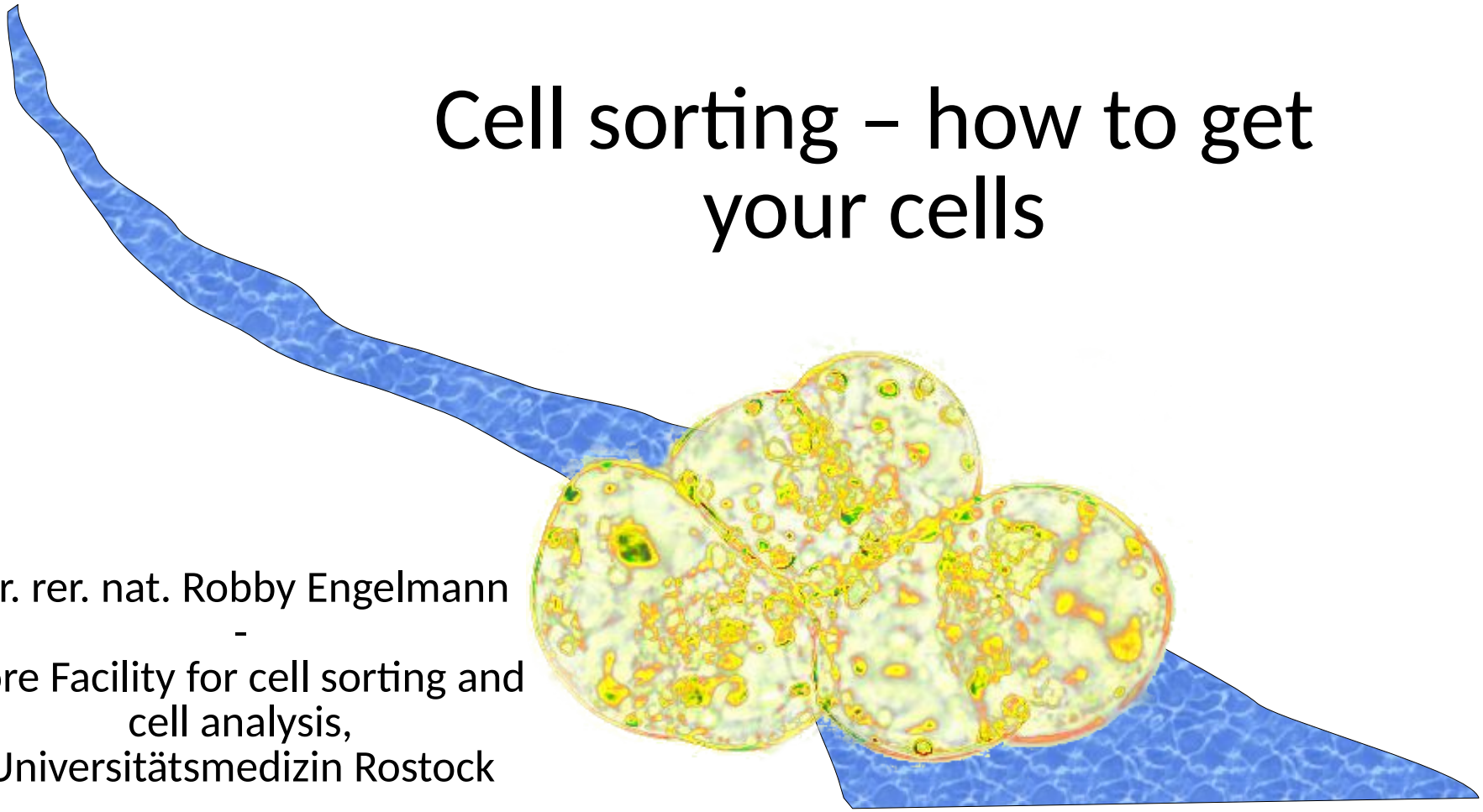
Universität Rostock Traditio et Innovatio

Universitätsmedizin Rostock

Cell sorting – how to get your cells



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Core Facility for cell sorting and cell analysis,
Universitätsmedizin Rostock

Overview

- magnetic cell sorting (MACS)
  - manual (MS or LS columns)
  - automated (autoMACS)
- fluorescence activated cells sorting (FACS)
  - sorter designs
  - steps to sort
  - think about

