FLOW CYTOMETRY IMMUNOPHENOTYPING OF HEMATOLOGICAL MALIGNANCIES: FROM THE PAST TO THE FUTURE

How did we arrive where we are ?

Cancer Research Centre & Dpt. Medicine
University Hospital of Salamanca
University of Salamanca

XVII CONGRESO CHILENO DE HEMATOLOGÍA & VII CONGRESO DE MEDICINA TRANSFUSIONAL
27 AL 30 DE OCTUBRE 2010, HOTEL DE LA BAHÍA, COQUIMBO , Chile

IMMUNOPHENOTYPING OF HAEMATOLOGICAL MALIGNANCIES

- 1953/1994: From the development of the instruments & techniques to the WHO classifications of haematological malignancies.

- 1994/2006: The ability to specifically identify leukaemic cells: from normal phenotypes to aberrant phenotypic profiles.

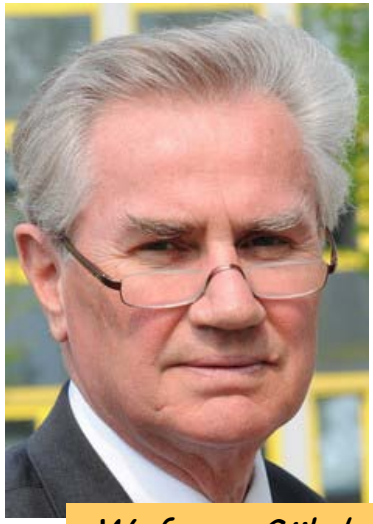
- 2006/-: Recent contributions of immuno-phenotyping of haematological malignancies: pointing to the future.

IMMUNOPHENOTYPING OF HAEMATOLOGICAL MALIGNANCIES

- 1953: The "Coulter" principle and instrument development
- 1965/68: Multiparameter and multicolour flow cytometry

Wallace Coulter

Wallace visiting manufacturing in the early years

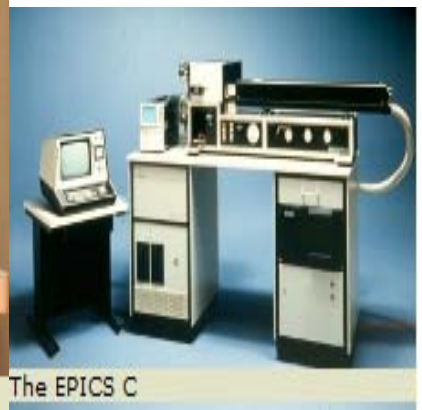


Wofgang Göhde

Marv van Dilla
H. Crissman

The TPS

The EPICS II



The EPICS C

Joe Gray
1970

IMMUNOPHENOTYPING OF HAEMATOLOGICAL MALIGNANCIES

- 1953: The "Coulter" principle and instrument development
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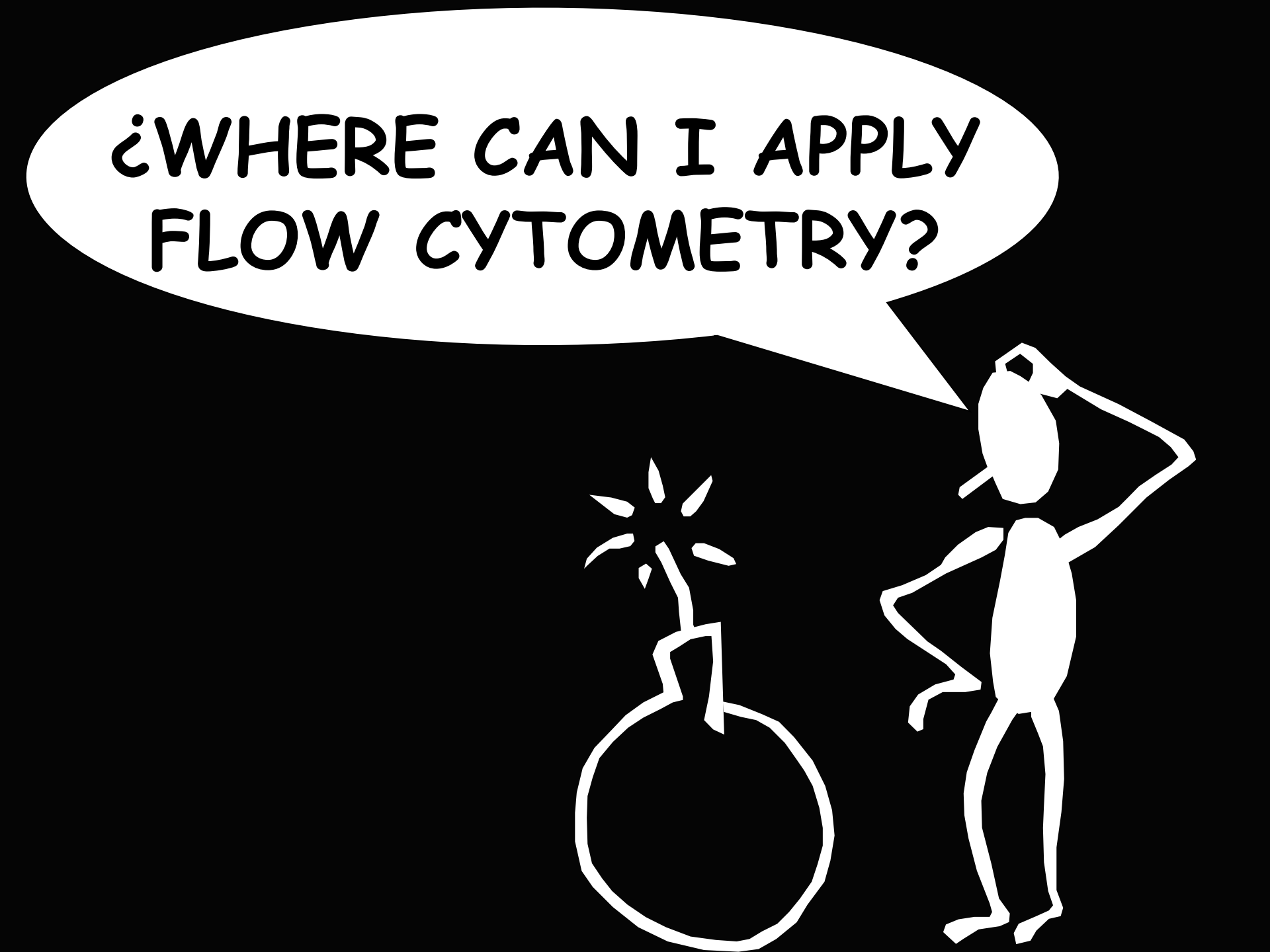
- 1970: FACS: "fluorescence activated cell sorter".

Len & Lee Herzenberg


¿WHERE CAN I APPLY FLOW CYTOMETRY?

IMMUNOPHENOTYPING OF HAEMATOLOGICAL MALIGNANCIES

- 1953: The "Coulter" principle and instrument development
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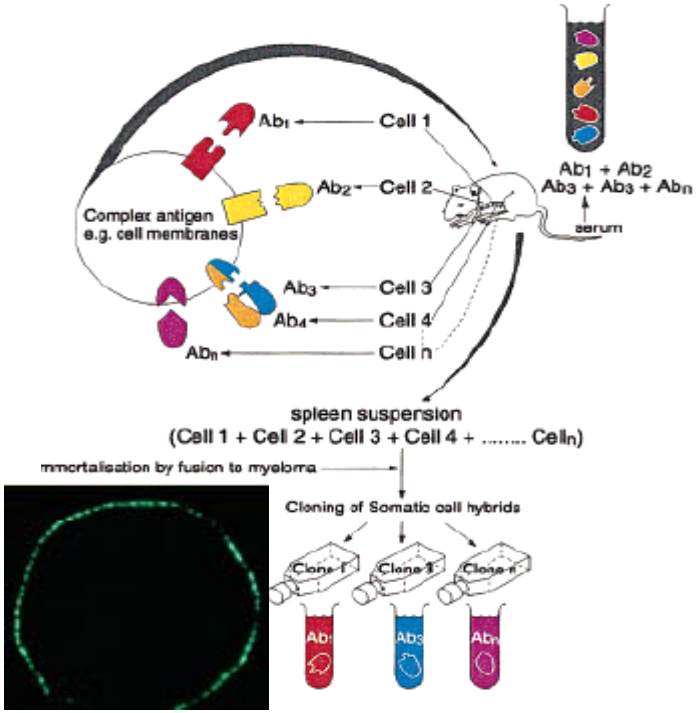
- 1970: FACS: "fluorescence activated cell sorter".
- 1975: Production of monoclonal antibodies

Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of pre-defined specificity. Nature 1975;256:495-497.



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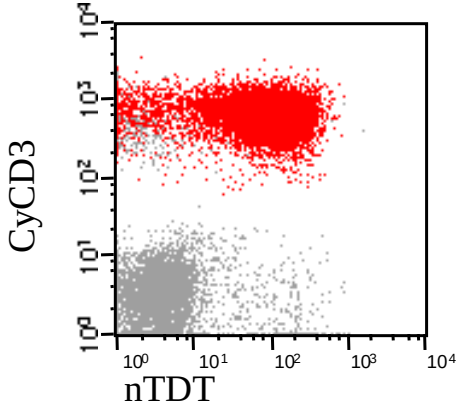
- 1953: The "Coulter" principle and instrument development
- 1965/68: Multiparameter and multicolour flow cytometry
- 1970: FACS: "fluorescence activated cell sorter".
- 1975: Production of monoclonal antibodies
- 1978/80: Immunophenotyping of leukemia cells
  Immunophenotypic classification of ALL
- 1981: Definition of aberrant marker expression
- 1985: Use of Immunophenotyping to classify FAB M7

Tabla 2.- Immunological classification of ALL

	Phenotype
B-ALL	Ig+
T-ALL	SER+
non-T non-B ALL	cALLA+
	cALLA-



FCM IMMUNOPHENOTYPING IN THE 80`S: PANELS OF REAGENTS AND TECHNIQUES

PANELS OF REAGENTS:

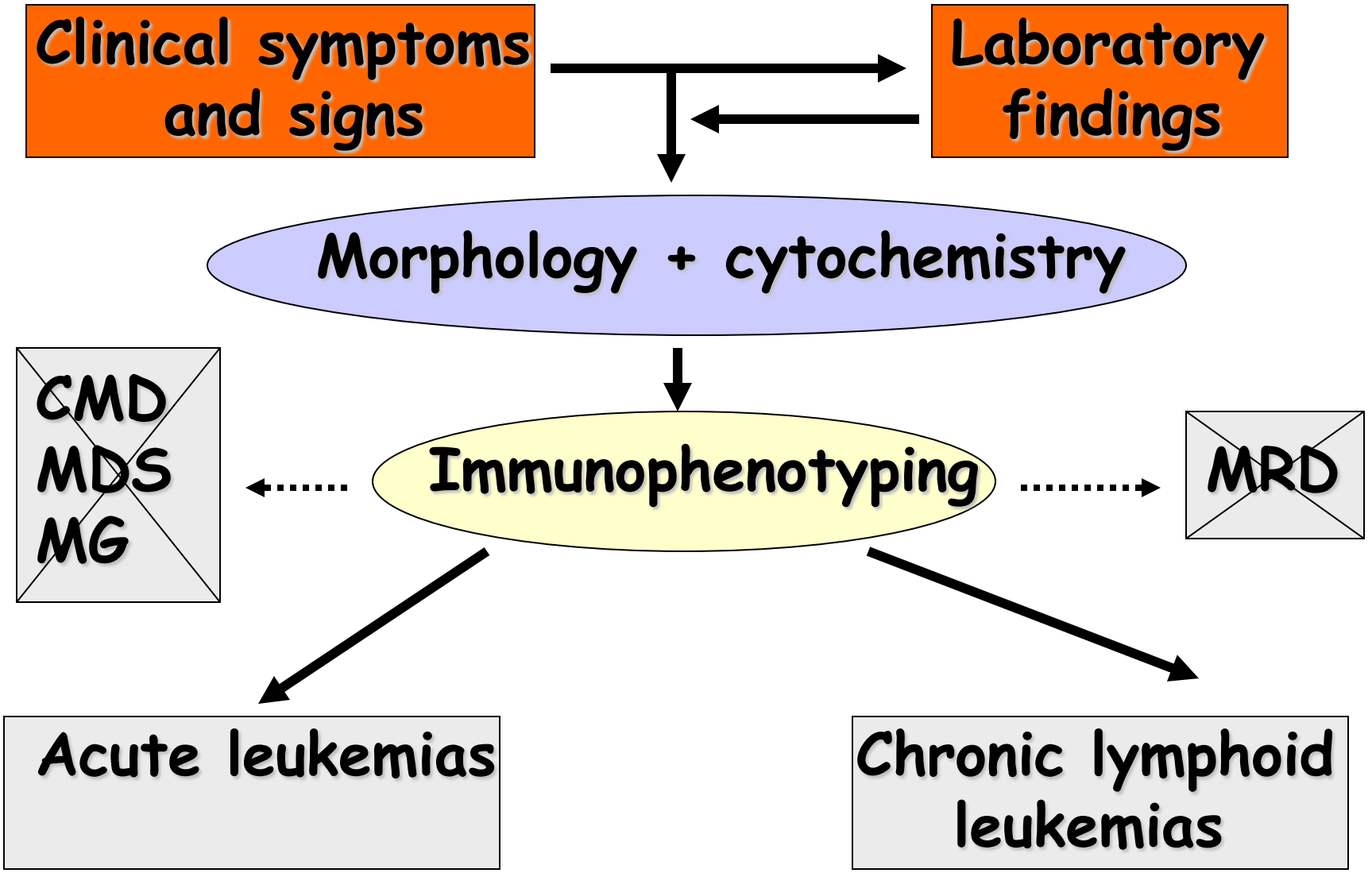
- Panels of relevant markers to support suspected diagnosis of:

