Introduction to Flow Cytometry

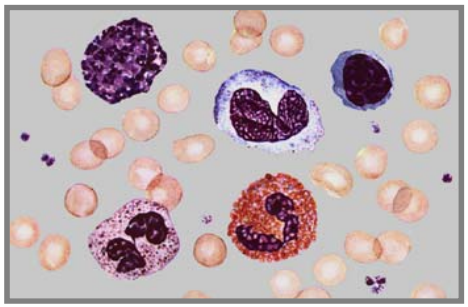
Overview

- Measurement of Cellular Parameters in Flow Cytometry
- The Optical System of Flow Cytometers
- Fluidics
- Electronics – Digital theory
- Sorting – An overview

Overview

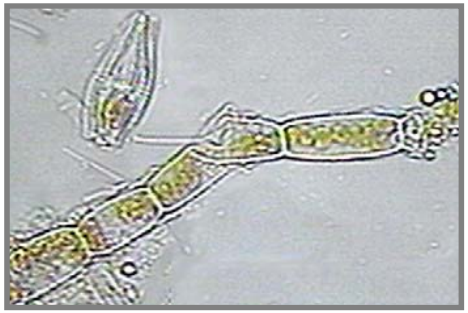
- Measurement of Cellular Parameters in Flow Cytometry
- The Optical System of Flow Cytometers
- Fluidics
- Electronics – Digital theory

Cellular Parameters:
These particles have something in common

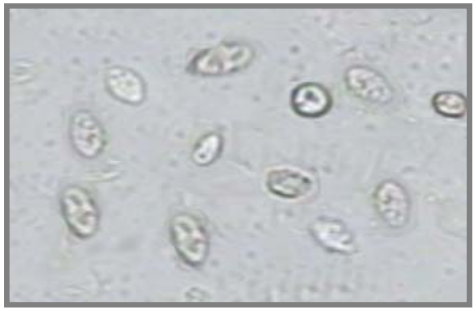


Blood cells

Chromosomes



Algae



Protozoa

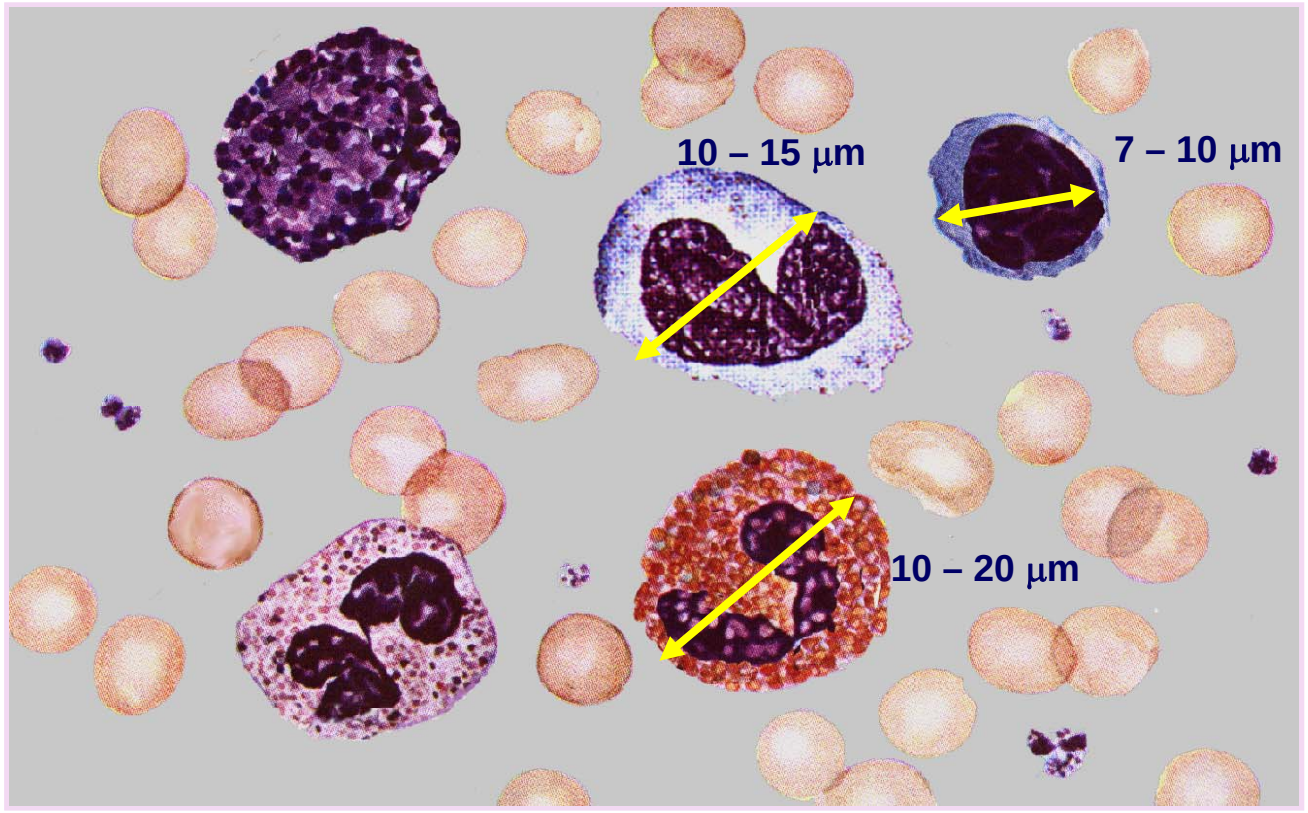
- Certain parameters of these particles can be measured with a flow cytometer

Cellular Parameters:
Relative Size and Complexity

Basophil Granulocyte

Erythrocytes

Eosinophil Granulocyte



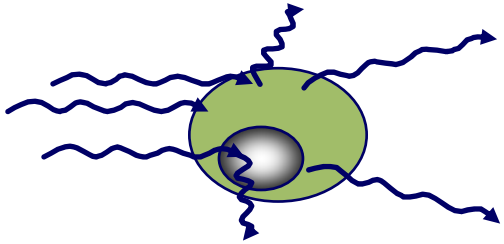
T cell

Monocyte

Neutrophil Granulocyte

- Morphological, cells are different in
  - Size
  - Complexity

Cellular Parameters:
Relative Size and Complexity



Forward scatter (FSC)

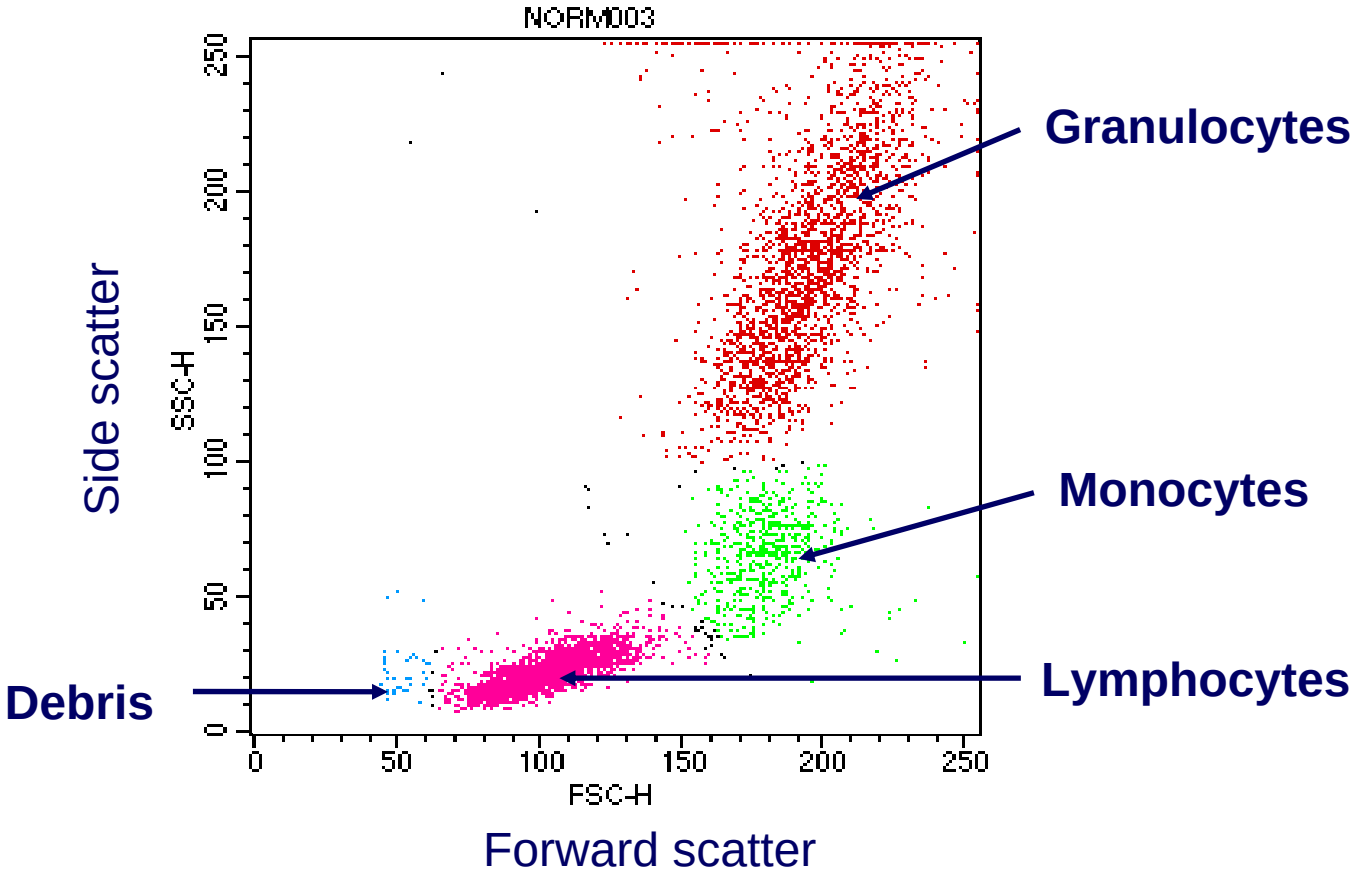
- measured along the axis of the incoming light
- proportional the the cell size / cell surface (only true for perfect round cells)

Side scatter (SSC)

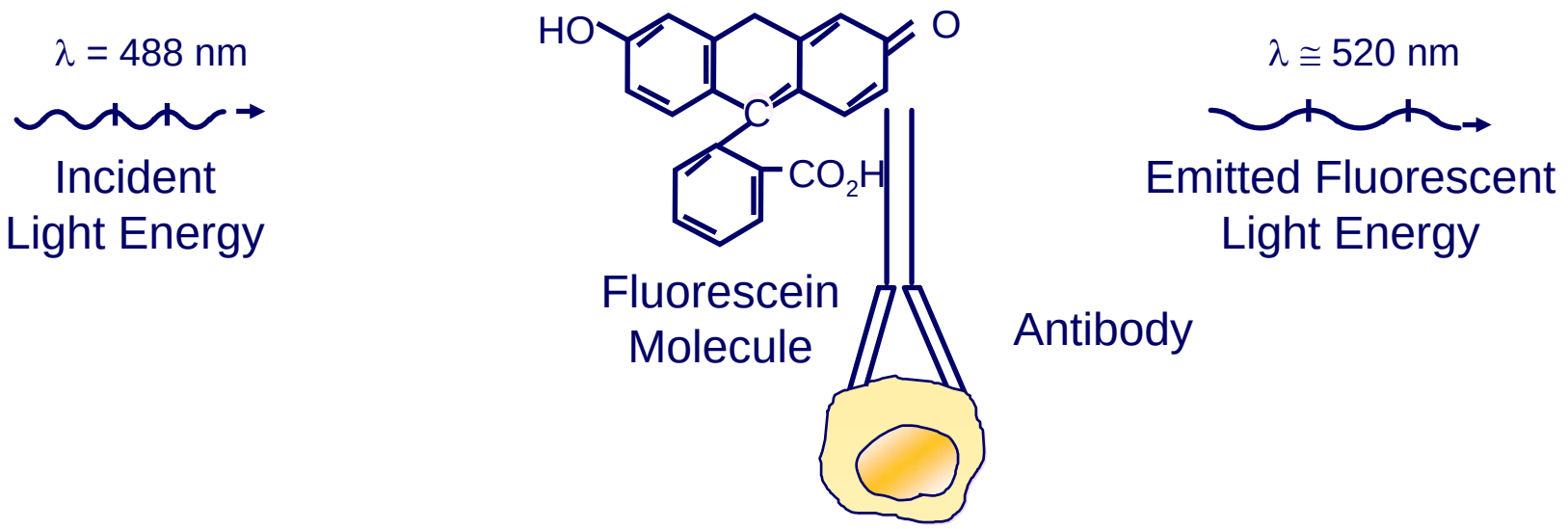
- measured in 90° direction to the excitation light
- proportional to cell „complexity“ or granularity

