Cell Sorting

An overview

Marcus Eich

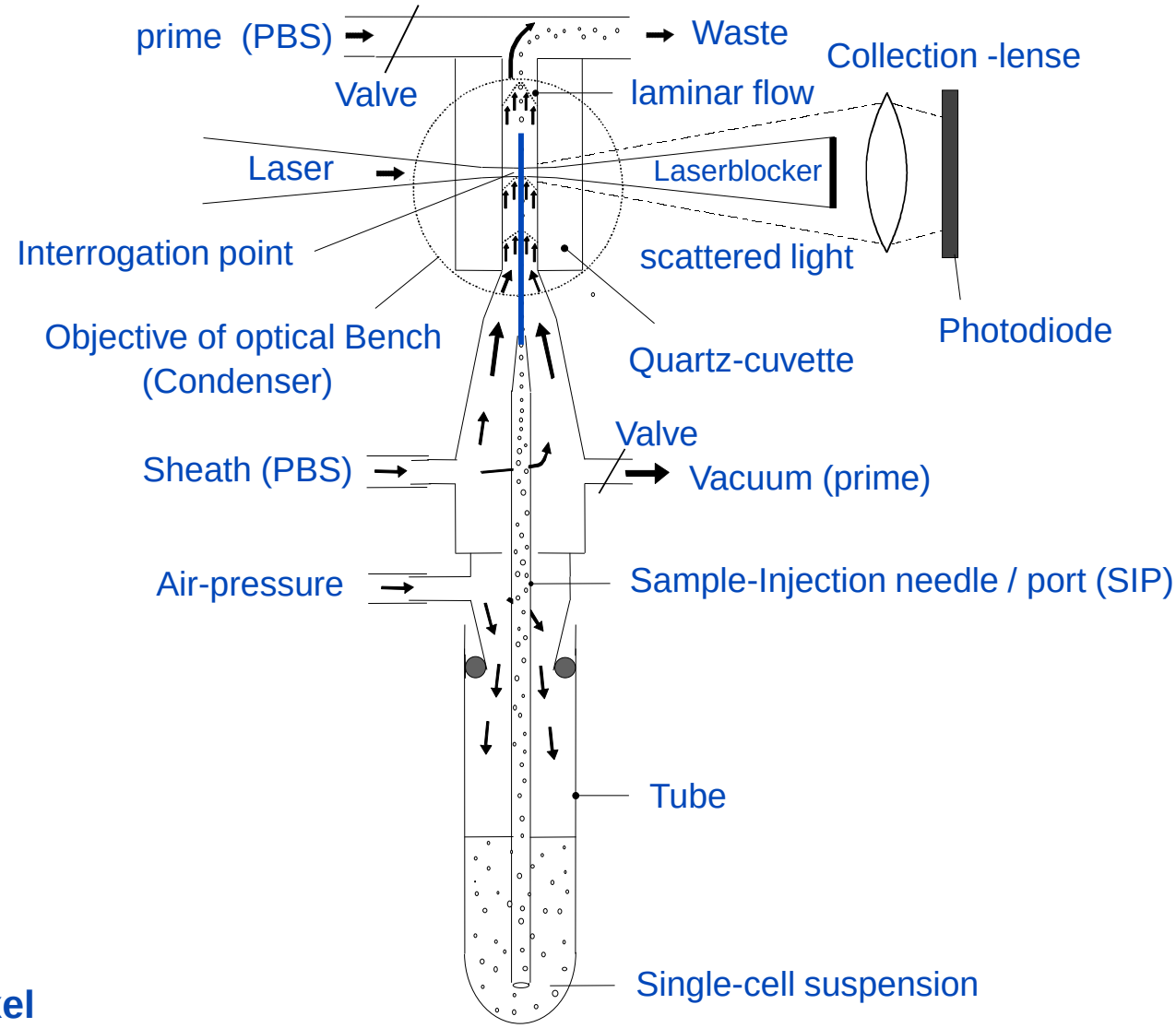
HI-STEM
HEIDELBERG INSTITUTE
FOR STEM CELL TECHNOLOGY
AND EXPERIMENTAL MEDICINE

dkfz.
DEUTSCHES
KREBSFORSCHUNGSZENTRUM
IN DER HELMHOLTZ-GEMEINSCHAFT

Possible applications

- RNA isolation, Single cell qPCR
- Protein isolation
- Discrimination of transfected cells (e.g. GFP)
- Single cell cloning
- Synchronization
- (Stem) Cell transplantation
- …

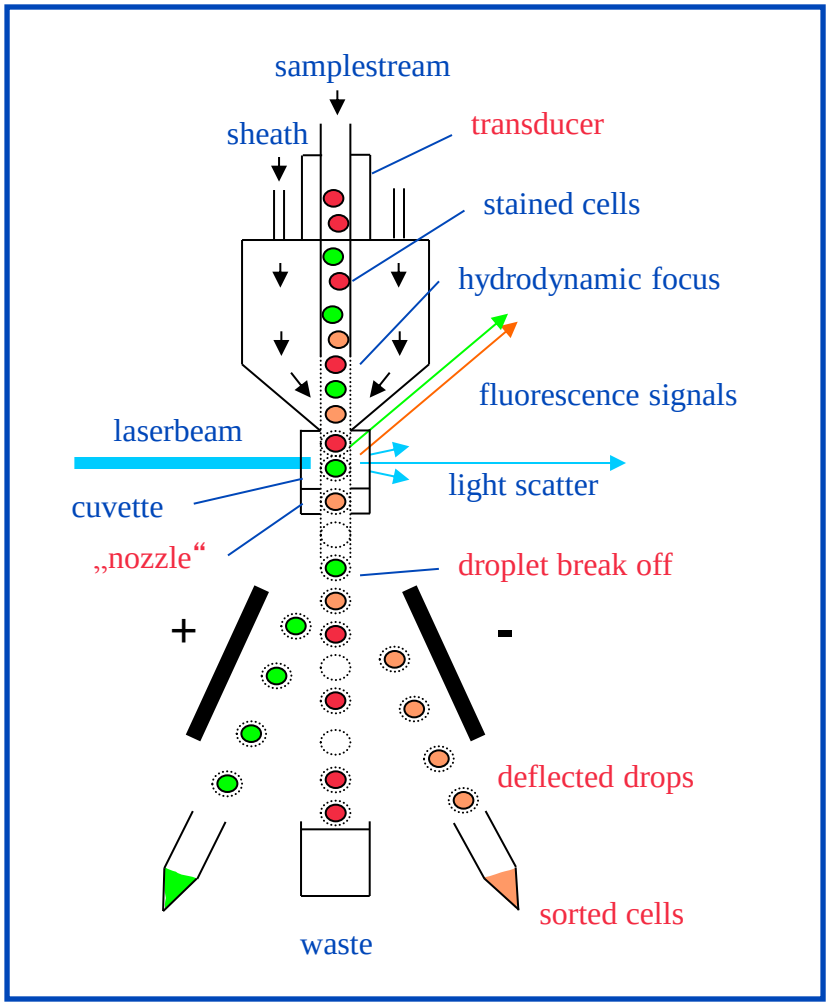
Cytometer basics



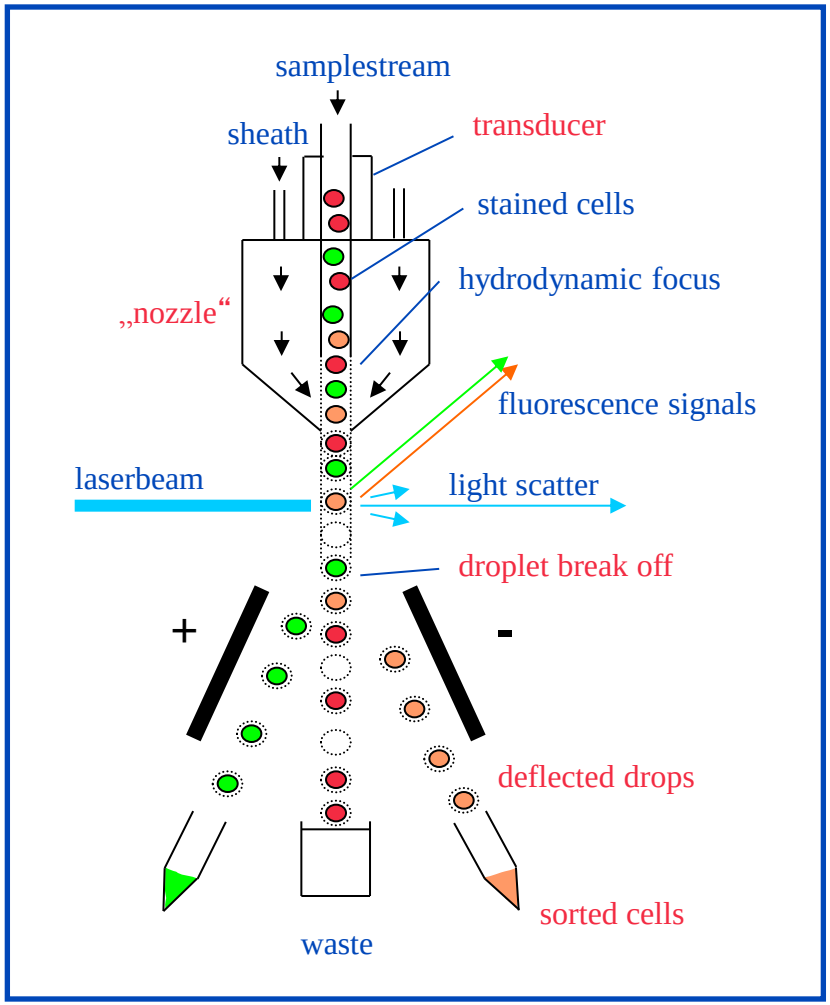
by Klaus Hexel

Principle of cell sorters

“Cuvette”

