



# Flow Cytometry in the Evaluation of Hematopoiesis and Hematological Neoplasms

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CAP-ACP Resident Review Course  
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# Disclosures



I HAVE NO CONFLICTS OF  
INTEREST OR FINANCIAL  
DISCLOSURES



NO OFF-LABEL OR  
INVESTIGATIONAL PRODUCT  
USE WILL BE DISCUSSED

# Objectives

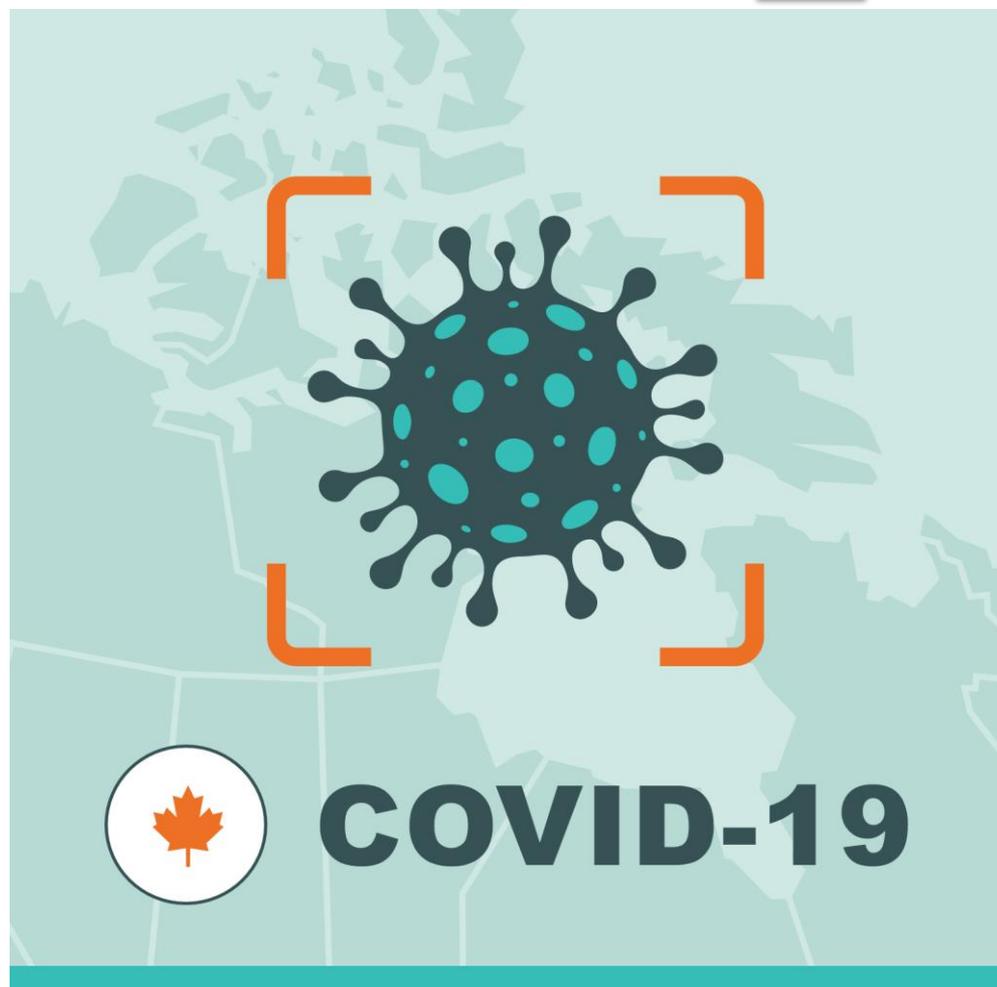
- ▶ Be Prepared!

Use first principles to approach problems

- ▶ Integrate all of the evidence/features

- ▶ Keep pattern recognition/common immunophenotypes in your back pocket

- ▶ We're in this together!



# Outline

## How flow cytometry works

ICCS-ICSH Guidelines

Technical sources of error and pitfalls to watch for!

## Immunophenotyping of hematopoietic populations

Normal hematopoiesis and maturation pathways

Classical immunophenotypes in malignant hematology

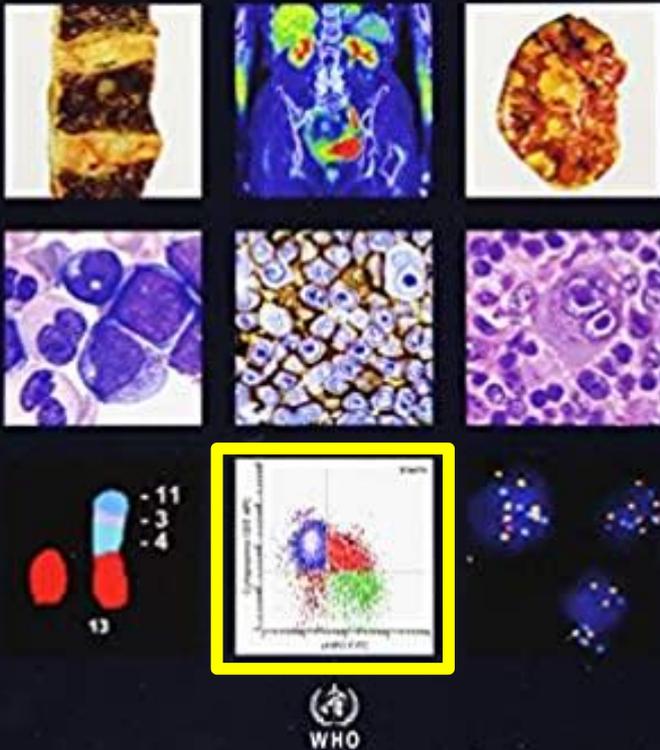
Considerations for high-sensitivity and minimal residual disease detection

?

# When morphology only goes so far...

## WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues

Steven H. Swerdlow, Elias Campo, Nancy Lee Harris, Elaine S. Jaffe, Stefano A. Pileri, Harald Stein, Jürgen Thiele, Daniel A. Arber, Robert P. Hasserjian, Michelle M. Le Beau, Attilio Orzi, Reiner Siebert



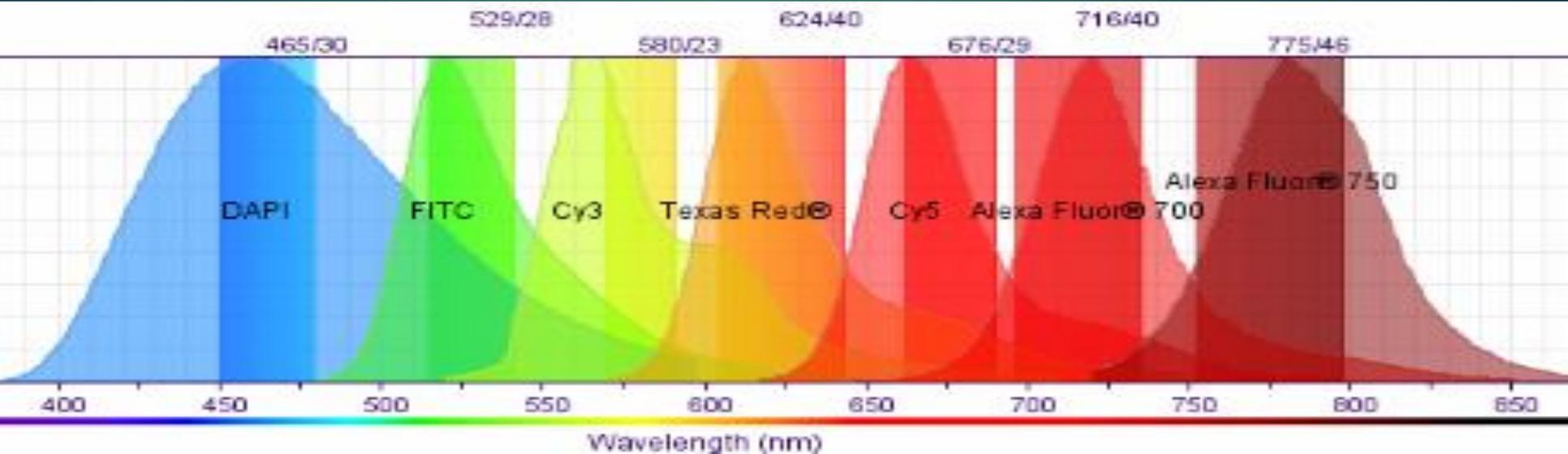
## An integrated approach:

- ▶ Blood and Aspirate cytology
  - ▶ **Flow Cytometry**
  - ▶ Molecular and Cytogenetics
- ▶ Trepine histology
  - ▶ Immunohistochemistry

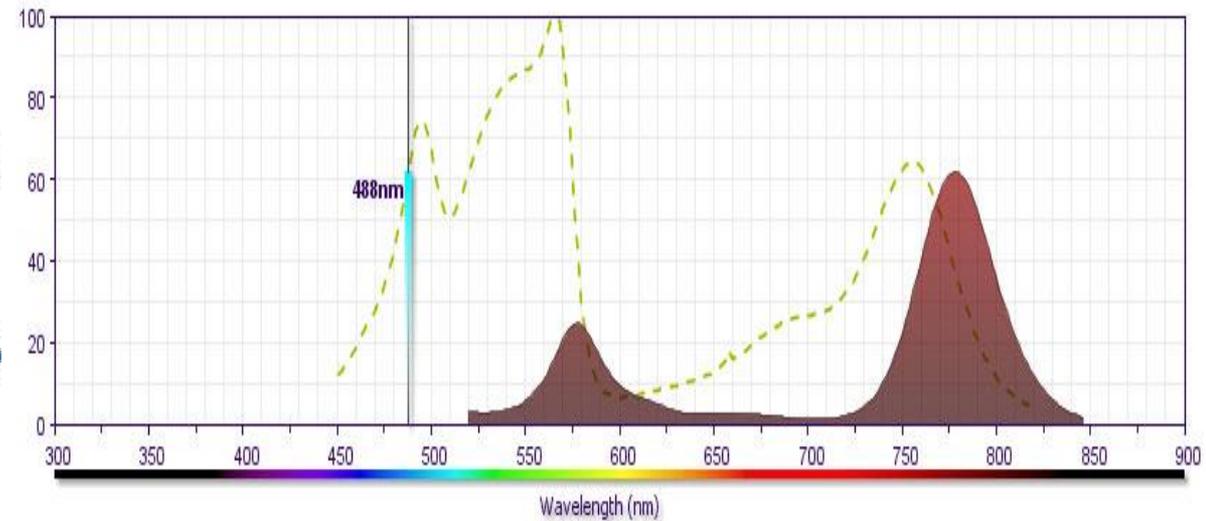
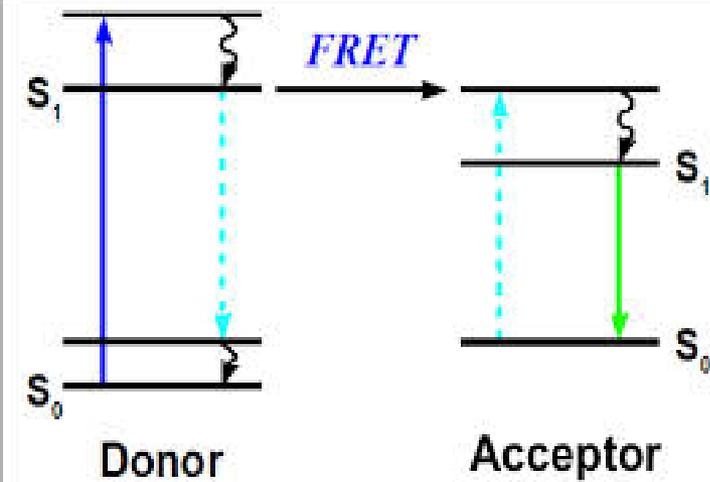
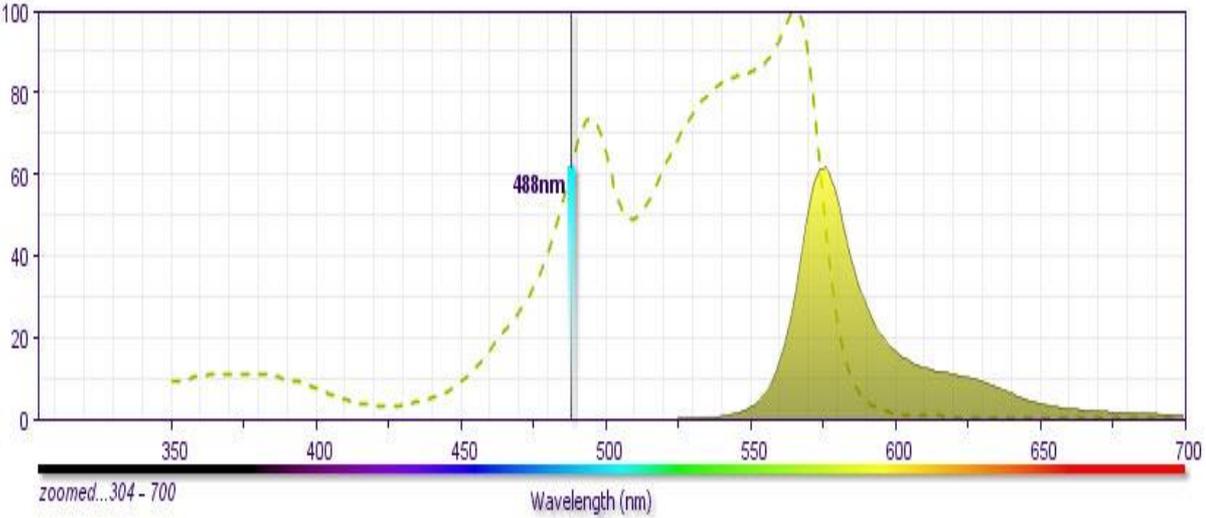
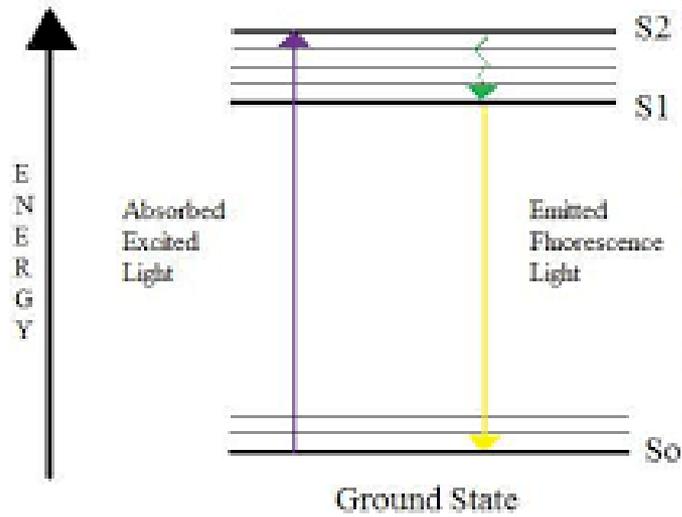
# Cytometry

## ▶ Cytometry: measuring cells

- ▶ FSC/FALS: forward scatter of light – reflective of cell size
- ▶ SSC/RALS: side scatter of light – reflective of complexity
- ▶ Fluorophore-conjugated antibodies/dyes/substrates (Fluorescence Cytometry)
- ▶ Metal ion-conjugated antibodies, dyes (Mass Cytometry)

