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What is wrong with my flow cytometry data?

Hints, tricks and pitfalls

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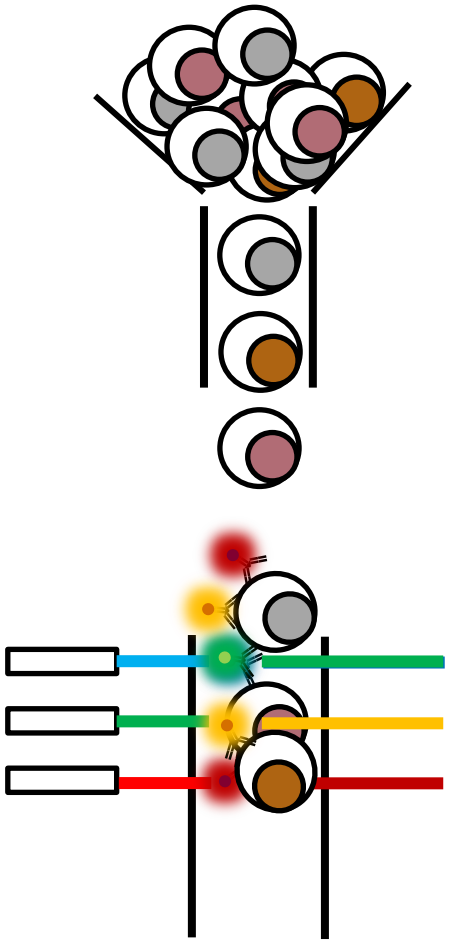
Webinar 5
06.06.2023

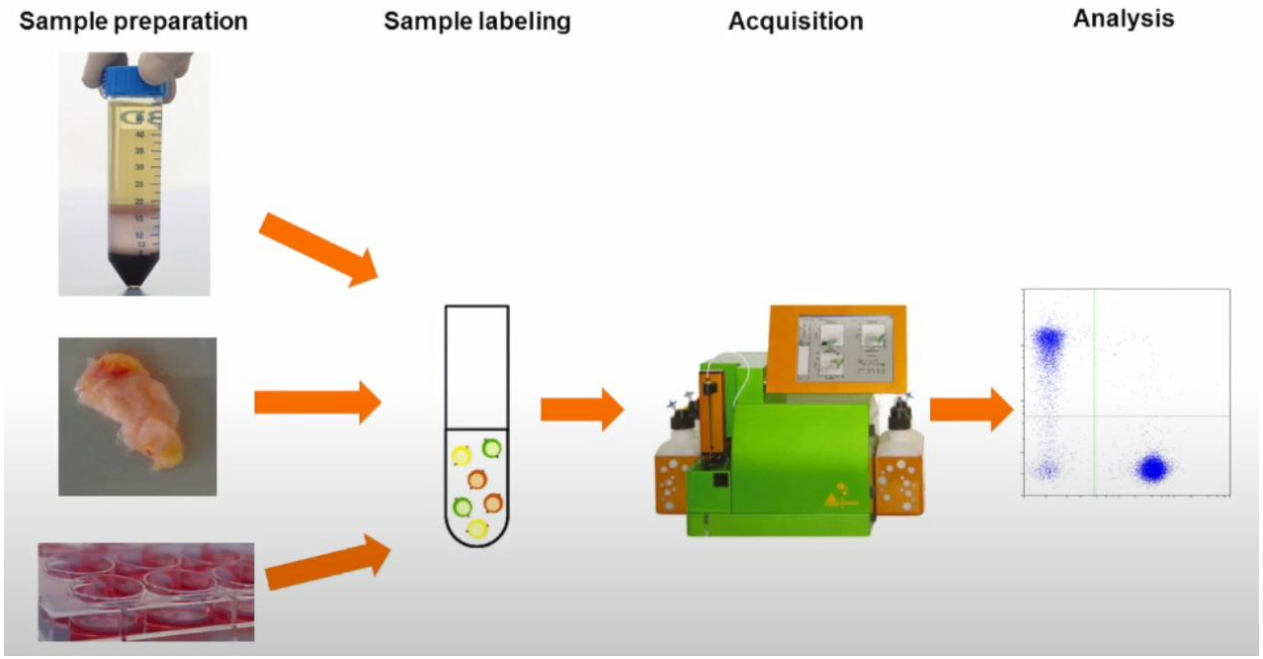
The alpha and omega of preparing cells for flow cytometry