Cellular Parameters:

An example for light scattering in whole blood



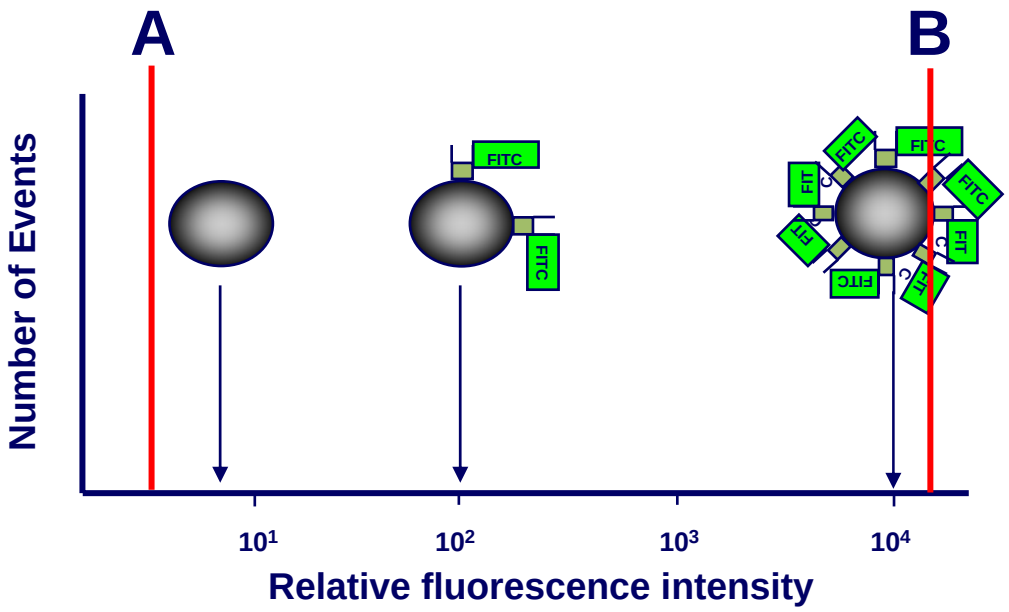
Cellular Parameters:
Fluorochrome Detection and Quantification



- The fluorochrome molecule absorbes the energy of the incoming light
- It releases the absorbed energy by:
  - vibration and dissipated heat
  - emission of a photon with a higher wavelength (= less energetic)

Cellular Parameters:
Fluorochrome Detection and Quantification

- Fluorescence-signals measured are proportional to numbers of fluorochromes bound to cells



Find „Min. Linearity Channel“ and „Max. Linearity Channel“ in the CS&T baseline report.

- IF these are within in the “Dynamic Range” of the detector
  - A) Minimal Linearity Channel
  - B) Maximal Linearity Channel

Cellular Parameters:
Fluorochrome Detection and Quantification

- Essential for quantification:

  Signals that are compared with each other have to be in the dynamic range

- Dynamic ranges are instrument specific and dependent on
  - Cleanness of the Flow cell
  - Performance of the PMT
  - etc

- What are detectors dynamic ranges on YOUR instrument?

- The CS&T baseline report can tell you!

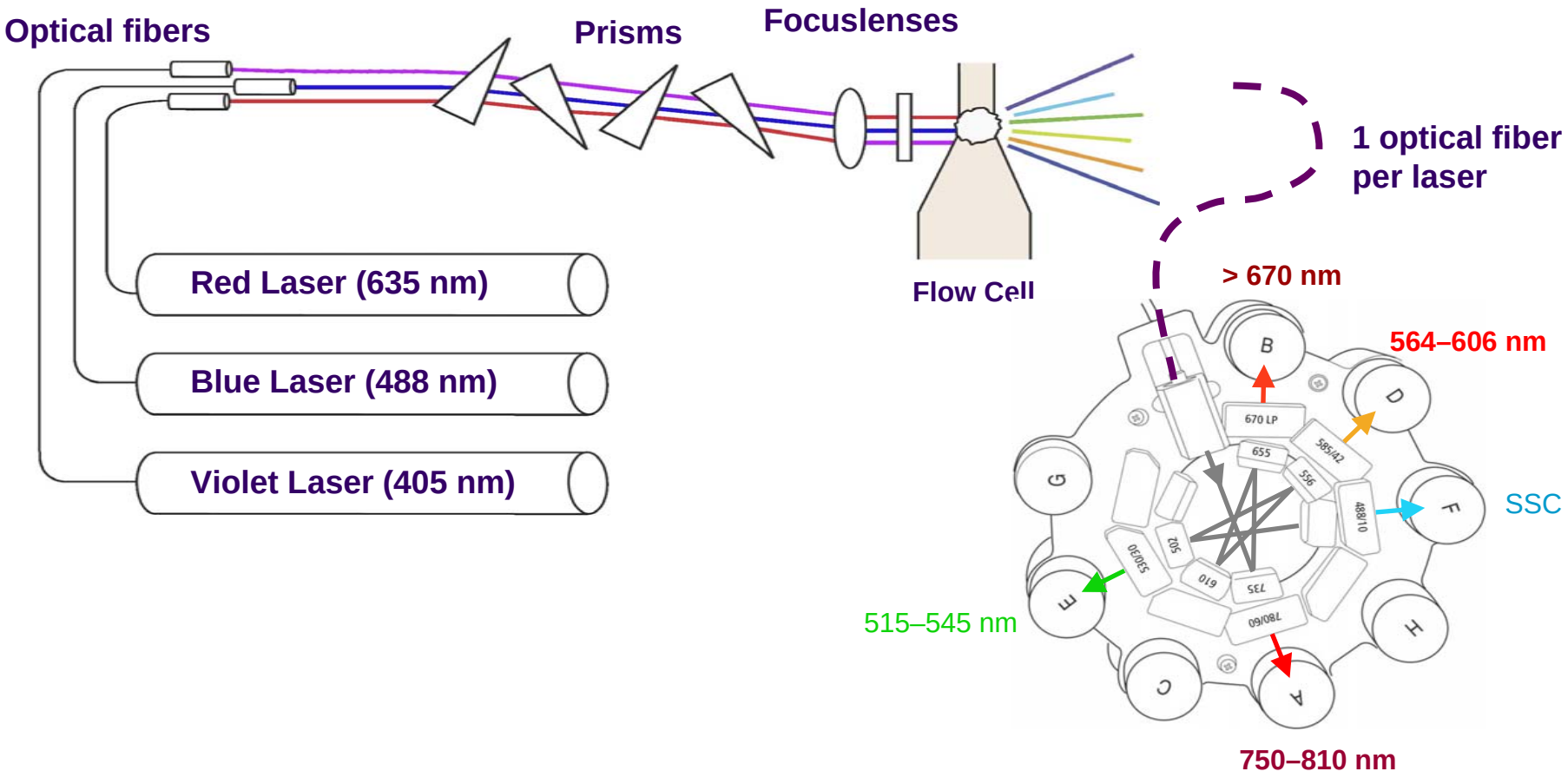
  You will learn tomorrow from the “baseline report” of CS&T!

Overview

- Measurement of Cellular Parameters in Flow Cytometry
- The Optical System of Flow Cytometers
  - Lasers
  - Filters and mirrors
  - Detectors
- Fluidics
- Electronics – Digital theory

