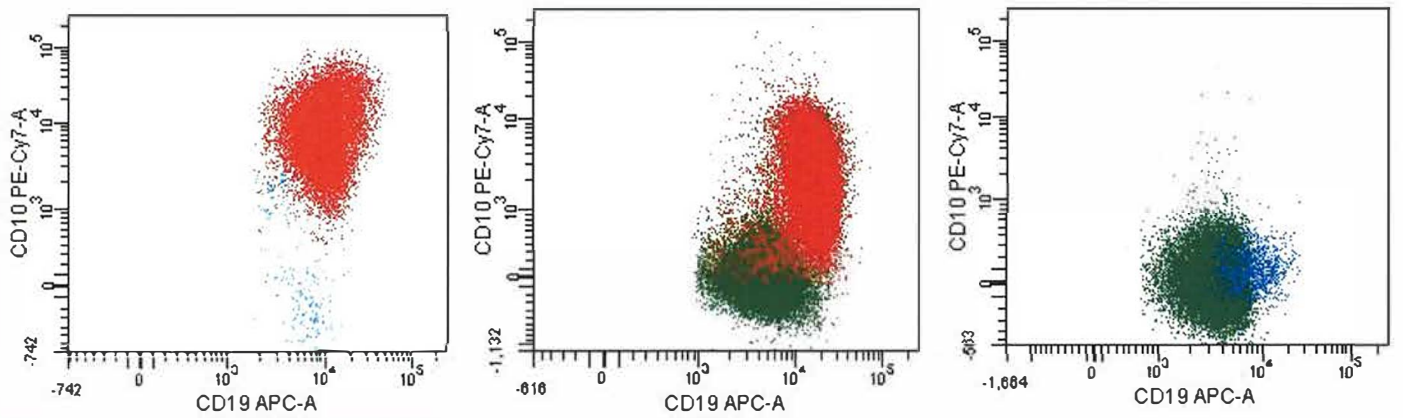
ONKOLOŠKI INŠTITUT LJUBLJANA
INSTITUTE OF ONCOLOGY LJUBLJANA

Slovensko združenje za pretočno citometrijo

DIAGNOSTIKA AKUTNE LIMFOBLASTNE LEVKEMIJE IN MINIMALNE REZIDUALNE BOLEZNI Z VEČ BARVNIM PRETOČNIM CITOMETROM

DIAGNOSING ACUTE LEUKEMIA AND MINIMAL RESIDUAL DISEASE WITH MULTIPARAMETRIC FLOW CYTOMETER



Onkološki inštitut Ljubljana
30. november 2015

Strokovni in organizacijski odbor:

doc. dr. Veronika Kloboves Prevodnik, dr.med.

dr. Jaka Lavrenčak, univ.dipl.biol.

Andreja Brožič, univ.dipl.biol.,prof.biol.

Simon Buček, univ.dipl.biokem.

Urednika zbornika:

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Andreja Brožič, univ.dipl.biol.,prof.biol.

Organizator in izdajatelj (založnik):

Oddelek za citopatologijo, Onkološki inštitut, Ljubljana

Slovensko združenje za pretočno citometrijo

Ljubljana, 2015

Program

14.00- 14.20 Flow cytometric immunophenotyping at the Institute of Oncology Ljubljana; historic overview *(Veronika Kloboves Prevodnik, Institute of Oncology Ljubljana, Slovenia)*

14.20-14.40 One year of flow cytometric diagnostics of MRD-ALL at the Institute of Oncology Ljubljana *(Andreja Brožič, Institute of Oncology Ljubljana Slovenia)*

14.40- 15.40 Eight- and 10-color flow cytometric diagnostics of acute leukemias and MRD in children, optimal panels of antibodies *(Michael Dworzak, St. Anna Kinderspital, Vienna, Austria)*

15.40-16.00 Clinical importance of MRD-ALL at 15thand 33th day and during the recurrent disease *(Janez Jazbec, Pediatric clinic Ljubljana, Slovenia)*

16.00-16.20 Coffee break

16.20-16.50 Differentiation between MRD-ALL and hematogones, difficulties and *pitfalls (Angela Schumich, St. Anna Kinderspital, Vienna, Austria)*

16.50-17.10 Set-up and compensation of 10-color flow cytometer in every day practice *(Dieter Prinz, St. Anna Kinderspital, Vienna, Austria)*

17.10-17.40 Set-up and compensation of 8-color flow cytometer; EuroFlow view *(Tomaš Kalina, Childhood Leukemia Investigation, Prague, Czech Republic)*

17.40-18.10 Set-up and compensation of 10-color flow cytometer; BD view *(Jiri Sinkora, BD Biosciences)*

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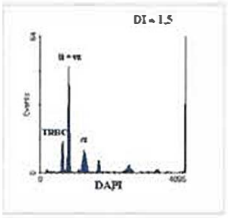
Flow cytometric immunophenotyping at the Institute of Oncology Ljubljana; historic overview

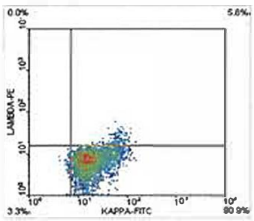
Veronika Kloboves Prevodnik
Department of Cytopathology
Institute of Oncology
Ljubljana, Slovenia

Flow Cytometry at the Institute of Oncology Ljubljana

- DNA period
  - Since 1988
  - DNA ploidy and proliferative activity



- FCI period
  - Since 1997
  - Diagnosing lymphomas



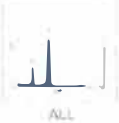
DNA analysis from 1988 to 2015

- 1988: **PAS I** (Partec)
- 1991: **PAS II** (Partec)
- 2001: **PAS** (Partec)
- 2009: **CyFlow Space** (Partec)


PAS


CyFlow Space

- DNA ploidy/proliferative activity
  - Research
    - Monitoring response to treatment
    - Prognostic value
  - Diagnostic
    - Neuroblastoma
    - ALL


ALL

FCI from 1997 to 2015 and beyond

- **1997: BD FACSCalibur (from 2 to 4 colors)**
  - 1997: 2-color FCI, standard protocols for sample preparation
  - 2000: in-house protocol for sample preparation
  - 2001: 3-color FCI
  - 2005: 4-color FCI
  - 2006: beginning of quantitative FCI
- **2007: BD FACSCanto II (6-colors)**
  - 2008: 5-color FCI
  - 2009: bone marrow
  - 2010: 6-color FCI
  - 2014: MRD-ALL
- **2015: BD FACSCanto 10c (10-colurs)**
  - Setup and compensation
  - Creating 8- and 10-color panels


BD FACSCalibur


BD FACSCanto II


BD FACSCanto 10c

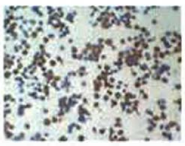
Cytopathological diagnostics of lymphomas at the Institute of Oncology Ljubljana

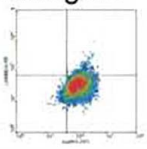
- Based on cell morphology, immunophenotype and molecular genetic features
- Well excepted method in diagnostic evaluation in lymphoma patients
- Not always like that!


Cervical lymphadenopathy

19 years ago

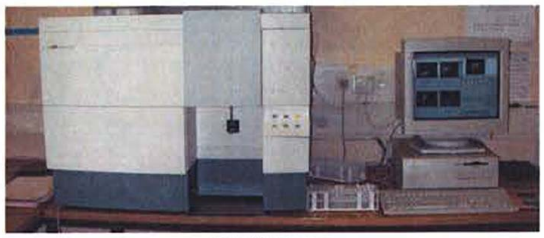
- Many discordant cytopathological and histopathological diagnoses
- Question
  - should cytopathological examination still be used in diagnostic evaluation of lymphoma patients?
- Solution → Prospective study
  - Which method should be used for the determination of lymphoma antigen?


Immunocytochemistry


Flow Cytometry

Conclusion

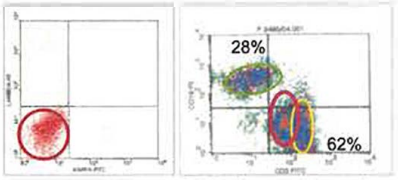
- Flow cytometric method more sensitive and specific than immunocytochemical method.
- Two-color flow cytometer was bought.



BD FACSCalibur

1997: Beginnings of FCI by BD FACSCalibur

- Problems
  - Low cellularity of cytological samples
  - Interpretation of results



s- negative CD3 dim positive cells

  - Low sensitivity of lymphoma detection

Solutions

1. International collaboration

Brian K. Hanton and Ian Brotherick

Solutions

2. To improve sensitivity and specificity

- In-house protocol for cytological sample preparation
  - Prevents cell loss during sample preparation
  - 150 000 cells per tube
- Upgrading cytometer with additional laser
  - From 2-color to 4-color FCI
- 4 tube initial B-cell lymphoma panel
  - 9 different antibodies
- Gating strategy
  - From FSC/SSC to SSC/ CD45 or CD19

Increased sensitivity but same specificity

Sensitivity and specificity

Author/year	Sensitivity	Specificity	Number of cases
Dunphy and Ramos, 1997	0.80	**1.0**	73
Young et al, 1998	0.80	**1.0**	100
Jeffers et al, 1998	0.86	**1.0**	46
Meda et al, 2000	0.95	**0.85**/1.00*	290
Dong et al, 2001	**0.76**	/	139
Zeppa et al, 2004	0.93	**1.0**	307
Bangerter et al, 2007	0.85	**1.0**	131
Swart et al, 2007	**0.97**	0.87	124
Zeppa et al, 2010	0.95	0.99	446
Institute of Oncology	**0.93**	**0.99**	**144**

2007: 6-color flow cytometer (BD FACSCanto II)

- Bone marrow and peripheral blood FCI
  - From 4-color to 6-color FCI
- Retrospective quantification of antigen expression
- Poorly cellular vitreous samples

Cytopathological examination and FCI of bone marrow

- 2008 - international guidelines for bone marrow examination


REVIEW ARTICLE INTERNATIONAL JOURNAL OF LABORATORY HEMATOLOGY

ICSH guidelines for the standardization of bone marrow specimens and reports

S.-H. LEE[1], W. N. ERBER[2], A. PORWIT[3], M. TOMONAGA[4], L. C. PETERSON[5] For The International Council for Standardization in Hematology

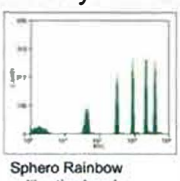
- 2009 - implementation of guidelines at our Institute
  - Aspiration and trephine biopsy of bone marrow performed simultaneously
  - Multidisciplinary diagnostic approach

Haematopathological conferences since 2011

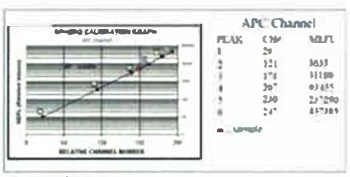
- **Aim:**
  - Discuss difficult lymphoma cases from different point of views to reach a more accurate diagnosis of lymphoma

Quantification of antigen expression

- The level of CD20 expression in B-cell lymphoma patients is crucial for planning the Rituximab containing therapy
  - immunohistochemical method
  - quantitative flow cytometric method



Sphero Rainbow calibration beads

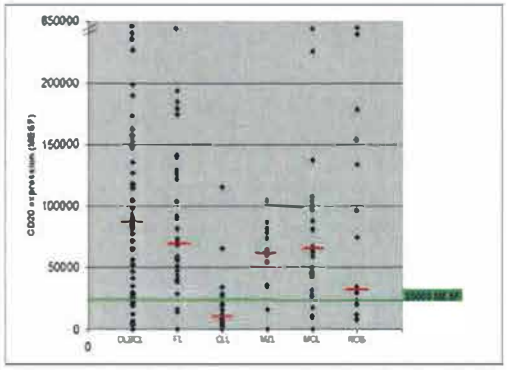


Regression plot

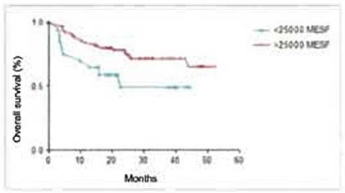
Immunohistochemical method

- generally used
- probably not precise enough
  - some patients with CD20 positive DLBCL do not respond to the rituximab treatment
  - when in this patients the level of CD20 expression was determined by quantitative flow cytometric method it was very low
  - CD 20 expression and response to the rituximab containing treatment are most probally connected

Quantitative flow cytometric method; CD20 expression in different B-cell lymphomas

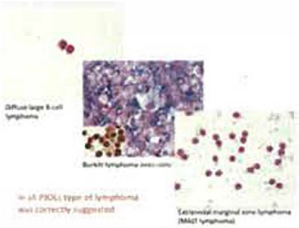


MESF...molecules of soluble fluorochrome, DLBCL...diffuse large B-cell lymphoma, FL...follicular lymphoma, CLL...chronic lympocytic leukemia, MCL...mantle cell lymphoma, MZL...marginal zone lymphoma, NOS...B- cell lymphoma unclassified,
– ... median values of CD 20 expression



Histological type of B-cell lymphoma	Number of patients below the cut-off value/ Total number of patients	% of patients below the cut-off value
DLBCL	11/64	17.2
FL	4/56	7.1
CLL	23/31	74.2
MCL	6/34	17.6
MZL	4/18	22.2
NOS	5/15	33.3
Total	53/114	34.5

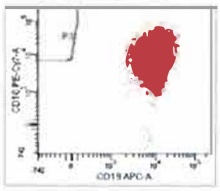
Diagnosing lymphomas from poorly cellular vitreous specimens



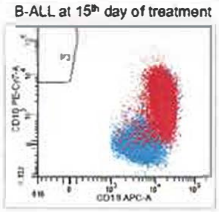
- 37th European Congress of Cytology, Croatia, 2012
  - Flow Cyrometric session with prof. Michael Dworzak and prof. Drago Batinić
  - Impressed by prof. Michael Dworzak lecture

Beginning of MRD-ALL project at the Institute of Oncology Ljubljana

B-ALL before treatment



B-ALL at 15th day of treatment



Jaka and Andreja

Ljubljana Flow Cytometric Meeting

2015: FACSCanto 10c and future development

- Improvement of sensitivity and specificity detection of lymphoma/leukaemia by FCI
- 8-10 color FCI measurements
- Experiments with 8 to 12 antibodies per tube

Ljubljana Flow Cytometric Meeting

Flow team

INSTITUTE OF ONCOLOGY LJUBLJANA

Mariana Matič, technician
Brigita Šturbej, technician
Jaka Lavrenčak, biologist, PhD
Andreja Brožič, biologist, PhD student
Simon Buček, biochemist
Ulrika Klopčič, cytopathologist
Sandra Jezeršek, cytopathologist
Veronika Kloboves Prevodnik, cytopathologist, PhD

Ljubljana Flow Cytometric Meeting

Ljubljana Flow Cytometric Meeting:
Diagnosing acute leukemia and minimal residual disease with multiparametric flow cytometer
Ljubljana, 30 November 2015

One year of flow cytometric diagnostics of MRD-ALL at the Institute of Oncology Ljubljana

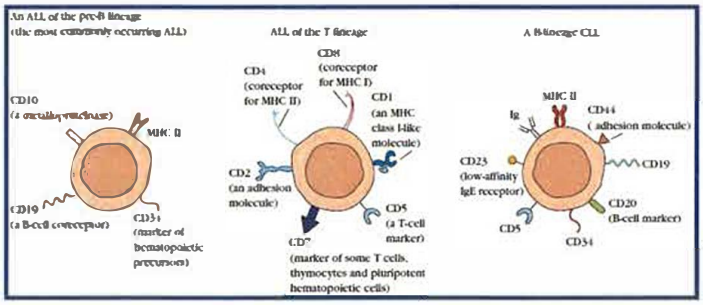
Andreja Brožič

ONKOLOŠKI INŠTITUT LJUBLJANA INSTITUTE OF ONCOLOGY LJUBLJANA

Slovensko združenje za pretočno citometrijo Slovenian Society For Flow Cytometry

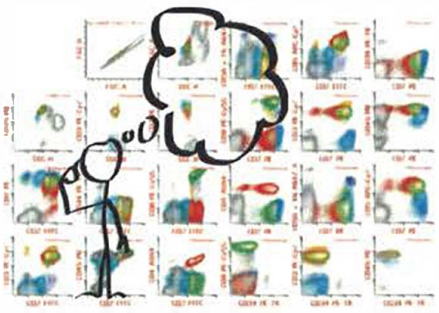
BEGINNING 2013

- Learn about:
  leukemia immunophenotyping
  MRD monitoring
- 4-color testing panels



BEGINNING 2013

- measuring reactive bone marrow samples (normal) with leukemia antibody panels
- measuring leukemia bone marrow testing samples
- training in Vienna
- defining 6-color panels



BEGINNING 2014

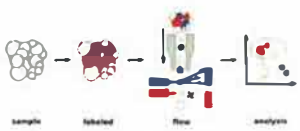
- 6-color panels measuring leukemia samples stained with leukemia antibody panel on BD FACSCanto II
- international collaboration
  - International Berlin-Frankfurt-Münster Study Group (I-BFM-SG)
  - supervised by laboratory in Vienna

LEARNING

- introducing with MRD monitoring
  - *Presence of leukemia cells that have avoided the action of antitumor drugs*
  - *The longer the tumor cells are retained during therapy, the worse the prognosis is*

LEARNING

- ALL IC-BFM protocol
  - cell input, staining, lysing, acquisition, ...



  - FC MRD: at least 30 blasts in 300 000 cell: MRD +

<0,1%	**FLR**
>0,1 and <10 %	**FMR**
>10 %	**FHR**

LEARNING

- Sensitivity of FC

  1 leukemia cell in 10 000 to 100 000 cells

- **How to find them**
- **Interpretation of results**

DEFINING PANELS

ALL

SURFACE

1. CD45 $_{APC\text{-}CY7}$
2. CD34 $_{FITC}$ /CD117 $_{PE}$ /CD33 $_{PerCpCy5,5}$ /HLA_DR $_{APC}$/CD14 $_{PE\text{-}CY7}$/CD45 $_{APC\text{-}CY7}$
3. CD3 $_{FITC}$ /CD19 $_{PE}$ / CD5$_{PerCpCy5,5}$ /CD20 $_{APC}$/ CD16+56 $_{PE\text{-}CY7}$/ CD45 $_{APC\text{-}CY7}$
4. CD38 $_{FITC}$ /CD34$_{PE}$ /CD19 $_{PerCpCy5,5}$ /CD20$_{APC}$ / CD10$_{PE\text{-}CY7}$/CD45 $_{APC\text{-}CY7}$

CYTOPLASMIC

5. CD45 $_{APC\text{-}CY7}$
6. c-TdT$_{FITC}$ /CD7$_{PE}$ / c-CD3 $_{PerCpCy5,5}$ /CD10 $_{APC}$/ CD19$_{PE\text{-}CY7}$/ CD45 $_{APC\text{-}CY7}$
7. c-MPO$_{FITC}$ /CD34$_{PE}$ / c-CD3 $_{PerCpCy5,5}$ /CD10 $_{APC}$/ CD19$_{PE\text{-}CY7}$/ CD45 $_{APC\text{-}CY7}$

DEFINING PANELS

8- LL

SURFACE

1. SYTO 16$_{FITC}$ / CD34$_{PE}$ /CD45 $_{PerCpCy5,5}$/ CD19$_{APC}$/CD10 $_{PE\text{-}CY7}$ /CD20$_{APC\text{-}CY7}$ (MRD- ALL)
2. CD58$_{FITC}$ / CD11a$_{PE}$ /CD45 $_{PerCpCy5,5}$/ CD19$_{APC}$/CD10 $_{PE\text{-}CY7}$ /CD20$_{APC\text{-}CY7}$ (MRD- ALL)

CYTOPLASMIC

3. c-kapa $_{FITC}$ /c-lamda $_{PE}$ /c-CD79a $_{PerCpCy5,5}$ /CD19 $_{APC}$ / CD10 $_{PE\text{-}CY7}$/CD45 $_{APC\text{-}CY}$
4. c-CD22$_{FITC}$ /c-μ(IgM)$_{PE}$ /c-CD79a $_{PerCpCy5,5}$ /CD19 $_{APC}$ /CD10 $_{PE\text{-}CY7}$/CD45 $_{APC\text{-}CY7}$
5. CD24 $_{FITC}$ /c-μ(IgM) $_{PE}$ /c-CD79a $_{PerCpCy5,5}$ /CD19$_{APC}$ /CD10 $_{PE\text{-}CY7}$/CD45 $_{APC\text{-}CY7}$
6. CD45 $_{APC\text{-}CY7}$
7. kapa $_{FITC}$ /lamda $_{PE}$ /CD79a $_{PerCpCy5,5}$ /CD19$_{APC}$ / CD10 $_{PE\text{-}CY7}$/CD45 $_{APC\text{-}CY7}$

T- LL

SURFACE

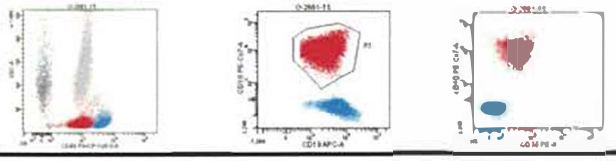
1. SYTO16$_{FITC}$ / CD99$_{PE}$ /CD3 $_{PerCpCy5,5}$/ CD7$_{APC}$/CD5 $_{PE\text{-}CY7}$ /CD45$_{APC\text{-}CY7}$ (MRD- ALL)
2. CD4$_{FITC}$ /CD8 $_{PE}$ / CD3 $_{PerCpCy5.5}$ /CD7$_{APC}$ / CD5$_{PE\text{-}CY7}$/CD45$_{APC\text{-}CY7}$ (MRD-T ALL)

CYTOPLASMIC

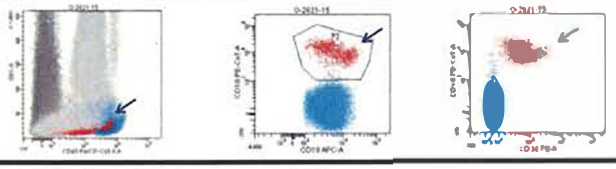
3. c- dT$_{FITC}$ /c- D3 $_{PE}$ / CD3 $_{PerCpCy5.5}$ /CD7$_{APC}$ / CD5$_{PE\text{-}CY7}$/CD45$_{APC\text{-}CY7}$ (MRD-T ALL)
4. c-CD3$_{FITC}$ /CD99 $_{PE}$ / CD3 $_{PerCpCy5,5}$ /CD7$_{APC}$ / CD16+56$_{PE\text{-}CY7}$/CD45$_{APC\text{-}CY7}$
5. c-CD3$_{FITC}$ /CD99 $_{PE}$ / CD3 $_{PerCpCy5,5}$ /CD4$_{APC}$ / CD8$_{PE\text{-}CY7}$/CD45$_{APC\text{-}CY7}$

