

Bi9393 Analytická cytometrie

Lekce 3



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Oddělení cytokinetiky
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Principy průtokové cytometrie a sortování

- sorting
- zpracování signálu
- analýza dat
- kompenzace signálu



Doležel (1999)

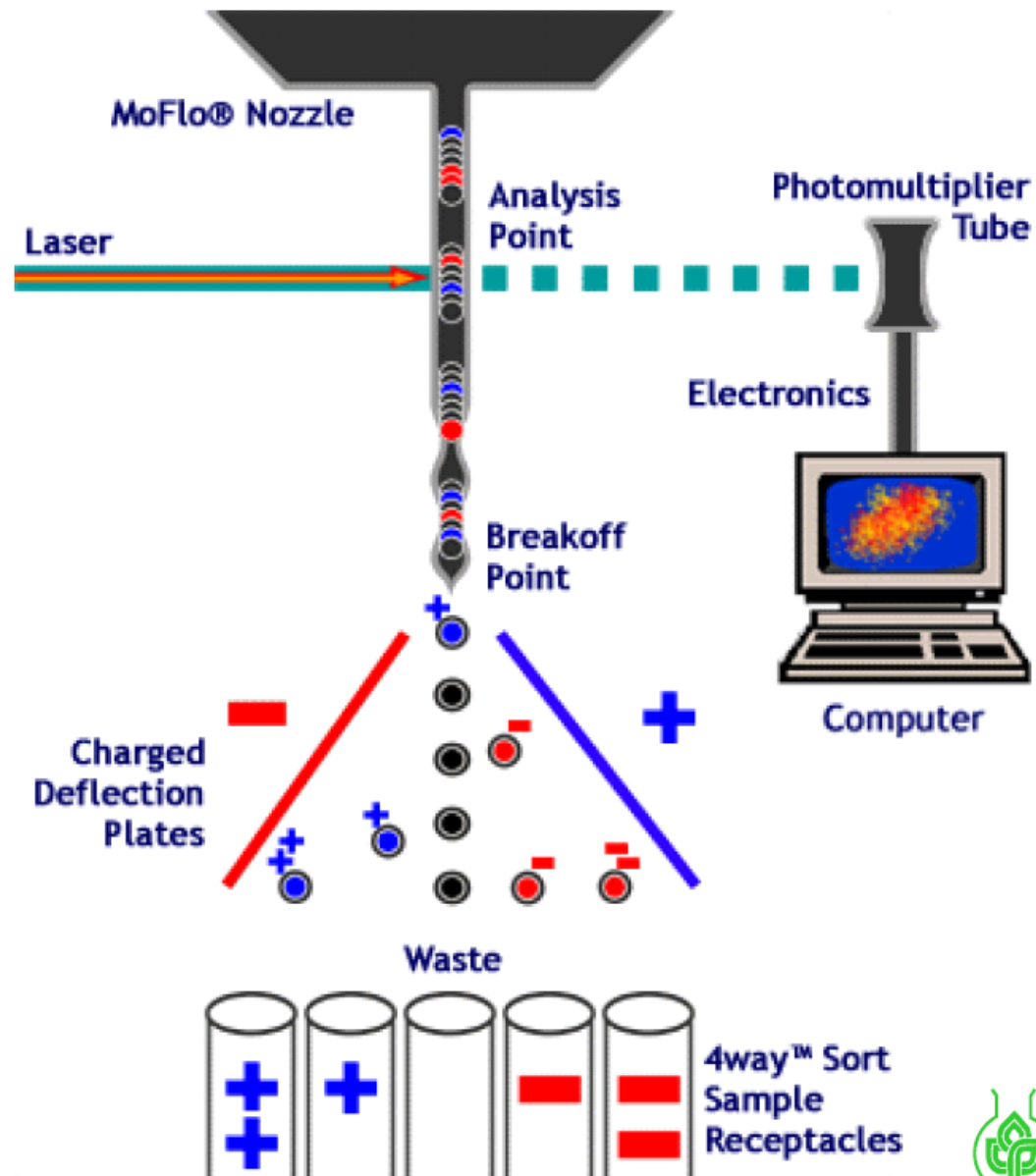


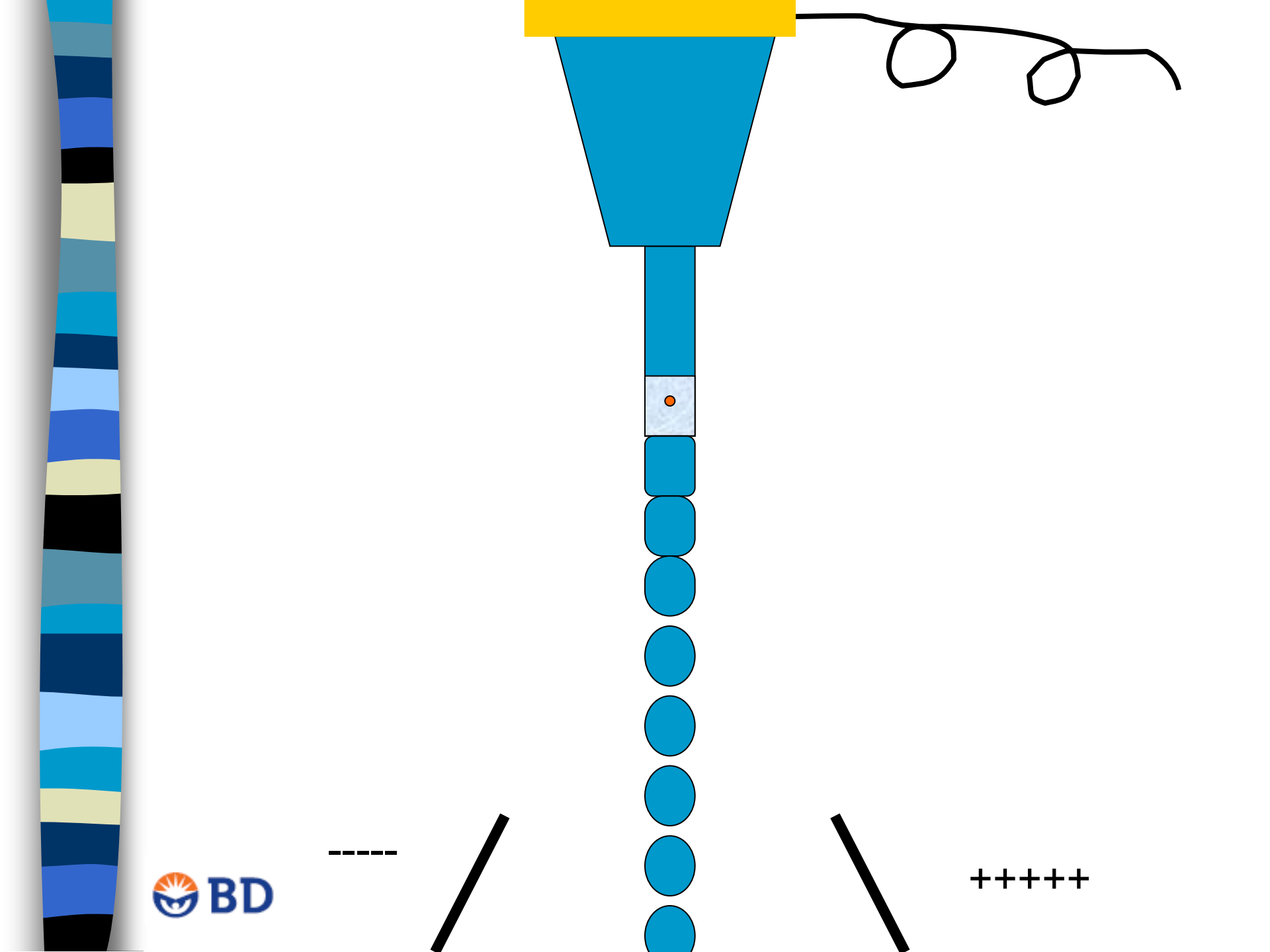
ELECTROSTATIC DROPLET SORTER

- High speed ($\sim 10^4/\text{sec}$)
 - Concentrated sorted fraction
 - Biosafety hazard
 - Mechanical shearing
- Problems to sort large particles

Used by:
Becton Dickinson
Beckman Coulter
Cytomation

Doležel (1999)

