Application of Flow Cytometry in a Clinical Laboratory: Near East University Hospital

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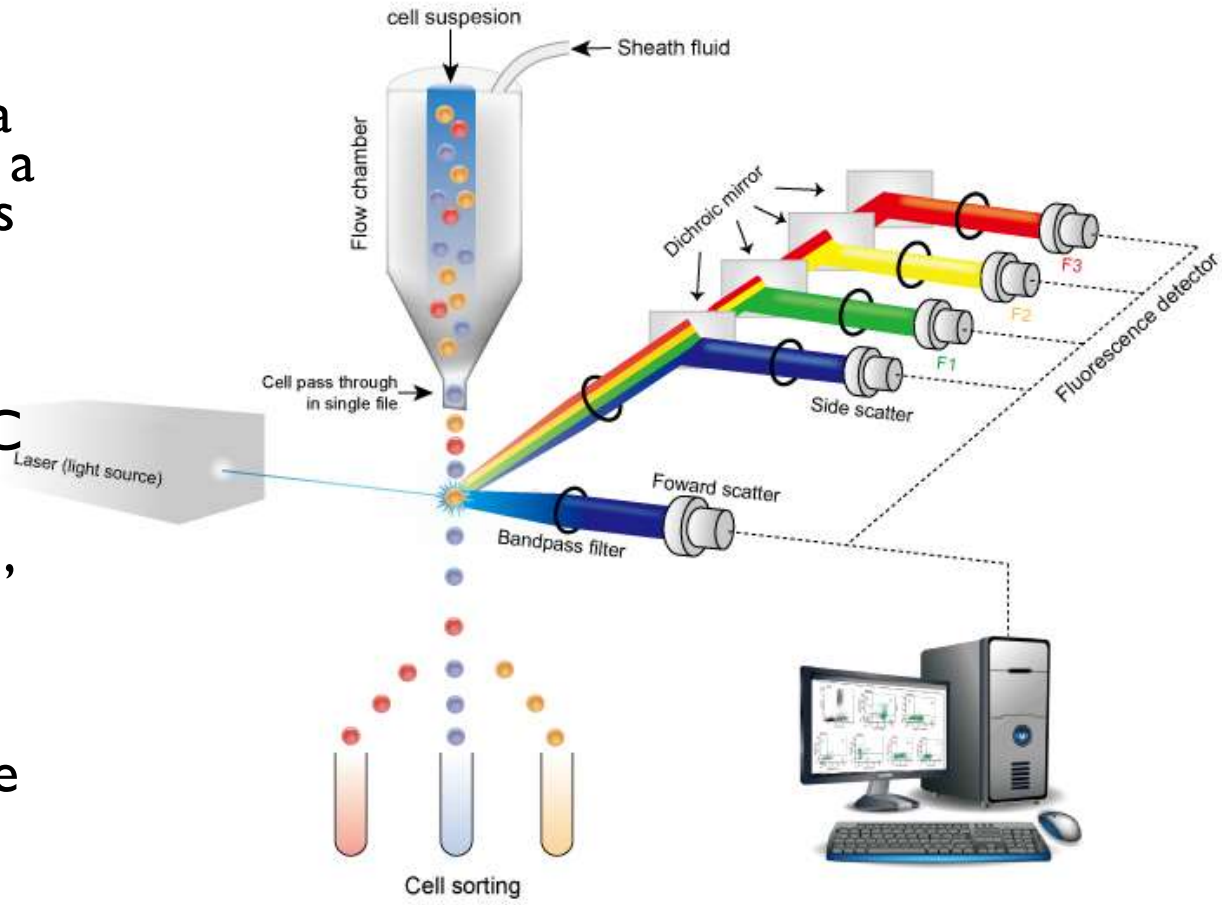
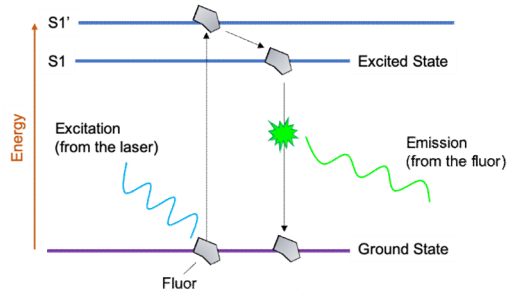
Basics of Flow Cytometry

- Flow Cytometry (FCM) is a technique, in which cells (or events) in a suspension "flow" in a fluid while intercepting a beam of a light source (laser), and the signals produced by this process are collected in "channels", and digitalized by computer.
- Forward Scatter, Side Scatter (FSC & SSC)
- Fluorescent Channels (FITC, APC, PE, etc.)
- For the fluorescent channels to receive signals, events must be marked with special fluorochrome dyes