  - AML, ALL
  - MM
  - B-CLPD, T-CLPD

TECHNIQUES:

- Isolation of MNC - Difficult to distinguish normal/leukemic cells
- Indirect and direct IF - Few fluorochrome conjugated MAb available
- Single stainings - Few fluorochrome available

DIAGNOSIS OF HAEMATOLOGICAL MALIGNANCIES



IMMUNOPHENOTYPIC CLASSIFICATION OF ACUTE LEUKAEMIAS

Leukemia (1995) 9, 1783–1786

OPEN FORUM

Proposals for the immunological classification of acute leukemias

European Group for the Immunological Characterization of Leukemias (EGIL): MC Bene[1], G Castoldi[2], W Knapp[3], WD Ludwig[4], E Matutes[5], A Orfao[6] and MB van't Veer[7]

[1]*Laboratoire d'Immunologie, Faculté de Médicine, Nancy, France;* [2]*Institute of Haematology, University of Ferrara, Italy;* [3]*Institute of Immunology, University of Vienna, Austria;* [4]*Free University of Berlin, Robert-Rossle-Clinic, Berlin, Germany;* [5]*Academic Department of Haematology and Cytogenetics, The Royal Marsden Hospital, London, UK;* [6]*Department of Cytometry, University of Salamanca, Spain; and* [7]*Department of Haematology, Dr Daniel den Hoed Cancer Center, Rotterdam, The Netherlands*

Table 1 Immunological classification of acute leukemias

3. Biphenotypic acute leukemias (BAL)

4. Undifferentiated acute leukemias

EGIL: DEFINITION OF BAL

Score	B-Lineage	T-Lineage	Myeloid lineage
2	cCD79a cIgM cCD22	c/mCD3 TCR	MPO Lisozyme
1	CD20	CD2, CD5 CD8, CD10	CD13, CD33 CD117, CDw65
0.5	Tdt, CD24	Tdt, CD7 CD1a	CD14, CD15 CD64

Criteria: > 2 points

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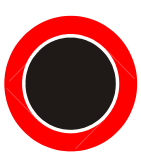
Identification of different granulocytic subpopulations in childhood BM



Myelo/monoblast

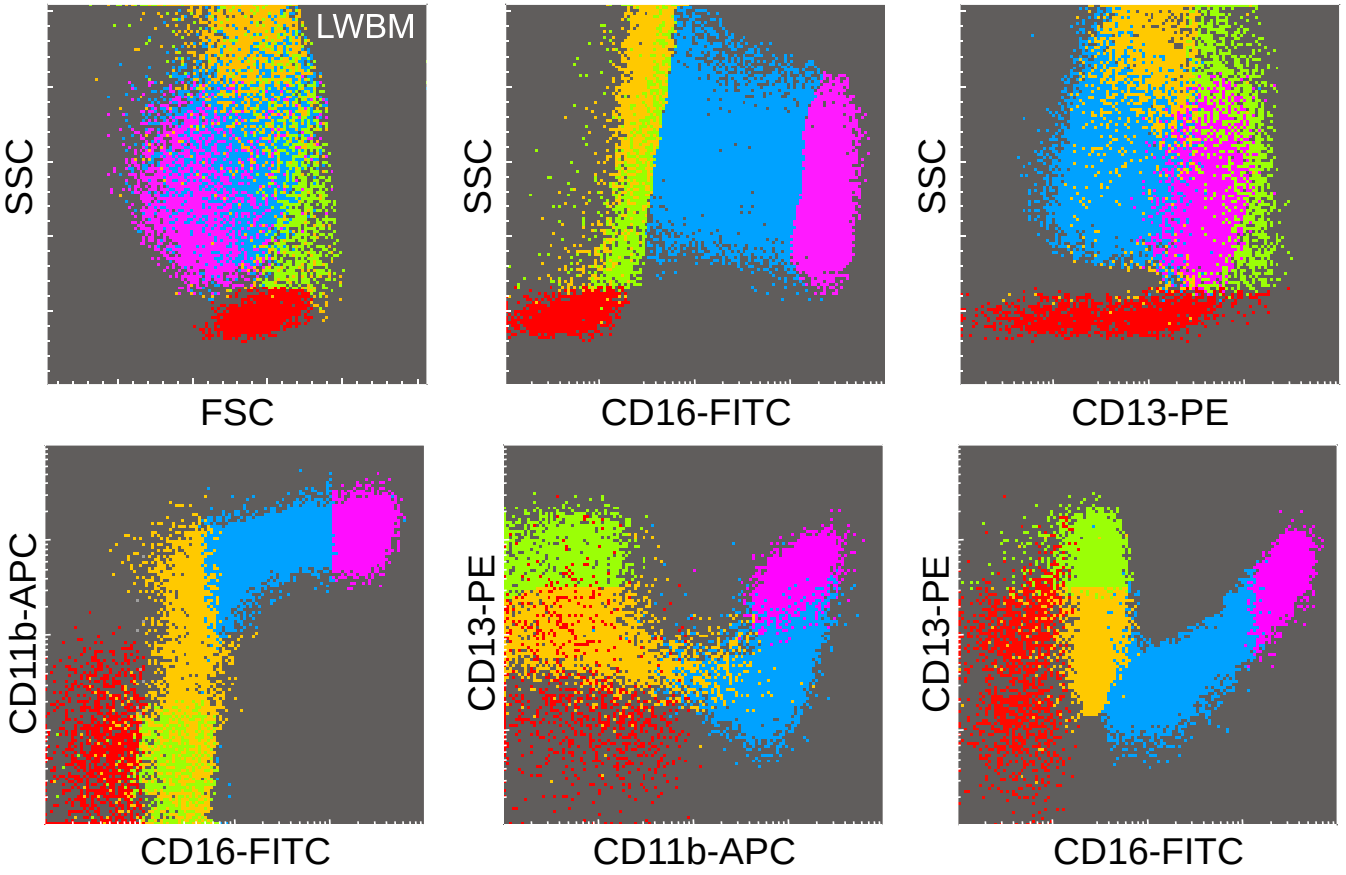
Promyelocyte

Myelocyte

Metamyelocyte

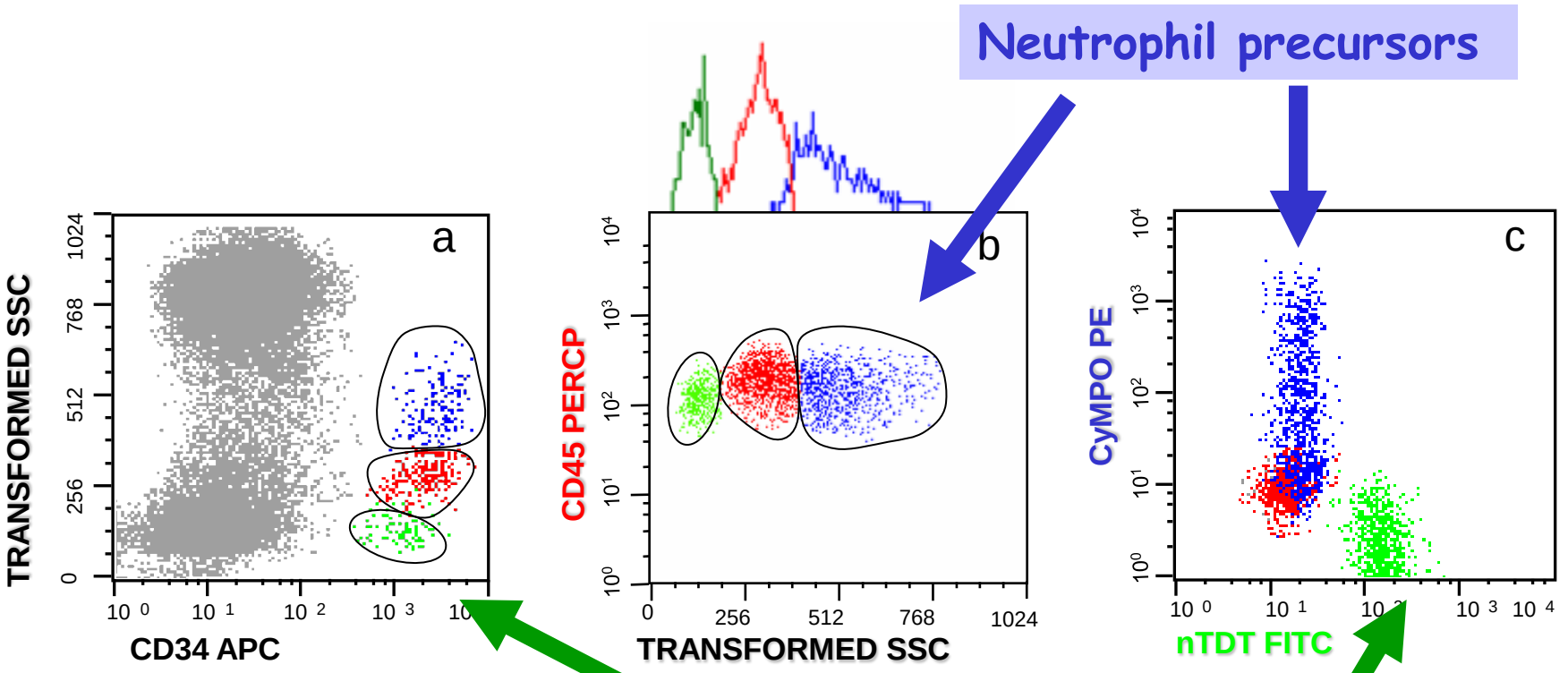
Neutrophill

staining 5
CD16/CD13/
CD45/CD11b



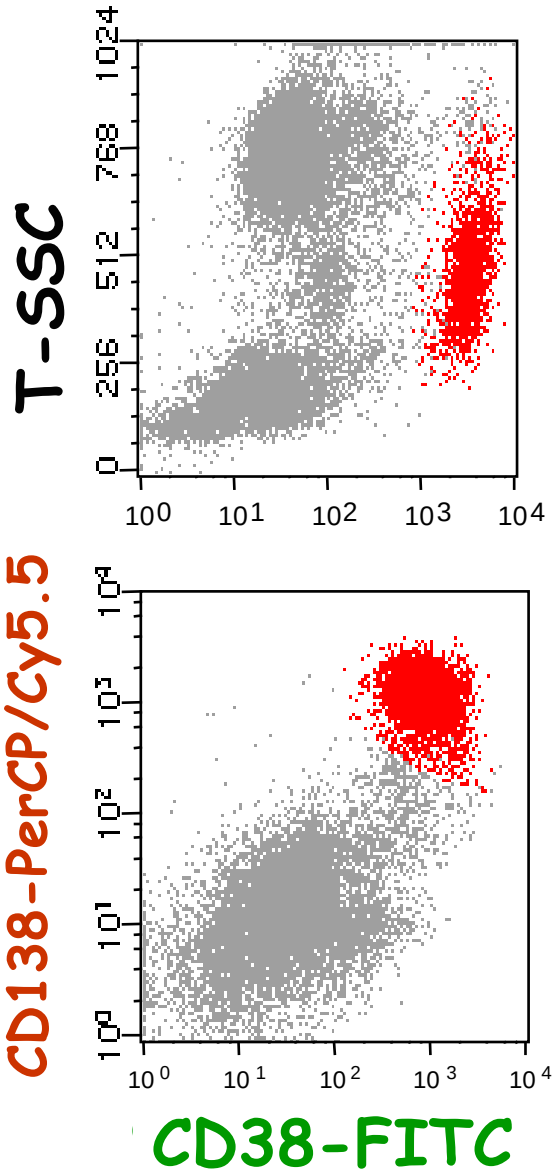
E.G. van Lochem et al., Cytometry Part B 2004; 60B: 1-13.

