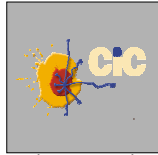


PAPEL DE LA CITOMETRIA DE FLUJO EN EL ANÁLISIS DE CÉLULAS Y SUS PRODUCTOS



CENTRO DE INVESTIGACIÓN DEL CÁNCER,
UNIVERSIDAD Y HOSPITAL UNIVERSITARIO
DE SALAMANCA

69º Congreso Argentino de Bioquímica
Buenos Aires, 30 de mayo de 2011

TECHNIQUES FOR THE ANALYSIS OF CELLS & CELL PRODUCTS

- Microbiology
- Biochemistry
- Molecular Biology
- Pathology
- Flow cytometry

CELL ANALYSIS TECHNIQUES

	Image Analysis	Flow Cytometry	Biochemistry/ Molecular biology
Information based on	per cell	per cell	Mean sample value
Localization in the cell	Yes	No	No
Speed	Low	High	High
Type of information	Qualitative	Qualitative Quantitative	Qualitative Quantitative
M-parametric	Yes	Yes	No

FLOW CYTOMETRY

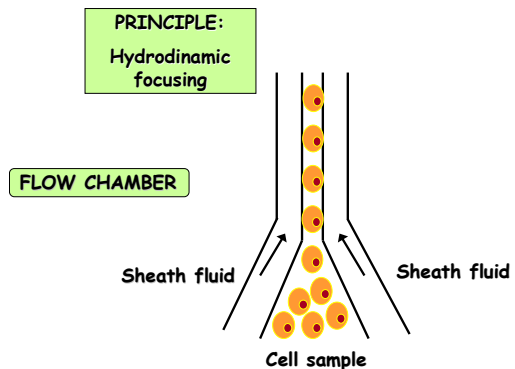
- Qualitative and quantitative information
- Sensitivity
- Objective and reproducible
- High speed and statistically reliable
- Multiparametric information

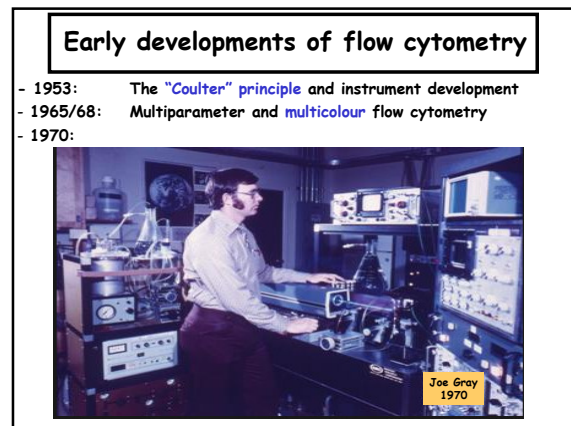
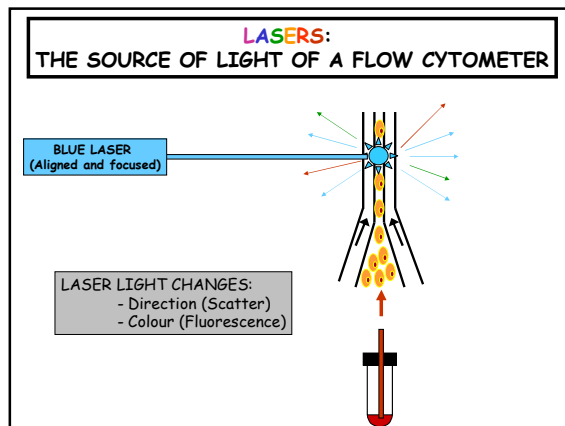
Early developments of flow cytometry

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



THE FLUIDIC SYSTEM OF A FLOW CYTOMETER

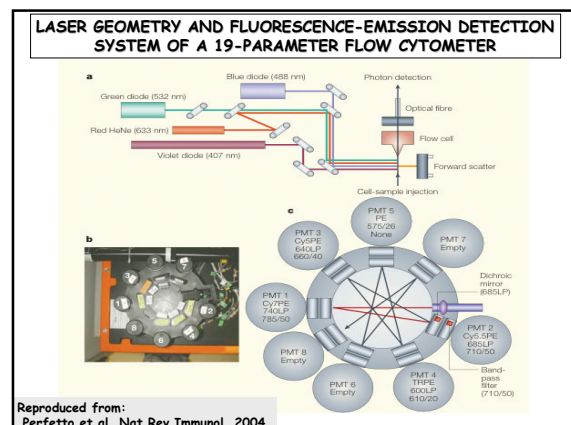




FLOW CYTOMETRY PARAMETERS

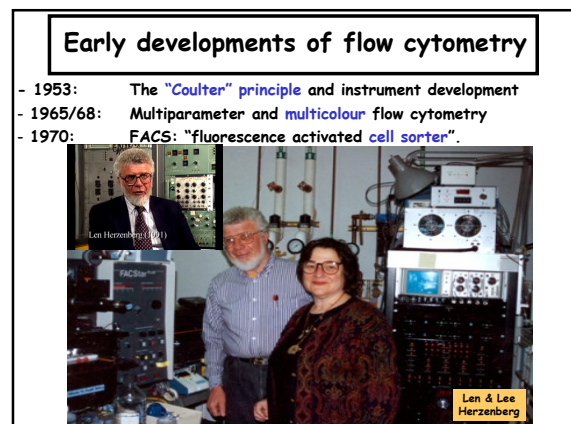
- **Light scatter:**
 - Low angle light scatter
 - Side (90°) scatter
- **Fluorescence emissions: ***
 - FL1
 - FL2
 - FL3
 - FL4