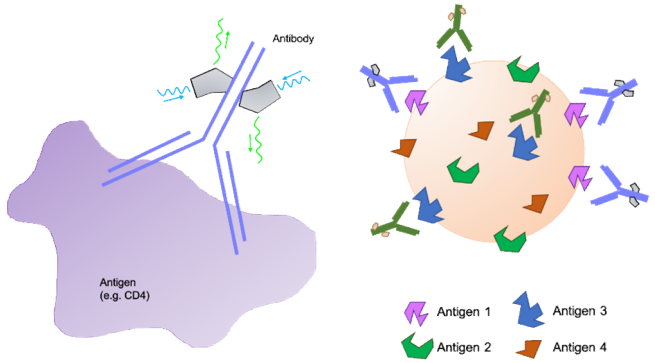


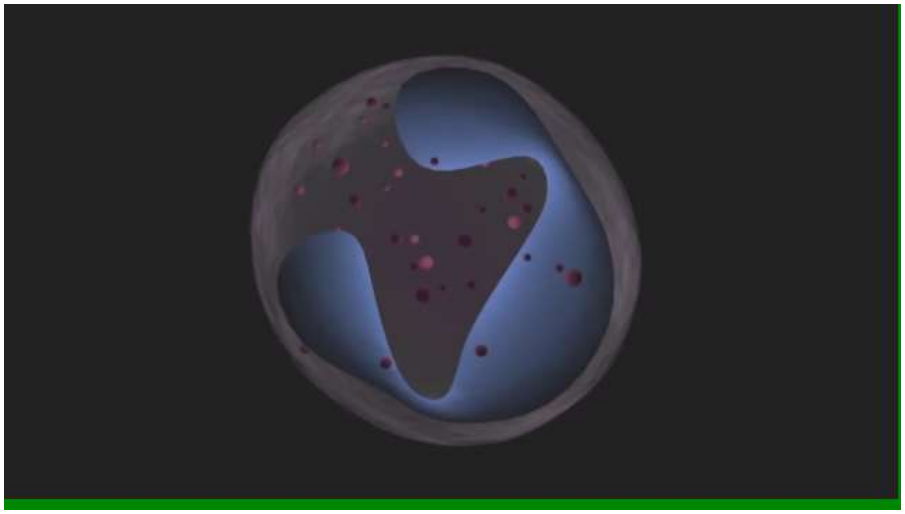
Basics of Flow Cytometry

- When the marked event passes through the laser, the fluorochrome becomes excited and emits light while returning to the ground state.
- Each fluorochrome has a specific excitation and emission wavelength.
- Antibodies specific to surface antigens or cellular components are conjugated with these fluorochromes.
- When they bind to their specific target structures in the analysis suspension, the event becomes marked.



(C) Marissa Fahlberg, PhD

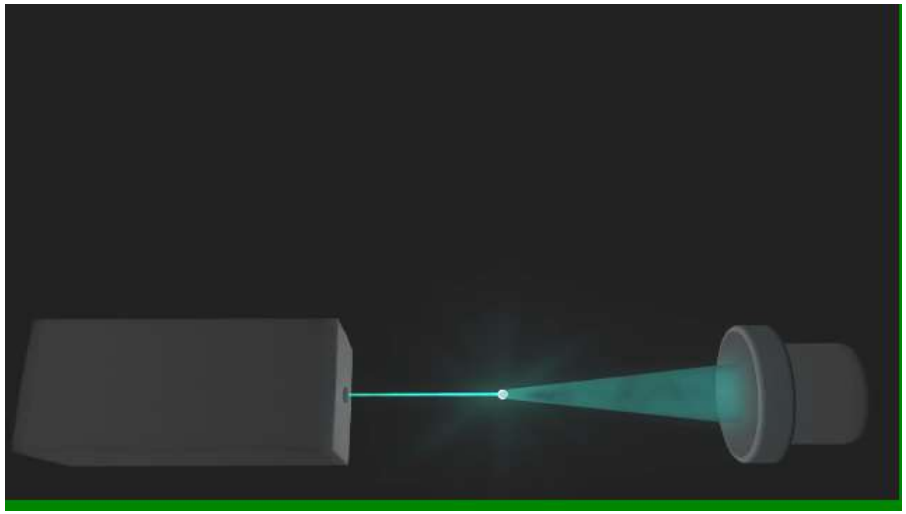




(C) Starcell Bioanimations

Basics of Flow Cytometry

- The expression of target molecules (i.e. antigens, eg. CD4 on the surface of a T lymphocyte) varies in each event
- This mixture of antibodies, conjugated with distinct fluorochromes, and events allows us to identify several different antigens, based on the emission spectrums of these fluorochromes



Clinical Applications of FCM

- Hematology
  - Immunophenotyping of leukemia
  - Analysis of cell cycle and DNA content
  - Hematological stem cell counting
  - Detection of cell viability and apoptosis studies
  - Evaluation of multiple drug resistance
  - Investigating the cellular immune response
  - Thrombocyte studies
- Immunology
  - Analysis of Lymphocyte subsets
  - CD4/CD8 ratio
- Microbiology
  - Molecular studies
  - Identification of surface molecules
  - Separation of Bacteria
  - Etc.
- Complete Blood Count
- Urinalysis
- …

Clinical Applications of FCM

- Immunophenotyping
  - Identification of hematological malignancies and cell typing
  - The term "cluster of differentiation" (CD) is used to define the cell surface antigens
  - Each antigen may be expressed in different quantities at different stages of maturation and this helps us understand, detect, and identify the malignant cell population
  - To evaluate and obtain comparable results in the FCM analysis, the study protocol and the data-obtaining format must be standardized
  - The most common way is expressing the data in %, and some other techniques allow quantifying cell populations by utilization of standard materials

Clinical Applications of FCM

- Hematopoietic stem cell (HSC) counting
  - CD34+ cells are counted
  - It is also possible to count subtypes of HSCs
- Cell viability
  - To detect viable cells, the DNA of the untreated cells is stained by fluorochromes, like 7AAD. The membrane permeability will be disrupted in dead cells and the dye will reach the DNA. However, the viable cells will not be stained because the dye wouldn`t enter the cell.

