Bi9393 Analytical cytometry
Lesson 3

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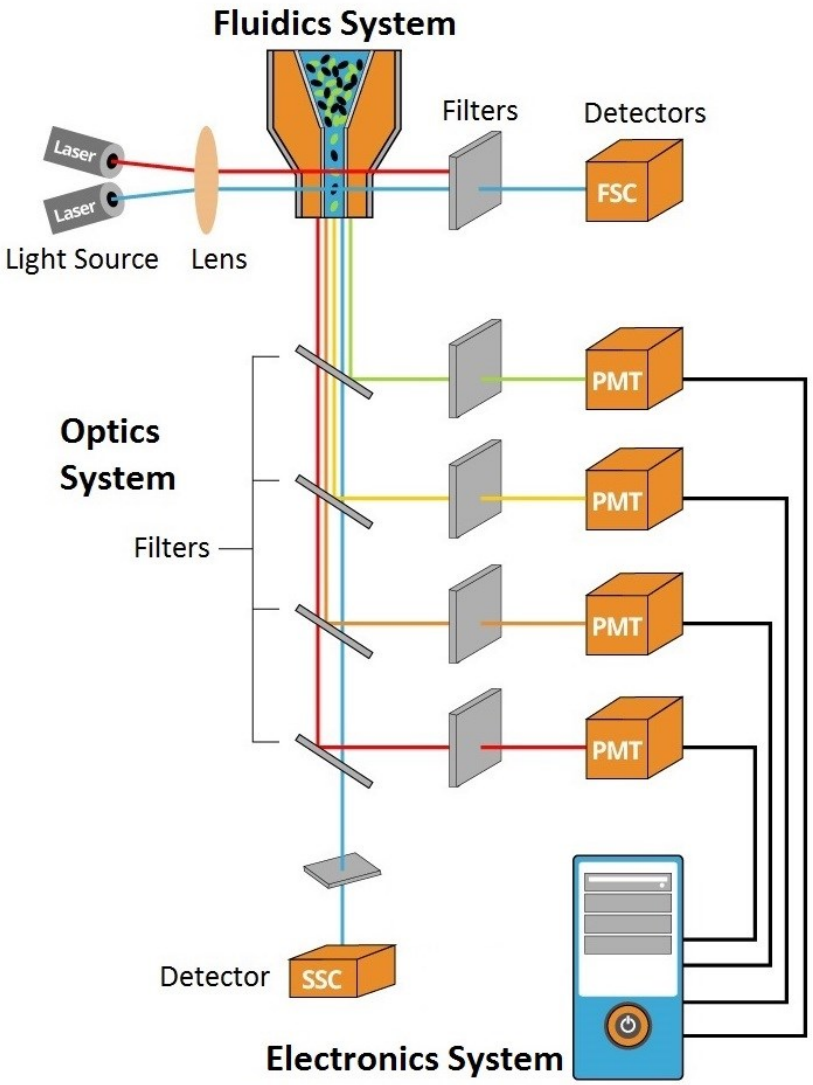
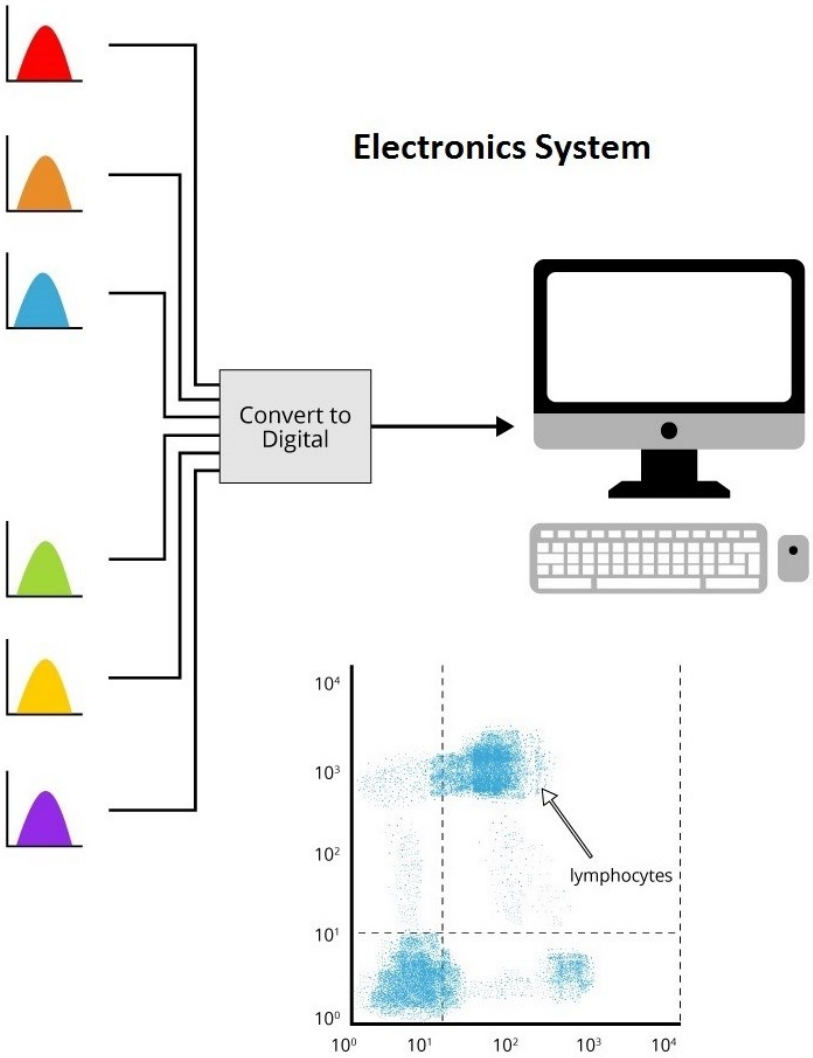


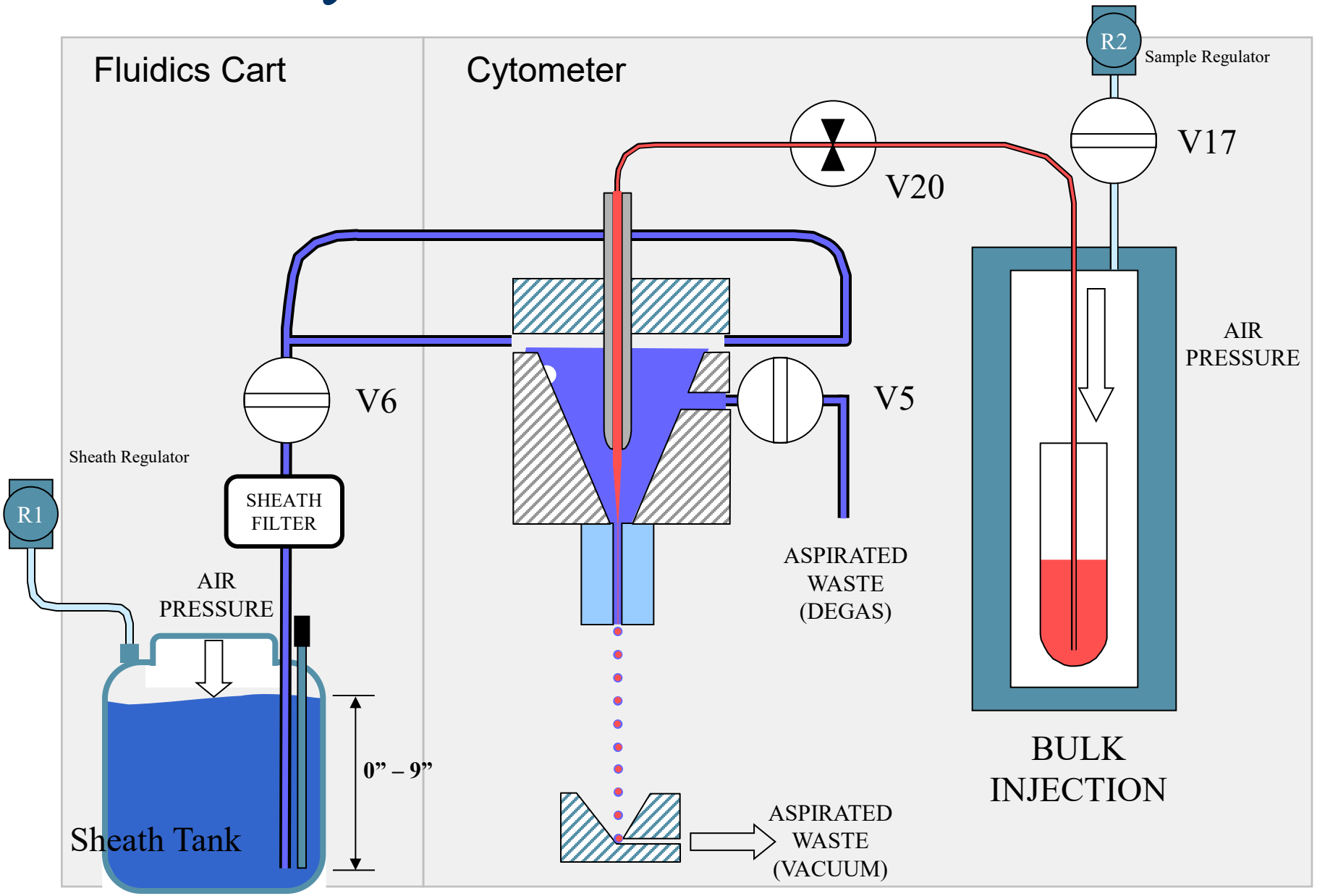


http[illegible]bosterbio.com/protocol-and-troubleshooting/flow-cytometry-principle

Fluidic systems and hydrodynamics

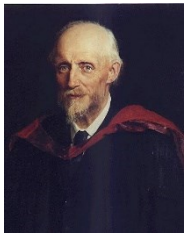
Getting the cells in the right place (at the right time)! (Shapiro, pp 133-143 - 3rd ed)

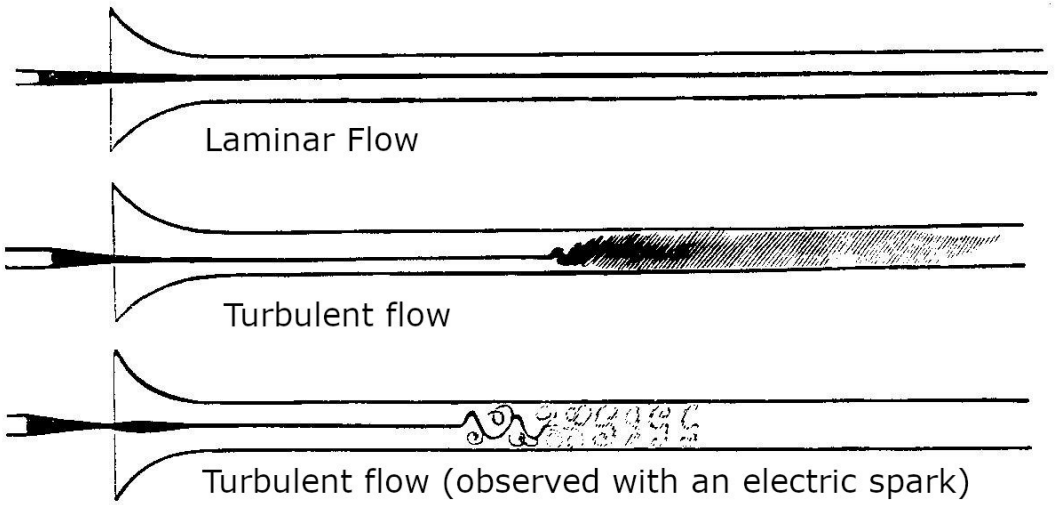
Fluid system : BD FACSAria II

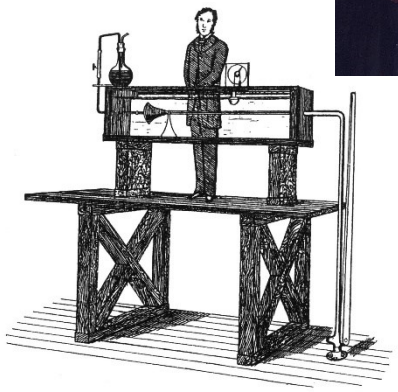


Reynolds number

Osborne Reynolds (1842-1912)







