Bi9393 Analytická cytometrie
Lekce 3

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Oddělení cytokinetiky
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Principy průtokové cytometrie a sortrování

- sorting
- zpracování signálu
- analýza dat
- kompenzace signálu

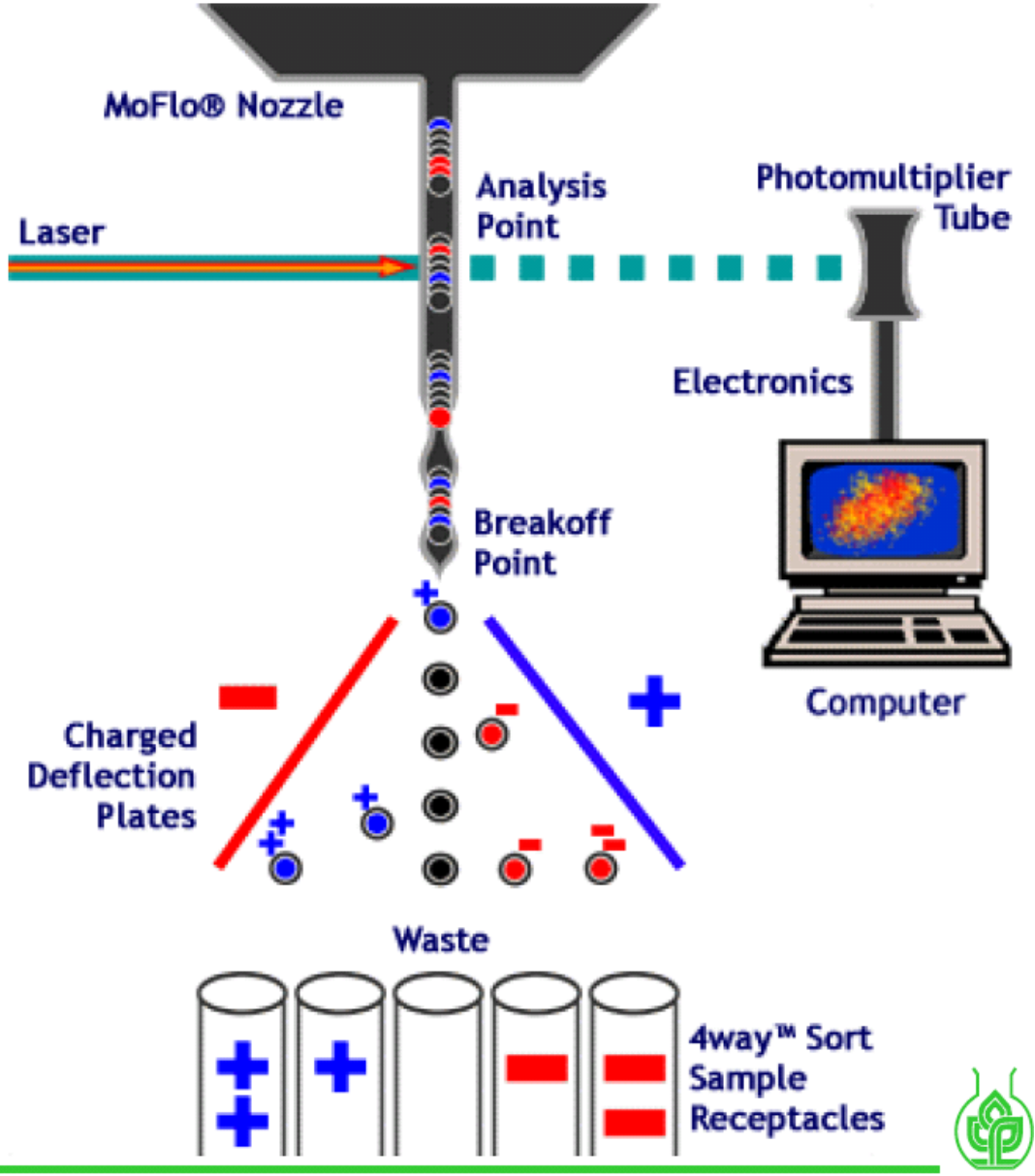
Doležel (1999)

ELECTROSTATIC DROPLET SORTER

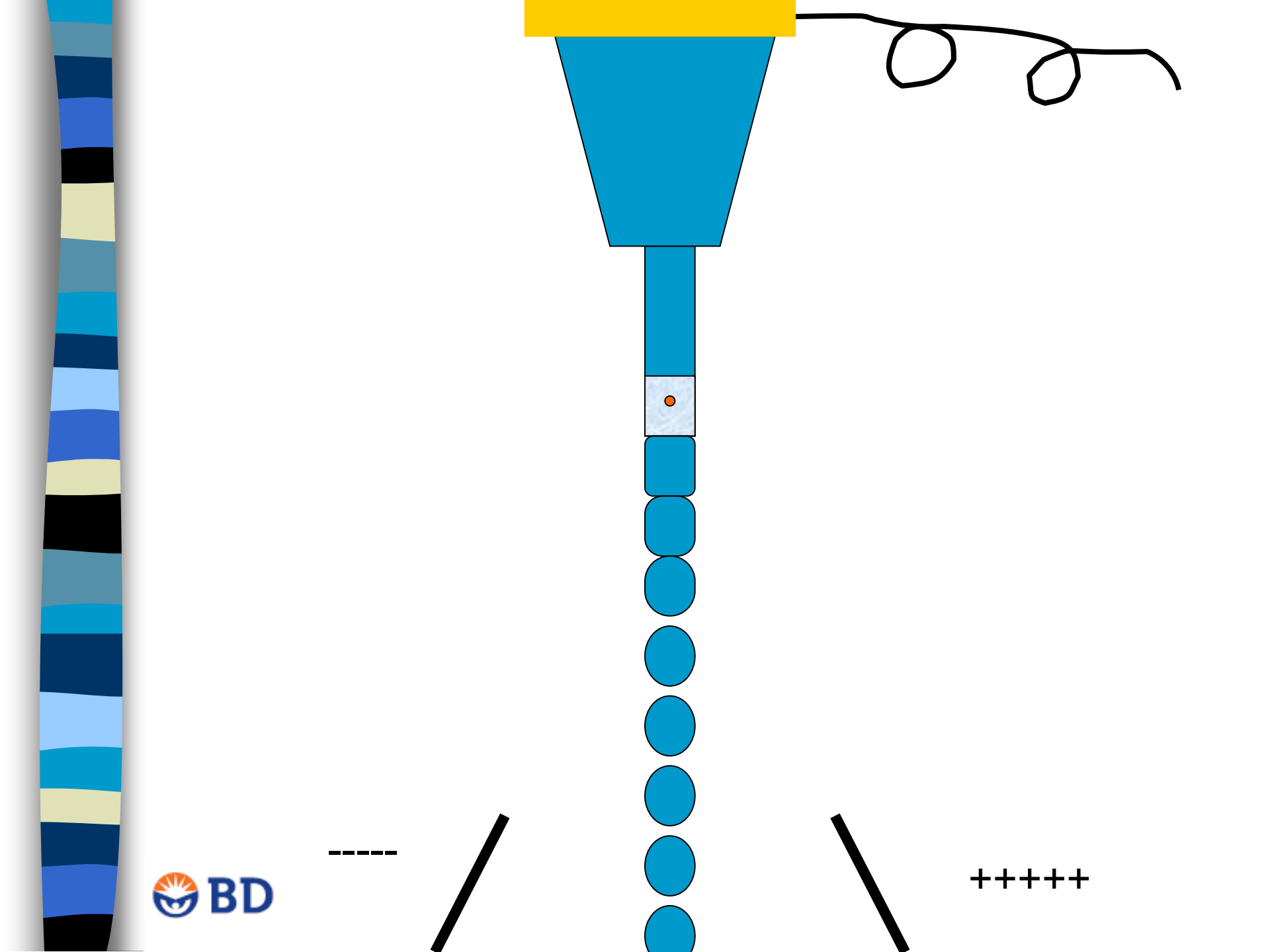
- High speed (~10^4/sec)
- Concentrated sorted fraction

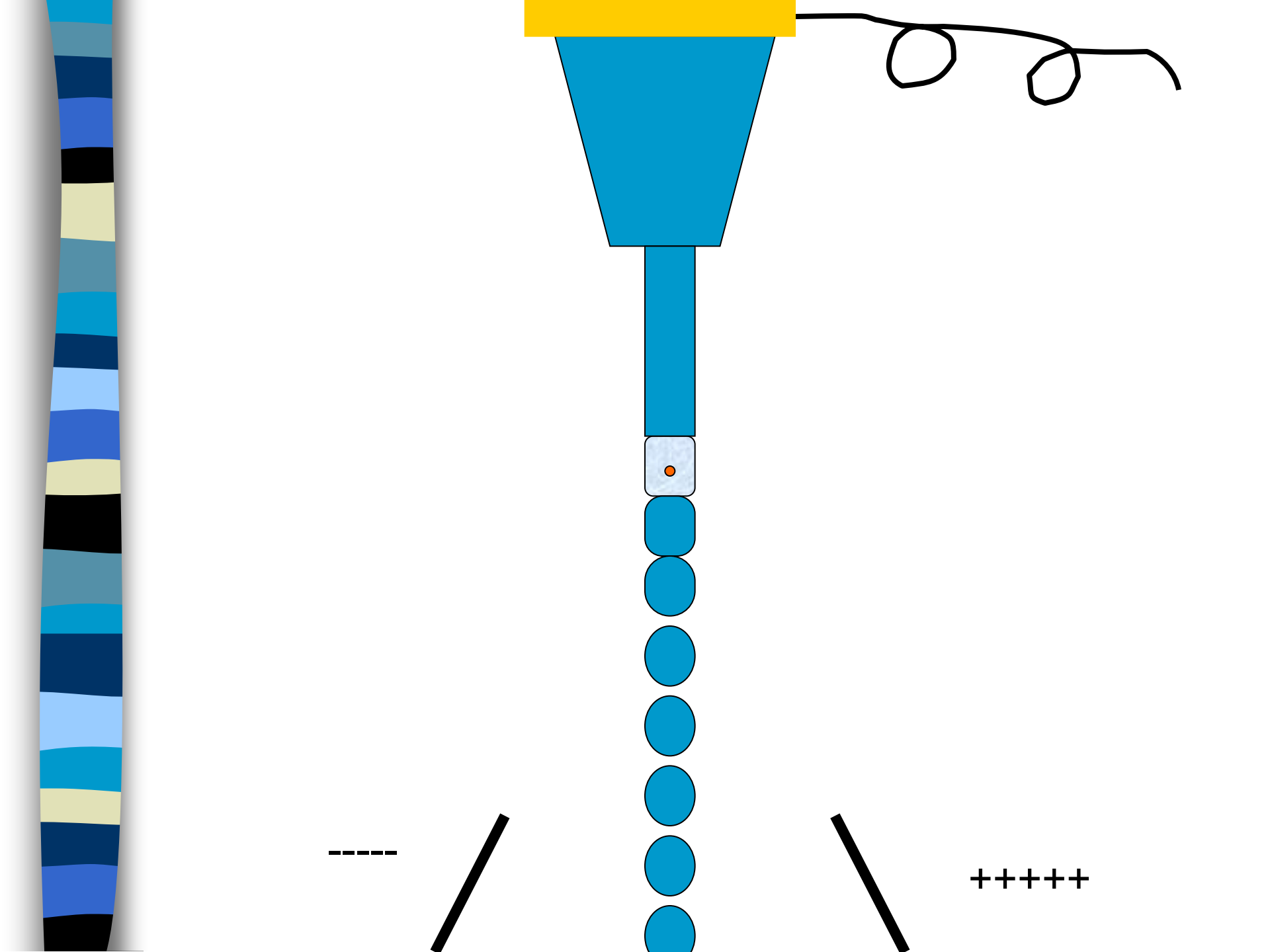
- Biosafefy hazard
- Mechanical shearing
- Problems to sort large particles

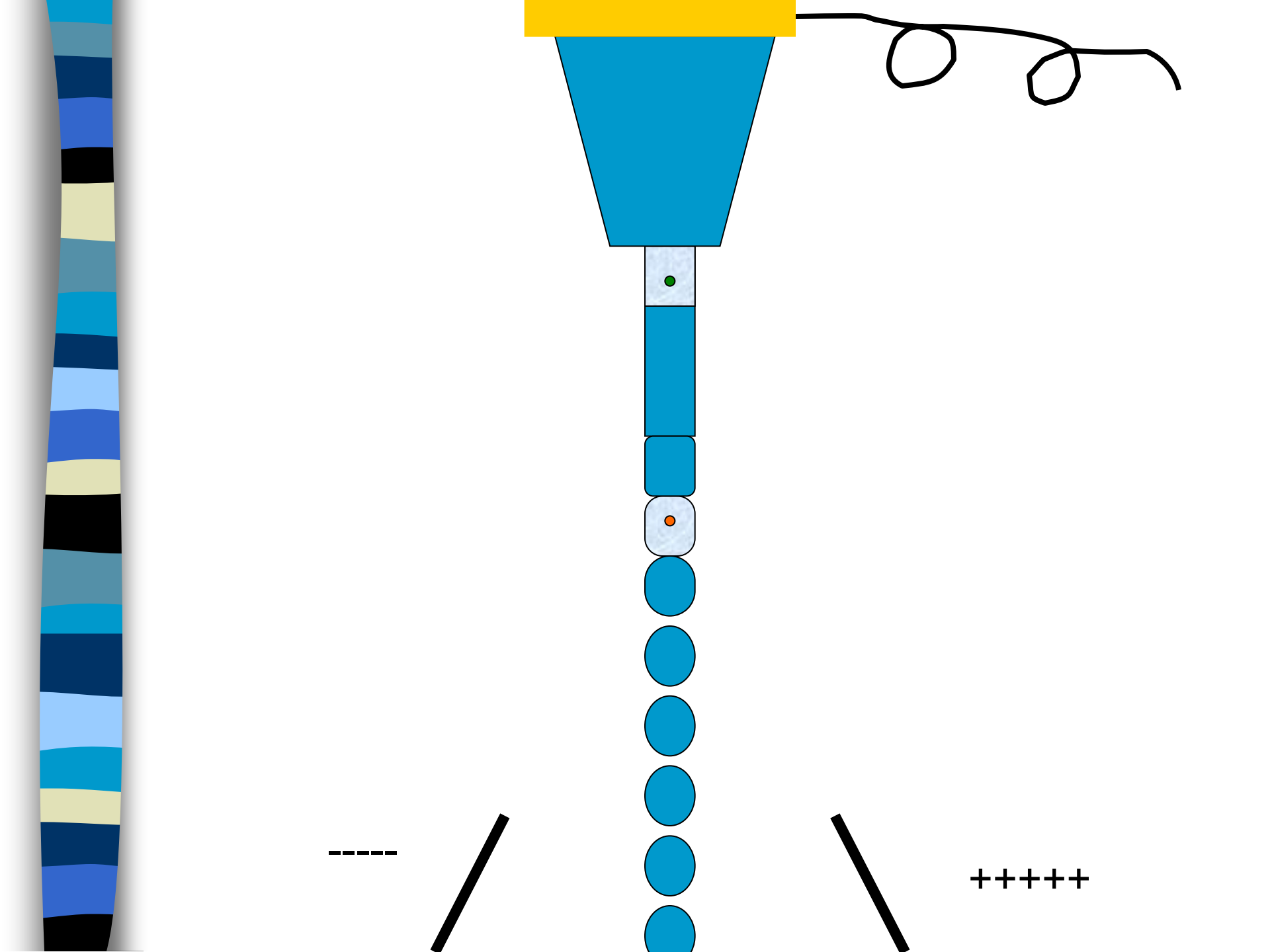
Used by:
Becton Dickinson
Beckman Coulter
Cytomation

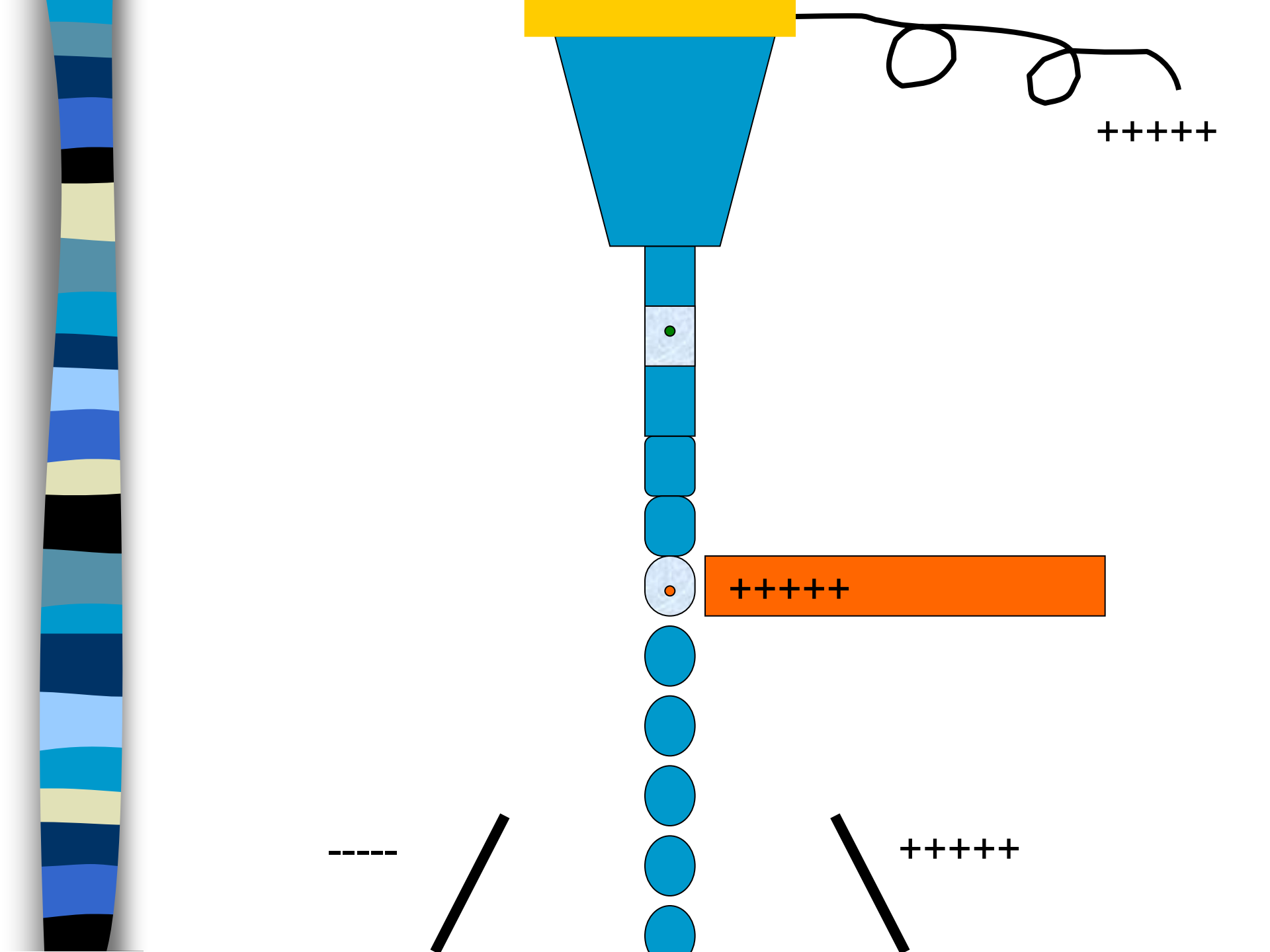


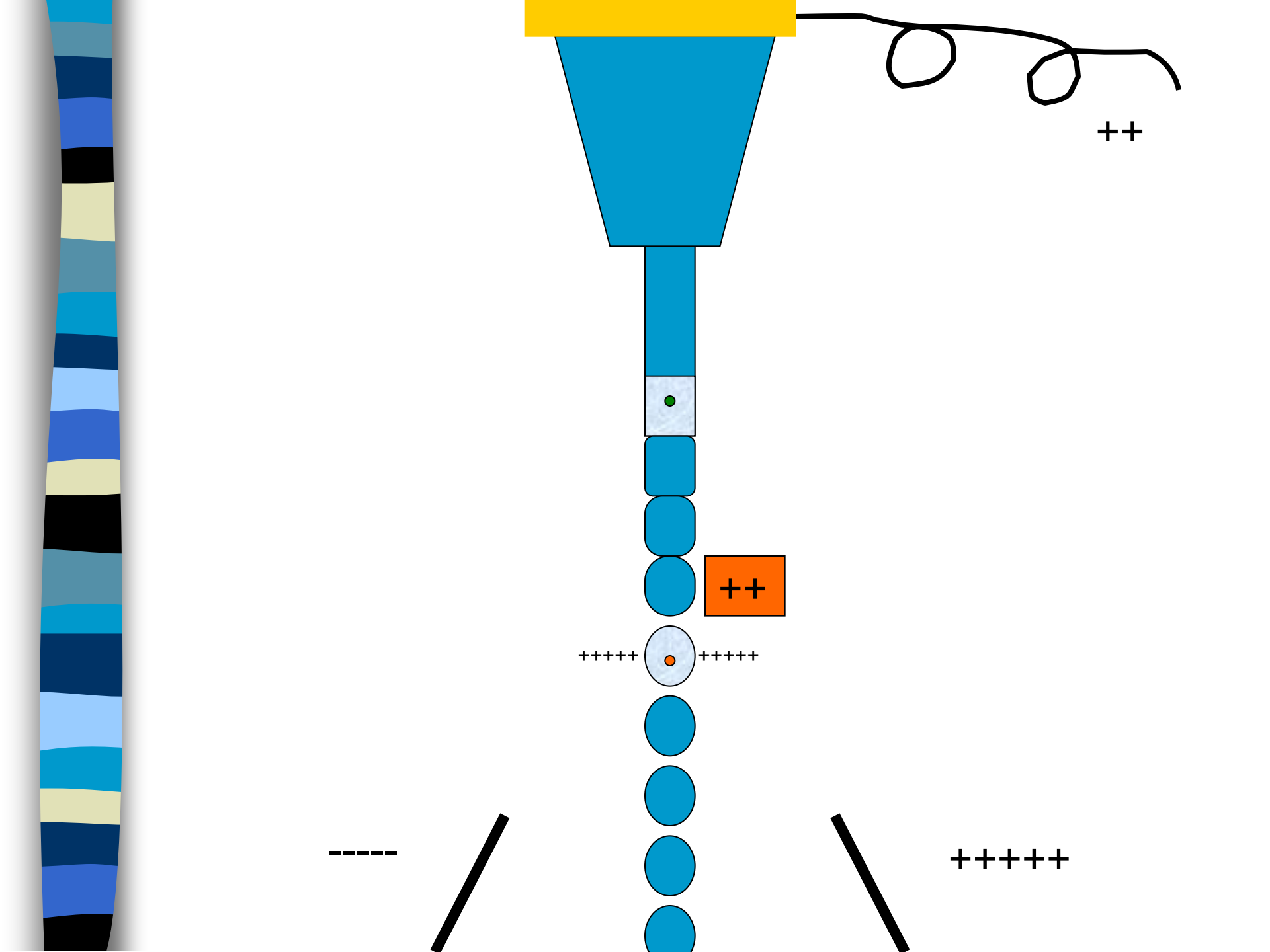
Doležel (1999)