Analysis of Lymphocyte Subsets with FCM

- It is the most common routine FCM analysis we perform in our laboratory (more than 70 cases in the last 18 months)
- This FCM procedure is used to determine the percentages and absolute counts of the mature human lymphocyte subsets in peripheral whole blood
- It is useful for the immunological assessment of normal individuals, and also patients with suspected or known immune deficiencies

Analysis of Lymphocyte Subsets with FCM

- Human lymphocytes are divided into three major populations according to their biological functions and cell surface antigen expressions:
  - T lymphocytes (CD3)
    - T-helper cells: CD4
    - T-cytotoxic cells:CD8
  - B lymphocytes (CD19 or CD20)
  - NK cells (CD16 or CD56 or CD57, expression varies)

Analysis of Lymphocyte Subsets with FCM

- Principles of the Analysis
  - The reagents are mixed with whole blood following a specifically designed procedure.
  - The BD FacsCalibur FCM analyzer is a six-parameter, 4-color device
    - FSC
    - SSC
    - FL1 – Green emission (filter: 530/30)
    - FL2 – Red/Orange emission (filter: 585/42)
    - FL3 – Red emission (filter: 670 LP)
    - FL4 – Far Red emission (filter: APC – 661/16 nm)

Fluorochrome	Fluorescence Emission Color
BD Horizon™ V450	Blue
Pacific Blue™	Blue
BD Horizon™ V500	Green
AmCyan	Green
Alexa Fluor® 488	Green
FITC	Green
PE	Yellow
PE-Texas Red®	Orange
Texas Red®	Orange
APC†	Red
Alexa Fluor® 647	Red
PE-Cy™5†	Red
PerCP	Red
PerCP-Cy™5.5	Far Red
Alexa Fluor® 700	Far Red
PE-Cy™7	Infrared
APC-Cy7	Infrared
BD APC-H7	Infrared

Analysis of Lymphocyte Subsets with FCM

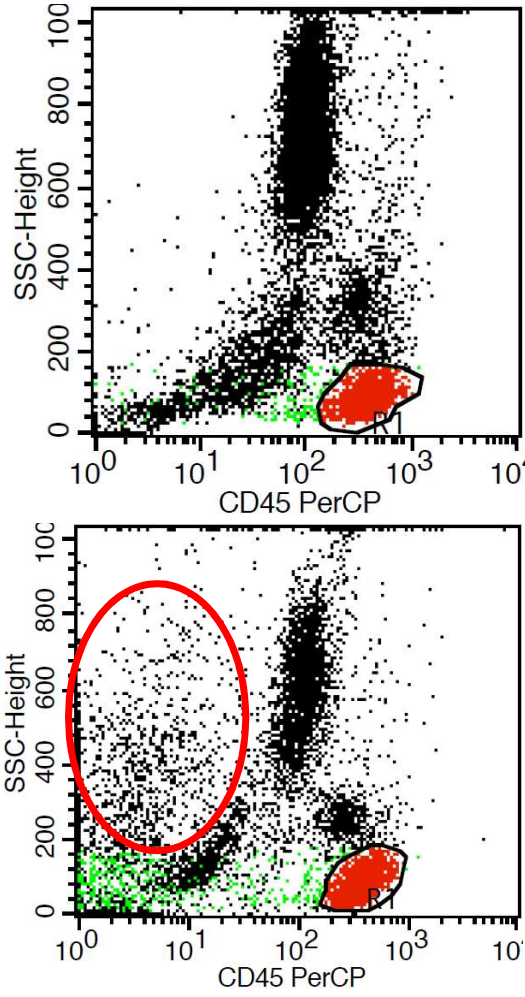
- We prepare three PPE test tubes (labeled 1, 2, and 3) before the analysis
  - 1st tube: CD45, CD3, CD19, CD16/56
  - 2nd tube: CD45, CD3, CD4, CD8
  - 3rd tube: CD3, HLA-DR, CD45 (optional)
- Always keep your fluorochrome antibodies away from direct light and perform the FCM studies in a dark room under dim and indirect illumination (they are excited by the light, so they may lose their activity)

Analysis of Lymphocyte Subsets with FCM

- The fluorochrome-labeled antibodies in the reagent bind specifically to leucocyte surface antigens in the whole blood.
- Pipette 10 µL of the desired fluorochrome in each tube
  - Tip 1: The official amount is 20 µL, but in most cases half of the officially stated amount of fluorochrome will be enough for the experiment. Perform a preliminary experiment first to determine the adequate amount of dye.
  - Tip 2: Always use a well-calibrated automatic pipette, and always pipette the fluorochrome dye first. Otherwise, you may be confused if you pipetted the fluorochrome or not!
- Pipette 50 µL of whole blood, cap the tubes and gently mix
- Incubate in the dark, at room temperature for 20 minutes