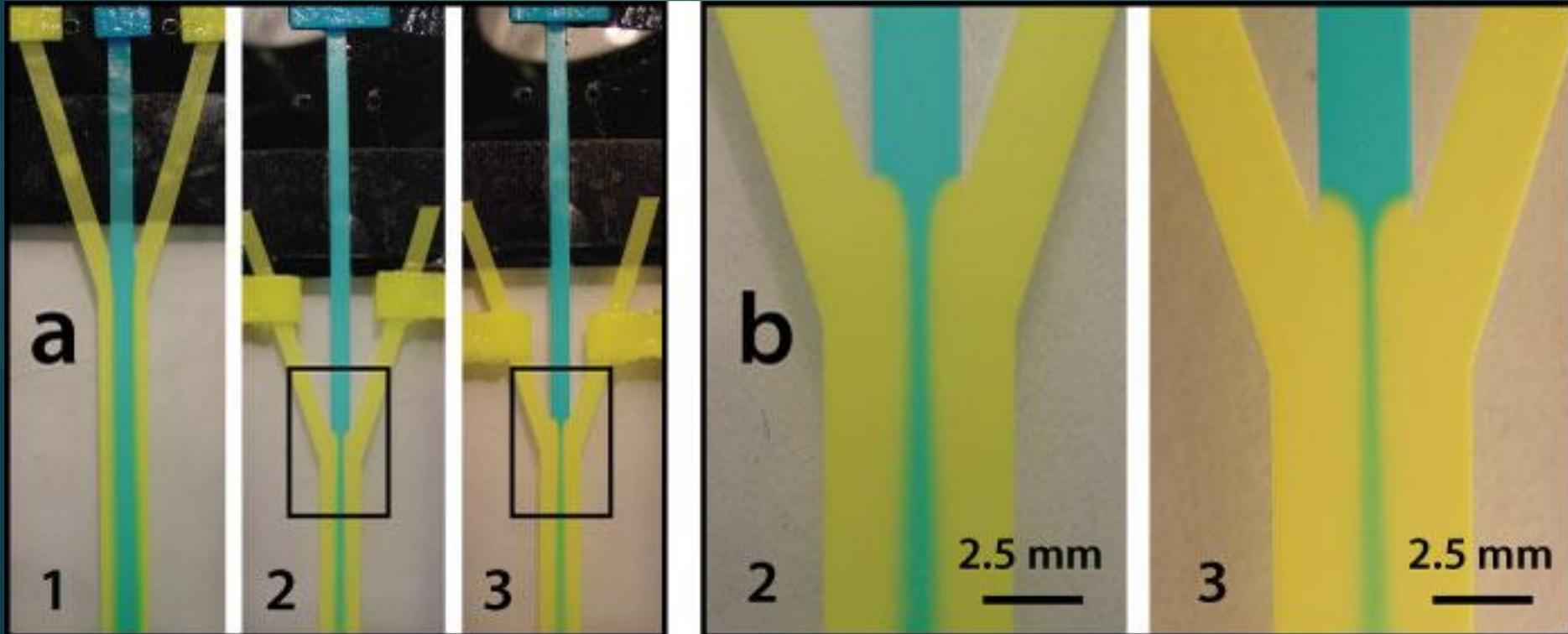


# Fluorescence

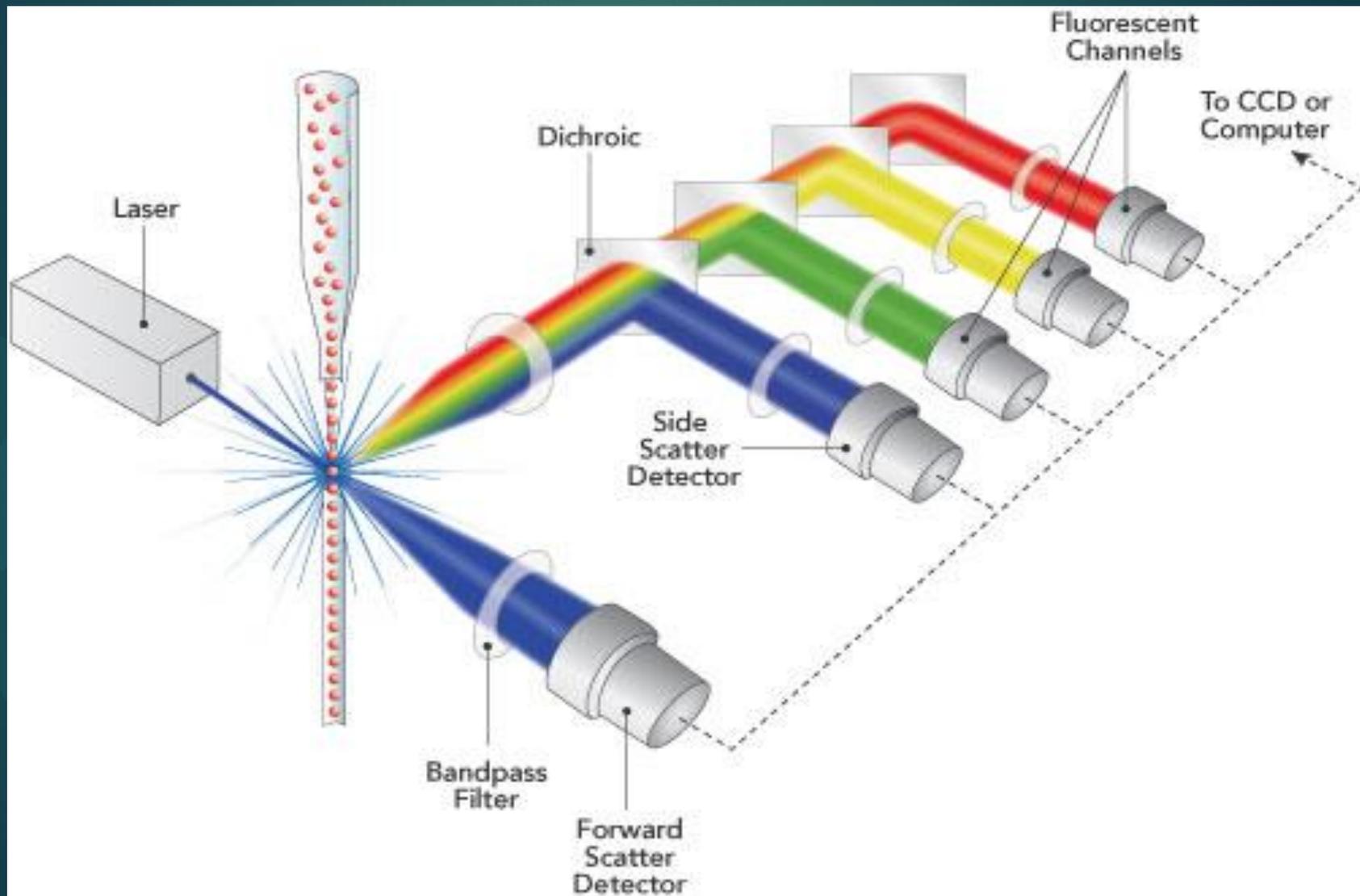


# Flow Cytometry

- ▶ Cells are analyzed in liquid phase using hydrodynamic focusing

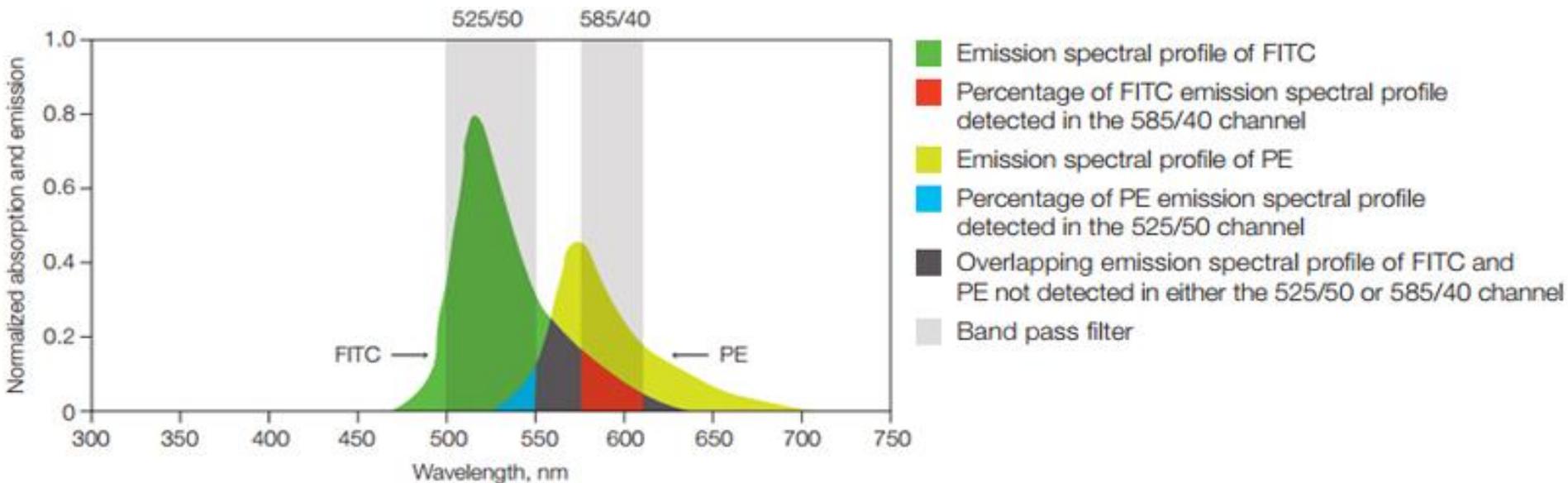


# Seeing the light



# Light is a bit messy...

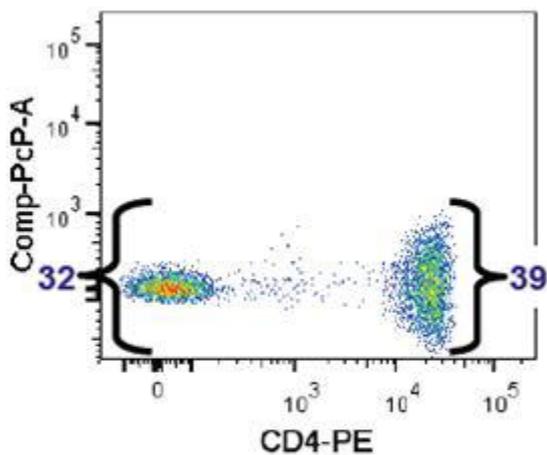
- ▶ Using dichroic mirrors and bandpass filters allows use to try to isolate the specific signal from each fluorophore
  - ▶ Trading off sensitivity for specificity
  - ▶ There is almost always an element of fluorescence spill



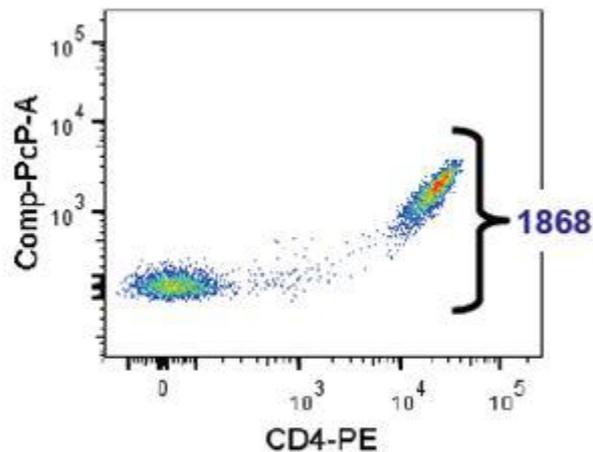
# The Goldilocks Zone

► Ideal compensation:

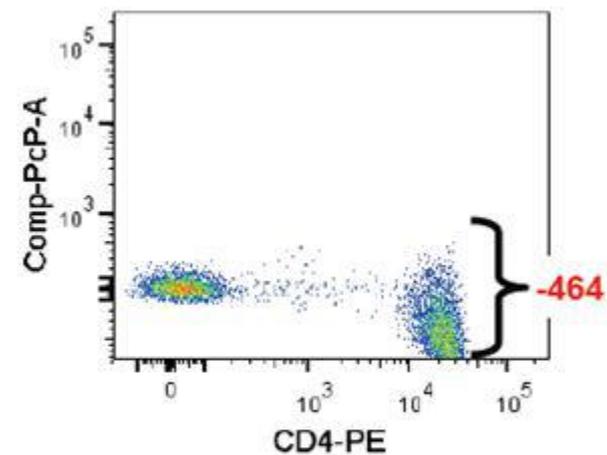
The median fluorescence intensity of a negative population should be independent of positive staining with other fluorophores



Properly compensated

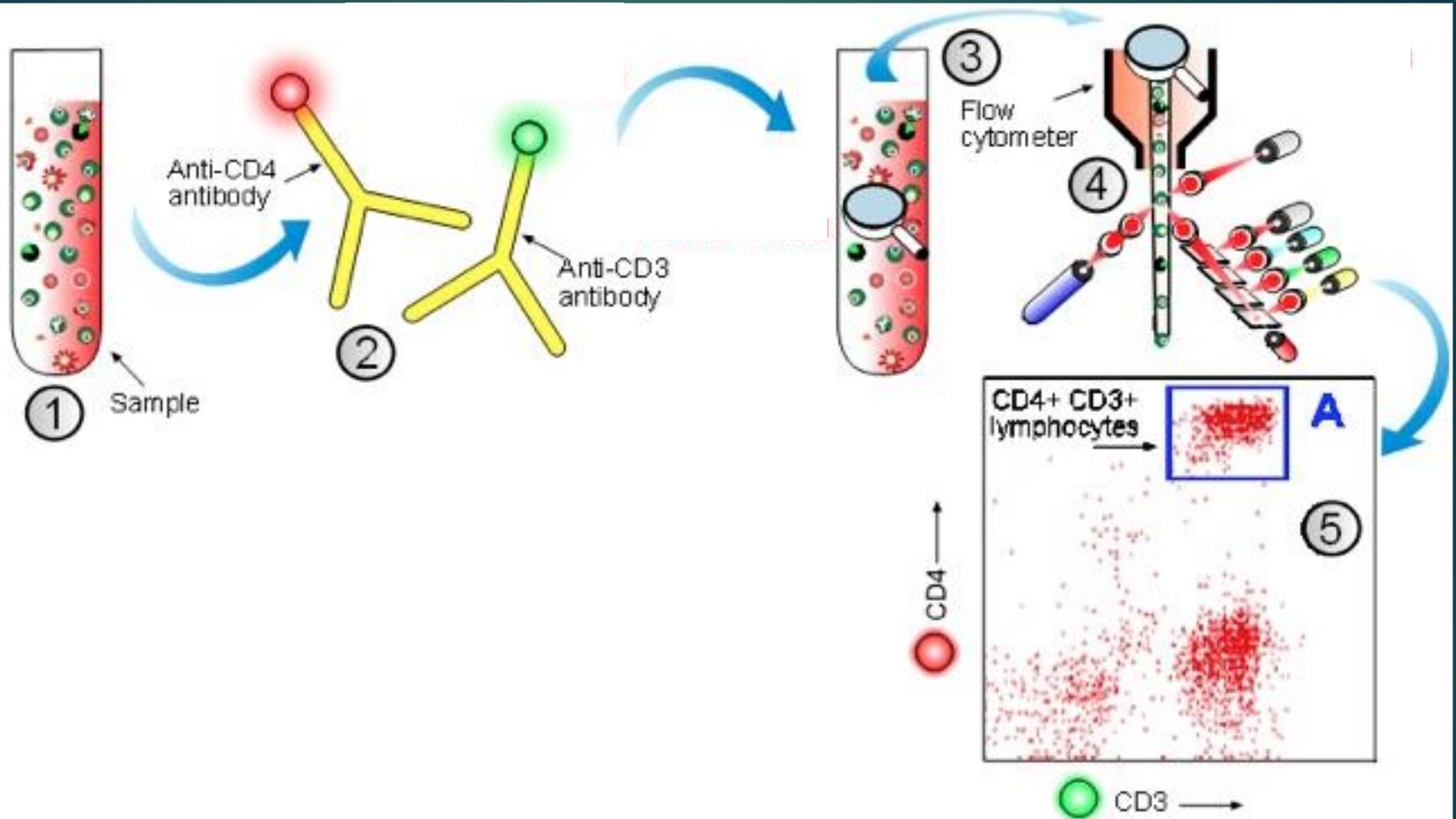


Under compensated



Over compensated

# Putting an experiment



# Analyzing Flow Data



FSC v SSC, CD45 v SSC,  
Lineage



Gating Strategies vrs  
Tragedies



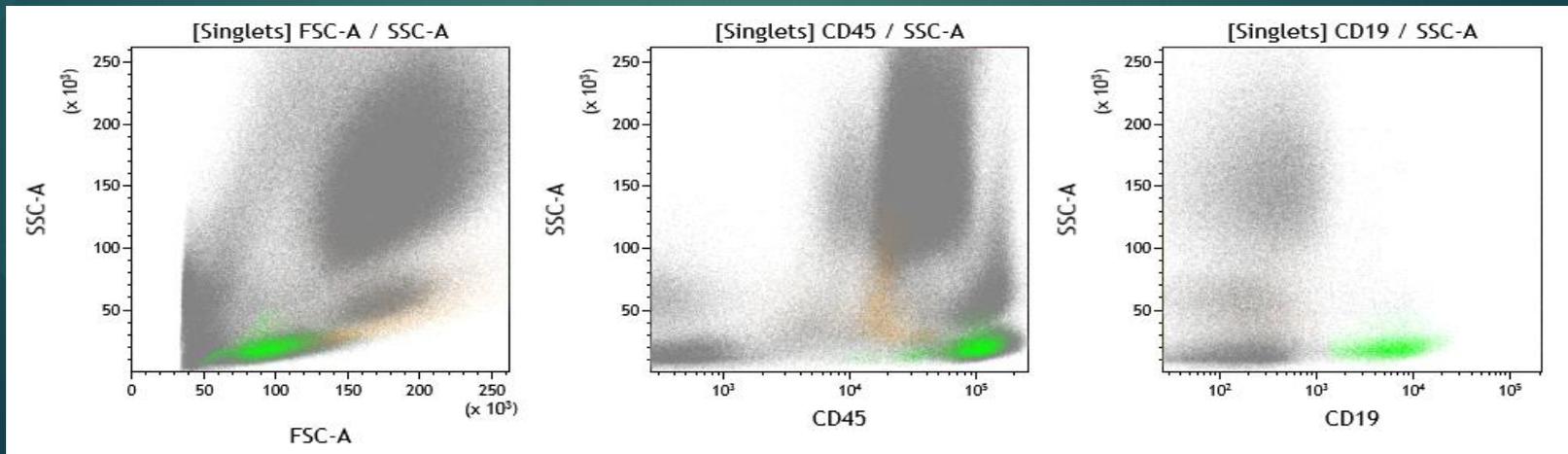
Analysis Pitfalls

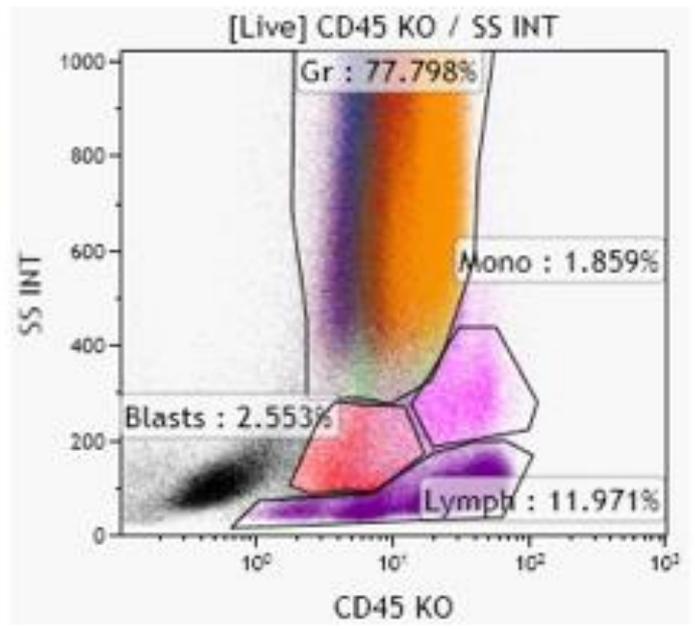
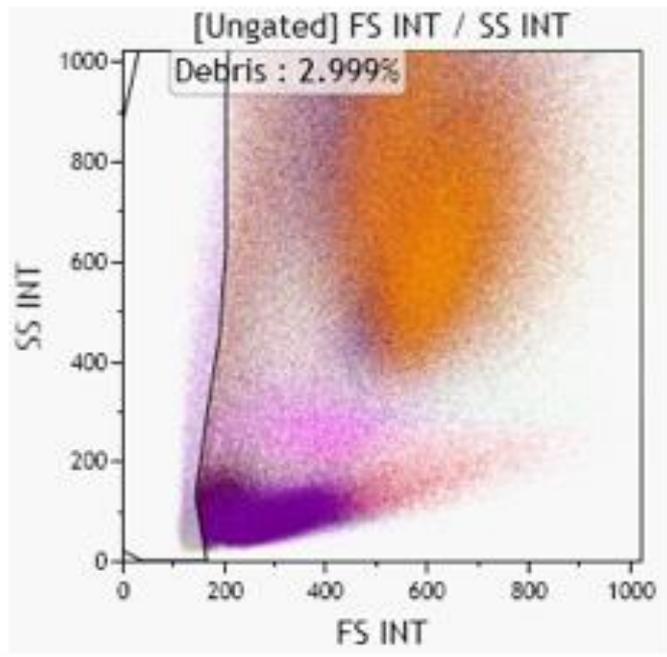


Quality and Validation

# Gating

- ▶ Gating can be used to identify, isolate, and quantitate specific populations/subsets of interest, as well as to remove debris and necrotic populations
- ▶ Classical 'initial' gating strategies include:
  - ▶ FSC ('size') x SSC (granularity/cytoplasmic 'complexity')
  - ▶ CD45 x SSC
  - ▶ Lineage marker x SSC (or CD45)



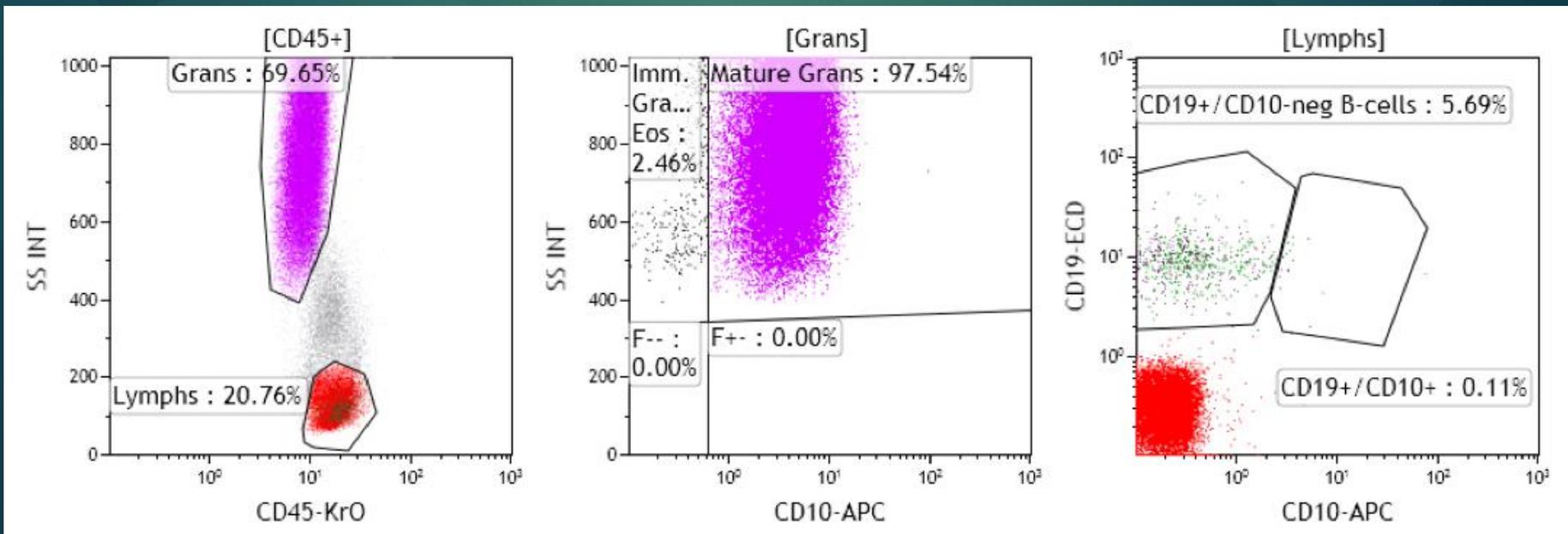


Who lives where?