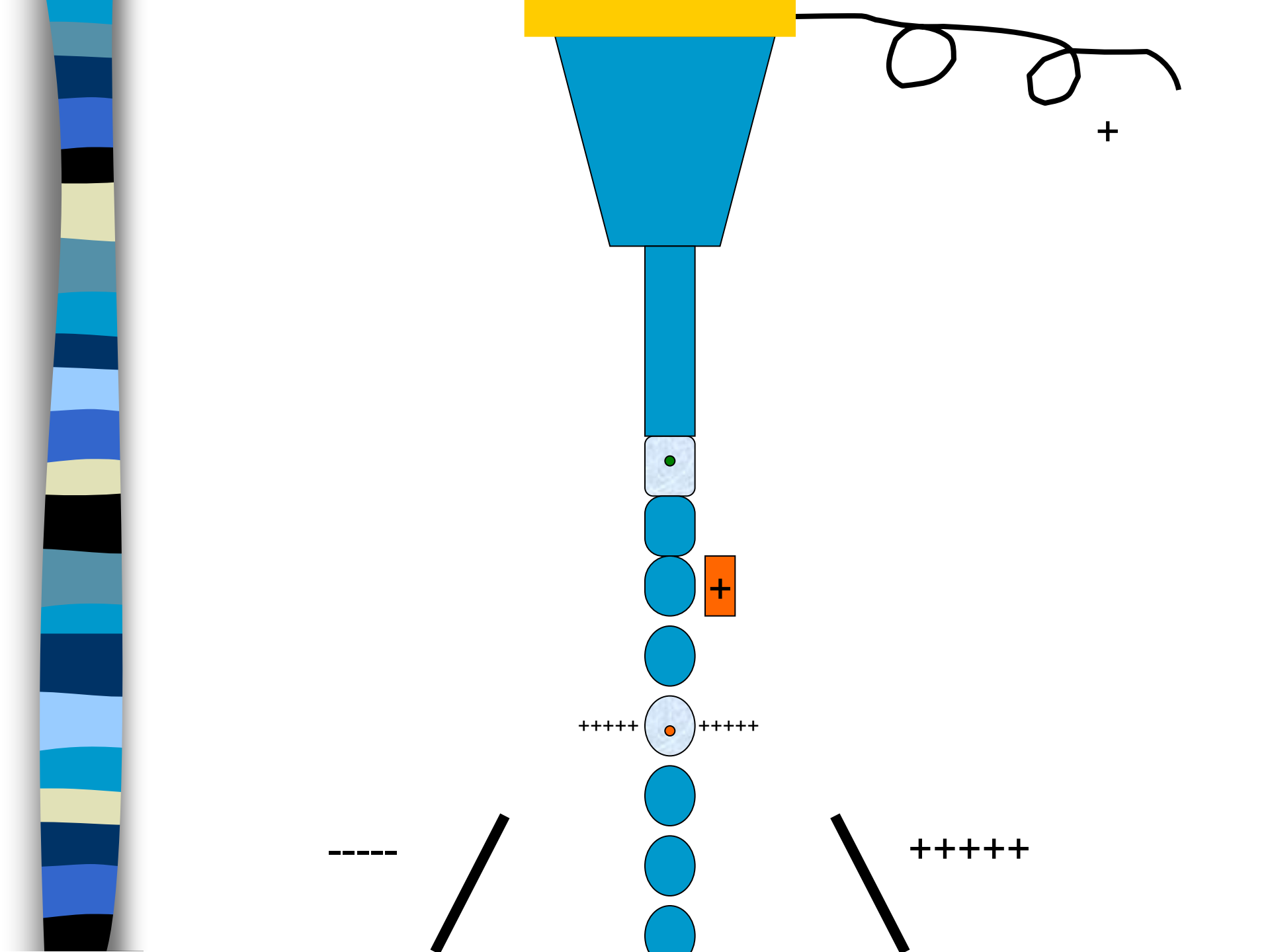


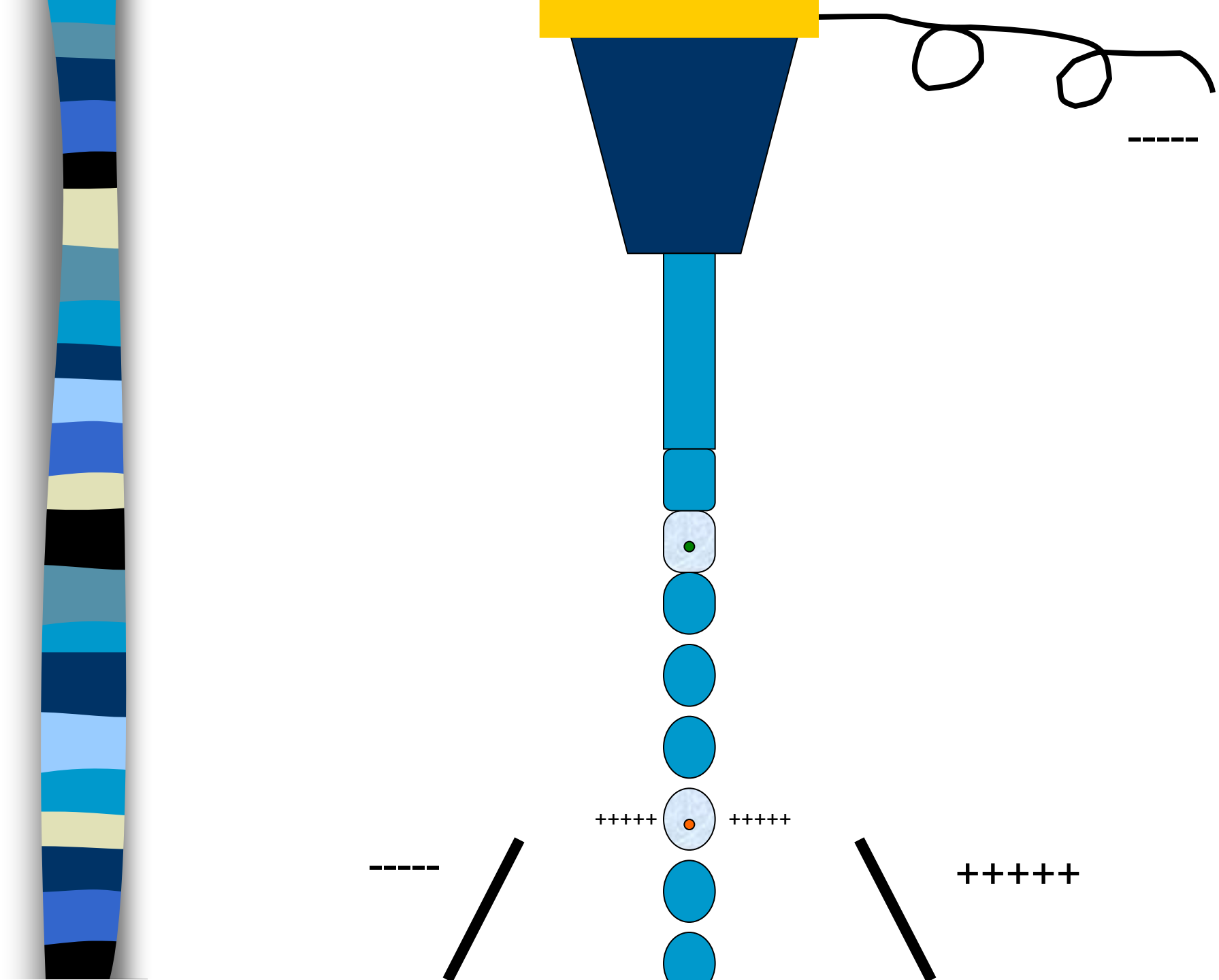


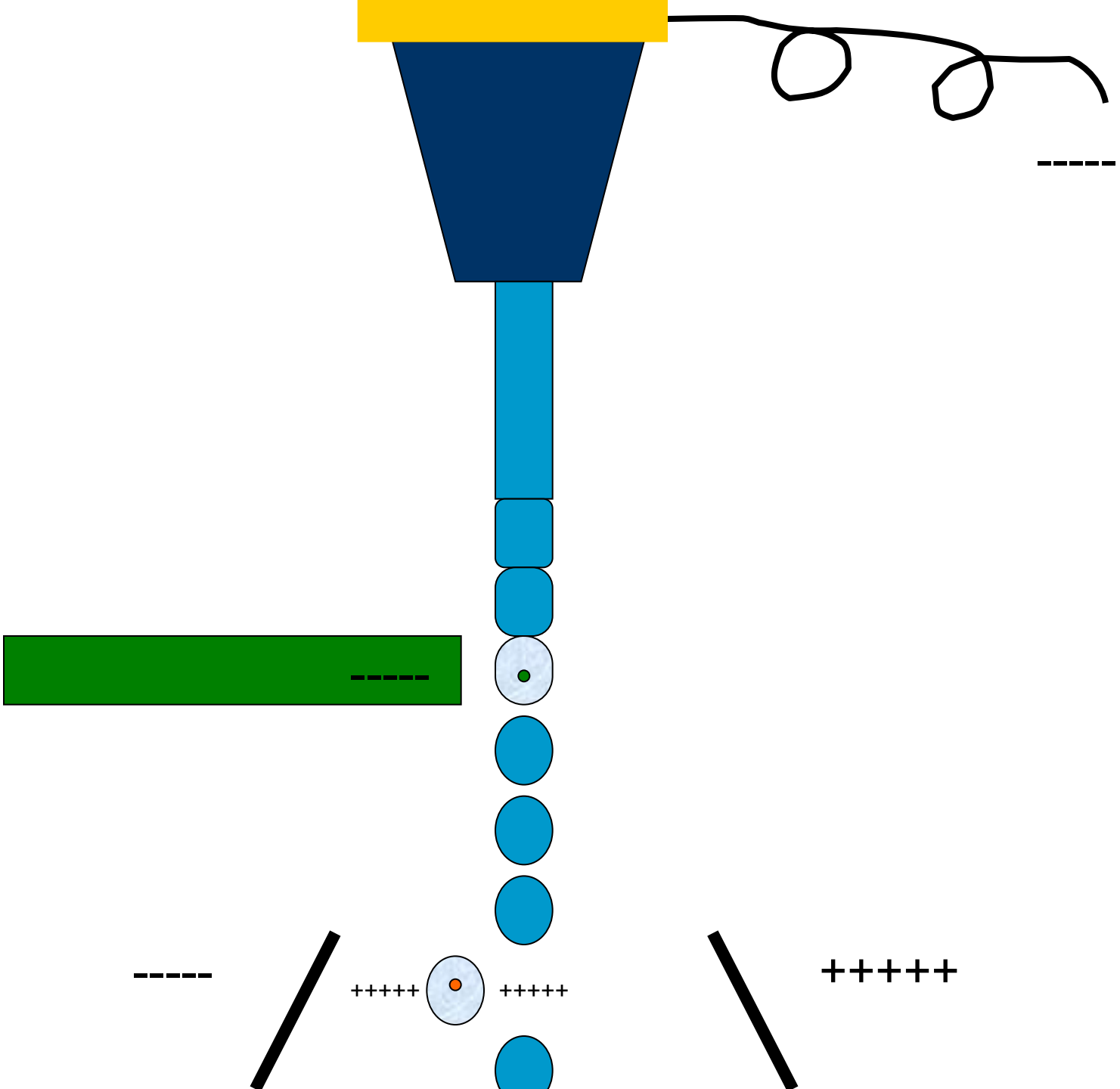


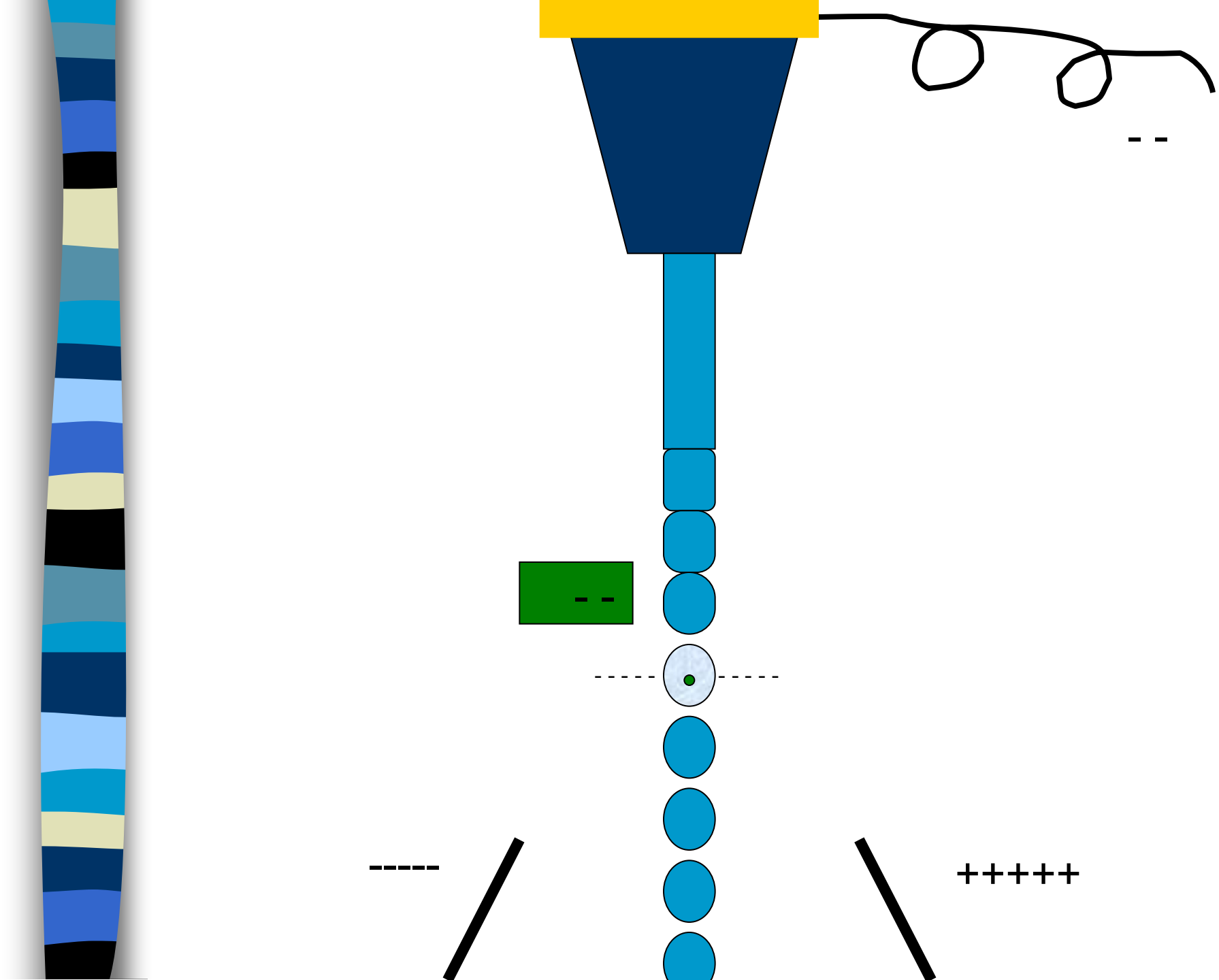


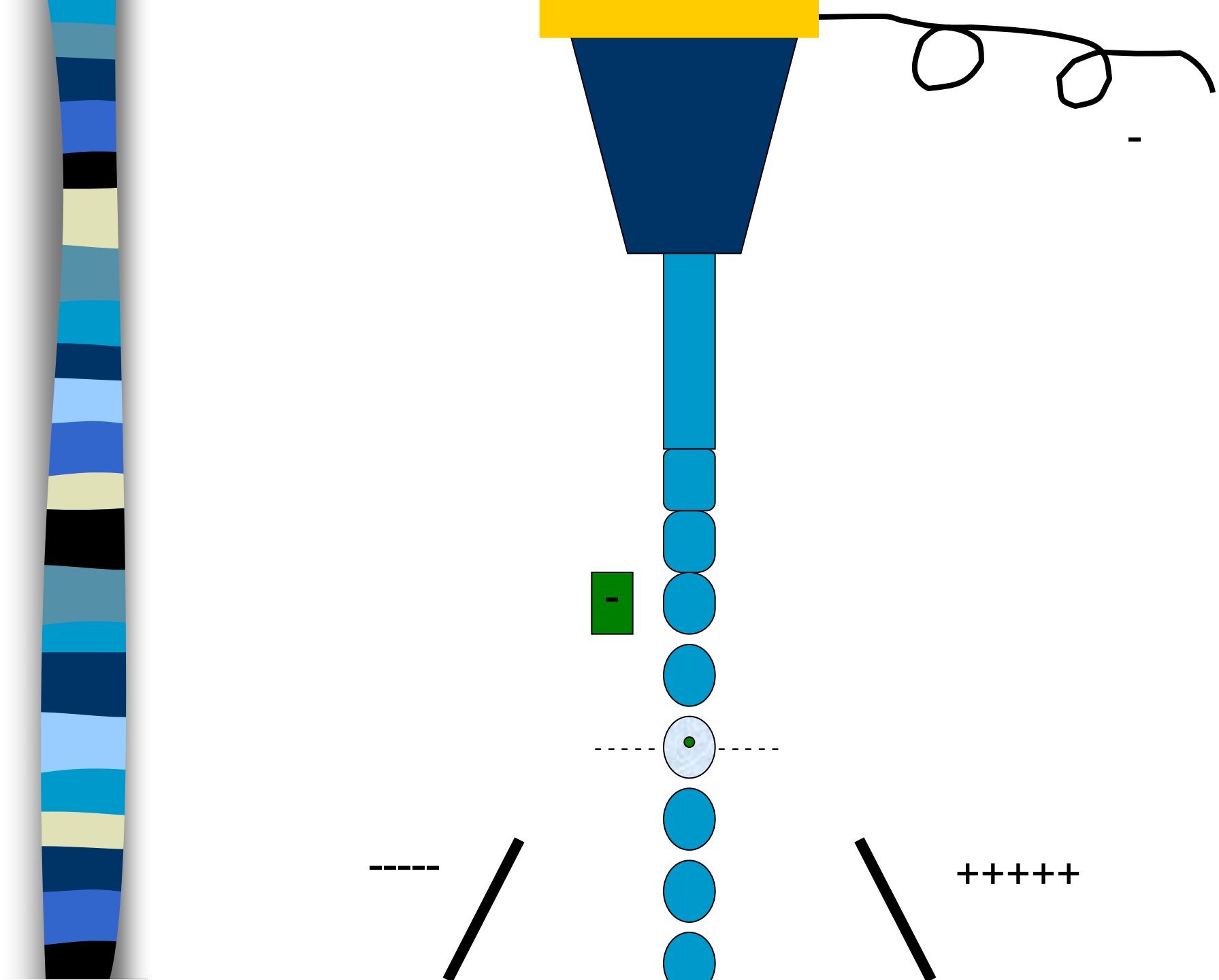


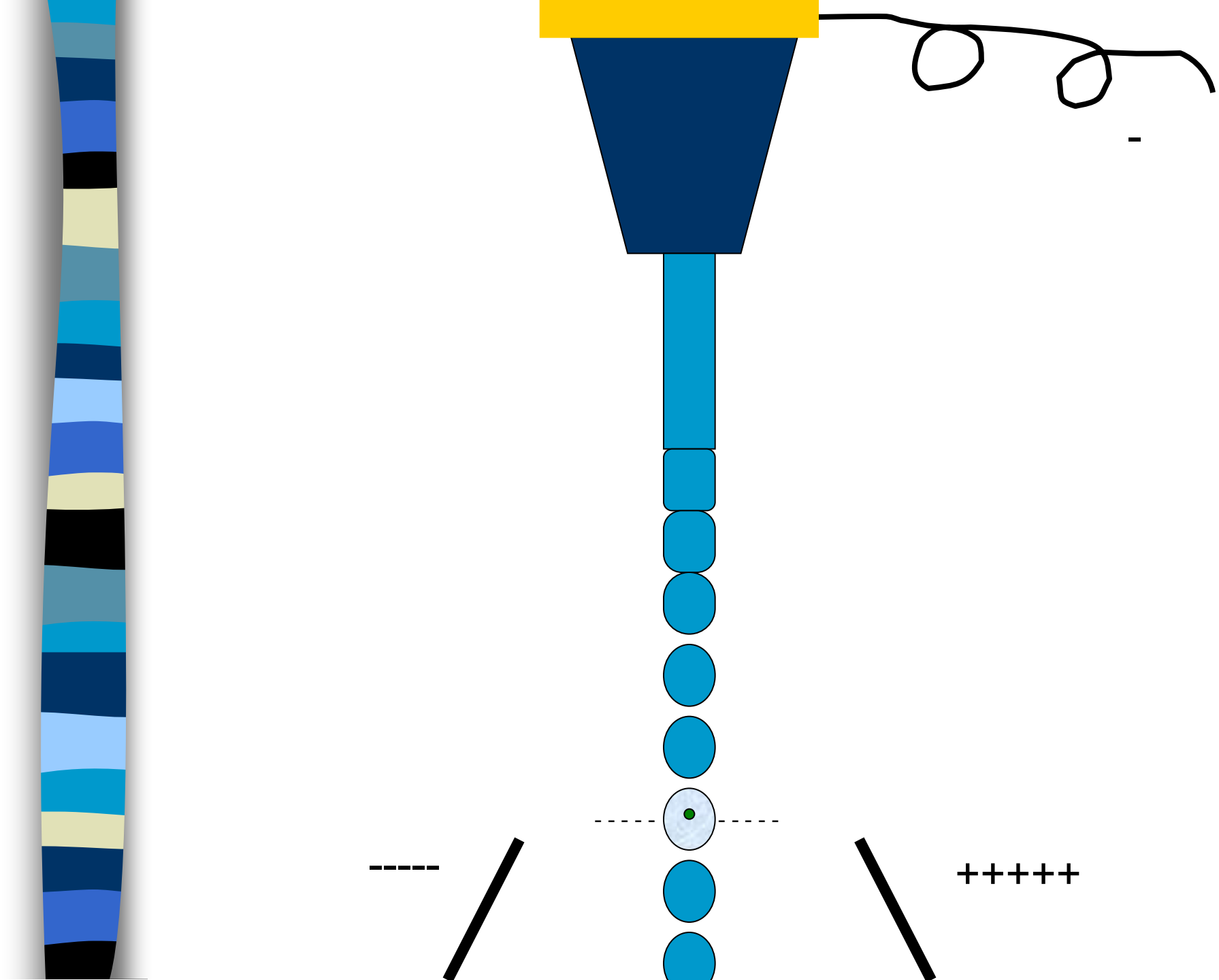


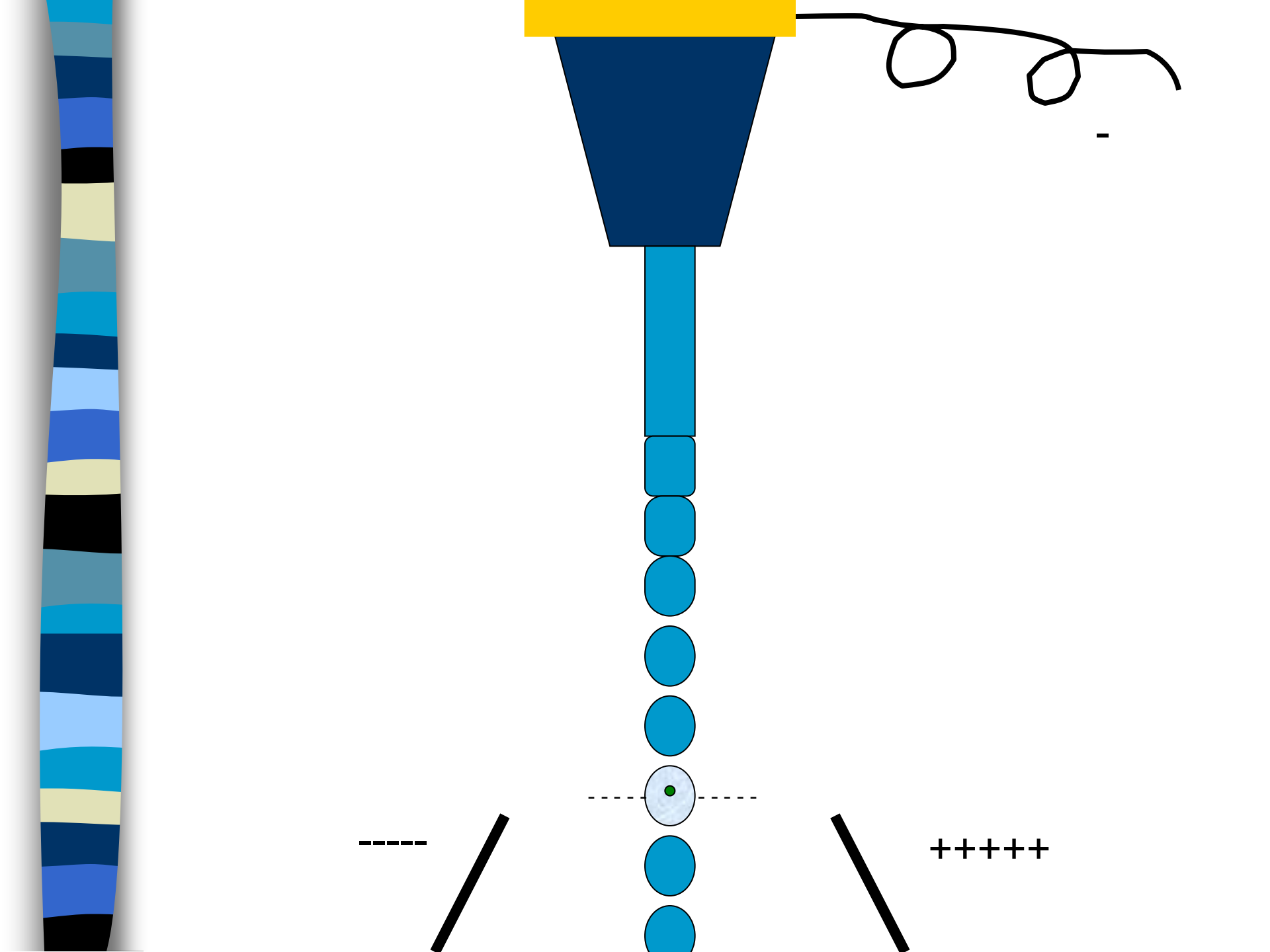


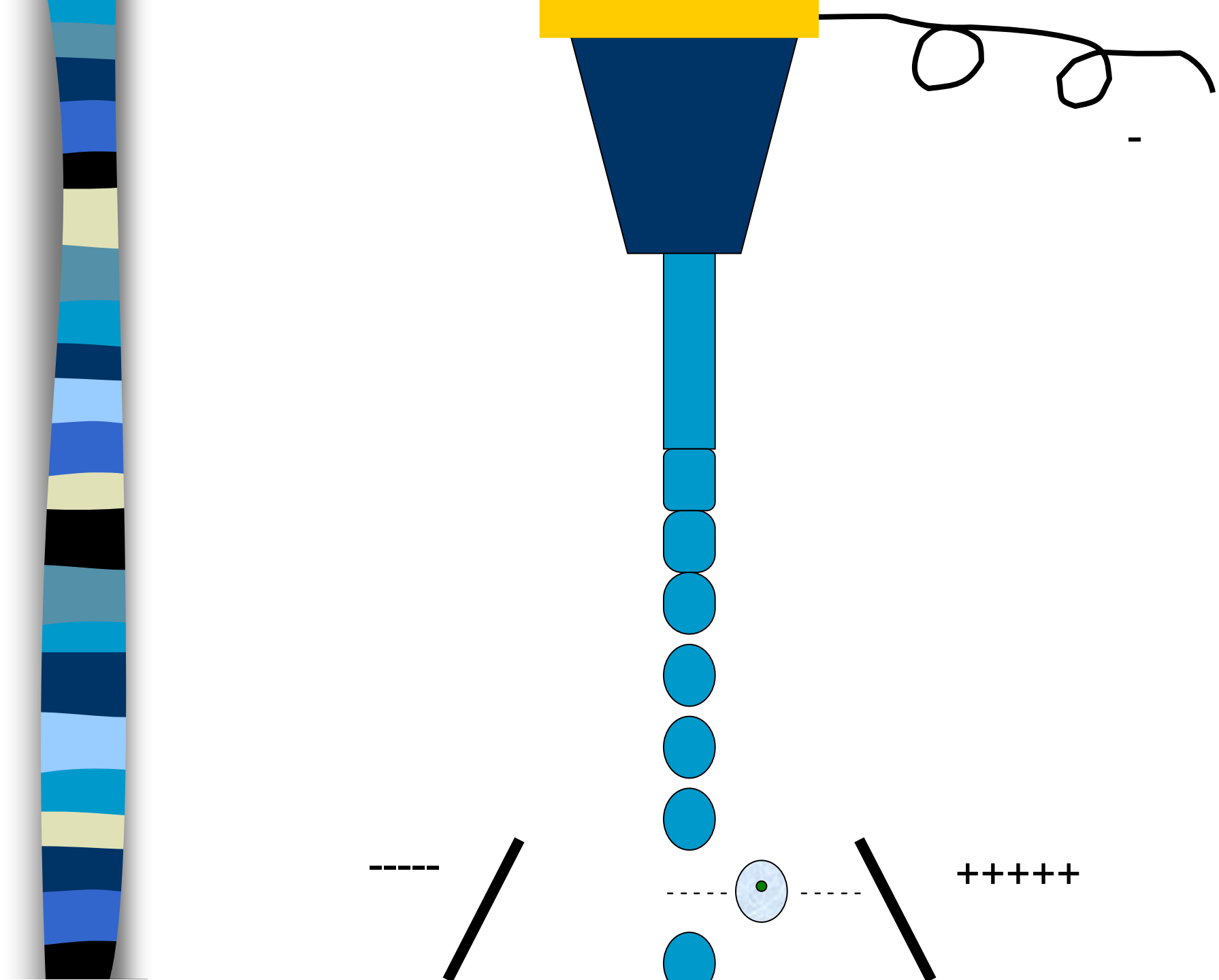








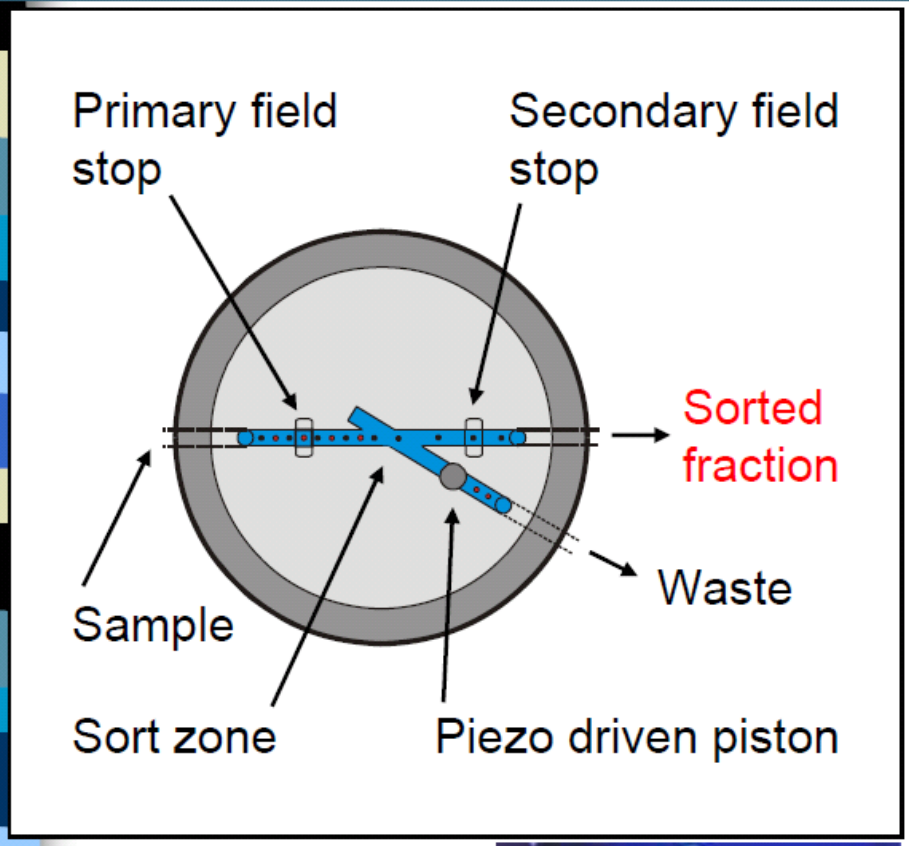




ISAC presents: Mack Fulwyler - Innovator, Inventor & Pioneer

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

FLUIDIC SWITCH SORTER

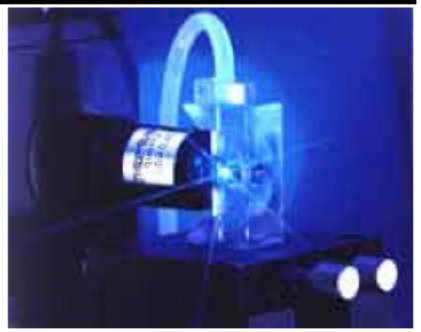


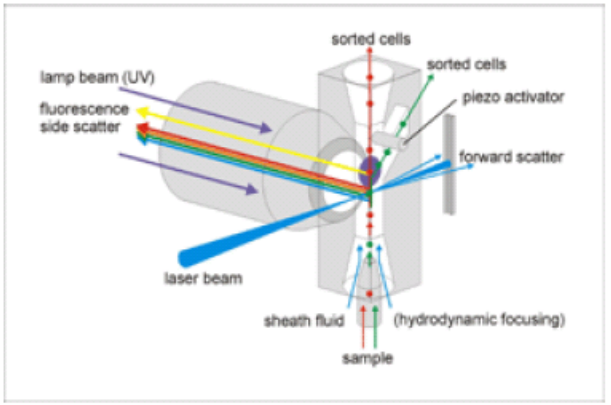
- **Safety (enclosed stream)**
- **Gentle to cells**
- **Sorting of large particles (>100 µm)**

Low speed (~100/sec)

- **Dilute sorted fraction**
- **Noisy**

Used by: Partec





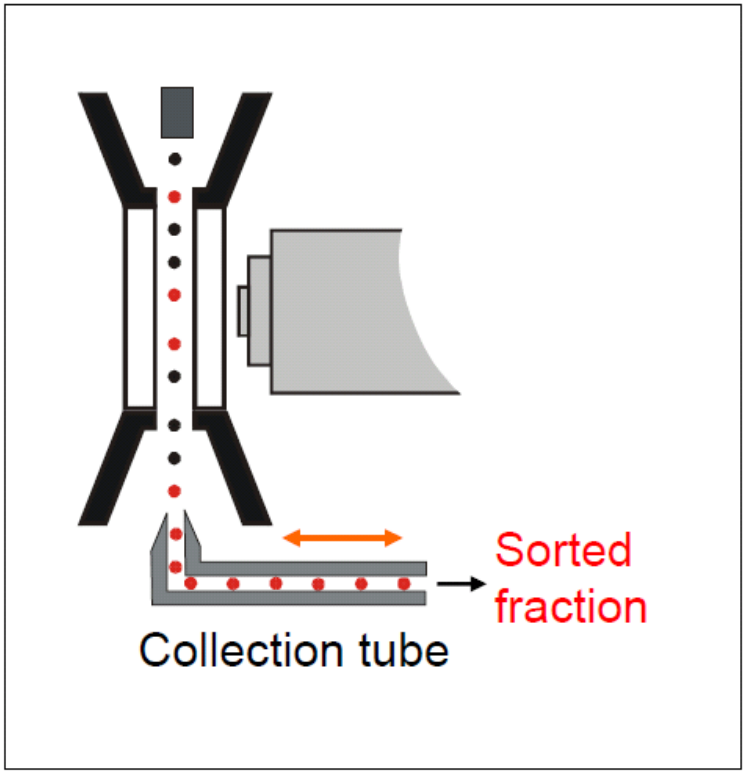
Doležel (1999)

FLUIDIC SWITCH SORTER

- Safety (enclosed stream)
- Gentle to cells

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

Used by: Becton Dickinson

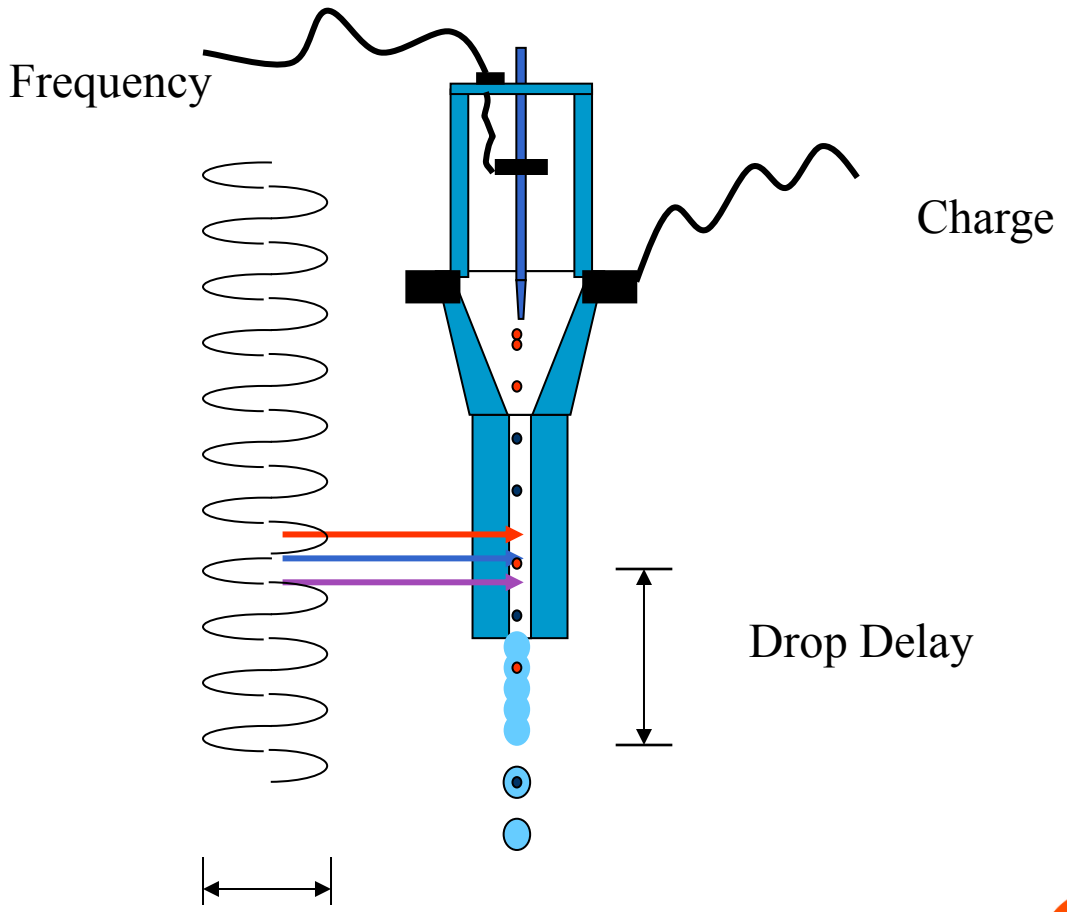


Doležel (1999)

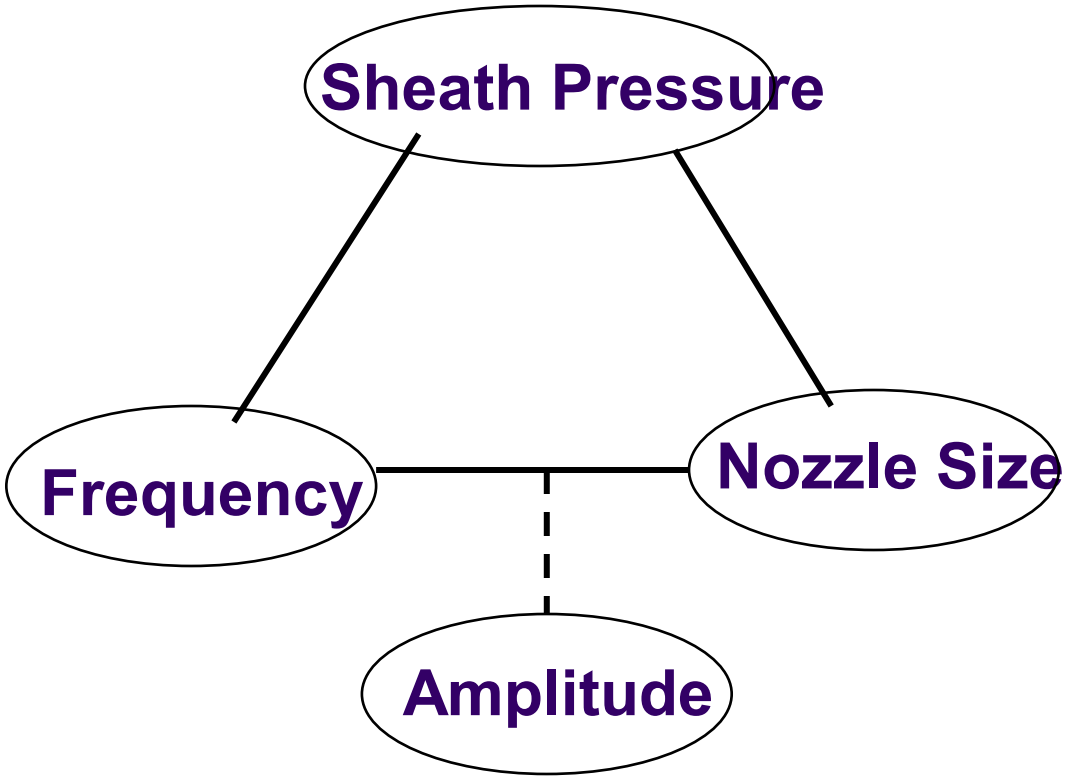
SORTING

BD

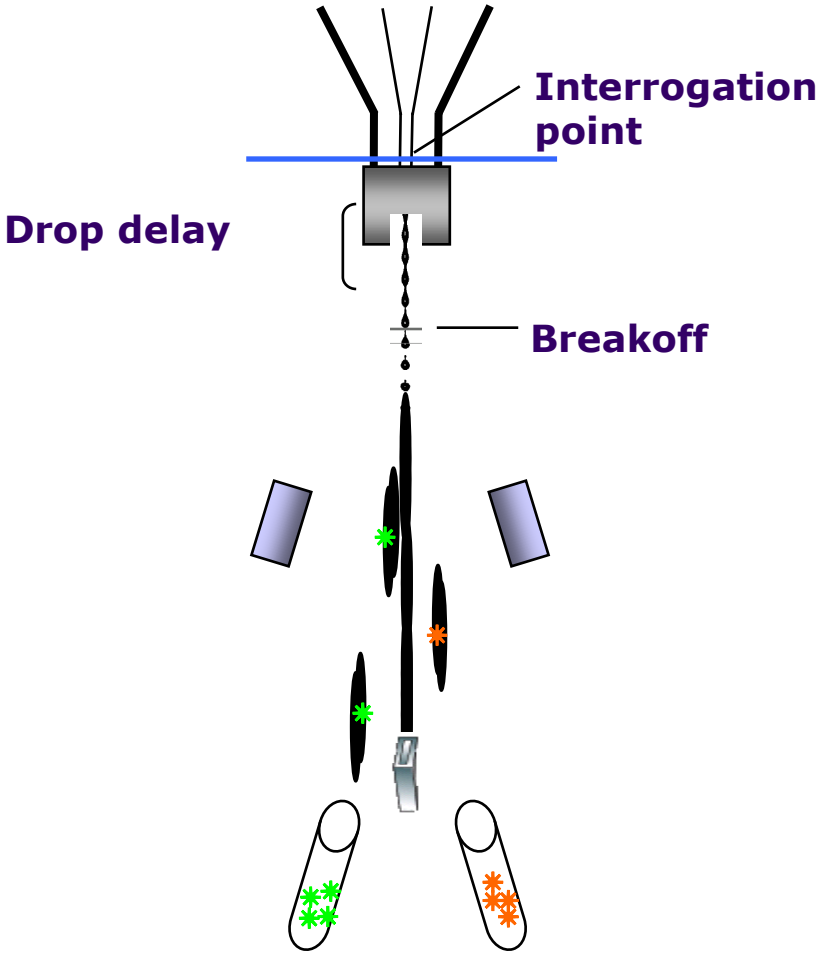
SORTING



SORTING



SORTING



SORTING

Each sort setup includes:

