

10 Farb -  
Immunphänotypisierung

# Bedingungen für die Multicolor – Durchflusszytometrie

## 3 Laser / 10 Farben mit Gallios / Navios

- Engere Bedingungen bei der Probenvorbereitung  
10 Antikörper davon 5 Tandem Konjugate
- Größeres Repertoire von Konjugaten  
mit unterschiedlichen Eigenschaften
- Grundlagen der Fluoreszenz Kompensation
- Zusammenstellen von Antikörperkombinationen und  
Fluoreszenzfarbstoffen mit komplexen spektralen Überlappungen



# Bedingungen bei der Probenvorbereitung

10 Antikörper davon 5 Tandem Konjugate

# „Neue“ Bedingungen bei der Probenvorbereitung

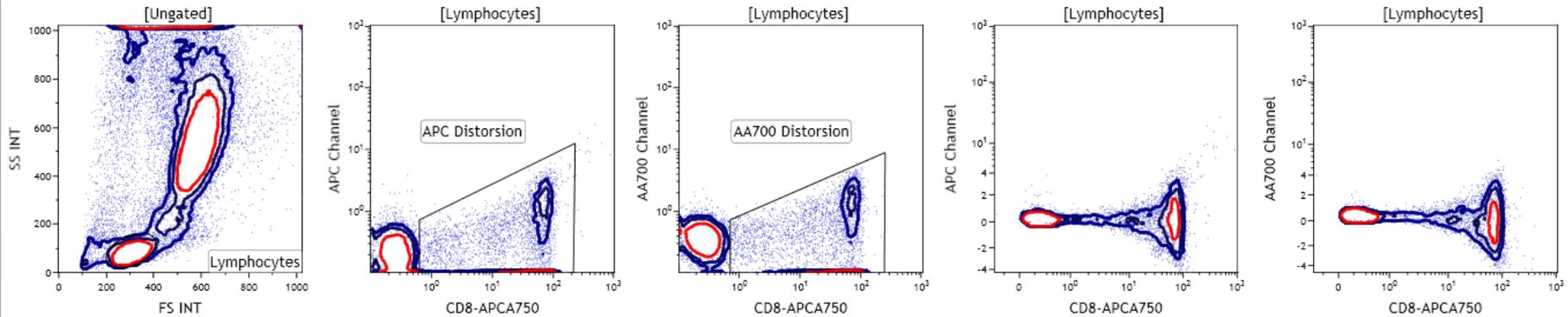
## Lichtempfindliche Fluorochrome !


$$E = h \times v$$

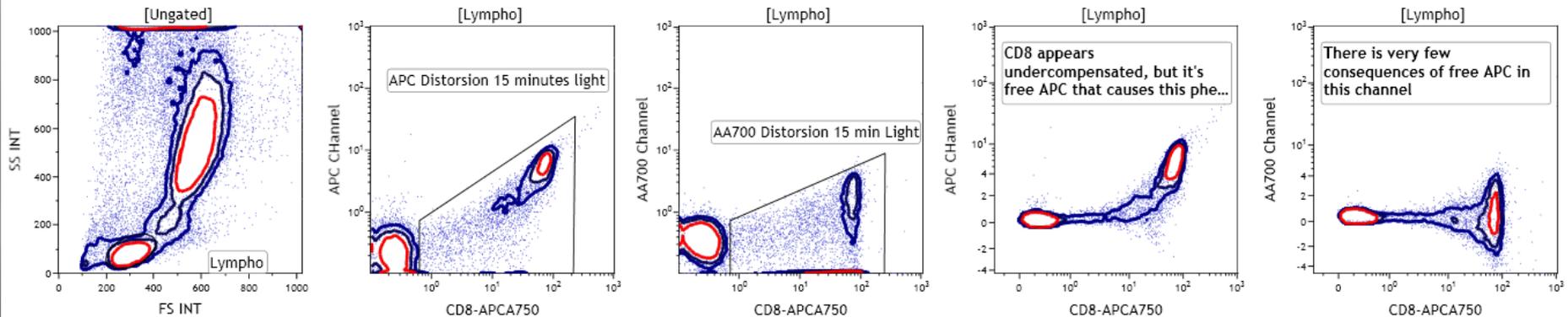
- Reduzierte Beleuchtung beim Pipettieren
- Inkubation im Dunkeln
- Lagerung der fertigen Proben dunkel und kühl

# „Neue“ Bedingungen bei der Probenvorbereitung

Data Set 1: 8APCA750 dark wash 00021776 010 • Event Count: 100 000



Data Set 2: 8APCA750 light wash 00021777 011 • Event Count: 100 000



CD8-APCA700 Aliquots: 15 min Exposition im Tageslicht vs dunkel gelagerte Probe

(data from Olivier Jaen / BC France)

# Biomek ACP



**Dark and cooled reagent racks**

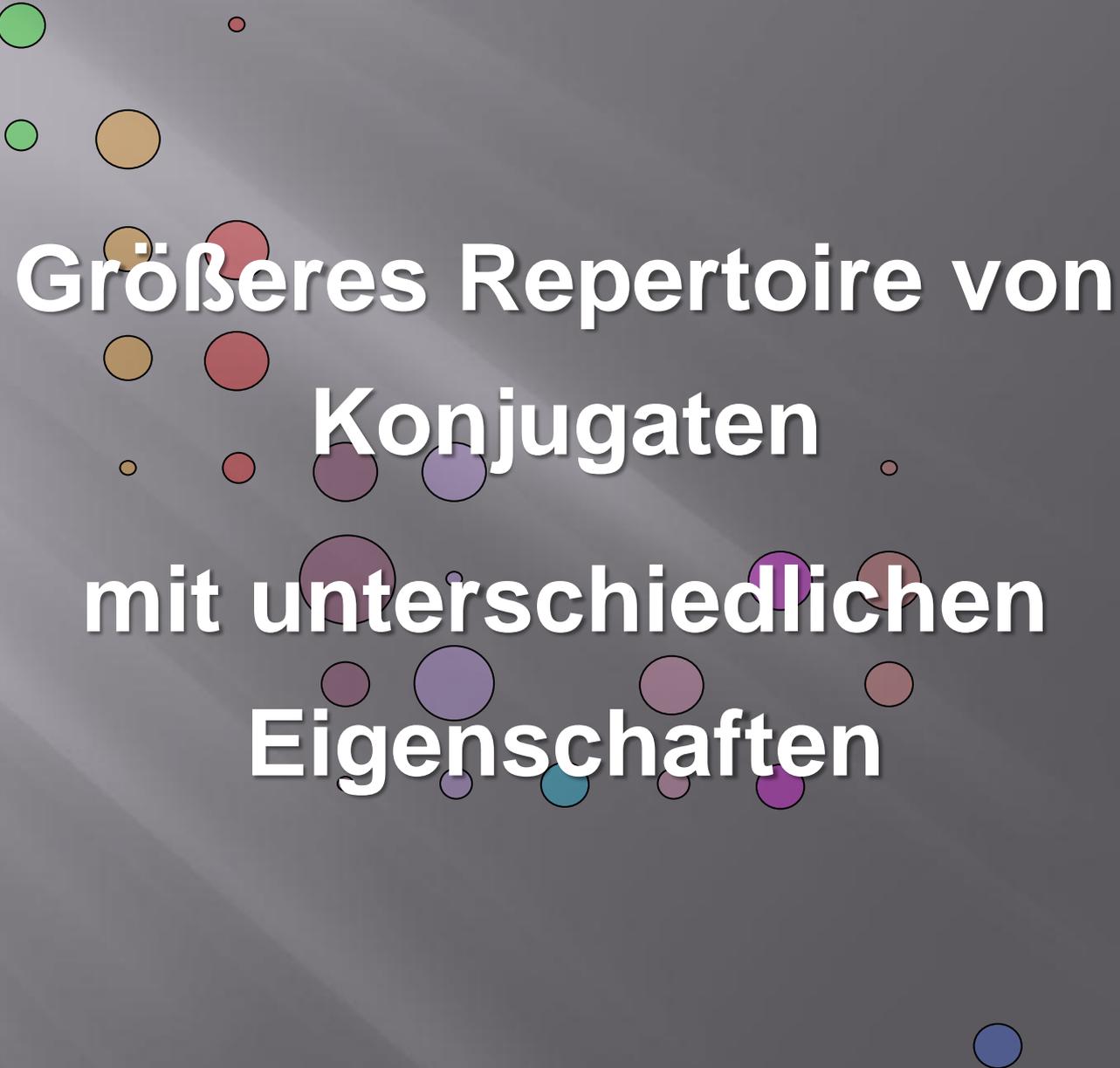
**Barcode tracked pipetting log and reagent administration**

**Automated hardware QC**

**10-min-to-learn interface**

**Surpasses manual preparation speed**

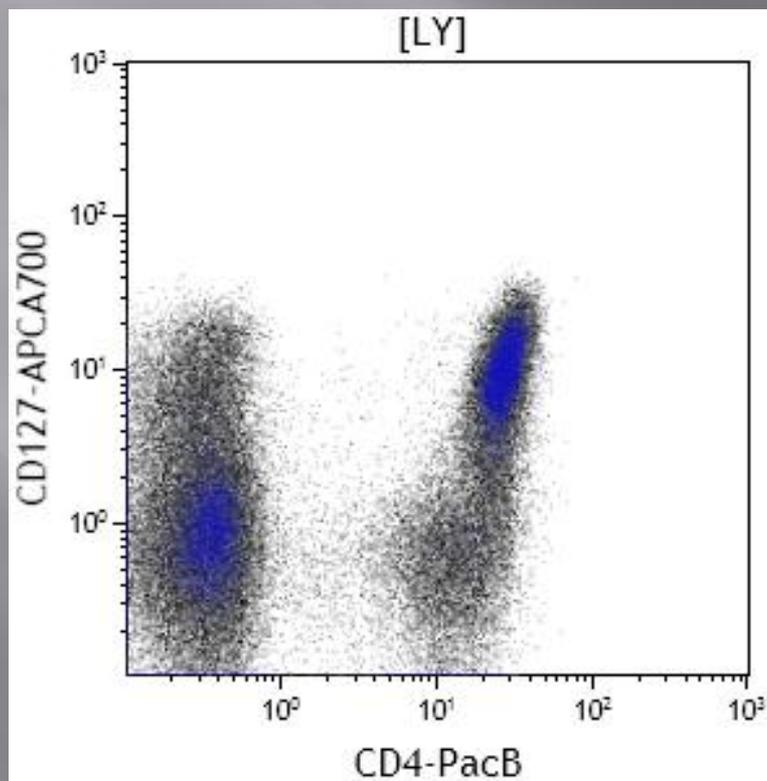
...



**Größeres Repertoire von  
Konjugaten  
mit unterschiedlichen  
Eigenschaften**

# Neue rot anregbare Fluoreszenzfarbstoffe:

## APC-AlexaFluor700

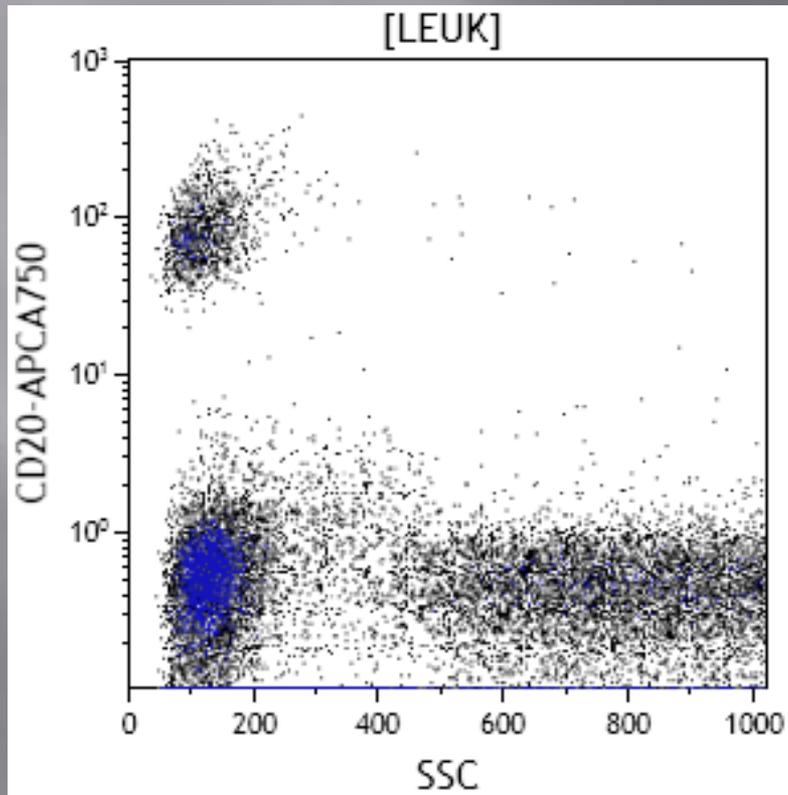


Spillover (%)			
	FL6	FL7	FL8
FL6		12,10	13,70
FL7	24,20		8,20
FL8	9,30	21,40	

**Brightness**  
 $\geq$  PE, APC

# Neue rot anregbare Fluoreszenzfarbstoffe:

## APC-AlexaFluor750

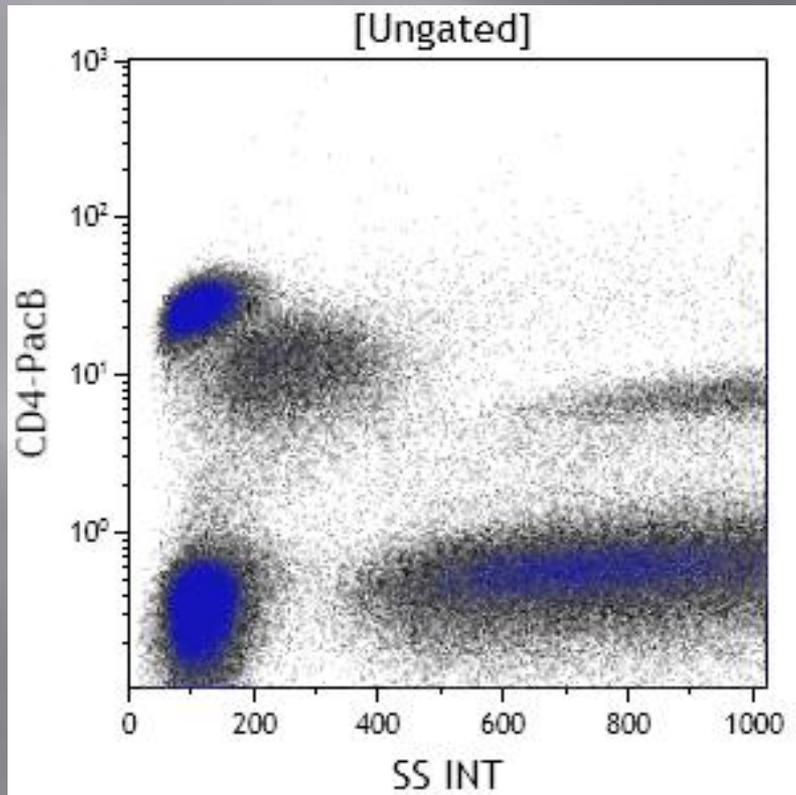


Spillover (%)			
	FL6	FL7	FL8
FL6		12,10	13,70
FL7	24,20		8,20
FL8	9,30	21,40	

Brightness  
= ECD

# Neue violett anregbare Fluoreszenzfarbstoffe:

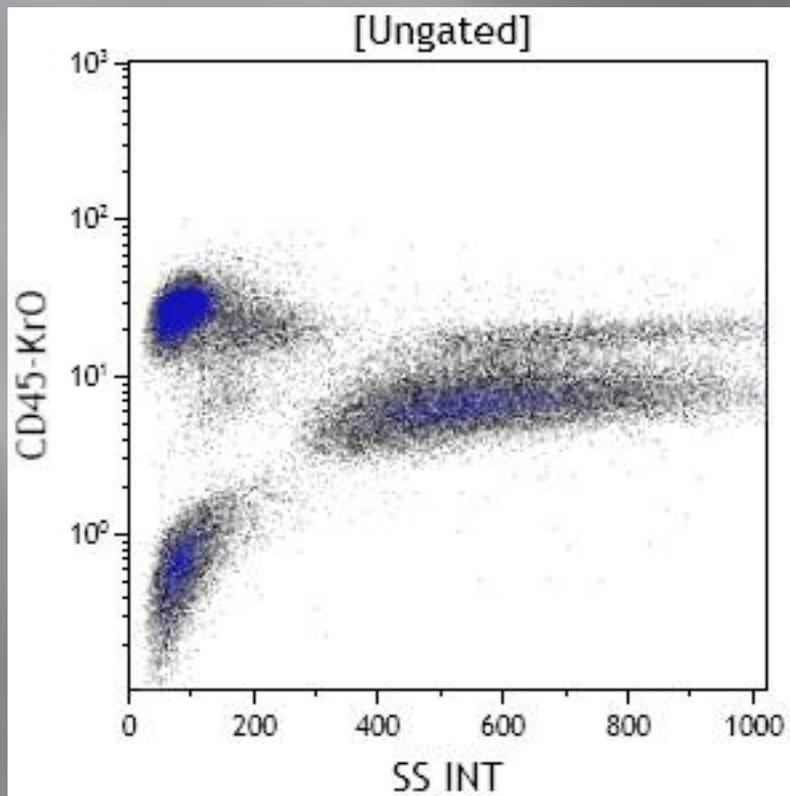
## Pacific Blue



Spillover (%)		
	FL9	FL10
FL9		1,60
FL10	18,90	

**Brightness  
= FITC**

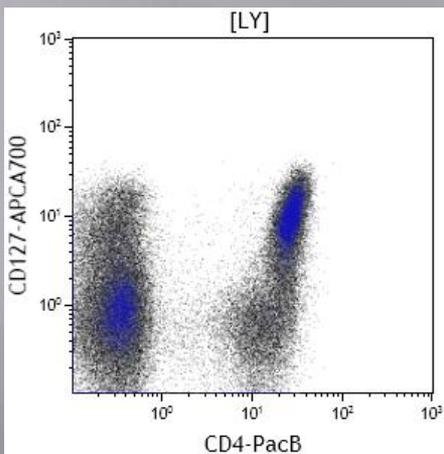
# Neue violett anregbare Fluoreszenzfarbstoffe: Krome Orange



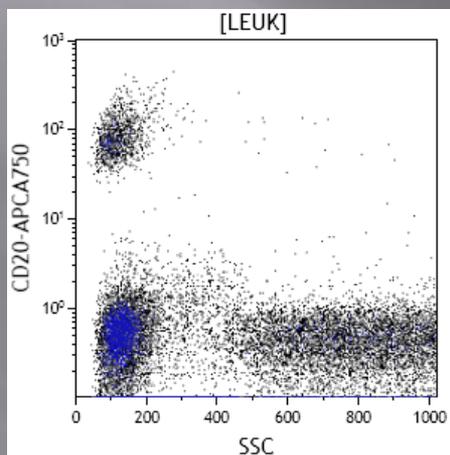
Spillover (%)		
	FL9	FL10
FL9		1,60
FL10	18,90	

Brightness  
 <FITC  
 >PacOrange  
 >V500

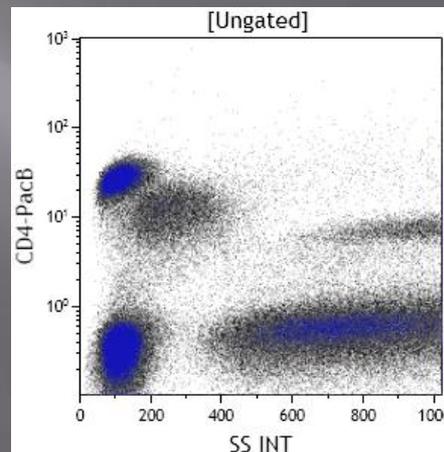
# Neue Fluoreszenzfarbstoffe



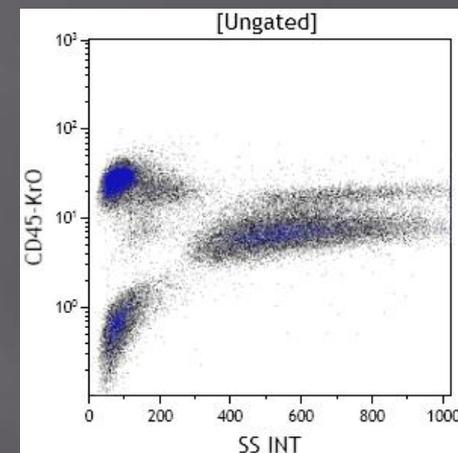
**APC-AlexaFluor700:**  
Tandem dye,  
excited at 638nm,  
brightness  $\geq$  PE, APC  
(wash)



**APC-AlexaFluor750:**  
Tandem dye,  
excited at 638nm,  
brightness  $\cong$  ECD (wash)



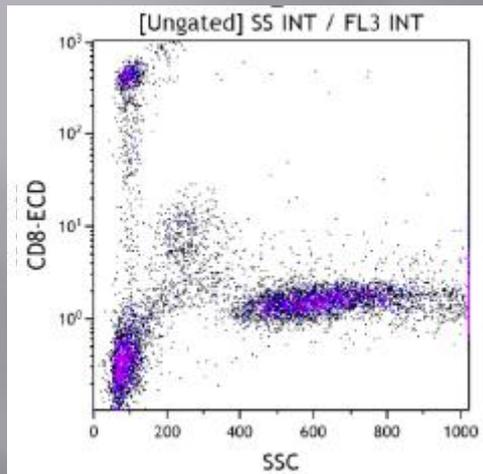
**Pacific Blue:**  
Synthetic dye  
excited at 405nm,  
brightness  $\cong$  FITC (wash)



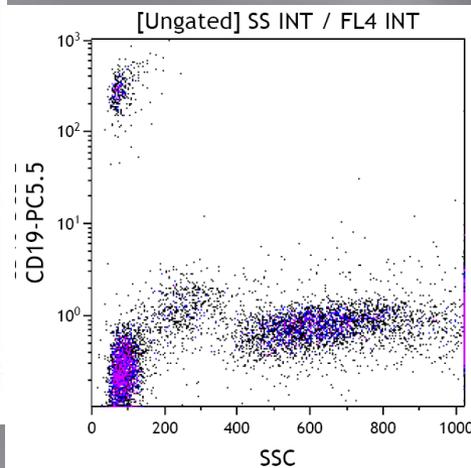
**Krome Orange:**  
Synthetic dye  
excited at 405nm,  
brightness  $<$  FITC (wash)  
 $>$ Pacific Orange

# Verbesserte Messempfindlichkeit am Gallios / Navios für einige „klassische Fluoreszenzfarbstoffe

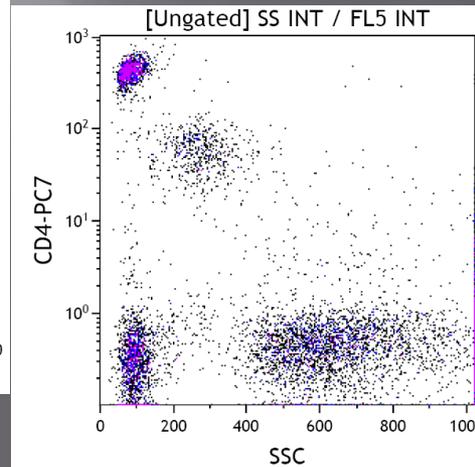
ECD, PC5.5, PC7, APC



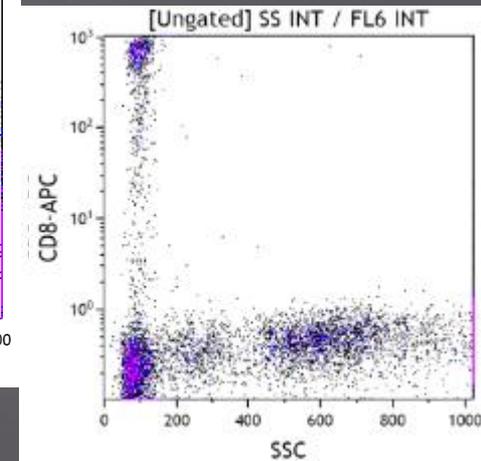
Beispiel: CD8-ECD



Beispiel: CD19-PC5.5

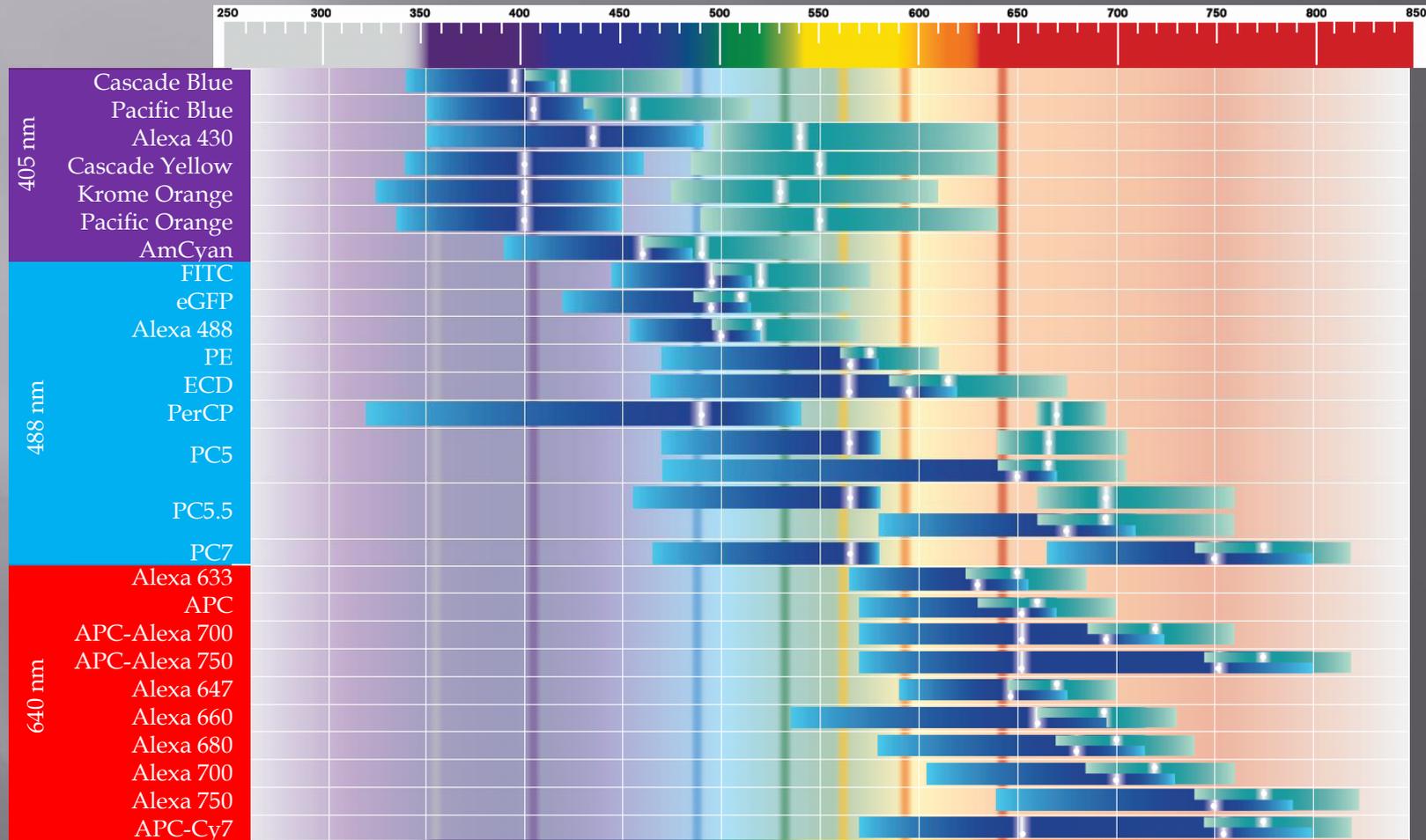


Beispiel: CD4-PC7



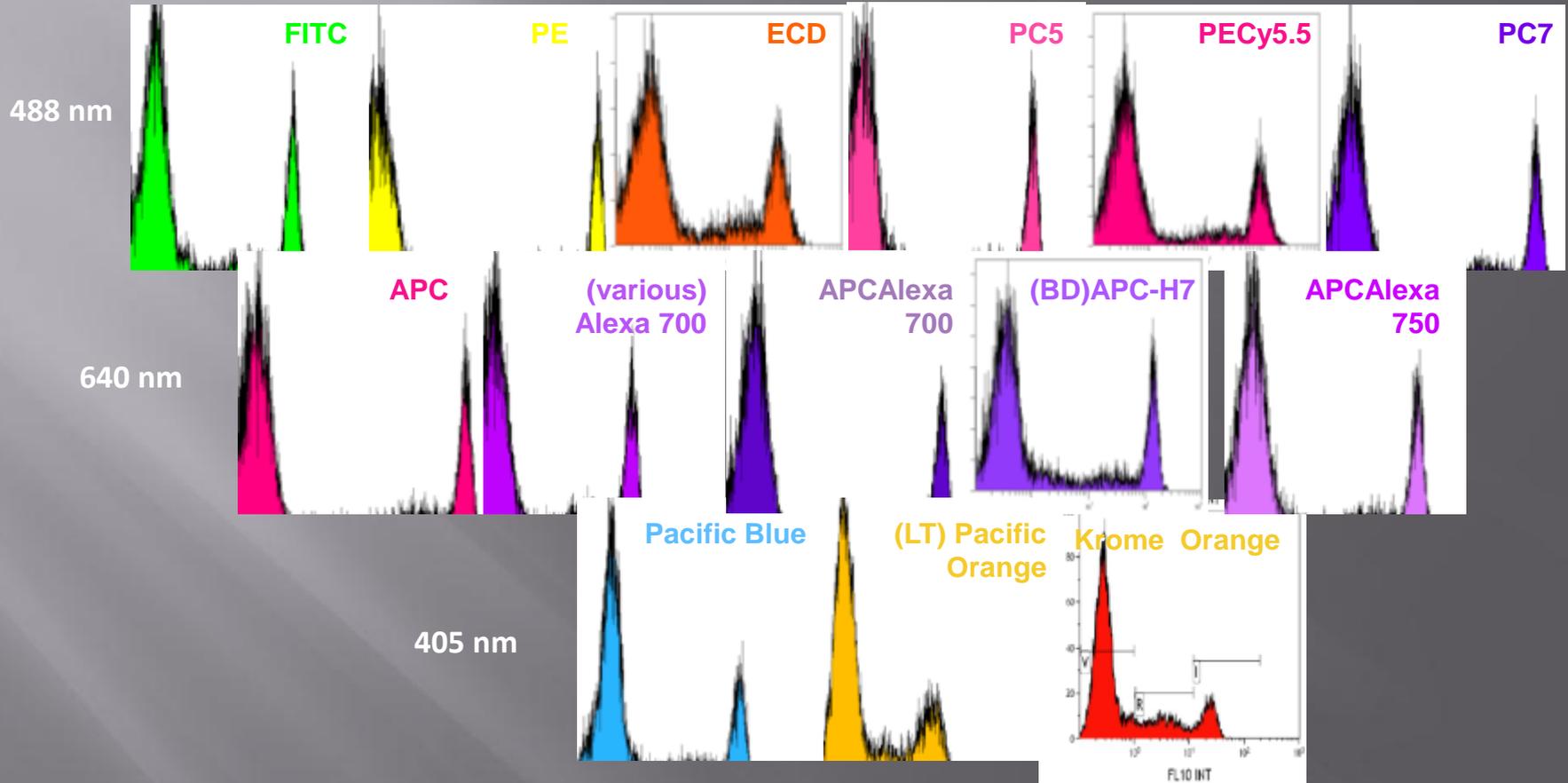
Beispiel: CD8-APC

# Fluoreszenzfarbstoffe



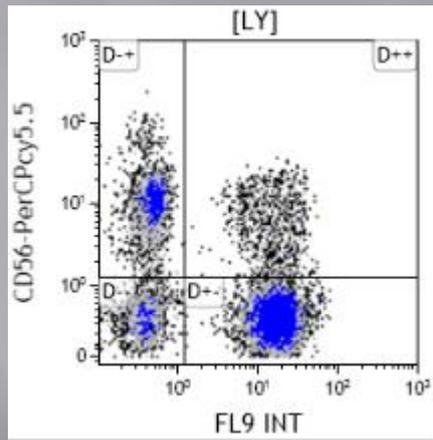
# Fluoreszenzfarbstoffe

Histogramme verschiedener CD8 - Konjugate

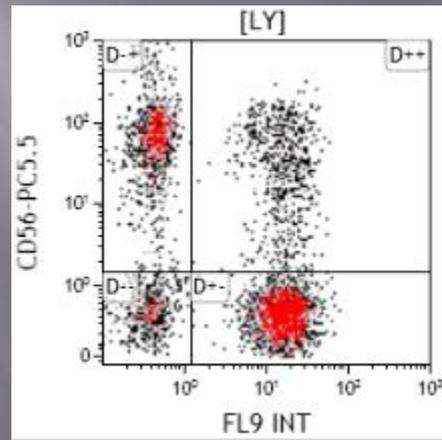


# CD56-Konjugate

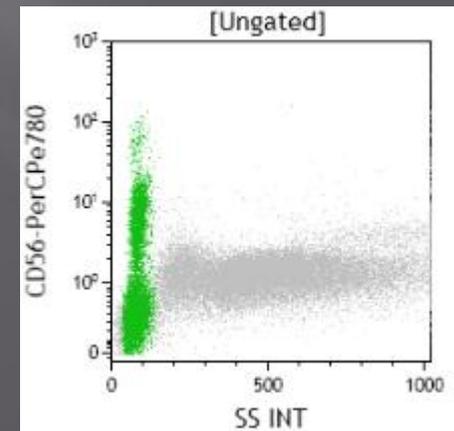
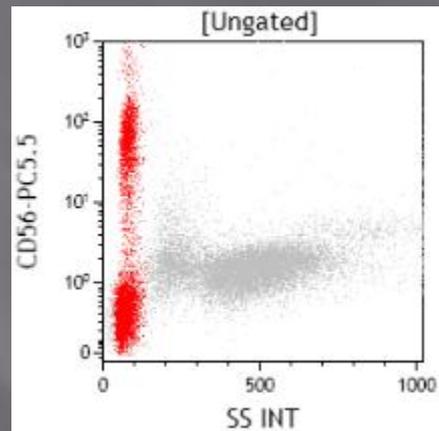
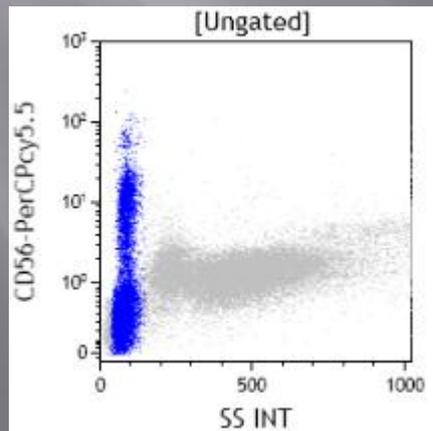
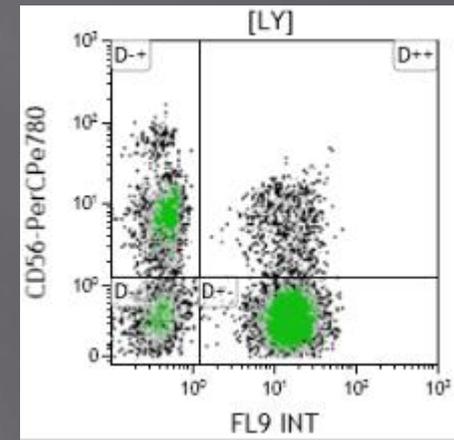
PerCPcy5.5