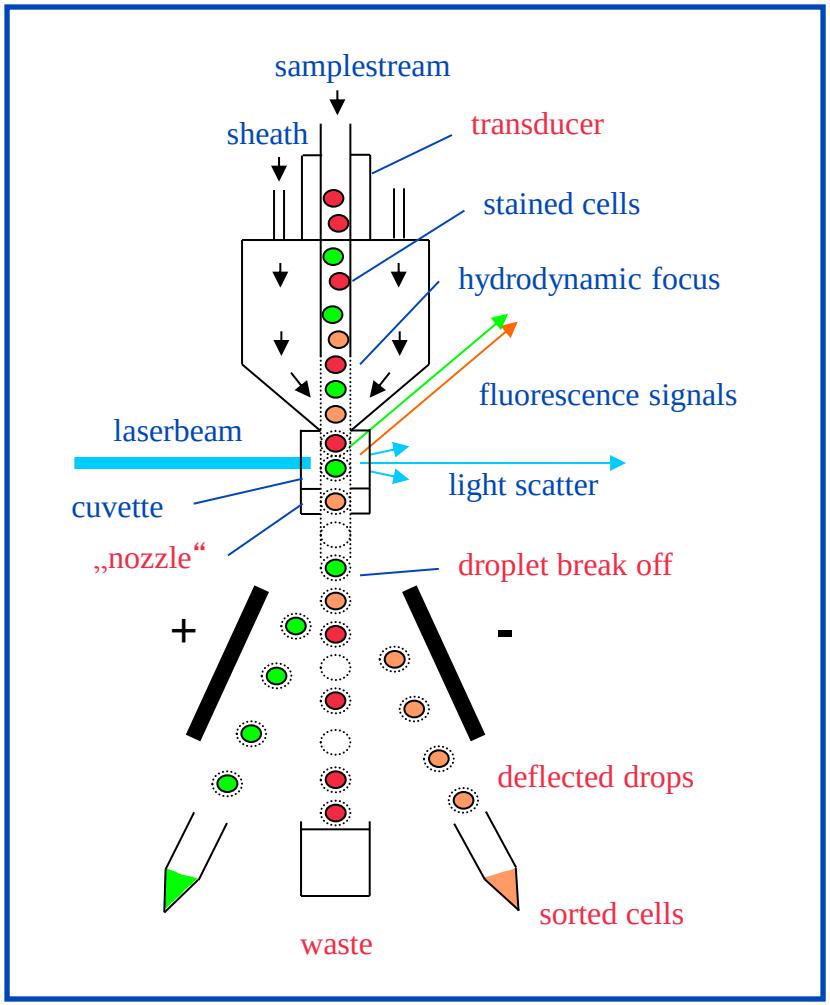


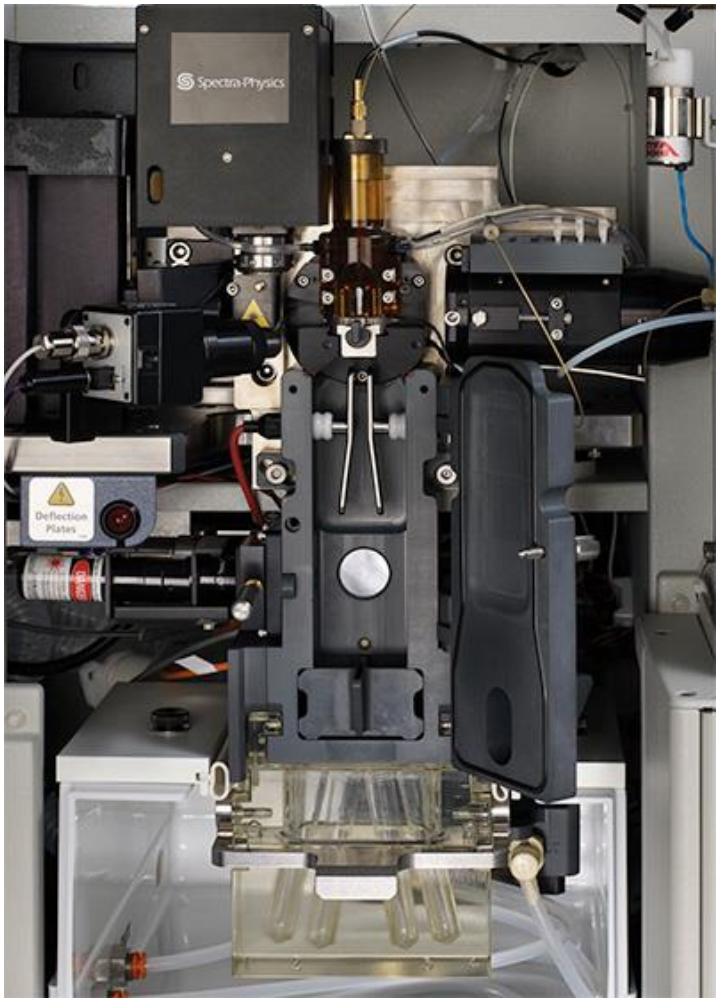
“Jet in Air”



Principle of cell sorters

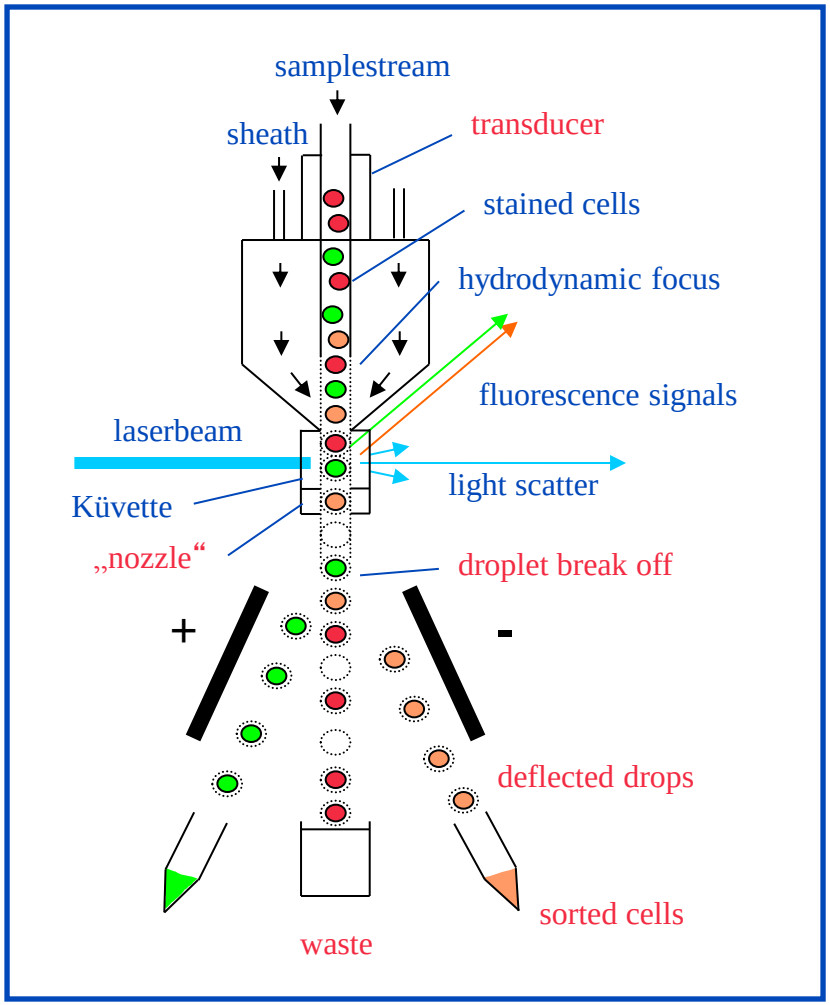
“Cuvette”

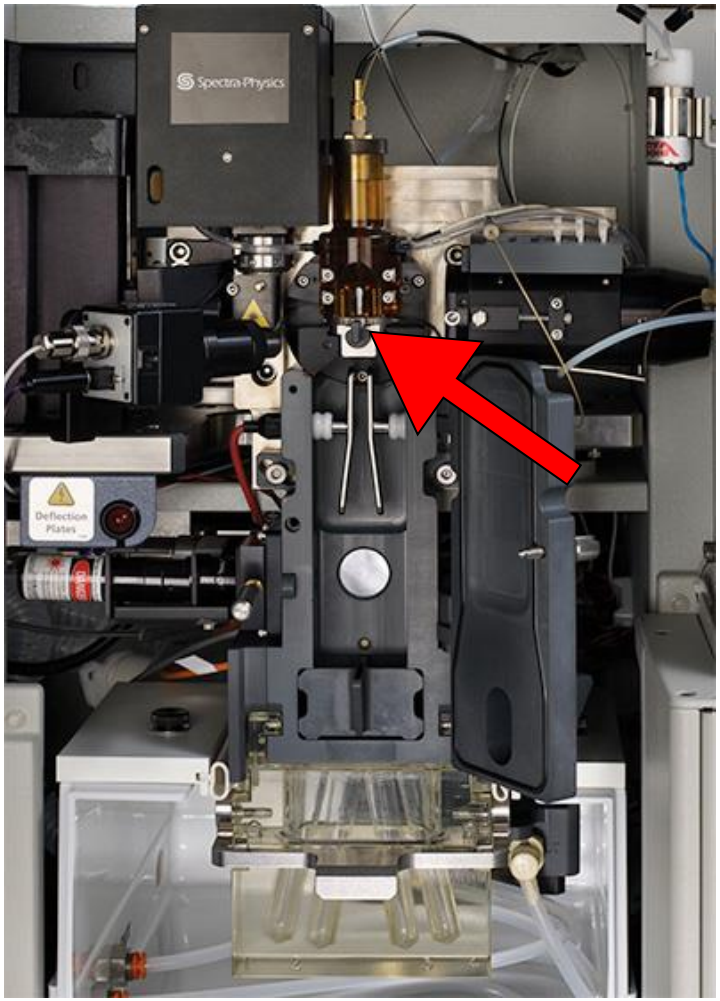




Principle of cell sorters

“Cuvette”





The nozzles

Sort speed increases ↑

- 70 µm → 87 KHz (87.000 drops/s)
- 85 µm → 47.7 KHz (47.700 drops/s)
- 100 µm → 28.2 KHz (28.200 drops/s)

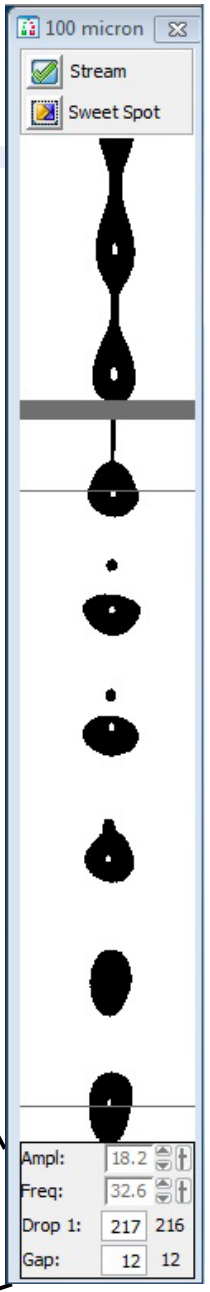
↓ Cell viability increases

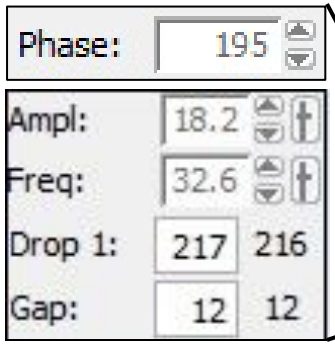
Nozzle diameter/3 → biggest cell size for sorting
Nozzle usage → depends on downstream assay

Stream & Drop Delay

How to set up the stream

- Set stream to a stable drop1 position having a constant gap by modulation of the phase and amplitude.
- Define drop delay under these constant conditions.





