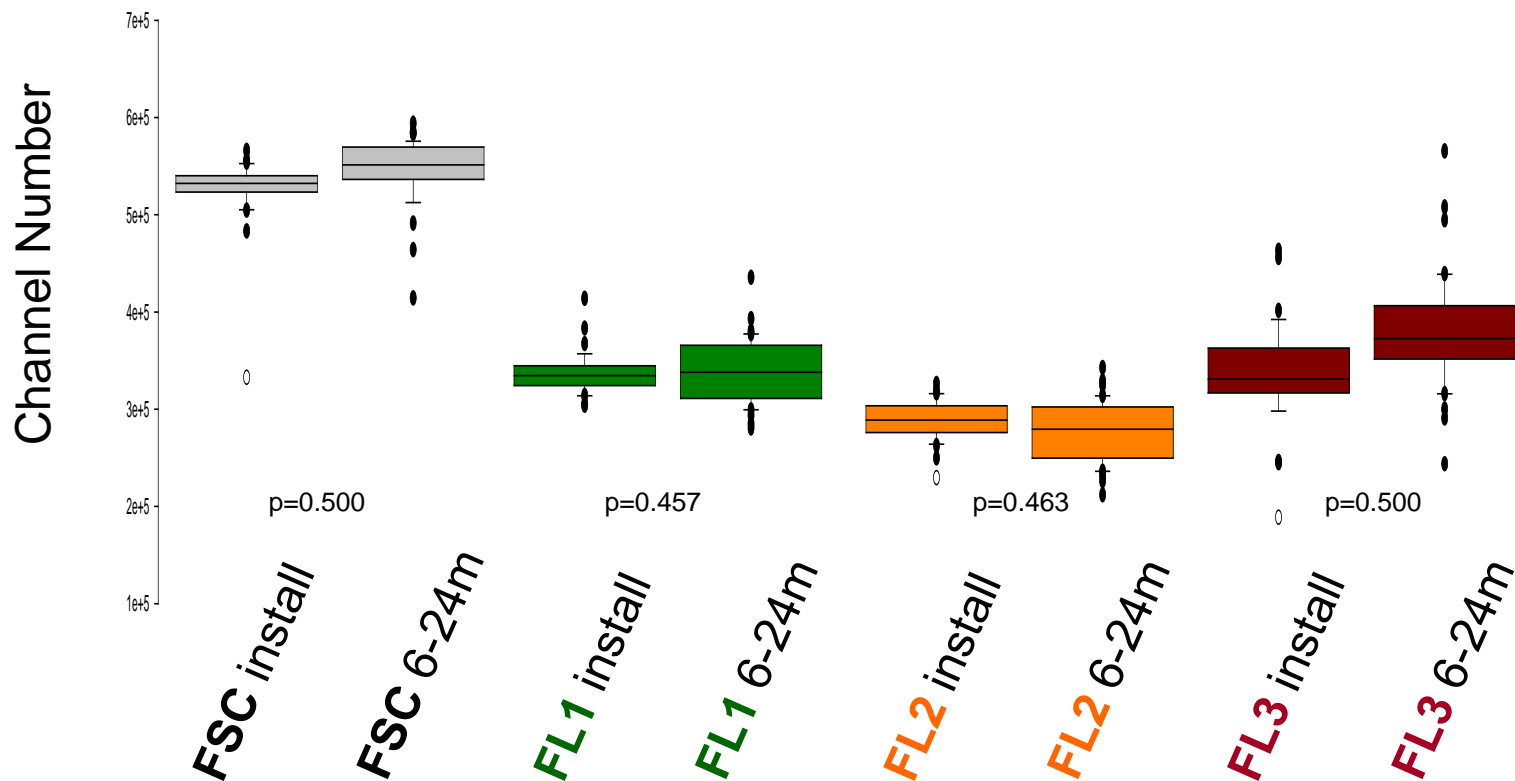
Detector voltage and optic alignment are set during manufacture to bead performance specifications



8-peak data from multiple C6 instruments manufactured over a six-month period.



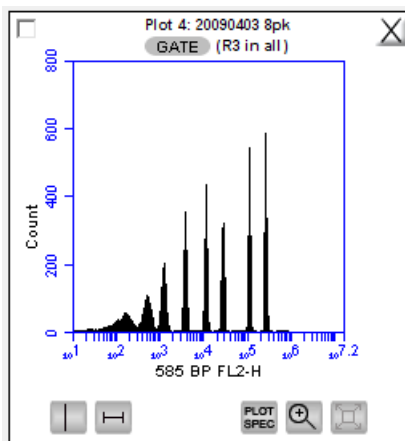
C6 instruments hold performance over time



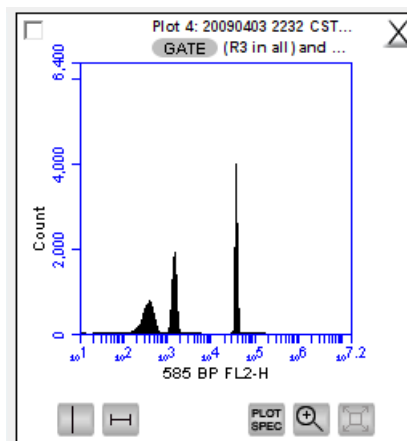
Top peak mean values (Spherotech 8-peak calibration beads) from 32 C6 instruments were compared from install to 6-24months. A 1-paired student's t-test was performed and showed no statistically significant difference in top peak mean values over time.

