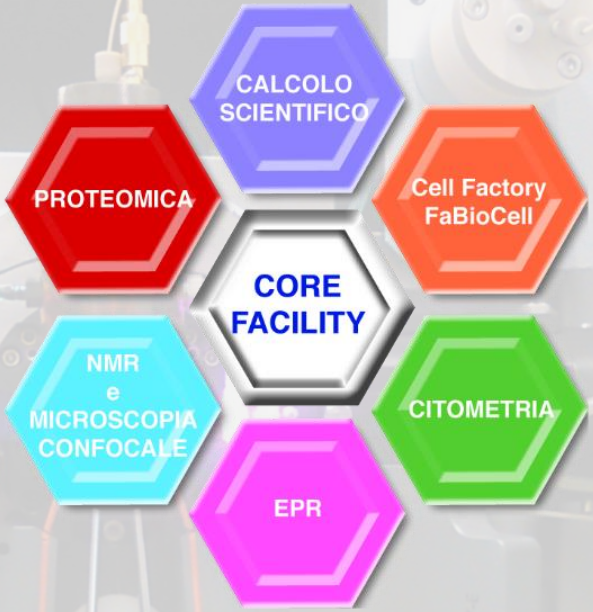
Servizio Grandi Strumentazioni e Core Facilities

Evoluzione della Citometria a flusso: dall'era policromatica alla massa

Valentina Tirelli **Luca Pasquini**

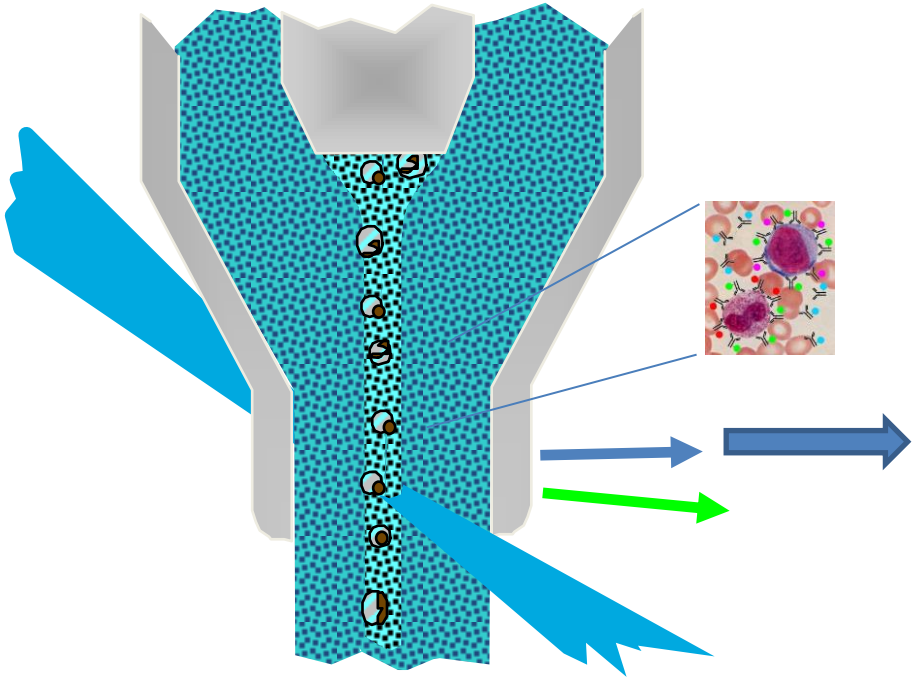


La citometria a flusso: princìpi ed applicazioni

Valentina Tirelli

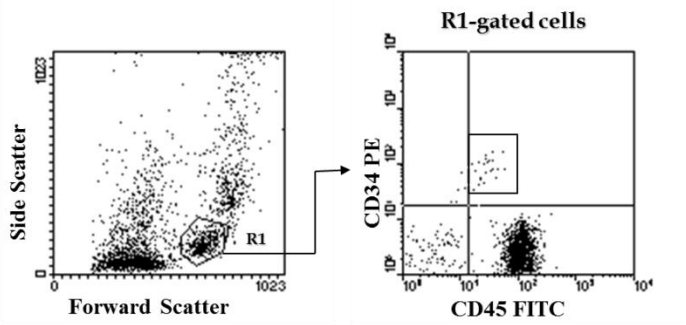
Flow Cytometry: principle

Flow cytometry measures multiple characteristics of individual particles flowing in single file in a stream of fluid.



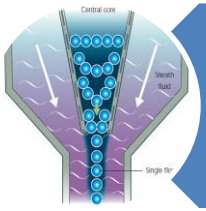
The basic principle of flow cytometry is the passage of cells in single file in front of a laser so they can be detected, counted and sorted

Light scattering at different angles can distinguish differences in size and internal complexity,
whereas light emitted from fluorescently labeled antibodies can identify a wide array of cell surface and cytoplasmic antigens



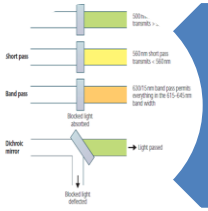
detection and enumeration of specific cell types in complex mixtures of cells, such as whole peripheral blood

How the flow cytometer works



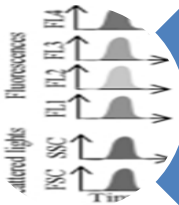
Fluidic system:

Cells pass one by one through one or more laser beam in a flow cytometer….



