

Bi9393 Analytická cytometrie

Lekce 3



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Fluidika

- potřebujeme buňky v suspenzi, protékající v jednom sloupci napříč osvětleným místem
- u většiny zařízení je toho dosaženo injekcí vzorku do proudu nosné kapaliny skrz malý otvor (50-300 μm)



Fluidika

- Pokud jsou podmínky optimální pak vzorek proudí středem bez směšování s nosnou kapalinou
- takový stav nazýváme laminární proudění (**laminar flow**)

Fluidika - Laminární vs. turbulentní proudění

- **Turbulentní** proudění je charakteristické chaotickými (stochastickými) změnami
- **Laminární** proudění – kapalina proudí v paralelních vrstvách které se vzájemně nemísí



Fluidika - Laminární vs. turbulentní proudění

- Osborne Reynolds (1842 -1912) definoval podmínky laminárního proudění (1883)



"http://en.wikipedia.org/wiki/Osborne_Reynolds"

Fluidika - Laminární proudění

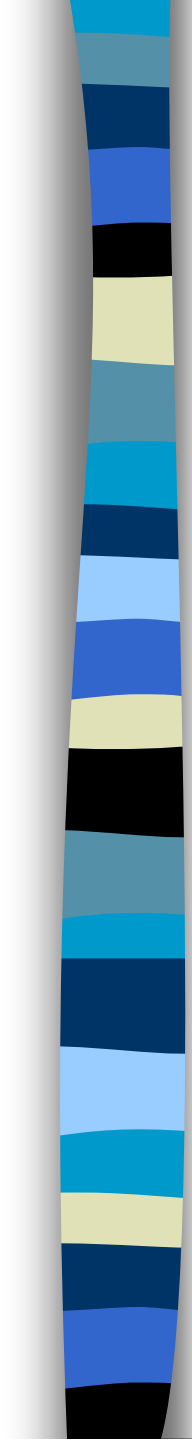
- Zda bude průtok laminární je možné určit pomocí **Reynoldova čísla**

$$Re = \frac{d\rho\bar{v}}{\eta}$$

where

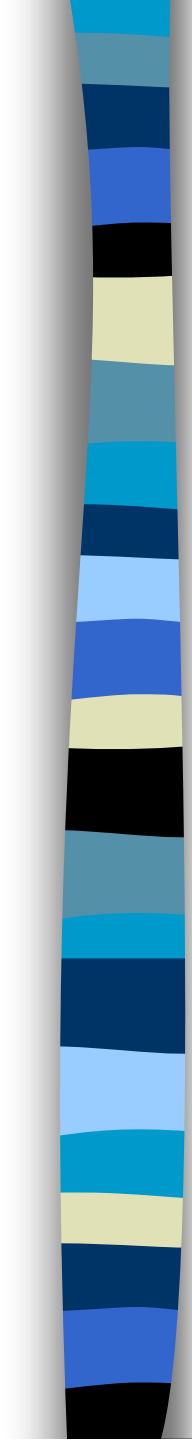
d = tube diameter
 ρ = density of fluid
 \bar{v} = mean velocity of fluid
 η = viscosity of fluid

- když $Re < 2300$, průtok je vždy **laminární** (v trubici)
- $Re > 2300$, průtok může být **turbulentní**



Fluidika – systém s rozdílným tlakem

- Tlak nosné kapaliny určuje objem v jakém proudí
- Rozdíl v tlaku mezi nosnou kapalinou a vzorkem určuje objem proudícího vzorku
- Kontrola není úplná – změny tření mohou způsobit změny v rychlosti proudění vzorku



Fluidika – systém s volumetrickou injekcí

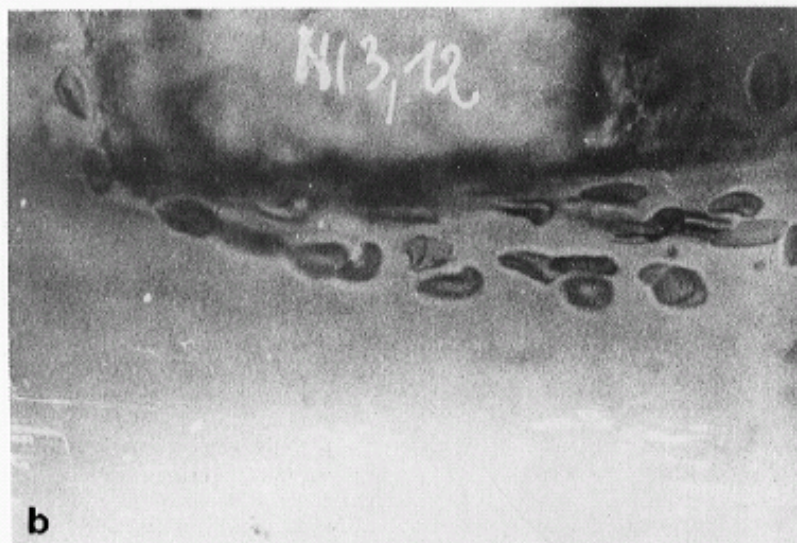
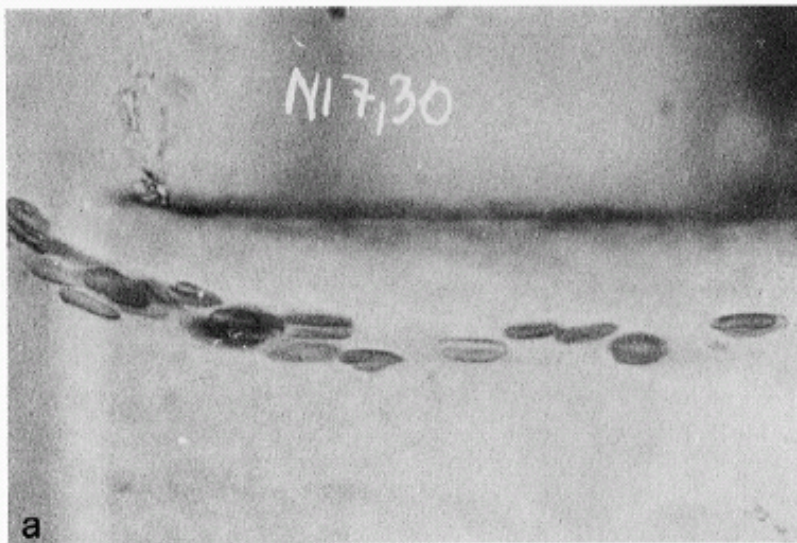
- Pomocí vzduchu tlakuje nosnou kapalinu
- Pomocí pístu injikuje vzorek
- **Objem proudícího vzorku** může být regulován rychlostí pohybu pístu
- Kontrola je úplná (za normálních podmínek)



Fluidika – orientace a deformace částic

- Během hydrodynamického ostření jsou buňky vystaveny třecímu stresu na různých místech jejich povrchu.
- Tření způsobuje jejich orientaci delším koncem ve směru proudění.
- Stres může také způsobit jejich deformaci.

Fluidika – orientace a deformace částic



“a: Native human erythrocytes near the margin of the core stream of a short tube (orifice). The cells are uniformly oriented and elongated by the hydrodynamic forces of the inlet flow.

b: In the turbulent flow near the tube wall, the cells are deformed and disoriented in a very individual way. $v > 3$ m/s.”

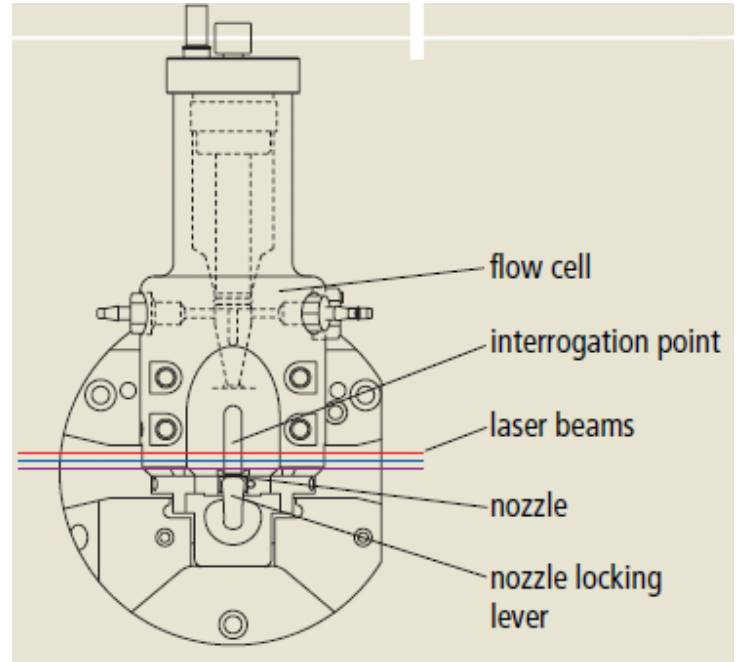
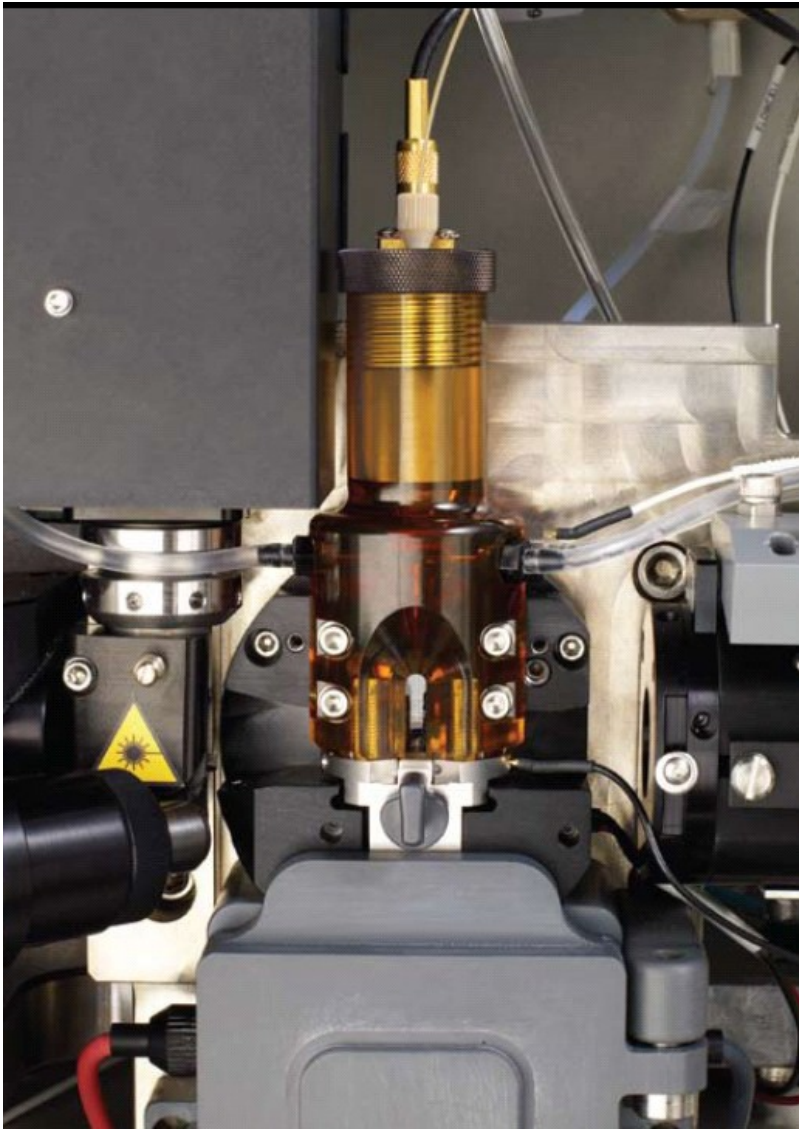
Image from V. Kachel, et al. – **Melamed**
Chapt. 3



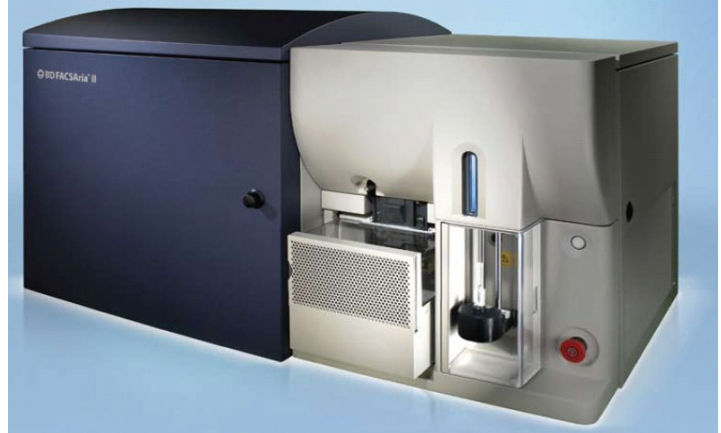
Fluidika – průtokové komory

■ Průtokové komory

- Určují osu a velikost průtoku nosné kapaliny a vzorku
- Vymezují místo pro hydrodynamické zaostření
- Slouží také jako místo kde dochází k ozáření buněk zdrojem světla



BD FACSAria II





Fluidika – průtokové komory

Základní typy průtokových komor

– **Jet-in-air**

- Nejlepší pro sortování, horší optické vlastnosti

– **Flow-through cuvette**

- Výborné optické vlastnosti, může být použita pro sortování

– **Closed cross flow**

- Nejlepší optické charakteristiky, nelze sortovat

– **Open flow across surface**

- Nejlepší optické charakteristiky, nelze sortovat



A



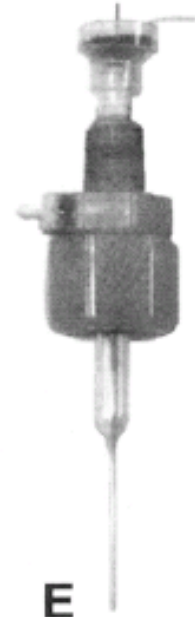
B



C

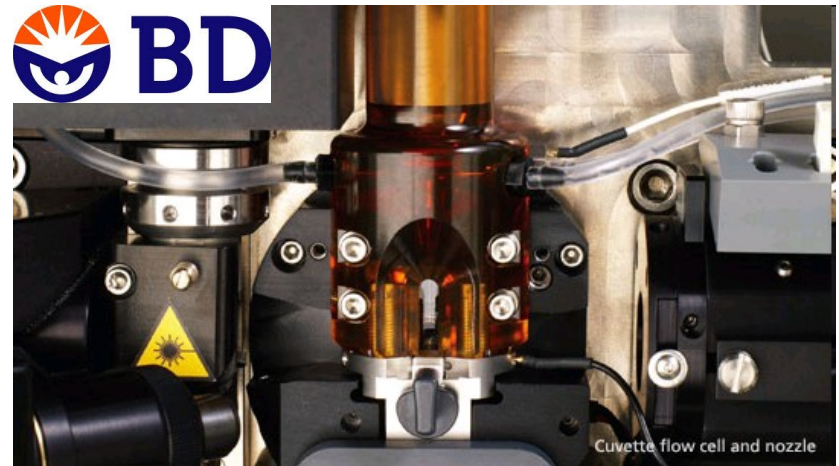
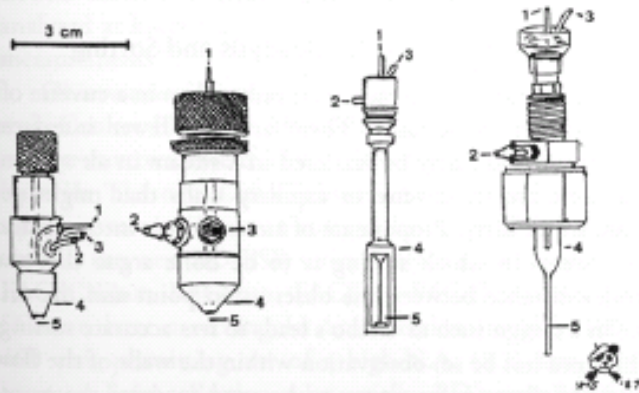


D



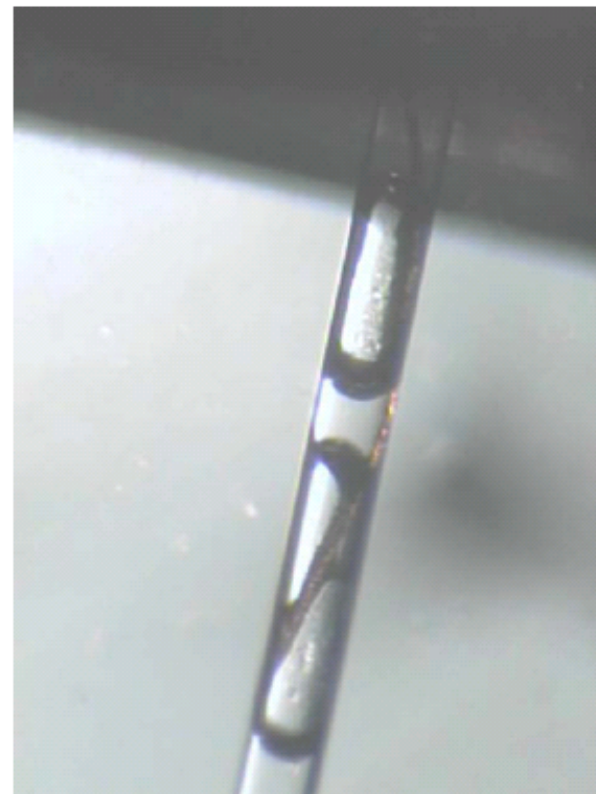
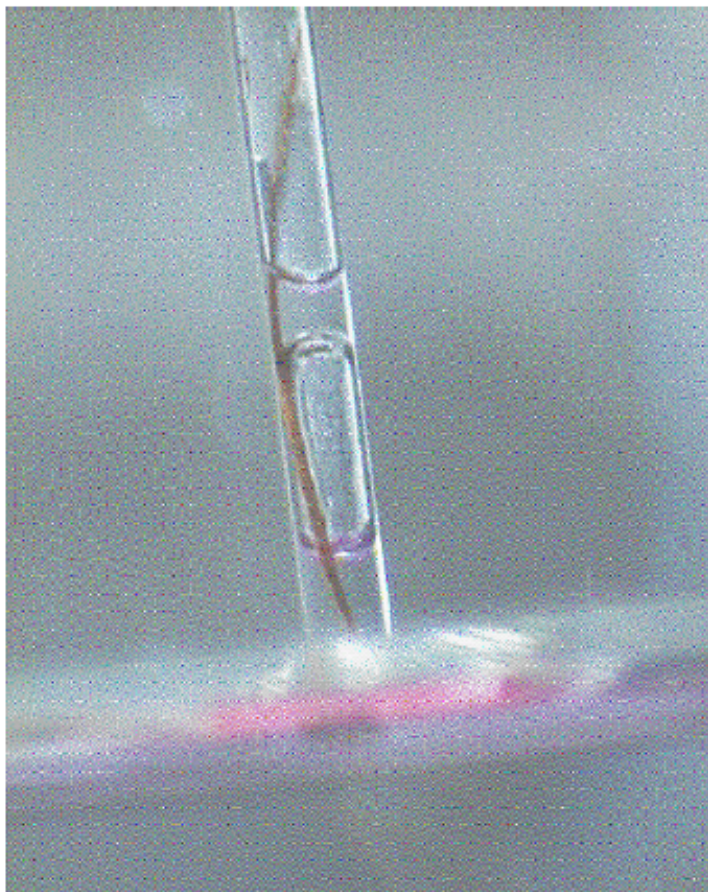
E

3 cm



Cuvette flow cell and nozzle

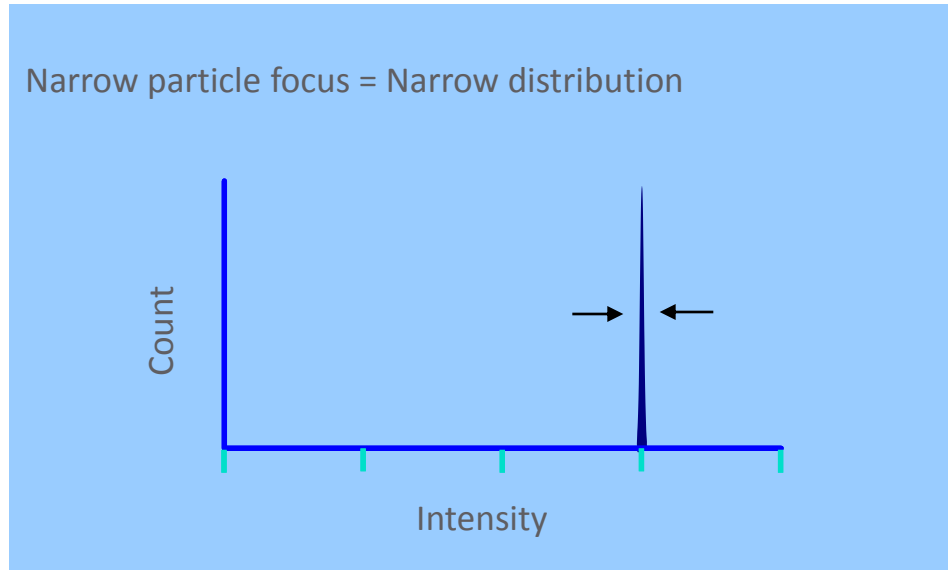
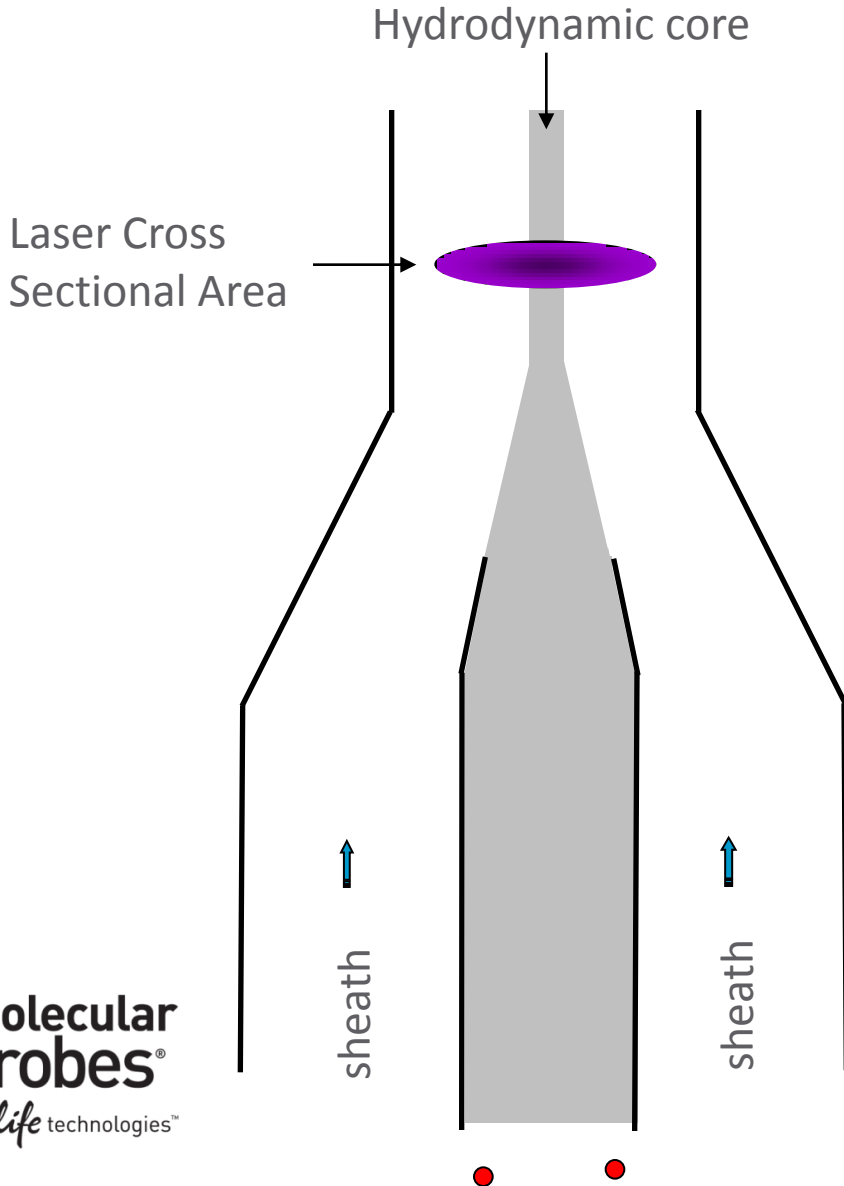
Zanesení průtokové komory



Lidský vlas zablokuje komoru a kompletně naruší kvalitu proudění.

Particle Delivery: Hydrodynamic Focusing

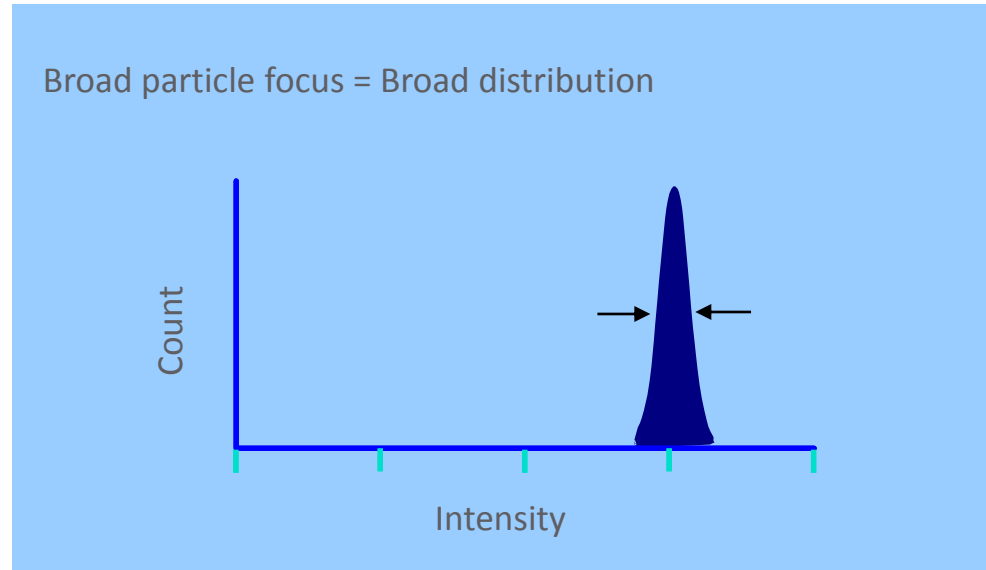
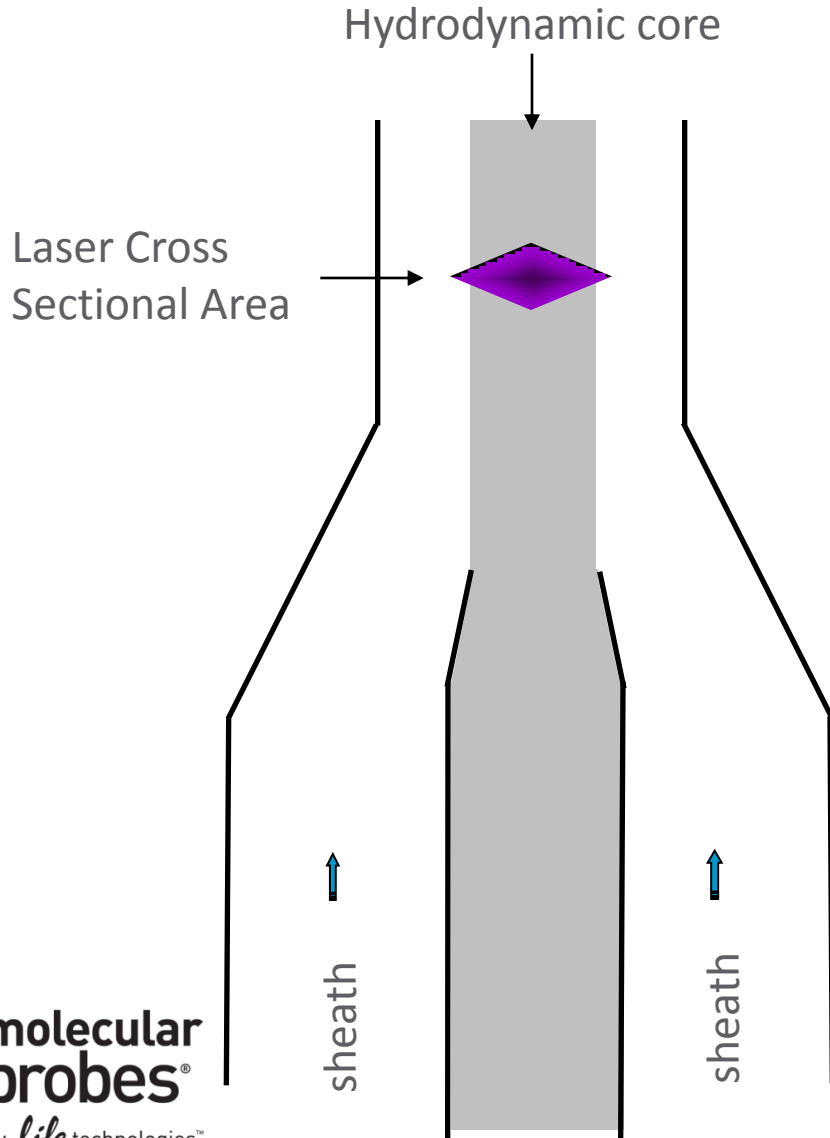
Conventional Instrumentation: **Low Flow Rates (12 μ L/min)**



- Sample core is 'pinched' by fast flowing sheath
- Sample volume ratios of 100 – 1000
- Large ratios => low sample inputs
- Resolution of particle populations

Particle Delivery: Hydrodynamic Focusing

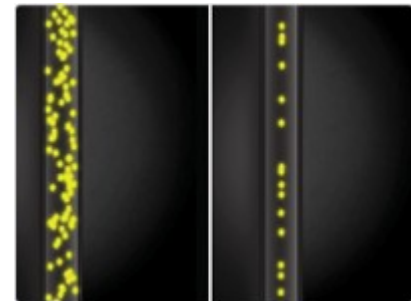
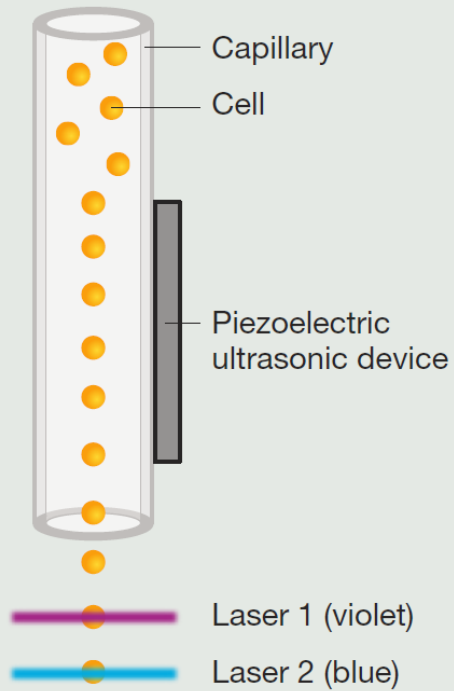
Conventional Instrumentation: **High Flow Rate (60 μ L/min)**



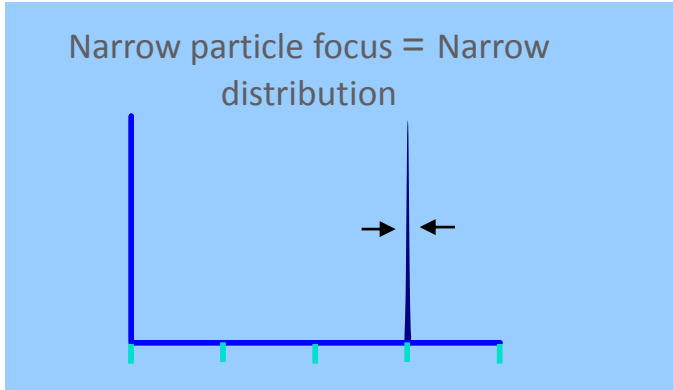
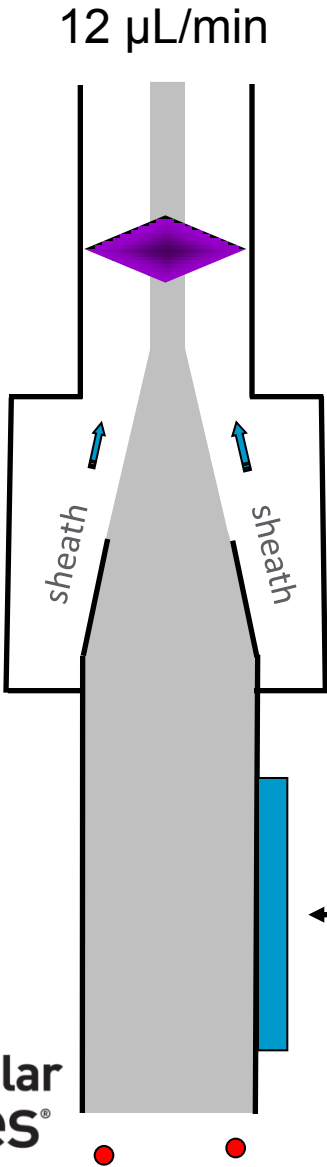
- Increased sample input = increase core size
- Particle distributions broadened, CVs increase
- Instrument resolution decreased
- Historically, low volumetric sample rates used (25 μ L/min – 150 μ L/min)

Attune® Acoustic Focusing Cytometer

Acoustic focusing cytometry

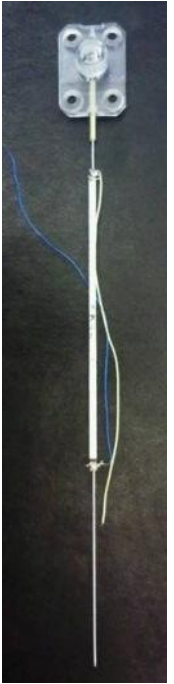
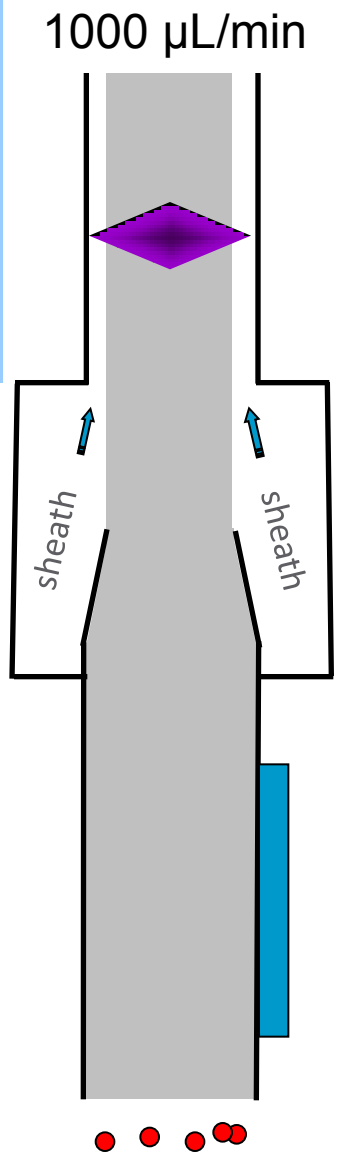


Acoustic Focusing = Better Precision



Acoustic focusing of particles occurs prior to mixing with sheath fluid

Acoustic focusing Module



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2. Curtis HW, Stephans EJ (1982) *IBM Technical Disclosure Bulletin* 25(1).
3. Yasuda K, Haupt SS, Unemura S (1997) *J Acoust Soc Am* 102:642–645.
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5. Kaduchak G, Goddard G, Salzman G et al. (2008) US Patent 7,340,957.



US007340957B2

(12) **United States Patent**
Kaduchak et al.

(10) **Patent No.:** US 7,340,957 B2
 (45) **Date of Patent:** Mar. 11, 2008

(54) **ULTRASONIC ANALYTE CONCENTRATION AND APPLICATION IN FLOW CYTOMETRY**

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(73) Assignee: **Los Alamos National Security, LLC**, Los Alamos, NM (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 143 days.

