What is wrong with my flow cytometry data?

Hints, tricks and pitfalls

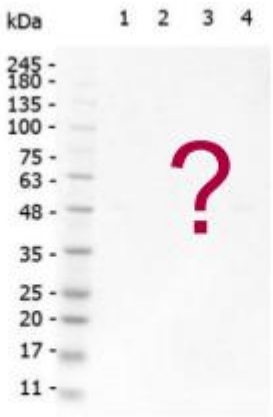
Universität Rostock Traditio et Innovatio

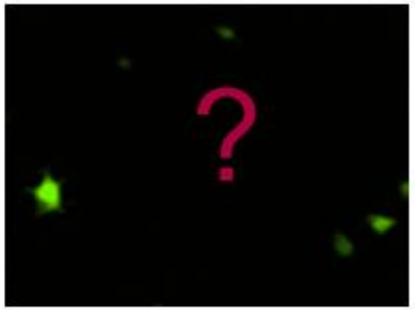
Universitätsmedizin Rostock

Webinar 8
05.09.2023

FACSorting – selecting the fit ones

The run for specific signals…





- samples often heterogenious: e.g. blood, spleen, organ and tumor biopsies
- not all cells react to stimulus equally (e.g. activation, proliferation, apoptosis)
- heterogenity can mask effects and reduce resolution
- reviewer might ask anyway: who is reacting/producing something?

…is the run for homogeneity.

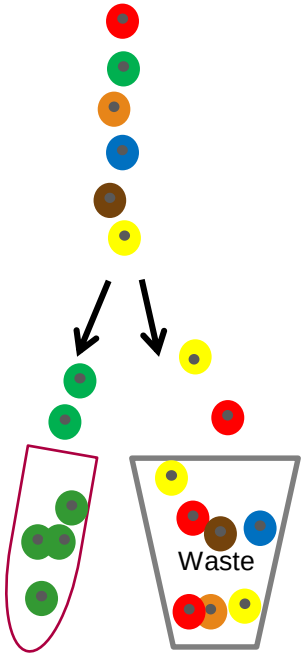
Cell sorting

+

- defined sample (homogenous) for downstream analysis, culture, …
- increased resolution of downstream applications
- extraction of rare cell types (e.g. stem cells, side populations,…)

