ESCCA 2017 Conference

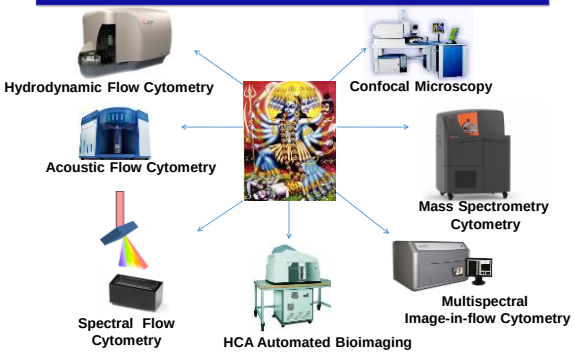
Clinical Diagnostic Cytometry Course

AN OVERVIEW ON CLINICAL FLOW CYTOMETRY

Bruno Brando & Arianna Gatti
Hematology Laboratory and
Transfusion Center
Western Milan Area Hospital Consortium
Legnano Hospital, Milano, Italy
e-mail: bruno.brando@asst-ovestmi.it

Sistema Socio Sanitario
Regione Lombardia
ASST Ovest Milanese

FROM CYTOLOGY TO CYTOMICS: TECHNOLOGY

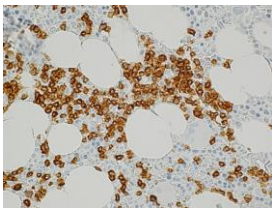


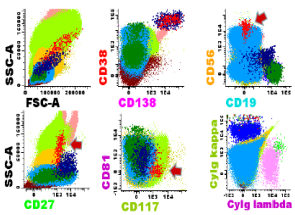
Multicolor Flow Cytometry: Strong Points

- Multiparametric Analysis with Full Cross-Correlation of Findings.
- Biological Markers of Different Meaning are Analyzed Simultaneously.
- Data From a Very Large Number of Cells Can Be Collected in a Short Time.
- Robust and Reliable Statistical Representation of Rare Events.
- Cells Are Examined in Their Original Milieu with Minimal Manipulation.
- Objective, Operator-Independent Measurement of Cell Parameters.
- High Level of Standardization and External Quality Assessment schemes.

Multicolor Flow Cytometry: Weak Points

- Monodispersed Cell Suspensions: Tissue Architecture Is Lost.
- The Cell Compartment That Generates the Signal Cannot Be Located.
- Some Cellular Markers that Work in Histochemistry Don't Work in Flow.
- Fluorescence Background and Signal Resolution With Some Cell Markers.
- "Just One Cell" is Not Enough for Data Interpretation.





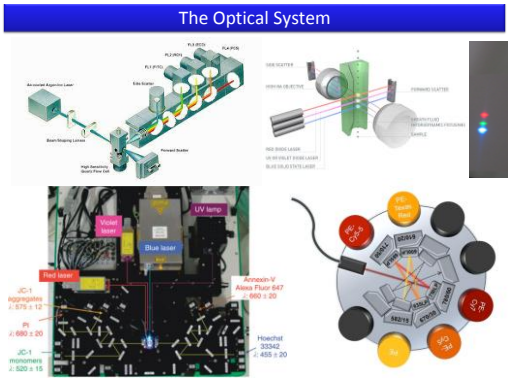
Immunohistochemistry:
One Cellular Marker at a Time.

- Cell Denominator Difficult to Enumerate
- Limited Statistical Robustness of Data
- Difficult correlation between markers
- Subjective, Operator-Dependent Analysis

Multicolor Flow Cytometry:
As Many Markers as You May Want.

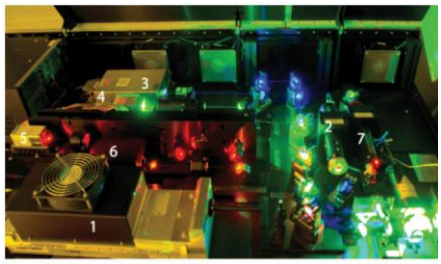
- Cell Denominator Precise
- Very High Statistical Robustness of Data
- Full cross-correlation among markers
- Objective, Operator-Independent Analysis

The Optical System



7-Laser 25-Color Configuration

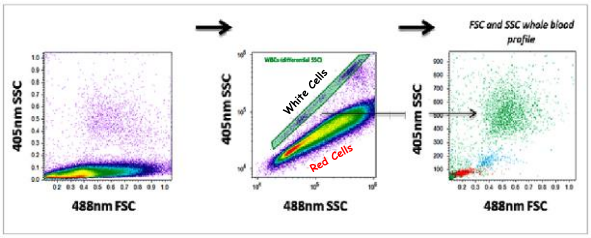
WOW !



	Laser	Wavelength [nm]	Style	Power [mw]	PMT Array	PMT
1	UV	355	Mode Lock	20	Trigon	2
2	Violet	405	Diode Pumped Solid State	50	Octagon	8
3	Blue	488	Diode Pumped Solid State	20-100	Octagon	4
4	Green	532	Diode Pumped Solid State	40-150	Octagon	5
5	Yellow	594	Diode Pumped Solid State	50	Trigon	2
6	Red	638	Diode Pumped Solid State	40	Trigon	3
7	IR	785	Diode Pumped Solid State	25	Trigon	1

Preffer F & Dombkowski D. Cytometry Part B 2009; 76B: 295 – 314.

The Availability of the 405nm (Violet) Laser Has Extended the Spectrum of Excitable Fluorochromes.
488 vs 405nm SSC Discriminates Red and White Blood Cells With a NO-YSE Technique.



- Diagrams are from the ATTUNE™ Acoustic Cytometer.
- 405 nm SSC can be implemented in new generation cytometers with a simple filter set.

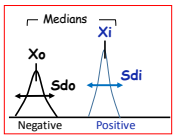
60+ Different Fluorochromes Available Today

Fluorochrome	Excitation max (nm)	Excitation lines (nm)	Emission max (nm)	Emission color
Propidium iodide	305, 540	350-360, 488	620	Yellow-orange
Indo-1 Ca bound	330	350-360	405	Violet
Indo-1 Ca free	346	350-360	480	Blue
LIVE/DEAD blue	350	350-360	450	Blue
Hoechst 33342	352	350-360	455	Blue
AMCA-X	353	350-360	442	Blue
DAPI	359	350-360	461	Blue
Marina Blue	365	350-360	460	Blue
Alexa Fluor 350	346	350-360	442	Blue
Pacific Orange	400	405, 407	551	Blue
Pacific Blue	404	405, 407	456	Blue
BD Horizon V450	416	405, 407	451	Blue
LIVE/DEAD violet	416	405, 407	451	Blue
Alexa Fluor 405	405	405, 407	420	Violet/blue
CellVue Lavendar	425	405, 407	461	Blue
ECFP	434	458, 488	477	Blue
AmCyan	458	405, 407	489	Blue
CellVue Jade	478	488	508	Cyan
EGFP	489	458, 488	508	Cyan
Alexa Fluor 430	430	405, 407	540	Green
CFSE	490	488	518	Green
PKH2 & PKH67	490	488	504	Green
LIVE/DEAD green	495	488	519	Green
Alexa Fluor 488	495	488	519	Green
FITC	494	488	519	Green
Rhodamine 123	507	488	529	Yellow-green
Ethidium monoazide	510	488	600	Yellow-orange
EYFP	514	488, 532	527	Yellow-green
Alexa Fluor 532	531	532	554	Yellow-green
PKH26	551	488, 532	567	Yellow-green
DsRed	558	568	583	Yellow
PE	496, 546	488, 532	578	Yellow
PE-Texas Red	496, 546	488, 532	615	Yellow-orange
PKH26	551	532, 561, 590, 594	567	Yellow-green
Alexa Fluor 594	590	594	617	Yellow-orange
LIVE/DEAD red	595	488	615	Yellow-orange
Texas Red	595	595	615	Yellow-orange
7-AAD	550	488, 532, 594	660	Red
DRAQ5	646	633	681	Red
CellVue Maroon	647	633	667	Red
APC	650	595, 633, 635, 647	660	Red
Alexa Fluor 647	650	595, 633, 635, 647	668	Red
PE-Cy5	496, 546	488, 532	667	Red
Cy5	640	633, 635	670	Red
PerCP	482	488, 532	675	Red
Alexa Fluor 660	663	647	690	Red
LIVE/DEAD far red	650	633, 635	665	Red
APC-Cy5.5	650	633, 635	694	Red
PerCP-Cy5.5	482	488, 532	695	Red
PE-Cy7	496, 546	488, 532	785	Infrared
APC-Cy7	650	595, 633, 635, 647	785	Infrared
APC-Alexa Fluor 750	650	595, 633, 635, 647	775	Infrared
HiLyte 750, no tandem	753	785	778	Infrared
IRDye 800CW	774	785	789	Infrared
CellVue NIR815	786	785	814	Infrared
Q-dots/FluorXuCad	Various	350-360, 407	Various	Various

Preffer F & Dombkowski D. Cytometry Part B 2009; 76B: 295 – 314.