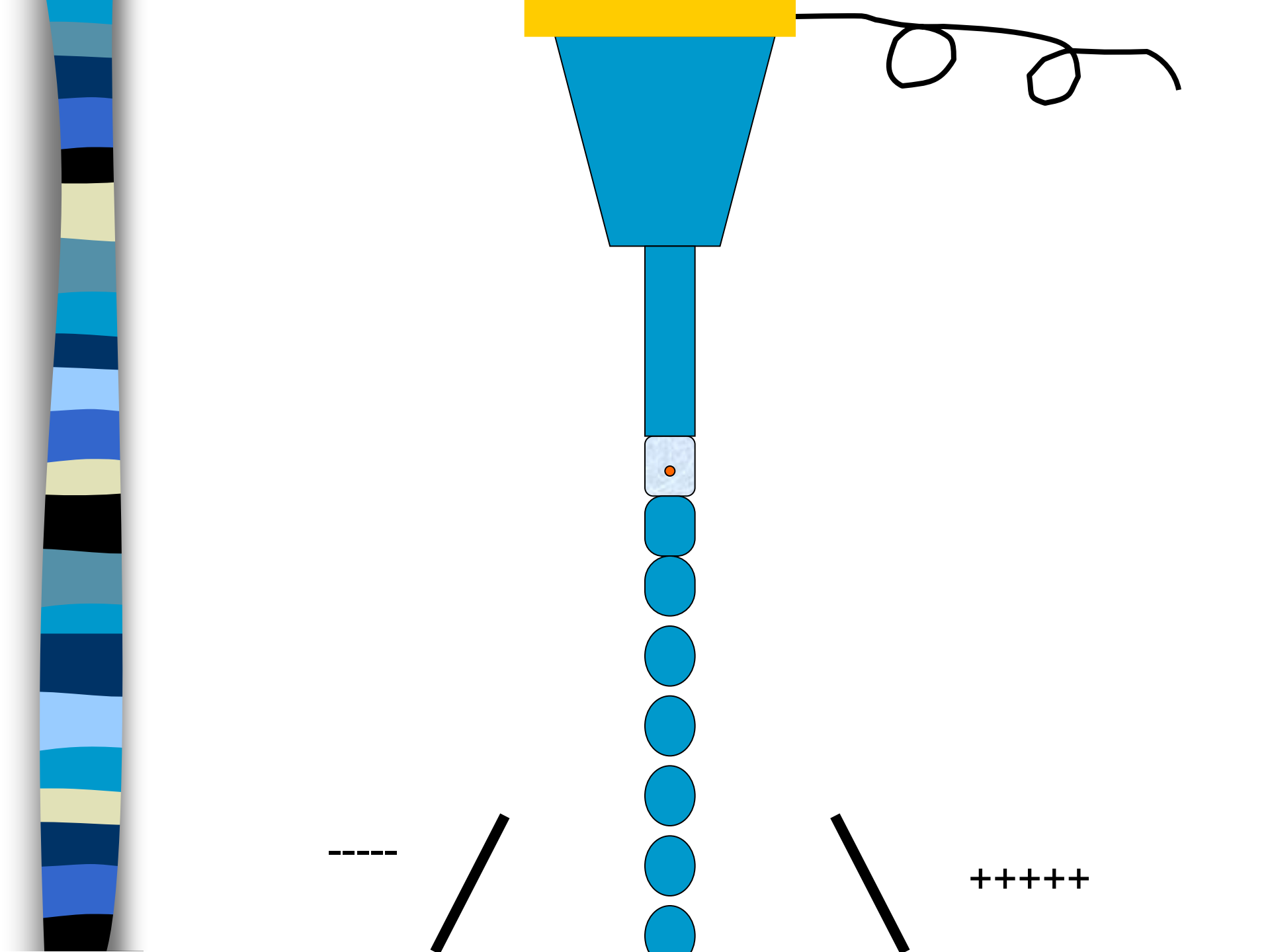


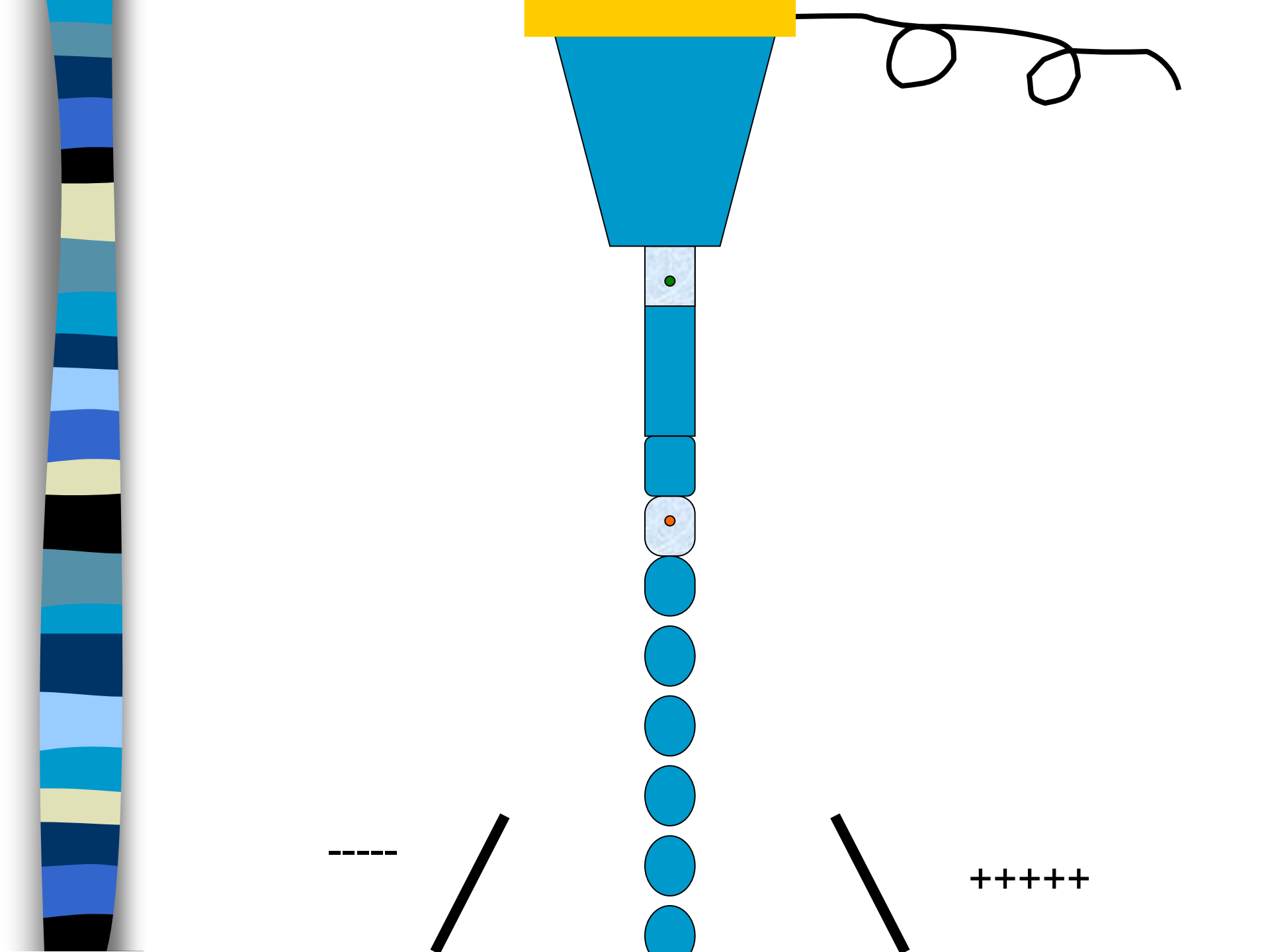


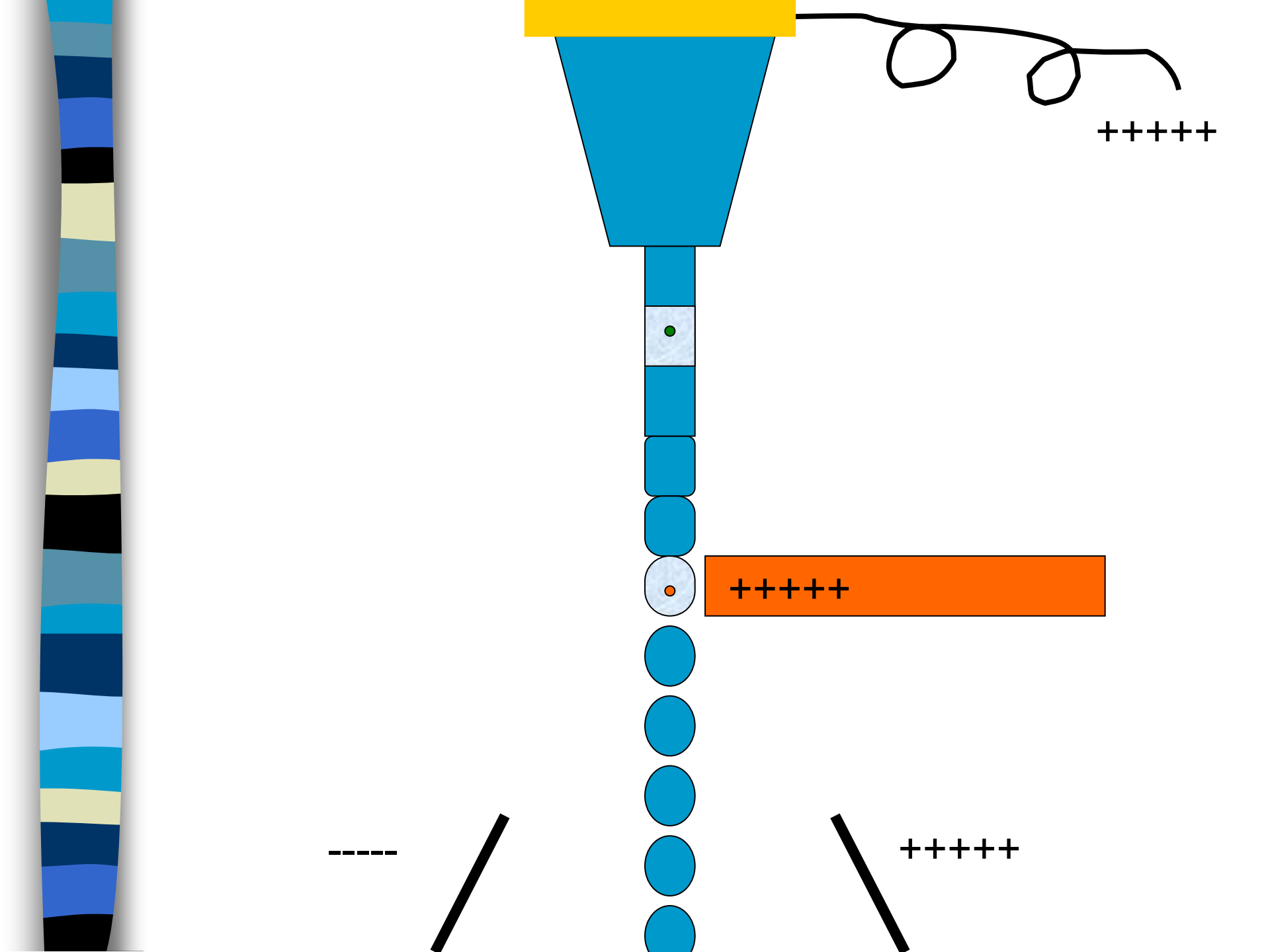


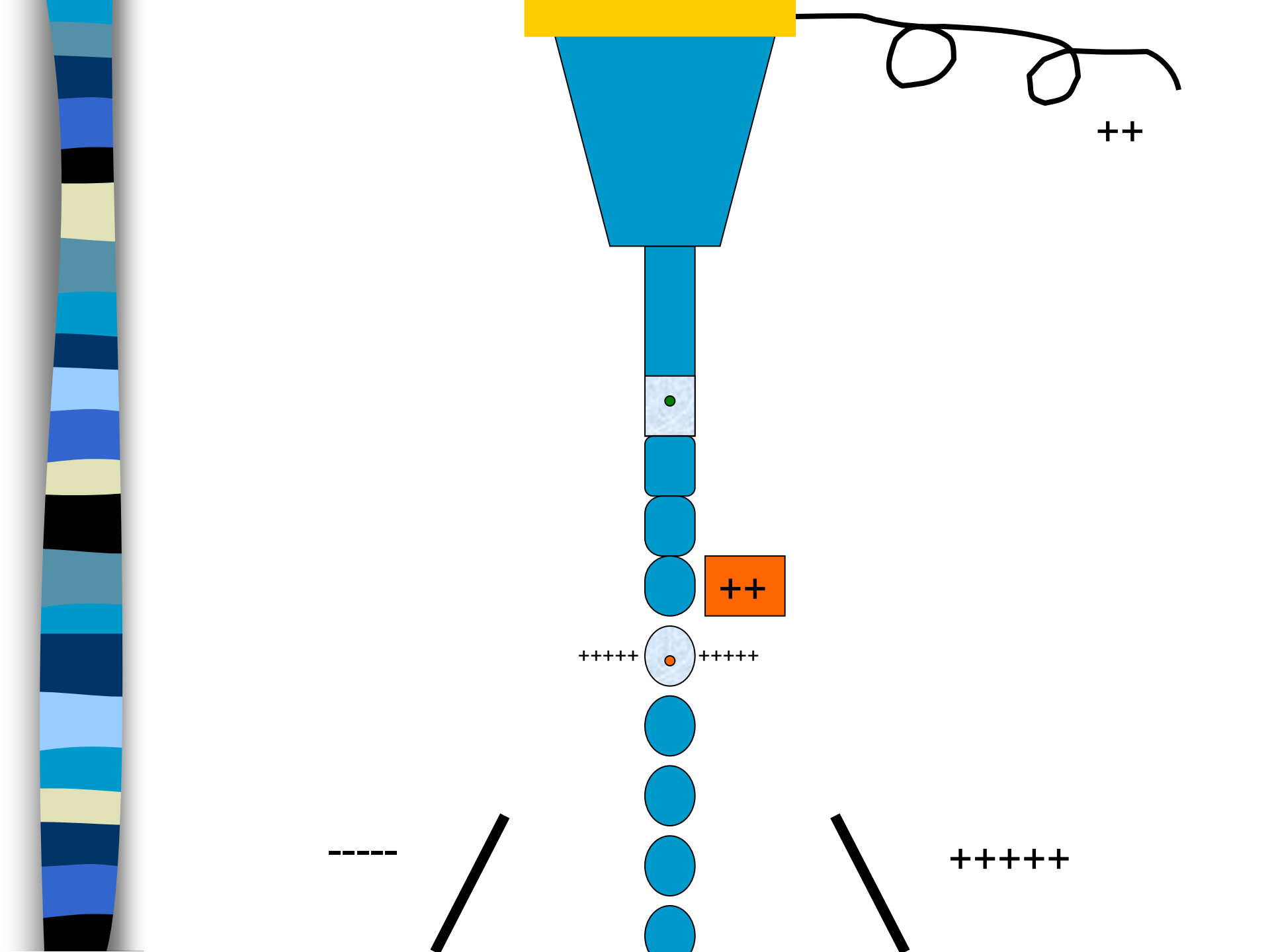
+++++

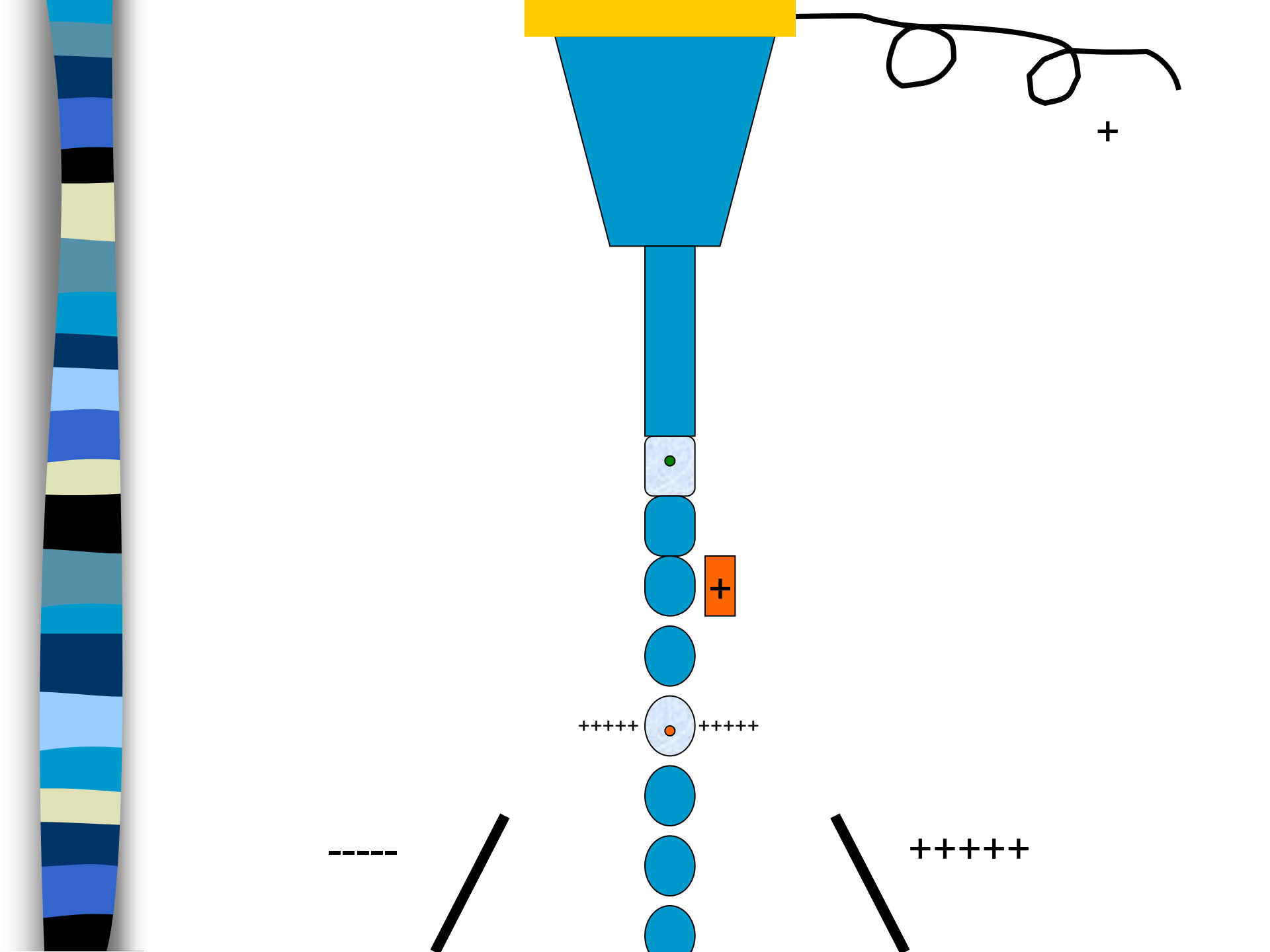


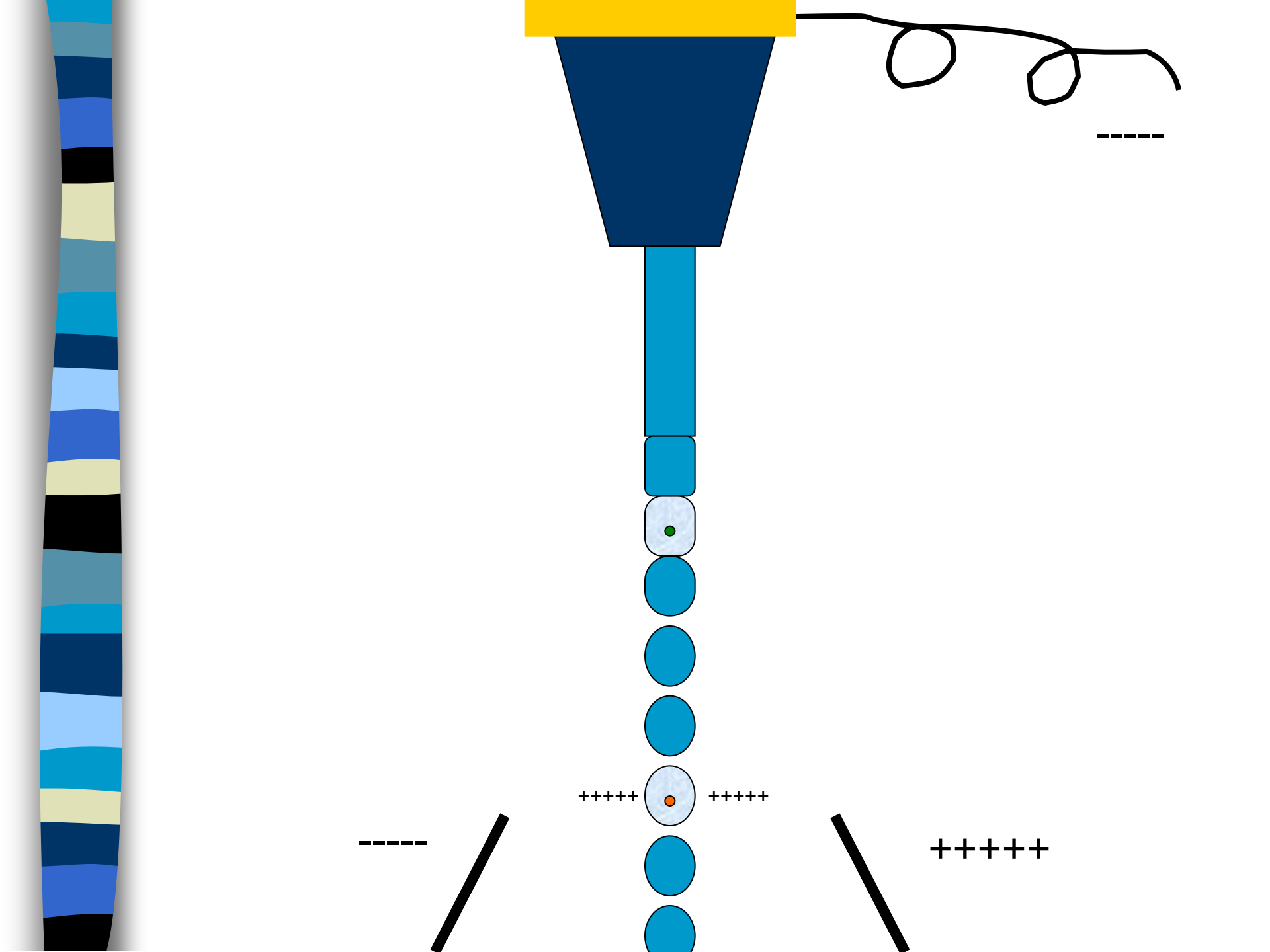


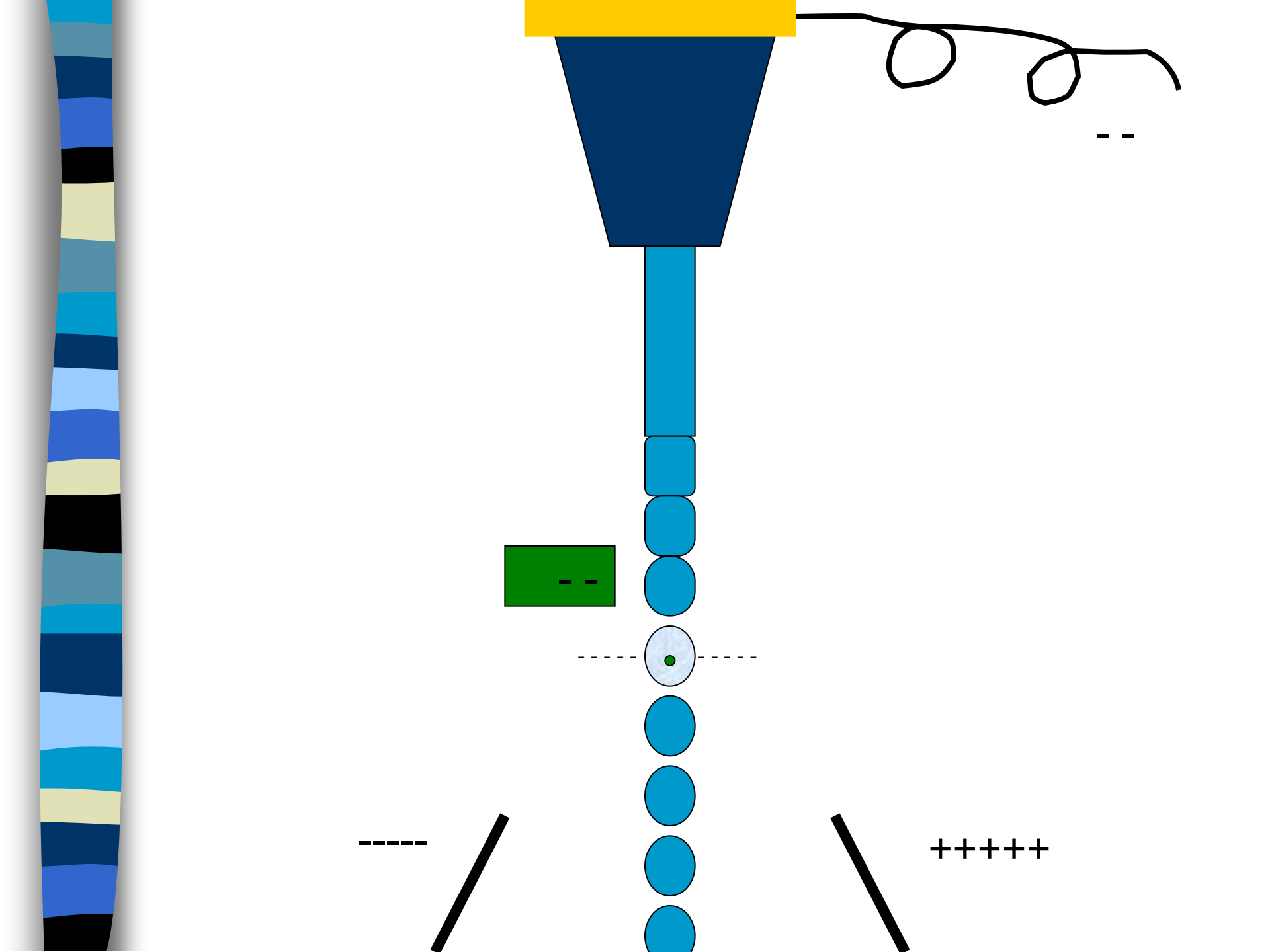


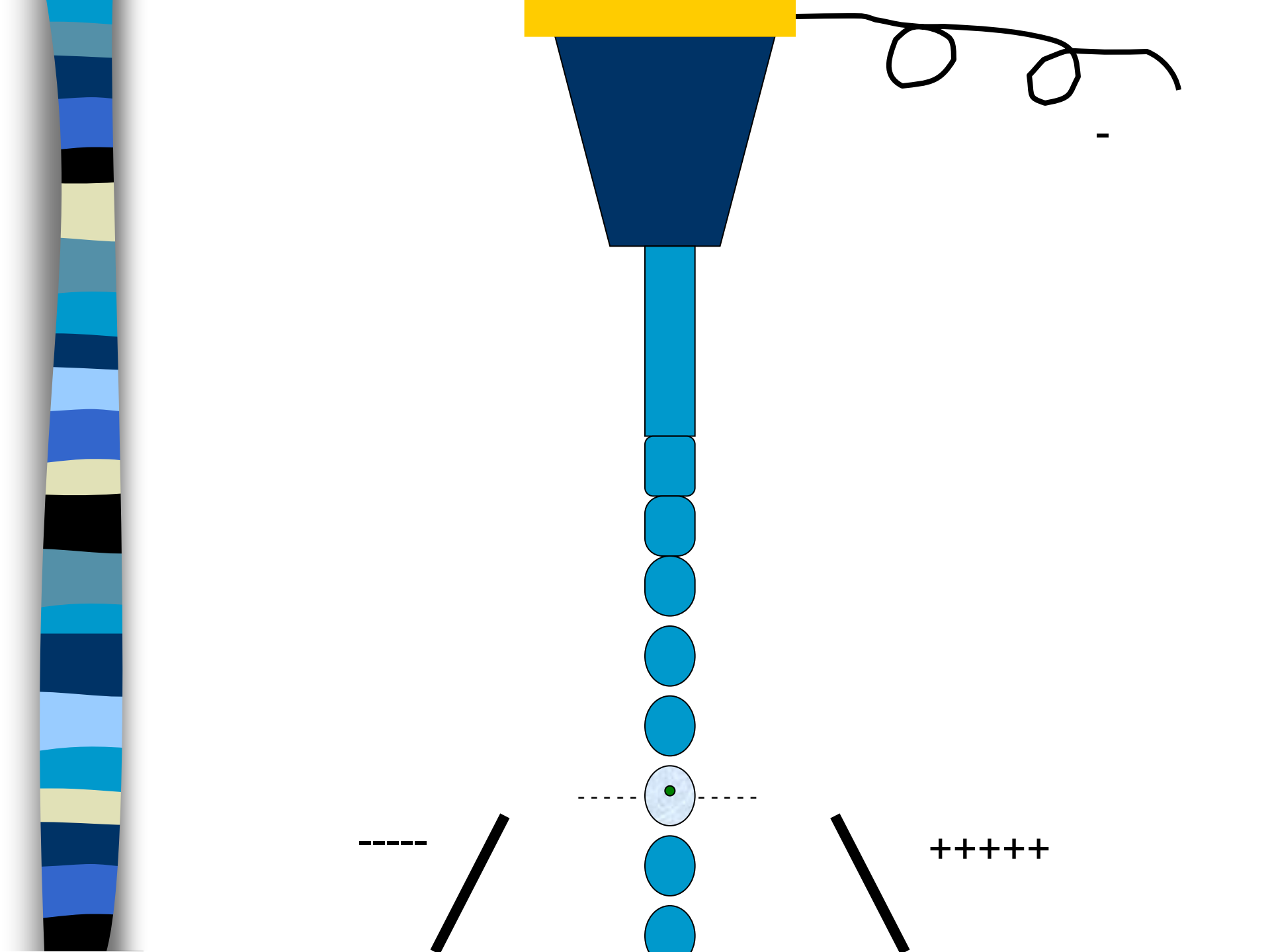


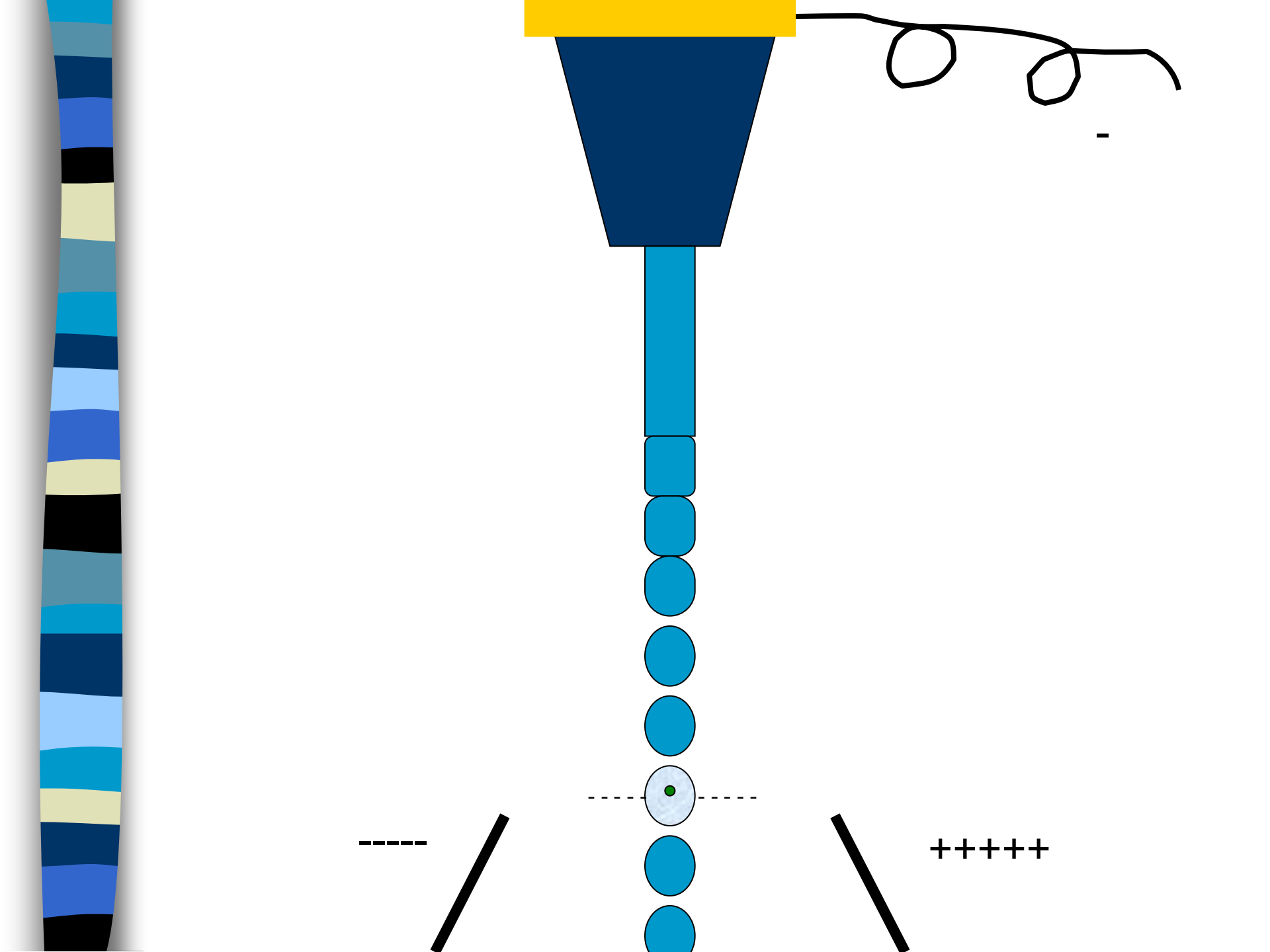


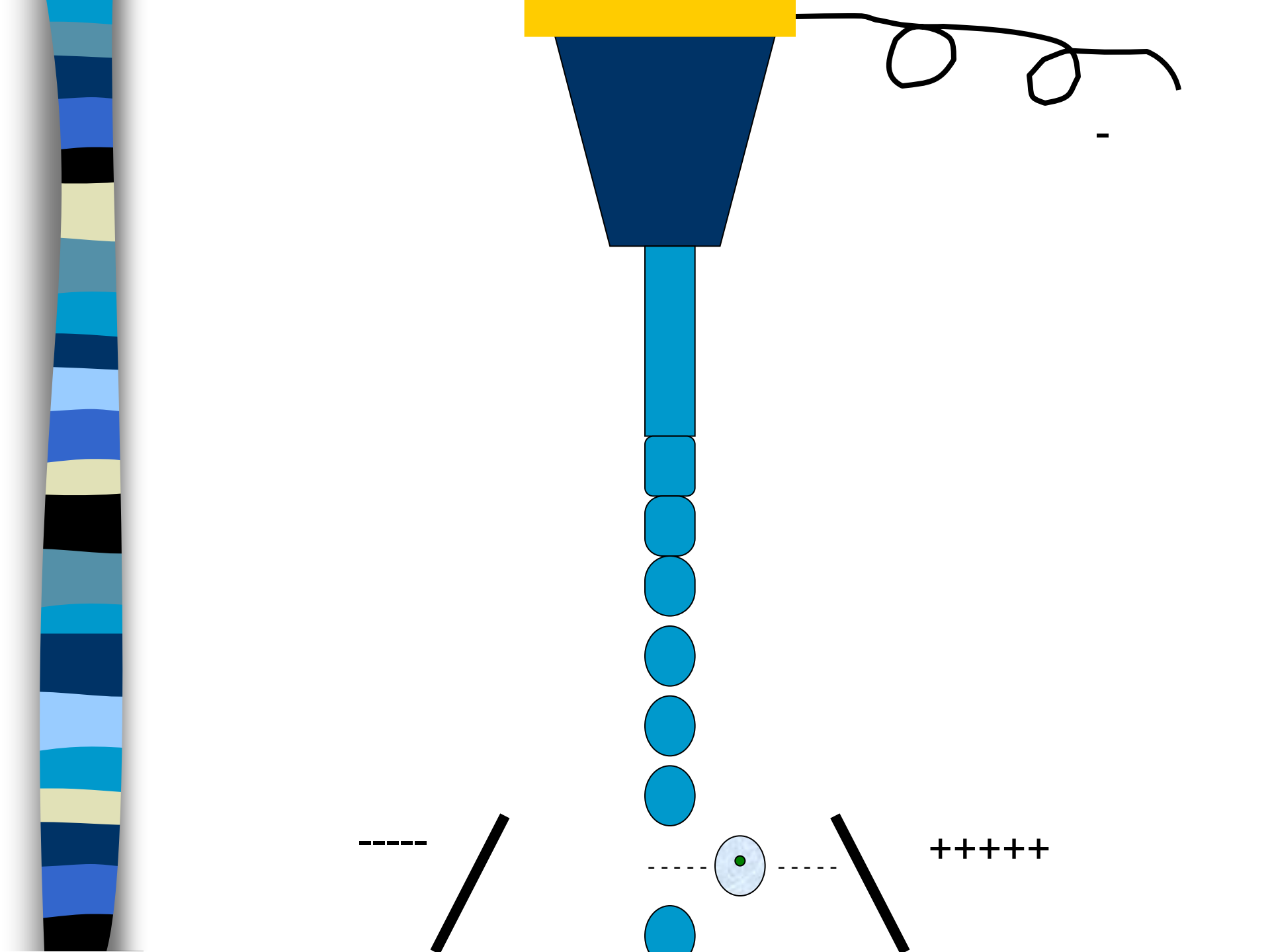










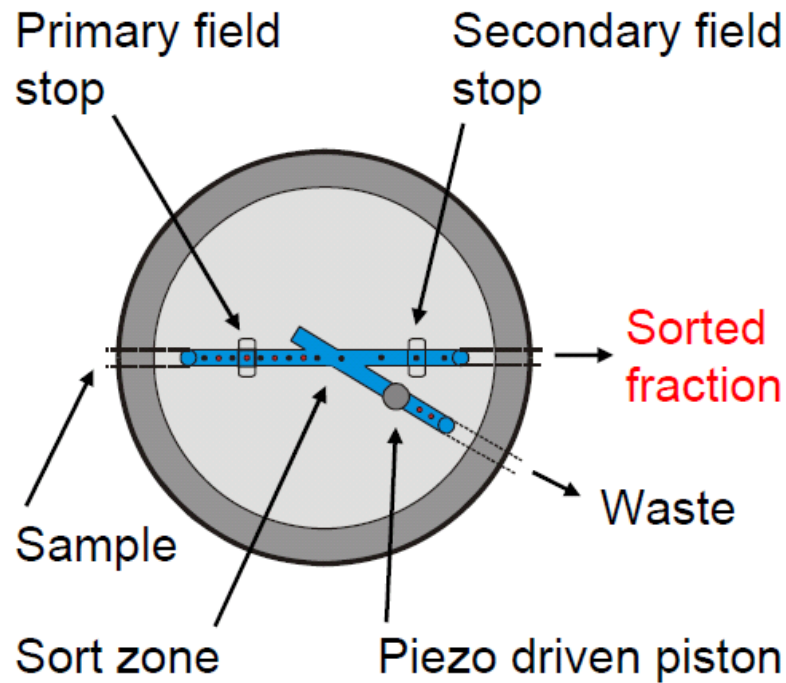


ISAC presents: Mack Fulwyler - Innovator, Inventor & Pioneer

<http://www.cyto.purdue.edu/cdroms/cyto10a/seminalcontributions/fulwyler.html>



FLUIDIC SWITCH SORTER

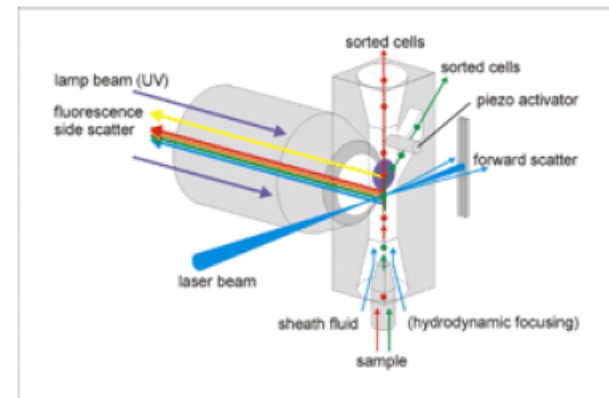
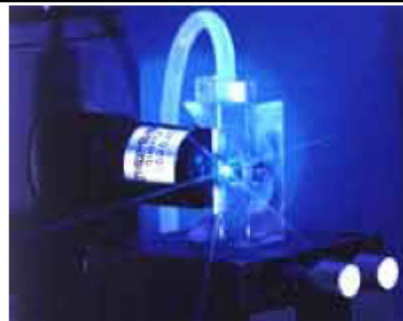


- Safety (enclosed stream)
- Gentle to cells
- Sorting of large particles ($>100 \mu\text{m}$)

Low speed ($\sim 100/\text{sec}$)

- Dilute sorted fraction
- Noisy

Used by: Partec

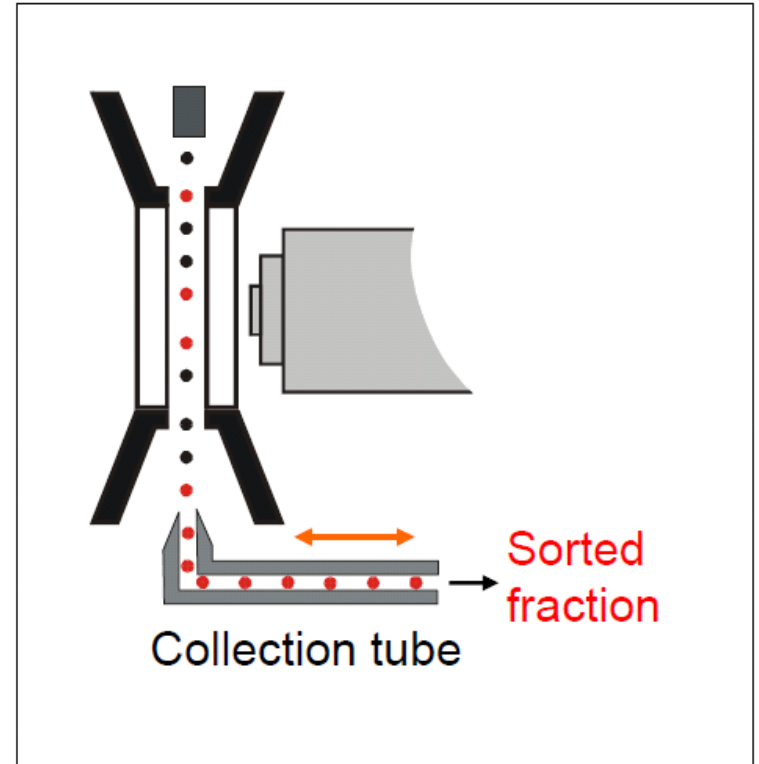


FLUIDIC SWITCH SORTER

- Safety (enclosed stream)
- Gentle to cells

- Low speed (~ 100 / sec)
- Dilute sorted fraction
- Noisy

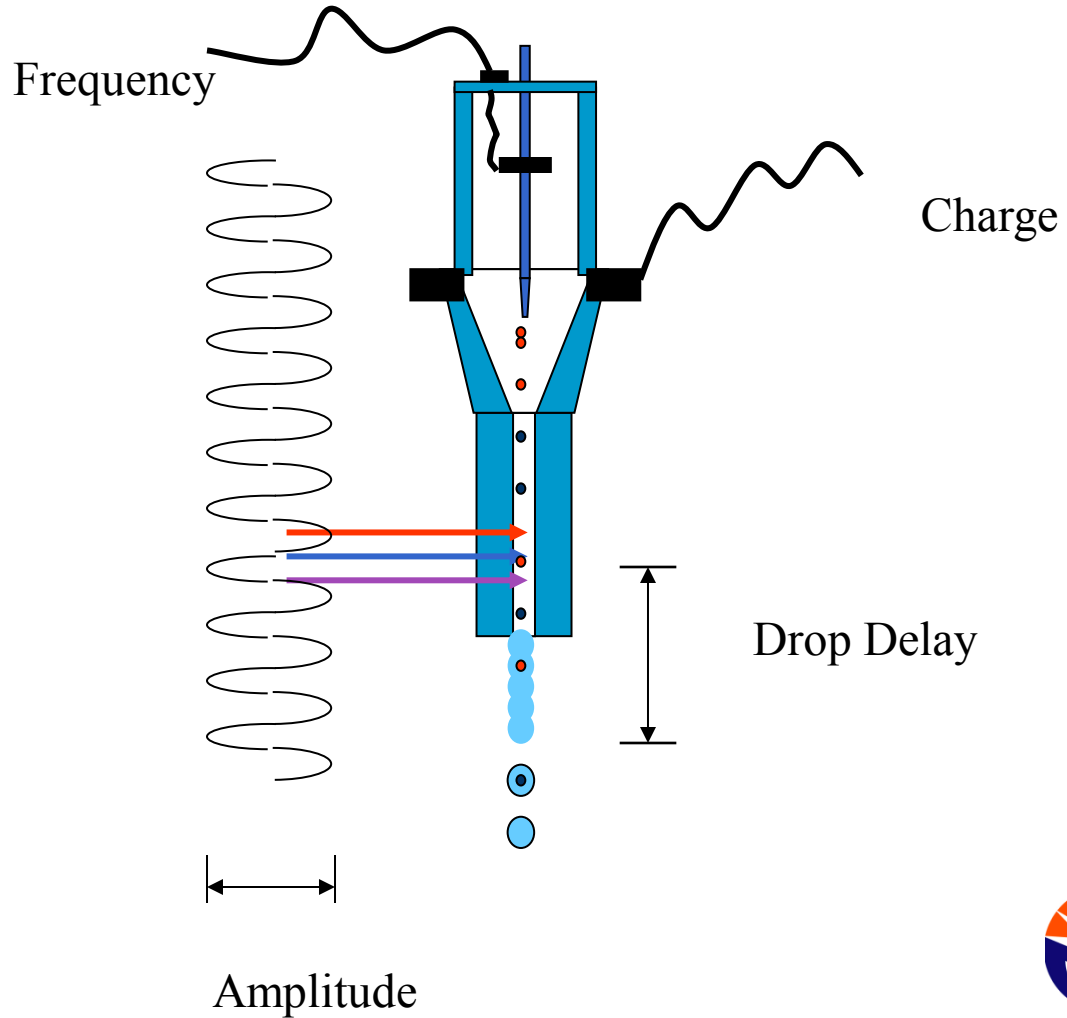
Used by: Becton Dickinson



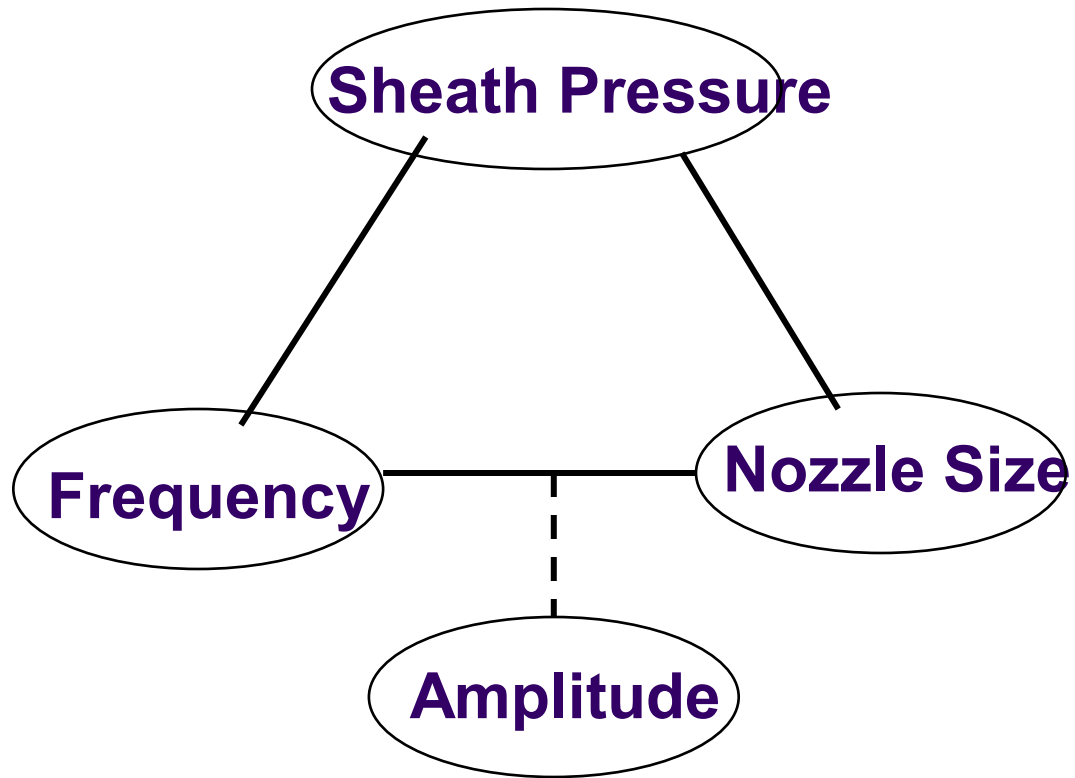
SORTING



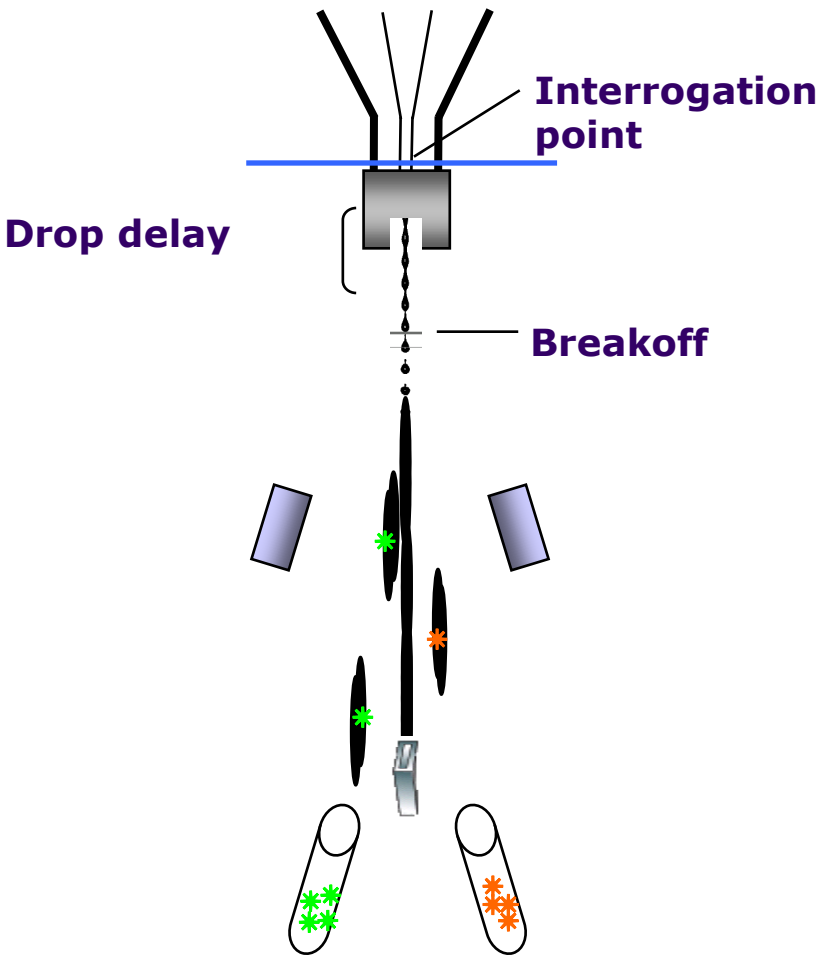
SORTING



SORTING



SORTING



SORTING

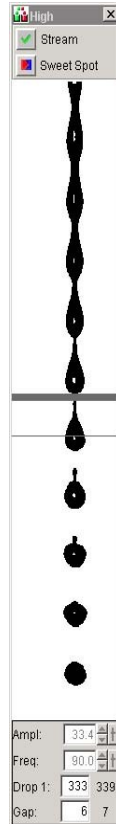
Each sort setup includes:
Sheath pressure
Breakoff window values
Side Stream window values

Table 3-2 Default Sort Setup values

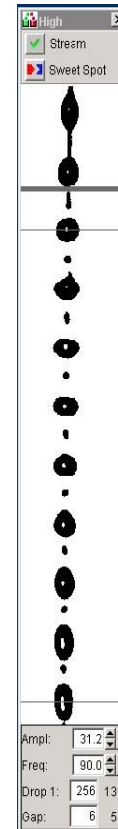
Setting	70 micron	85 micron	100 micron	130 micron
Sheath Pressure	70	45	20	10
Amplitude	60	32	12	24
Frequency	87	47	30	12
Drop 1	150	150	150	150
Gap (upper limit)	6 (14)	7 (17)	10 (21)	12 (21)
Attenuation	Off	Off	Off	Off
Drop Delay	47.00	30.00	27.00	16.00
Far left voltage	100	100	80	60



SORTING - Streams

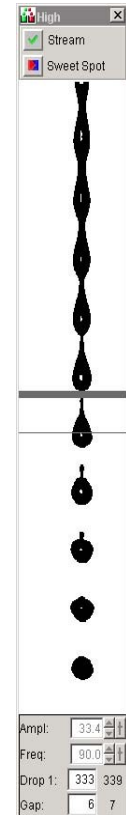
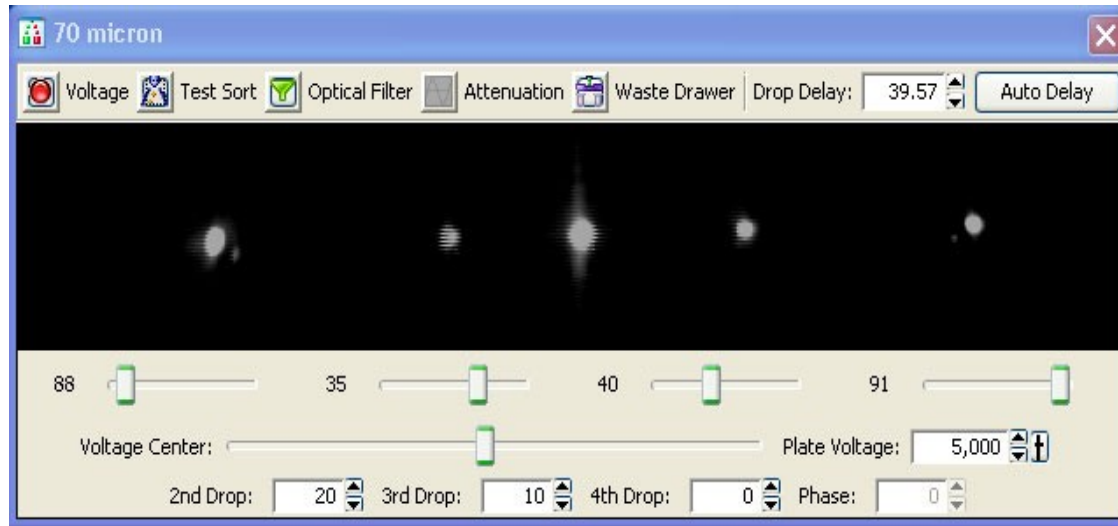