–

- preparation before and afterwards (time and reagents)
- costs
- cell loss

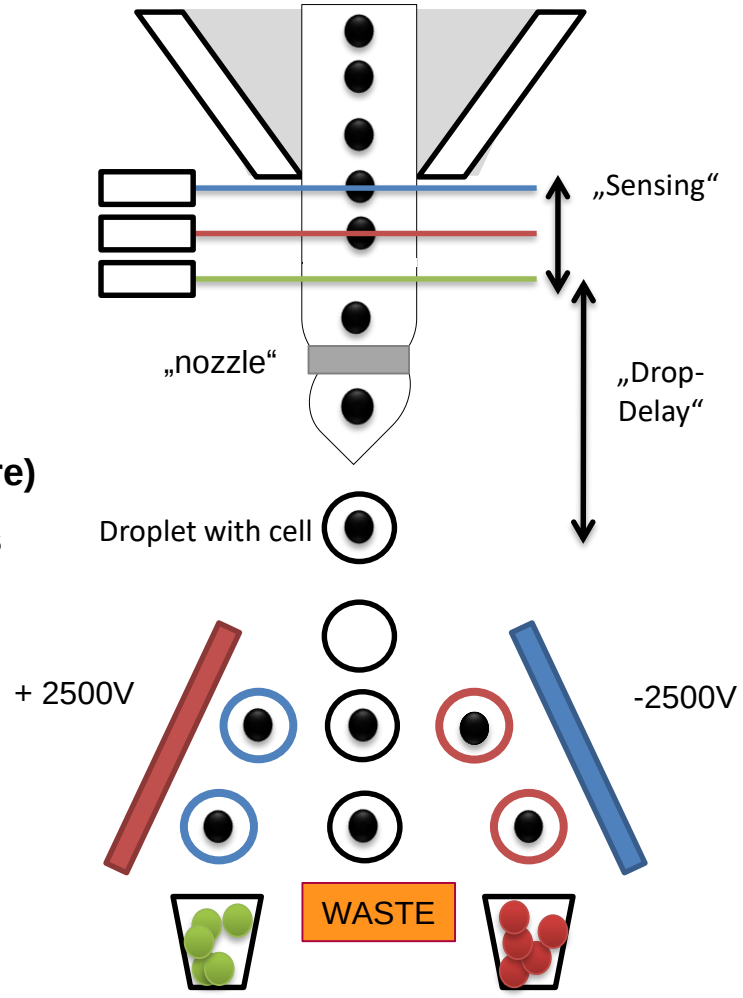


Cell separation systems

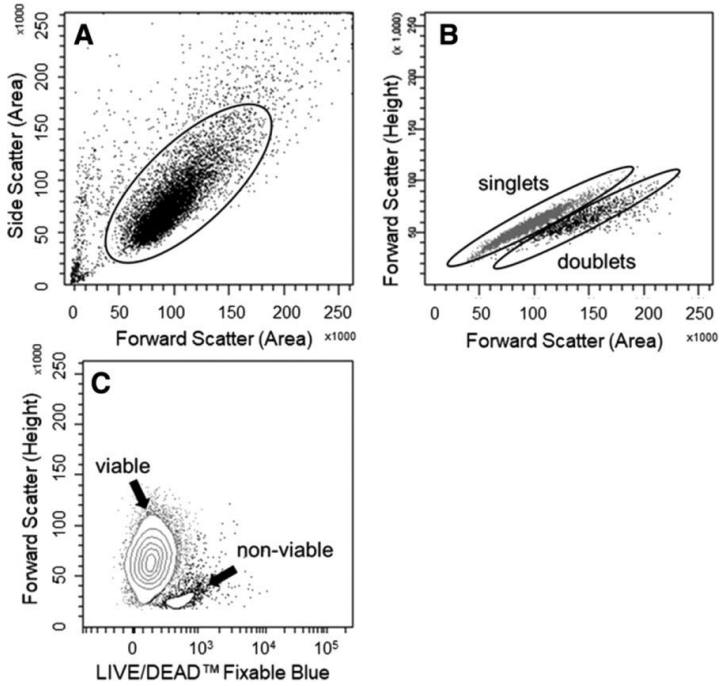
	Density gradient centrifugation	Pluribead	MACS	FACS
				
principle	cell size	bead based, size exclusion	magnetic antibody-labelled beads	fluorescence labeled antibodies
parameters fractions	1 ≤ 3	1 2 (+/-)	1 2 (+/-)	16 ≤ 4
time	+	+	+	(+/-)
costs	++	++	++	+ (depends on project)
+	SOP no add. equipment	low cell stress no add. equipm.	reproducible, scalable	flexible, different plates/tubes

Fluorescence-activated cell sorting **FACS**

- data-based cell sorting
- droplets created after nozzle
- droplets are electrically charged and depending on the signal directed towards positive or negative plates
- sorting in tubes or plates
- **high purity, but also high cell stress (EM-fields, fragmentation after droplet creation, sheath pressure) and possible activation of e.g. T cells or neutrophils**



Practical considerations



- exclude doublets → would impact purity
- include live-dead-stain (discriminate already disturbed cells)
- single cell suspension (not too dense, not too diluted) needed → always! filtered 30-50 µM nylon mesh
- bring cells in buffer **w/o** azide or medium **w/o** phenol red but **w/** EDTA or DNase
- bring collection devices (plate, tubes, etc) with high serum-medium (or lysis buffer if desired) → will be diluted
  - HEPES-buffered and conditioned media for single-cell sorting
- good panel for analysis ≠ good panel for sorting (reduce necessary markers to increase speed and resolution = higher sorting efficiency)