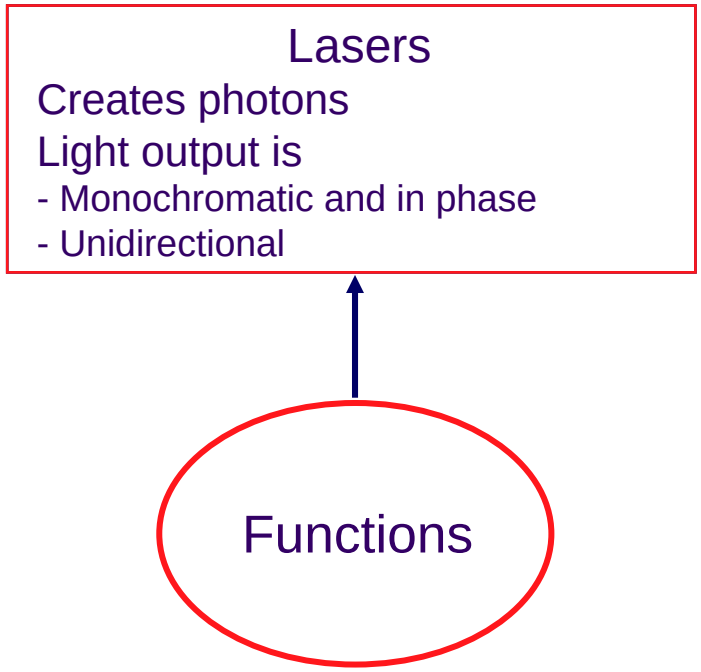
The optical system of Flow Cytometers

- Overview on the optical system



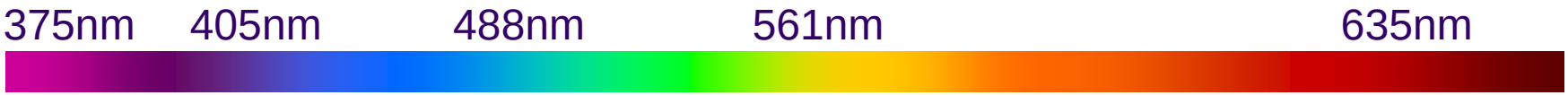
The optical system of Flow Cytometers

- Components of the optical system



The optical system of Flow Cytometers: *Lasers*

- Excitation of fluorochromes by different lasers



AF®350

BD Horizon™V450
BD Horizon™V500
Pacific Blue
AmCyan

FITC / AF®488
PerCP / PerCP-Cy™5.5

Excitation-efficiency approx.60%

PE
PE-TxRed
PE-Cy™5
PE-Cy™7

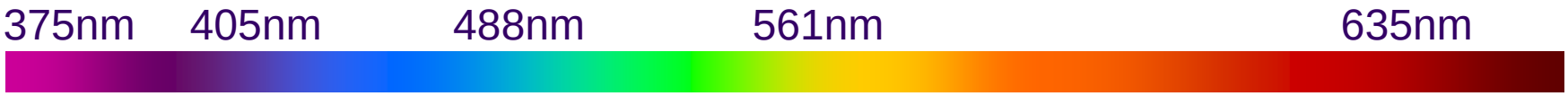
PE
PE-TxRed
PE-Cy5
PE-Cy7

Excitation-efficiency > 90%

APC / AF®647
AF®700
APC-Cy™7 /
BD™APC-H7

The optical system of Flow Cytometers: *Lasers*

- Excitation of fluorescent dyes by different lasers



Hoechst33265
Indo-1

DAPI

SYTOX Blue

ECFP

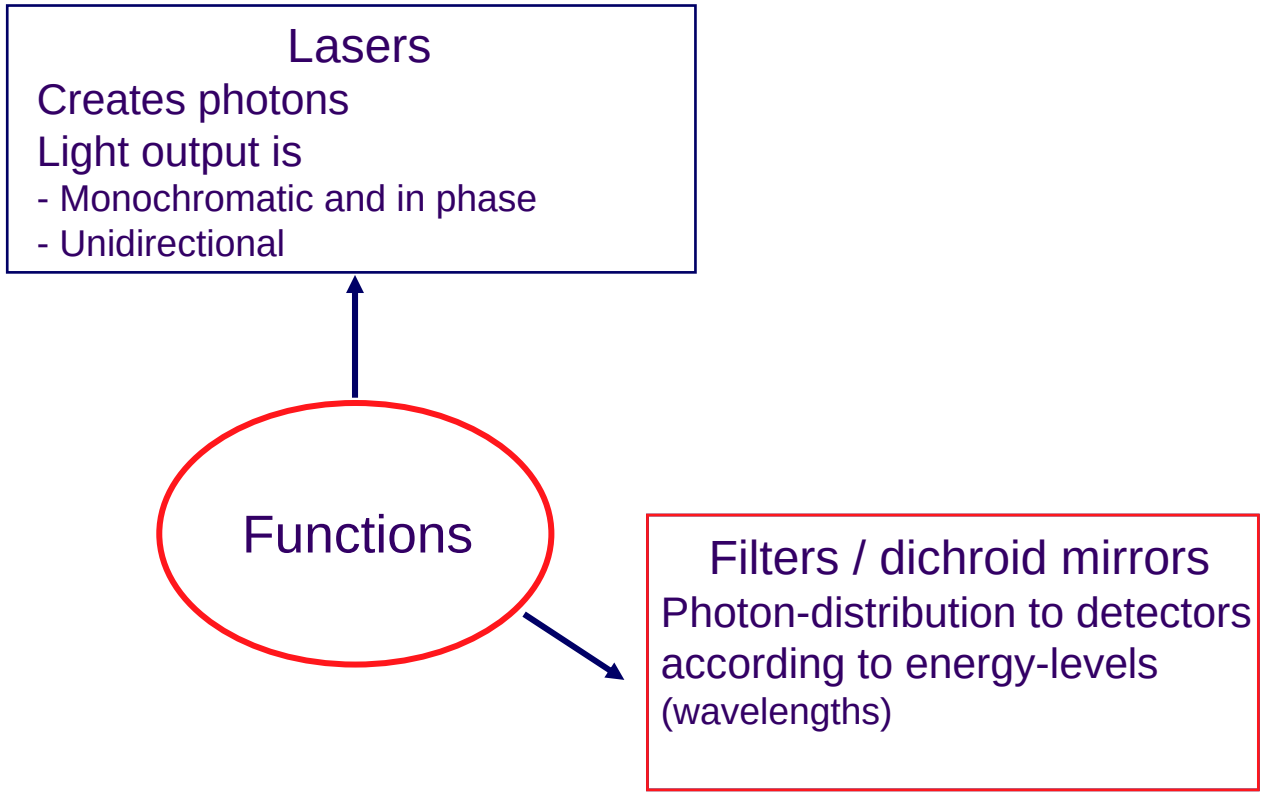
EGFP / EYFP
CFSE
7-AAD / PI
dsRed
SYTOX Green
SYBR Green

7-AAD / PI
mCherry
mTomato
mPlum
mOrange

DRAQ5
SYTOXRed

The optical system of Flow Cytometers

- Components of the optical system

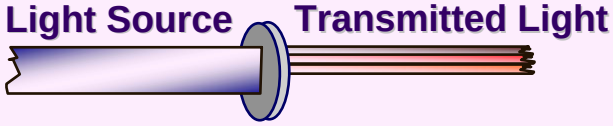


The optical system of Flow Cytometers:
Filters / Mirrors

- Distribution of photons to detectors is filter-dependent

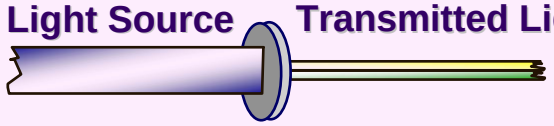
Filters in Flow Cytometers

Long-pass Filters (580nm LP)



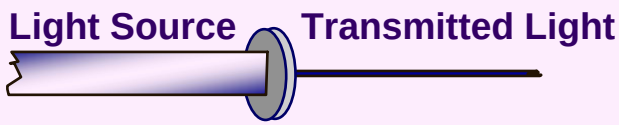
> 580 nm Light

Short-pass Filters (580nm SP)



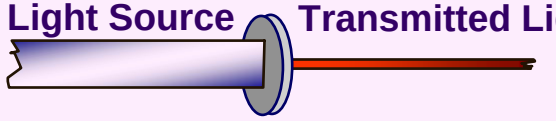
< 580 nm Light

Neutral-density Filters (ND1)



488 nm light (absorbs 90% of light)

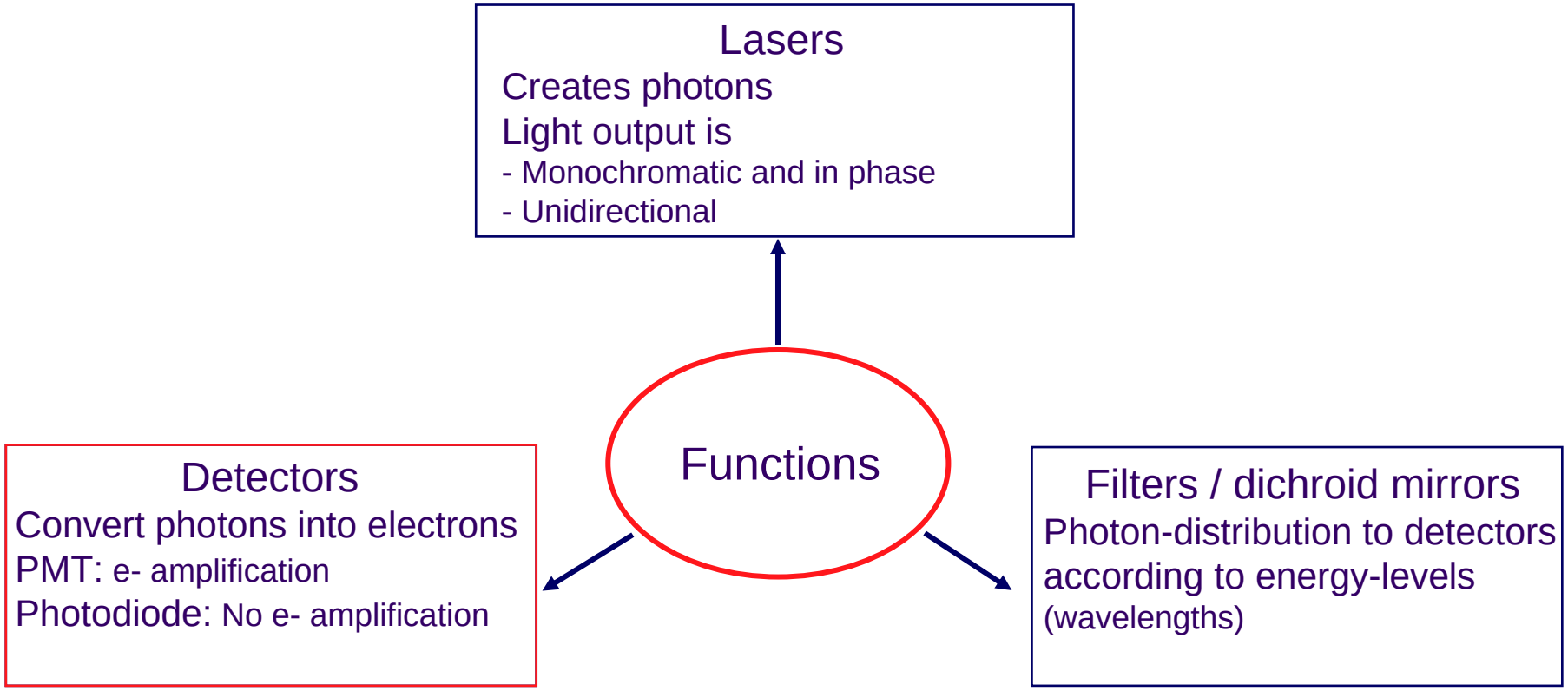
Band-pass Filters (660/20 BP)



650 – 670 nm light

The optical system of Flow Cytometers

- Components of the optical system

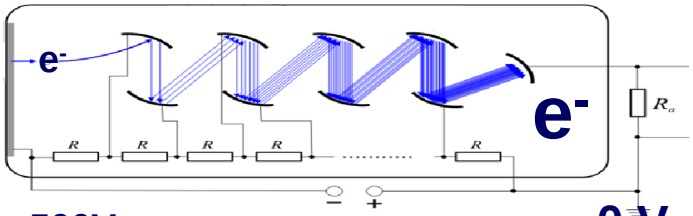


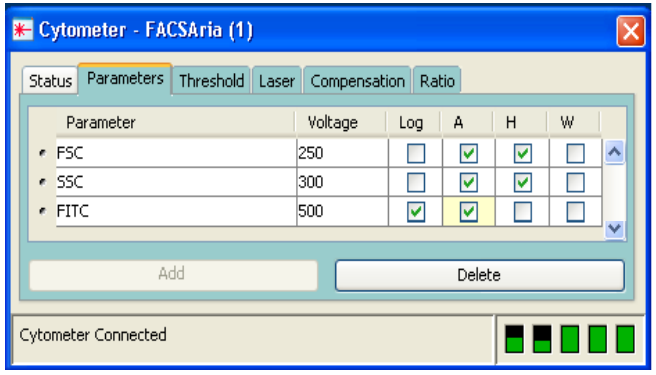
The optical system of Flow Cytometers: Detectors

- Photons (scattered from cells or emitted from fluorochromes) have to be converted into electrons (electronic signal) to become analyzed
  - Photodiodes (on conventional flow cytometers)
    - Detected parameter: FSC
    - Direct and proportional 1:1 conversion of photons into electrons
    - No amplification inside the photodiode
  - Photomultiplier Tube (PMT)
    - Detected parameters: SSC, fluorochromes
    - Efficiency of photon to electron conversion is wavelengths-dependent
    - Amplification inside PMT via Dynodes

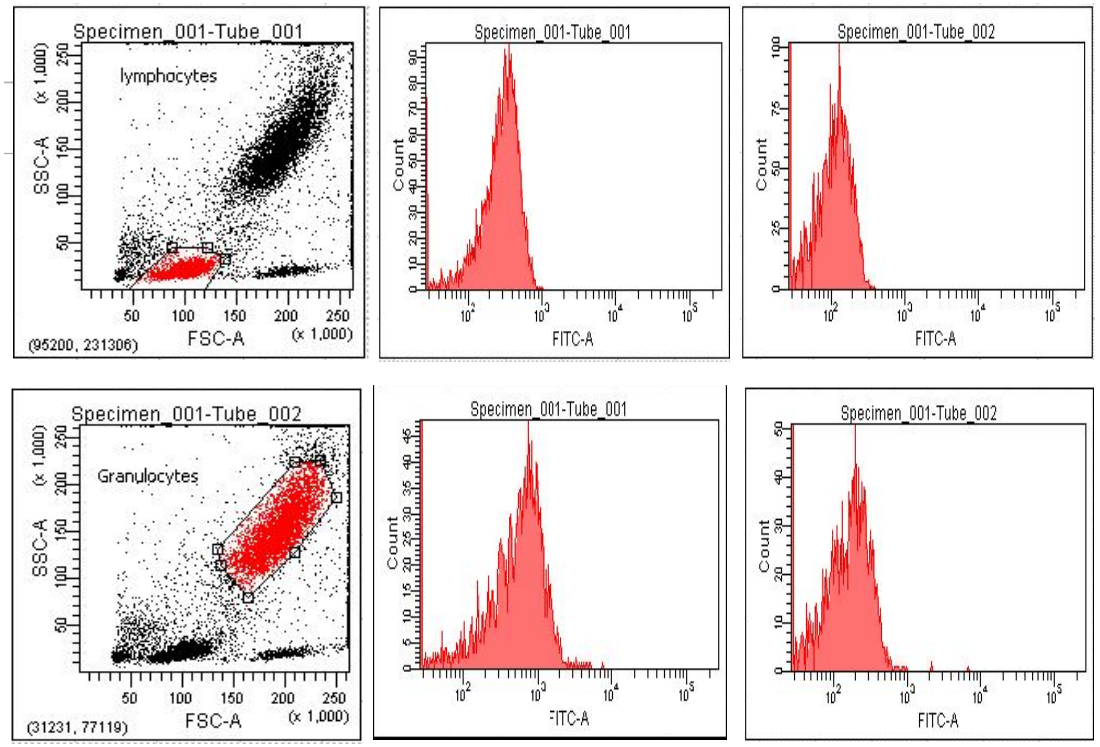
The optical system of Flow Cytometers: *Detectors*

- Instrument Settings: Sample-dependent adaption of the PMT V to set unstained cells on scale to distinguish positive from negative samples.