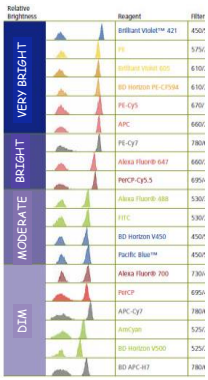
Relative Brightness of Fluorochromes
Ranking of Fluorochromes by the Resolution Index

$$\text{Resolution Index (Z)} = \frac{Xi - Xo}{\sqrt{Sdi^2 + Sdo^2}}$$



Resolution Index (Z) of various fluorochromes calculated from the staining of human PBMCs with anti-CD4 clone RPA-T4 on a BD FACSCanto™ II

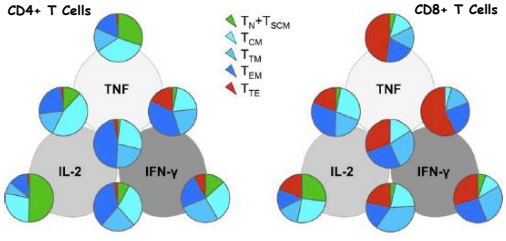
http://www.bdbiosciences.com/documents/Multicolor_Fluorochrome_Guide.pdf



Why do we need so many colors?

Summary of CD4+ and CD8+ T Cell average subset distribution and their cytokine profiles in healthy adults

CD3
CD4
CD7
CD8
CD27
CD28
CD31
CD45RA
CD45RO
CD62L
CD95
CCR7
TNF-α
IL-2
IFN-γ



Over 250 different functional subsets can be identified within the T Cell compartment

Mahnke YD. Eur J Immunol 2013; 43: 2797-2809

B Cell Ontogeny and B Cell Functional Subsets

FITC	CD3/CD14
PerCP-Cy5-5	CD19
APC	CD27
BV786	IgG
BV711	CD38
BV605/Qdot 605	IgM
Aqua	LiveDead
VioBlue	IgA
PE	IgD
PE-Dazzle 594	CD24

Unraveling the Complexity of B Cell Maturation and of the Development of B Cell Functional Subsets

Regulatory B Cells

CD1d
CD5
CD19
CD21
CD23
CD24
CD93
IgM

CD19
CD20
CD27
CD28
CD38
CD56
CD81
CD117
CD138
K / λ

Normal and Abnormal Plasmacells

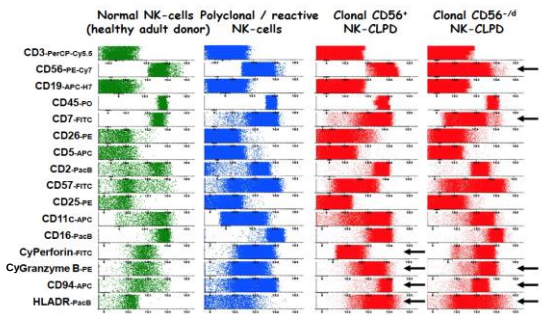
Transitional B Cells

FITC	IgD
PE	IgM
PerCP-Cy5-5	CD10
PE-Cy7	CD21
APC	BAFF-R
APC-Cy7	CD27
Aqua	LiveDead
PB	CD20

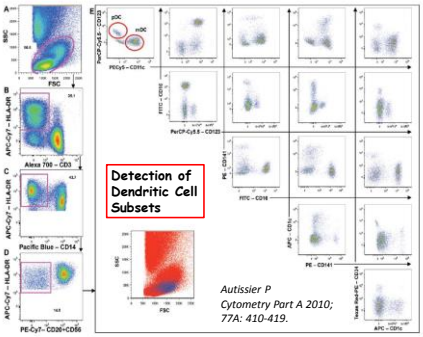
Why do we need so many colors?

Why do we need so many colors?

IMMUNOPHENOTYPIC PROFILE OF NORMAL, REACTIVE AND CLONAL NK CELLS



Why do we need so many colors?

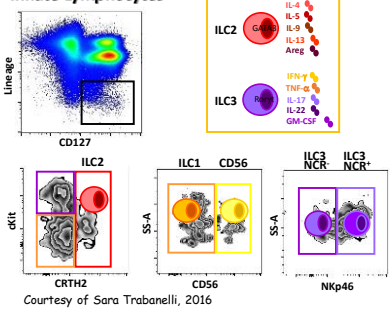


APC	CD1c
Alexa700	CD3
AmCyan	CD4
QDot655	CD8
PE-Cy5	CD11c
PacB	CD14
FITC	CD16
APC-Cy7	CD20
ECD	CD34
PerCP-Cy5.5	CD123
PE	CD141
APC-Cy7	HLADR

A **12-Color Panel** to enumerate Dendritic Cell Subsets, Monocyte and Lymphocyte Populations

Why do we need so many colors?

Innate Lymphocytes



Courtesy of Sara Trabanelli, 2016

Identification through the combined use of surface phenotypic markers, intracellular transcription factors and cytokine synthesis.

CD3	IFN-γ
CD4	TNF-α
CD8	IL-4
CD14	IL-5
CD15	IL-17
CD16	IL-22
CD19	
CD20	
CD33	
CD34	
CD56	
CD127	
CD203c	
FceRI	
CRTH2	
NKp46	

Leukemia Stem Cell Detection for Diagnostic Purposes: Discrimination Between Leukemia and Hematopoietic Stem Cells

(1 tube, 8 colors, 13 markers)

Tube	FITC	PE	PerCP-CY5.5	PeCy7	APC	APC-H7	BV421	HV500C
	CD45ra	CLL-1 TIM-3 CD7 CD11b CD22 CD56	CD123	CD33	CD38	CD44	CD34	CD45

PE "Lineage (dump) Channel" contains markers that are NEGATIVE on HSC

- 1 tube for LSC detection at diagnosis and follow-up
- Identifies almost all CD34+CD38- LSC
- Needs no extensive experience in BM immunophenotyping
- Multi-institutional approach possible
- Can be extended with other markers in the PE-channel

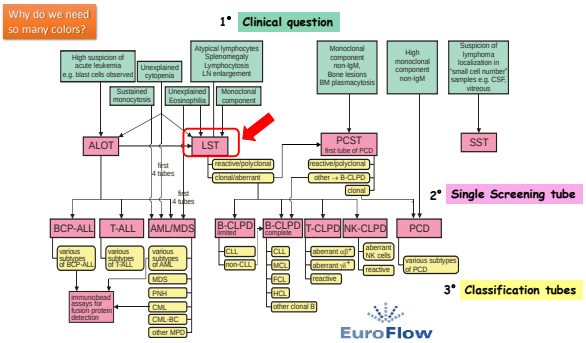
Why do we need so many colors?

Courtesy of GJ Schuurhuis, 2016

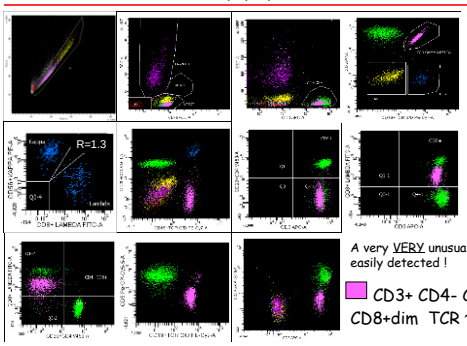
- Zeijlemaker W. Leukemia 16 Sept 2016. doi:10.1038/leu.2015.255
- Zeijlemaker W. Leukemia 16 Sept 2015. doi:10.1038/leu.2015.252

Algorithm for EuroFlow Antibody Panels in Hemato-Oncology

Why do we need so many colors?