Good



Bad

SORTING – Setup Side Streams

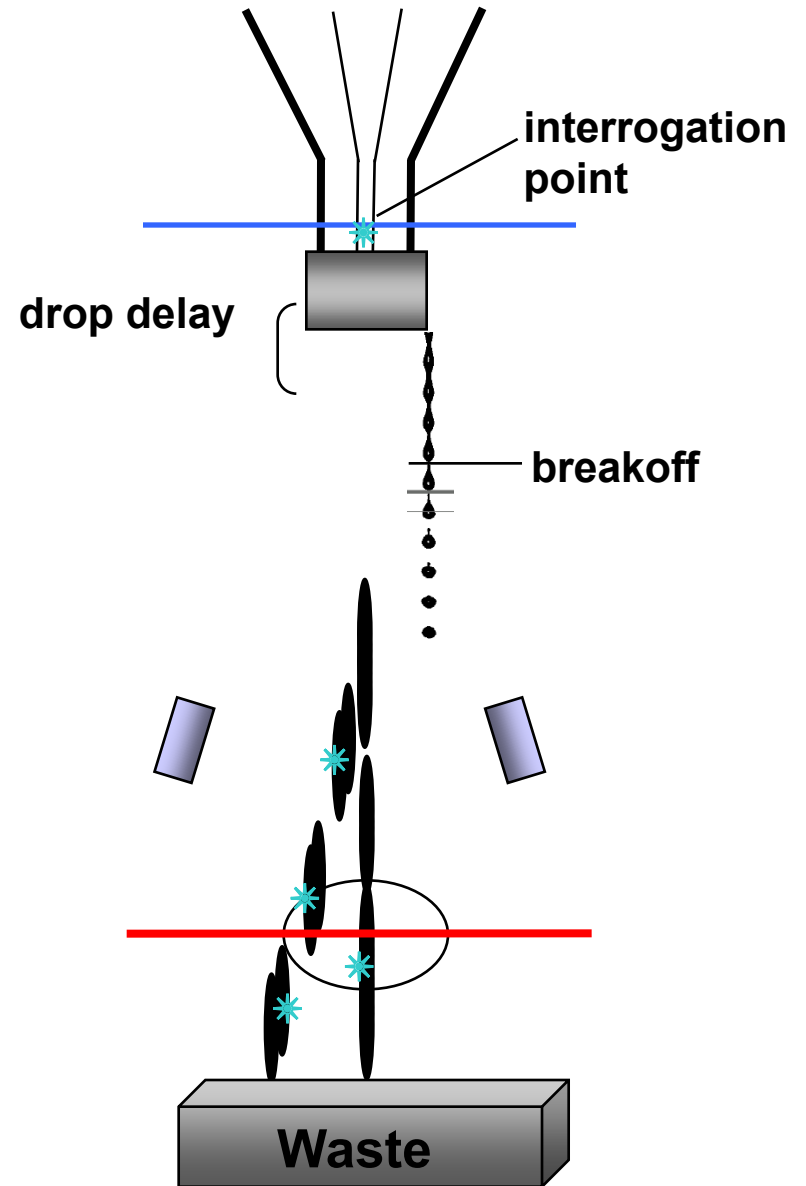


Drop Delay

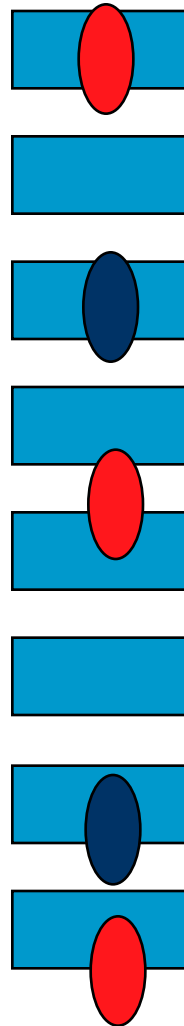
BD FACS™

Accudrop
technology

- Accudrop beads
- Diode laser
- Camera
- Optical filter

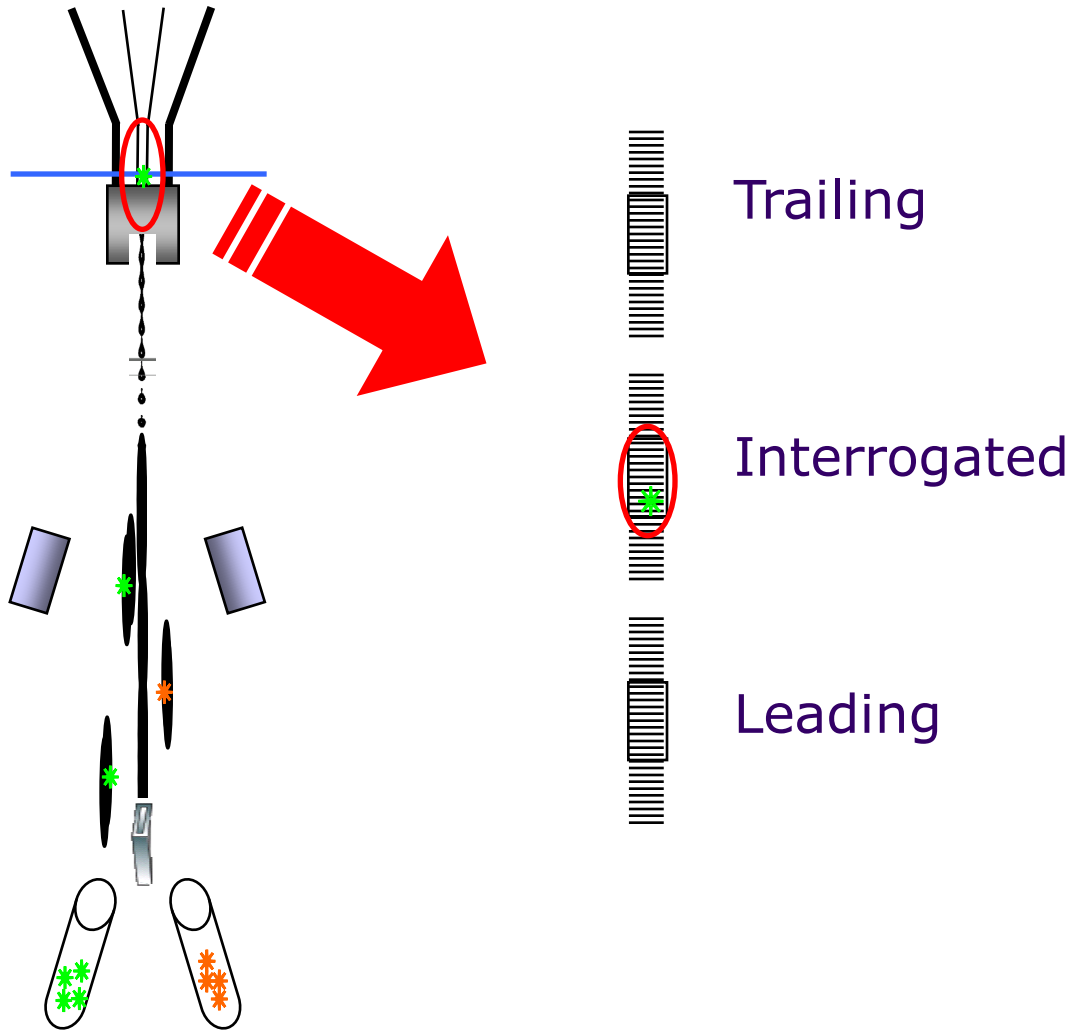


Sorting - Sort Masks



Cells are randomized
distributed over the stream

Sorting - Sort Masks



Mask

- A region of the stream monitored for the presence of cells
- Determines how drops will be deflected if a sorting conflict occurs
- Measured in 1/32 drop increments



Conflict Resolution

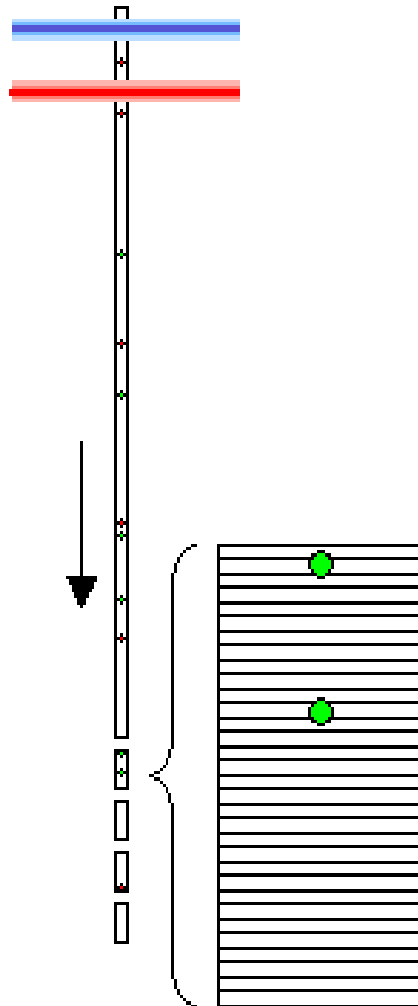
- Precision modes include three types of masks

- Yield
- Purity
- Phase

	Precision Mode				
	Purity	Yield	Single Cell	Initial	Fine Tune
Yield Mask:	32	32	0	32	0
Purity Mask:	32	0	32	0	0
Phase Mask:	0	0	16	0	0
Single Cell:	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Sorting - Sort Masks

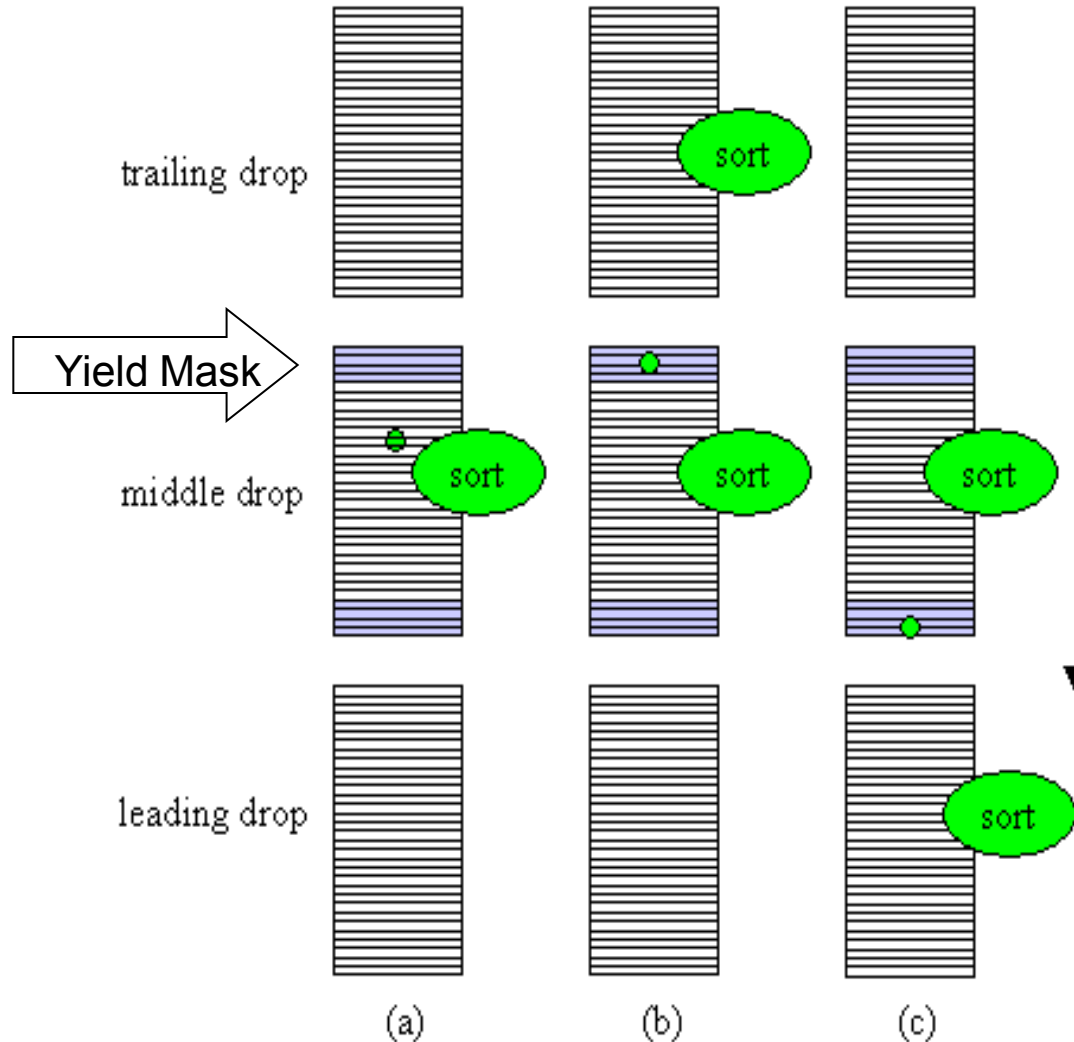
Sort decisions are determined by sort masks



Target particles in a drop with
1/32-drop resolution

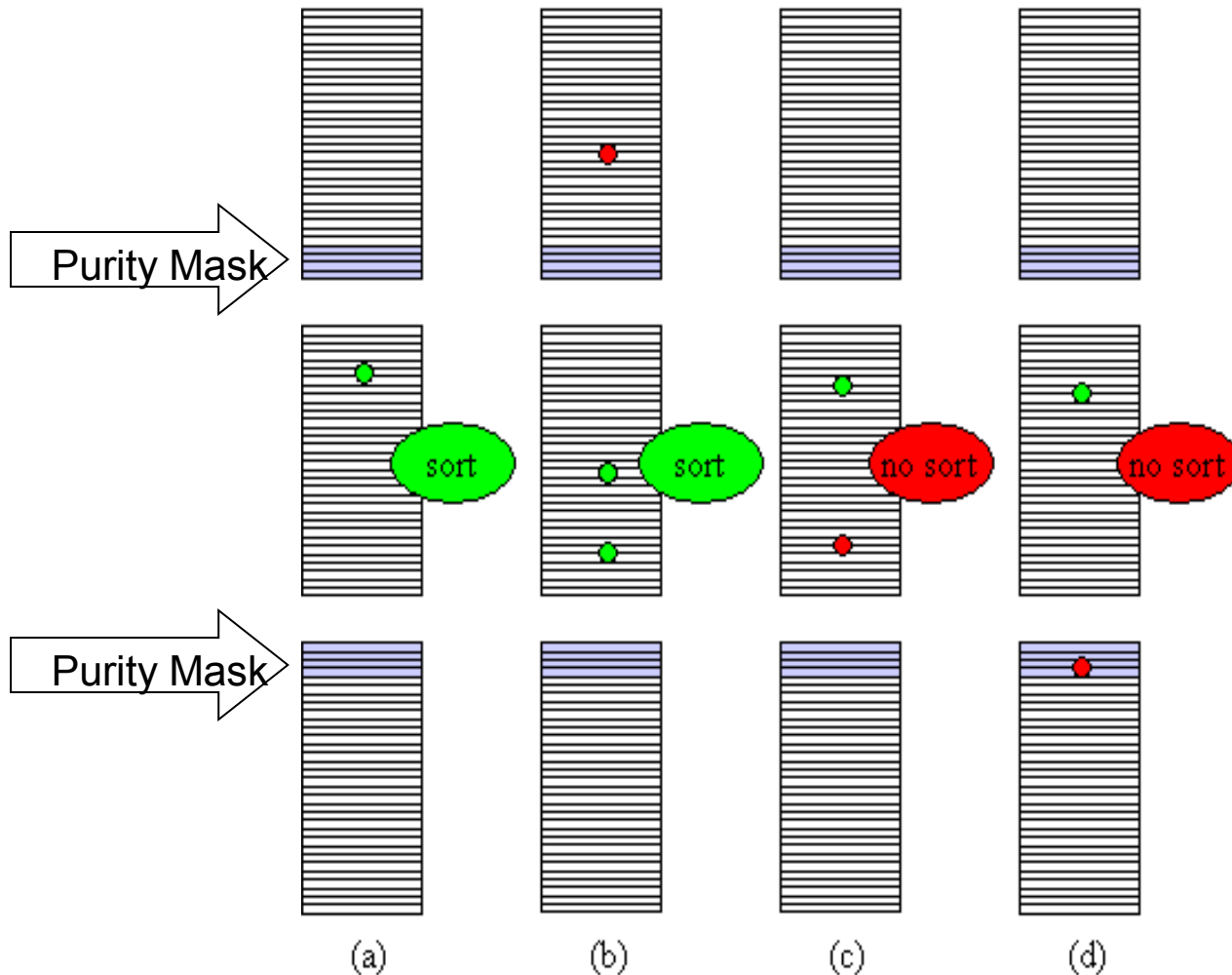
Sorting - Yield Mask

The yield mask defines how many drops will be sorted. Yield mask of 8/32 indicated in blue; target particle shown in green



Sorting - Purity Mask

Purity mask of 8/32 in blue, 4/32 in each adjacent drop;
target particles in green, non-target particles in red





Elektronika

- Zpracování signálu z detektorů
 - Předzesílení
 - zesiluje signál pro přenos z detektorů do centralní elektronické části
 - Zesílení
 - úprava intensity signálu
 - lineární nebo logaritmické
 - Generování integrálu a šířky pulsu
 - Analog-digital konverze



Sběr dat

- Data jsou sbírána jako “list” hodnot, pro každý “parametr” a pro každou “event” (buňku)
- každé měření z každého detektoru je označeno jako “parameter”

Flow Cytometry Standard data file format. FCS 3.1

http://www.isac-net.org/images/stories/documents/Standards/fcs3.1_normativespecification_20090813.pdf

Spidlen, J. *et al. Cytometry. Part A : the journal of the International Society for Analytical Cytology* 77, 97-100, (2010).

Date: 17-JUL-2015
 System: Windows XP 5.1
 Cytometer: FACS Aria II SORP (FACS Aria II)
 File: 150717_DU145 Ctrl.fcs
 File URI: file://C:/Users/user/Desktop/install/Infinicyt/150717_DU145%20Ctrl.fcs

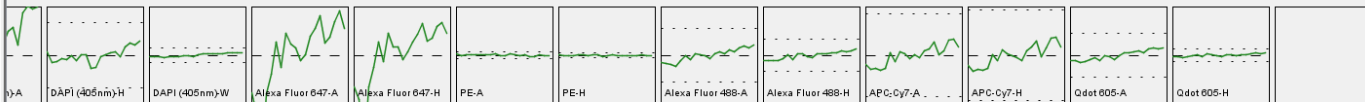
\$BEGIN ANALYSIS: 0
 \$BEGIN DATA: 4148
 \$BEGIN TEXT: 0
 \$BTIM: 13:25:01
 \$BYTE ORD: 4,3,2,1
 \$CYT: FACS Aria II SORP (FACS Aria II)
 \$DATATYPE: F
 \$DATE: 17-JUL-2015
 \$END ANALYSIS: 0
 \$END DATA: 6055267
 \$END TEXT: 0
 \$ETIM: 13:28:55
 \$FIL: 150717_DU145 Ctrl.fcs
 \$INST: IBP
 \$MODE: L
 \$NEXT DATA: 0
 \$OP: fedr
 \$PAR: 19
 \$SRC: 150717
 \$SYS: Windows XP 5.1
 \$TIMESTEP: 0.01
 \$TOT: 79620
 APPLY COMPENSATION: TRUE
 AUTOBS: TRUE
 CREATOR: BD FACSDiva Software Version 6.1.3
 CST BASELINE DATE: 03_24_2015 12:52:48 PM
 CST BEADS LOT ID: 91725
 CST SETUP DATE: 03_25_2015 03:01:55 PM
 CST SETUP STATUS: SUCCESS WITH WARNING
 CYTNUM: P5Y500001
 CYTOMETER CONFIG CREATE DATE: 05_13_2013 01:32:45 PM
 CYTOMETER CONFIG NAME: RF_85u 45 psi_SORP Aria_5-laser (Zuv-6v-3b-5yg-3r)
 EXPERIMENT NAME: DU145_POPRO1_LDYellow_AF488_AF594_PE_APCcy7
 EXPORT TIME: 17-JUL-2015-14:30:11
 EXPORT USER NAME: fedr
 FJ_FCS_VERSION: 3
 FSC ASF: 0.57
 GUID: dc7612a3-65af-4520-bc0f-51d53273ebea
 LASER1ASF: 0.86
 LASER1DELAY: 0.00
 LASER1NAME: Blue
 LASER2ASF: 0.86
 LASER2DELAY: -38.47
 LASER2NAME: Red
 LASER3ASF: 1.02
 LASER3DELAY: 77.49
 LASER3NAME: UV
 LASER4ASF: 0.63
 LASER4DELAY: 45.00
 LASER4NAME: Violet
 LASER5ASF: 0.83
 LASER5DELAY: -76.49
 LASER5NAME: YG
 P10BS: 602
 P10DISPLAY: LOG
 P10MS: 0
 P11BS: 38
 P11DISPLAY: LOG
 P11MS: 0
 P12BS: 5
 P12DISPLAY: LOG
 P12MS: 0
 P13BS: 1118
 P13DISPLAY: LOG

Compensation Matrix

	Alexa Fluor 594-A	DAPI (405nm)-A	Alexa Fluor 647-A	PE-A	Alexa Fluor 488-A	APC-Cy7-A	Qdot 605-A
Alexa Fluor 594-A	100	0.42	1.53	1.94	0.02	0.32	9.95
DAPI (405nm)-A	1.1	100	0.27	0.05	0.01	0.08	0.98
Alexa Fluor 647-A	2.45	22.87	100	0.1	0.08	15.14	0.85
PE-A	440.67	0	0.14	100	8.03	0.03	32.23
Alexa Fluor 488-A	-0.01	0.09	0.01	0	100	0	0.05
APC-Cy7-A	0.01	0.04	2.67	0	0.05	100	0.01
Qdot 605-A	0	41.05	0	0	2.34	0	100

Parameters and Stains

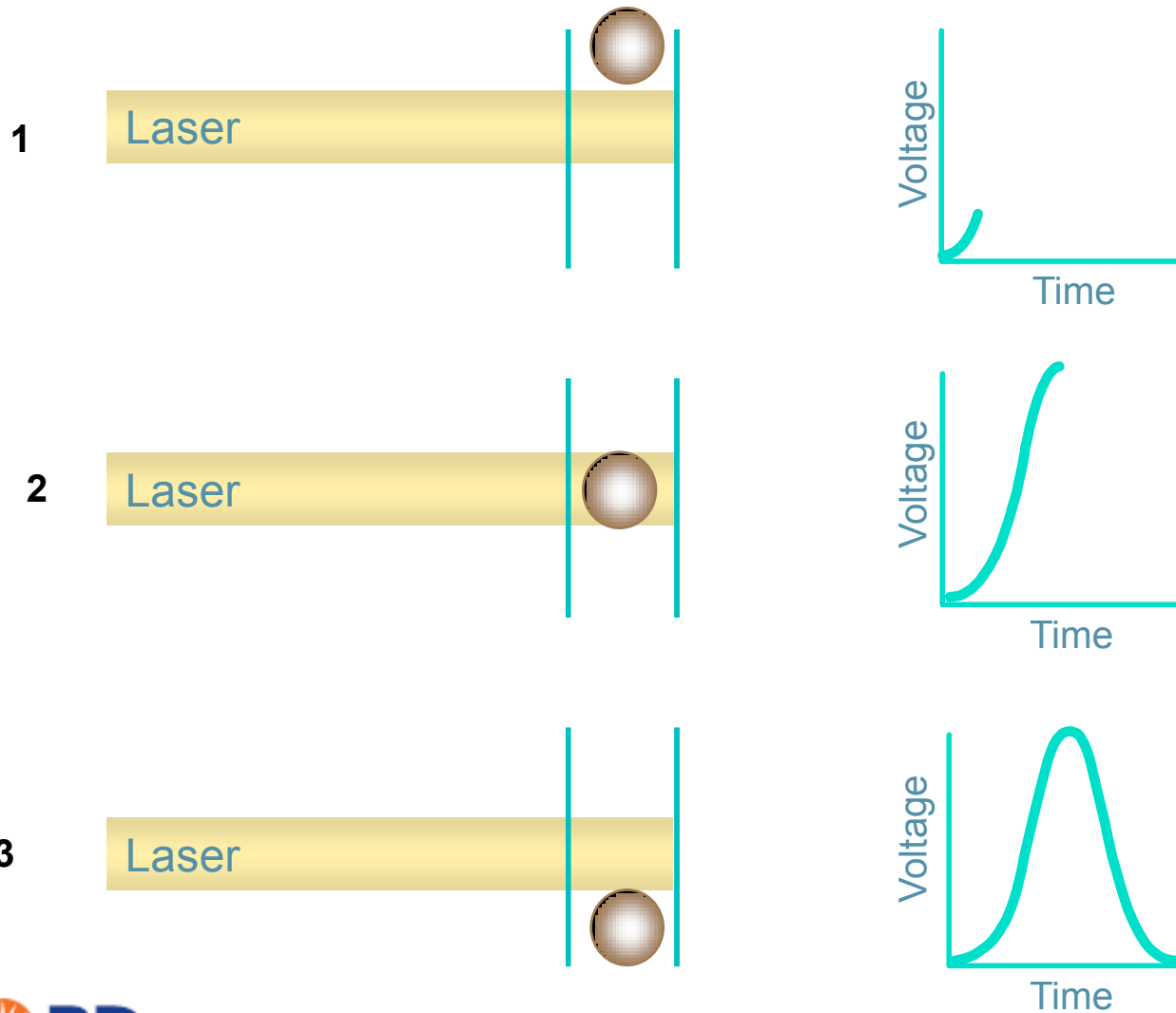
Parameter (\$PnI)	Stain (\$PnS)	Range (\$PnR)	Bits (\$PnB)	Decades (\$PnE)	Gain (\$PnG)	Voltage (\$PnV)	Derived From
FSC-A		262144	32	0.0	1.0	280	
FSC-H		262144	32	0.0	1.0	280	
SSC-A		262144	32	0.0	1.0	210	
Alexa Fluor 594-A		262144	32	0.0	1.0	460	
Alexa Fluor 594-H		262144	32	0.0	1.0	460	
DAPI (405nm)-A		262144	32	0.0	1.0	650	
DAPI (405nm)-H		262144	32	0.0	1.0	650	
DAPI (405nm)-W		262144	32	0.0	1.0	650	
Alexa Fluor 647-A		262144	32	0.0	1.0	538	
Alexa Fluor 647-H		262144	32	0.0	1.0	538	
PE-A		262144	32	0.0	1.0	330	
PE-H		262144	32	0.0	1.0	330	
Alexa Fluor 488-A		262144	32	0.0	1.0	366	
Alexa Fluor 488-H		262144	32	0.0	1.0	366	
APC-Cy7-A		262144	32	0.0	1.0	700	
APC-Cy7-H		262144	32	0.0	1.0	700	
Qdot 605-A		262144	32	0.0	1.0	410	
Qdot 605-H		262144	32	0.0	1.0	410	
Time		262144	32	0.0	0.01		
Comp-Alexa Fluor 594-A		262144					
Comp-DAPI (405nm)-A		262144					
Comp-Alexa Fluor 647-A		262144					
Comp-PE-A		262144					
Comp-Alexa Fluor 488-A		262144					
Comp-APC-Cy7-A		262144					
Comp-Qdot 605-A		262144					



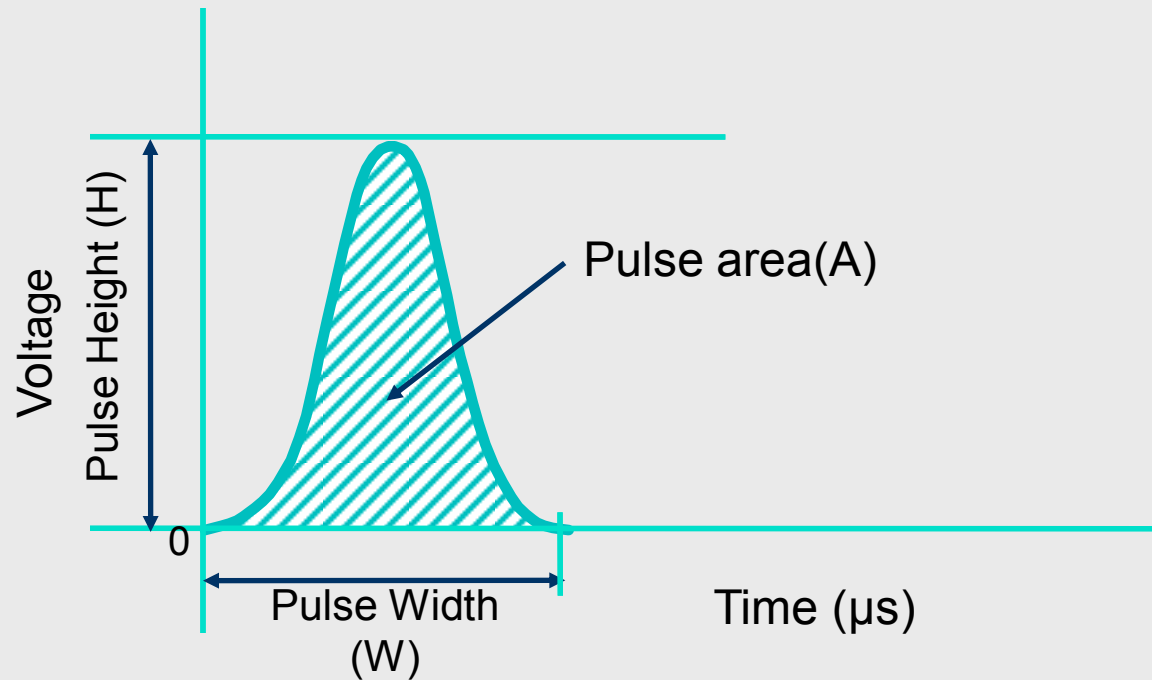
Data Acquisition - Listmode

<i>Event</i>	<i>Param1</i> <i>FS</i>	<i>Param2</i> <i>SS</i>	<i>Param3</i> <i>FITC</i>	<i>Param4</i> <i>PE</i>
1	50	100	80	90
2	55	110	150	95
3	110	60	80	30