The impact of cell size on…

https://expert.cheekyscientist.com/improve-flow-cytometry-cell-sorting-speed/

The impact of cell size on … time and speed

Cell Type	Average Volume (μm^3)	Average Diameter (µm)	Recommend Nozzle Size (µm)
sperm cell	30	3.86	70
red blood cell	100	5.76	70
lymphocyte	130	6.29	70
neutrophil	300	8.31	70
fibroblast	2000	15.63	85
HeLa cell	3000	17.89	100
osteoblast	4000	19.69	130

Nozzle diameter	Sheath pressure	Frequency
50 µm	60-80 psi	120-160 KHz
70 µm	45-60 psi	65-100 KHz
80 µm	35-50 psi	45-80 KHz
100 µm	16-30 psi	28-45 KHz
130 µm	10-15 psi	16-25 KHz
150 µm	6-10 psi	7-12 KHz

= droplets / sec

https://expert.cheekyscientist.com/improve-flow-cytometry-cell-sorting-speed/

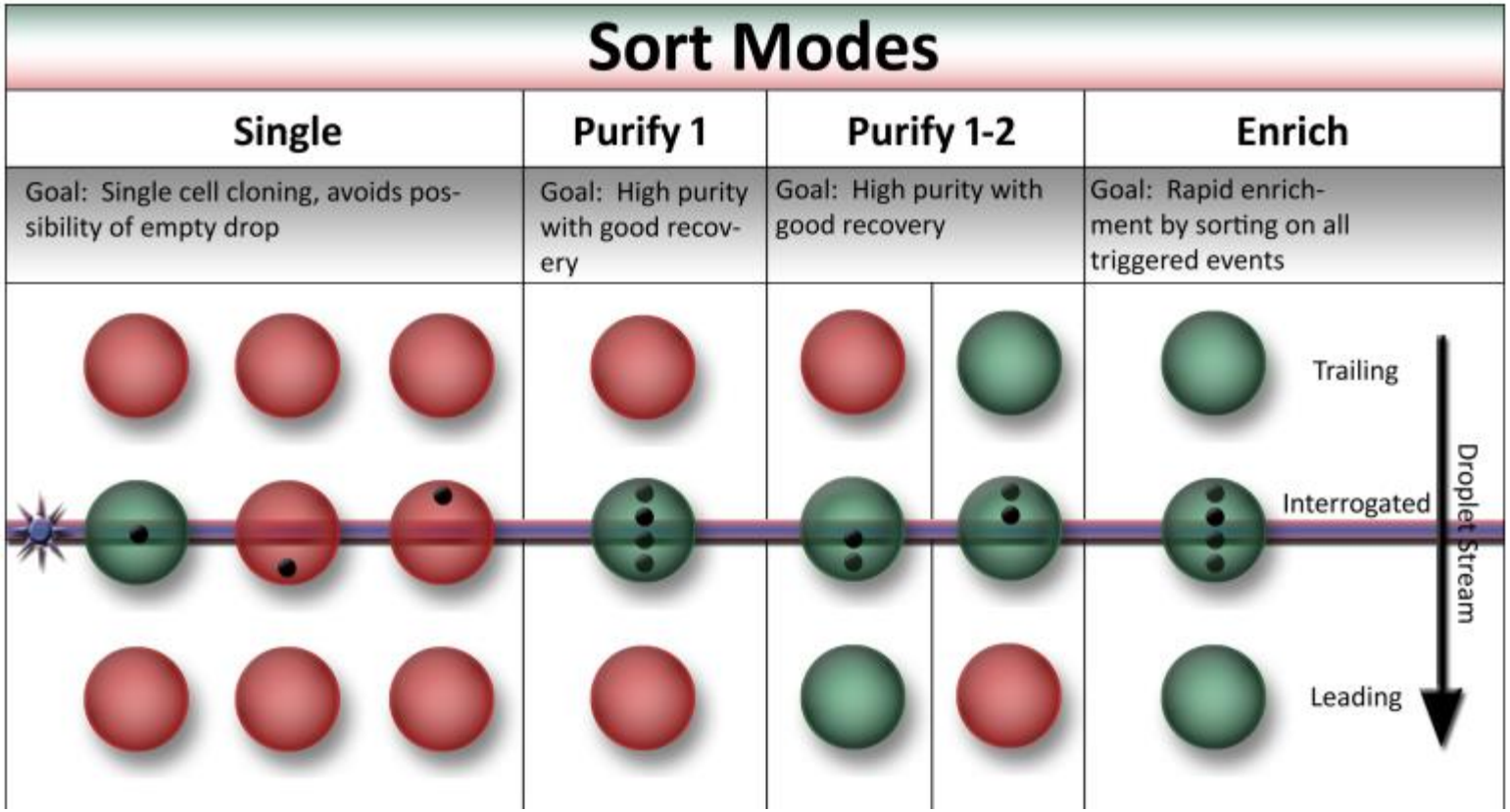
The impact of cell size on… cell number

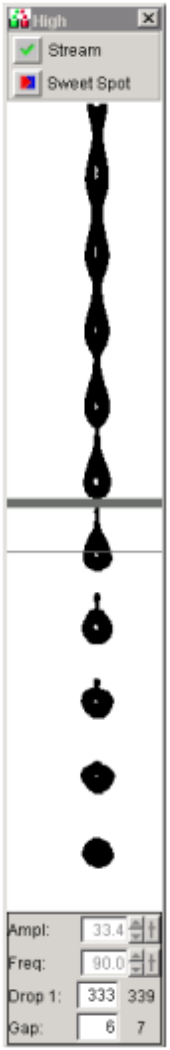
					Time to Sort (Min)		