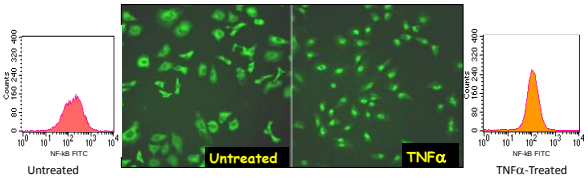
8-Color/12-MoAbs LST Tube: Lymphoproliferative Disorders Screening



A very VERY unusual case easily detected !

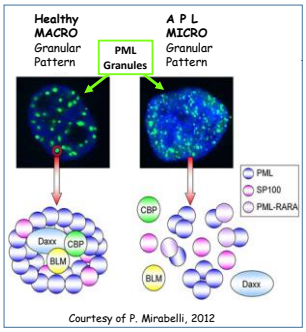
CD3+ CD4- CD5- CD8+dim TCR γ/δ+

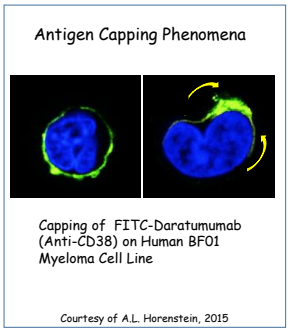
Flow Cytometric Analysis Cannot Locate Where the Signal is Generated

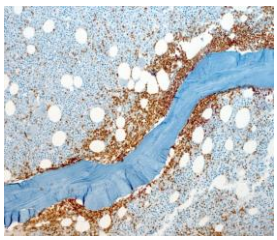


TNFα-Induced Shift of **NF-kB** From Cytoplasm to Nucleus of HeLa Cells

Other Signal Pattern Issues That Cannot be Appreciated by FCM

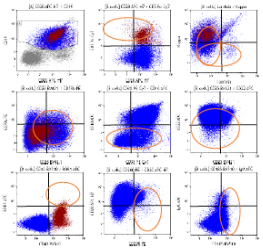






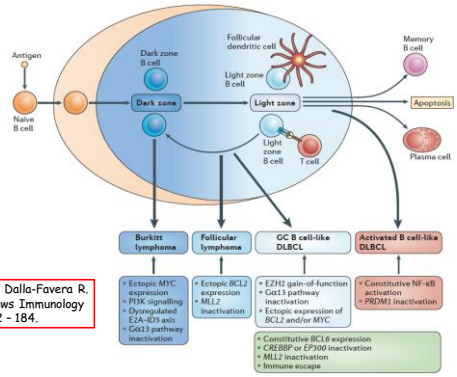
IHC - Bone Marrow Trephine Biopsy:
Paratrabecular Lymphocyte Infiltrate

Architecture of cellular clustering has a clinical meaning (i.e. Follicular Lymphoma)



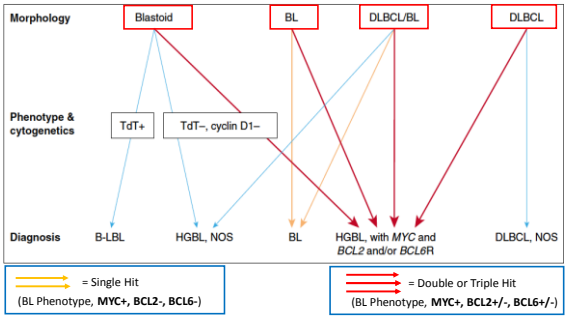
FCM Analysis of Bone Marrow Aspirate:
Dissociated Particles-Homogeneous Suspension

Abnormal cells diluted and admixed with normal lymphocytes. Clonality may not emerge.



Basso K and Dalla-Favera R. Nature Reviews Immunology 2015; 15: 172 – 184.

Diagnostic Approach to Aggressive, High-Grade B Cell Lymphomas (**HGBL**)



Swerdlow SH. Blood 2016; 127: 2375-2390.

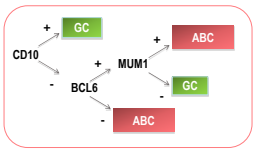
Some Important Diagnostic Markers that Cannot be Detected by FCM

- **Nuclear Cyclin-D1:** Translocation CCND1/IGH-t(11;14) Mantle Cell Lymphoma, Myeloma
- **Nuclear Cyclin-D2:** Multiple Myeloma
- **BCL-6 (**By FISH**):** DLBCL
- **IGH/MYC/CEP8:** (MYC-BCL2= Double Hit) MYC-Translocated DLBCL: 30% OS at 24mos MYC-wild type: 70% OS at 24 mos.
- **IRF4/MUM1:** Cell of Origin DLBCL ABC vs GC
- **SOX11:** Mantle Cell Lymphoma In situ vs Leukemized
- **EBV Products (EBER** By FISH): DLBCL EBV-Related, Primary Effusion Lymphoma
- **MIB-1 Quantitation: Cutoff** >90% or <90% DLBCL vs Burkitt
- **TP53 Mutations:** Multiple Myeloma, Chronic Lymphocytic Leukemia

The '**Cell-of-Origin**' of Lymphomas Cannot be Established by FCM

Mature, Clonal, Highly Proliferating B Cells (CD19+ CD20+ CD22+ CD79b+ sIg+ Ki67>40%)

- **Germinal Center (GC) Type DLBCL:** { **CD10+** *or* **CD10- BCL6+ MUM1-**
- **Post-GC Activated Phenotype (ABC):** { **CD10- BCL6-** *or* **CD10- BCL6+ MUM1+**



Such analysis can be accomplished by Immuno-Histochemistry (IHC) Only

In IHC slides are processed at HIGH TEMPERATURE, so antigens undergo structural changes

Histology and Immunohistochemistry of Lymph Nodes in Lymphoma Diagnosis: *Is There Any Role For Multicolor FCM Analysis?*

- It is estimated that in **10-15%** of Non-Hodgkin Lymphoma cases the conventional Histology/IHC analysis of Lymph Nodes may be **inconclusive**, thus requiring additional analytical steps.
- The 2016 WHO revision (S. Swerdlow. Blood 2016; 127: 2375-2390) highlights the need of a more cautious usage of the term '*Lymphoma*' since cases of in-situ neoplasms and borderline or atypical reactive lesions may occur.
- Multicolor FCM can be of help as an ancillary technique in NHL diagnosis, especially in **T-Cell Lymphomas, Composite Lymphomas, Dendritic Plasmocytoid neoplasms, PTGC** (Progressive Transformation of Germinal Center), **In-Situ Follicular Lymphoma**, **Thyroid Aspirates**, to approach 100% diagnostic accuracy.
- Coupling FCM to Histology/IHC of Lymph Nodes requires a tight cooperation between operators (organization, technicalities, logistics)

But Multicolor Flow Cytometry is Unbeatable in Detecting and Characterizing Difficult and Unusual Leukemias

Blastic Plasmacytoid Dendritic Cell Neoplasm

Acute Leukemias of Ambiguous Lineage:

- Acute Undifferentiated Leukemia
- Mixed Phenotype Acute Leukemia (MPAL) with t(9;22)(q34.1;q11.2); BCR-ABL1
- MPAL with t(v;11q23.3); KMT2A Rearranged
- MPAL, B/Myeloid, NOS
- MPAL, T/Myeloid, NOS

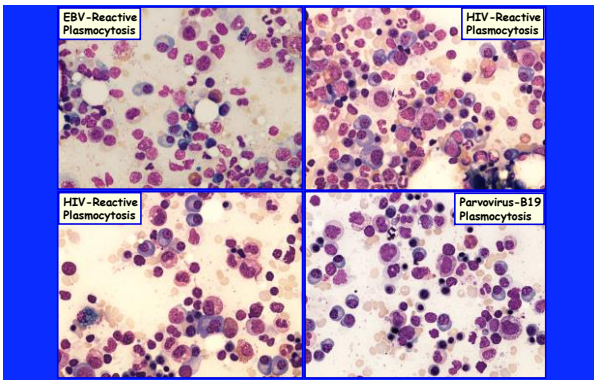
Criteria for Lineage Assignment for a Diagnosis of MPAL

Myeloid Lineage
MPO (flow cytometry, immunohistochemistry, or cytochemistry)
or Monocytic differentiation (at least 2 of the following: nonspecific Esterase cytochemistry, CD11c, CD14, CD64, Lysozyme)
T-Lineage
Strong cytoplasmic CD3 (with antibodies to CD3 ε chain)
or Surface CD3
B-Lineage
Strong CD19 with at least 1 of the following strongly expressed: CD79a, cytoplasmic CD22, or CD10
or Weak CD19 with at least 2 of the following strongly expressed: CD79a, cytoplasmic CD22, or CD10
Arber DA. Blood 2016; 127: 2391-2405.