B-ALL

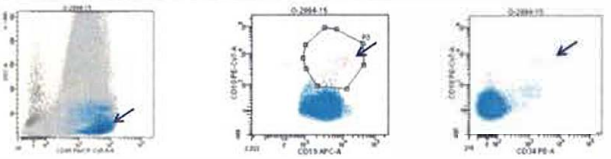
Diagnose:
70,0% blasts



Day 15:
2 populations of blasts
Shift in a expression

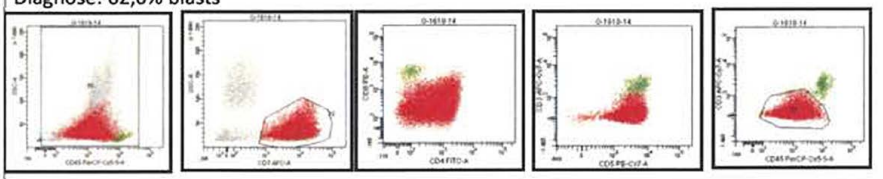


Day 33:
Same atypical
population
Shift in a expression

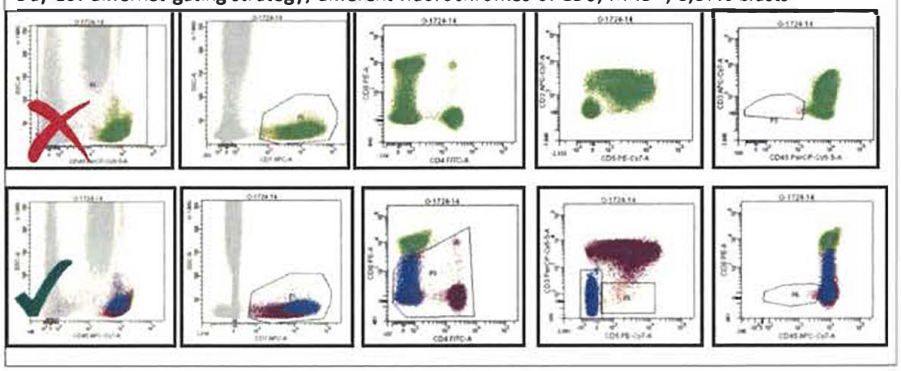


T-ALL

Diagnose: 62,6% blasts

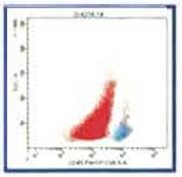


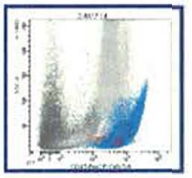
Day 15: differnet gating strategy; different fluorochromes of CD3; MRD+; 0,07% blasts

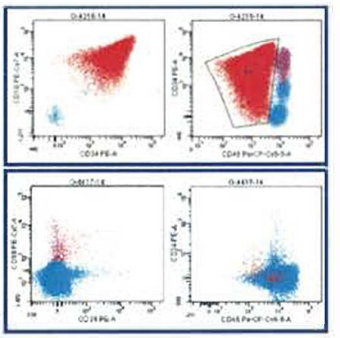


PITFALLS

- Problems with antibodies: CD3 APC-CY7, CD22,...
  skipping unnecessarily, change clone, fluorochrome
- Atypical populations – blasts?
- Down modulation - CD34



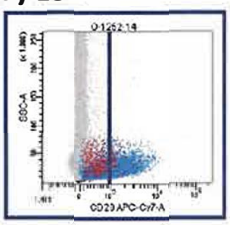




PITFALLS

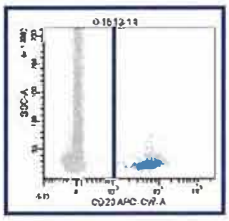
- Dilution of antibodies: correct volume cell – antibodies

Day 15



Low cellularity
High cell volume
Not enough antibody

Day 33

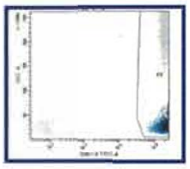


Usual cellularity
Usual cell volume (titrated)
Enough antibody

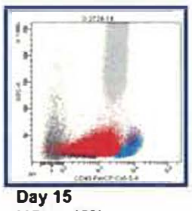
- Low cellularity samples – one tube

PITFALLS

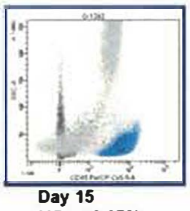
- Syto16 – to much; wash step overspill in red



- Number of cells depends on MRD (if high-less if low-more) sensitivity 0,01 % (1 dot in 10 000)



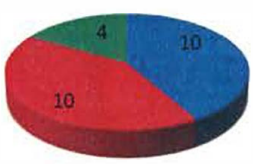
Day 15
MRD+ 45%



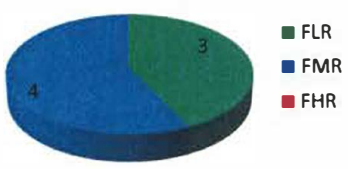
Day 15
MRD+ 0,07%

STATISTICS

B-ALL



T-ALL



FUTURE WORK 2015 -2016

- Measuring bone marrow samples stained with leukemia antibody panel on:
  - -BD FACSCanto II and
  - -BD FACSCanto 10-color
- Defining 8-color (10-color) panels
- Collaboration

Ljubljana Flow Cytometry Meeting
Nov 30th & Dec 1st, 2015; Institute of Oncology Ljubljana, Slovenia

iBFM-FLOW

Multicolor flow cytometric diagnostics and MRD in acute leukemias in children

Panel optimization and more...

Michael N. Dworzak
St. Anna Children's Cancer Research Institute
Vienna, Austria, EU

Up-front considerations

iBFM-FLOW

- Technical issues and standardization
  - Pre-analytics (sampling, transportation etc.)
  - Pre-acquisition issues (sample preparation, staining & panel set-up)
  - Acquisition issues (machine set-up and QC, acquisition standards)
  - Post-acquisition issues (data analysis, software, gating strategy)
- Data interpretation and diagnostic rules (EGIL, WHO, LeukemiaNet, ...)

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Up-front considerations

MPAL can be identified using the recommended panel of the European LeukemiaNet[2] or other comprehensive combinations.[3] Importantly, several markers specific for the myelo/monocytic lineage as well as for the B- and T-lymphoid lineages must be tested, excluding a restrictive strategy of quick orientation followed by selected lineage-specific markers.

This proposal relied on 26 antibodies to perform a proper score, yet published reports seldom applied such extensive panels.

Am J Clin Pathol 2015;144:361-376
Anna Porwit, MD, PhD,[1] and Marie C. Béné, PharmSciD, PhD[2]

Up-front considerations

WHO 2008 Criteria for MPAL Definition

Myeloid lineage:
Myeloperoxidase (flow cytometry, immunohistochemistry, or cytochemistry)
or
Monocytic differentiation (NSE, CD11c, CD14, CD64, or lysozyme)

T-lineage:
Cytoplasmic CD3 (flow cytometry with antibodies to CD3 epsilon chain; immunohistochemistry using polyclonal anti- CD3 antibody may detect CD3 zeta chain, which is not T-cell specific)
or
Surface CD3 (rare in mixed phenotype leukaemias)

B-lineage (multiple antigens required):
Strong CD19 with at least 1 of the following strongly expressed: CD79a, cytoplasmic CD22, CD10
or
Weak CD19 with at least 2 of the following strongly expressed: CD79a, cytoplasmic CD22, CD10

Note: Monocytic differentiation requires positivity of ≥2 of these antigens;
The T-cell component is recognized by bright expression of cCD3, either on the entire blast population or on a separate subpopulation of leukemic cells ... should be as bright or nearly as bright as that of normal residual T cells present in the sample.

Up-front considerations

Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia *Lancet Oncol* 2009; 10: 147-56

Elaine Coustan-Smith, Charles G Mullighan, Mihaela Onciu, Frederick G Behm, Susana C Raimondi, Deqing Pei, Cheng Cheng, Xiaoping Su, Jeffrey E Rubnitz, Giuseppe Basso, Andrea Biondi, Ching-Hon Pui, James R Downing, Dario Campana

Development of an ETP-ALL scoring system

ETP-ALL shows a distinctive immature immunophenotype characterized by lack of CD1a and CD8 expression, weak CD5 expression with <75% positive blasts, and expression of one or more of the following myeloid or stem cell antigens on at least 25% of lymphoblasts: CD117, CD34, HLA-DR, CD13, CD33, CD11b and/or CD65 (Coustan-Smith *et al*, 2009).

Choice of the iBFM FLOW-nw

- Extensive single- latform panel for acute leukemia in children

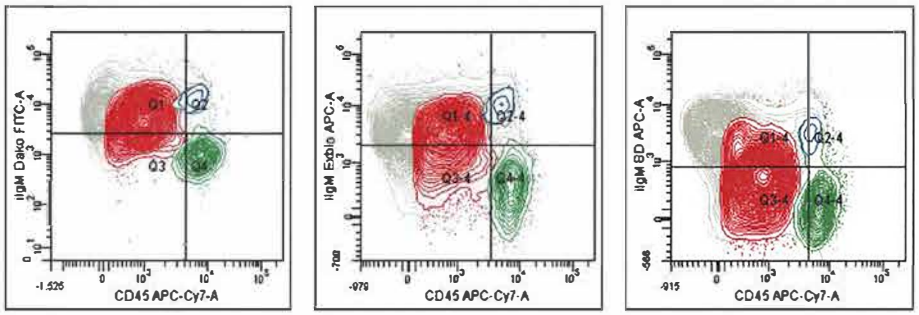
	Markers (each combined with CD45)
Intracellular*	iCD3, iCD22, iCD79a, iIgM (μ-chain), iLysozyme, iMPO
Surface	CD2[‡], CD3, CD5, CD7, CD10, CD19, CD20, CD11c, CD11b, CD13, CD14, CD15, CD33, CD64, CD65[A], CD117, CD34, (CD45), CD56, HLA-DR if T-ALL: CD1a, CD4, CD8, TCRαβ, TCRγδ if B-IV suspected: κ-chain, λ-chain (surface staining after pre-washing or intracellular)
Optional / Recommended	all cases: NG2[‡], Clec12A[‡§] if BCP-ALL: CD11a[‡], CD22, CD24, CD38, CD44, CD58, CD66c, CD123[‡], CRLF2[‡*] if T-ALL: CD99, iTdT if BAL according to general panel: CD24, iTdT

→ compatible with WHO 2008 and EGIL score

* prefix "i" stands for intracellular staining
‡ phycoerythrin-conjugate (PE) recommended
§ available only labelled with fluorescein isothiocyanate (FITC)
‡ clone 7.1, Beckman-Coulter, Miami, FL USA
A clone 50C1, Becton Dickinson Biosciences (BD), San Jose, CA USA
* clone 1D3-PE, BioLegend, San Diego, CA USA

iIgM and light-chains: *fault-prone* with influence on subclassification (and potential impact on therapy in case of BIV!)

Dako (pc) | Exbio (mc) | BD (mc)



Ambiguities remaining with MPAL:

Impact of methodology on MPAL-diagnosis?

- ❖ WHO 2008 does not define weak/strong!
- ❖ What is positive? (EGIL rules date back to 1995)
- ❖ Impact of procedural peculiarities (permeabilization, choice of antibody clones)
- ❖ Adaptation to multi-color flow cytometry is lacking!

WHO 2008 Criteria for MPAL Definition

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OPEN FORUM

Proposals for the immunological classification of acute leukemias

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

There was general consensus on the cut-off point to consider a marker positive and this was set up at 20% of cells stained with the monoclonal antibody (MoAb) whether using indirect immunofluorescence with microscope or flow cytometry or immunocytochemical techniques. An exception was made for anti-MPO, CD3 and CD79a due to their high degree of specificity, as well as TdT, being the cut-off point for these markers set up to a minimum of 10% of blast cells stained, providing that confirmation of blasts stained with the antibody is made by light microscopy examination. These cut-off points are applicable to both diagnosis of the acute leukemias and classification of the various ALL and AML subtypes.

There was concern on a number of technological aspects, eg different pattern of staining when using directly conjugated phycoerythrin MoAb vs fluorescein conjugated or unlabelled, adequate gating, quantification of the antigenic molecules, cytoplasmic staining by flow cytometry, etc. However, all these aspects were beyond the aims of the group and will likely be considered as a topic in the future.

Leukemia (1995) 9, 1783–1786

bjh short report

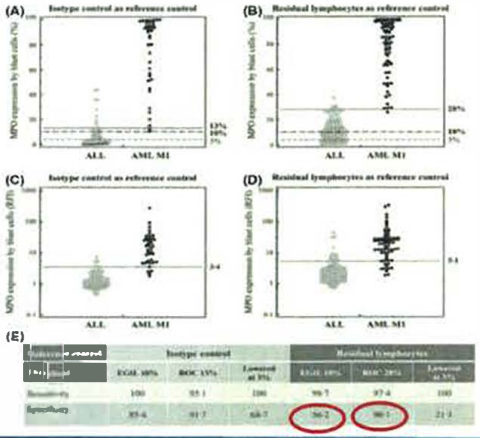
Flow cytometry thresholds of myeloperoxidase detection to discriminate between acute lymphoblastic or myeloblastic leukaemia

Julien Guy,[1] Iléana Antony-Debré,[2,3] Emmanuel Benayoun,[2] Isabelle Arnoux,[4] Chantal Fossat,[4] Magali Le Garff-Tavernier,[5] Anna Raimbault,[5] Michèle Imbert,[2,3] Marc Maynadié,[1] Francis Lacombe,[6] Marie C Béné[7] and Orianne Wagner-Ballon[2,3] on behalf of the GEIL (Groupe d'Etude Immunologique des Leucémies)

Summary

The World Health Organization 2008 Classification emphasizes myeloperoxidase (MPO) detection as sufficient for assigning a blast population to the myeloid lineage. Published MPO positivity thresholds are 10% for flow cytometry [EGIL] and 3% for cytochemistry. Here we re-evaluated the FCM-MPO threshold by comparing retrospectively 128 acute lymphoblastic leukaemias and 75 acute myeloid leukaemias without maturation, all assessed by benzidine-based cytochemistry. A 13% threshold was found to be relevant using an isotype control as background-reference (sensitivity 95·1%, specificity 91·7%). Residual normal lymphocytes proved to be an advantageous alternative reference: a threshold of 28% yielding improved 97·4% sensitivity and 96·1% specificity.

After permeabilization, the cells were stained with 5 µl of anti-MPO- fluorescein isothiocyanate (FITC)-labelled monoclonal antibody (Dako or Immunotech) or isotype controls (for 96/128 ALL and 40/75 AML M1 samples).



Flow cytometric detection of intracellular myeloperoxidase, CD3 and CD79a
Interaction between monoclonal antibody clones, fluorochromes and sample preparation protocols

János Kappelmayer[a,*], Jan W. Gratama[b], Éva Karászi[a], Pablo Menéndez[c], Juana Ciudad[c], Rosana Rivas[c], Alberto Orfao[c]

Pairwise comparison of FITC and PE conjugates of the same clone revealed, that PE conjugates yielded significantly higher MFI than FITC conjugates (MPO-7, $P=0.01$; H-43-5, $P=0.009$). The percentages of MPO+ cells (expressed as a fraction of total leukocytes) in normal PB and BM samples, as well as in AML samples, did not differ significantly between the five fixation–permeabilization systems (data not shown). Finally, no false-positive reactions of any of the anti-MPO clones (irrespective of fluorochrome) was seen on precursor B-ALL and T-ALL blasts prepared with any fixation–permeabilization kit except following use of Cytofix/Cytoperm™ (Table 2), where all three anti-MPO clones stained B-ALL and T-ALL blasts dimly.

Journal of Immunological Methods 242 (2000) 53–65

Comparative Analysis of Different Permeabilization Methods for the Flow Cytometry Measurement of Cytoplasmic Myeloperoxidase and Lysozyme in Normal and Leukemic Cells

Francesco Lanza,* Angela Latorraca, Sabrina Moretti, Barbara Castagnari, Luisa Ferrari, and Gianfuigi Castoldi
Section of Hematology, University of Ferrara, Ferrara, Italy

used an FITC-conjugated anti-MPO McAb (clone MPO-7, IgG1 isotype, from DAKO, Glostrup, Denmark), whereas only the F&P reagent was characterized by a good specificity and sensitivity in detecting the two granule constituents (MPO, lysozyme) on leukocytes taken from healthy subjects. The remaining two permeabilization techniques (OPF and FLy) were characterized (at least under our experimental conditions) by a lower specificity in detecting MPO and, to a lesser extent, lysozyme antigens;

trials dealing with leukemias. A standardization of cytofluorimetric analysis of intracellular antigens is needed in order to improve the reproducibility and comparability of results in multicenter studies.

Cytometry (Communications in Clinical Cytometry) 30:134–144 (1997)

MPO: *difference by choice of mAb !!*

Arber et al / MYELOPEROXIDASE-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA *Am J Clin Pathol* 2001;116:25-33

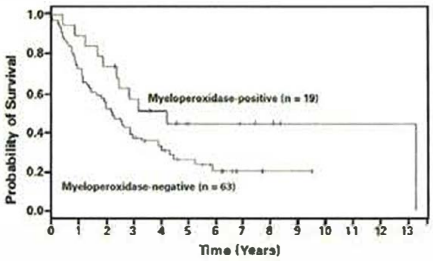


Figure 1 Overall survival from date of diagnosis in patients with polyclonal myeloperoxidase–positive or polyclonal myeloperoxidase–negative acute lymphoblastic leukemia. $P = .07$ (log-rank test). Vertical marks represent censored data.

The 19 pMPO-positive cases were all of precursor B-cell lineage ($P = .06$) and, therefore, had a higher frequency of CD10, CD19, and CD20 expression than did the pMPO-negative group **(Table 3)**. No difference in aberrant CD33 expression was identified between the 2 groups ($P = 1.00$), but the expression of either CD13 or CD15 was increased significantly in the pMPO-positive group (53% vs 21%; $P = .01$). Immunocytochemical studies performed on cytocentrifuged preparations of frozen cells from 8 cases, including 4 pMPO-positive and 4 pMPO-negative ALLs using a monoclonal MPO antibody, were negative in all cases.

studied with a monoclonal MPO antibody (clone MPO-7; 1:100 dilution; DAKO) on methanol-fixed cytocentrifuged

Mixed-phenotype acute leukemia: clinical and laboratory features and outcome in 100 patients defined according to the WHO 2008 classification

Estella Matutes,[1] Winfried F. Pickl,[2] Mars van't Veer,[3] Ricardo Morilla,[1] John Swansbury,[1] Herbert Strobl,[4] Andishe Attarbaschi,[4] Georg Hopfinger,[5] Sue Ashley,[6] Marie Christine Bene,[7] Anna Porwit,[8] Alberto Orfao,[9] Petr Lernez,[10] Richard Schabath,[11] and Wolf-Dieter Ludwig[11]

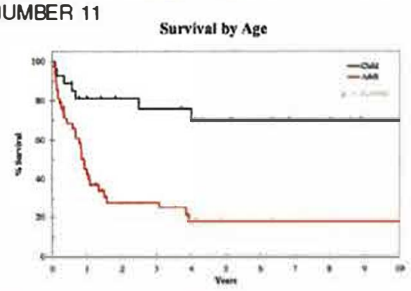
BLOOD, 17 MARCH 2011 • VOLUME 117, NUMBER 11

iBFM-FLOW

Most cases were from United Kingdom (64), Austria (21), and Holland (8), and a minority were from France (3), Sweden (2), Spain (1), and Czech Republic (1).

According to each center's protocols, multiparameter immunostaining with fluorochrome directly labeled monoclonal antibodies (MoAbs) was performed.

Pediatric patients: antiMPO clone 8E2 Caltag AdG

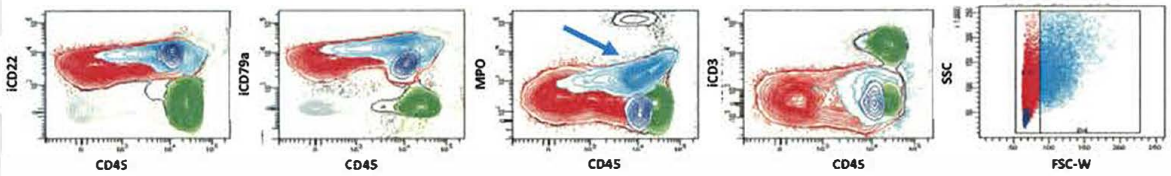


No expressed information on the anti-MPO mAb clone given In the following papers on MPAL & BAL:

- Matutes et al., Blood 2011
- Gerr et al., BJH 2010
- Al Seraihy et al., Haematologica 2009
- Rubnitz et al., Blood 2009
- Aribi et al., BJH 2007

MPO: *false positivity by non-exclusion of doublets*

iBFM-FLOW



AIEOP-BFM immunophenotyping study – part I

„BD-study" LOCAL vs. COMMON panel

CONCLUSIONS 1

iBFM-FLOW

- Lineage assignment: complete concordance between panels
- COMMON-panel is valid for lineage assignment of pediatric AL
- **AIEOP-BFM thresholds for weak/strong distinction (CD19, iCD3) are very well set**

- Subclassification (EGIL): high degree of concordance between panels
- Few discordances related to iIgM readings
- COMMON-panel is generally valid for subclassification of pediatric AL

- BAL/MPAL: relevant divergences occurred!
- Discordances related to MPO and other myeloid marker readings
- Local panels seem to overestimate MPAL incidence: MPAL 6% vs 2%
- If MPAL/BAL assessment is relevant within a collaborative trial and/or for therapy assignment, certain involved markers and/or procedures should be standardized to avoid inter-center bias
- **AIEOP-BFM dominant lineage strategy is VERY relevant to limit influence of fault-prone myeloid marker „expression"**

- ETP: all 4 cases correctly assigned by all panels
- COMMON-panel seems valid for ETP assignment (limited evidence: few cases tested)

AIEOP-BFM immunophenotyping study – part I

„BD-study" LOCAL vs. COMMON panel

CONCLUSIONS 2

- AIEOP-BFM thresholds for weak/strong distinction (CD19, iCD3) are very well set
- iCD79a is more reliable than ICD22
- Discordances in
  - IgM: quite random – seem related to several factors: sample quality, antibody and/or permeabilization
  - iCD22: permeabilization dependent – procedure-optimization warranted!
    sCD22 is frequently more easily detectable – but – can be weak/negative in BI w MLLr & BIV
  - MPO: most probably antibody-related (clone)
  - Myeloid markers: due to weak expressions and to higher background (fluorescence or cellular)

AIEOP-BFM immunophenotyping study – part II

„RT-pheno 29 cases" post-acquisition analysis concordance

CONCLUSIONS 1

- Lineage assignment: very high degree of full concordance between 8 centers (26/29)
- Subclassification (EGIL): relevant divergences occurred (in 11 of 29 cases; (≥7/8: 5 cases)
- Most discordances related to B-II vs. B-III (3 cases), T-II vs. T-IV (4 cases), MPAL (2 cases)
- BAL/MPAL/ETP/blast cell heterogeneity: relevant divergences occurred!
- Only 1 of 7 BAL cases judged fully or ≥7/8 concordant
- None of 2 MPAL cases judged fully or ≥7/8 concordant
- None of 2 ETP cases judged fully concordant (≥7/8 concordant: 1 ETP)
- None of 2 cases with >1 phenotypic blast clone judged fully or ≥7/8 concordant

AIEOP-BFM ALL 2009: immunophenotype survey 12/2013

blast cell heterogeneity

10) IMMUNOPHENOTYPIC HETEROGENEITY OF BLAST SUBPOPULATIONS:

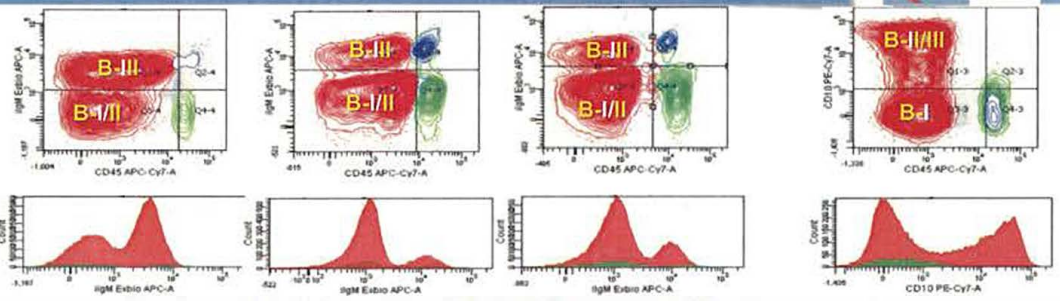
This is defined by the existence of ≥2 immunophenotypically distinct subpopulations of leukemic cells in a single leukemia case. Cases which contain more than one blast population should be clearly flagged in the data base and the type of heterogeneity should be described in the descriptive summary of the clinical report.