PC5.5



PerCPe780

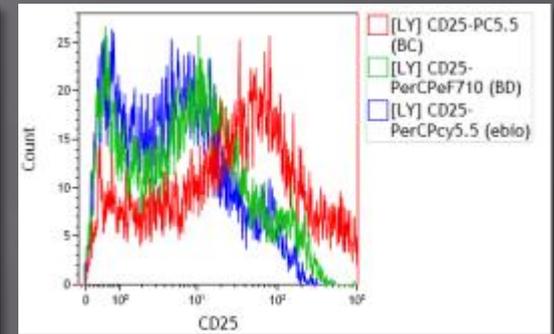
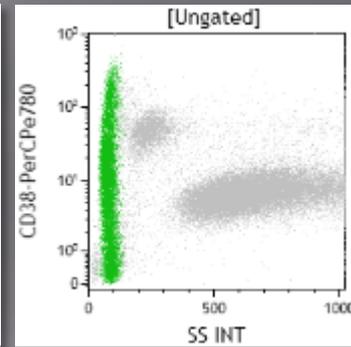
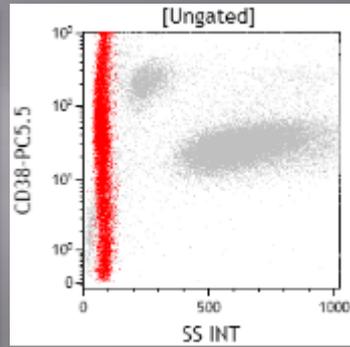
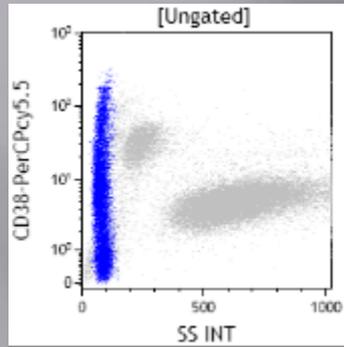


PerCPcy5.5

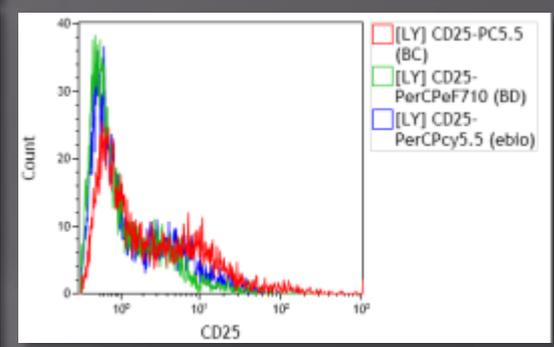
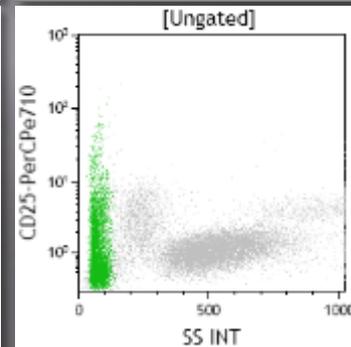
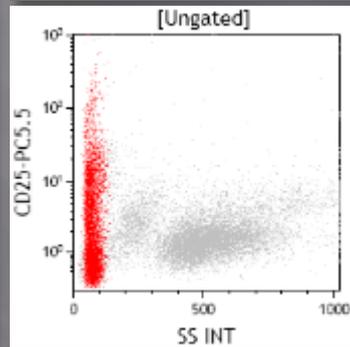
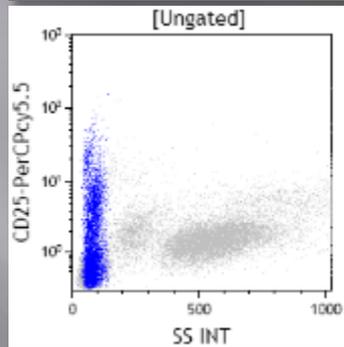
PC5.5

PerCPe780

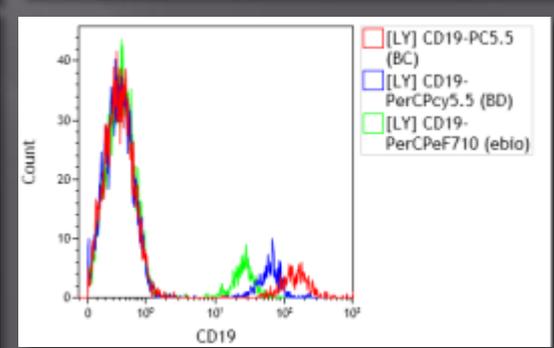
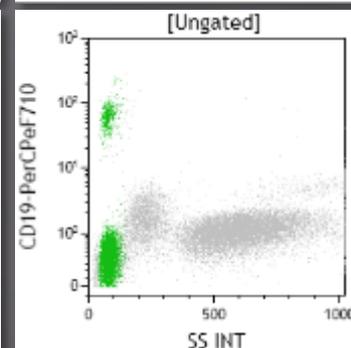
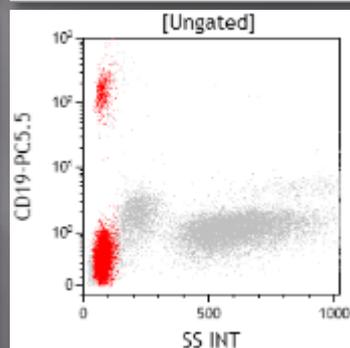
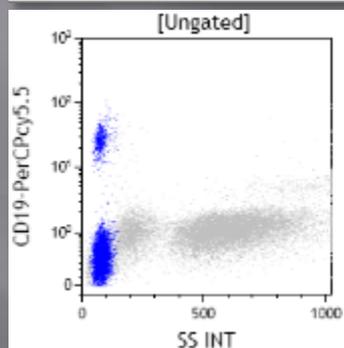
CD38



CD25



CD19

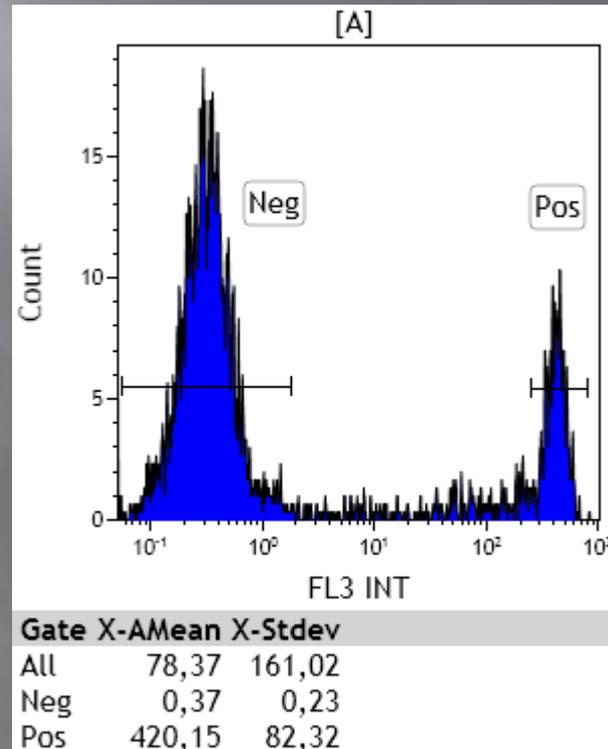


# Signal / Rauschabstand und Auflösung

Signal-to-Noise Ratio:

$MFI(pos) / MFI(neg),$

$420.15 / 0.37 = \underline{1136}$



Staining Index:

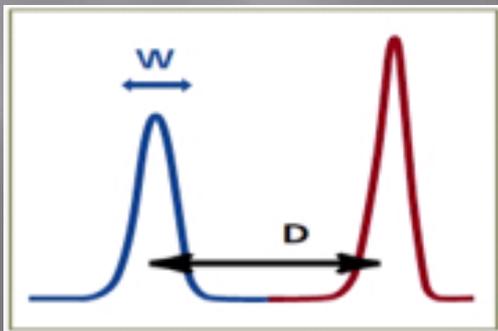
$(MFI(pos)-MFI(neg))/2*SD(neg),$

$(420.15-0.37)/2*0.23 = \underline{913}$

# Signal / Rauschabstand und Auflösung

CD8-Markierung mit verschiedenen Fluoreszenzfarbstoffen

$$\text{Stain index} = D / W$$

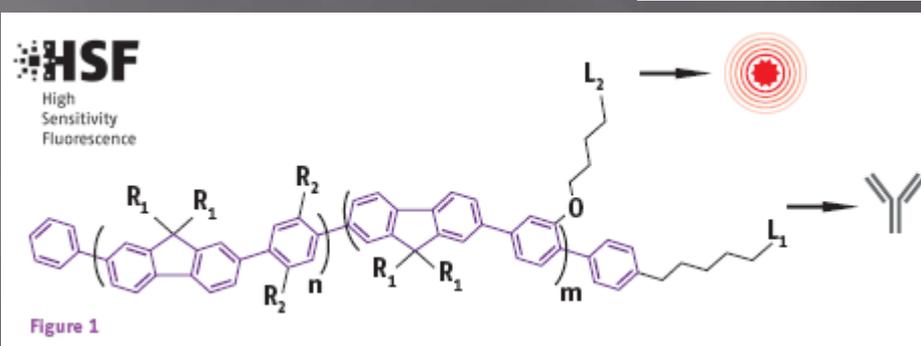
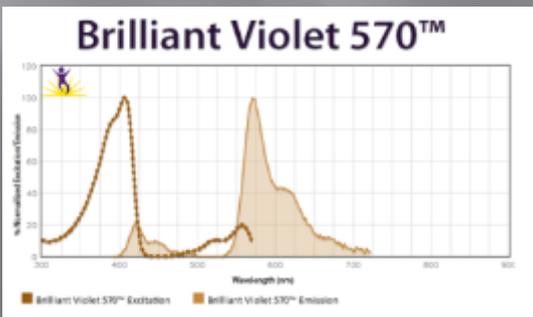
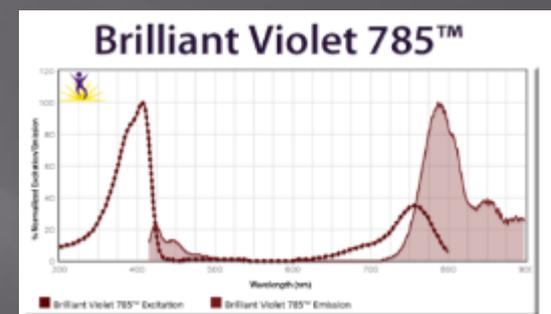
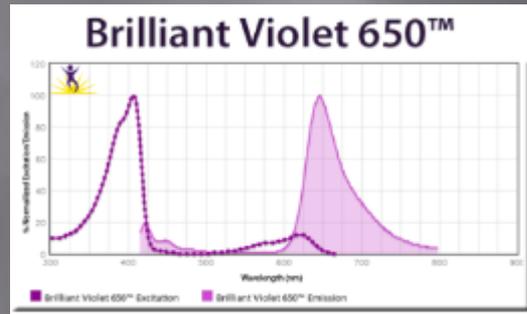
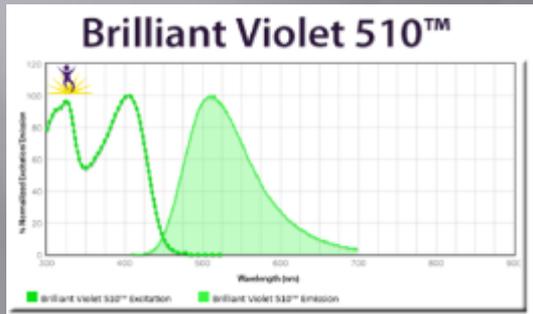
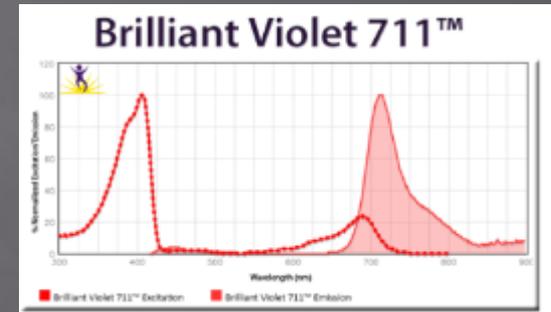
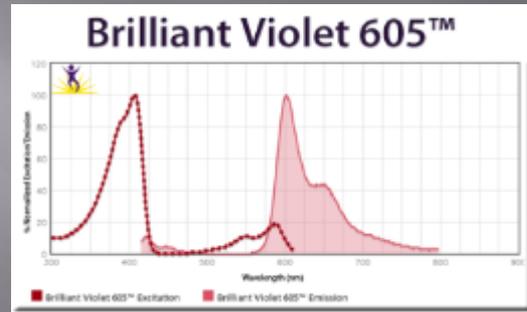
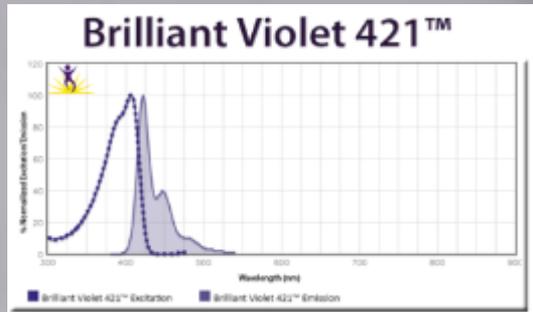


(Quelle: BD Biosciences 'Multicolor Fluorochrome Guide')

Fluorochrome	Laser	Filter	PMT	Stain Index	Gallios
PE	blue	575/30 nm band pass	FL-2	617	
PE-Cy7	blue	755 nm long pass	FL-5	532	
Brilliant Violet-421	violet	450/40 nm band pass	FL-9	476	
APC	red	660/20 band pass	FL-6	440	
Alexa Fluor 647	red	660/20 band pass	FL-6	307	
APC-Cy7	red	755 nm long pass	FL-8	196	
APC eFluor-780	red	755 nm long pass	FL-8	174	
Alexa Fluor 488	blue	525/40 nm band pass	FL-1	133	
PerCP-Cy5.5	blue	675/20 nm band pass	FL-4	118	
FITC	blue	525/40 nm band pass	FL-1	97	
eFluor-650NC	red	660/20 band pass	FL-6	71	
BD Horizon-V450	violet	450/40 nm band pass	FL-9	61	
Pacific Blue	violet	450/40 nm band pass	FL-9	44	
PerCP	blue	675/20 nm band pass	FL-4	32	
eFluor-605NC	blue	620/30 nm band pass	FL-3	27	

Quelle: Dartlab; <http://www.dartmouth.edu/~dartlab/>

# Brilliant Violet Farbstoffe (biolegend) sind Tandem Farbstoffe

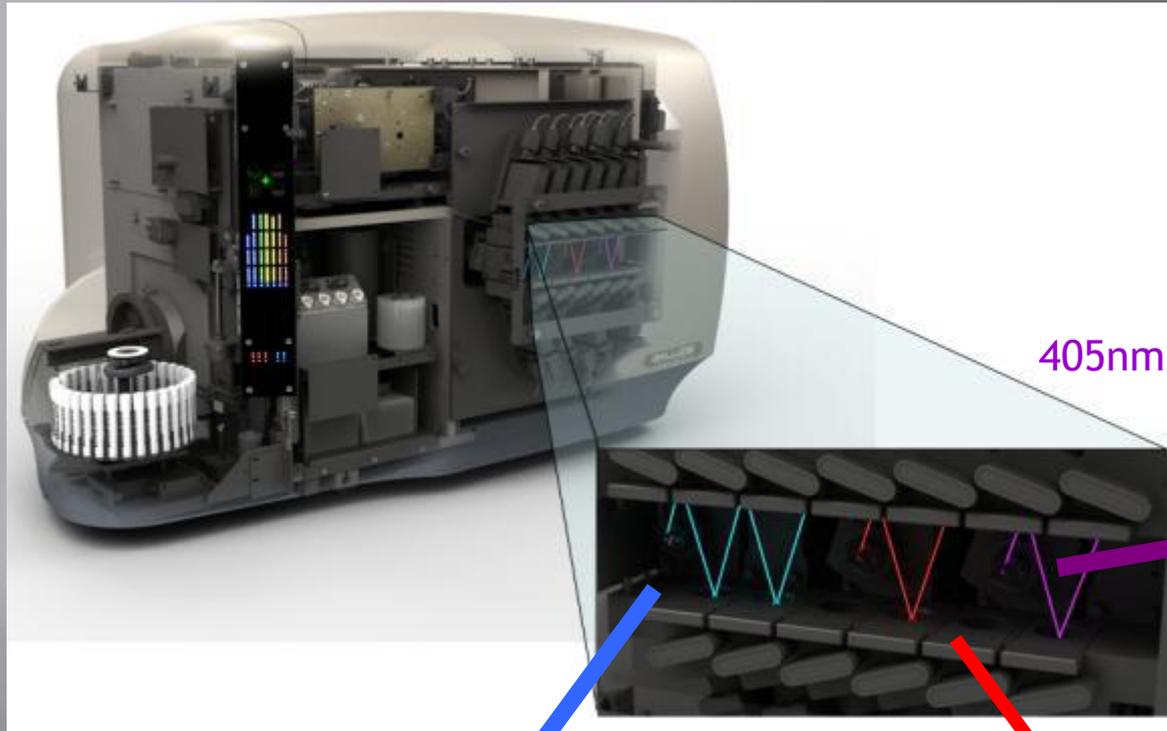


- Grundlagen der
- Fluoreszenz –
- Kompensation

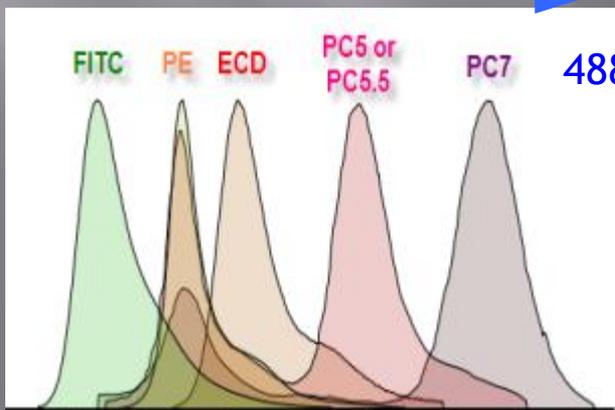
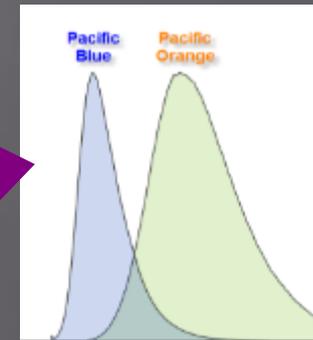


A decorative graphic consisting of numerous circles of various colors (green, brown, red, purple, blue, cyan) and sizes, scattered across the lower half of the slide. Some circles are larger and more prominent, while others are smaller and more subtle.

# 3 Laser / 10 Farben mit Gallios / Navios

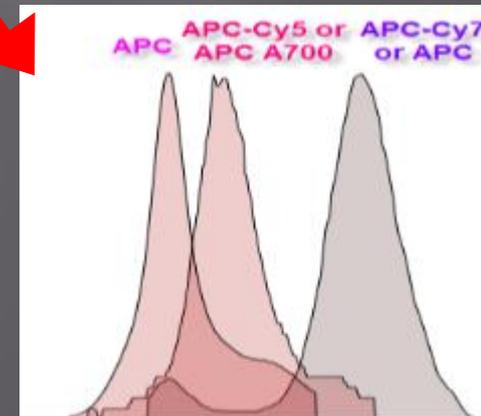


3 Laser foki  
5+3+2 Detektoren



488nm

638nm



# Optische Filter des Gallios / Navios

<b>Laser</b>	<b>Dye Options</b>	<b>Peak Emission</b>	<b>Dichroic</b>	<b>Collection Filter</b>
<b>488nm</b>	<b>FITC</b>	<b>520</b>	<b>550 DCSP</b>	<b>525/38 BP</b>
	<b>PE</b>	<b>575</b>	<b>600 DCSP</b>	<b>575/30 BP</b>
	<b>ECD</b>	<b>620</b>	<b>655 DCSP</b>	<b>620/30 BP</b>
	<b>PE-Cy5</b>	<b>680</b>	<b>730 DCSP</b>	<b>695/30 BP</b>
	<b>PE-Cy5.5</b>	<b>695</b>		
	<b>PE-Cy7</b>	<b>770</b>		<b>755 LP</b>
<b>638nm</b>	<b>APC</b>	<b>670</b>	<b>700 DCSP</b>	<b>675/20 BP</b>
	<b>APCAlexaFluor700</b>	<b>720</b>	<b>745 DCSP</b>	<b>725/20 BP</b>
	<b>APCAlexaFluor750</b>	<b>780</b>		<b>755 LP</b>
	<b>APCCy7</b>	<b>780</b>		
<b>405nm</b>	<b>Pacific Blue</b>	<b>450</b>	<b>480 DCSP</b>	<b>430/40 BP</b>
	<b>Pacific Orange</b>	<b>550</b>		<b>550/40 BP</b>

# Grundlagen der Fluoreszenz Kompensation

„Intralaser“  
Kompensation

Cytometer Control

Acquisition Setup Compensation

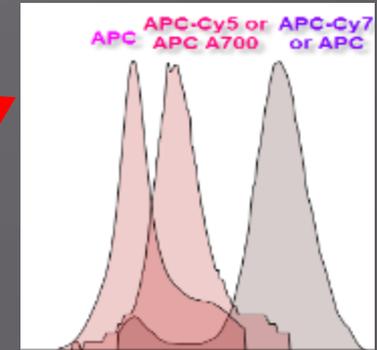
	FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8	FL9	FL10
FL1		1.2	0.1	0.4	0.4	0.2	0.1	0.3	0.4	0.0
FL2	15.8		6.3	2.6	1.4	0.3	0.1	0.2	0.2	0.0
FL3	7.4	53.2		1.6	0.9	0.7	0.1	0.2	0.2	0.0
FL4	2.1	14.2	38.6		0.5	1.2	0.4	0.2	0.1	0.0
FL5	0.2	1.1	3.7	37.9		0.2	0.5	0.8	0.1	0.0
FL6	0.1	0.1	0.3	2.3	0.1		7.6	8.6	0.1	0.0
FL7	0.1	0.2	0.3	30.2	0.8	25.6		5.8	0.1	0.0
FL8	0.1	0.2	0.2	17.2	20.5	10.7	42.8		0.1	0.0
FL9	0.1	0.1	0.1	0.1	0.1	0.8	0.0	0.1		0.0
FL10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	

Select the column which contains the Fluorochrome creating spectral overlap in the PMT (row).

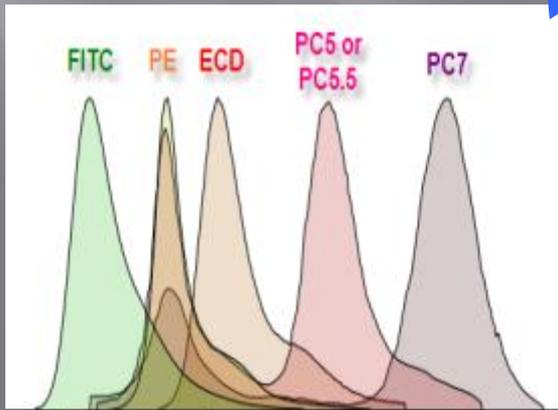
Clear

Close Help

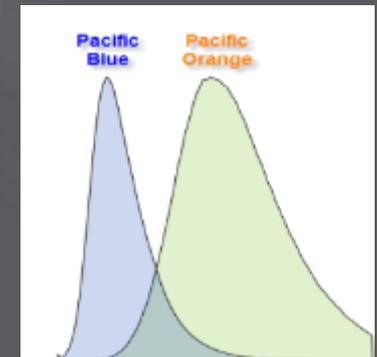
638 nm



488 nm



405 nm



# Grundlagen der Fluoreszenz Kompensation

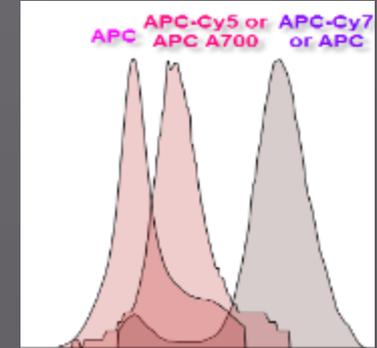
Cytometer Control

Acquisition Setup Compensation

	FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8	FL9	FL10
FL1		1.2	0.1	0.4	0.4	0.2	0.1	0.3	0.4	0.0
FL2	15.8		6.3	2.6	1.4	0.3	0.1	0.2	0.2	0.0
FL3	7.4	53.2		1.6	0.9	0.7	0.1	0.2	0.2	0.0
FL4	2.1	14.2	38.6		0.5	1.2	0.4	0.2	0.1	0.0
FL5	0.2	1.1	3.7	37.9		0.2	0.5	0.8		0.0
FL6	0.1	0.1	0.3	2.3	0.1		7.6	8.6	0.1	0.0
FL7	0.1	0.2	0.3	30.2	0.8	25.6		5.8	0.1	0.0
FL8	0.1	0.2	0.2	17.2	2.8	42.8			0.1	0.0
FL9	0.1	0.1	0.1	0.1	0.1	0.8	0.1			0.0
FL10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		

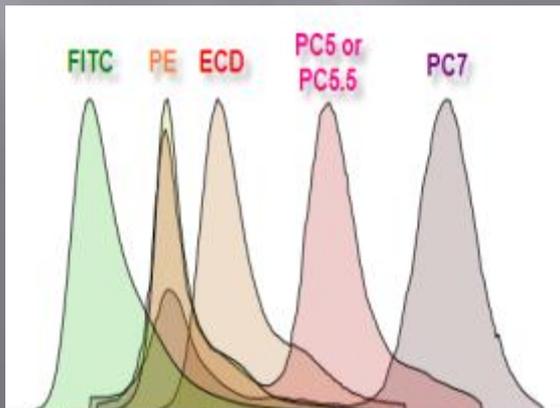
Select the column which contains the Fluorochrome creating spectral overlap in the PMT (row).