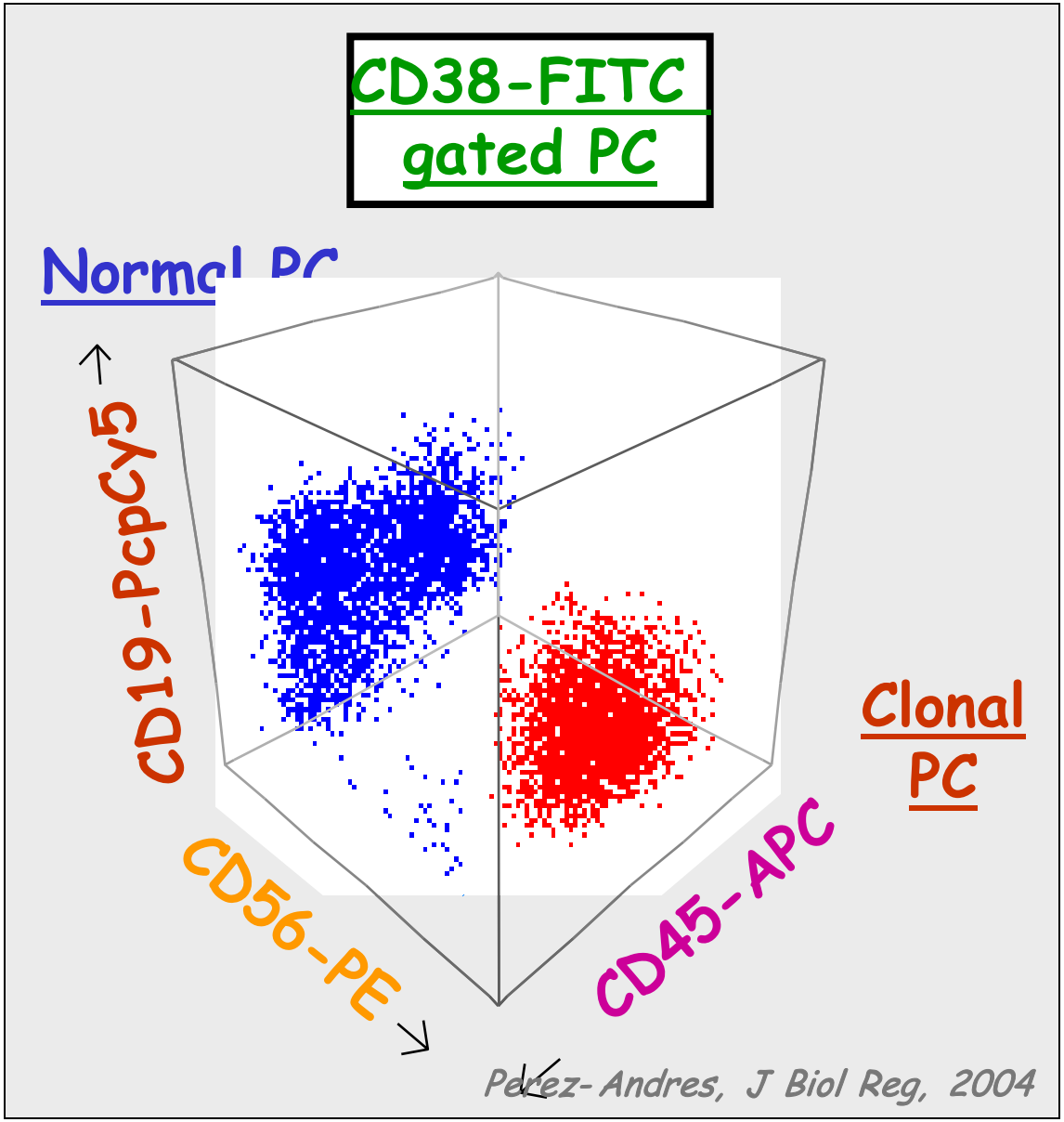
IMMUNOPHENOTYPIC IDENTIFICATION OF LINEAGE COMMITMENT OF CD34+ BM CELLS



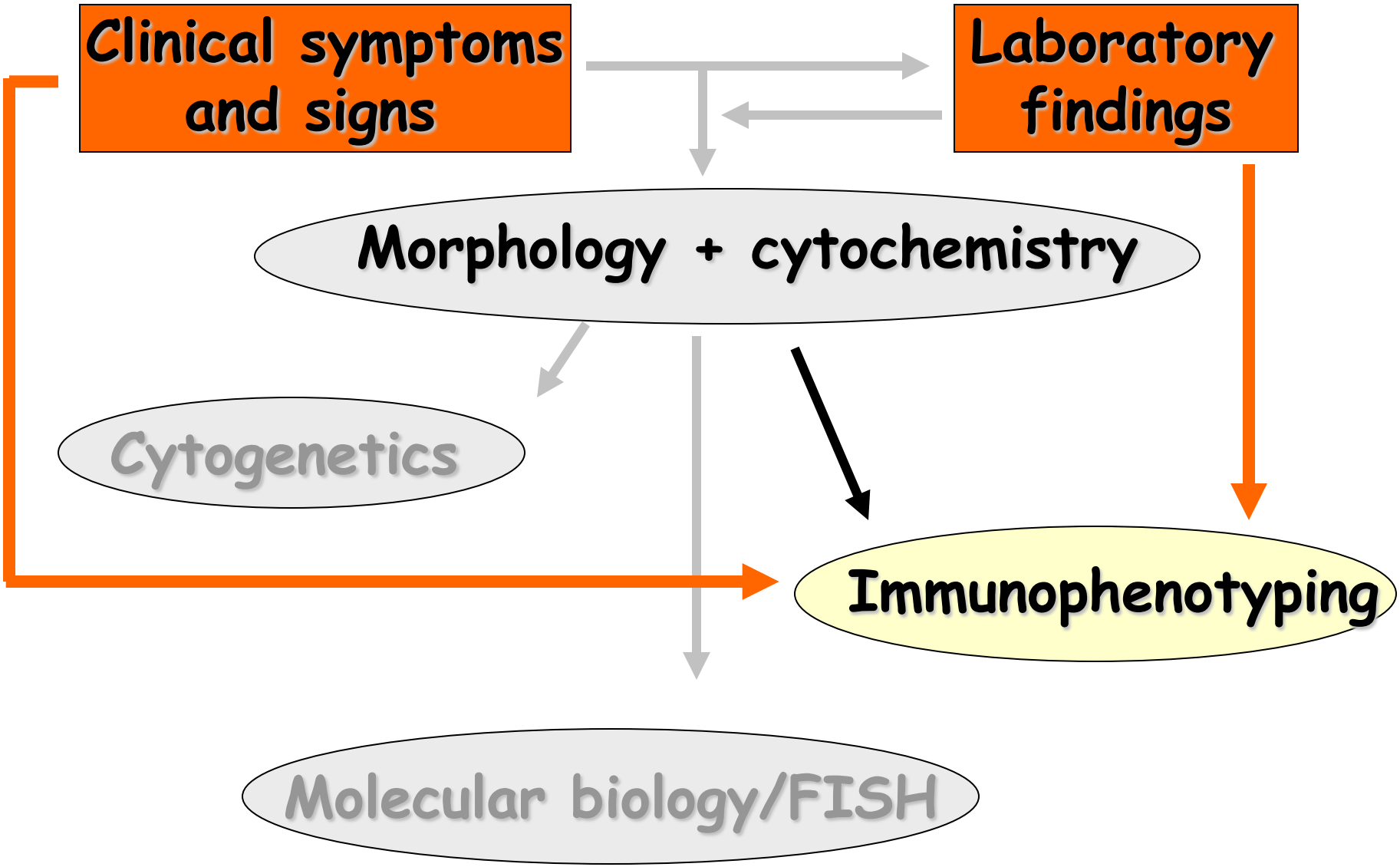
Matarraz S et al. Leukemia 2008

MONOCLONAL GAMMOPATHIES: IDENTIFICATION OF CLONAL PLASMA CELLS

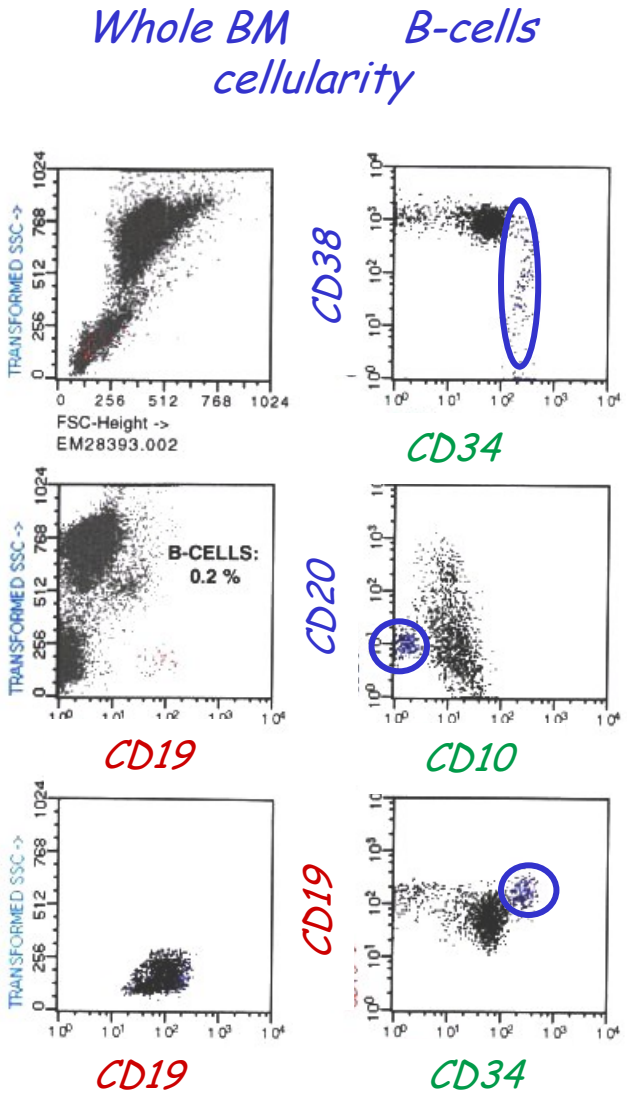




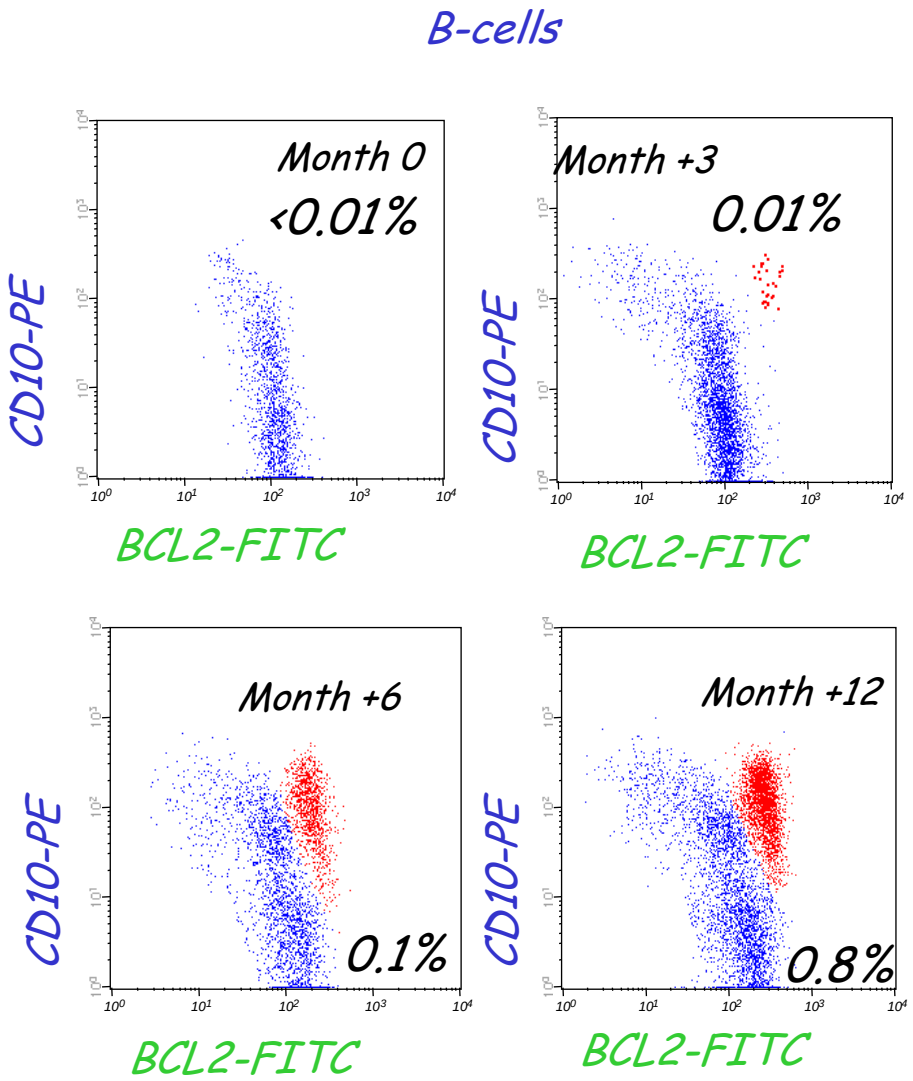
DIAGNOSIS OF HAEMATOLOGICAL MALIGNANCIES