- Cases fulfilling criteria of different lineages – in the sense of bilineal leukemia – as per WHO MPAL or EGIL BAL definitions.
- Leukemia cases with maturation, i.e. classical admixture of maturing cells to immature blasts (e.g. AML M2, M4), also fall into this category.
- Also heterogeneity in blast appearance within single-lineage cases of ALL will be recorded. Such significant differences are defined by unambiguous partial positivity (NOT by dim expression) among the total blast population with at least one marker of the following which are essential for ALL-subtype definition: CD10, IgM, CD1a, CD3, and CD5. This heterogeneity (in order to be counted as such) must lead to divergent subtype designations (including ETP) when assessing blast cell subsets separately.

AIEOP-BFM ALL 2009: immunophenotype survey 12/2013

blast cell heterogeneity

iBFM-FLOW



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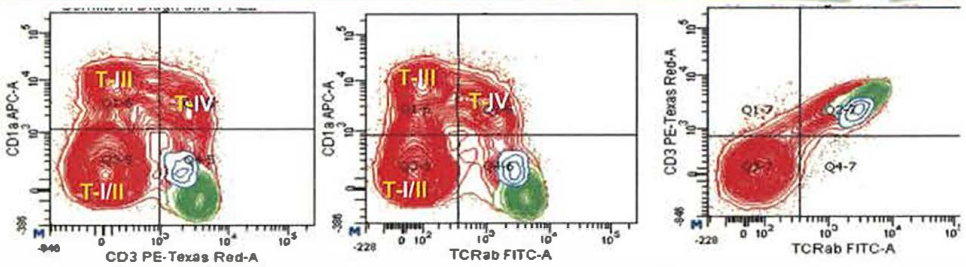
AIEOP-BFM ALL 2009: immunophenotype survey 12/2013

blast cell heterogeneity – also in T-ALL

iBFM-FLOW



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iBFM-FLOW

TABLE 4: THE AIEOP-BFM SUBCLASSIFICATION OF ALL[#]

Subtype	Discriminators	Remarks
B-I (pro-B)	$CD10^{neg}$	BCP-ALL lineage criteria fulfilled
B-II (common)	$CD10^{pos}$	/
B-III (pre-B)	IgM^{pos}	$CD10^{neg\ or\ weak\ pos}$ may occurs
B-IV (mature B)	κ- or λ-$chain^{pos}$	may occur with FAB L1/L2 morphology[&]
T-I (pro-T)[$]	only $cCD3^{pos}$ and $CD7^{pos}$	T-ALL lineage criteria fulfilled
T-II (pre-T)	≥1 of $CD2^{pos}$, $CD5^{pos}$, $CD8^{pos}$	surface (s) $CD3^{weak\ pos}$ allowed[*]
T-III (cortical T)	$CD1a^{pos}$	$sCD3^{weak}$ may occur[*]
T-IV (mature T)	$CD1a^{neg}$ and $sCD3^{pos}$	$sCD3^{strong}$, or $sCD3^{weak\ pos}$ with TCR^{pos}
ETP (only additive to T-I or T-II)	$CD1a^{neg}$, $CD8^{neg}$ usually $CD5^{neg\ or\ weak\ pos}$ and ≥1^{pos} of HLADR, CD11b,13,33,34,65,117	if $CD5^{strong\ pos}$: ≥2^{pos} of HLADR, CD11b,13,33,34,65,117; $sCD3^{weak\ pos}$ may occur[*]

[#] adapted from refs. 8 & 9.
[!] $CD10^{neg\ or\ weak}$ B-III is frequently associated with MLL-rearrangements (12).
[&] light-$chain^{pos}$ cases without FAB L3-morphology and without *MYC*-translocation are eligible for conventional ALL treatment, and thus must be separated from Burkitt-type mature B-ALL (40-43).
[$] T-I is very rare and can be reported together with T-II (as T-I/II)
[*] Dim or even more frequently partial surface positivity with CD3 (e.g. in a minor blast subpopulation) occurs when sensitive methodology is used and should not mislead to diagnose mature T-ALL in the absence of TCR expression.

Another issue with blast cell heterogeneity

MPAL - BIL

Cytometry Part B (Clinical Cytometry) 86B:152–153 (2014)

Editorial

Mixed Phenotype Acute Leukemia

Michael J. Borowitz*
Professor of Pathology and Oncology, Johns Hopkins Medical Institutions, Baltimore, Maryland

criteria for T/Myeloid MPAL (mixed phenotype acute leukemia) can be met in one of two ways. The criterion most are familiar with requires the expression of the most specific markers for each lineage—in this case cytoplasmic CD3 and myeloperoxidase.

However, less frequently recognized is the fact that expression of these specific markers only applies to the situation in which there is a single population of blasts: criteria for identifying a myeloid component are also met "...when there are two or more distinct populations of leukaemic cells, one of which would meet immunophenotypic criteria for acute myeloid leukaemia (with the exception that this population need not comprise 20% of all nucleated cells)..."

Simple co-expressing MPAL

Bi-lineal MPAL

Cytometry Part B (Clinical Cytometry) 86B:152–153 (2014)

Editorial

Mixed Phenotype Acute Leukemia

Michael J. Borowitz*
Professor of Pathology and Oncology, Johns Hopkins Medical Institutions, Baltimore, Maryland

establish a diagnosis of MPAL. However, we would reject the use of 10% as a definitive criterion. We do not believe that any percentage threshold is an accurate measure of biology. Nine percent MPO positive myeloblasts with an aberrant phenotype would clearly establish an MPAL diagnosis, while 15% MPO positive normal myeloblasts that

leukemia. The WHO specifically and deliberately does not put a lower limit on the number of myeloblasts that must be present to permit a diagnosis of MPAL. This is

MPAL – or even bilineal?

- **Initial diagnoses of truly bilineal acute leukemia with a non-lymphoblastic blast component** (irrespective of clonal proportions), **AML, AUL** (according to above mentioned criteria), or other unusual AL as above are **NOT** considered apt for inclusion **into study AIEOP-BFM ALL 2009** as protocol cases.
- In contrary, cases with a **single blast population with ALL-type dominant Immunophenotype** (according to the AIEOP-BFM lineage assignment criteria adapted from Mejstrikova et al. 2010) **but fulfilling MPAL/BAL criteria** (below) are apt to be **included into study AIEOP-BFM ALL 2009** as protocol patients.

AIEOP-BFM ALL FLOW-SG

Consensus lineage assignment

Immunophenotyping standardization – our future?!

❖ Standardizing the most relevant issues of divergence.....

- (i)IgM (e.g. Exbio clone CH2)
- iCD22 (e.g. Invitrogen clone RFB4)
- iMPO (mind clone differences, e.g. Invitrogen 8E2 vs. Dako MPO-7 or BD 5B8)
- Permeabilization (e.g. Intrasure™)
- Doublet exclusion
- Blast clone heterogeneity (as distinct from an antigen expressed heterogeneously...)

Switch pB-ALL

Leukemia (2014) 1–12
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www.nature.com/leu

iBFM-FLOW

ORIGINAL ARTICLE

CD2-positive B-cell precursor acute lymphoblastic leukemia with an early switch to the monocytic lineage

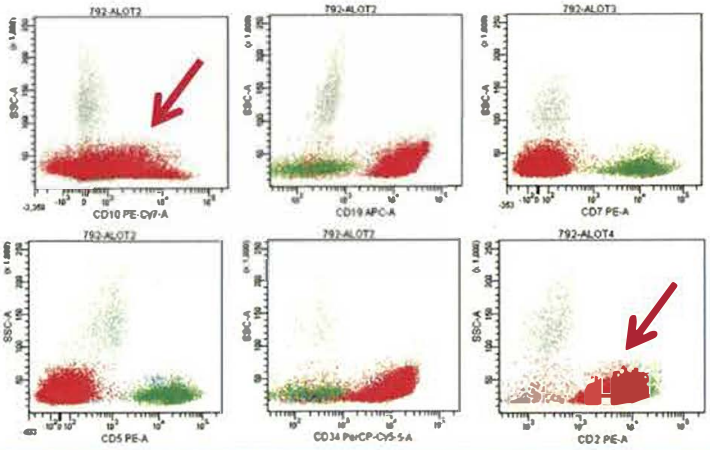
L Slamova[1], J Starkova[1], E Fronkova[1], M Zaliova[1], L Reznickova[1], FW van Delft[2], E Vodickova[3], J Volejnikova[3], Z Zemanova[4], K Polgarova[1], G Carlo[5], M Figueroa[6], T Kalina[1], K Fiser[1], JP Bourquin[7], B Bornhauser[7], M Dworzak[8], J Zuna[1], J Trka[1], J Stary[1], O Hrusak[1] and E Mejstrikova[1]

Switches from the lymphoid to myeloid lineage during B-cell precursor acute lymphoblastic leukemia (BCP-ALL) treatment are considered rare and thus far have been detected in MLL-rearranged leukemia. Here, we describe a novel BCP-ALL subset, switching BCP-ALL or swALL, which demonstrated monocytosis early during treatment. Despite their monocytic phenotype 'monocytoids' share immunoreceptor gene rearrangements with leukemic B lymphoblasts. All swALLs demonstrated BCP-ALL with CD2 positivity and no MLL alterations, and the proportion of swALLs cases among BCP-ALLs was unexpectedly high (4%). The upregulation of CEBPα and demethylation of the CEBPA gene were significant in blasts at diagnosis, prior to the time when most of the switching occurs. Intermediate stages between $CD14^{neg}CD19^{neg}CD34^{pos}$ B lymphoblasts and $CD14^{pos}CD19^{neg}CD34^{neg}$ 'monocytoids' were detected, and changes in the expression of PAX5, PU1, M-CSFR, GM-CSFR and other genes accompanied the switch. Alterations in the Ikaros and ERG genes were more frequent in swALL patients; however, both were altered in only a minority of swALLs. Moreover, switching could be recapitulated in vitro and in mouse xenografts. Although children with swALL respond slowly to initial therapy, risk-based ALL therapy appears the treatment of choice for swALL. SwALL shows that transdifferentiating into monocytic lineage is specifically associated with CEBPα changes and CD2 expression.

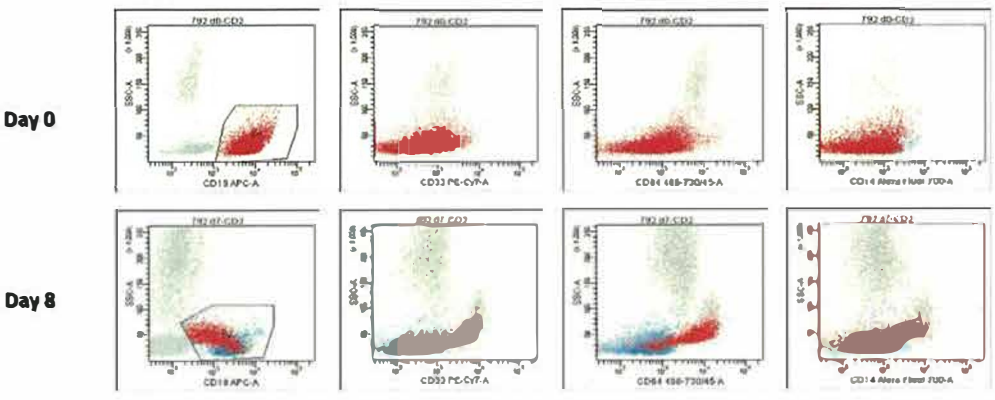
Leukemia advance online publication, 14 January 2014; doi:10.1038/leu.2013.354

Keywords: minimal residual disease; lineage switch; CCAAT/enhancer-binding protein alpha; acute lymphoblastic leukemia; Monocytes; CD2 Antigen

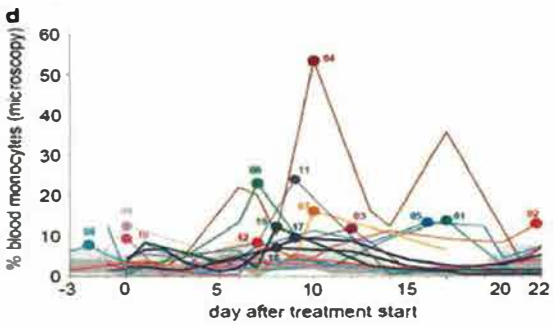
Switch – pB-ALL – a new entity
Typical phenotype at diagnosis

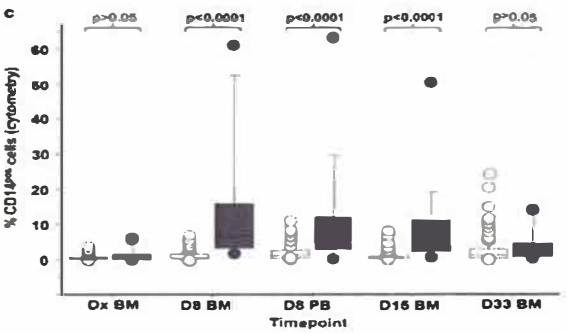


Switch – pB-ALL CD10dim CD2+
Phenotype modulation in vivo – during steroid prephase



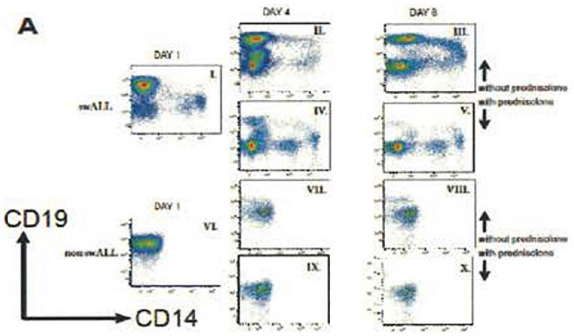
Switch – pB-ALL CD10dim CD2+
Phenotype modulation in vivo – period of aberrant monocytes

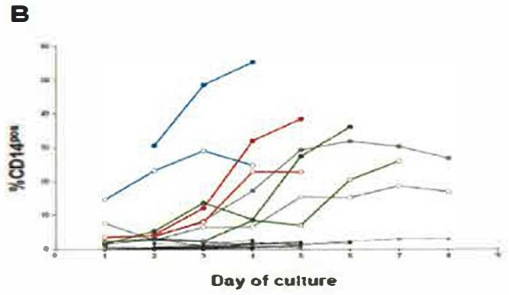




Slamova et al., Leukemia 2014

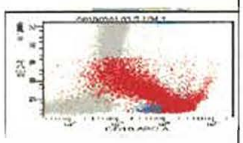
Switch – pB-ALL CD10dim CD2+
Phenotype modulation in vitro

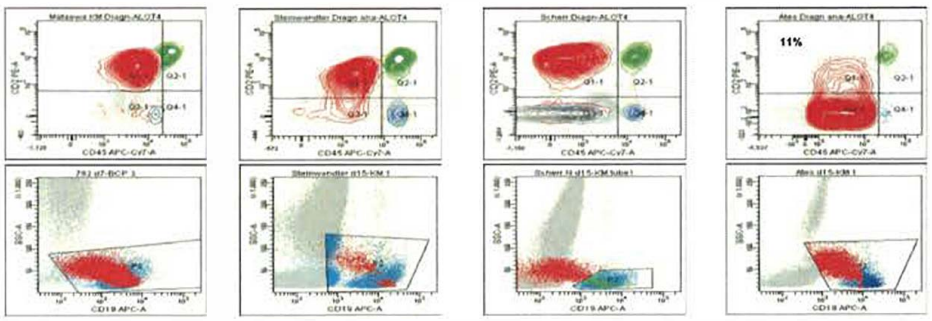




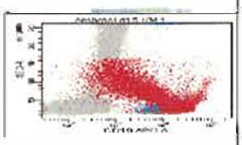
Slamova et al., Leukemia 2014

Switch – pB-ALL CD10dim CD2+
Heterogeneity of CD2 but uniform transdifferentiation

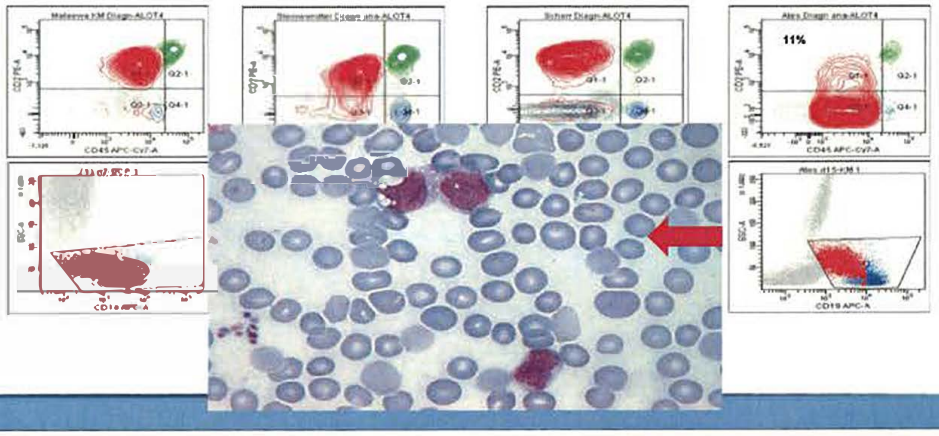




Switch – pB-ALL CD10dim CD2+
Heterogeneity of CD2 but uniform transdifferentiation



CD2 draw-backs: Weak expression in ~40% of swALL cases
Positive not only in swALL cases



iBFM-FLOW

ETP and MPAL

iBFM-FLOW

Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia

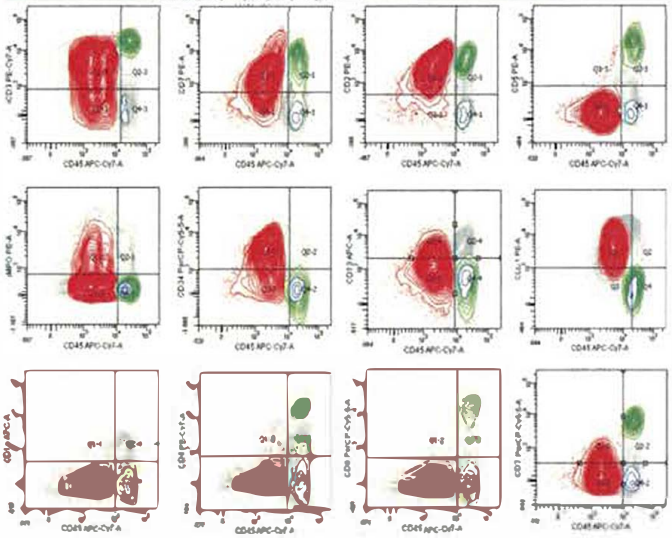
Elaine Coustan-Smith, Charles G Mullighan, Mihaela Onciu, Frederick G Behm, Susana C Raimondi, Deqing Pei, Cheng Cheng, Xiaoping Su, Jeffrey E Rubnitz, Giuseppe Basso, Andrea Biondi, Ching-Hon Pui, James R Downing, Dario Campana

Thus, in the broadest sense, ETP ALL is a kind of "T/myeloid" leukemia. From a definitional perspective, however, MPO expression excludes ETP ALL, while the great majority of cases of MPAL are MPO positive. In addition, the T cell component of T/myeloid leukemia frequently would meet criteria for ETP ALL. Thus, these two leukemias appear more alike than different, although because of the central importance of MPO to labeling something as myeloid, and the way leukemia treatment protocols are structured, they are typically treated differently. Unfortunately, this may make it difficult ever to understand whether these do in fact constitute different leukemic entities. It will be interesting to see how this situation will be treated in the next iteration of the WHO classification.

Cytometry Part B (Clinical Cytometry) 86B:152–153 (2014)

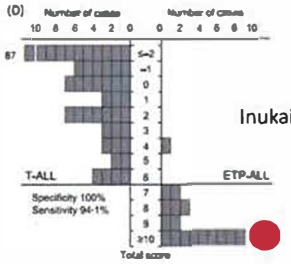
Michael J. Borowitz*
Professor of Pathology and Oncology,
Johns Hopkins Medical Institutions,
Baltimore, Maryland

Subtype	Discriminators	Remarks
ETP (only additive to T-I or T-II)	CD1a^{neg}, CD8^{neg} usually CD5$^{neg\ or\ weak\ pos}$ and ≥1^{pos} of HLADR, CD11b,13,33,34,65,117	if CD5$^{strong\ pos}$; ≥2^{pos} of HLADR, CD11b,13,33,34,65,117; sCD3$^{weak\ pos}$ may occur*



iBFM-FLOW

Dgn.: T-ALL
EGIL: TI/II
ETP: yes
MPAL: yes
BAL: yes
Blast clone heterogeneity: yes



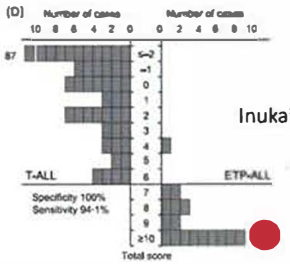
Inukai- core

Subtype	Discriminators	Remarks
ETP (only additive to T-I or T-II)	CD1a^{neg}, CD8^{neg} usually CD5$^{neg\ or\ weak\ pos}$ and ≥1^{pos} of HLADR, CD11b,13,33,34,65,117	if CD5$^{strong\ pos}$; ≥2^{pos} of HLADR, CD11b,13,33,34,65,117; sCD3$^{weak\ pos}$ may occur*

iBFM-FLOW

Scoring system based on 11 marker expression				
Score	–2	–1	+1	+2
CD5	≥75%			<75%
CD8	≥5%			<5%
CD13			≥25%	≥75%
CD33			≥25%	≥75%
CD34			≥25%	≥75%
HLA-DR			≥25%	≥75%
CD2		≥75%	<20%	
CD3		≥75%	<20%	
CD4		≥75%	<20%	
CD10		≥75%	<20%	
CD56			≥20%	

Dgn.: T-ALL
EGIL: TI/II
ETP: yes
MPAL: yes
BAL: yes
Blast clone heterogeneity: yes



Inukai- core

Immunophenotyping standardization – the future starts now!

iBFM-FLOW

- New markers: e.g. CLL- (CD371), CRLF2, ...

