Universität Rostock Traditio et Innovatio

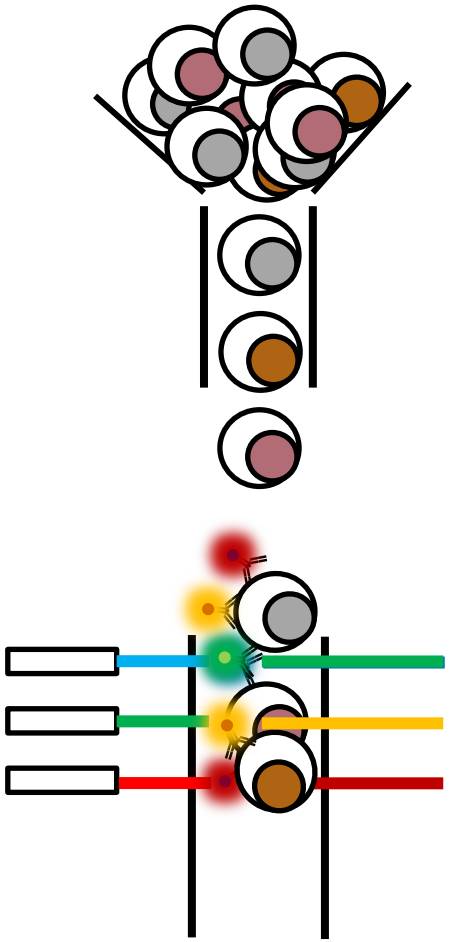
Universitätsmedizin Rostock

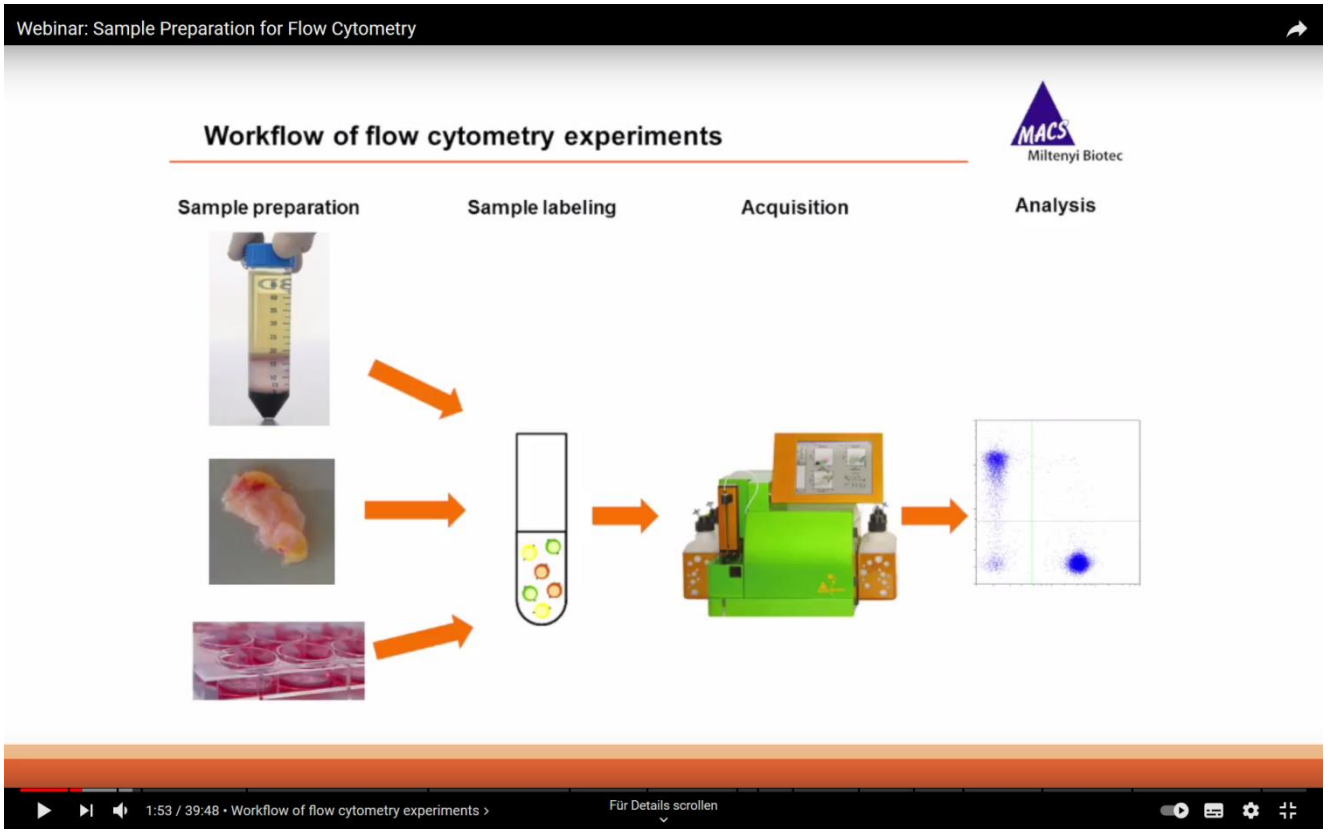
5.) „The alpha and omega of preparing cells“

