Universität Rostock Traditio et Innovatio

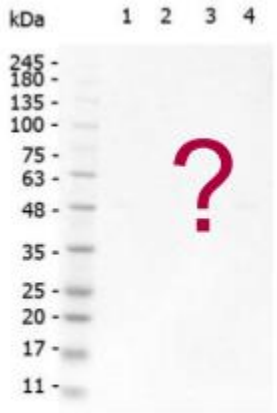
Universitätsmedizin Rostock

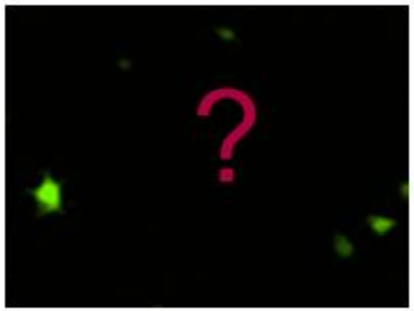
7.) „FACSorting – selecting the fit ones“

7.)„FACSorting – die Fitten in`s Töpfchen “

cell sorting: why is it important?

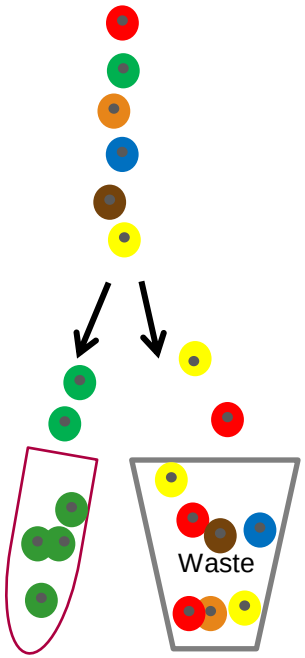
- samples often heterogenious: e.g. blood, spleen, organ biopsies, tumor biopsies (CSCs, TIL, cancer-associated fibroblast)
- not all cells react to certain stimulus (e.g. activation, proliferation, apoptosis)
- heterogenity can mask effects, reduces resolution
- reviewer might ask anyway: who is reacting/producing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





cell sorting: pros and cons

- pros:
  - better defined sample for downstream analysis, culture, …
  - increased resolution
  - extraction of rare cell types (e.g. stem cells, single clones)
- cons:
  - additional pipetting (time and reagents)
  - costs
  - cell loss



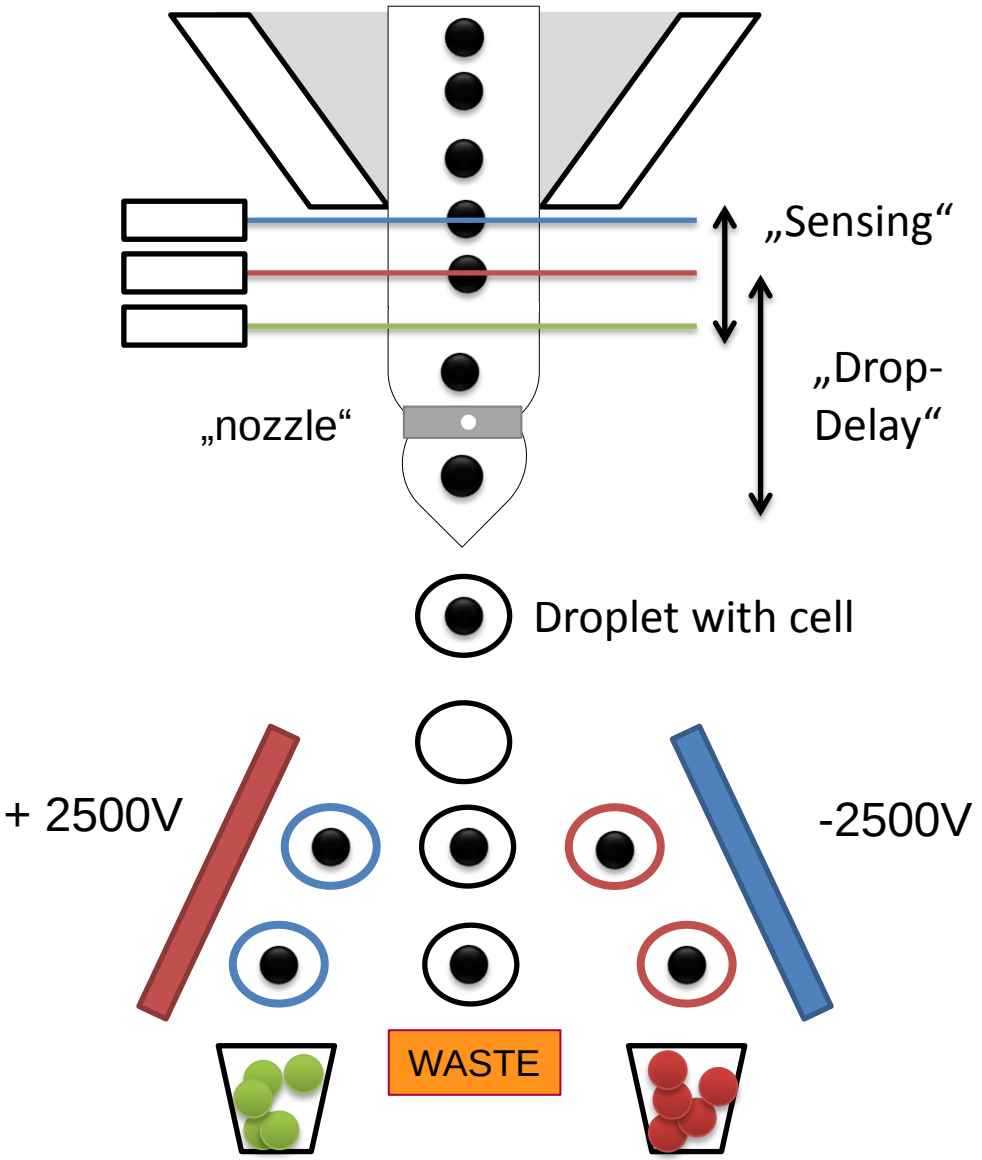
cell sorting: different types/principles

