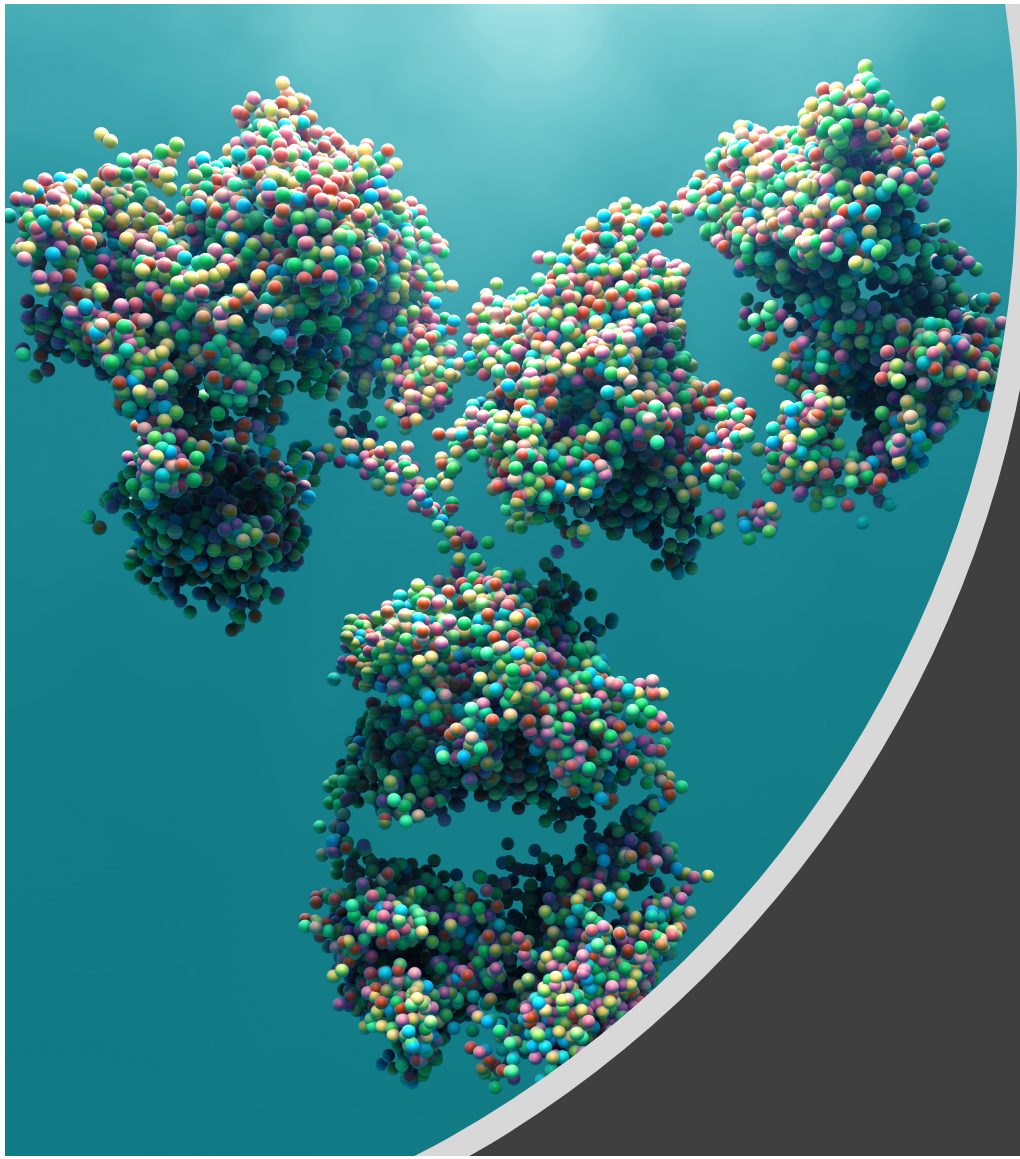
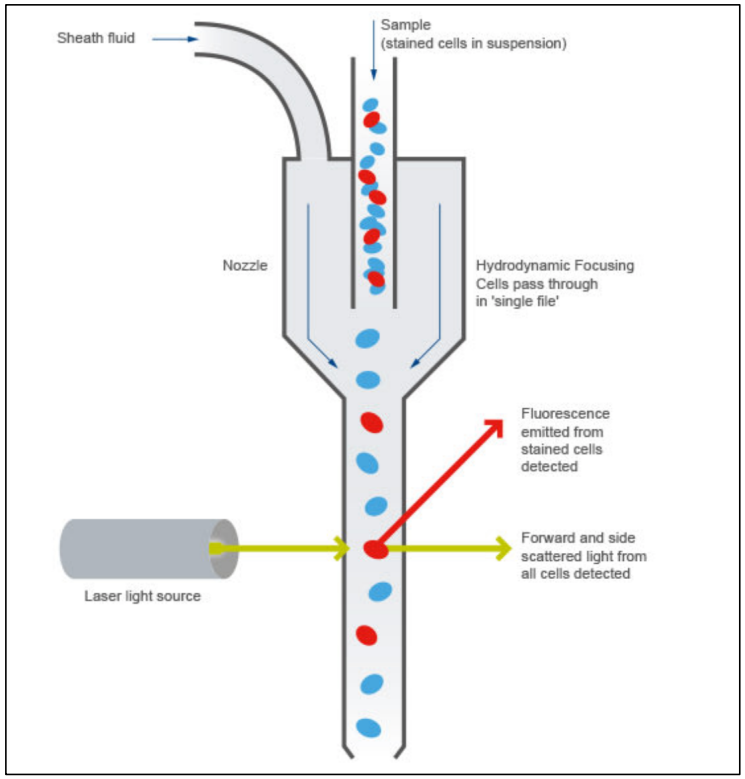


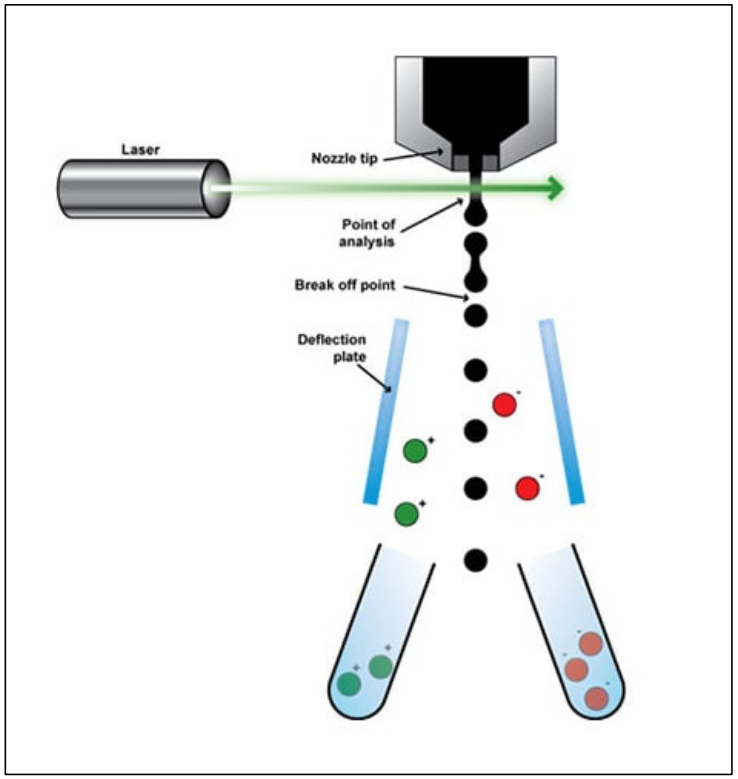
Flow cytometry for diagnosis and analysis

Refresher: Flow cytometry and FACS

Flow cytometry: analyze cells based on physical and fluorescent qualities



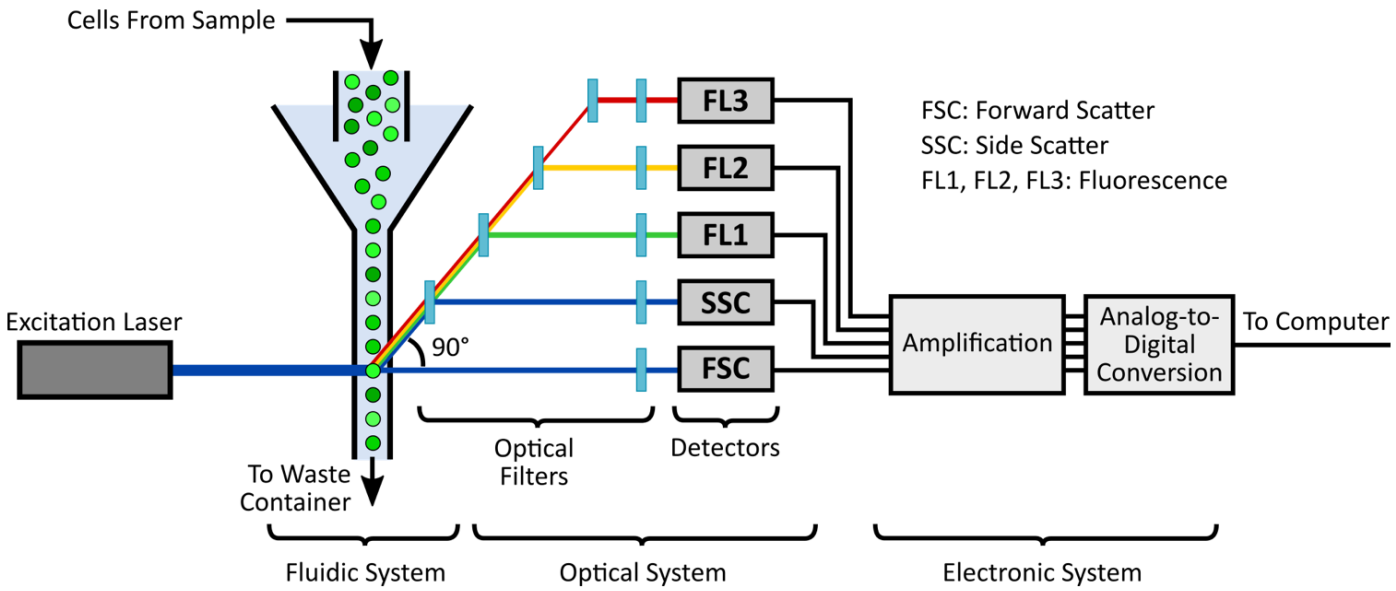
FACS: extension of flow cytometry, allows for cells to be sorted based on specified characteristics