What we need to talk **about**

1. Single cells
2. Preparation: blood, cells, tissues
3. Mechanical vs. enzymatic disintegration of tissues or cell layers
4. Doublets and dead cell exclusion
5. Fixation
6. Surface staining
7. Intracellular staining

All about **single cells…**





https://www.youtube.com/watch?v=mpOlRplZhPg

Viability and yield max!

The A and O of **sample preparation**

- better sample preparation leads to better results

and easy data analysis

https://www.youtube.com/watch?v=mpOlRplZhPg

- standard procedure:

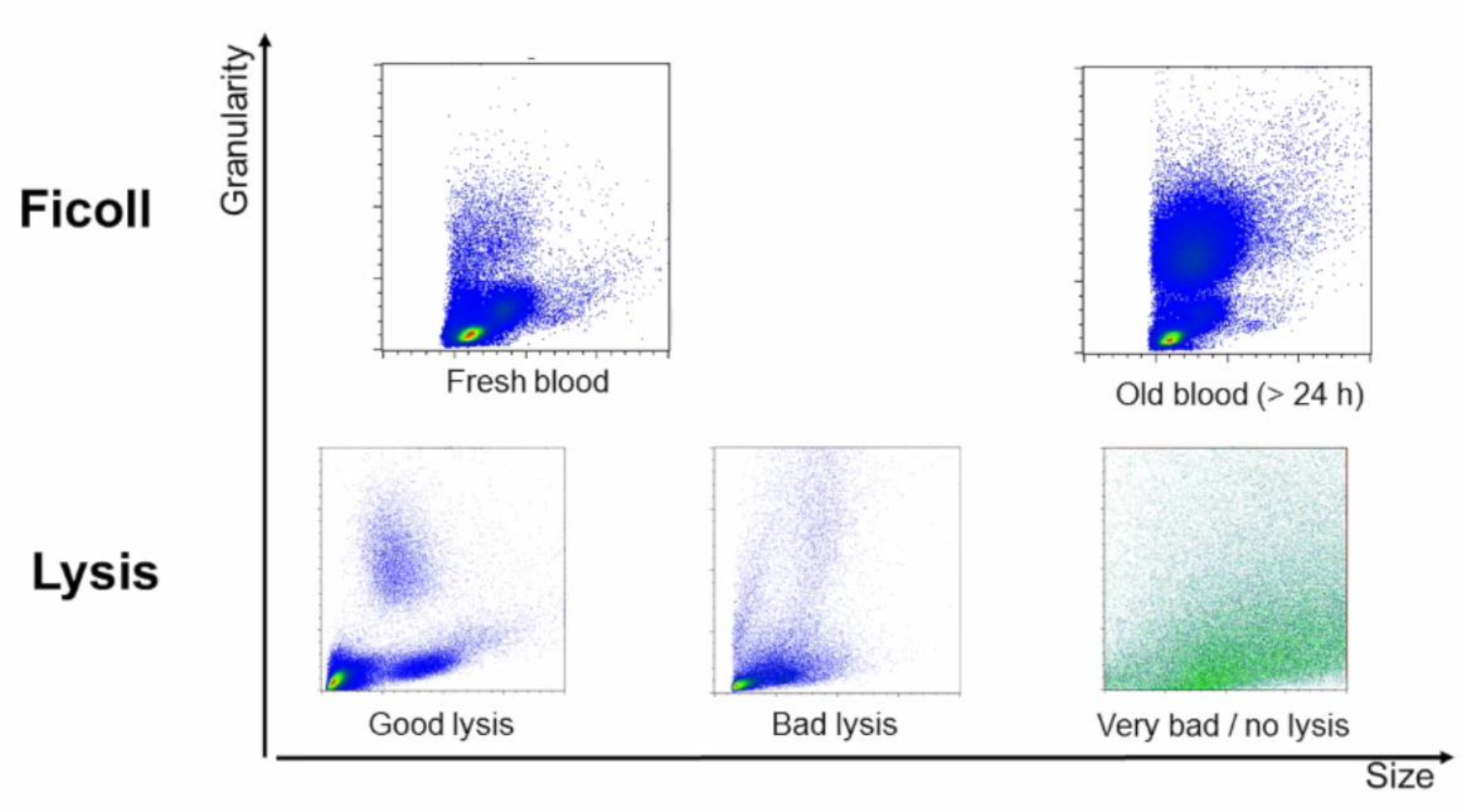
- sampling: blood, tissues, freeze/thaw, cell harvest

