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иММУНОЛОГИЯ ГЕМОПОЭЗА

HÆMATOPOÏESIS iMMUNOLOGY

2/2011

European Consortia in hemato-oncology

ИММУНОЛОГИЯ ГЕМОПОЭЗА

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Настоящий номер «Иммунологии гемопоэза» необычен. Он практически полностью посвящен научным разработкам и диагностическим подходам авторитетного Европейского консорциума EUROFLOW. Профессора Жак Ван Донген (Нидерланды) и Альберто Орфао (Испания) подробно осветили достижения EUROFLOW на конференции в Санкт-Петербурге 17-19 мая 2011 г. Конференция была организована компаниями Becton Dickinson и Биолайн. Для российского читателя столь подробное освещение направлений работы EUROFLOW приводится впервые и будет, несомненно, полезным в совершенствовании иммунодиагностики гемобластозов и минимальной остаточной болезни в онкогематологии.

Выражаем огромную благодарность профессорам J.J.M. van Dongen и A.Orfao за предоставленные для публикации материалы.

Редакция «Иммунологии гемопоэза»

This *Haemopoiesis Immunology* issue is unusual. It practically entirely addresses scientific developments and diagnostic approaches of the European Consortium EUROFLOW. Professors Jack Van Dongen (The Netherlands) and Alberto Orfao (Italy) described in detail EUROFLOW's achievements at the St. Peterburg conference of May 17—19, 2011. The conference was organized by Becton Dickinson and Bioline. For Russian readers such a detailed coverage of EUROFLOW's fields of activities is made for the first time and will undoubtedly be very useful for improvement of immunodiagnosis in hematology malignancies and minimal residual disease in cancer hematology.

Editorial board of "Haematopoiesis Immunology" is very grateful to Professors J.J.M. van Dongen and A.Orfao for presentation of their articles.

Editors

Prof. J.J.M. van Dongen, M.D., Ph.D.

Jacques J.M. van Dongen studied Medicine at the Erasmus University Rotterdam (Rotterdam, the Netherlands) and received his M.D. degree in 1981. From then on he worked in the field of immunology research, with special focus on human T- and B-cell differentiation as well as lymphoid malignancies and immunodeficiencies. Together with Herbert Hooijkaas, he initiated the immunodiagnostic laboratory of the Erasmus *university* Medical Center Rotterdam in 1985, which has developed into one of the leading centers for immunodiagnostics and translational research of lymphoproliferative diseases and immunodeficiencies in Europe. In 1990 he received his Ph.D. degree (thesis: "*Human T-cell differentiation: Basic aspects and their clinical applications*"). Since 1991 he has been full Professor of Immunology at the Erasmus MC/Erasmus University Rotterdam. His translational research focuses on molecular genetic processes during normal, malignant, and immunodeficient lymphoid differentiation and on the development and clinical evaluation of new immunodiagnostic methods in lymphoproliferative diseases and primary immunodeficiencies. He contributed to ~550 manuscripts, including ~360 international SCI publications (number of citations: ~14,300; H-factor: 61). He is/was coordinator of seven European networks in the field of diagnostics in hemato-oncology and immunology, such as the currently ongoing EuroClonality, EuroMRD, and EuroFlow networks.

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March 2011

Профессор J.J.M. van Dongen

«Европейские консорциумы в онкогематологии.

Цели и достижения».

Russian flow cytometry conference, St Petersburg, Russia
17-19 March 2011

European Consortia in hemato-oncology

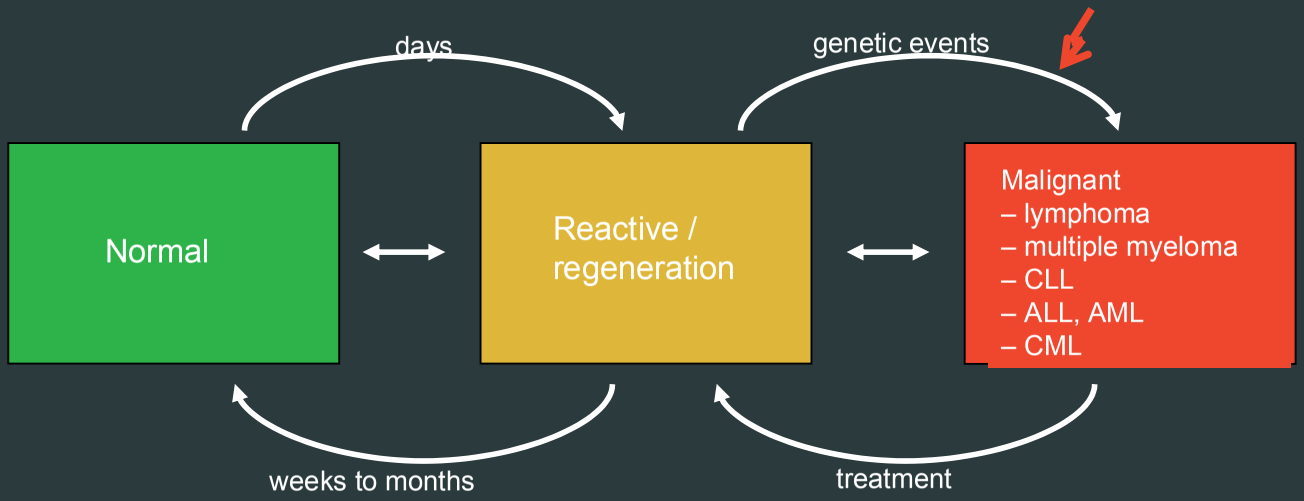
Aims and achievements

J.J.M. van Dongen
on behalf of

ESLHO
european scientific foundation of laboratory hemato oncology

EuroFlow

Discrimination between normal and malignant immune cells in blood, bone marrow and lymphoid tissues



Laboratory methods:

- Cytomorphology and immunophenotyping
- Molecular diagnostics, e.g. PCR-based clonality diagnostics and molecular classification via detection of oncogenetic defects

Diagnostics for hematological malignancies

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)

European networks since 1994

15 year experience with international collaboration

1. MRD networks (since 1994)

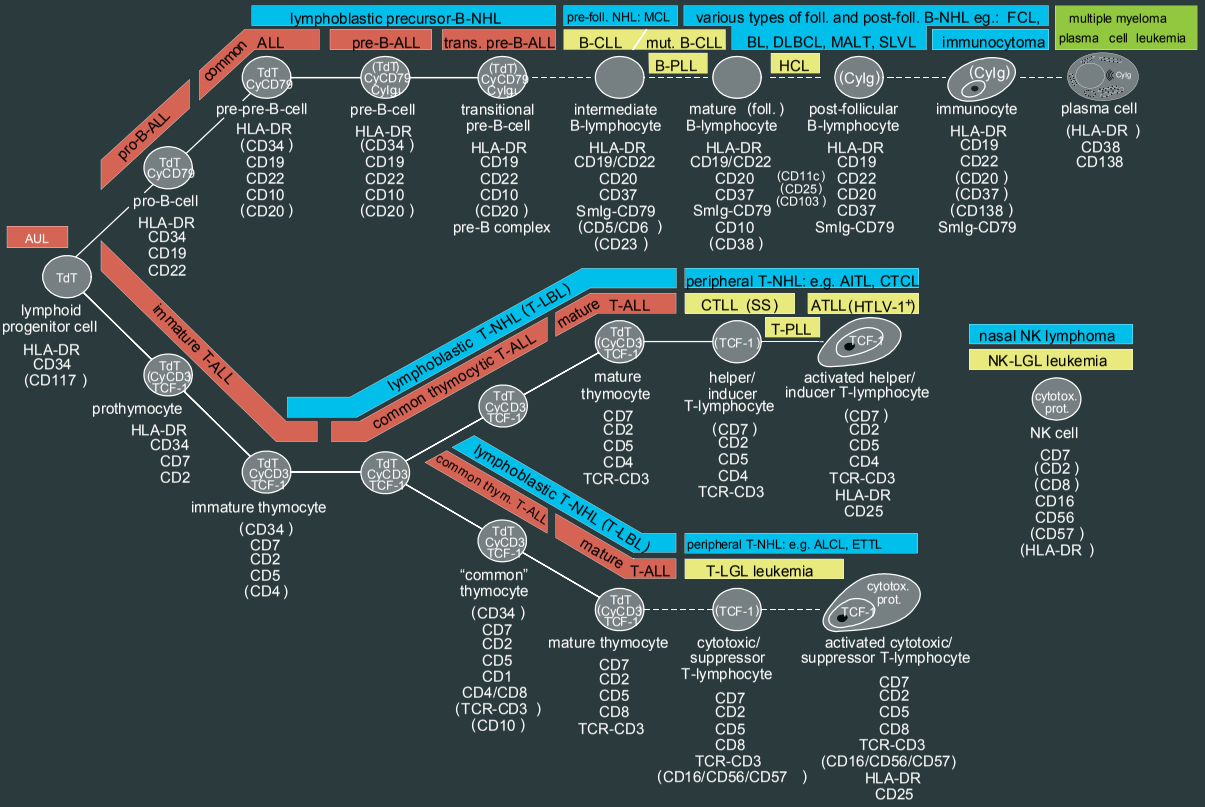
- BIOMED-1 Concerted Action (1994-1998):
  - flow cytometry
  - PCR analysis of fusion gene transcripts
  - PCR analysis of patient-specific Ig/TCR junctional regions
- I-BFM-SG MRD study group (1994- 2001)
  PCR analysis of patient-specific Ig/TCR junctional regions
- Europe Against Cancer (1998- 2003)
  RQ-PCR analysis of fusion gene transcripts (TaqMan technology)
- EuroMRD (ESG-MRD) (2001- up till now)
  RQ-PCR analysis of patient-specific Ig/TCR junctional regions

2. PCR-based clonality diagnostics (since 1996)

- BIOMED-2 Concerted Action (1998-2004)

3. Advanced flow cytometry (since 1994)

- BIOMED-1 Concerted Action (1994-1998): original focus on MRD
- EuroFlow (2006- up till now): diagnosis, classification and monitoring
- ERA-NET PRIOMEDCHILD (2011-2014): 8-color flow cytometry for MRD detection in ALL



J.J.M. van Dongen et al, Neth J Med 1988;33:298-314

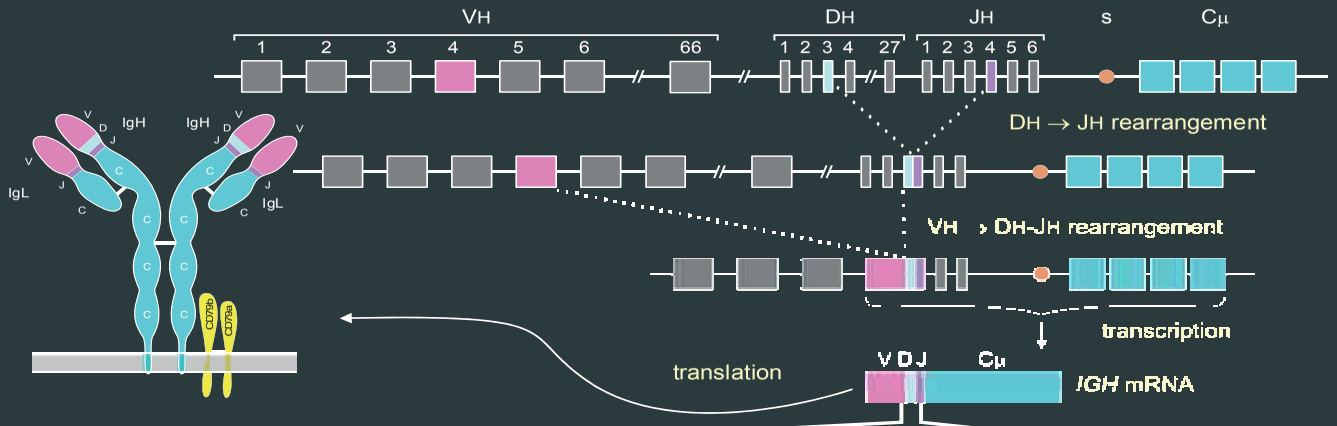
Molecular diagnostics in suspected lymphoproliferations

Normal ↔ reactive ↔ malignant

In 10 to 15% of suspect lymphoproliferations no reliable discrimination between reactive and malignant can be made.

→ Molecular clonality diagnostics via Ig/TCR genes

From Ig gene to Ig molecule



VH3-21 (germline)	insertion	DH3-3 (germline)	insertion	JH4-1 (germline)
TGTATTACTGTGCGAGA		GTATTACGATTTTTGGAGTGGTTATTATACC		ACTACTTTGACTACT
TGTATTACTGT	AGGC	CGATTTTTGGAGTGGTTATTATA	GTCCA	TGACTACT
TGTATTACTGTGCG	TATCCGGA	TTACGATTTTTGGAGTGGTTATTATAC	CGATCG	CTTTGACTACT
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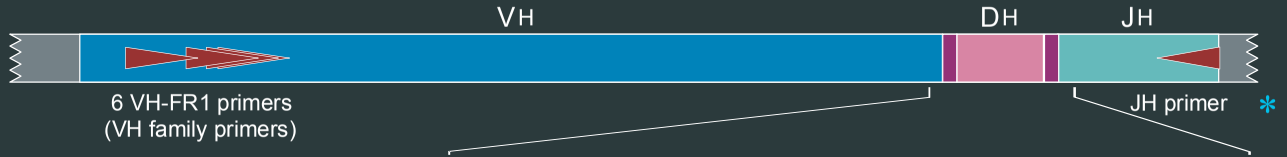
TGTATTACTGTGCGA	CCGG	TTACGATTTTTGGAGTGGTTATTATACC	CGGAGC	TACTTTGACTACT

EuroClonality

BIOMED-2 Concerted Action BMH4-CT98-3936:
PCR-based clonality studies



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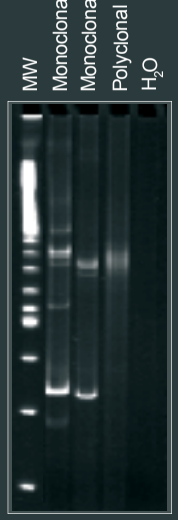
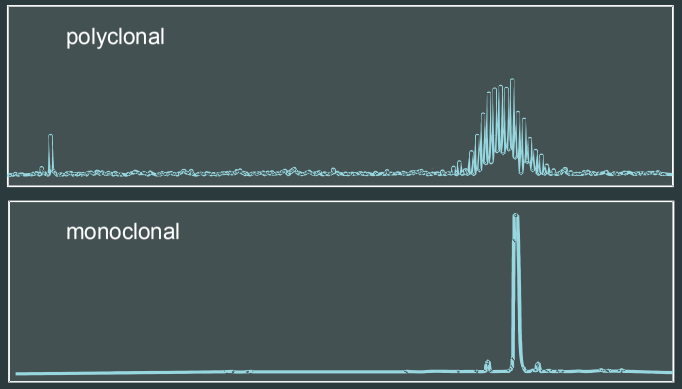
Heteroduplex analysis



GeneScan analysis



BIOMED-2 report:
J.J.M. van Dongen et al.
Leukemia 2003; 17: 2257-2317

Analysis of *IGH* gene rearrangements

IGH tube A

			5'	3'
VH1-FR1	(1-2)	(-252)	GGCCTCAGTGAAGGTCTCCTGCAAG	
VH2-FR1	(2-5)	(-284)	GTCTGGTCCTACGCTGGTGAAACCC	
VH3-FR1	(3-7)	(-256)	CTGGGGGGTCCCTGAGACTCTCCTG	
VH4-FR1	(4-4)	(-256)	CTTCGGAGACCCTGTCCCTCACCTG	
VH5-FR1	(5-51)	(-255)	CGGGGAGTCTCTGAAGATCTCCTGT	
VH6-FR1	(6)	(-263)	TCGCAGACCCTCTCACTCACCTGTG	

IGH tube B

VH1-FR2	(1-2)	(-192)	CTGGGTGCGACAGGCCCCTGGACAA
VH2-FR2	(2-5)	(-190)	TGGATCCGTCAGCCCCCAGGGAAGG
VH3-FR2	(3-7)	(-189)	GGTCCGCCAGGCTCCAGGGAA
VH4-FR2	(4-4)	(-188)	TGGATCCGCCAGCCCCCAGGGAAGG
VH5-FR2	(5-51)	(-190)	GGGTGCGCCAGATGCCCGGGAAAGG
VH6-FR2	(6)	(-194)	TGGATCAGGCAGTCCCCATCGAGAG
VH7-FR2	(7)	(-192)	TTGGGTGCGACAGGCCCCTGGACAA

IGH tube C

VH1-FR3	(1-2)	(-55)	TGGAGCTGAGCAGCCTGAGATCTGA
VH2-FR3	(2-5)	(-54)	CAATGACCAACATGGACCCTGTGGA
VH3-FR3	(3-7)	(-57)	TCTGCAAATGAACAGCCTGAGAGCC
VH4-FR3	(4-4)	(-48)	GAGCTCTGTGACCGCCGCGGACACG
VH5-FR3	(5-51)	(-69)	CAGCACCGCCTACCTGCAGTGGAGC
VH6-FR3	(6)	(-63)	GTTCTCCCTGCAGCTGAACTCTGTG
VH7-FR3	(7)	(-69)	CAGCACGGCATATCTGCAGATCAG

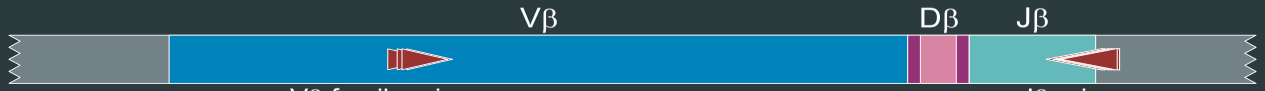
IGH tubes A, B, and C

3'	5'		
CCAGTGGCAGAGGAGTCCATTC	(+57)	JH consensus	

BIOMED-2 report:
J.J.M. van Dongen et al.
Leukemia 2003; 17: 2257-2317

Analysis of *TCRB* gene rearrangements

EuroClonality



Vβ family primers

Jβ primers

TCRB tubes A and B

		5'		3'
Vβ2	(-204)		AACTATGTTTTGGTATCGTCA	
Vβ4	(-201)		CACGATGTTCTGGTACCGTCAGCA	
Vβ5/1	(-197)		CAGTGTGTCCTGGTACCAACAG	
Vβ6a/11	(-201)		AACCCTTTATTGGTACCGACA	
Vβ6b/25	(-201)		ATCCCTTTTTTGGTACCAACAG	
Vβ6c	(-201)		AACCCTTTATTGGTATCAACAG	
Vβ7	(-198)		CGCTATGTATTGGTACAAGCA	
Vβ8a	(-201)		CTCCCGTTTTCTGGTACAGACAGAC	
Vβ9	(-198)		CGCTATGTATTGGTATAAACAG	
Vβ10	(-201)		TTATGTTTACTGGTATCGTAAGAAGC	
Vβ11	(-198)		CAAAATGTACTGGTATCAACAA	
Vβ12a/3/13a/15	(-198)		ATACATGTACTGGTATCGACAAGAC	
Vβ13b	(-198)		GGCCATGTACTGGTATAGACAAG	
Vβ13c/12b/14	(-198)		GTATATGTCCTGGTATCGACAAGA	
Vβ16	(-201)		TAACCTTTATTGGTATCGACGTGT	
Vβ17	(-198)		GGCCATGTACTGGTACCGACA	
Vβ18	(-201)		TCATGTTTACTGGTATCGGCAG	
Vβ19	(-201)		TTATGTTTATTGGTATCAACAGAATCA	
Vβ20	(-193, inv)		CAACCTATACTGGTACCGACA	
Vβ21	(-201)		TACCCTTTACTGGTACCGGCAG	
Vβ22	(-201)		ATACTTCTATTGGTACAGACAAATCT	
Vβ23/8b	(-201)		CACGGTCTACTGGTACCAGCA	
Vβ24	(-197)		CGTCATGTACTGGTACCAGCA	

TCRB tubes A and C: Jβ A primers

3'		5'	
GTGGTCTAAGTGTCAACATCCATTC	(+53)		Jβ1.1
CTGGTCCAATTGGCAACATCCATTC	(+53)		Jβ1.2
TTCAACCGAGTGACAACATCCATTC	(+55)		Jβ1.3
CTTGGGTCGAGAGACAGAACCCATAC	(+56)		Jβ1.4
CTGAGCTGAGAGGTAGGATCCATTC	(+55)		Jβ1.5
GTCCGAGTGACACTGTCCATAC	(+58)		Jβ1.6
TCCGACTGGCATGACCCATTC	(+56)		Jβ2.2
TCCGACTGGCACGACCCGCTC	(+58)		Jβ2.6
GTCCGAGTGCCAATGTCCATTC	(+52)		Jβ2.7

TCRB tubes B and C: Jβ B primers

3'		5'	
AGTGGCACGATCCATTCTTCC	(+59)		Jβ2.1
ACTGTCACGAGCCATTCGCCC	(+58)		Jβ2.3
AGAGTCACGACCCATTCGACC	(+59)		Jβ2.4
CACGAGCCACACGCGC	(+57)		Jβ2.5

Dβ1 primer

Dβ2 primer

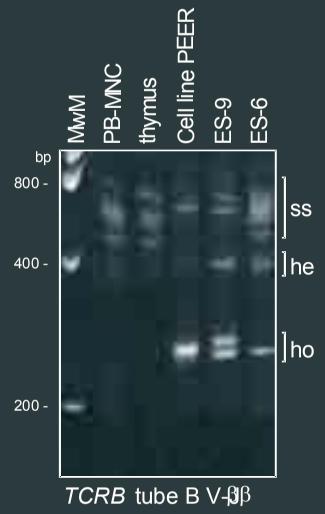
Jβ primers

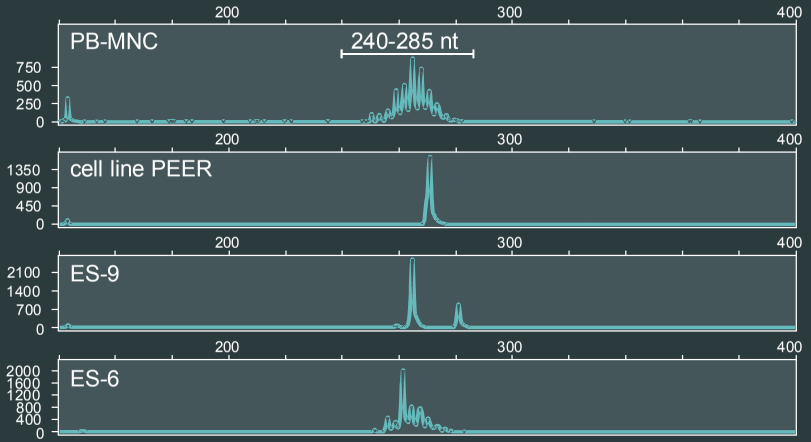
TCRB tube C

		5'		3'			5'		3'
Dβ1	(-252)		GCCAAACAGCCTTACAAAGAC		Dβ2	(-137)		TTTCCAAGCCCCACACAGTC	

BIOMED-2 report: Leukemia 2003; 17: 2257-2317

BIOMED-2 multiplex *TCRB* tube B: Vβ-Jβ





BIOMED-2 report: J.J.M. van Dongen et al. Leukemia 2003; 17: 2257-2317

EuroClonality/BIOMED-2 Concerted Action: BMH4-CT98-3936

Complementarity of Ig/TCR targets for PCR-based clonality detection

B-cell malignancies	*IGH* VH-JH + DH-JH	*IGK* Vκ-Jκ + Kde	*IGH* + *IGK*	T-cell malignancies	*TCRB* Vβ-Jβ + Dβ-Jβ	*TCRG* Vγ-Jγ	*TCRB* + *TCRG*
MCL (n=54)	100%	100%	100%	T-PLL (n=33)	100%	94%	100%
B-CLL/SLL (n=56)	100%	100%	100%	T-LGL (n=28)	96%	96%	100%
FL (n=109)	86%	84%	100%	PTCL-U (n=47)	98%	94%	100%
MZL (n=41)	95%	83%	100%	AILT (n=37)	89%	92%	95%
DLBCL (n=109)	85%	80%	98%	ALCL (n=43)	74%	74%	79%*
TOTAL (n=369)	91%	88%	99%	TOTAL (n=188)	91%	89%	94%* (99%)

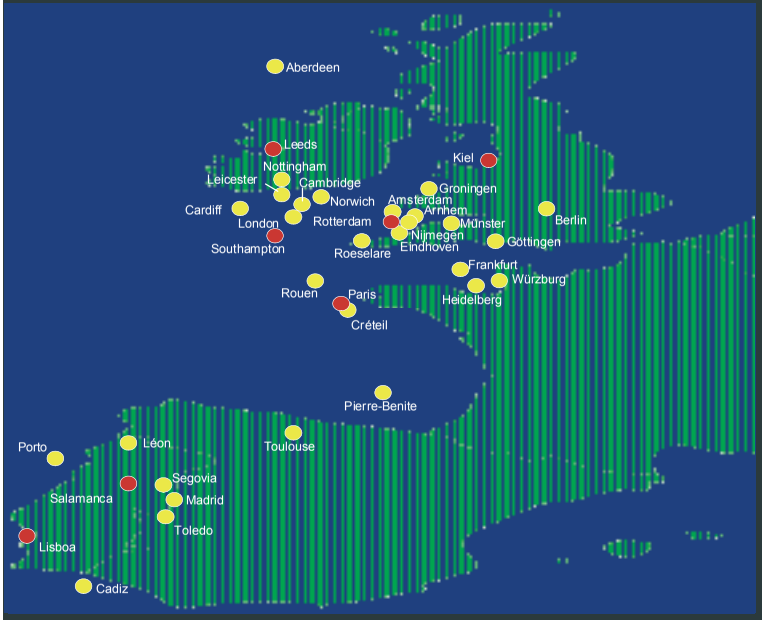
* 20% to 25 % of anaplastic large cell lymphomas do not have TCR gene rearrangements (null-ALCL)

EuroClonality

BIOMED-2 summary: JHJM van Krieken et al, Leukemia 2007;21:201-206
BIOMED-2 B-cell malignancy report: PAS Evans et al, Leukemia 2007;21:207-241
BIOMED-2 T-cell malignancy report: M Brüggemann et al, Leukemia 2007;21:215:221

EuroClonality/BIOMED-2 Concerted Action: BMH4-CT98-3936

EuroClonality



PCR-based clonality studies for early diagnosis of lymphoproliferative disorders

Chairman: J.J.M. van Dongen

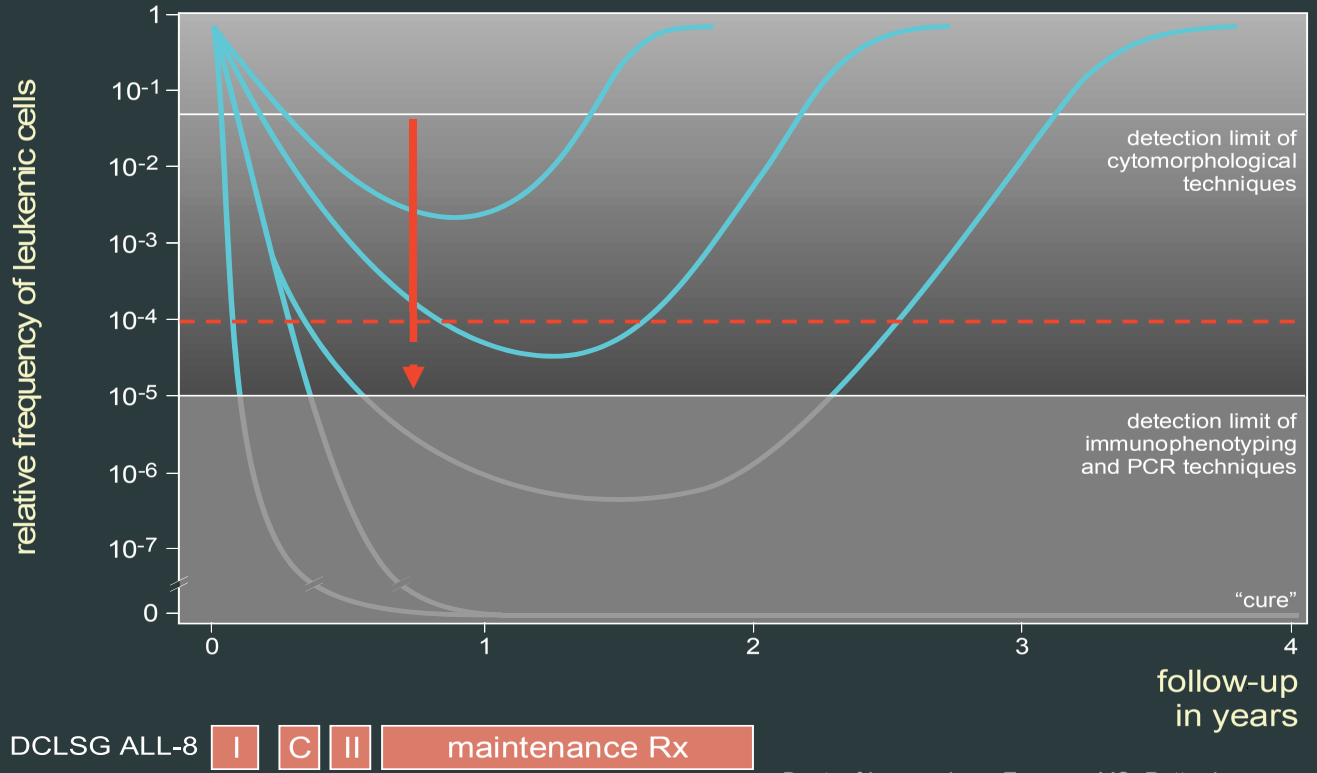
47 laboratories in 8 countries

www.euroclonality.org

For reprints, contact:
b.vanbodegom@erasmusmc.nl

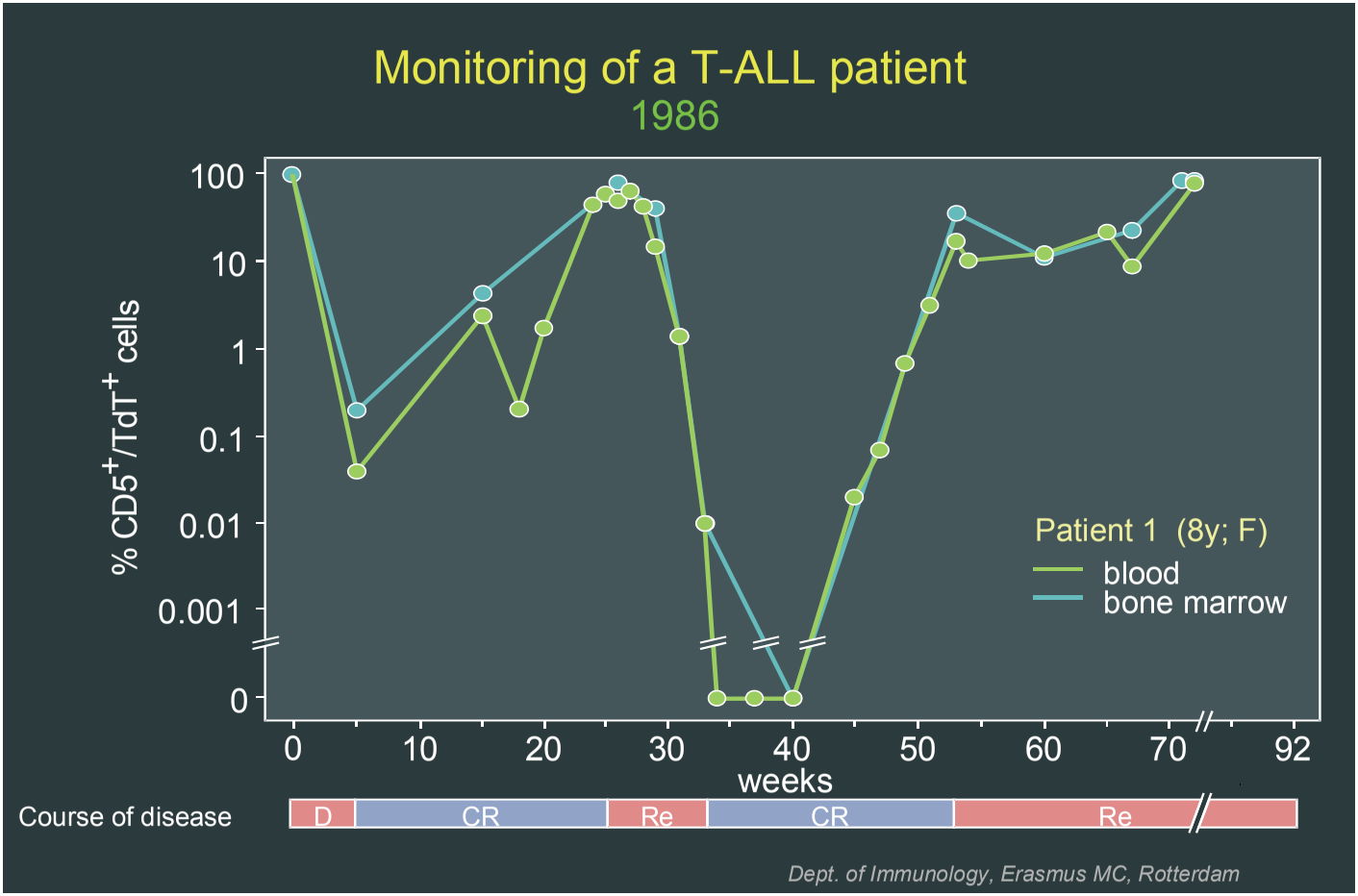
Availability of BIOMED-2 multiplex tubes:
InVivoScribe Technologies,
San Diego, CA
www.invivoscribe.com

Detection of minimal residual disease (MRD)



DCLSG ALL-8 | I | C | II | maintenance Rx

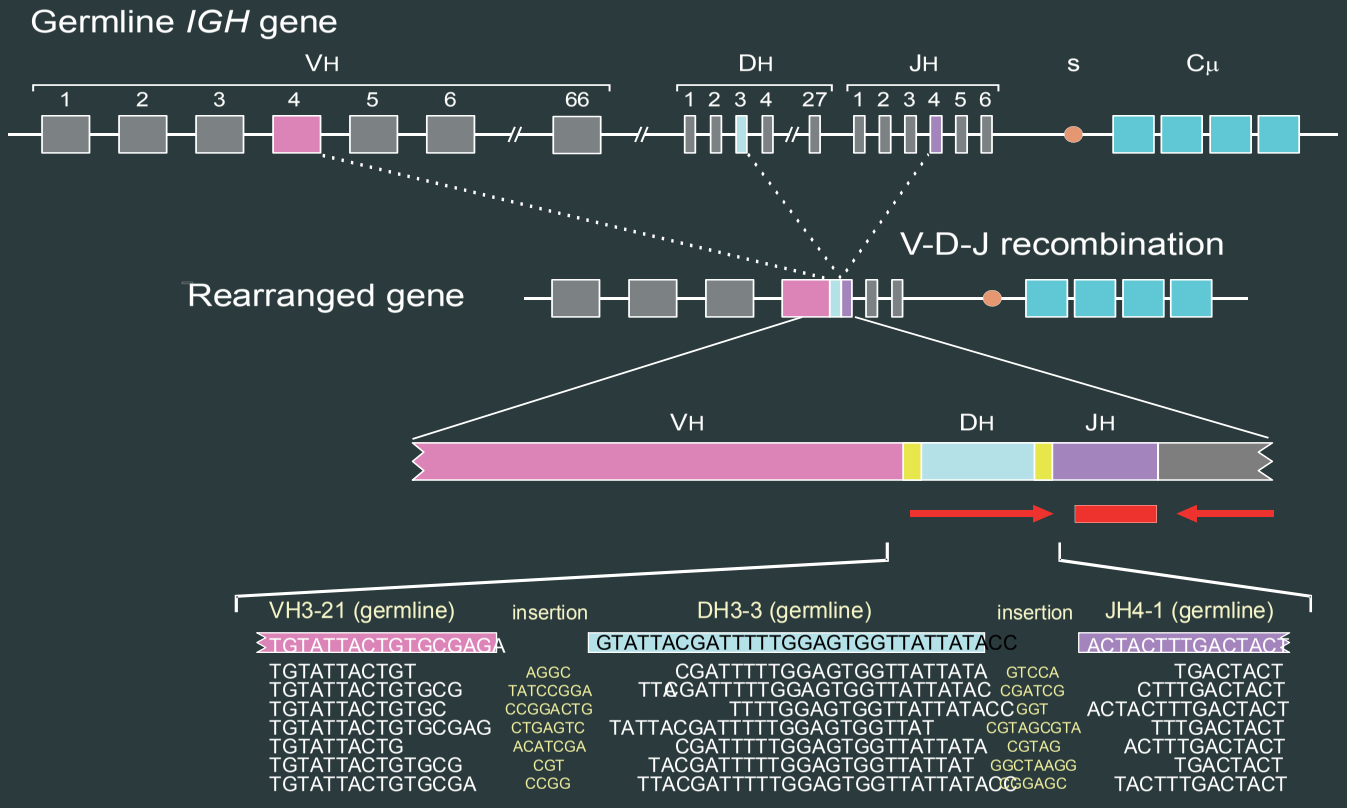
Dept. of Immunology, Erasmus MC, Rotterdam


Monitoring of a T-ALL patient
1986
% CD5+/TdT+ cells
100
10
1
0.1
0.01
0.001
0
0
10
20
30
40
50
60
70
92
weeks
Patient 1 (8y; F)
blood
bone marrow
Course of disease
D
CR
Re
CR
Re
Dept. of Immunology, Erasmus MC, Rotterdam

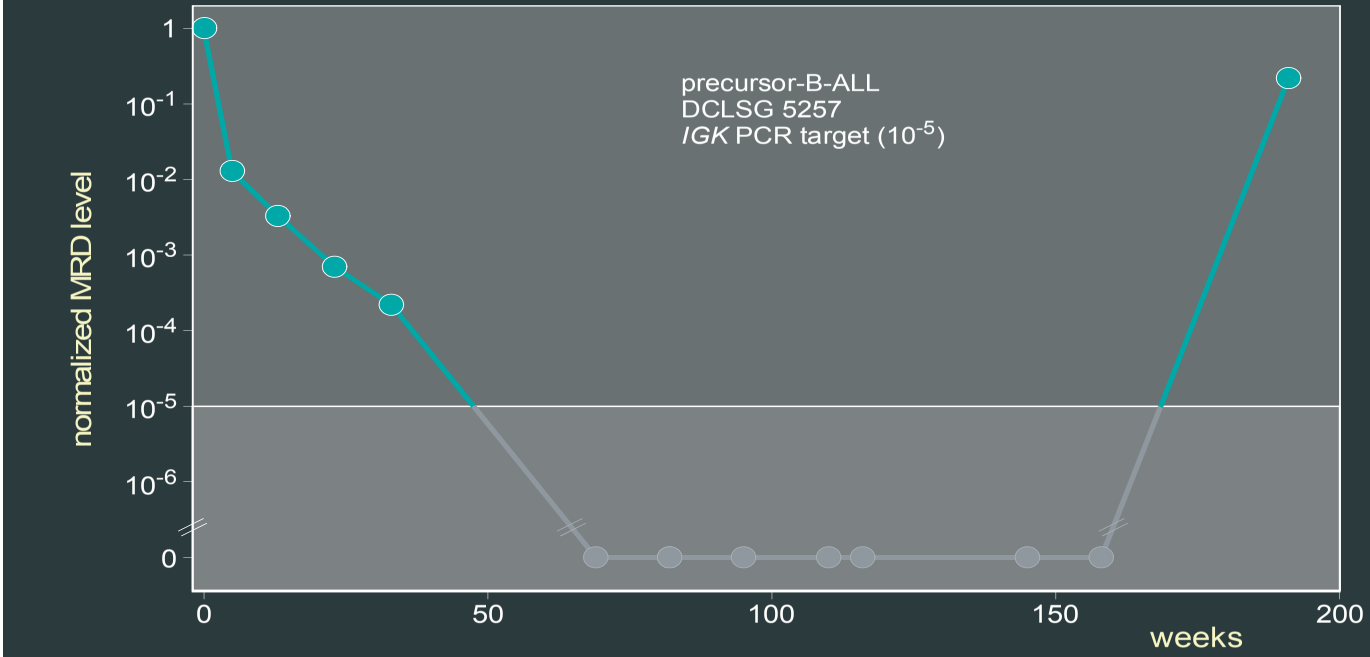
Detection of minimal residual disease in hemato-oncology

Technique	Detection limit	Remark
Cytomorphology	5×10^{-2}	Difficult discrimination between malignant blasts and normal (regenerating) blasts
Cytogenetica	5×10^{-2}	Differential proliferation of malignant (metaphases) and normal cells
FISH	10^{-2}	Only applicable if split-signal FISH is used (not fusion-signal FISH)
Immunophenotyping (3 to 4 color flow cytometry)	(10^{-3}-) 10^{-4}	Fast, but variable sensitivity because of similarities between normal (regenerating) cells and malignant cells
PCR of Ig/TCR genes	10^{-4}-10^{-5}	Time consuming and expensive, but applicable in ≥ 95% of lymphoid malignancies
PCR of fusion gene transcripts	10^{-4}-10^{-6}	Application in >98% of CML, but in only 40% of ALL, 25% of AML, and 5% of NHL

PCR analysis of Ig/TCR genes



RQ-PCR based MRD monitoring in a precursor-B-ALL patient



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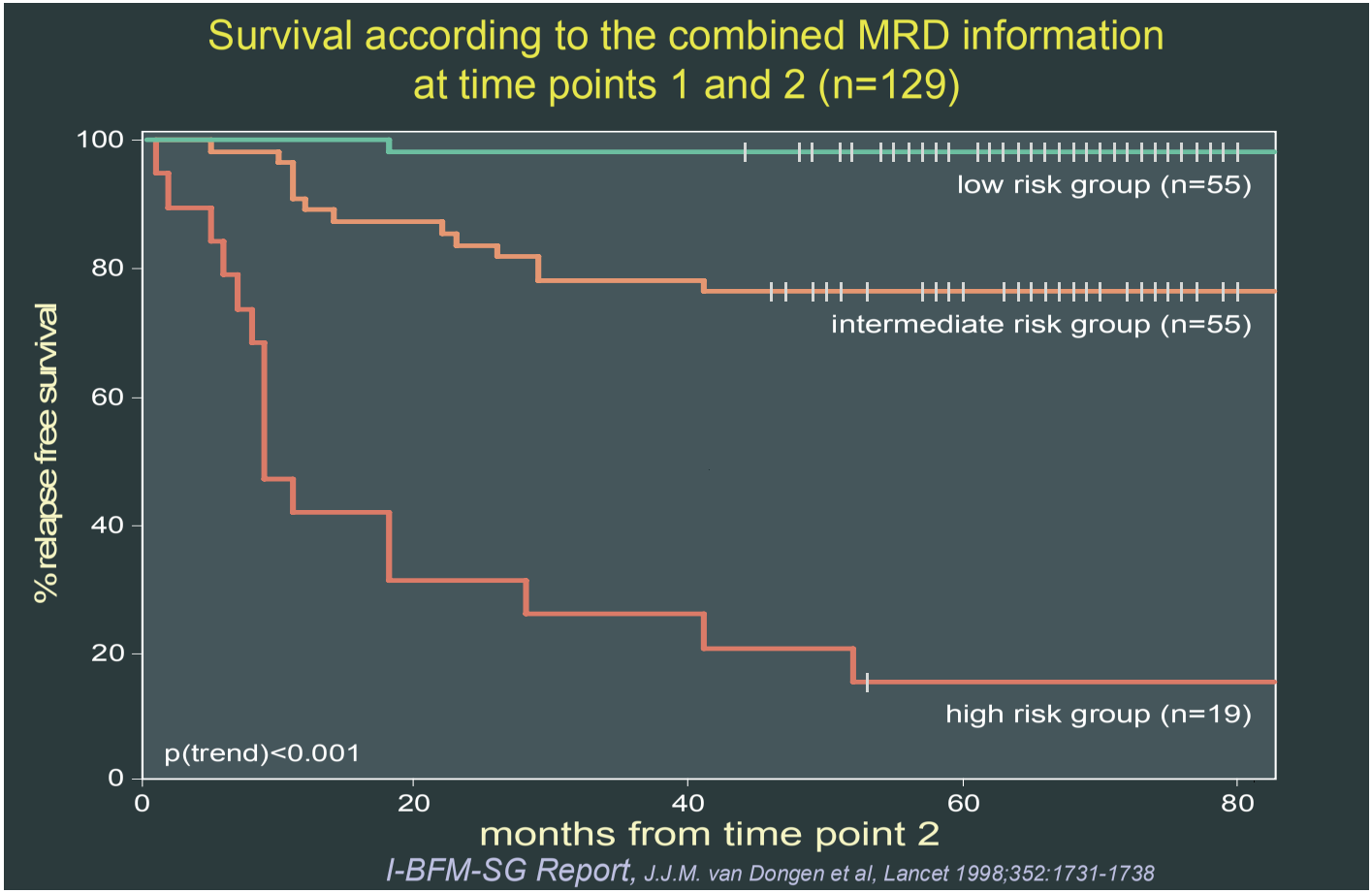
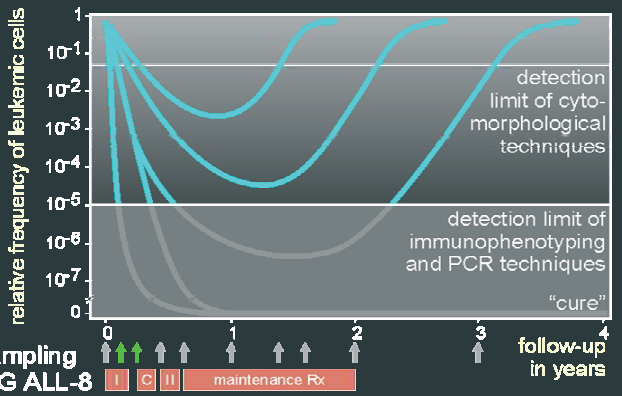
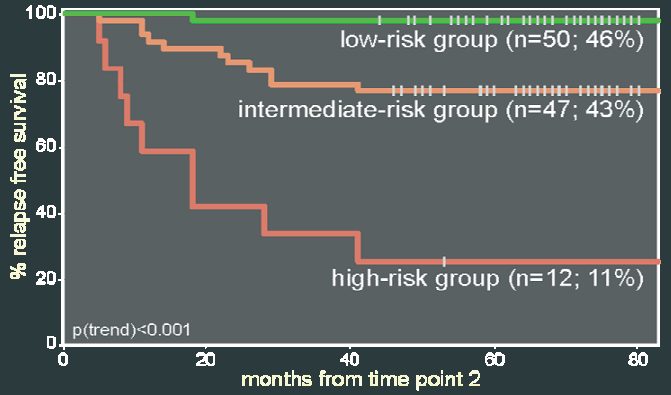
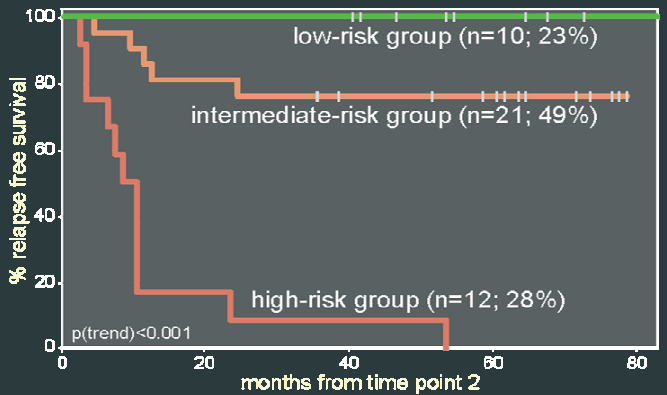

Survival according to the combined MRD information
at time points 1 and 2 (n=129)
100
80
60
40
20
0
% relapse free survival
low risk group (n=55)
intermediate risk group (n=55)
high risk group (n=19)
p(trend)<0.001
0
20
40
60
80
months from time point 2
I-BFM-SG Report, J.J.M. van Dongen et al, Lancet 1998;352:1731-1738

MRD-based risk groups

according to MRD status at
time points 1 and 2
- low risk: MRD negative at TP1/TP2
- intermediate: MRD^{+}, but <10^{-3} at TP2
- high risk: MRD ≥10^{-3} at TP1/TP2

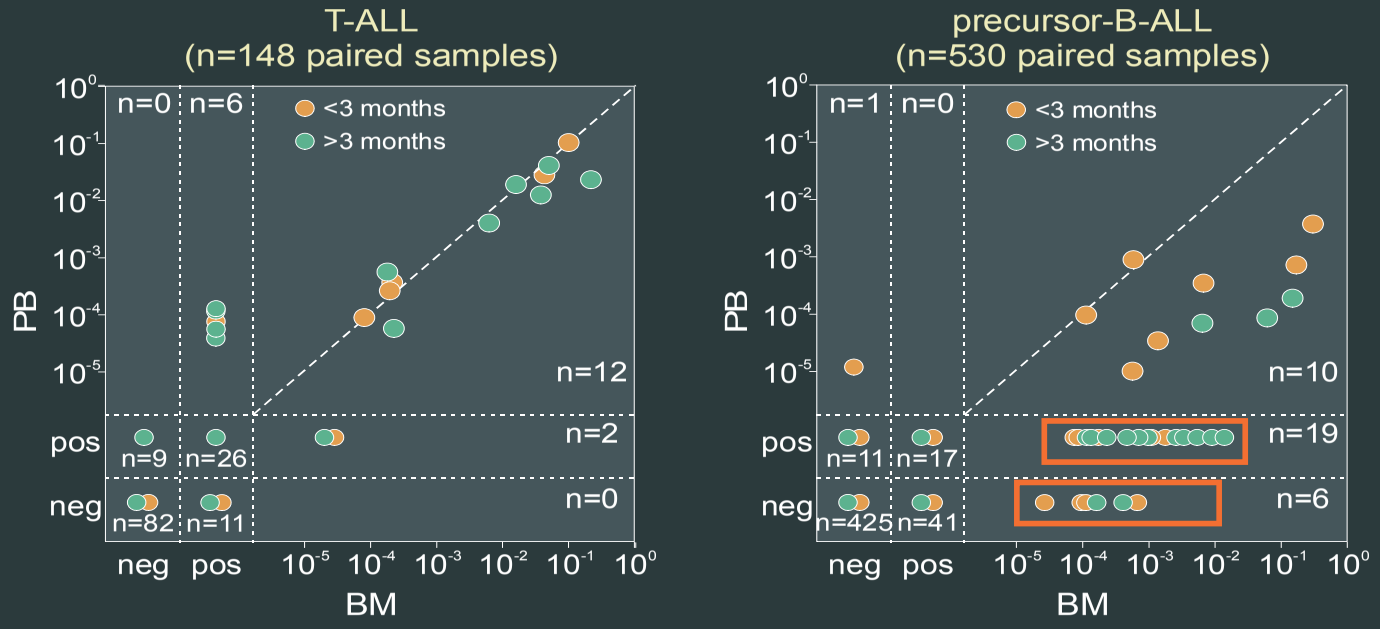






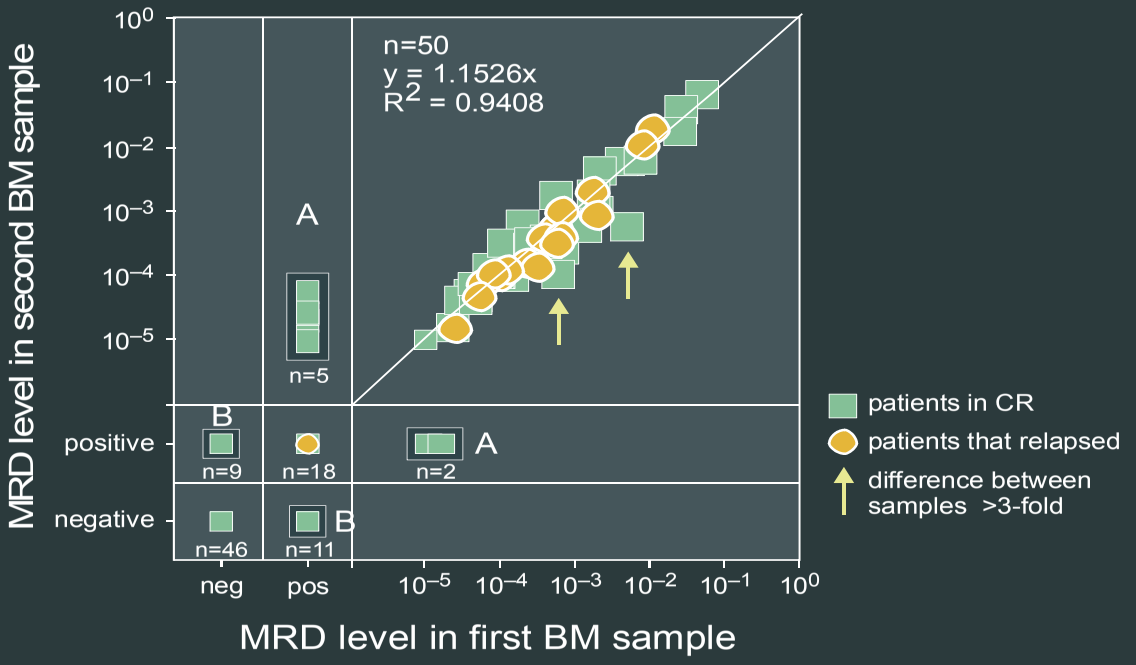
I-BFM-SG Report: Willemse et al., Blood 2002;99:4386-4393

RQ-PCR based MRD detection in paired BM-PB samples in precursor-B-ALL and T-ALL



Van der Velden et al., Leukemia 2002;16:1432-1436.