All commonly used beads are on scale

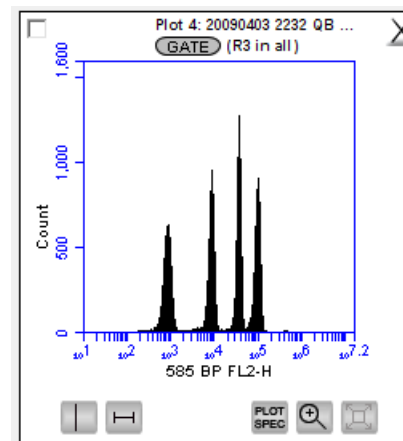
8-Peak Validation Beads



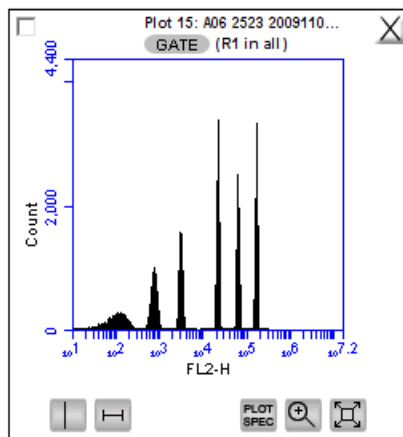
BD™ CS&T



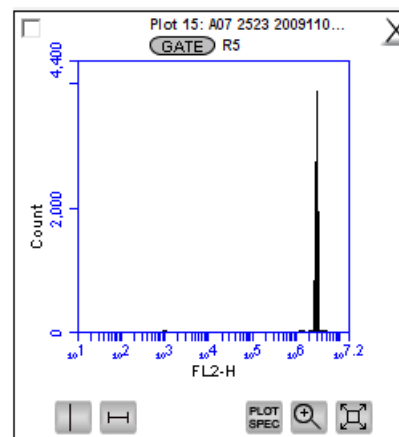
BD QuantiBrite™ PE



Thermo Cyto-Cal™



BEC Flow-Check™

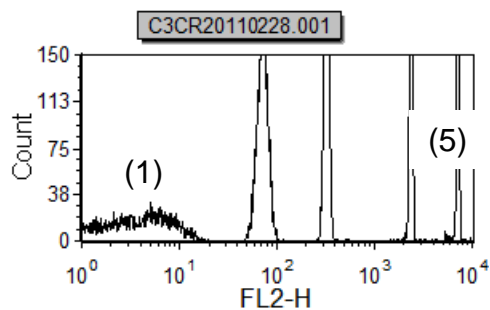


Broad dynamic range: 24 bit DSP

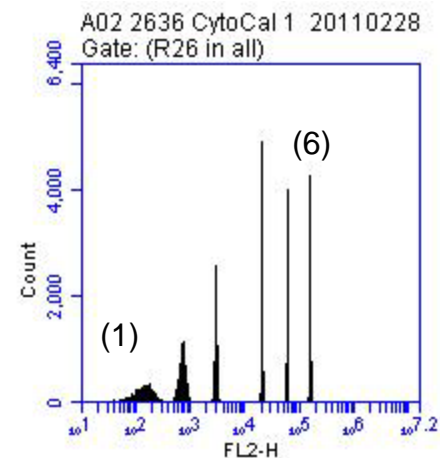
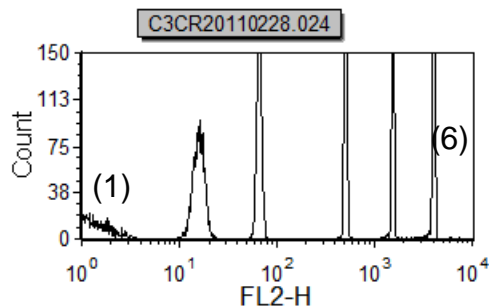
10 bit, 1024 channel system

24 bit, 1.6×10^7 channel system

v = A



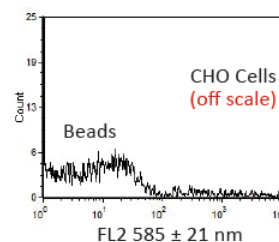
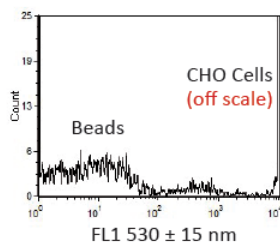
v = B



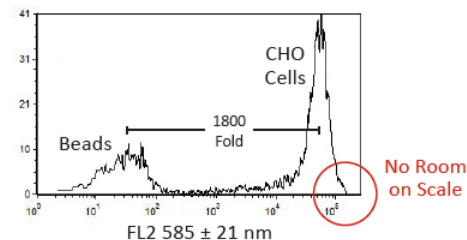
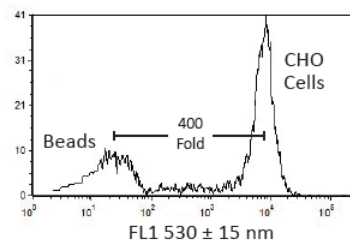
Is the range *really* broader?

Dynamic Range Comparison of 3 Cytometers with Different Signal Processing (DSP) Resolution

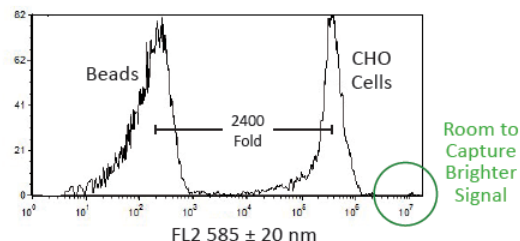
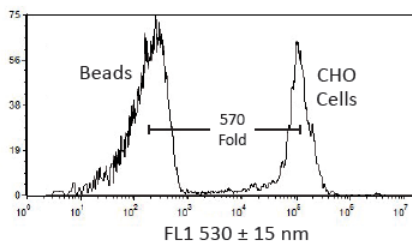
10-bit
1024 channels
(Adjustable Voltage)



18-bit
2.62 x10⁵ Channels
(Adjustable Voltage)

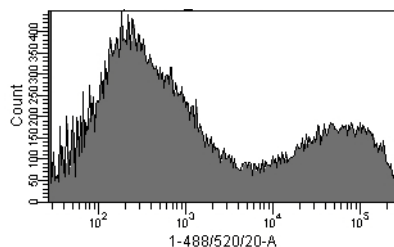


24-bit
16.7 x10⁶ Channels
(Fixed Voltage)

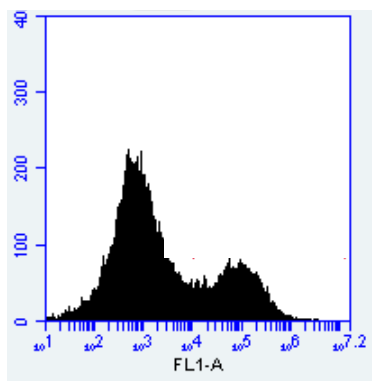


Q: Do we really need such a broad range?
 A: You do if you hold voltage constant.