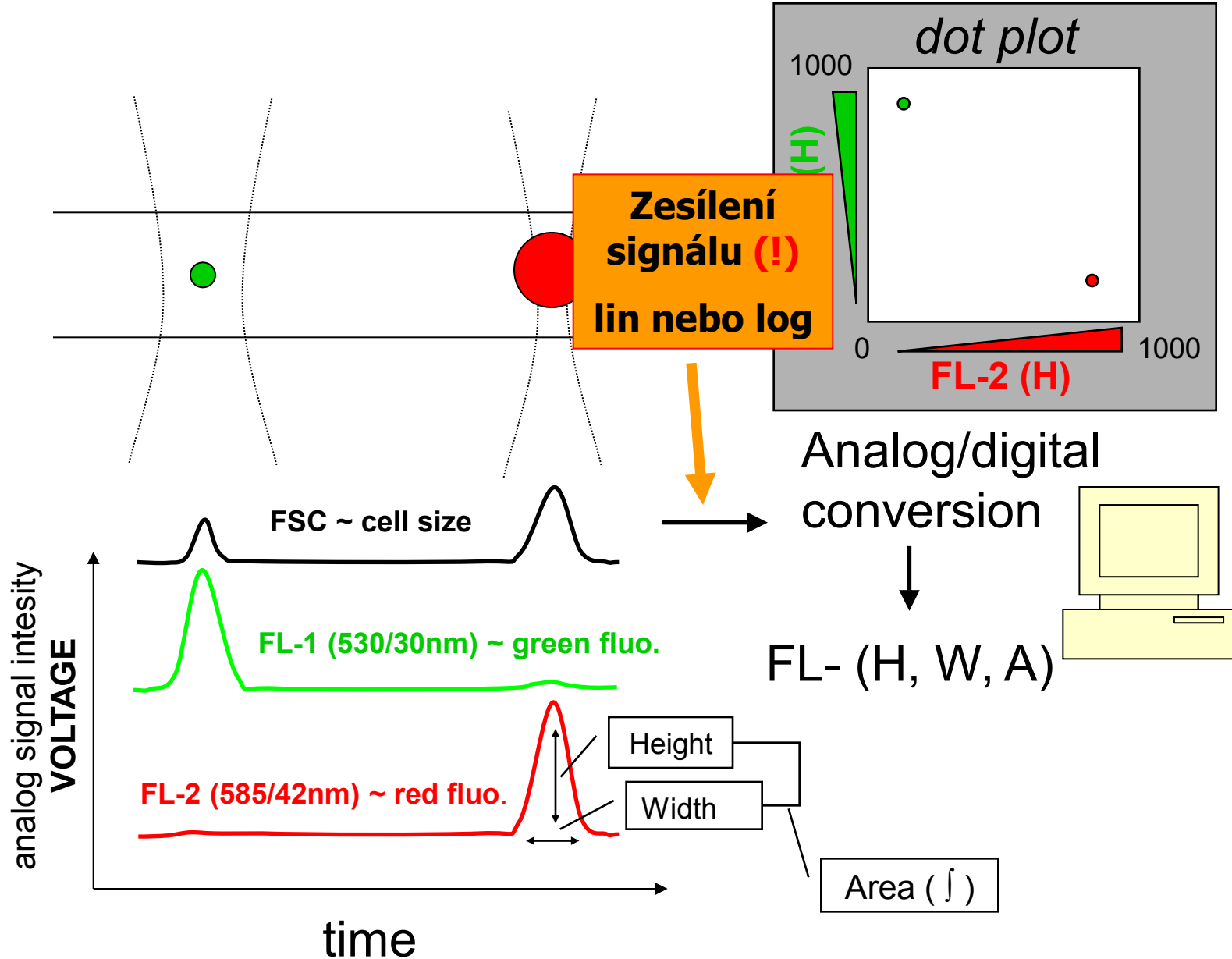
Creation of a Voltage Pulse



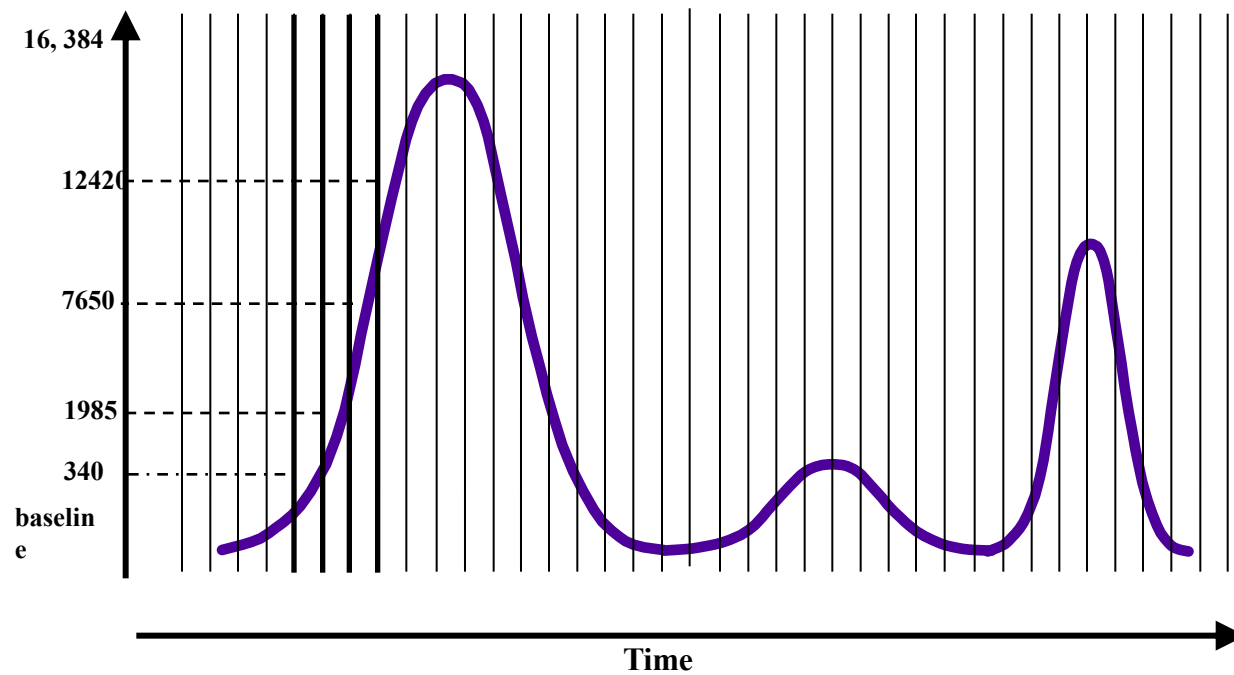
Height, Area, and Width



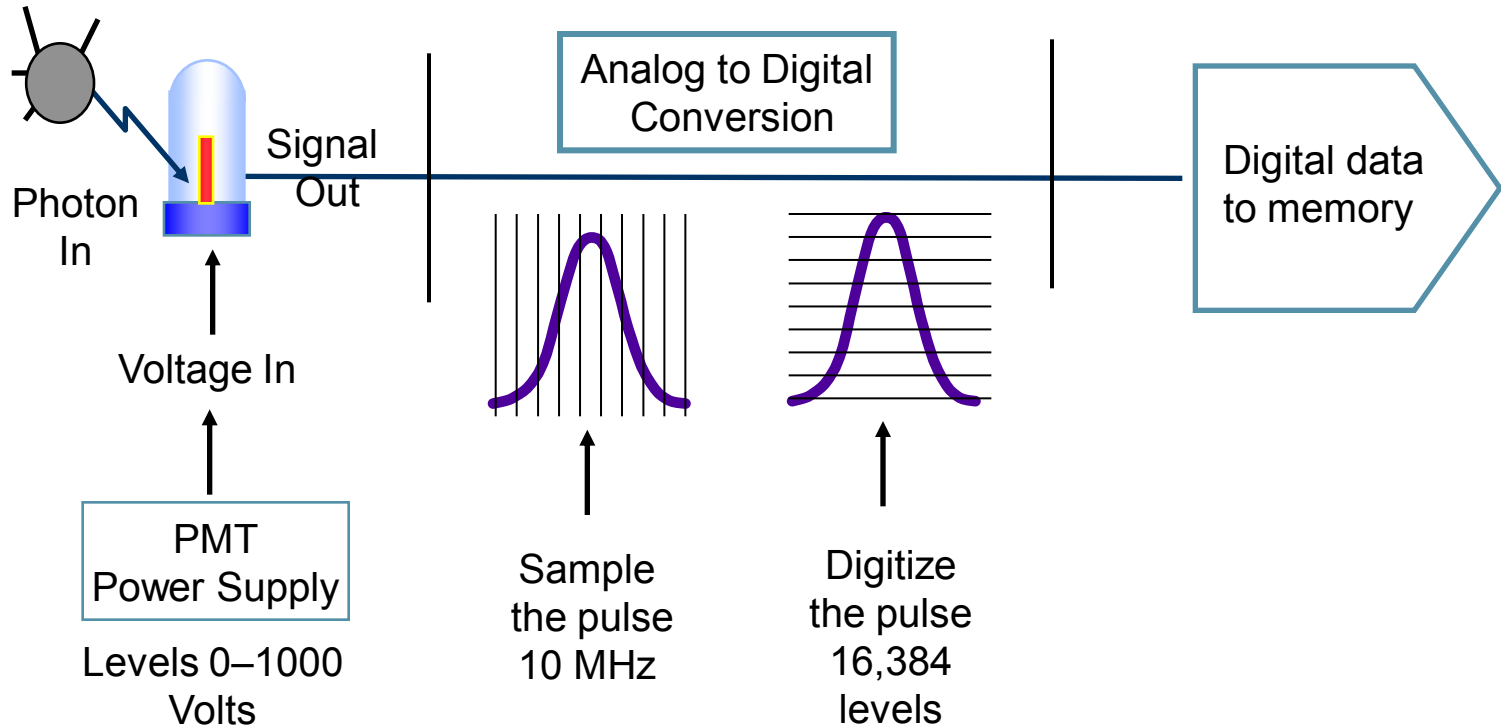
Signal processing



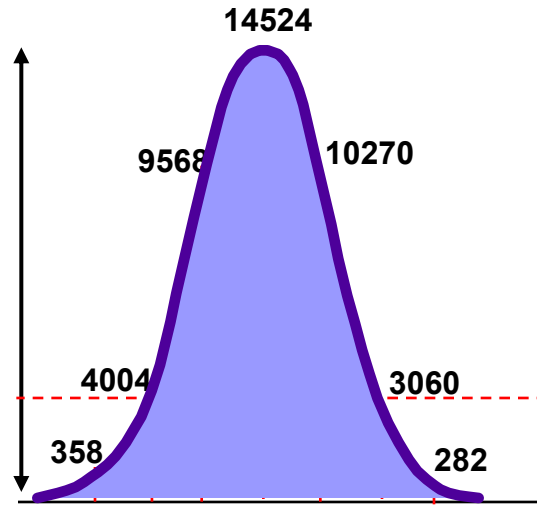
Analog to Digital Converter



Analog to Digital Converter



Parameters



- Area: Sum of all height values
- Height: Maximum digitized value
X 16
- Width: Area/Height X 64K

Data is displayed on 262,144 scale

$$2^8 = 256$$

$$2^{10} = 1024$$

AD převodníky

Počet bitů	# kanálů	rozlišení
8	256	39.1 mV
10	1024	9.77 mV
12	4096	2.44 mV
14	16384	610 μ V
16	65536	153 μ V
18	262144	38.1 μV
20	1048576	9.54 μ V
22	4194304	2.38 μ V
24	16777216	596 nV

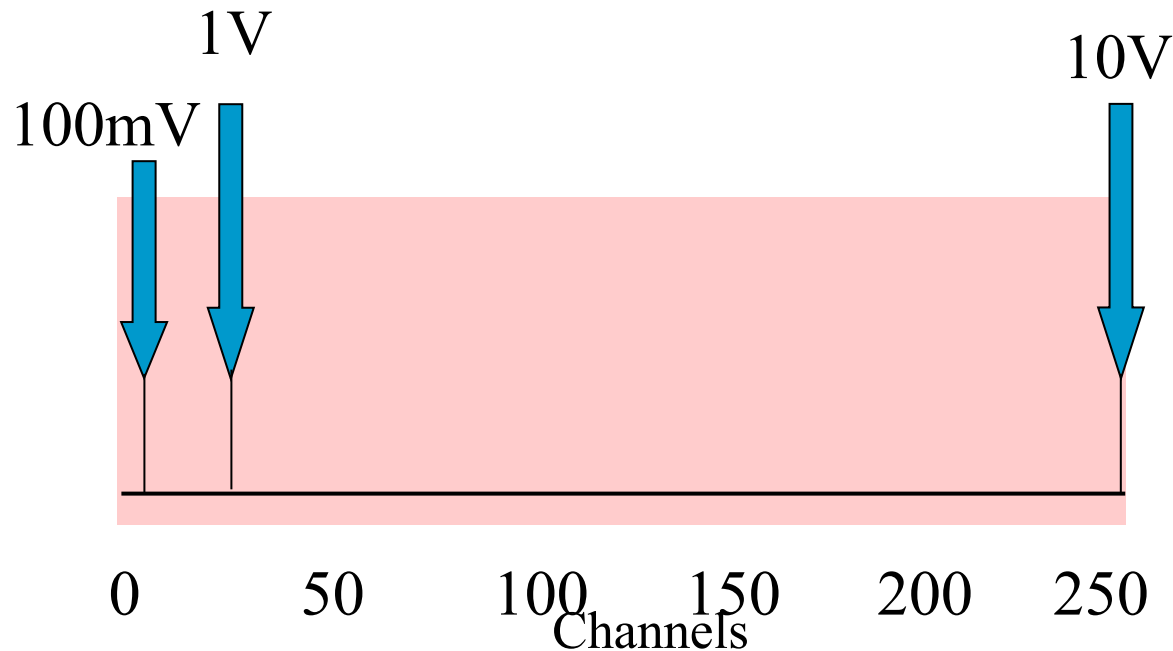
Full scale measurement range = 0 to 10 volts

ADC resolution is 12 bits: $2^{12} = 4096$ quantization levels

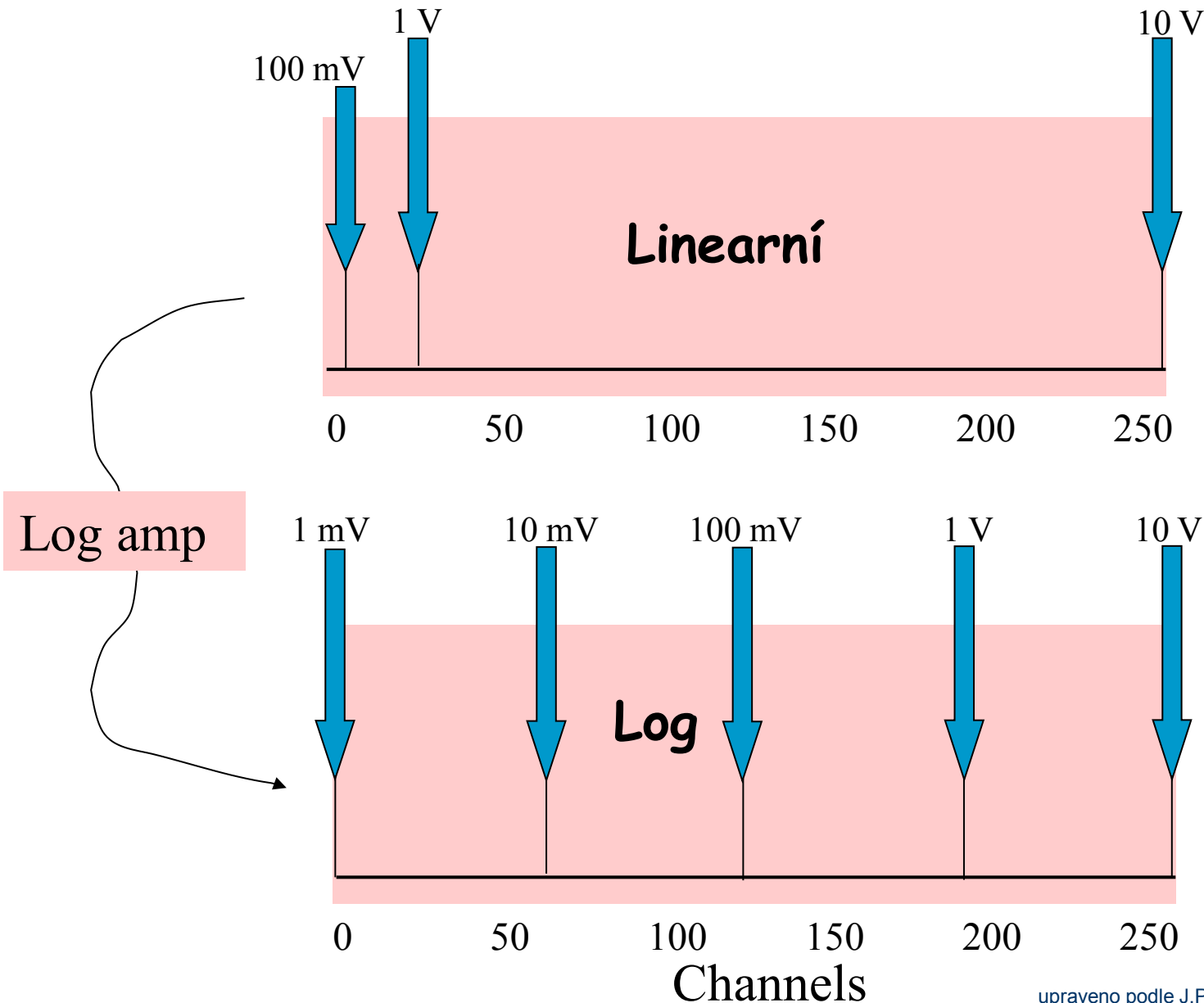
ADC voltage resolution is: $(10-0)/4096 = 0.00244$ volts = 2.44 mV

Kolik bitů?

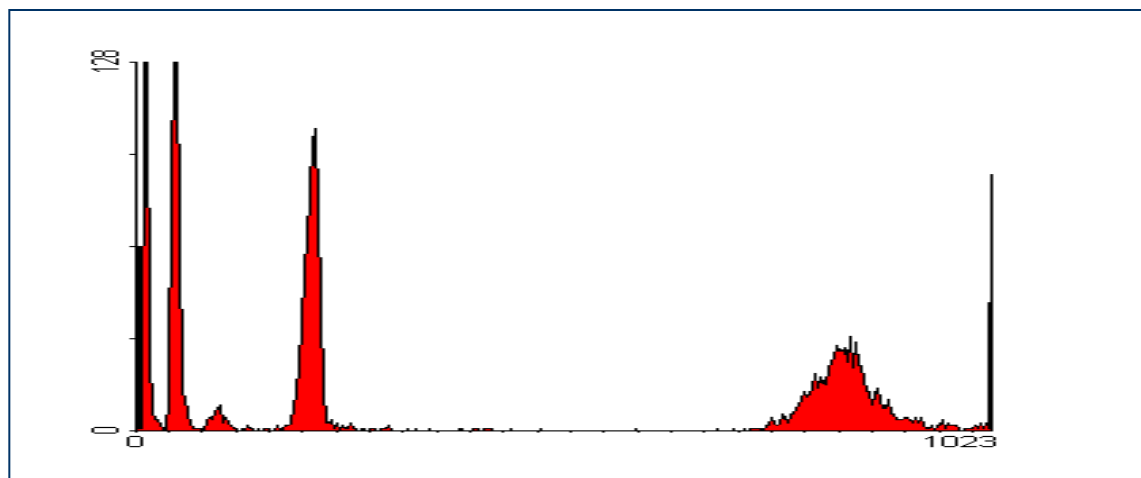
- Pokud konvertujeme analogový signál pomocí 8 bitového ADC – máme 256 kanálů ($2^8=256$) odpovídajících rozsahu 0-10 V
- Rozdíl mezi kanály je $10/256 \approx 40\text{mV}$



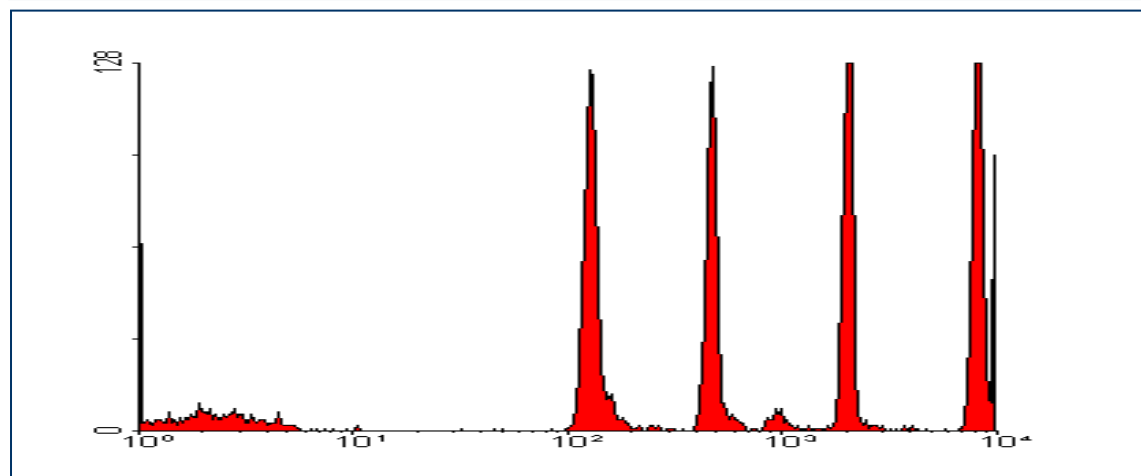
Ideální logaritmický zesilovač



Logaritmické zesílení & dynamický rozsah



lin



log



Charakteristiky pulsu

- Pulsy detekované na průtokovém cytometru jsou analogové jevy detekované pomocí analogových zařízení
- Tyto pulsy trvají několik mikrosekund
- Pokud nemůžeme digitalizovat tento puls v reálném čase musíme kombinovat analog-digitalní zpracování pulsu
- běžně trvalo několik mikrosekund digitalizovat puls – to nebylo dostatečně průchodné pro vysokorychlostní sběr dat
- Nové – plně digitální systémy mohou digitalizovat puls přímo pomocí MHz frekvence



Kompenzace fluorescenčního signálu

...později



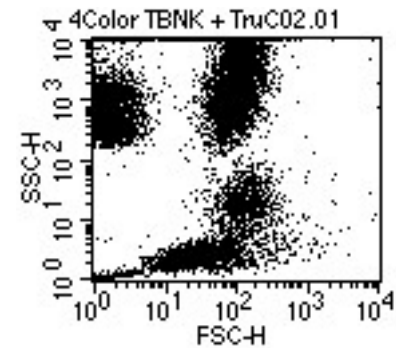
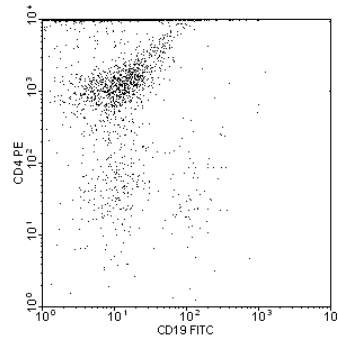
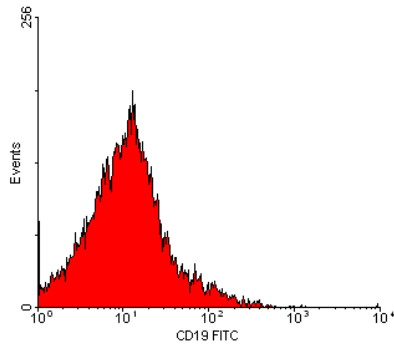
Analýza dat

■ Zobrazení dat

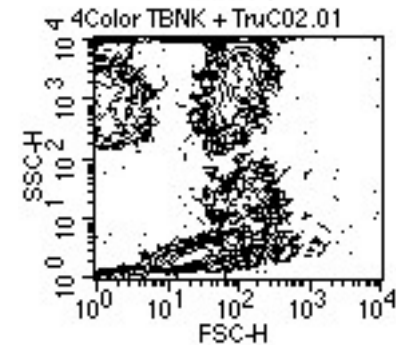
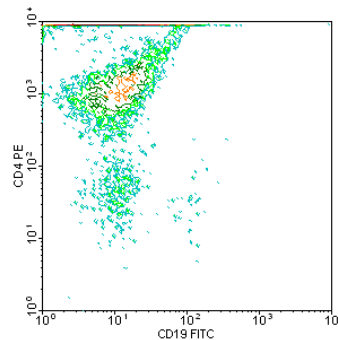
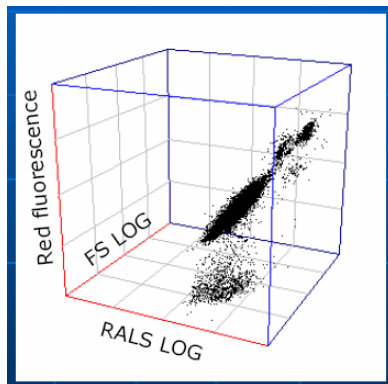
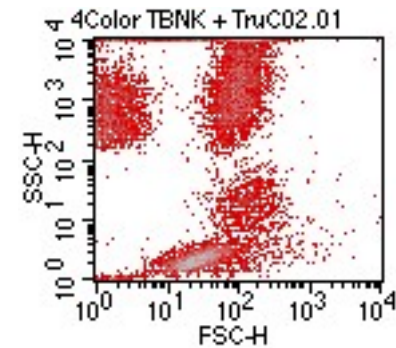
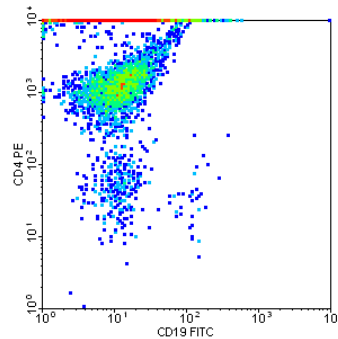
- histogram
- dot plot
- isometric display
- contour plot
- chromatic (color) plots
- 3 D projection

■ Gating

Způsoby pro zobrazení dat

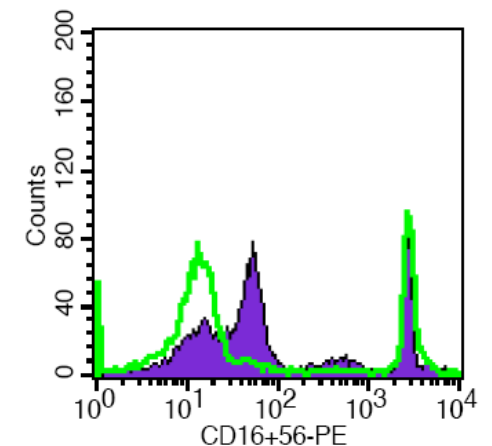
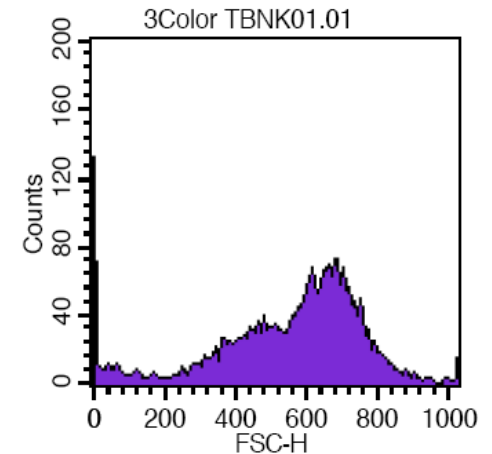


4Color TBNK + TruCO2.01



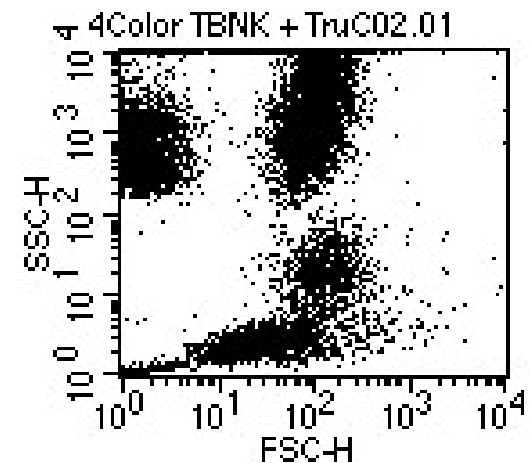
Histogram distribuce četnosti

- Histogram zobrazuje četnost částic pro jeden parametr
- Jednoduchý výstup
- Nekoreluje s dalším parametrem
- Problém s identifikací populací



Dot plot

- Zobrazuje korelaci dvou libovolných parametrů
- Jednotlivé tečky představují konkrétní změřené buňky (částice)
- Hodnoty pro řadu částic mohou ležet ve stejném místě
- Nemáme informaci o relativní densitě částic
- Problémy s vykreslením v případě velkých objemů dat



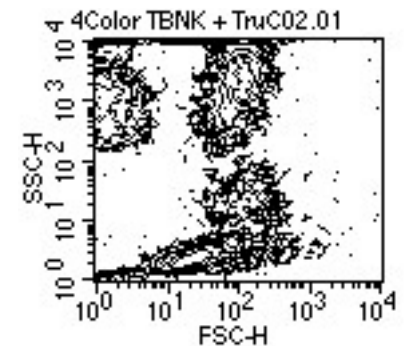
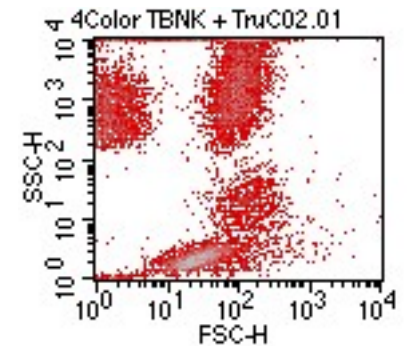
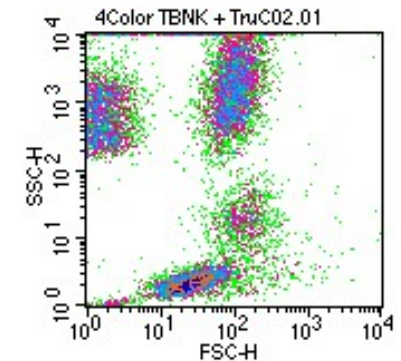
Density & contour plot

Density plot:

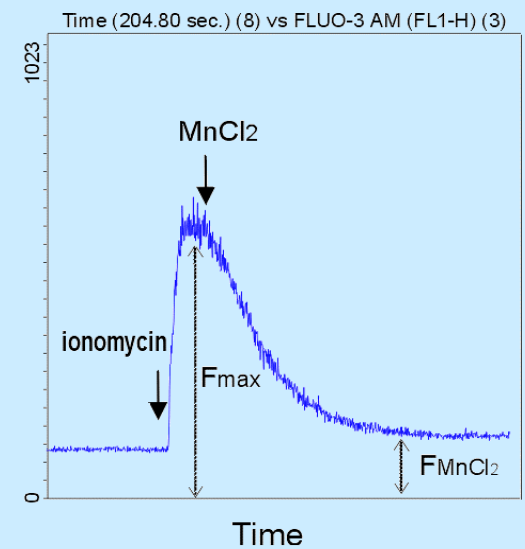
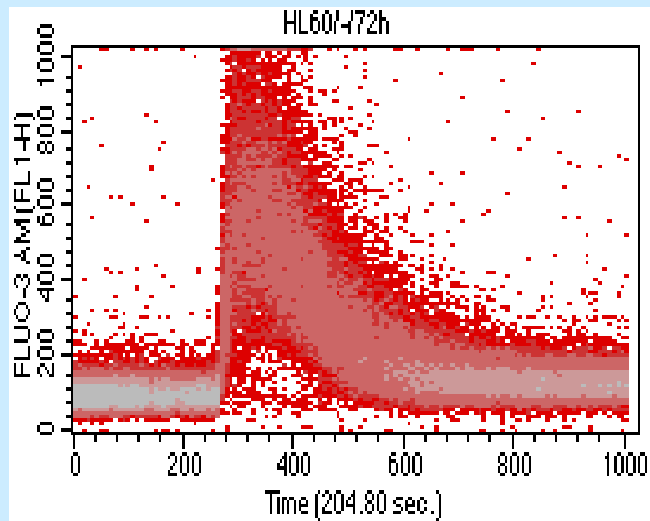
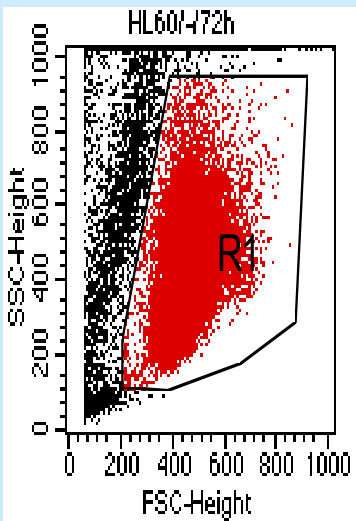
- Zobrazuje dva parametry jako frekvenci četnosti
- barva a nebo její odstín odpovídá četnosti částic

Contour plot:

- spojnice spojuje body (částice) se stejnou hodnotou signálu
- V podstatě simulujeme 3D graf – třetí rozměr je frekvence



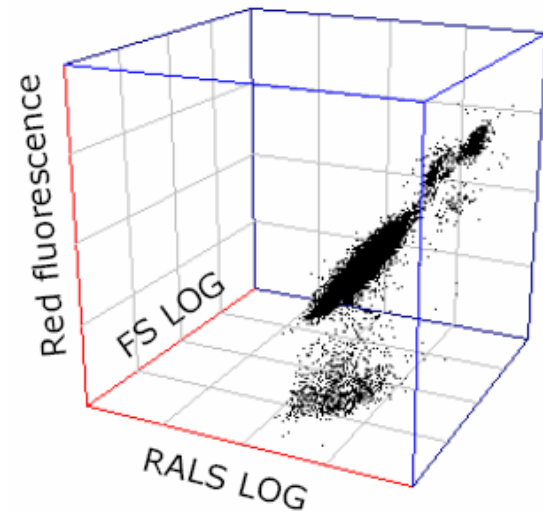
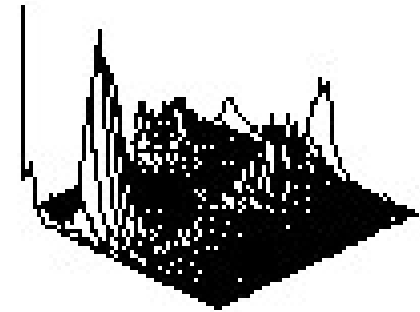
Čas jako jeden z parametrů



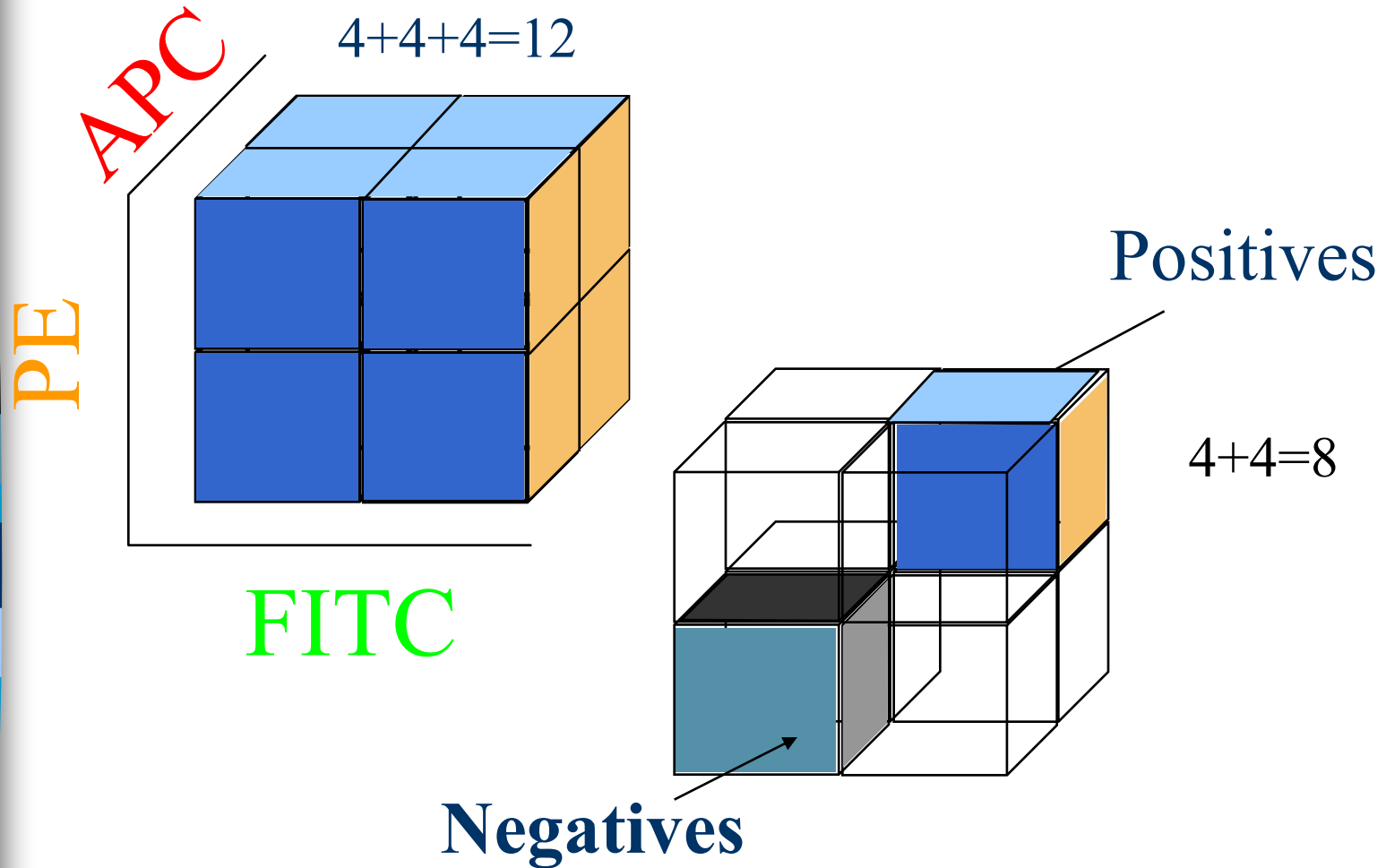
3D zobrazení

- 2 parametry + četnost
- 3 parametry společně

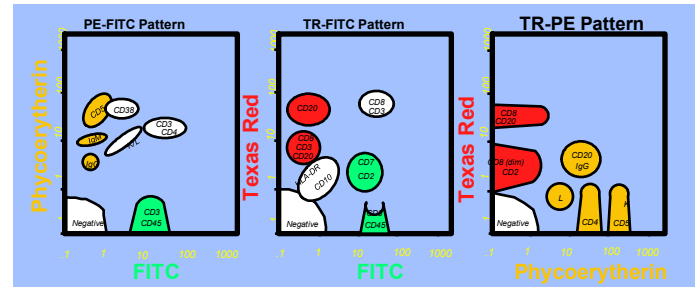
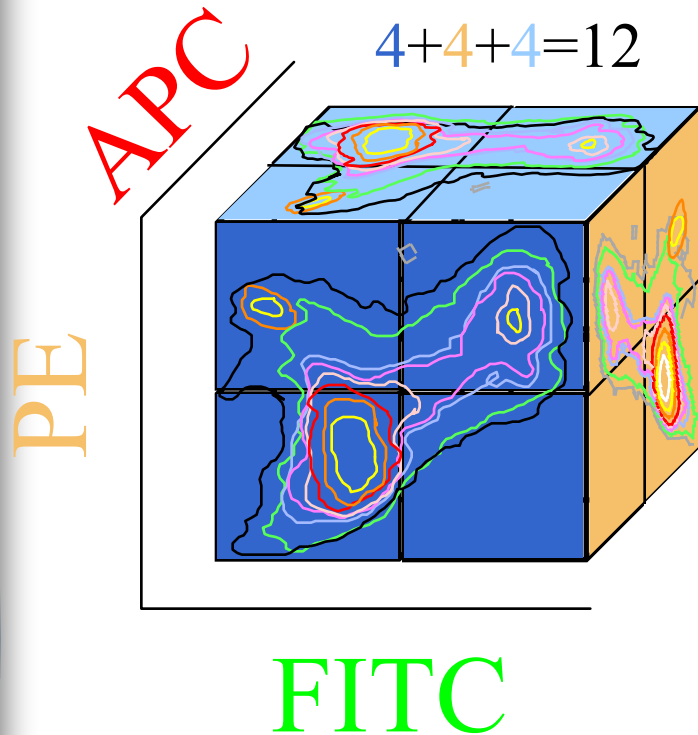
4Color TBNK + TruCO2.01



