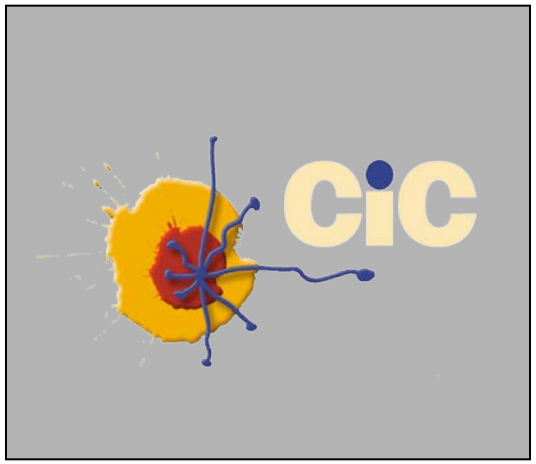
Diagnóstico y clasificación de los sindromes linfoproliferativos crónicos B



CENTRO DE INVESTIGACIÓN DEL CANCER & SERVICIO DE CITOMETRIA UNIVERSIDAD DE SALAMANCA

EuroFlow

Buenos Aires; Argentina. 30-31 mayo 1 junio 2011

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.

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

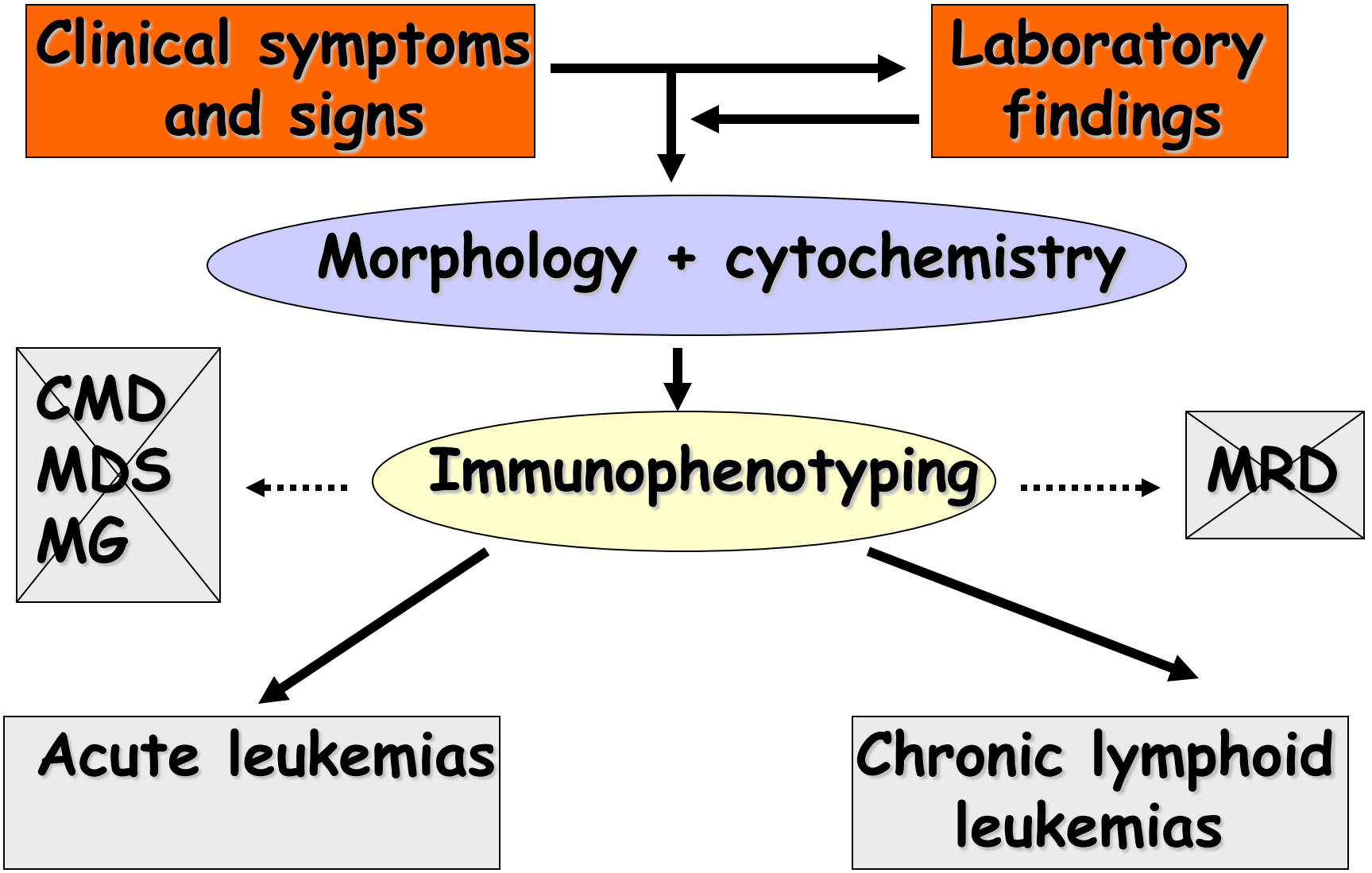
MATUTES et al SCORE FOR B-CLL

MARKER	EXPRESSION	SCORE
CD5	positive	1
CD23	positive	1
FMC7	negative	1
sIg	dim	1
CD22/CD79b	dim/dim	1

Scores in CLL range from 3 to 5, while in other B-CLPD range between 0 and 2

Matutes et al, Leukemia 1994

DIAGNOSIS OF HAEMATOLOGICAL MALIGNANCIES



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.

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

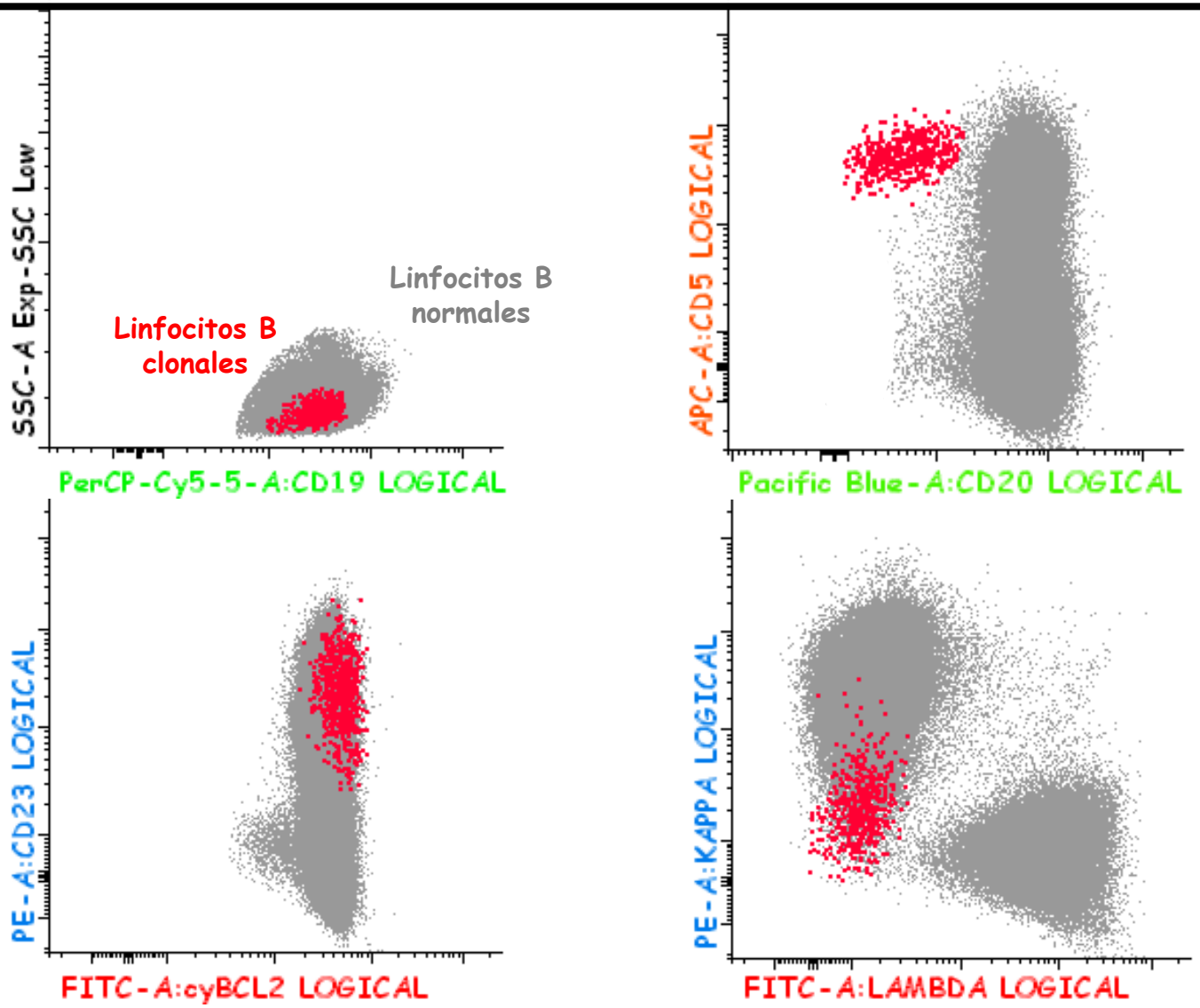
Diagnosis & follow-up of MRD in acute leukaemias, CLPD & MM

TECHNIQUES:

- Non-NRBC lysis
- Direct IF
- Multiple stainings

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

Immunophenotypic identification of PB B-cells with a CLL-like phenotype



*0.35% of all B-cells & 0.03% of all leucocytes

MINIMAL RESIDUAL DISEASE IN B-CLL

	Aberrant criteria	Sensitivity	Prognostic value
Brugiatelli M *et al.* (Cancer 1989)	sIgκ$^+$/sIgλ$^+$ ratio	10^{-2}	Yes
Robertson LE *et al.* (Blood 1992)	sIgκ$^+$/sIgλ$^+$ ratio	10^{-2}	Yes
Leonormand B *et al.* (Leukemia 1994)	CD19$^+$/CD5$^+$	10^{-3}	Yes
Cabezudo E *et al.* (Leukemia, 1997)	CD19$^+$/CD5$^+$	10^{-3}	Yes
García-Vela A *et al.* (Leukemia, 1999)	CD19$^+$/CD79b^{+d}	10^{-4}	Yes
Rawstron AC *et al.* (Blood 2001)	CD19$^+$/CD20^{+d}/CD5$^+$/CD79b^{+d}	10^{-4}	Yes
Maloum K *et al.* (Br J Haematol 2002)	CD19$^+$/CD20^{+d}/CD5$^+$/CD79b^{+d}	10^{-4}	Yes
Gupta R *et al.* (Am J Clin Pathol 2004)	CD19$^+$/CD5$^+$	10^{-3}	Not analyzed
Bottcher S *et al.* (Leukemia 2004)	CD19$^+$/CD5$^+$/CD43$^+$/CD20^{+d}	10^{-4}	Not analyzed
Moreton P *et al.* (J Clin Oncol 2005)	CD19$^+$/CD20^{+d}/CD5$^+$/CD79b^{+d}	10^{-5}	Yes
Montillo M *et al.* (Cancer Invest 2005)	CD19$^+$/CD20^{+d}/CD5$^+$/CD79b^{+d}	10^{-5}	Yes

Origen de los SLPC-B según su célula homóloga normal

SANGRE PERIFERICA

LEZM:
CD19+, CD20+, CD22+, CD79b+, sIg+, FMC7+, CyBcl2+, sIg+, CD11c+, CD103-, HC2-, CD25-, CD5-, CD10-, CD23-, bcl6-, (CD38 y CD43 variable)

TL:
CD19++, FMC7+, CD79b+, CD20+++, CD22++, sIg++, CD72+, CD11c++, CD25+, CD103+, LAIR-1++, CD123++, HC2+, CD5-, CD10-, CD23-, CD24-

LPL:
CD19+, CD20+, FMC7+, CD22+, CD79b+, sIgM y/o sIgD+, CD5-/+, CD23-/+, CD25-/+, CD103-, CD11c y LAIR-1 variable.

Célula B de memoria

LLC-B

Tejido MALT

Zona Marginal

Zona del Manto

GANGLIO LINFÁTICO

Centro Germinal (CG)

MALT:
sIg+, CD19+, CD20+, CD22+, CD79b+, CxCR3+, CD5-, CD23-, CD10-, CD27+ y CD45RO-, CD11c y CD43 variable.

Célula B naïve

Célula B del CG

LLC-B:
CD19+, CD23+, CD200+, CD21+, CD24+, CD25+, CD27+, sIgM+, CD39+, CD62L+, CD40+, CD45RA+, CD5+, CD20+d, CD22+d, CD79b+d, CD81+d, sIg-/+, CD10-, FMC7-

Plasmablasto

MÉDULA ÓSEA

Célula plasmática

LF:
CD19-/+débil, sIgM-/+, CD20/FMC7+, CD22+, CD79b+, sIg+, CD10+, bcl6+, CD38+, CyBcl2+++, CD5-, CD11c-, CD23-, CD43-, CD103-, CD200-, HC2-

LDCG:
CD19++, CD20/FMC7++, CD79b++, CD22++, sIg++, CD10-/+, CD200/+-, CD5-, CD23-.

LB:
CD19+, CD20+, CD22+, CD79b+, sIg+, CD10+, CD77+, CD43+, HLADR+, bcl6+, CD38+++, CyBcl2+d, CD5-, CD23-, TdT-

LLP/MW:
CD19+, CD24+, CD25+, CD20/FMC7+, CD22+, CD79+, cyIg+, CD54+, sIgM+, CD5-, CD10-, CD23-, (CD38 variable)

MM:
sIg+d, CD20+d, CD38++, CD138++, CyIg++, CD56++, CD22-, CD5-, CD23-, CD45-, CD19-, CD117-/+, CD27-/+, CD28-/+, CD33-/+, CD81-/+, CD126-/+, CD229-/+, CD86-/+

LCM:
CD19+, CD20+, CD22+, CD24+, CD79b+, CD5+, CD43+, bcl1, sIgM y/o sIgD+, CD18+, FMC7+, CD54+, CD79b+ CD10-, CD23-, CD200-, CD38 variable

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

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)

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)

DIAGNOSIS OF CLONALITY: CLINICAL QUESTIONS

- Is this lymphocytosis clonal?

