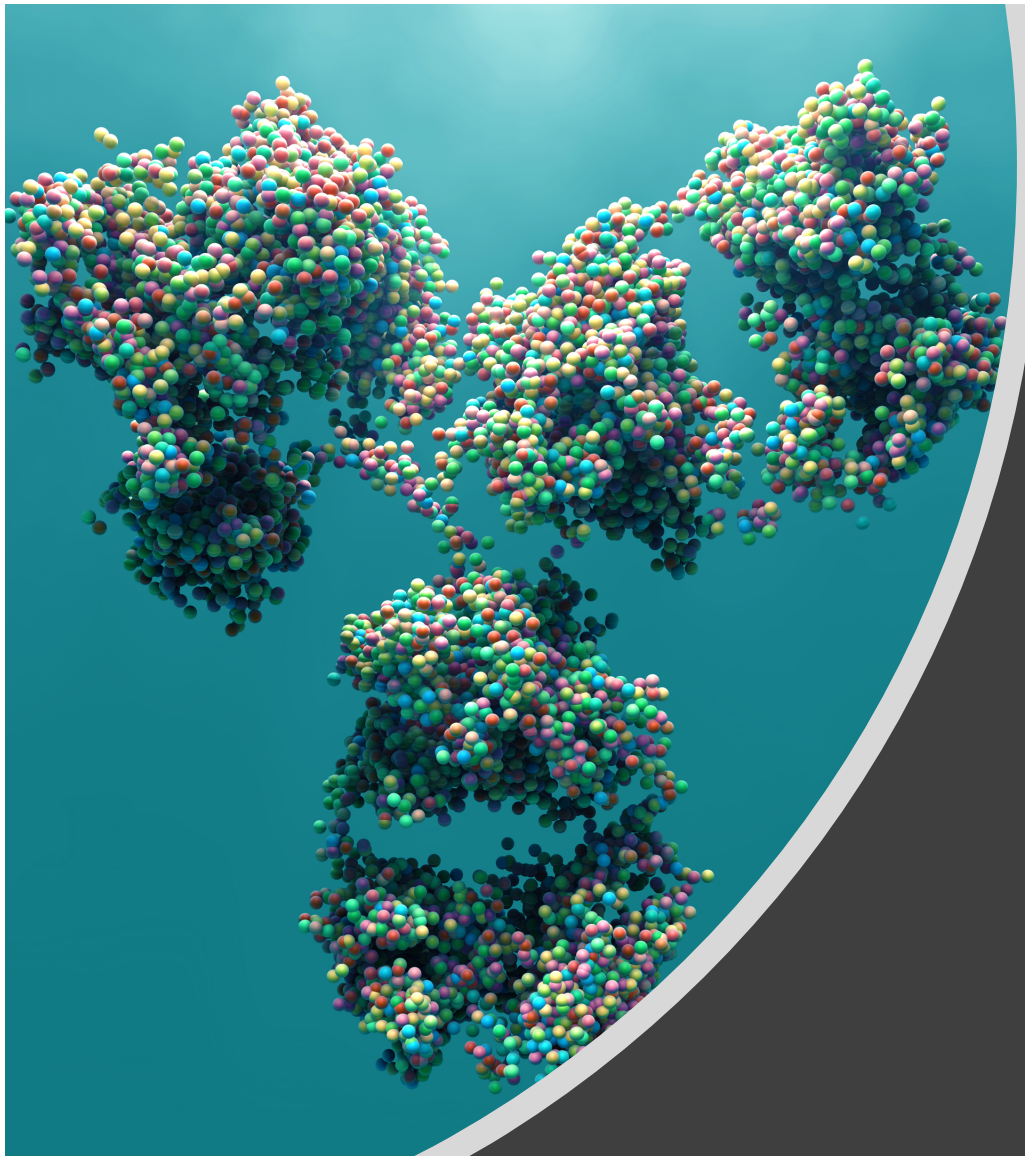
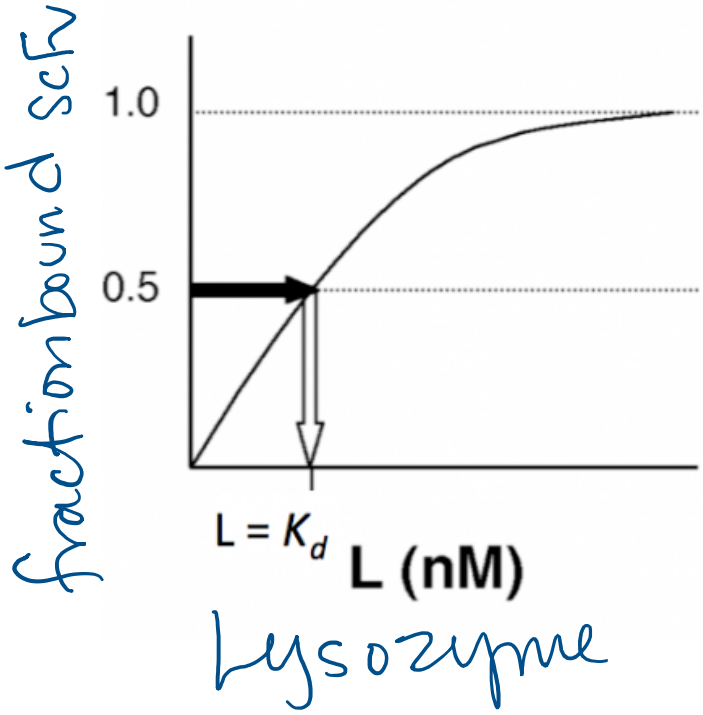


Flow cytometry for analysis and diagnosis

RECAP-Biomolecular binding interaction at equilibrium:

The antibody dissociation constant (K_d) is equal to the antigen concentration at which 50% antibody is bound to antigen.