638 nm

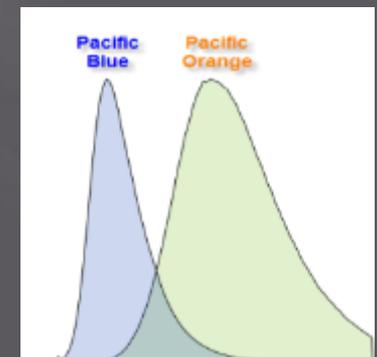


„Interlaser“  
Kompensation

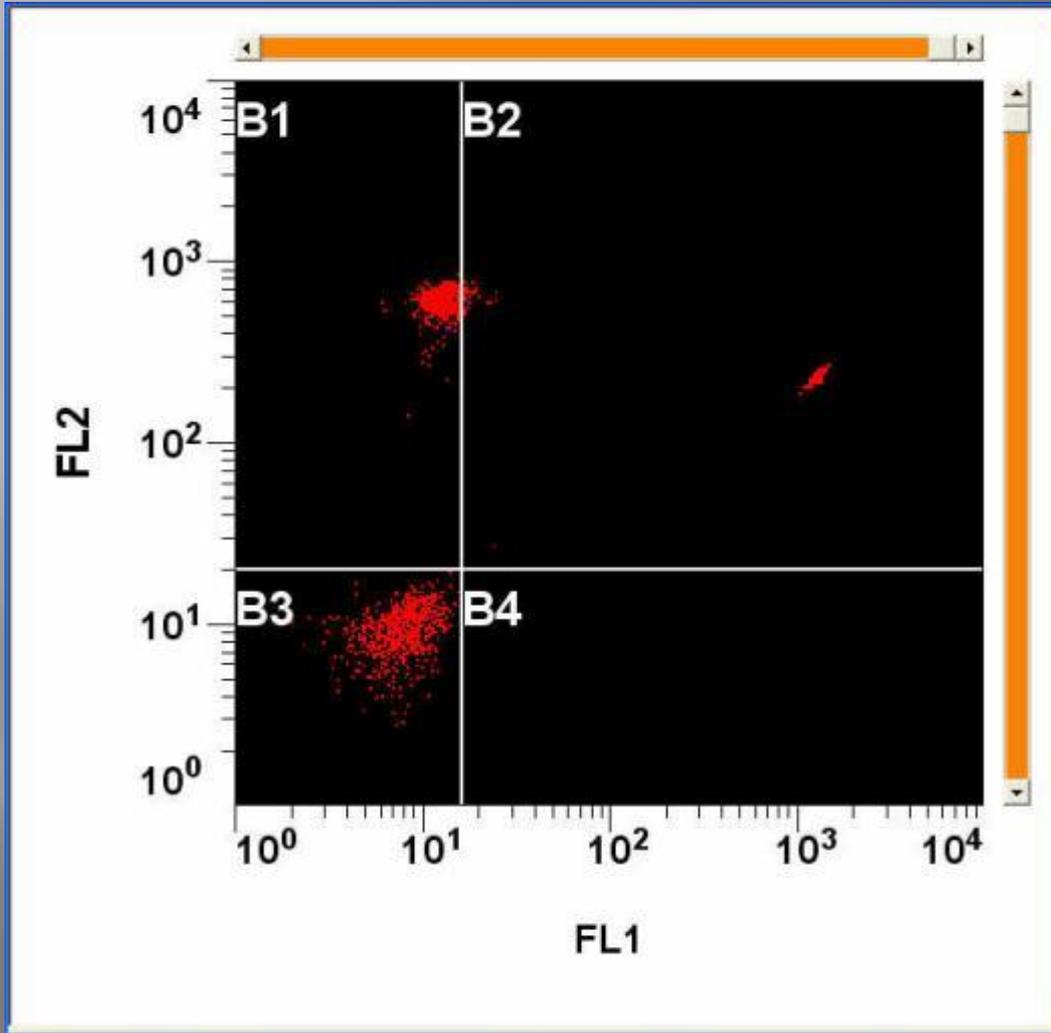
488 nm



405 nm



# Klassische Kompensation

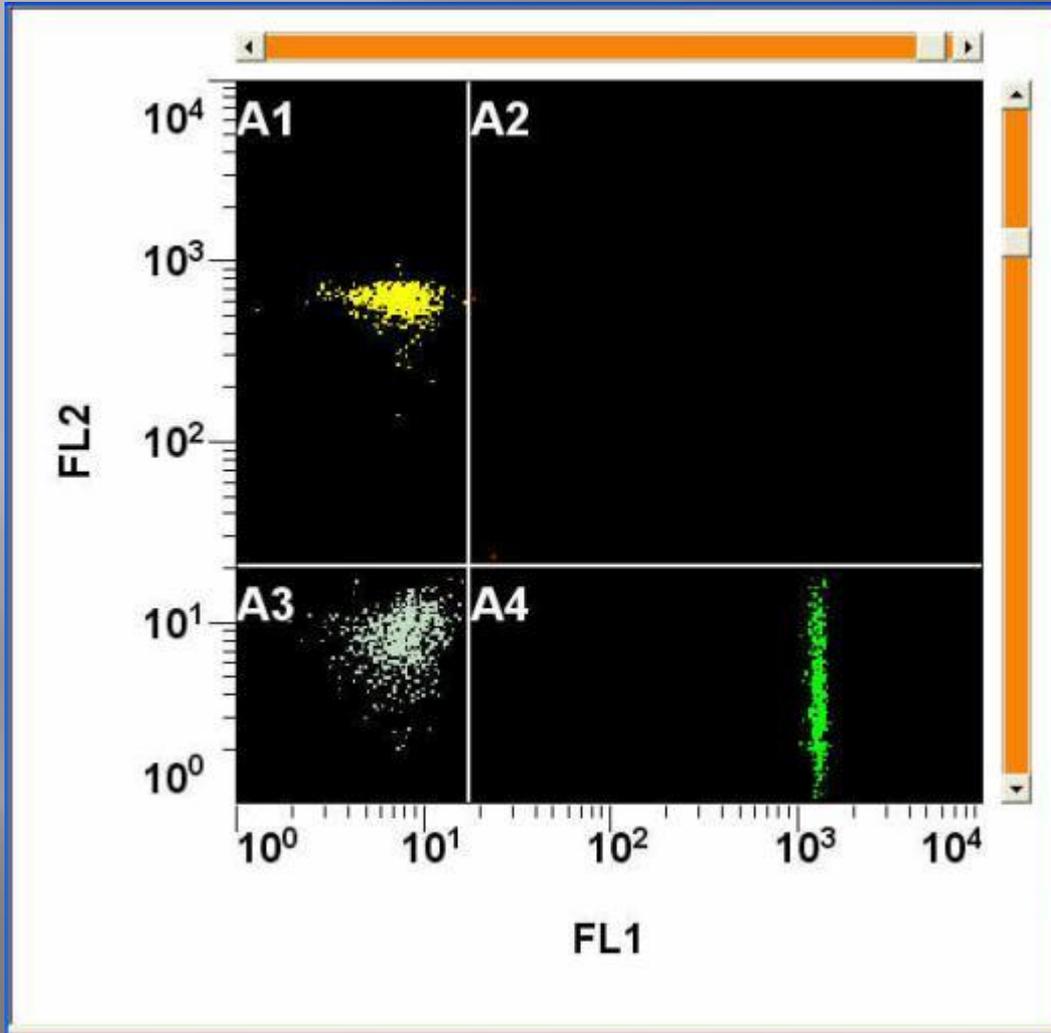


Unkompensiert

Calibright Beads

Blank / FITC / PE

# Klassische Kompensation



Region	X-Mean	Y-Mean
ALL	334	231
A1	7.46	631
A2	331	339
A3	7.85	8.89
A4	1.28e+003	4.93

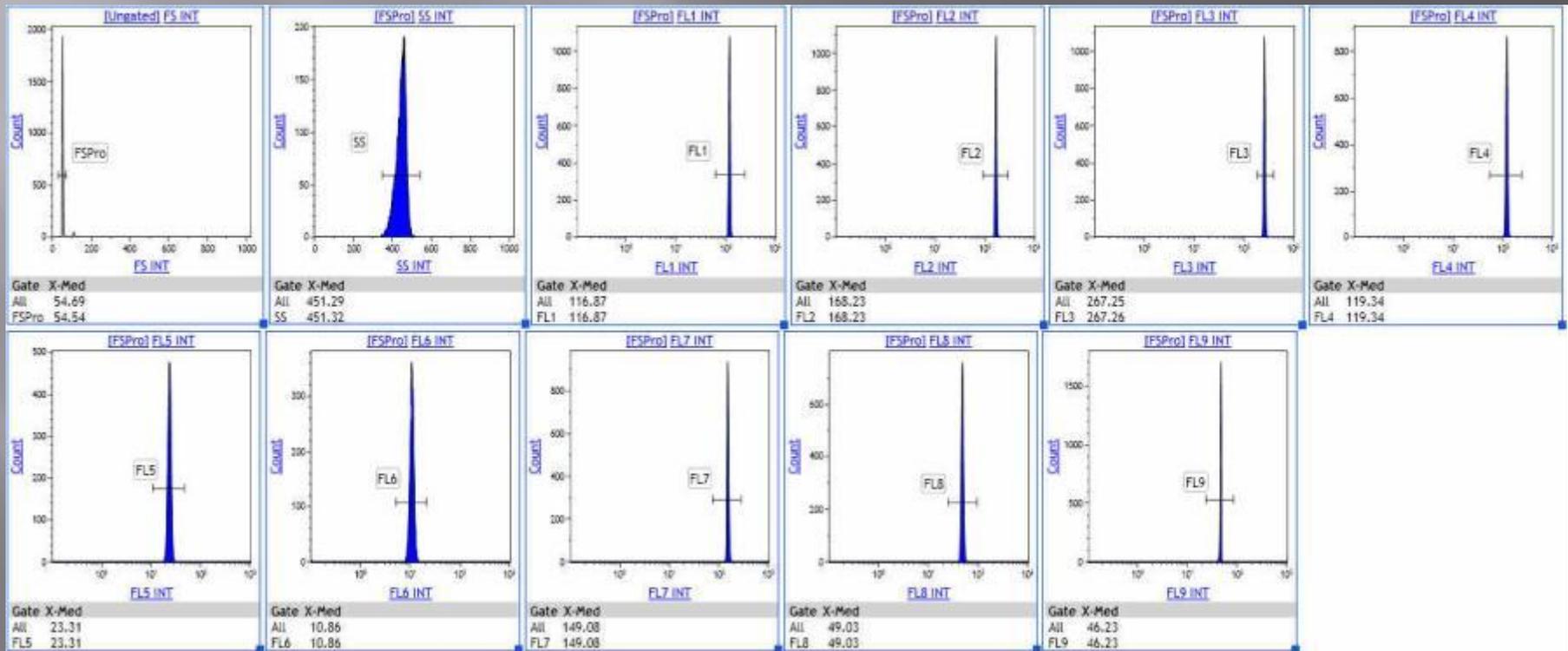
## Übersprech Matrix

	FL1	FL2
FL1		0.9
FL2	18.5	

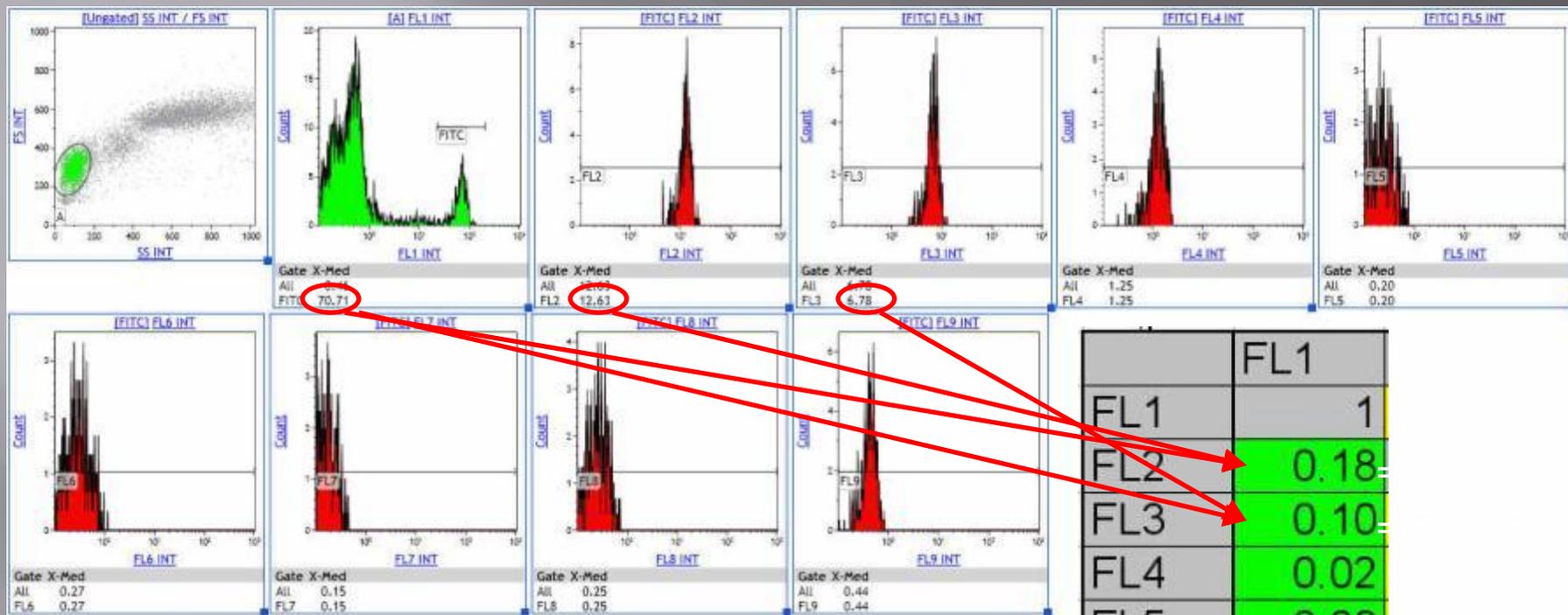
$$FL2_{\text{true}} = FL2_{\text{measured}} - k * FL1$$

# Kalibration und Standardisierung

Applikations-spezifische und Farbstoff-spezifische  
Einstellung der Verstärkung (PMT-Hochspannung)



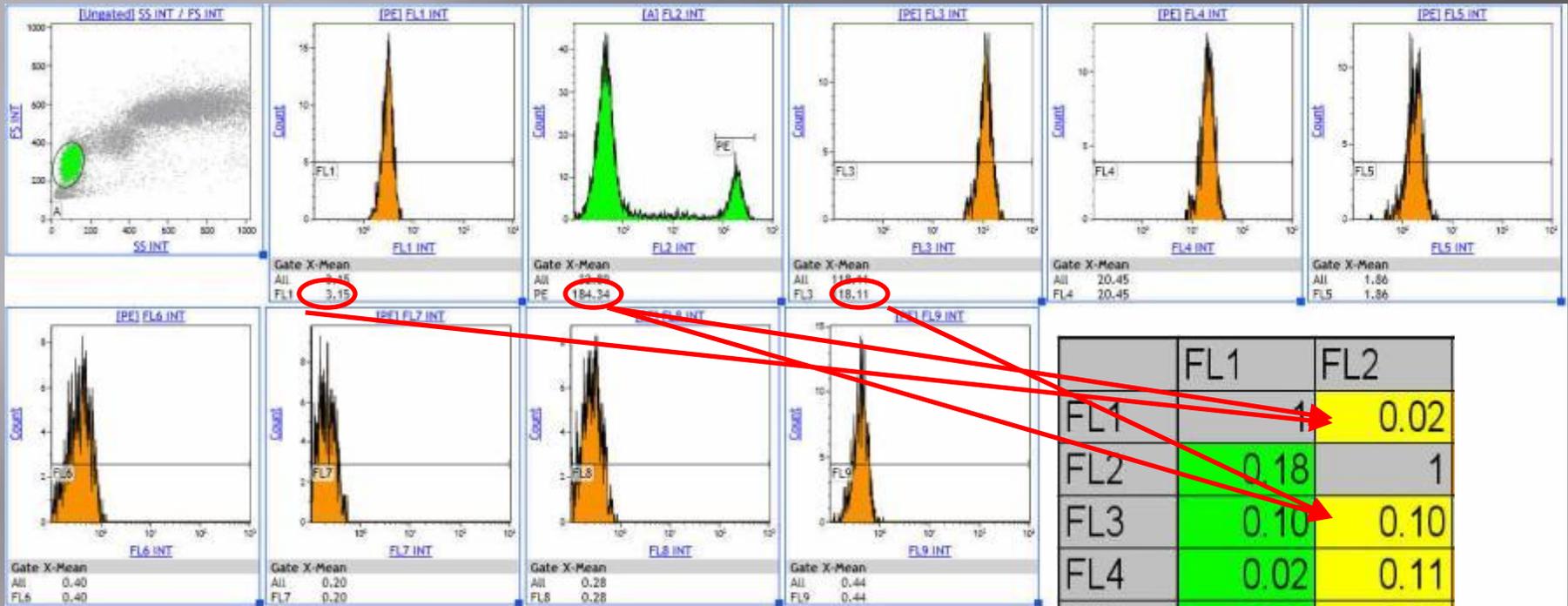
# Automatische Berechnung der relativen Übersprechanteile in die benachbarten Messkanäle



	FL1
FL1	1
FL2	0.18
FL3	0.10
FL4	0.02
FL5	0.00
FL6	0.00
FL7	0.00
FL8	0.00
FL9	0.01

Übersprech - Matrix

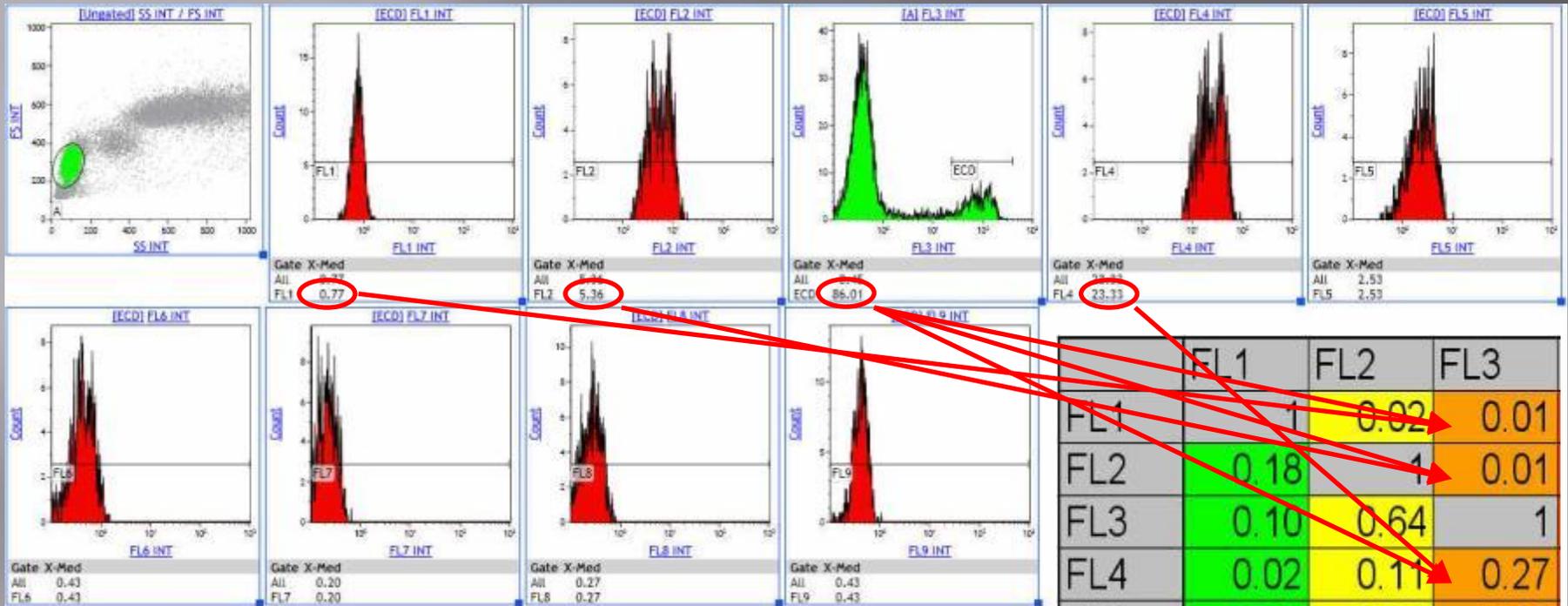
# Automatische Berechnung der relativen Übersprechanteile in die benachbarten Messkanäle



	FL1	FL2
FL1	1	0.02
FL2	0.18	1
FL3	0.10	0.10
FL4	0.02	0.11
FL5	0.00	0.01
FL6	0.00	0.00
FL7	0.00	0.00
FL8	0.00	0.00
FL9	0.01	0.00

Übersprech - Matrix

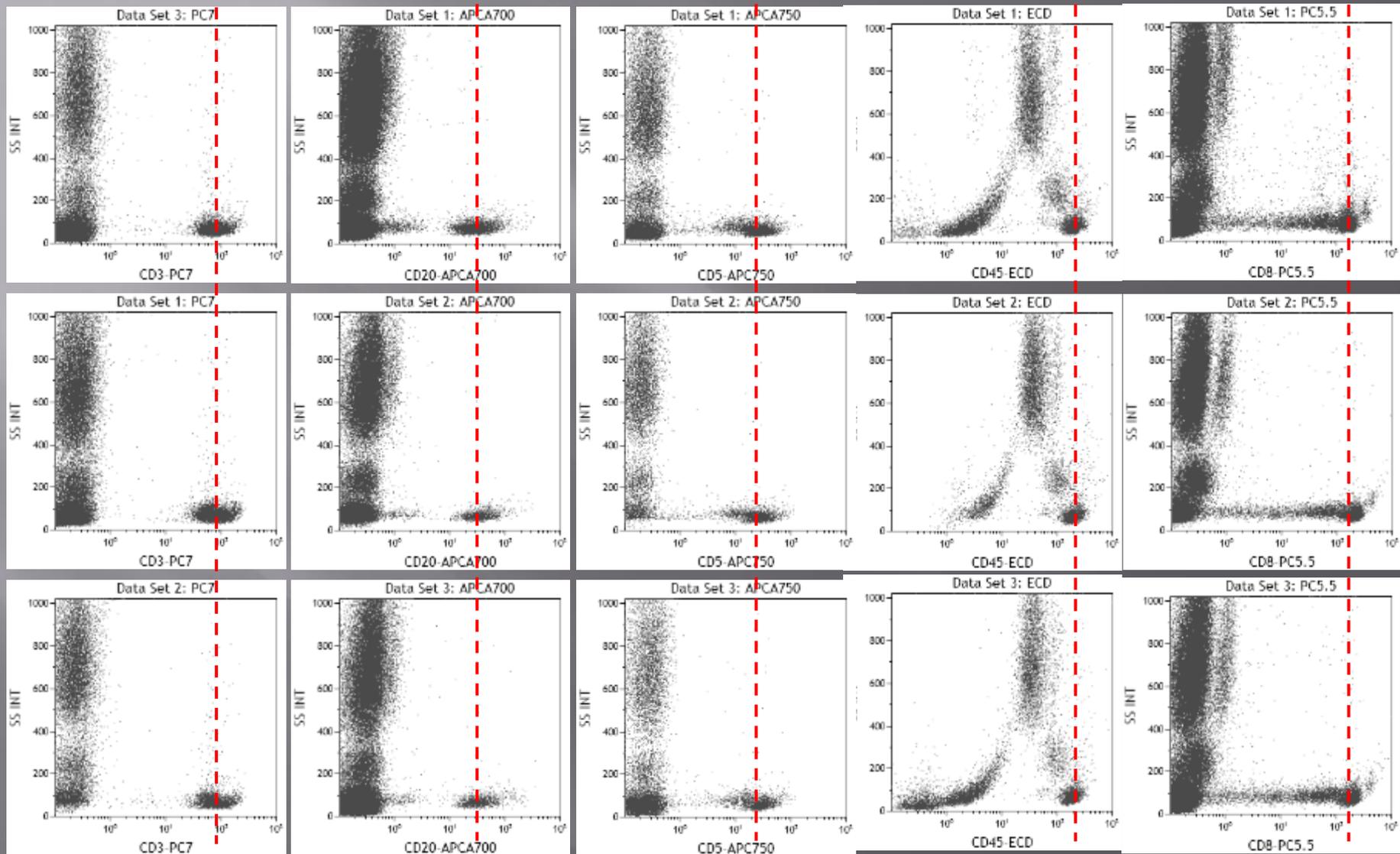
# Automatische Berechnung der relativen Übersprechanteile in die benachbarten Messkanäle



	FL1	FL2	FL3
FL1	1	0.02	0.01
FL2	0.18	1	0.01
FL3	0.10	0.64	1
FL4	0.02	0.11	0.27
FL5	0.00	0.01	0.03
FL6	0.00	0.00	0.00
FL7	0.00	0.00	0.00
FL8	0.00	0.00	0.00
FL9	0.01	0.00	0.00

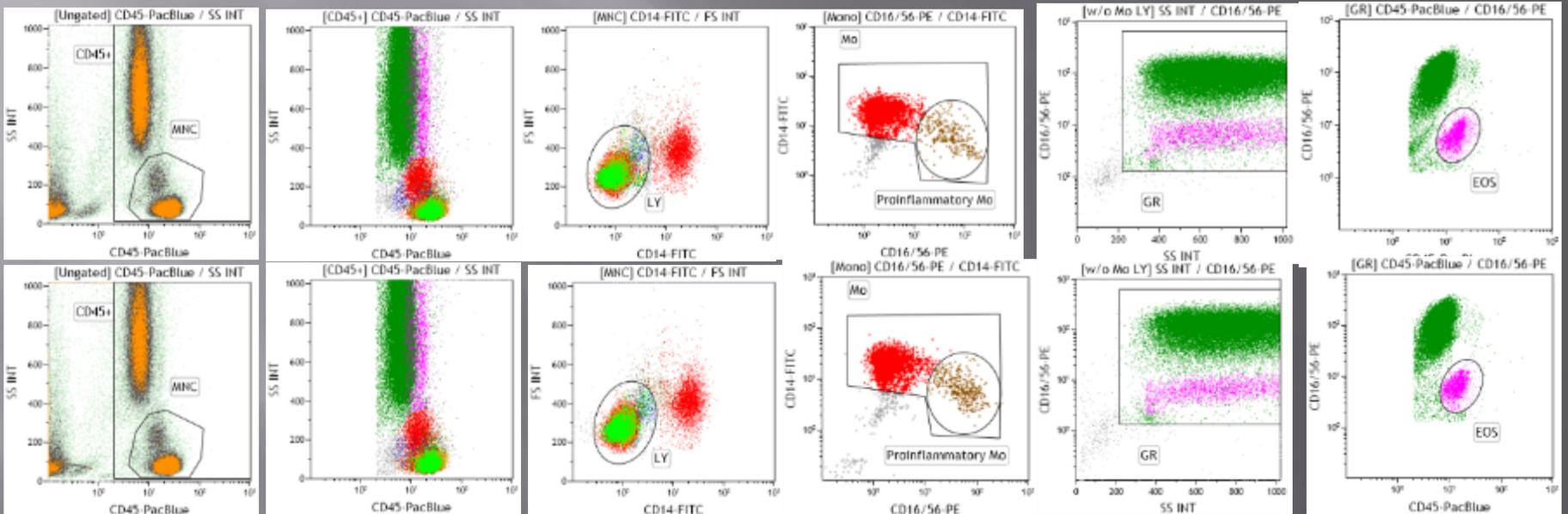
Übersprech - Matrix

# Einzelfärbungen auf 3 Geräten: AN26093 / AS19123 / AT13106



	CD14-FITC
	CD16/56-PE
	CD4-ECD
	CD19-PC5.5
	CD8-APC
	CD3-APCA750
	CD45-PacBlue
CYTOMETERID	AT15121
DATE	10-Jan-12

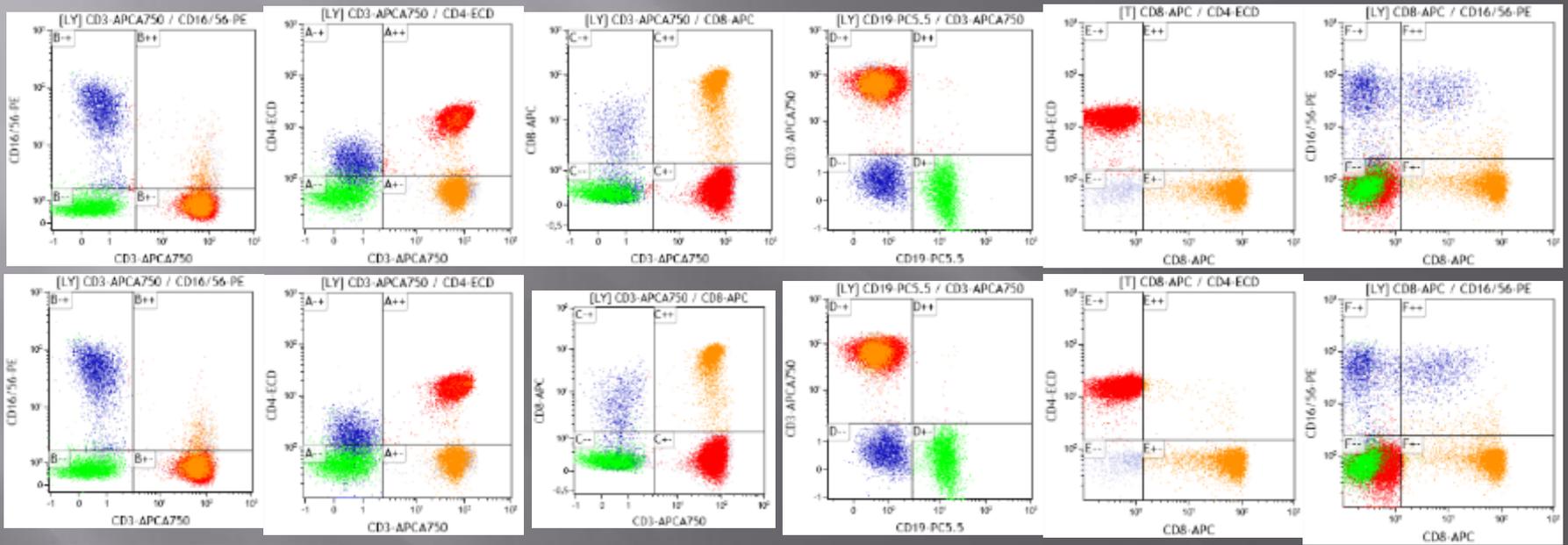
## Standardization of a 7 Color Panel over Time (Berlin Campus Charite, Streck Lab controls)



	CD14-FITC
	CD16/56-PE
	CD4-ECD
	CD19-PC5.5
	CD8-APC
	CD3-APCA750
	CD45-PacBlue
CYTOMETERID	AT15121
DATE	01-Mar-12

# Standardisierung eines 7 Color Panels über einen längeren Zeitraum (Berlin Campus Charite, Streck Lab Kontrollen)

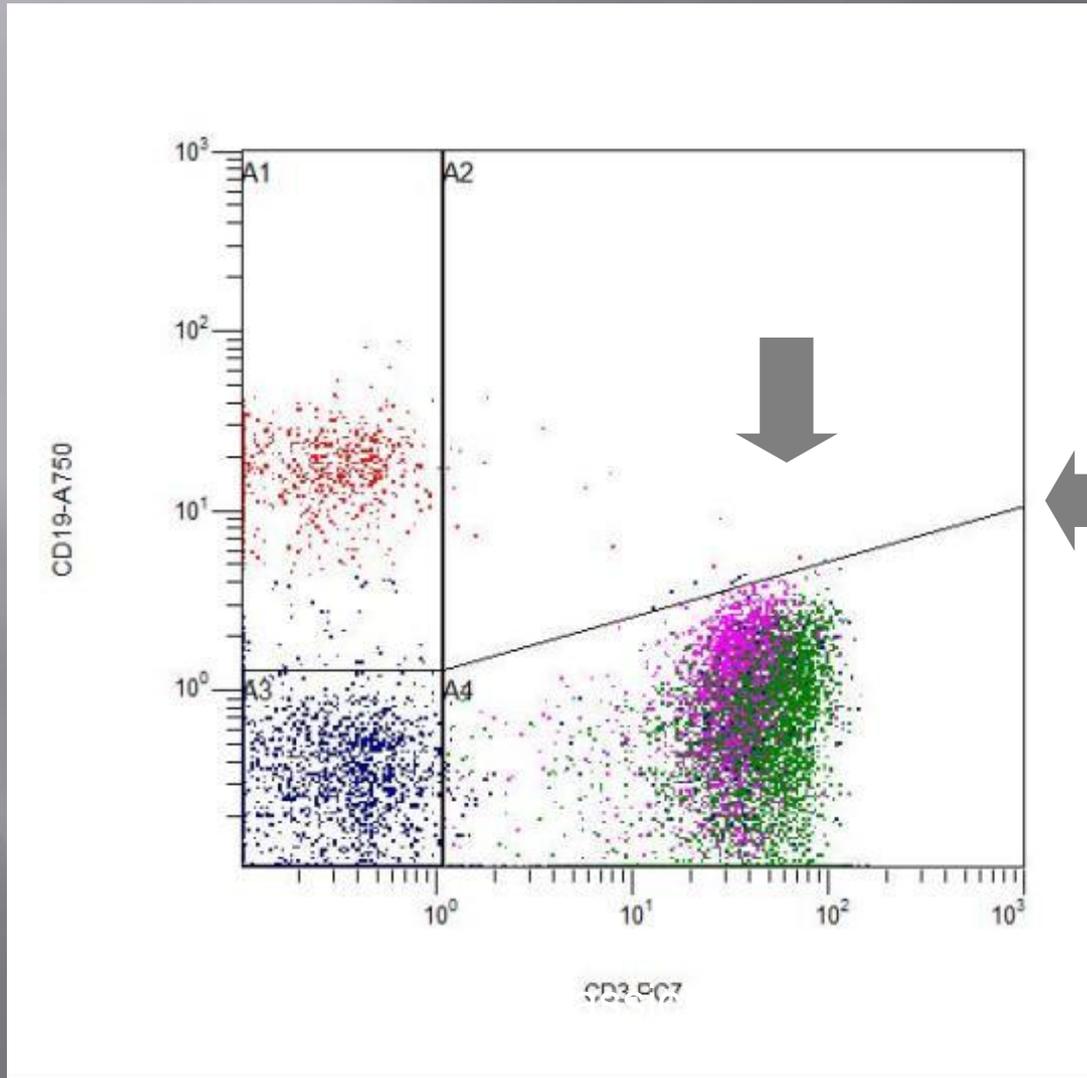
	CD14-FITC
	CD16/56-PE
	CD4-ECD
	CD19-PC5.5
	CD8-APC
	CD3-APCA750
	CD45-PacBlue
CYTOMETERID	AT15121
DATE	10-Jan-12



	CD14-FITC
	CD16/56-PE
	CD4-ECD
	CD19-PC5.5
	CD8-APC
	CD3-APCA750
	CD45-PacBlue
CYTOMETERID	AT15121
DATE	01-Mar-12

# Darstellung der Daten

Klassisches Dotplot 4 Dekaden ???