	Ficoll/Percoll gradient centrifugation	**Pluribead**	**Miltenyi AutoMACS or „manual MACS“**	**FACS (BD FACSAria IIIu)**
				
principle	cell size	bead based, cell size	magnetic beads	fluorescence labeled antibodies
separation parameters	1 3 fractions	1 2 populations +/-	1 2 populations +/-	up to 14 + FSC/SSC 4 populations
time	+	+	+	(+/-)
costs	++	++	+	+ depends on project
pros	SOP, low costs	low cell stress	reproducible, scalable	flexible, different plates/tubes

other methods, but not available at ZSA:
microfluidic chips, laser microdissection

FACS

- data-based cell sorting
- droplets created after nozzle Düse
- 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**

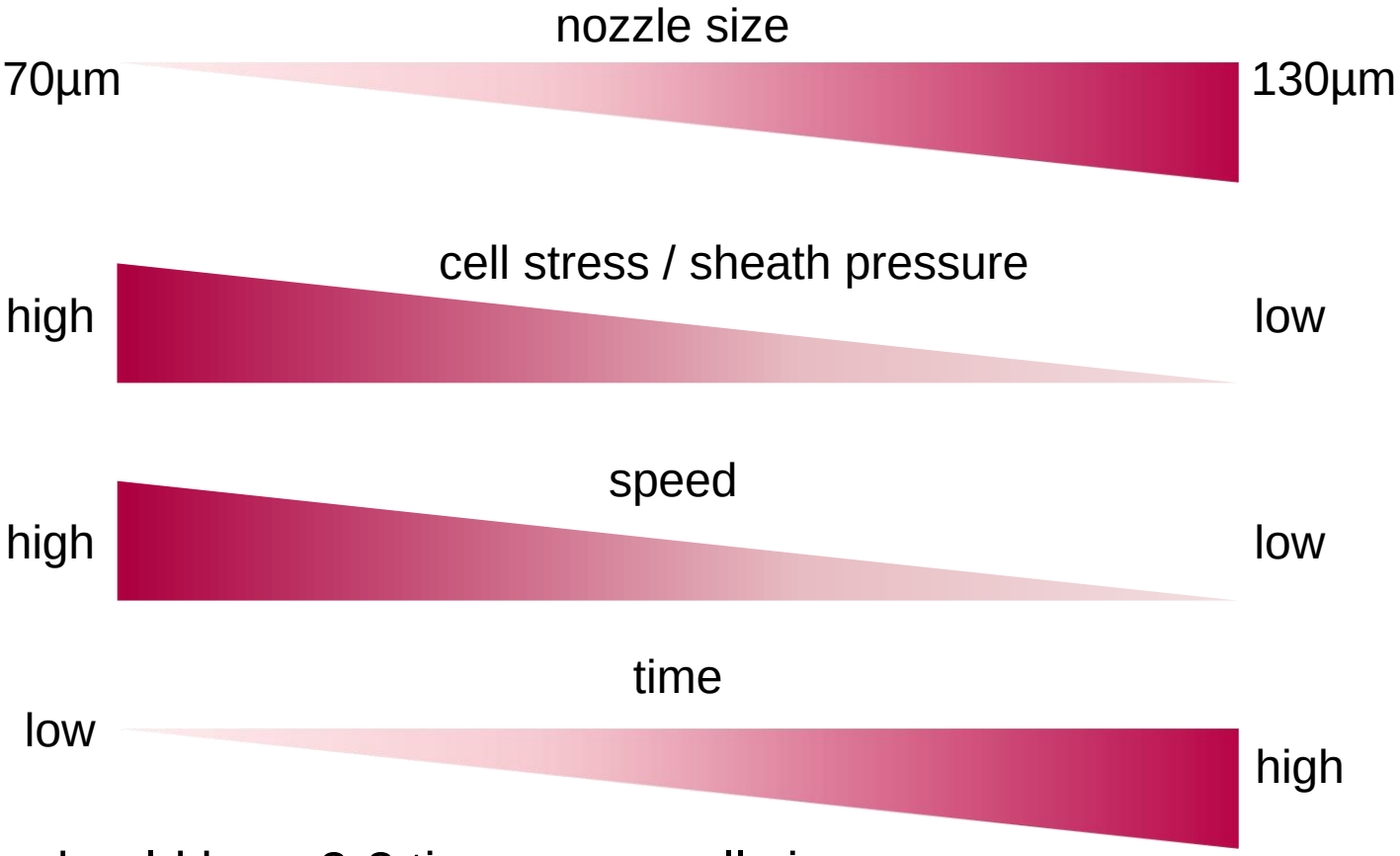


FACS – practical considerations

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

FACS – nozzle size

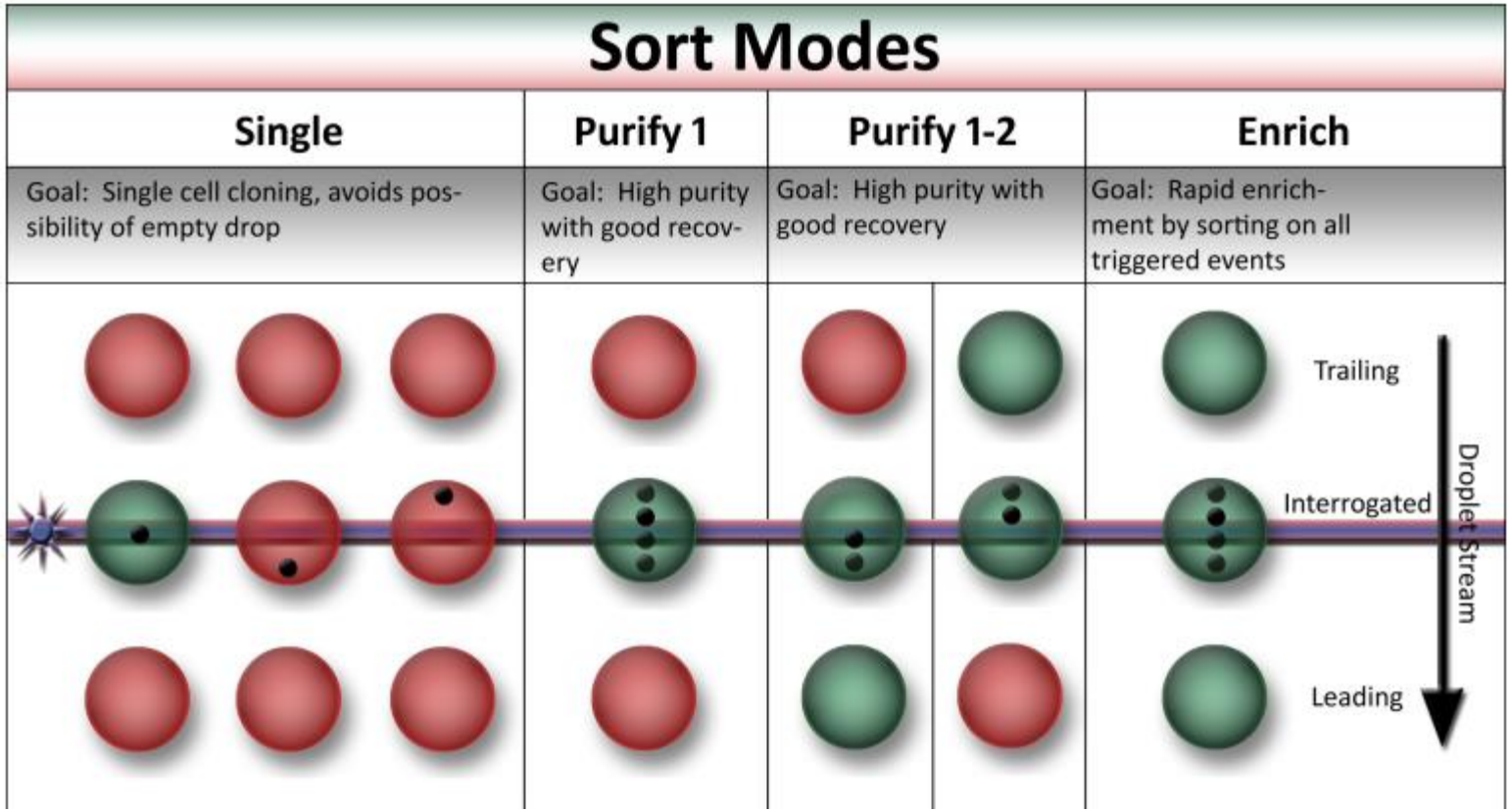
- what is the size of your cells → different nozzles available (70µm, 85µm, 100µm, 130µm)



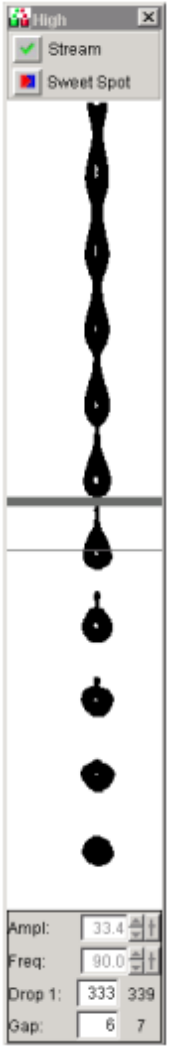
