FLOW CYTOMETRY - FROM ORIGINS TO TWENTY SECOND CENTURY PERSPECTIVES

KEYNOTE

ACS2024 – HOBART

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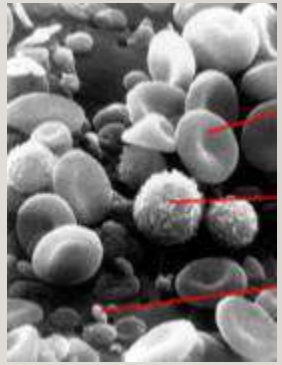
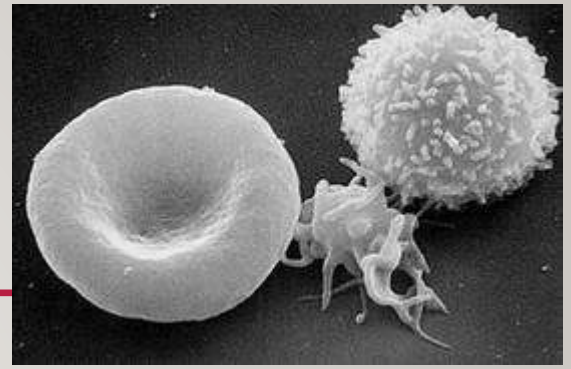
CYTOMETRY : DEFINITION

- « Measure cells»
- Measure what?
  - Size
  - Structure
  - Type
  - Number
  - Populations
  - Status : viabiity, quiescence, activation, proliferation….
  - Health and disease.

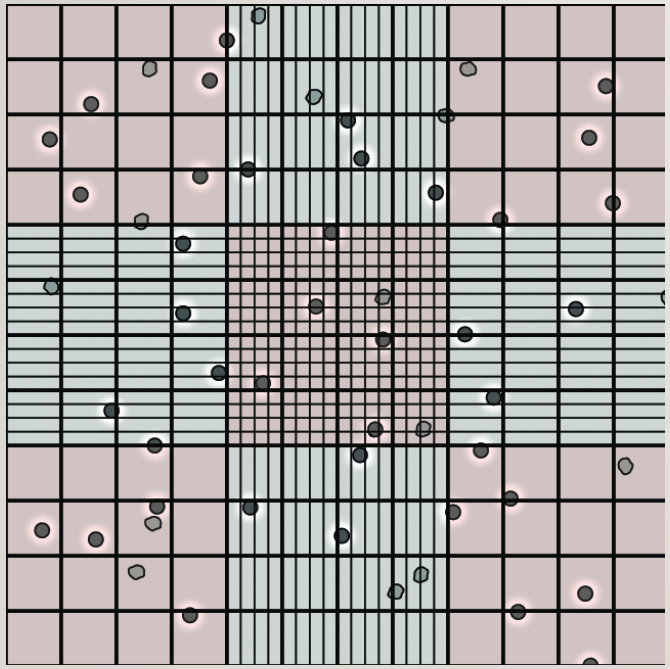


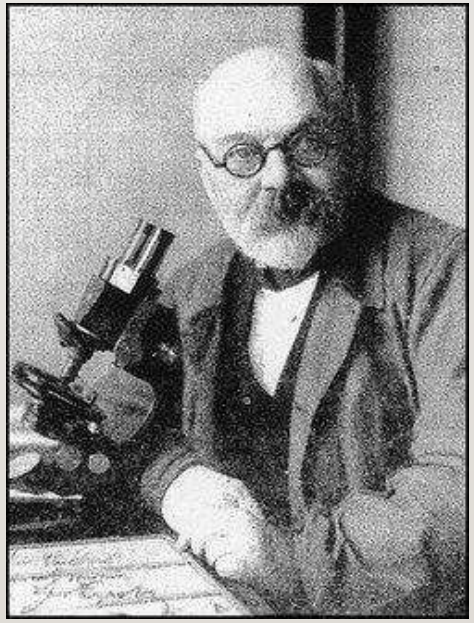
CYTOMETRY : WHICH CELLS?



- Bacteria, yeast, algae….
- Blood
  - Mature cells
  - Erythrocytes
  - Leukocytes : Polymorphonuclears, Monocytes, Lymphocytes
  - Platelets
- Bone marow
  - Immature cells, hematopoiesis
- Fluids
  - CSF
  - Vitré
  - Effusions : pleura, synovial fluid
  - Broncho alveolar lavage
- Tissues : dilacerated to obtain cell suspensions
- Cell lines…

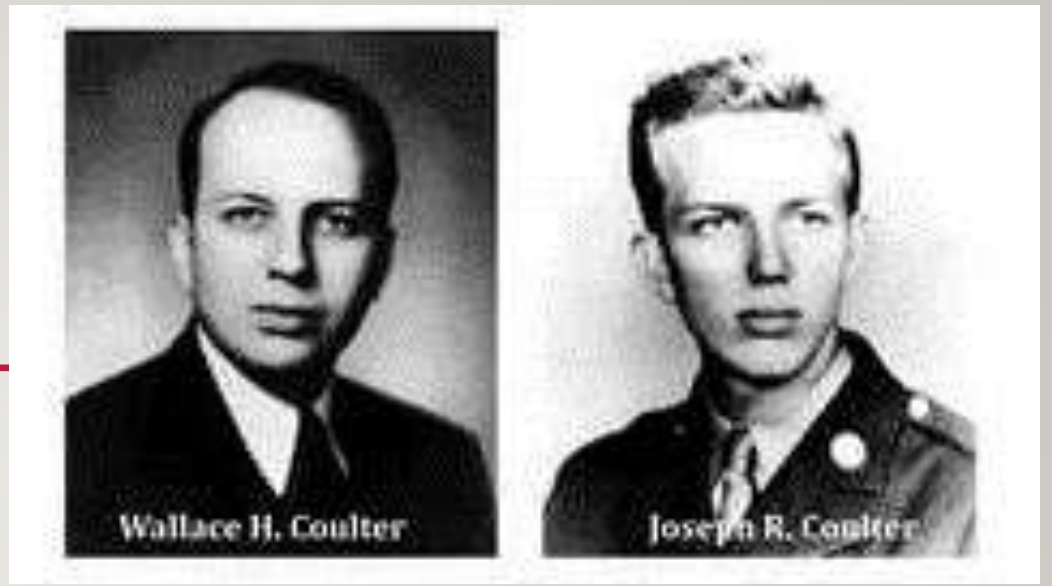
THE FIRST CYTOMETERS



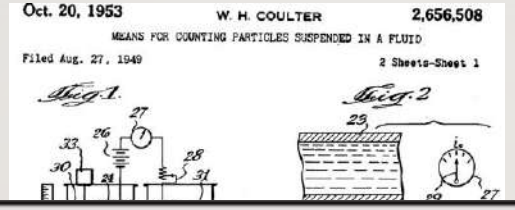


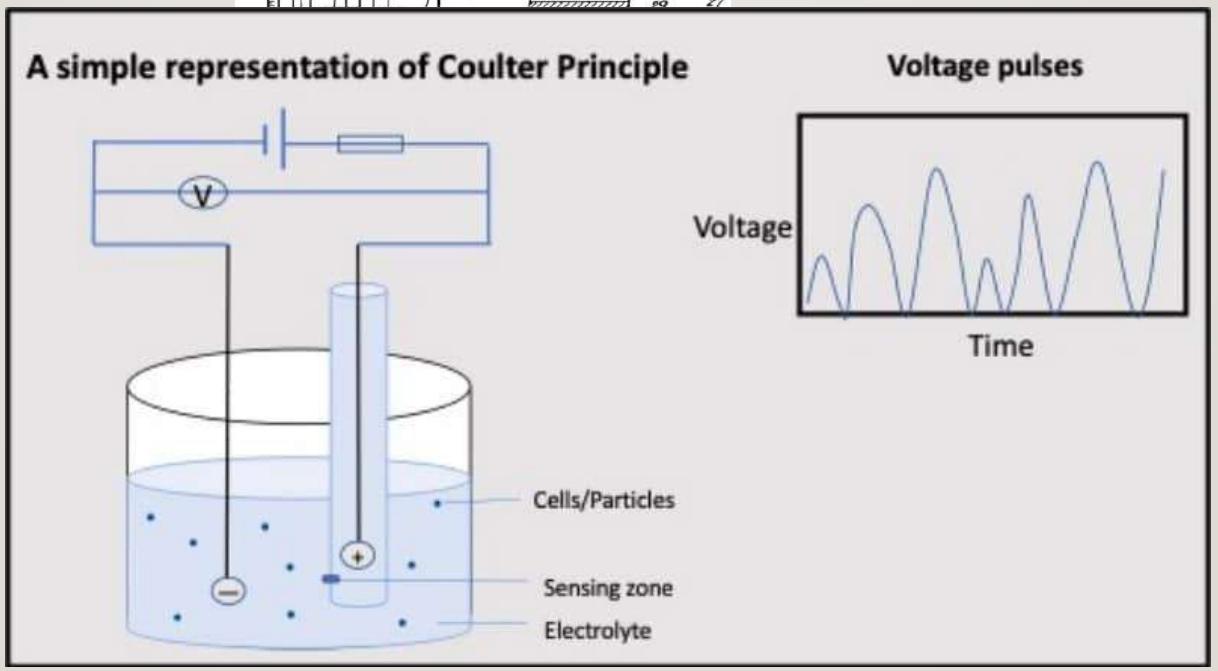
Jean Nageotte

COUNTING CELLS? THE FASCINATING STORY OF THE COULTER BROTHERS…



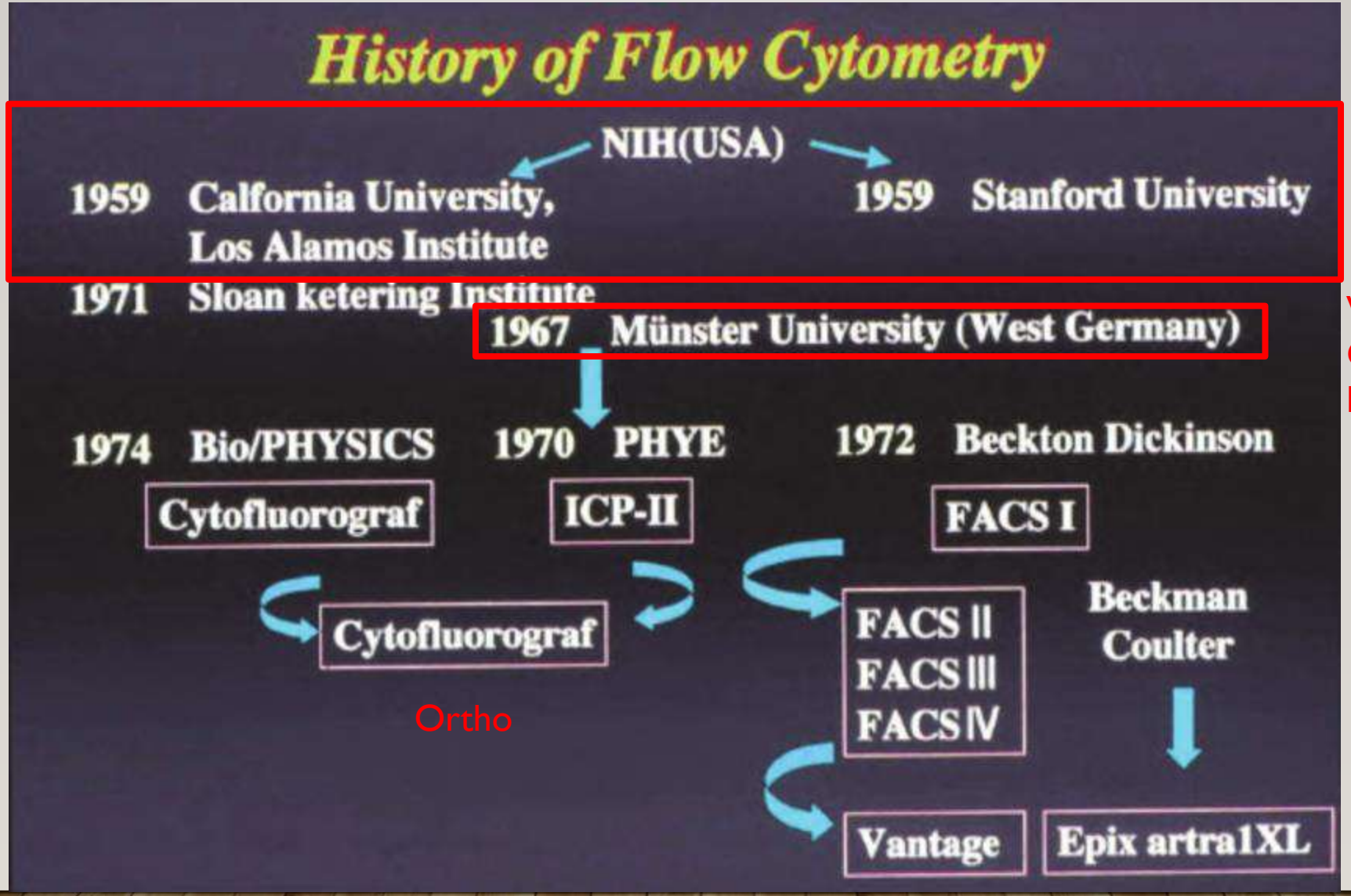
- 1948
- Winter in Chicago…
- First patent 1950
- 85 patents
- ~Almost all automated cell counters
- 5 diff for blood
- Bought by Beckman Inc. In 1997
- **Here comes Flux!**


Oct. 20, 1953 W. H. COULTER 2,656,508
MEANS FOR COUNTING PARTICLES SUSPENDED IN A FLUID
Filed Aug. 27, 1949 2 Sheets-Sheet 1
Fig. 1. Fig. 2



pHmeter inventor in1939 Arnold Beckman

MEANWHILE…



Wolfgang Göhde Partec

AT THE BEGINNING…

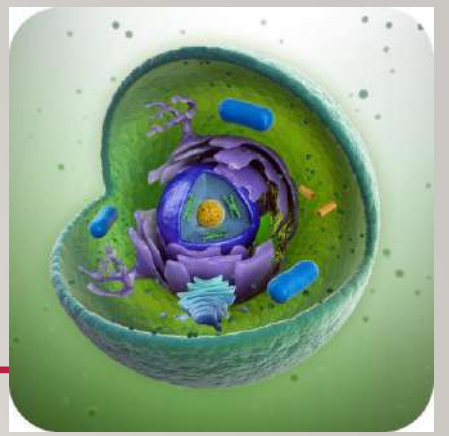
- Electronic separation by volume: Mack Fulwyler1965
- Cell sorter 1967
- Lasers 1969
- Bio/Physics Systems early Cytofluorograf **1971**
- Fluorescence activated cell sorter : FACS Len Herzenberg1972
- Creation of ISAC Schloss Elmau (Bavaria) 1978

Front Row: Leon Wheeless, Ted Young, Marvin Van Dilla, Dennis Rutovitz, Tom Jovin. Back Row: Myron Melamed, Scott Cram, Sandy Cole, Mort Mendelsohn, Hans Aus, Klaus Goerttler, Jim Tucker, Paul Mullaney, Volker Kachel, Unidentified, Brian Mayall, Mack Fulwyler

Picot et al., 2011

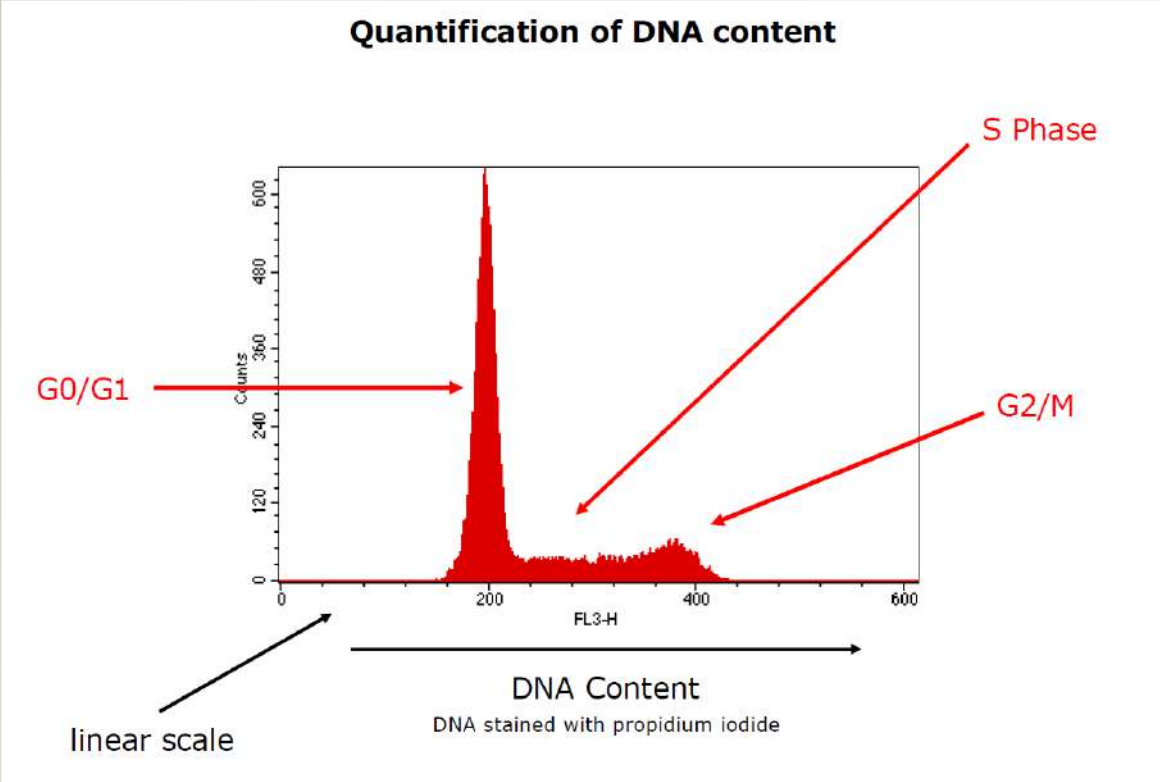
FLOW CYTOMETRY WITH DYES…

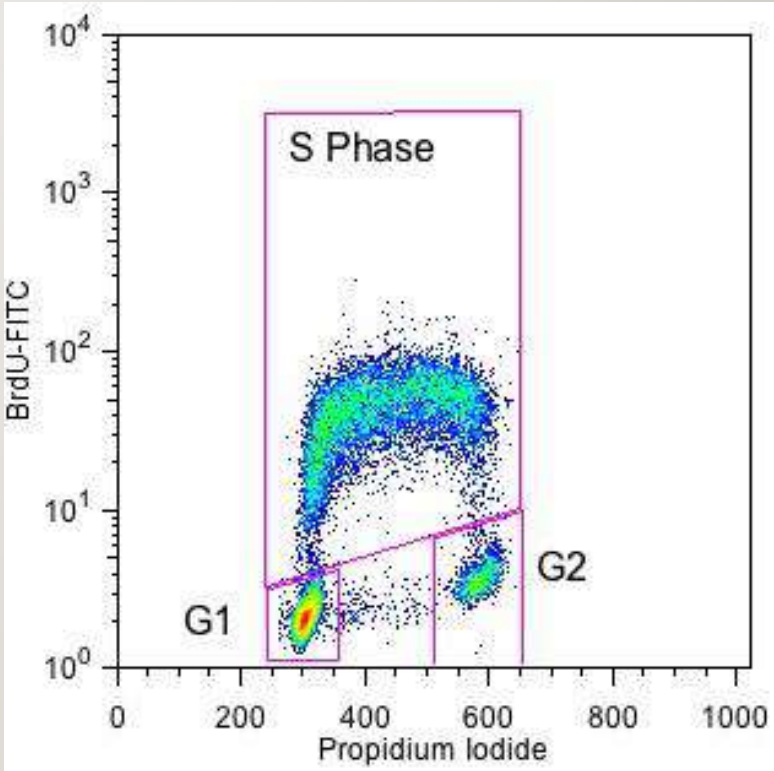
MANY APPLICATIONS IN CELL BIOLOGY



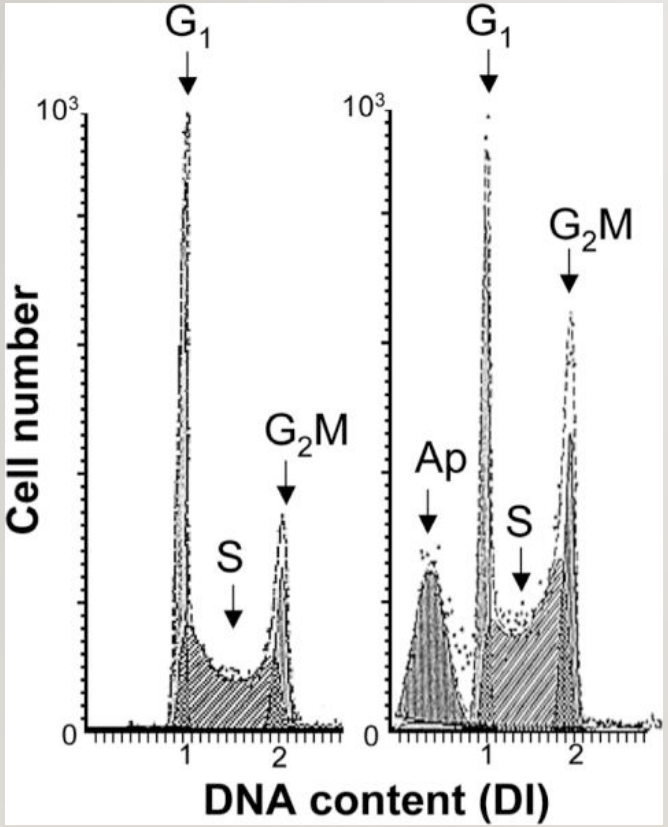
- Protein content : FITC
- Production of intracellular oxidative species, neutrophil oxidative burst : derivatives of rhodamine and ethidium
- Phagocytosis : stained beads or bacteria
- Calcium flux : fluo-3 and fura red
- Drug uptake and multi-drug resistance associated protein (MRP)
- Green fluorescent protein
- Cell proliferation: CSFE
- Intracellular pH
- Mitochondria
- Nucleic acids : acridin orange, Hoechst, propidium iodide, thiazole orange

