Multicolor panel design: demystifying the complexity

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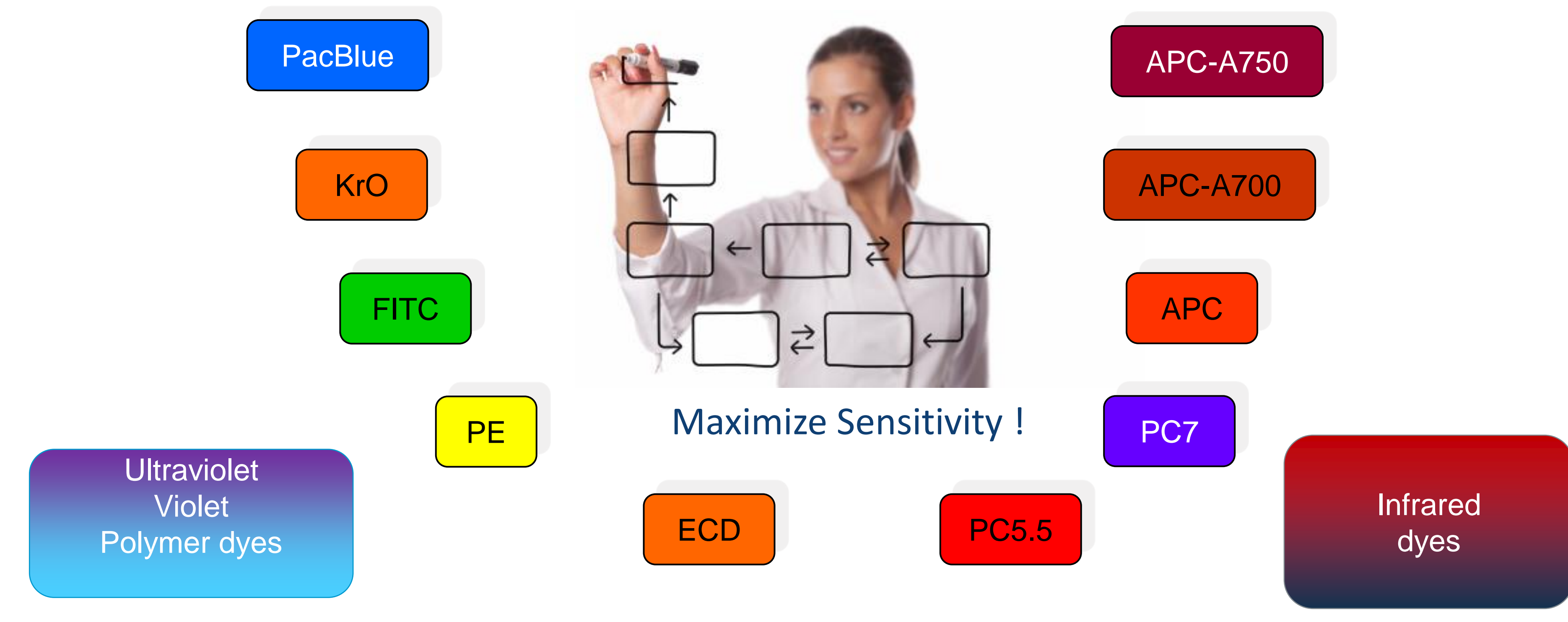
Multicolor phenotyping :

Multiple markers can be analyzed simultaneously in the same sample, more and better information, less sample required



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Overview Brightness Of Popular Dyes

Laser				
NUV 375nm		BUV395 BUV496	BUV661	
Violet 405nm	PacBlue Krome Orange		SN605 SN785	SN428 SN650
Blue 488nm	PerCP	FITC	ECD	
Yellow 561nm				PE, PC5.5 PC5, PC7
Red 638 nm			APCA750	APC APCA700
IR 808 nm	ALEXA792			

Brightness : dim / medium / bright / bright+

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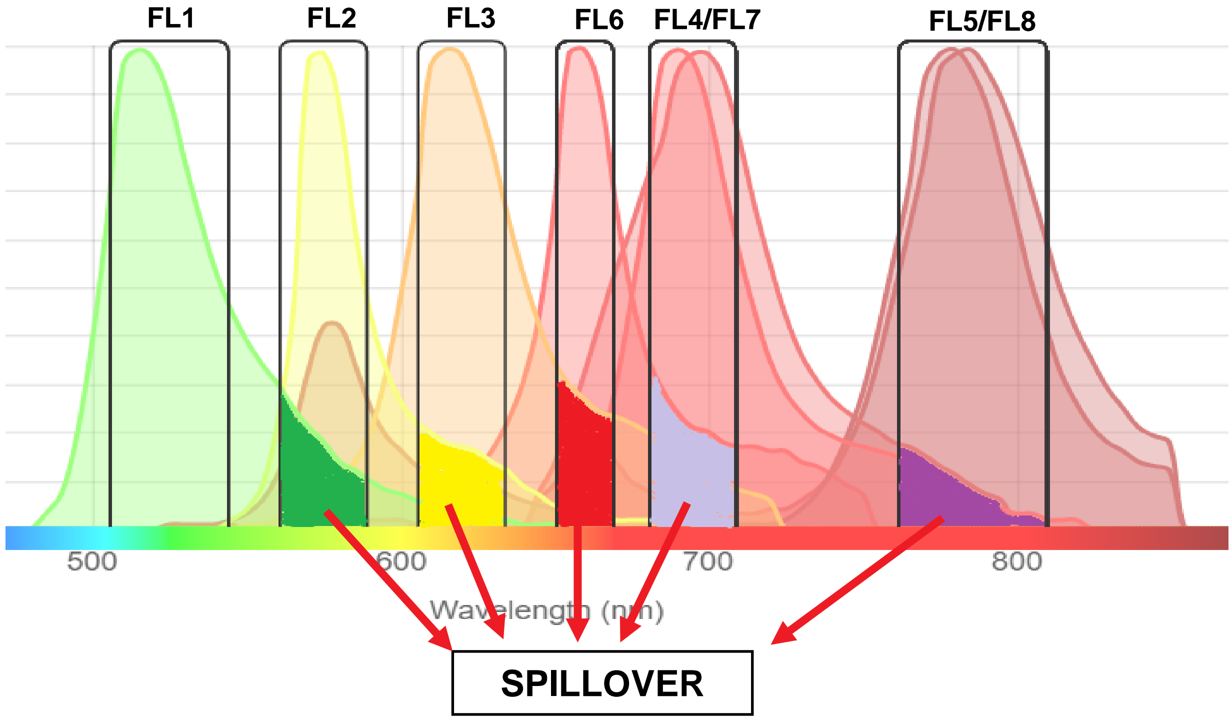
Fluorescence Spillover: Why do we need compensation?