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

where

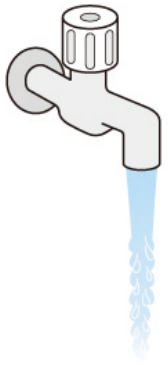
d = tube diameter

ρ = density of fluid

$\bar{v}$ = mean velocity of fluid

η = viscosity of fluid

(a) Laminar flow

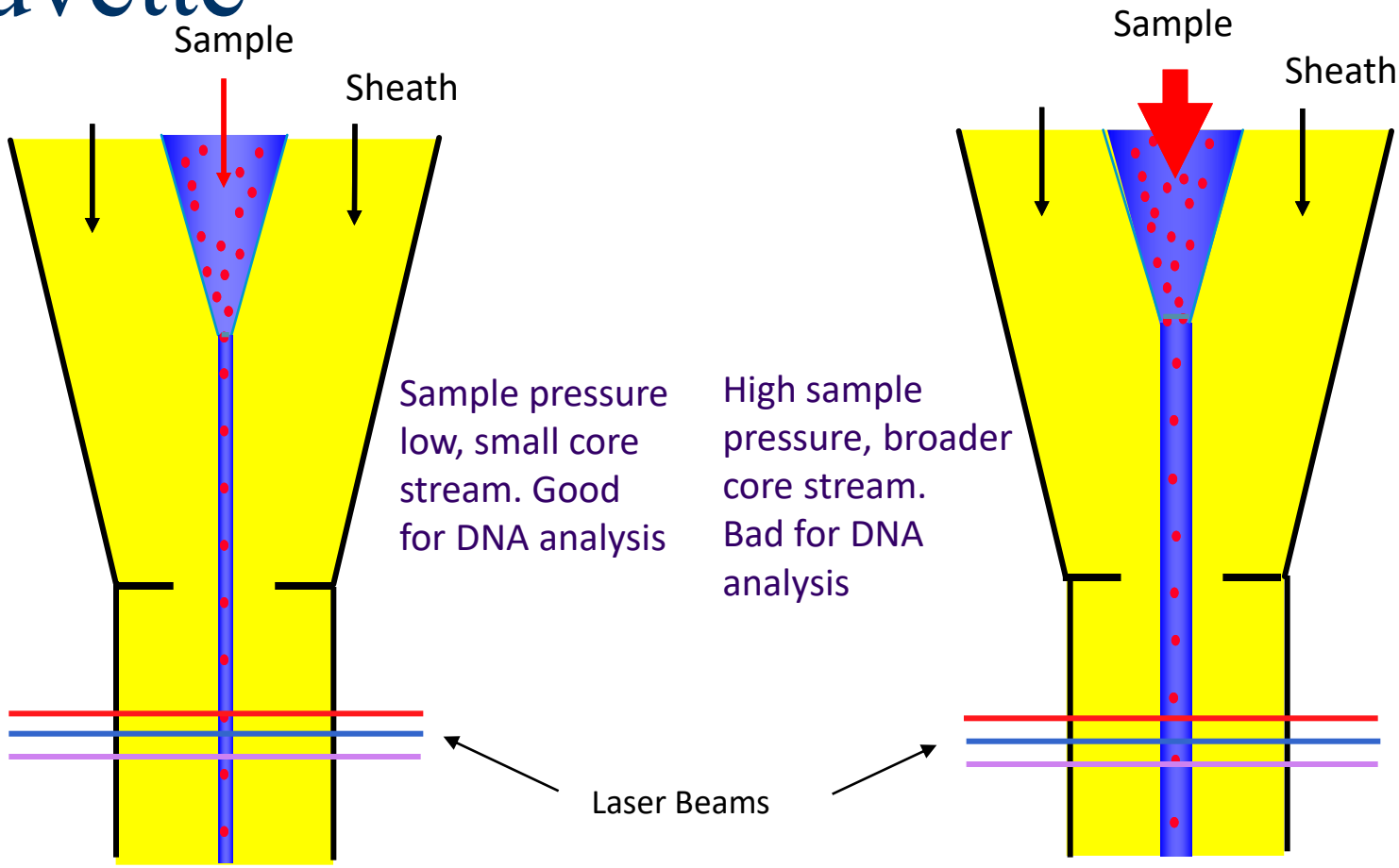


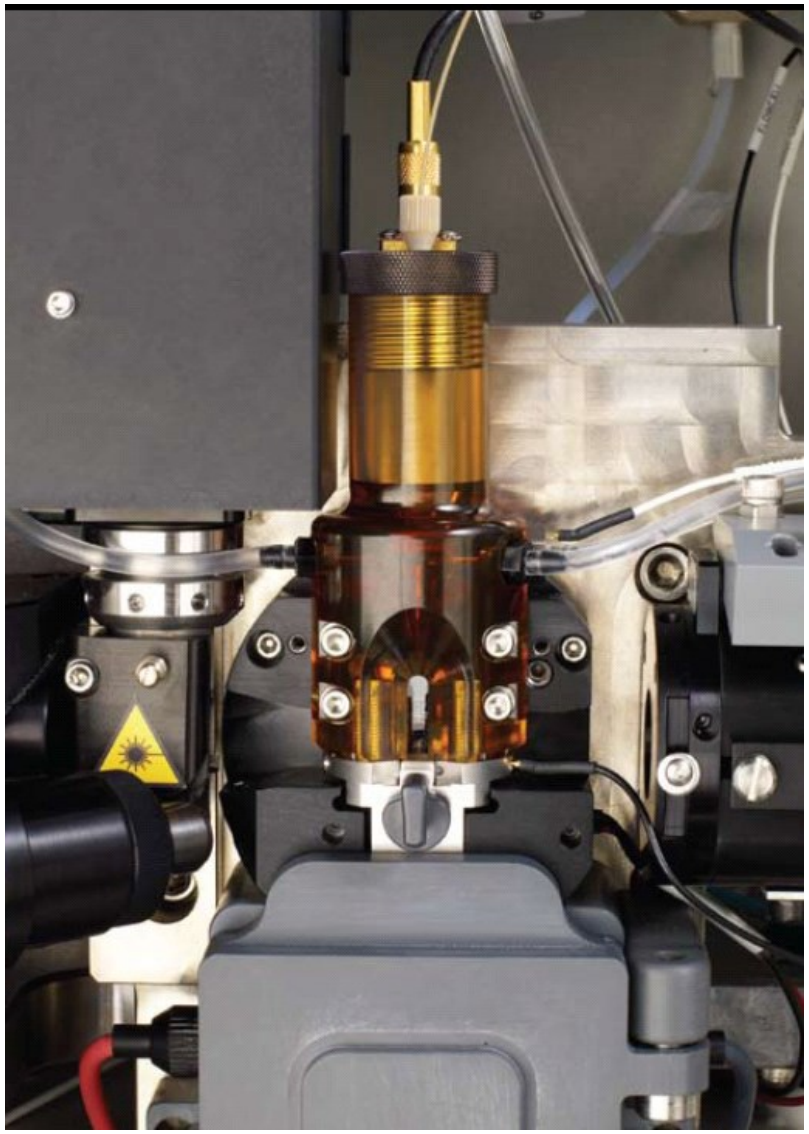
(b) Turbulent flow

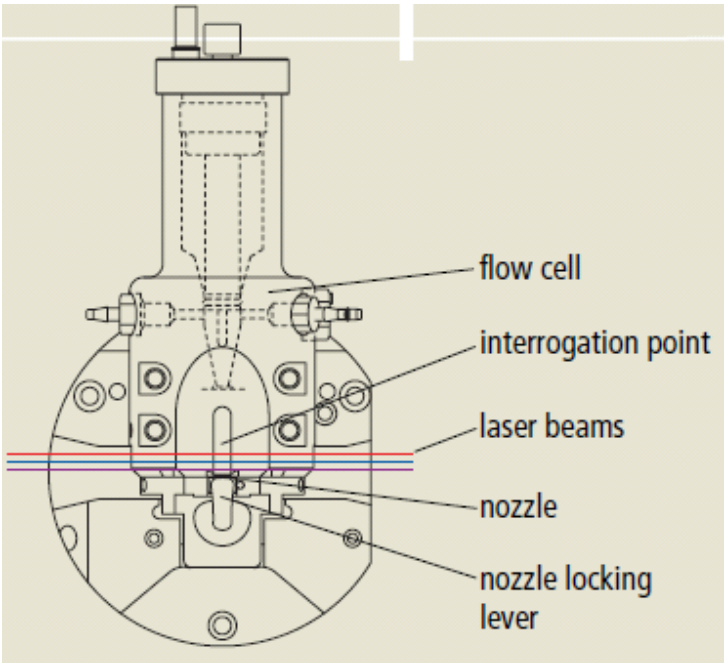
$R_e < 2300$

$R_e > 2300$

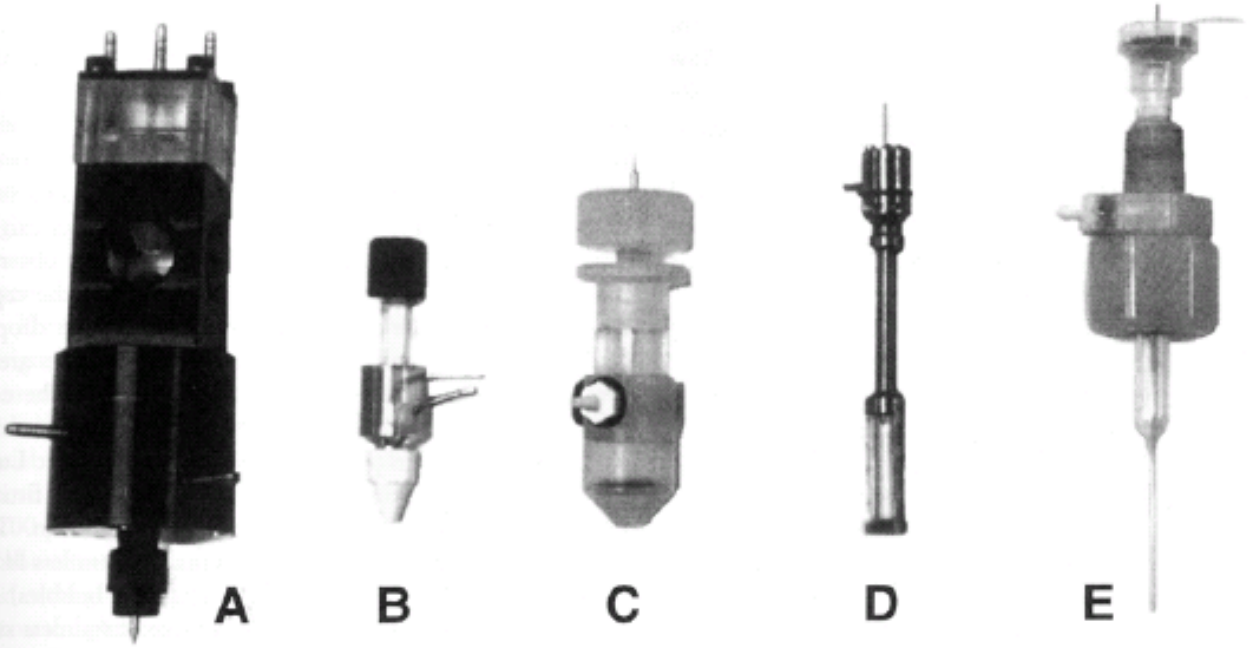
Hydrodynamic focusing in the cuvette

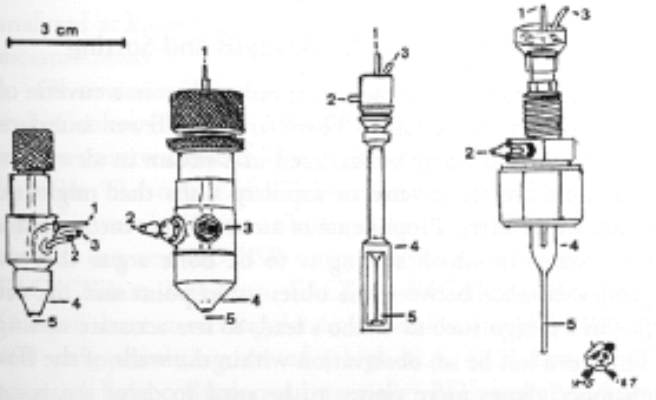


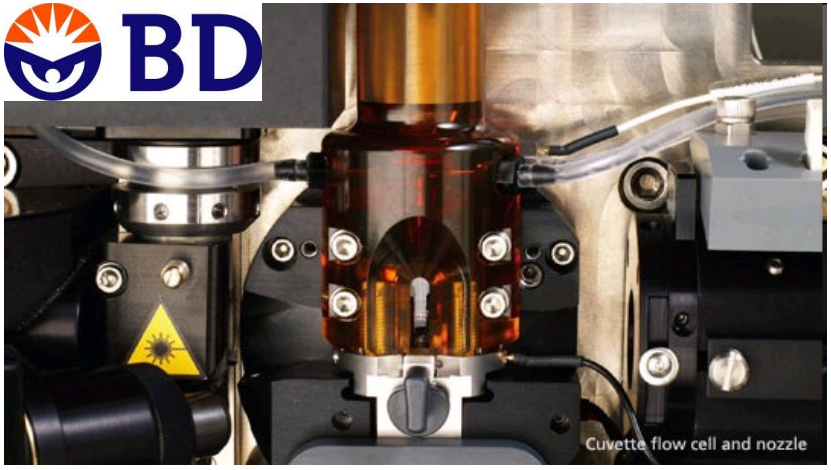



flow cell
interrogation point
laser beams
nozzle
nozzle locking lever


BD FACSAria II



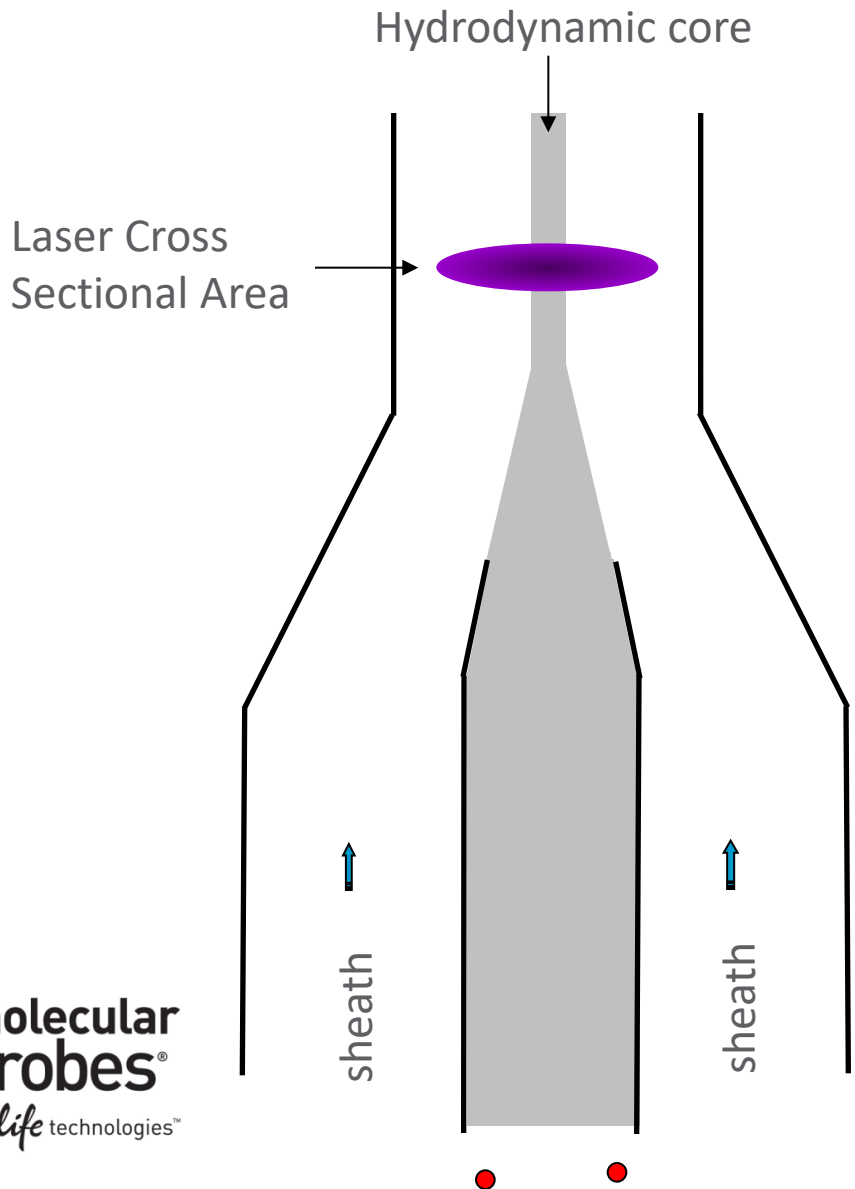


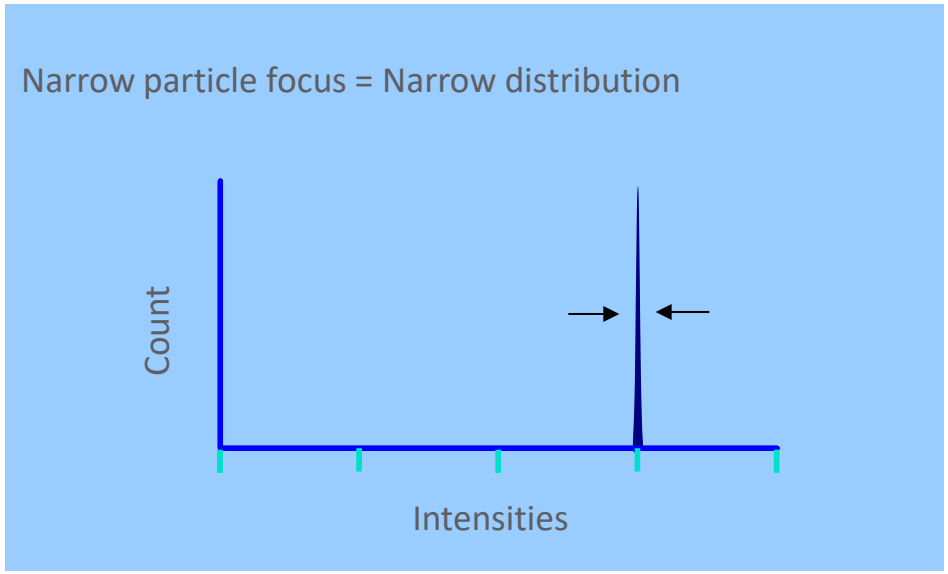


Shapiro 4.ed.p 169

Particle Delivery: Hydrodynamic Focusing

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





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

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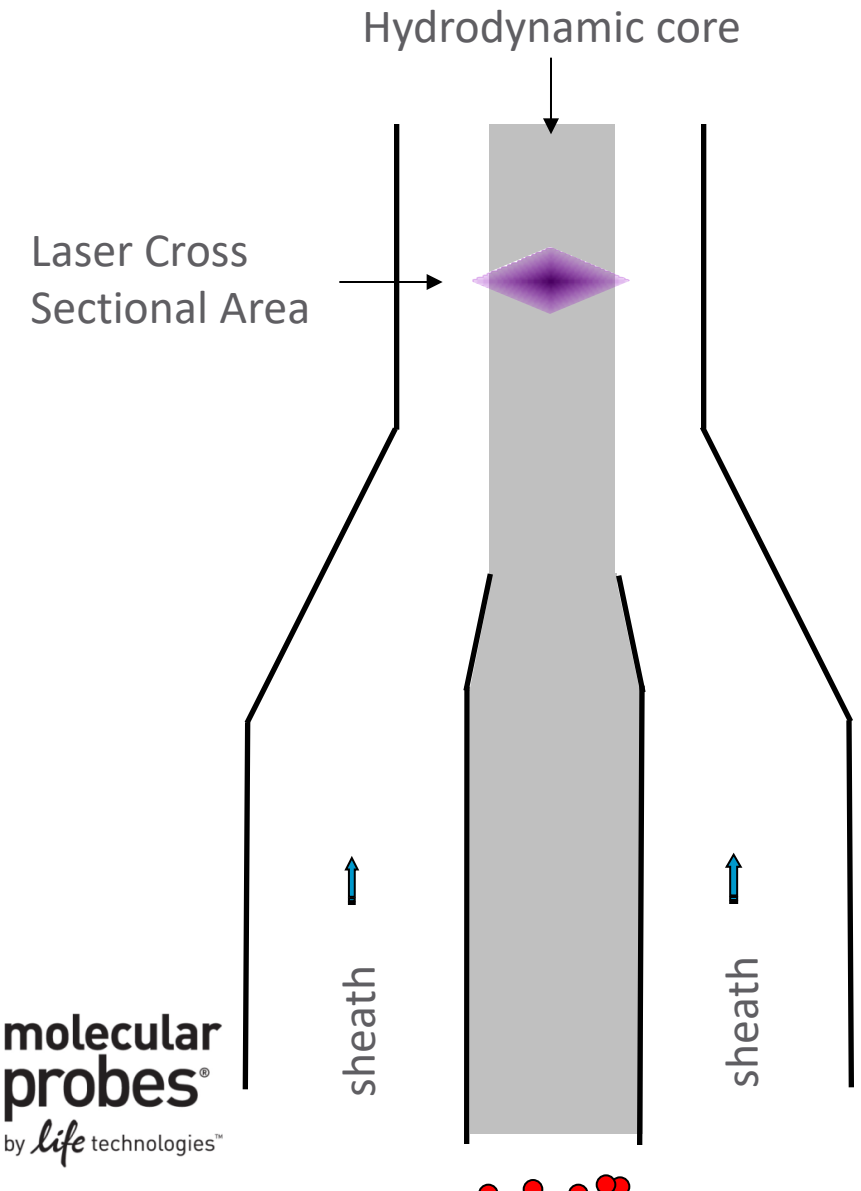
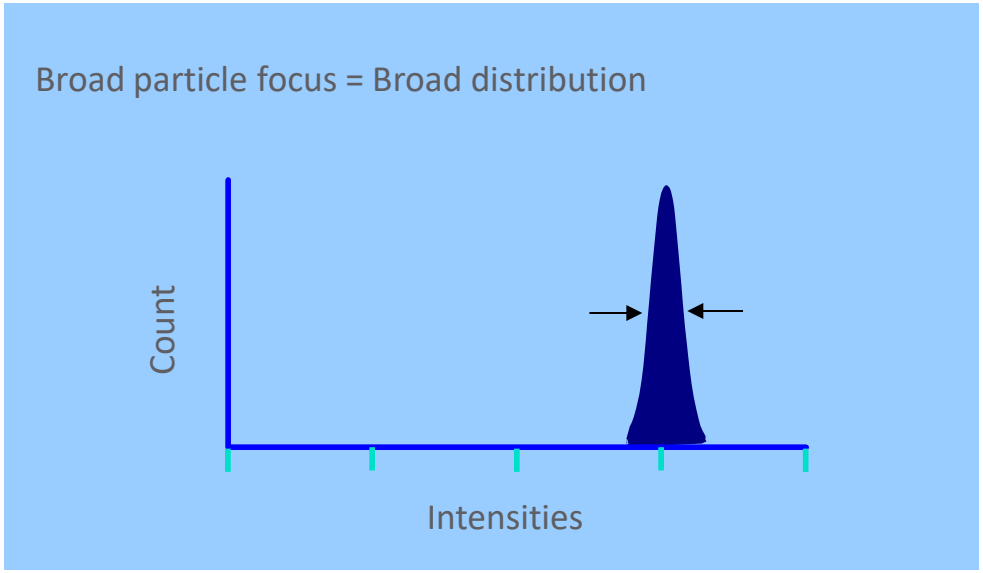
Particle Delivery: Hydrodynamic Focusing

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





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

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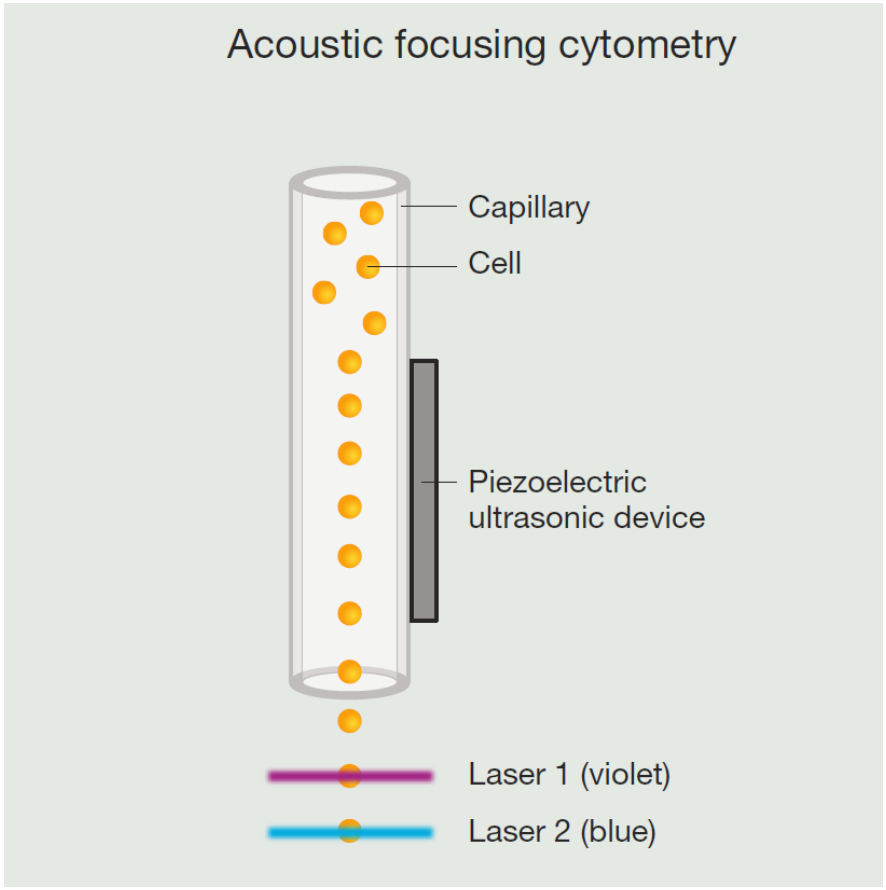
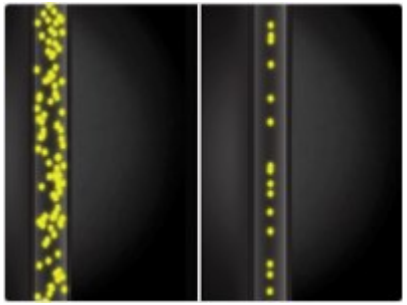
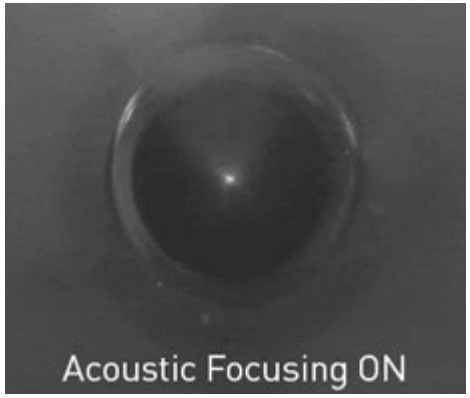
Attune® Acoustic Focusing Cytometer

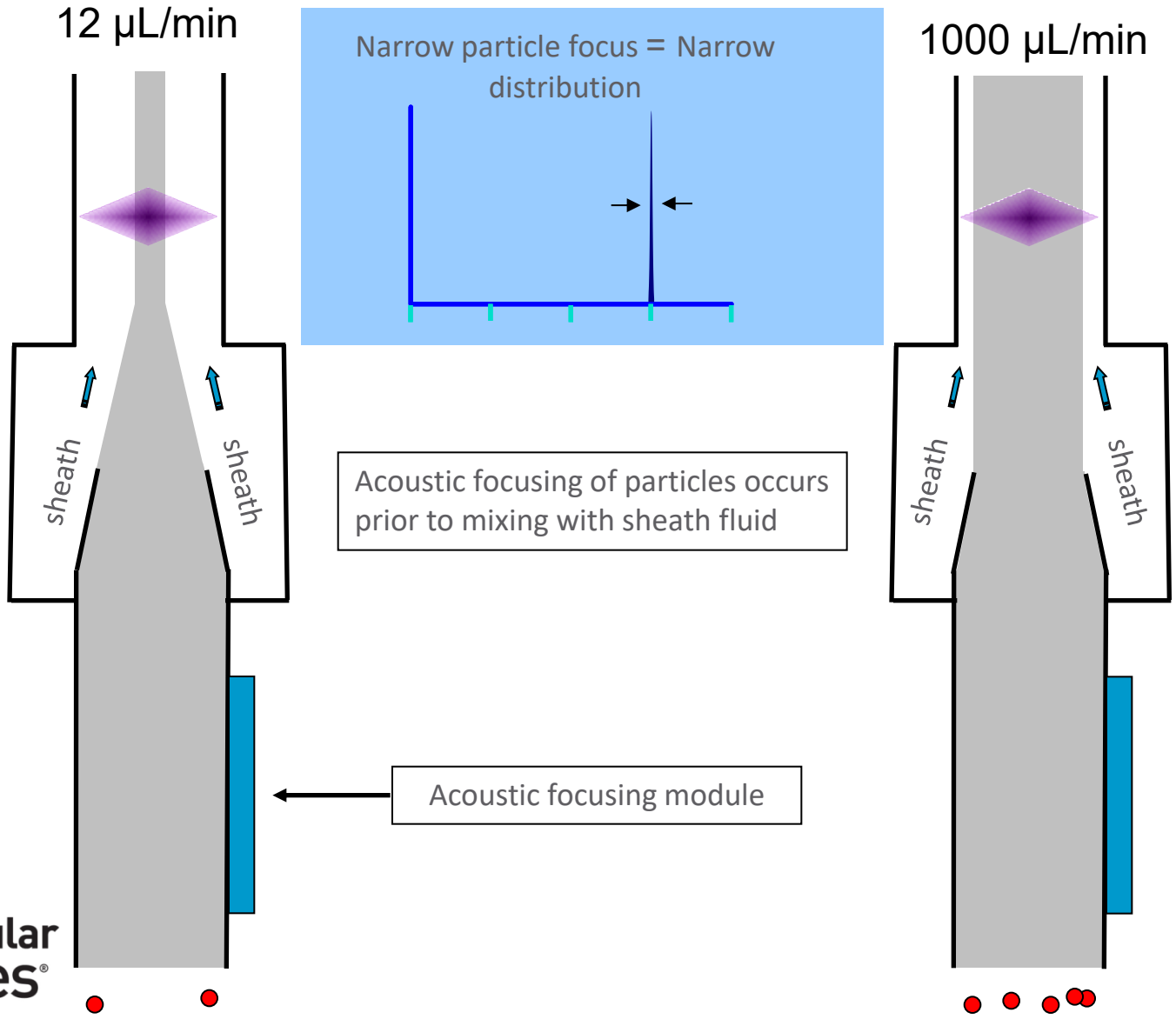


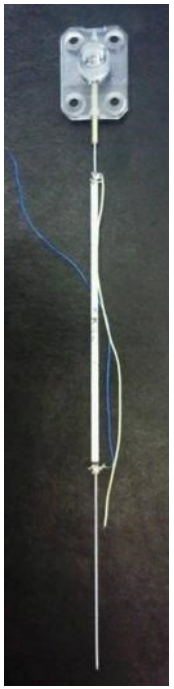




ThermoFisher
SCIENTIFIC

Acoustic Focusing = Better Precision





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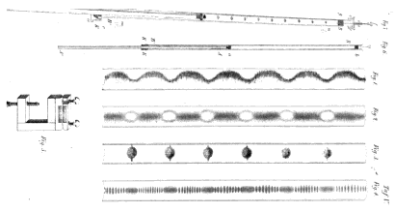
5. Kaduchak G, Goddard G, Salzman G et al. (2008) US Patent 7,340,957.



