IMMUNOLÓGIAI ÉS BIOTECHNOLÓGIAI INTÉZET

5th practice: Flow cytometry, cell separation methods

Basic Immunology

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Flow cytometry practice 1.

Steps of the practice:

1. Obtaining anticoagulated peripheral blood. (already done)
2. **Surface labeling of cells with fluorochrome-conjugated antibodies**: Add 50 µl of blood to the tubes which already contain the antibodies and incubate for 30 minutes!
3. **Hemolysis**: Add 1 ml of hemolysis buffer to the sample and incubate for 10 minutes!
4. Washing: Add 2 ml PBS to the samples then centrifuge them with 1000 rpm for 5 minutes!
5. Discard the supernatant and resuspend the pellet in 500 µl FACS-Fix solution! (FACS-Fix: PBS + 0,5% paraformaldehyde, **POISONOUS**)
6. Measure your samples with the BD FACSCalibur™ flow cytometer and analyze the results with the BD CellQuest™ program.

WEAR GLOVES!

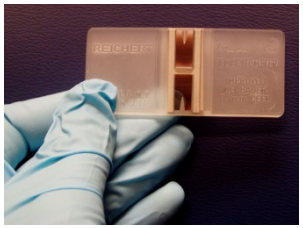
Flow cytometry practice 2.

- Content of the prepared tubes:
  1. Tube: anti-CD3-FITC + anti-CD4-PE
  2. Tube: anti-CD3-FITC + anti-CD8-PE
  3. Tube: anti-CD19-FITC + anti-CD5-PE
  4. Tube: doesn't contain antibodies = autofluorescence control
  5. Tube: doesn't contain antibodies = autofluorescence control
- Used fluorochromes (see later):
  - **FITC: Fluorescein isothiocyanate**
  - **PE: Phycoerythrin**

BD FACSCalibur™ flow cytometer

Definition of flow cytometry

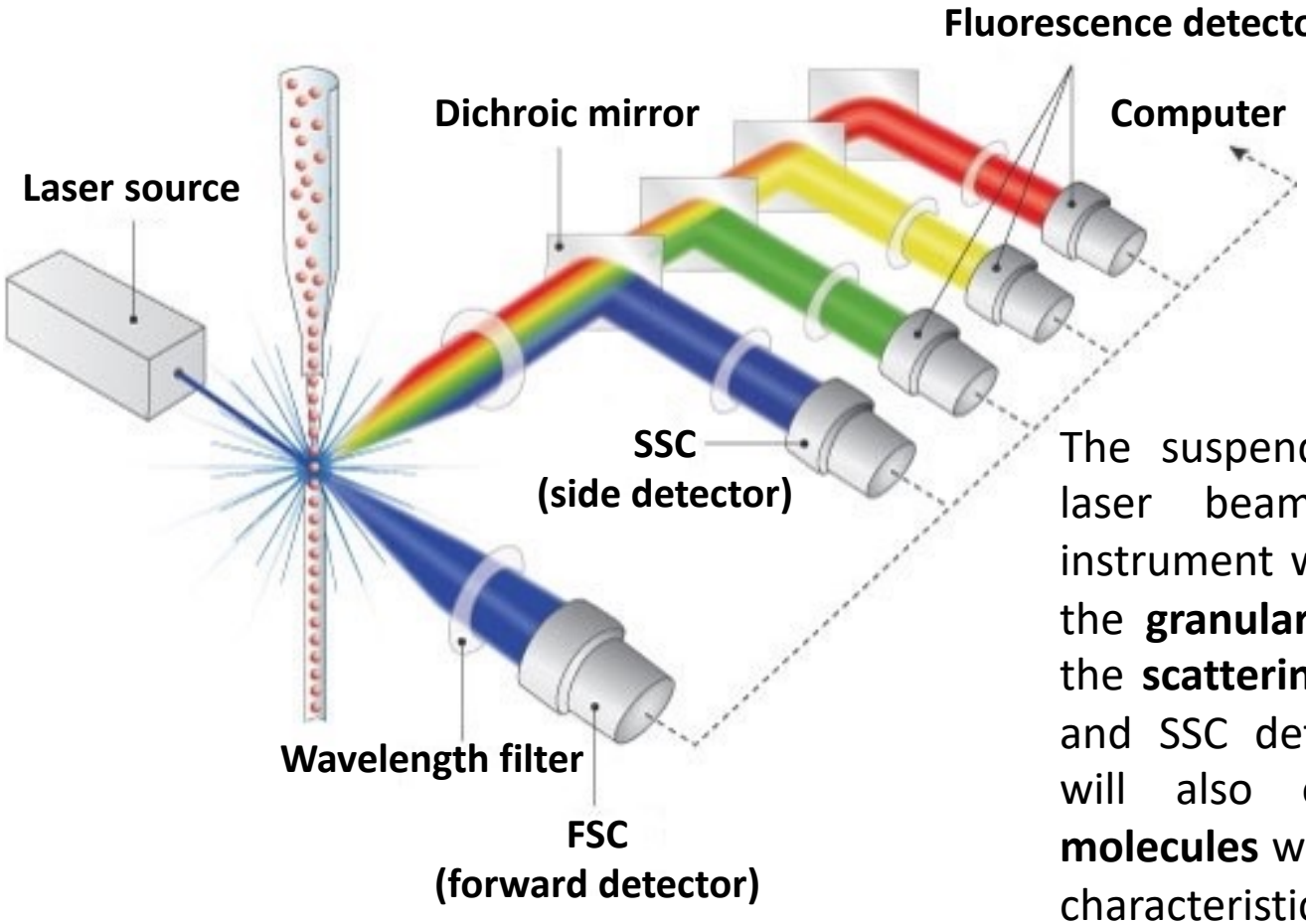
- Why cytometry?
  - A method capable of analyzing certain parameters of cells. (e.g. cell numbers, cell size, granularity, presence of certain proteins on the cell surface or inside the cytoplasm, DNA content, etc., see on the other slides)
- Why flow cytometry?
  - It is based on the flow of suspended particles (e.g cells) that are organized to create a stream that cross a laser beam. (see later)
- What can it be used for?
  - It is capable of the **multiparametric** analysis of large numbers of cells **quickly**. This means that it will analyze various parameters of a each measured cell simultaneously. (size, granularity, others depending on labeling, see later)



A hemocytometer:
The researcher/clinican may count 100-200 cells per minute.

BD FACSCanto™ II flow cytometer:
Can measure up to 10.000 cells in a second.[1.]

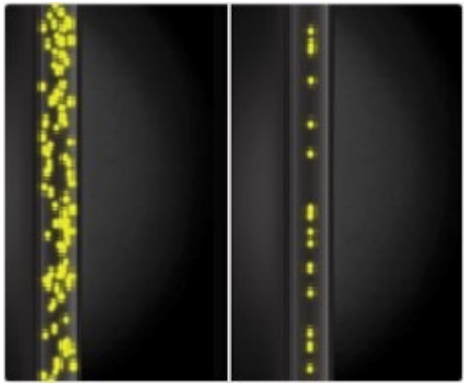
The basic principle of flow cytometry



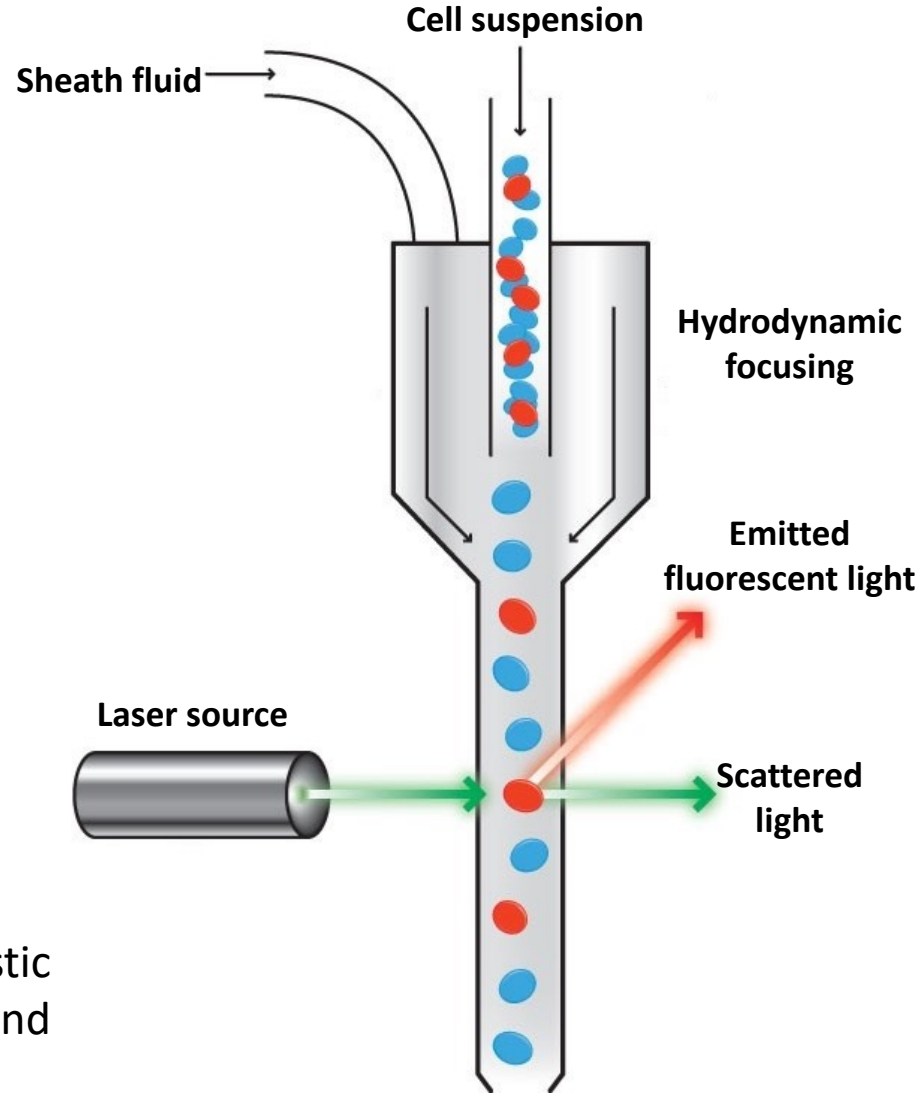
The suspended cells will cross the laser beam **one by one**. The instrument will calculate the **size** and the **granularity** of the cell based on the **scattering of the light** in the FSC and SSC detectors. The laser beam will also **excite** the **fluorescent molecules** which will **emit light** with a characteristic spectrum. The emitted fluorescent light is deflected to the corresponding **detectors** by **dichroic mirrors** or filters.[2.]

Hydrodynamic focusing

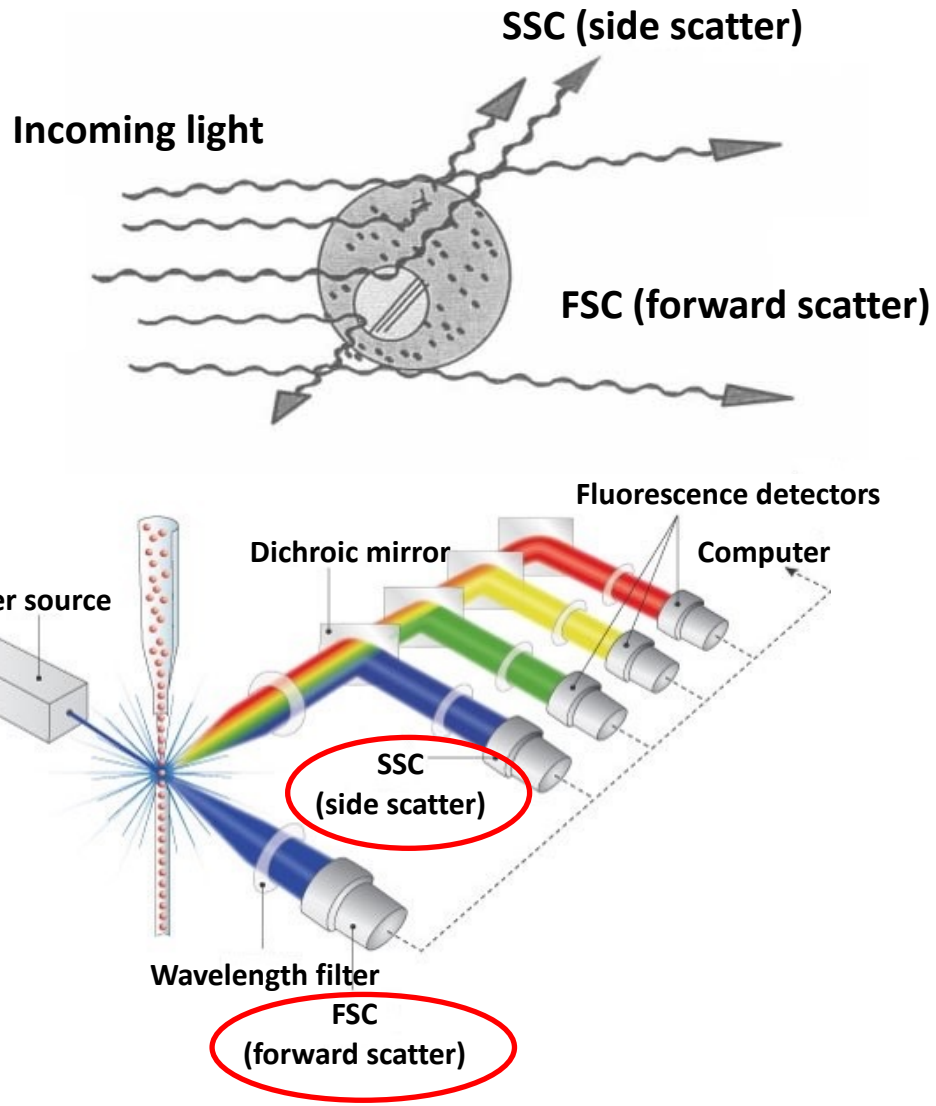
- The **sheath fluid** (usually PBS) will align the suspended cells into a line. The cells flow **one by one** in a straight line inside the **capillary**. The **laser ray** is focused on this capillary.
- Ultrasound may also be used for the focusing of the cells (acoustic focusing):



The Attune® flow cytometer which uses acoustic focusing with focusing turned off (left) and focusing turned on (right).