5.) Das A und O der Probenpräparation: Von Asservierung bis Zielzelle

Core Facility for Cell sorting and Cell analysis

Flow cytometry needs single cells



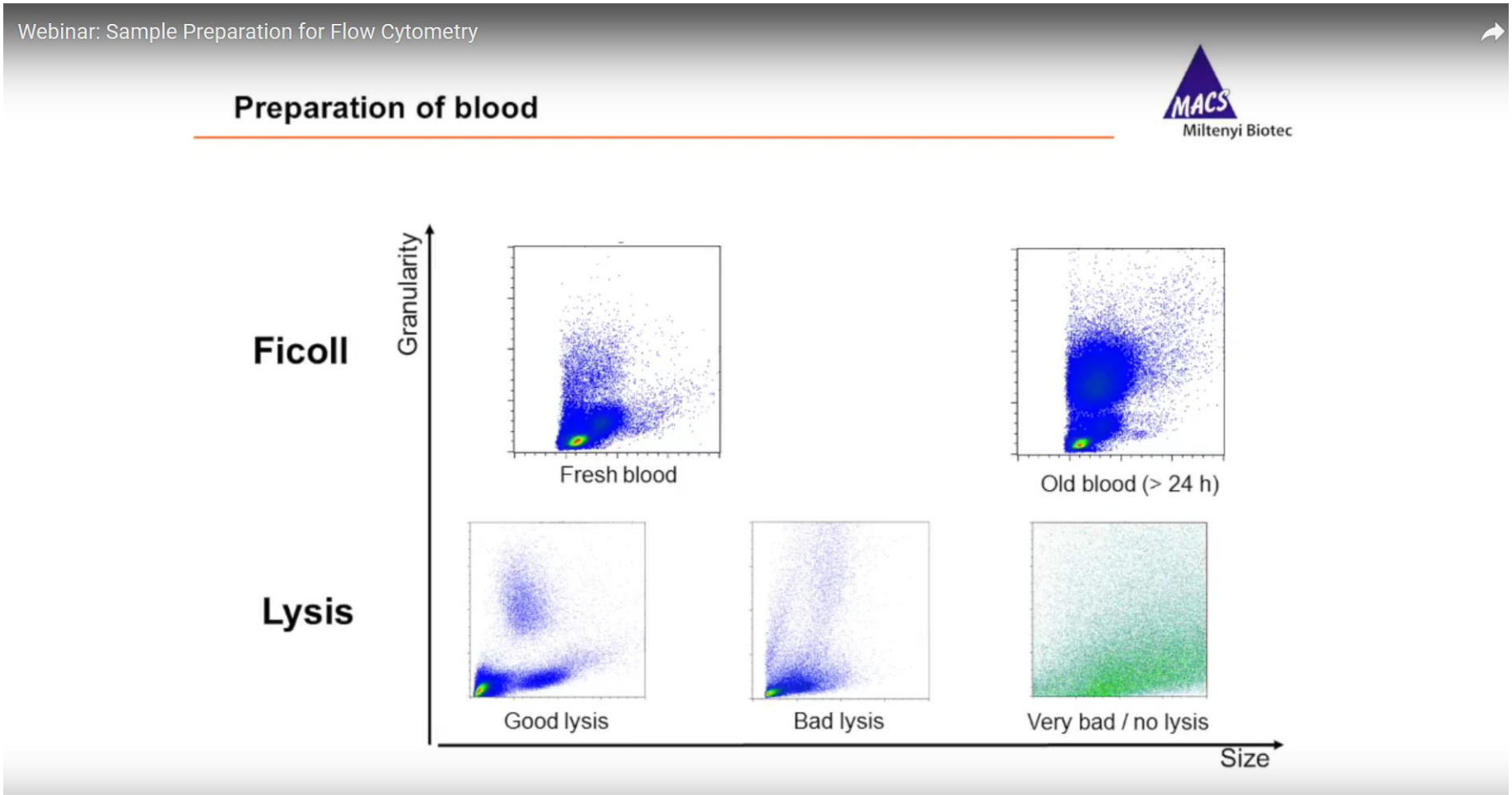


https://www.youtube.com/watch?v=mpOlRplZhPg

sample preparation

- better sample preparation leads to better results and easy data analysis
- standard procedure:
  - trypsinize cells or lyse blood
  - wash with cell stain buffer (1x PBS 1-2% BSA, 2mM EDTA, 2mM NaN_3
  - stain and measure
- changing BSA and/or EDTA conc. might reduce doublets