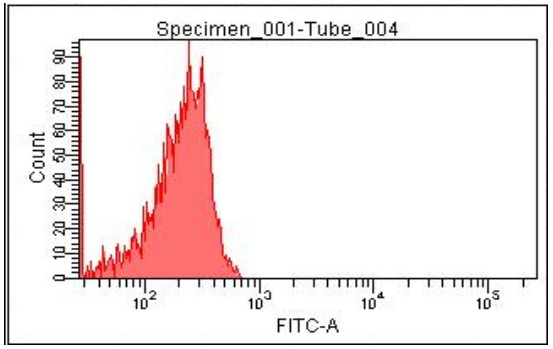
PMT V = 500V **PMT V = 470V**



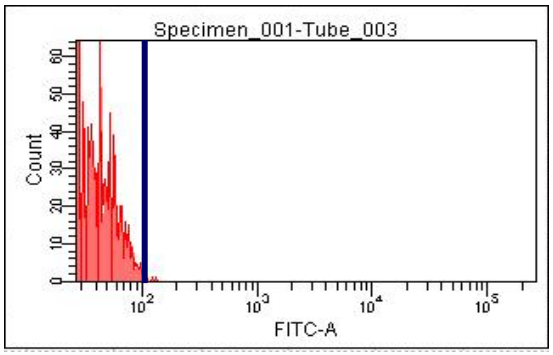
The optical systrem of Flow Cytometers: Detectors

- But how are the instrument settings adjusted “properly” ?

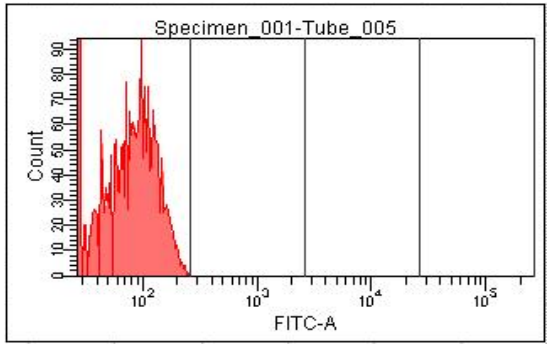
As YOU like?



According to 1st decade?



According to grids?



- According to the “electronic noise” that is individual for each instrument!
- But what is the electronic noise of YOUR instrument?

You will learn tomorrow from the “baseline report” of CS&T!

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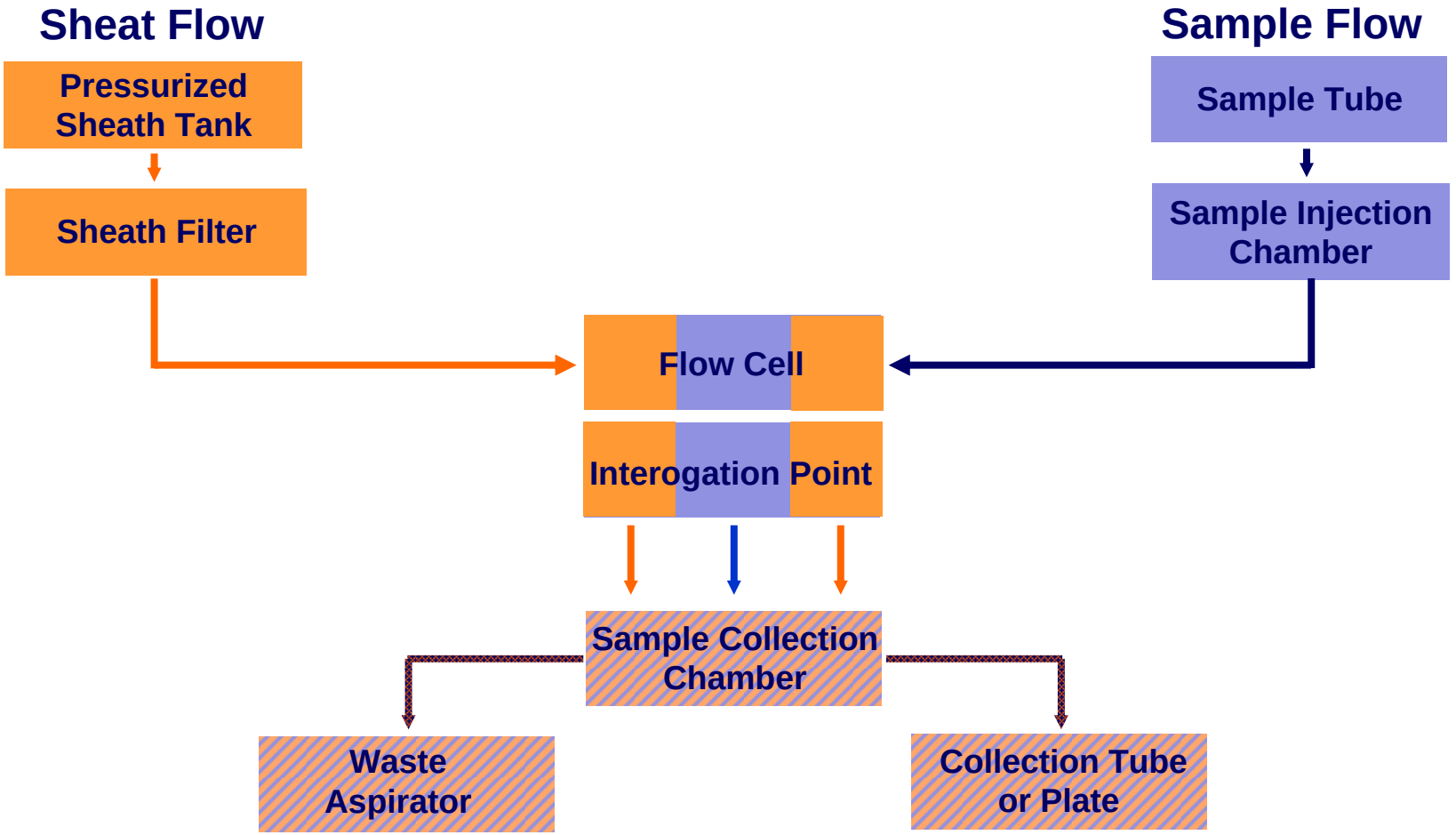
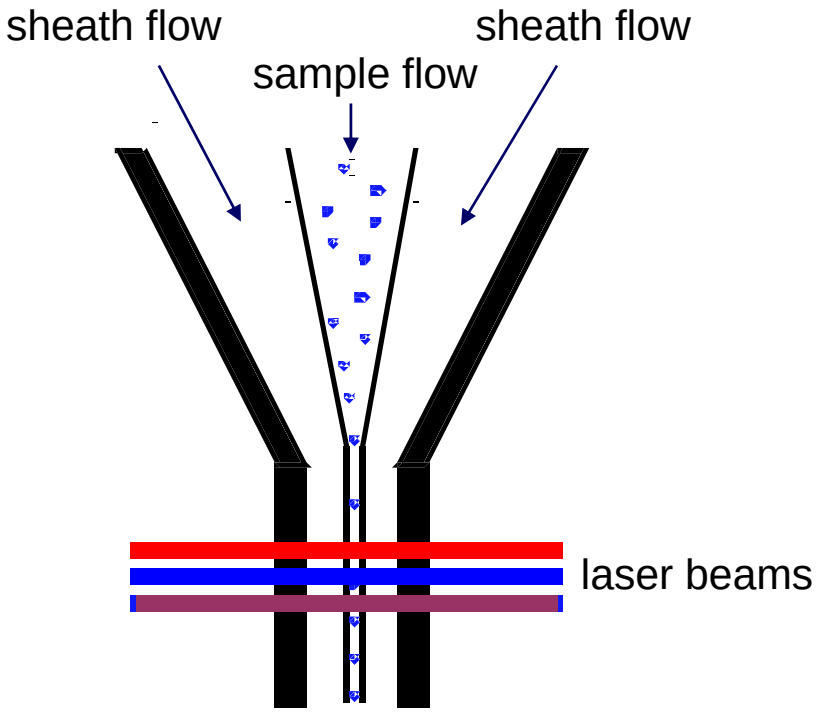
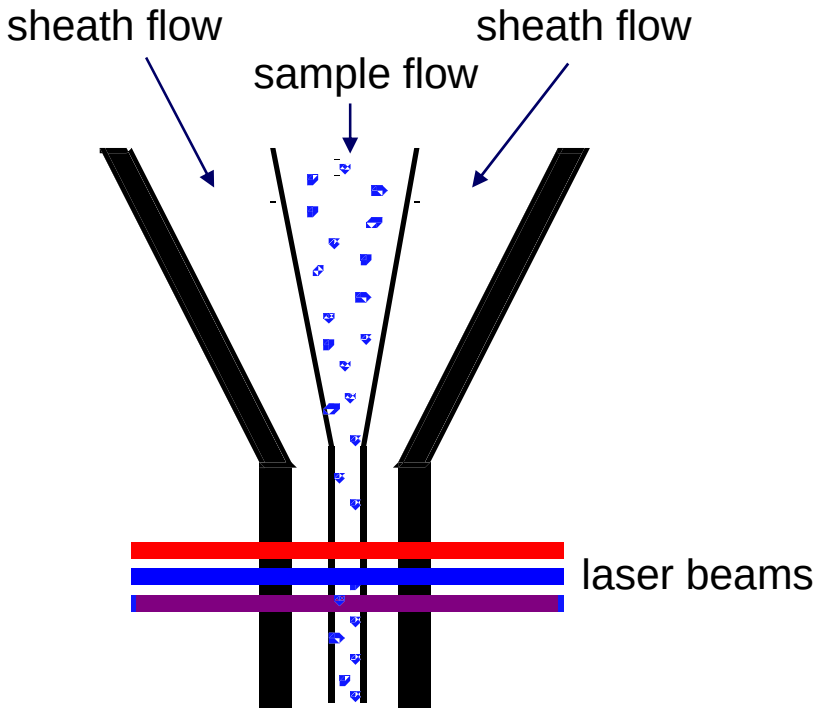
Fluidics:
Overview on the BD FACS Aria Fluidic System



Fluidics:
Hydrodynamic Focusing



low sample pressure



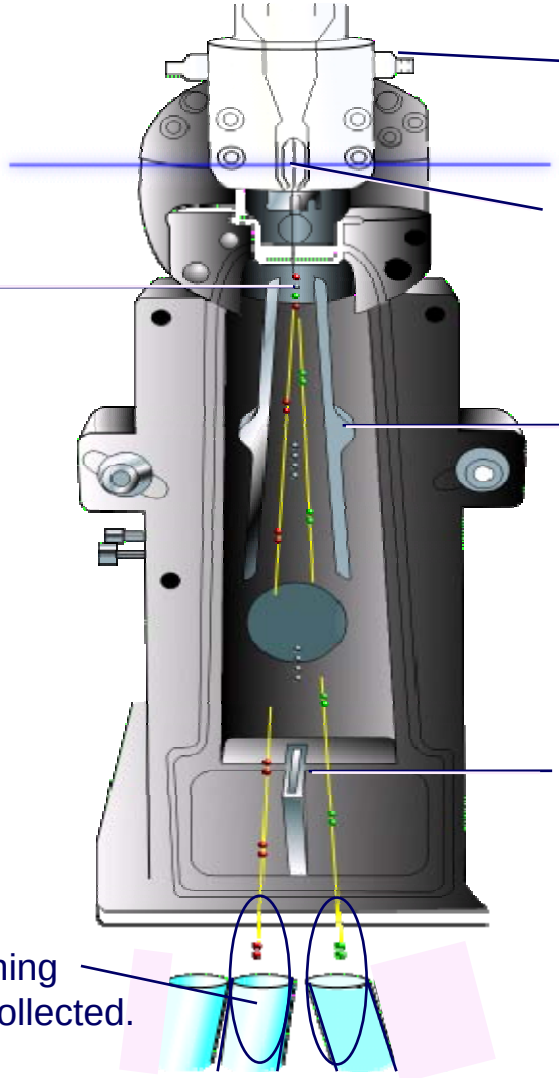
high sample pressure

Fluidics:
Summary

- Sheath Pressure: Drives sheath buffer through the cuvette.
- Sample Pressure: Higher than Sheat Pressure. Delivers sample to Cuvette. Determines the Flow Rate.
- Cuvette: Hydrodynamic Focussing align cells while passing the interception point for analysis.