(21) Appl. No.: 10/979,065

(22) Filed: Nov. 2, 2004

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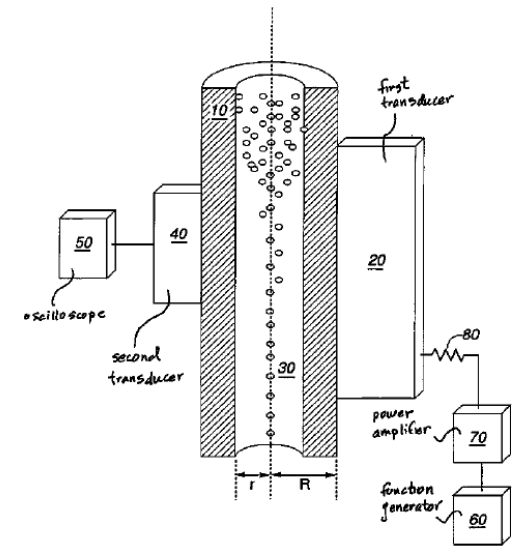
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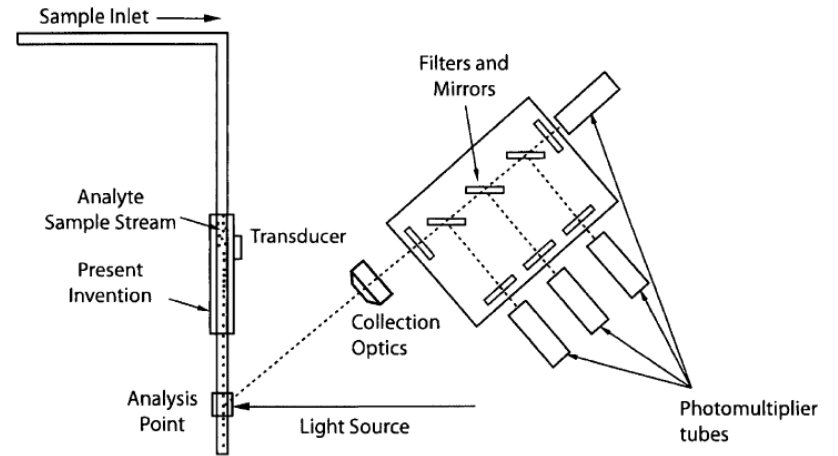
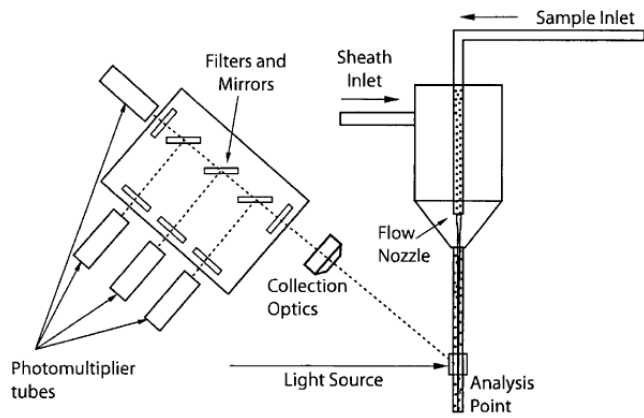
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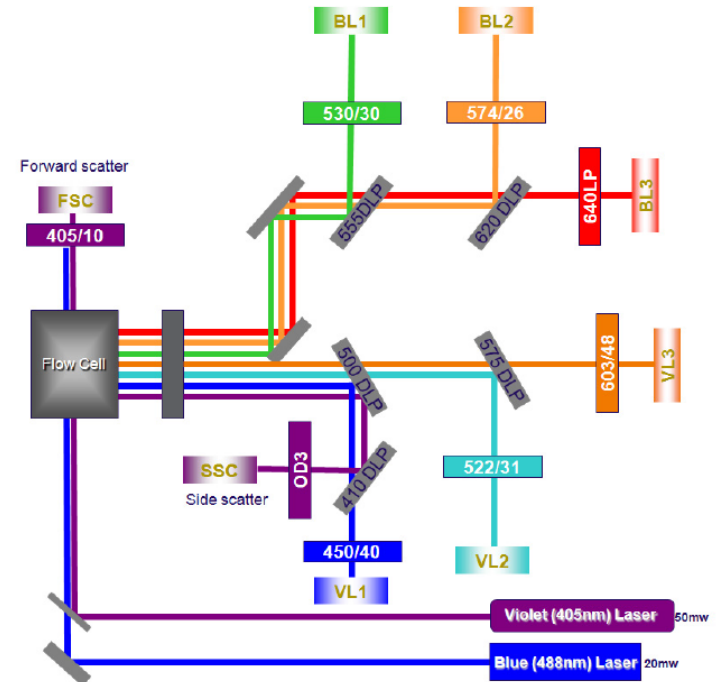
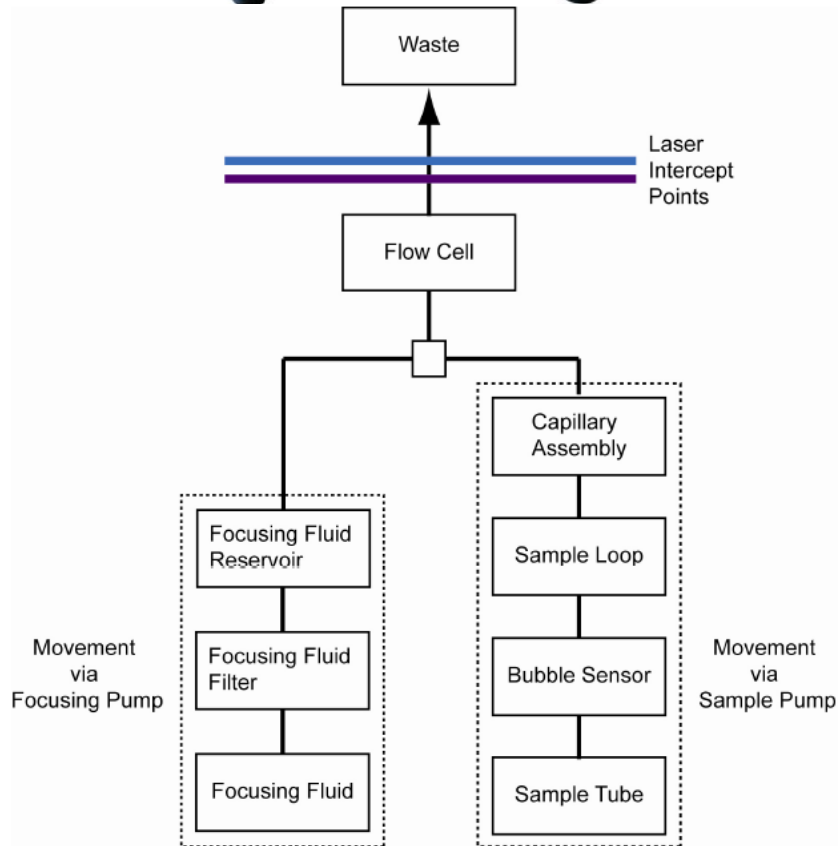
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(Continued)



U.S. Patent **Mar. 11, 2008** **US 7,340,957 B2**





Attune (2 lasers, 6 detectors setup)

laser	detector	fluorochrome
405		
	VL1 450/50	Pacific Blue, Alexa Fluor 405, Brilliant Violet 421
	VL2 522/31	Horizon V500, LIVE/DEAD Aqua
	VL3 603/48	LIVE/DEAD Yellow, Qdot 605
488		
	BL1 530/30	FITC, GFP, Alexa Fluor 488, Calcein, LIVE/DEAD Green, ALDEFLUOR, HiLyte 488
	BL2 574/26	PE, propidium iodide, Hilyte 555
	BL3 640 LP	PerCP, Pe-Cy7, PerCP-eFluor 710, LIVE/DEAD Red, 7-AAD



Attune - Key performance features include:

- Breakthrough acoustic technology that focuses cells or particles
- **Highest sample delivery rates commercially available (up to 1,000 μ L/minute)**
- User-selectable collection rates
- Equipped with: Blue/Violet: 488 nm (20 mW) and 405 nm (50 mW) lasers
- 8 parameters—6-color detection plus side scatter and forward scatter
- **User-changeable bandpass and dichroic filters**
- Simplified fluorescence compensation
- Manual and automated compensation
- Adjustable PMT voltage settings
- Detection of up to 20,000 events/sec and 20 million events/file
- **Calibrated delivery volumes for volumetric analysis and absolute cell counts**
- Electronic resolution of 6 decades
- Low fluid consumption (about 1 L/day); self-contained fluids
- Countertop instrument—fits on standard lab bench or in laminar

Software:

- **No software licensing fees**
- Output file format: FCS 3.0
- Live gating with automatic saving
- User and administrator log-in

Cancer

The Attune® Acoustic Focusing Cytometer, with its fast acquisition times and increased precision, overcomes the technological hurdles involved in analyzing CECs.

Stem Cells

Side Population Analysis

In this study, we demonstrate the ability of the Attune® Acoustic Focusing Cytometer with the blue/ violet laser configuration to quickly analyze a large number of events in search of very rare populations of stem cells.

Human Mesenchymal Stem Cells (hMSCs)

Adult human mesenchymal stem cells (hMSCs) are rare fibroblast-like cells capable of differentiating into a variety of cell tissues, including bone, cartilage, muscle, ligament, tendon, and adipose.

Cell Cycle Analysis

Cell cycle analysis is just one example of an application in which precise detection of differences in fluorescence intensity between multiple cell populations is critical...

Cell Proliferation

Successful proliferation analysis by dye dilution requires sensitive instrumentation and an extremely bright dye to accurately distinguish fluorescently labeled cells from autofluorescence after several cell divisions...

Marine Sample Analysis

Flow cytometry is a powerful tool for studying the biology, ecology, and biogeochemistry of marine photosynthetic picoplankton...

Immunophenotyping

The Attune® Acoustic Focusing Cytometer exhibits excellent segregation of populations in immunophenotyping experiments (with up to 6 colors)...

Apoptosis

The Attune® Acoustic Focusing Cytometer is compatible with a broad offering of reagents and kits for flow cytometric apoptosis testing...

GFP & RFP Detection

Data for GRP and RFP detection were collected from the Attune® Acoustic Focusing Cytometer using human osteosarcoma cells (U2OS) and BacMam CellLight® reagents...

Microbiological Applications

In recent years the application of flow cytometry to the study of various microbiological phenomena has increased, finding utility in studies that include detection and quantification...

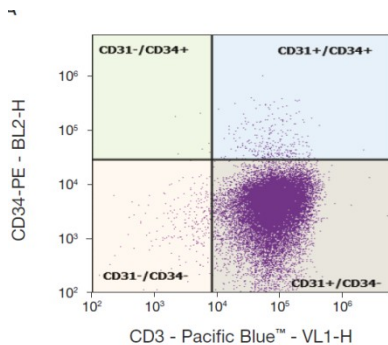
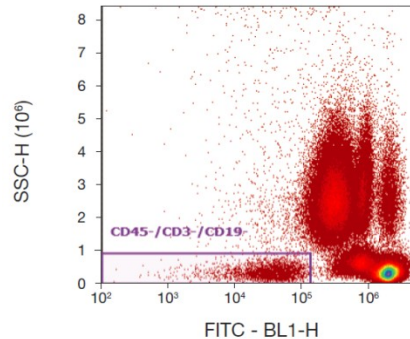
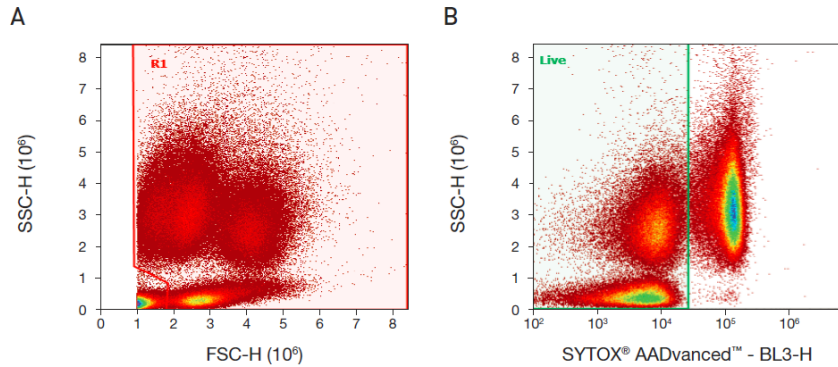


Example: Detecting human circulating endothelial cells using the Attune® Acoustic Focusing Cytometer



Circulating endothelial cells (CECs) are mature cells shed from blood vessel walls during the natural process of endothelial cell turnover. Elevated levels of CECs have been reported in a host of pathological conditions including cardiovascular disorders, infectious diseases, immune disorders, post transplantation analysis, and cancer.

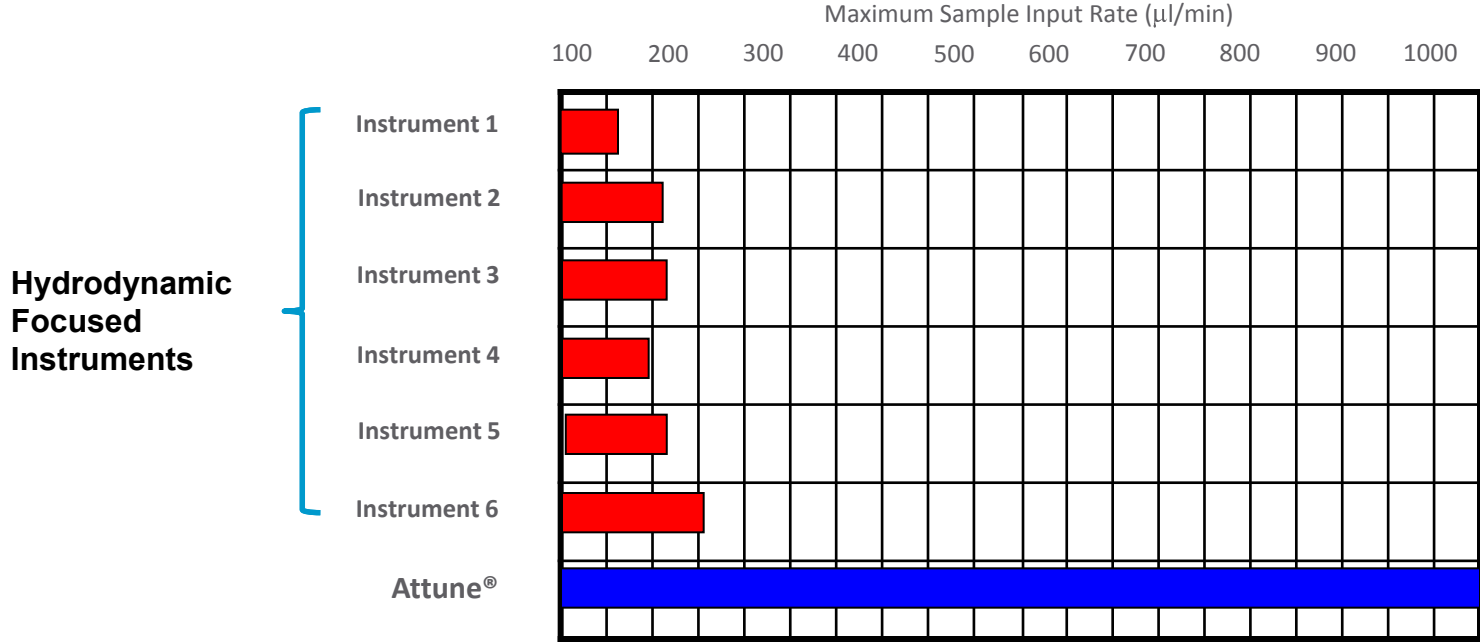
CECs are reported to be present in very low numbers: 0.01%–0.0001% of all peripheral blood mononuclear cells



B

Name	Event Count	% Parent	% Total
All Events	8,600,152	---	100.000%
R1	7,372,407	85.724%	85.724%
Live	4,029,176	54.652%	46.850%
CD45-/CD3-/CD19-	76,852	1.907%	0.894%
CD31-/CD34+	24	0.031%	0.000%
CD31+/CD34+	444	0.578%	0.005%
CD31-/CD34-	1,442	1.876%	0.017%
CD31+/CD34-	74,942	97.515%	0.871%

Attune® Throughput Compared to Hydrodynamic Focused Instruments



- Attune® can analyze at sample rates from 25µL/min to 1000µL/min without losing accuracy
- Traditional Flow Cytometers can only run at most 150µL/min and will sacrifice data quality
- Higher sample rates enable dilution of limited samples and analysis of Rare Events Faster

Table 1. Comparison of acquisition times using a hydrodynamic focusing cytometer or the Attune® Acoustic Focusing Cytometer. A blood sample was taken from an individual with aplastic anemia, and each instrument had a stop gate set on 1 million granulocyte events.

Instrument (collection rate)		Relative rate compared to hydrodynamic focusing
Hydrodynamic focusing cytometer (high)	63 min 33 sec	–
Attune® Acoustic Focusing Cytometer (200 µL/min)	13 min 20 sec	4.8x
Attune® Acoustic Focusing Cytometer (500 µL/min)	5 min 47 sec	11.0x
Attune® Acoustic Focusing Cytometer (1,000 µL/min)	3 min 13 sec	19.7x



Attune souhrn

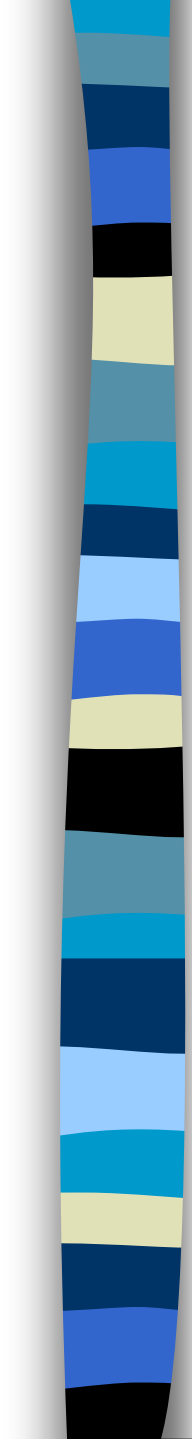
Výhody:

- rychlost měření
- jednoduché ovládání
- sw licence bez omezení
- snadná výměna emisních filtrů
- nízká spotřeba nosné kapaliny (cca 1L denně)

Limitace:

- jen dva lasery (6 barev)
- pouze originální roztoky
- dlouhý (i když automatický) shutdown
- sw nedokáže importovat FCS data
- nutnost nastavit určitý akviziční objem vzorku

Note: The minimum sample analysis volume is 50 μ L. However, the system requires that at least 220 μ L is used to account for the analysis volume + boost volume + safety volume.



Fluidika - shrnutí

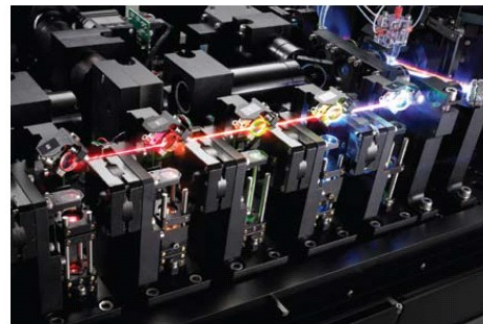
- Průtok musí být laminární (Reynoldovo #)
 - $R_e < 2300$, flow je vždy **laminární**
- Vzorky mohou být injikovány a nebo proudit na základě rozdílných tlaků
- Existuje mnoho typů průtokových komor
- Pro přesnost měření je nutné odstranit a zabránit ucpání komory



Fluidika – shrnutí 2

- tlak nosné (oplašťující) kapaliny vede pufr kyvetou a vyšší tlak ve zkumavce se vzorkem zavádí vzorek do kyvety.
- Princip hydrodynamického zaostření zarovná buňky v kyvetě „jako perly na šňůrce“ předtím než dojdou do bodu kde protnout paprsek laseru.
- Hydrodynamické zaostření nemůže oddělit buněčné agregáty. Průtoková cytometrie vyžaduje suspenzi jednotlivých buněk!

Image Stream & Flowsight Amnis – kombinace průtokové cytometrie a analýzy obrazu

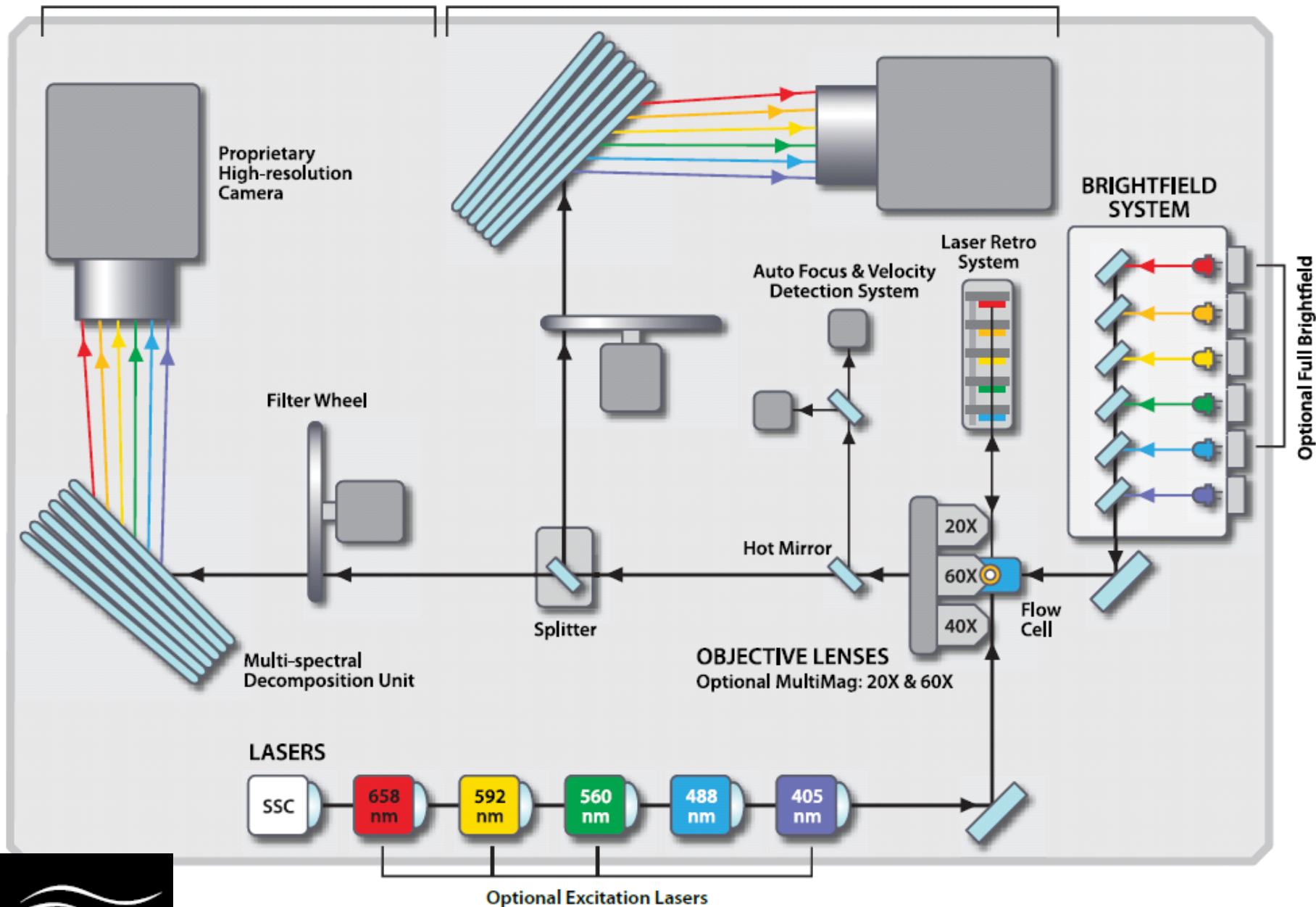


STANDARD COLLECTION SYSTEM

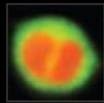
Image Channels: 1-6

OPTIONAL COLLECTION SYSTEM

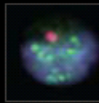
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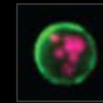
Amnis - aplikace



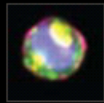
Cell Signaling



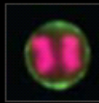
DNA Damage and Repair



Cell Death



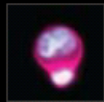
Co-localization



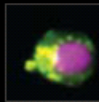
Cell Cycle and Mitosis



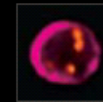
Parasitology



Cell-Cell Interactions



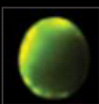
Autophagy



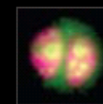
Microbiology



Morphology



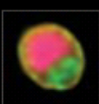
Targeted Immunotherapy



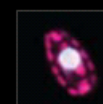
Oncology



Internalization



Stem Cell Differentiation



Oceanography

The logo for Amnis, featuring a stylized white wave above the word "amnis" in a white, lowercase, sans-serif font, all set against a black rectangular background.



Principy průtokové cytometrie a sortování

- sorting
- zpracování signálu
- analýza dat
- kompenzace signálu



Doležel (1999)

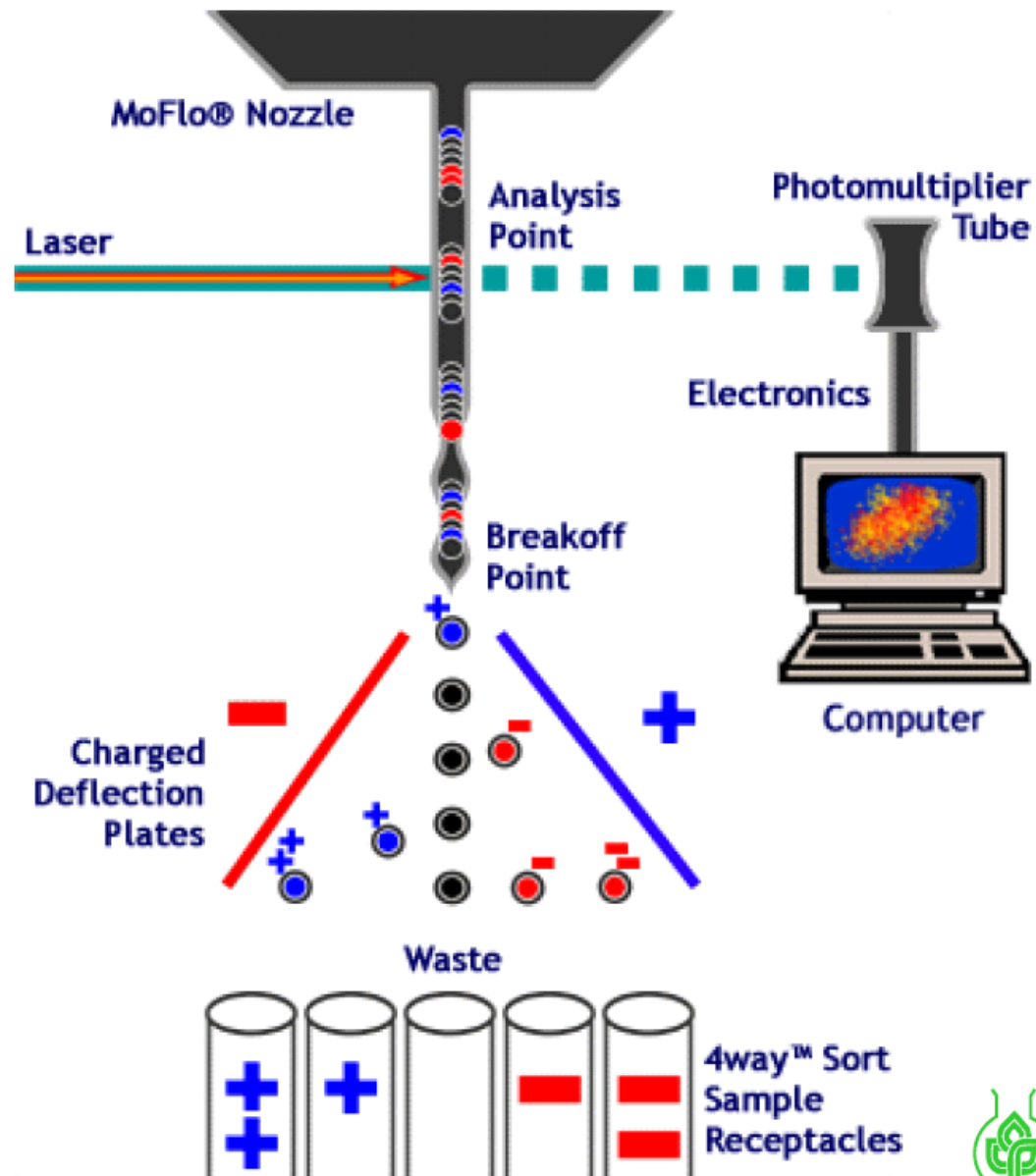


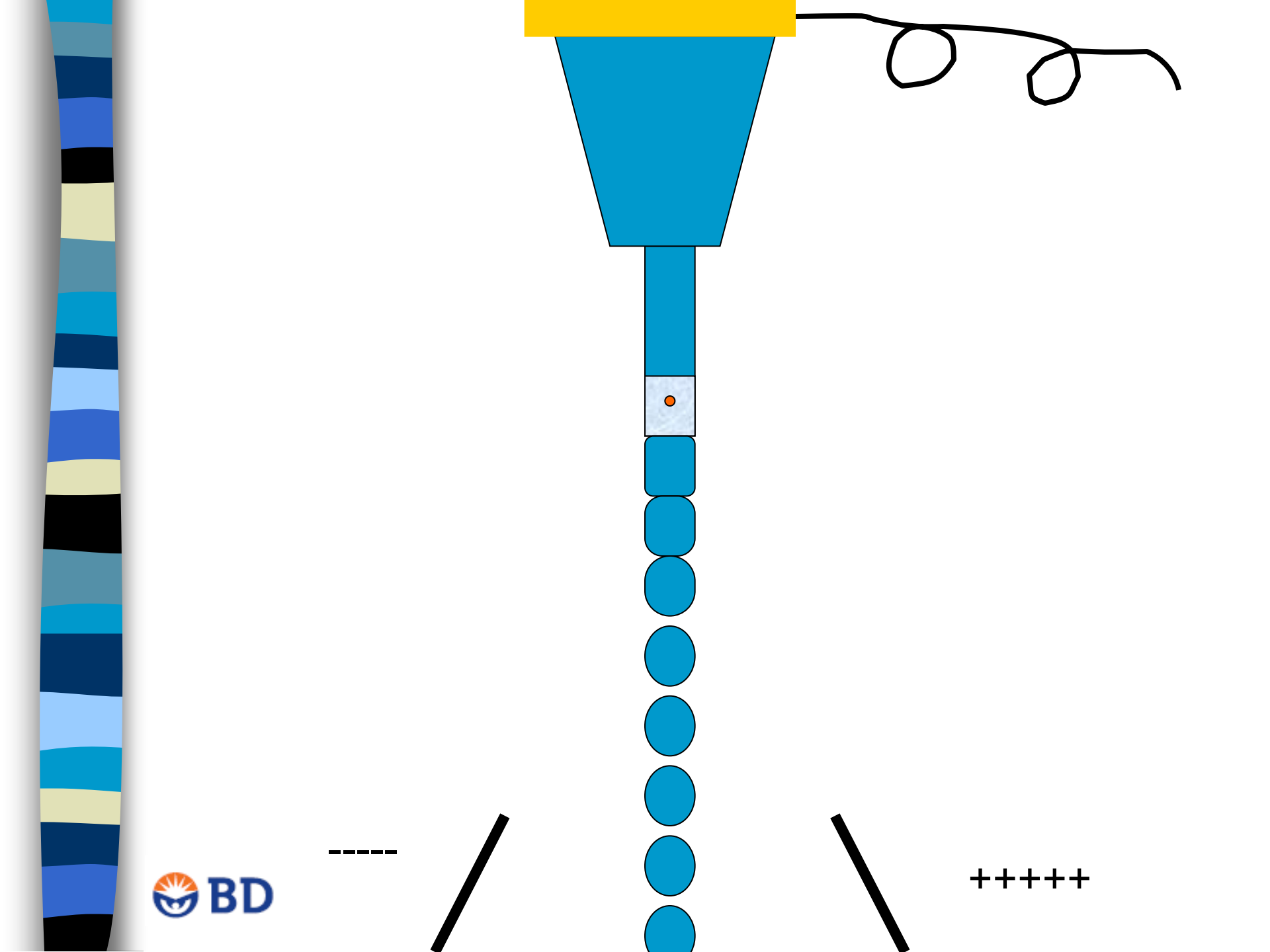
ELECTROSTATIC DROPLET SORTER

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

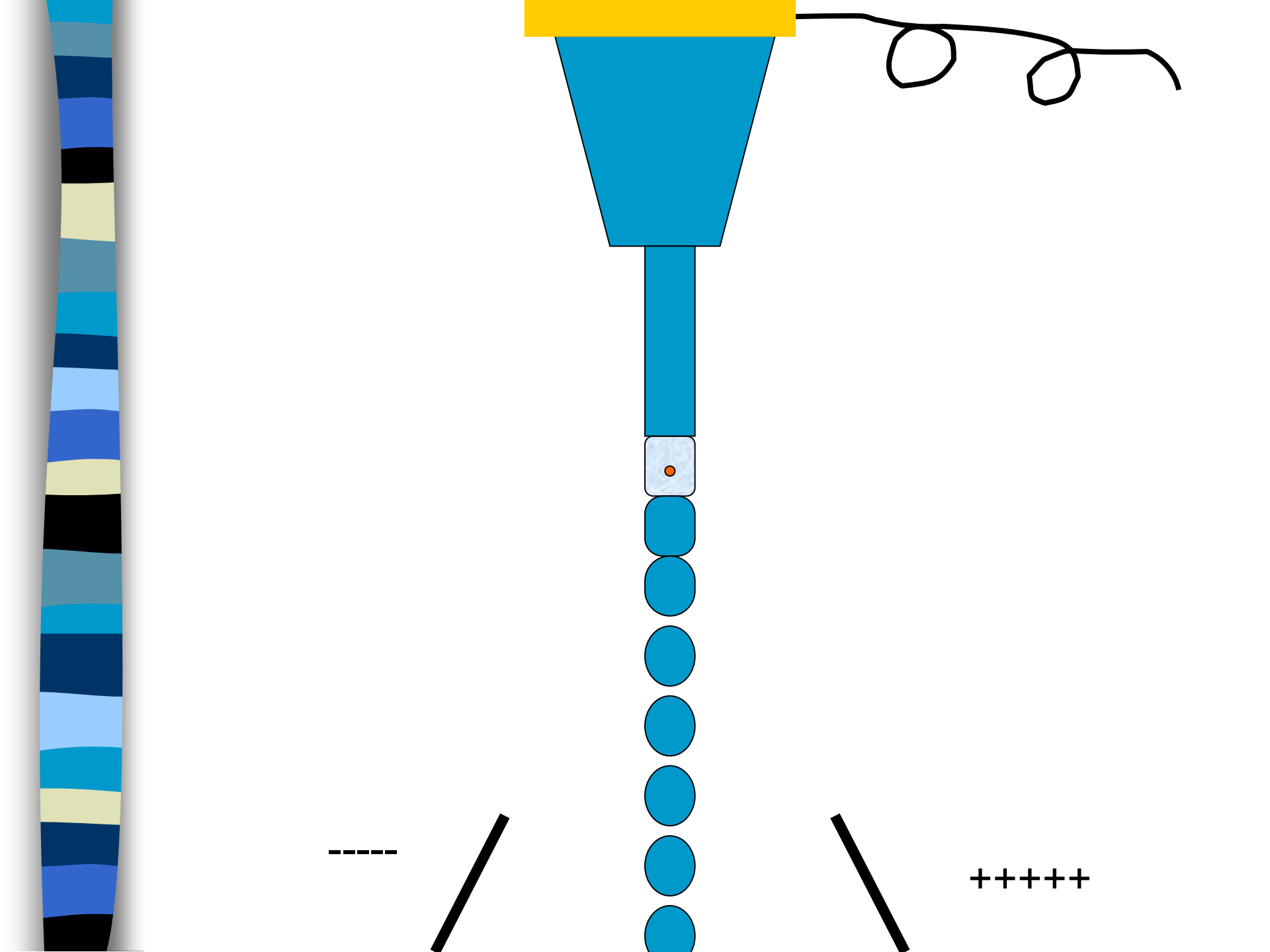
Used by:
Becton Dickinson
Beckman Coulter
Cytomation

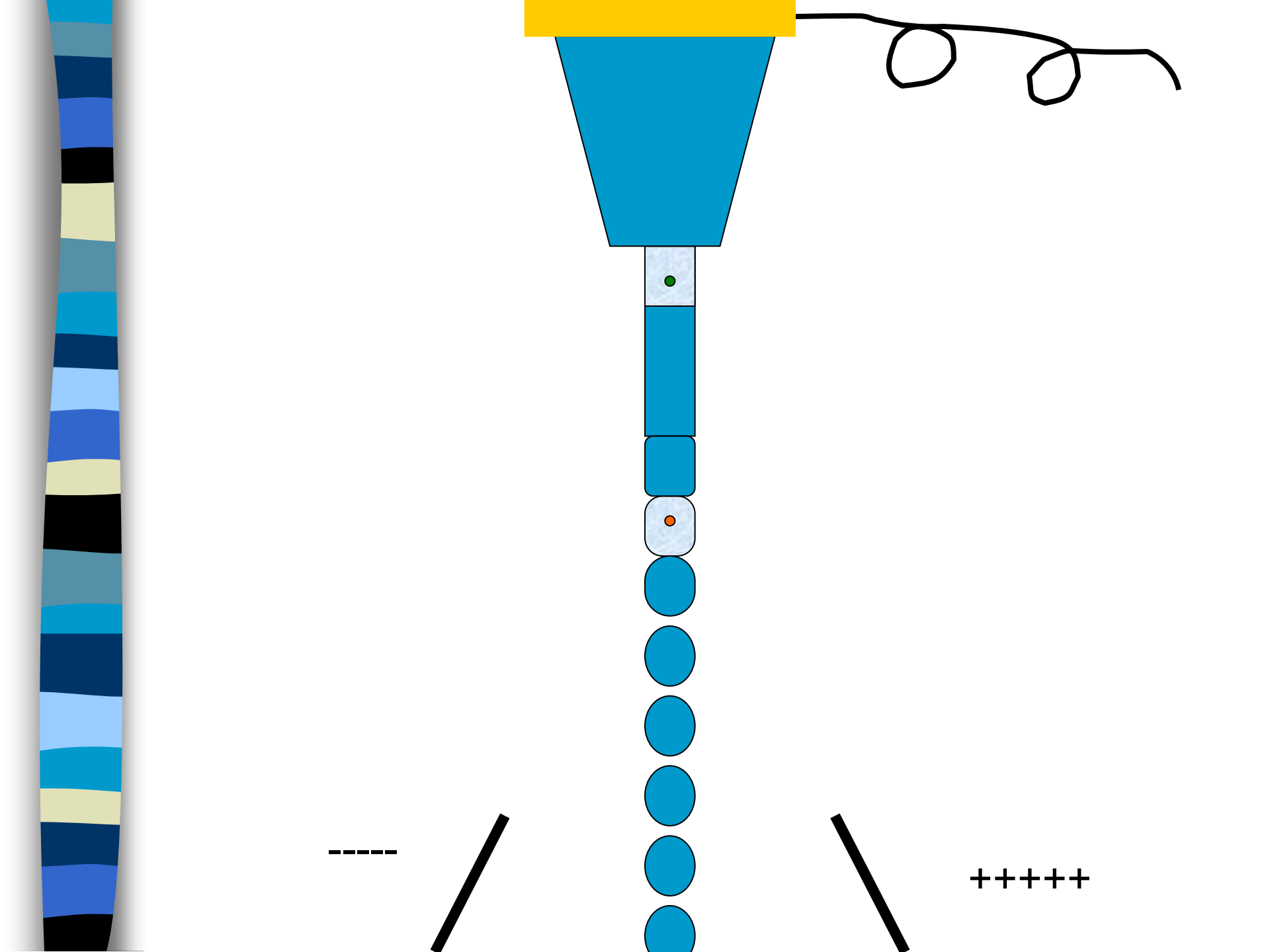
Doležel (1999)