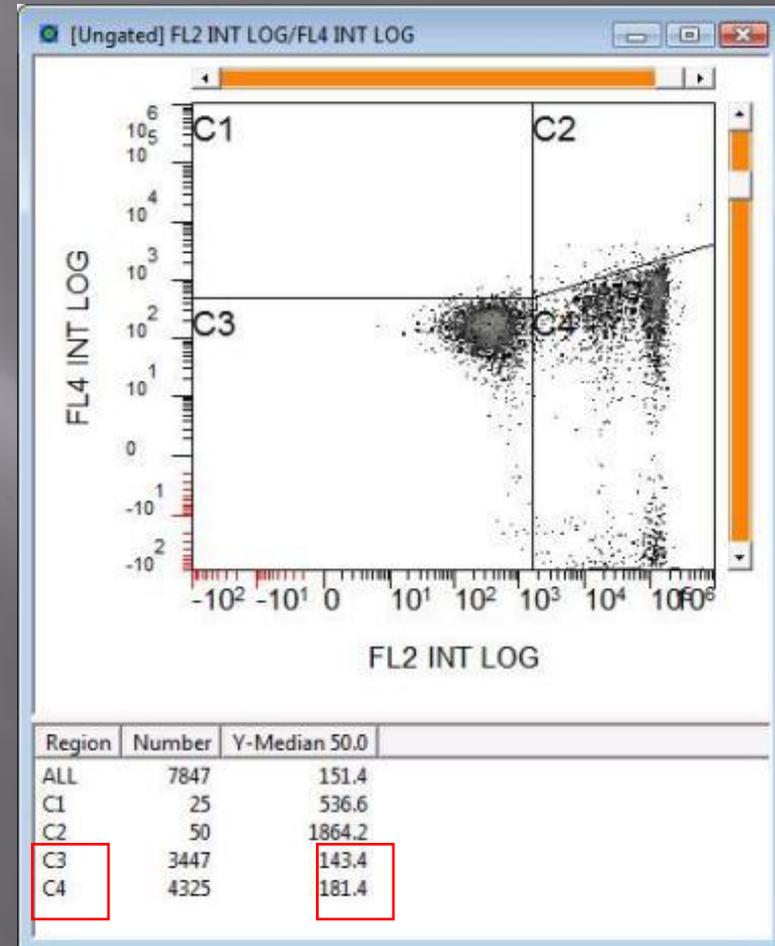
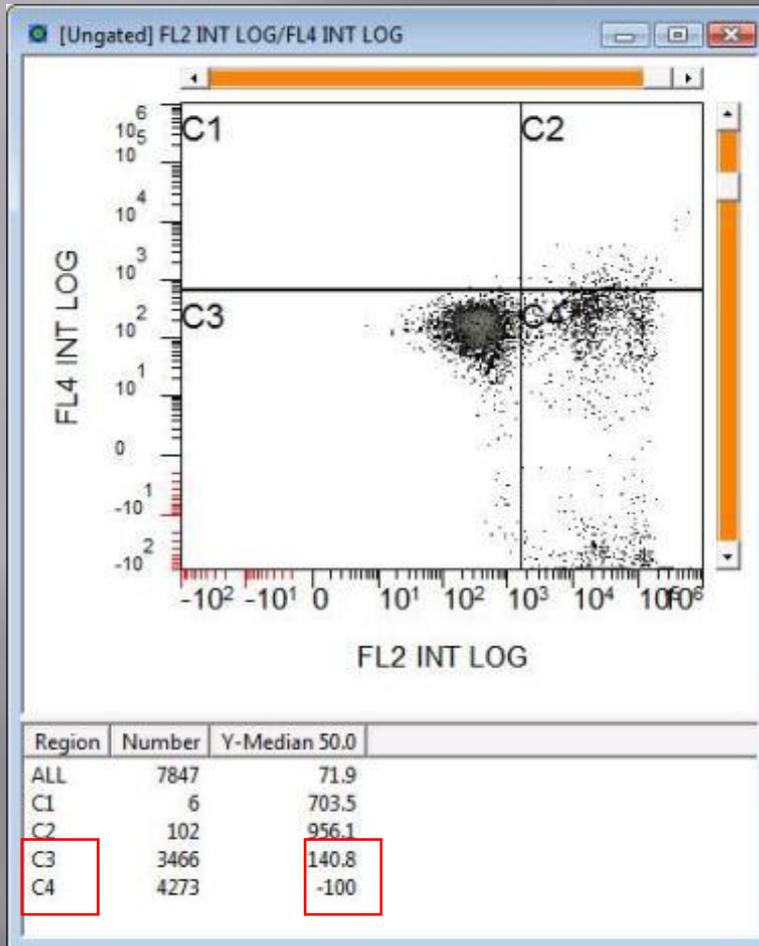


Kompensationsfaktor  
zu gering?

Schrägant ???

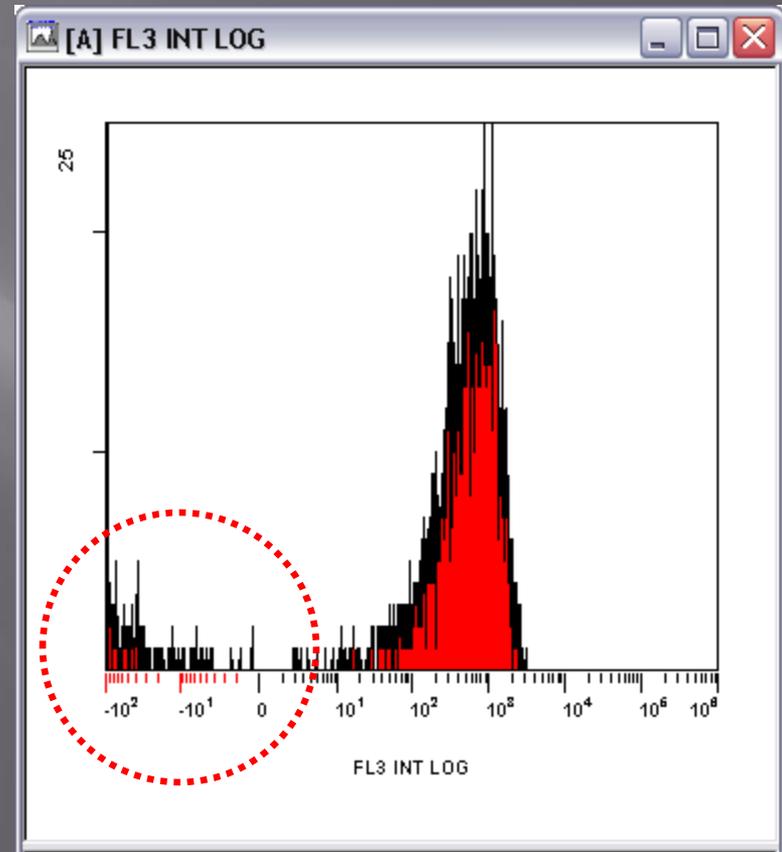
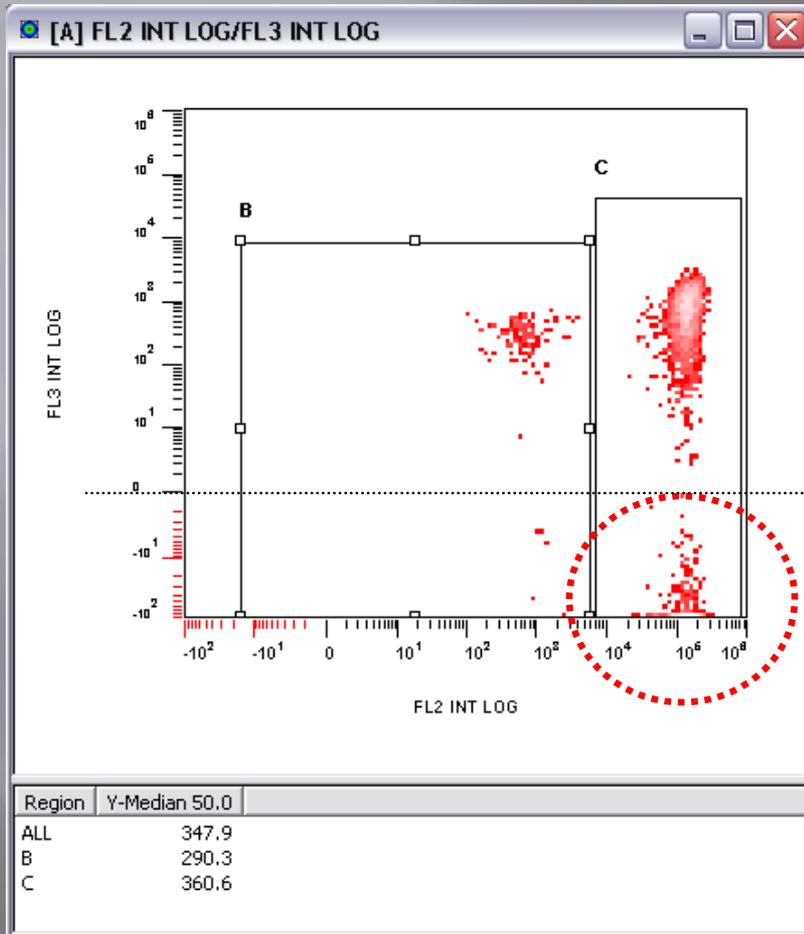
# Vergleich der Mittelwerte der Verteilungen

“True View” Darstellung



Hohe Auflösung und großer Störspannungsabstand erlaubt genaue Bestimmung der Mittelwerte

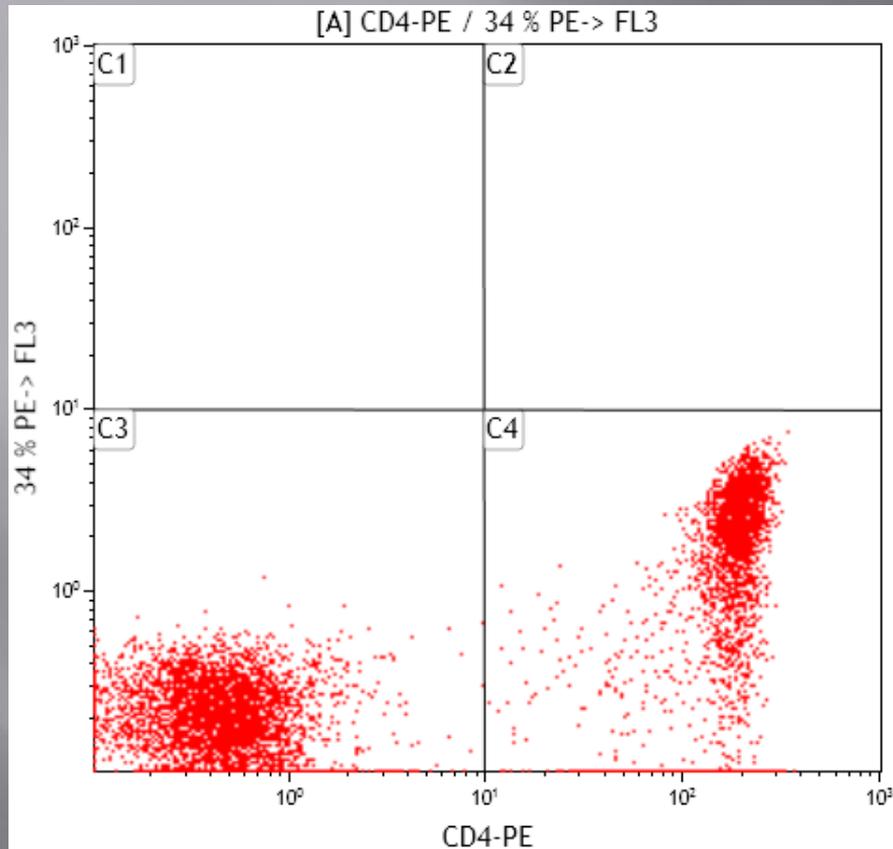
# Gallios / Navios Multicolor Practice - Basic Considerations on Compensation



...and artificially generate negative fluorescence values in a bimodal distribution !

# Dotplots mit neuer Darstellung

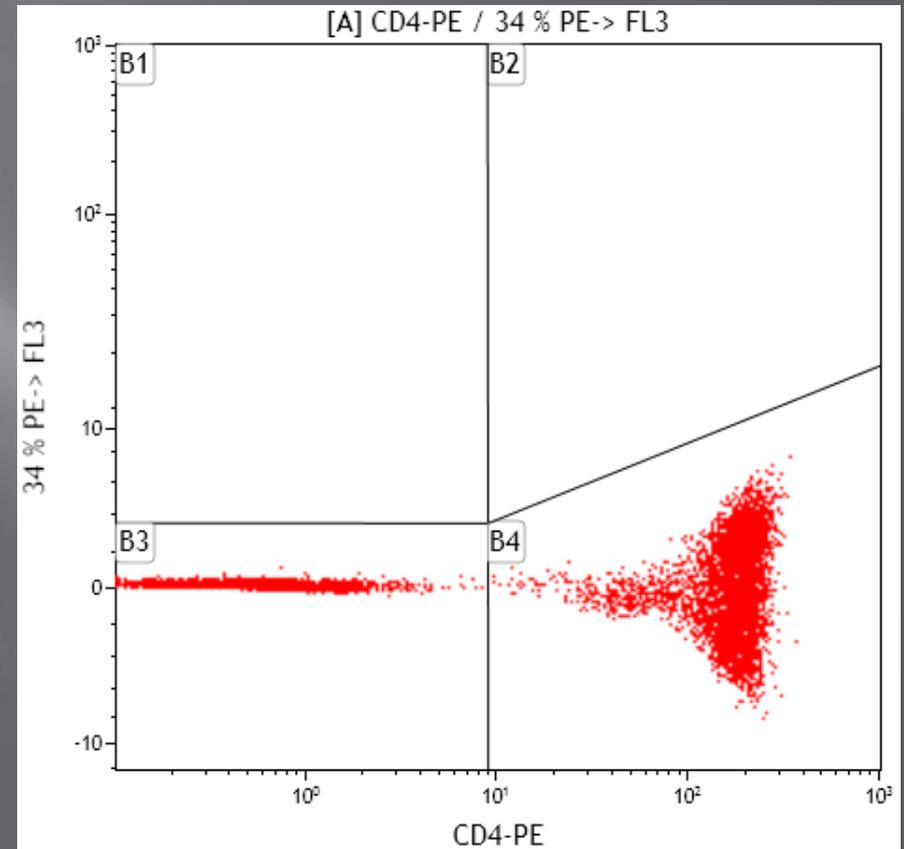
## Logarithmisch, 4 Dekaden



Gate Y-Med

All	0,19
C1	N/A
C2	N/A
C3	0,19
C4	0,19

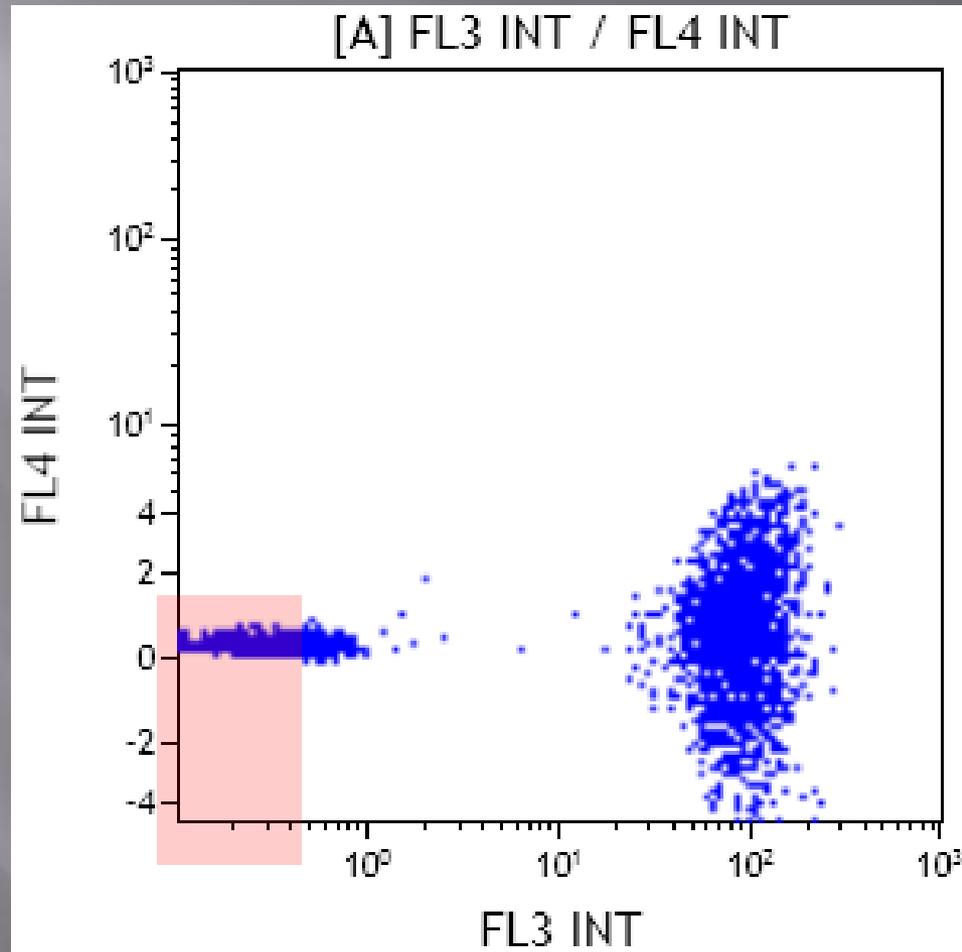
## logical



Gate Y-Med

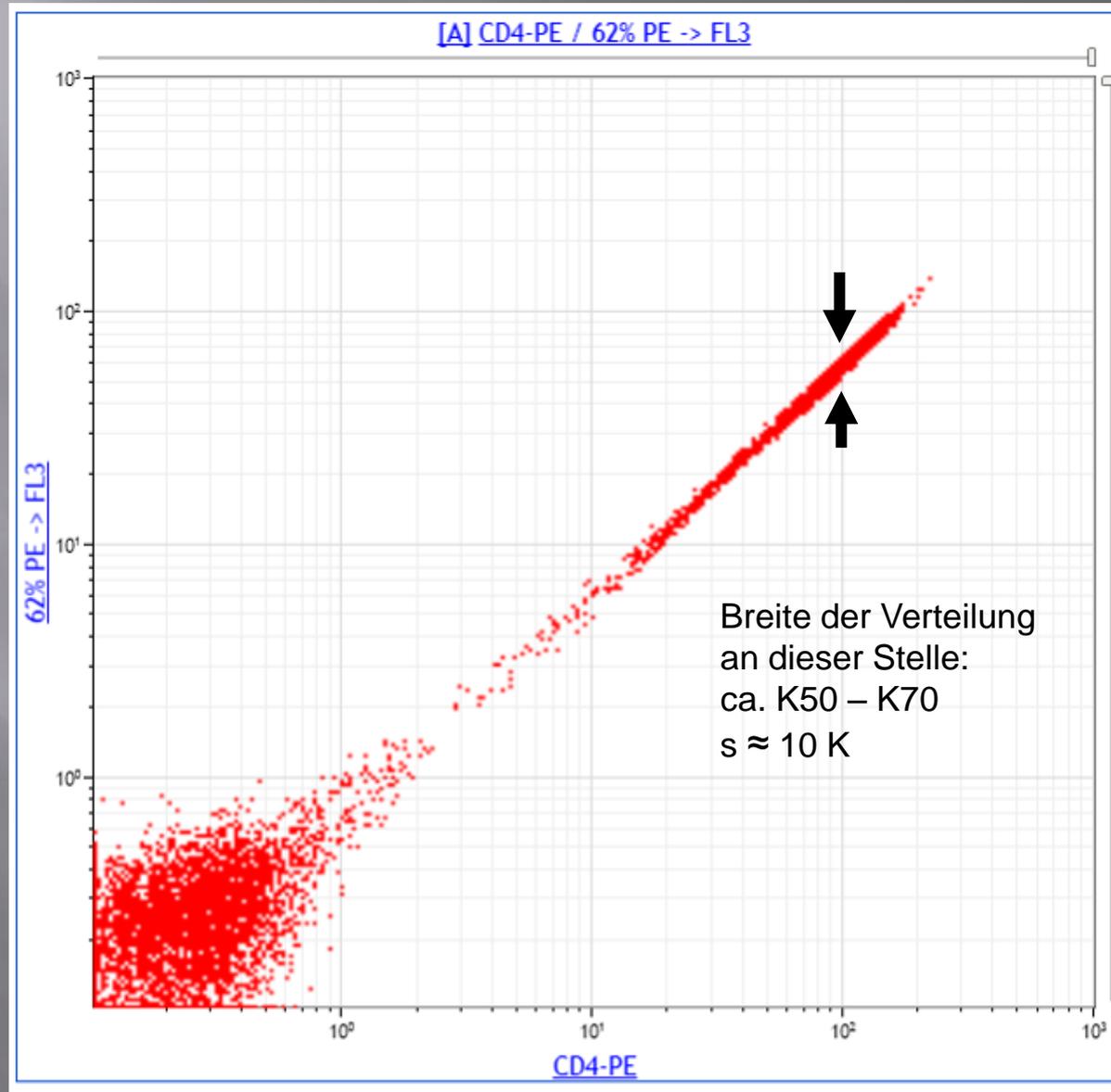
All	0,19
B1	N/A
B2	N/A
B3	0,19
B4	0,19

## Dotplots mit neuer Darstellung

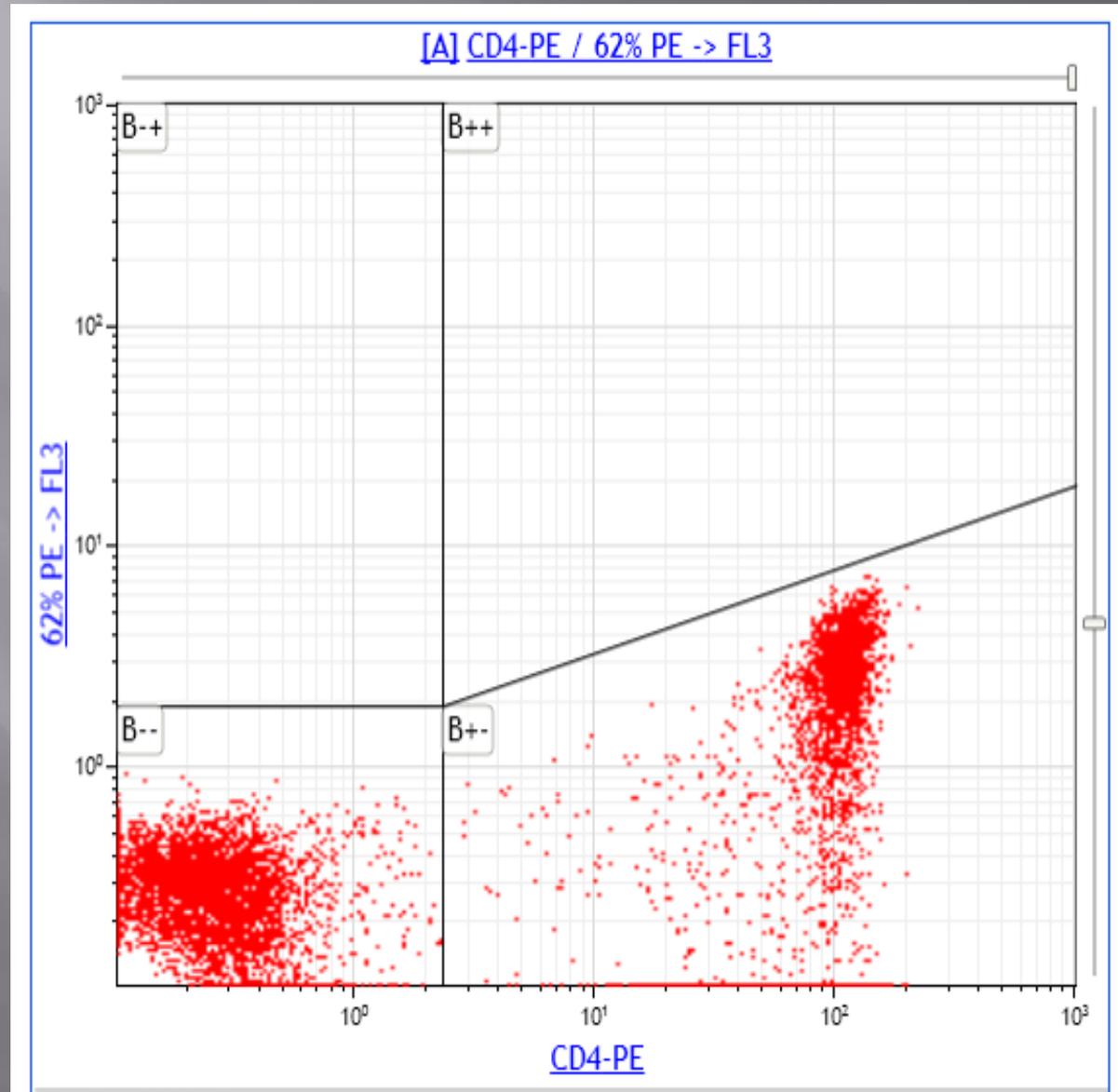


Die logarithmische Skalierung wird unterbrochen und die negativen Werte werden linear dargestellt

# Standardabweichung



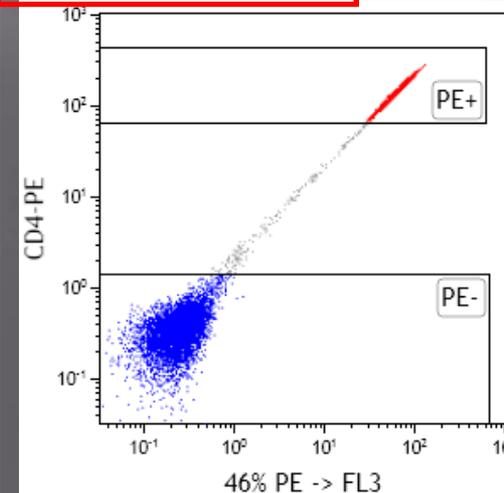
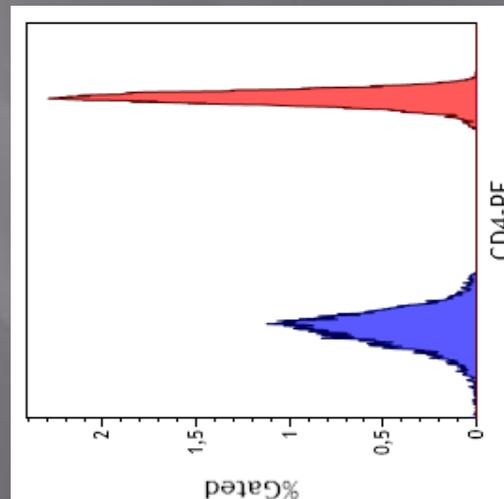
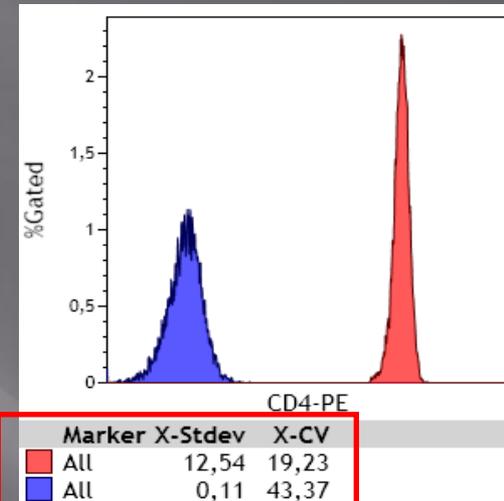
# Grundlagen der Kompensation Standardabweichung und Variationskoeffizient



# Grundlagen der Kompensation

## Standardabweichung und Variationskoeffizient

Autofluoreszenz und  
übersprechende Verteilung haben  
den gleichen Variationskoeffizient CV  
aber unterschiedliche  
Standardabweichung SD

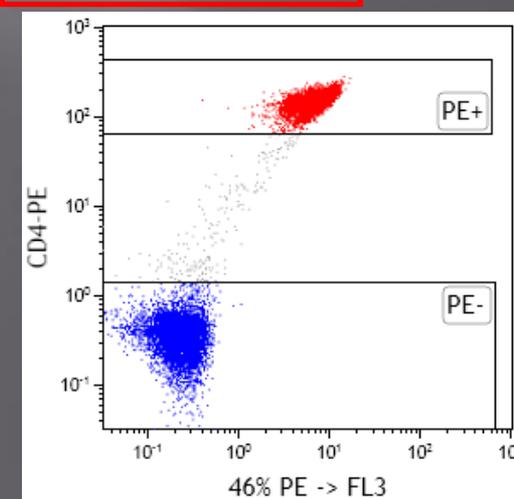
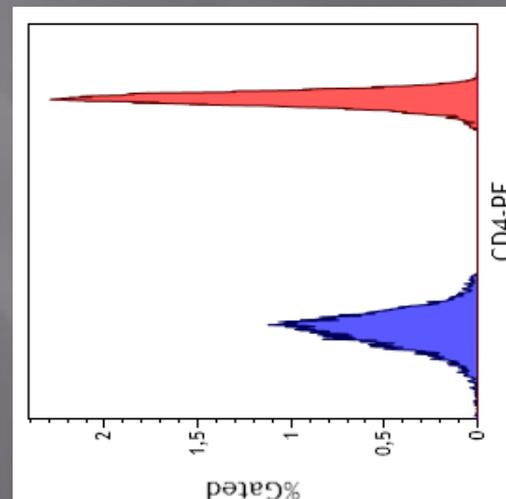
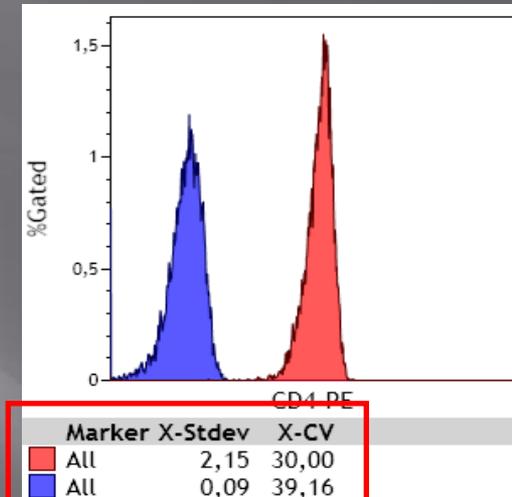


# Grundlagen der Kompensation

## Standardabweichung und Variationskoeffizient

Bei der Kompensation der übersprechenden Verteilung bleibt die Standardabweichung unverändert

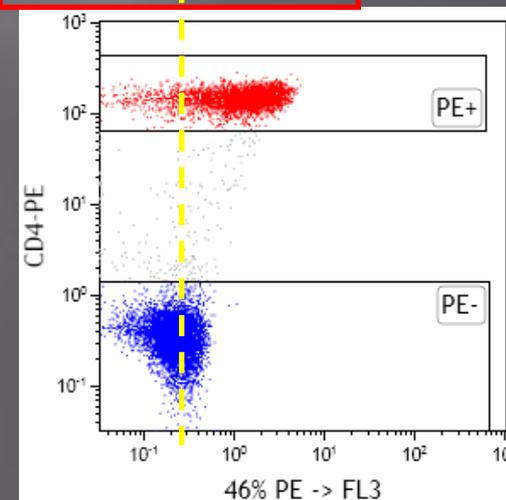
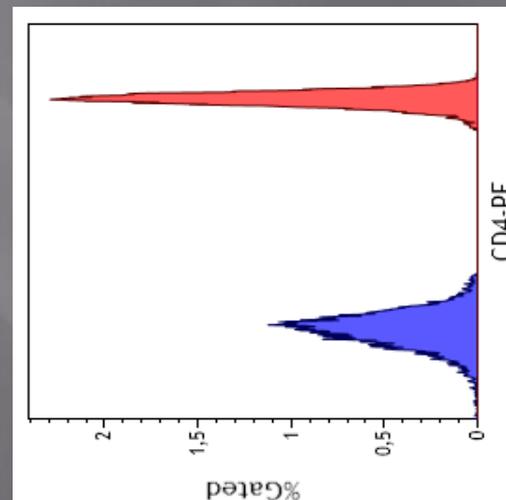
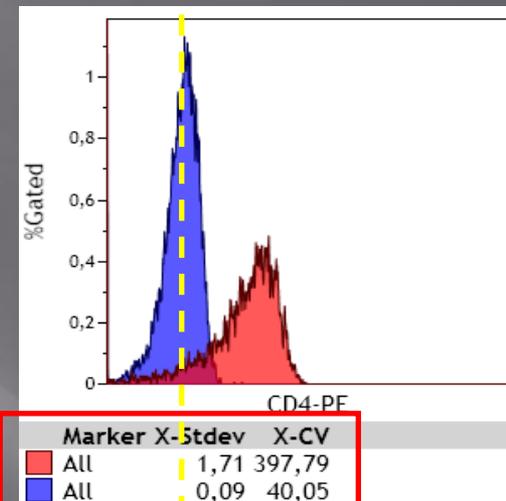
> der CV erhöht sich proportional zur Verschiebung



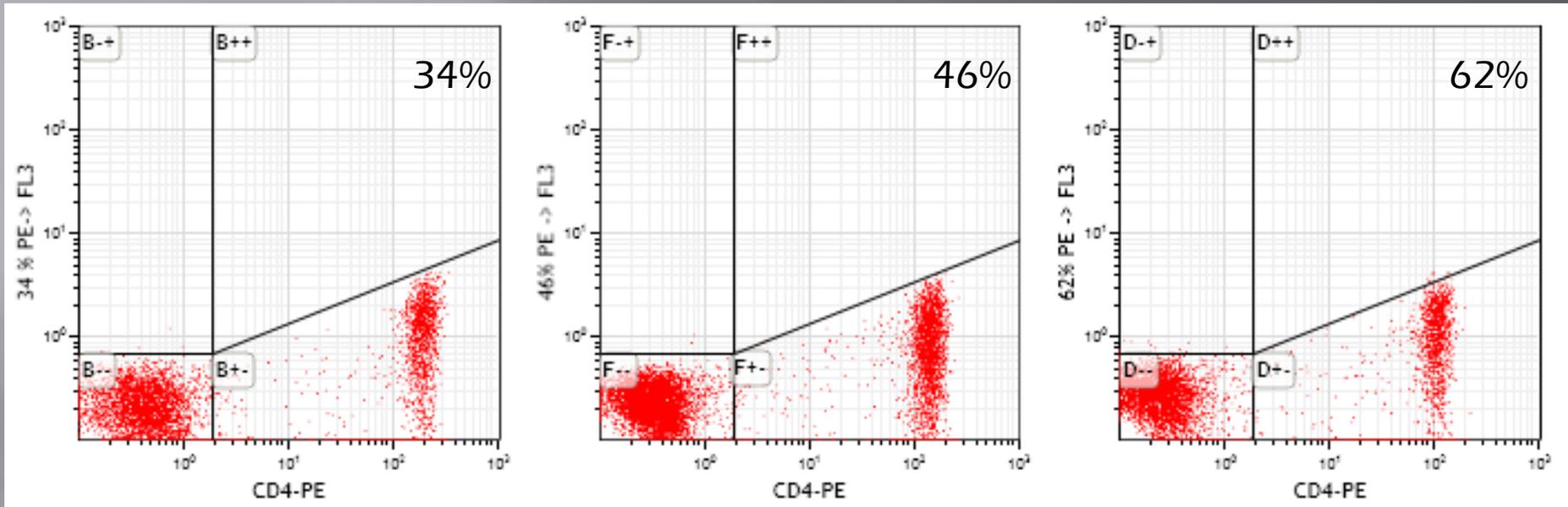
# Grundlagen der Kompensation

## Standardabweichung und Variationskoeffizient

Am unteren Ende der logarithmischen Skala erreicht die linke Seite der kompensierten Verteilung negative Werte und können nicht mehr dargestellt werden.