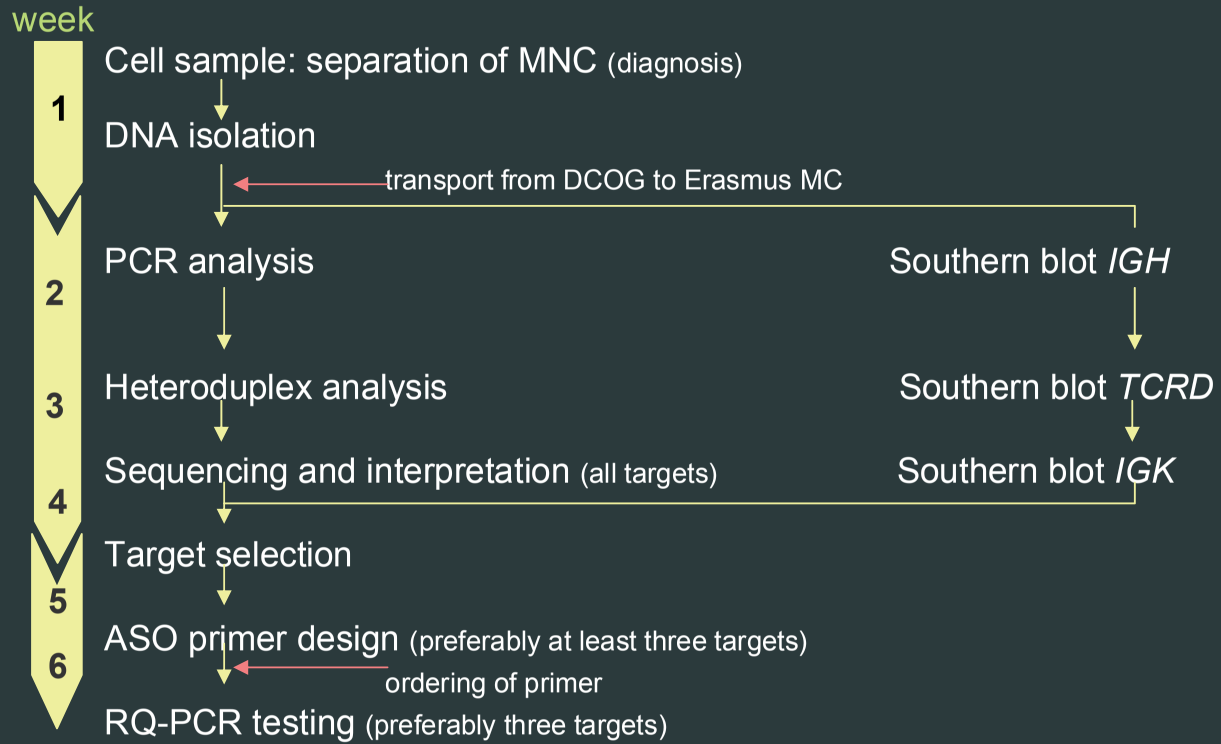
Comparison of MRD levels in paired collected BM samples



V.H.J. van der Velden et al. Br J Haematol 2006; 133: 382-388.

Ig/TCR gene rearrangements as MRD-PCR target

Time consuming and labor intensive (4 to 6 weeks)



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Problems and pitfalls of MRD-PCR detection via Ig/TCR genes

1. Time consuming, labor intensive and expensive
   - Target identification, selection and testing: 3-4 weeks
   - RQ-PCR analysis of follow-up samples:1-2 weeks
2. Extensive knowledge and experience needed:
   - Structure of Ig/TCR genes and rearrangement processes
   - Ig/TCR gene rearrangement patterns in ALL (precursor-B-ALL ↔ T-ALL; children ↔ adults)
3. International comparability of MRD-PCR results
   - Between MRD-PCR centers of same treatment protocol
   - Between treatment protocols

→ Standardization, guidelines and quality control

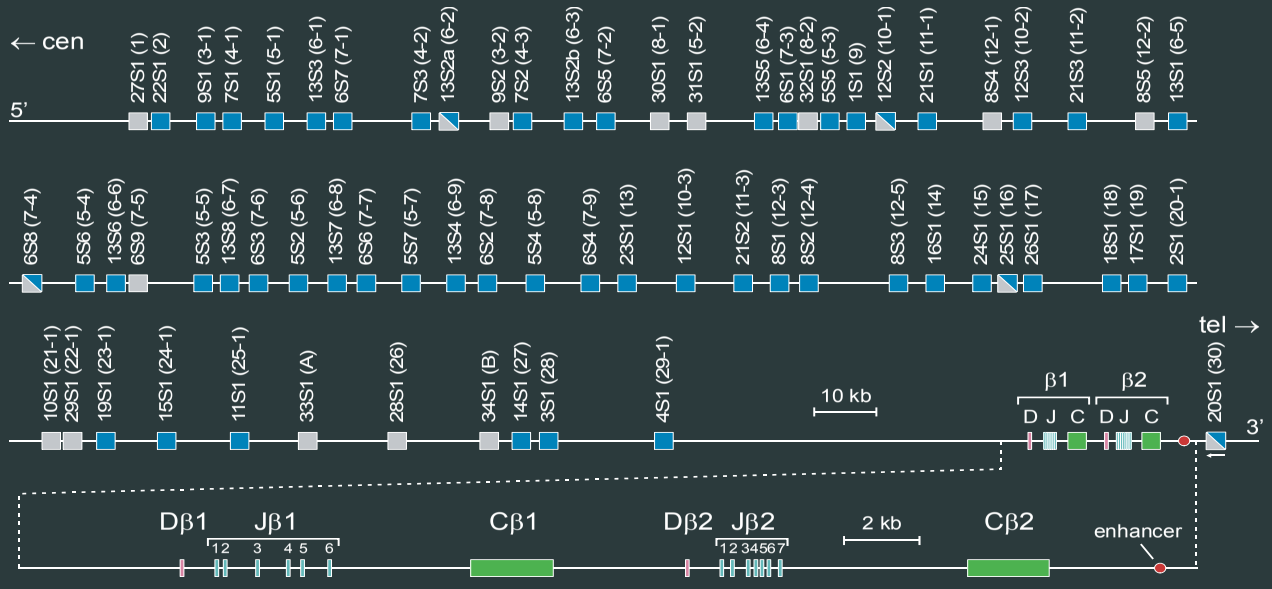
EuroMRD

Frequencies and stability of MRD-PCR targets in childhood precursor-B-ALL and T-ALL

Gene	Rearrangement type	Precursor-B-ALL				T-ALL	
		frequency	oligoclonality	stability		frequency	stability
				monoclonal	oligoclonal		
IGH	VH-JH	93%	30-40%	88%	47%	5%	NT
	DH-JH	20%	50-60%	57%	38%	23%	NT
	total *IGH*	98%	~40%	85%	44%	23%	NT
IGK	Vκ-Kde	45%	5-10%	95%	40%	0%	NA
	intron RSS-Kde	25%	5-10%	86%	0%	0%	NA
	total Kde	50%	5-10%	95%	40%	0%	NA
TCRB	Vβ-Jβ	21%	10-15%	89%	60%	77%	79%
	Dβ-Jβ	14%	10-15%	67%	0%	55%	80%
	total *TCRB*	33%	10-15%	81%	43%	92%	80%
TCRG	Vγ-Jγ	55%	~15%	75%		95%	86%
TCRD	Vδ-Jδ or Dδ-Jδ1	<1%	NA	NA	NA	50%	100%
	Vδ2-Dδ3 or Dδ2-Dδ3	40%	20-25%	86%	26%	55%	100%
	total *TCRD*	40%	20-25%	86%	26%	55%	100%
TCRD/A	Vδ2-Jα	46%	~45%	86%	43%	NT	NT

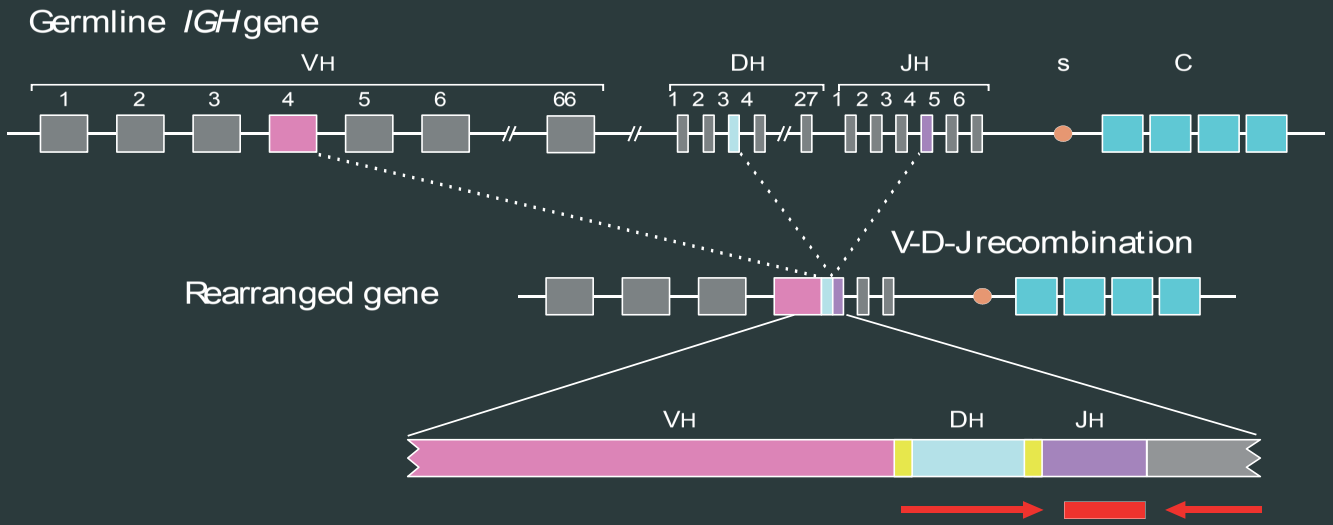
Human *TCRB* gene complex (#7q34)

67 Vβ, 2 Dβ, and 13 Jβ rearrangeable gene segments



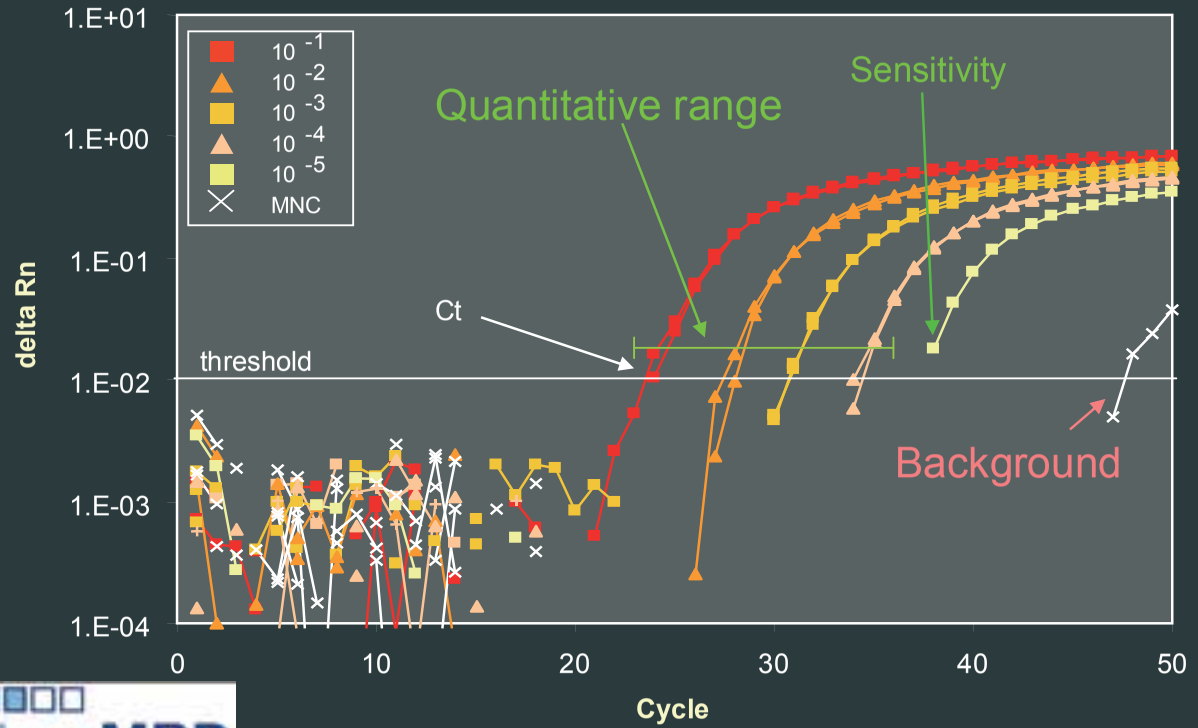
Adapted from IMGT database, M.P. Lefranc, Leukemia 2003; 17: 260-266

PCR analysis of Ig/TCR genes



High levels of standardization required

RQ-PCR analysis of TCR/Ig gene rearrangements



EuroMRD

(see EuroMRD Report: Van der Velden, Leukemia 2007)

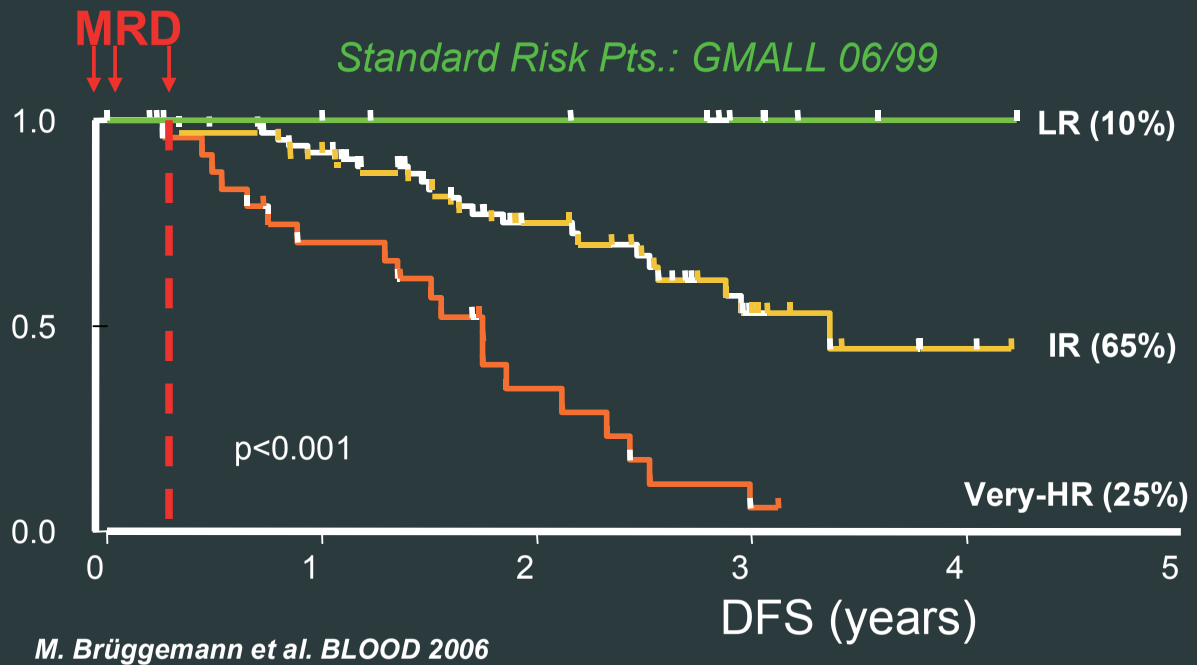
European Study Group on MRD detection

Chairman: J.J.M. van Dongen

42 laboratories in 17 countries

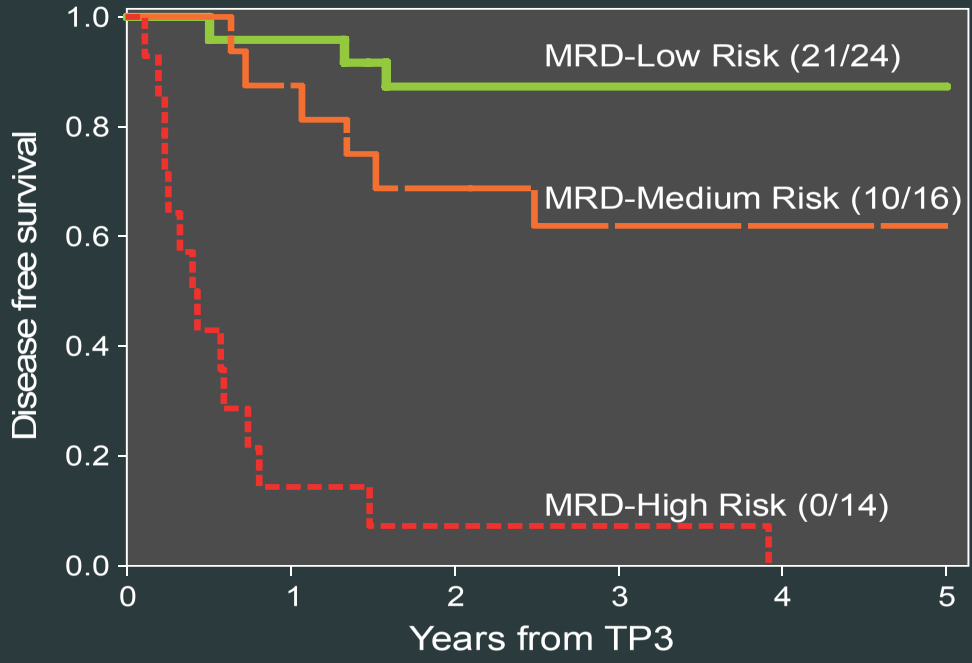
DFS according to MRD-based Risk Groups

defined by day +11, day +24 and week +16



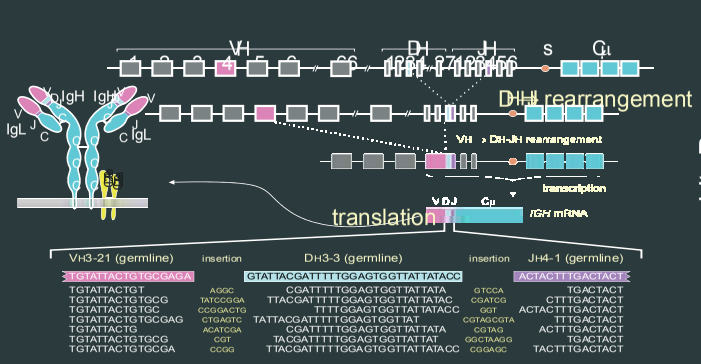
M. Brüggemann et al. BLOOD 2006

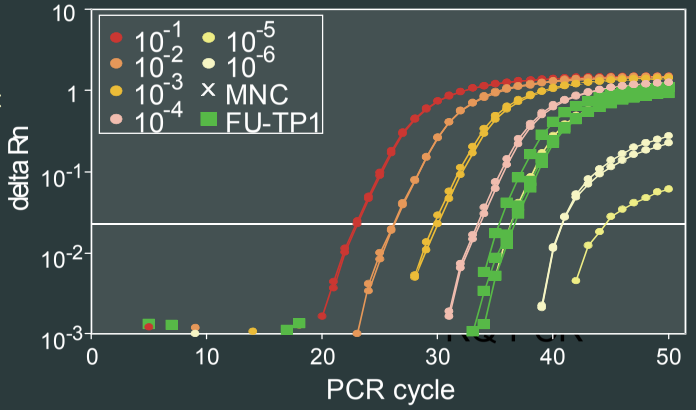
MRD diagnostics in infant ALL (Interfant-99 protocol)



Van der Velden et al., Leukemia 2009;23:1073-1079

Current MRD technique in lymphoid malignancies





Disadvantages of Ig/TCR-based MRD-PCR techniques:

- labor intensive (junctional regions per patient);
- require specialized laboratories;
- time consuming (target identification: 4 to 6 weeks))

→ Faster technique needed: 8-color flow cytometry ?

Comparison between molecular techniques and flow cytometry in hematological malignancies

	Molecular techniques	Flow cytometry
Speed	2-3 days (up to weeks)	fast: 1-2 hours !!
Target	DNA or RNA (RNA is an instable target)	protein/cells ("end-product")
Applicability	depends on disease (chromosome aberrations)	broad
Multiplexing	technically demanding	relatively easy (even 25 to 100 tests per tube)
Accuracy	semi-quantitative	quantitative
Focus	all cells in sample (or: prior purification)	any subpopulation
Facilities	special laboratories needed (pre-PCR lab, PCR lab, etc)	only standard lab needed (+ flow cytometer)

Achievements in flow cytometry by EuroFlow

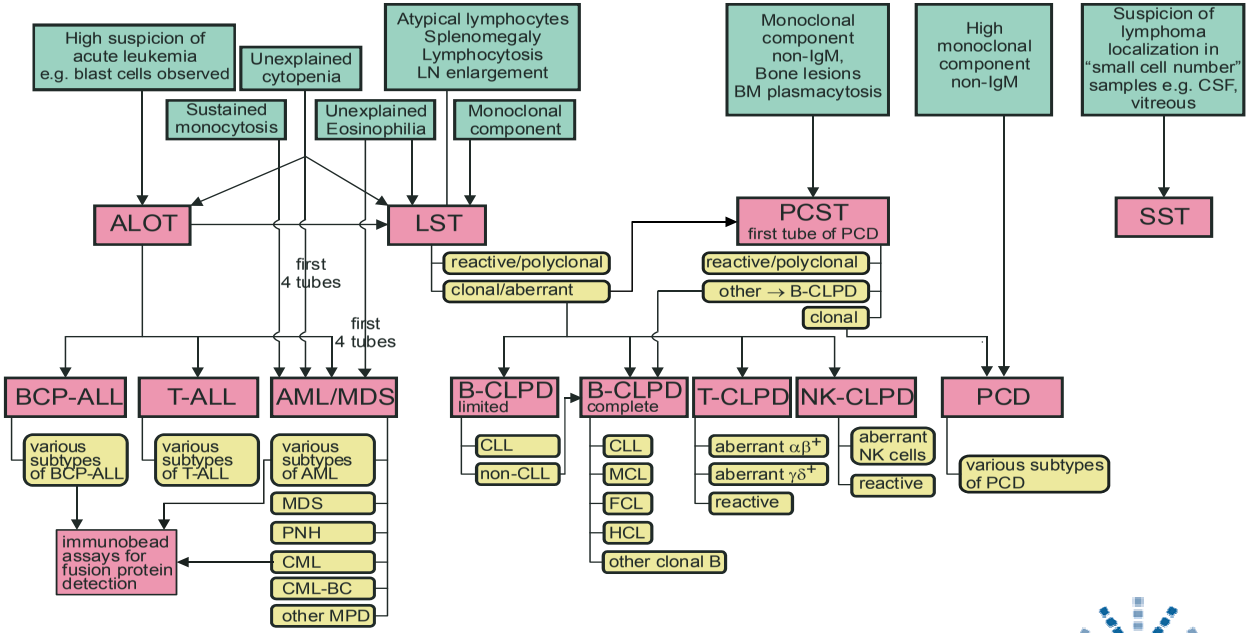
Immunobeads
- special immunobead assay for detection of fusion proteins in leukemias
- multiplex approach for fusion protein detection per disease category

Standardized multicolor flow cytometry (≥8 colors)
- inclusion of violet laser and selection of 8 appropriate fluorochromes
- Standardization of instrument settings, compensation settings, sample preparation, immunostaining procedures (including intracellular targets)

Development of novel software (INFINICYT) for complex pattern recognition
- "Merging": automated fusion of results from multiple multi-color labelings
- "Calculation": combining results of multiple tubes at the single cell level
- Multidimensional comparison of multiple samples: APS view
- Comparison of new diagnosis (and follow-up) data with large data base of earlier obtained samples (leukemia or normal/control)

Development of 8-color antibody protocols for diagnosis, classification and monitoring of hematological malignancies
- screening tubes (include recognition of normal leukocyte subsets)
- multi-tube panels for diagnosis and classification per disease category
- special tubes for MRD monitoring per disease category

Algorithm for EuroFlow antibody panels in hemato-oncology



EuroFlow

EuroFlow

EuroFlow is an independent scientific consortium, which aims at innovation in flow cytometry for improvement of diagnostic patient care

Chairmen:
J.J.M. van Dongen & A. Orfao

20 laboratories in 14 countries

www.euroflow.org

EU-supported	Period	Participating institutes	Results	Patents	Lisence & Annual networks royalty income
BIOMED-1 MRD techniques	1994 - 1998 continued as EuroMRD	14 - PCR Ig/TCR	- flow cytometry	None	-
BIOMED-2 EuroClonality	1998-2002 continued	47	- PCR for clonality - Ig and TCR gene sets	One patent	IVS Technologies
EAC RQ-PCR of FGT	1998-2003	24	- RQ-PCR of FGT Goes to Université	One patent	Ipsogen stopped Mediterané
EuroFISH FISH probe development	2003- 2006 reactivated	15	- split-signal FISH - leukemias & lympomas	One patent	DAKO
EuroChimerism Chimerism studies	2002- 2005 stopped	12	- STR primers - special SCT sets	One patent	Mylteni (just implemented)
EuroFlow Novel flowcytometry	2006-2009 continued	14 →21	- novel flow software - 8-color Ab protocols - large EU data base.	2 patents 1 in prep	BD Biosciences Cytognos

ESLHO and its divisions
EuroFlow, EuroClonality and EuroMRD

EuroClonality

Clonality Patent

"Nucleic acid amplification primers for PCR-based clonality studies"

Priority number:	US20020417779P	
Priority date:	11 October 2002	
Publication numbers:	EP1549764	22 April 2004
	WO2004033728	22 April 2004
Applicant:	Erasmus University Rotterdam, Rotterdam, NL	
Inventors (n=16):	J.J.M. van Dongen	G.J. Morgan
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	E.M.D. Schuuring	M. Kneba
	J.F. San Miguel	M. Hummel
	R. Garcia-Sanz	E.A. Macintyre
	A. Parreira	C. Bastard
	J.L. Smith	F.B.L. Davi
	F.L. Lavender	M. Brüggemann
Representative: (attorney)	B. van Wezenbeek (Vereenigde, The Hague, NL)	

EuroFlow

EuroFlow Patent

"Methods, reagents and kits for flow cytometric immunophenotyping"

Priority number:	EP09161870.2
Priority date:	3 June 2009
Publication numbers:	EP 2259065 8 December 2010
Applicant:	Erasmus University Medical Center Rotterdam, Rotterdam, NL
Inventors (n=15):	J.J.M. van Dongen J.A. Orfao J. Flores-Montero J.M. Almeida Parra V.H.J. van der Velden S. Böttcher A.C. Rawstron R.M. de Tute L.B.S. Lhermitte V. Asnafi E. Mejstrikova T. Szczepanski P.J. Lucio M.M. Ayuso C.E. Pedreira
Representative: (attorney)	A. Tepper (Vereenigde, The Hague, NL)

www.euroflow.org

EuroFlow participants

EuroFlow

University Institutes / Medical Schools

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IMM, Lisbon, PT	P. Lucio, A. Mendonça, A. Parreira a.o.
UNIKIEL, Kiel, DE	M. Kneba, S. Böttcher, M. Ritgen, M. Brüggemann a.o.
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SAM, Zabrze, PL	T. Szczepanski, L. Sedek a.o.
DCOG, The Hague, NL	E. Sonneveld, A. van der Sluijs-Gelling a.o.
KUL, Leuven, BE	N. Boeckx a.o.
HGSA, Porto, PT	M. Lima, AH Santos
UFRJ, Rio de Janeiro, BR	C. Pedreira, E.S. Costa

Companies (SME's)

DYNOMICS, Rotterdam, NL	E. Dekking, F. Weerkamp a.o.
CYTOGNOS, Salamanca, ES	M. Martin, J. Bensadon, J. Hernandez, M. Muñoz a.o.

European network for laboratory diagnostics
Scientifically independent consortium

Innovation
- development of new technologies or technical strategies, using the most suited and advanced equipment
- protection of new intellectual property (IP) by filing of patents on behalf of the consortium

(collective IP, collective ownership, collective revenues)

Standardization
- standardization of methods, including primer sequences, instrument settings, antibodies, fluorochromes, etc
- guidelines for interpretation of results

AIM: fully identical results in all participating laboratories (essential for international treatment protocols)

Quality Control
- regular quality control rounds (twice per year)
- certificates for participation and performance

Dissemination and continuous education
- Educational Workshops for dissemination of knowledge and experience
- educational lectures on new developments during consortium meetings

Профессор J.J.M. van Dongen

«Обнаружение аберраций,
происходящих вследствие слияния генов
при острых лейкозах,
методом проточной цитометрии.
Возможности использования
вместо молекулярной диагностики»

Russian flow cytometry conference, St Petersburg, Russia
17-19 March 2011

Detection of fusion gene aberrations in acute leukemia by flow cytometry

Possibilities for replacing molecular diagnostics

Jacques J.M. van Dongen
on behalf of

EuroFlow

Diagnostics in hematological malignancies

1. Making the diagnosis
   Normal ↔ reactive/regenerating ↔ malignant
   Annually > 300,000 new patients with a hematological malignancy in developed countries

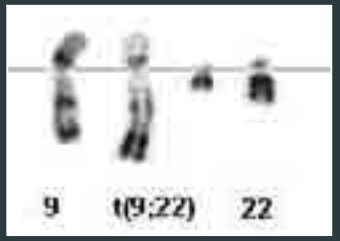
2. Classification of hematopoietic malignancies
   - relation with prognosis
   - relevance of risk-group definition in treatment protocols

   → 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)

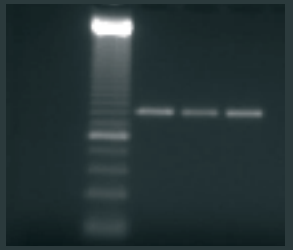
Current detection of genetic aberrancies

including overexpressed oncogenes and fusion genes

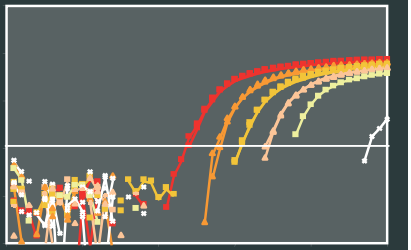


Cytogenetics

FISH



PCR

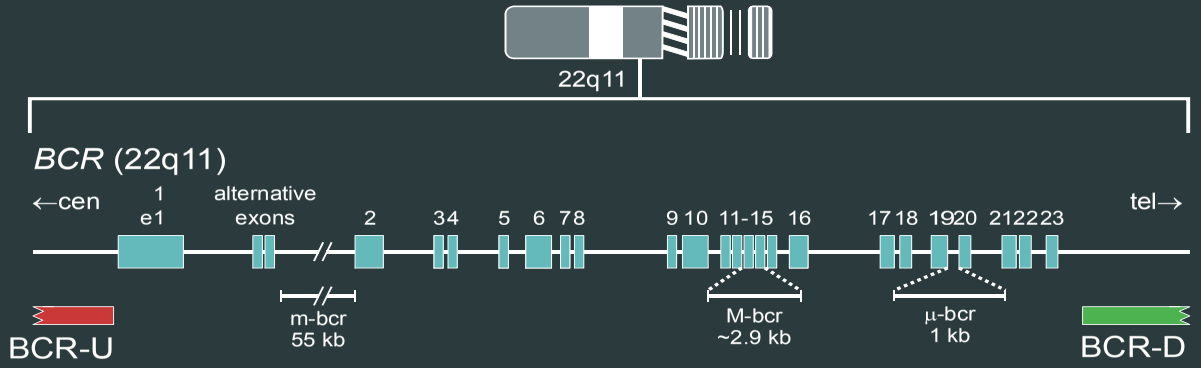


RQ-PCR

Advantages of molecular techniques:

- Generally well-established;
- Cytogenetics screens total genome for visible structural aberrations;
- FISH: screening of all relevant breakpoints of targeted genes;
- PCR: most variant breakpoints are identified (size differences);
- RQ-PCR: highly sensitive and reproducible: Useful for MRD diagnostics
- Microarray, CGH, SNP: promising, but to be established !

Split-signal FISH of *BCR* gene



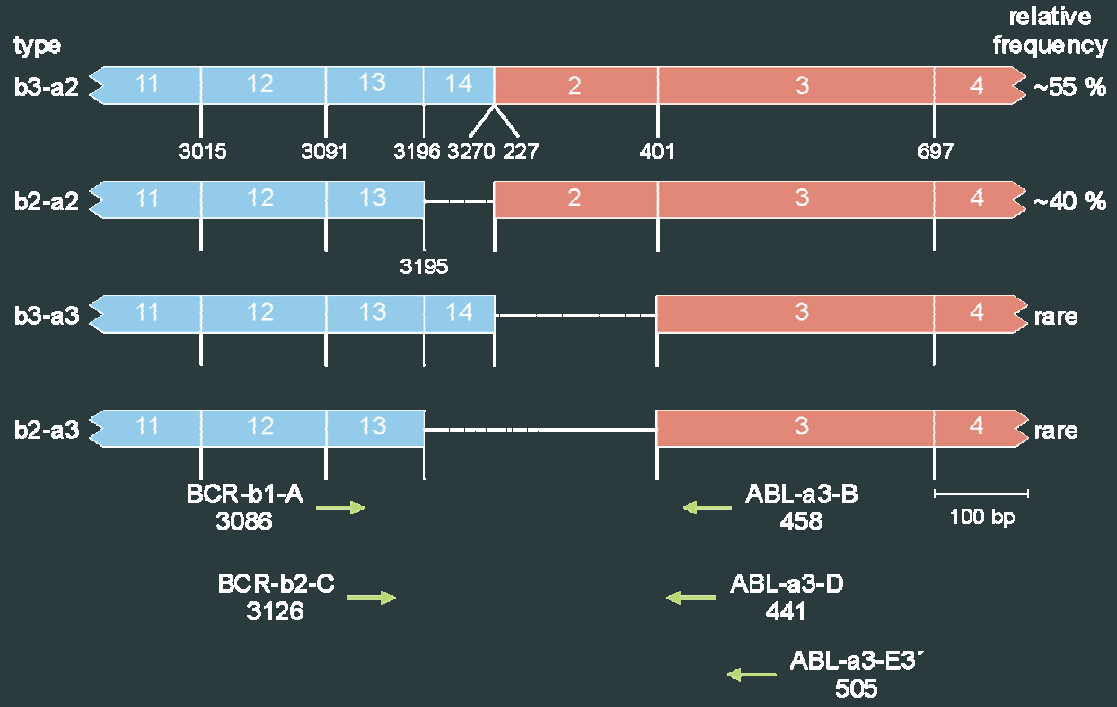
normal

translocation involving m-bcr

translocation involving M-bcr

M. van der Burg et al, Leukemia 2004; 18: 895-908.

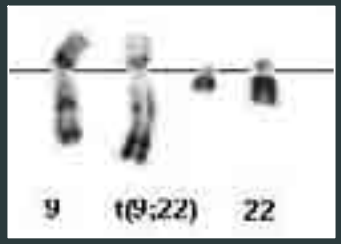
RT-PCR analysis of t(9;22)(q34;q11) with *BCR-ABL* p210 transcripts



J.J.M. van Dongen et al, BIOMED-1 Report, Leukemia 1999; 13: 1901-1928.

Current detection of genetic aberrancies

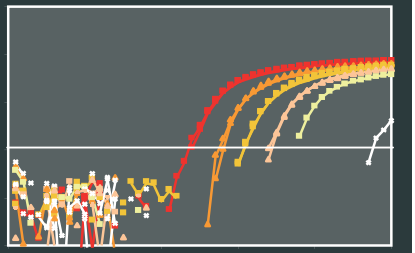
including overexpressed oncogenes and fusion genes



Cytogenetics

FISH

PCR



RQ-PCR

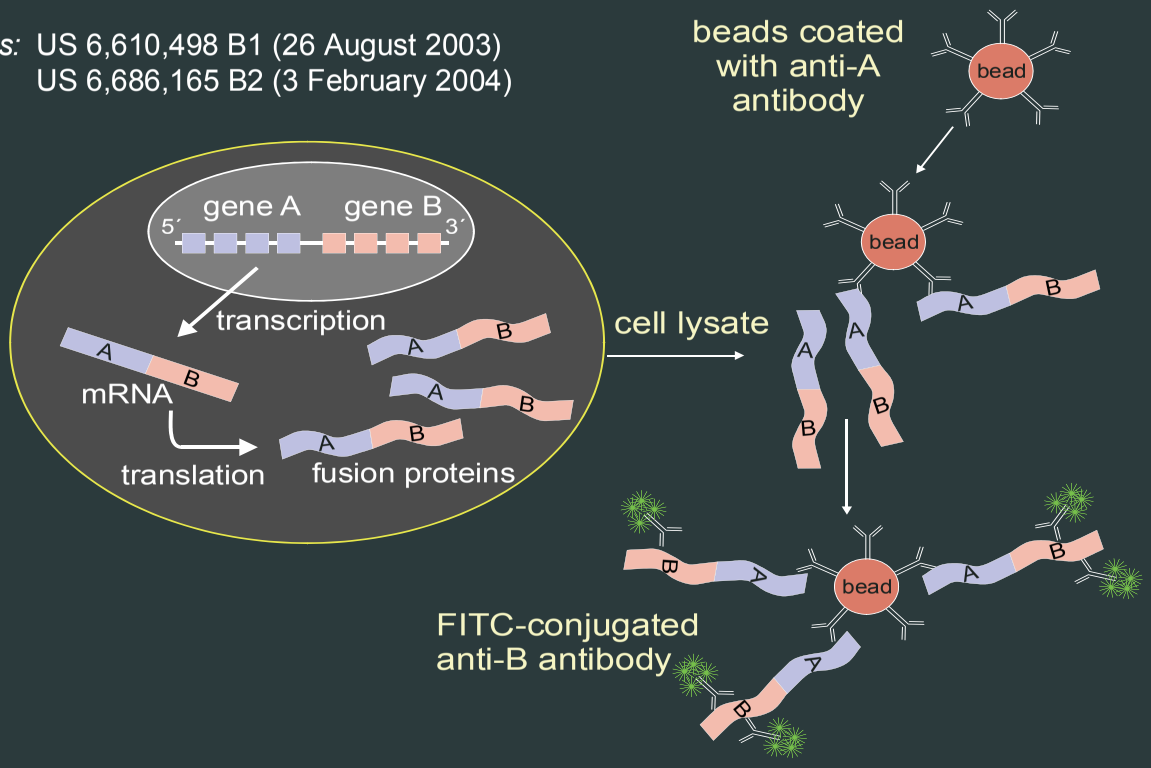
Disadvantages of molecular techniques:

- labor intensive;
- require specialized laboratories;
- time consuming (2-3 days, up to a -week)

→ Faster technique needed: at protein level?

Bead-based flow cytometric assay for detection of fusion proteins

Patents: US 6,610,498 B1 (26 August 2003)
US 6,686,165 B2 (3 February 2004)



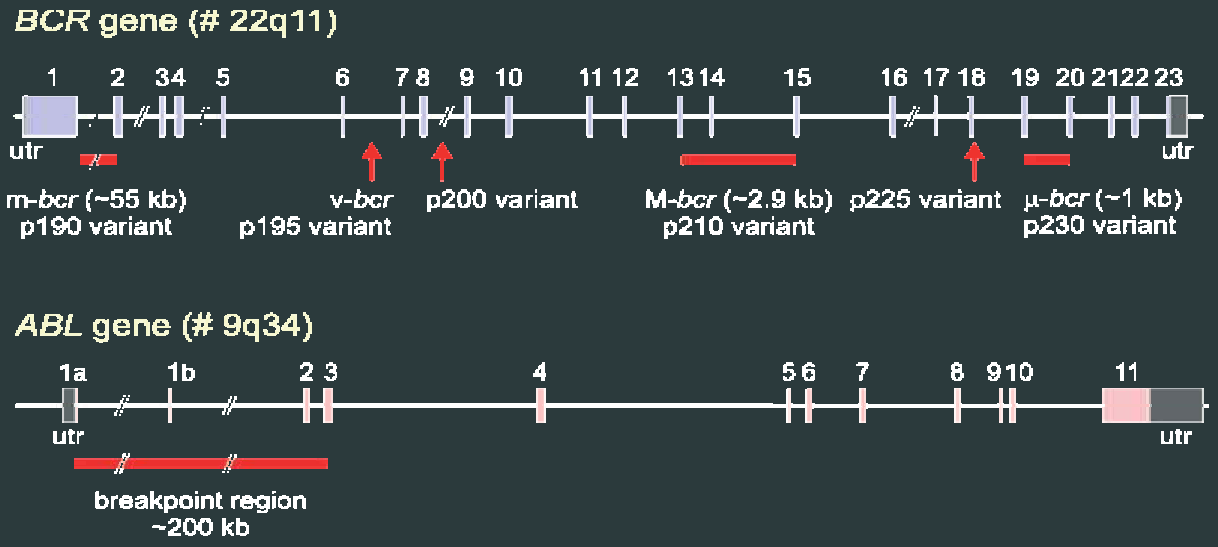
Dept. of Immunology, Erasmus MC, Rotterdam

Chromosome aberrations and fusion genes in acute leukemias

Chromosome aberration	Fusion genes	Relative frequency per type of acute leukemia				
		Precursor-B-ALL		AML		
		children	adults	children	adults <60 y	adults >60y
t(1;19)(q23;p13)	*E2A-PBX1*	5-8%	3-4%	-	-	-
t(4;11)(q21;q23)	*MLL-AF4*	3-5%[a]	3-4%	<1%	<1%	<1%
t(9;22)(q34;q11)	*BCR-ABL* p190	3-5%	15-30%	<1%	<1%	<1%
	BCR-ABL p210	1-2%	10-15%	<1%	<1%	<1%
t(12;21)(p13;q22)	*TEL-AML1*	25-30%	<2%	-	-	-
t(8;21)(q22;q22)	*AML1-ETO*	-	-	10-14%	6-8%	2-3%
t(15;17)(q22;q21)	*PML-RARA*	-	-	8-10%[b]	5-15%[b]	2-6%[b]
inv(16)(p13;q22)	*CBFB-MYH11*	-	-	5-7%	5-6%	3-4%
	TOTAL	40-45%	40-45%	25-30%	20-25%	10-12%

[a] In infant ALL, the frequency of t(4;11) can be as high as 70%.
[b] In southern European regions (ES, FR, and IT) the frequency of t(15;17) with *PML-RARA* is essentially higher than in northern European regions.