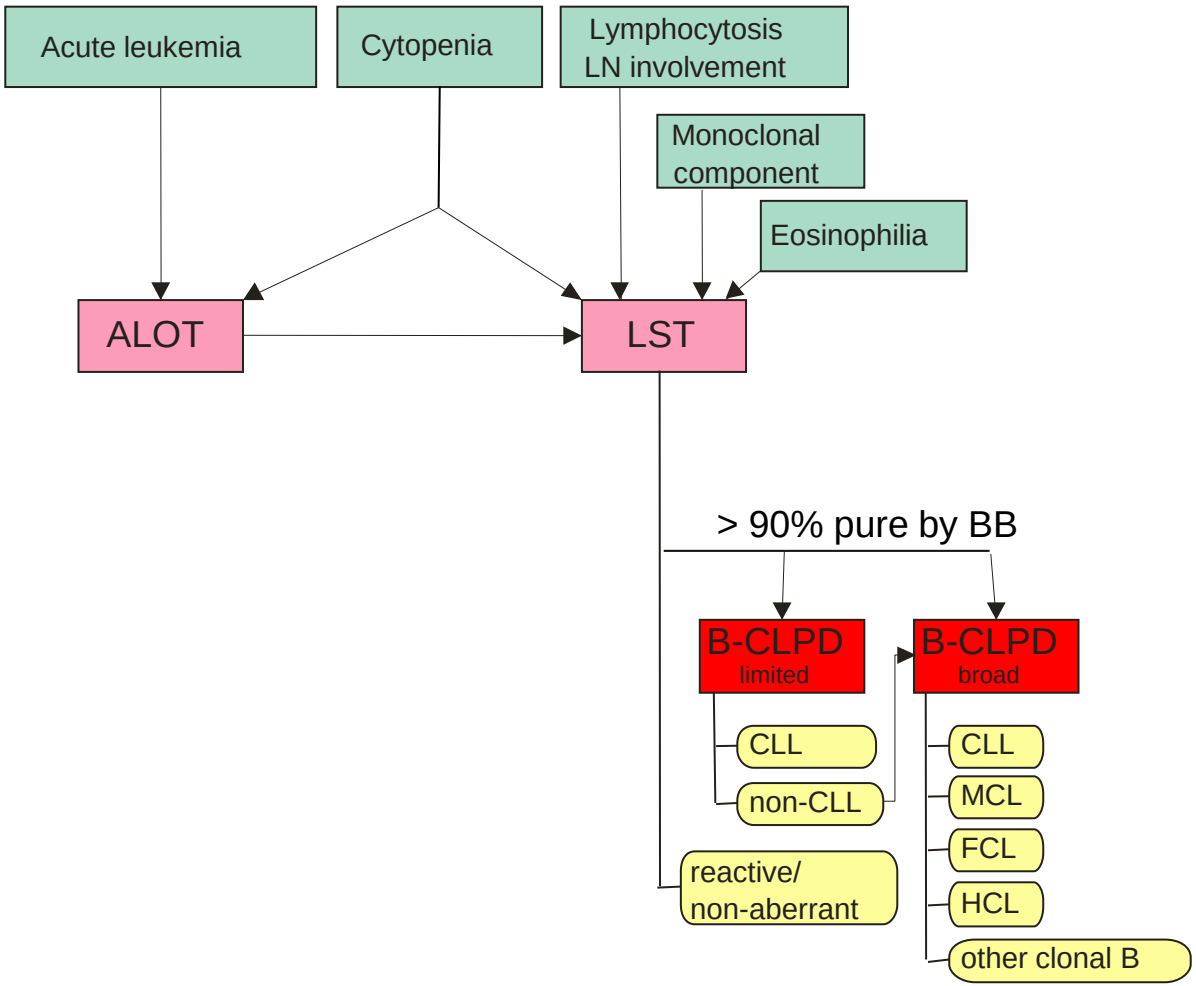
-Is this tissue enlarged because of an underlying clonal LPD?

-Are there clonal lymphocytes in this "apparently" normal tissue?/Is there minimal disease?

THE B-CLPD EuroFlow PANEL

Prepared by Sebastian Böttcher for

B-CLPD: Diagnostic work-flow



EuroFlow

Lymphocytosis Screening Tube (LST)

Aim: *DIAGNOSTIC SCREENING OF B, T & NK CELL CLPD to identify the cell lineage involved (in the lymphocytosis), to detect the most common aberrant phenotypes (CD5), and to assess B-cell clonality*

Pacific Blue	Pacific Orange	FITC	PE	PerCP Cy5.5	PECy7	APC	APC-H7
CD20 CD4	CD45	CD8 Anti-Igλ	CD56 Anti-Igκ	CD5	CD19 Anti-TCRγδ	sCD3	CD38

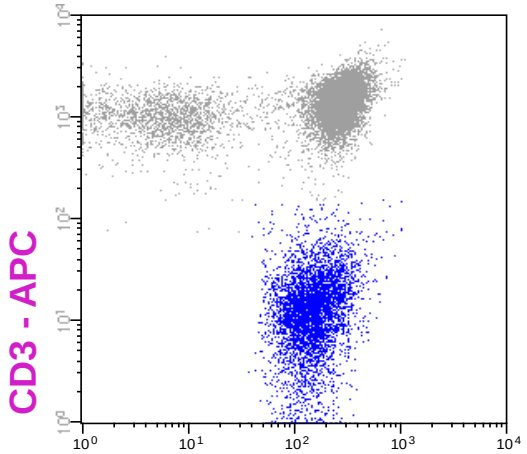
- Markers aiming at identification of B-lineage cells, common B-cell aberrancies and to assess B-cell clonality
- Markers aiming at identification of T cells and T-cell subsets:
  - .- TCRγδ vs TCRαβ (non-TCRγδ)
  - .- CD4+/CD8-; CD4-/CD8+; CD4+/CD8+; CD4-/CD8-

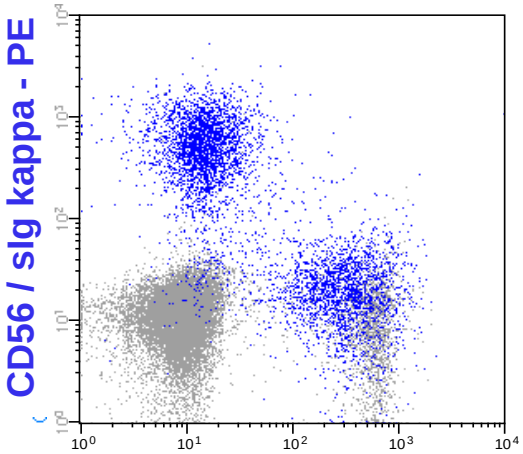
  and T-cell aberrancies
- Markers aiming at identification of NK cells, and NK-cell subsets

EuroFlow

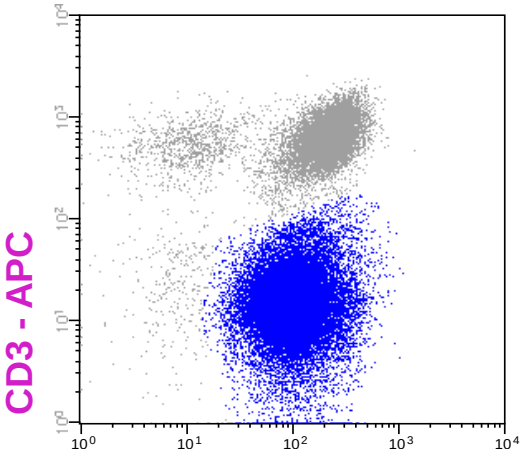
RAPID SCREENING OF LYMPHOID MALIGNANCIES IN FNA FROM TISSUE SAMPLES

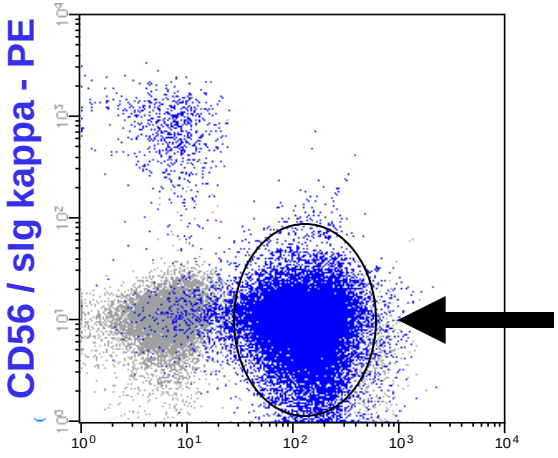
NORMAL LYMPH NODE





B-CLPD LYMPH NODE





RAPID FCM SCREENING OF CLONAL B-CELLS: ABSOLUTE LYMPHOCYTOSIS (> 3.5 x10^9/L)

	Conventional Morphology	Flow Cytometry	Final Diagnosis
Reactive	25	32	32
Doubtful	21	0	
Neoplastic	63	77	77

Sensitivity	95%	100%
Specificity	75%/100%*	100%

*Considering only the neoplastic group

Costa et al, Leukemia, 2006

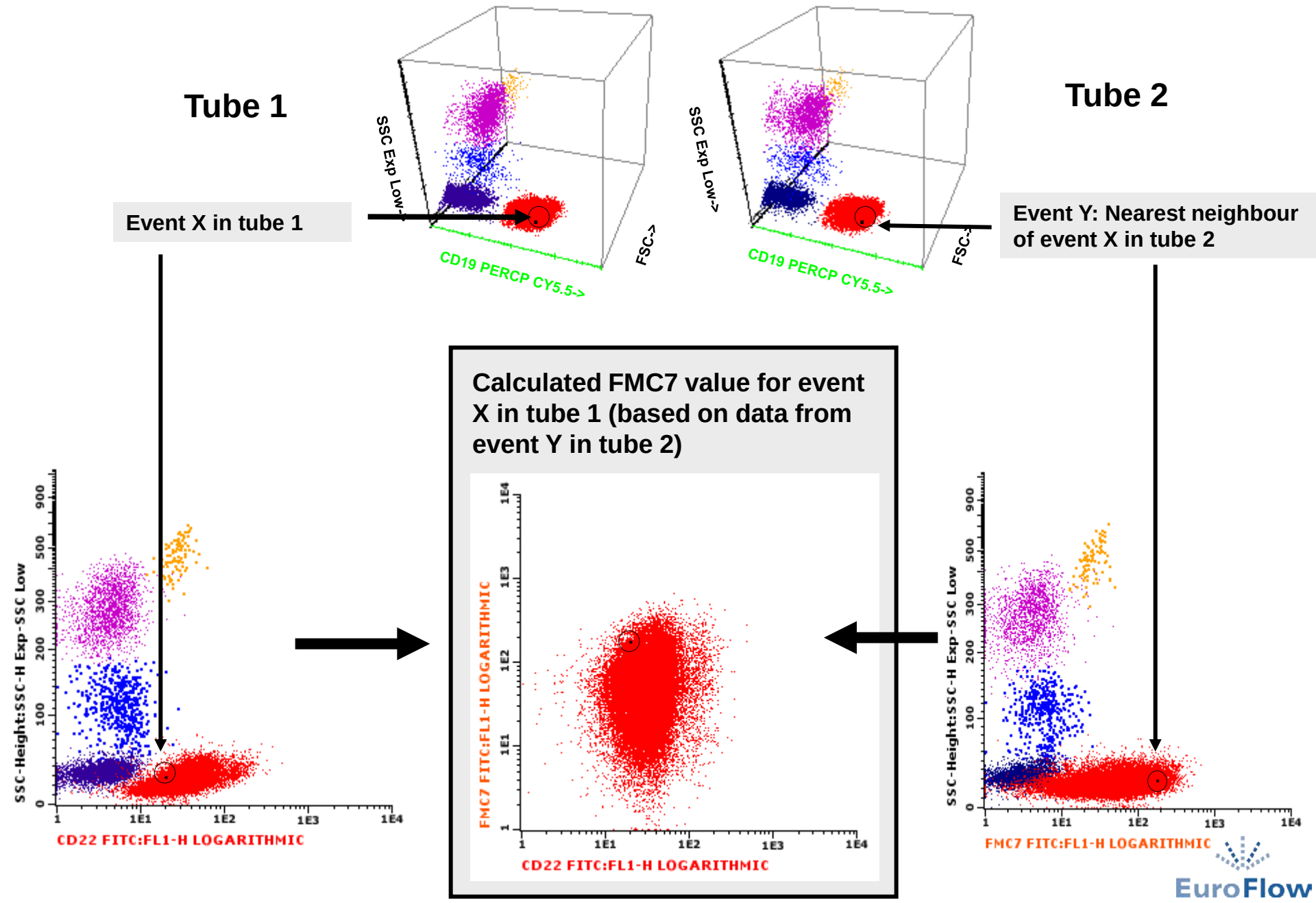
B-CPLD panel

	Pac Blue	Pac Orange	FITC	PE	PerCP-Cy5.5	PECy7	APC	APC-H7
1	CD20	CD45				CD19		
2	CD20	CD45				CD19		
3	CD20	CD45				CD19		
4	CD20	CD45				CD19		
5	CD20	CD45				CD19		

1= LST	CD20 / CD4	CD45	λ/CD8	κ/CD56	CD5	CD19 / TCRγ/δ	CD3	CD38
2	CD20	CD45	CD23	CD10	CD79b	CD19	CD200	CD43
3	CD20	CD45	CD31	LAIR	CD11c	CD19	IgM	CD81
4	CD20	CD45	CD103	CD95	CD22	CD19	CXCR5	CD49d
5	CD20	CD45	CD62L	CD39	HLA-DR	CD19	CD27	