Cells needed	Frequency of population	Expected recovery (Sorter)	Expected recovery (Sample Processing)	Approximate # cells to stain	20 KHz	45 Khz	87 KHz
100,000	10%	50%	80%	2,500,000	6.67	2.96	1.53
100,000	1%	50%	80%	25,000,000	66.67	29.63	15.33
100,000	0.10%	50%	80%	250,000,000	666.67	296.30	153.26
100,000	10%	25%	80%	5,000,000	13.33	5.93	3.07
100,000	10%	50%	80%	2,500,000	6.67	2.96	1.53
100,000	10%	75%	80%	1,666,667	4.44	1.98	1.02
100,000	10%	90%	80%	1,388,889	3.70	1.65	0.85
100,000	10%	50%	40%	5,000,000	6.67	2.96	1.53
100,000	10%	50%	60%	3,333,333	6.67	2.96	1.53
100,000	10%	50%	80%	2,500,000	6.67	2.96	1.53

- pre-enrichmend recommended

https://expert.cheekyscientist.com/improve-flow-cytometry-cell-sorting-speed/

Purity or yield



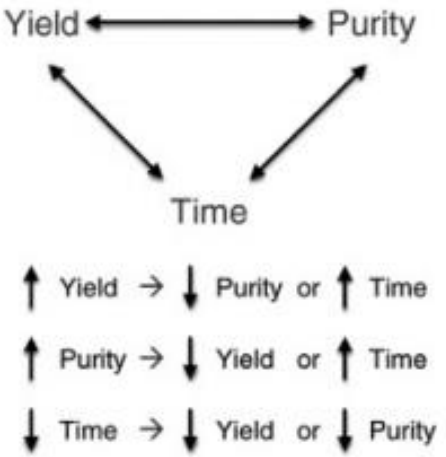


purity yield

https://www.beckman.com/gated-media?mediaId=%7B97FC53EE-1699-4DEA-964C-71AC13F23CED%7D

Long, longer, longest…

- how many cells you need?
- what is the % of target cells in your sample?