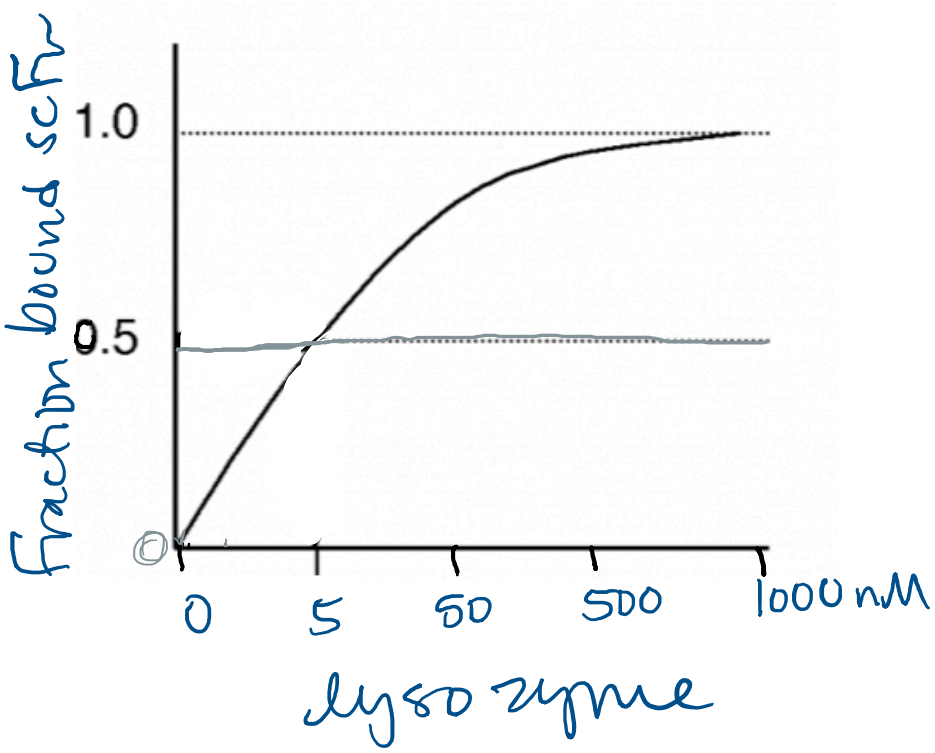
$A + B \rightleftharpoons AB$

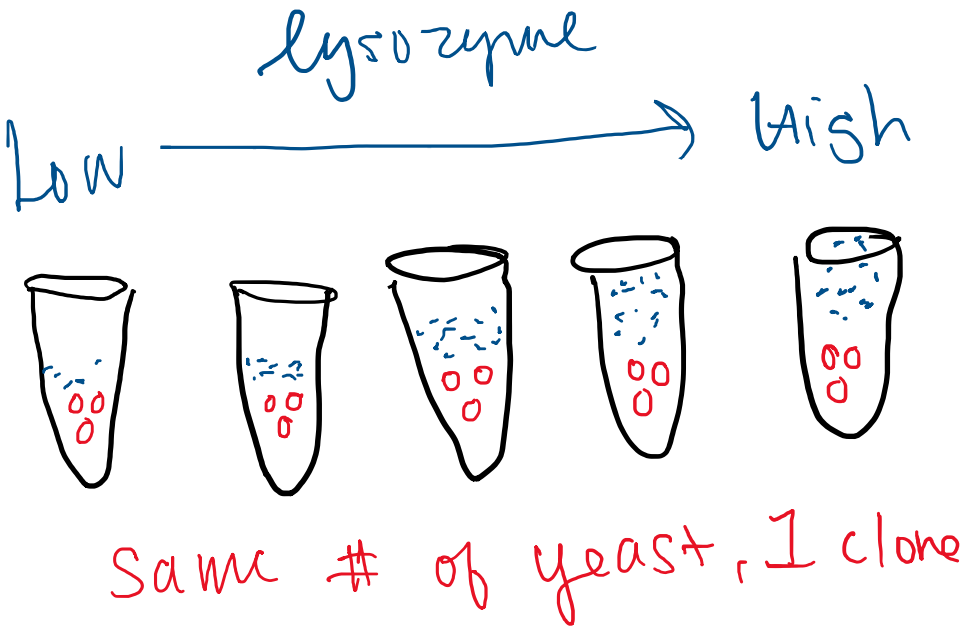
Lysozyme + scFv ⇌ lysozyme-scFv

when $K_d = [A]$

fraction B bound $= \frac{1}{2}$

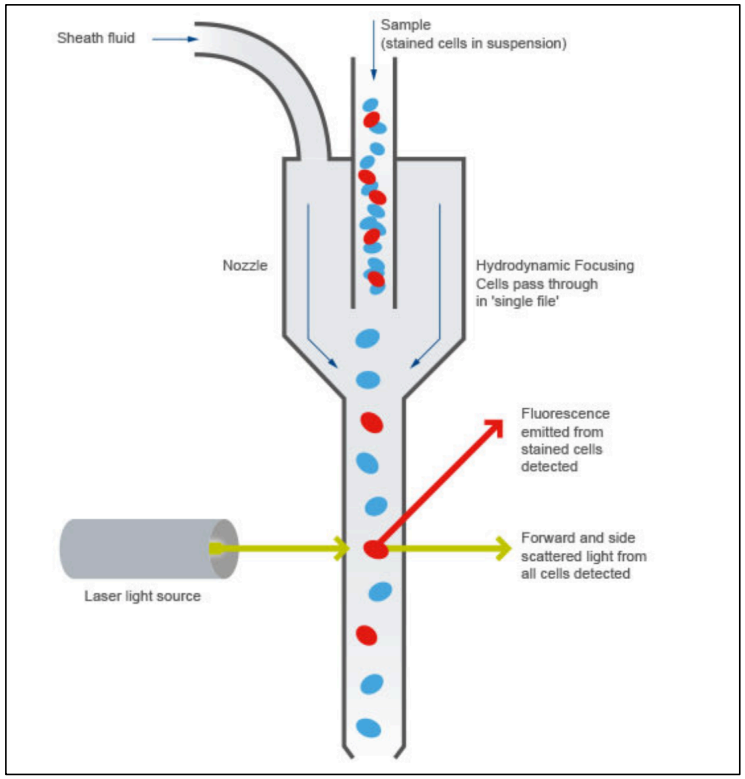
RECAP-Practically how will we measure the strength of our lysozyme and scFv interaction





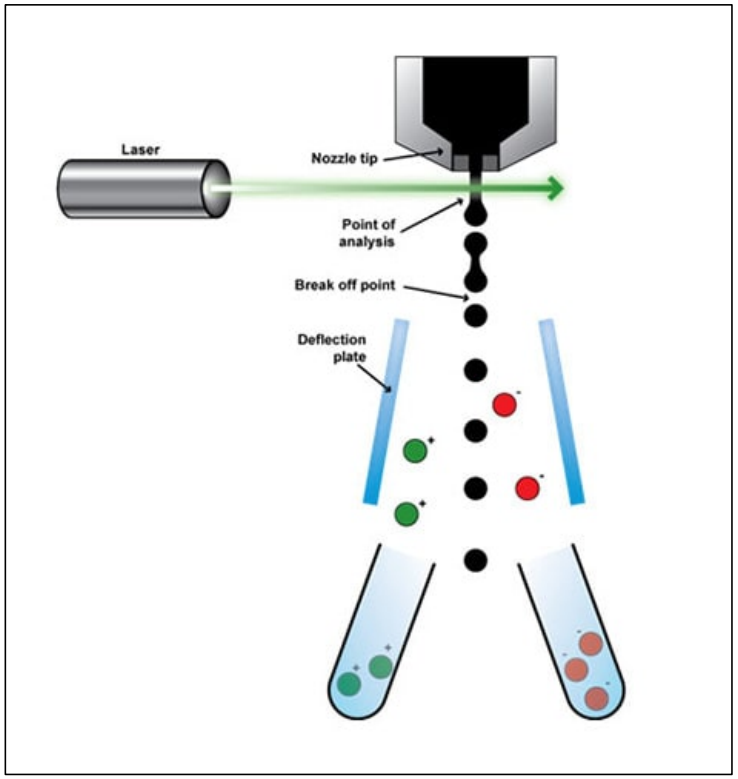
Flow cytometry and FACS: related but distinct experimental approaches

Flow cytometry: analyze cells based on physical and fluorescent qualities



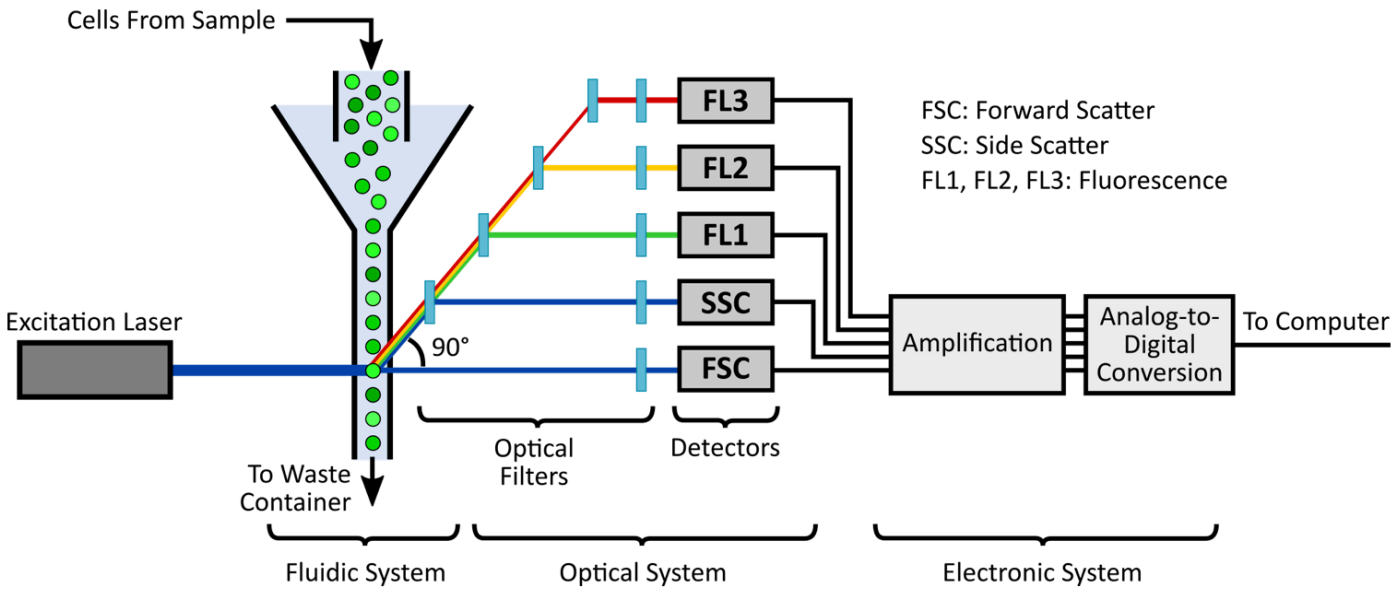
https://www.jove.com/science-education/10494/flow-cytometry-fluorescence-activated-cell-sorting-facs-isolation

FACS: extension of flow cytometry, cells can be sorted based on specified characteristics

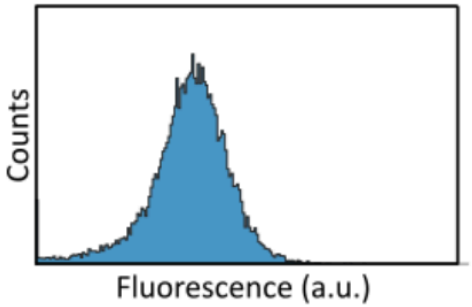


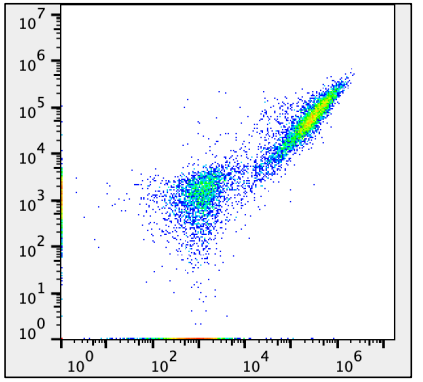
Images: abcam.com/protocols/introduction-to-flow-cytometry