18-Bit System



24-Bit System



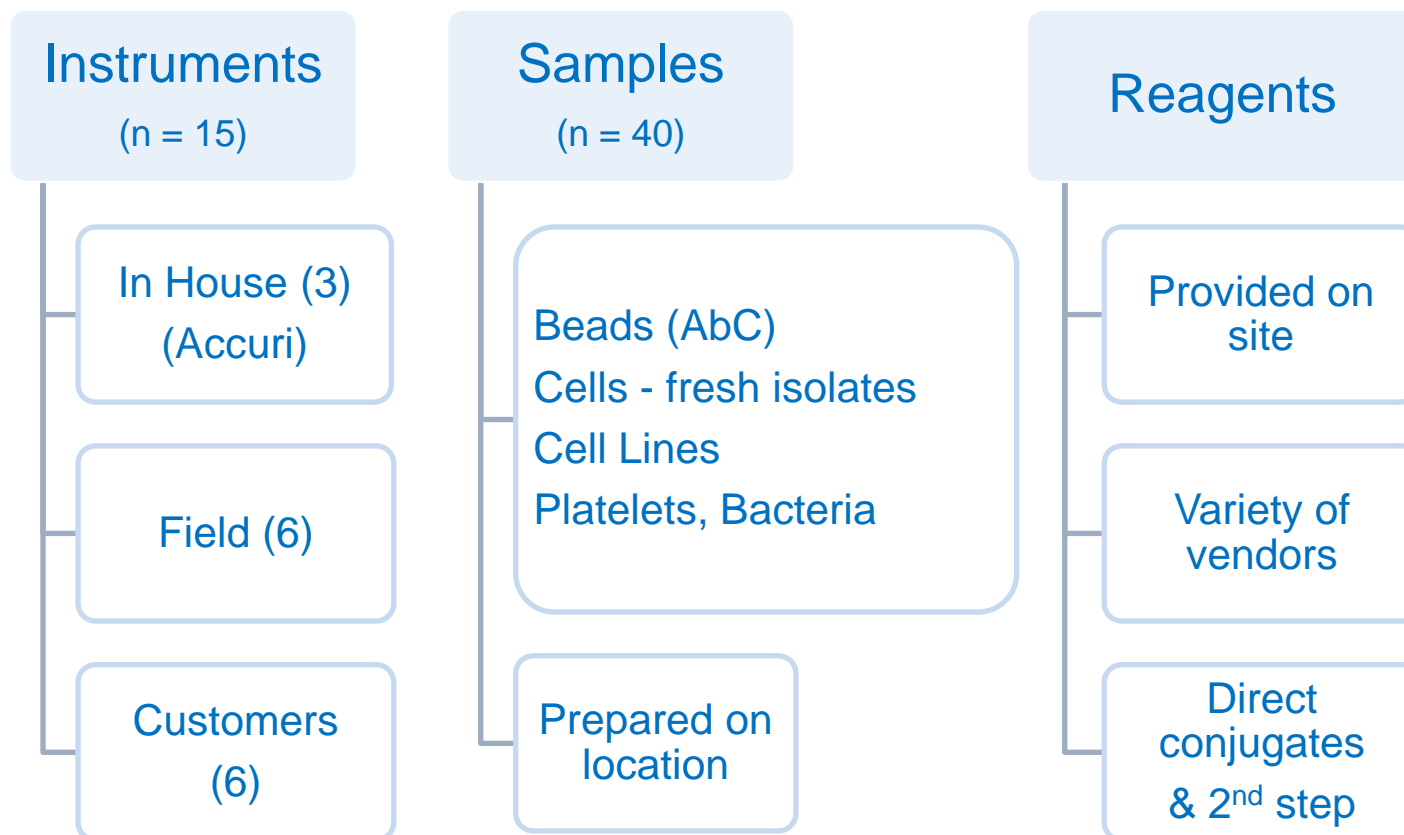
“A GFP signal that was off scale on our other flow cytometer gave us a distribution that was contained within the 24-bit scale on the C6.”

*Ian Dimmick, Flow Cytometry Core Facility Manager,
 Institute of Human Genetics
 Newcastle University, UK*



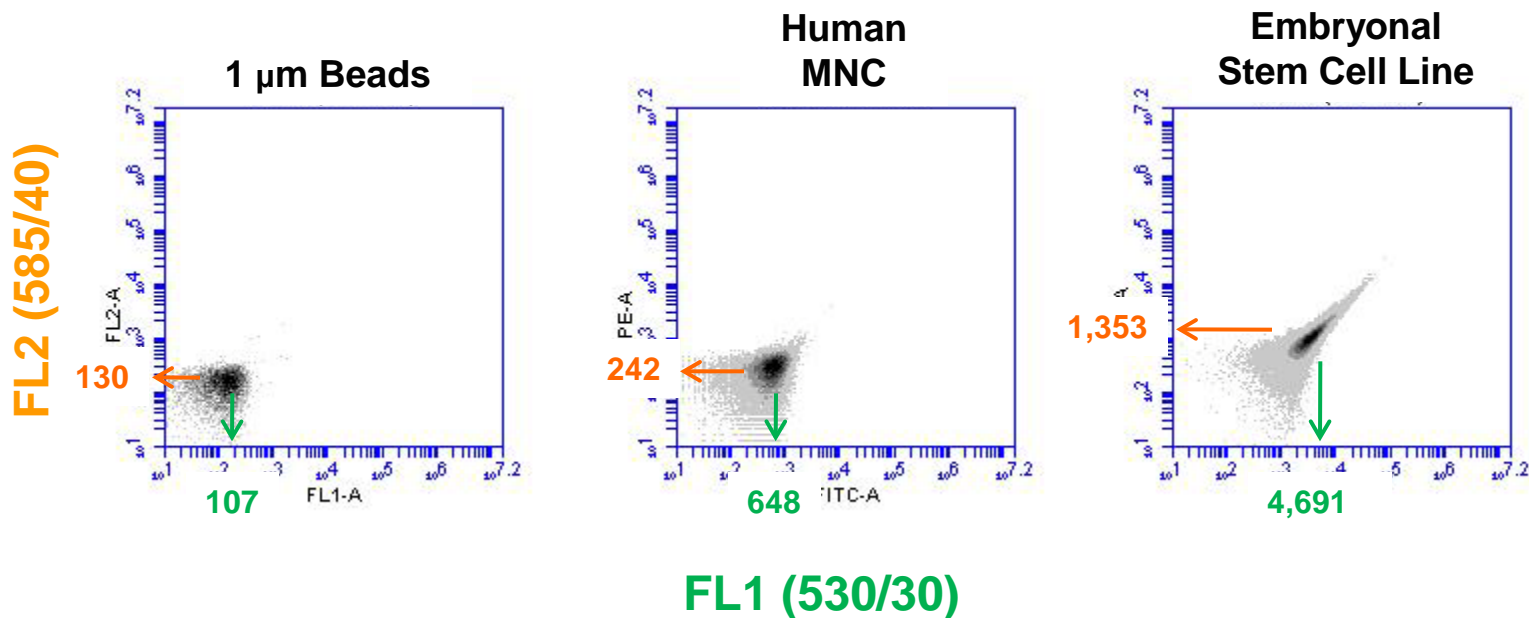
Standardized fluorescence measurement should result in predictable spillover

Experiment: 40 sets of FITC/PE samples containing unstained and single stained positive controls