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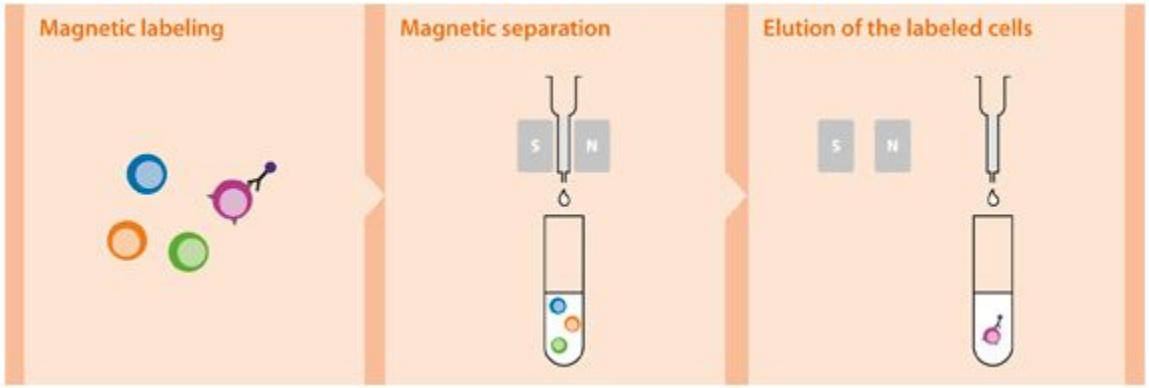