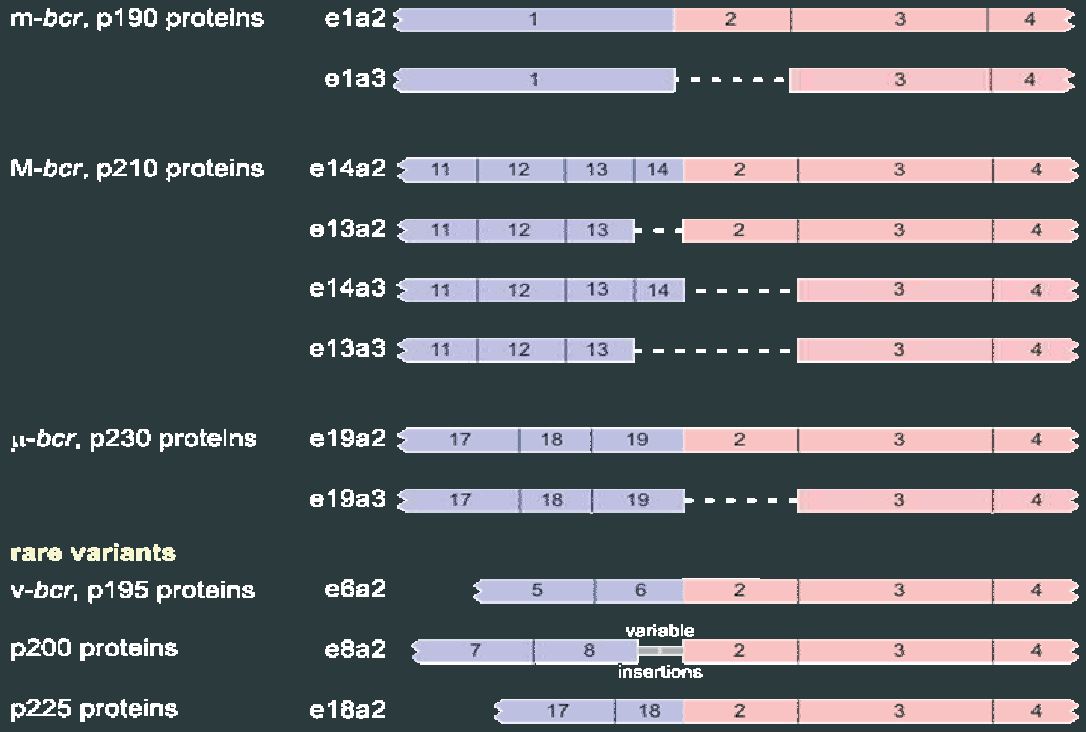
Breakpoint regions in t(9;22)(q34;q11) with *BCR* and *ABL* genes



EuroFlow

F. Weerkamp, et al. Leukemia 2009; 23: 1106-1117.

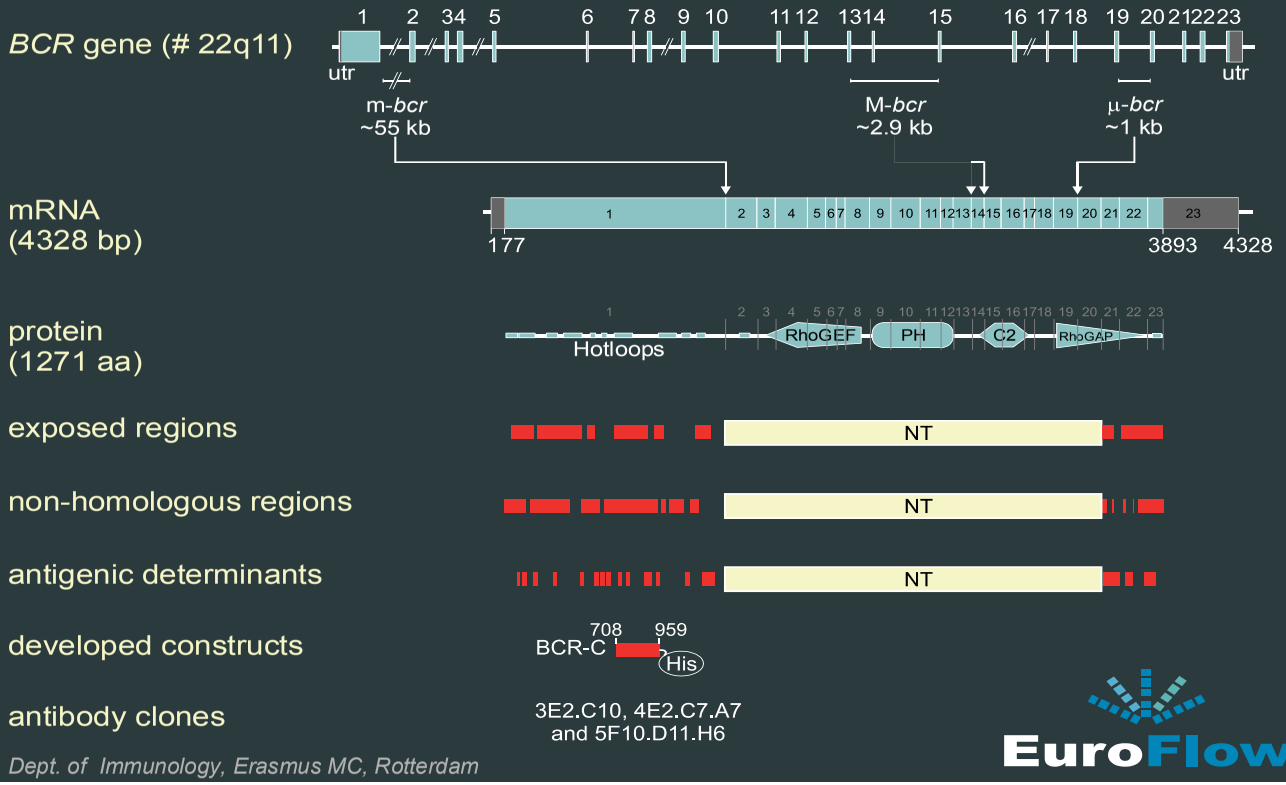
Multiple variants of *BCR-ABL* transcripts caused by multiple different *BCR* breakpoint regions



EuroFlow

F. Weerkamp, et al. Leukemia 2009; 23: 1106-1117.

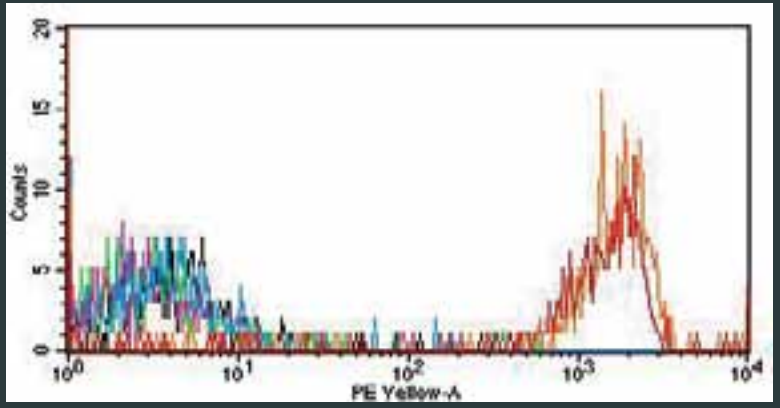
Design of anti-*BCR* antibodies for fusion protein beads



Dept. of Immunology, Erasmus MC, Rotterdam

EuroFlow

BD Biosciences platform for immunobeads detection of BCR-ABL t(9;22) fusion protein

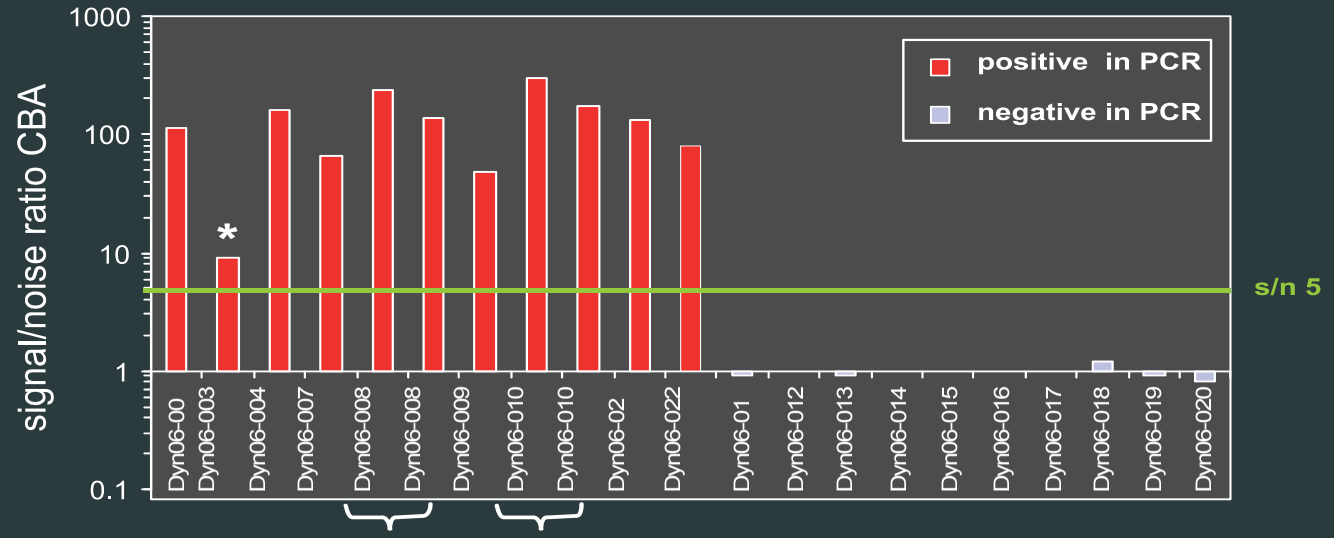


Black: PBMC (neg. control)
Green: 697 (E2-PBX1; neg. control)
Purple: REH (TEL-AML1; neg. control)
Blue: RS4,11 (MLL-AF4; neg. control)
Orange: K562 (BCR-ABL p210)
Red: Lama-84 (BCR-ABL p210)

Catching antibody: anti-ABL
Bead: BD-Flex bead (A7)
Detection antibody: anti-BCR (biotinylated)

EuroFlow

BCR-ABL CBA for precursor B-ALL (diagnosis)



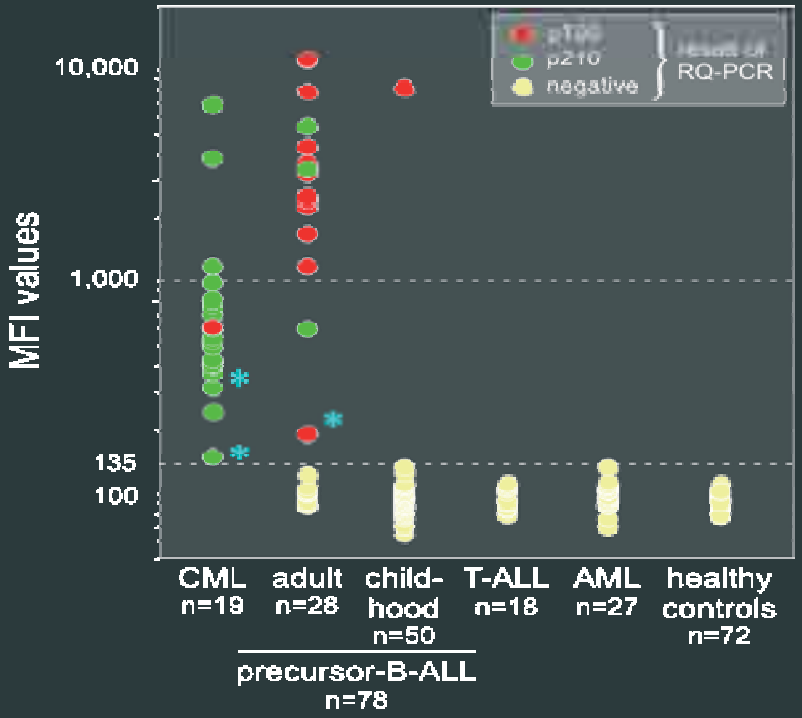
same sample tested in 2 separate experiments

* patient with mutation close to a ABL-binding site

Only frozen samples tested

EuroFlow

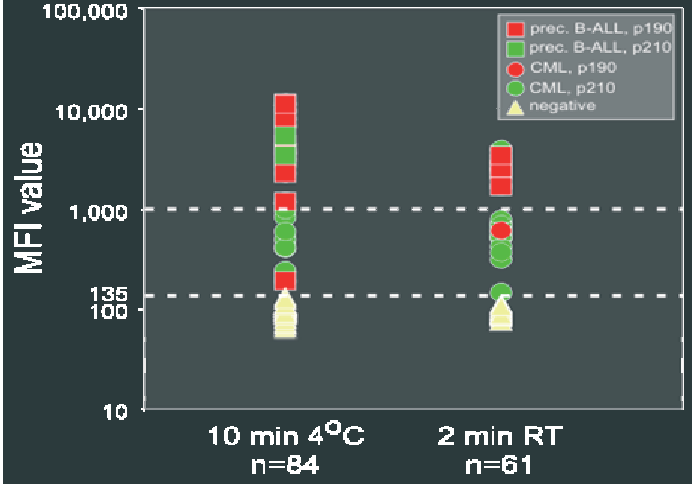
Results of the BCR-ABL RUO testing by the EuroFlow laboratories

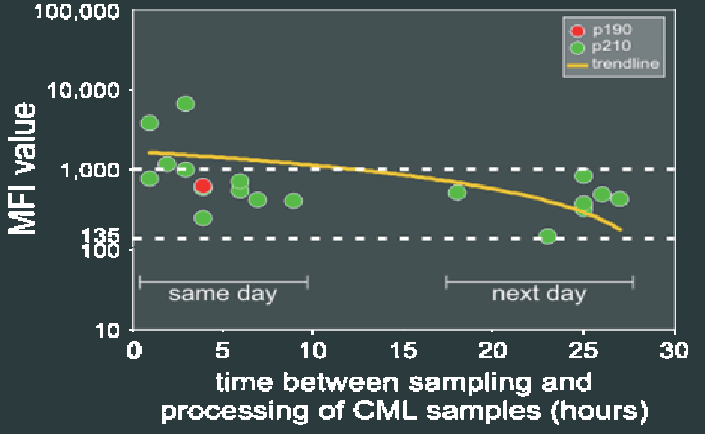


EuroFlow

F. Weerkamp, et al. Leukemia 2009; 23: 1106-1117.

Stability fo BCR-ABL fusion proteins
influence of temperature and transportation-processing time

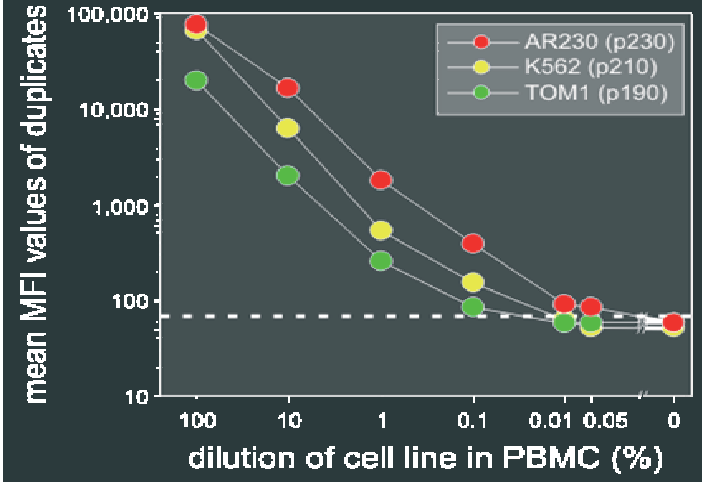


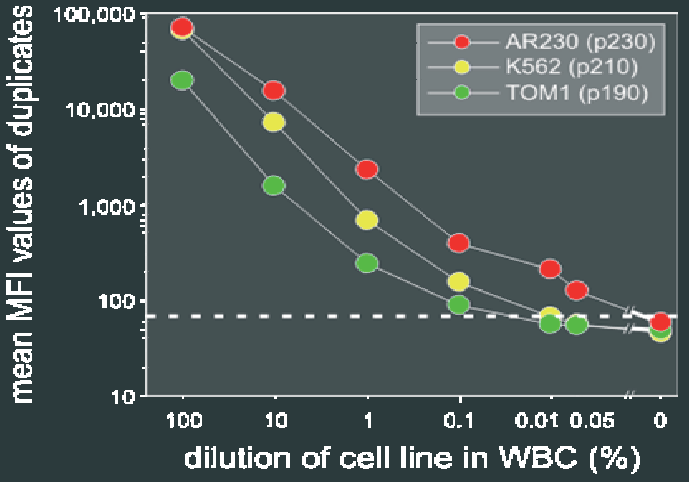


EuroFlow

F. Weerkamp, et al. Leukemia 2009; 23: 1106-1117.

Sensitivity of the BCR-ABL RUO immunobead assay

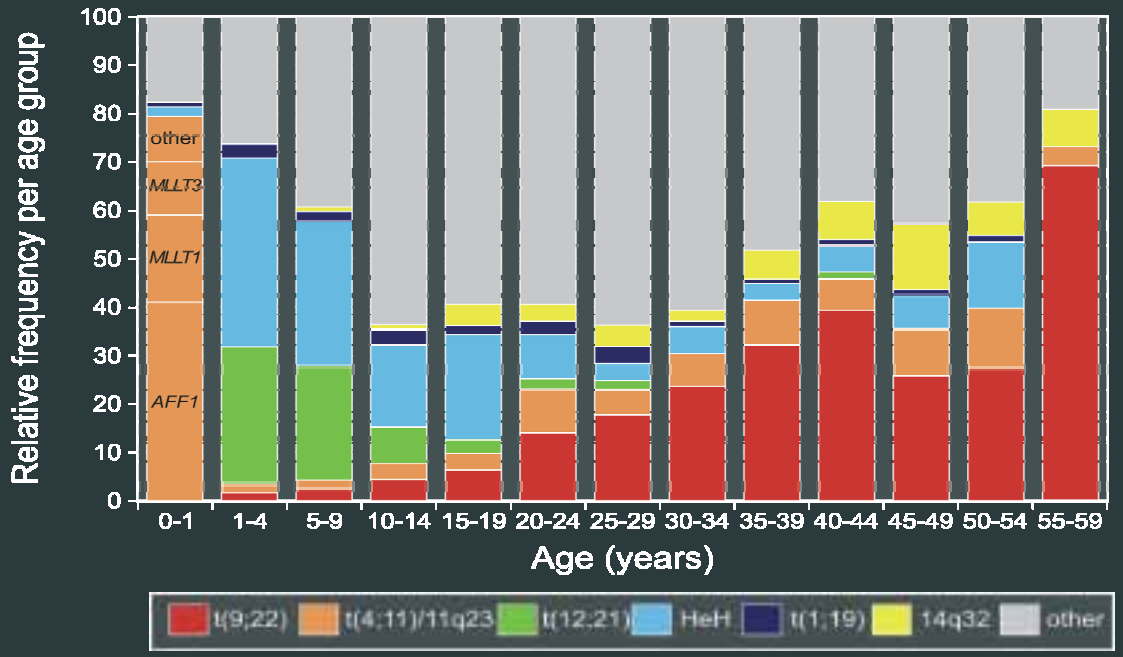




EuroFlow

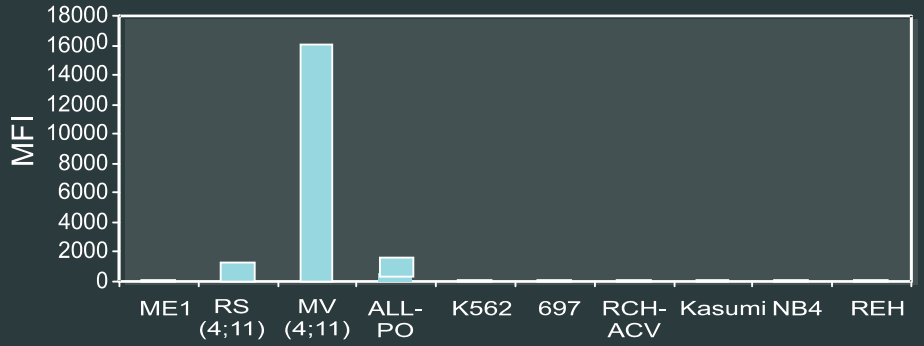
F. Weerkamp, et al. Leukemia 2009; 23: 1106-1117.

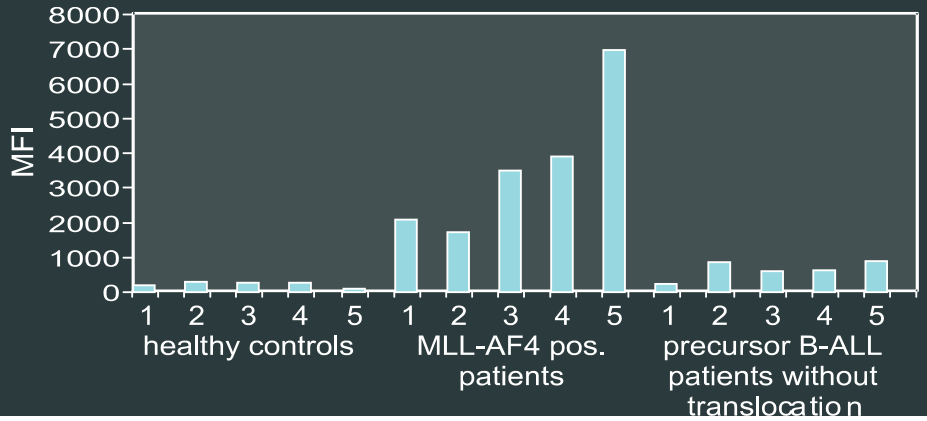
Relative frequency of well-defined genetic aberrations in ALL according to age groups



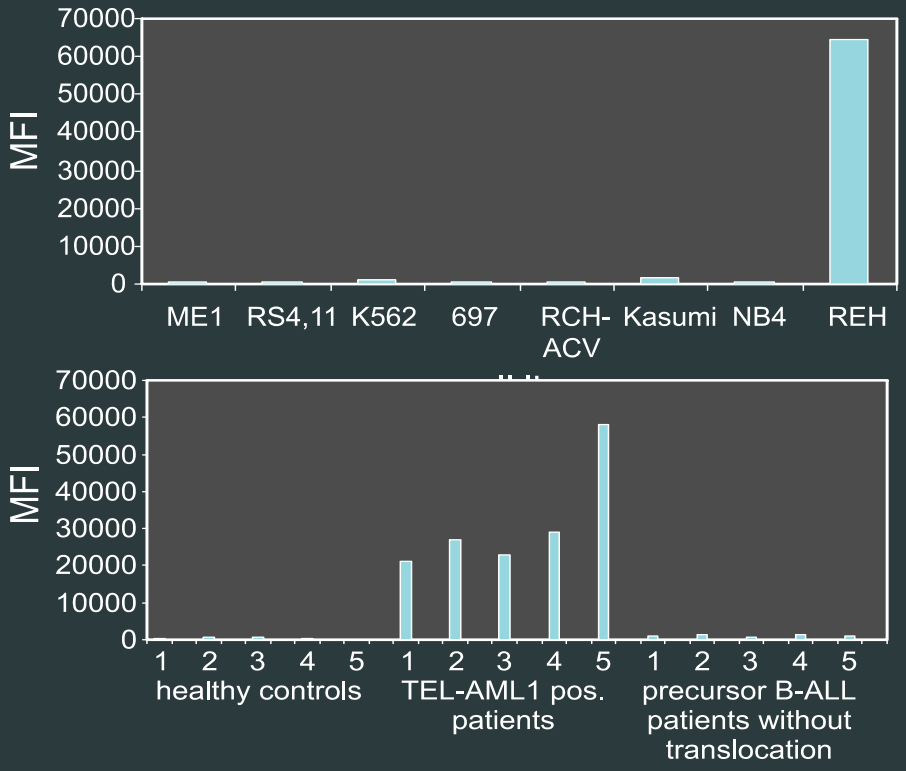
T. Szczepanski, et al. Lancet Oncology, 2010; 11:880-889

Flow cytometric MLL-AF4 immunobead assay





Flow cytometric TEL-AML1 immunobead assay

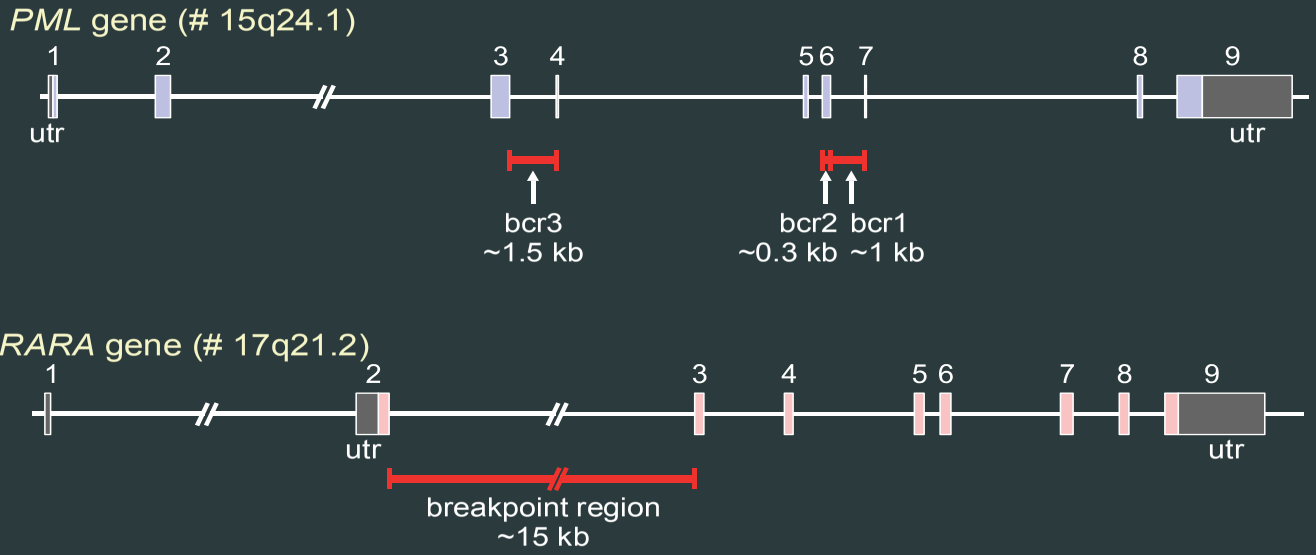


Chromosome aberrations and fusion genes in acute leukemias

Chromosome aberration	Fusion genes	Relative frequency per type of acute leukemia				
		Precursor-B-ALL		AML		
		children	adults	children	adults <60 y	adults >60y
t(1;19)(q23;p13)	*E2A-PBX1*	5-8%	3-4%	-	-	-
t(4;11)(q21;q23)	*MLL-AF4*	3-5%[a]	3-4%	<1%	<1%	<1%
t(9;22)(q34;q11)	*BCR-ABL* p190	3-5%	15-30%	<1%	<1%	<1%
	BCR-ABL p210	1-2%	10-15%	<1%	<1%	<1%
t(12;21)(p13;q22)	*TEL-AML1*	25-30%	<2%	-	-	-
t(8;21)(q22;q22)	*AML1-ETO*	-	-	10-14%	6-8%	2-3%
t(15;17)(q22;q21)	*PML-RARA*	-	-	8-10%[b]	5-15%[b]	2-6%[b]
inv(16)(p13;q22)	*CBFB-MYH11*	-	-	5-7%	5-6%	3-4%
	TOTAL	40-45%	40-45%	25-30%	20-25%	10-12%

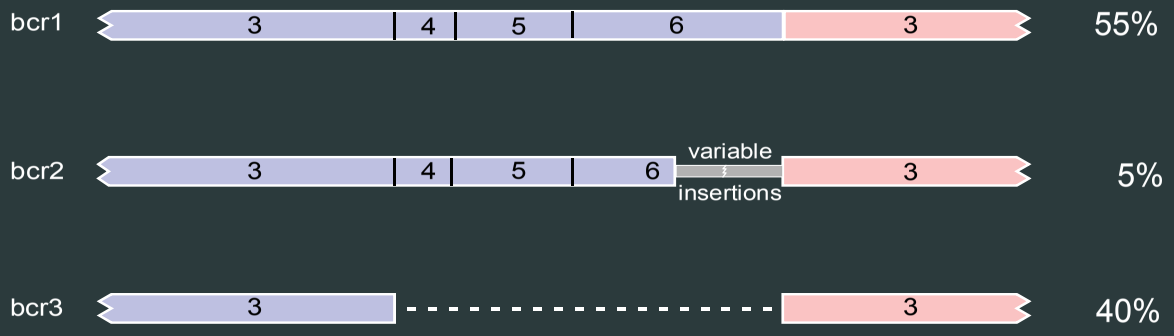
[a] In infant ALL, the frequency of t(4;11) can be as high as 70%.
[b] In southern European regions (ES, FR, and IT) the frequency of t(15;17) with *PML-RARA* is essentially higher than in northern European regions.

Breakpoint regions in t(15;17)(q24;q21) with *PML* and *RARA* genes



EuroFlow

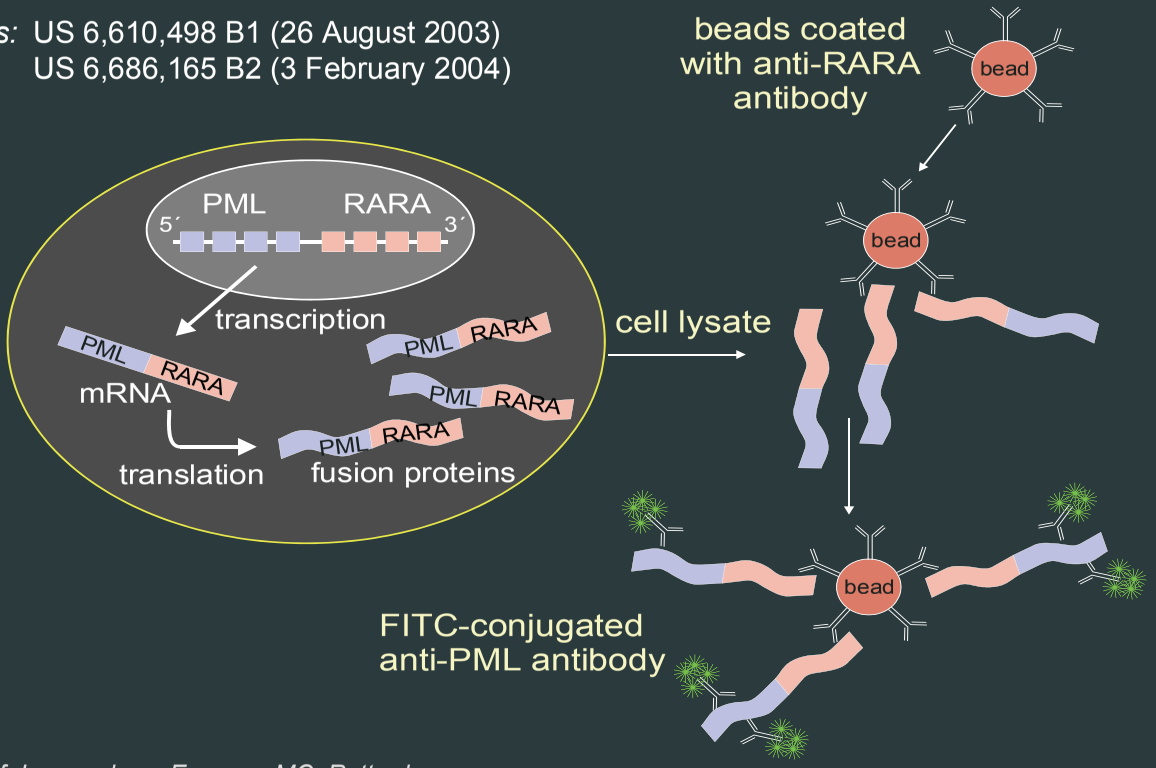
Variants of *PML-RARA* transcripts caused by different *PML* breakpoint regions



EuroFlow

Bead-based flow cytometric assay for detection of fusion proteins

Patents: US 6,610,498 B1 (26 August 2003)
US 6,686,165 B2 (3 February 2004)



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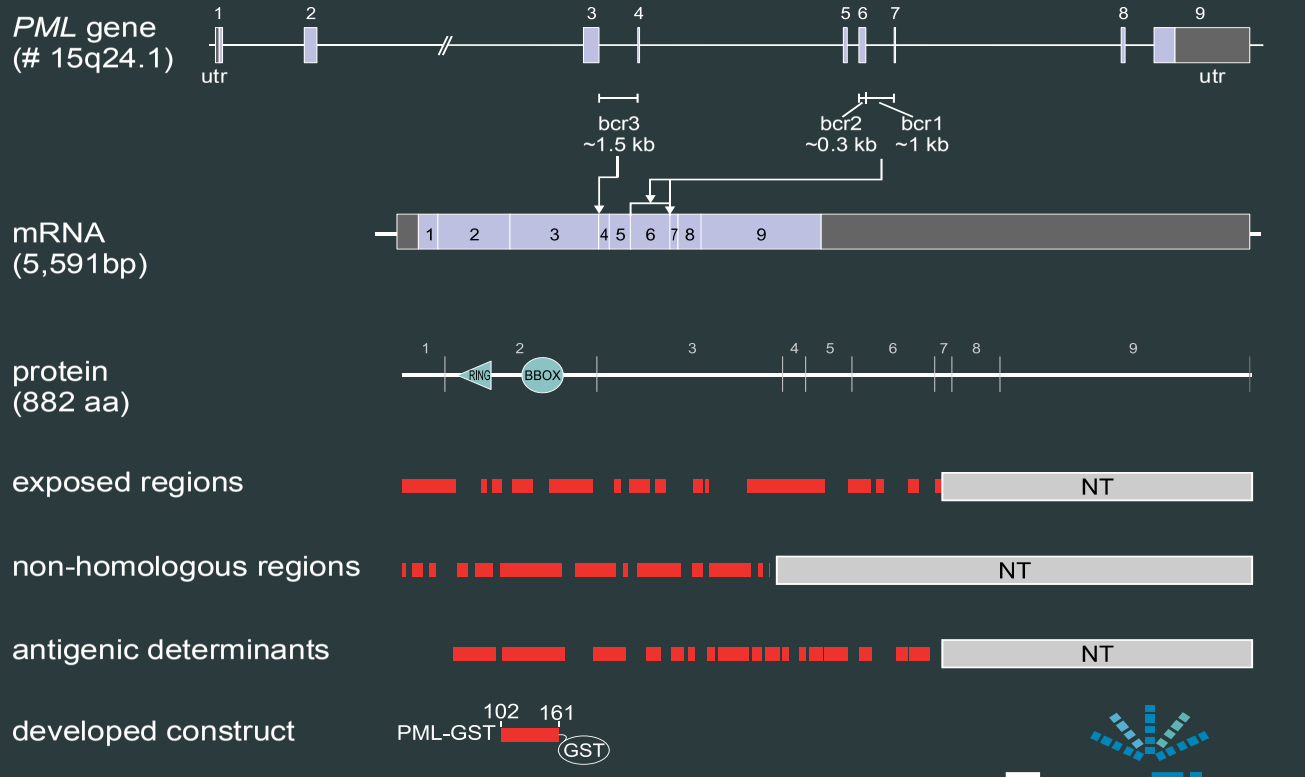
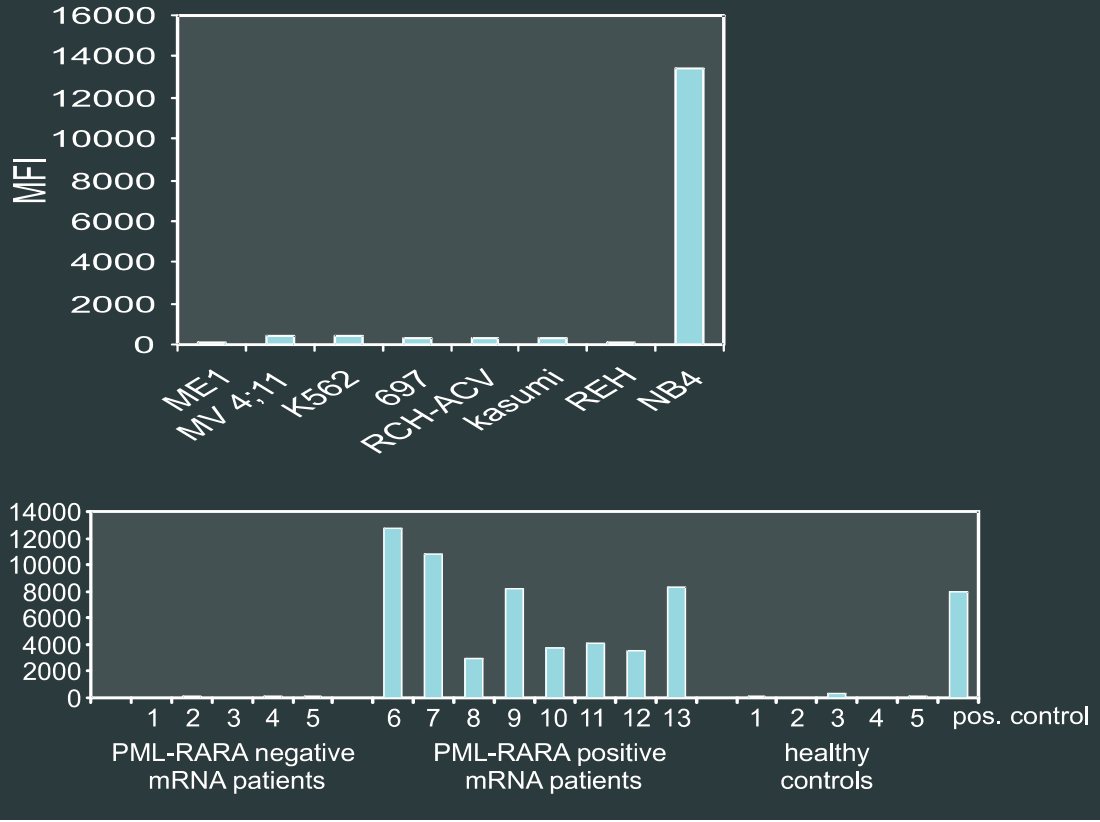
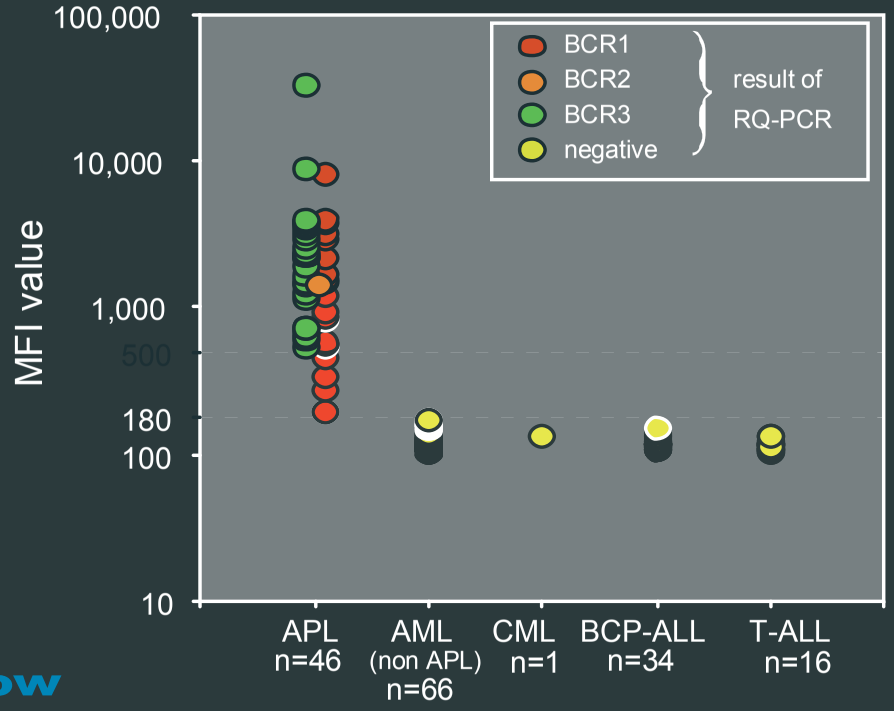
Design of anti-*PML* antibodies for fusion protein beads



Flow cytometric PML-RARA immunobead assay

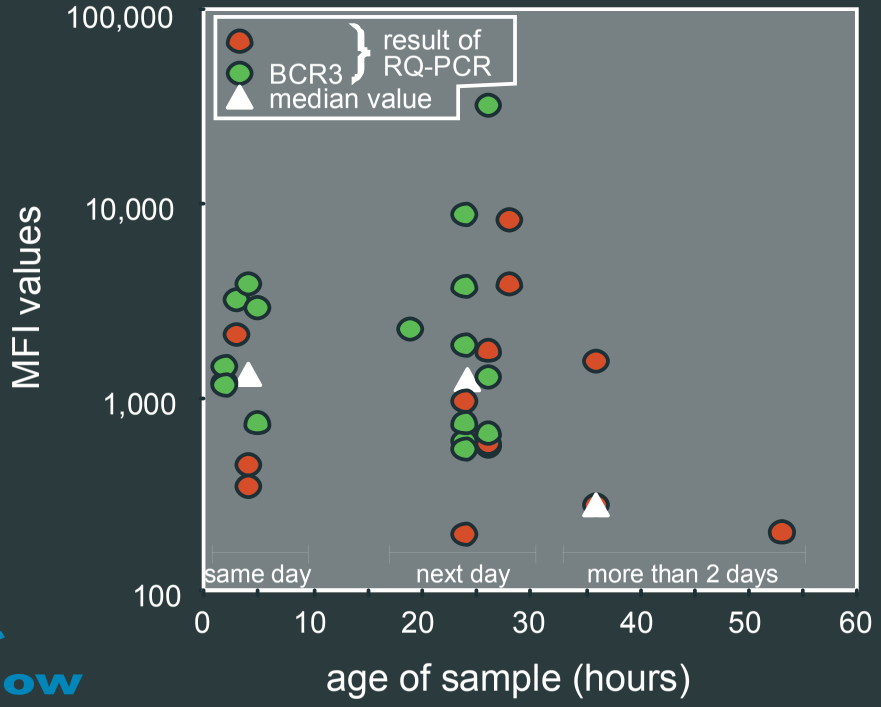


Results of PML-RARA fusion protein detection using the immunobead assay



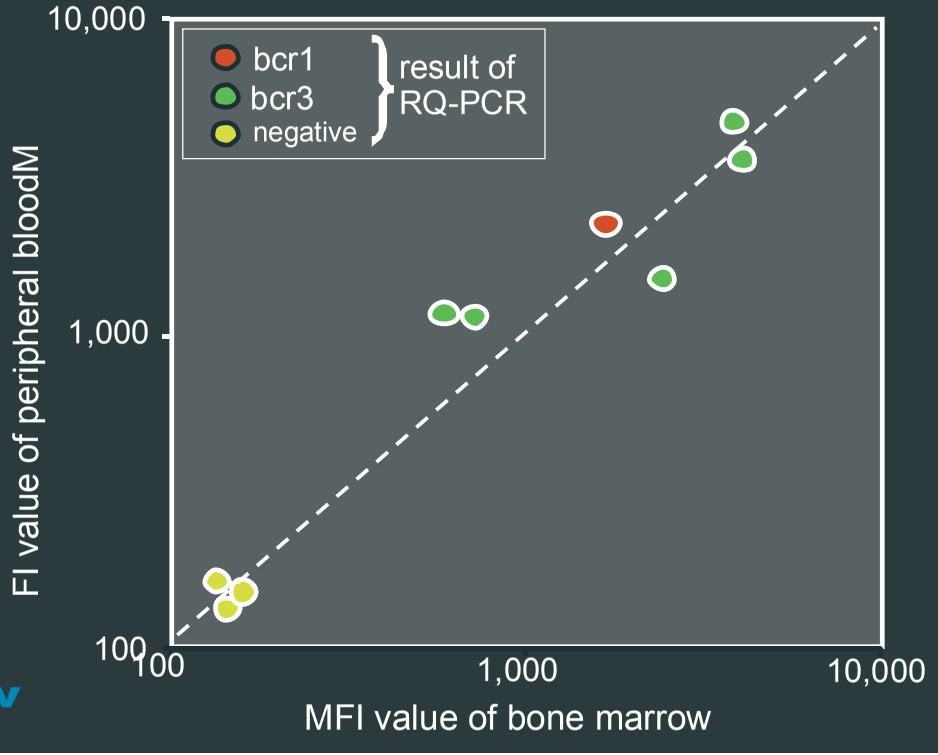
EuroFlow

Level of PML-RARA fusion protein expression in APL versus time lapse in sample processing



EuroFlow

Comparison of PML-RARA fusion protein levels between paired BM and PB samples of 9 acute leukemias at diagnosis



EuroFlow

At this moment the technical developments for 7 well-defined fusion proteins have (virtually) been completed

• CML	–	BCR-ABL	: completed	RUO kit launched and published
• precursor-B-ALL	–	TEL-AML	: completed	Precursor-B-ALL tube Multiplex tube: prototype completed
	–	E2A-PBX1	: completed	
	–	MLL-AF4	: completed	
• AML	–	AML1-ETO	: completed	Core-Binding-Factor tube Multiplex tube: prototype completed
	–	CBFB-MYH11:	completed	
AML/APL	–	PML-RARA	: completed	prototype testing completed

Erasmus MC
Universitair Medisch Centrum Rotterdam

dynomics

BD

EuroFlow

From invention through development and production to final diagnostic testing

Advantages of CBA system, when compared to classical molecular techniques for fusion gene detection

- Easy and reliable technique for fusion protein detection
- Independent of breakpoint position in the involved genes
- Multiplex possibilities by use of differential labeling of beads
- Fast technique: provides results within several hours
- No need for special laboratory facilities (only routine flow cytometer)
- Can be run in parallel with standard immunophenotyping (saves technician time!)
- The danger of protease activity requires integrity checking via an ubiquitous (protease sensitive) “household protein”

Conclusion: The CBA technique can contribute to fast and easy diagnosis and classification of leukemias and other malignancies.
If sufficient sensitivity is reached, MRD diagnostics becomes possible as well.

EuroFlow

EuroFlow is an independent scientific consortium, which aims at innovation in flow cytometry for improvement of diagnostic patient care

Chairmen:
J.J.M. van Dongen & A. Orfao

20 laboratories in 14 countries

www.euroflow.org

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Профессор J.J.M. van Dongen

«Консорциум EuroFlow и его достижения.

Новая концепция диагностической проточной цитометрии»

Russian flow cytometry conference, St Petersburg, Russia
17-19 March 2011

EuroFlow Consortium and its achievements

Novel concept in diagnostic flow cytometry

Jacques J.M. van Dongen
on behalf of

EuroFlow

Diagnostics for hematological malignancies

EuroFlow

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)

Flow cytometric immunophenotyping of normal and malignant leukocytes
Gaps and areas for improvement (Status in 2005)

- no technical standardization in flow cytometry
- no guidelines for selection of the appropriate antibody clones
- virtually no new markers introduced over a decade (stand-still in development)
- many oncoproteins (including fusion proteins) not yet included in immunostaining protocols;
- 3- and 4-color flow cytometry has many limitations: limited sensitivity and limited specificity
- management of large data files from multiple samples is complex and time-consuming; new software needed for:
  - fast and easy analysis of data;
  - automated patient reports;
  - introduction of flow data into electronic hospital systems.