Optic system:

…photodiode and photomultiplier convert and amplify light signals (photons) to an electric signals…

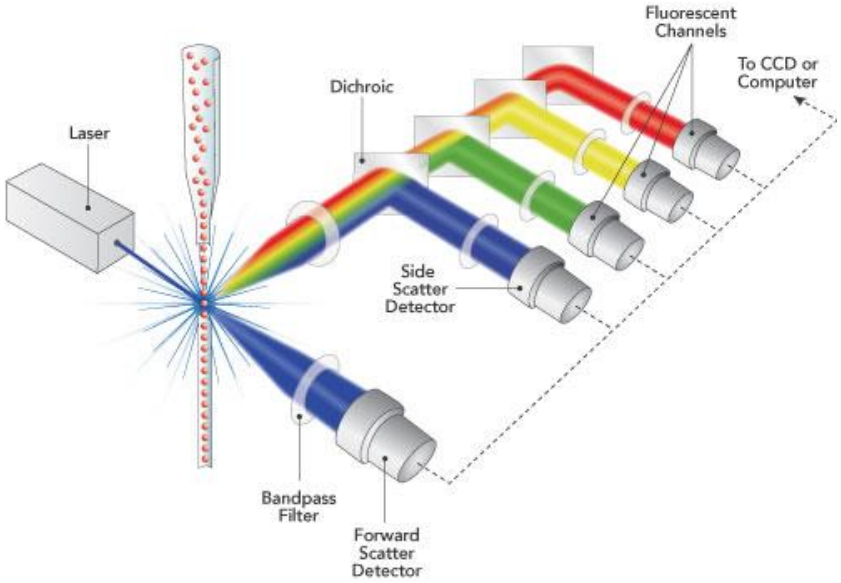


Electronic system

…this pulse is digitalized, recorded to be analyzed …

Data analysis

…with specific computer software



Advantages

Flow cytometry provides **rapid** analysis of **multiple characteristics** of single cells

Acquisition of **high rate of events** (>10^6 cells)

Polychromatic flow cytometry

Increasing the number of markers that distinguish rare cells can lead to enhanced assay sensitivity and specificity

Gating strategy

To maximize detection of the rare event, more than one fluorescence parameter should be used to positively identify the cell of interest (compound gating). Likewise, there should be at least one fluorescence parameter for which the rare event is negative (negative gating)

Rare-cell populations

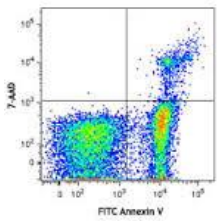
stem cells, circulating endothelial cells, circulating tumor cells, and residual disease cells

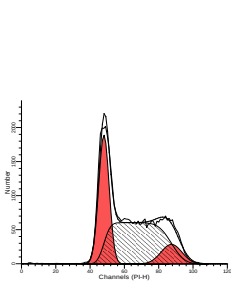
The information obtained is both **qualitative and quantitative**

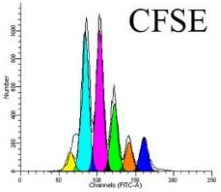
Possibility to physically separate sub-populations (**cell sorting**)

Measurable parameters

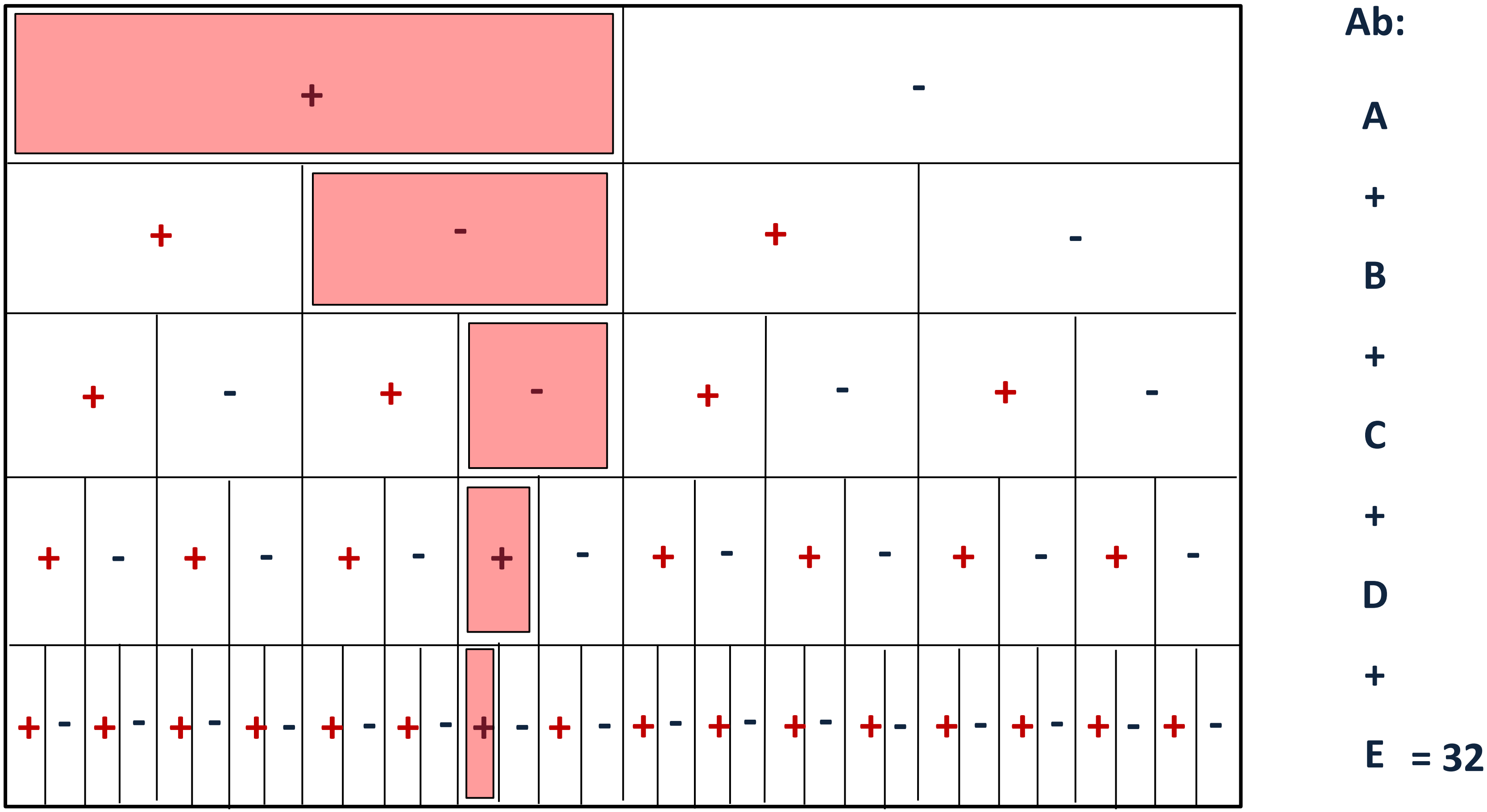
Cell surface antigens (CD markers)
DNA (cell cycle analysis, cell kinetics, proliferation etc.)
RNA
Chromosome analysis and sorting (library construction, chromosome paint)
Proteins
Intracellular antigens (cytokines, secondary mediators etc.)
Nuclear antigens
Enzymatic activity
pH, intracellular ionized calcium, magnesium, membrane potential
Membrane fluidity
Apoptosis (measurement of DNA degradation, mitochondrial membrane potential, permeability changes, caspase activity)
Cell viability
Monitoring electropermeabilization of cells
Oxidative burst
Characterising multi-drug resistance (MDR) in cancer cells
Cellular pigments (Chlorophyll or phycoerythrin)