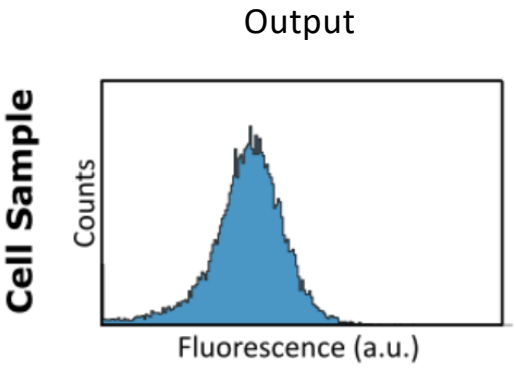


Images: abcam.com/protocols/introduction-to-flow-cytometry

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

Components of a flow cytometer





benchling.com/pub/tabor-flowcal

Our flow cytometer: Accuri C6

BD Accuri™ C6 Flow Cytometer Instrument Manual

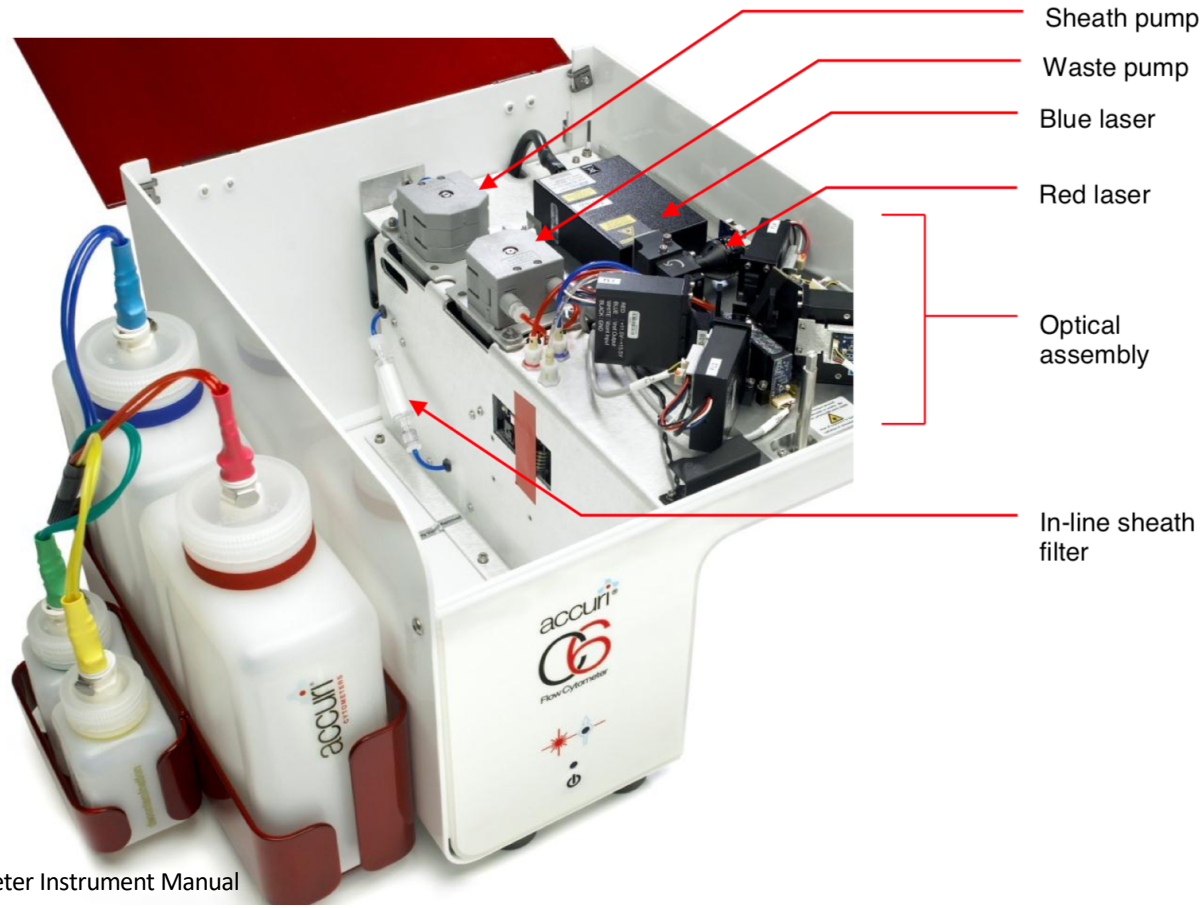
Benefits

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

Downside

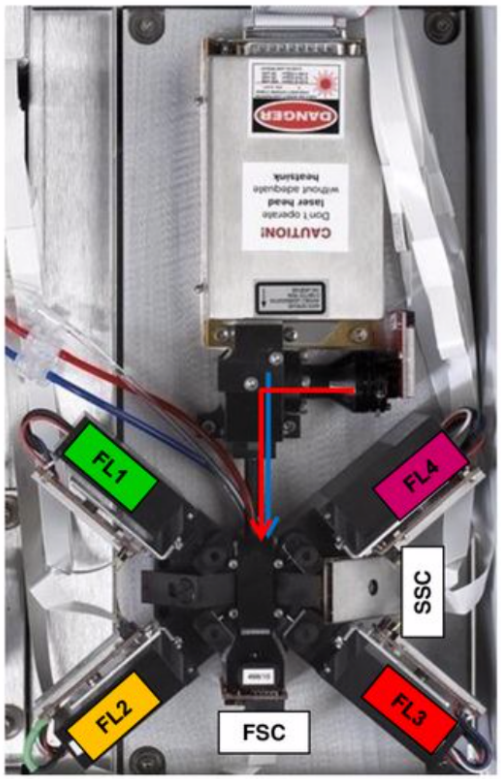
- Still needs technical service
- Limited to four color fluorophores
- Can’t sort cells after analysis

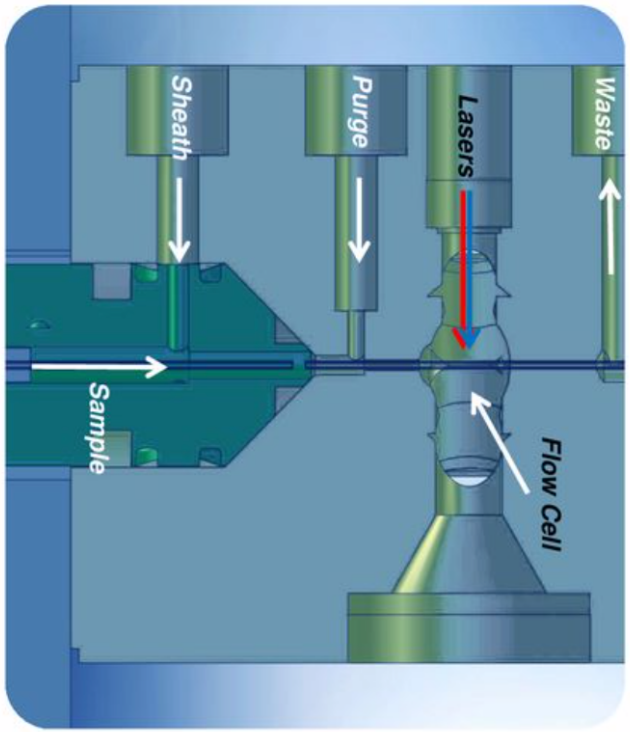
Major components of the fluidics and optics subsystems



BD Accuri™ C6 Flow Cytometer Instrument Manual

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

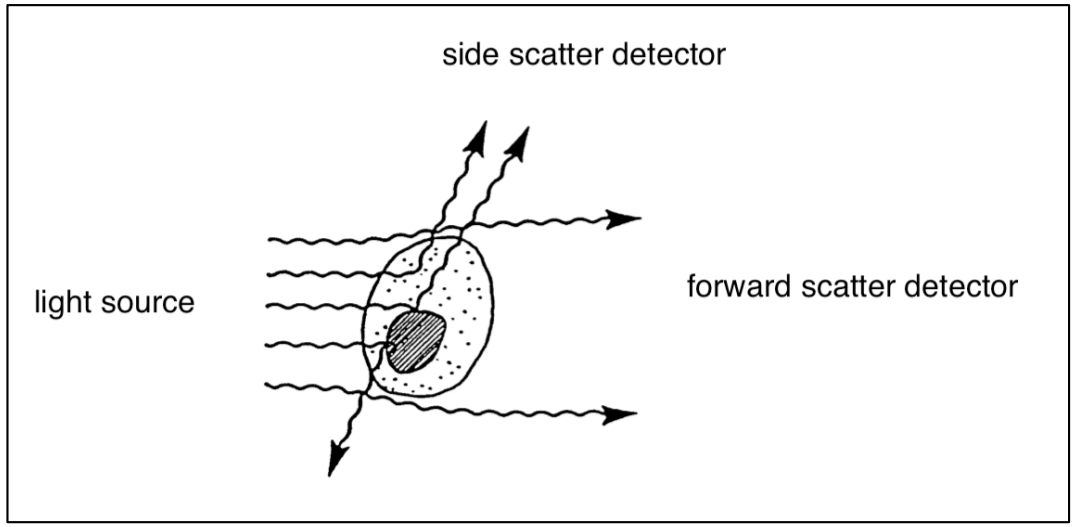




- The sample stream (cells) is pressurized upward through an optically clear region of the flow cell
- Cells within sheath fluid pass through the laser beam while in the flow cell
- Four fluorescence detectors and two scatter detectors process the signal