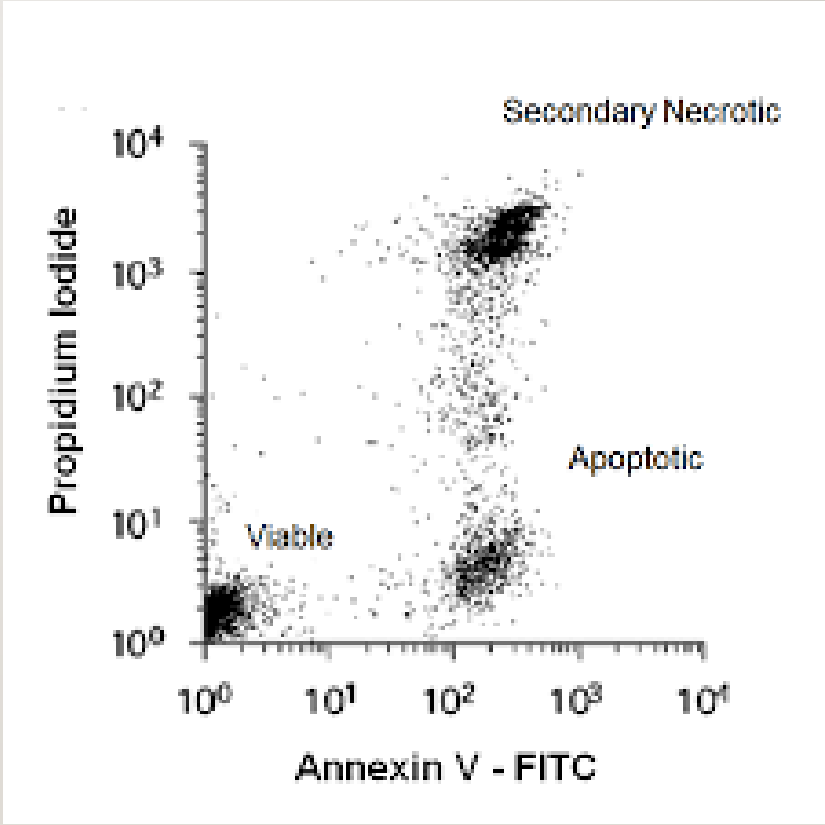
A LOT OF WORK ON DNA: CELL CYLE



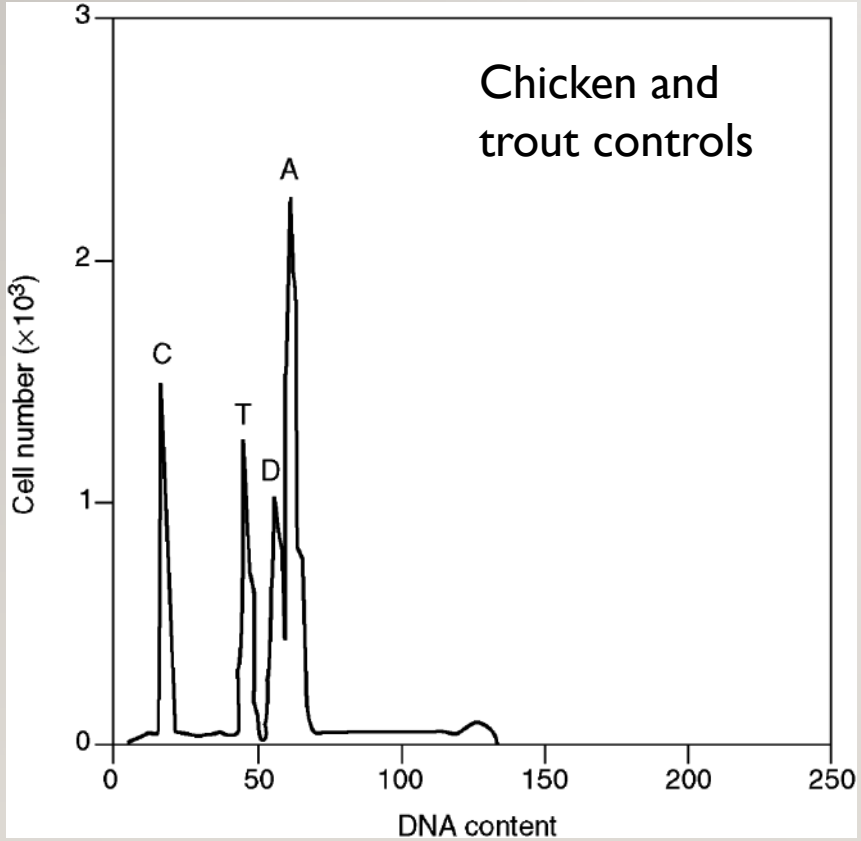


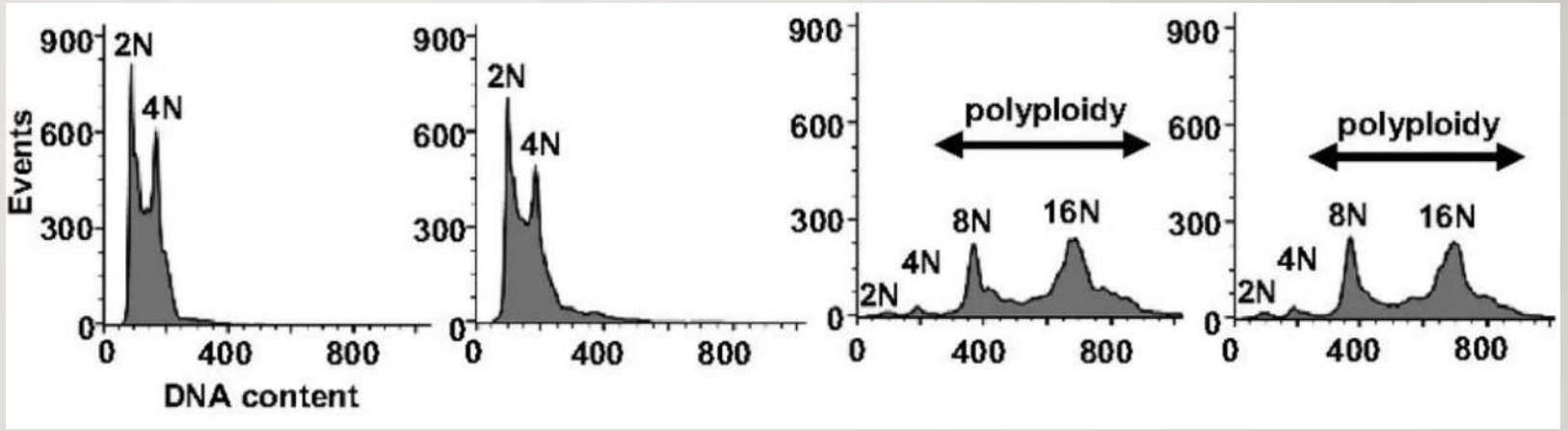
APOPTOSIS : SUB G1 OR MORE?





A LOT OF WORK ON DNA: PLOIDY (TUMORS)



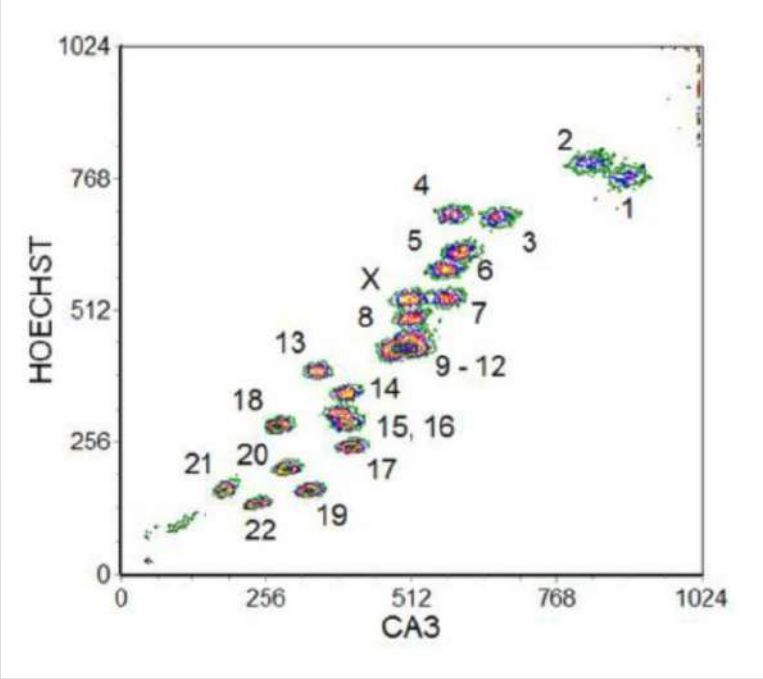


Increase of ploidy (and apoptosis) by inhibition of polo-like kinase 4

Zbignew Darczynkiewicz

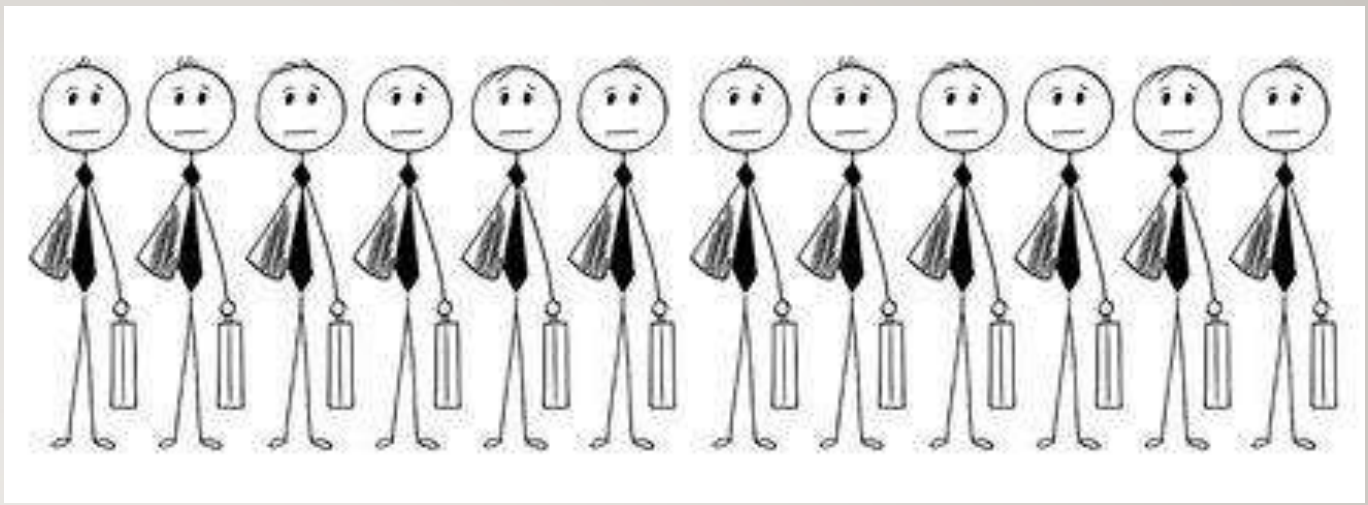
Masanori Kawakami

CHROMOSOME IDENTIFICATION AND SORTING

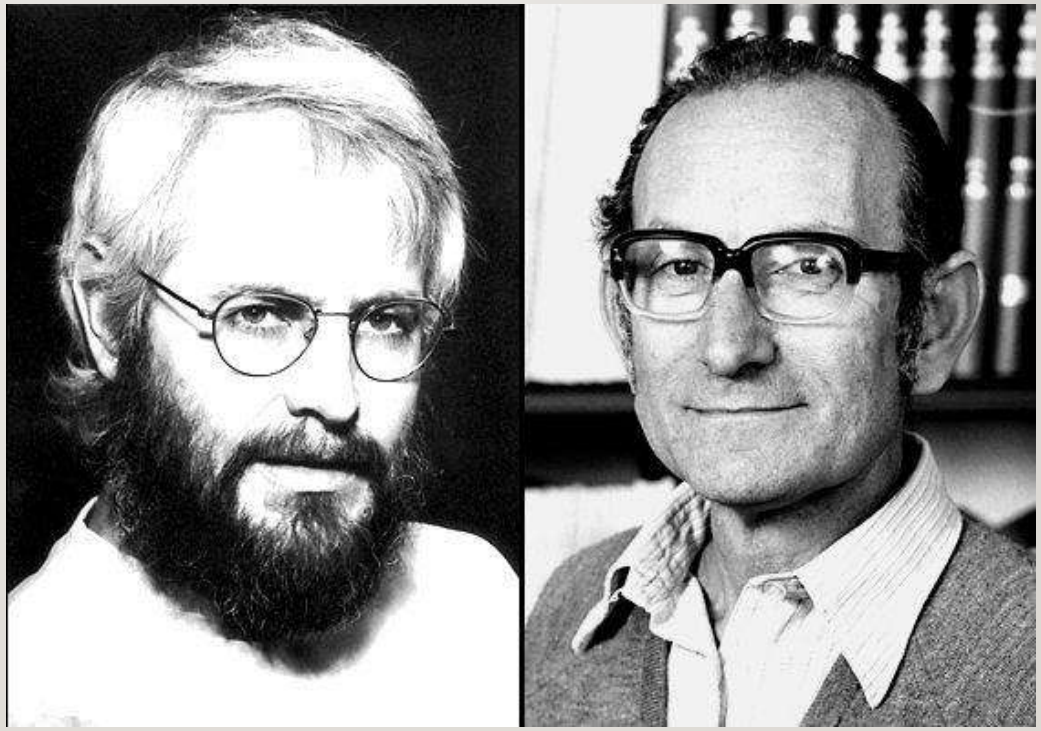


Cordelia Langford and Nigel Carter, The Sanger Centre, Cambridge, UK

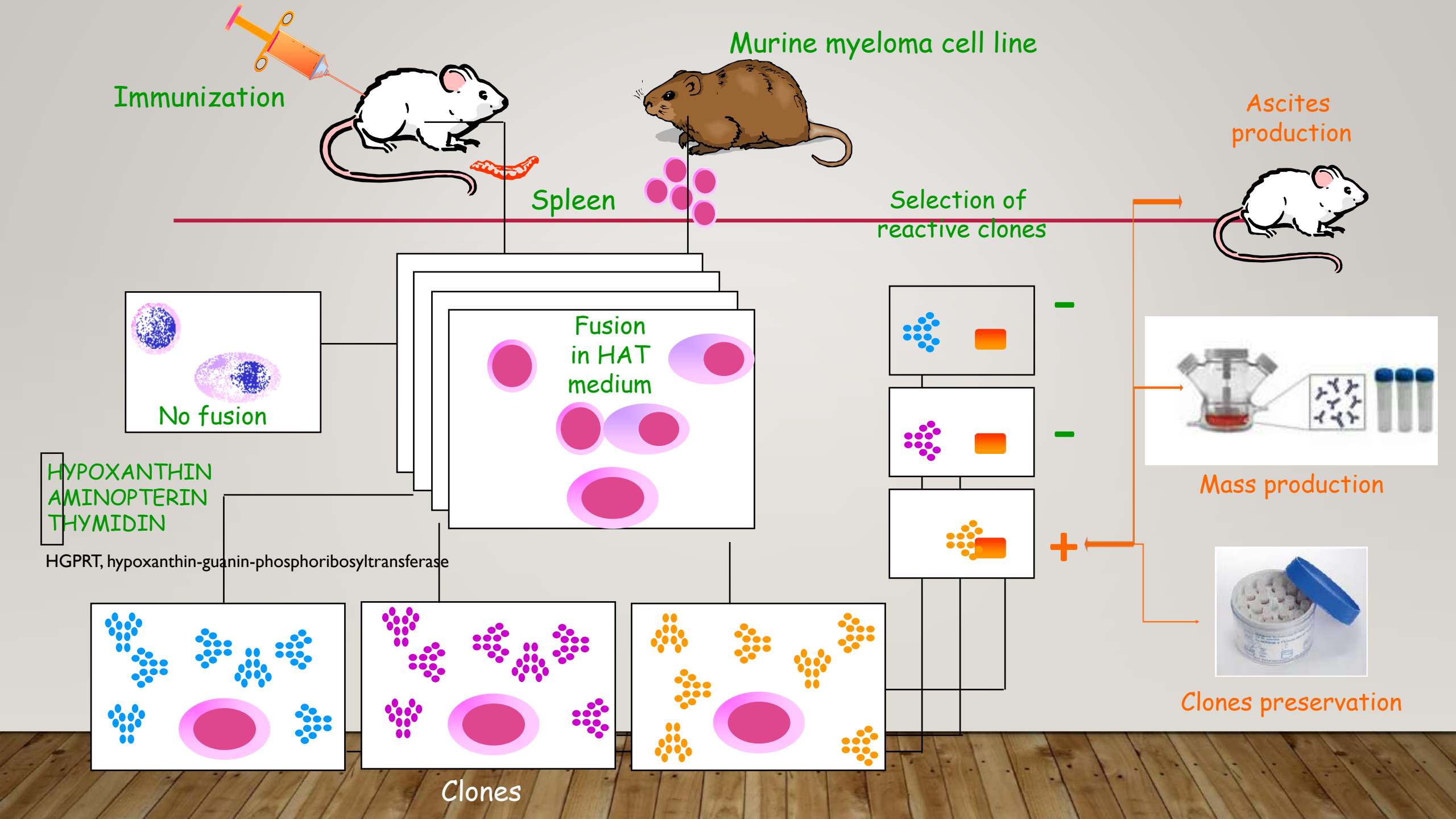
1975: THE REVOLUTION



MONOCLONAL ANTIBODIES!



Georges KOHLER César MILSTEIN 1984


Immunization
Murine myeloma cell line
Spleen
Selection of reactive clones
Ascites production
No fusion
Fusion in HAT medium
HYPOXANTHIN
AMINOPTERIN
THYMIDIN
HGPRT, hypoxanthin-guanin-phosphoribosyltransferase
Mass production
Clones preservation
Clones

SUCCESS ….AND CHAOS!

INTERNATIONAL EXPLOSION

- Production of monoclonal antibodies in numerous laboratories : mostly mouse ascites
- Identification on known/available cells in producing laboratories
- Absolutely new unknown molecules
- High level of redundancy
- Necessary organization
  - Heirloom of HLA (Jean Dausset)
  - Workshop culture (wet labs)
  - Alain Bernard and Laurence Boumsell
  - First HLDA 1982

Human Leukocyte Differentiation Antigens
then HCDM : Human Cell Differentiation Molecules

NOTION OF CD
CATHERINE HILL

- « Clusters of differentiation »
  - Groups of monoclonal antibodies recognizing the same molecule
  - By extension, this molecule
  - CD+ number ± letter : ex CD1a

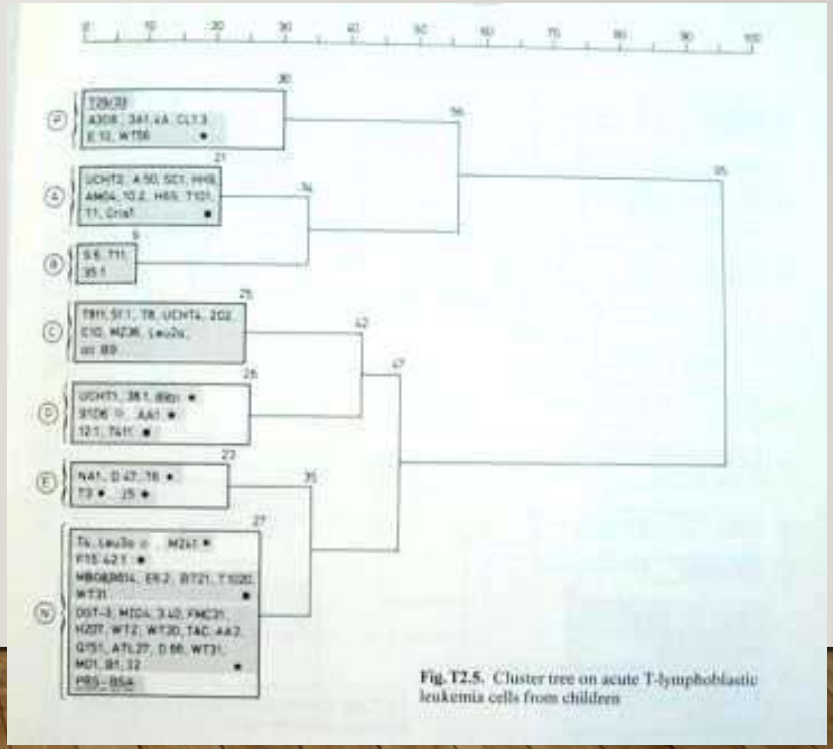
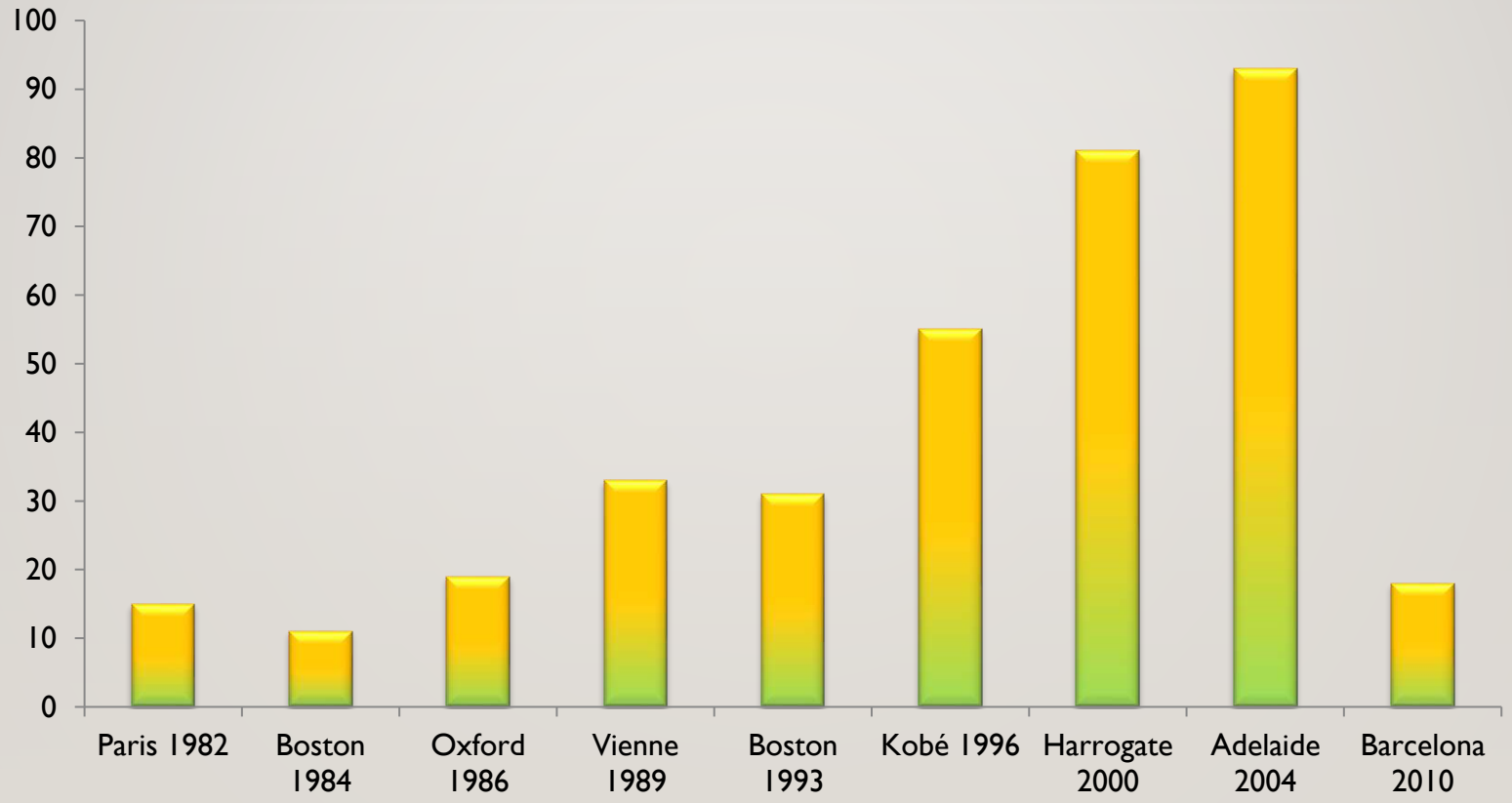
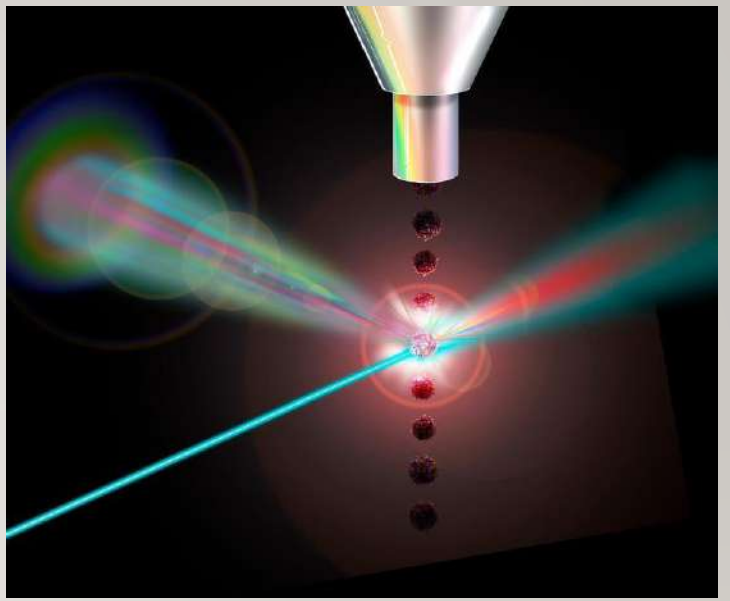