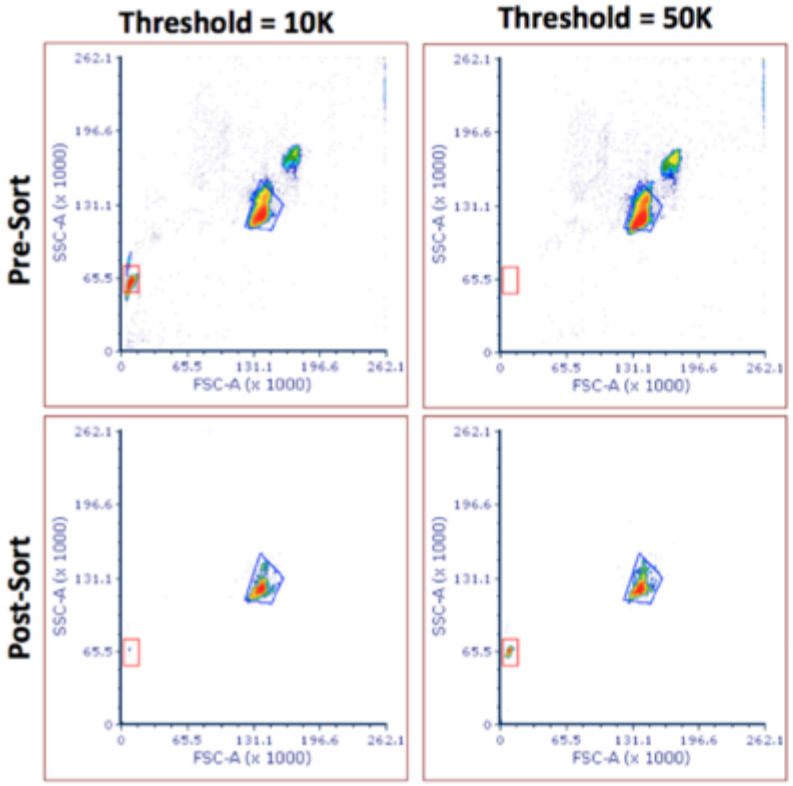


time calculation example: assuming 3.000 evt/s, 10^7 cells per hour

% of + cells in sample at start	0,1%	1%	5%	10%	20%
No. of cells requested					
1.000	5,5 min	33 s	6,5 s	3,3 s	0,17 s
10.000	55 min	5,5 min	1,1 min	33 s	17 s
100.000	9,8 h	55 min	11 min	5,5 min	2,8 min
1.000.000	3,8 d	9,2 h	1,8 h	55 min	28 min
10.000.000	38 d	3,8 d	18 h	9,2 h	4,6 h

Cossarizza, A et al. “Guidelines for the use of flow cytometry and cell sorting in immunological studies” (second edition). (2019), Eur. J. Immunol., 49: 1457-1973. https://doi.org/10.1002/eji.201970107

Threshold



10K Threshold	Small gate	Sort gate
Pre-sort	28.8%	58.3%
Post-sort	1.10%	97.5%

50K Threshold	Small gate	Sort gate
Pre-sort	0%	85.2%
Post-sort	11.9%	86.1%

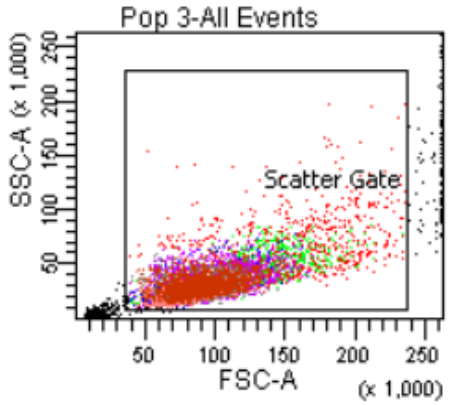
in contrast to analyzers!: lower threshold is better to receive higher purity

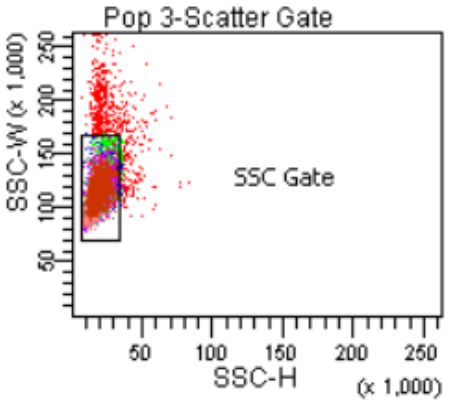
source: https://expert.cheekyscientist.com/improve-flow-cytometry-cell-sorting-speed/