- Emission spectrum of dyes can extend into multiple channels: **Spillover**

- The spilled light has to be subtracted to get the correct amount of light in a channel : **Compensation**

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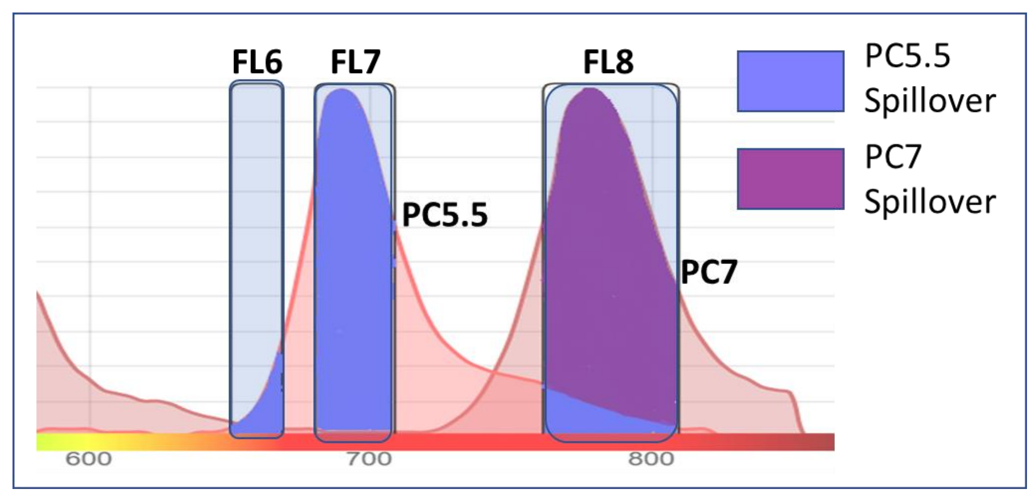
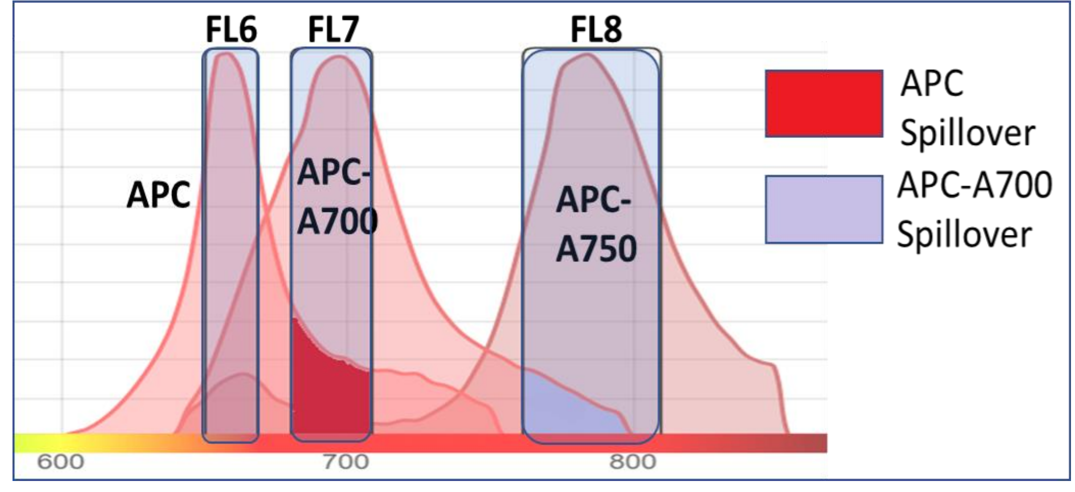
Fluorescence Spillover: Why do we need compensation?



- **This problem is significant with PC5.5 and PC7 spilling into FL7 and FL8.**
- **Mainly due to the fact that PC5.5 and PC7 can be excited by both 488 and 638nm lasers.**

Data spread in the red channels FL6, FL7, FL8



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Fluorochromes, Lasers and Filters – Distortion Matrix

CytoFLEX (APD)

		488nm laser					633nm laser			405 laser				
	FROM	FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	SN v421	KrO	SN v605	SN v650	SN v786
488nm laser	FITC													
	PE													
	ECD													
	PC5.5													
	PC7													
633nm laser	APC													
	APC-A700													
	APC-A750													
405 laser	SNv421													
	KrO													
	SNv605													
	SNv650													
	SNv786													

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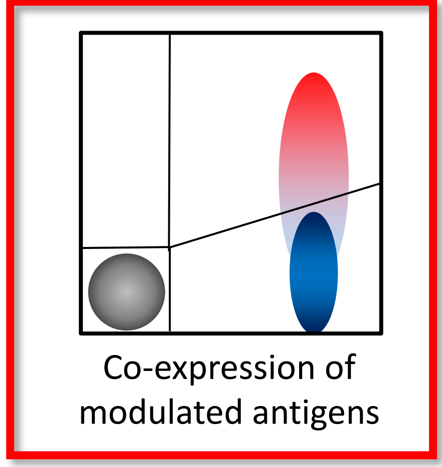
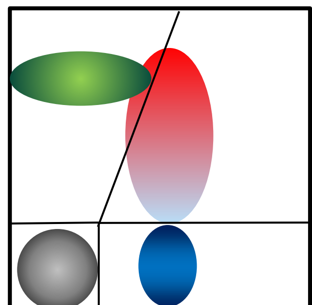
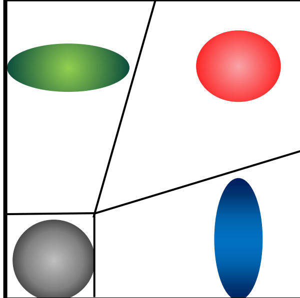
Relationship Is Everything



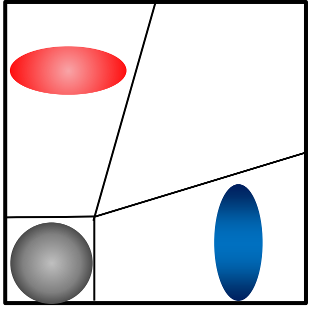
Co-expression of modulated antigens



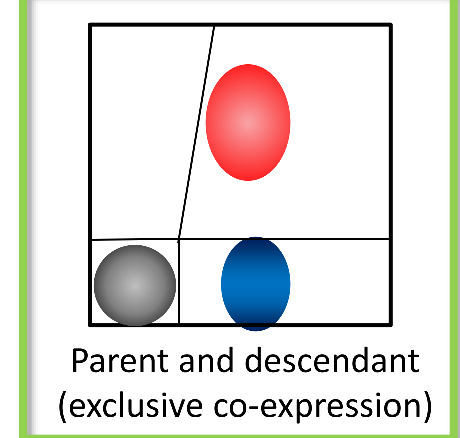
Co-expression of crosstalking antigens (non-exclusive co-expression)



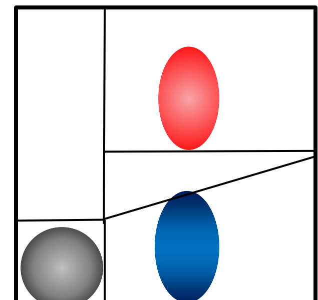
Co-expression of discrete antigens



Exclusion



Parent and descendant (exclusive co-expression)



Positive threshold for co-expressed discrete antigen

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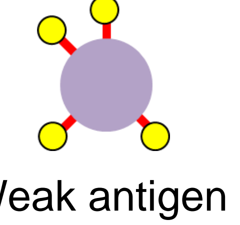
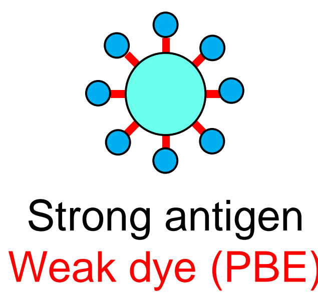
Rules to Maximize Effective Sensitivity

Weakly expressed antigen works best on ‘untouched’ channel, strongly expressed antigen works best with ‘silent’ dye

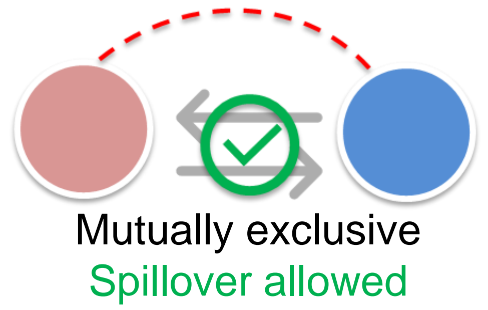


Weak antigen
Bright dye (PE)



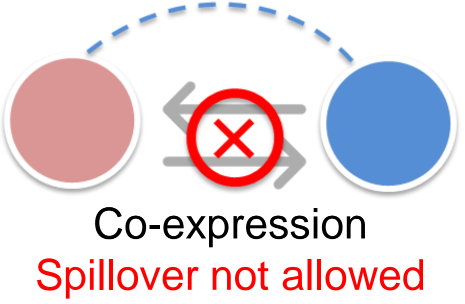
Strong antigen
Weak dye (PBE)