3 Color Combinations



3 Color Combinations





„Gating“

- Real-time gating vs. softwarový „gating“
- Určení regionů
- Strategie „gatingu“
- Analýza kvadrantů
- Boolean „gating“
- zpětný „gating“

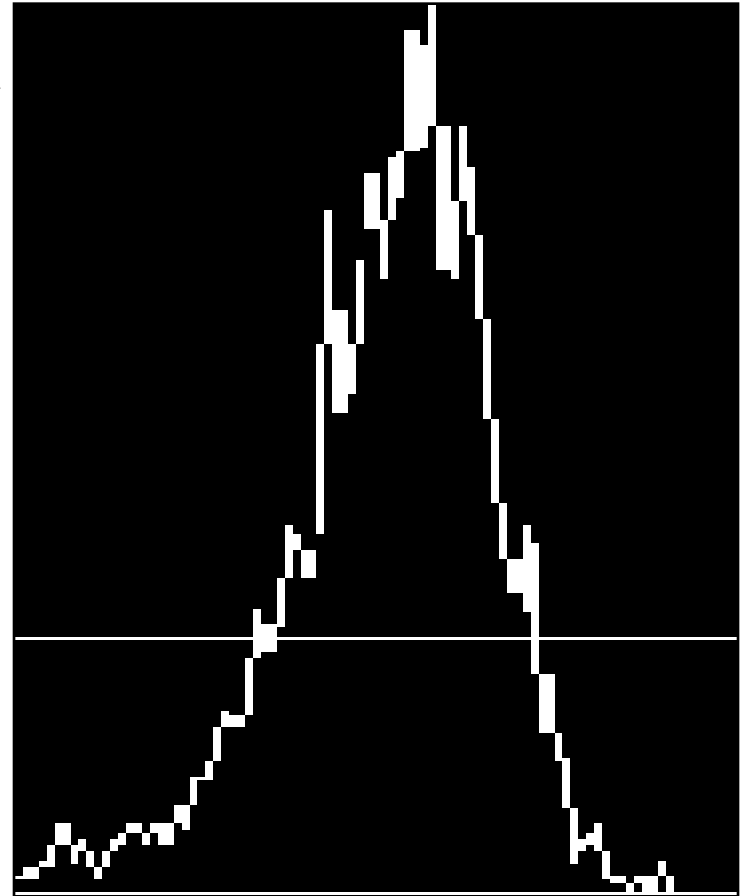


Real-Time vs. Software Gating

- Real-time (live) gating:
 - omezuje akceptovaná data během měření
- Software (analysis) gating:
 - vyřazuje určitá data během následné analýzy

Určení regionů

- Objektivní nebo subjektivní?
 - školení/schopnosti/trénink
- Možné tvary:
 - obdelník
 - elipsa
 - “free-hand” (polygon)
 - kvadrant
- Statistika
 - počet
 - podíl (%)
 - průměr, medián, S.D., CV,



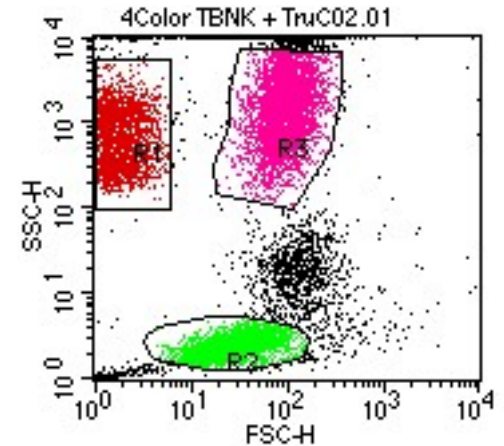
Region vs. gate

Region

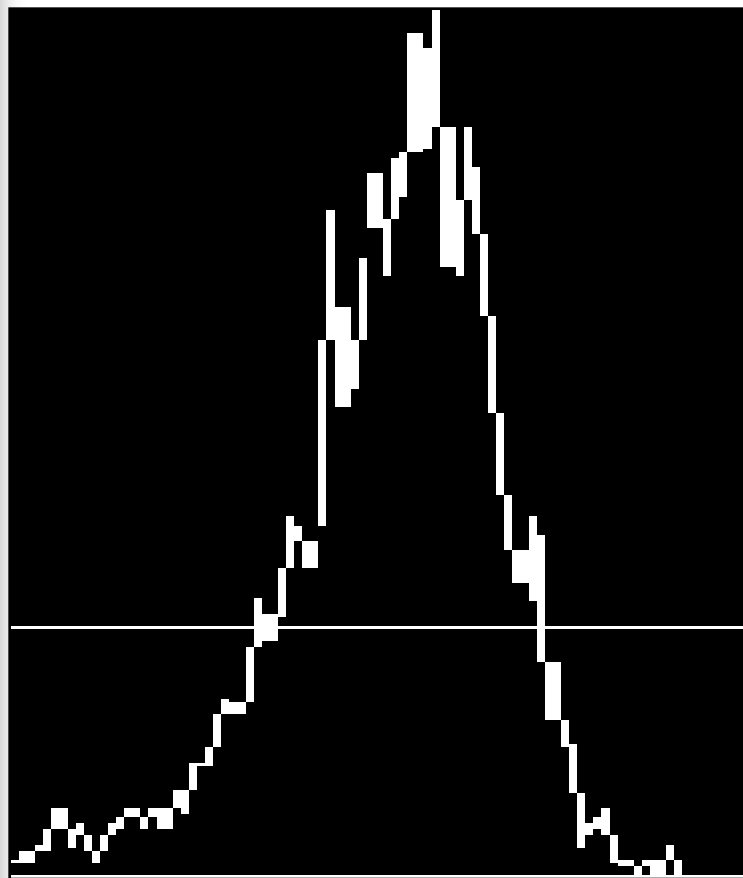
- oblast (plocha) v grafu definovaná uživatelem
- mnoho regionů v jedné grafu
- ohraničujeme pomocí nich populace našeho zájmu
- je možné je barevně odlišit
- je definován stejně pro všechny vzorky v analýze

Gate

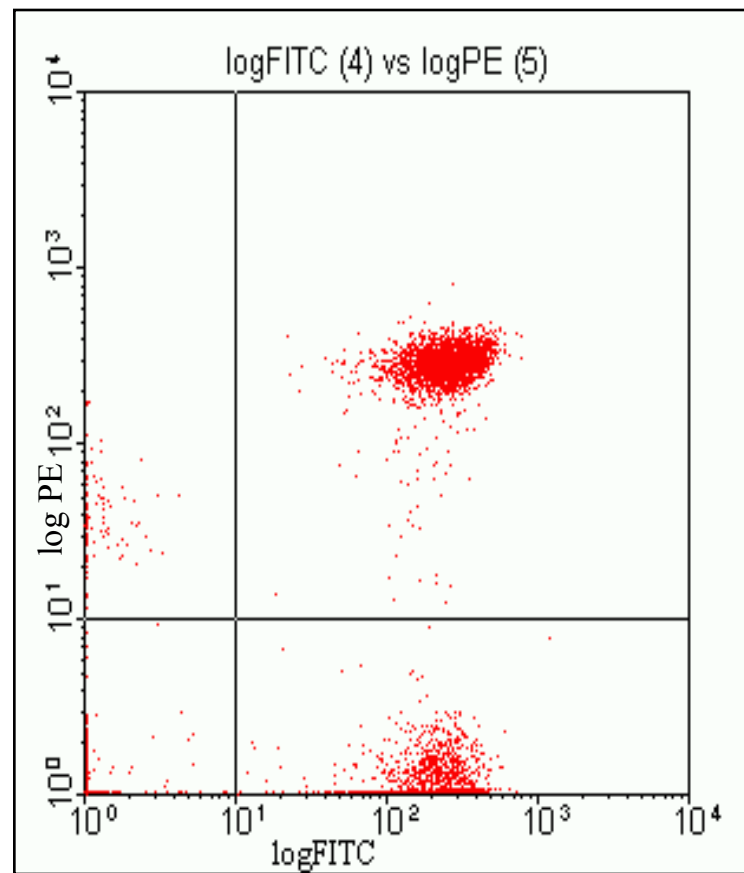
- je definován jako jeden a nebo více regionů zkombinovaných pomocí logických operátorů (AND, OR, NOT; Booleova logika)



Using Gates



Region 1 established



Gated on Region 1

Statistika

- Aritmerický průměr
- Geometrický průměr
- Medián
 - odhad střední hodnoty
 - není ovlivněn extrémními hodnotami
- Směrodatná odchylka
- Koeficient variance
- Modus – nejčastější hodnota

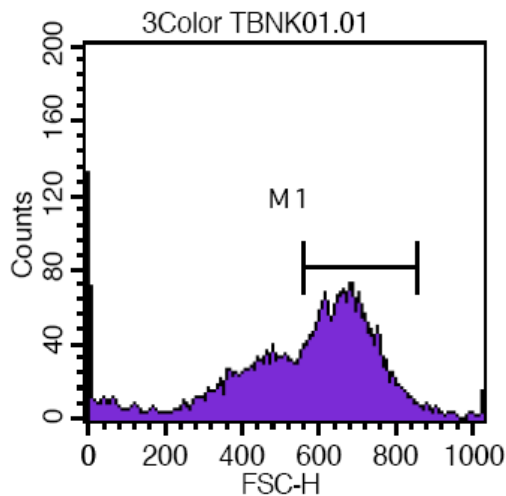
$$\bar{x} = \frac{1}{n} (x_1 + x_2 + \dots + x_n) = \frac{1}{n} \sum_{i=1}^n x_i$$

$$(a_1 \cdot a_2 \cdot \dots \cdot a_n)^{\frac{1}{n}} = \sqrt[n]{a_1 \cdot a_2 \cdot \dots \cdot a_n} = \left(\prod_{i=1}^n a_i \right)^{\frac{1}{n}}$$

$$\int_{-\infty}^m f(x) dx = 0,5$$

$$\bar{x} = \frac{1}{N} \sum_{i=1}^N x_i$$

Statistika pro histogram



Histogram Statistics

File: 3Color TBNK01.01

Sample ID:

Tube: CD3/CD4/CD45

Acquisition Date: 21-Apr-98

Gated Events: 15000

X Parameter: FSC-H (Linear)

Log Data Units: Linear Values

Patient ID:

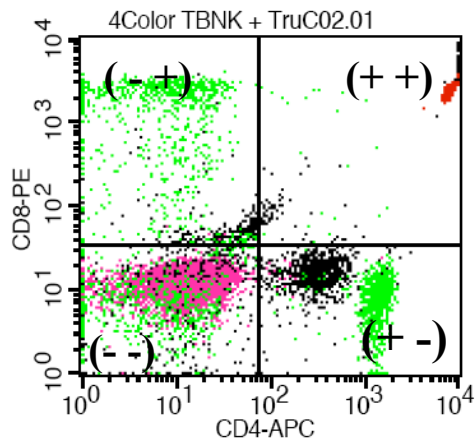
Panel: 3 Color TBNK

Gate: No Gate

Total Events: 15000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	15000	100.00	100.00	570.49	500.40	29.98	612.00	0
M1	559, 855	9306	62.04	62.04	670.83	667.81	9.56	667.00	672

Analýza kvadrantů



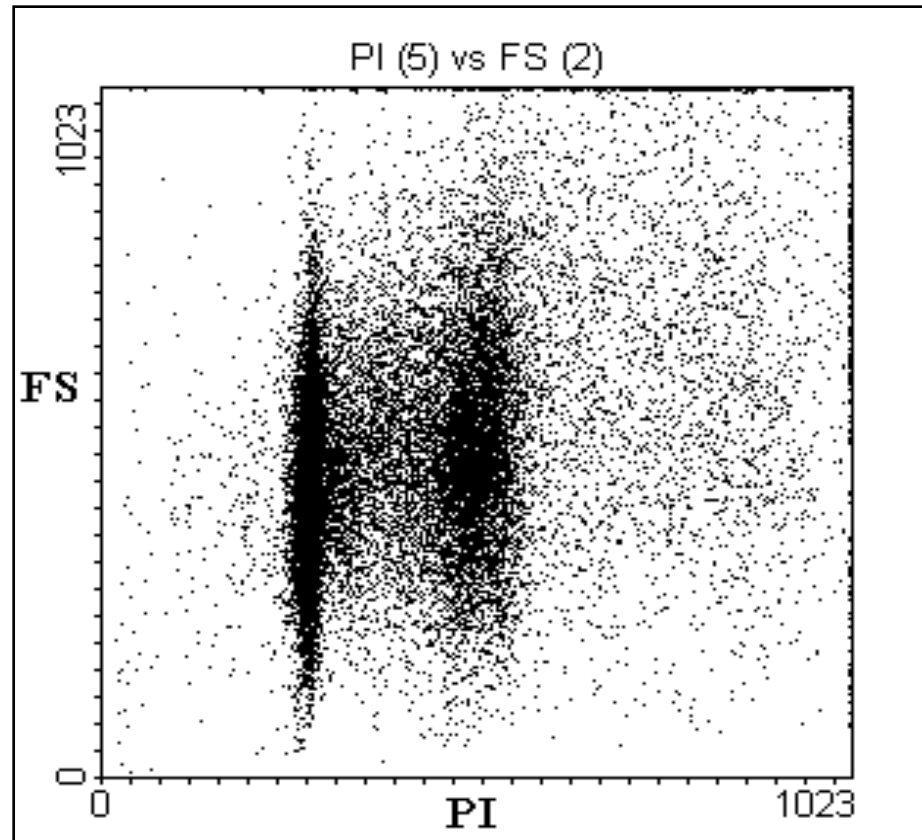
Quadrant Statistics

File: 4Color TBNK + TruC02.01 Log Data Units: Linear Values
 Sample ID: Patient ID:
 Tube: CD8/CD8/CD45/CD4 TruC Panel: 4 Color TBNK + TruC
 Acquisition Date: 08-Oct-98 Gate: No Gate
 Gated Events: 10000 Total Events: 10000
 X Parameter: CD4-APC (Log) Y Parameter: CD8-PE (Log)
 Quad Location: 74, 35

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	1149	11.49	11.49	16.67	9.14	1474.42	618.99
UR	2222	22.22	22.22	7621.69	6806.34	2386.22	2160.04
LL	4783	47.83	47.83	15.00	10.87	12.01	10.64
LR	1846	18.46	18.46	879.87	646.31	12.24	10.28

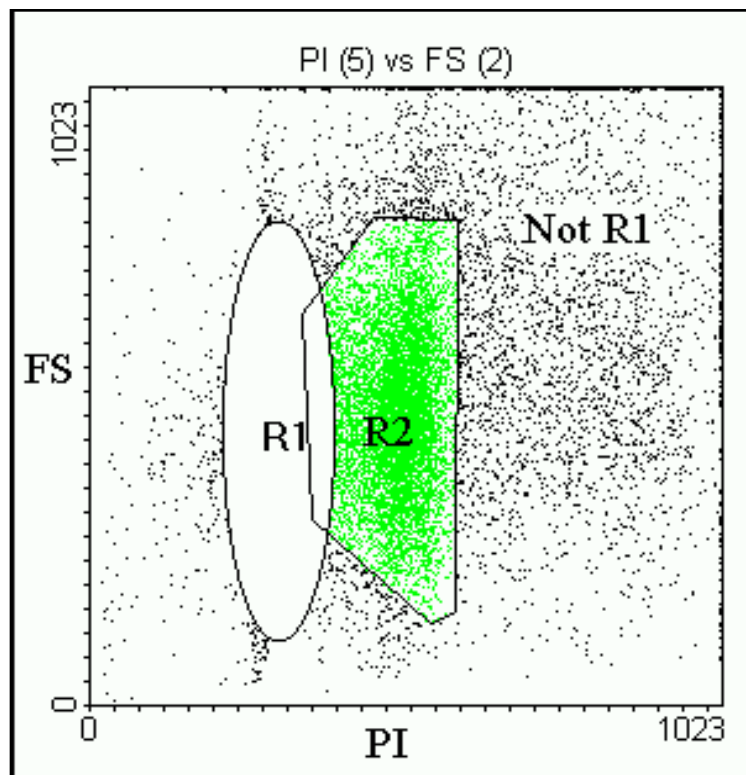
Logický „Gating“ (Booleova logika)

S překrývajícími se oblastmi máme mnoho možností:



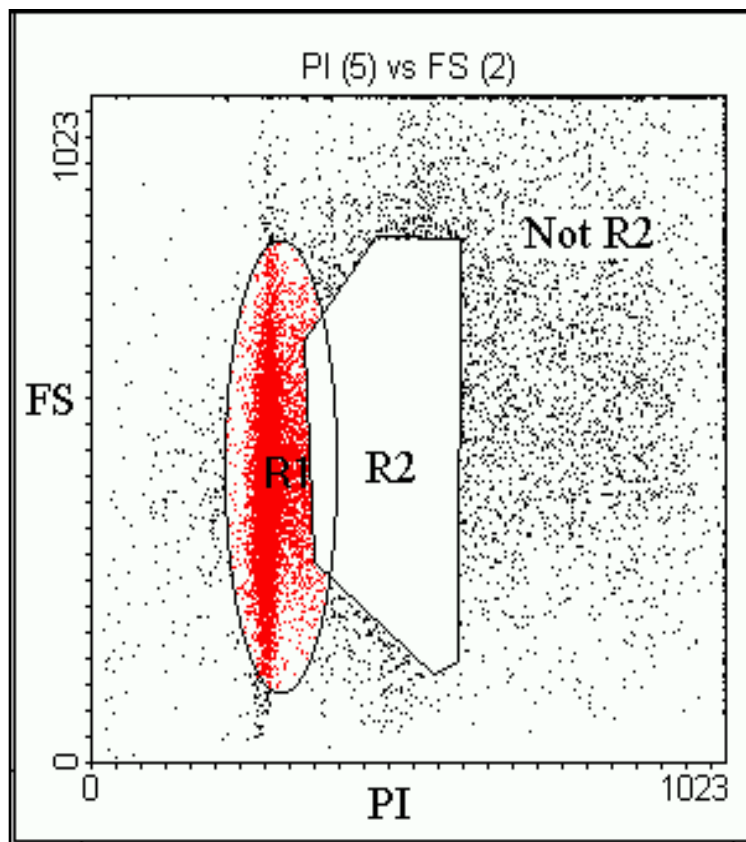
Boolean Gating

Not Region 1:



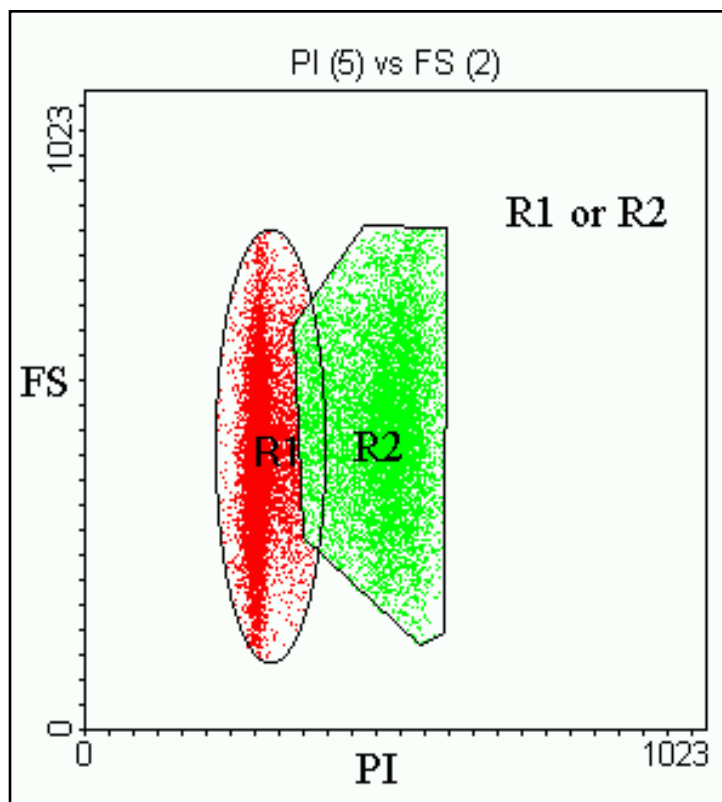
Boolean Gating

Not Region 2:



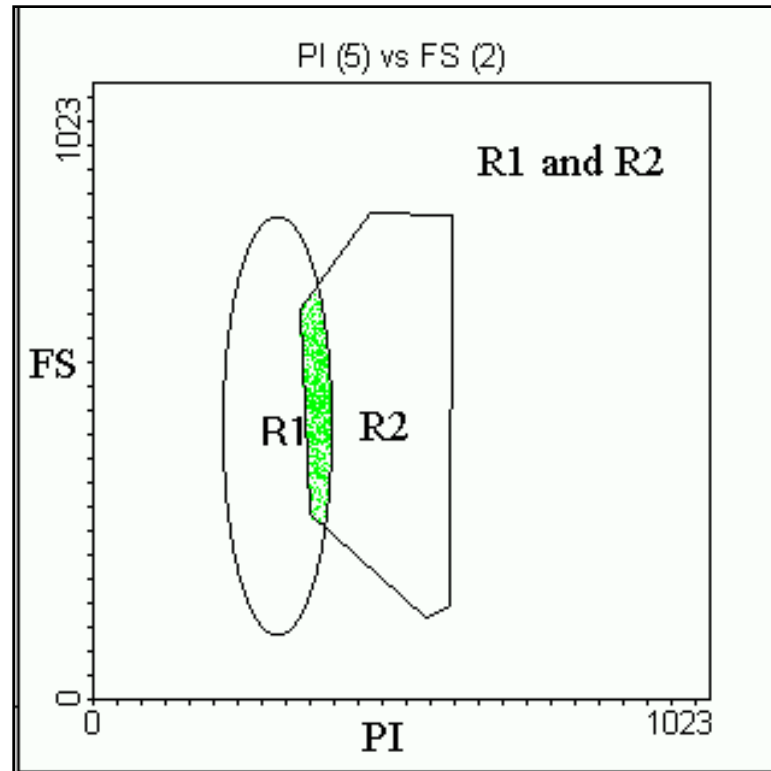
Boolean Gating

Region 1 or Region 2:



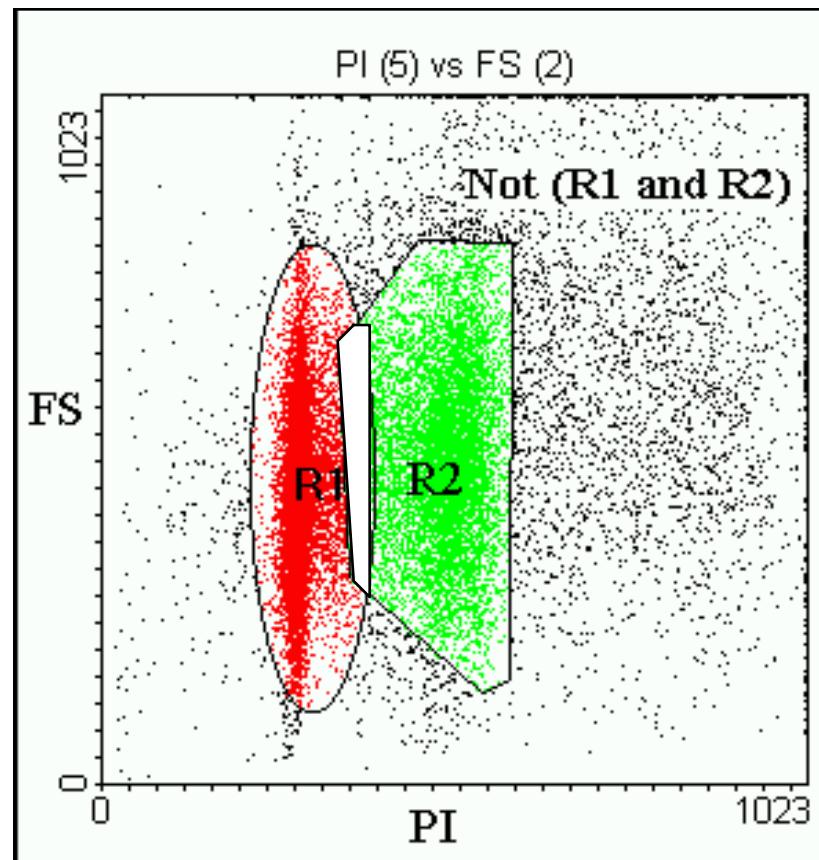
Boolean Gating

Region 1 and Region 2:

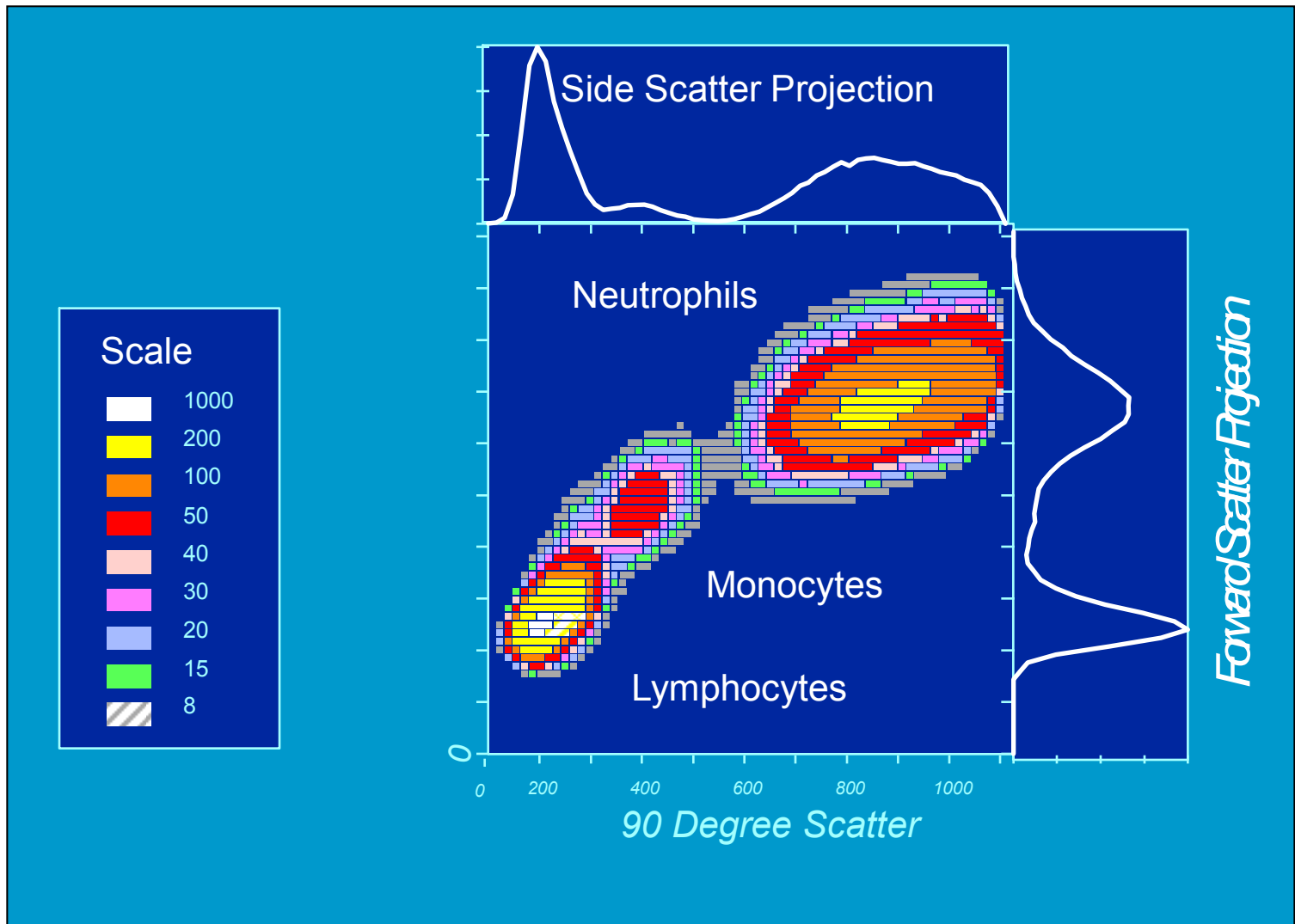


Boolean Gating

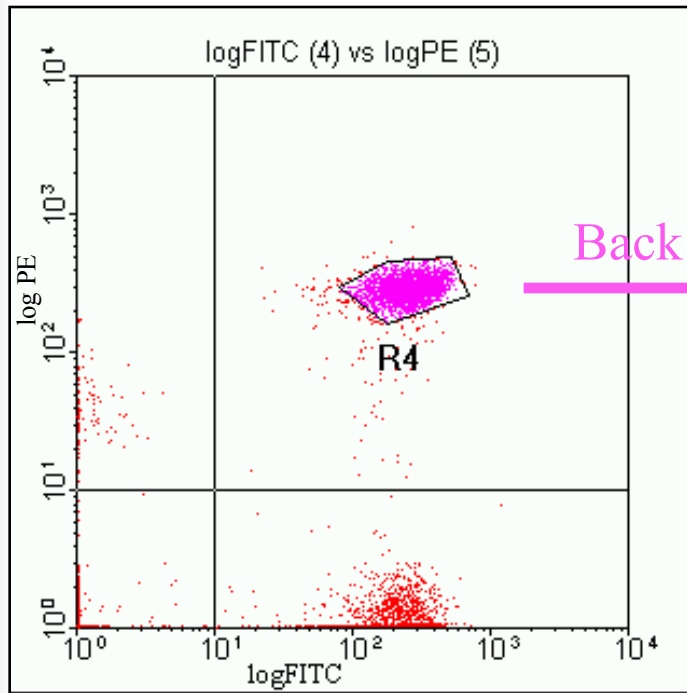
Not (Region1 and Region 2):