  Important: The hydrodynamic focusing can not separate cell agregates!

Fluidics:
Drop Formation and Charging



2. Charge is applied via the stream-charging wire.

1a. Sample generates light scatter and fluorescent signals; signals are analyzed.
1b. Sorting decision is done according to the gating strategy and coincidences of events.

3. Charged droplets break off.

4. Deflection plates attract or repel charged droplet.

5. Uncharged droplets pass to waste.

6. Charged drops containing particles of interest are collected.

Fluidics:
Drop Formation and Charging

- Drop Formation
  - Amplitude:

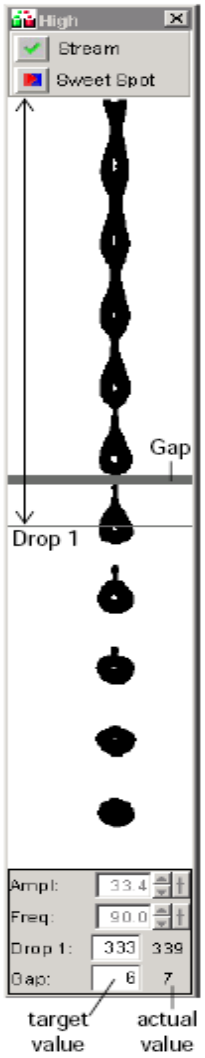
    Intensity of the drop drive
  - Frequency:

    Number of drops formed per second
  - Drop1:

    Number of pixels from the top of the image

    to the center of the first disconnected drop
  - Gap:

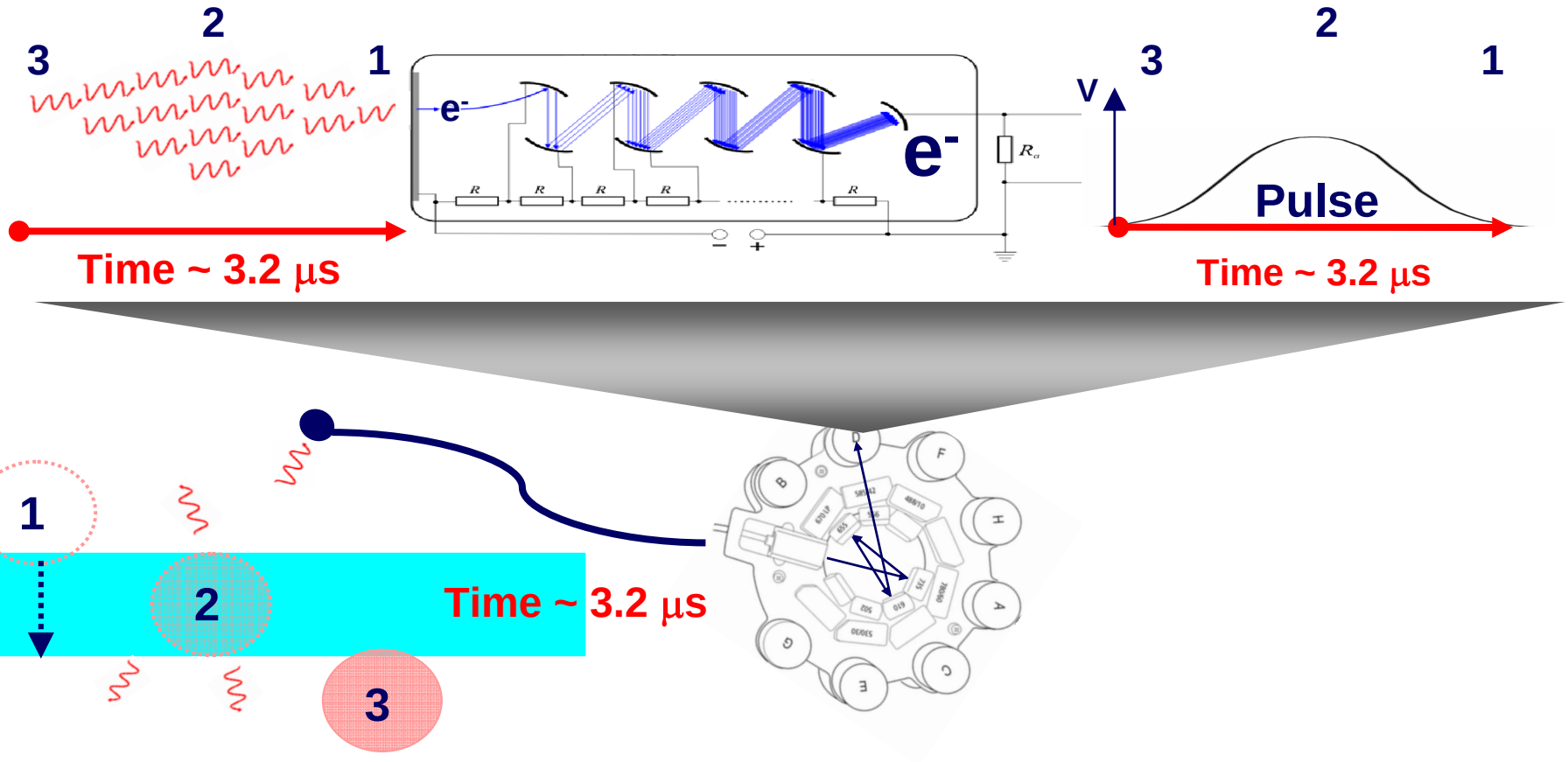
    Number of pixels between the stream breakoff and the first drop



Overview

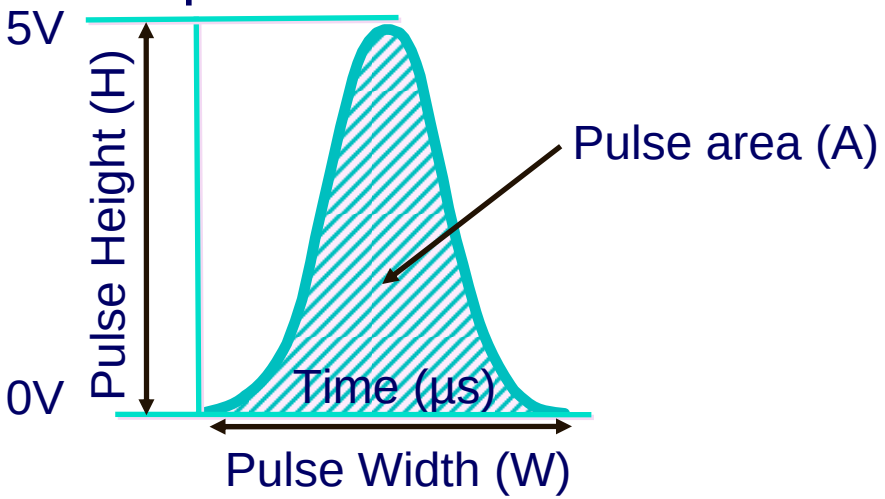
- Measurement of Cellular Parameters in Flow Cytometry
- The Optical System of Flow Cytometers
- Fluidics
- Electronics – Digital theory
  - Pulse generation
  - Data generation, storage and display
  - Doublete discrimination
  - Thresholds

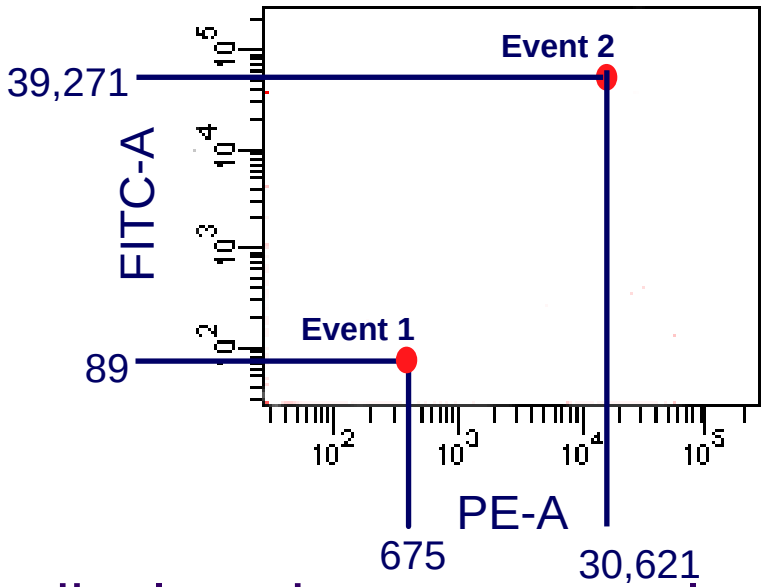
Electronics – Digital Theory:
Pulse Generation



Electronics – Digital Theory:
Data Generation

- Pulse parameters

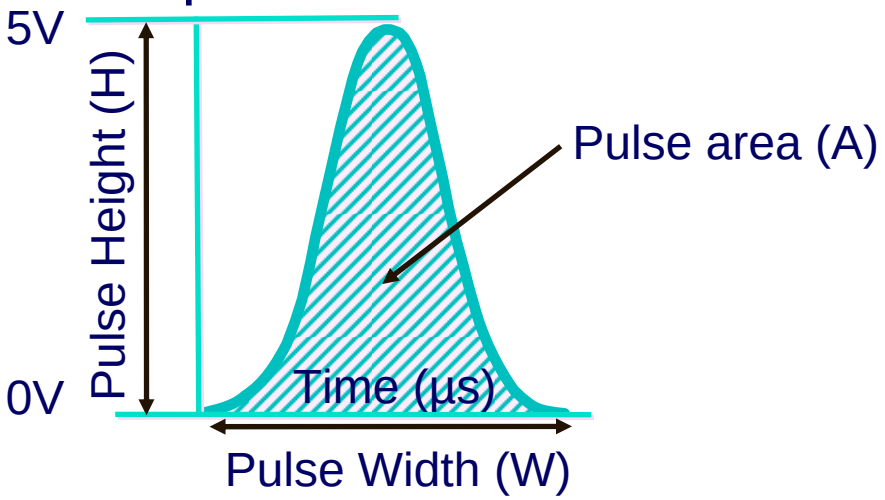


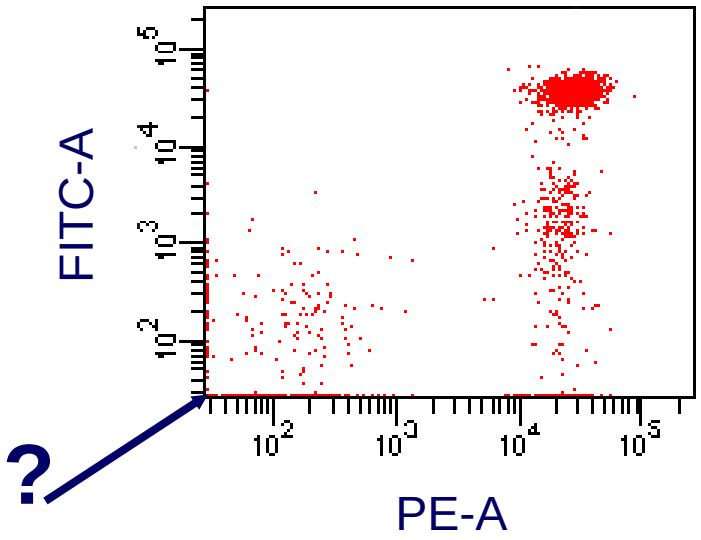


- Data for all pulse parameters are displayed on same scale with 262.144 channels
  - Default parameter to display is Area (H and W have to be selected actively)
  - Data are calculated and displayed in linear numbers