→ International collaboration between academia and industry

Russian flow cytometry conference,
St Petersburg, Russia,17-19 March 2011

EuroFlow

Achievements of the EuroFlow Consortium

Multicolor flow cytometry (≥8 colors) with full technical standardization
- inclusion of violet laser and selection of appropriate fluorochromes
- standardization of instrument settings and laboratory protocols
- detailed testing and comparison of antibody clones and conjugated antibodies (multiple companies)

Implementation and development of novel software
- fast and easy handling of large data files (including automated pattern recognition)
- combining multiple tubes: calculation and APS view
- mapping of diagnosis and follow-up leukemia samples against templates of "normal/control" samples

Development of 8-color antibody protocols for diagnosis, classification and monitoring of hematological malignancies
- screening tubes (include recognition of normal leukocyte subsets)
- multi-tube panels for diagnosis and classification per disease category
- special tubes for MRD monitoring per disease category

Standardization in diagnostic flow cytometry

Standardization according to literature generally refers to:

- lists of CD codes and markers per disease category
- rarely a specific antibody is recommended and (almost) never a fluorochrome is proposed

HOWEVER: Standardization according to GLP guidelines demands for much higher levels of standardization

EuroFlow standardization aims at:

- usage of comparable flow cytometers (3 lasers and ≥ 8 colors)
- full standardization of instrument settings (e.g. based on standard beads)
- standardized laboratory protocols and immunostaining procedures (SOP's)
- careful selection of optimal antibody clones per marker/CD code
- selection of optimal 8-color antibody combinations and fluorochromes
- design of combinations of multiple 8-color tubes: estimation and APS view
- new software for fast and easy data analysis with automated pattern recognition
- recognition of normal and abnormal leukocyte subsets (complete differentiation pathways) with the same immunostaining protocols
- mapping of new patient samples against large data base of earlier collected patient samples, analyzed with the same immunostaining protocol

EuroFlow

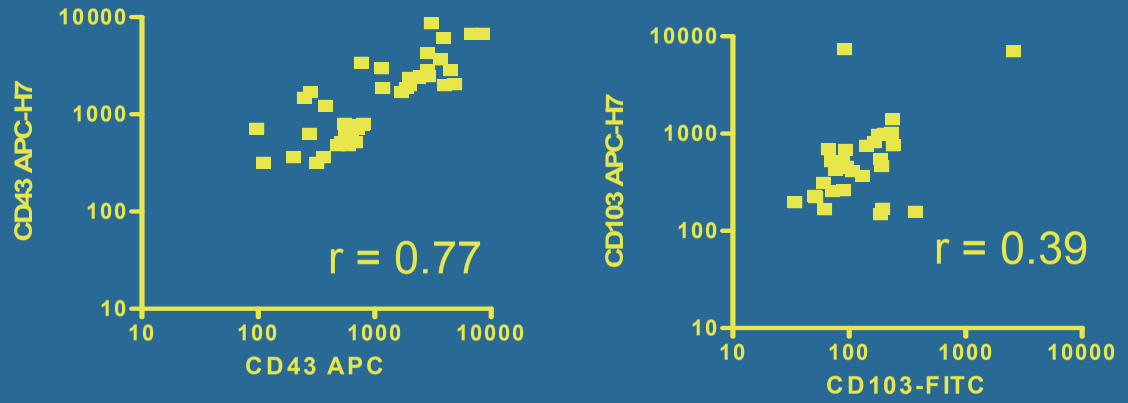
Fluorochromes for 8-color flow cytometric immunophenotyping

Fluorochrome	Excitation Peak (nm)	Emission Peak (nm)	Lasers		
			Violet	Argon	Helium-Neon
Pacific Blue/Horizon	405	455	+		
AmCyan	405	490	+		
Pacific Orange/Horizon	405	550	+		
Marina Blue	365	460		+	
FITC	495	520		+	
Phycoerythrin (PE)	565	575		+	
PE Texas Red	565	615		+	
PerCP	488	678		+	
PerCP-Cy5.5	488	695		+	
PE-Cy7	565	770		+	
Allophycocyanin (APC)	650	660			+
Alexa 700	635	720			+
APC-H7	650	770			+

Construction of EuroFlow panels

Fluorochrome conjugates, antibody panels, and antibody combinations

Fluorochrome‘s choice

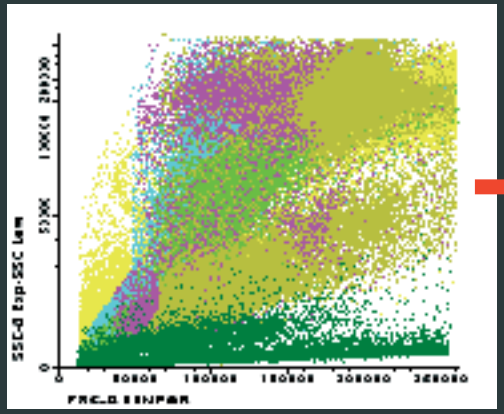


Responsible scientist: Sebastian Bottcher

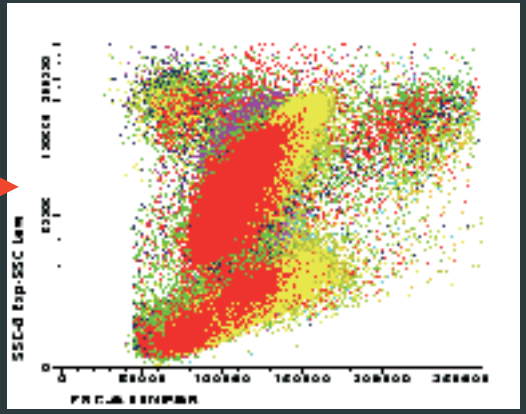
EuroFlow

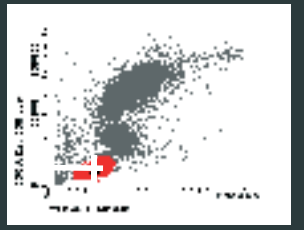
Synchronized light scatter experiments

"Local" settings



EuroFlow settings





7 different normal PB samples acquired in 7 different centers

Normal PB samples processed according to
the standardized EuroFlow sample preparation protocol

EuroFlow

5th EuroFlow Educational Workshop, Paris, FR, 9 March 2011

EuroFlow

Achievements of the EuroFlow Consortium

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Immunophenotypic classification & identification of LAIP

EuroFlow

TdT+ / CD19+ / CD38+

Tube

Tube

Tube

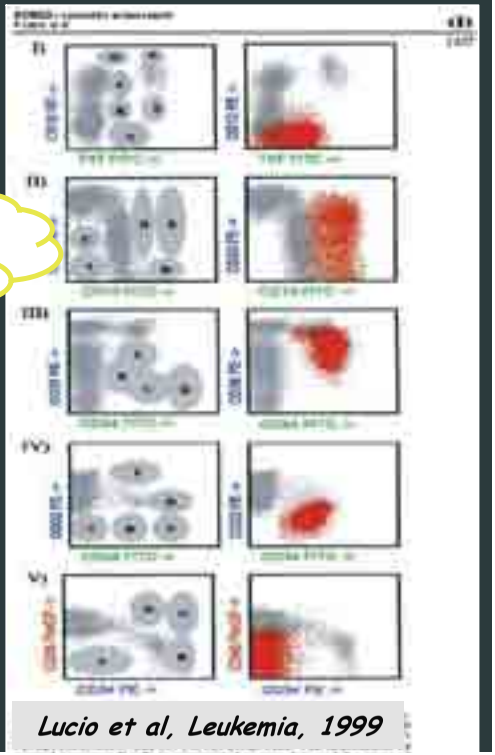
Precursor -B-ALL

LAIP:

CD45⁻ TdT⁺

alyse (2D)

Analyse (2D)



Lucio et al, Leukemia, 1999

JJM van Dongen Department of Immunology, Erasmus MC

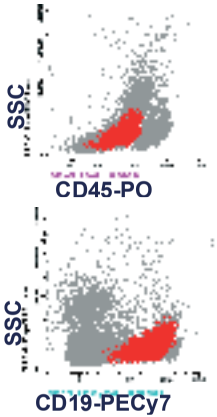
IMMUNOPHENOTYPIC CHARACTERISTICS OF NORMAL vs LEUKEMIC B-CELLS

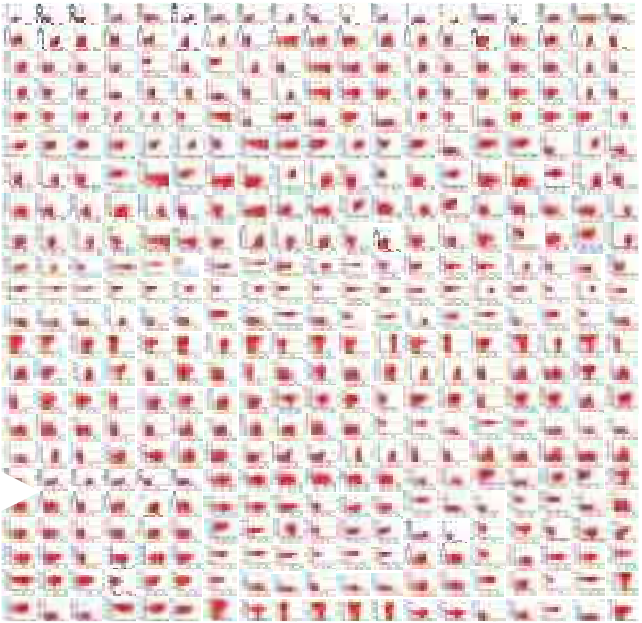
EuroFlow

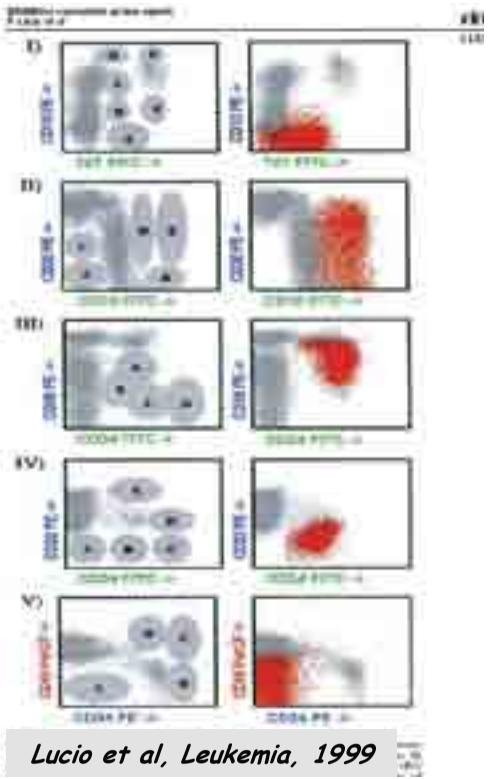
CD19+
B-CELLS

8- COLOR flow cytometry: Bcp-ALL

EuroFlow panel (450 bivariate plots)

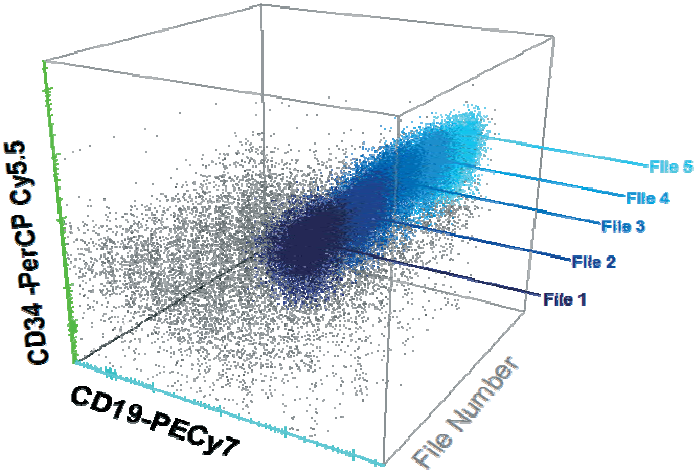




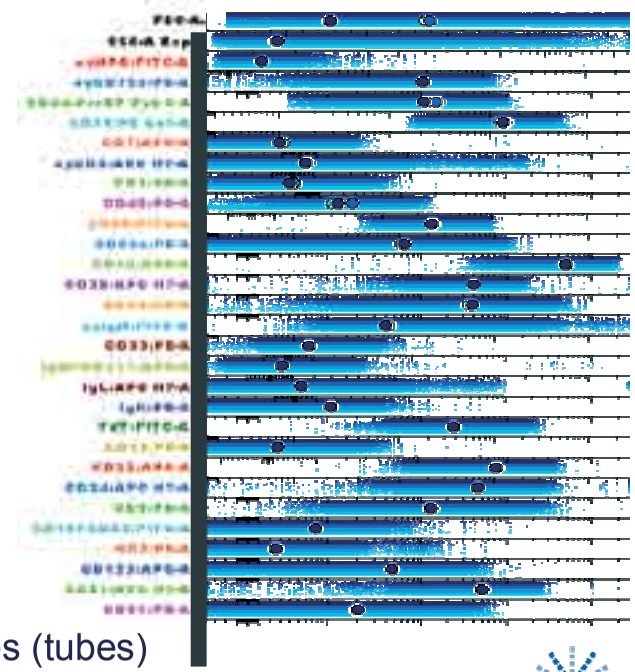


Lucio et al, Leukemia, 1999

MERGED DATA FILES FOR SINGLE STEP GATING



Full phenotypic profile



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

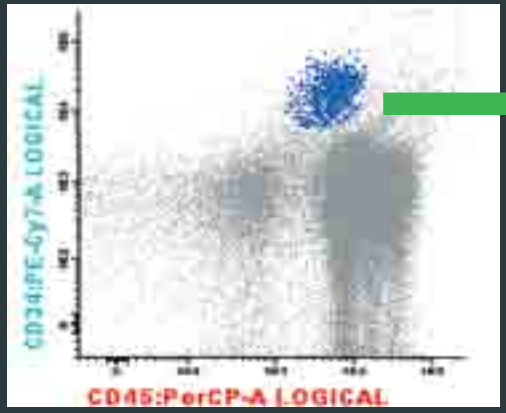
Responsible scientist: Marta Martin-Ayuso

EuroFlow

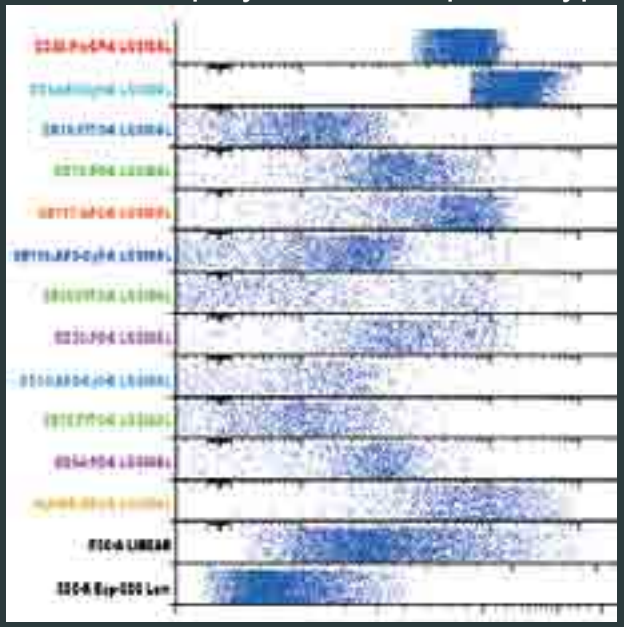
Merging of data files

EuroFlow

A single gating step for five different data files (merged)



Simultaneous display of immunophenotype



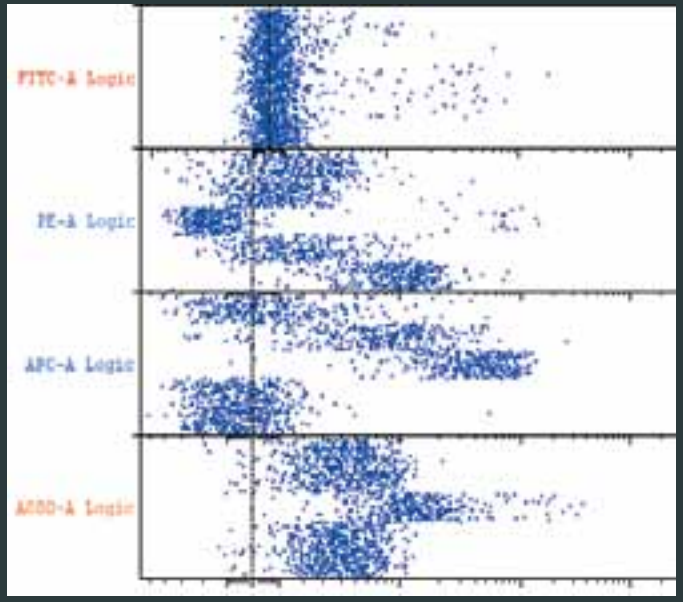
Responsible scientist: Marta Martin-Ayuso

Pedreira et al, Cytometry 2008;73:834

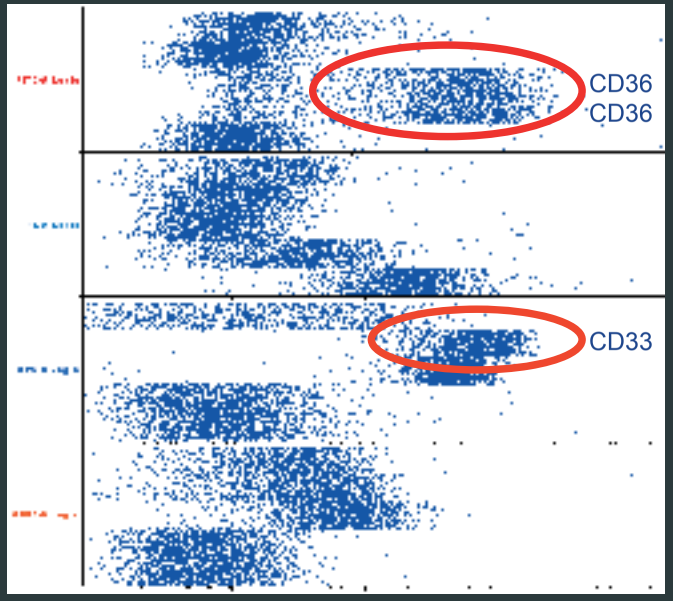
Multicolor analysis: “bar code”

EuroFlow

normal



MDS



Blast cells (CD34+/CD45+)

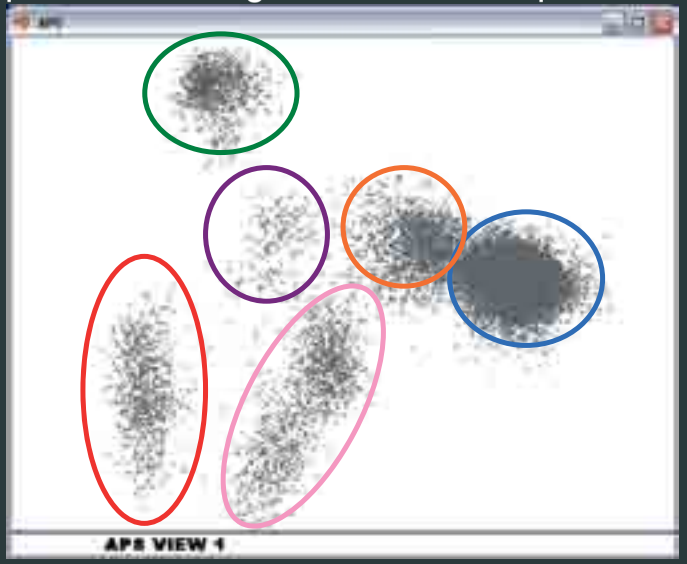
Responsible scientist: Vincent van der Velden

Automatic identification of populations

EuroFlow

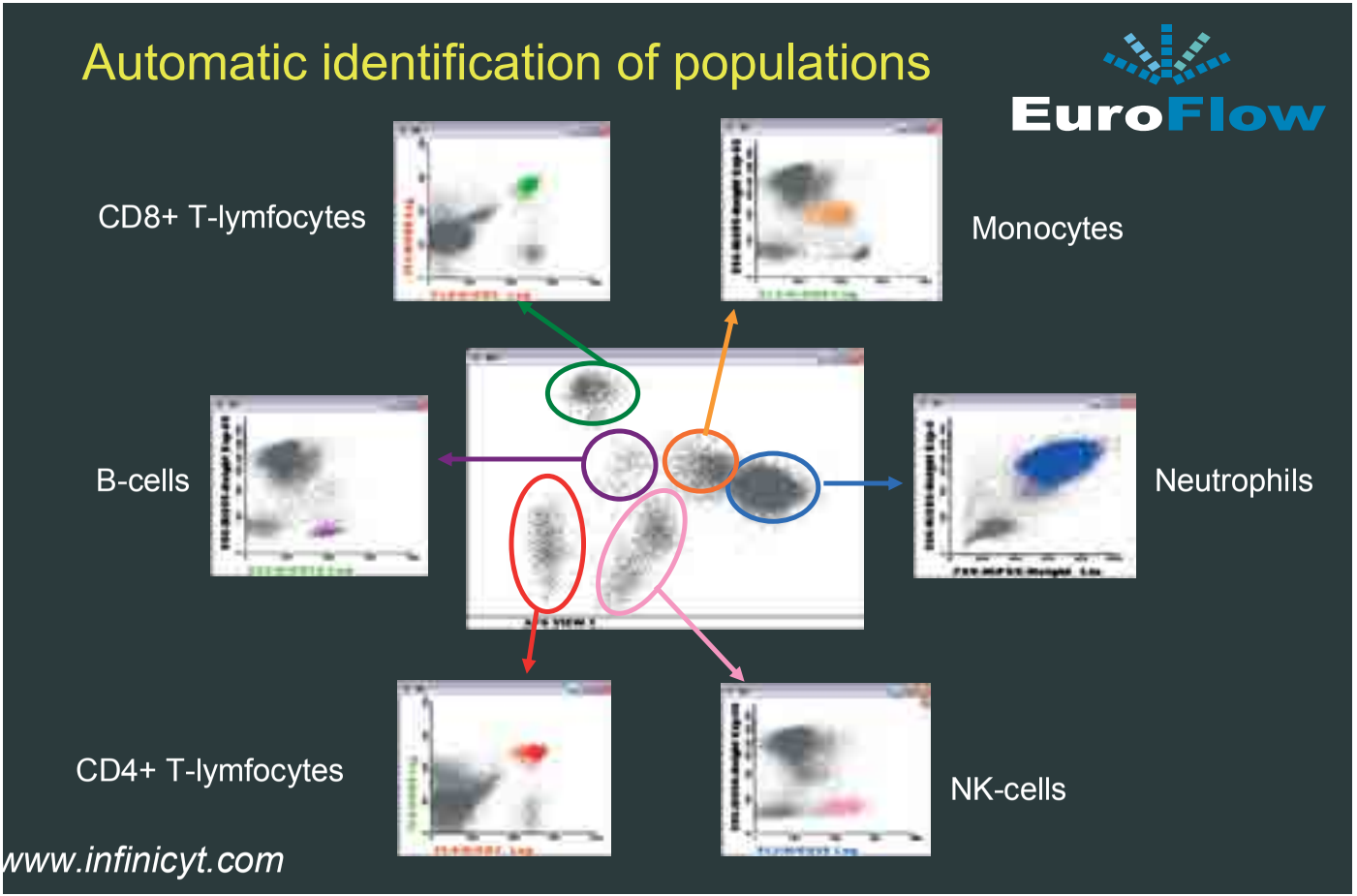
Multidimensional analysis:

Automated Separation among different cell Populations (APS view)



Automatic identification of populations

EuroFlow



Infinicyt

CYTOGNOS

APS Procedure for AUTOMATIC ANALYSIS

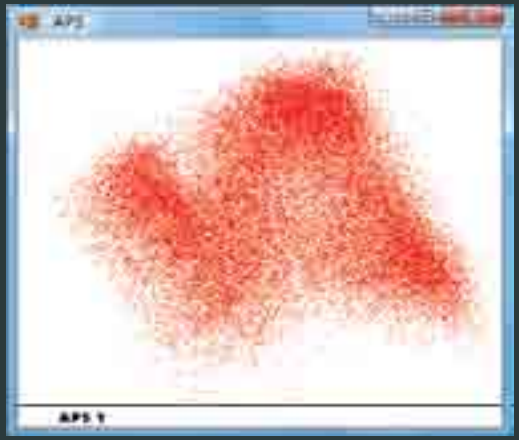
Visualization options

Dots **Dots / Mean** **Mean**

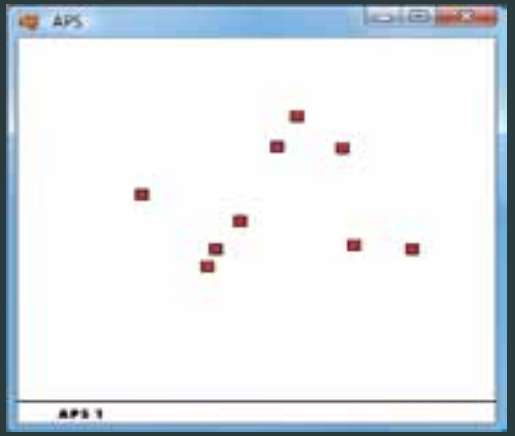
Infinicyt

SYTOGNOS

APS procedure for groups of patients



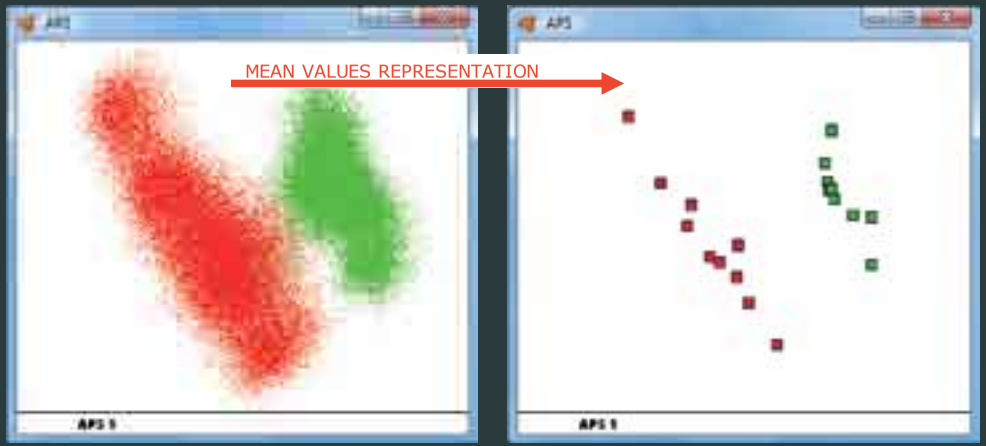
APS Dots View



APS Means View

Group of patients with the same panel/protocol applied and same disease category

APS procedure for groups of patients



Each dot

=

patient/sample

Group of patients with same panel/protocol applied and 2 different disease categories

Results of synchronized experiments

APS view of 30 merged data files from different centers

CD14+ Monocytes

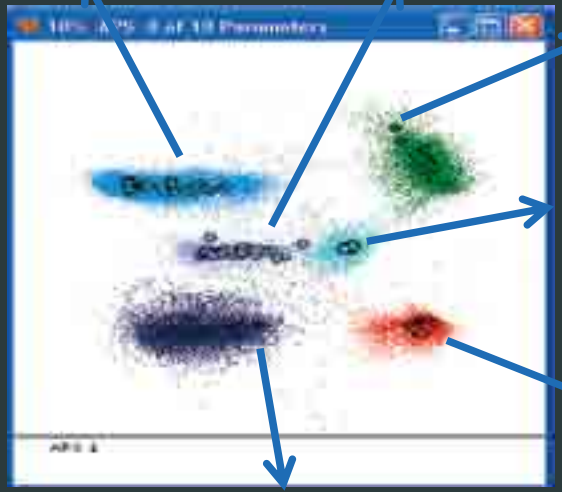
CD8+ CD3- T cells

CD3+ CD4+ T-cells

CD3+ CD8- CD4- T cells

CD3+ CD8+ T cells

CD19+ CD20+ B-cells



Local normal donors

CV MFI subset

PacBlue channel	Lym - CD20pos	16,90%
PerCp channel	Lym - CD45 pos	15,52%
FITC channel	Lym - CD3+ CD8pos	16,93%
PE channel	Lym - CD3+ CD27pos	27,95%
PerCPCy55 channel	Lym - CD3+ CD4pos	28,39%
PECy7 channel	Lym - CD19 pos	15,40%
APC channel	CD15pos - CD11 pos	22,58%
APC-H7 channel	Lym - CD3 pos	48,35%

EuroFlow

Responsible scientist: Tomas Kalina

Russian flow cytometry conference,
St Petersburg, Russia,17-19 March 2011

EuroFlow

Achievements of the EuroFlow Consortium

Multicolor flow cytometry (≥8 colors) with full technical standardization
- inclusion of violet laser and selection of appropriate fluorochromes
- standardization of instrument settings and laboratory protocols
- detailed testing and comparison of antibody clones and conjugated antibodies (multiple companies)

Implementation and development of novel software
- fast and easy handling of large data files (including automated pattern recognition)
- combining multiple tubes: calculation and APS view
- mapping of diagnosis and follow-up leukemia samples against templates of "normal/control" samples

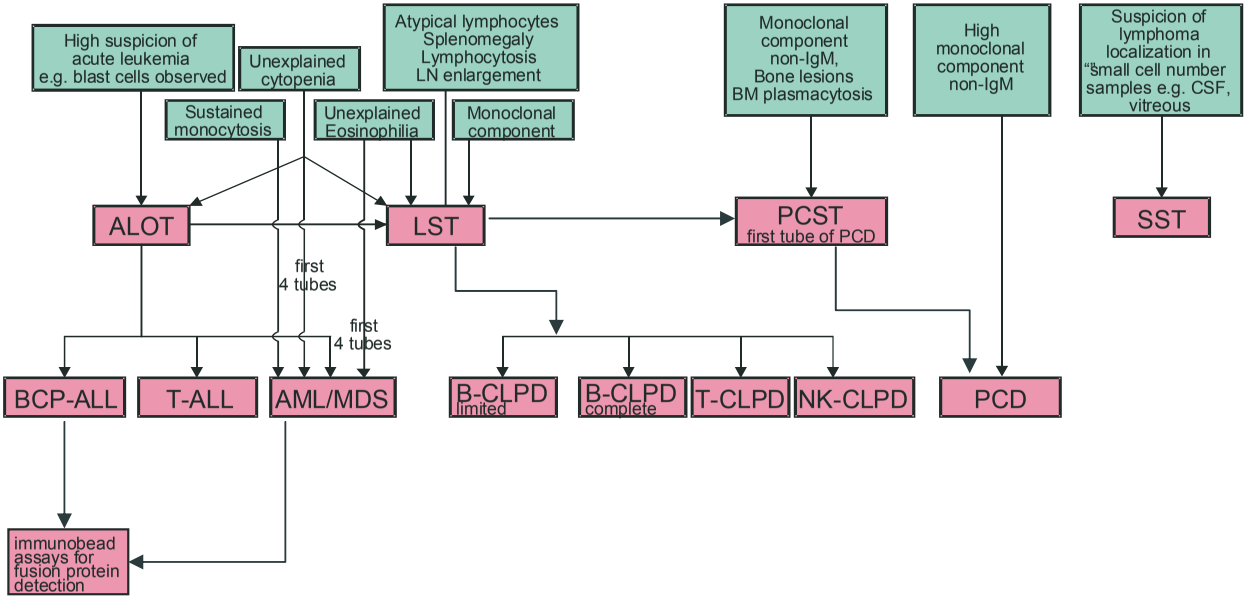
Development of 8-color antibody protocols for diagnosis, classification and monitoring of hematological malignancies
- screening tubes (include recognition of normal leukocyte subsets)
- multi-tube panels for diagnosis and classification per disease category
- special tubes for MRD monitoring per disease category

EuroFlow antibody protocols

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

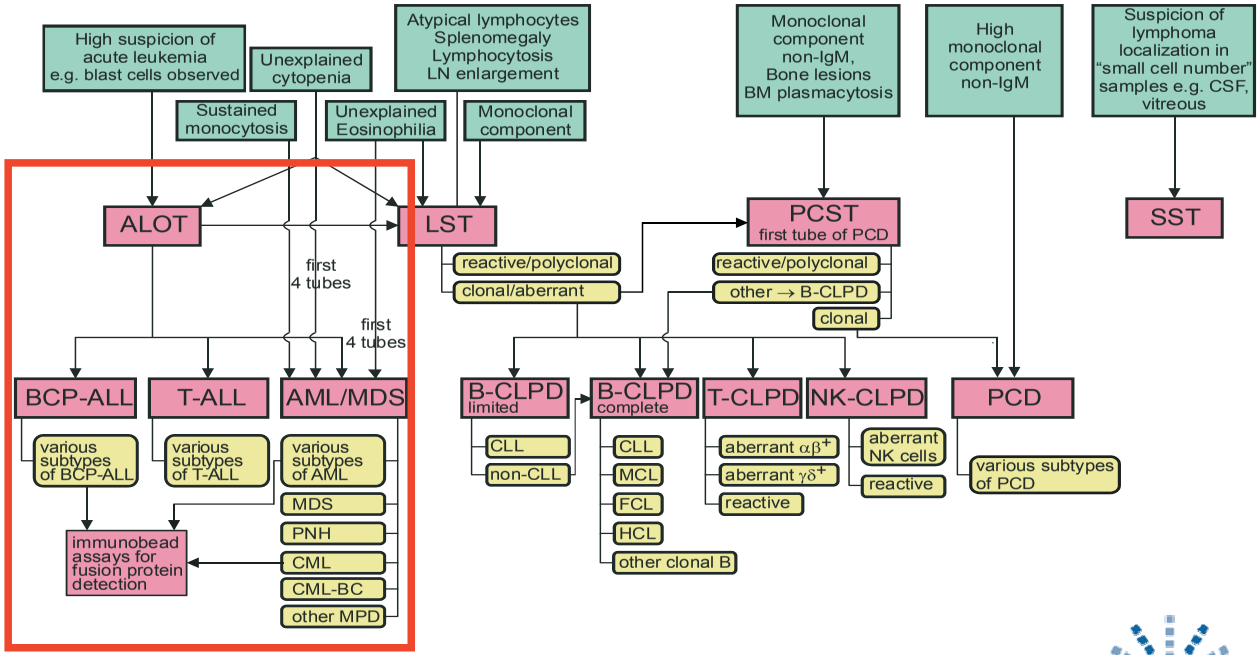
1. Screening tubes (include recognition of normal leukocyte subsets)
   - Acute leukemia orientation tube (ALOT): 1 tube (L Lhermitte)
   - Lymphoid screening tube (LST): 1 tube (J Flores Montero)
   - Small sample screening tube (SST): 1 tube (AW Langerak)
   - Plasma cell dyscrasia tubes (PCD): 2 tubes (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)

Algorithm for EuroFlow antibody panels in hemato-oncology



EuroFlow

Algorithm for EuroFlow antibody panels in hemato-oncology



EuroFlow

Single tube EuroFlow screening tube for acute leukemias

Acute Leukemia Orientation Tube (ALOT)*

Responsible scientist: L Lhermitte

Pacific Blue	Pacific Orange	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7
cyCD3	**CD45**	cyMPO	cyCD79a	**CD34**	**CD19**	CD7	**smCD3**

* Backbone markers are indicated in bold; cy= cytoplasmic; sm= surface membrane.

EuroFlow

Multi-tube EuroFlow classification panel for B-cell precursor ALL (BCP-ALL)*

Responsible scientist: L Lhermitte

Tube	Pacific Blue	Pacific Orange	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7	Aim**
1	CD20	**CD45**	CD58	CD66c	**CD34**	**CD19**	CD10	CD38	Diagnosis and classification of BCP-ALL; Detection of LAP markers; Detection of phenotypes associated with molecular aberrations
2	smIgκ	**CD45**	cylgμ	CD33	**CD34**	**CD19**	smIgμ and CD117	smIgλ	Diagnosis and classification of BCP-ALL;
3	CD9	**CD45**	nuTdT	CD13	**CD34**	**CD19**	CD22	CD24	Diagnosis and classification of BCP-ALL; Detection of phenotypes associated with molecular aberrations; Detection of LAP markers
4	CD21	**CD45**	CD15 and CDw65	NG2	**CD34**	**CD19**	CD123	CD81	Subclassification of BCP-ALL; Detection of LAP markers; Detection of phenotypes associated with molecular aberrations

* Backbone markers are indicated in bold; cy= cytoplasmic.

** The described marker combinations can also be applied for disease staging and monitoring of treatment effectiveness (MRD diagnostics).

EuroFlow

Multi-tube EuroFlow classification panel for T-ALL.*

Responsible scientist: V Asnafi

Tube	Pacific Blue	Pacific Orange	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7	Aim**
1	**cyCD3**	**CD45**	nuTdT	CD99	CD5	CD10	CD1a	**smCD3**	Diagnosis and classification of BCP-ALL; Detection of LAP markers; Detection of phenotypes associated with molecular aberrations
2	**cyCD3**	**CD45**	CD2	CD117	CD4	CD8	CD7	**smCD3**	Diagnosis and classification of BCP-ALL;
3	**cyCD3**	**CD45**	TCR$\gamma\delta$	TCR$\alpha\beta$	CD33	CD56	cyTCRβ	**smCD3**	Diagnosis and classification of BCP-ALL; Detection of phenotypes associated with molecular aberrations; Detection of LAP markers
4	**cyCD3**	**CD45**	CD45	CD13	HLADR	CD45RA	CD123	**smCD3**	Subclassification of BCP-ALL; Detection of LAP markers; Detection of phenotypes associated with molecular aberrations

* Backbone markers are indicated in bold; cy= cytoplasmic; sm= surface membrane; nu= nuclear.

** The described marker combinations can also be applied for disease staging and monitoring of treatment effectiveness (MRD diagnostics).

EuroFlow

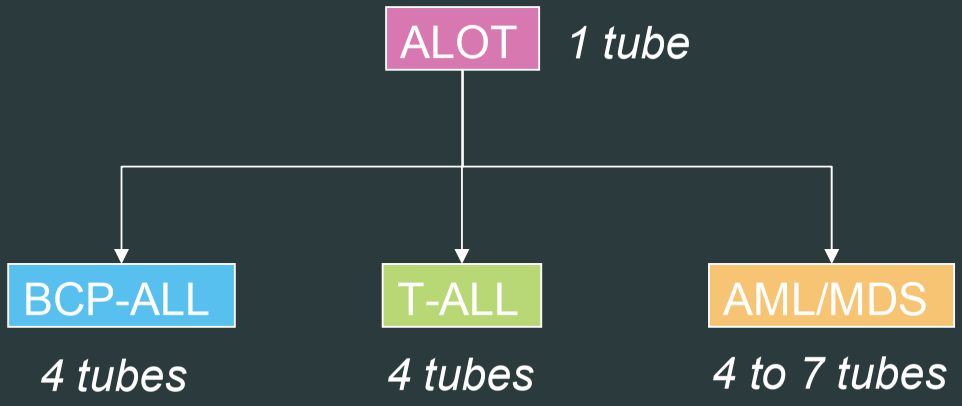
Multi-tube EuroFlow classification panel for AML/MDS

Responsible scientist: VHJ van der Velden

Tube	Pacific Blue	Pacific Orange	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7	Aim**
AML/ MDS									
1	**HLADR**	**CD45**	CD16	CD13	**CD34**	**CD117**	CD11b	CD10	Diagnosis and subclassification of AML and PNH especially focused on neutrophilic lineage
2	**HLADR**	**CD45**	CD35	CD64	**CD34**	**CD117**	IREM2	CD14	Diagnosis and subclassification of AML and PNH especially focussed on monocytic lineage
3	**HLADR**	**CD45**	CD36	CD105	**CD34**	**CD117**	CD33	CD71	Diagnosis and subclassification of AML especially focused on erythroid lineage
4	**HLADR**	**CD45**	nuTdT	CD56	**CD34**	**CD117**	CD7	CD19	Aberrant expression of lymphoid-associated markers and abnormal lymphoid maturation

* Further information about the markers and the availability of hybridoma clones is summarized in Appendix A. Backbone markers are indicated in bold; nu= nuclear.
** The described marker combinations might also be applied for disease staging and monitoring of treatment effectiveness (MRD diagnostics)

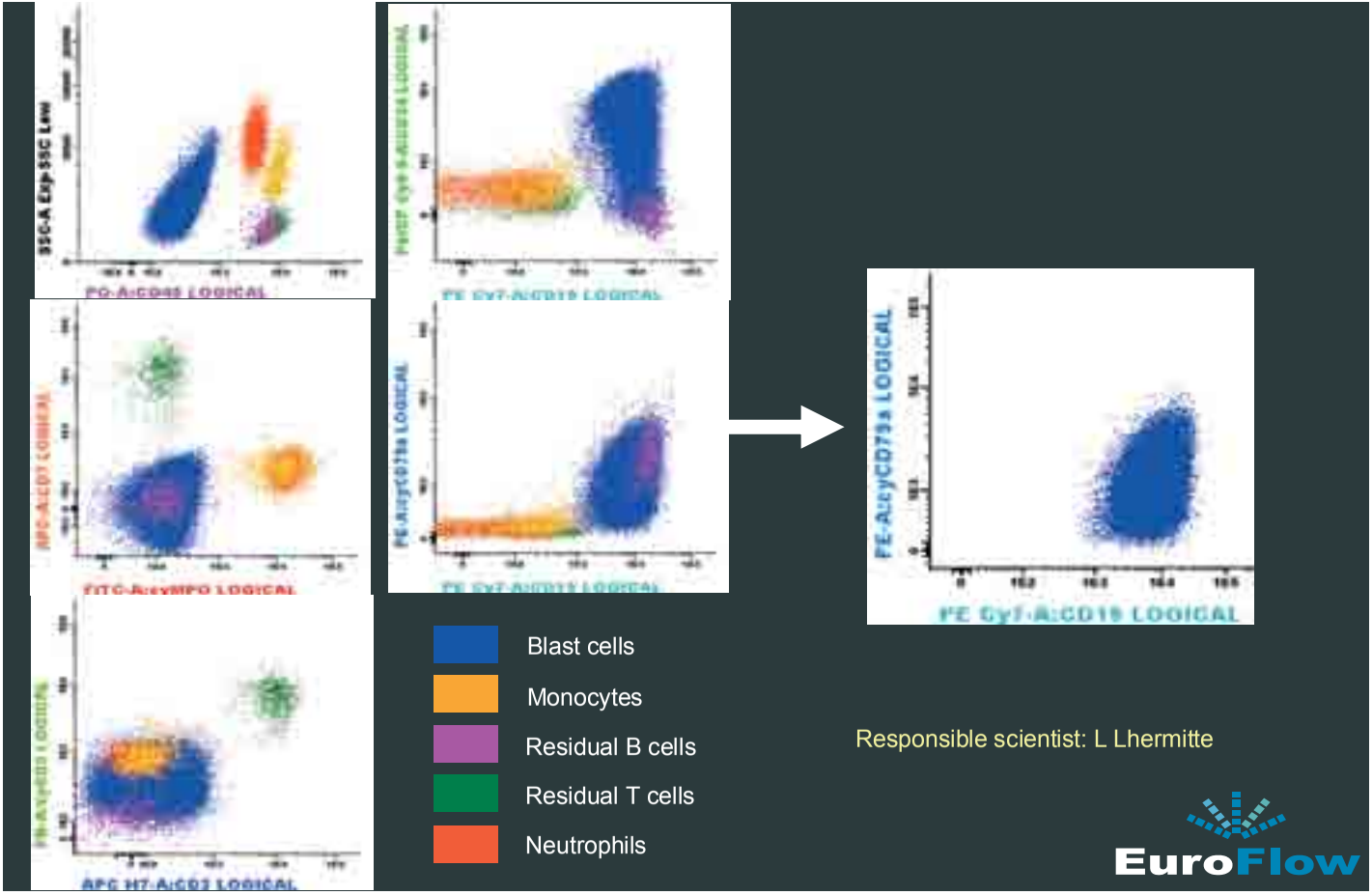
Acute leukemia orientation tube (ALOT)

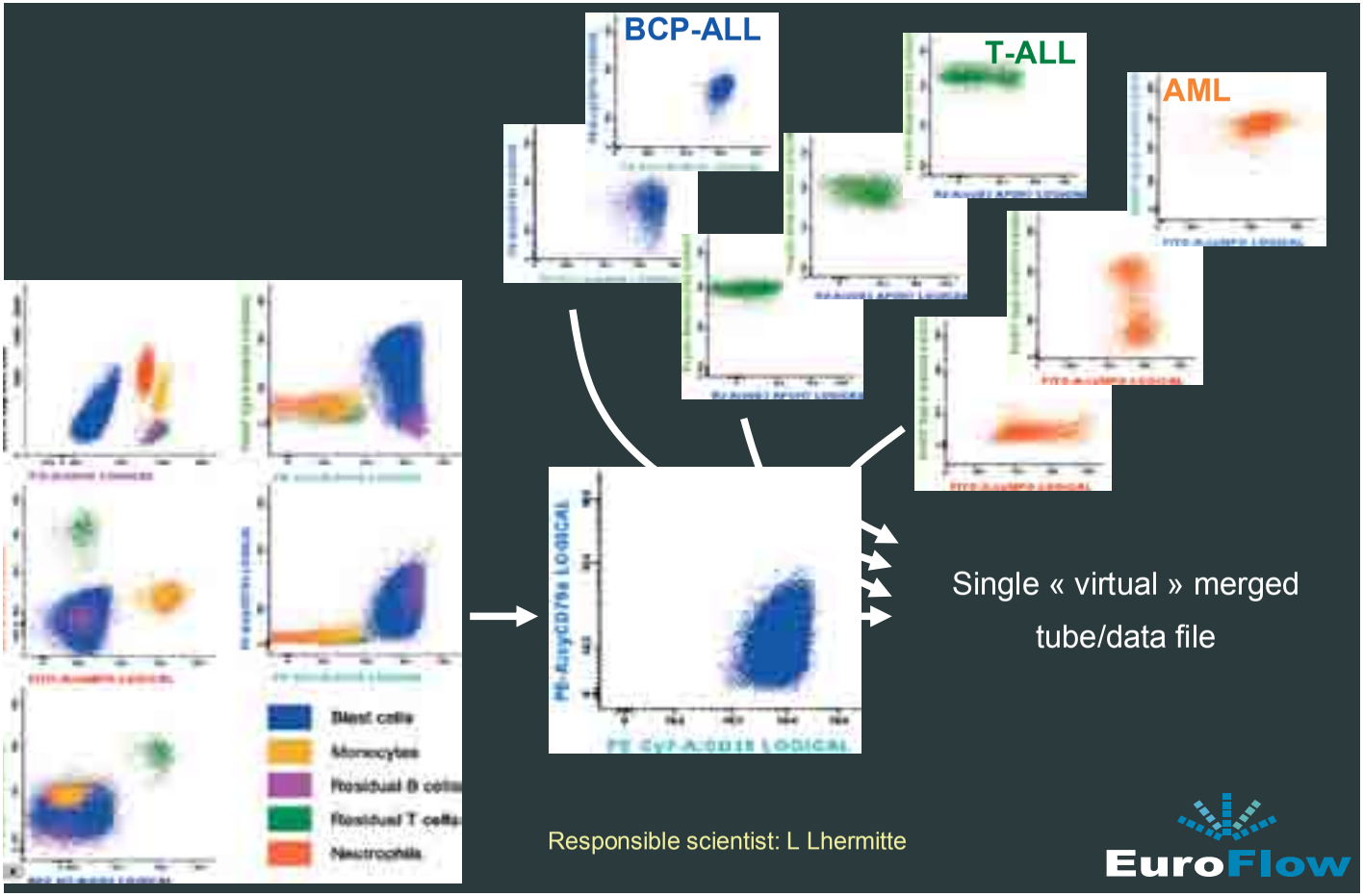


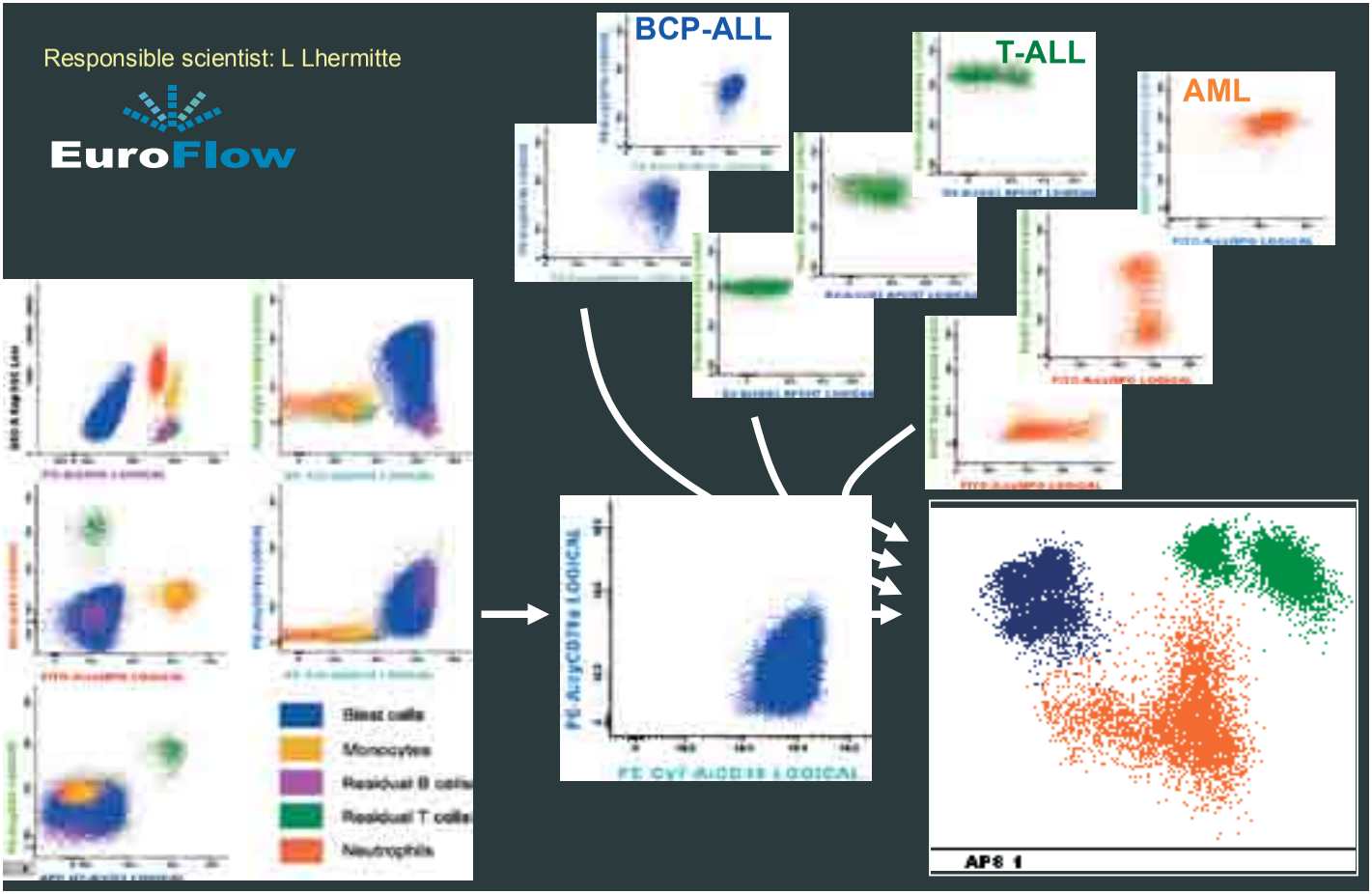
Pacific Blue	Pacific Orange	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7
cyCD3	**CD45**	cyMPO	cyCD79a	**CD34**	**CD19**	CD7	**smCD3**

EuroFlow

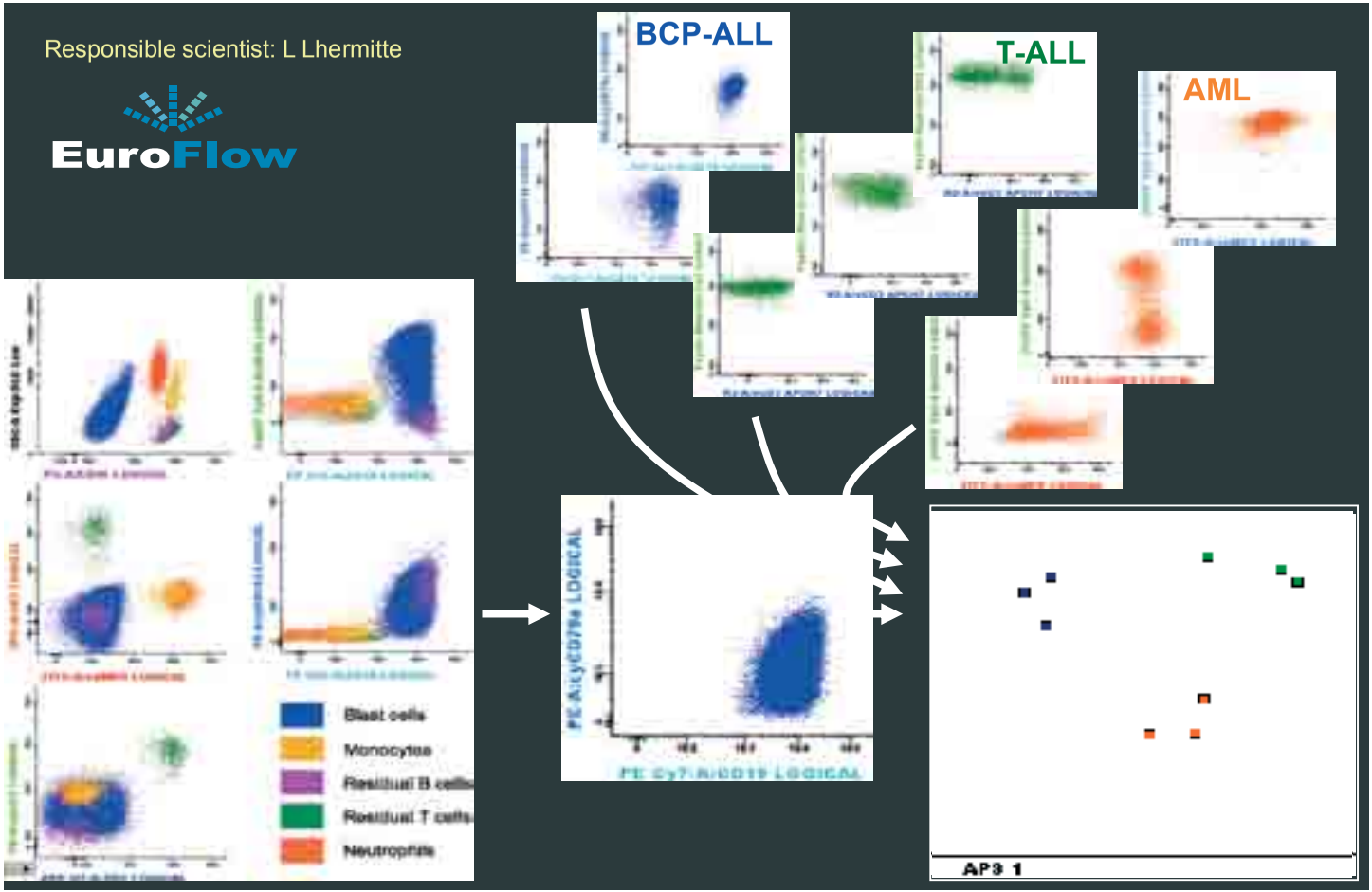
Responsible scientist: L Lhermitte

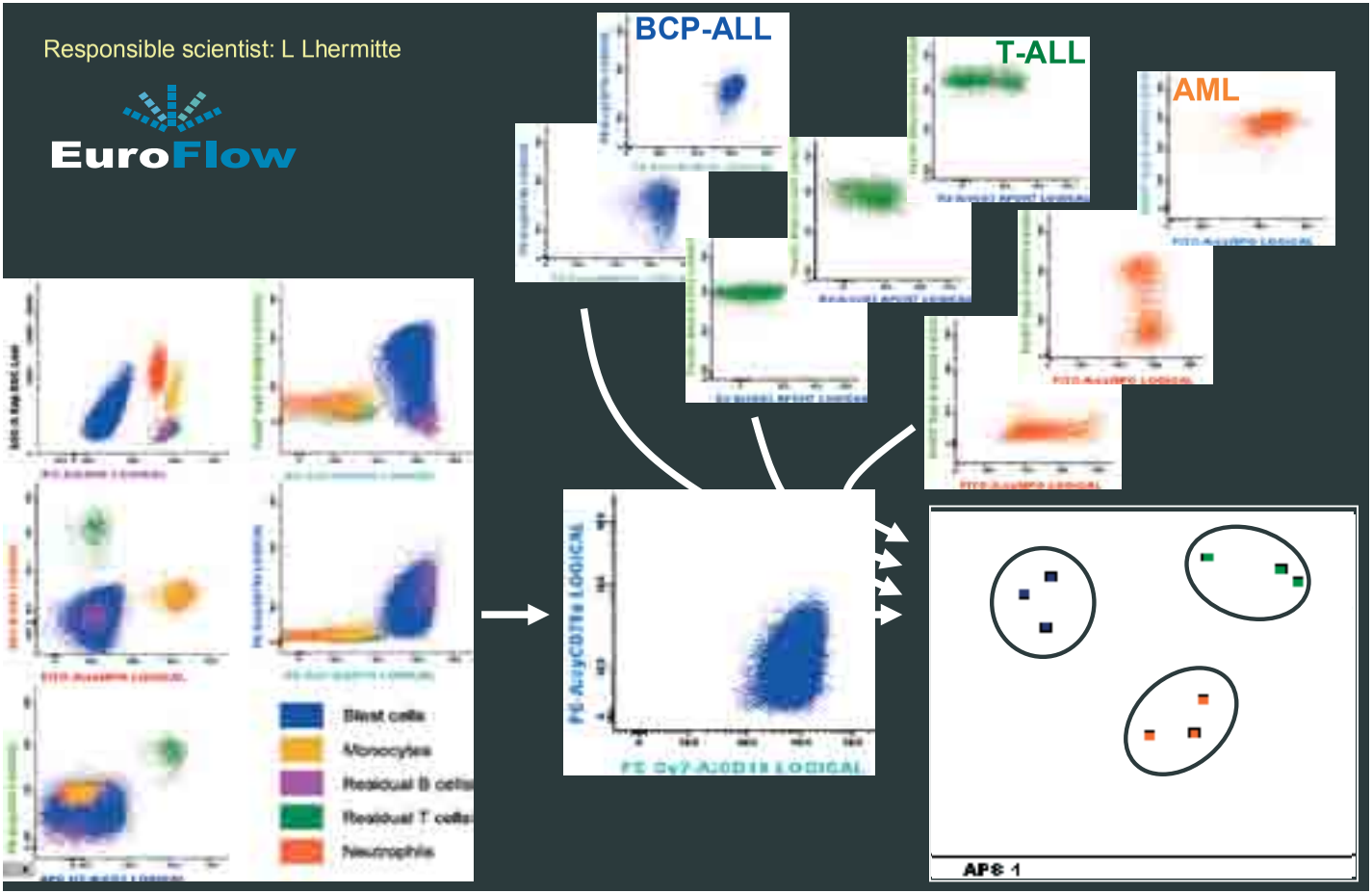

SSC-A Exp SSC Low
PO-A:CD45 LOGICAL
APC-A:CD7 LOGICAL
FITC-A:cyMPO LOGICAL
APC H7-A:CD3 LOGICAL
PE-A:cyCD79a LOGICAL
PE Cy7-A:CD19 LOGICAL
Blast cells
Monocytes
Residual B cells
Residual T cells
Neutrophils
Responsible scientist: L Lhermitte
EuroFlow