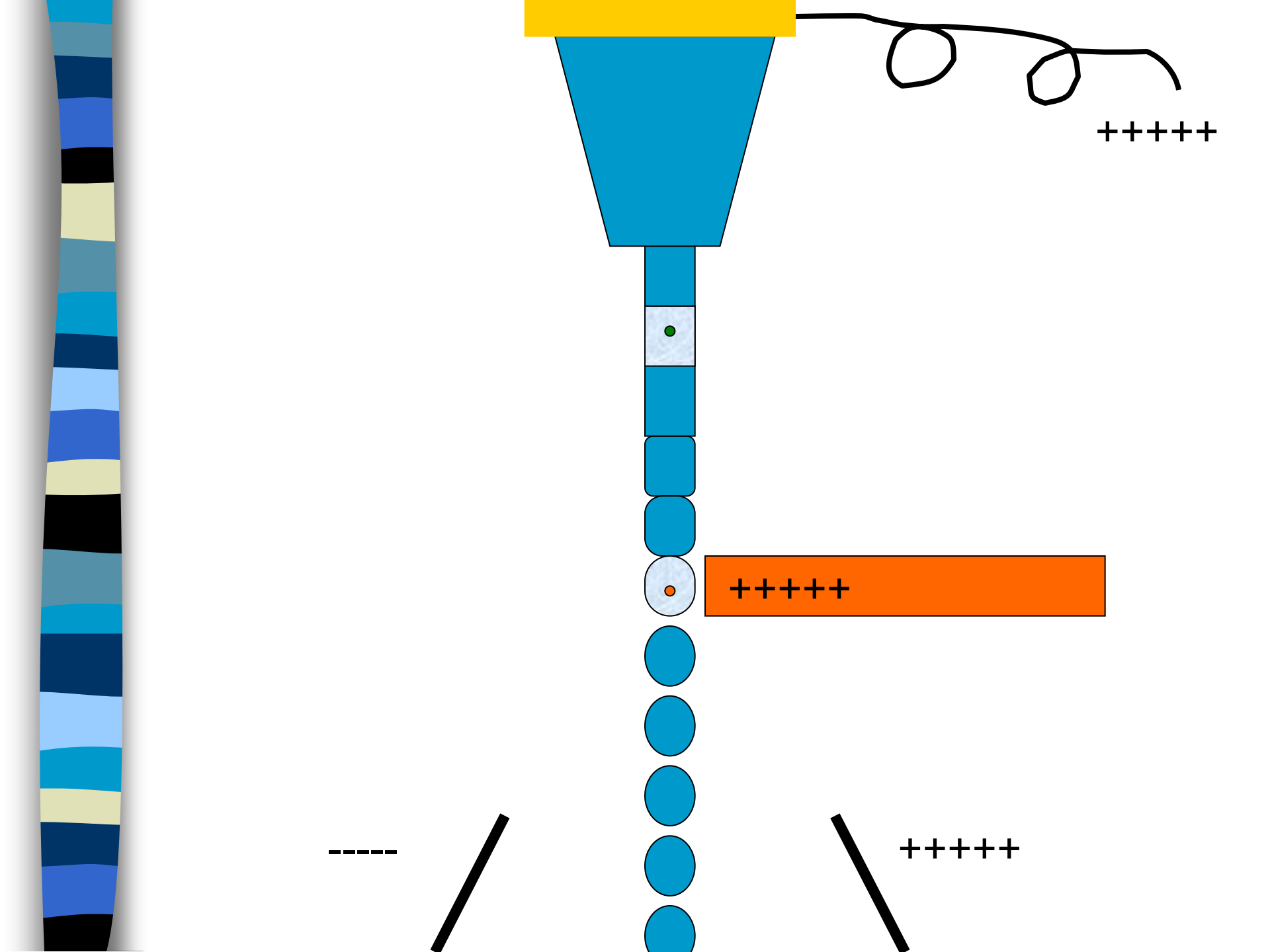


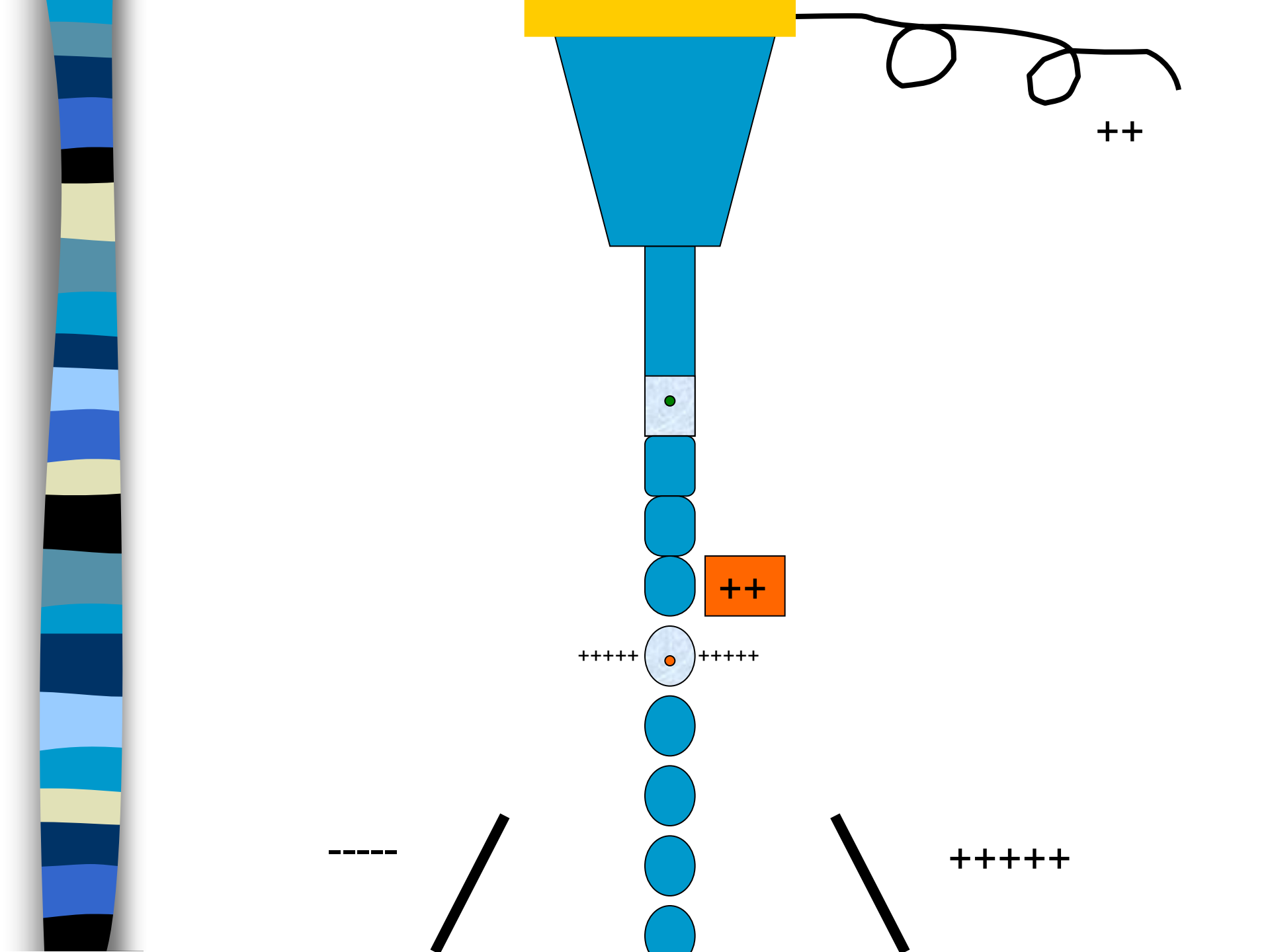


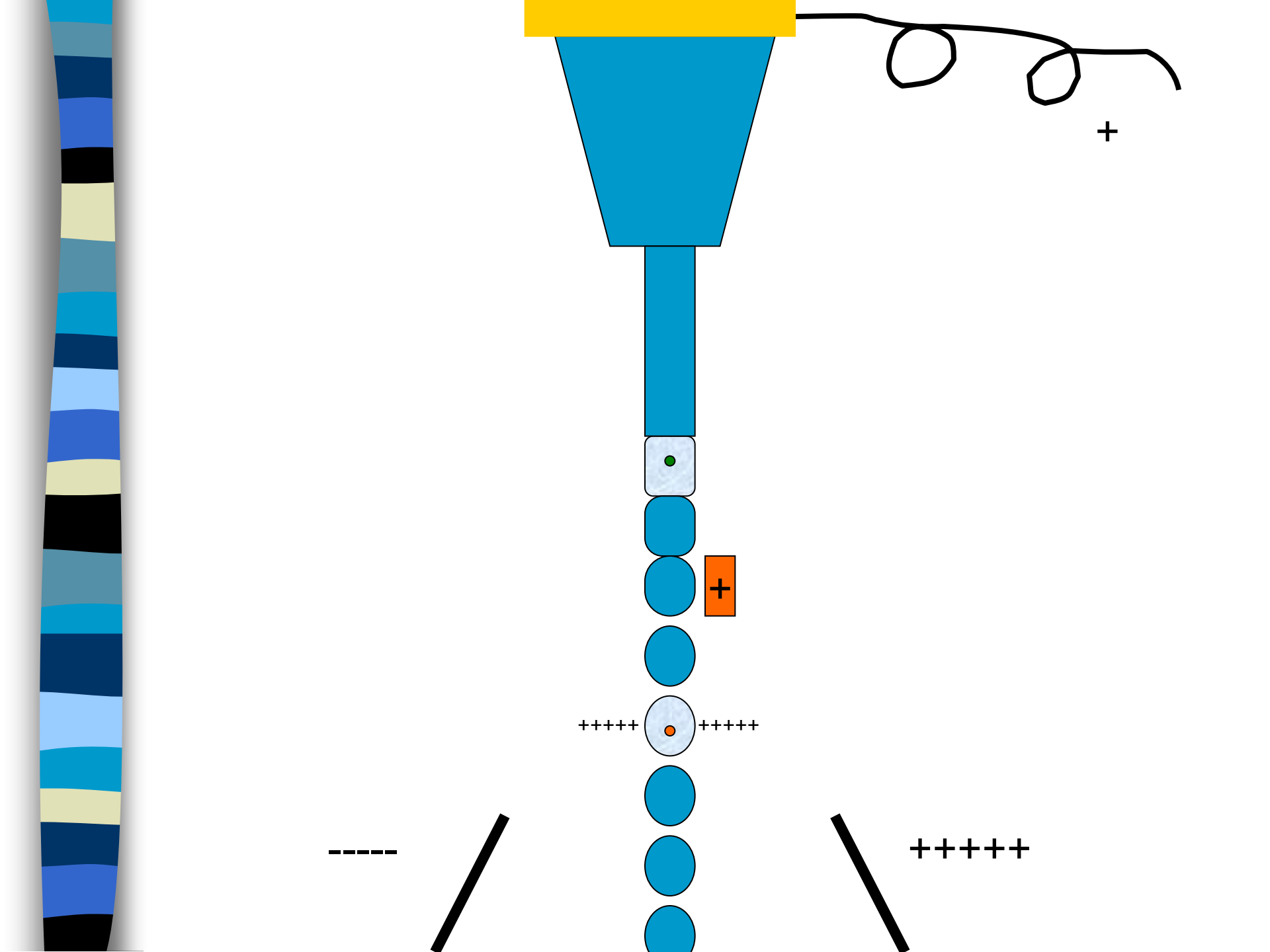
+++++

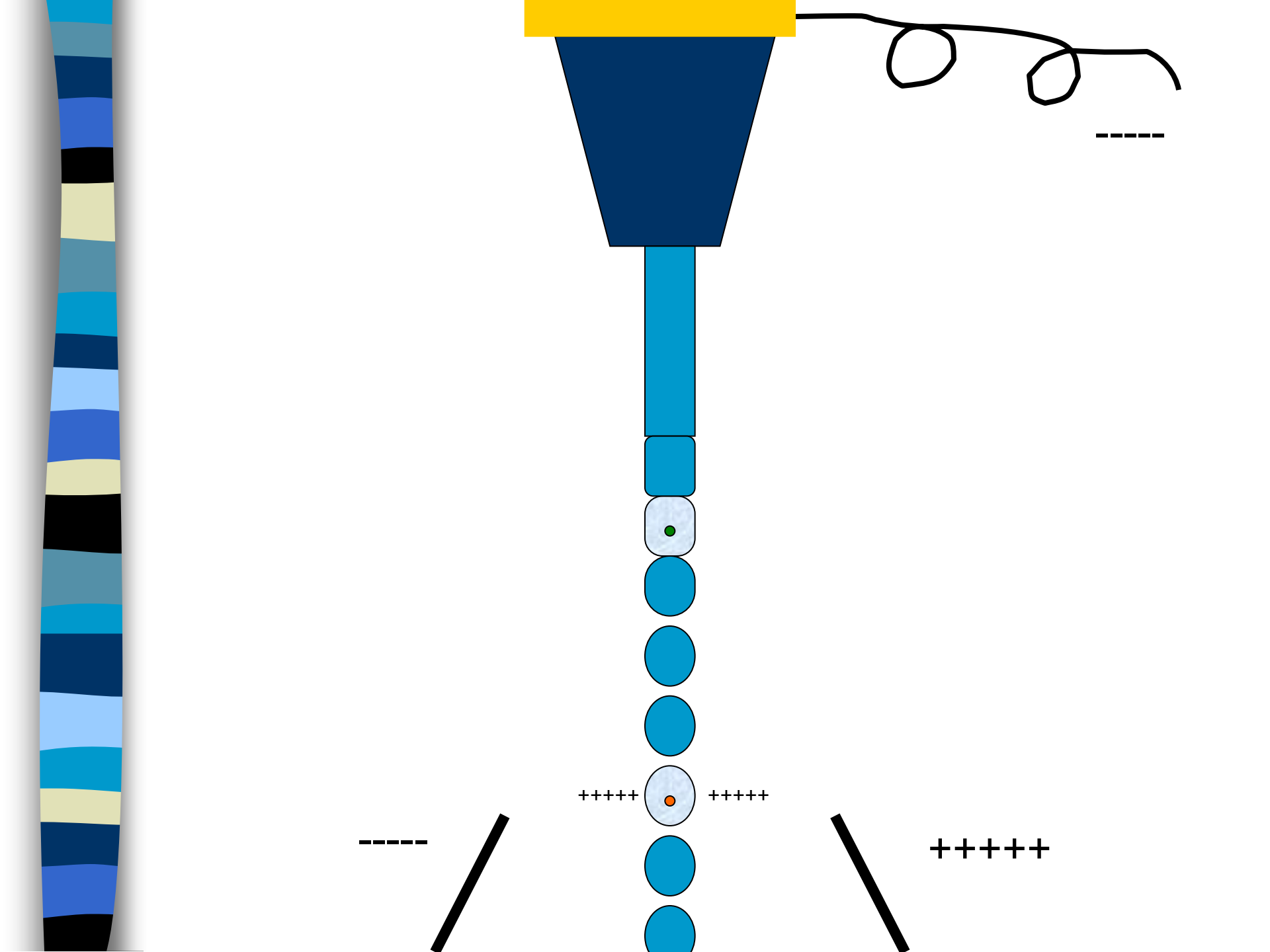


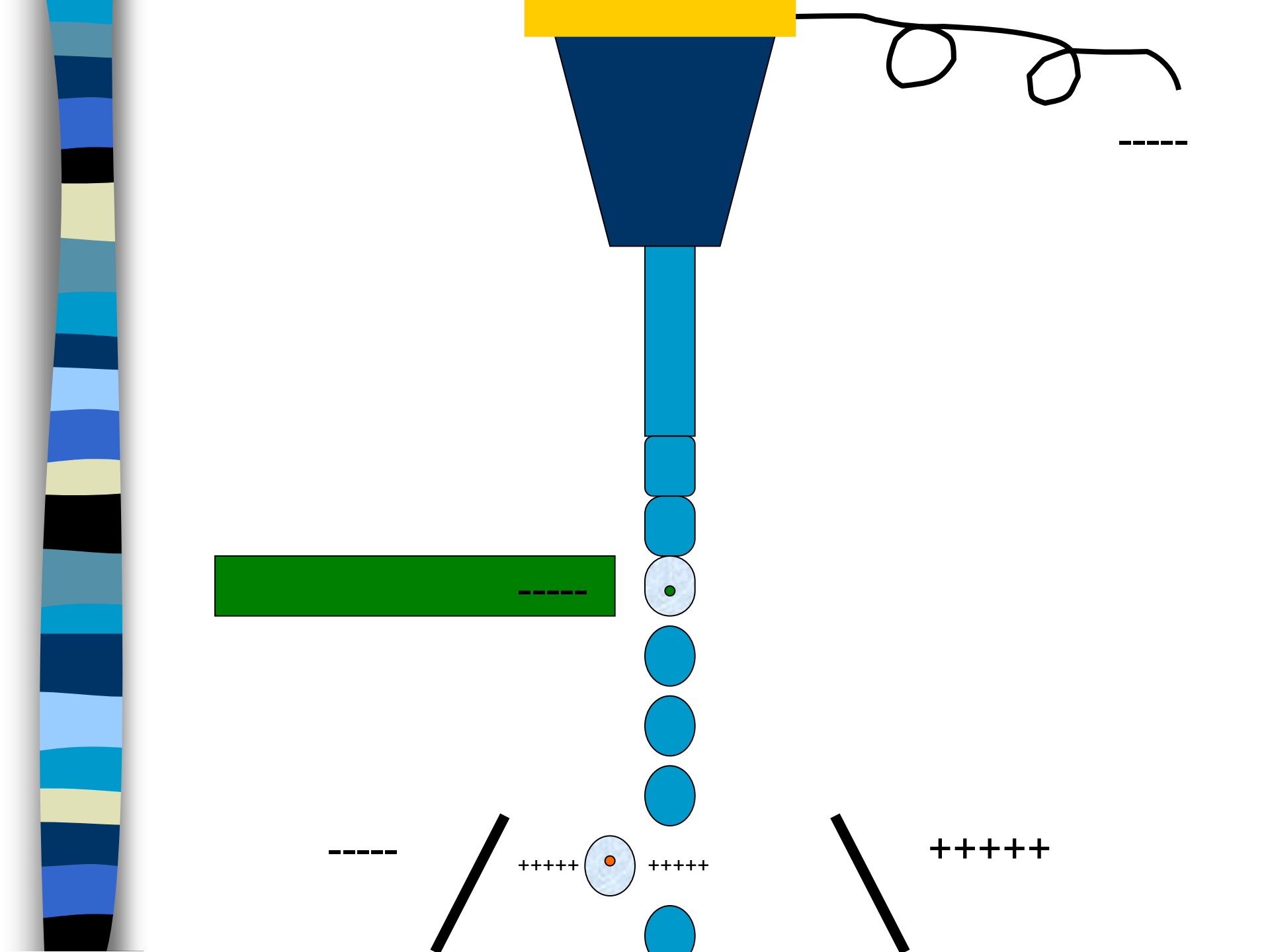


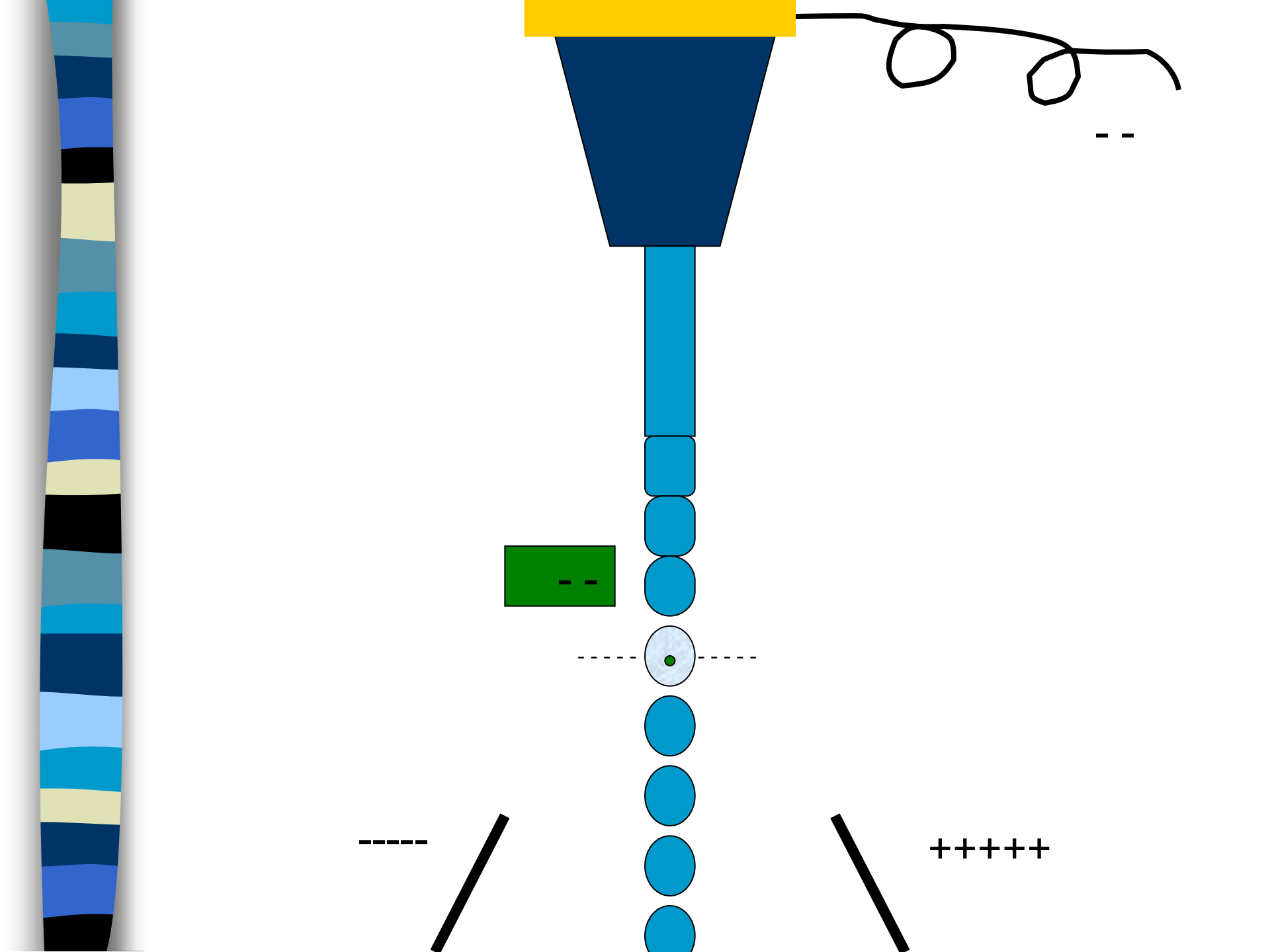


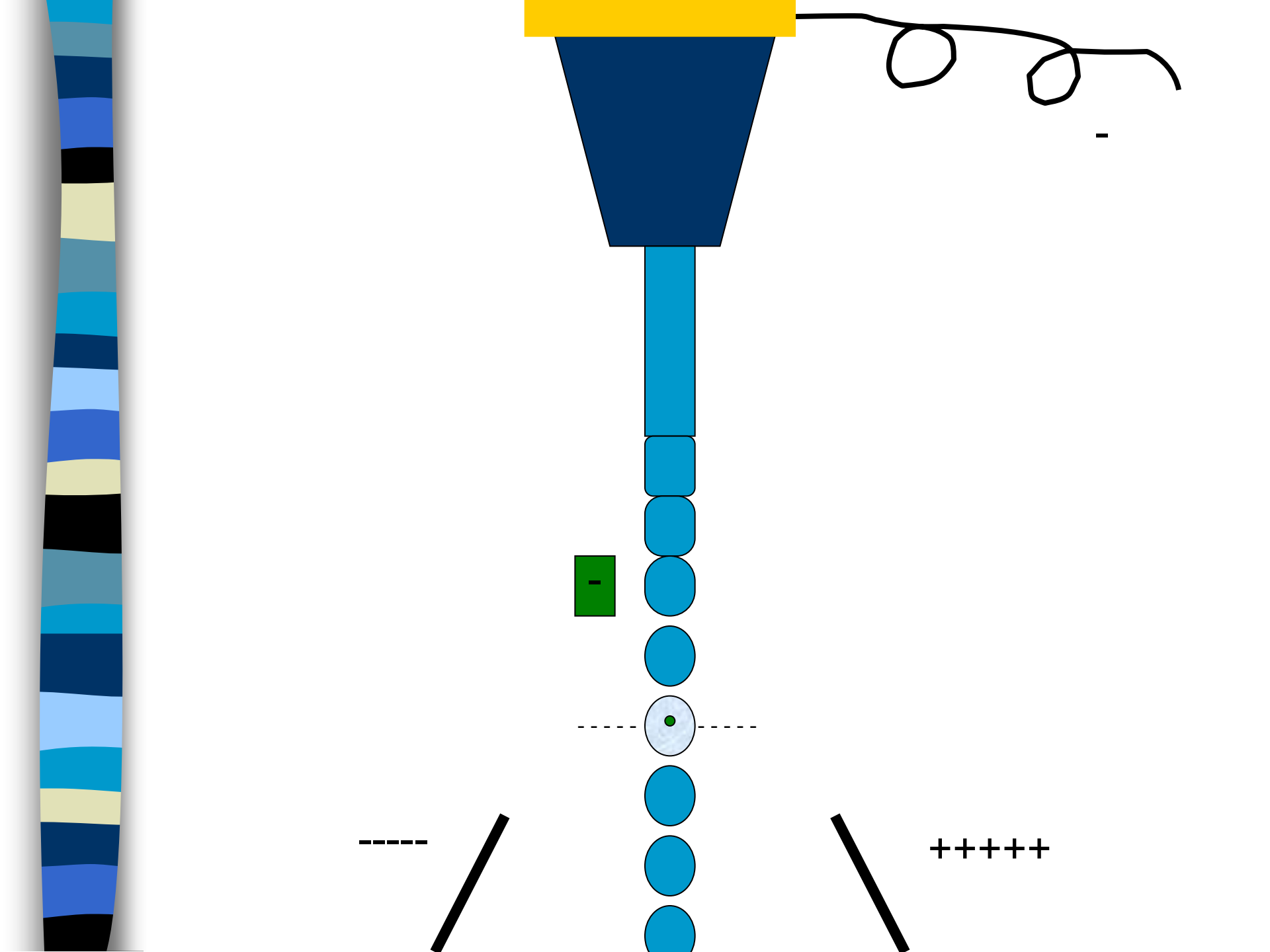


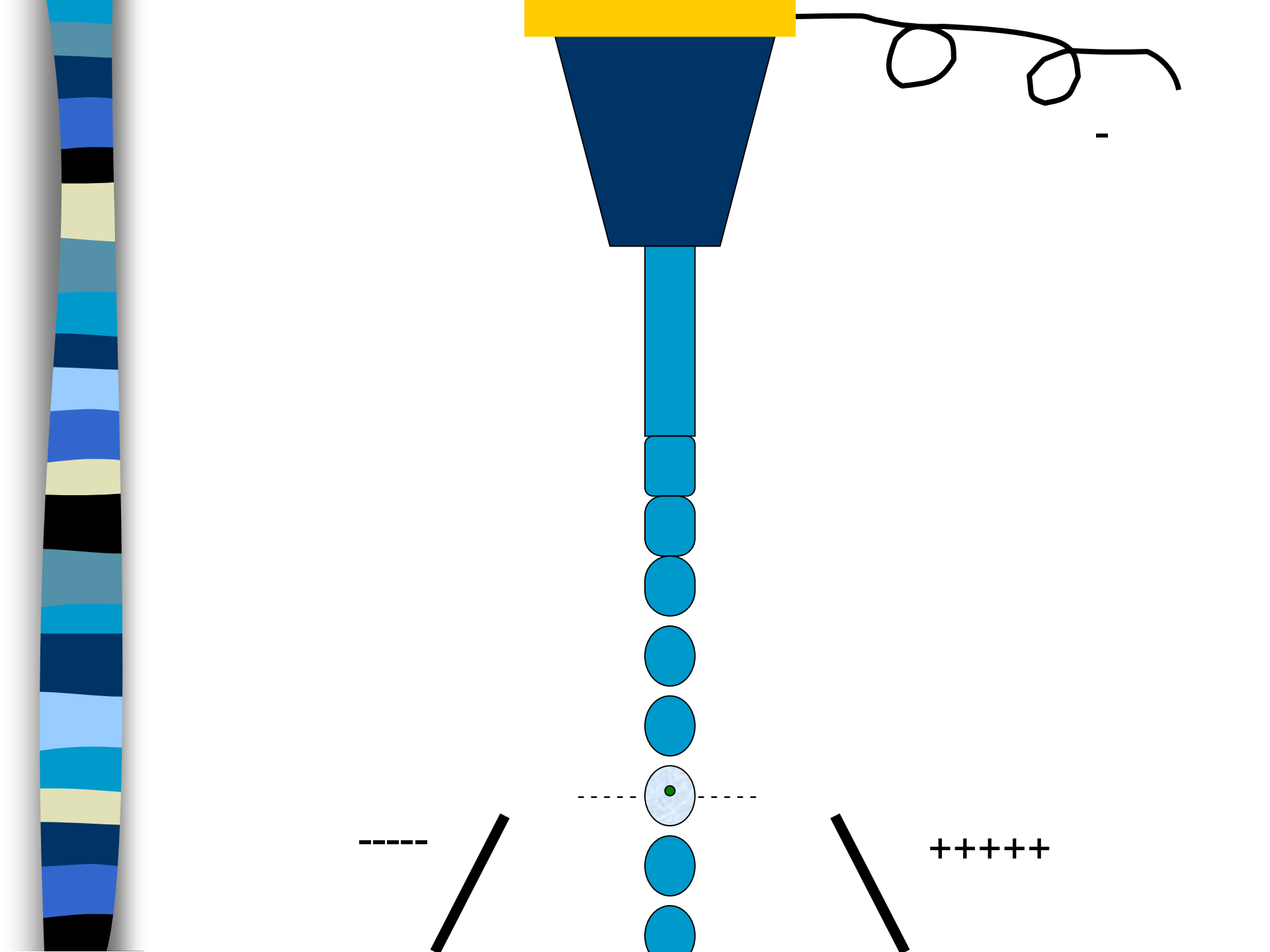


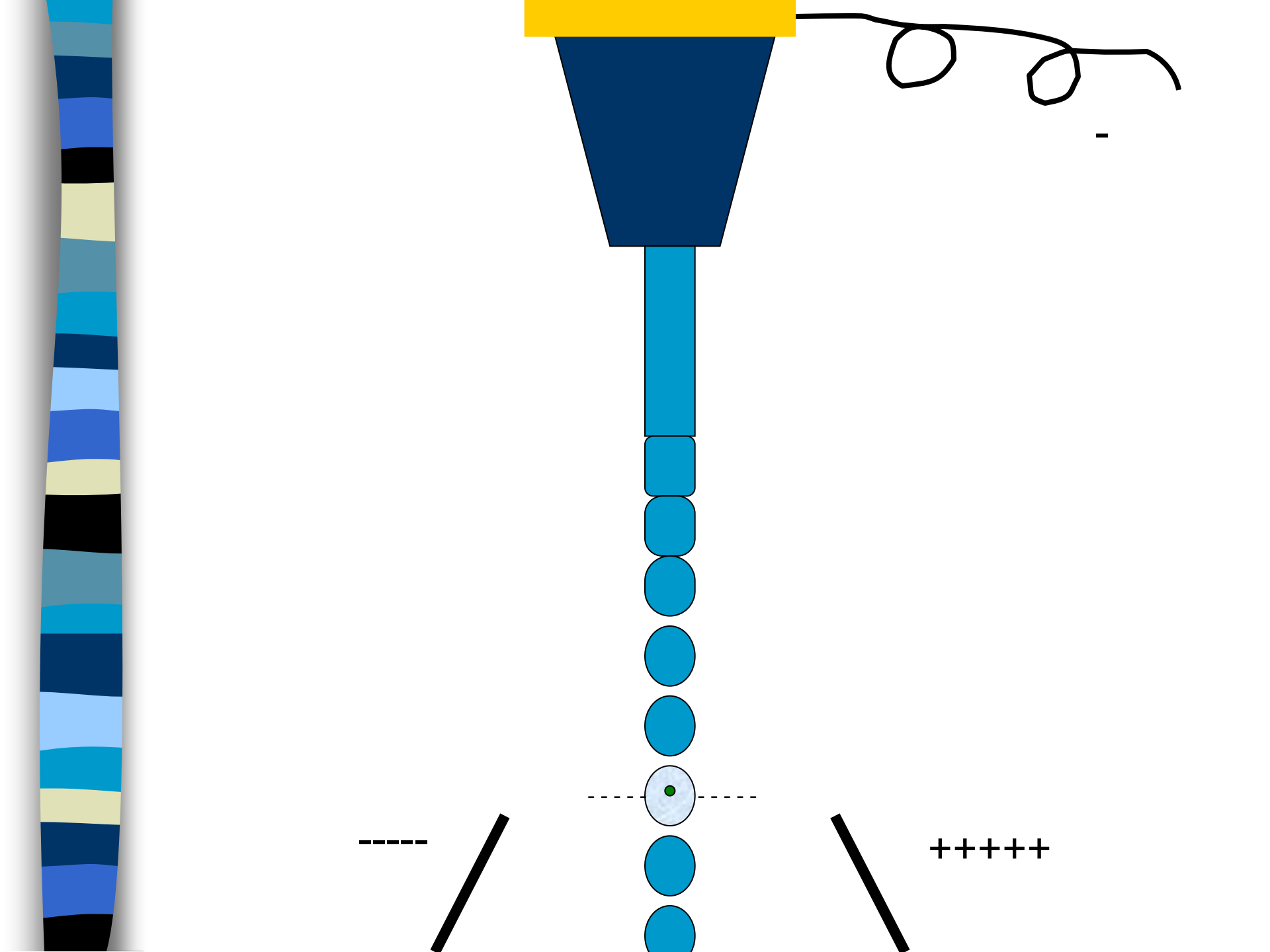


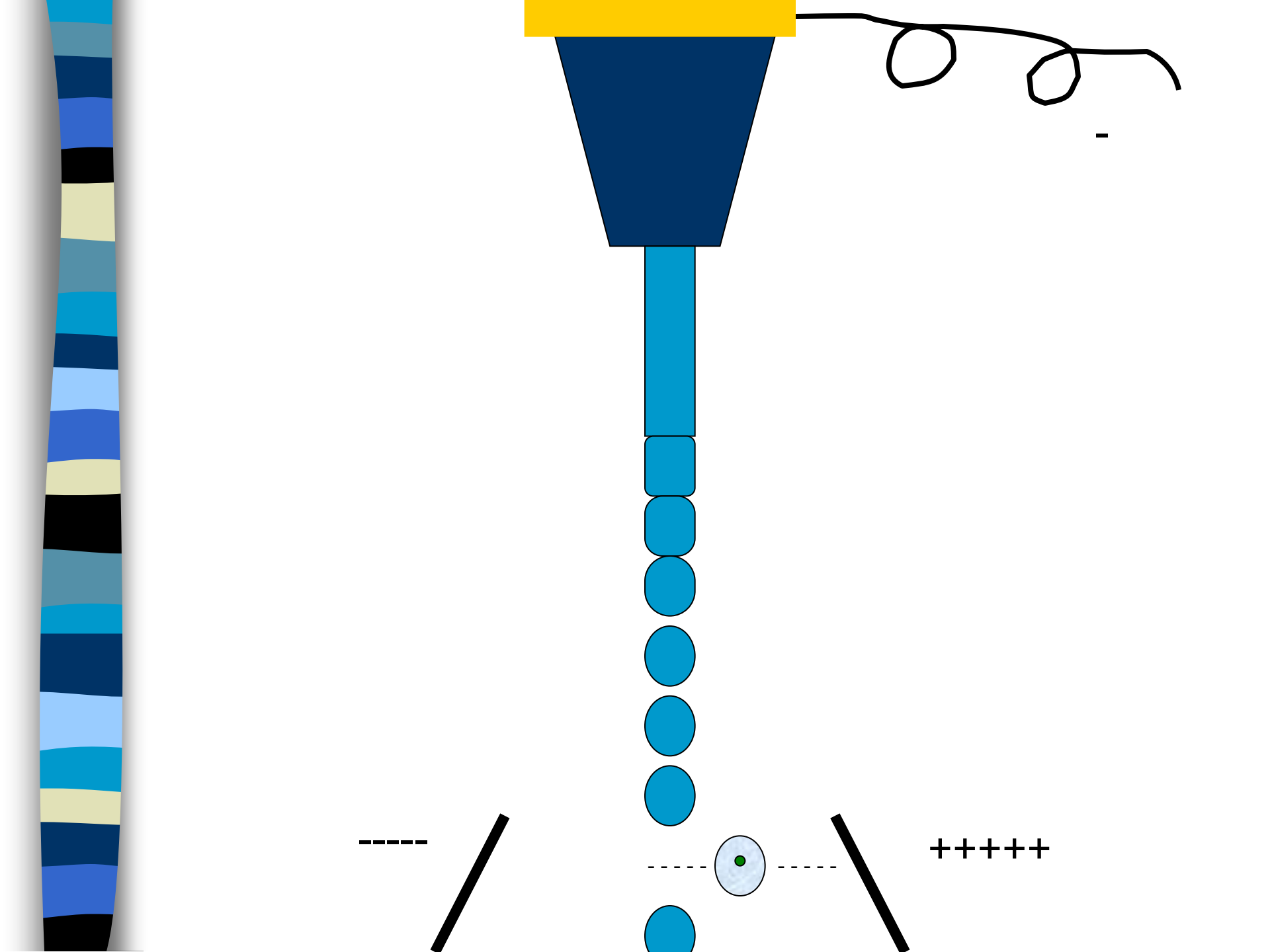










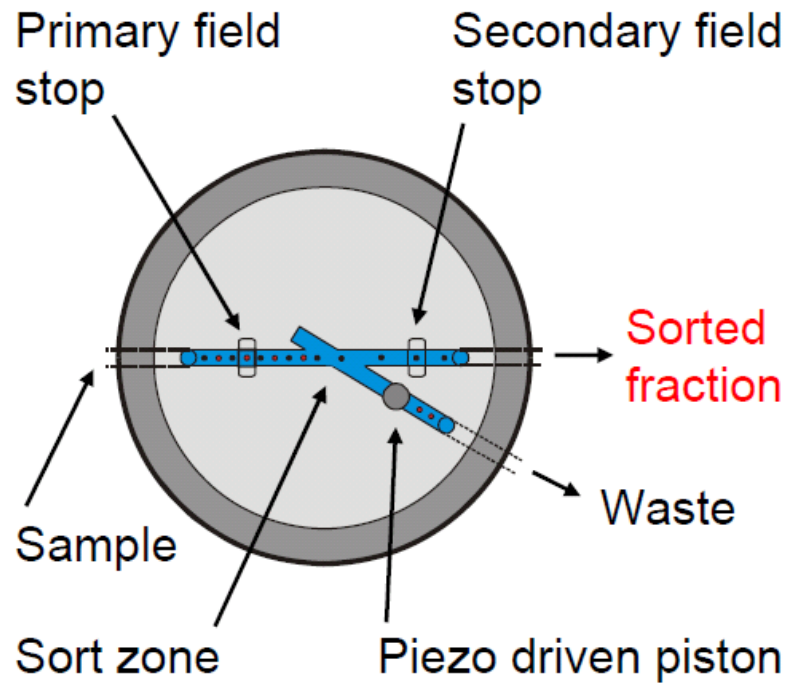


ISAC presents: Mack Fulwyler - Innovator, Inventor & Pioneer

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



FLUIDIC SWITCH SORTER

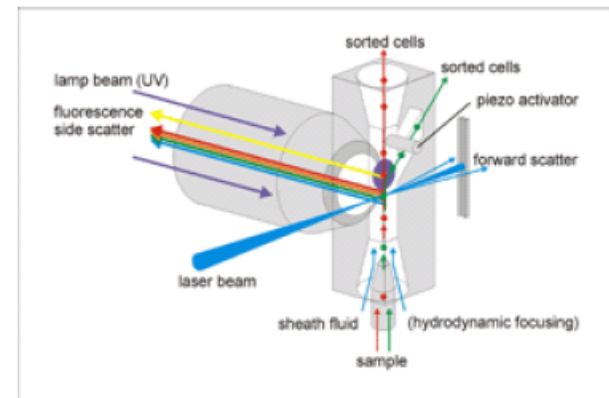
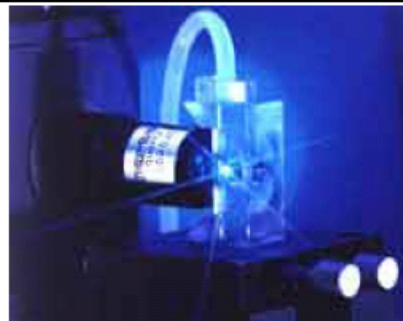


- Safety (enclosed stream)
- Gentle to cells
- Sorting of large particles ($>100\ \mu\text{m}$)

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

- Dilute sorted fraction
- Noisy

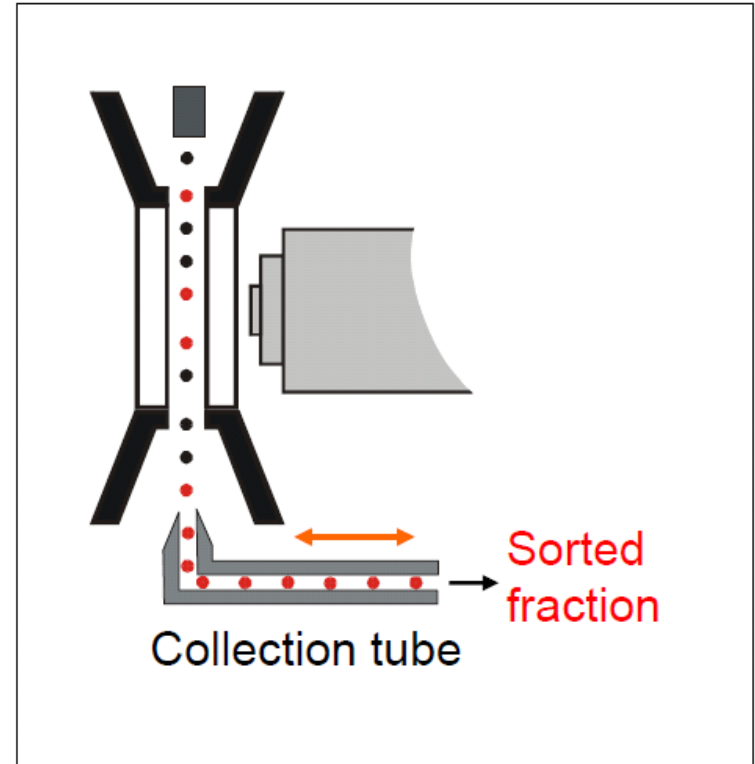
Used by: Partec



FLUIDIC SWITCH SORTER

- Safety (enclosed stream)
- Gentle to cells
- Low speed (~ 100 / sec)
- Dilute sorted fraction
- Noisy