- nozzle should be ~ 2-3 times your cell size

FACS – sorting modes

- what is the goal? yield or purity?



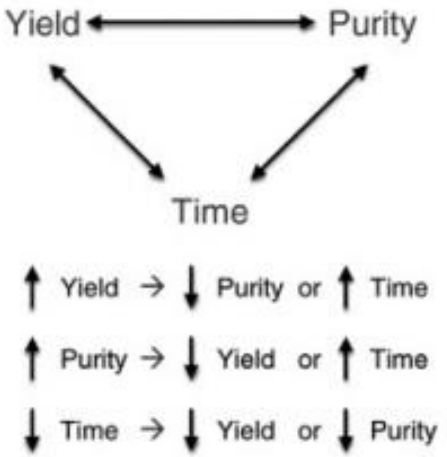
BD Mode: ‚single cell sorting' ‚4-way purity' ‚purity' ‚yield'



Source of picture: https://www.google.com/url?sa=i&url=https%3A%2F%2Fwww.beckman.com%2Fgated-media%3FmediaId%3D%7B97FC53EE-1699-4DEA-964C-71AC13F23CED%7D&psig=AOvVaw3X7GnbIxIeg2eTCq0eV6wQ&ust=1628592995764000&source=images&cd=vfe&ved=2ahUKEwigzp7p46PyAhXVgv0HHSQVAKIQr4kDegQIARAa