MULTIPARAMETRIC Immunophenotyping

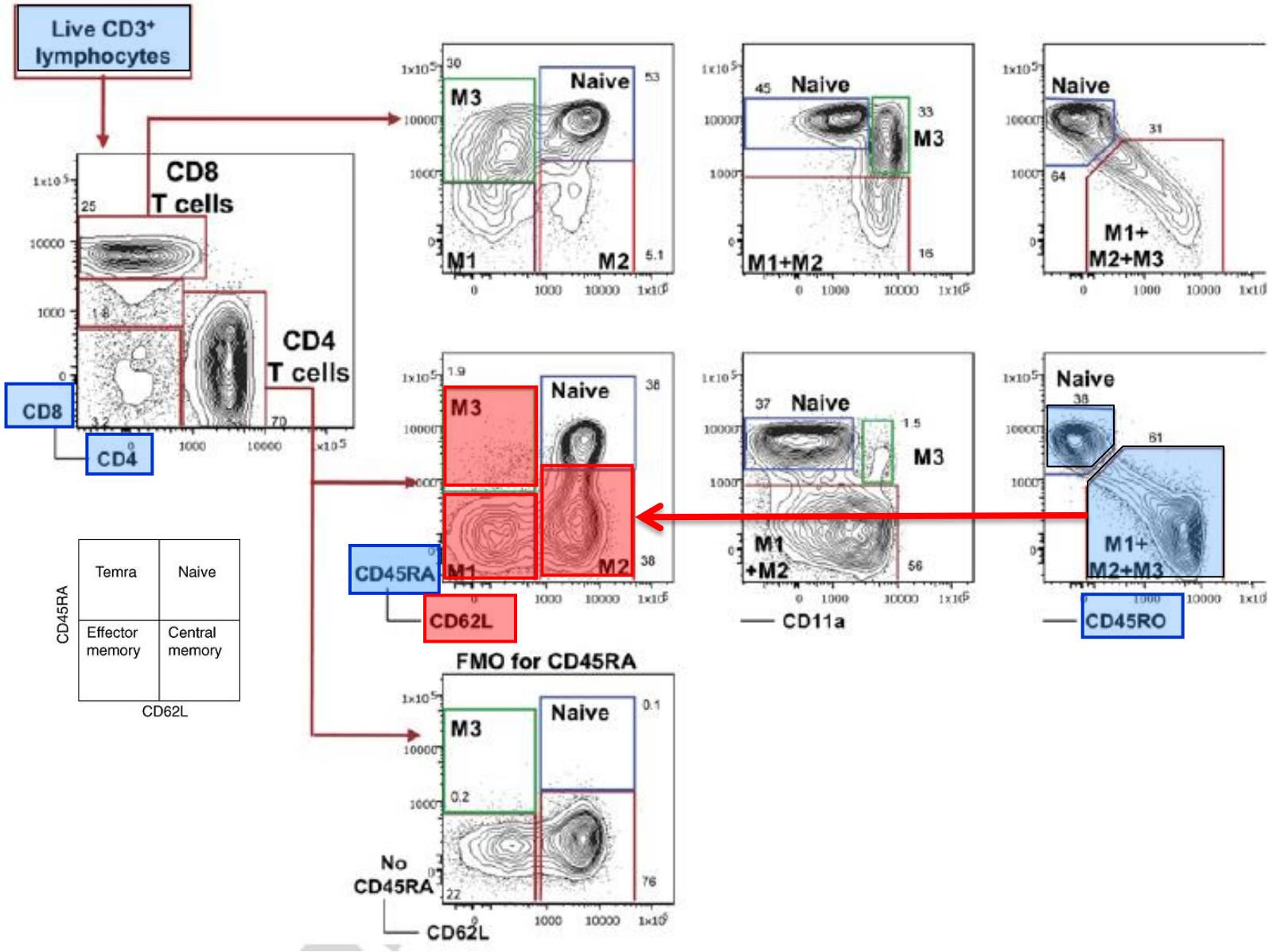


phenotype: A+B-C-D+E+

a^n

a = 2 n = number of antibodies

MULTIPARAMETRIC Immunophenotyping



Gating strategy to detect rare population

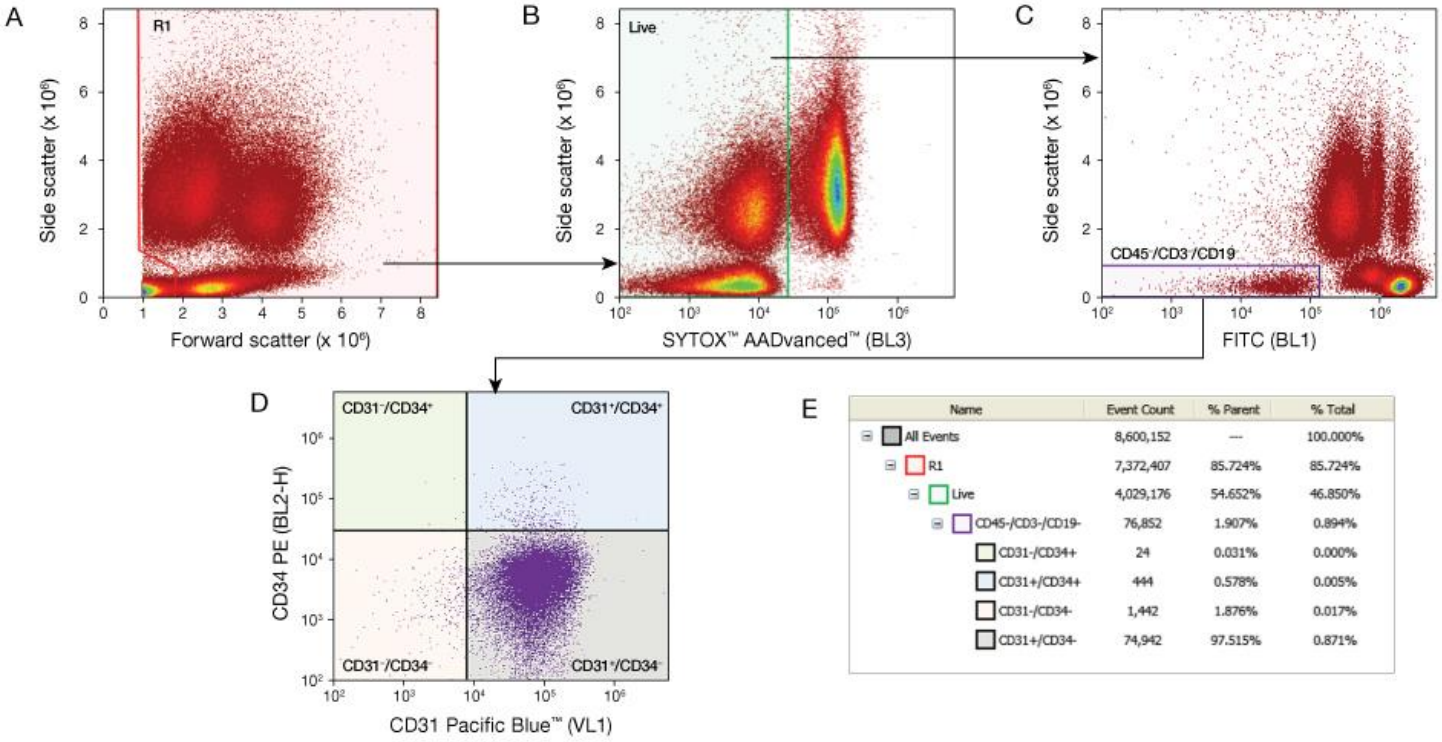
Multiparameter flow cytometry (FCM) is ideal for rapid processing of high numbers of cells per second and is commonly utilized to quantify CECs and EPCs

CECs and EPCs are rare in healty subjects and increase in different tumor

Tool for monitoring clinical outcome and tumor treatment (prognostic marker in specific tumor types)