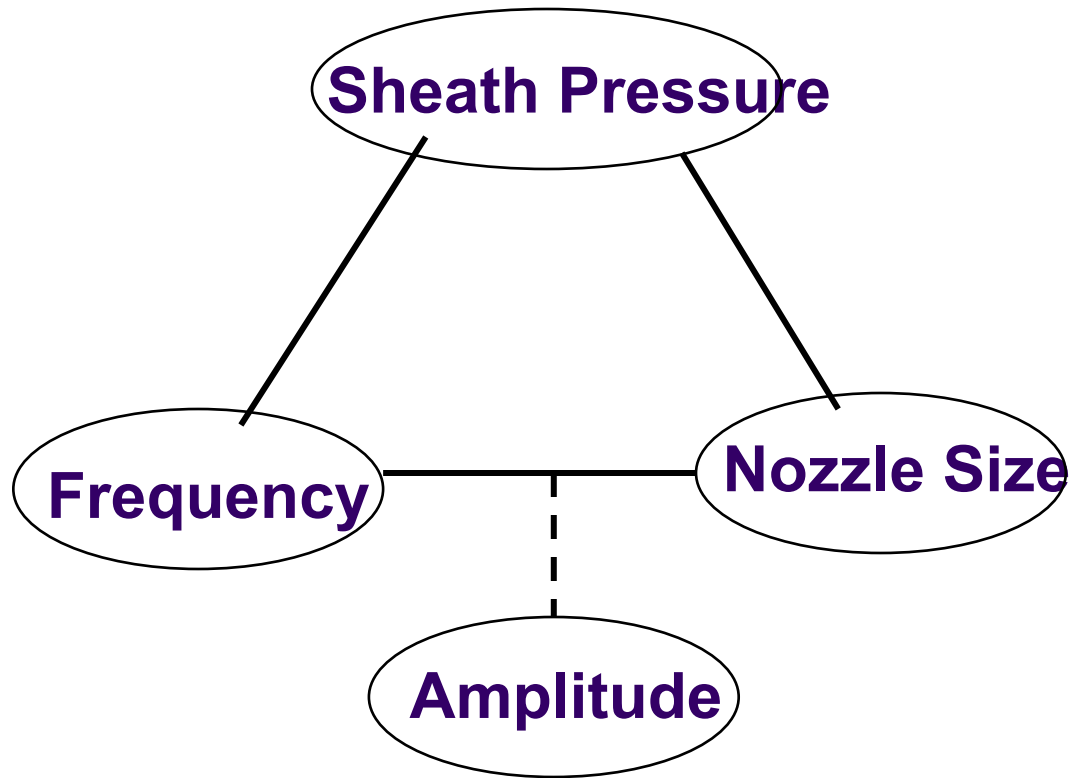
Used by: Becton Dickinson



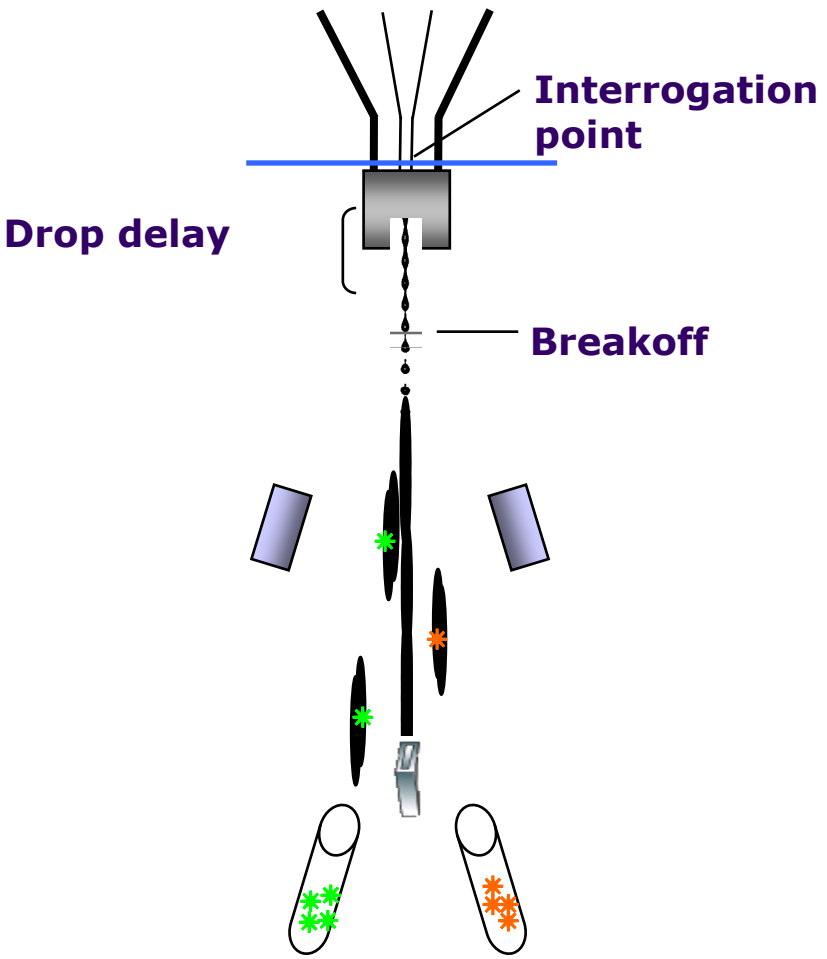
SORTING



SORTING



SORTING



SORTING

Each sort setup includes:

Sheath pressure

Breakoff window values

Side Stream window

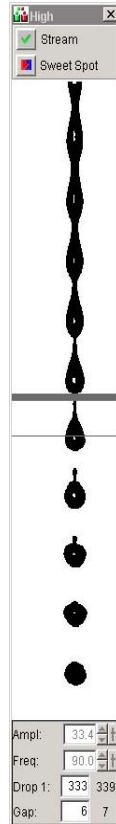
values

Table 3-2 Default Sort Setup values

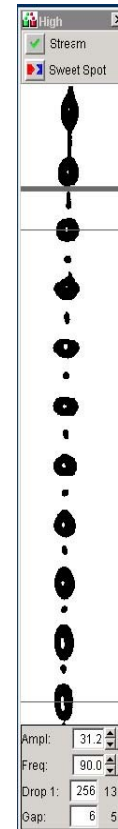
Setting	70 micron	85 micron	100 micron	130 micron
Sheath Pressure	70	45	20	10
Amplitude	60	32	12	24
Frequency	87	47	30	12
Drop 1	150	150	150	150
Gap (upper limit)	6 (14)	7 (17)	10 (21)	12 (21)
Attenuation	Off	Off	Off	Off
Drop Delay	47.00	30.00	27.00	16.00
Far left voltage	100	100	80	60



SORTING - Streams

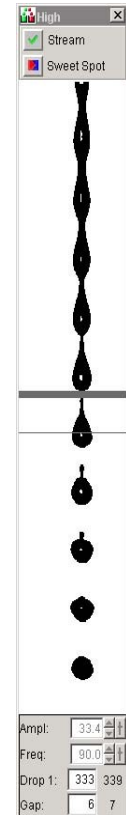
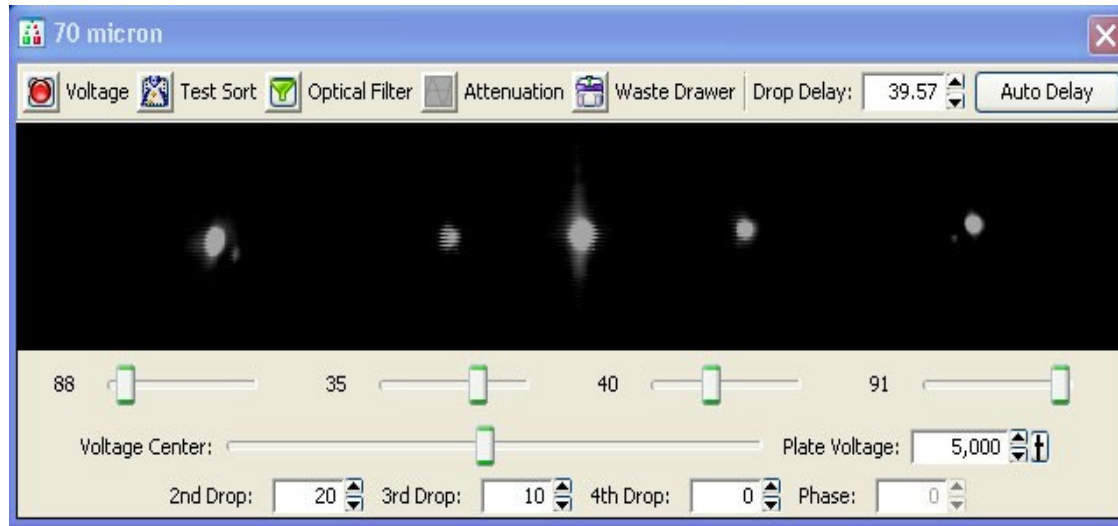


Good



Bad

SORTING – Setup Side Streams

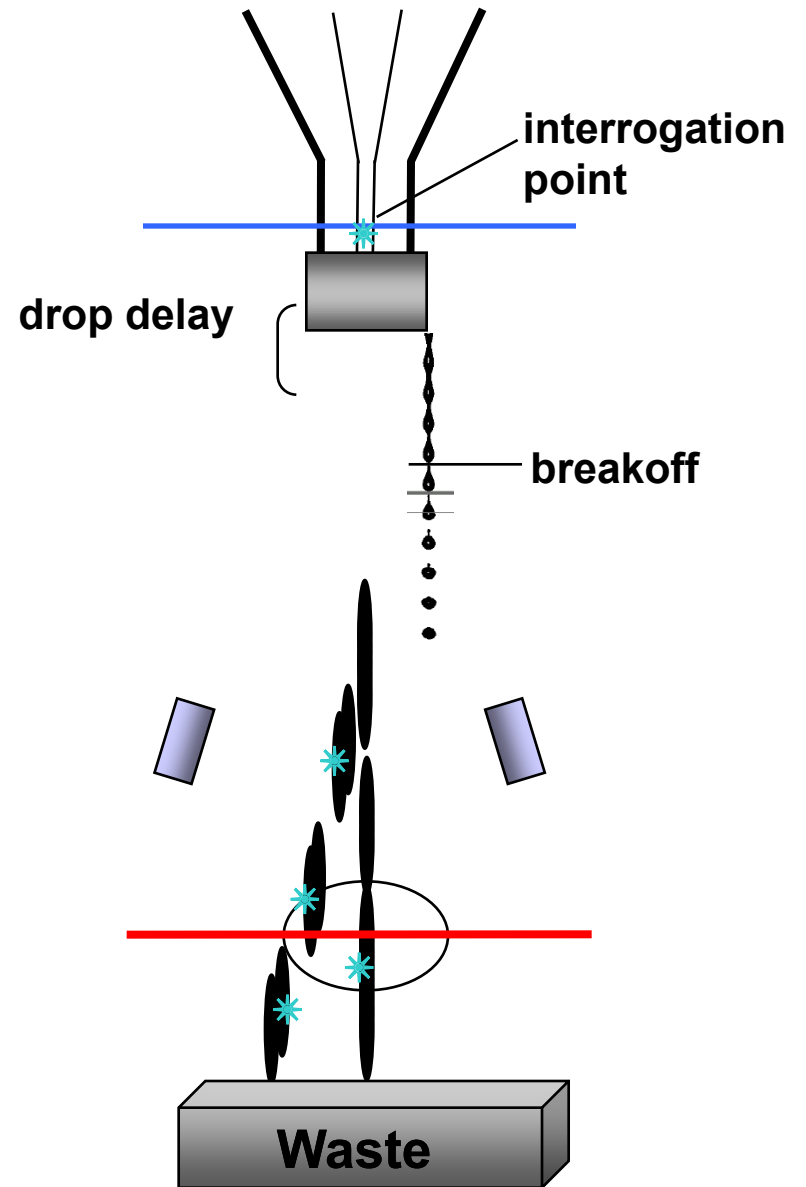


Drop Delay

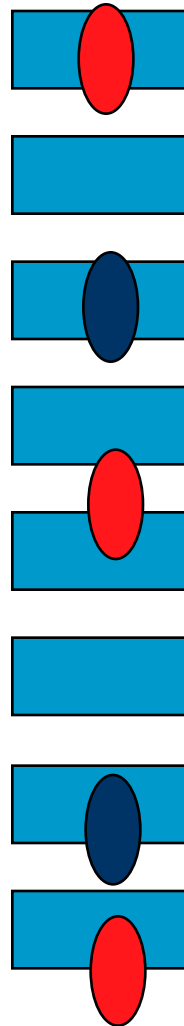
BD FACS™

Accudrop
technology

- Accudrop beads
- Diode laser
- Camera
- Optical filter

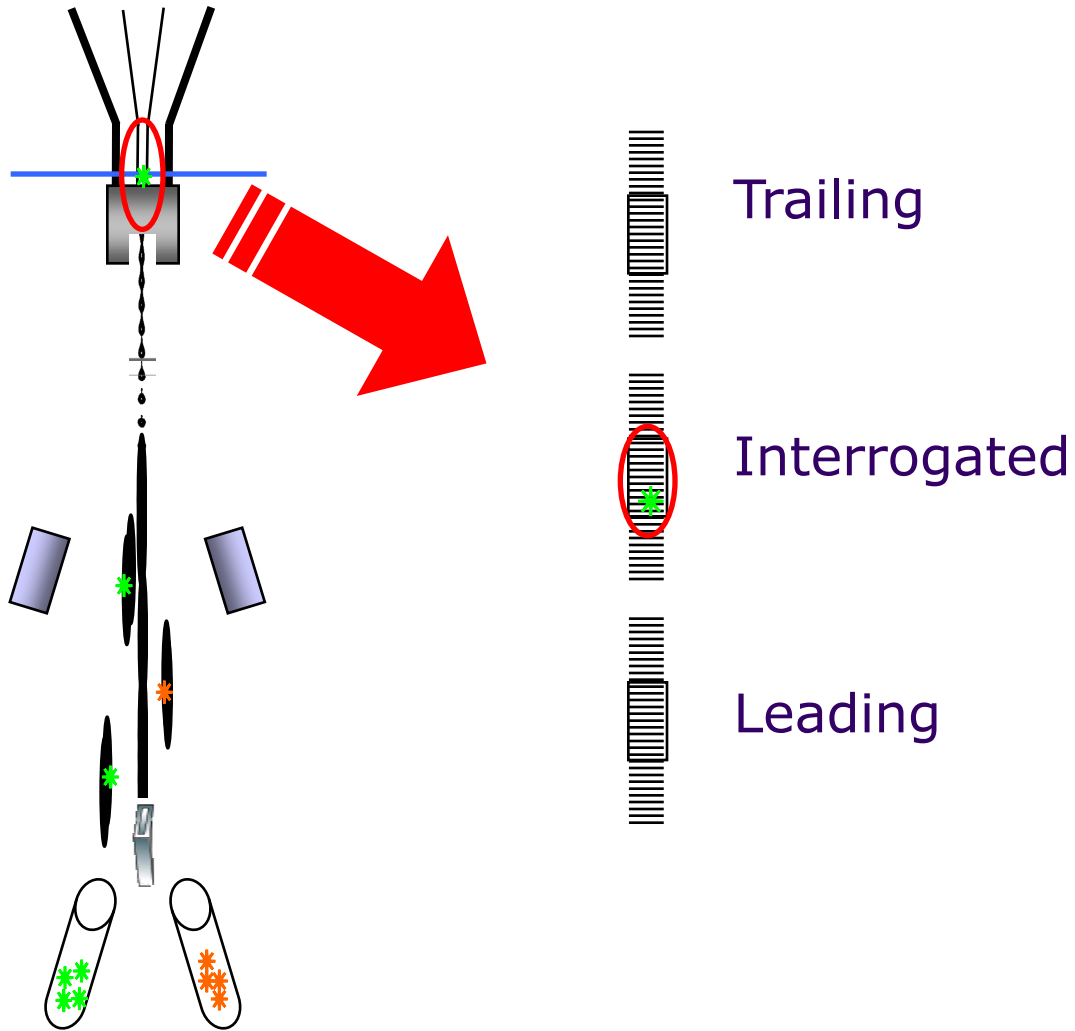


Sorting - Sort Masks



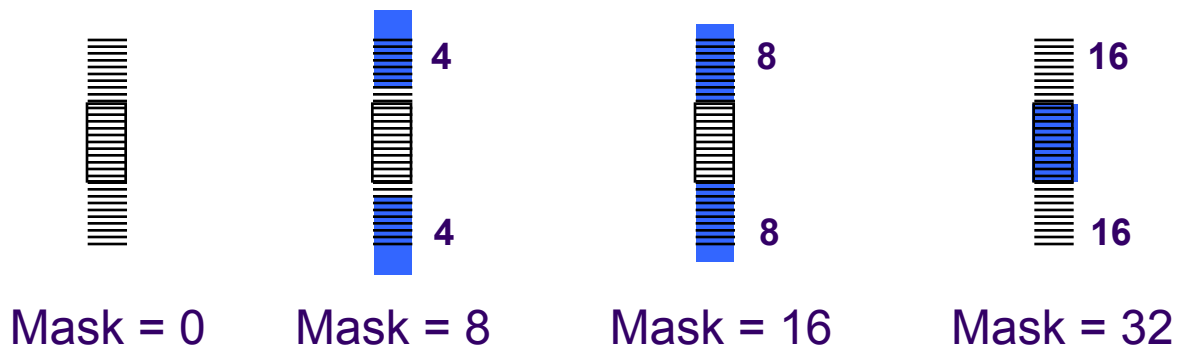
Cells are randomized
distributed over the stream

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



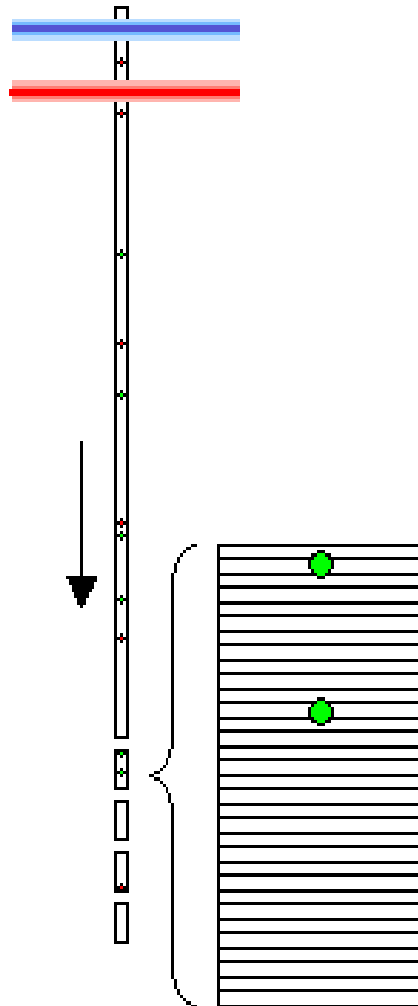
Conflict Resolution

- 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

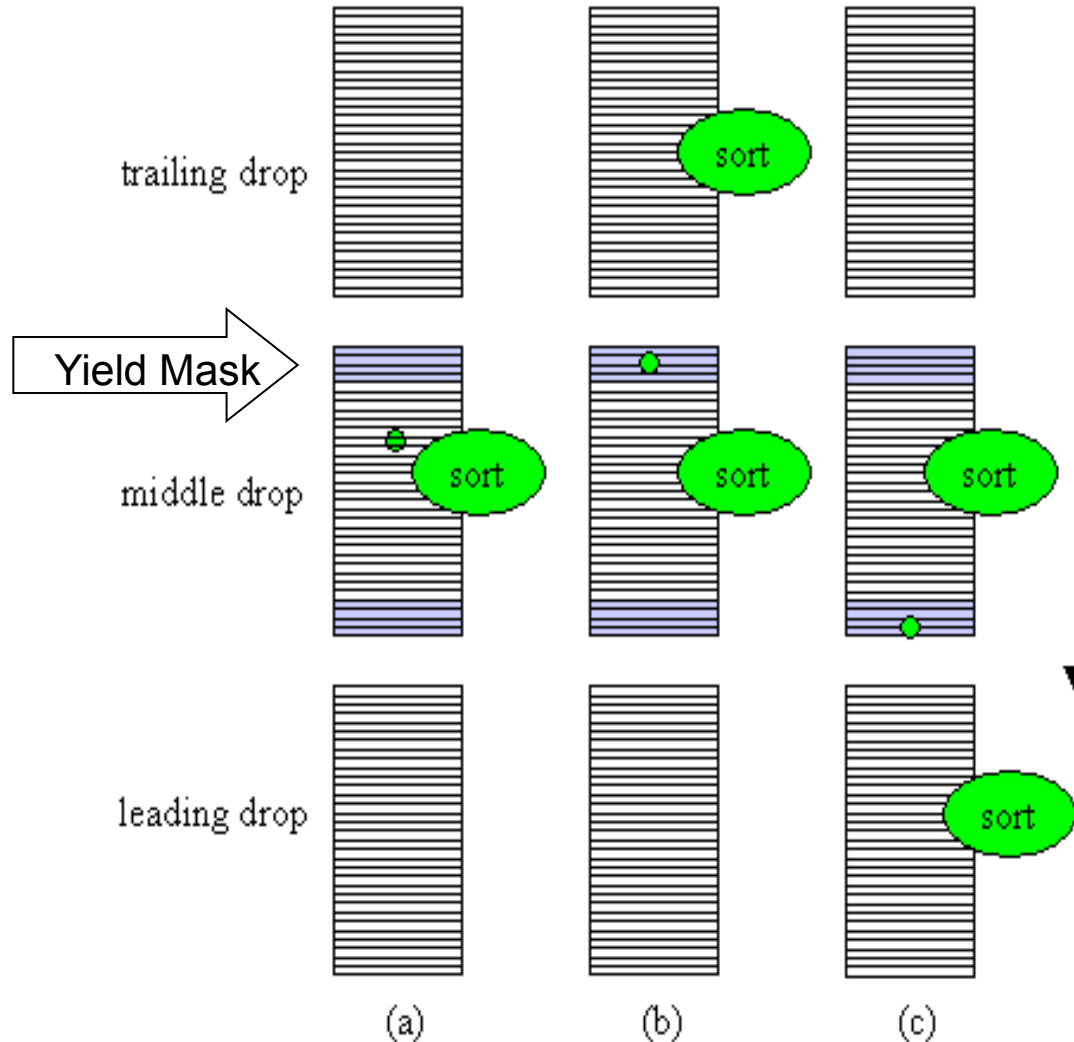
Sort decisions are determined by sort masks



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

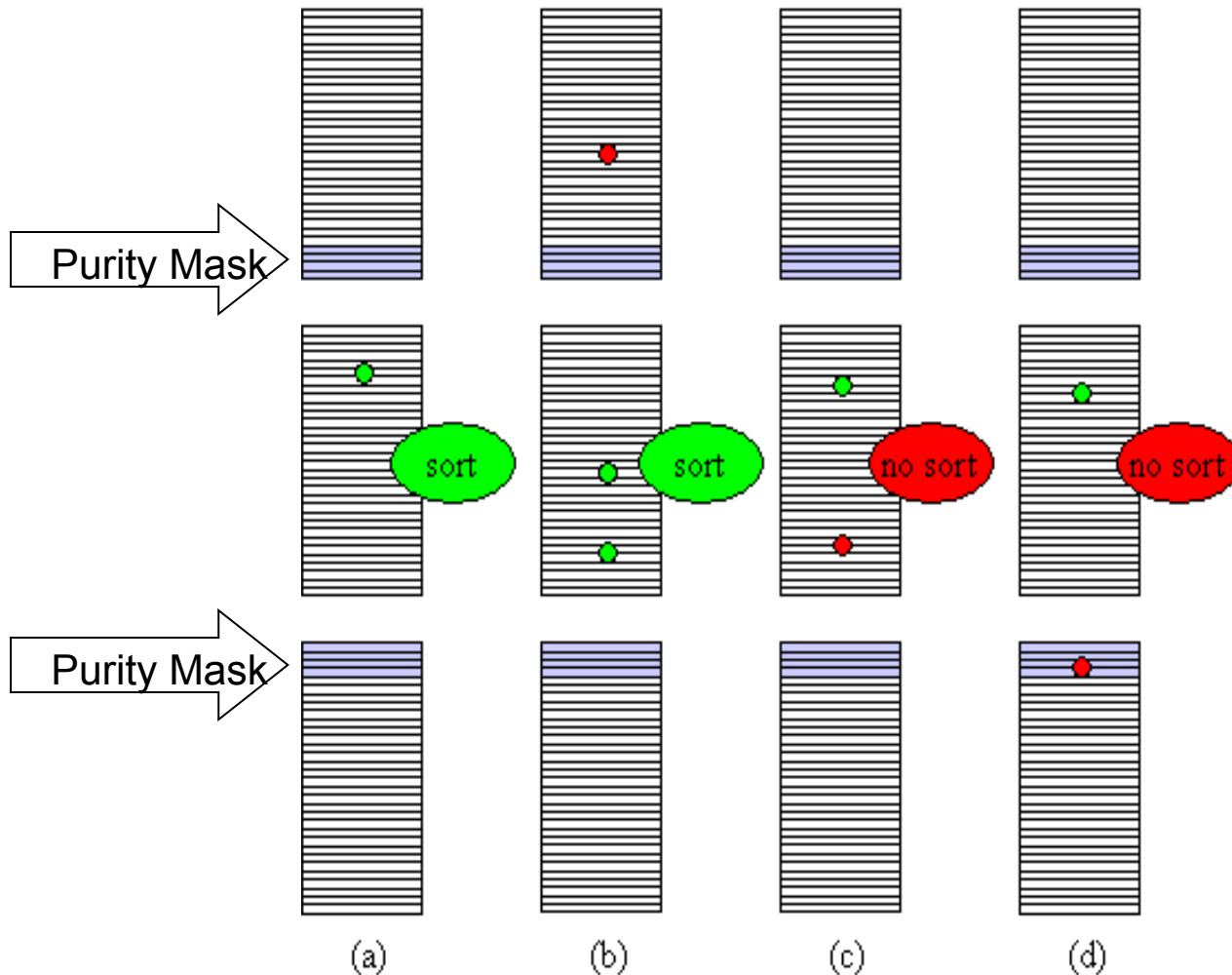
Sorting - Yield Mask

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

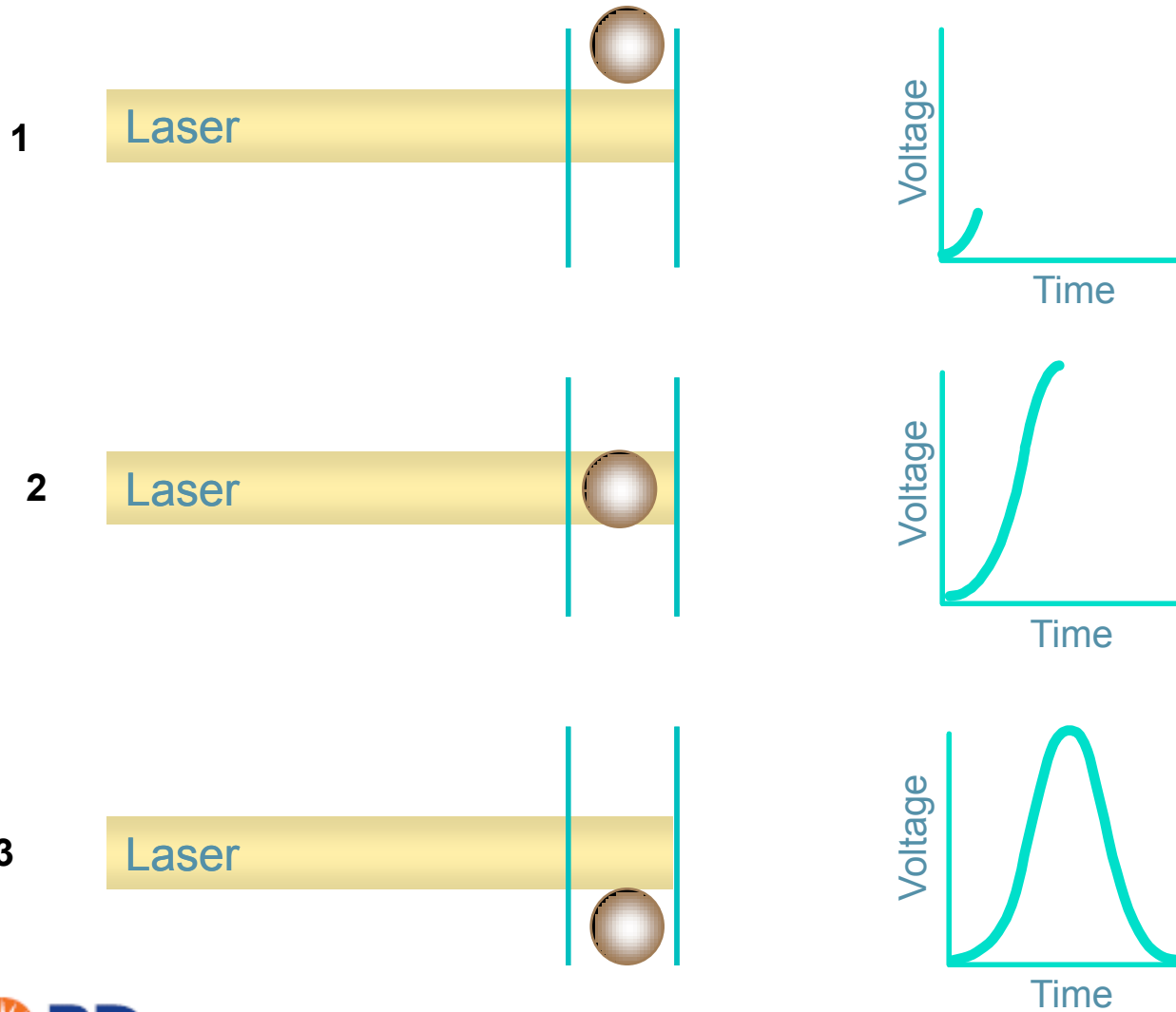
- Zpracování signálu z detektorů
 - Předzesílení
 - zesiluje signál pro přenos z detektorů do centralní elektronické části
 - Zesílení
 - úprava intensity signálu
 - lineární nebo logaritmické
 - Generování integrálu a šířky pulsu
 - Analog-digital konverze