# Grundlagen der Kompensation



Die Kompensationskoeffizienten repräsentieren nur die Verstärkungseinstellungen

Emissionsspektrum des jeweiligen Farbstoffs und  
des Transmissionspektrum der optische Filter sind invariant

# Grundlagen der Kompensation

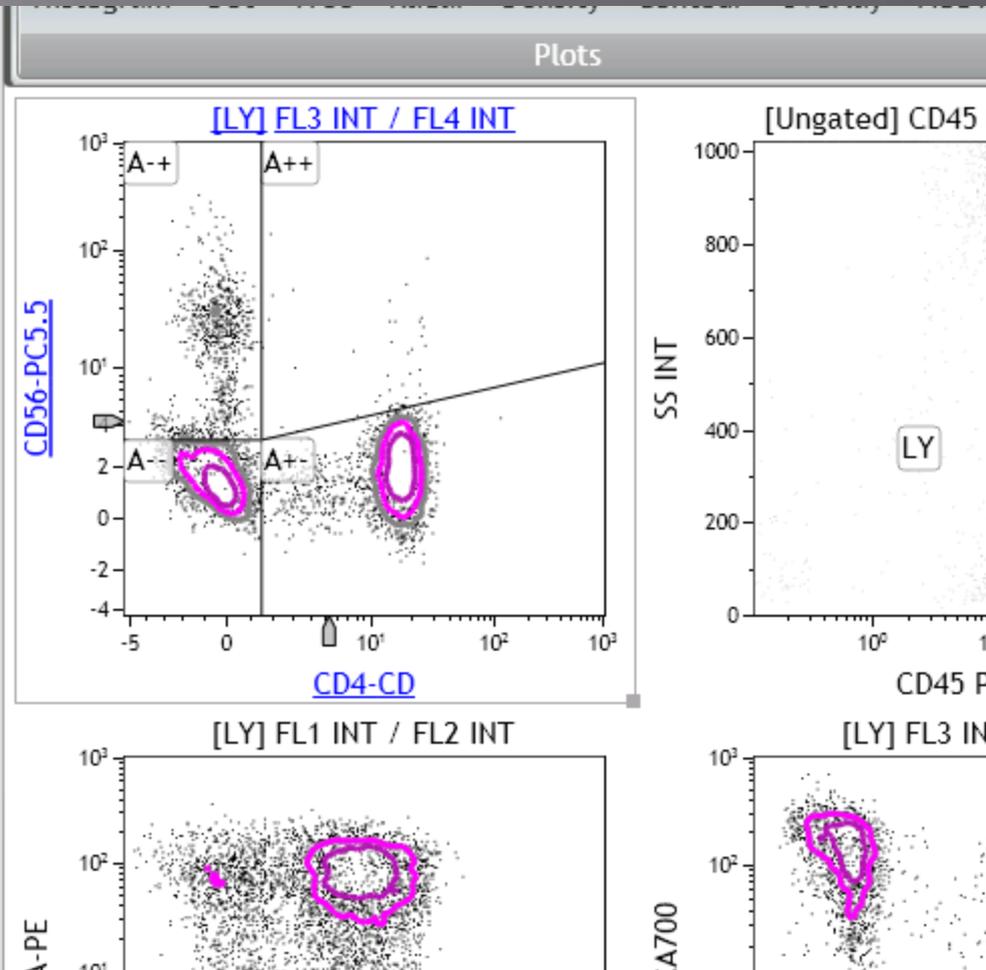
Spillover (%)										
	FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8	FL9	FL10
FL1		1.70	0.20	0.30	0.20	0.00	0.00	0.00	0.10	0.00
FL2	19.30		6.60	1.30	1.00	0.00	0.00	0.00	0.00	0.00
FL3	7.30	54.30		0.70	0.50	0.00	0.00	0.00	0.00	0.00
FL4	1.90	16.51	47.50		0.30	0.00	0.00	0.00	0.00	0.00
FL5	0.40	2.10	10.30	40.70		0.00	0.10	2.80	0.00	0.00
FL6	0.10	0.00	0.50	1.20	0.10		5.00	19.50	0.00	0.00
FL7	0.00	0.00	0.20	27.60	0.40	35.40		17.70	0.00	0.00
FL8	0.00	0.00	0.00	5.20	3.80	5.30	13.70		0.00	0.00
FL9	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00		0.00
FL10	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	9.90	

Alles nur Zahlen !

Das Problem sind die spektralen Eigenschaften der Farbstoffe

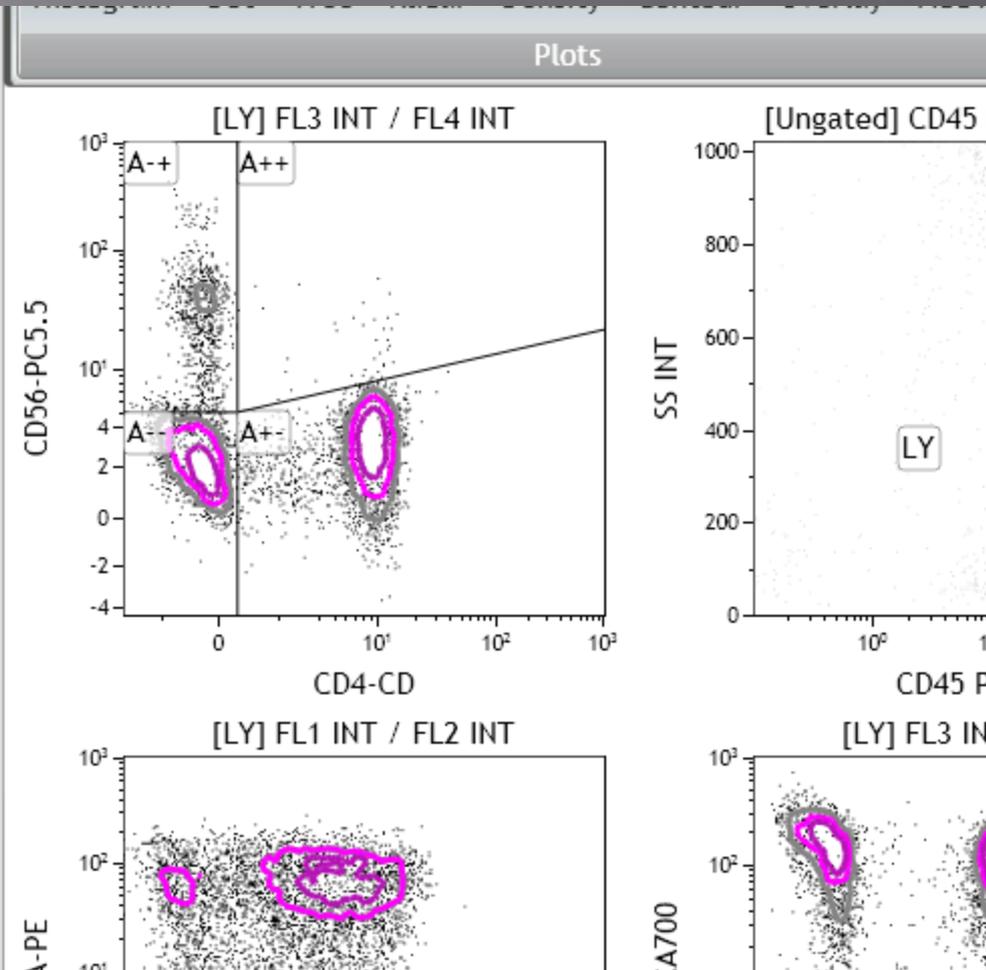
# Grundlagen der Kompensation

Spillover (%)				
	FL1	FL2	FL3	FL4
FL1		0,80	0,70	
FL2	22,10		28,90	
FL3	2,20	12,20		
FL4	0,80	5,20	69,30	
FL5	0,10	0,70	13,60	
FL6	0,00	0,00	0,60	
FL7	0,00	0,00	0,10	
FL8	0,00	0,00	0,30	
FL9	0,00	0,00	0,20	



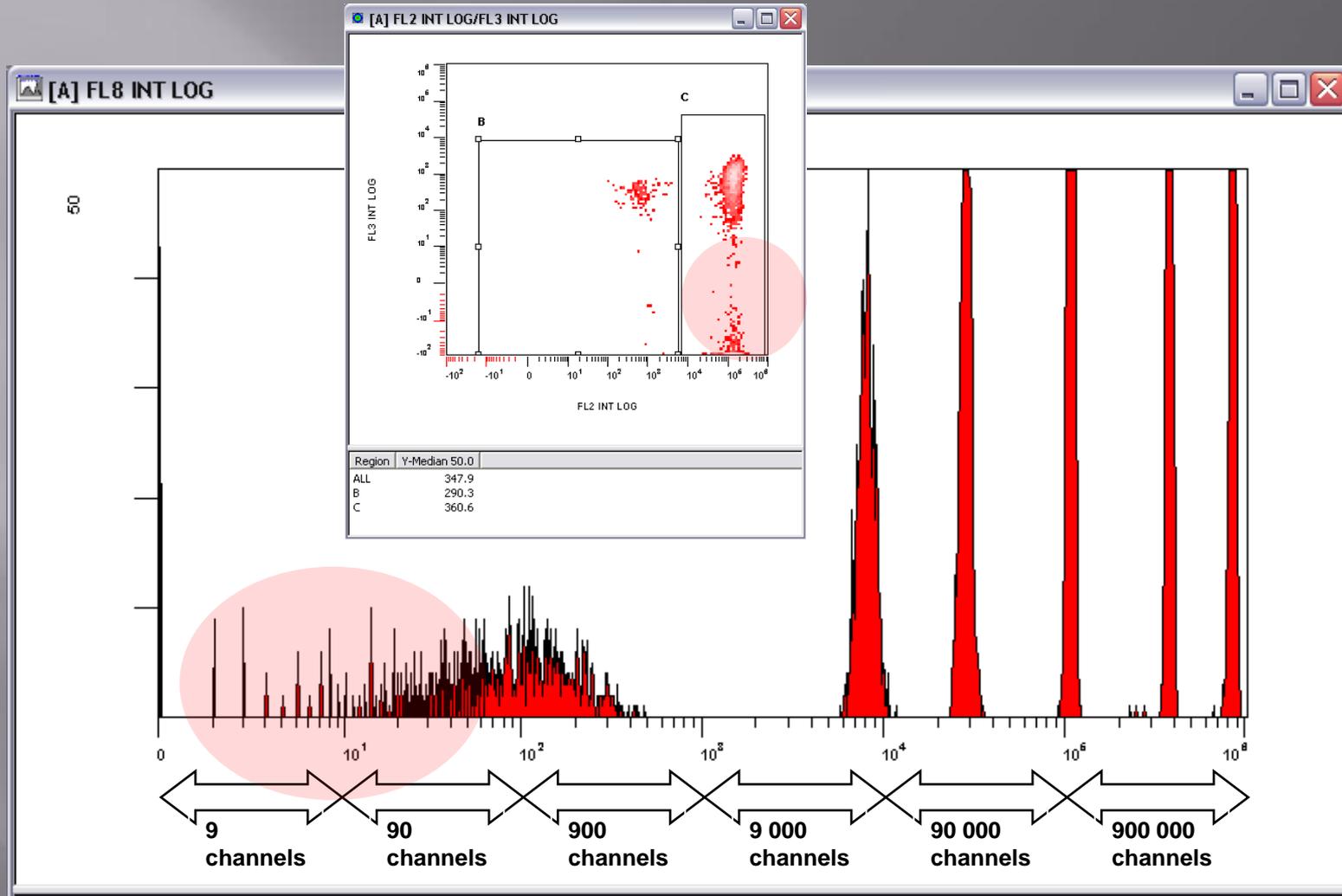
# Grundlagen der Kompensation

Spillover (%)				
	FL1	FL2	FL3	FL4
FL1		0,98	3,27	
FL2	22,58		41,66	
FL3	1,65	8,40		
FL4	1,59	8,89	<b>167,82</b>	
FL5	0,33	1,13	24,64	
FL6	0,15	0,20	3,72	
FL7	0,16	0,21	3,00	
FL8	0,26	0,32	3,13	
FL9	0,26	0,32	3,04	



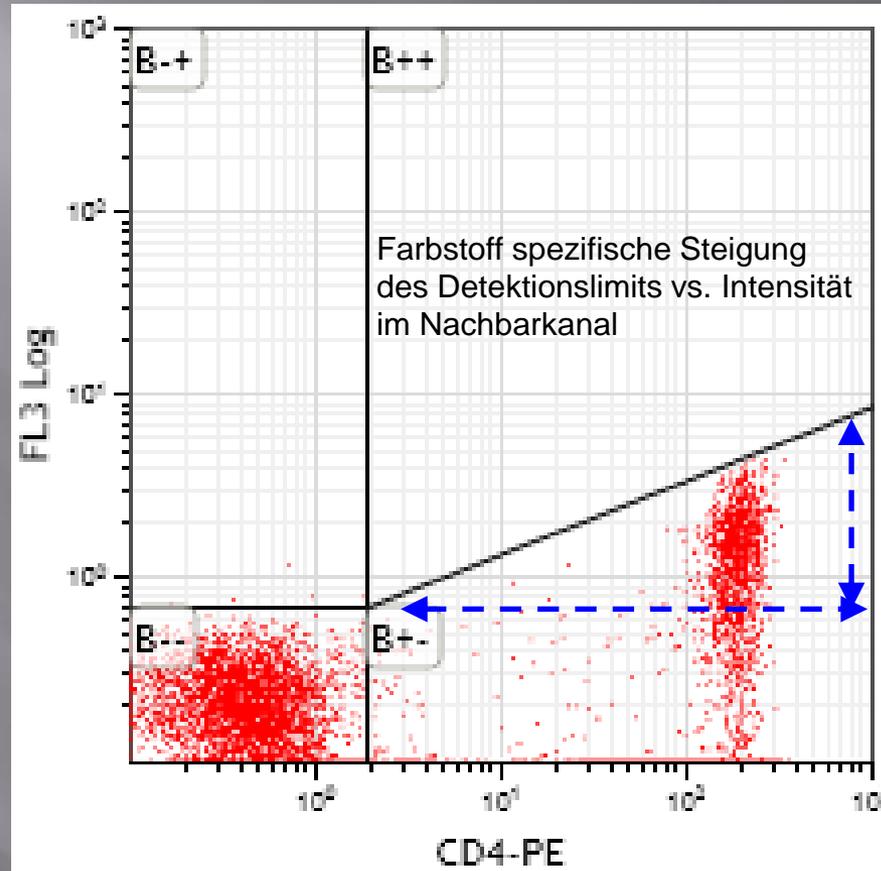
# Grundlagen der Kompensation

## Auflösung der logarithmischen Skalierung



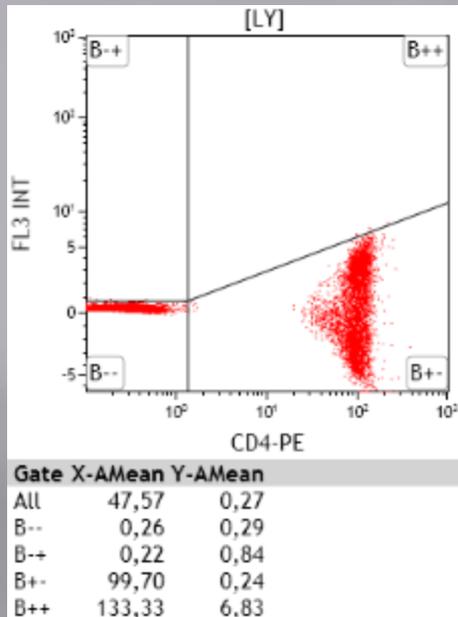
Die geringe Auflösung in den unteren Dekaden erzeugen Lücken

# Grundlagen der Kompensation



Übersprechen erhöht das Detektionslimit der betroffenen Kanäle

# Crosstalk Index ist ein Maß für den Verlust an Messempfindlichkeit



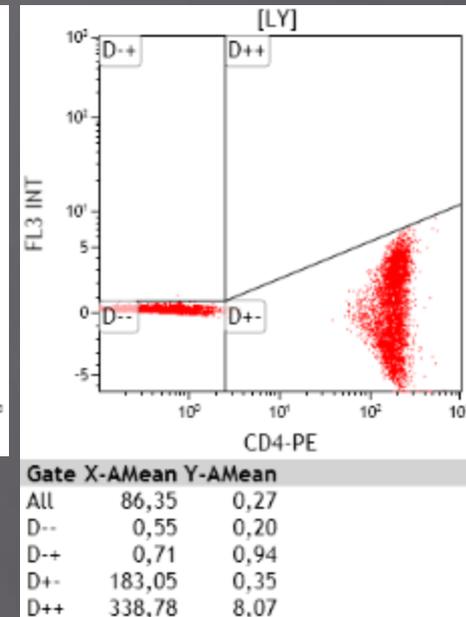
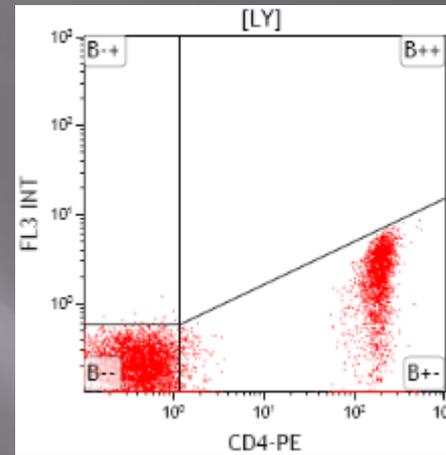
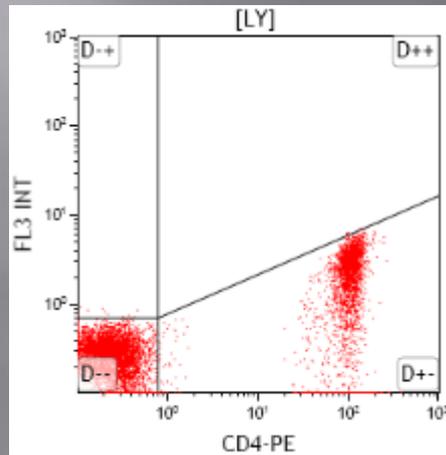
Kompensationsfaktor = 59.5 %

Crosstalk Index = 0.918

$$\text{Crosstalk Index} = \frac{\text{LOG}_{10}(\text{SNR}(\text{sekundärer Messkanal}))}{\text{LOG}_{10}(\text{SNR}(\text{primärer Messkanal}))}$$

$$\text{LOG}_{10}(\text{SNR}(\text{primärer Messkanal}))$$

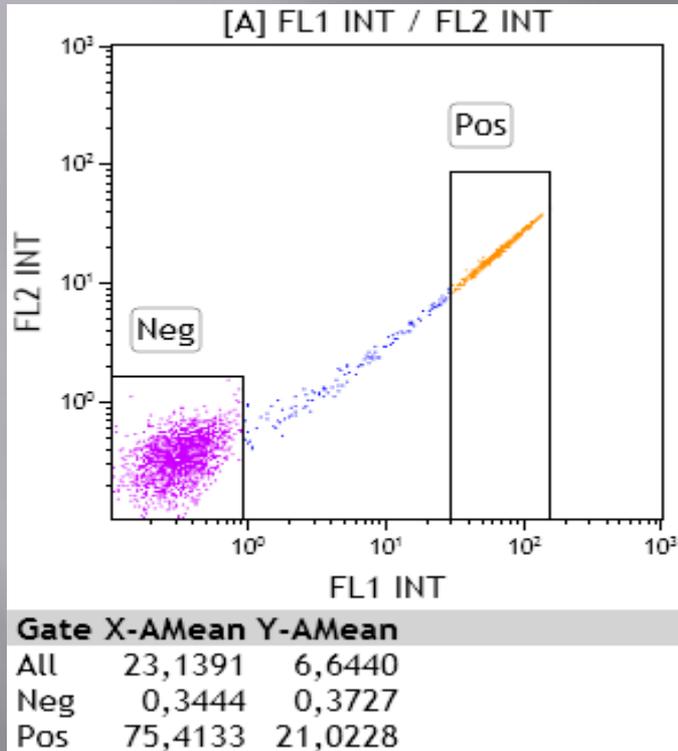
(SNR = signal-to-noise ratio)



Kompensationsfaktor = 32.5 %

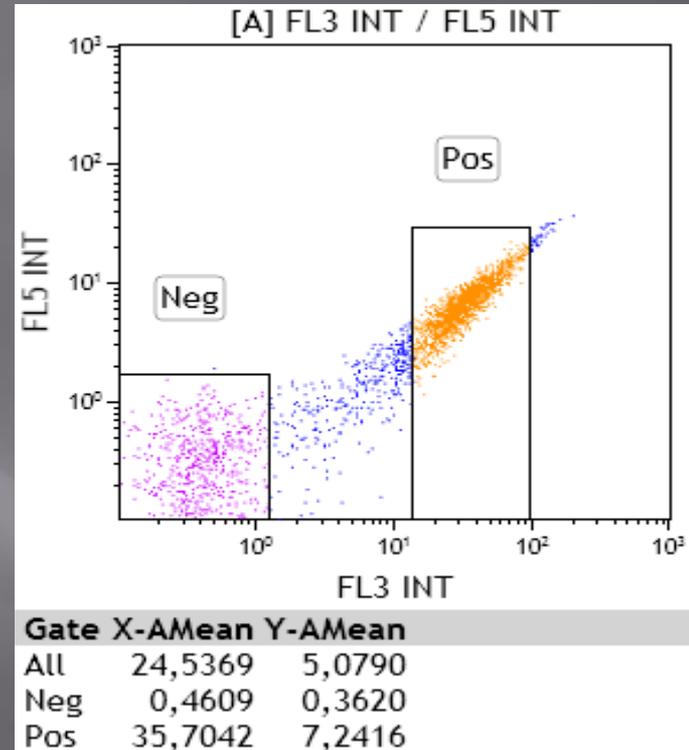
Crosstalk Index = 0.926

# Crosstalk Index



Kompensationsfaktor = 27.5 %

Crosstalk Index = 0.748



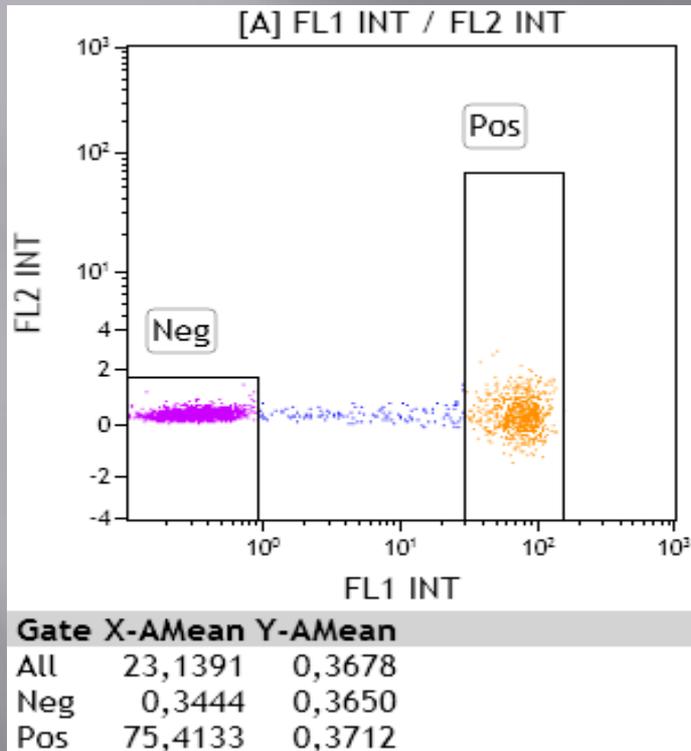
Kompensationsfaktor = 19.55 %

Crosstalk Index = 0.698

$$\text{Crosstalk Index} = \frac{\text{LOG}_{10}(\text{SNR}(\text{sekundärer Messkanal}))}{\text{LOG}_{10}(\text{SNR}(\text{primärer Messkanal}))}$$

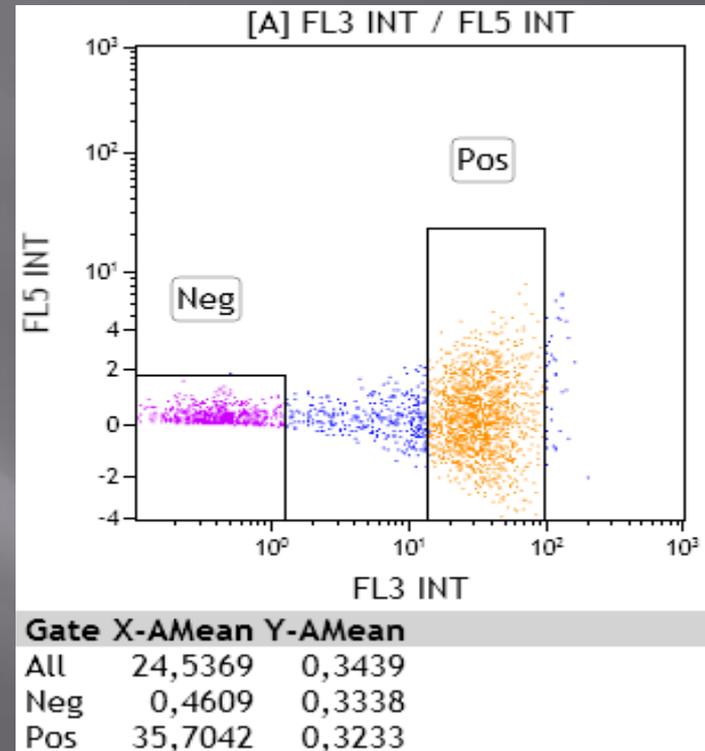
(SNR = signal-to-noise ratio)

# Crosstalk Index



Kompensationsfaktor = 27.5 %

Crosstalk Index = 0.748



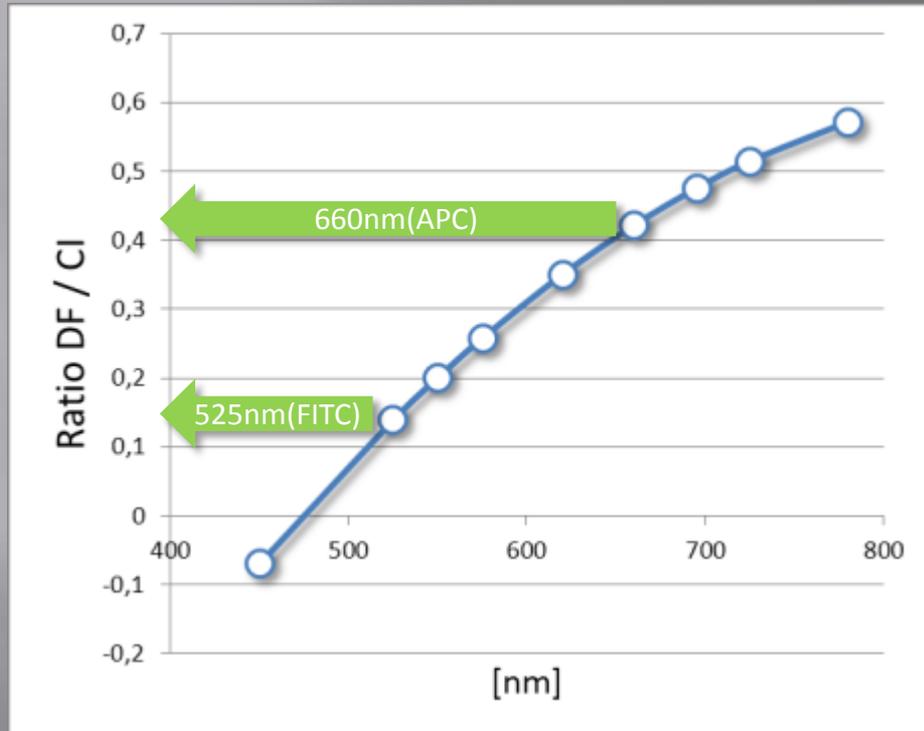
Kompensationsfaktor = 19.55 %

Crosstalk Index = 0.698

$$\text{Streuung der Messwerte} = \frac{\text{LOG}_{10}(\text{SNR}(\text{sekundärer Messkanal}))}{\text{LOG}_{10}(\text{SNR}(\text{primärer Messkanal}))} \times (f(\lambda)) \text{ „Bandpass Temperature“}$$

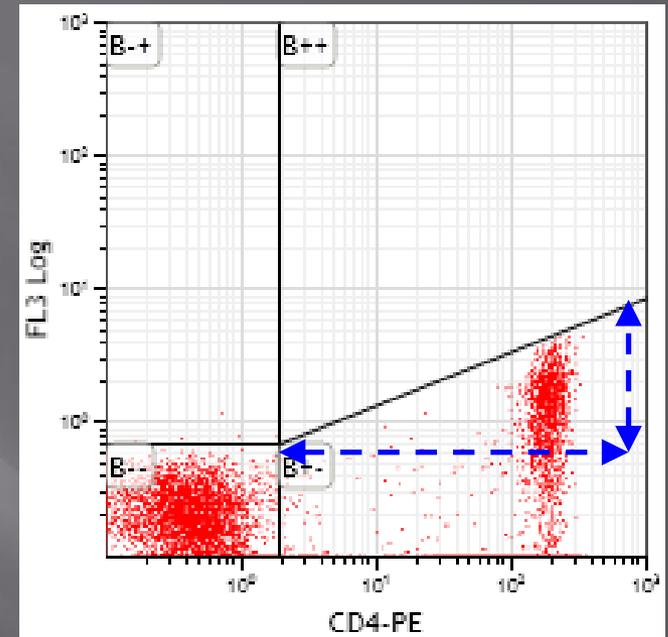
(SNR = signal-to-noise ratio)