Flow Cytometry in Hematologic Malignancies

Some abnormal phenotypes can be highly predictive of genetic defects:

- **Acute Myeloid Leukemia** CD34+ CD56+ CD15+/- CD19+ → t(8;21)
- **Mantle Cell Lymphoma** CD5+ CD23- CD79b+ CD200- → t(11;14)
- **Burkitt Lymphoma** CD10++ CD38++ CD43++ CD81++ → t(8;14)
- **Atypical B-CLL** CD5+ CD20++ CD23+/- CD49d+ → trisomy 12
- **Multiple Myeloma** CD20+ → t(11;14) - Standard Risk
- **Multiple Myeloma** CD28+++ CD27- → t(14;16) - High Risk
- **Multiple Myeloma** CD28- CD27- → t(4;14) - High Risk
- **Pediatric ALL** CD123+ → Hyperdiploidy
- **Pediatric ALL** CD66c+ → Hyperdiploidy, BCR/ABL
- **Pediatric ALL** CD44- → TEL/AML1
- **Mature B-ALL** CD44- → MYC-Translocation
- .....and many other.....



Four Expert Pathologists Were Asked to Analyze **50 Established MDS Cases**: High Inter-Observer Variability of Morphological Evaluation of Blasts.

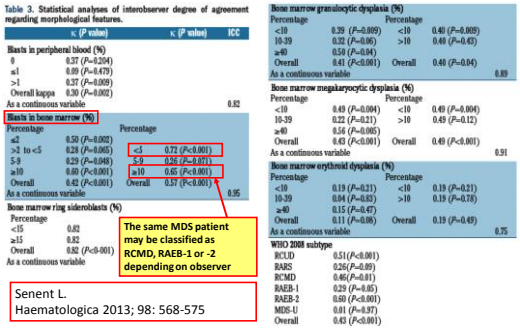
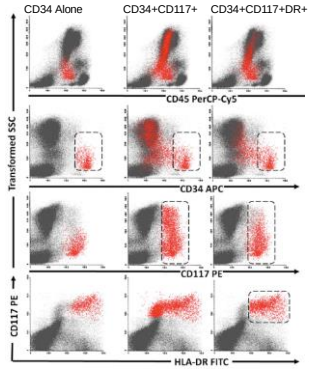


Table 3. Statistical analyses of interobserver degree of agreement regarding morphological features.

	κ (P value)	κ (P value)	ICC
Blasts in peripheral blood (%)			
0	0.37 (P=0.204)		
≥1	0.09 (P=0.479)		
>1	0.37 (P=0.009)		
Overall kappa	0.30 (P=0.002)		
As a continuous variable			0.82
Blasts in bone marrow (%)			
Percentage		Percentage	
≤2	0.50 (P=0.002)		
>2 to <5	0.28 (P=0.065)	<5	0.72 (P<0.001)
5-9	0.29 (P=0.048)	5-9	0.26 (P=0.071)
≥10	0.60 (P<0.001)	≥10	0.65 (P<0.001)
Overall	0.42 (P<0.001)	Overall	0.57 (P<0.001)
As a continuous variable			0.95
Bone marrow ring sideroblasts (%)			
Percentage			
<15	0.82		
≥15	0.82		
Overall	0.82 (P<0.001)		
As a continuous variable			
Bone marrow granulocytic dysplasia (%)			
Percentage		Percentage	
<10	0.29 (P=0.009)	<10	0.40 (P=0.009)
10-39	0.32 (P=0.06)	>10	0.40 (P=0.43)
≥40	0.50 (P=0.04)		
Overall	0.41 (P<0.001)	Overall	0.40 (P=0.04)
As a continuous variable			0.89
Bone marrow megakaryocytic dysplasia (%)			
Percentage		Percentage	
<10	0.49 (P=0.004)	<10	0.49 (P=0.004)
10-39	0.22 (P=0.21)	>10	0.49 (P=0.12)
≥40	0.56 (P=0.005)		
Overall	0.45 (P<0.001)	Overall	0.49 (P<0.001)
As a continuous variable			0.91
Bone marrow erythroid dysplasia (%)			
Percentage		Percentage	
<10	0.19 (P=0.21)	<10	0.19 (P=0.21)
10-39	0.04 (P=0.82)	>10	0.19 (P=0.78)
≥40	0.15 (P=0.47)		
Overall	0.11 (P=0.08)	Overall	0.19 (P=0.49)
As a continuous variable			0.75
WHO 2008 subtype			
RCUD	0.51 (P<0.001)		
RARS	0.26 (P=0.09)		
RCMD	0.46 (P=0.01)		
RAEB-1	0.29 (P=0.05)		
RAEB-2	0.60 (P<0.001)		
MDS-U	0.01 (P=0.87)		
Overall	0.43 (P<0.001)		

The same MDS patient may be classified as RCMD, RAEB-1 or -2 depending on observer

Senent L.
Haematologica 2013; 98: 568-575



Counting BM Blasts by FCM

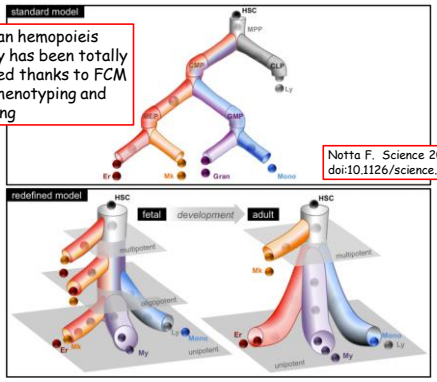
BM Blast enumeration by FCM using CD34+ CD117+ HLADR+ displays the BEST CORRELATION with morphological blast count as performed by expert readers.

Cell denominator is made by CD45+ cells, **excluding erythroid precursors**.

Sandes AF. Clinical Cytometry 2013; 84B: 157-166.

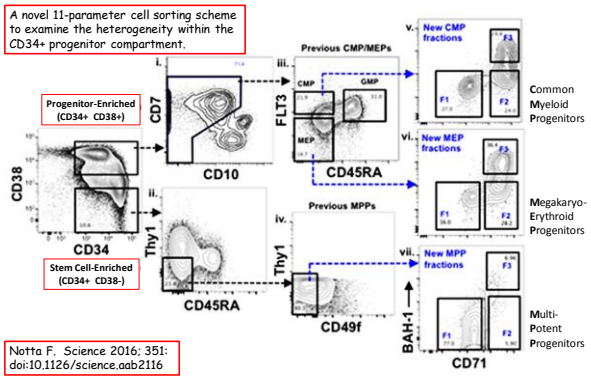
Phenotype changes with Age, Exposure & Environment

The human hemopoieis hierarchy has been totally redesigned thanks to FCM immunophenotyping and cell sorting



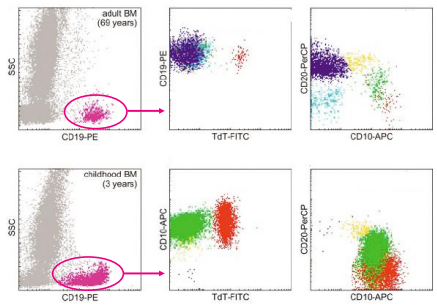
Notta F. Science 2016; 351: doi:10.1126/science.aab2116

A novel 11-parameter cell sorting scheme to examine the heterogeneity within the CD34+ progenitor compartment.