Fig. T2.5. Cluster tree on acute T-lymphoblastic leukemia cells from children

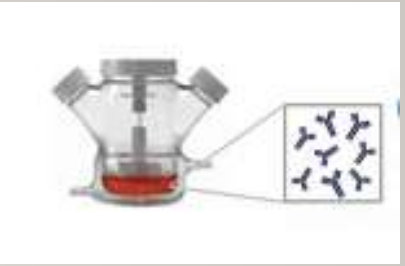
HLDA WORKSHOPS – NEW CDS





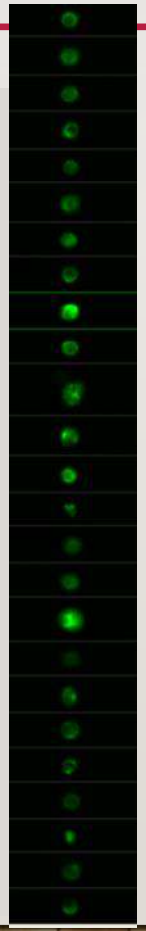
BACK TO FLOW CYTOMETRY

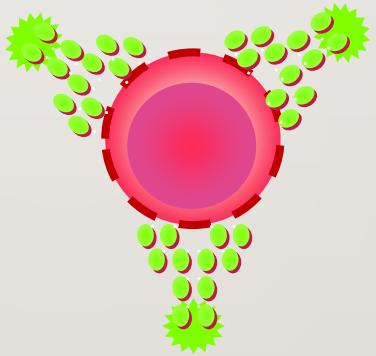
HOW TO USE MONOCLONAL ANTIBODIES?

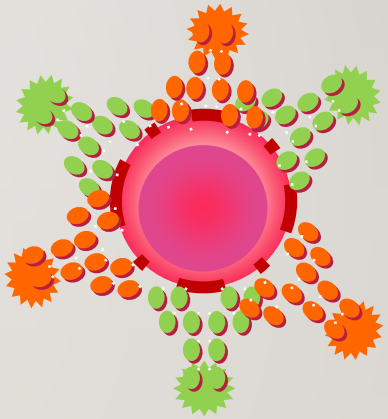


- Coupling to fluorochromes
- Excitation
- Observation of emitted light
- Can be done with a UV light microscope
- Better with an instrument:
  - More cells
  - Faster
  - In silico

Direct immunofluorescence





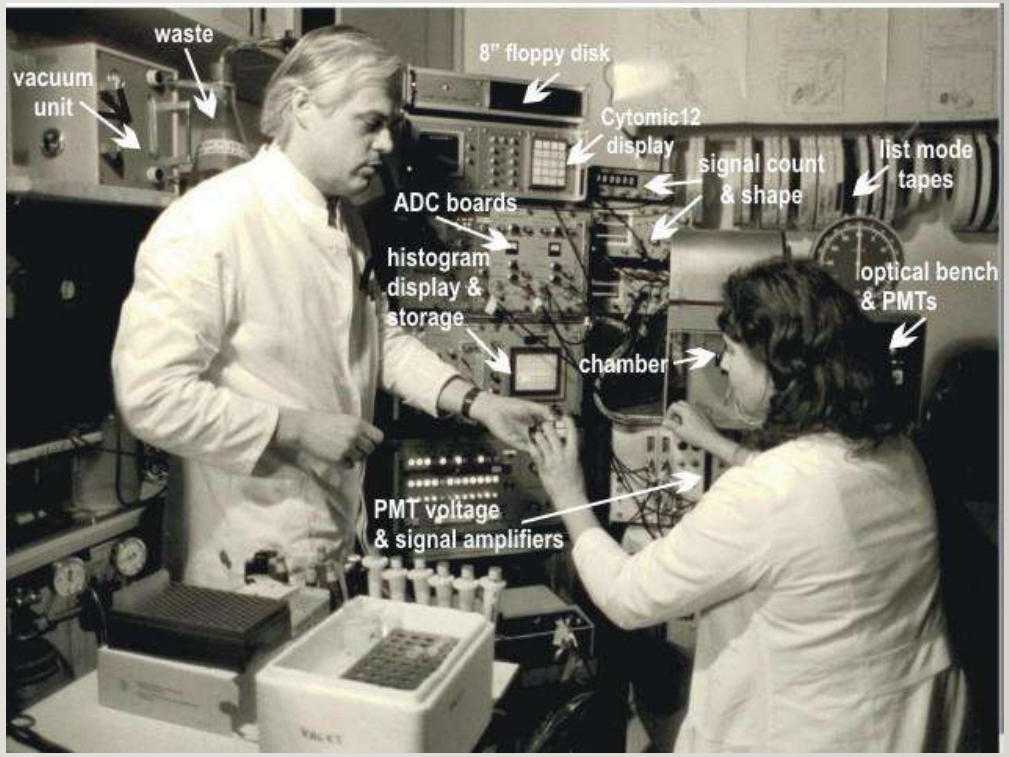


Multiparametric immunofluorescence

FROM BIG INSTRUMENTS

Howard Shapiro
1985

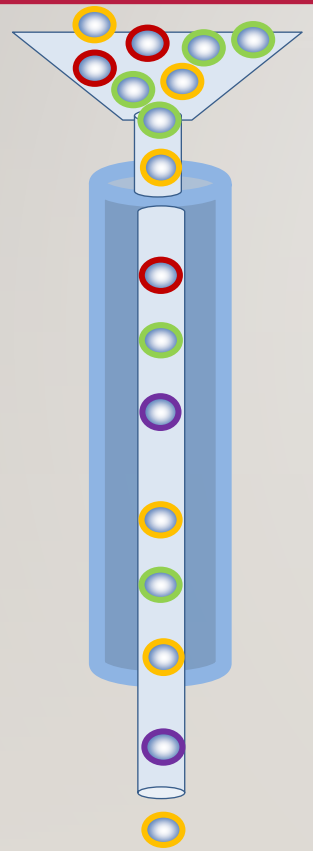
Günther Valet
1995



TO SMALLER AND SMALLER ONES

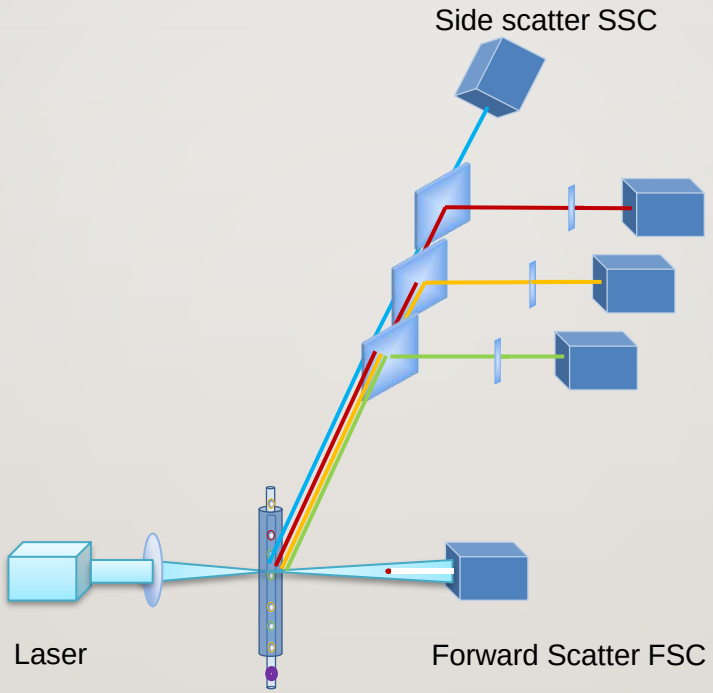
REQUIREMENTS

FLUIDICS



Hydrofocalization

OPTICS



Diffusion (FSC) and diffraction (SSC) plus emitted light for each fluorochrome

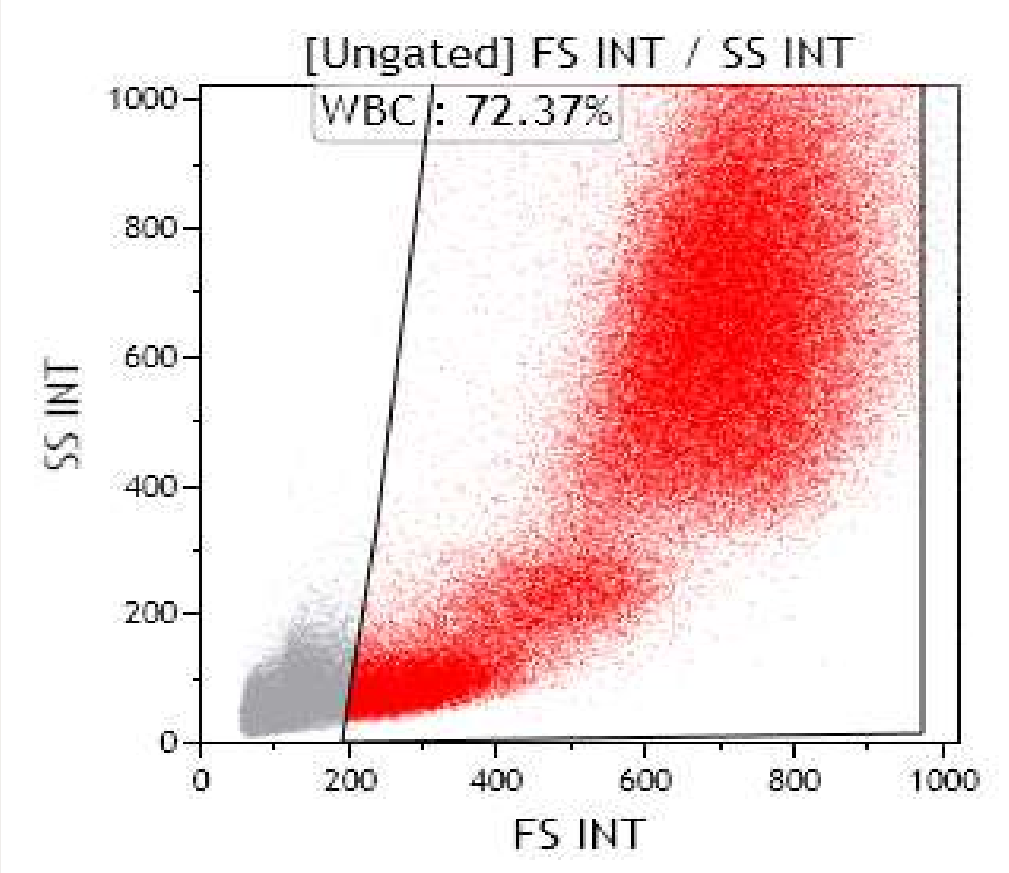
INFORMATICS

001010101011
101010100101
011101010

Voltage des photomultiplicateurs :
Compensations de fluorescence
Pour chaque cellule:
- Taille (FSC)
- Granularité (SSC)
- Fluorescence à chaque longueur d'onde d'émisssion (FL)

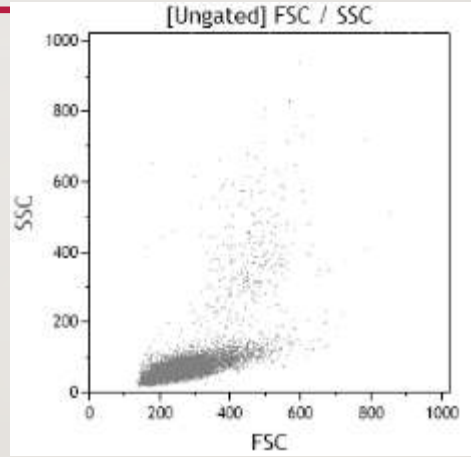
Fenêtrage
Graphiques
Hiérarchie de fenêtrage
Couleurs
Backgating

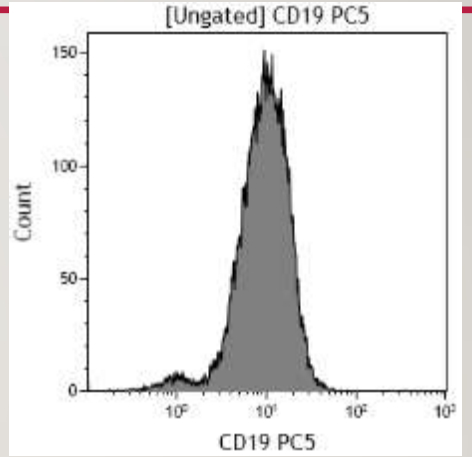
UNSTAINED BLOOD

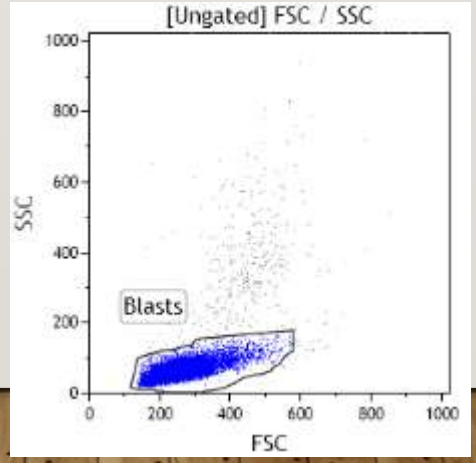


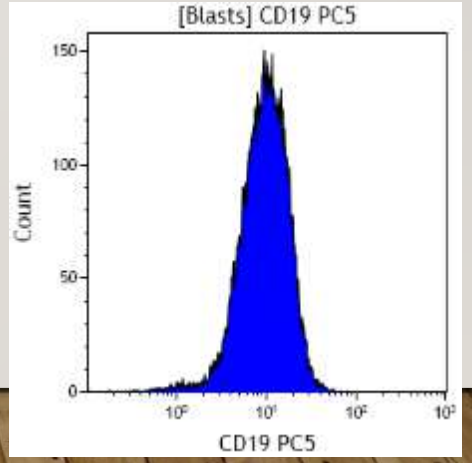
FIRST DATA ANALYSES (EXAMPLE OF LEUKEMIA)

- The simplest
  - FSC (size)
  - SSC (granularity)
  - One marker
  - Gating and coloring
  - OK if large population

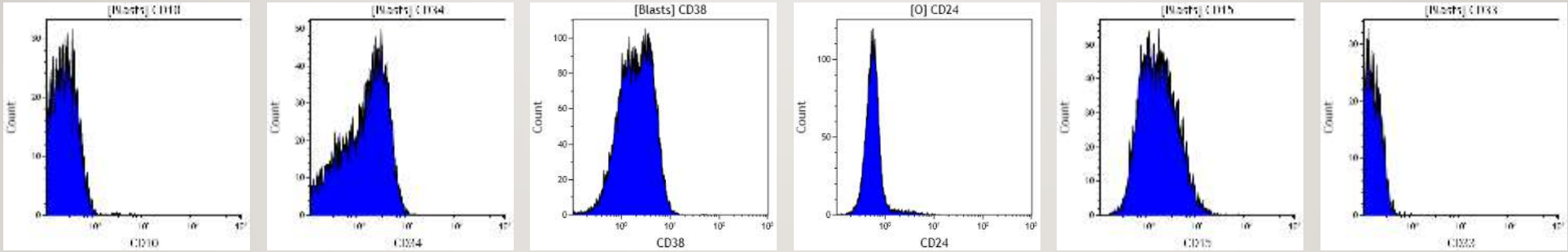




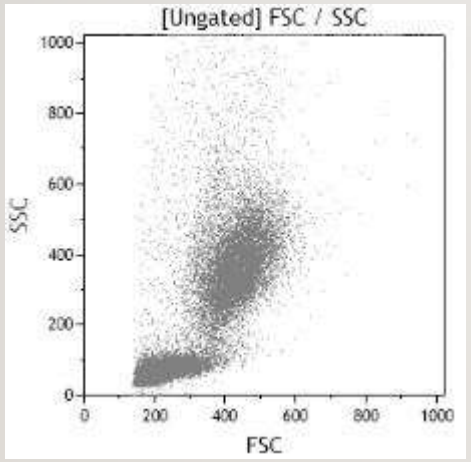


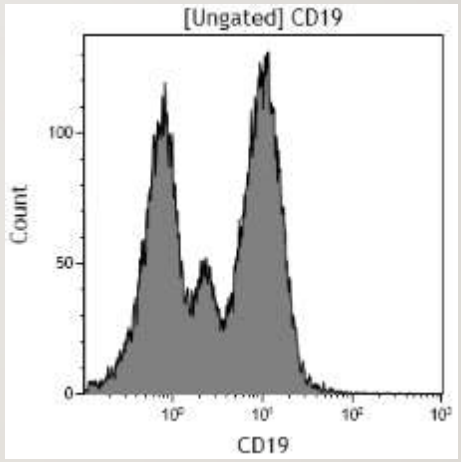


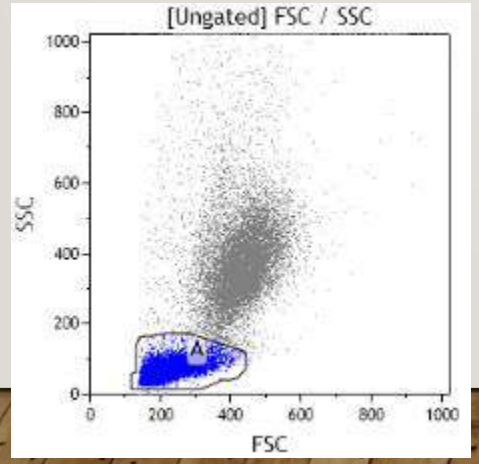
REPEAT FOR EACH MARKER….