# Internal Control Plots

“Check yo’ self before you wreck yo’self” – Ice Cube

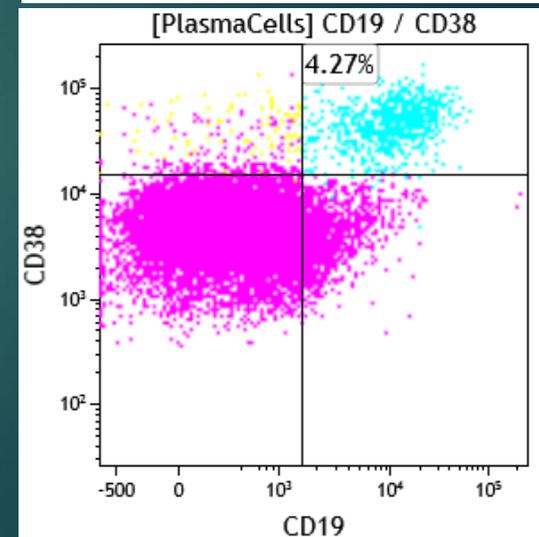
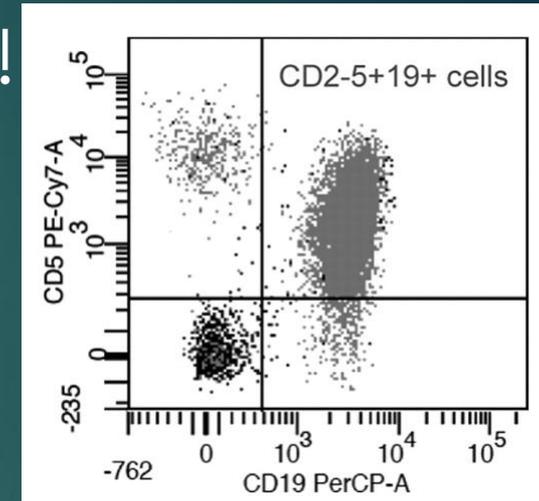
- ▶ Always use backgating and/or check plots to verify you’ve gated correctly!
- ▶ Use internal control populations (positive and negative!) to verify the performance of the tube



# Setting Gates: Why Gate There?!

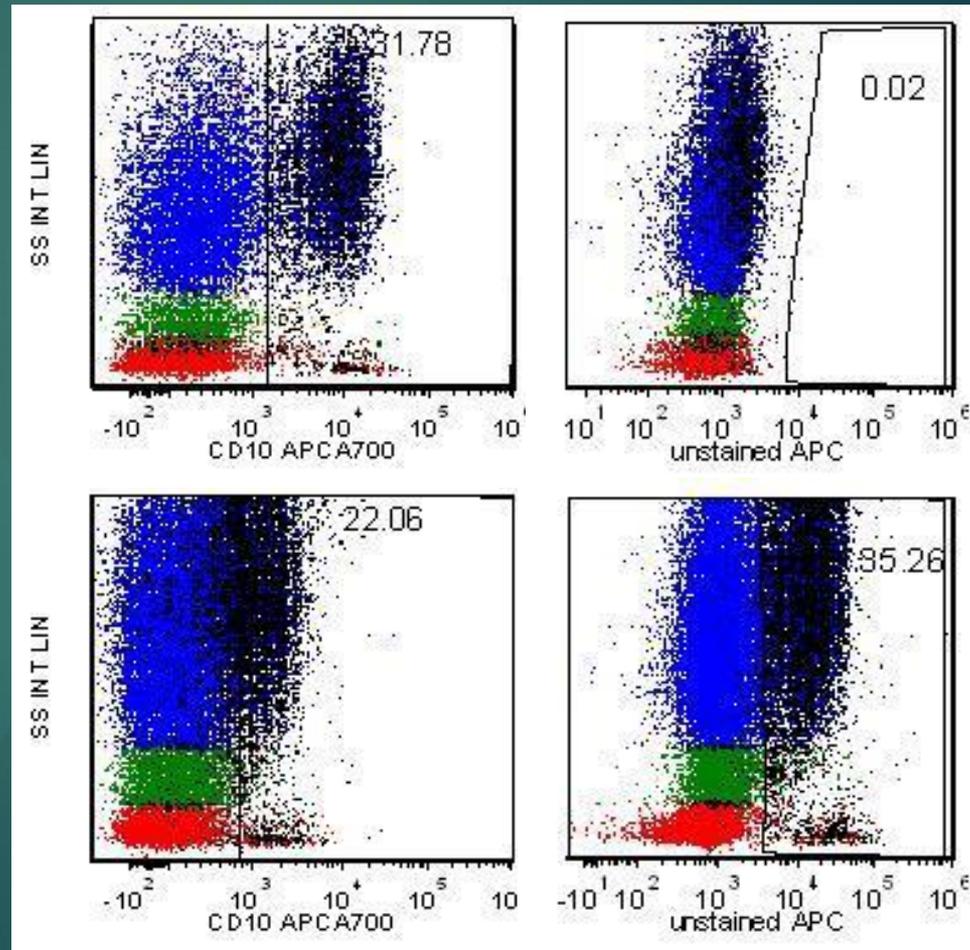
Gate positioning should be rational!

- ▶ Quadrant gating internal NEGATIVE populations
  - ▶ Emphasize abnormal expression
    - ▶ eg: CD5+ in CLL
- ▶ Quadrant gating internal POSITIVE population
  - ▶ Emphasize abnormally weak expression
    - ▶ eg: dim CD38 and loss of CD19 in myeloma



# Tandem Breakdown

What would happen if CD117 was normally cocktail'd on APC?



# Preventing Gating Tragedies

## Every plot should make sense

- Gated on meaningful population(s)
- Axes chosen to separate and accentuate meaningful populations
  - Junk/compensation error/tandem breakdown
  - Lineage
  - Maturation pathways
  - Aberrancy

## Every drawn gate should be rationally chosen

- Remove debris/coincident events ('doublets')/bubble noise
- Differentiate/isolate/quantitate populations
  - Sequential gating: further subdividing related populations
  - Eg: FSC/SSC: viable, subgate CD45/SSC: lymphs, subgate CD3/CD19: T cells
- Compare/quantitate expression of markers
  - Boolean gating: combining gates that are not sequential
    - Logical expressions to selectively include/exclude populations
    - eg: (CD138+ OR CD19+) - include both B cells and plasma cells
    - eg: (CD34+ OR "Lymph") - include both blasts and lymphocytes

# Recap Quiz

A population of small, mature lymphocytes appears to express both CD19 (APC) and CD3 (APC-H7/A750).

The LEAST LIKELY cause would be:

- ▶ A) Wrong antibodies in tube
- ▶ B) Tandem breakdown
- ▶ C) Fluidics/hydrodynamic focusing issues
- ▶ D) Dichroic/bandpass breakdown/errors
- ▶ E) Compensation error
- ▶ F) Gating error
- ▶ G) Bilineal lymphoma

# ICSH/ICCS Guidelines

- ▶ Preanalytical factors:
  - ▶ Sample labeling
    - ▶ Labeled at collection: at least two unique identifiers, date and time
  - ▶ Anticoagulants/media/fixatives (labile antigens)
    - ▶ EDTA, NaHeparin, ACD, RPMI+, fixatives
  - ▶ Transport time (viability, labile antigens)
  - ▶ Transportation conditions (temperature)

# Analytic Validation

- ▶ PMT standardization/laser stability
  - ▶ QC: Levy-Jennings
- ▶ Panel design, antibody/dye titration, compensation matrix
- ▶ Suggested that 50% validation samples normal, 50% abnormal:  
Spanning the clinically relevant range
- ▶ Carry-over
- ▶ Precision, resolution, linearity, sensitivity, dynamic range
- ▶ Inter-instrument comparison/calibration
- ▶ Post-analytics:
  - ▶ Analysis, interpretation, reporting of the data, eQA

# Quality Matters!

## Doing

Doing the right test on the right sample

- Blood/Marrow/Tissue/Fluid
- Sample age
- Validated anticoagulant type

## Using

Using the right method

- Optimized instrument settings
- Titrated antibodies in validated panels
- Optimized staining procedure
- Optimized compensation matrix

# Quality Matters

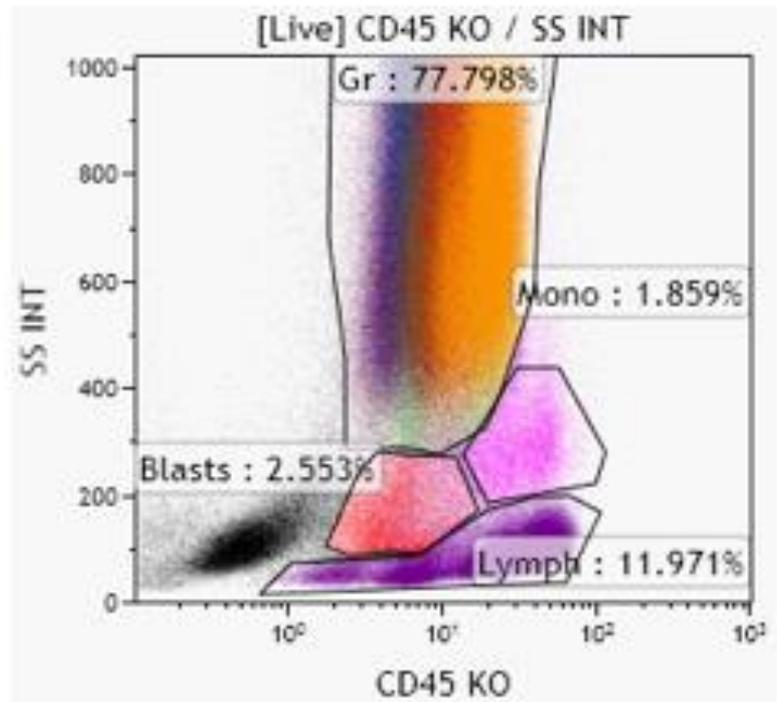
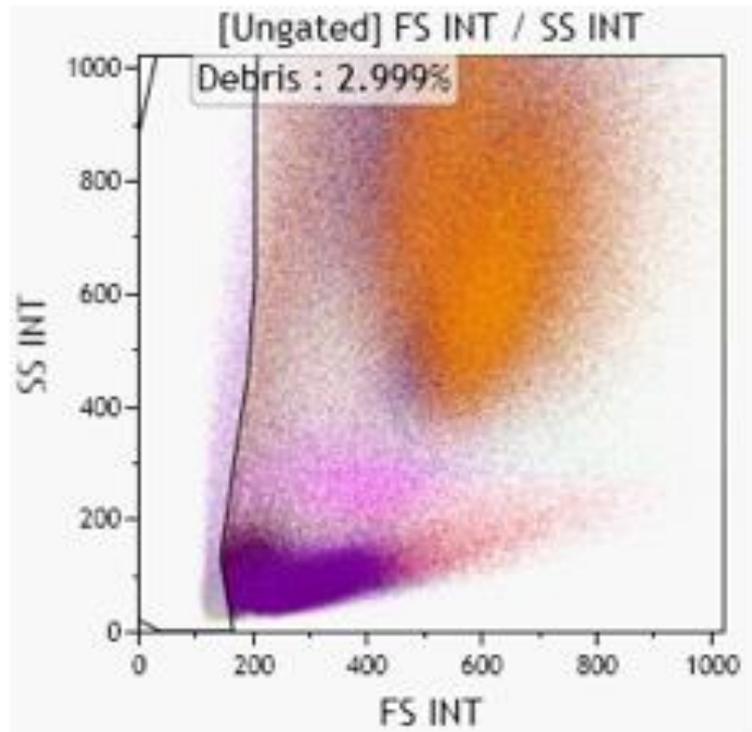
- ▶ Doing the right analysis
  - ▶ Verify your staining: POS/NEG internal controls (where possible!)
  - ▶ Justify your gating strategy: POS/NEG gating controls (where possible!)
  - ▶ Compensation is CRITICAL
    - ▶ Flow is not theatre: No smiles/frowns in flow data!
- ▶ Reporting the right results
  - ▶ One obvious abnormality doesn't mean there aren't two (or more!)
    - ▶ May need add-on panels based on analysis of the first round of data!
  - ▶ Any 'stats' reported should be meaningful
    - ▶ No bisecting continuous populations!
    - ▶ Describe populations, not percentages: don't let noise drown out reason

# Analyzing Flow Data

Granulocytic Maturation Pattern

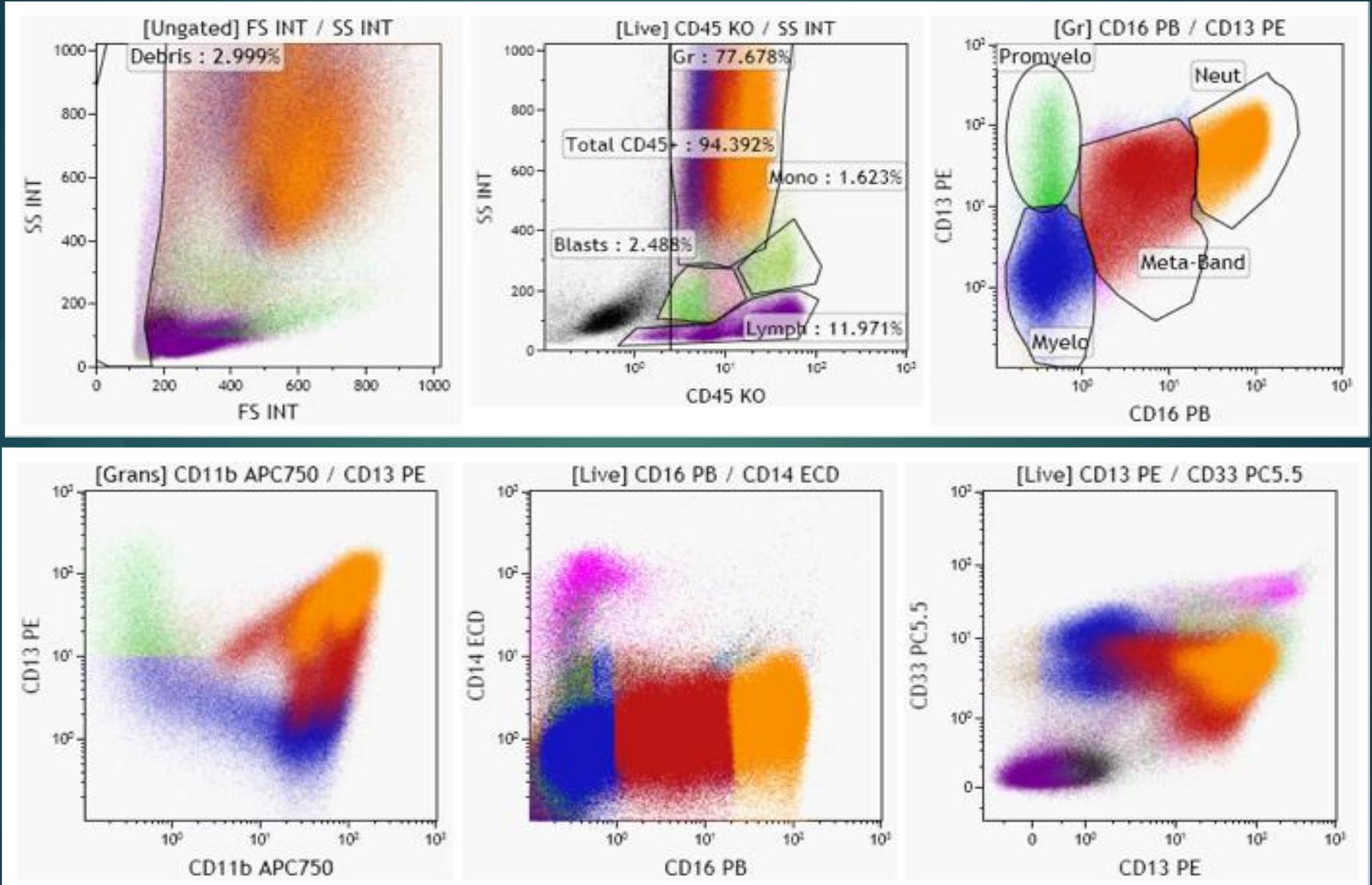
B Cell Maturation Pattern

Common Patterns of Aberrancy



Who lives where?

# Myeloid Maturation



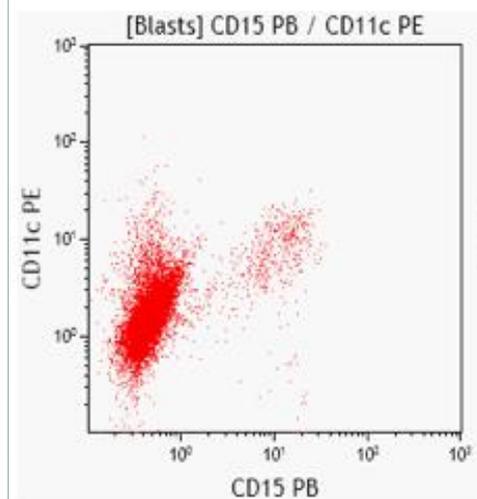
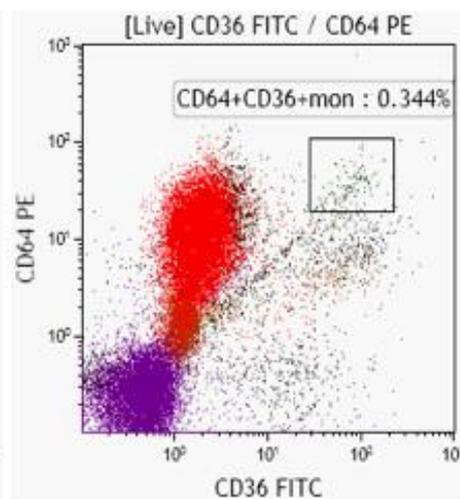
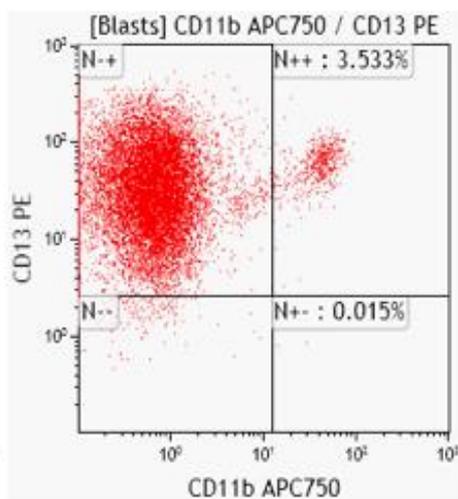
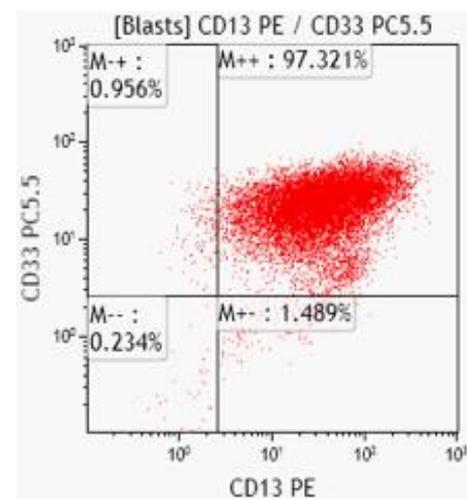
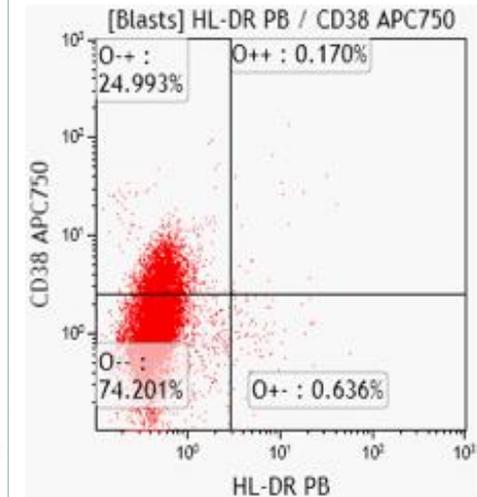
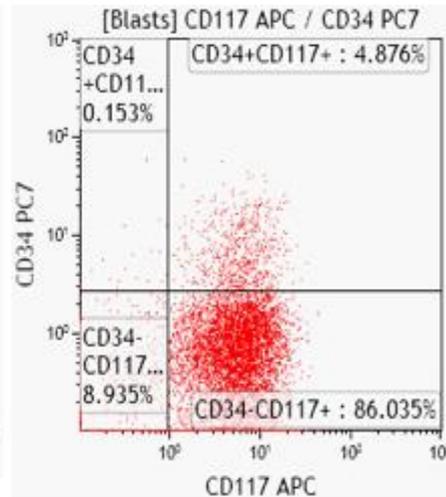
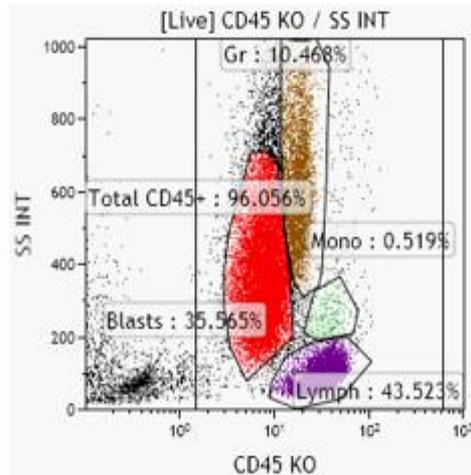
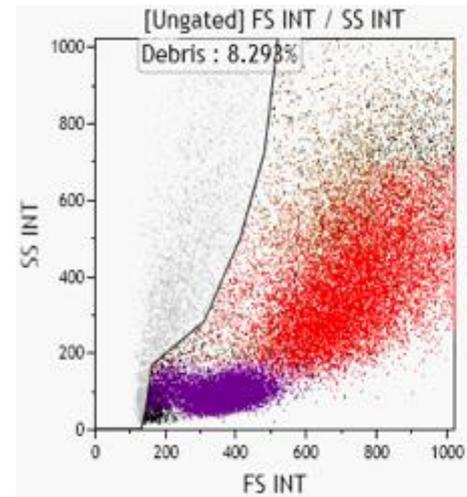
# Lineage and Subset Markers