Electronics – Digital Theory:
Data Generation

- Pulse parameters





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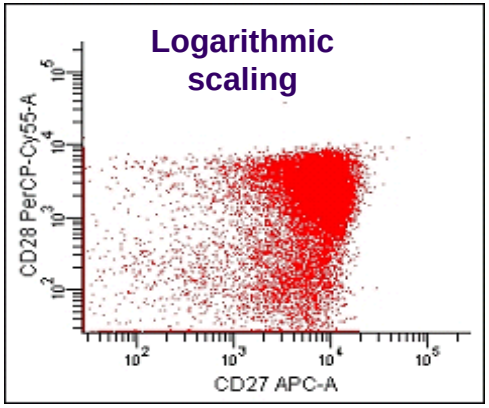
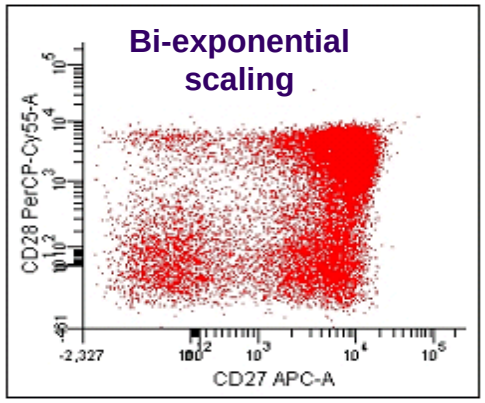
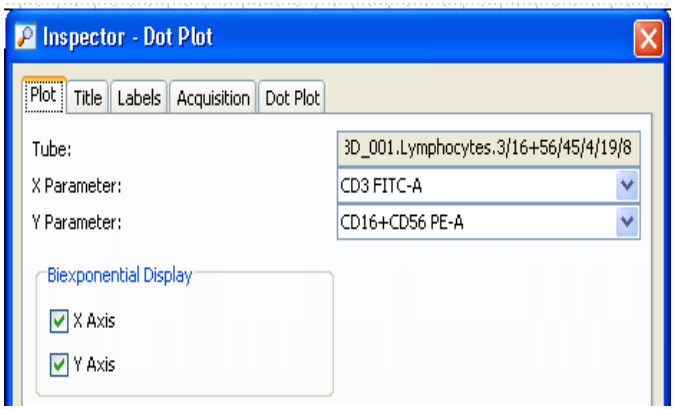
  - Default parameter to display is Area (H and W have to be selected actively)
  - Data are calculated and displayed in linear numbers

Electronics – Digital Theory:
Data Display

- In digital systems positive and negative numbers can be displayed in the „bi-exponential display“





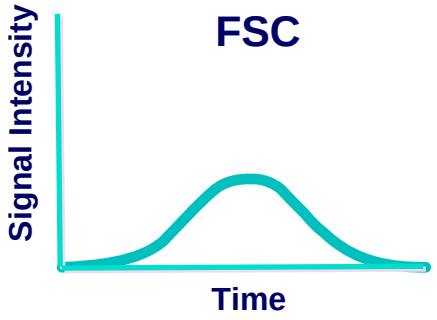


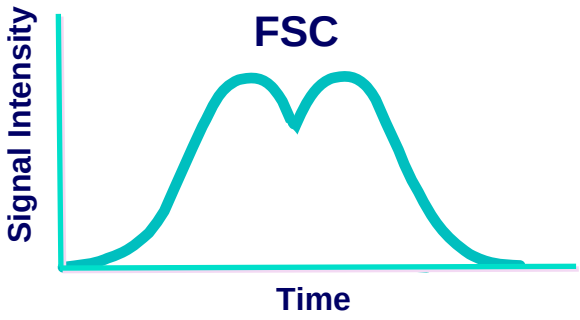
- In a system working only with linear numbers, negative values are as „good“ as positives – they are just smaller

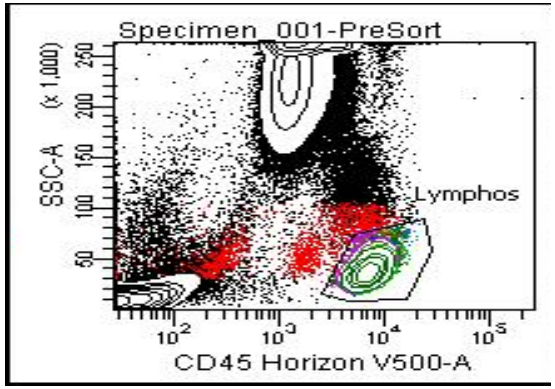
Electronics – Digital Theory:
Doublet Discrimination

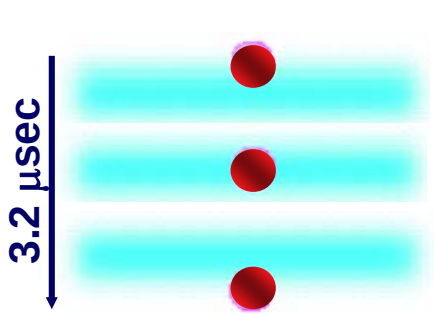
- Doublets passing the laser beam will generate one pulse!

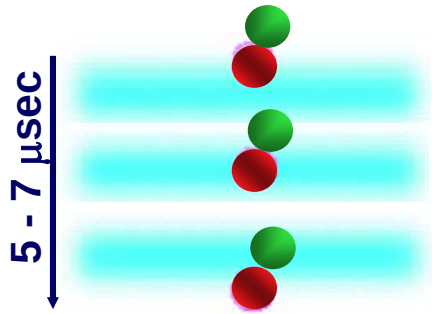
How can a doublet be discriminated from a single cell?

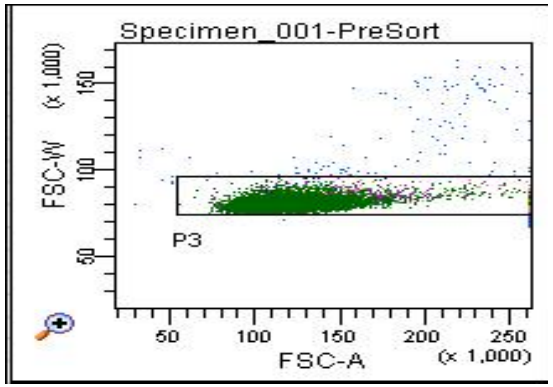












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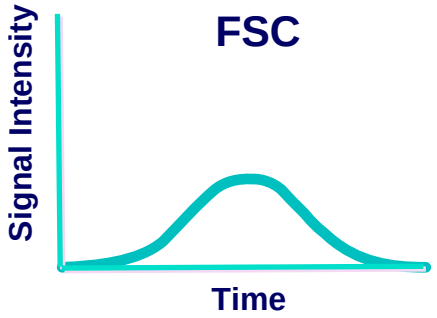
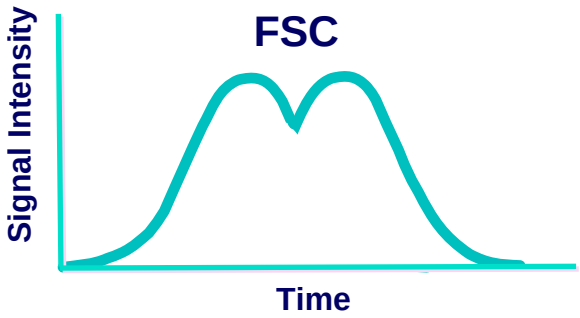
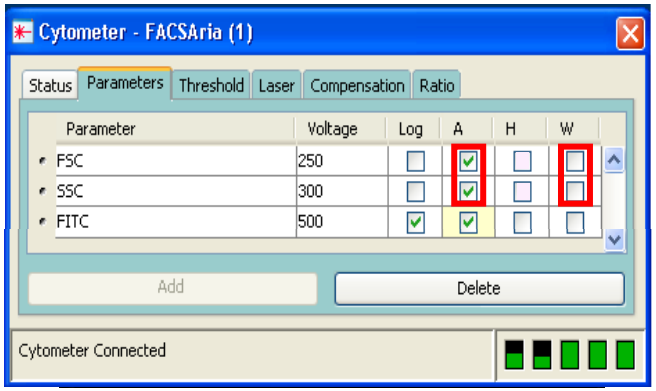
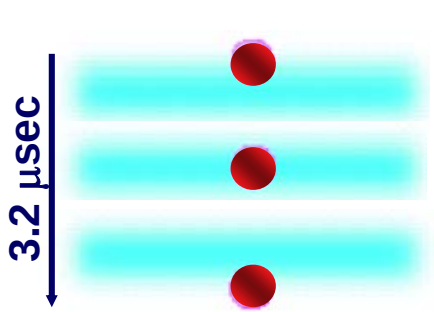
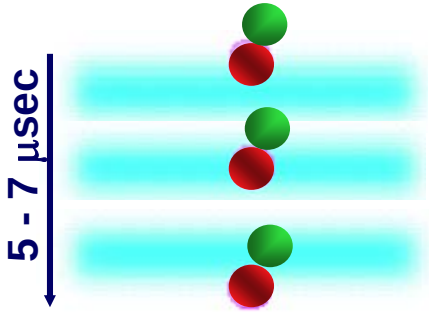
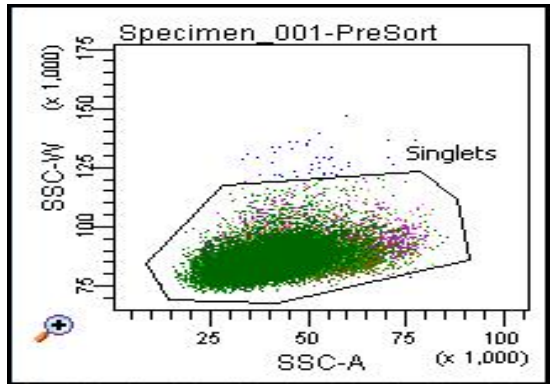






