Spillover Spread

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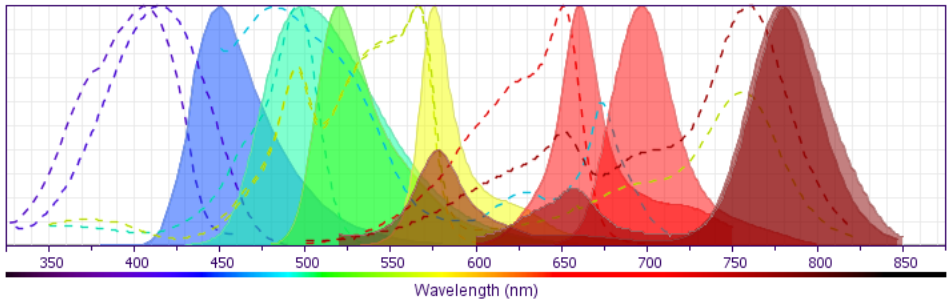


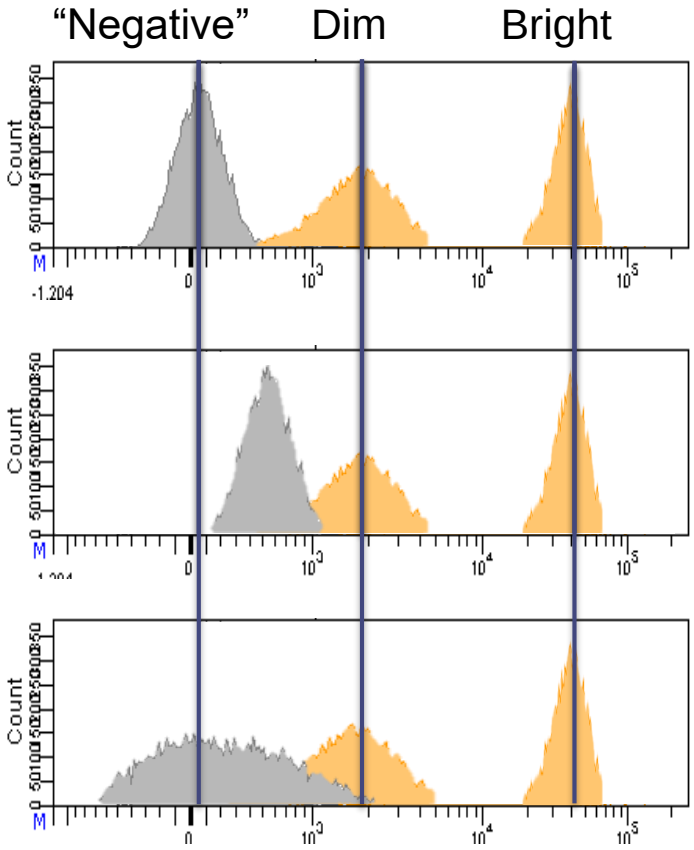
Resolution and Spread

Important in multicolor flow cytometry is ability to resolve populations: the degree to which a flow cytometer can distinguish unstained from dimly stained in a mixture.

Negative population has low background
populations well resolved

Negative population has high background
populations not resolved

Negative population ha slow background but high rSD (spread)
populations not resolved



Resolution is a function of
position of the positive population
position ***and*** spread of the negative population.

Factors affecting Background Position and Spread

Instrument factors
Electronic noise
Optical background
Gain settings (PMT)

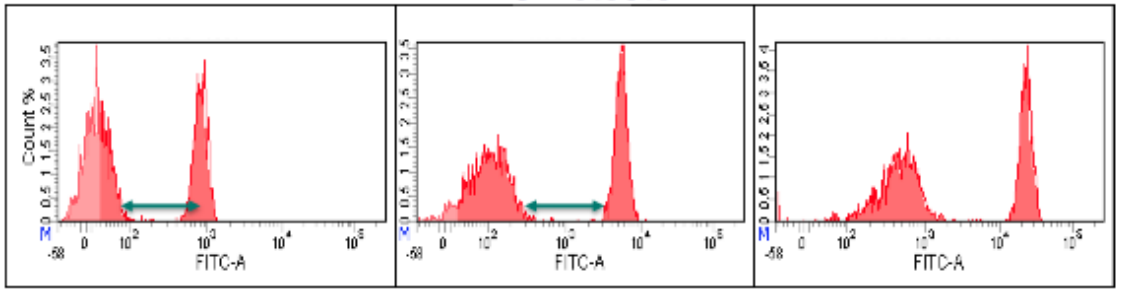
Cell and staining factors
Spread due to spillover
Fluorochrome
Antigen density
Autofluorescence
Nonspecific staining

Reminder: Titration and gain optimization

Antibody concentration and detector gains will affect background spread:
Your antibodies should be titrated and your PMT gains optimized for each color

- To maximize sensitivity and resolution
- To decrease background spread

Adjusting PMTV To Maximize SI



PMT Voltage	373	473	573
Stain Index	15	39	42
MFI Pos Cells	750	5072	21183
MFI Neg Cells	15	94	415
rSD Neg Cells	24	64	245

BD

Factors affecting Background Position and Spread

Instrument factors

Electronic noise
Optical background
Gain settings (PMT)

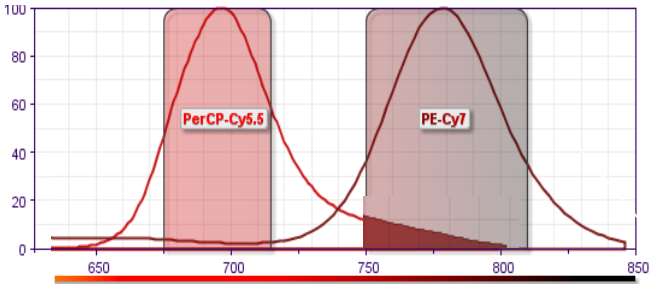
Cell and staining factors

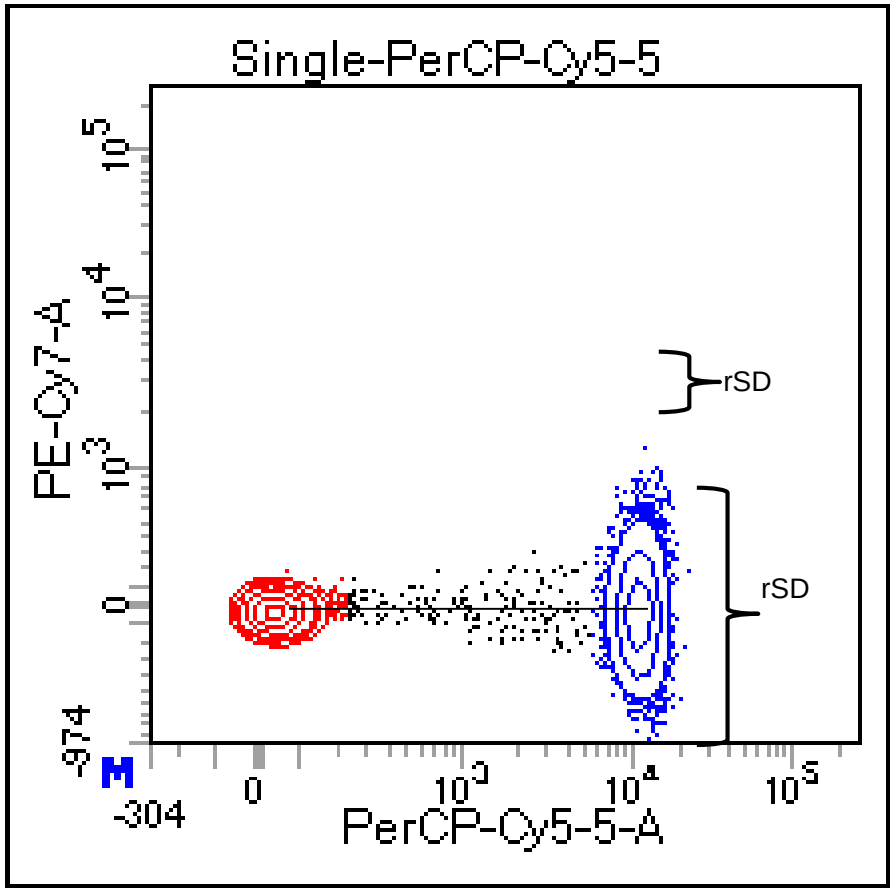
Spread due to spillover
Fluorochrome
Antigen density