*artificial or autofluorescence

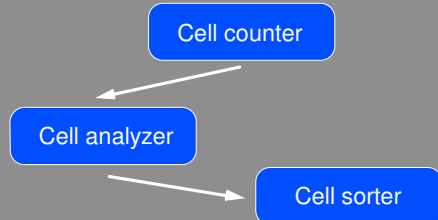


SETS OF FLUOROCHROMES FOR FLOW CYTOMETRY IMMUNOPHENOTYPING

Fluorochrome	Excitation Peak (nm)	Emission Peak (nm)	Argon	Lasers Violet	Helium-Neon
Marina Blue	365	460	+		
FITC	495	520	+		
Phycoerythrin (PE)	565	575	+		
PE Texas Red	565	615	+		
Per-CP	490	670	+		
PE-Cy7	565	770	+		
Pacific Blue	405	455		+	
AmCyan	405	490		+	
Pacific Orange	405	550		+	
Allophycocyanin (APC)650		660			+
Alexa 700	635	720			+
APC-Cy7	650	770			+



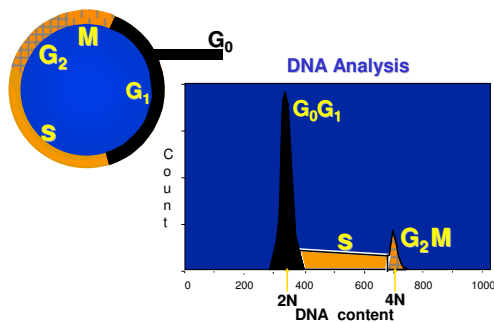
FUNCTIONAL FEATURES OF A FLOW CYTOMETER



APPLICATIONS OF FLOW CYTOMETRY

- Immunophenotype of cells
- DNA, RNA, protein cell contents
- Enzyme analysis
- Ca⁺⁺ and other ion measurements
- Intracellular pH
- Drug effects and drug resistance
- Apoptosis and cell death
- Phagocytosis
- Chromosome analysis
- Quantitation of soluble proteins
- Study of cell organelles
- Cell/chromosome sorting

Cell Cycle Analysis by FCM



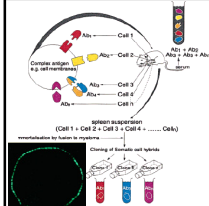
PROGNOSTIC VALUE OF DNA STUDIES BY FCM

Tumor type	DNA variable	Clinical impact
Bladder	Aneuploid	Aggressive
Bone	S-phase	Aggressive
Breast	Aneuploid	Short survival
	Aneuploid	Short survival?
	S-phase	Relapse, short surv
Colorectal	Aneuploid	Short survival?
	S-phase	Relapse, short surv
Gastric	Aneuploid	Short survival
Cervix	Aneuploid	Invasive, short surv
Kidney	Aneuploid	Short survival
Lung	Aneuploid	Short survival
Prostate	Aneuploid	Short survival
Neuroblastoma	Aneuploid	Long survival

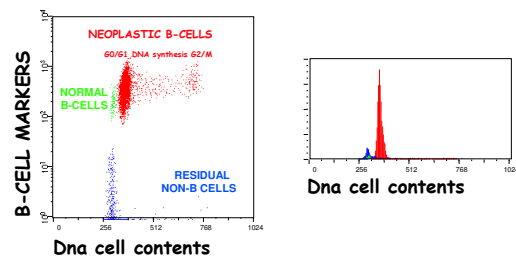
Early developments of flow cytometry

- 1953: The "Coulter" principle and instrument development
- 1965/68: Multiparameter and multicolour flow cytometry
- 1970: FACS: "fluorescence activated cell sorter".
- 1975: Production of monoclonal antibodies

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



ALL: DNA PLOIDY & CELL CYCLE ANALYSIS



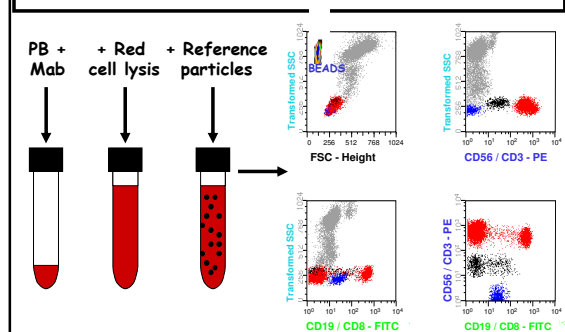
FLOW CYTOMETRY: TYPE OF INFORMATION

- **Identification** of cell populations
- **Quantification** of cell numbers
- **Characterization** of cell populations
- **Isolation/purification** of cells