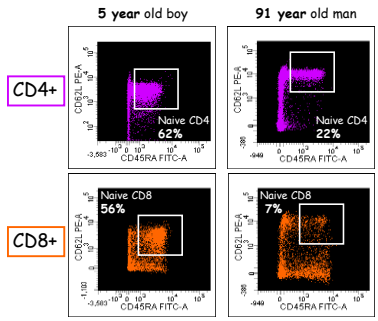
Notta F. Science 2016; 351: doi:10.1126/science.aab2116

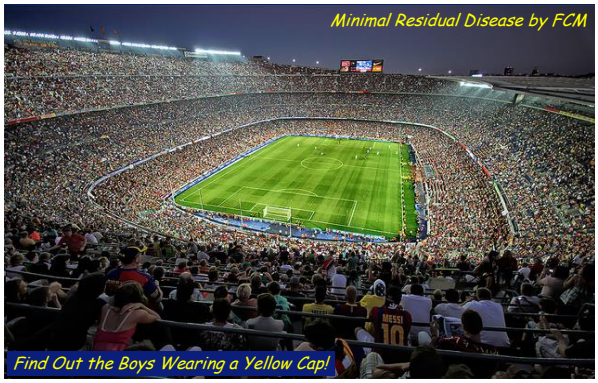
Age-related changes in the normal Bone Marrow (B Cell Lineage)



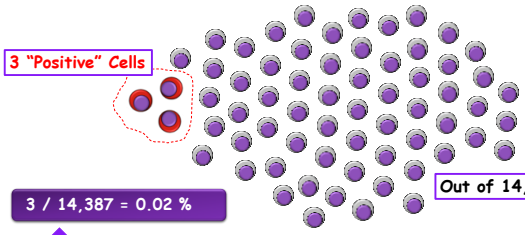
Van Lochem EG. Clinical Cytometry 2004; 60B: 1-13.

Naive T Cells and Aging





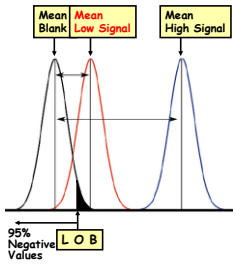
Rare Event Detection and Enumeration by FCM – The Minimal Residual Disease



Is it a correct approach? ➡ NO!

Enumeration of rare cell events (i.e. < 10^{-2}) **is NOT a mere arithmetical calculation.** Stringent technical and statistical criteria are required (both for 'positive' and 'denominator').

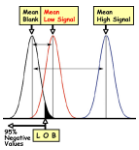
- *Lower* **Limit of Blank (LLOB):** The highest signal in the absence of the measurand. (Mean Blank + SD x 1.65). 95% of negative values are below this limit.
- *Lower* **Limit of Detection (LLOD):** (Mean Blank + SDlow x 1.65). 95% of negative values are above this limit. 5% false negatives and 5% false positives are assumed
- *Lower* **Limit of Quantitation (LLOQ):** The lowest level of measurand that can be reliably quantitated at a predefined criterion for precision and accuracy (clinical utility value). Never lower than LOD.



Such concepts derive from clinical chemistry and can be directly applied to Flow Cytometry for INTENSITY measurements only.

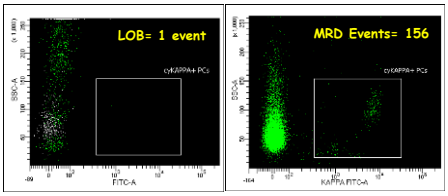
Wood B. ICSH/ICCS. Cytometry Part B 2013; 84B: 315-323.

Consensus on MRD Detection in Multiple Myeloma



In FCM MRD studies, the LLOB / LLOD / LLOQ concepts must be appropriately **translated:**

LLOB = Background events in the blank (to be subtracted)
LLOD = > **30** Events detected (> 0.001% or >10^{-5})
LLOQ = > **50** Events quantitated (> 0.001% or 10^{-5})
[30 and 50 events derive from statistical estimations]



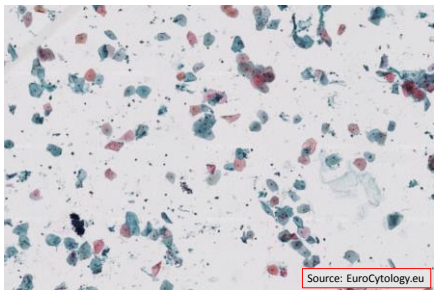
FCM MRD Studies: Estimated LLOD & LLOQ According to the Total Number of Acquired Cells

Total Number of Acquired Cells (Excluding Erythroid)	LLOD % ≥ **30** Events	LLOQ % ≥ **50** Events
100,000	0.03	0.05
200,000	0.015	0.025
500,000	0.006	0.01
1,000,000	0.003	0.005
2,000,000	0.0015	0.0025
3,000,000	**~ 0.001**	**~ 0.0017**
5,000,000	**~ 0.0006**	**~ 0.001**

The specific LLOD for the total amount of acquired cells should be reported.

Arroz M. Cytometry Part B 2016; 90B: 31-39.

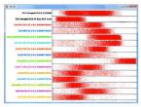
When examining a **PAP Smear** (around 300,000 cells), **JUST ONE ABNORMAL CELL** may be enough to classify the sample as SUSPECT, therefore requiring expert review or resampling.



A Limitation of FCM Analysis: JUST ONE CELL (Event) IS NOTHING

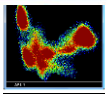
Next Generation FCM: Extended Multiparamater Analysis by *Infinicyt* ™

Infinicyt™ is a software for data integration and multidimensional analysis of flow cytometry files. The main features of **Infinicyt™** are:



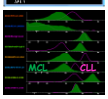
The Merge Process

Two or more datafiles from the same sample or from different samples with some common 'backbone' markers are merged. The resulting file sums up limitless cell phenotypes as if they were generated by a virtual single cell sample.



The Automatic Population Separator (APS)

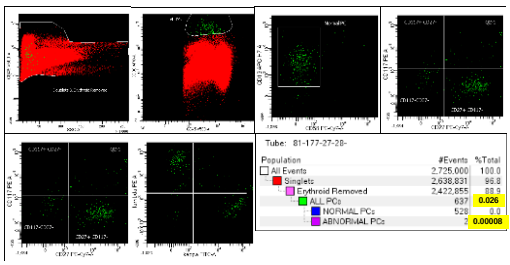
An automatic separator of events using the 'Principal Component Analysis'. Multidimensionally identified cell subsets are rendered in a 2-dimensional format.



The Internal or External Reference Case Database

Cell analyses can be compared to reference datasets generated internally (i.e. at disease onset) or to an external case library, if analyzed by the same criteria.

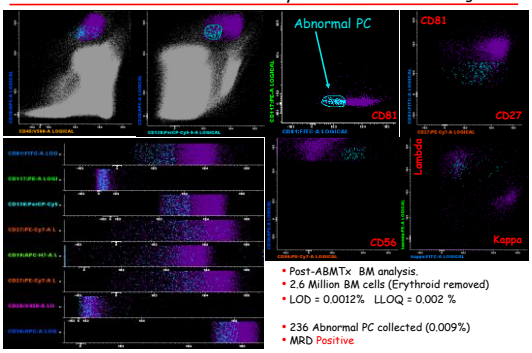
Conventional Flow-MRD Assay: MRD - Negative Case



Consensus on a standardized, robust MM-MRD gating protocol with **conventional FCM analysis** softwares is still lacking.

