FACS in the bioanalysis

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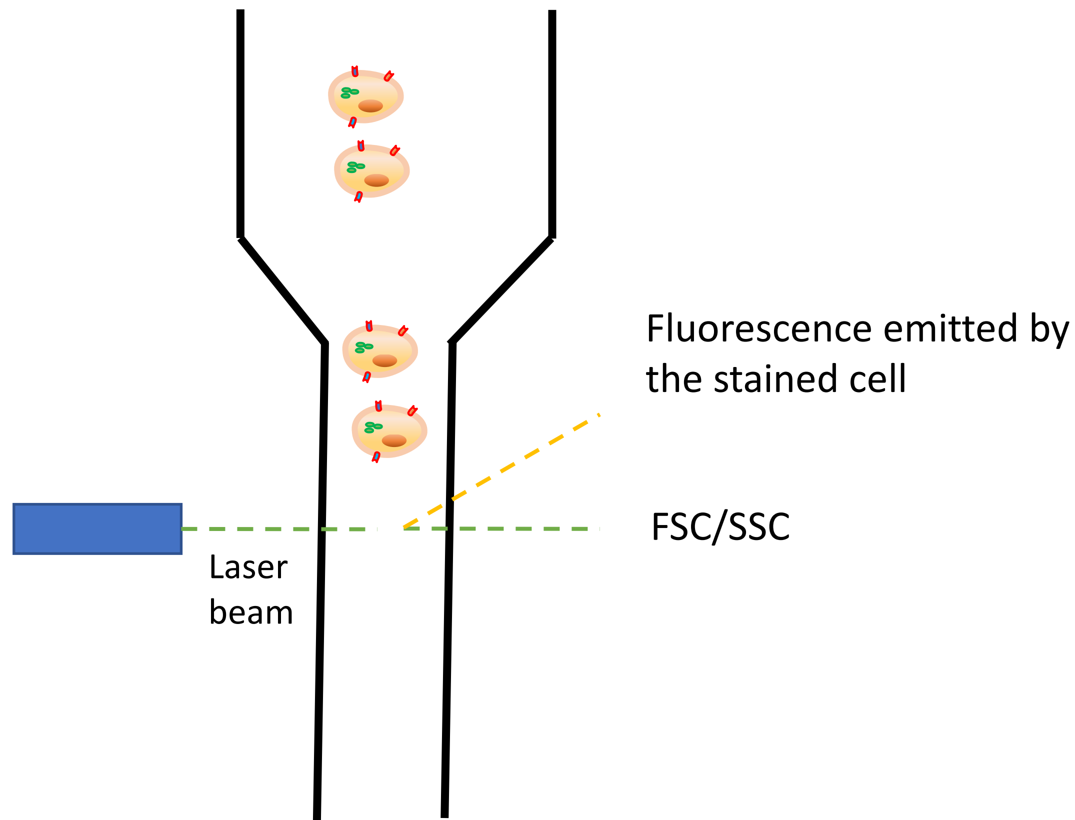
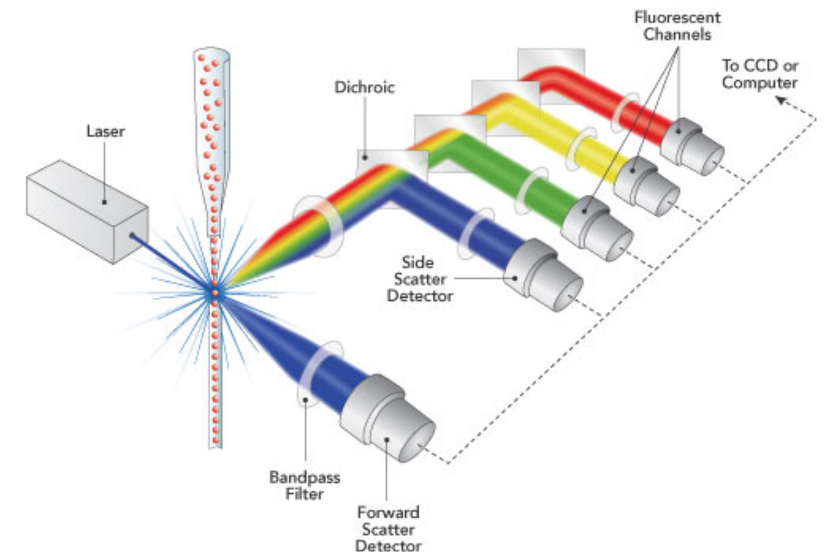
Swiss BioQuant AG

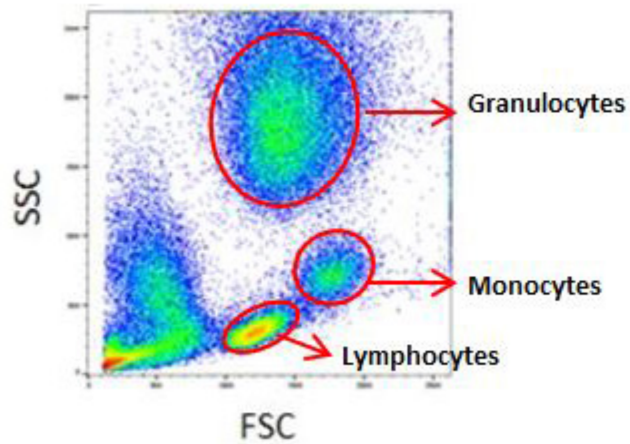
What is Flow Cytometry?