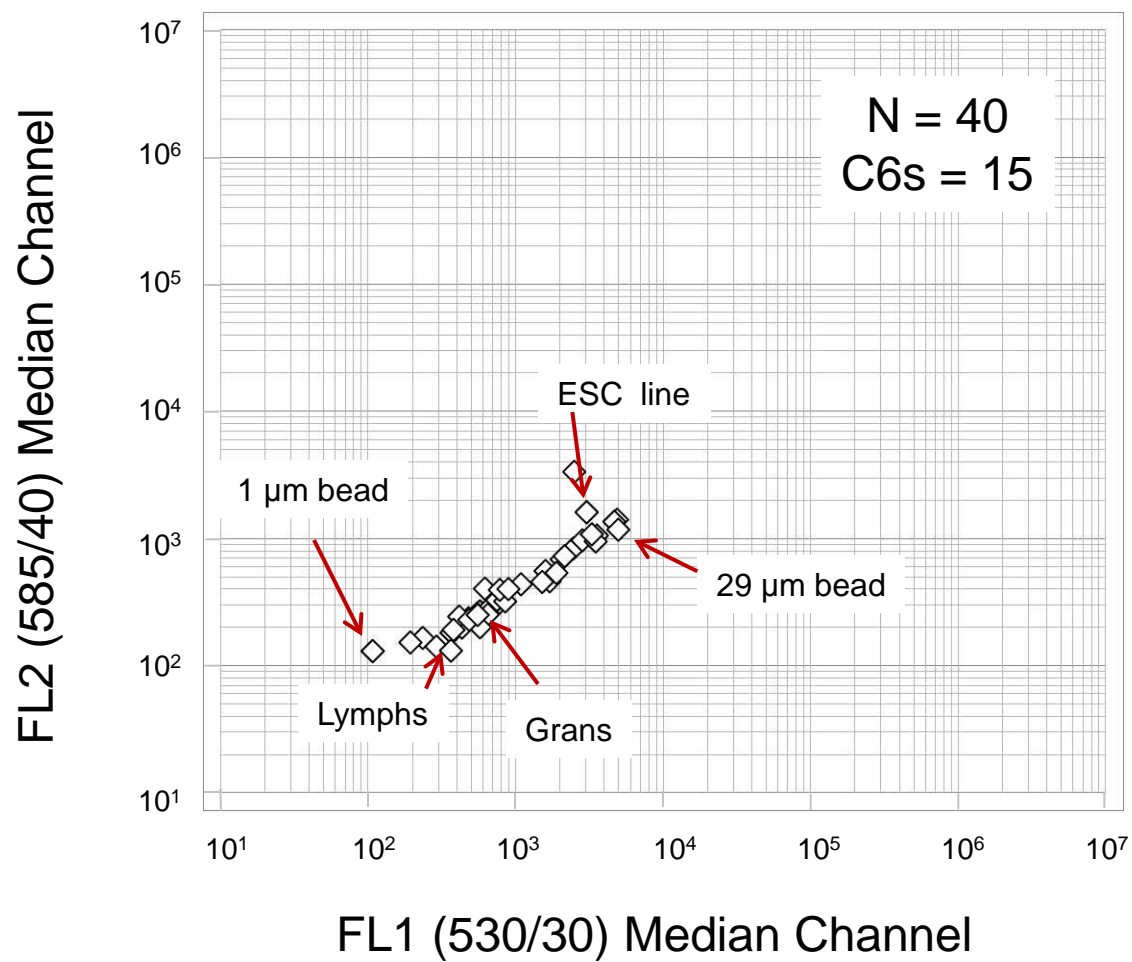


Reducing the Data

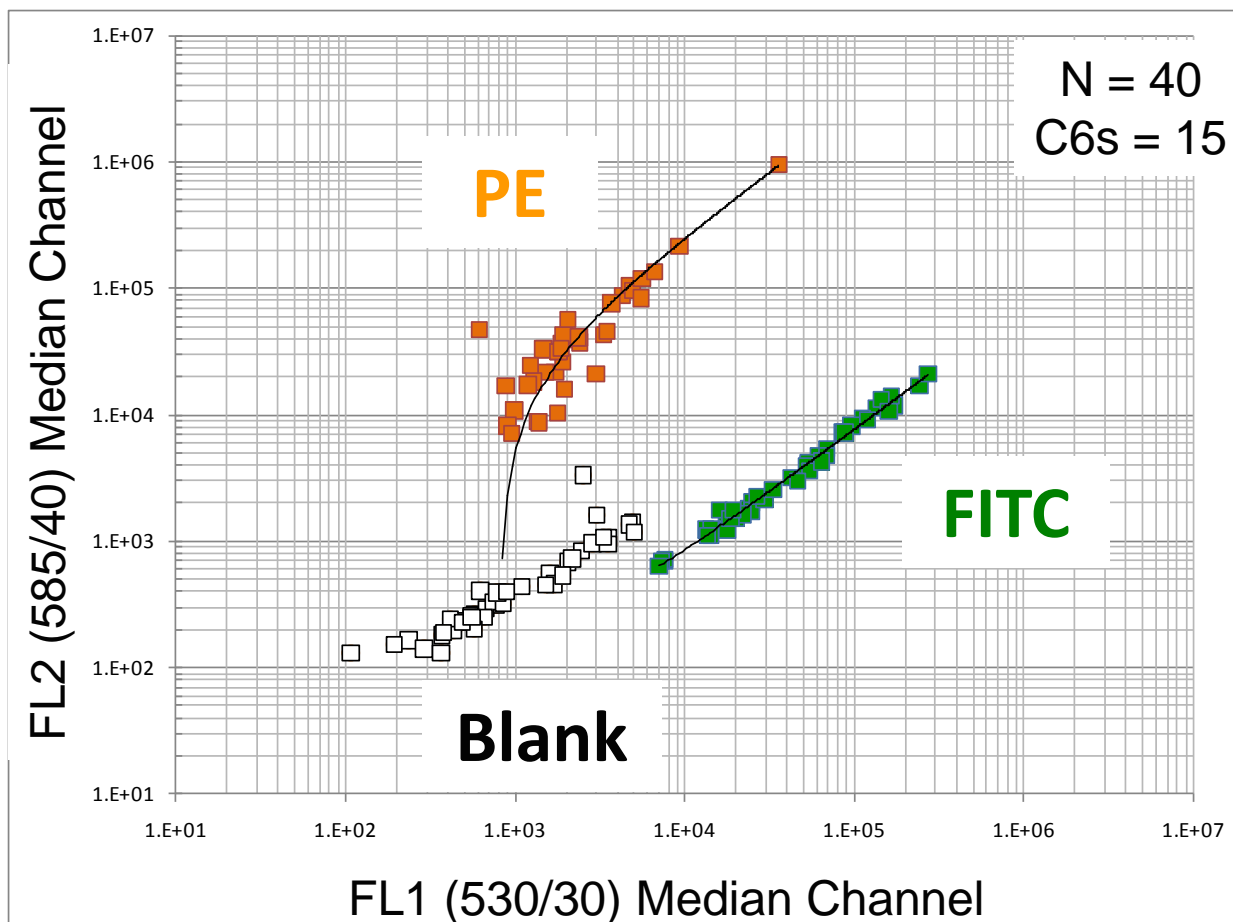
Determine X and Y median co-ordinates for unstained and single stained populations to collapse data from 120 FCS files to a single data set