exclusion of debris and dead cells

Gating the negative population



FITC dump channel
Since CECs are negative for all three of these markers, all positive cells can be eliminated from further analysis using only one fluorescence channel

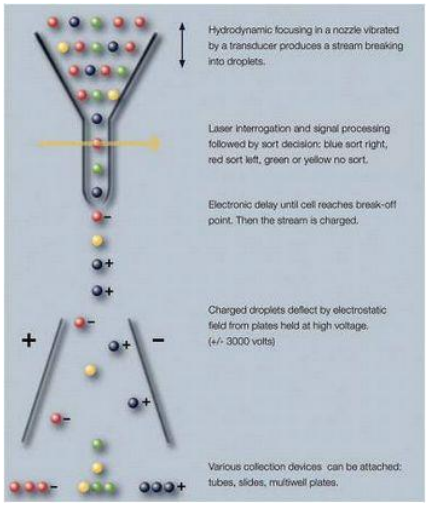
Widemann A, Sabatier F, Arnaud L et al. (2008) J Thromb Haemost 6:869–876.
Roodhart JM, Langenberg MH, Vermaat JS et al. (2010) Neoplasia 12:87–94.
Khan SS, Solomon MA, McCoy JP Jr (2005) Cytometry B Clin Cytom 64:1–8.