→ single cell suspension

- wash with cell stain buffer (1X PBS + 1-2% BSA + 2-5 mM EDTA + 2mM NaN_3)
- stain and measure

- changing BSA and/or EDTA conc. might reduce doublets

Sample prep: **Blood**



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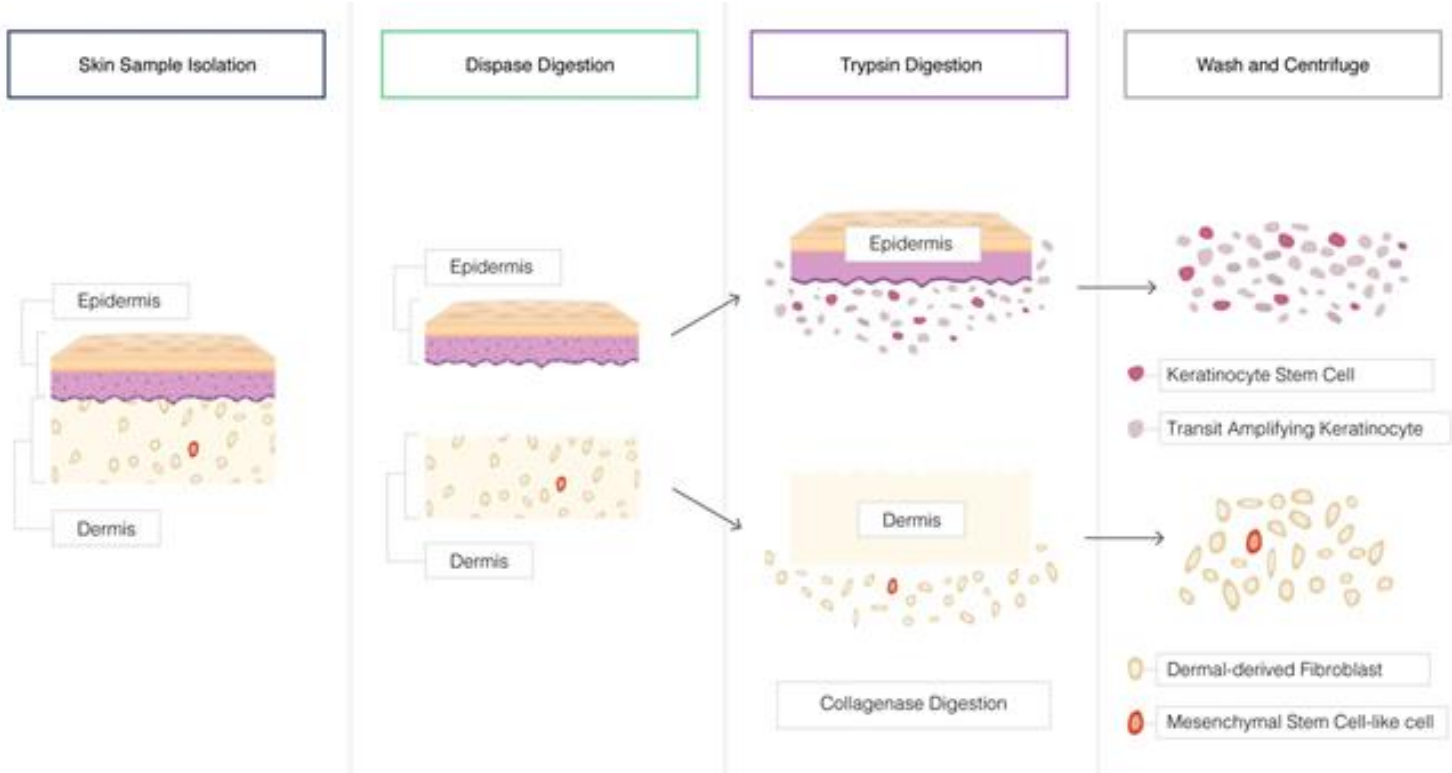
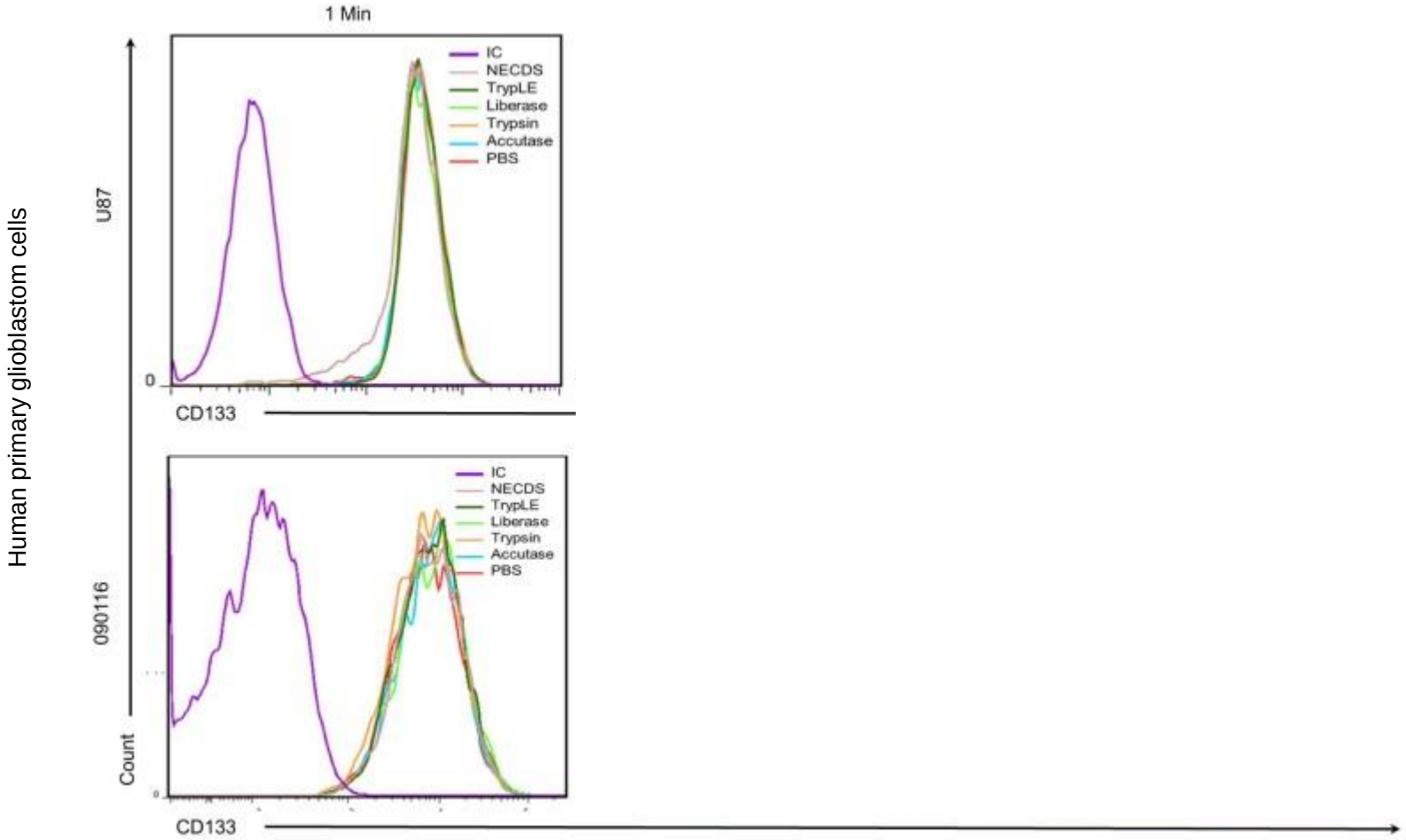
Sample prep: **Tissues**