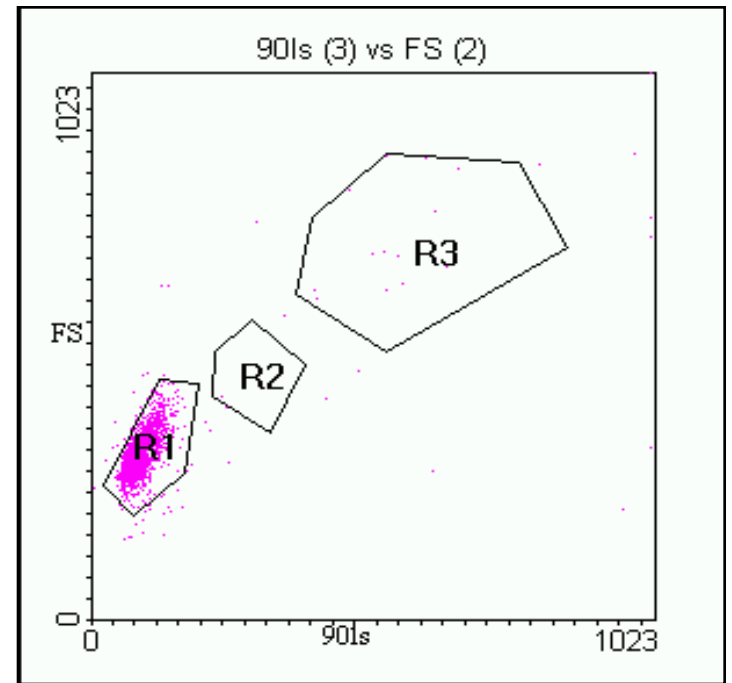
Light Scatter Gating



Back Gating

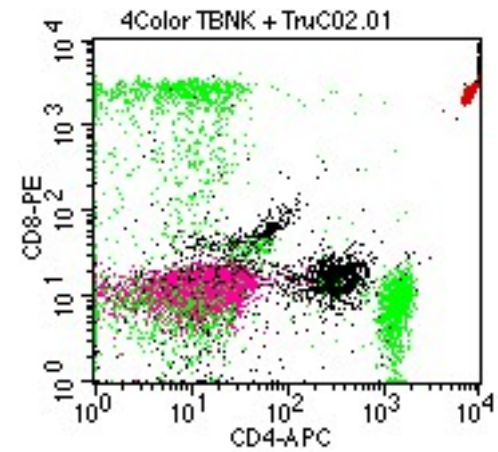
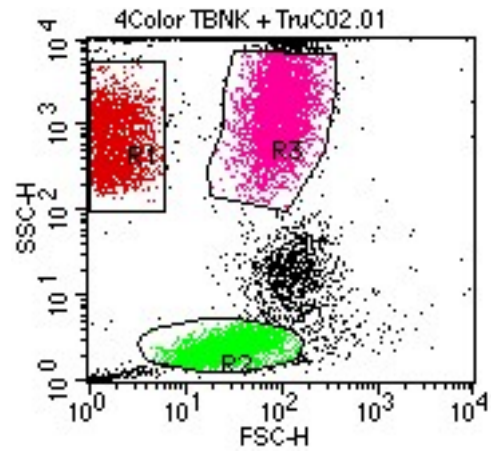


Region 4 established

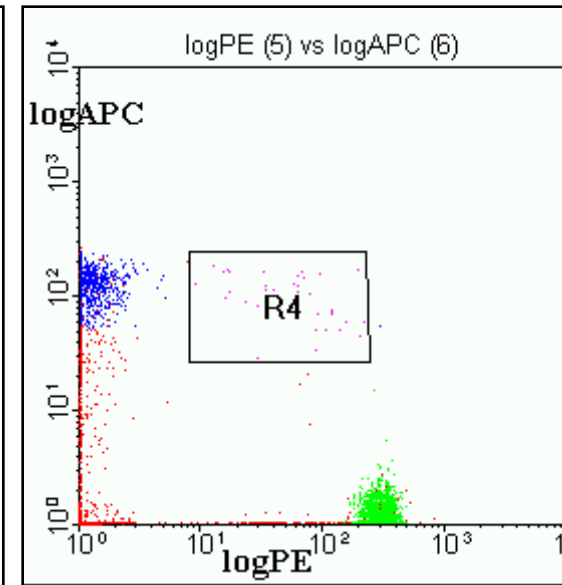
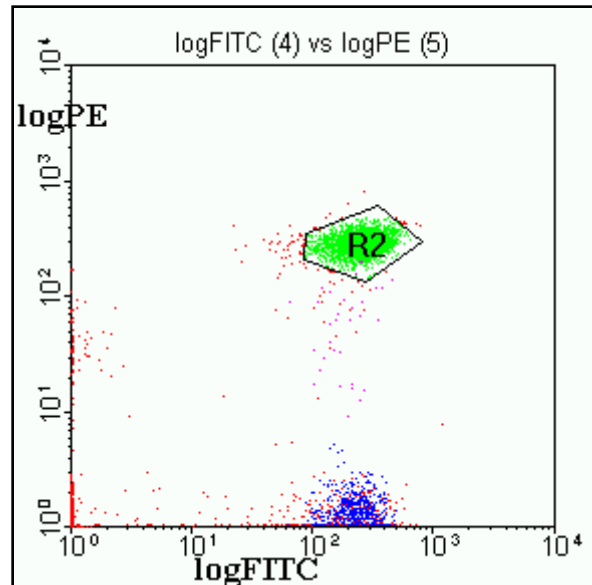
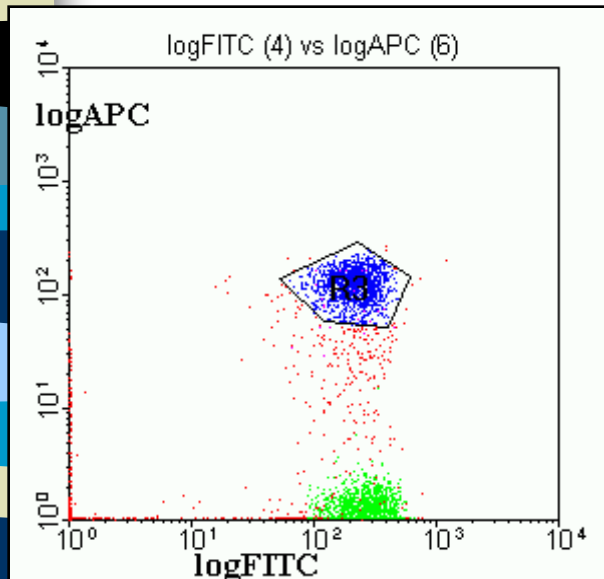
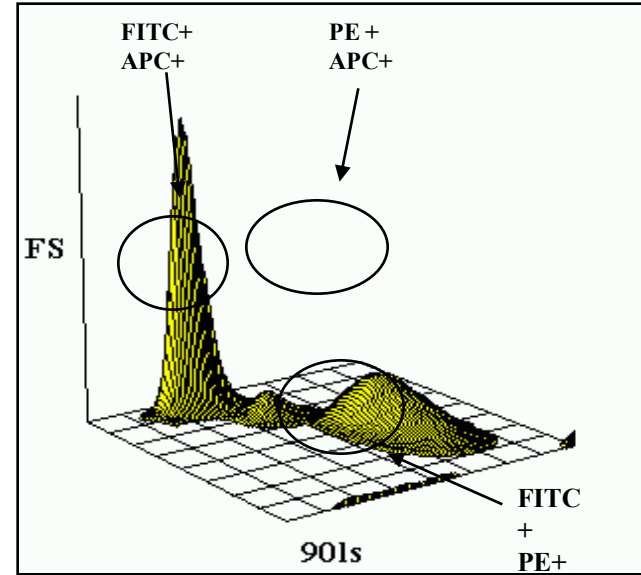
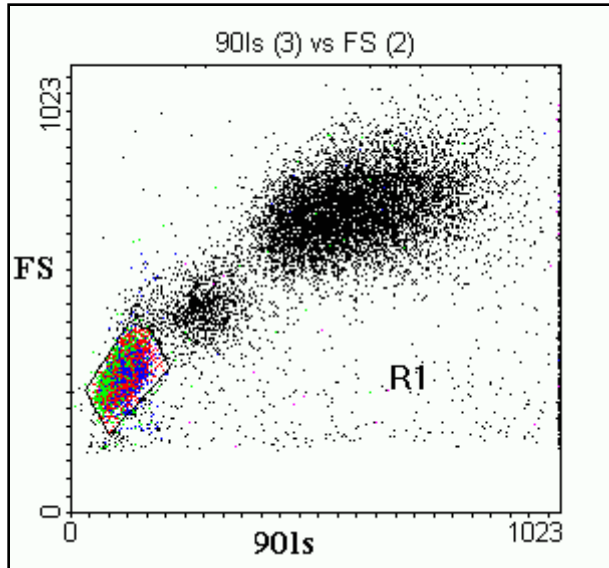


Back-gating using Region 4

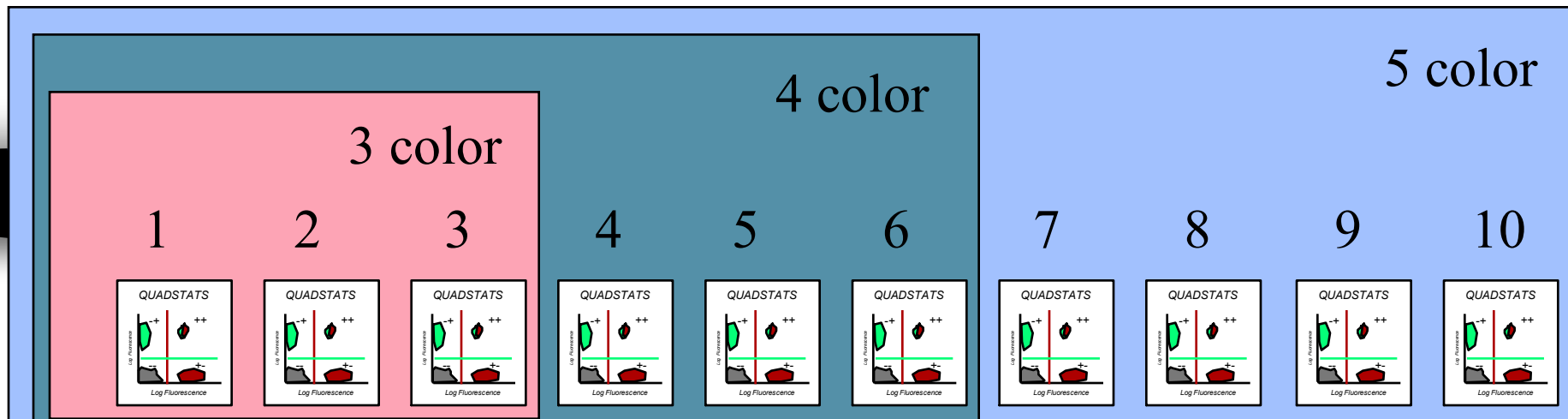
Back Gating

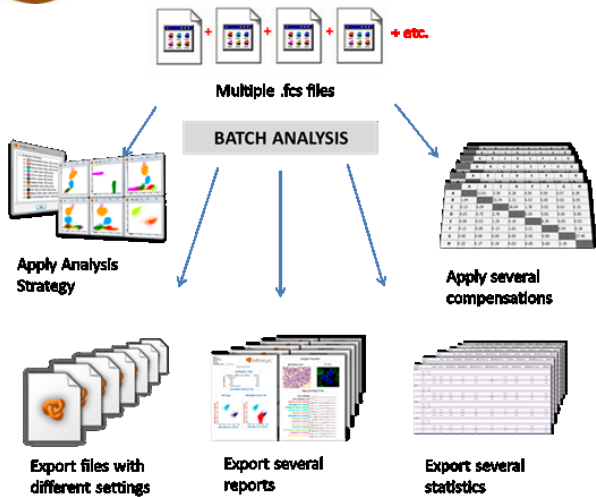


3 Parameter Data Display

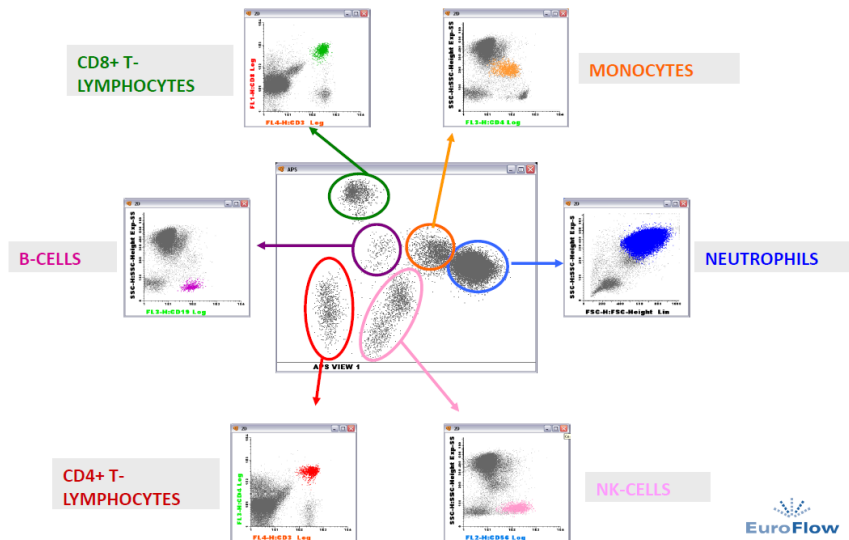


Vícebarevné analýzy generují mnoho dat...





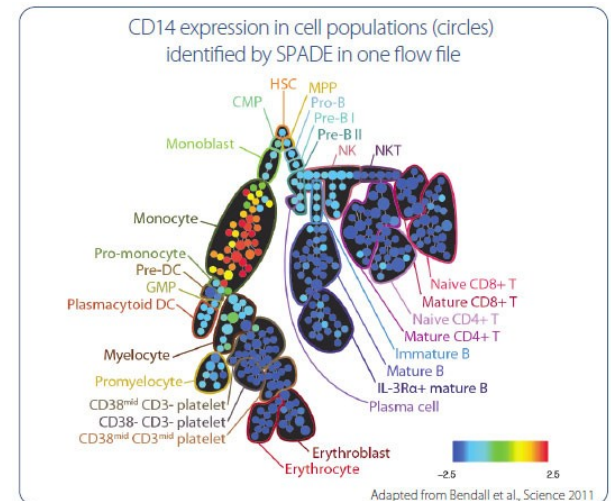
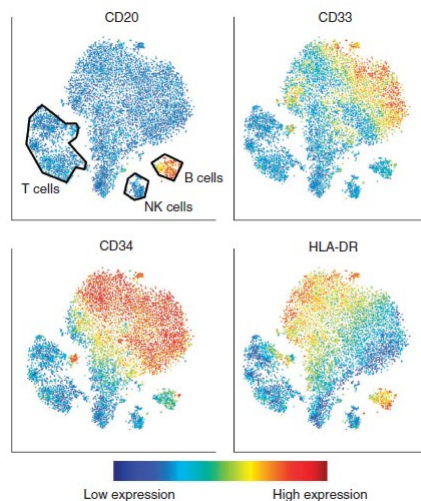
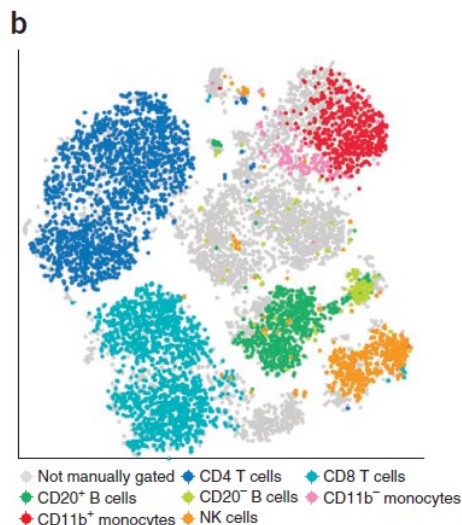
Automatic Population Separator

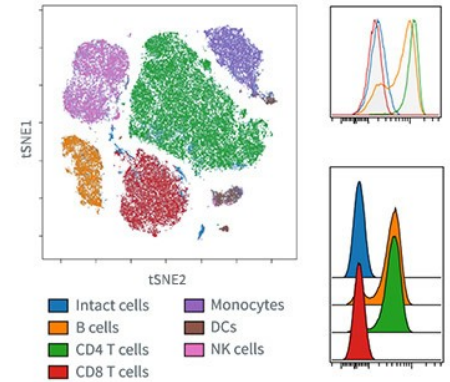
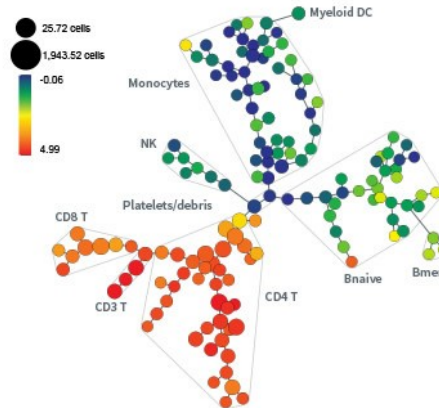
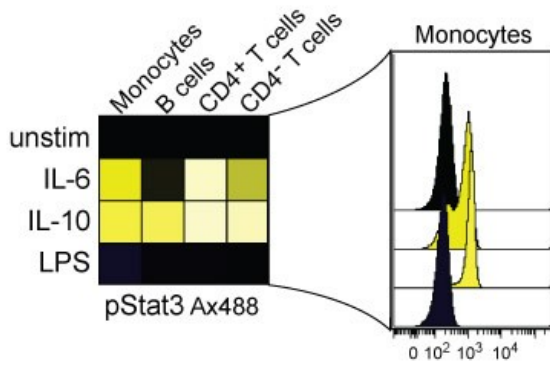
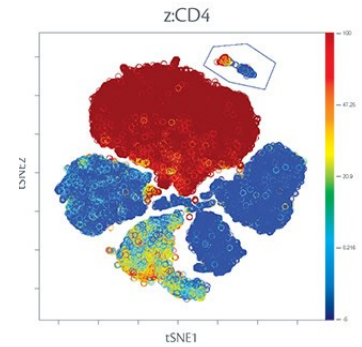
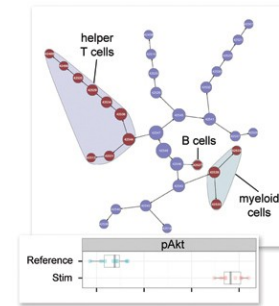
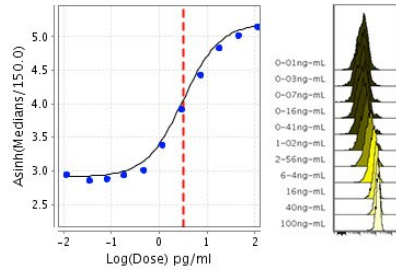
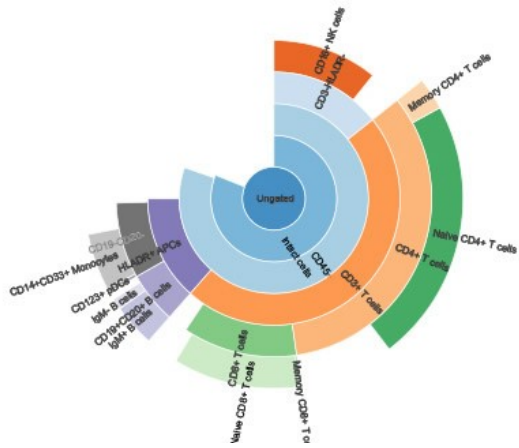


Další způsoby vizualizace vícerozměrných dat



- t-SNE, viSNE
 - t-Distributed Stochastic Neighbor Embedding
 - viSNE is a tool for reducing high-parameter data down to two dimensions
 - visually identify interesting and rare biological subsets
 - allow to gate single cell events across different samples.
- SPADE
 - Spanning-tree Progression Analysis of Density-normalized Events
 - way to automatically identify populations in multidimensional flow cytometry data files
 - clusters cells into populations and then projects them into a tree



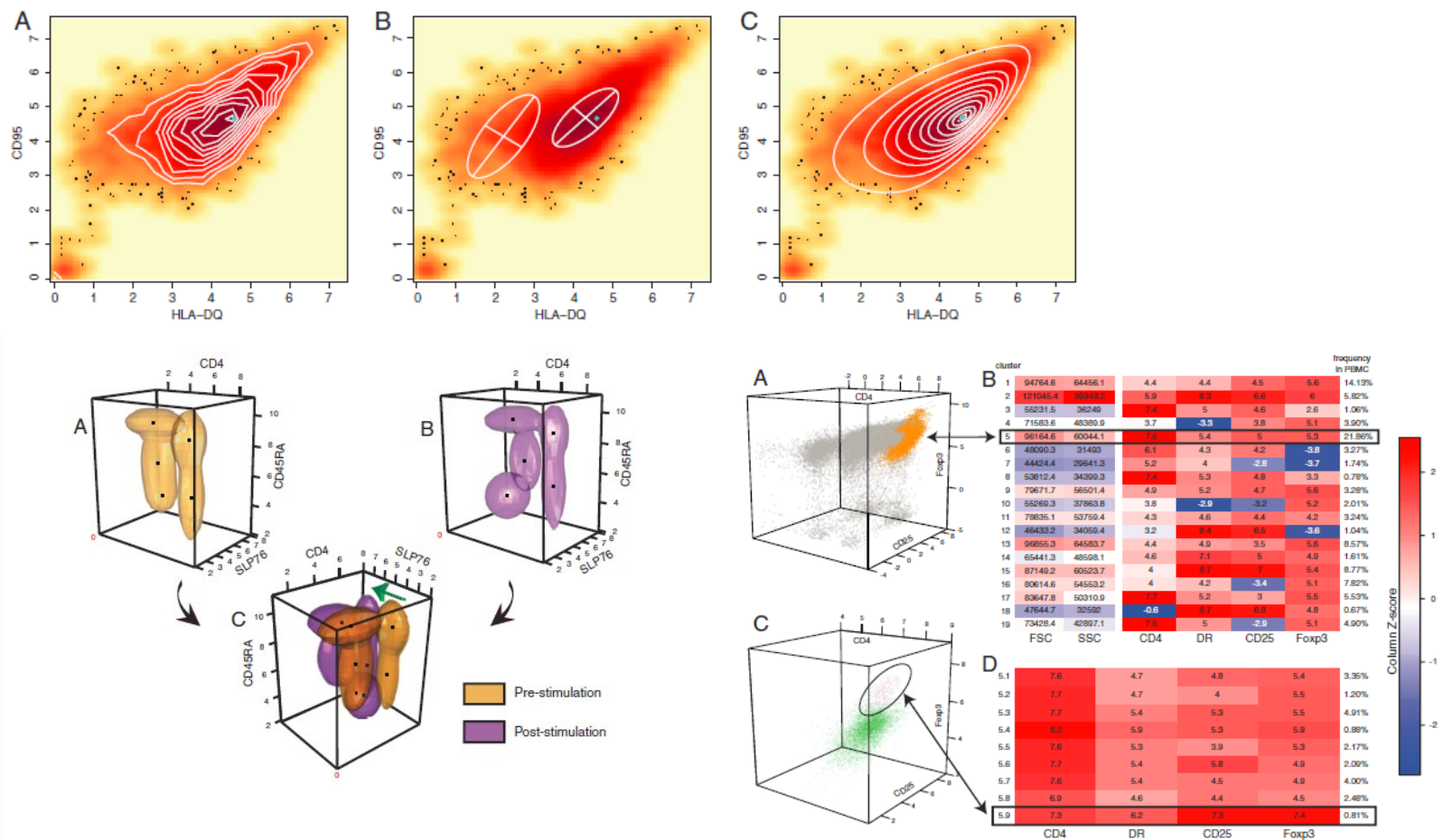


Automated high-dimensional flow cytometric data analysis

Saumyadipta Pyne^a, Xinli Hu^{a,1}, Kui Wang^{b,1}, Elizabeth Rossin^{a,1}, Tsung-I Lin^c, Lisa M. Maier^{a,d}, Clare Baecher-Allan^d, Geoffrey J. McLachlan^{b,e}, Pablo Tamayo^a, David A. Hafler^{a,d,2}, Philip L. De Jager^{a,d,f,3}, and Jill P. Mesirov^{a,2,3}

^aBroad Institute of MIT and Harvard, 7 Cambridge Center, Cambridge MA 02142; ^bDepartment of Mathematics and ^cInstitute for Molecular Bioscience, University of Queensland, St. Lucia, Queensland, 4072, Australia; ^dDepartment of Applied Mathematics, National Chung Hsing University, Taichung 402, Taiwan; ^eDivision of Molecular Immunology, Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115; and ^fPartners Center for Personalized Genetic Medicine, Boston, MA 02115

Communicated by Peter J. Bickel, University of California, Berkeley, CA, April 3, 2009 (received for review December 28, 2008)





The Flow Cytometry: Critical Assessment of Population Identification Methods (FlowCAP)

The goal of FlowCAP is to advance the development of computational methods for the identification of cell populations of interest in flow cytometry data. FlowCAP will provide the means to objectively test these methods, first by comparison to manual analysis by experts using common datasets, and second by prediction of a clinical/biological outcome.

Critical assessment of automated flow cytometry data analysis techniques

Nima Aghaeepour¹, Greg Finak², The FlowCAP Consortium³, The DREAM Consortium³, Holger Hoos⁴, Tim R Mosmann⁵, Ryan Brinkman^{1,7}, Raphael Gottardo^{2,7} & Richard H Scheuermann^{6,7}

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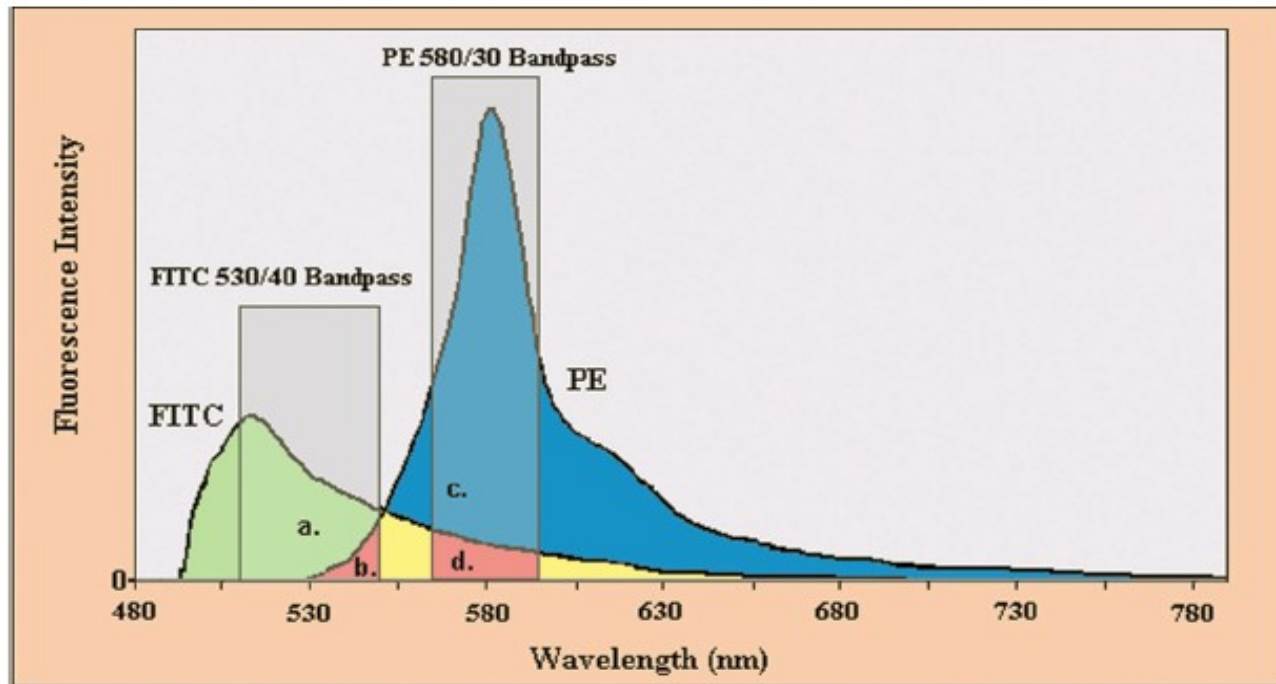
Způsoby pomocí kterých lze upravit výsledky:

1. Odstranění „doublets“
2. Čas jako parametr pro kontrolu kvality

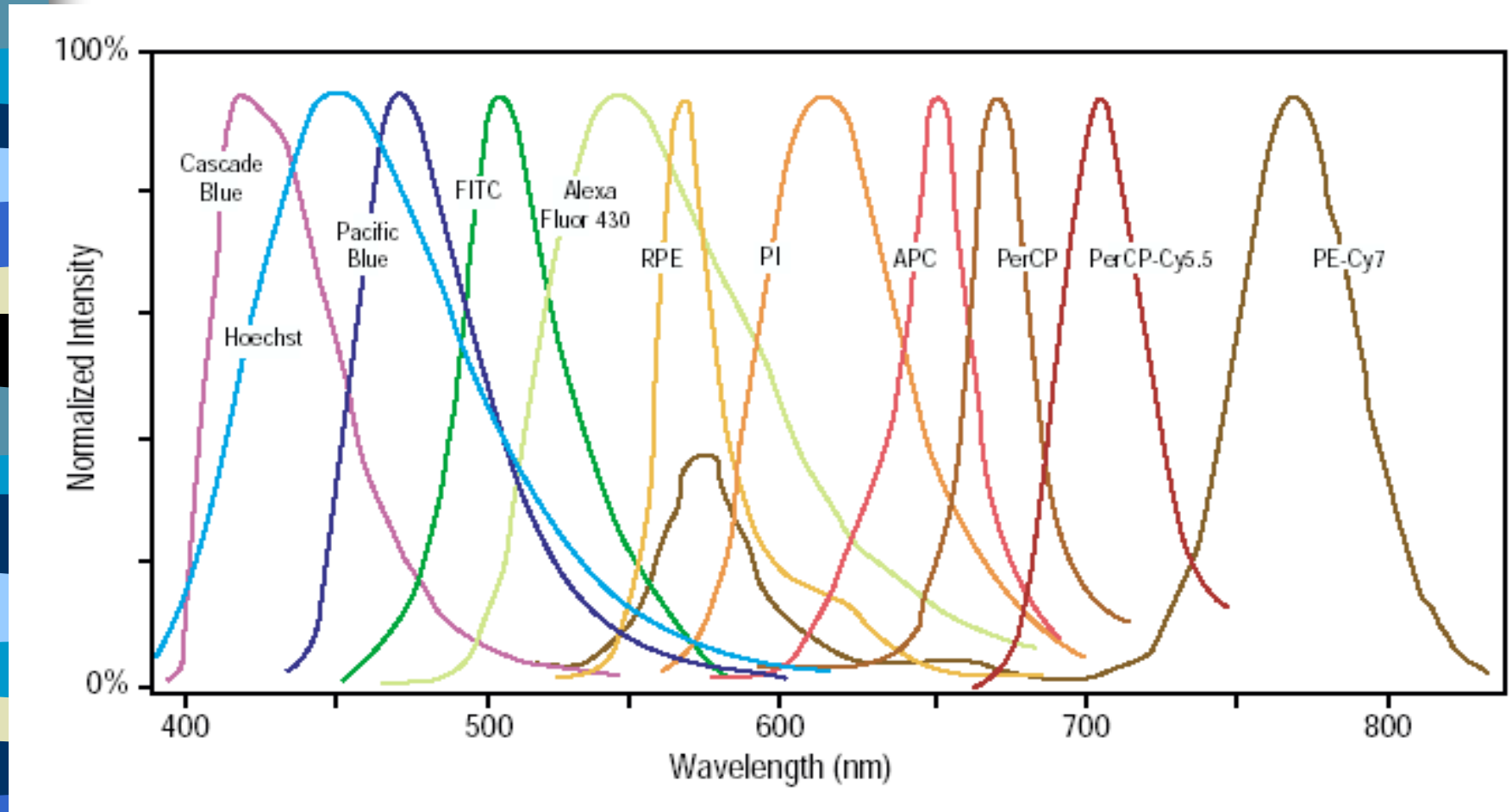
Příklad - pro DNA analýzu je třeba:

- odstranit „debris“ a shluky
- odstranit „doublets“
- udržovat konstantní průtok

Co je problém při vícebarevné detekci?



Emission Spectra–Spectral Overlap

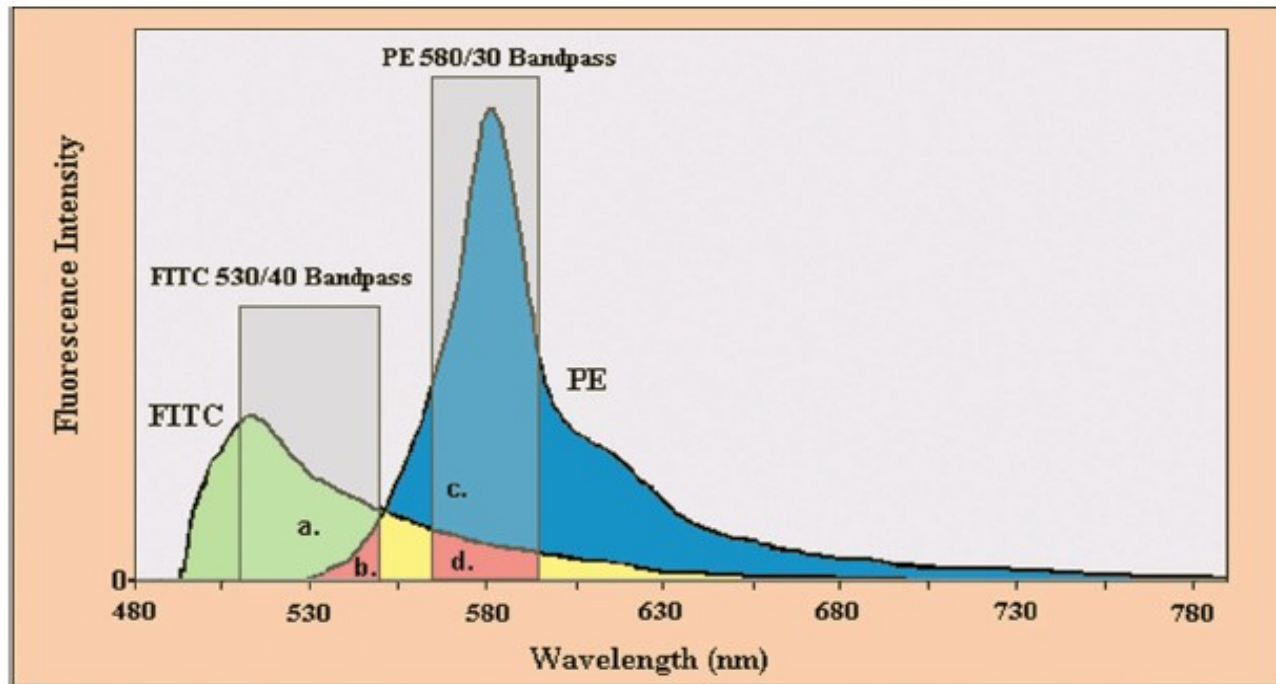




Kompenzace fluorescenčního signálu při vícebarevné detekci

- Proces při kterém dochází k eliminaci všech fluorescenčních signálů kromě signálu z fluorochromu který má být na příslušném detektoru detekován
- Nastavení pomocí mixu mikročástic či buněk označených/neoznačených příslušnými fluorochromy.