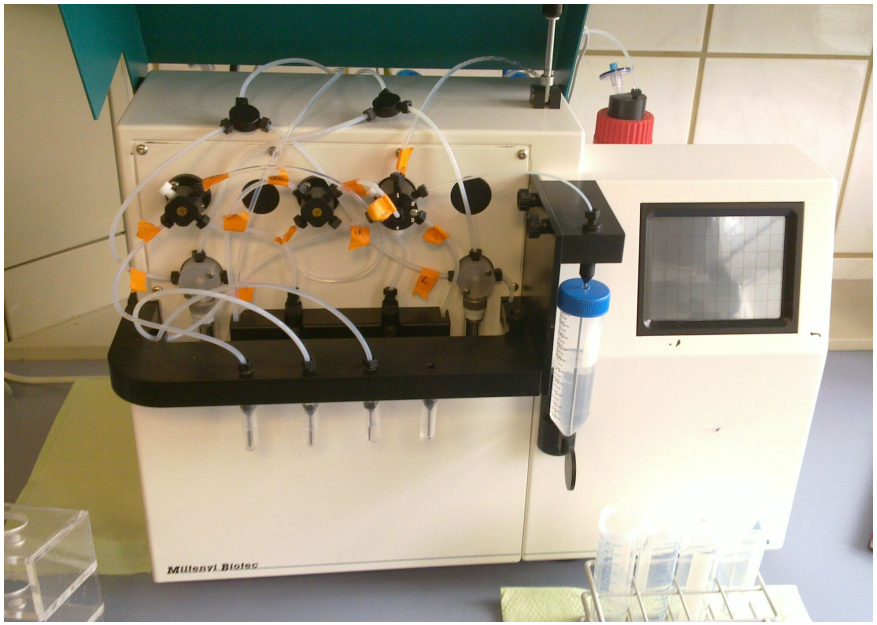
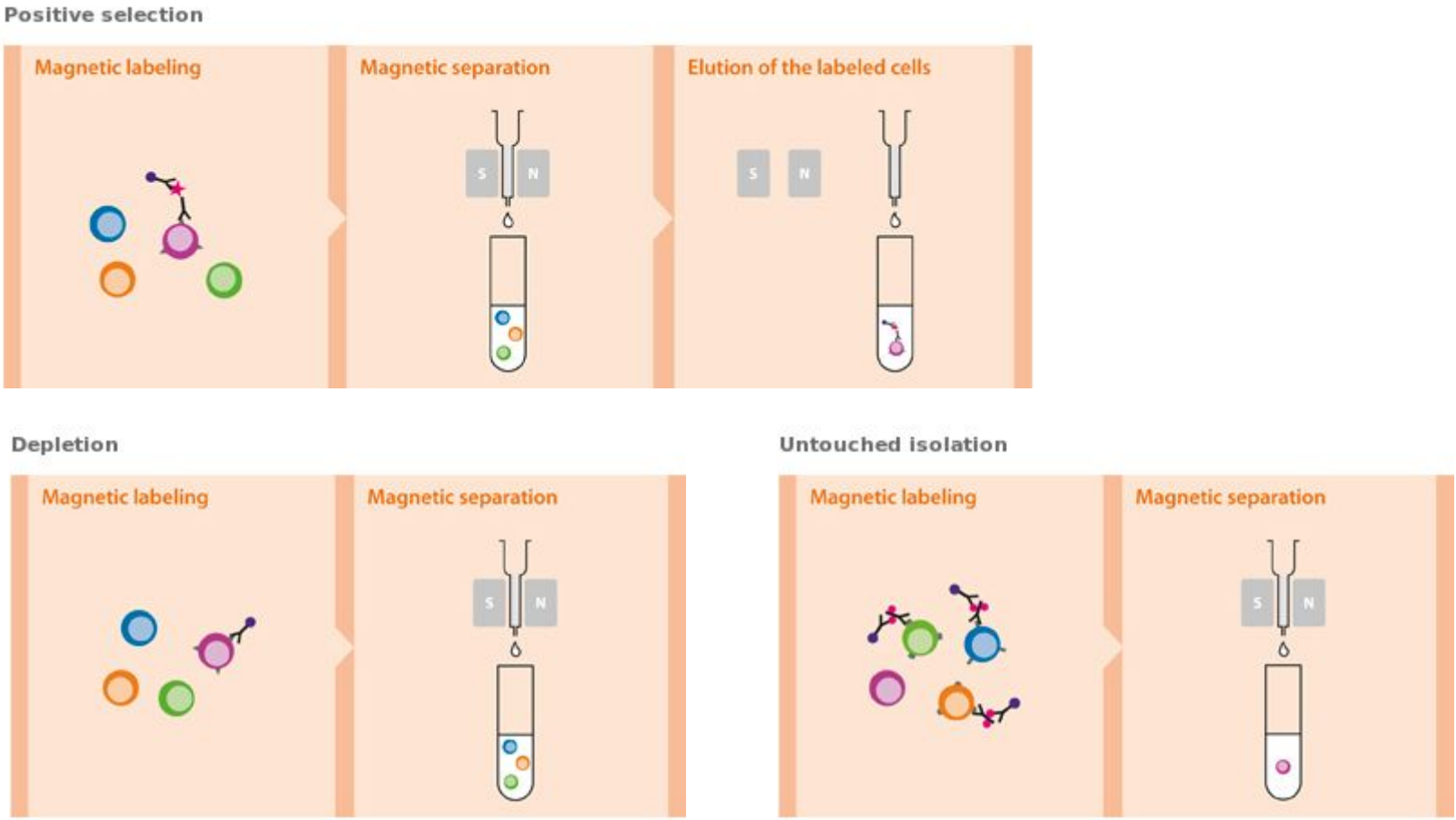
magnetic cell sorting