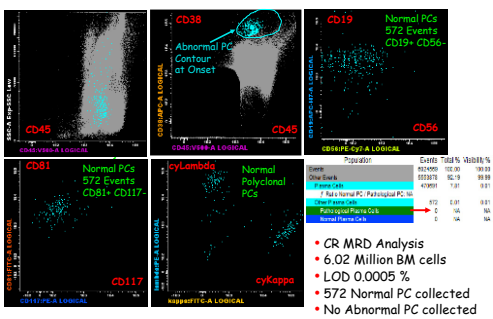
- MRD Study - 1 Tube, Conventional Analysis
- 2.725 M BM cells acquired & erythroid removed
- LOD = 0.0012%
- 2 Abnormal PCs collected (0.00008%)
- MRD Negative

Next Generation Flow-MRD Assay: 2 EuroFlow Tubes Merged



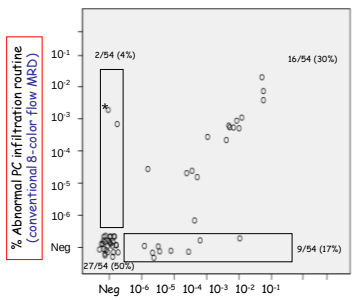
- Post-ABMTx BM analysis.
- 2.6 Million BM cells (Erythroid removed)
- LOD = 0.0012% LLOQ = 0.002 %
- 236 Abnormal PC collected (0.009%)
- MRD Positive

Next Generation Flow-MRD Assay: 2 EuroFlow Tubes Merged



- CR MRD Analysis
- 6.02 Million BM cells
- LOD 0.0005 %
- 572 Normal PC collected
- No Abnormal PC collected
- MRD Negative

Next Generation Flow MRD vs Conventional 8-color Flow MRD



* Proven polyclonal by CyIg staining

Courtesy of Alberto Orfao, 2015

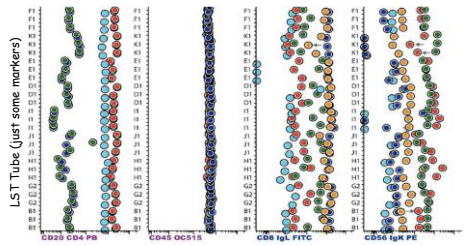
Next-Generation Flow-MRD is now considered equivalent to ASO-qPCR and NGS

	Allele-specific oligonucleotide qPCR	MFC	*VDJ* sequencing
Applicability	60-70%	Nearly 100%	>90%
Need for baseline sample	Yes, requires production of patient-specific probes	Not required; aberrant plasma cells can be identified in any sample by their distinct immunophenotypic pattern vs normal plasma cells	Baseline samples required for identification of the dominant clonotype; alternatively, a stored sample from a time point with detectable disease can be used to define baseline status
Sample requirements	<1 million cells	>5 million cells	<1 million cells; higher numbers improve sensitivity
Sample processing	Can be delayed; can use both fresh and stored samples	Needs assessment within 24–48 h; requires a fresh sample	Can be delayed; can use both fresh and stored samples
Sample quality control	Not possible. Additional studies required	Immediate with global bone marrow cell analysis	Not possible. Additional studies required
Sensitivity	≥1 in 10^5	≥1 in10^5	≥1 in 10^5
Information regarding sample composition	No further information available	Detailed information available on leucocyte subsets and their relative distribution	Information about immunoglobulin gene repertoire of B cells in the studied patient samples
Turnaround and complexity	Labour intensive; requires the development of patient-specific primers/probes; can take several days	Can be done in a few hours; automated software available	Can take several days for turnaround; requires intense bioinformatics support. Use of local laboratories could speed up this limitation
Standardisation	Has been done for other diseases (EuroMRD), can be done for myeloma as well	Standardised by the EuroFlow consortium	In process
Availability	Wide*	Most hospitals with four-colour flow cytometry. Eight or more-colour flow cytometry requires more experienced centres/laboratories. Many laboratories have adopted the EuroFlow laboratory protocols and use the EuroFlow MRD tubes	So far limited to one company/platform

Multicolor Flow Cytometry

New International Myeloma Working Group (IMWG) Response Criteria
Kumar S. Lancet Oncology 2016; Aug (17): e328-e346

EUROFLOW: Long-Term Monitoring of Instrumental Standardization **(MedFI)**



- When the analysis output is an **Intensity of Expression** variable, the control of consistency and stability of **MedFI over time** is mandatory.
- Despite Instrument setup, Calibration microspheres, and Reagents are fully homogeneous, some markers still display a high grade of intrinsic expression variability.

EuroFlow

Kalina T. Cytometry Part A 2015; 87A: 145-156.

STANDARDIZATION vs HARMONIZATION IN FLOW CYTOMETRIC ANALYSES

Standardization

Tchaikovsky's Piano Concert N. 1 in Bb Major op. 23

Notes are always exactly the same at every performance

The Tchaikovsky's 1st Piano Concert in Bb is immediately recognized **BECAUSE** the notes are always the same.

Harmonization

A 12-bar Jazz Blues in F

Notes vary greatly among the various performers

A 12-bar Jazz Blues in F is immediately recognized **DESPITE** the notes are different at each performance.

STANDARDIZATION vs HARMONIZATION IN FLOW CYTOMETRIC ANALYSES

Standardization

- Rather easy to implement for simple, repetitive analyses (i.e. CD4, CD34, CD64, FMH, Low Level Leucocytes...)
- Requires a lot of time for validation of MoAb recipes. Scientific evidence may change meanwhile, making guidelines rapidly obsolete (esp. in Leukemia).
- The strict rules may cause excessive selection of labs according to their available technologies.
- Everyone invoke 'standardization' in FCM, but once technical guidelines are published criticism and variations become quickly the rule.

Harmonization

- Requires a strong agreement on basic general principles and technical operating procedures.
- Detailed recipes of antibody mixtures are not strictly necessary.
- Guidelines can be applied more flexibly, even in labs not equipped with state-of-the-art technology.
- It doesn't matter if a cat is black or white, as long as it catches mice. *(Deng Xiaoping, 1960)*

OPTIMIZATION OF THE TWO 8-COLOR MM MRD ANTIBODY COMBINATIONS

Panel version	Tube	PacB	PacO	FITC	PE	PerCP Cy5.5	PE Cy7	APC	APC H7
1	1	CD45	CD138	CD38	CD56	CD27	CD19	CD117	CD81
	2	CD45	CD138	CD38				CyIgk	CyIgl
			HV500-C						
2	1	CD45	CD138	CD38	CD56	CD27	CD19	CD117	CD81
	2	CD45	CD138	CD38	CD56	CD229	CD19	CyIgk	CyIgl
									APC C750
3	1	CD45	CD138	CD38	CD56	CD27	CD19	CD117	CD81
	2	CD45	CD138	CD38	CD56	CD229	CD19	CyIgk	CyIgl
		HV450							
4	1	CD138	CD27	CD38	CD56	CD45	CD19	CD117	CD81
	2	CD138	CD27	CD38	CD56	CD45	CD19	CyIgk	CyIgl
		BV421	BV510						
5	1	CD138	CD27	CD38 (ME)	CD56	CD45	CD19	CD117	CD81
	2	CD138	CD27	CD38 (ME)	CD56	CD45	CD19	CyIgk	CyIgl

ME= Multi-Epitope CD38

EuroFlow

Courtesy of Alberto Orfao, ESCCA Course 2016

REPORTING IN HEMATOLOGICAL MALIGNANCIES: A Matter of Communication

An example of a modern, integrated hematological report.

The results of all the various technical approaches are summarized **in the same** report file, each one signed by the respective responsible academic.

A unified, collegial clinical conclusion is offered.

Johansson U.
Brit J Haematol 2014; 165: 455-488

Leukemia / Lymphoma Report

Report to: Dr Bloggs, St Elsewhere Address

Sample Date: Day-Month-Year
Date received: Day-Month-Year
Lab number: 1234567
Referral lab no: 345678

Patient Name: Joe Bloggs
Date of Birth: Day-Month-Year
Hosp No: 11223344
Referring Clinic: St Elsewhere

LEUKAEMIA DIAGNOSIS – INTERGRATED REPORT
Clinical Details: None stated

FBC/Film morphology: WBC: 669·3×10⁹/l Hb: 82 g/l Plts: 129×10⁹/l Ly: 656·0×10⁹/l

Sample Investigated: Peripheral Blood and Bone Marrow Aspirate

Morphology/Cytochemistry (Name of Section Head, phone number)
The vast majority of cells are variable sized, largely mature lymphoid cells. Many have a single prominent cleft.
(Initials of reporter/s)

Immunophenotyping (Name of Section Head, phone number)
There is a moderately bright kappa clone of CD19+/CD5+ B cell present which accounts for 98% of the total nucleated cells in this sample. Many of the lymphoid cells show an increase in forward scatter signal, indicating a larger cell size.
The B cell phenotype is FMC7 wk-mod/CD23-/CD79b wk/CD20++/CD10-/CD43-/CD200wk/sIgM-/sIgD variable. KI67 is positive on 15% of the B cells. CD38 was not tested for.
This is unlikely to be CLL, the CLL score is 1-2/5. Await FISH for IgH/CCND1 gene rearrangement to test for mantle cell lymphoma.
(Initials of reporter/s)

Cytogenetics/molecular Cytogenetics (Name of Section Head, phone number)
Result from bone marrow sample taken (date)
Probe hybridised: (company name) IGH-CCND1 dual fusion.
Sample hybridised: bone marrow cultured.
Result: positive.
All 100 nuclei examined showed a signal pattern consistent with an IGH-CCND1 gene rearrangement.
This is consistent with the diagnosis of mantle cell lymphoma.
(Initials of reporter/s)

Comments and Conclusion
Mantle cell lymphoma (9861/3)
(Initials of reporter)

Molecular Genetics and Cytogenetics Seem to Dominate in the Field of Hematologic Malignancies

Table 1. Genetic abnormalities that affect acute myeloid leukemia (AML) classification.