FACS – sorting time

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

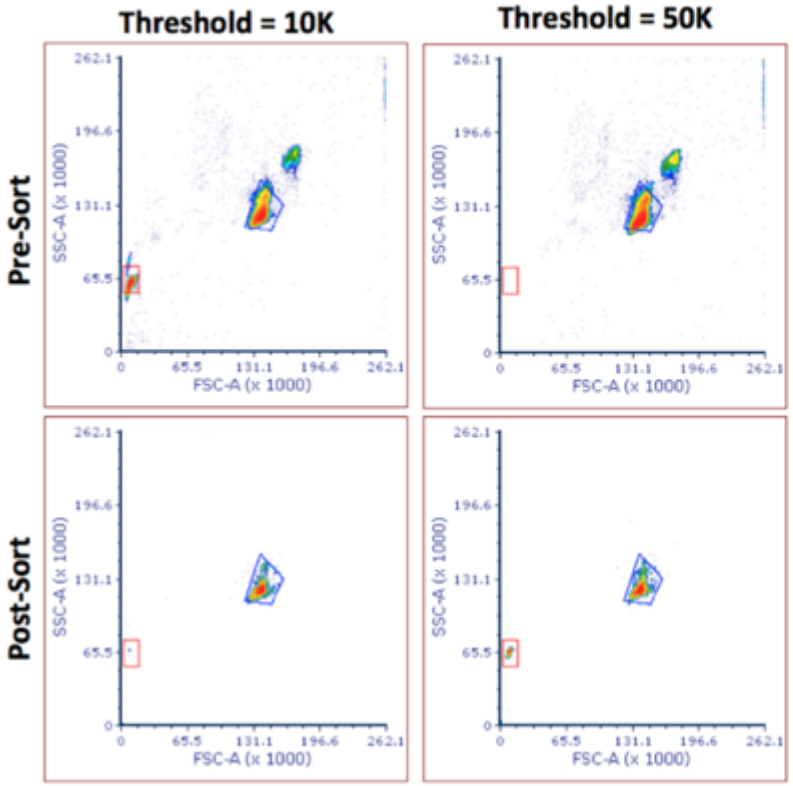


time calculation example: assuming 3.000 evt/s, 10^7 cells per hour

% 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

FACS – 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%

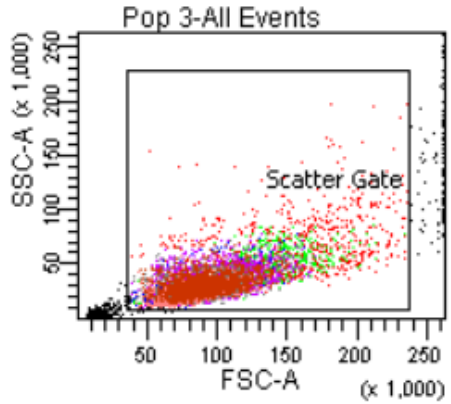
in contrast to analyzers!: lower threshold is better to receive higher purity

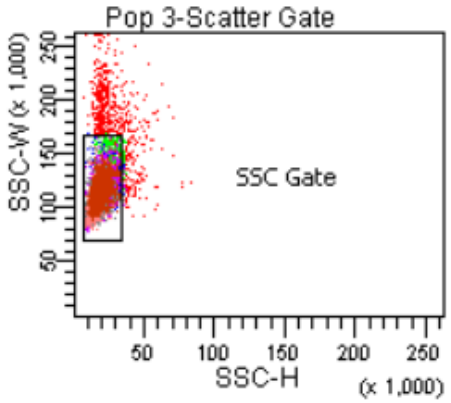
source: https://expert.cheekyscientist.com/improve-flow-cytometry-cell-sorting-speed/