magnetic cell sorting





sorting strategies in MACS



FACS

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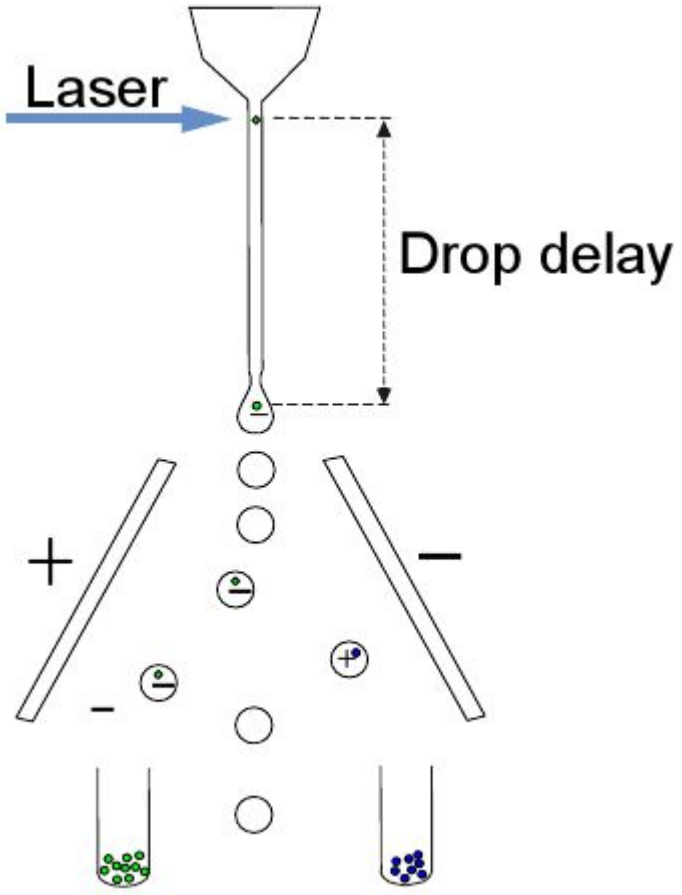
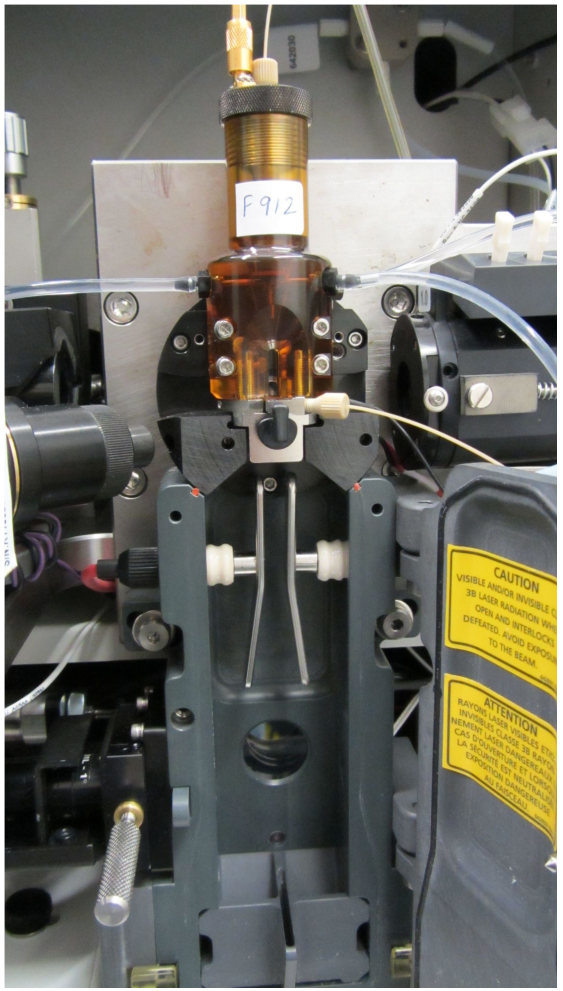
FACS – sorter designs



- stream-in-air
  - interrogation after droplet formation
  - cells fragmented during droplet formation are not sorted
- cuvette-based sorter
  - interrogation within a quartz cuvette
  - measurement more accurate
  - cells fragmented during droplet formation cannot be excluded

FACS – cuvette based sorter – FACS Aria II



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FACS Aria II – technical specifications

- optics
  - 3 lasers (405nm, 488nm, 633nm)
  - up to 9 fluorescence channels
  - recording of up to 70,000 events/sec
- sorting
  - nozzles with 70µm, 85µm, 100µm and 130µm for different cell sizes
  - up to 80,000 drops/sec generated → up to 20,000 cells/sec can be sorted
  - sort into 5ml FACS tubes, 15ml Falcon tubes, well-plates and slides

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Steps to your cells

- **sorting is NOT analysing ! ! !**

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Steps to your cells

- sample preparation
- Fluidics startup (Ethanol → sheath fluid)
- CS&T (quality control, baseline mapping)
- select appropriate nozzle size
- setup stream → stable stream
- setup PMT-V (your unstained cells) and compensation (beads)
- measure your cells
- define drop delay (beads)
- sort your cells
- reanalyse purity

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Steps to your cells

ZSA

- sample preparation
- Fluidics startup (Ethanol → sheath fluid)
- select appropriate nozzle size
- setup stream → stable stream
- CS&T (quality control, baseline mapping)
- setup PMT-V (your unstained cells) and compensation (stained beads)
- measure and analyse your cells
- set sort gates
- define drop delay (beads)
- sort your cells
- reanalyse purity

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Steps to your cells – sample preparation

- how many cells do I need for my downstream experiment?
- how many cells do I need to start with?
- pre-enrichment?