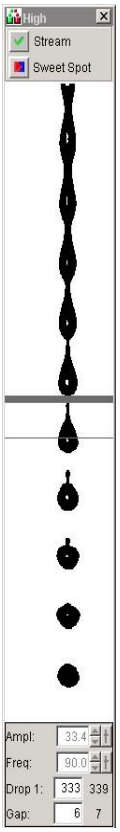
- Sheath pressure
- Breakoff window values
- Side Stream window values

Table 3-2 Default Sort Setup values

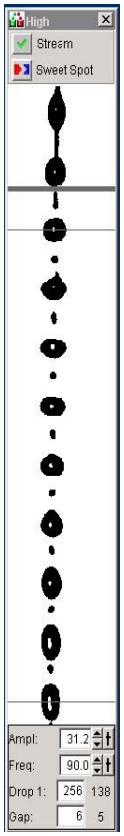
Setting	70 micron	85 micron	100 micron	130 micron
Sheath Pressure	70	45	20	10
Amplitude	60	32	12	24
Frequency	87	47	30	12
Drop 1	150	150	150	150
Gap (upper limit)	6 (14)	7 (17)	10 (21)	12 (21)
Attenuation	Off	Off	Off	Off
Drop Delay	47.00	30.00	27.00	16.00
Far left voltage	100	100	80	60

BD

SORTING - Streams



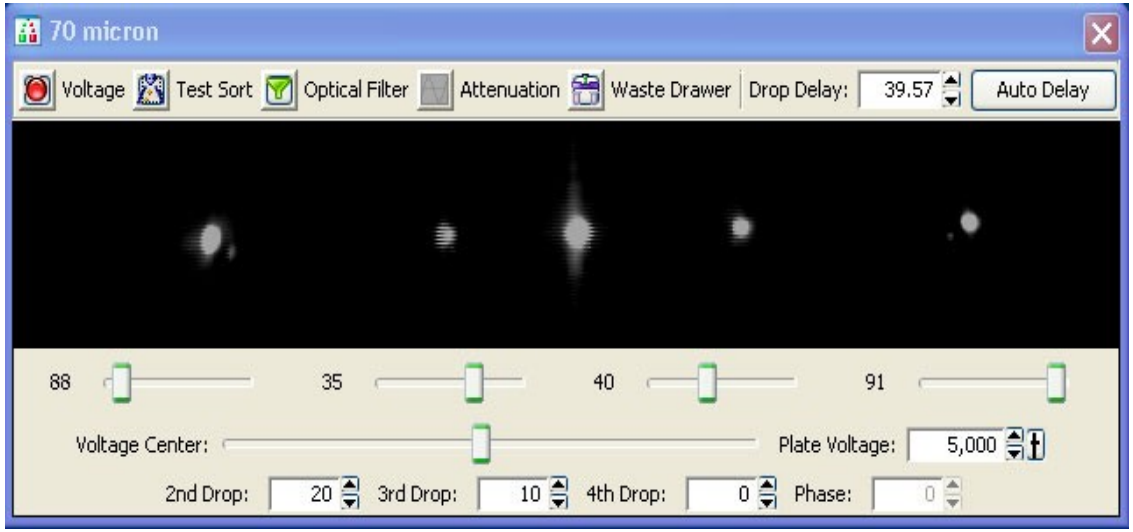
Good

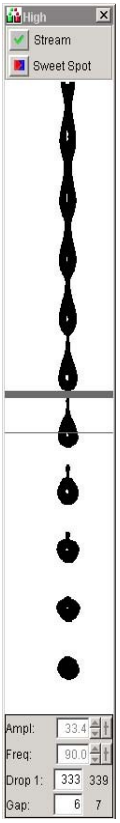


Bad

BD

SORTING – Setup Side Streams





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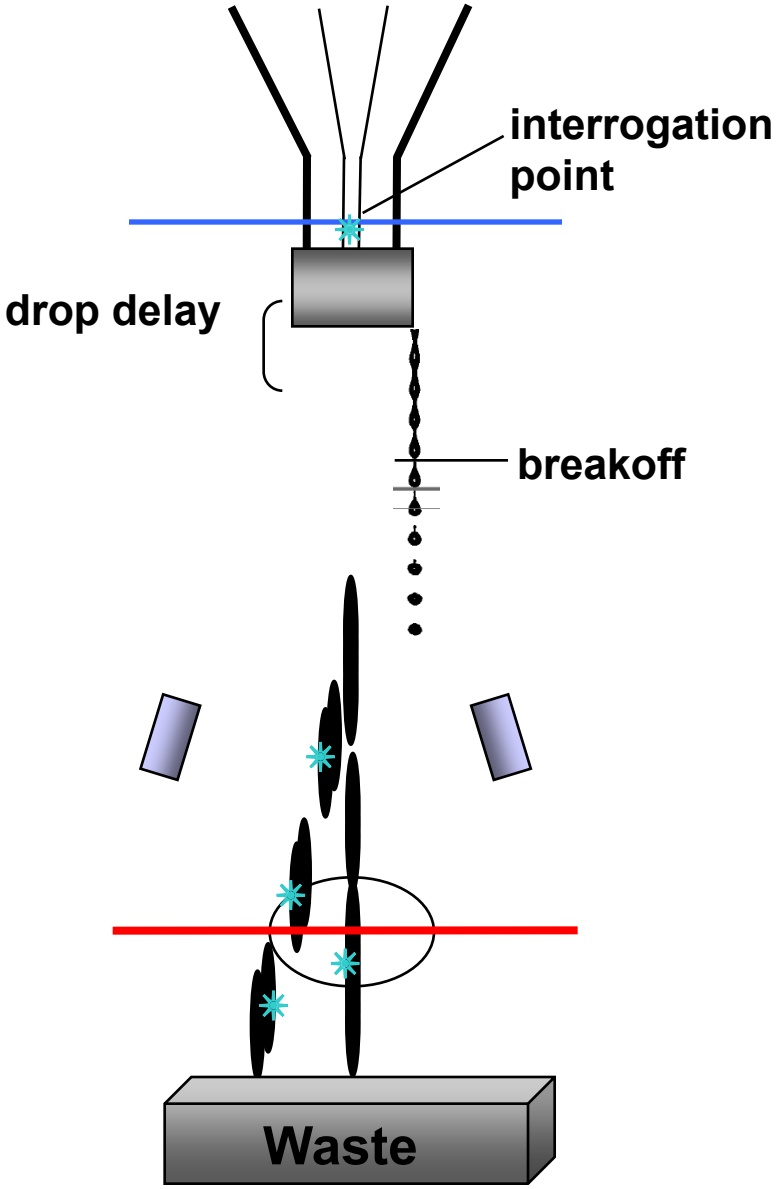
Drop Delay

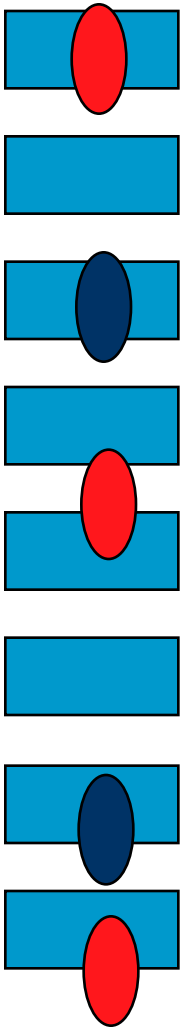
BD FACS™ Accudrop technology

- Accudrop beads
- Diode laser
- Camera
- Optical filter



BD

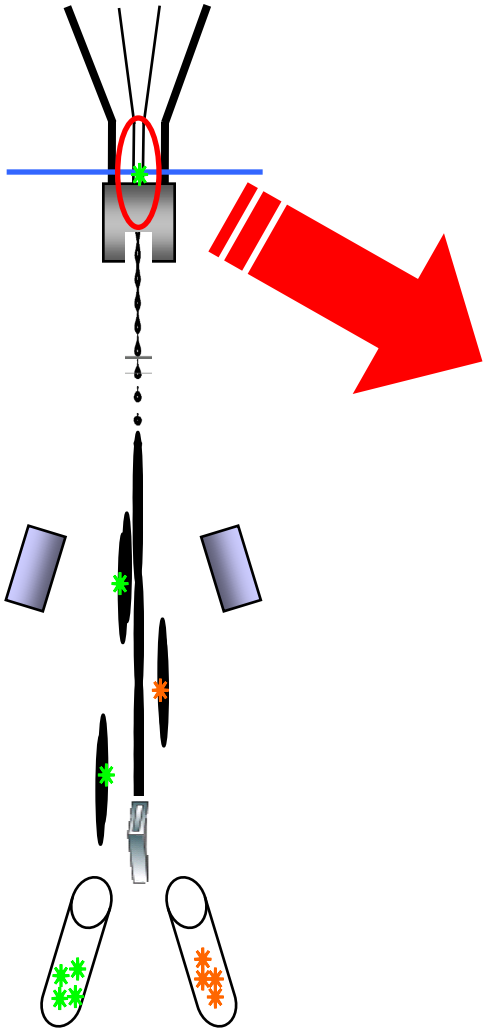
Sorting - Sort Masks



Cells are randomized distributed over the stream

BD

Sorting - Sort Masks



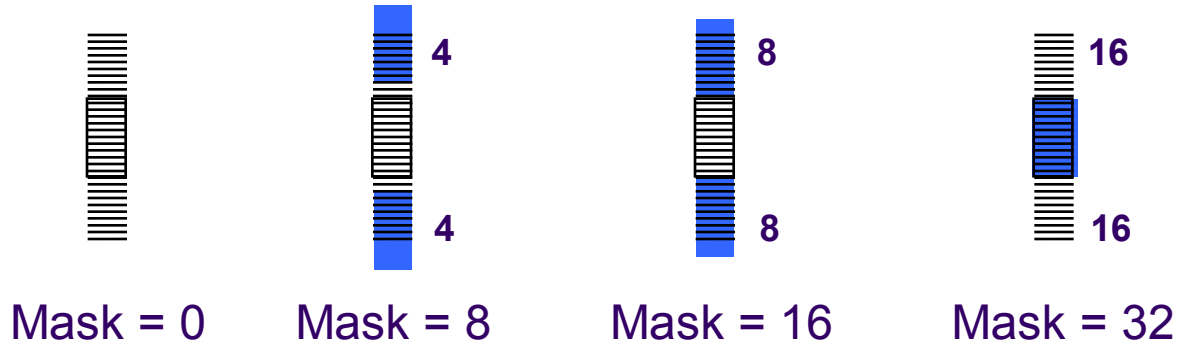
Trailing

Interrogated

Leading

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



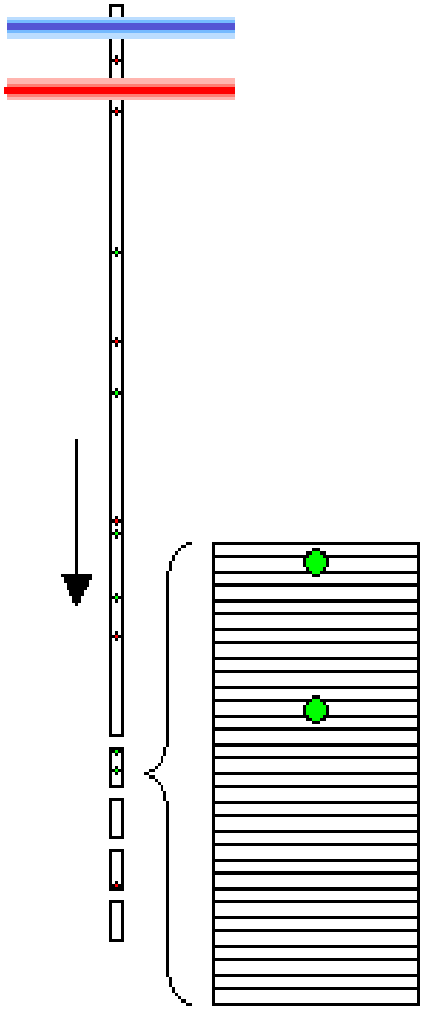
Conflict Resolution

- 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:	☐	☐	☒	☐	☐

Sorting - Sort Masks

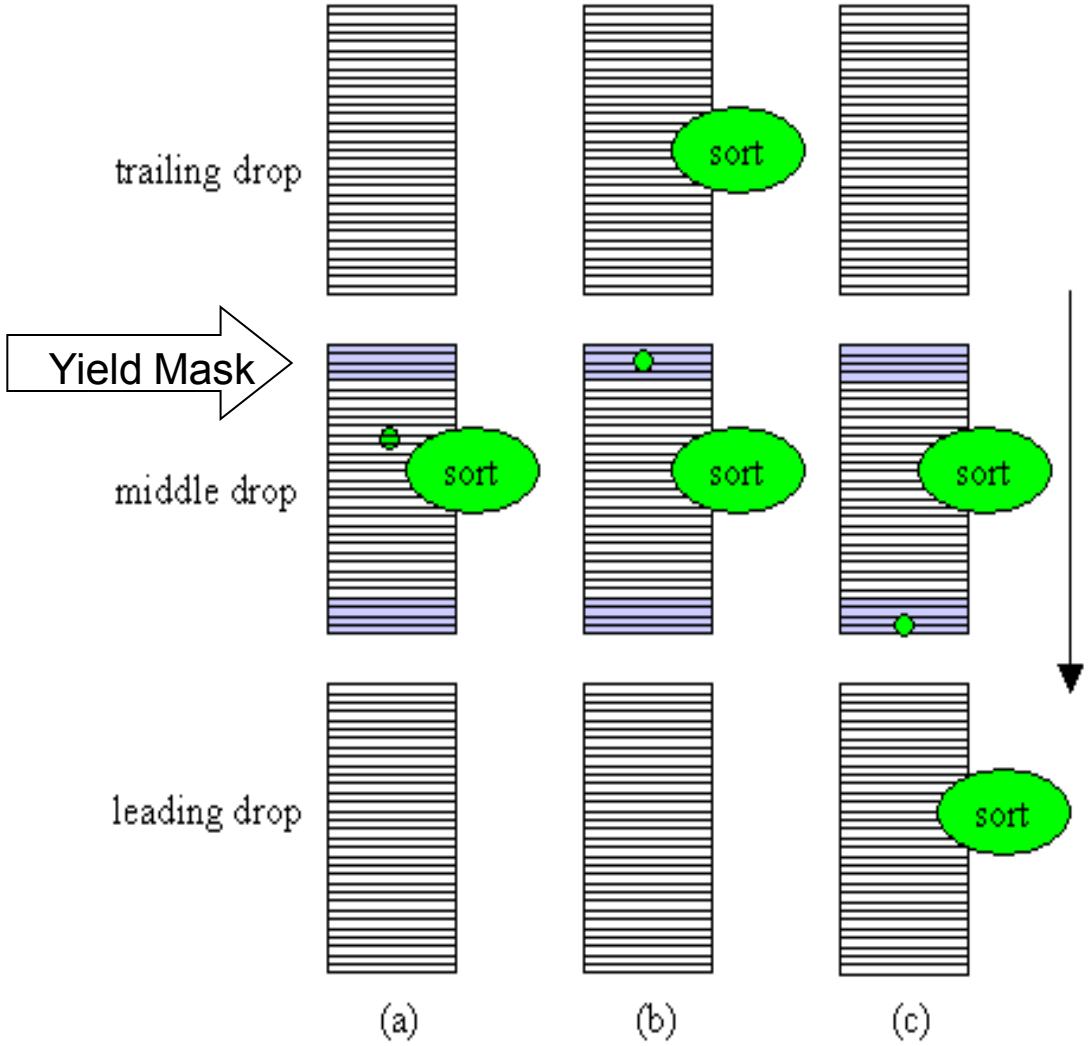
Sort decisions are determined by sort masks



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

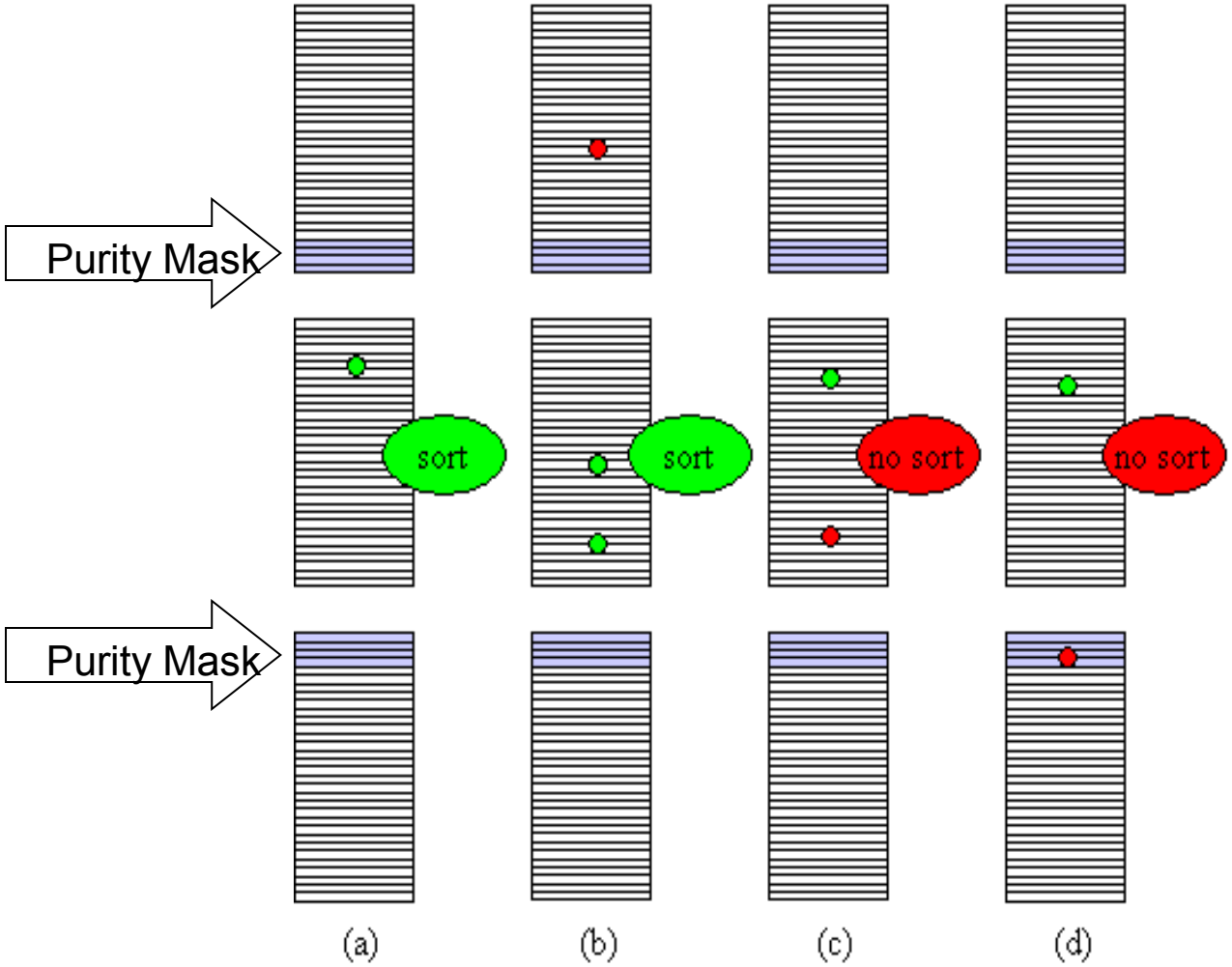
Sorting - Yield Mask

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
    - linearní 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).***

Properties:150717_DU145 Ctrl.fcs

Help

OK

Date:17-JUL-2015
System:Windows XP 5.1
Cytometer:FACSAriaII SORP (FACSAriaII)
File:150717_DU145 Ctrl.fcs
File URI:file:/C:/Users/user/Desktop/install/Infinicyt/150717_DU145%20Ctrl.fcs
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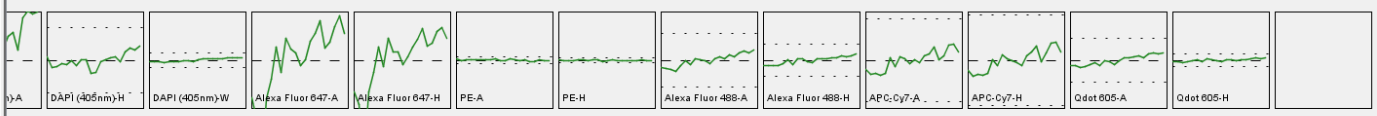
$BEGINANALYSIS: 0
$BEGINDATA: 4148
$BEGINSTEXT: 0
$BTIM: 13:25:01
$BYTEORD: 4,3,2,1
$CYT: FACSAriaII SORP (FACSAriaII)
$DATATYPE: F
$DATE: 17-JUL-2015
$ENDANALYSIS: 0
$ENDDATA: 6055267
$ENDSTEXT: 0
$ETIM: 13:28:55
$FIL: 150717_DU145 Ctrl.fcs
$INST: IBP
$MODE: L
$NEXTDATA: 0
$OP: fedr
$PAR: 19
$SRC: 150717
$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 (2uv-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	0,96
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

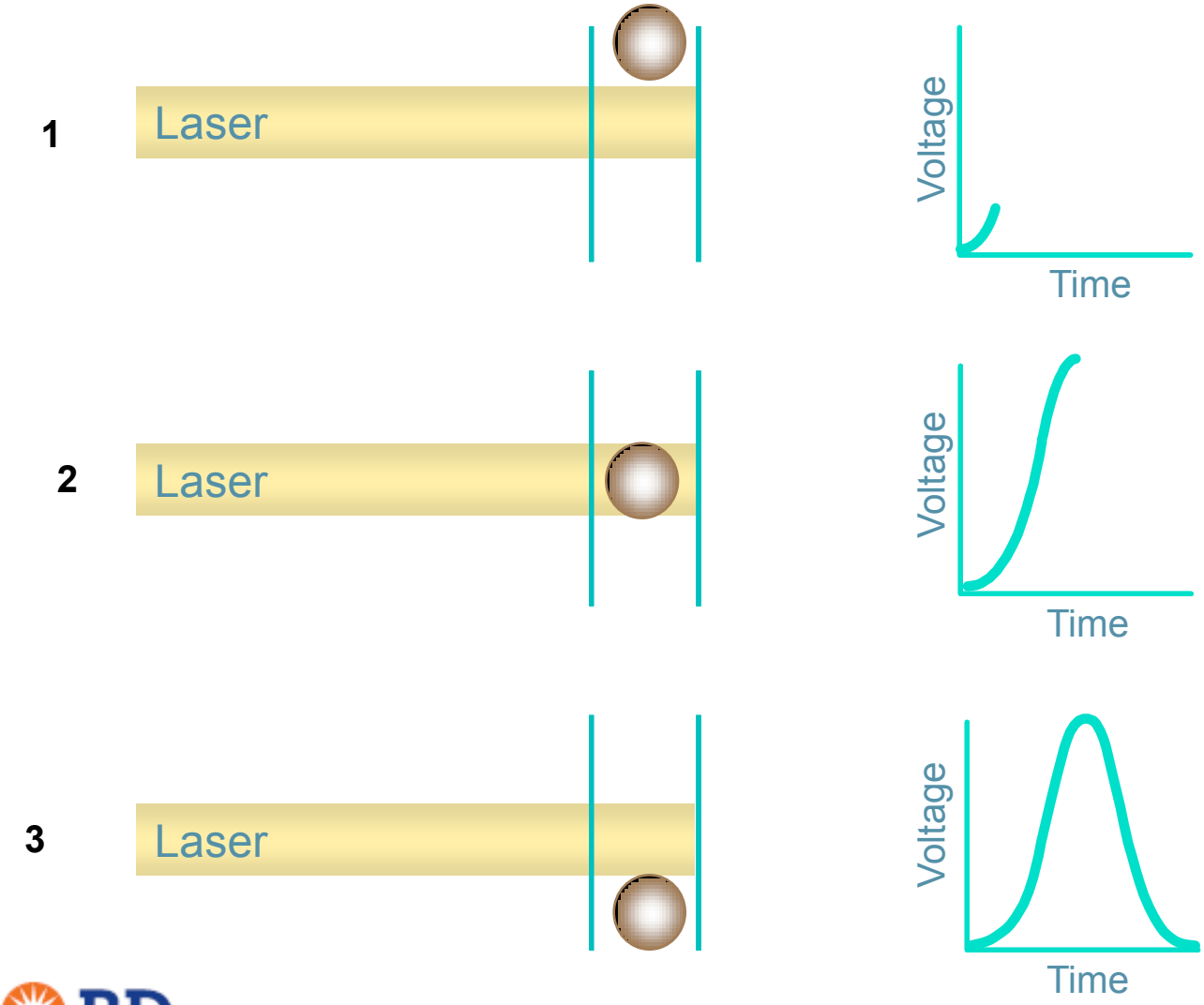
Parameter ($PnN)	Stain ($PnS)	Range ($PnR)	Bits ($PnB)	Decades ($PnE)	Gain ($PnG)	Voltage ($PnV)	Derived From
FSC-A		262144	32	0,0	1.0	280	
FSC-H		262144	32	0,0	1.0	280	
SSC-A		262144	32	0,0	1.0	210	
Alexa Fluor 594-A		262144	32	0,0	1.0	460	
Alexa Fluor 594-H		262144	32	0,0	1.0	460	
DAPI (405nm)-A		262144	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

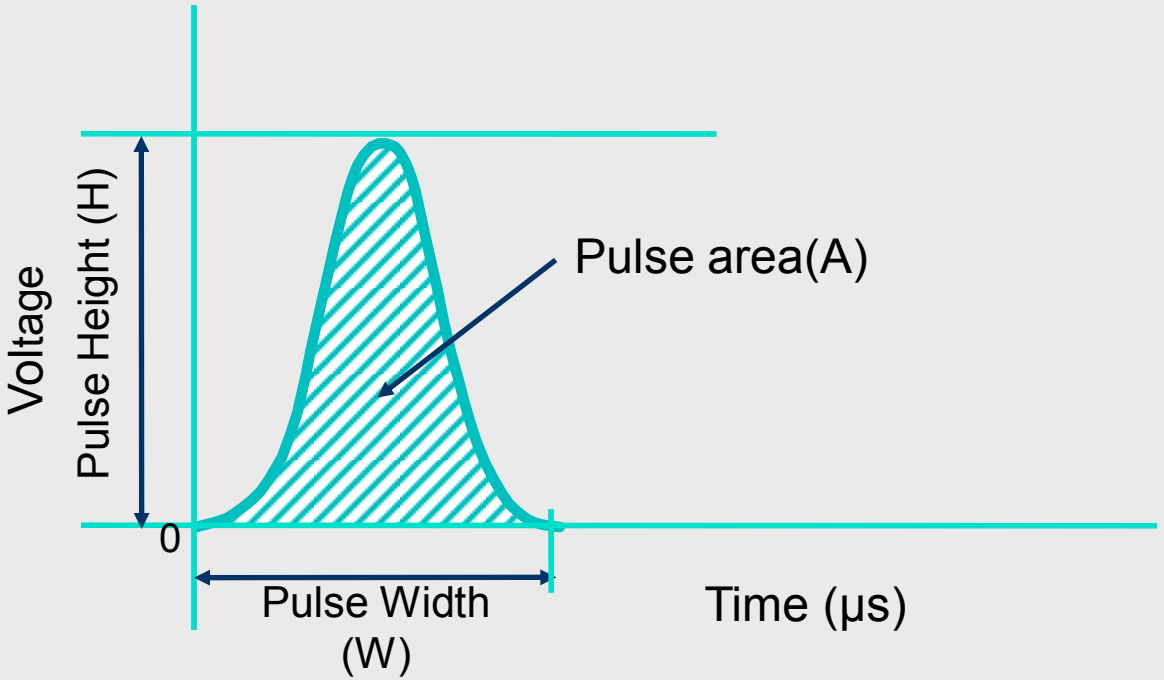
Event	*Param1 FS*	*Param2 SS*	*Param3 FITC*	*Param4 PE*
1	50	100	80	90
2	55	110	150	95
3	110	60	80	30