EuroFlow

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-A	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
LAIR	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

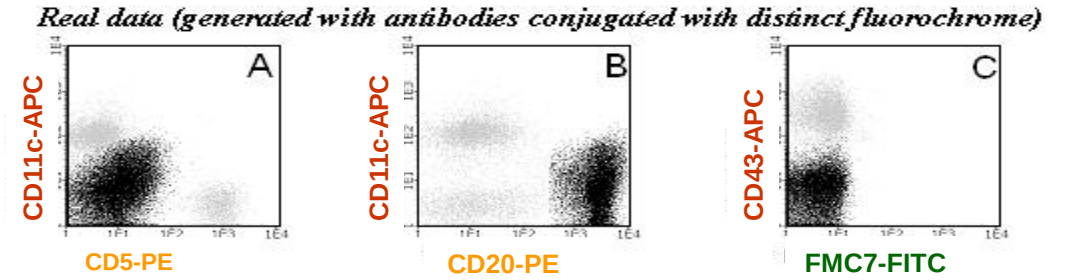
E = Calculated

EuroFlow

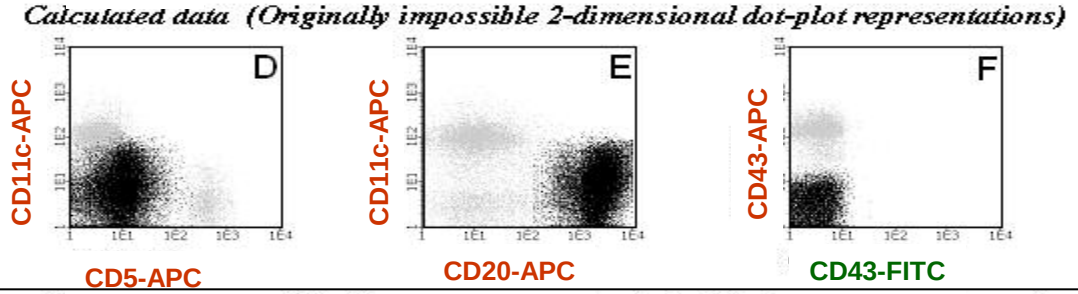
CALCULATED VS MEASURED DATA

FIGURE 4

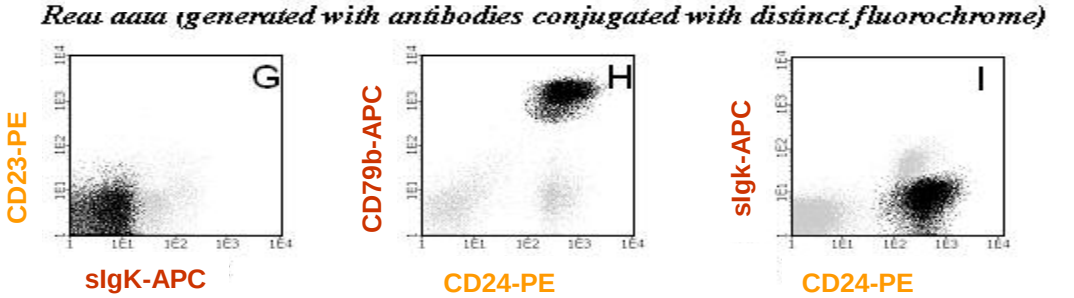
Measured



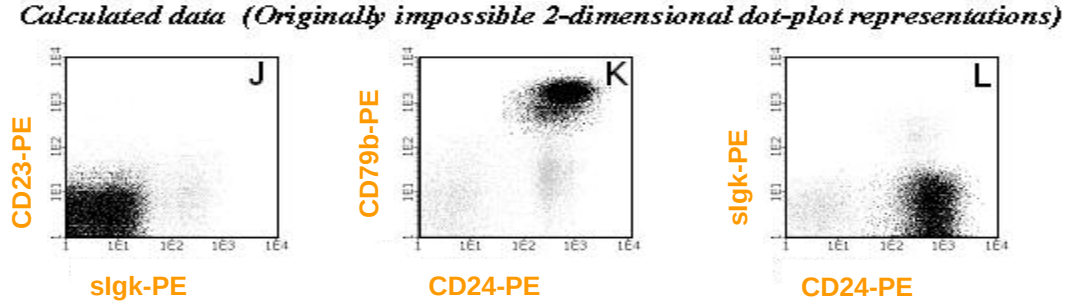
Calculated



Measured



Calculated



Pedreira et al, Cytometry A, 2008

REFERENCE DATAFILES FOR CLL B-CELLS

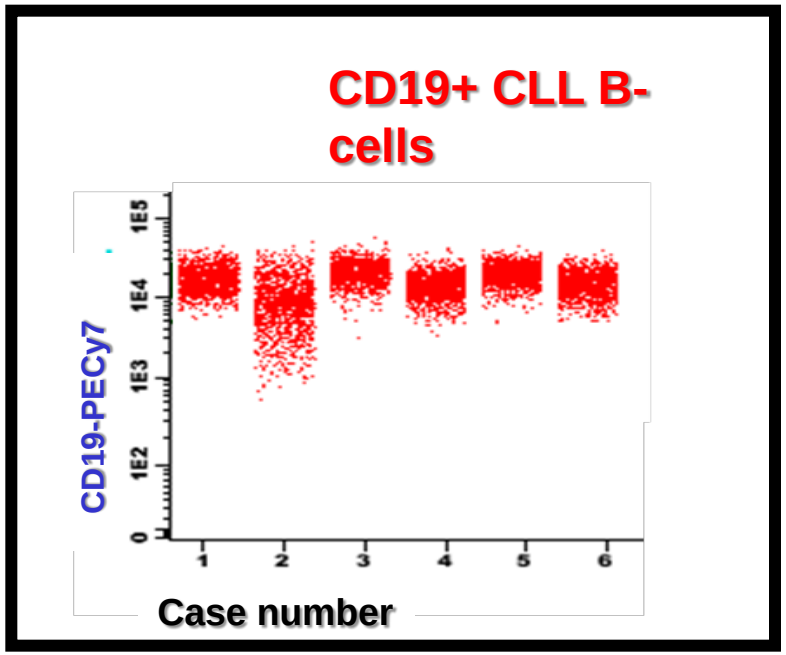
CLL case 1

CLL case 2

CLL case 3

CLL case 4

Merge Calculated Data files



Responsible scientist: Sebastian Bottcher

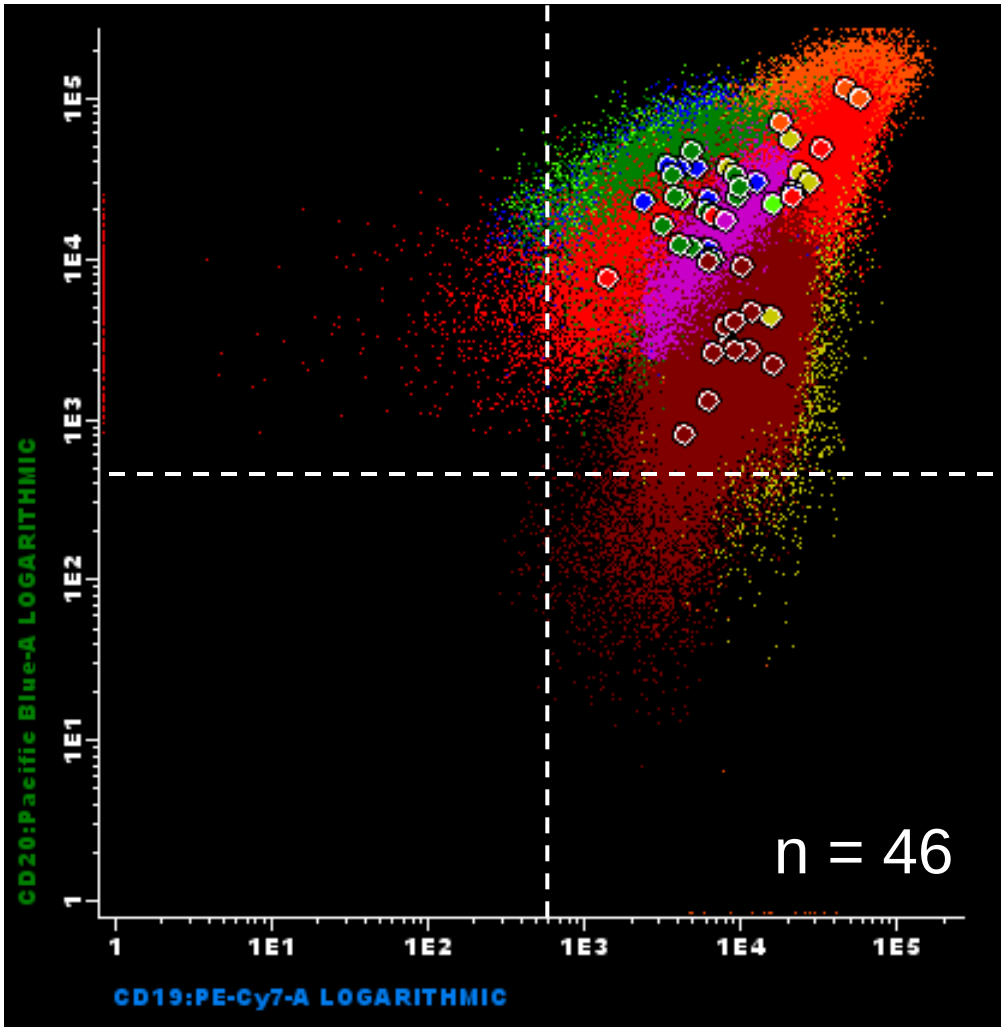
EuroFlow

Backbone markers

- Backbone markers should identify all B cells
- Aberrant underexpression of CD19 and/or CD20 frequently observed
- κ/CD37/λ/CD19/CD22/CD20 tested in 69 B-NHL cases
- Conclusion: CD37 & CD22 redundant, as CD20 Pacific Blue plus CD19 PE-Cy7 sufficient to identify all malignant B cells in all cases

EuroFlow

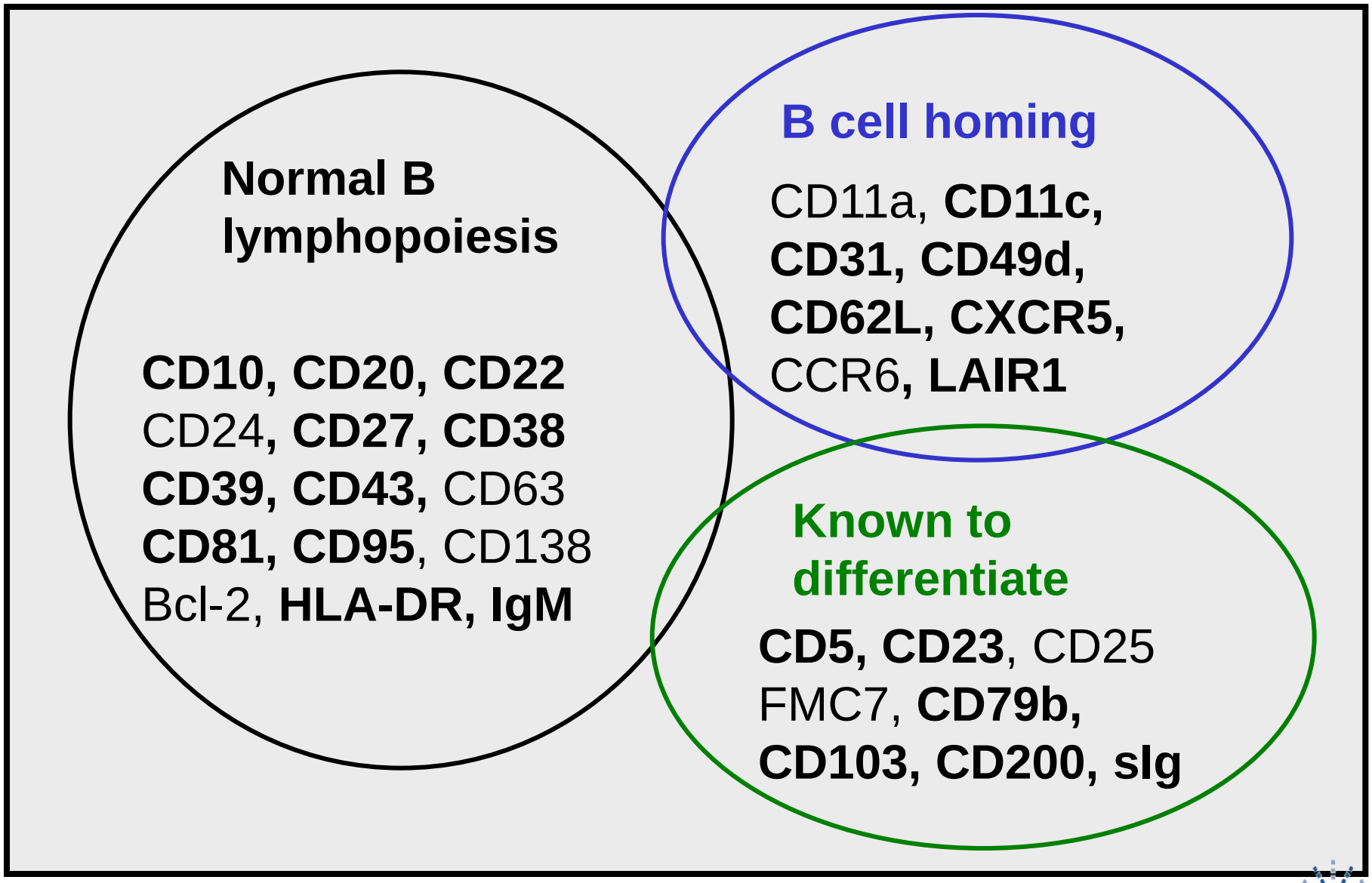
Backbone markers



No problems to include malignant clone in 190 cases

EuroFlow

Characterization markers



Responsible scientist: Sebastian Bottcher

EuroFlow

Characterization markers

Taken out:

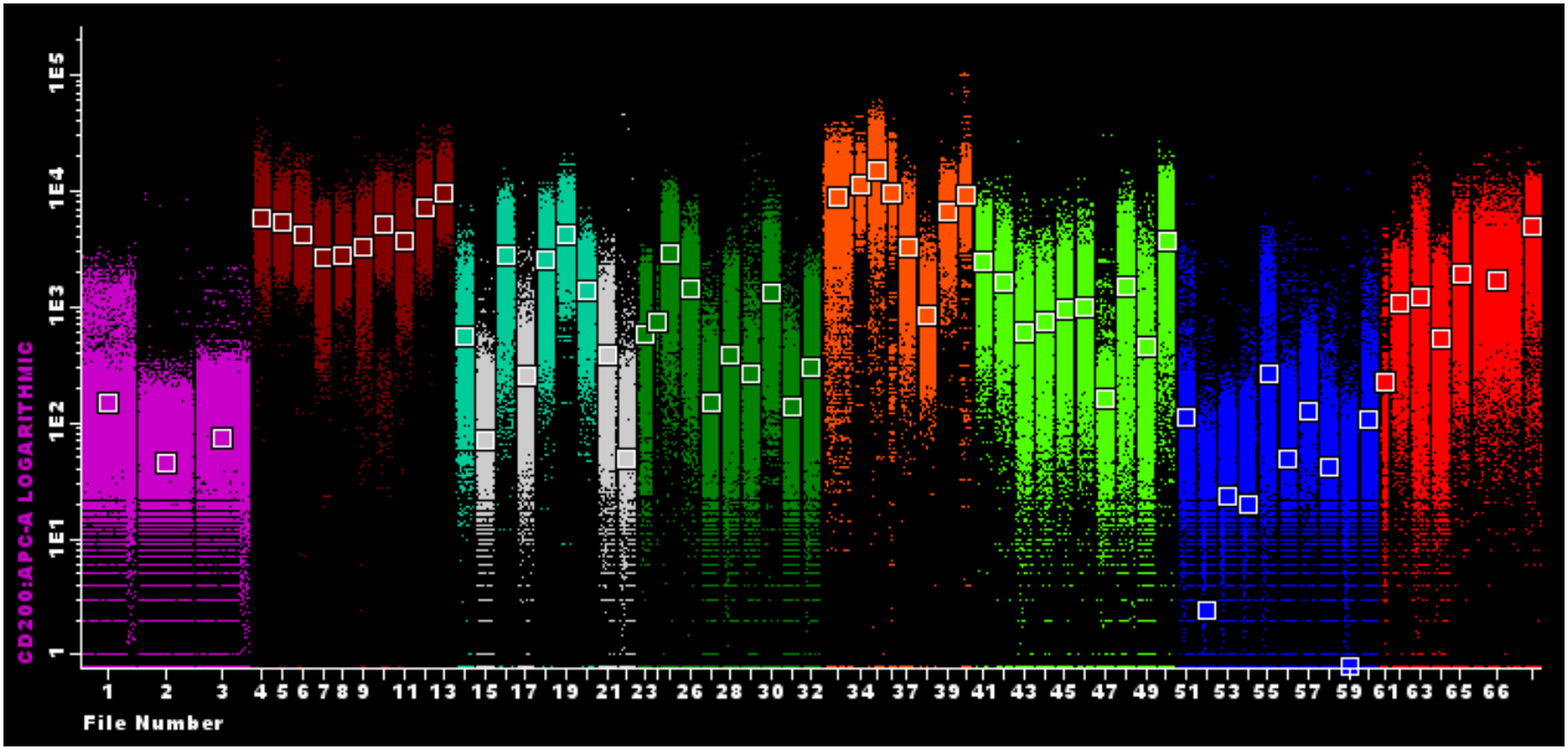
- univariate analysis: CD11a, CD63, CD138
- multivariate analysis (INFINICYT software):
  - bcl-2
  - CD24
  - CD25

  (no significant contribution for 1 x 1 differential diagnosis between any two B-CLPD entities)