Drop Delay

Distance from interrogation point to drop1 position

Droplet characteristics

Droplet size

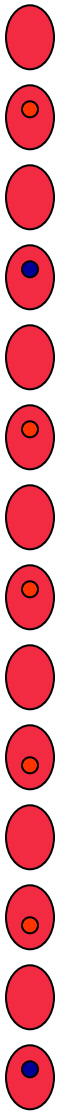
Nozzle/ pressure	Volume flow rate $\dot{V}$	Current velocity v	Droplet size V
70 µm/ 70 psi	95,0 $\frac{\mu L}{s}$	24,7 $\frac{m}{s}$ (88,9 $\frac{km}{h}$)	(*f = 87,0 kHz*) **1,1 nL**
85 µm/ 45 psi	118,8 $\frac{\mu L}{s}$	20,9 $\frac{m}{s}$ (75,4 $\frac{km}{h}$)	(*f = 47,7 kHz*) **2,5 nL**
100 µm/ 20 psi	101,0 $\frac{\mu L}{s}$	12,9 $\frac{m}{s}$ (46,3 $\frac{km}{h}$)	(*f = 28,2 kHz*) **3,2 nL**

Approximate droplet count per tube

Nozzle/ pressure	1,5 mL	5,0 mL	15 mL	per mL
70 µm/ 70 psi	1 363 637	4 545 454	13 636 363	*~900 000*
85 µm/ 45 psi	600 000	2 000 000	6 000 000	*~400 000*
100 µm/ 20 psi	468 750	1 562 500	4 687 500	*~310 000*

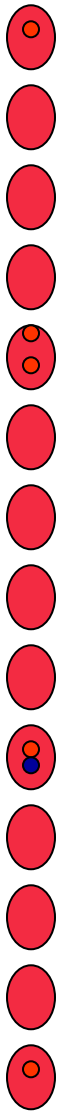
Calculation by Tobias Rubner

Distributions of cells in stream



In reality there is no equal distribution of particles.

Under this condition even rare particles could be separated without problems from other events.



A random distribution is reality.

Therefore, it becomes difficult to find rare events completely free of unwanted companions.

Max. sort speed with 70 µM

25.000 cells / sec

1.5 x 10^6 cells / min

9 x 10^7 cells / h

2,1 x 10^9 cells / d

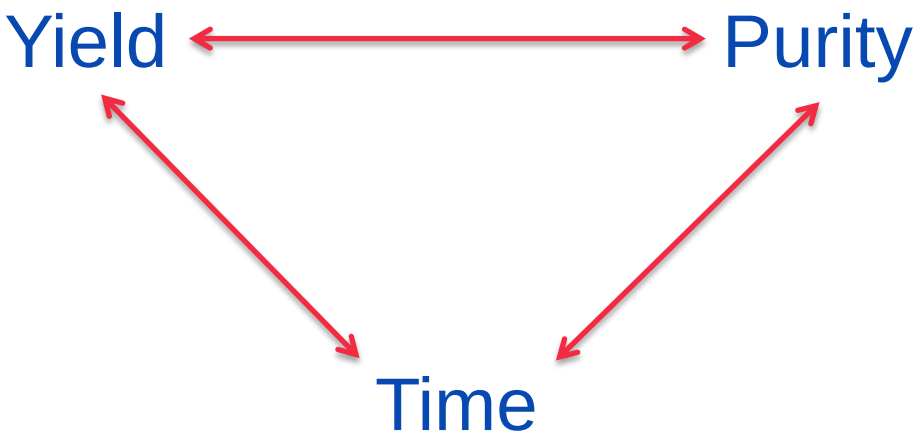
That means for a 0.1% population → 2x 10^6 cells / d

Possible targets

- 5 ml FACS tubes → 4 populations
- 1.5 ml tubes → 4 populations
- 15 ml tubes → 2 populations
- 6-well plates
- 24-well plates
- 96- well plates
- 384-well plates
- Glas slides

- Continuous cells
- Specific cell number

Limitations



Yield ↑ → Purity ↓ Time ↑
Purity ↑ → Yield ↓ Time ↑
Time ↓ → Yield ↓ Purity ↓

Time ↑ → Flowrate ↓

What is the aim of the sort?

Before cell sorting some considerations sould to be made:

- Which downstream assay is planned?
- What is the desired number of cells?
- Is high purity an issue?
- How may cells do you have to start with?
- What is the percentage of cells of interest?
- Is pre-enrichment or depletion (MACS, Ficoll, etc.) possible?

Calculation of the yield

10% cells of interest are in the sort gate

100.000 cells are analysed

10.000 cells could have been sorted

9.000 cells were sorted into the tube

Yield is 90%

Recovery

Mostly: 70% - 80% of the sort counts

Reason:

- Cells die and get fragmented → Cell sorting is a very stressful process for the cell
- Cells stick to the tube → Material of target is important
- Sample processing after sorting

Why does the cell sorter not detect all cells, which were counted after harvesting?

- Loss of cells during sample preparation before sorting: Centrifugation, Transfer, Washing, Errors during counting

Predefined Sort options

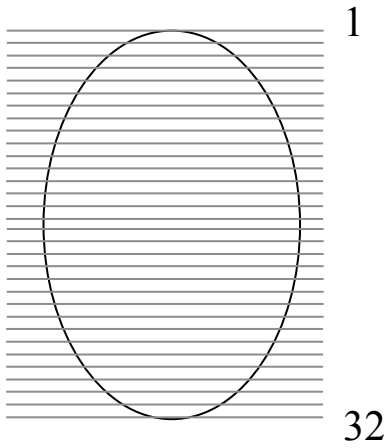
Sort precision mode:

- Yield → low purity depending on the flow rate
- Purity → low yield depending on the flow rate
- Single Cell → highest purity, very low yield

→ Determined by **sort masks**

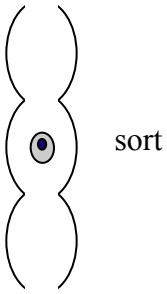
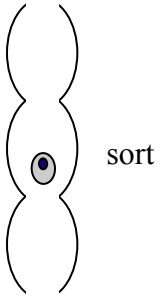
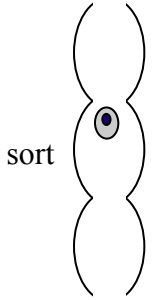
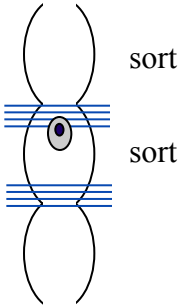
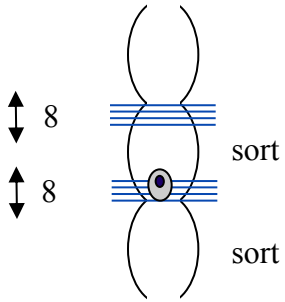
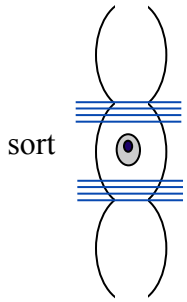
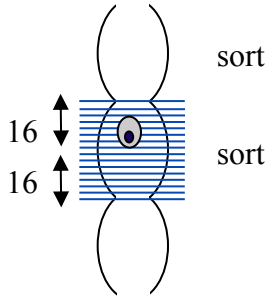
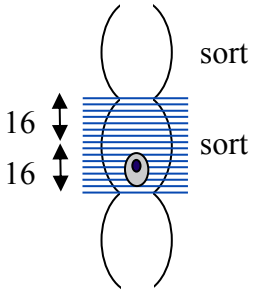
Sort-Masks

- There are **3 types of sort masks**, which in combination define the sort precision modes:
  - Yield mask
  - Purity mask
  - Phase mask

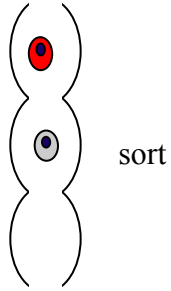
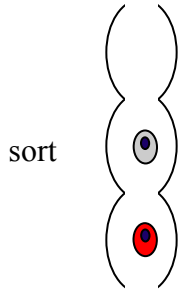
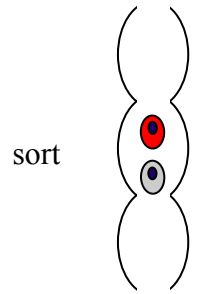
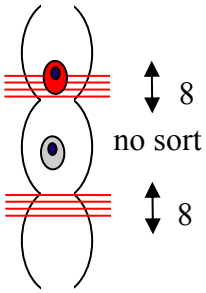
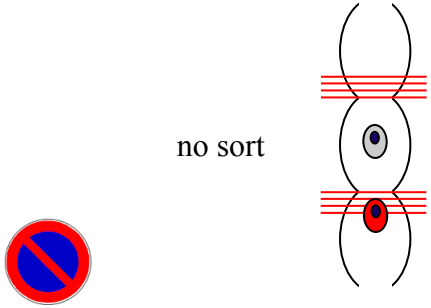
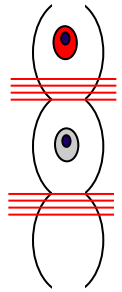
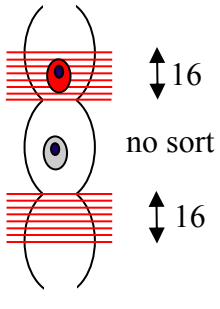
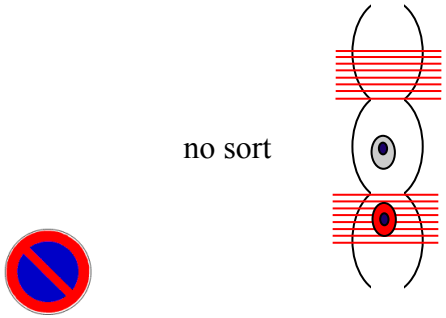


- Every drop is virtually divided in 32 segments.
- A cell can be located in one or more ogf these segments
- The relative position of the cell inside the drop influences the sort decision.