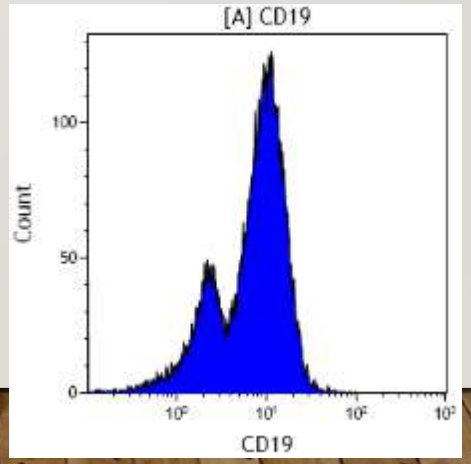


NOT ALWAYS SO SIMPLE



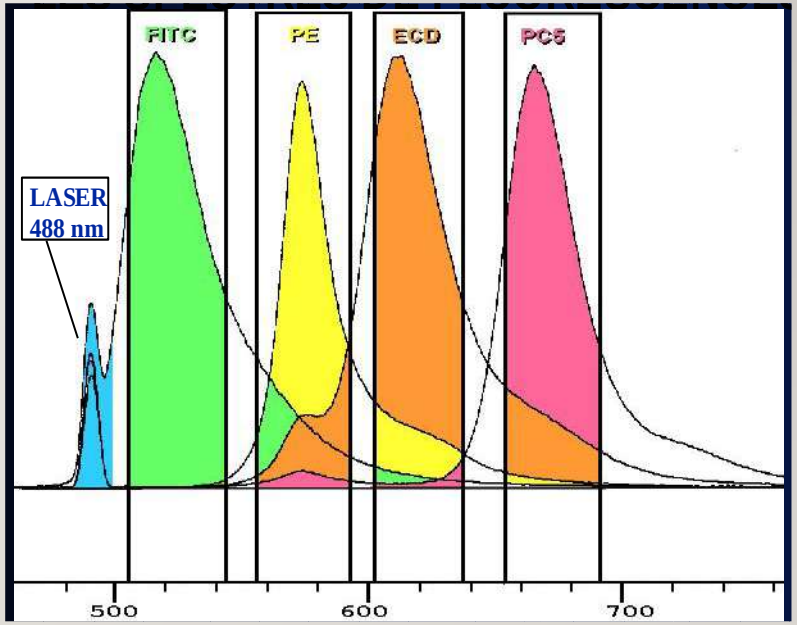


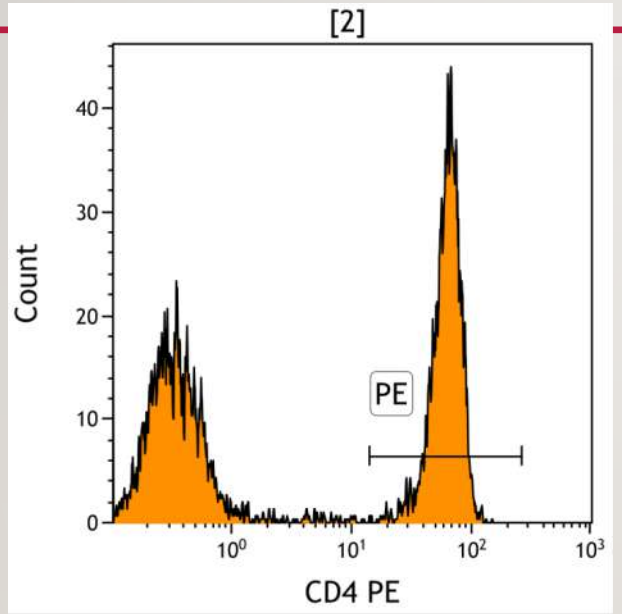




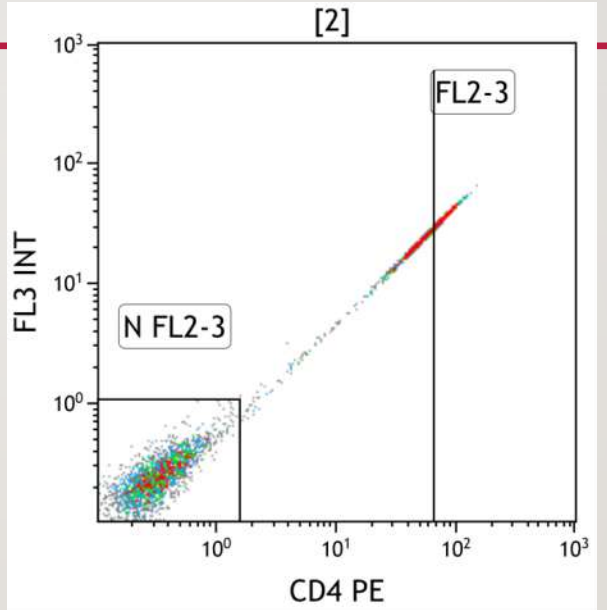
MULTIPARAMETRIC FLOW CYTOMETRY

- Several antibodies with different fluorochromes
- Identification of
  - Multiple subsets
  - Coexpressions
- Warning : fluorescence compensation required



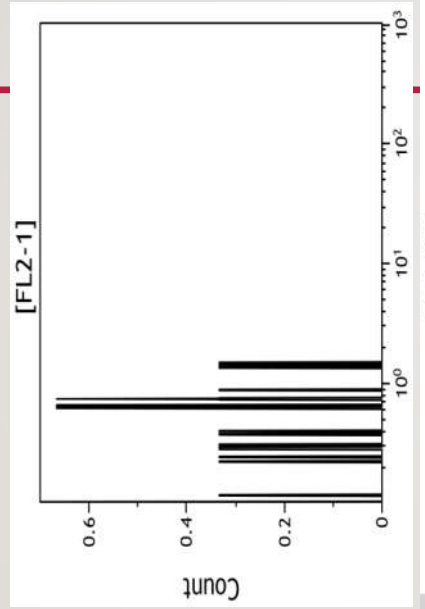


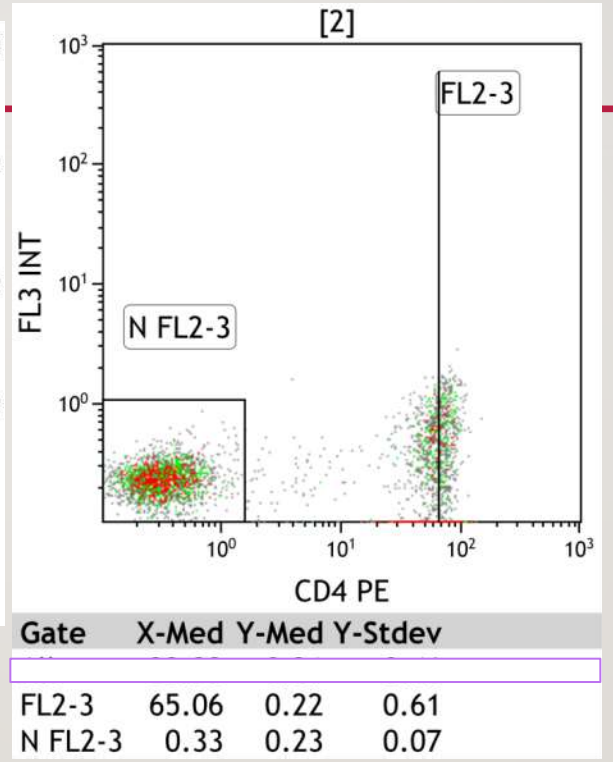
Gate	X-Med
All	33.32
PE	64.33



Gate	X-Med	Y-Med	Y-Stdev
All	33.32	14.49	14.88
FL2-3	65.06	28.72	0.63
N FL2-3	0.33	0.24	0.12

Projection of FL2-3 on the Y axis

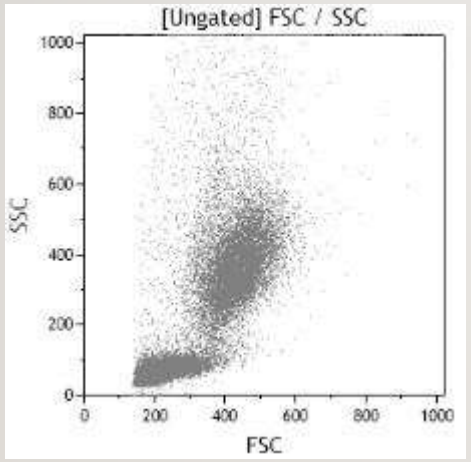


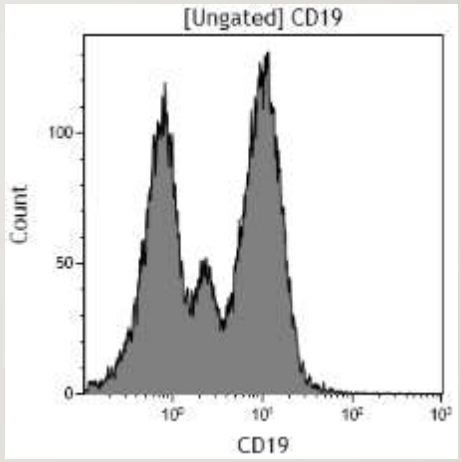


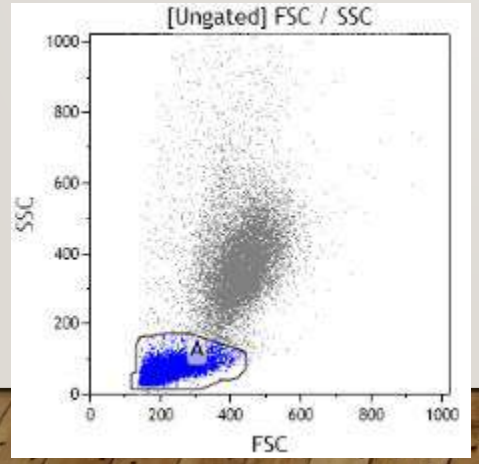
Gate	X-Med	Y-Med	Y-Stdev
FL2-3	65.06	0.22	0.61
N FL2-3	0.33	0.23	0.07

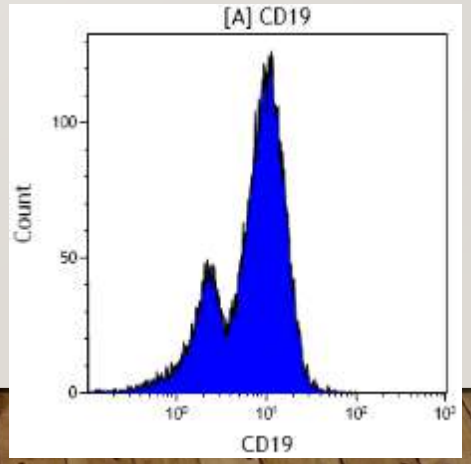
Compensation

BACK TO THIS ALL CASE

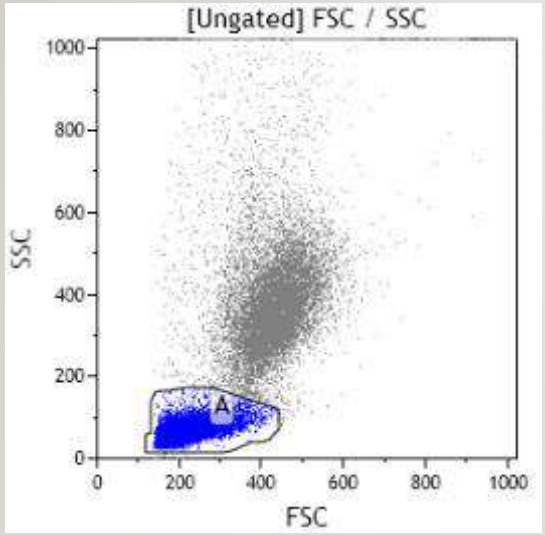


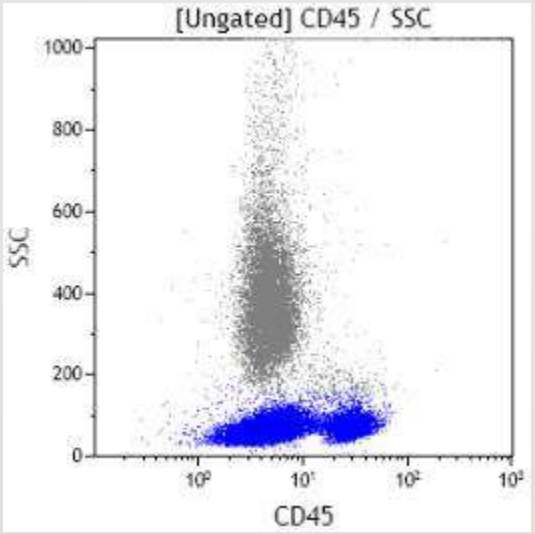


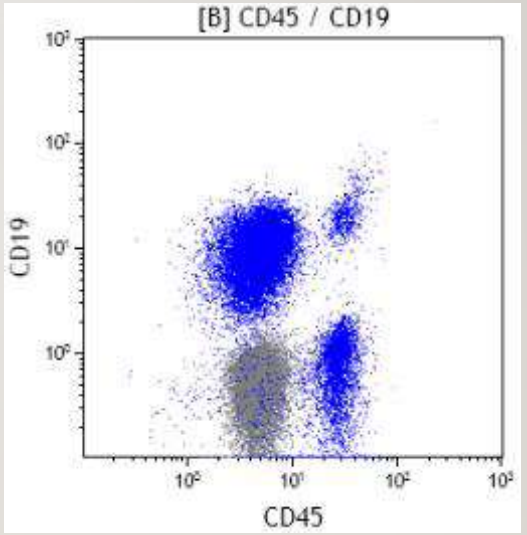




THE MAGIC OF CD45 (LACOMBE 1995)

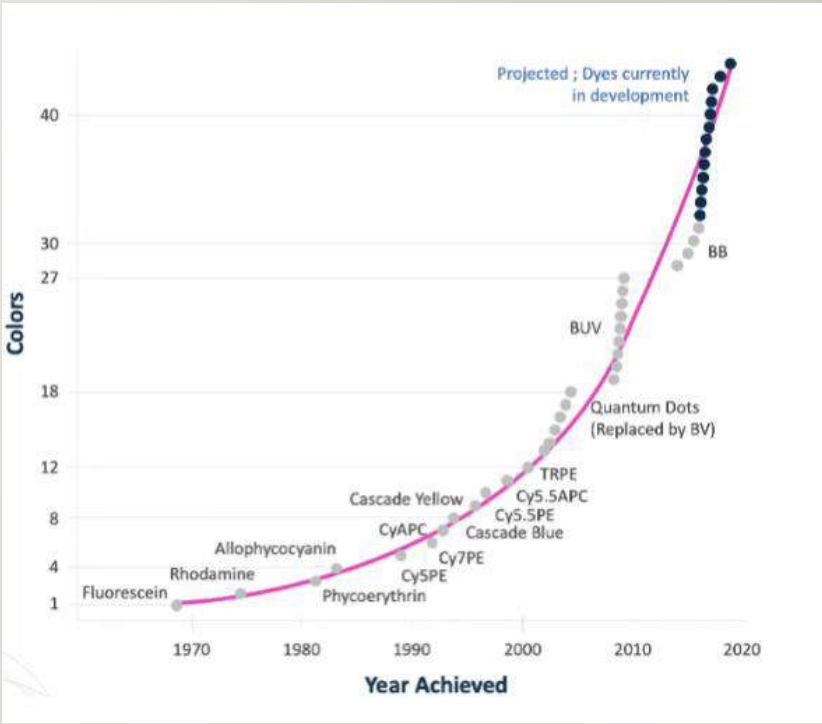


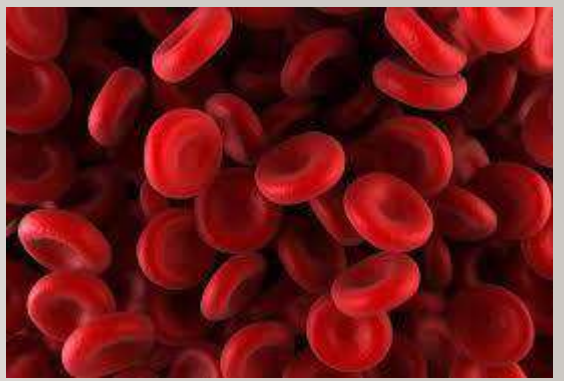




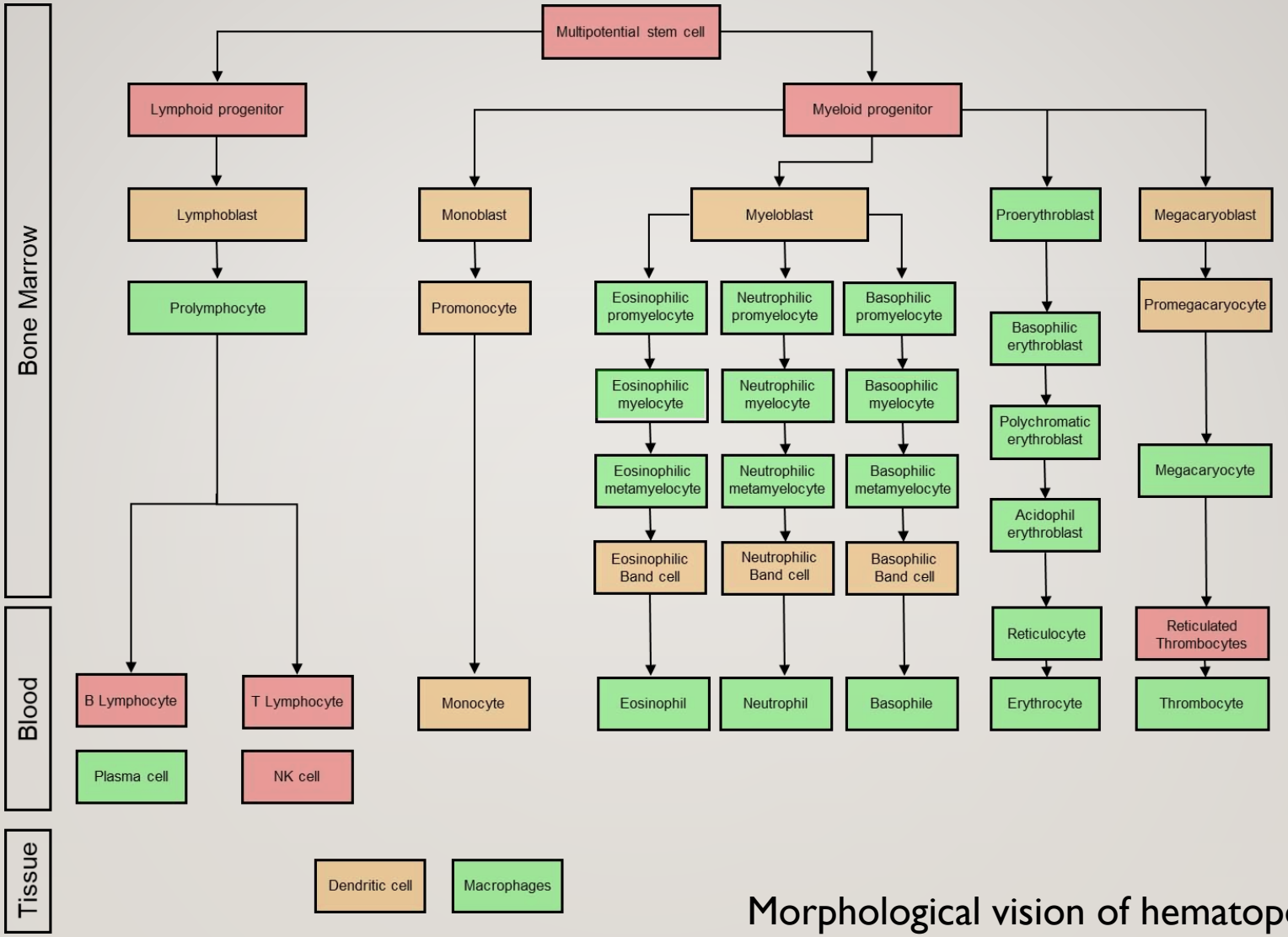
EXPANSION OF FLOW CYTOMETRY

- Expansion of panels
- Clinical/diagnosis breakthroughs
  - Viral infections
  - Normal bone marrow, leukemias, LPDs, myelodysplasias…
  - Many more: sepsis, platelets, PNH…
- Discovery of new fluorochromes
  - Sophisticated technology
  - Analysis skills
- Compensation wizards



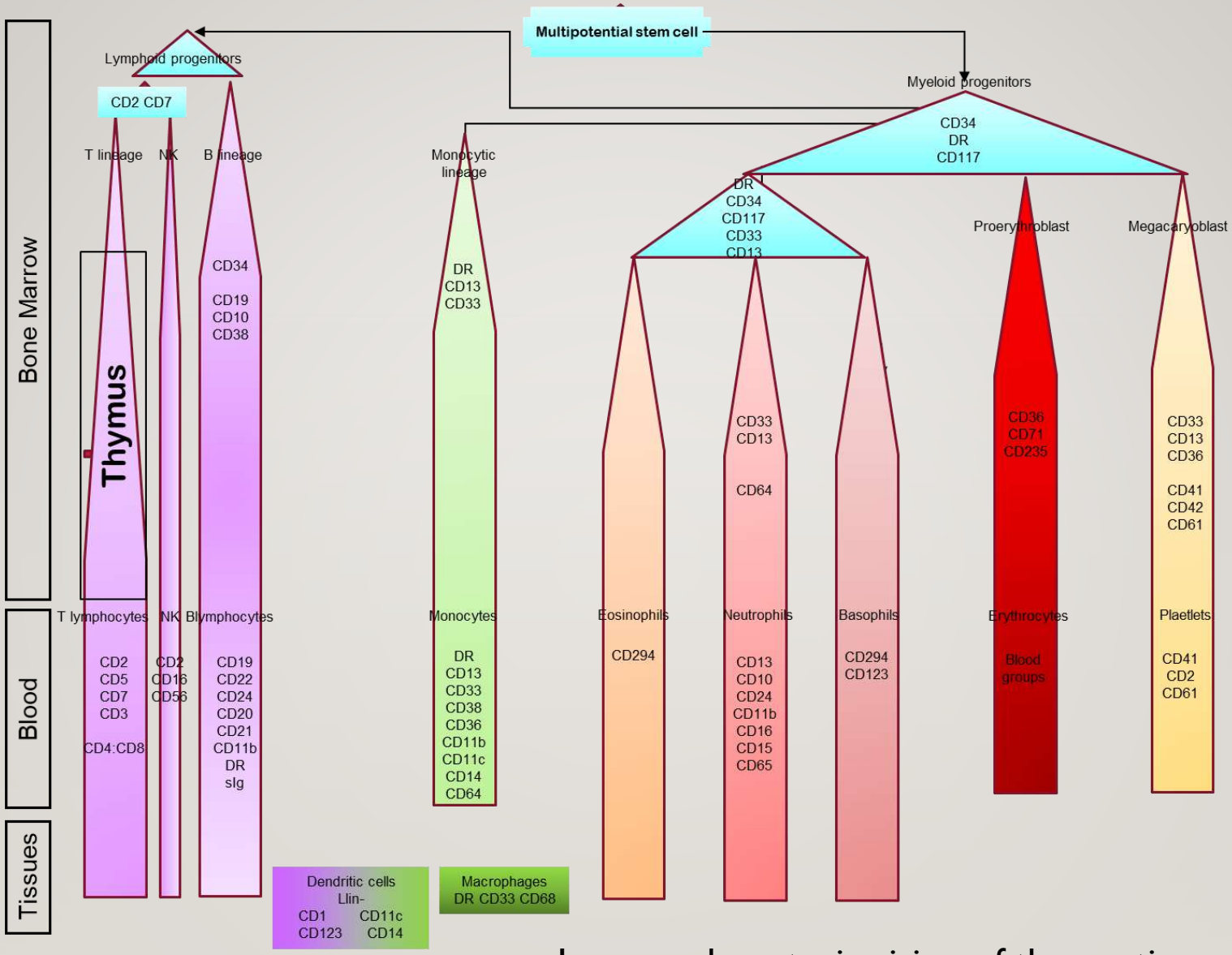


SOME APPLICATIONS IN HEMATOLOGY



Morphological vision of hematopoiesis

Laboratory Hematology Practice, Kotte Marchand & Davis, 2012



Immunophenotypic vision of the continuum of hematopoiesis

Laboratory Hematology Practice, Kotte Marchand & Davis, 2012

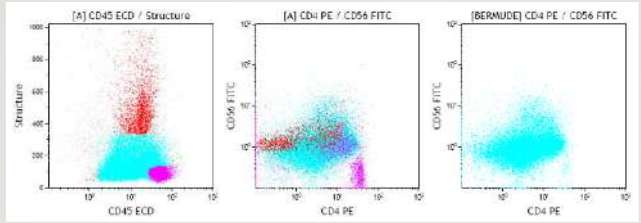
EGIL CLASSIFICATION OF ACUTE LEUKEMIAS

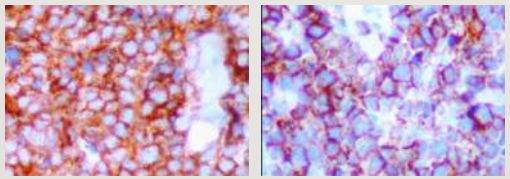
	cCD79a CD19 c/s CD22	CD10	cμ	sIg
B-I (proB)	+	-	-	-
B-II (common)	+	+	-	-
B-III (pre-B)	+	+/-	+	-
B-IV (mature)	+	+/-	+/-	+