EuroFlow

UTILITY OF THE B-CLPD EuroFlow PANEL

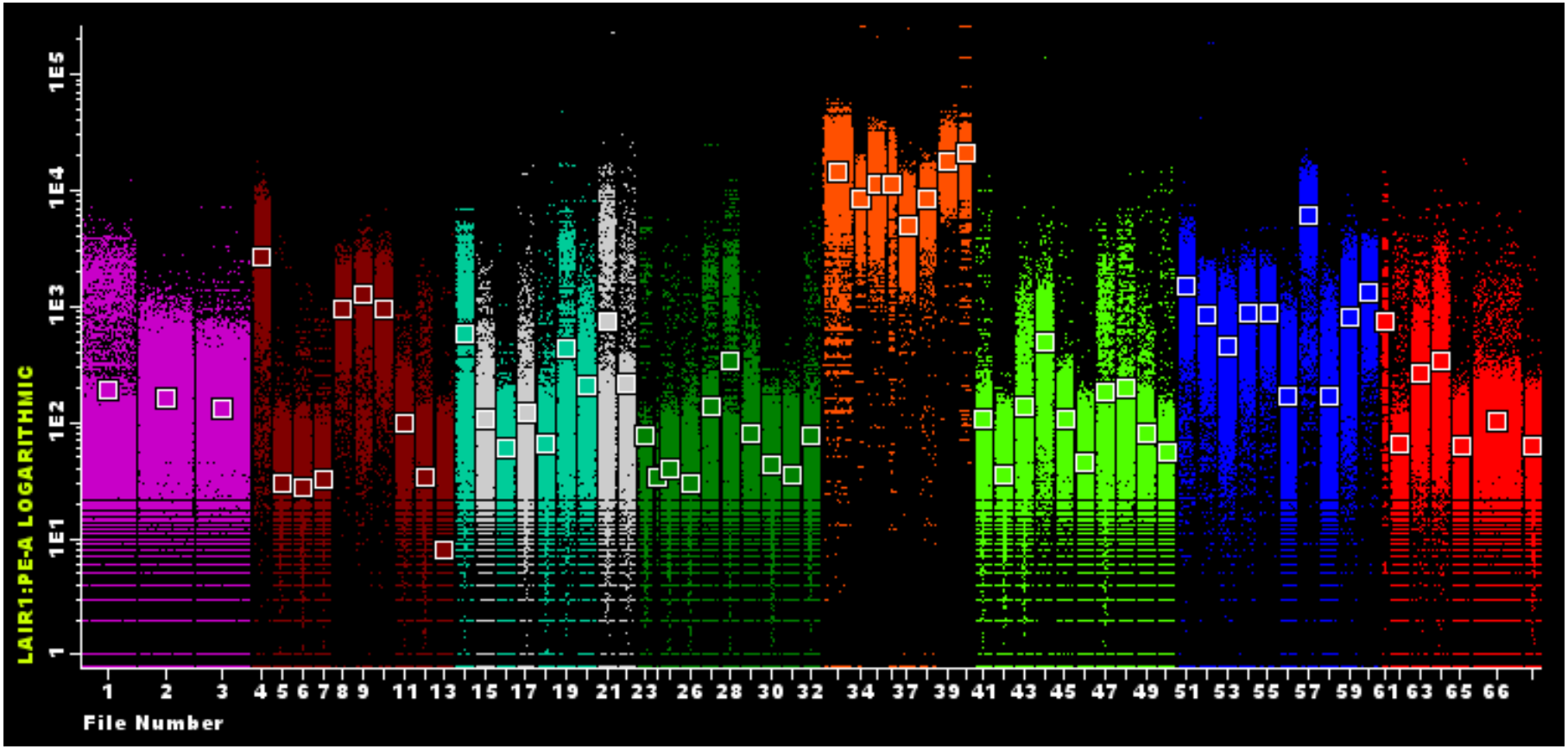
Characterization markers:
CD200



BL CLL DLBCL FL HCL LPL MCL MZL

EuroFlow

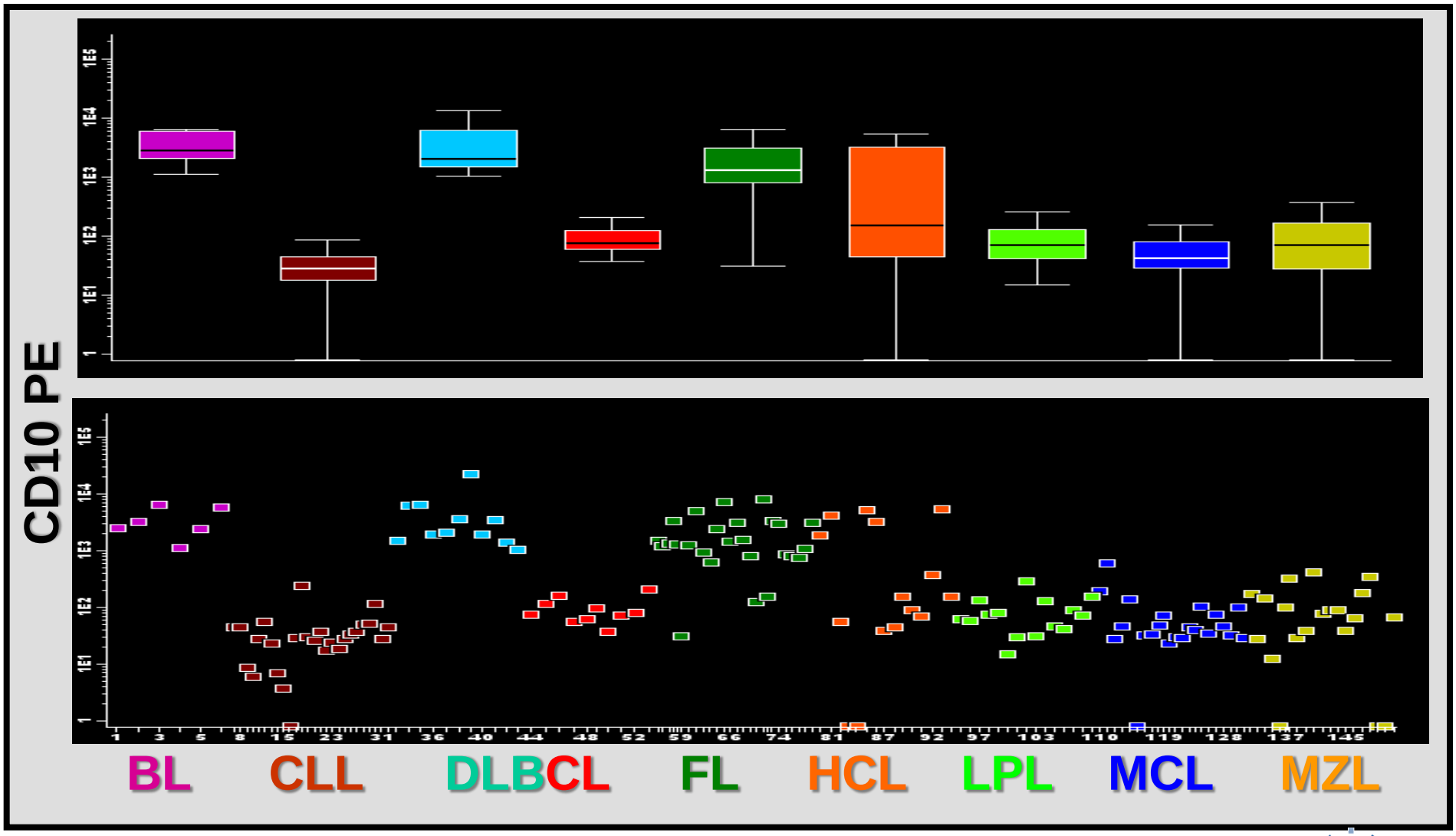
Characterization markers:
LAIR1



BL CLL DLBCL FL HCL LPL MCL MZL

EuroFlow

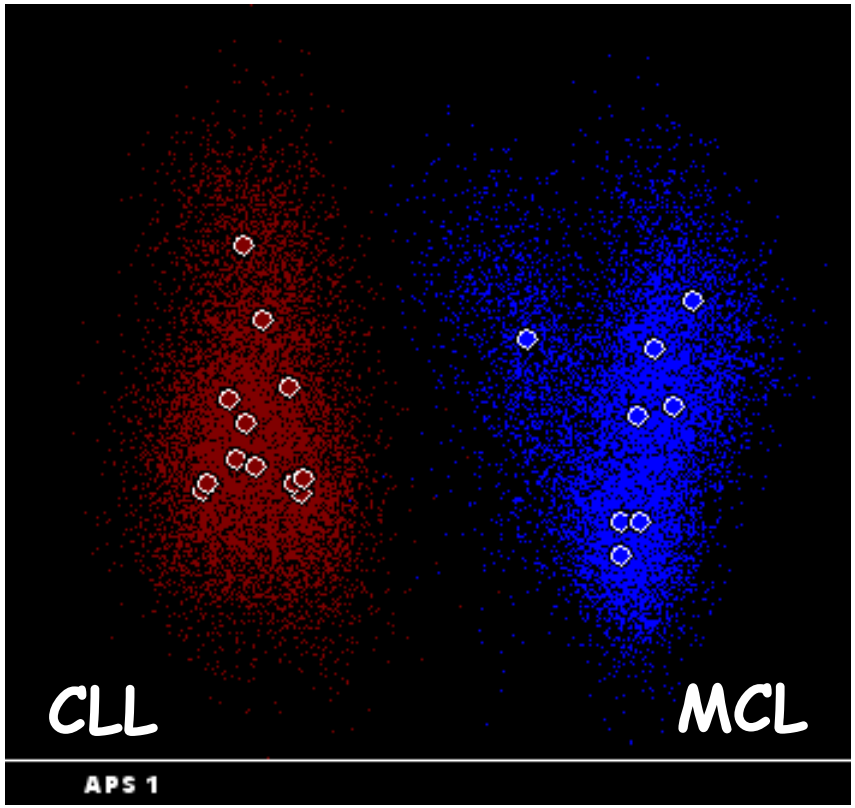
Characterization markers:
CD10



Responsible scientist: Sebastian Bottcher

EuroFlow

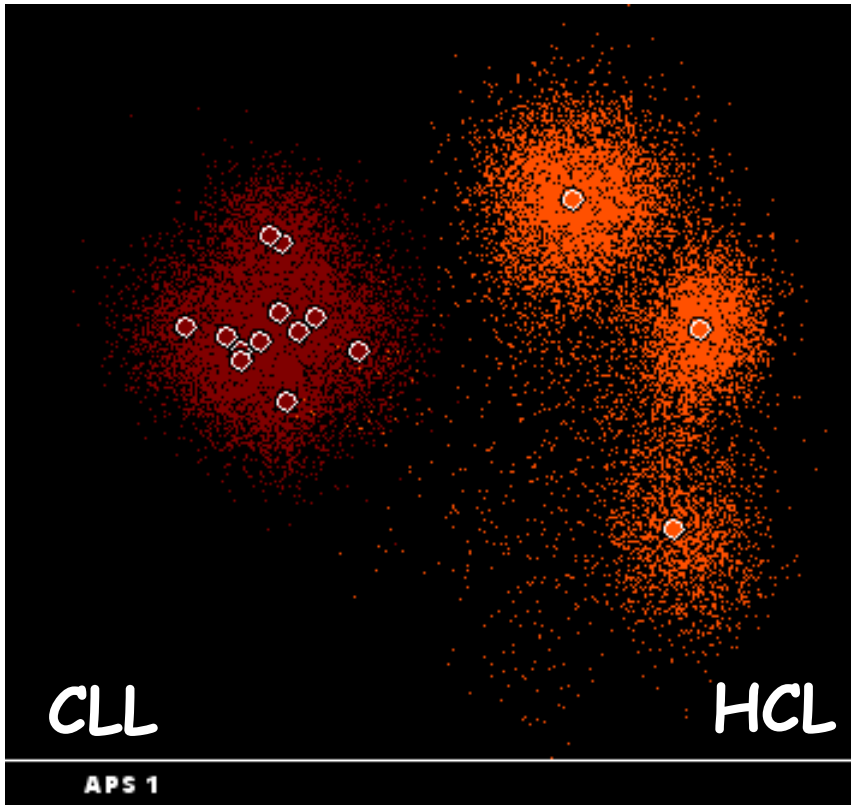
B-CLPD panel: utility



1	IgM	14.02
2	CD200	13.76
3	CD79b	11.94
4	CD23	8.57
5	CD38	7.73

EuroFlow

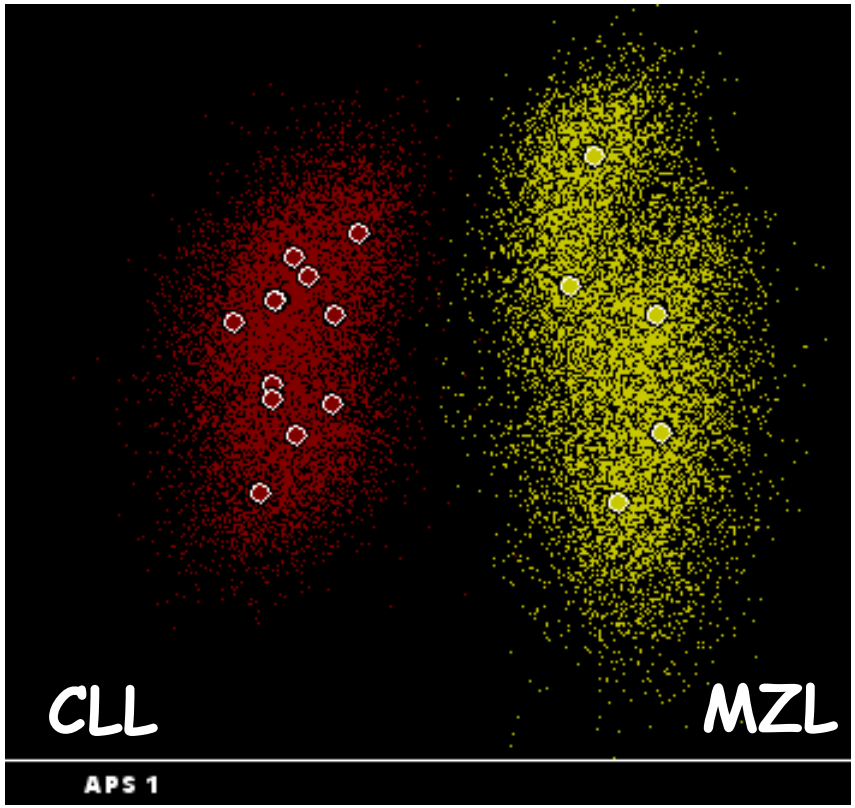
B-CLPD panel: utility



1	CD11c	10.48
2	LAIR1	9.75
3	CD22	8.89
4	CD79b	8.26
5	CD20	7.7

EuroFlow

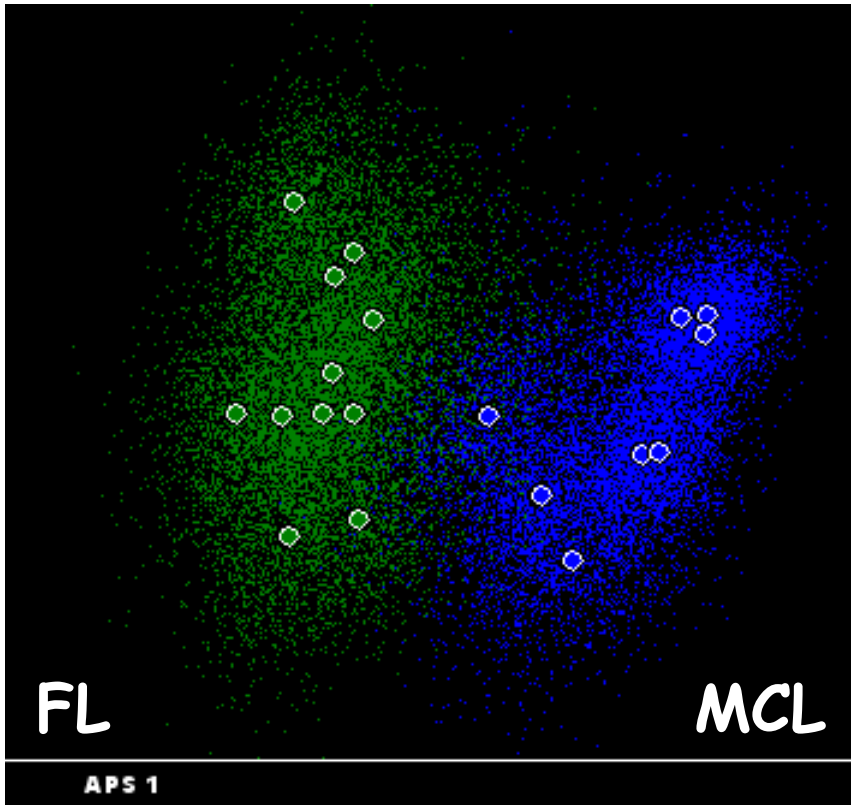
B-CLPD panel: utility



1	CD79b	9.84
2	CD5	8.86
3	CD200	7.35
4	CD43	7.3
5	CD49d	7.28

EuroFlow

B-CLPD panel: utility



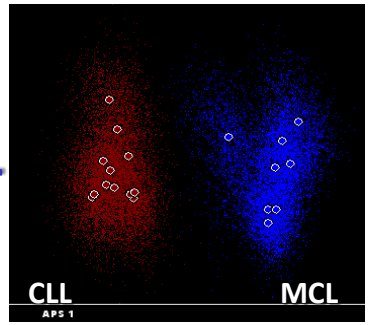
1	LAIR1	11.73
2	CD5	11.28
3	IgM	8.69
4	CD31	8.09
5	CD10	7.5

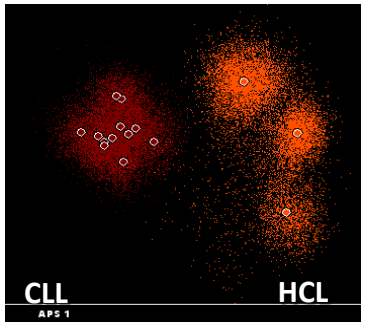
EuroFlow

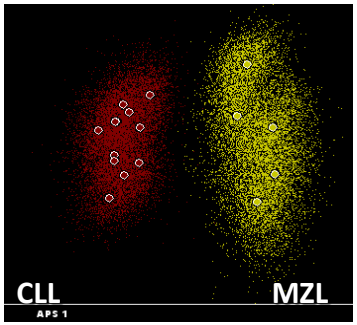
B-NHL classification panel – modular design

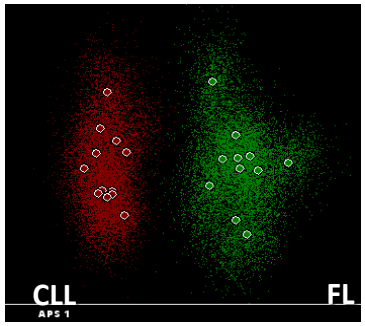
EuroFlow

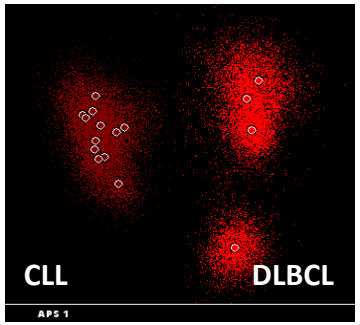
Full panel



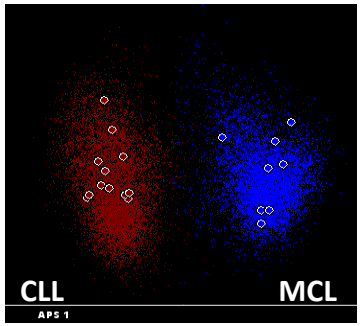


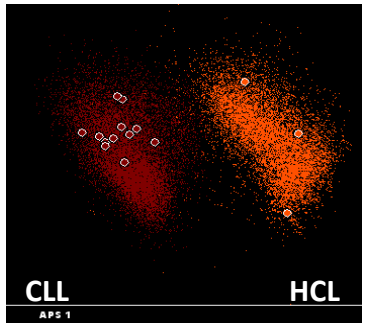


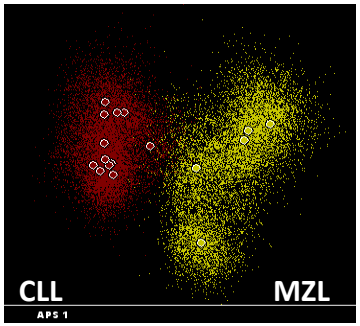


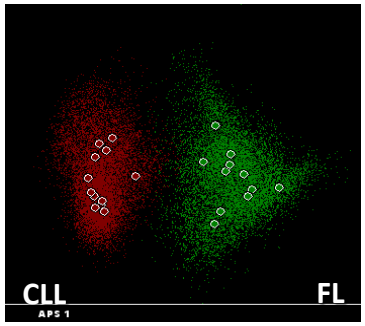


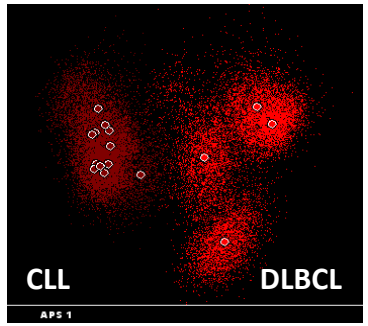
Tubes 1 & 2 only





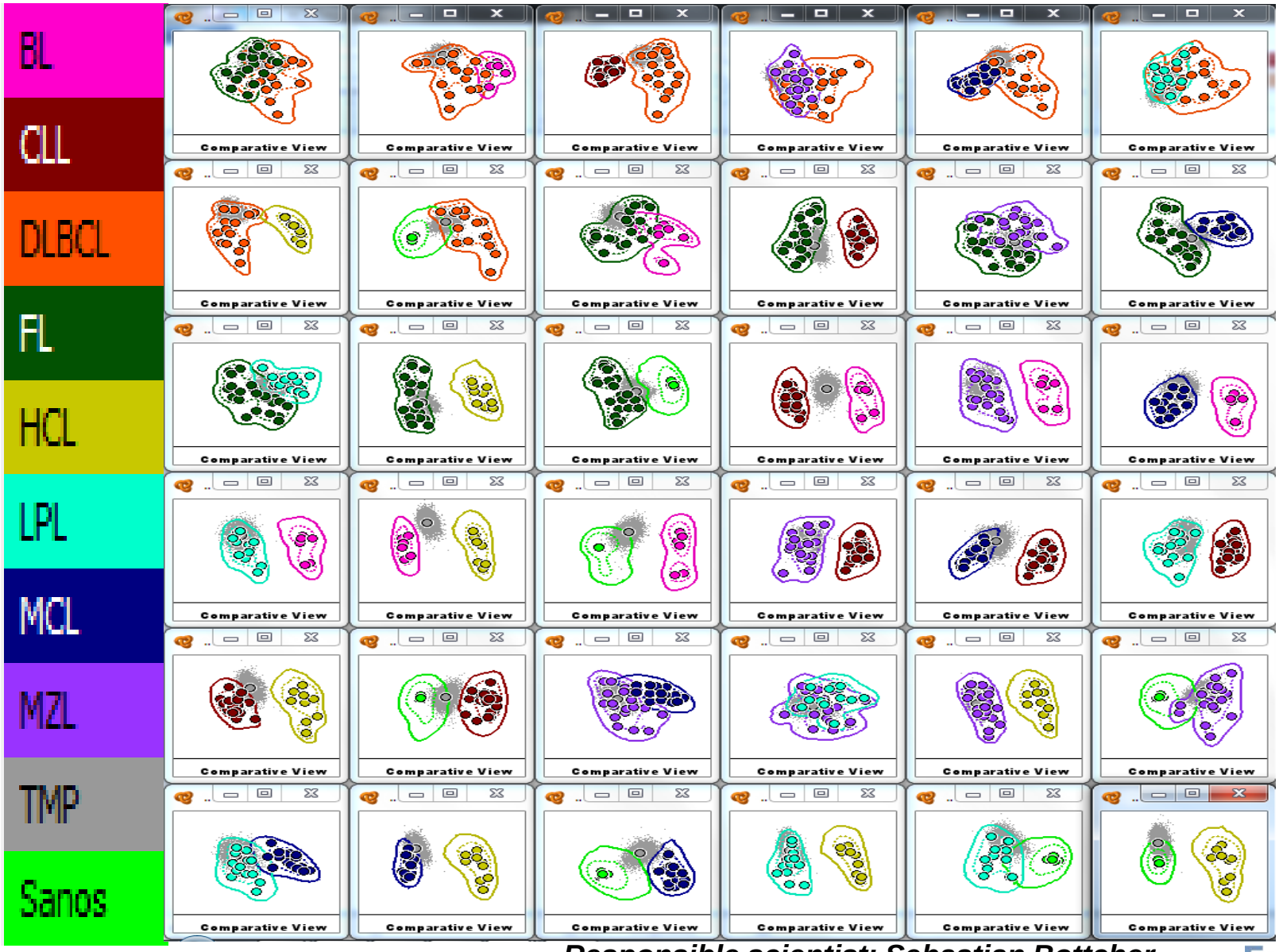






Responsible scientist: Sebastian Bottcher

BCLPD panel: classification of an atypical case vs the reference WHO diagnostic groups