- ▶ Lineage defining markers:
  - ▶ Neutrophils and Precursors: Myeloperoxidase
    - ▶ Common markers: CD33
    - ▶ Maturation markers: CD117, CD11b, CD13, CD15, CD16, CD10
  - ▶ Monocytes:
    - ▶ Common markers: CD33, HLA-DR, CD11c, CD64, CD36, CD4
    - ▶ Maturation markers: CD123, CD14, CD163, CD300e
    - ▶ Subsets: CD16-CD14++, CD16+CD14+
  - ▶ Basophils: CD123+ HLA-DR- CD33+ CD36-
  - ▶ Plasmacytoid dendritic cells: CD123+ HLA-DR+ CD33- CD36+
  - ▶ Eosinophils:
    - ▶ Common markers: CD33, CD13, CD11b, CD15, SSC-higher
    - ▶ Negative for CD16 and CD10

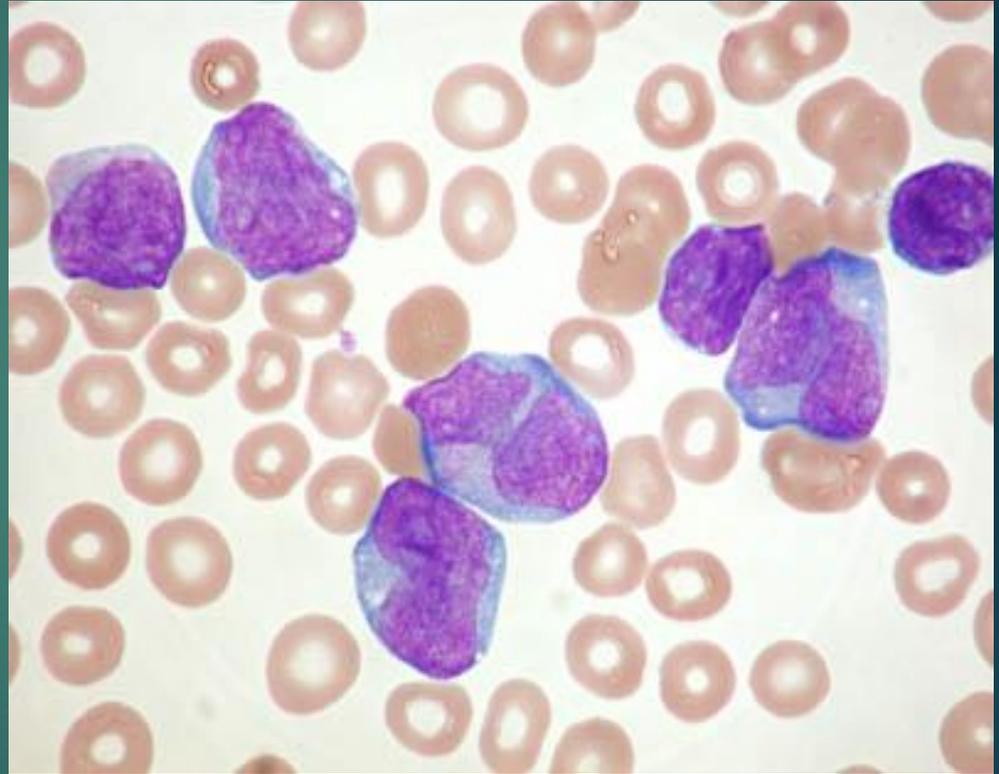
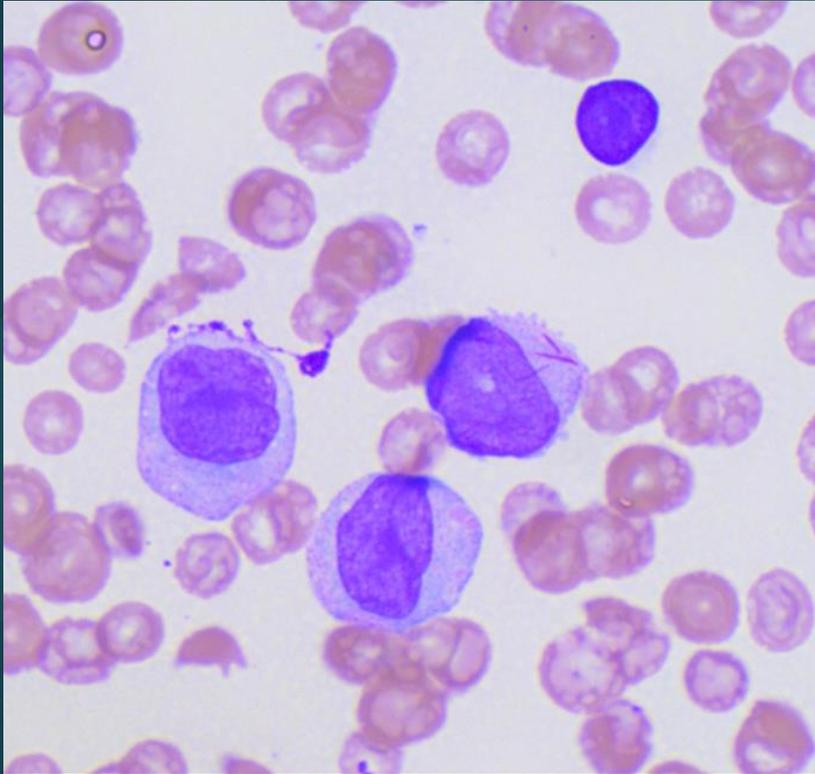
# Problems with Myeloid Maturation

- ▶ Cytometric evidence of disordered myelopoiesis
  - ▶ Decreased SSC
  - ▶ Abnormally increased or decreased antigen intensity
    - ▶ eg, CD45, CD38, CD14
  - ▶ Loss of normal antigens
    - ▶ eg, loss of CD11b, CD16
  - ▶ Gain of abnormal antigens
    - ▶ eg, gain of lymphoid markers: CD19, CD5, CD7, CD2
  - ▶ Dyssynchronous antigen expression
    - ▶ eg, CD34 AND CD15 or CD11b

# 23y M, Pancytopenia. Excessive bleeding post-BMBx



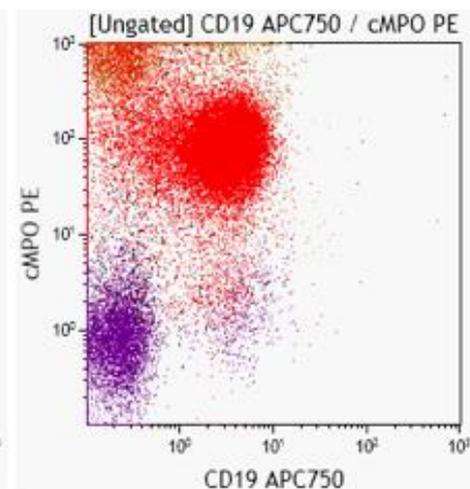
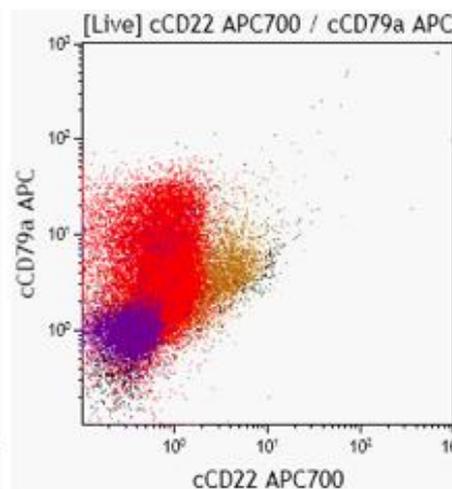
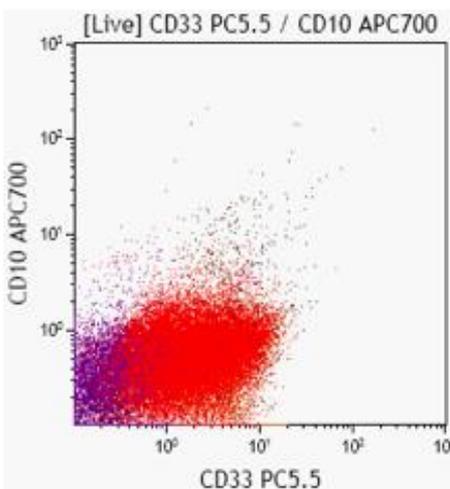
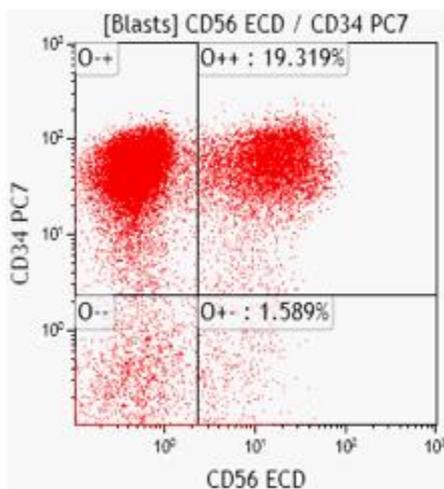
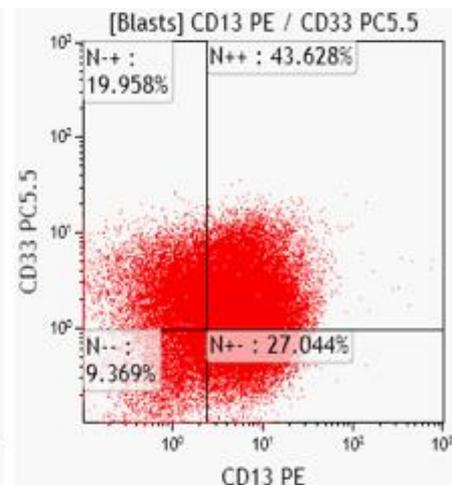
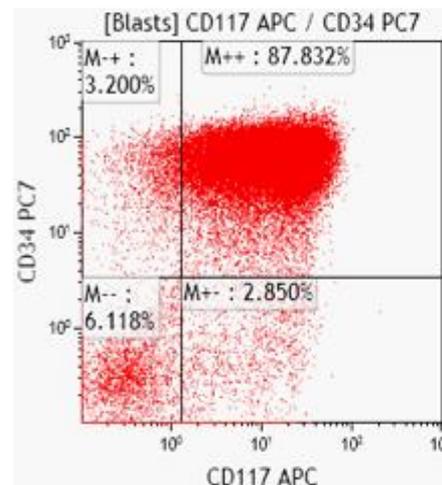
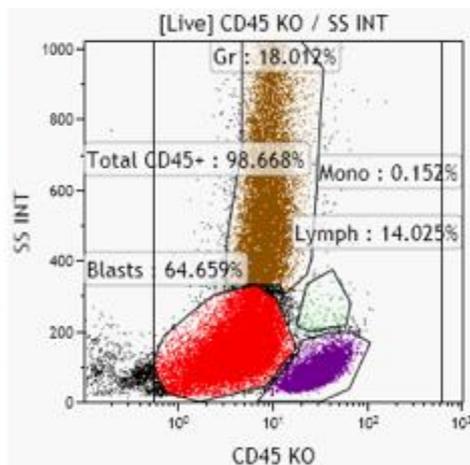
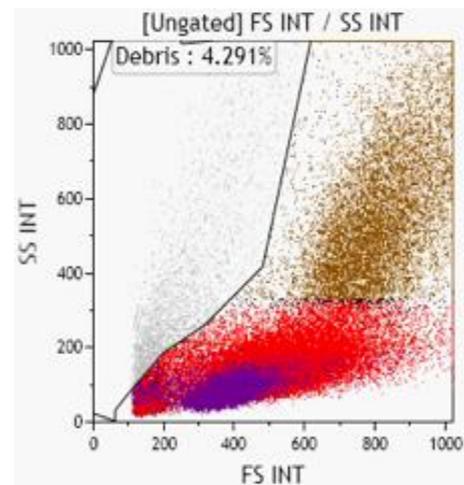
# Acute Promyelocytic Leukemia t(15;17): PML-RARA



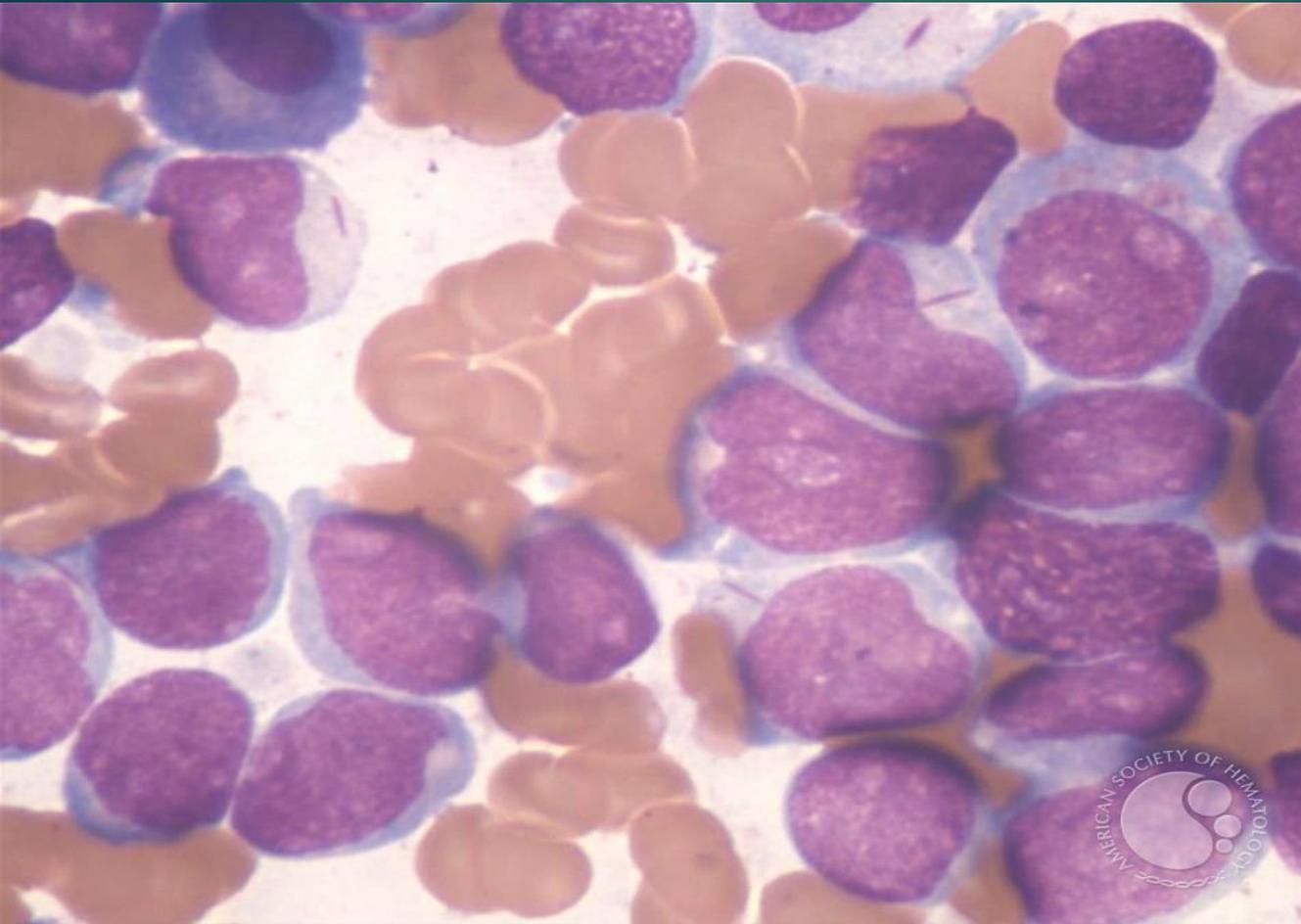
Positive for CD117, CD33, CD13 (variable), CD64, MPO, (sometimes CD2, CD56)  
Negative for CD34, HLA-DR, CD11b, CD11c, CD15, CD16, CD10

<http://www.fmshk.com.hk/hkabth/em/200406.htm>

# 35y F with a breast mass, Circulating blasts



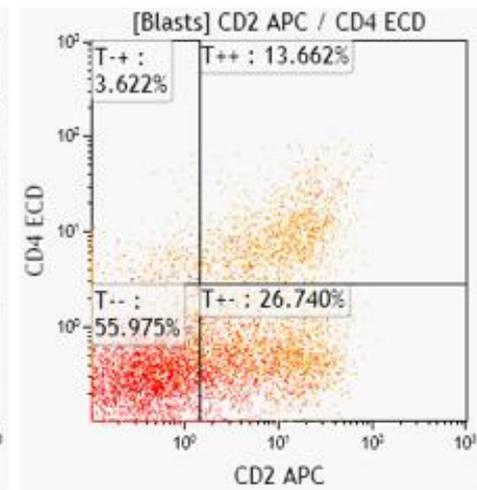
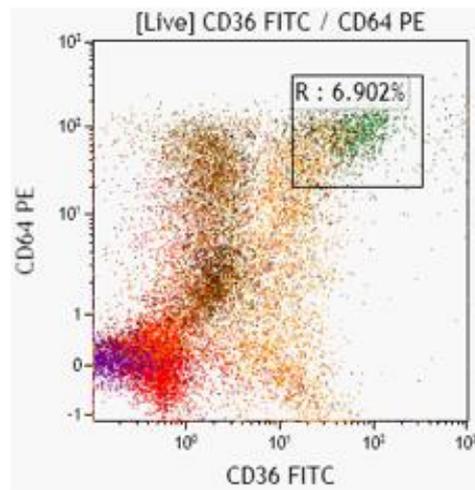
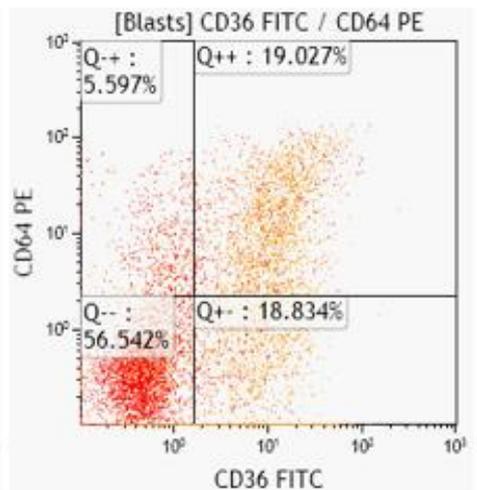
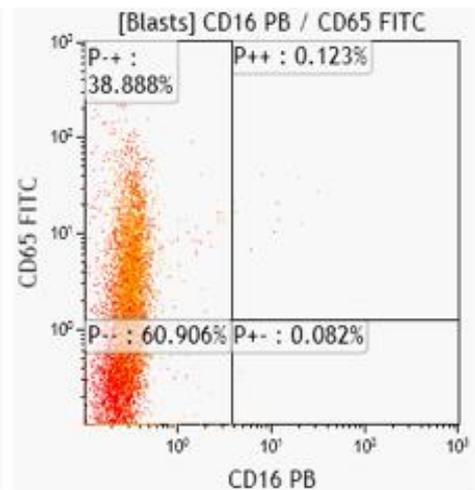
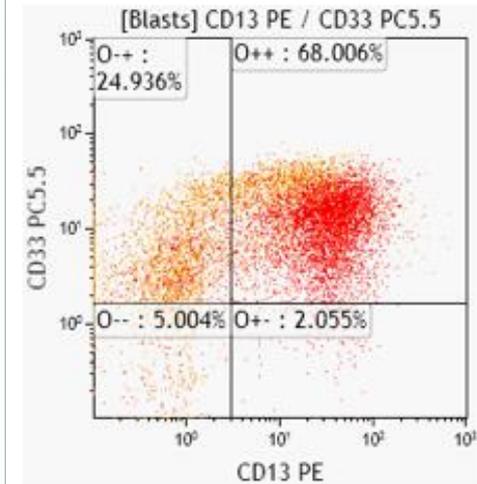
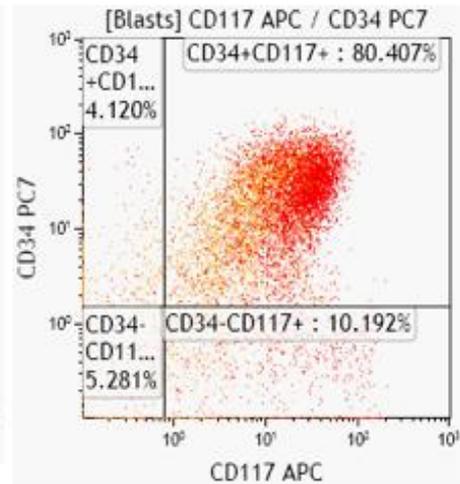
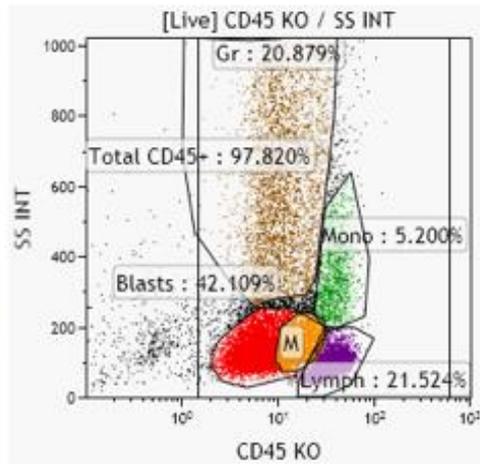
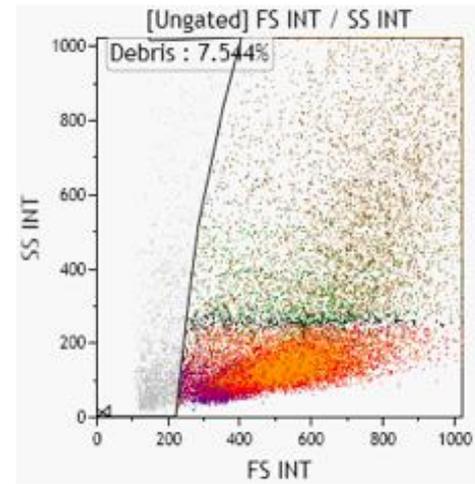
# AML with t(8;21) RUNX1-RUNX1T1