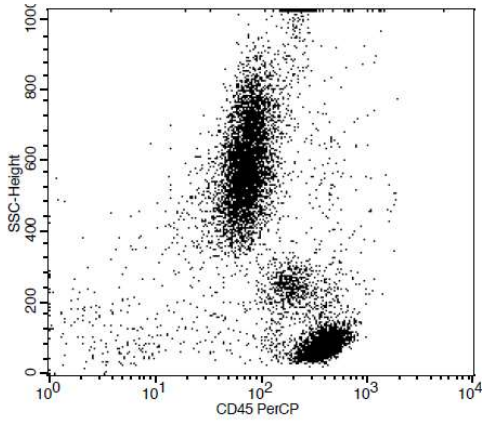
Analysis of Lymphocyte Subsets with FCM

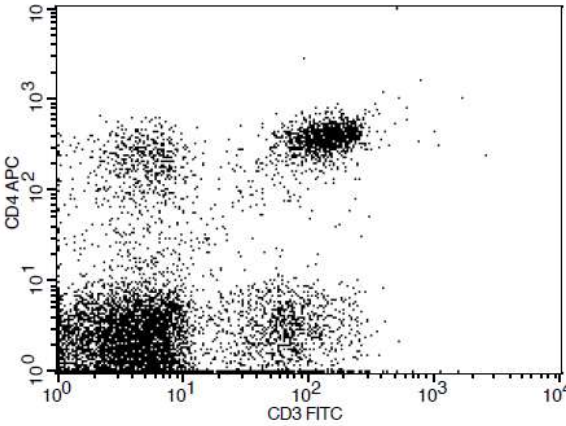
- Treat the stained samples with the lyse solution to remove the erythrocytes
  - We don`t want them to interfere with the analysis
- Centrifuge and wash the tubes
  - A proper washing procedure helps us get rid of the "debris", i.e. the unwanted events.
- Fill the tubes with an appropriate buffer solution, and load into the cytometer
- All the channels receive the signals generated by these events that pass through the interrogation point, and the data is converted to digital format and saved

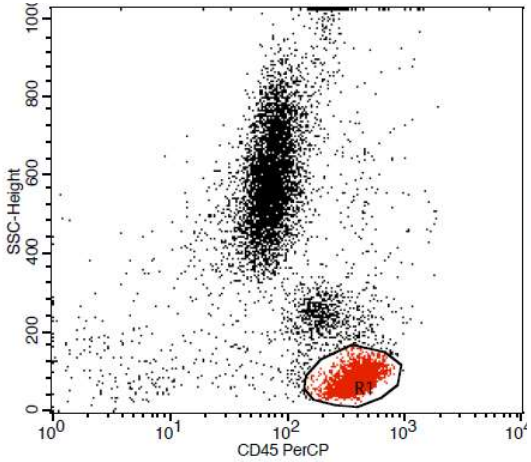


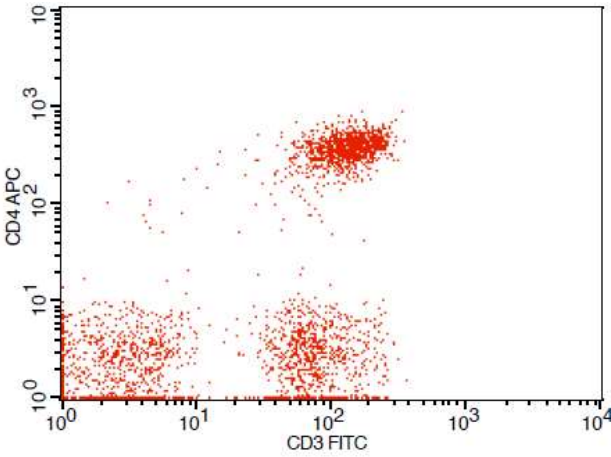
Analysis of Lymphocyte Subsets with FCM

- Tip: Don't increase the reading speed above 1200 events per second
- The readings are further examined and evaluated using specialized software
- Gating:
  - Selecting a group of events based on one of their common attributes and continuing the analysis on these selected events.