# Bandpass “Temperatur”



525nm: absolute distortion =  $0.15 \times \text{LOG}(\text{SNR}(\text{secondary}))$   
i.e. 0.15 decades distortion per decade of secondary signal intensity

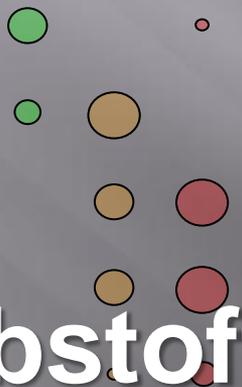
660nm: absolute distortion =  $0.42 \times \text{LOG}(\text{SNR}(\text{secondary}))$   
i.e. 0.42 decades distortion per decade of secondary signal intensity



# Zusammenstellen

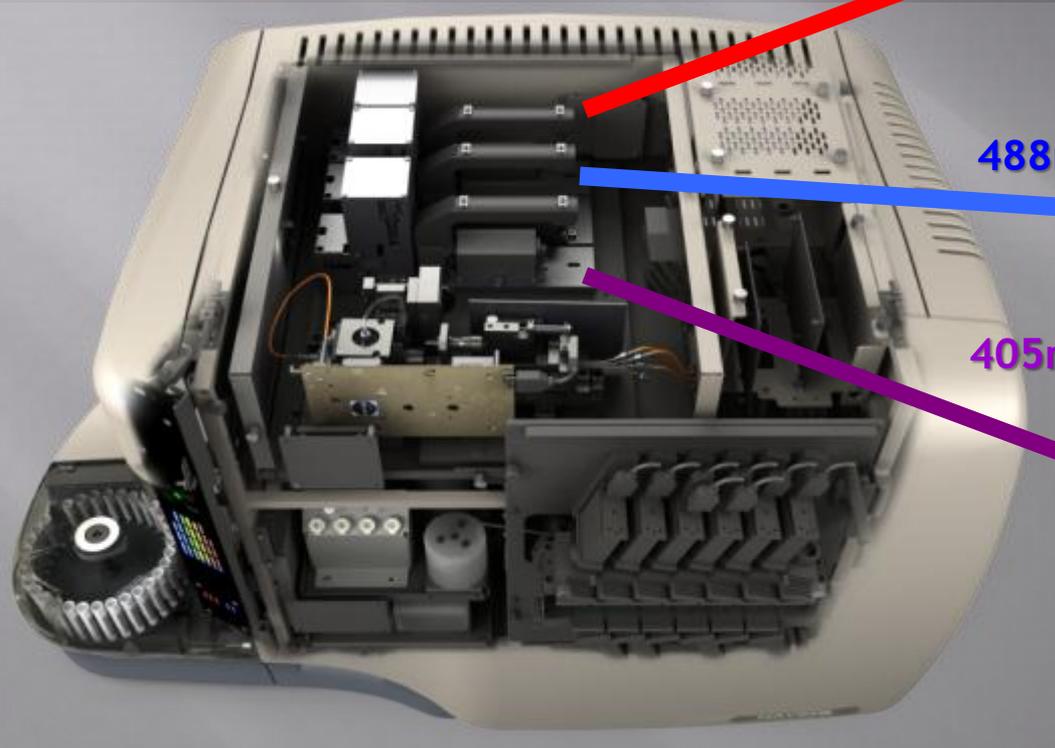
von

Farbstoffkombinationen



# Kombinationen < 10 Farben ohne Kompensation

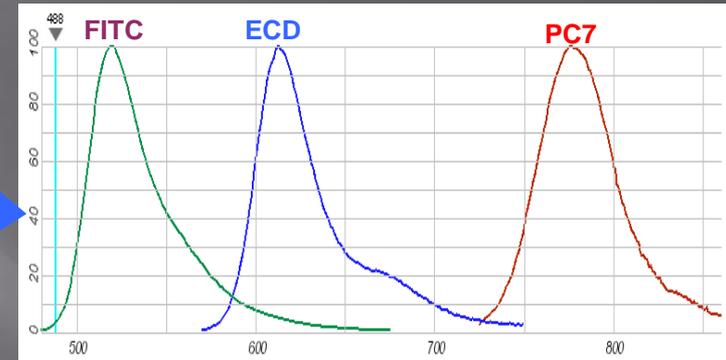
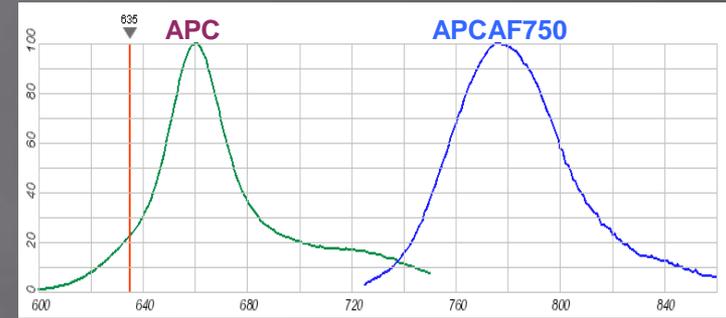
3 Laser 10 Color (5+3+2)



640nm

488nm

405nm



(Für ECD, PC7 und APCAF750 müssen ggf. Donorfluoreszenzen berücksichtigt werden)

# Die Übersprech – Matrix für Gallios / Navios 3 Laser / 10 Farben

		488 Excitation					633 Excitation			405 Excitation		
		FITC	PE	ECD	PC5	PC5.5	PC7	APC	APC-AF700	APC-AF750	Pacific Blue	Krome Orange
488 Excitation	FITC	Grey										
	PE	Green circle	Grey	Red dot								
	ECD	Green circle	Orange circle	Grey								
	PC5		Orange circle	Red circle	Grey	Grey						
	PC5.5		Orange circle	Red circle	Grey	Grey						
	PC7		Small orange dot	Red dot	Dark purple circle	Purple circle	Grey			Small red dot		
633 Excitation	APC				Dark purple circle	Small purple dot		Grey	Purple circle	Red circle		
	APC-AF700				Dark purple circle	Purple circle		Pink circle	Grey	Red circle		
	APC-AF750				Small dark purple dot	Small purple dot	Cyan circle	Pink circle	Purple circle	Grey		
405 Excitation	Pacific Blue										Grey	
	Krome Orange										Blue circle	Grey

Diese Klassifikation ist spezifisch für jedes Konjugat bei gegebenen Transmissionsspektren der optischen Filter

Sie ist unabhängig von Kompensations – und Verstärkungseinstellungen

# “Silent” Farbstoffe und “Untouchable” Kanäle

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

**Untouchable** =  
kein signifikanter Eintrag  
von anderen Farbstoffen  
(freie Zeile)

**Silent** =  
Kein signifikanter Übertrag  
in andere Kanäle  
(freie Spalte)



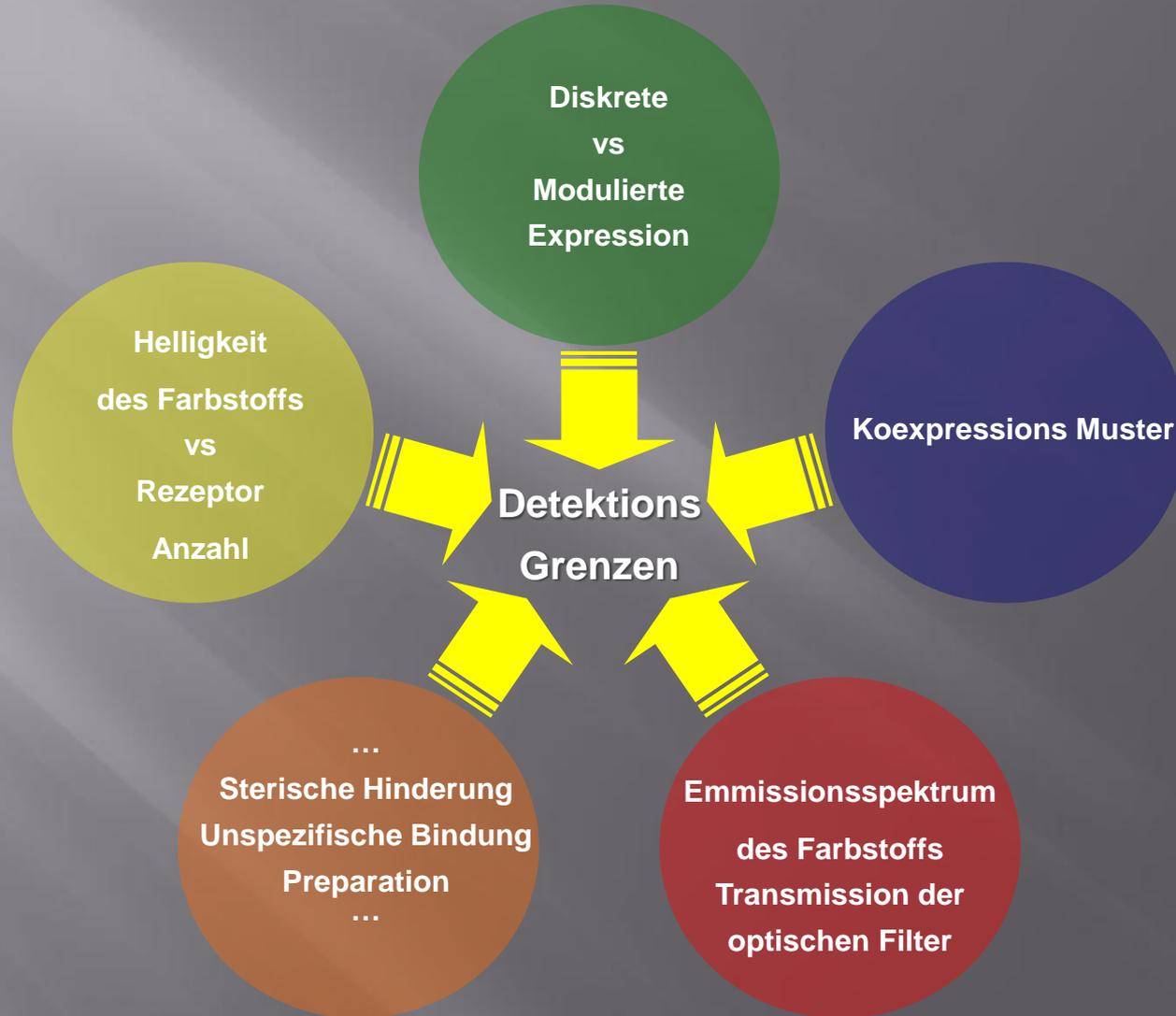
# Gallios / Navios Multicolor Praxis

„Silent“ und „untouchable“ Kanäle in einer 7 Farb - Kombination

		488 Excitation			633 Excitation			405 Excitation	
		FITC	PE		PC7	APC	APC- AF700	Pacific Blue	Krome Orange
488 Excitation	FITC			UNTOUCHABLE					
	PE								
			N T		N T			N T	
633 Excitation	PC7		E	UNTOUCHABLE				E	
	APC		L		L			L	
	APC- AF700		I		I			I	
405 Excitation	Pacific Blue		S	UNTOUCHABLE				S	
	Krome Orange								

Die Kombination großer Lücken und engen Nachbarkanälen eröffnet erstaunliche Möglichkeiten

# Gallios / Navios Multicolor Praxis - Einflussgrößen der Detektionsgrenzen



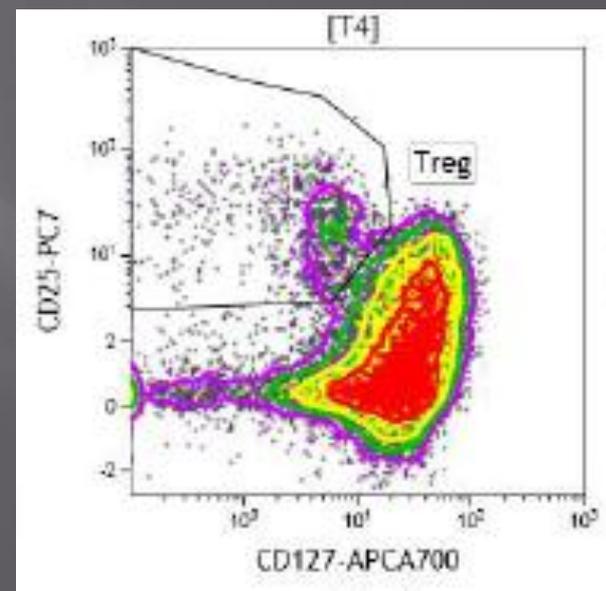
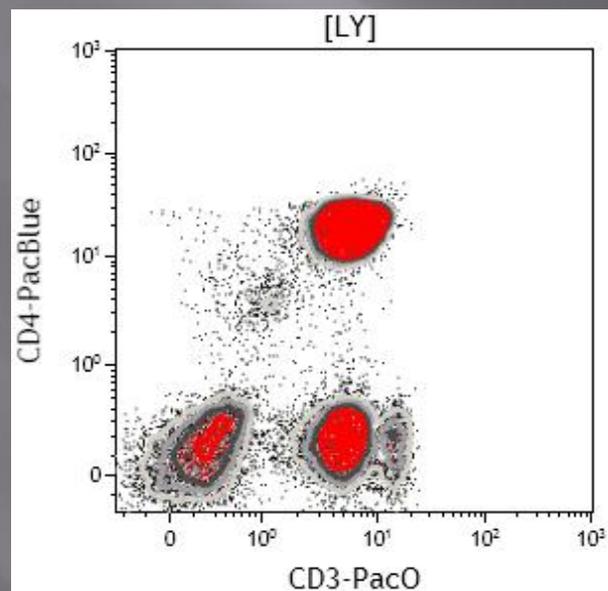
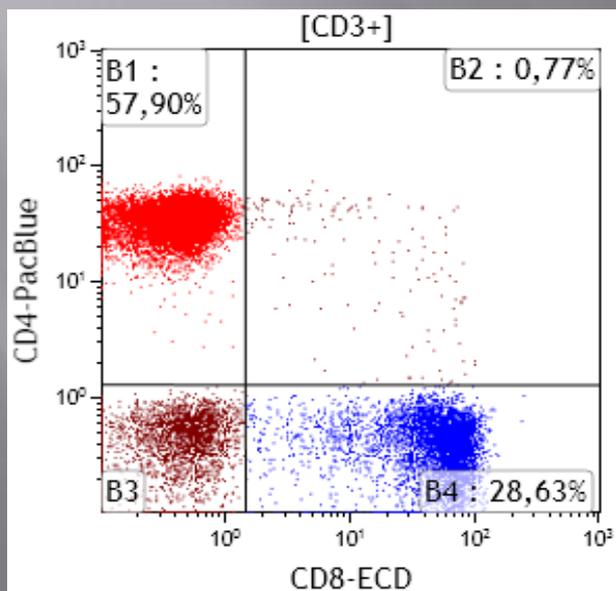
# Kombination der Marker und Optimierung der Detektionsgrenzen

Exklusiv exprimiert

gegenseitig sich  
ausschließende  
Antigene

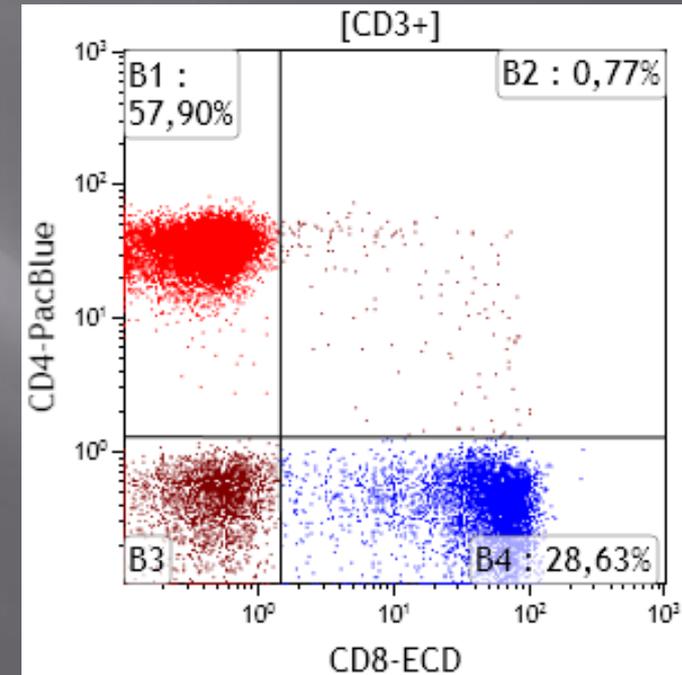
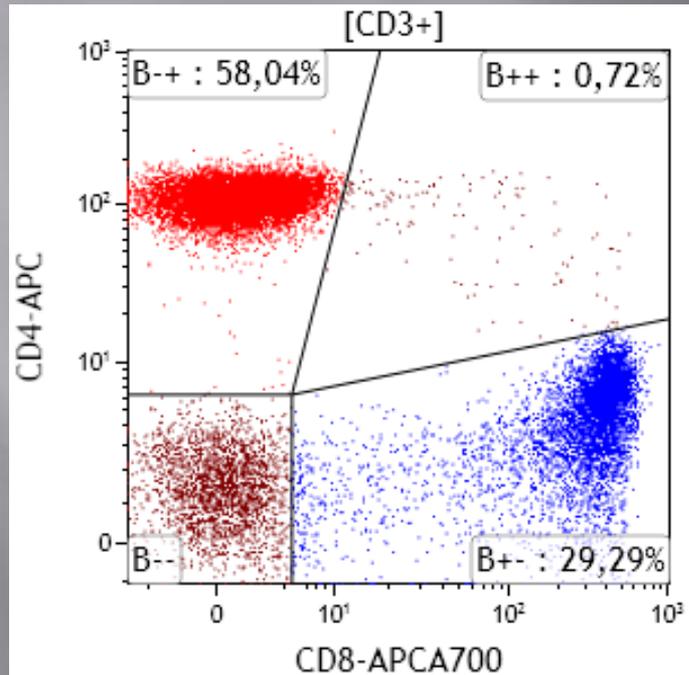
Stamm - und Subpopulation

Koexpression



# Gallios / Navios Multicolor Praxis

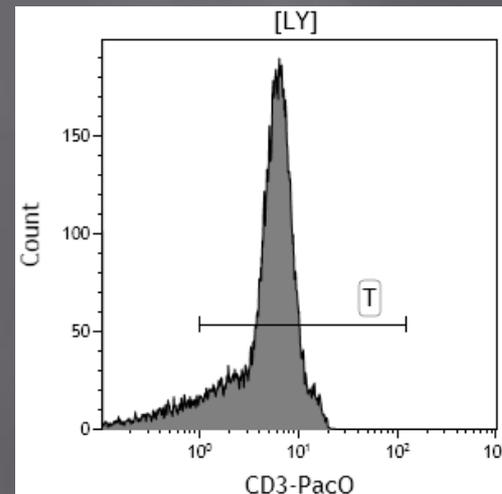
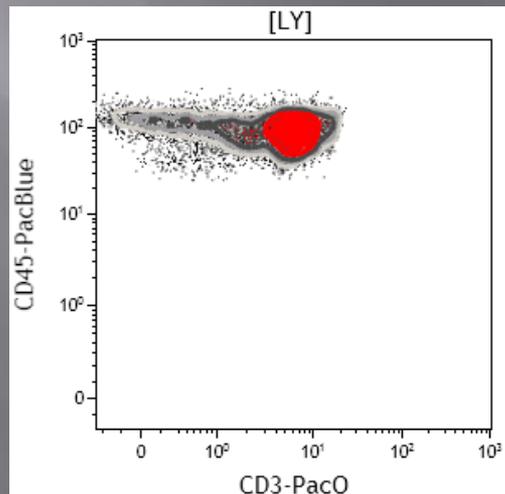
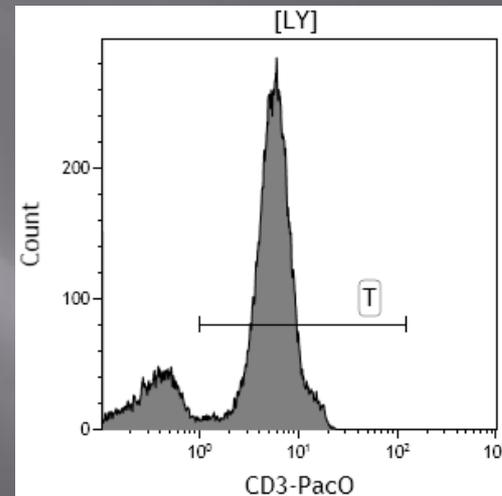
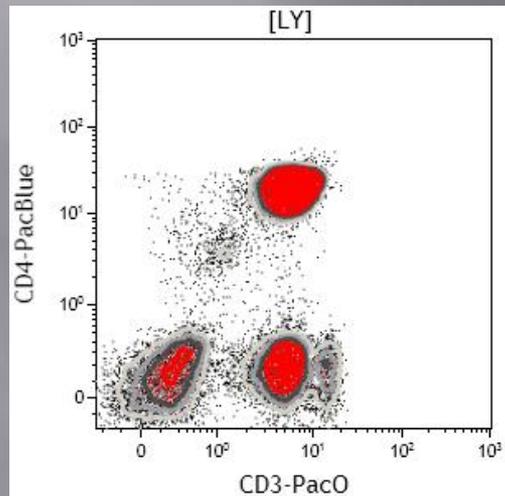
gegenseitig sich ausschließende Antigene



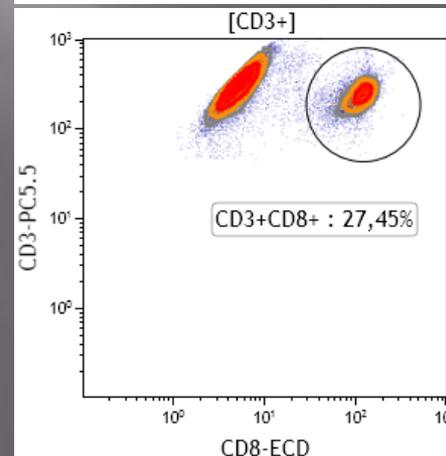
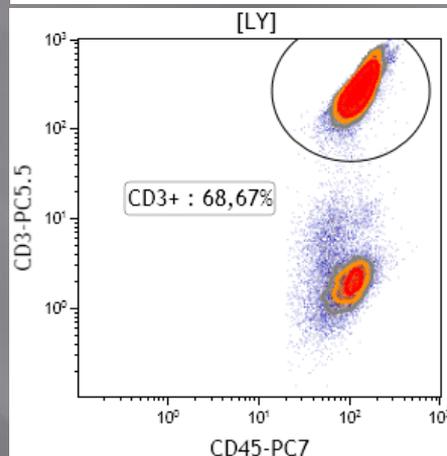
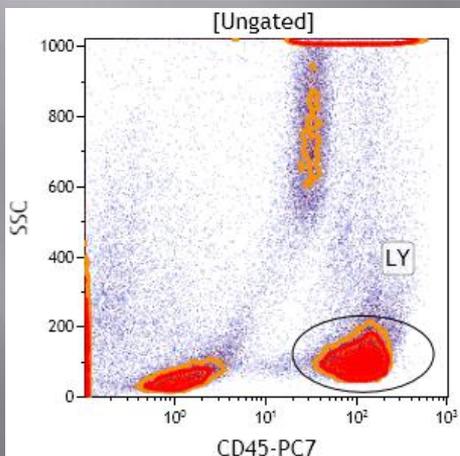
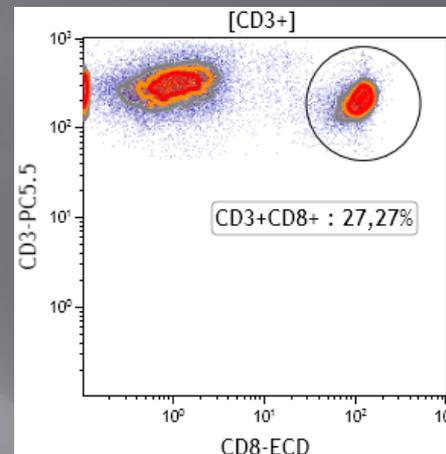
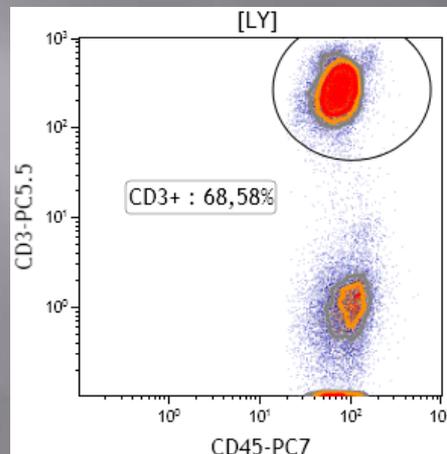
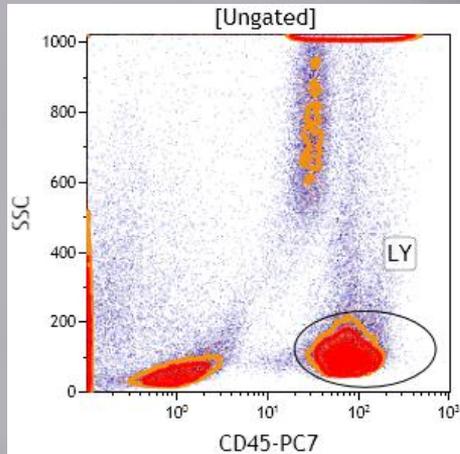
Übersprechen zwischen exklusiv exprimierten Antigenen kann toleriert werden

# Gallios / Navios Multicolor Praxis

Übersprechen eines Markers einer Subpopulation zur Stamm - Population kann toleriert werden - jedoch nie umgekehrt



# Übersprechen mehrerer Marker von einer Subpopulation zur Stamm - Population CD45 > CD3 > CD8



Spillover (%)			
	FL3	FL4	FL5
FL3		0,90	1,10
FL4	30,90		0,60
FL5	3,40	19,80	

**kompensiert**

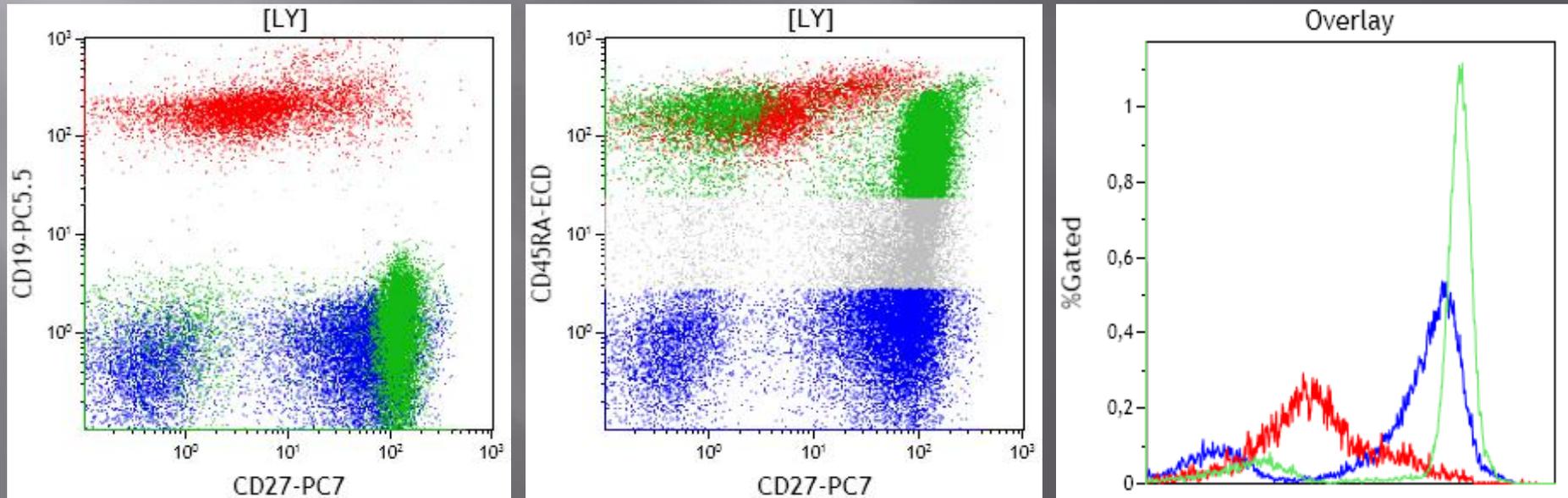
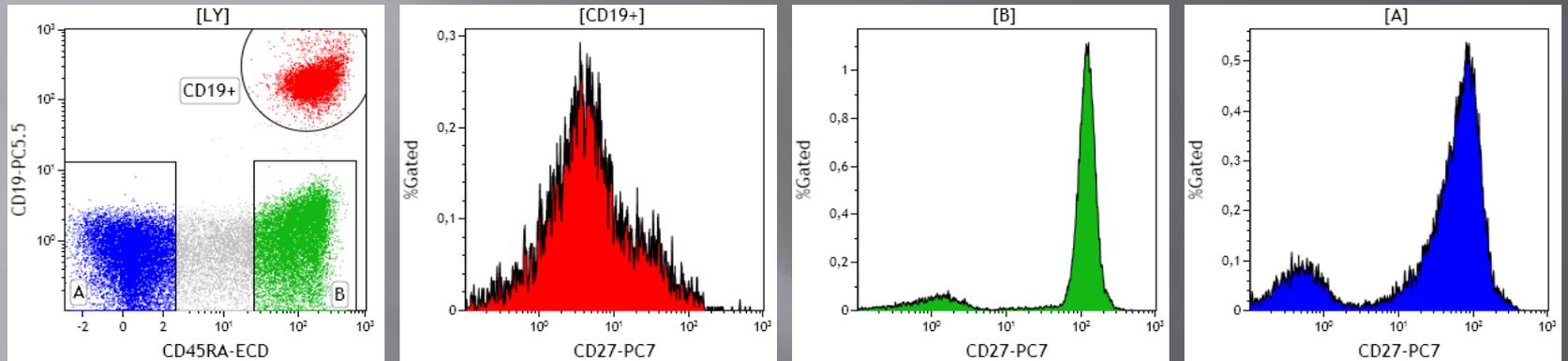
Spillover (%)			
	FL3	FL4	FL5
FL3		0,00	0,00
FL4	0,00		0,00
FL5	0,00	0,00	

**Unkompensiert**

**Garantie für einen robusten Assay !**

# Gallios / Navios Multicolor Praxis

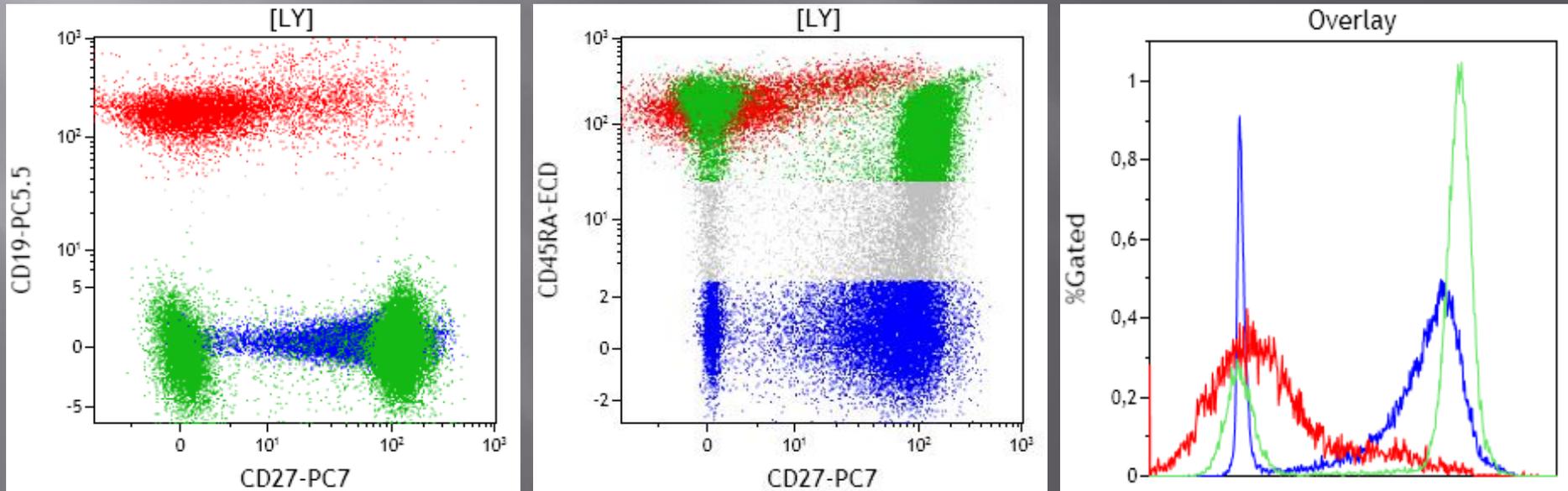
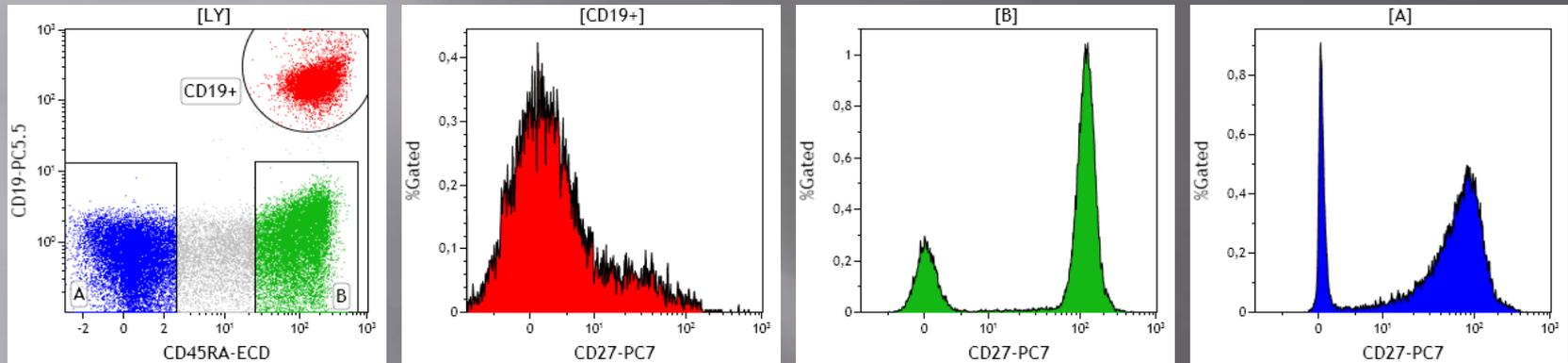
Übersprechen zwischen koexprimierten Antigenen ist zu vermeiden