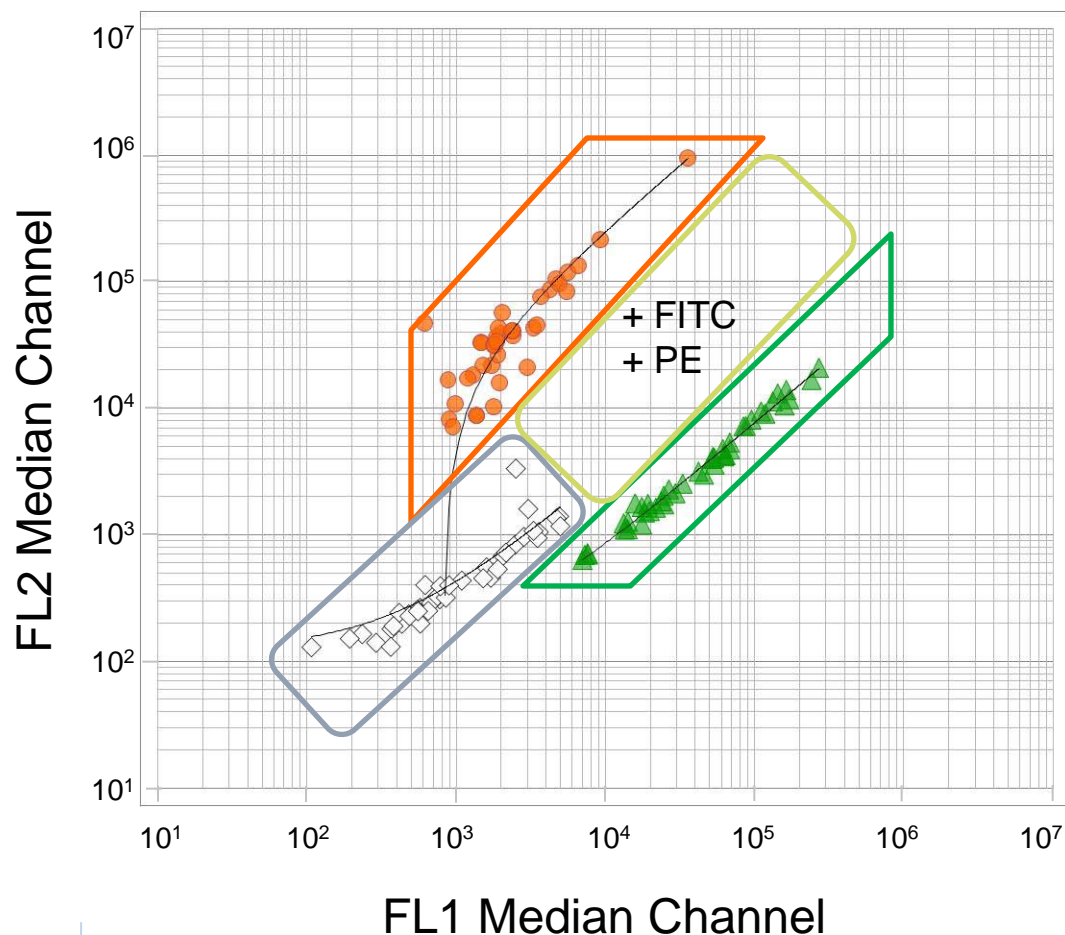
Master Plot: Unstained Samples



Master Plot Including FITC and PE



FITC and PE Regions are Predictable



Mathematical Descriptions

FITC Only:

$$y = 0.0757x + 103 \quad (r^2 = 0.98)$$

PE Only:

$$y = 0.0367x + 892 \quad (r^2 = 0.98)$$

Unstained:

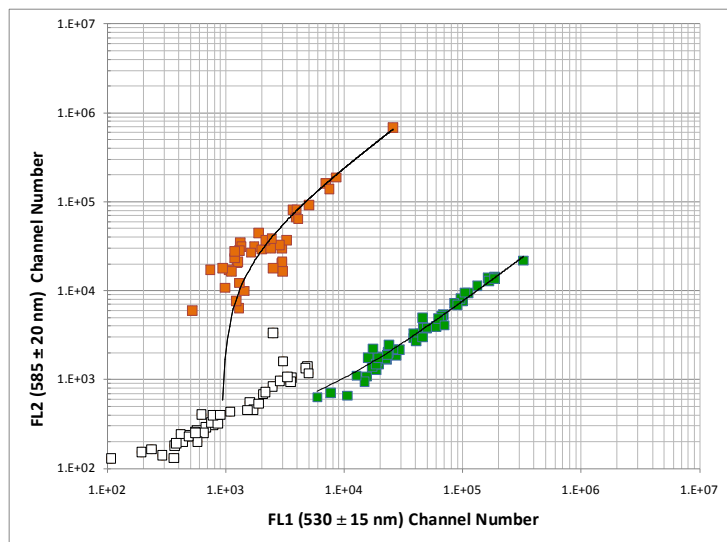
$$y = 0.3057x + 124 \quad (r^2 = 0.51)$$

(Slope of Line = Spillover)

By adding in variance or spread information, regions can be assigned for given fluorochromes.

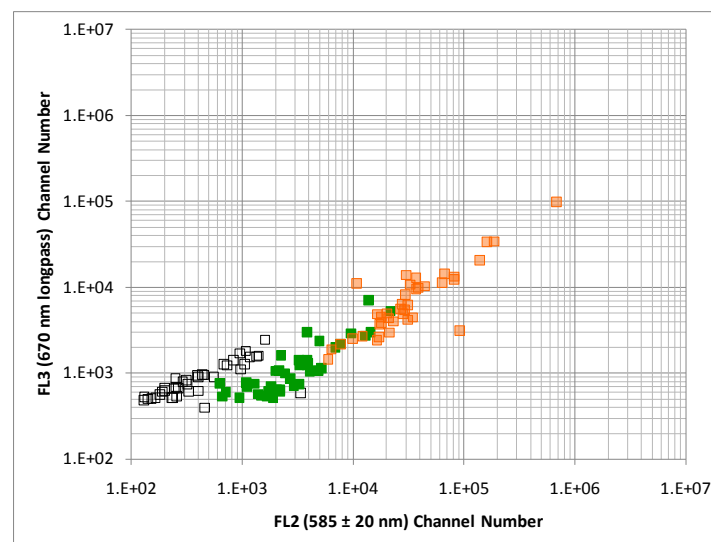
Following the spillover

FL2: 585/40



FL1: 530/30

FL3: 670 LP



FL2: 585/40

How does predictable spillover help you?

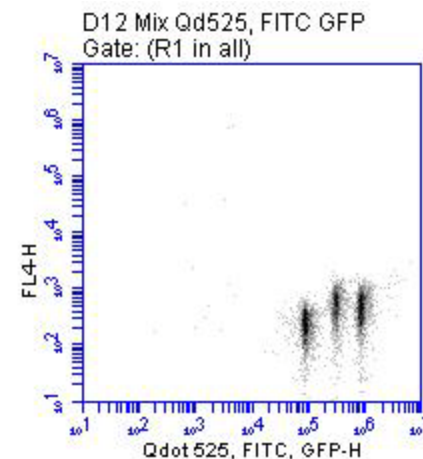
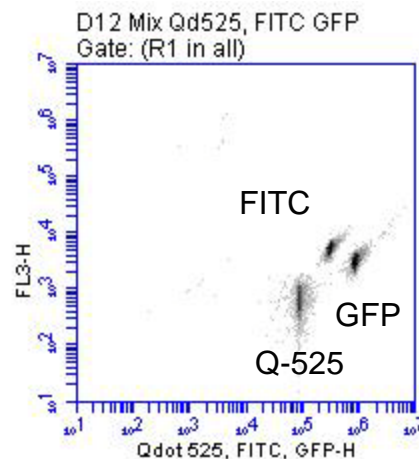
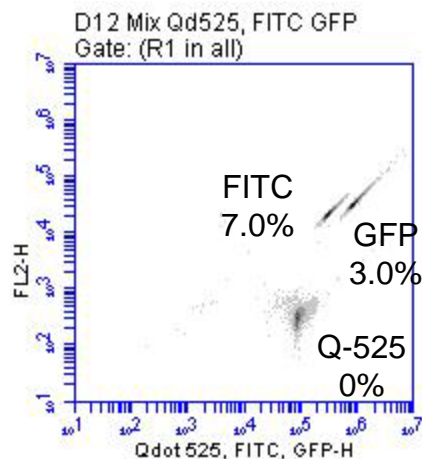
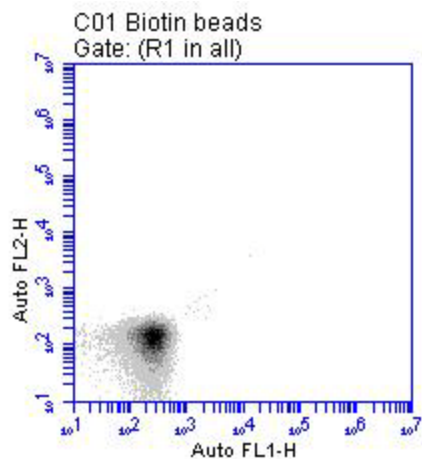
- Predictable compensation values for certain fluors