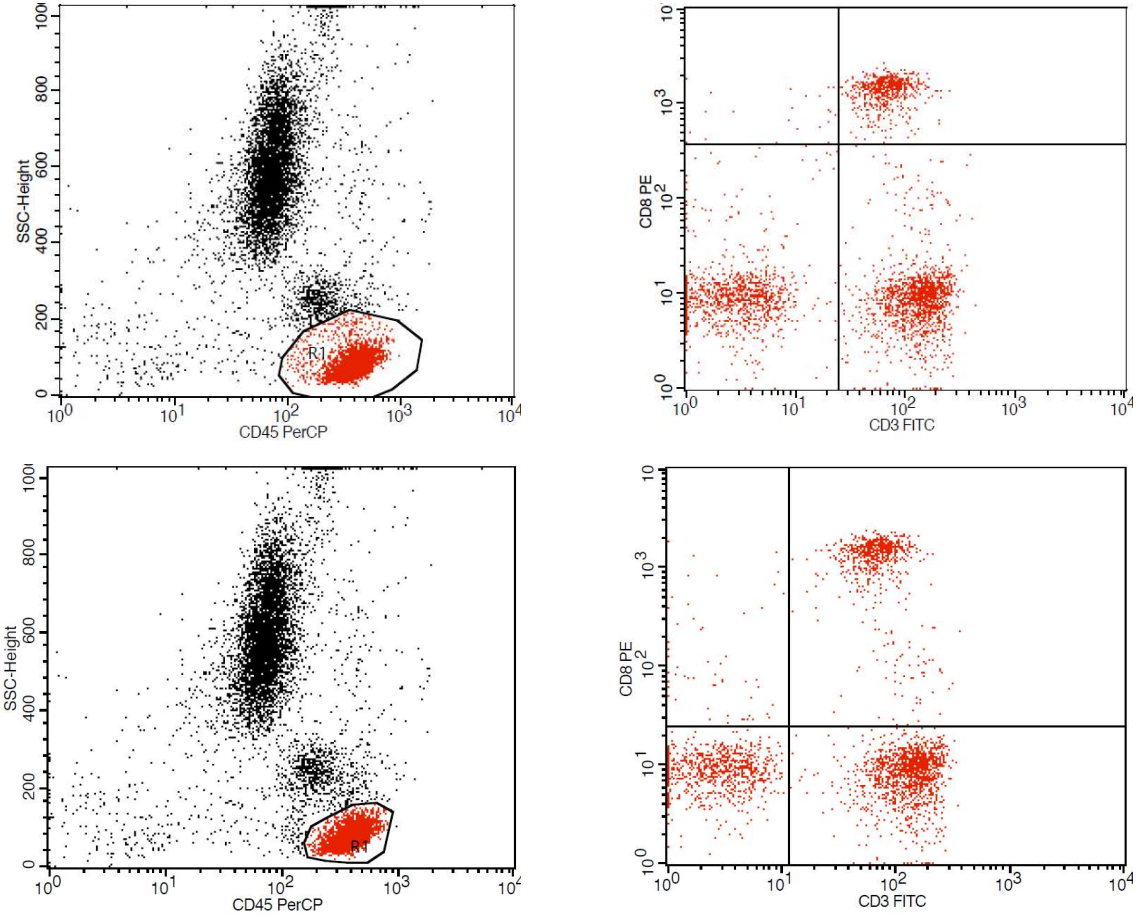




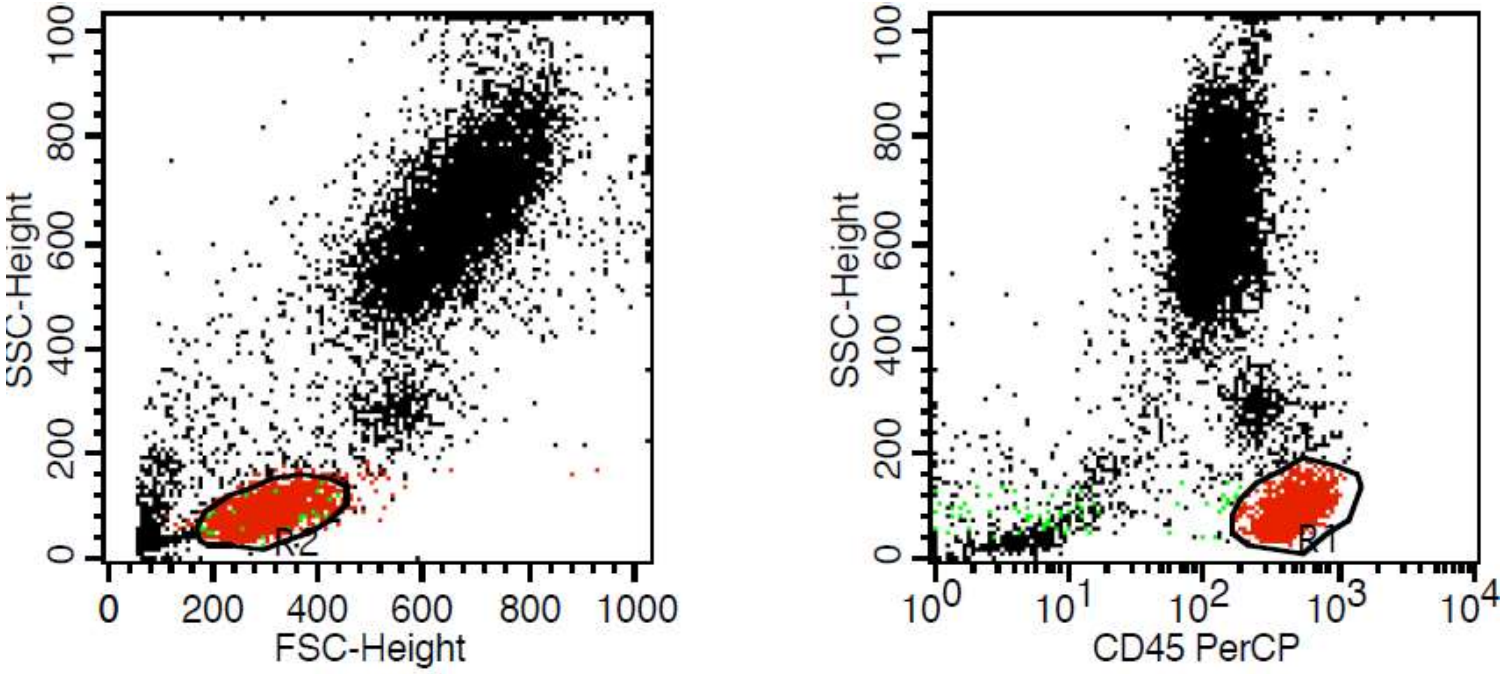


Analysis of Lymphocyte Subsets with FCM

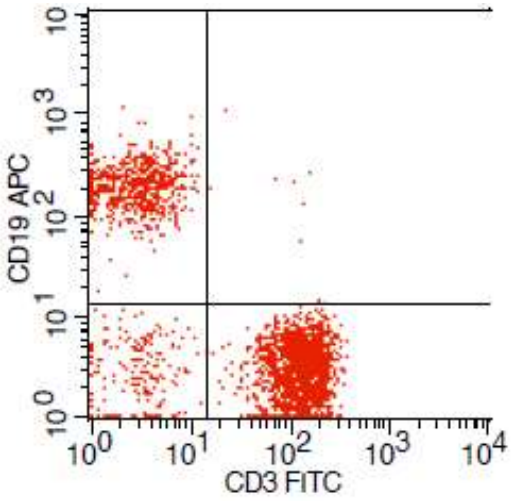
- How to distinguish a negative population from the positive one?
  - Draw the line or take the gate just after the negative population