BD Accuri™ C6 Flow Cytometer Instrument Manual

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

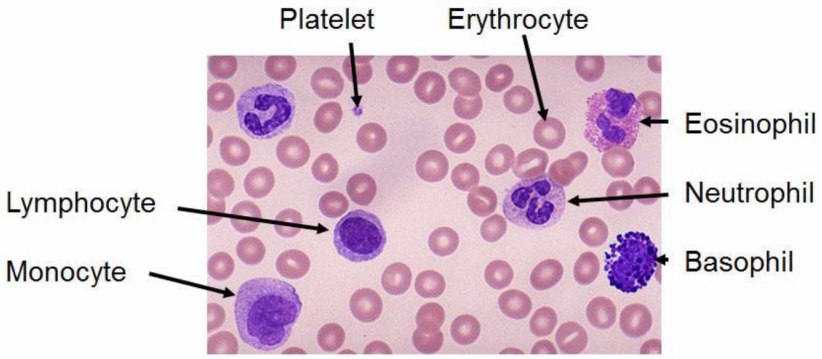


Light scattering occurs when a particle deflects incident laser light

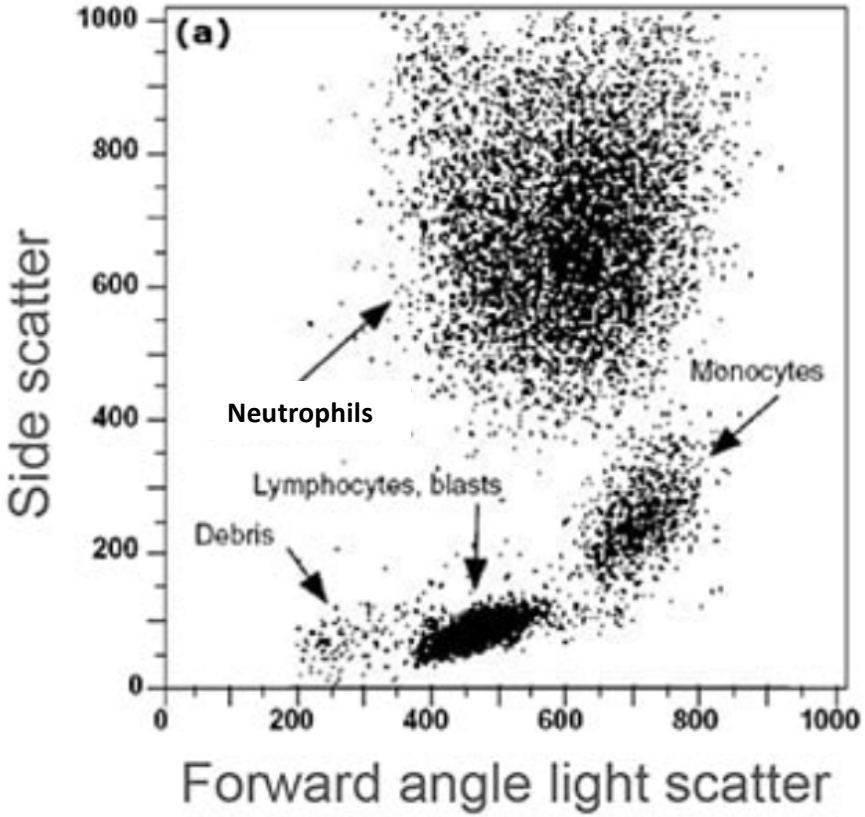
- Forward scatter, is refraction of light, proportional to cell-surface area or size
- Side scatter, is proportional to cell granularity or internal complexity of the cell

benchling.com/pub/tabor-flowcal

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

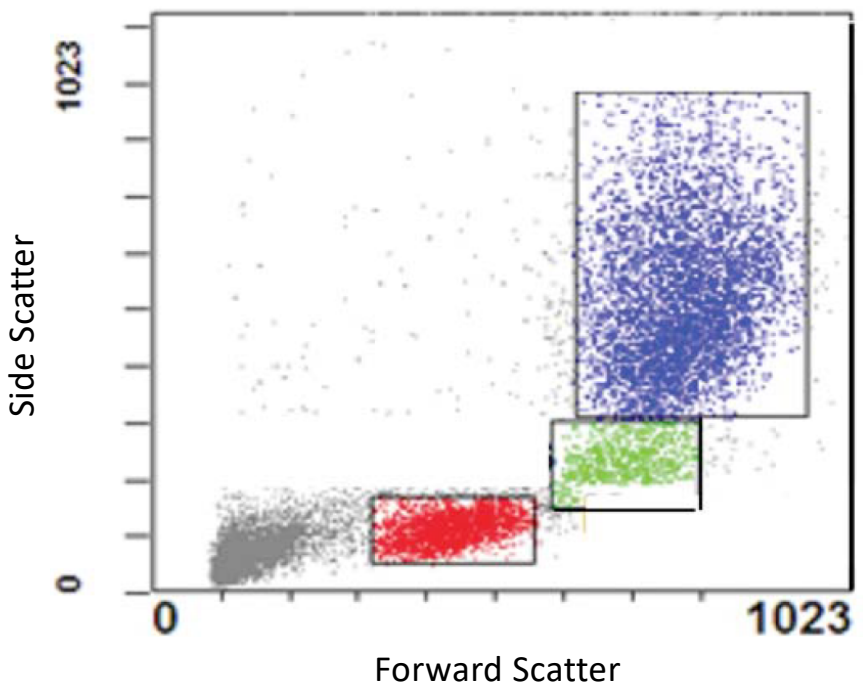


http://extralymey.com



benchling.com/pub/tabor-flowcal

Scatterplot of healthy whole blood, no labeling



- Identify the three populations of cells:
  - Lymphocytes:
    - red
  - Neutrophils:
    - blue
  - Monocytes:
    - green

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

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

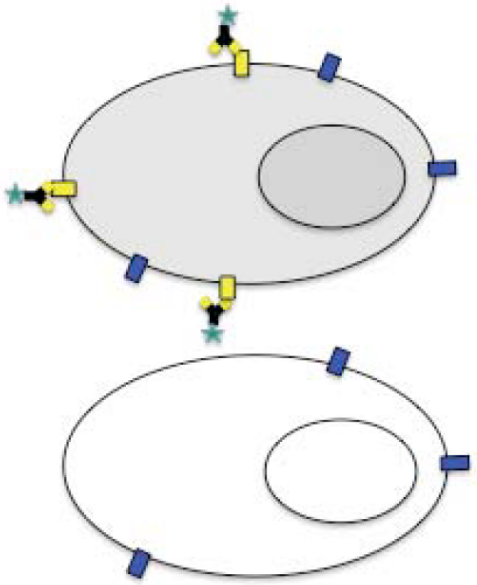
- Key technologies in this approach-
  - What can be measured with flow cytometry?
  - How are fluorophores incorporated?
  - What role do antibodies play in this diagnostic test?

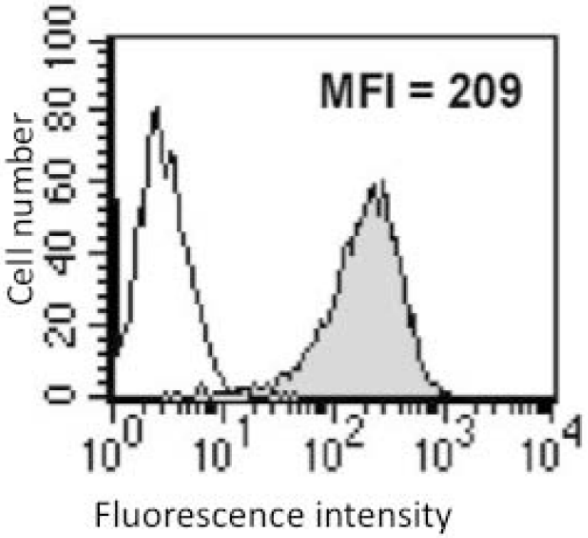
Histogram plot appropriate for a single parameter

B cell specific protein marker

Kappa light chain B cell Receptor

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

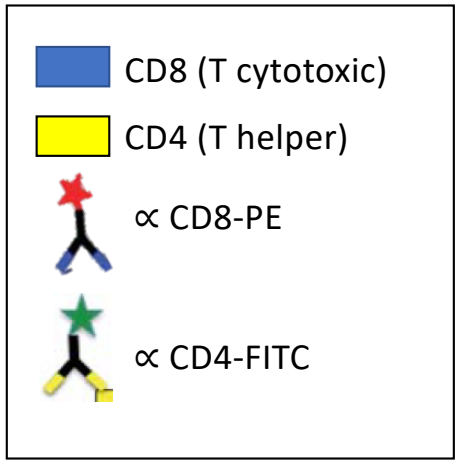


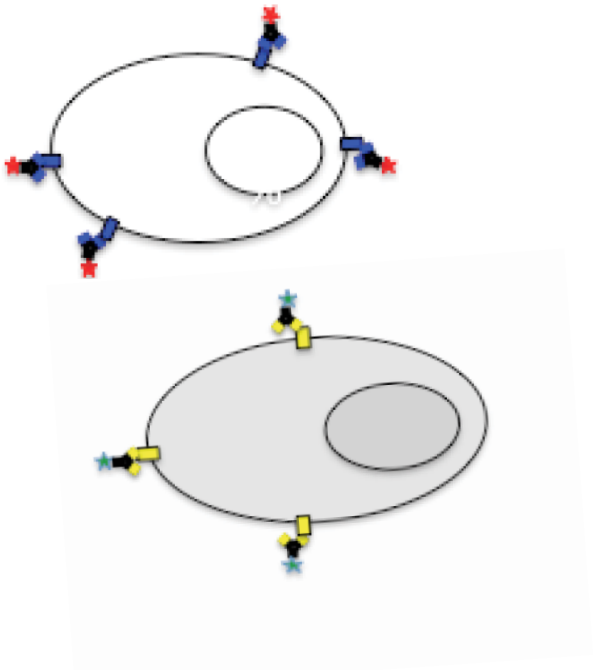


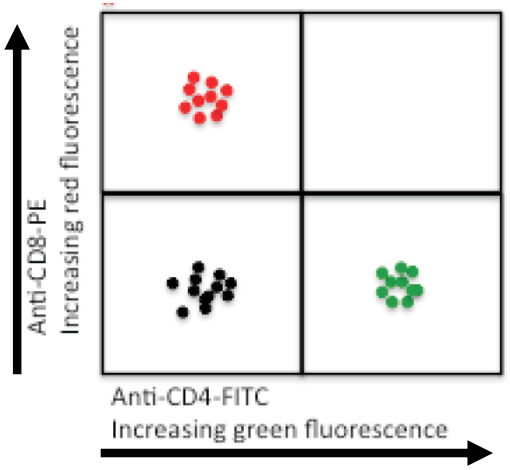
What results does this test provide?

Scatterplot can distinguish multiple parameters

What is PE?
What is FITC?