https://www.flowmetric.com/cytometry-blog/sample-preparation-for-flow-cytometry

- complex enzymatic digestion needed
- combination of enzymatic and mechanical protocols, include DNase
- goal: single cells with > 50% vitality and intact epitopes

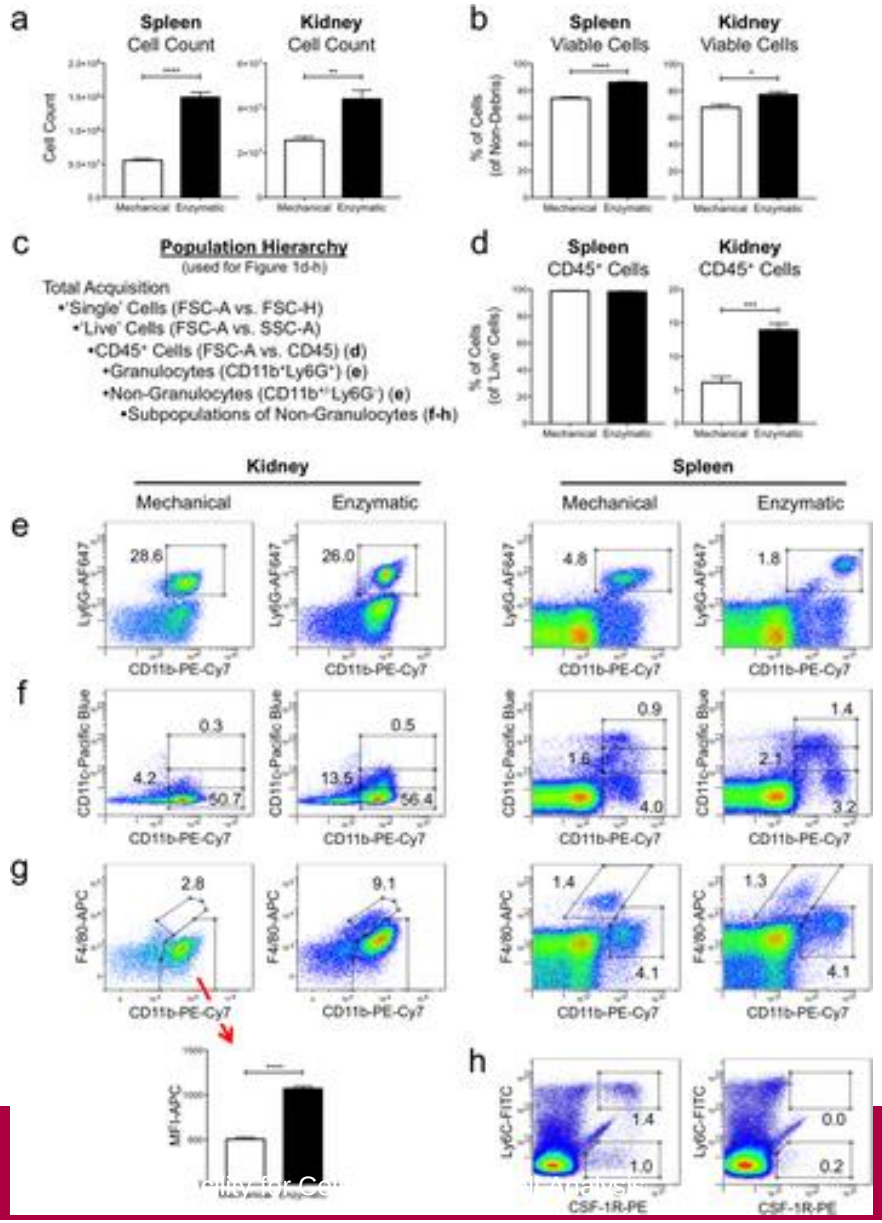
Sample prep: **Enzymatic digestion**

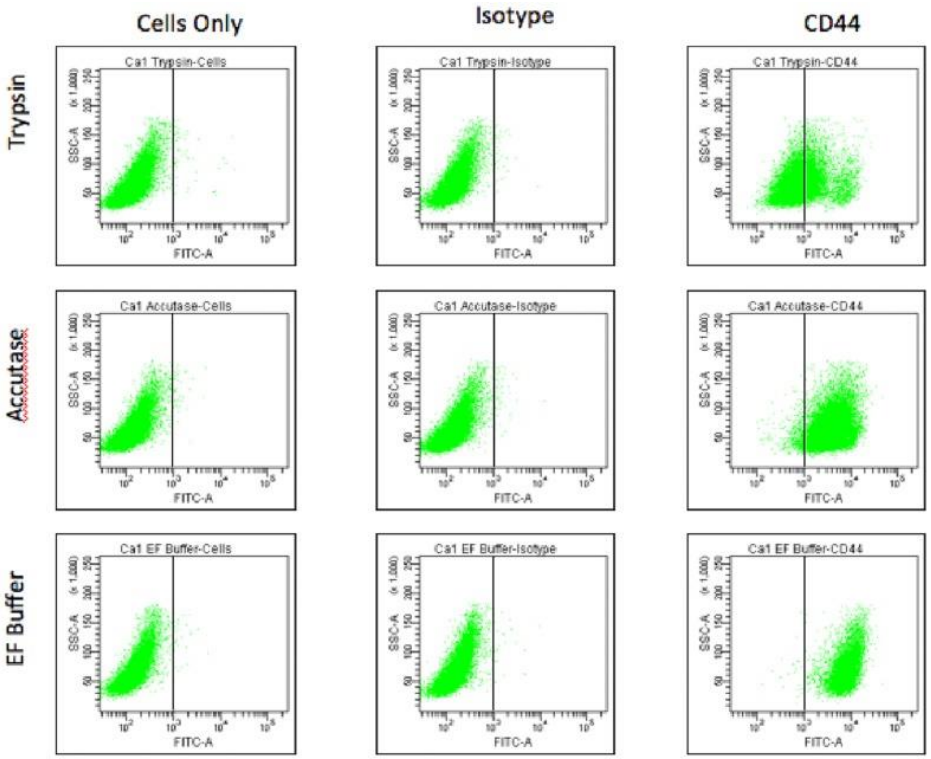


NECDS (non-enzymatic cell dissociation solution): detachment of primary cells, EDTA based