Allow spillover between excluding antigens



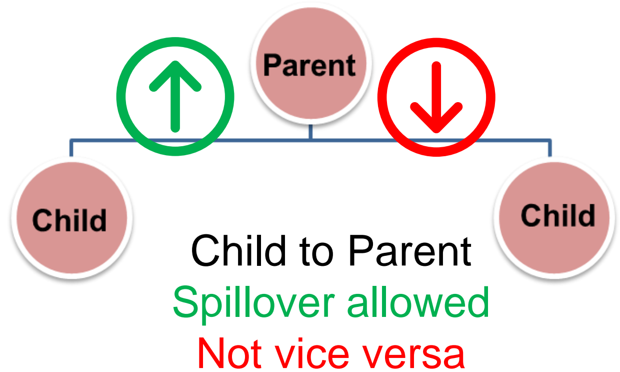
Mutually exclusive
Spillover allowed

Avoid spillover between non-exclusively co-expressed antigens



Co-expression
Spillover not allowed

Allow spillover from descendant antigens to parent antigens, avoid vice-versa



Child to Parent
Spillover allowed
Not vice versa

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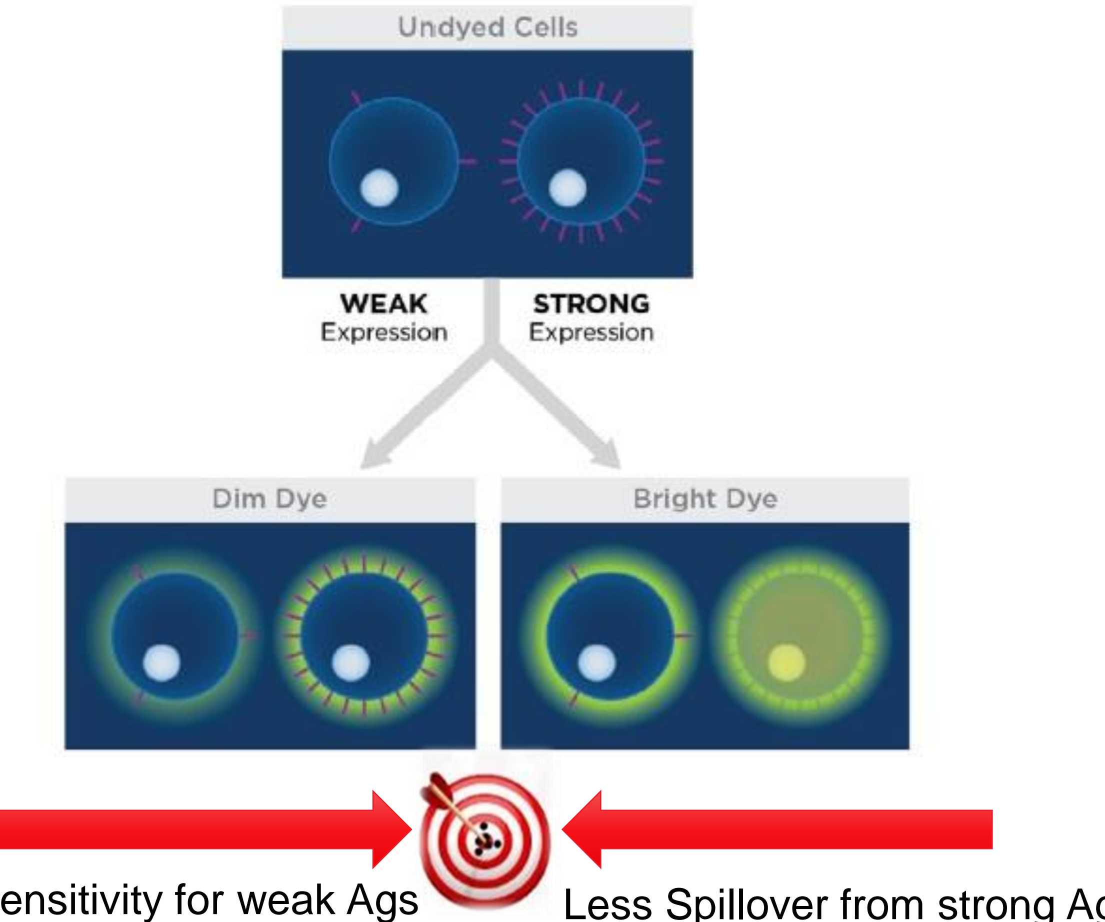
Match Brightness and Antigen Density



Better Sensitivity for weak Ags

Less Spillover from strong Ags

Cell	Antigen	Molecules per Cell
T cell	TCR	100,000
	CD2	55,000
	CD3	124,000
	CD5	90,000
	CD7	20,000
	CD45	>200,000
CD4+ T cell	CD4	100,000
	CD28	20,000
	CCR5	4,000-24,000
CD8+ T cell	CD8	90,000
	CD28	15,000
B cell	CD19	18,000
	CD20	109,000
	CD21	210,000
	CD22	14,000
	HLA-DR	85,000
	CD11a	10,000
	CD40	2,000
	CD86	16,000
	CD80	2,000
Dendritic cell	CD11a	27,000
	CD40	17,000
	CD80	132,000
	CD86	208,000
Monocyte	CD14	110,000
	CD32	21,000
	CD64	13,000
Neutrophil	CD14	3,500
	CD16	225,000
NK cell	CD56	10,000
Red Blood Cell	Glycophorin A	340,000
Basophil	CD23	15,000

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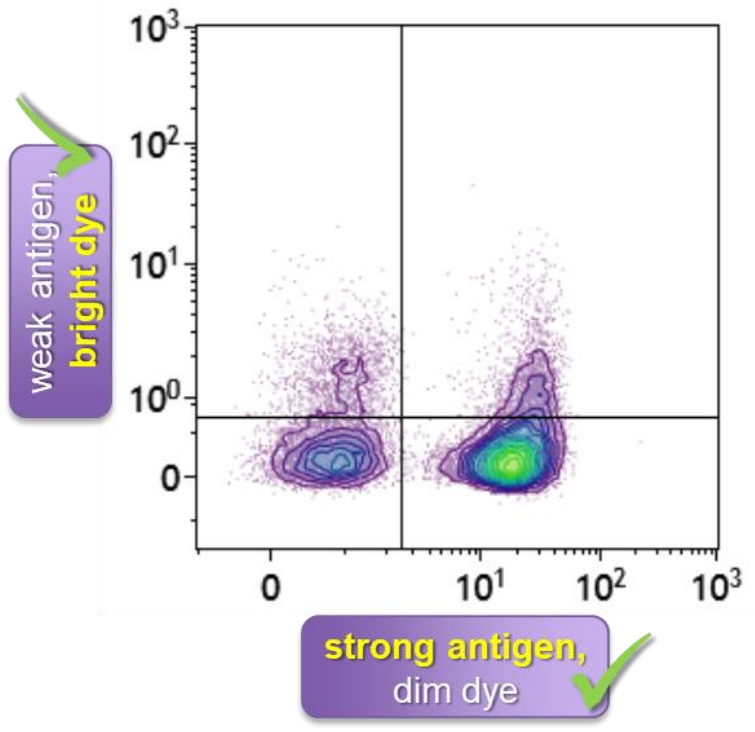
Appropriate Dye Brightness - Example



Dim and/or modulated expression:
the brighter, the better !