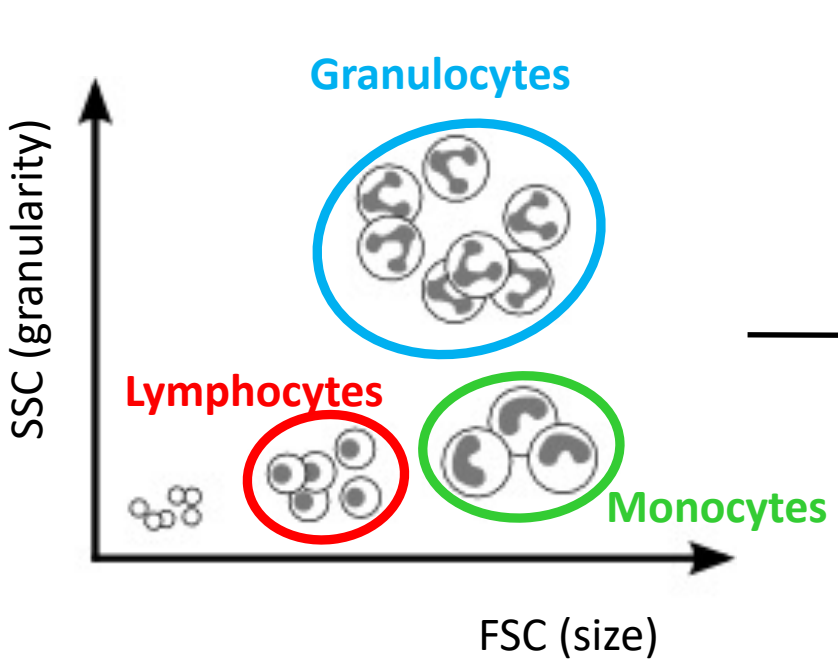
The FSC and SSC detectors



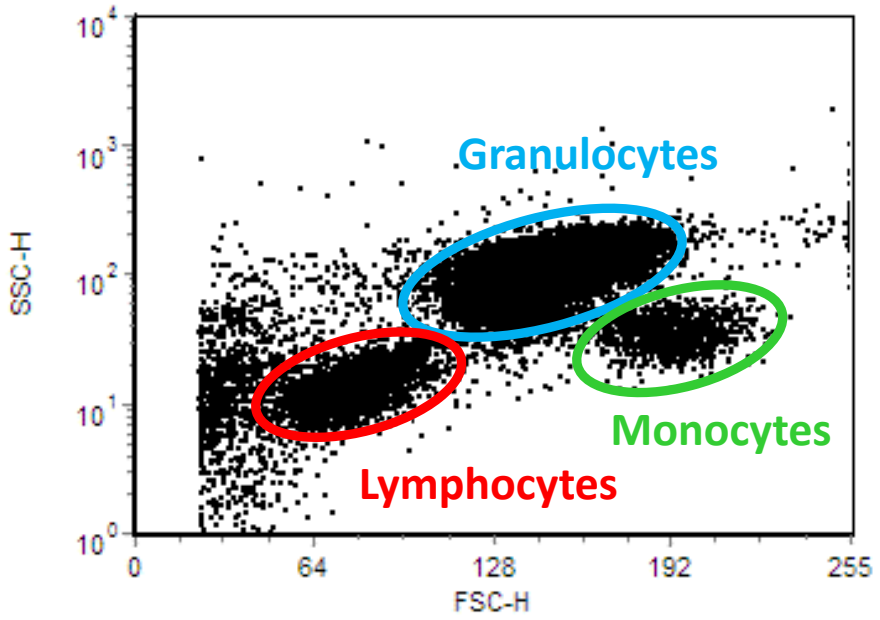
- The **FSC** (forward scatter) detector is located in the axis of the laser source and detects the forward-scattered light which is **proportional to size of the cell**. Forward diffracton is due to the cell's surface membrane.
- The **SSC** (side scatter) detector is roughly 90 degrees to the laser beam. Side-scattering of the light is caused by lamellar structures within the cell (e.g. mitochondria, granules, vesicles, etc.) and it is **proportional to the granularity** of the cell.

Introduction to dot plots based on FSC and SSC

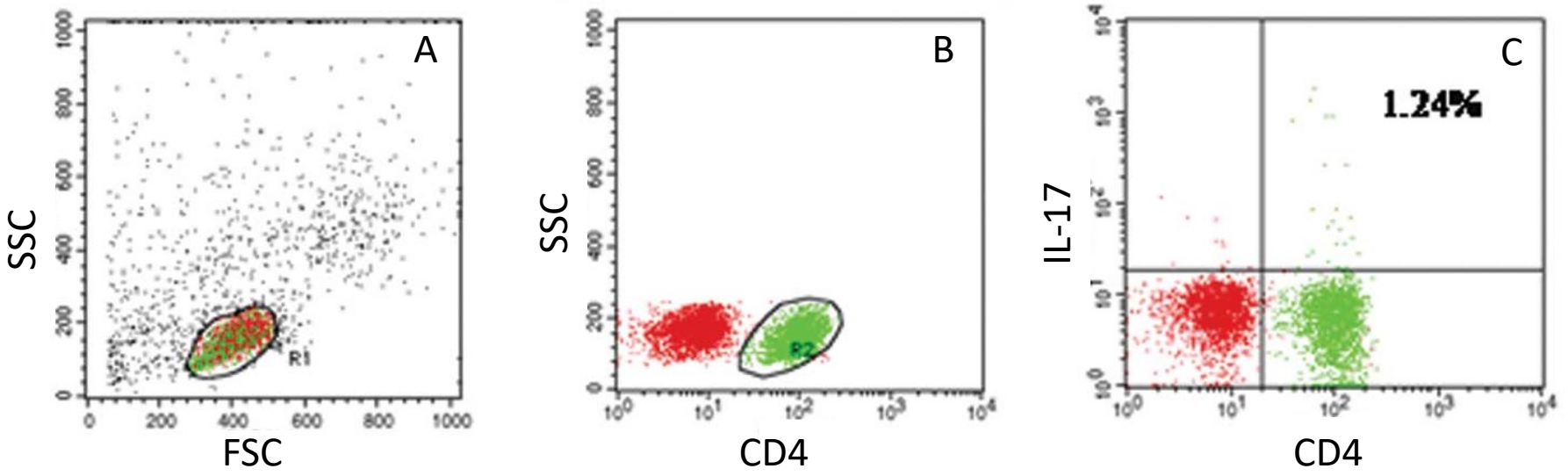
The **dot plot** is the most frequently used way to visualize the data of flow cytometric measurements. It shows the cells in a **two-dimensional coordinate system** where each plot represents an individual cell and the axes show specific parameters set by the investigator. Cells with similar parameters form groups called **cell populations**. These cell populations can be selected („**gating**") and selectively investigated for other parameters.



Dot plot of human peripheral blood:



Gating example

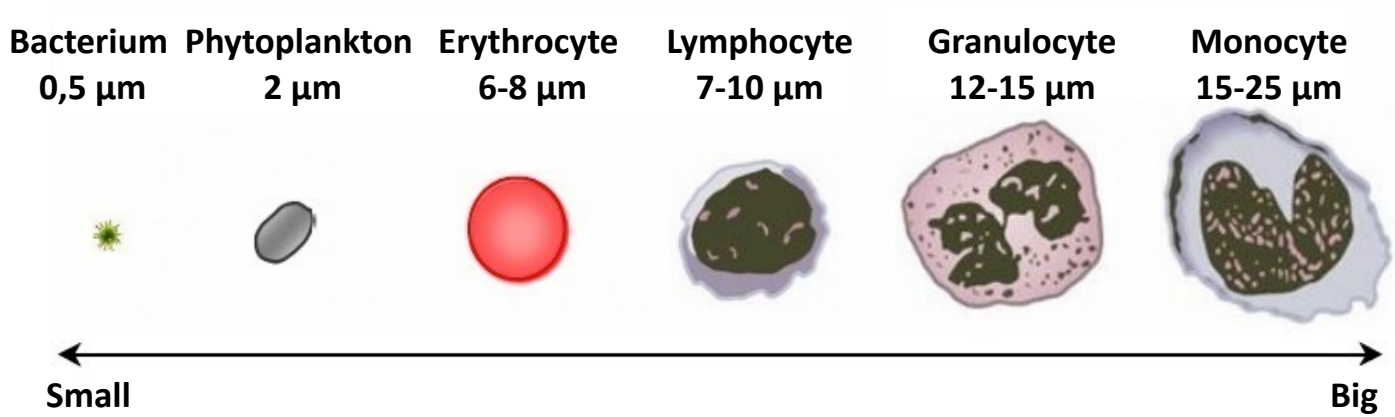


They wanted to determine the ratio of Th17 cells (see 2nd practice) in the sample. The blood was labeled with fluorochrome-conjugated antibodies (see later) for CD4 and IL-17 then the samples were measured with a flow cytometer.

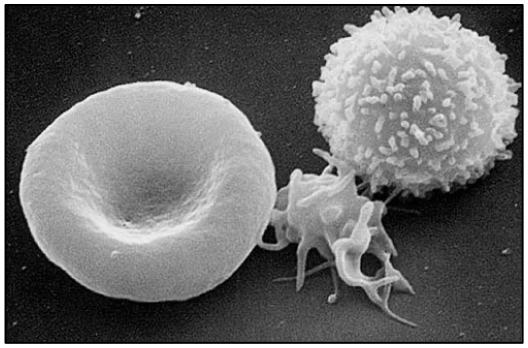
- **A**: Lymphocytes were selected based on their size (FSC) and granularity (SSC). → (R1 gate)
- **B**: CD4+ cells were selected within the lymphocyte population. (R2, green gate)
- **C**: They measured the ratio of CD4+ and IL-17+ cells within the lymphocyte gate. (the upper right quadrant, 1.24% of all lymphocytes.)

Flow cytometry and erythrocytes

- Erythrocytes should be removed from the samples because:
  - **Their size is similar** to that of lymphocytes.
  - They are far more abundant in the blood compared to lymphocytes and therefore block the signals coming from lymphocytes.