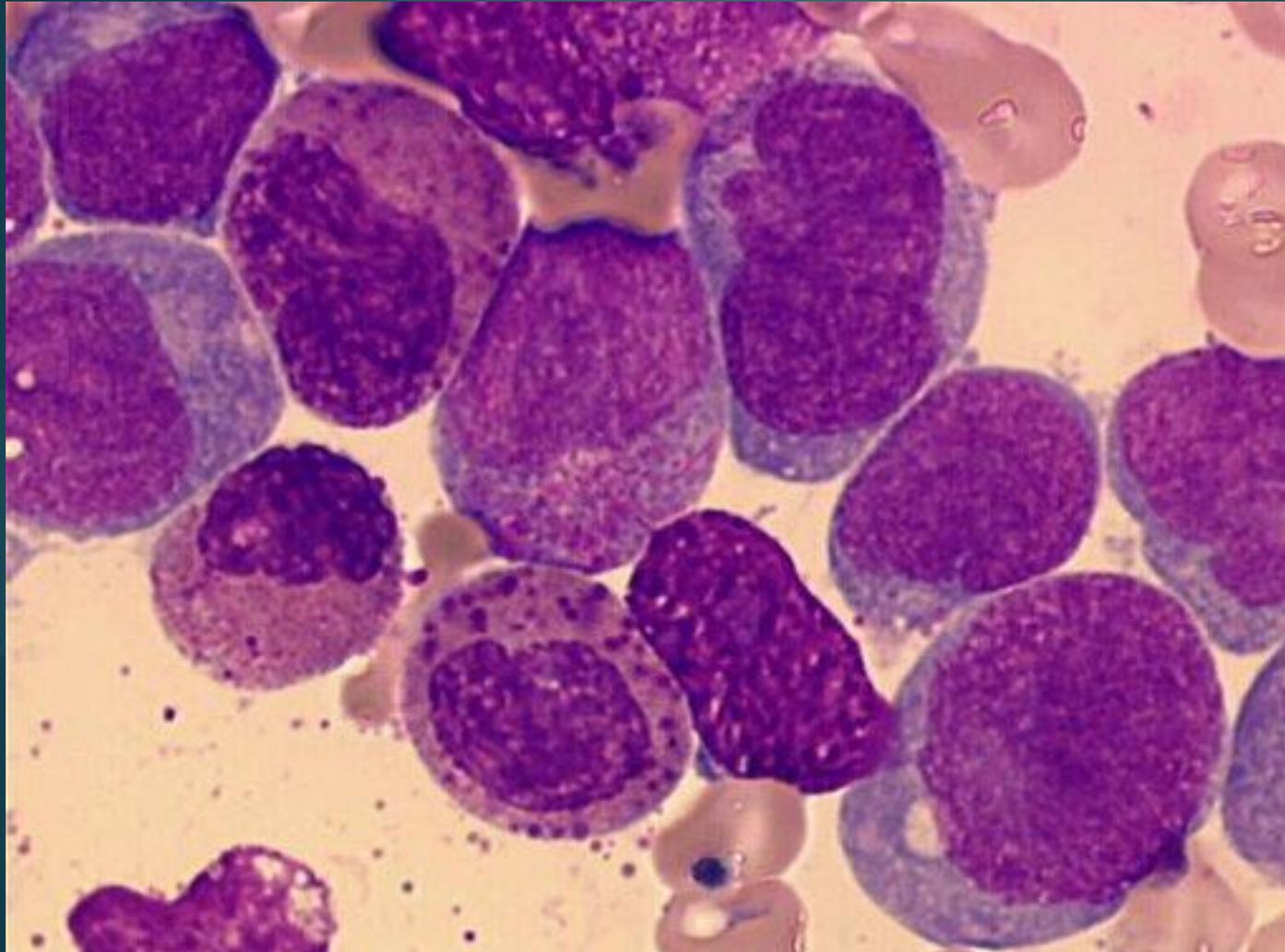
Positive for:  
CD34, CD117, CD33  
(dim), CD13, CD15,  
MPO, CD19 (dim),  
cCD79a (dim),  
sometimes CD56

Negative for:  
CD11b, CD16, CD10,  
CD22, TdT

# 15y M, fatigue, rare blast



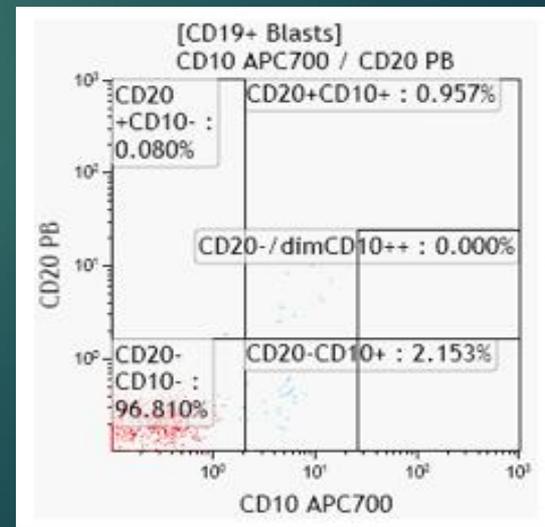
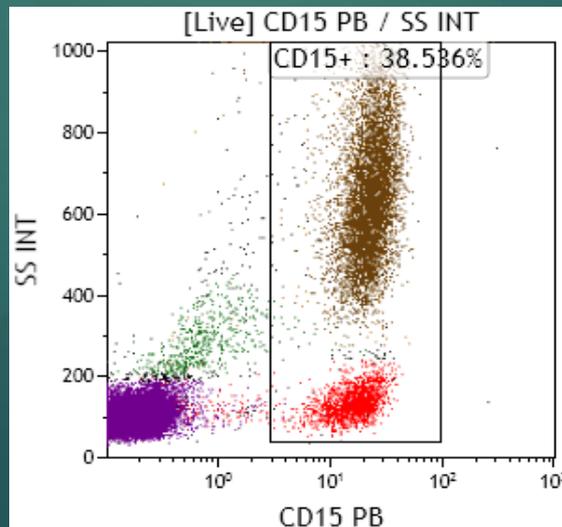
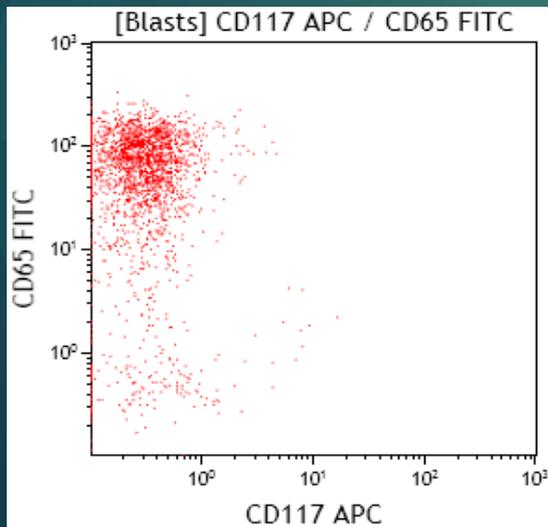
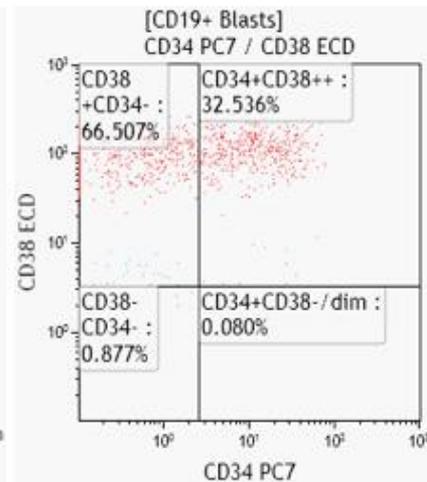
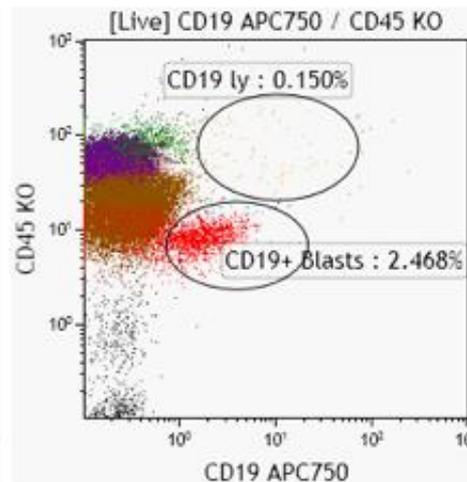
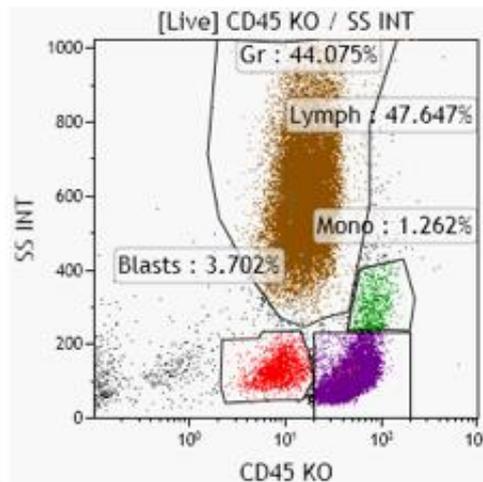
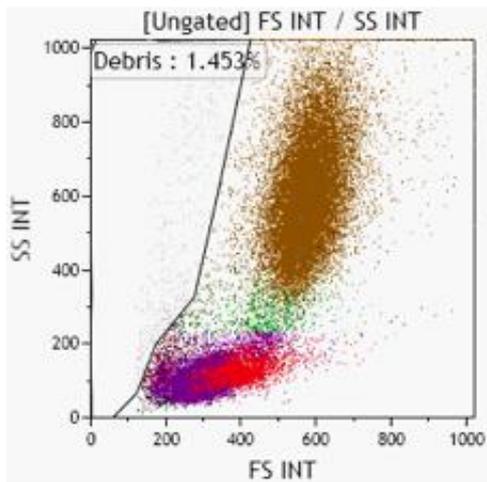
# AML with $inv(16)$ CBFB-MYH11



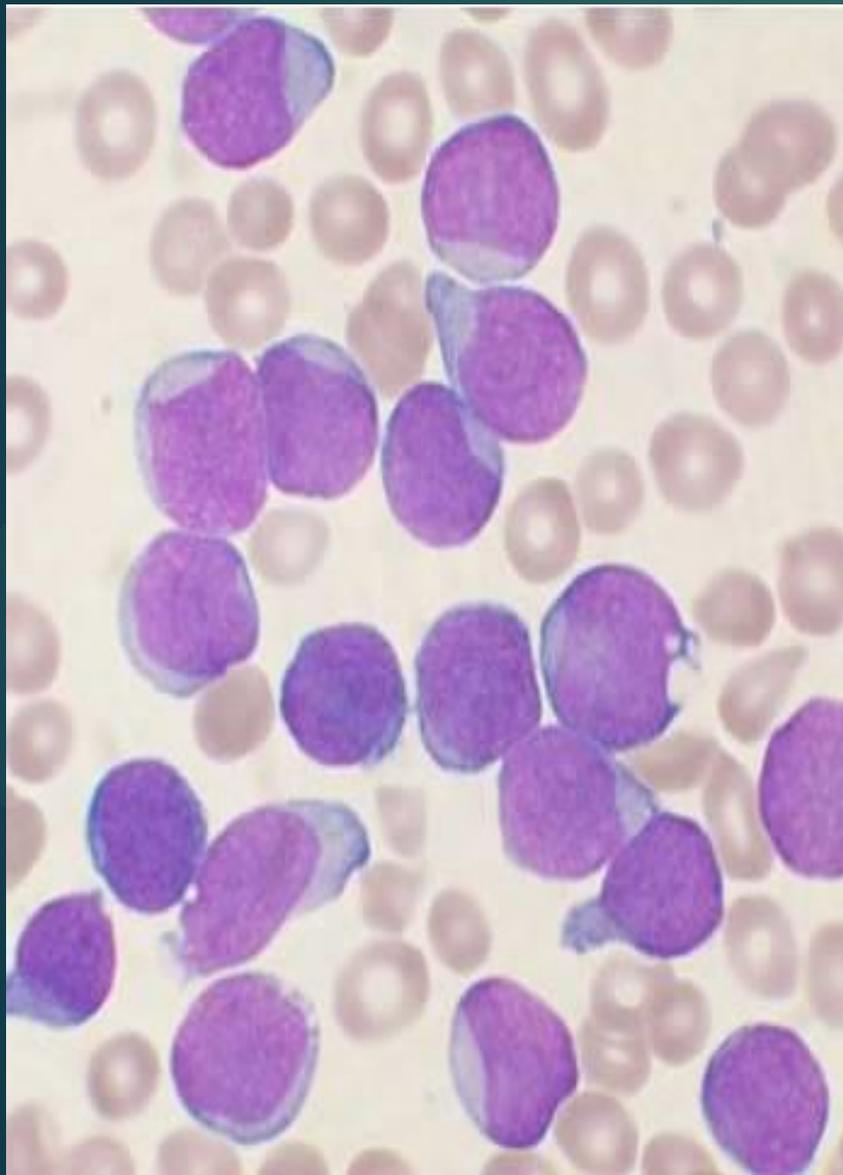
Two diverging populations  
Positive for:  
CD34, CD117,  
CD33, CD13,  
CD11b, CD15,  
CD65, MPO

CD4, CD36,  
CD64, CD14  
(variable), often  
CD2

# 50y F, 4 years post-Tx BrCa, Now with circulating blasts



# B-ALL with t(11;19) (KMT2A-MLLT1) (Therapy-related)



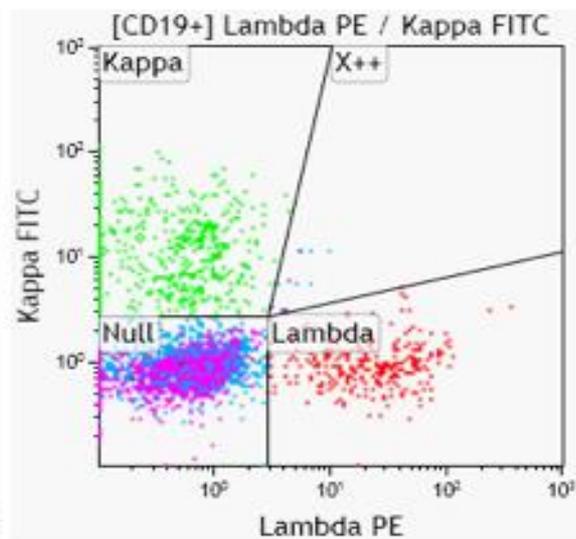
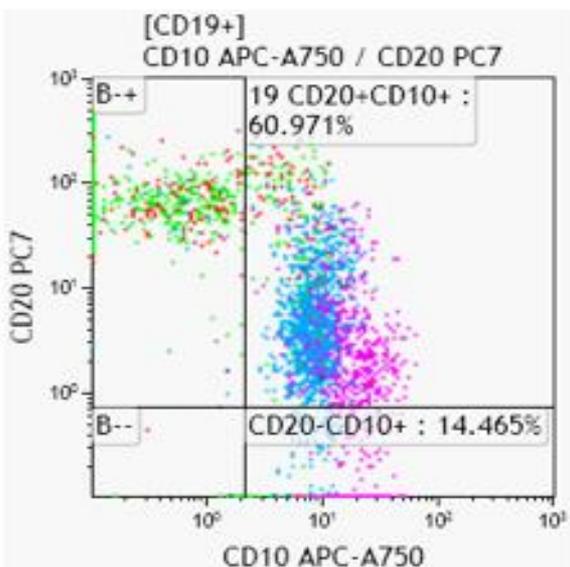
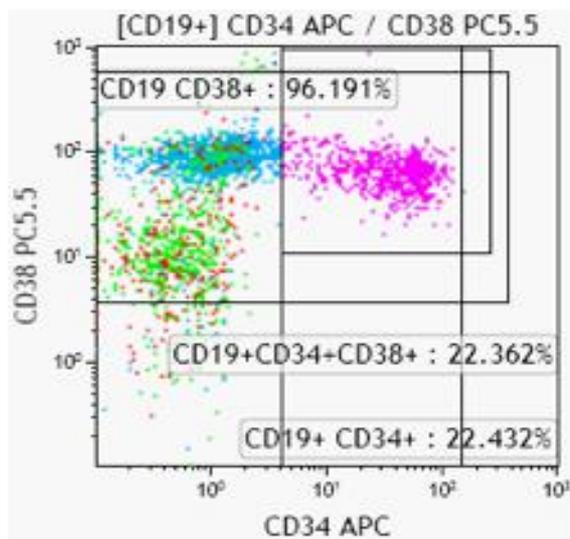
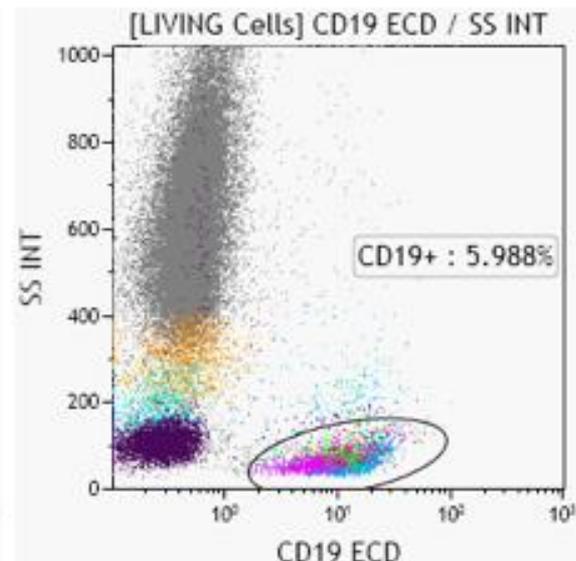
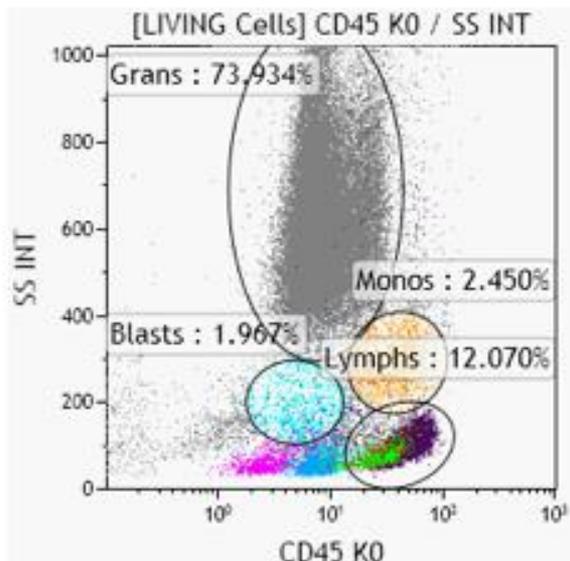
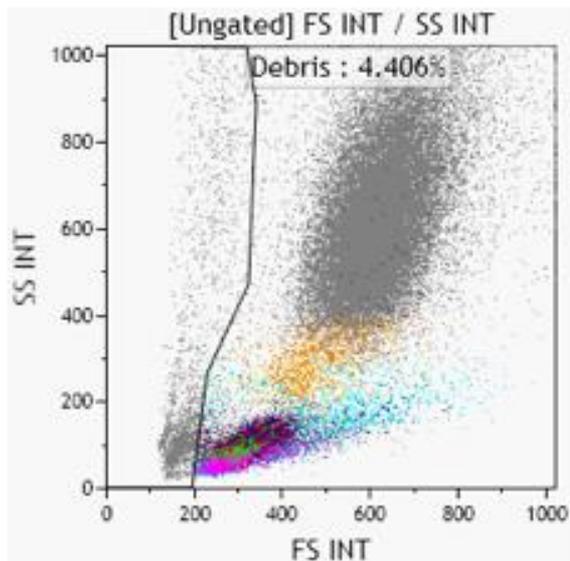
Watch for Lineage Infidelity vrs mixed phenotype in acute leukemias with KMT2A translocations

B-ALLs: Positive for CD19, CD38 (bright), CD79a, CD22, TdT, but often negative for CD34 and CD10,