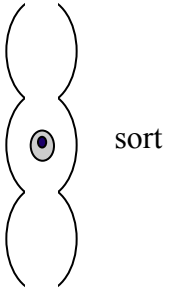
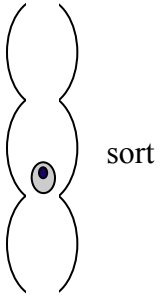
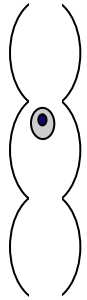
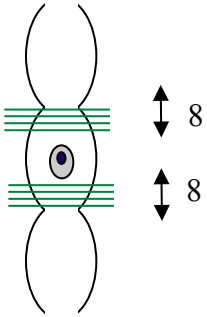
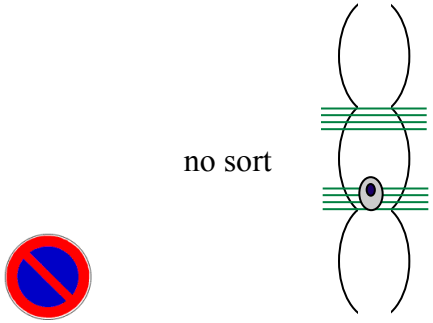
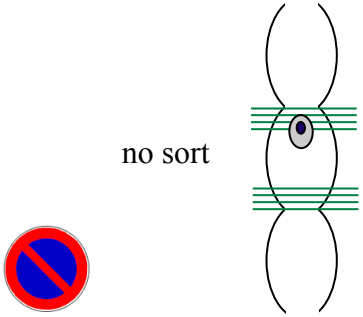
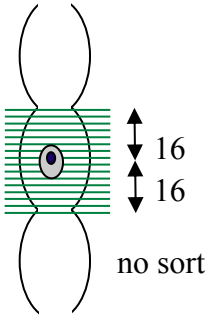
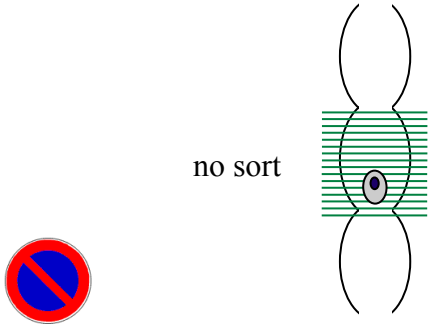
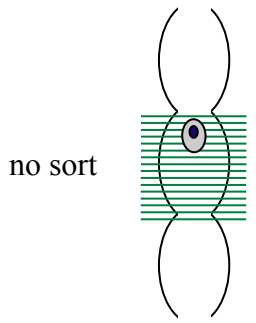
Yield Mask

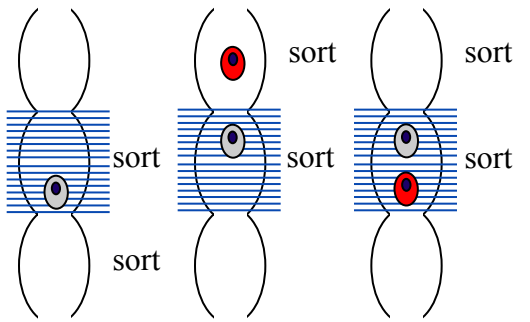
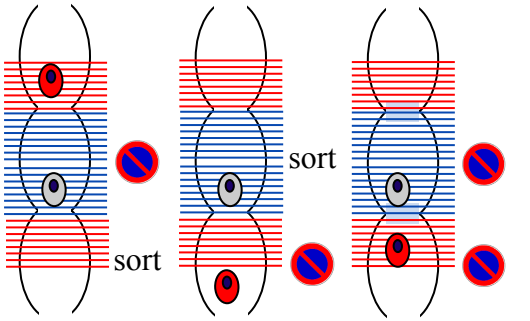
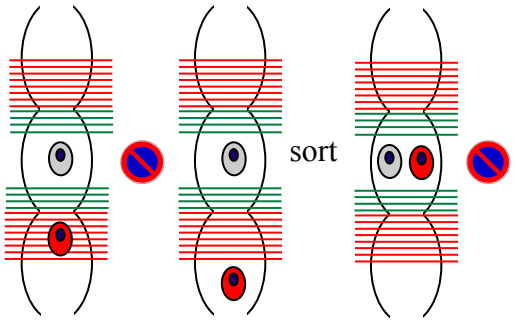
Sort envelope	Determinination of number of sorted drops, Non-target cells are not considered		
0	Only the calculated drop is sorted 		
16	If the cell lies at The border of the drop, 2 drops are sorted 		
32	Always 2 drops are sorted 		

Purity Mask

Sort envelope	Control for particles in neighboring drop, Non-target cells are considered		
0	No coincidence: Cell is sorted regardless the presence of an unwanted cell 		
16	Cells in the neighboring drop cause an abort (coincidence) 		
32	Cells in the neighboring drop cause an abort (coincidence) 		Non-Target cells located in the same drop like (sort envelope 16/32)

Phase Mask

Sort envelope	Cell position inside a drop determines the sort decision, Non-target cells are not considered		
0	Position of a cell inside a drop has no influence of sort decision 		
16	If cell is in the middle of the drop, drop is sorted 		
32	No cell is sorted, because it is always in the phase mask 		

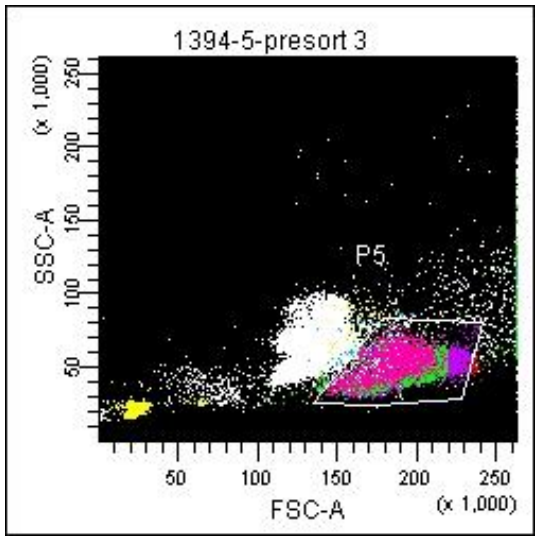
Sort precision	Explanation	Yield mask	Purity mask	Phase mask	Examples
Yield	Always two drops are sorted regardless of contaminating cells. No cells are lost.	**32**	**0**	**0**	
Purity	If possible two drops are sorted without contamination of non-target cells in the neighboring drop.	**32**	**32**	**0**	
Single cell	Drop is sorted, if the cell is in the middle of a drop. If the cell lies in the border a sort abort occurs.	**0**	**32**	**16**	

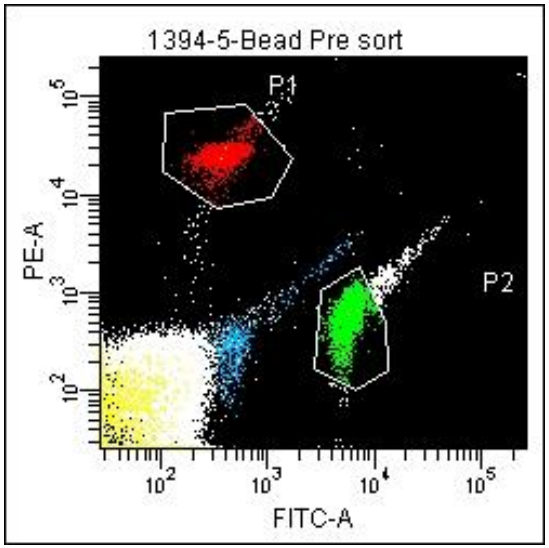
How to increase the sort quality

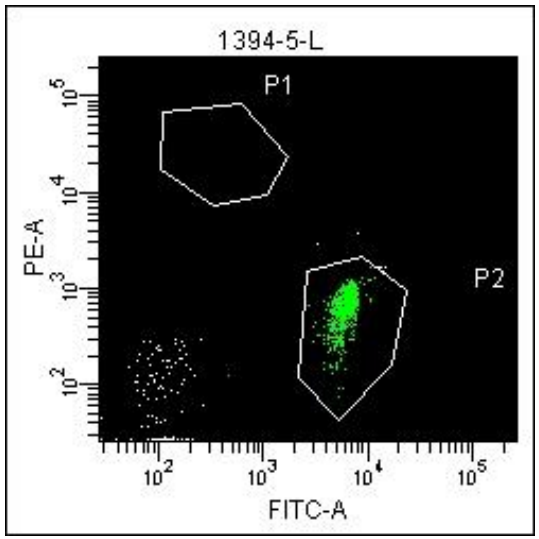
- Duplet exclusion
- Influence of target medium during reanalysis
- Multiple thresholds
- Reanalysis of „negative“ cells