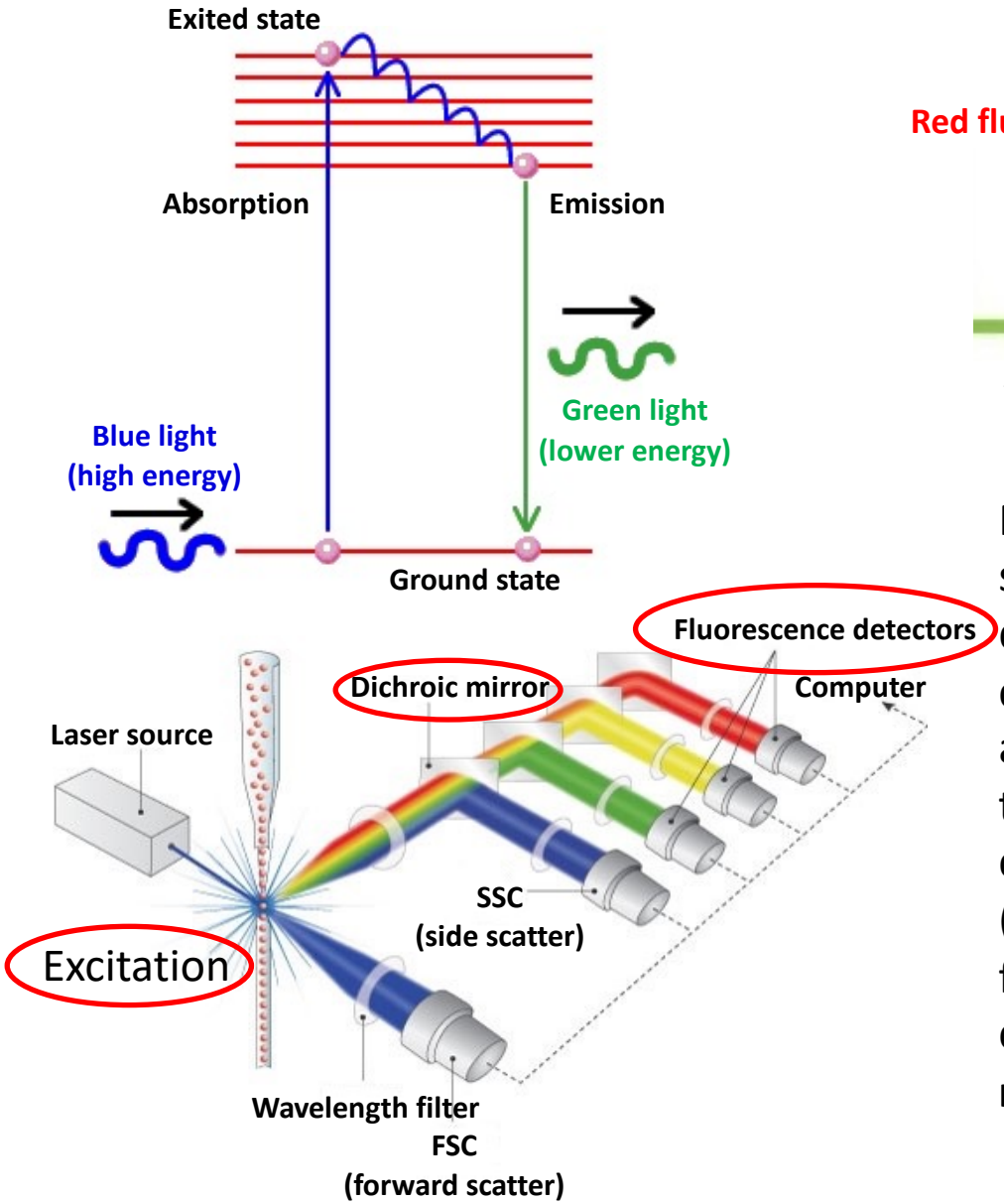


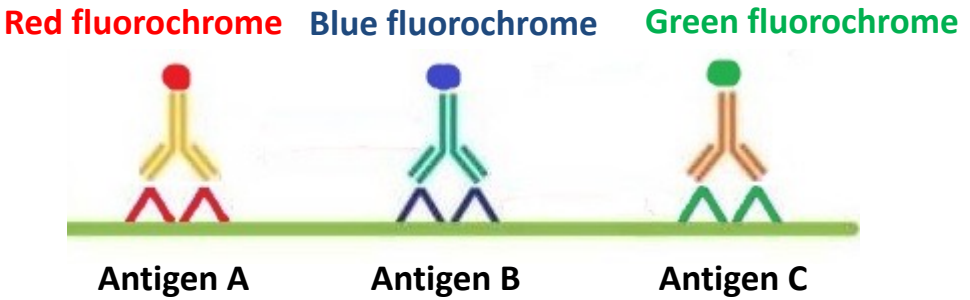
- Methods to eliminate erythrocytes:
  - **Hemolysis**
  - **Ficoll-gradient centrifugation** (see later)



RBC, TCT and a lymphocyte

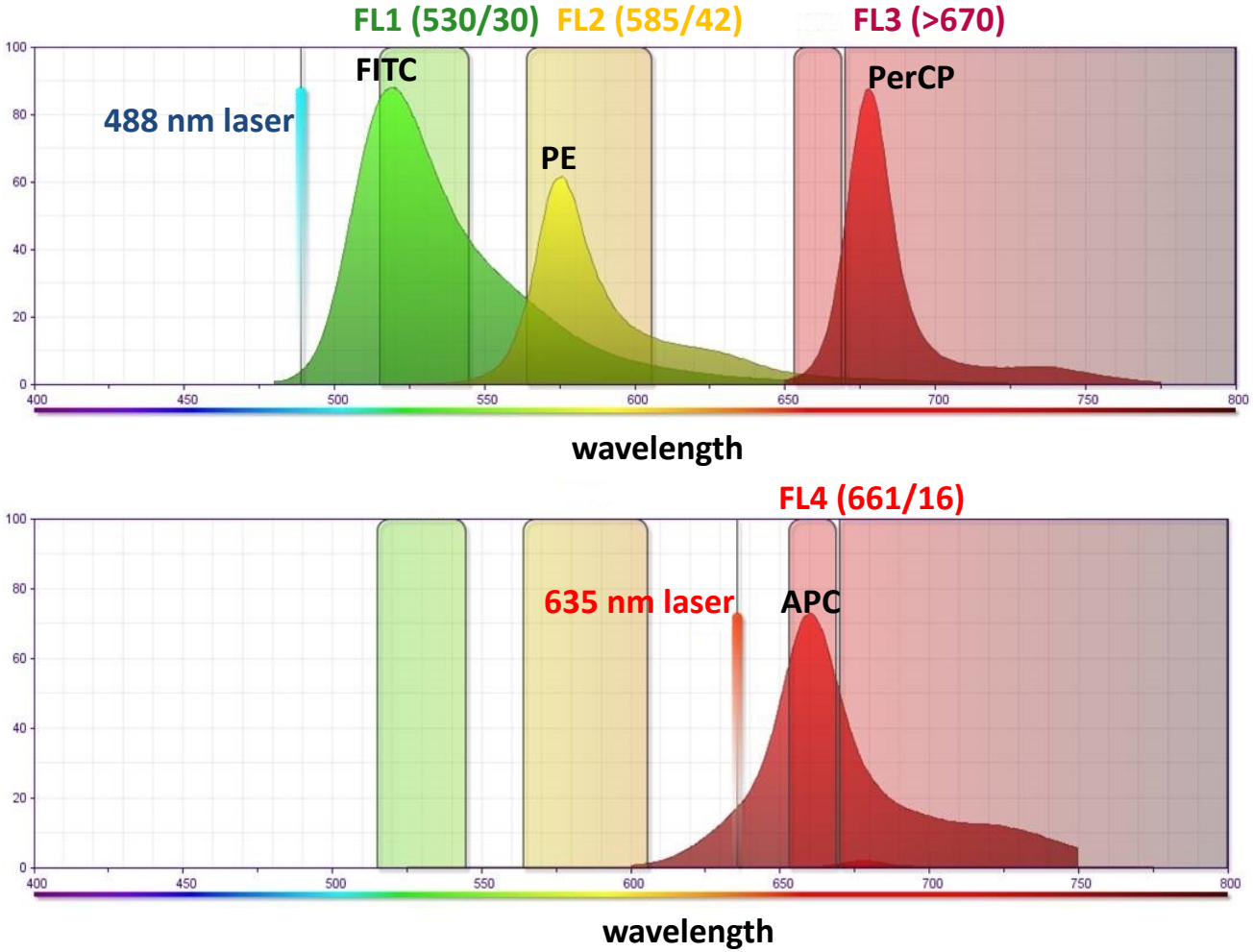
Immunofluorescence labeling





Not only can the instrument calculate the size and granularity of a cell, it can also detect the **fluorescence** emitted by the cell. The laser will **excite** the fluorochrome attached to the labeling antibody which then will emit fluorescent light characteristic for the used fluorochrome (**emission spectrum**). The emitted fluorescent light is deflected to the corresponding **detectors** by **dichroic mirrors** or filters.

The fluorescence spectrum



Lasers of BD FACSCalibur:[3.]
488 nm argon laser
635 nm red diode laser

Detectors of BD FACSCalibur:
FL1: 515-545 nm (530/30)
FL2: 564-606 nm (585/42)
FL3: >670 nm
FL4: 653-669 nm (661/16)

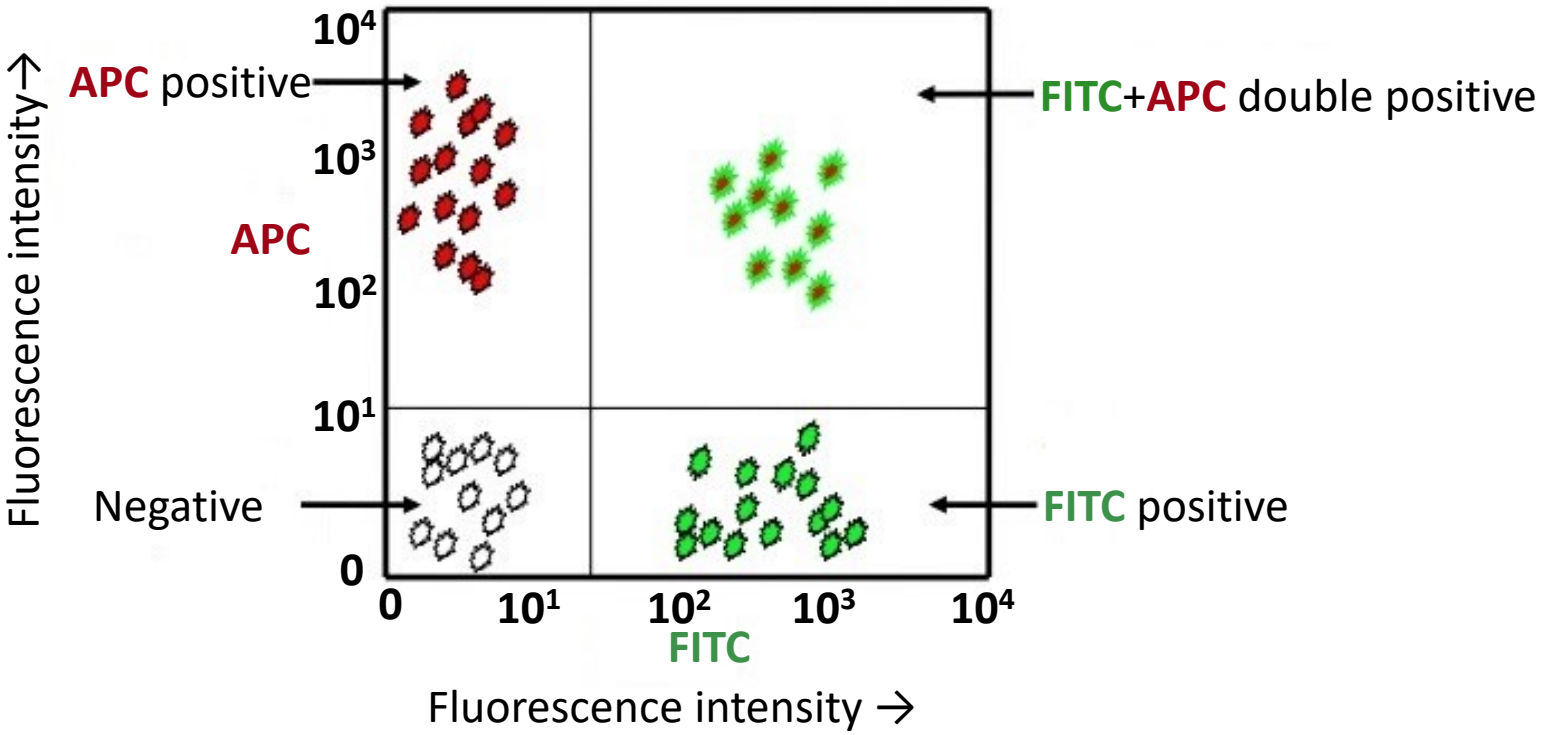
Unlike lasers which emit light of a single wavelength the fluorochromes emit a **spectrum of light**. These frequently **overlap** with each other.

Fluorochromes

Fluorochrome	Color	Excitation wavelength (nm)	Laser for excitation (nm)	Emission wavelength (nm)
Hoechst 33342	Blue	350	355, 375	461
Brilliant Violet™ 421	Blue	407	405	421
BD Horizon™ V450	Blue	404	405	448
Pacific Blue™	Blue	401	405	452
BD Horizon™ V500	Green	415	405	500
AmCyan	Green	457	405	491
Alexa Fluor® 488	Green	495	488	519
FITC	Green	494	488	519
PE	Yellow	496, 564	488, 532, 561	578
Brilliant Violet™ 605	Orange	407	405	602
BD Horizon™ PE-CF594	Orange	496, 564	488, 532, 561	612
PI	Orange	351	488, 532, 561	617
7-AAD	Red	543	488, 532, 561	647
APC[1]	Red	650	633, 635, 640	660
Alexa Fluor® 647	Red	650	633, 635, 640	668
PE-Cy™5[1]	Red	496, 564	488, 532, 561	667
PerCP	Red	482	488, 532	678
PerCP-Cy™5.5	Far Red	482	488, 532	695
Alexa Fluor® 700	Far Red	696	633, 635, 640	719
PE-Cy™7	Infrared	496, 564	488, 532, 561	785
APC-Cy7	Infrared	650	633, 635, 640	785
BD APC-H7	Infrared	650	633, 635, 640	785

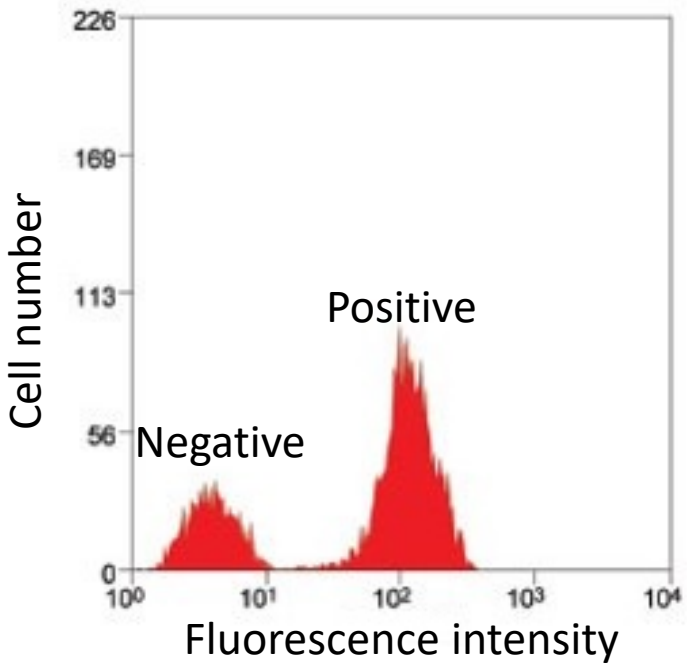
Flurochromes that are excited by the same laser and that emit light of the same spectrum should not be used together in a sample as the labeled antigens will not be distinguishable. (Frequently used combination: **FITC** + **PE** + **APC**)

Fluorescence on dot plots



The measurement is **quantitative**, the detected fluorescence intensities can be given in numbers. (=One can also tell the **level of positivity**.)