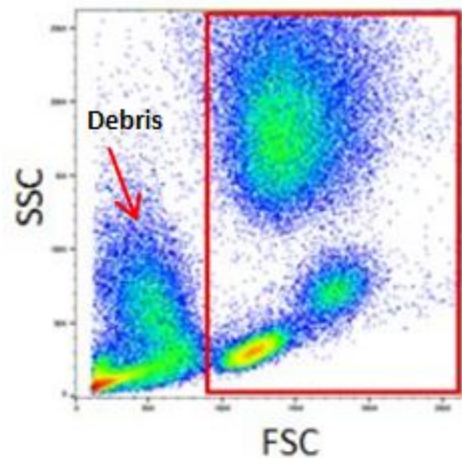


It is a technique used to perform multi-parametric analysis at the single-cell level.



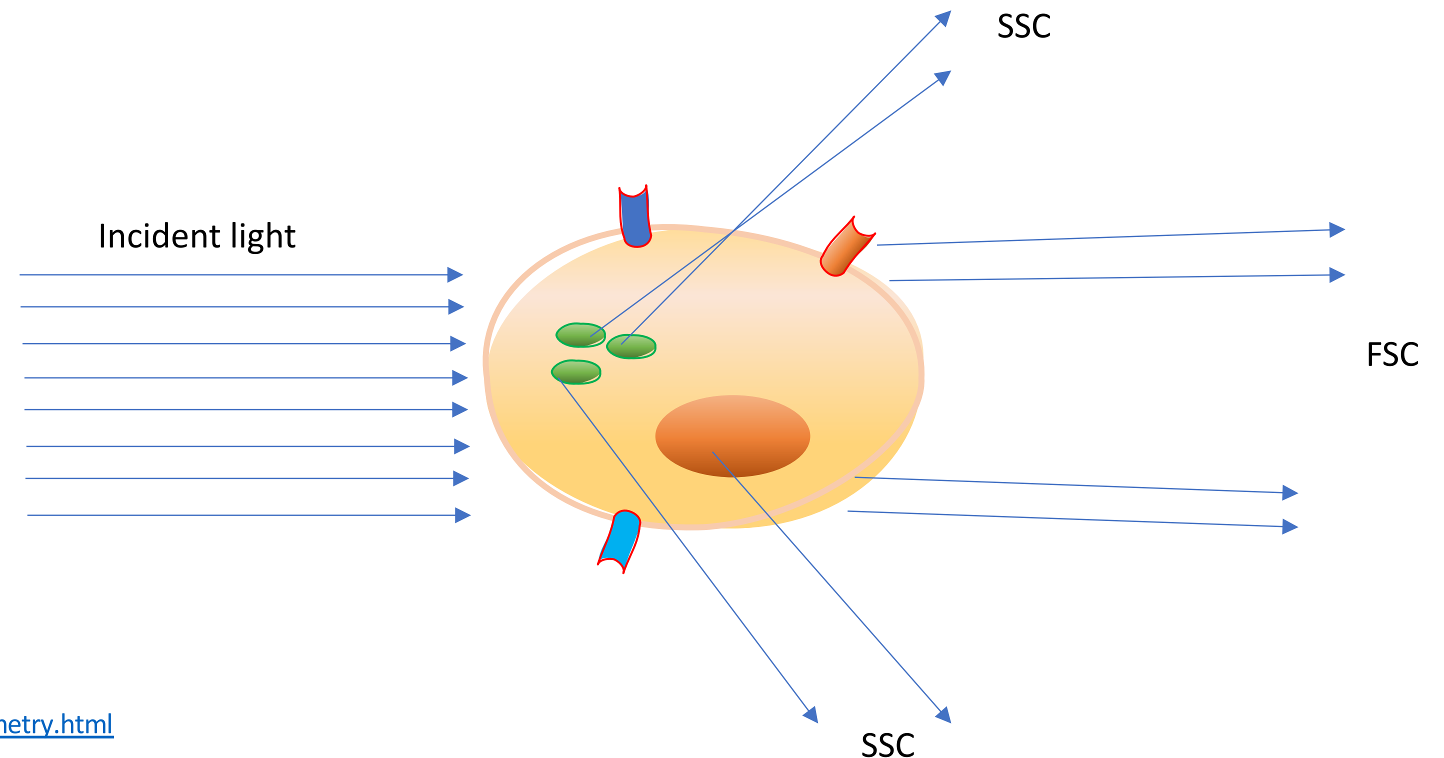
Forward and side scatter





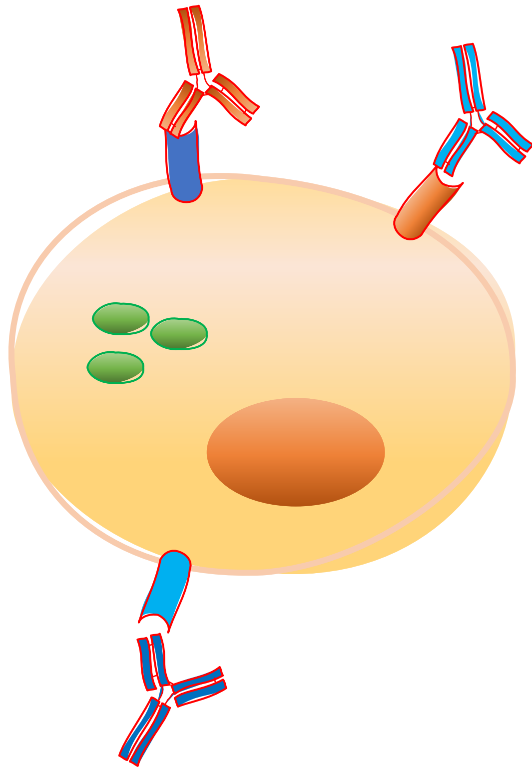
The plotting of FSC vs SSC is often the first step during the FACS analysis of the samples.