TrypLE (Gibco): animal origin-free, recombinant enzymes for dissociating adherent mammalian cells → substitutes trypsin, superior performances
Liberase (Merck): Kollagenase I / II + Thermolysin (Protease)
Accutase: animal origin-free, proteolytisch und kollagenolytisch

CD133 – prominin-1 HSC,CSC marker

Sample prep: **Enzyme vs. scissors**





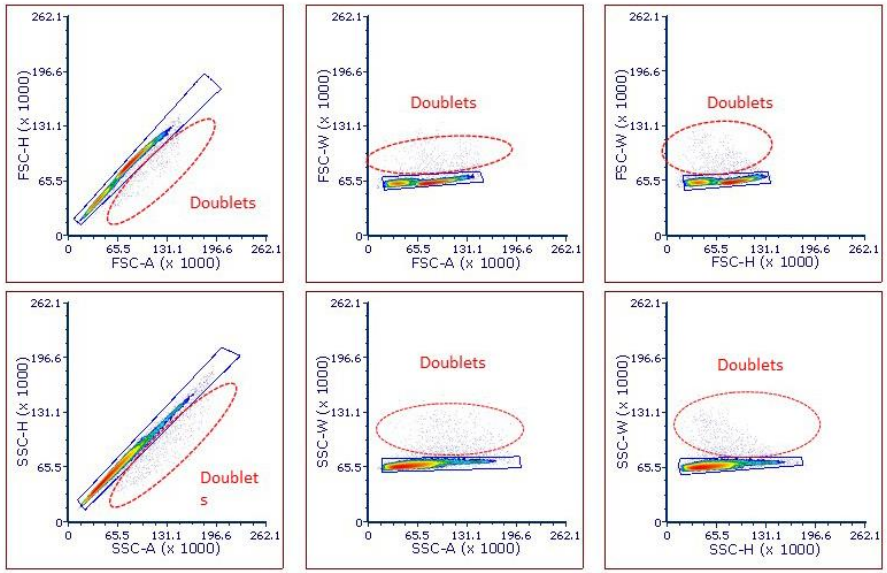
EF buffer = enzyme-free cell dissociation buffer (Invitrogen #13151-014)