Time Taken to Sort a Given Number of Cells in an Ideal Situation Assuming a High Pressure Sorter Running at 10,000 Cells/s (3.6×10^7/h)

Number of cells requested	Percentage of population				
	0.1%	1%	5%	10%	40%
1000	1.7 min	10 s	5 s	1 s	0.25 s
10,000	17 min	1.7 min	50 s	10 s	2.5 s
100,000	2.8 h	17 min	8.3 min	1.7 min	25 s
1,000,000	28 h	2.8 h	1.4 h	17 min	4.2 min

With the time taken to set up the sorter and cell losses due to aborts, it is reasonable to assume a 50% increase in the actual time needed.

Steps to your cells – sample preparation

- how many cells do I need for my downstream experiment?
- how many cells do I need to start with?
  - goal is to sort 100.000 target cells
  - 110.000 10% loss on tube wall
  - 130.000 20% loss using purity mask (dependent on sort speed)
  - 150.000 10% loss on filtering
  - 180.000 20% loss for 2x washing
  - 200.000 10% Reserve
  - → stain approx. double the cells you need

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Steps to your cells – sample preparation

- What is the optimal cell density
  - resuspend at a density of 1-2*10^7 cells/ml for normal sorts of lymphoid cells
  - resuspend at a density of 5-8*10^6 cells/ml for normal sorts of sticky cells
  - resuspend at a density of 3-5*10^6 cells/ml for normal sorts of large cells (>15 µm in diameter → use 130µm nozzle)
  - resuspend at a density of <1.5*10^6 cells/ml for single cell sorts
- Accutase gently detached cells, no need to neutralize like trypsin, preserves epitopes
- centrifuge cells at slowest possible speed in a round bottom tube to remove small particles (cell debris, platelets)

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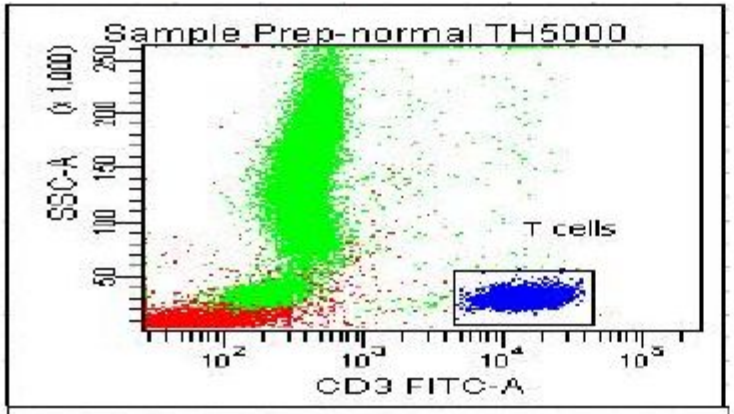
Steps to your cells – sample preparation

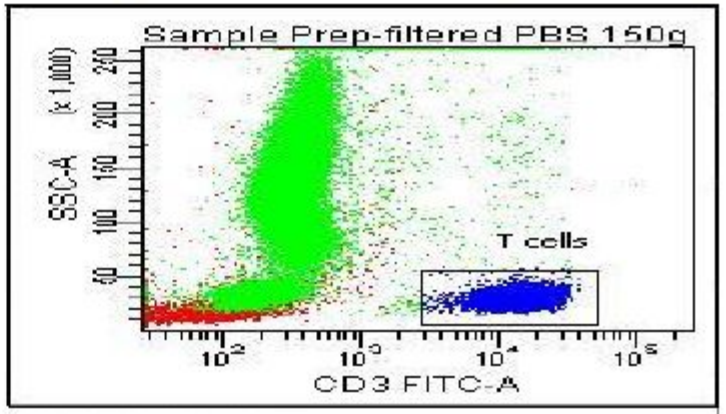
- **Analysis (original protocol):**
- 2x centrifugation 250g
- Washing 2ml PBS + 1% FBS



Tube: normal TH5000

Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
Debris + Platelets	25,673	51.3	51.3
nucleated cells + Erys	24,327	48.7	48.7
T cells	3,504	7.0	7.0