IMMUNOPHENOTYPIC INVESTIGATION OF MRD



B-cell precursor ALL



Follicular lymphoma

Diagnostics in hemato-oncology

1. Making the diagnosis

   Normal ↔ reactive/regenerating ↔ malignant

   Annually > 300,000 new patients with a hematological malignancy in developed countries

2. Classification of hematopoietic malignancies
   - relation with prognosis
   - relevance of risk-group definition in treatment protocols

   → Based on differentiation characteristics and particularly on chromosome aberrations, resulting in fusion gene transcripts or aberrantly (over) expressed genes

3. Evaluation of treatment effectiveness

   Detection of minimal residual disease (MRD):

   MRD-based risk-group stratification (treatment reduction or treatment intensification)

   Annually > 400,000 follow-up samples in leukemia patients (ALL, AML, CML)

FCM IMMUNOPHENOTYPING IN THE 90`S: PANELS OF REAGENTS AND TECHNIQUES

PANELS OF REAGENTS:

- Panels of informative combinations of markers for:
  - AML, ALL, BAL
  - MM, WM, MGUS
  - B-CLPD, T-CLPD
  - MDS

Diagnosis & follow-up of MRD in acute leukaemias, CLPD & MM

TECHNIQUES:

- Non-NRBC lysis
- Direct IF
- Multiple stainings

- Distinct normal vs leukemic phenotypes
- Many fluorochrome conjugated MAb available
- Increased number of fluorochrome available

CLINICAL APPLICATIONS OF FLOW CYTOMETRY

Microscopy | **Flow cytometry**

70s-90s

Hybridoma technology
Monoclonal antibodies
Fluorochrome-conjugates

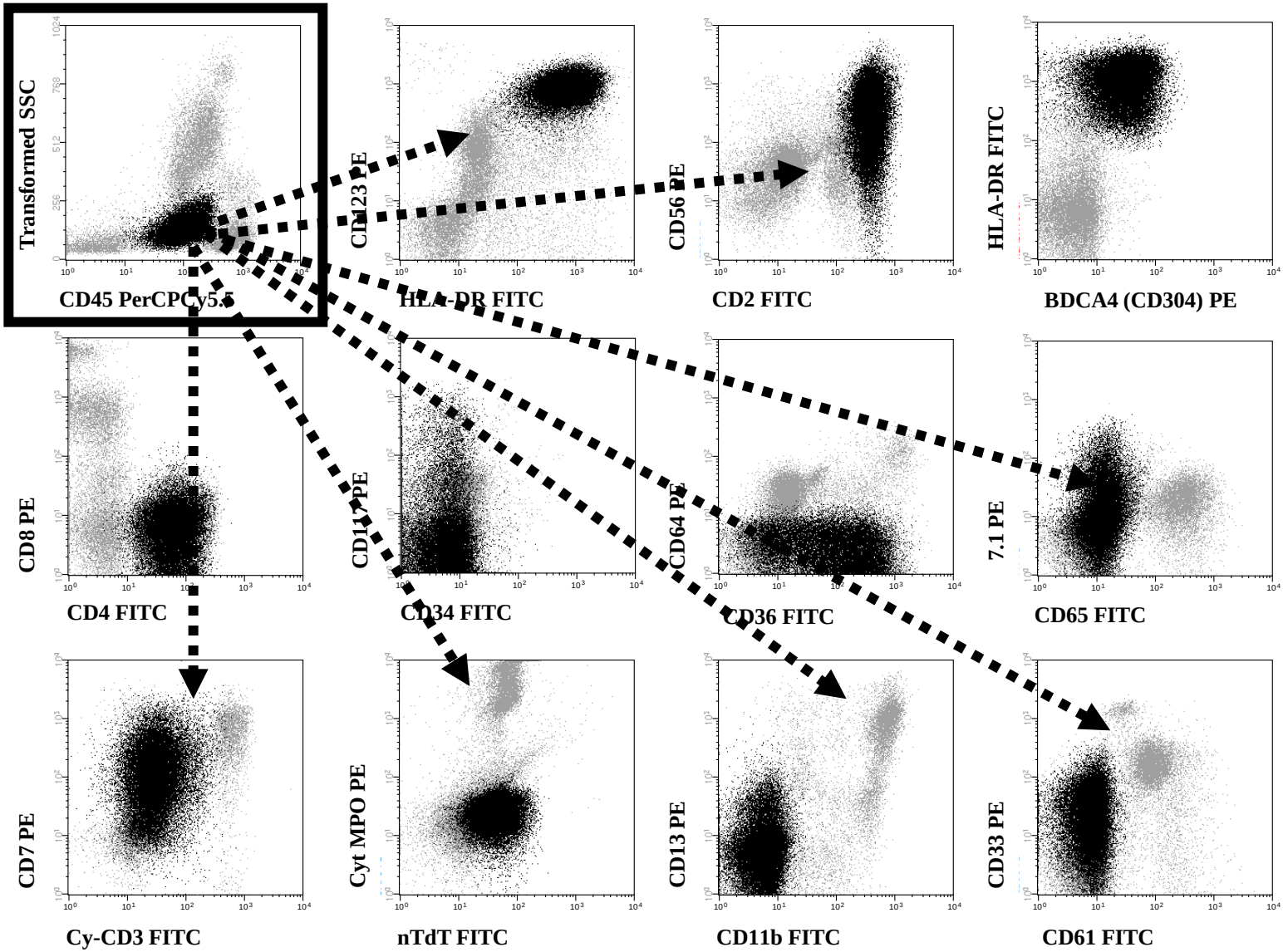
From research laboratories to clinical diagnostics

XXI century

Digital instruments
>4 color flow cytometers
Higher analytical speed

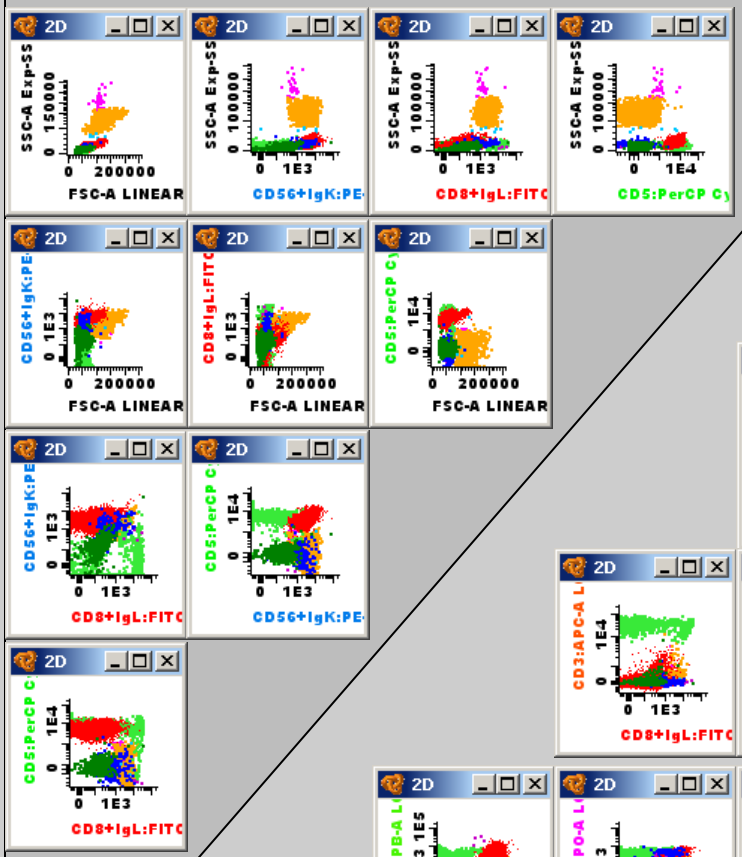
Exponentially growing amount of complex information/data

IMMUNOPHENOTYPIC FEATURES OF NEOPLASTIC CELLS (pDCs)

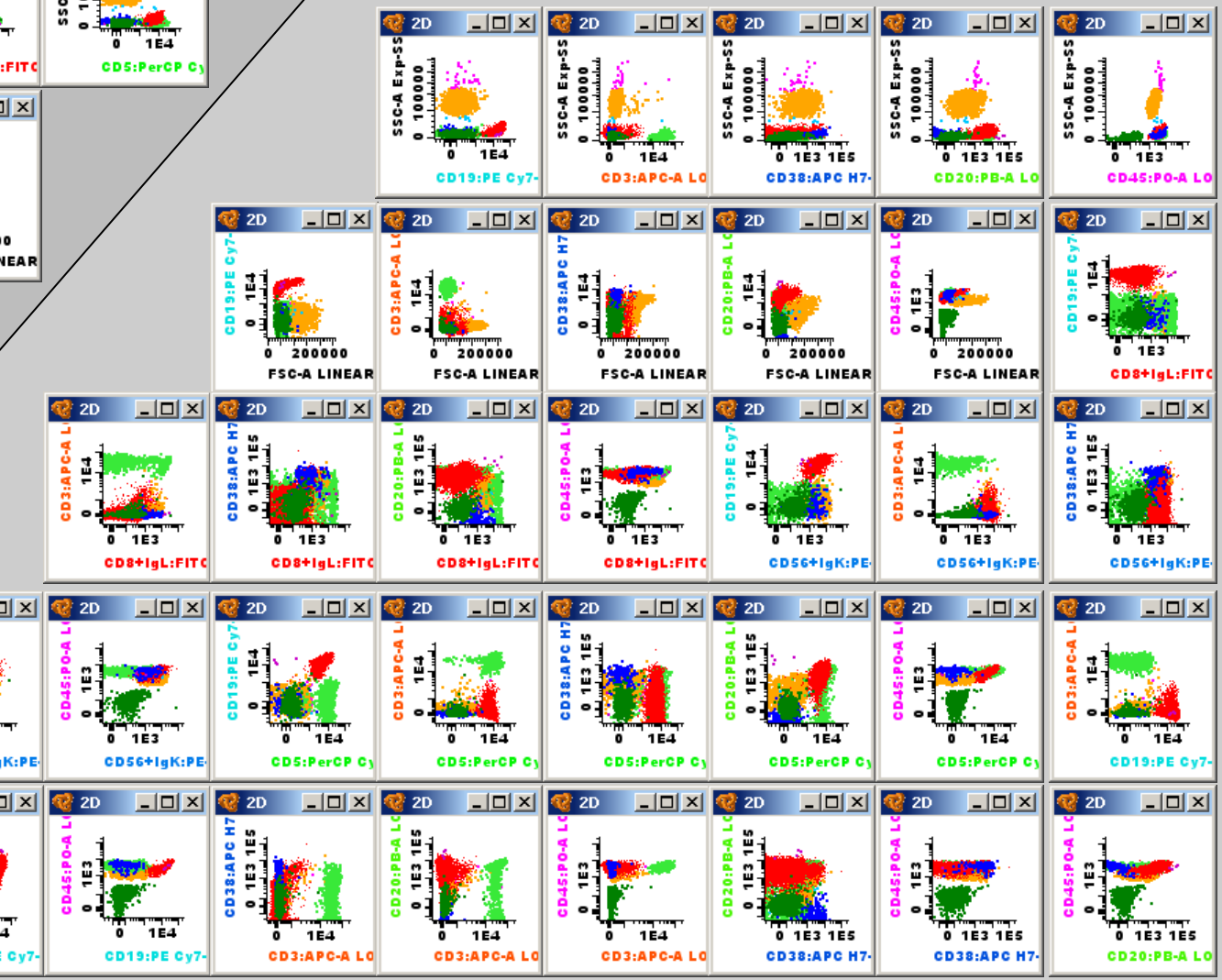


FCM DATA COMPLEXITY & SUBJECTIVITY

3- COLOR flow cytometry: 10 bivariate dot plots



8- COLOR flow cytometry: 45 bivariate dot plots



STANDARDIZATION EFFORTS FOR IMMUNOPHENOTYPIC STUDIES

- **CLSI** (Clinical Laboratory Standards Institute):

  - Stetler-Stevenson et al.: Clinical flow cytometric analysis of neoplastic hematolymphoid cells; Approved guideline. CLSI document H43-A2. CLSI, 2007

- **CCS** (Clinical Cytometry Society):

  - Davis et al: 2006 Bethesda International Consensus recommendations on the flow cytometric immunophenotypic analysis of hematolymphoid neoplasias. Clin Cytometry, 72B, 2007.