1. Gating out Duplets

Sort P5 + P2 (Scatter + Fluorescence) → 1% Target population



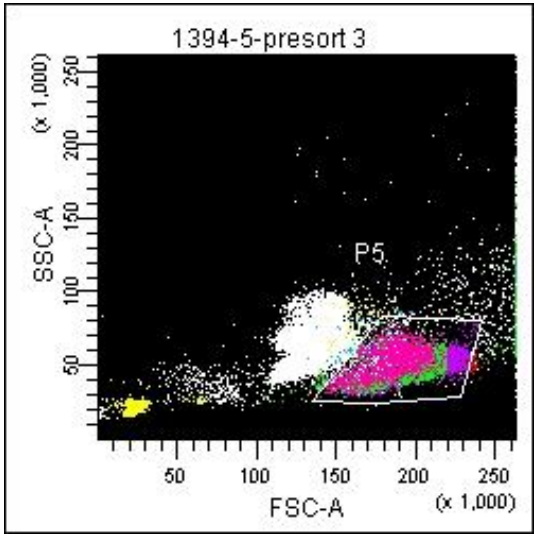


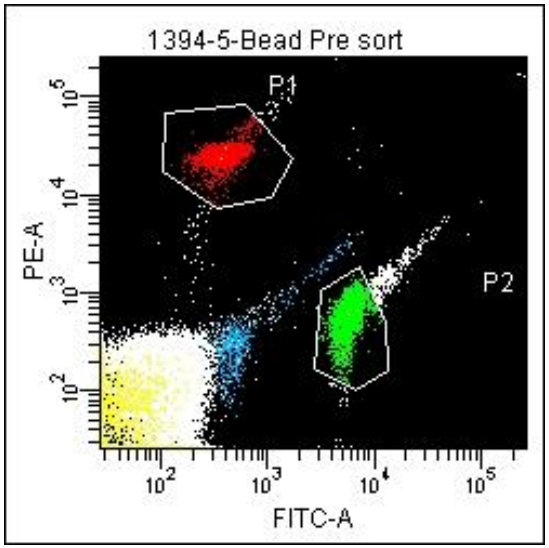


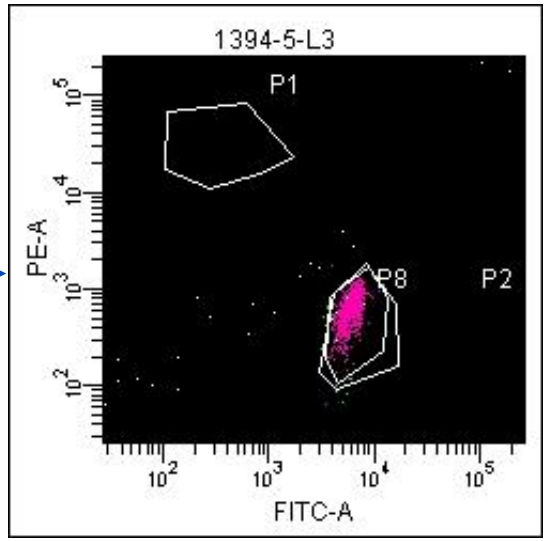
89% Purity
(not good enough!)

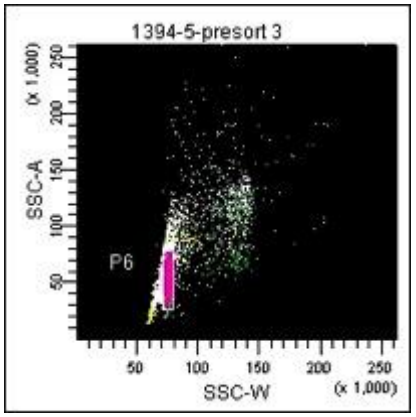
1. Gating out Duplets

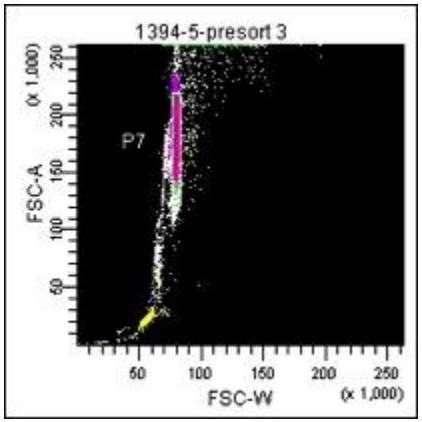
P5+P6+P7+P2 (Scatter + Duplets + Fluorescence) → 1% Target population





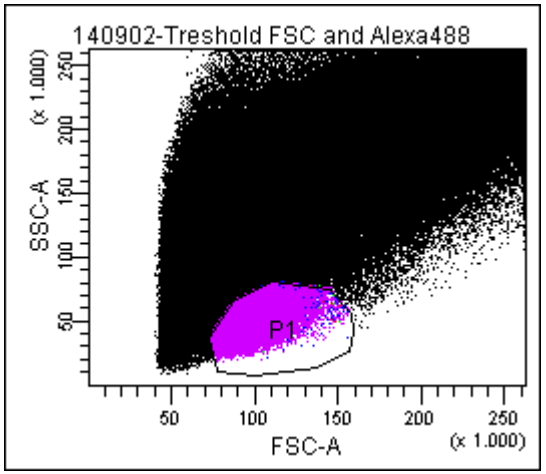


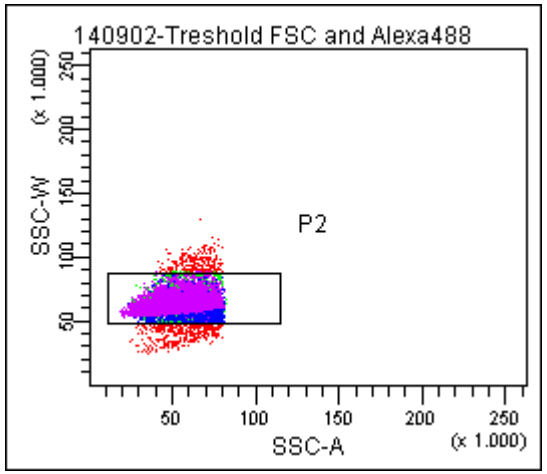


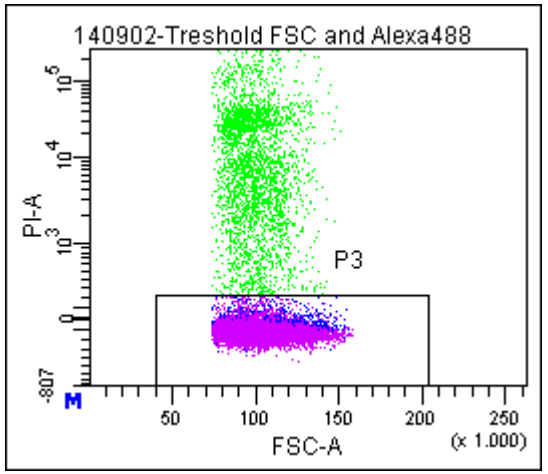


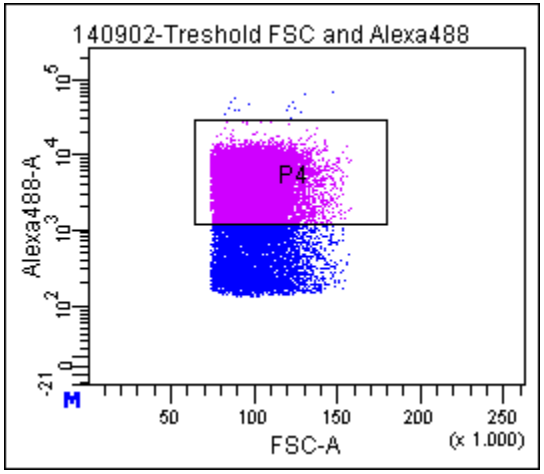
99% Purity

2. Influence of target medium





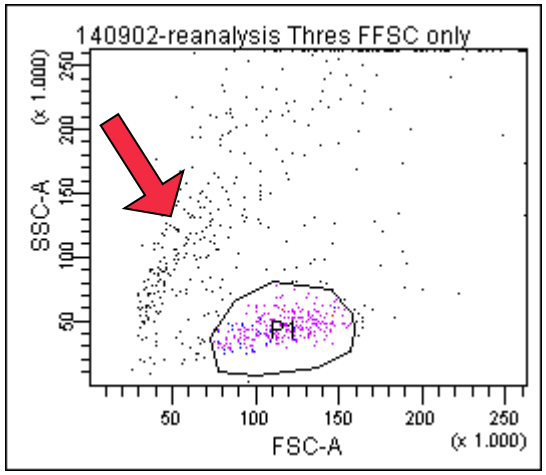


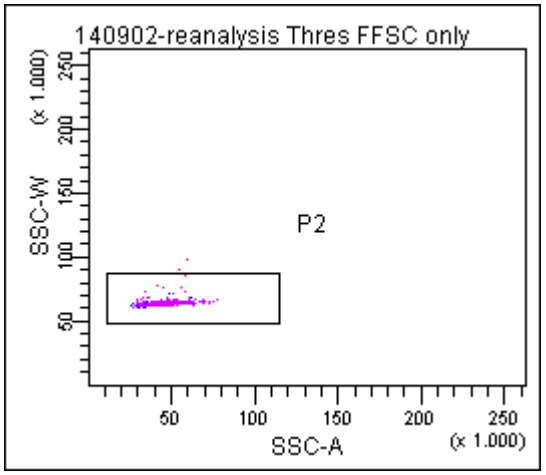


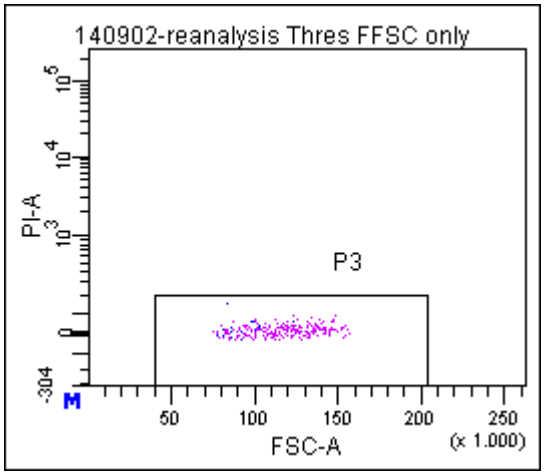
Tube: Treshold FSC and Alexa488

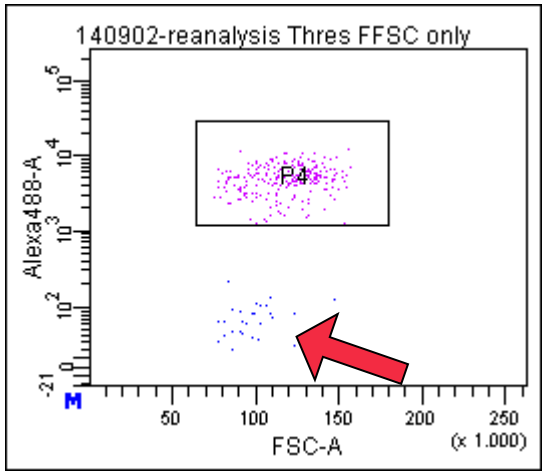
Population	#Events	%Parent	%Total
All Events	401.648	####	100,0
P1	34.427	8,6	8,6
P2	33.674	97,8	8,4
P3	30.676	91,1	7,6
P4	24.642	80,3	6,1