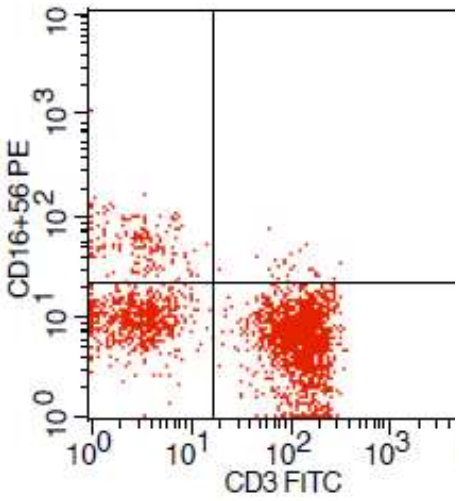
A normal case…



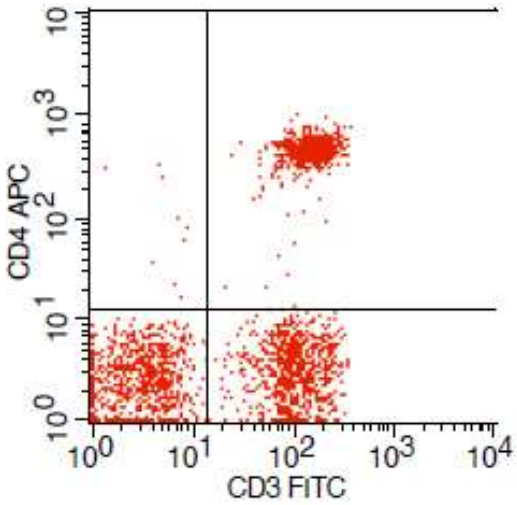
A normal case…



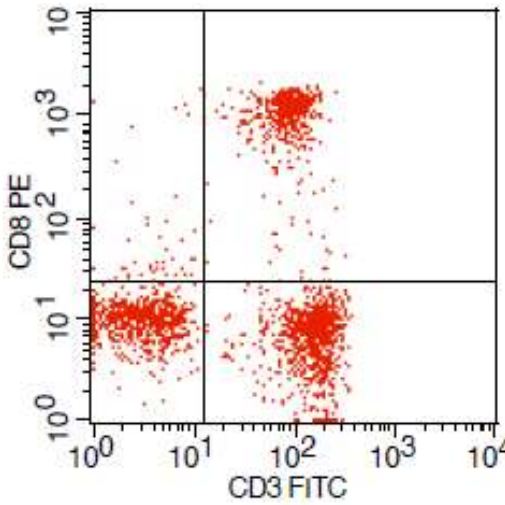
Quad	Events	% Gated	% Total
UL	602	24.83	6.02
UR	10	0.41	0.10
LL	181	7.47	1.81
LR	1631	67.29	16.31



Quad	Events	% Gated	% Tot
UL	168	6.93	1.6
UR	23	0.95	0.2
LL	617	25.45	6.1
LR	1616	66.67	16.1