Using acoustic radiation force as a concentration method for erythrocytes

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(Received 20 May 1996; accepted for publication 7 March 1997)

We investigated the potential damage in icted on erythrocytes by acoustic radiation force when the cells are concentrated by a 500-kHz ultrasonic standing wave at the pressure node. The extent of the damage was estimated from the concentrations of potassium ions, iron complexes, and lactate dehydrogenase released from the cells. After 2 min of ultrasound irradiation at 12.8 mJ/m^3, the cells concentrated on the pressure node, with a cell distribution half-width of 138 μm; no significant release of intracellular components was detected, even after 15 min of irradiation. The results indicate that even small ions like potassium are not released as a result of ultrasound irradiation on cell membranes without cavitation, and they demonstrate the potential use of acoustic radiation force for concentrating living cells in biomedical applications. © *1997 Acoustical Society of America.* [S0001-4966(97)01407-0]

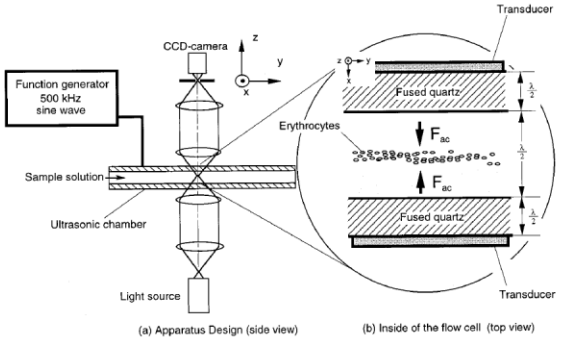
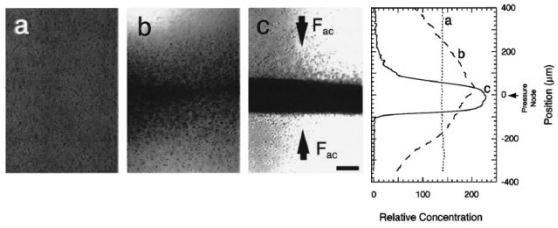
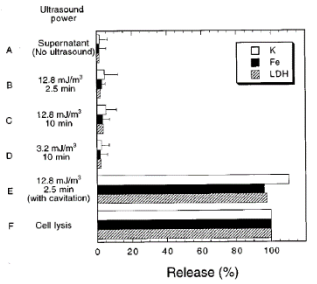


FIG. 1. Schematic diagram of the apparatus for concentration of erythrocytes.





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**

(75) Inventors: **Gregory Kaduchak**, Los Alamos, NM (US); **Greg Goddard**, Los Alamos, NM (US); **Gary Salzman**, White Rock, NM (US); **Dipen Sinha**, Los Alamos, NM (US); **John C. Martin**, Los Alamos, NM (US); **Christopher Kwiatkowski**, Los Alamos, NM (US); **Steven Graves**, San Juan Pueblo, NM (US)

(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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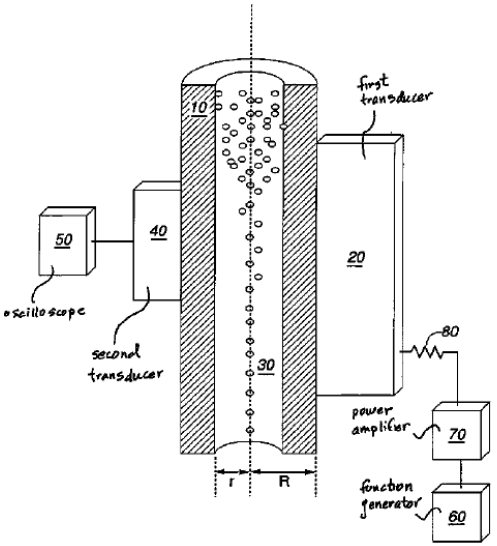

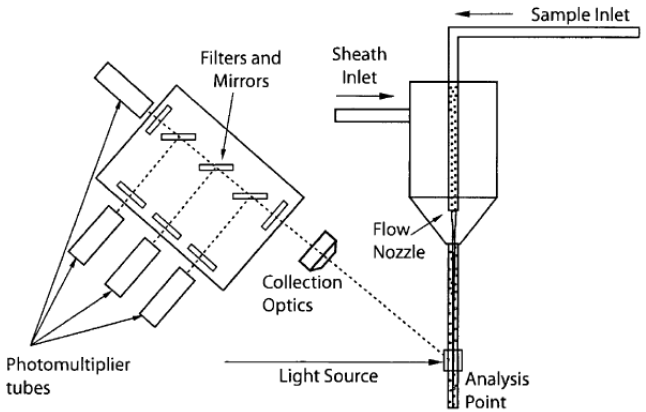

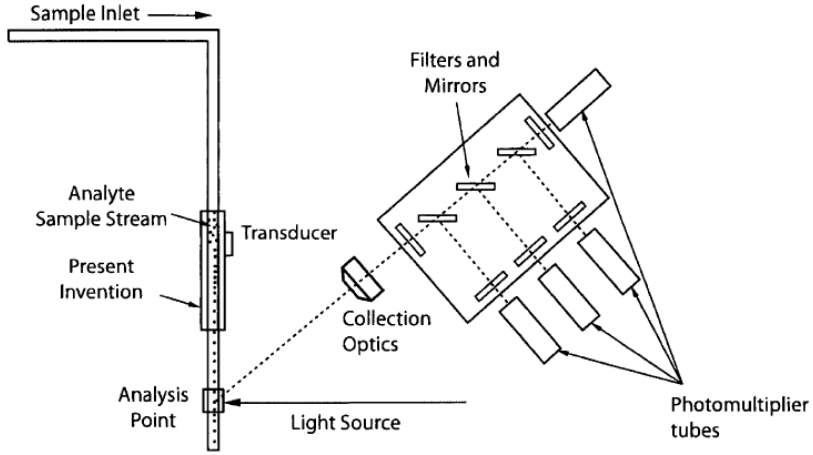

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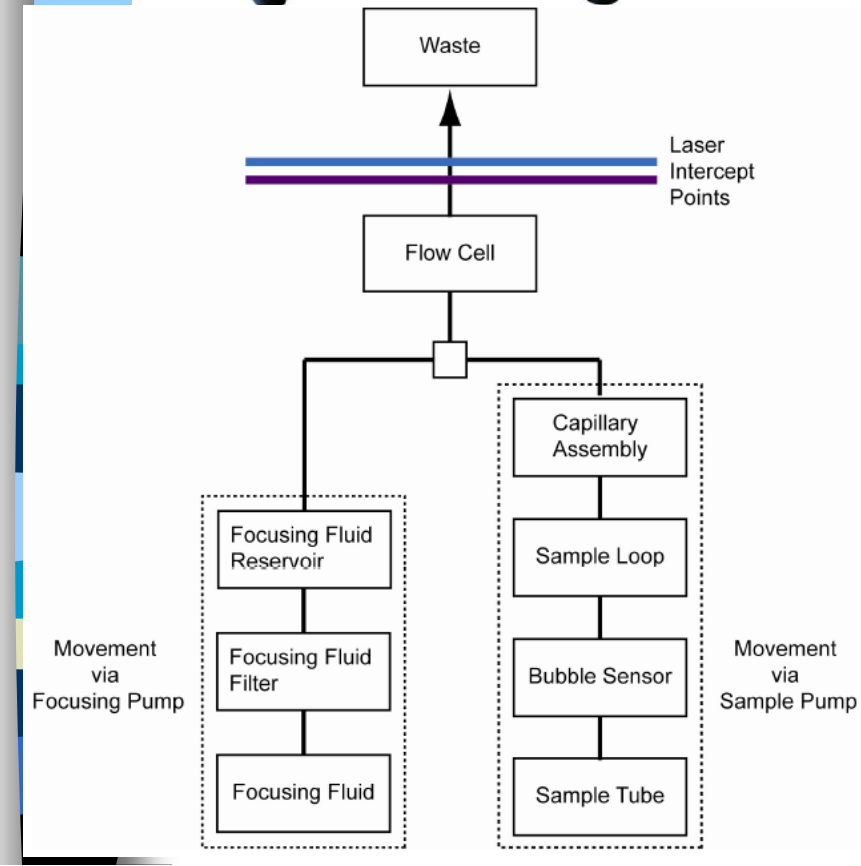
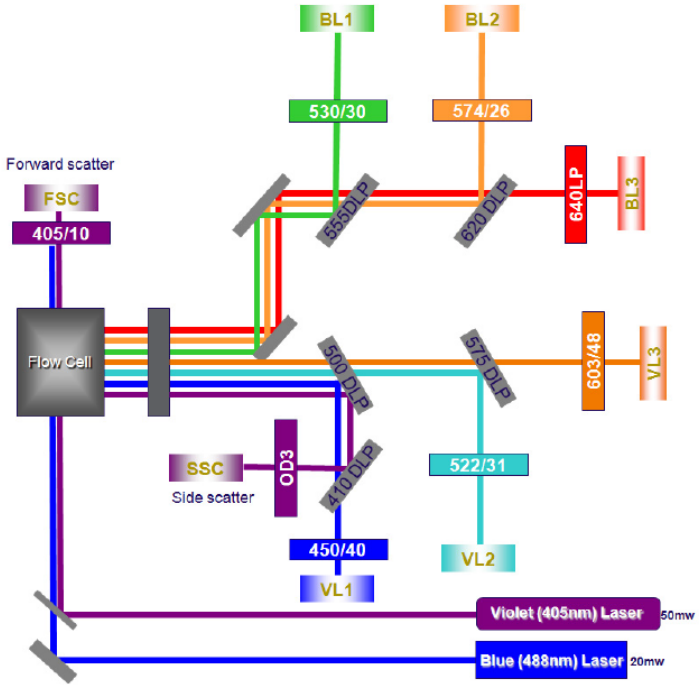




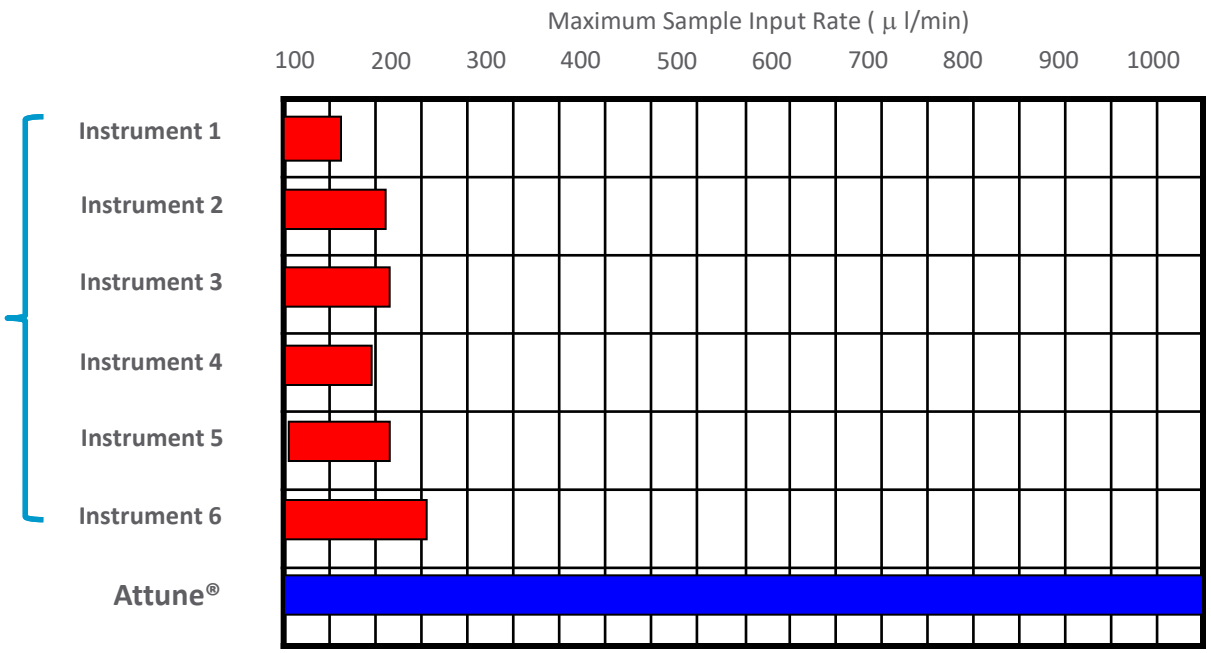
Attune NxT (2nd generation)

ThermoFisher SCIENTIFIC

Lasers	Laser configuration (Cat. No.)	Violet 405 nm	Blue 488 nm	Yellow 561 nm	Green 532 nm	Red 637 nm	Total detection channels*
1	Blue (A24864)	Available as upgrade	4	Available as upgrade	Available as upgrade	Available as upgrade	6
2	Blue/green (A28995)	Available as upgrade	3	–	4	Available as upgrade	9
	Blue/yellow (A24861)	Available as upgrade	3	4	–	Available as upgrade	9
	Blue/violet (A24862)	4	4	Available as upgrade	Available as upgrade	Available as upgrade	10
	Blue/red (A24863)	Available as upgrade	4	Available as upgrade	Available as upgrade	3	9
3	Blue/green/red (A28997)	Available as upgrade	3	–	4	3	12
	Blue/green/violet (A28999)	4	3	–	4	Available as upgrade	13
	Blue/red/yellow (A28993)	Available as upgrade	3	4	–	3	12
	Blue/violet/yellow (A24859)	4	3	4	–	Available as upgrade	13
	Blue/red/violet (A24860)	4	4	Available as upgrade	Available as upgrade	3	13
4	Blue/red/violet/ green (A29001)	4	3	–	4	3	16
	Blue/red/yellow/ violet (A24858)	4	3	4	–	3	16

* Includes forward scatter (FSC) and side scatter (SSC).

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

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Fluidics – summary

• the pressure of the carrier (sheathing) liquid drives the buffer through the cuvette and the higher pressure in the sample tube introduces the sample into the cuvette.

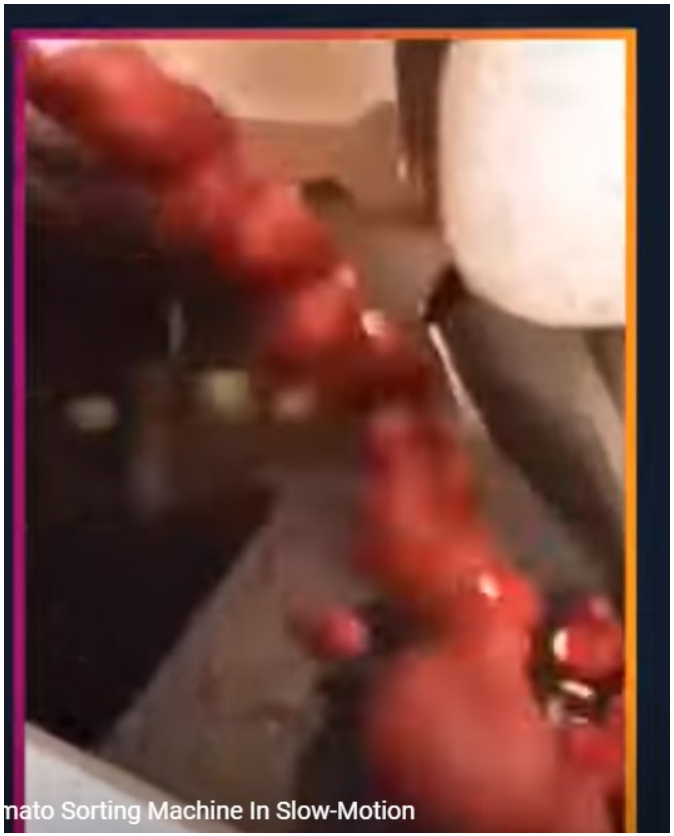
• The principle of hydrodynamic focusing aligns the cells in the cuvette "like pearls on a string" before they reach the point where the laser beam intersects.

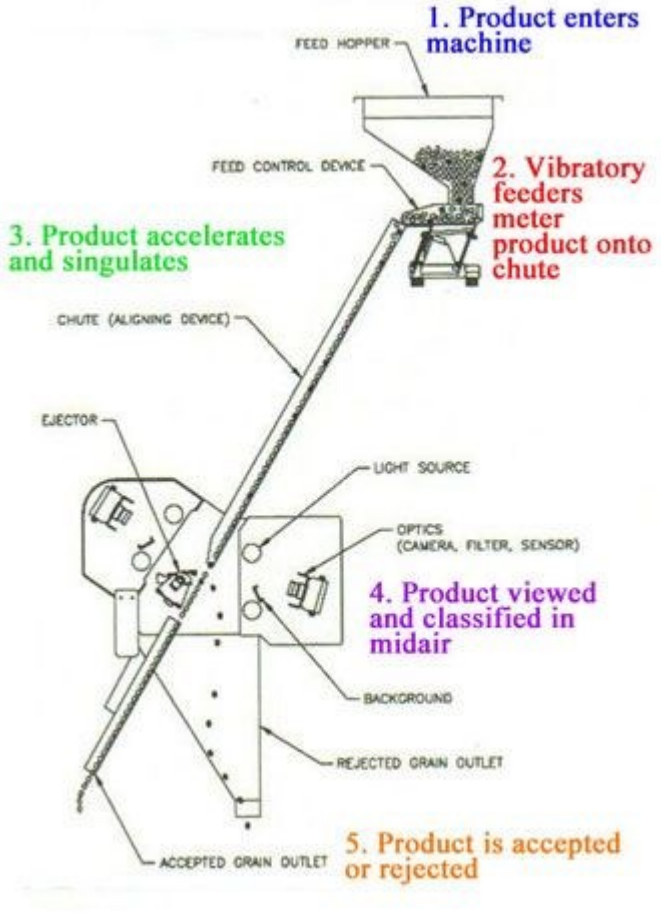
•Hydrodynamic focusing cannot dissociate cell aggregates. Flow cytometry requires a suspension of single cells!