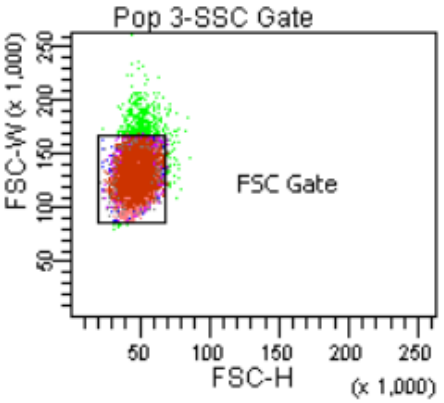
Sample – before FACS

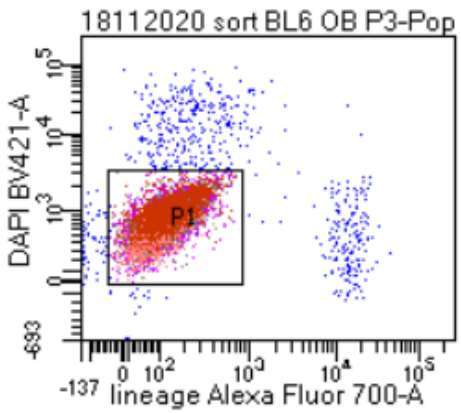
Tube: Pop 3

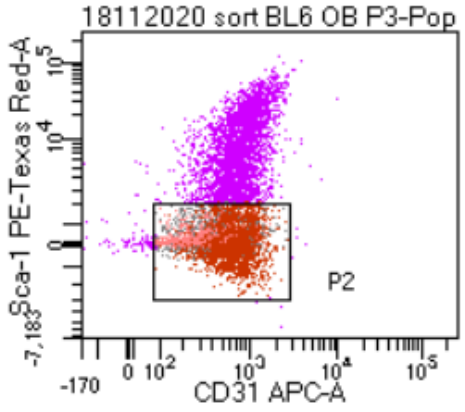
Population	#Events	%Parent	%Total
All Events	100,000	####	100.0
Scatter Gate	96,015	96.0	96.0
SSC Gate	90,273	94.0	90.3
FSC Gate	85,732	95.0	85.7
P1	79,944	93.2	79.9
P2	48,431	60.6	48.4
Osteobl	8,538	17.6	8.5
Fibrobla	18,437	38.1	18.4
Rest of All Events	3,985	4.0	4.0