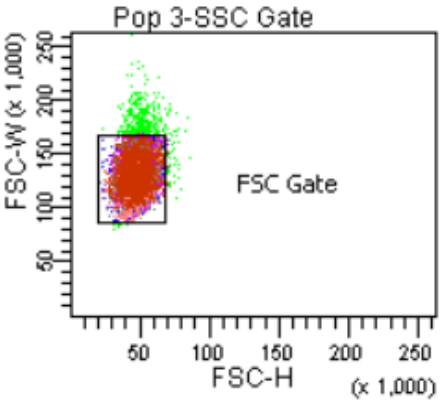
Example BEFORE

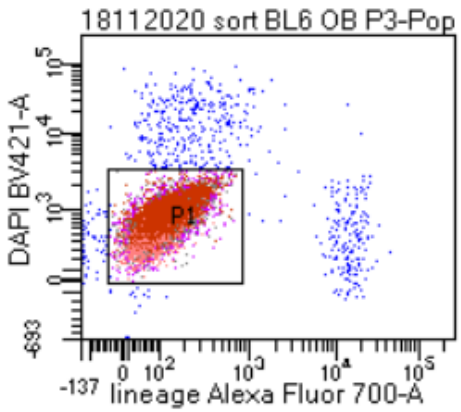
Tube: Pop 3

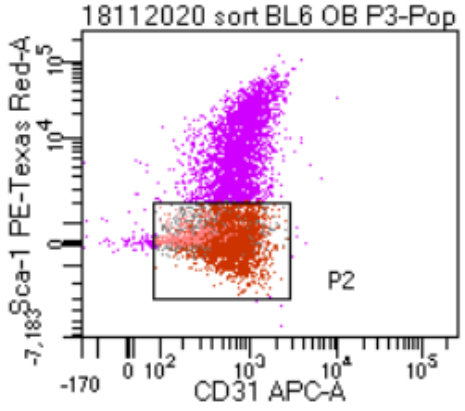
Population	#Events	%Parent	%Total
All Events	100,000	####	100.0
Scatter Gate	96,015	96.0	96.0
SSC Gate	90,273	94.0	90.3
FSC Gate	85,732	95.0	85.7
P1	79,944	93.2	79.9
P2	48,431	60.6	48.4
Osteobl	8,538	17.6	8.5
Fibrobla	18,437	38.1	18.4
Rest of All Events	3,985	4.0	4.0