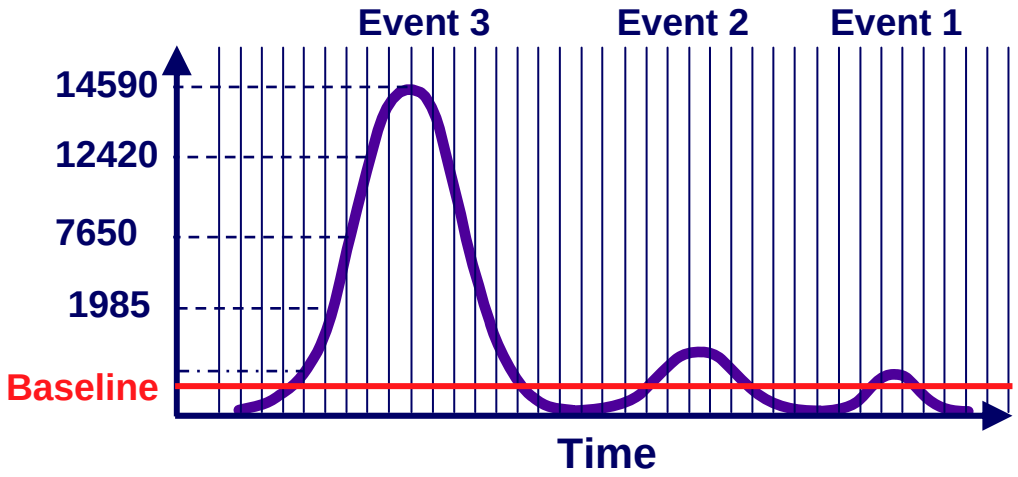


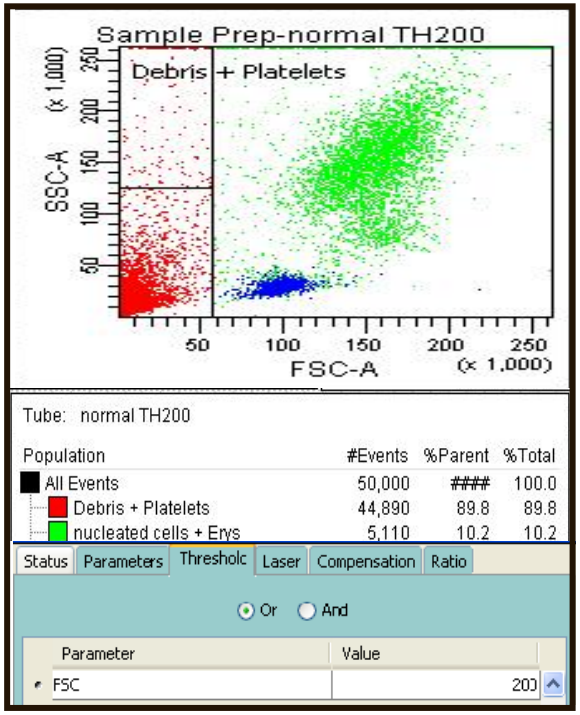




Electronics – Digital Theory:
Threshold

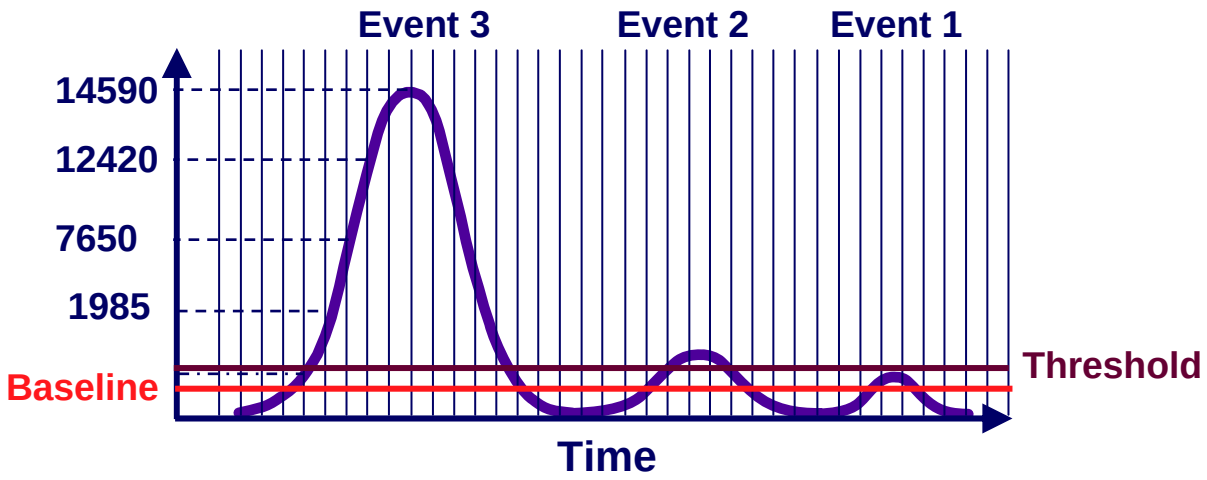
- Area values for pulses are calculated by addition of single Height values for an event that exceeds above the „baseline“.

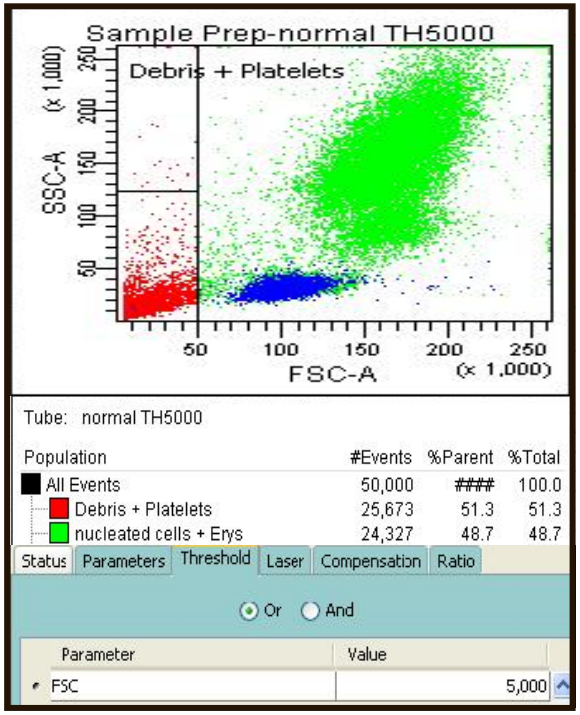




Electronics – Digital Theory:
Threshold

- To exclude events (e.g.: debris) you set the „Threshold“ as a 2nd value that has to be exceeded





Electronics – Digital Theory:
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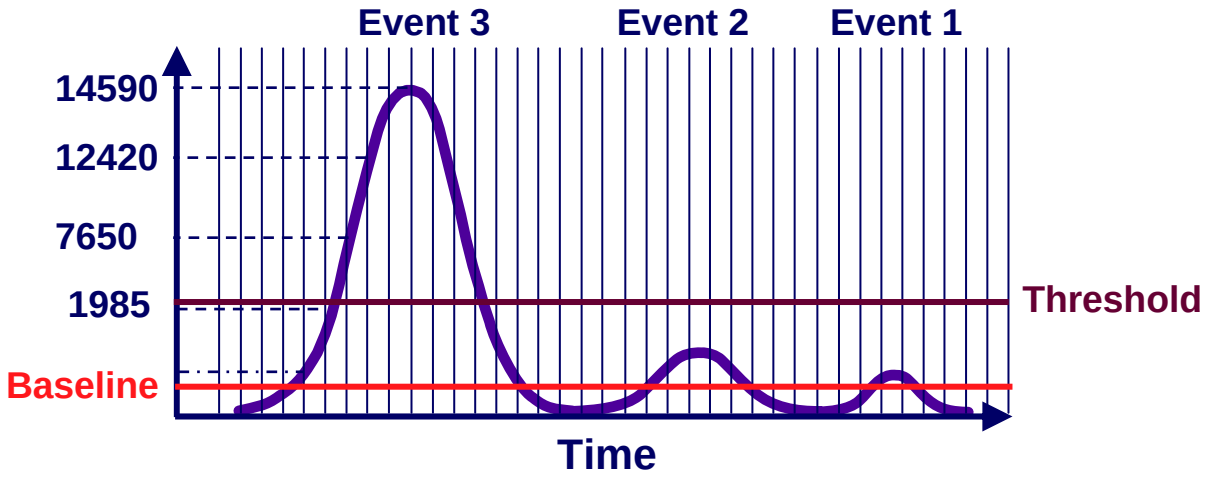
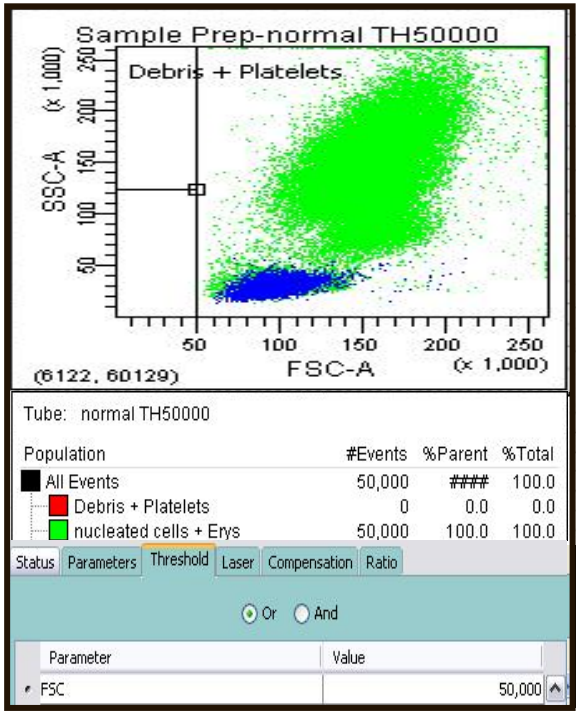




- Pulses that do not exceed the threshold are not stored. The cytometer becomes „blind“ for these events!

Overview

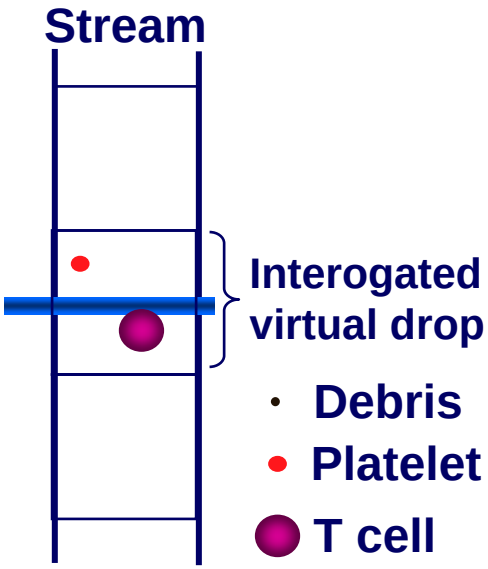
- Measurement of Cellular Parameters in Flow Cytometry
- The Optical System of Flow Cytometers
- Fluidics
- Electronics – Digital theory
- Sorting – An overview
  - Coincidences and Sort Decisions
  - Drop Formation and Charging

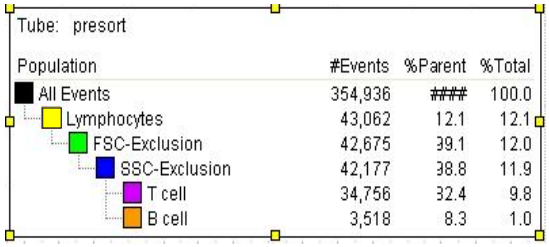
Sorting – An Overview
Coincidences and Sort Decisions

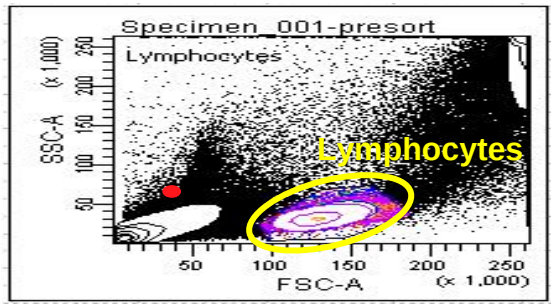
- Your sort decision is done according to your gating strategy

  Be aware: You do NOT sort cells! You sort drops!

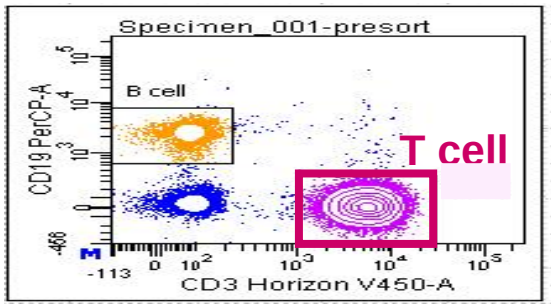
  You sort a drop if one event in the drop fulfills the gating strategy!







Will you sort the T cell ?



Sorting – An Overview
Coincidences and Sort Decisions

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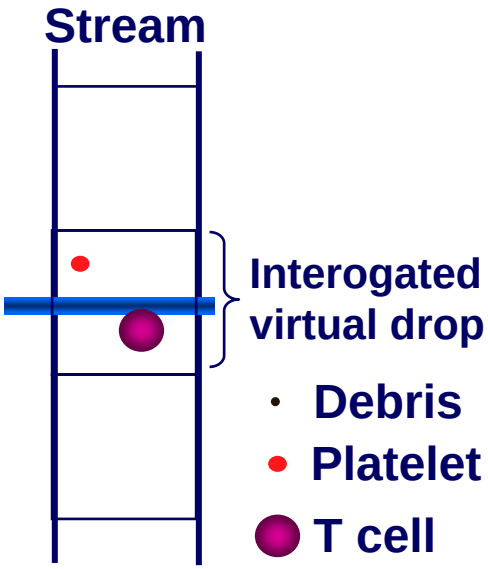
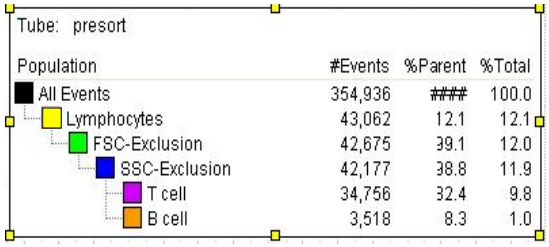
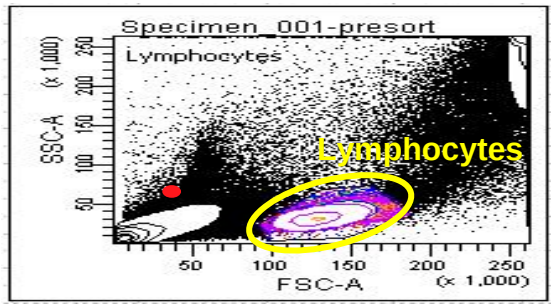
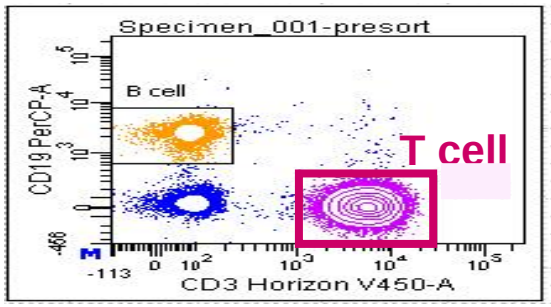