Components of a flow cytometer



Output: Histogram or Scatterplot





benchling.com/pub/tabor-flowcal

Our flow cytometer: Accuri C6

BD Accuri™ C6 Flow Cytometer Instrument Manual

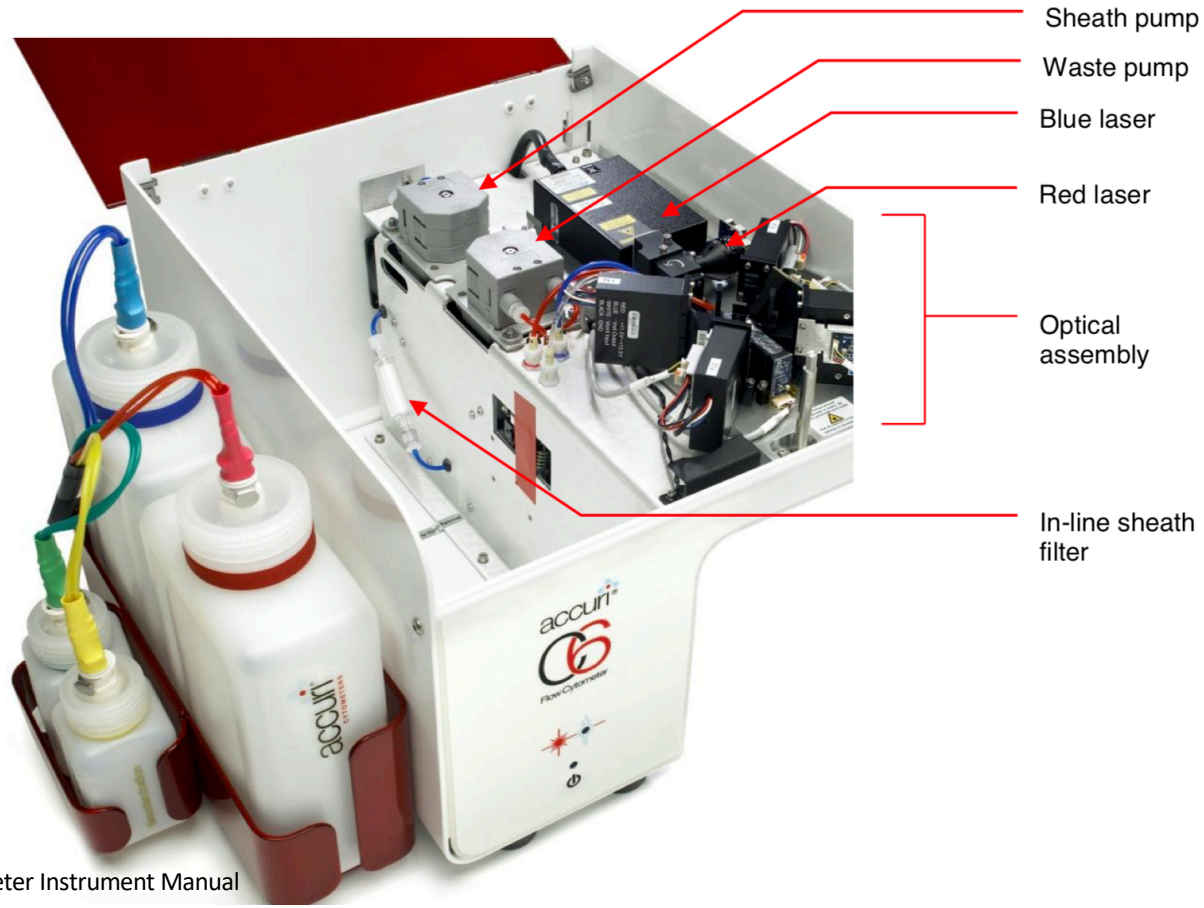
Benefits

- Compact and easy to use
- Open configuration
- Easily add samples by placing tube under the sample stage
- Low price (~$30-50K)

Downside

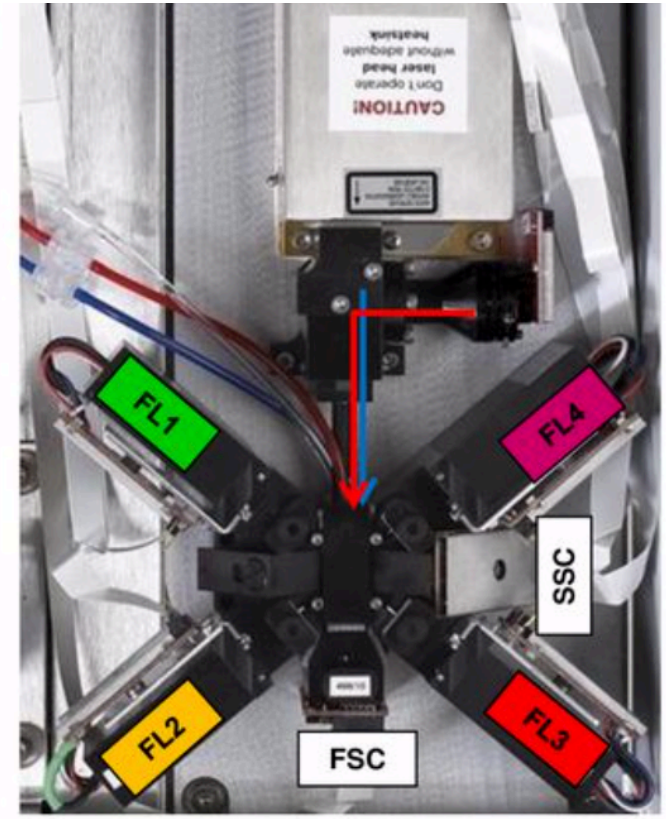
- Limited to four color fluorophores
- Analysis precision slightly lower
- Can’t sort cells after analysis

Major components of the fluidics and optics subsystems



BD Accuri™ C6 Flow Cytometer Instrument Manual

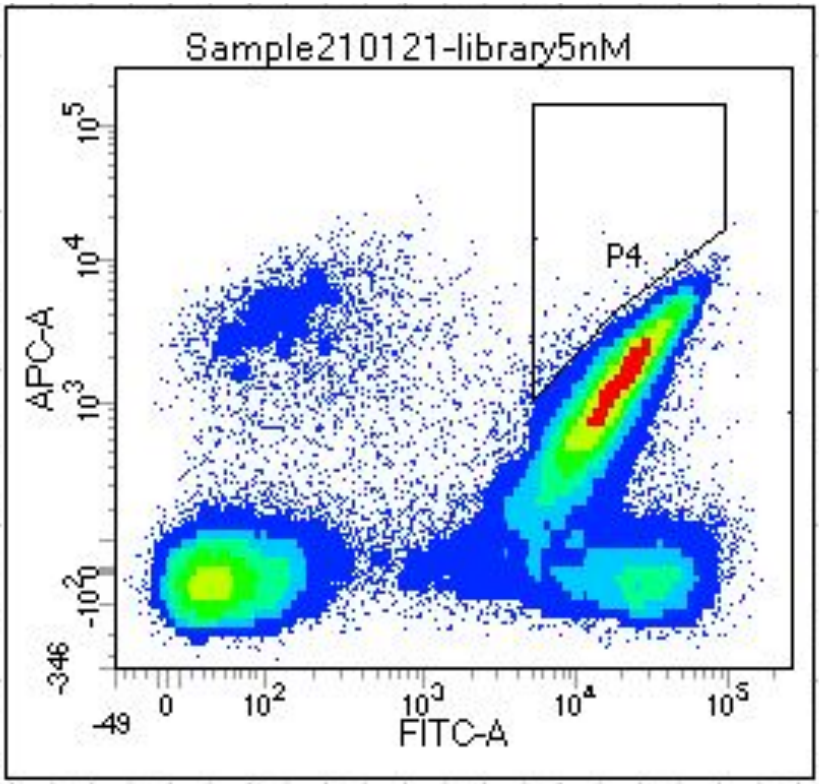
Cells pass through lasers in a flow cell and emission is measured by detectors



BD Accuri™ C6 Flow Cytometer Instrument Manual

- The sample stream (cells) is pressurized upward through an optically clear region of the flow cell
- Cells within sheath fluid pass through the laser beam (2 lasers) while in the flow cell
- Four fluorescence detectors and two scatter detectors process the signal
  - Forward scatter, side scatter
  - **488 excitation (blue laser)**
    - FL1 (530±15nm) – AlexaFluor 488/FITC
    - FL2 (585±20nm) – PE/PI
    - FL3 (>670nm) – PI
  - **640 excitation (red laser)**
    - FL4(675±12.5nm) – AlexaFluor 647/APC

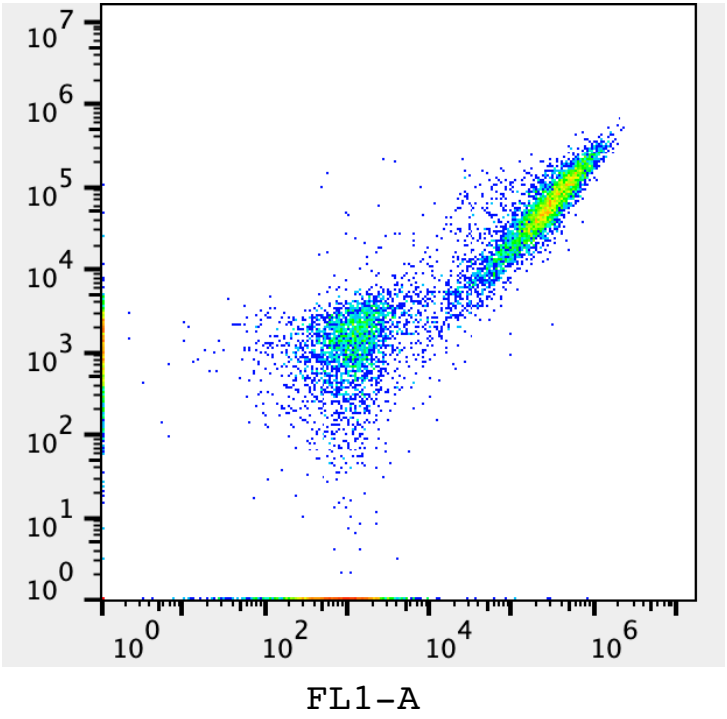
Review: FACS scatterplot represents ~1 million clones



- FACS Controls: library+ no staining, library+ fluorophore 488 only, library+ fluorophore 647 only, a positive control clone (strong binder)
- Controls are necessary to draw a precise gate
- Experiment carried out with one concentration of lysozyme
- **The cell sort is only as good as the precision of the gates!**

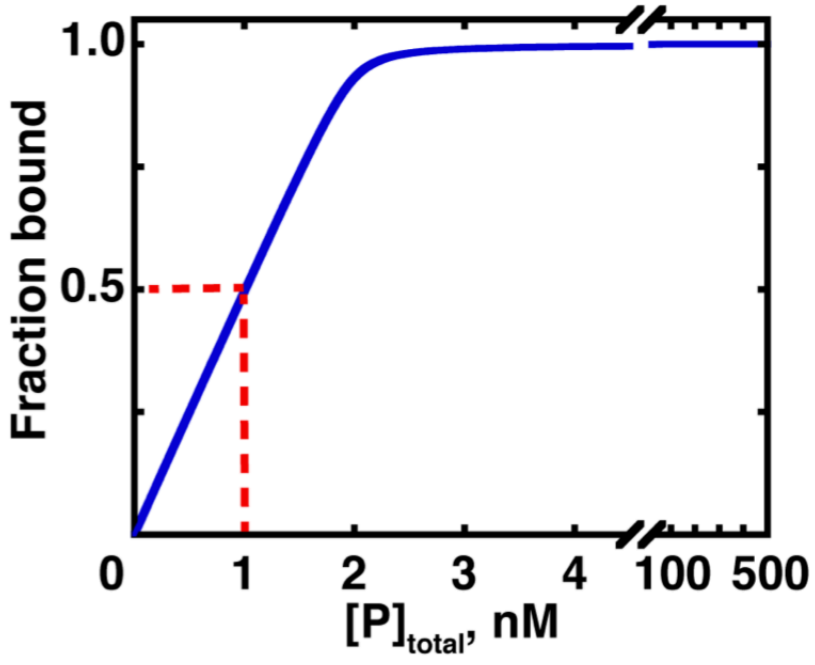
Flow cytometry scatterplots represent a single clone