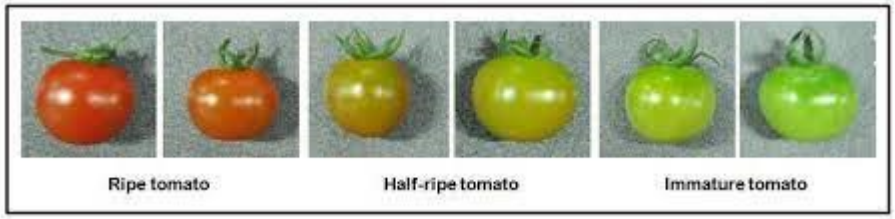
Principles of flow cytometry and sorting

- sorting

Doležel (1999)


mato Sorting Machine In Slow-Motion


1. Product enters machine
FEED HOPPER
FEED CONTROL DEVICE
2. Vibratory feeders meter product onto chute
3. Product accelerates and singulates
CHUTE (ALIGNING DEVICE)
EJECTOR
LIGHT SOURCE
OPTICS (CAMERA, FILTER, SENSOR)
4. Product viewed and classified in midair
BACKGROUND
REJECTED GRAIN OUTLET
ACCEPTED GRAIN OUTLET
5. Product is accepted or rejected

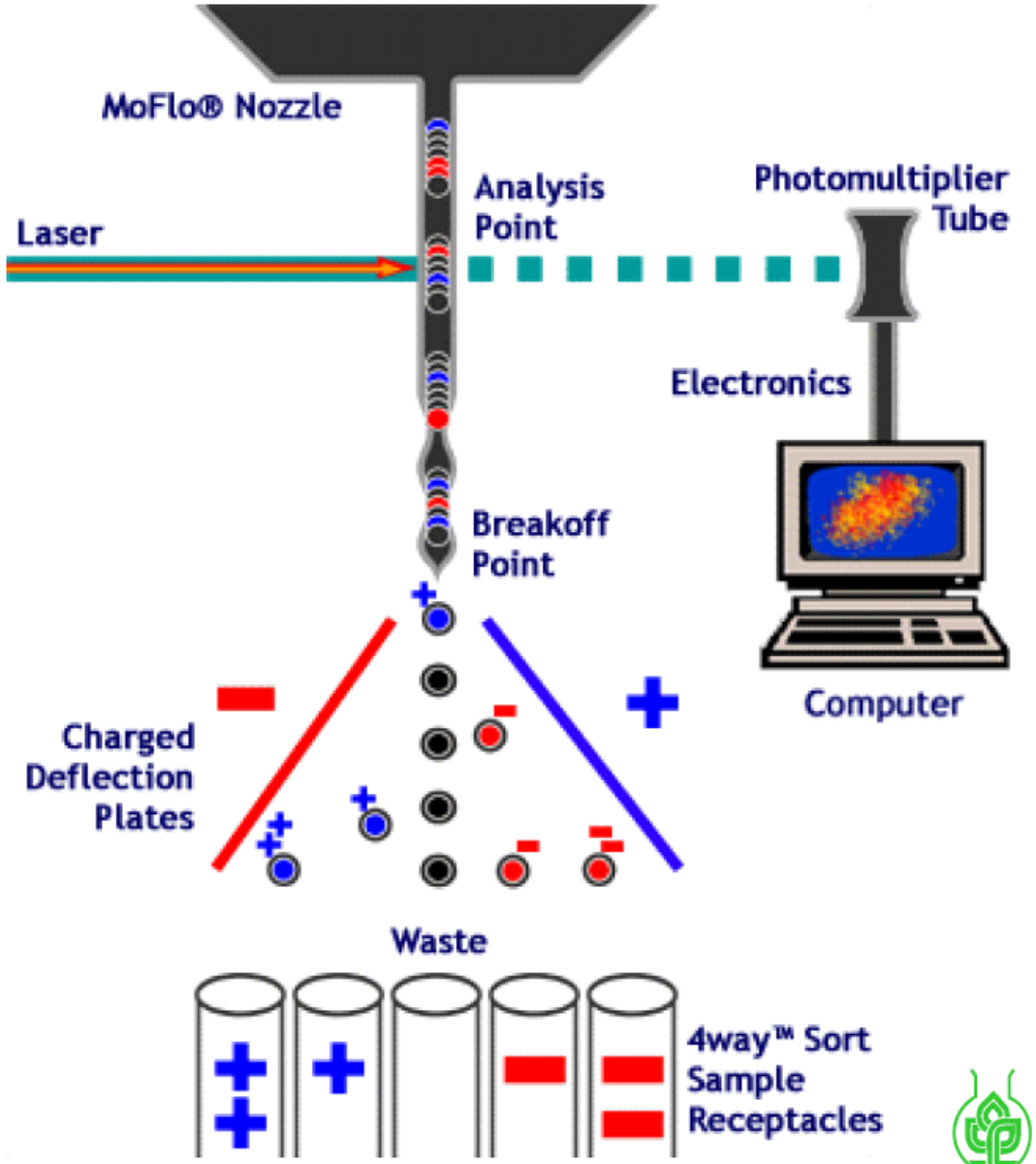

Ripe tomato
Half-ripe tomato
Immature tomato

ELECTROSTATIC DROPLET SORTER

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

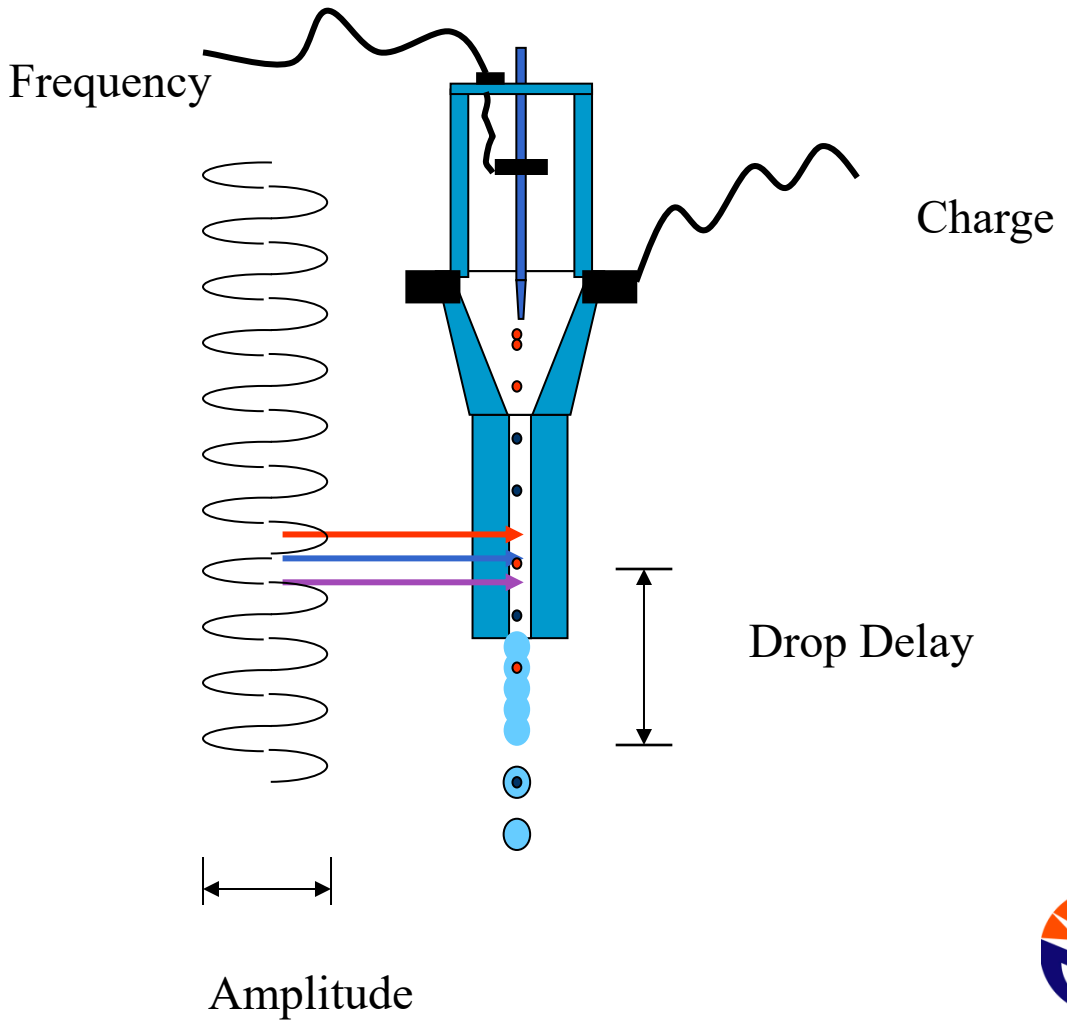
- Biosafefy hazard
- Mechanical shearing
- Problems to sort large particles

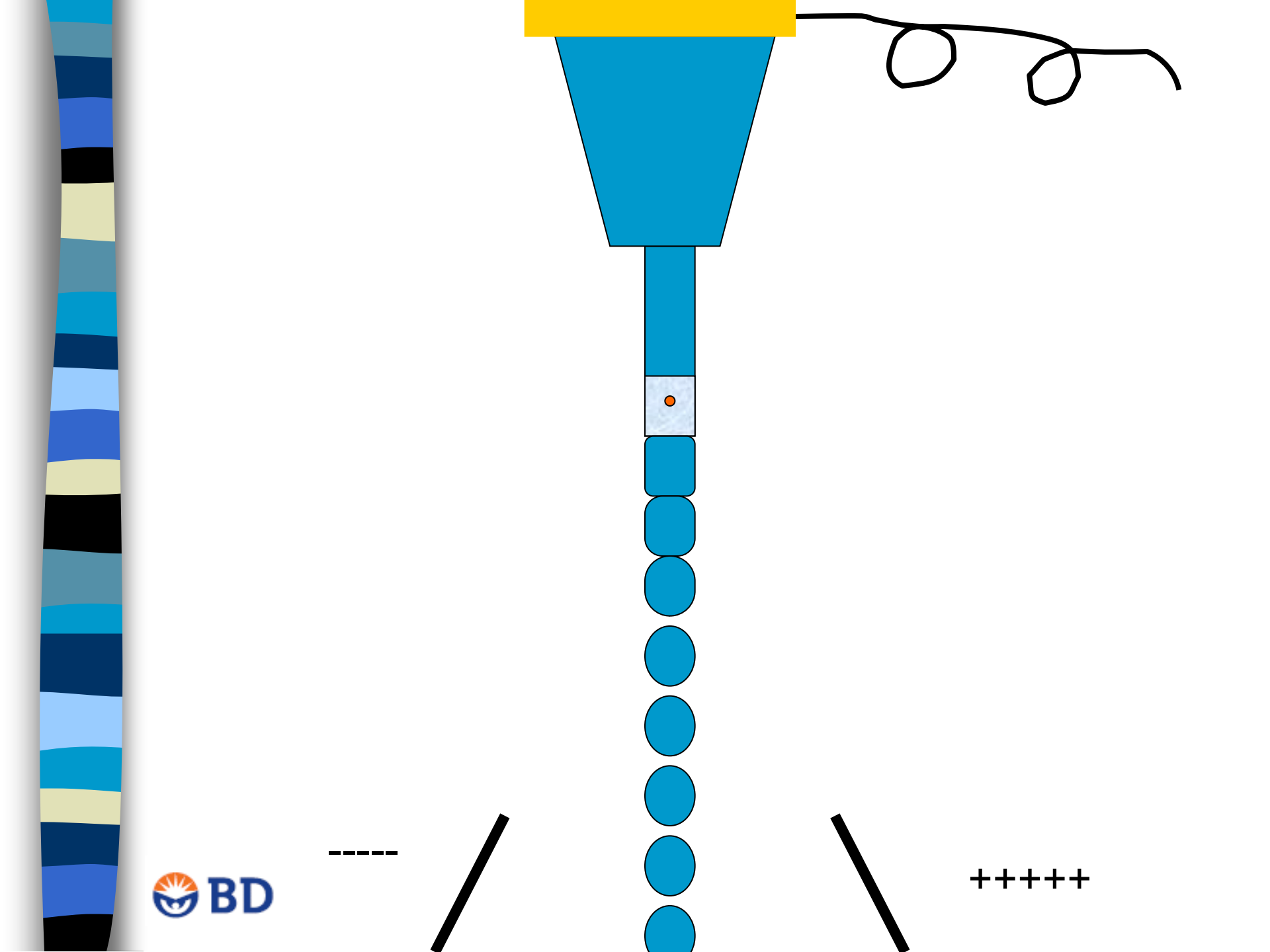
Used by:
Becton Dickinson
Beckman Coulter
Cytomation

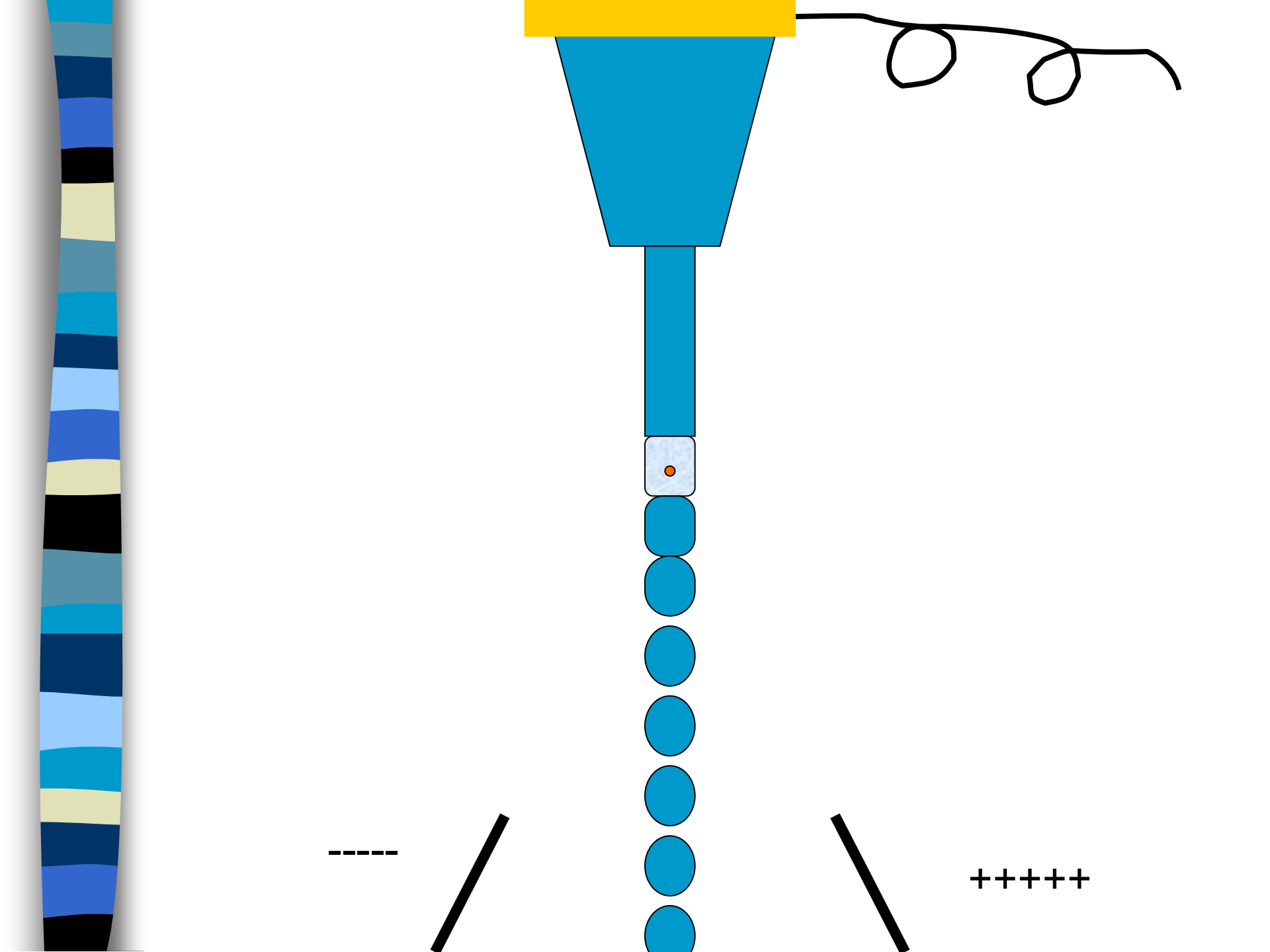


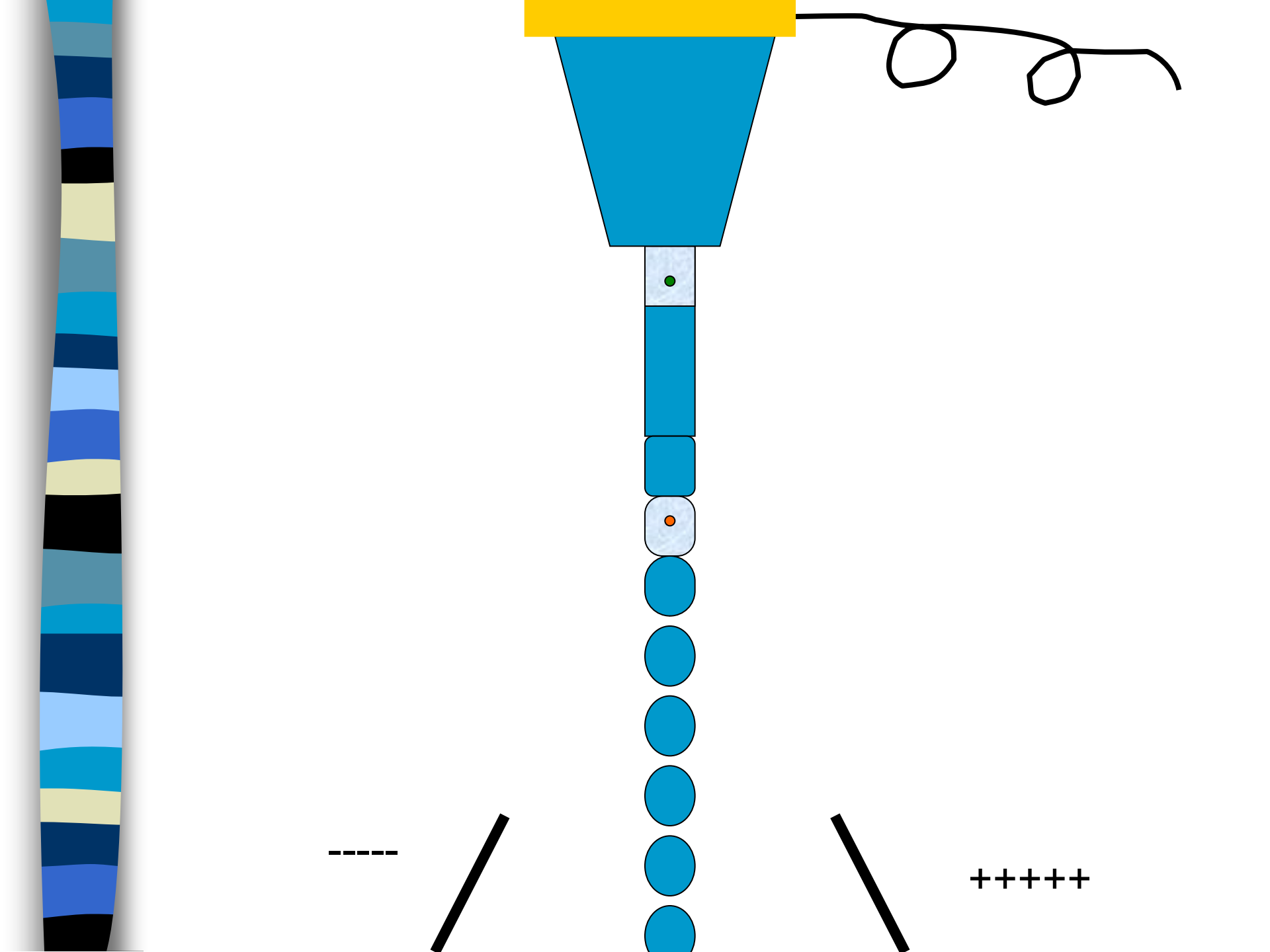
Doležel (1999)