Sample prep: **dead/ doublet cell ex**

Doublet cells

- wrong buffer composition
- clumps do clog the cytometer
- doublets cause false-positive signals

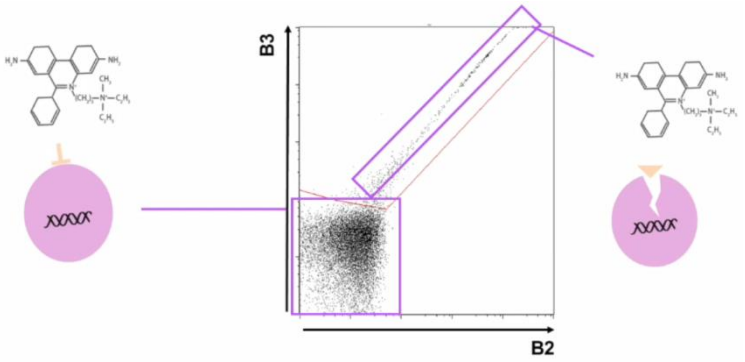
→ resuspend, filter (30-70 nm), vortex



Dead cells

- density gradient:
- high cell loss (≤ 50%)
- toxic
- increase of autofluorescence

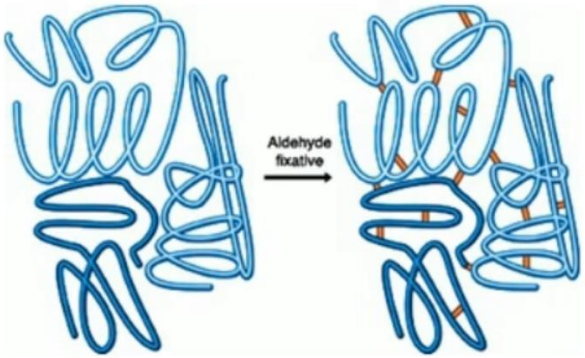
→ DNase treatment

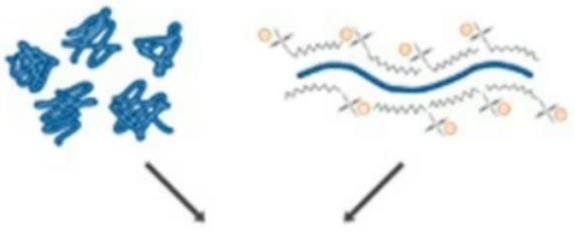


Sample prep: **Fixation**

- fixation BEFORE staining - epitope alteration
- fixation AFTER staining - fluorophore instability
  - GFP, cyanine- and protein based fluorophors: signal loss after fixation (APC-Cy7< APC-H7, PerCP, FITC, etc)
  - but some phospho-epitopes needs methanol demasking

- Fixation
  - Formaldehyde
    - Create bonds between lysine residues to cross link proteins
    - Used between 0.5% and 4%
    - Stable for 1-2 weeks but should be kept in PBS after initial fixation