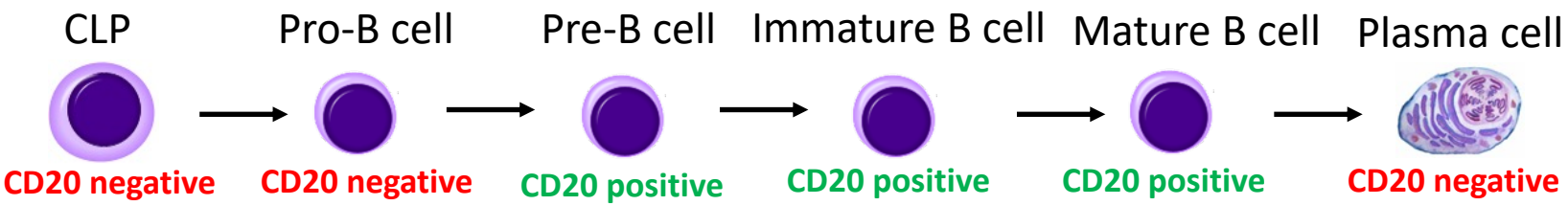
Data shown on histograms



Ordinary histogram: The horizontal axis shows the fluorescence intensity (=strength of the signal) that was measured by the detector, while the vertical axis represents the number of measured cells. In the example above, two cell populations can be distinguished one being negative, the other being positive for the tested marker.

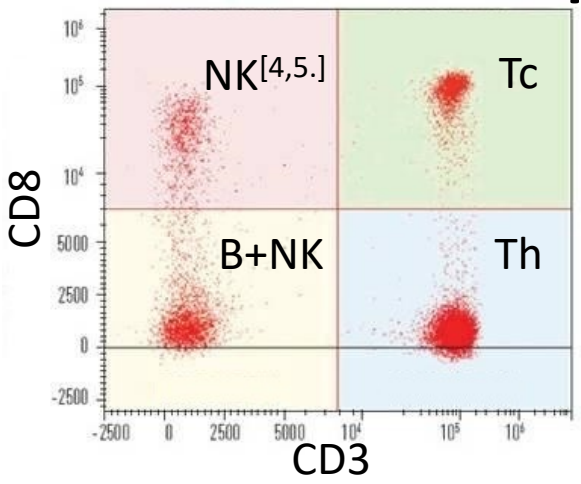
Types of CD markers

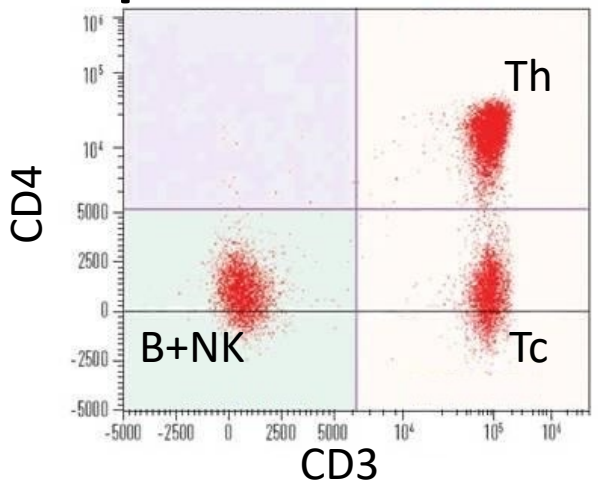
- **Lineage markers**: Molecules expressed exclusively on certain cell lineages.
  - E.g.: CD3 → found on all T cells CD19 → found on all B cells
- **Maturation markers:** The immunophenotype might differ in the phases of cell maturation, certain molecules are only expressed on immature cells, others on mature, fully functioning cells, etc.
  - E.g.: CD20 (It is also a lineage marker of B cells, cannot be found on any other cells)[1.]

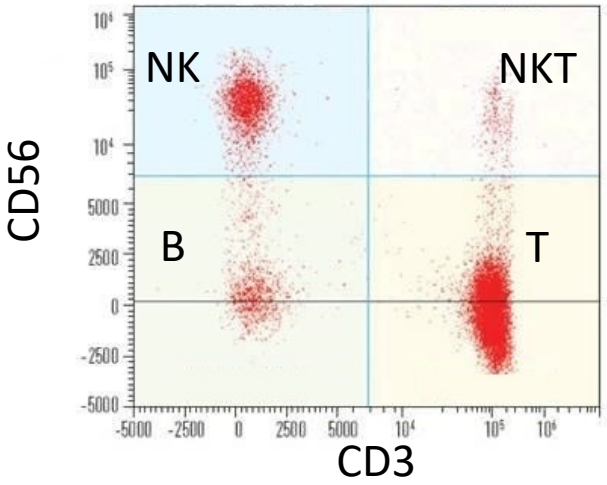


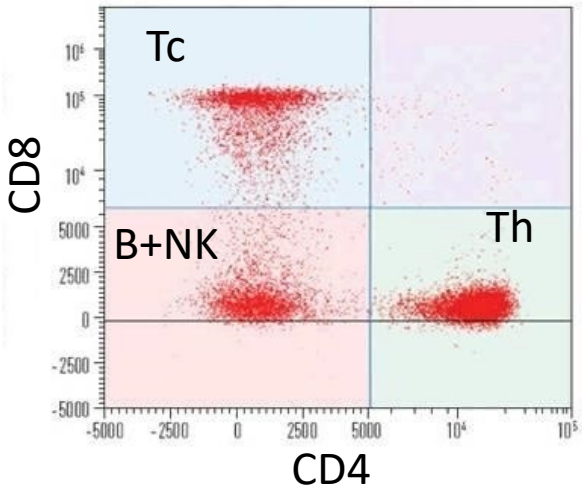
- **Activation markers:** Molecules expressed by activated cells, whereas resting cells either lack them completely or express them at low levels, e.g.:
  - CD25 (The alpha chain of the interleukin-2 receptor, IL-2Rα, see later)
  - CD80 and CD86 (B7-1 and B7-2, so-called costimulatory molecules expressed by activated antigen presenting cells, see later)

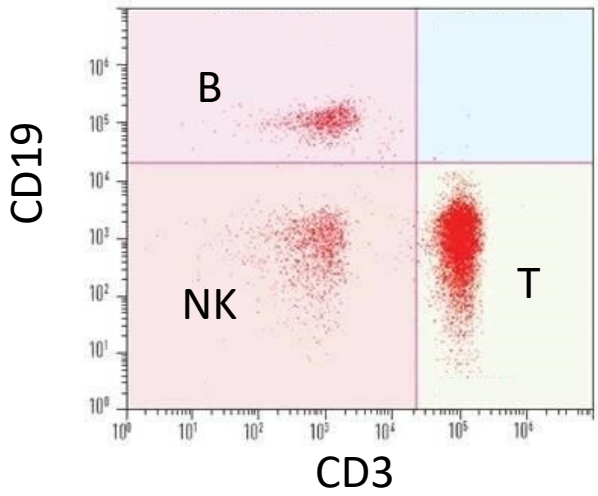
Lymphocyte populations of normal human peripheral blood

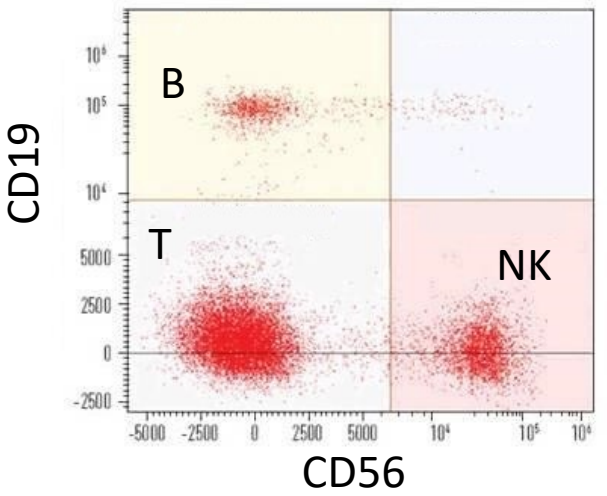






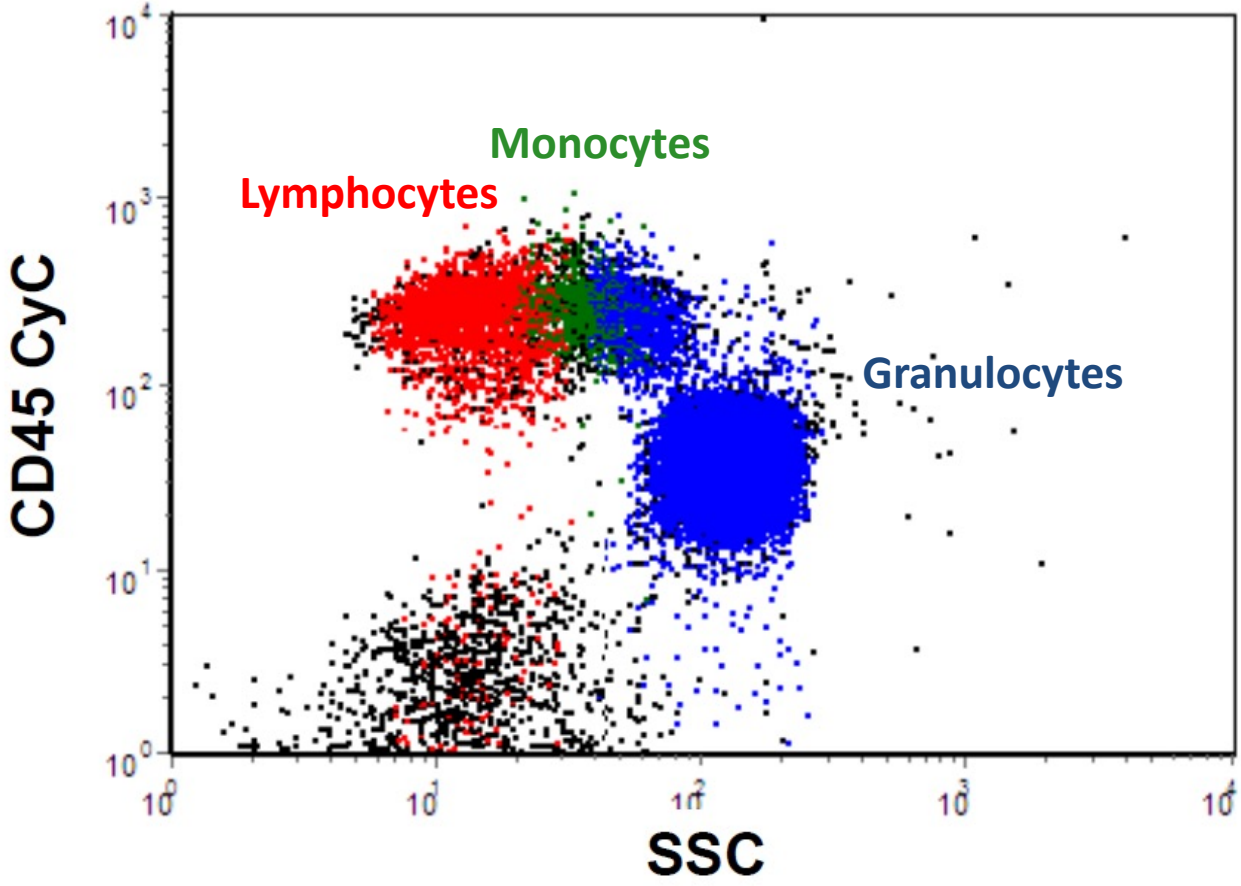






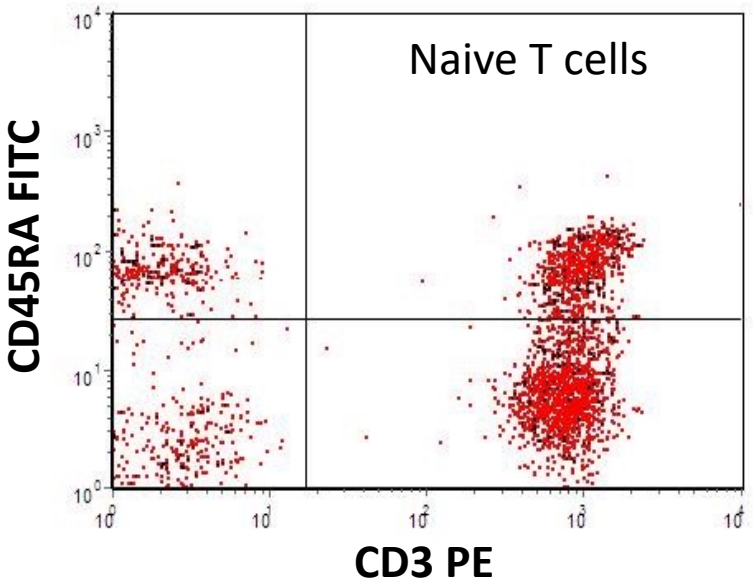
Markers: **CD3** → T cell, **CD4** → Th cell, **CD8** → Tc cell, **CD19** → B cell, **CD56** → NK cell