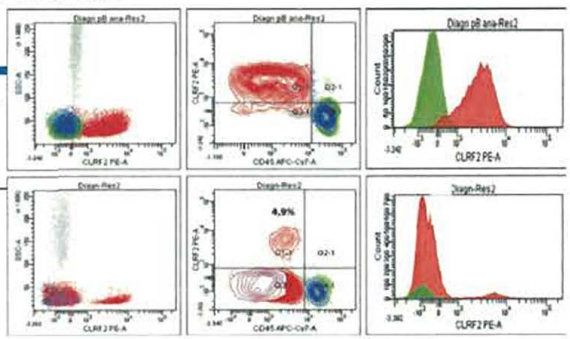
haematologica 2015; 100:e

Fine tuning of surface CRLF2 expression and its associated signaling profile in childhood B-cell precursor acute lymphoblastic leukemia

Cristina Bugarin,[1] Jolanda Sarno,[1] Chiara Palmi,[1] Angela Maria Savino,[1] Geertruy te Kronnie,[1] Michael Dworzak,[2] Angela Shumich,[2] [illegible] Oscar Maglia,[1] Simona Salo,[1] [illegible] Jean-Pi[illegible] Dr [illegible] An[illegible] Giovanni Cazzaniga[1] and Giuseppe Gaipa,[1] on behalf of the I-BFM study group

P2RY8-CRLF2+

IGH@-CRLF2+



Immunophenotyping standardization – the future starts now!

- New markers: e.g. CLL- (CD371), CRLF2, ...
- New subclassifications, new entities: e.g. ETP, switch pB- LL
- MPAL – (cross- ineage) blast subclones and aberrant maturation

MRD

FLOW-standardization: challenges and pitfalls

1) Pre-analytical sample quality

2) Staining panel, procedure optimization, definitions

3) Marker (in)stability and background

4) Human factor (experience – standardization)

Towards one voice in ALL FLOW-MRD:
I-BFM standards

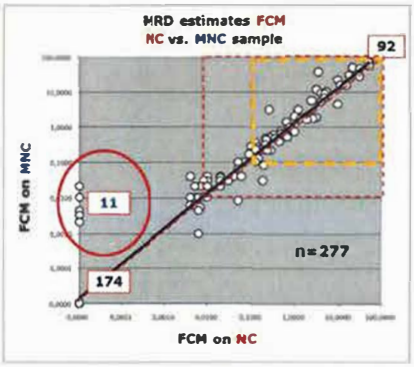
- acquisition: minimum 3x 10^5 cells of interest
- gating hierarchy/calculation basis:
  1. ntact nucleated cells
  2. IN/SSC (i. . D19, CD7)
  3. mmature LIN/SSC (e.g. CD10)
  4. mmature LIN/aberration marker (e.g. CD10 vs CD45)
- cluster gating
- positivity criteria: reproducible cluster of ≥10 cells with related characteristics
- criteria for quantifiable positivity: cluster of ≥30 cells
- target sensitivity: 1 in 10^4 (i. . 0 in 3x 10^5 cells!)

Time point-dependent concordance of flow cytometry and real-time quantitative polymerase chain reaction for minimal residual disease detection in childhood acute lymphoblastic leukemia

Giuseppe Gaipa,[1] Giovanni Cazzaniga,[2] Maria Grazia Valsecchi,[3] Renate Panzer-Grümayer,[2] Barbara Buldini,[4] Daniela Silvestri,[3] Leonid Karawajew,[5] Oscar Maglia,[1] Richard Ratei,[5] Alessandra Benetello,[1] Simona Sala,[1] Angela Schumich,[3] Andre Schrauder,[6] Tiziana Villa,[1] Marinella Veltroni,[7] Wolf-Dieter Ludwig,[6] Valentino Conter,[8] Martin Schrappe,[9] Andrea Biondi,[1] Michael N. Dworzak,[2] and Giuseppe Basso[4]

high-resolution FLOW-MRD
- 7- or 8-colors
- higher cell input/tube
- fewer tubes

- increased sensitivity
- more events acquired
- higher LAIP complexity
- reduced costs



The overall correlation of MRD estimates by PCR and by FCM with both cell preparations is shown in Figure 2. The concordance between the two FCM assays was 96% (255/266) in positive-negative correlations, and 91% (242/266; Figure 2) using the cut-off of 0.01%. Of 24 divergent samples at the 0.01% cut-off, 11 samples were negative by FCM^{NC} but positive by FCM^{MNC}, mostly at very low levels of MRD. When limiting the FCM^{MNC} assessment to only 3×10^5 cells, as for the FCM^{NC} assay, seven of these 11 samples were MRD negative. Hence, most of the increase in sensitivity was related to the number of cells acquired. The MRD levels measured by FCM^{MNC} had a 1.85 times higher mean than those obtained by FCM^{NC} (SD 1.86; among 86 paired positive samples).

haematologica | 2012; 97(10)

Different methodology: NC is input 3 x10^6 (4 colors)
MNC is input 0.75 - 1 x10^6 (7 colors)

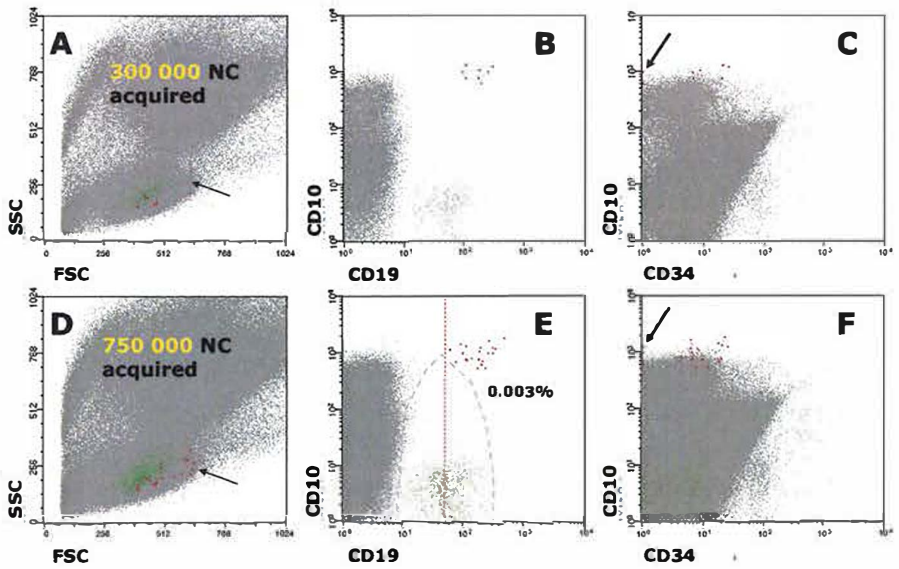
Enhanced sensitivity of flow cytometry for routine assessment of minimal residual disease

Esther Domingo,[1] Cristina Moreno,[1] Alfonso Sánchez-Iberrola,[1] Carlos Panizo,[1] José Antonio Páramo[2] and Juana Merino[1]

haematologica | 2010; 95(4)

In a recent paper by Béné and Kaeda,[1] technical approaches for minimal residual disease (MRD) assessment are extensively reviewed. PCR-based studies have proved to be 1-log more sensitive than flow cytometry (FC). For this reason, they are increasingly being preferred for MRD analysis, especially at the end of therapy or post hematopoietic stem cell transplantation.[1,2] It would be valuable to develop MRD flow cytometry assays with this level of sensitivity that could be applied routinely. In the present work, we analyzed MRD+ samples with a level of infiltration below the limit of detection of routine FC, which is accepted as 10^{-4} and comes from the standard acquisition of 2-5x10^6 leukocytes.[3-11] At least 10-fold more leukocytes must be acquired to increase sensitivity by 1-log; this large number of leukocytes can be acquired easily in digital cytometers by acquiring several individual tubes stained with the same combination of monoclonal antibodies, and putting them in a single file. Because the time of acquisition for each individual tube is not increased, no problems of cellular aggregation arise.

In summary, acquiring 6 million leukocytes is feasible with a digital cytometer on a routine basis. Because detection of 50-60 malignant cells is required to get a CV less than 15%, a sensitivity of 1×10^{-5} is achieved. Being able to routinely apply MRD FC assays with high sensitivity would be very valuable, especially in cases where molecular techniques cannot be used.



iBFM-FLOW

homogeneous appearance: not with all markers!

Towards one voice in ALL FLOW-MRD:

➡ I-BFM standards

iBFM-FLOW

- erythrocyte lysis
- staining panel: minimum 2 tubes per sample
  constant backbone of 2-3 markers
  for at least 4-color flow cytometry
  minimum different 6-8 markers in set-up
- B-LIN obligatory markers: CD10, 19, 20, 34, 38, 45, 58
  optional markers: CD9, 11a, 66c, 123, ...
- T-LIN obligatory markers: cyCD3, sCD3, CD5, 7, 45, 99, TdT
  optional markers: CD1a, 4, 8, 10, 34, 56/16, 117...
- panel rules: CD10 with strong label: PE, PE-Cy7 preferred
  cyCD3 and sCD3: same moAb clone
  „lack of expression": strong label (CD11a, CD38)

New markers for MRD – are they necessary?

iBFM-FLOW

Aberrant Underexpression of CD81 in Precursor B-Cell Acute Lymphoblastic Leukemia

Utility in Detection of Minimal Residual Disease by Flow Cytometry

Tariq Muzaffar, MBBS,[1] L. Jeffrey Medeiros, MD,[1] Su A. Wang, MD,[1] Archana Brahmandam, MS,[1] Deborah A. Thomas, MD,[2] and Jeffrey L. Jorgensen, MD, PhD[1]

Overexpression of CD123 correlates with the hyperdiploid genotype in acute lymphoblastic leukemia

Miroslav Djokic,[1*] Elisabet Björklund,[1] Elisabeth Blennow,[2] Joanna Mazur,[1] Stefan Söderhäll,[4] and Anna Porwit[1]

Leukemia (1999) 13, 550–56

A limited antibody panel can distinguish B-precursor acute lymphoblastic leukemia from normal B precursors with four color flow cytometry: implications for residual disease detection

EG Weir, K Cowan, P LeBeau and MJ Borowitz

CD9

Brief Communication

Overexpression of CD49f in Precursor B-cell Acute Lymphoblastic Leukemia: Potential Usefulness in Minimal Residual Disease Detection

Joseph A. Ducksworth,[1,2] Sheila G. Fuller,[1] and Michael J. Borowitz[1]

CD304 Is Preferentially Expressed on a Subset of B-Lineage Acute Lymphoblastic Leukemia and Represents a Novel Marker for Minimal Residual Disease Detection by Flow Cytometry

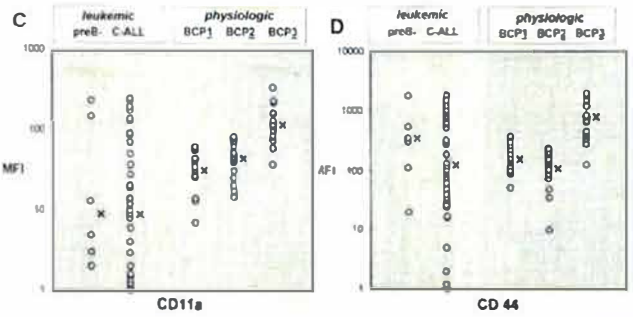
Françoise Solly,[1,2] Fanny Angelot,[1,2] Richard Garand,[1] Christophe Ferrand,[1,2] Estelle Scillin,[1,2] Françoise Schillinger,[1] Agnès Decobecq,[1] Maryse Billot,[1] Fabrice Larosa,[1] Emmanuel Plouvier,[1] Eric Deconinck,[3,4] Faezeh Legrand,[3] Philippe Saas,[1,3] Pierre-Simon Rohrlich,[3,4] Francine Garnache-Ottou[1,2]

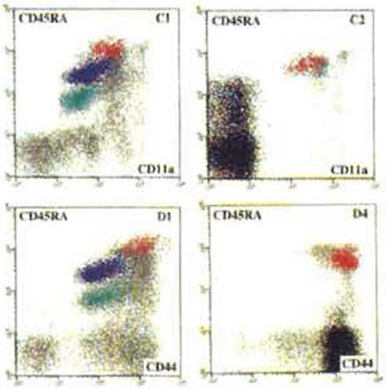
Experimental Hematology 26:305–313 (1998)

Comparative phenotype mapping of normal vs. malignant pediatric B-lymphopoiesis unveils leukemia-associated aberrations

Michael N. Dworzak, Gerhard Fritsch, Christine Fleischer, Dieter Printz, Gertraud Fröschl, Petra Buchinger, Georg Mann, Helmut Gadner

Children's Cancer Research Institute, St. Anna Kinderspital, Vienna, Austria





Experimental Hematology

iBFM-FLOW

New markers for MRD – are they necessary?

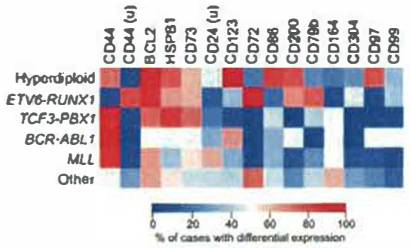
iBFM-FLOW

blood

Prepublished online April 12, 2011;
doi:10.1182/blood-2010-12-324004

New markers for minimal residual disease detection in acute lymphoblastic leukemia

Elaine Coustan-Smith, Guangchun Song, Christopher Clark, Laura Key, Peixin Liu, Mohammad Mehrpooya, Patricia Stow, Xiaoping Su, Sheila Shurtleff, Ching-Hon Pui, James R. Downing and Dario Campana

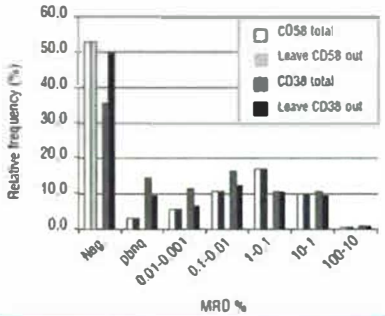


Of the 30 markers, 22 (CD44, BCL2, HSPB1, CD73, CD24, CD123, CD72, CD86, CD200, CD79b, CD164, CD304, CD97, CD102, CD99, CD300a, CD130, PBX1, CTNNA1, ITGB7, CD69, CD49f) were differentially expressed in up to 81.4% of ALL cases; expression of some markers was associated with the presence of genetic abnormalities.

Minimal residual disease analysis by eight-color flow cytometry in relapsed childhood acute lymphoblastic leukemia

Leonid Karawajew,[1] Michael Dworzak,[2] Richard Ratei,[1] Peter Rhein,[1] Giuseppe Gaipa,[2] Barbara Buldini,[2] Giuseppe Basso,[3] Ondrej Hrusak,[4] Wolf-Dieter Ludwig,[1] Günter Henze,[5] Karl Seeger,[5] Arend von Stackelberg,[6] Ester Mejstrikova,[4] and Cornelia Eckert[5]

iBFM-FLOW



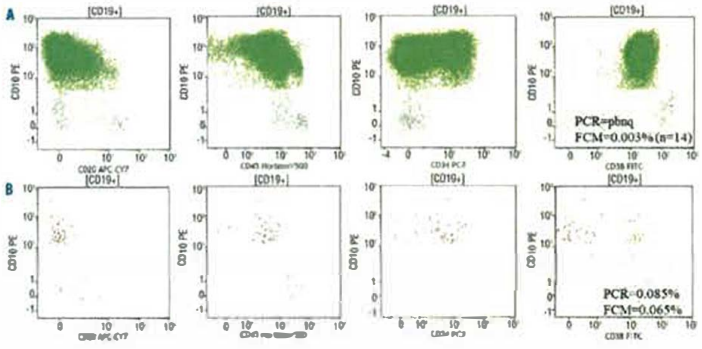


Figure 3. Histograms displaying the distribution of different MRD levels within testing series. The height of the bar (y-axis) corresponds to the relative frequency of the samples falling within the indicated MRD interval (x-axis). The series using experimental CD58-tubes comprised 159 samples, the series using the CD38-tube comprised 104 samples.

DuraClone Reagents

DURACLONE

Take dry unitized dried-down reagent tube | Add blood sample | Vortex & incubate | Add lysis solution & incubate | Acquire & analyze by flow cytometry

- **DuraClone** is BEC's proprietary line of dry reagent cocktails which can be used on several cytometry platforms.
- Shelf- table (at least 1 year) at room temperature, don't require cold chain.
- These are unitized, ready- o- se, affordable and accurate.
- Simplified work flow, minimum hands- n- ime and robust results

BECKMAN COULTER

Minimal residual disease-directed therapy for childhood acute myeloid leukaemia: results of the AML02 multicentre trial

Jeffrey E Rubnitz, Hirato Inaba, Gary Dahl, Raul C Ribeiro, W Paul Bowman, Jeffrey Taub, Stanley Pounds, Bassem I Razzouk, Normanj Lacaya, Xueyuan Cao, Soheil Meshinchi, Barbara Degar, Gladstone Airewele, Susana C Raimondi, Mihaela Onciu, Elaine Coustan-Smith, James R Downing, Wing Leung, Ching-Hon Pui, Dario Campana

www.thelancet.com/oncology **Published online May 6, 2010**

Table S1. Set of markers used to monitor MRD in AML02 and number of patients studied with each set

Marker combination[1]	Number of Patients Studied (%)[2]
CD13 / CD33	84 (41.2)
CD13 / CD33	67 (32.8)
CD117 / CD34 / CD33	62 (30.4)
CD13 / CD34 / CD33	60 (29.4)
CD13 / CD34 / CD33	55 (27.0)
CD13 / CD34 / CD33	52 (25.5)
CD117 / CD34 / CD33	47 (23.0)
CD13 / CD34 / CD33	30 (14.7)
CD13 / CD34 / CD33	28 (13.7)
CD13 / CD34 / CD33	20 (9.8)
CD13 / CD117 / CD34 / CD33	20 (9.8)
CD13 / CD117 / CD34 / CD33	20 (9.8)
CD13 / CD34 / CD33	18 (8.8)
CD13 / CD34 / CD33	15 (7.4)
CD13 / CD34 / CD33	15 (7.4)
CD117 / CD34 / CD33	13 (6.4)
CD13 / CD34 / CD33	13 (6.4)
CD13 / CD34 / CD33	10 (4.9)
CD13 / CD117 / CD34 / CD4	10 (4.9)
CD65 / CD13 / CD34 / CD33	10 (4.9)
CD117 / CD34 / CD33	10 (4.9)
CD65 / CD13 / CD34 / CD33	8 (3.9)
CD13 / CD34 / CD33	8 (3.9)
CD13 / CD34 / CD33	6 (2.9)
CD13 / CD117 / CD34 / CD33	5 (2.5)
CD13 / CD34 / CD65 / CD33	4 (2.0)
CD13 / CD34 / CD65 / HLA-Dr	4 (2.0)
CD13 / CD13 / CD34 / CD33	3 (1.5)
CD13 / CD13 / CD34 / CD33	1 (0.5)

4-color FLOW

Marker panels for AML-MRD analyses

comparison of different group approaches = consensus markers

backbones	colors	FAB-tubes	tubes / pt.	D45	CD34	CD117	CD33	HLADR	CD38	CD4
St. ude	8 (9)	no	2							
Virgo UK	6 (7)	yes	2 (3)							
Tierens NOPHO	8	(yes)	? (5)							
EUROFLOW	8	yes	? (8)							
BFM	7	yes	2 (3)							
AIEOP	5	no	1 to 3							

additional	n=	per tube	CD7	CD56	CD19	CD2	NG2	CD11b	CD14	CD64	CD16	CD13	CD15	CD133	CD41
St. Jude	11	2													
Virgo UK	6	2													
Tierens NOPHO	15	4													
EUROFLOW	26	4													
BFM	7	4													
AIEOP	13	2													

additional	CD235	CD36	CD96	CD123	CD135	CD184	CD10	CD22	TDT	CD35	IREM2	CD105	CD71	CD42a	CD42b	CD61	CD9	CD25	CD203	CD11a
St. ude																				
Virgo UK																				
Tierens NOPHO																				
EUROFLOW																				
BFM																				
AIEOP																				

AML-FLOW-MRD in the iBFM-FLOW-network

1) Equivalent panels are needed:

- 10 colour tube design
- Extensive 8 colour back-bone (different-from-normal detection)
- 2 variable slots (patient-specific LAIPs)
- Innovative markers in new combinations
- Stable cocktail formulations for the consortium (e.g. Duraclone BC; possibly also from BD)
- Single batch antibodies to variable markers for the consortium (e.g. Exbio)
- Towards automated software support

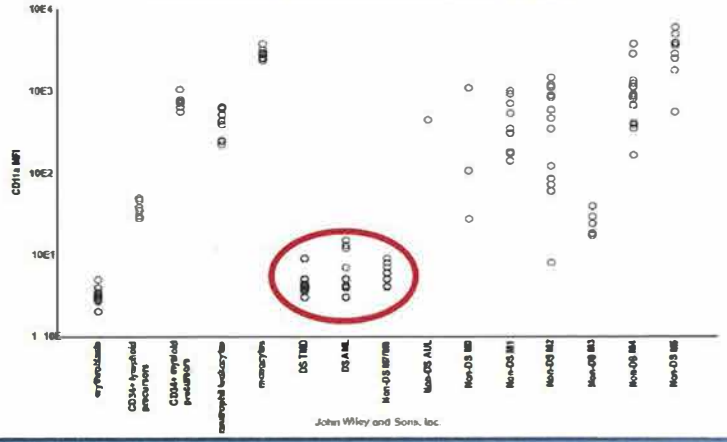
2) QC program including NEQAS AML-MRD trials