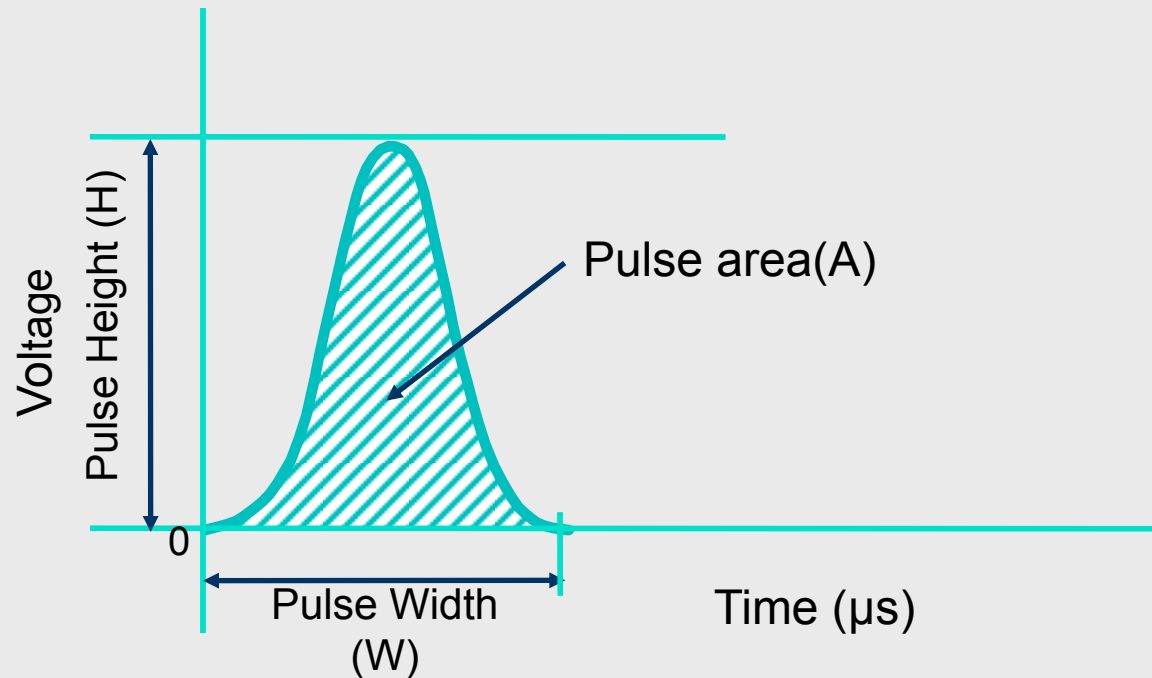
Sběr dat

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

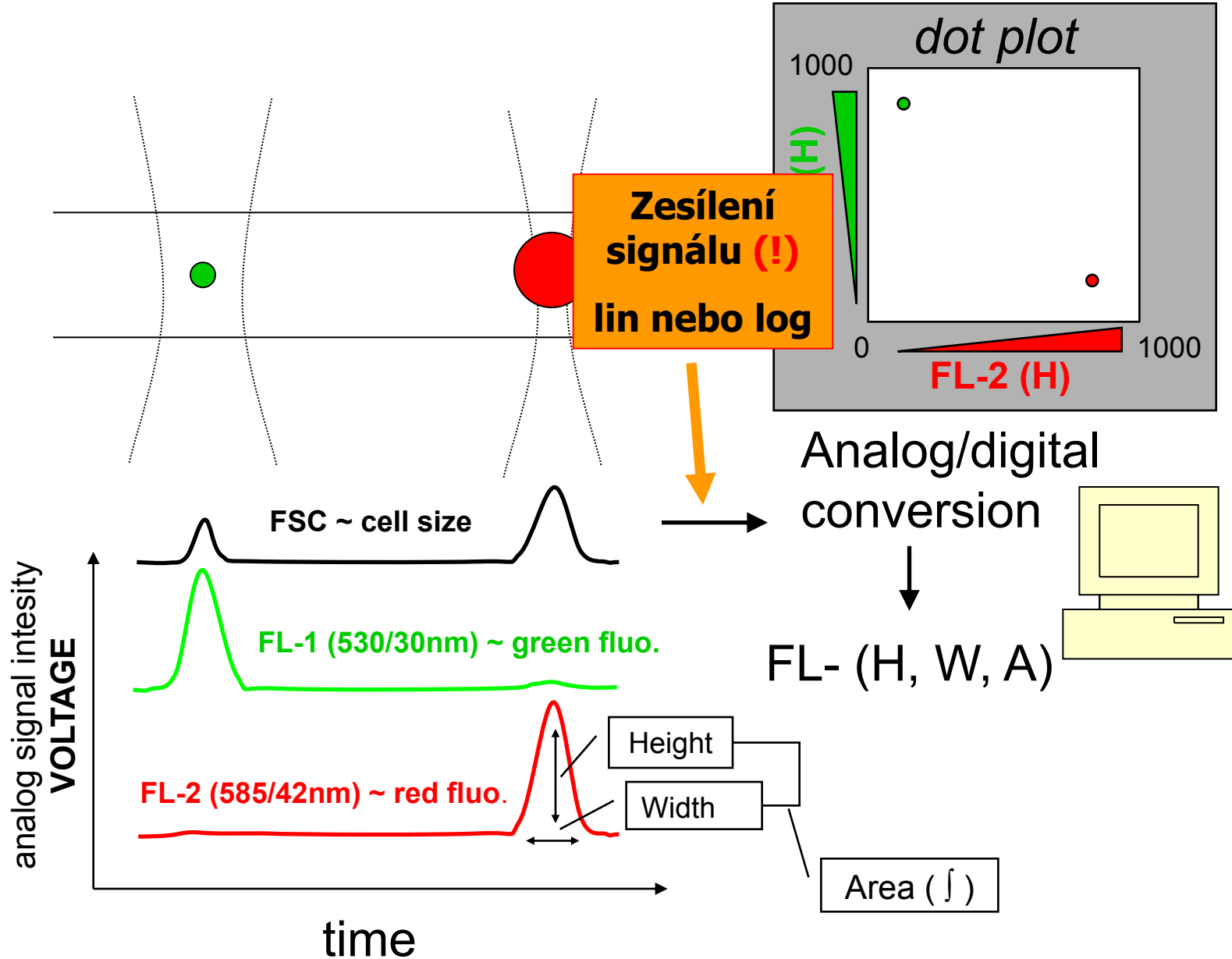
Creation of a Voltage Pulse



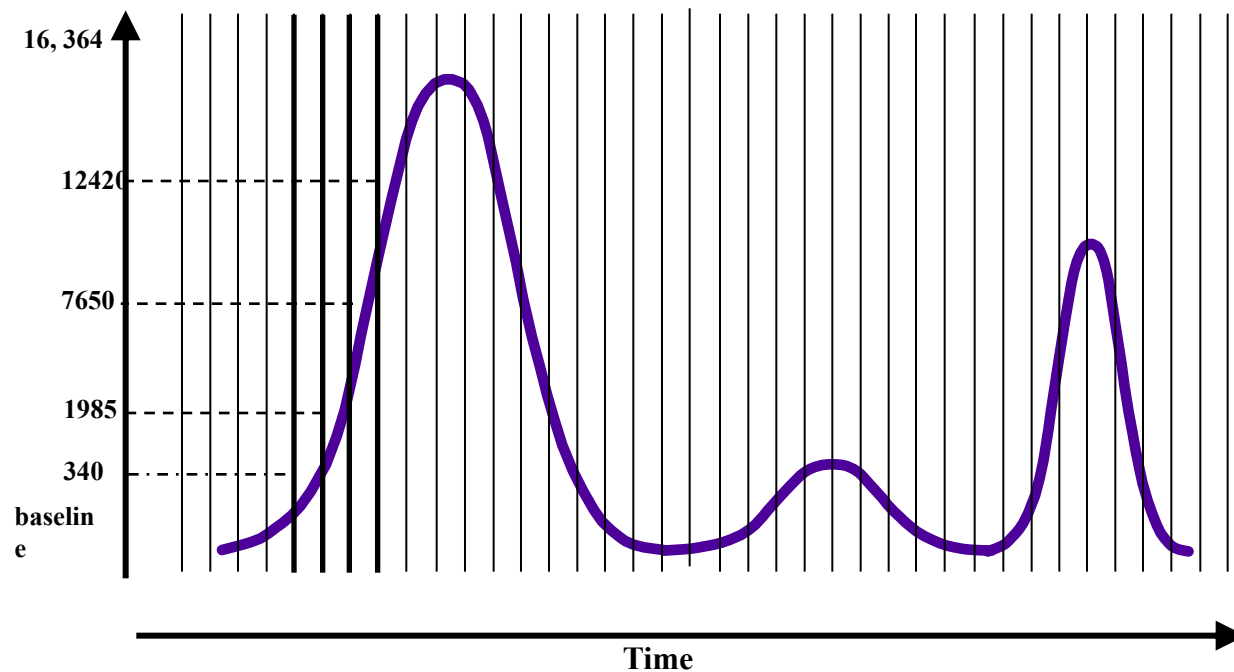
Height, Area, and Width



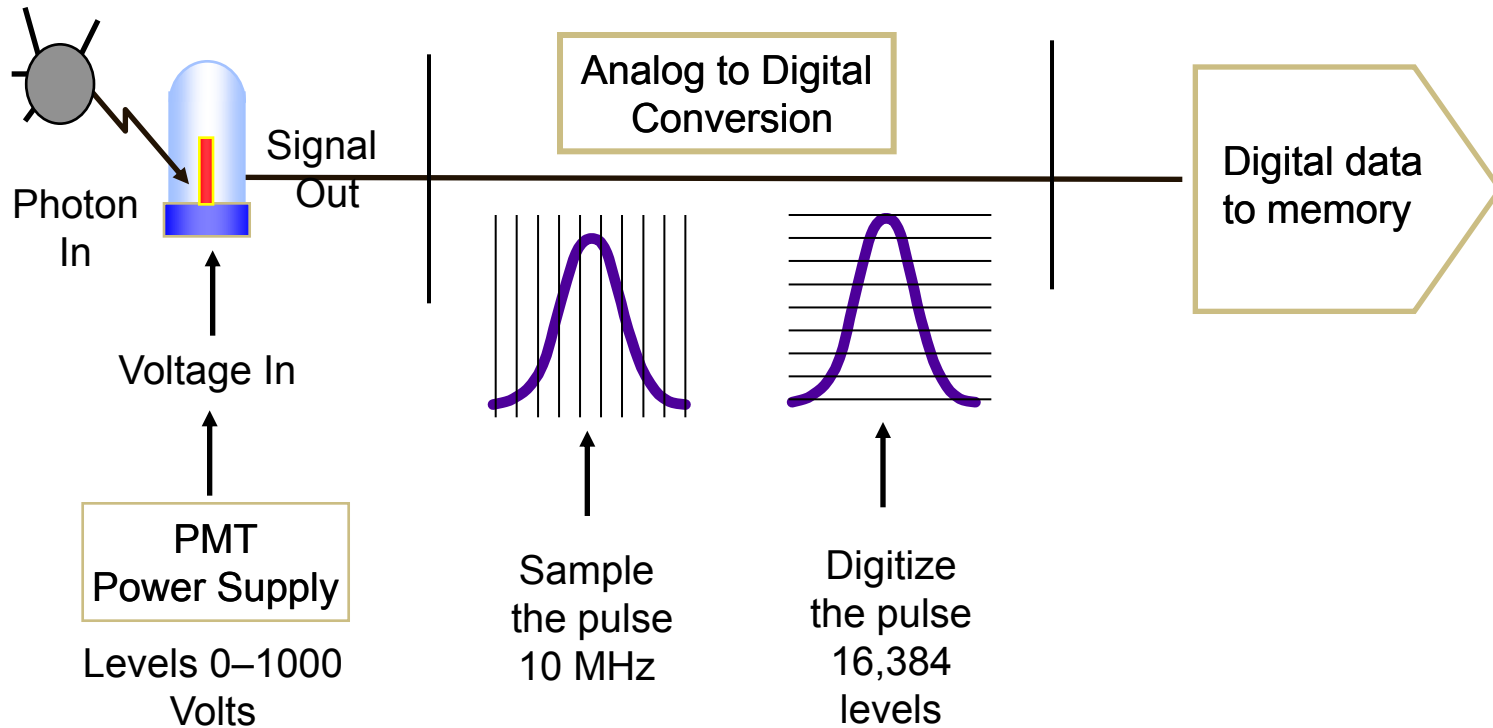
Signal processing



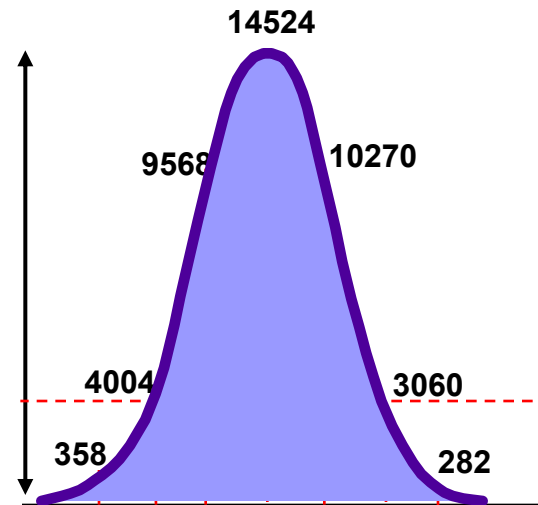
Analog to Digital Converter



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

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



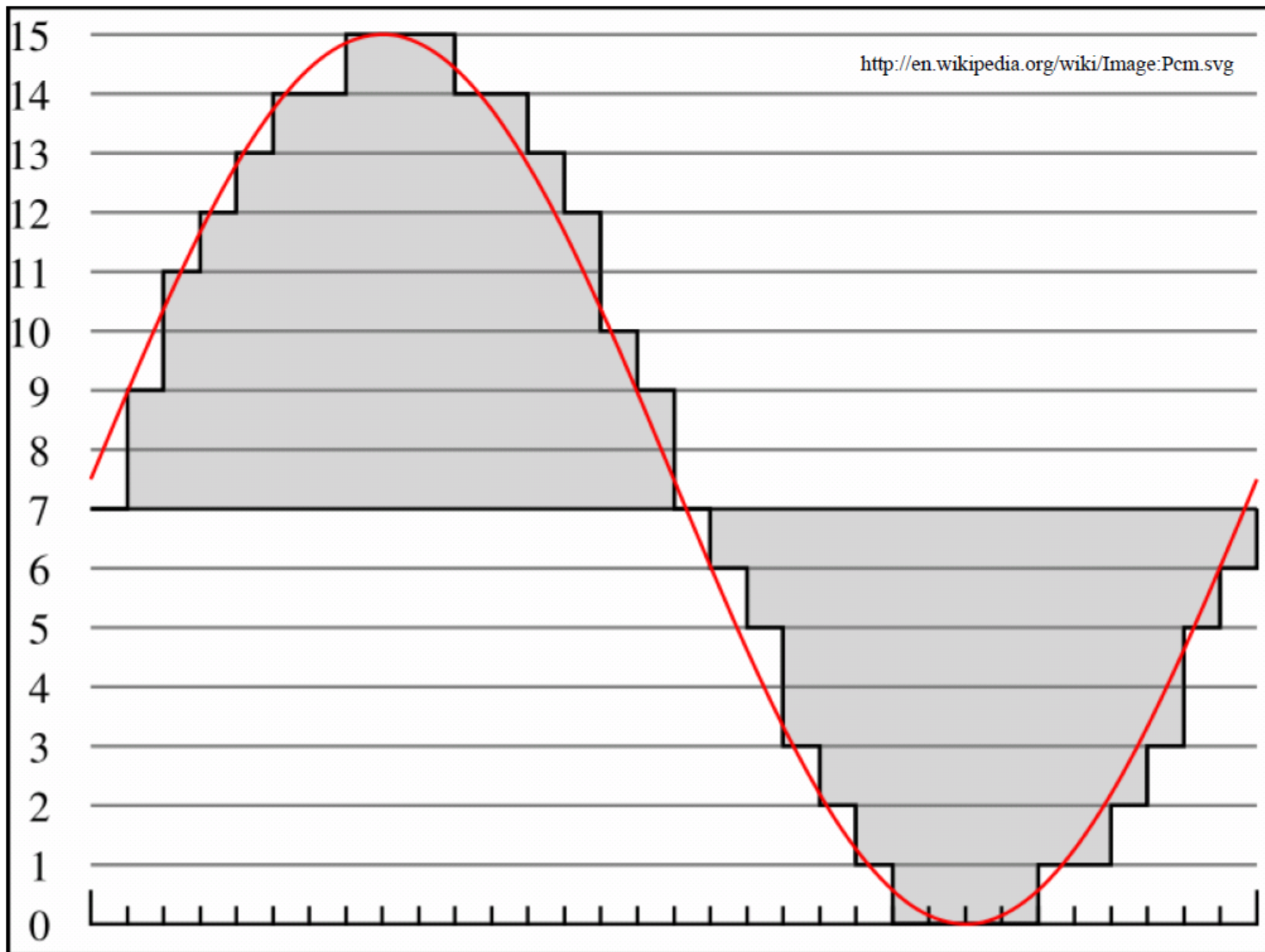
Charakteristiky pulsu

- 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



Analogově-digitální převod

- Analogový signál musí být konvertován na digitální puls, aby bylo umožněno jeho další zpracování
- Pomocí analog-digitální konverze (ADC) – analogový vstup/digitální výstup
- Obvod rozděljuje signál do předvolených kanálů
- 8 bitů = 256 kanálů
- 10 bitů = 1024 kanálů



4 bitová pulzně kódová modulace analogového signálu

$$2^4=16$$



Lineární a logaritmický obvod

- lineární
- logaritmický
- kompenzace fluorescenčního signálu

$$2^8 = 256$$

$$2^{10} = 1024$$

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

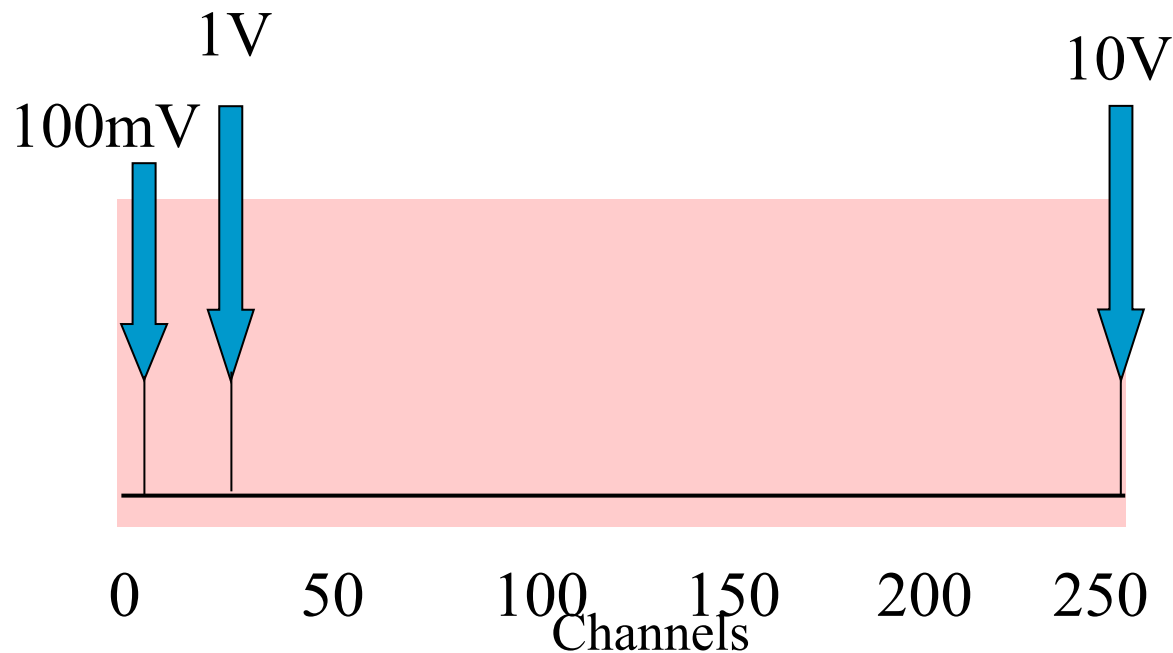
Full scale measurement range = 0 to 10 volts

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

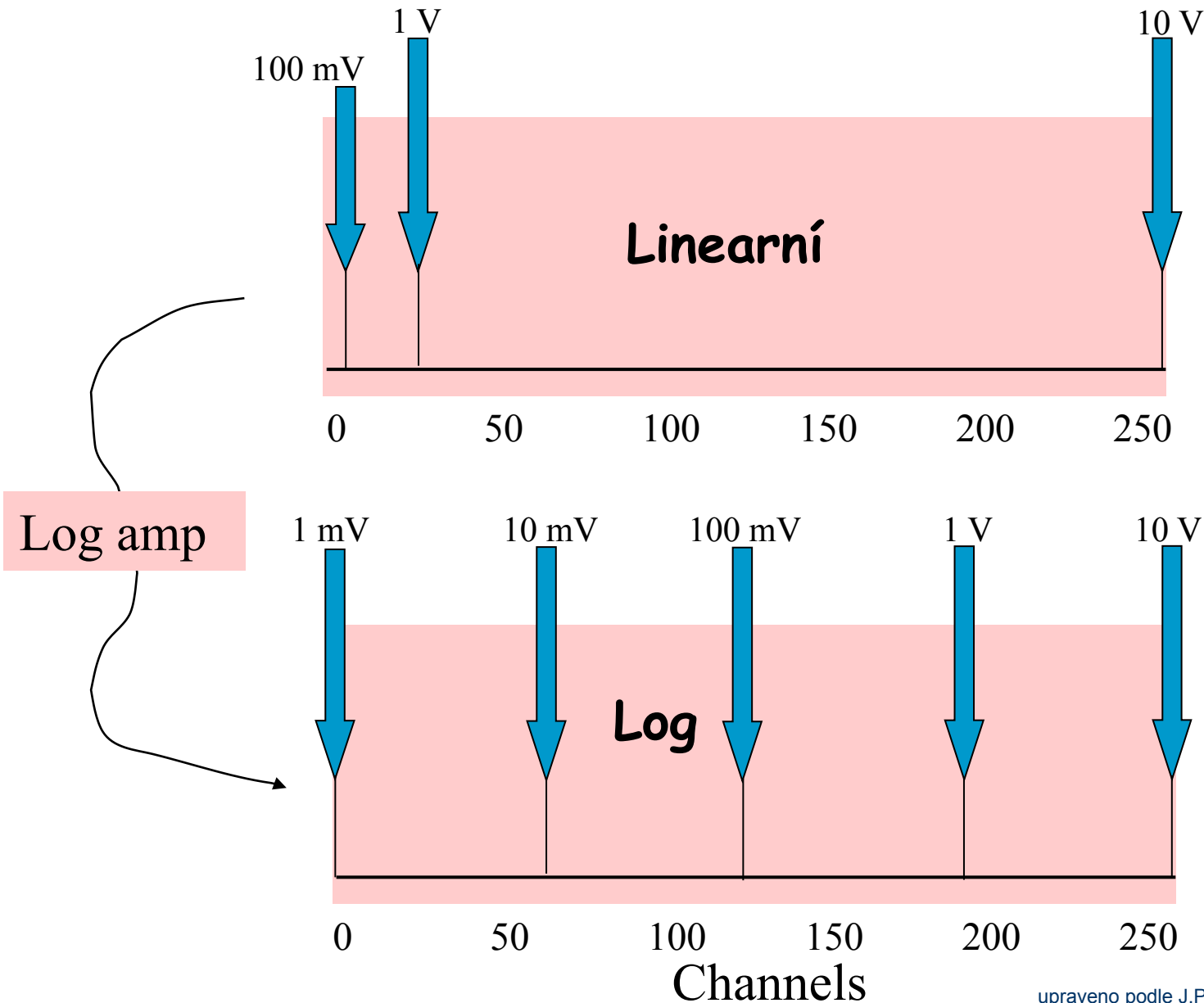
ADC voltage resolution is: $(10-0)/4096 = 0.00244$ volts = 2.44 mV

Kolik bitů?

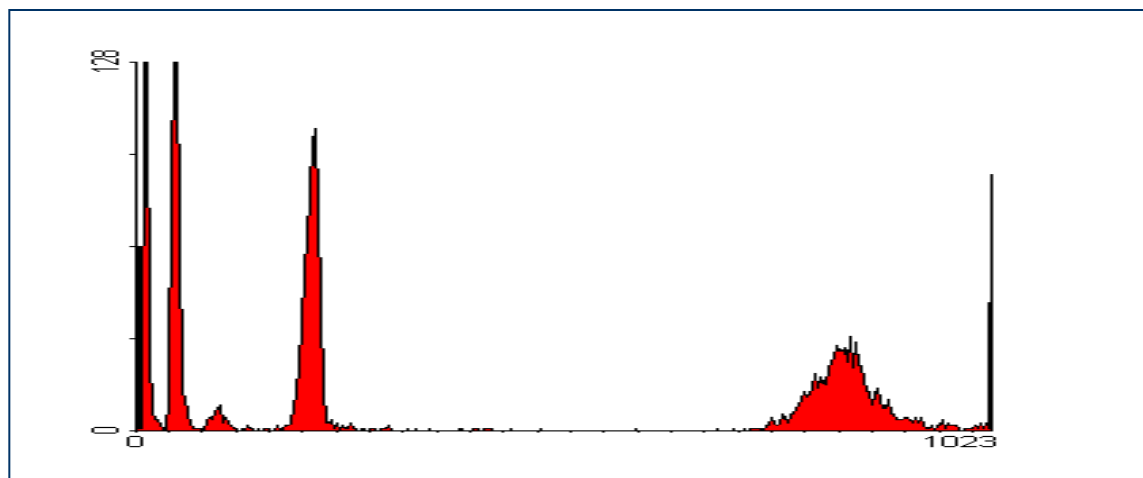
- Pokud konvertujeme analogový signál pomocí 8 bitového ADC – máme 256 kanálů ($2^8=256$) odpovídajících rozsahu 0-10 V
- Rozdíl mezi kanály je $10/256 \approx 40\text{mV}$



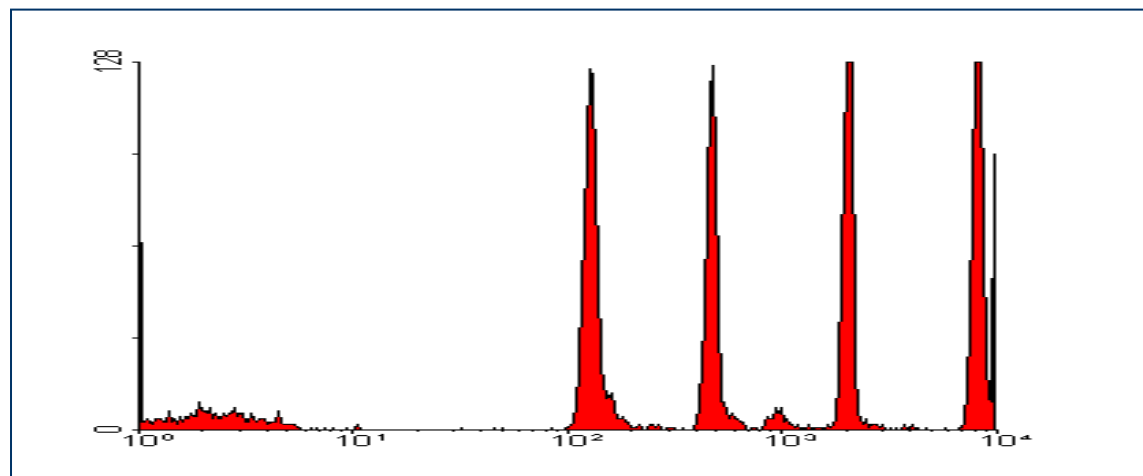
Ideální logaritmický zesilovač



Logaritmické zesílení & dynamický rozsah



lin



log



Kompenzace fluorescenčního signálu

...později



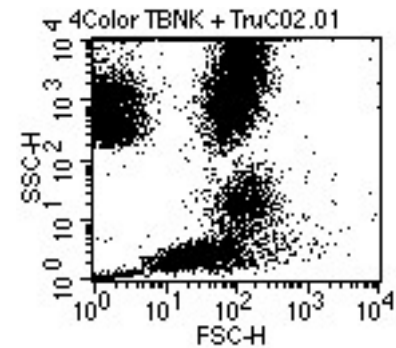
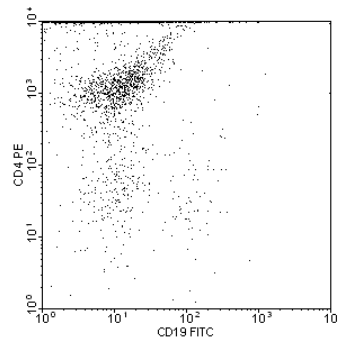
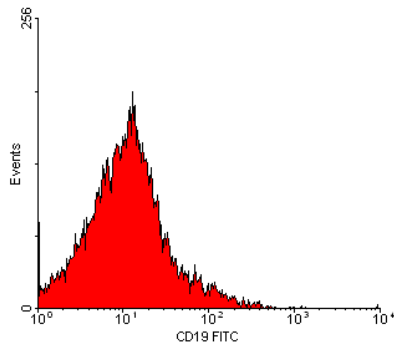
Analýza dat

■ Zobrazení dat

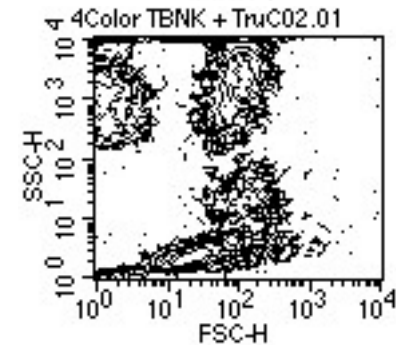
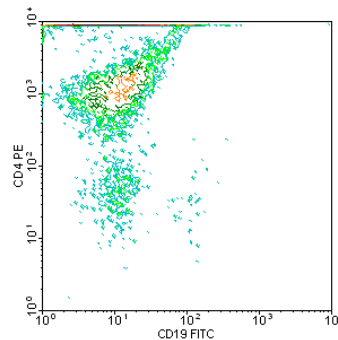
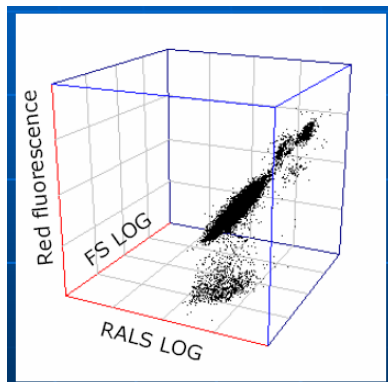
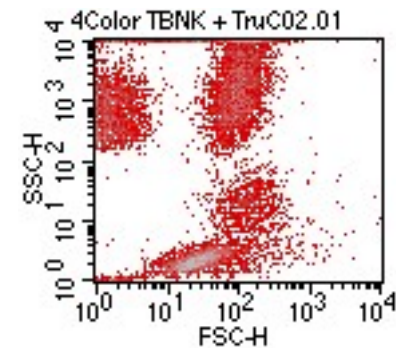
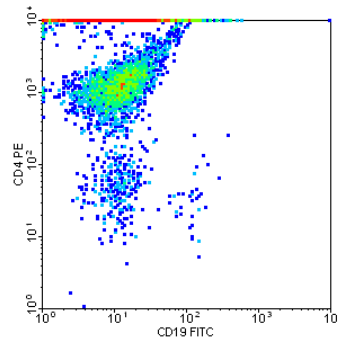
- histogram
- dot plot
- isometric display
- contour plot
- chromatic (color) plots
- 3 D projection

■ Gating

Způsoby pro zobrazení dat

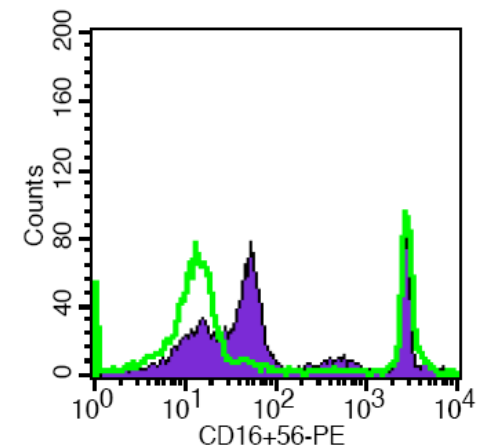
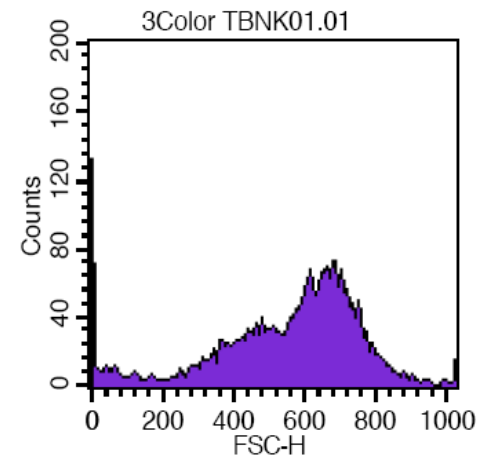


4Color TBNK + TruC02.01



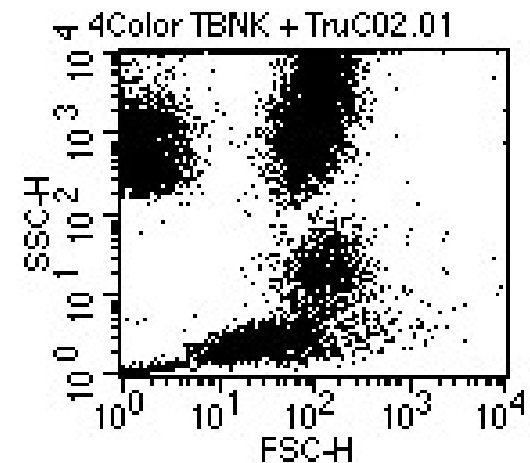
Histogram distribuce četnosti

- Histogram zobrazuje četnost částic pro jeden parametr
- Jednoduchý výstup
- 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 denzitě částic
- Problémy s vykreslením v případě velkých objemů dat



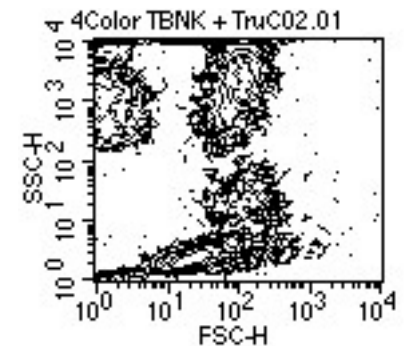
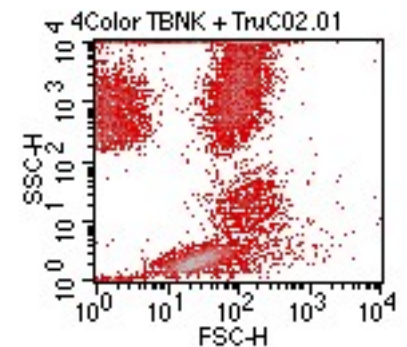
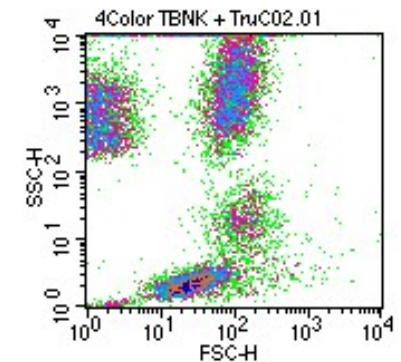
Density & contour plot

Density plot:

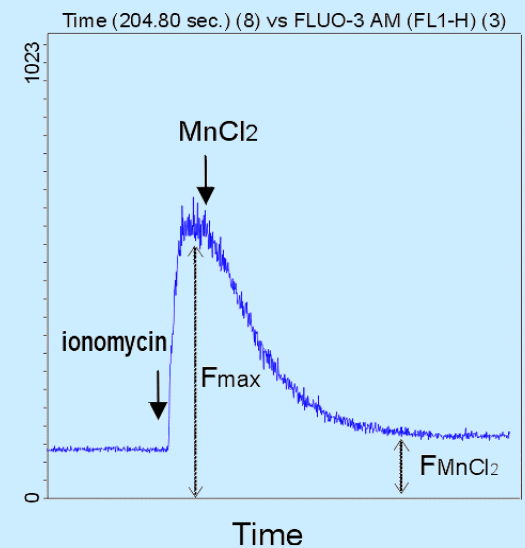
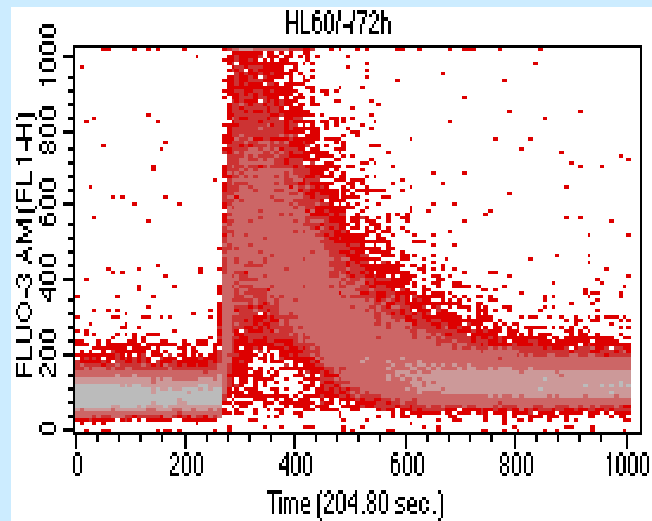
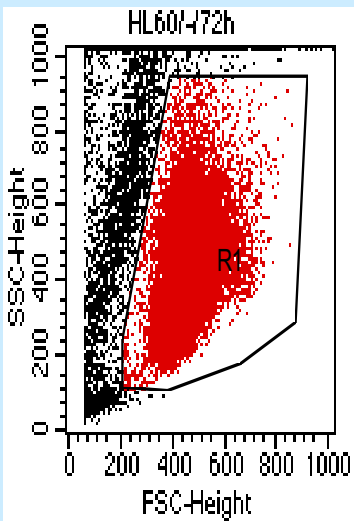
- Zobrazuje dva parametry jako frekvenci četnosti
- barva a nebo její odstín odpovídá četnosti částic

Contour plot:

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



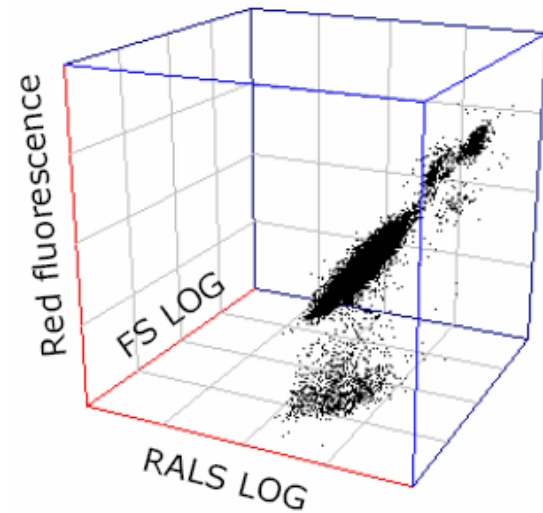
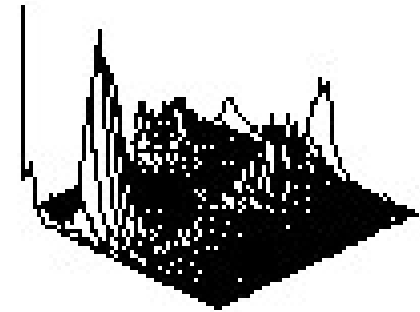
Čas jako jeden z parametrů



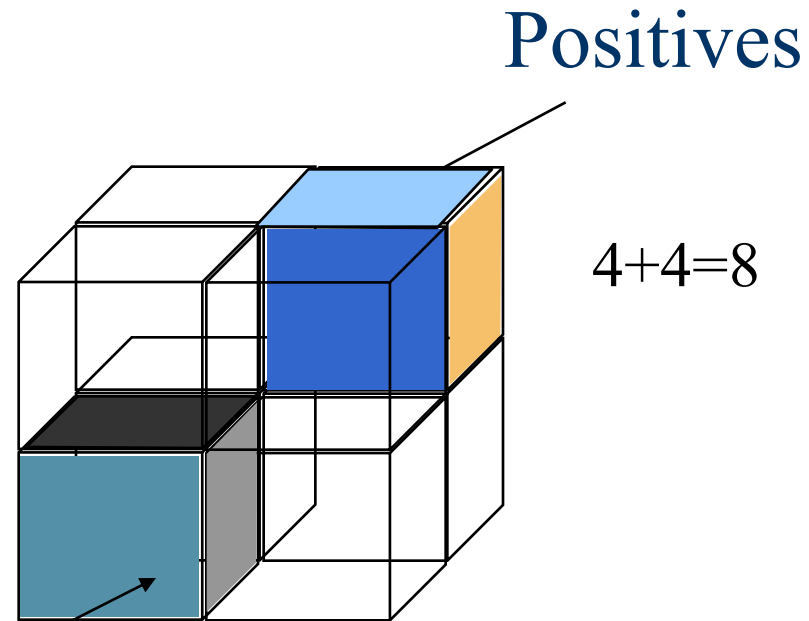
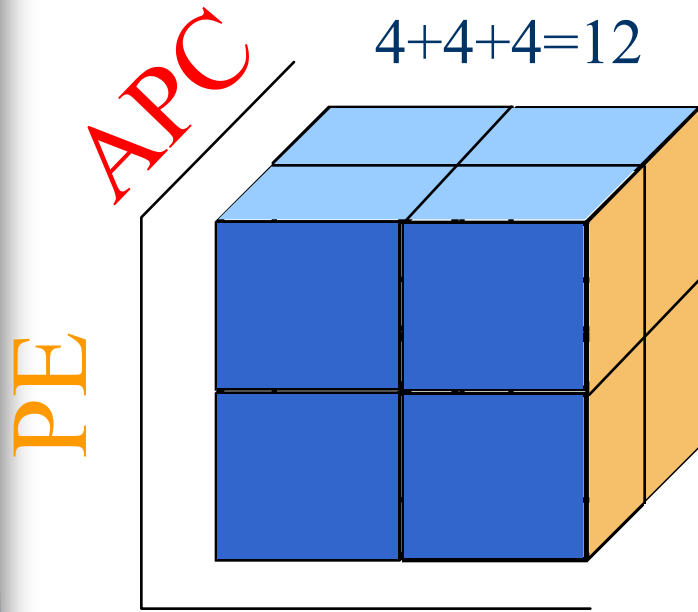
3D zobrazení

- 2 parametry + četnost
- 3 parametry společně

4Color TBNK + TruCO2.01

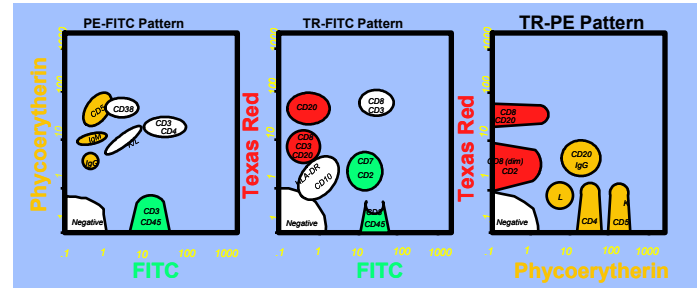
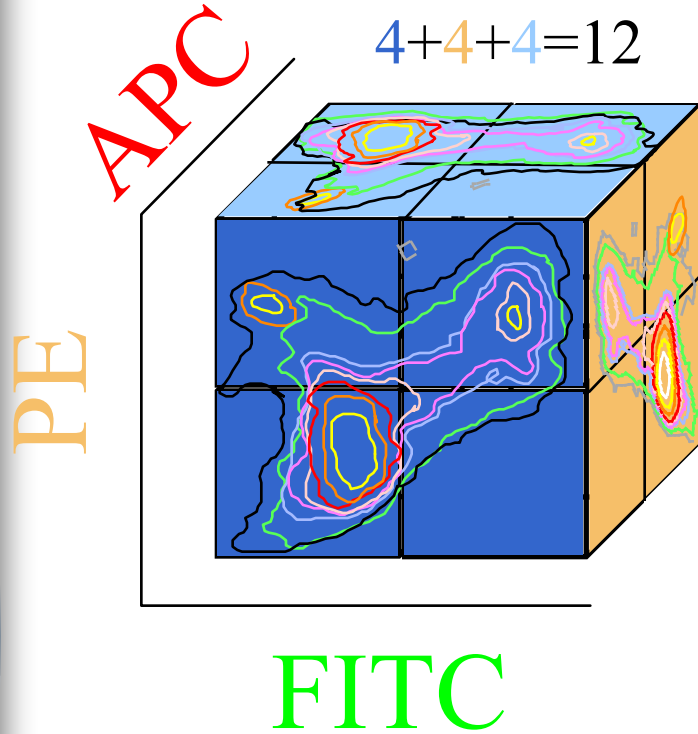


3 Color Combinations



Negatives

3 Color Combinations





„Gating“

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

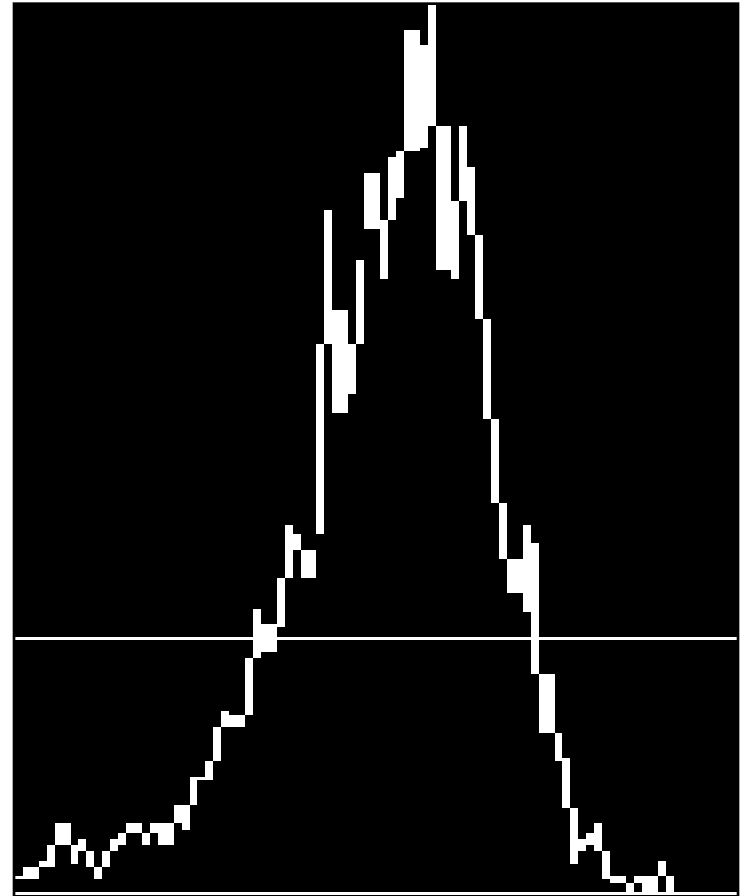


Real-Time vs. Software Gating

- 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,



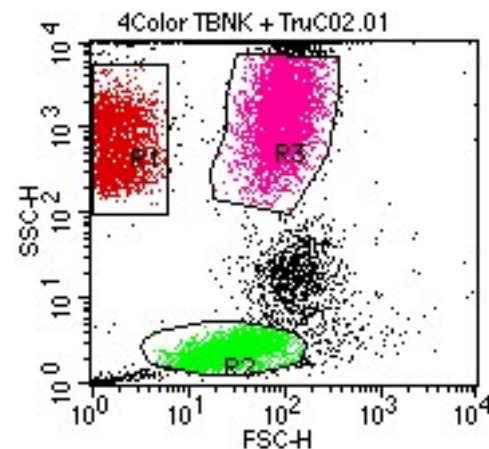
Region vs. gate

Region

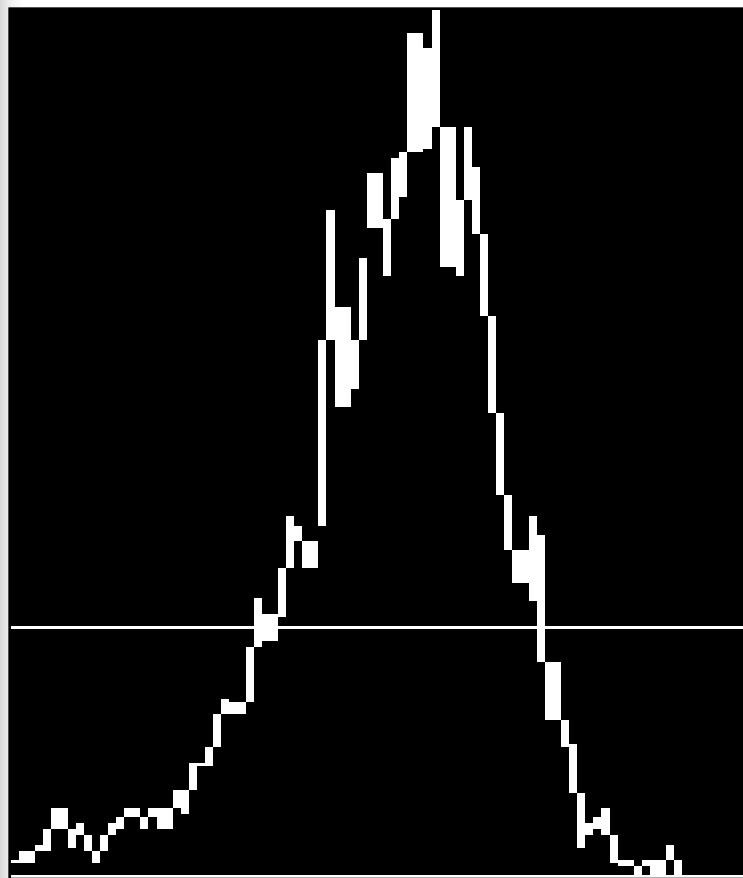
- oblast (plocha) v grafu definovaná uživatelem
- mnoho regionů v jedné grafu
- ohraničujeme pomocí nich populace našeho zájmu
- je možné je barevně odlišit
- je definován stejně pro všechny vzorky v analýze