BCP-ALL
T-ALL
AML
Blast cells
Monocytes
Residual B cells
Residual T cells
Neutrophils
Single « virtual » merged tube/data file
Responsible scientist: L Lhermitte
EuroFlow

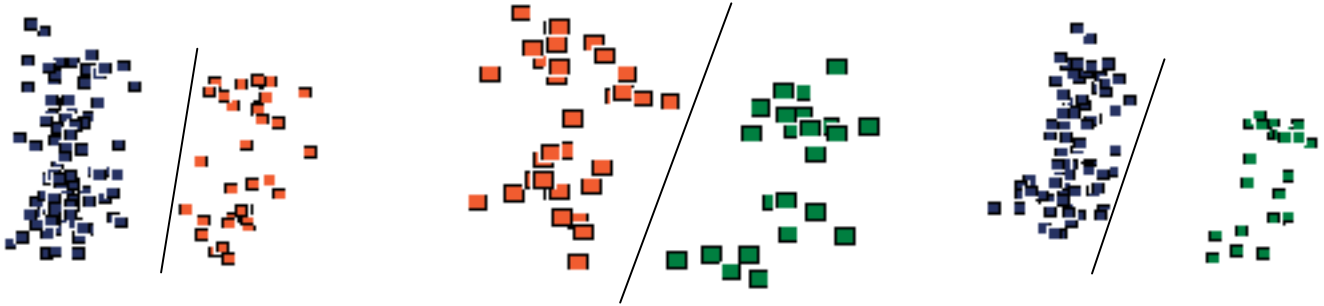

Responsible scientist: L Lhermitte
EuroFlow
BCP-ALL
T-ALL
AML
Blast cells
Monocytes
Residual B cells
Residual T cells
Neutrophils
AP8 1

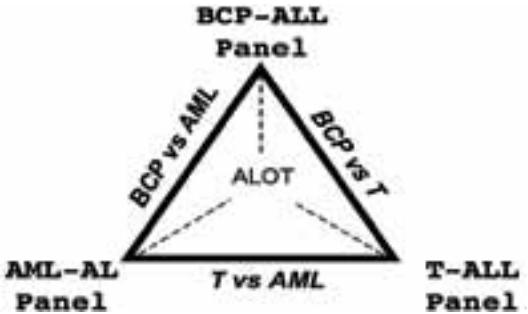
Responsible scientist: L Lhermitte




Responsible scientist: L Lhermitte
EuroFlow
BCP-ALL
T-ALL
AML
Blast cells
Monocytes
Residual B cells
Residual T cells
Neutrophils
APC-1

ALOT *(Acute Leukemia Orientation Tube)*





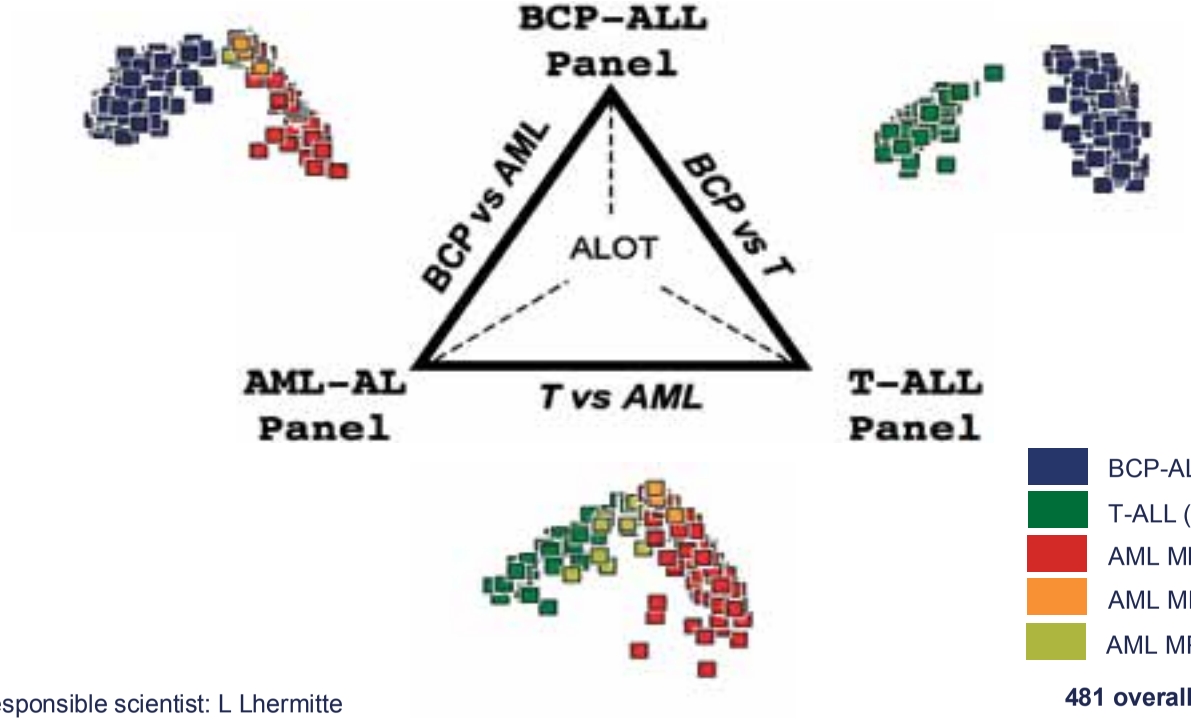
BCP-ALL cases (n=89)

T-ALL cases (n=27)

AML cases (n=36)

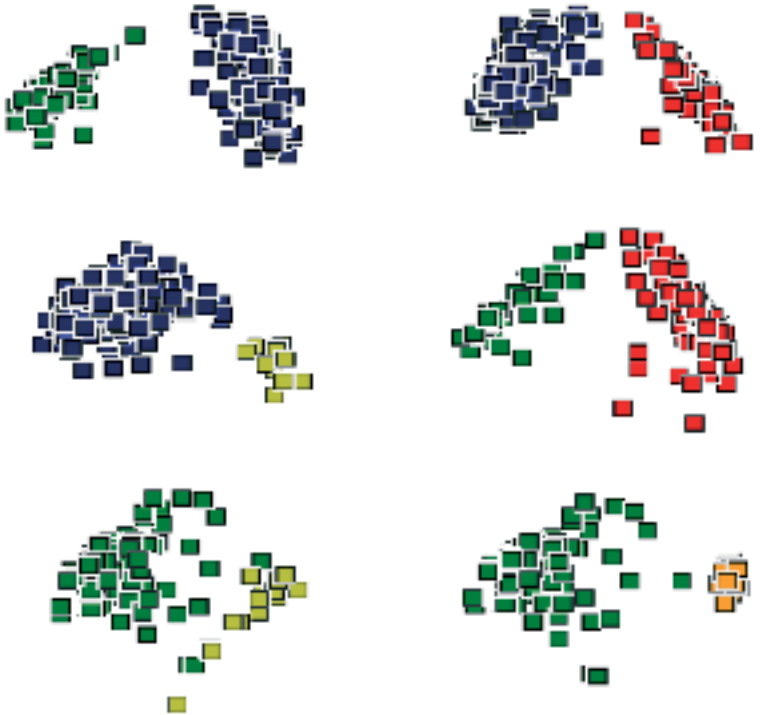
Responsible scientist: L Lhermitte

ALOT *(Acute Leukemia Orientation Tube)*



Responsible scientist: L Lhermitte

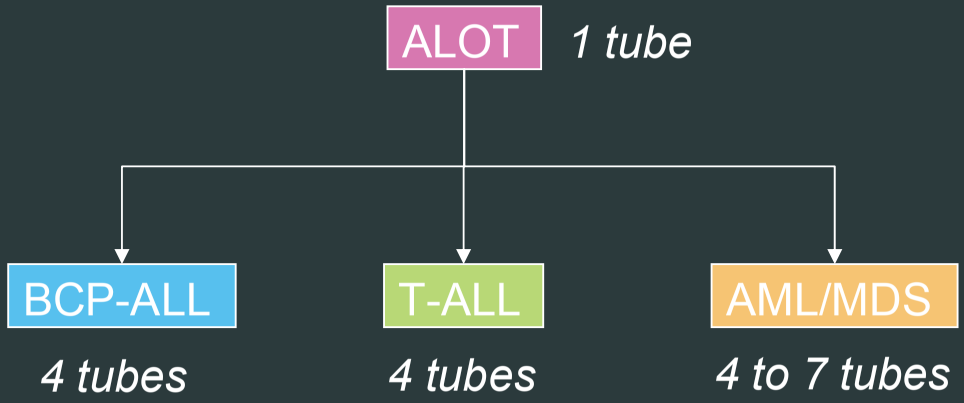
ALOT *(Acute Leukemia Orientation Tube)*



Responsible scientist: L Lhermitte

481 overall cases

Acute leukemia orientation tube (ALOT)



Pacific Blue	Pacific Orange	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7
cyCD3	**CD45**	cyMPO	cyCD79a	**CD34**	**CD19**	CD7	**smCD3**

EuroFlow

Responsible scientist: L Lhermitte

Multi-tube EuroFlow classification panel for B-cell precursor ALL (BCP-ALL)*

Responsible scientist: L Lhermitte

Tube	Pacific Blue	Pacific Orange	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7	Aim**
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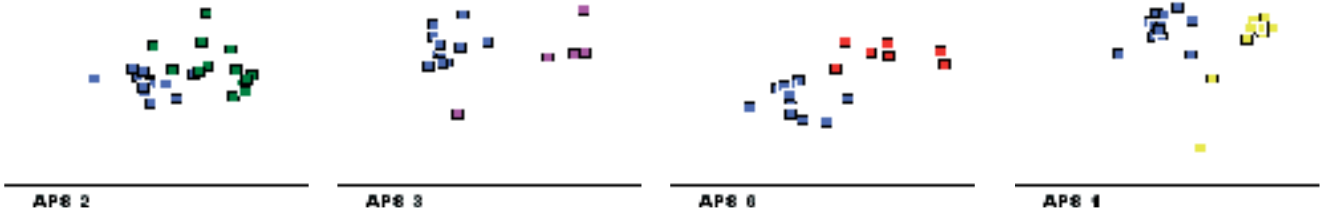
4	CD21	**CD45**	CD15 and CDw65	NG2	**CD34**	**CD19**	CD123	CD81	Subclassification of BCP-ALL; Detection of LAP markers; Detection of phenotypes associated with molecular aberrations

* Backbone markers are indicated in bold; cy= cytoplasmic.

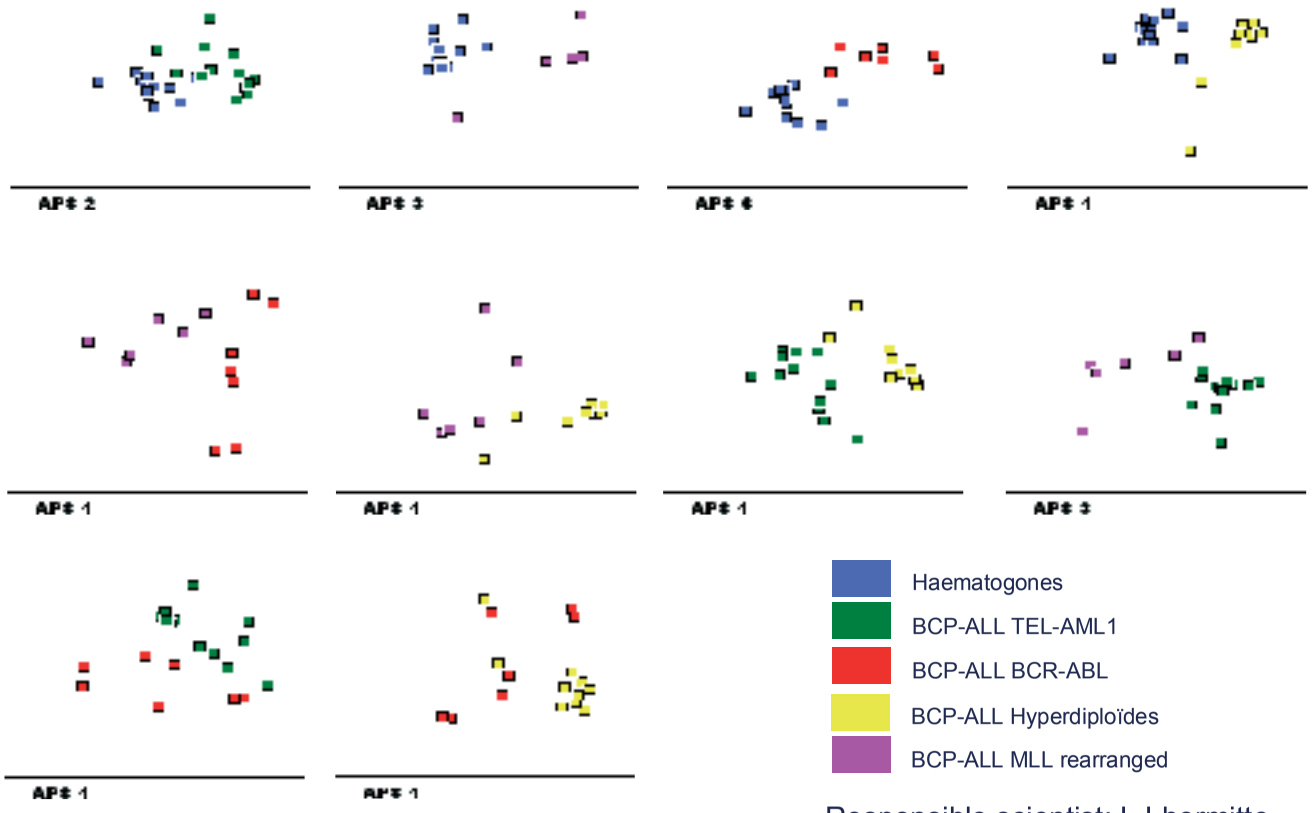
** The described marker combinations can also be applied for disease staging and monitoring of treatment effectiveness (MRD diagnostics).

EuroFlow



Haematogones
BCP-ALL TEL-AML1
BCP-ALL BCR-ABL
BCP-ALL Hyperdiploïdes
BCP-ALL MLL rearranged

Responsible scientist: L Lhermitte



Responsible scientist: L Lhermitte

Russian flow cytometry conference,
St Petersburg, Russia,17-19 March 2011

EuroFlow

Achievements of the EuroFlow Consortium

Multicolor flow cytometry (≥8 colors) with full technical standardization
- inclusion of violet laser and selection of appropriate fluorochromes
- standardization of instrument settings and laboratory protocols
- detailed testing and comparison of antibody clones and conjugated antibodies (multiple companies)

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- combining multiple tubes: calculation and APS view
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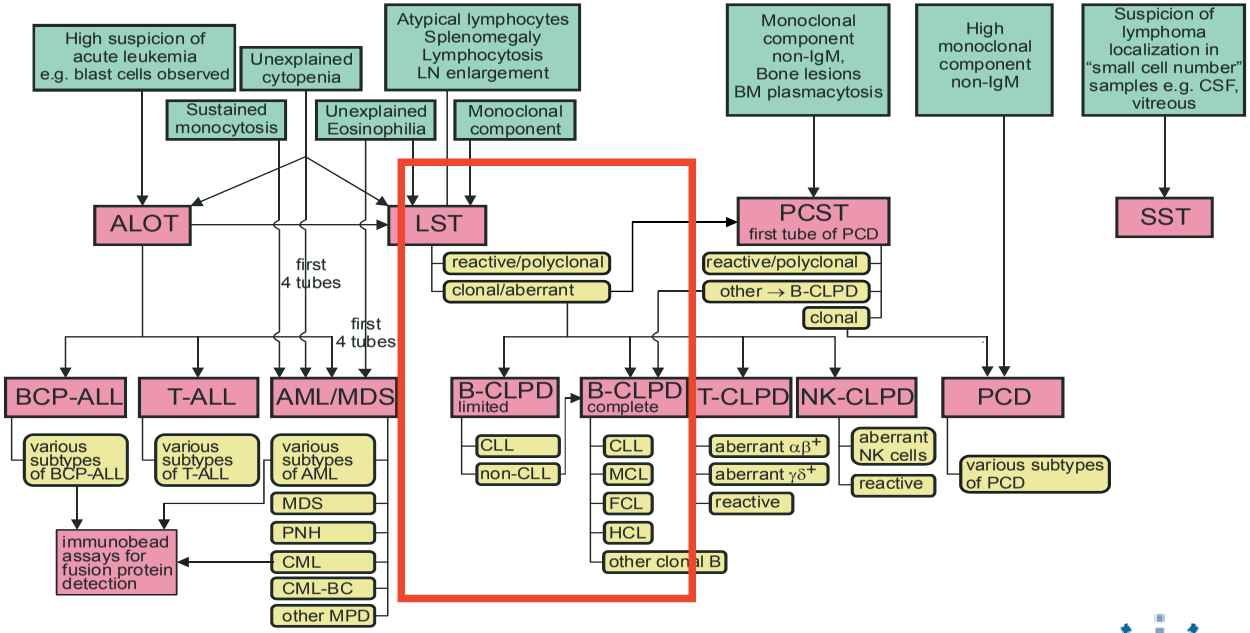
Development of 8-color antibody protocols for diagnosis, classification and monitoring of hematological malignancies
- screening tubes (include recognition of normal leukocyte subsets)
- multi-tube panels for diagnosis and classification per disease category
- special tubes for MRD monitoring per disease category

Algorithm for EuroFlow antibody panels in hemato-oncology



EuroFlow

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]

On behalf of the EuroFlow Consortium (EU-FP6, LSHB-CT-2006-018708)

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;

To be published in: Leukemia 2011; 25: xxxx-xxxx

EuroFlow

EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes

J.J.M. van Dongen, L. Lhermitte, S. Böttcher, J. Almeida, V.H.J. van der Velden, J. Flores-Montero, A. Rawstron, V.Asnafi, Q. Lécrevisse, P. Lucio, E. Mejstrikova, T. Szczepański, T. Kalina, R. de Tute, M. Brüggemann, L. Sedek, M. Cullen, A.W. Langerak, A. Mendonça, E. Macintyre, M. Martin-Ayuso,O. Hrusak, M.B. Vidriales, and A. Orfao

On behalf of the EuroFlow Consortium (EU-FP6, LSHB-CT-2006-018708)

1, Department of Immunology, Erasmus MC, Rotterdam, NL;
2, Department of Hematology, Hôpital Necker, University of Paris Descartes, AP-HP, Paris, FR
3, Medical Clinic II, University Medical Center Schleswig-Holstein,Campus Kiel, Kiel, DE;
4, Department of Medicine, Cancer Research Centre (IBMCC-CSIC-USAL) and Cytometry Service, University of Salamanca, Salamanca, ES;
5, St. James University Hospital, Leeds, UK;
6, Department of Hematology, Instituto Portugues de Oncologia , Lisbon, PT;
7, Department of Pediatric Hematology and Oncology, Charles University, Prague, CZ;
8, Department of Pediatric Hematology and Oncology, Medical University of Silesia, Zabrze, PL;
9, Cytognos SL, Salamanca, ES;
10,Department of Hematology, University Hospital, Salamanca, ES.

To be published in: Leukemia 2011; 25: xxxx-xxxx

EuroFlow

Achievement of the EuroFlow Consortium: New concept in diagnostic flow cytometry

EuroFlow

- Full technical standardization of multicolor flow cytometry (≥8 colors)
  - standardization of instrument settings and laboratory protocols
  - selection of fluorochromes and selection of antibody clones per marker
  - EuroFlow protocols work on all tested ≥8 colors flow cytometers:
    - DAKO Cyan, LSR-II, FACS Canto-II;
    - "late arrivals" (Navios and Gallios) still to be tested

- Implementation and further development of novel software: Infinicyt
  - fast and easy data handling with automated pattern recognition
  - combining multiple tubes: calculation and APS (principle component analysis
  - mapping of diagnosis and follow-up leukemia samples against templates of "normal/control" samples

- Development of 8-color antibody protocols for diagnosis, classification and monitoring of hematological malignancies
  - 8-color panels are based on recognition of normal cells & differentiation pathways
  - diagnosis and classification tubes are ready; MRD tubes in development
  - flexibility within panels: deletion and inclusion of markers and tubes is possible

- Large EuroFlow data base linked to Infinicyt software

European network for laboratory diagnostics

EuroClonality (BIOMED-2)

EuroClonality

EuroMRD
ALL, infant ALL and NHL

EuroMRD

EuroFlow

EuroFlow

Participants: based on experience and participation in (inter)national clinical trails

Aims:
- Innovation and standardization of laboratory diagnostics
- Quality control
- Continuous education

EuroFlow

EuroFlow is an independent scientific consortium,
which aims at innovation in flow cytometry
for improvement of diagnostic patient care

www.euroflow.org

Профессор J.J.M. van Dongen

«EuroFlow достижения и проблемы.
Новая концепция
проточно-цитометрического обнаружения
минимальной остаточной болезни при острых
лейкозах»

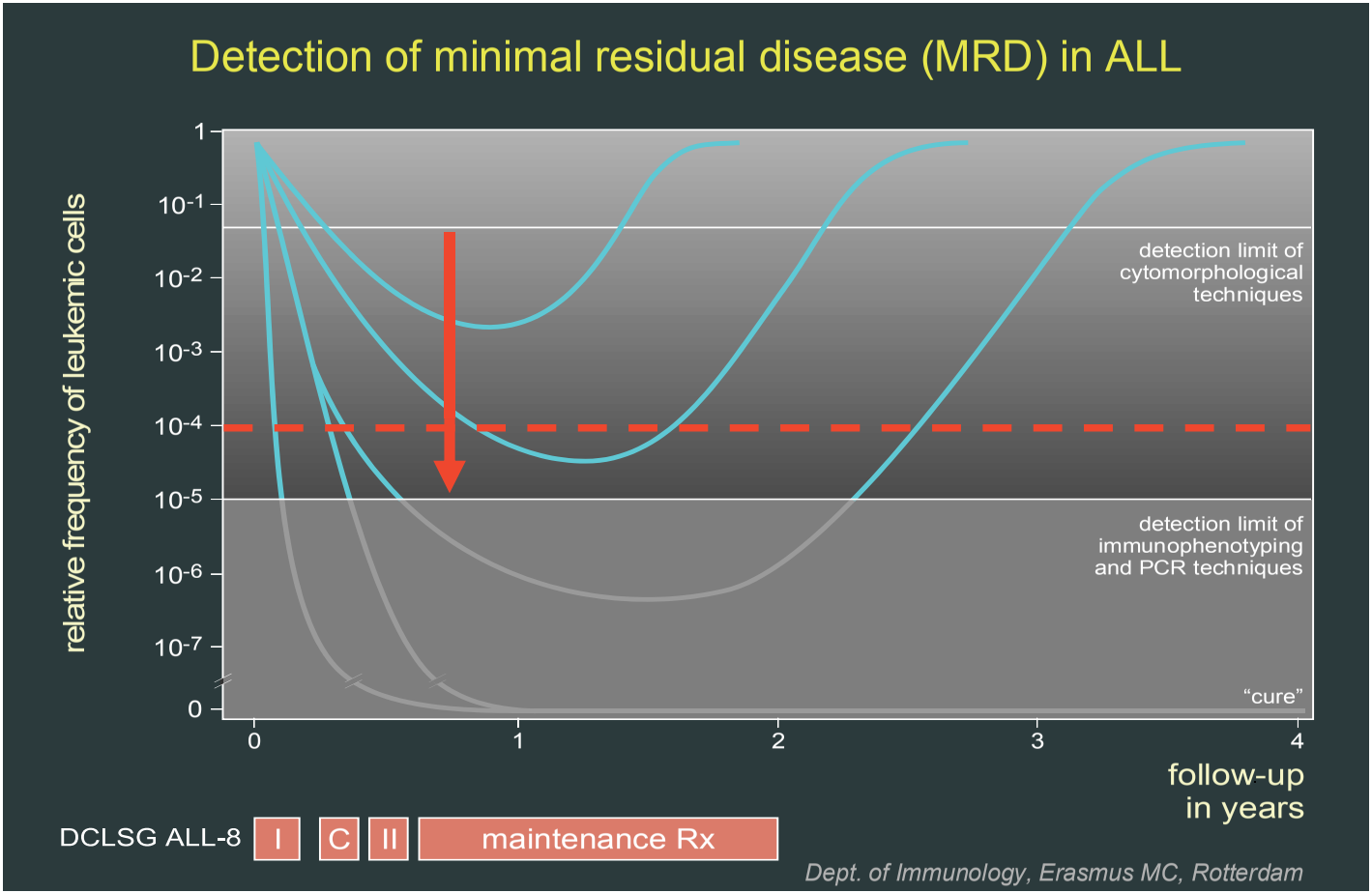
Russian flow cytometry conference, St Petersburg, Russia
17-19 March 2011

EuroFlow achievements and challenges

Novel concept for flow cytometric MRD detection in acute leukemia

Jacques J.M. van Dongen
on behalf of

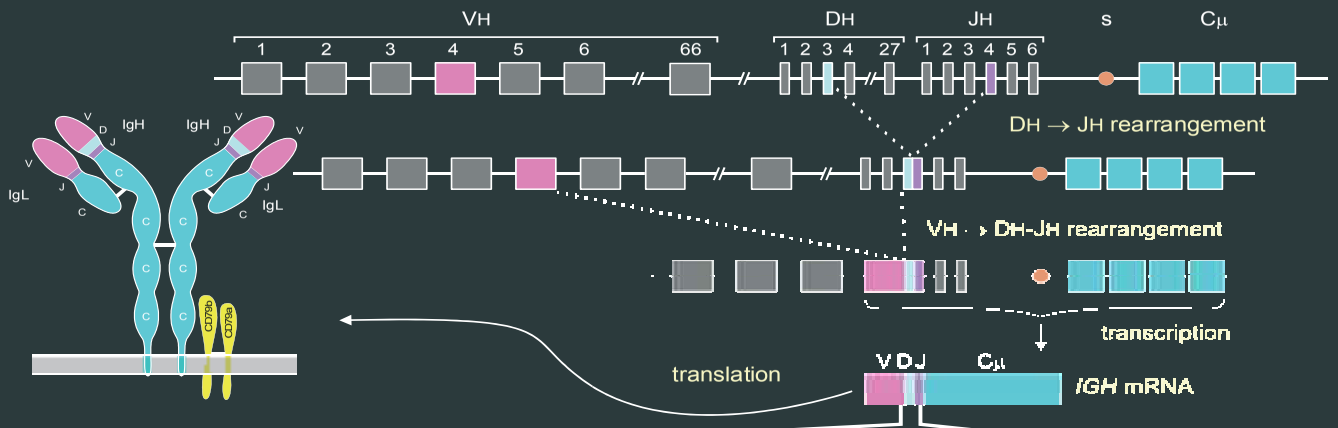
EuroFlow


Detection of minimal residual disease (MRD) in ALL
relative frequency of leukemic cells
1
10-1
10-2
10-3
10-4
10-5
10-6
10-7
0
detection limit of cytomorphological techniques
detection limit of immunophenotyping and PCR techniques
"cure"
0
1
2
3
4
follow-up in years
DCLSG ALL-8
I
C
II
maintenance Rx
Dept. of Immunology, Erasmus MC, Rotterdam

Detection of minimal residual disease in acute leukemia

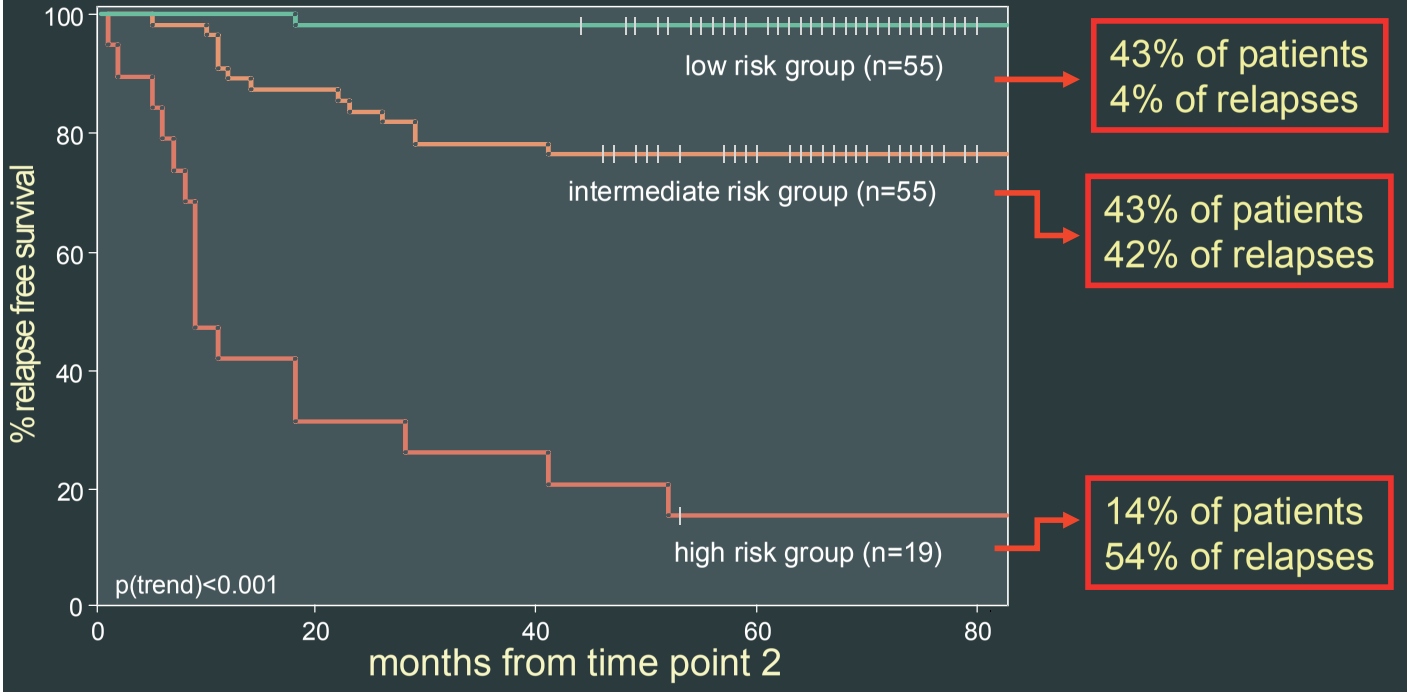
Technique	Applicability	Detection limit	Remark
Flow cytometry (4 colors)	BCP-ALL: 85% T-ALL: 90% AML: 60-80%	(10^{-3}-) 10^{-4}	Fast, but variable sensitivity because of similarities between normal (regenerating) cells and malignant cells
PCR of Ig/TCR genes	BCP-ALL: 95% T-ALL: 95% AML: 10-15%	10^{-4}-10^{-5}	Time consuming and relatively expensive (junctional region sequencing), but applicable in ≥ 95% of lymphoid malignancies
PCR of fusion transcripts	BCP-ALL: 40% T-ALL: 25% AML: 25-40%	10^{-4}-10^{-6}	Limited applicability in ALL, but potentially useful in specific subgroups, e.g. BCR-ABL cases in specific protocols

From Ig gene to Ig molecule

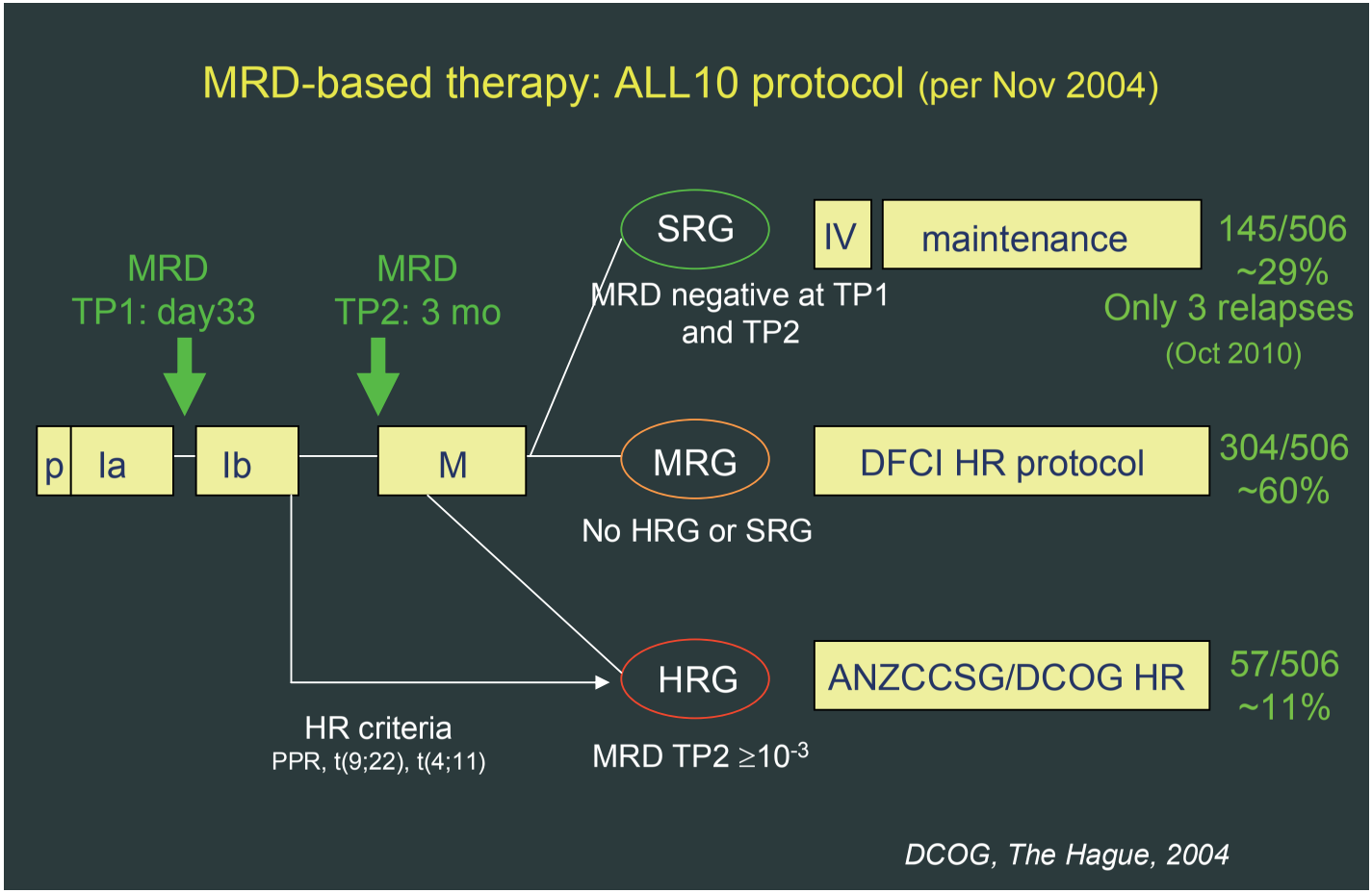


VH3-21 (germline)	insertion	DH3-3 (germline)	insertion	JH4-1 (germline)
TGTATTACTGTGCGAGA		GTATTACGATTTTTGGAGTGGTTATTATACC		ACTACTTTGACTACT
TGTATTACTGT	AGGC	CGATTTTTGGAGTGGTTATTATA	GTCCA	TGACTACT
TGTATTACTGTGCG	TATCCGGA	TTACGATTTTTGGAGTGGTTATTATAC	CGATCG	CTTTGACTACT
TGTATTACTGTGC	CCGGACTG	TTTTGGAGTGGTTATTATACC	GGT	ACTACTTTGACTACT
TGTATTACTGTGCGAG	CTGAGTC	TATTACGATTTTTGGAGTGGTTAT	CGTAGCGTA	TTTGACTACT
TGTATTACTG	ACATCGA	CGATTTTTGGAGTGGTTATTATA	CGTAG	ACTTTGACTACT
TGTATTACTGTGCG	CGT	TACGATTTTTGGAGTGGTTATTAT	GGCTAAGG	TGACTACT
TGTATTACTGTGCGA	CCGG	TTACGATTTTTGGAGTGGTTATTATACC	CGGAGC	TACTTTGACTACT

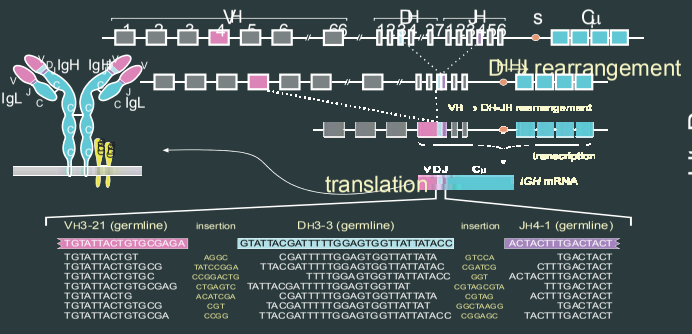
Relapse free survival in I-BFM-SG study according to the combined MRD information at time points 1 and 2 (n=129)

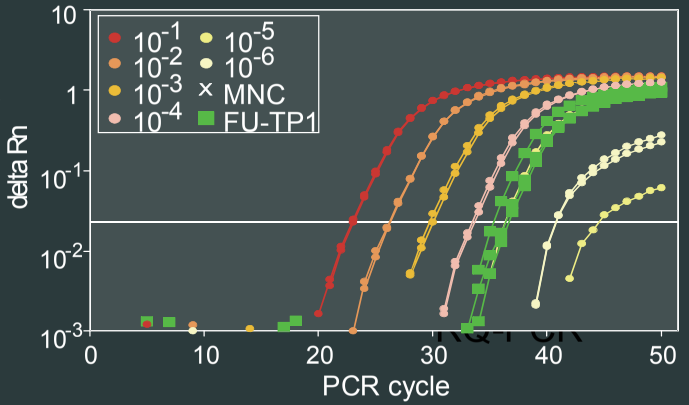


I-BFM-SG Report, J.J.M. van Dongen et al, Lancet 1998;352:1731-1738


MRD-based therapy: ALL10 protocol (per Nov 2004)
MRD
TP1: day33
MRD
TP2: 3 mo
p
Ia
Ib
M
SRG
MRD negative at TP1
and TP2
IV
maintenance
145/506
~29%
Only 3 relapses
(Oct 2010)
MRG
No HRG or SRG
DFCI HR protocol
304/506
~60%
HRG
MRD TP2 ≥10-3
ANZCCSG/DCOG HR
57/506
~11%
HR criteria
PPR, t(9;22), t(4;11)
DCOG, The Hague, 2004

Current MRD technique in lymphoid malignancies





Disadvantages of Ig/TCR-based MRD-PCR techniques:

- labor intensive (junctional regions per patient);
- require specialized laboratories;
- time consuming (target identification: 4 to 6 weeks))

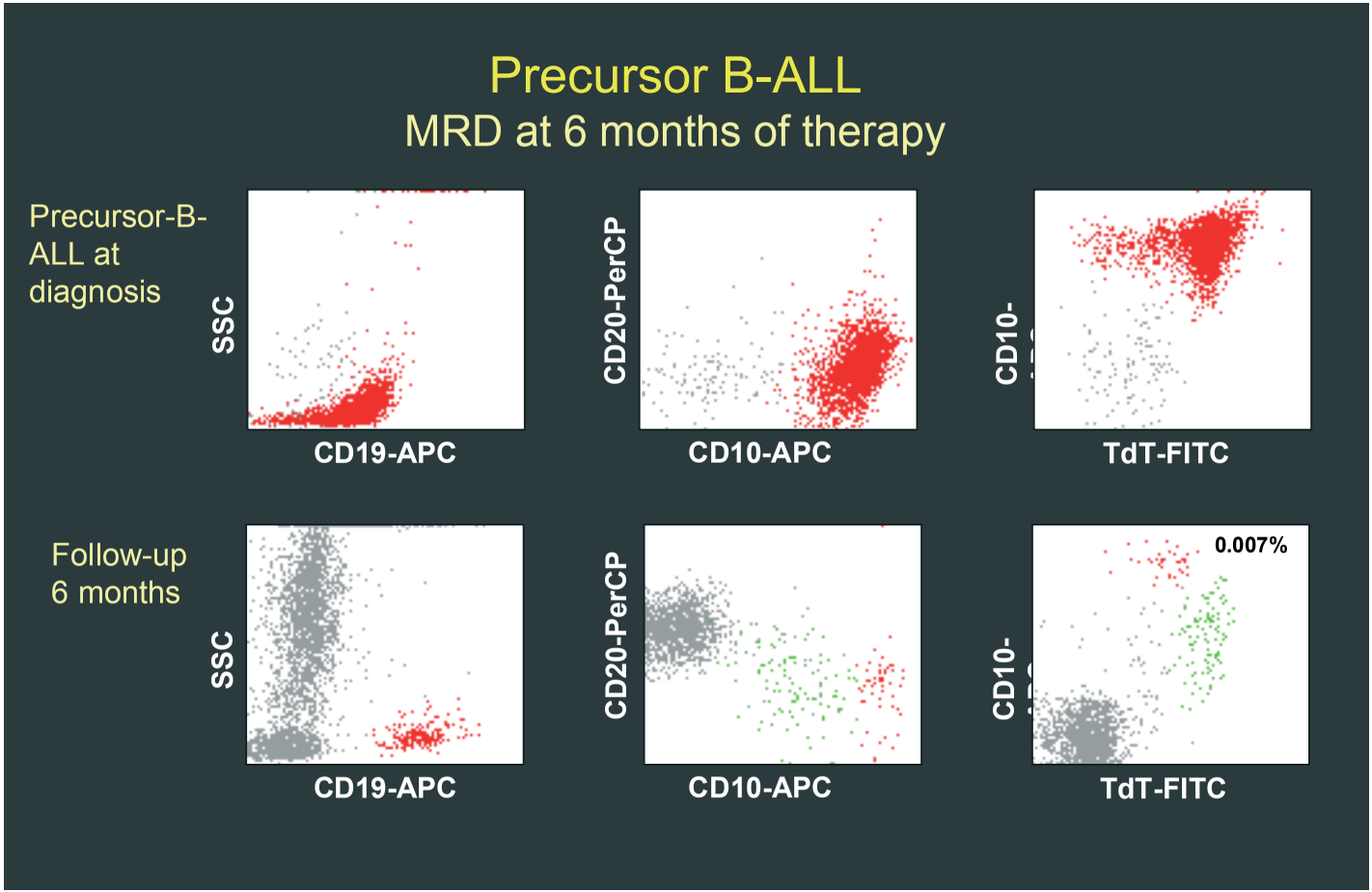
→ Faster technique needed: 8-color flow cytometry ?