EU-AML CONSENSUS „ROME" 10-2014

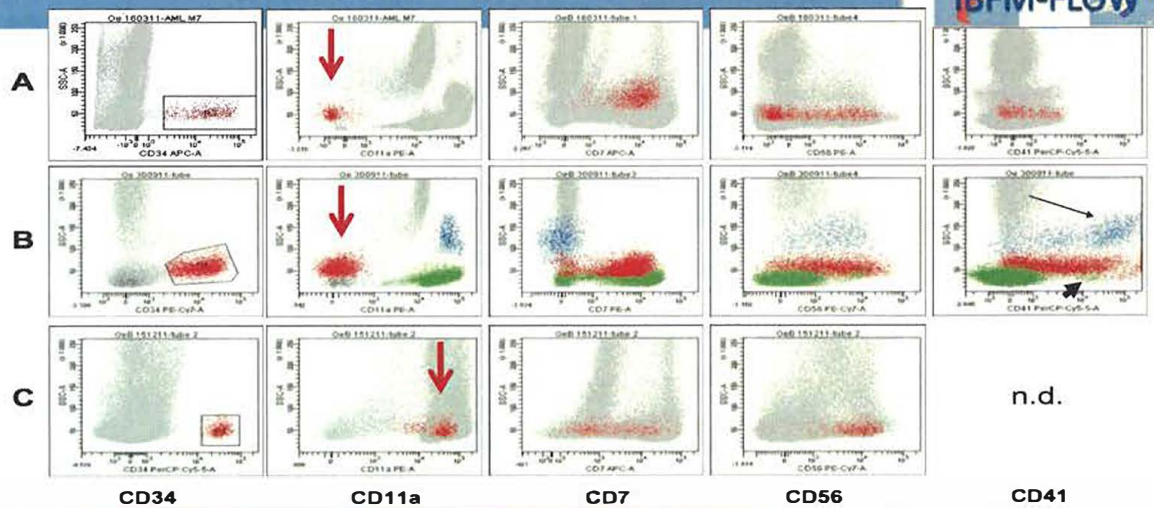
Blast Cell Deficiency of CD11a as A Marker of Acute Megakaryoblastic Leukemia and Transient Myeloproliferative Disease in Children with and Without Down Syndrome

Heidrun Boztug,[1]* Angela Schumich,[1] Ulrike Pötschger,[1] Sora Mühlegger,[1] Alexandra Kolenova,[2] Katarina Reinhardt,[3] and Michael Dworzak[1]*

Cytometry Part B (Clinical Cytometry) 99998:00–00 (2013)

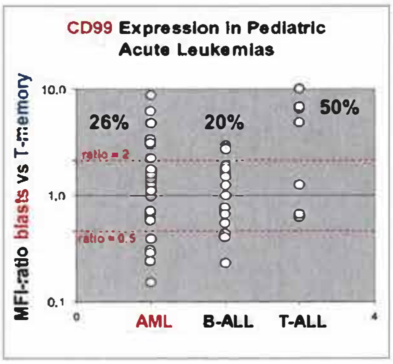


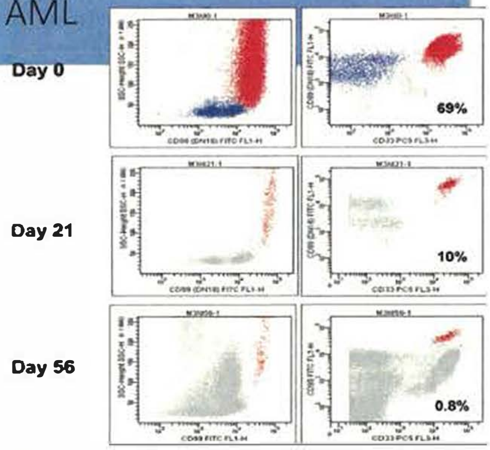
CD11a is a valuable MRD-marker in AML M7 and TMD



GATA1 analysis: K. Reinhardt, Hannover

MRD detection by CD99 in AML





All-*trans* retinoic acid and arsenic trioxide resistance of acute promyelocytic leukemia with the variant *STAT5B-RARA* fusion gene

Leukemia (2013) **27**, 1606–1610; doi:10.1038/leu.2012.371

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bjh research paper

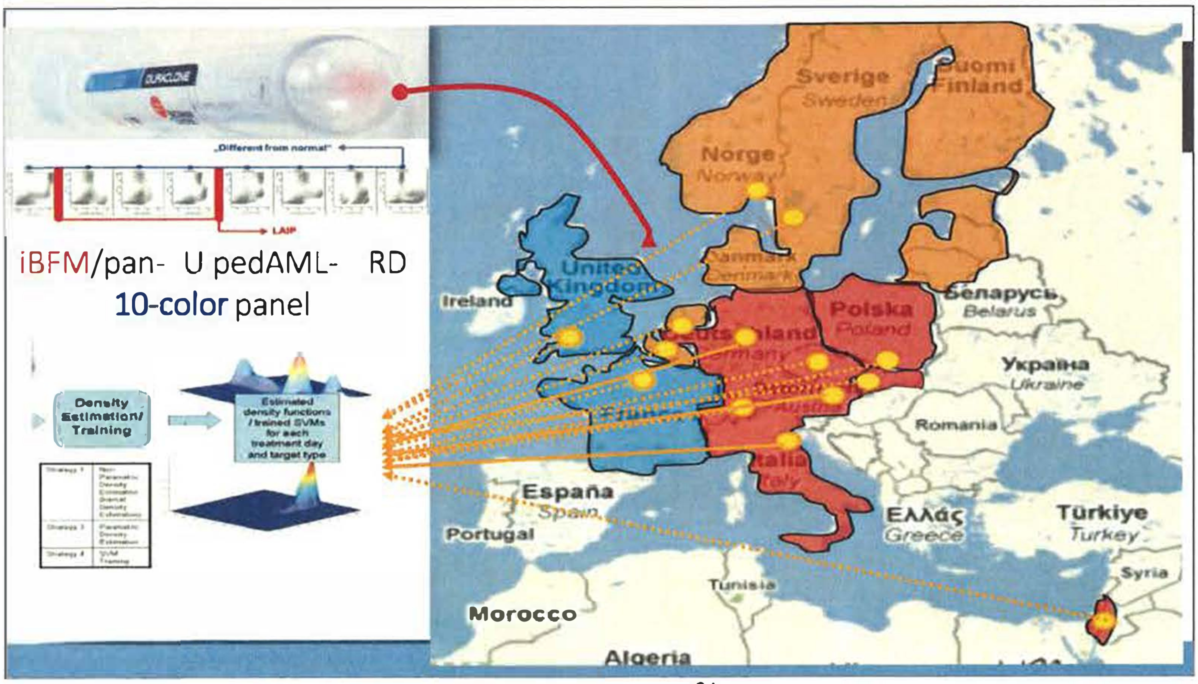
hMICL and CD123 in combination with a CD45/CD34/CD117 backbone – a universal marker combination for the detection of minimal residual disease in acute myeloid leukaemia

British Journal of Haematology, 2014, **164**, 212–222

hMICL = CLL-1

Spiking experiments revealed that the assay could detect MRD down to 10^{-4} in normal bone marrow with sensitivities equalling those of validated qPCR assays. Moreover, it provided at least one MFC MRD marker in 62/69 patients (90%). High levels of hMICL/CD123 LAIPs at the post-induction time-point were a strong prognostic marker for relapse in patients in haematological complete remission ($P < 0.001$). Finally, in post induction samples, hMICL/CD123 LAIPs were strongly correlated ($r = 0.676$, $P = 0.0008$) to applied qPCR targets. We conclude the hMICL/CD123-based MFC assay is a promising MRD tool in AML.

Anne S. Roug,[1] Hanne Ø. Larsen,[1] Line Nederby,[1] Tom Just,[2] Gordon Brown,[3] Charlotte G. Nyvold,[1] Hans B. Ommen[1] and Peter Hokland[1]



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G. Gaipa, O. Maglia, S. Sala (Monza)
O. Hrusak, E. Mejstrikova (Prague)
L. Karawajew, R. Ratei, S. Groeneveld-Krentz, J. Hoffmann (Berlin)

ALL IC strategy for patients with relapsed ALL

Janez Jazbec
UKC Ljubljana
Children's Hospital

- Currently achieved rates of event-free survival (EFS) in 1st complete remission (CR) are 70-80% with multi-drug chemotherapy

VOLUME 32 · NUMBER 3 · JANUARY 20 2014
JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Intensive Chemotherapy for Childhood Acute Lymphoblastic Leukemia: Results of the Randomized Intercontinental Trial ALL IC-BFM 2002

Jan Stary, Martin Zimmermann, Myriam Campbell, Luis Castillo, Eduardo Dibar, Svetlana Donska, Alejandro Gonzalez, Shai Izraeli, Dragana Janic, Janez Jazbec, Josip Kanja, Emilia Kaiserova, Jerzy Kowalczyk, Gabor Kovacs, Chi-Kong Li, Edina Magyarszy, Alexander Popa, Batia Stark, Yahia Jabali, Jan Trka, Ondrej Hrusak, Hansjörg Riehm, Giuseppe Masera, and Martin Schrappe

See accompanying editorial on page 169

- ALL IC-BFM 2002 study enrolled 5,060 children with ALL in 15 countries on 3 continents.
- is a good example of international collaboration in pediatric oncology.
- A wide platform of countries able to run randomized studies in ALL has been established.
- Alternative DI did not improve outcome compared with standard treatment and the overall results are worse than those achieved by longer established leukemia groups, the national results have generally improved

What about patients who relapsed on ALL-IC 2002

- The question not a part of study
- No sistematic data collection
- In Prague 2013 BFM meeting – request for common strategy for ALL-IC "countries"

Relapses in ALL IC 2002 study

- 5060 patients in ALL IC 2002 study
- By november 2010 – 830 relapses registered

- Great variability in data

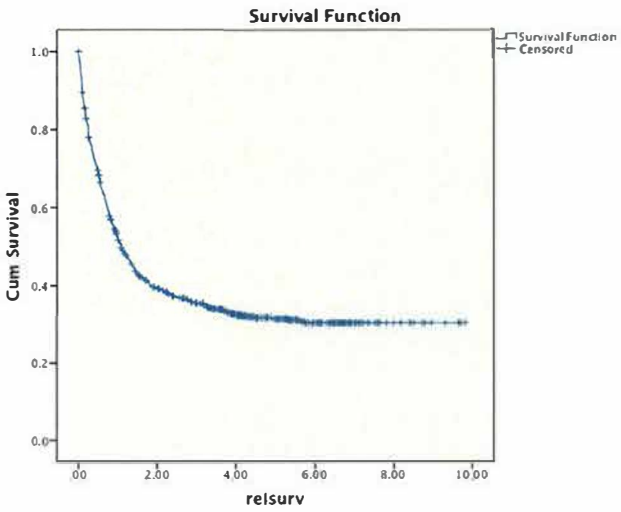
- Mean time to relapse: 2,19 years (0,11 to 6,59)

- By the end of 2013: 289 (35%) alive
  542 (65%) dead

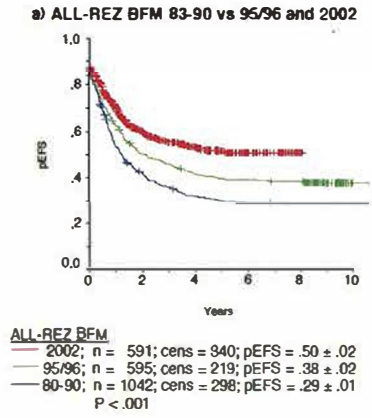
What about treatment?

ALL REZ 2002	178	24%
ARRIETA?		
BADER+FLAG		
BFM Rez 96	19	2,5%
CCG		
CLOFARABINE		
CTX,IT		
CTX+IT		
FLAG		
FLAG,RIVERRA		
HyperCVAD +		
Individual t		
IT+STEROIDS		
LA-REZ-2002		
LPU		
LLA-REZ-2002	129	16,9%
LLA-REZ-95 T	79	10,3%
Modified ALL	23	3,0%
no data	244	31,9
NO TREATMENT		
OTHER		
palliativ		
palliative t		
SAHOP	53	6,9%
sec AML		
treatment re		
VCR,DEXA,PEG		

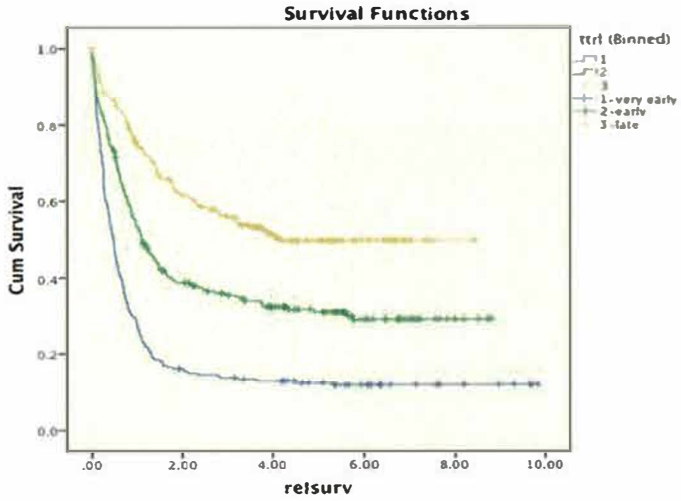
Overall survival



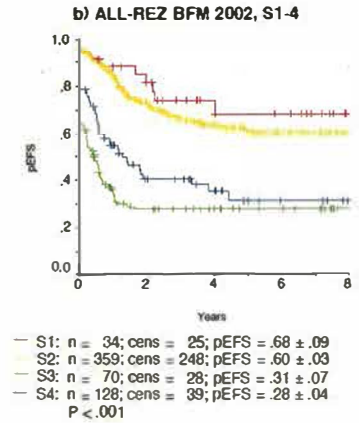
OS by BFM Rez protocol



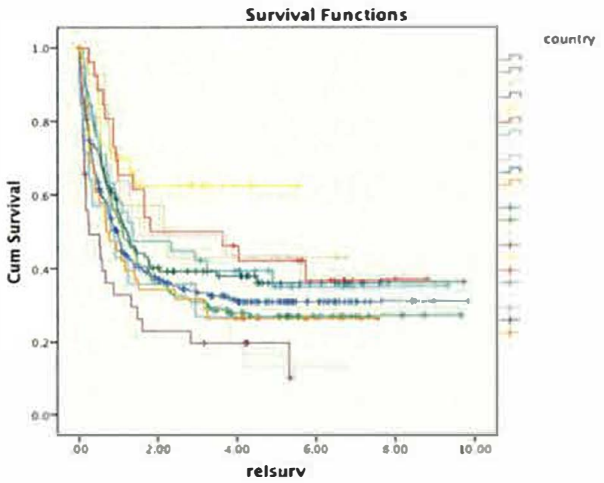
Survival by the time to relapse



OS by risk group



Survival by country



ALL-IC options

- Run your own trial (Argentina, Chile)
- Join existing (IntReALL??)
- New single arm registry trial

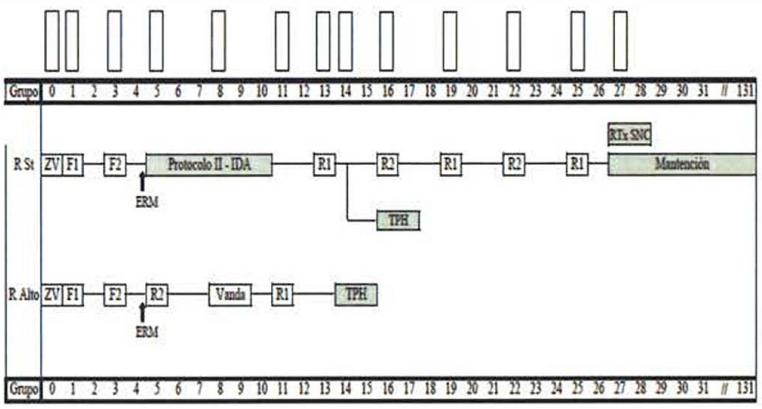
ALL-IC relapsed ALL protocol

- Prospective non-randomised observation study
- Goal is to set up a large international study group platform allowing for optimization of standard treatment strategies
- The main goal of this study is to improve the outcome of children and adolescents with first relapsed acute lymphoblastic leukemia.

The aim of the proposal

- to develop a single arm treatment guidelines for the treatment of children with relapsed ALL
- employ the combination of drugs that are already available
- NO randomization in the first stage
- to homogenize the diagnostic criteria and the treatment

ALL REZ PINDA 2013



Flow MRD

- Flow-cytometric determination of minimal residual disease was already an integral part of ALL-IC 2002 and 2009 study
- it is proposed to use Flow-cytometry for estimation of minimal residual disease at the end of the fourth week of induction (after F2 block) with threshold point of 10^{-3} to identify subgroup of patients with sub-optimal response to induction treatment.

Problem

- Aplastic D15 post F2 marrow
- The question of the timing of post-F2 bone marrow aspiration has not been finalized yet. Some argued for d15 sampling for easier laboratory analysis and evaluation.
- Vaskar Saha and Arend von Stackelberg advised for sampling at the time of bone marrow recovery based on the similar timing found prognostic with PCR MRD measurement

Cornelia Eckert:

- Within the ALL-REZ BFM 2002 trial we had it sometimes, that two weeks after finishing F1/F2 induction treatment the BM was still aplastic, therefore a second BM aspiration after F1/F2 was performed before start of the new treatment.
- Regarding indication for HSCT, we always used the second BM aspiration. Interestingly, in most cases the PCR MRD results was concordant.

Other issues omments

- 1) Nelarabine .It could be optional and in that case we agree to uniffy how to use it.
- 2) TYA (teenage and young adult group). Can we change the inclusion age criteria from under 18 to under 24 or > under 30 years?
- 3) Do we involve relapsed lymphoblastic lymphoma patients?
- 4) Tyrosine kinase inhibitors (TKIs).

Vanda?

Drug	Route	Dosage	Days						
Dexamethasone (DEXA)	p.o.	20 mg/m²/day; divided into 3 doses	1	2	3	4	5		
Cytosine arabinoside (ARA-)*	3 h i.v. infusion	2000 mg/m²/dosage, ? doses/day	1	2					
Mitoxantrone	1 h i.v. infusion	8 mg/m²/day			3	4			
Etoposide (VP-16)	1 h i.v. infusion	150 mg/m²/day			3	4	5		
Peg-Asparaginase (Peg- SP)	6 h i.v. infusion	1000 unite/m²							7
Methotrexate (MTX)	intrathecal	based on age	1						
Cytarubine (ARA-C)	intrathecal	based on age	1						
Dexamethasone (DEXA)	intrathecal	based on age	1						

DRUG	ROUTE	DOSAGE	DAYS
Dexamethasone (DEXA)*	p.o.	6 mg/m²/day	1 to 14
Vincristine (VNC)	i.v.	1.5 mg/m²/day, max 2 mg	1, 8, 15, 22
Idarubicine (IDA)	1 h i.v. infusion	6 mg/m²/day	1, 8, 15, 22
Peg-Asparaginase (Peg-ASP)	2 h i.v. infusion	1000 U/m²/day	1, 11
Cyclophosphamide (CPM)	1 h i.v. infusion	1000 mg/m²/dosage	29
Mesna	0' and 4' h of CPM	500 mg/m²/dosage	29
Cytarabine (ARA-C)	1 h i.v. infusion	75 mg/m²/dosage	31, 32, 33, 34, 38, 39, 40, 41
6-Tioguanine (6-TG)	p.o.	60 mg/m²/day	29 to 43
Methotrexate (MTX)	intrathecal	based on age	1, 15, 31, 38
Cytarabine (ARA-C)	intrathecal	based on age	1, 15, 31, 38
Dexamethasone (DEXA)	intrathecal	based on age	1, 15, 31, 38

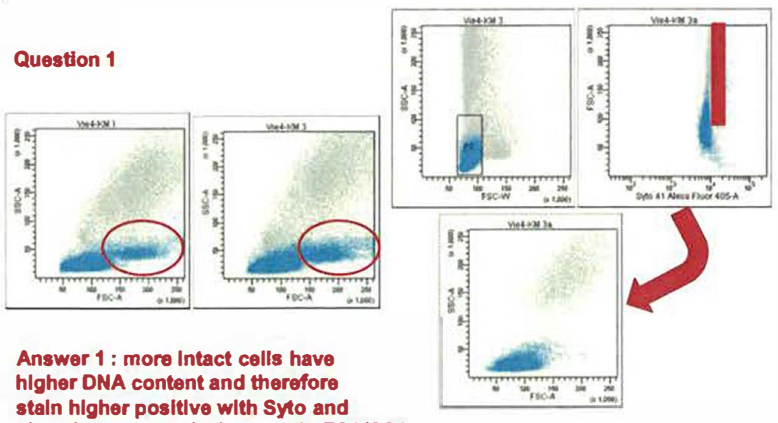
Differentiation between MRD-ALL and Hematogones .....

Angela Schumich
Children Cancer Research Institute Vienna
Ljubljana Flow Cytometric Meeting
Ljubljana December 2015

.....difficulties and pitfalls

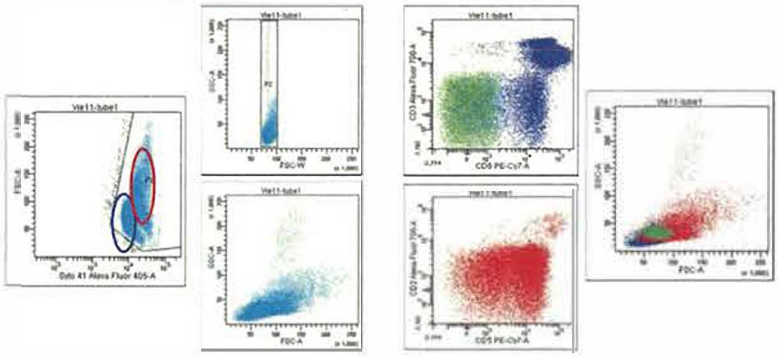
- Most frequent questions
- Most frequent failures
- BCP-ALL
- T- LL

Question 1



Answer 1 : more intact cells have higher DNA content and therefore stain higher positive with Syto and show less apoptotic features in FSC/SSC Do not exclude!

Question 1 another example



Do not exclude !!

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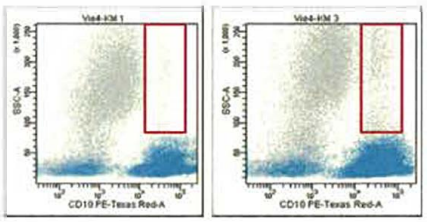
Question 2



Answer 2 : Granulocytes may stain unspecific positive with any AB prominent visible because of high cellnumber display Include only small FSC/SSC cells in Lymphogate !