Suggested Compensation Values for the Accuri C6						
	FITC	PE	PerCP	PerCPCy5.5	PE-Cy7	APC
FL1 (530BP)	---	3.2	0.00	0.0	0.50	0.0
FL2 (585 BP)	7.5	---	0.00	0.00	1.50	0.0
FL3 (670 LP)	1.0	19.5	---	---	---	0.8
FL4 (675 BP)	0.0	0.0	3.00	12.00	0.00	---

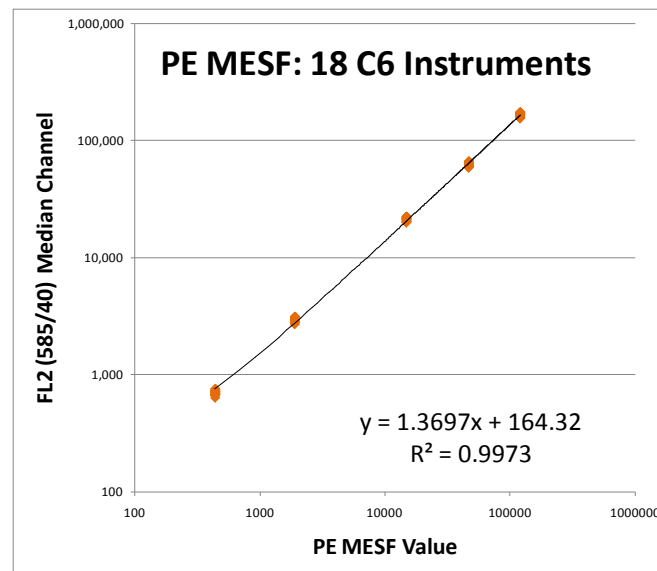
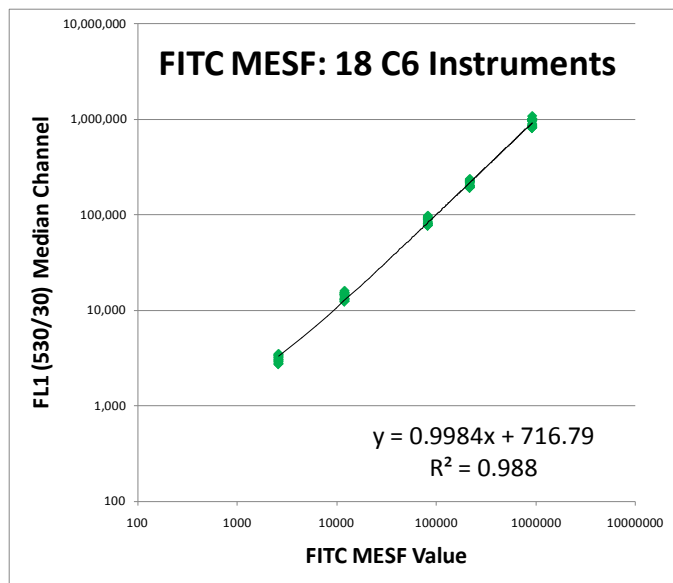
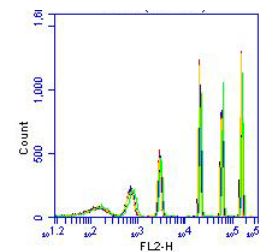
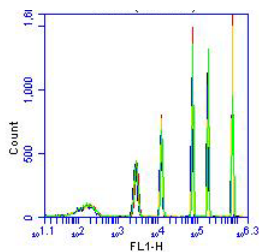


How does predictable spillover help you?

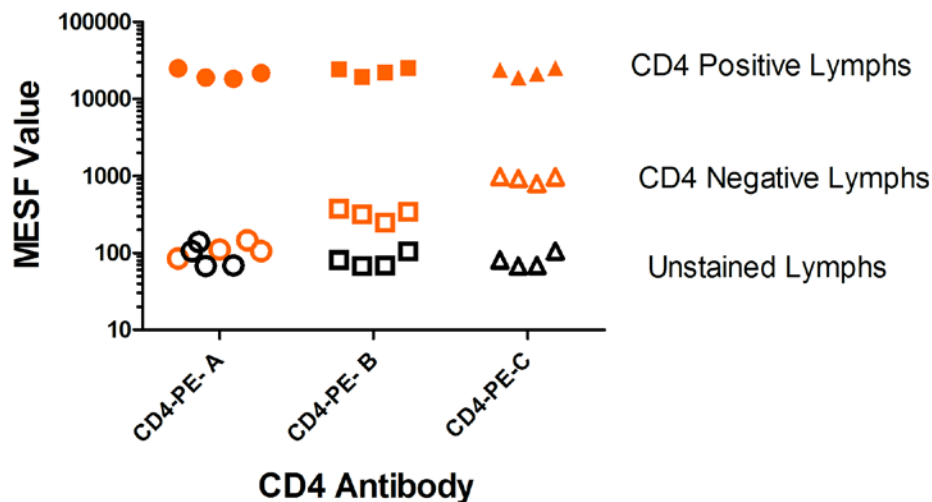
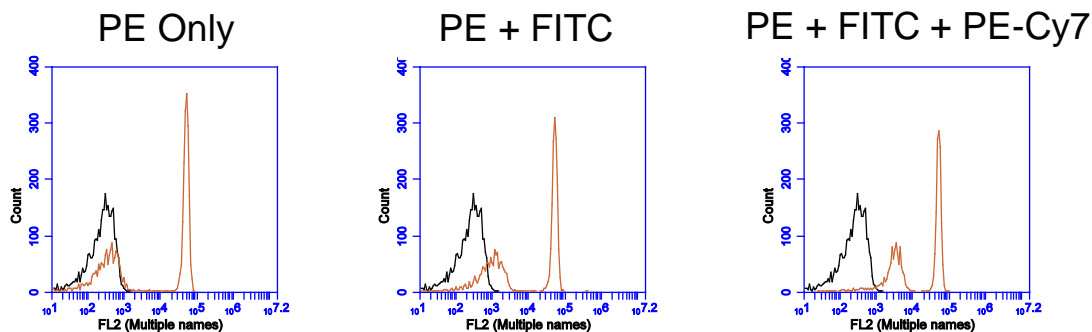
- Spillover is fluor specific
- Aides in identifying staining errors