Often aberrantly positive for CD15 and CD65, without other myeloid markers especially \*NO MPO\*

B/Myeloid MPALs: variable mix of myeloid and B cell markers, often with diverging populations. Must have MPO (myeloid) and CD19 with at least one other B cell marker (two if CD19 weak): CD10, CD79a, CD22

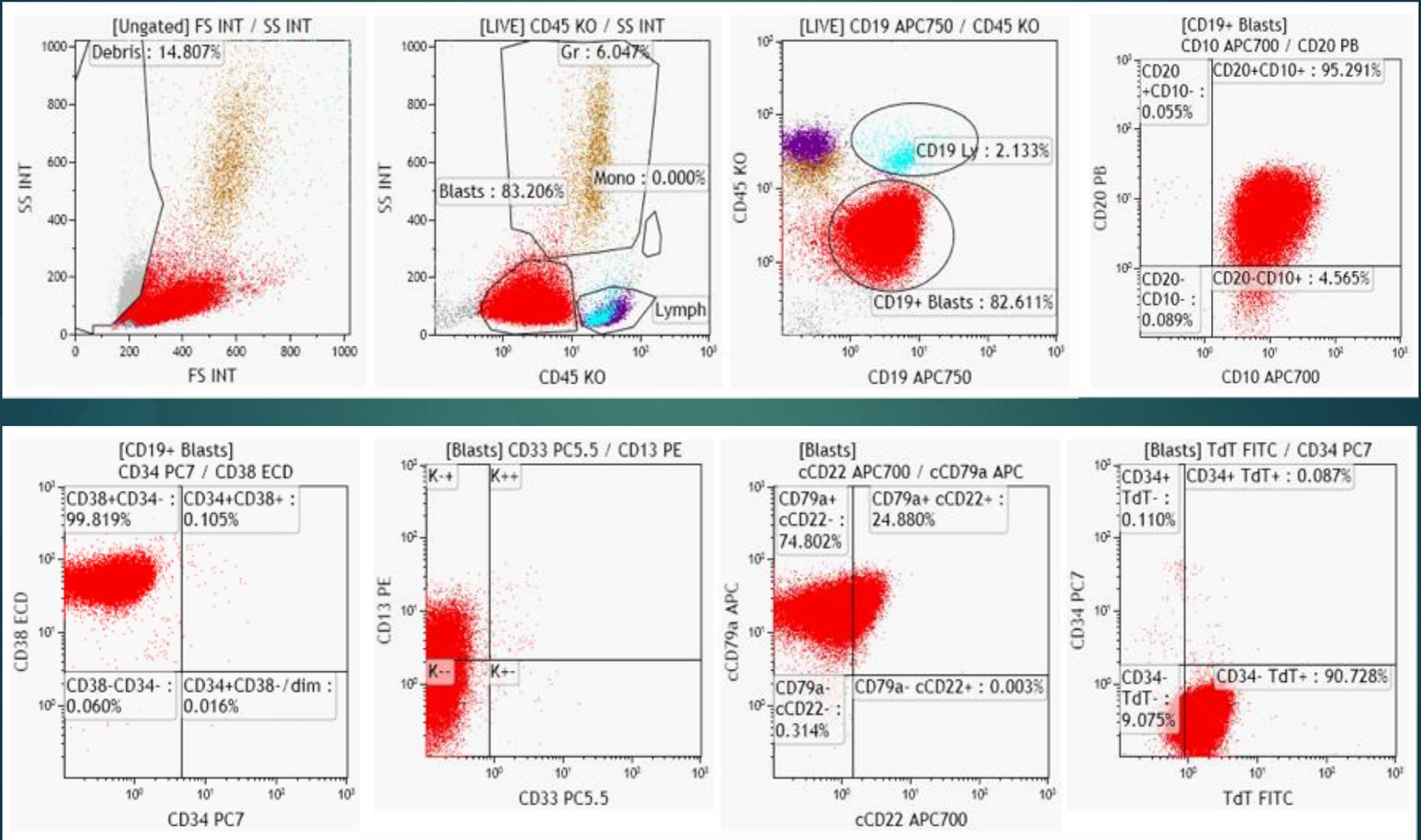
# B cell Maturation



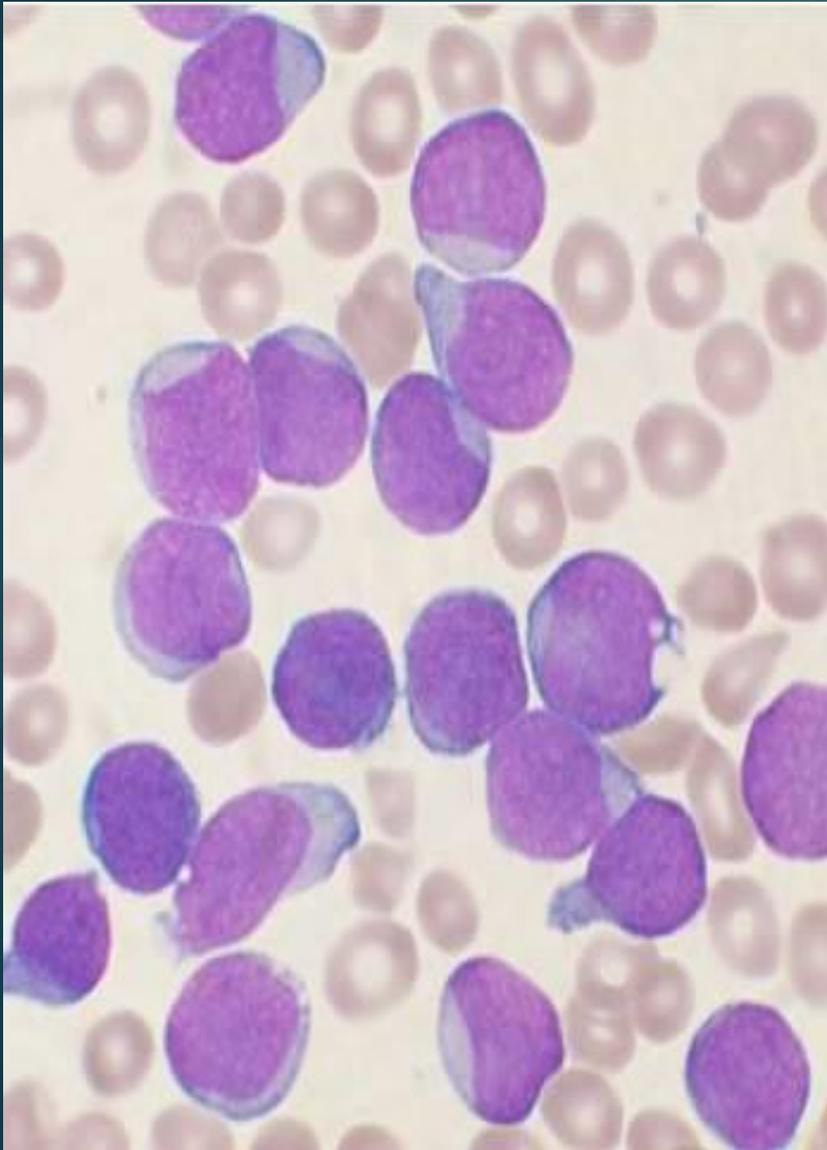
# Lineage and Subset Markers

- ▶ Lineage defining markers:
  - ▶ B cells: CD19+ and (CD22 and/or CD79a and/or CD10)
    - ▶ Maturation markers: TdT, CD10, CD38, CD43, CD20, IgM
    - ▶ Normal subsets: Kappa or Lambda
      - ▶ Sub-subsets: CD27, CD23, CD38, CD11c, CD25
  - ▶ T cells: cCD3+
    - ▶ Common T cell markers: CD2, CD5, CD7
    - ▶ Maturation markers: TdT, sCD3, CD4-CD8-, CD4+CD8+, CD1α, CD99
    - ▶ Normal subsets: CD4+, CD8+, TCRαβ or TCRγδ
      - ▶ Sub-subsets: CD11c/CD16/CD56, CD57, CD25, HLA-DR, CD38
  - ▶ NK cells: CD3- CD19- and (CD56+ and/or CD16+) and CD45++
    - ▶ Common NK cell markers: CD2, CD7, CD38, negative for CD5
    - ▶ Subsets: CD56++CD16-dim, CD56-dim/CD16++, CD11c, CD57

# 74y M, marked fatigue, bruising



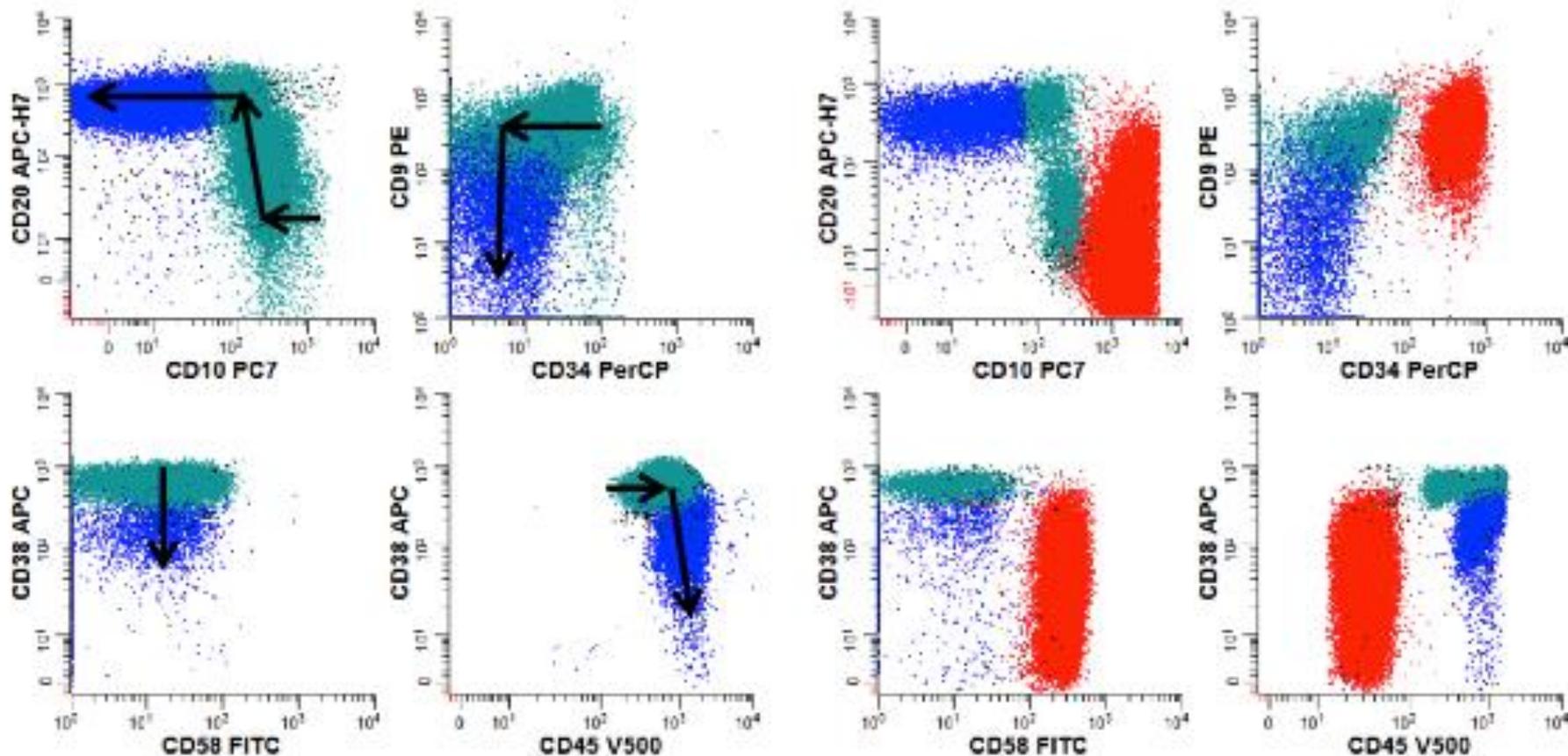
# B-ALL with t(9;22) BCR-ABL



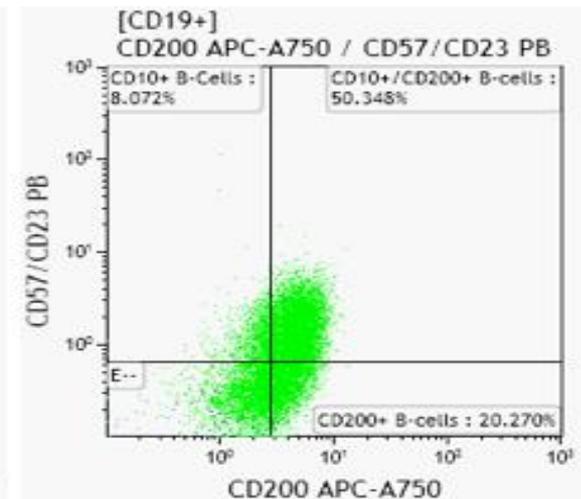
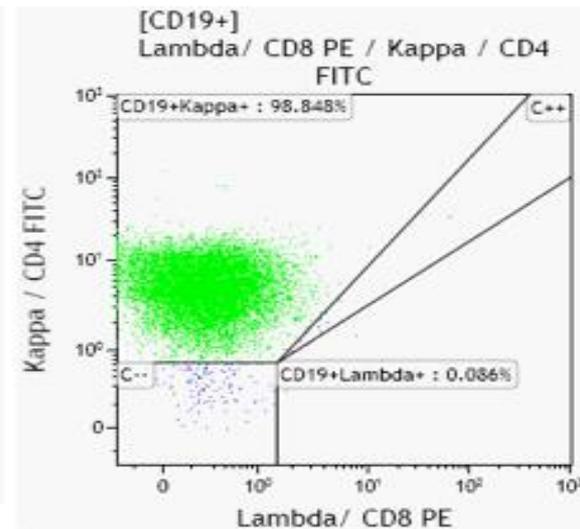
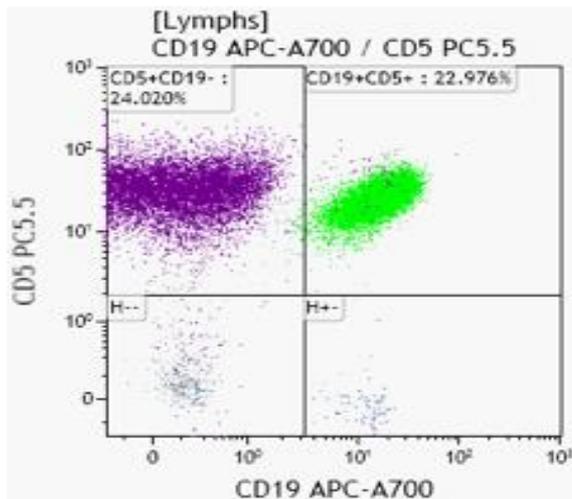
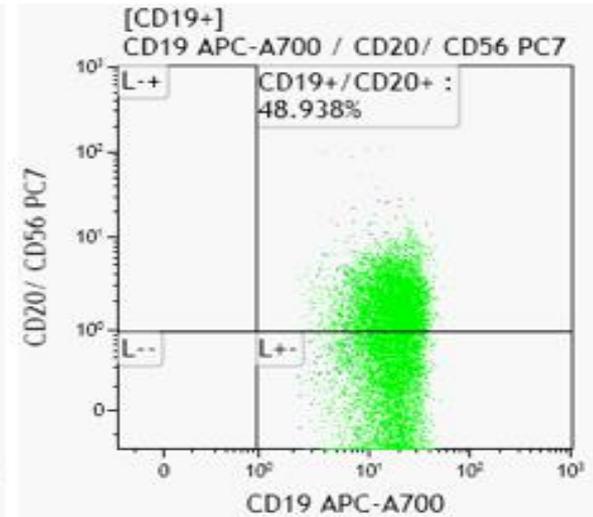
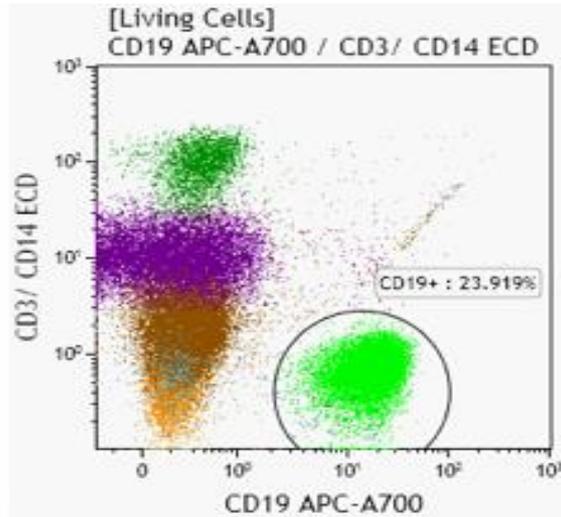
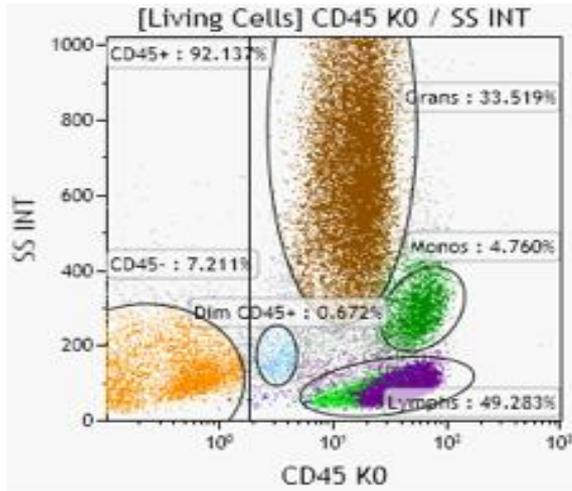
- ▶ Usually CD34+CD10+, often with dimmer CD38 than normal
- ▶ Often aberrant expression of CD13 and/or CD33
- ▶ Often positive for CD25, CD66c, CD123
- ▶ Often CRLF2/TSLPR over-expression in BCR-ABL-like B-ALL

# B cell Maturation: Difference-From-Normal/MRD Gating

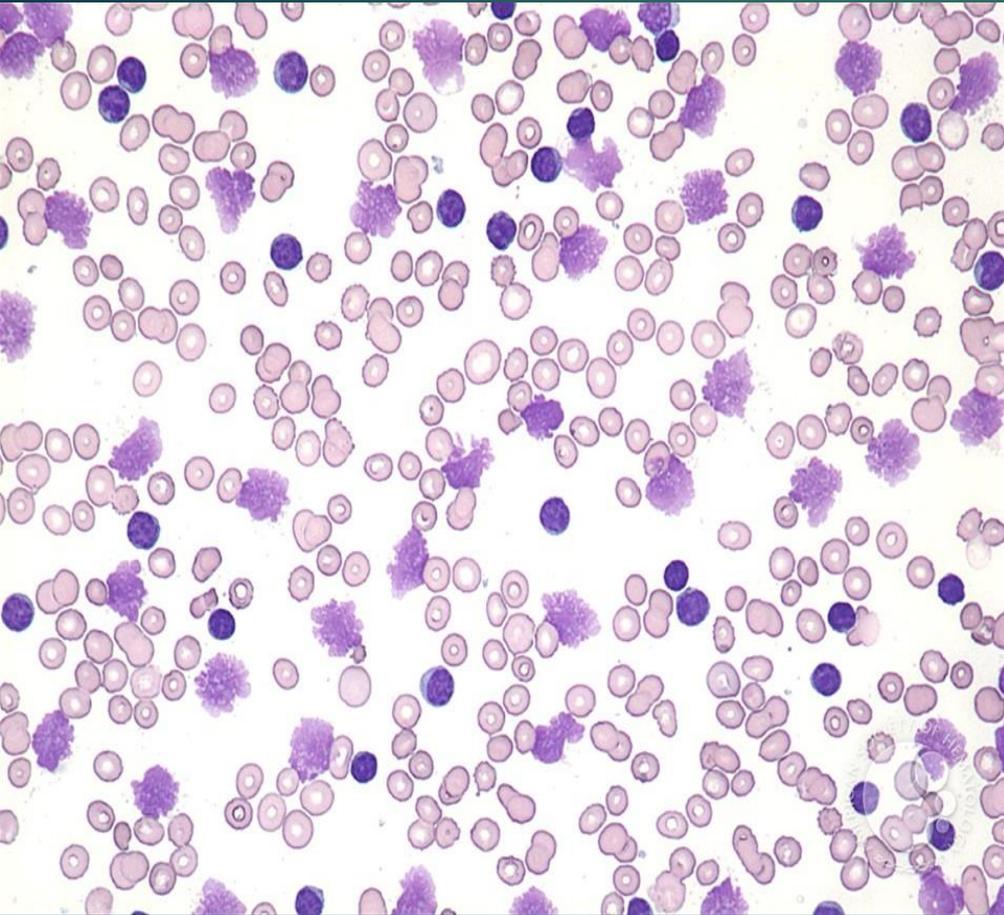
- ▶ Polygon mask/gate(s): set to normal populations/maturation
- ▶ Emphasize deviation from normal, based on density