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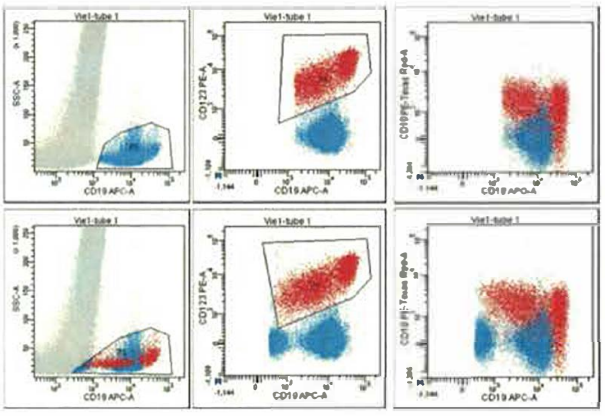
Failure 1 and 2



Correct is:

Two MRD-fractions are possible but not frequent

Try to include all cells

In this case 0,59% /0,66%

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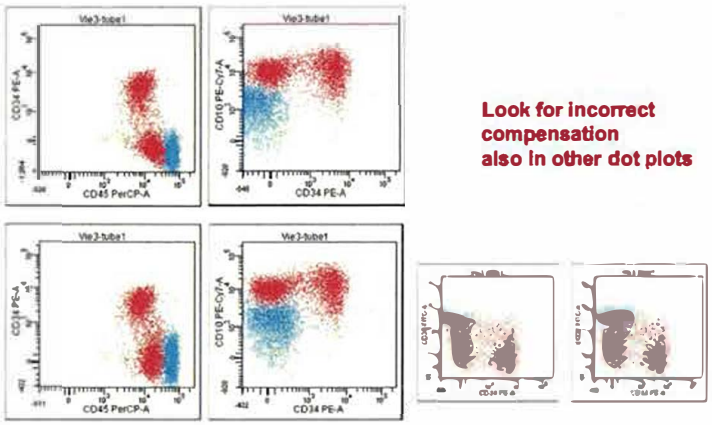
Failure (?) 3a



Look for incorrect compensation also in other dot plots

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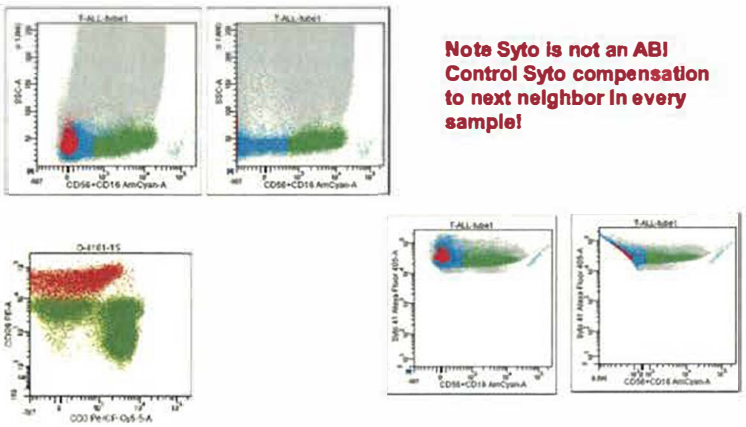
Failure (?) 3b



Note Syto is not an ABI Control Syto compensation to next neighbor in every sample!

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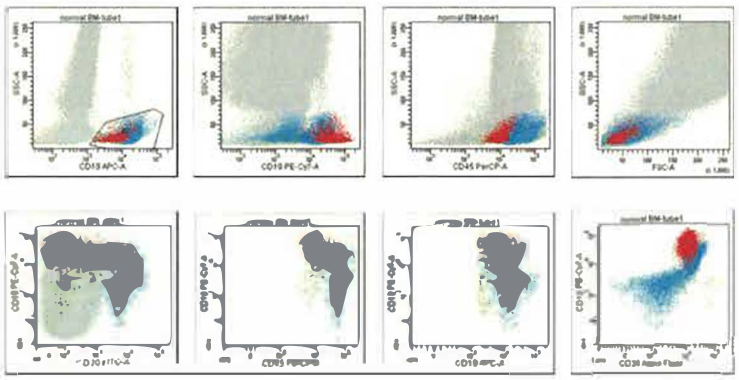
BCP- normal BM



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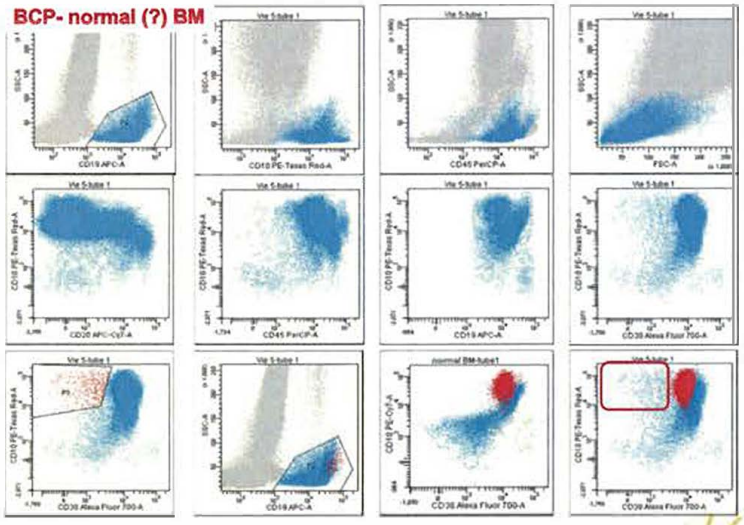
BCP- normal (?) BM



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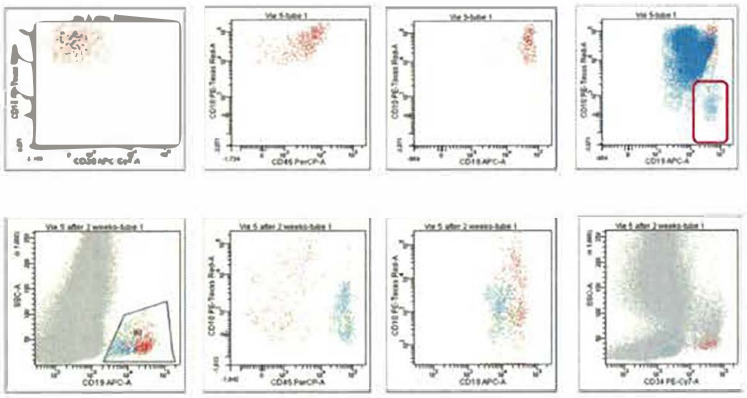

Early TP: 647 suspicious out of 1.000.000 cells (0,06%)
Later TP: 0,06% but no B-hematogones

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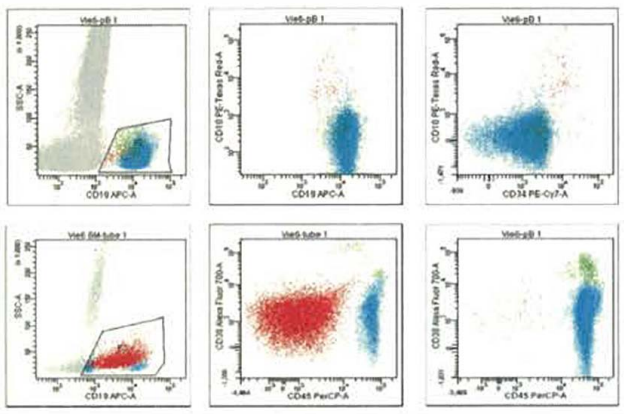

0,06%

We can detect ALL in pB if we stain sufficient number of cells

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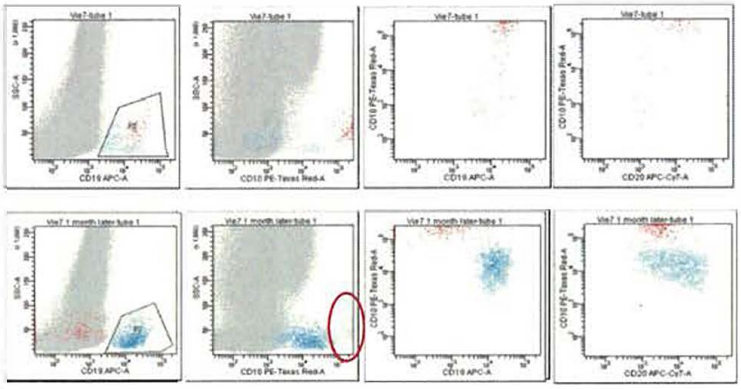

Look for aberrant appearance!
Note: Loss of CD19 under Blinatumomab treatment!

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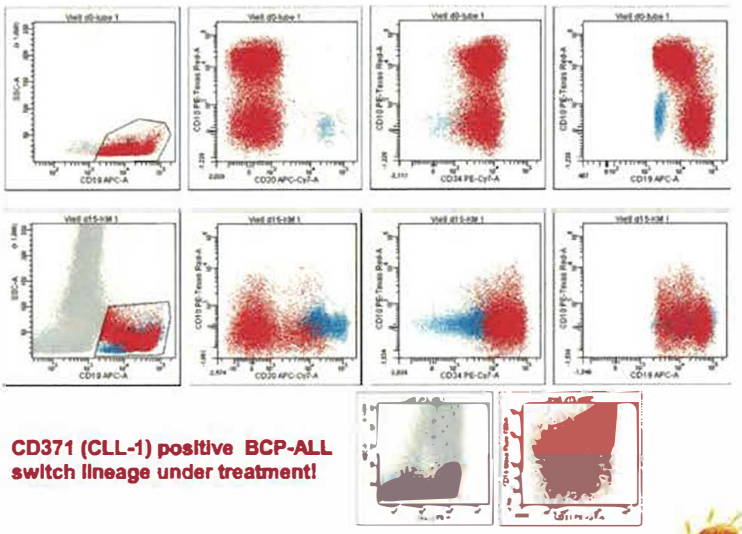

CD371 (CLL-1) positive BCP-ALL
switch lineage under treatment!

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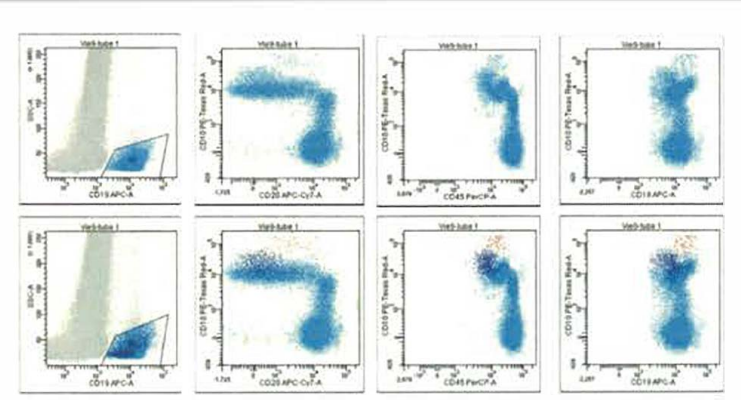

0,01% leukemic cells
0,08% BCP1 early B-cell-precursor

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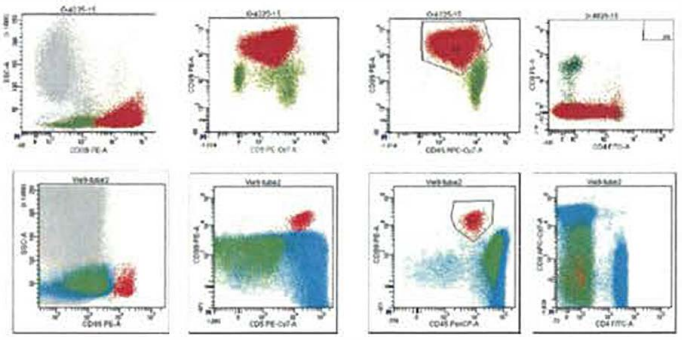
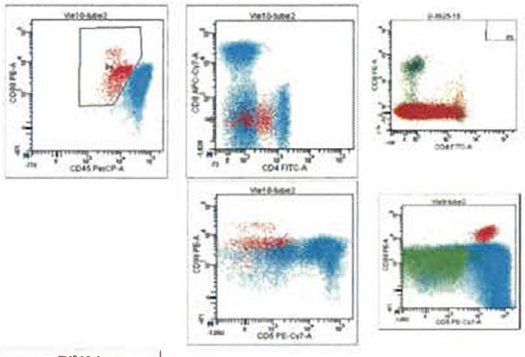
T-ALL

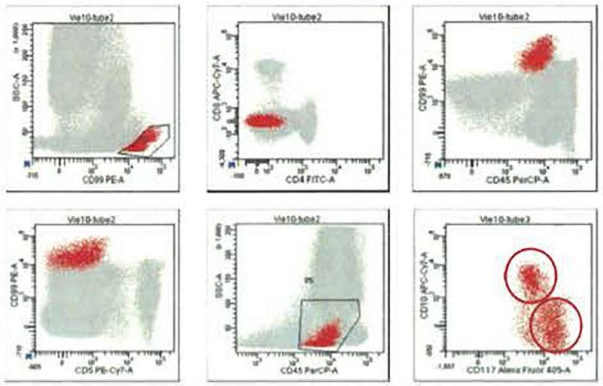


Upper row T-ALL d15
Lower row T-ALL relaps



CD99 positive cells in BM? T-ALL?
Of note: no T-hematogones in BM!

Majority of T-ALL is CD5 positive!



In healthy BM CD99 correlates with CD34
CD99 is positive on B and Myeloid precursers

Next time I may tell something about myeloid precursers

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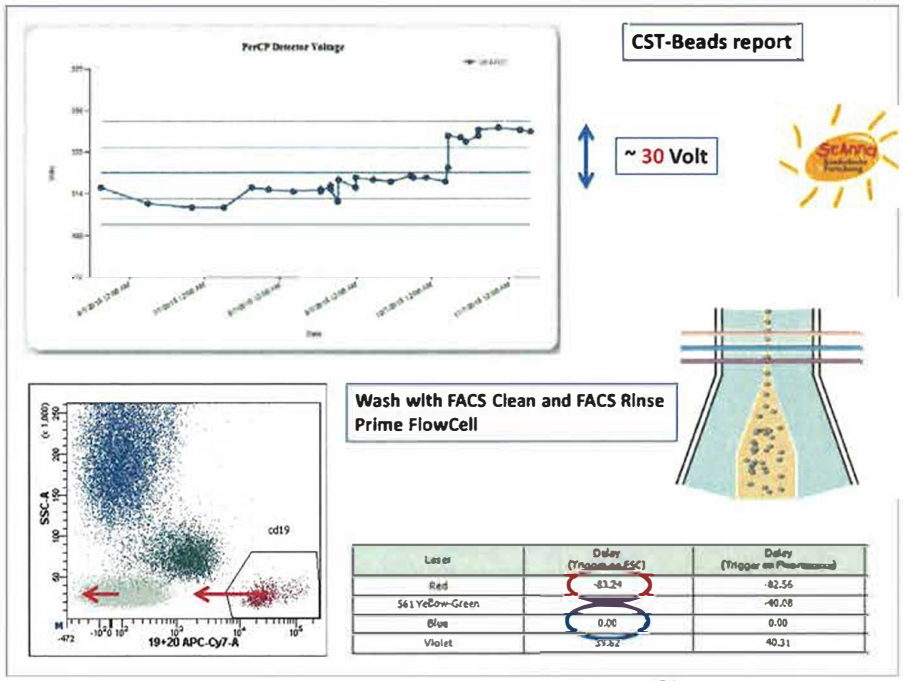
Angela Schumich

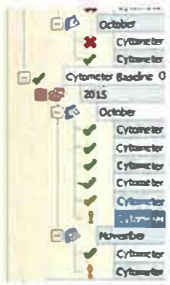
What could we do every day?!

- *Instrument controls*
- Staining controls
- Setting controls
- Compensation controls

Dieter Printz
FACS Core Unit
Children Cancer Research Institute Vienna



CST-Beads report

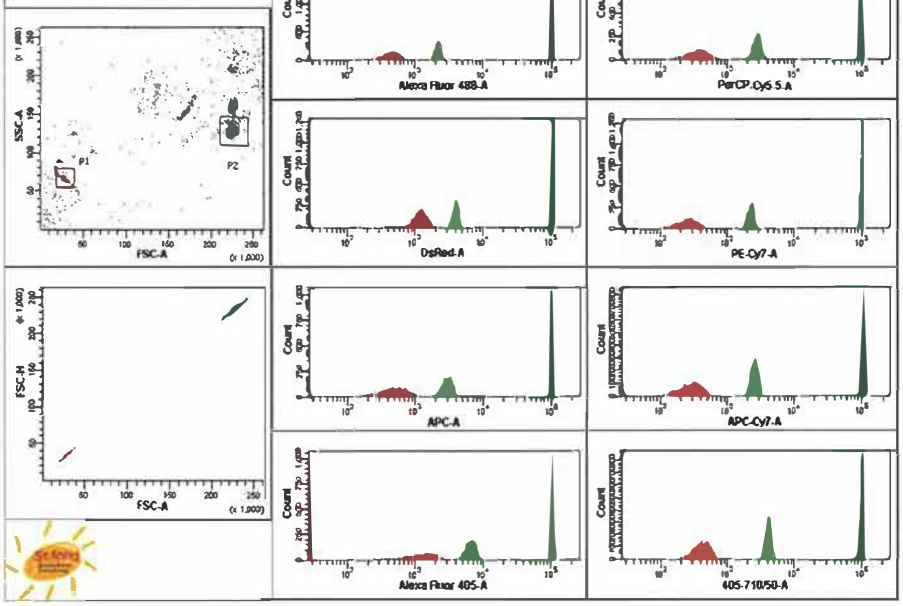


Parameter	Target Value	Actual Target Value	% Difference Target Value	Bright Bead % Robust CV
FSC	1 125000	125438	0	0.1
SSC	125000	121060	-4	4.7
Alexa Fluor 488	4980	4960	-1	3.99
PerCP	13773	13675	-1	5.67
Per CP-eFL710	21536	21635	0	5.16
APC	16197	15887	-2	4.6
Alexa Fluor 700	17127	17421	1	6.48
APC-Cy7	15521	15248	-2	6.66
BV421	2814	2750	-3	8.55
AmCyan	10342	10549	-3	4.4
BV605	18835	18604	-2	7.59
BV650	14061	14047	-1	5.62
BV711	8408	8204	-3	5.63
BV786	13242	12981	-2	6.93
PE	18566	17869	-4	4.69
PE-CF594	16944	16387	-4	4.77
PE-Cy5-5	17475	16682	-5	5.86
PE-Cy7	18805	18002	-5	6.32

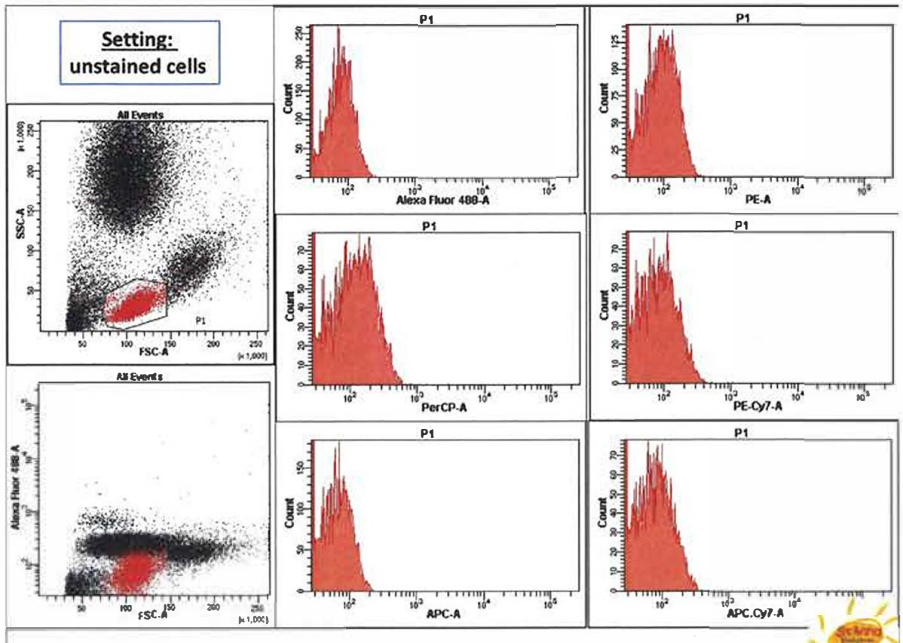
Parameter	Dim Bead Median Channel	Dim Bead % Robust CV	PMTV	Δ PMTV
FSC	8598	10.93	535	-18
SSC	54721	6.53	320	3
Alexa Fluor 488	16	118.61	430	0
PerCP	49	85.44	547	3
Per CP-eFL710	77	48.35	606	9
APC	94	23.67	424	2
Alexa Fluor 700	61	26.61	375	18
APC-Cy7	51	29.65	360	11
BV421	18	90.02	423	5

Wash with FACS Clean and FACS Rinse
Prime FlowCell

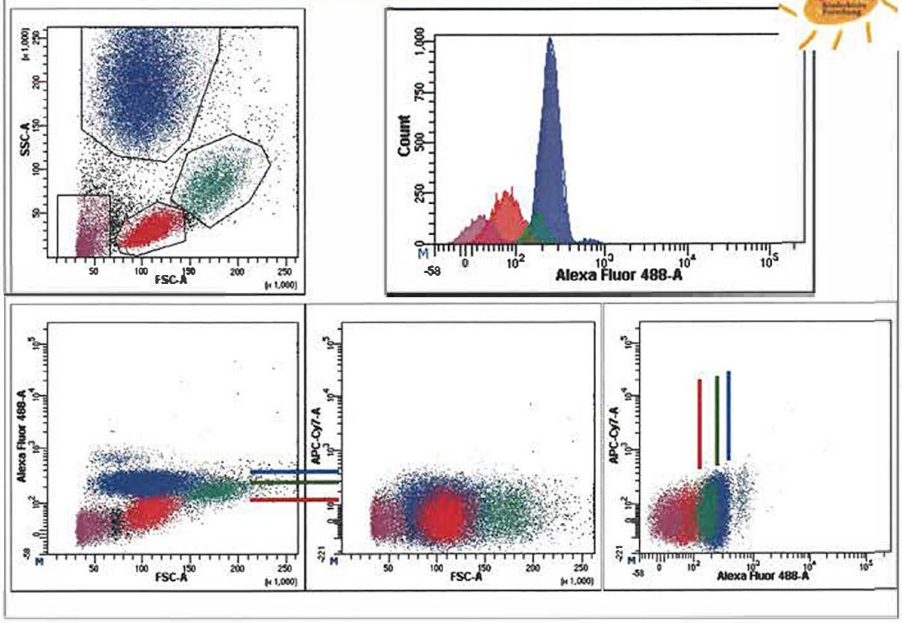
CST Beads manually



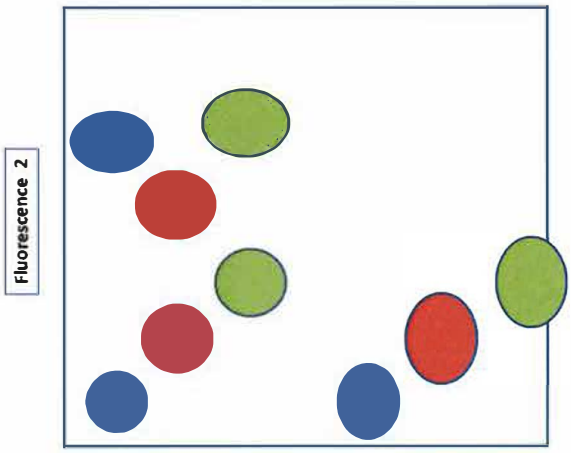
Setting: unstained cells



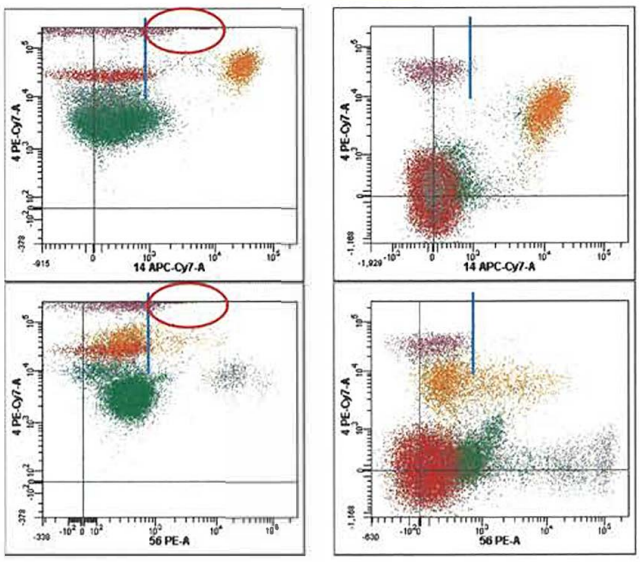
Setting: unstained cells - different autofluorescence