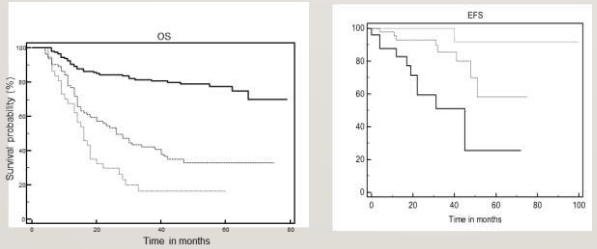
	cCD3 CD7	CD2 CD5	CD1a	sCD3 CD1a-
T-I (pro-T)	+	-	-	-
T-II (pre-T)	+	+	-	-
T-III (cortical)	+	+	+	-
T-IV (mature)	+	+	-	+

Leukemia, 1995

Blastic plasmacytoid dendritic cells neoplasms *Blood 2002*







MRD in acute leukemias
Hematol Oncol, 2017, 2018

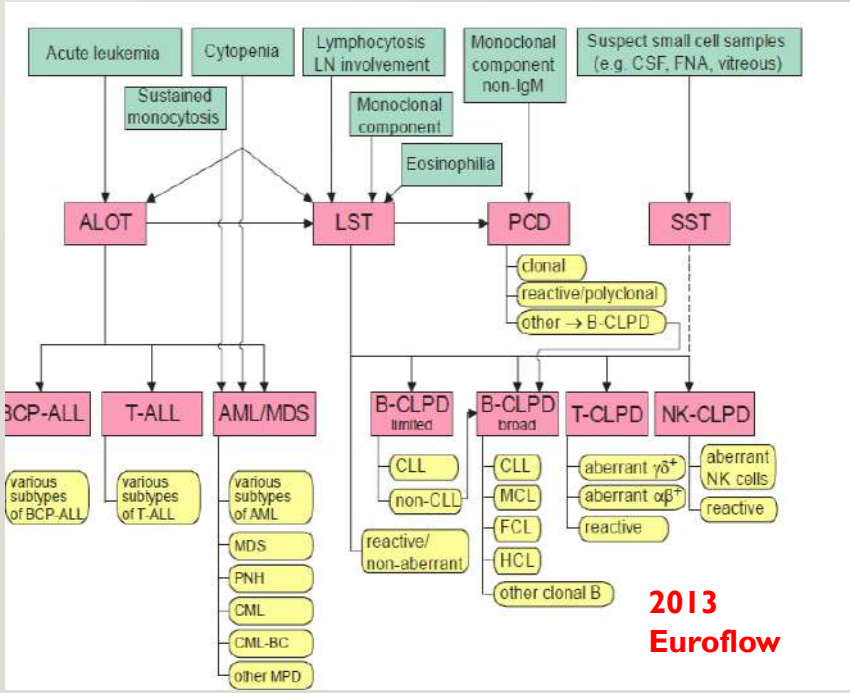
CONSENSUAL EUROPEAN PANEL, EUROPEAN LEUKEMIANET (2005 & LEUKEMIA 2011)

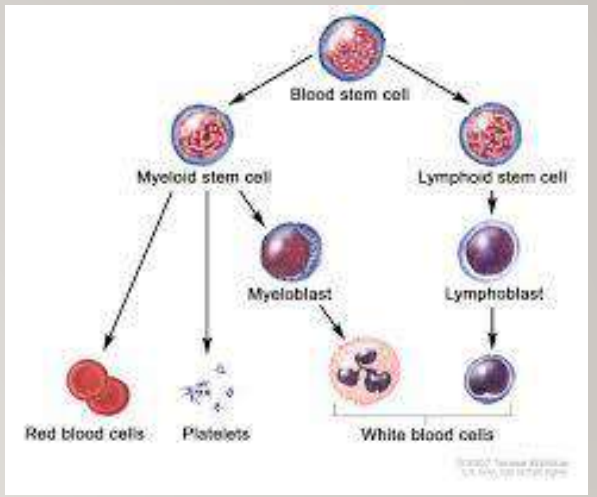
For quick orientation or paucicellular samples

- cCD3, MPO, cCD79a, TdT
- CD7, CD2, CD10, CD19, CD22 (s or c), sIg, CD13, CD33, CD34
- CD45 for gating purposes

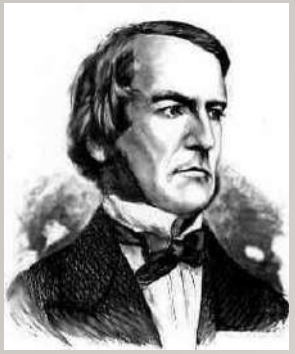
Sublineage classification and definition of clinical entities (also with adapted gating strategy)

- DR, CD1a, CD4, CD5, CD8, CD3 (m), IgM (c), CD14, CD117, CD56, CD65, CD41 or CD61, RBC marker such as glycophorin A
- *Additional useful markers (addendum) especially for BAL identification*
- TCR, CD20, CD24, CD15, CD64



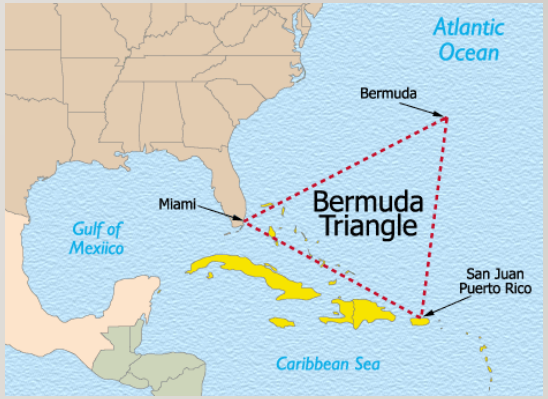


HOW TO FIND THE PROGENITORS?

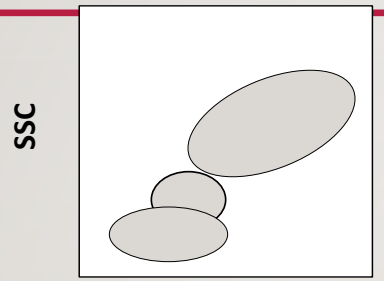


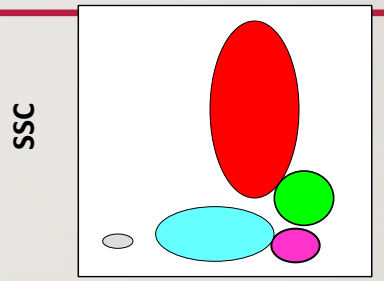
WITH THE HELP OF GEORGE BOOLE
ARNOULET ET AL. 2010

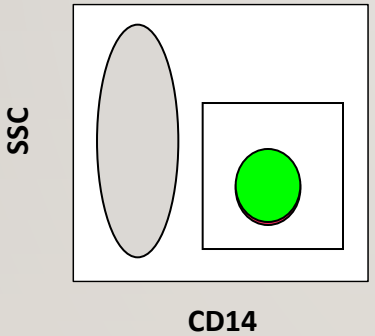
- Positive identification of bone marrow mature cells
- Color code
  - CD14/CD11b : monocytes
  - CD16/CD11b : granulocytes (NK, B cells)
  - CD45 bright : lymphocytes
- Boolean selection
  - NOT monocytes AND NOT granulocytes AND NOT lymphocytes
  - What is left : « bermudes »