# 76y F, well, lymphocytosis



# CLL/SLL/MBL

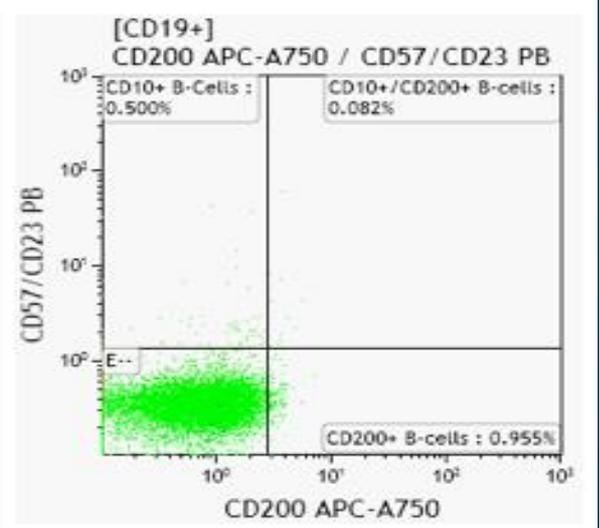
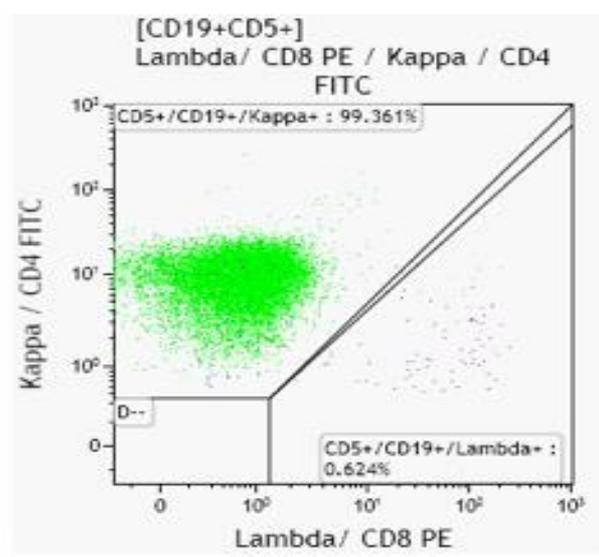
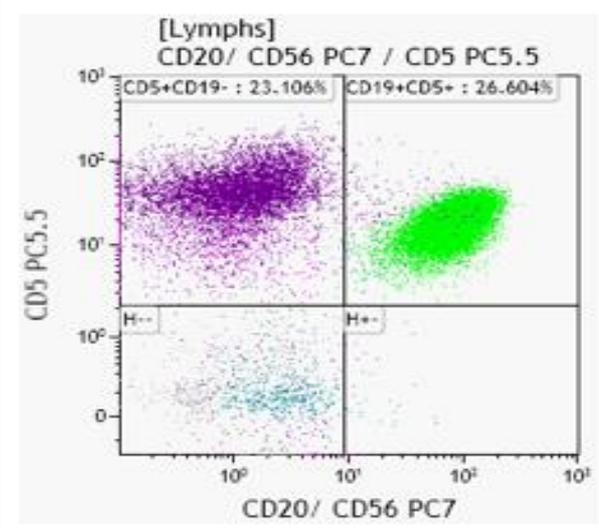
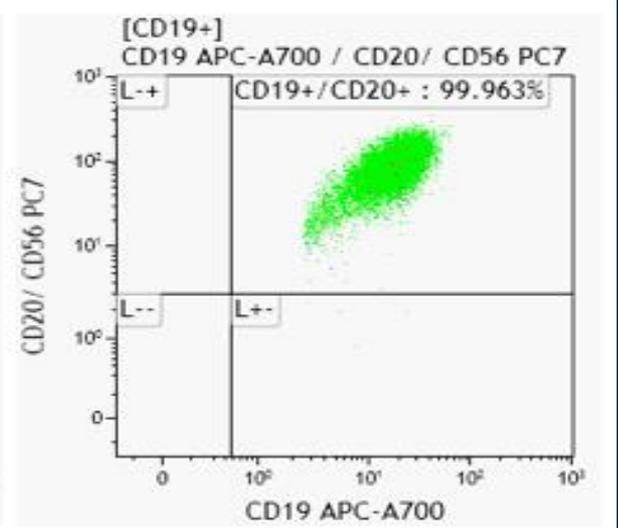
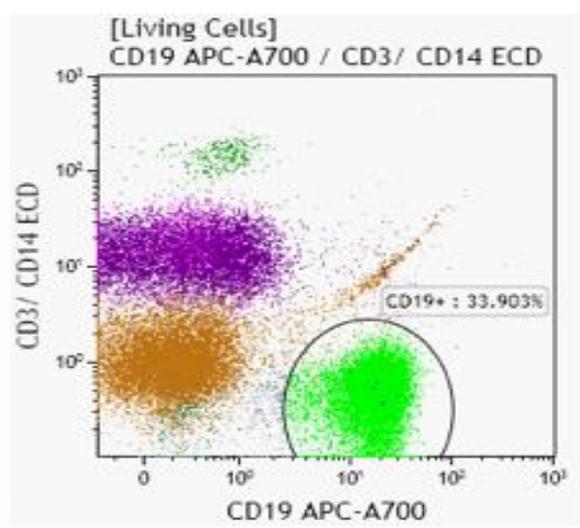
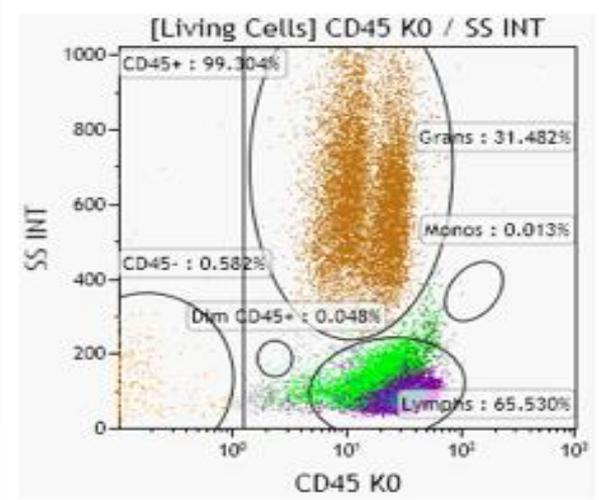


Aberrant expression of CD5, CD43

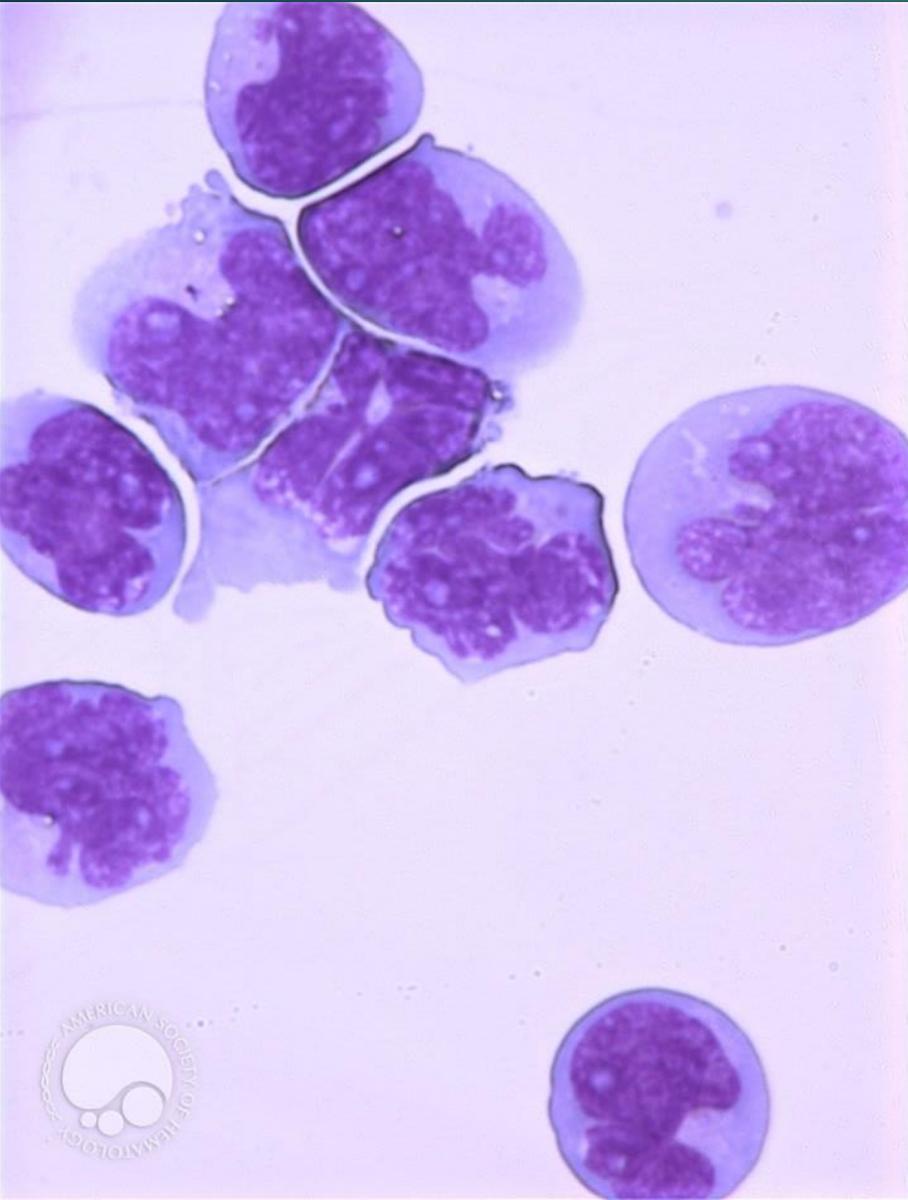
Dimmed expression of light chains & CD20 (FMC7), CD22, CD79b, CD81

Positive for CD23, CD27, CD200

# 55y M, colon mass, lymphocytosis



# Mantle Cell Lymphoma (IGH-BCL1)



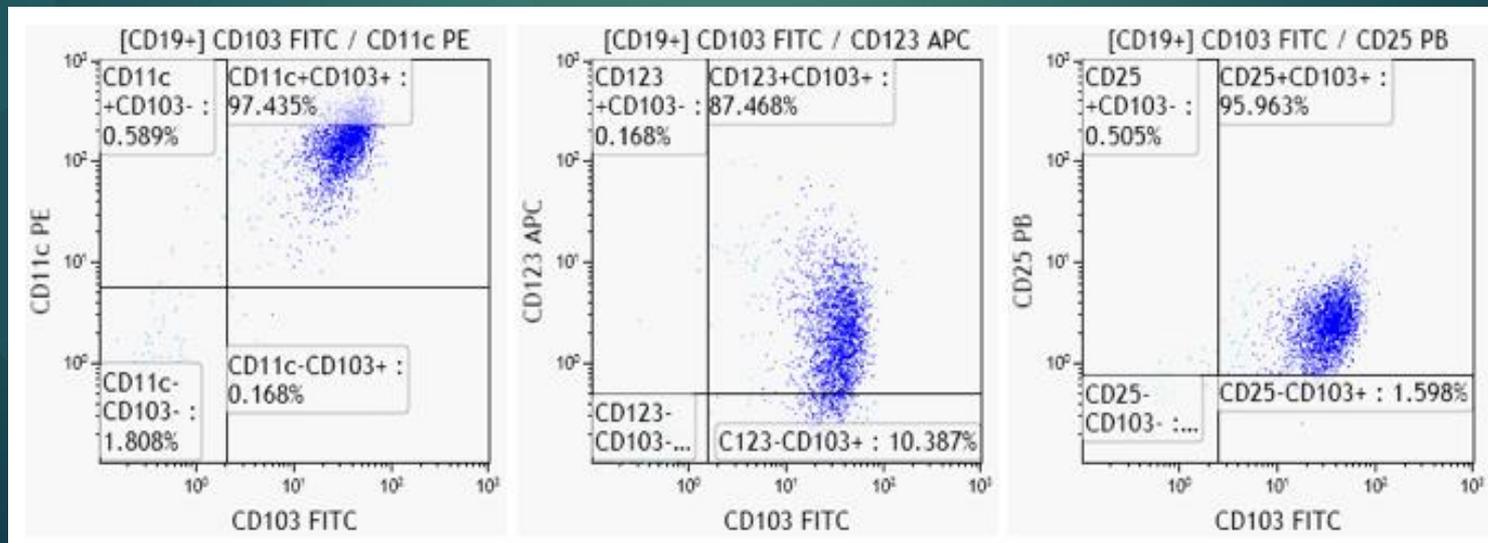
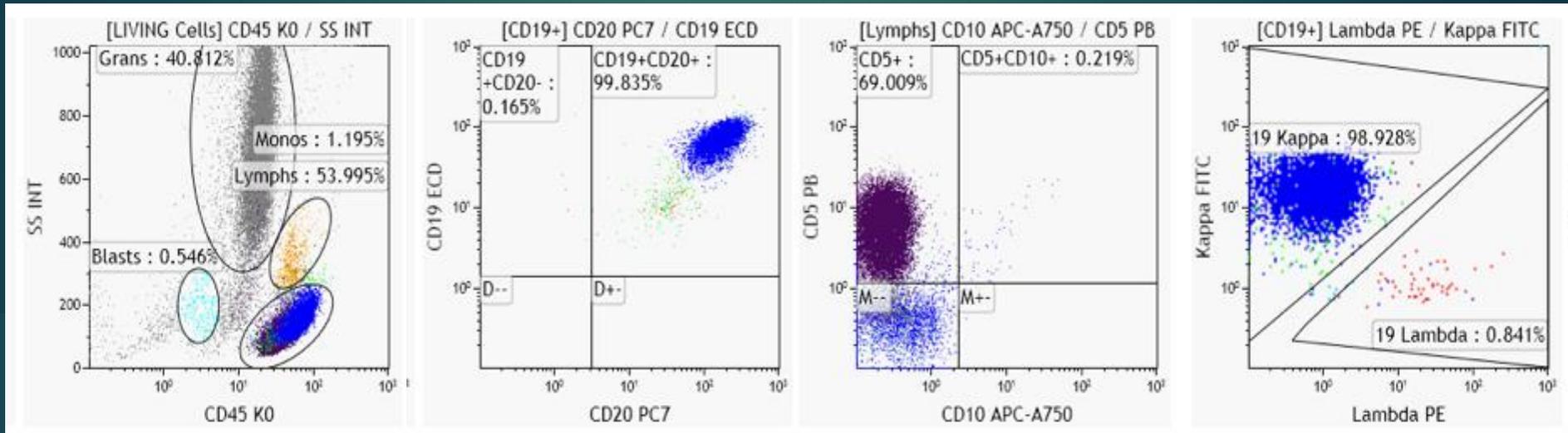
Aberrant expression of CD5, CD43