- **ESCCA** (European Society for Clinical Cell Analysis: www.escca.eu)

- **European Leukemia Net** (www.leukemia-net.org)

- **Consenso Latinoamericano** (Clin Cytometry, 1998 y 2006)

What problems are we experiencing?

- Many reagents: costly and complex

- Need expertise in normal (& reference) cell populations

- Time consuming

- Technical limitations

- Many (my) strategies to reach a similar result but suboptimal

- Not standardized: reproducibly harmonized?

- Partial and more limited clinical utility than expected

Required developments in clinical flow cytometry

Multicolor flow cytometry: >4-6 colors

- inclusion of solid state violet laser
- compare conjugated antibodies (multiple companies)

Immunobeads

- introduce combined cellular/immunobead assays
- special immunobead for leukemias

Novel antibodies

- test new (academic) antibodies for application in intracellular stainings
- development of new antibodies

Development of novel software

- novel software for pattern recognition: mapping of diagnosis and follow-up leukemia samples against templates of "normal/control" samples

EuroFlow

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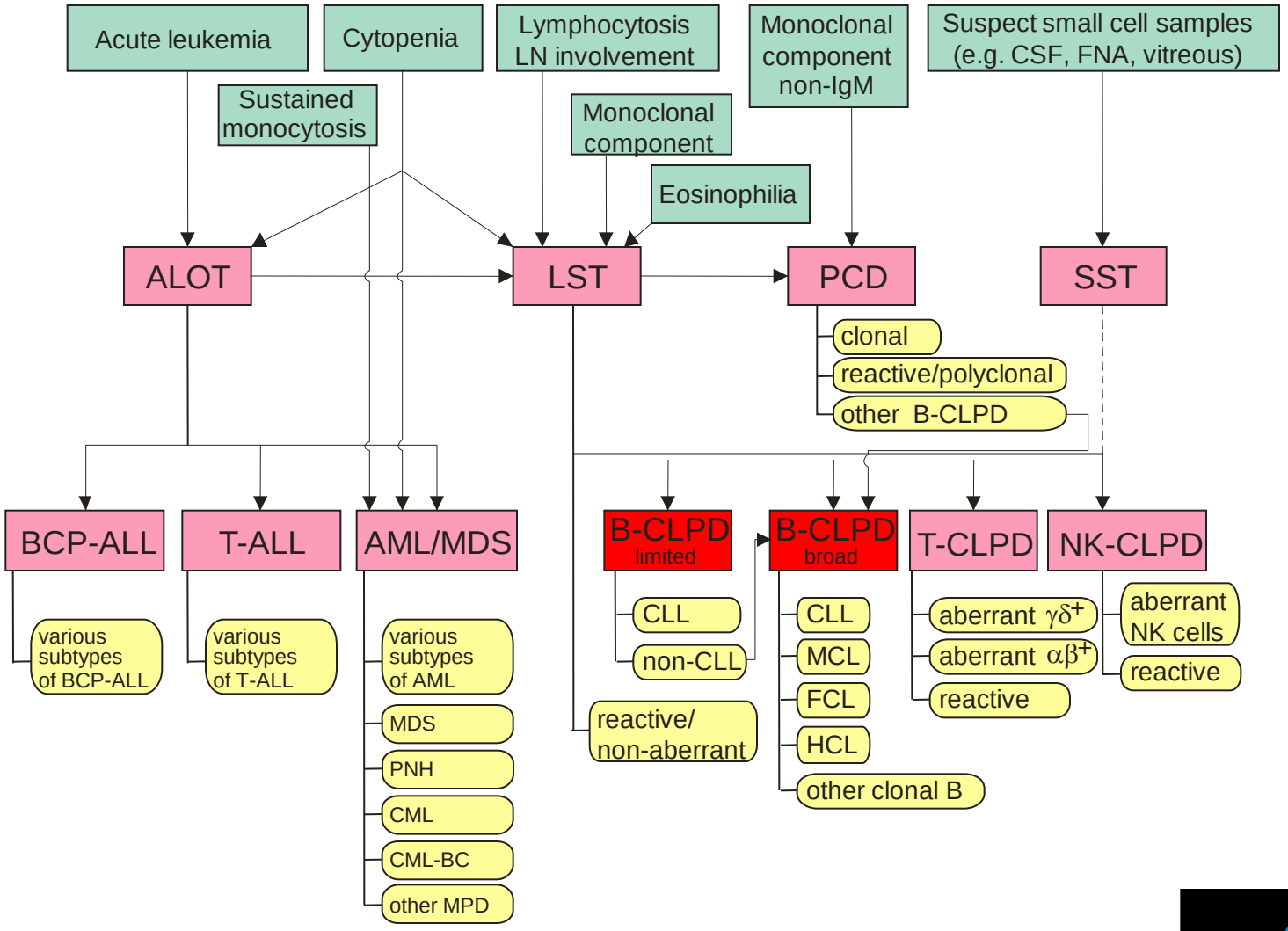
Technical aspects of EuroFlow protocols: instrument settings, fluorochrome choice, standardization

T. Kalina[1], J. Flores-Montero[2], Q. Lecrevisse[2], M. Cullen[3],L. Lhermitte[4], L. Sedek[5], A. Mendonca [6], S. Bötcher[7], J. te Marvelde[8], Mejstříková, O. Hrušák[1], J.J.M. van Dongen[8], and A. Orfao[2]

1, Department of Pediatric Hematology and Oncology, Charles University, Prague, Czech Republic;
2, Department of Medicine, Cancer Research Centre and Cytometry Service, University of Salamanca, Salamanca, ES;
3, St. James University Hospital, Leeds, UK;
4, Department of Hematology, Hôpital Necker, Paris, FR
5, Department of Pediatric Hematology and Oncology, Medical University of Silesia, Zabrze, PL;
6, Department of Hematology, Instituto Portugues de Oncologia , Lisbon, PT;
7, 2nd Department of Medicine, University Klinik Schleswig-Holstein, Kiel, DE;
8, Department of Immunology, Erasmus MC, Rotterdam, NL;

Prepared by Thomas Kalina on behalf of the EuroFlow Consortium (EU-FP6, LSHB-CT-2006-018708)