AML with Recurrent Genetic Abnormalities	AML with Myelodysplasia Related Changes
RUNX1-RUNX1T1 t(8;21)(q22;q22)	Complex karyotype (≥3 unrelated abnormalities)
CBFB-MYH11 inv(16)(p12.1q22) or t(16;16)(p13.1;q22)	−7/del(7q), −5/del(5q) — **Unbalanced**
PML-RARA t(15;17)(q22;q12)	−13/del(13q), del(11q), del(12p)/t(12p), del(9q)
MLLT3-MLL/KMT2A t(9;11)(q22;q23)	i(17q)/t(17p), idic(X)(q13)
DEK-NUP214 t(6;9)(p23;q34)	t(5;12)(q33;p12), t(5;7)(q33;q11.2) t(5;17)(q33;p13), t(5;10)(q33;q21)
RPN-EVI1 inv(3)(q21q26.2) or t(3;3)(q21;q26.2)	t(1;3)(p36.3;q21.2), t(3;5)(q25;q34)
RBM15-MKL1 t(1;22)(p13;q13)	t(11;16)(q23;p13.3) *, t(3;21)(q26.2;q22.1) *
NPM1 gene mutation, WT1, EVI1	t(2;11)(p21;q23) *
Mutated *CEBPA*	**Balanced**

Plus: SANGER (Kit, DNMT£A, IDH1/2, CEBPA. NRAS/KRAS)
NGS (RUNX1, TET2, TP53, ASXL1, EZH2)
RT-PCR (MLL-PTD)

...but....

Therapeutic and Diagnostic MoAbs

To date **105+ Monoclonal antibodies are registered and commercially available for diagnosis and therapy in patients** *(June 2017)*

Such molecules interact with target cell surface molecules or with soluble factors

Many issues related to MoAb usage and its effects on the target should be carefully monitored *(by FCM, of course!)*

MoAb MOLECULE	Commercial Name	Biological Target
Abciximab	ReoPro	CD41 (integrin alpha-IIb)
Adalimumab	Humira, Trudexa	TNF-α
Adalimumab-Atto	Amjevita	TNF-α Biosimilar
Alefacept	Amevive	LFA-3 (CD58)
Alemtuzumab	Lemtrada, Campath	CD52
Alirocumab	Praluent	PCSK9
Altumomab pentetate	Hybri-ceaker	CEA
Arcitumomab	CEA-Scan	CEA
Atezolizumab	Tecentriq	CD274 (PD-L1)
Atlizumab	RoActemra	IL-6 receptor
Avelumab	Bavencio	PD-L1
Basiliximab	Simulect	CD25 (α chain of IL-2 receptor)
Bectumomab	LymphoScan	CD22
Belatacept	Nulojix	CTLA-4
Belimumab	Benlysta, LymphoStat-B	BAFF
Besilesomab	Scintimun	CEA-related antigen
Bevacizumab	Avastin	VEGF-A
Bezlotoxumab	Zinplava	Clostridium difficile
Biciromab	FibriScint	fibrin II, beta chain
Blinatumomab	Blincyto	CD19
Brentuximab vedotin	Blentreа	CD30 (TNFRSF8)
Brodalumab	Siliq	IL-17
Canakinumab	Ilaris	IL-1β
Capromab pendetide	Prostascint	Prostatic carcinoma cells
Catumaxomab	Removab	EpCAM, CD3
Certolizumab pegol	Cimzia	TNF-α
Cetuximab	Erbitux	EGFR
Citvatuzumab tetraxetan	hPAM4-Cide	MUC1
Daclizumab	Zenapax, Zinbryta	CD25 (α chain IL-2 R)
Daratumumab	Darzalex	CD38 (cyclic ADP ribose hydrolase)
Denosumab	Prolia, Xgeva	RANKL
Dinutuximab	Unituxin	GD2 ganglioside
Eculizumab	Soliris	C5
Edrecolomab	Panorex	EpCAM
Efalizumab	Raptiva	LFA-1 (CD11a)
Efungumab	Mycograb	Hsp90
Elotuzumab	Empliciti	SLAMF7
Ertumaxomab	Rexomun	HER2/neu, CD3
Etaracizumab	Abegrin	integrin αvβ3
Evolocumab	Repatha	PCSK9
Fanolesomab	NeutroSpec	CD15
FBTA05	Lymphomun	CD20
Fontolizumab	HuZAF	IFN-γ
Gemtuzumab ozogamicin	Mylotarg	CD33
Girentuximab	Rencarex	carbonic anhydrase 9 (CA-IX)
Golimumab	Simponi	TNF-α
Ibritumomab tiuxetan	Zevalin	CD20
Idarucizumab	Praxbind	Dabigatran
Igovomab	Indimacis-125	CA-125
Imciromab	Myoscint	Cardiac myosin

To follow

Continued

MoAb MOLECULE	Commercial Name	Biological Target
Infliximab	Remicade, Flixabi, Inflectra, Remsima	TNF-α
Ipilimumab	Yervoy	CD152
Ixekizumab	Taltz	IL-17A
Labetuzumab	CEA-Cide	CEA
Mepolizumab	Bosatria, Nucala	IL-5
Motavizumab	Numax	Respiratory Syncytial Virus
Moxetumumab Pasudotox	Moxetumomab	CD22
Muromonab-CD3	Orthoclone OKT3	CD3
Natalizumab	Tysabri	integrin α4
Necitumumab	Portrazza	EGFR
Nimotuzumab	Theracim, Theraloc	EGFR
Nivolumab	Opdivo	PD-1
Nofetumomab Merpentan	Verluma	EpCAM
Obiltoxaximab	Anthim	Bacillus anthracis anthrax
Obinutuzumab	Gazyva	CD20
Ocrelizumab	OCRE	CD20
Ofatumumab	Arzerra	CD20
Olaratumab	Lartruvo	PDGF-R α
Omalizumab	Xolair	IgE Fc region
Oregovomab	OvaRex	CA-125
Palivizumab	Synagis, Abbosynagis	F protein Resp Syncytial Virus
Panitumumab	Vectibix	EGFR
Pembrolizumab	Keytruda	PD-1
Pemtumomab	Theragyn	MUC1
Pertuzumab	Omnitarg, Perjeta	HER2/neu
Ramucirumab	Cyramza	VEGFR2
Ranibizumab	Lucentis	VEGF-A
Raxibacumab	Raxibacumab	Anthrax toxin, protective antigen
Reslizumab	Cinqair	IL-5
Rituximab	MabThera, Rituxan, Truxima	CD20
Rovelizumab	LeukArrest	CD11, CD18
Ruplizumab	Antova	CD154 (CD40L)
Secukinumab	Cosentyx	IL 17A
Siltuximab	Sylvant	IL-6
Sulesomab	LeukoScan	NCA-90 (granulocyte antigen)
Tacatuzumab tetraxetan	AFP-Cide	alpha-fetoprotein
Tamtuvetmab	Tactress	CD52
Tecnemab K1	Tecnemab	Melanoma
Tefibazumab	Aurexis	clumping factor A
Teprotumumab	Gennab	Insulin-like GF Receptor
Tisotumab Vedotin	HuMax TF ADC	Tissue Factor
Tocilizumab	RoActemra	IL-6 receptor
Tositumomab	Bexxar	CD20
Trastuzumab	Herceptin	HER2/neu
Trastuzumab Emtansine TDI	Kadcyla	HER2/neu
TRB507	Ektomab	GD2 ganglioside
Ustekinumab	Stelara	IL-12, IL-23
Vedolizumab	Entyvio	Integrin α4β7
Visilizumab	Nuvion	CD3
Votumumab	HumaSPECT	tumor antigen CTAA16.88
Zalutumumab	HuMax-EGFr	EGFR
Zanolimumab	HuMax-CD4	CD4