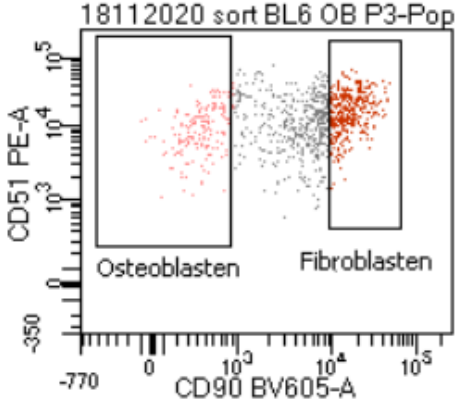








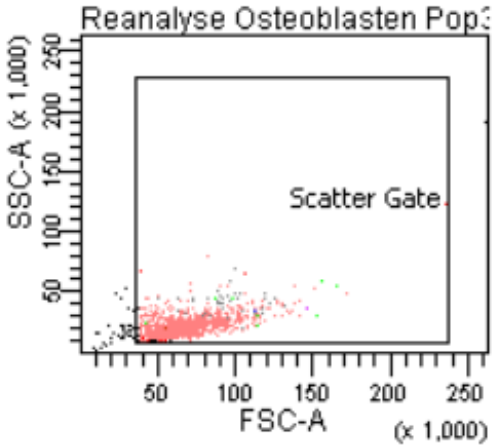


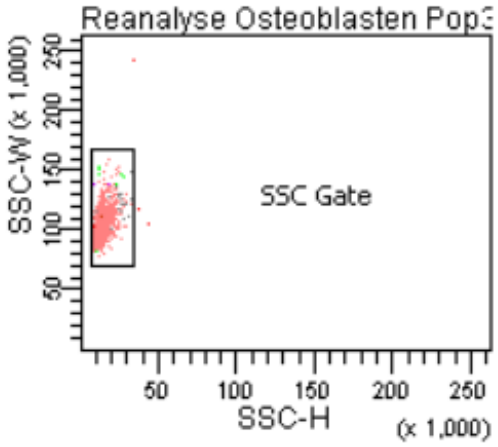


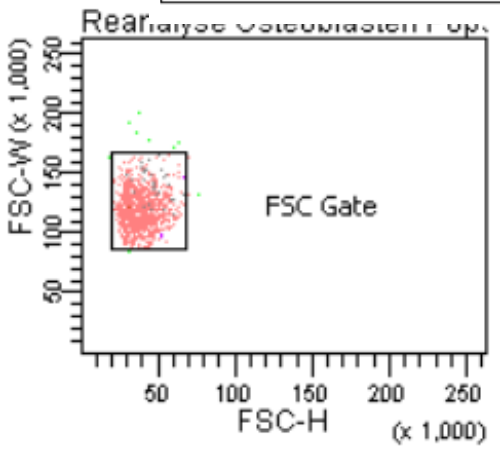
Reanalysis – after FACS

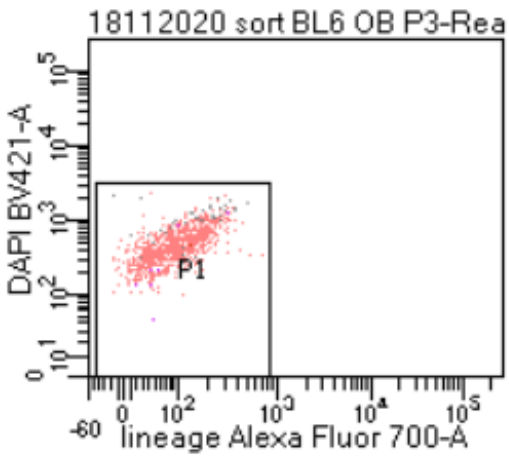
Tube: Reanalyse Osteoblasten Pop3

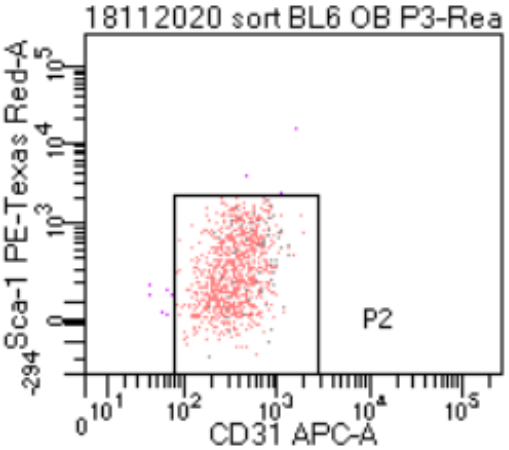
Population	#Events	%Parent	%Total
All Events	1,000	####	100.0
Scatter Gate	936	93.6	93.6
SSC Gate	924	98.7	92.4
FSC Gate	911	98.6	91.1
P1	910	99.9	91.0
P2	899	98.8	89.9
Osteobl	821	91.3	82.1
Fibrobla	1	0.1	0.1
Rest of All Events	64	6.4	6.4