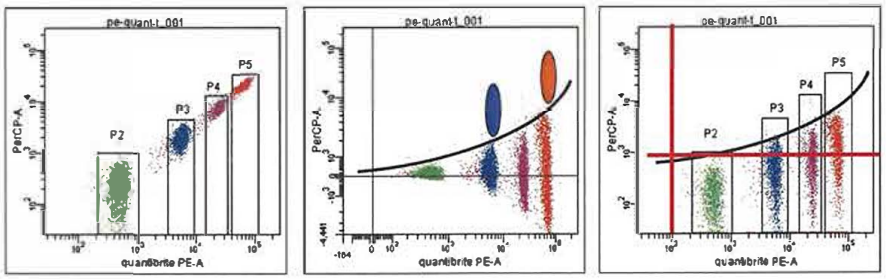
Compensation



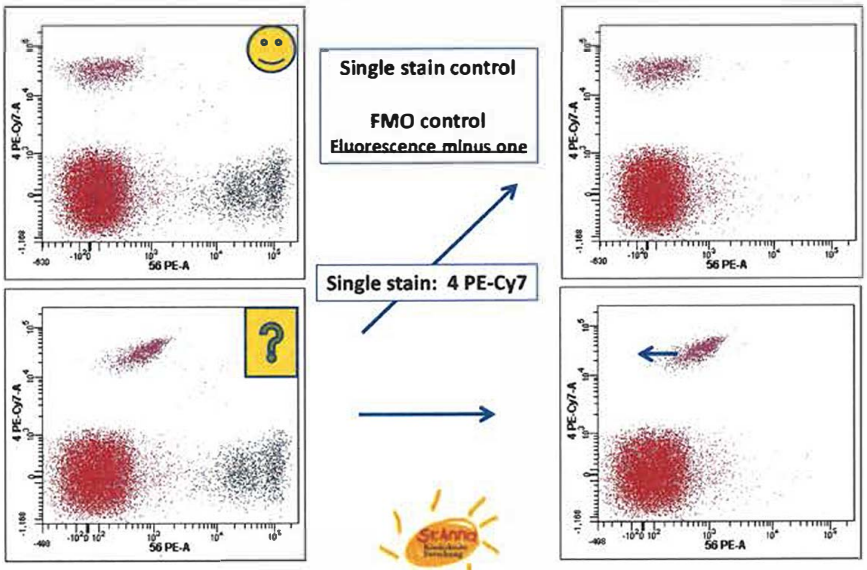
Signals are Off-scale



Dataspreading



Correct Compensation ?



THERE'S NO MAGIC !!!

Ljubljana Flow Cytometric Meeting
Ljubljana, 2015

Set-up and compensation of 8-color flow cytometer; EuroFlow view

Tomáš Kalina

on behalf of EuroFlow

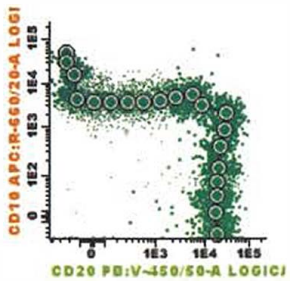
Charles University, 2nd Faculty of Medicine,
Prague, Czech Republic
Dpt. of Pediatric Hematology and Oncology

- *Childhood Leukemia Investigation Prague*

EuroFlow

What is the key feature of FCS data?

Patterns
- Fluorescence intensity
- Relative fluorescence



Factors setting the fluo intensity:
- PMT settings
- Reagent (clone, fluorochrome)
- Compensation (artefacts?)
- Sample preparation
- Errors

Flow cytometry data interpretation

FCS data:
- Pattern analysis
- Errors – is it for real?
- Comparison to reference

Can you interpret somebody else's FCS data?

What is limiting inter-laboratory collaboration?

Variability (pre-analytical and analytical)
- day-by-day,
- instrument-to-instrument
- lab-to-lab

Immunophenotyping workflow

- Cytometer settings
  - (PMT, compensation, daily checks)
- SOPs
  - Sample preparation
  - Panel composition
    - tubes, mAb + fluorochrome positions, titration)
- Analysis
- Blast gating and export
- Merge and interlab. analysis

Presentation content

1. Standardization – How to?
2. Compensation
3. Remarks

EuroFlow

8-color flow cytometry of hematological malignancies

Leukemia (2012) 26, 1986–2010

www.nature.com/leu

Open

SPECIAL REPORT

EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols

T Kalina[1,11], J Flores-Montero[2,11], VHJ van der Velden[2], M Martin-Ayuso[4], S Böttcher[5], M Ritgen[5], J Almeida[3], L Lhermitte[6], V Asnafi[6], A Mendonça[7], R de Tute[8], M Cullen[8], L Sedek[9], MB Vidriales[10], JJ Pérez[10], JG te Marvelde[2], E Mejstrikova[1], O Hrusak[1], T Szczepanski[9], JJM van Dongen[2] and A Orfao[2] on behalf of the EuroFlow Consortium (EU-FP6, LSHB-CT-2006-018708)

www.euroflow.org

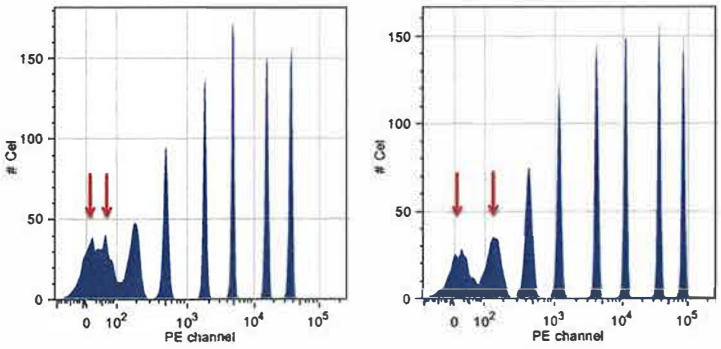
EuroFlow

EuroFlow Instruments Setup & compensation Protocol

Index:

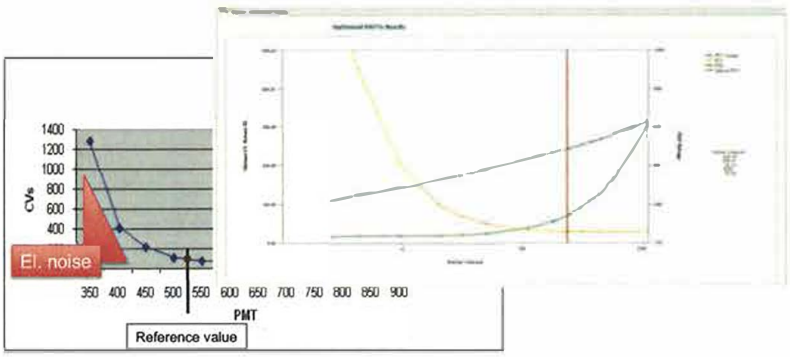
Optimization of PMT voltage

1. Best resolution of dim -> put PMTv above noise



Less noise more peaks !!
= sensitive to low expression

Optimization of PMT voltage above noise

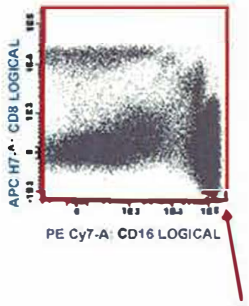


- Reference values in the plateau part of the curve in all instruments

Ref. :Maecker, H. T. and J. Trotter (2006). Cytometry A.

EuroFlow

Optimization of PMT voltage

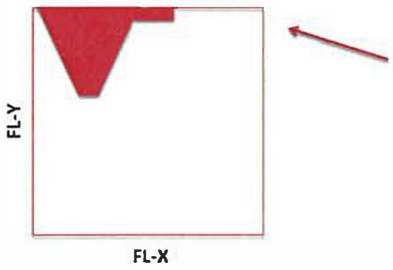


- No bright positive population out of the window of analysis

Ref. :Maecker, H. T. and J. Trotter (2006). Cytometry A.

EuroFlow

Beware of Martini effect!



- No bright positive population out of the window of analysis

FL-X true = **FL-X** measured - (0.15) x **FL-Y** measured

EuroFlow

Optimization -> Standardization

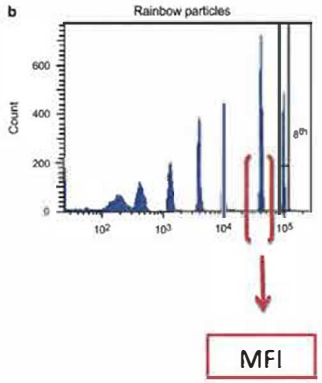
Once optimized -> fix it

Two approaches

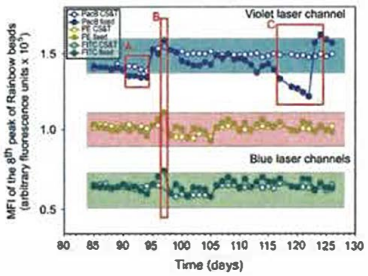
- Fixed voltages – record MFI
- Fixed MFI – record voltage
  - EuroFlow – manual check
    - manual adjust if needed be
  - CS & T – automated check and adjust

Standardization and instrument QA

- Mean fluorescence intensity **(MFI)** of the bright peak
- **CV** of the bright peak



Daily QC with Rainbow
x CS&T beads by BDB



EuroFlow target values

Daily check Set-up Daily check

Lot:		Target MFI	
EAB01	*Lower MFI (-±5%)*	*Rainbow 7-peak (DiVa)*	*Upper MFI (+15%)*
Pacific Blue	100,452	118,178	135,905
Pacific Orange	93,871	110,436	127,002
FITC	28,752	33,826	38,900
PE	32,381	38,095	43,809
PerCP	66,846	78,642	90,438
PC7	8,316	9,783	11,250
APC	158,639	186,634	214,629
APC-H7	64,194	75,522	86,850

Rainbow 8-peak beads (Spherotech) – **EuroFlow lot** ("EAB01", "EAC01"...)

Check www.euroflow.org for the new lots

Fluorochrome selection - EuroFlow

Laser	Fluorochromes	Equivalent reagents	2. generation MRD panels
Violet	Pacific Blue	HV450	BV421
Violet	Pacific Orange	HV500	BV510
Blue	FITC		
Blue	PE		
Blue	PerCP Cy5.5		
Blue	PE Cy7		
Red	APC		
Red	APC H7	APC C750 APC Ax750	

EuroFlow

Presentation content

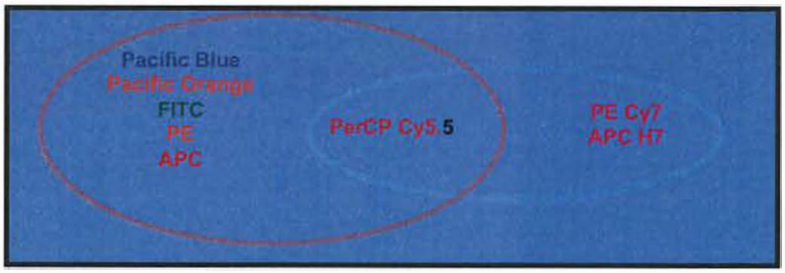
① Standardization – How to?

② Compensation

③ Remarks

Fluorochromes

Single fluorochrome dyes **Tandem dyes**

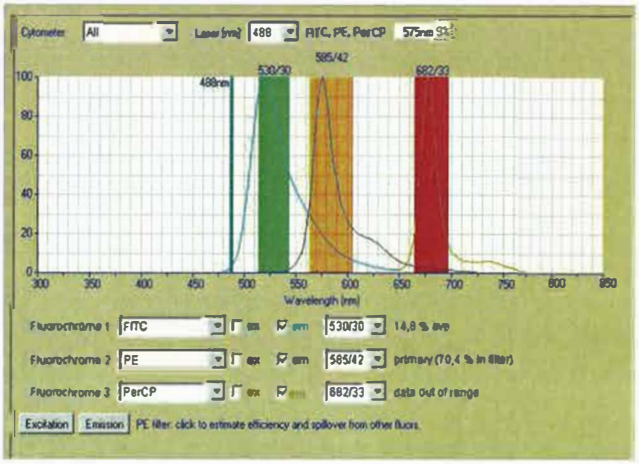


Generic reagent independent FL compensation

Reagent conjugate & lot specific needs

EuroFlow

Compensation



PEtrue = PEmeasured - (0.15) x FITCmeasured

www.bdbiosciences.com/spectra /

Compensation – what you need

- CAVEAT : Still the most frequent error in execution of standardized protocol
- Understand the compensation theory and the DiVa software
  - Diva Manual
  - EuroFlow SOP
  - Single stained tubes
  - Common sense and practice

Three Rules for Compensation Controls

The Daily Dongle

First and foremost, there must be a single stained control for every parameter in the experiment!

In addition, there are three rules for "good" compensation controls:

1) Controls need to be at least as bright or brighter than any sample the compensation will be applied to

2) Background fluorescence should be the same for the positive and negative control

Lympocytes ≠ beads ≠ monocytes

3) Compensation controls MUST match the exact experimental fluorochrome

Pacific Orange ≠ HV-500

http://flowjo.typepad.com/the_daily_dongle/2011/09/three-rules-for-compensation-controls.html

Generic fluorochromes and fluorochrome tandems

Generic fluorochromes		Tandem fluorochromes			
Generic targets	Positive target (Bead or cell) population	PECy7 targets	Positive (target Bead or cell) population	APCH7 targets	Positive target (Bead or cell) population
CD20 PacB	B-cells	CD2 PECy7	CD2⁺ T/NK-cells	CD3 APCH7	T-cells
CD45 PacO	Lymphocytes	CD8 PECy7	CD8⁺ T	CD4 APCH7	CD4⁺ T-cells
CD8 FITC	CD8⁺ T-cells	CD10 PECy7#	CompBead	CD8 APCH7	CD8⁺ T-cells
CD8 PE	CD8⁺ T-cells	CD16 PECy7	NK-cells	CD9 APCH7#	CompBead
CD5 PerCPCy5.5*	CD5⁺ T-cells	CD19 PECy7	B-cells	CD10 APCH7#	CompBead
CD8 APC	CD8⁺ T-cells	CD45RA PECy7	CD45RA⁺ T-cells	CD14 APCH7*	Monocytes
		CD45RO PECy7	CD45RO⁺ T-cells	CD19 APCH7	B-cells
		CD56 PECy7	NK- & CD16⁺ T-cells	CD24 APCH7	B-cells
		CD117 PECy7#	CompBead	CD38 APCH7	CD38⁺ Lymphocytes
		HLADR PECy7	B- & HLADR⁺ T-cells	CD43 APCH7	T-cells
				CD49d APCH7	T-cells
				CD71 APCH7#	CompBead
				CD81 APCH7	B-cells
				Bmig APCH7#	CompBead

*This tandem reagent requires generic compensation
#: Negative CompBead used as negative reference population
*: Artificialy CD14⁺ monocytes created by "appending" 5000 events from the unstained tube to this tube acquisition

EuroFlow SOP

Compensation - Automatic Method

Automatic compensation in the Diva software offers a fast, easy and reliable method to set the correct compensation.

- First, elect „Create Compensation Tubes" from the Instrument Menu:

Compensation - Automatic Method

The software automatically creates a list of single color tubes, based on your instrument setting.

Reagent specific controls for tandem dyes

Figure 3: Illustrating screen printout of the list of fluorophores and corresponding labels used to define the compensation controls.

EuroFlow compensation overview

Compensation experiment (monthly)

(all comp. reagents at once)

Compensation catalog

(same name, rewritten by each new comp exp.)

EuroFlow panels

ALOT
BCP-ALL
AML
T-ALL

MRD
SST
LST

In house panels

over 20 panels

(all panels use names matching with catalog)

Presentation content

① Standardization – How to?

② Compensation

③ Remarks

Problems

- Reagent ageing
  - > Use as few tandems as possible, use them up quick
  - > Use stable lyo or dried reagents

- Errors in compensation experiment
  - Mostly minor overcompensation in non-overlapping channels (PacB vs APC-H7), often single stained tube handling issues, gating problems.

  –> Manual check (not in SOP, hard to objectively setup)
  –> Automated compensation check (generic min-max values, constrains by past experiments)

Future directions

- If signal is kept standardized (Rainbow or CS&T)
  - Re-compensation is not necessary due to setup

But:
Reagents must be identical
Batch to batch variation (improving, but in the hands of companies)
Stability over time (improving, lyo or dried reagents)
Reagent control instead of re-compensation?
-> reagent QC tool Infinicyt

More colors, more instruments

- 16 color setup / Target values EuroFlow members only

  Difficulties:
  All emission filters must be identical
  (or spectrally matched beads should be used)

- 8 color setup for Navios / Rainbow Targets given

  Difficulties:
  Using Navios filters Targets are valid for PacB and OC515 for whoch it was developed
  (or spectrally matched beads should be used)

Anticipated questions

Do you really see no need to adjust compensation a little bit for each particular sample?

How good is good enough and how much we pay for perfection?

If the small imperfection is not changing my interpretation (e.g. negative becomes dim) -> I don't care

If it does -> I should change my panel becuase it is not robust

Why don't you use CS&T instead of Rainbow?

We work with Rainbow beads for ages, CS&T only simplifies it, the improvement of the data is not visible (CS&T are equally good though!)

EuroFlow 2006 – 2015 *..and going on*

Standardization in Flow Cytometry

Set-up and Compensation of 10-color Flow Cytometer BD view

Jiří Šinkora, BD Biosciences
Ljubljana Flow Cytometric Meeting
30th November and 1st December 2015
Institute of Oncology, Ljubljana

Standardization in Flow Cytometry

Goal of Standardization:

To get **consistent, reproducible results**

on **samples**

stained with **Standard reagents**

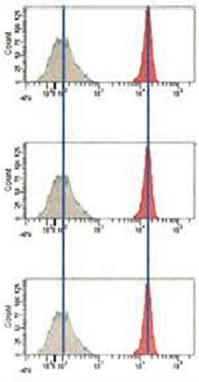
using **Standard protocols**

Lyse / Wash (LW)
Lyse No Wash (LNW)
EuroFlow SOP
.....

on **Standardized flow cytometers**

Standardization in Flow Cytometry

"Identical" results across multiple users and days on every single cytometer



Day 1
User 1, 2, 3....

Day 2
User 1

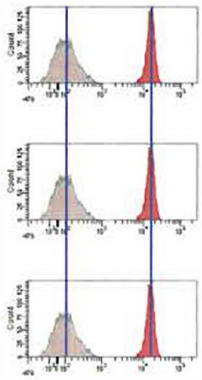
Another day(s)
User 1, 2, 3....

User-independent data consistency in time

Standardized flow cytometer

Standardization in Flow Cytometry

"Identical" results across cytometers and sites



Site 1
Cytometer 1

Site 1
Cytometer 2

Site 2
Any cytometer

Inter-instrument data consistency

Standardized flow cytometers

Standardization in Flow Cytometry

Do you need it?
How to do it?

"Identical" (as similar as possible) results across cytometers and sites on different days for all users

Standardized flow cytometers

Standardization in Flow Cytometry

How to do it?

By bringing the selected **cell population**
(e.g. lymphocytes in PBL preparations)
to the same **FSC and SSC** intensity channels.

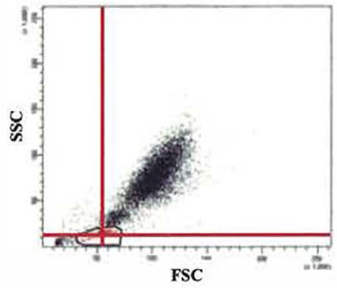
By bringing **brightly fluorescent objects**
(e.g. polychromatic beads or cells stained with anti-CD8 conjugates)
to the same intensity channels in all **fluorescence** detectors

Standardized flow cytometers

Standardization in Flow Cytometry

Example: EuroFlow

By bringing the selected **cell population**
(e.g. lymphocytes in PBL preparations)
to the same **FSC and SSC** intensity channels.



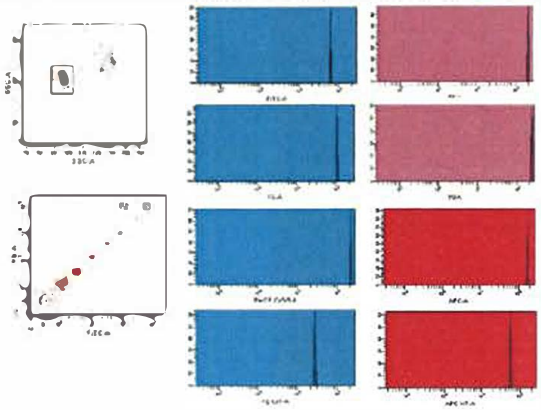
FSC_{MED} = 55 000
SSC_{MED} = 13 000

7

Standardization in Flow Cytometry

Example: EuroFlow

By bringing **brightly fluorescent objects**
(8 peak polychromatic Rainbow beads)
to selected (by experience) channels in 8 **fluorescence** detectors



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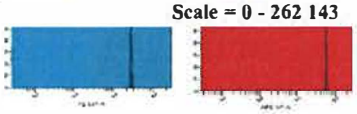
Standardization in Flow Cytometry

Example: EuroFlow

By bringing **brightly fluorescent objects**
(8 peak polychromatic Rainbow beads)
to selected (by experience) channels in 8 **fluorescence** detectors

	Lower MFI (-15%)	Target MFI Rainbow 8-peaks (DiVa)	Upper MFI (+15%)
PacB	166,236	195,572	224,908
PacO	196,575	231,265	262,143
FITC	50,638	59,574	68,510
PE	86,615	101,900	117,185
PerCPCy5.5	183,654	216,064	248,474
PECy7	23,343	27,462	31,581
APC	150,263	176,780	203,297
APCH7	47,971	56,437	64,903

Scale = 0 - 262 143



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Standardization in Flow Cytometry

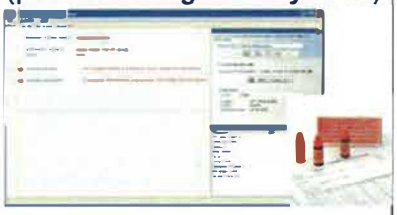
How to standardize?