Responsible scientist: Sebastian Bottcher

EuroFlow

EuroFlow B-CLPD panel: summary and perspectives

- B-CLPD panel allows unequivocal classification of most mature B-cell malignancies according to WHO classification

- All differential diagnoses achieved efficiently except for the following 1 vs 1 comparisons:

FL vs DLBC

MZL vs LPL

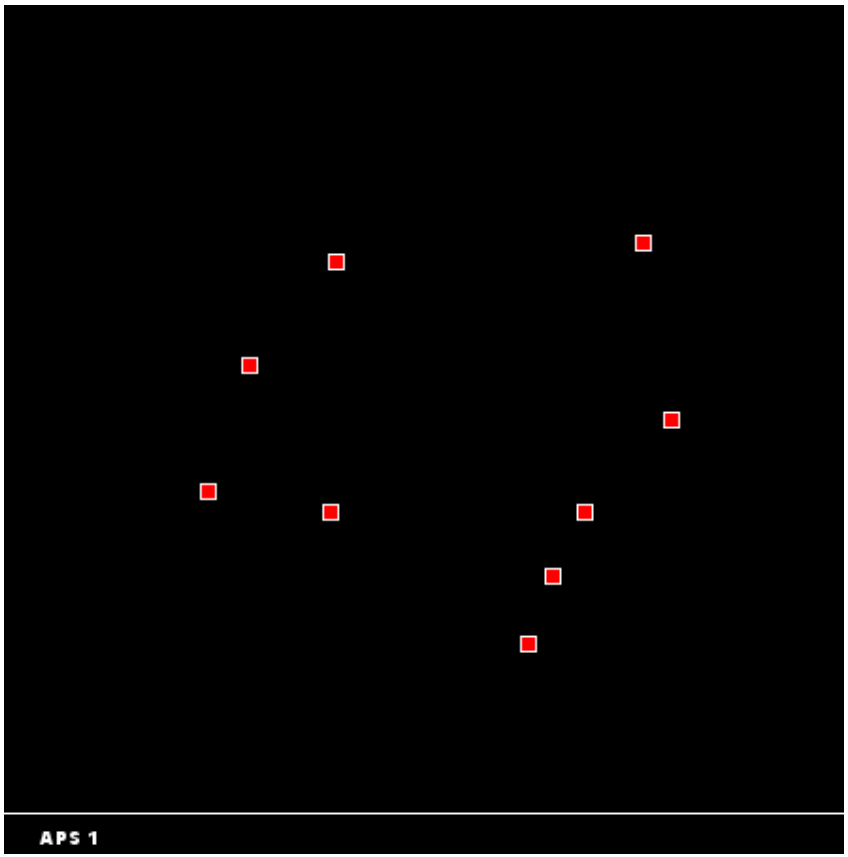
LPL vs DLBCL

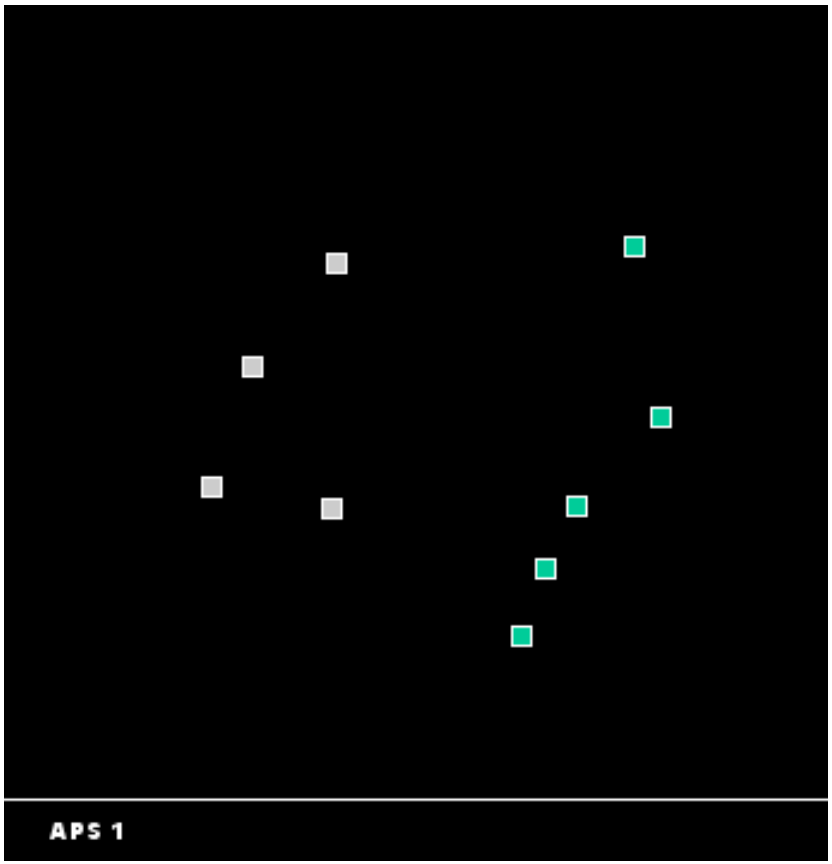
MZL vs DLBCL

By S. Böttcher

EuroFlow

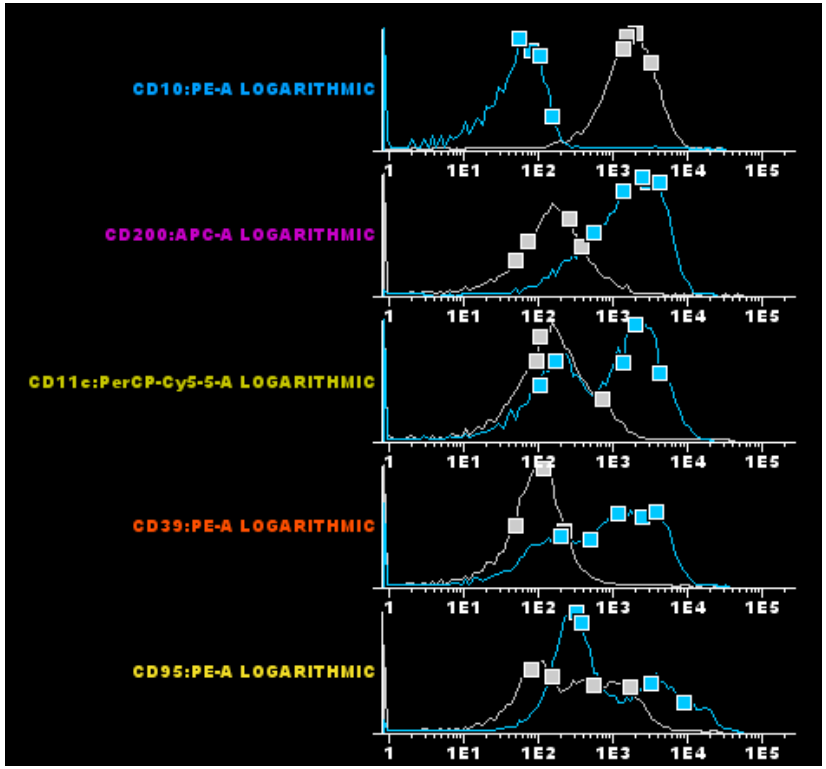
B-CLPD panel: subclassification of DLBCL

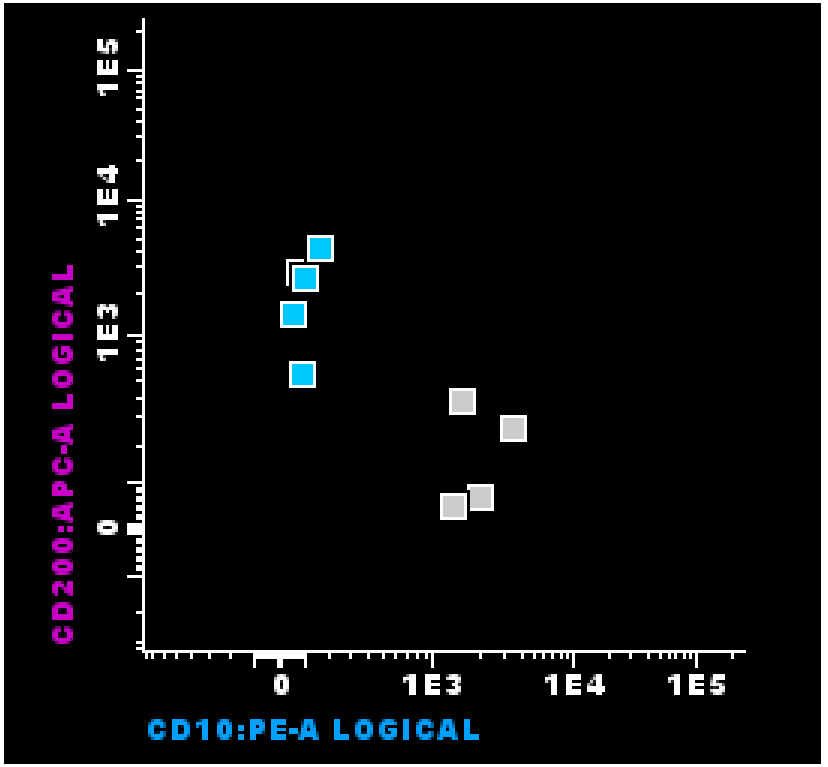




EuroFlow

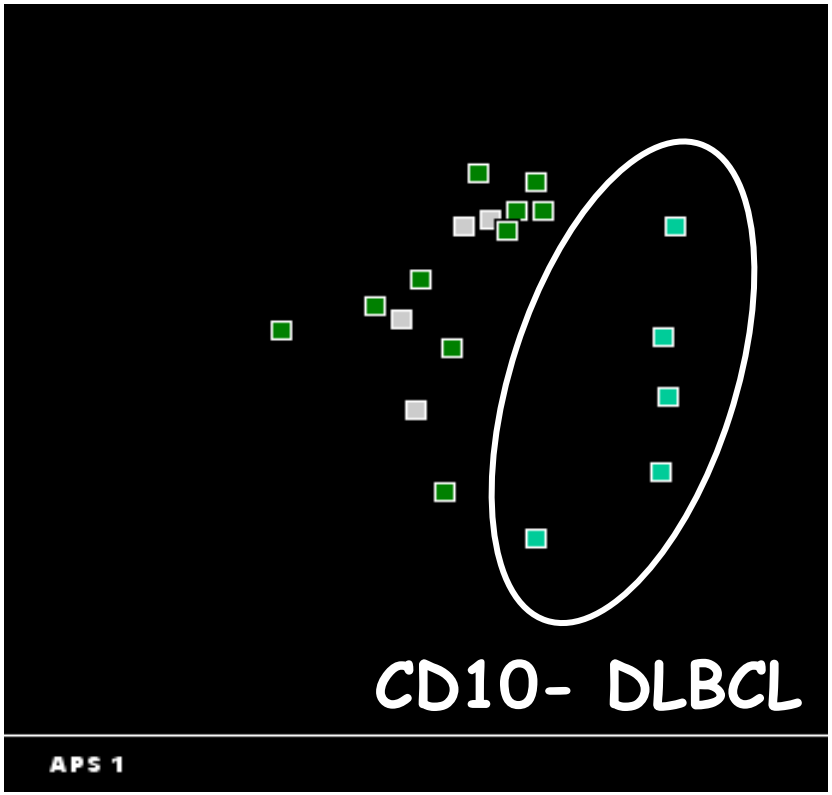
B-CLPD panel: subclassification of DLBCL