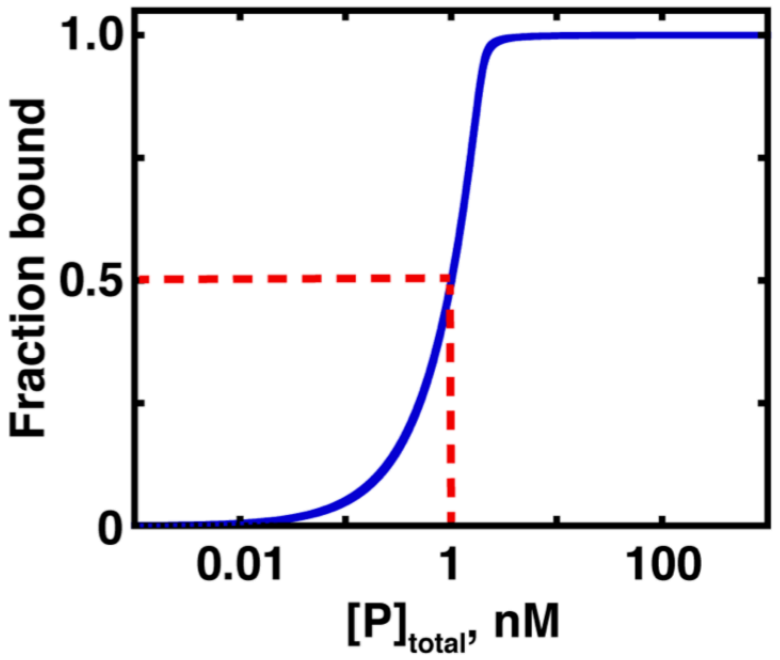
Why does this scatterplots look different from the FACS scatterplot?



- Today we will carry out a **titration** with lysozyme (8 concentrations) and a scFv clone
  - Titration: process of analysis in which the quantity of some constituent of a sample is determined by adding to the measured sample an exactly known quantity of another substance with which the desired constituent reacts in a known proportion
- [Lysozyme] range should ideally span two orders of magnitude both above and below the estimated K_d
- Titration Controls: clone+ no staining and clone+ both (488,647) secondaries only
- Gates can be drawn after the experiment is complete (why?)

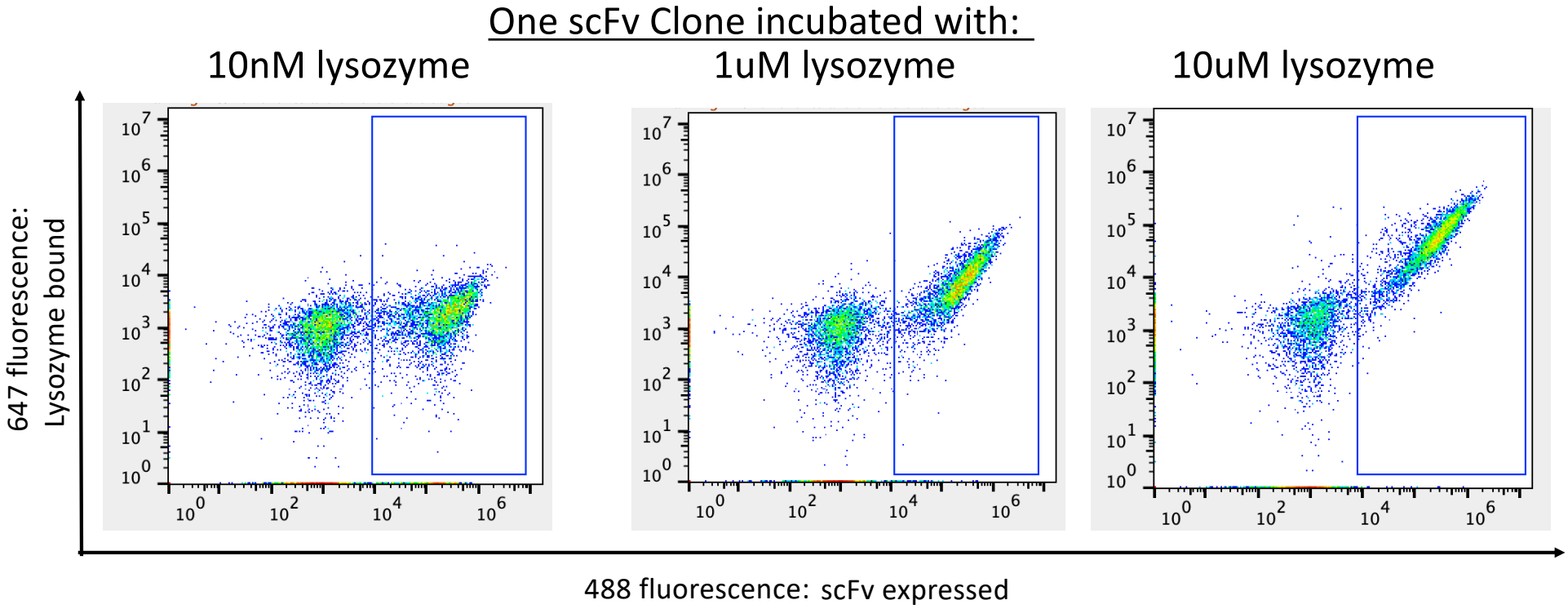
Fraction bound vs. Linear scale or Log scale antigen concentration: ease of K_d visualization





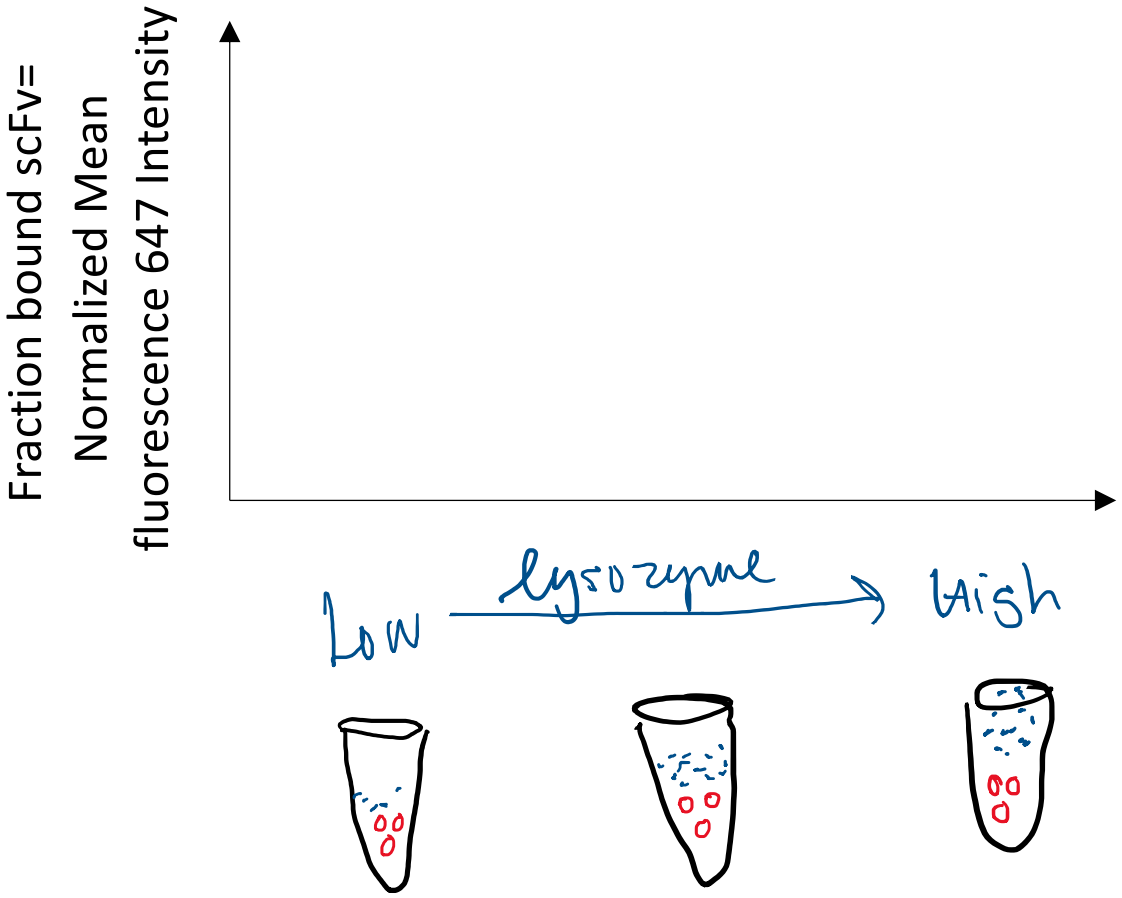
Jarmoskaite, J. et al. How to measure and evaluate binding affinities. eLife. (2020)

Median 647 fluorescent intensity of scFv positive gate is used to calculate fraction bound