Gate

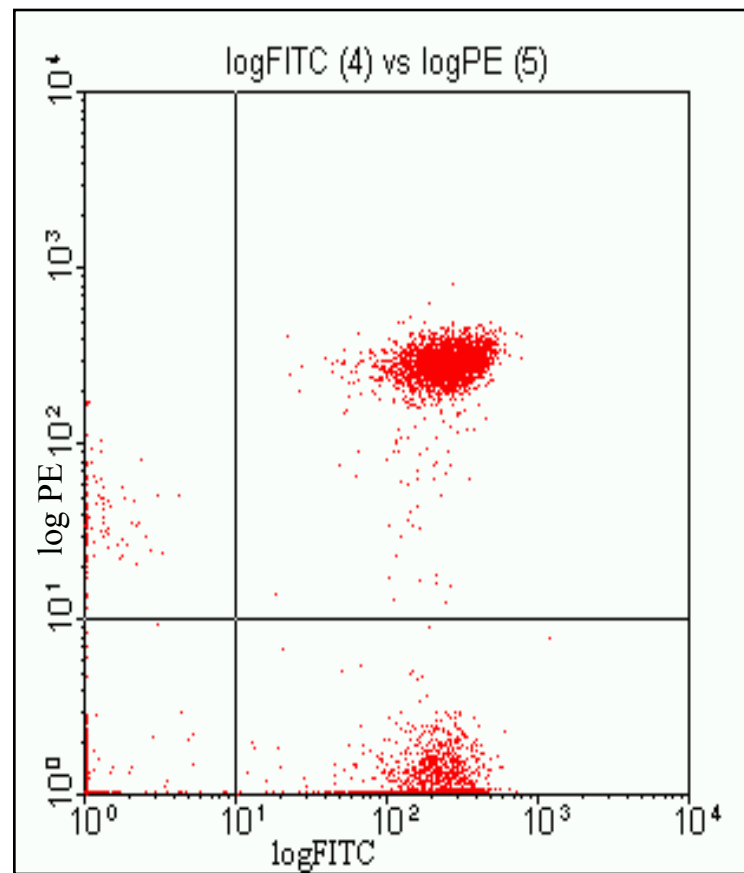
- je definován jako jeden a nebo více regionů zkombinovaných pomocí logických operátorů (AND, OR, NOT; Booleova logika)



Using Gates



Region 1 established



Gated on Region 1

Statistika

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

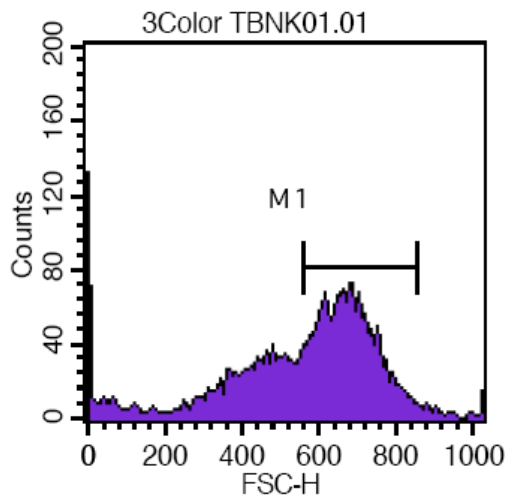
$$\bar{x} = \frac{1}{n} (x_1 + x_2 + \dots + x_n) = \frac{1}{n} \sum_{i=1}^n x_i$$

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

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

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

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:

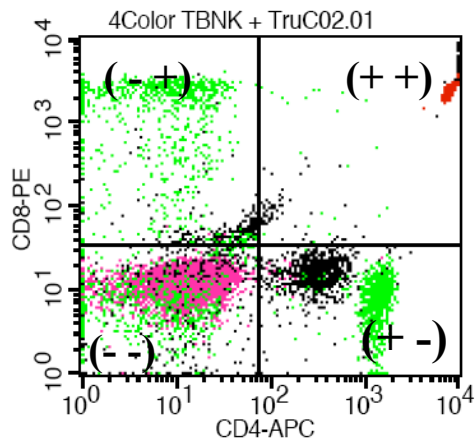
Panel: 3 Color TBNK

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



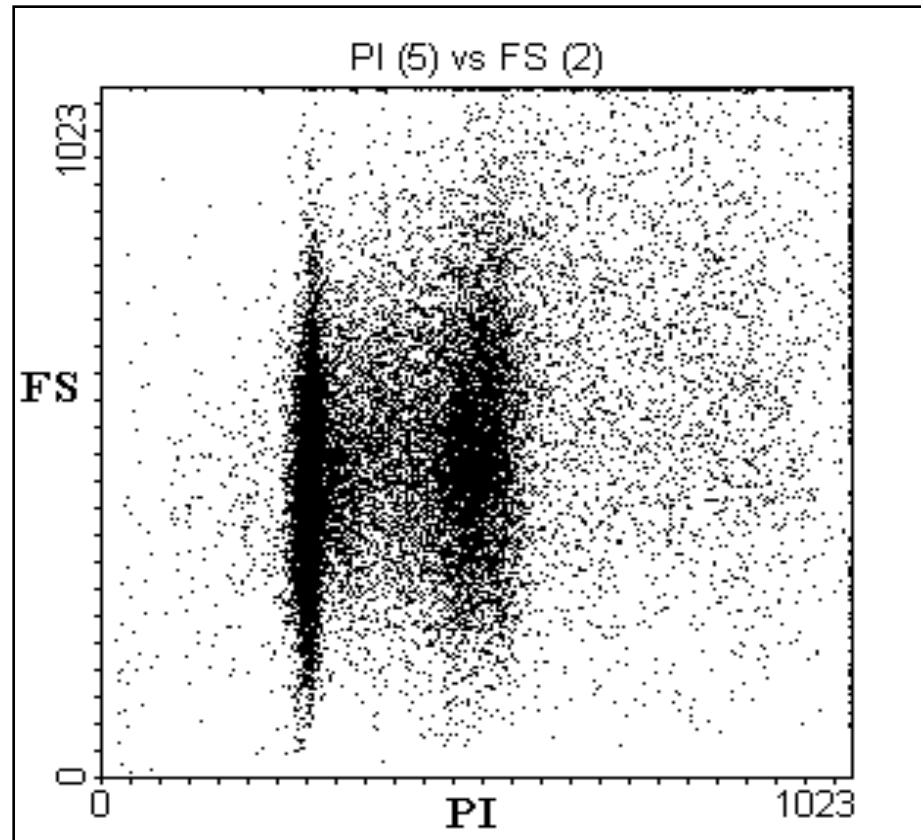
Quadrant Statistics

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

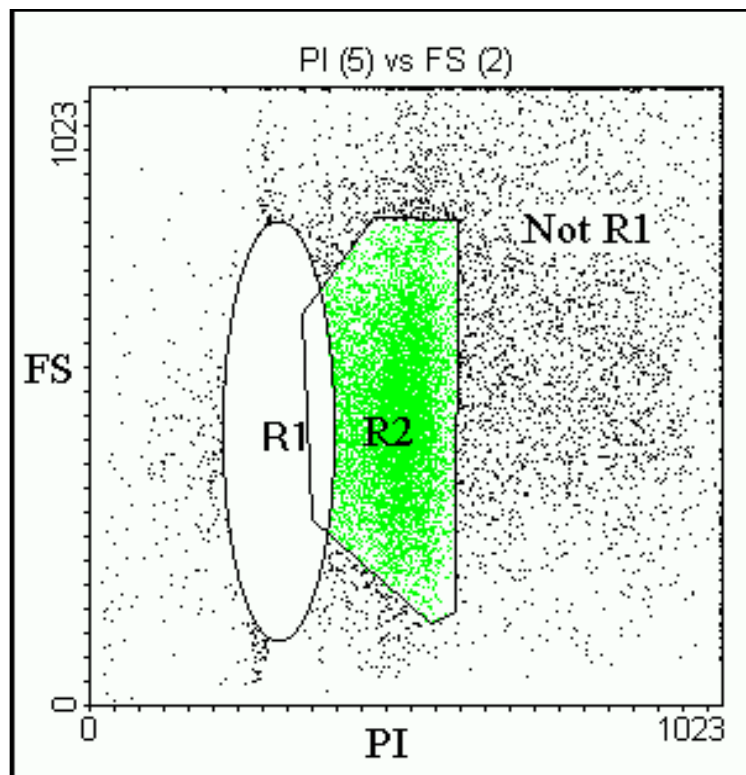
Logický „Gating“ (Booleova logika)

S překrývajícími se oblastmi máme mnoho možností:



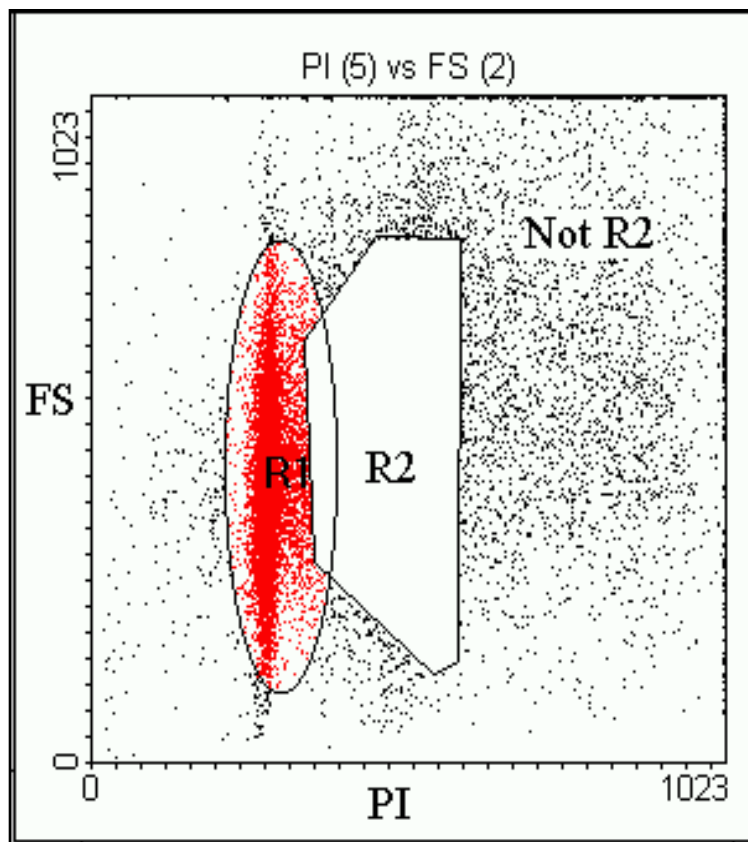
Boolean Gating

Not Region 1:



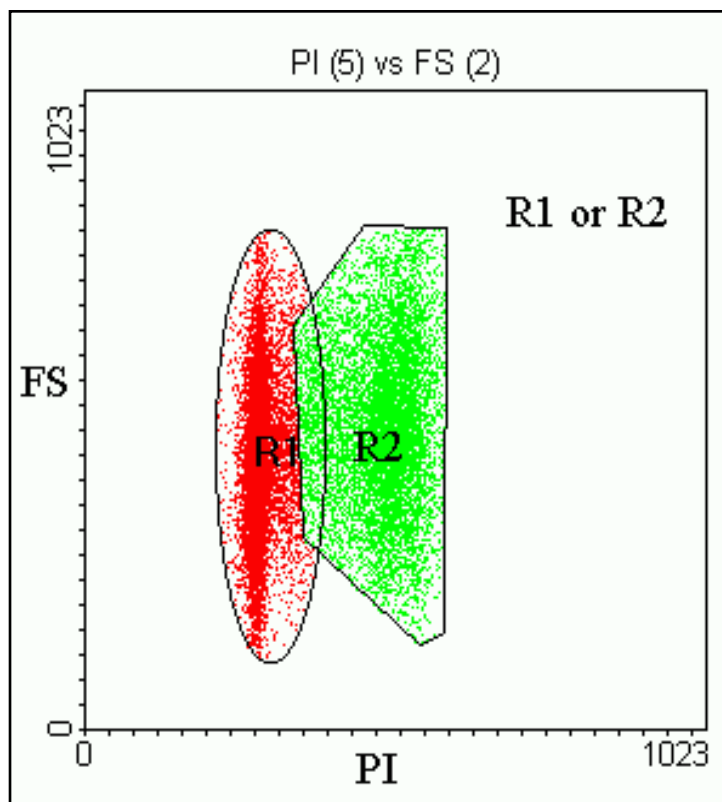
Boolean Gating

Not Region 2:



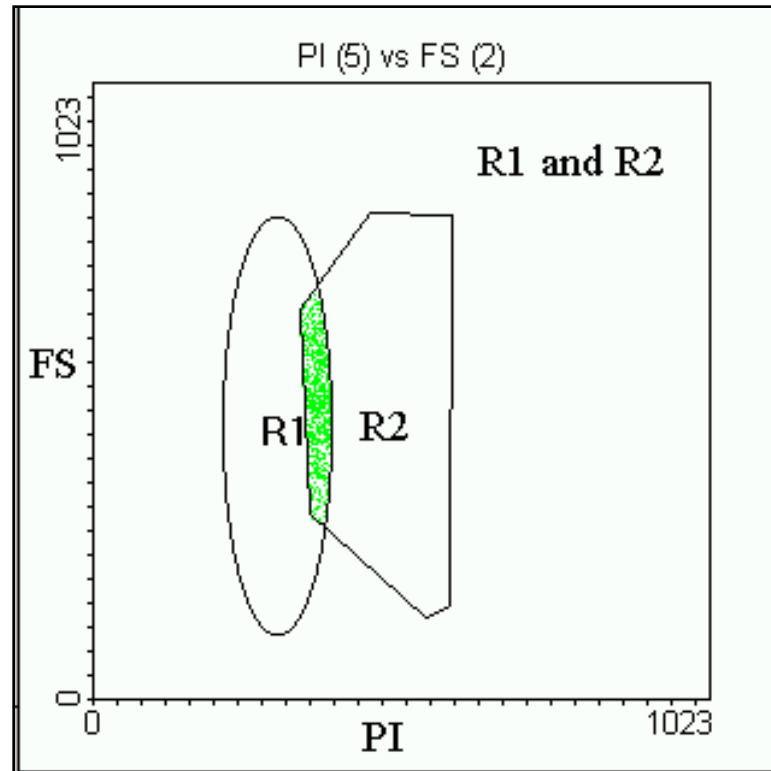
Boolean Gating

Region 1 or Region 2:



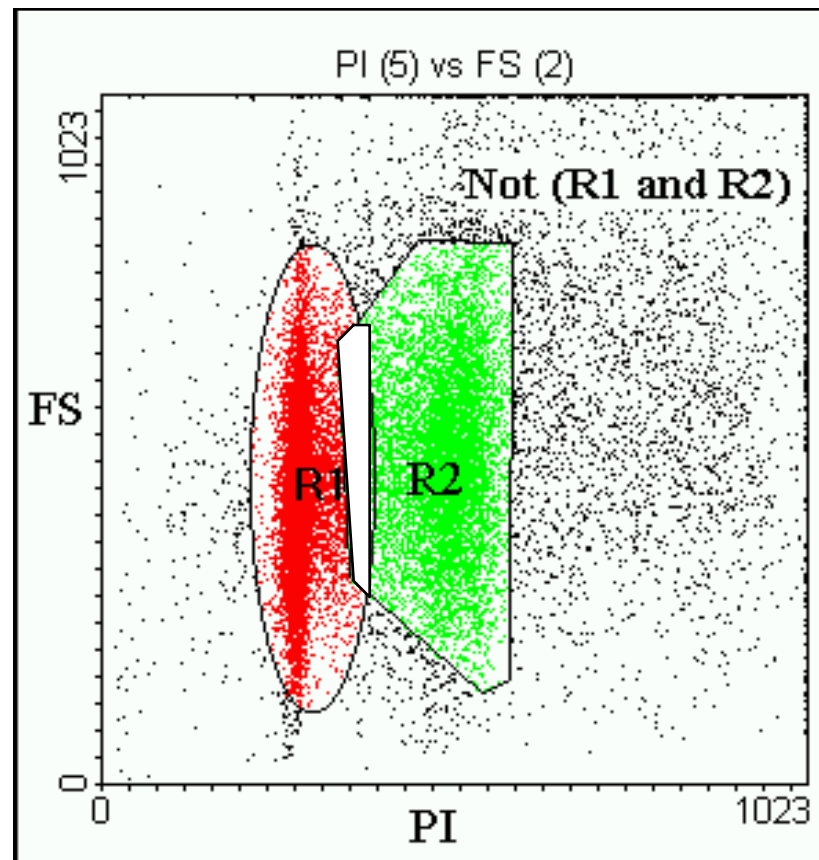
Boolean Gating

Region 1 and Region 2:

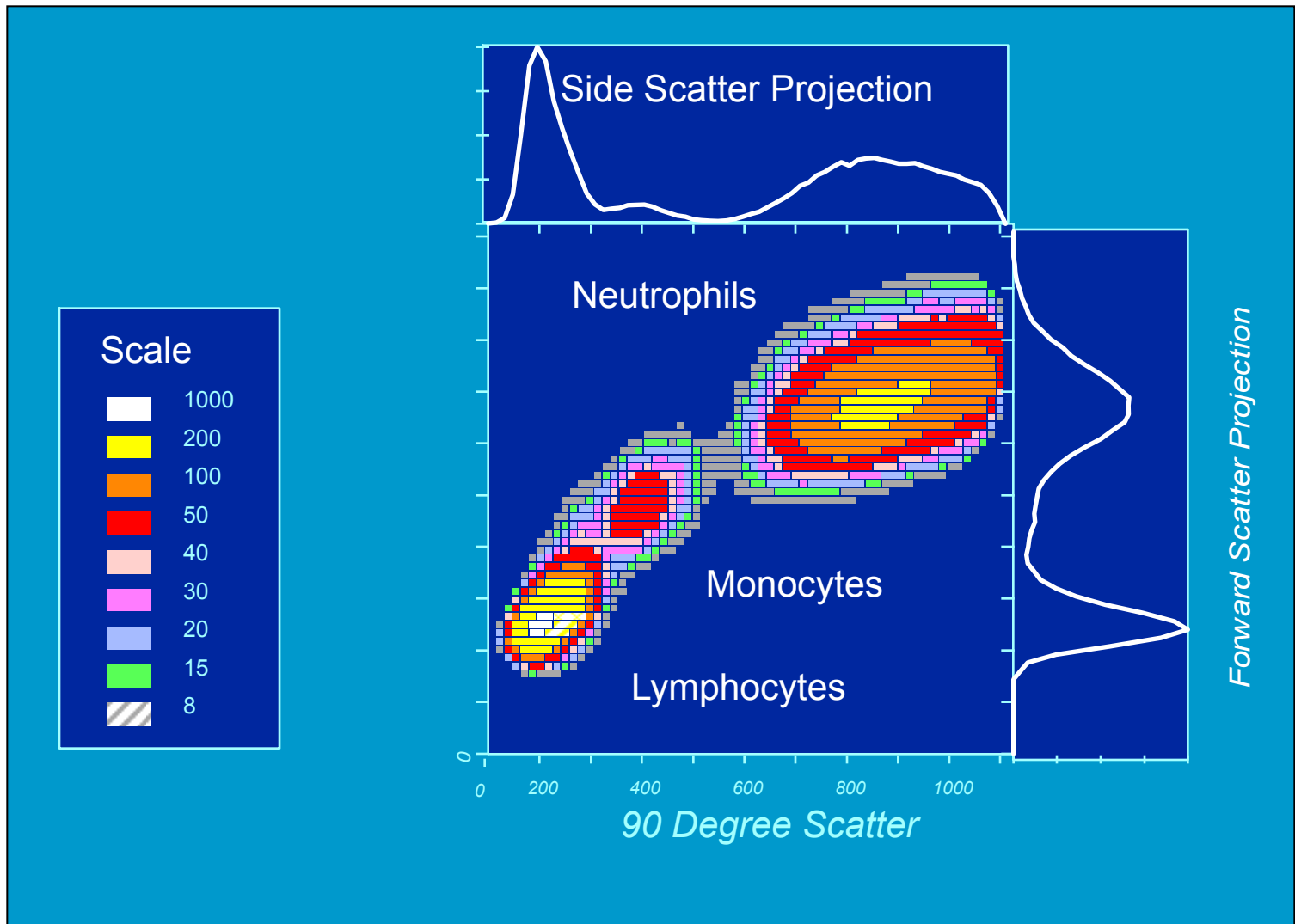


Boolean Gating

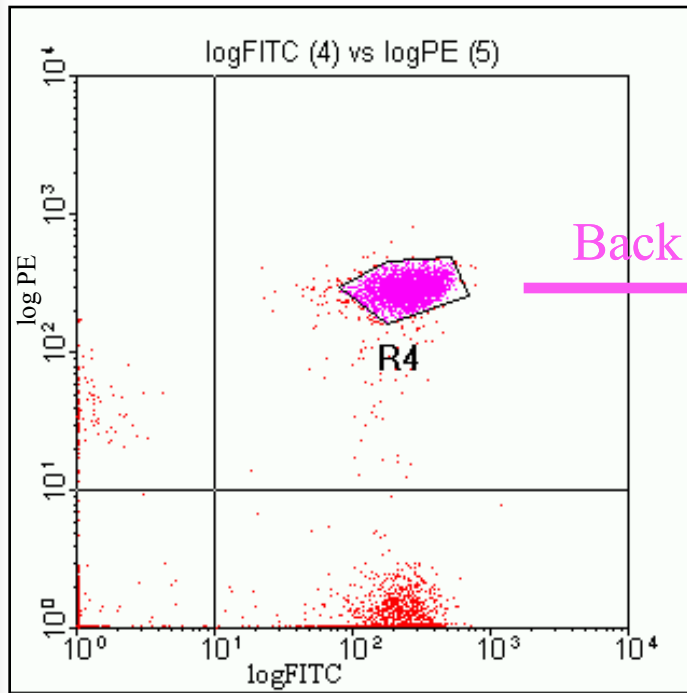
Not (Region1 and Region 2):



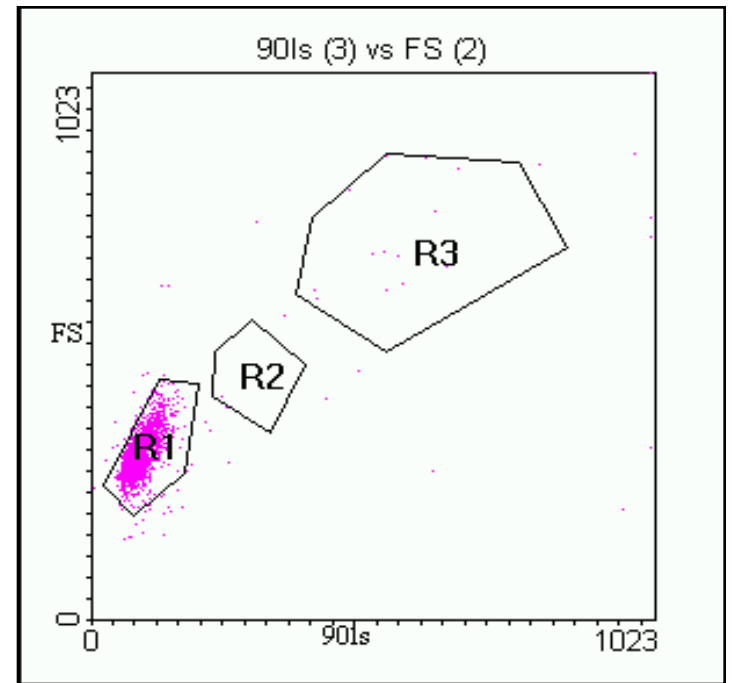
Light Scatter Gating



Back Gating

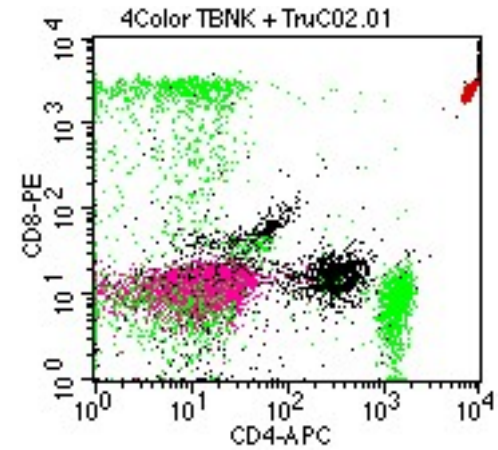
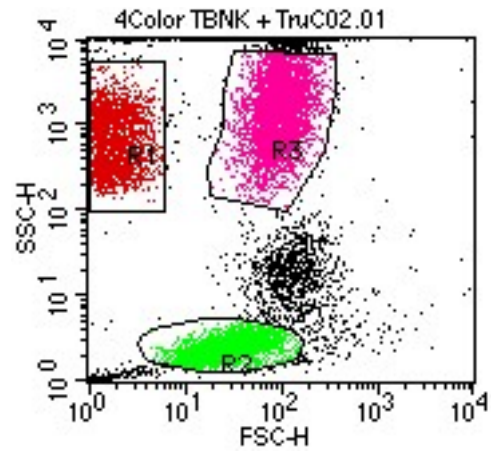


Region 4 established

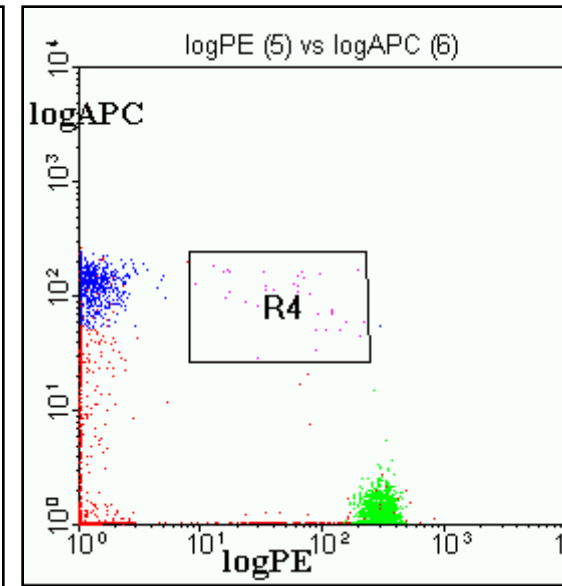
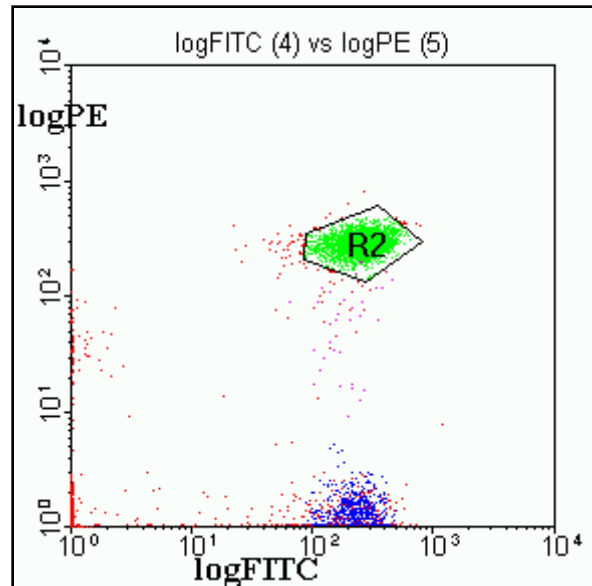
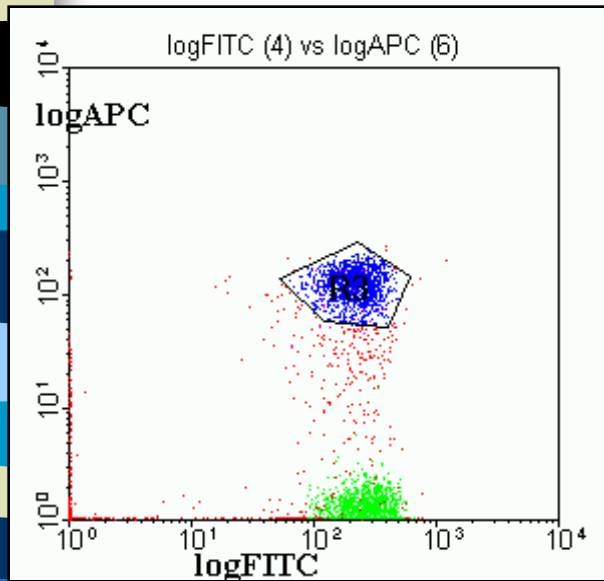
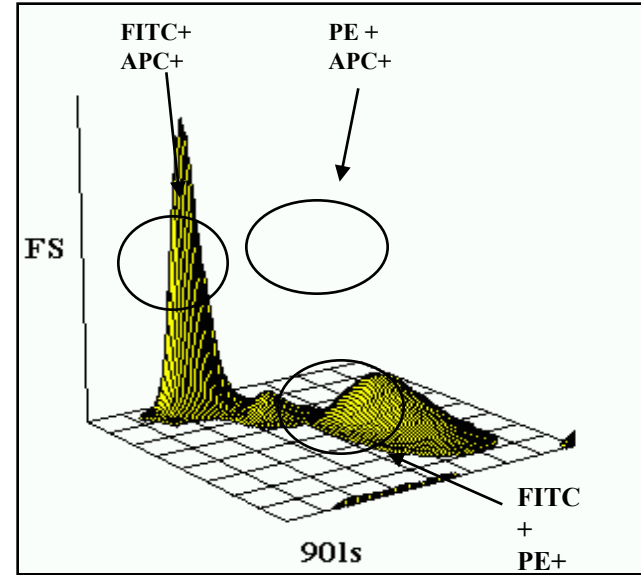
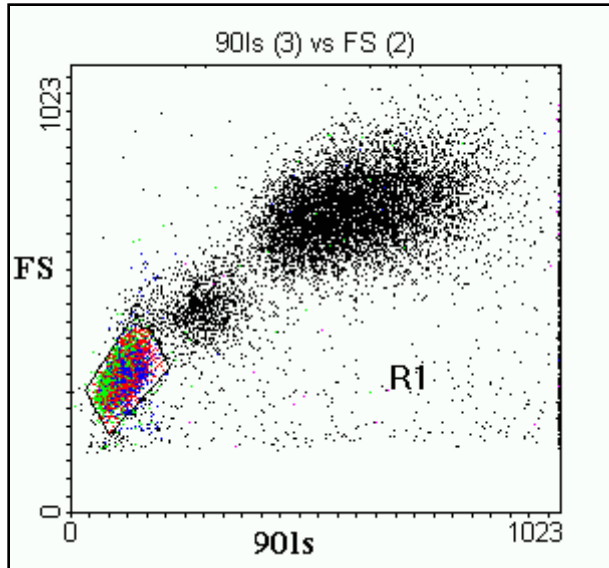


Back-gating using Region 4

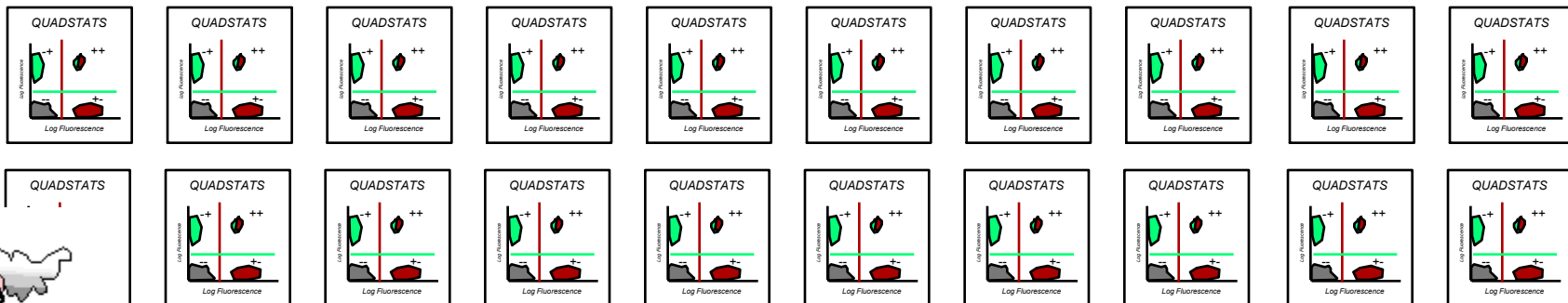
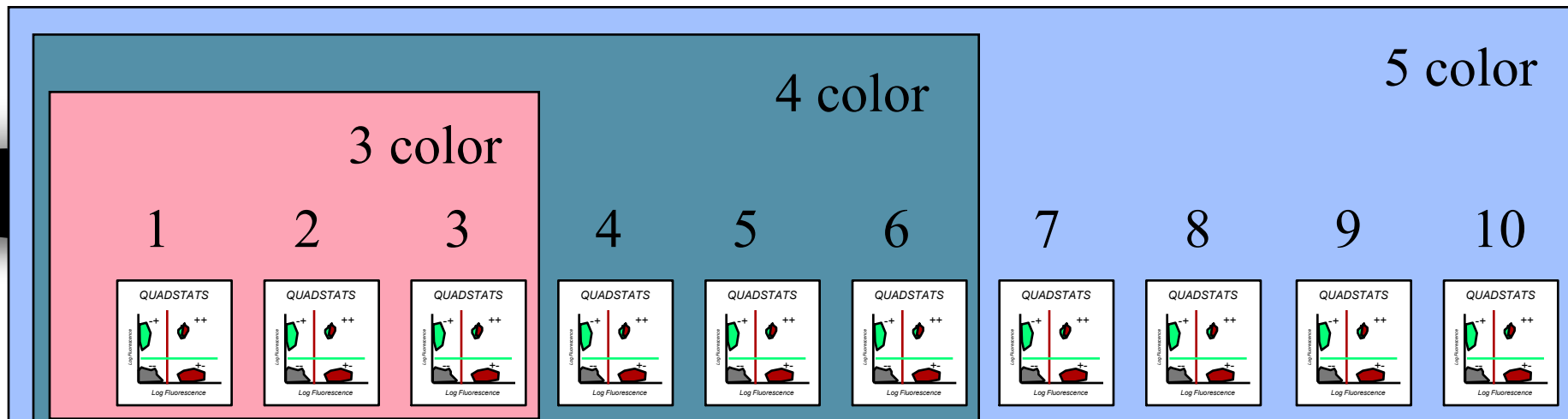
Back Gating

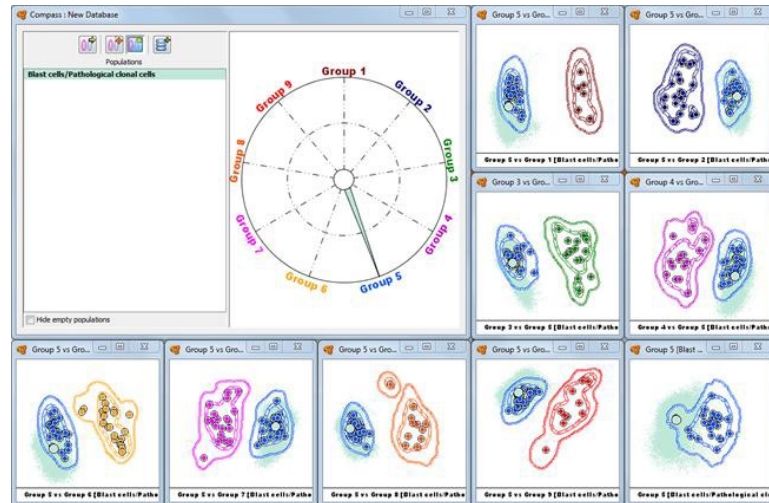
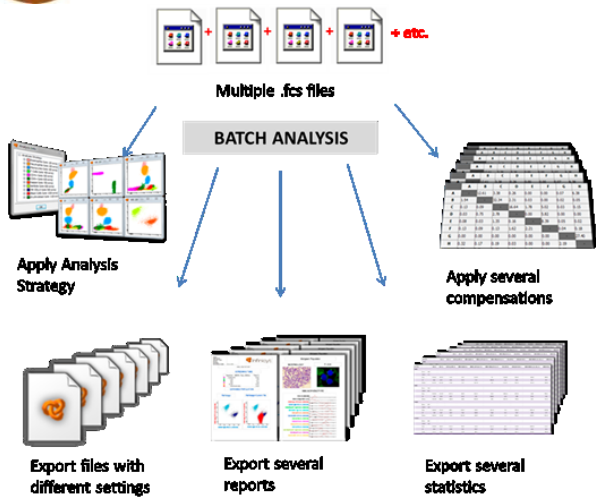


3 Parameter Data Display

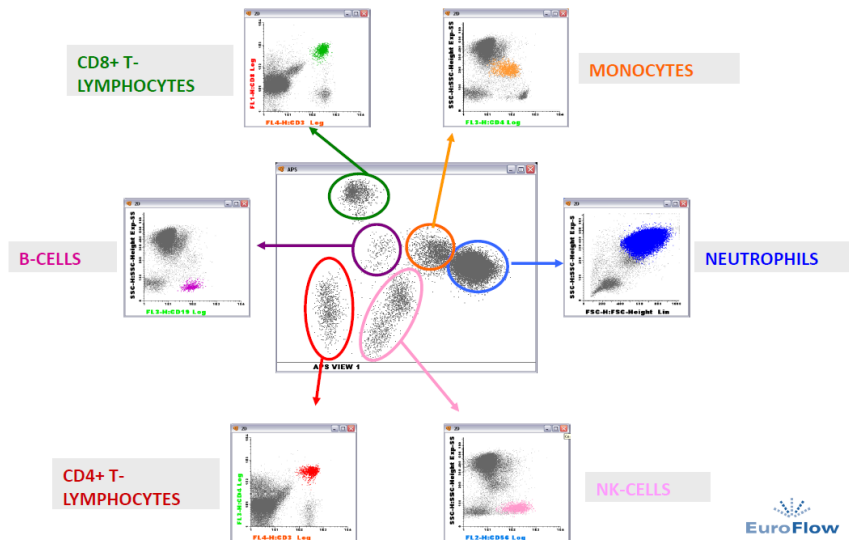


Vícebarevné analýzy generují mnoho dat...