- Alcohols
  - Usually 70% ethanol or methanol
  - Precipitating/denaturing fixatives
  - Dissolve lipids so permeabilize at the same time
  - Coagulates proteins so may hide epitope
  - Stable for months at 4°C or -20°C

https://www.youtube.com/watch?v=oeqiCa3nEwc

Sample prep: **Surface staining**

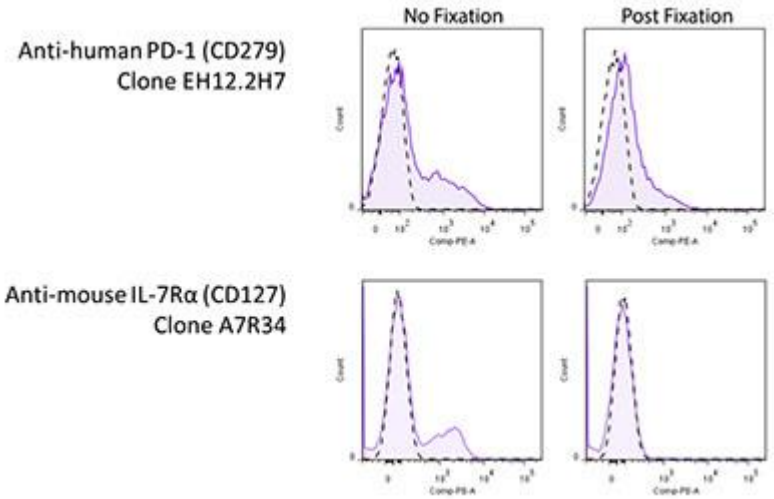
- coaggulant (e.g. EDTA, Heparin) interfere with some epitopes
- check enzyme activity and whether targets are „nibbled off“
- Ficoll and RBC Lysis buffer also interfere with surface epitopes
- blocking of Fc receptors when working with PBMCs or any other cell types expressing FcR

→ exception: Fc-staining on monocytes, Ig-staining on B cells

- in case of fixation: check tandem fixability
- stimulation reduces or increases expression? →PMA/Iono causes internalization
- no azide in functional assays → ROS detection, Ca^{2+}-flux, …

Sample prep: **Intracellular staining**

- also block intracellularly
- dilute antibodies in saponin-based buffer (reversible)
- use bright fluorophors since expression is often weak
- use (FMO) controls



https://www.biolegend.com/en-us/blog/fix-now-fix-later-considerations-for-the-use-of-paraformaldehyde-fixation-in-flow-cytometry

- wash the cells adequately after each antibody incubation step (unbound antibodies are trapped in the cells in the case of intracellular staining)
- perform all protocol steps at 4°C
- permeabilize on ice

Sample prep: **Take home message**

- the better the preparation and protocol of your samples the better your results/analysis is
- coaggulants, Ficoll, lysis buffer, enzymes, fixation buffer interfere with epitope access and reduce signal of flurophors
- in multi-colour panels optimization towards high signal, low doublet rate, high viability is the goal!

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Thank you for your attention.

See you next month: **4th July**

Next topic
When to use which buffer system

Enzymes for tissue dissociation

MACS Miltenyi Biotec

Enzyme	Type	Source	Target	
Trypsin	serine protease	bovine/porcine pancreas	predominantly at c-terminal side of AA Lys or Arg	relatively harsh
Papain	cysteine protease	*Carica papaya*	cleaves basic AAs	relatively harsh, but less digestive than Trypsin
Collagenase	protease, includes also phospholipases or neuraminidases	*Clostridium histolyticum*	peptide bonds in collagen; Different types available, depending on supplier Roche: A, B, D, H, P Sigma-Aldrich: I-IV, Worthington: Type 1-4	different collagenase types with different levels of unspecific activities
Liberase Blendzymes	blend of purified collagenases with neutral proteases	*Clostridium histolyticum* and *Bacillus polymyxa* or *B. thermoproteolyticus*	Supplied by Roche, more defined activity and composition	developed for pancreatic islet transplantation research
Dispase	protease	*Bacillus polymyxa*	fibronectin, collagen IV (collagen I)	separation of dermis and epidermis
DNase	nuclease DNase I: neutral DNase II; functions optimally at acidic pH	bovine pancreas	DNA	avoid cell clumping

https://www.youtube.com/watch?v=mpOlRplZhPg