Creation of a Voltage Pulse

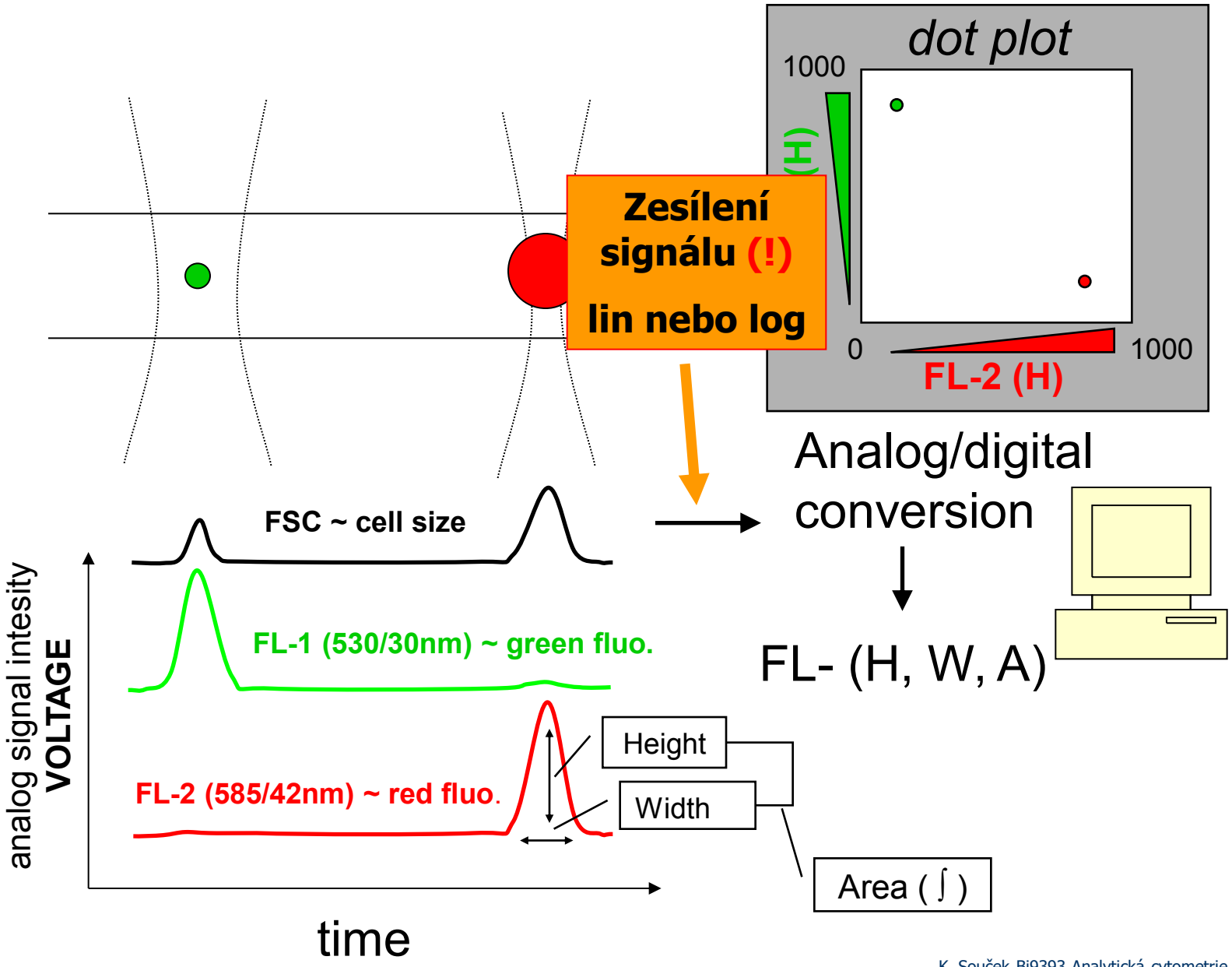


BD

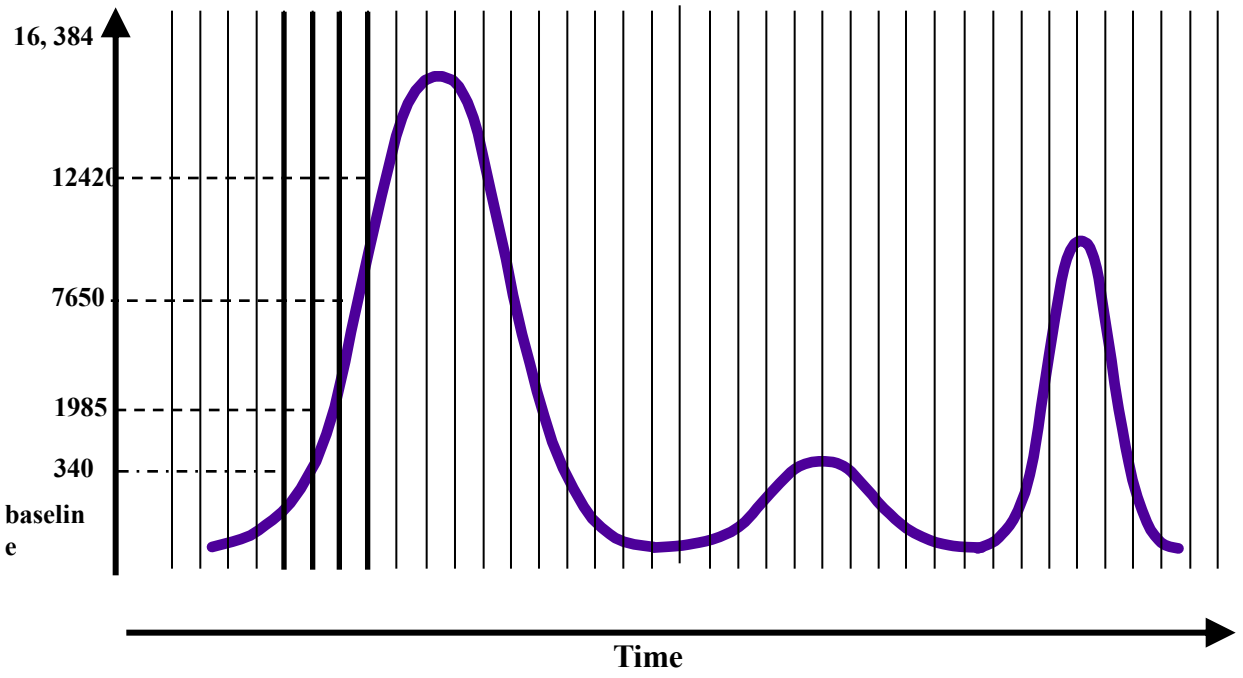
Height, Area, and Width



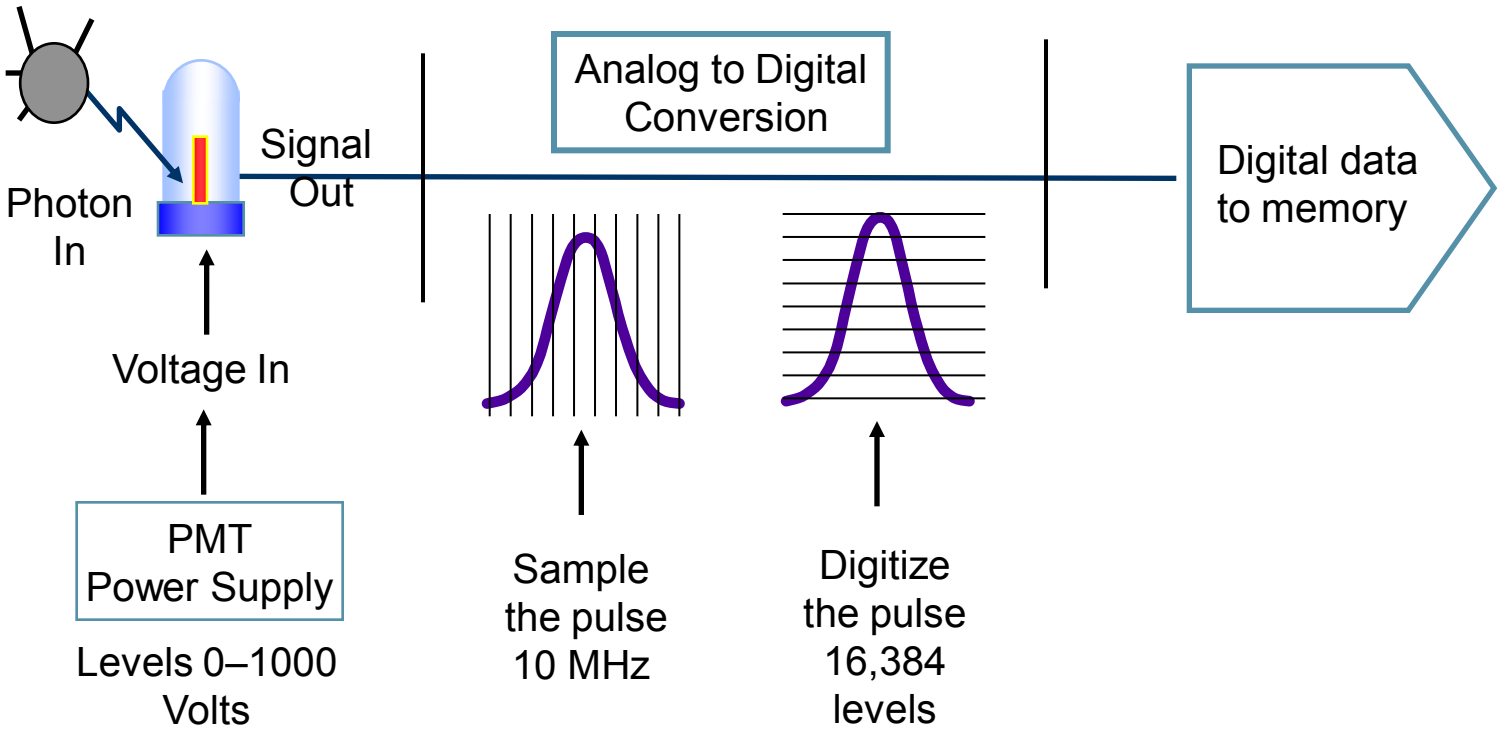
Signal processing



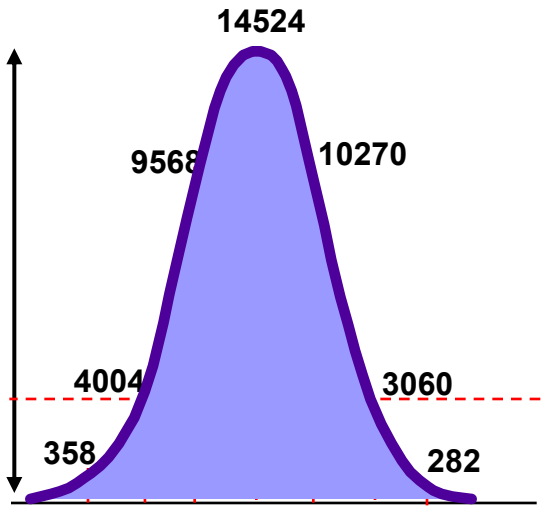
Analog to Digital Converter



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

$2^8 = 256$
$2^{10} = 1024$
.
.
.

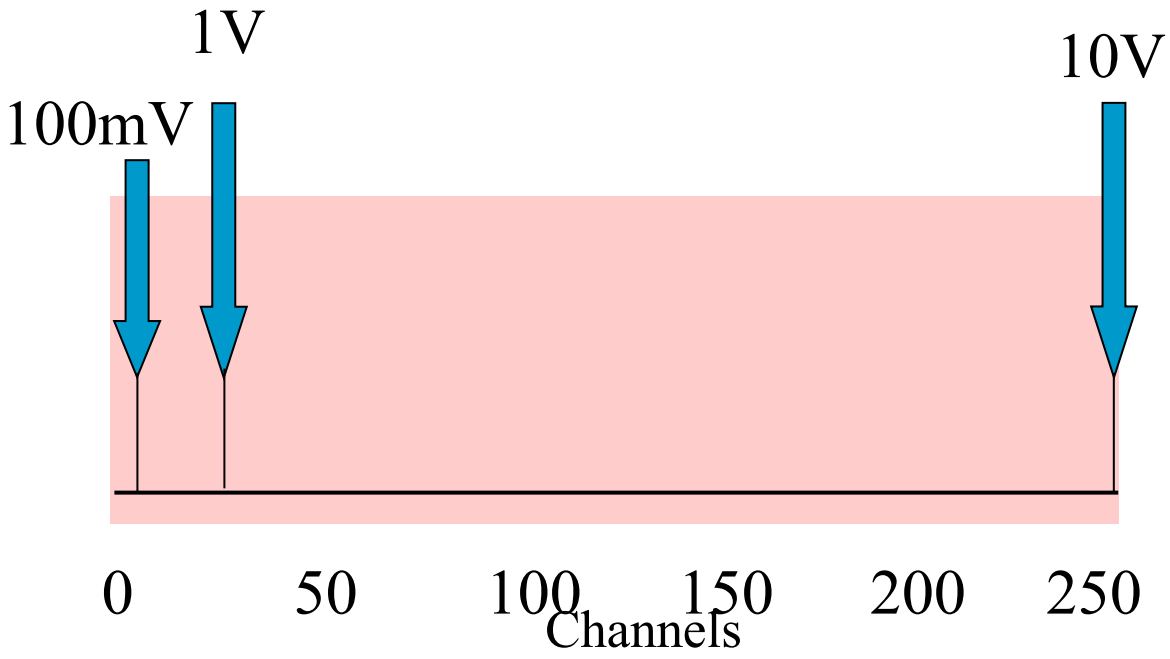
AD převodníky

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

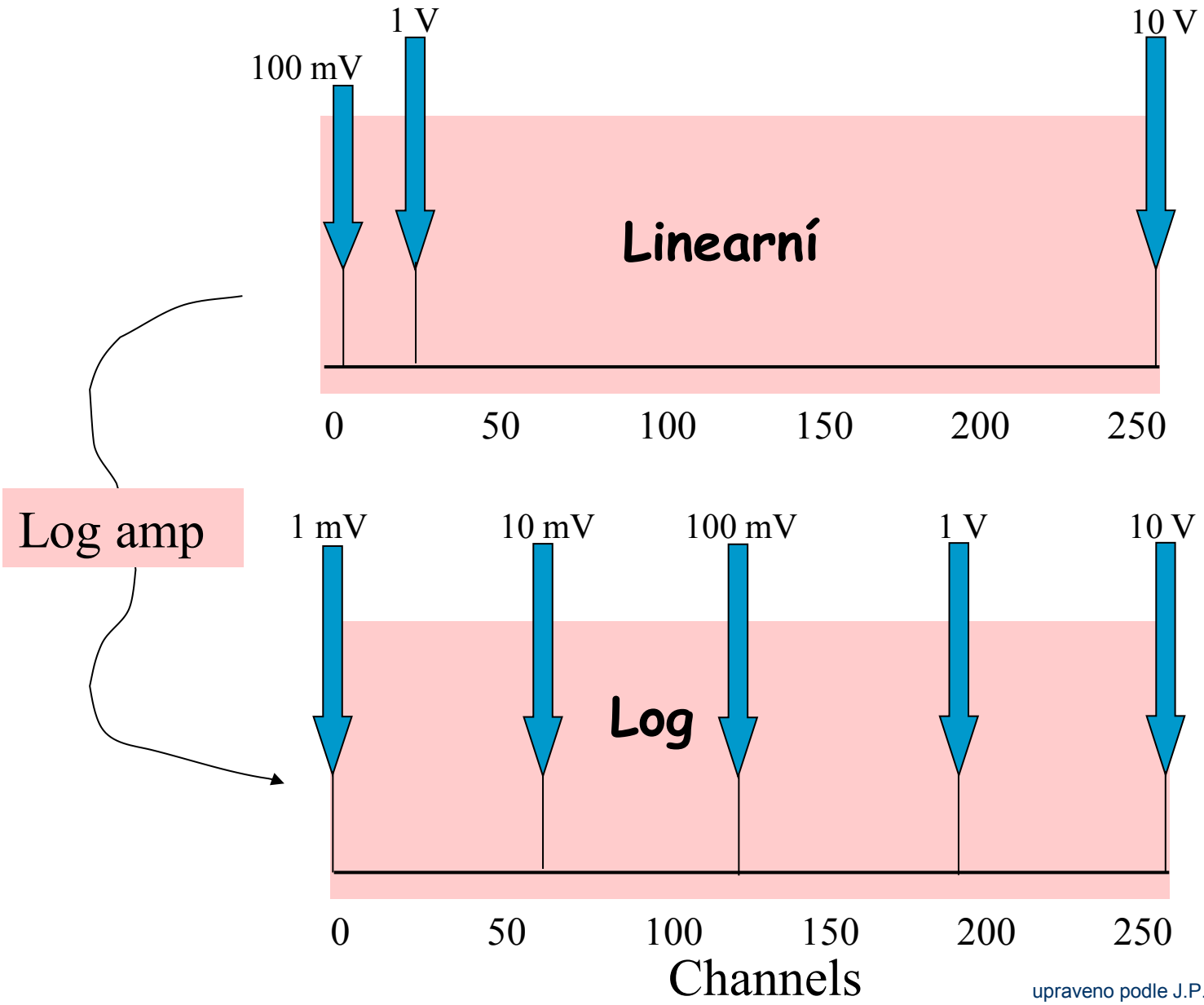
Full scale measurement range = 0 to 10 volts
ADC resolution is 12 bits: 212 = 4096 quantization levels
ADC voltage resolution is: (10-0)/4096 = 0.00244 volts = 2.44 mV

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=~40mV



Ideální logaritmický zesilovač



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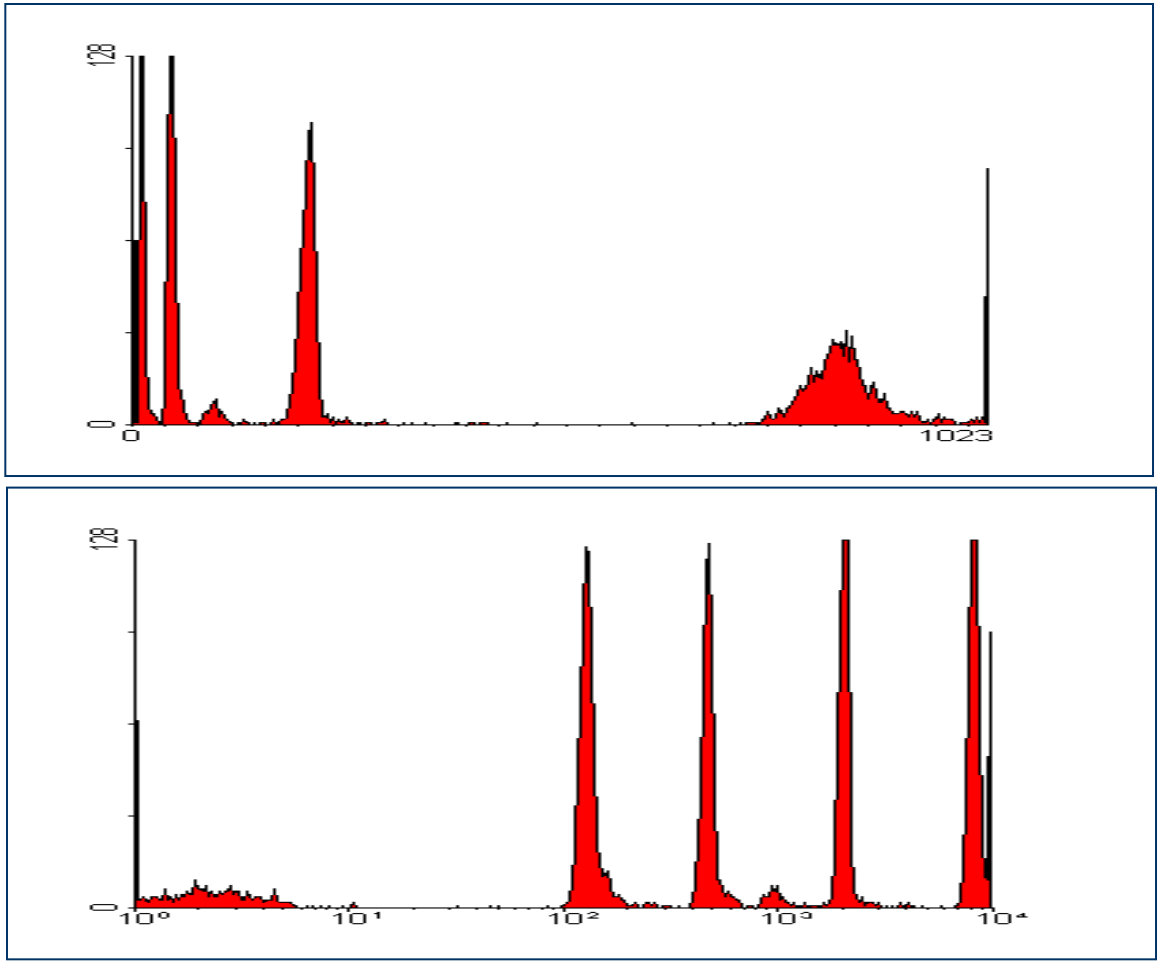
Logaritmické zesílení & dynamický rozsah



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log

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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

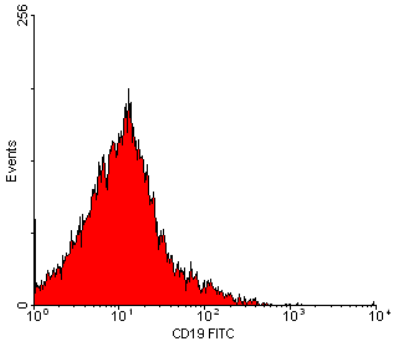
Kompenzace fluorescenčního signálu

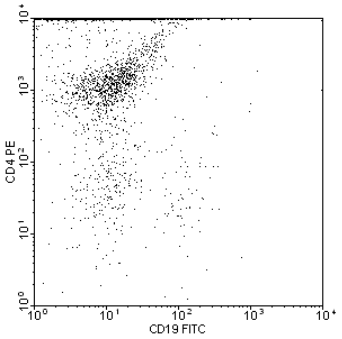
…později

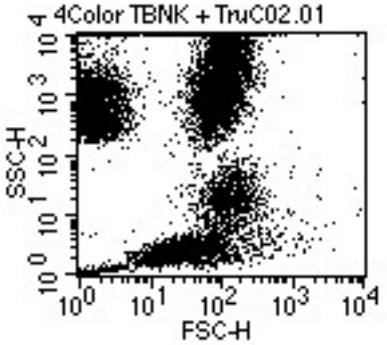
Analýza dat

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

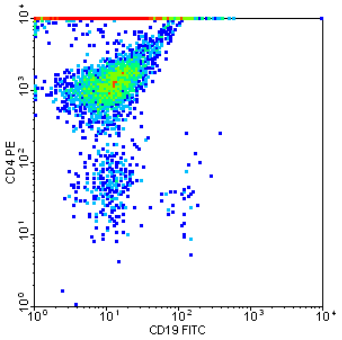
Způsoby pro zobrazení dat

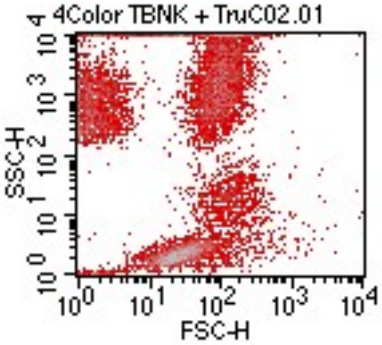


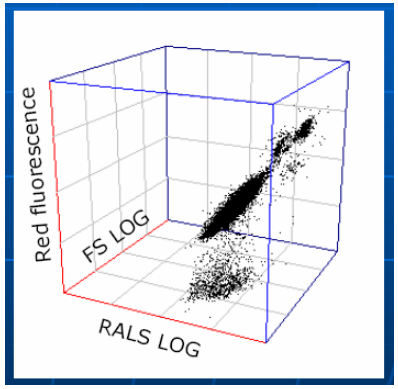


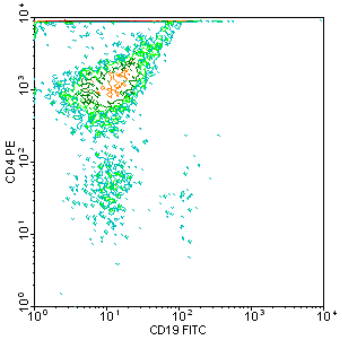


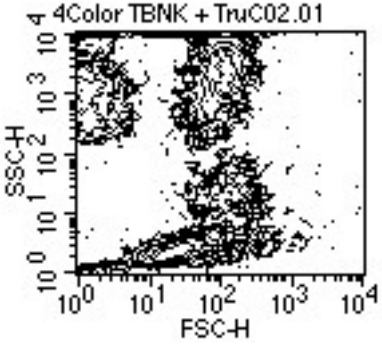
4Color TBNK + TruC02.01





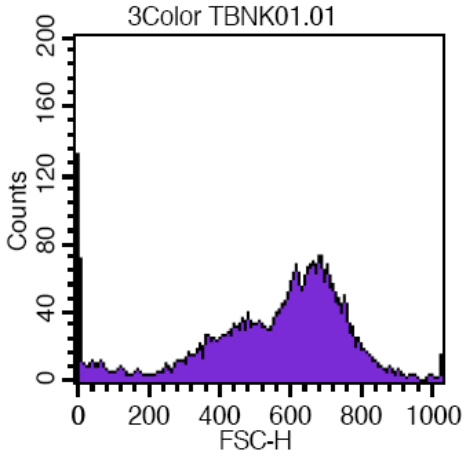


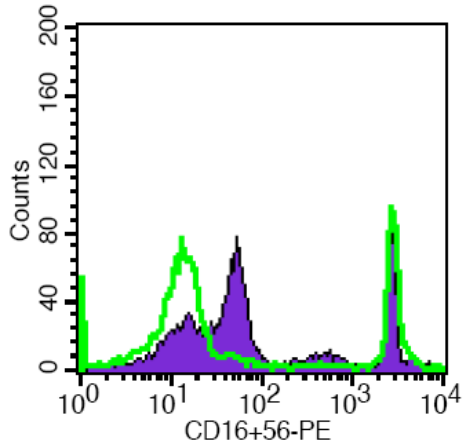




Histogram distribuce četnosti

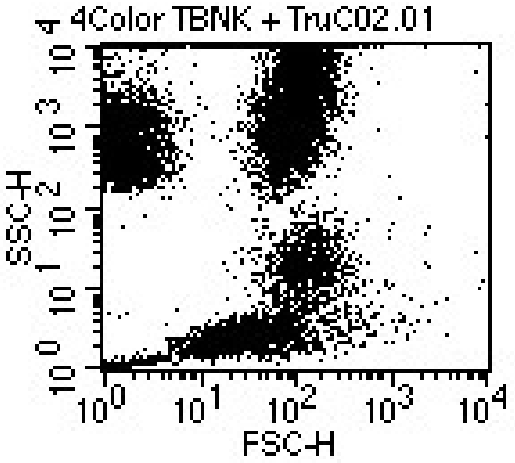
- Histogram zobrazuje četnost částic pro jeden parametr
- Jednoduchý výstup
- Nekorelujeme s dalším parametrem
- Problém s identifikací populací



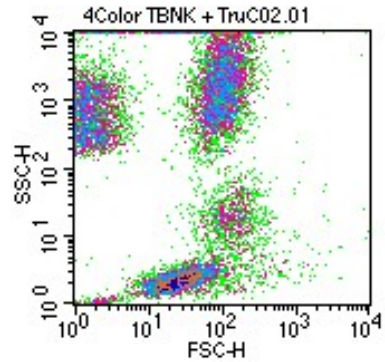


Dot plot

- 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 denzitě částic
- Problémy s vykreslením v případě velkých objemů dat

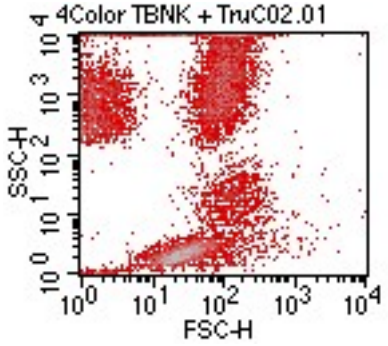


Density & contour plot



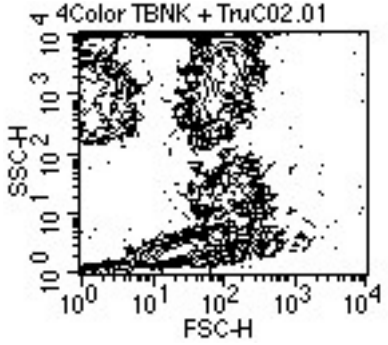
Density plot:

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



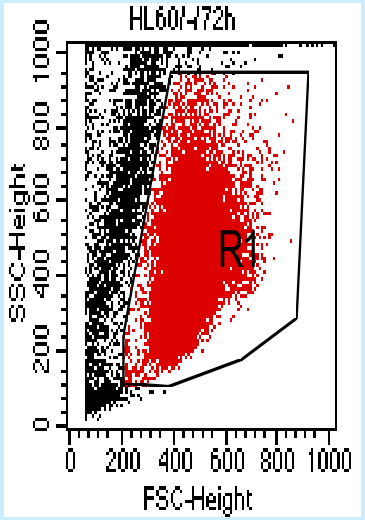
Contour plot:

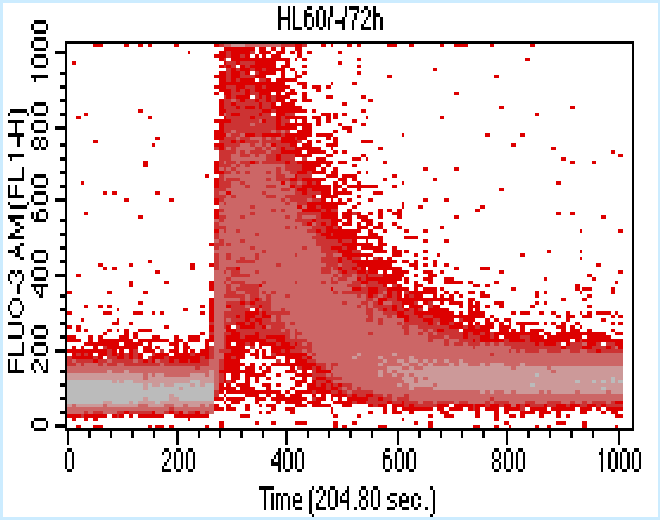
- spojnice spojuje body (částice) se stejnou hodnotou signálu

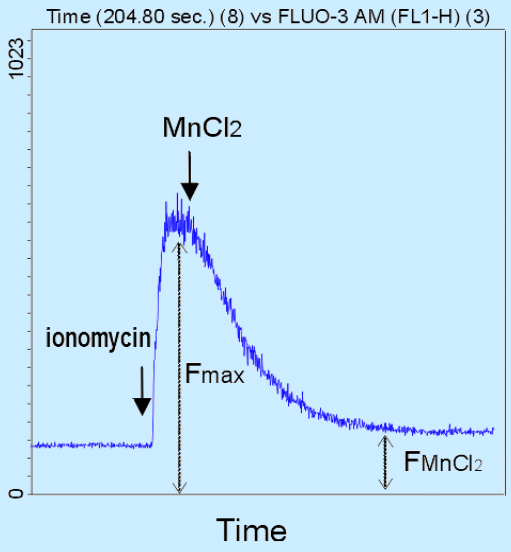


- V podstatě simulujeme 3D graf – třetí rozměr je frekvence

Čas jako jeden z parametrů

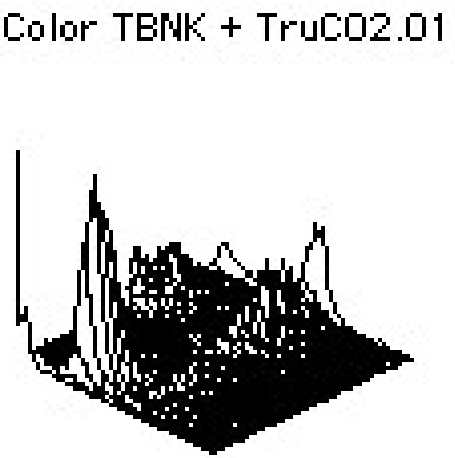




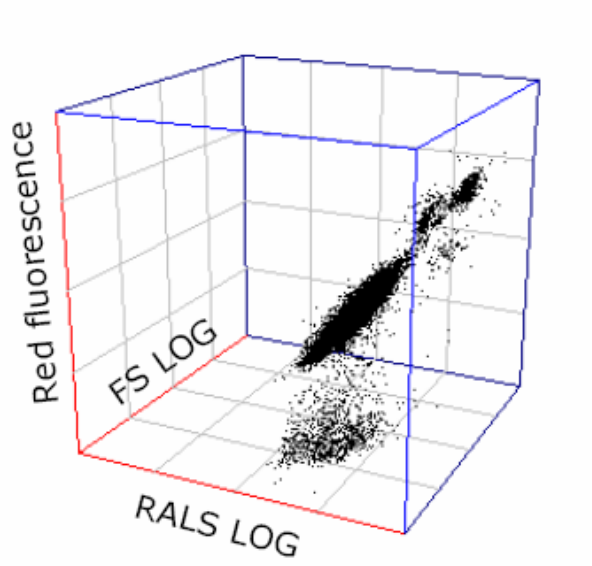


3D zobrazení

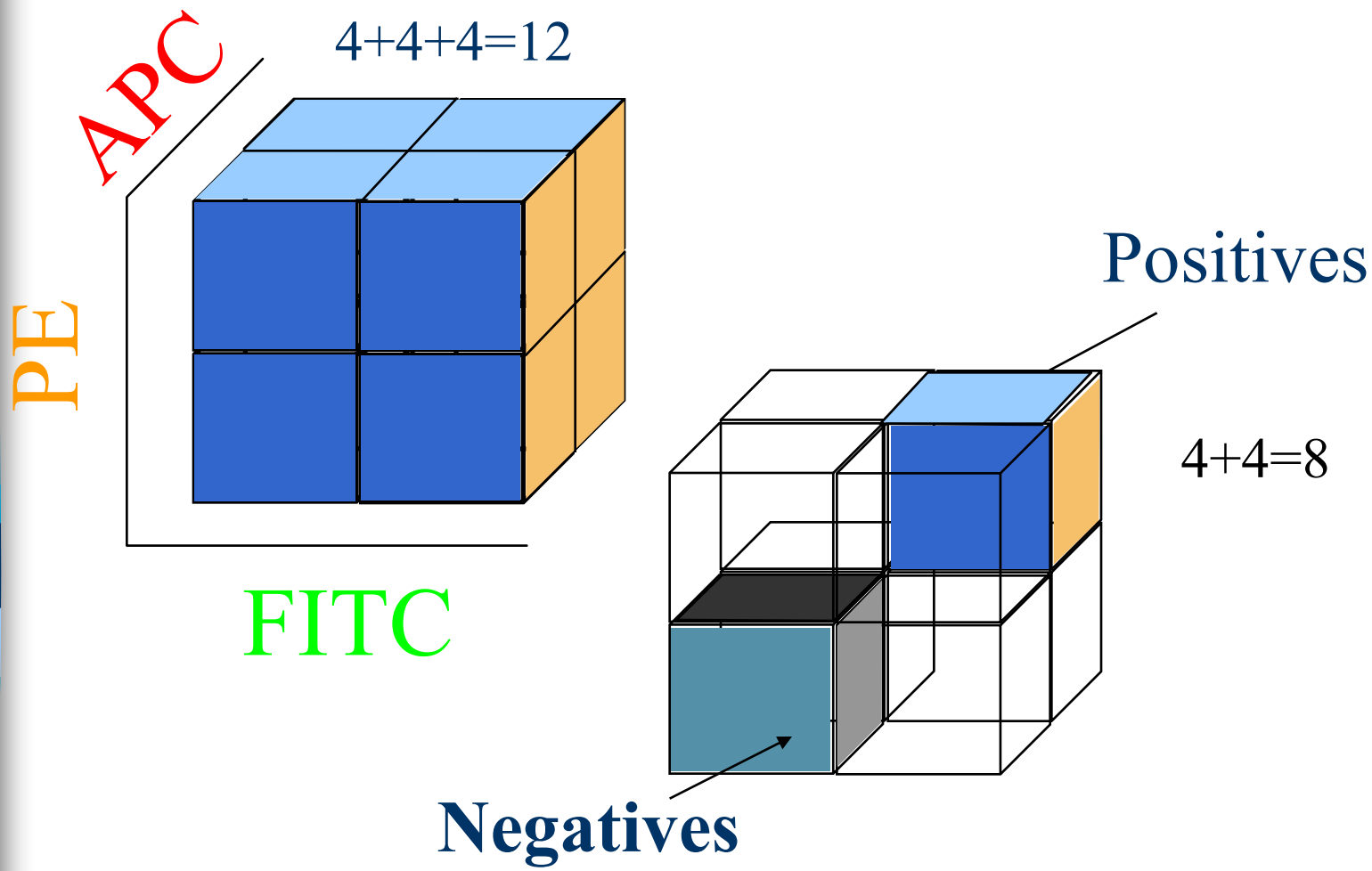
- 2 parametry + četnost



- 3 parametry společně



3 Color Combinations



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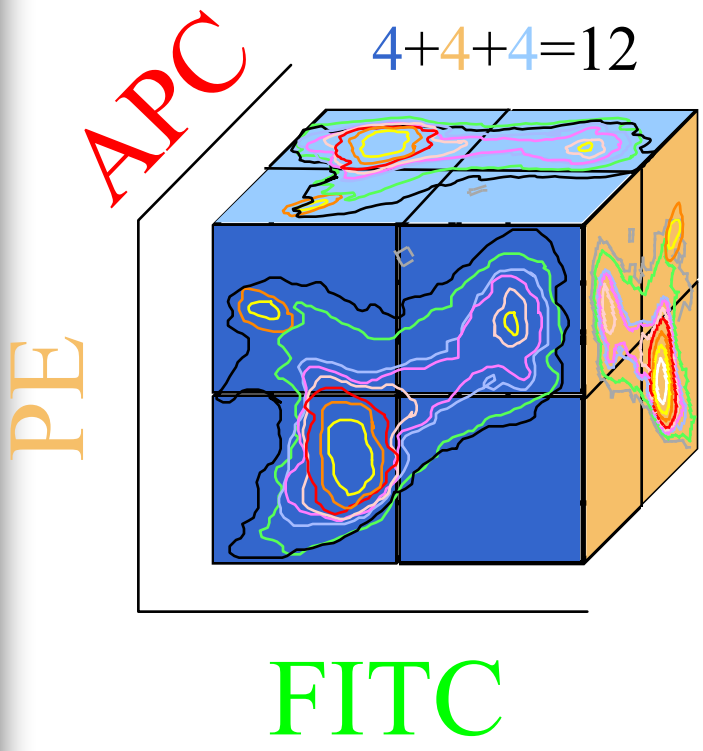
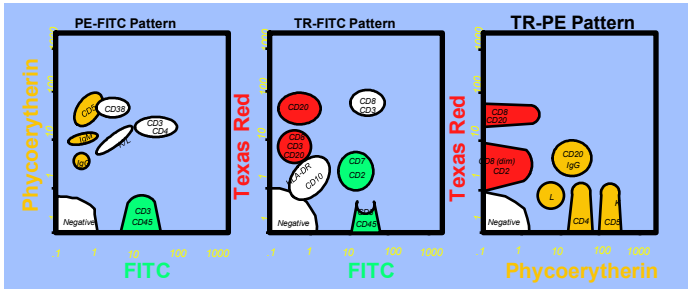
3 Color Combinations