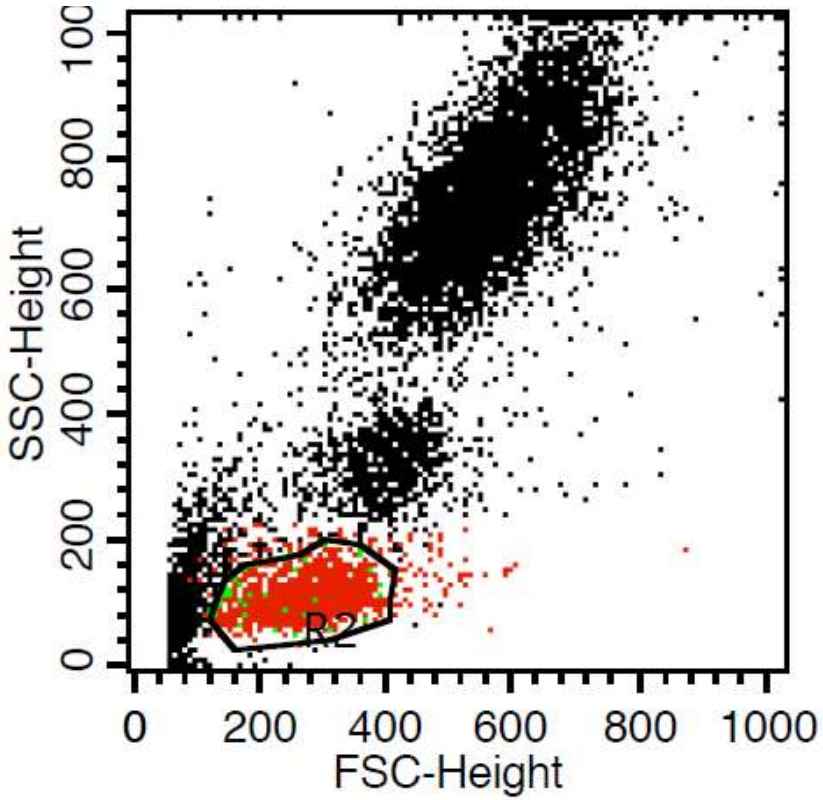


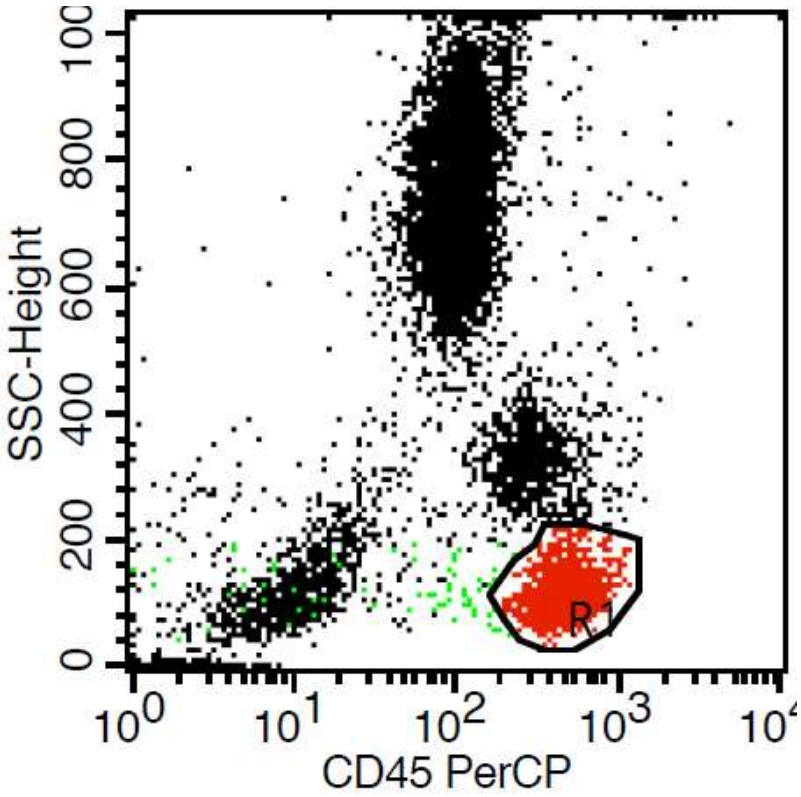
Quad	Events	% Gated	% Total
UL	9	0.40	0.09
UR	688	30.43	6.88
LL	760	33.61	7.60
LR	804	35.56	8.04



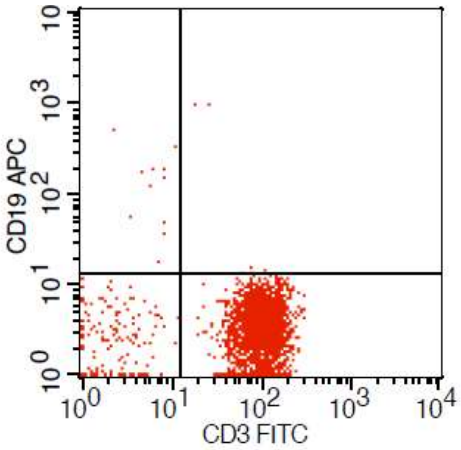
Quad	Events	% Gated	% Total
UL	46	2.03	0.46
UR	581	25.70	5.81
LL	719	31.80	7.19
LR	915	40.47	9.15

Case-1

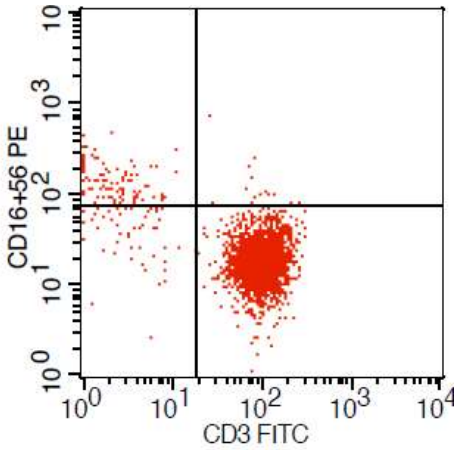

SSC-Height
0
200
400
600
800
1000
0
200
400
600
800
1000
FSC-Height
R2


SSC-Height
0
200
400
600
800
1000
10^0
10^1
10^2
10^3
10^4
CD45 PerCP
R1

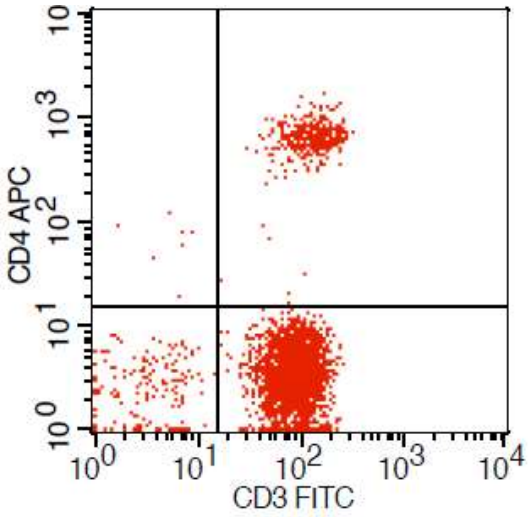
Case-1



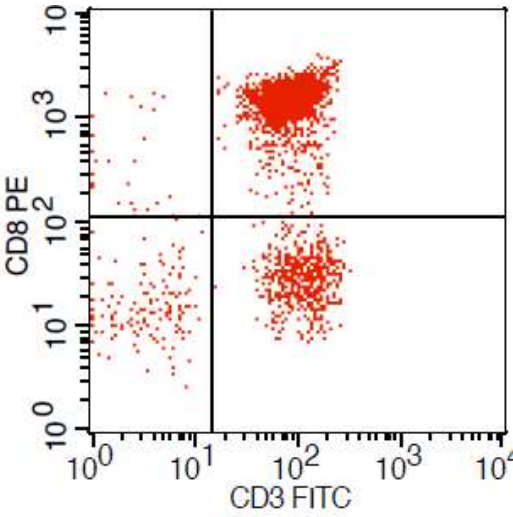
Quad	Events	% Gated	% Total
UL	11	0.43	0.11
UR	5	0.20	0.05
LL	136	5.31	1.36
LR	2408	94.06	24.08



Quad	Events	% Gated	% Total
UL	94	3.67	0.94
UR	16	0.62	0.16
LL	53	2.07	0.53
LR	2397	93.63	23.97