- FSC indicates the size of the cells (or beads)
- SSC is influenced by the granularity (complexity)



Source: https://www.bio-rad-antibodies.com/blog/a-guide-to-gating-in-flow-cytometry.html

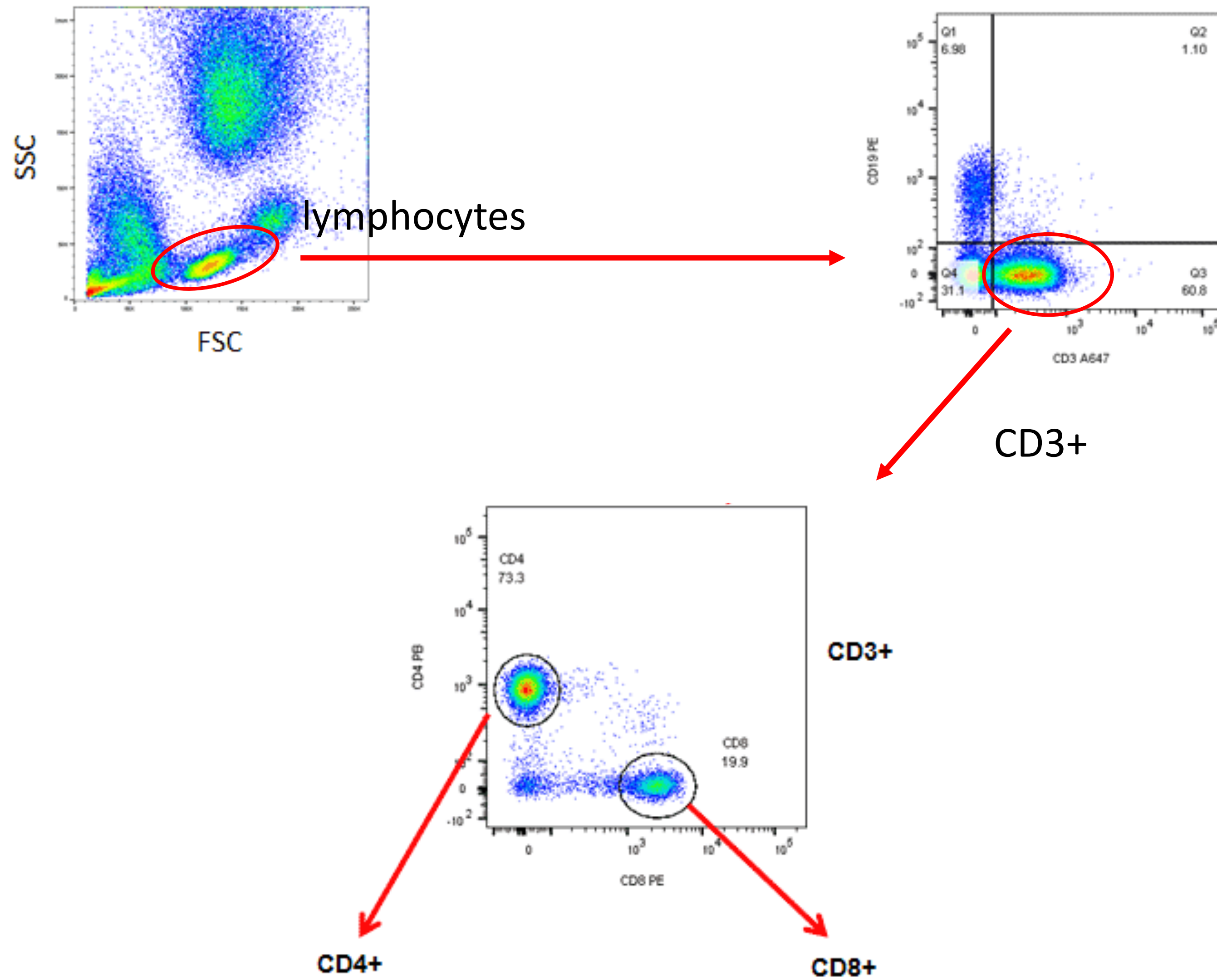
Staining of the cells



Staining panel:

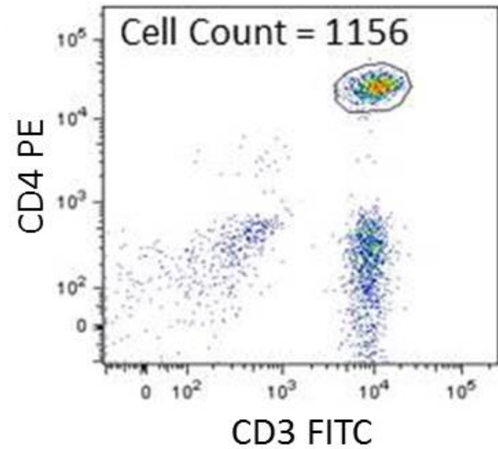
- CD3 (T-cell marker)
- CD19 (B-cell marker)
- CD8
- CD4

Gating strategy



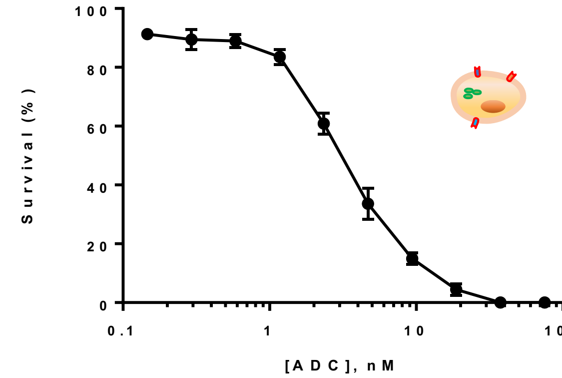
Source: https://www.bio-rad-antibodies.com/flow-cytometry-gating-strategies.html

Fields of application... (Some of them)

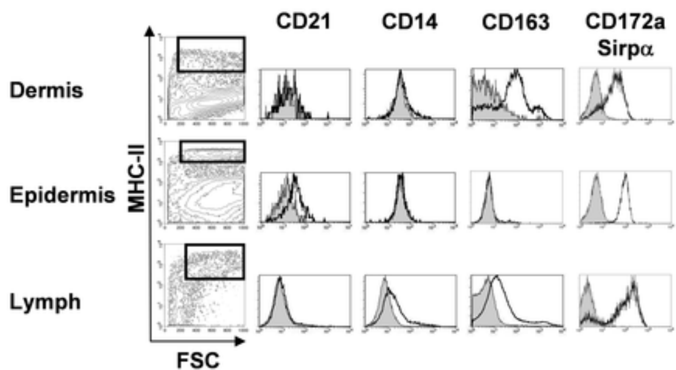


Cell count

Source:https://www.biolegend.com/en-us/protocols/precision-count-beads-protocol-and-applications

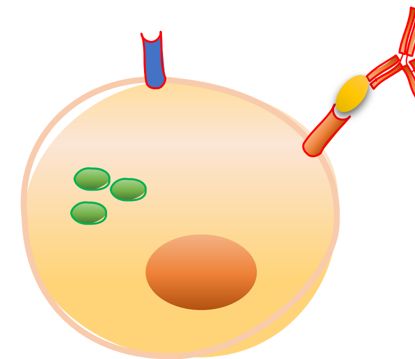


Potency assay



Marquet et al.