Discrete / bimodal antigen expression (e.g. lineage markers):
*dim dyes are equivalent……**or even better ?***

Weakly expressed antigen works best with bright dye, strongly expressed antigen works with all dyes („old school“)

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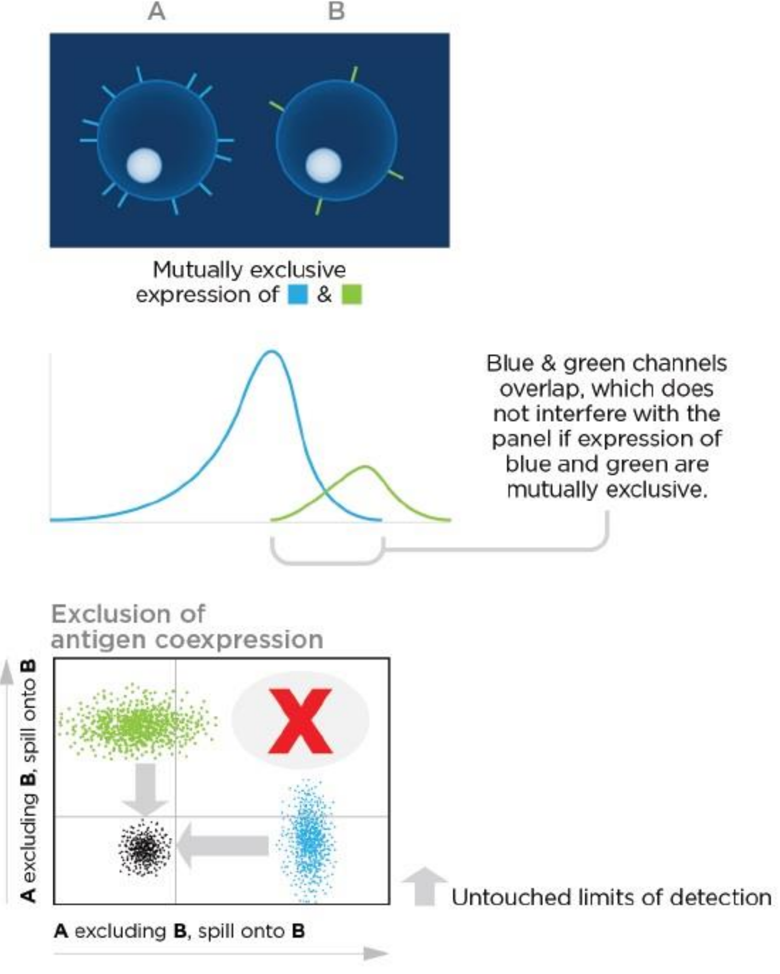
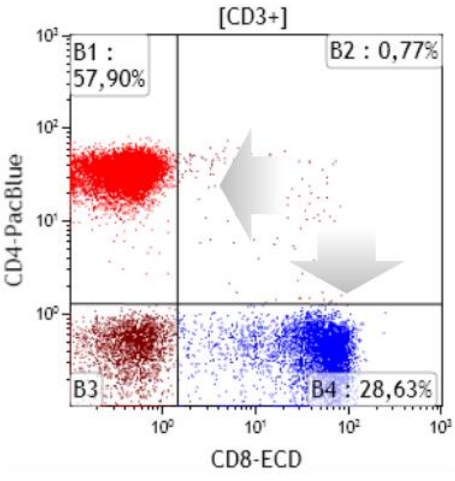
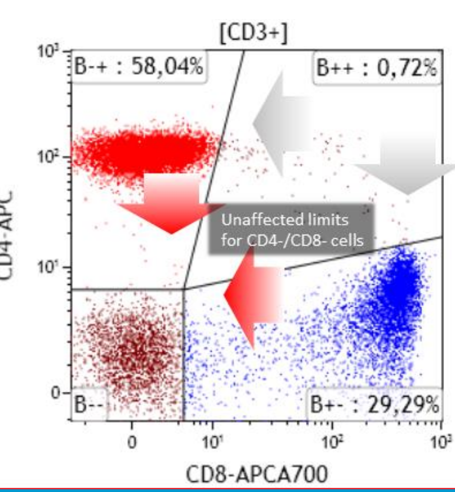
Expression Patterns – Exclusion







Allow spillover between mutually exclusive antigens

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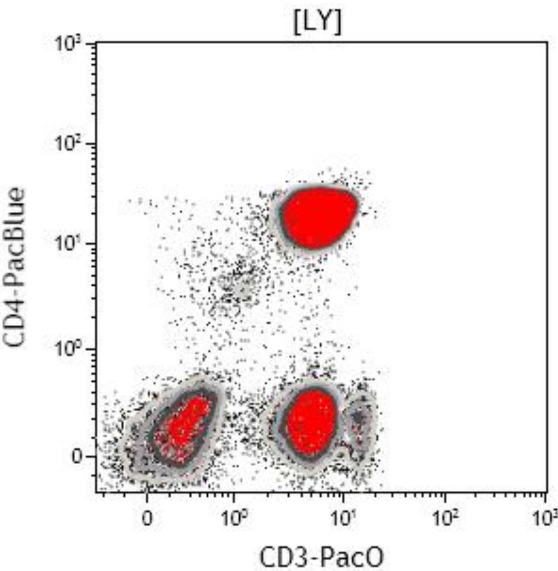
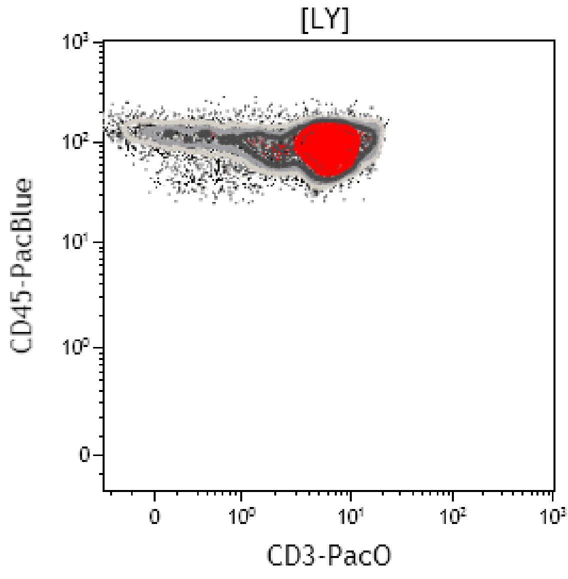
Expression Patterns – Parent descendent





CD3 Parent
CD4 Descendent
Allow CD4 to spill into CD3

CD45 Parent
CD3 Descendent
Improper separation

Allow spillover from descendant antigens to parent antigens, avoid vice-versa

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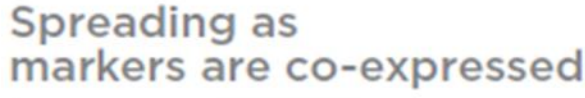
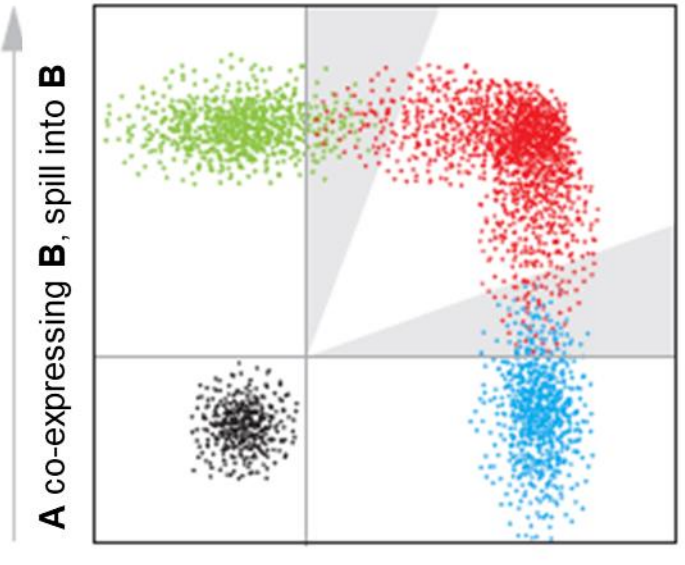
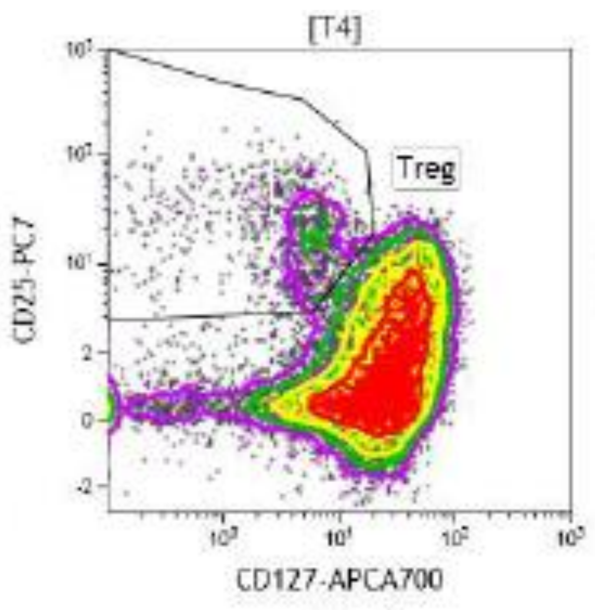
Expression Patterns: co-expression