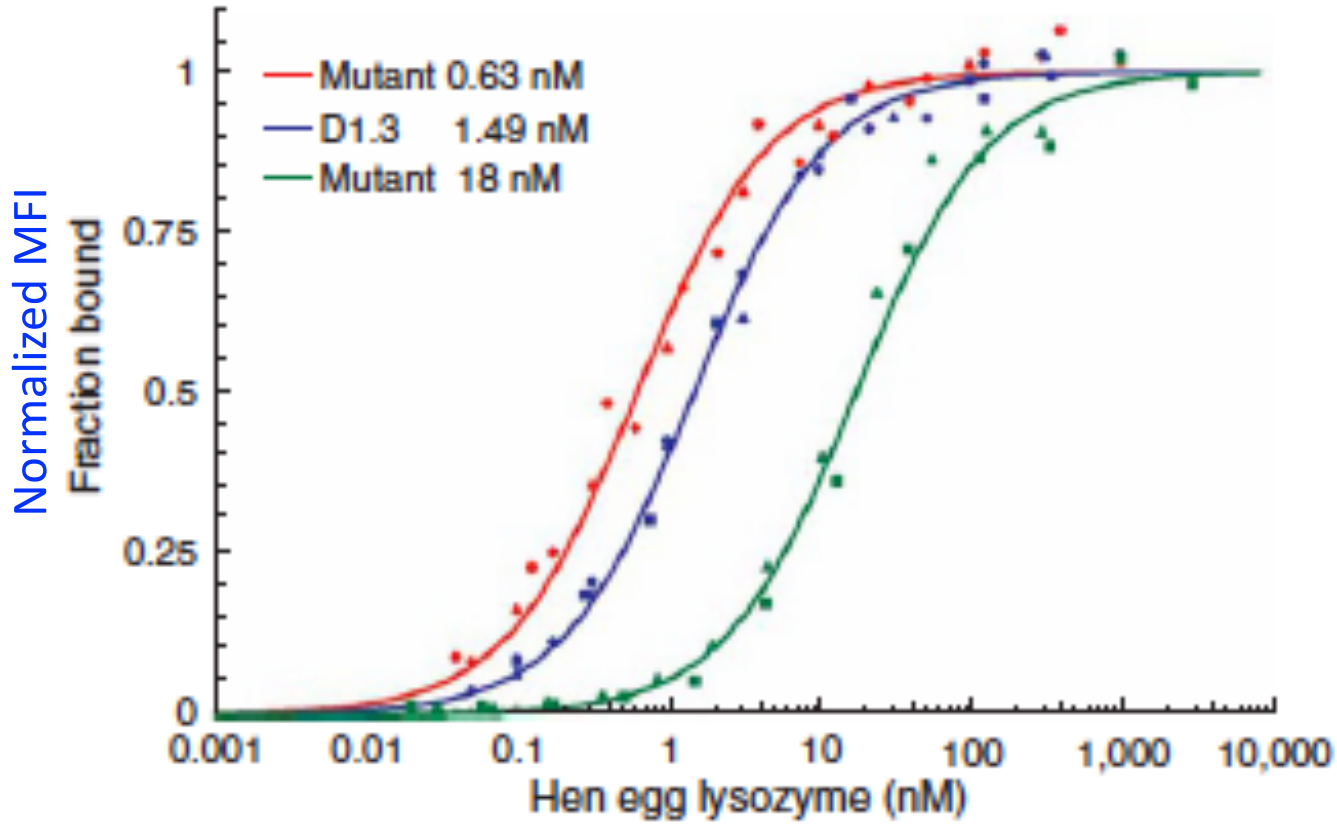
Why does “gated median 647 fluorescent intensity” represent fraction bound?

Normalized mean fluorescent intensity(MFI) equates fraction bound scFv

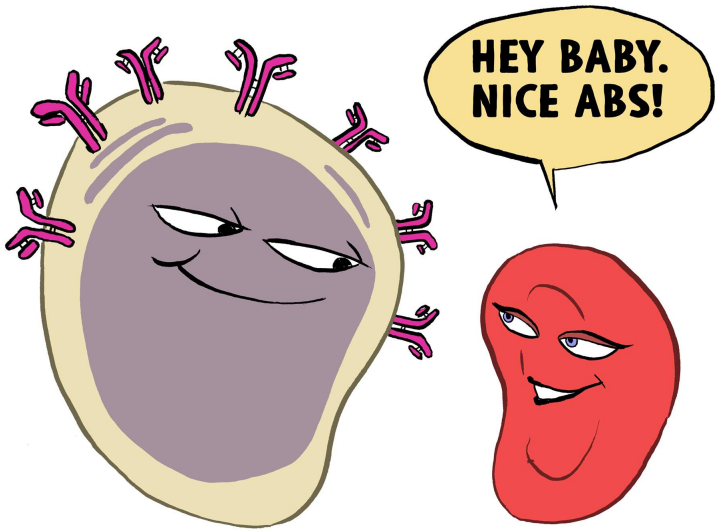


How do we normalize the signal?

We can directly compare parental clone and mutants by normalized MFI



How will we know (and illustrate) if we find a clone that's a stronger binder to lysozyme than our parental clone?

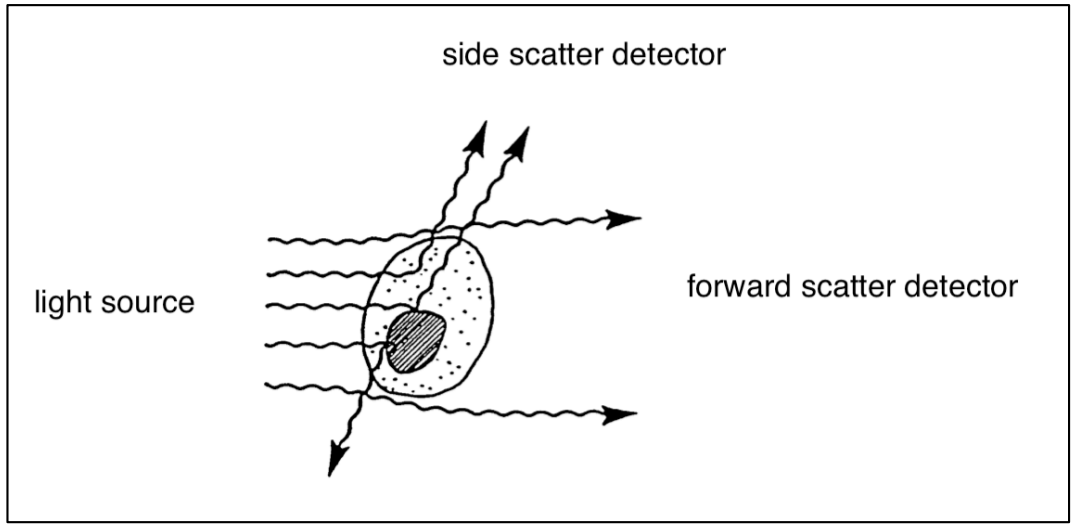


Too many B cells: CLL and the role of flow cyotmetry

By Debby R. Walser-Kuntz, Biology Dept., Carleton College

National Center for Case Study Teaching in Science

Forward scatter and side scatter gives information about cell size and internal complexity

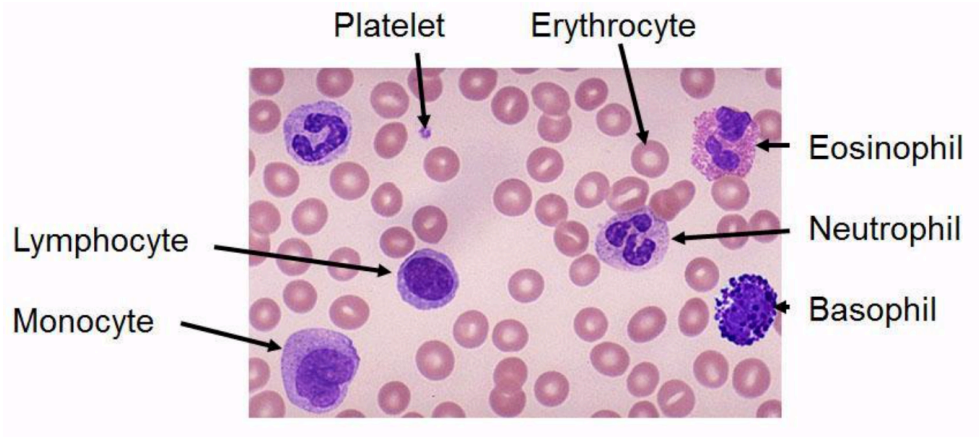


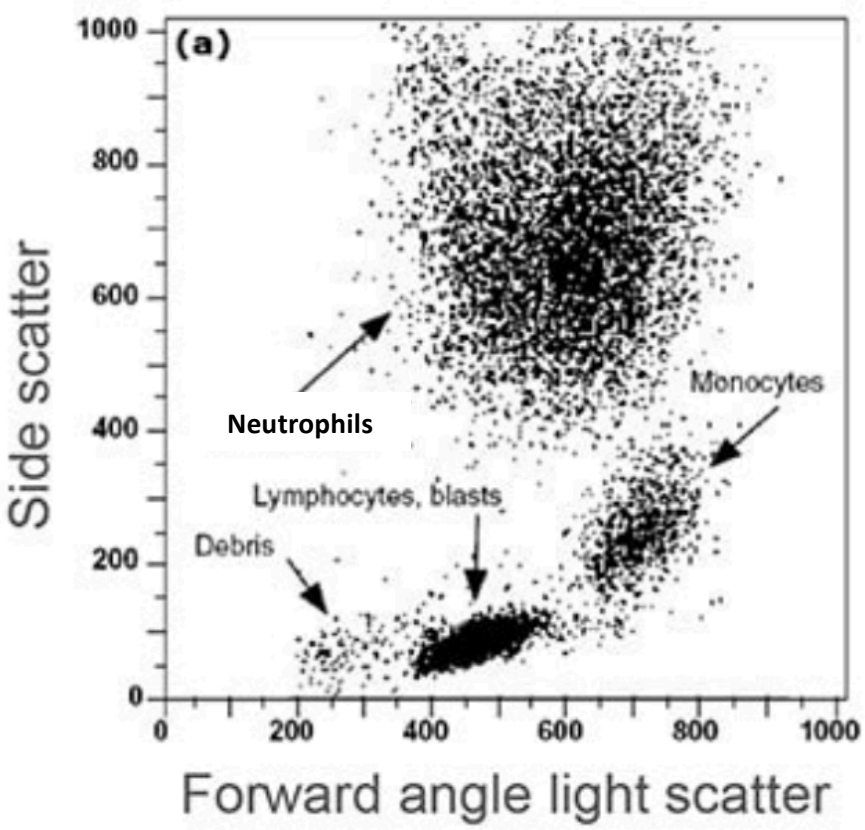
Light scattering occurs when a particle deflects incident laser light

- Forward scatter, is refraction of light, proportional to cell-surface area or size
- Side scatter, is proportional to cell granularity or internal complexity of the cell

benchling.com/pub/tabor-flowcal

FSC and SSC can allow for differentiation of cell types in a heterogeneous cell population