Comparison between molecular techniques and flow cytometry in hematological malignancies

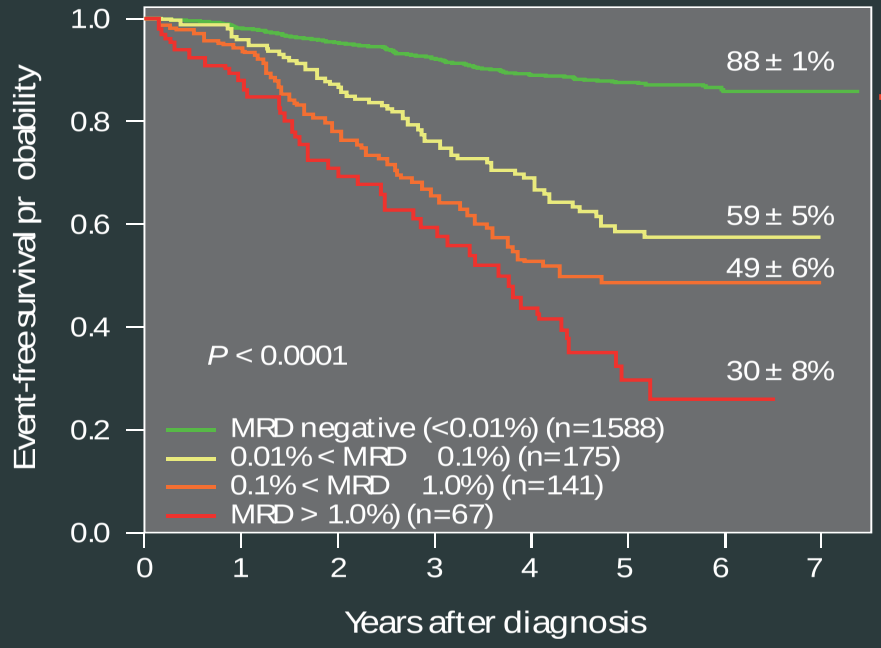
	Molecular techniques	Flow cytometry
Speed	2-3 days (up to weeks)	fast: 1-2 hours !!
Target	DNA or RNA (RNA is an instable target)	protein/cells ("end-product")
Applicability	depends on disease (chromosome aberrations)	broad
Multiplexing	technically demanding	relatively easy (even 25 to 100 tests per tube)
Accuracy	semi-quantitative	quantitative
Focus	all cells in sample (or: prior purification)	any subpopulation
Facilities	special laboratories needed (pre-PCR lab, PCR lab, etc)	only standard lab needed (+ flow cytometer)

Precursor B-ALL

MRD at 6 months of therapy

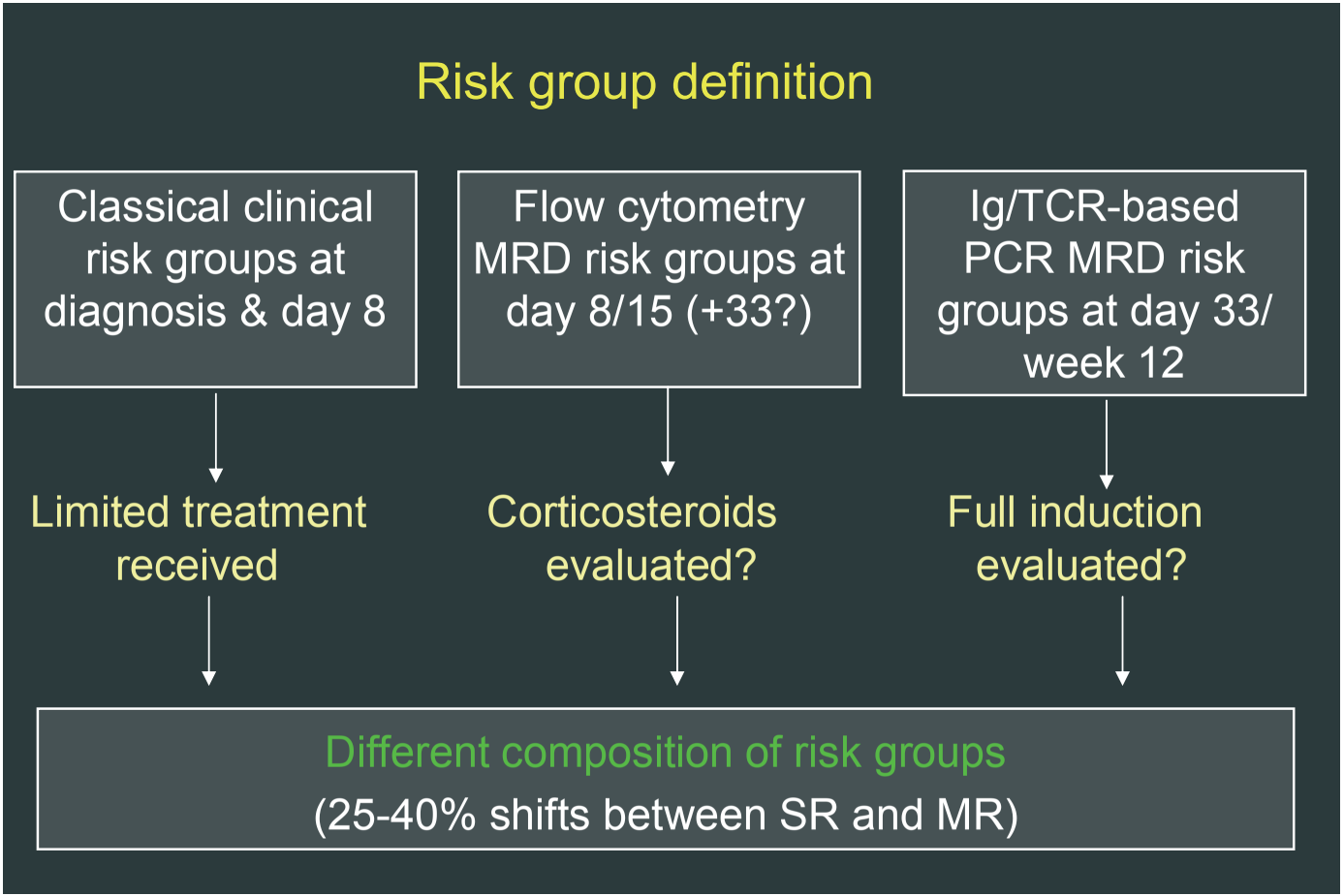


EFS of MRD-based risk groups (FCM at day 29) in COG protocol

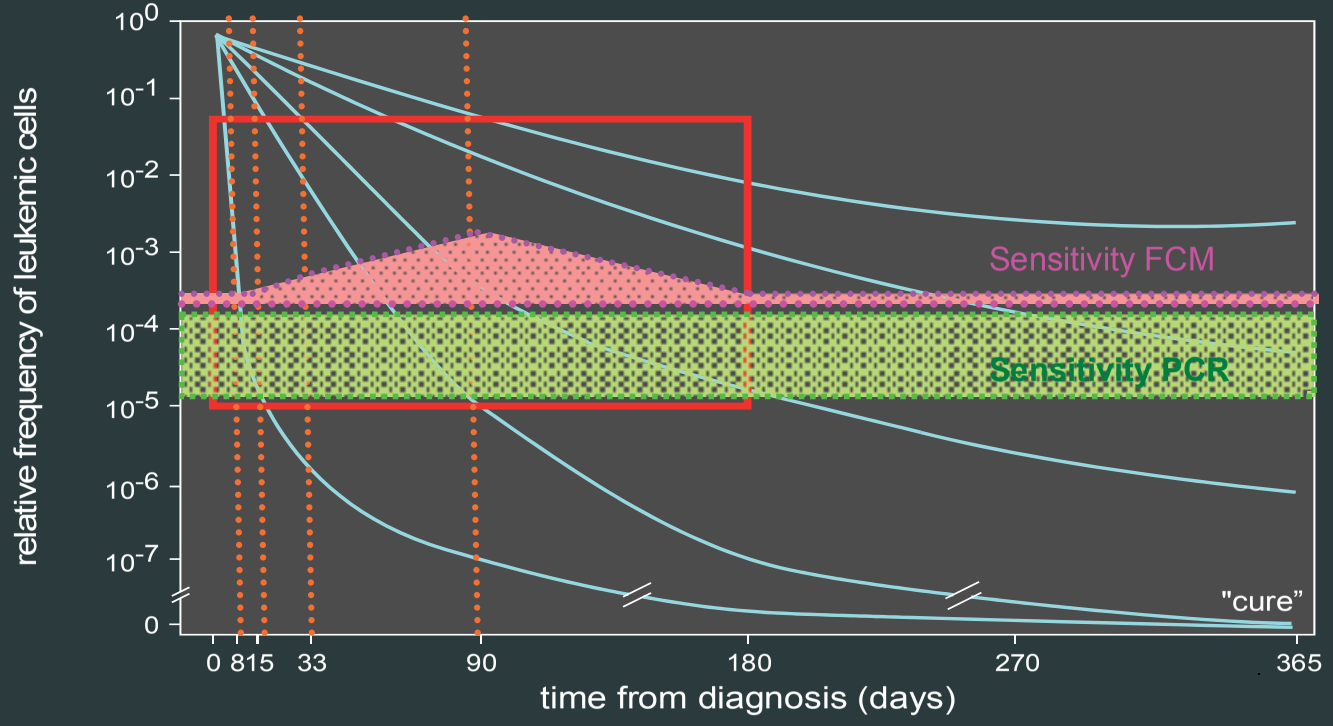


80% of patients
51% of relapses

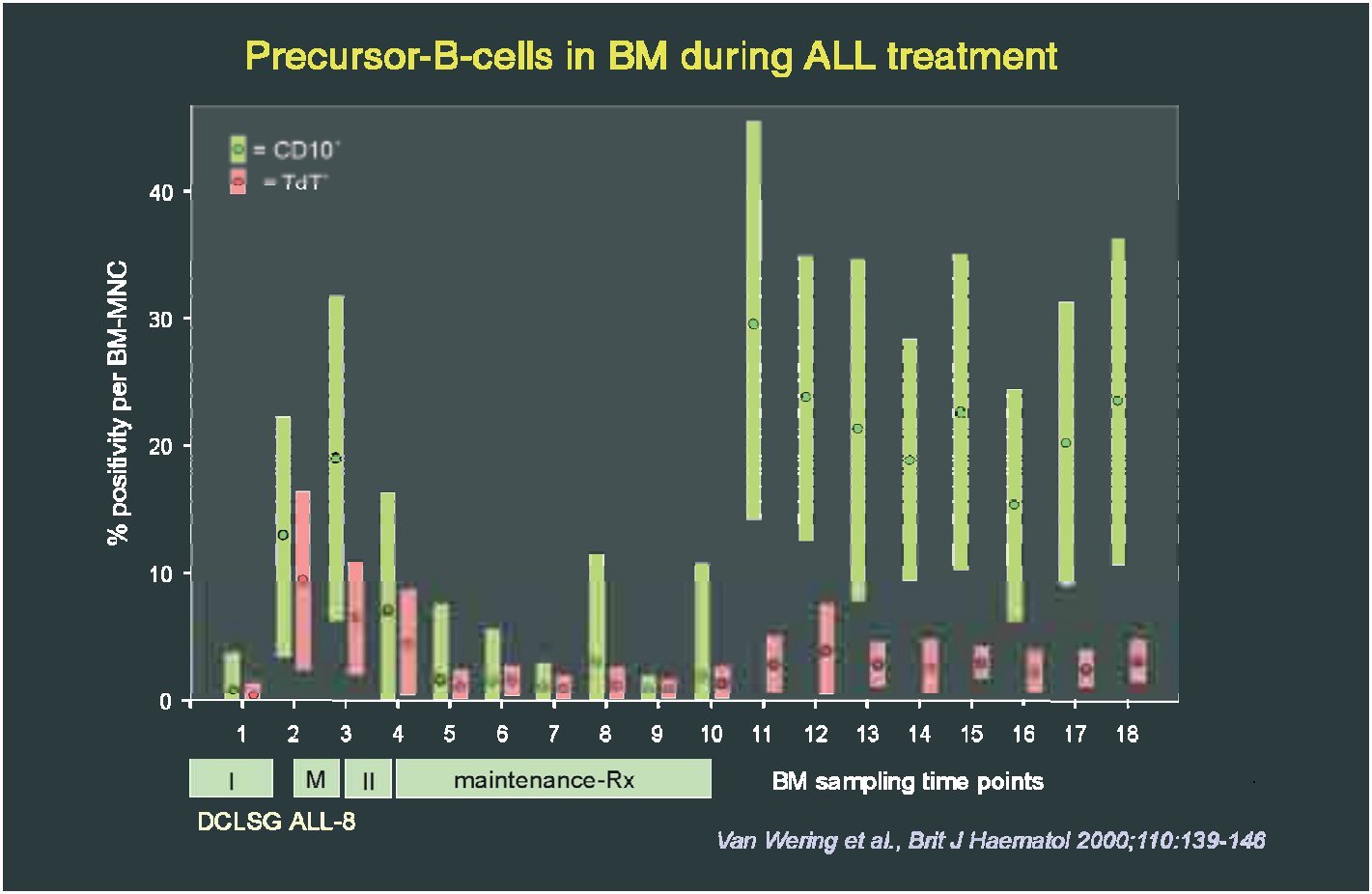
Borowitz et al. , Blood 2008 ; 111: 5477-5485 .


Risk group definition
Classical clinical risk groups at diagnosis & day 8
Flow cytometry MRD risk groups at day 8/15 (+33?)
Ig/TCR-based PCR MRD risk groups at day 33/ week 12
Limited treatment received
Corticosteroids evaluated?
Full induction evaluated?
Different composition of risk groups
(25-40% shifts between SR and MR)

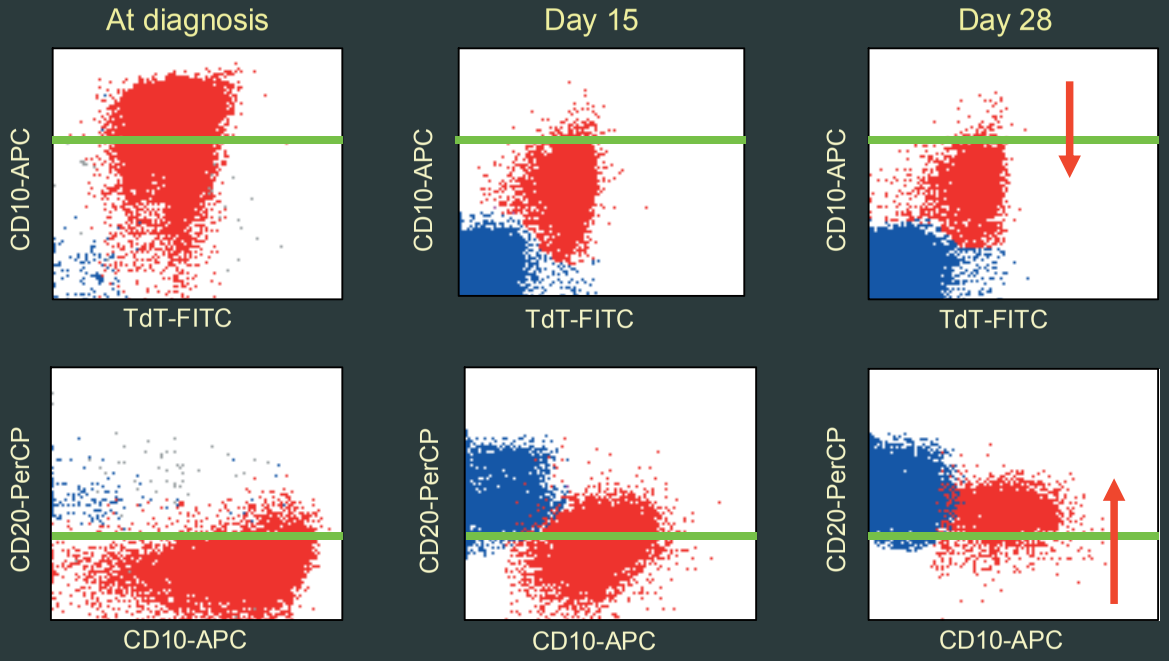
MRD window, time points, MRD techniques and QR & sensitivity



Dept. of Immunology, Erasmus MC, Rotterdam

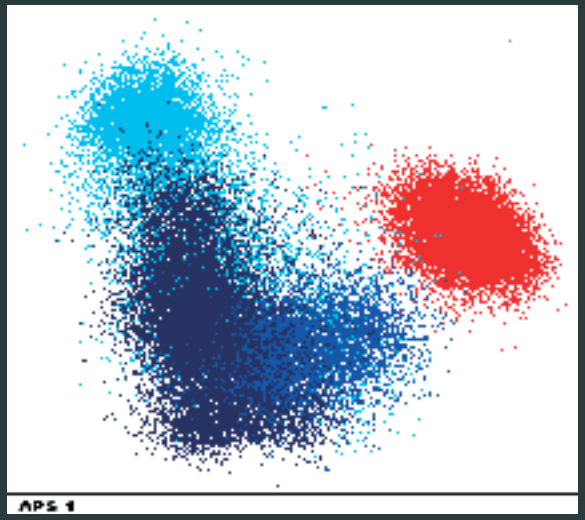

Precursor-B-cells in BM during ALL treatment
= CD10+
= TdT+
% positivity per BM-MNC
0
10
20
30
40
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
I
M
II
maintenance-Rx
BM sampling time points
DCLSG ALL-8
Van Wering et al., Brit J Haematol 2000;110:139-146

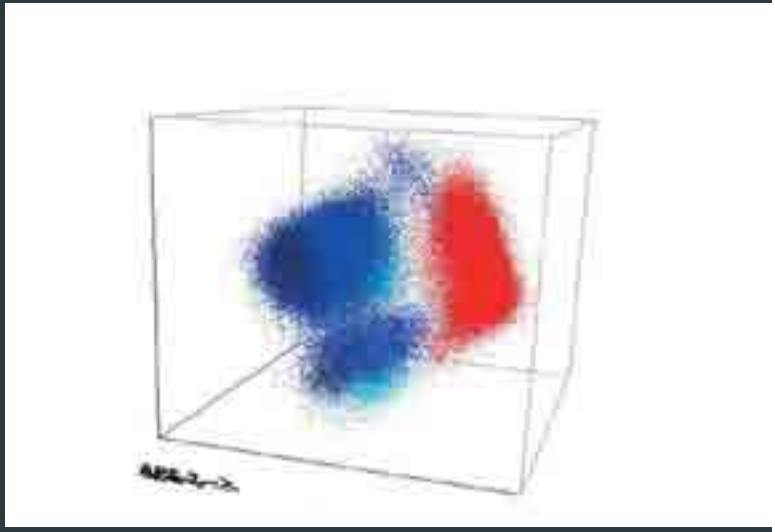
Therapy-induced immunophenotypic shifts



Van der Sluijs et al, LEUKEMIA 2005; 19: 1845-1847

BCP-ALL panel





Mix of 3 different regenerating B cell populations (Haematogones)

BCP-ALL blast cells

Responsible scientist: L Lhermitte

EuroFlow

Identification of different granulocytic subpopulations in childhood BM


Myelo/monoblast

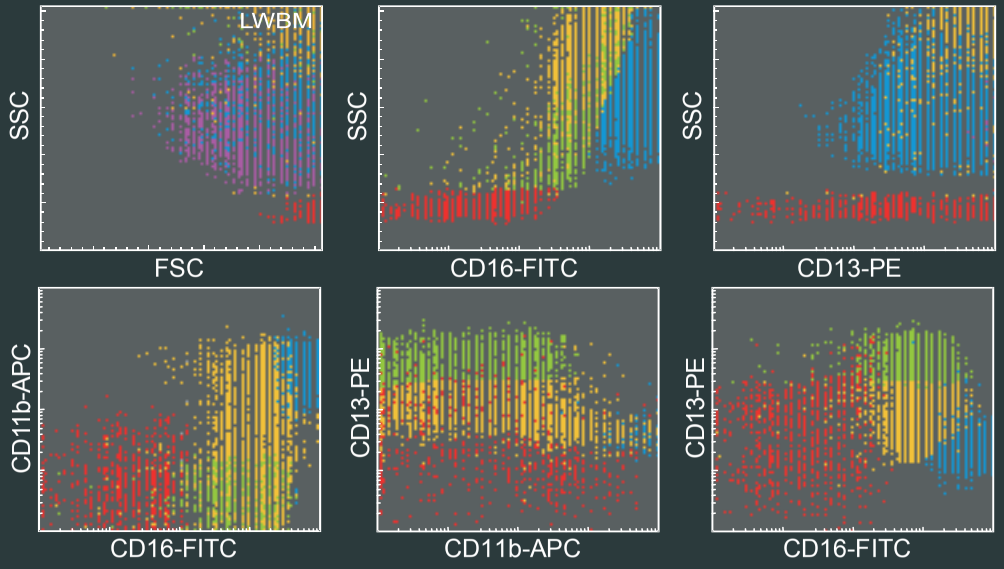

Promyelocyte


Myelocyte


Metamyelocyte

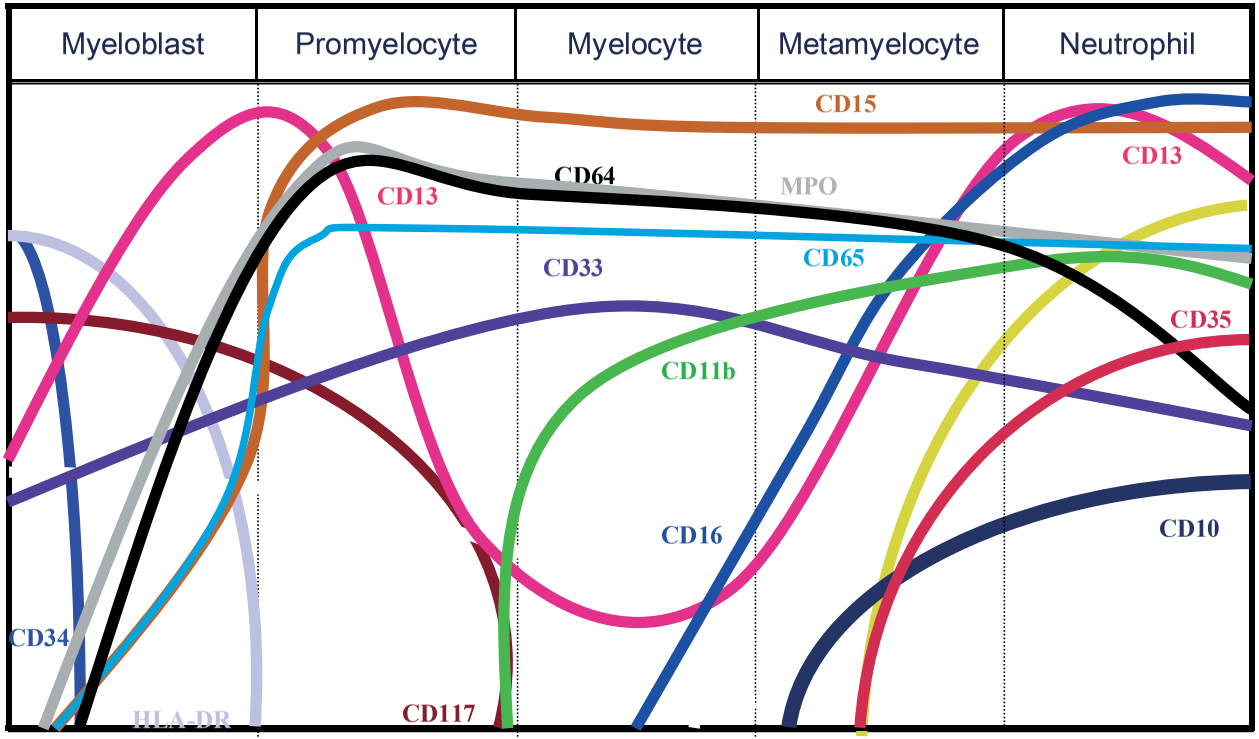

Neutrophill

staining 5
CD16/CD13/
CD45/CD11b



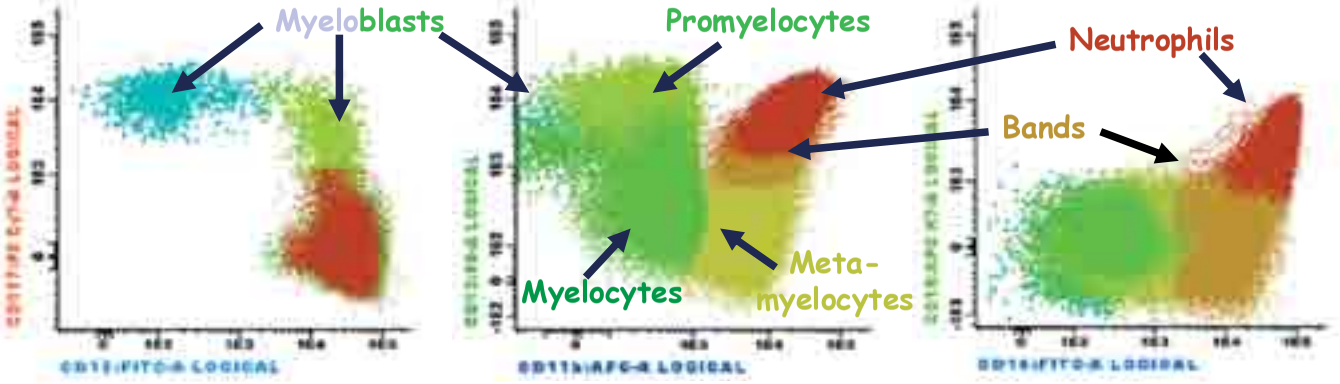
E.G. van Lochem et al., Cytometry Part B 2004; 60B: 1-13.

Phenotypic changes during normal neutrophil differentiation



Responsible scientist: A. Orfao

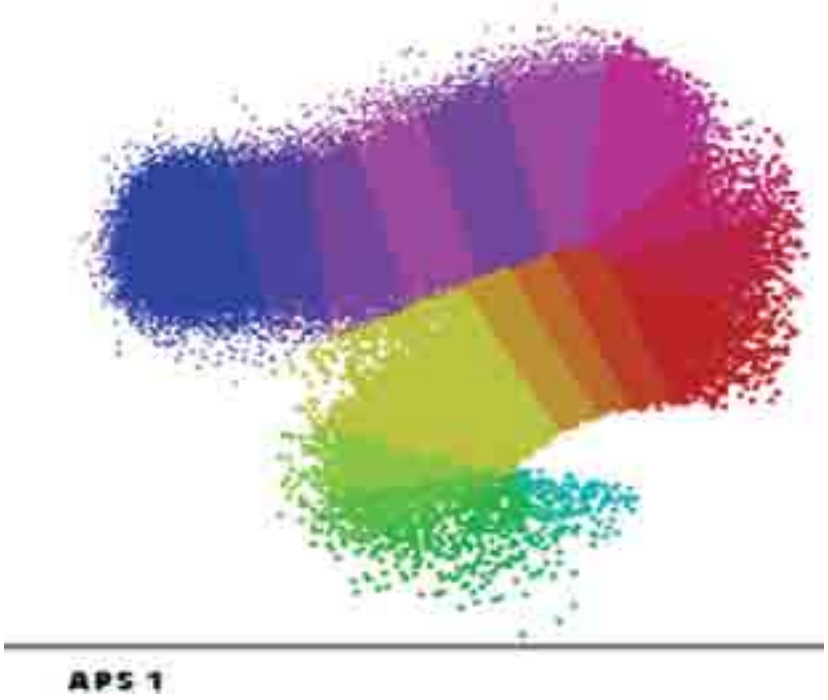
Maturation of neutrophil precursors in normal BM



Responsible scientist: V.H.J. van der Velden

EuroFlow

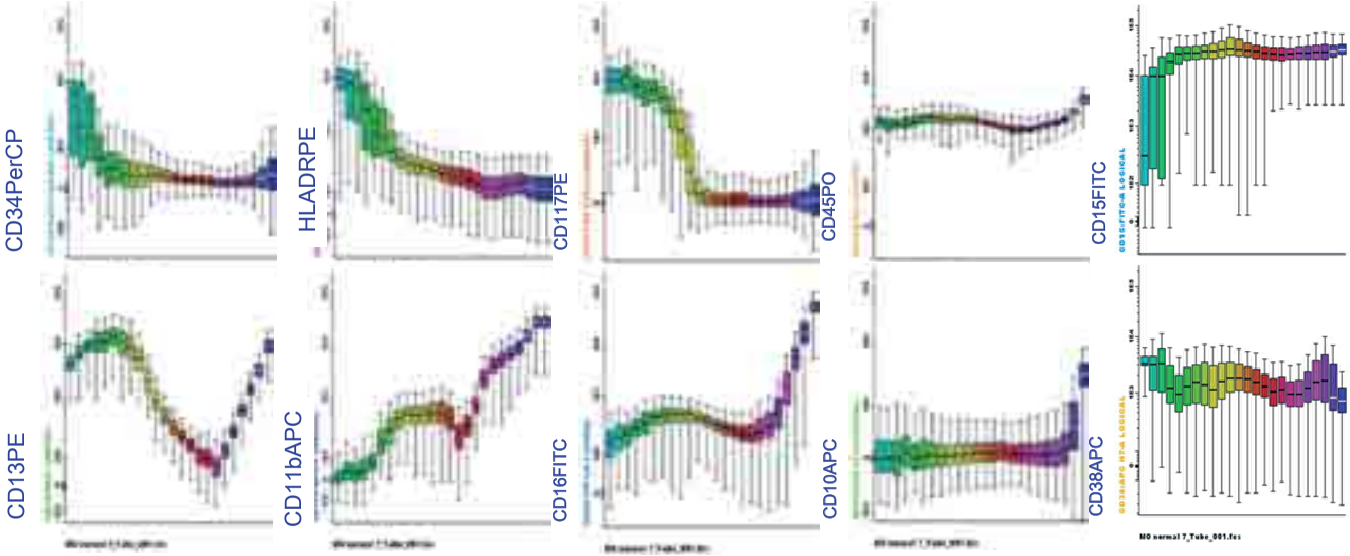
Maturation stage of neutrophil precursors in normal BM



Responsible scientist: A. Orfao

EuroFlow

Maturation stage of neutrophil precursors in normal BM



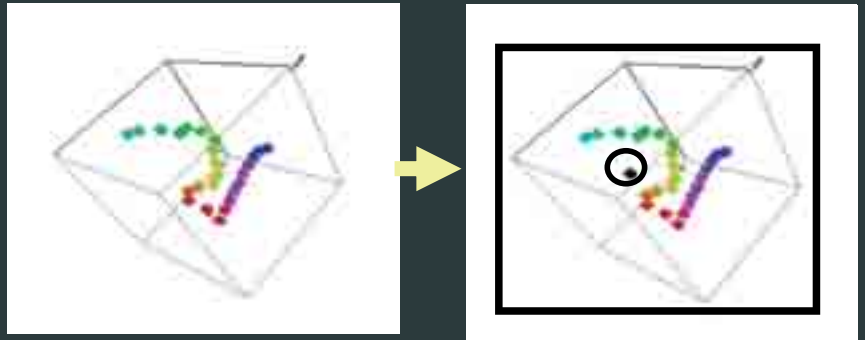
Responsible scientist: A. Orfao

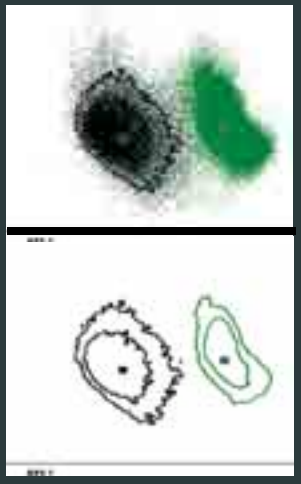
EuroFlow

N-dimensional neutrophil maturation in normal BM versus AML

Maturation stage of AML blasts

Aberrant phenotype of AML blasts



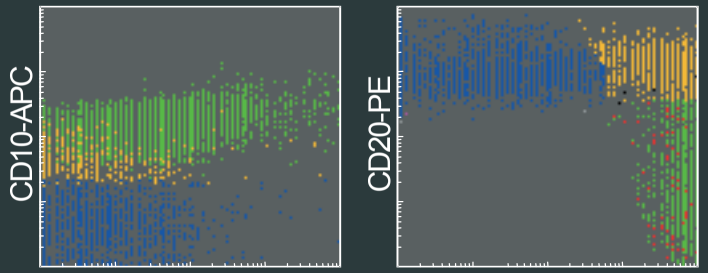


Aberrant markers

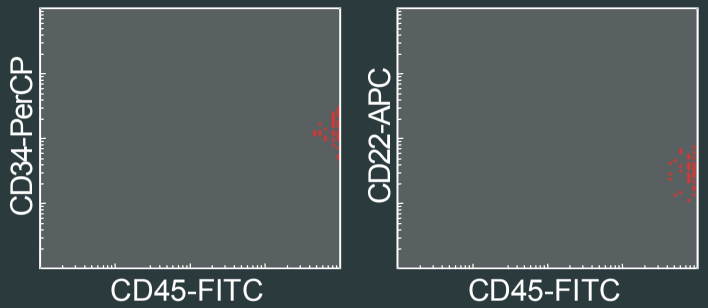
CD15	26.6
CD117	19.4
SSC	19.2
FSC	19.0
CD11b	6.0

Identification of different (precursor)B-cell subpopulations in BM

staining 1
TdT/CD19/CD20/CD10
(CD19 $^{+}$lymphogate)

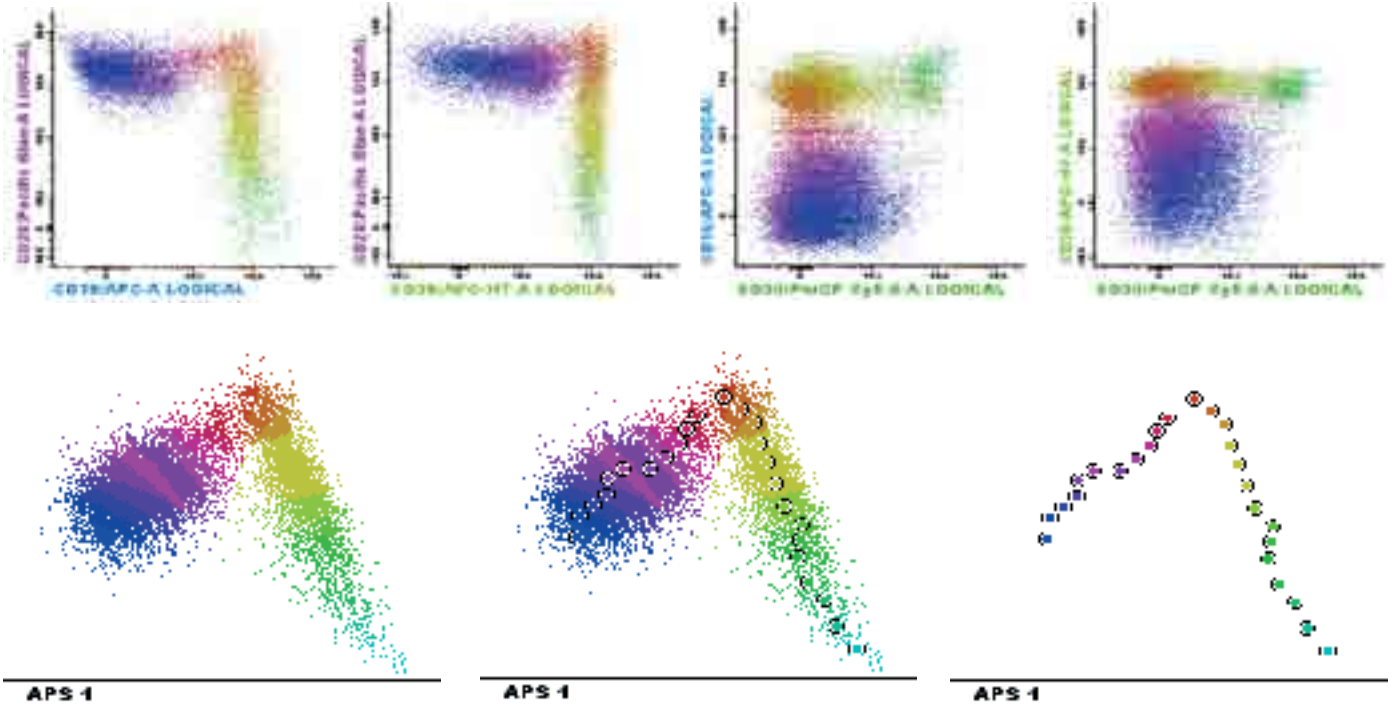


staining 2
CD45/CD34/CD19/CD22
(CD19 $^{+}$lymphogate)



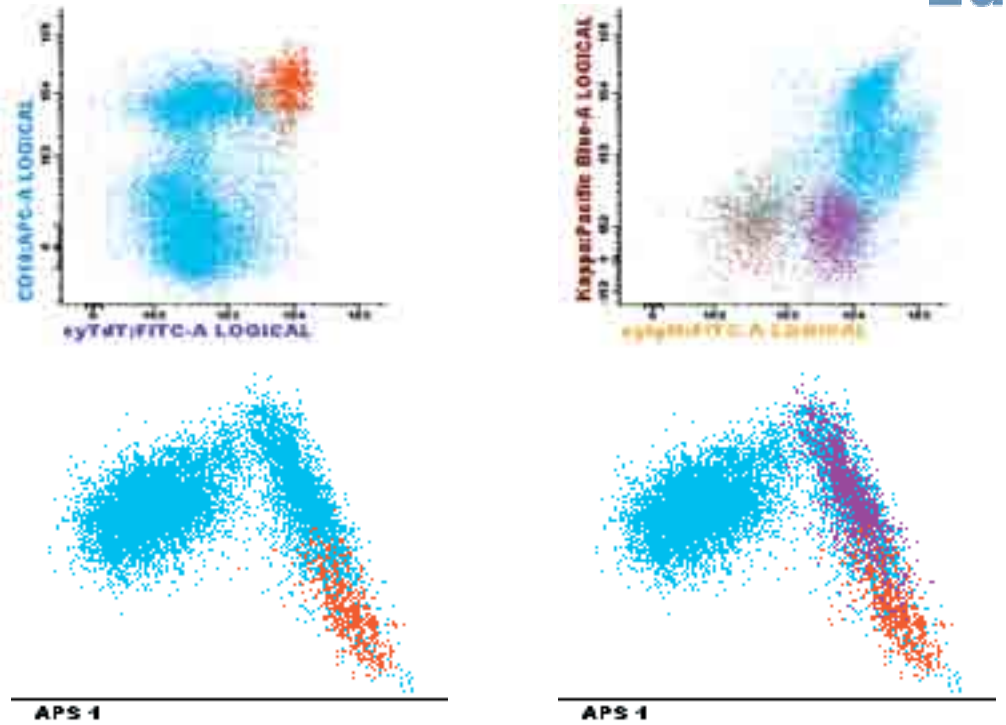
E.G. van Lochem et al., Cytometry Part B 2004; 60B: 1-13.

Dissection of normal precursor-B-cell differentiation



Responsible scientists: V.H.J. van der Velden and E. Mejstrikova

Dissection of normal precursor-B-cell differentiation

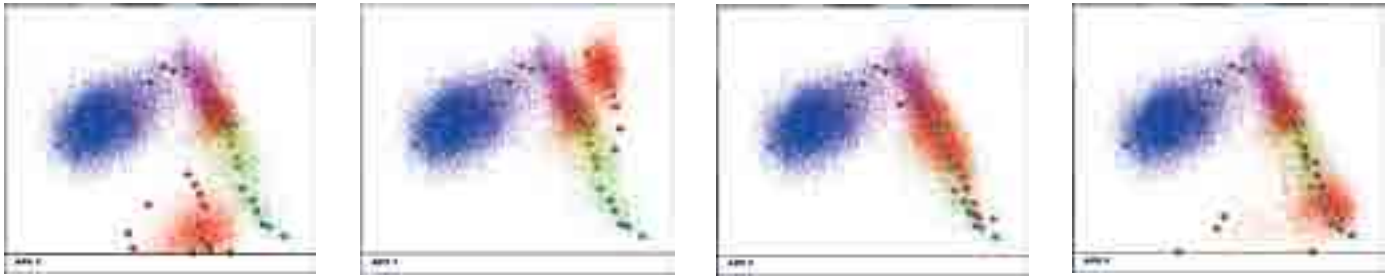


Responsible scientists: V.H.J. van der Velden and E. Mejstrikova

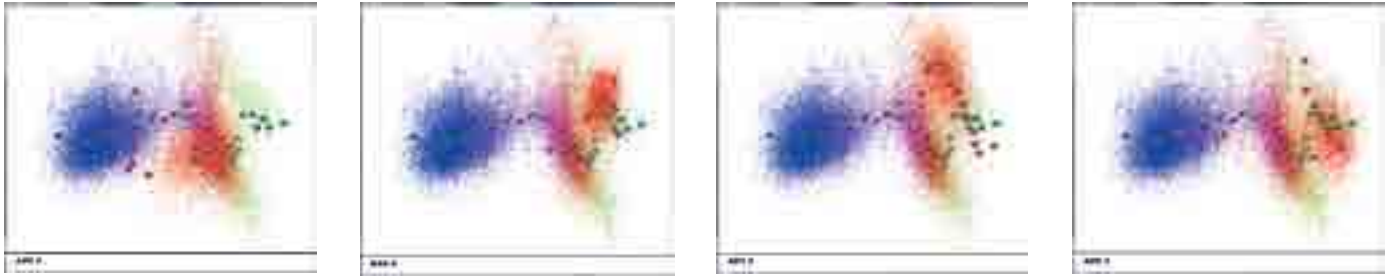
Four BCP-ALL cases vs normal precursor B-cells

EuroFlow

APS view 1



APS view 2

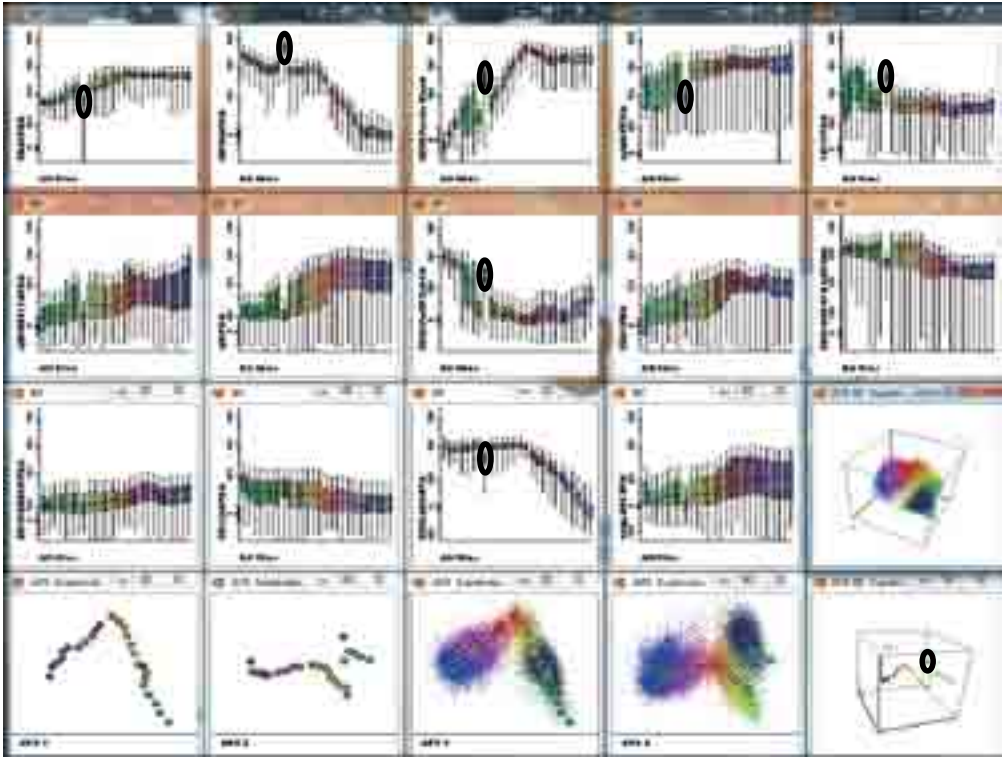


Case 1 Case 2 Case 3 Case 4

Responsible scientists: V.H.J. van der Velden and E. Mejstrikova

Designed by: A. Orfao & Q Lecrevisse

BCP-ALL (Case 4) vs normal precursor B-cell differentiation



Designed by: A. Orfao & Q Lecrevisse

EuroFlow

Russian flow cytometry conference, St Petersburg, RUS, 17-19 March 2011

Conclusions

1. PCR-based MRD diagnostics (IG/TCR genes or fusion genes) is currently the gold standard in most European ALL protocols
2. Differences in MRD value between protocols are mainly caused by application of different non-standardized MRD techniques, which also differ in sensitivity (e.g. current flow cytometry does not reach ≤ 10^{-3}).
3. PCR-based MRD diagnostics can potentially be replaced by 8-color flow cytometry, based on new concepts, such as discrimination of normal-regenerating cells from aberrant blasts cells via PCA (see APS tool developments by EuroFlow)
4. Standardization, regular Quality Control, and guidelines for data interpretation and data reporting are essential for international comparability of MRD results (within and between treatment protocols).

Collaborative networks are essential for innovation, reliable technical developments, standardization & quality control

Factors involved in treatment e• ectiveness in ALL patients

Factors	Relative contribution	Solutions
Treatment compliance e.g. - duration of Rx - side e• ects (e.g. allergy)	35%??	- Psychosocial care - Safer drugs
In vivo drug distribution e.g. - gastrointestinal absorption - distribution in body (e.g. CNS) - drug metabolism (e.g. polymorphisms in enzymes) - liver excretion - kidney excretion	30%??	Adaptation of drug dosage (based on measurement of therapeutic levels?)
Characteristics of tumor cells e.g. - prednison response - *in vitro* drug sensitivity gene expression pro• le	35%??	Adaptation of drugs? New drugs?

Evaluation of overall treatment e• ectiveness by MRD diagnostics

Di• erent treatment arms

-

Development of 8-color MRD panels

EuroFlow

Single-tube antibody EuroFlow MRD protocols under evaluation

1. Acute leukemias (includes recognition of normal precursors)
   - Acute myeloid leukemia panel (AML-MRD): 1 tube per pathway (A. Orfao)
   - B-cell precursor (BCP-ALL-MRD): 1 tube (V. van der Velden, T. Szczepañski, E. Mejstrikova)
   - T-cell ALL (T-ALL-MRD): 1 tube (V.Asnafi)

2. Chronic lymphoproliferative disorders (includes recognition of normal cells)
   - Chronic lymphocytic leukemia (CLL-MRD): 1 tube (A. Langerak)
   - Hairy cell leukemia (HCL-MRD): 1 tube (E. MacIntyre)
   - Mantle cell lymphoma (MCL-MRD): 1 tube (S. Böttcher)
   - Follicular lymphoma (FL-MRD): 1 tube (S. Böttcher)
   - Marginal zone lymphoma (MZL-MRD): 1 tube (P.Lucio)
   - Lymphoplasmacytic lymphoma (LPL-MRD): 1 tube (P. Lucio)
   - Diffuse large B-cell lymphoma (DLBCL-MRD): 1 tube (P. Lucio)
   - Burkitt lymphoma (BL): 1 tube (L.Lhermitte)
   - T-chronic lymphoproliferative diseases (T-CLPD-MRD): 1 tube (J. Almeida)
   - Multiple myeloma (MM): 1 tube (J. Flores)

EuroFlow

EuroFlow is an independent scientific consortium,
which aims at innovation in flow cytometry
for improvement of diagnostic patient care

www.euroflow.org

Alberto Orfao was born on 15 July 1960. He received his M.D. Degree at both the University of Salamanca, Spain (1984) and the Nova University of Lisbon, Portugal (1985) and obtained the Ph.D. Degree at the University of Salamanca in 1987. He is currently Full Professor of the Department of Medicine and the Director of the General Cytometry Service at the University of Salamanca, as well as one of the Principal Investigators at the Cancer Research Center of Salamanca. He leads the Spanish National DNA Bank since its creation in 2004. His main research interest is in translational medicine, mainly focused on hematological malignancies and the relationship between immune system and cancer. At present he has published more than 400 scientific papers and has co-authored 25 patents. He has received more than 30 awards including the Berend Howen and the Wallace Coulter awards of the International Society for Laboratory Hematology and the International Society for Clinical Cytometry.

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
José Alberto Orfao de Matos	Director, National Bank of DNA, Cytometry Service, Associate Professor of Immunology of USAL

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Salamanca	MD	1984	Medicine / Surgery
University of Salamanca	PhD	1987	Medicine / Surgery

A. Positions and Honors.

RESEARCH AND PROFESSIONAL EXPERIENCE:

2004-Present - Director of the National DNA Bank of Spain, at the University of Salamanca

2000-Present - Principal Investigator at the Cancer Research Center of Salamanca

1996- Present - Professor of Immunology, Department of Medicine, at the University of Salamanca

1990- Present - Director of the Central Cytometry Service of the University of Salamanca (Spain).

1987: Ph.D. Title of Ph.D. Thesis: Immunological phenotype and clinical-biological correlation in chronic lmphocytic leukemia

1985: MD degree at the University of Lisbon

1984: MD degree at the University of Salamanca

Other Experience and Professional Memberships

Member of the Scientific Committee of the National Genotyping Center of Spain (2004- Present)

Councilor of the executive board of the International Society for Analytical Cytology (1996-2000)

Member of European Group for the Immunological Characterization of leukemias

Member of European BIOMED Group for the study and standarization of minimal residual disease in acute leukemias on Clinical Cell Analysis.

Chairman of the standarization Committee of the International Federation of Clinical Chemistry (1994-2000).

Secretary of Iberian Society Cytometry (1999- 2003)

President of Iberian Society Cytometry (1995-1999)

Tesaurer of The Iberian Society of Cytometry (1993-1995)

Member of the External advisory board of the CICS (University of Beira Interior, Portugal) (2007-present)

Member of the Research Council of the University of Salamanca (2004-2008)

Member of the advisory board of the Brasilian Studies Institute of the University of Salamanca (2008-present)

HONORS & AWARDS

"Mecenas" Prize of the University of Salamanca. January 2001

Prize of The Royal Academy of Medicine of Salamanca. Feb 2000

Iberoamerican Prize of Flow Cytometry of The Mexican Society of Cytofluorografy. March 1998

Annual Prizes of the Spanish Society of Hematology for several different scientific works: Nov 1989, Nov 1990, Oct 1991, Nov 1993, Nov 1995, Oct 1999, Oct 2001

Spanish Cancer Association Prize for best Ph.D. thesis in Cancer. Oct 1987

Wallace Coulter Prize of the Clinical Cytometry Society (USA, 2001)

EDITORIAL BOARD OF THE JOURNALS

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B. Peer-reviewed publications (in chronological order).

The publications listed below were selected from a total of 399 publications in international peer-reviewed journals

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IF: 9.77

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C. Research Support.

1.- "Standardization of flow cytometric assays for clinical cell analysis"
Principal investigator of one of the groups of research
Agency: European Comission BIOMED 2 Concerted Action – European Union
Period: 1997-2000

2.- "Central facility for the production of stabilized cellular reference standards and external quality assessment in clinical flow cytometry (Eurostandards)"
Principal investigator of one of the groups of research
Agency: RTD Actions (Research and Technology Development projects)"-"European Comission Research Directorate", European Union
Period: 2000-2003

3.- " Mastocytosis's Spanish network (REMA): study of molecular routes of proliferation and response of mast cells and his modulation for medicaments to achieve a standard diagnostic and therapeutic high place of the mastocytosis ".
Principal investigator of one of the integral nodes of the network.
Agency: G03/007 for the development of thematic networks of cooperative research of the Fund of Sanitary Researches Institute of Health Carlos III of the Ministry of Health and Consumption.
Period: 2003 – 2006

4.- " Multiple Myeloma and other gammopathies: of the genesis to the therapeutic one "..
Principal investigator of one of the research groups of the node of the Cancer Research Centre of Salamanca.
Agency: G03/136 for the development of thematic networks of cooperative research of the Fund of Sanitary Researches, Institute of Health Carlos III of the Ministry of Health and Consumption.
Period: 2003 – 2006.

5.- " Genomic of cancer ".
Principal investigator of one of the integral groups of the network.
Agency: Institute of Health Carlos III. Ministry of Health and Consumption. Help G03/10 for the development of thematic networks of cooperative research of the Fund of Sanitary Researches, Institute of Health Carlos III of the Ministry of Health and Consumption.
Period: 2003 – 2006

6.- " Phenotypic and functional characterisation of the neoplasic cell in leukemic T/NK Chronic lymjphoproliferative disorders and his implications in the biological behavior of the disease ".
Member of the research team.
Agency: Institute of Health Carlos III. Ministry of Health and Consumption.
Period: 2003 – 2006

7.- " National Bank of DNA ".
Principal Investigator
Agency: Foundation Genome Spain.
Period: 2004 – 2005

8.- "European Leukemia Net" - Network of Excellence
Principal investigator of one of the groups of research
Agency: "Research Directorate-General, Commission of the European Communities". European Union
Period: 2004-2006

9.- " Multiple Myeloma and other gammopathies: of the genesis to the therapeutic one ".
Principal investigator of one of the groups of the node of the Cancer Research Centre of Salamanca.
Agency: Project of research of the Program of Promotion of the Biomedical Research and in Sciences of the Health of the Fund of Sanitary Researches, Institute of Health Carlos III of the Ministry of Health and Consumption.

Period: 2006

10.- “Flow cytometry for fast and sensitive diagnosis and follow-up of haematological malignancies (EUROFLOW)”
Subcoordinator of the project and Principal Investigator of one of the groups of research
Agency: Research Directorate-General of European Union.
Period: 2007-2009.

11.- " Clonal B-cell lymphocytosis of undetermined significance: determination of its frequency, analysis of the phenotypic and genetic characteristics of the B-cell clone and identification of possible factors involved in his ontogenesis ".
Principal Investigator.
Agency: Project PI060824 of the Fund of Sanitary Researches of the Institute of Health Carlos III of the Ministr Health and Consumption (Spain).
Period: 2007 – 2009

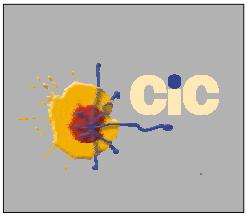
12.- “Myeloma Stem Cell Network: A translational programme identifying and targeting the myeloma stem cell (MSCNET)”
Principal investigator of one of the groups of research
Agency: Research Directorate-General of European Union.
Period: 2006-2009

13.- " Thematic network of research in cancer ".
Principal investigator of one of the integral groups of the network.
Institute of Health Carlos III. Ministry of Health and Consumption. Help RD06/0020/0035 for the developmer thematic networks of cooperative research (RETICS) of the Fund of Sanitary Researches, Institute of Health Carlo of the Ministry of Health and Consumption.
Period: 2007 - 2010

Профессор А.Орфао

«Стратегии EuroFlow и инструменты анализа данных при злокачественных гематологических опухолях»

EUROFLOW STRATEGIES & TOOLS FOR DATA ANALYSIS IN HEMATOLOGICAL MALIGNANCIES



CANCER RESEARCH CENTER, UNIVERSITY & UNIVERSITY HOSPITAL of SALAMANCA (SPAIN)

Conference on "Flow cytometry in Oncology and Oncohematology".
Saint Petersburg, 17th-19th of May, 2011

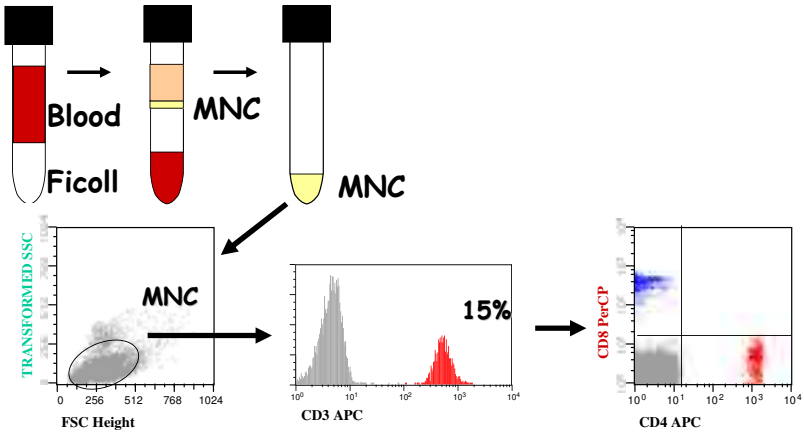
IMMUNOPHENOTYPING OF HAEMATOLOGICAL MALIGNANCIES

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

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

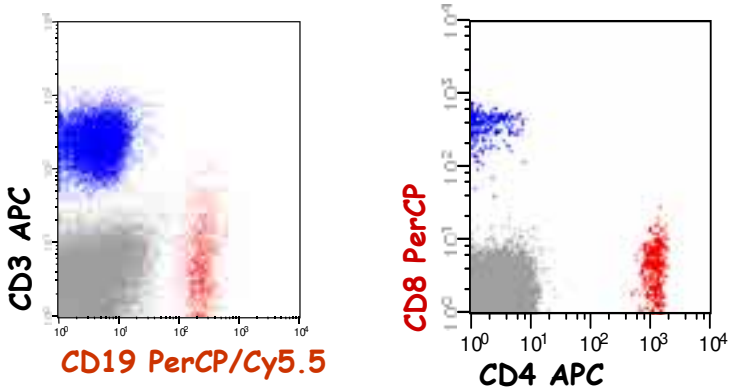
- 2006/-: Recent contributions of immuno-phenotyping of haematological malignancies: pointing to the future.