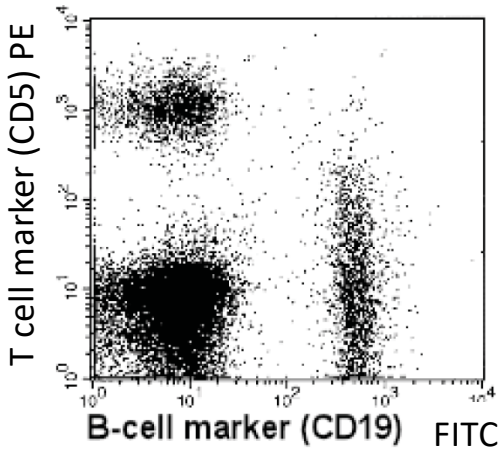




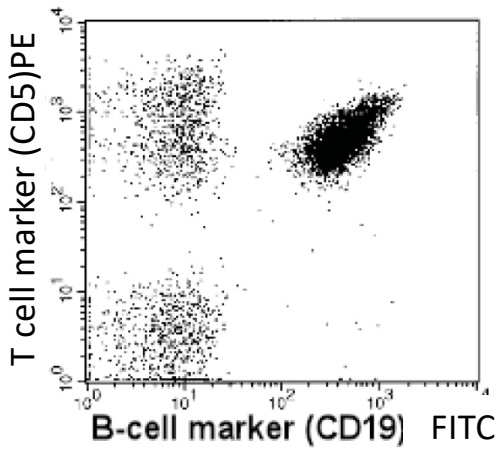
Taylor's test results

- Plasma from healthy donor and Taylor incubated with ∝ CD5 (T cell marker) and and ∝ CD19 (B cell marker)

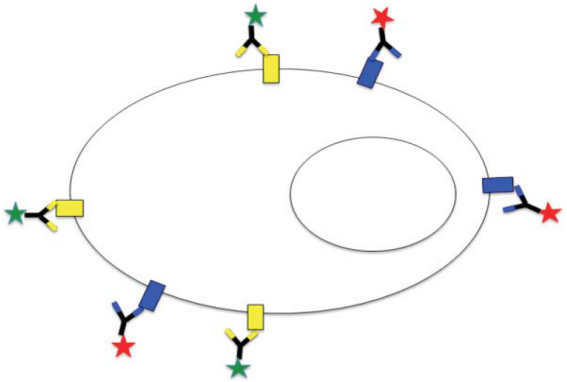
Healthy donor

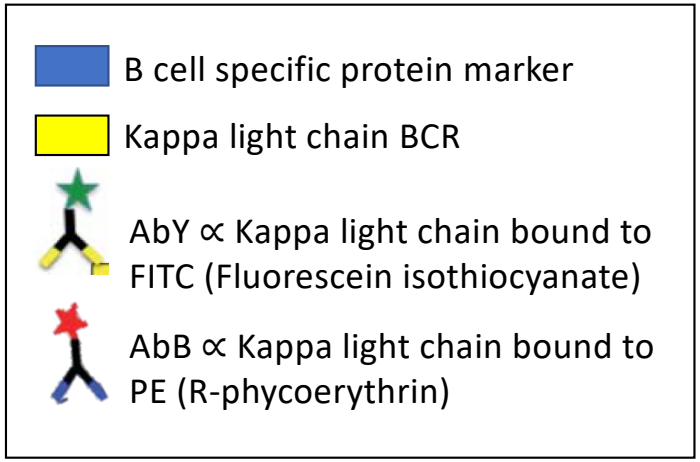


Taylor's sample



Ratio of kappa to lamda light chains is a B cells diagnostic marker for CLL

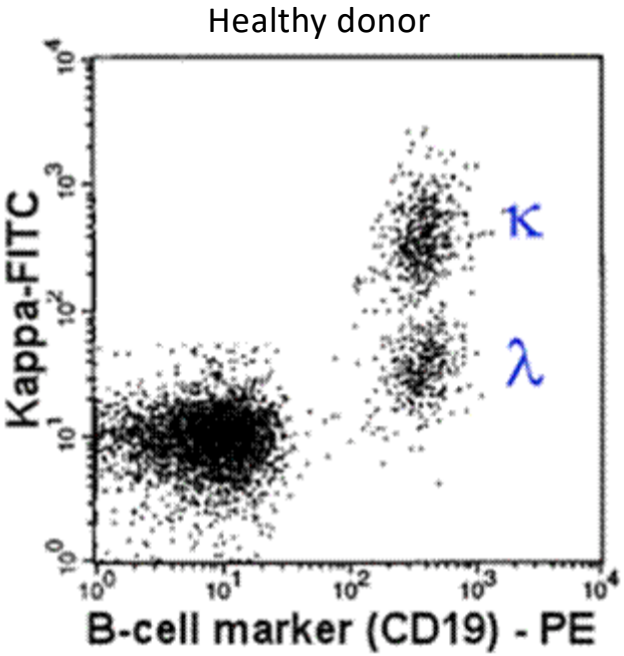




- In healthy people, kappa light chains are expressed on 2/3 of B cells.
- Taylors plasma is incubated with AbY to kappa and AbB to a B cell marker
- The plasma is then run on a flow cytometer
- What result does this test provide?

Scatterplot of Kappa staining

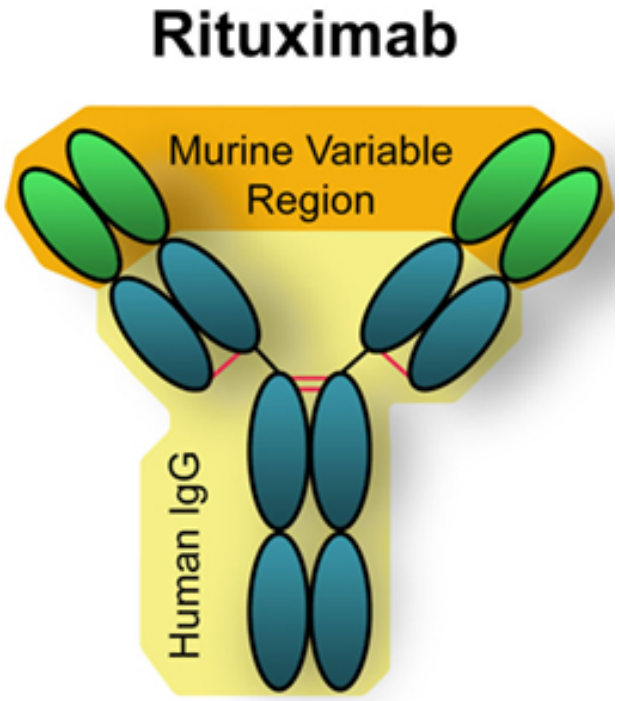
- Plasma is incubated with ∝ kappa-FITC and ∝ CD19-PE



How would you interpret the ratio?

How would you quantify this?

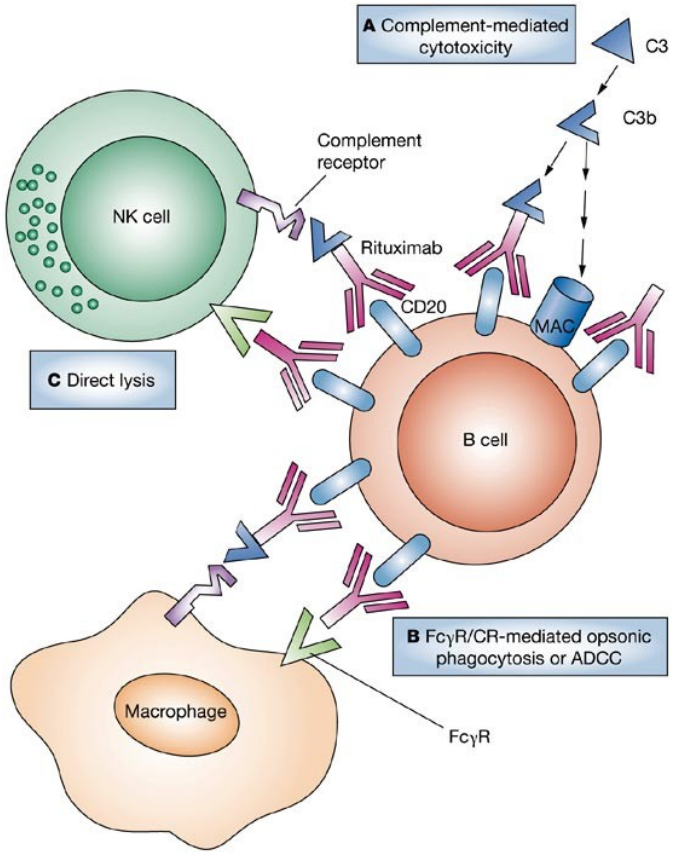
Rituximab is treatment for CLL



- 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

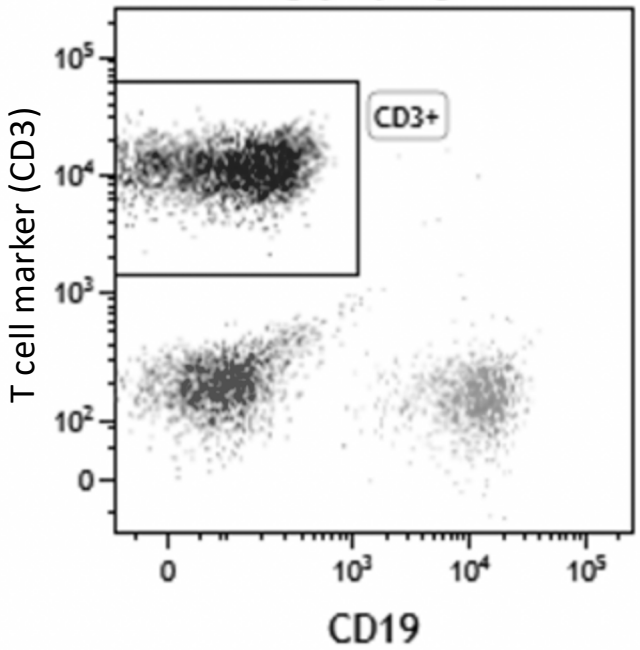


- Binding of Rituximab to CD20+ cells results in B cell lysis
- flow cytometric assay may be used to monitor B-cell depletion by assessing the percentage and absolute count of CD19+ B-cells and/or CD20+ B-cells