Autofluorescence
Nonspecific staining

Spillover Introduces Background Spread Into Other Detectors

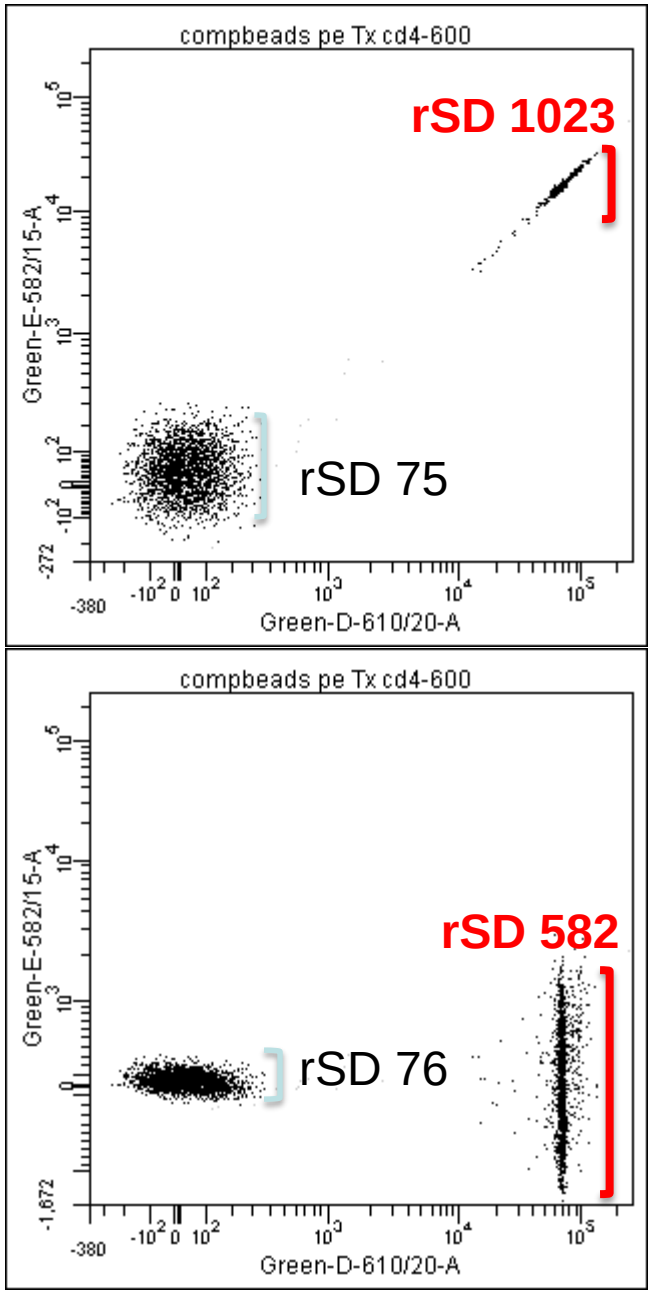
We can compensate for fluorochrome spillover but we cannot eliminate spread





	Negative		Positive	
	MFI	rSD	MFI	rSD
No Comp	12	29	3098	291
Comp	4	29	3	289

Spread is caused by spillover
not removed by compensation
reduces resolution of double positives



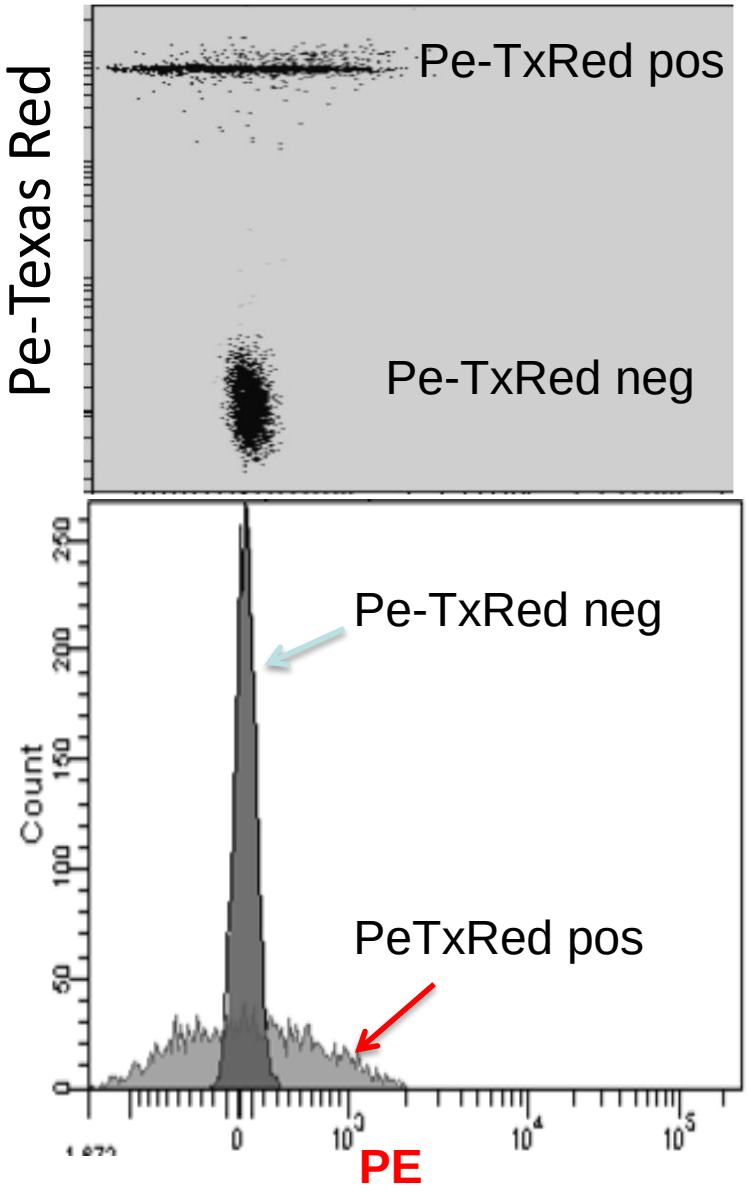
PE

PE

PE-Texas Red

Although this peak looks tight
This is in log scale:
range and spread
is over 10,000 channels up here

This range and spread
Is maintained after compensation
but is much more apparent
In the low channels



The effect of spread
can been seen in the width of
the Pe-TxRed positive population
in the **PE** channel

A double positive population
would have to be above
channel 2000 to be detectable

No problem for the Pe-TxRed negative
population

What affects spread?