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„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

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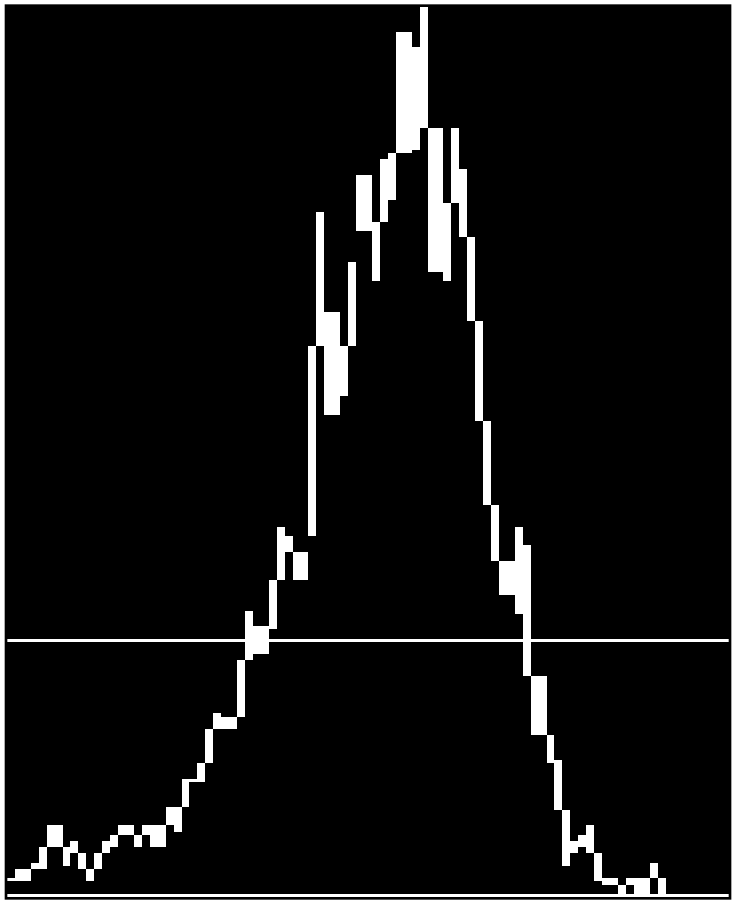
Určení regionů

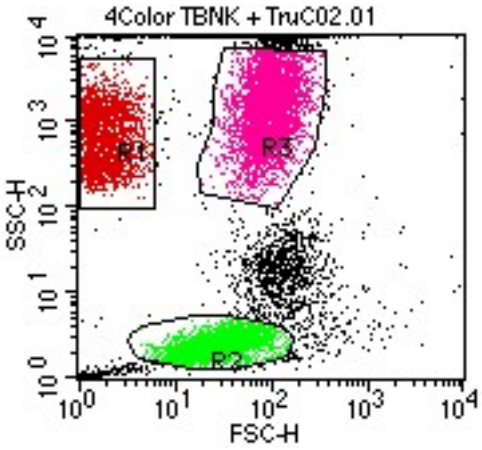
- Objektivní nebo subjektivní?
  -školení/schopnosti/trénink

- Možné tvary:
  -obdelník
  -elipsa
  -"free-hand" (polygon)
  -kvadrant

- Statistika
- počet
- podíl (%)
- průměr, medián, S.D., CV, ….



Region vs. gate



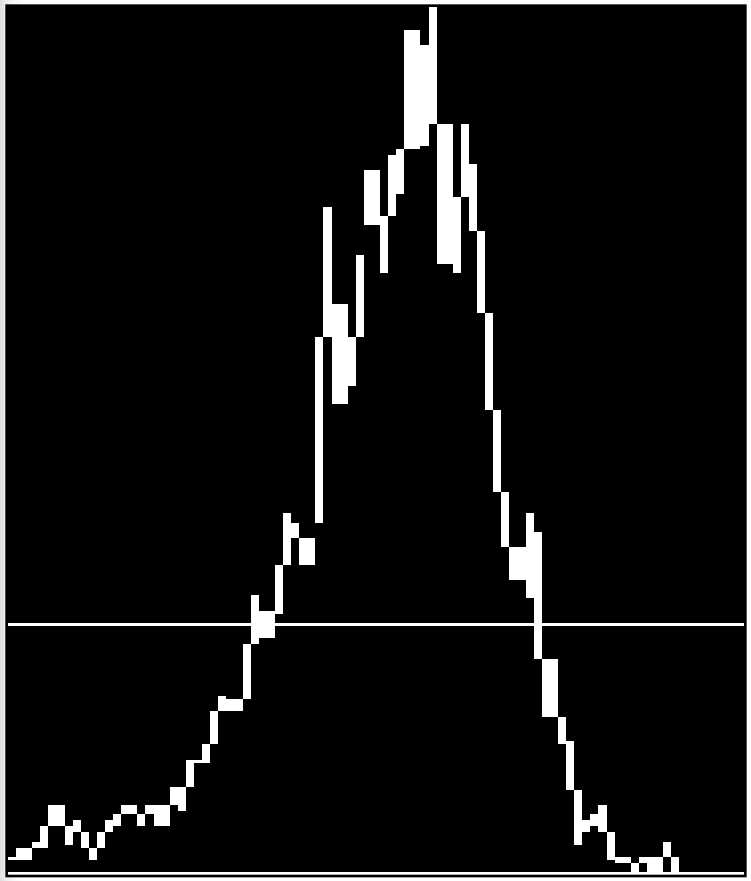
Region

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

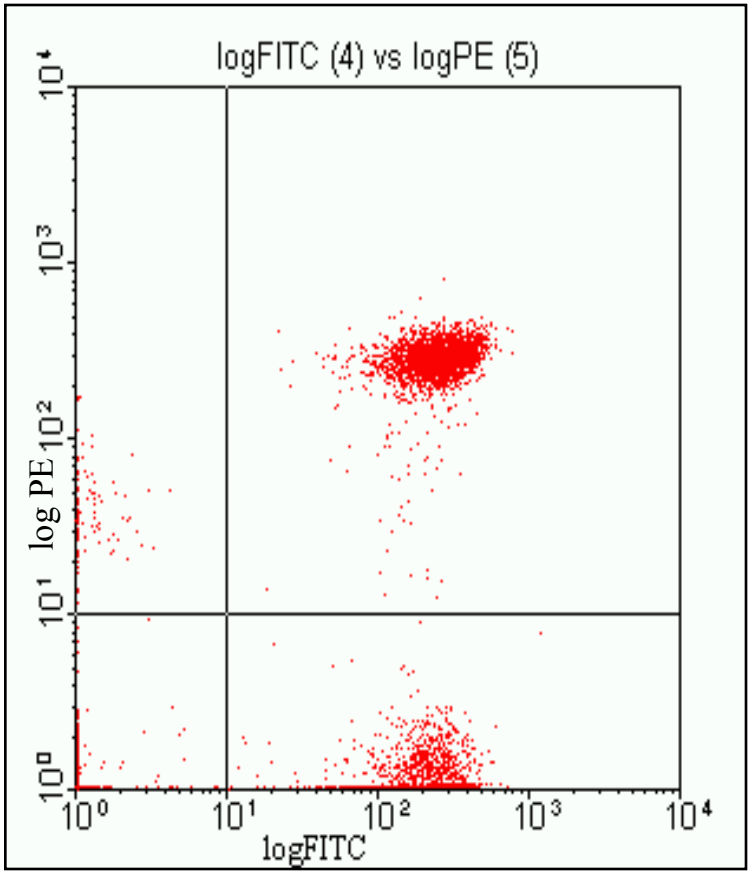
Gate

- 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

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Statistika

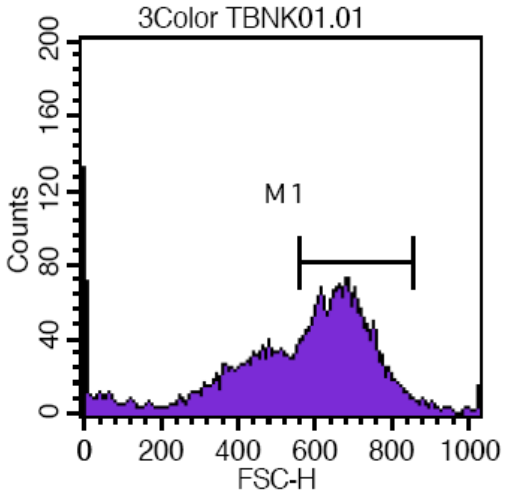
- Aritmerický průměr $\bar{x} = \frac{1}{n}(x_1 + x_2 + \ldots + x_n) = \frac{1}{n}\sum_{i=1}^{n} x_i$
- Geometrický průměr $(a_1 \cdot a_2 \cdots a_n)^{\frac{1}{n}} = \sqrt[n]{a_1 \cdot a_2 \cdots a_n} = \left(\prod_{i=1}^{n} a_i\right)^{\frac{1}{n}}$
- Medián $\int_{-\infty}^{m} f(x)dx = 0,5$

– odhad střední hodnoty

– není ovlivněn extrémními hodnotami

- Směrodatná odchylka $\overline{x} = \frac{1}{N}\sum_{i=1}^{N} x_i$
- Koeficient variance
- Modus – nejčastější hodnota

Statistika pro histogram



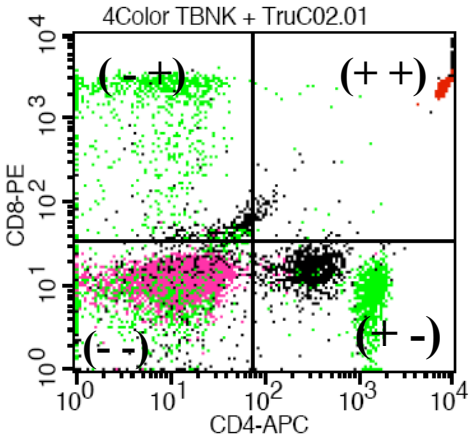
Histogram Statistics

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

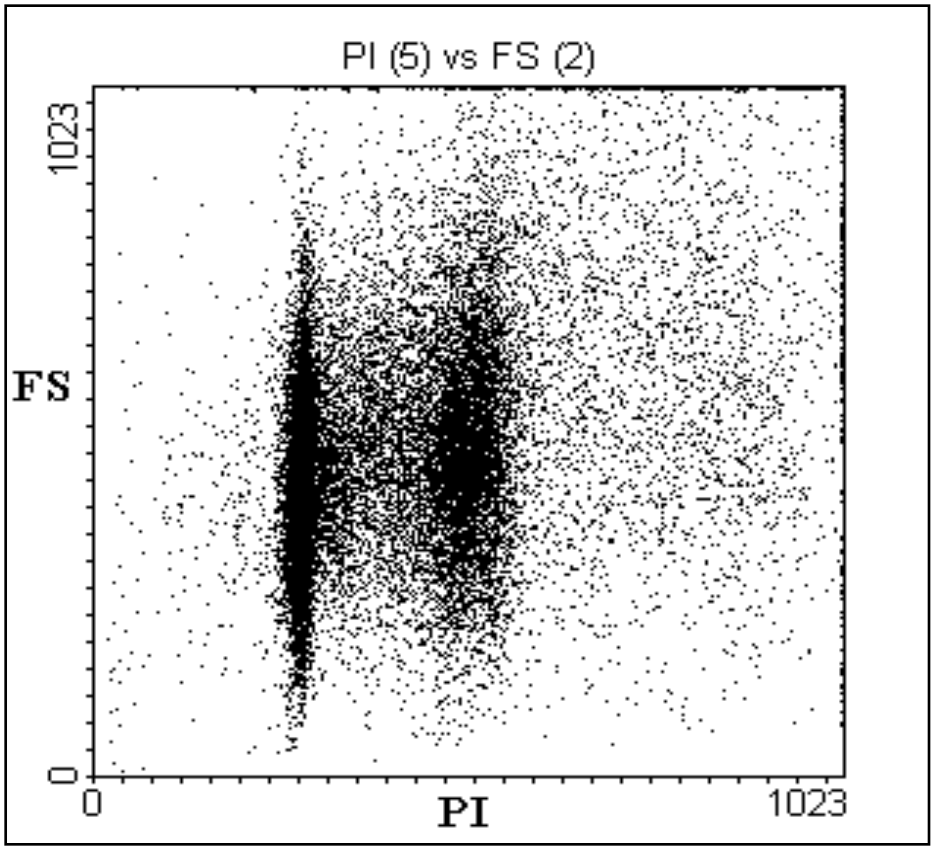
File: 4Color TBNK + TruC02.01
Sample ID:
Tube: CD3/CD8/CD45/CD4 TruC
Acquisition Date: 08-Oct-98
Gated Events: 10000
X Parameter: CD4-APC (Log)
Quad Location: 74, 35

Log Data Units: Linear Values
Patient ID:
Panel: 4 Color TBNK + TruC
Gate: No Gate
Total Events: 10000
Y Parameter: CD8-PE (Log)

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:



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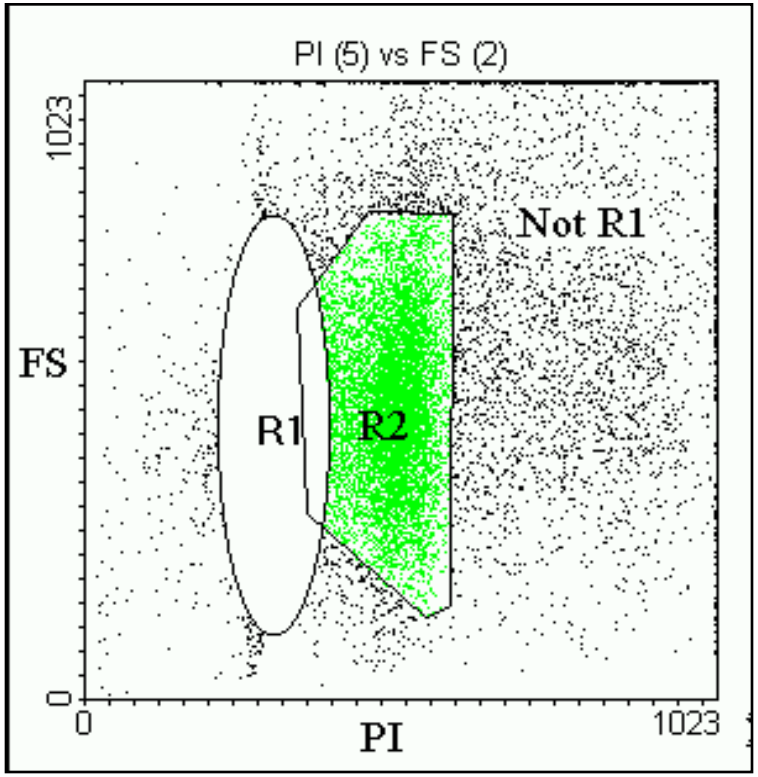
Boolean Gating

Not Region 1:



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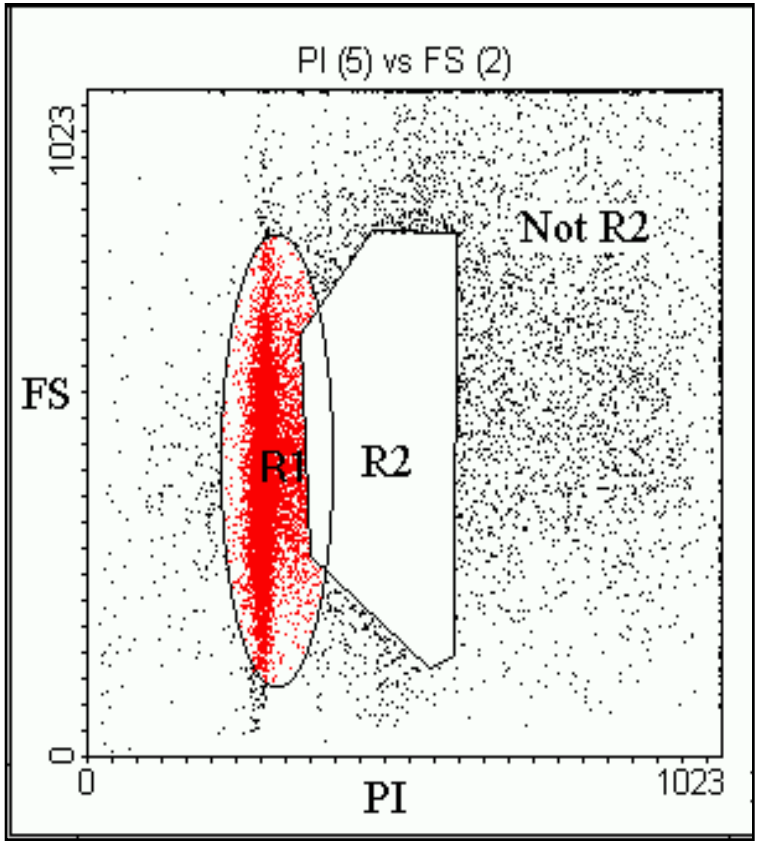
Boolean Gating

Not Region 2:



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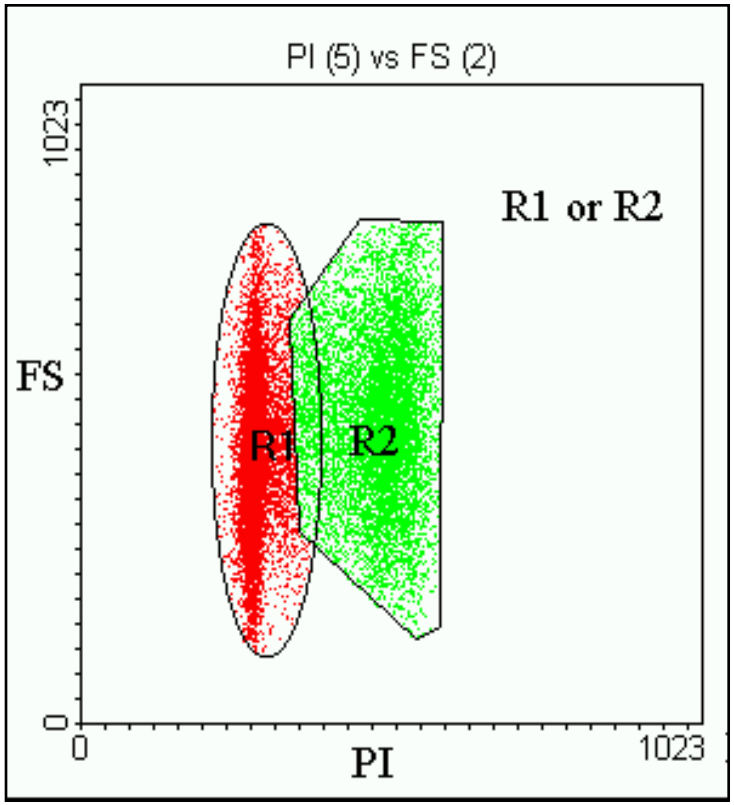
Boolean Gating

Region 1 or Region 2:



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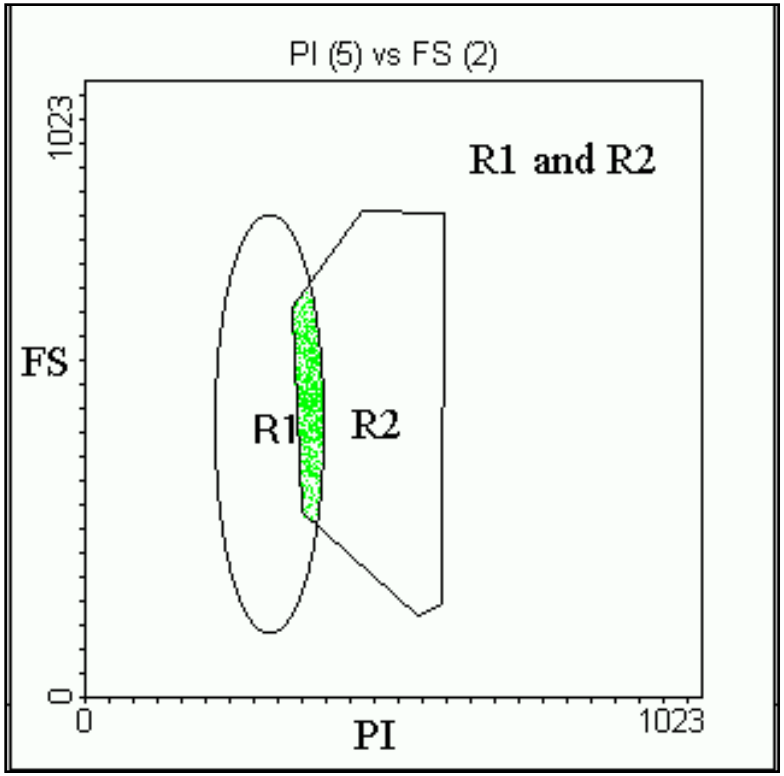
Boolean Gating

Region 1 and Region 2:



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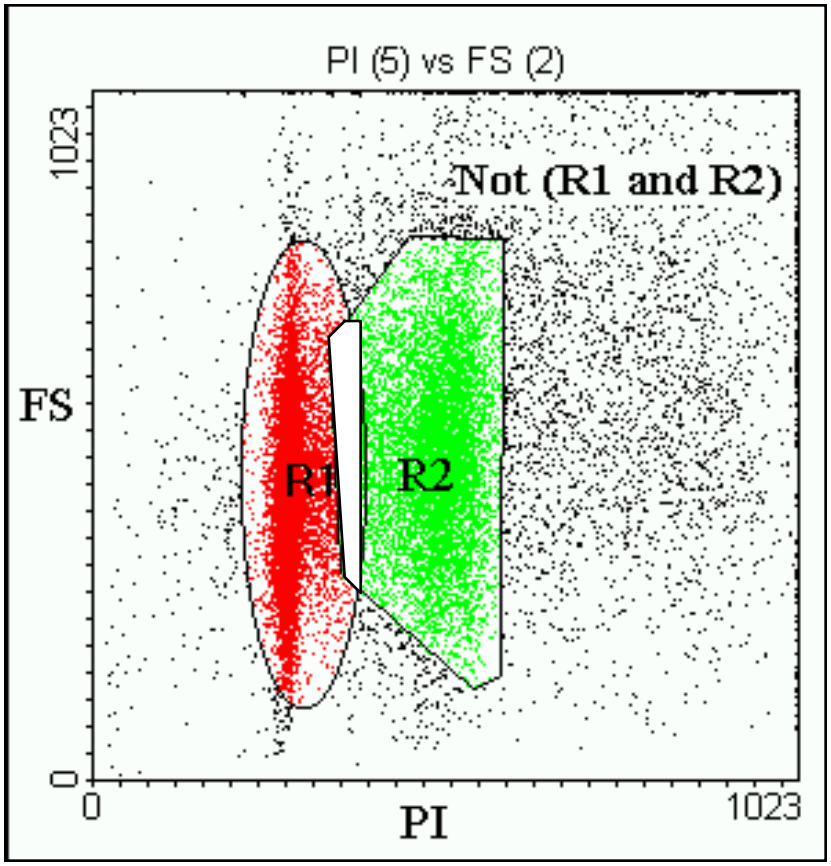
Boolean Gating

Not (Region1 and Region 2):



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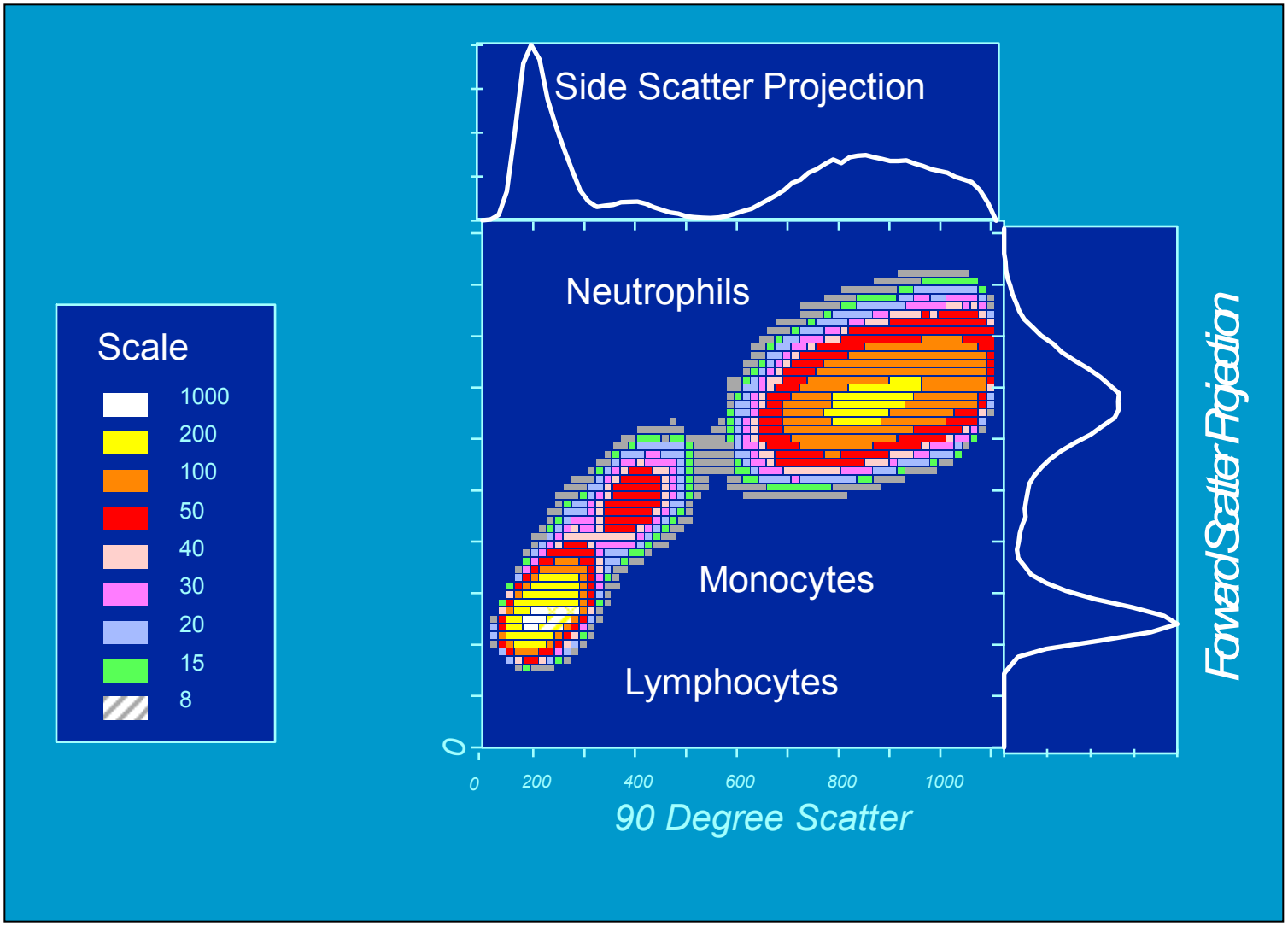
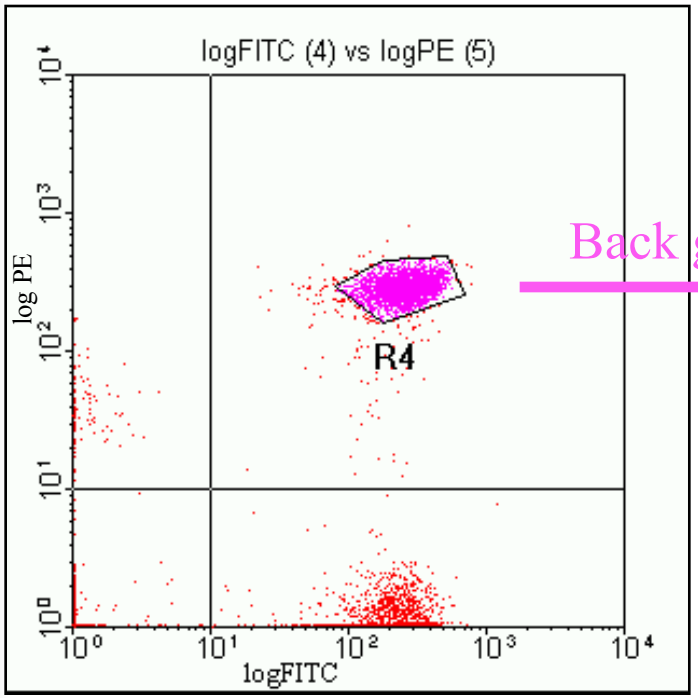
Light Scatter Gating