Diagnostic work-flow



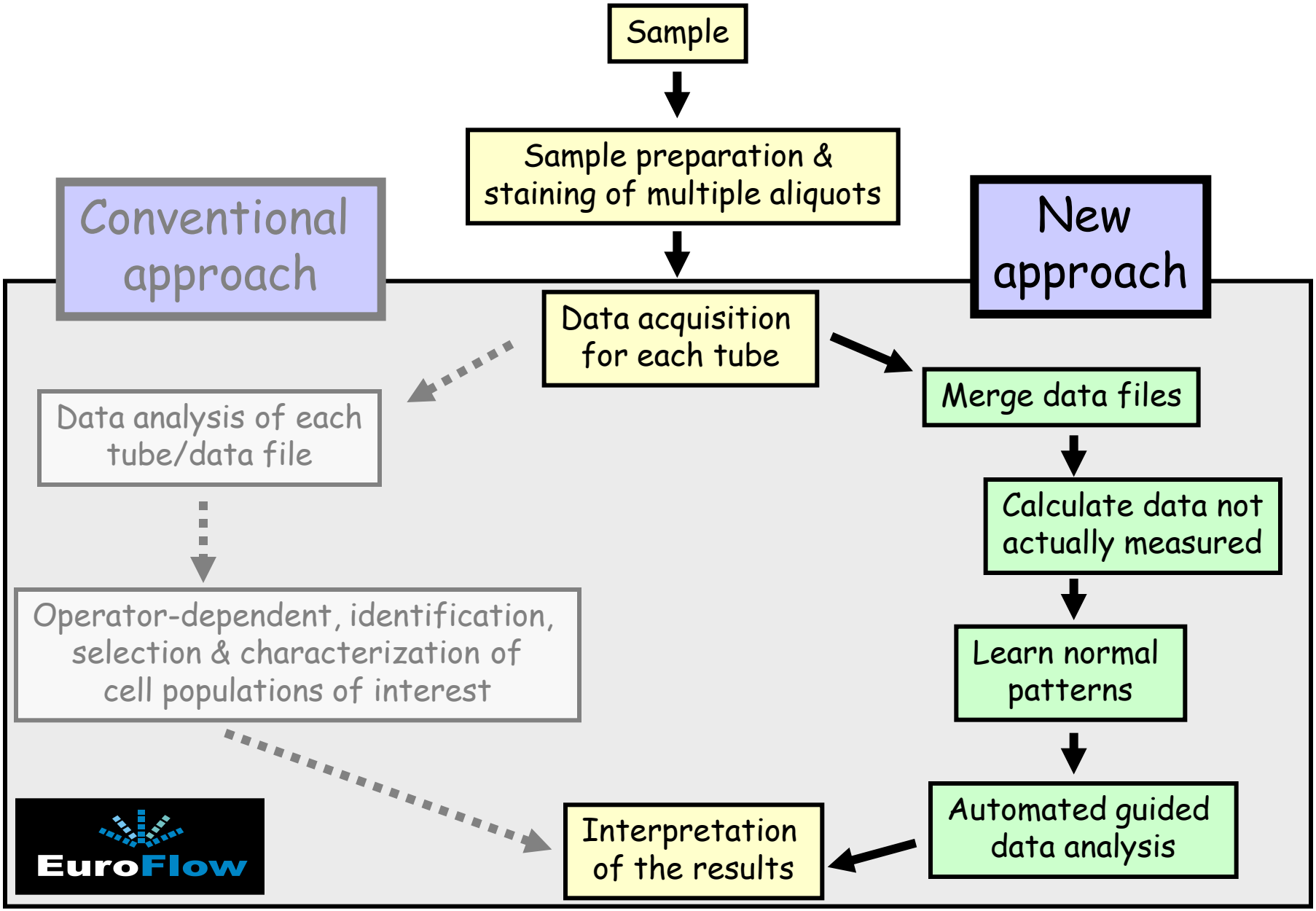
EuroFlow

EuroFlow antibody protocols

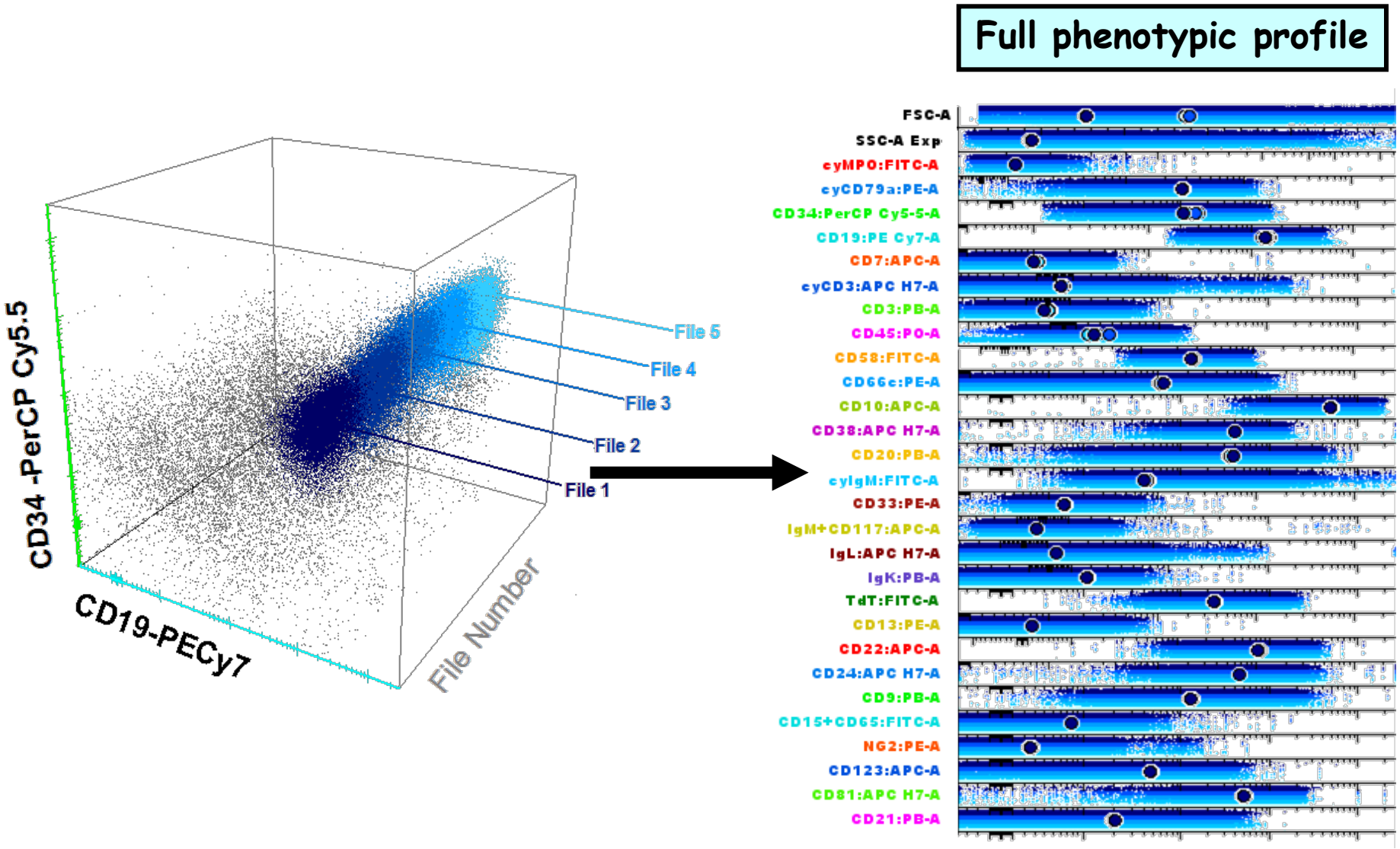
Development of 8-color multi-tube antibody protocols
(3 or 4 antibodies in common per tube in each protocol)

1. **Screening tubes (include recognition of normal leukocyte subsets)**
   - Acute leukemia orientation tube (ALOT): 1 tube (L Lhermitte)
   - Lymphoid screening tube (LST): 1 tube (J Flores Montero)
   - Small sample screening tube (SST): 1 tube (AW Langerak)
   - Plasma cell dyscrasia tubes (PCD): 2 tubes (J Flores Montero)
2. **Multi-tube panels for characterization per disease category**
   - B-cell precursor ALL (BCP-ALL) protocol: 4 tubes (L Lhermitte)
   - T-cell ALL (T-ALL) protocol: 4 tubes (V Asnafi)
   - AML/MDS protocol: 7 tubes (VHJ van der Velden)
   - B chronic lymphoproliferative diseases (B-CLPD): 5 tubes (S Böttcher)
   - T chronic lymphoproliferative diseases (T-CLPD): 6 tubes (J Almeida)
   - NK chronic lymphoproliferative diseases (NK-CLPD): 3 tubes(J Almeida)

FLOW CYTOMETRY IMMUNOPHENOTYPING APPROACH



MERGED DATA FILES FOR SINGLE STEP GATING



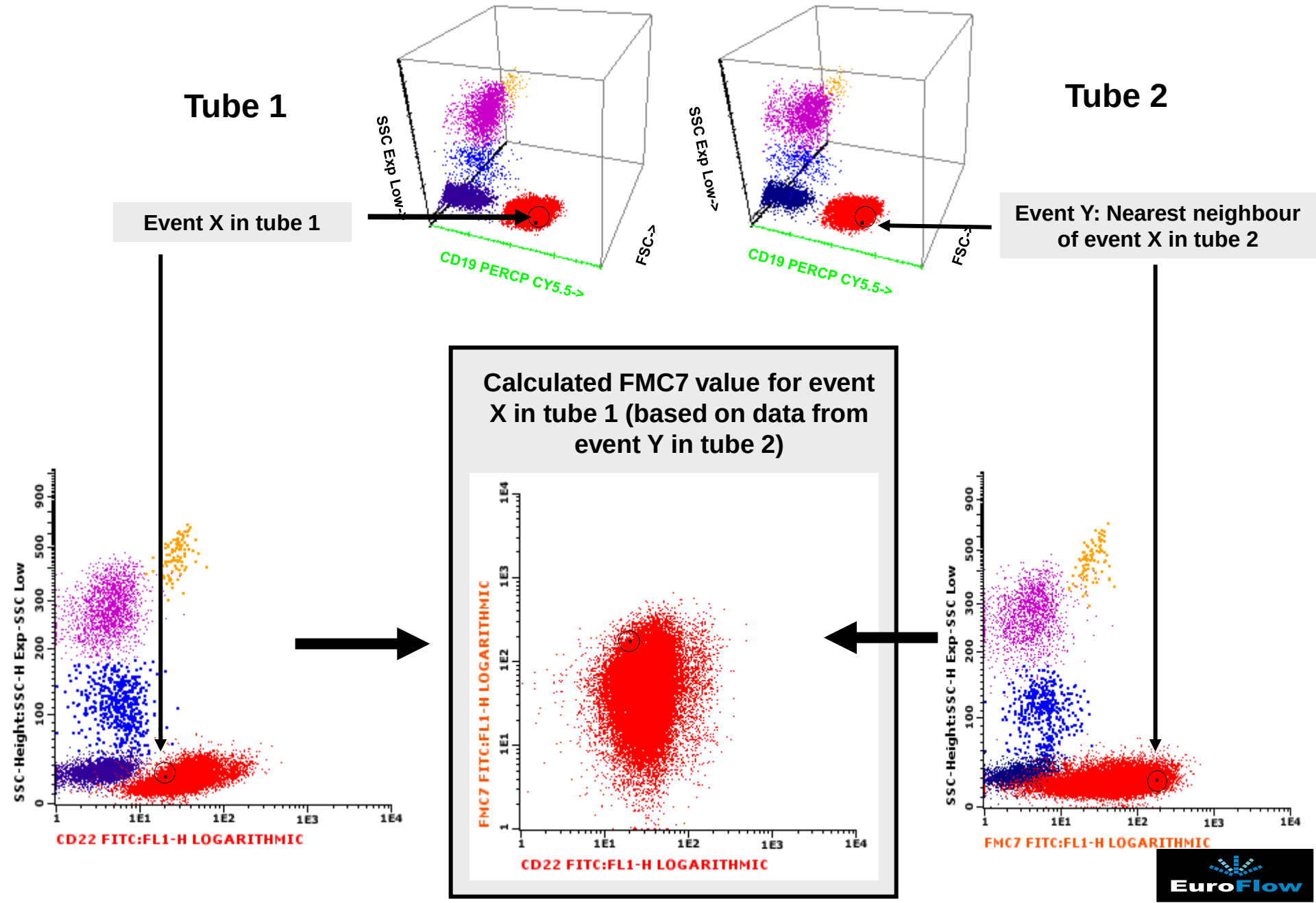
A single gating step for 5 different data files (tubes)

EuroFlow

DATA IN A MERGED DATA FILE

	TUBE No								
PARAMETER	1	2	3	4	5	6	7	8	9
FSC-HEIGHT	C	C	C	C	C	C	C	C	C
SSC-HIGHT	C	C	C	C	C	C	C	C	C
CD11b-FITC	R								
CD13-PE	R								
CD45-PerCP	C	C	C	C	C	C	C	C	C
CD34-APC	C	C	C	C	C	C	C	C	C
CD2-FITC		R							
CD56-PE		R							
HLADR-FITC			R	R					
CD117-PE			R						
CD123-PE				R					
CD15-FITC					R				
CD16-PE					R				
CD22-FITC						R			
CD25-PE						R			
CD65-FITC							R		
7.1-PE							R		
CD61-FITC								R	
CD33-PE								R	
CD71-FITC									R
Glyphorin-PE									R

DATA CALCULATION IN THE INFINICYT™ SOFTWARE



MERGED & CALCULATED LISTMODE DATA FILE

Files

Parameters

Parameters\File	1	2	3	4	5	6
FSC-A	C	C	C	C	C	C
SSC-A	C	C	C	C	C	C
KAPPA:FITC-A	R	E	E	E	E	E
LAI	R	E	E	E	E	E
CD	R	R	R	R	R	E
CD	C	C	C	C	C	C
IgM	R	E	E	E	E	E
CD	C	C	C	C	C	C
CD	C	C	C	C	C	C
CD45-PO:AmCyan-H	C	C	C	C	C	C
CD103:FITC-A	E	R	E	E	E	E
CD10:PE-A	E	R	E	E	E	E
CD43:APC-A	E	R	E	E	E	E
CD81:FITC-A	E	E	R	E	E	E
CD79b:PE-A	E	E	R	E	E	E
CD2	E	E	R	E	E	E
CD3	E	E	E	R	E	E
CD6	E	E	E	R	E	E
CXC	E	E	E	R	E	E
CD2	E	E	E	E	R	E
LAIP	E	E	E	E	R	E
CD11a:APC-A	E	E	E	E	R	E
CD38:FITC-A	E	E	E	E	E	R
CD25:PE-A	E	E	E	E	E	R
CD138:PerCP-Cy5-5-A	E	E	E	E	E	R

6 Files x

10 parameters x

100.000 events

1 File x

25 parameters x

600.000 events

Parameter coding

- C = Common
- R = Measured
- E = Calculated

EuroFlow

CAN WE IMPROVE DISCRIMINATION BETWEEN NORMAL AND NEOPLASTIC CELLS ?

Merged and calculated datafiles

(matched for several markers)

Datafile 1

Neoplastic sample

(e.g.: B-CLPD PB)

Datafile 2

Matched normal sample

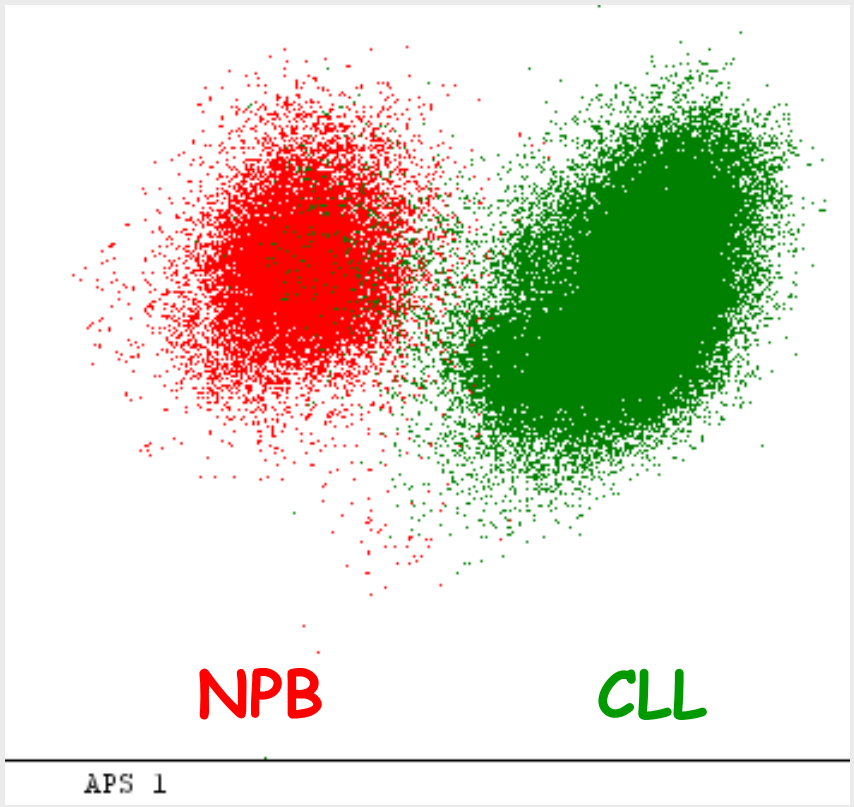
(e.g.: Normal PB from X donors)