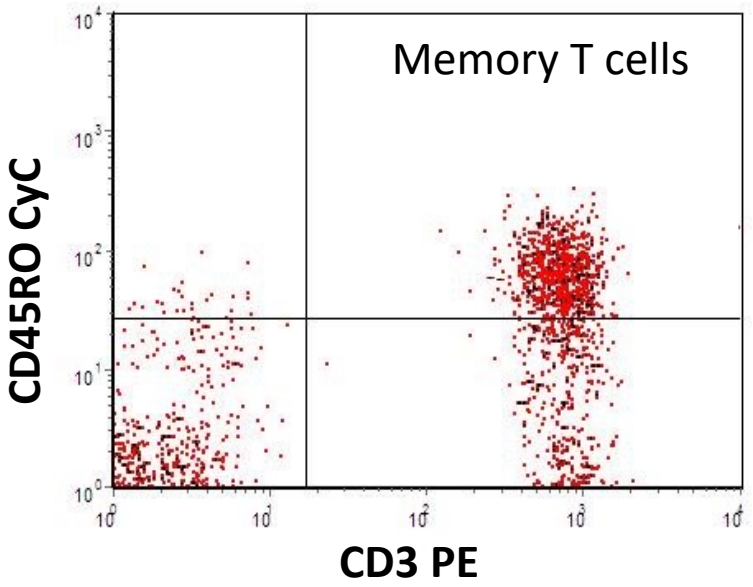
CD45 in human peripheral blood



The **CD45** molecule can be found on **all leukocytes**. It is therefore considered a **pan-leukocyte marker.**

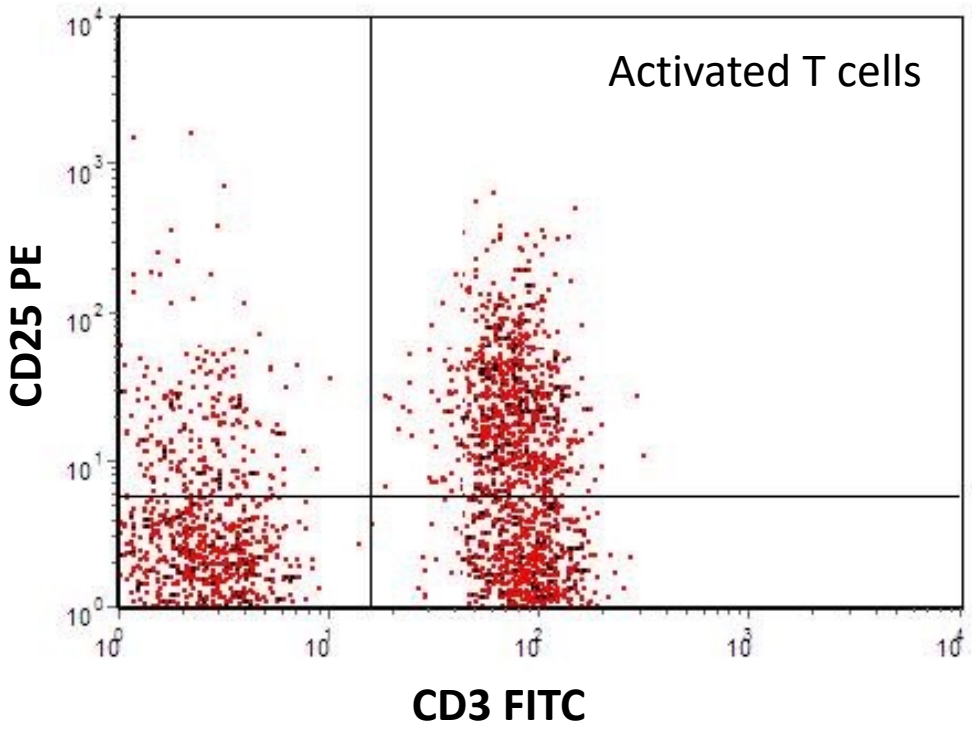
CD45RA and CD45RO isoforms in normal human blood





Function of **CD45**: It is a **phosphatase** embedded in the cell membrane. It has various isoforms, **CD45RA** can be found on **naive T cells** while **CD45RO** is expressed by **memory T cells**.[6.] (see the lectures for more details)

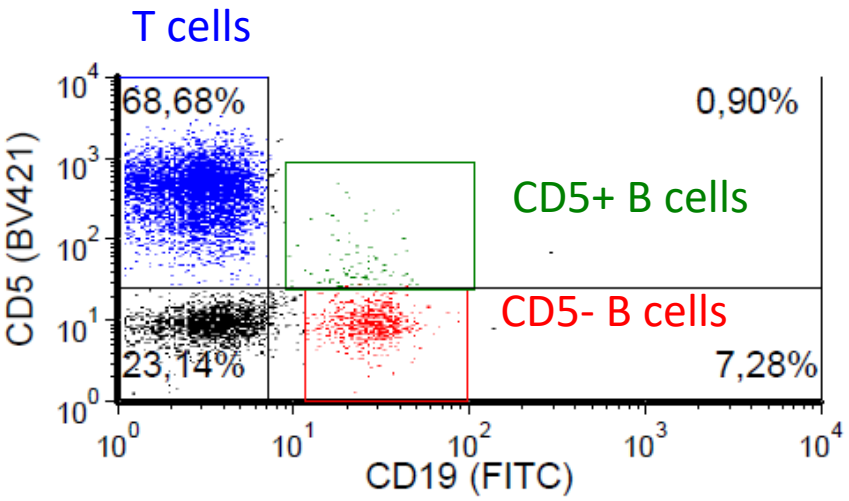
CD25 expression in T cells found in the human blood



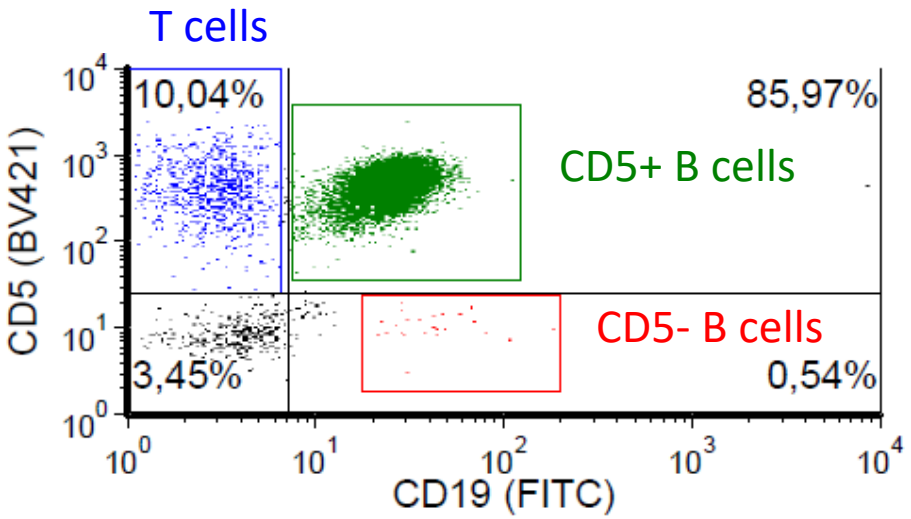
CD25: It is the alpha chain of the interleukin-2 receptor (IL-2Rα) which only appears on activated lymphocytes.[7.] (see the lectures for more details)

Human CD5+ B cells

Peripheral blood of a healthy donor:



B-cell chronic lymphocytic leukemia (B-CLL):



Significance of flow cytometry 1.

- Can determine the immunophenotype of the cells in the sample (**immunophenotyping**):
  - Diagnostics and differential diagnosis of **malignant hematological diseases**[8, 9.]
  - Diagnostics and differential diagnosis of **immunodeficiencies** [10.]
  - Monitoring of **autoimmune disorders**
  - Monitoring of pre- and post-**transplantation** state[11.]
  - Determining **HLA haplotypes**[12.] (see later)
  - Diagnostics and monitoring of **infectious disease**
- **Quantitative** measurements:
  - Determining the **ratio of cells** that express the antigen of interest
  - Determining the **level of antigen expression** in the investigated cell population
- Determining the **DNA and RNA** content:[13.]
  - Investigating apoptosis
  - Investigating the cell cycle
  - Determining the ratio of reticulocytes
  - Detection of aneuploidy

Significance of flow cytometry 2.

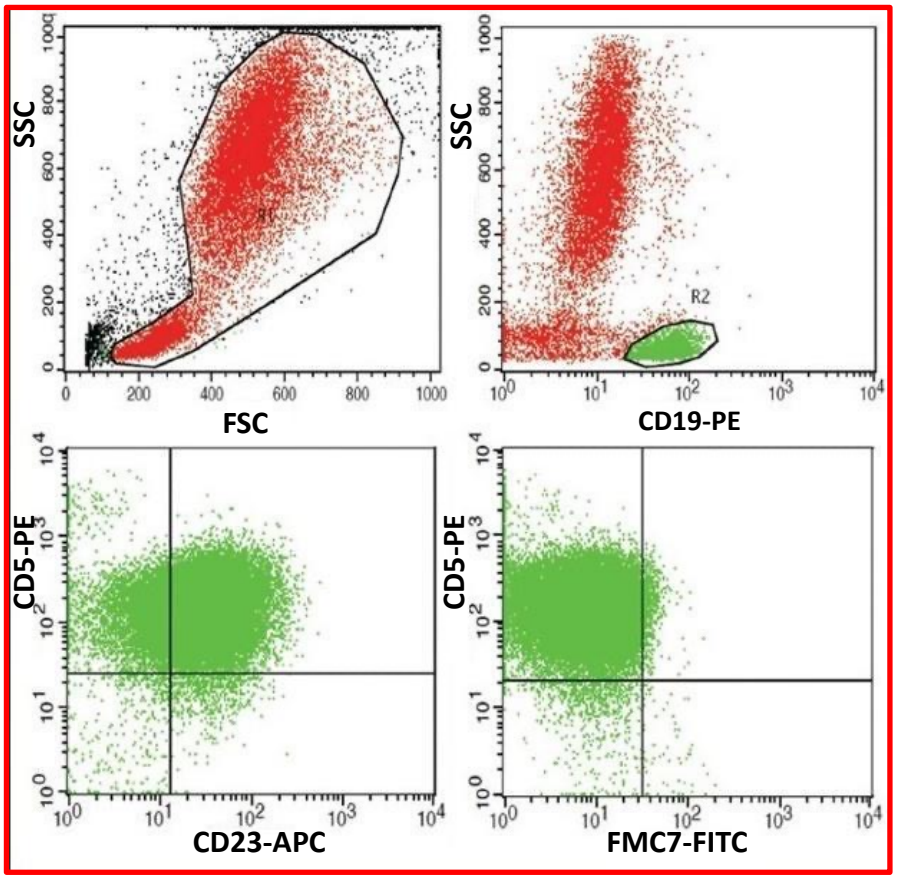
- **Functional tests**:
  - Measuring **phagocytosis**[14.]
  - Measuring **intracellular calcium levels**[15.]
  - Measuring intracellular pH
  - Measuring the quantity and activity of enzymes
  - Investigating chemotaxis
  - Measuring the production of reactive oxygen species
  - Measuring the proliferative index
- **Measuring of cytokine levels** [16.] (CBA, see later among the serological tests)
- **Cell separation**[17.] (cell sorting, see later)
- Automated hematology analyzers→ **blood counts** (without fluorescence labeling)

The next few slides will show you examples of various possible applications of flow cytometry. They are for illustration only, you **do not have to learn/know** the material presented on them but you must be able to **analyze dot plots** and **name the applications** of flow cytometry!

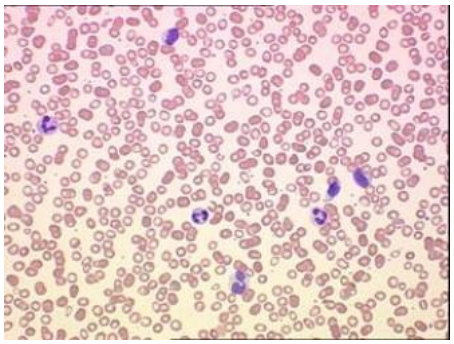
Immunophenotyping example 1.

A routine blood test revealed abnormally high lymphocyte numbers. (lymphocytosis)

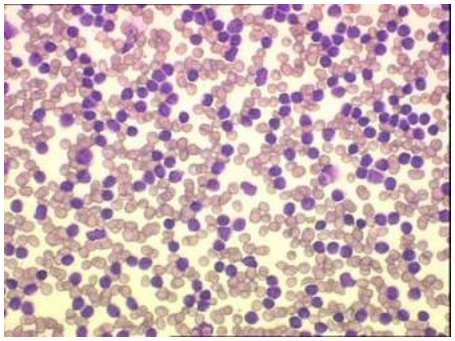
Flow cytometry →



Question: What kind of cells are these?