Danova et al. (2016) MOLECULAR AND CLINICAL ONCOLOGY 4: 909-917

Mancuso P et al. (2014) PLoS One 9(12):e114713

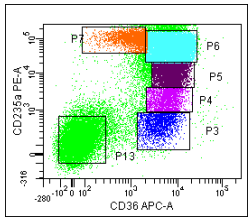
Cell sorting

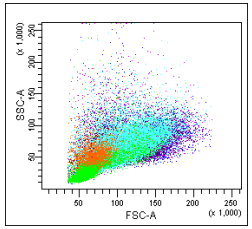
Cell sorting principle



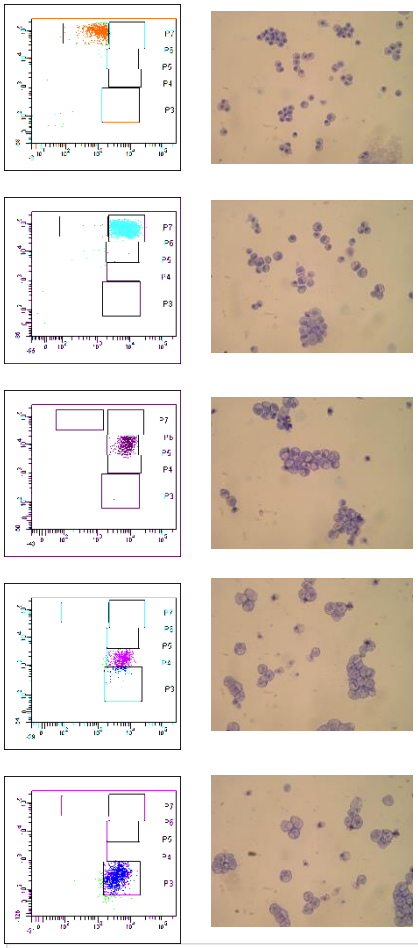
BD FACS Aria I

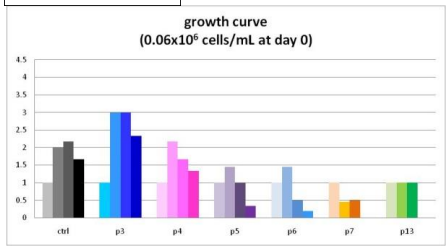
Day 10





Sorted populations
Purity >95%

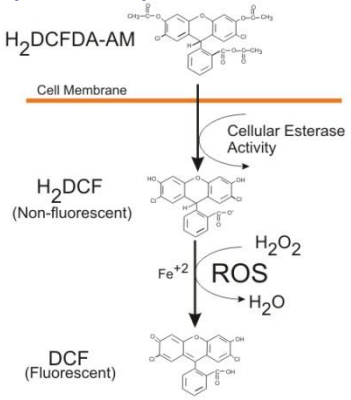




ROS detection

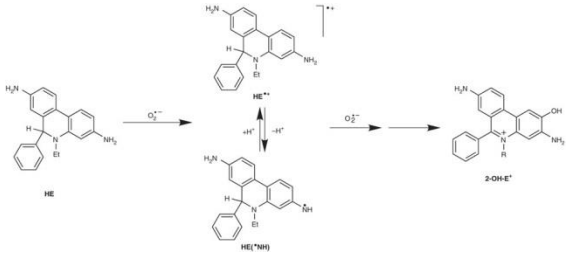
Fluorescent dyes used frequently for measuring hydrogen peroxide, superoxide, and peroxynitrite in biological systems.

Dichlorodihydrofluorescein (DCFH-DA)



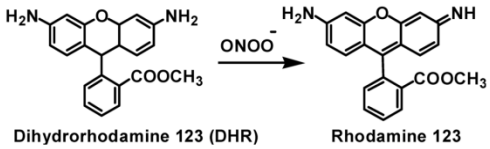
Free Radic Biol Med. 2012 Jan 1; 52(1): 1–6.

Hydroethidine (HE) and Mito-SOX or Mito-HE

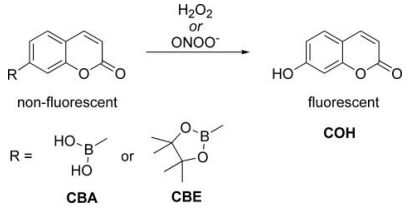


Nature Protocols 3, - 8 - 21 (2008)

dihydrorhodamine (DHR)



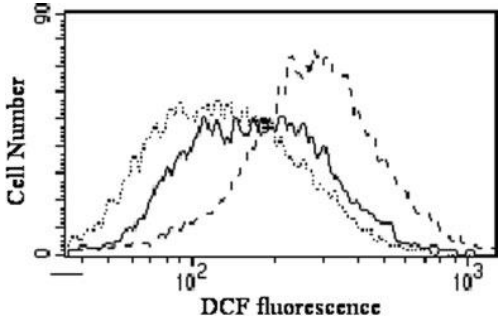
Boronate-containing fluorophores (e.g., coumarin boronate)



J Biol Chem. 2010 May 7; 285(19): 14210–14216.

The generation of reactive oxygen and nitrogen species has been implicated in the onset and progression of several diseases (e.g., atherosclerosis, cancer, diabetes, neurodegeneration)

Representative histograms showing ROS levels in peripheral blood mononuclear cells of euthyroid (——), hypothyroid ( ), and hyperthyroid patients (- - - -).



Cytometry Part B (Clinical Cytometry) 70B:20–23 (2005)

Proliferation assay

Table 1: Some Common Cell Proliferation Dyes

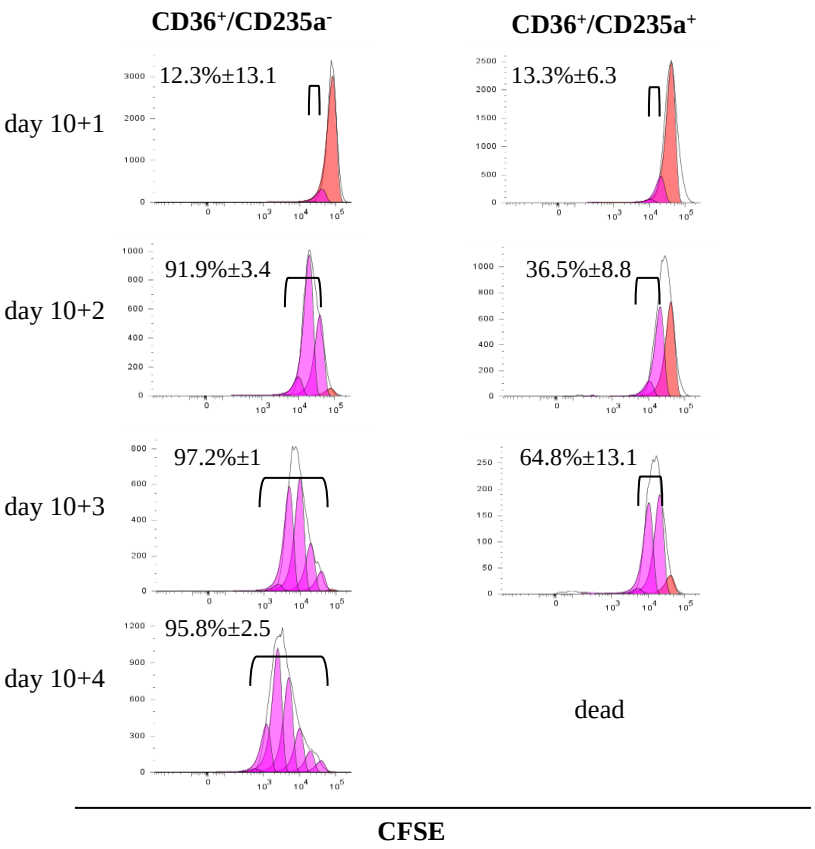
Dye	Cellular Location	Excitation Laser	Emission Peak
CFSE	intracellular	488 nm	~519 nm
CellTrace Violet	intracellular	405 nm	~455 nm
eFluor670 proliferation dye	intracellular	633 nm	~670nm
PKH26	membrane	488, 532, 561 nm	~567 nm
PKH67	membrane	488 nm	~502 nm
CellVue Claret	membrane	633 nm	~675 nm