1. Amount of spillover between fluorochromes

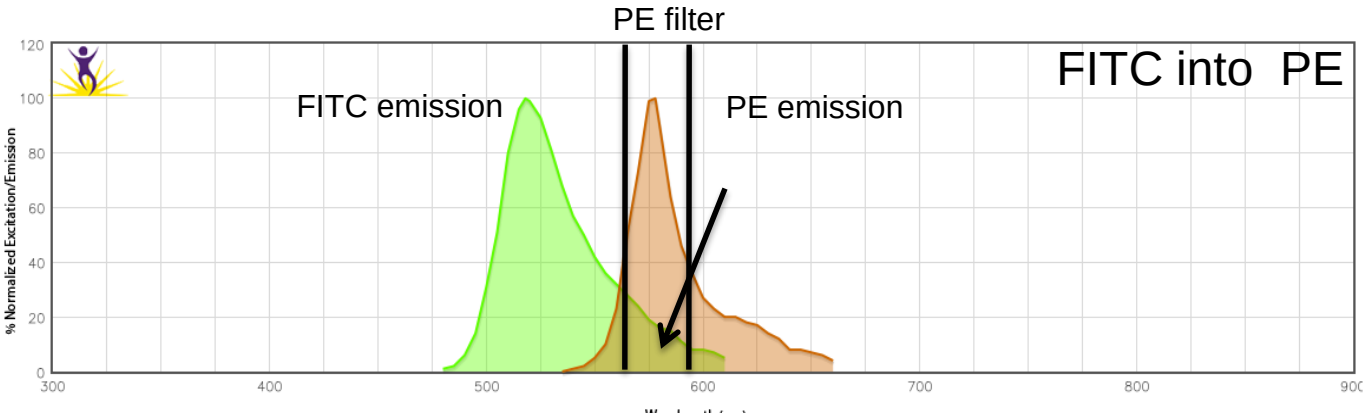
2. Staining intensity, which depends on:
    A. Fluorochrome brightness
    B. Antigen Density

3. The Cytometer
    Stain Index and spread values differ from cytometer to cytometer
    Due to: laser wavelength and power
    filters
    detectors

1.Is there Spillover? Look at your spectra!