- **Sorting (adapted protocol):**
- 3x centrifugation 150g (aspirate carefully!)
- Washing 2ml PBS + 1% FBS (FILTERED)



Tube: filtered PBS 150g 3x

Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
Debris + Platelets	3,182	6.4	6.4
nucleated cells + Erys	46,818	93.6	93.6
T cells	6,765	13.5	13.5

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Steps to your cells – sample preparation

- basic sorting buffer
  - 1x PBS (Ca/Mg++ free)
  - 1mM EDTA
  - 25 mM HEPES ph 7.0
  - 1% FCS (heat-inactivated)
  - filtered to remove small particles
- for sticky cells
  - increase EDTA up to 5mM
  - Accumax breaks up aggregates in suspension culture

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Steps to your cells – sample preparation

- How viable / stressed are my cells prior to the sort?
  - add an viability dye (e.g. 7-AAD)
  - perhaps it is a bad idea to sort transfected cells shortly after transfection
  - same true for cells after tissue digestion
- sorting is enough stress for your cells
  - pressures up to 70 psi
  - rapid acceleration to 20m/s
  - exiting a small opening
  - returning to atmospheric pressure
  - passing through laser beams
  - charging a few hundred volts
  - passing through an electric field of several kV/cm
  - hitting a liquid surface still traveling 20m/s

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Steps to your cells – sample preparation

- Which antibodies should I use for staining?
  - avoid antibodies that activate your cells or block downstream stimulations (carefully read datasheets!)
  - follow basic rules for panel design → optimal population separation is crucial
  - a panel good enough for analysis is not necessarily good for sorting

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Steps to your cells – setup PMT-V & Comp.

- PMT-V
  - your unstained cells
- compensation
  - CompBeads stained with your antibodies
  - care: tandem dyes

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Steps to your cells – setup PMT-V & Comp.

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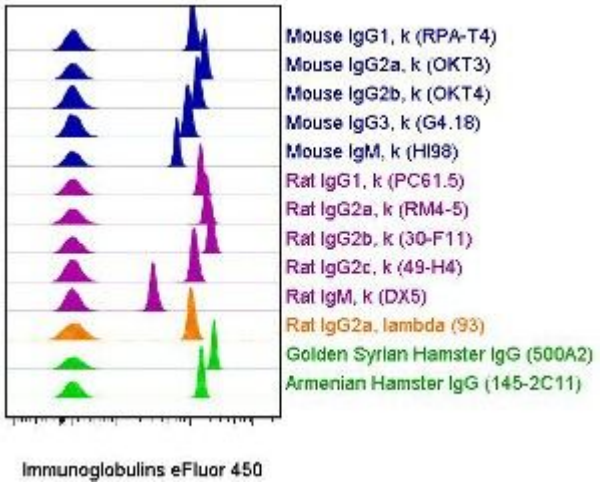
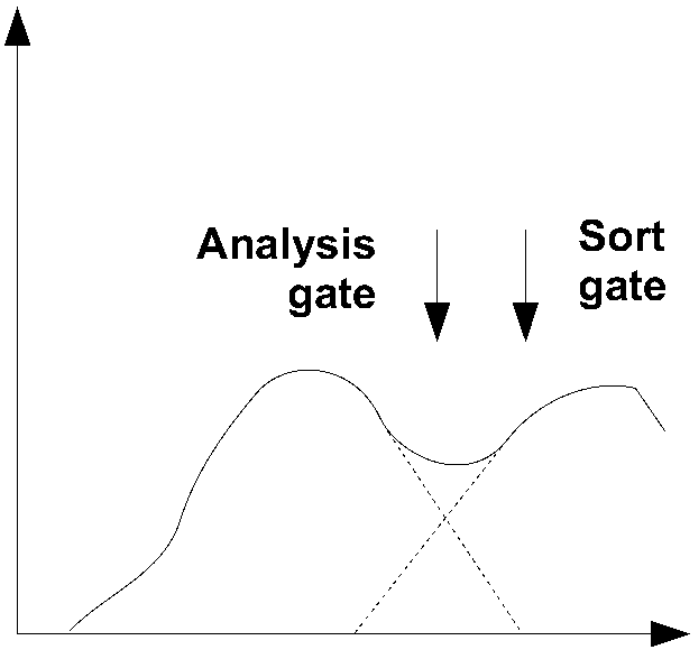
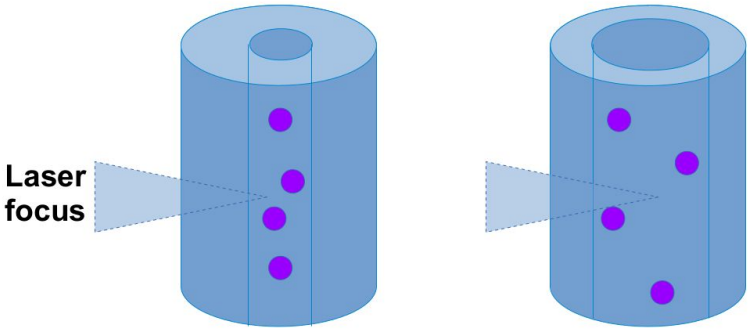