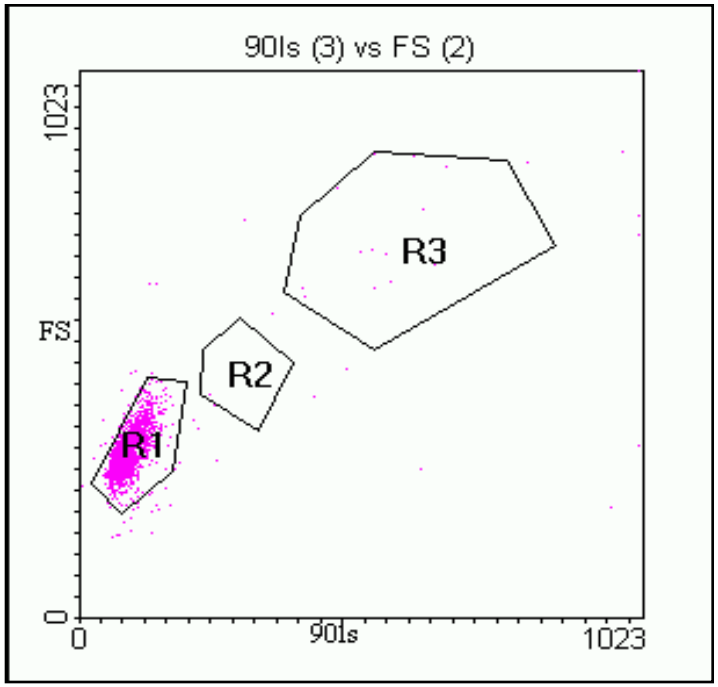


upraveno podle J.P.Robinson

Back Gating



Back gate

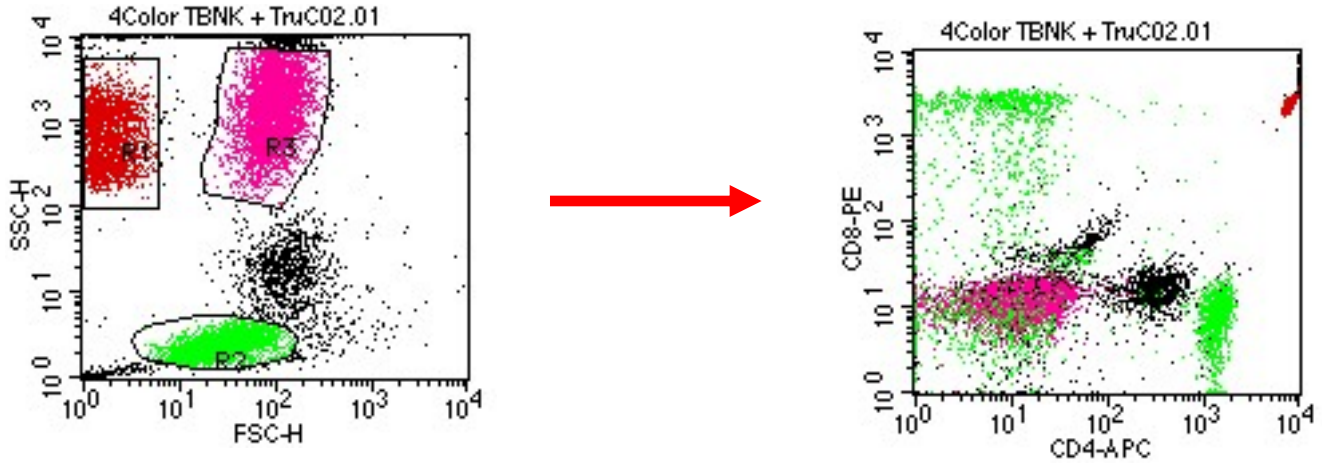


Region 4 established

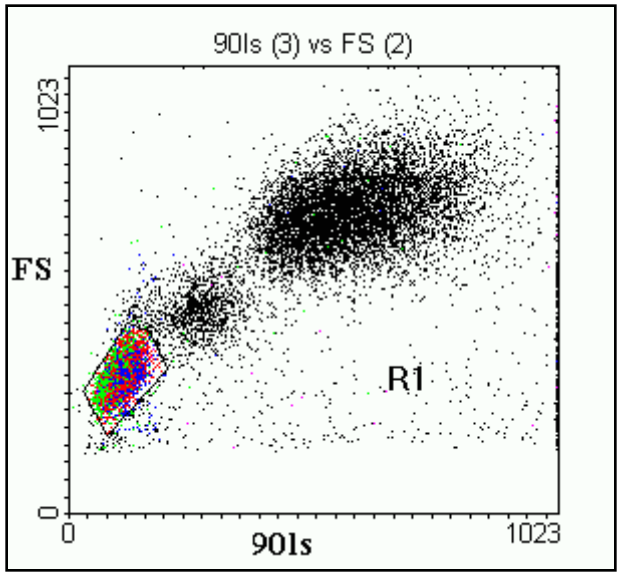
Back-gating using Region 4

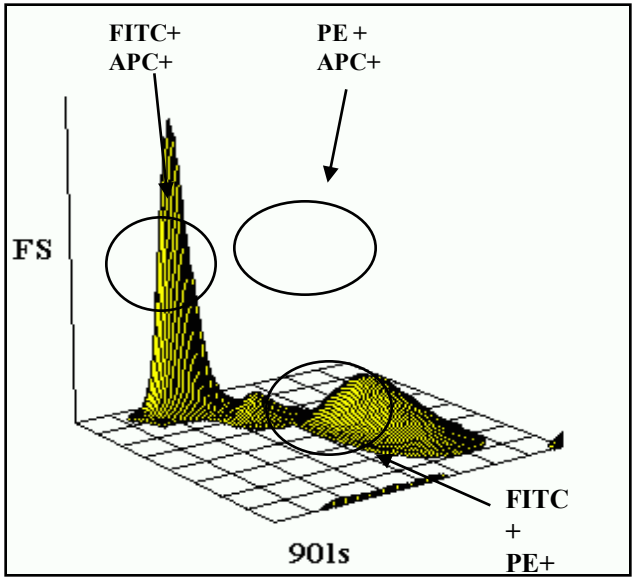
upraveno podle J.P.Robinson

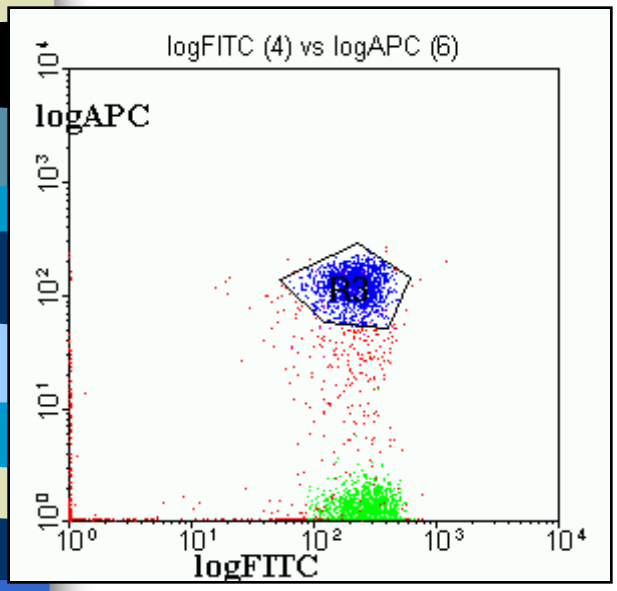
Back Gating

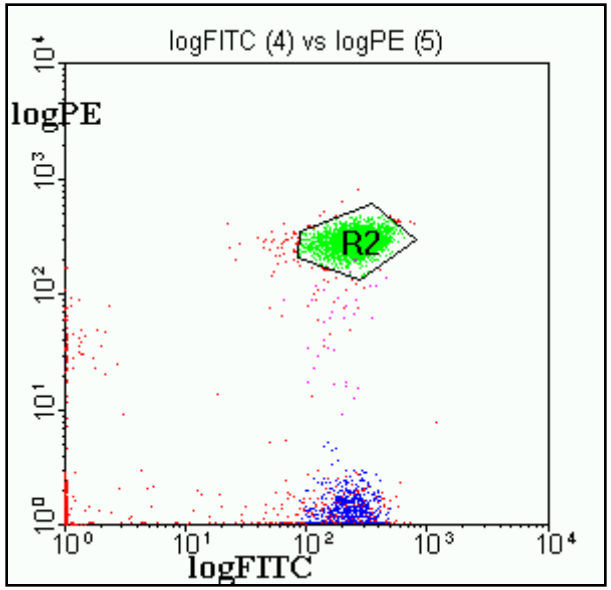

4Color TBNK + TruC02.01
SSC-H
FSC-H
R1
R3
R2
4Color TBNK + TruC02.01
CD8-PE
CD4-APC

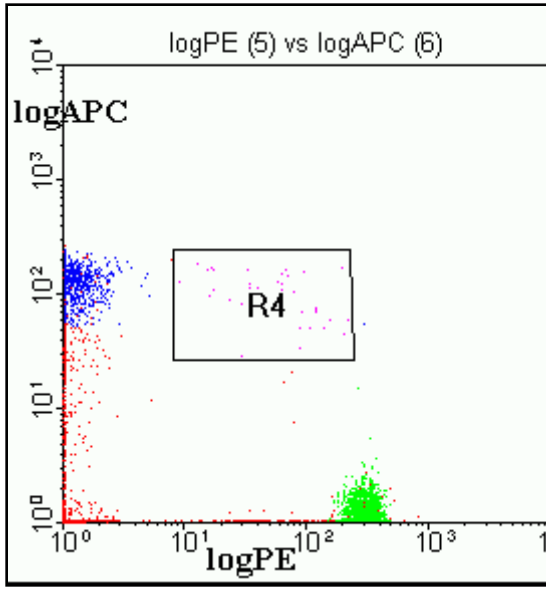
3 Parameter Data Display











upraveno podle J.P.Robinson

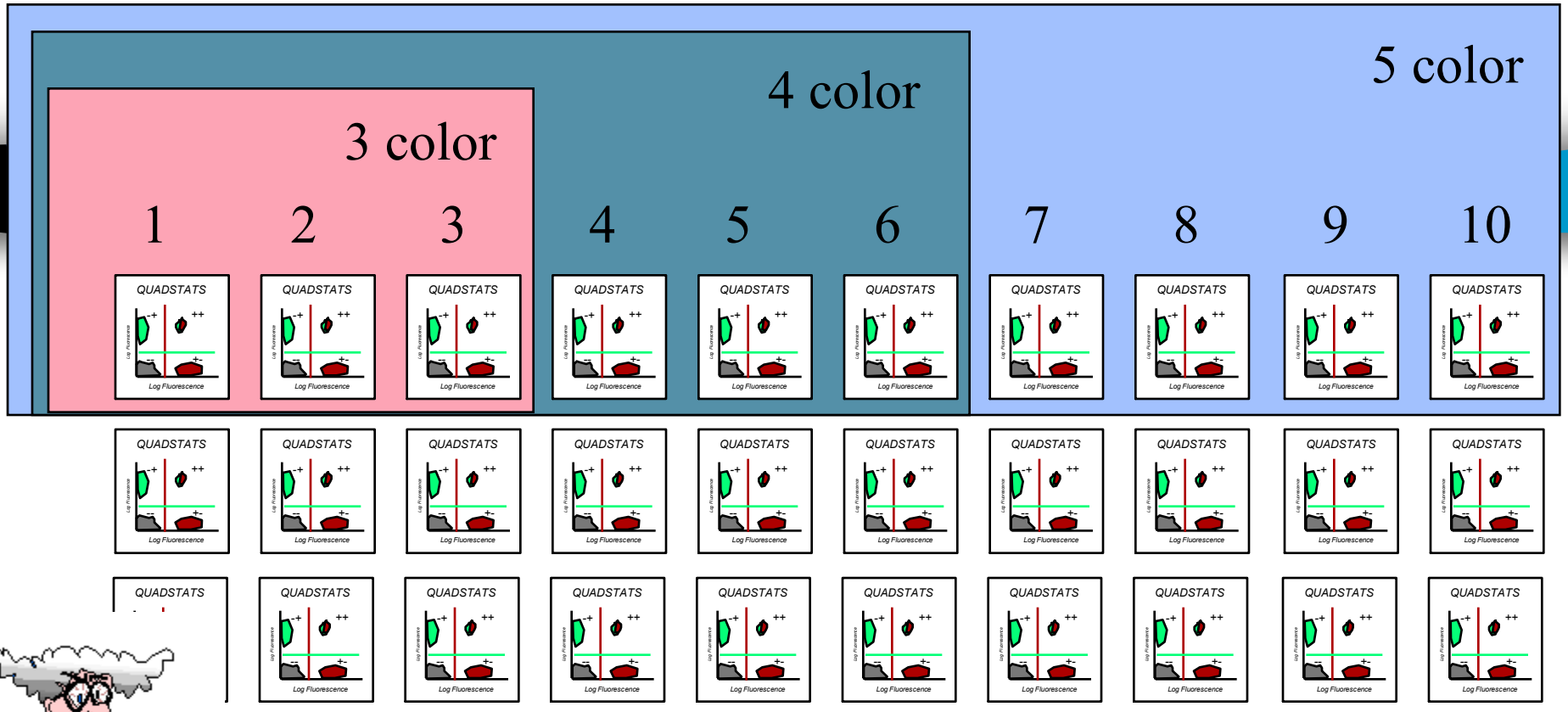
Nástroje pro analýzu dat

- Výrobci HW
  - Beckman Coulter
    - Kaluza
  - Becton Dickinson
    - FACSDiva
    - FACSSuite
    - FlowJo
  - BioRad
  - Sony
  - Milteney
  - …
- Univerzální platformy
  - Komerční
    - FlowJo
    - FCS Express
    - …
  - Freeware
    - Flowing Software
    - Cyflogic
    - BioConductor - Flowcore

De Novo™ Software

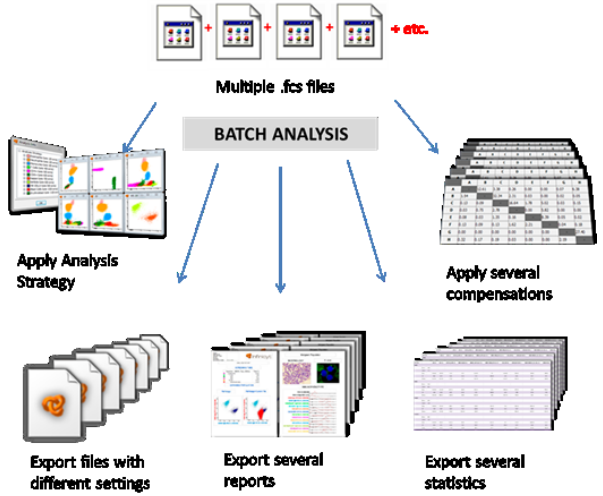
Turning Cytometry Data Into Results

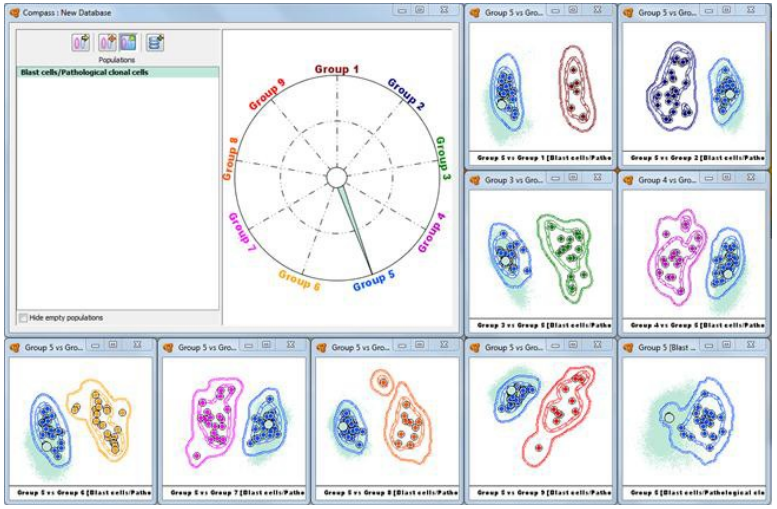
Vícebarevné analýzy generují mnoho dat…



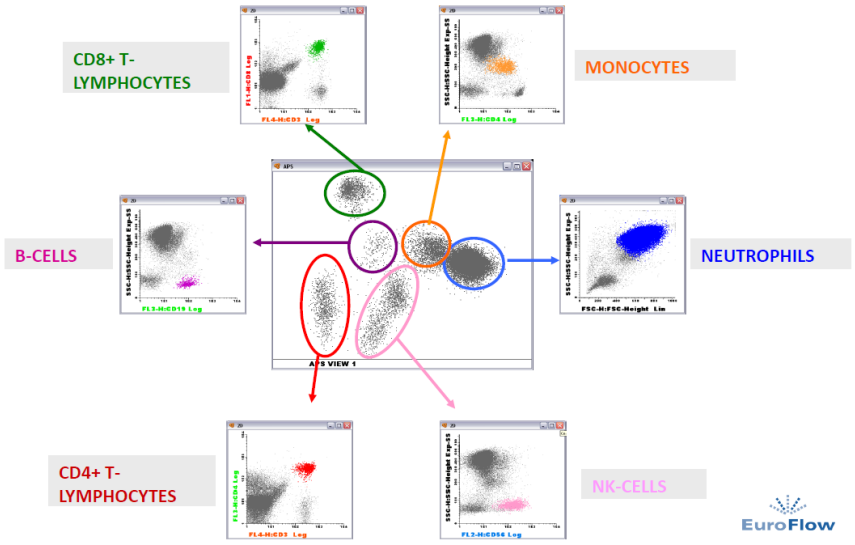
upraveno podle J.P.Robinson

Infinicyt™



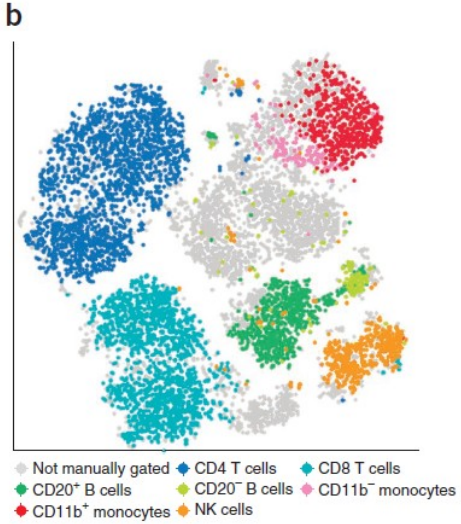


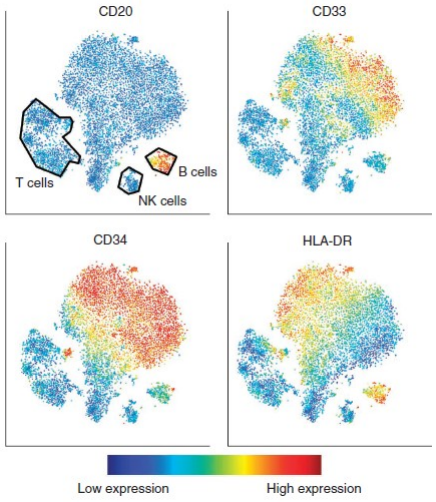
Automatic Population Separator

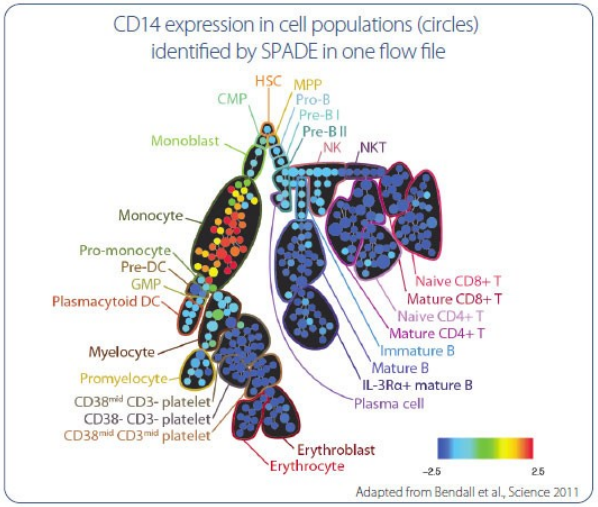


Další způsoby vizualizace vícerozměrných dat

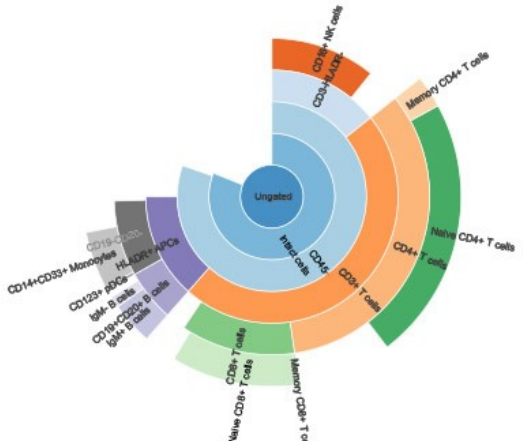
- t-SNE, viSNE
  - t-Distributed Stochastic Neighbor Embedding
  - viSNE is a tool for reducing high-parameter data down to two dimensions
  - visually identify interesting and rare biological subsets
  - allow to gate single cell events across different samples.
- SPADE
  - Spanning-tree Progression 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

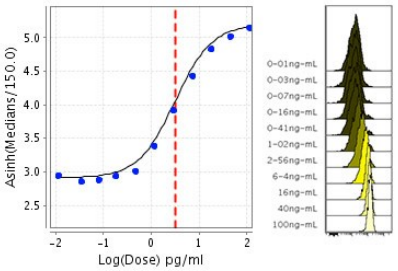


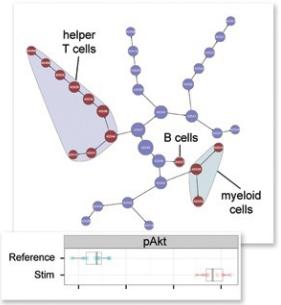


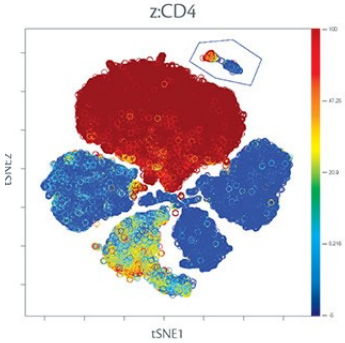


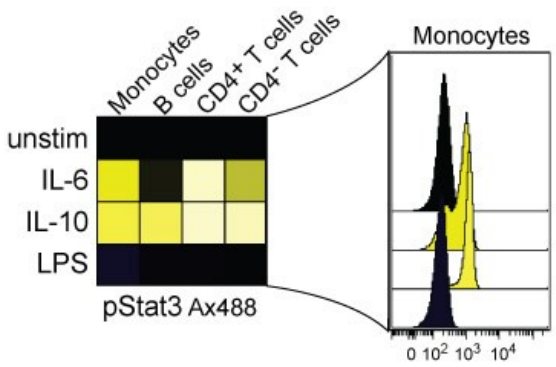
Cytobank

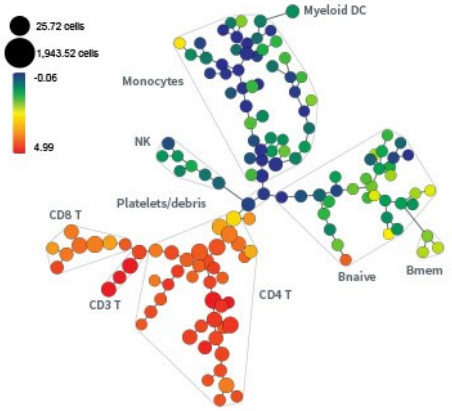

Ungated
Intact cells
CD45+
CD3+ T cells
CD4+ T cells
CD8+ T cells
Memory CD4+ T cells
Naive CD4+ T cells
Memory CD8+ T cells
Naive CD8+ T cells
CD3-/CD56+ NK cells
CD3-/HLADR+
CD14+CD33+ Monocytes
HLADR+ APCs
CD123+ pDCs
IgM- B cells
CD19+CD20+ B cells
IgM+ B cells

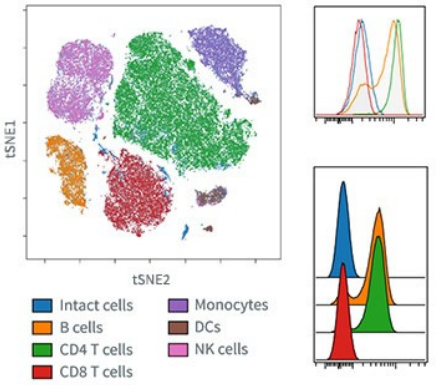

Asinh(Medians/150.0)
Log(Dose) pg/ml
0-01ng-mL
0-03ng-mL
0-07ng-mL
0-16ng-mL
0-41ng-mL
1-02ng-mL
2-56ng-mL
6-4ng-mL
16ng-mL
40ng-mL
100ng-mL


helper T cells
B cells
myeloid cells
pAkt
Reference
Stim


z:CD4
tSNE1
tSNE2


Monocytes
B cells
CD4+ T cells
CD4- T cells
unstim
IL-6
IL-10
LPS
pStat3 Ax488
Monocytes
0 10^2 10^3 10^4


25.72 cells
1,943.52 cells
-0.06
4.99
Myeloid DC
Monocytes
NK
Platelets/debris
CD8 T
CD3 T
CD4 T
Bnaive
Bmem

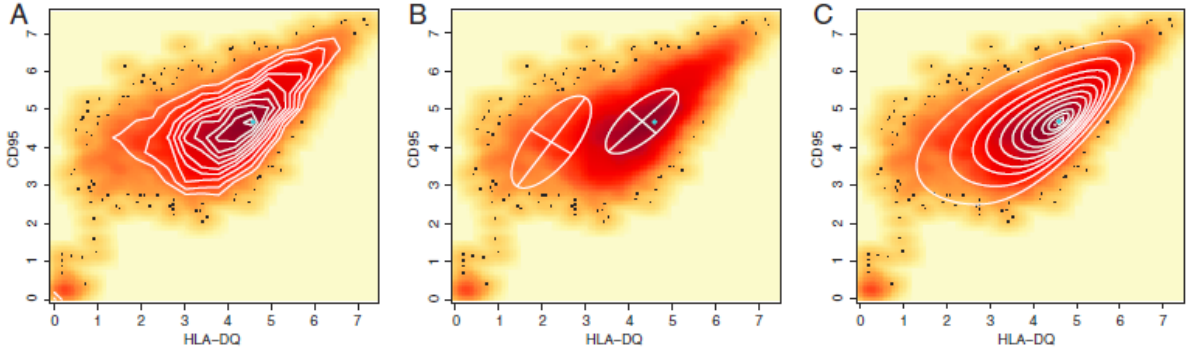

tSNE1
tSNE2
Intact cells
B cells
CD4 T cells
CD8 T cells
Monocytes
DCs
NK cells

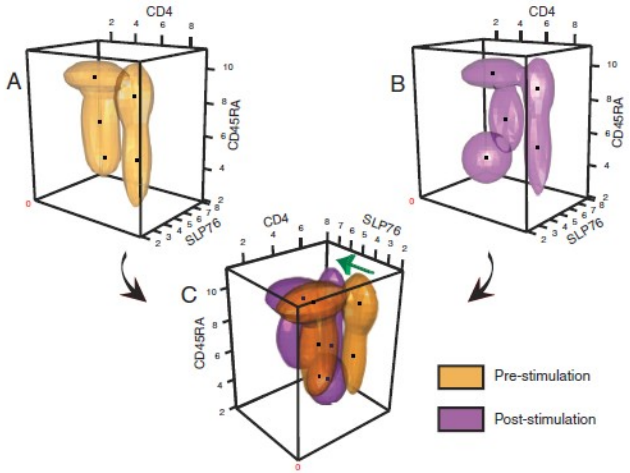
Automated high-dimensional flow cytometric data analysis

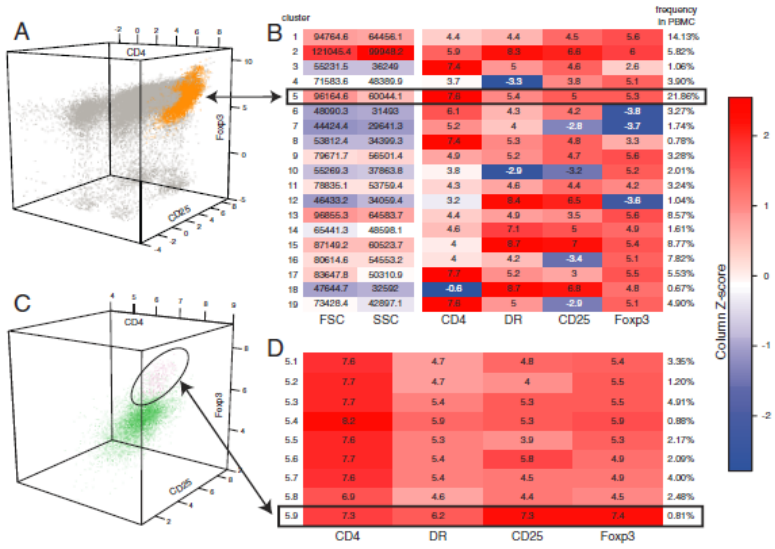
Saumyadipta Pyne[a], Xinli Hu[a,1], Kui Wang[b,1], Elizabeth Rossin[a,1], Tsung-I Lin[c], Lisa M. Maier[a,d], Clare Baecher-Allan[d], Geoffrey J. McLachlan[b,e], Pablo Tamayo[a], David A. Hafler[a,d,2], Philip L. De Jager[a,d,f,3], and Jill P. Mesirov[a,2,3]

[a]Broad Institute of MIT and Harvard, 7 Cambridge Center, Cambridge MA 02142; [b]Department of Mathematics and [e]Institute for Molecular Bioscience, University of Queensland, St. Lucia, Queensland, 4072, Australia; [c]Department of Applied Mathematics, National Chung Hsing University, Taichung 402, Taiwan; [d]Division of Molecular Immunology, Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115; and [f]Partners 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 Aghaeepour[1], Greg Finak[2], The FlowCAP Consortium[3], The DREAM Consortium[3], Holger Hoos[4], Tim R Mosmann[5], Ryan Brinkman[1,7], Raphael Gottardo[2,7] & Richard H Scheuermann[6,7]

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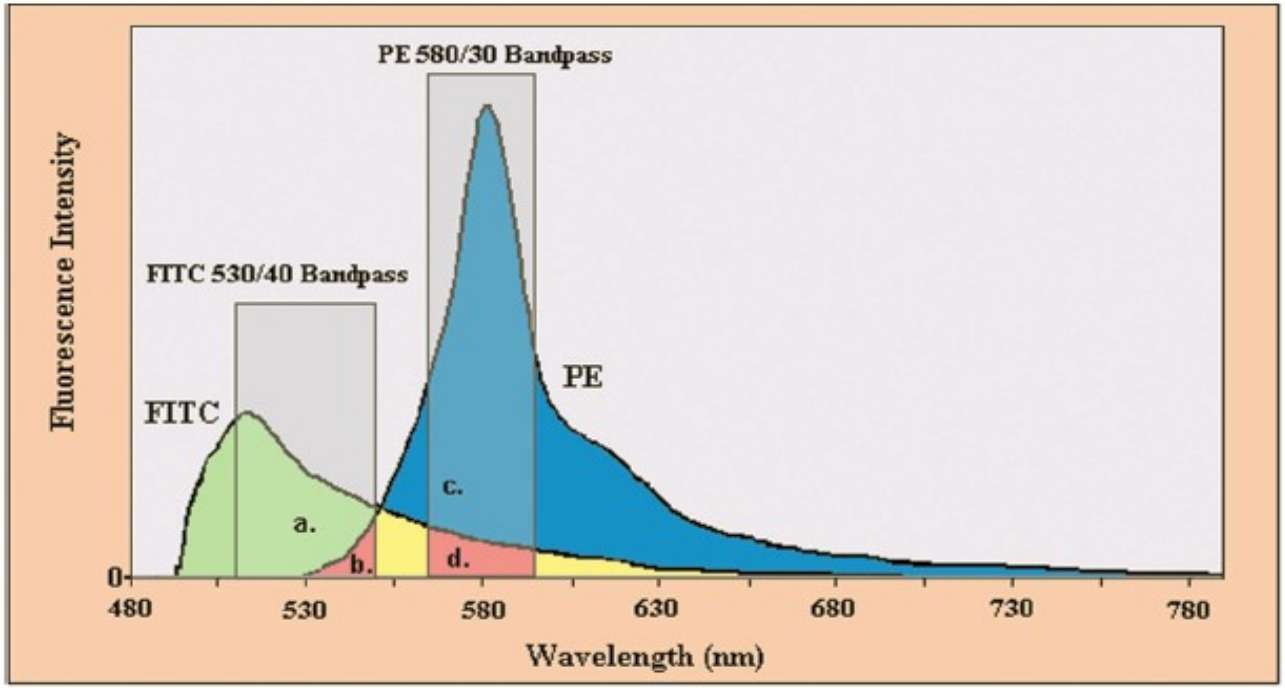
Způsoby pomocí kterých lze upravit výsledky:

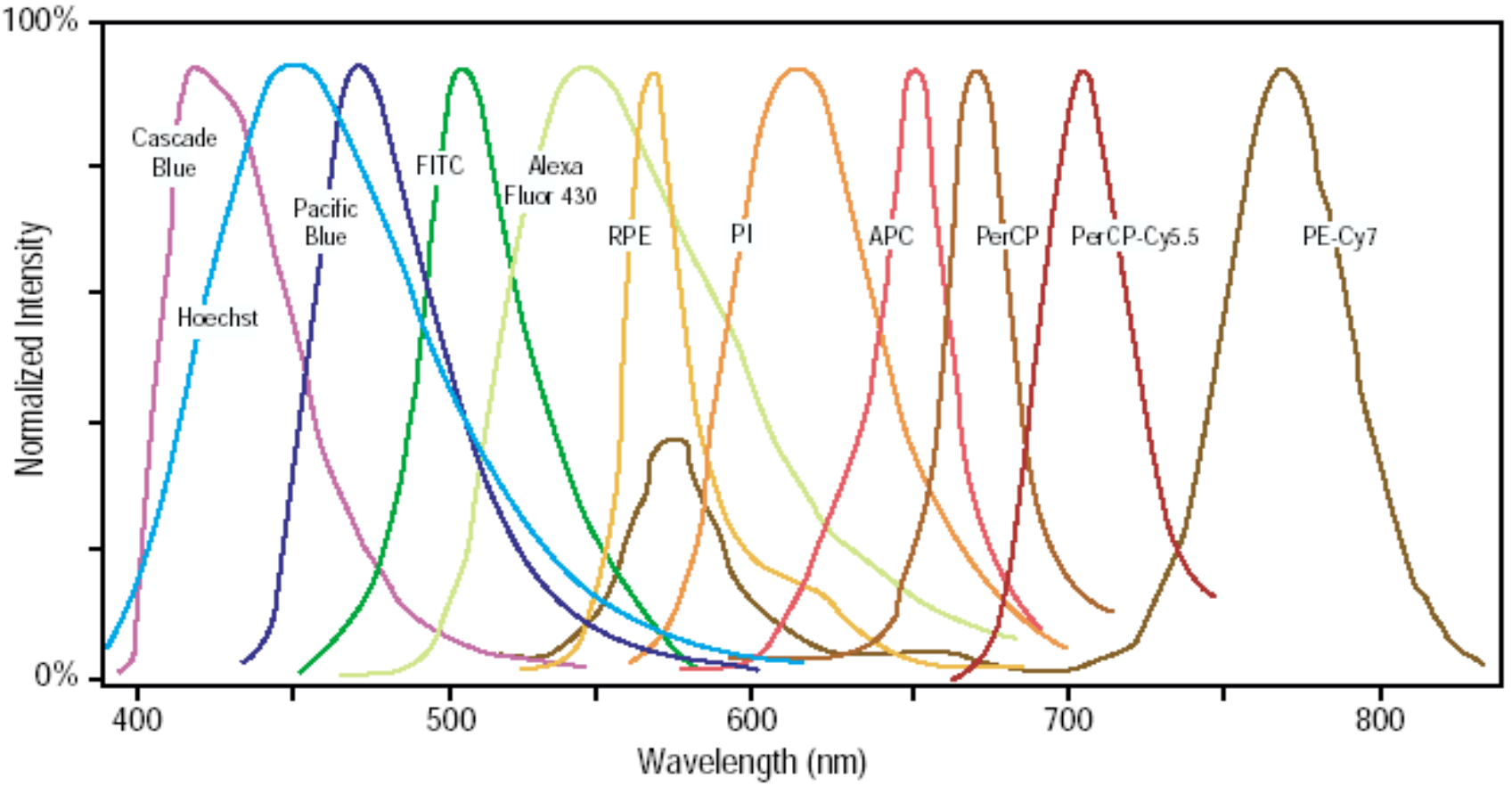
1. Odstranění „doublets“
2. Čas jako parametr pro kontrolu kvality

Příklad - pro DNA analýzu je třeba:
- odstranit „debris“ a shluky
- odstranit „doublets“
- udržovat konstantní průtok

Co je problém při vícebarevné detekci?