Normal blood smear



Blood smear of the patient

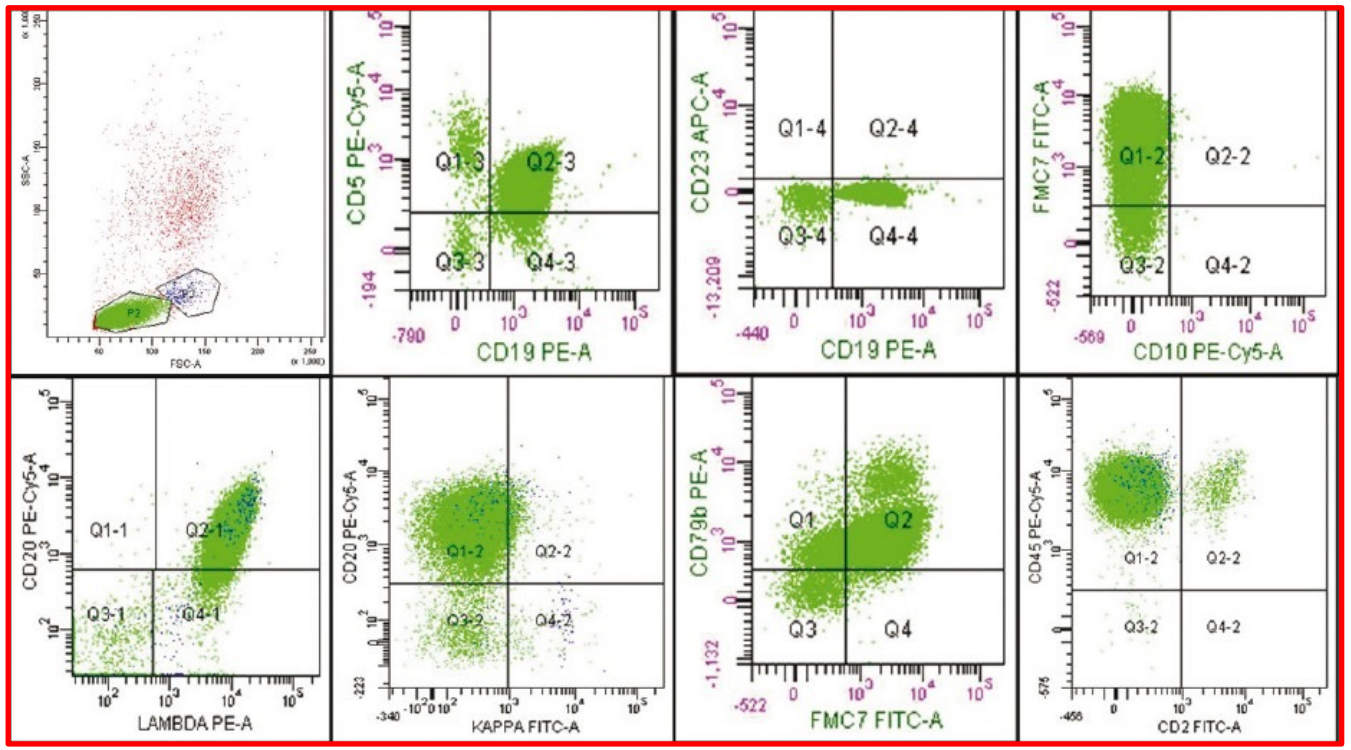
Immunophenotype:
CD5+/CD23+/CD19+/FMC7-

↓

Diagnosis: B-cell chronic lymphocytic leukemia (B-CLL)

Immunophenotyping example 2.

The patient was investigated because of lymphocytosis and enlarged lymph nodes (lymphadenomegaly).

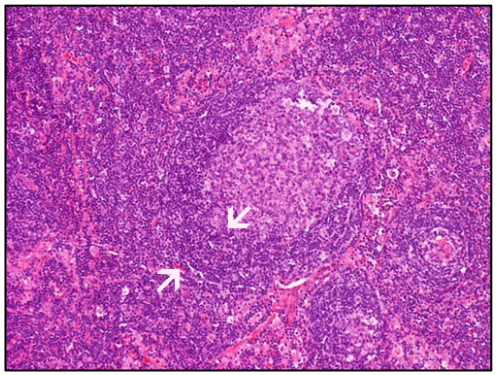


Question: What kind of cells are these?

Immunophenotype:

- **CD5+**
- **CD19+**
- CD20+
- CD45+
- CD79b+
- **FMC7+**
- Lambda+
- **CD10-**
- **CD23-**

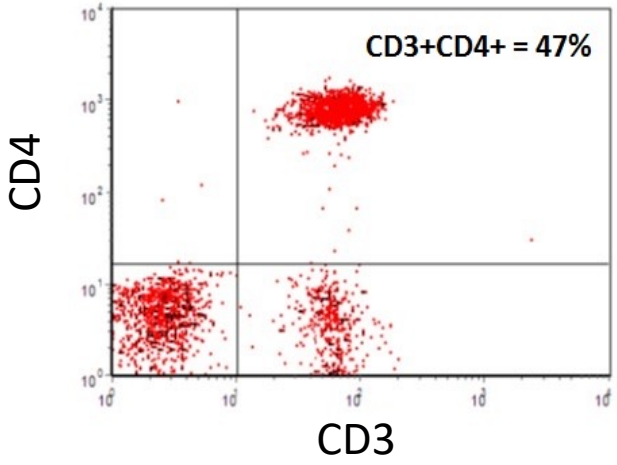
Diagnosis: Mantle cell lymphoma (MCL) in leukemic phase

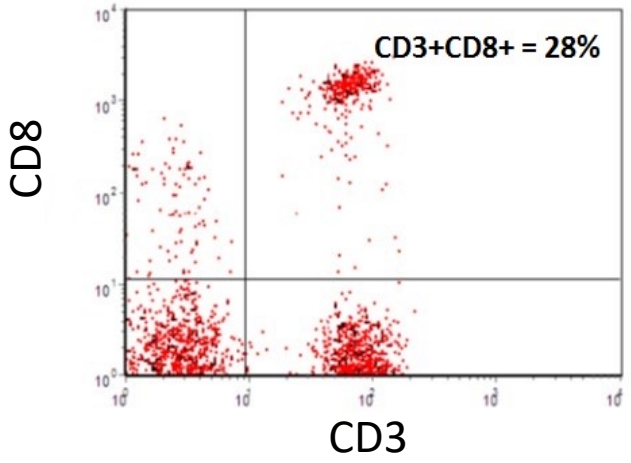


Widened mantle zone (marked by white arrows) around a lymphoid follicle in MCL. (Lymph node, H&E staining)

Follow-up of a patient with immunodeficiency

CD4/CD8 ratio in the blood of a healthy donor:

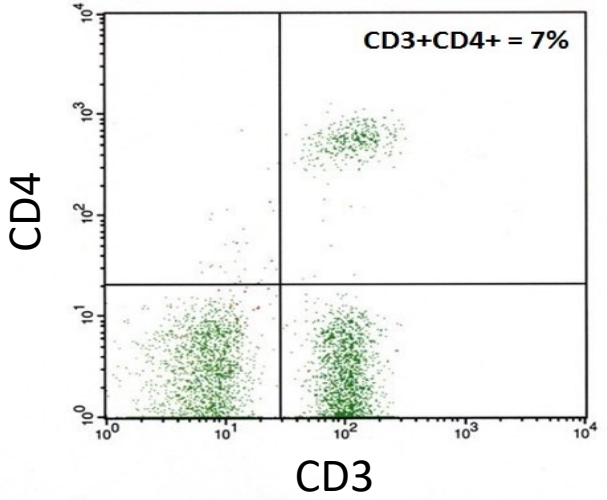


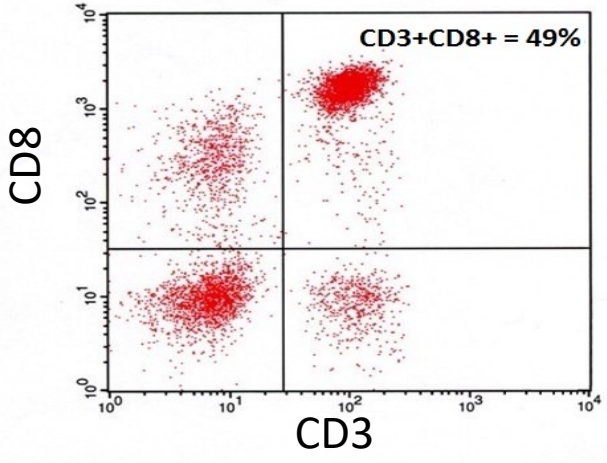


Helper T cells

Cytotoxic T cells

Pathological CD4/CD8 ratio in the blood of a patient with HIV:

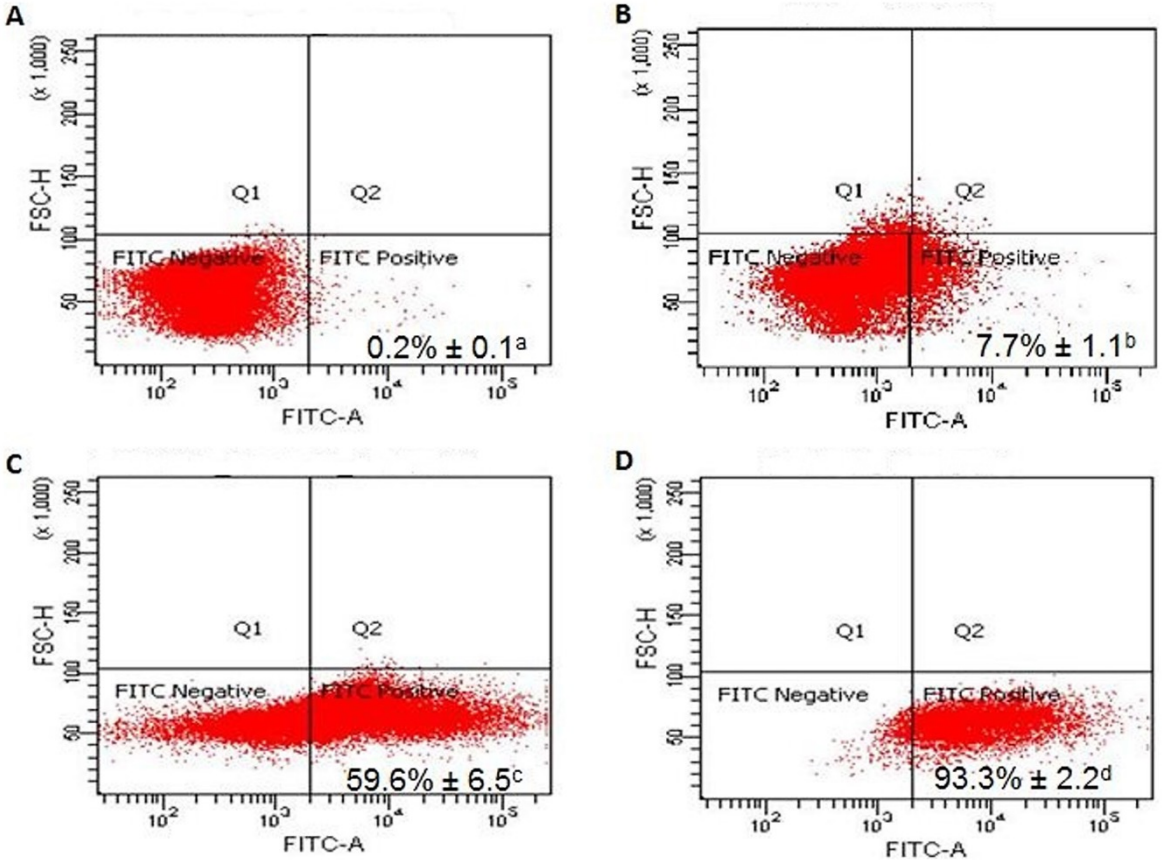




Measuring phagocytosis

A: Untreated macrophages incubated without FITC-conjugated beads

B: Untreated macrophages incubated with FITC-conjugated beads



Treating the macrophages increased their phagocytosing capabilities.

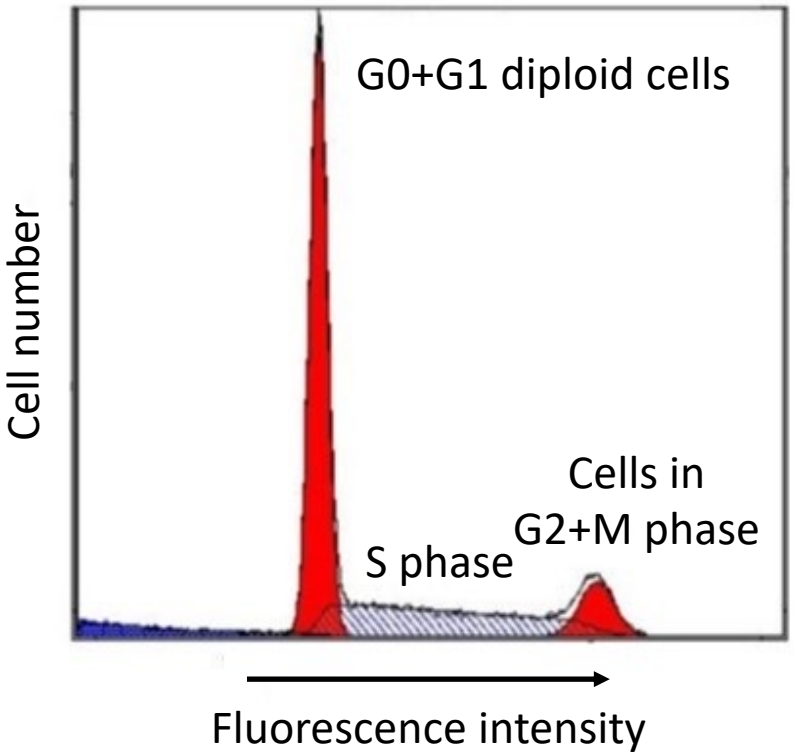
C: Macrophages treated with plant polysaccharides incubated with FITC-conjugated beads