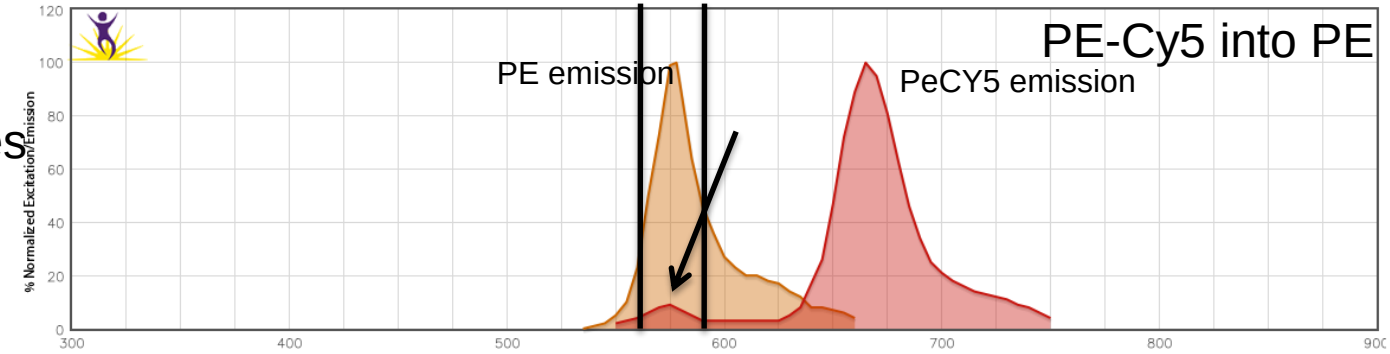
1

Adjacent spectra



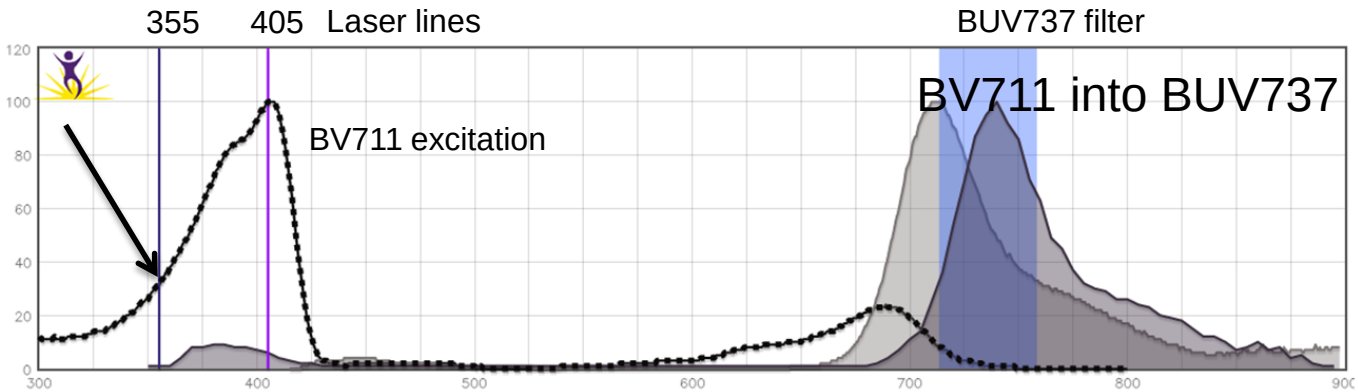
2

Tandems and their bases



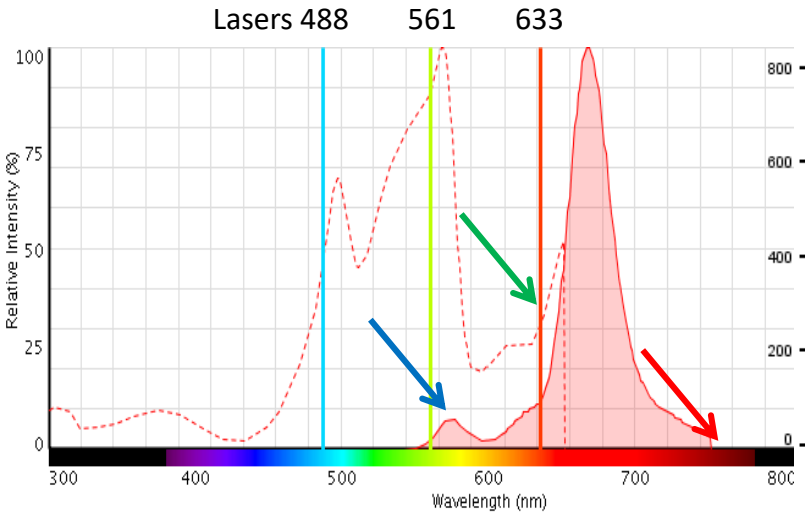
3

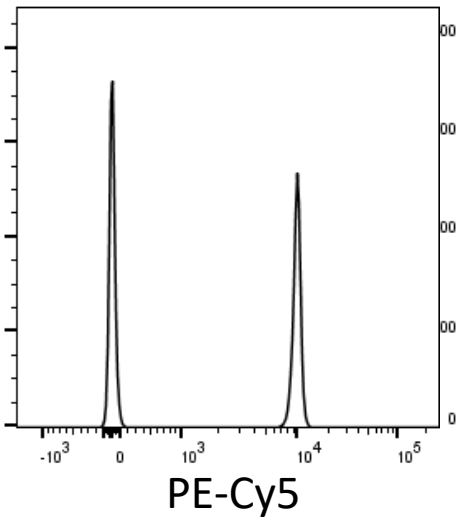
Cross laser excitation



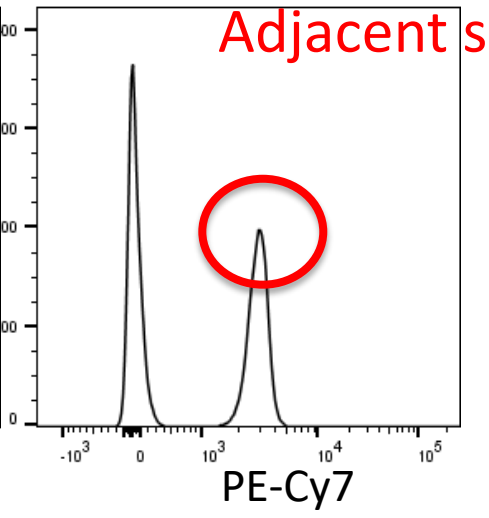
1. Spillover: PE-Cy5 Single Color

You can a good idea of where there will be a spillover problem by looking at the excitation and emission spectra