APPLICATIONS OF FCM TO TRANSFUSION MEDICINE

- **Cell analysis:**
 - Leucocyte contamination of transfusion products
 - Fetomaternal hemorrhage
 - Monitoring of transfused red cells /platelets
 - Red cell chimerism or mosaicism
 - Mutant/variant RBC
 - Platelet activation & functionality
 - Immunophenotypic analysis of blood cells
- **Specific protein studies:**
 - Proteins bound to blood cells (i.e. platelet & red cell associated Igs /C3)
 - Anti-platelet antibodies
 - Anti-leucocyte antibodies

PHENOTYPIC ANALYSIS OF PB LYMPHOCYTE SUBSETS IN HIV: CURRENT APPROACHES



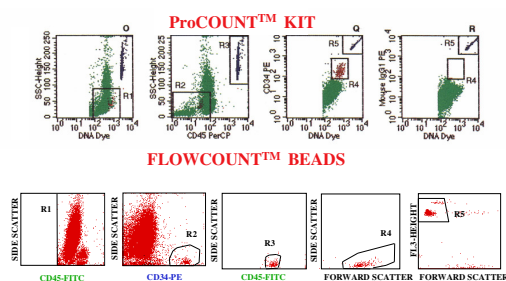
PROGNOSTIC MARKERS IN HIV INFECTION

- Number of PB **CD4+ T-cells**
- **Viral load** measured in the serum
- **Mean number of CD38 molecules** expressed by CD8+ T-cells
- **Other lymphoid subsets/serologic markers:** CD8/CD45ro, CD8/CD28, CD95/CD4, CD95/CD8, sCD95, sCD8,