D: Macrophages treated with **LPS** incubated with FITC-conjugated beads

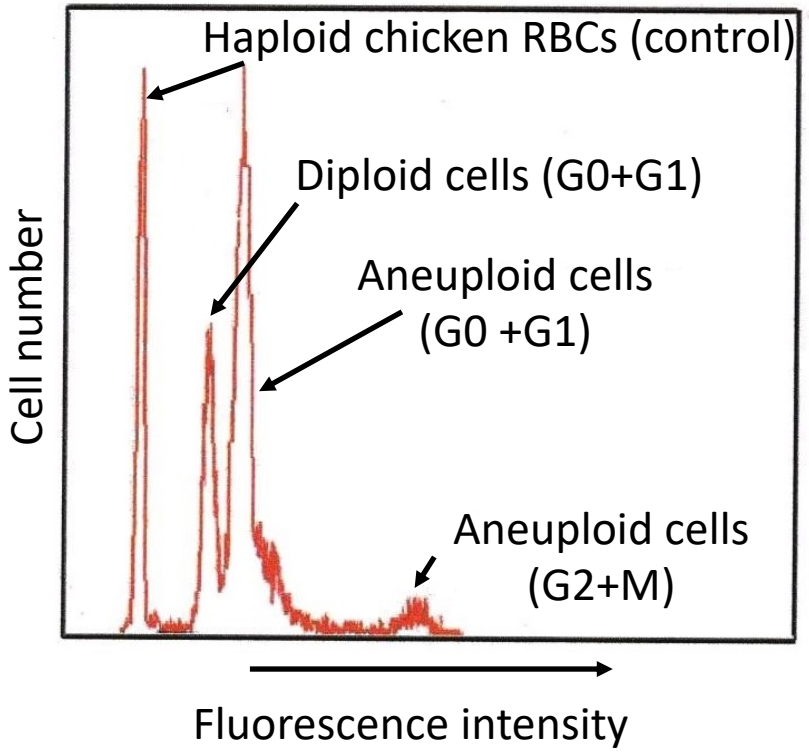
Investigating DNA content in tumors

The cells are incubated with fluorochromes that bind DNA and then the emitted fluorescence intensity is measured with flow cytometry.

Normal:



Biopsy from hepatocellular carcinoma:[18.]

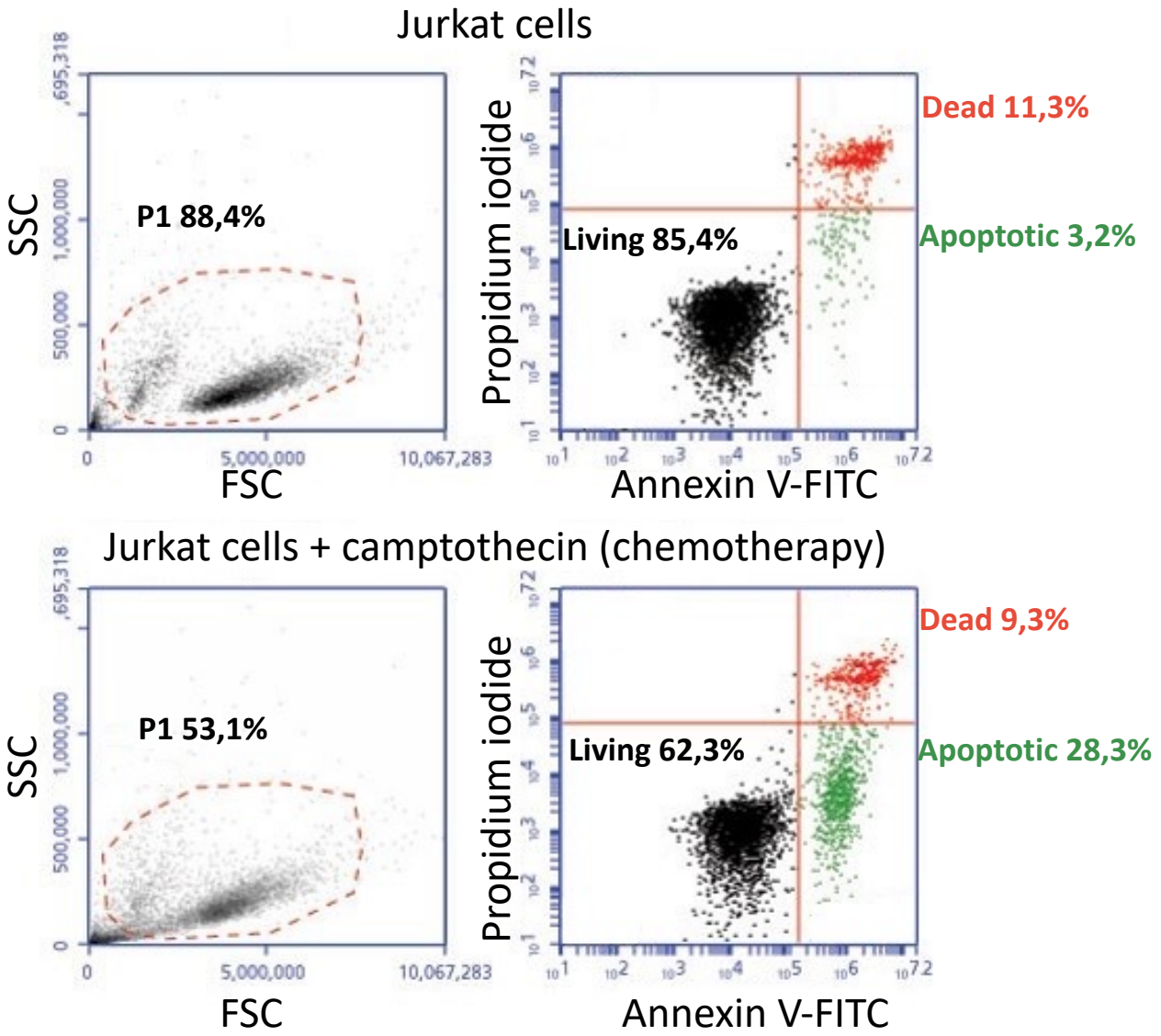


Aneuploidy: Abnormal number of chromosomes (e.g. 60 instead of 46 in cells that are in the G0 phase)

Cell viability test

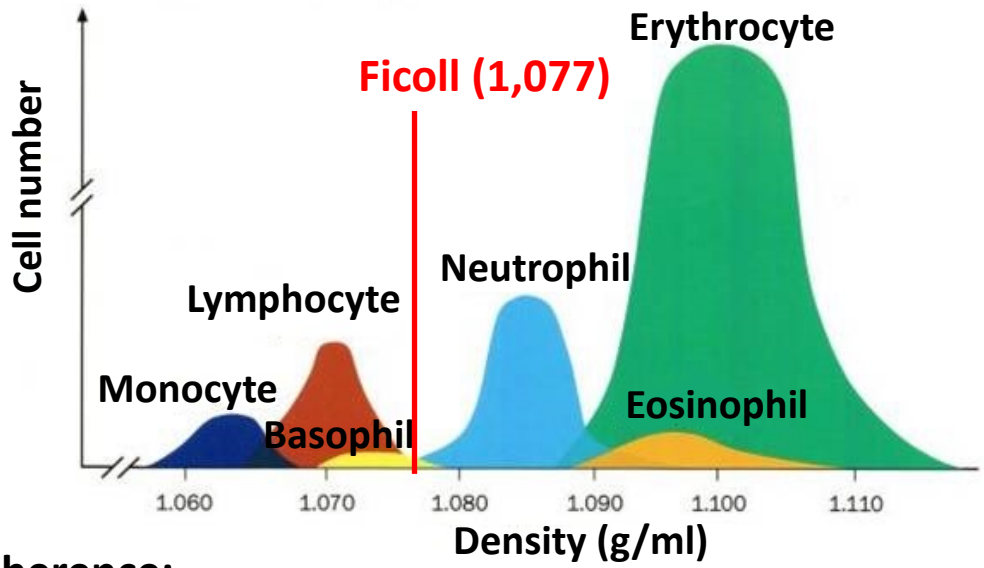
Propidium iodide: A fluorescent molecule that binds DNA which cannot cross the intact cell membranes of living cells.

Annexin V: It binds phosphatidylserine which can be found in the cell membranes of apoptotic cells.



Isolation of mononuclear cells

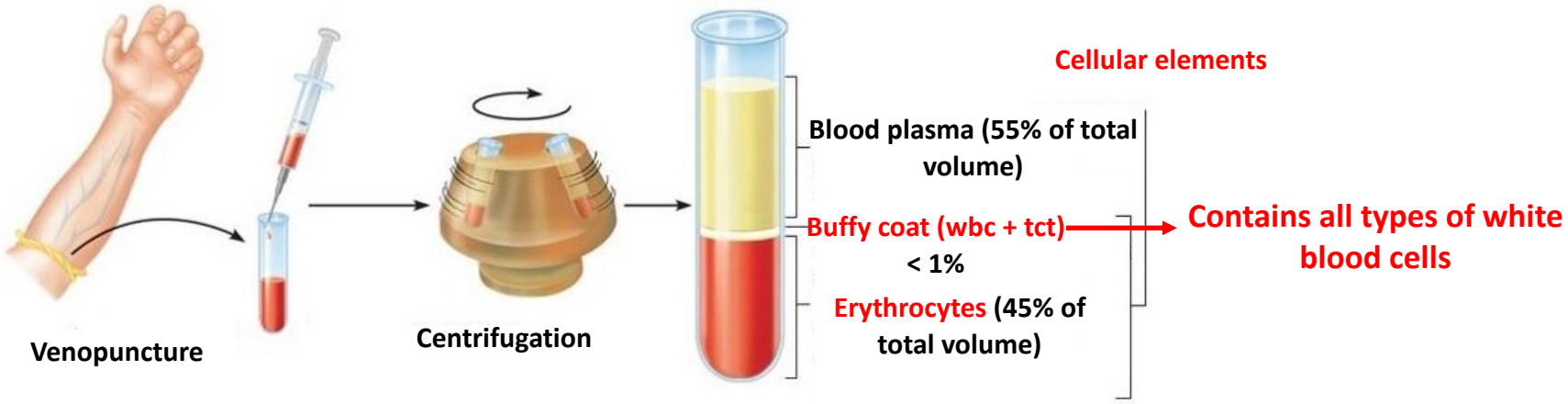
- **Procedures based on physical parameters:**[19.]
  - Filtration (based on the different sizes of blood cells)
  - Density gradient centrifugation (e.g. **Ficoll gradient centrifugation**, based on the different density of blood cells)



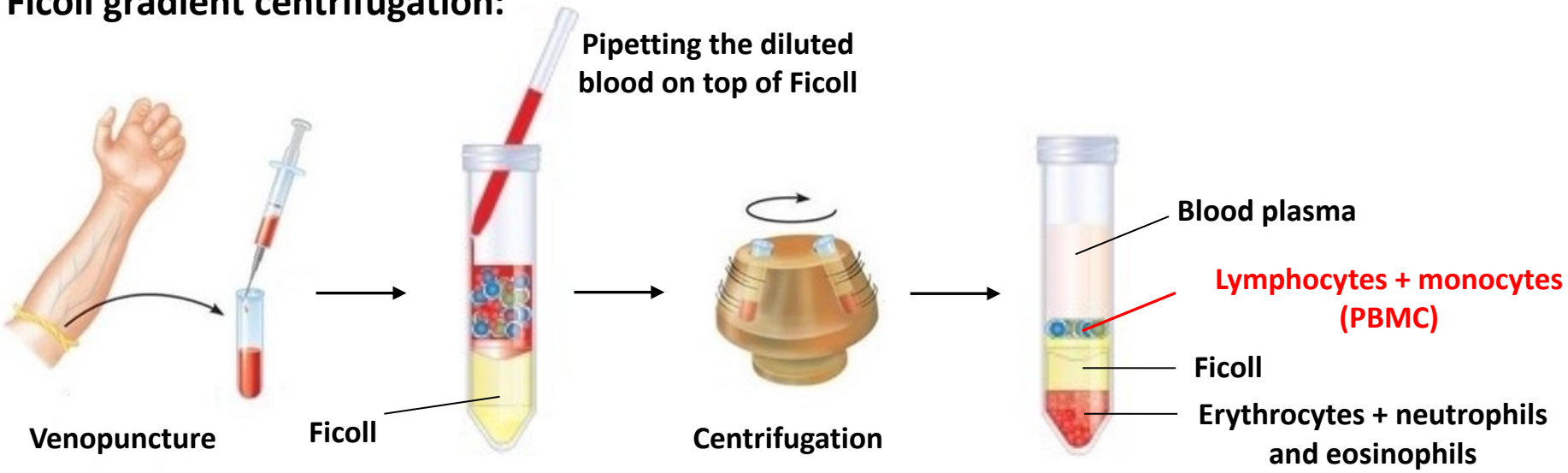
- **Procedures based on leukocyte adherence:**
  - Nylon wool: monocytes and B cells will adhere to the nylon
  - Plastic/glass surfaces: monocytes will adhere to the surface, lymphocytes can be removed

Ficoll gradient centrifugation 1.