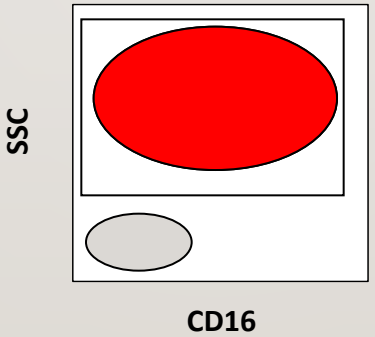


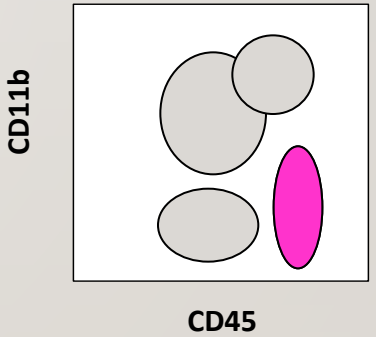
Application

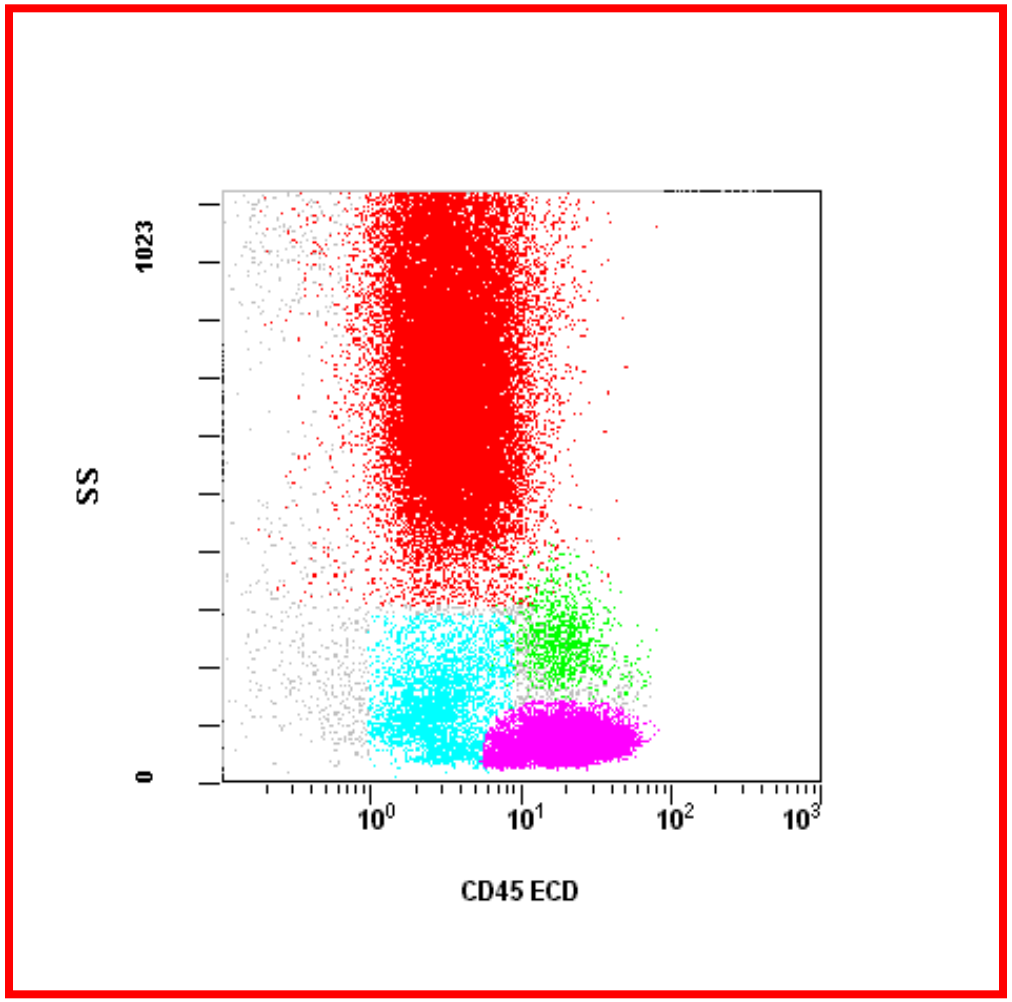




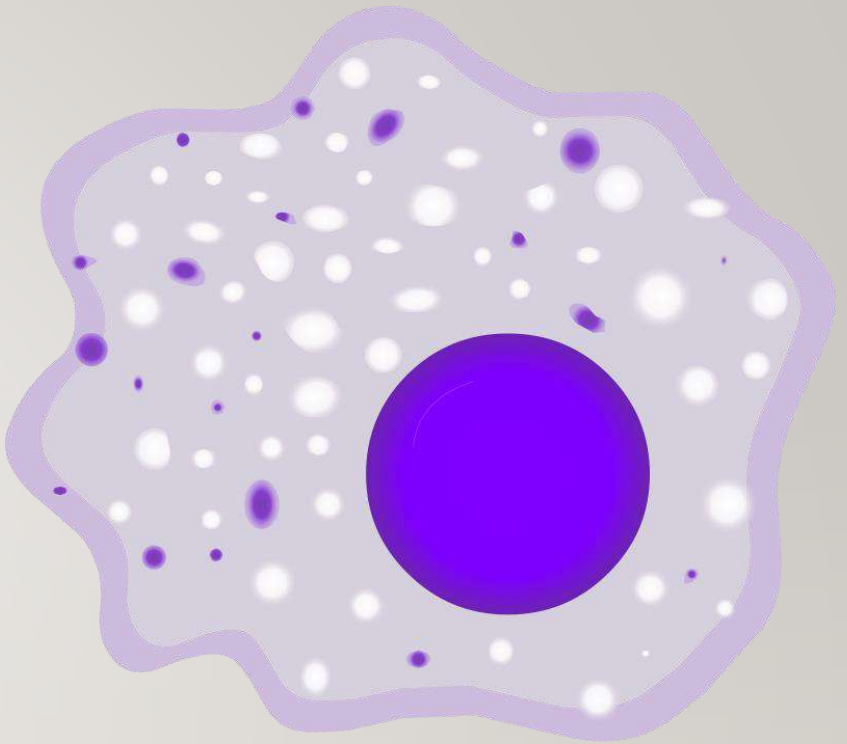




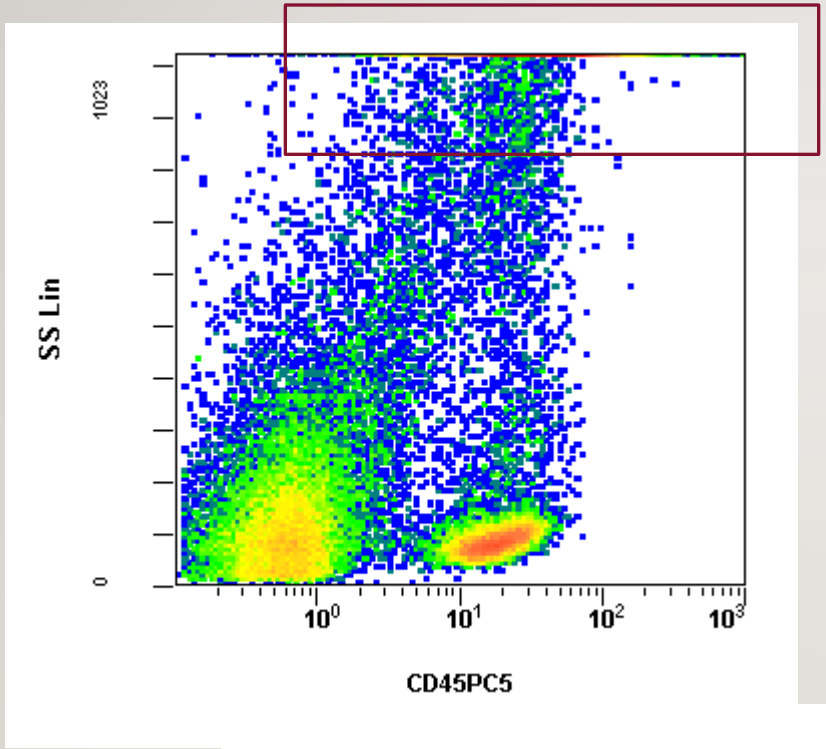


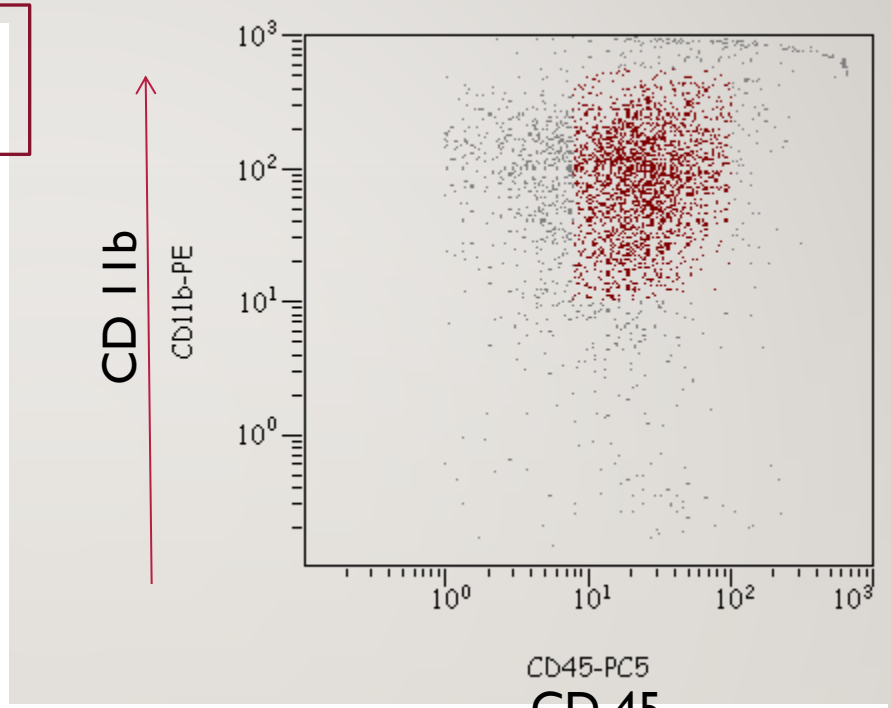

SS
1023
0
10^0
10^1
10^2
10^3
CD45 ECD

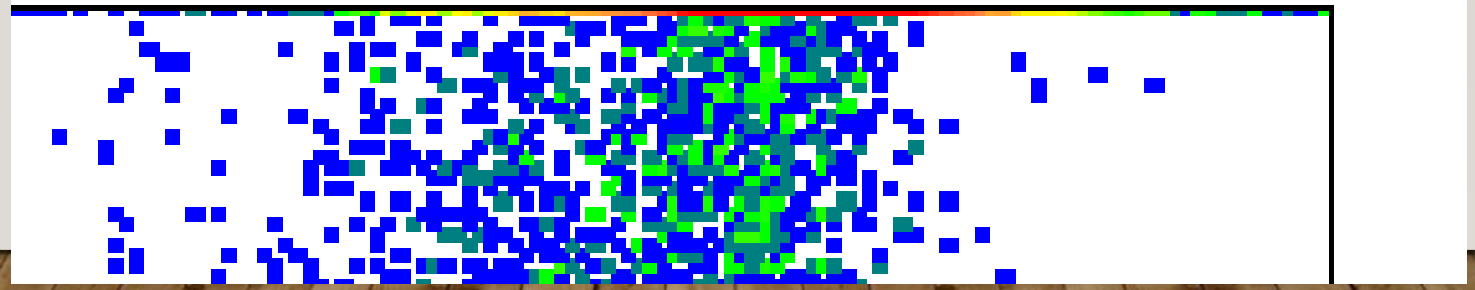
BIG CELLS



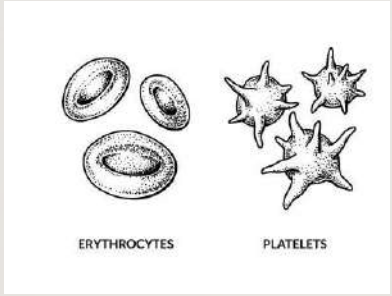
BAL ELUSIVE MACROPHAGES…



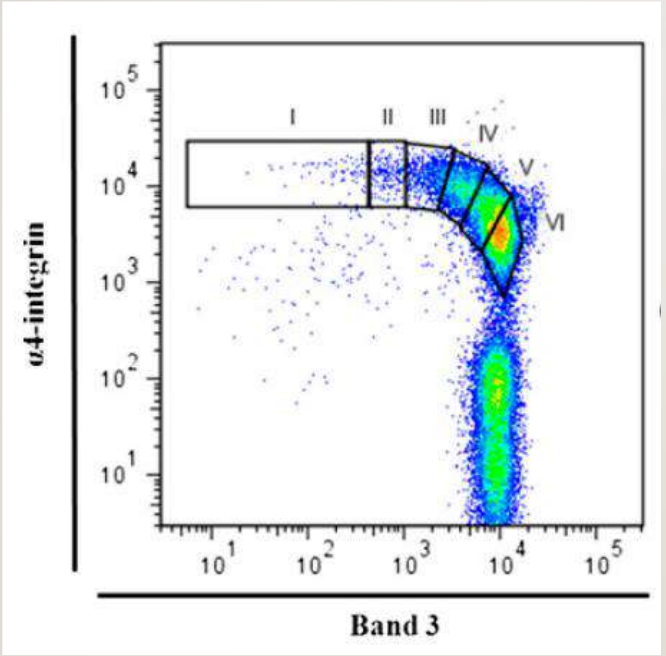


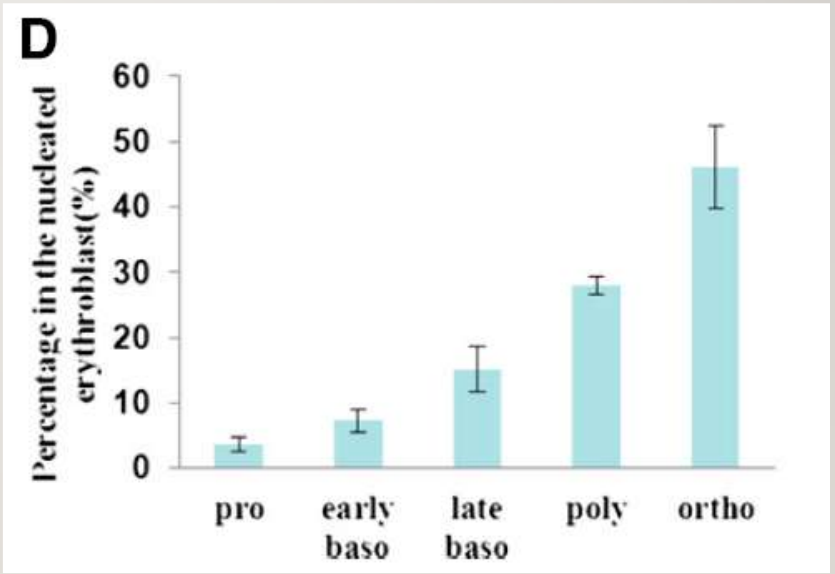


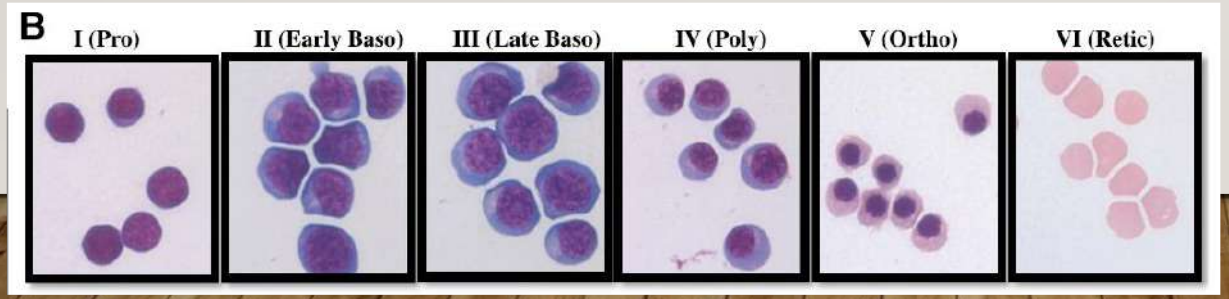
SMALL CELLS



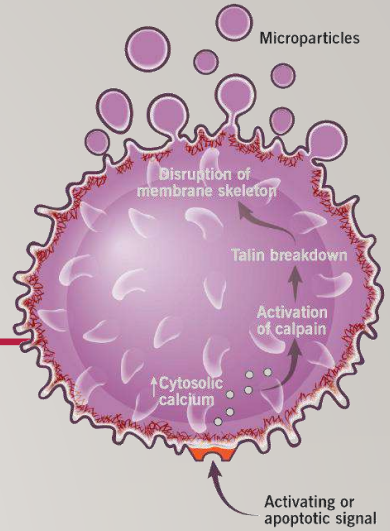
ERYTHROID MATURATION

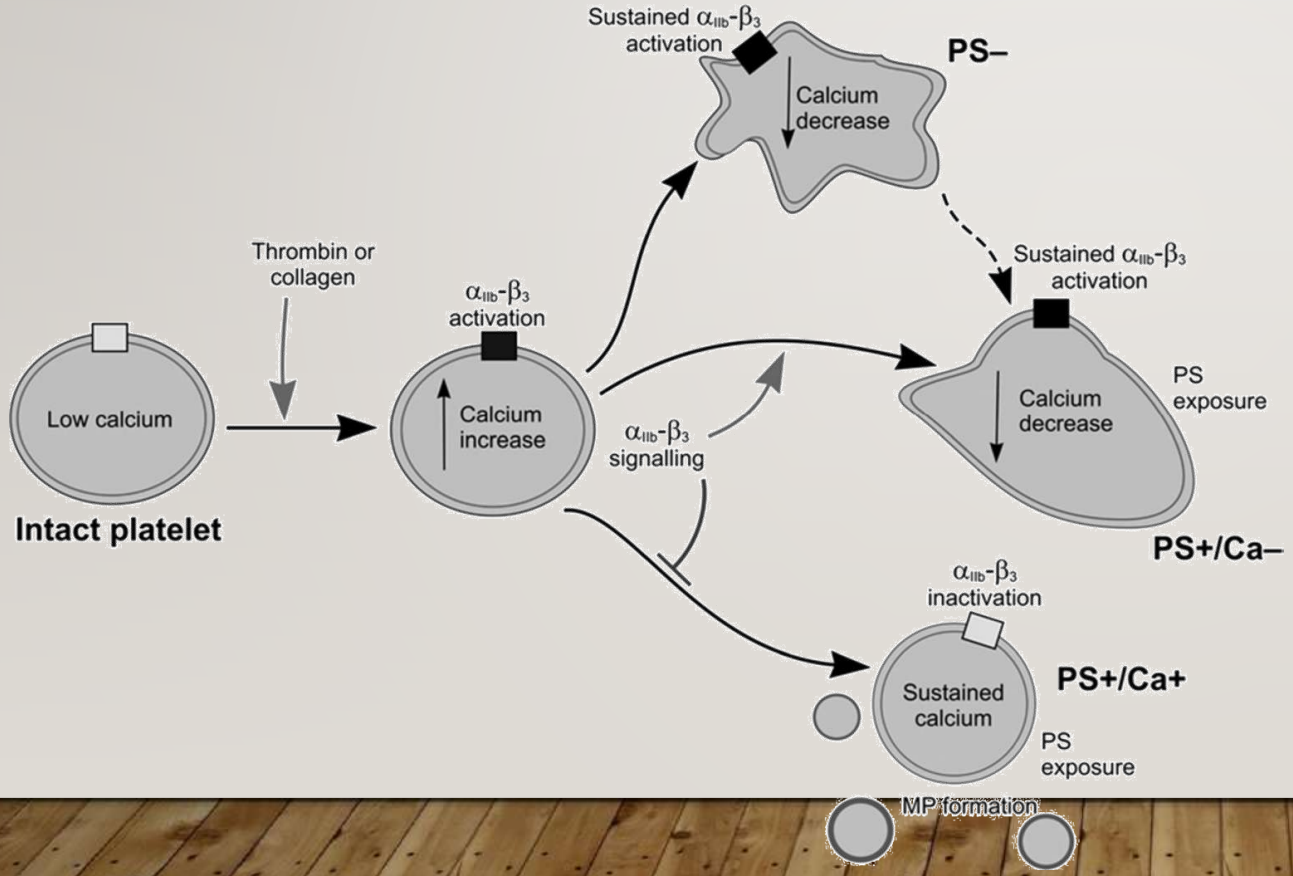


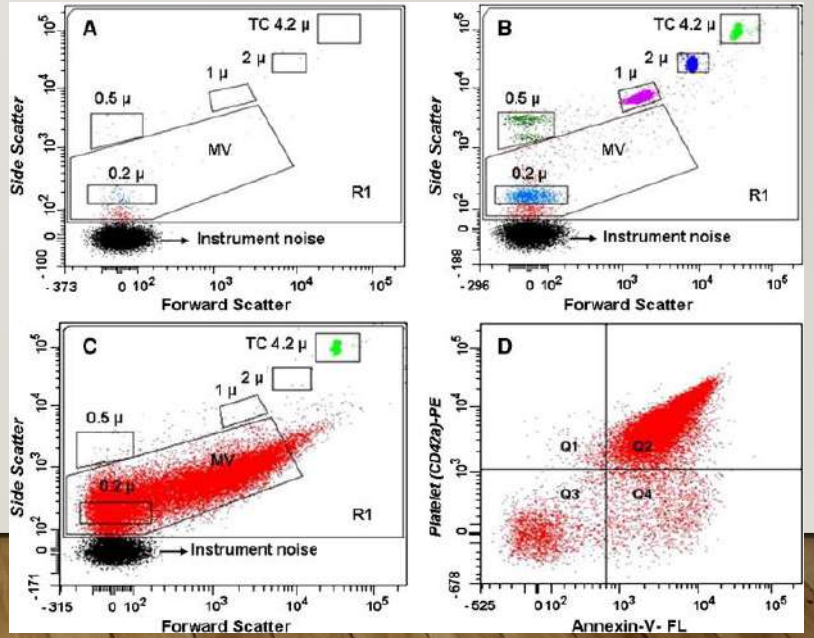




PLATELETS AND MICROPARTICLES…



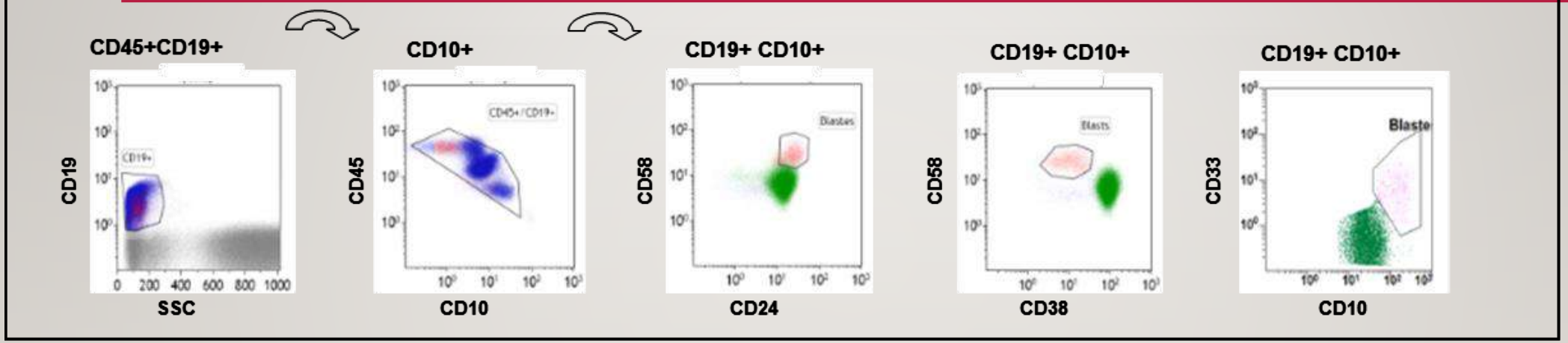




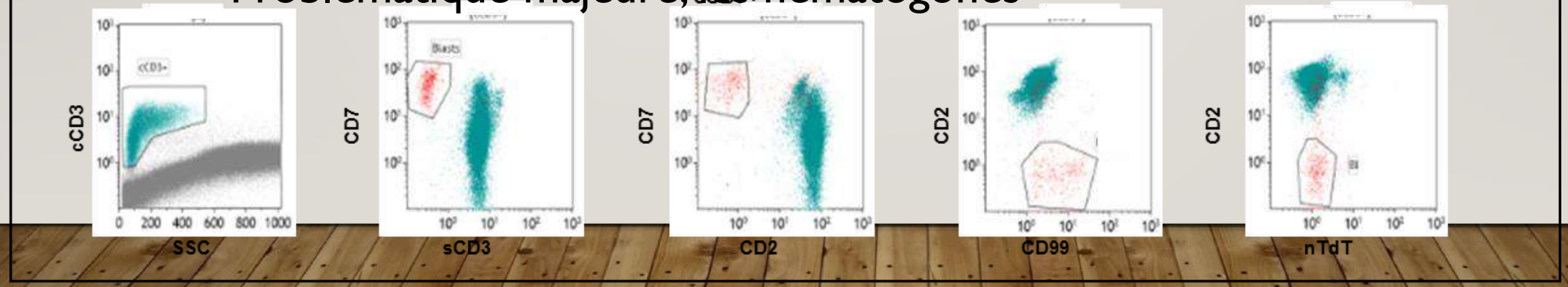
SMALL NUMBERS OF CELLS: MEASURABLE RESIDUAL DISEASE

A LONG STORY!

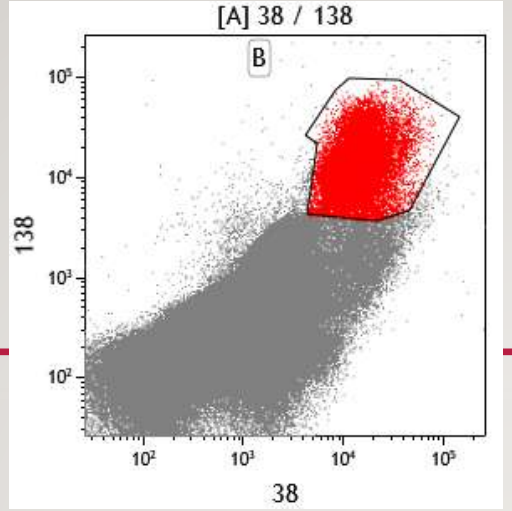
MRD IN ALL (FOSSAT ET AL. 2014)

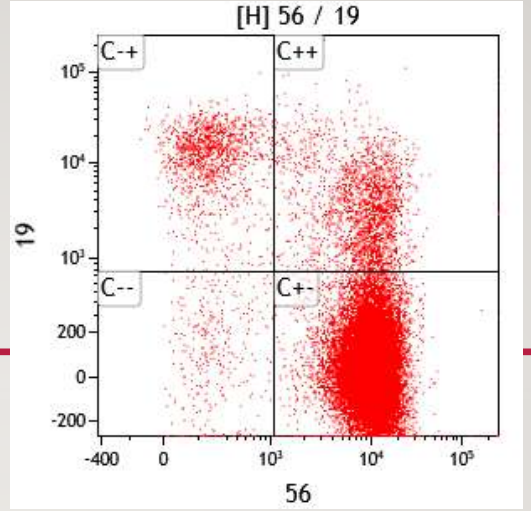


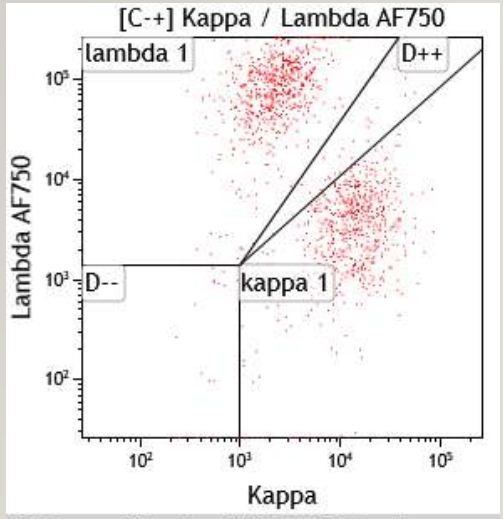
Problématique majeure, les hématogones



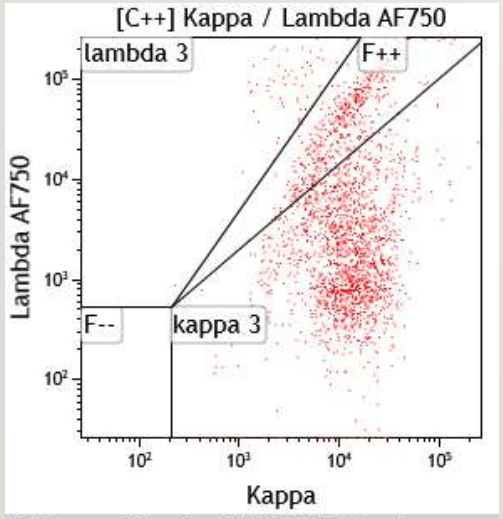
MRD IN MM
Robillard, 2015



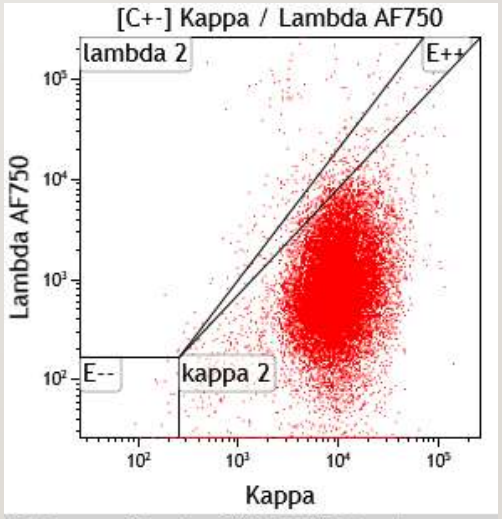




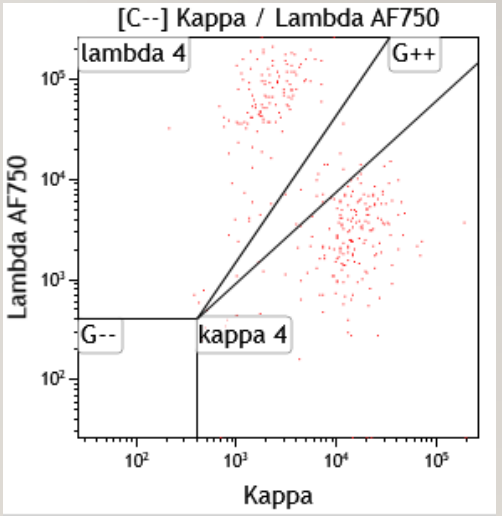
Gate	Number	%Total	%Gated
All	1 286	0,14	100,00
D--	21	0,00	1,63
D++	28	0,00	2,18
kappa 1	613	0,07	47,67
lambda 1	624	0,07	48,52



Gate	Number	%Total	%Gated
All	2 277	0,25	100,00
F--	1	0,00	0,04
F++	479	0,05	21,04
kappa 3	1 740	0,19	76,42
lambda 3	57	0,01	2,50

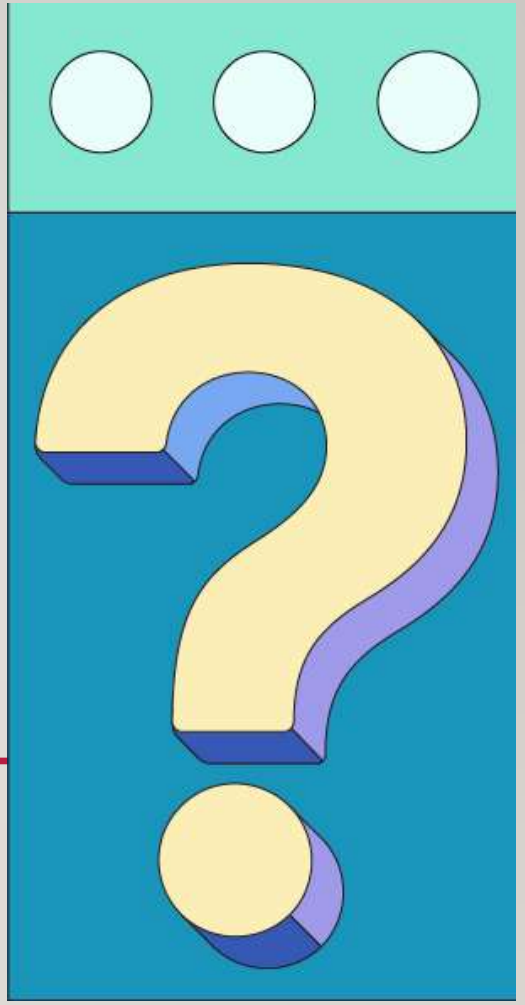


Gate	Number	%Total	%Gated
All	26 716	2,95	100,00
E--	26	0,00	0,10
E++	269	0,03	1,01
kappa 2	26 268	2,90	98,32
lambda 2	153	0,02	0,57



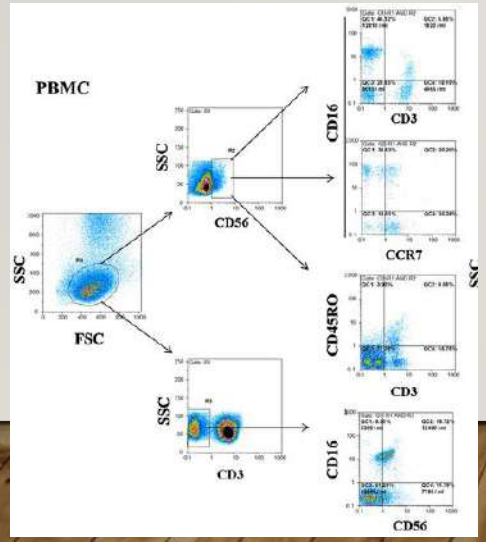
Gate	Number	%Total	%Gated
All	327	0,04	100,00
G--	0	0,00	0,00
G++	16	0,00	4,89
kappa 4	184	0,02	56,27
lambda 4	127	0,01	38,84

HOWEVER…



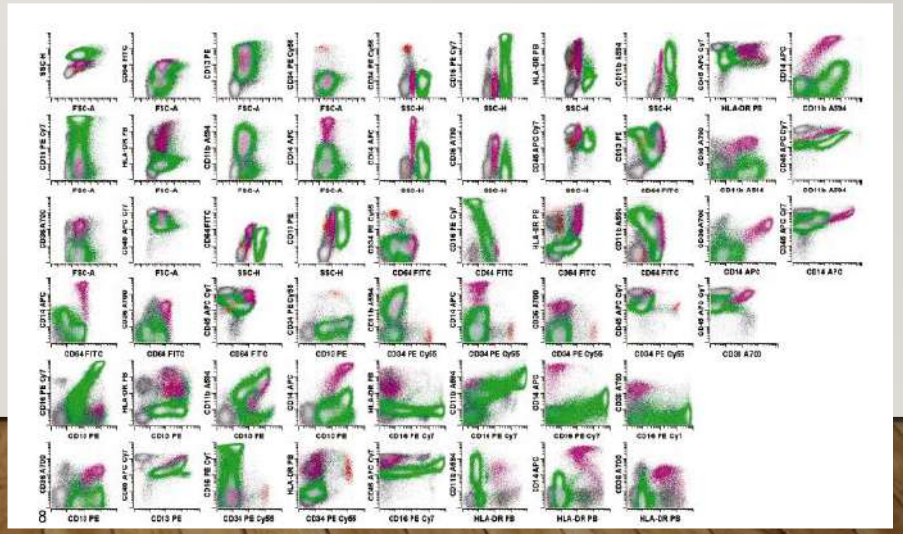
THE CURSE OF MUTIDIMENSIONALITY

- Biparametric display with increasing colors and channels (currently >=13)
- Increase in the number of histograms to consider !
- Complexity of manual gating