SINGLE PLATFORM METHODS

REFERENCE FLUORESCENCE BEADS

CD34 HPC ABSOLUTE COUNT → FLOW CYTOMETRY



IMPACT OF THE COMPOSITION OF THE LEUKAPHERESIS PRODUCTS ON THE HEMATOPOIETIC RECOVERY AFTER AUTOLOGOUS TRANSPLANT

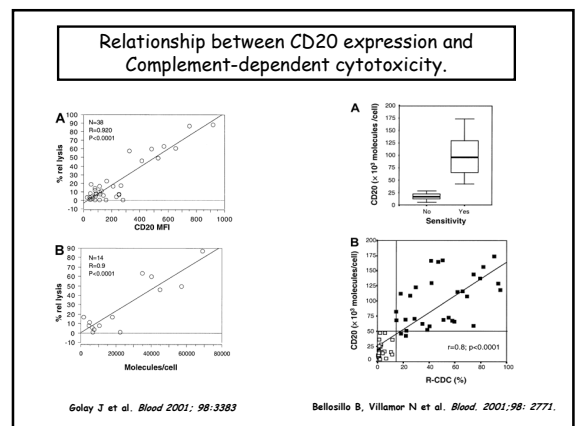
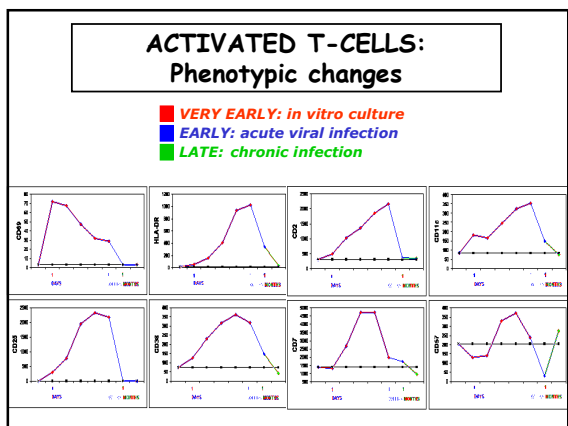
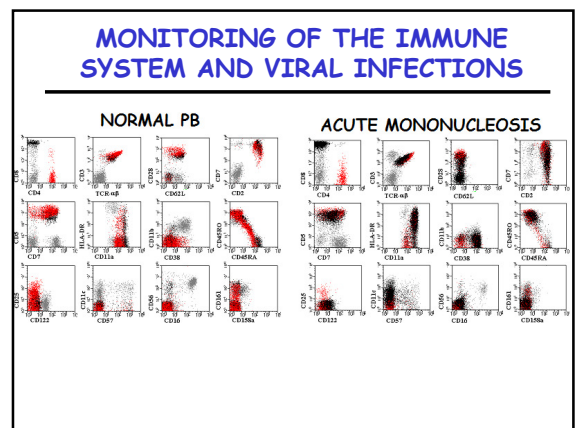
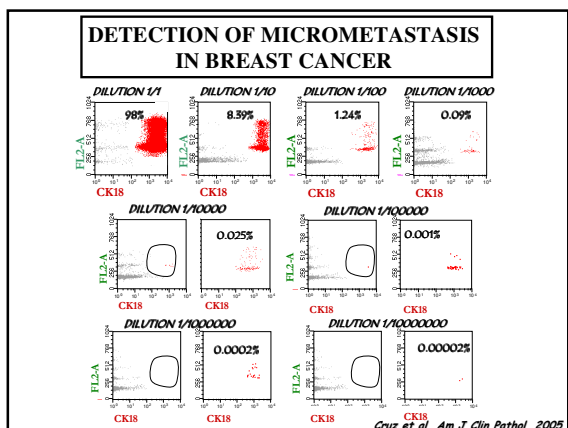
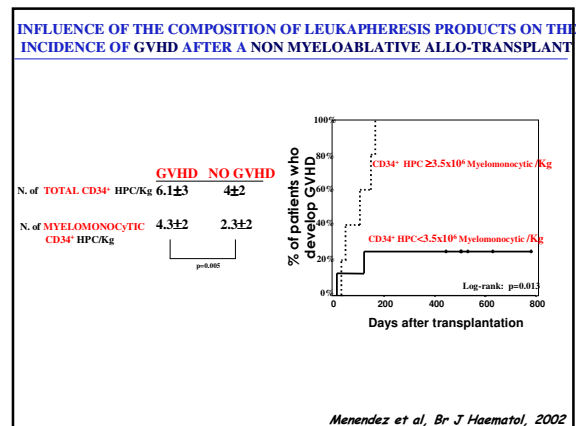
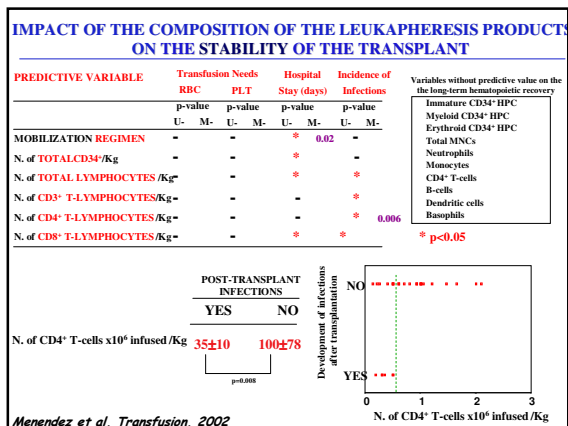
PREDICTIVE VARIABLE	ANC >0.5x10 ⁹ /L		ANC >1x10 ⁹ /L		PLT >20x10 ⁹ /L		PLT >50x10 ⁹ /L	
	p-value		p-value		p-value		p-value	
	U-	M-	U-	M-	U-	M-	U-	M-
MOBILIZATION REGIMEN	*		*		*		*	
N. of TOTALCD34 ⁺ HPC/Kg	*		*		*		0.006	0.001
N. of MYELOID CD34 ⁺ HPC/Kg	0.007		0.009		-		-	
N. of NEUTROPHILS /Kg	*		*		-		-	
N. of Total LYMPHOCYTES /Kg	*		*		-		-	
N. of CD3 ⁺ T-LYMPHOCYTES /Kg	*		*		-		-	
N. of CD8 ⁺ T-LYMPHOCYTES/Kg	*		*		-		-	
N. of NK Cells /Kg	*		*		-		-	

Variables without predictive value on the short-term hematopoietic recovery:

Immature CD34⁺ HPC, erythroid CD34⁺ HPC, Total Monocytes, CD4⁺ T-cells, B-cells, Dendritic cells, basophils

* p<0.05

Menendez et al, Transfusion, 2002



**EXPRESSION OF CD52 (CAMPATH-1) BY
NORMAL PB LEUKOCYTES**

CD52 Antigen Binding Capacity (ABC)

Monocytes, Neutrophils, Eosinophils, B-1a Memory, B-2a Memory, CD4+ CD45RO+ CD45RA- T Cells, CD4+ CD45RO- CD45RA- T Cells, CD8+ CD8+ T Cells, CD8+ CD8- T Cells, CD4+ CD4+ T Cells, CD4+ CD4- T Cells, CD8+ CD8+ T Cells, CD8+ CD8- T Cells, NK Cells

Hernández-Campo et al. 2005 (submitted)

Hernández-Campo et al. 2005 (submitted)