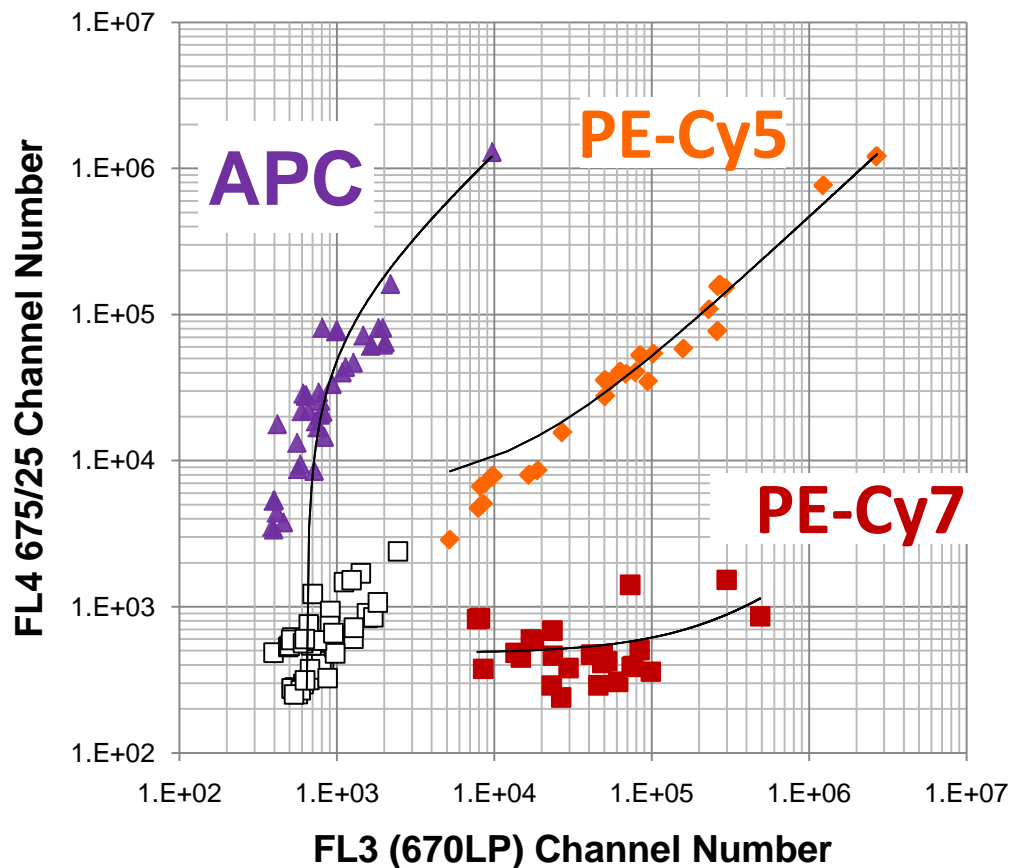
Simplifies fluorescence quantification



Aides in multi-color experimental planning



Consider using spillover to get more out of available 2- and 3-D analytic “space”

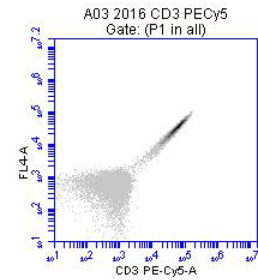
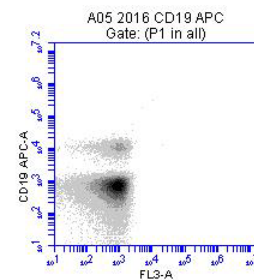
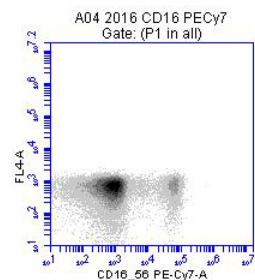


Data is from multiple C6 units, various reagents and cell systems, collected with standard C6 filters and lasers.



Predictable spillover: allows definition of a “3rd” detector

Fluorochrome Spillover	Slope of graphed data	Average Compensation
FITC into FL2	0.0770	7.00%
PE into FL1	0.0370	3.50%
PE-Cy7 into FL4	0.0013	0.00%
PE-Cy5 into FL4	0.4613	47.00%
APC into FL3	0.0075	1.00%