Re-Analysis of sorted events





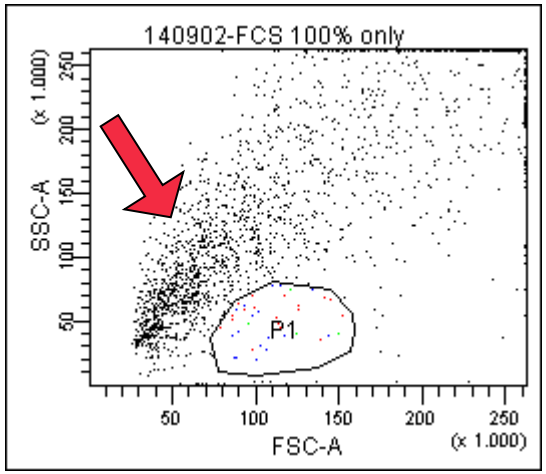


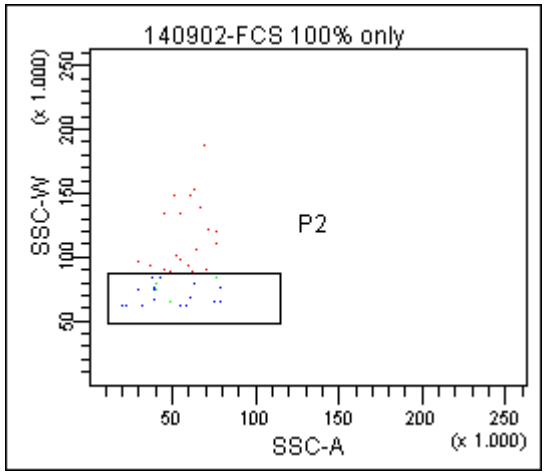


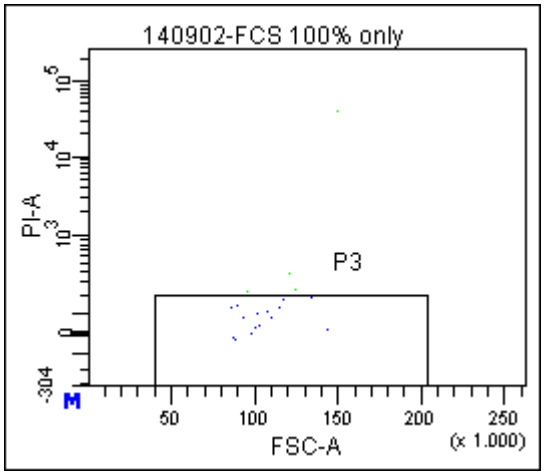
Tube: reanalysis Thres FFSC only

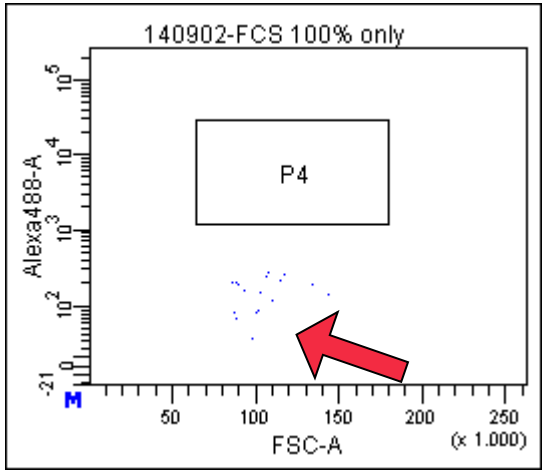
Population	#Events	%Parent	%Total
All Events	761	####	100,0
P1	268	35,2	35,2
P2	266	99,3	35,0
P3	266	100,0	35,0
P4	239	89,8	31,4

Control: FCS alone





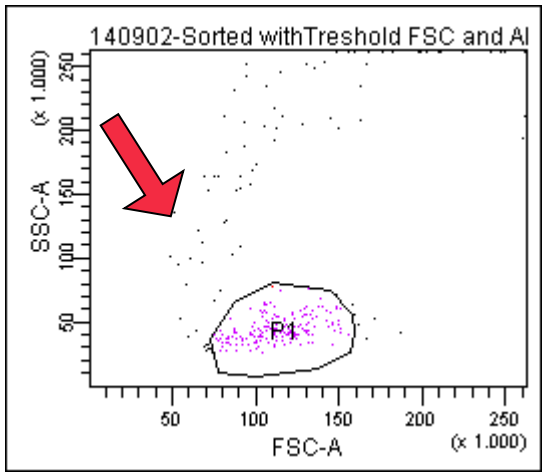


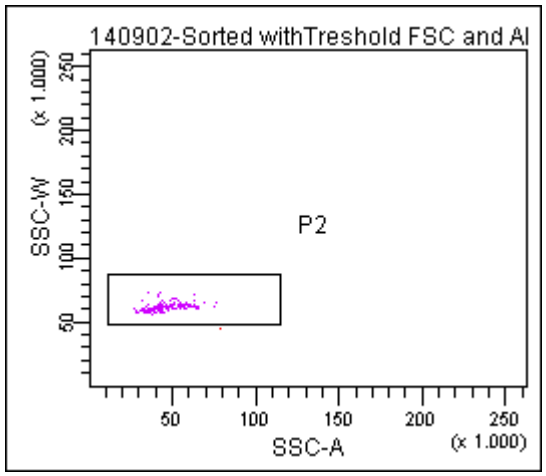


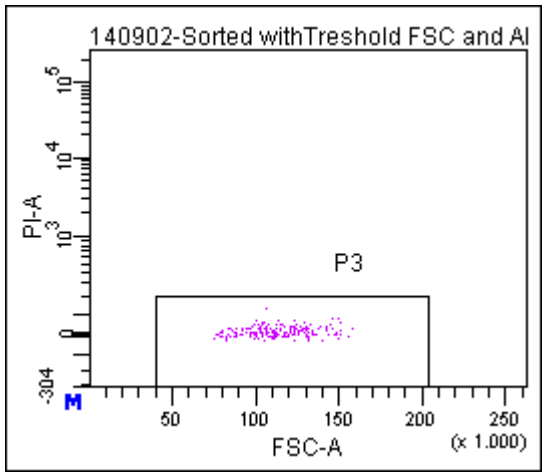
Tube: FCS 100% only

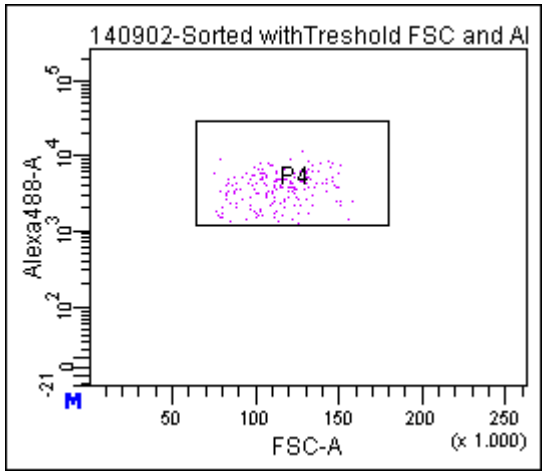
Population	#Events	%Parent	%Total
All Events	2.161	####	100,0
P1	42	1,9	1,9
P2	21	50,0	1,0
P3	17	81,0	0,8
P4	0	0,0	0,0

Reanalysis without FCS









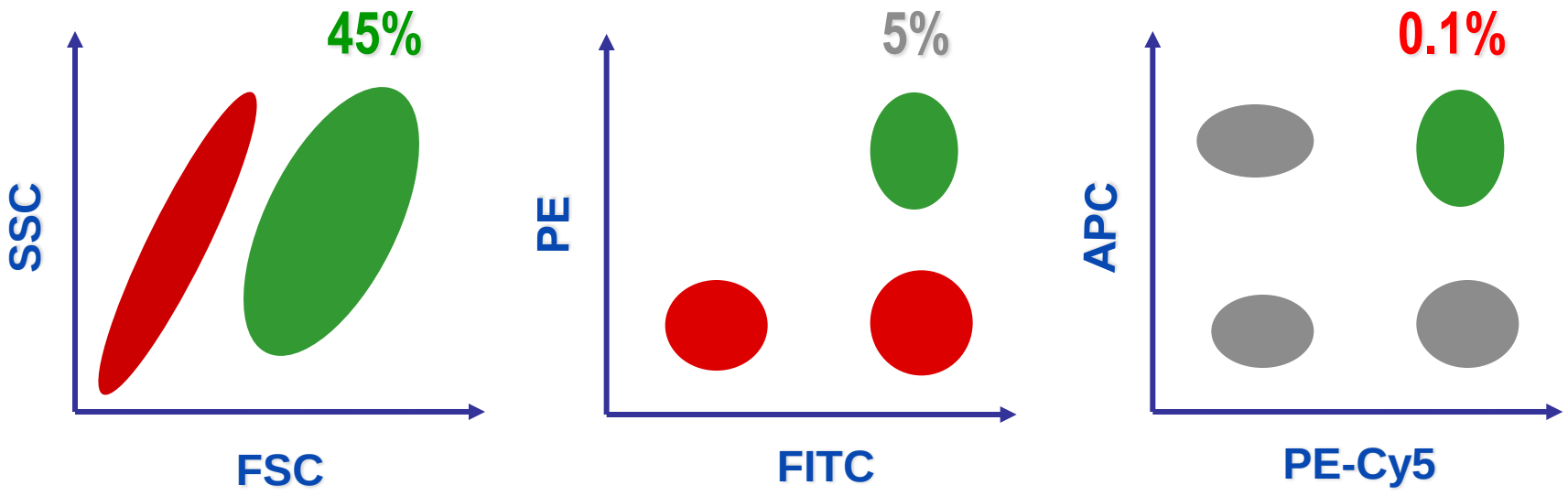
Tube: Sorted withTreshold FSC and Alexa488_001

Population	#Events	%Parent	%Total
All Events	293	####	100,0
P1	184	62,8	62,8
P2	183	99,5	62,5
P3	183	100,0	62,5
P4	183	100,0	62,5

3. Multiple Thresholds

Thresholds can be set for each parameter separately.