Phenotyping



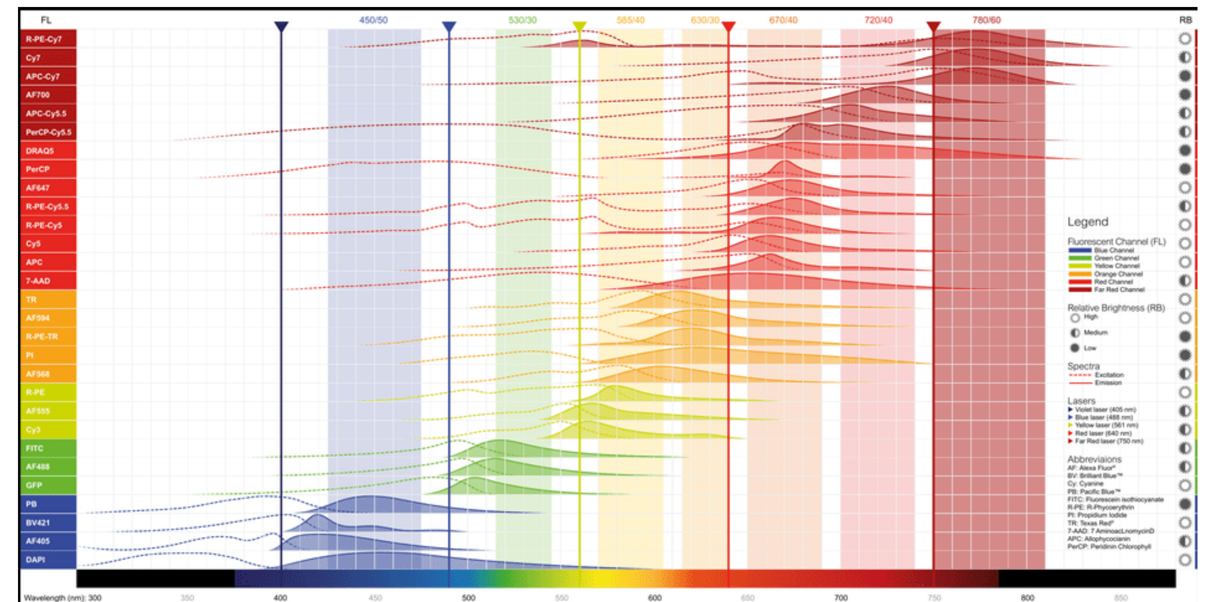
Receptor occupancy

Main challenges

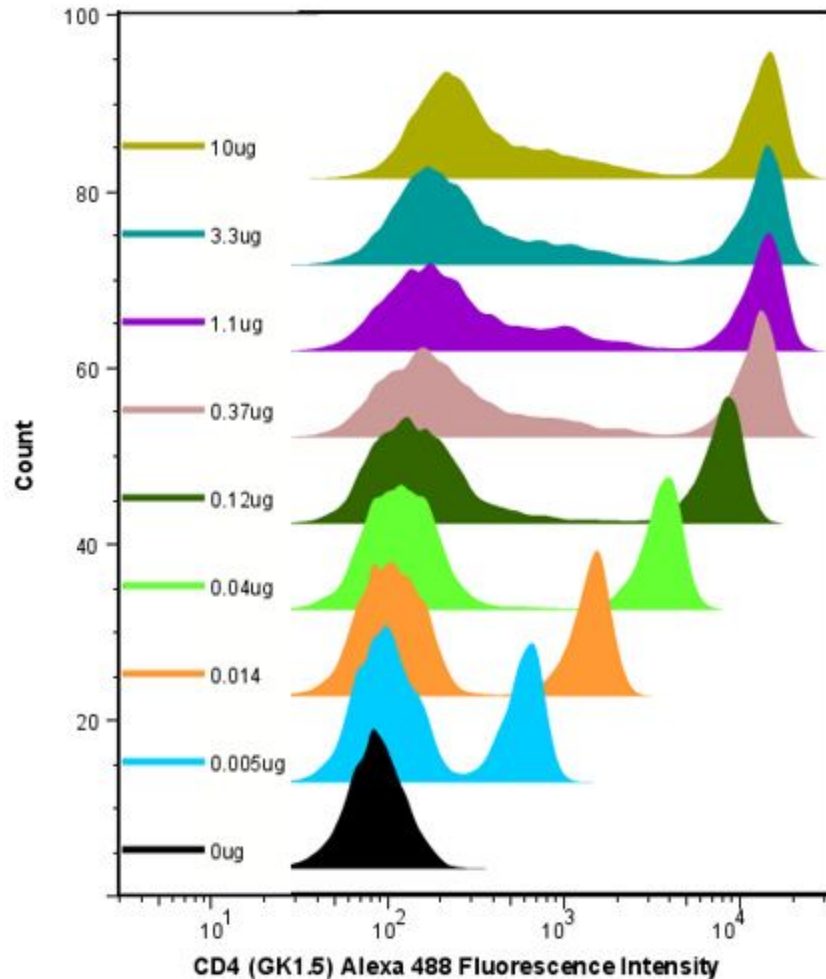
- Absence of a standard cellular reference material
- No guidelines for the validation of flow cytometry methods to be used for pre-clinical studies
- Difficult to fulfill criteria in case of rare events
- Selection of the right antibodies
- Stability of the samples

Before validation: Panel design

1. **Know your FACS**
    - Lasers and filters present in the instrument, affect the type of fluorochromes of choice
2. **Know your cell population and antigen**
    - Use bright color for low expressed antigens and less bright for high expressed antigens
3. **Minimize the «spill over»**
    - Try to select colors which emit at wavelenghts far from each other
4. **Include controls**
    - Unstained, FMO, live/death markers are keys to success
5. **Optimize the staining protocol**
    - Important to reach a good signal/noise separation