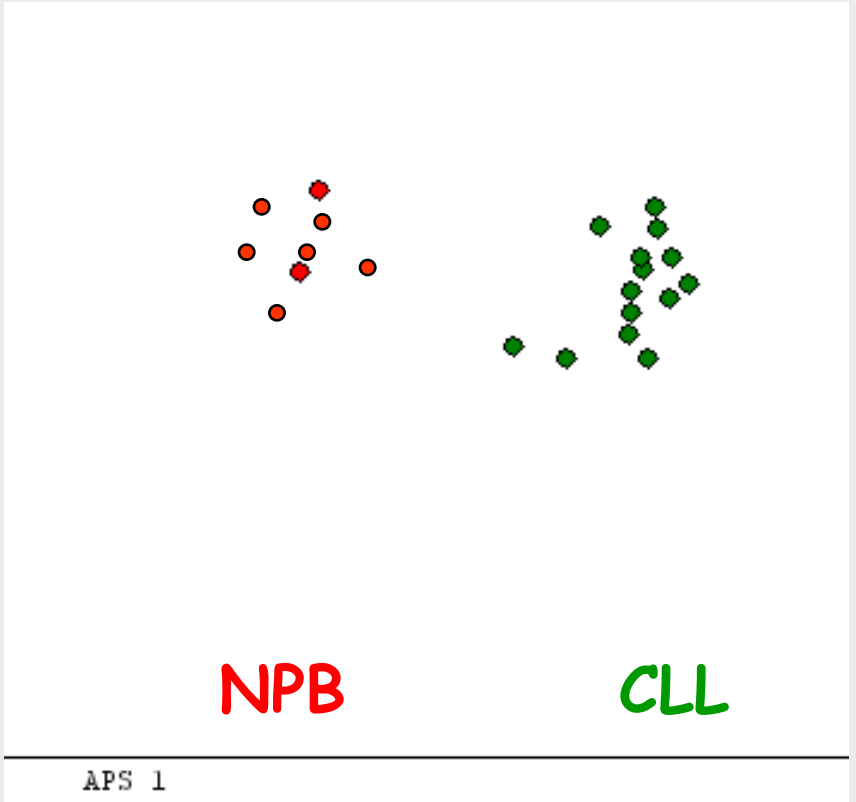
Merge datafiles 1 & 2

Select cell subset of interest (e.g.: B-cells) common to datafiles 1 & 2 in markers common to both datafiles

(e.g.: SSC^{lo}, FSC^{lo}, $CD19^{+}$ events)

GATED CD19+ NORMAL PB VS CLL B-CELLS: Improving Expert-based selection of CLL cells





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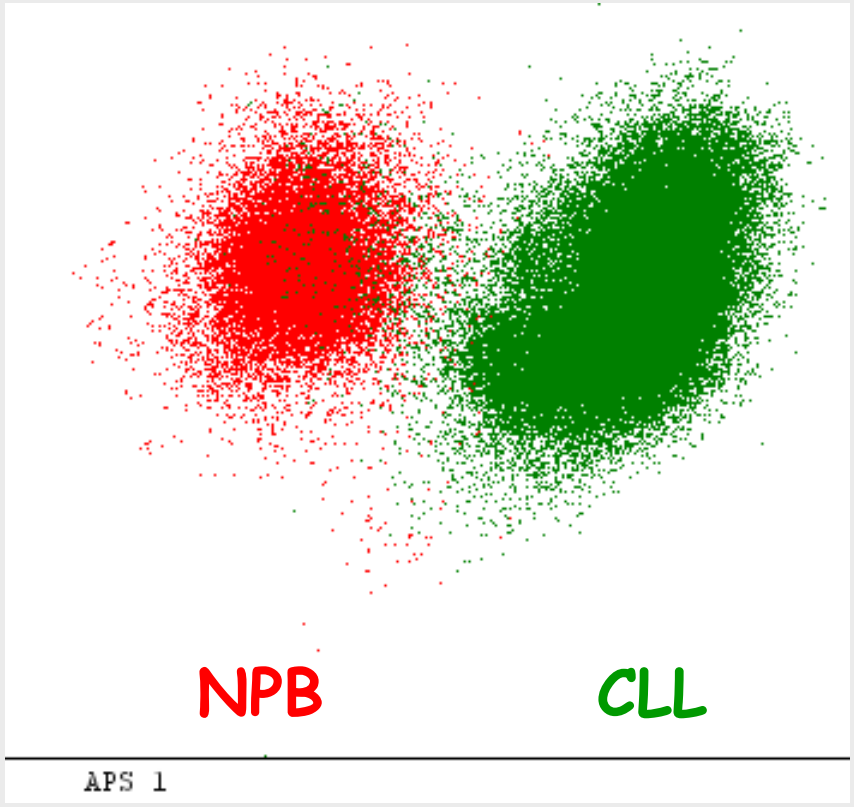
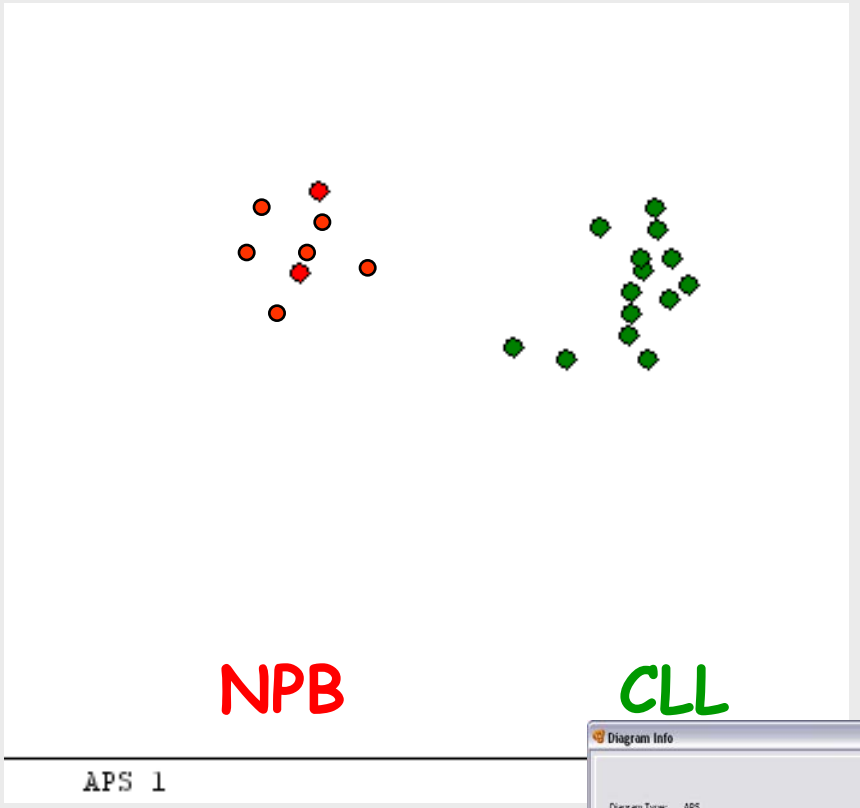
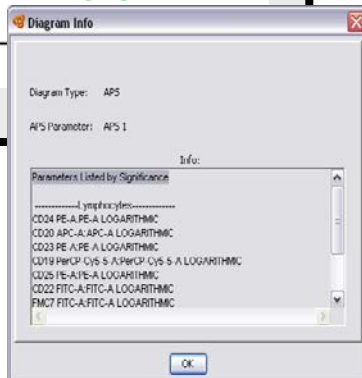






Information about the most discriminating parameters: (Most informative panels for the follow-up of MRD)

B-CLPD: ESTIMATED SENSITIVITY FOR MRD STUDIES BY FLOW CYTOMETRY

- Simulated dilution of progressively low numbers of neoplastic B-cells in a normal sample:

N. of neoplastic CD19+ B-cells:

1000, 900, 800… 100, 90, 80… 10, 9… 1, 0

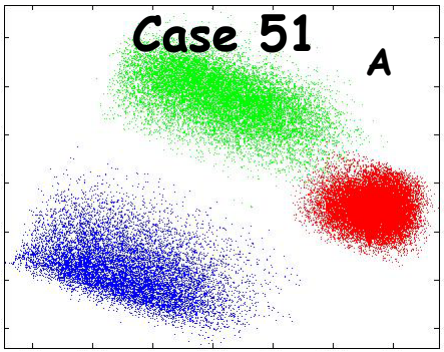
Total number of normal cells: 10^7

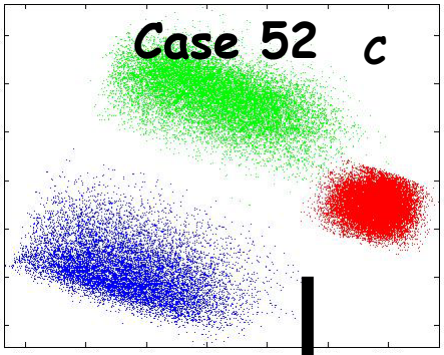
Sensitivity of $<10^{-6}$ in 80% of the cases

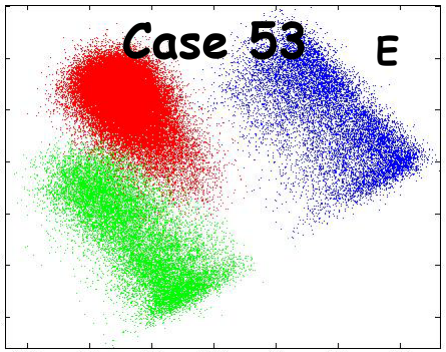
B-CLPD: AUTOMATED IDENTIFICATION OF ABERRANT PHENOTYPES AT DIAGNOSIS

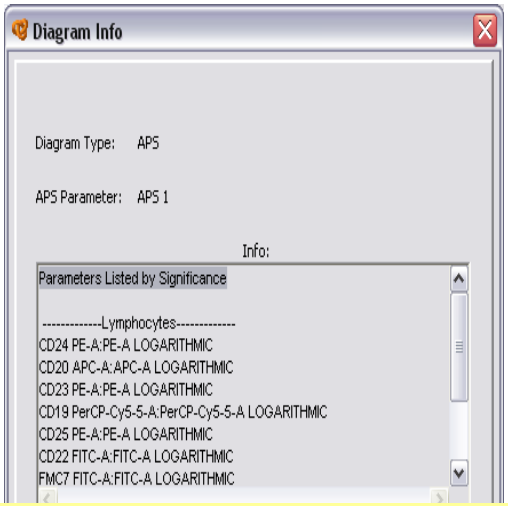
(Only CD19+ B-cells are displayed)

PB SAMPLES AT DIAGNOSIS









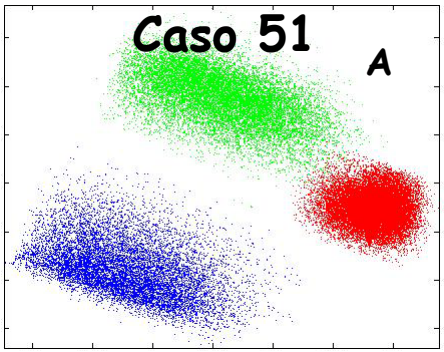
Information about the most discriminating parameters: (Most informative panels for the follow-up of MRD)

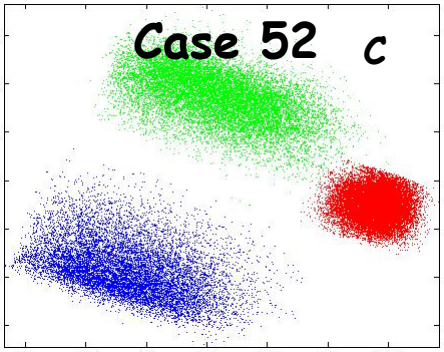
Pedreira et al, CytometryA 2008

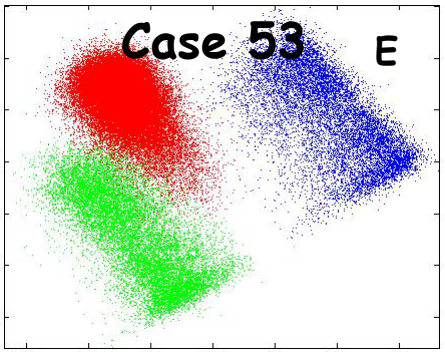
B-CLPD: AUTOMATED IDENTIFICATION OF MRD

(Only CD19+ B-cells are displayed)