Logarithmic view

# Gallios / Navios Multicolor Praxis

Übersprechen zwischen koexprimierten Antigenen ist zu vermeiden



Logicle view

# Gallios / Navios Multicolor Praxis

Beispiel für eine perfektes 7 Farb Kombination

		488 Excitation		633 Excitation			405 Excitation		
		FITC	PE		PC7	APC	APC- AF700	Pacific Blue	Krome Orange
488 Excitation	FITC			UNTOUCHABLE					
	PE	●							
		<i>Exc</i>	<i>ision</i>		<i>N</i>	<i>T</i>		<i>Parent</i>	
633 Excitation	PC7		●	UNTOUCHABLE					
	APC		<i>L</i>		<i>L</i>	<i>HIGH</i>		<i>Subpop/Parent</i>	
	APC- AF700		<i>S</i>		<i>S</i>	<i>SENS</i>		<i>Subpop/Parent</i>	<i>S</i>
405 Excitation	Pacific Blue			UNTOUCHABLE					
	Krome Orange						●		

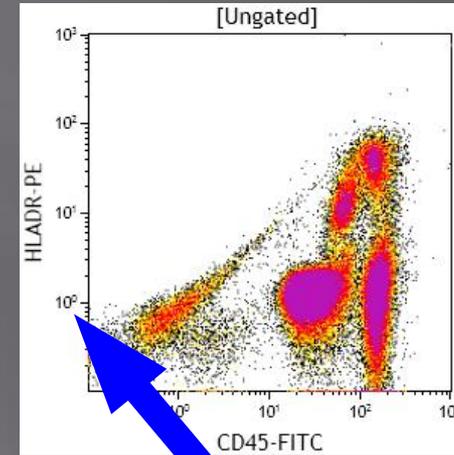
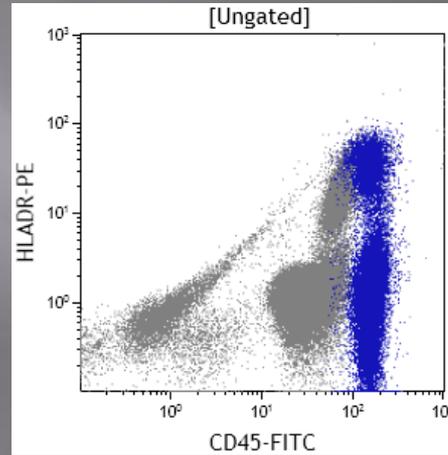
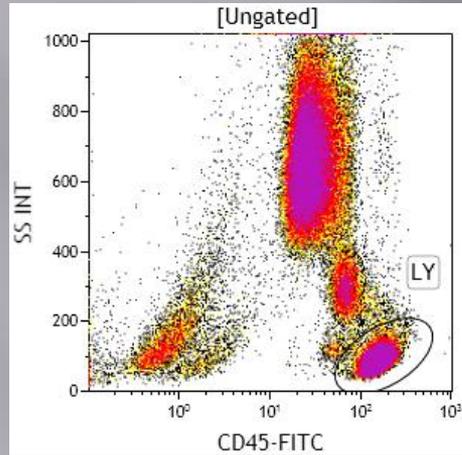
# Gallios / Navios Multicolor Praxis

## Allgemeine Regeln

- **Schwach exprimierte Marker mit Fluorochromen hoher Quantenausbeute kombinieren**  
Stark exprimierte Antigene funktionieren mit allen Farbstoffen
- **Schwach exprimierte Antigene möglichst auf „untouchable“ Messkanäle legen, Stark exprimierte Antigene auf „silent“ Messkanäle**  
Die spektrale Isolation schwacher Marker von hellen Linienmarker kann eventuell gegenüber der Verwendung von Fluorochromen mit hoher Quantenausbeute zu bevorzugen sein
- **Übersprechen zwischen sich ausschließenden Antigenen ist erlaubt**
- **Übersprechen zwischen sich koexprimierten Antigenen ist zu vermeiden**
- **Übersprechen eines Markers einer Subpopulation zur Stamm - Population kann toleriert werden jedoch nie umgekehrt**

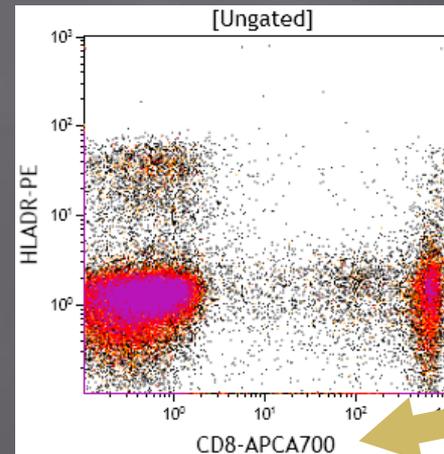
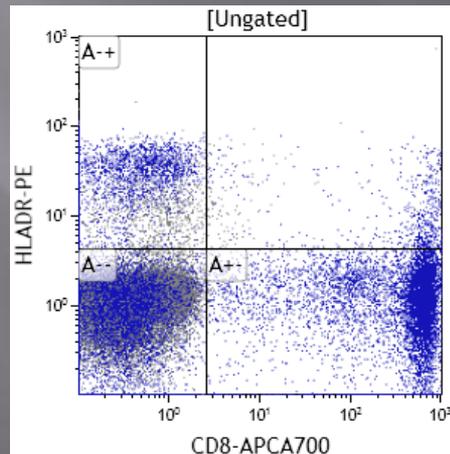
# Gallios / Navios Multicolor Praxis

Schwache Expression / heller Farbstoff / „untouched“ Kanal vs starke Expression / schwacher Farbstoff / „silent“ Kanal



schwacher / modulierende Expression:  
heller Farbstoff  
Aber: Messkanal nicht „untouched“

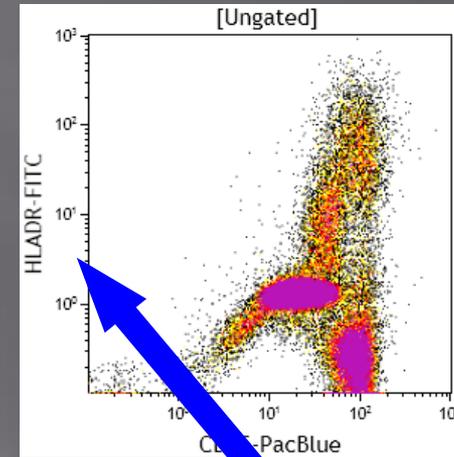
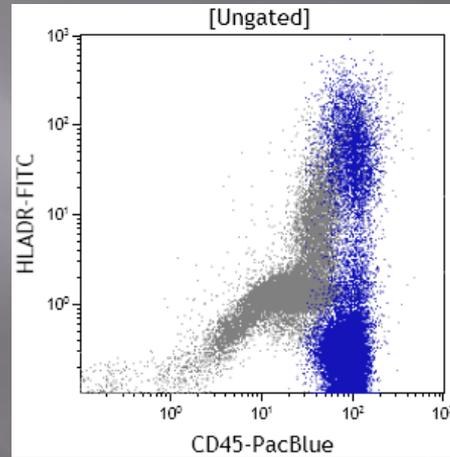
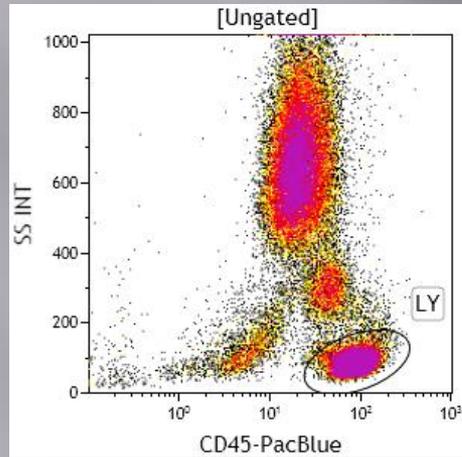
starke Expression:  
Schwacher Farbstoff  
aber nicht „silent“,



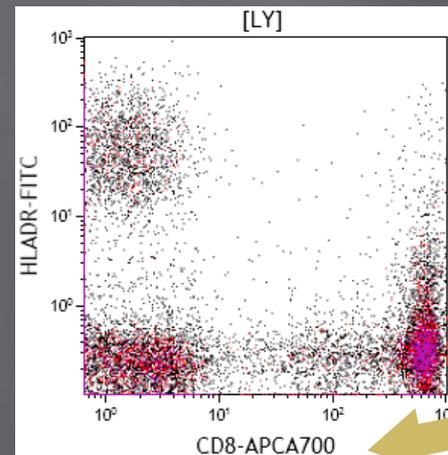
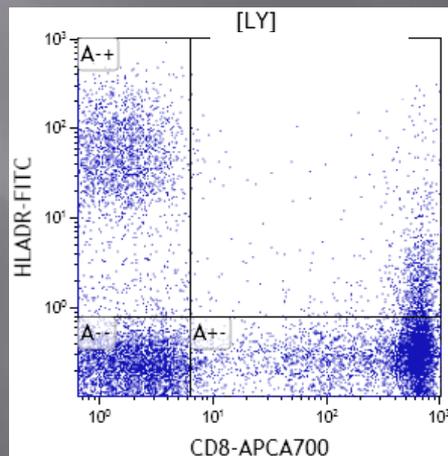
starke Expression:  
„silent“ Kanal,  
heller Farbstoff

# Gallios / Navios Multicolor Praxis

Schwache Expression / schwacher Farbstoff / „untouched“ Kanal vs starke Expression / schwacher Farbstoff / „silent“ Kanal



starke Expression:  
schwacher Farbstoff  
aber „silent“



schwacher /  
modulierende  
Expression:  
Schwacher Farbstoff  
(+ geringe sterische  
Behinderung)  
Isolierter Kanal

starke Expression:  
heller Farbstoff,  
„silent“

# Gallios / Navios Multicolor Praxis

## Quiz

Schwache /  
modulierende  
Expression:

CD25/IL2R,  
CD57,  
CD69, CD127/IL7R,  
CD184/CXCR4,  
CD279/PD-1

		488 Excitation					633 Excitation			405 Excitation		
		FITC	PE	ECD	PC5	PC5.5	PC7	APC	APC- AF700	APC- AF750	Pacific Blue	Krome Orange
488 Excitation	FITC											
	PE	●		●								
	ECD	●	●									
	PC5		●	●								
	PC5.5		●	●								
	PC7		●	●	●	●	●			●		
633 Excitation	APC				●	●			●	●		
	APC- AF700				●	●			●	●		
	APC- AF750				●	●	●	●	●			
405 Excitation	Pacific Blue											
	Krome Orange										●	

Minimierte  
Detektionsgrenzen  
für T4 / T8

Panel 1

FITC	PE	ECD	PC5.5	PC7	APC	APCAF700	APCA750	PacBlue	KrOrange
CD4	CD184	CD69	CD25	CD279	CD8	CD127	CD3	CD57	CD45

Panel 2

FITC	PE	ECD	PC5.5	PC7	APC	APCAF700	APCA750	PacBlue	KrOrange
CD57	CD184	CD8	CD25	CD279	CD69	CD127	CD45	CD4	CD3

# Gallios / Navios Multicolor Praxis

☞ ☞ ☞ **Der Gewinner ist ....** ☞ ☞ ☞

Schwache /  
modulierende  
Expression:

CD25/IL2R,  
CD57,  
CD69, CD127/IL7R,  
CD184/CXCR4,  
CD279/PD-1

	488 Excitation						633 Excitation			405 Excitation	
	FITC	PE	ECD	PC5	PC5.5	PC7	APC	APC- AF700	APC- AF750	Pacific Blue	Krome Orange
488 Excitation	FITC										
	PE	●	●								
	ECD	●	●								
	PC5		●	●							
	PC5.5		●	●							
	PC7		●		●	●			●		
633 Excitation	APC			●	●			●	●		
	APC- AF700			●	●		●		●		
	APC- AF750			●	●	●	●	●			
405 Excitation	Pacific Blue										
	Krome Orange								●		

Minimierte  
Detektionsgrenzen  
für T4 / T8

Panel 1

FITC	PE	ECD	PC5.5	PC7	APC	APCAF700	APCA750	PacBlue	KrOrange
CD4	CD184	CD69	CD25	CD279	CD8	CD127	CD3	CD57	CD45

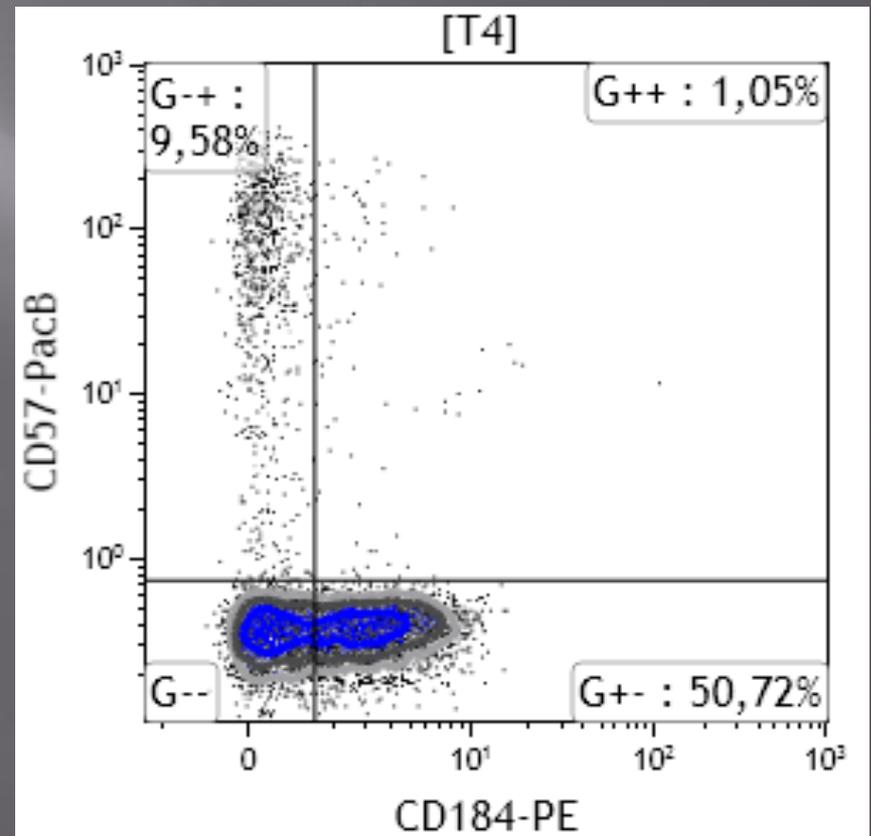
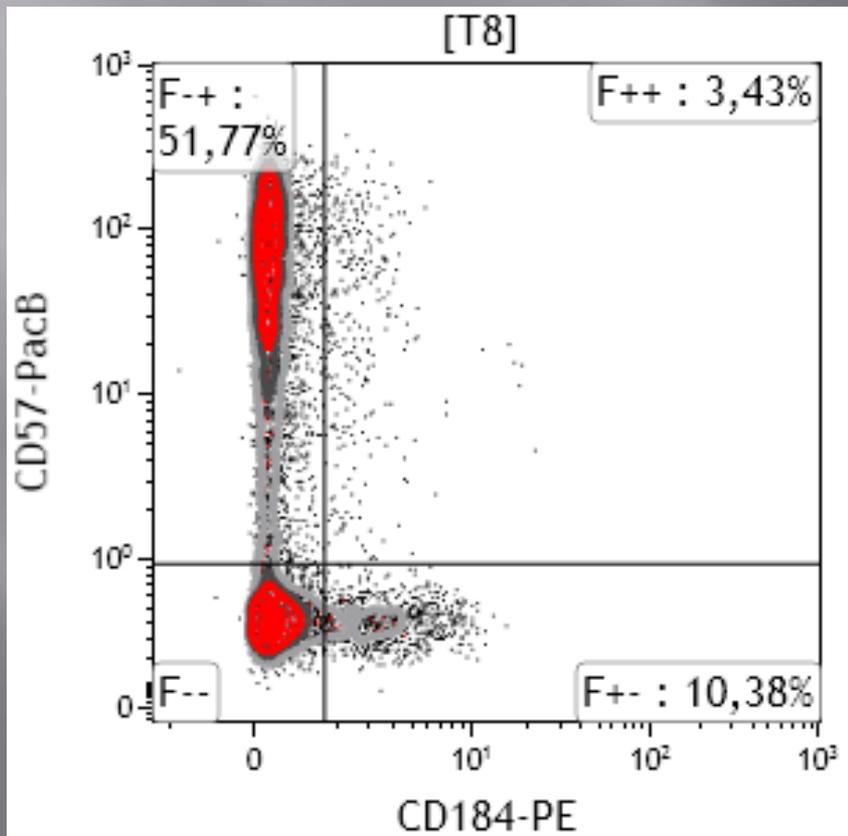
Panel 2

FITC	PE	ECD	PC5.5	PC7	APC	APCAF700	APCA750	PacBlue	KrOrange
CD57	CD184	CD8	CD25	CD279	CD69	CD127	CD45	CD4	CD3

# Gallios / Navios Multicolor Praxis

## Optimierung der Detektionsgrenzen

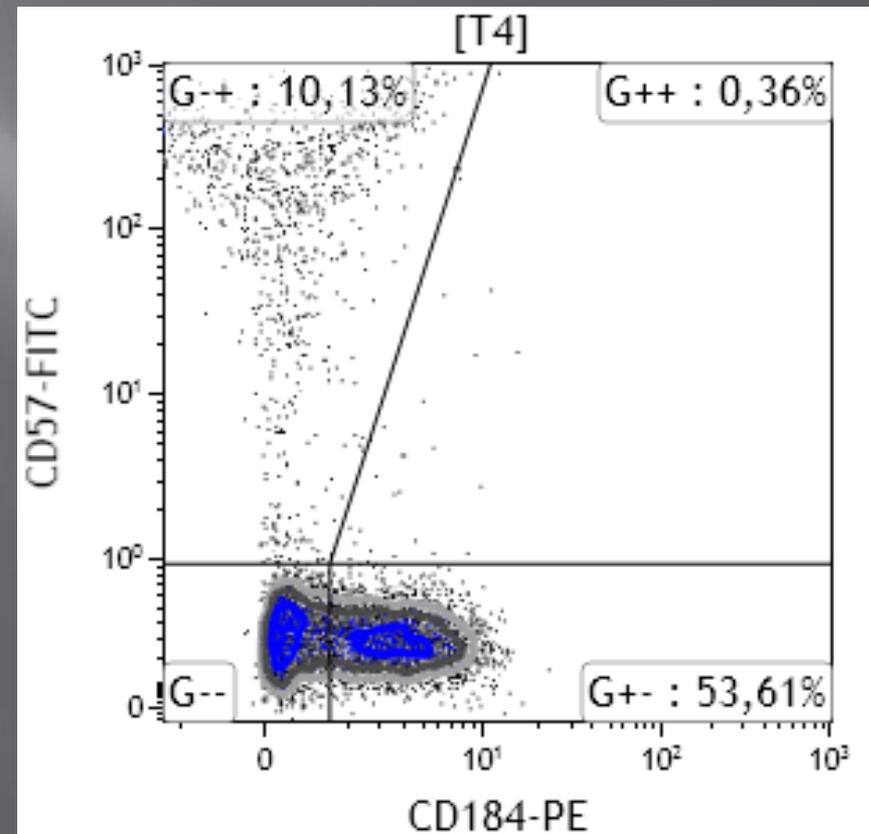
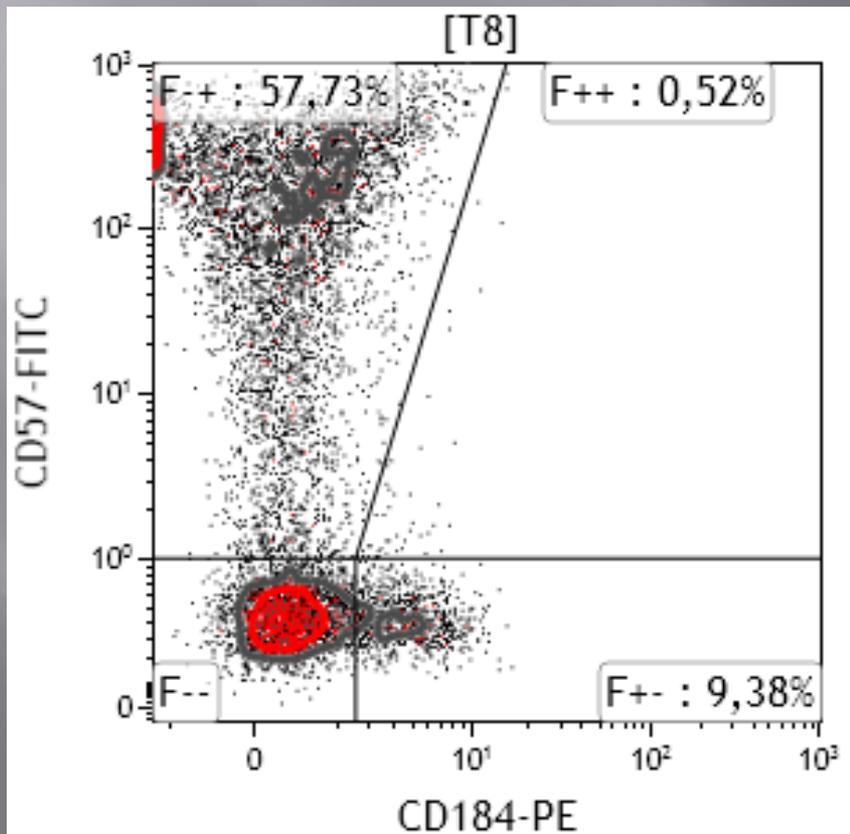
### Panel 1



# Gallios / Navios Multicolor Praxis

## Optimierung der Detektionsgrenzen

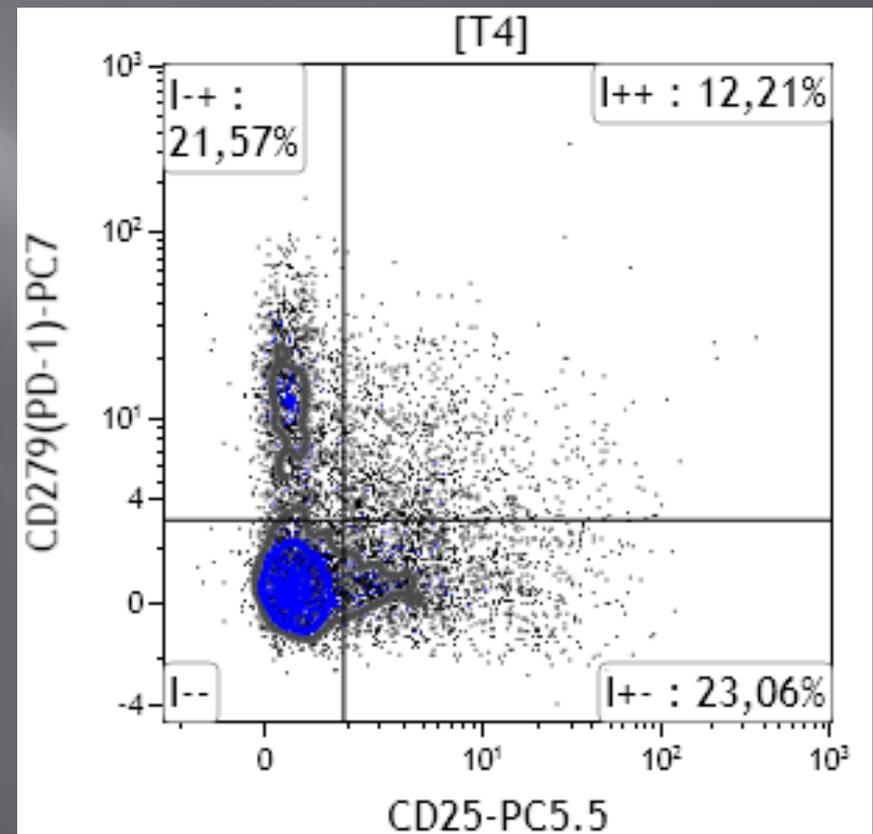
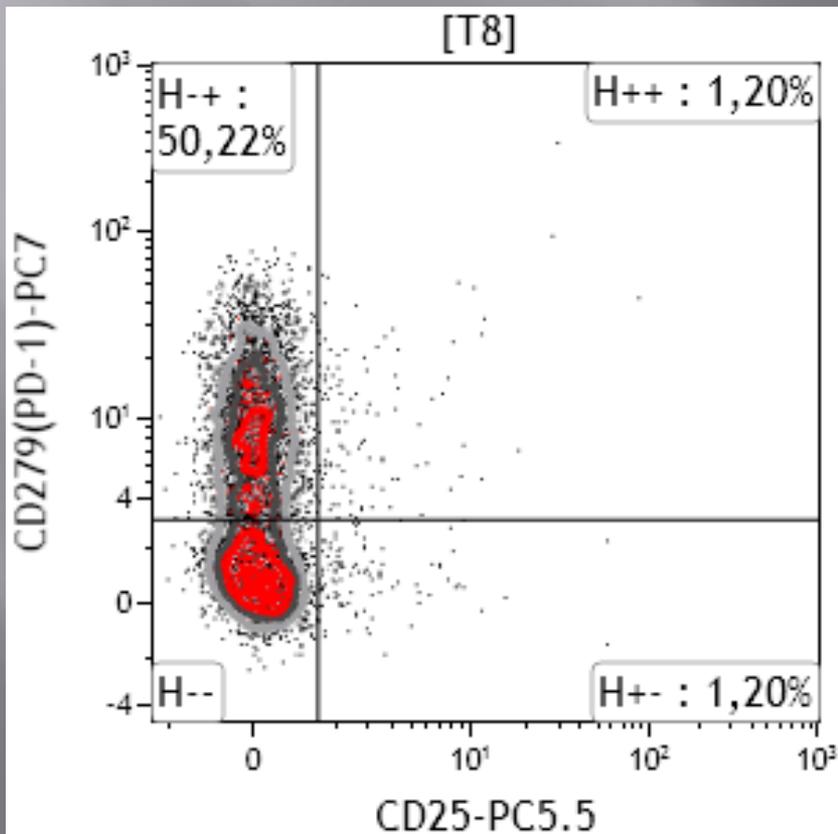
### Panel 2



# Gallios / Navios Multicolor Praxis

## Optimierung der Detektionsgrenzen

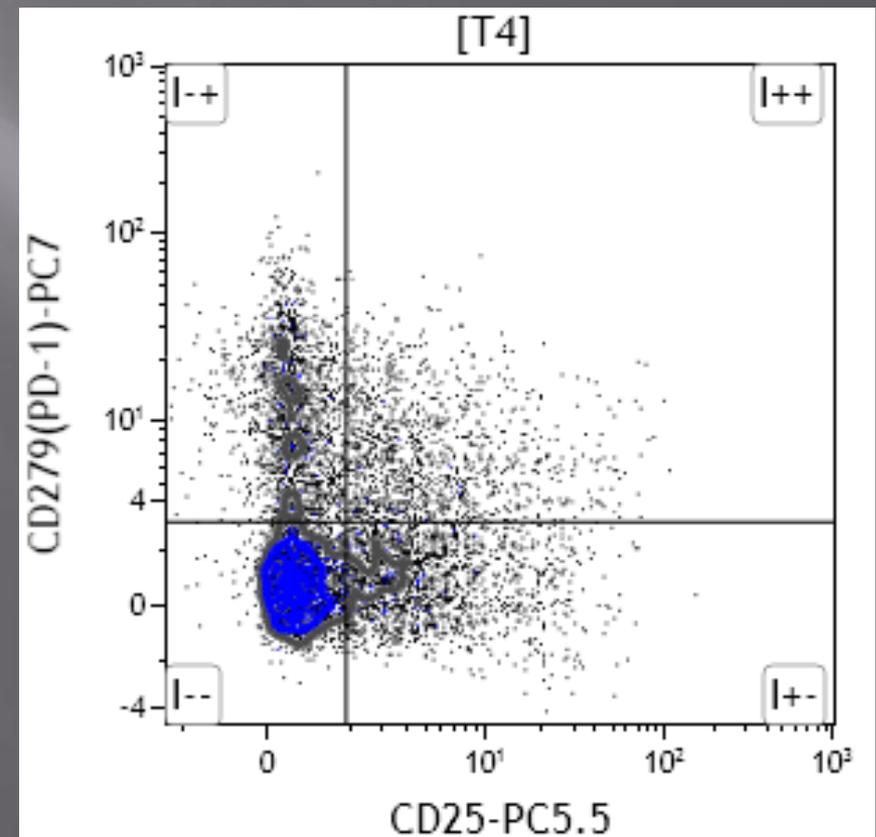
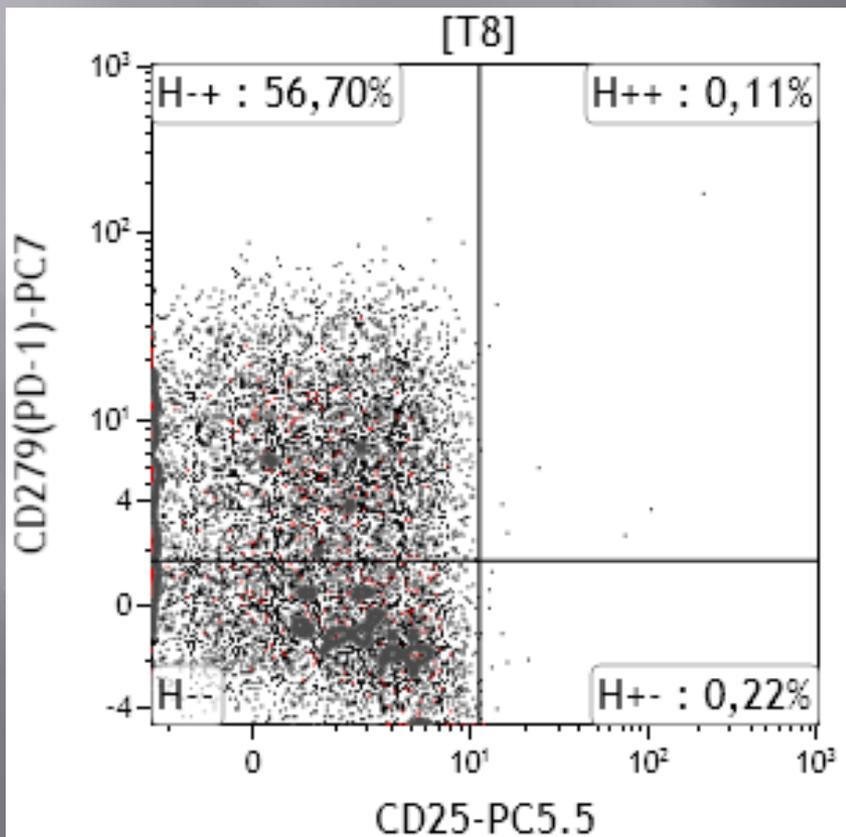
### Panel 1