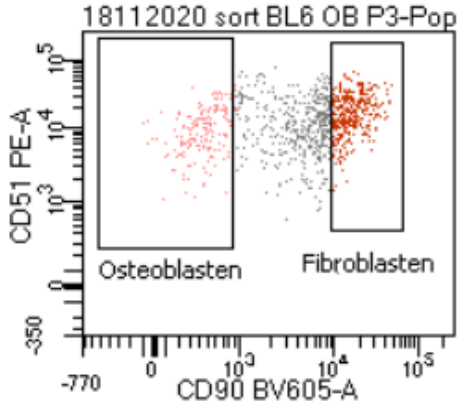




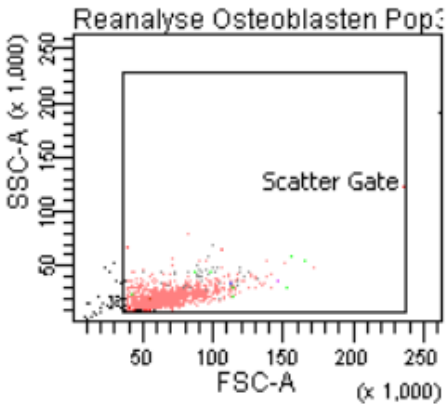


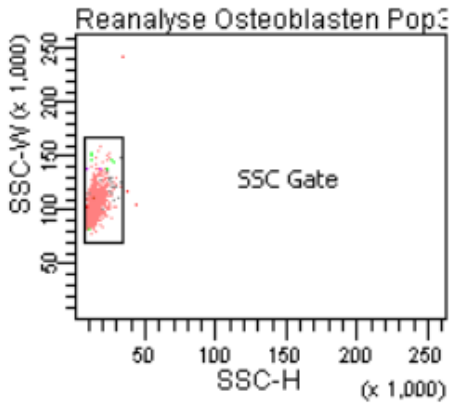


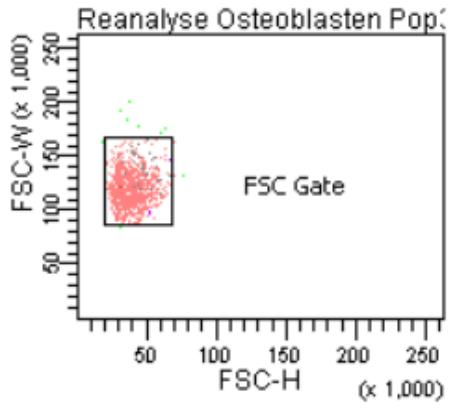


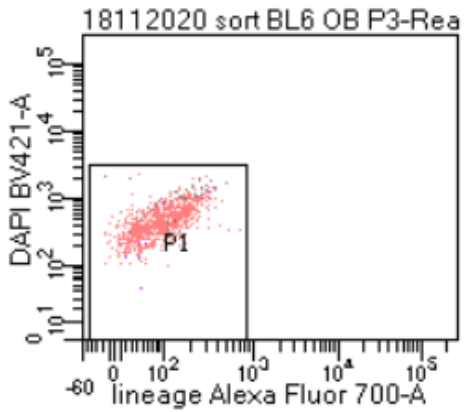


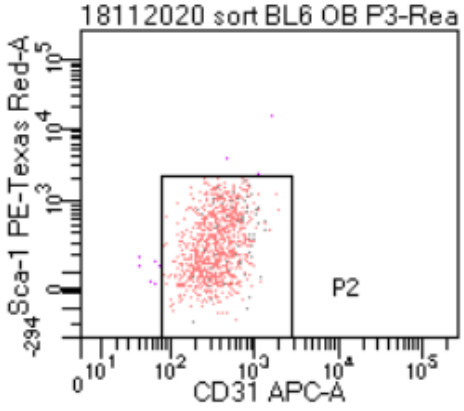
Example AFTER

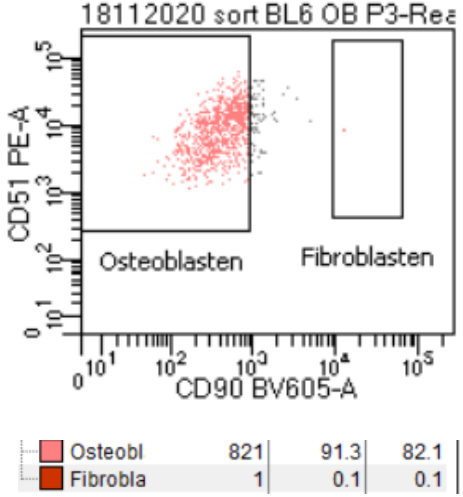


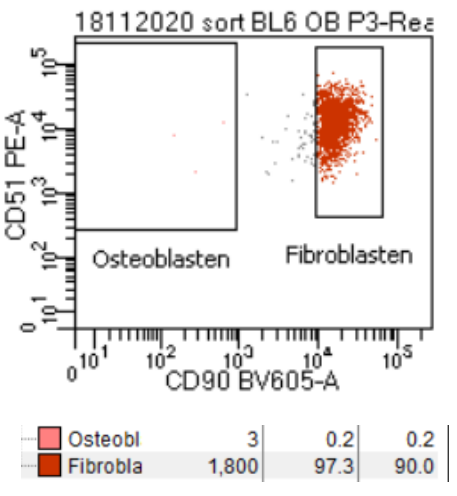












TAKE HOME MESSSAGE:

- cell sorting useful to get more specific results in downstream methods
- FACS sorting most advanced and flexible technology
- needs proper planning
  - markers?
  - cell number?
  - sample availability?
  - **purity, time or yield** most important?
- careful panel setup has to be done in advance
- live-dead-stain, filtration, doublet exclusion raises purity and resolution

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Thank you for your attention.

See you next month: **10th October**

Next topic:
FlowJo Basics – How to analyse your flow data