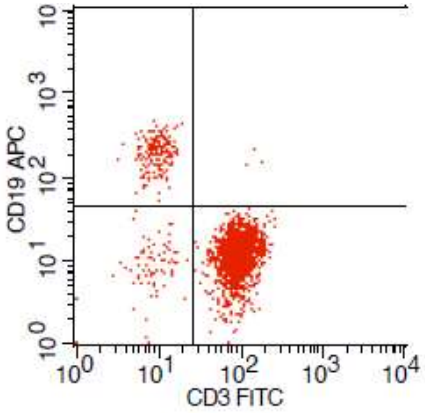


Quad	Events	% Gated	% Total
UL	7	0.24	0.07
UR	360	12.48	3.60
LL	152	5.27	1.52
LR	2365	82.00	23.65

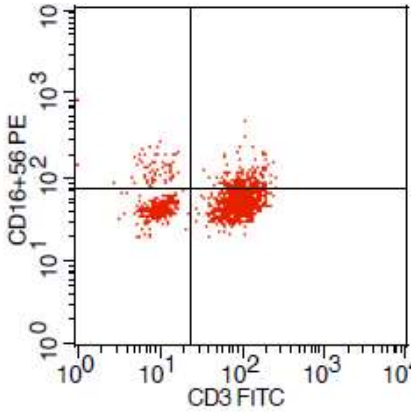


Quad	Events	% Gated	% Total
UL	27	0.94	0.27
UR	2242	77.74	22.42
LL	131	4.54	1.31
LR	484	16.78	4.84

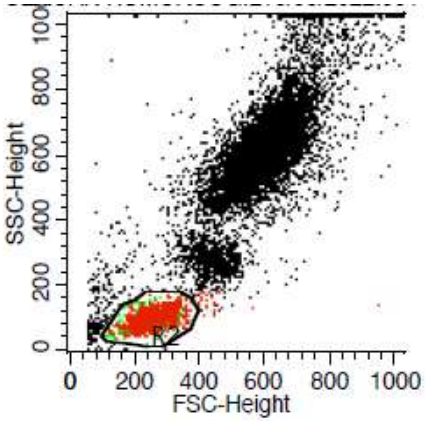
Case-2

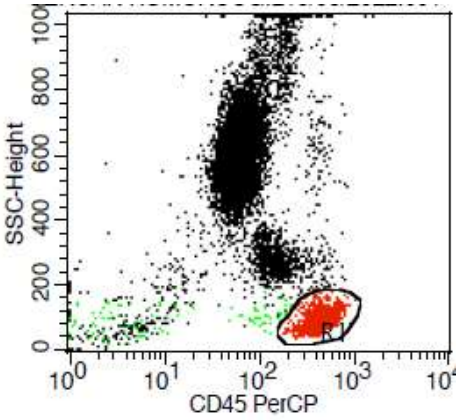


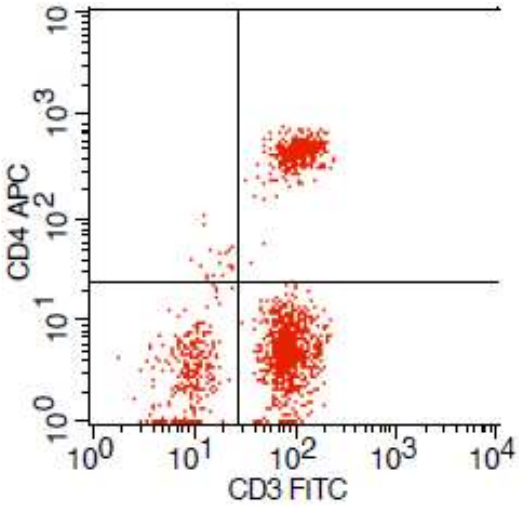
Quad	Events	% Gated	% Total
UL	198	12.51	1.98
UR	3	0.19	0.03
LL	73	4.61	0.73
LR	1309	82.69	13.09



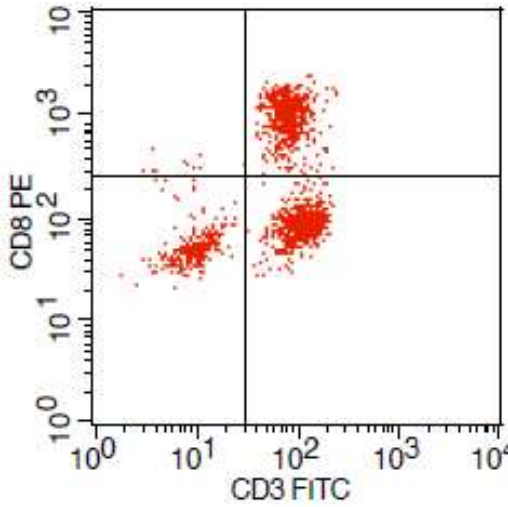
Quad	Events	% Gated	% Total
UL	60	3.79	0.60
UR	202	12.76	2.02
LL	211	13.33	2.11
LR	1110	70.12	11.10







Quad	Events	% Gated	% Total
UL	18	1.25	0.18
UR	415	28.84	4.15
LL	257	17.86	2.57
LR	749	52.05	7.49



Quad	Events	% Gated	% Total
UL	10	0.69	0.10
UR	598	41.56	5.98
LL	266	18.49	2.66
LR	565	39.26	5.65

Future of FCM in Near East University Hospital

- Implementation of new panels
  - Multiple Myeloma
  - PNH
  - Chronic lymphocytic leukemia
- Taking part in doctoral and post-doctoral studies