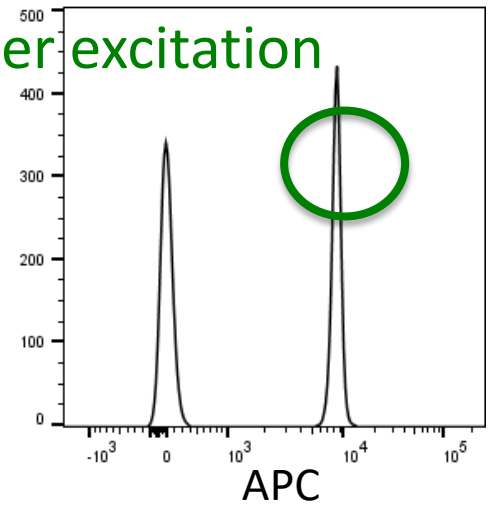


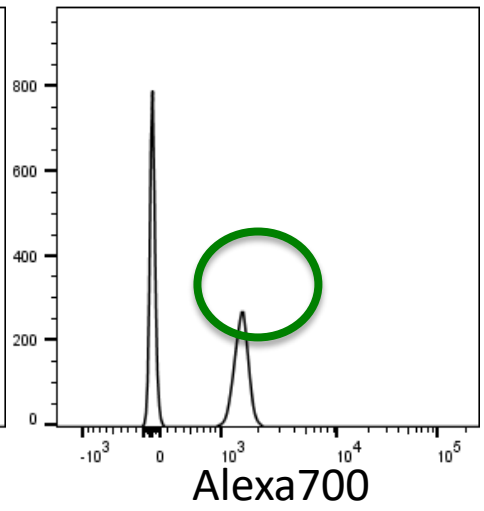


Adjacent spectra

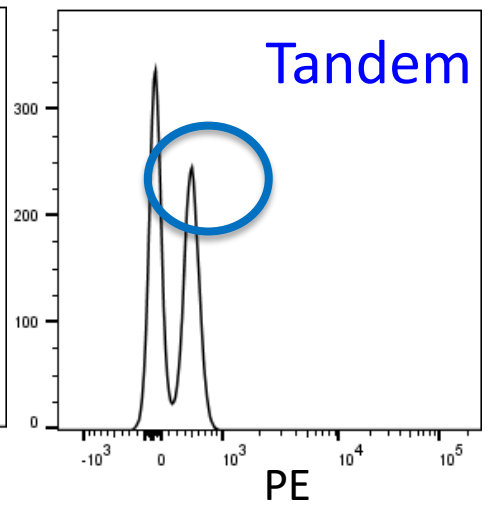


Cross laser excitation





Tandem base



2. Staining intensity

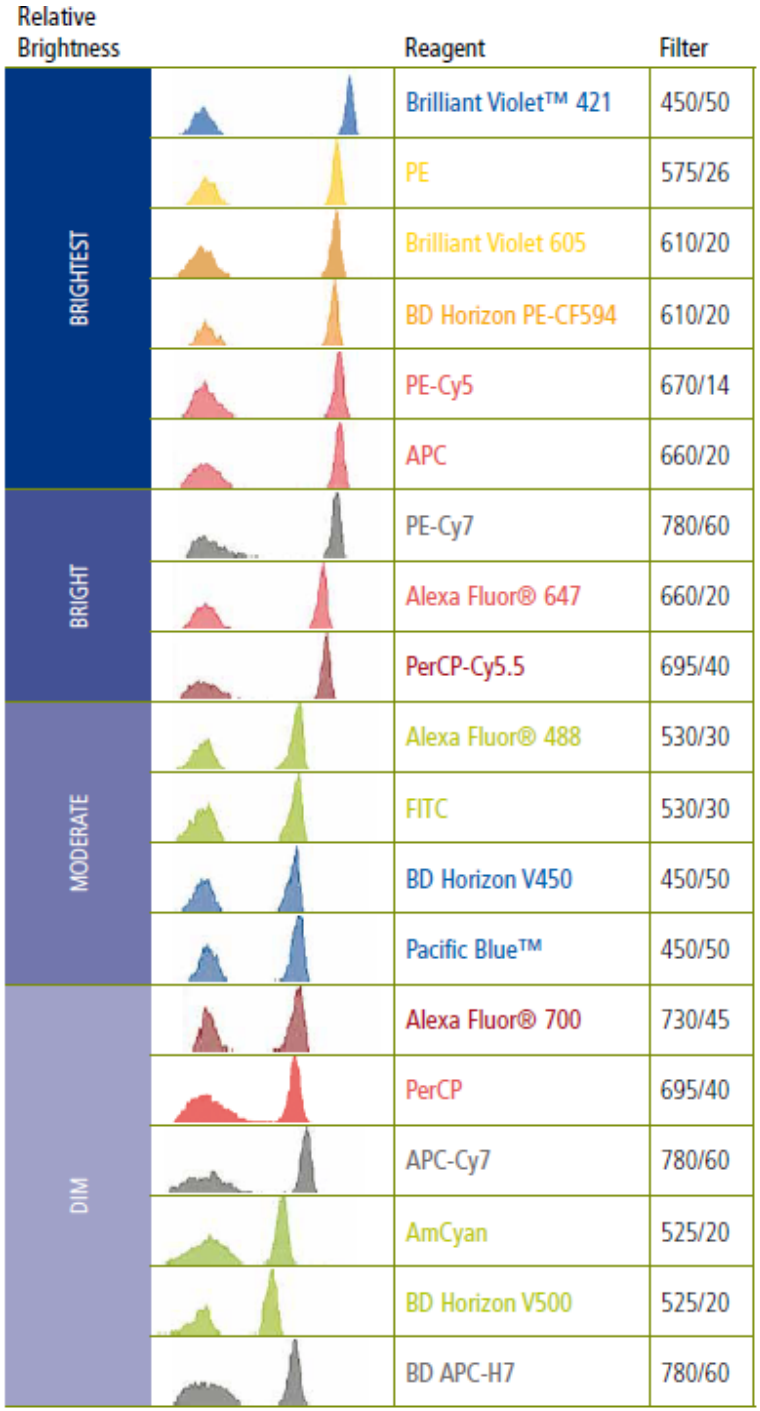
A. Fluorochrome Brightness

Despite differences across cytometers
Fluorochromes can be grouped into:
Brightest
Bright
Moderate
Dim

Some general information on brightness at:

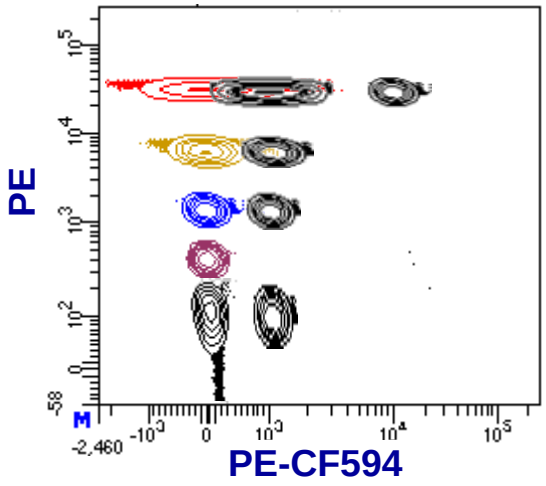
http://www.biolegend.com/brightness_index

http://static.bdbiosciences.com/documents/multicolor_fluorochrome_laser_chart.pdf?_ga=1.193693357.1447862526.1480066966



2. Staining intensity

B. Antigen Density



Here it can be seen that

as the intensity of PE staining increases
due to increased antigen density

so does the PE spread into the PE-CF594 channel

This makes it difficult to distinguish bright PE positive cells that are also dimly positive for PE-CF594.

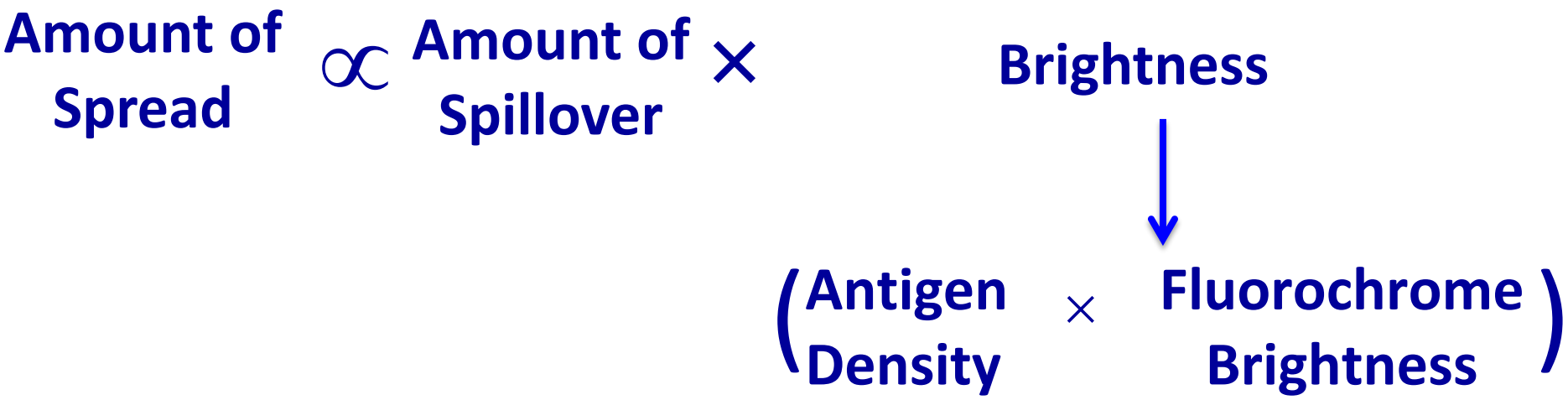
The PE-CF594 cells must be very positive to be resolved.

3. Cytometer differences

Fluorochrome brightness varies from one cytometer to the next
Even in a supposedly identical cytometer

Fluorochrome	Brightness x 10^5	Brightness relative to PE	Stain index	
			Cytometer 1	Cytometer 2
PE	16	100%	348	262
PeCy5	NA	NA	180	131
APC	4.7	29%	238	281
FITC	0.4	2%	132	61
BV421	16	100%	264	145