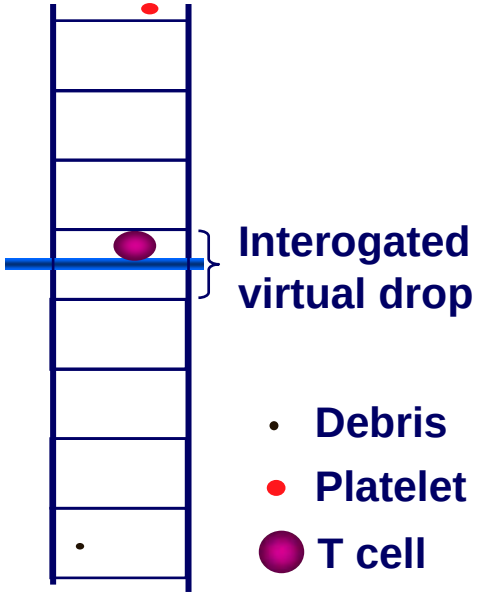


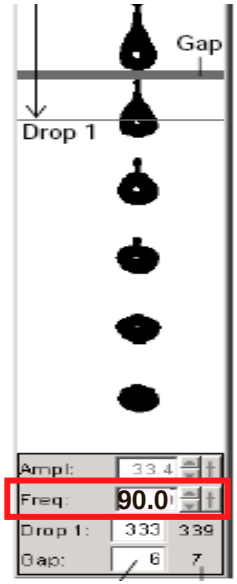
Yes,
even if the platelet might screw up your cytokine assay!

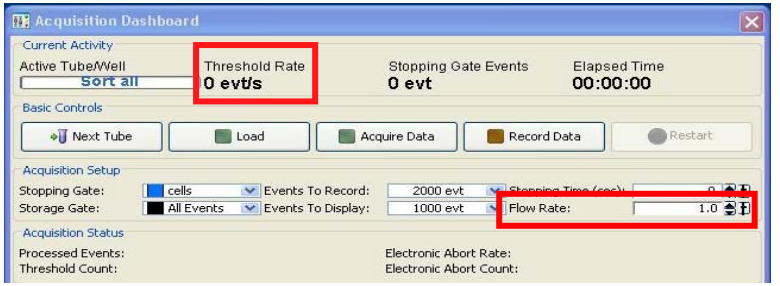


Sorting – An Overview
Coincidences and Sort Decisions

- To reduce the chance of coincidences, we adapt the number of events we sort to the number of drops we produce
  - Frequency / 4 is highest Threshold Rate (# of events / sec)
  - Flow Rate for sorting is maximal 6

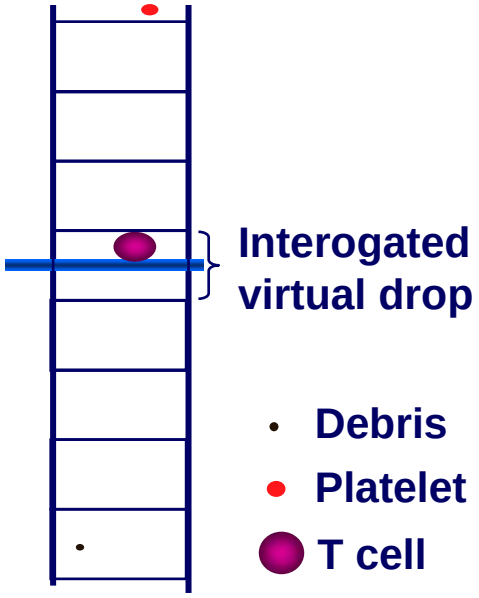


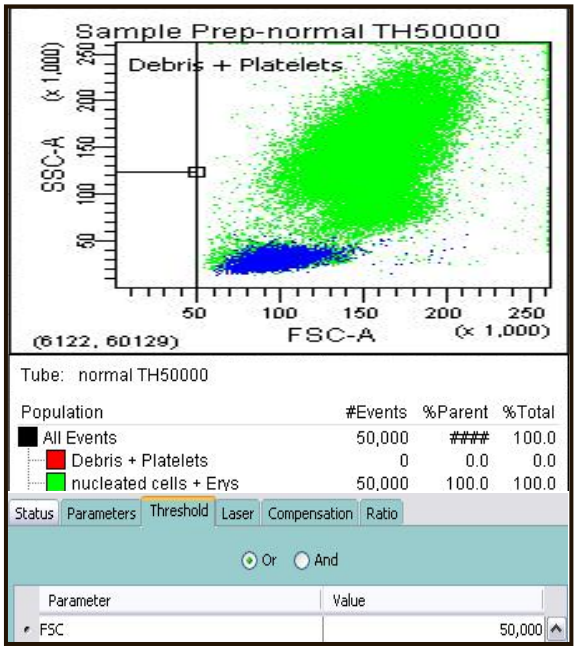




Sorting – An Overview
Coincidences and Sort Decisions

- Also pieces of debris are „events“ that will enhance due to their high numbers the sorting time significantly
  - Enhance the threshold on FSC: events below threshold become „invisible“





Sorting – An Overview
Coincidences and Sort Decisions

- Also pieces of debris are „events“ that will enhance due to their high numbers the sorting time significantly
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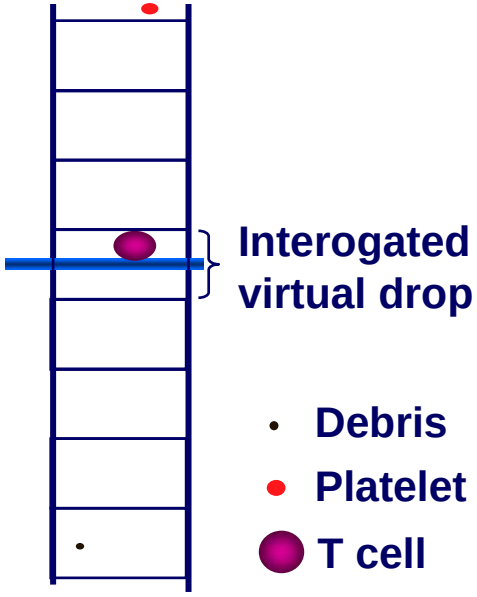
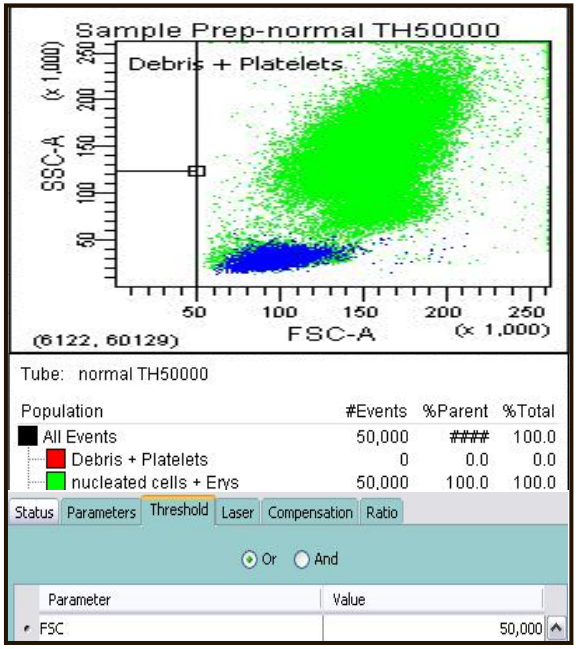
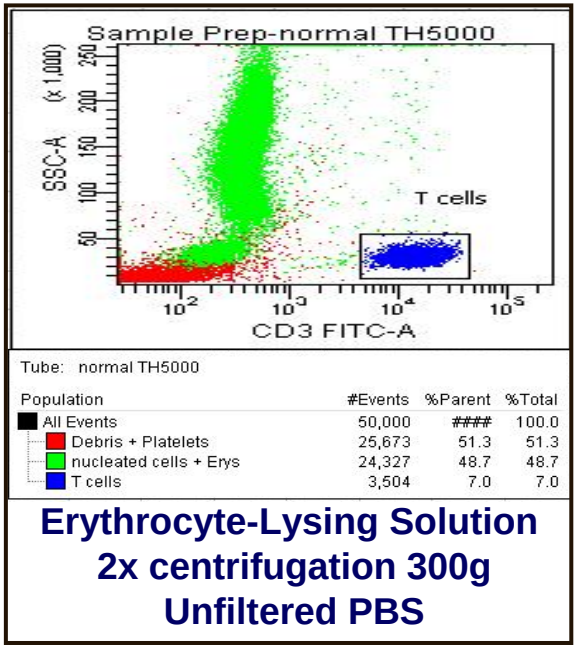
  - Optimize sample-preparation: reduce numbers of debris or platelets







Erythrocyte-Lysing Solution
2x centrifugation 300g
Unfiltered PBS

Sorting – An Overview
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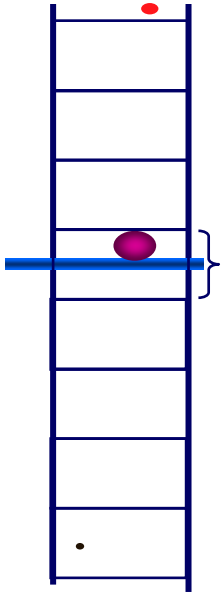

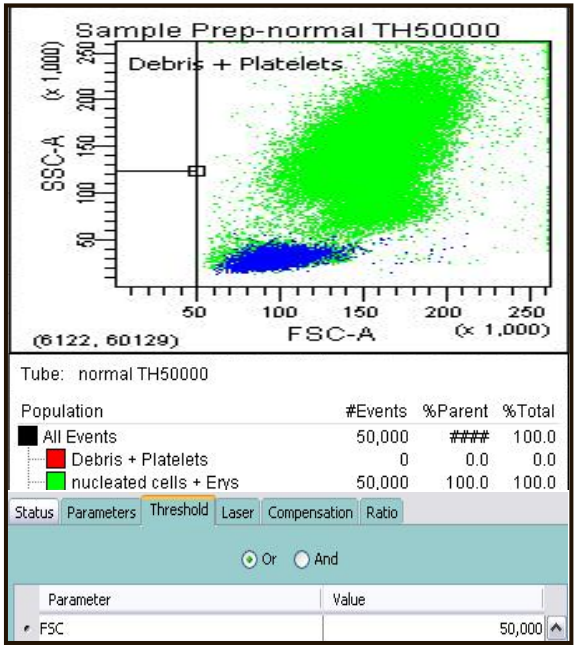

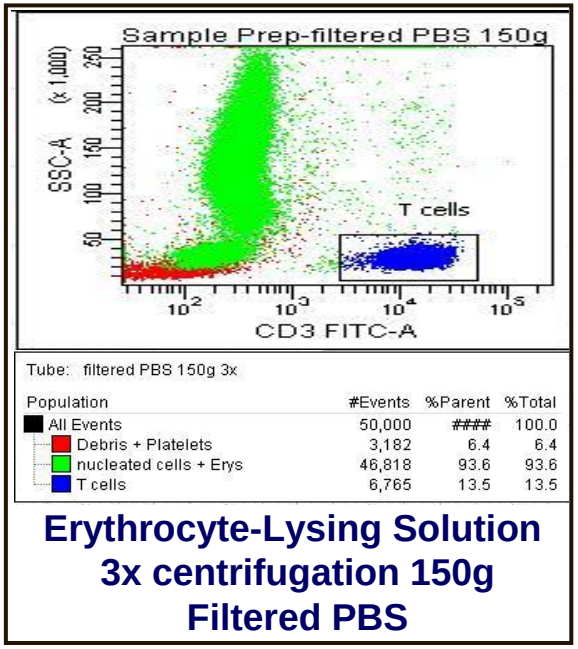

Erythrocyte-Lysing Solution
3x centrifugation 150g
Filtered PBS