# Gallios / Navios Multicolor Praxis

## Optimierung der Detektionsgrenzen

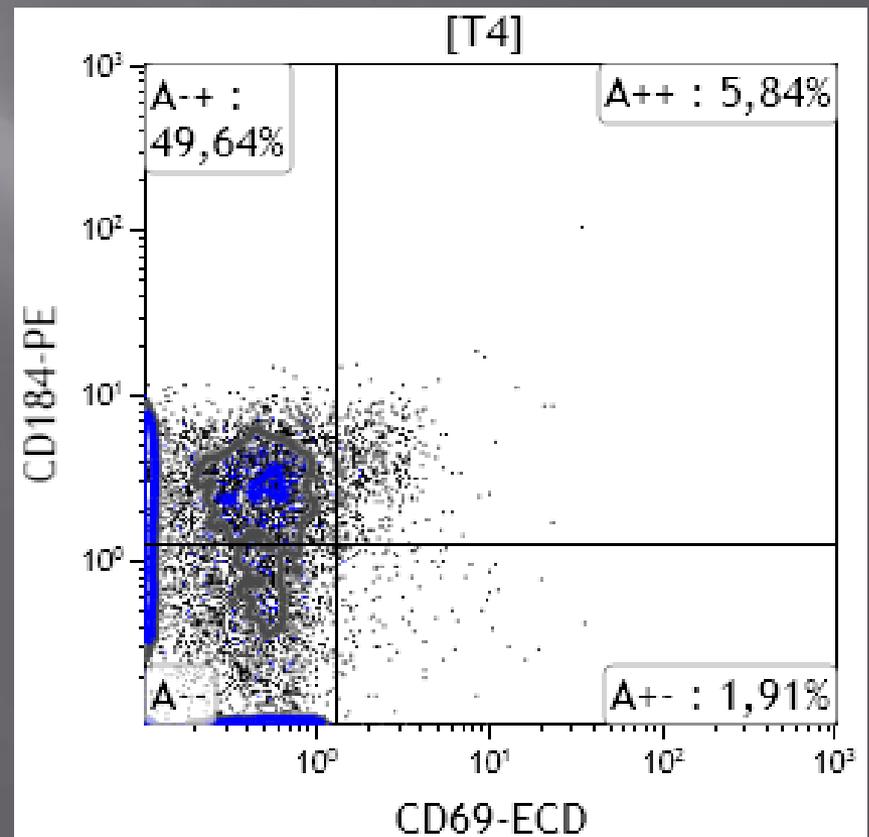
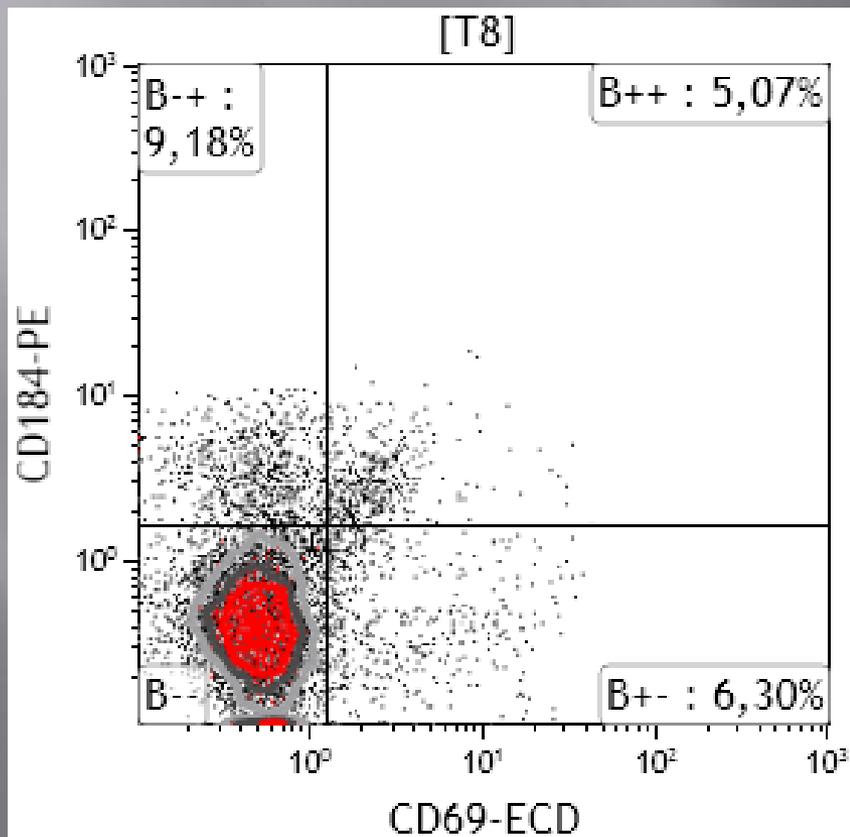
### Panel 2



# Gallios / Navios Multicolor Praxis

## Optimierung der Detektionsgrenzen

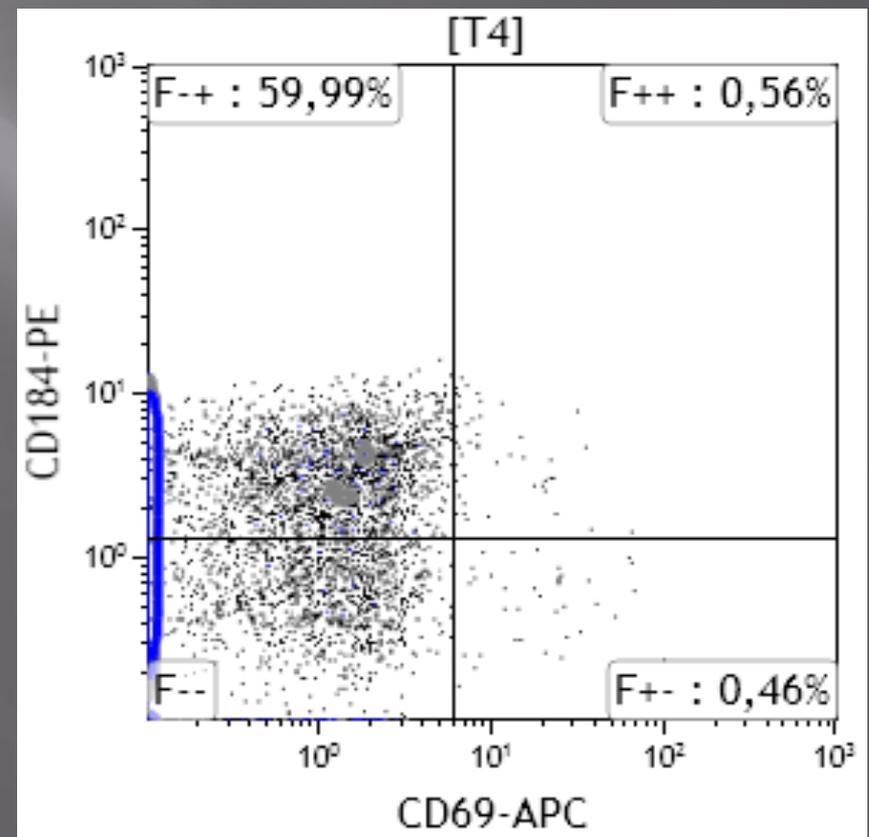
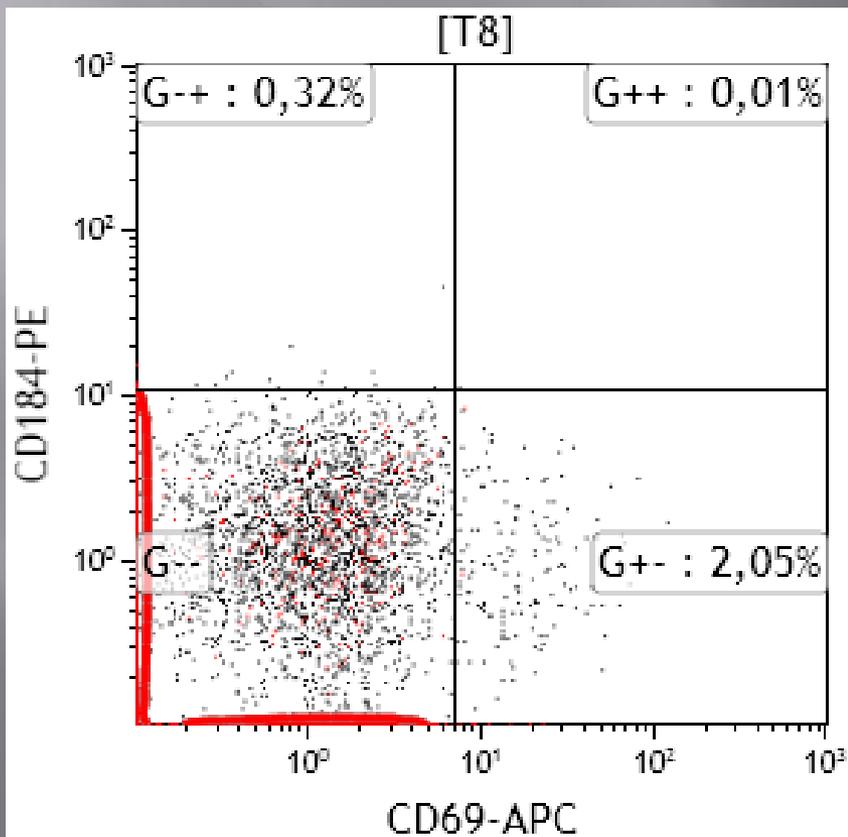
### Panel 1



# Gallios / Navios Multicolor Praxis

## Optimierung der Detektionsgrenzen

### Panel 2



# Gallios / Navios Multicolor Praxis

## 10 Farben “to go” Tips und Tricks

		488 Excitation					633 Excitation			405 Excitation		
		FITC	PE	ECD	PC5	PC5.5	PC7	APC	APC-AF700	APC-AF750	Pacific Blue	Krome Orange
488 Excitation	FITC											
	PE	●		●								
	ECD	●	●									
	PC5		●	●								
	PC5.5		●	●	<i>FL4-determined channels</i>							
	PC7		●	●	●	●				●		
633 Excitation	APC				●	●			●	●		
	APC-AF700				●	●		●		●		
	APC-AF750				●	●	●	●				
405 Excitation	Pacific Blue											
	Krome Orange										●	

PC5.5 und PC5 sind auch vom roten Laser anregbar

Sie stören damit alle roten Messkanäle (Dominoeffekt)

PC7,  
APC,  
APCA700,  
APCA750

# Gallios / Navios Multicolor Praxis

## 10 Farben "to go" Tips und Tricks

		488 Excitation					633 Excitation			405 Excitation		
		FITC	PE	ECD	PC5	PC5.5	PC7	APC	APC-AF700	APC-AF750	Pacific Blue	Krome Orange
488 Excitation	FITC											
	PE											
	ECD											
	PC5											
	PC5.5											
	PC7											
633 Excitation	APC											
	APC-AF700											
	APC-AF750											
405 Excitation	Pacific Blue											
	Krome Orange											

Sinnvolle Paarung für Stamm - Subpopulationen oder sich ausschließende

Marker:

PE - ECD,

ECD - PC5.5(PC5),

PC5 - APC,

PC5.5 – APC700,

PC7 - APC750...

# Gallios / Navios Multicolor Praxis

## 10 Farben “to go” Tips und Tricks

		488 Excitation						633 Excitation			405 Excitation	
		FITC	PE	ECD	PC5	PC5.5	PC7	APC	APC- AF700	APC- AF750	Pacific Blue	Krome Orange
488 Excitation	FITC											
	PE	•		•								
	ECD	•	•									
	PC5		•	•								
	PC5.5		•	•								
	PC7		•	•	•	•				•		
633 Excitation	APC				•	•			•	•		
	APC- AF700				•	•		•		•		
	APC- AF750				•	•	•	•				
405 Excitation	Pacific Blue											
	Krome Orange										•	

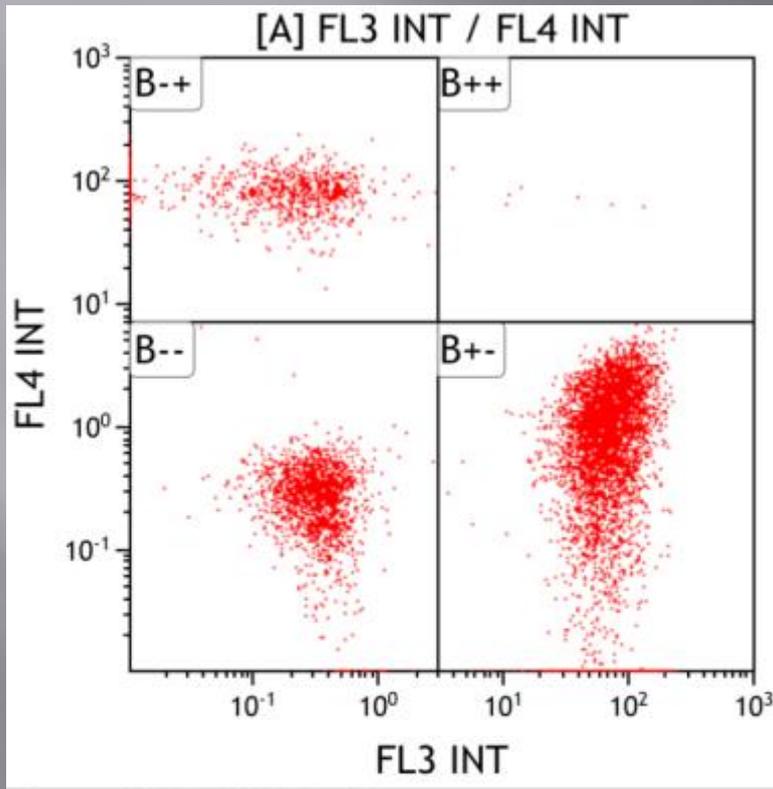
„No wash“  
 Probenvorbereitung  
 erhöhter Hintergrund  
 überstrahlt den  
 Übersprechanteil

> klassische orthogonale  
 Anordnung der Cluster

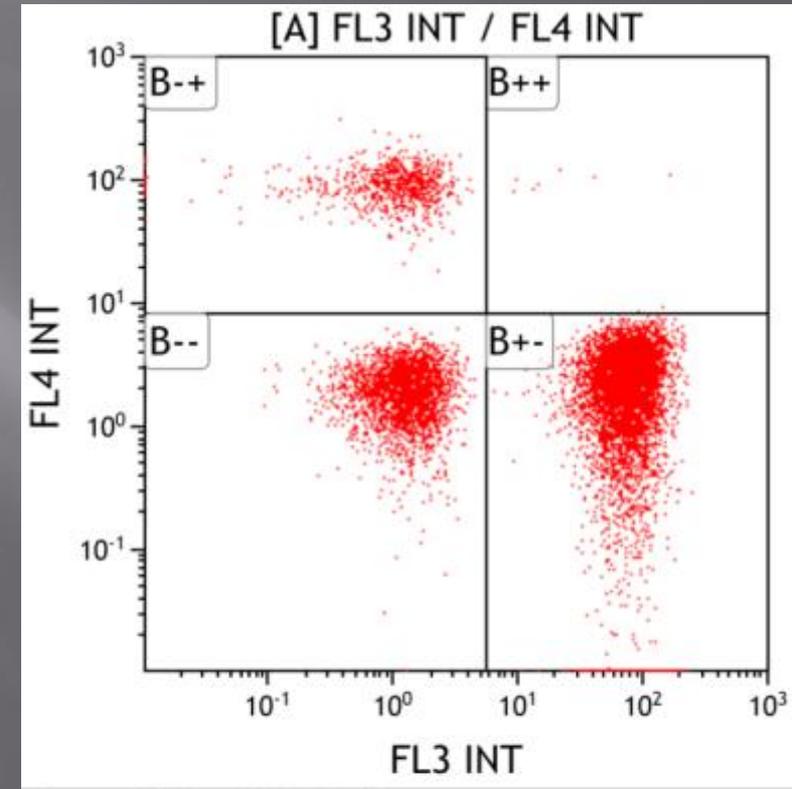
# Waschen der Probe eliminiert den Background der negativen Population

wash

no wash



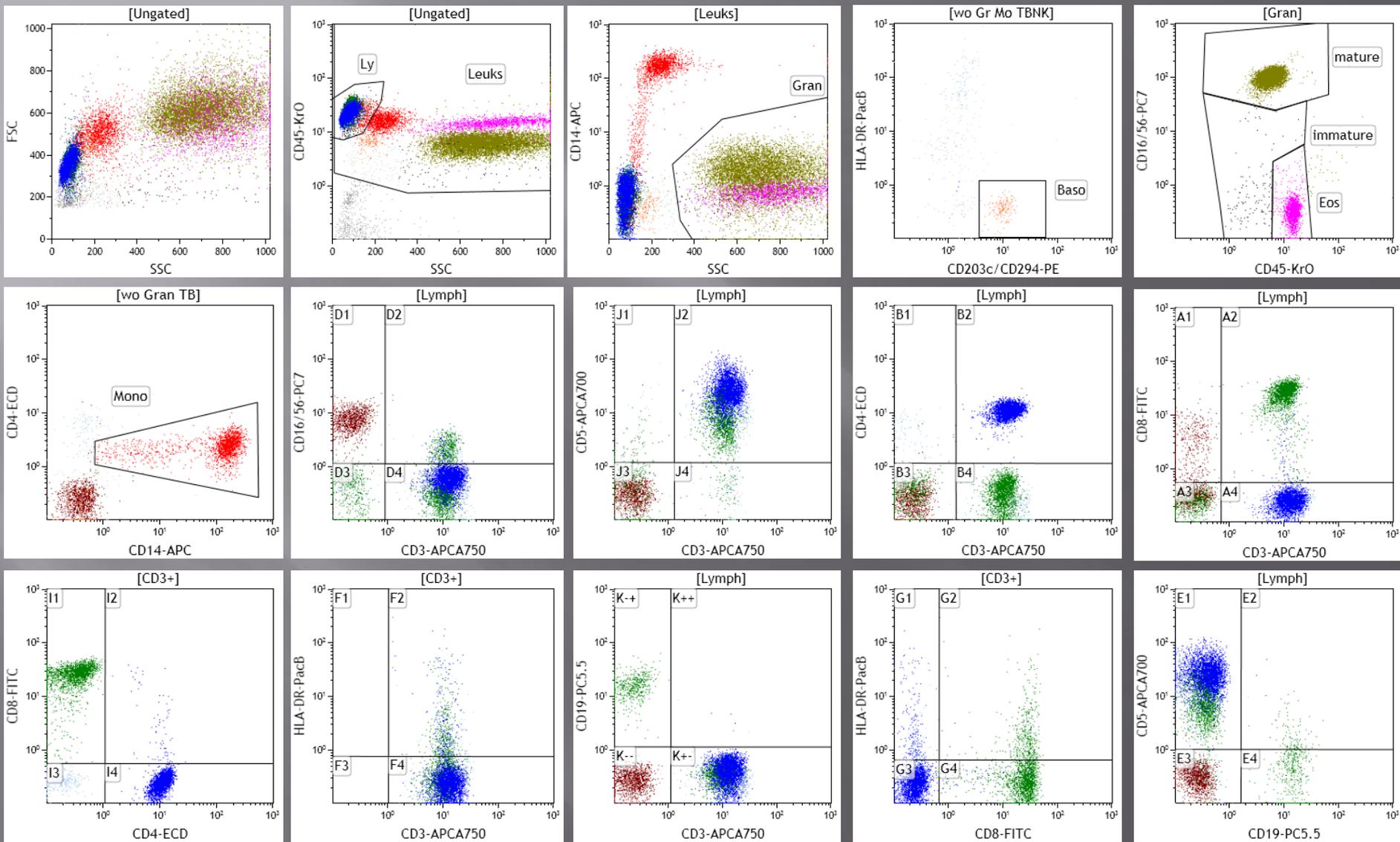
Gate	X-Med	Y-Med
All	56,25	0,36
B--	0,33	0,29
B-+	0,17	83,43
B+-	67,63	0,29
B++	13,81	72,10



Gate	X-Med	Y-Med
All	55,07	2,12
B--	1,28	1,98
B-+	1,18	90,17
B+-	74,13	1,98
B++	40,18	90,28

# Gallios / Navios Multicolor Praxis

## 10 Colors “no wash”



# Gallios / Navios Multicolor Praxis

## 10 Farben “to go” Tips und Tricks

		488 Excitation					633 Excitation			405 Excitation		
		FITC	PE	ECD	PC5	PC5.5	PC7	APC	APC- AF700	APC- AF750	Pacific Blue	Krome Orange
488 Excitation	<i>Untouchable Backbone</i>											
	PE	●	■	●								
	ECD	●	●	■								
	PC5		●	●	■	■						
	PC5.5		●	●	■	■						
	PC7		●	●	●	●	●			●		
633 Excitation	APC				●	●		■	●	●		
	APC- AF700				●	●		●	■	●		
	APC- AF750				●	●	●	●	●	■		
405 Excitation	Pacific Blue										■	
	Krome Orange										●	■

„Untouchable“ Backbones  
sichern konsistente  
Expressionsmuster  
unabhängig von der  
Expression anderer  
Antigene

# Gallios / Navios Multicolor Praxis

## 10 Farben "to go" Tips und Tricks

		488 Excitation						633 Excitation			405 Excitation	
		FITC	PE	ECD	PC5	PC5.5	PC7	APC	APC- AF700	APC- AF750	Pacific Blue	Krome Orange
488 Excitation	<i>Untouchable Backbone</i>											
	PE	●		●								
	ECD	●	●									
	PC5		●	●								
	PC5.5		●	●								
	PC7		●	●	●	●	●			●		
633 Excitation	APC				●	●			●	●		
	APC- AF700				●	●		●		●		
	APC- AF750				●	●	●	●				
405 Excitation	Pacific Blue											
	Krome Orange										●	

„Untouchable“ Backbones  
sichern konsistente  
Expressionsmuster  
unabhängig von der  
Expression anderer  
Antigene

*Untouchable Backbone*

# Gallios / Navios Multicolor Praxis

## 10 Farben "to go" Tips und Tricks

### "Untouchable" Backbone

[LEUK]

CD23-PE

CD5-FITC

CD19-ECD

CD5-FITC

CD23-PE

CD19-ECD

CD25-PC5

HLA-DR-PC7

CD38-APC

CD20-APCA750

CD45-PacBlue

[LEUK]

CD23-PE

CD5-FITC

CD19-ECD

CD5-FITC

CD23-PE

CD19-ECD

CD38-PC5

CD25-PC7

HLA-DR-APC

CD45-APCA750

CD20-PacBlue

[LEUK]

CD23-PE

CD5-FITC

CD19-ECD

CD5-FITC

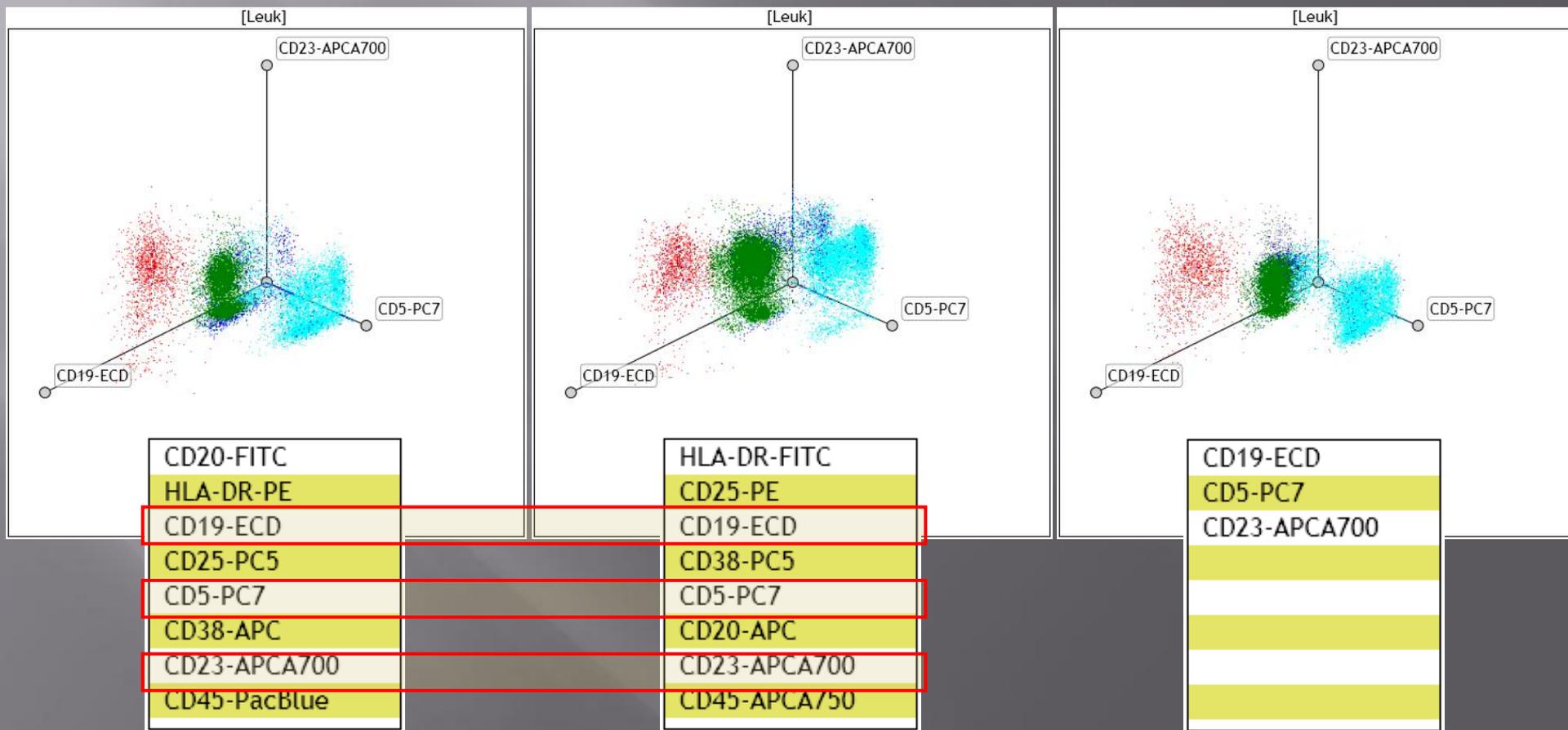
CD23-PE

CD19-ECD

# Gallios / Navios Multicolor Praxis

## 10 Farben "to go" Tips und Tricks

### "Touchable" Backbone



# Gallios / Navios Multicolor Praxis

## 10 Farben “to go” Tips und Tricks

		488 Excitation						633 Excitation			405 Excitation	
		FITC	PE	ECD	PC5	PC5.5	PC7	APC	APC- AF700	APC- AF750	Pacific Blue	Krome Orange
488 Excitation	FITC											
	PE	●		●								
	ECD	●	●									
	PC5		●	●								
	PC5.5		●	●								
	PC7		●	●	●	●				●		
633 Excitation	APC				●	●			●	●		
	APC- AF700				●	●		●	●	●		
	APC- AF750				●	●	●	●	●	●		
405 Excitation	Pacific Blue											
	Krome Orange										●	

Duch „Silent“ Backbones werden die Nachweisgrenzen für koexprimierte Marker nicht erhöht

# Gallios / Navios Multicolor Praxis

## 10 Farben "to go" Tips und Tricks

		488 Excitation						633 Excitation			405 Excitation	
		FITC	PE	ECD	PC5	PC5.5	PC7	APC	APC- AF700	APC- AF750	Pacific Blue	Krome Orange
488 Excitation	FITC											
	PE	●		●								
	ECD	●	●									
	PC5		●	●								
	PC5.5		●	●								
	PC7		●	●	●	●				●		
633 Excitation	APC				●	●			●	●		
	APC- AF700				●	●		●		●		
	APC- AF750				●	●	●	●	●			
405 Excitation	Pacific Blue										<i>Silent</i>	
	Krome Orange										●	<i>Backbone</i>

Duch „Silent“ Backbones  
werden die Nachweisgrenzen  
für koexprimierte Marker  
nicht erhöht

# Wichtungen für Separation und Messempfindlichkeit



## Immunomonitoring

Zahlreiche Linienmarker  
Bekannte Phänotypen



## Lymphome

1-2 Linienmarker

bekannte Phänotypen,  
aber atypische Ausnahmen

einige sich gegenseitig  
ausschließende Phänotypen



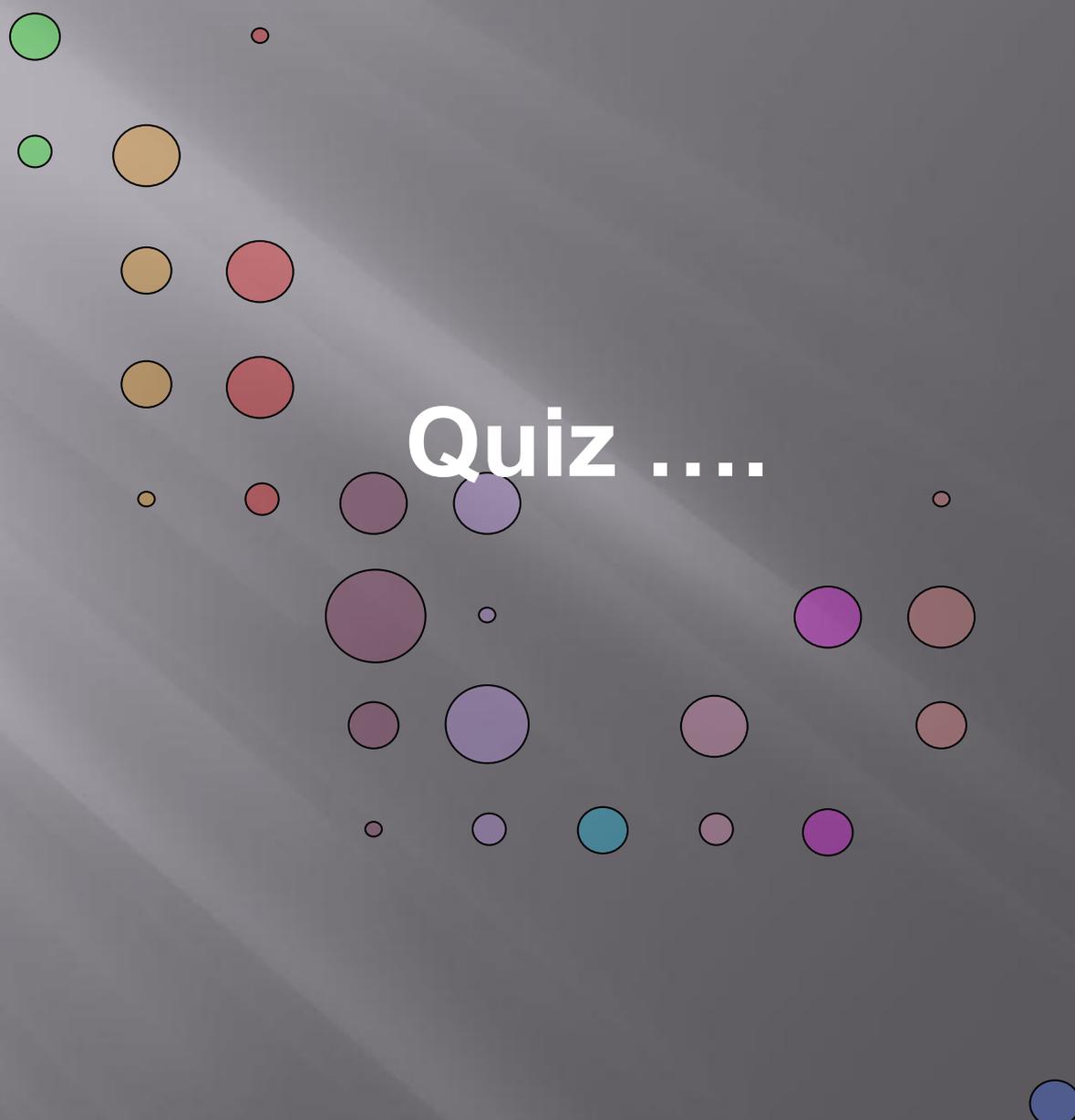
## Leukemia

Abnorme Linien Antigene  
Große Variation der Expression  
Große Vielfalt an Phänotypen



# Noch Fragen

# ?



Quiz ....