EXPRESSION OF ALLERGEN-INDUCED ACTIVATION MARKERS ON BASOPHILS

Identificación CDs y MONOCITOS de SP

PRODUCCIÓN DE TNF- α por las CD5 de SP en pacientes VIH-1+

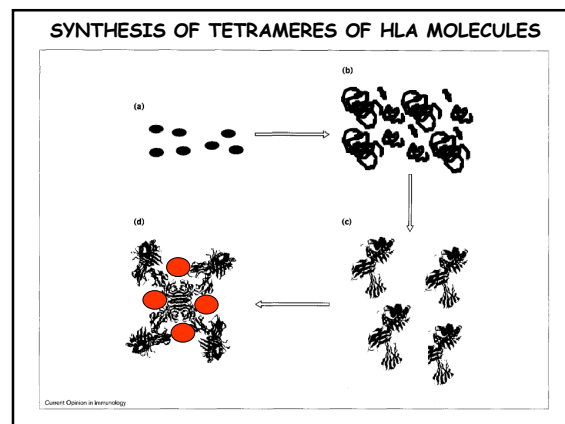
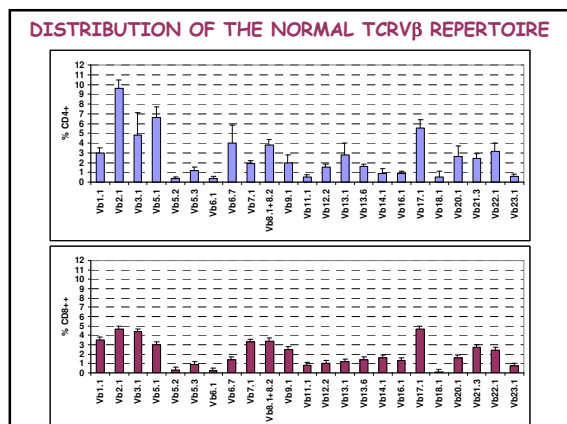
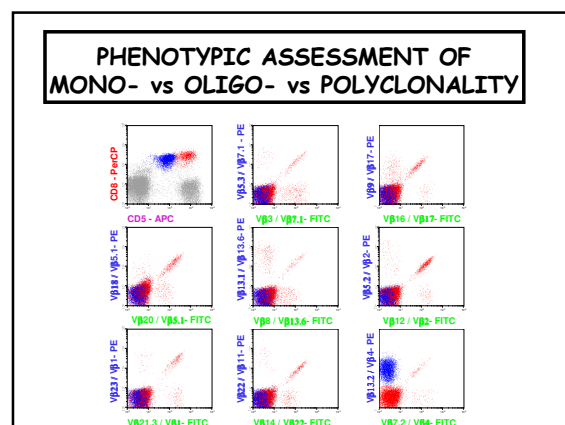
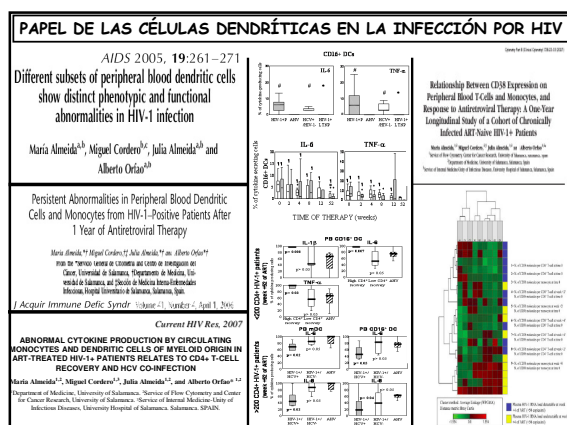
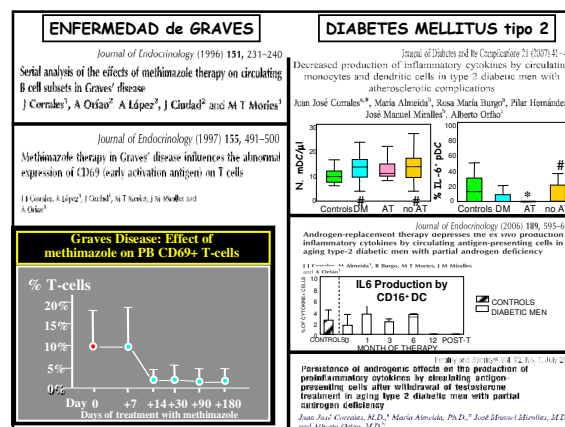
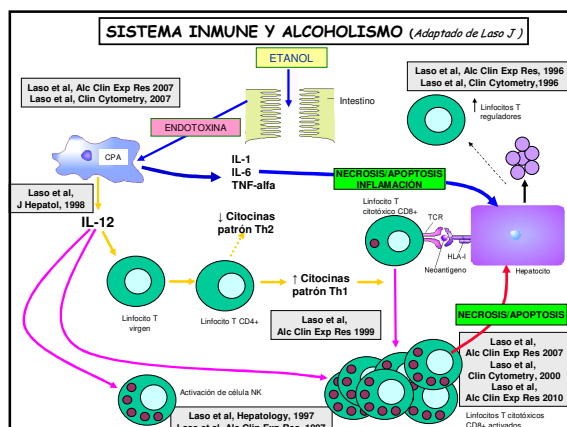
Normal BM pre-pDC subsets