sample preparation – lysing blood



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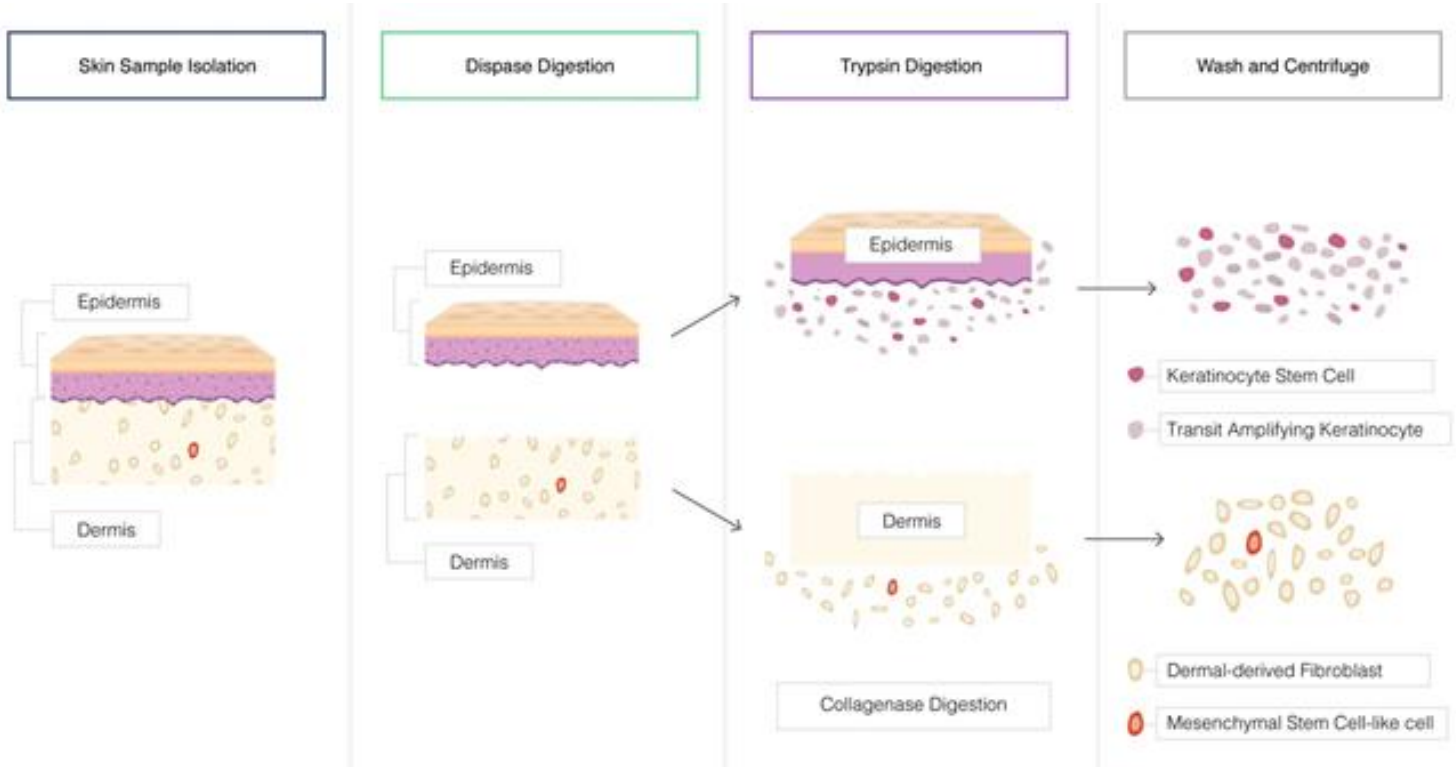
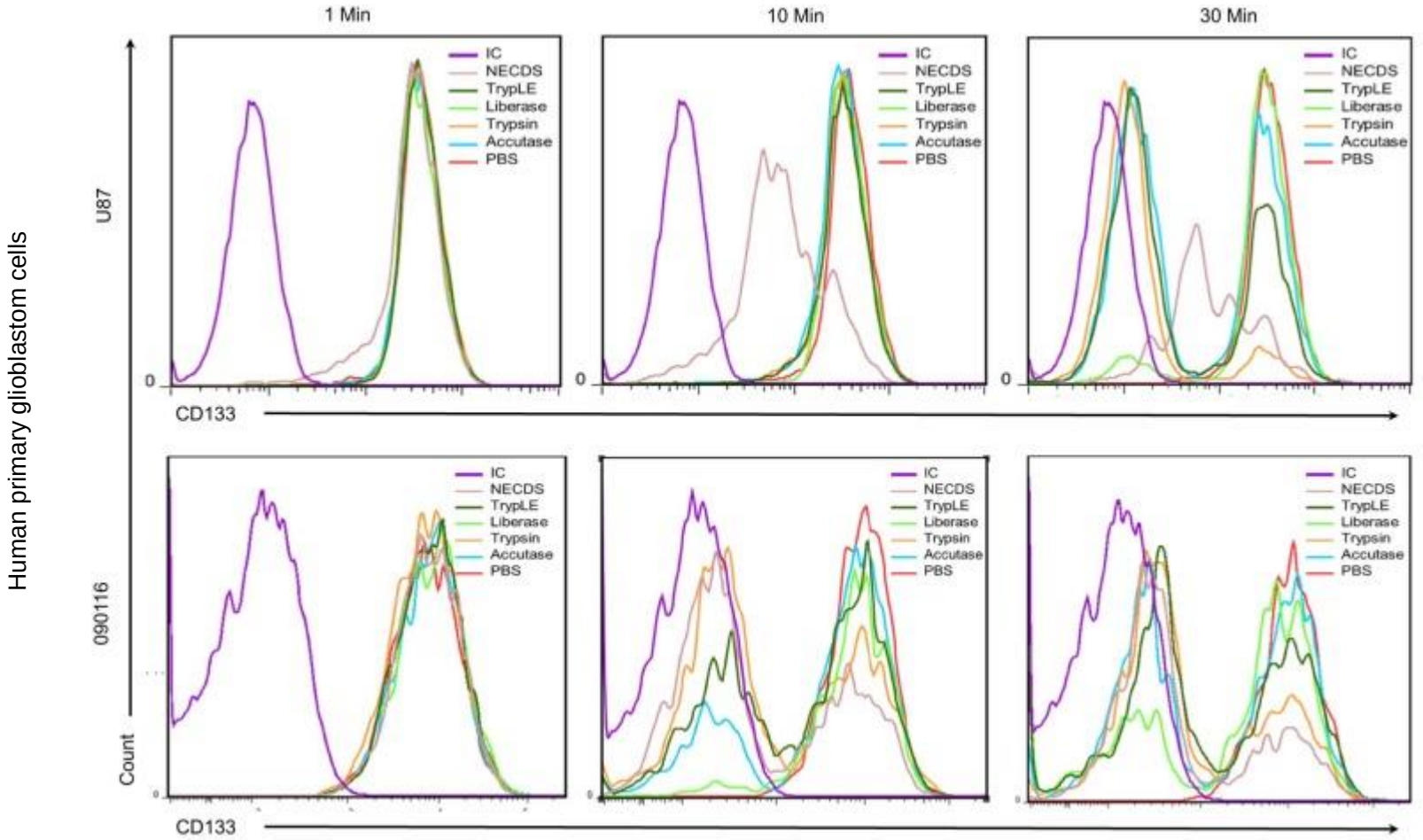
sample preparation - tissues



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

- complex enzymatic digestion needed
- goal: single cells with >50% vitality and intact epitopes