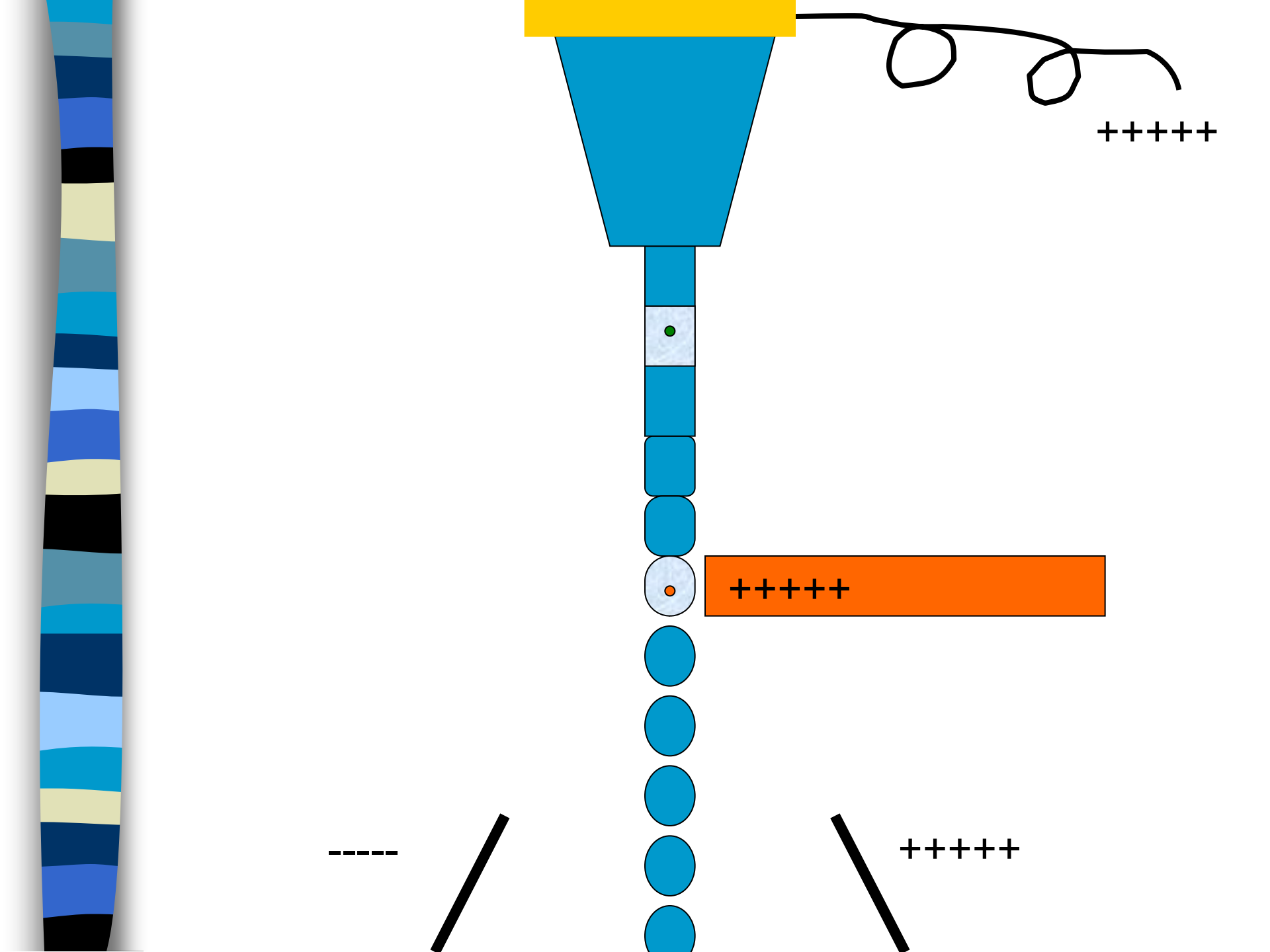
SORTING

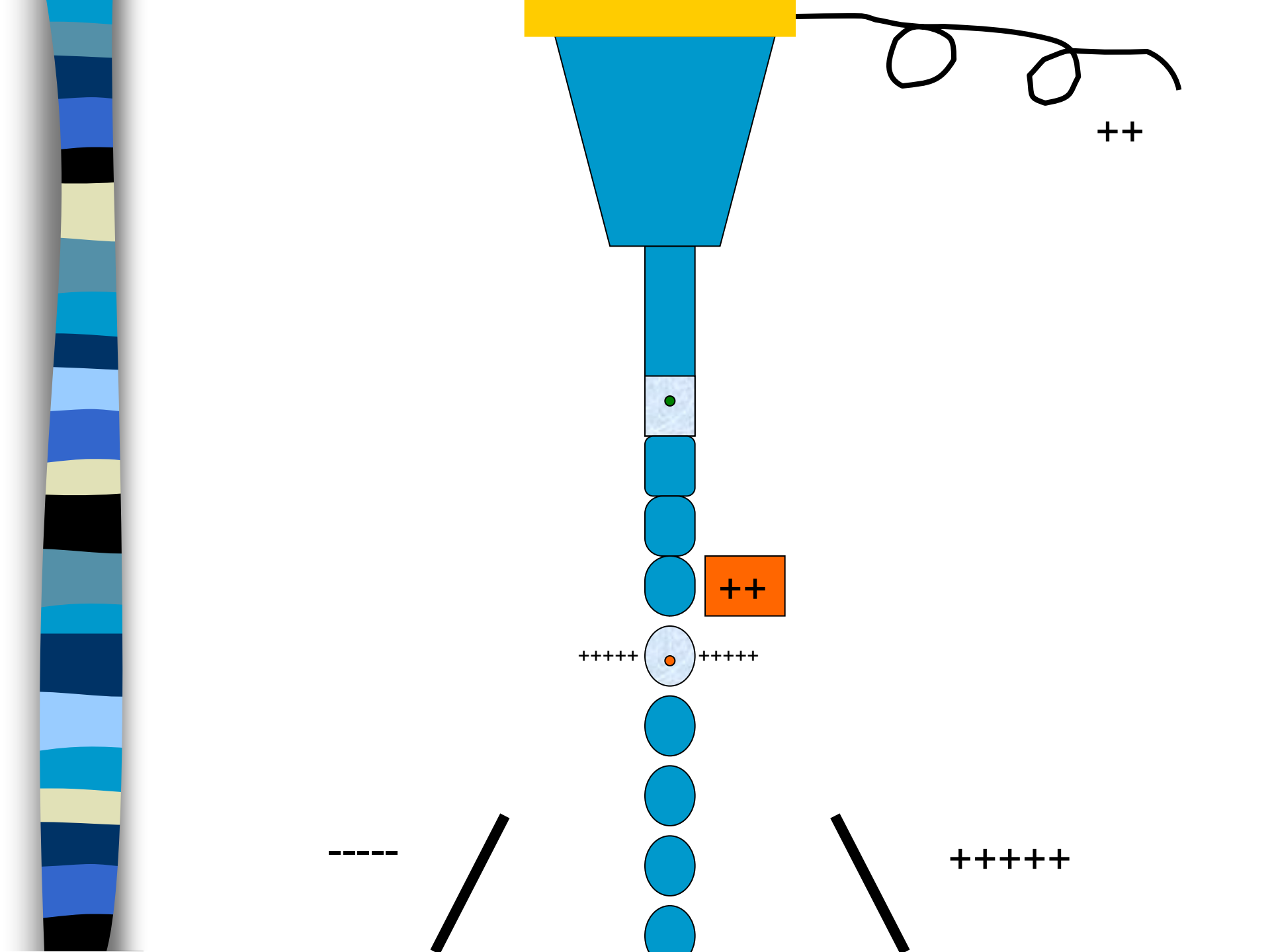


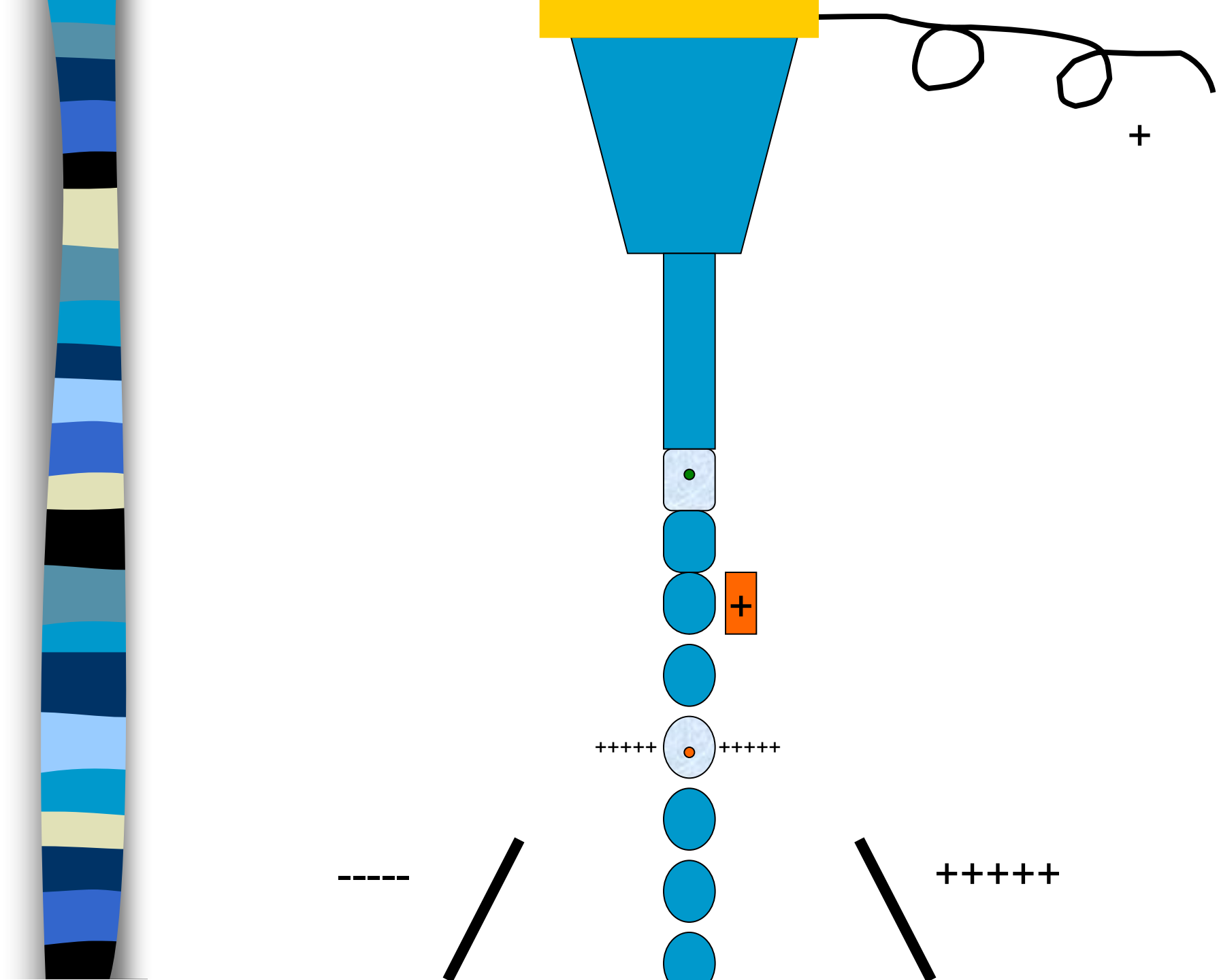


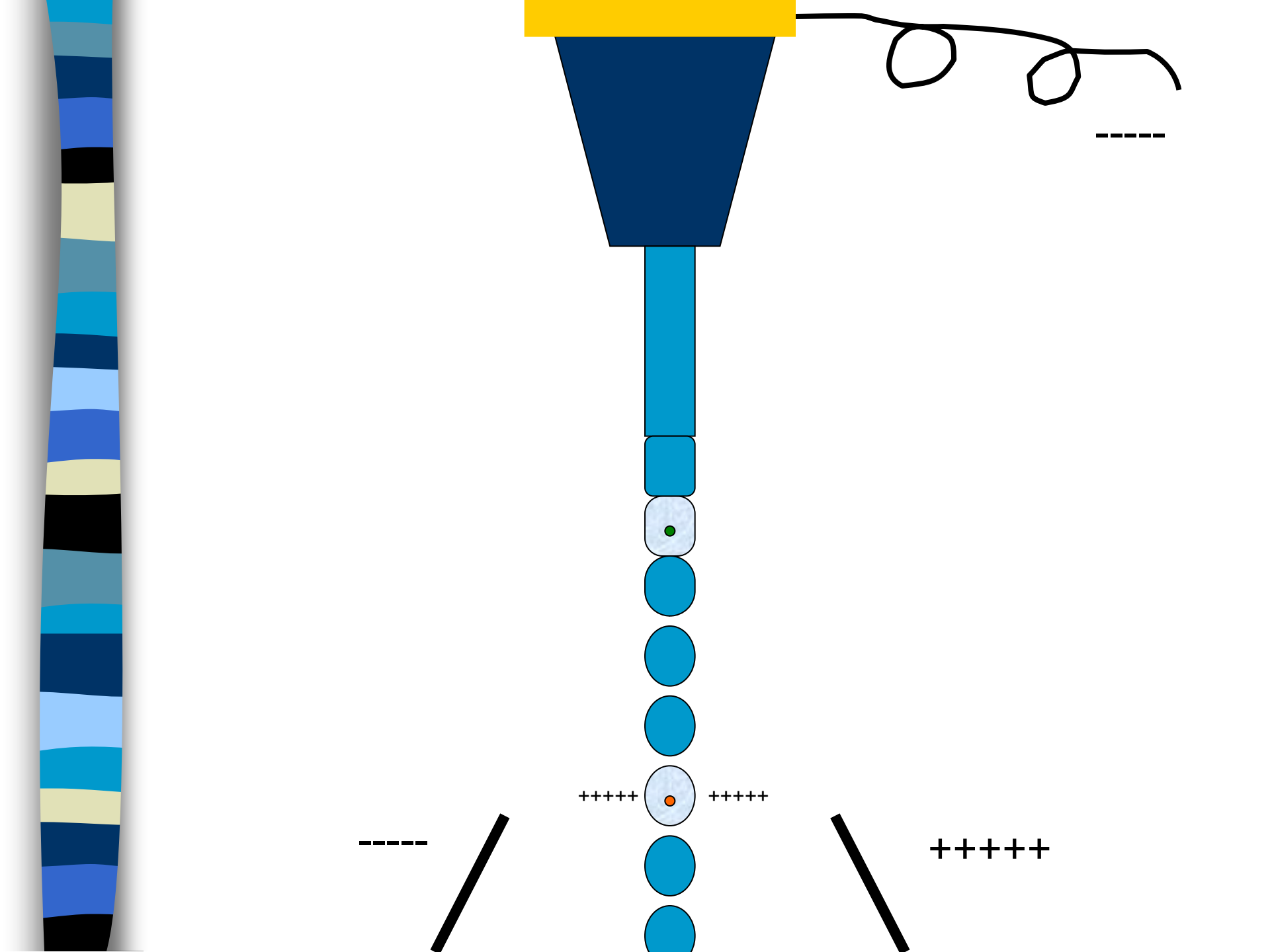


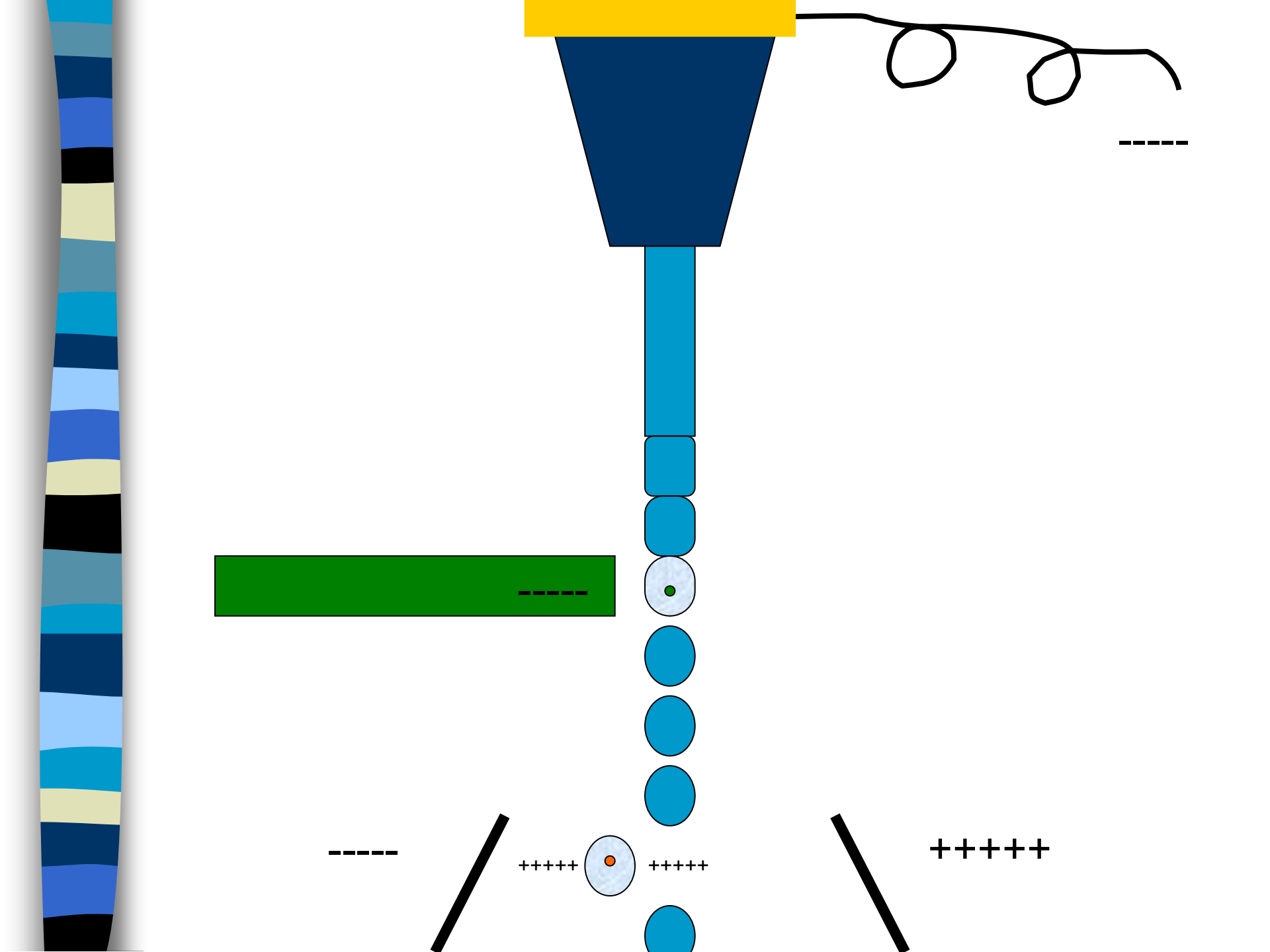


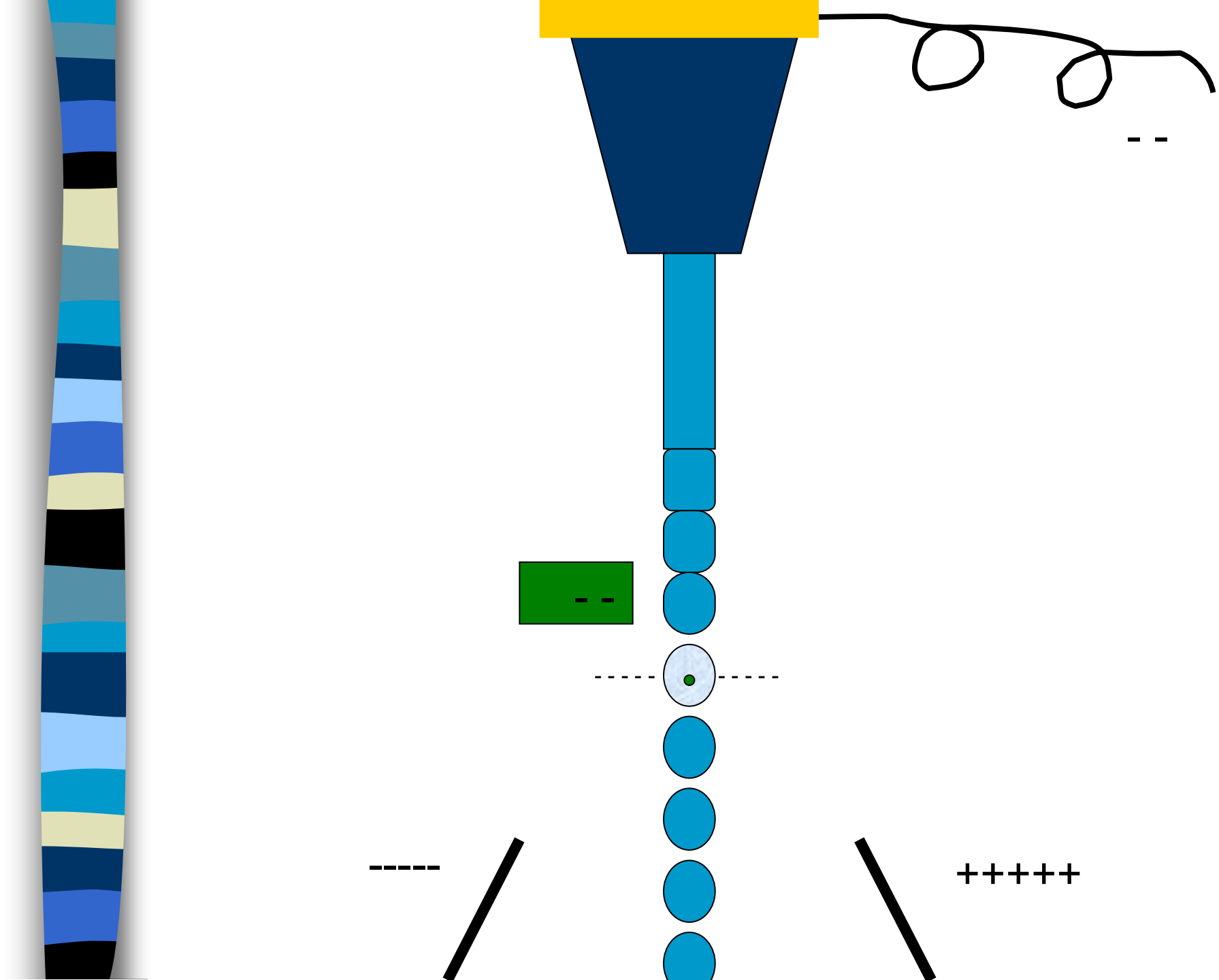


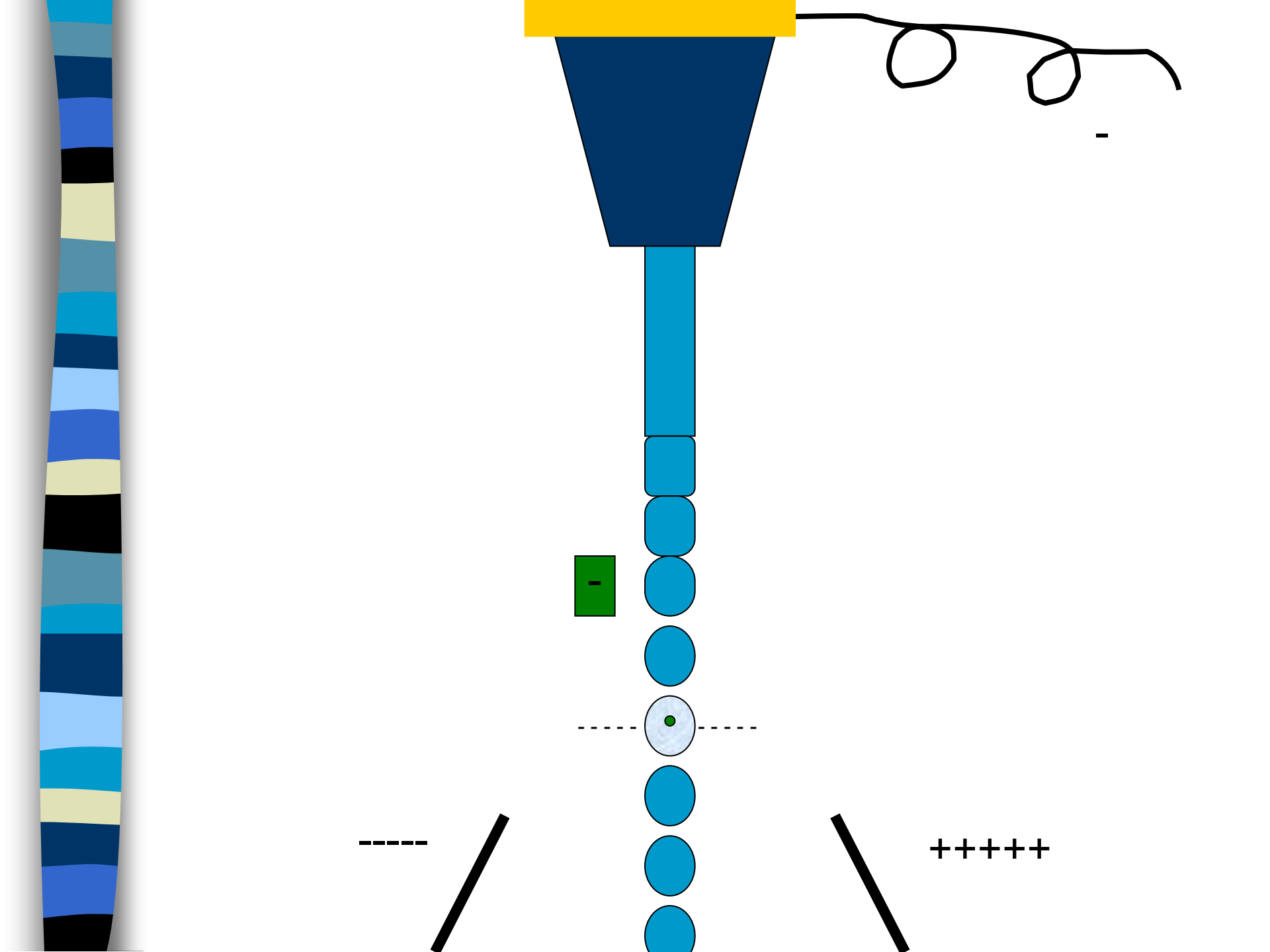


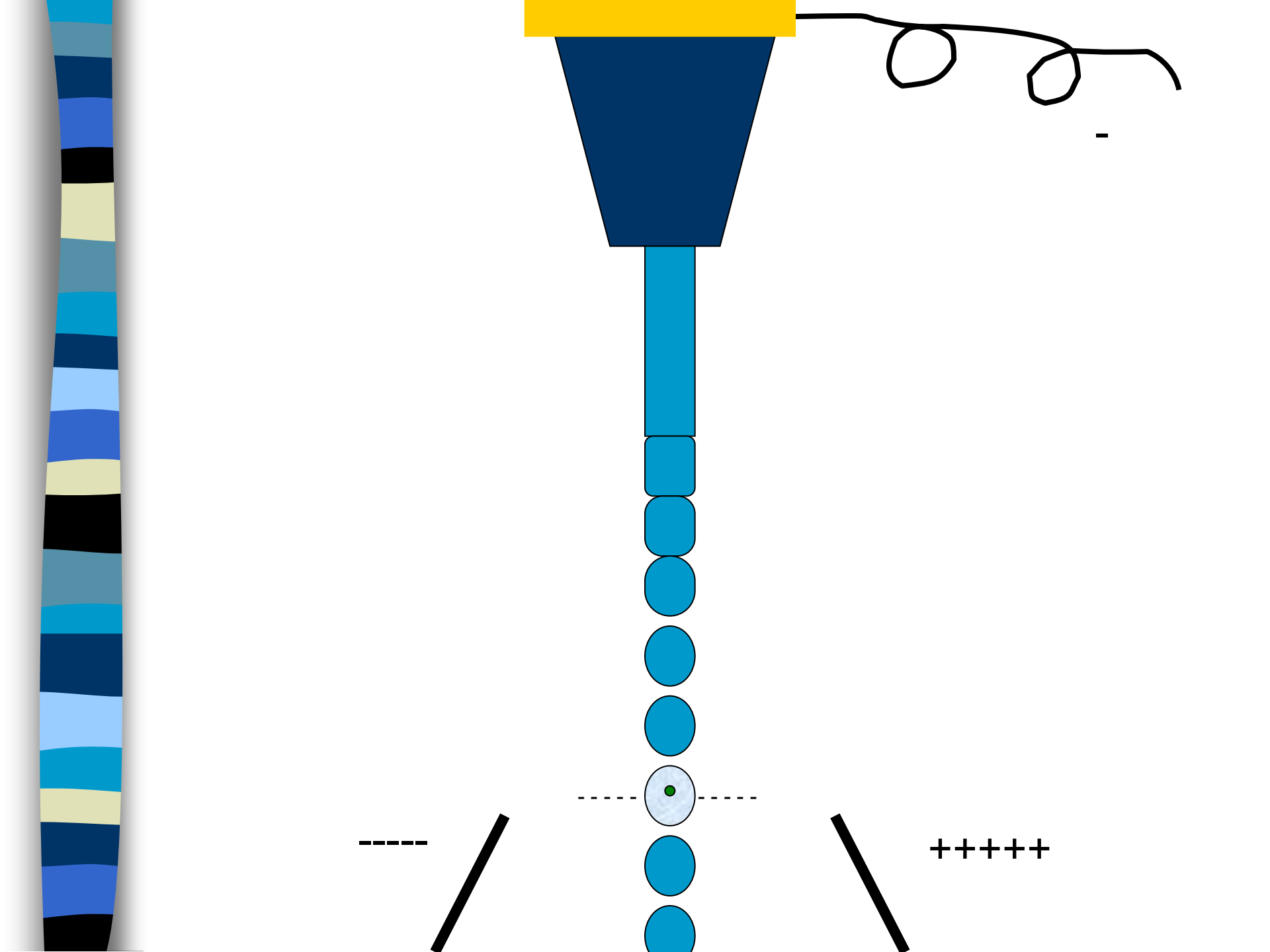


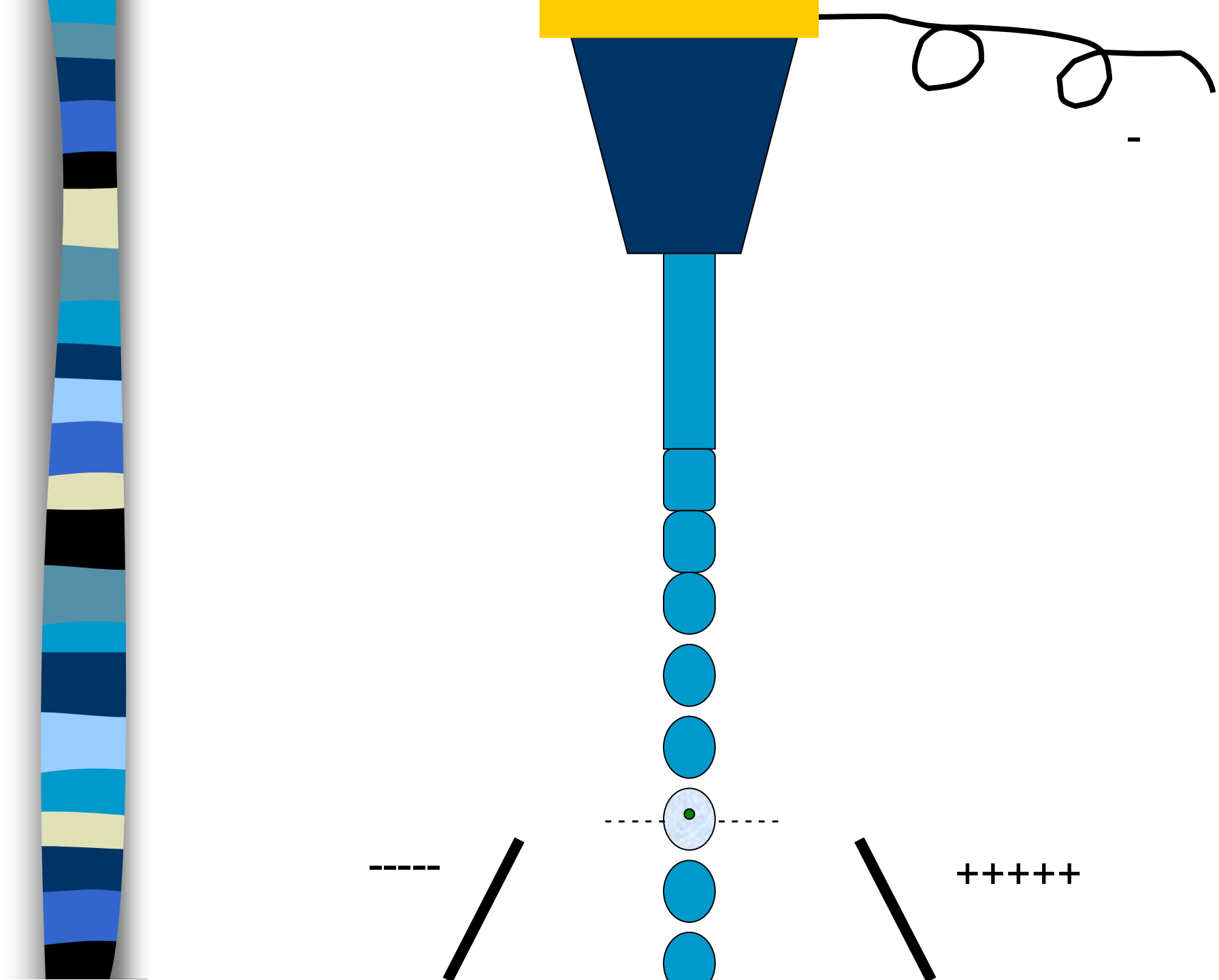


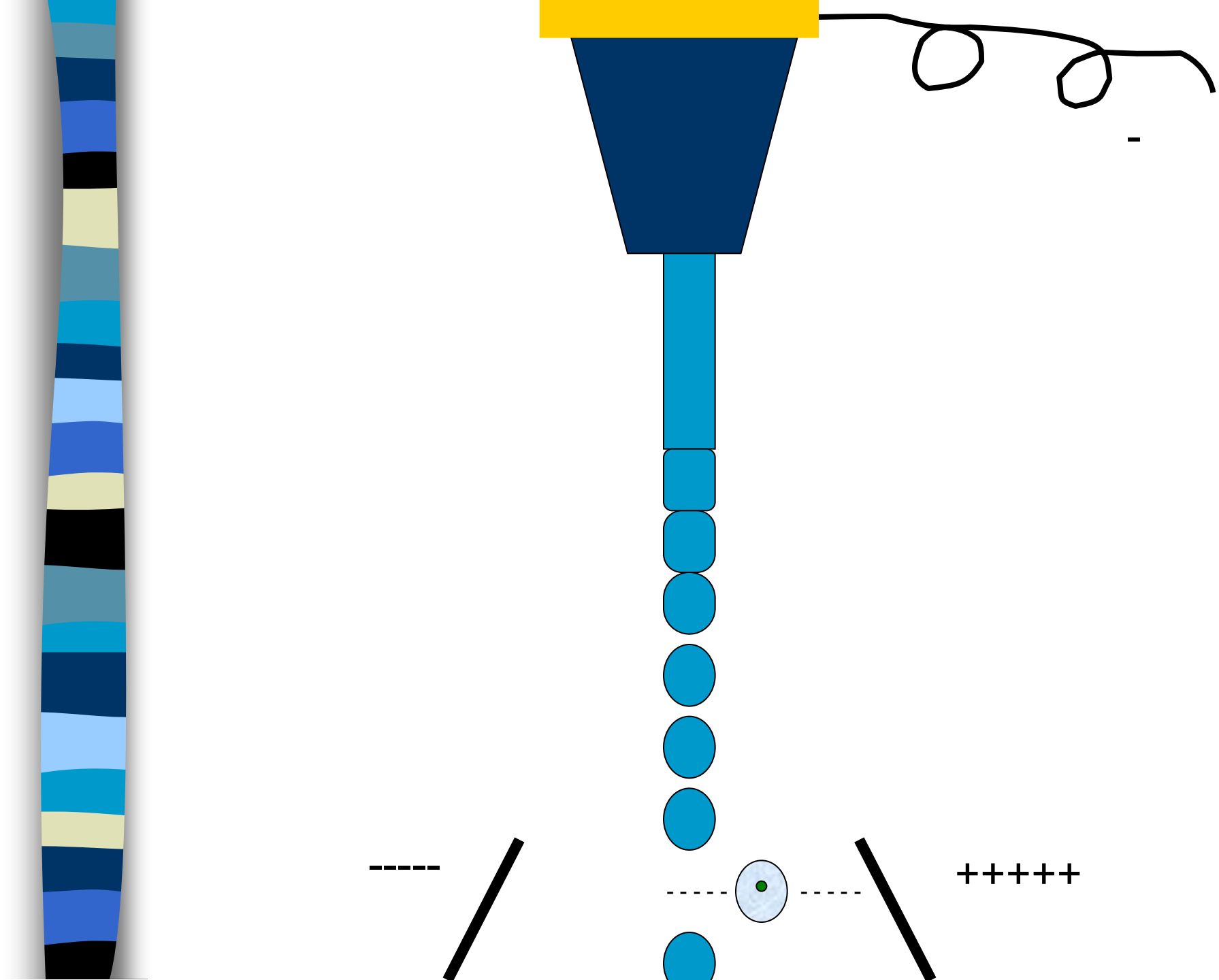








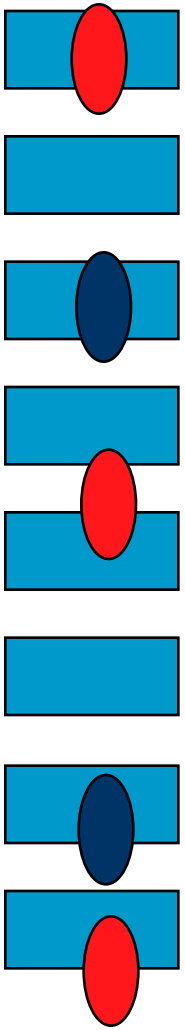




ISAC presents: Mack Fulwyler - Innovator, Inventor & Pioneer