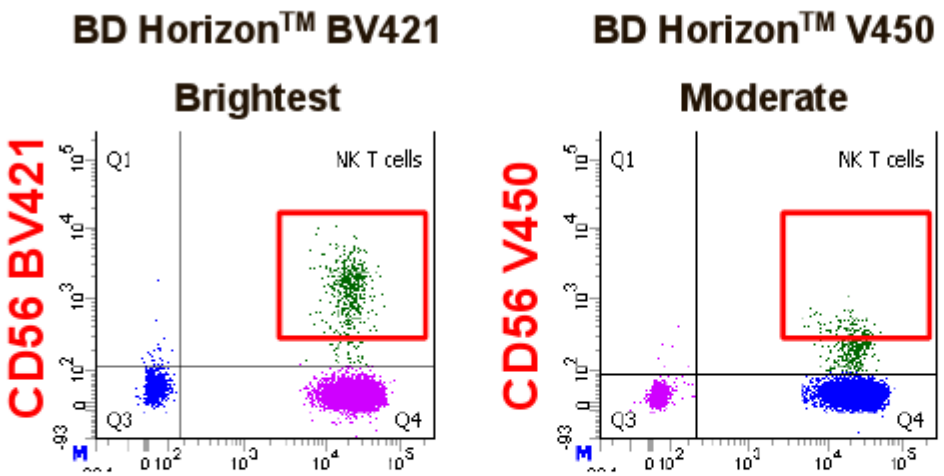
Steps to your cells – sorting gates



- Bad population separation will result in a loss of target cells!



Steps to your cells – sorting gates



Assumption:

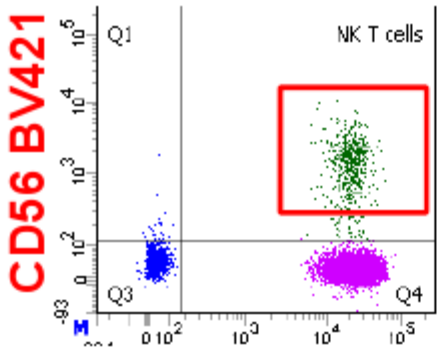
1 Mio nucleated cells in 100 µl whole blood

% NK-T cells		CD3 APC	
from total cells	: 0.27%	0.08%	
100.000 NK-T cells	: 37 Mio	125Mio	total cells
Volume blood	: 3.7 ml	12.5 ml	to be stained

Steps to your cells – sorting gates

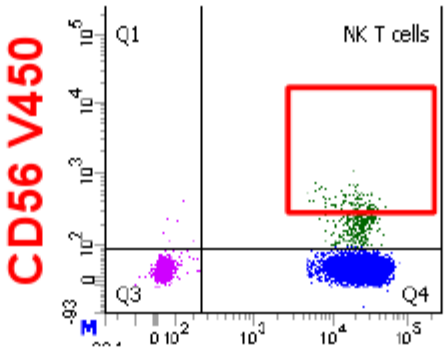
BD Horizon™ BV421

Brightest



BD Horizon™ V450

Moderate



Assumption:

Aria III, 70µm, 50psi, 90kHz

0 – 16 – 0

Concentration: 20.000 evt / sec

Calculate Sort-Times according to Poissons Statistics

% NK-T cells	**CD3 APC**	
from total cells	**: 0.27%**	**0.08%**
Time:	**48min**	**2.8hrs** **to sort 100.000 NK-T cells**

Steps to your cells – sorting gates

questions & discussion

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