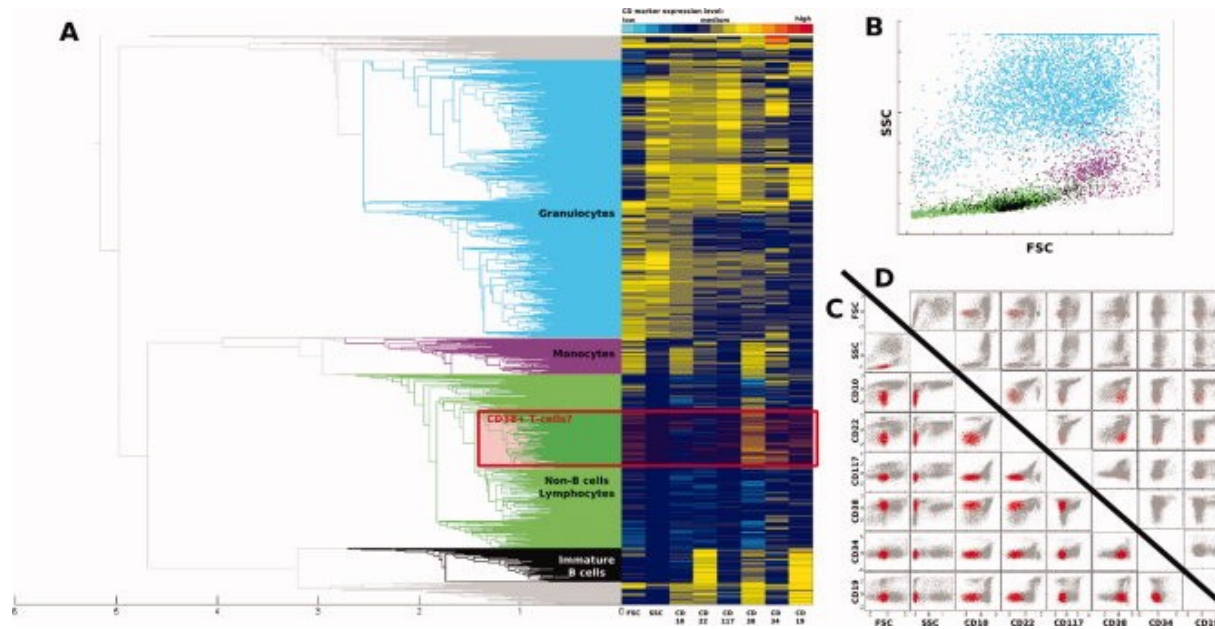




Automatic Population Separator



Detection and monitoring of normal and leukemic cell populations with hierarchical clustering of flow cytometry data

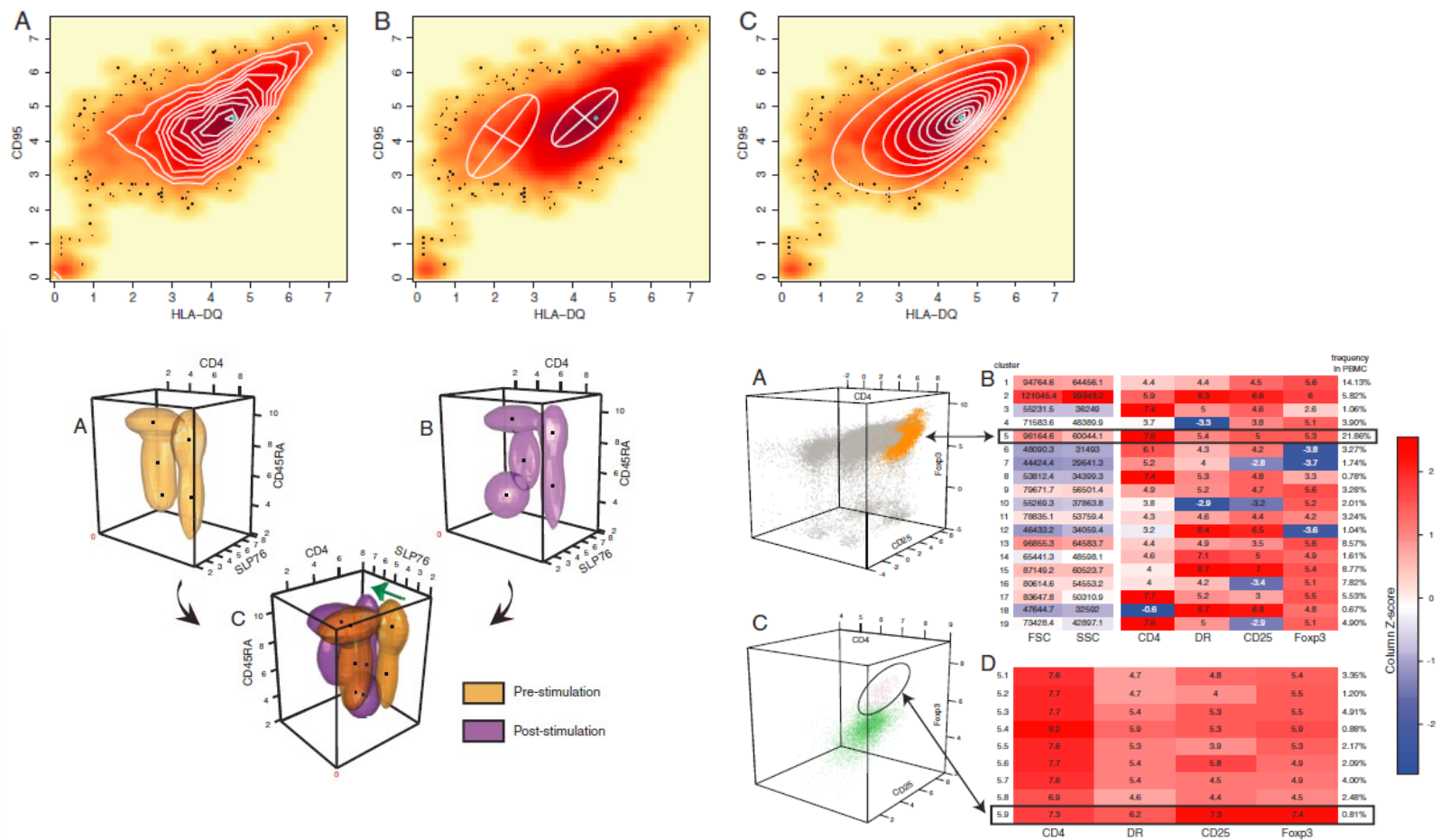


Automated high-dimensional flow cytometric data analysis

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}



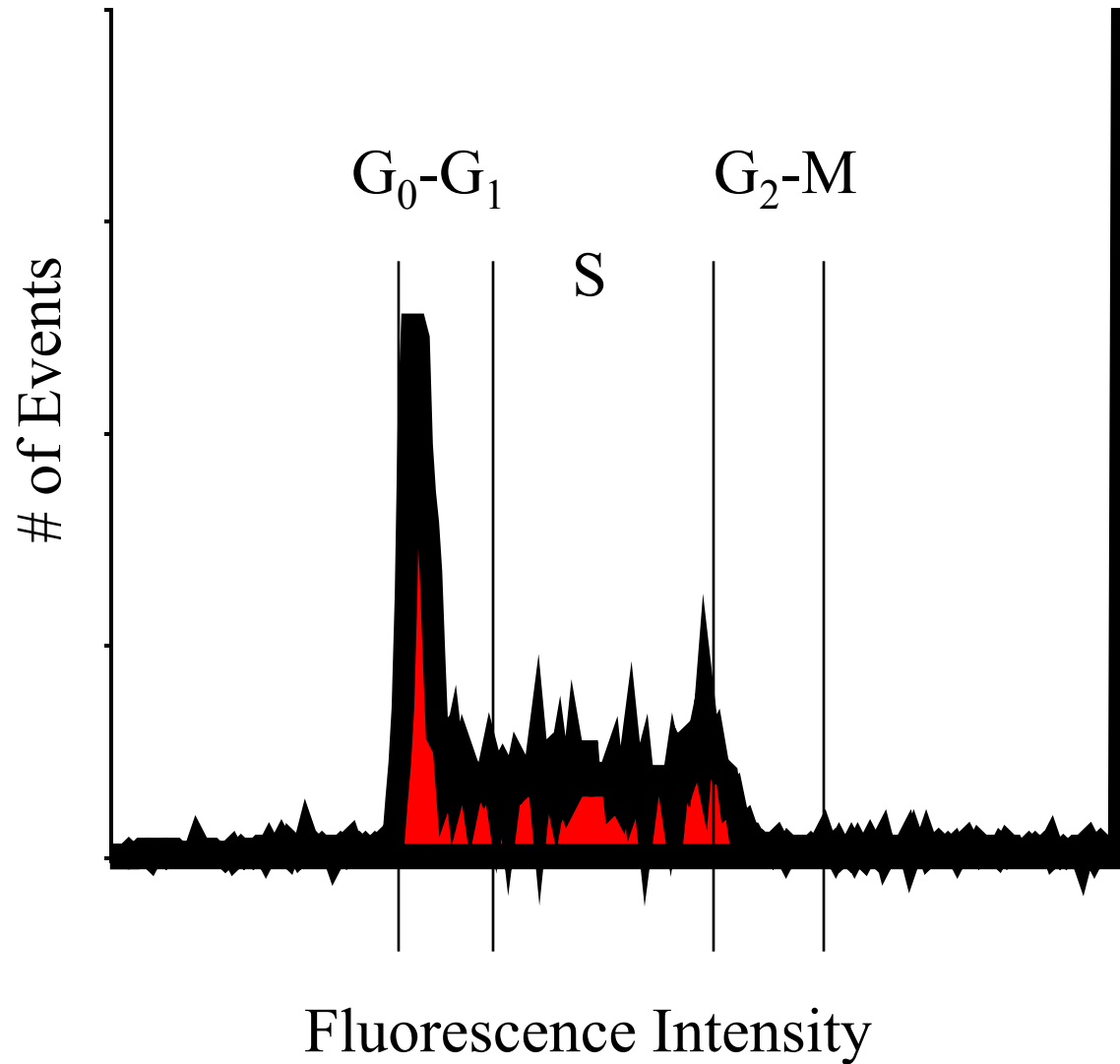
Způsoby pomocí kterých lze upravit výsledky:

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

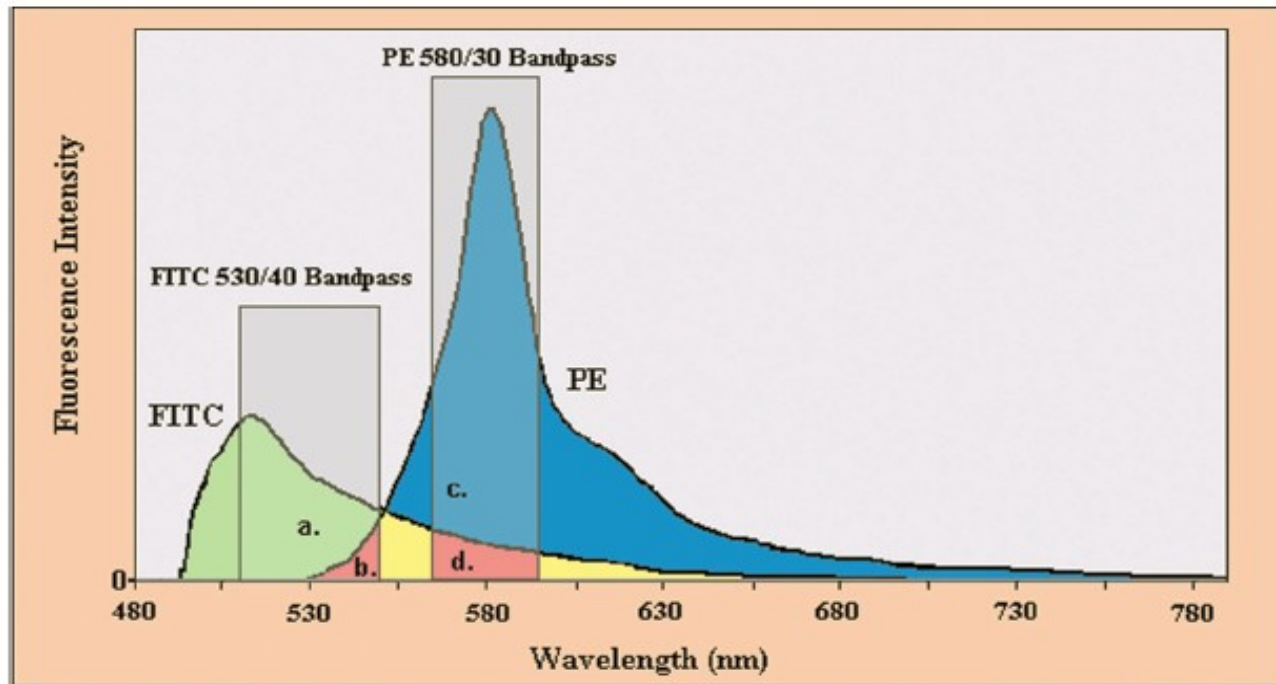
Příklad - pro DNA analýzu je třeba:

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

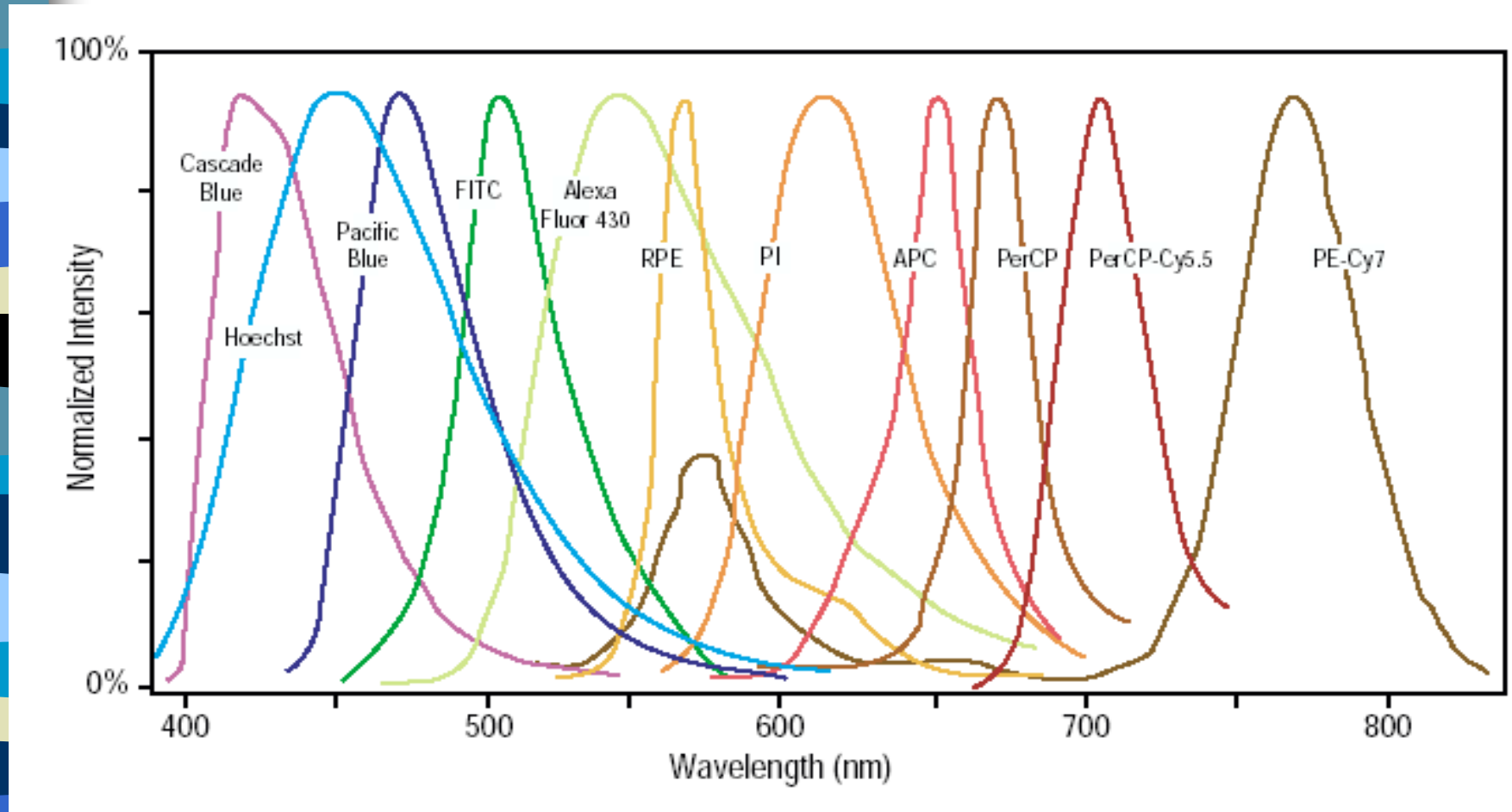
DNA Histogram



Co je problém při vícebarevné detekci?



Emission Spectra–Spectral Overlap

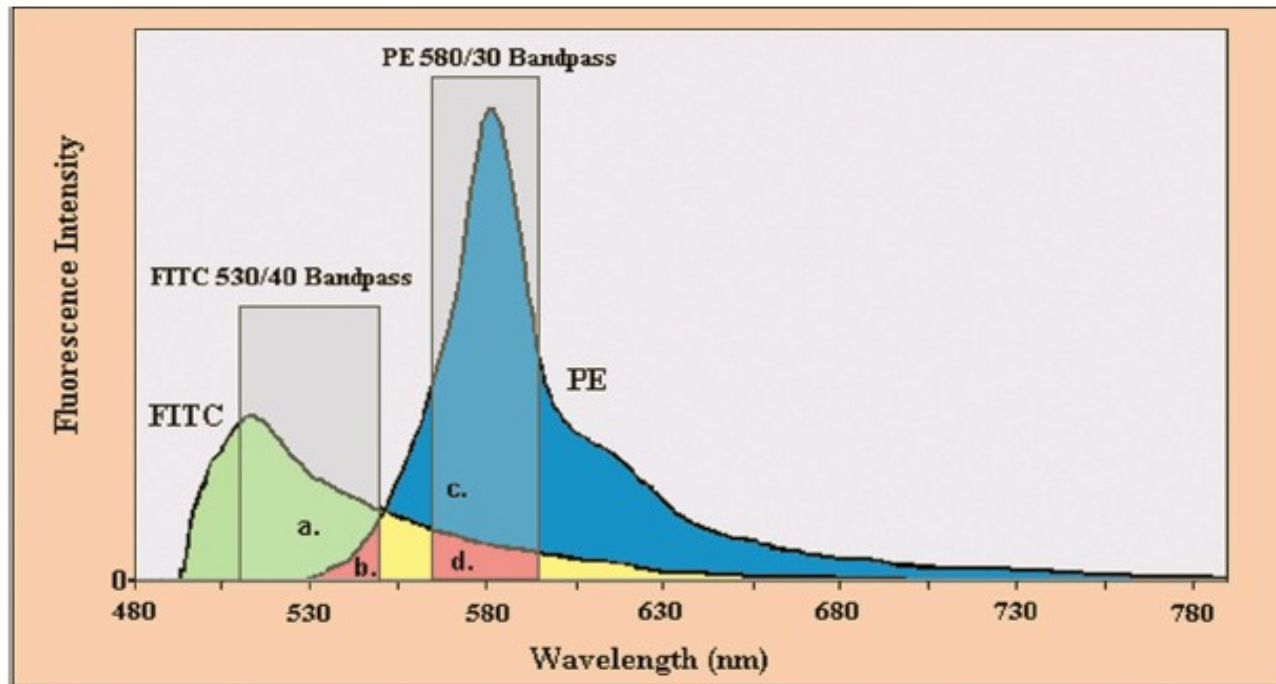




Kompenzace fluorescenčního signálu při vícebarevné detekci

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

Co je problém při vícebarevné detekci?



Kompenzace fluorescenčního signálu při vícebarevné detekci

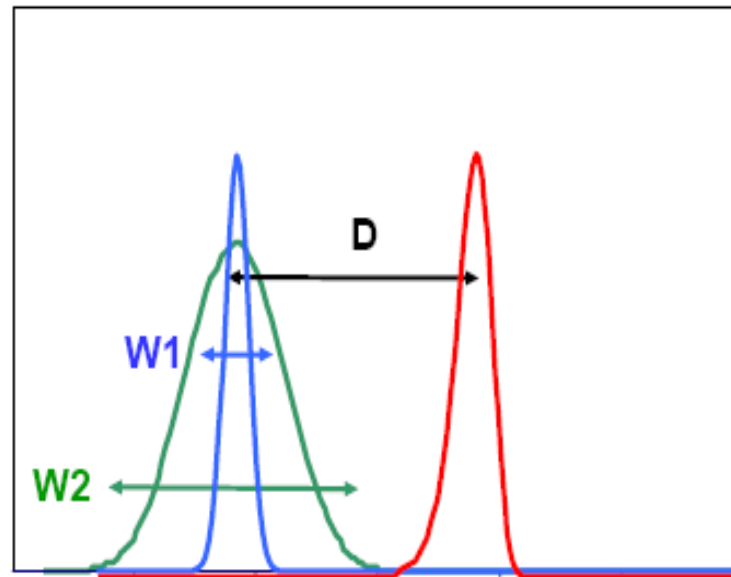
Table 1.14.1 Typical Spillover Matrix for a Three-Color Compensation^a

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



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

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...

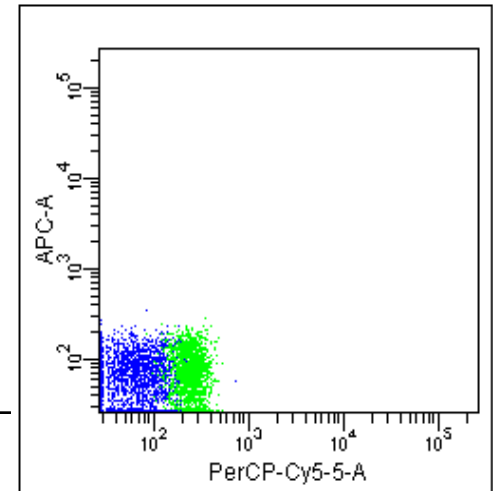
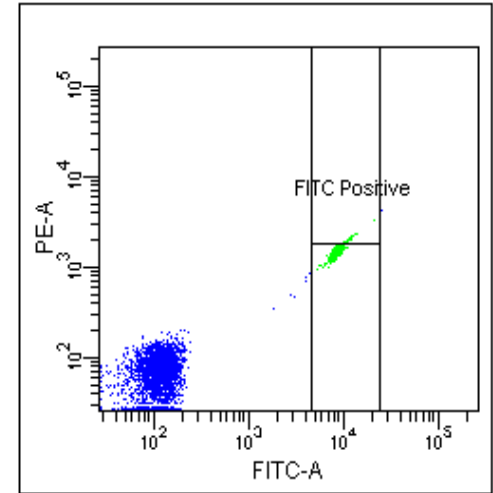
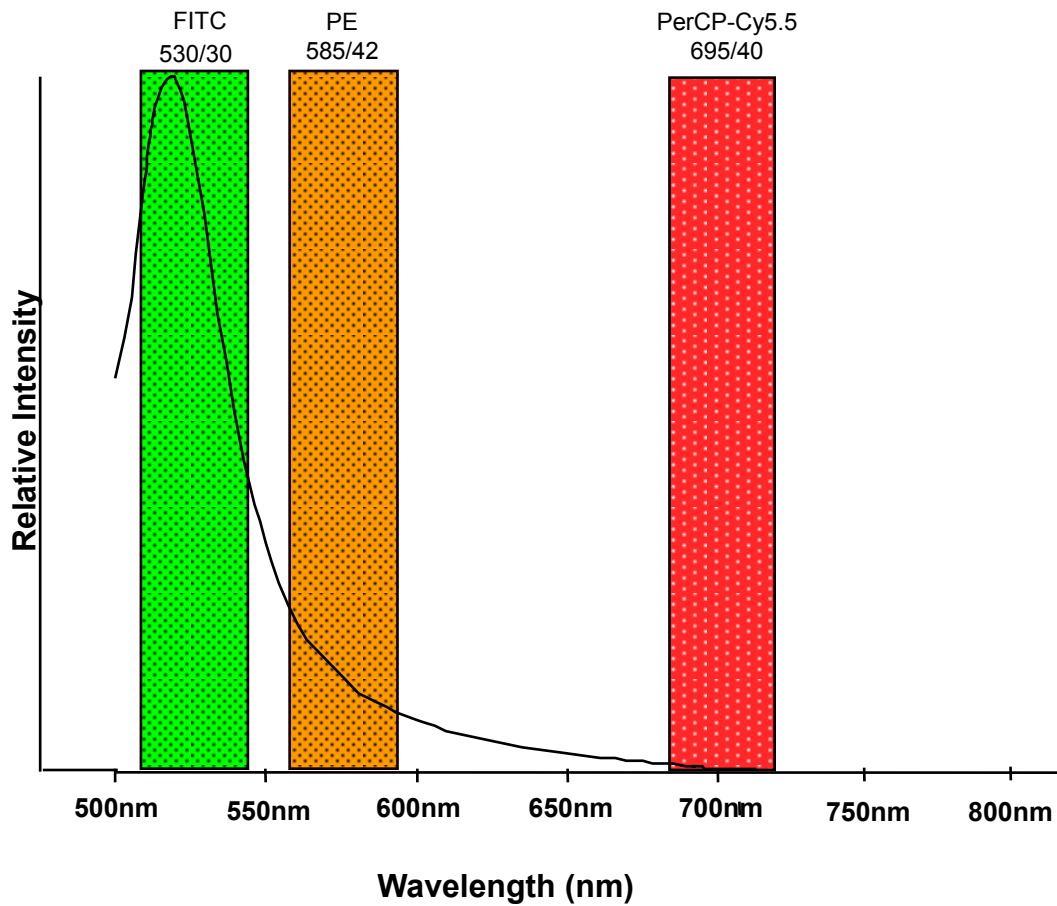
Fluorochrome selection considerations

“Bright” antibodies go on “dim”
fluorochromes

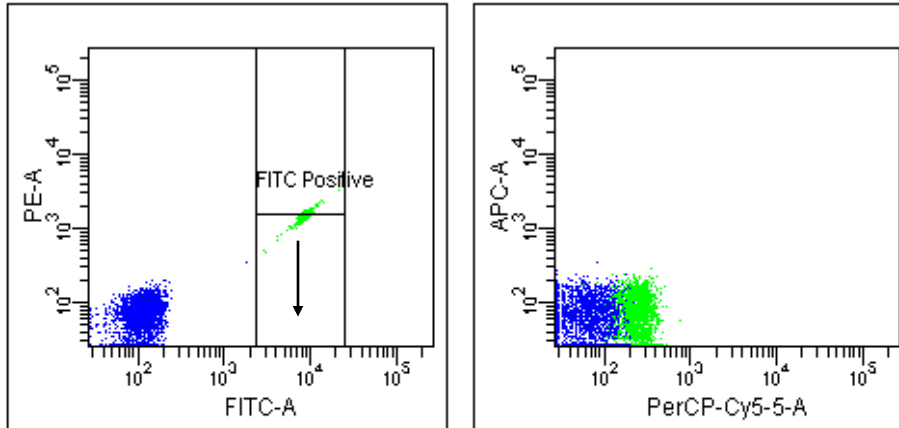
Avoid spillover from bright cell populations
into channels requiring high sensitivity

Beware of tandem dye degradation

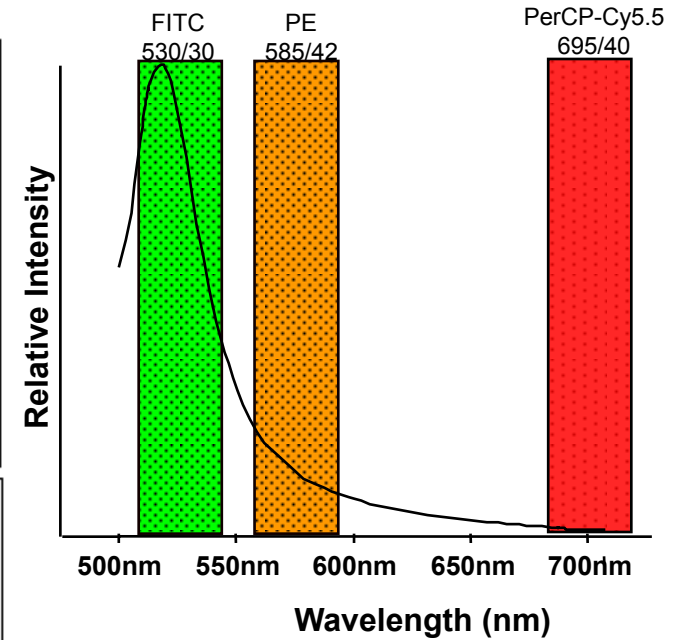
FITC Spillover



FITC Compensation

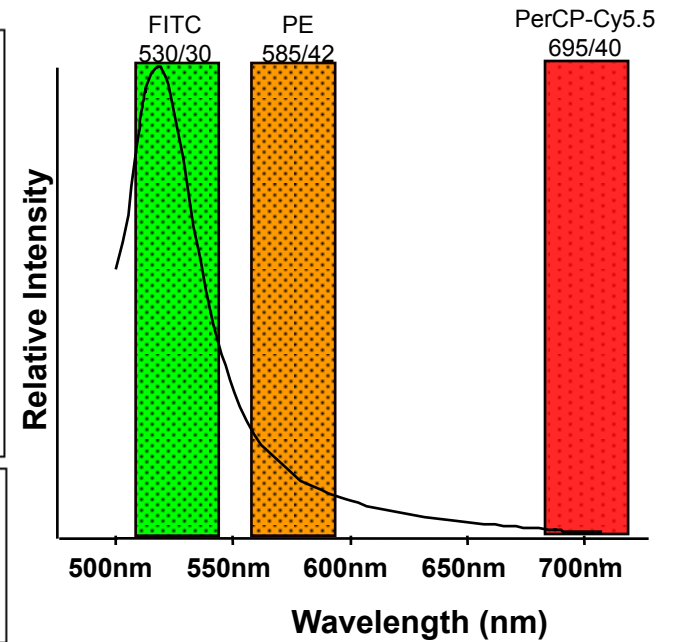
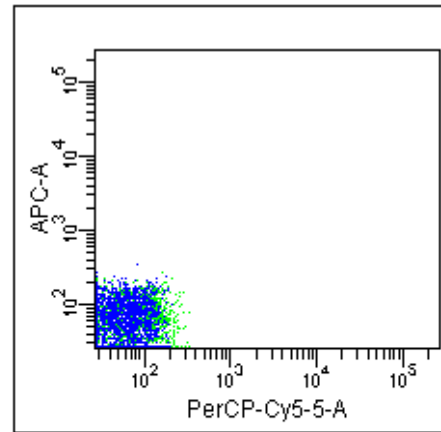
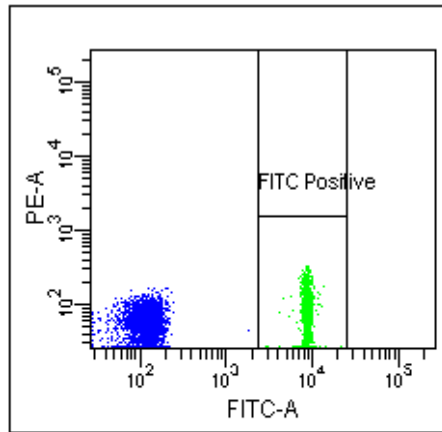


Population	FITC-A Median	PE-A Median	PerCP-Cy5-5-A Median	APC-A Median
■ FITC Positive	8,776	1,499	226	63
■ FITC Negative	113	70	52	56



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

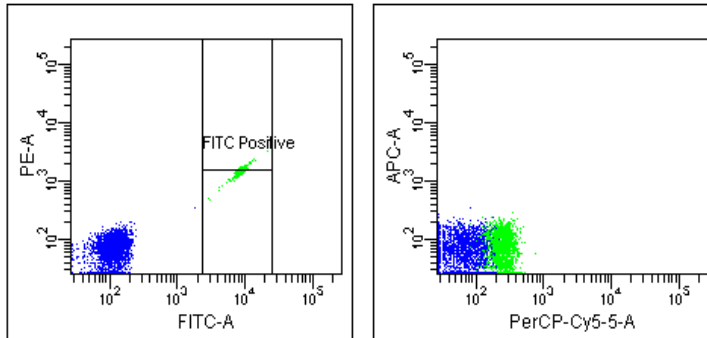
FITC Compensation



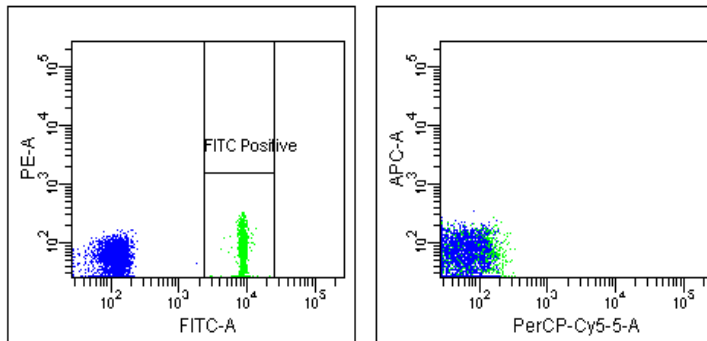
Population	FITC-A Median	PE-A Median	PerCP-Cy5-5-A Median	APC-A Median
■ FITC Positive	8,776	54	49	53
■ FITC Negative	113	50	49	56

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

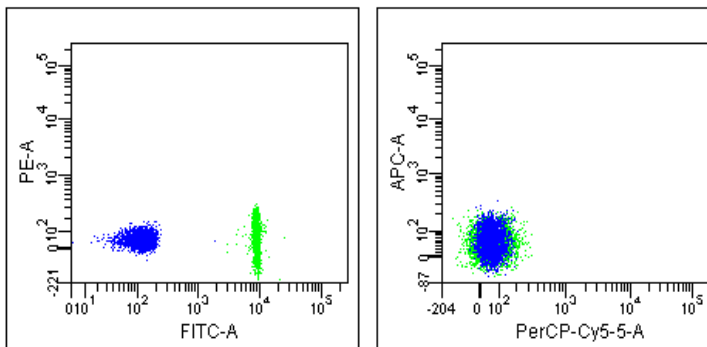
FITC Compensation



Dot plot showing uncompensated FITC data

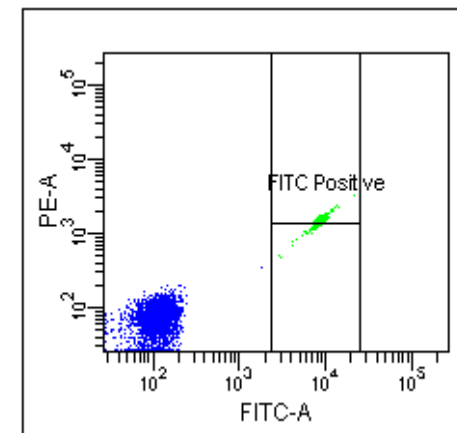
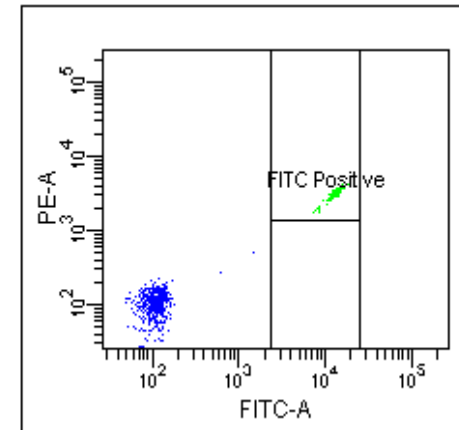
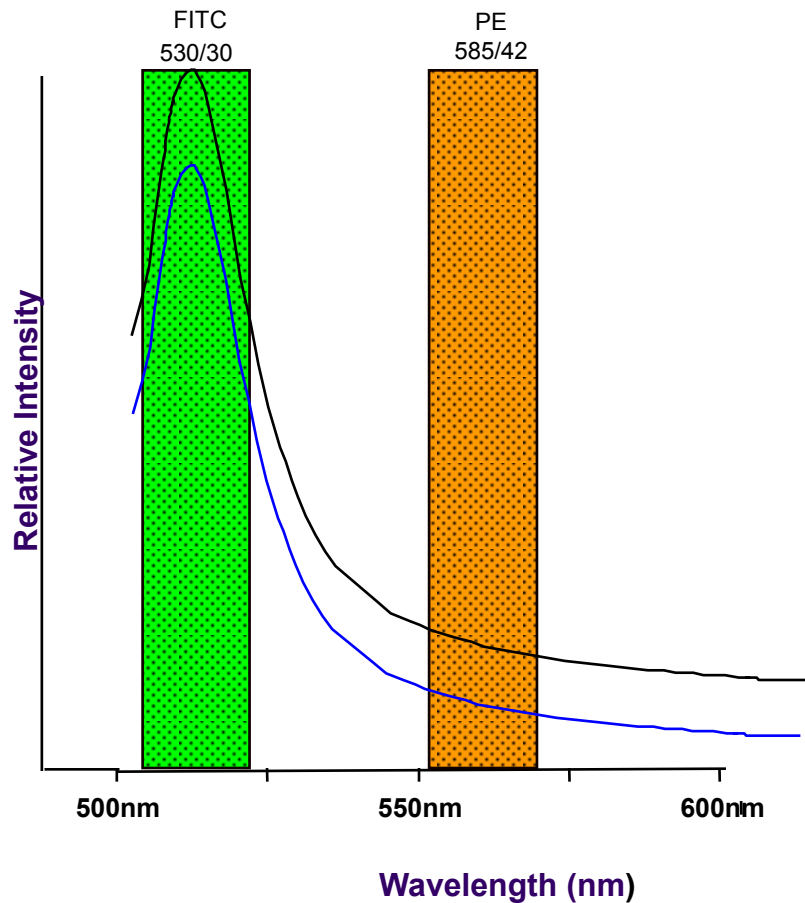