Co je problém při vícebarevné detekci?



Kompenzace fluorescenčního signálu při vícebarevné detekci

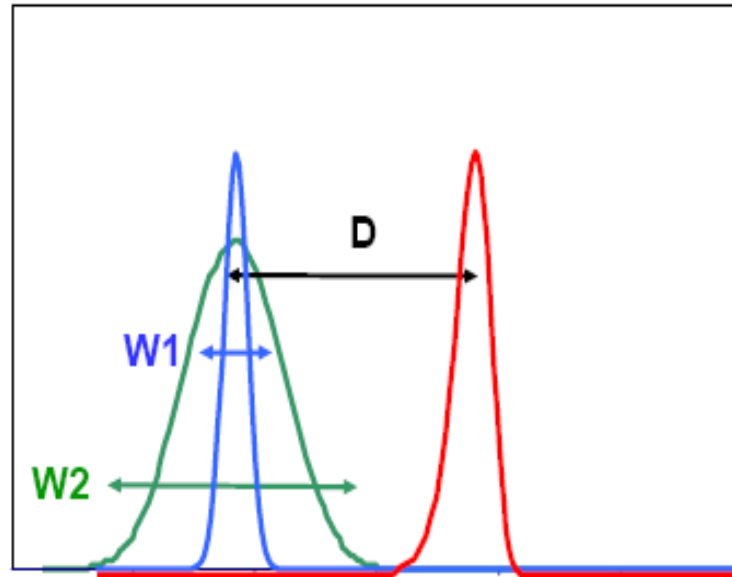
Table 1.14.1 Typical Spillover Matrix for a Three-Color Compensation^a

Fluorophore	Detector		
	Green	Orange	Red
FITC	1.000	0.180	0.040
PE	0.009	1.000	0.213
PE-Cy5	0.005	0.029	1.000

^aNote: The diagonal elements are 1, since the contribution of each fluorophore to its cognate detector is defined to be 100%. In this table, the FITC into PE spillover is 18%; the PE into FITC spillover is 0.9%.

Current Protocols in Cytometry

“Bright” = good resolution sensitivity



$$\text{Stain Index (SI)} = \frac{D}{W}$$

Various fluorochromes-stain index

Reagent	Clone	Filter	Stain Index
PE	RPA-T4	585/40	356.3
Alexa 647	RPA-T4	660/20	313.1
APC	RPA-T4	660/20	279.2
PE-Cy7	RPA-T4	780/60	278.5
PE-Cy5	RPA-T4	695/40	222.1
PerCP-Cy5.5	Leu-3a	695/40	92.7
PE-Alexa 610	RPA-T4	610/20	80.4
Alexa 488	RPA-T4	530/30	75.4
FITC	RPA-T4	530/30	68.9
PerCP	Leu-3a	695/40	64.4
APC-Cy7	RPA-T4	780/60	42.2
Alexa 700	RPA-T4	720/45	39.9
Pacific Blue	RPA-T4	440/40	22.5
AmCyan	RPA-T4	525/50	20.2

Choices for 6,- 8,- 10,- and more colors

6-color	8-color	10-color	Additional
FITC or Alexa 488	FITC or Alexa 488	FITC or Alexa 488	FITC or Alexa 488
PE	PE	PE	PE
		PE-Texas Red or PE-Alexa 610	PE-Texas Red or PE-Alexa 610
PerCP-Cy5.5	PerCP-Cy5.5	PerCP-Cy5.5	PerCP-Cy5.5
PE-Cy7	PE-Cy7	PE-Cy7	PE-Cy7
APC or Alexa 647	APC or Alexa 647	APC or Alexa 647	APC or Alexa 647
		Alexa 680 or 700	Alexa 680 or 700
APC-Cy7	APC-Cy7	APC-Cy7	APC-Cy7
	AmCyan	AmCyan	AmCyan
	Pacific Blue	Pacific Blue	Pacific Blue
			Q-dot 655, 705...



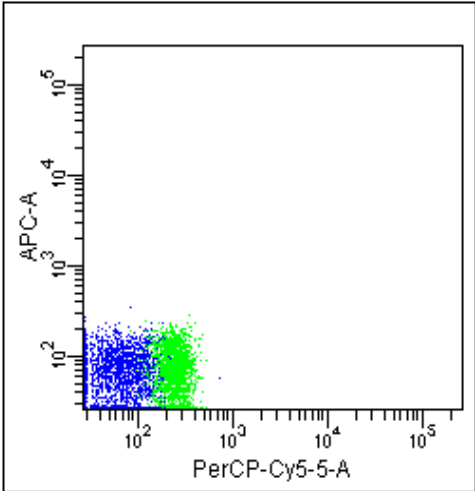
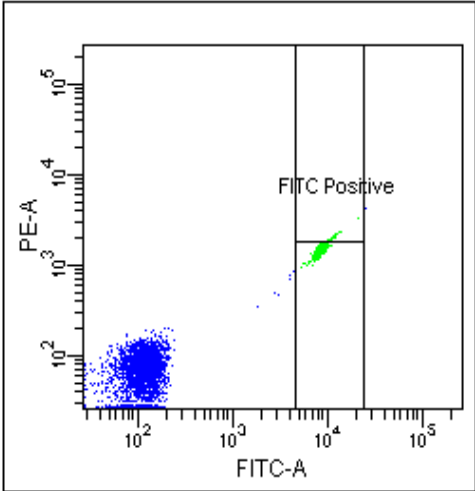
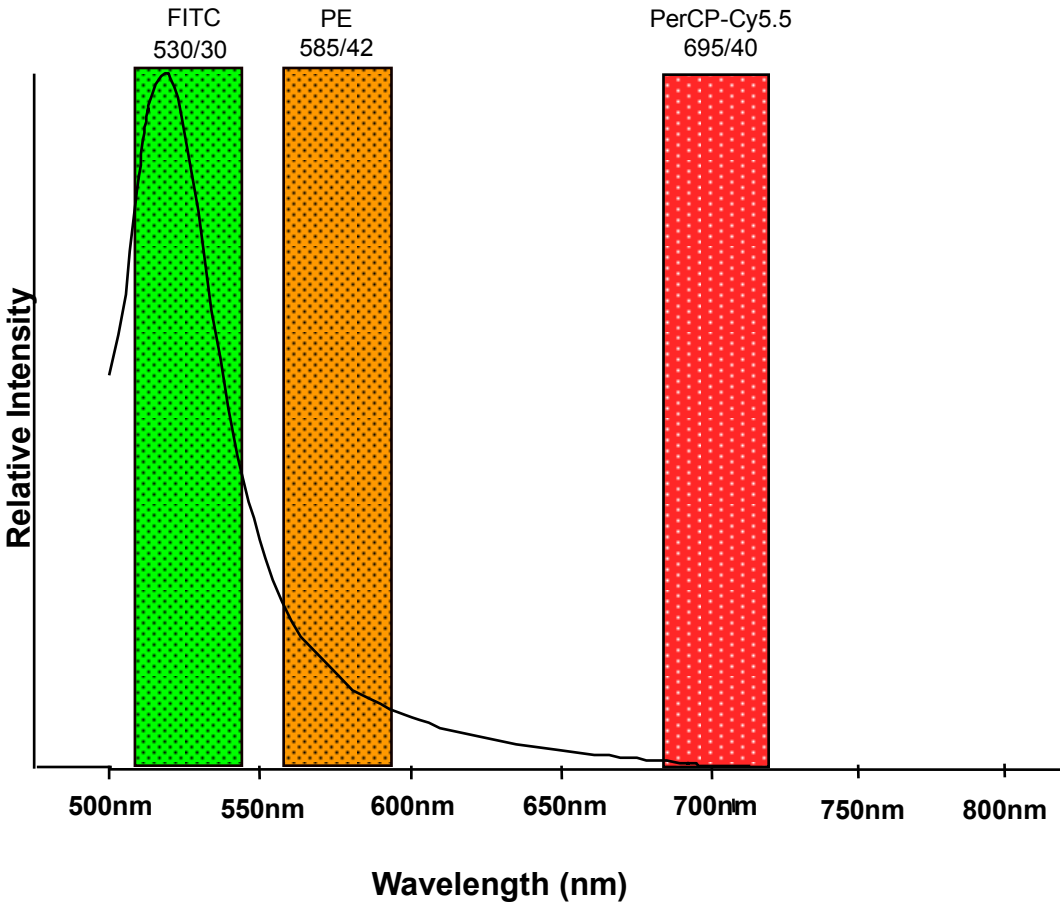
Fluorochrome selection considerations

“Bright” antibodies go on “dim”
fluorochromes

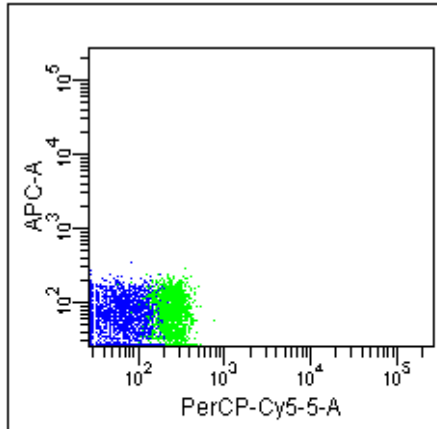
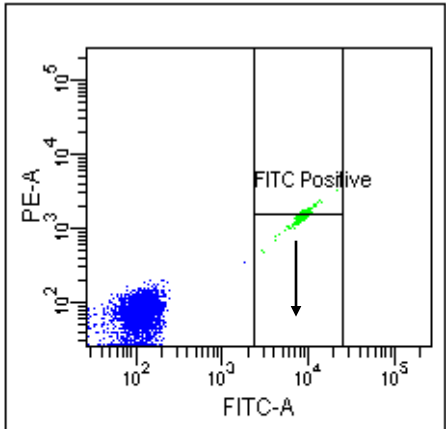
Avoid spillover from bright cell populations
into channels requiring high sensitivity

Beware of tandem dye degradation

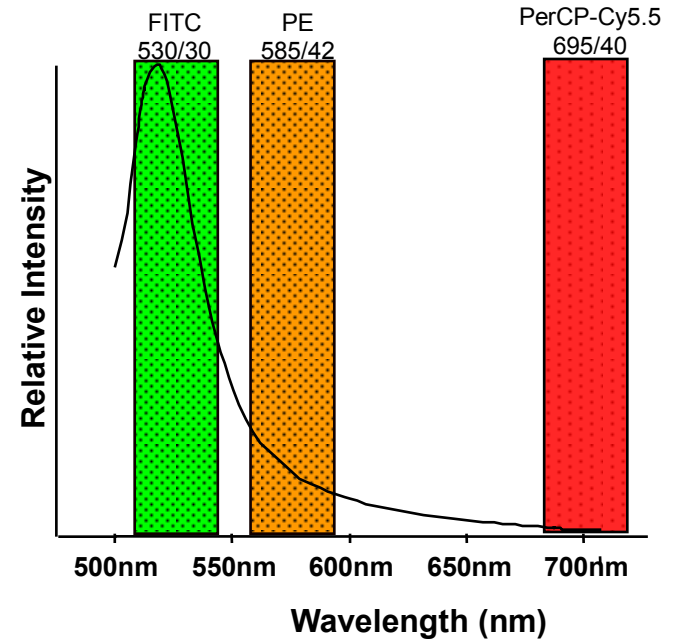
FITC Spillover



FITC Compensation

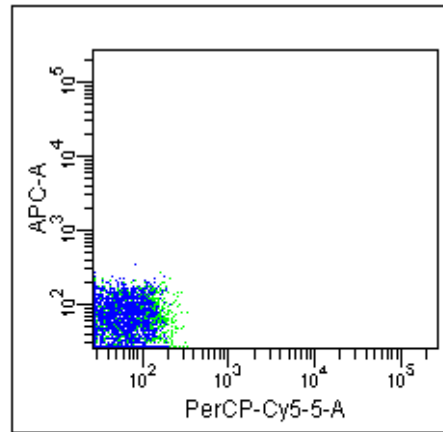
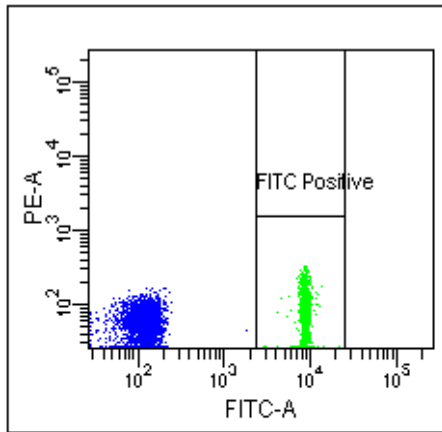


Population	FITC-A Median	PE-A Median	PerCP-Cy5-5-A Median	APC-A Median
■ FITC Positive	8,776	1,499	226	63
■ FITC Negative	113	70	52	56

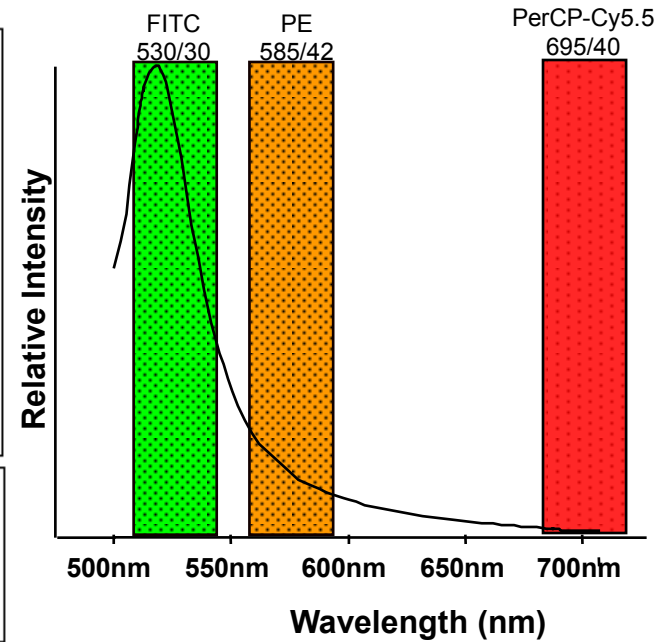


	Fluorochrome	- % Fluorochrome	Spectral Overlap
•	PE	FITC	0.00
•	PerCP-Cy5-5	FITC	0.00
•	APC	FITC	0.00

FITC Compensation

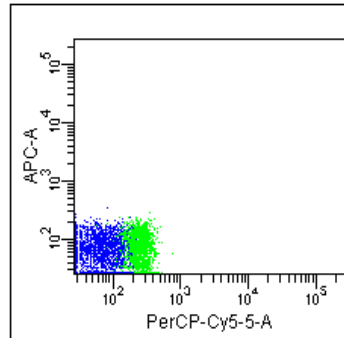
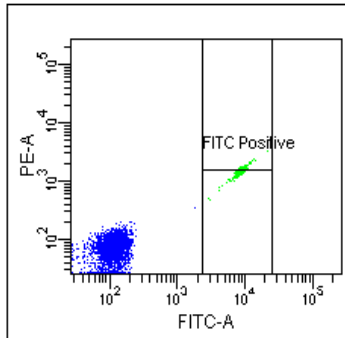


Population	FITC-A Median	PE-A Median	PerCP-Cy5-5-A Median	APC-A Median
■ FITC Positive	8,776	54	49	53
■ FITC Negative	113	50	49	56

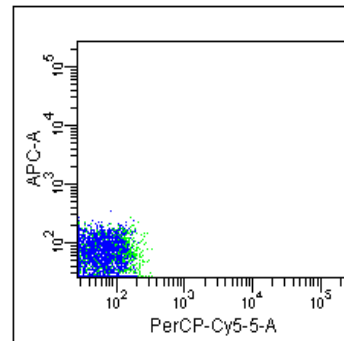
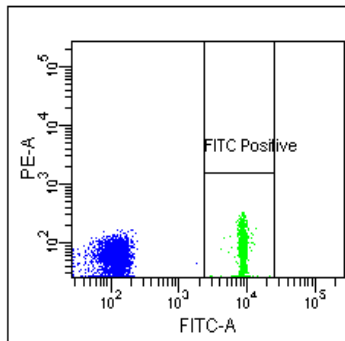


	Fluorochrome	- % Fluorochrome	Spectral Overlap
•	PE	FITC	16.50
•	PerCP-Cy5-5	FITC	2.00
•	APC	FITC	0.11

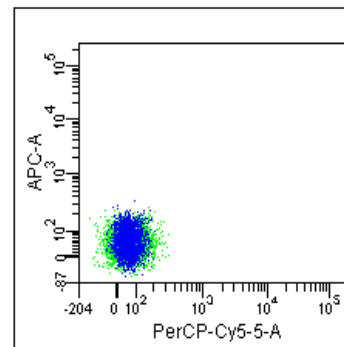
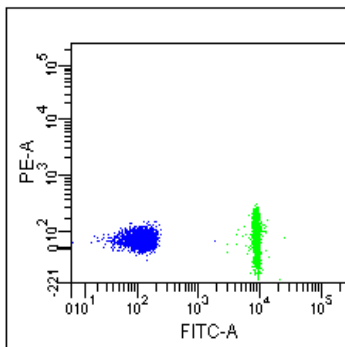
FITC Compensation



Dot plot showing uncompensated FITC data

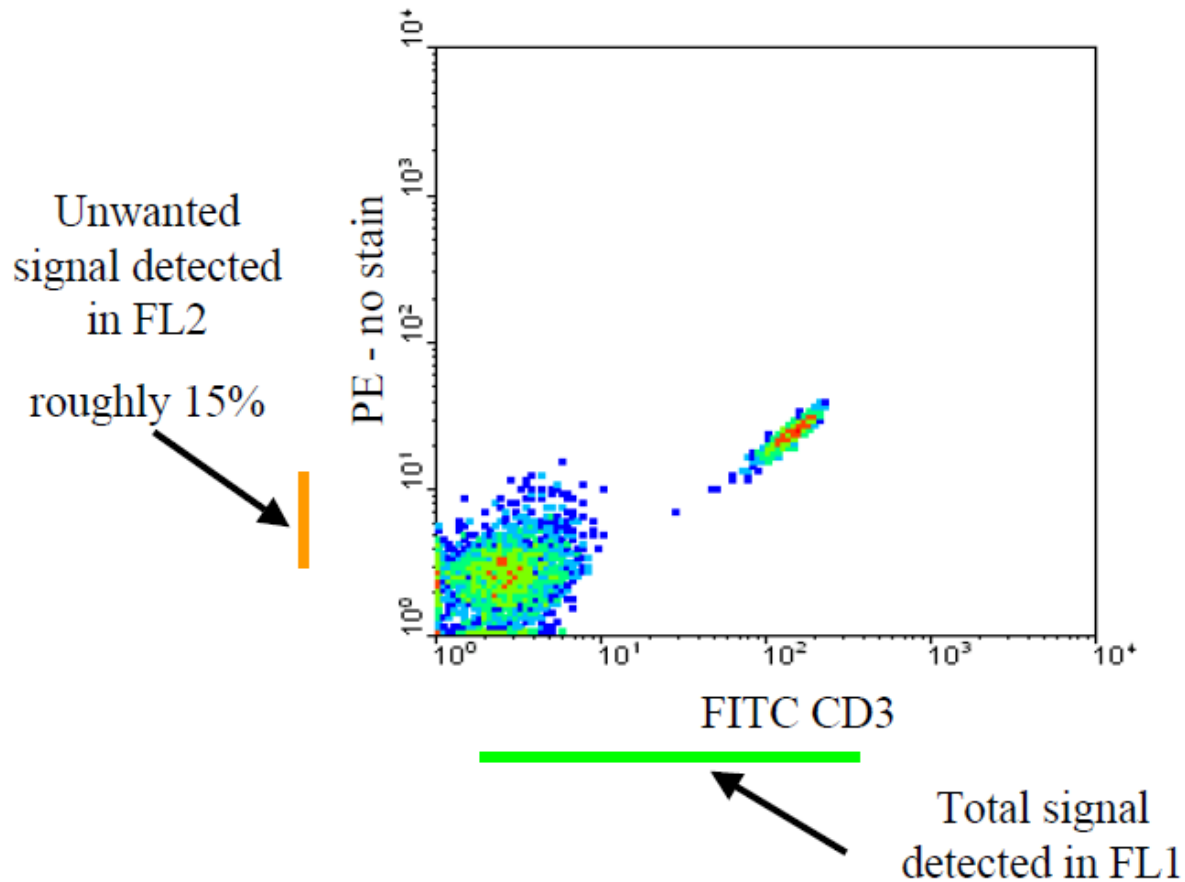


Dot plot showing compensated FITC data



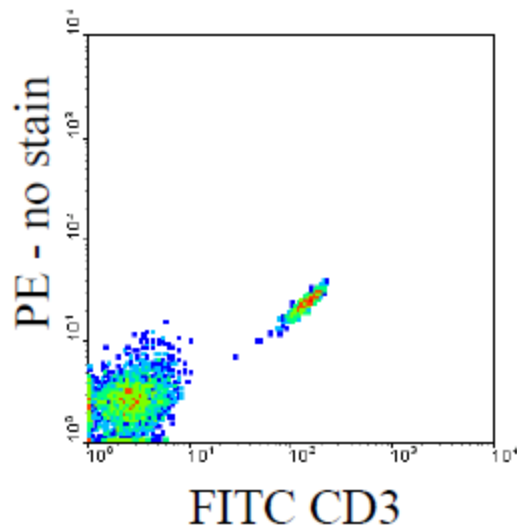
Biexponential dot plot showing compensated FITC data

Uncompensated FITC Single stain Control



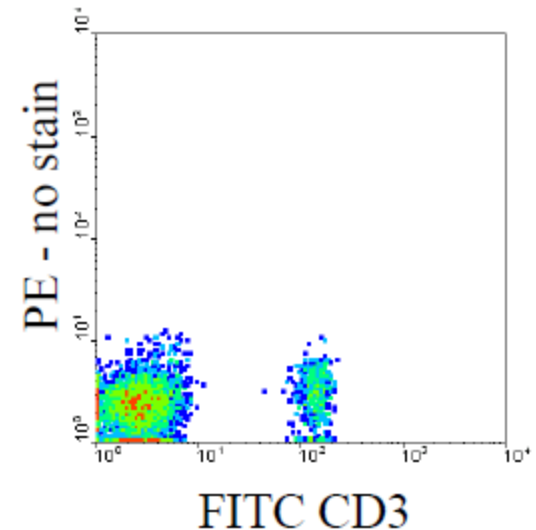
FITC Single Stain Control

Uncompensated

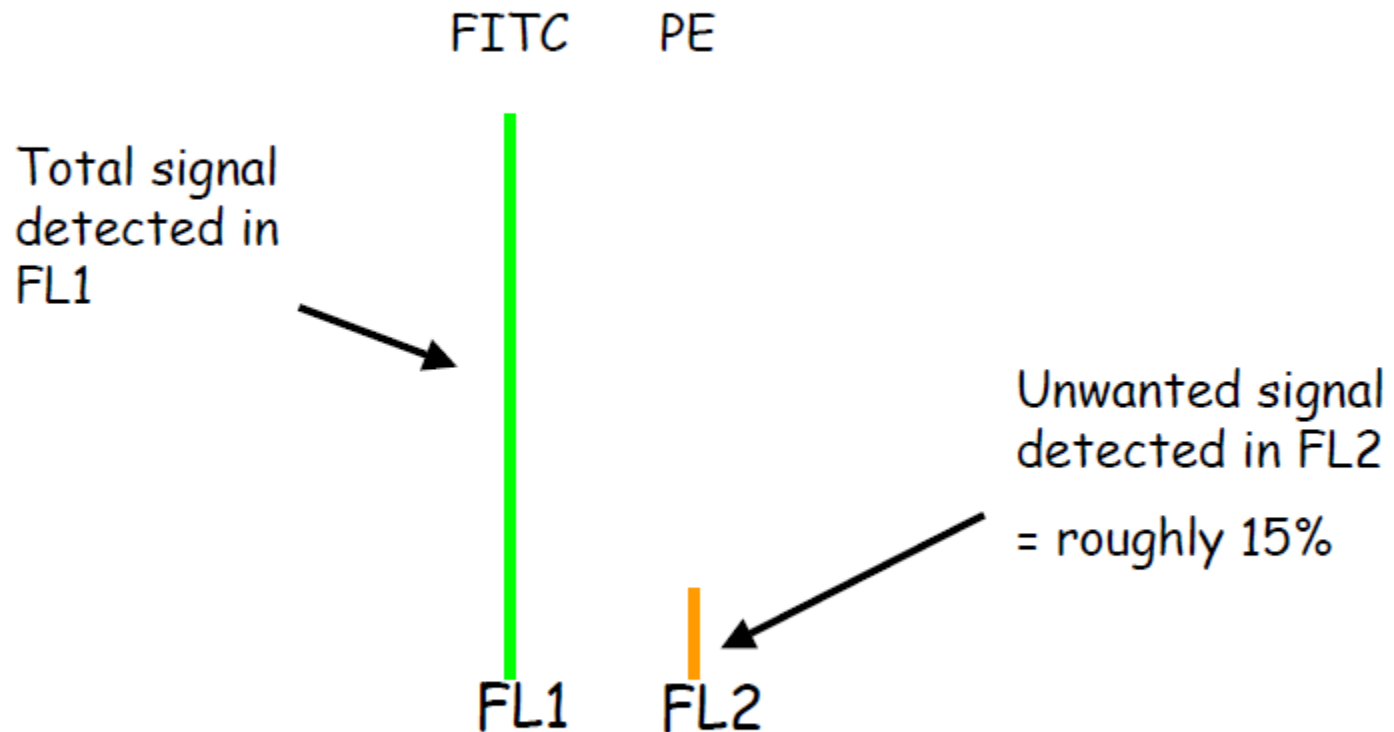


FL2-15%FL1

Compensated



FITC Single Stain Control



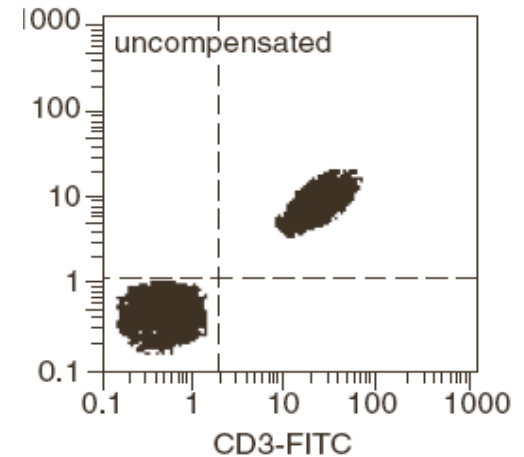
$$\text{True PE} = \text{Total FL2} - 15\% \text{ FL1}$$

Kompenzace fluorescenčního signálu

#2

FITC positive & negative

PE negative beads



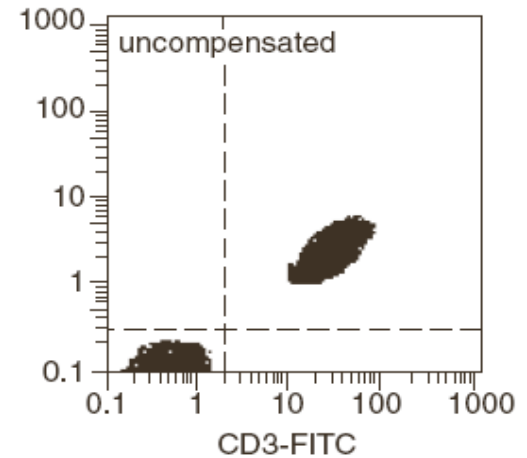
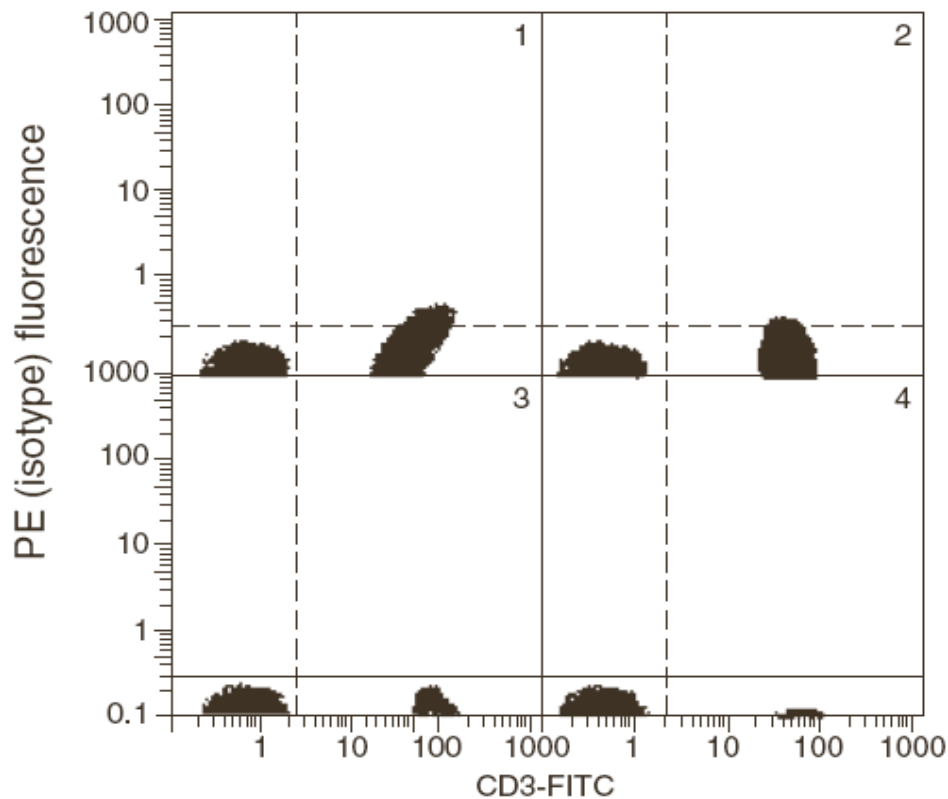
Current Protocols in Cytometry

Kompenzace fluorescenčního signálu

FITC positive & negative

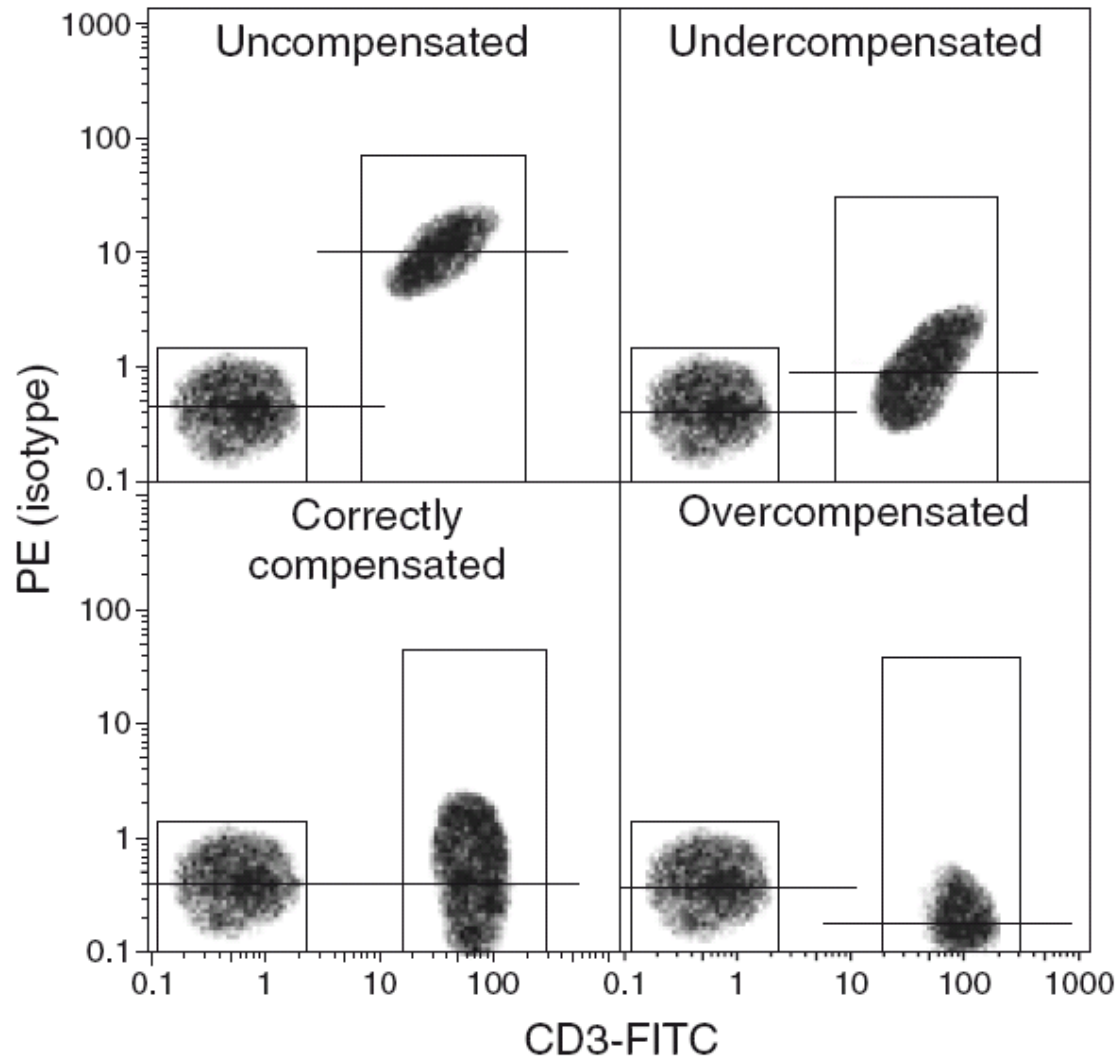
PE negative beads

NONE!