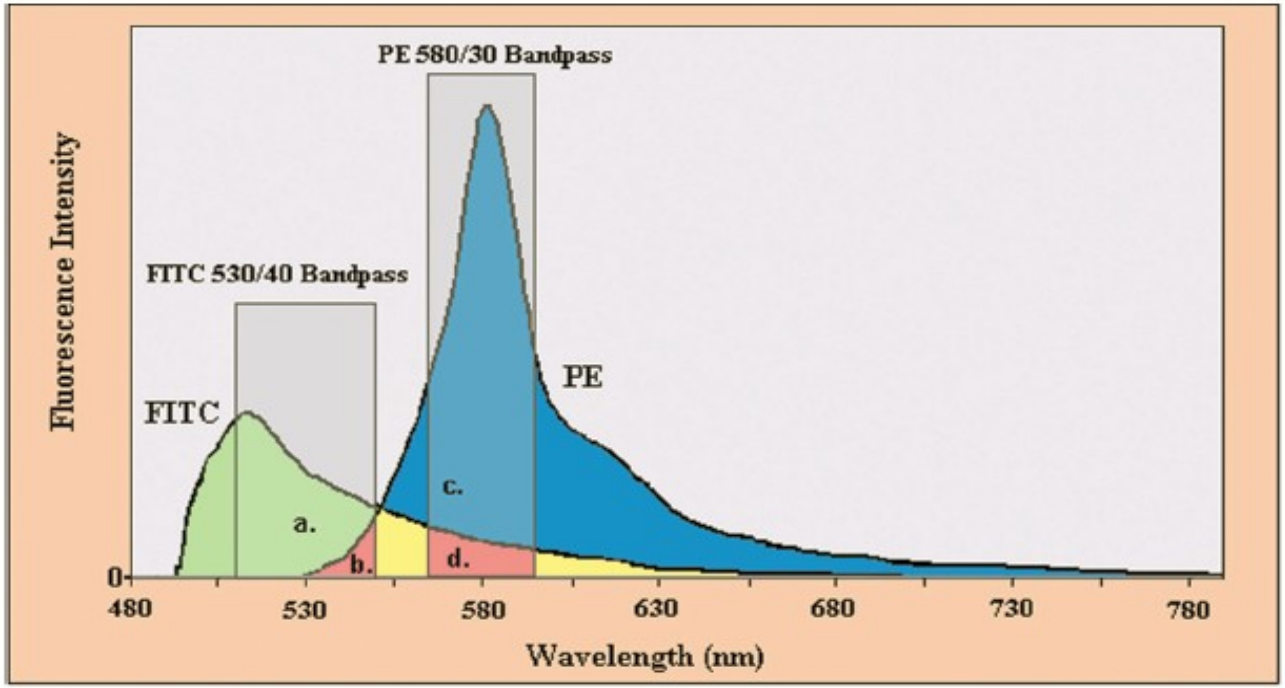
Emission Spectra–Spectral Overlap



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

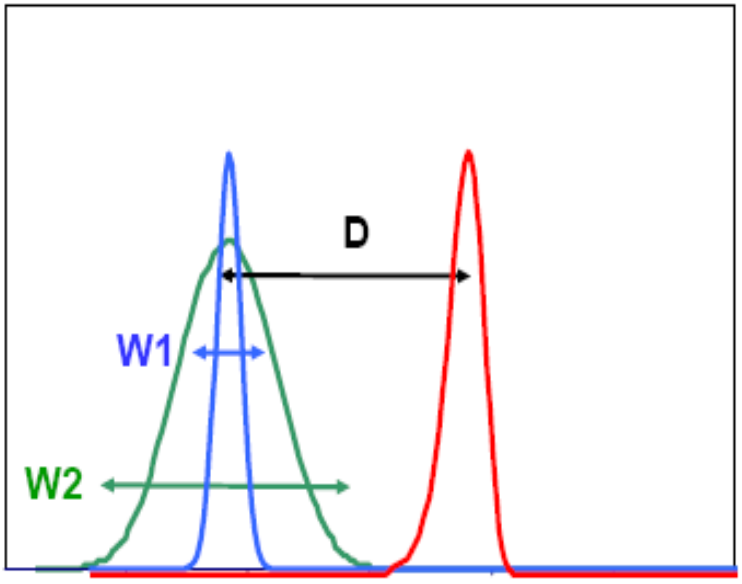
Table 1.14.1 Typical Spillover Matrix for a Three-Color Compensation[a]

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

[a]Note: 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



$$\text{Stain Index (SI)} = {}^{D}\!/_{W}$$

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	7801/60	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…

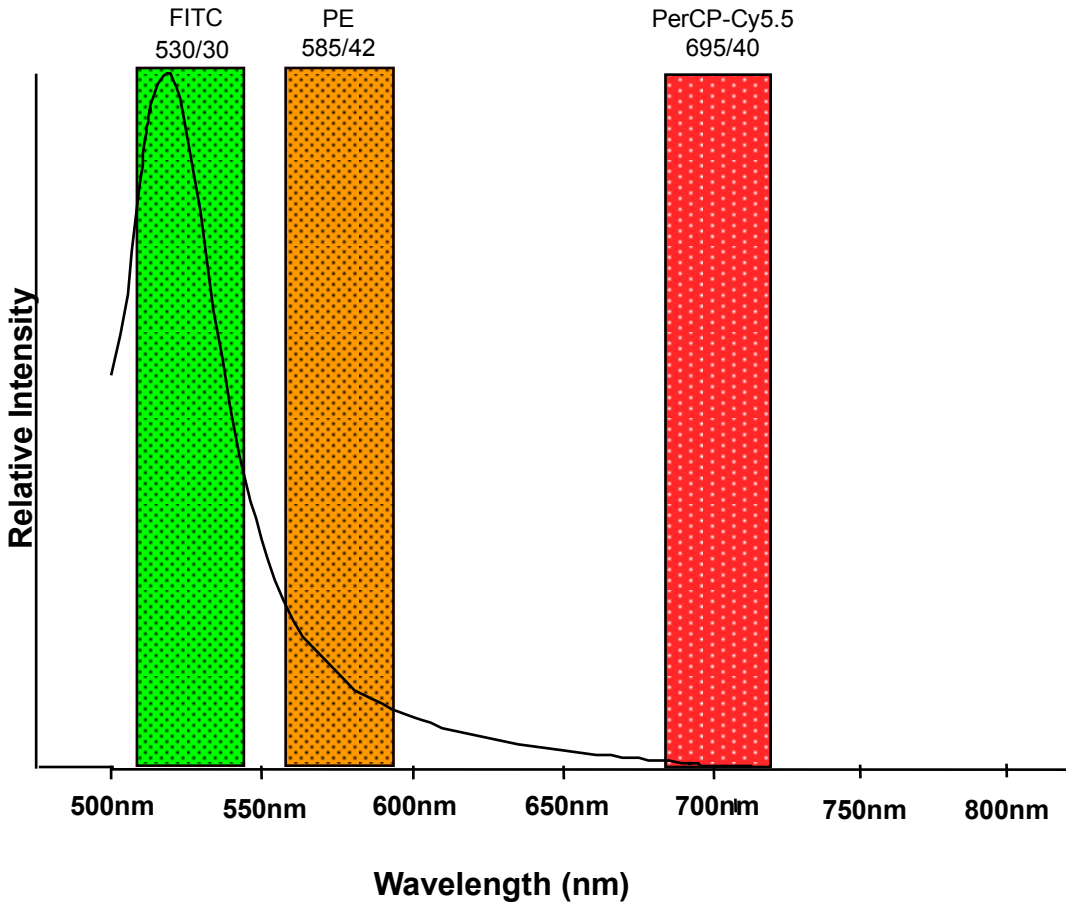
Fluorochrome selection considerations

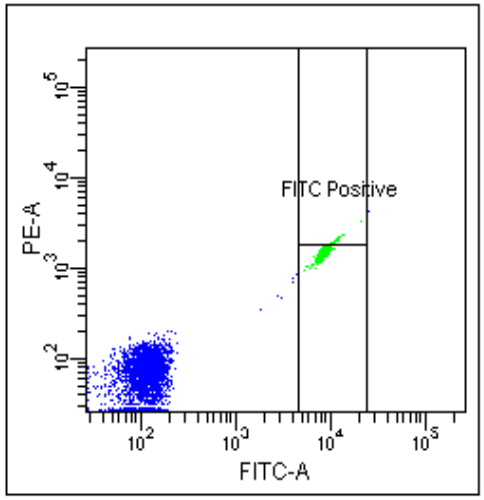
"Bright" antibodies go on "dim" fluorochromes

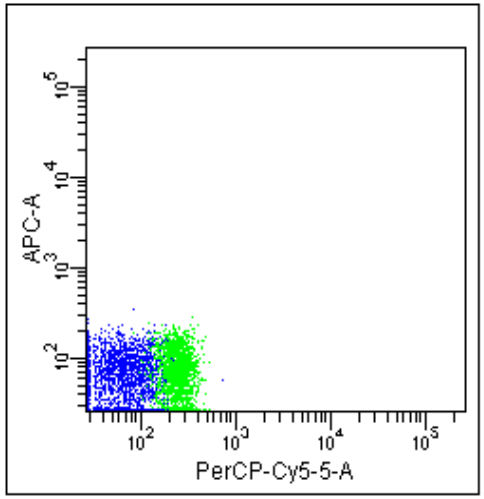
Avoid spillover from bright cell populations into channels requiring high sensitivity

Beware of tandem dye degradation

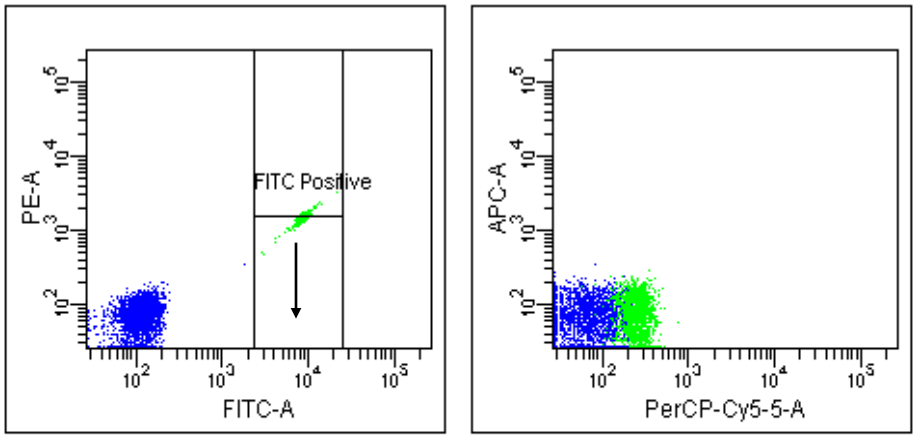
FITC Spillover



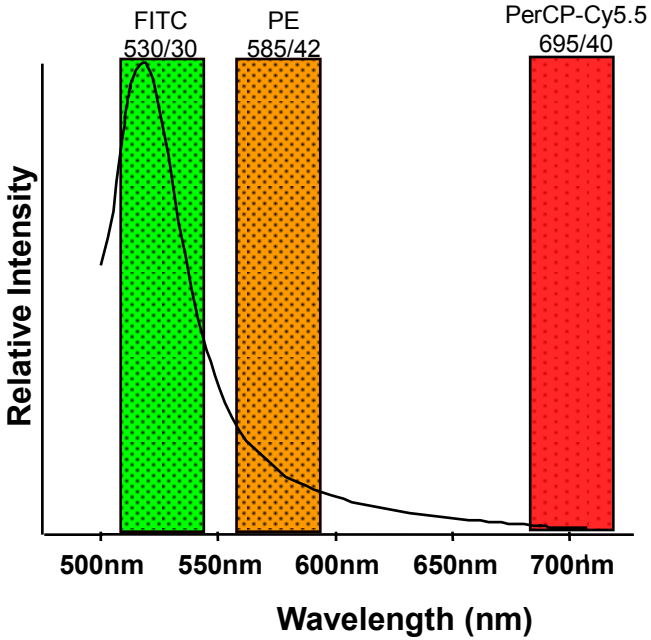




FITC Compensation

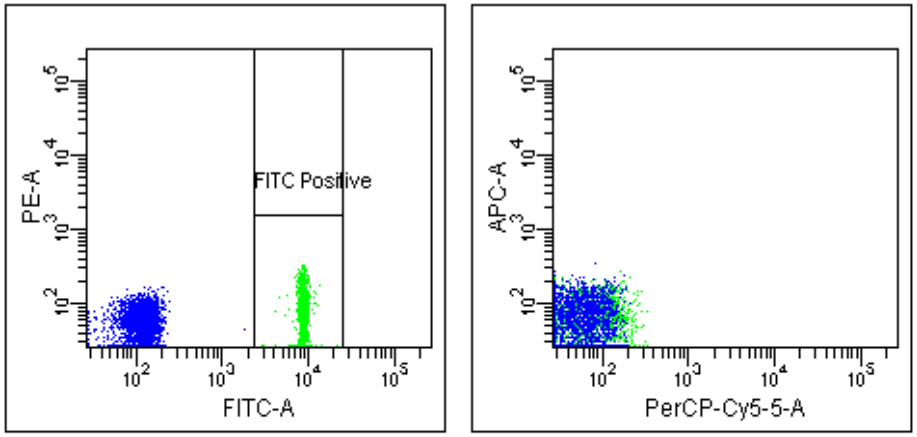


Population	FITC-A Median	PE-A Median	PerCP-Cy5-5-A Median	APC-A Median
FITC Positive	8,776	1,499	226	63
FITC Negative	113	70	52	56

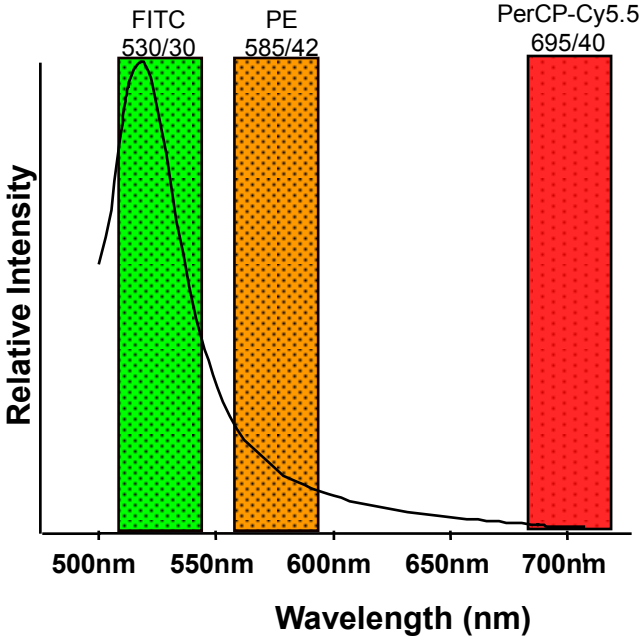


	Fluorochrome	- % Fluorochrome	Spectral Overlap
•	PE	FITC	0.00
•	PerCP-Cy5-5	FITC	0.00
•	APC	FITC	0.00

FITC Compensation

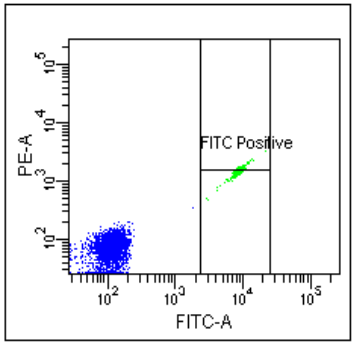


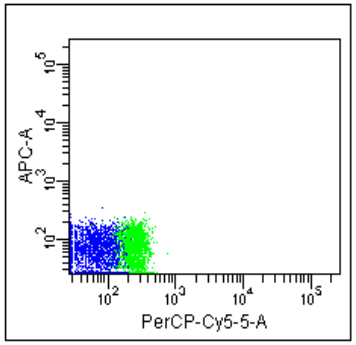
Population	FITC-A Median	PE-A Median	PerCP-Cy5-5-A Median	APC-A Median
FITC Positive	8,776	54	49	53
FITC Negative	113	50	49	56



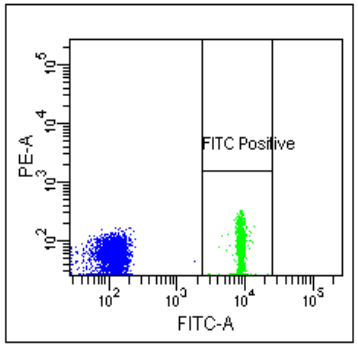
	Fluorochrome	- % Fluorochrome	Spectral Overlap
•	PE	FITC	16.50
•	PerCP-Cy5-5	FITC	2.00
•	APC	FITC	0.11

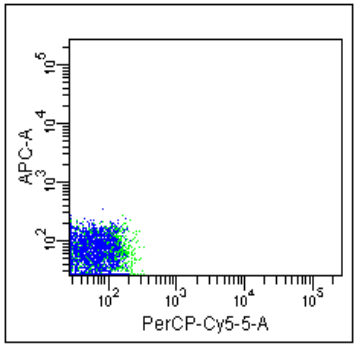
FITC Compensation



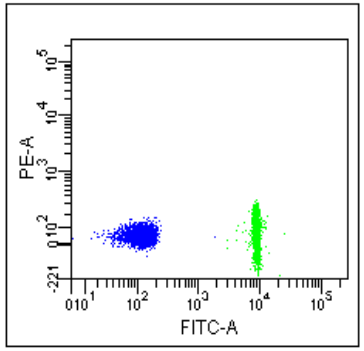


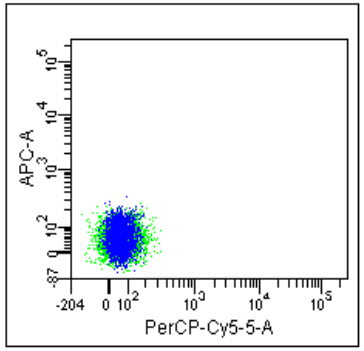
Dot plot showing uncompensated FITC data





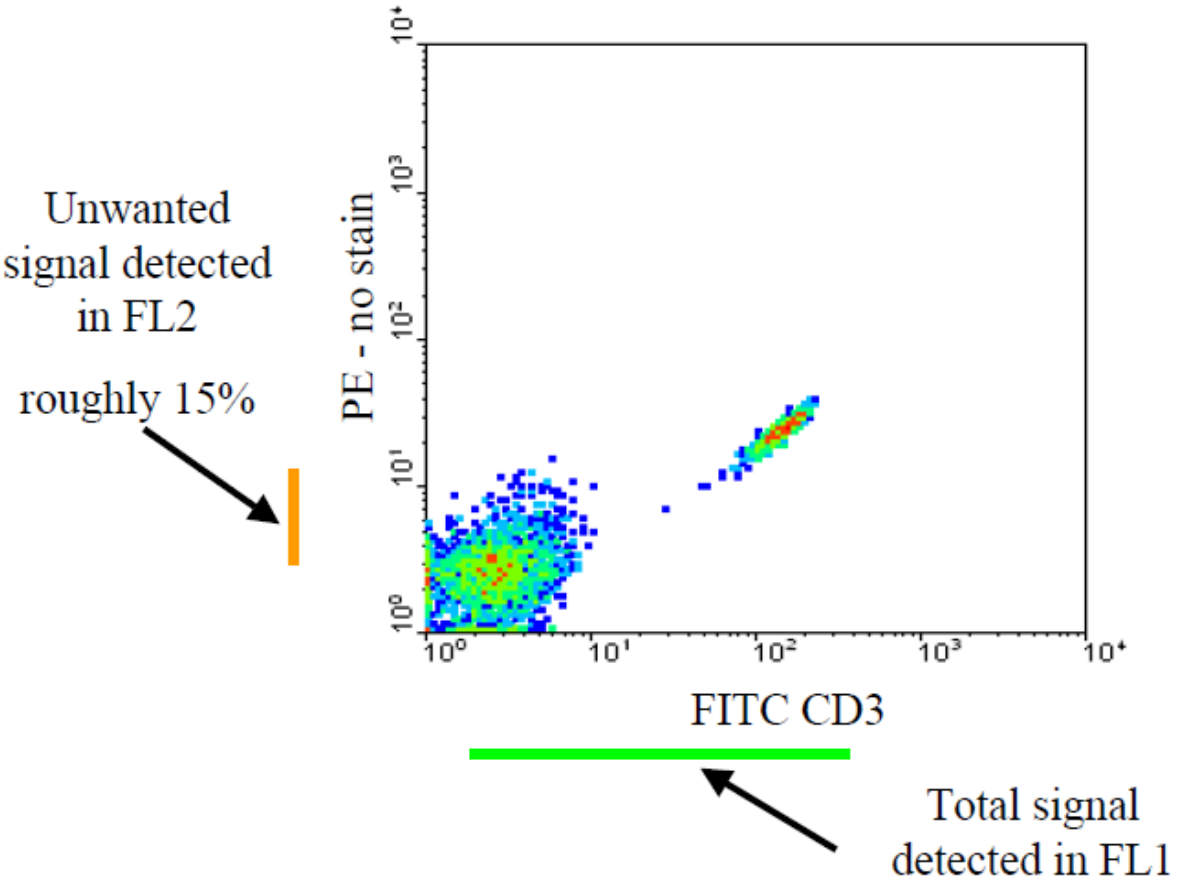
Dot plot showing compensated FITC data





Biexponential dot plot showing compensated FITC data

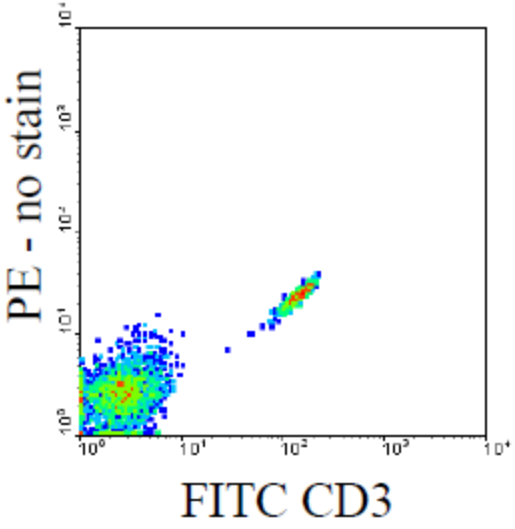
Uncompensated FITC Single stain Control



Jennifer Wilshire, PhD
jennifer@flowjo.com

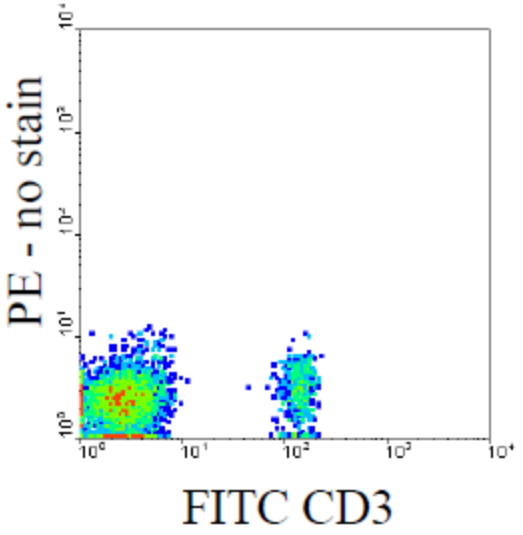
FITC Single Stain Control

Uncompensated



FL2-15%FL1

Compensated



Jennifer Wilshire, PhD
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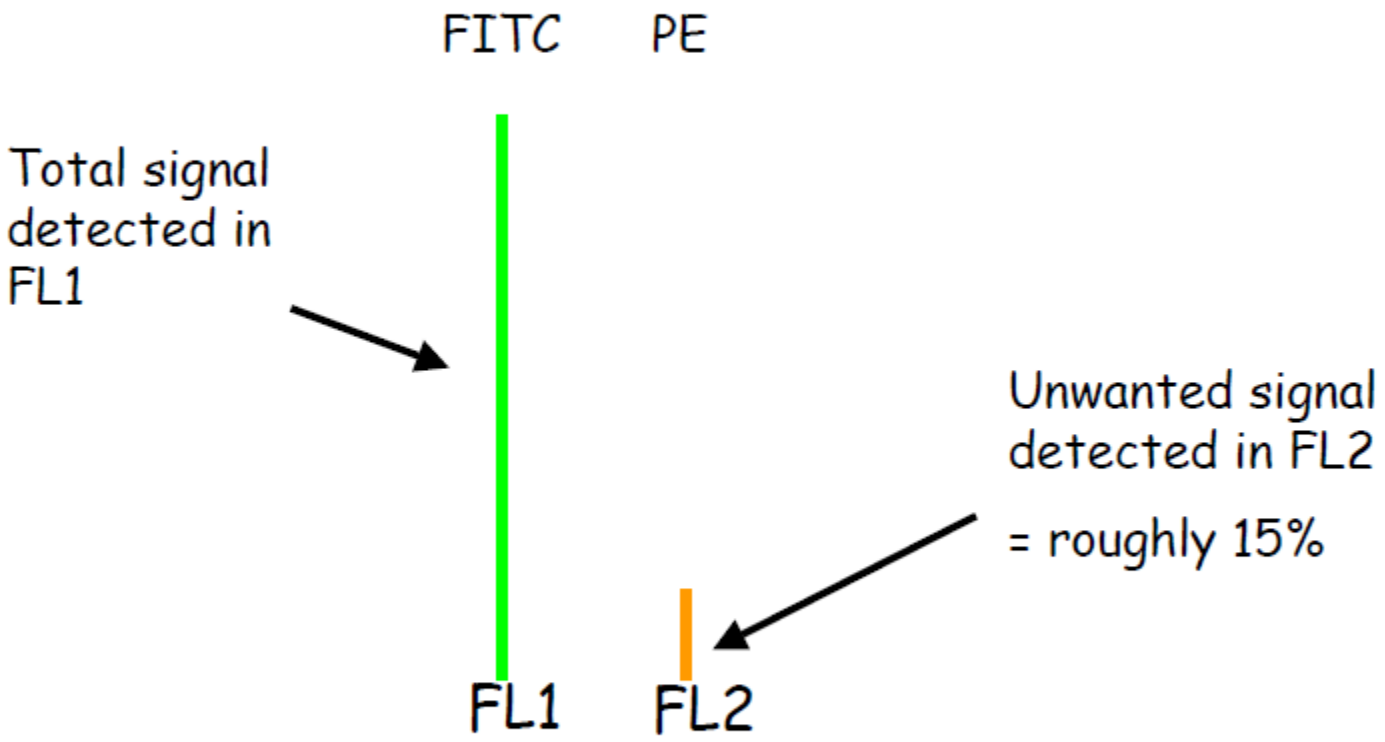
FITC Single Stain Control



True PE = Total FL2 – 15% FL1

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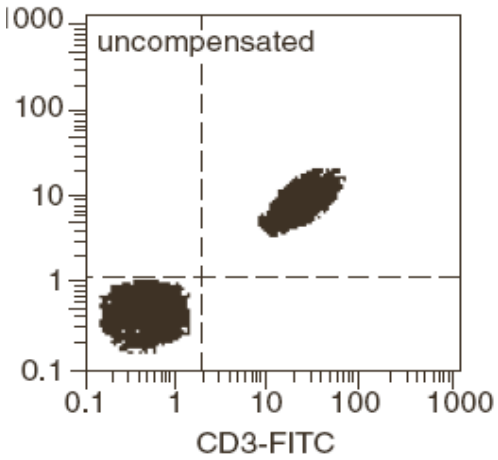
Kompenzace fluorescenčního signálu