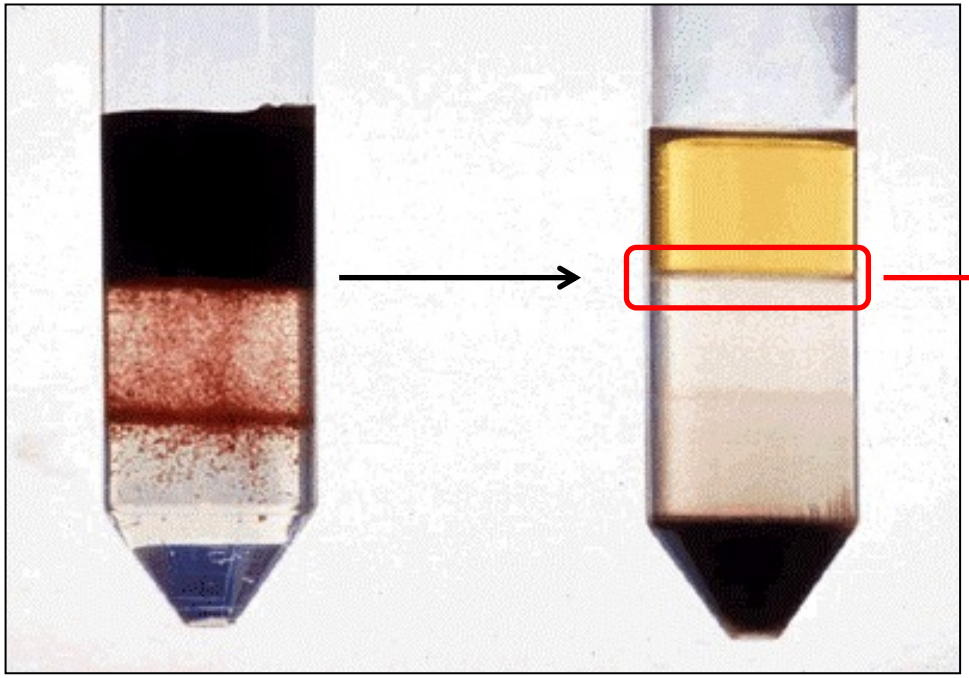
Ordinary centrifugation:

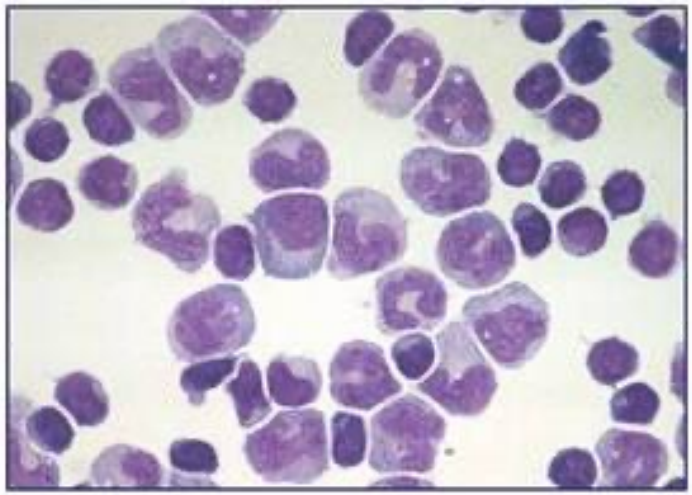


Ficoll gradient centrifugation:



Ficoll gradient centrifugation 2.





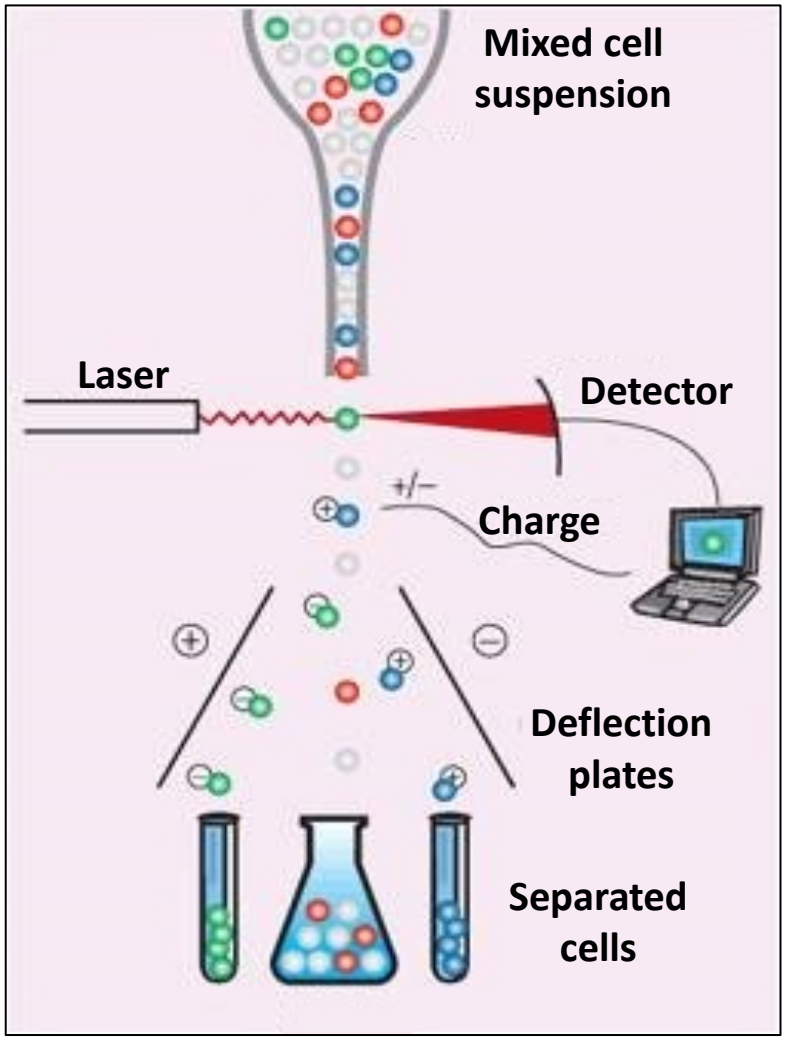
Peripheral blood mononuclear cells (**PBMC**, leukocytes with a non-segmented cell nucleus): **Lymphocytes, monocytes** (may also contain basophil granulocytes)[20.]

Sorting

Sorting: A method of **cell separation** based on flow cytometry.

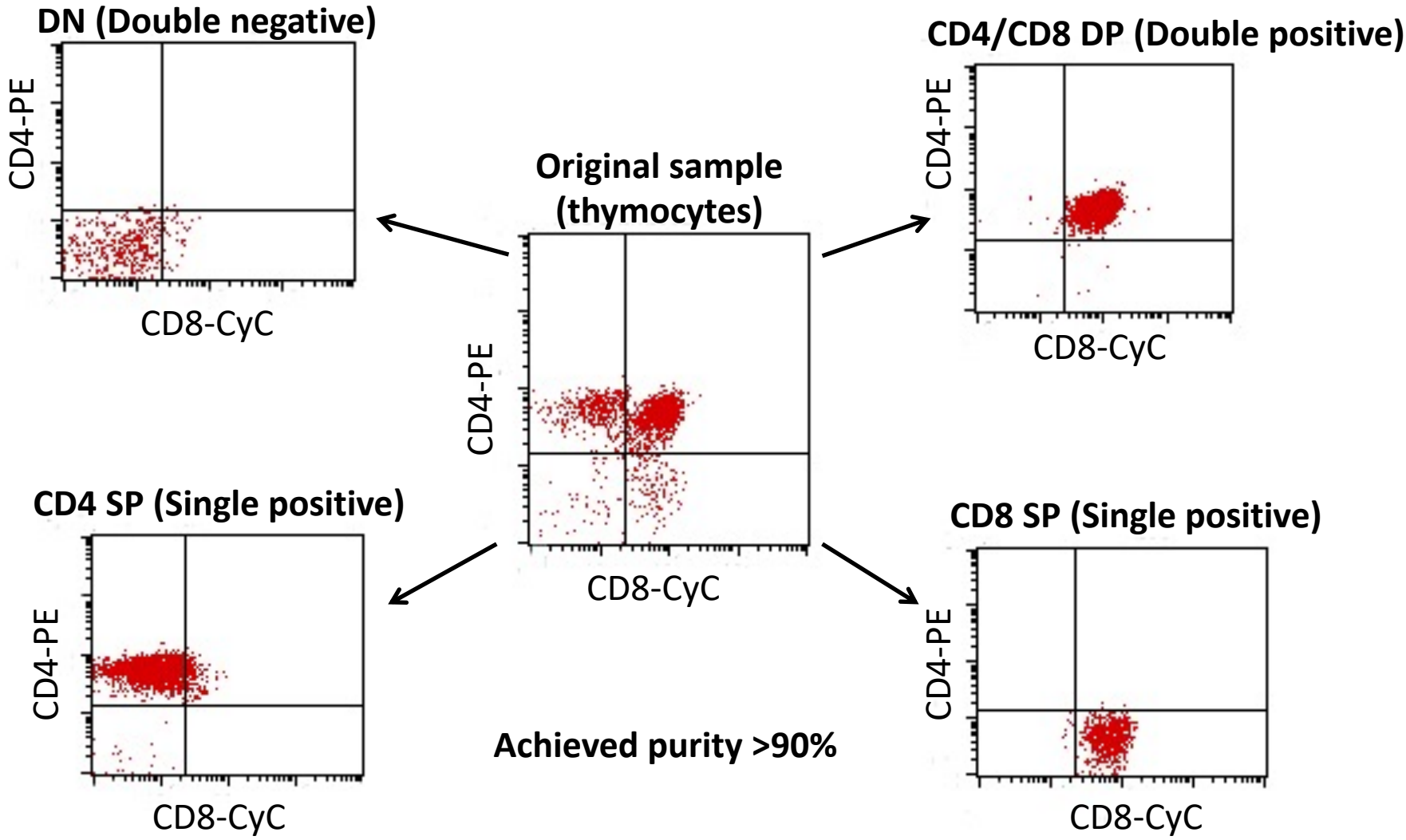
FACS: Fluorescence activated cell sorting

1. Each cell is put into a single droplet.
2. The cell will cross the laser beam, the fluorescent molecules will emit light that will reach the detectors.
3. The droplets will get electric charges based on the pre-defined parameters.
4. The charged droplets are deflected by charged deflection plates.
5. The deflected droplets containing the cells are collected by different tubes.

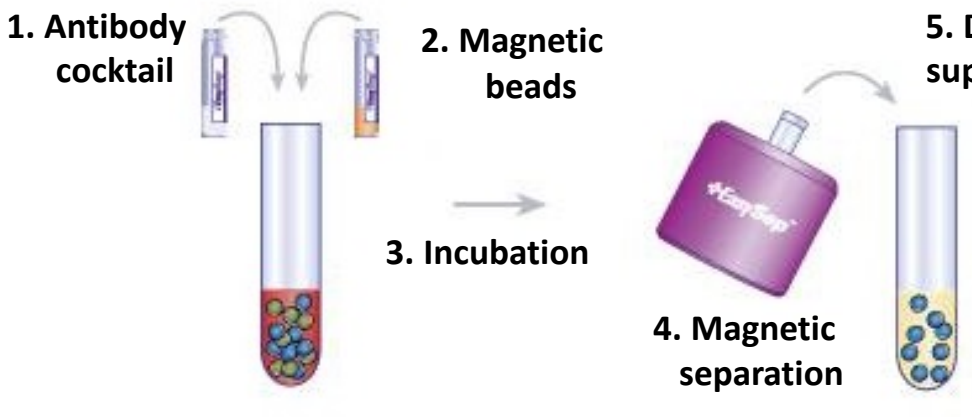


The separated cells are viable

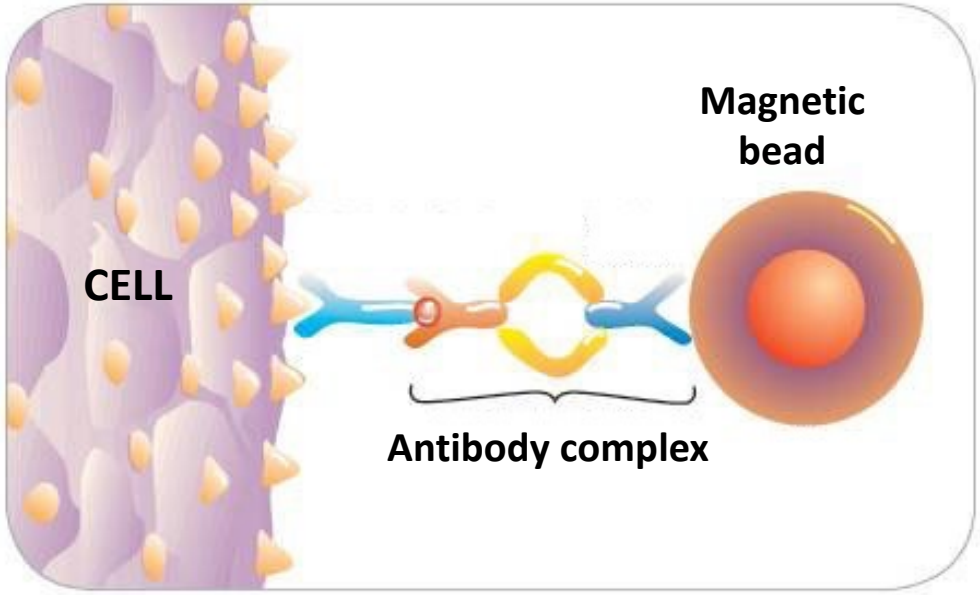
Sorting from a murine thymus



Immunomagnetic cell separation (MACS)



Positive selection: Labeled cells that attached to the wall of the tube
Negative selection: Unlabeled cells in the supernatant



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