Possible applications:

- Proliferation of immune cells in response to stimulation
- Self-renewal of stem cells
- Biological homeostasis
- Tumor cell proliferation

Cell proliferation assays are widely used in cell biology to measure cellular metabolic activity in response to stimuli such as growth factors, cytokines and other media components. The development of new treatments for cancer, for example, target the proliferation of the cancer cells,

Proliferation assay

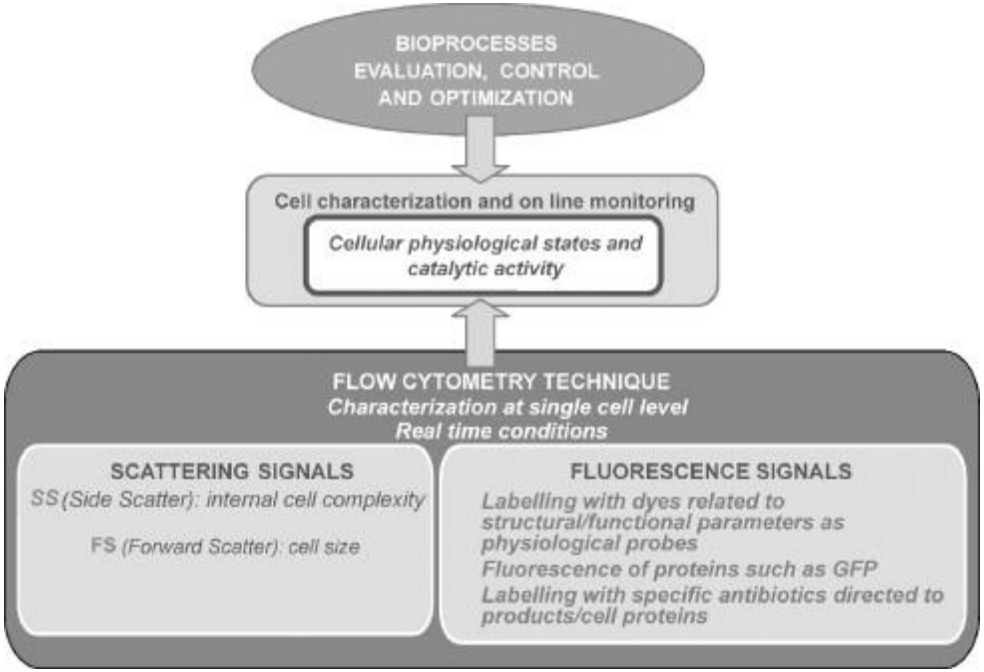


	Day			
	10+1	**10+2**	**10+3**	**10+4**
Proliferation index				
iEBs	1.02±0.01	1.53±0.03	2.35±0.07	2.76±0.21
mEBs	1.07±0.04	1.12±0.07	1.41±0.34	b.d.
Division index				
iEBs	0.04±0.01	1.32±0.08	2.25±0.07	2.65±0.27
mEBs	0.14±0.07	0.4±0.08	0.8±0.11	b.d.

Table II. Proliferation and Division indexes values of 10 days sorted iEBs and mEBs populations in Hema culture conditions

The Percent Divided – the measure of the percent of the input cells that entered division.
The Division Index – a measure of the average number of divisions which includes the undivided cells.
The Proliferation Index – the average number of divisions that exclude the undivided cells.

Application of flow cytometry to industrial microbial bioprocesses



more efficient and less time-consuming methods to assess the presence of microorganisms, as well as their viability for bioprocesses control and improvement.

Rapid detection of microorganism in sample → More accurate quality control
Optimization of bioprocess

Industrial microbial bioprocesses application:
dairy industry, beverages industry, environmental and water system

Biochemical Engineering Journal, Volume 48, Issue 3, 2010, 385–407

Application of flow cytometry to industrial microbial bioprocesses

Industrial microbial bioprocesses application: dairy industry, beverages industry, environmental and water system

Table 5
Some FC applications to water and environmental microbiology.

Application	Microorganism	Functional or structural parameter	Reference
Characterization of freshwater and marine waters	*Bacterial cells*	DNA content, Membrane integrity and esterase activity	[16]
Cell functionality after peracetic acid treatments	*S. Typhimurium*	DNA	[43]
Cell enumeration in marine and ballast water samples	*Escherichia coli* and *Bacillus cereus*	Nucleic acid content	[92]
Characterization of activated sludge	*Comamonas testoteroni, Paracoccus pantotrophus, Escherichia coli*	Respiratory activity	[109]
Enumeration of viable cells in water after chlorination	*Legionella pneumophila*	Esterase activity	[120]
Cell survival in seawater during starvation	*Escherichia coli* and *S. Typhimurium*	Membrane integrity and potential	[134]
Effectiveness of disinfection treatments	*Escherichia coli, Salmonella Typhimurium, Shigella flexneri, Enterococcus faecalis*	Membrane integrity and potential, pump activity	[138,151]
Estimation of total bacteria in live and fixed samples from fresh and saline waters	*Salmonella typhimurium*	Nucleic acids	[252]
Cell characterization in tropical marine environmets	Bacterioplankton	DNA	[257]
Assessment of marine bacterial death	Natural bacterioplankton	Nucleic acids staining	[258]
Cell viability of in different types of water	*Aeromonas hydrophila*	DNA content and membrane integrity	[259]
Detection and viability assessment of water pathogens	*Giardia lamblia*	Fluorescent antibodies and membrane integrity	[260]
Chlorination treatment	*Nitrosospira* spp.	Membrane integrity and esterase activity	[262,263]
Evaluation of different environmental conditions and disinfection processes on VBNC cells in water	*Legionella pneumophila*	Esterase activity	[264]
Drinking water treatment processes	Heterotrophic bacteria	Nucleic acids detection	[266]
Determination of assimilable organic carbon in drinking water	*Pseudomonas fluorescens*	Nucleic acids	[267]
Assessment of water quality in papermaking	*Bacterial cells*	DNA content and membrane integrity	[268]