STRONG expression of light chains & CD20 (FMC7), CD22 CD79b, CD81

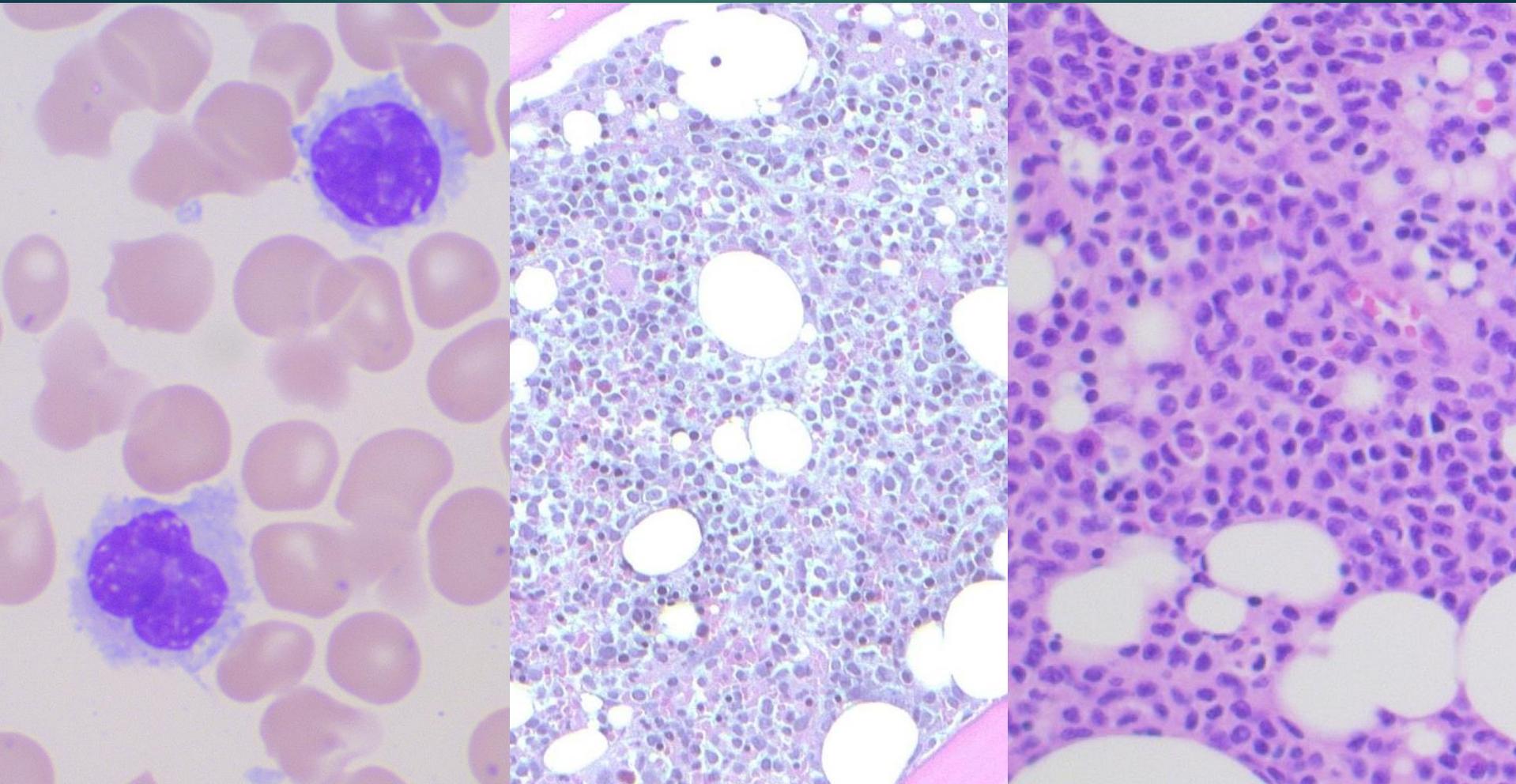
NEGATIVE for CD23, CD200

<http://imagebank.hematology.org/AssetDetail.aspx?AssetID=11722>

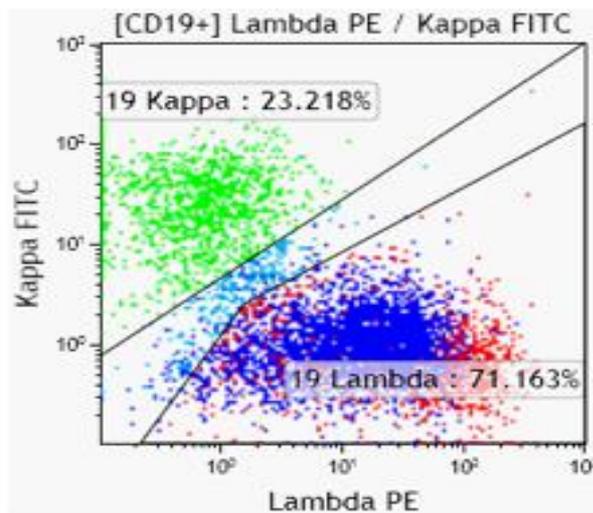
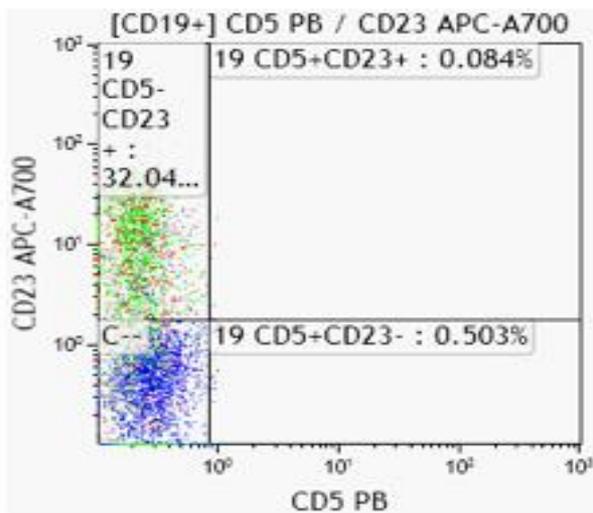
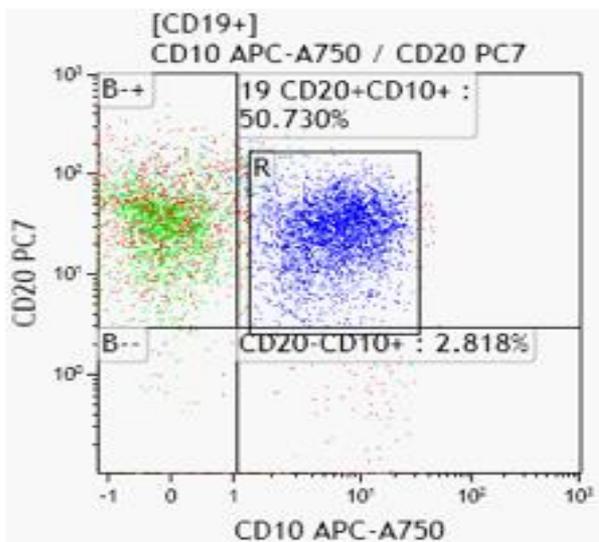
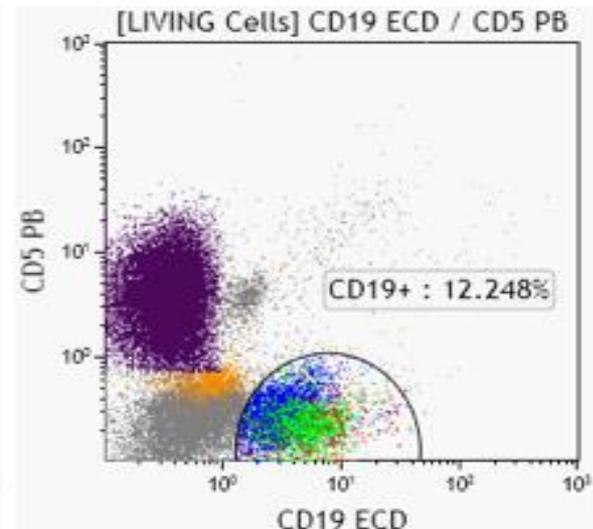
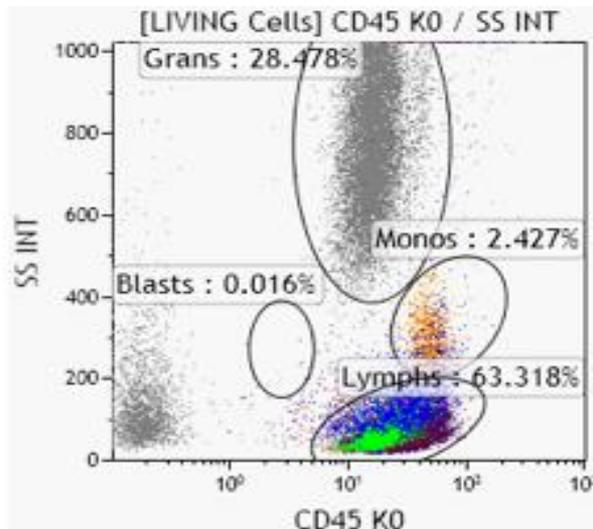
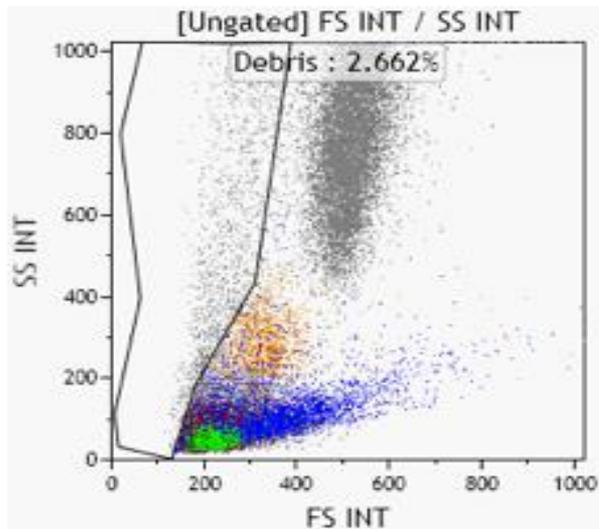
# 57y M, LUQ pain, pancytopenia



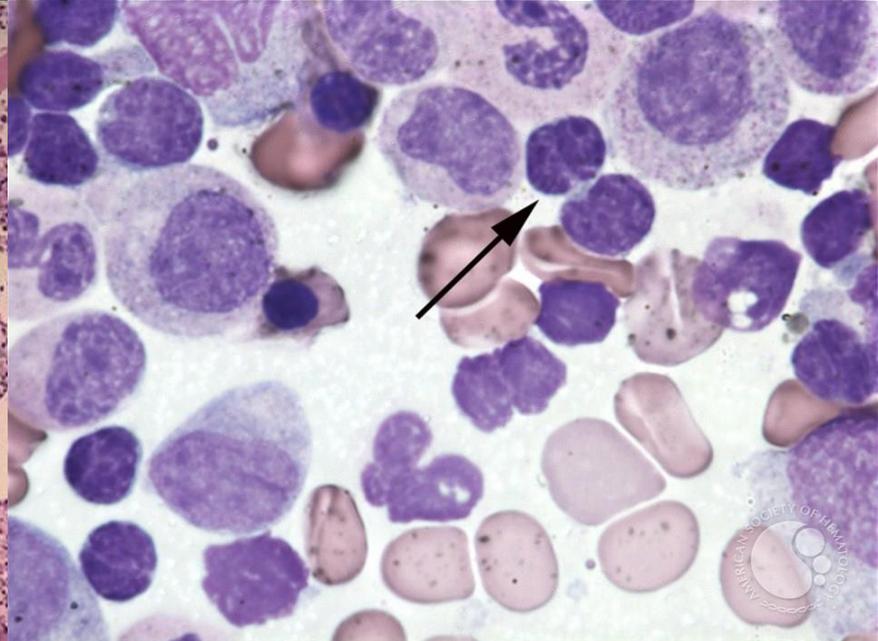
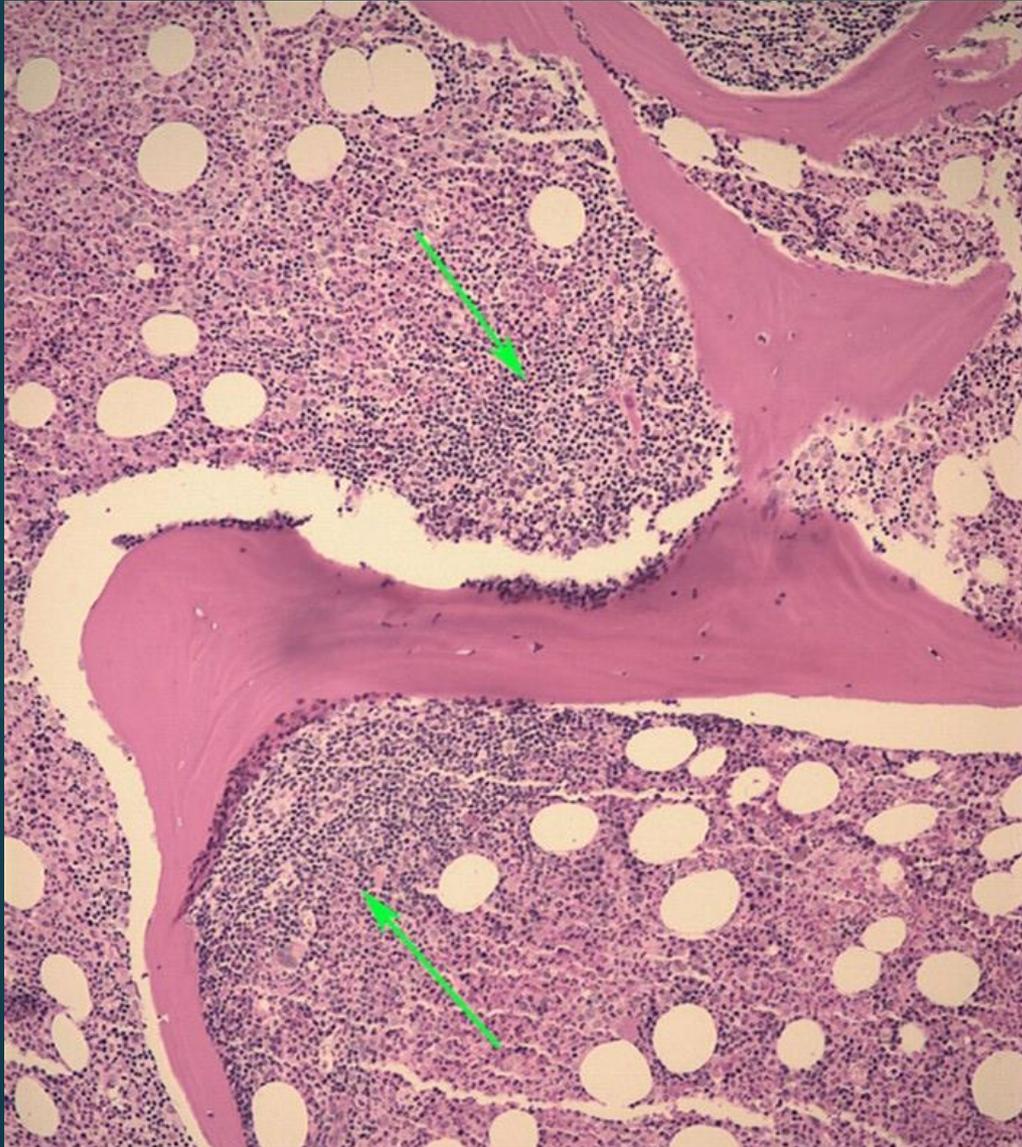
# Hairy Cell Leukemia



# 33 F, inguinal mass



# Follicular Lymphoma (IgH-BCL2)

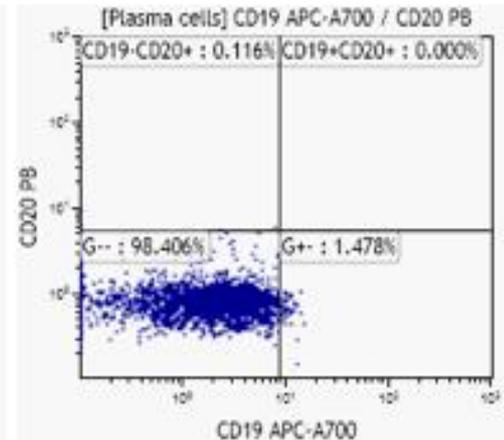
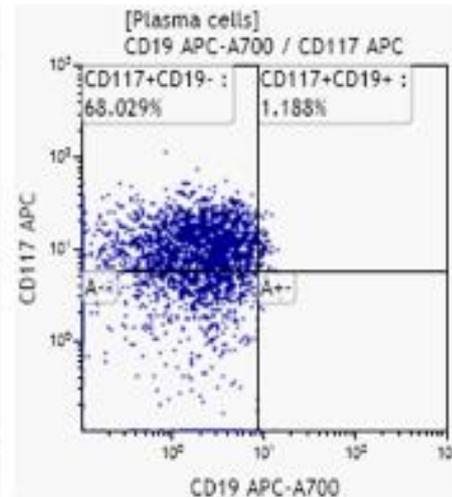
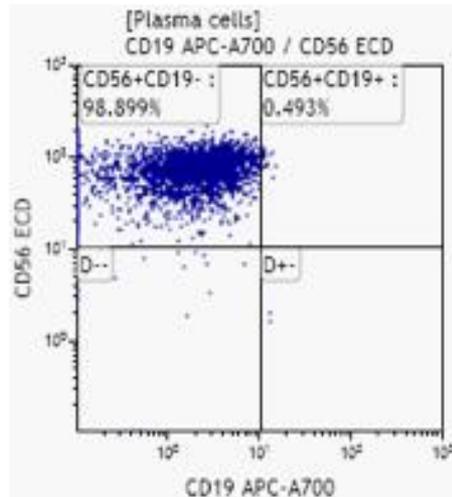
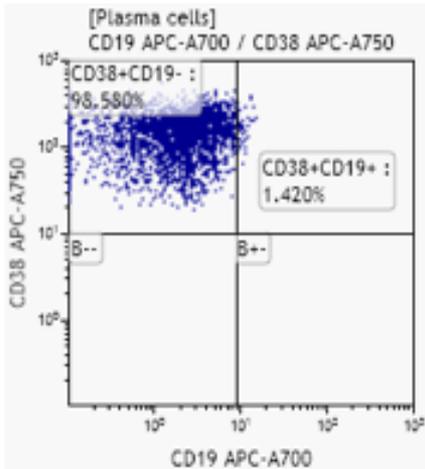
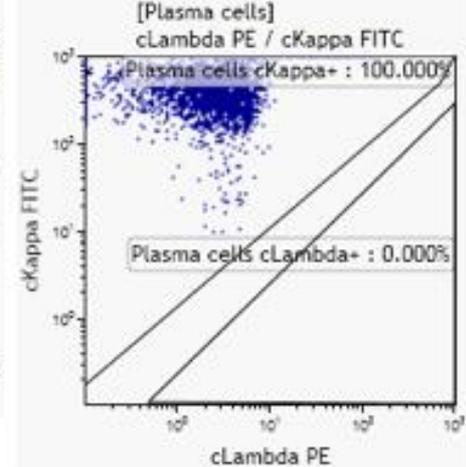
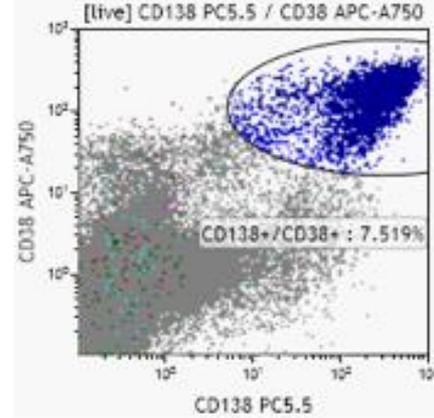
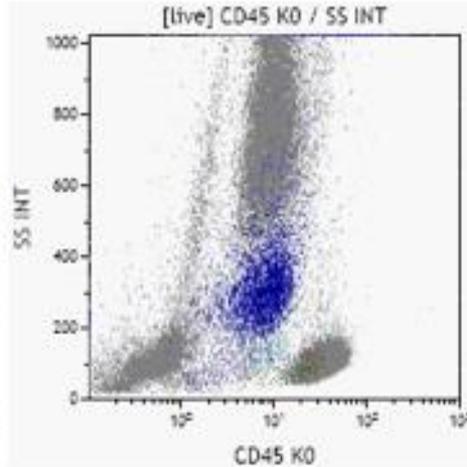
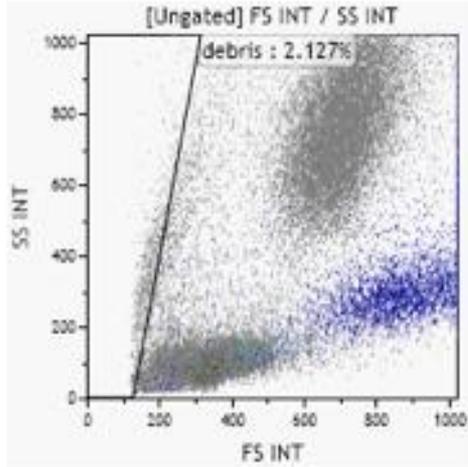


Positive for CD10, CD20,  
Often dimmed CD19 and CD38  
Negative for CD43

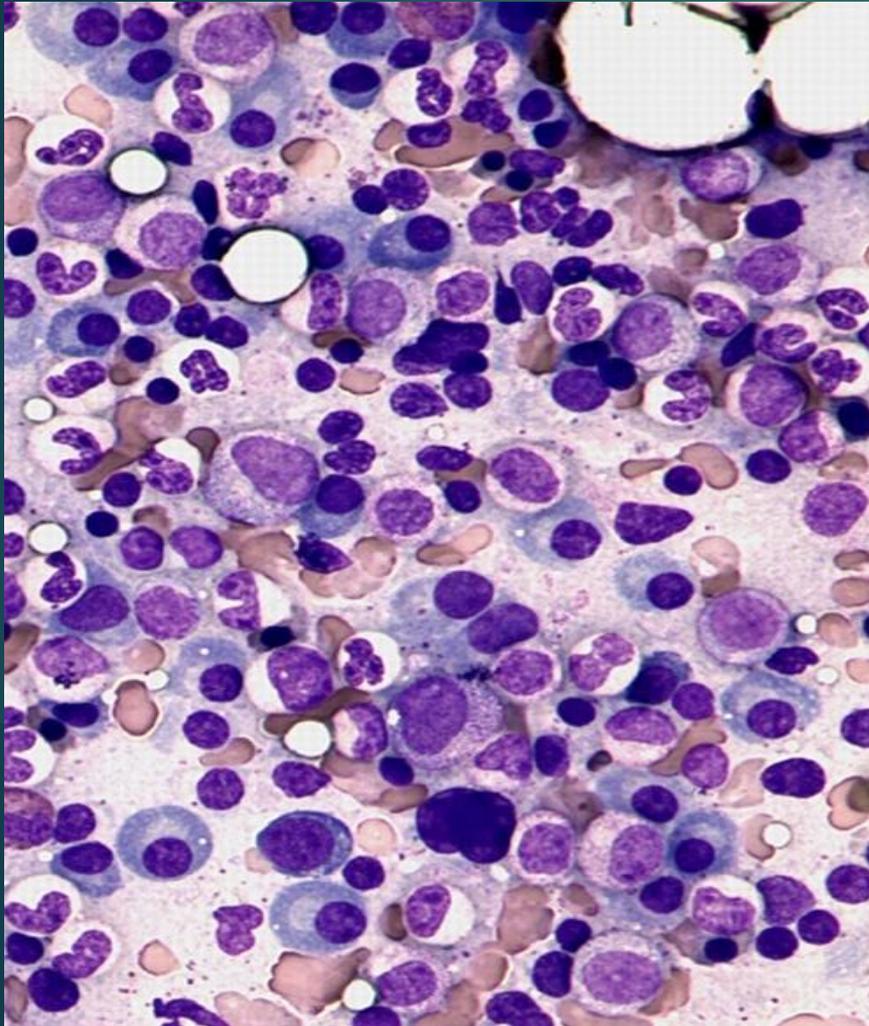
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[http://imagebank.hematology.org/Content%5C904%5C1030%5C1030\\_full.JPG](http://imagebank.hematology.org/Content%5C904%5C1030%5C1030_full.JPG)

# 52y M, pathologic hip fracture, anemia



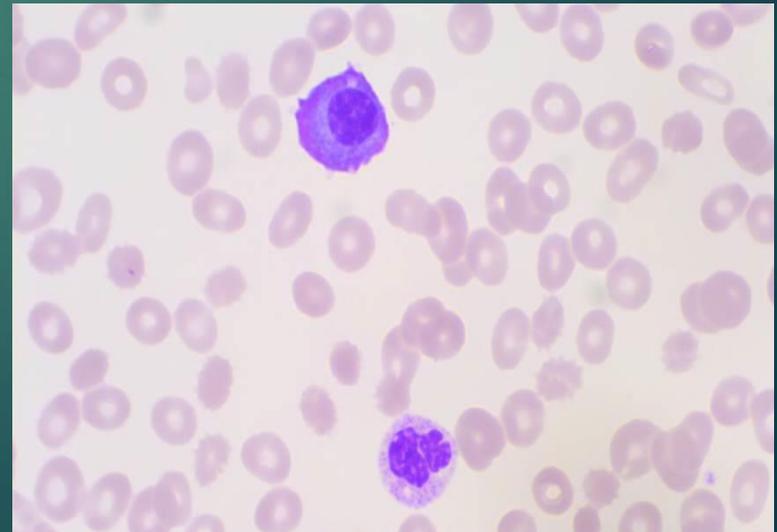
# Plasma Cell Myeloma



Often loss of CD19, CD45, CD27, CD81  
May dim CD38  
Gain of CD56, CD117, CD200

Gain of CD20, CD23 may suggest IGH-  
CCND1

Plasmacytic differentiation of B-LPD  
typically maintains CD19, CD45, but  
may express CD20



# Summary

- ▶ Flow Cytometry: Quality in = Quality out
  - ▶ Technical principles of cytometry
  - ▶ Cossarizza, A. et al. Eur J Immunol 2017. 47:1584-1797
  - ▶ Validation, Pre-analytic/Analytic-to-Post-analytic
  - ▶ ICCS-ICSH Guidelines: <https://icsh.org/guidelines>
- ▶ Compensation, Gating, Pitfalls
- ▶ Normal populations and maturation patterns
- ▶ Classic abnormal immunophenotypes



**Everything  
is  
AWESOME**

# Questions?

Graeme.Quest@kingstonhsc.ca



INTERNATIONAL COUNCIL FOR  
STANDARDIZATION IN HAEMATOLOGY