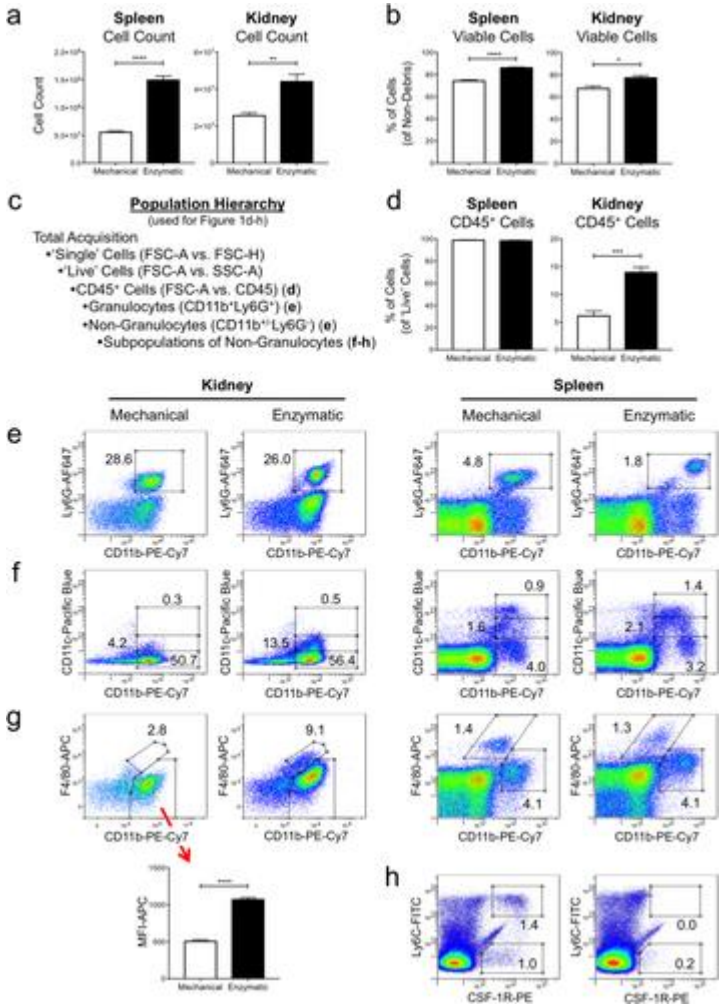
sample preparation - enzymatic digestion



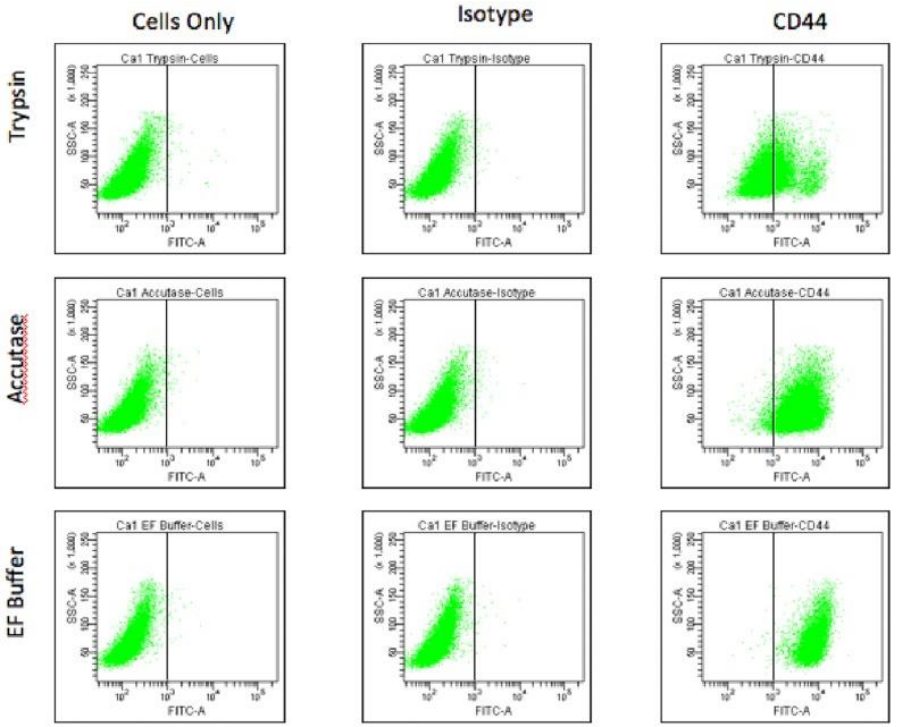
NECDS (non-enzymatic cell dissociation solution): detachment of primary cells, EDTA based
TrypLE (Gibco): animal origin-free, recombinant enzymes for dissociating adherent mammalian cells → replaces trypsin, superior performances
Liberase (Merck): Kollagenase I / II + Thermolysin (Protease)
Accutase: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