Spread variables

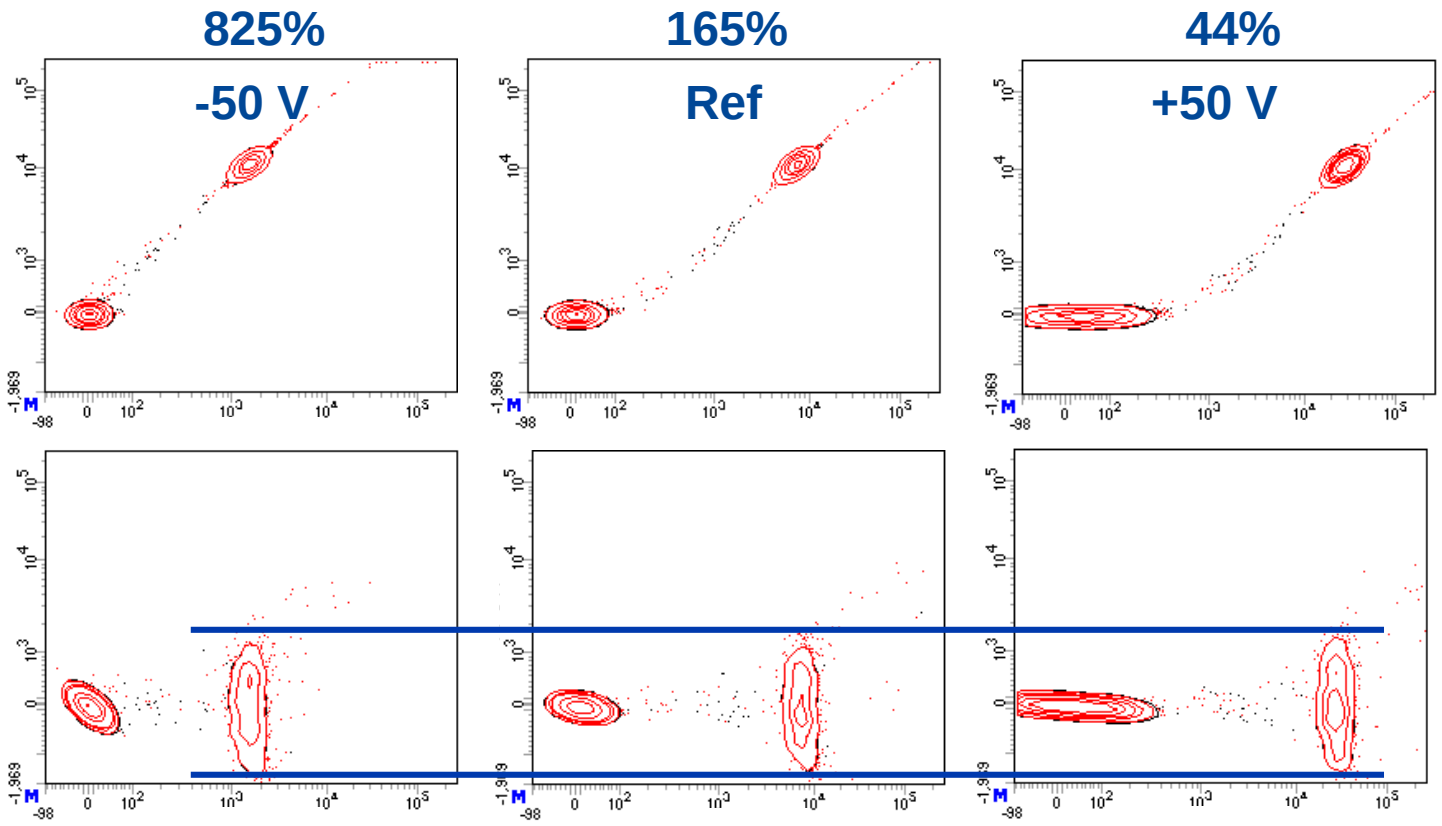


More colors = more spread = more problems

Spread is additive:

the sum of all fluorochrome spreads into each channel

Spread is not affected by PMT or Comp values

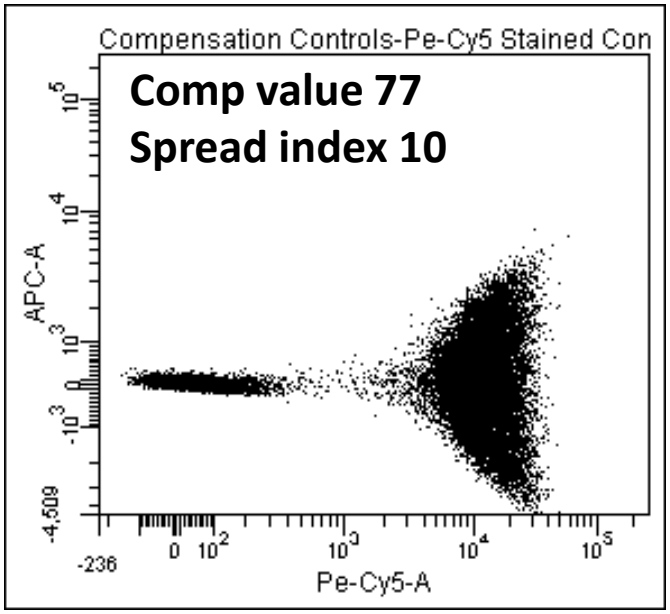


These is the same tube run at 3 different PMT Voltages

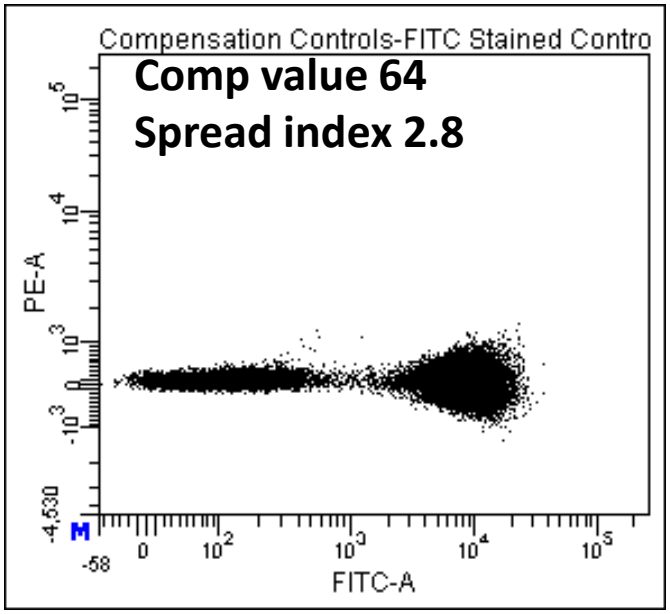
They have the same spread into the other detector

Spillover Spread index vs Compensation

Pe-CY5 into APC detector



FITC into PE detector



Spread is not necessarily reflected by the compensation value

can have similar compensation value between different parameters

but very different SSI!

How to assess Spread due to Spillover

Calculation of Spillover Spread is very useful to evaluate potential problems due to spread in the choice of fluorochromes for your own cytometer.

- Manual calculation according to Nguyen et al
- Automatic calculation using Flowjo 9

Both methods use compensated single color controls

Generation of a Spillover Spread Matrix (SSM) across all detectors

SSM valid across compensation and gain changes

SSM valid until laser, filter or detector change

TECHNICAL NOTE

Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

Quantifying Spillover Spreading for Comparing Instrument Performance and Aiding in Multicolor Panel Design

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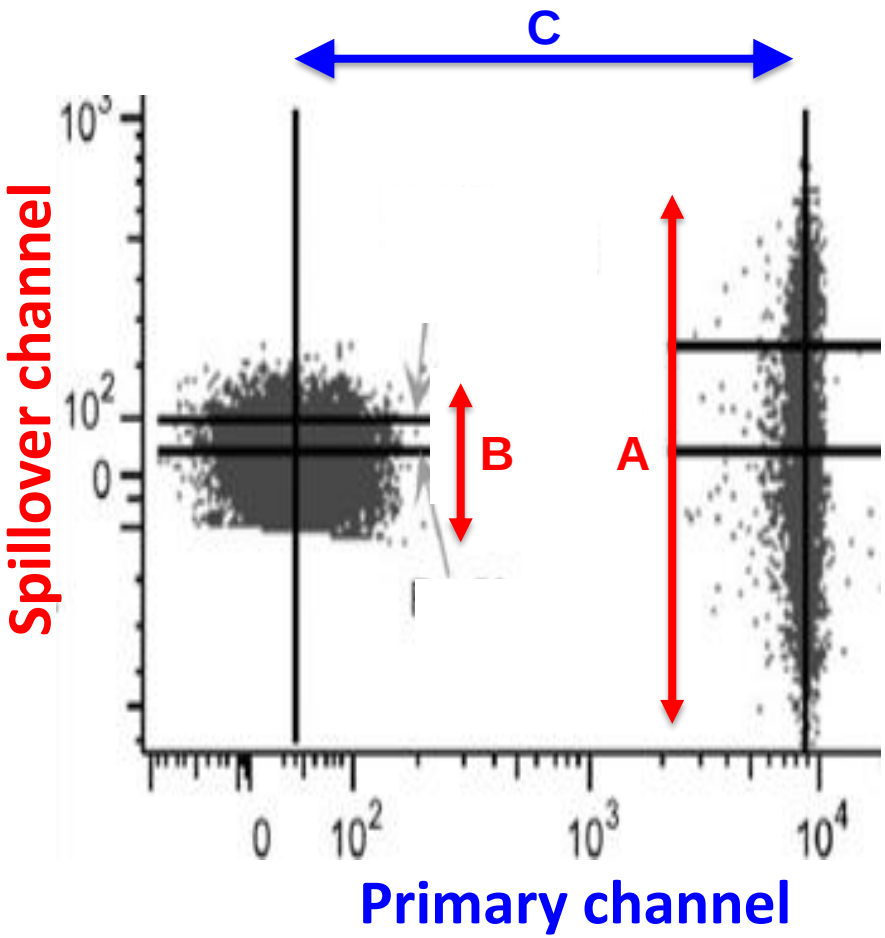
• Abstract