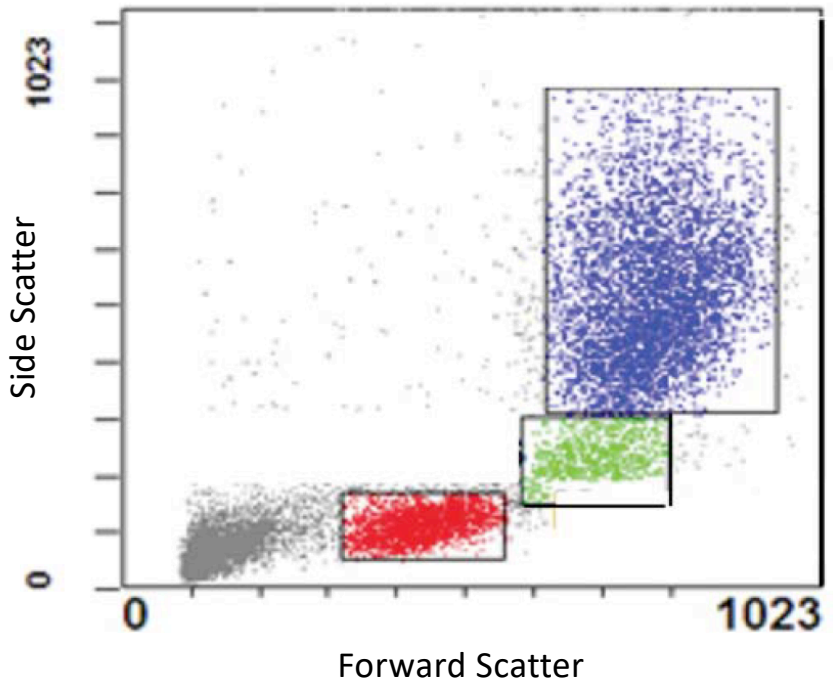


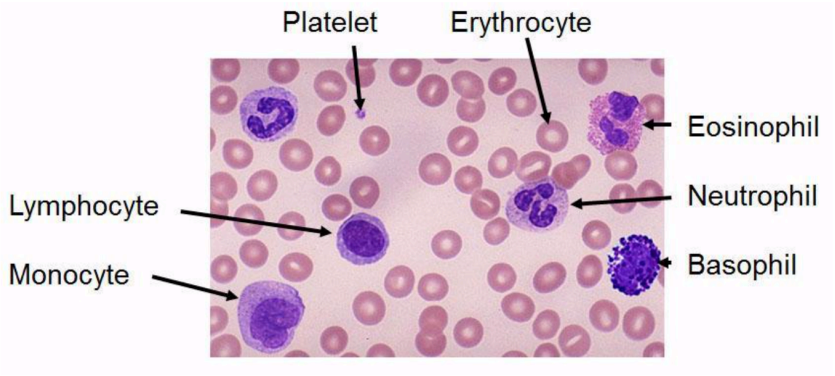


http://extralymey.com

benchling.com/pub/tabor-flowcal

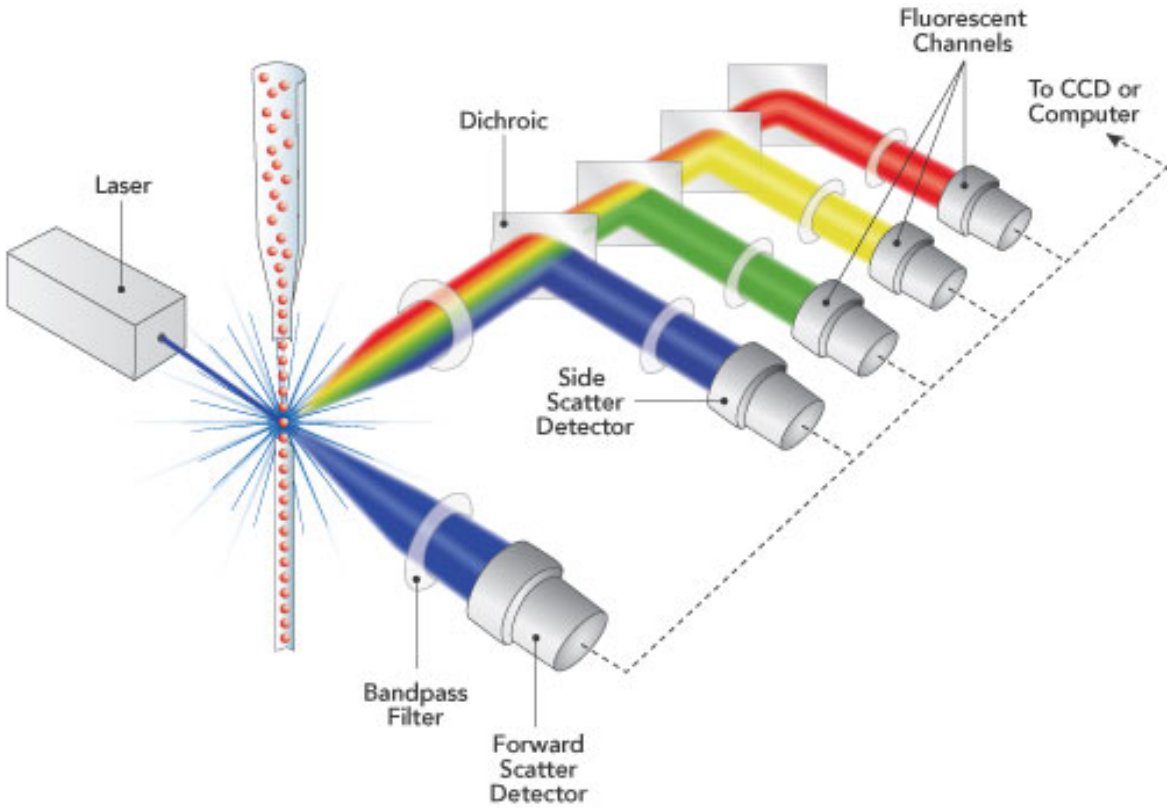
Scatterplot of healthy whole blood, no labeling





- Identify the three populations of cells:
  - Lymphocytes:
  - Neutrophils:
  - Monocytes:

Flow cytometry can be used to quantify and analyze characteristics of fluorescently labeled cells



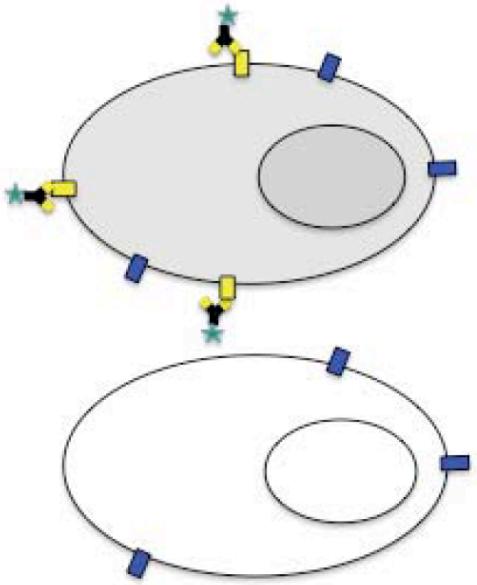
https://www.semrock.com/flow-cytometry

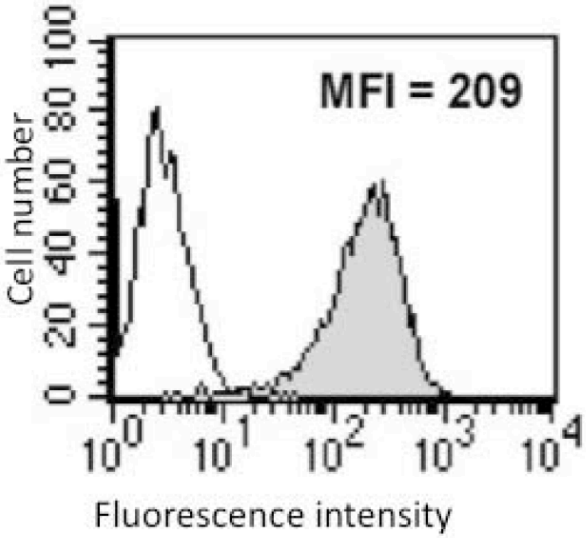
Histogram plot appropriate for analyzing a single parameter

B cell specific protein marker

Kappa light chain B cell Receptor

AbY ∝ Kappa light chain bound to FITC (Fluorescein isothiocyanate)

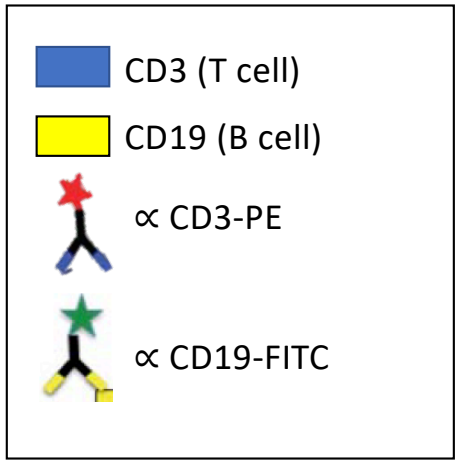


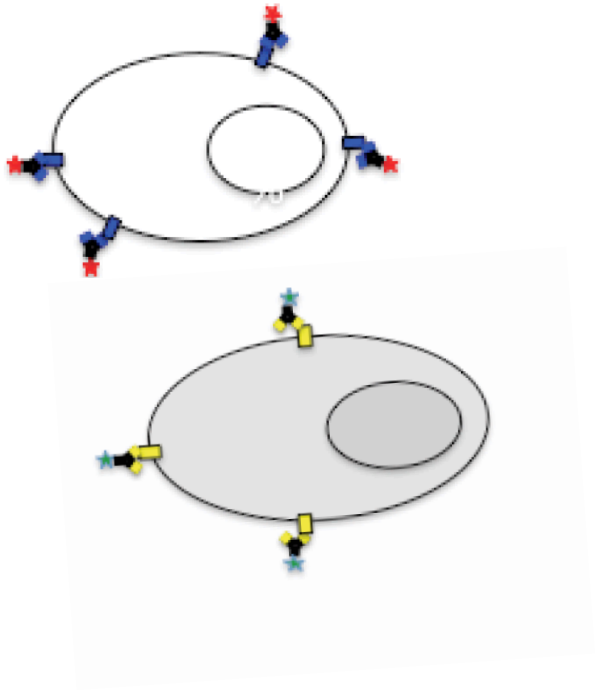


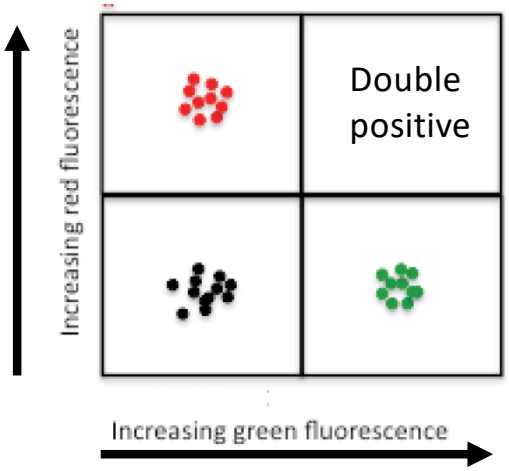
What results does this histogram analysis provide?