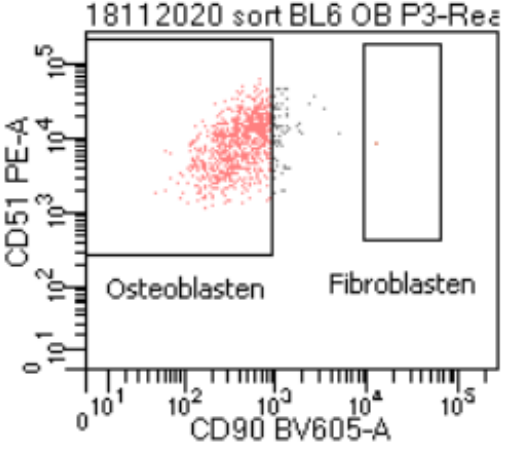








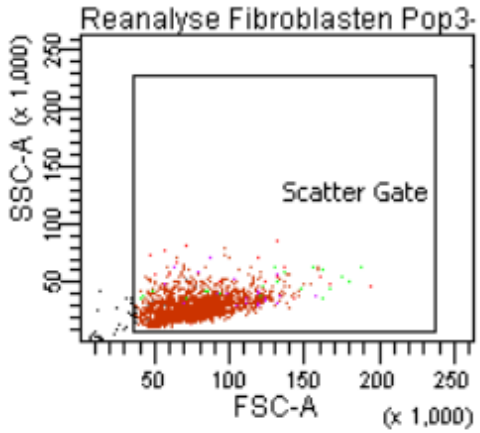


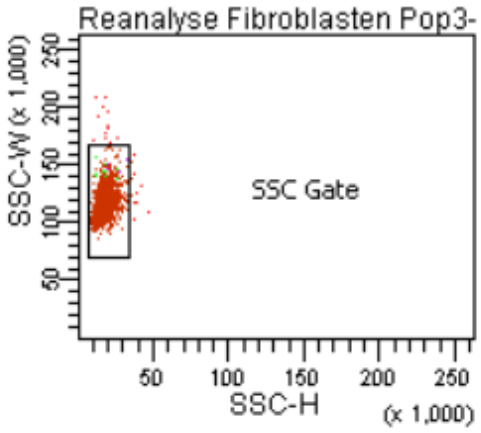


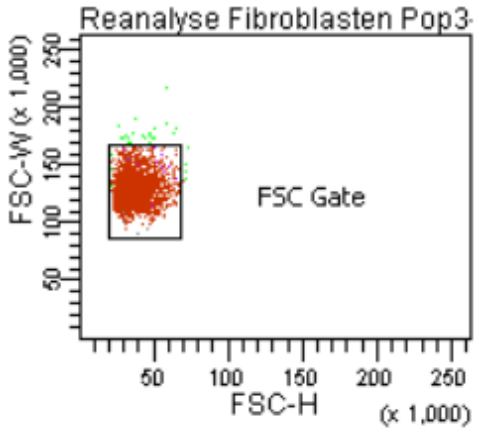
Reanalysis – after FACS

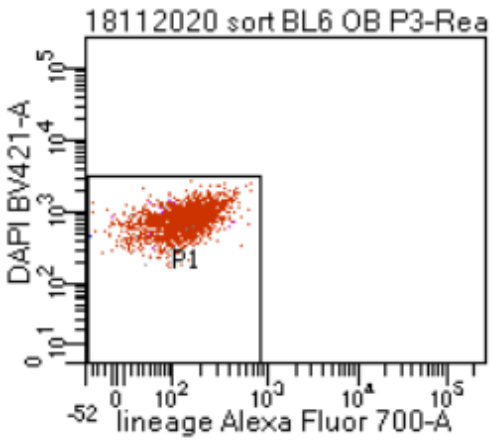
Tube: Reanalyse Fibroblasten Pop3

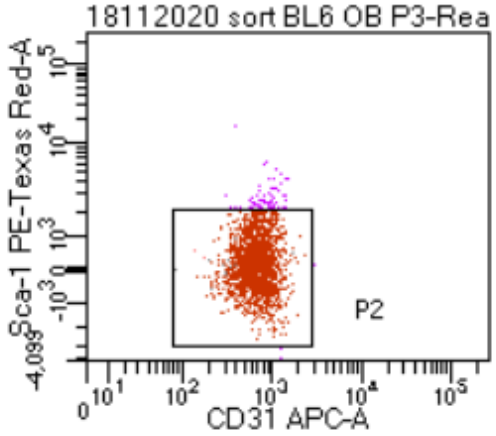
Population	#Events	%Parent	%Total
All Events	2,000	####	100.0
Scatter Gate	1,968	98.4	98.4
SSC Gate	1,948	99.0	97.4
FSC Gate	1,913	98.2	95.6
P1	1,912	99.9	95.6
P2	1,849	96.7	92.4
Osteobl	3	0.2	0.2
Fibrobla	1,800	97.3	90.0
Rest of All Events	32	1.6	1.6

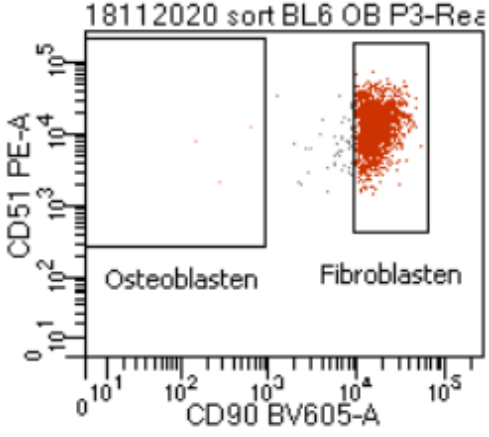












TAKE HOME MESSSAGE:

- cell sorting useful to get better results in downstream methods
- FACS sorting most advanced and flexible technology
- needs proper planning (which markers? cell number I need? how much sample available? **purity, time or yield** most important?)
- 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!

Next topic (7th September):
„FlowJo – how to analyse your flow data – the basics“

Nächstes Mal (am 07. September):
„FlowJo – wie man FCS-Daten auswertet – die Grundlagen“

→ will be longer talk (1,5-2h)