#2

FITC positive & negative

PE negative beads



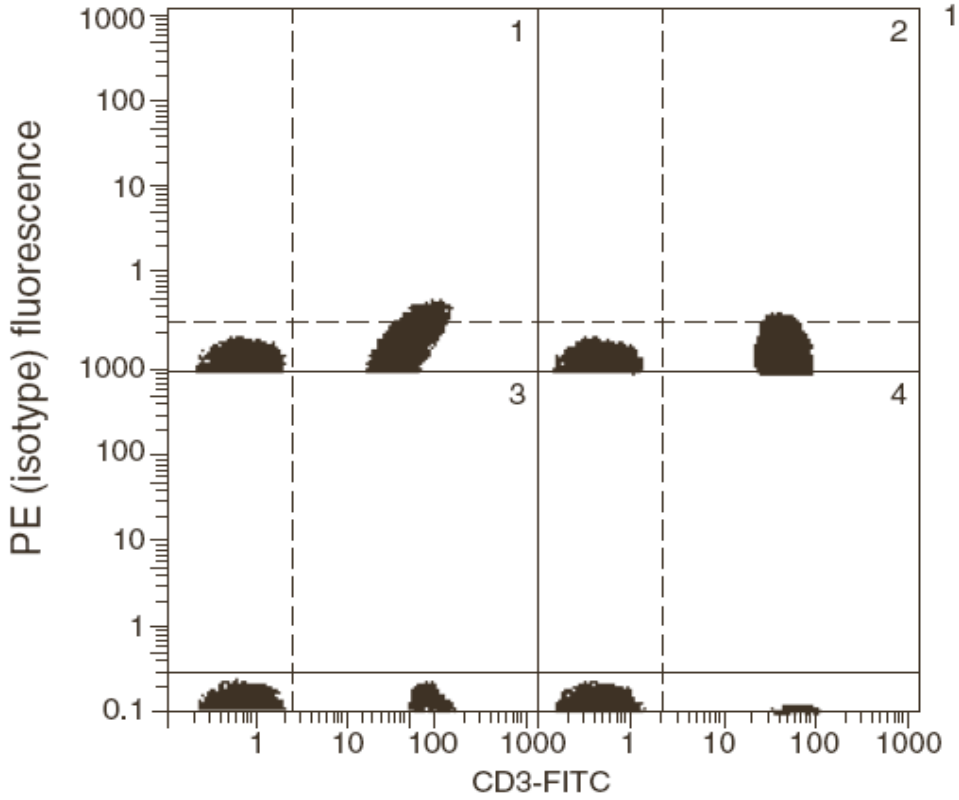
Current Protocols in Cytometry

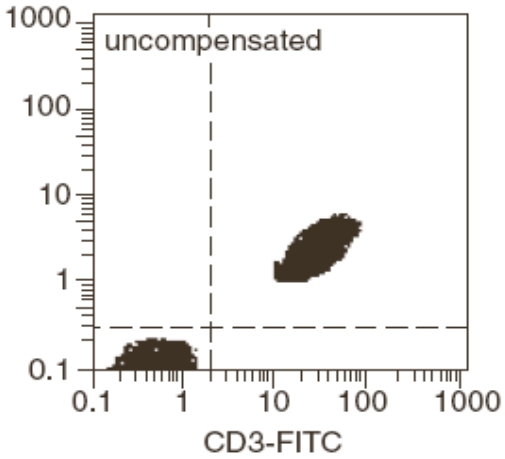
Kompenzace fluorescenčního signálu

FITC positive & negative

PE negative beads

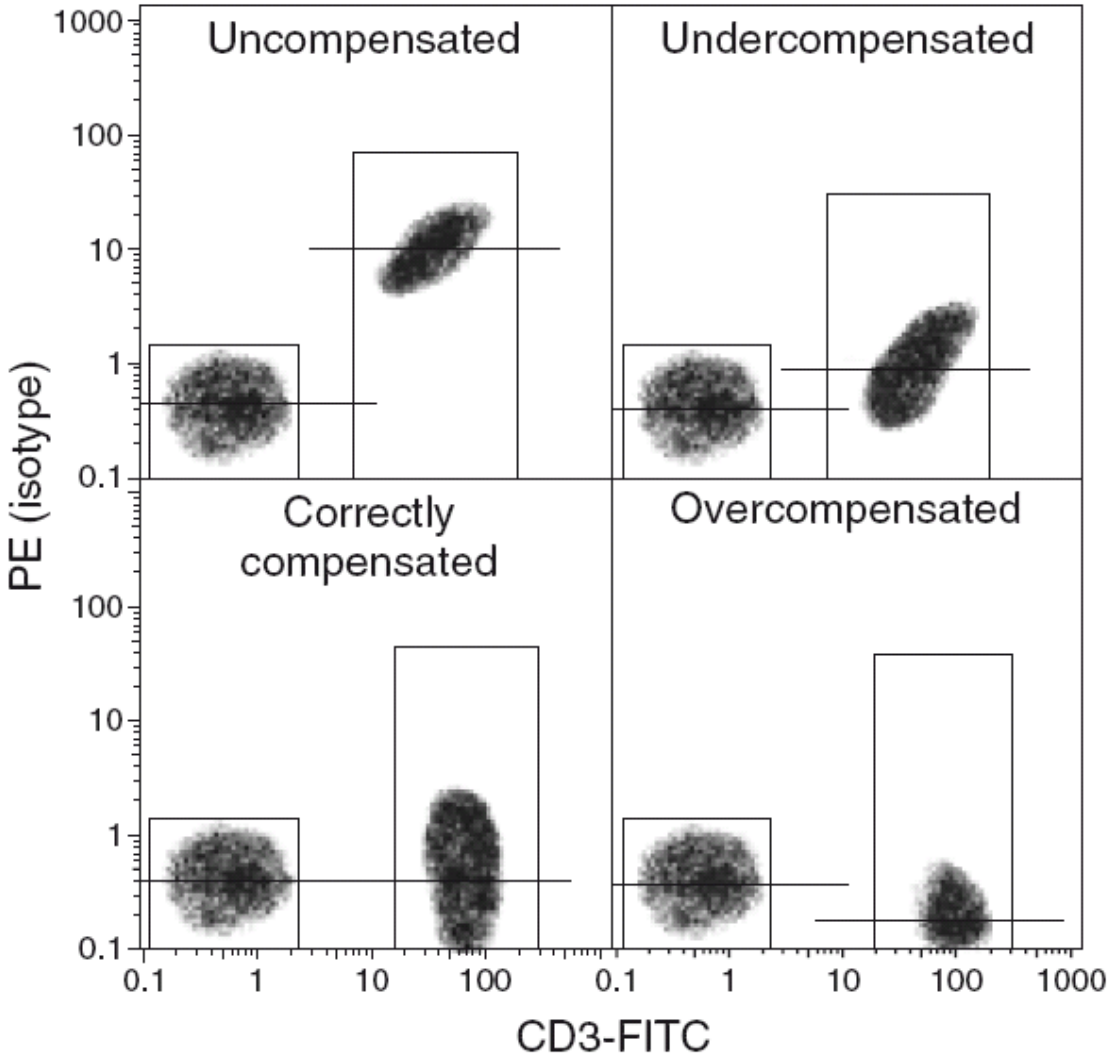
NONE!





Current Protocols in Cytometry

Kompenzace fluorescenčního signálu



Nastavení kompenzací

- značené mikročástice – pro běžně konjugované fluorochromy

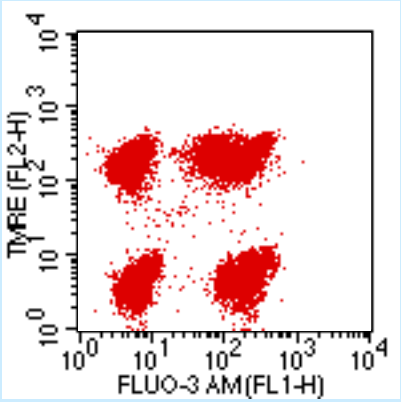
BD

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

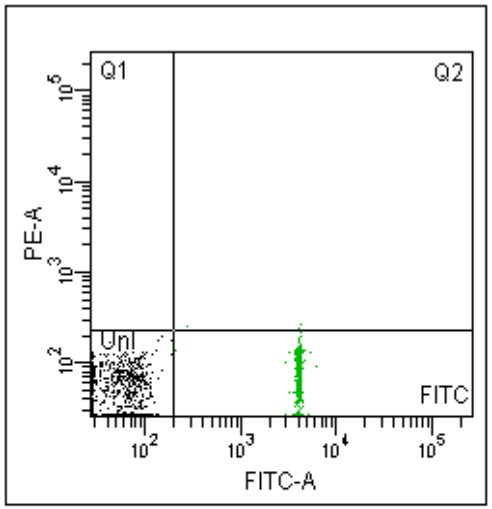
- značené buňky – pro vitální značení



parametr - detektor amp.
FL1 - 544
FL2 - 434
kompenzace
FL1 - 1.1%FL2
FL2 - 17.5%FL1

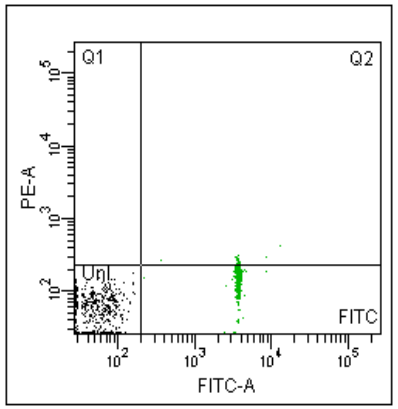
Effects of Changing PMT Values

Correct Compensation



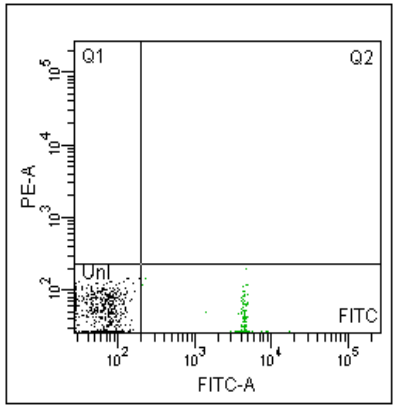
Population	PE-A Mean
Unl	69
FITC	64

FITC Voltage Decreased by 5 V



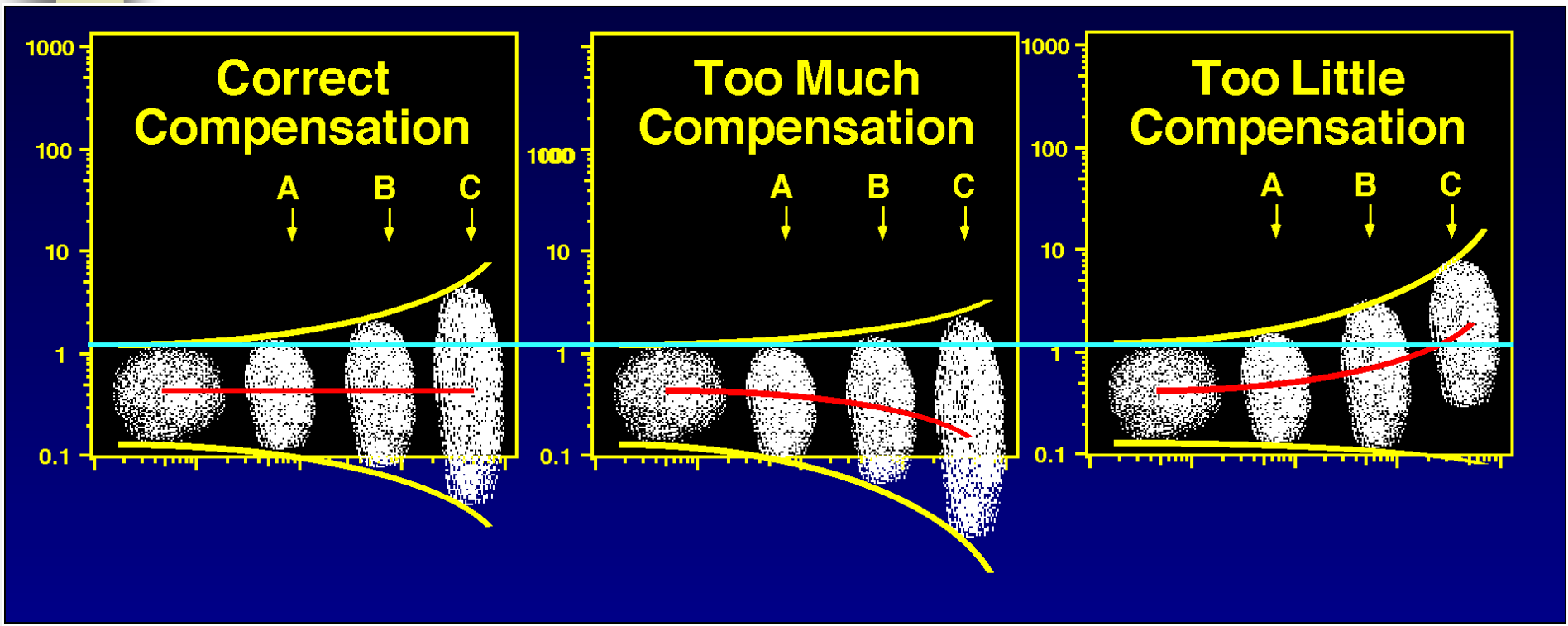
Population	PE-A Mean
Unl	68
FITC	132

FITC Voltage Increased by 5 V



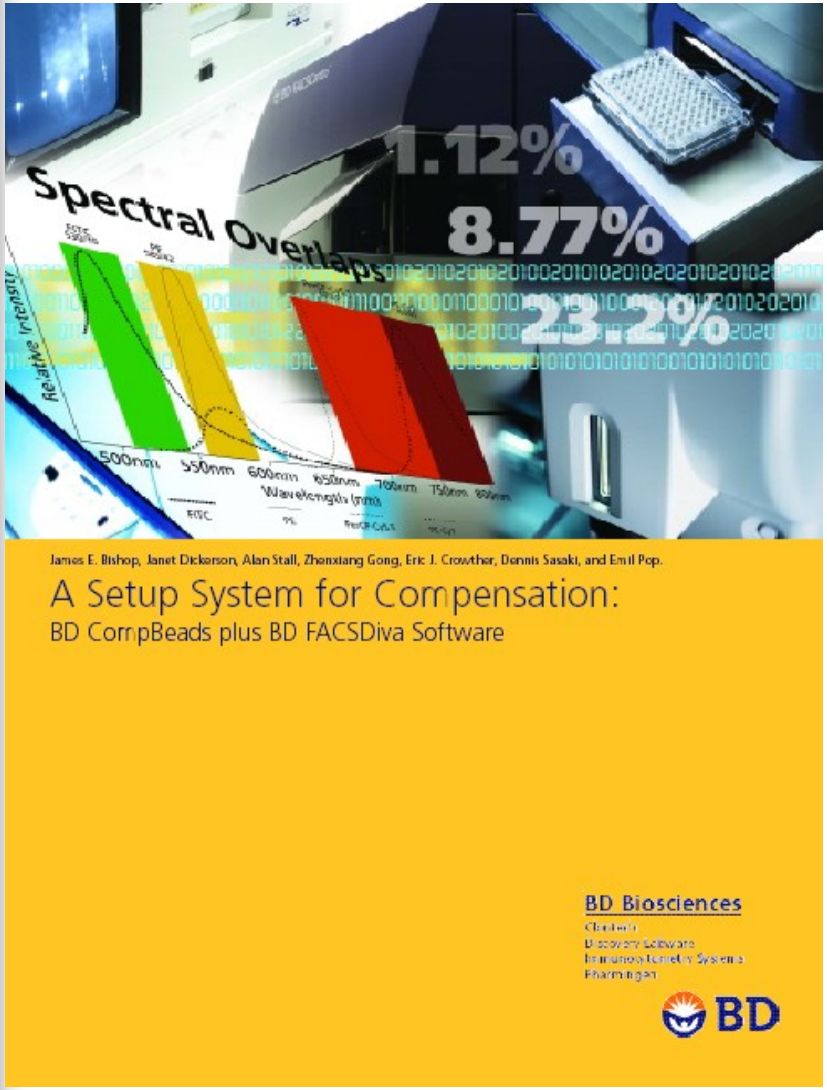
Population	PE-A Mean
Unl	67
FITC	49

Which marker for compensation?



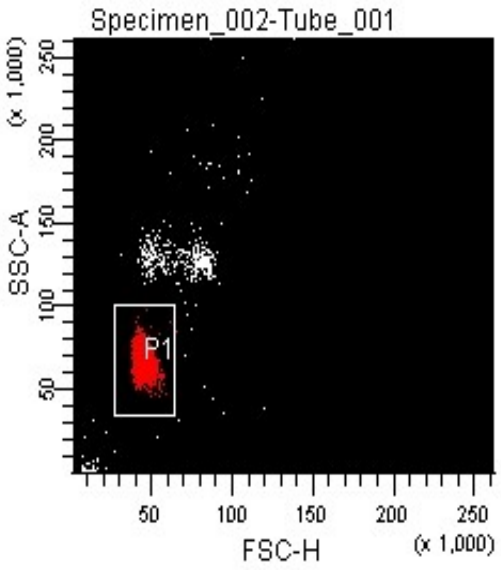
Small errors in compensation of a dim control (A) can result in large compensation errors with bright reagents (B & C).
Use bright markers to setup proper compensation.

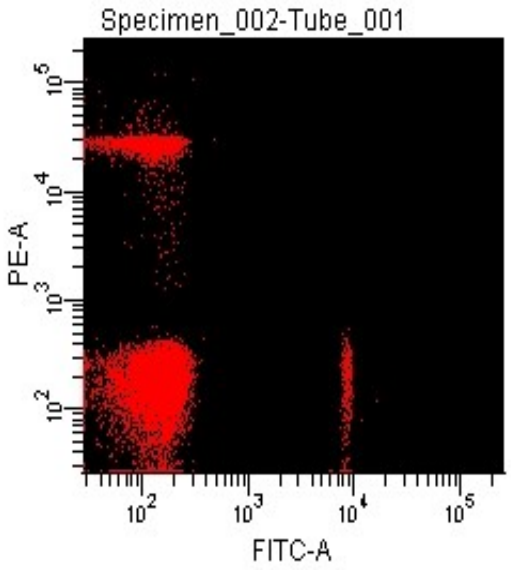
BD Comp Beads

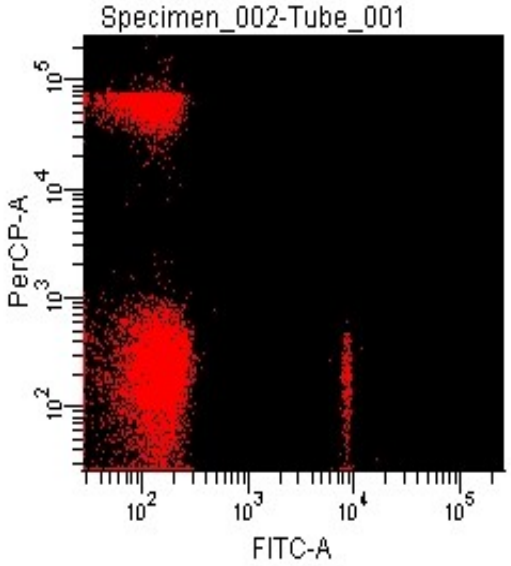


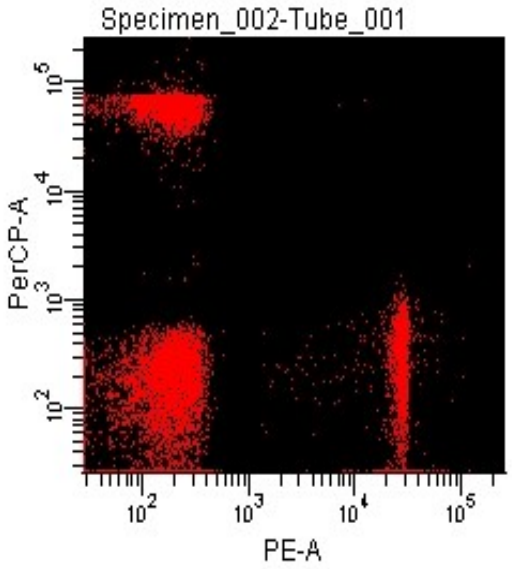
- Always positive
- Bright staining
- Save sample (HIV patients)
- Use the same antibody for compensation and the real experiment

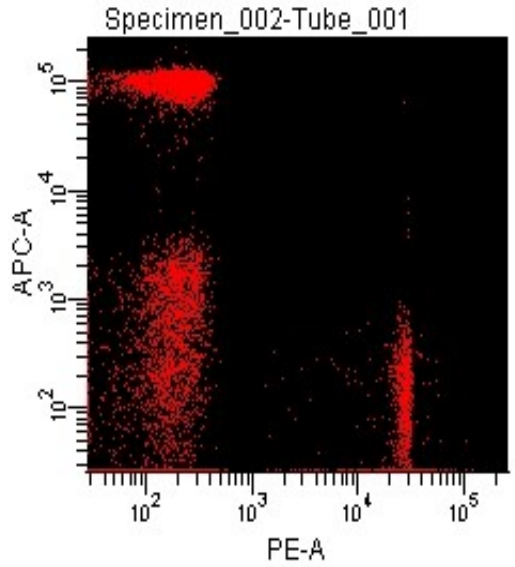
BD Comp Beads







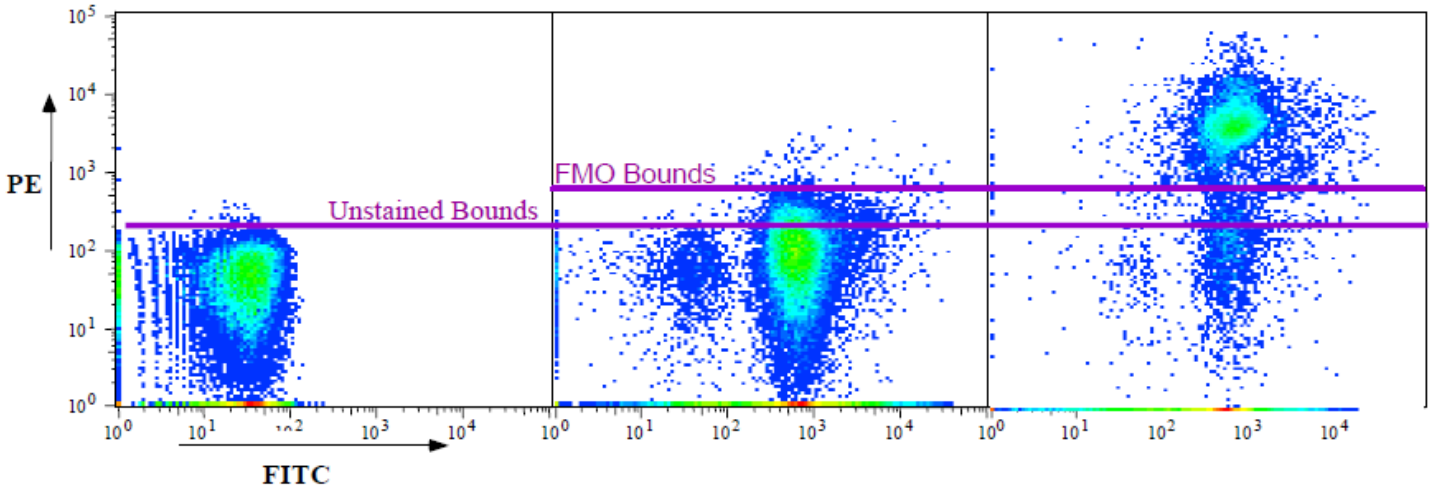




Fluorescence Minus One

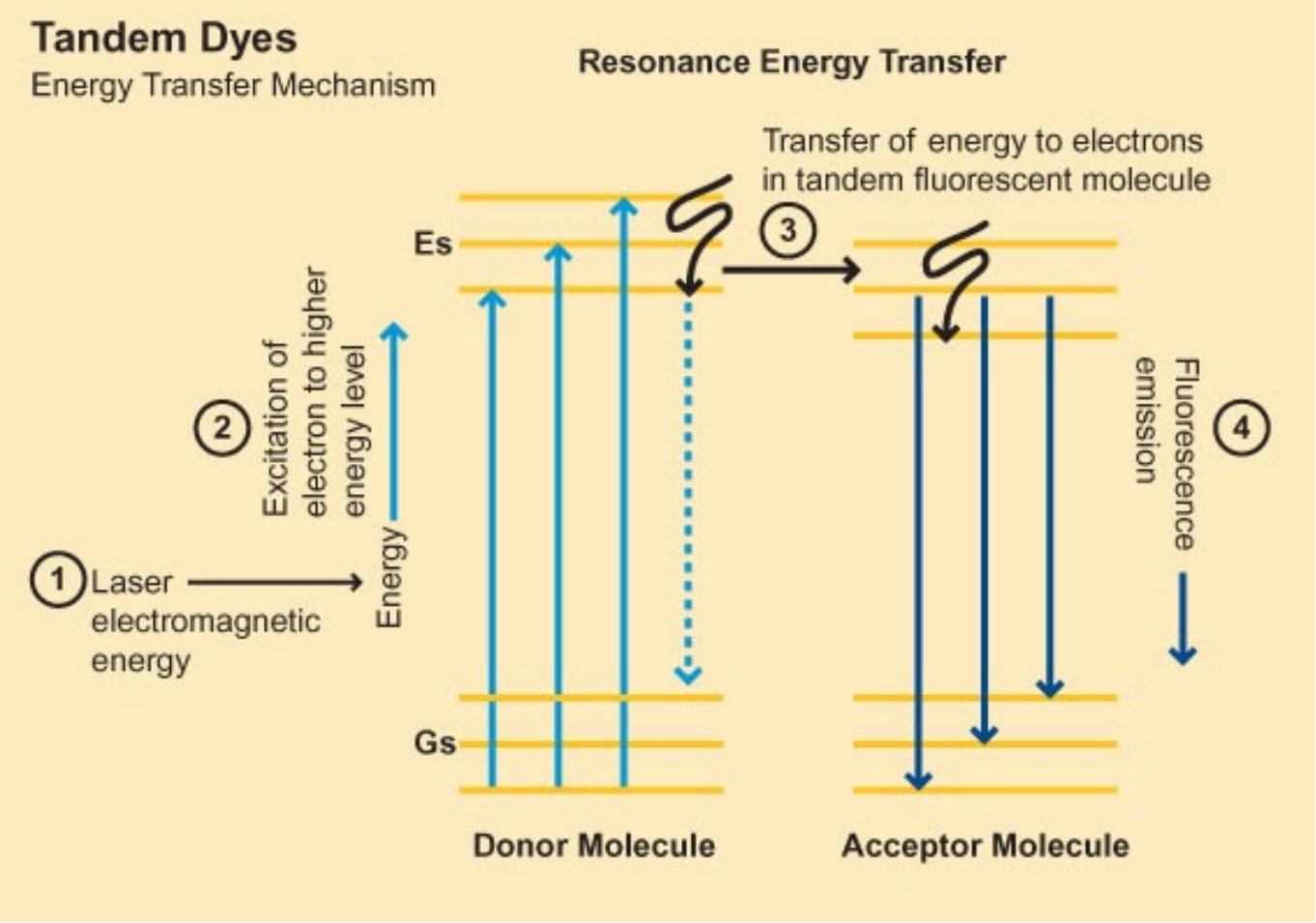
PBMC were stained as shown in a 3-color experiment. Compensation was properly set for all spillovers

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

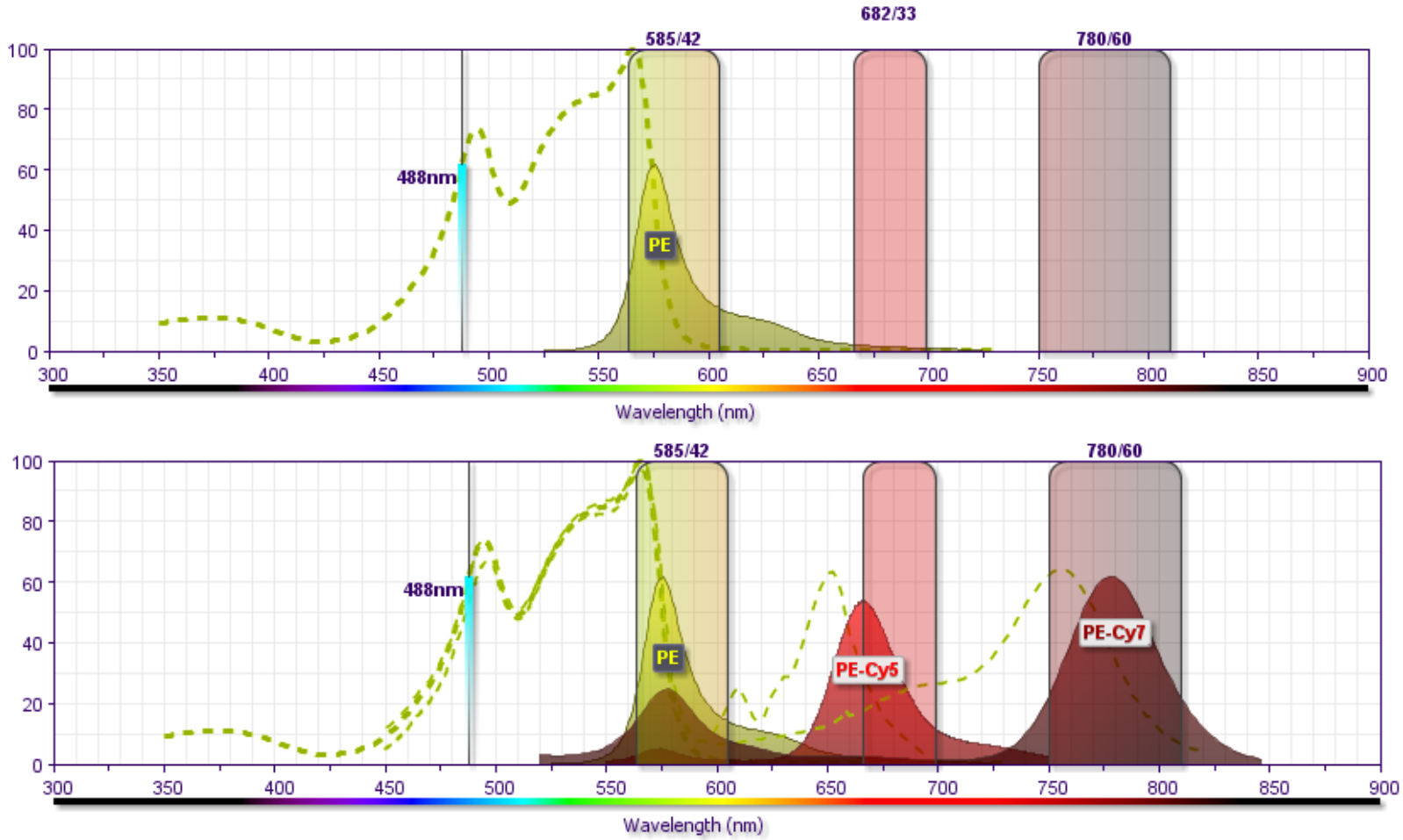


Courtesy Mario Roederer

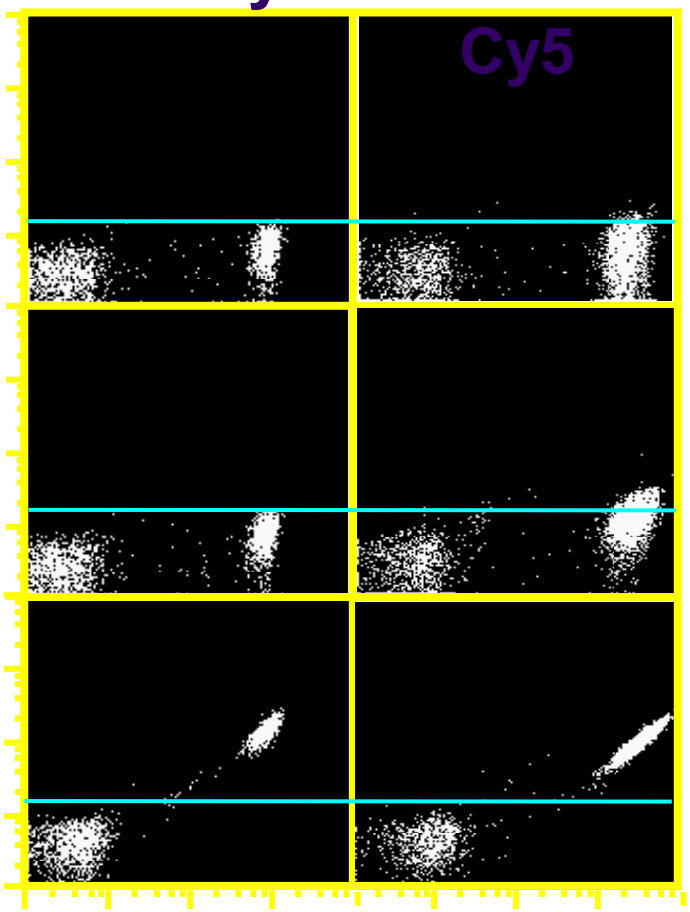
Tandemové značky



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. Znát 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.