EuroFlow

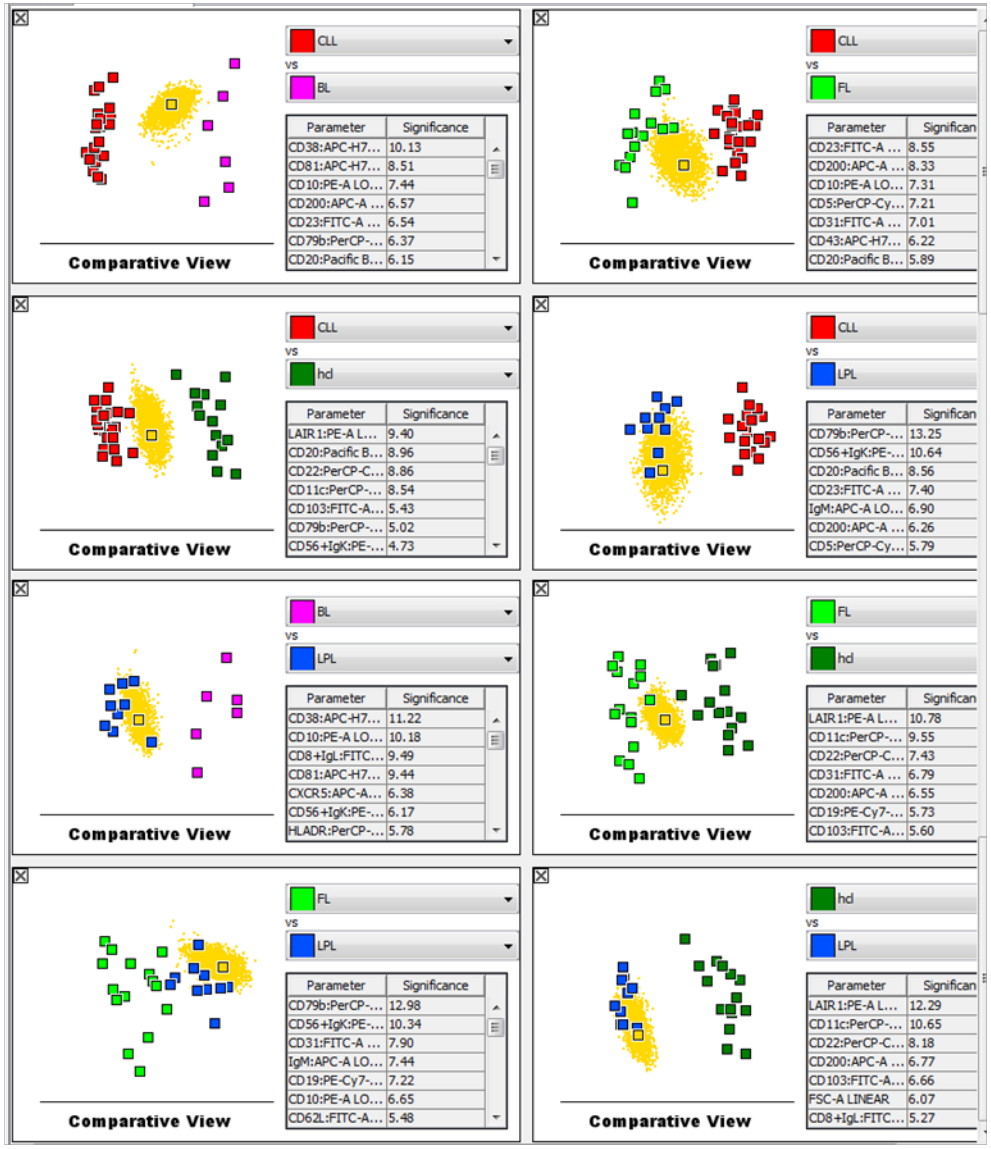
B-CLPD panel: subclassification of DLBCL vs FL

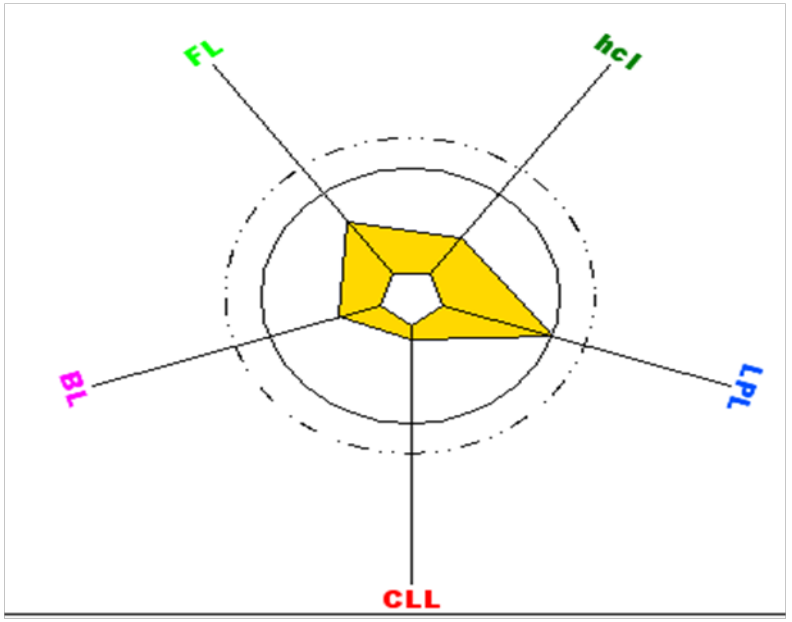


	APS Significance
IgM-APC	11.4
CD10-PE	9.7
CD200-APC	9.1
CD39:PE	8.4

EuroFlow

B-CLPD:Comparative analysis of "our case" vs multiple reference groups



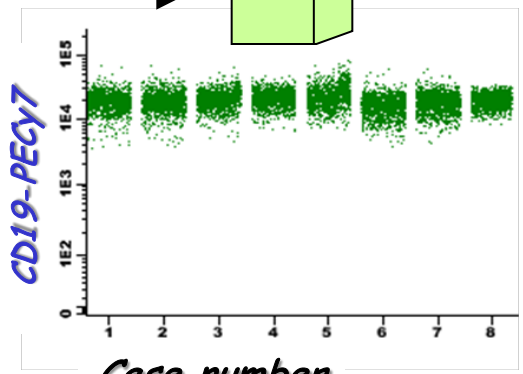


Responsible scientists: Sebastian Bottcher

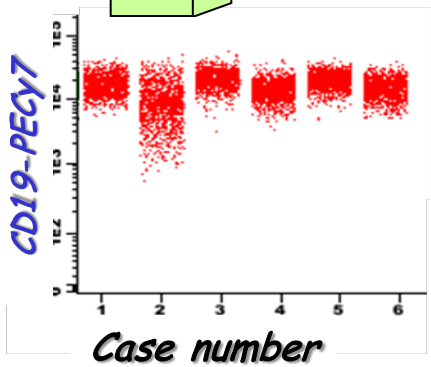
REFERENCE DATAFILES: NORMAL vs. CLL B-CELLS

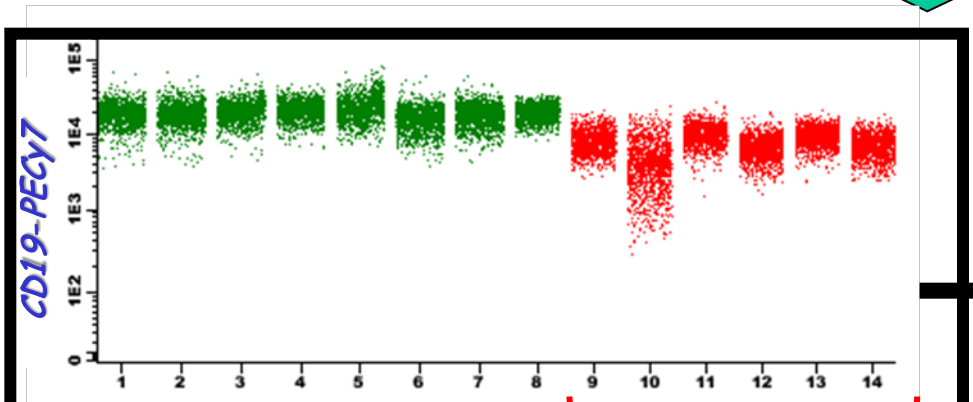
EuroFlow

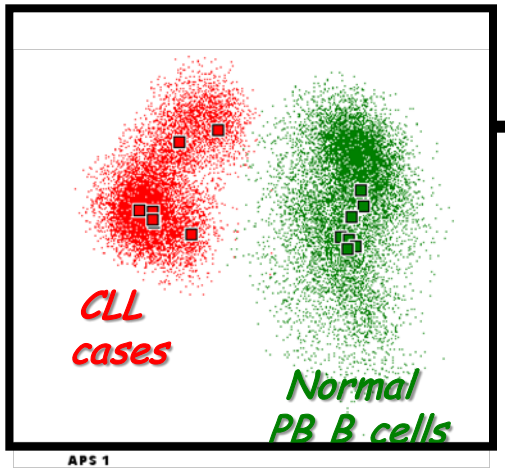
Normal PB
CD19+ B-cells



CD19+ CLL
B-cells



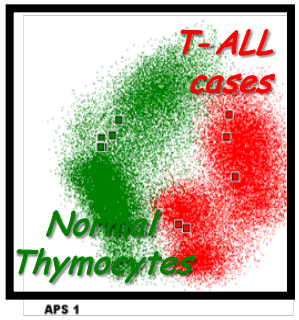
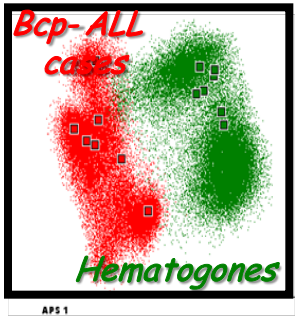
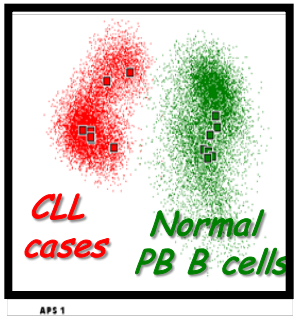
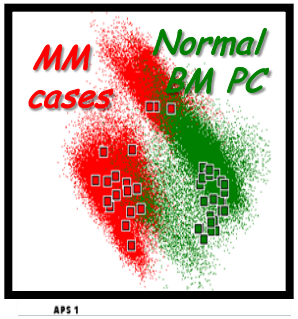
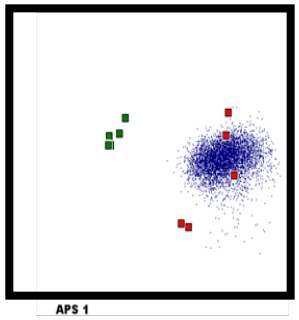
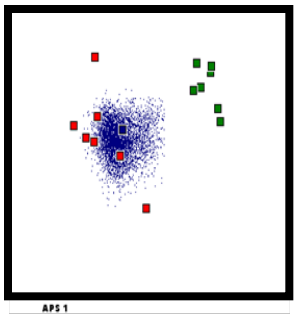
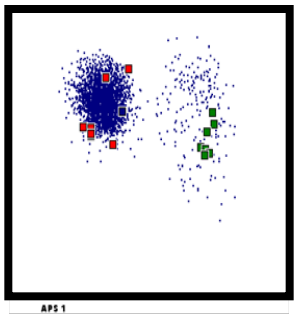
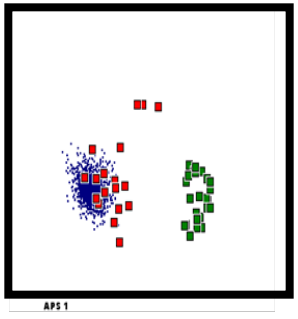
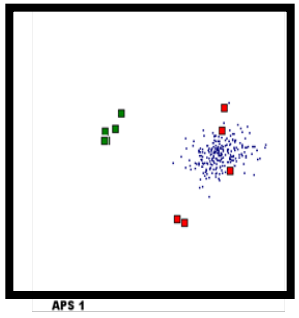
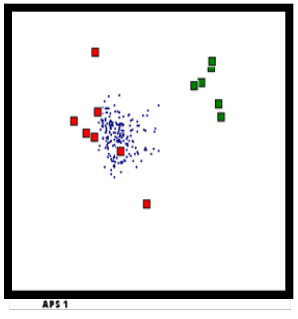
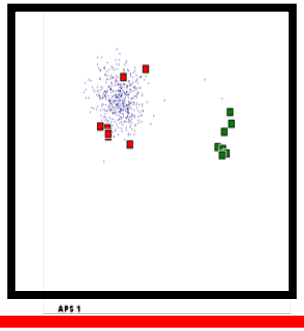
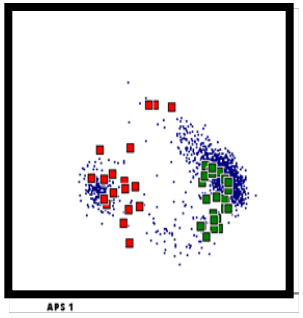




LAIR1	9.3
CD5	8.6
CD79b	8.2
IgM	8.1

COMPARE A CASE VS NORMAL & NEOPLASTIC B-CELLS

EuroFlow

	T-ALL	B-cell precursor ALL	CLL	Multiple myeloma
Reference data files				
Diagnostic samples vs. Reference data files				
MRD samples vs. Reference data files				