These can be combined multiple thresholds.

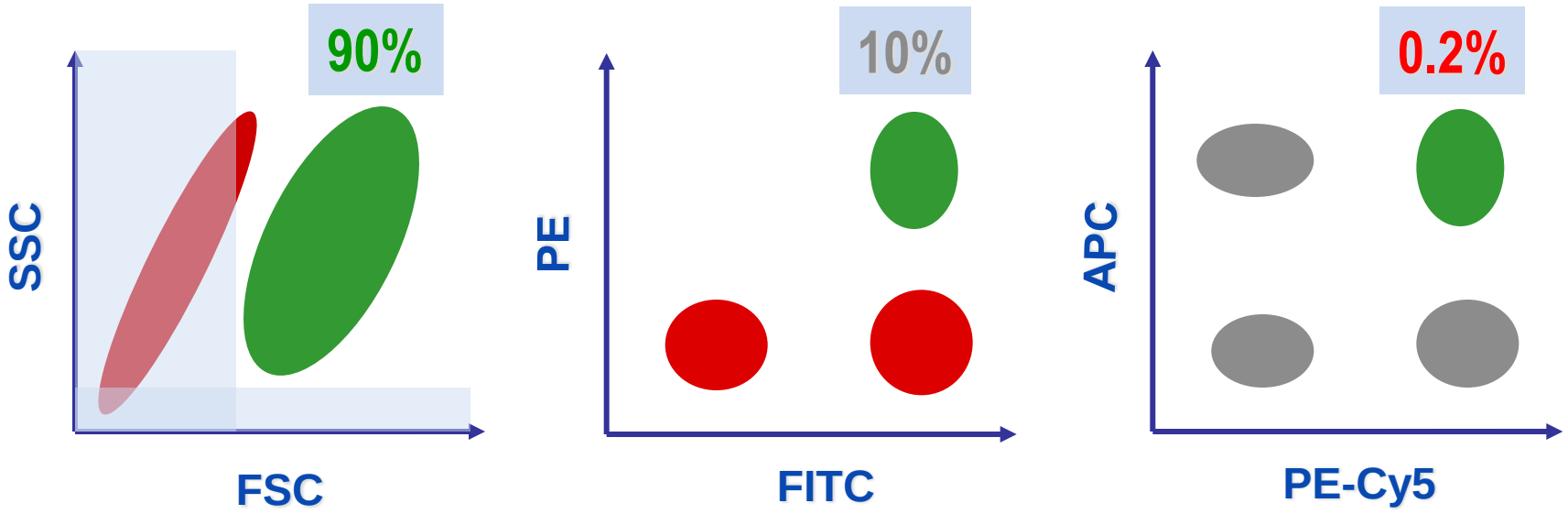


by Jochen Barths

3. Multiple Thresholds

Thresholds can be set for each parameter separately.

These can be combined multiple thresholds.



by Jochen Barths

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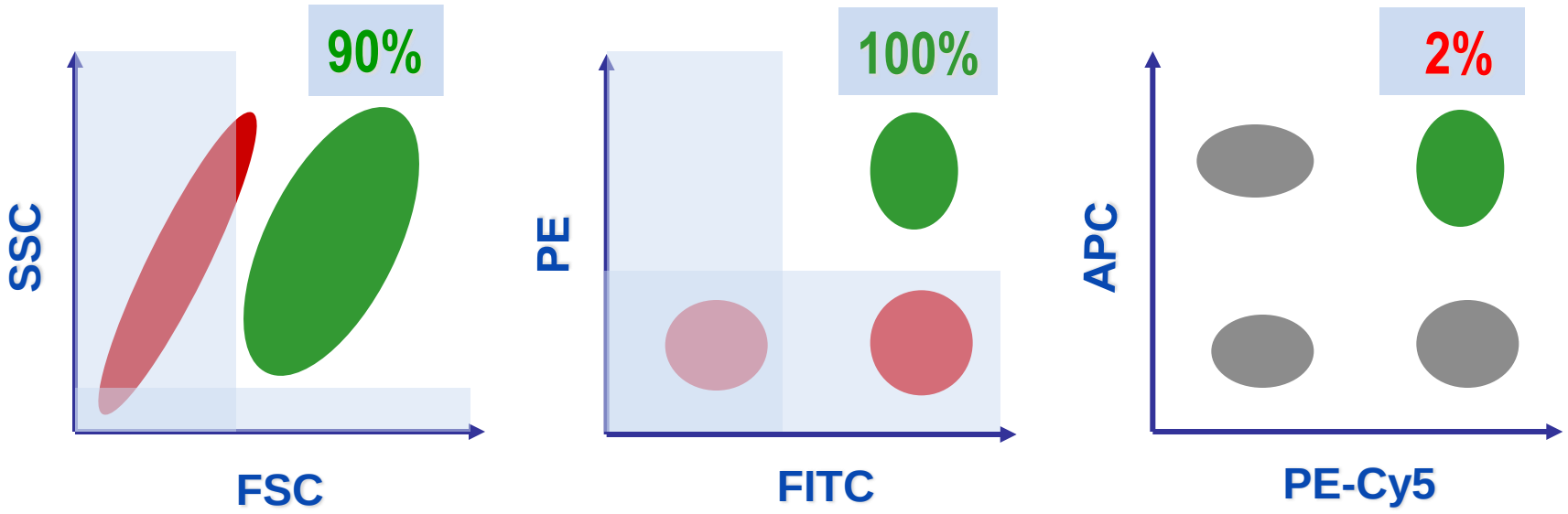

by Jochen Barths

3. Multiple Thresholds

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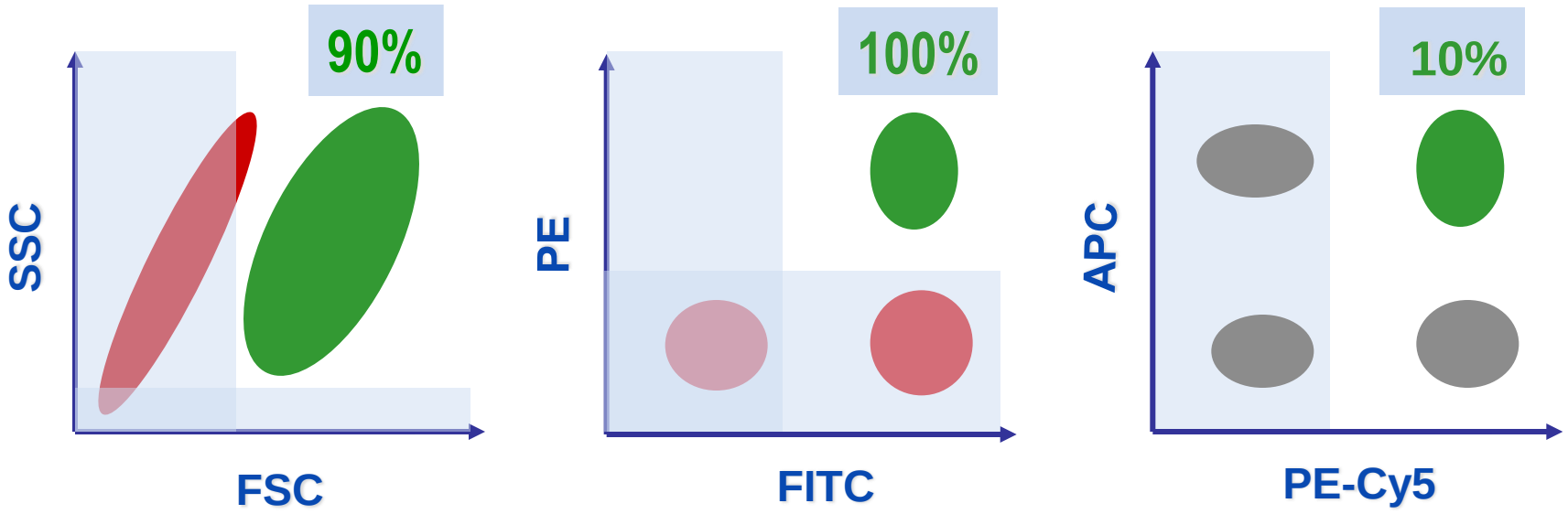


by Jochen Barths

3. Multiple Thresholds

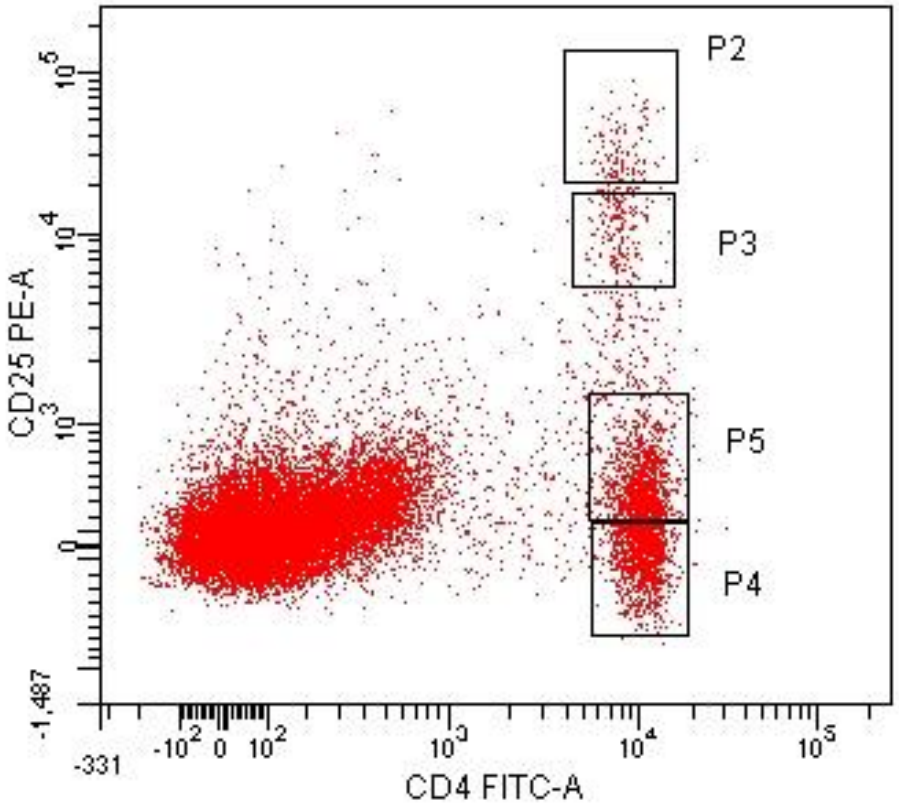
Advantages

- Analysis only of the cells of interest at a faster rate
- Rare cell populations become better visible on the plots
- High throughput sorting
- Isolation of rare cells at high speed with high yield