By bringing the selected **cell population**
(e.g. lymphocytes in PBL preparations)
to the same **FSC and SSC** intensity channels.

By bringing **brightly fluorescent objects**
(e.g. polychromatic beads or cells stained with CD8-conjugates)
to the same intensity channels in all **fluorescence** detectors

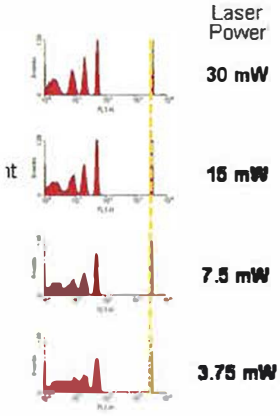
Manually
(e.g. /- 15%)

Automatically
(precise settings is easy to do)



Standardization in Flow Cytometry

Benefits of Standardization



Laser power was intentionally decreased and the median of the bright bead population was subsequentally placed to TV

Decreased sensitivity on detuned cytometers affects low-end resolution

However,
intensity patterns remain similar

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Standardization in Flow Cytometry

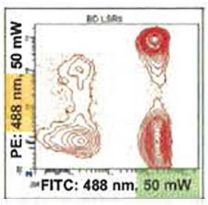
Benefits of Standardization

Instruments equipped with lasers with different

Power

Wavelength

provide results that are "as similar as possible"



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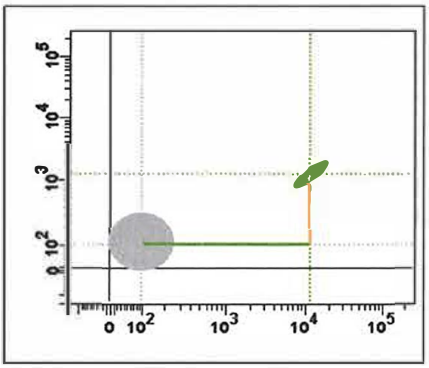
Standardization in Flow Cytometry

Benefits of Standardization



Spectral Overlay Value
(Compensation Matric Element)
is calculated as:

$$100\ \% \times \frac{\text{Overlay}}{\text{FL signal}}$$

Standard patterns = identical compensation values

!!! until optical characteristics of the cytometer change !!!

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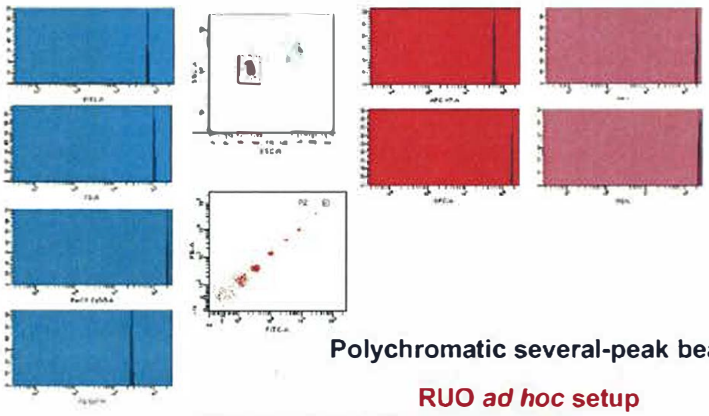
Standardization in Flow Cytometry

All Flow Cytometers



Polychromatic several-peak beads

RUO *ad hoc* setup

How many peaks are needed?

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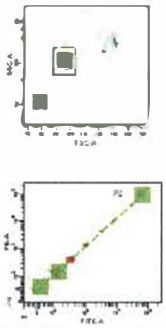
Standardization in Flow Cytometry

Beads for General Use



Dim: Electronic noise
Sensitivity

Dim + Medium: Resolution

Bright: Alignment (CV)
Reproducibility (TV)
Synchronization (TV)

Medium + Bright for measured linearity

Median (Bright) / Median (Medium) = const.

How many peaks are needed?

3 intensities: Dim, Medium, High + 2 sizes

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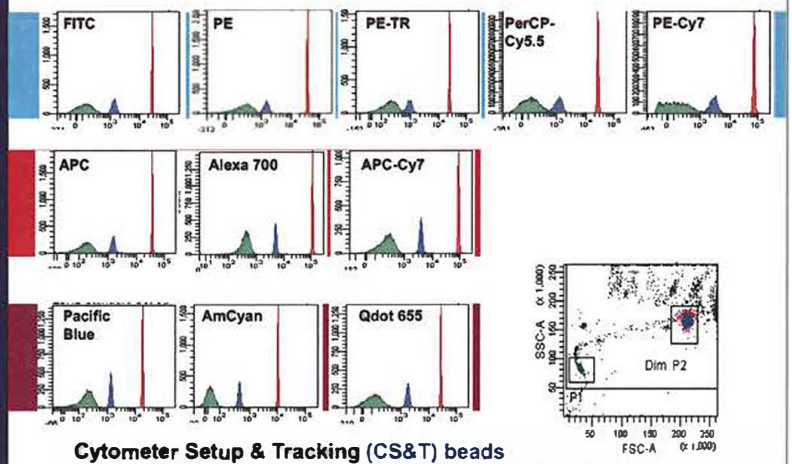
Standardization in Flow Cytometry

Beads for General Use



Cytometer Setup & Tracking (CS&T) beads

3 intensities: Dim, Medium, High + 2 sizes

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Standardization in Flow Cytometry

BD Multicolor Digital Analyzers

RUO **CE-IVD**

BD FACSDiva Acquisition / Analysis SW

Cytometer Setup & Tracking (CS&T) module & beads

BD LSR Fortessa

BD FACSCanto II

BD FACSVerse

BD FACSuite Acquisition / Analysis SW

Cytometer Setup & Tracking (CS&T) beads

BD FACSCanto

New CE-IVD to come

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Standardization in Flow Cytometry

BD FACSDiva and CS&T

Calibration Beads

Automatic SW module

Instrument Characterization (Baseline)

Daily Quality Control (Performance Check)

Automatic Data Reproducibility (Application Settings)

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Standardization in Flow Cytometry

BD FACSDiva and CS&T

Instrument Characterization (Baseline)

Expires in 1 Year

LASER DELAYS (LD)~ distance between lasers
AREA SCALING FACTORS (ASF) ~ 1 / laser beam height
LIGHT DETECTION EFFICIENCY (Qr) FOR EVERY SINGLE DETECTOR
OPTICAL BACKGROUND (Br) FOR ALL DETECTORS
COEFFICIENT OF VARIATION (rCV) FOR ALL BEADS AND ALL DETECTORS
TARGET VALUES FOR BRIGHT CS&T BEADS FOR ALL DETECTORS
SIGNAL LINEARITY FOR ALL DETECTORS AND THEIR ELECTRONICS
ELECTRONIC NOISE FOR ALL DETECTORS AND THEIR ELECTRONICS

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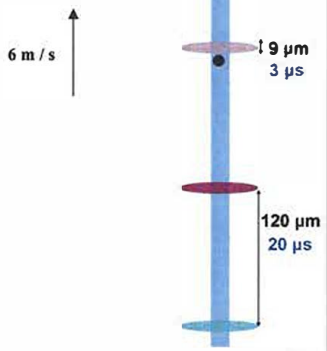
Standardization in Flow Cytometry

BD FACSDiva and CS&T

Instrument Characterization (Baseline)

LASER DELAYS (LD)~ distance between lasers
AREA SCALING FACTORS (ASF) ~ 1 / laser beam height



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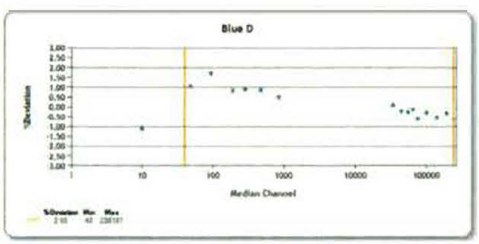
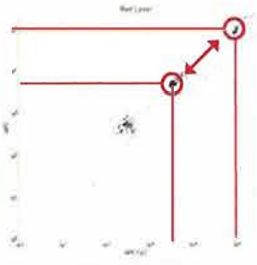
Standardization in Flow Cytometry

BD FACSDiva and CS&T

Instrument Characterization (Baseline)

SIGNAL LINEARITY FOR ALL DETECTORS AND THEIR ELECTRONICS





RATIO of MEDIANS

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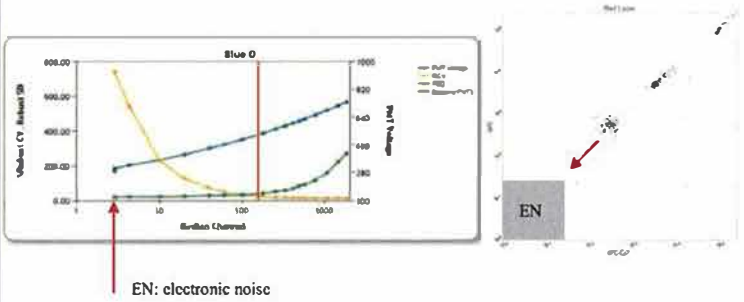
Standardization in Flow Cytometry

BD FACSDiva and CS&T

Instrument Characterization (Baseline)

ELECTRONIC NOISE FOR ALL DETECTORS AND THEIR ELECTRONICS



EN: electronic noise

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Standardization in Flow Cytometry

BD FACSDiva and CS&T

Daily Quality Control (Performance Check)

Expires in 1 Day

LASER DELAYS (LD)~ distance between lasers

AREA SCALING FACTORS (ASF) ~ 1 / laser beam height

LIGHT DETECTION EFFICIENCY (Qr) FOR EVERY SINGLE DETECTOR

OPTICAL BACKGROUND (Br) FOR ALL DETECTORS

COEFFICIENT OF VARIATION (rCV) FOR ALL BEADS AND ALL DETECTORS

TARGET VALUES FOR BRIGHT CS&T BEADS FOR ALL DETECTORS

~~**SIGNAL LINEARITY FOR ALL DETECTORS AND THEIR ELECTRONICS**~~

~~**ELECTRONIC NOISE FOR ALL DETECTORS AND THEIR ELECTRONICS**~~

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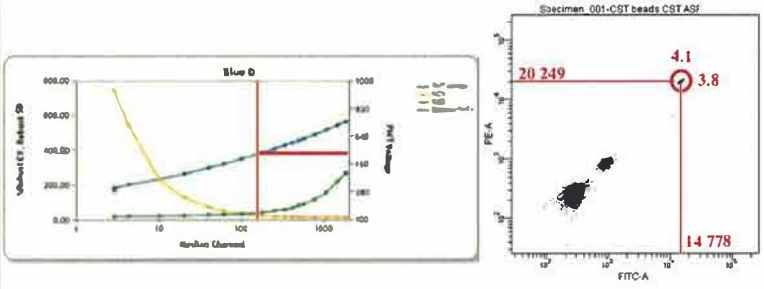
Standardization in Flow Cytometry

BD FACSDiva and CS&T

Daily Quality Control (Performance Check)



COEFFICIENT OF VARIATION (rCV) FOR ALL BEADS AND ALL DETECTORS

TARGET VALUES FOR BRIGHT CS&T BEADS FOR ALL DETECTORS

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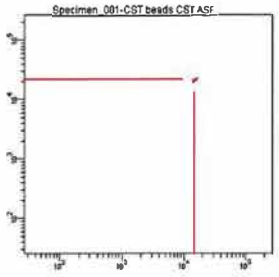
Standardization in Flow Cytometry

BD FACSDiva and CS&T

Daily Quality Control (Performance Check)



Bright CS&T bead population

Baseline position

TARGET VALUES FOR BRIGHT CS&T BEADS FOR ALL DETECTORS

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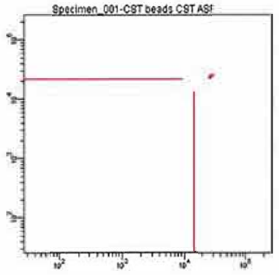
Standardization in Flow Cytometry

BD FACSDiva and CS&T

Daily Quality Control (Performance Check)



Bright CS&T bead population

Newly found position

TARGET VALUES FOR BRIGHT CS&T BEADS FOR ALL DETECTORS

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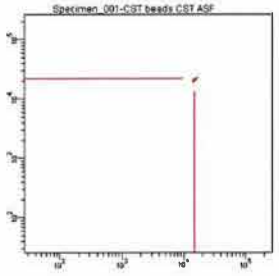
Standardization in Flow Cytometry

BD FACSDiva and CS&T

Daily Quality Control (Performance Check)



Bright CS&T bead population

Re- et position by modifying PMTV

TARGET VALUES FOR BRIGHT CS&T BEADS FOR ALL DETECTORS

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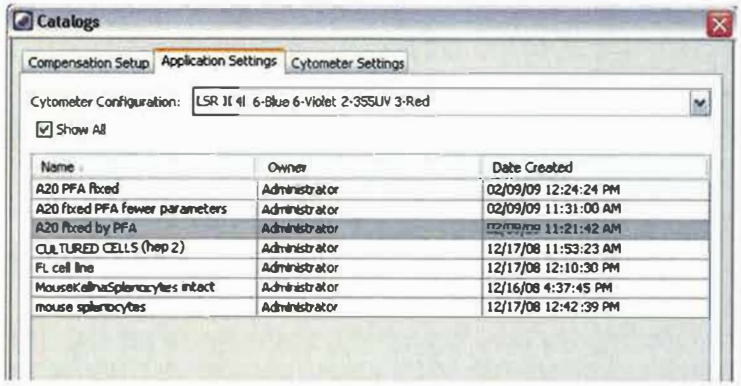
Standardization in Flow Cytometry

BD FACSDiva and CS&T

Automatic Data Reproducibility (Application Settings)



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Standardization in Flow Cytometry

BD FACSDiva and CS&T

Automatic Data Reproducibility (Application Settings)

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Standardization in Flow Cytometry

BD FACSuite and CS&T system

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Standardization in Flow Cytometry

BD FACSuite and CS&T

Calibration Beads

FACSuite Workspace

FACSuite:

Characterization Quality Control (CQC)

Performance Quality Control (PQC)

Integrated Reproducibility (Tube Target Values, TTV)

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Standardization in Flow Cytometry

BD FACSuite and CS&T

Characterization Quality Control (CQC)

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Standardization in Flow Cytometry

BD FACSuite and CS&T

Cytometer Performance QC Report
4-Blue 2-Red 2-Violet (RUO)

Cytometer:		User	Admin User
Cytometer Name:		Institution:	None
Serial Number:	Z651155P030		
Fluidics Mode:	Normal		
Last Characterization QC	8/19/2011 2:08 PM		

Configuration Name: 4-Blue 2-Red 2-Violet (RUO) — Last Modified: 8/19/2011 1:47 PM

SUMMARY: PASSED

DETECTOR SETTINGS

List Info	Detector				PMTV		Bright Bead		Linearity (±3%)		Resolution			
	Name	Mirror	Filter	Position	Actual	Δ	Median	%rCV	Min Channel	Max Channel	Sensitivity Actual	Sensitivity % Diff	Qr (x10³)	Br
LASER: Blue (Wavelength = 488nm)														
X	FSC	-	-	FSC	450.9	-1.4	127096	0.6	N/A	N/A	174	18	N/A	N/A
X	SSC	10	488/15	F	326.4	-1.3	124623	1.4	N/A	N/A	2055	6	N/A	N/A
X	FITC	507LP	527/32	F	580.9	-2.4	100264	1.2	82	232578	523	-4	103.2	130
X	PE	580LP	586/42	D	585.5	-2.7	100743	1.1	115	231248	1301	0	711.9	157
X	PerCP-Cy5.5	685LP	700/54	B	580.9	-3.7	99927	1.9	87	231210	612	15	41.4	21
X	PE-Cy7	752LP	783/56	A	660.5	-3.1	100416	3.3	213	234293	2909	6	84.9	2
LASER: Red (Wavelength = 640nm)														
X	APC	660	660/10	E	612.8	-4.0	101371	1.4	77	226734	841	5	89.4	29
X	APC-Cy7	752LP	783/56	A	482.1	-1.0	101170	1.7	89	227096	197	2	42.8	213
LASER: Violet (Wavelength = 405nm)														
X	V450	648	448/45	E	621.1	-0.9	101961	3.3	78	220619	124	-6	153.9	3939
X	V500	500LP	528/45	A	551.7	-1.5	101375	3.1	92	230481	147	-16	87.2	1576

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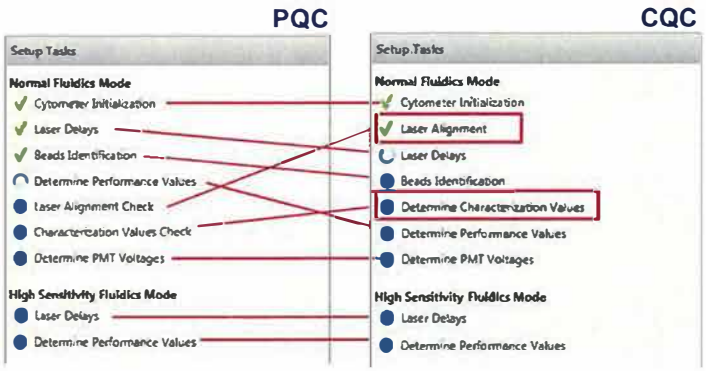
Standardization in Flow Cytometry

BD FACSuite and CS&T



Performance Quality Control (PQC)

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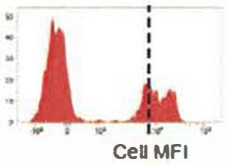
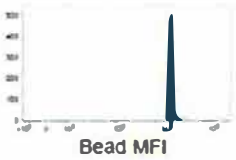
Standardization in Flow Cytometry

BD FACSuite and CS&T



Setup FL pattern for cells



At the same settings
Run CS&T beads = Get TTV

Integrated Reproducibility (Tube Target Values, TTV)

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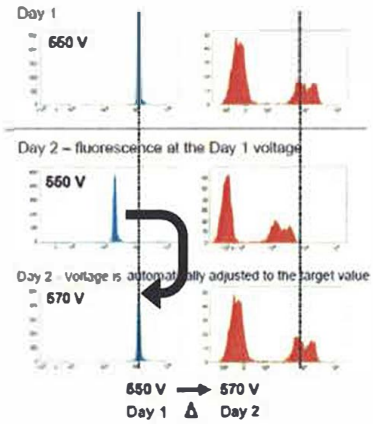
Standardization in Flow Cytometry

BD FACSuite and CS&T



Integrated Reproducibility (Tube Target Values, TTV)

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Standardization in Flow Cytometry

Progress in Standardization:

FACSDiva:

User settings (Applications Settings) are calculated from results of measurements of CS&T beads in the CS&T module under standard (Baseline defined conditions). Compensation matrix is duplicated (unchanged).
PMTV increment = min. 1 V

FACSuite

Tube settings (TTV) are measured using CS&T beads ofr every signle settings. Compensation matrix is recalculated for all smallest imprecisssions.
PMTV increment = min. 0.1 V

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BD Set-up of 10-color Flow Cytometer

PMTV

BD Lyse Wash (LW) and Lyse No Wash (LNW) available in
6 colors in FACSDiva
8 colors in FACSuite
FITC, PE, PerCP(-Cy5.5), PE-Cy7, APC, APC-Cy7 (or H7), V450, V500c using Calibration beads (7-color Setup Beads or CS&T beads)

For special applications, expert (group) Target Values (TV) using recommended setup materials are followed: EuroFlow setup (8 colors)

For *ad hoc* settings (e.g. 8 color EuroFlow and9th and 10th color on FACSCanto) the 2.5 x SDEN rule is recommended while paying attention to balancing detector PMTVs (CS&T module provides 10x SDEN values)

If possible, one set of PMTV per "Experiment" in FACSDiva

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BD Set-up of 10-color Flow Cytometer

Threshold

On analyzers, the Threshold value should be set to include all relevant events In analysis and to see the "closest" part of debris/irrelevant events as well.

If fully quantitative (peak Area) measurement is done, signal intensity depends on appropriate Threshold and Window Gate Extension (WE) selection.
The most recent recommendations are:
FSC Threshold ~ 5% of the scale (13 000) and WE = 3

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BD Set-up of 10-color Flow Cytometer

Compensation

FACSDiva:
Automatic Compensation at the beginning
(Link and Save, Compensation Setup Catalogue)
Repeated monthly

Manual Compensation
(visual or by comparing median FL of positive and negative events) between the automated procedure
namely for tandem conjugates (labels): periodically or with a new lot

FACSuite:
Adding fluorochromes and updating compensation for label-specific reagents (tandem conjugates) when needed.

On standardized instruments:
Compensation matrix does not significantly change. Within the time, only Spectral characteristics of a cytometer (optical filters) play a significant role in compensation matrix changes, such processes normally take years before any significant difference is observed.

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BD Set-up of 10-color Flow Cytometer

Practical Approach

FACSDiva:

The most conveneient procedure
of setting up 10 color FACSCanto
and maintaining it standardized
(keeping Target Values and Maintaining Compensation)
will be demonstrated during the practical part on day 2

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BD OneFlow

BD OneFlow

Instrument Setup (EuroFlow TV)

1 peak

Very fast TV
setup and maintenance

Target values for
monthly (+/-2%)
daily (+/- 15%)
setup

CE-IVD template
in FACSDiva 8.0.1

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BD OneFlow

BD OneFlow

Compensation

All components included

No pipetting

Controls for 5 months (or more)

Highly reproducible

No label-specific controls for OneFlow diagnostics

Generic single-stained control candidate when OneFlow is used in combination with other cocktails

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BD OneFlow

BD OneFlow

Multicolor cocktails

8-color (12 antibodies) LST Tube:

FITC	CD8, Lambda
PE	CD56, Kappa
PerCP- y5.5	CD5
PE-Cy7	CD19, TCRgd
APC	CD3
APC-H7	CD38
V450	CD4. CD20
V500c	CD45

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BD OneFlow

BD OneFlow

Multicolor cocktails

All (dry) mAbs included = no pipetting error

Storage at RT

No expiration mismatch

Highly reproducible

No label-specific controls needed

Full CE-IVD compliance in FACSDiva 8.0.1

More is coming .....

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