Biochemical Engineering Journal, Volume 48, Issue 3, 2010, 385–407

FCM in drinking water

The Federal Council

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Drinking water unexpectedly rich in microbial life

Dübendorf, 24.01.2013 - Flow cytometry (FCM) can now be officially used for the quantification of microbial cells in drinking water. The new analytical method – developed at Eawag and extensively tested both in Switzerland and abroad – has been incorporated into the Swiss Food Compendium (SLMB) by the Federal Office of Public Health (FOPH). FCM provides much more realistic results than the conventional method, in which bacterial colonies are grown on agar plates. The results demonstrate that even good-quality drinking water harbours 100 to 10,000 times more living cells than the conventional plate count method would suggest.

Real time quality programs could lead to substantial industrial cost reductions

FCM in Aquatic microbiology

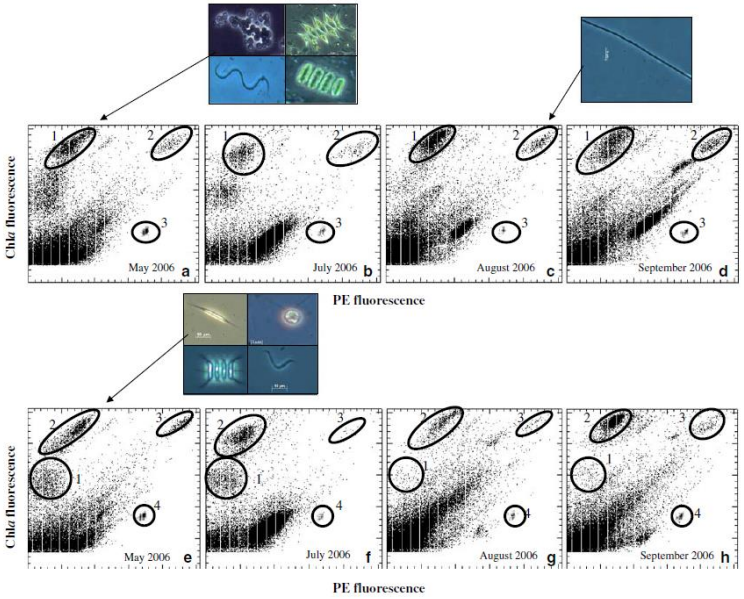


Fig. 2 PE vs. Chl*a* fluorescence cytograms showing the seasonal evolution of the phytoplankton composition (**a–d**) for two clusters isolated from station 501 of the Reservoir Marne (*1* mixed green algae community, *2 Pseudanabaena limnetica* (picture of a filament), *3* beads) and **e–h** for three clusters isolated from station 553 of the Reservoir Marne (*1 Chlorella vulgaris*, *2* mixed green algae community, *3* mixed green algae community, *4* beads). Pictures of the mixed green algae communities with *Chlorella vulgaris*, *Scenedesmus acuminatus*, *Hyaloraphidium contortum*, *S. linearis* (upper left, from up left to right down) and with *Ankyra lanceolata*, *Chlamydomonas* sp., *Scenedesmus spinosus*, *Monoraphidium* sp. (middle, from up left to right down)

Cellammare M., et al. (2009)J Appl Phycol

Naturally fluorescence pigments in phytoplankton

Fluorophore	Exciting Laser	Major Emission Wavelength
Chlorophyll *a,b*	488	>640 nm
Phycoerythrin	488	575 nm
C-phycocyanin	640	650 nm
R-phycocyanin	640	646 nm
Allophycocyanin	640	660 nm

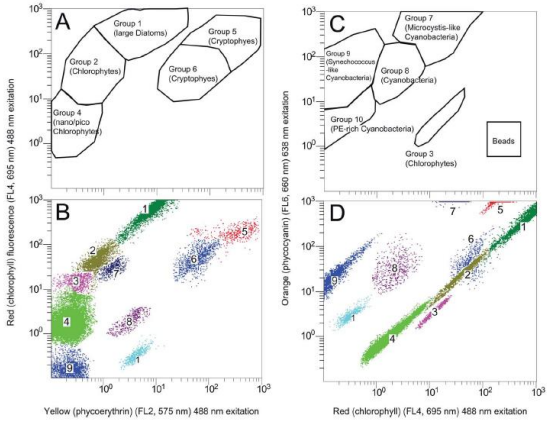
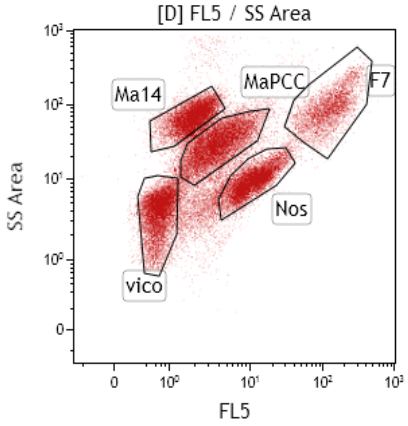


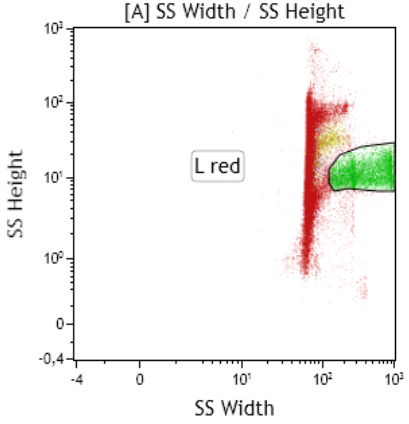
Fig. 1 Flow cytometry panels showing gating profiles for phycoerythrin (FL2 – 575 nm) against chlorophyll (FL4 – 695 nm) fluorescence (a) and chlorophyll (FL4 – 695 nm) against phycocyanin (FL6 – 660 nm) (c). Composite plots (data from different samples consolidated) showing example data for phytoplankton groups from the River Thames at Wallingford, UK (b and d).