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

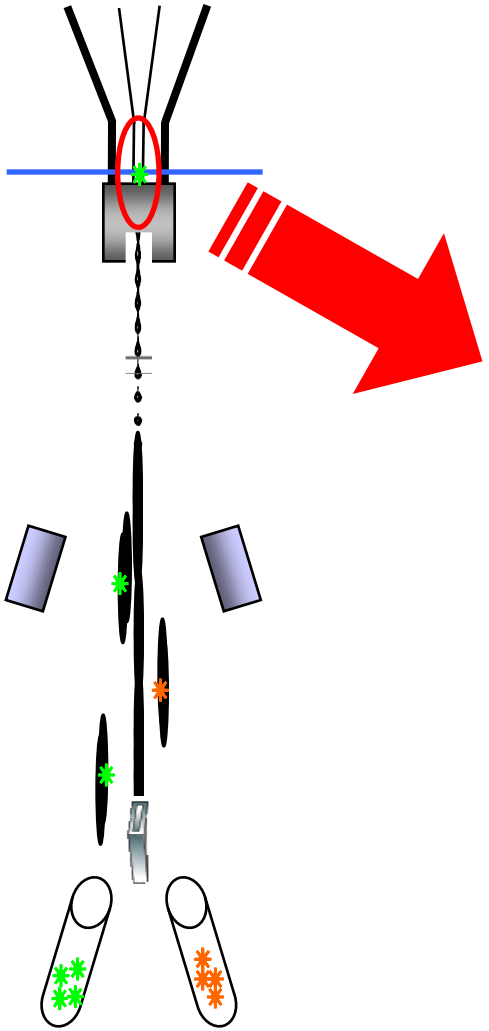
Sorting - Sort Masks



Cells are randomized distributed over the stream

BD

Sorting - Sort Masks



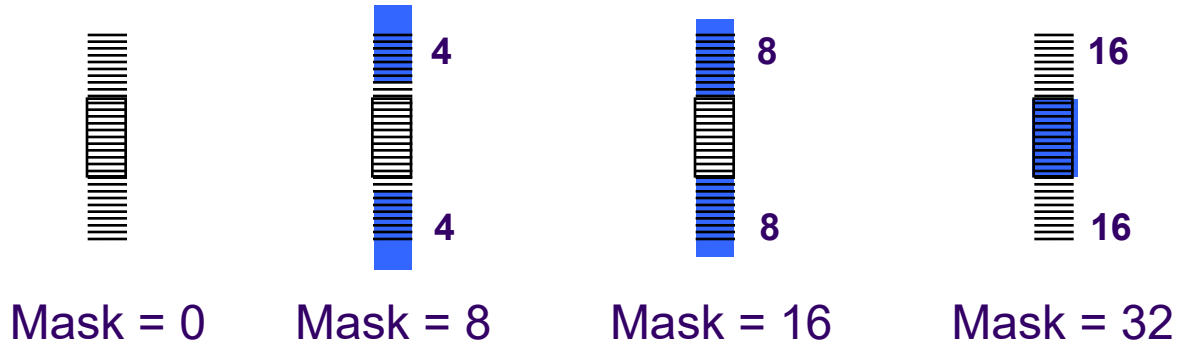
Trailing

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



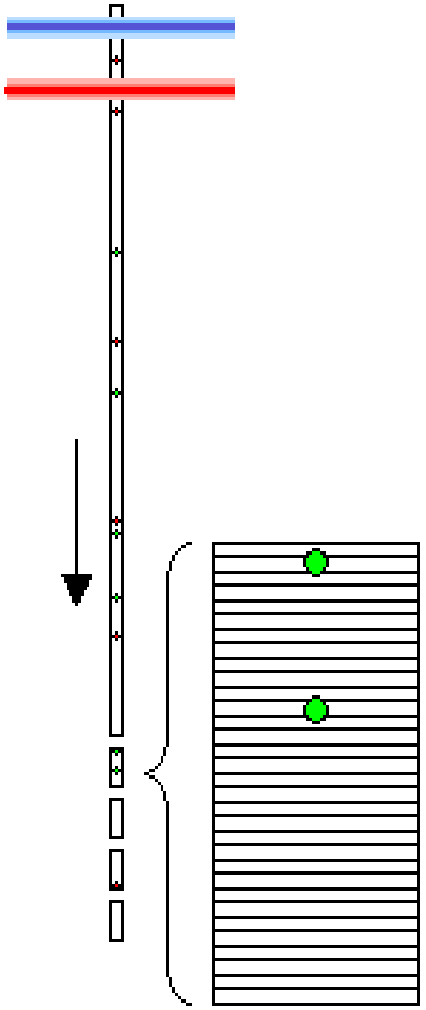
Conflict Resolution

- 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

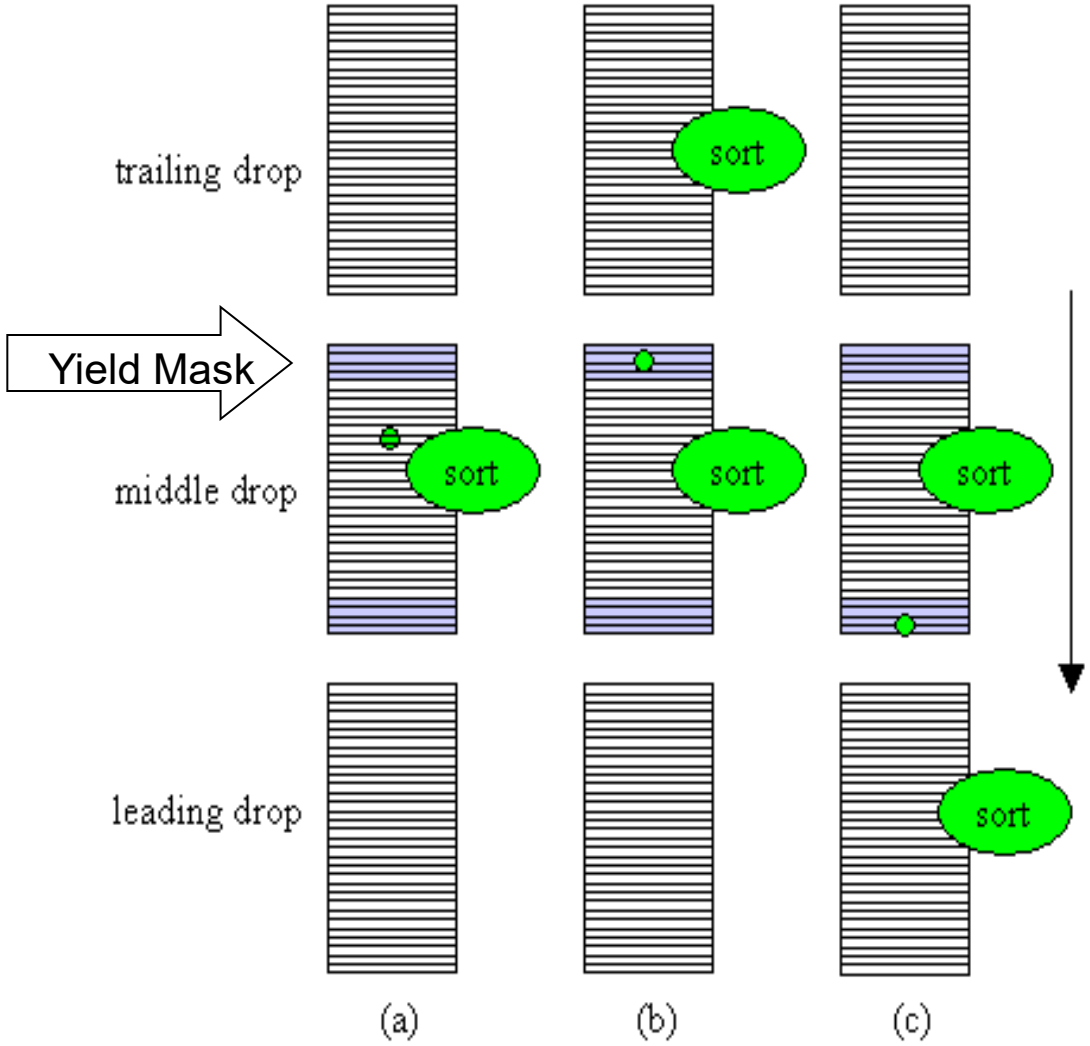
Sort decisions are determined by sort masks