After compensation, the measurement errors arising from multiple fluorescences spilling into each detector become evident by the spreading of nominally negative distributions. Depending on the instrument configuration and performance, and reagents used, this "spillover spreading" (SS) affects sensitivity in any given parameter. The degree of SS had been predicted theoretically to increase with measurement error, i.e., by the

Manual Calculation (Nguyen)

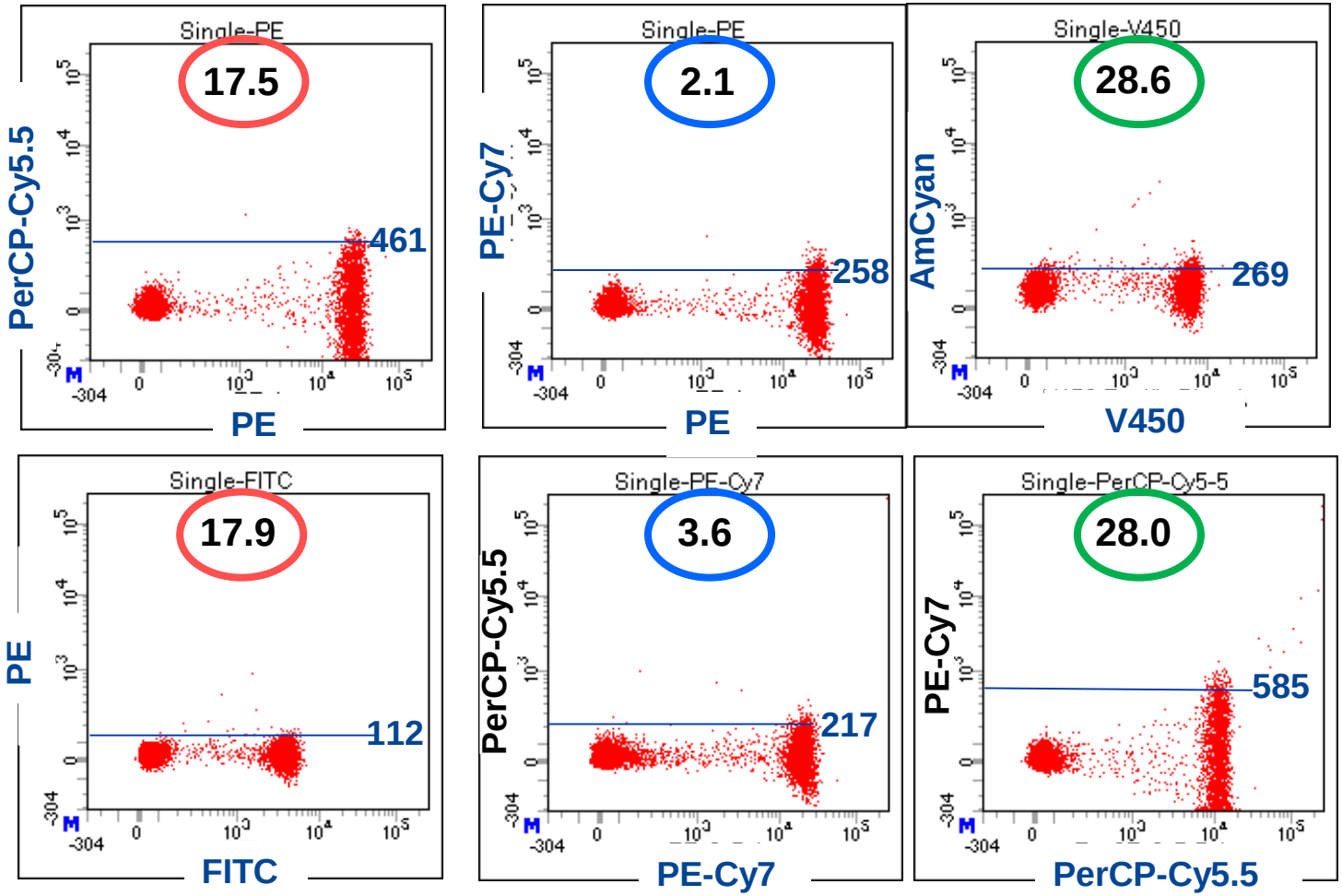


takes in account:

1. difference in spread of the (A) positive vs (B) negative populations in the spillover channel

2. normalized to the (C) median intensity difference between the two populations in the primary channel.

Similar compensation: different spread



Just because you have a high compensation value does not mean you have high spread

Spillover Spread Matrix (Flowjo)

Detector / Fluorochrome	Stain Index	FITC B527/32	PE B586/42	PerCP B700/54	PE-Cy7 B783/56	APC R660/10	APC-H7 R783/56	V450 V448/45	V500 V528/45
FITC	583		1.41	0.37	0.72		0.11		2.77
BB515	569		1.50	0.26	0.48				1.10
PE	2402	0.21		1.11	1.76				0.37
PE-Cy5	2558		0.36		10.14	96.57	30.39		
PerCP	174		0.15		5.86	2.20	2.01		
PerCP-Cy5.5	355				11.05	2.26	13.78		
PE-Cy7	1409		0.16	0.12			10.32		
APC	2072			0.84	0.80		2.67		
Alexa 647	1875			0.68	0.58		4.45		
APC-Cy7	578				3.50	0.93			
APC-H7	826				2.46	0.33			
Pac Blue	100								2.18
V450	103					0.12	0.19		1.86
BV421	680								1.67
V500									
BV510	154	0.26	0.13		0.16				

Optimize multicolor panels for fluorochrome-combinations that show only low spread in channels with co-expressed markers (especially for highly expressed antigens)

Be careful using fluorochrome-combinations with significant or high spread into other detectors for antigens that are expressed on the same cell.

If combination cannot be avoided, try to use markers that are not co-expressed.