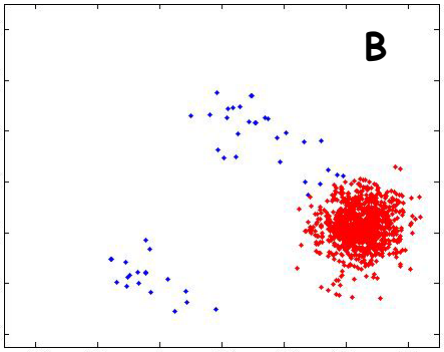
PB SAMPLES AT DIAGNOSIS

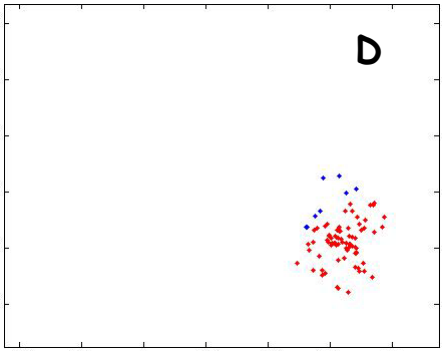


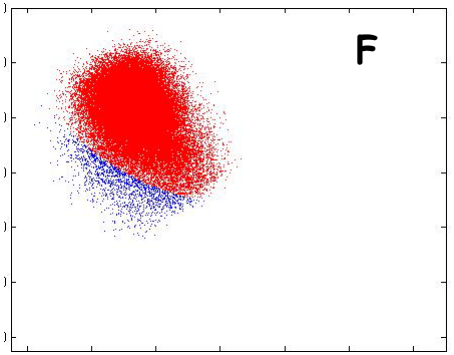




PB SAMPLES AFTER THERAPY







% of neoplastic B-cells correctly classified: 99,1%.

% of neoplastic B-cells correctly classified: 97,4%

% of neoplastic B-cells correctly classified: 92%

Case 51 **Case 52** **Case 53**

Pedreira et al, CytometryA 2008

FLOW CYTOMETRY IMMUNOPHENOTYPING OF LEUKEMIA & LYMPHOMA

Phase 1:

Question 1.- Is it clinically useful?

(IVD test?)

Question 2.- Are its results going to be clinically applied?

(Real usage)

Phase 2:

Question 3.- Why are samples submitted for immunophenotyping?

(Medical indications)

Question 4.- What information shall be given back from the lab?

(Report conclusions)

Question 5.- How can we better reach the right conclusion?

LEUKEMIA/LYMPHOMA TYPING

Phase 2 (continued):

Question 5.- How can we better reach the right conclusion?

(Type of sample)

↓

(Selection of algorithms)

↓

(Panels of reagents)

↓

(Sample preparation techniques)

↓

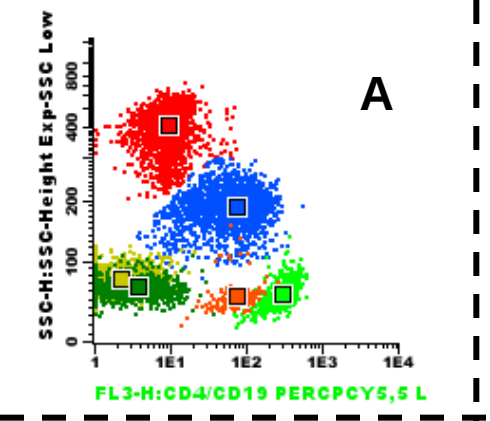
(Instrument set up procedure)

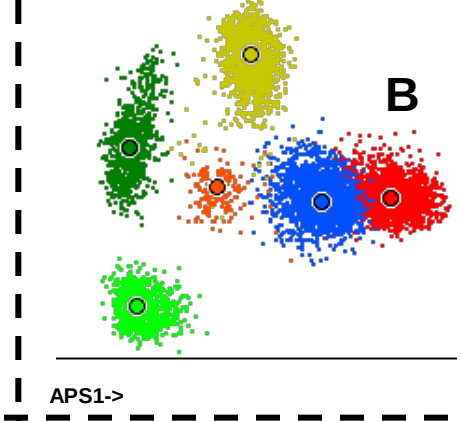
↓

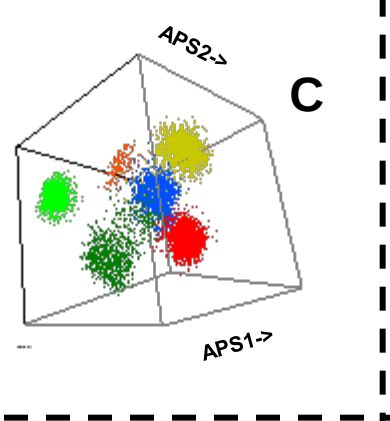
(Data acquisition)

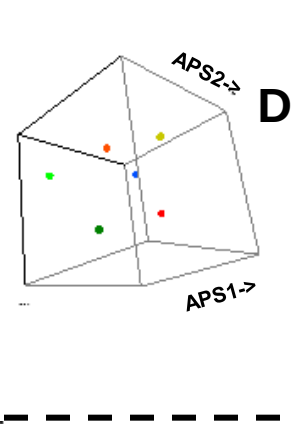
↓

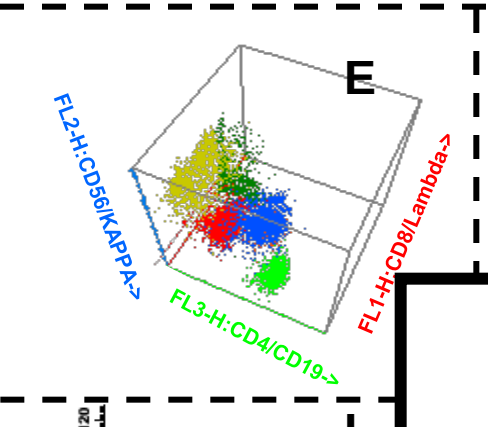
(Analytical approach)

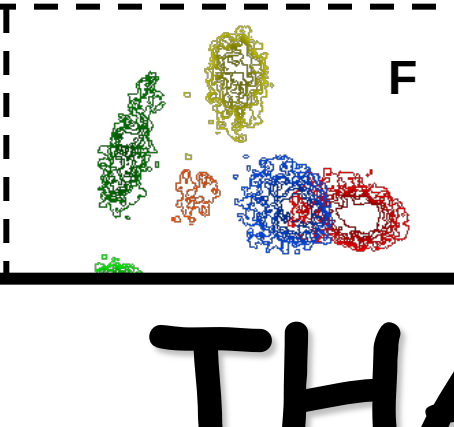

A
SSC-H:SSC-Height Exp-SSC Low
FL3-H:CD4/CD19 PERCPCY5,5 L

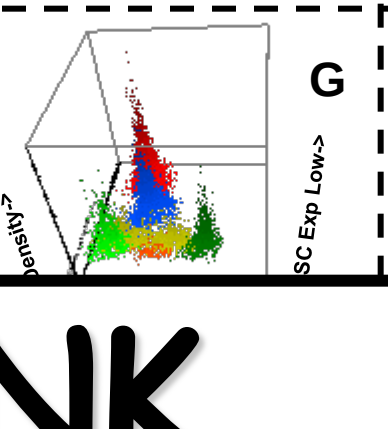

B
APS1->

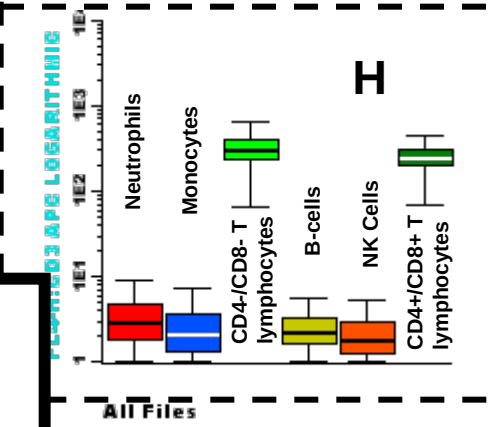

C
APS2->
APS1->


D
APS2->
APS1->

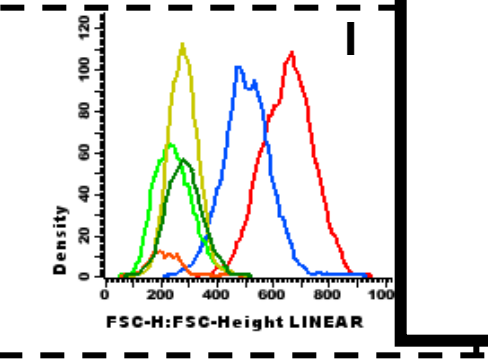

E
FL2-H:CD56/KAPPA->
FL3-H:CD4/CD19->
FL1-H:CD8/Lambda->

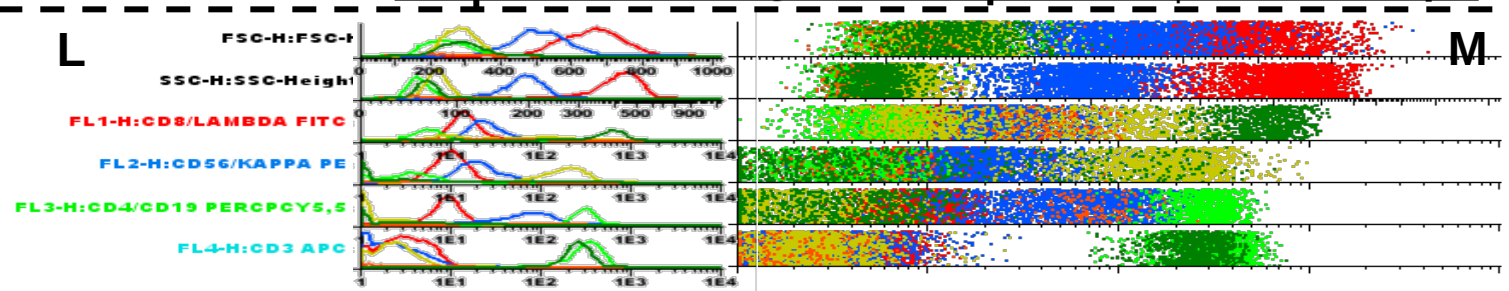

F

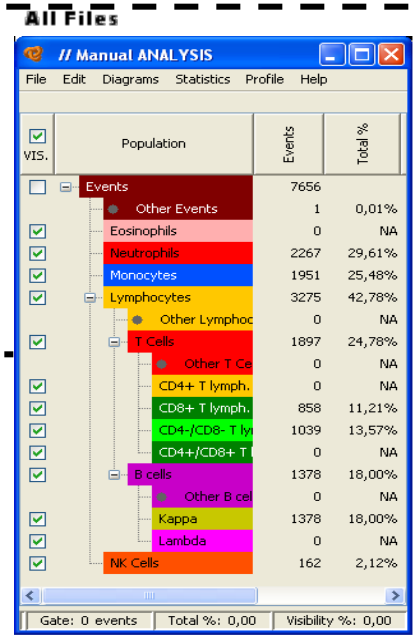

G
SC Exp Low->


H
Neutrophils
Monocytes
CD4-/CD8- T lymphocytes
B-cells
NK Cells
CD4+/CD8+ T lymphocytes

THANK YOU


I
Density
FSC-H:FSC-Height LINEAR


L
FSC-H:FSC-H
SSC-H:SSC-Height
FL1-H:CD8/LAMBDA FITC
FL2-H:CD56/KAPPA PE
FL3-H:CD4/CD19 PERCPCY5,5
FL4-H:CD3 APC
M


All Files
// Manual ANALYSIS
File Edit Diagrams Statistics Profile Help
VIS.
Population
Events
Total %
Events 7656
Other Events 1 0,01%
Eosinophils 0 NA
Neutrophils 2267 29,61%
Monocytes 1951 25,48%
Lymphocytes 3275 42,78%
Other Lymphoc 0 NA
T Cells 1897 24,78%
Other T Ce 0 NA
CD4+ T lymph. 0 NA
CD8+ T lymph. 858 11,21%
CD4-/CD8- T ly 1039 13,57%
CD4+/CD8+ T 0 NA
B cells 1378 18,00%
Other B cel 0 NA
Kappa 1378 18,00%
Lambda 0 NA
NK Cells 162 2,12%
Gate: 0 events
Total %: 0,00
Visibility %: 0,00