Tube: DNT

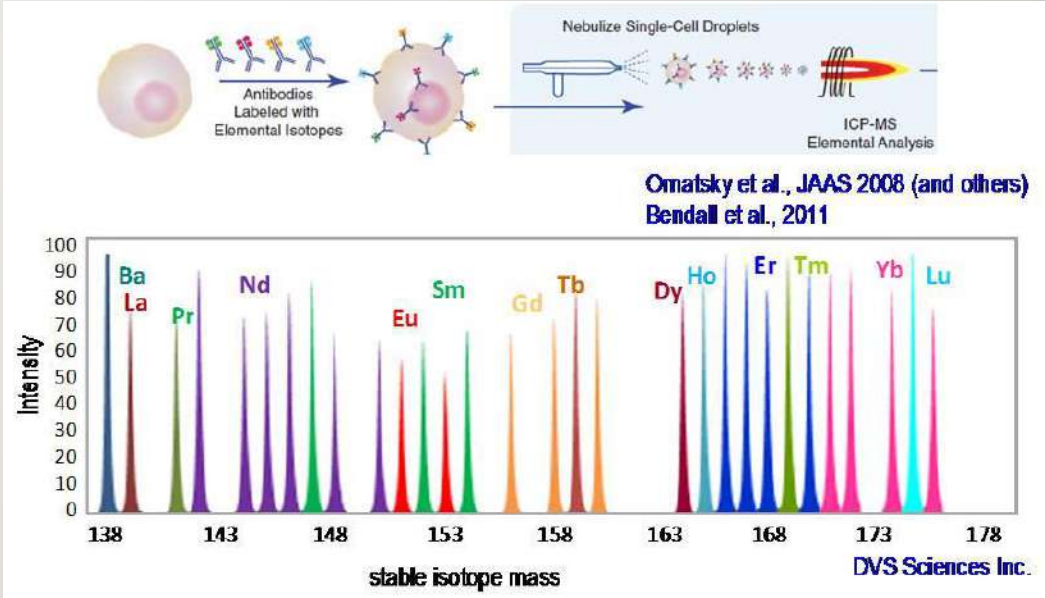
Population	#Events	%Parent	%Total
All Events	1,221,486	####	100.0
SINGLETS	486,237	39.8	39.8
NDCE	125,015	25.7	10.2
LYMPH	53,314	42.6	4.4
CD3 + T-CELLS	18,792	35.2	1.5
ALPHABETA POSITIVE T	18,380	97.8	1.5
Q1	9,131	49.7	0.7
Q2	80	0.4	0.0
DNT	889	4.8	0.1
Q4	8,280	45.0	0.7
CD19 + B-CELLS	23,999	45.0	2.0
MONO	31,482	25.2	2.6
GRANS	32,432	25.9	2.7



GROWING TECHNOLOGY

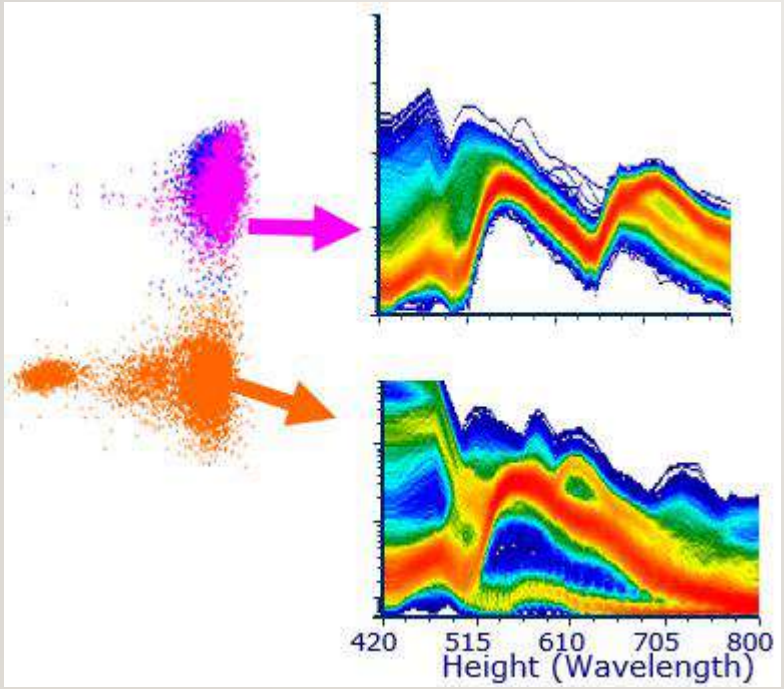
MASS SPECTROMETRY CYTOF (2009)

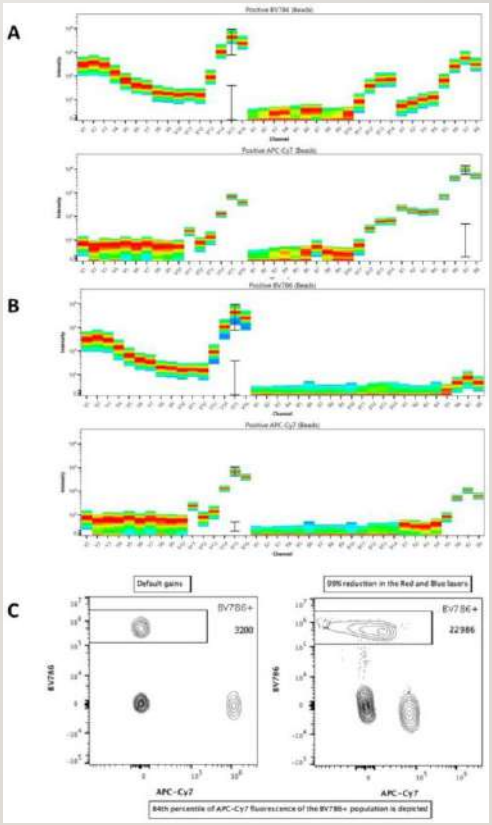
- Up to 40 antibodies
- Big data
- New strategies
- Applications
  - Immunology
  - Drug testing
  - Data comparison…



GETTING SPECTRAL?

- Entering routine laboratories since ~2015

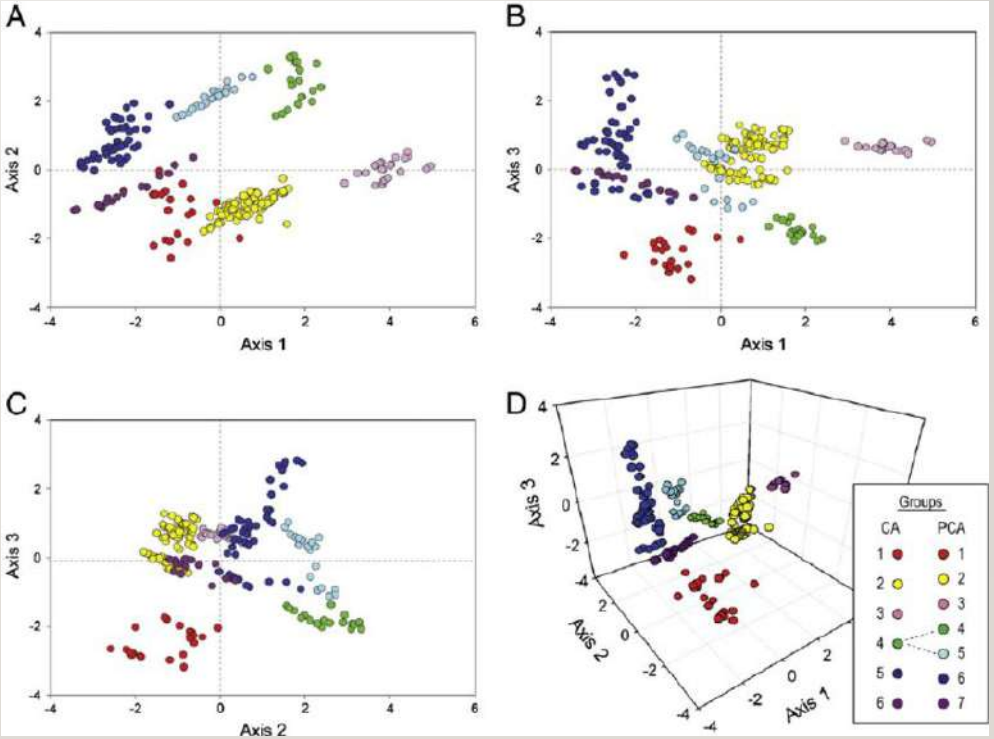




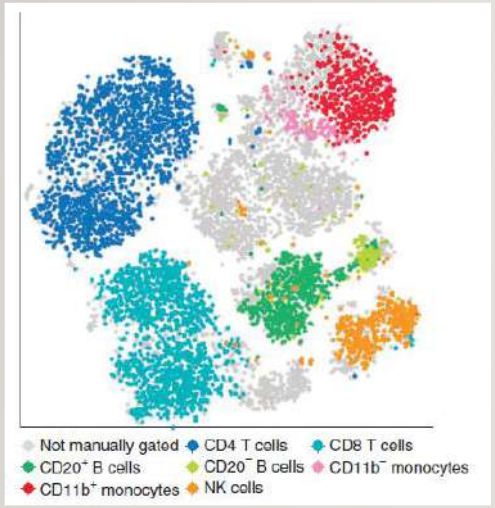
HERE COMES ARTIFICIAL INTELLIGENCE

MATHEMATICAL ANALYSISOF DIFFERENCES/SIMILARITIES

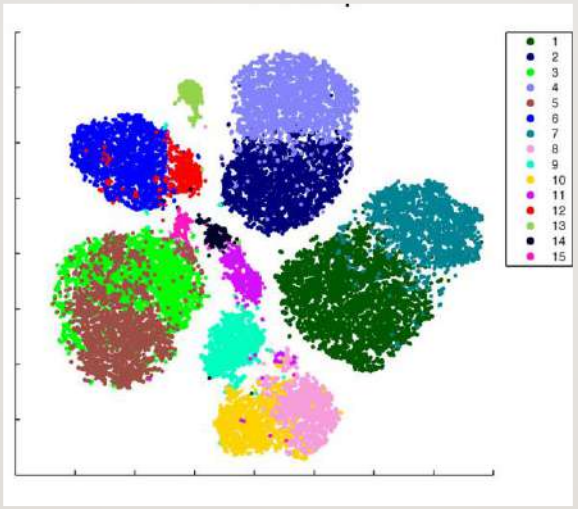
- Principal component analysis (PCA)
  - Most different clusters
  - 2D/ 3D reprsentations
  - Notion of « dimension »



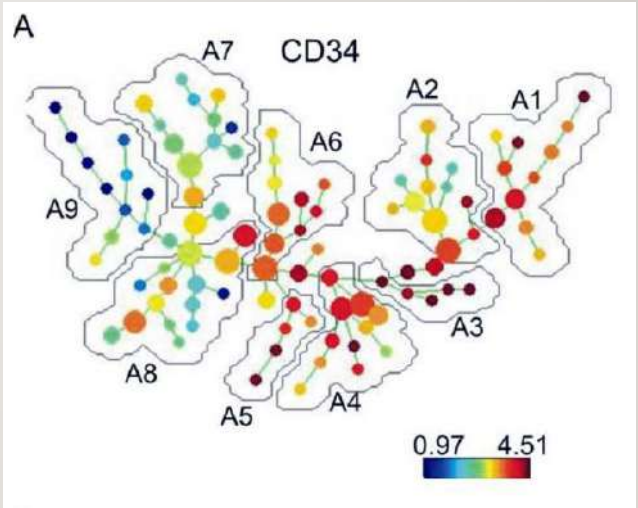
EXEMPLES OF AML ANALYSES IN CYTOF



VISNE
Amir ED et al. 2013



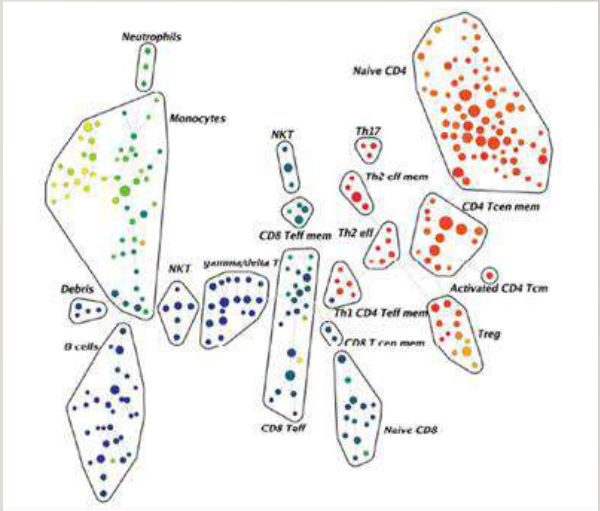
Phenograph
Levine J et al. 2015



SPADE
Han L et al. 2015

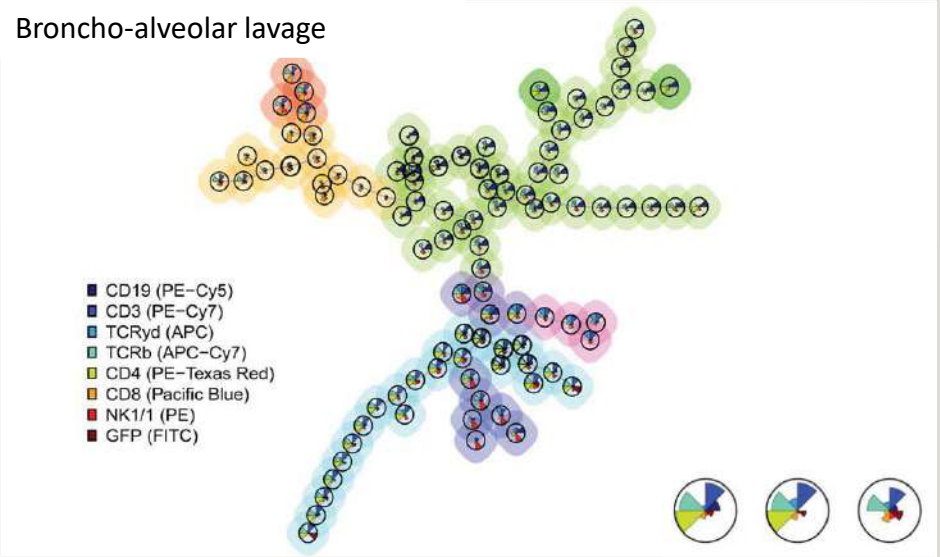
MANY SOLUTIONS

- SPADE : Spanning-tree Progression Analysis of Density-normalized Events
- t-SNE : t-Stochastic Neighbourhood Embedding
- VISNE : VIsualization of Stochastic Neighbor Embedding
- Phenograph
- UMAP : Uniform Manifold Approximation and Projection for Dimension Reduction
- …



APPLCATION TO CONVENTIONAL MFC?

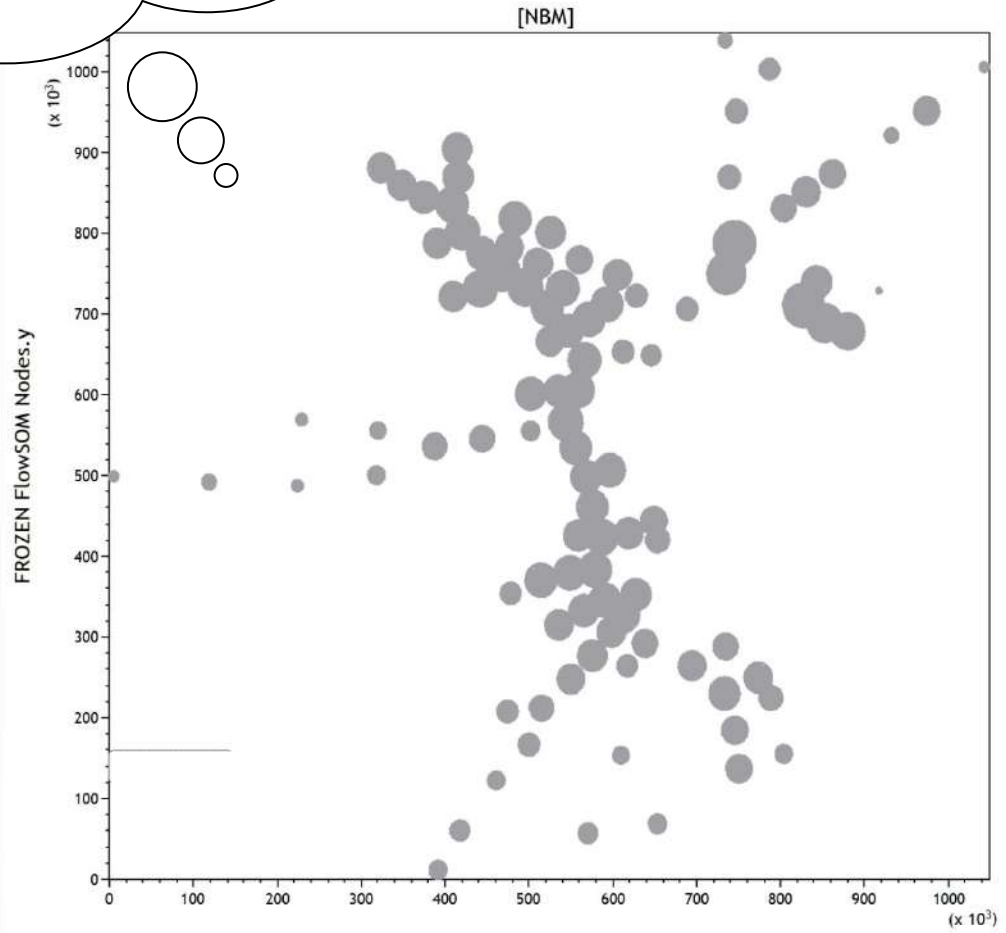
- Sofie van Gassen et al. (Cytometry 2015)
- FlowSOM: Self Organizing Maps



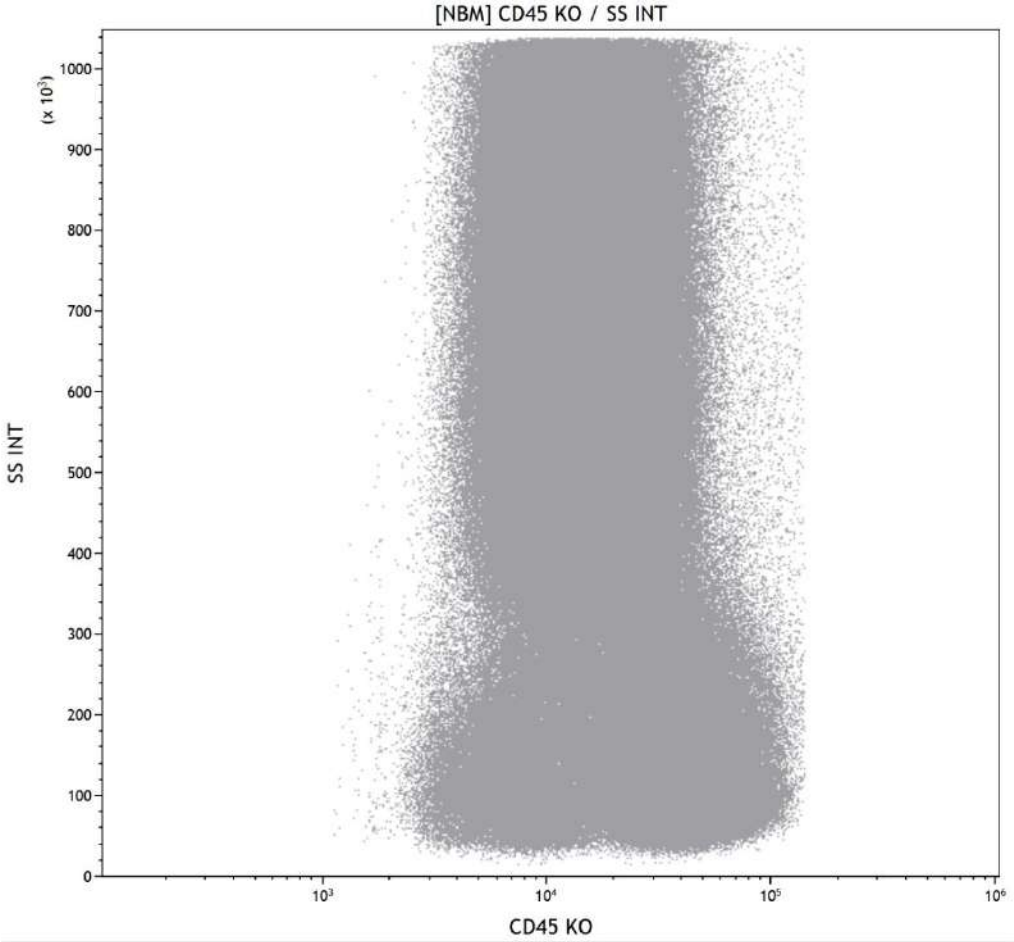
OK FlowSOM?

100 nodes!