Ability to induce T-cell proliferation

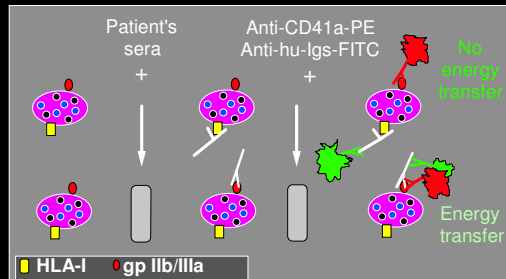
Negative control non-stimulated CFDA-SE - instead of T cells	Stage I pre-pDC	Stage II pre-pDC	Stage III pre-pDC	Positive control (PHA)
Allergic CD4⁺ T cells	~ 0%	3.6%	6.9%	69%
→ CFDA-SE - FITC →				
Allergic CD8⁺ T cells	~ 0%	6.2%	8.8%	71%
→ CFDA-SE - FITC →				

Anti-CD3 induced T-cell activation (intracellular Ca^{++} mobilization)

The graph displays the ratio of intracellular calcium concentration (RATIO [a.u./mg]) on the y-axis (0 to 1000) against time in seconds on the x-axis (0 to 180). The data shows a baseline ratio of approximately 200-400 until 22 seconds, after which it rises sharply to a plateau of approximately 600-1000. A yellow arrow points to the rising edge of the curve, labeled "Calcium Release".

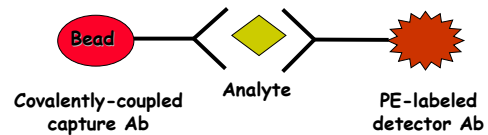


FCM ANALYSIS OF THE SPECIFICITY OF ANTI-PLATELET ANTIBODIES



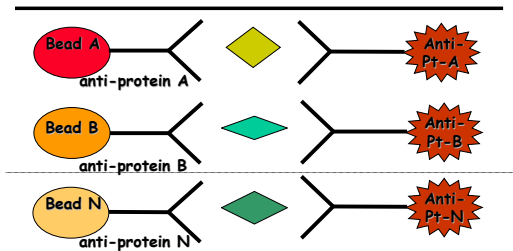
CYTOMETRY BEAD ARRAYS

Direct Sandwich Assay

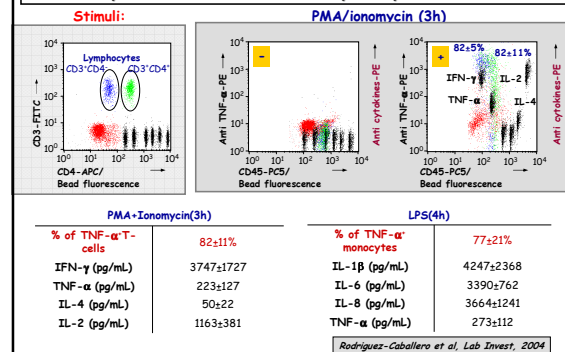


MULTIPLEXED BEAD ARRAYS

Bead-coupled with capture Ab Analyte Fluorochrome-labeled detector Ab



Simultaneous identification of activated cells and quantification of multiple cytokines



TYPES OF PROBES ON BEADS

- NUCLEOTIDES:

- Maximum of 16-20 bases
- Hybridization decreased with 1/15 mismatch

- ANTIBODIES & OTHER PROTEINS:

- All isotypes
- Oriented or not
- Variable amounts (i.e. spacers)

APPLICATIONS OF FLOW CYTOMETRY BASED BEAD-ARRAY SYSTEMS

- Protein analysis:

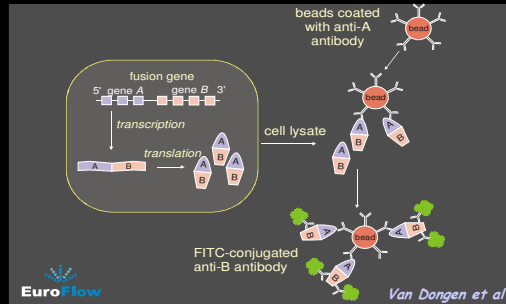
- Cytokine measurements
- Viral serology
- Allergy tests
- Hormone measurements
- Cardiovascular markers
- Immunoglobulin isotypes
- Phosphorylated proteins
- Mutated and fusion proteins

- Study of oligonucleotide sequences:

- HLA typing
- SNP analysis

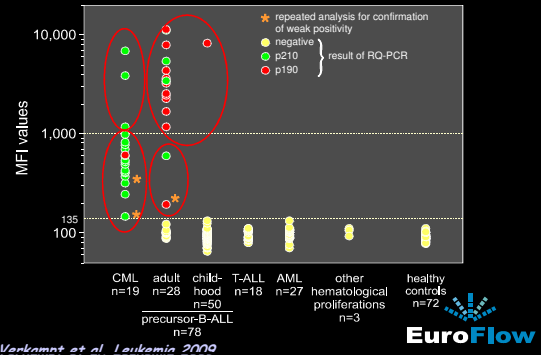
DETECTION OF FUSION PROTEINS DERIVED FROM CHROMOSOMAL TRANSLOCATIONS BY FCM

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

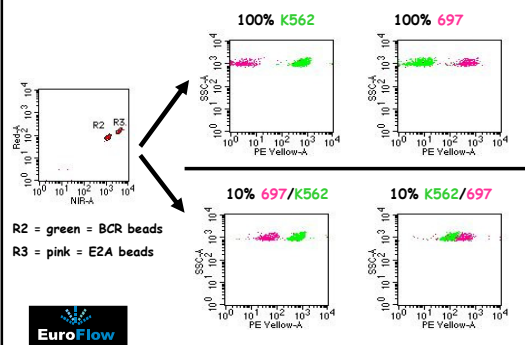


BCR-ABL RUO testing by EuroFlow

MFI values of the different cell samples (n=217)



SIMULTANEOUS DETECTION OF BCR-ABL & E2A-PBX FUSION PROTEINS



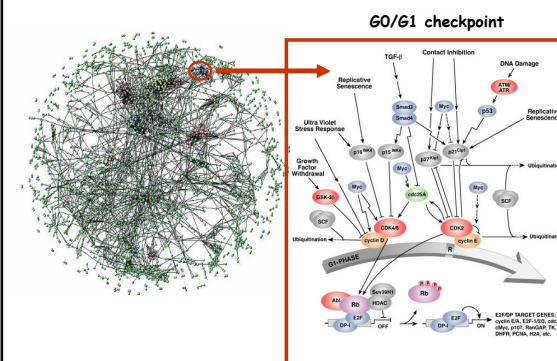
At this moment the technical developments for 7 well-defined fusion proteins have (virtually) been completed:

CML:	BCR-ABL	: completed	RUO kit launched and published
precursor-B-ALL:	BCR-ABL	: completed	} Precursor-B-ALL tube Multiplex tube close to completion
	TEL-AML	: completed	
	E2A-PBX2	: completed	
	MLL-AF4	: completed	
AML:	AML-ETO	: completed	} Core-factor tube Multiplex tube: testing ongoing
	CBFB-MYH11	: completed	
	PML-RARA	: completed	RUO testing ongoing