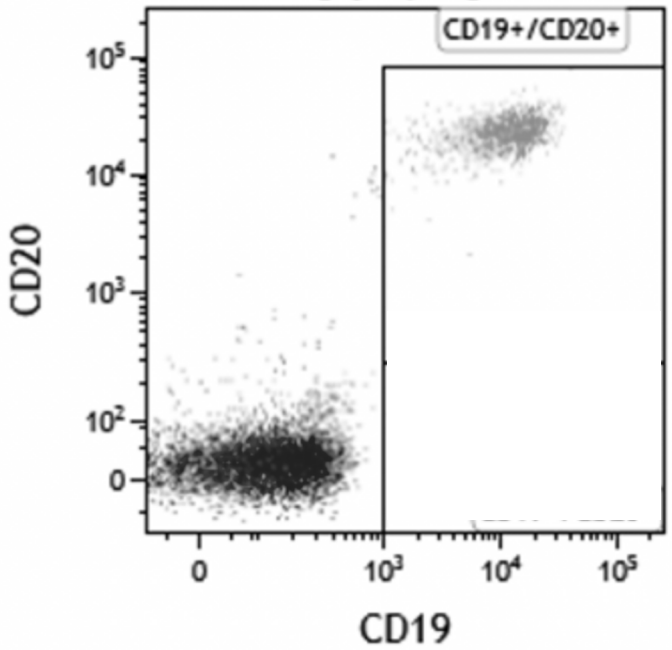
Taylor RP et al. Drug insight: the mechanism of action of rituximab in autoimmune disease. Nat Clin Pract Rheumatol. 2007 Feb;3(2):86-95.

Flow cytometry of lymphocytes of patient with CLL

lymphocytes



B cells

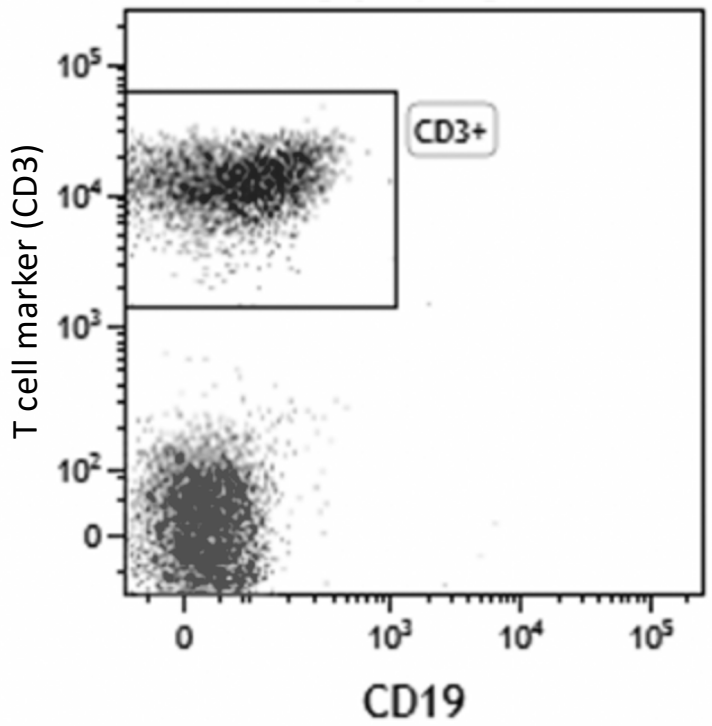


- assay usually includes CD19, CD20, and other T/NK cell markers to confirm the B-cell lineage
- Percentages and absolute counts of CD19, CD20, and others are included in the report

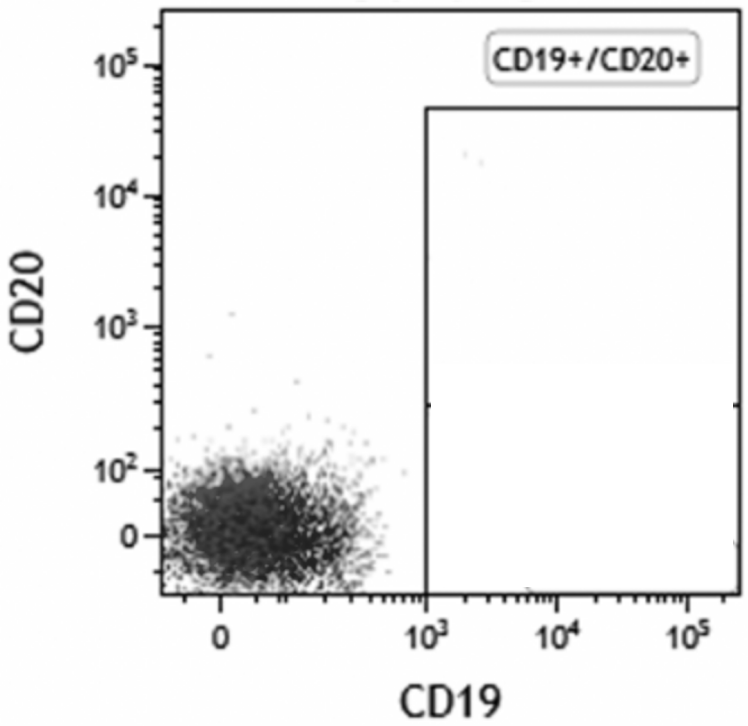
www.cytometry.org

Lymphocytes of patient with CLL after Rituximab treatment

lymphocytes



B cells

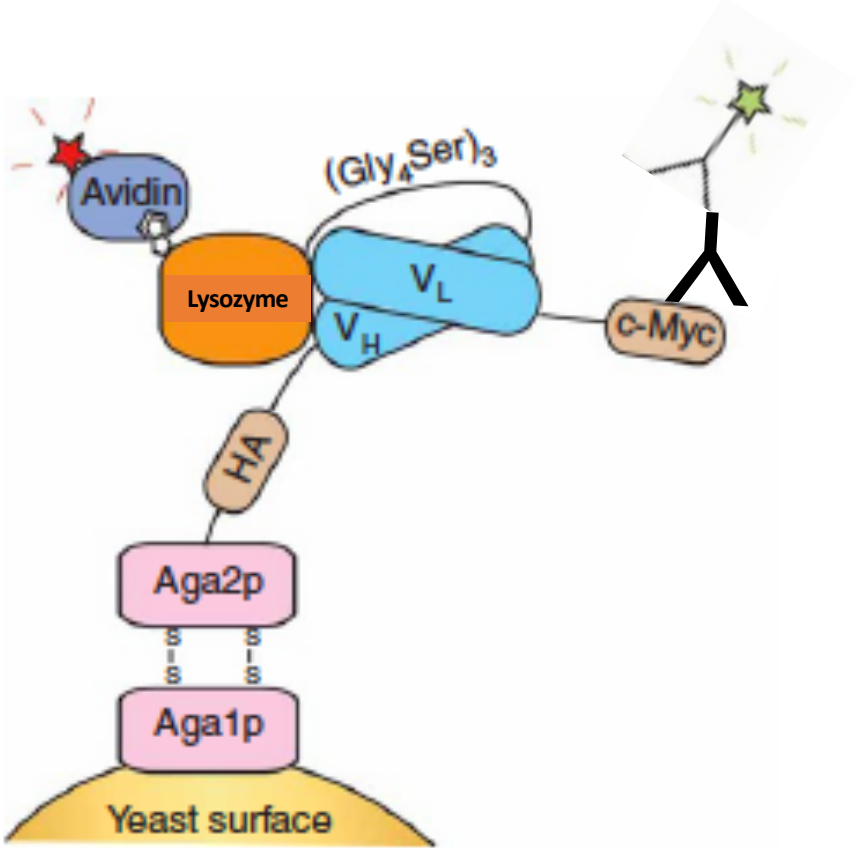


www.cytometry.org

Too many B cells Recap:

- What can be measured with flow cytometry? Name at least two things
- What role do antibodies play in this diagnostic test?
- How are fluorophores incorporated?

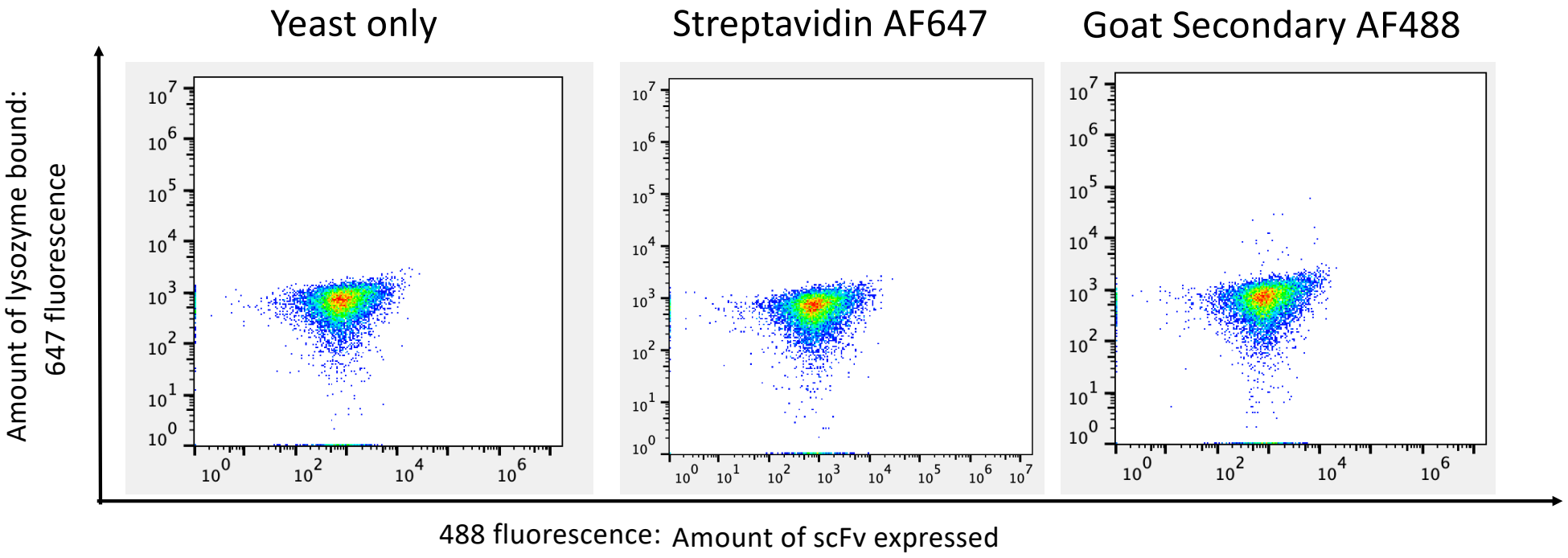
Fluorescently labeled antibodies and streptavidin identify scFv expression and antigen binding respectively