Scatterplot can distinguish multiple parameters

What are PE and FITC?





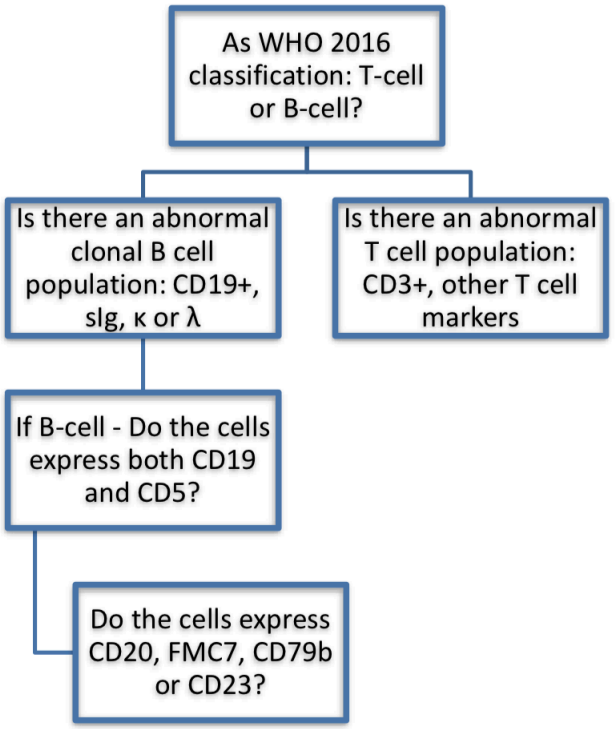


Case study details:

- Patient: Taylor (female)
- Symptoms: tired, swollen lymph nodes, elevated lymphocytes
- Possible prognosis: Chronic lymphocytic leukemia
- Test patient's blood using flow cytometry

- Key technologies in this approach: flow cytometer, antibodies and fluorophores
  - What can be measured with flow cytometry?
  - What role do antibodies play in this diagnostic test?
  - How are fluorophores incorporated?

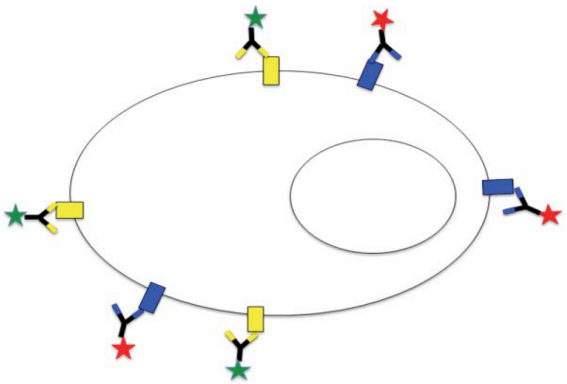
Chronic lymphoproliferative disorders

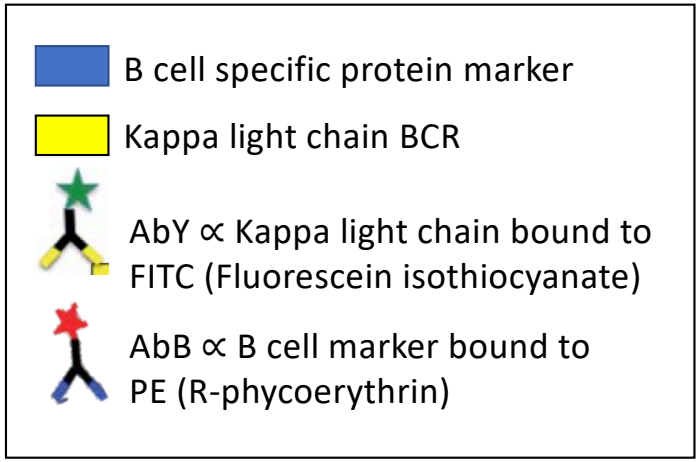


Mantle cell lymphoma and Chronic lymphocytic leukaemia are the 2 major B cell chronic lymphoproliferative diseases that express CD5 and CD19.

https://lymphoma-action.org.uk

Ratio of kappa to lambda light chain receptors is a B cell diagnostic marker for CLL

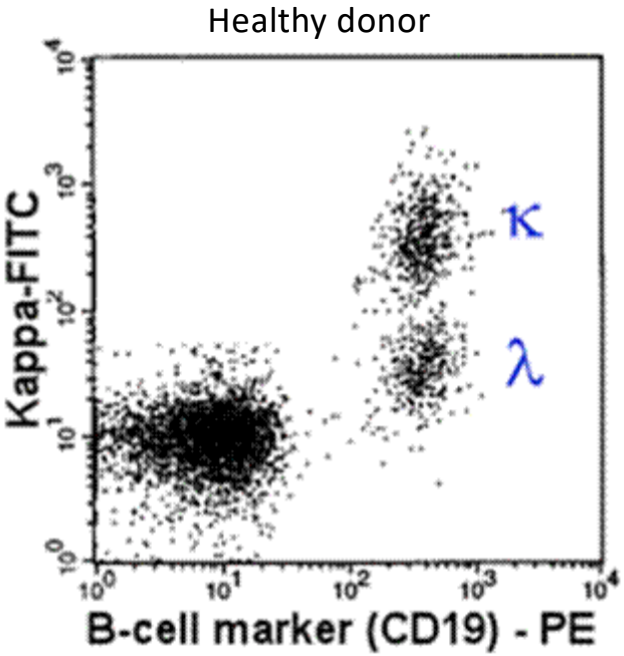




- In healthy people, kappa (κ) light chains are expressed on 2/3 of B cells and lambda (λ) light chains are on 1/3 of B cells
- This ratio of receptors can get skewed in CLL
- Taylor's plasma is incubated with antibodies to kappa and antibodies to a B cell marker
- The plasma is then run on a flow cytometer

Taylor's test results: Scatterplot of Kappa staining

- Plasma is incubated with ∝ kappa-FITC and ∝ CD19-PE
- Lambda population (λ) is inferred



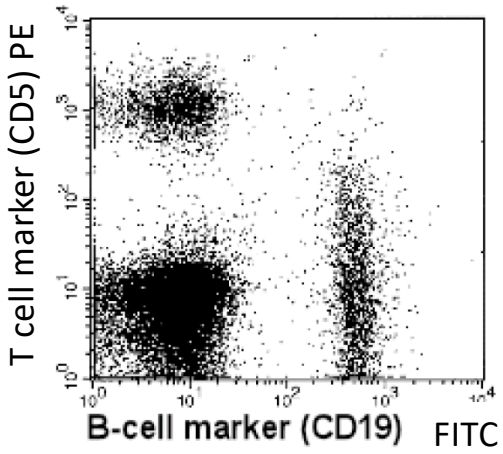
How would you interpret the ratio? Is kappa 2/3 of B cells?

How would you quantify this with flow cytometry analysis?

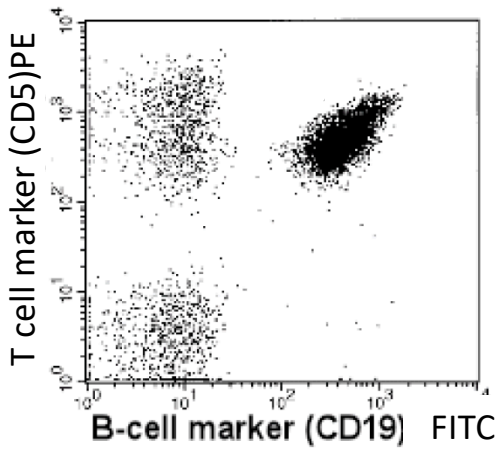
Taylor's test results: Scatterplot of CD5 and CD19

- Plasma from healthy donor and Taylor incubated with ∝ CD5 (T cell marker) and and ∝ CD19 (B cell marker)
- Do Taylor's cells express both CD5 and CD19?