Validation parameters: Antibody titration

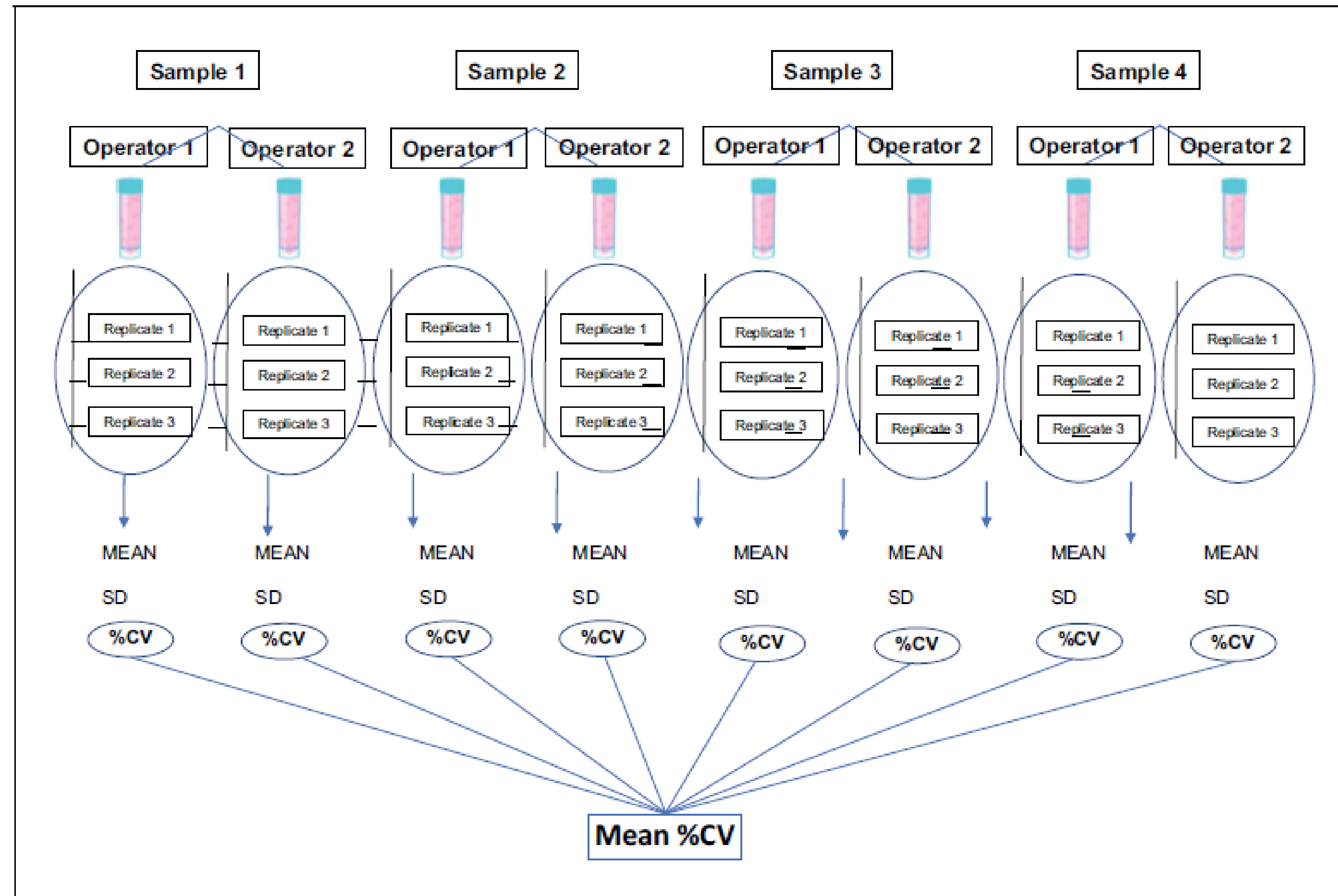


The optimal antibody titers are evaluated by compairing the staining of populations which overexpress the marker against negative populations. The optimal concentration has the best separation of the two populations.

An excess of antibody could lead to an increase of the background signal (non-specific staining of the negative population). Therefore, the selection of the right titer is crucial for the assay.

Source: https://www.pinterest.ch/pin/106397609916502386/

Validation parameters: Precision of the assay



Number of Samples 3-6

Number of Replicates 3-5

Number of Runs 2 or more (This enables determination of intra-precision, inter-precision and inter-analyst variability)

Number of Operators 1-2

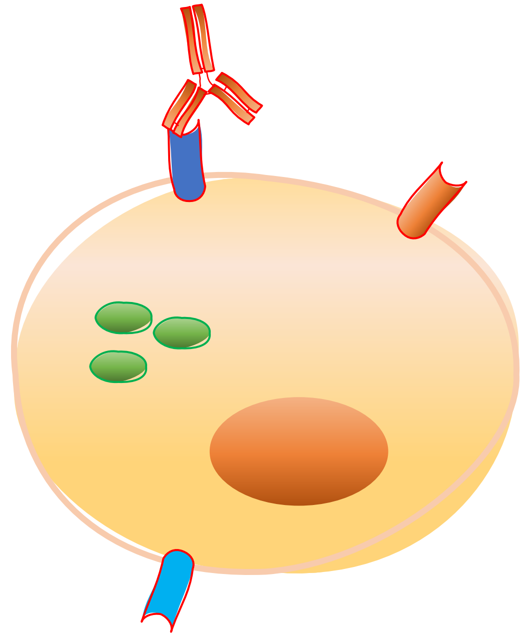
Statistical Evaluation

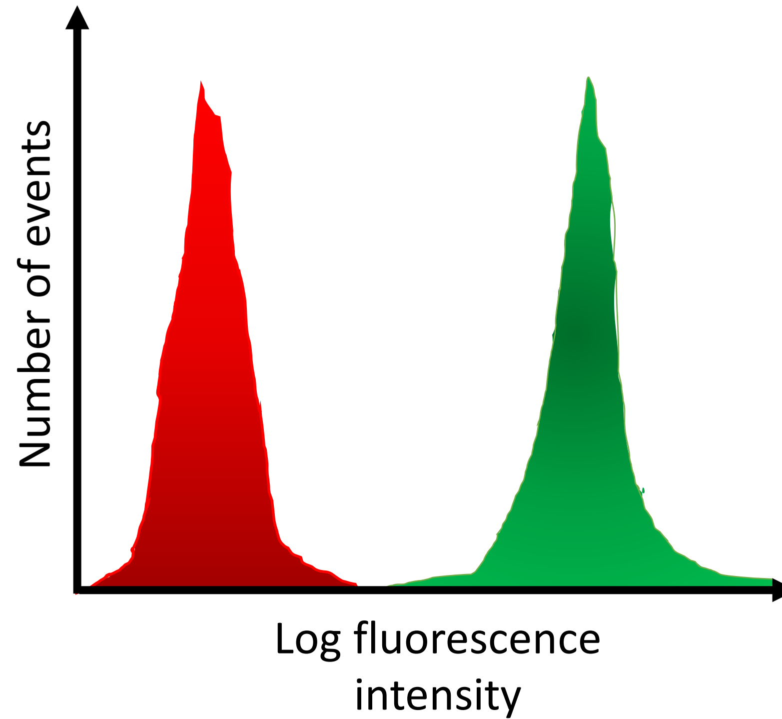
- Calculate the mean, SD, and %CV for each reportable result from each sample where all three replicates are >LLOQ, if established
- Calculate the mean of the %CV for each reportable result from all samples (Mean %CV). The intra-assay precision for each reportable result should be reported as the Mean %CV followed by the CV range.