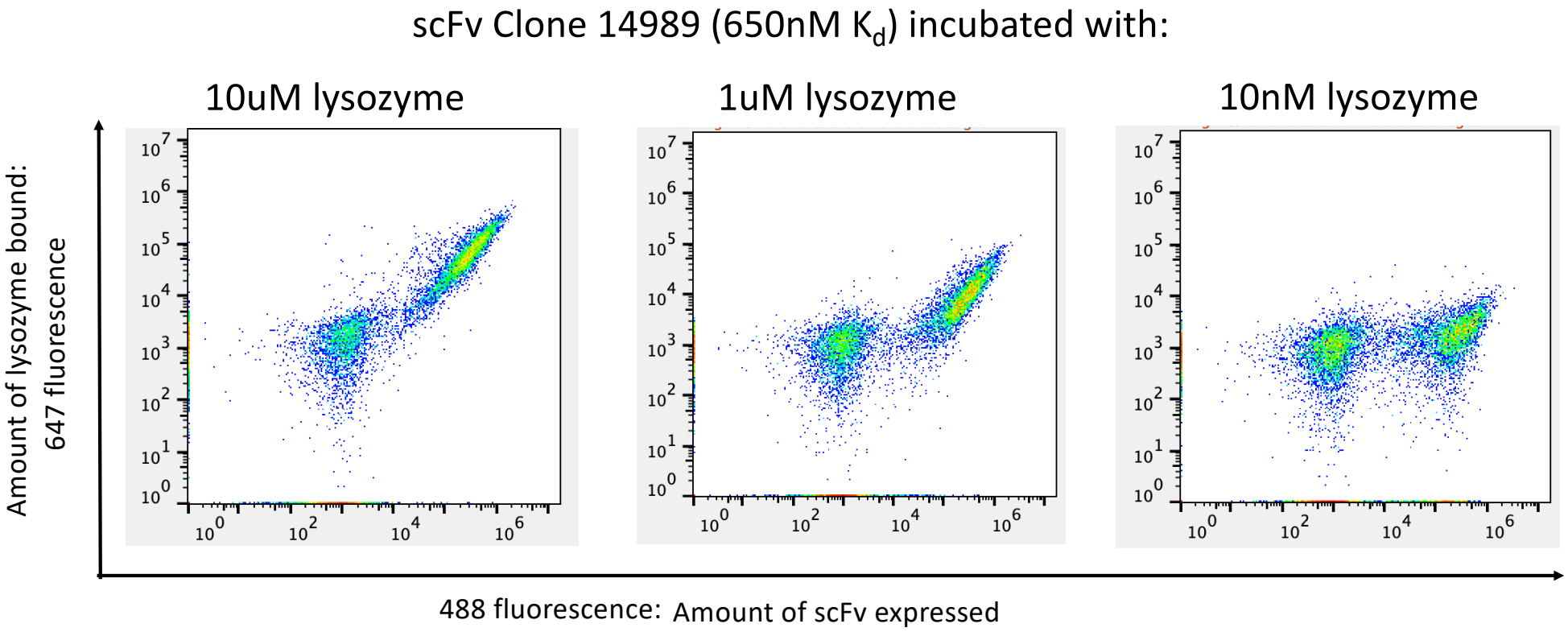
- To measure scFv expression we use a primary antibody to c-MYC and fluorescently labeled secondary antibody against primary antibody constant region
- To measure lysozyme binding we use fluorescently labeled streptavidin

Controls: how “sticky” are our fluorophores?

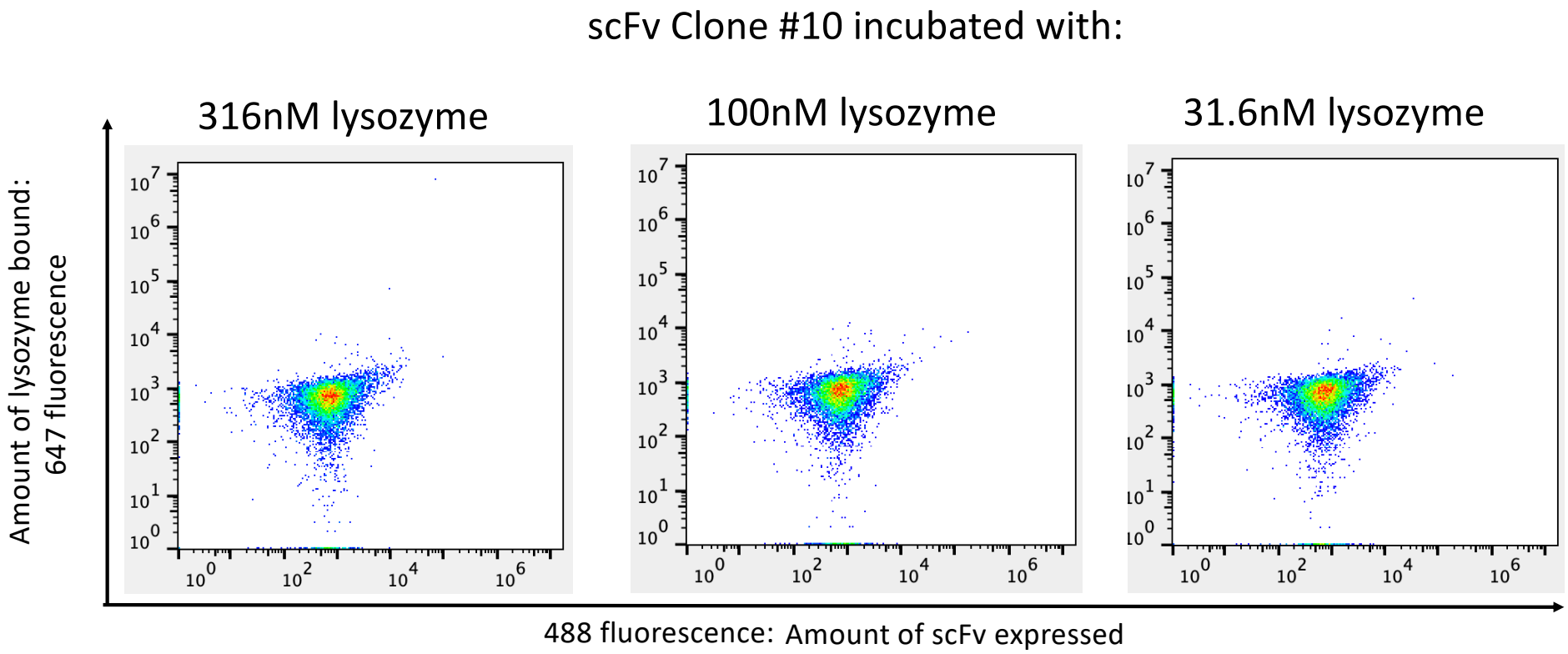
scFv Clone 14989 (650nM K_d) incubated with:



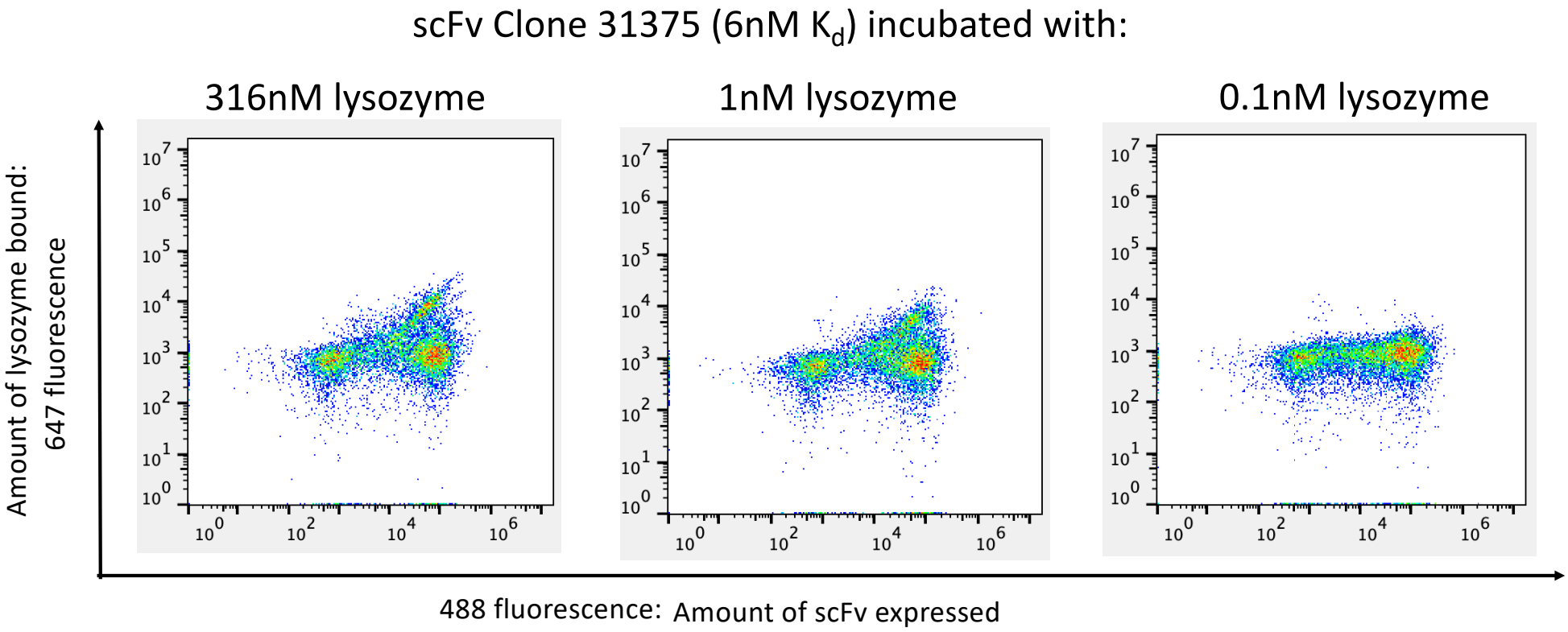
Characterized clone 14989 binds lysozyme



scFv clone #10, What do you think happened?