Loss of sensitivity - Dim positive events cannot be detected



Bright CD25 in PC7 is essential in distinguishing the Treg population (CD25+ CD127-)
Weaker CD25 population will not separate clearly

Avoid spillover between non-exclusively coexpressed antigens

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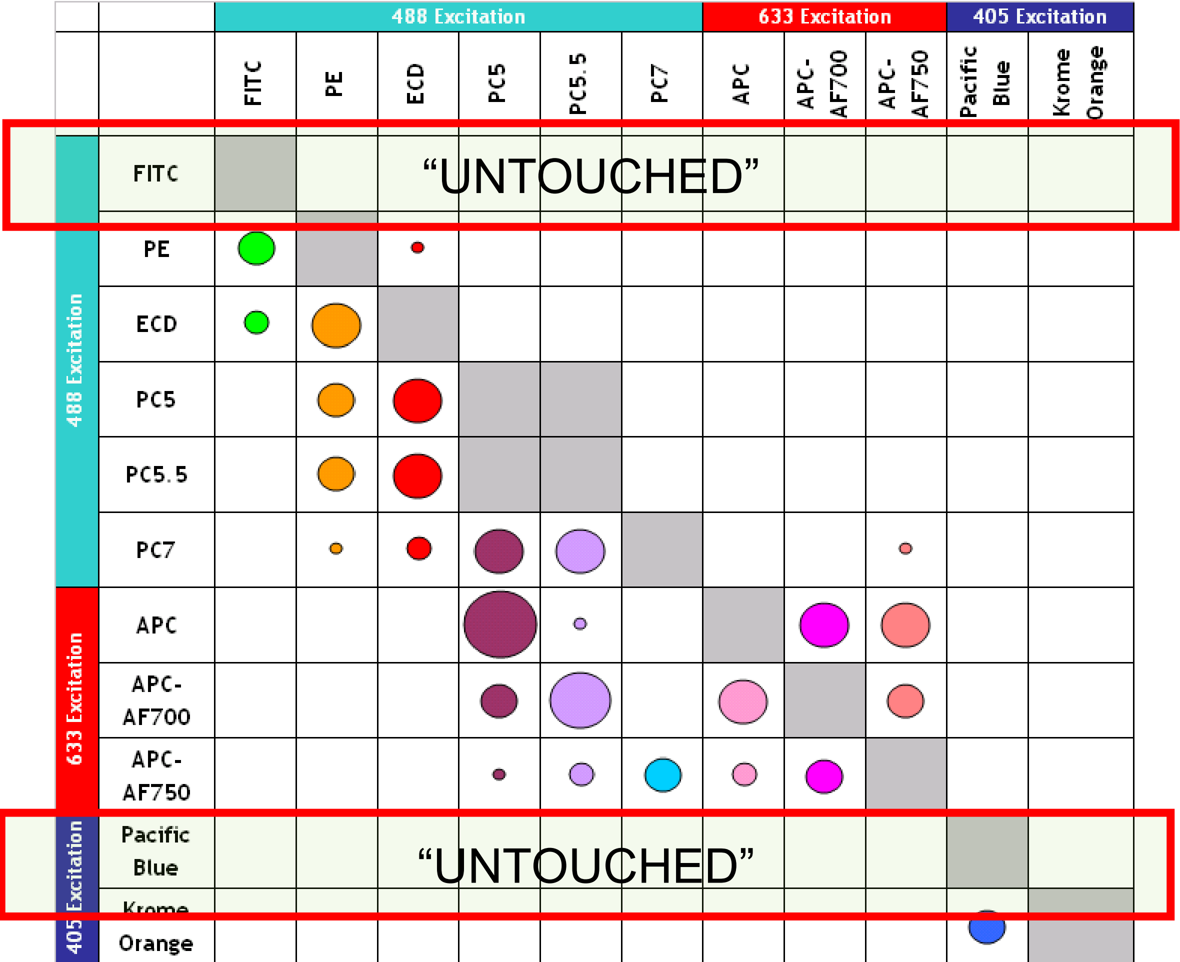
Fluorochromes, Lasers and Filters – Distortion Matrix



Untouchable: No overspill from other dyes (clean row)

Classification is specific for each combination of antibodies and conjugated dyes on a given hardware configuration

Weakly expressed antigen works best on “untouchable“ channel,

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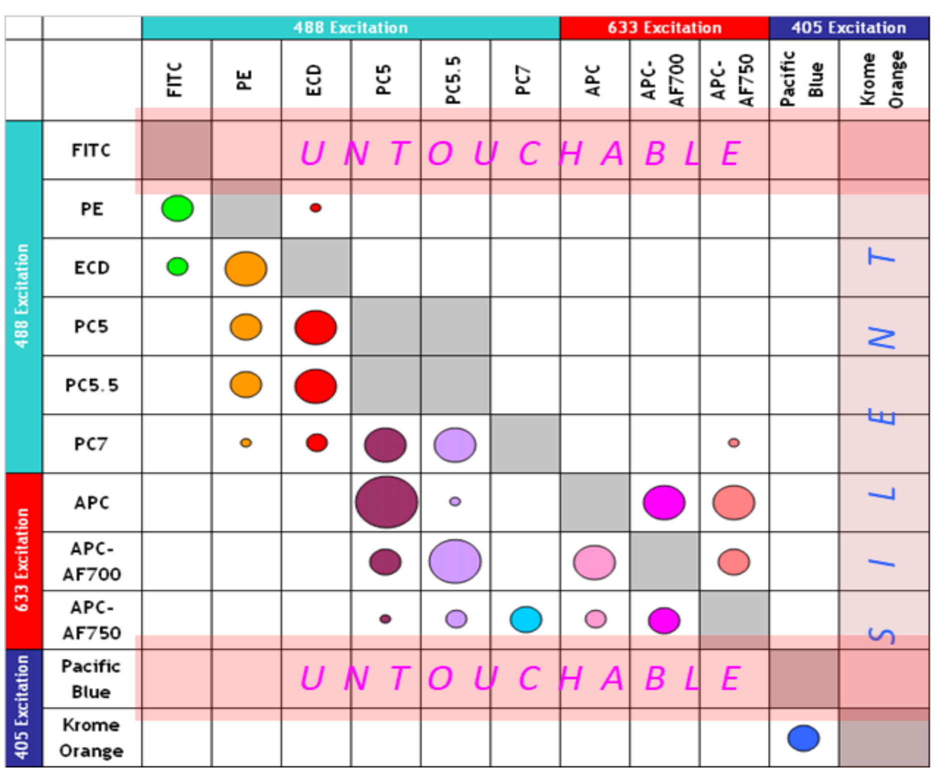

Untouchable: No overspill from other dyes (clean row)

Silent: No overspill into other channels (clean column)

Classification is specific for each combination of antibodies and conjugated dyes on a given hardware configuration