Acceptance Criteria

- 10% CV is desired, up to 25% CV is acceptable
- When the population or antigen is less well defined (rare events), 30% to 35% CV is acceptable
- It is not necessary for each sample to meet the criteria as long as the Mean %CV meets the criteria.

Validation parameters: Specificity



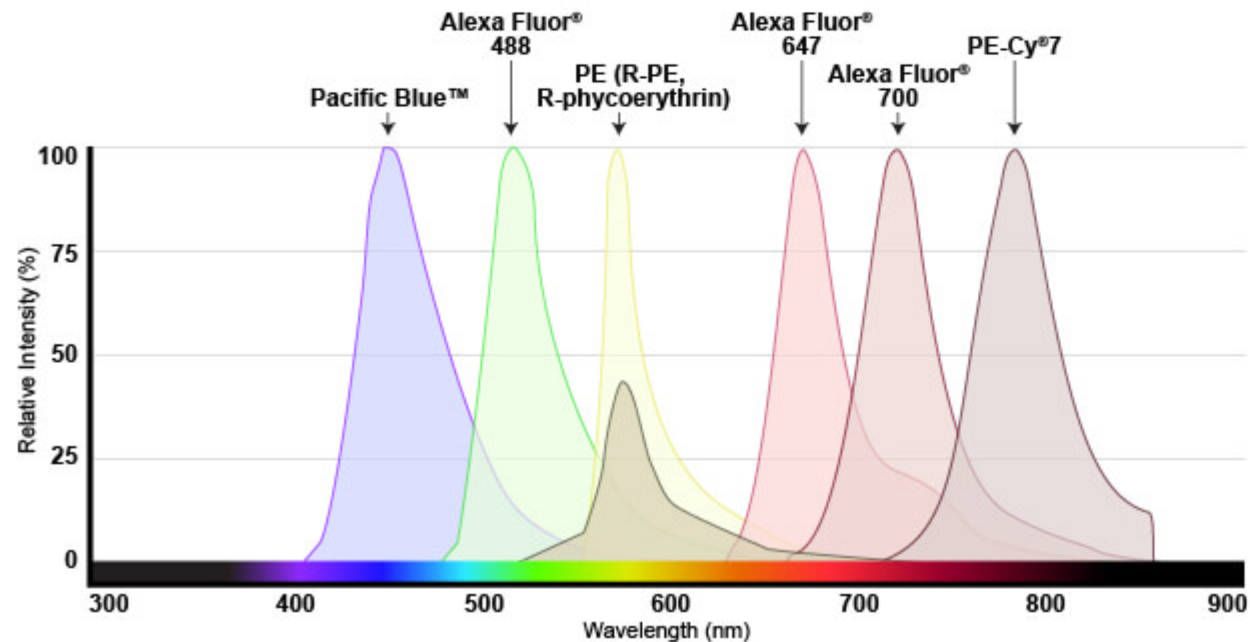


An isotype control is an antibody raised against an antigen not present on the cell type being analyzed and has been specifically developed to determine the level of background surface staining.

The best isotype control has to share the following characteristics with the actual staining antibody:

- Host species
- Fluorophore
- Ig subclass

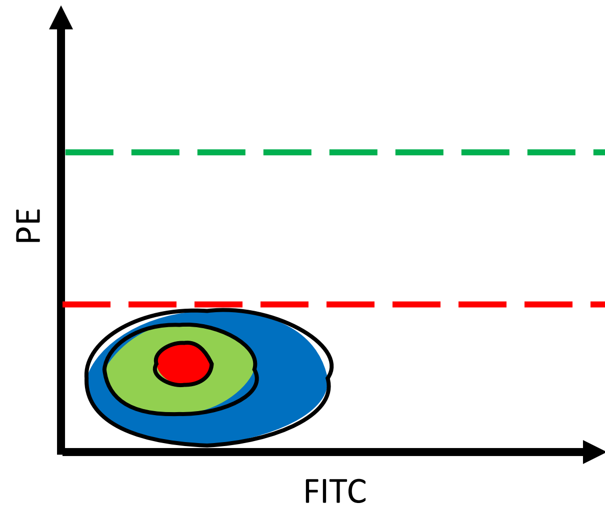
Compensation



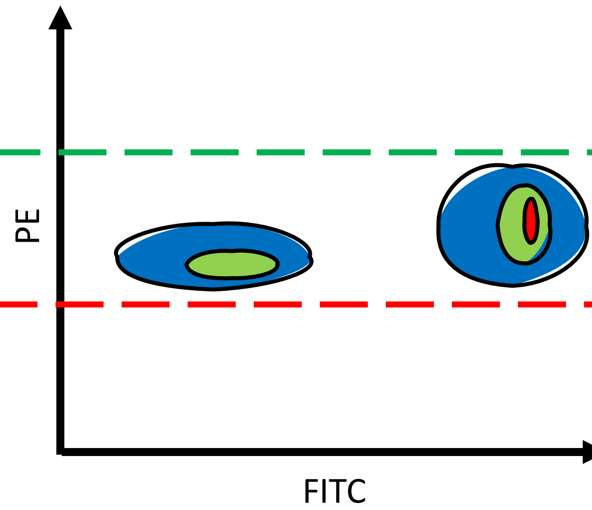
The photons emitted by fluorochromes have different energies and wavelengths and as flow cytometers use photomultiplier tubes (PMT) in order to convert the phonons into electrons, the detector can register the signal from more than one fluorochrome. This creates a signal overlap (spillover) which cannot be removed by the optical system and has to be corrected electronically

Validation parameters: Interaction between antibodies from the same panel

Unstained control

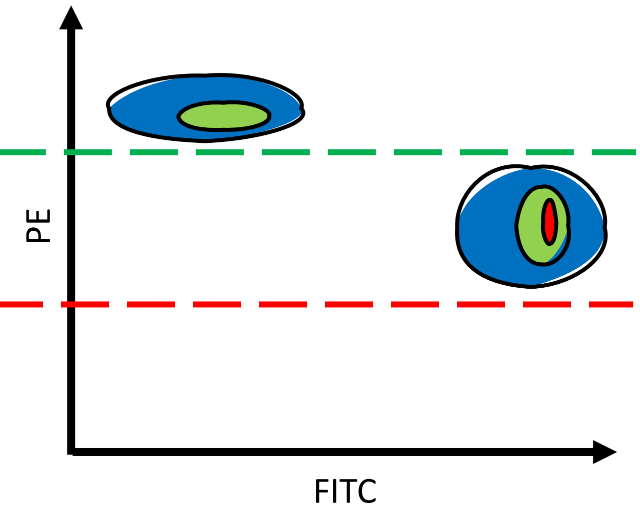


FMO control



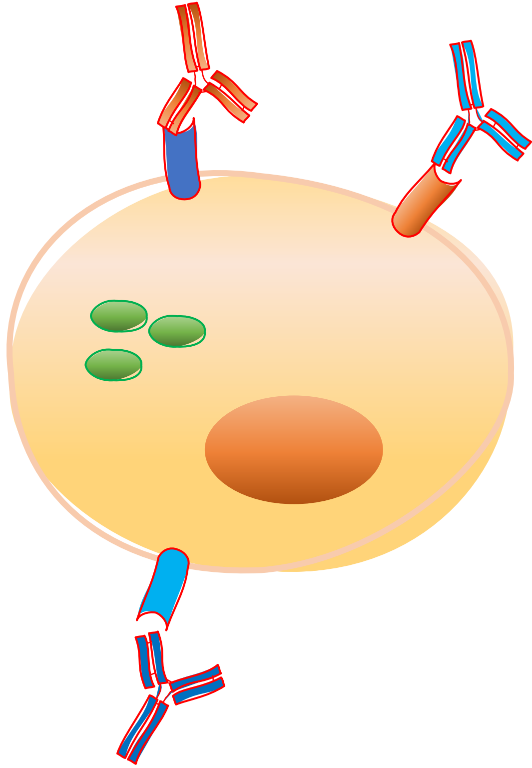
Stainings:
- FITC

Fully stained



Stainings:
- FITC
- PE

Validation parameters: Stability



Sample stabilization

PROS:

- Stabilization of surface markers between 72 and 96 hours (up to 14 days for lymphocytes)
- FDA-approved

CONS:

- Can only be used if cell stimulation is not required during the analysis
- Possible alteration of the antigen expression

Conclusions

- Flow cytometry is a powerful tool which allows multi-parametric analysis at the single-cell level.
- Compared to a classic large molecule method (e.g. ELISA) new challanges and peculiarities must be taken into account-
- Since accuracy cannot be determined, more importance has to be given to the precision.
- Standardization of different cytometers configuration is crucial to achieve comparable results.
- Until new stabilization reagents aren’t available, it is best to analyse the samples within 24 hours from collection.

THANKS FOR YOUR ATTENTION!

References

- *“Current Practices and Challenges in Method Validation”*, Florence Poitout et al., Toxicologic Pathology 2018
- *“Best practices in performing flow cytometry in a regulated environment: feedback from experience within the European Bioanalysis Forum”*, Barry van der Strate et al., Bioanalysis 2017
- *“Approaching stability challenges for flow cytometry in a regulated bioanalytical environment”,* Anamica Muruganandham et al., Bioanalysis 2019
- *“Flow cytometry method validation protocols”*, Nithianandan Selliah et al., Current Protocols in Cytometry 2018