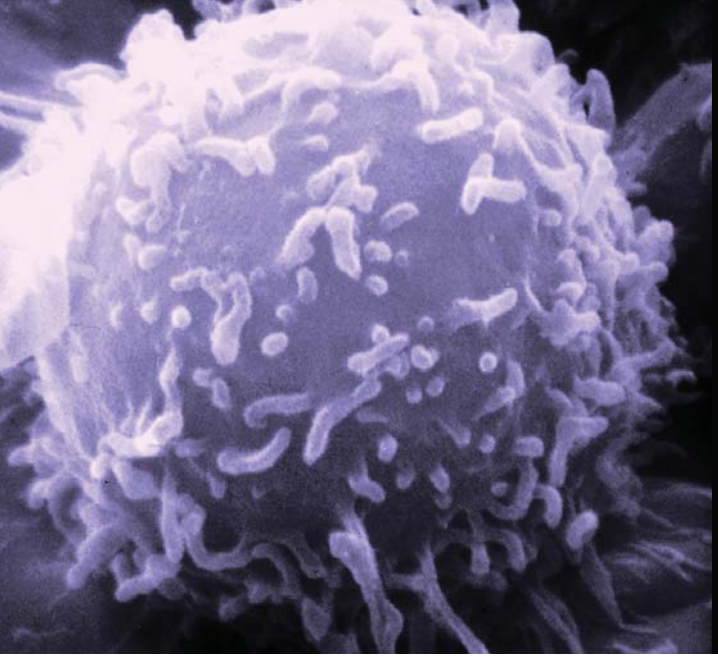


Clone 31375, more than one plasmid?



Today in “lab”

- Research Proposal Pitch
- 2-5min each team
- No visuals or slides necessary
- Ideas don’t have to be final or complete



NATIONAL CENTER FOR CASE STUDY TEACHING IN SCIENCE

Too Many B Cells: Chronic Lymphocytic Leukemia and the Role of Flow Cytometry

by
Debby R. Walser-Kuntz
Biology Department
Carleton College, Northfield, MN

Taylor goes in to see her doctor, Dr. Chavez, for an annual exam and, when asked, admits that she has been feeling tired and has had several colds recently. Since she works full-time and has two young children, she didn't think these symptoms unusual. However, her meticulous doctor notices swollen lymph nodes in her neck and decides to draw blood to perform a white blood cell count. Taylor gets a call from her doctor the next day with the news that her white blood cell count shows an elevated number of lymphocytes.

At her return appointment, Taylor is told by Dr. Chavez that she needs to have more blood drawn and that this time the blood will be tested using a more sophisticated test called flow cytometry. When asked why, the doctor replies that flow cytometry is a common test for chronic lymphocytic leukemia, or CLL, a cancer typically affecting older adults. CLL is characterized by a slow increase in the number of lymphocytes, most often the B lymphocytes, the antibody-producing cells of the immune system.

Dr. Chavez continues to explain that in CLL, the B cells mature from stem cells located in the bone marrow, and they rearrange and express cell surface B cell antigen receptors following the same process as normal B cells. However, in CLL, the mature B cells continue to divide without undergoing cell death; the result is high numbers of circulating B cells in the blood and swelling of the lymph nodes or spleen. B cells from the circulation also return to the site of their synthesis—the bone marrow. The excess number of B cells in the bone marrow disrupts the production of red blood cells; this reduction in oxygen-carrying red blood cells leaves the individual feeling weak and tired. Because the cancerous B cells grow in an unregulated manner, they replace other healthy cells, including normal B cells. Without normal levels of B cells, the individual is not well protected from infection, and individuals with CLL suffer from frequent infections. Additional symptoms of CLL include fevers and unexplained weight loss.

At this point, Taylor asks Dr. Chavez to explain what flow cytometry is and why this type of test will be useful in her diagnosis. Taylor adds that she majored in biology in college and would like as many details as possible to fully understand the technique.

Dr. Chavez begins by explaining that a *flow cytometer* is an instrument that simultaneously measures multiple properties of an individual cell, including cell size, number of particles in the cell's cytoplasm (also called granularity), and fluorescence. By analyzing thousands of cells in a short time period, flow cytometry also gives a picture of the distinct subpopulations of cells found within a sample. Cells are drawn into the flow cytometer and enter a narrow channel where they are forced into a single file stream. A nozzle shakes this stream of cells into droplets containing no more than one cell per droplet (think inkjet printer). Each individual droplet passes through a laser, a process that allows a single cell to be analyzed individually.

As the cell passes through the laser, the light is scattered in all directions, with some of the light being scattered in the forward direction. The forward scattered light is picked up by a detector and converted to a voltage pulse. *Forward*

light scatter (FSC) is proportional to cell size; a cell with a large diameter gives a large voltage pulse and a cell with a small diameter gives a smaller voltage pulse. As each cell passes through the laser, light is also scattered to the sides, and a cell containing many cytoplasmic granules will have a higher side scattered light (SSC) reading than cells with low granularity. Different types of cells within a sample, including white blood cells, can often be distinguished according to their size or granularity. Neutrophils are phagocytic cells containing many cytoplasmic granules; the granules contain enzymes and other proteins that participate in the destruction of phagocytosed microbes—in fact, another name for neutrophils is granulocytes. Unstimulated B and T lymphocytes are small cells with minimal cytoplasm, and they can be distinguished from monocytes, which are much larger cells that do have some cytoplasmic granules. When activated, T cells produce cytokines, the chemical messengers of the immune system, and distinct T cell subsets either play a role in activating B cells to produce antibodies or in killing virally infected cells.

Unlike the intrinsic factors of cell size and granularity, both of which are able to be detected by light scatter, specific cellular proteins are detected and quantified using antibodies labeled with *fluorophores*. A fluorophore is a functional group in a molecule that absorbs light energy of a specific wavelength and is excited to a higher energy state. The higher energy state cannot be sustained and, by emitting light of a longer wavelength, the fluorophore loses energy and returns to the ground state. This process is known as *fluorescence*. Each fluorophore is optimally excited at one wavelength and emits light at another wavelength. This characteristic allows for the simultaneous use of multiple fluorophores in flow cytometry—depending of course on the flow cytometer having the correct lasers to excite the fluorophores. Fluorescein isothiocyanate (FITC) and phycoerythrin (PE) are two commonly used fluorophores that have been available for many years. Many newer fluorophores are now available, and sophisticated flow cytometers simultaneously distinguish as many as 15 "colors."

In flow cytometry, fluorophores are typically conjugated to antibodies; antibodies can be thought of as protein probes that bind with high specificity to cellular proteins. Antibodies (also called immunoglobulins) are composed of two types of polypeptides known as heavy and light chains based on their molecular weight (see Figure 1). The antibody molecule, frequently depicted as a Y, is a mirror image of itself containing two identical heavy and two identical light chains. There is both a variable and a constant region on each of the heavy and light chains; the variable region is highly diverse and functions to tag the specific antigen (shown as a yellow rectangle in Figure 1). Due to the Y shaped nature of the molecule, each antibody contains two identical antigen binding sites and both the heavy and light chains contribute to forming the antigen binding site.

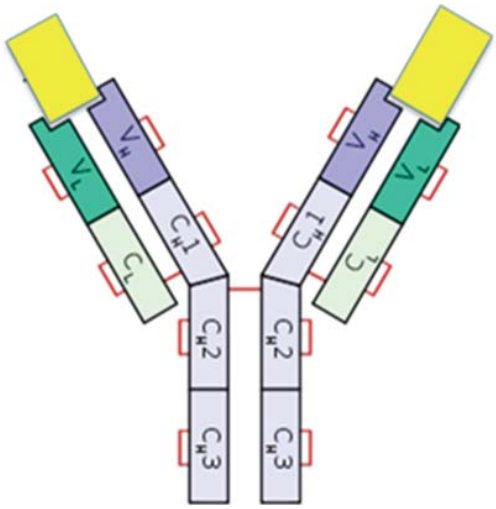


Figure 1. Antibody structure. The heavy chain (purple) is composed of a variable (V_H) and a constant region (C_H). The light chain (green) also has a variable (V_L) and constant (C_L) region. The light chain constant regions can be either kappa or lambda. The variable region of the antibody molecule functions to bind the antigen (yellow rectangles). The red lines represent disulfide bonds that covalently link the chains together. *Source:* Image modified from http://en.wikipedia.org/wiki/File:AntibodyChains.svg, CC-BY-SA 3.0.

Both the heavy and the light chain also contain constant regions. Humans have five "flavors" or types of heavy chain constant regions called M, D, A, E, and G; the type of heavy chain constant region allows specific interactions between the antibody and other cells or molecules. These interactions lead to elimination of the antigen or the transport of the antibody molecule within the body. For example, one "flavor" of antibody, known as Immunoglobulin G (or IgG), binds via the heavy chain's G constant region to receptors located on cells of the placenta. The binding of IgG to receptors allows the mother's IgG to be transported across the placenta, thus protecting the fetus from specific antigens. Humans have only two options, or "flavors," for the light chain constant regions: kappa or lambda. Although the light chain constant regions do not play a role in either transport of the antibody molecule or in clearing the antigen, they are critical in the proper folding and function of the antibody molecule.

Taylor remembers that the doctor had told her previously that antibodies are secreted by a type of white blood cell, the B cell, produced in the bone marrow. Dr. Chavez now further explains that as each B cell develops in the bone marrow, it produces multiple copies of one unique antibody molecule having a distinct variable region. The developing B cell first expresses the antibody molecule as a transmembrane receptor known as the *B cell receptor.* Antigen binding to the variable region of the B cell receptor activates the B cell, causing the B cell to divide and differentiate into a plasma cell; it is the plasma cell that secretes antibodies. The secreted antibodies function to tag the antigen and help clear infections.

The B cell receptor also serves as a marker uniquely distinguishing B cells from other white blood cells. In healthy adult humans, kappa light chains are expressed on about 2/3 of B cells, and lambda light chains on the remaining 1/3 of B cells. At this point, Taylor's doctor emphasizes that the ratio of kappa to lambda light chains can be skewed in an individual with cancer, and thus the ratio serves as a useful diagnostic tool.