Weakly expressed antigen works best on “untouchable“ channel and strongly expressed antigen works best with “silent“ dye

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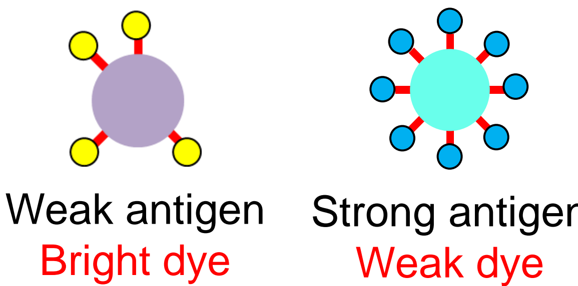
Rules to Maximize Effective Sensitivity

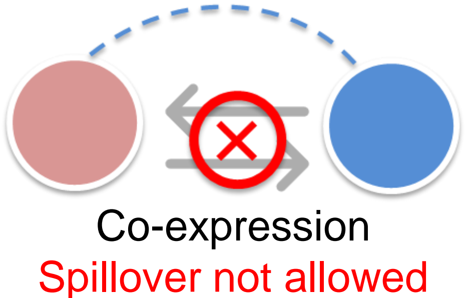
Weakly expressed antigen works best on „untouched“ channel, strongly expressed antigen works best with „silent“ dye



Weak antigen
Bright dye

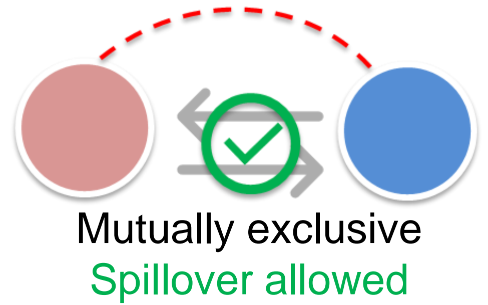
Strong antigen
Weak dye

Avoid spillover between non-exclusively co-expressed antigens



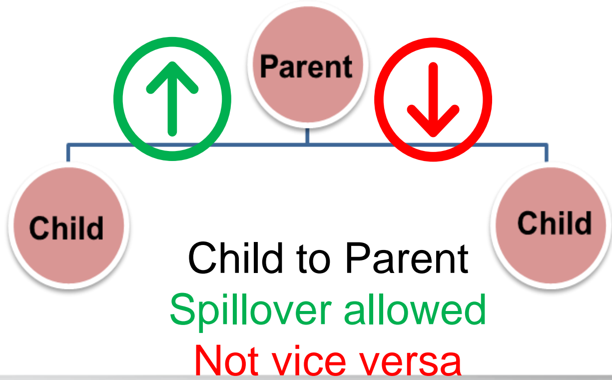
Co-expression
Spillover not allowed

Allow spillover between excluding antigens



Mutually exclusive
Spillover allowed

Allow spillover from descendant antigens to parent antigens, avoid vice-versa



Child to Parent
Spillover allowed
Not vice versa

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CD45
CD3
CD4
CD28
CD45RA
CD27
CD127
CCR7
PD-1
CD25
CD95
FoxP3
CD39

Panel Design Interdependencies (PanelMate)

18

Range of density

dim — bright

negative correlation

Co-expression

Antigens

CD45
CD27 — CD3 — CD95 — CD45RA
CCR7 — CD4 — CD28 — PD-1
CD25 — CD127
FoxP3
CD39

Range of density

dim — bright

Instrument

	488 Excitation						633 Excitation			405 Excitation	
	FITC	PE	ECD	PC5	PC5.5	PC7	APC	APC-AF700	APC-AF750	Pacific Blue	Krome Orange
FITC		U	N	T	O	U C	H	A	B L E		
PE											
ECD											T
PC5											N
PC5.5											E
PC7											L
APC											
APC-AF700											I
APC-AF750											S
Pacific Blue		U	N	T	O	U C	H	A	B L E		
Krome Orange											

Dyes

PE
APC
PC5.5 PC7 APCA700
PC5
A647 A700[$]
APCA750 ECD
FITC A488[$]
PacBlue
Krome Orange

Brightness

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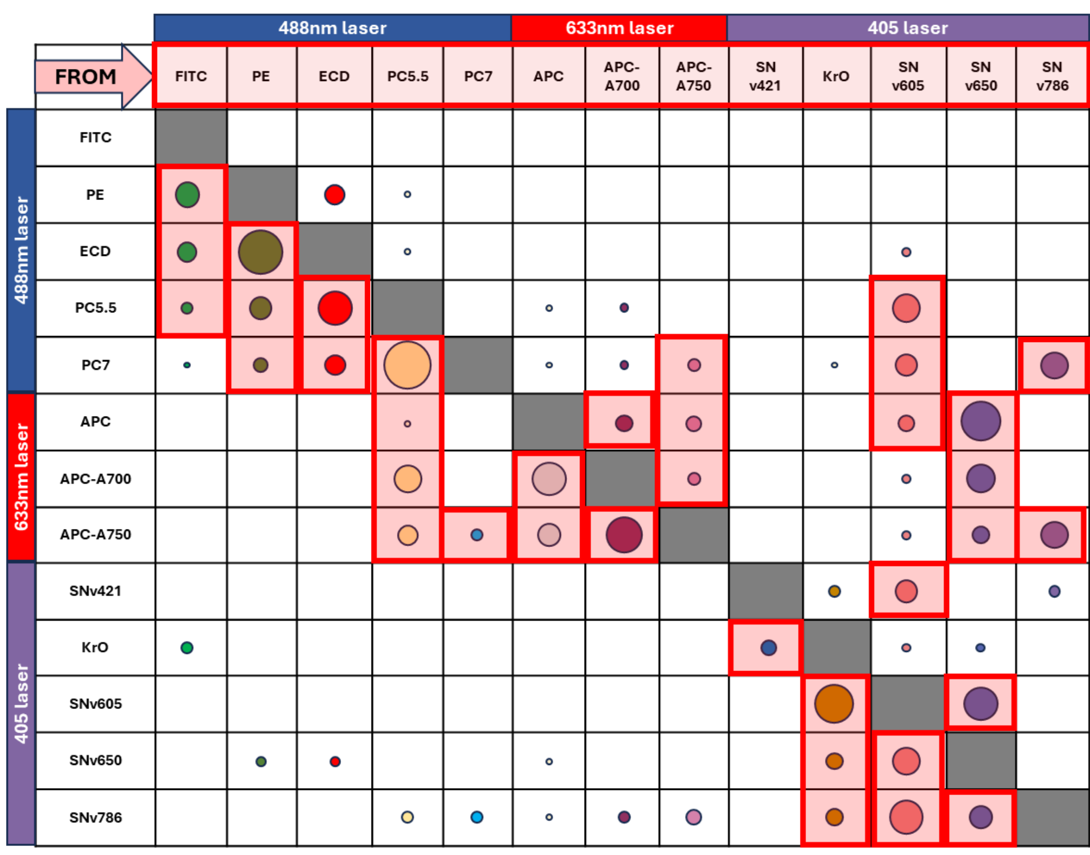
Panel design guide

- Start with AA700 and AA750, put parent markers
- Otherwise put exclusive markers on AA700 and PC5.5, and AA750/PC7
- Markers for analysis on FITC, PE, PC5.5, PC7 and APC
- Rest are mainly for gating markers.
- Refer to existing/working panels for guidance.
- Good sub/parent or exclusion pairs
- ECD-PC5.5
- PC5.5-AA700
- PC7-AA750
- PE-ECD



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Examples

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Panel configuration example 1

Combination of cytoplasmic and surface markers:
nTDT, CMPO, cCD79a, cCD3, cCD22, CD19, CD34, CD3, CD15, CD45

(Differentiation in the cytoplasmic and surface markers will be done with the sample staining procedure)

- Strongest antigen on the silent channel/weakest dye
  CD45 on Krome Orange (silent channel). No spillover into other channels

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
									CD45

- Data spreading high in the Red channels
- Start with AA700 and AA750, put parent markers
  Put CD3 (surface) in AA750 parent markers for all T Cells can tolerate spillover from PC5.5, PC7.
  Does not spill into other channels
  Put cCD22 in AA700, parent marker for all B cells. Mutually exclusive to CD3.