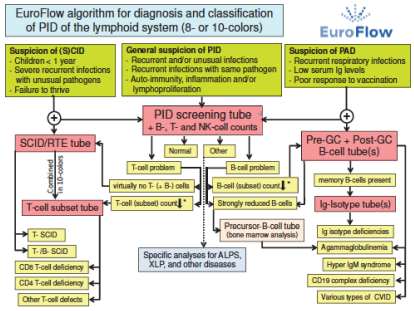
Therapeutic and Diagnostic MoAbs

Laboratory Support plays an ever increasing role in MoAb therapy, to monitor:

- **The development of the correct mechanism of action of the MoAb.**
- **The kinetics of target cell disappearance and reappearance.**
- **The monitoring strategies determined by the changes induced by the MoAb on its target.**
- **The development of escape mechanisms that may hamper the clinical effects of the MoAb.**

Not Only Leukemia

A Comprehensive FCM Approach to Primary Immunodeficiencies



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Flow Cytometry Studies of Immune System

- **An attempt to standardize a comprehensive antibody panel to study all immune system cell functional and regulatory subsets:**
  - **T Cells:** Naive, Memory, Central & Effector memory, TH1/TH2/TH17, Treg, Activated
  - **B Cells:** Naive, Memory IgD+/-, Transitional, Plasmablasts
  - **NK Cells:** 56+low NK, 56+high NK
  - **Monocytes:** Classical, Intermediate and Non-Classical Monocytes
  - **Dendritic Cells:** Myeloid DC, Plasmacytoid DC

	T cell	Treg	B cell	DC/mono/NK	Th1/2/17
FITC	dead	dead	dead	dead	dead
PE	CCR7 (150503)	CD25 (2A3)	CD24 (ML5)	CD56 (B159)	CXCR3 (1C6/CXCR3)
PerCP-Cy5.5	CD4 (SK3)	CD4 (SK3)	CD19 (SJ25C1)	CD123 (7G3)	CD4 (SK3)
PE-Cy7	CD45RA (L48)	CCR4 (1G1)	CD27 (M-T271)	CD11c (B-LY6)	CCR6 (11A9)
APC	CD38 (HIT2)	CD127 (HIL-7R-M21)	CD38 (HIT2)	CD16 (B73.1)	CD38 (HIT2)
APC-H7	CD8 (SK1)	CD45RO (UCHL1)	CD20 (2H7)	CD3+19+20 (SK7, SJ25C1, 2H7)	CD8 (SK1)
V450	CD3 (UCHT1)	CD3 (UCHT1)	CD3 (UCHT1)	CD14 (MPHIP9)	CD3 (UCHT1)
V500	HLA-DR (G46-6)	HLA-DR (G46-6)	IgD (IA6-2)	HLA-DR (G46-6)	HLA-DR (G46-6)

- Maecker HT. Nature Review Immunology 2012; 12: 191-200.
- Finak G. Nature Scientific Reports 2015. 6:20686, DOI: 101038/srep20686

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Flow Cytometry of the Red Cell Compartment

- **Normal & Abnormal Red Cells as Seen by FCM:**
  - Blood Counter Measurements vs FCM Findings
  - Detection of normal & abnormal RBC Subpopulation
  - RBC aging in vivo and Eryptosis
  - FCM and RBC Osmotic Fragility
  - Congenital Spherocytosis and Pyro-Poichilocytosis
  - Feto-Maternal Hemorrhage Analysis, not Rh/D related
  - Detection of RBC PNH Clones
  - Detection of Blood Doping in Athletes
  - Detection of Fluorocytes in Protoporphyria
- **Bone Marrow Maturation of Red Cells by FCM:**
  - Normal and Dysplastic Maturation Patterns
  - Intracellular Isoferritins
- **The Never Ending Story:**
  - Detection of Malaria Parasites by FCM

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Flow Cytometry in the Blood Bank

- **Quality Control of Blood Components:**
  - Low-Level Leucocyte Count in Leucoreduced products
  - Control of Platelet Function and Sterility in PLT Bags
  - Study of the RBC 'Storage Lesions'
- **Control of CD34+ PBSC Units for Transplantation:**
  - Evaluation of Apheresis Yield and PBSC Collection
  - Enumeration of Viable PBSC at Freezing
  - Evaluation of Viable PBSC after Thawing
- **Management of Feto-Maternal Hemorrhage (Anti-D):**
  - FCM to Guide the Anti-D Therapy
- **Experimental Applications:**
  - Anti-RBC Antibody Studies
  - Monitoring the kinetics of transfused RBC survival
  - Large-Scale in-Vitro Production of Red Blood Cells

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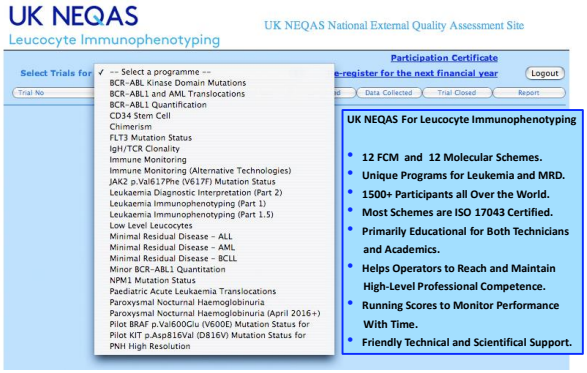
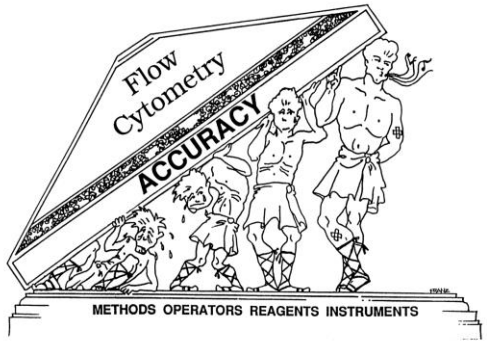
Bronchoalveolar Lavage: Clinically Important Questions To Be Answered by the FCM Analysis of the Recovered Immune Cells

- Overall Cellular Concentration:
  - Hypercellular BAL indicates active disease.
- Lymphocyte Percentage:
  - Relative lymphocytosis indicates an ongoing immune process.
- T Lymphocyte Subset Distribution:
  - Prevalence of T CD4+ or T CD8+ cells varies in different diseases.
- Presence of Other Cell Types:
  - PMNs, Eosinophils, CD1a+ Histiocytes (sometimes disease-specific).
  - Leukemia/Lymphoma Cells (Lung involvement in hematological malignancies).
  - Activated Lymphocytes, TH1/TH2/TH17 Cells, Dendritic Cells (experimental)





An Overview of Clinical Flow Cytometry (Conclusions)

- **New developments in medicine require extensive patient monitoring in many different diseases** *(Hematology/Oncology, Autoimmune Diseaseses, Immuno-deficencies, Infectious Diseases, Reproductive and Cardiovascular Medicine).*
- **Changes in diagnostic strategies are needed to follow the new targeted therapies** *(Humanized Antibodies, TKI- and BCL2-blockers, CAR-T Cells etc.)* **also by implementing new data analysis systems.**
- **The new (immune)therapeutic agents may induce prolonged overall survival and better quality of life for patients, thus making the clinical follow-up longer and made of complex sequential runs of therapy** *(i.e. Myeloma).*
- **The more targeted the therapy, the higher the chance of mutations and consequent resistance. An eary detection of therapy resistance will be increasingly important.**
- **As a consequence, Flow Cytometry Monitoring is the answer!**