Taylor now wonders how flow cytometry can be used to distinguish whether her B cells have a normal or skewed ratio of light chains. Dr. Chavez explains that linking a distinct fluorophore to an antibody specific for a cell surface protein allows the user to distinguish between multiple cell types and/or cell surface molecules simultaneously. The real power of flow cytometry is its ability to measure multiple parameters of a single cell simultaneously. To help make a diagnosis for Taylor, multiple cell surface markers will be analyzed by flow cytometry to first determine which type of white blood cell is overabundant (i.e., the cancer cells). Figure 2 shows how a cell bearing two distinct cell surface proteins can be labeled with two antibodies. Each antibody is composed of a unique variable region and each is labeled with a different fluorophore. In Taylor's case, the antibodies could be recognizing the kappa light chain of the BCR (yellow rectangle) and a B cell specific marker protein (blue rectangle). This test would provide the following information: (a) the percentage of Taylor's blood cells expressing the B cell marker protein and (b) the percentage of B cells expressing the kappa light chain.

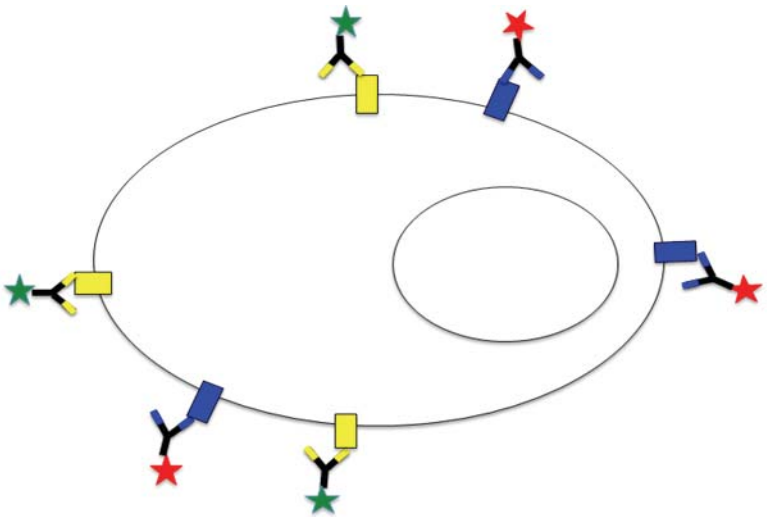


Figure 2. A cell expresses two distinct cell surface proteins (yellow and blue rectangles). Each antibody (Y) is labeled with a fluorophore: FITC (green star) or PE (red star). Each antibody recognizes a unique cell surface protein and has a unique variable region (shown by either the yellow or blue antigen-binding site).

Taylor's doctor explains how flow cytometry data may take the form of either a histogram or a dot plot/quadrant. Histograms suffice when a single parameter is being measured, such as the expression of an individual surface protein. A histogram gives information about whether or not a population of cells is expressing this particular protein, with fluorescence intensity being displayed on the x-axis and cell number on the y-axis.

For example, let's look at a population of cells shown below in Figure 3. The gray cells express the protein of interest, displayed as a yellow rectangle on the cell surface, whereas the white cells lack this protein. The FITC-labeled antibody recognizes and binds to the protein; cells bound by the fluorophore-labeled antibody will fluoresce when they pass through the laser—on the histogram we say the cells "shift to the right." Each of the two peaks shown on the histogram in Figure 3 represent a population of cells; the peak on the left represents the cells that do NOT express our protein of interest (the white cells in our example), and we refer to these as the negative population. These cells do, however, show a low level of fluorescence known as autofluorescence. All cells contain certain molecules that have the property of fluorescing under UV light, but the level of fluorescence is typically low. In comparison, the population of cells in the peak that shifted to the right express the protein of interest, and thus, the fluorescence intensity increases (these are the gray cells in our example). The flow cytometer calculates the percentage of positive cells in the population (about 55% in the gray peak in our example) and, although not shown here, the percentage is often displayed on the histogram.

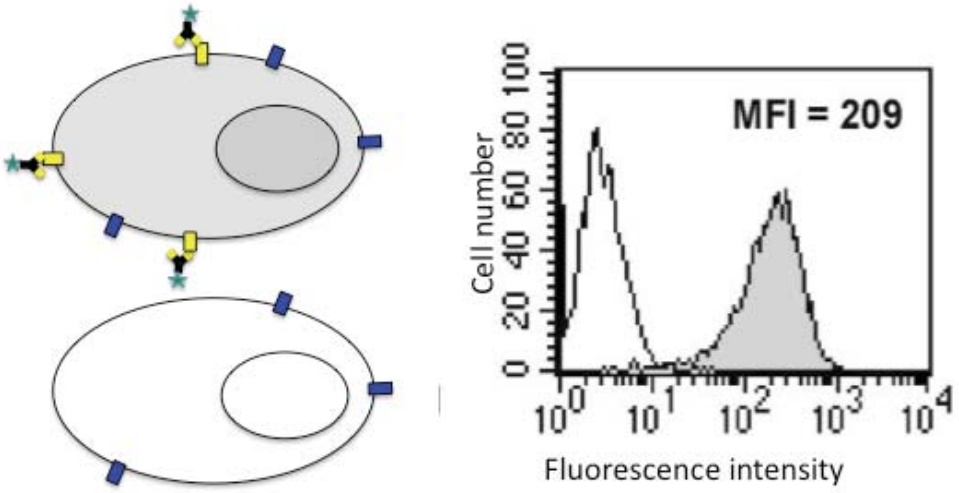


Figure 3. Histogram showing the population of cells expressing the protein of interest. Fluorophore-labeled antibody binds to the cells expressing the protein of interest, shifting the peak to the right on the histogram.

Note that we can get additional information from the histogram above. The shape of the histogram shows us that individual cells vary in the amount of protein expressed on the cell surface—in other words, there is a distribution. When the protein is highly expressed on a cell, more antibody binds, and the fluorescence intensity increases. A population of cells will almost always show a range of protein expression. MFI stands for *Mean Fluorescence Intensity* (note the log scale on the X axis) and allows a comparison of the degree of protein expression between two samples.

The dot plot display is useful when you want to analyze cells using two markers simultaneously, such as determining the percentage of B and T cells in an individual's blood sample. During differentiation, cells are programmed to express a subset of proteins unique to that particular cell type. Almost all B cells, for example, express the CD19 and CD20 cell surface proteins, whereas all T cells express the CD3 molecule. T helper and T cytotoxic cells can be distinguished based on the cell surface expression of CD4 and CD8 molecules, respectively. In the example shown in Figure 4, the yellow rectangle on the gray cell represents the CD4 molecule, and the blue rectangle on the white cell represents the CD8 molecule. Fluorescence intensity from FITC-labeled anti-CD4 antibody binding is displayed on

the x-axis and PE-labeled anti-CD8 antibody binding is displayed on the y-axis (the PE fluorophore is represented by the red star).

Dot plots are often divided into quadrants; each quadrant displays the individual cells that were tagged with fluorophore-labeled antibodies. Cells that were not labeled by antibody appear in the bottom left quadrant, and cells labeled by both FITC- and PE-labeled antibodies are located in the upper right quadrant. As shown in Figure 4, each cell expressing the CD4 molecule is represented as a green dot in the lower right quadrant. Each cell expressing the CD8 molecule appears in the upper left quadrant as a red dot. The bottom left quadrant contains cells lacking both the CD4 and CD8 molecules (shown here by black dots). There are no cells in our sample that express both the CD4 and CD8 molecules; therefore, the upper right quadrant is empty. Often the percentage of cells found in each quadrant is also displayed on the dot plot. Some flow cytometers are capable of sorting cells, and these instruments allow the user to separate and collect the CD4+ and/or CD8+ cells for additional experiments requiring purified subpopulations of cells.

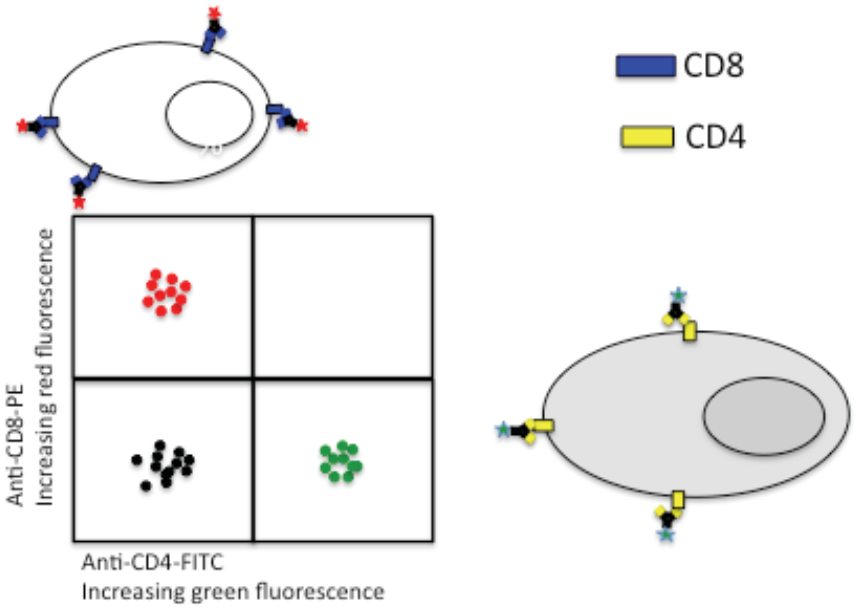


Figure 4. A dot plot showing cells incubated with monoclonal antibodies to both the CD4 and CD8 cell surface markers. Cells that are recognized by the FITC-labeled monoclonal antibody are found in the lower right quadrant; these cells express only the CD4 marker and would be characterized as T helper cells. Cells that are recognized by the PE-labeled monoclonal antibody are found in the upper left quadrant; these cells express only the CD8 marker and are characterized as T cytotoxic cells. Cells found in the lower left quadrant do not express either the CD4 or CD8 molecules. If a cell type expressed both the CD4 and the CD8 markers, it would be bound by both types of antibody and show both red and green fluorescence; these cells would be found in the upper right quadrant.

Taylor and her doctor have talked for a long time about flow cytometry and decide they will wait until Taylor gets her results before discussing the topic any longer. Taylor has blood drawn and sets up an appointment for the end of the week to return and see the results of the flow cytometry test.