TRADITIONAL FCM PHENOTYPING



Criteria for positivity: >20%

MULTICOLOR FLOW CYTOMETRY vs SINGLE-STAININGS IN ONE TUBE



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

PANELS OF REAGENTS:

- Panels of relevant markers for the diagnostic classification of patients suspected of:
  - AML, ALL
  - B-CLPD, T-CLPD

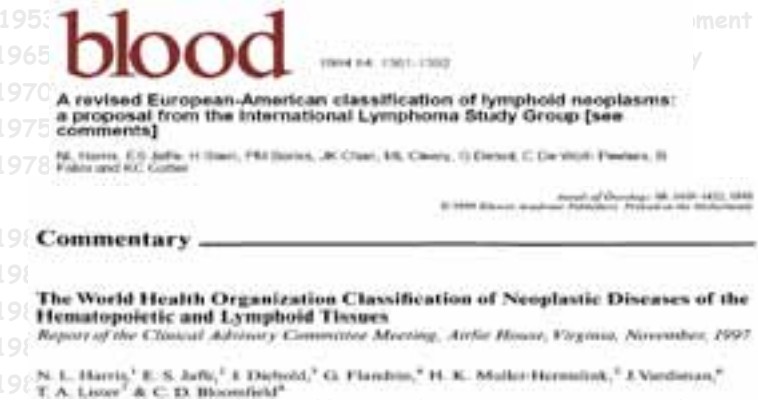
TECHNIQUES:

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

IMMUNOPHENOTYPING OF HAEMATOLOGICAL MALIGNANCIES

- 1953/1994: From the development of the instruments & techniques to the WHO classification 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


blood

A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group [see comments]

Commentary

The World Health Organization Classification of Neoplastic Diseases of the Hematopoietic and Lymphoid Tissues

Report of the Clinical Advisory Committee Meeting, Airlie House, Virginia, November, 1997

- 1988: Second MIC classification of haematological malignancies
- 1993: CD45-based blast cell gating/identification
- 1994: REAL classification of lymphoid neoplasias
- 1997: WHO classification of lymphoid neoplasias

IMMUNOPHENOTYPING OF HAEMATOLOGICAL MALIGNANCIES

Am J Clin Pathol 1993. Nov, 100 (5). 534-40

Immunophenotyping of acute leukemia by flow cytometric analysis. Use of CD45 and right-angle light scatter to gate on leukemic blasts in three-color analysis.

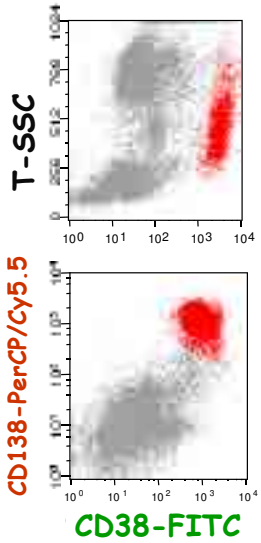
Borowitz MJ, Guenther KL, Shults KE, Stelzed GT.

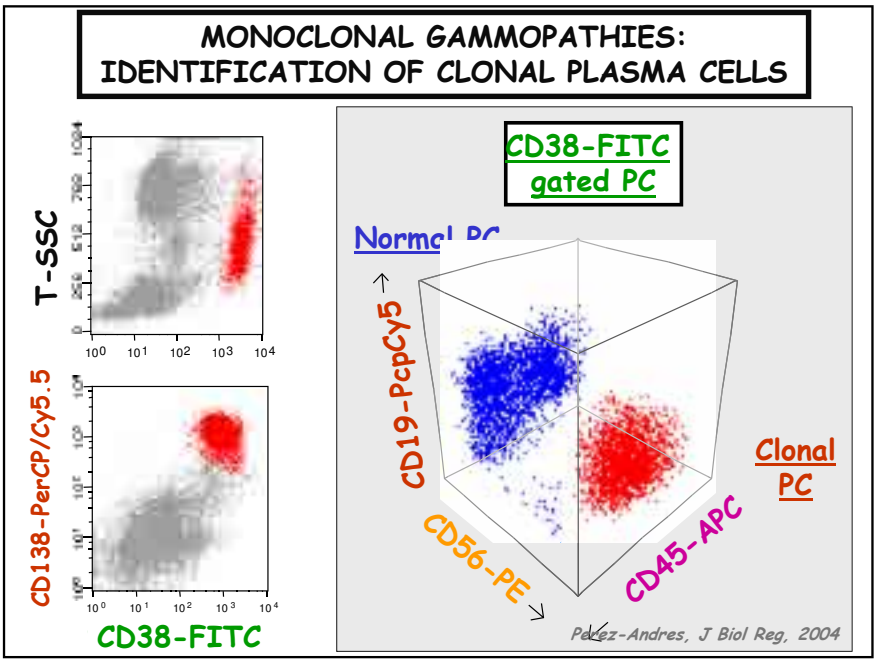
- 1988: Second MIC classification of haematological malignancies
- 1993: CD45-based blast cell gating/identification
- 1994: REAL classification of lymphoid neoplasias
- 1997: WHO classification of lymphoid neoplasias

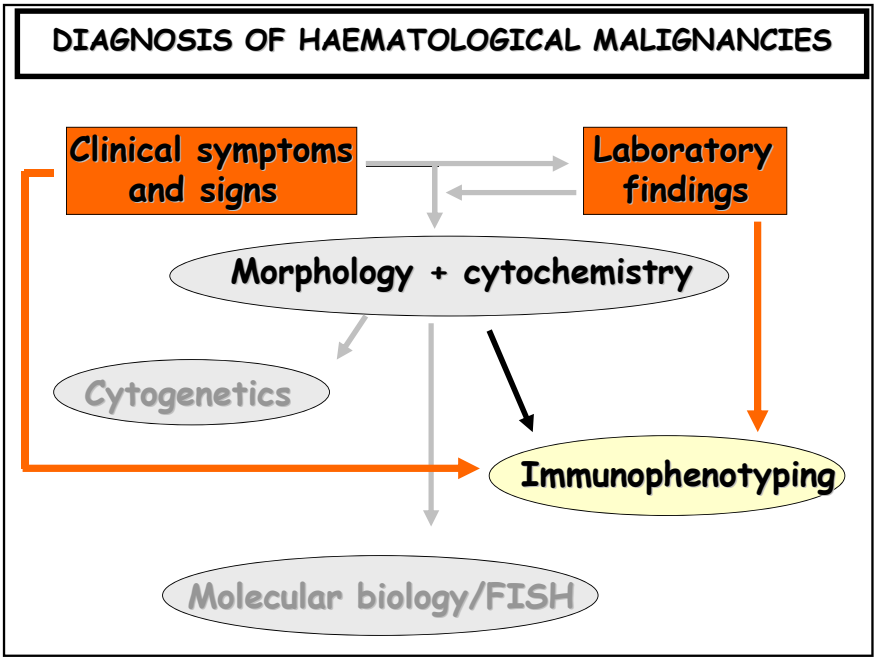
FLOW CYTOMETRY: TYPE OF INFORMATION

- **Identification** of cell populations
- **Enumeration** of cell numbers
- **Characterization** of cell populations

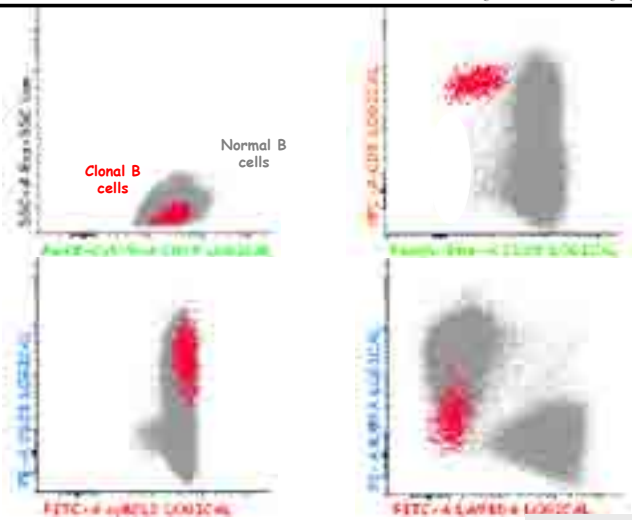
MONOCLONAL GAMMOPATHIES: IDENTIFICATION OF CLONAL PLASMA CELLS




MONOCLONAL GAMMOPATHIES:
IDENTIFICATION OF CLONAL PLASMA CELLS
T-SSC
CD138-PerCP/Cy5.5
CD38-FITC
CD38-FITC
gated PC
Normal PC
CD19-PcpCy5
CD56-PE
CD45-APC
Clonal
PC
Pérez-Andres, J Biol Reg, 2004


DIAGNOSIS OF HAEMATOLOGICAL MALIGNANCIES
Clinical symptoms and signs
Laboratory findings
Morphology + cytochemistry
Cytogenetics
Immunophenotyping
Molecular biology/FISH

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



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

Nieto et al, Blood 2009

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

PANELS OF REAGENTS:

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

Diagnosis, classification & 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

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

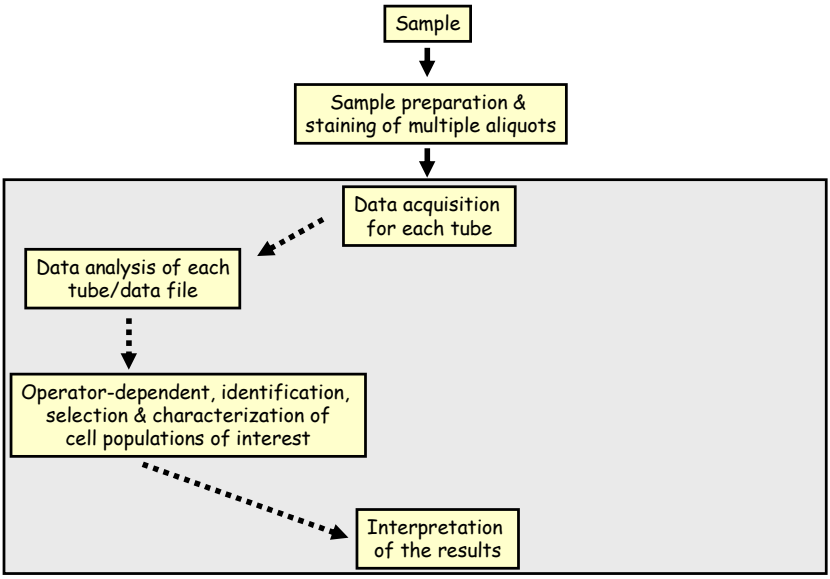
Detection of minimal residual disease (MRD):

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

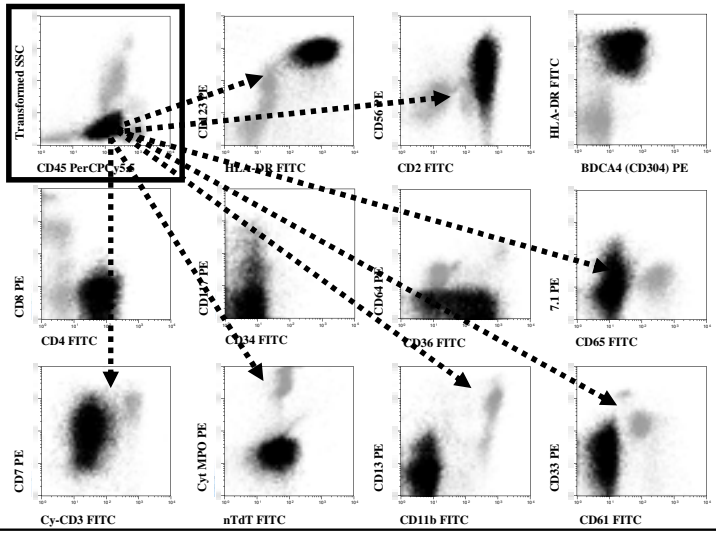
Annually > 400,000 follow-up samples in leukemia patients (ALL, AML, CML)

Prepared by JJM van Dongen

FLOW CYTOMETRY IMMUNOPHENOTYPING APPROACH



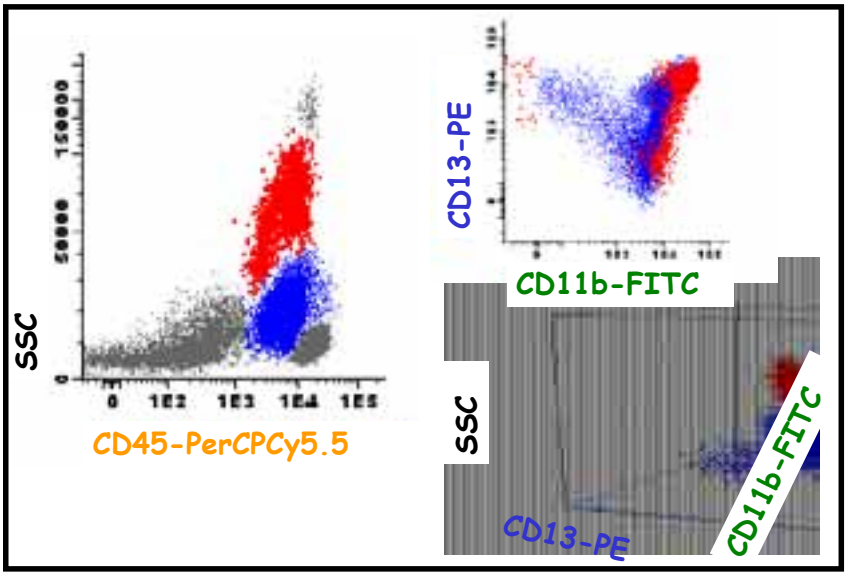
IMMUNOPHENOTYPIC FEATURES OF NEOPLASTIC CELLS



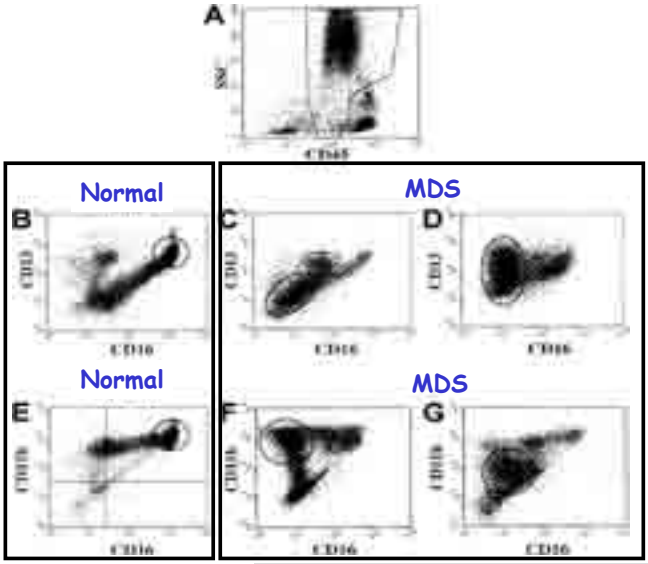
MDS/AML: 3-COLOR STAINING PANEL

- FITC	PE	PerCP/Cy5.5
- cCont.	cCont.	CD45
- nTdt	cMPO	CD45
- cCD3	CD7	CD45
- CD19	cCD79a	CD45
- sCont	sCont	CD45
- HLADR	CD117	CD45
- HLADR	CD123	CD45
- CD11b	CD13	CD45
- CD15	CD16	CD45
- CD36	CD64	CD45
- CD33	CD61	CD45
- CD71	GphA	CD45
- CD65	7.1	CD45
- CD2	CD56	CD45

MDS: COEXISTENCE OF NORMAL & HYPOGRANULAR MATURING NEUTROPHILS

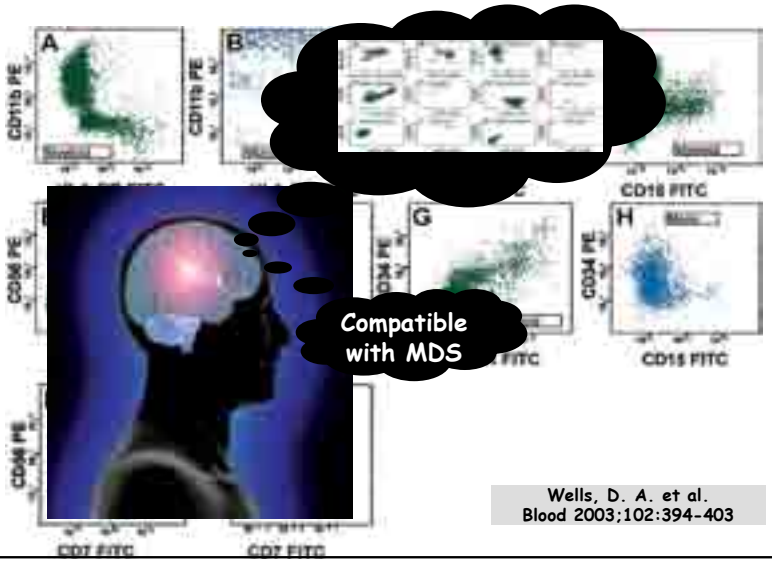


Immunophenotypic Myeloid Abnormalities in MDS



Stetler-Stevenson M et al, Blood 2001;98:979-987

Examples of various myeloid and monocytic aberrant antigenic patterns in MDS



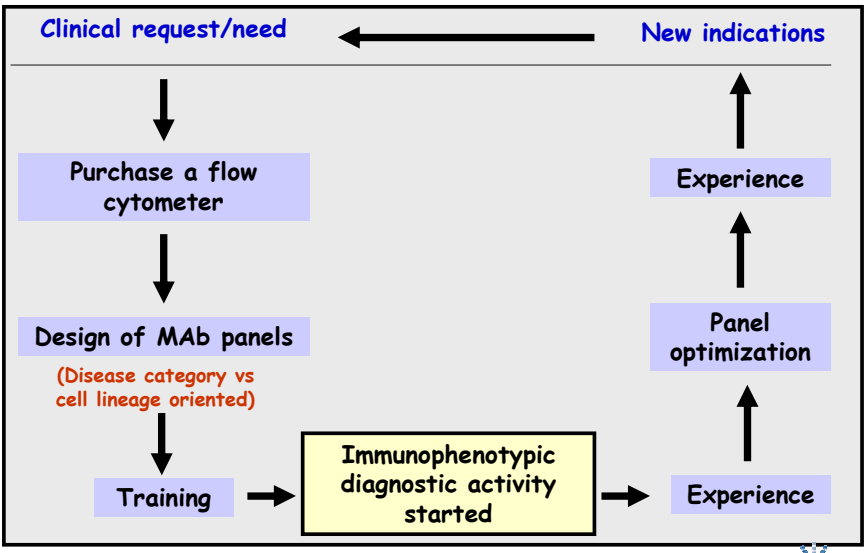
Wells, D. A. et al.
Blood 2003;102:394-403

WHICH PROBLEMS ARE WE FACING ?

- Many reagents: costly and complex panels
- Need expertise in normal (reference) cell populations
- Time consuming
- Technical limitations
- Many (my) suboptimal strategies to reach a similar result
- Not standardized: reproducibly harmonized?
- Partial and more limited clinical utility than expected

EuroFlow

SET UP OF A FCM LABORATORY FOR LEUKEMIA /LYMPHOMA TYPING: CONVENTIONAL PANEL DESIGN

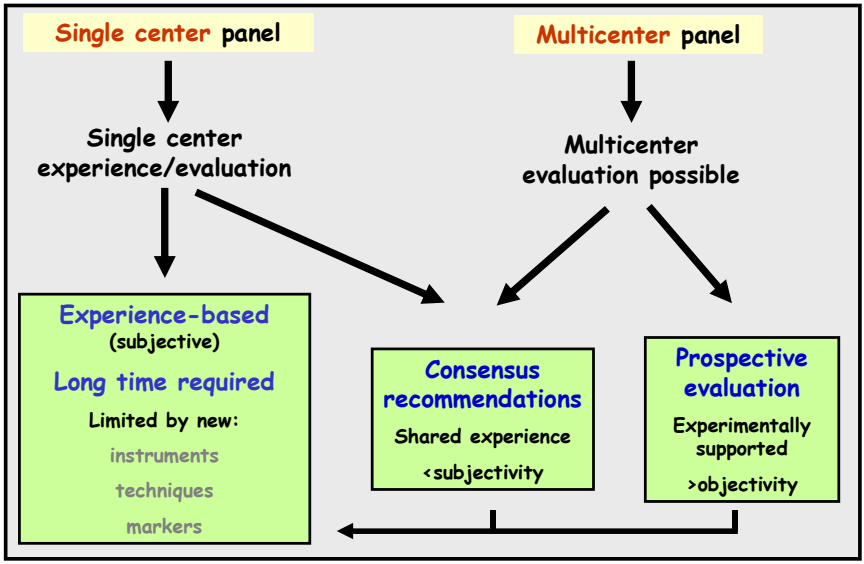


STANDARDIZATION EFFORTS FOR IMMUNOPHENOTYPIC STUDIES

EuroFlow

- 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)

LEUKEMIA /LYMPHOMA IMMUNOPHENOTYPING: EVALUATION OF ANTIBODY PANELS



REQUIRED DEVELOPMENTS IN FLOW CYTOMETRY

(status in 2005)

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

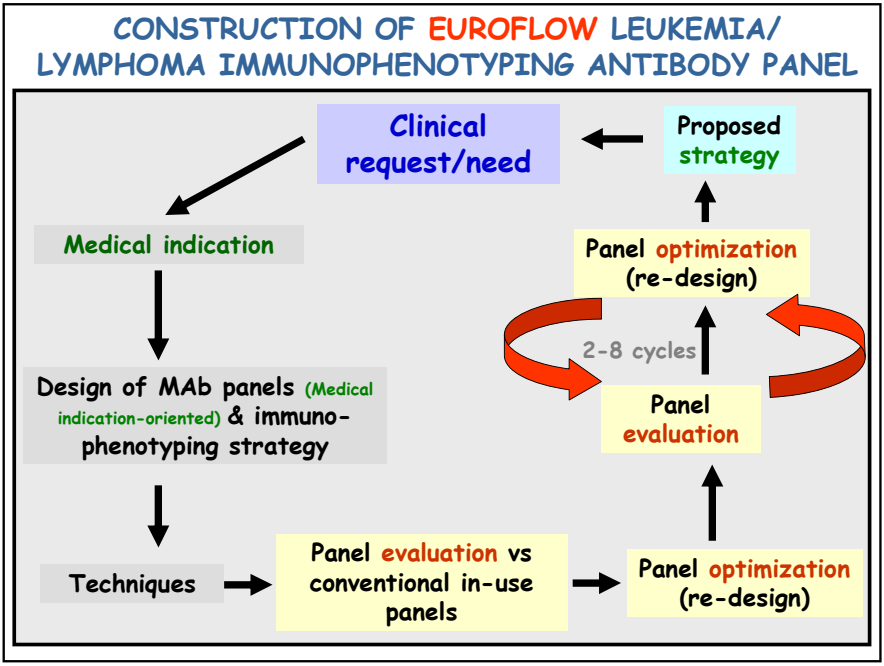
Novel antibodies
- test new (academic) antibodies for application in intracellular stainings
- development of new antibodies against oncoproteins and aberrant signalling pathways

Multicolor flow cytometry: ≥8 color comprehensive panels
- inclusion of solid state violet laser
- selection of appropriate fluorochromes
- compare conjugated antibodies (multiple companies)

Development of novel software for complex pattern recognition
- combining multiple tubes: calculate data & multivariate analyses
- mapping of diagnosis and follow-up leukemia samples against templates of reference "normal/control" samples

EuroFlow


EuroFlow
EuroFlow consortium aims at innovation in flow cytometry
www.euroflow.org
1st EuroFlow meeting, Salamanca (Spain), April 2006


CONSTRUCTION OF EUROFLOW LEUKEMIA/ LYMPHOMA IMMUNOPHENOTYPING ANTIBODY PANEL
Clinical request/need
Proposed strategy
Medical indication
Panel optimization (re-design)
2-8 cycles
Design of MAb panels (Medical indication-oriented) & immuno-phenotyping strategy
Panel evaluation
Techniques
Panel evaluation vs conventional in-use panels
Panel optimization (re-design)

THE EUROFLOW APPROACH TO LEUKEMIA/LYMPHOMA IMMUNOPHENOTYPING

Clinical question

↓

Diagnostic screening tube

↓

"Diagnostic classification" panel

↓

MRD monitoring

Knowledge

Experience Evaluation

Reference profiles

14 Major groups

154 Nosologic entities

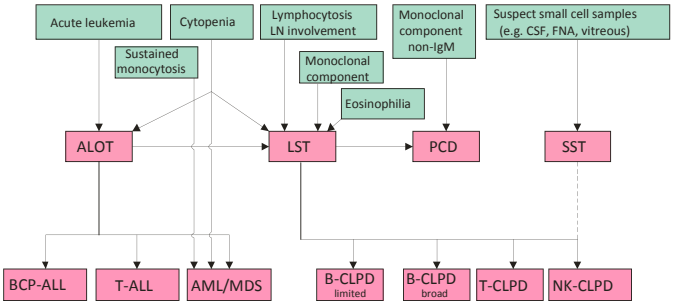
Majority of diseases?

Majority of cases?

New disease entities?

EuroFlow

CONSTRUCTION OF EUROFLOW PANELS: MEDICAL INDICATION ENTRIES AND ORIENTATION/SCREENING PANELS

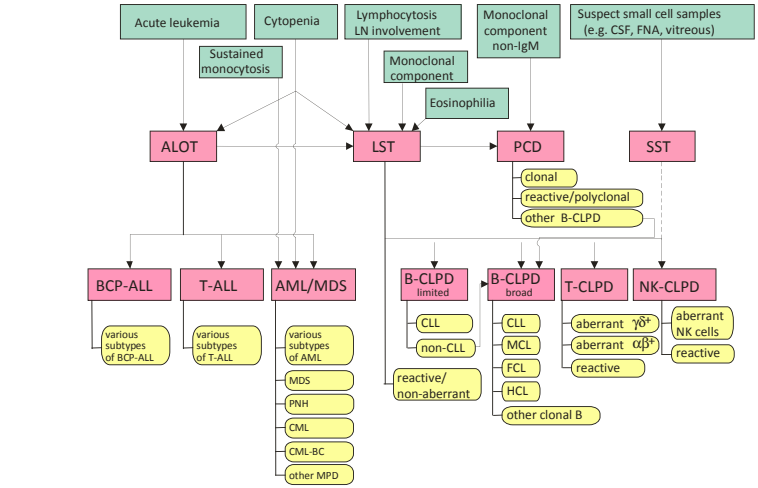


Comprehensive network of panels aimed at the phenotypic diagnosis and characterization of the major WHO entities

Van Dongen et al: EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. To be published in: Leukemia 2011;

EuroFlow

CONSTRUCTION OF EUROFLOW PANELS: MEDICAL INDICATION ORIENTATION/SCREENING & CLASSIFICATION PANELS



Van Dongen et al: EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. To be published in: Leukemia 2011

EuroFlow

CONSTRUCTION OF EUROFLOW ANTIBODY PANELS

Step 0: Design strategy

↓

Step 1: Selection of fluorochromes

↓

Step 2: Selection of markers

↓

Step 3: Selection of antibody reagents

↓

Step 4: Selection of antibody combinations

↓

Step 5: Panel constructed

EuroFlow

CONSTRUCTION OF EUROFLOW ANTIBODY PANELS: STRATEGY & TYPE OF MARKERS

. **Backbone markers (2-4 markers required)** *for the identification of the cell populations of interest:*

- Antibodies placed **in every tube** in a panel
- Essential for simultaneous **evaluation of complete profile** in every cell within a population *(merge-calculation functions)*

. **Characterization markers (> 4 markers/tube)** *for detailed immunophenotypic characterization of the cell populations of interest:*

- **Lineage** assessment,
- **Maturation** stage,
- **Cytogenetic** subgroups,
- **Aberrant** markers for MRD,
- Clinical impact

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FLUOROCHROME SELECTION

Initial selection

FL Channel	Laser	Commonly available fluorochromes
1	Violet	Pacific Blue HORIZON V450
2*	Violet	AmCyan Pacific Orange HORIZON V500
3	Blue	FITC Alexa Fluor 488
4	Blue	PE
5	Blue	PE-TxRed
6	Blue	PerCP Cy5.5 PerCP
7	Blue	PE Cy7
8	Red	APC Alexa Fluor 647
9	Red	APC Cy7 APC H7 Alexa Fluor 700

Further comparisons

FL Channel	Laser	Commonly available fluorochromes
1	Violet	Pacific Blue HORIZON V450
2	Violet	AmCyan* Pacific Orange HORIZON V500
3	Blue	FITC Alexa Fluor 488
4	Blue	PE
5	Blue	PE-TxRed
6	Blue	PerCP Cy5.5 PerCP
7	Blue	PE Cy7
8	Red	APC Alexa Fluor 647
9	Red	APC Cy7 APC H7 Alexa Fluor 700

**Alternative new additional fluorochromes currently under evaluation*

Responsible scientists: T.Kalina, J.Flores

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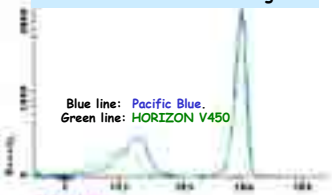
Fluorochrome selection: further comparisons

Laser Channel	Fluorochrome conjugate	MFI*	SI*	Main Overlap	Availability
Violet 1	CD4 Pacific Blue (BD Biosciences)	9,474	61.8	Violet 2 (AmCyan/ Pacific Orange/ Horizon V500)	★
	CD4 HORIZON V450 (BD Biosciences)	9,195	66.6	Violet 2 (AmCyan/ Pacific Orange/ Horizon V500)	★

* Intensity of staining and staining index (SI) obtained for reagents evaluated in normal PB samples (n=5).

Overlay histograms of normal PBL stained with both reagents



% FL compensation requirements in other fluorescence channels

Channel	PB	HV450
1	-	-
2	24.5	20
3	0	0
4	0	0
6	0	0
7	0	0
8	0	0
9	0	0

Responsible scientists: T.Kalina, J.Flores

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B-CLPD panel

Backbone markers:

- Should identify all B cells
- Aberrant underexpression of CD19 and/or CD20 frequently observed
- sIgκ/CD37/sIgλ/CD19/CD22/CD20 tested in 151 B-NHL cases

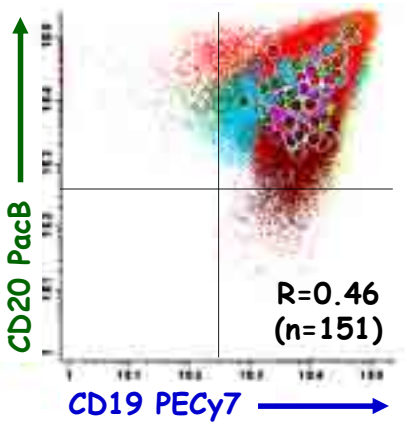
Responsible scientist: Sebastian Bottcher

EuroFlow

B-CLPD panel

Backbone markers:

- Should identify all B cells
- Aberrant underexpression of CD19 and/or CD20 frequently observed
- sIgκ/CD37/sIgλ/CD19/CD22/CD20 tested in 69 B-NHL cases
- **Conclusion**: CD37 & CD22 redundant, as CD20 PacB plus CD19 PE-Cy7 were sufficient to identify all malignant B cells in all cases



Responsible scientist: Sebastian Bottcher

EuroFlow

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		

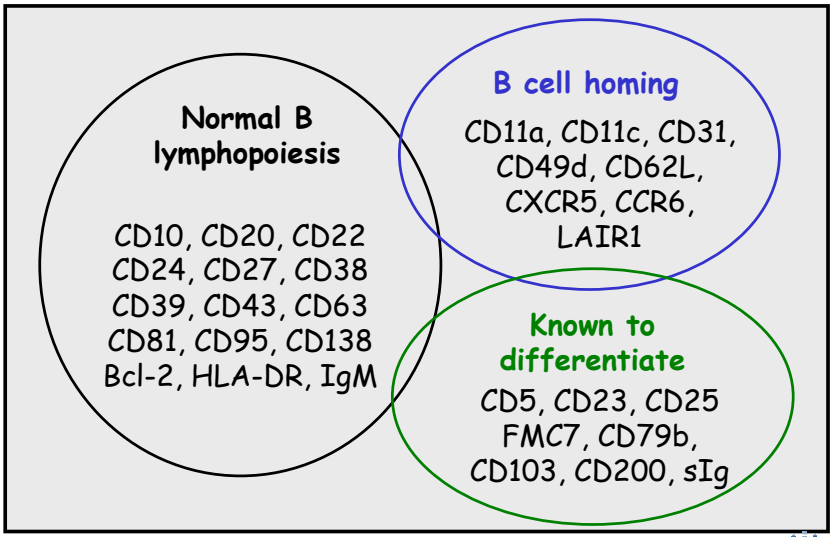
Responsible scientists: Juan Flores and Sebastian Bottcher

EuroFlow

IMMUNOPHENOTYPIC PATTERNS OF DIFFERENT TYPES OF B-CLPD (*Orfao et al, In: "B-CLL".Humana Press, 2004*)

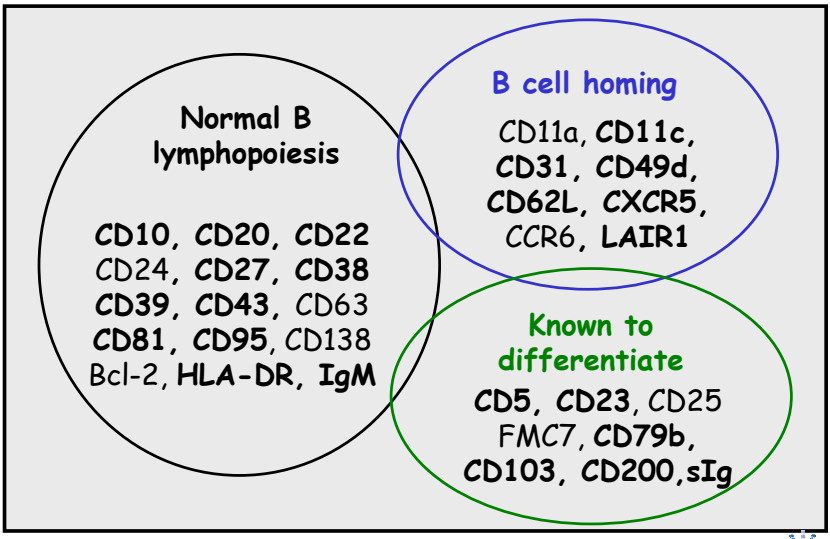
	sIg	CD5	CD10	CD20	CD11c	CD23	CD24	CD25	CD38	CD43	CD79b	CD103	FMC7
B-CLL	d	+	-	d	-/+	++	+	+	-/+	+	d	-	-
PLL	+	-/+	-	+	-/+	-/+	+	-/+	-/+	-/+	+	-	+
HCL	+	-	-	++	++	-	-/+	++	-	-	+	+	+
SMZL	+	-/+	-	+	+	-	+	-/+	-	-	+	-/+	+
LPL	+	-	-	+	-	-	+	+	-/+	-	+	-	-/+
MCL	+	+	-	+	-/+	-	+	-/+	-	+	+	-	-/+
FL	+	-	+	+	-/+	-/d	+	-/+	+	-	+	-	+
LDBCL	+	-	-	+	-/+	-	-/+	-	+	-	+	-	+
BL	-/+	-	+	+	-	-	+	-	++	-/+	-/+	-	+

Characterization markers



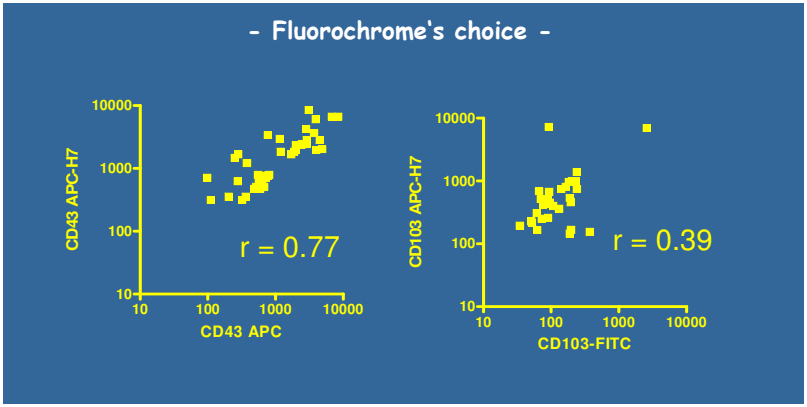
Responsible scientist: Sebastian Bottcher EuroFlow

Characterization markers



Responsible scientist: Sebastian Bottcher EuroFlow

CONSTRUCTION OF EUROFLOW PANELS:
FLUOROCHROME CONJUGATES AND ANTIBODY COMBINATIONS



Responsible scientist: Sebastian Bottcher EuroFlow

BCLPD classification panel

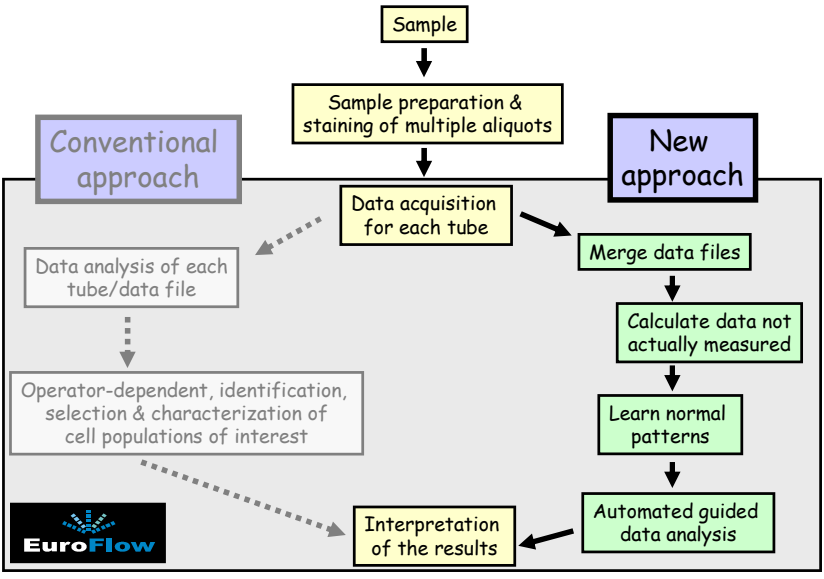
	Pac Blue	Pac Orange	FITC	PE	PerCP-Cy5.5	PECy7	APC	APC-H7
1= LST	CD20 /CD4	CD45	sIgλ /CD8	sIgK /CD56	CD5	CD19 /TCRγδ	CD3	CD38
2	CD20	CD45	CD23	CD10	CD79b	CD19	CD200	CD43
3	CD20	CD45	CD31	LAIR	CD11c	CD19	sIgM	CD81
4	CD20	CD45	CD103	CD95	CD22	CD19	CXCR5	CD49d
5R	CD20	CD45	CD62L	CD39	HLA-DR	CD19	CD27	

CD20/CD4/CD45/sIgl/sIgK/CD8/CD56/CD5/CD19/CD38/CD23/CD10/CD79b/CD200/CD43/CD31/LAIR1/CD11c/sIgM/CD81/CD103/CD95/CD22/CXCR5/CD49d/CD62L/CD39/HLA-DR/CD19/CD27

30-colors flow cytometry !

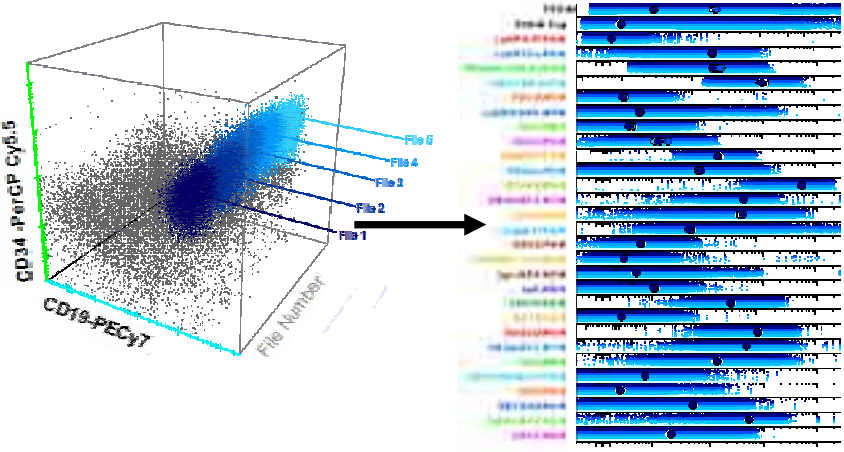
Responsible scientist: Sebastian Bottcher EuroFlow

FLOW CYTOMETRY IMMUNOPHENOTYPING APPROACH



MERGED DATA FILES FOR SINGLE STEP GATING

Full phenotypic profile



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

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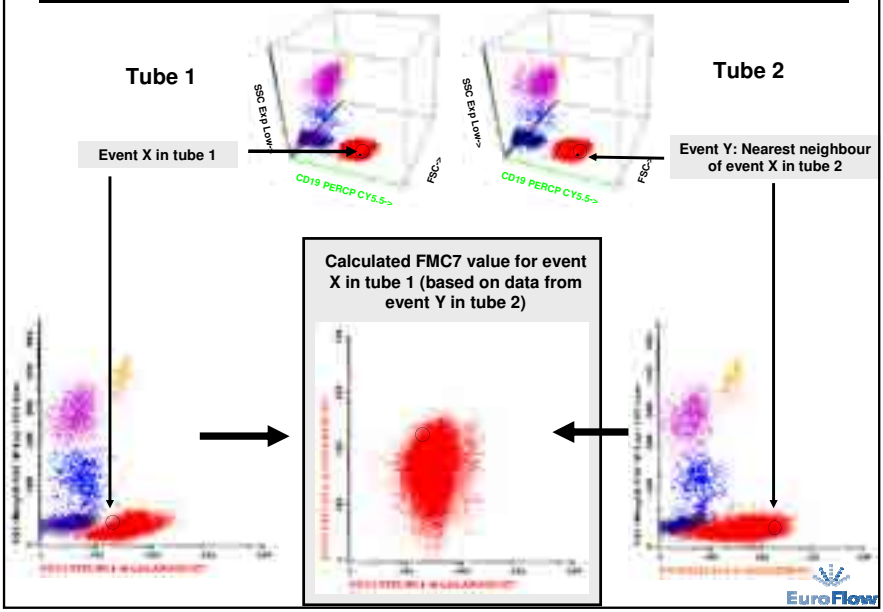
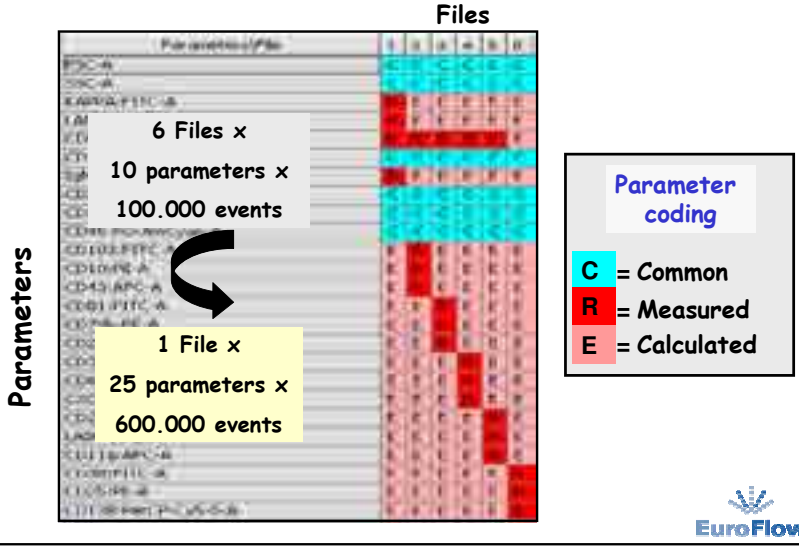
DATA IN A MERGED DATA FILE

	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

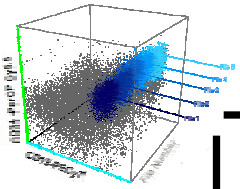
DATA CALCULATION IN THE INFINICYT™ SOFTWARE



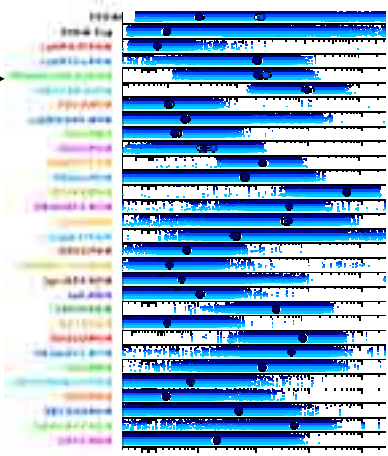
MERGED & CALCULATED LISTMODE DATA FILE



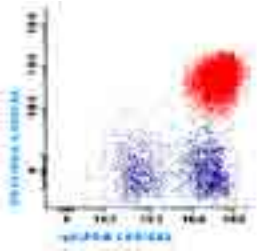
MERGED AND CALCULATED DATA FILE



Full phenotypic profile



Imposible phenotypic profile



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REFERENCE DATAFILES FOR CLL B-CELLS

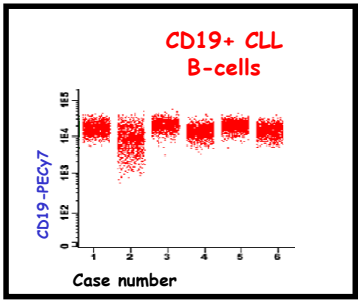
CLL case 1

CLL case 2

CLL case 3

CLL case 4

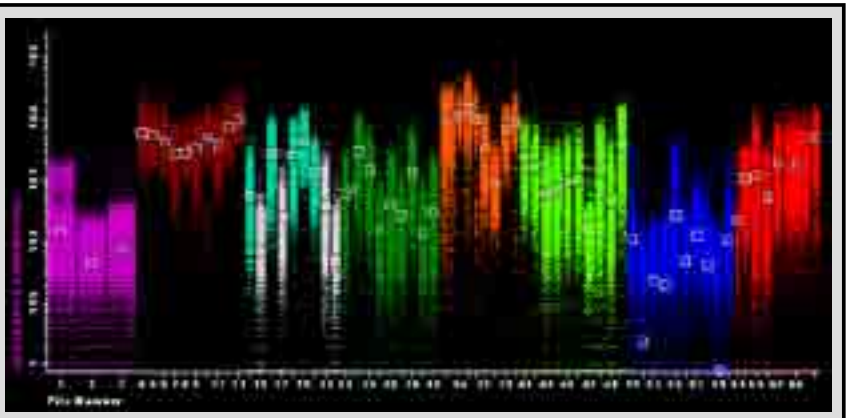
Merge Calculated Data files



Responsible scientist: Sebastian Bottcher

EuroFlow

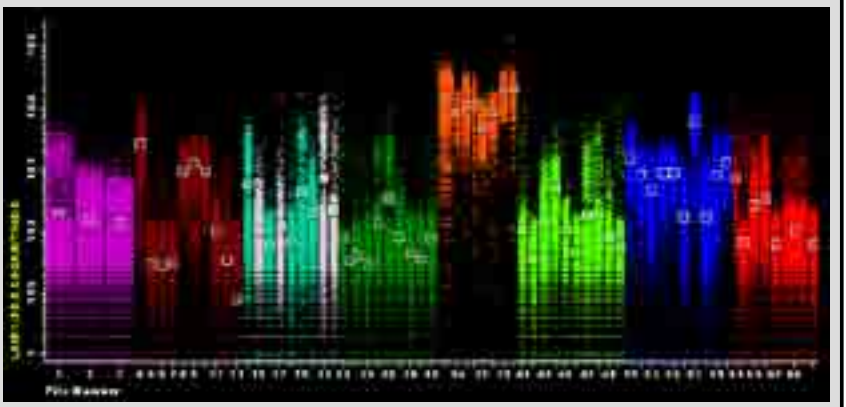
Characterization markers: CD200



BL CLL DLBCL FL HCL LPL MCL MZL

Responsible scientist: Sebastian Bottcher EuroFlow

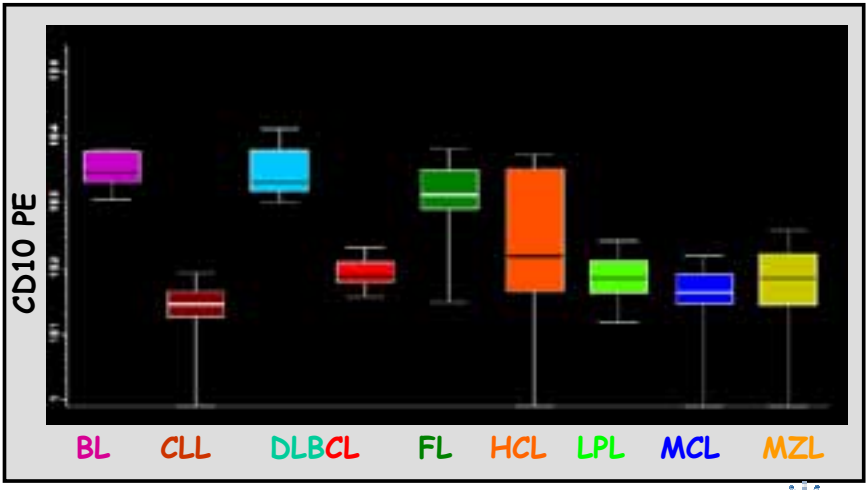
Characterization markers: LAIR1(CD305)