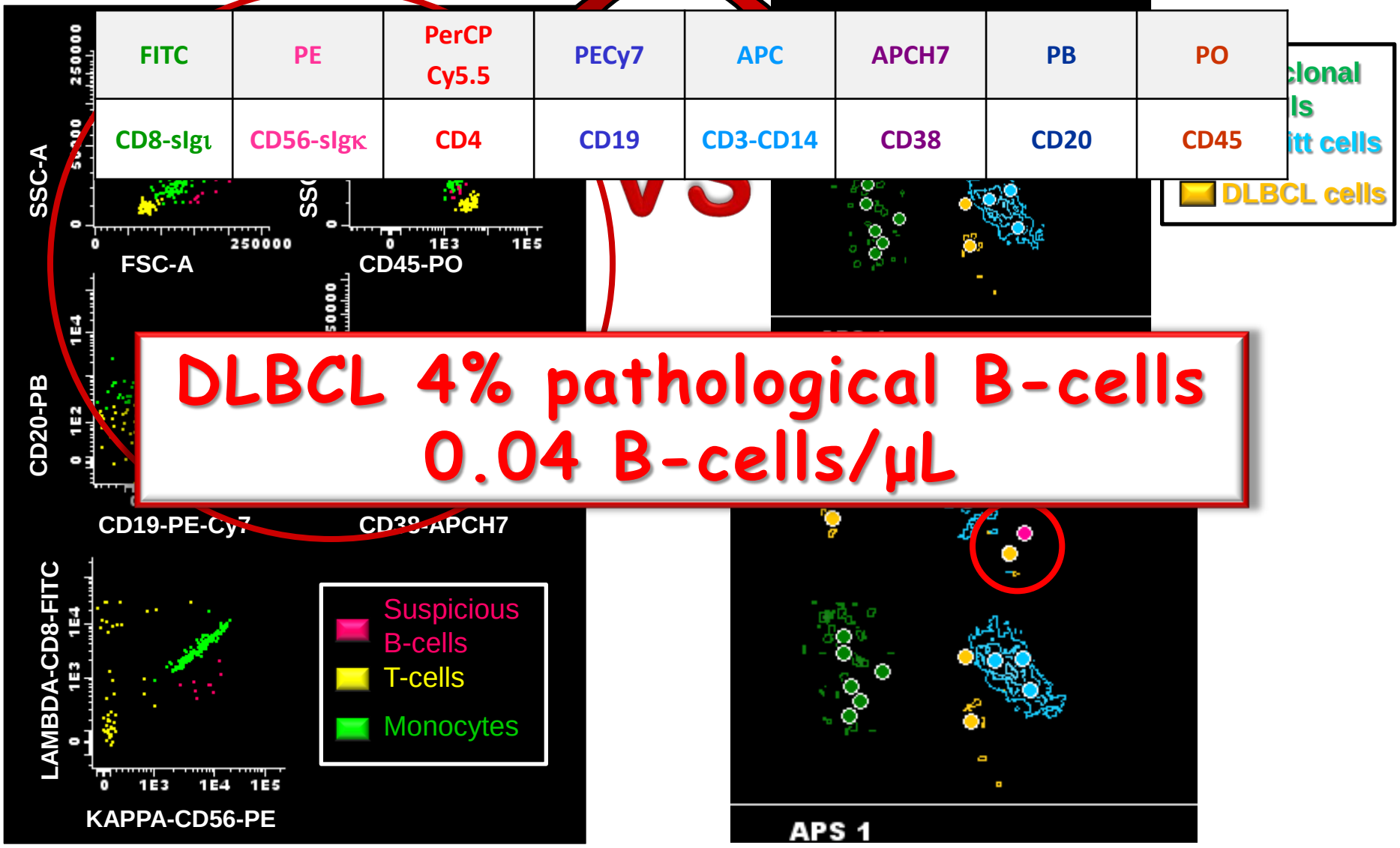
LEPTOMENINGEAL DISEASE IN HEMATOLOGICAL MALIGNANCIES

- **Clinical parameters** used to define risk for CNS involvement/relapse in B-NHL, ALL and AML are found to be of **limited predictive value**

- Although **cytologic analysis** of CSF has a great specificity, it has a limited **sensitivity** with a reported **false-negative rate** of between 20%-60%
- Recent single center studies suggest that **FCM** can increase the sensitivity of conventional cytology for the **identification of CSF involvement**, particularly in B-NHL

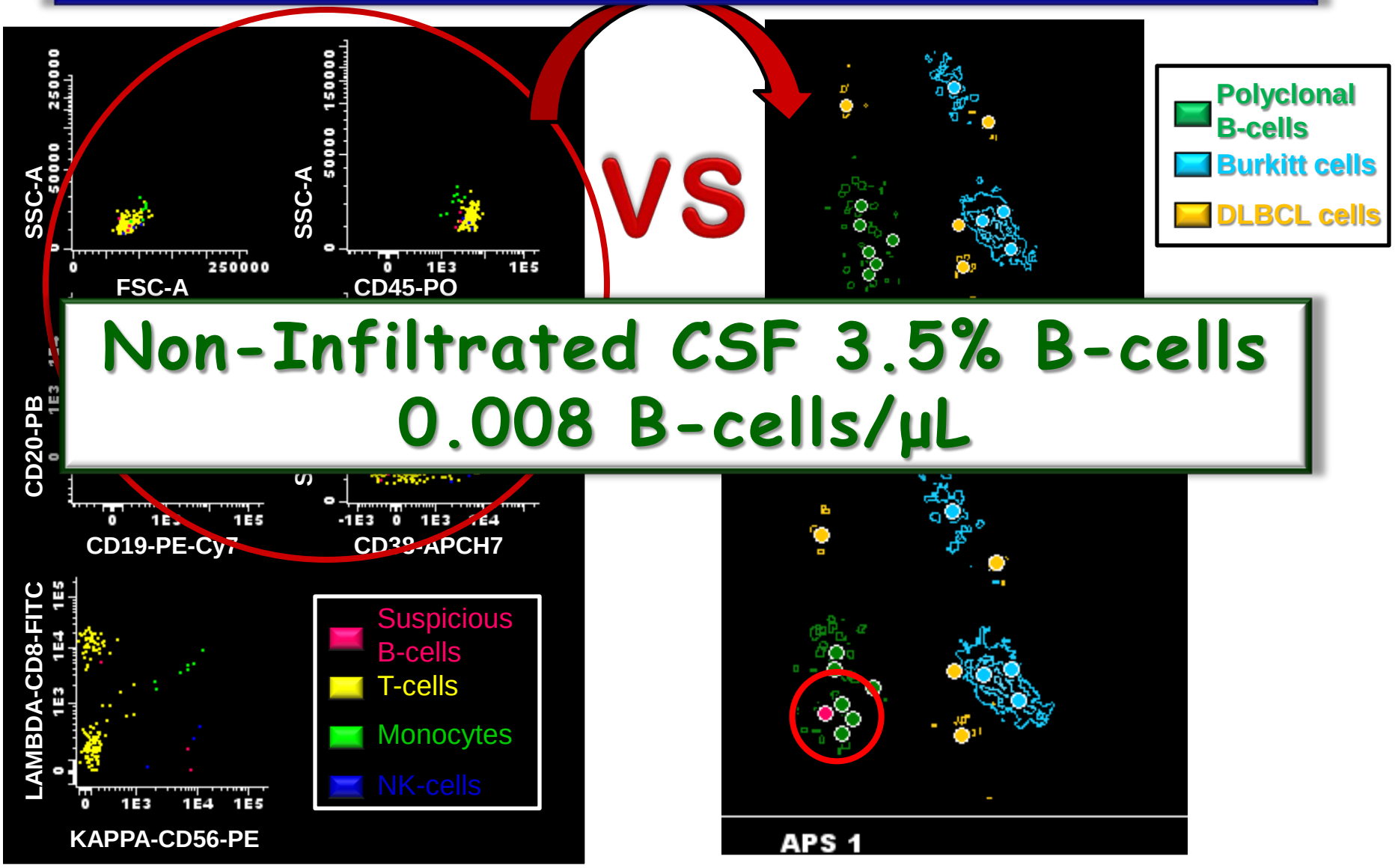
SUSPICIOUS B-CELLS VS REFERENCE LIBRARY: COMPASS ANALYSIS

FITC	PE	PerCP Cy5.5	PECy7	APC	APCH7	PB	PO
CD8-sIgλ	CD56-sIgκ	CD4	CD19	CD3-CD14	CD38	CD20	CD45



DLBCL 4% pathological B-cells
0.04 B-cells/µL

SUSPICIOUS B-CELLS VS REFERENCE LIBRARY: COMPASS ANALYSIS



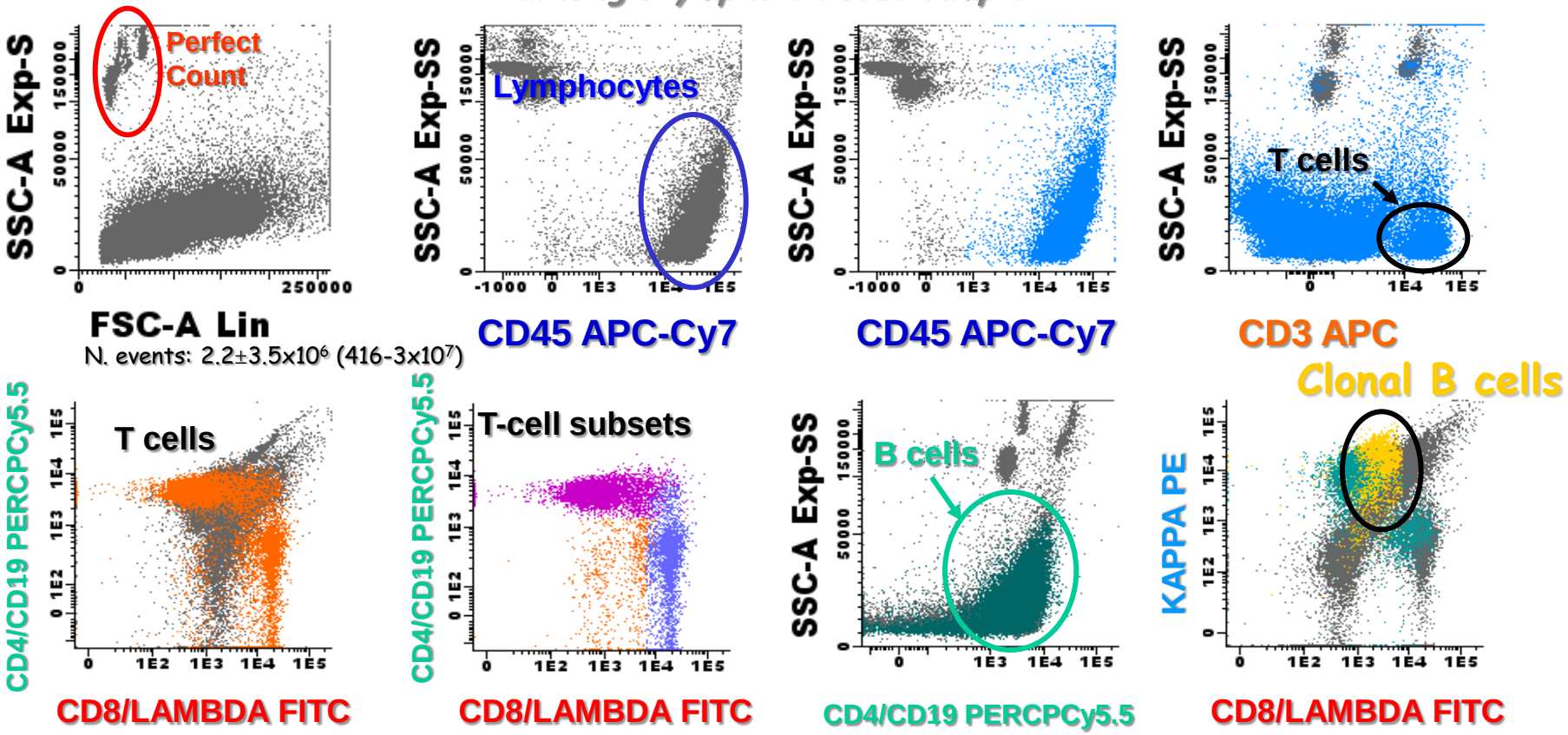
Non-Infiltrated CSF 3.5% B-cells
0.008 B-cells/µL

FC on FNA samples as a screening diagnostic tool

GATING STRATEGY for the immunophenotypic identification of lymphoid cells

CD8+anti-λ FITC / anti-κ PE / CD4 + CD19 PerCPCy5.5 / CD3 APC / CD45 APCCy7

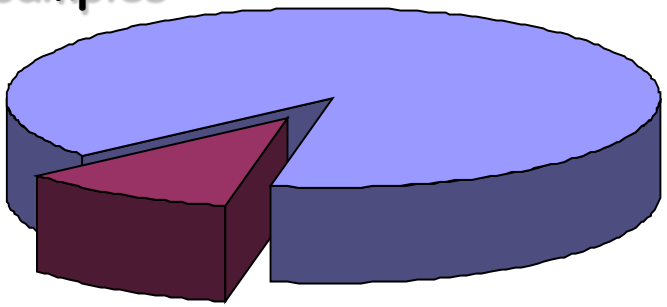
Pathologic lymphoid tissue sample



FC on FNA samples as a screening diagnostic tool

Comparison of cytology, FC and histology

Total FNA samples:
n=446
(from 400 cases)



No valuable samples
n=48 (10.5%)
(from 41 patients)

Only for cytology: 35
Only for FCM: 3
For FCM & cytology: 10

<3%

Usually due to scanty cellularity or PB contamination

Valuable samples
n= 398 (89.5%)
(from 359 patients)

Degree of concordance between FNAC, FC and histology:

Concordant samples:
373/398 (94%)
from 336 cases
84% from the total samples

Discordant samples:
25/398 (6%)
from 23 cases
5.5% from the total samples

Conclusions

- FCM is useful for the study of B-CLPD for: (i) the diagnosis (of clonality, NHL staging, and CSF infiltration), (ii) classification into WHO groups and (iii) MRD monitoring

- The new strategies developed by the EuroFlow Consortium opens the door for all applications of multiparameter flow cytometry for which a large number of parameters are needed and for the automation of FCM data analysis

Contributors - Acknowledgements

Michael Kneba
Monika Brüggemann
Sebastian Böttcher

Stephen Richards
Paul Evans
Matt Cullens
Ruth de Tute
Andy Rawstron

Elizabeth MacIntyre
Vahid Asnafi
Ludovic Lhermitte

Paulo Lucio
Andreia Mendonça
Evan Jensen

Tomek Szczepanski
Lukasz Sedek

Ondrej Hrusak
Tomas Kalina
Ester Mejstrikova

Jacques van Dongen
Vincent van der Velden
Jeroen Te Marvelde
Alita van der Sluijs

Juan Flores-Montero
Julia Almeida
Quentin Lecrevisse

Photo Lukasz Sedek

EuroFlow