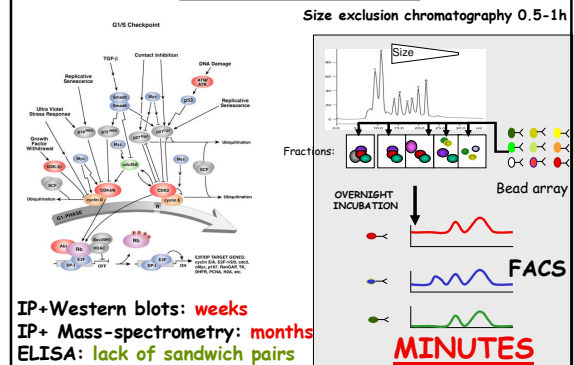


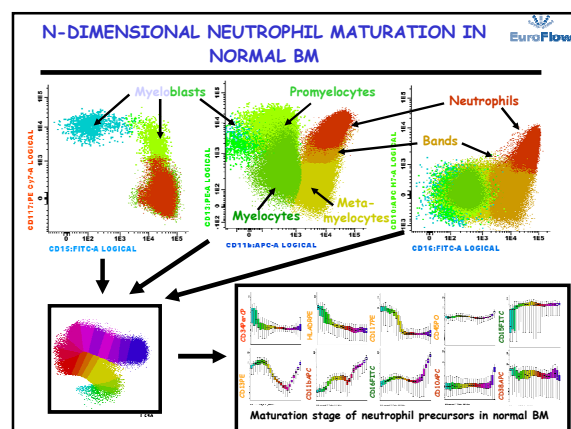
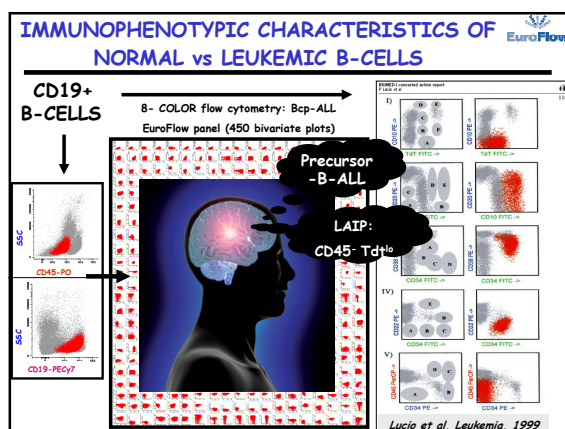
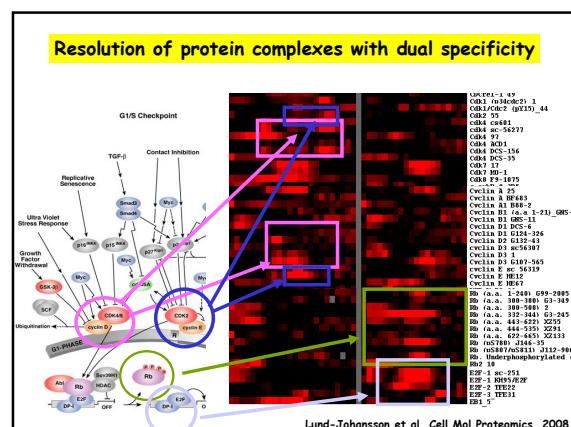
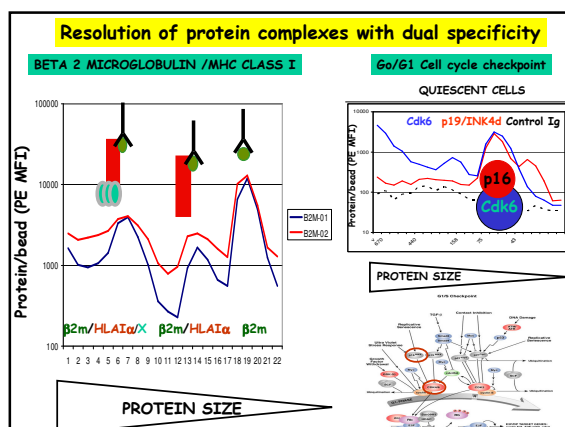
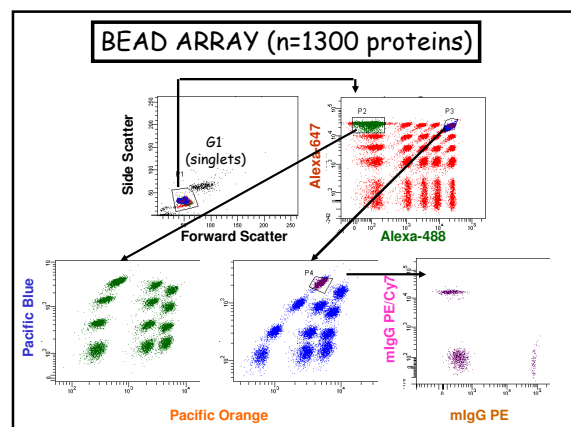
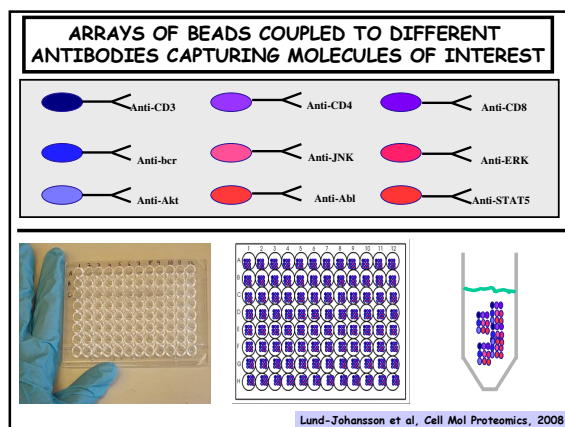
BD Biosciences

PROTEOMICS: THE WORLD OF PROTEINS

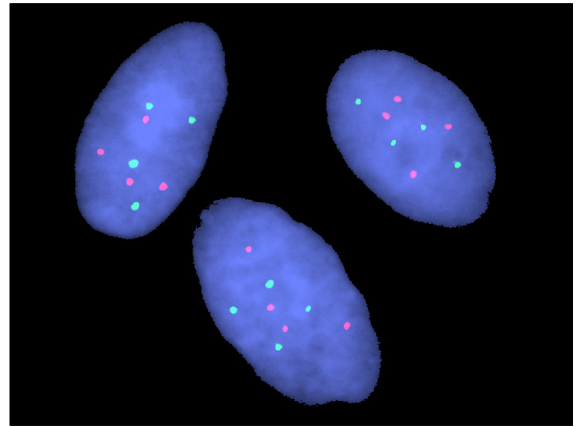
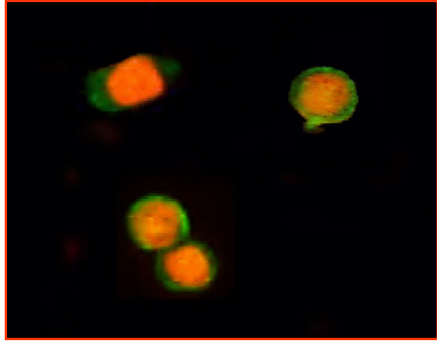


Proteomics 2009





CK18+ BREAST CANCER CELLS



SERVICIO DE CITOMETRIA, DEPARTAMENTO DE MEDICINA Y
CENTRO DE INVESTIGACIÓN DEL CÁNCER (IBMCC)
UNIVERSIDAD DE SALAMANCA




EuroFlow



EuroFlow consortium aims at
innovation in flow cytometry

**MUCHAS
GRACIAS**