Current Protocols in Cytometry

Kompenzace fluorescenčního signálu



Nastavení kompenzací

- značené mikročástice – pro běžně konjugované fluorochromy



CaliBRITE Beads

CaliBRITE 3 three-color kit–Catalog No. 340486

CaliBRITE two-color kit–Catalog No. 349502

CaliBRITE PerCP Beads–Catalog No. 340497

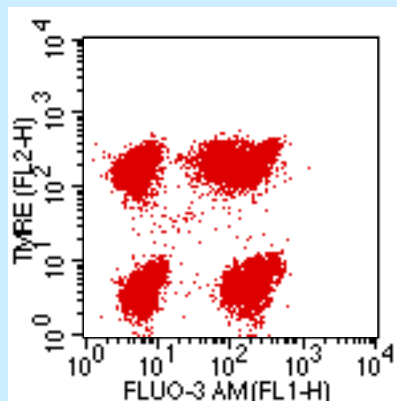
CaliBRITE APC Beads–Catalog No. 340487

CaliBRITE PerCP-Cy5.5 Beads with Bead Dilution Buffer–Catalog No. 345036

For In Vitro Diagnostic Use with FACS brand flow cytometers

Setup	Tube ^a	Unlabeled	FITC	PE	PerCP or PerCP-Cy5.5 ^b	APC
two-color	A	1 drop				
	B	1 drop	1 drop	1 drop		
three-color	A	1 drop				
	B	1 drop	1 drop	1 drop	1 drop	
four-color	A	1 drop				1 drop
	B	1 drop	1 drop	1 drop	1 drop	1 drop

- značené buňky – pro vitální značení



parametr - detektor amp.

FL1 - 544

FL2 - 434

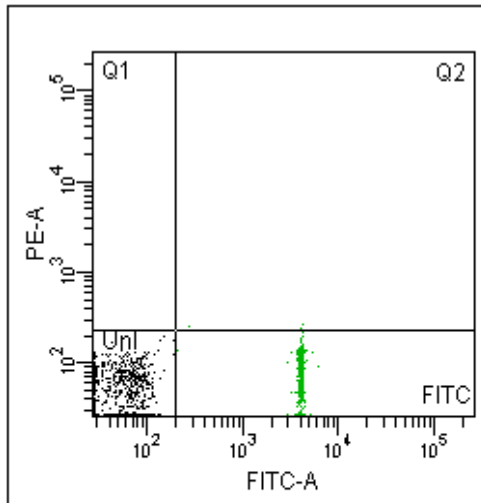
kompenzace

FL1 - 1.1%FL2

FL2 - 17.5%FL1

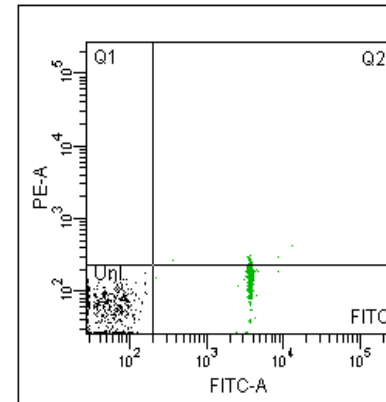
Effects of Changing PMT Values

Correct Compensation



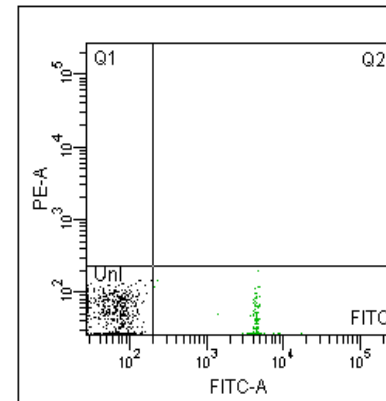
Population	PE-A Mean
Unl	69
FITC	64

FITC Voltage Decreased by 5 V



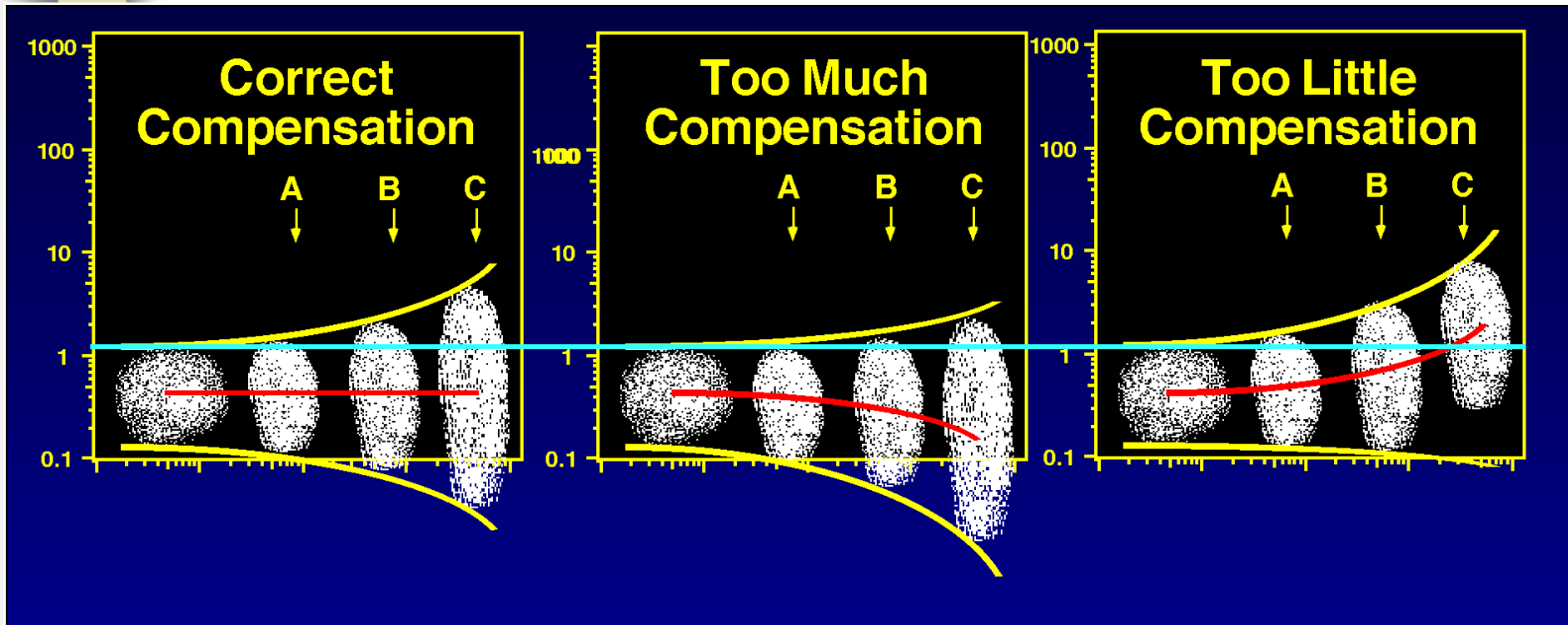
Population	PE-A Mean
Unl	68
FITC	132

FITC Voltage Increased by 5 V



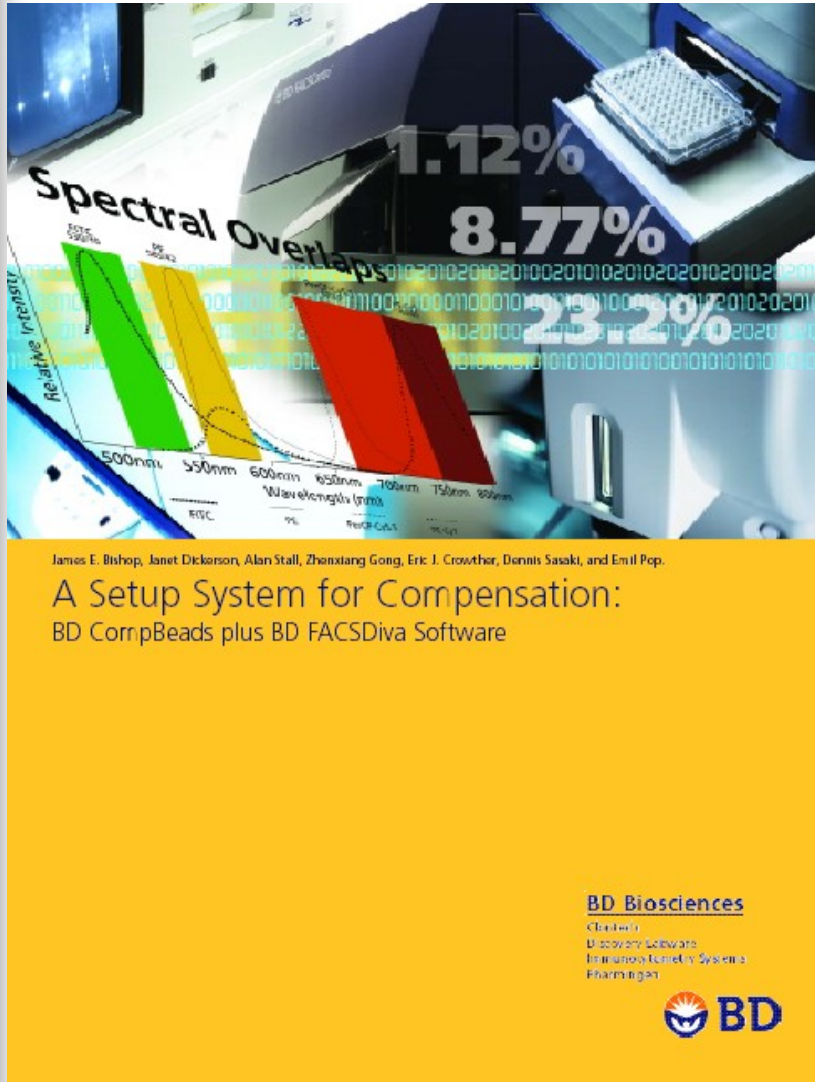
Population	PE-A Mean
Unl	67
FITC	49

Which marker for compensation?



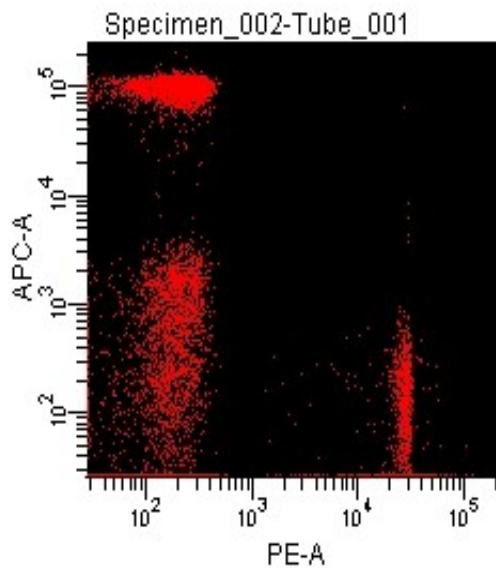
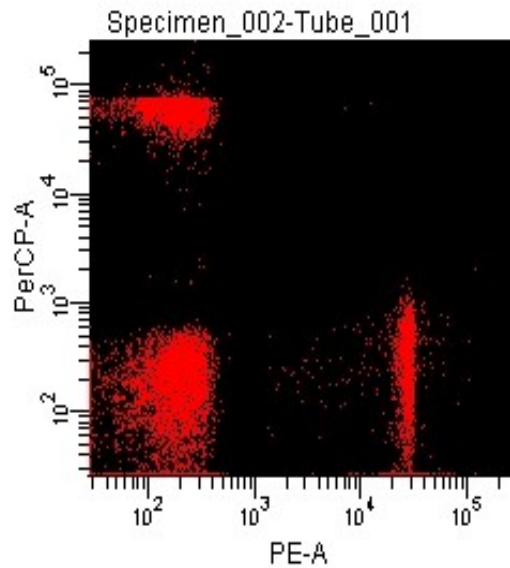
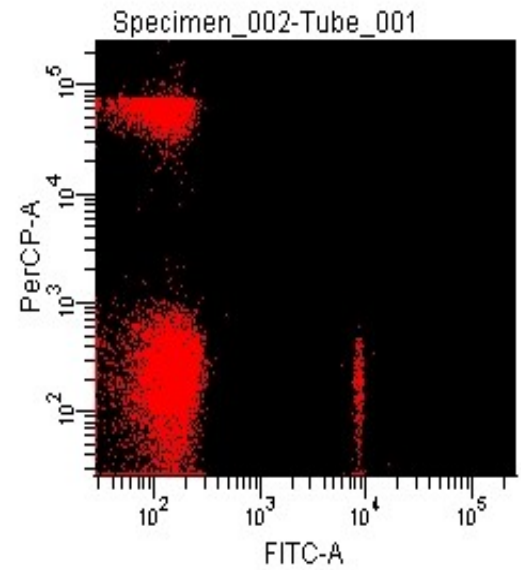
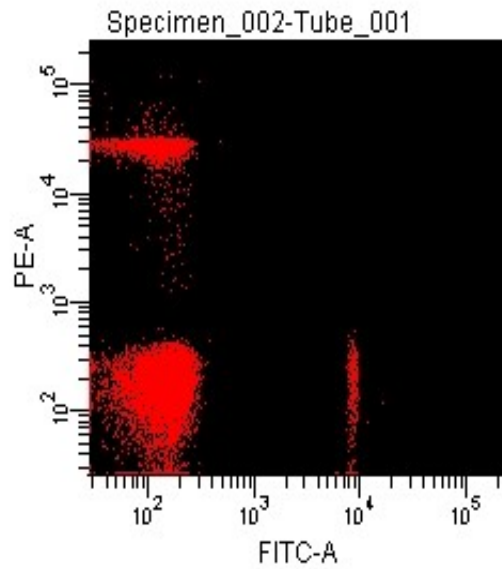
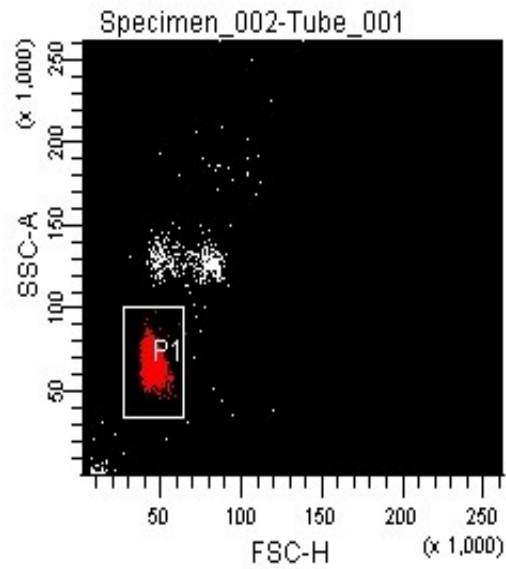
Small errors in compensation of a dim control (A) can result in large compensation errors with bright reagents (B & C).
Use bright markers to setup proper compensation.

BD Comp Beads



- Always positive
- Bright staining
- Save sample (HIV patients)
- Use the same antibody for compensation and the real experiment

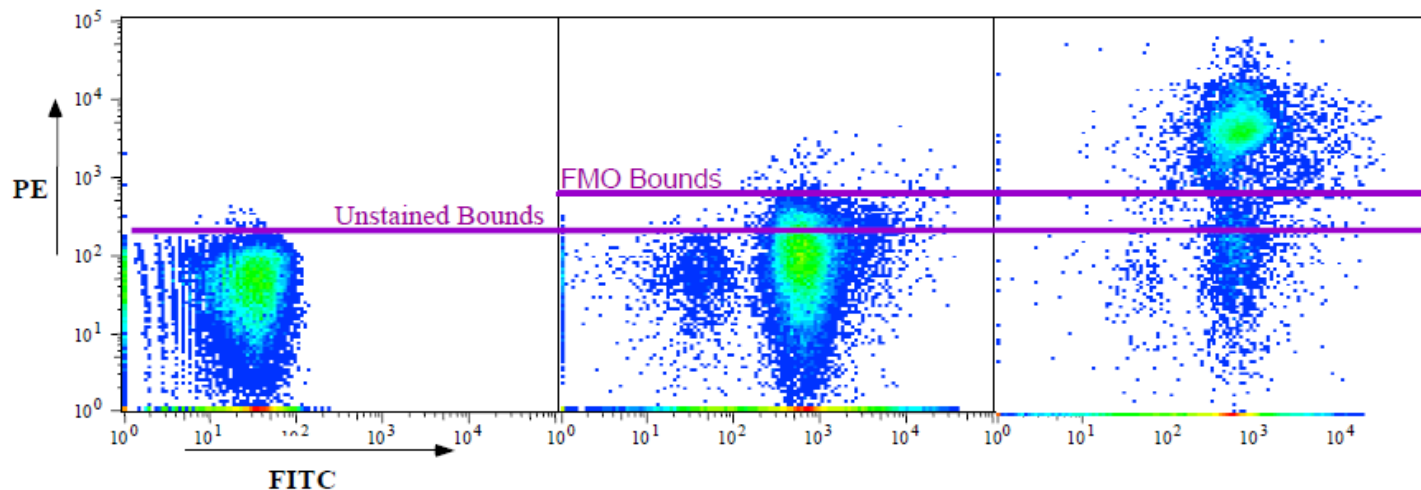
BD Comp Beads



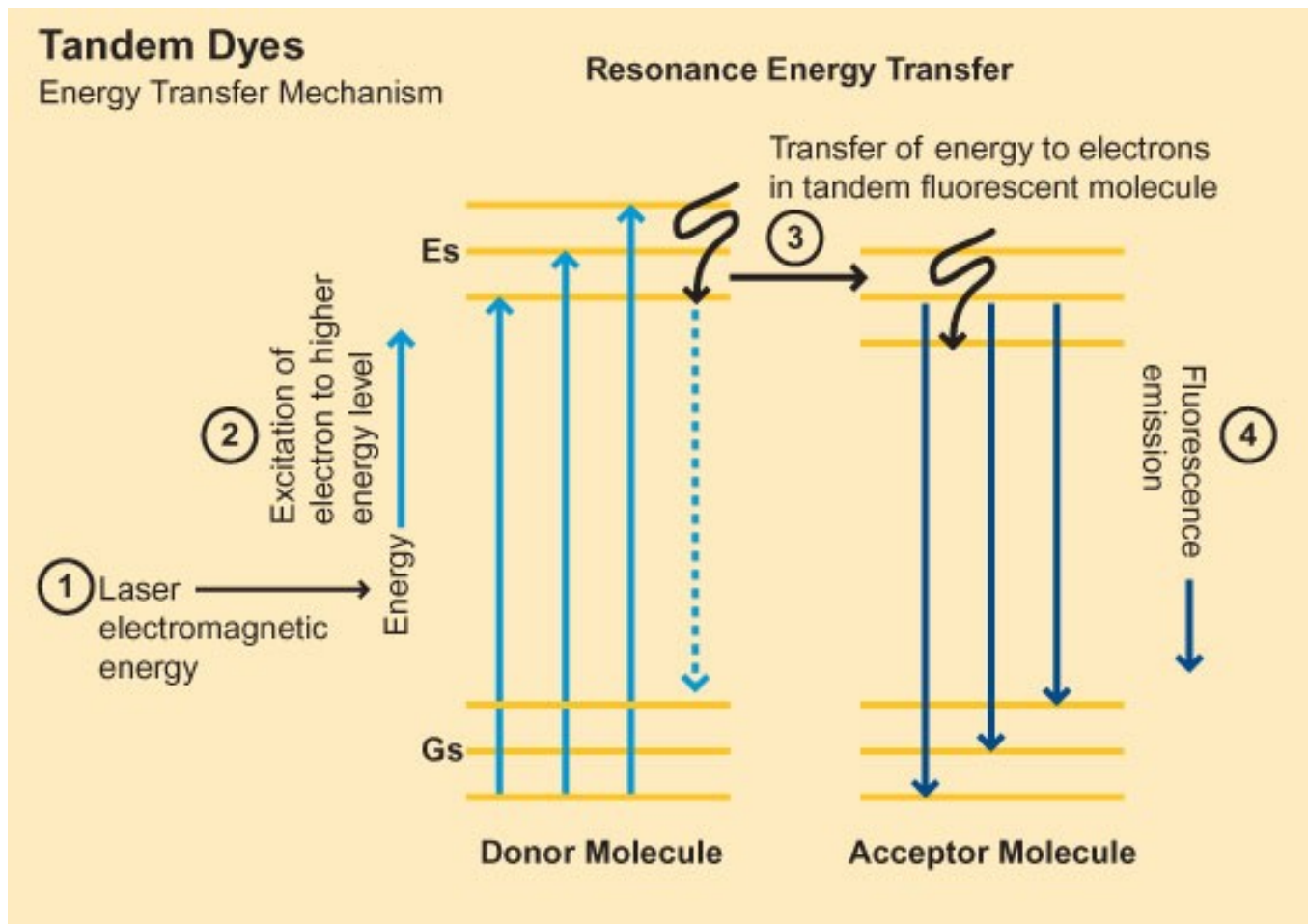
Fluorescence Minus One

PBMC were stained as shown in a 3-color experiment. Compensation was properly set for all spillovers

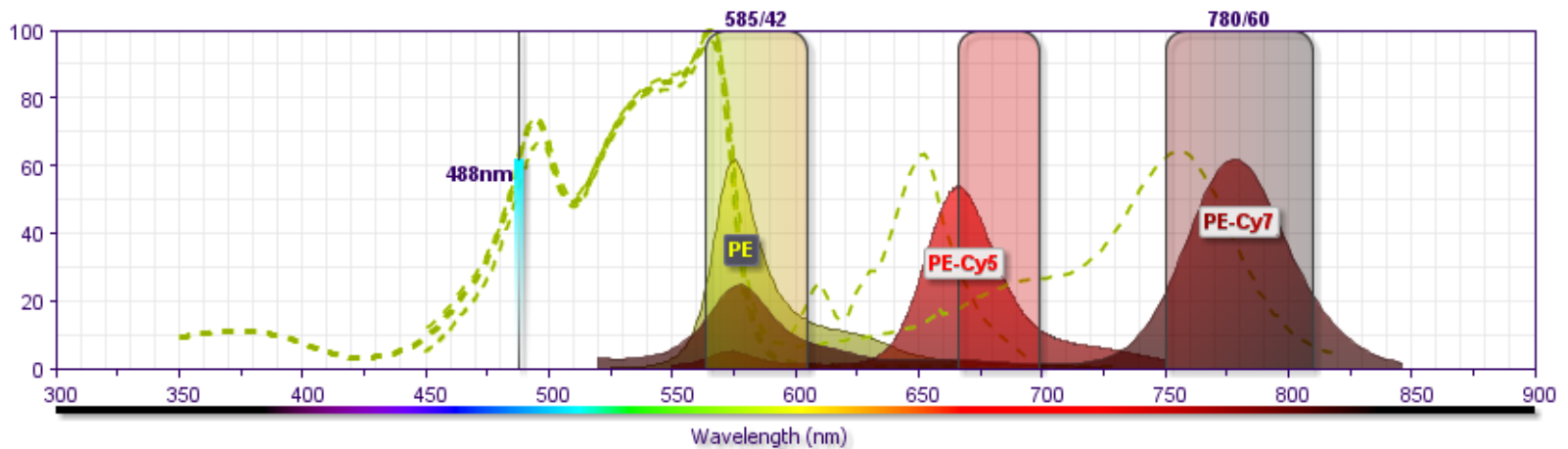
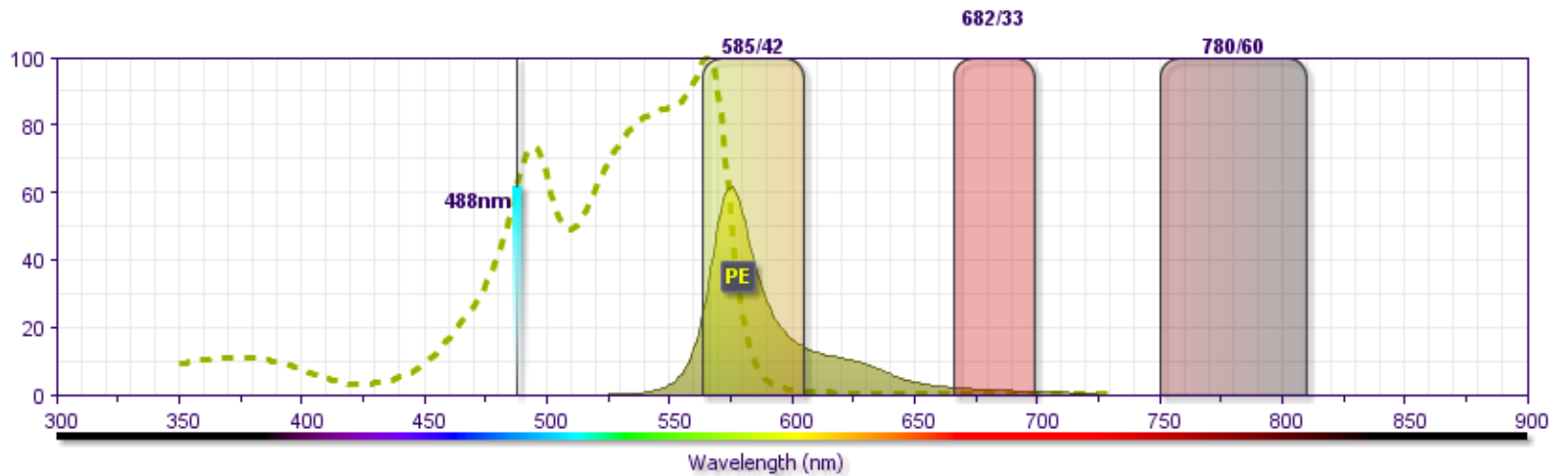
	Unstained Control	FMO Control	Fully Stained
FITC	-	CD3	CD3
PE	-	-	CD4
Cy5PE	-	CD8	CD8



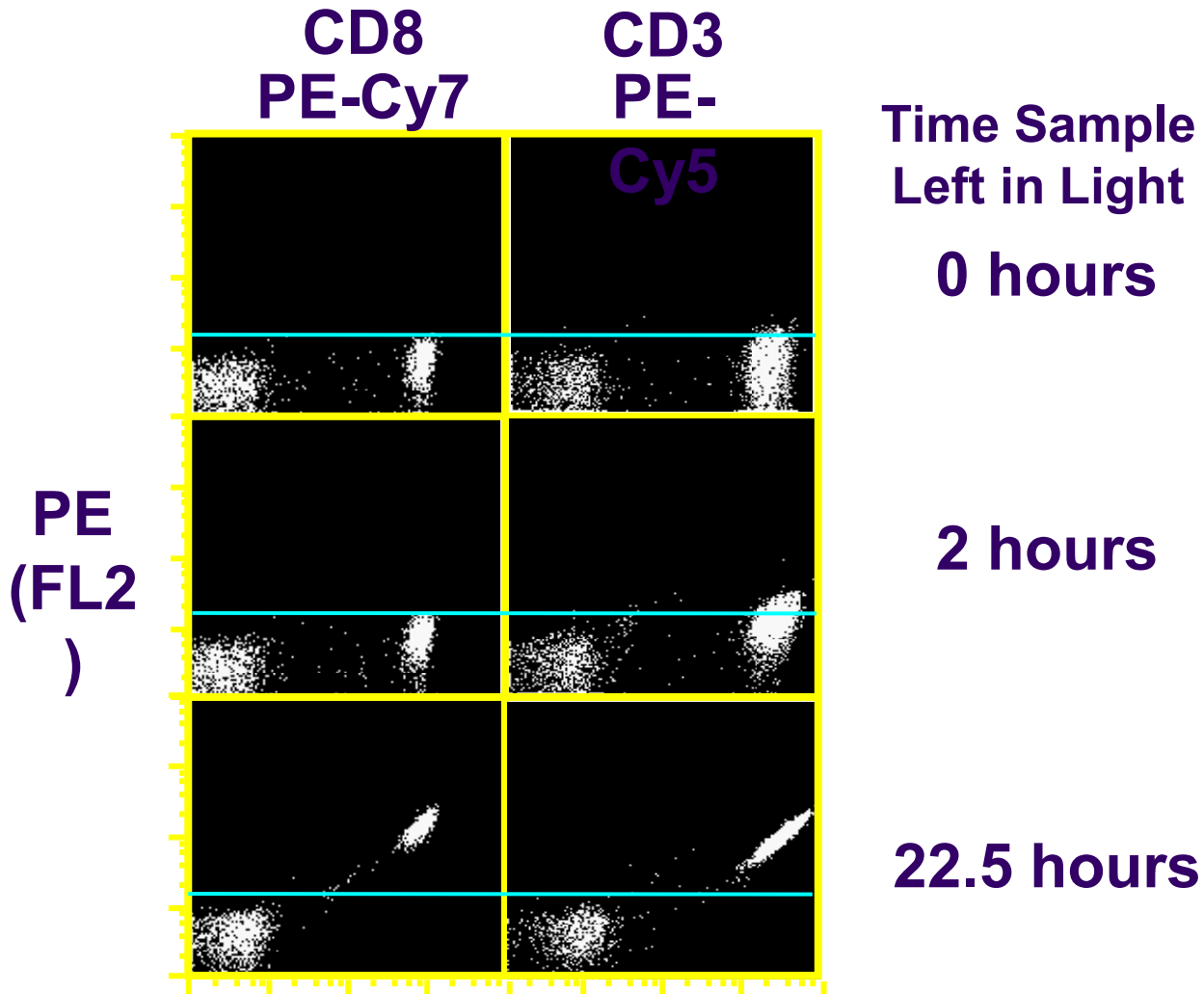
Tandemové značky



Tandemové značky - příklad



Tandems are light sensitive





Kompenzace - literatura

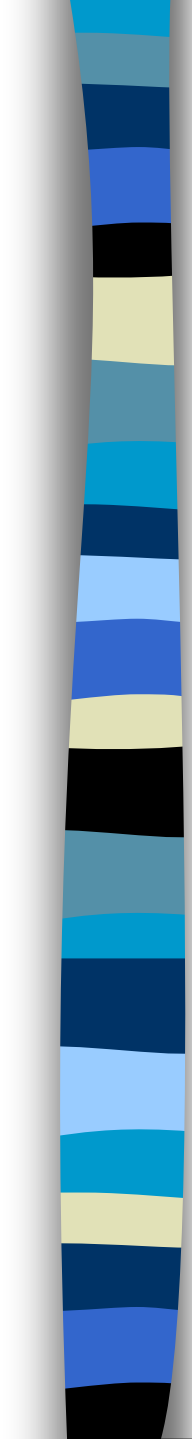
Mario Roederer - Compensation in Flow Cytometry
Current Protocols in Cytometry (2002) 1.14.1-1.14.20 John Wiley & Sons, Inc.

M. Loken, D. R. Parks, & L. A. Herzenberg (1977). Two-color immunofluorescence using a fluorescence-activated cell sorter. *J. Histochem. Cytochem.* **25**:899-907.

M. Roederer & R. F. Murphy (1986). Cell-by-cell autofluorescence correction for low signal-to-noise systems: application to EGF endocytosis by 3T3 fibroblasts. *Cytometry* **7**:558-565.

S. Alberti, D. R. Parks, & L. A. Herzenberg (1987). A single laser method for subtraction of cell autofluorescence in flow cytometry. *Cytometry* **8**:114-119.

C. B. Bagwell & E. G. Adams (1993). Fluorescence spectral overlap compensation for any number of flow cytometry parameters. *in: Annals of the New York Academy of Sciences*, **677**:167-184.



*No Data Analysis
Technique Can Make
Good Data Out of
Bad Data!*

Shapiro's 7th Law of Flow Cytometry



Shrnutí

- sorting
- zpracování signálu
- vizualizace dat a „gating“
- kompenzace

Na konci dnešní přednášky byste měli:

1. Znat základní principu sortování,
2. popsat způsob zpracování signálu,
3. rozumět lin / log zesílení signálu,
4. rozeznat jednotlivé způsoby vizualizace dat,
5. chápat základní principy „gatingu“,
6. znát princip kompenzace signálu při vícebarevné detekci.