CD133 – prominin-1 HSC,CSC marker

enzymatic vs. mechanical dissoziation



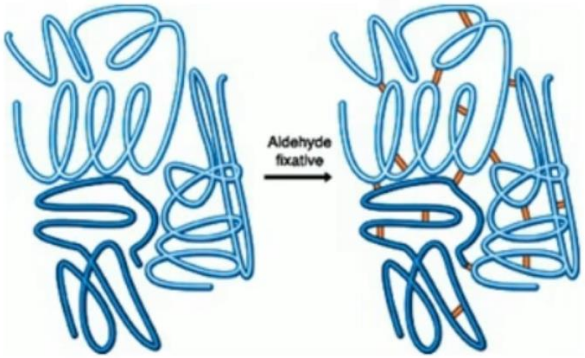
- Single cell suspension
- Method of harvest: trypsin, accutase, EDTA
  → crucial for successful staining

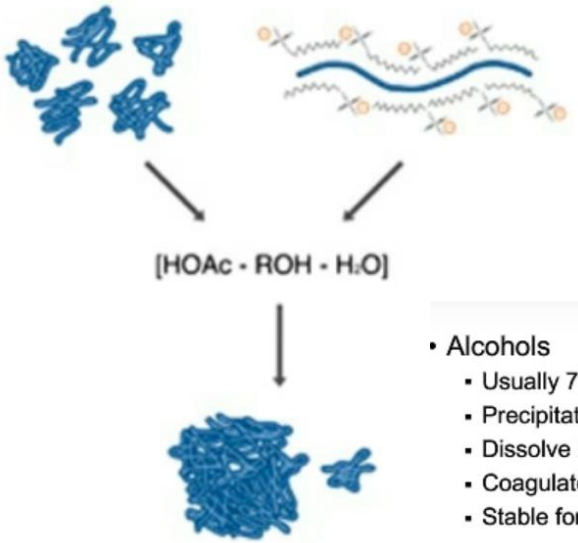


fixation of cells

- fixation BEFORE staining - epitope alteration
- GFP, PerCP, fluorescence loss after fixation (APC-Cy7< APC-H7)
- fixation AFTER staining - fluorophore stability
- but pSTAT 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

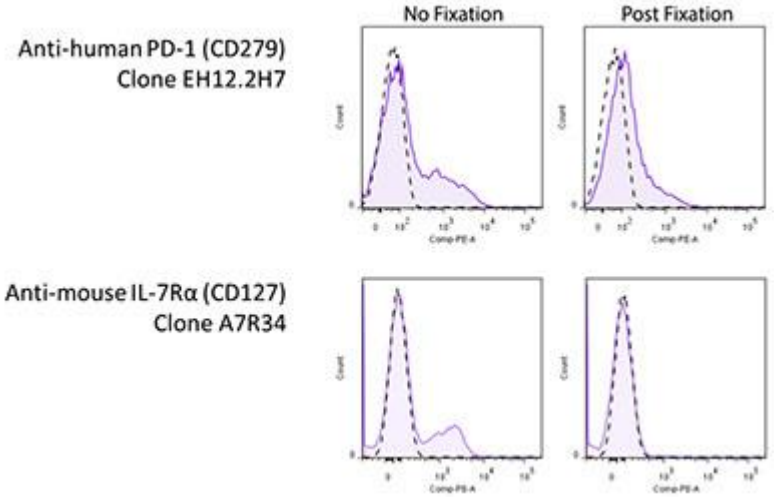
https://www.youtube.com/watch?v=oeqiCa3nEwc

what to consider - 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 who express 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

what to consider - intracellular staining

- also block intracellularly
- dilute antibodies in perm buffer when perm buffer is saponin (o.a.) based (reversible)
- use bright fluorophors (background/autofluorescence increases always)
- use (FMO) controls



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

Unbound antibodies are trapped in the cells in the case of intracellular staining

•Wash the cells adequately after each antibody incubation step and include Tween or Triton X in wash buffers.

The surface antigen is getting internalized

•Perform all protocol steps at 4°C and use ice cold reagents. We recommend you permeablize on ice.

TAKE Home Message

- the better the preparation and protocol of your samples
- the better results/analysis you get
- coaggulants, Ficoll, lysis buffer, enzymes, fixation buffer interfere with epitopes and reduce signal
- in multi-colour panels optimization towards high signal, low doublets, high viability is the goal!

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Thank you for your attention.

Next topic (6th July): „The golden rules of panel design“

Nächstes Mal (am 6. Juli): „Die goldenen Regeln des Paneldesigns“

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