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
						CD22	sCD3		CD45

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Panel configuration example 1

Combination of cytoplasmic and surface markers:

nTDT, CMPO, cCD79a, cCD3, cCD22, CD19, CD34, CD3, CD15, CD45

- TdT and MPO are available only in FITC and PE

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
nTdT	cMPO					cCD22	sCD3		CD45

- Weakest antigens on the brightest dyes
  cCD3, cCD79a, CD34 to be placed in PC5.5, PC7, APC
- cCD79a is a weak marker and cannot tolerate any spillover, so APC
- CD34 is available in PC7 but not in PC5.5
- cCD3 in PC5.5

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
nTdT	cMPO		cCD3	CD34	cCD79a	cCD22	sCD3		CD45

- CD19 is placed in ECD, as it is too weak for PB

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
nTdT	cMPO	CD19	cCD3	CD34	cCD79a	cCD22	sCD3		CD45

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Panel configuration example 1

- CD15 is placed in PB, it is a relatively bright marker and available only in PB

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
nTdT	cMPO	CD19	cCD3	CD34	cCD79a	cCD22	sCD3	CD15	CD45

Combination of cytoplasmic and surface markers:
nTDT, CMPO, cCD79a, cCD3, cCD22, CD19, CD34, CD3, CD15, CD45

Differential surface and cytoplasmic staining (suggested procedure)

- Place all surface markers in the same tube (CD45, CD19, sCD3, CD34, CD15)
- Stain cells with surface markers, wash the cells
- Fix the cells with formaldehyde
- Permeabilize and stain with cytoplasmic markers (nTdT, cMPO, cCD3, cDD79a, cCD22)

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Panel configuration example 2

Myeloid, NK, lymphs cell markers
Myeloid markers: CD64, CD117, CD10, CD34, CD14
NK markers: CD7
Lymph markers: CD5, CD7, CD34, CD20, CD10, CD3

- Strongest antigen on the silent channel/weakest dye
  CD45 on Krome Orange (silent channel). No spillover into other channels

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
									CD45

- Data spreading high in the Red channels
- Start with AA700 and AA750, put parent markers
  CD3 (T), CD14 (Mono), CD20 (B) can tolerate spillover from PC5.5, PC7.
  Does not spill into other channels
  Put CD14 in AA750, and CD20 in AA700. CD3 can also be put in PB

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
						CD20	CD14	CD3	CD45

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Panel configuration example 2

- Weakest antigen with the brightest dyes
  CD117, CD64 and CD10 in PC5.5, PC7 and APC
- CD64 can tolerate spillover the best, so CD64 in PC7 (descendant to CD14-AA750)
- CD10 in APC, can tolerate spillover the least
- CD117 in PC5.5

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
			CD117	CD64	CD10	CD20	CD14	CD3	CD45

- Among CD5, CD7 and CD34, the weakest is CD7
- CD7 in PE

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
	CD7		CD117	CD64	CD10	CD20	CD14	CD3	CD45

- Among CD5 and CD34,
- ECD is brighter than FITC, so CD34-ECD and CD5-FITC

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
CD5	CD7	CD34	CD117	CD64	CD10	CD20	CD14	CD3	CD45

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Panel configuration example 3

T cell marker panel
CD2, CD5, CD7, TCRab (Pan alpha beta), TCRgd (Pan gamma delta), CD57
Gating markers: CD3, CD4, CD8, CD45

- Strongest antigen on the silent/weakest dye
  CD45 on Krome Orange (silent channel). No spillover into other channels

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
									CD45

- Data spreading high in the Red channels
- Start with AA700 and AA750, put parent markers
  CD3, CD4, CD8 can tolerate spillover from PC5.5, PC7.
  Does not spill into other channels
  Put CD3 in AA750, and CD8 in AA700.

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
						CD8	CD3		CD45

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Panel configuration example 3

- Weakest antigen with the brightest dye
  CD7, TCRab. So CD7 in PE and TCRab in APC, not in PC7, cannot tolerate spillover

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
	CD7				TCRab	CD8	CD3		CD45

- CD57 is available as PB
- Among CD4, CD5, CD4, TCRgd; CD5 in FITC, untouched, clear discrimination
- CD4 in ECD as parent of CD7-PE

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
CD5	CD7	CD4			TCRab	CD8	CD3	CD57	CD45

- CD2, TCRgd in PC5.5 and PC7

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
CD5	CD7	CD4	TCRgd	CD2	TCRab	CD8	CD3	CD57	CD45

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Panel configuration example 4

Myeloid cell markers:
CD15, CD123, CD11b, CD16, CD13, CD33, CD14
Activation markers:
CD38, HLA-DR

- Parent markers: CD14 (mono), CD16 (NK and myeloid cells)
  CD14 in AA750 and CD16 in AA700

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
						CD16	CD14		CD45

- Weak markers: CD13, CD33, CD11b, CD123
- CD13/CD33 is a convenient combination, can tolerate spillover, classic PC5.5/PC7

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
			CD33	CD13		CD16	CD14		CD45

- CD123 in PE and CD11b in APC. CD11b can be also be placed in PB

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
	CD123		CD33	CD13	CD11b	CD16	CD14		CD45

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Panel configuration example 4

- CD15, CD38, HLA-DR are medium/weak markers,
- Can be placed in FITC, ECD, PB

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
	CD123		CD33	CD13	CD11b	CD16	CD14		CD45

- HLA-DR is expressed, can be placed in FITC, ECD, PB
- HLA-DR is an activating marker and will spill into all cell types, ideally placed in an “untouched” channel with no spillover issues, like PB

Other possibilities:

CD15 is available only as FITC or PB

1

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
CD15	CD123	CD38	CD33	CD13	CD11b	CD16	CD14	HLA-DR	CD45

2

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
CD38	CD123	HLA-DR	CD33	CD13	CD11b	CD16	CD14	CD15	CD45

- CD38 can also be placed in AA700, moving CD16 to AA750, CD14 in ECD

3

FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PacBlu	KrO
CD15	CD123	CD14	CD33	CD13	CD11b	CD38	CD16	HLA-DR	CD45

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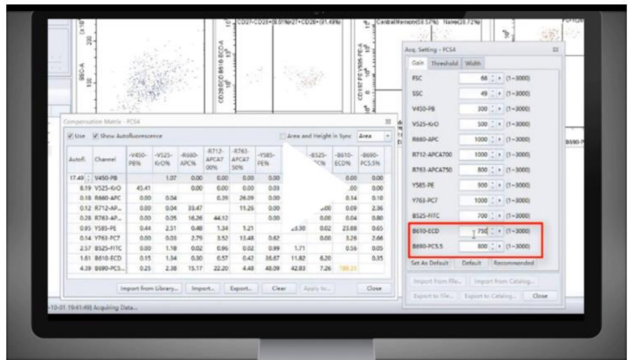
Gain Independent Compensation

A feature in CytExpert for CytoFLEX software is called Gain Independent Compensation. In a CytExpert compensation matrix, the gain value is recorded along with the spillover values. When the gain is adjusted during the experiment, the compensation matrix is updated to reflect the change in the spillover values.



Clip from CytExpert Compensation Workflow Video demonstrating Gain Independent Compensation.

This feature is made possible by the types of detectors used and how they were implemented in the CytoFLEX platform. Due to the reproducible semiconductor manufacturing process, the gains of the detectors used in the CytoFLEX platform can be calibrated for a linear response. Measured intensities are linear to the detector gain setting across a wide range of gain settings. This means the fluorescence measurement increases or decreases linearly based on the gain adjustment. (Each instrument has its own gain/fluorescence curve for each detector).