Spread Matrix: Fortessa X-20: PE-Cy5

Detector		BV421	BV510	BV605	BV650	BV711	BV786	FITC	PerCP	BUV395	BUV496	APC	Alexa 700	APC-H7	PE	PE-CF594	PE-Cy5	PE-Cy7
Fluorochrome	Stain Index	V450/50	V525/50	V610/20	V670/30	V710/50	V780/60	B525/50	B695/40	UV1	UV2	R670/30	R730/45	R780/60	YG586/15	YG610/20	YG670/30	YG780/60
Pac Blue	29		1.9	1.6	1.2	0.8	0.6				0.6		0.5		0.3	0.3		0.2
V450	44		1.6	1.4	0.6	0.9	0.9				0.9		0.8	0.4			0.4	
BV421	268		1.0	0.8	0.6	0.4	0.4		0.1		0.4		0.4			0.1		0.1
V500	41	0.3		3.4	2.9	2.0	1.4	0.3	0.4		0.9		1.4			0.8		
BV510	127	0.8		5.0	4.5	3.2	2.5		0.7	0.2	2.3	0.7	2.7		0.2	1.3	0.2	
BV605	110	0.7			7.4	5.6	3.8		3.3		0.3	3.0	4.7	0.2	1.6	4.8	2.7	1.8
BV650	188	0.7		3.0		7.5	5.0		2.3		0.3	5.3	7.9	1.2	0.1	1.0	1.9	1.2
BV711	118	0.8		0.5	2.6		9.6		3.6		0.4	2.5	16.6	3.1			0.5	1.4
BV786	201	1.0	0.2	0.4	0.7	1.3			0.3		0.2	0.6	1.7	2.6				1.2
FITC	38		0.7	0.6	0.7	0.6	0.4		1.8		0.7		0.5				0.4	
BB515	82		0.4	0.3	0.3	0.3	0.2		1.0		0.7							0.2
PerCP	16			2.2	13.0	5.5	4.2				0.7	8.1	7.3	1.2		0.3	5.6	2.7
PerCP-Cy5.5	42			1.0	5.5	7.5	5.3				0.4	5.4	8.7	2.3			2.3	3.3
BUV395	159			0.2	0.5	0.3			0.3		0.9				0.1		0.3	
BUV496	107	0.3	3.0	1.6	1.1	0.8	0.6	1.2	0.2	2.9			0.8		0.1	0.4		
APC	149			0.4	2.4	1.7	1.0		3.4		0.2		11.9	2.5		0.3	3.1	1.6
Alexa 647	182			0.1	0.4	0.5	0.4		1.4		0.1		10.6	2.6			1.1	1.1
Alexa 700	23			0.2	0.3	1.3	1.3		0.7		0.3	1.8		3.0			0.3	1.4
APC-Cy7	27				0.4	0.5	2.9		0.7			3.2	5.0				0.7	5.2
APC-H7	58				0.4	0.5	2.9		0.7		0.3	1.3	3.2			0.2	0.4	4.5
PE	313		0.2	2.9	2.3	2.4	0.8	1.5	6.1		0.2	4.2	1.1			10.7	4.0	1.9
PE-CF594	253	0.2		2.4	2.5	2.8	1.0		7.7		0.2	5.3	1.4	0.2	1.6		4.8	2.7
PE-Cy5	265	0.4		1.4	8.5	7.0	2.0		20.4			18.8	12.4	2.5	0.6	0.9		5.4
PE-Cy7	461				0.1	0.3	3.0		0.5		0.2	0.3	1.0	2.1	0.4	0.6	0.3	

PE-Cy5: high spread into a lot of channels, receives some spread
Al700: receives a lot of spread

Spread Matrix: Fortessa X-20: BV421

Detector		BV421	BV510	BV605	BV650	BV711	BV786	FITC	PerCP	BUV395	BUV496	APC	Alexa 700	APC-H7	PE	PE-CF594	PE-Cy5	PE-Cy7
Fluorochrome	Stain Index	V450/50	V525/50	V610/20	V670/30	V710/50	V780/60	B525/50	B695/40	UV1	UV2	R670/30	R730/45	R780/60	YG586/15	YG610/20	YG670/30	YG780/60
Pac Blue	29		1.9	1.6	1.2	0.8	0.6				0.6		0.5		0.3	0.3		0.2
V450	44		1.6	1.4	0.6	0.9	0.9				0.9		0.8	0.4			0.4	
BV421	268		1.0	0.8	0.6	0.4	0.4		0.1		0.4		0.4			0.1		0.1
V500	41	0.3		3.4	2.9	2.0	1.4	0.3	0.4		0.9		1.4			0.8		
BV510	127	0.8		5.0	4.5	3.2	2.5		0.7	0.2	2.3	0.7	2.7		0.2	1.3	0.2	
BV605	110	0.7			7.4	5.6	3.8		3.3		0.3	3.0	4.7	0.2	1.6	4.8	2.7	1.8
BV650	188	0.7		3.0		7.5	5.0		2.3		0.3	5.3	7.9	1.2	0.1	1.0	1.9	1.2
BV711	118	0.8		0.5	2.6		9.6		3.6		0.4	2.5	16.6	3.1			0.5	1.4
BV786	201	1.0	0.2	0.4	0.7	1.3			0.3		0.2	0.6	1.7	2.6				1.2
FITC	38		0.7	0.6	0.7	0.6	0.4		1.8		0.7		0.5				0.4	
BB515	82		0.4	0.3	0.3	0.3	0.2		1.0		0.7							0.2
PerCP	16			2.2	13.0	5.5	4.2				0.7	8.1	7.3	1.2		0.3	5.6	2.7
PerCP-Cy5.5	42			1.0	5.5	7.5	5.3				0.4	5.4	8.7	2.3			2.3	3.3
BUV395	159			0.2	0.5	0.3			0.3		0.9				0.1		0.3	
BUV496	107	0.3	3.0	1.6	1.1	0.8	0.6	1.2	0.2	2.9			0.8		0.1	0.4		
APC	149			0.4	2.4	1.7	1.0		3.4		0.2		11.9	2.5		0.3	3.1	1.6
Alexa 647	182			0.1	0.4	0.5	0.4		1.4		0.1		10.6	2.6			1.1	1.1
Alexa 700	23			0.2	0.3	1.3	1.3		0.7		0.3	1.8		3.0			0.3	1.4
APC-Cy7	27				0.4	0.5	2.9		0.7			3.2	5.0				0.7	5.2
APC-H7	58				0.4	0.5	2.9		0.7		0.3	1.3	3.2			0.2	0.4	4.5
PE	313		0.2	2.9	2.3	2.4	0.8	1.5	6.1		0.2	4.2	1.1			10.7	4.0	1.9
PE-CF594	253	0.2		2.4	2.5	2.8	1.0		7.7		0.2	5.3	1.4	0.2	1.6		4.8	2.7
PE-Cy5	265	0.4		1.4	8.5	7.0	2.0		20.4			18.8	12.4	2.5	0.6	0.9		5.4
PE-Cy7	461				0.1	0.3	3.0		0.5		0.2	0.3	1.0	2.1	0.4	0.6	0.3	

BV421: very little spread into other channels, receives very little spread

Spillover Spread Summary

The amount of spread due to **spillover** depends on

- Amount of spillover between fluorochromes
- Staining intensity:
  - Fluorochrome brightness and antigen density
- Cytometer characteristics
  - spread differs from cytometer to cytometer