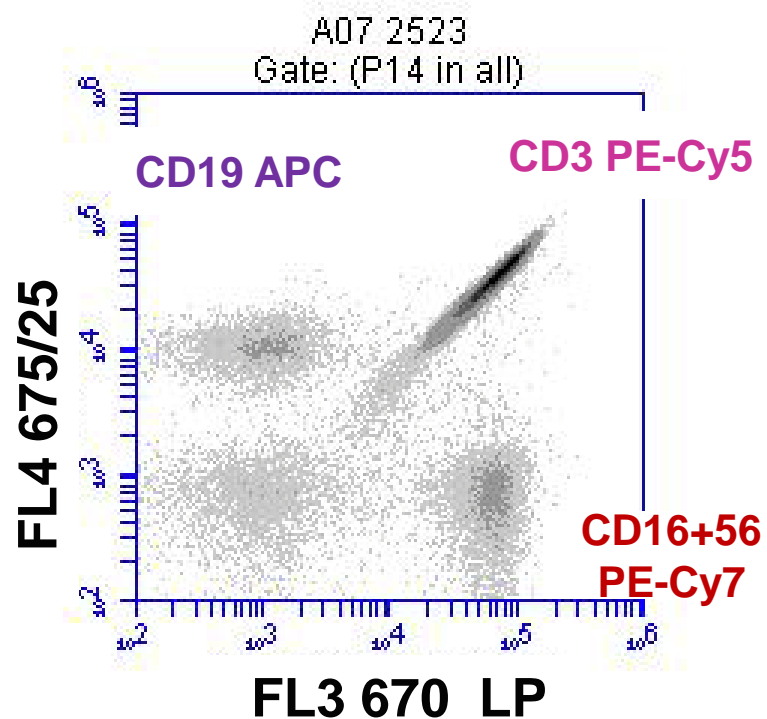


- PE-Cy7 limited to FL3
- PE-Cy5 in FL3 and FL4 **equally**
- APC limited to FL4

In this fluorochrome context, we now have FL3, FL4 and **FL3/4**

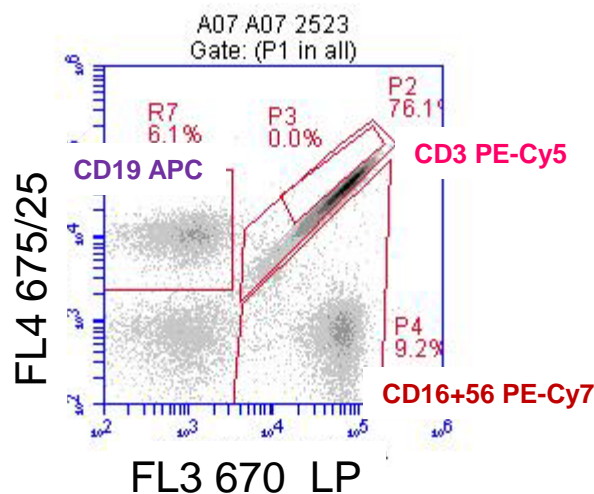


Mutually exclusive epitopes CD19, CD3, CD16+56

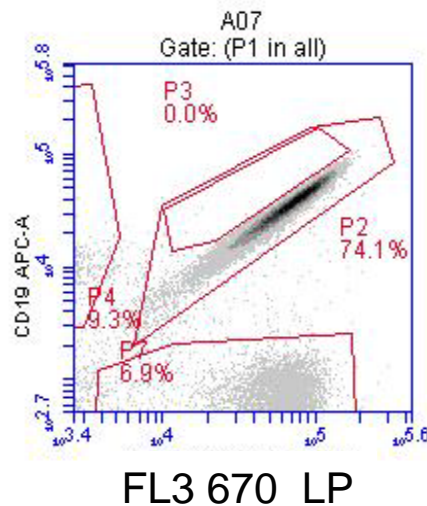


Subsets of the main populations can be added: example: CD4+ CD3+ cells

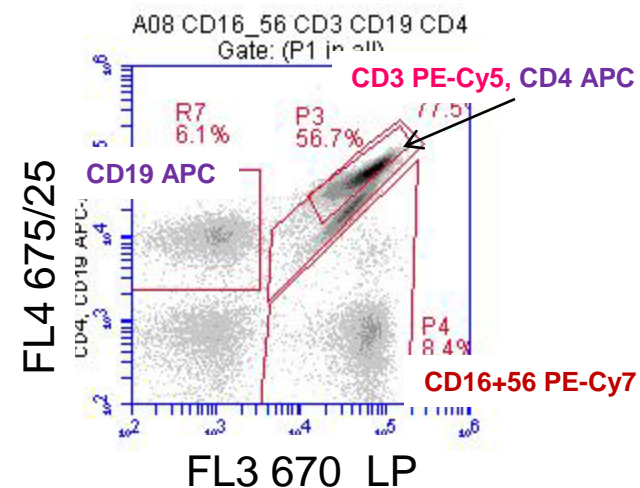
Control for lymphocyte subsets



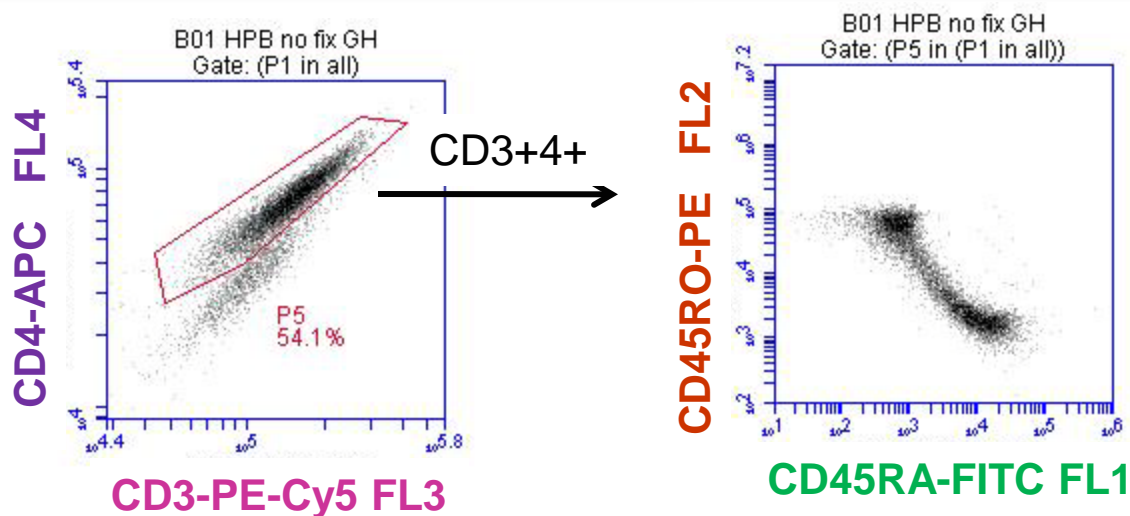
Zoom in to gate



Plus CD4-APC



FL1 and FL2 are available to phenotype the CD3+4+ population

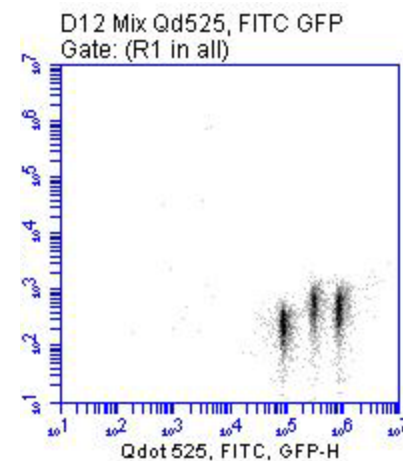
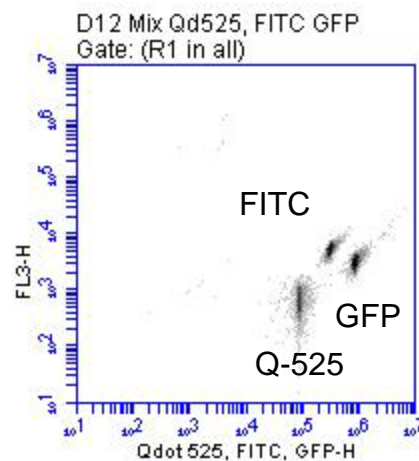
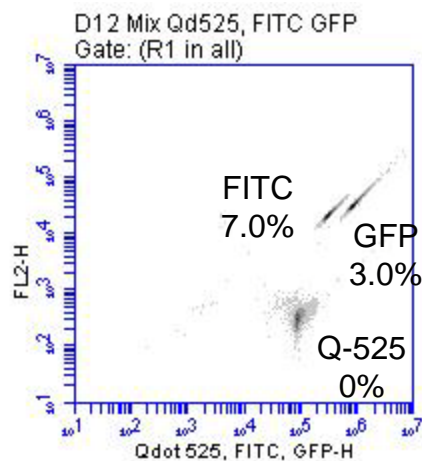
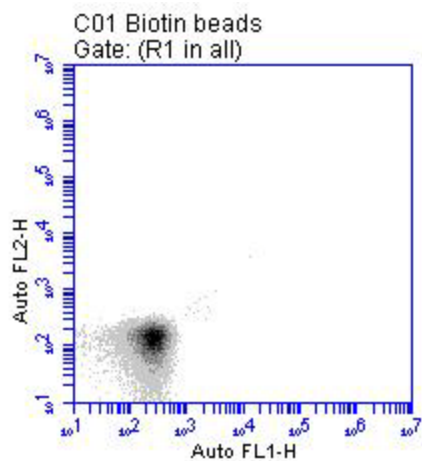


CD19-APC
CD16+56-PE-Cy7
CD3-PE-Cy5

CD4-APC

CD45RA-FITC, CD45RO-PE

Why not use Qdot-525 and FITC together?



Conclusions

- Optimizing fluorescence intensity measurements using a bead standard results in:
 - Instrument to instrument comparability
 - Consistent performance with other fluorescence standards for MESF calibration
 - Predictable fluorescence spillover: possibility for mathematical modeling
 - Identification of fluors, and fluor combinations, based on location in multidimensional space
 - Aides in experimnetal planning and trouble-shooting
 - Multiplexing of fluors to get more analysis out of fewer detectors