Gain Independent Compensation is one of the features that helps users new to flow cytometry implement multicolor panels into their experimental programs and laboratories.

WHITE PAPER

CytoFLEX Platform Gain Independent Compensation Enables New Workflows

Objectives

- See example data showing different compensation workflow scenarios
- Understand the role of detector adjustments on the compensation spillover values
- Learn about CytExpert software automatic compensation algorithm and Gain Independent Compensation

Introduction

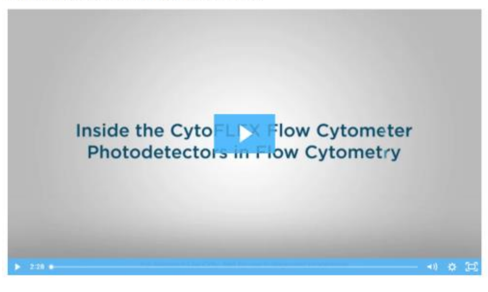
In flow cytometry, we use fluorochromes to label markers of interest on cells. These fluorochromes emit light (fluorescence) in a spectrum. Some of the fluorescence will be collected in the detection channel of interest, but some of the fluorescence will overlap with other channels.

Compensation is the process that we use to remove the unwanted light signals from all detectors except the one devoted to that fluorochrome. It is a mathematic procedure based on a fundamental constant proportional relationship between the two signals, Figure 1.

Figure 1. Proportionality of Signals. For a given cytometer configuration, "spillover" can be observed where signal from a fluorochrome will be captured in channels other than the designated channel used to measure it. The spectrograph shows the theoretical emission characteristics of FITC into two detection channels, 525/40 (primary channel) and 585/42 (spillover), panel A. The bivariate plot shows observed spillover using FITC beads acquired on the CytoFLEX V-B-R Flow Cytometer without spectral compensation, panel B. The equation shows the constant relationship between the Primary signal and Spillover Signal.

Visualize the Possibilities | 1

Photodetectors in Flow Cytometry



Related Videos

CytExpert Compensation Workflow

Describes fundamentals of fluorescence spillover and the process of compensation in flow cytometry. CytExpert software compensation setup and multicolor flow cytometry set up is shown along with a demonstration of gain independent compensation available in the CytoFLEX flow cytometry platform. →

Generating a Compensation Matrix using CytoFLEX

- Gain Independent Compensation (CytoFLEX ecosystem reinforcement webpage)
- CytoFLEX Platform Gain Independent Compensation Enables New Workflows (webpage)
- CytExpert Compensation Workflow Video
- Photodetectors in Flow Cytometry Video

Home > Resources > Reading Material > Whitepapers > CytoFLEX Platform Gain Independent Compensation Enables New Workflows

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INTRODUCTION

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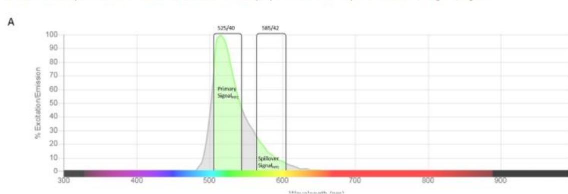

Gain Independent Compensation

Two types of photon detectors are used to amplify and then convert light into electronic signals in flow cytometry. The traditional detector is the photomultiplier tube (PMT). Avalanche Photodiode (APD) are another type of photon detector.

A voltage is applied that accelerates the electron through the detector to amplify the signal. Increasing the voltage increases the energy of the electrons and thereby amplifies the signal. A measure of the amplification is a unitless quantity, called the Gain.

Signal Intensity versus Gain: Photomultiplier Tube Versus Avalanche Photodiode

In a PMT, the gain isn't linear. For example, if the voltage is increased by a factor of 2, the resulting signal, or MFI, does not double. Non-linear detection means that measurements taken at different voltages cannot be compared. Consequently, compensation needs to be empirically measured at every setting for each experiment.

Due to the reproducible semiconductor manufacturing process the gains in an APD can be calibrated for a linear response. Measured intensities are linear to the detector gain setting. This linearity means that a compensation matrix obtained at one gain setting can be used for experiments at different gain settings.

CytExpert for CytoFLEX Acquisition and Analysis Software Compensation Import

1. Use Suggested Gain settings to create compensation. Use beads to acquire the single color stains. Save to the Compensation Library or export to a Comp file.
2. Import the compensation and gains into the New Experiment.
3. Then adjust gains on the sample if needed, compensation will automatically adjust.

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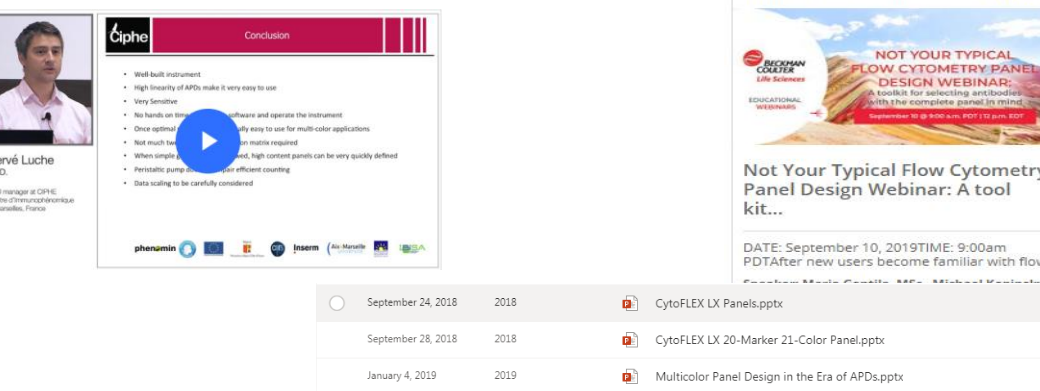
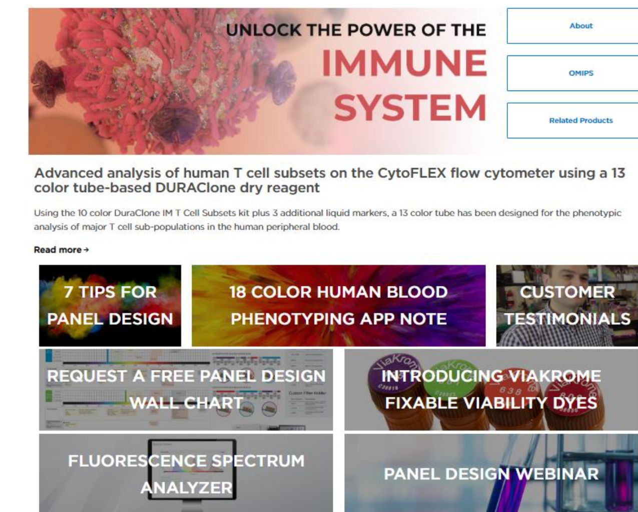
Beckman Coulter Life Sciences



ORIGINAL ARTICLE

Deep Phenotyping of Immune Cell Populations by Optimized and Standardized Flow Cytometry Analyses

Fabien Pitoiset,[1,2†] Lydie Cassard,[3†] Karim El Soufi,[1,2] Lisa Boselli,[3] Jonathan Grivel,[3] Alexandra Roux,[1,2] David Klatzmann,[1,2] Nathalie Chaput,[3,4†] Michelle Rosenzwajg[1,2†*]



"Increased usage and performance of flow cytometry experiments by lab personnel; beginner – intermediate flow cytometry users are attempting to design more complex multi-color experiments

— Chris Corkum, Research Assistant, Memorial University of Newfoundland

- Herve Luche LX Evaluation Webinar
- Kapinsky, Gentile: Panel Design Webinar
- Parks et all Cytometry Publication
- Immunophenotyping Web Section
- Tech Validate Testimonials (Chris Corkum)
- Applications Flipbook chapter Immunophentotyping

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Thank you for your attention

Questions?

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