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

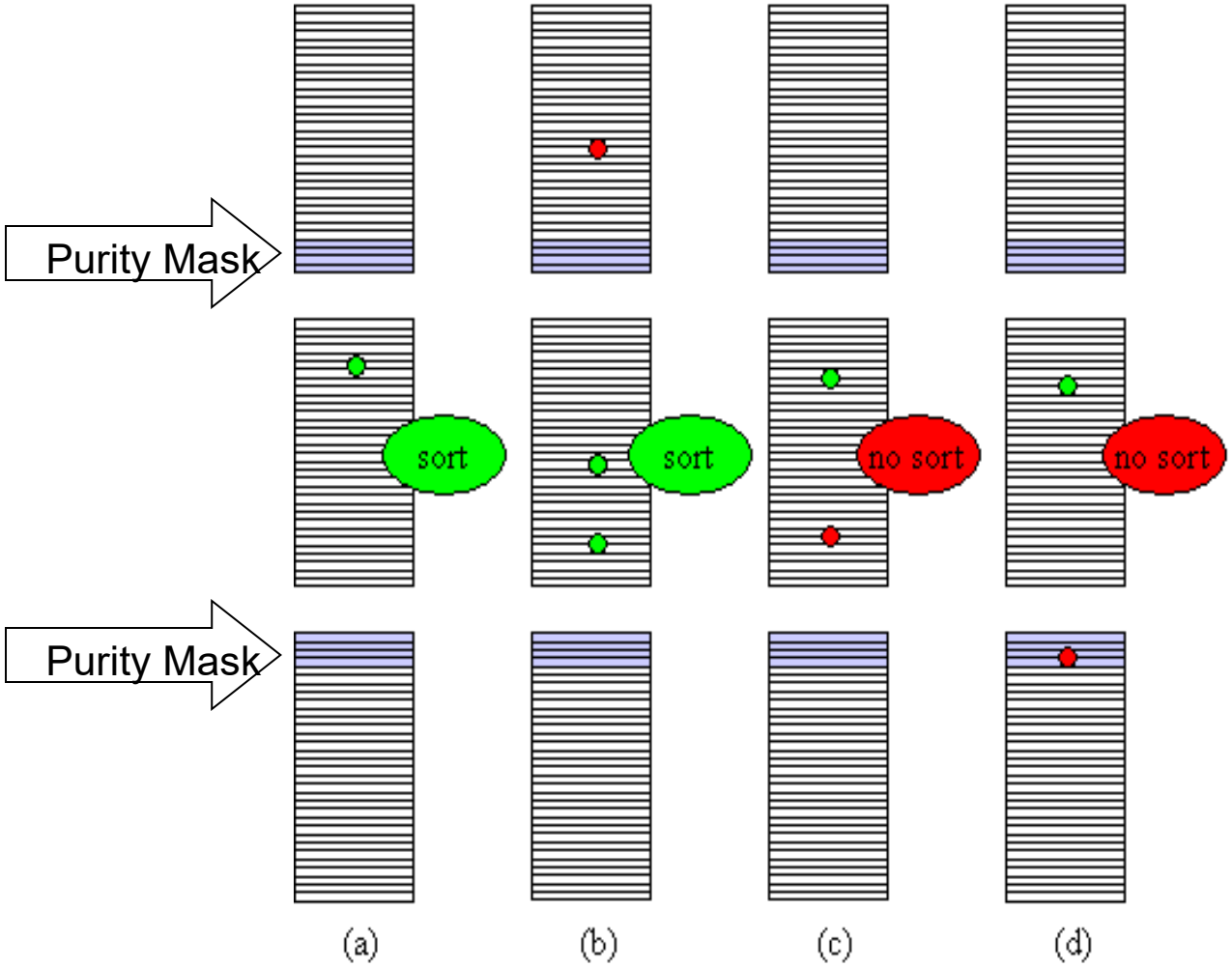
Sorting - Yield Mask

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

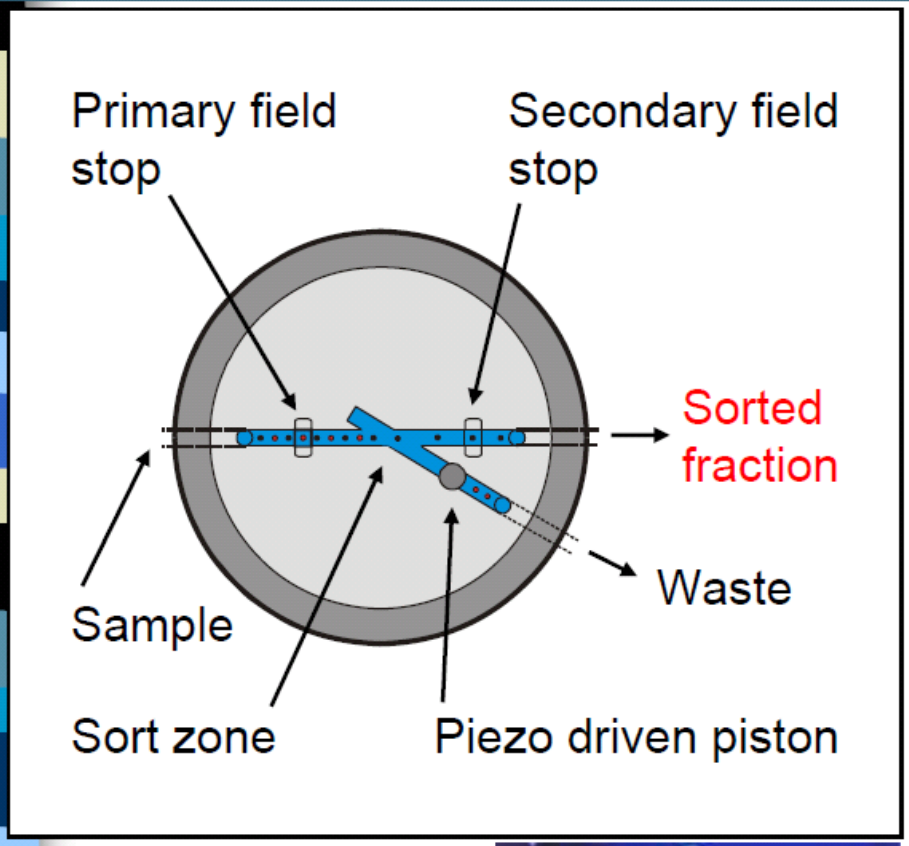


Sorting - Purity Mask

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



FLUIDIC SWITCH SORTER

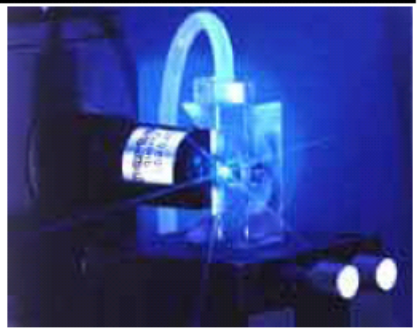


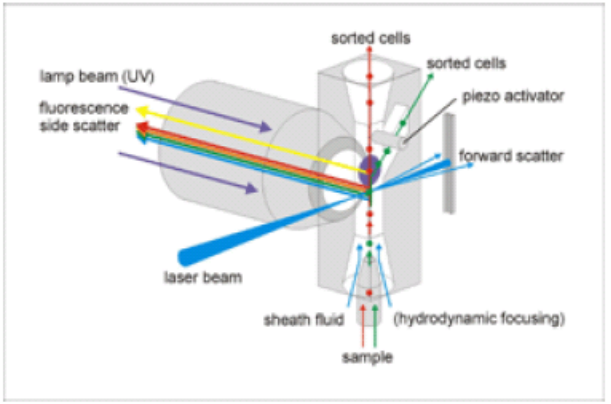
- **Safety (enclosed stream)**
- **Gentle to cells**
- **Sorting of large particles (>100 µm)**

Low speed (~100/sec)

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

Used by: Partec





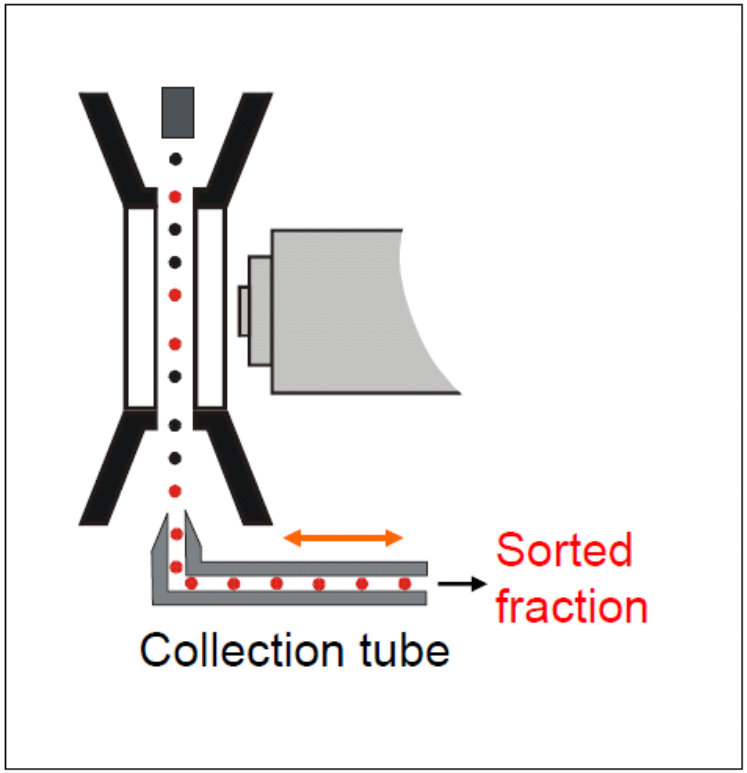
Doležel (1999)

FLUIDIC SWITCH SORTER

- Safety (enclosed stream)
- Gentle to cells

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

Used by: Becton Dickinson



Doležel (1999)

Why Choose the Cytek® Aurora CS?

So Many Colors

40 colors demonstrated including fluorochromes with emission spectra in close proximity to each other.

Exceptional Sensitivity and Resolution

Sensitivity redefined using state-of-the-art optics and low-noise electronics.

Extract autofluorescence and improve resolution of highly autofluorescent samples.

New Levels of Flexibility

No need to reconfigure optical filters for different fluorochromes.

Use any commercially available fluorochrome excited by the onboard lasers.

Choose from a variety of sample input and collection devices including 5 and 15 mL tubes for input and 96-well plates, 1.5 and 5 mL tubes for collection.

Seamless Sorting Experience

Automated drop delay, sort monitoring, and clog detection for a reliable sorting experience.

Comprehensive sort reports automatically record settings used from every sort.

Assay transferability from the Cytek Aurora system or conventional flow cytometers.

Predefined and Custom Sort Modes

Select one of Cytek's predefined sort modes or create a custom defined sort mode to meet the needs of each user's sorting application.

Purity

Isolate the population of interest with little to no contaminants from other populations

Enrich

Prioritize retrieving a high number of the target population with reduced sort purity

Multiway

Intended for 4- or 6-way sorting for efficient drop deflection

Single Cell

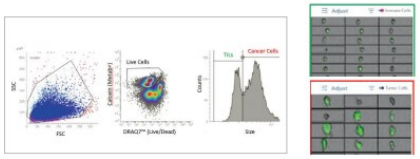
Isolate single cells into 96-well plates

Mixed

A combination of Purity and Enrich modes

Custom

Adjust the sort decision settings to meet your application needs



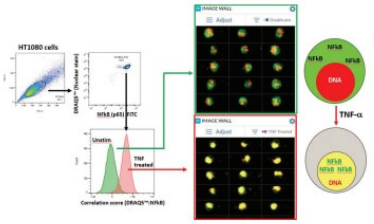
Label-free identification of TILs and cancer cells based on size

Label-free sorting

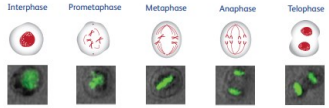
Minimize sample preparation and sort precious, sensitive and transiently expressing cells using image-enabled FSC, SSC and light loss detectors to enable accurate cell characterization without fluorescent antibody labeling.

Fluorescent localization

Reveal the spatial context of fluorescent signals hidden in flow cytometry. Track the subcellular movement of a protein across organelle boundaries within the cell, such as the NFκB translocation from the cytoplasm to the nucleus.



Configurations				
Number of spectral lasers	3	4	4	5
Number of fluorescent detectors	44	56	66	78
Total detectors	52	64	74	86
Lasers				
Ultraviolet laser (349 nm)			●	●
Violet laser (405 nm)	●	●	●	●
Blue laser (488 nm)	●	●	●	●
Yellow-green laser (561 nm)		●		●
Red laser (638 nm)	●	●	●	●

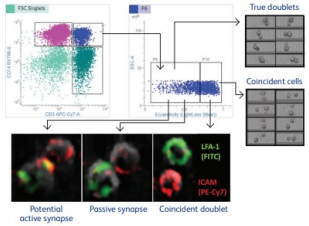


Cell cycle analysis

Flow cytometry methods only rely on a single indicator of DNA content for cell cycle classification, which is incomplete. Image feature analysis can provide insight into DNA distribution information to differentiate the phases of the cell cycle.

Cell-cell interaction

Reveal the spatial context of cells using image feature analysis to identify combinations of engaged cells. Distinguish between two cells that are coincident (passed through the interrogation point in close proximity) and true doublets (cells that are actually touching each other). Further image analysis can reveal receptor accumulation at the site of the cell-cell synapse (active synapse).



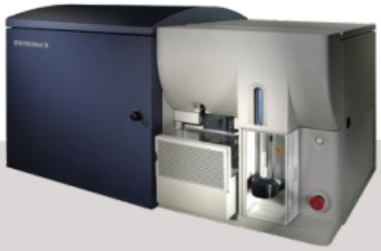
Cell sorting - trends

- Easy operation
- Careful handling
  - On-chip technology
- Size ↓ and security ↑
- Microfluidic-based cell sorting
- Spectral cell sorting
- Image-based sorting

- Buoyancy Activated Cell Sorting (BACS™)
  - a method that uses low-density particles (microbubbles) for flotation separation.

Electronics and data

File size considerations



FCS data
- Saved for selected channels only
- H/W optional
- 10,000 events → ~2 MB for an 8-color experiment

Data management is vitally important for spectral and imaging cytometers

FCS data
- Saved for all channels
- A/H/W/TTP always saved
- 10,000 events → ~17 MB for **any** experiment

Image files
- All imaging features saved for selected imaging channels
- Larger cells will generate larger image files
- 10,000 events → ~1.5 GB for a PBMC experimen

Properties:150717_DU145 Ctrl.fcs

Help

OK

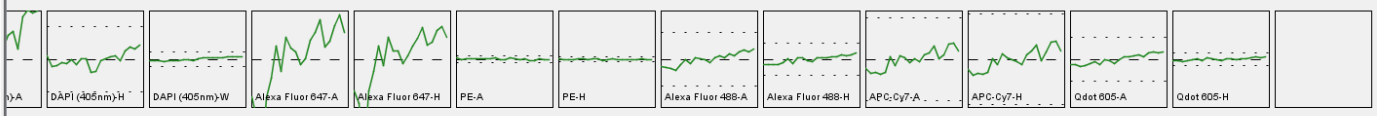
Date:17-JUL-2015
System:Windows XP 5.1
Cytometer:FACSAriaII SORP (FACSAriaII)
File:150717_DU145 Ctrl.fcs
File URI:file:/C:/Users/user/Desktop/install/Infinicyt/150717_DU145%20Ctrl.fcs
---
$BEGINANALYSIS: 0
$BEGINDATA: 4148
$BEGINSTEXT: 0
$BTIM: 13:25:01
$BYTEORD: 4,3,2,1
$CYT: FACSAriaII SORP (FACSAriaII)
$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

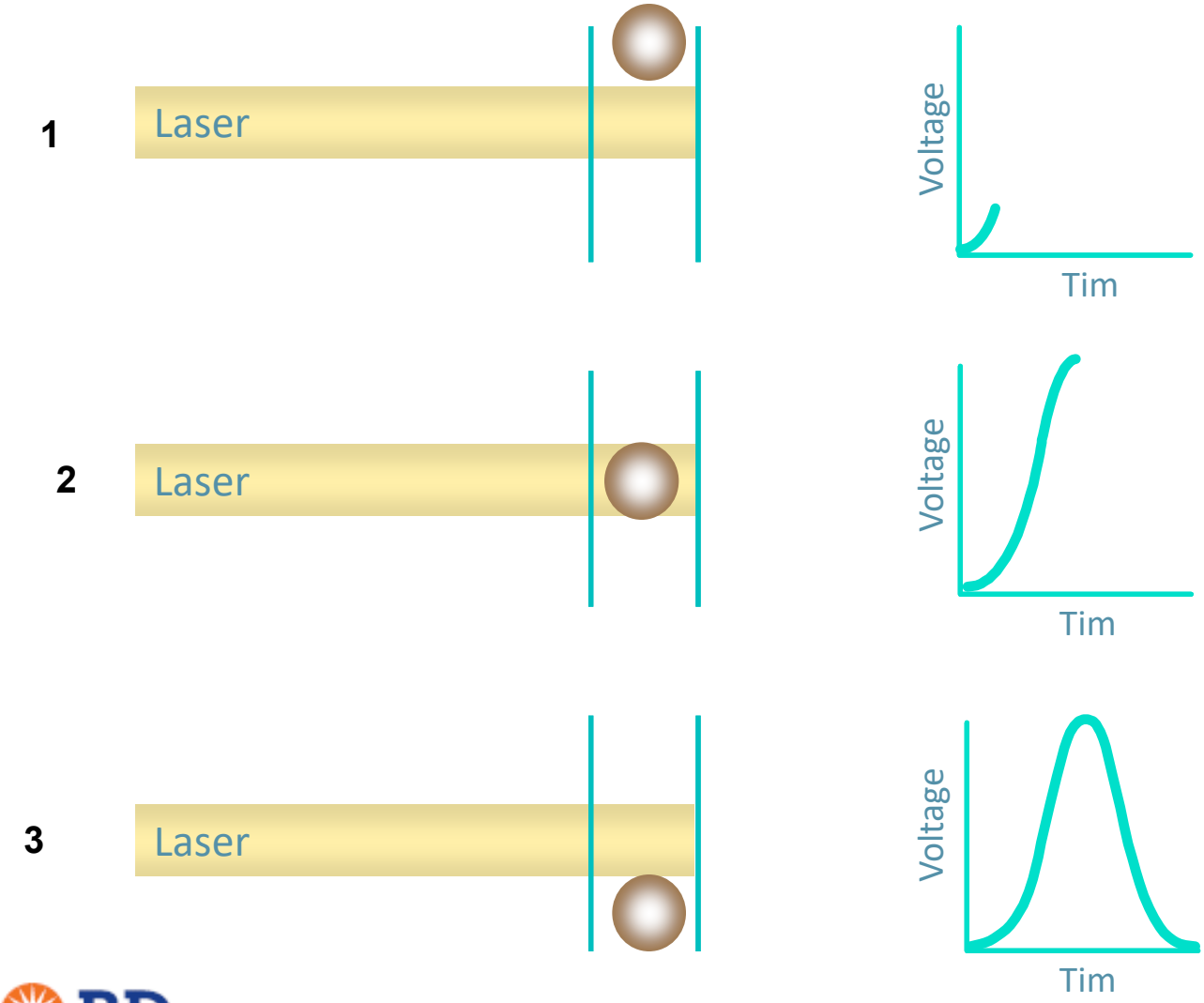
Parameter ($PnN)	Stain ($PnS)	Range ($PnR)	Bits ($PnB)	Decades ($PnE)	Gain ($PnG)	Voltage ($PnV)	Derived From
FSC-A		262144	32	0,0	1.0	280	
FSC-H		262144	32	0,0	1.0	280	
SSC-A		262144	32	0,0	1.0	210	
Alexa Fluor 594-A		262144	32	0,0	1.0	460	
Alexa Fluor 594-H		262144	32	0,0	1.0	460	
DAPI (405nm)-A		262144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