19 MERGED NORMAL BONE MARROWS

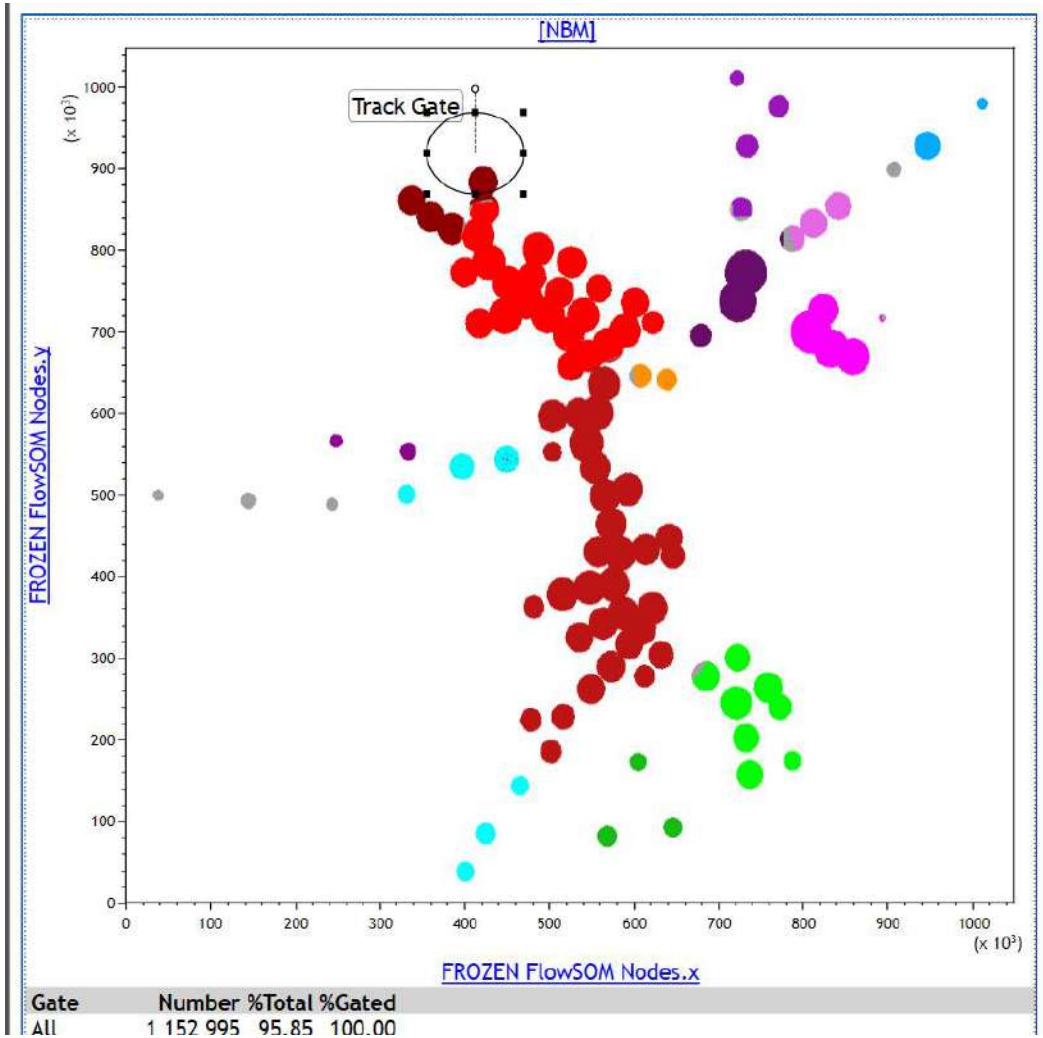


Gate	Number	%Total	%Gated
All	1 152 995	98.14	100.00
FSG MRD FP	0	0.00	0.00



Gate	Number
All	1 152 995

AND THEN?



Gate	Number	%Total	%Gated
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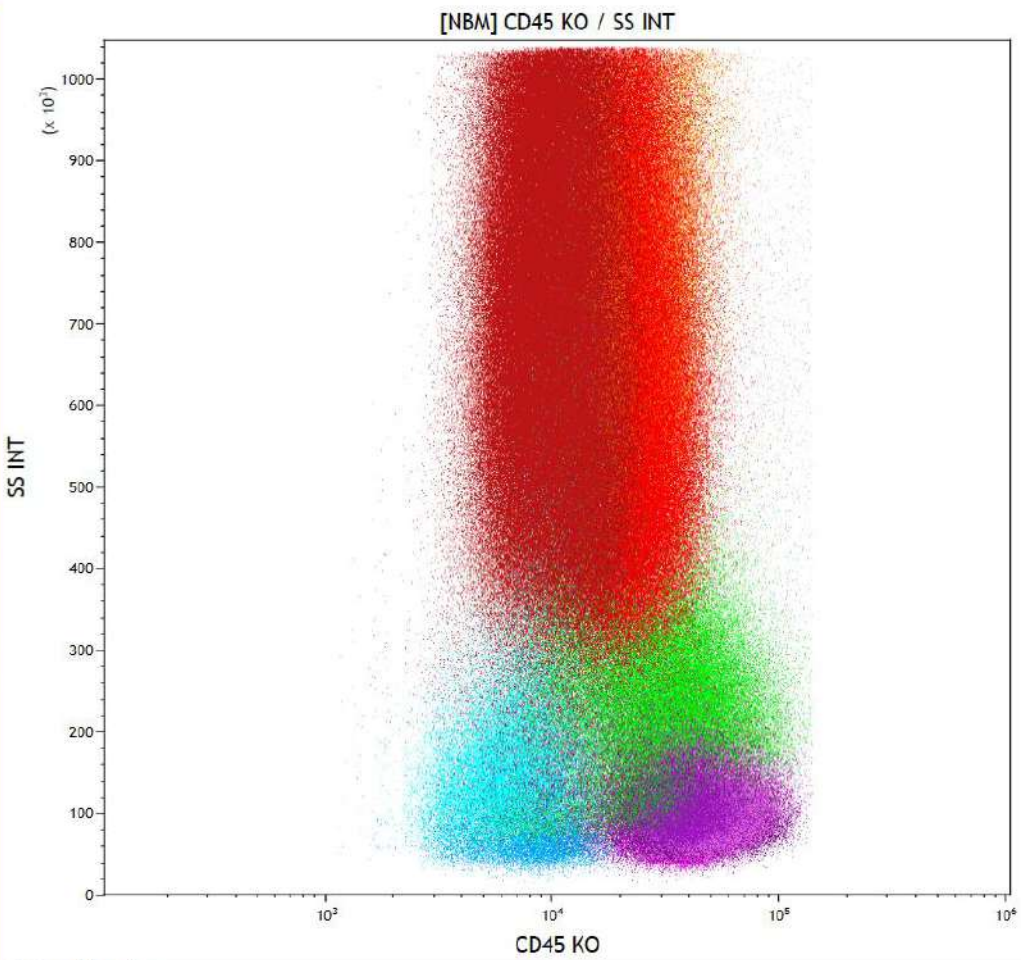
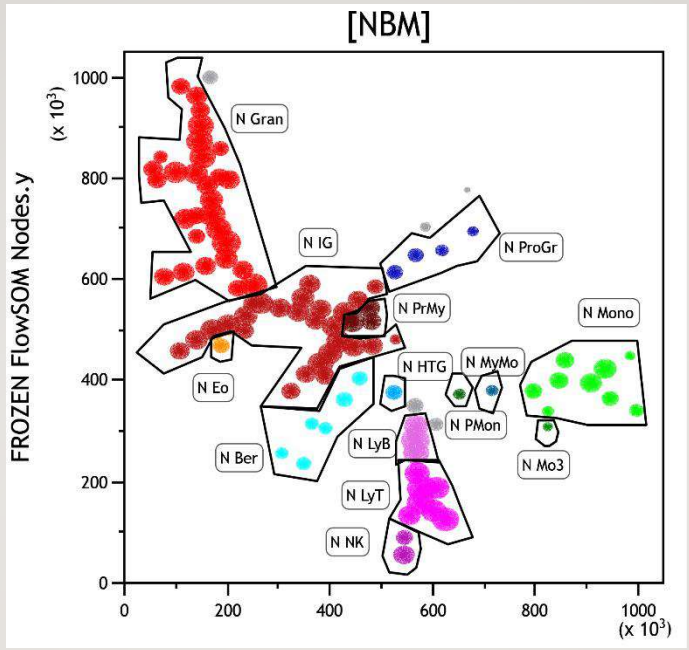
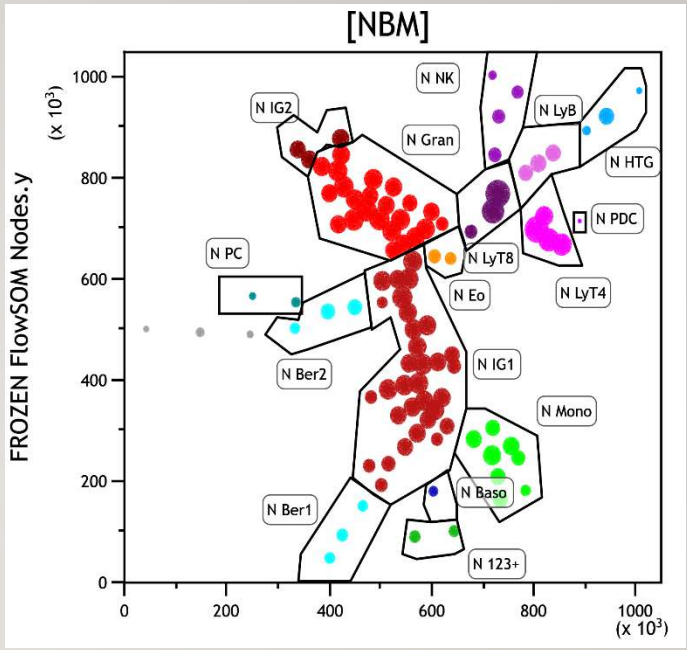
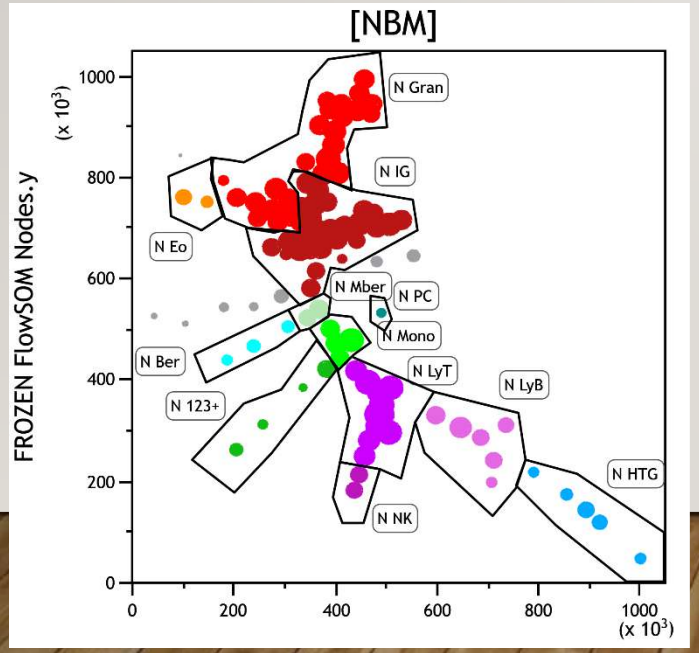


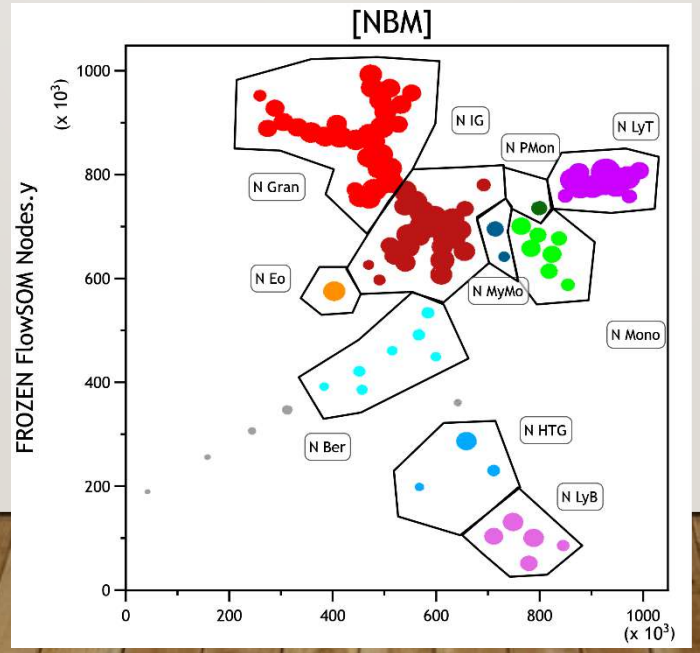
Gate	Number
All	1 152 995

Panel dependent…..



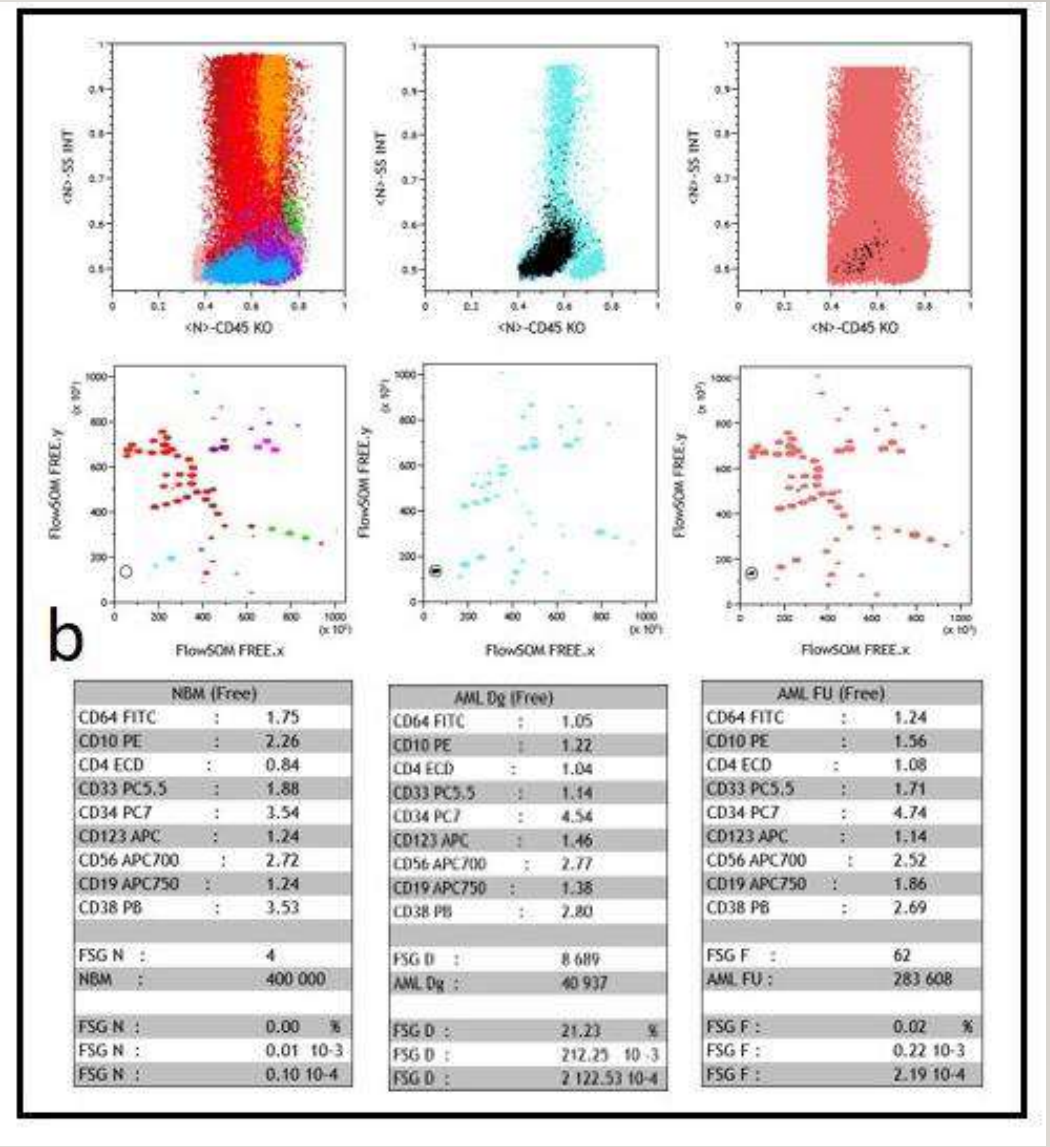






FLOWSOM MRD IN AML

- Merged normal bone marrows
- Directly compared to
- Diagnosis
- And Follow-up
- Example of an FU subset
- absent from normal bone marrow



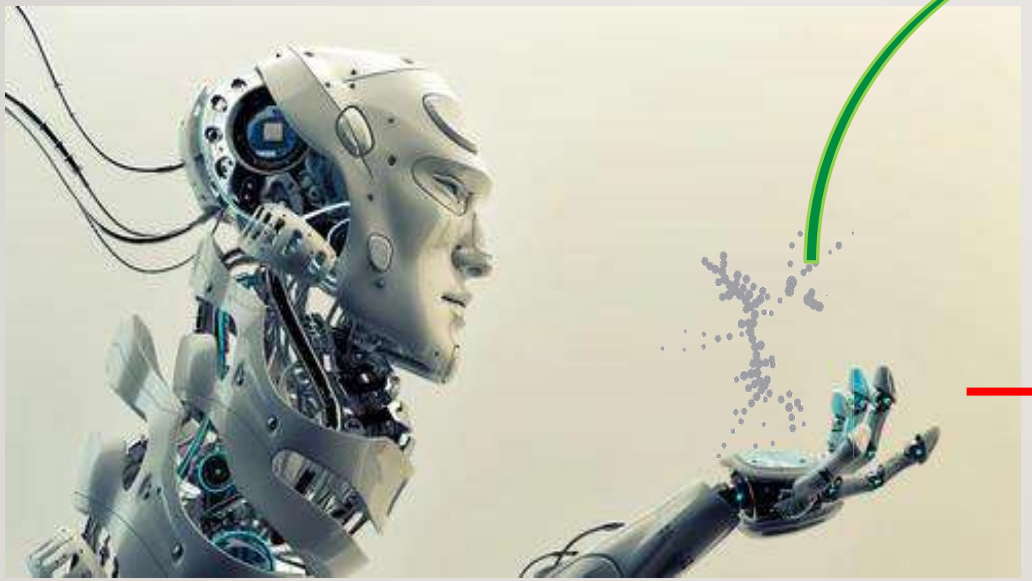
NBM (Free)		
CD64 FITC	:	1.75
CD10 PE	:	2.26
CD4 ECD	:	0.84
CD33 PC5.5	:	1.88
CD34 PC7	:	3.54
CD123 APC	:	1.24
CD56 APC700	:	2.72
CD19 APC750	:	1.24
CD38 PB	:	3.53
FSG N	:	4
NBM	:	400 000
FSG N	:	0.00 %
FSG N	:	0.01 10-3
FSG N	:	0.10 10-4

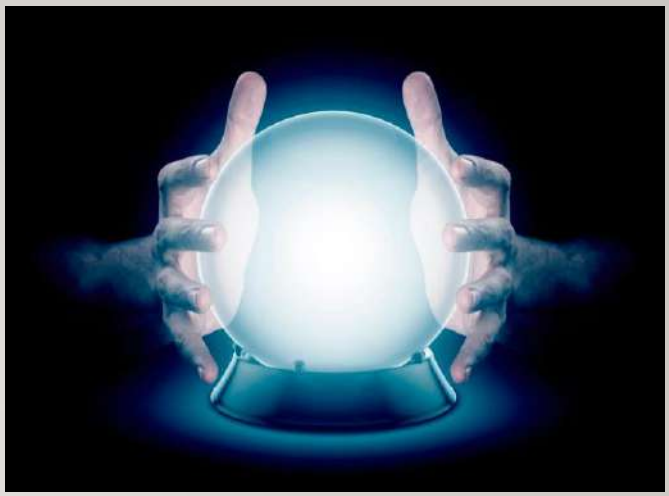
AML Dg (Free)		
CD64 FITC	:	1.05
CD10 PE	:	1.22
CD4 ECD	:	1.04
CD33 PC5.5	:	1.14
CD34 PC7	:	4.54
CD123 APC	:	1.46
CD56 APC700	:	2.77
CD19 APC750	:	1.38
CD38 PB	:	2.80
FSG D	:	8 689
AML Dg	:	40 937
FSG D	:	21.23 %
FSG D	:	212.25 10-3
FSG D	:	2 122.53 10-4

AML FU (Free)		
CD64 FITC	:	1.24
CD10 PE	:	1.56
CD4 ECD	:	1.08
CD33 PC5.5	:	1.71
CD34 PC7	:	4.74
CD123 APC	:	1.14
CD56 APC700	:	2.52
CD19 APC750	:	1.86
CD38 PB	:	2.69
FSG F	:	62
AML FU	:	283 608
FSG F	:	0.02 %
FSG F	:	0.22 10-3
FSG F	:	2.19 10-4

Lacombe, 2019. Vial 2021

TAKE-HOME-MESSAGE





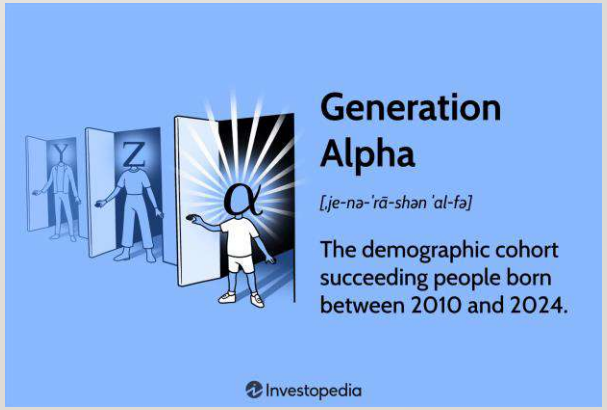
WHAT NEXT (I.E. 2100….)

WHAT IS IN STORE?

- Same basic principles
- Same clinical utility : morpholgy may decline, MFC will remain
- Less human intervention in sample preparation
- Unsupervised analyses and AI interpretation tools
- Absolute necessity of supervision, no black boxes!!
- Molecular flow cytometry (i.e. single cell DNA amplification…on the way already)
- Mixed techniques : intergrative neural networks

A BRIGHT FUTURE?

- Already a bright and fascinating past
- Innumerable applications of a tool allowing to dive deeper in biology (broadly)
- …or more (particles)
- A new job for us and them!!



THANK YOU FOR YOUR ATTENTION!