BL CLL DLBCL FL HCL LPL MCL MZL

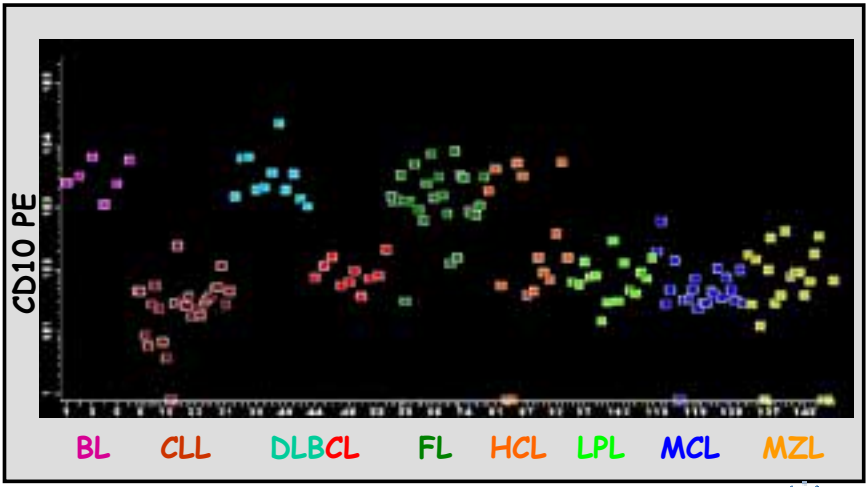
Responsible scientist: Sebastian Bottcher EuroFlow

Characterization markers: CD10



Responsible scientist: Sebastian Bottcher EuroFlow

Characterization markers: CD10



Responsible scientist: Sebastian Bottcher EuroFlow

B-CLPD panel: clinical utility

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

Responsible scientist: Sebastian Bottcher EuroFlow

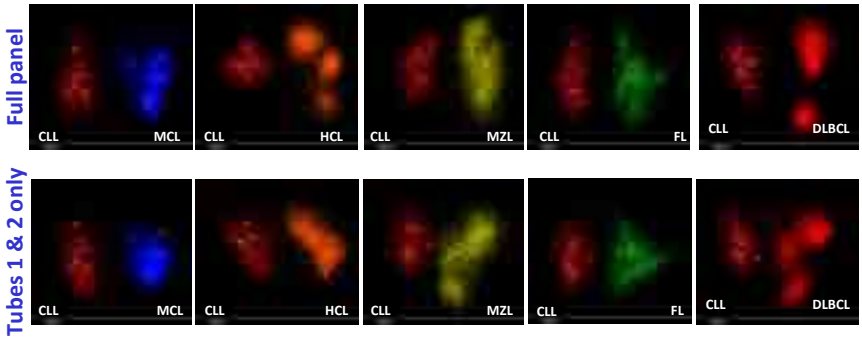
B-CLPD panel: modular design

Full panel

Informative markers only

Responsible scientist: Sebastian Bottcher EuroFlow

BCLPD classification panel: modular design



Responsible scientist: Sebastian Bottcher

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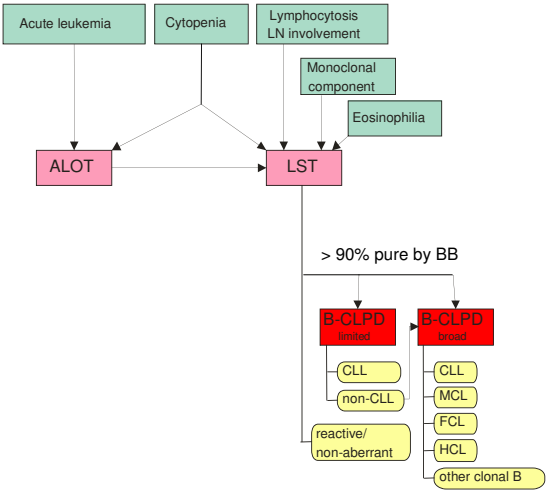
B-CLPD: Diagnostic work-flow



Responsible scientists: Juan Flores and Sebastian Bottcher

EuroFlow

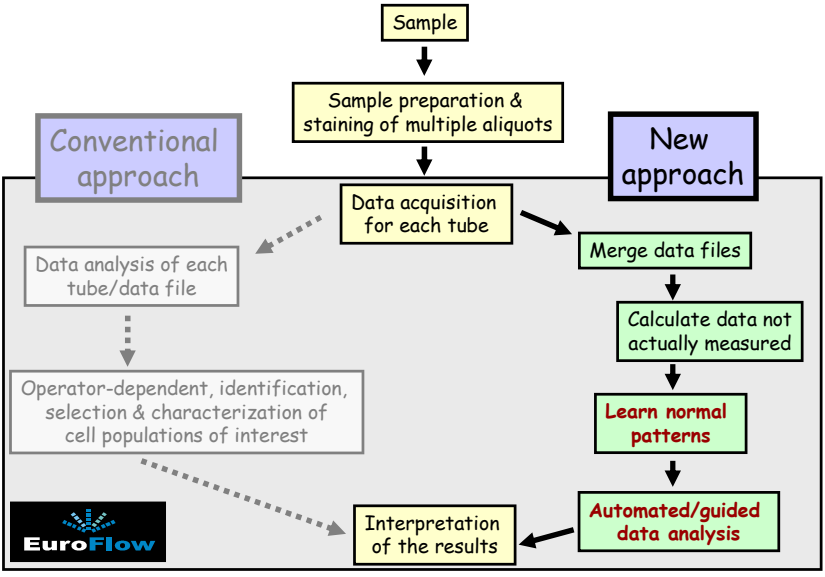
EuroFlow B-CLPD panel: summary

- B-CLPD panel allows unequivocal classification of most mature B-cell malignancies according to WHO
- Most differential diagnoses achieved (n=32/36) efficiently except for the following 1 vs 1 comparisons:

FL vs DLBC
MZL vs LPL
LPL vs DLBCL
MZL vs DLBCL

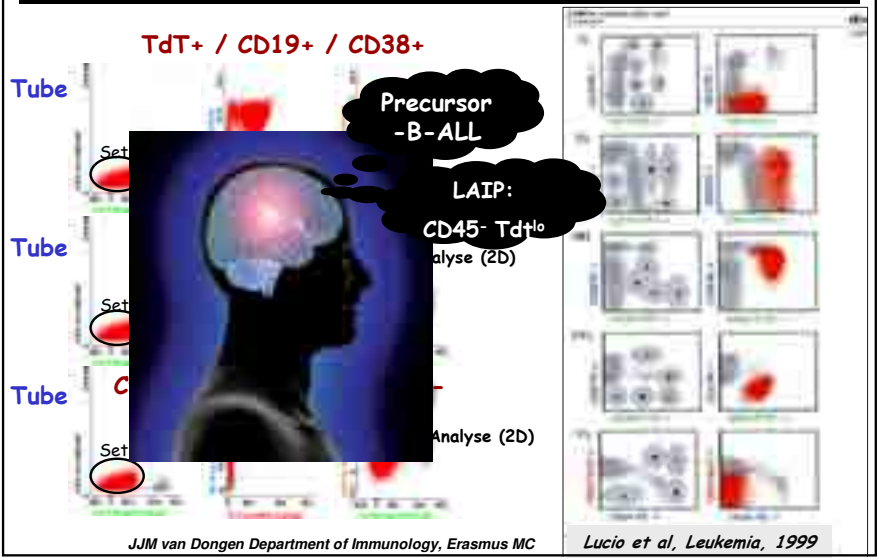
Responsible scientist: Sebastian Bottcher EuroFlow

FLOW CYTOMETRY IMMUNOPHENOTYPING APPROACH



Identification of leukemia-associated immunophenotypes

EuroFlow



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

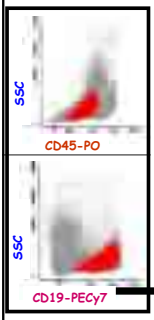
Exponentially growing amount of complex information/data

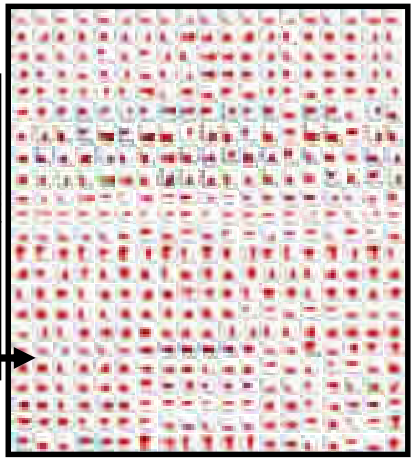
IMMUNOPHENOTYPIC CHARACTERISTICS OF NORMAL vs LEUKEMIC B-CELLS

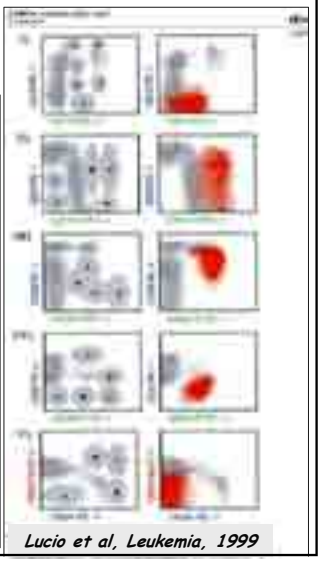
EuroFlow

CD19+ B-CELLS

8- COLOR flow cytometry: Bcp-ALL EuroFlow panel (450 bivariate plots)







Lucio et al, Leukemia, 1999

EuroFlow

HOW TO SIMPLIFY DATA ANALYSIS

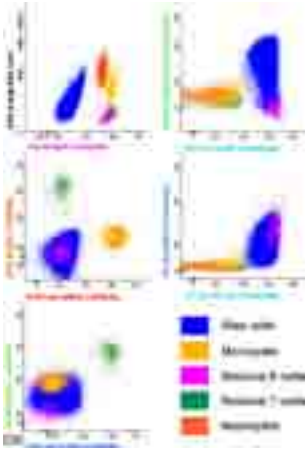
- **Improve the design of antibody panels** for a greater efficiency and higher reproducibility.

- **Construct reference data files** for normal and pathologic cell populations (e.g.: per disease category)

- Multi-n-dimensional **comparison of normal vs pathologic cell populations** (e.g.: at diagnosis and follow-up):

  - Automated PCA-guided approach for homogeneous cell populations (e.g. Blood lymphocyte subsets)
  - Maturation tools for heterogeneous cell populations (e.g. Maturing BM B-cell precursors)

ALOT: B-cell precursor ALL



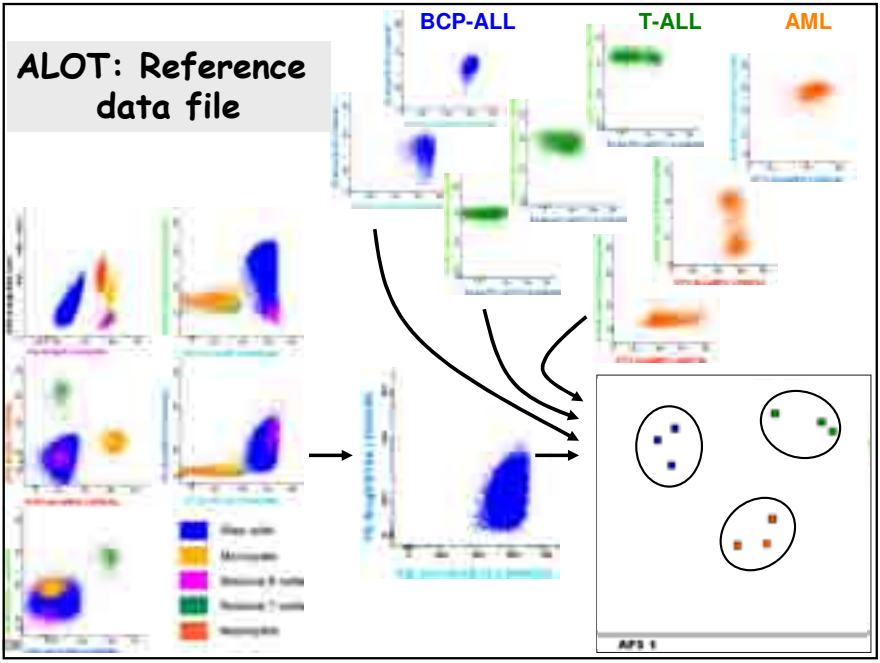
BM stained with
ALOT 8-color tube

CyCD3
CD7
sCD3
CD19
CyCD79a
CyMPO
CD45
CD34

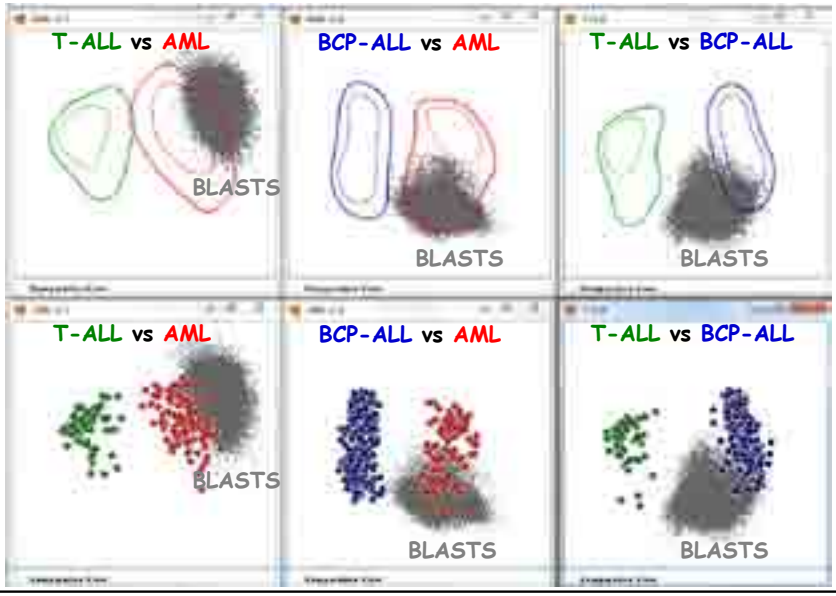
Responsible scientist: Ludovic Lhermitte

EuroFlow

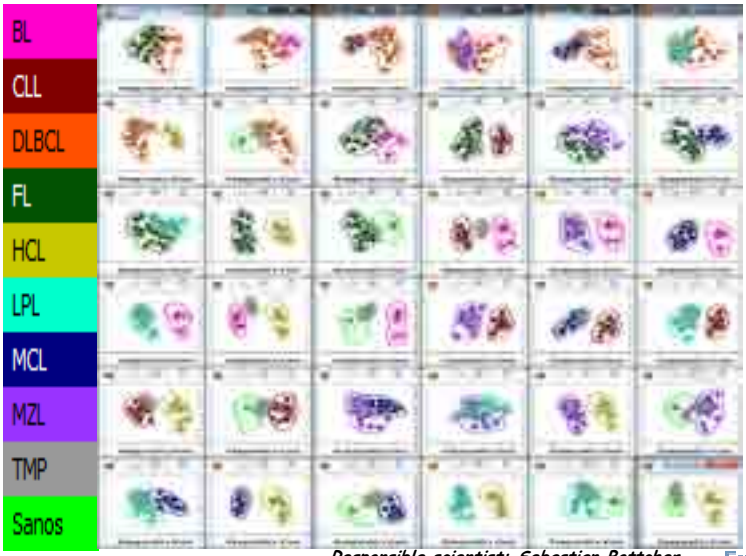
ALOT: Reference data file



ALOT: REFERENCE DATA FILES FOR IMMUNOPHENOTYPIC CLASSIFICATION OF BLASTS



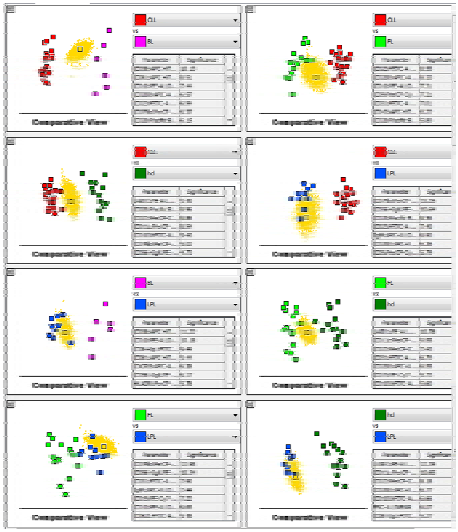
BCLPD panel: classification of an atypical case vs the reference WHO diagnostic groups



Responsible scientist: Sebastian Bottcher

EuroFlow

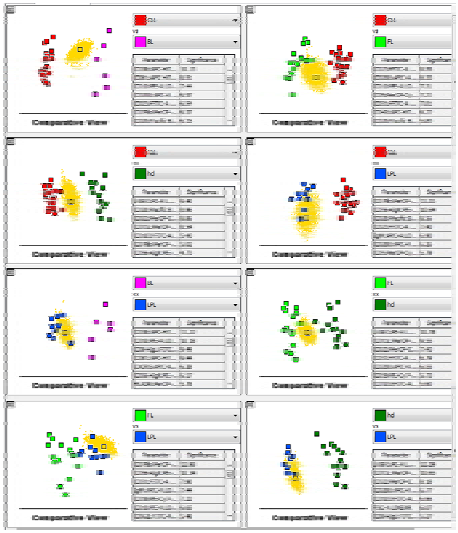
B-CLPD: Comparative analysis of "our case" vs multiple reference groups

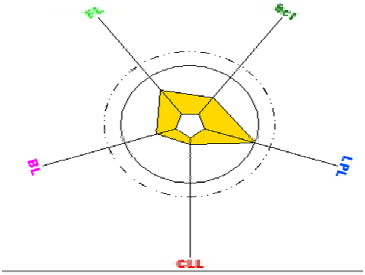


Responsible scientists: Sebastian Bottcher

Costa et al, Leukemia, 2010

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





Responsible scientist: Sebastian Bottcher

Costa et al, Leukemia, 2010

DEVELOPMENT OF 8-COLOR EUROFLOW MRD ANTIBODY PANELS

Single-tube antibody EuroFlow MRD protocols under evaluation

1. **Acute leukemias (include recognition of normal precursors)**
   - Acute myeloid leukemia panel (AML-MRD): 1 tube (M. Cullen)
   - B-cell precursor (BCP-ALL-MRD): 1 tube (V. van der Velden, E. Mejstrikova)
   - T-cell ALL (T-ALL-MRD): 1 tube (V.Asnafi)

2. **Chronic lymphoproliferative disorders**
   - Chronic lymphocytic leukemia (CLL-MRD): 1 tube (L. Lhermitte)
   - Hairy cell leukemia (HCL-MRD): 1 tube (E. Mcentyre)
   - Mantle cell lymphoma (MCL-MRD): 1 tube (S. Böttcher)
   - Follicular lymphoma (FL-MRD): 1 tube (S. Böttcher)
   - Marginal zone lymphoma (MZL-MRD): 1 tube (R. de Tute)
   - Lymphoplasmacytic lymphoma (LPL-MRD): 1 tube (R de Tute)
   - Diffuse large B-cell lymphoma (DLBCL-MRD): 1 tube (P. Lucio)
   - Burkitt lymphoma (BL): 1 tube (R. de Tute)
   - T-chronic lymphoproliferative diseases (T-CLPD-MRD): 1 tube (J. Almeida)
   - Multiple myeloma (MM): 1 tube (J. Flores)

HOW TO SIMPLIFY MRD STRATEGIES

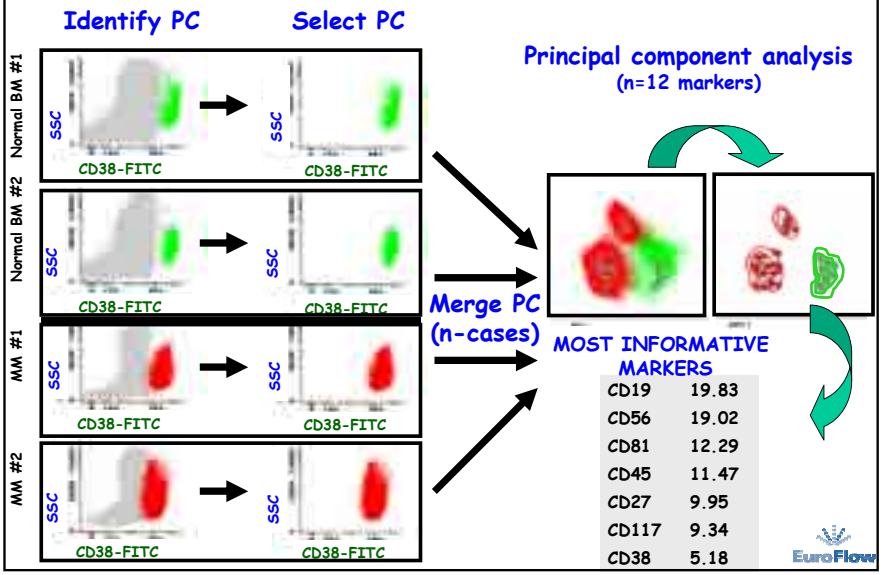
- Improve the design of MRD panels for a greater efficiency and higher reproducibility.

- **Construct reference data files for normal and neoplastic cells (e.g.: per disease category)**

- Multi-n-dimensional comparison of normal vs neoplastic cell populations (e.g.: at diagnosis and follow-up):

  - Automated PCA-guided approach for homogeneous cell populations (e.g. lymphoid)
  - Maturation tools for heterogeneous cell populations (e.g. myeloid)

CONSTRUCTION OF EuroFlow MRD PANELS: MM



Aim of Euroflow MRD approach for B-CLPD

- To find a marker combination to discriminate all neoplastic B-cells in every B-CLPD case vs all normal/reactive PB and BM mature B-cells/hematogones

NOT an individualized approach per patient

↓

the approach should work without knowledge of the exact initial immunophenotype

BCLPD classification panel

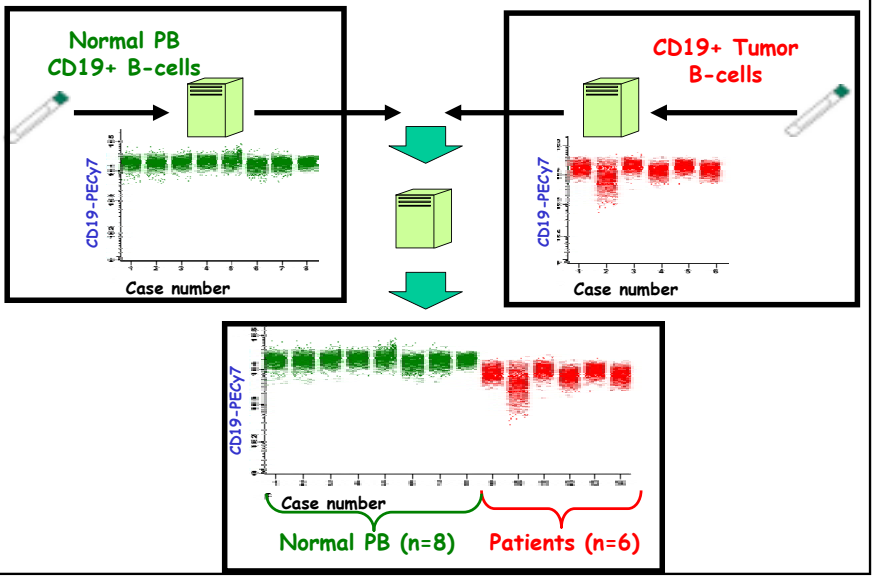
	Pac Blue	Pac Orange	FITC	PE	PerCP-Cy5.5	PECy7	APC	APC-H7
1= LST	CD20 /CD4	CD45	sIgλ /CD8	sIgK /CD56	CD5	CD19 /TCRγδ	CD3	CD38
2	CD20	CD45	CD23	CD10	CD79b	CD19	CD200	CD43
3	CD20	CD45	CD31	LAIR	CD11c	CD19	sIgM	CD81
4	CD20	CD45	CD103	CD95	CD22	CD19	CXCR5	CD49d
5R	CD20	CD45	CD62L	CD39	HLA-DR	CD19	CD27	

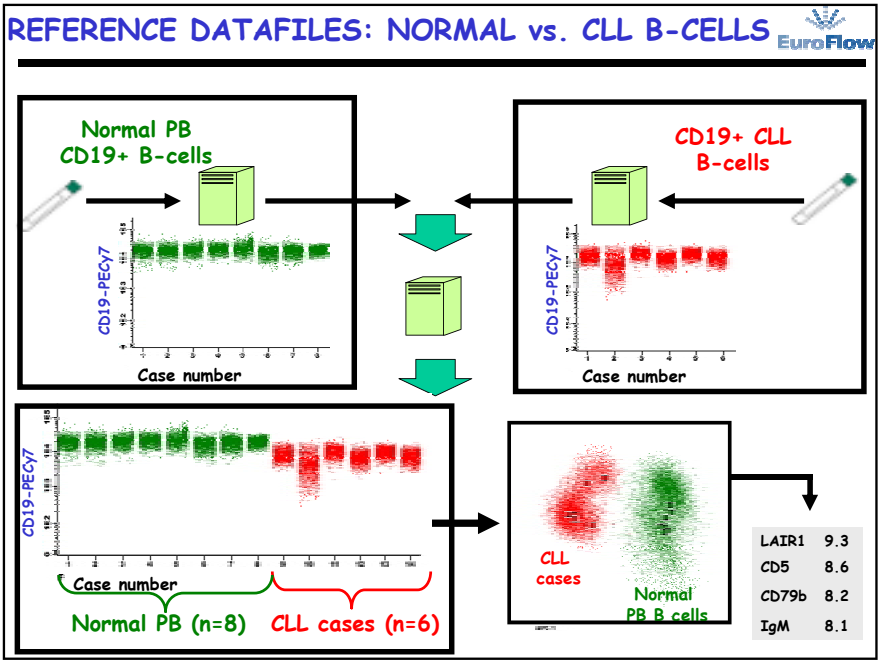
CD20/CD4/CD45/sIgl/sIgK/CD8/CD56/CD5/CD19/CD38/CD23/CD10/CD79b/CD200/CD43/CD31/LAIR1/CD11c/sIgM/CD81/CD103/CD95/CD22/CXCR5/CD49d/CD62L/CD39/HLA-DR/CD19/CD27

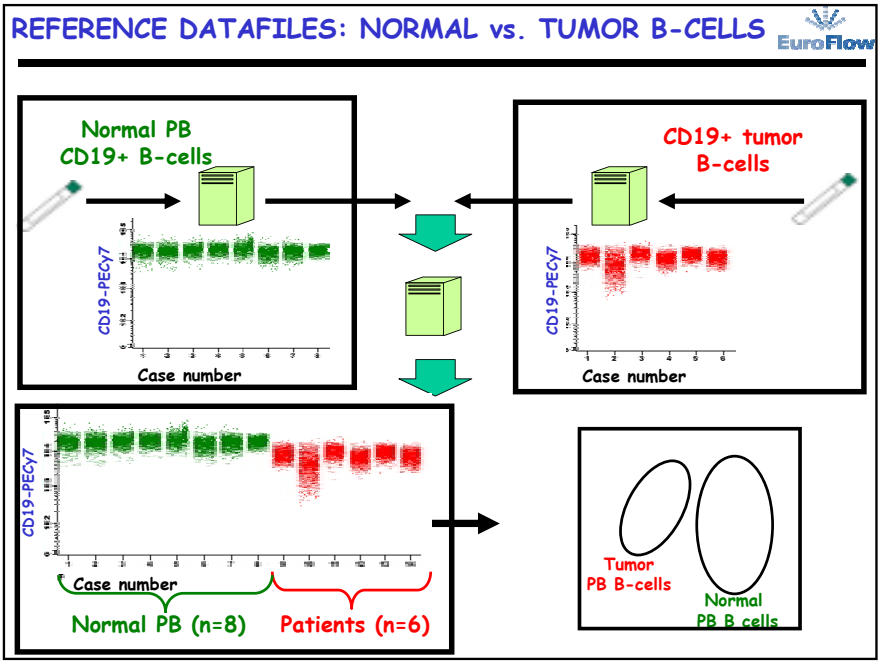
30-colors flow cytometry !

Responsible scientist: Sebastian Bottcher EuroFlow

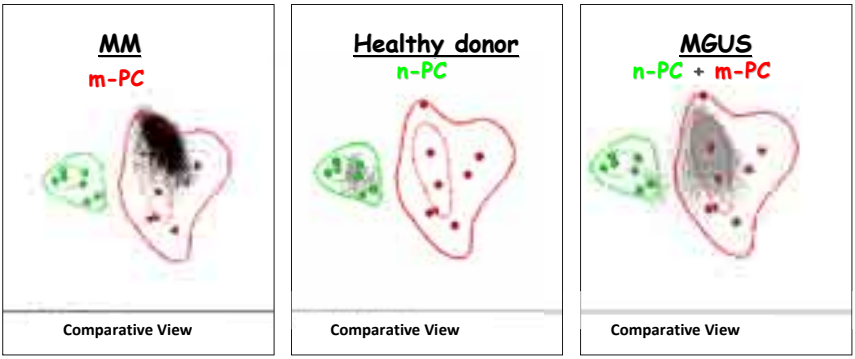
REFERENCE DATAFILES: NORMAL vs Tumor B-CELLS EuroFlow




REFERENCE DATAFILES: NORMAL vs. CLL B-CELLS
EuroFlow
Normal PB
CD19+ B-cells
CD19-PECy7
Case number
CD19+ CLL
B-cells
CD19-PECy7
Case number
CD19-PECy7
Case number
Normal PB (n=8)
CLL cases (n=6)
CLL
cases
Normal
PB B cells
LAIR1 9.3
CD5 8.6
CD79b 8.2
IgM 8.1


REFERENCE DATAFILES: NORMAL vs. TUMOR B-CELLS
EuroFlow
Normal PB
CD19+ B-cells
CD19-PECy7
Case number
CD19+ tumor
B-cells
CD19-PECy7
Case number
CD19-PECy7
Case number
Normal PB (n=8)
Patients (n=6)
Tumor
PB B-cells
Normal
PB B cells

Automated classification of normal vs malignant plasma cells in MM, healthy donors and MGUS



EuroFlow

HOW TO SIMPLIFY DATA ANALYSIS

- **Improve the design of antibody panels** for a greater efficiency and higher reproducibility.

- **Construct reference data files** for normal and pathologic cell populations (e.g.: per disease category)

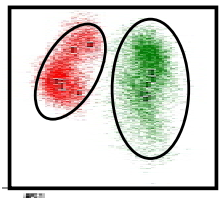
- Multi-n-dimensional **comparison of normal vs pathologic cell populations** (e.g.: at diagnosis and follow-up):

  - Automated PCA-guided approach for homogeneous cell populations (e.g. Blood lymphocyte subsets)
  - Maturation tools for heterogeneous cell populations (e.g. Maturing BM B-cell precursors)

COMPARE A CASE VS NORMAL & NEOPLASTIC B-CELLS

EuroFlow

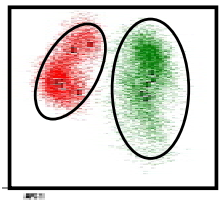
REFERENCE DATA FILES



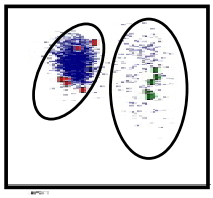
COMPARE A CASE VS NORMAL & NEOPLASTIC B-CELLS

EuroFlow

REFERENCE DATA FILES



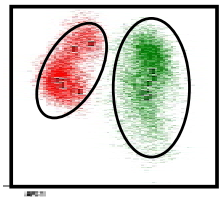
DIAGNOSTIC SAMPLES
vs.
REFERENCE DATA FILES

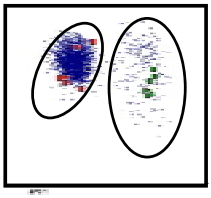


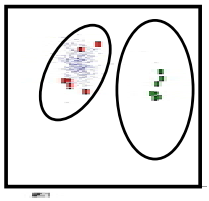
COMPARE A CASE VS NORMAL & NEOPLASTIC B-CELLS

EuroFlow

REFERENCE DATA FILES	DIAGNOSTIC SAMPLES vs. REFERENCE DATA FILES	MRD SAMPLES vs. REFERENCE DATA FILES

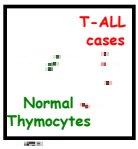
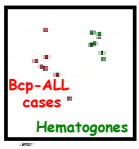
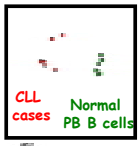
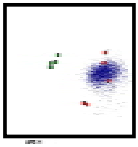
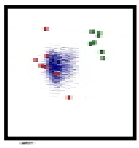
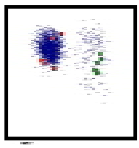
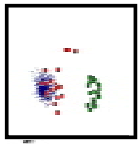
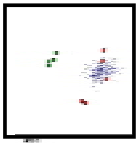
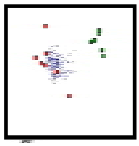
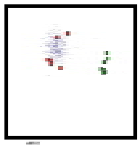
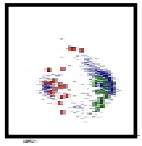




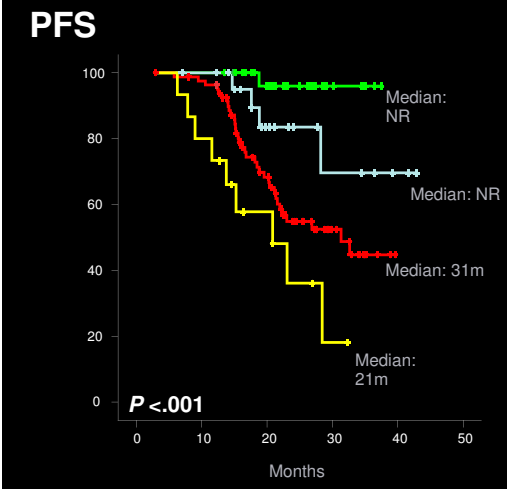


COMPARE A CASE VS NORMAL & NEOPLASTIC B-CELLS

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				

Impact on PFS of the depth of response after induction therapy (n=153)



MRD negative	97% at 3y
CR (IFx-)	70% at 3y
nCR + PR	45% at 3y
MR or SD	0% at 3y

Paiva et al; J Clin Oncol. 2011

EuroFlow

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- Improve the design of antibody panels for a greater efficiency and higher reproducibility.

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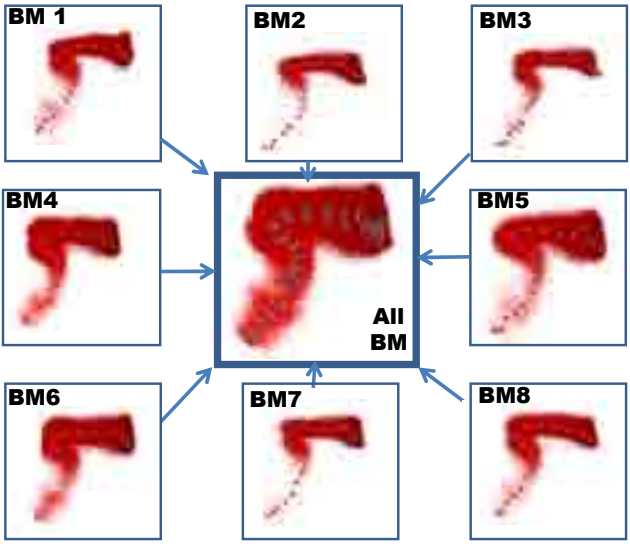
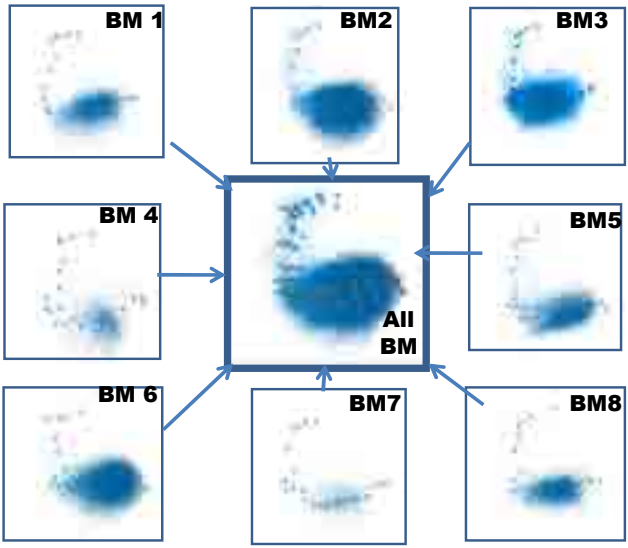
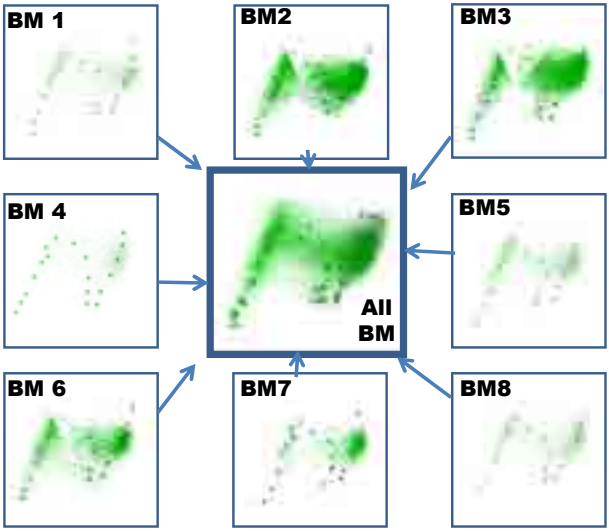
Neutrophil maturation in normal BM: definition of maturation stages based on principal component analysis (PCA) Of 10 parameters



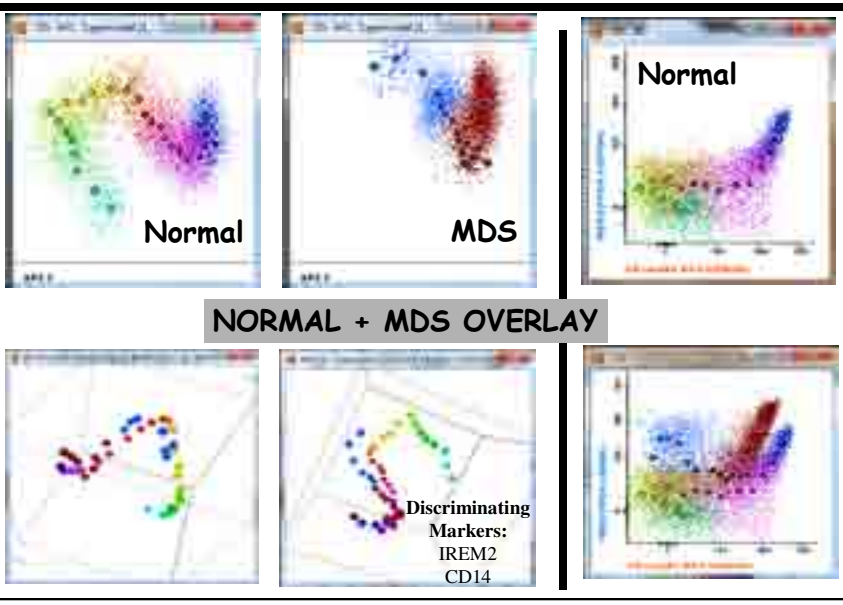
Erythroid maturation in normal BM: definition of maturation stages based on principal component analysis (PCA) of data on 10 parameters



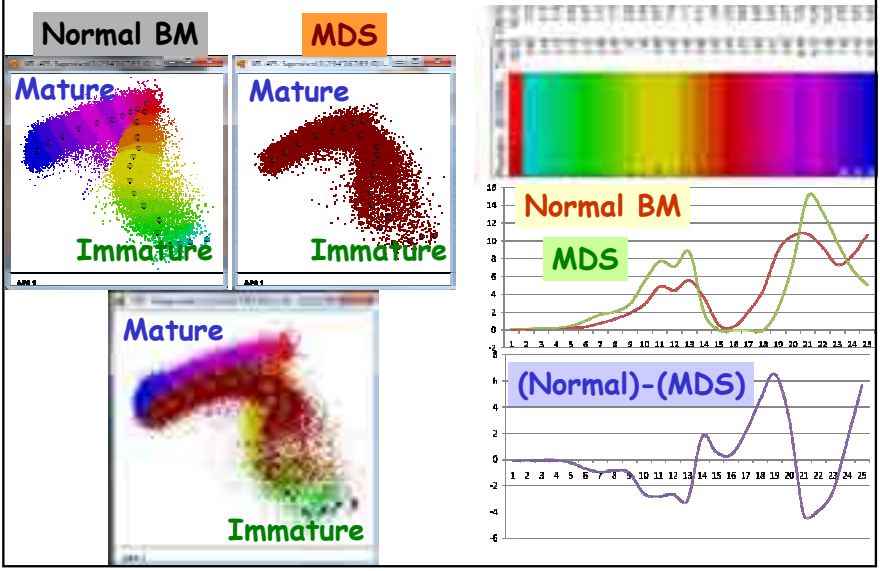
Monocytic maturation in normal BM: definition of maturation stages based on principal component analysis (PCA) of data on 10 parameters



NEUTROPHIL MATURATION IN NORMAL VS MDS BM



NEUTROPHIL MATURATION IN NORMAL VS MDS BM



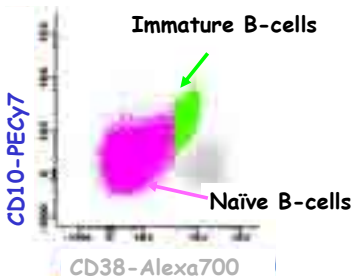
CIRCULATING NORMAL PB B-CELL COMPARTMENTS

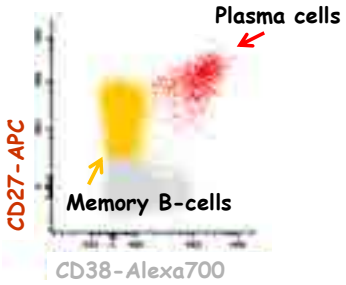
PRE-GC PB B-CELLS

Immature + naïve sIgM non-SHM B-cells in human PB.

POST-GC CD27 PB B-CELLS

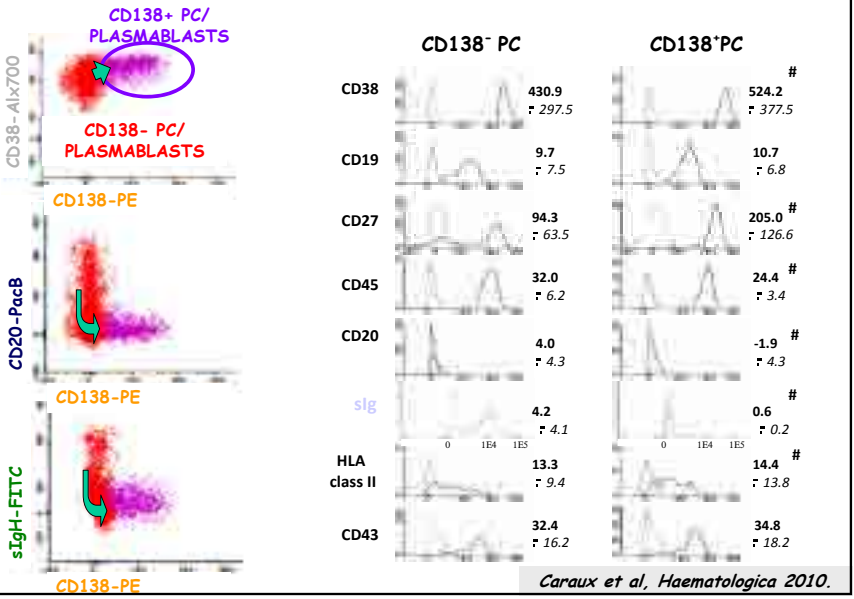
CD27^{+} human PB B-cells include CD38^{-} memory B-cells & CD38^{hi} PC





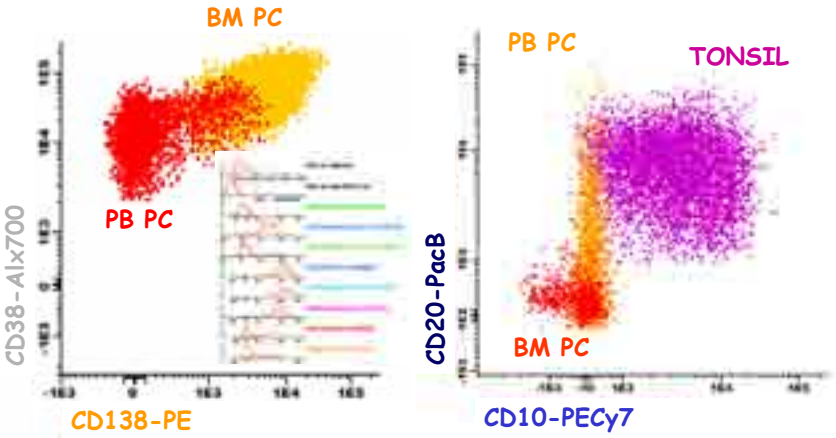
Sims et al, Blood 2005; Lee et al J. Immunol 2009 Klein et al, J Exp Med 1998; Tangye et al, J Exp Med 1998

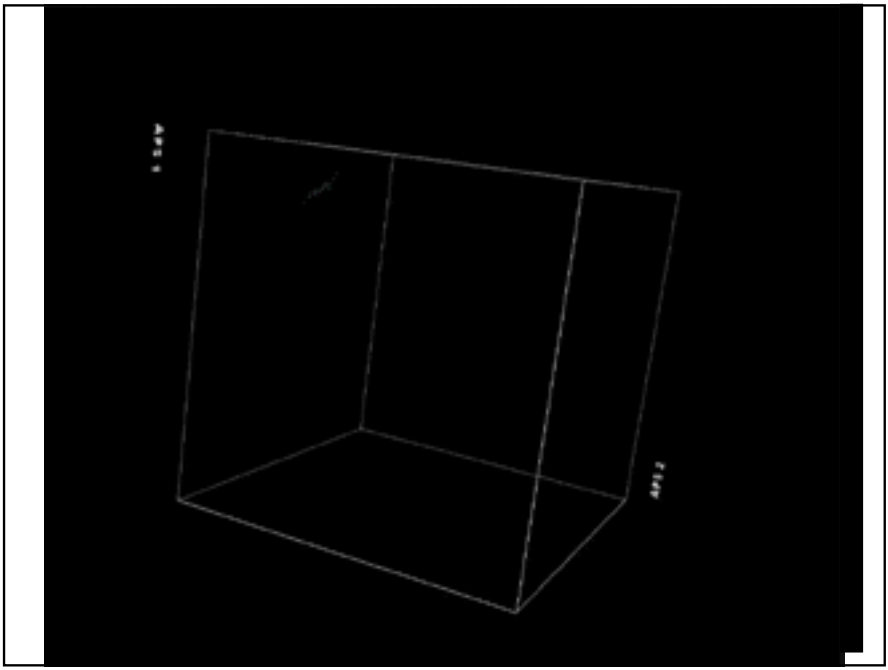
CIRCULATING PB PLASMABLASTS/PLASMA CELLS



Caraux et al, Haematologica 2010.

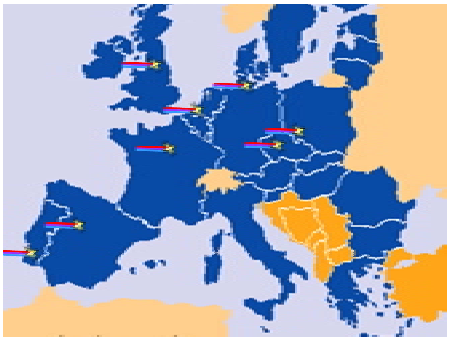
IMMUNOPHENOTYPIC PROFILE OF PB vs BM PLASMABLASTS/ PLASMA CELLS





UTILITY OF THE NEW SOFTWARE TOOLS
Standardization of FCM immunophenotyping

- How to get optimal and comparable measurements?
- Which are the most appropriate fluorochromes?
- What is the optimal sample preparation protocol?



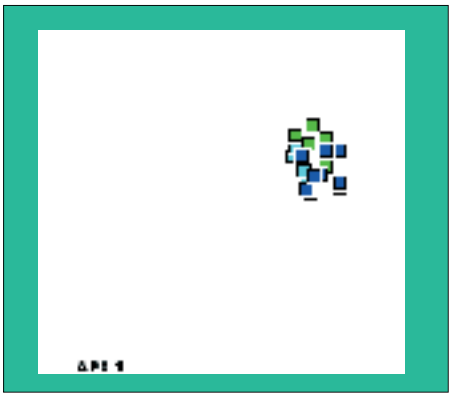
REPRODUCIBLE & OBJECTIVE RESULTS

EuroFlow

Results of standardized experiments

EuroFlow Panel for Plasma Cell Disorders: Can we identify the center?

Normal BM PC from 24 samples from different centres, acquired over a one year period.



EuroFlow

Which problems are we facing?

- Many reagents (costly and complex)
- Need expertise (reference profiles)
- Time consuming
- Technical limitations
- Many (my) strategies (suboptimal)
- Not standardized (reproducible?)
- Limited clinical utility

Which are we solving?

- No redundancy
- Computer expertise
- Fast
- Real multi-D FCM
- Harmonized
- Reproducible
- Higher clinical utility

www.euroflow.org

EuroFlow participants

EuroFlow

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HGSA, Porto, PT	M. Lima, AH Santos
UFRJ, Rio de Janeiro, BR	C. Pedreira, E.S. Costa

Companies (SME's)

DYNOMICS, Rotterdam, NL	E. Dekking, F. Weerkamp …
CYTOGNOS, Salamanca, ES	M. Martin, J. Bensadon, J. Hernandez, M. Muñoz …

THANK YOU