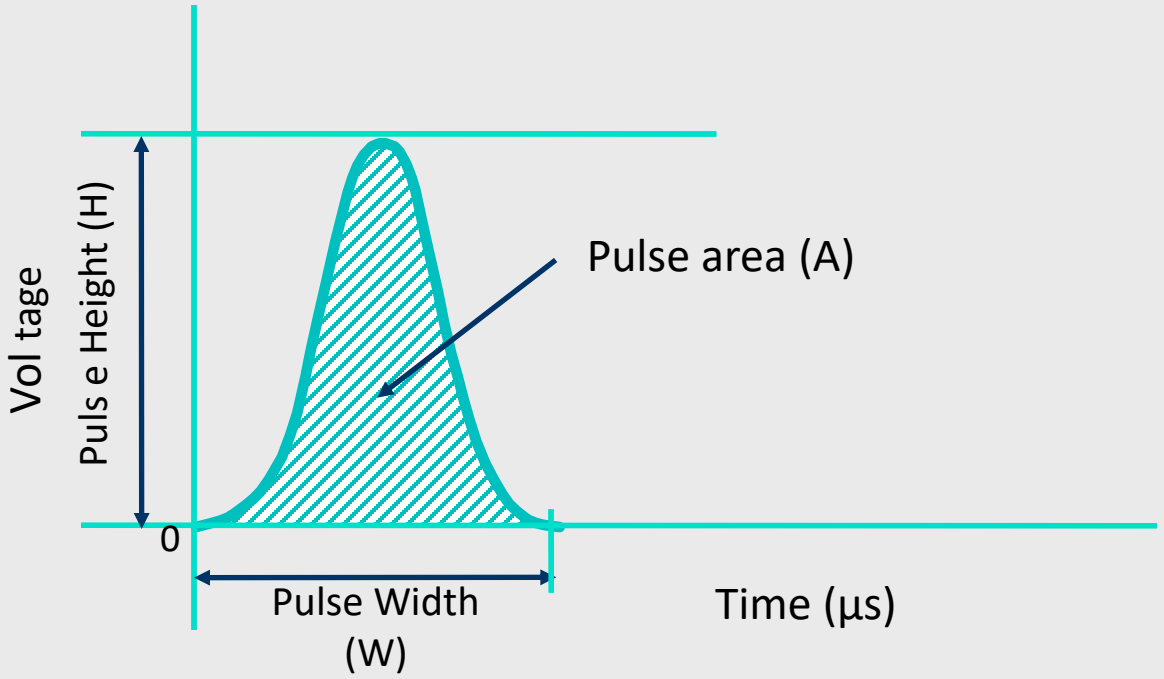
Event	*Param1 FS*	*Param2 SS*	*Param3 FITC*	*Param4 PE*
1	50	100	80	90
2	55	110	150	95
3	110	60	80	30

Creation of a Voltage Pulse

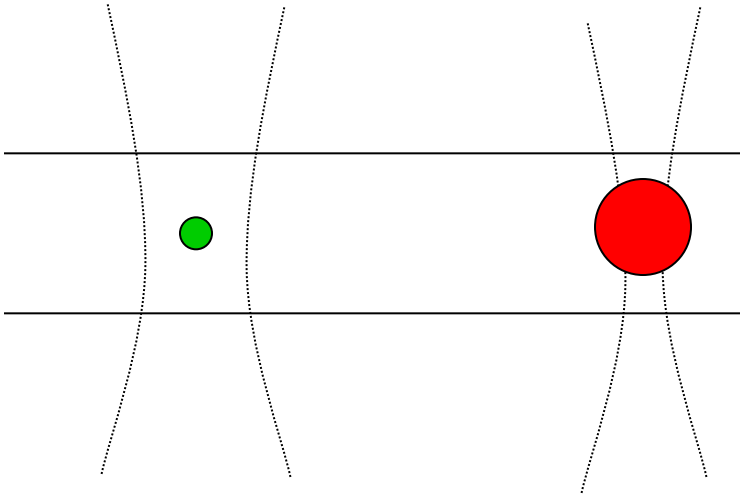


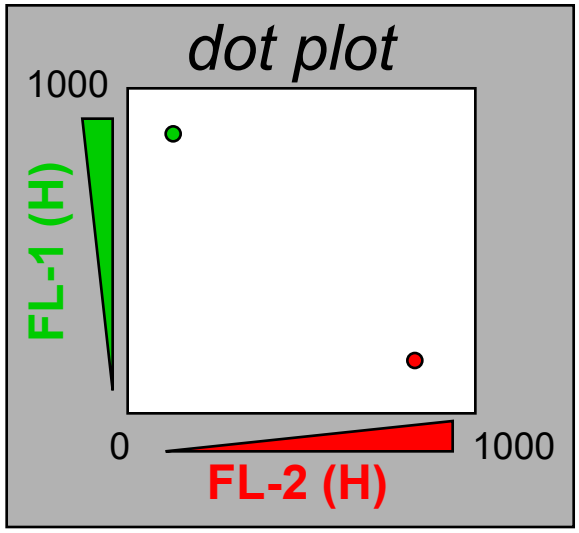
BD

Height, Area, and Width

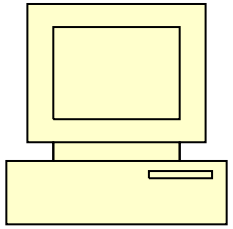


Signal processing

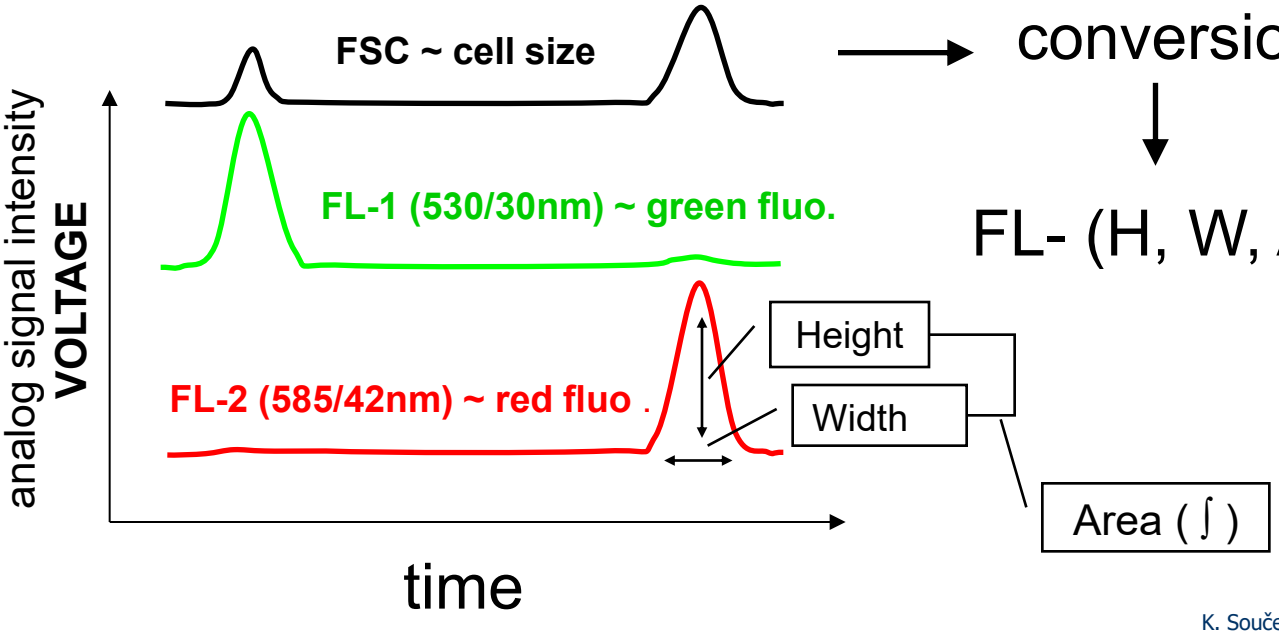




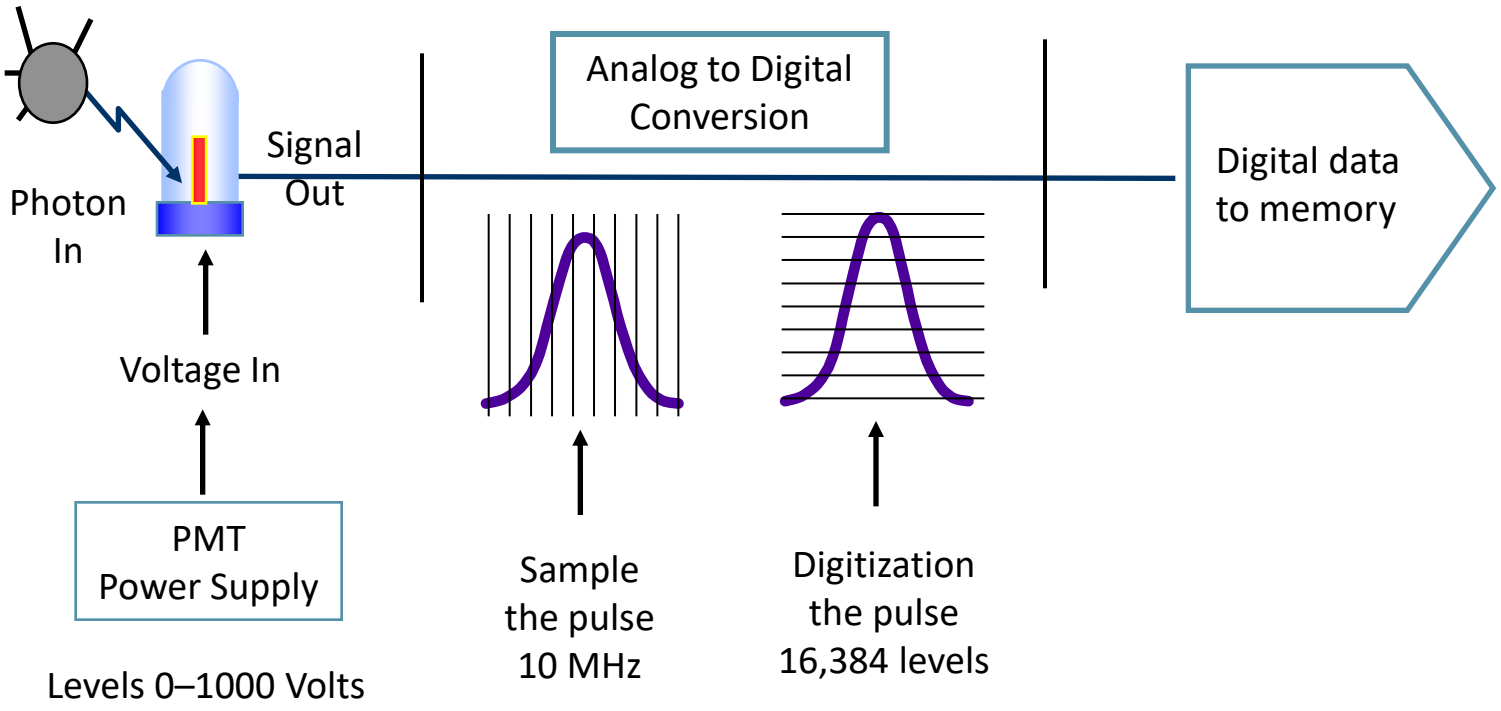
Analog/digital conversion



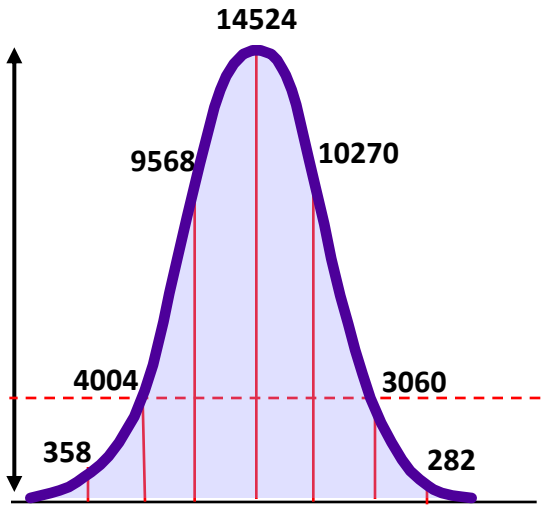
FL- (H, W, A)



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 a 262,144 scale

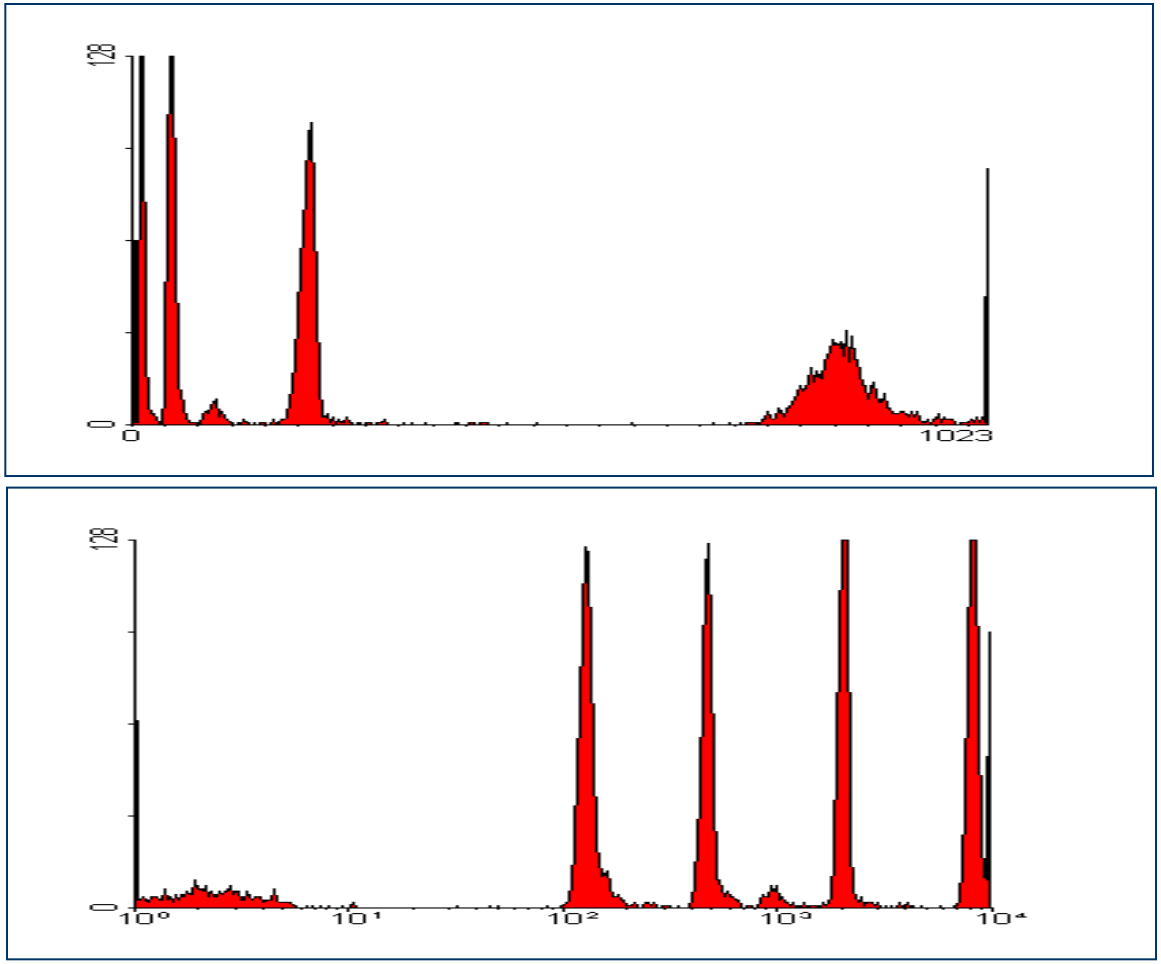
$2^{8} = 256$
$2^{10} = 1024$
.
.
.

AD converters

Number of bits	# channels	distinction
8	256	39.1 mV
10	**1024**	**9.77 mV**
12	4096	2.44 mV
14	16384	610 μ asl
16	65536	153 μ E
18	**262144**	**38.1 μ E**
20	1048576	9.54 μ V
22	4194304	2.38 μ H
24	16777216	596 AD

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

Logarithmic gain & dynamic range

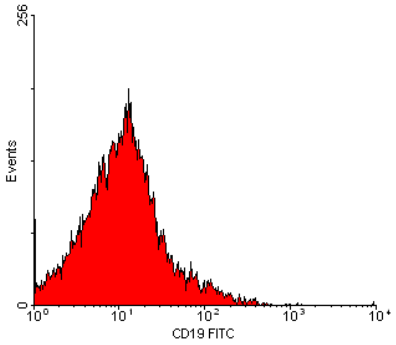


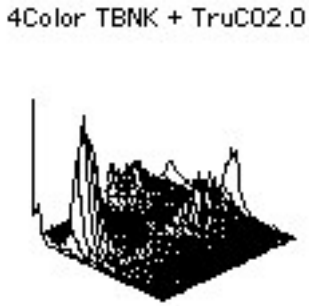
adapted from JPRobinson

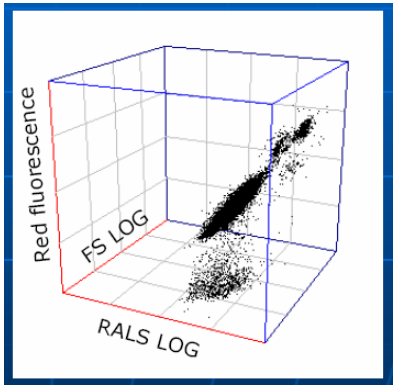
Data analysis

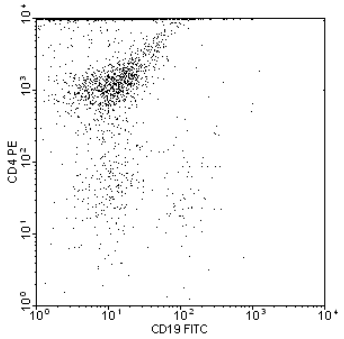
- View data
  - histogram
  - dot plot
  - isometric display
  - contour plot
  - chromatic (color) plots
  - 3D projection
- Gating

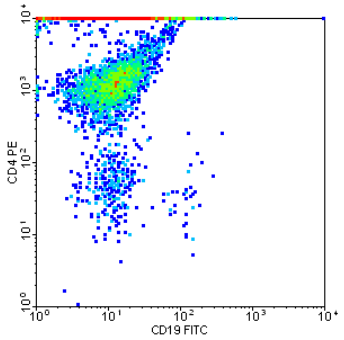
Basic ways to display data

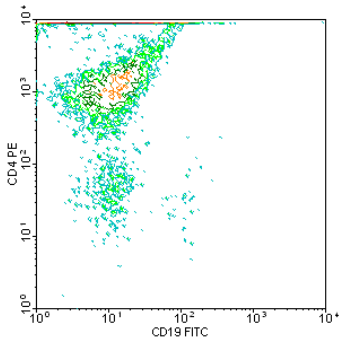


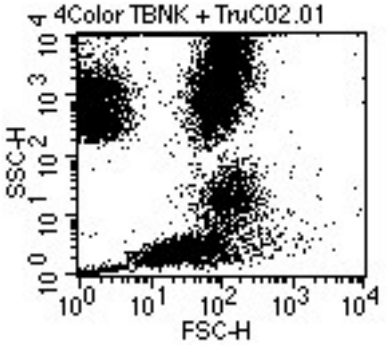


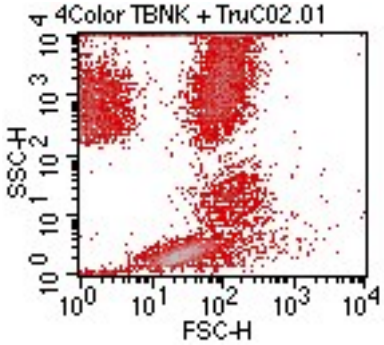


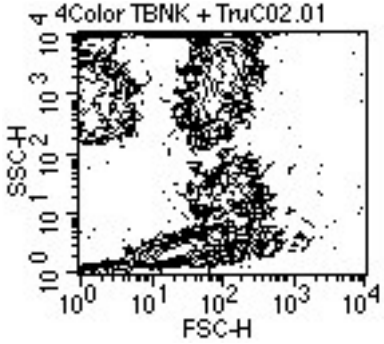












Summary

- Fluid systems
- Sorting
- Signal, data – basic principle

At the end of today's lecture you should :

1. Know the basic principles of light scattering
2. and fluorescence;
3. to know what light sources are used in flow cytometry;
4. and how it is detected;
5. know the basic principles of fluid systems and laminar flow.
6. Know the basic principle of data processing and visualization