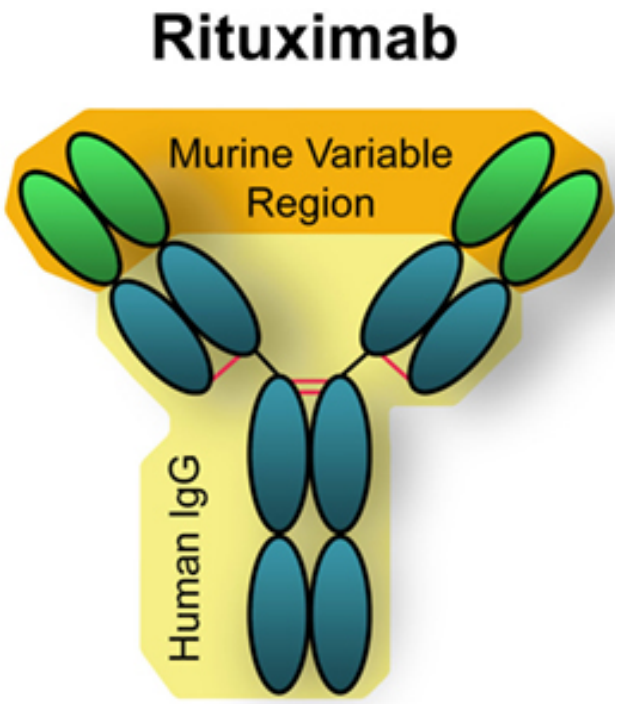
Healthy donor



Taylor's sample



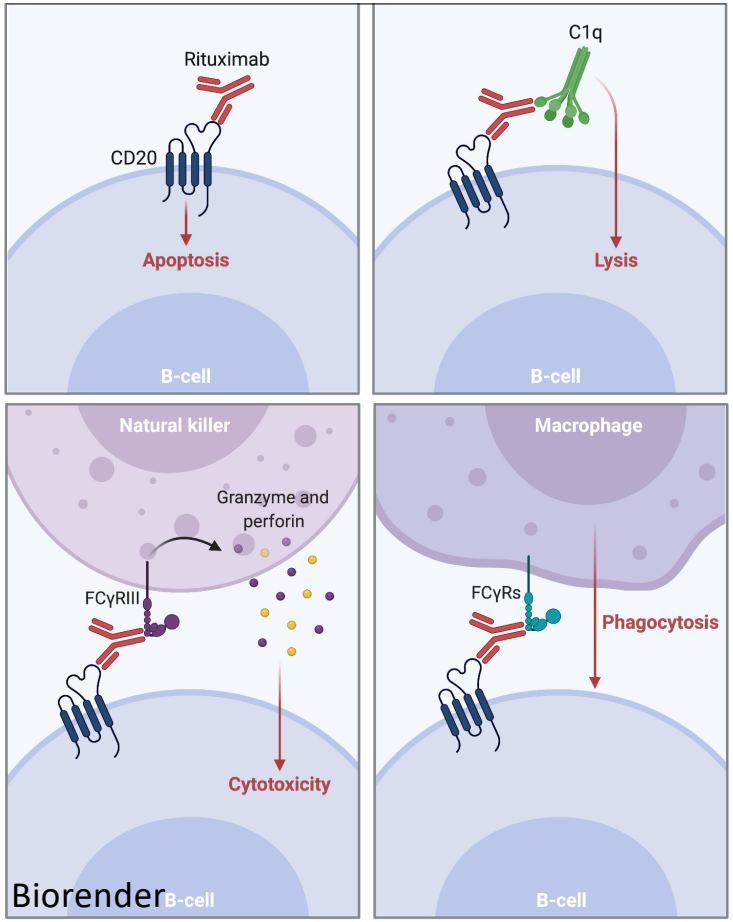
Rituximab is treatment for CLL



- Genetically engineered chimeric antibody directed against CD20 antigen
- Expressed on the surface of B-cells from pre-B cell (intermediate and late hematogones) through memory B-cell stages
  - not on hematopoietic stem cells, pro-B cells (early hematogones), and normal plasma cells.
- CD20 is expressed on more than 90% of B-cell CLL

Pierpont TM et al. Past, Present, and Future of Rituximab.Front Oncol. 2018 Jun 4;8:163.

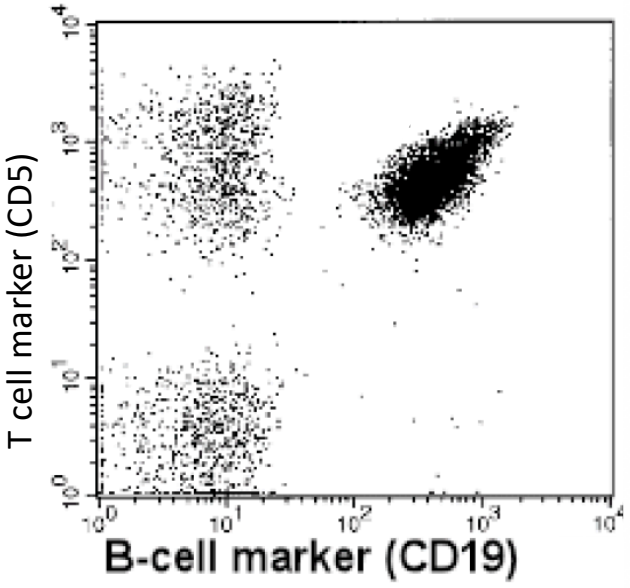
Rituximab targets and kills CD20+ B cells



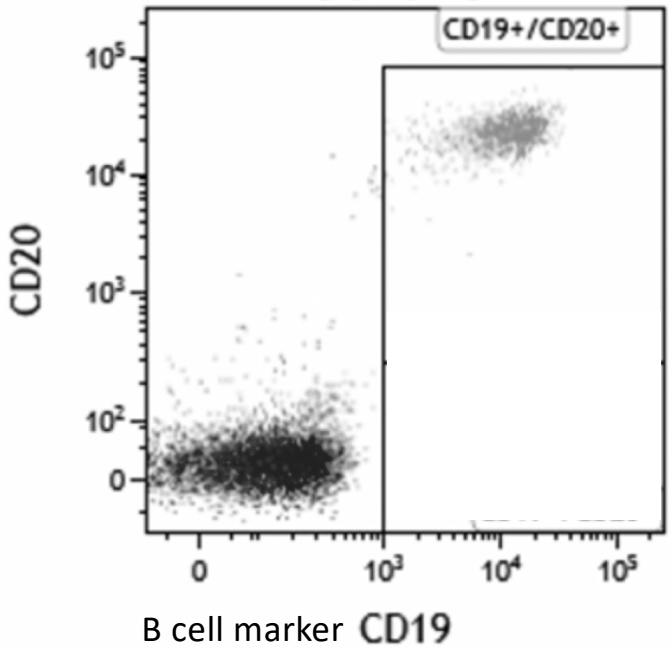
- Binding of Rituximab to CD20+ cells results in B cell lysis
  - CD20 mediated apoptosis
  - Complement-dependent cytotoxicity (direct lysis)
  - antibody-dependent cell-mediated cytotoxicity (Natural Killer cell)
  - antibody-dependent phagocytosis (macrophage phagocytosis)
- flow cytometric assay may be used to monitor B-cell depletion by assessing the percentage and absolute count of CD20+ /CD19+ B-cells

Flow cytometry of lymphocytes of patient with CLL

lymphocytes



B cells

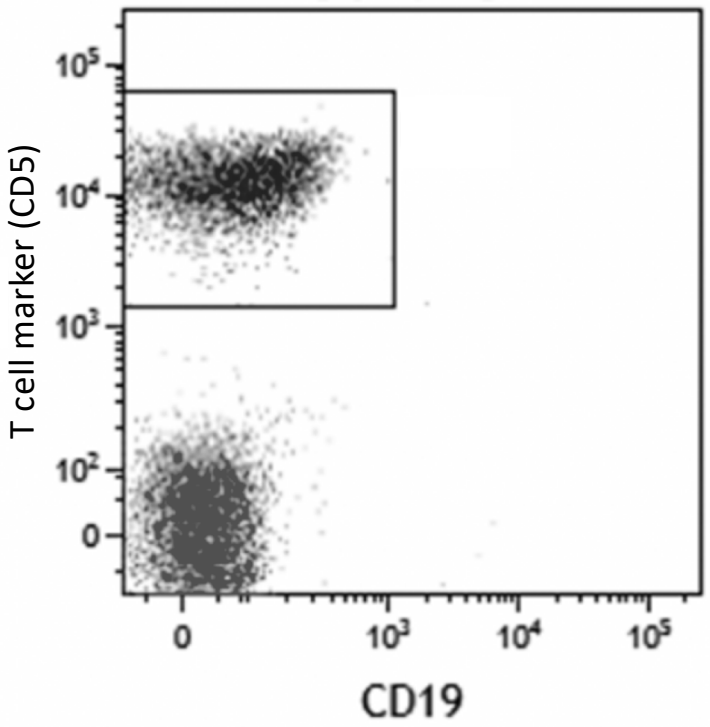


- To confirm CLL, diagnostic analysis usually includes CD19+ and T cell markers (CD5) to confirm the lineage
- Percentages and absolute counts of CD20+ / CD19+ are included in the diagnosis

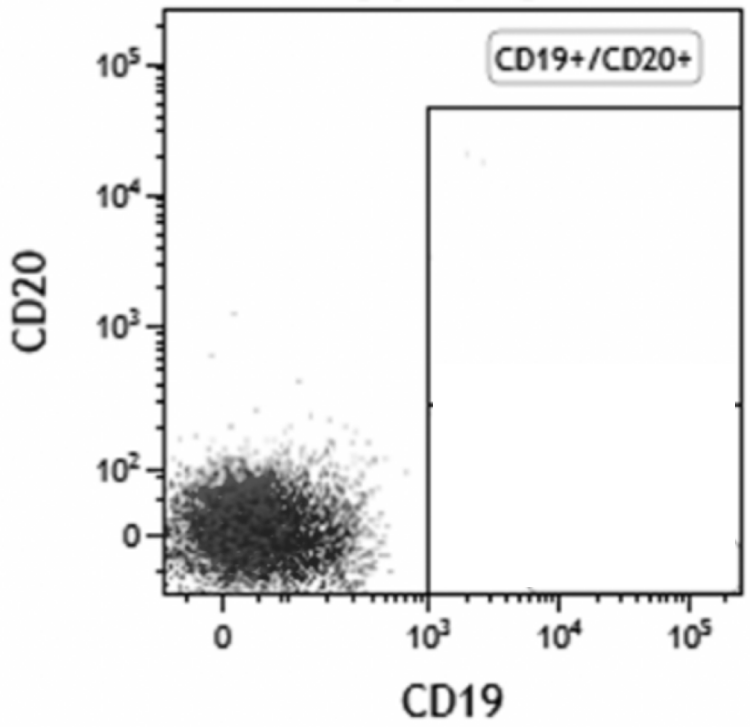
www.cytometry.org

Lymphocytes of patient with CLL after Rituximab treatment

lymphocytes



B cells



- Loss of CD20+ / CD19+ B cells indicate effective CLL treatment!
- T cells are not killed by the Rituximab treatment

www.cytometry.org

Today in lab,M1D6: Characterize scFv clone-lysozyme binding using flow cytometry

Set up titration of lysozyme with yeast, and allow binding to come to equilibrium, then measure by binding by flow cytometry.