Dot plot showing compensated FITC data

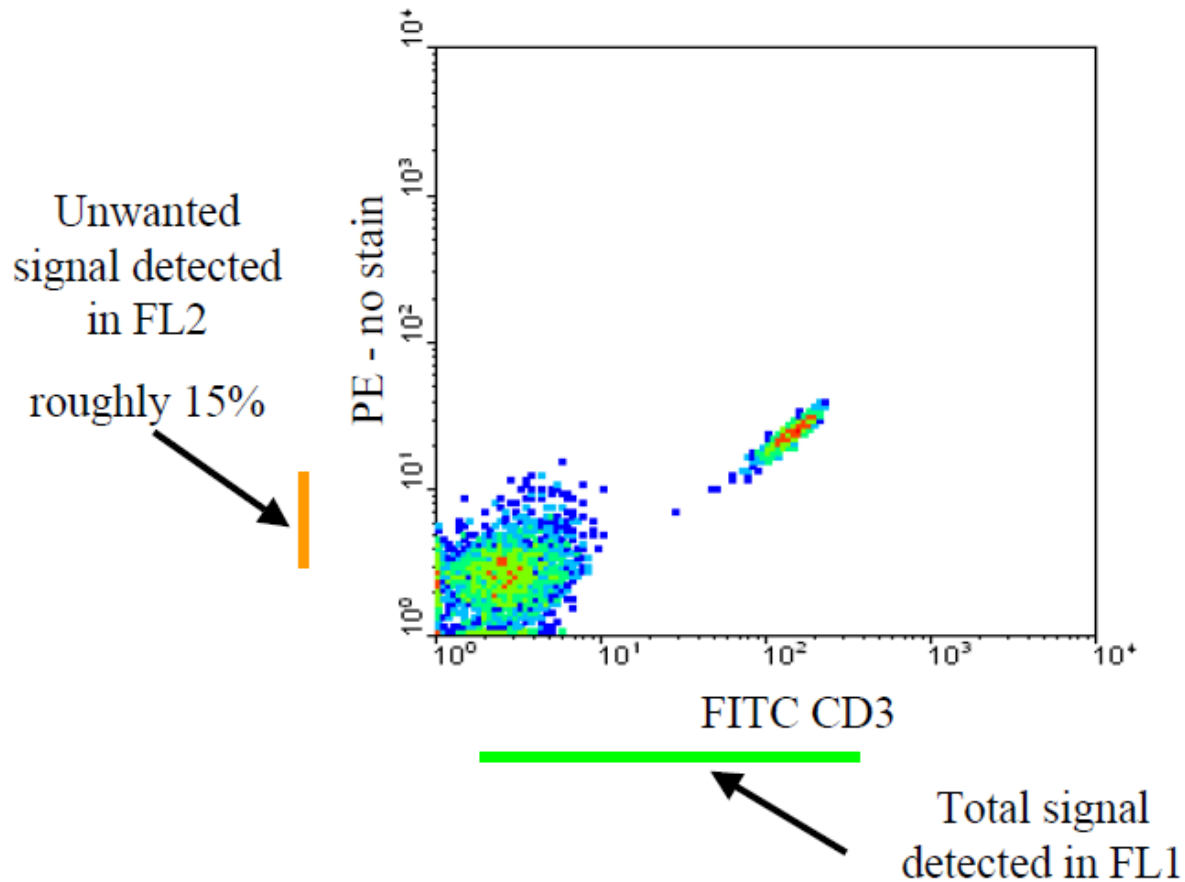


Biexponential dot plot showing compensated FITC data

FITC Spillover

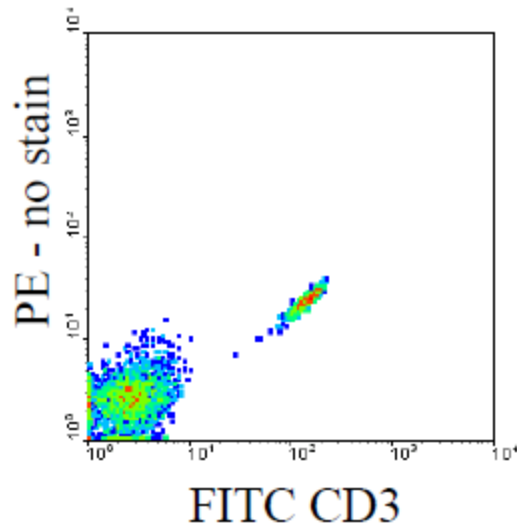


Uncompensated FITC Single stain Control



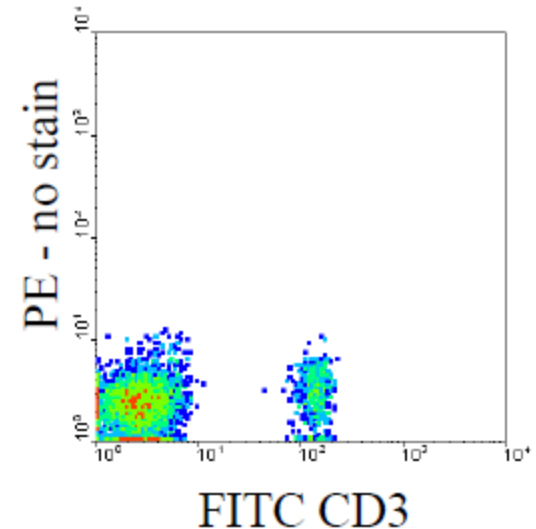
FITC Single Stain Control

Uncompensated

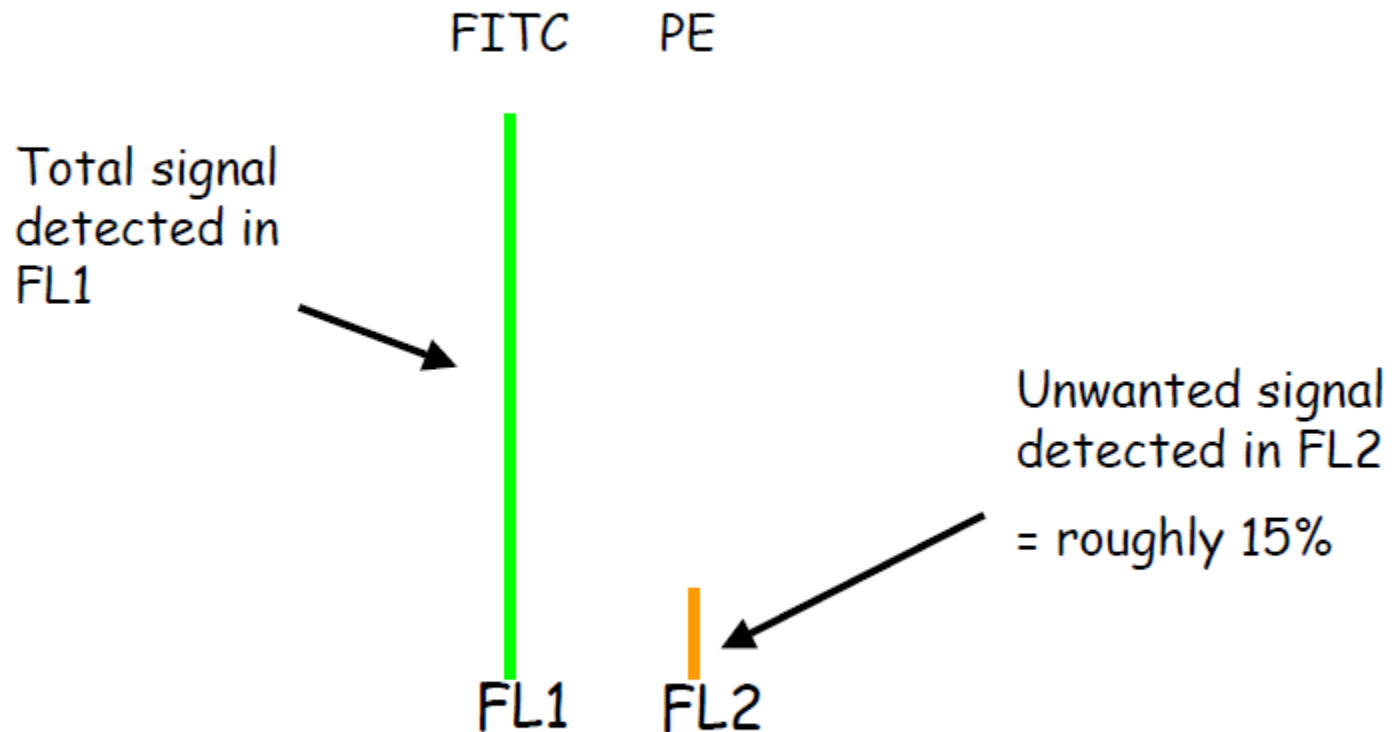


FL2-15%FL1

Compensated



FITC Single Stain Control



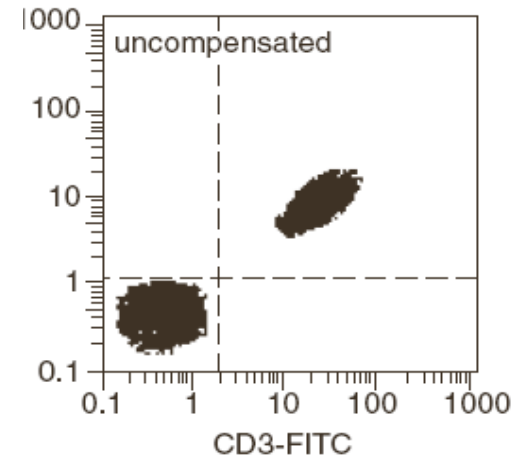
$$\text{True PE} = \text{Total FL2} - 15\% \text{ FL1}$$

Kompenzace fluorescenčního signálu

#2

FITC positive & negative

PE negative beads



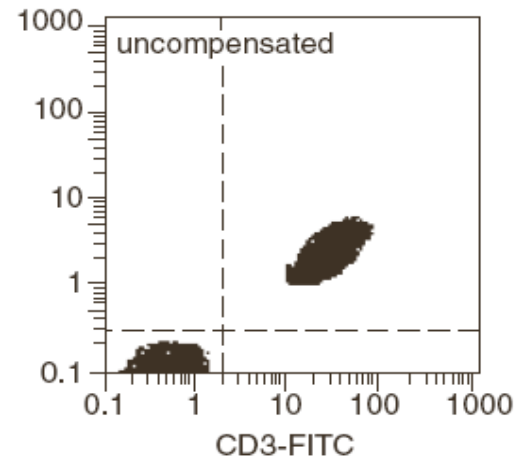
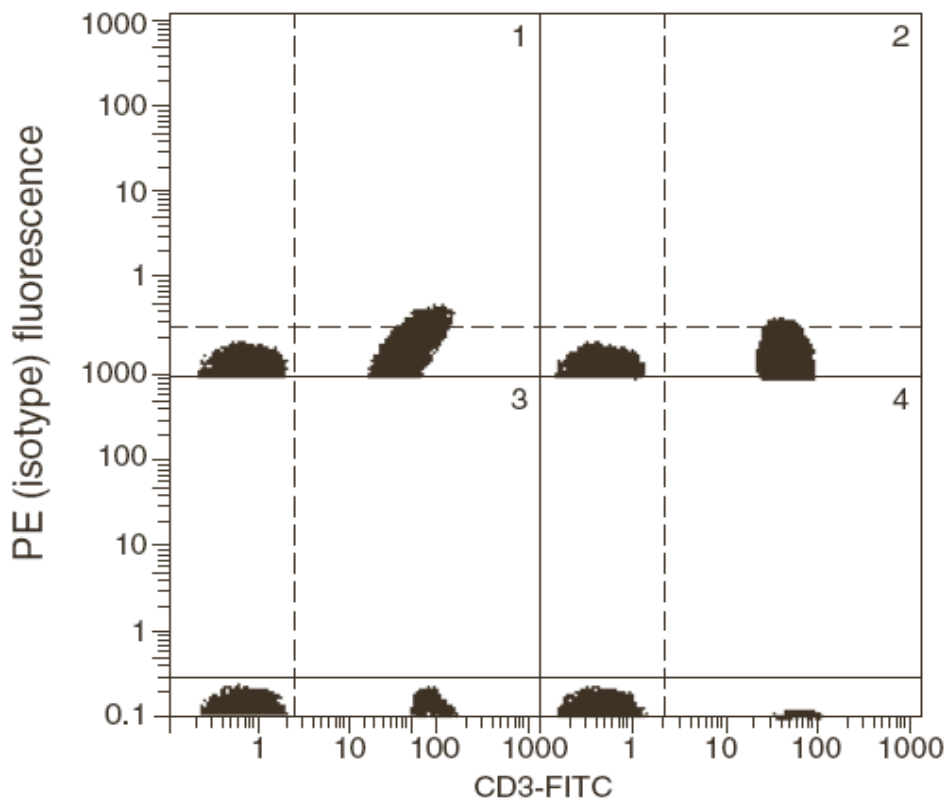
Current Protocols in Cytometry

Kompenzace fluorescenčního signálu

FITC positive & negative

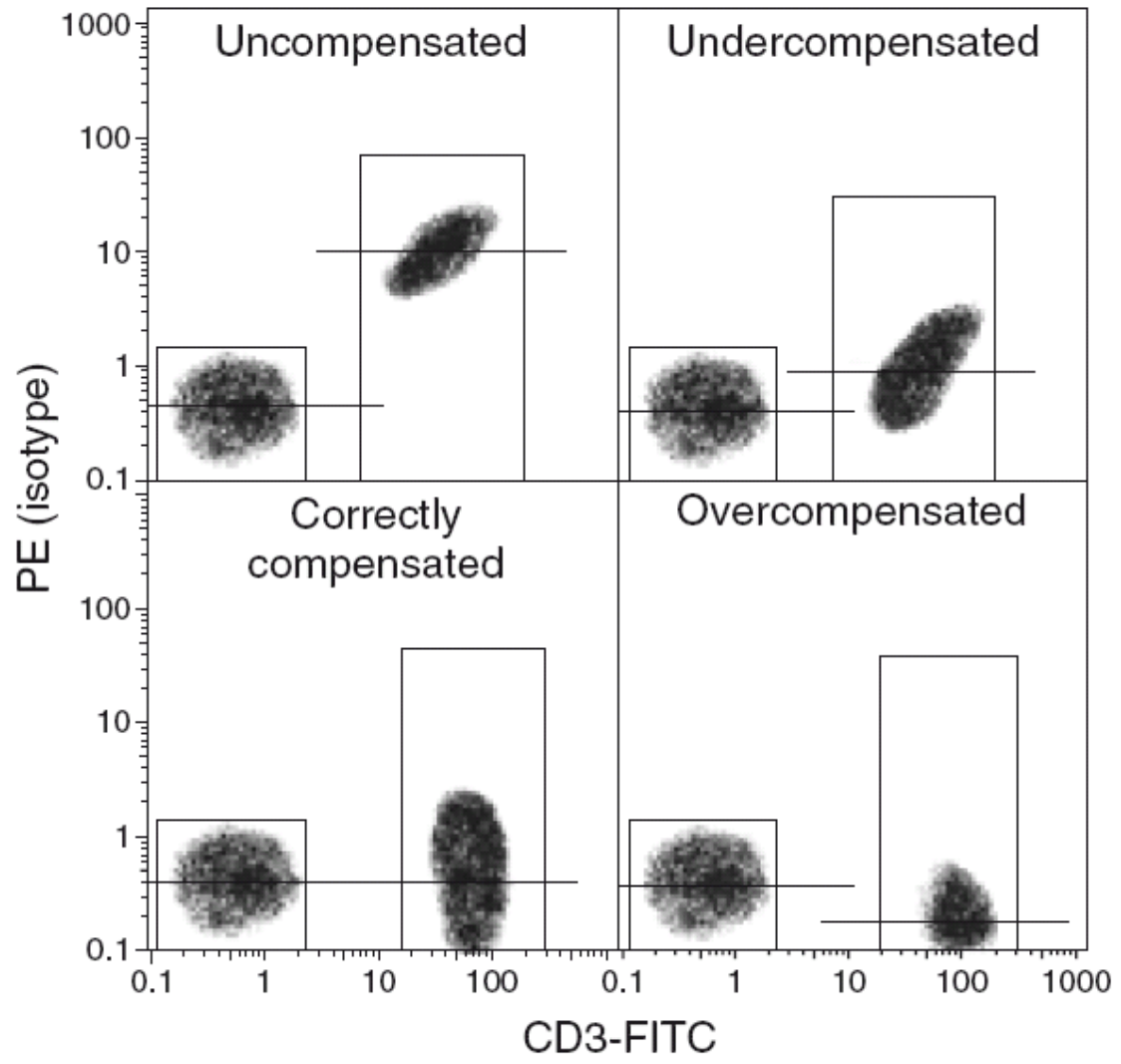
PE negative beads

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

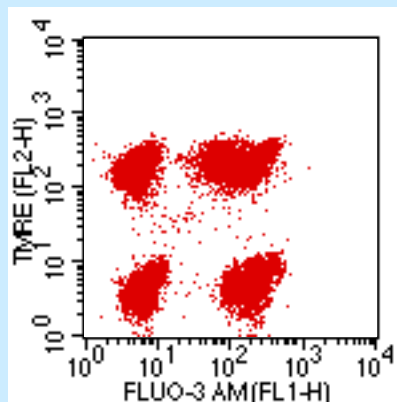
CaliBRITE APC Beads–Catalog No. 340487

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

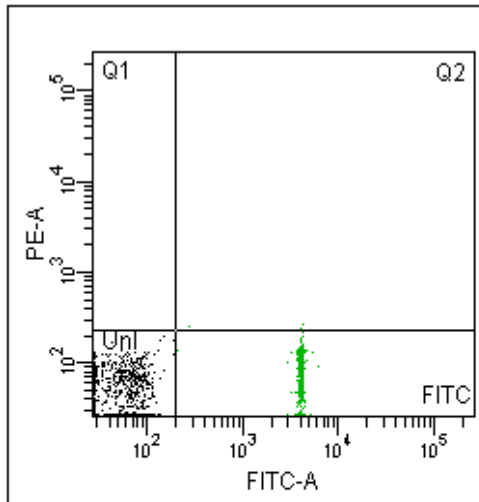
kompenzace

FL1 - 1.1%FL2

FL2 - 17.5%FL1

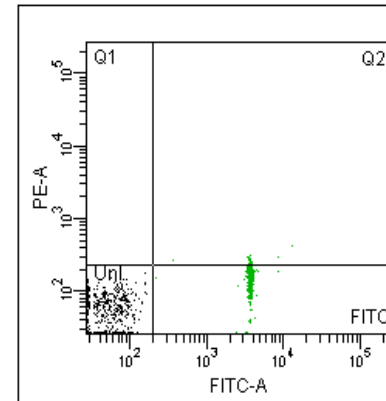
Effects of Changing PMT Values

Correct Compensation



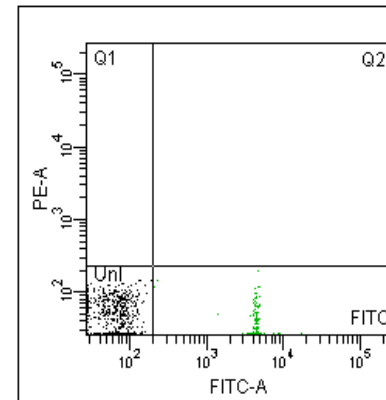
Population	PE-A Mean
Unl	69
FITC	64

FITC Voltage Decreased by 5 V



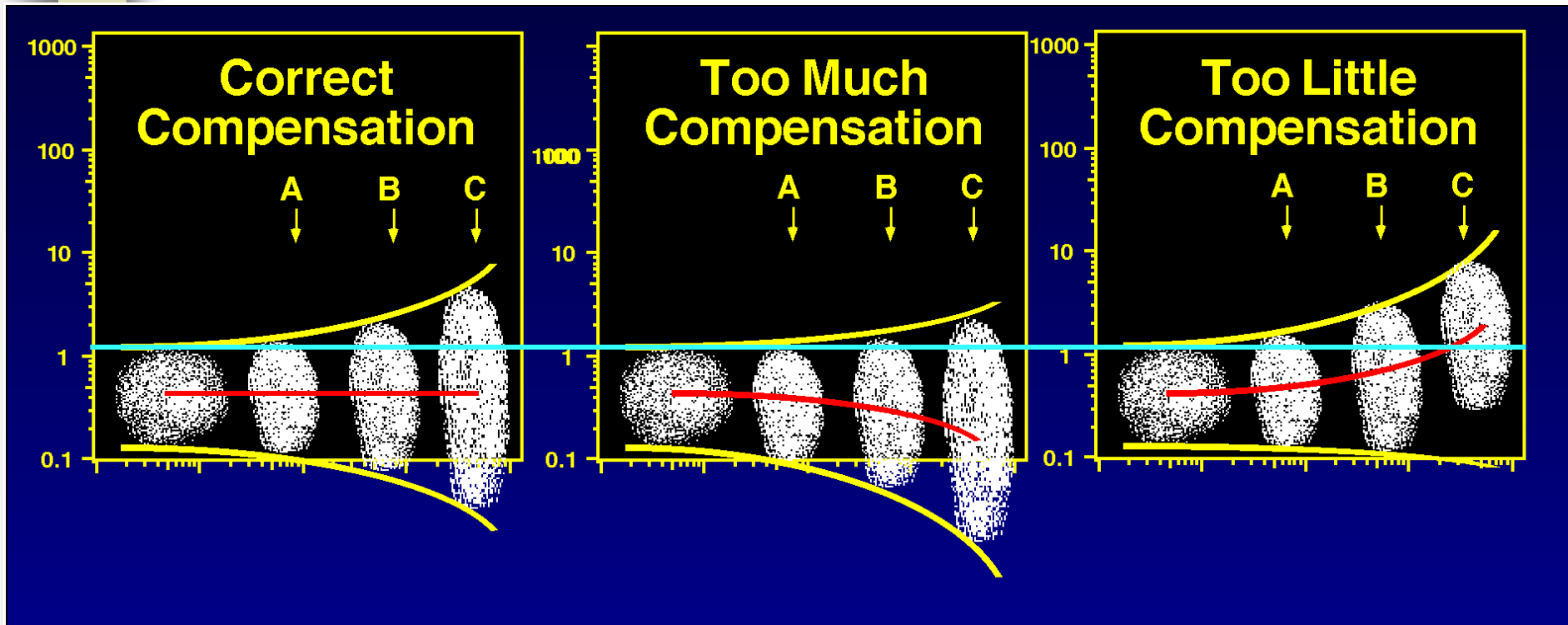
Population	PE-A Mean
Unl	68
FITC	132

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



James E. Bishop, Janet Dickerson, Alan Stall, Zhenxiang Gong, Erik J. Crowther, Dennis Sasaki, and Emil Pop.

A Setup System for Compensation:
BD CompBeads plus BD FACSDiva Software

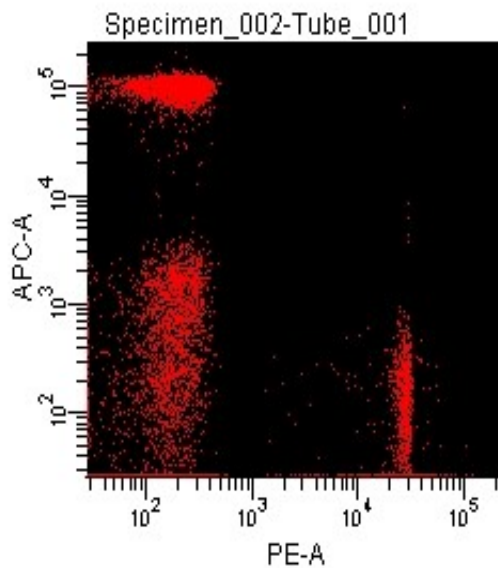
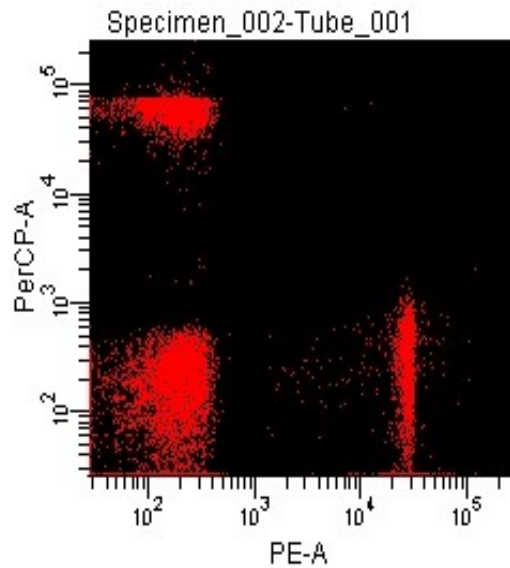
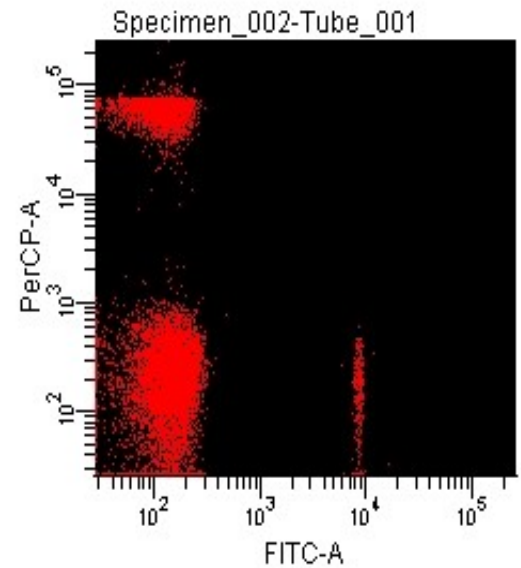
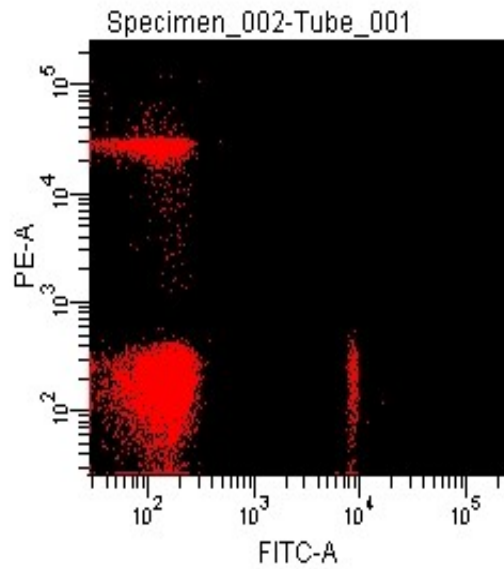
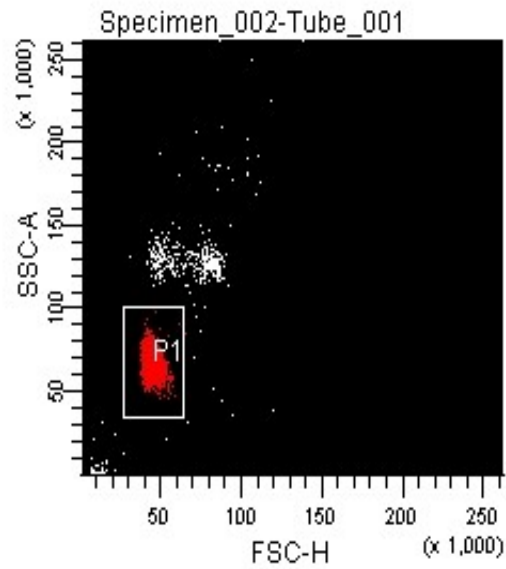
BD Biosciences

Cellular
Discovery Software
Immunology & Cell Biology Systems
Pharmaceutical



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

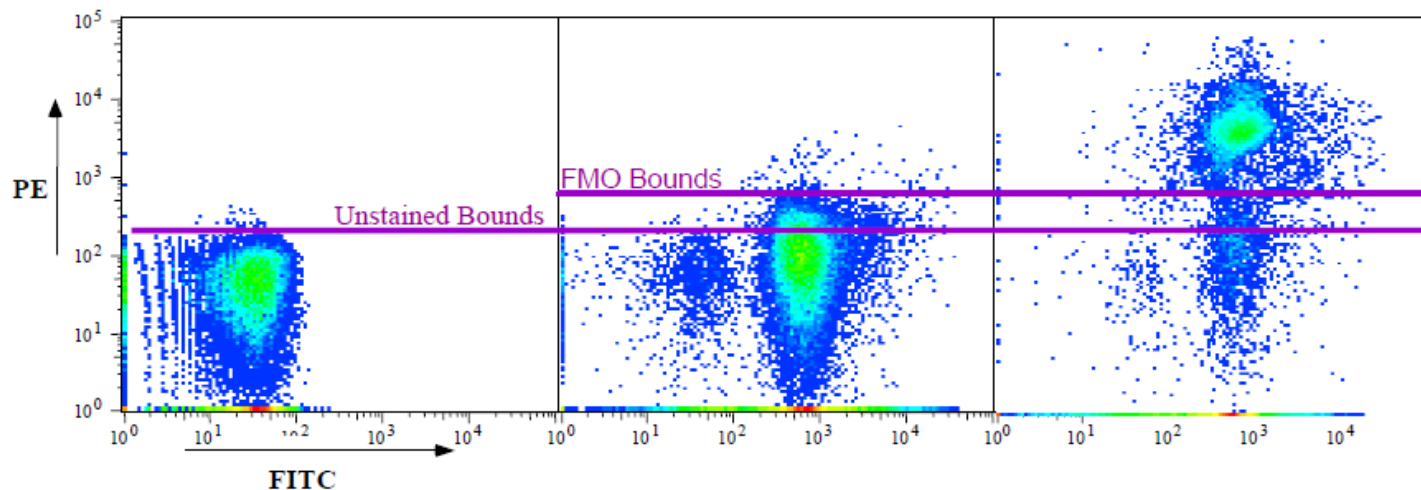
BD Comp Beads



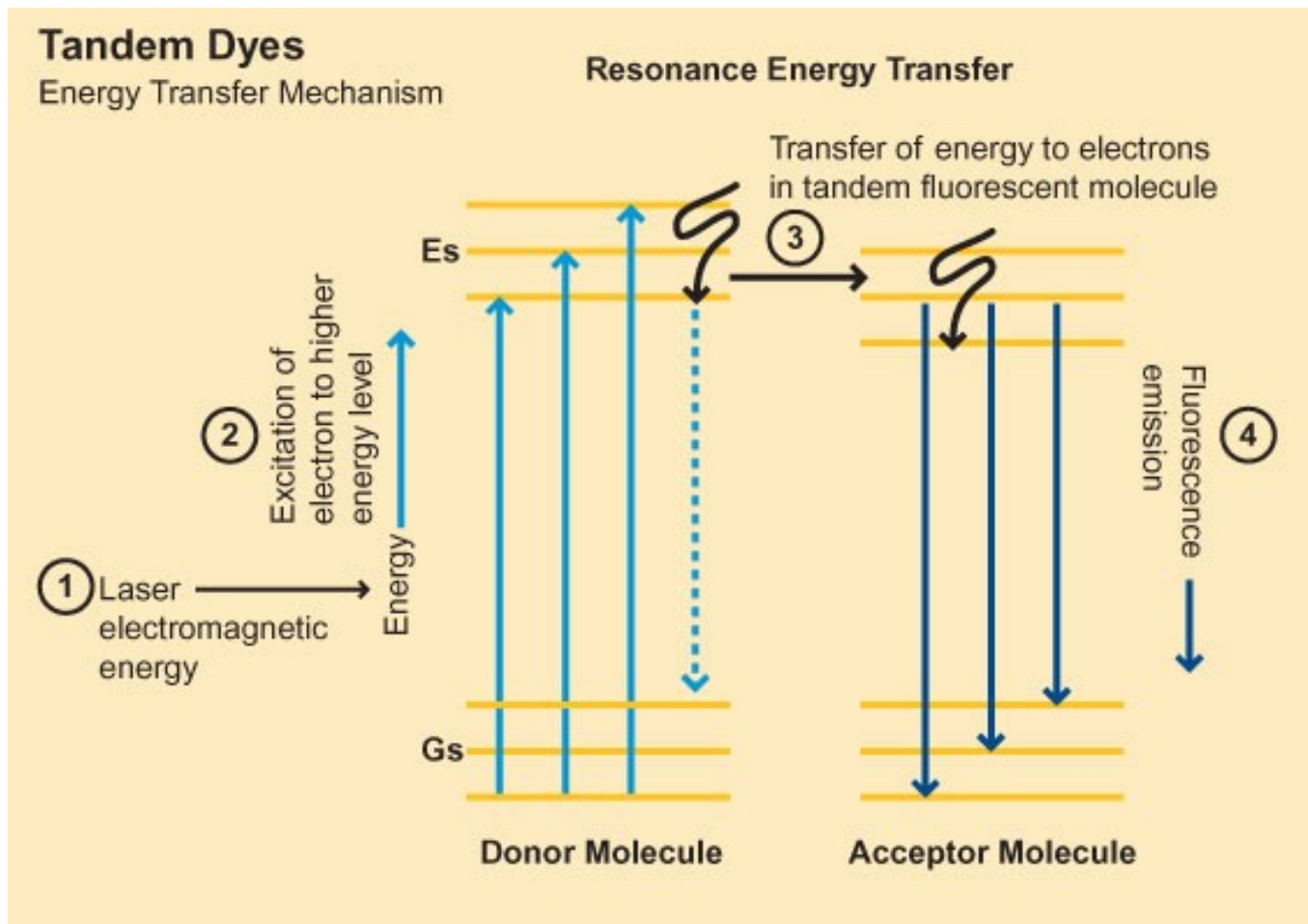
Fluorescence Minus One

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

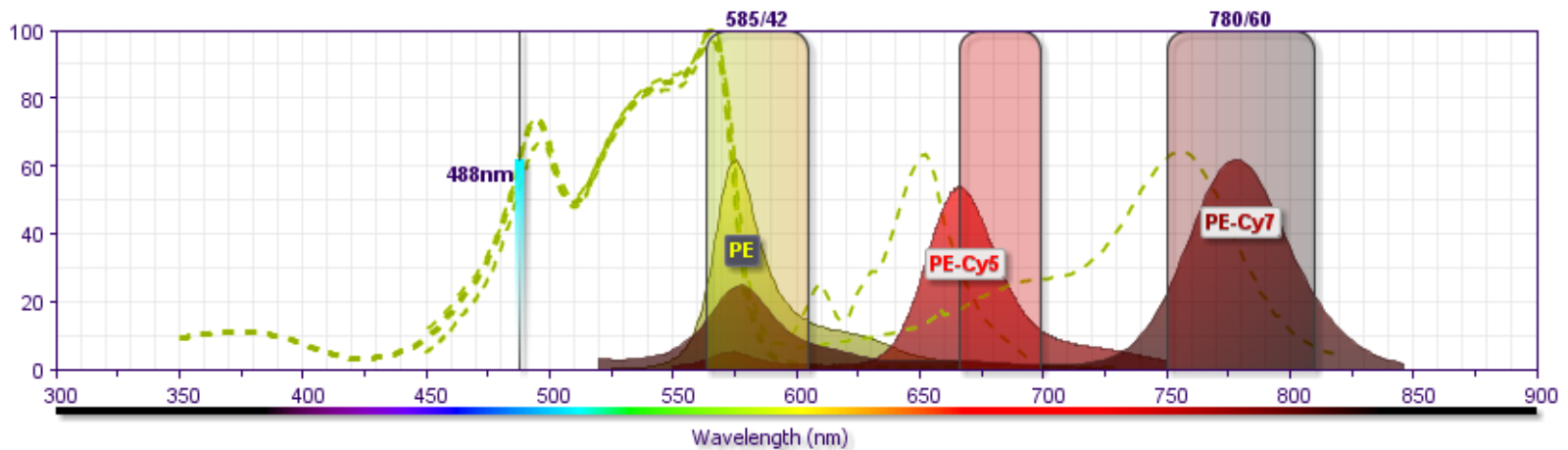
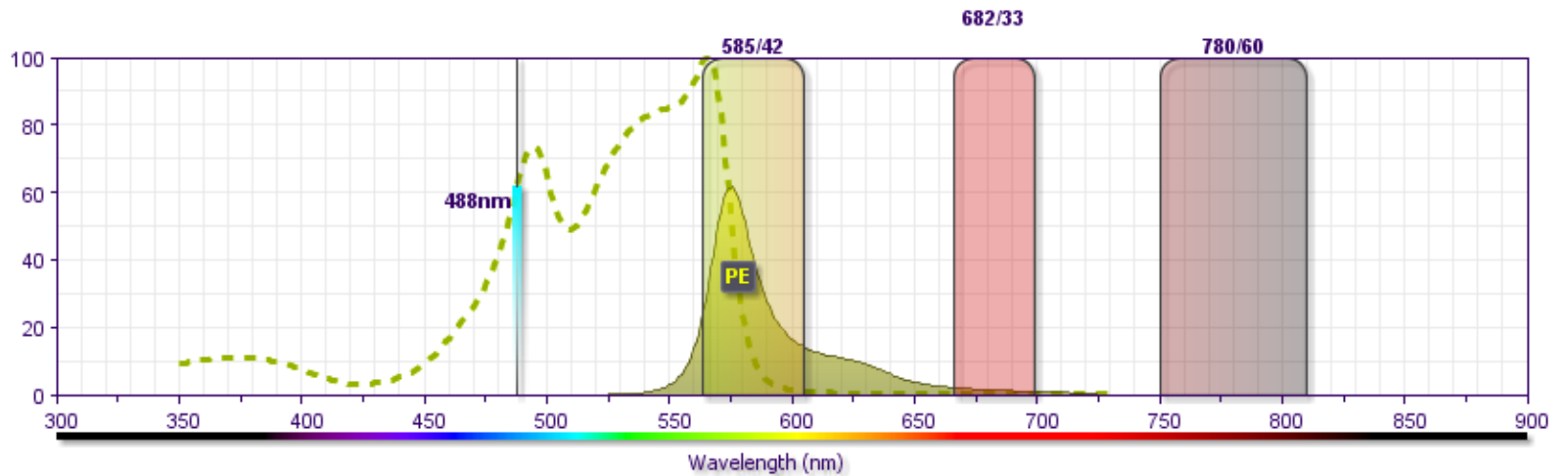
	Unstained Control	FMO Control	Fully Stained
FITC	-	CD3	CD3
PE	-	-	CD4
Cy5PE	-	CD8	CD8



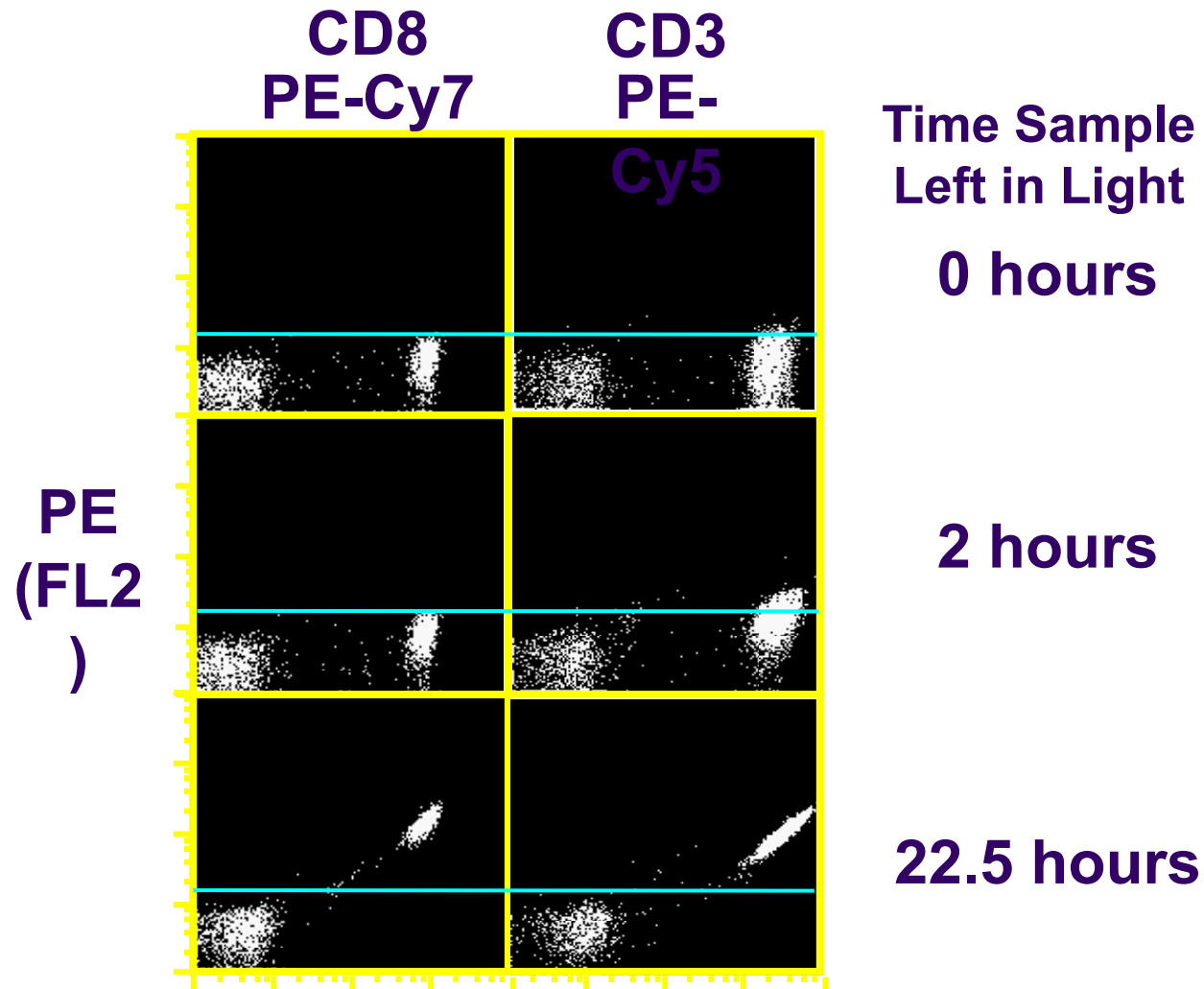
Tandemové značky



Tandemové značky - příklad



Tandems are light sensitive





Kompenzace - literatura

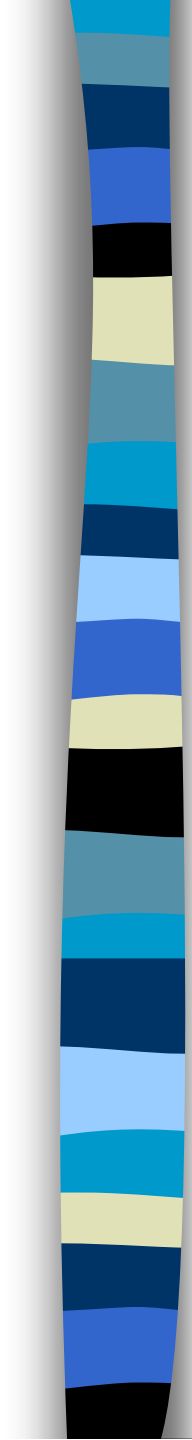
Mario Roederer - Compensation in Flow Cytometry
Current Protocols in Cytometry (2002) 1.14.1-1.14.20 John Wiley & Sons, Inc.

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 přednášky

- 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.