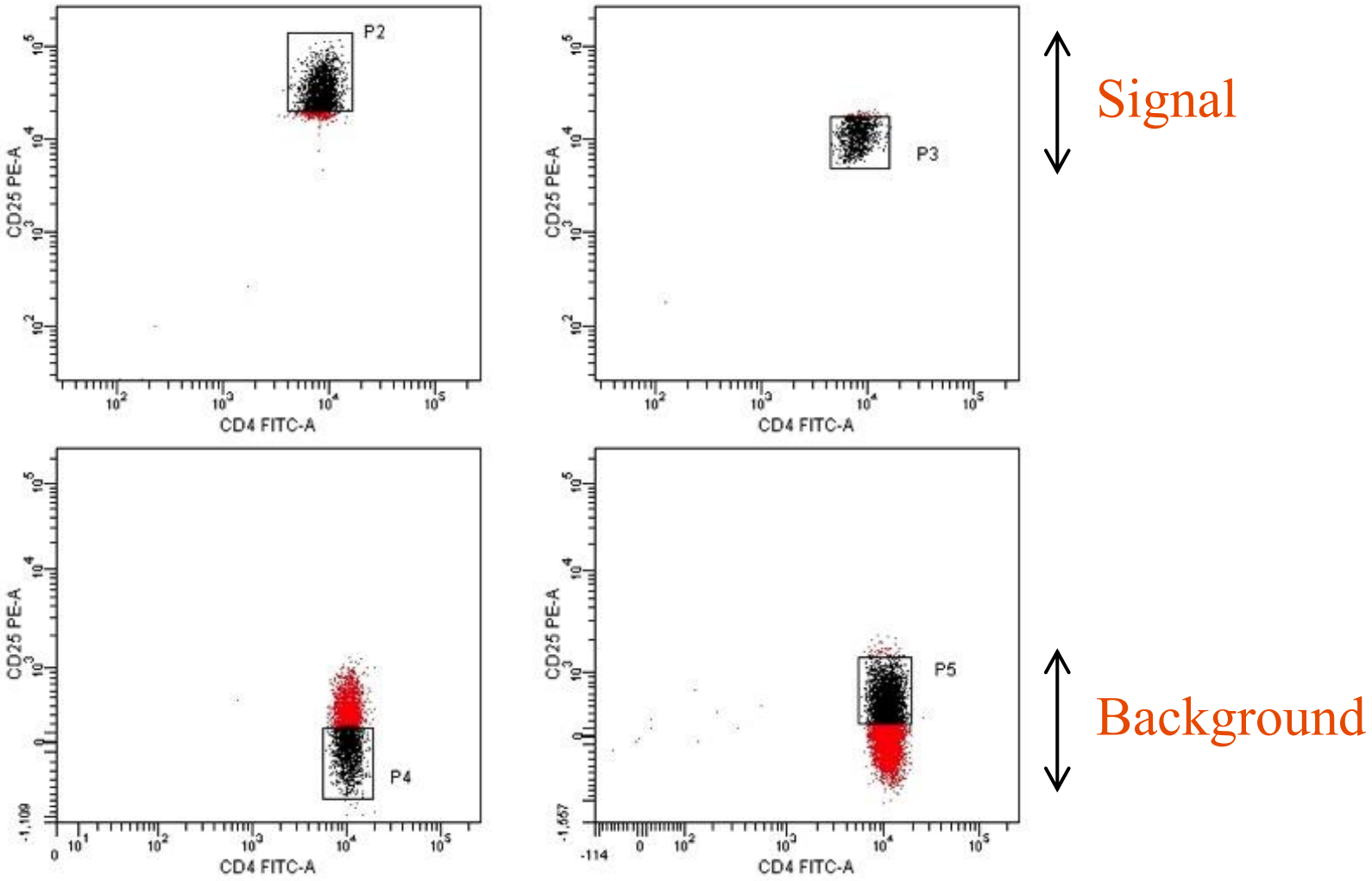
Disadvantage

- Purity cannot be achieved, because events under the thresholds are not analysed and evaluated for the sort decision

4. Another sort example . . .

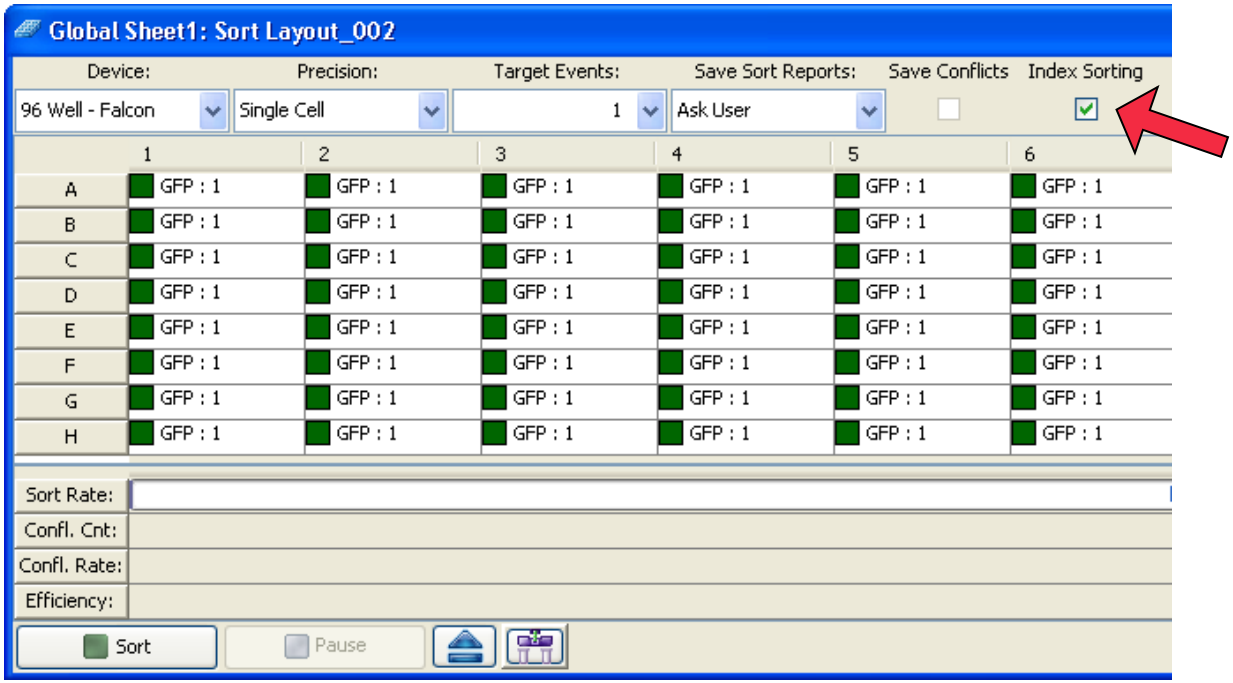


Reanalysis: Is it pure ?



Index sort

- Sorting of single cells into single wells.
- Detection of the sorted cells on histograms, plots and in the statistics



Index sort

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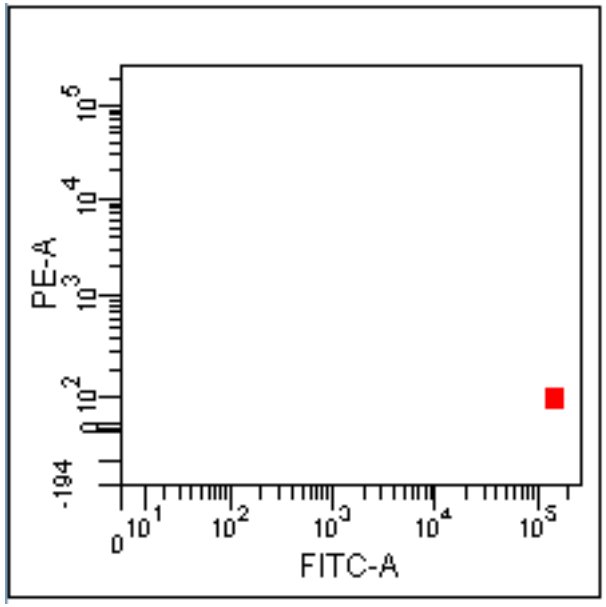

	A	B	C
1	Experiment Name	Experiment_028	
2	Specimen Name	Tube_001	
3	Tube Name	Tube_1000-50	
4	Record Date	3/20/2012 12:39	
5	Operator		
6	GUID	fa8e74f2-a681-4dc9-ab98-309db7bb094d	
7	Date Analyzed	5/22/2012 10:40	
8			
9	Sort Type	Index Sort	
10	Sort Setup	100 micron	
11	Precision	Single Cell	
12	Device	Slide - Frosted End	
13	Yield Mask	0	
14	Purity Mask	32	
15	Phase Mask	16	
16	Well	PE OR FITC FITC-A Mean	PE OR FITC PE-A Mean
17	A1	10,207	39,359
18	A2	9,511	35,718
19	A3	143,557	98
20	B1	147,000	91
21	B2	9,481	39,525
22	B3	134,630	88
23	C1	9,546	37,958
24	C2	141,284	83
25	C3	132,301	90
26	D1	11,261	41,732
27	D2	9,756	40,026
28	D3	9,684	38,868
29	E1	10,162	40,435
30	E2	9,384	40,557
31	E3	146,927	88
32	F1	9,670	37,902
33	F2	132,584	99

Experiment_028IS Results5