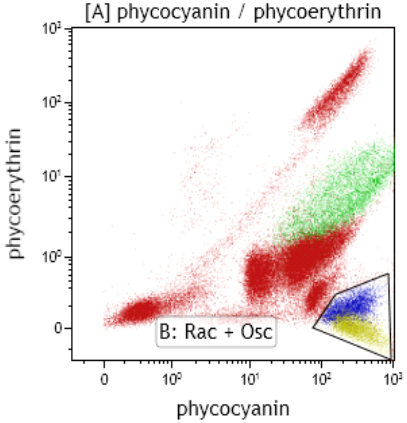
Environ. Sci.: Processes Impacts, 2014, 16, 594-603

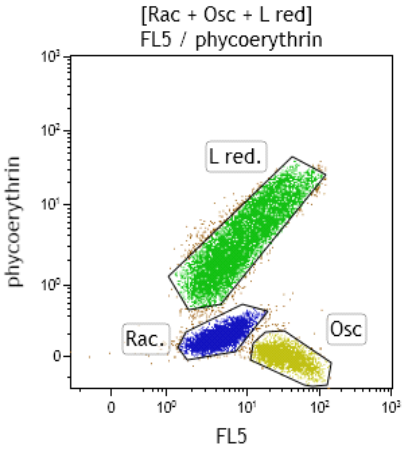
FCM in Aquatic microbiology

Merge campioni acquisiti

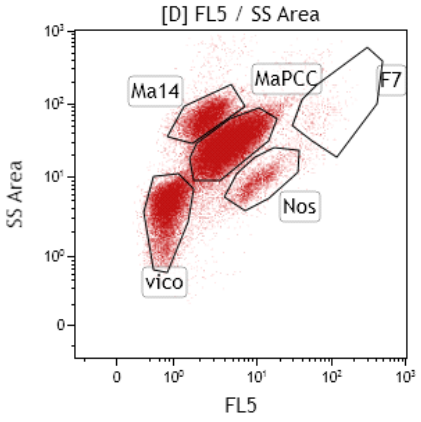


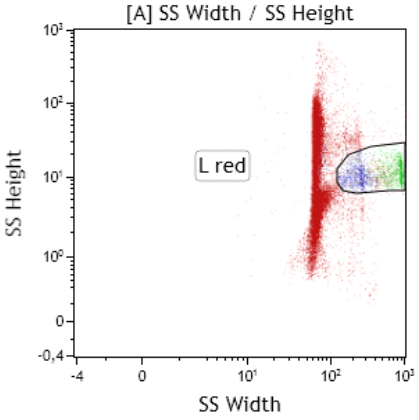


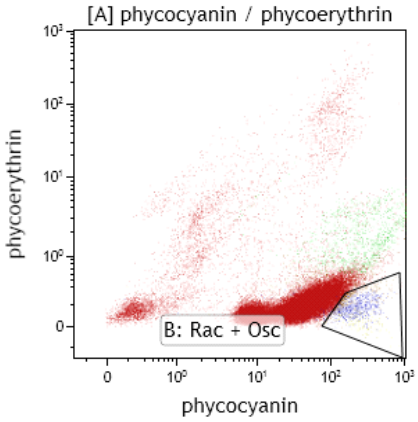


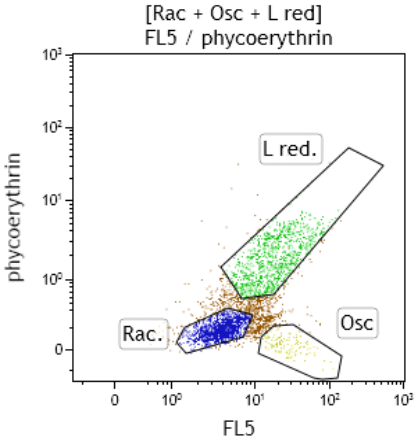


Mix colture









Multicolor flow cytometry: choice of fluorochromes

Multicolor flow cytometry rapidly reveals a large amount of biological information from a single sample.
Over the past few years, the number of parameters (and consequently colors) simultaneously analyzed in typical flow cytometry experiments has increased

One consideration when performing multicolor fluorescence studies is the possibility of **spectral overlap between fluorophores** and when making decisions about which fluorochromes to use in your experiments, you'll want to know their relative **emission spectra**

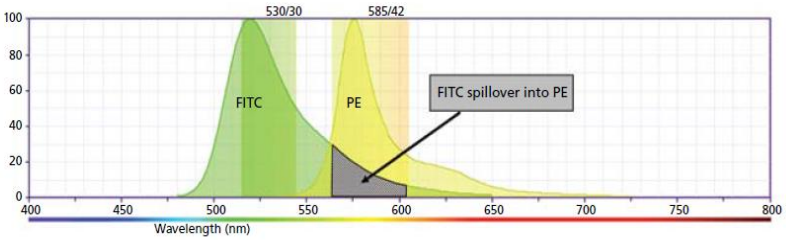
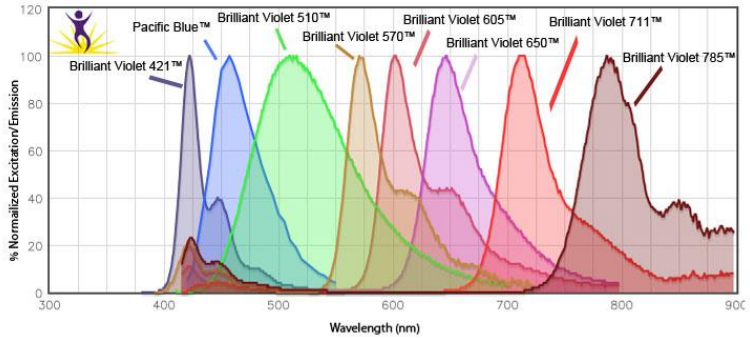


Figure 1. Example of FITC spillover into the PE channel.

need for compensation

Alternatively you can combine fluorophores that can only be activated by specific individual laser lines, (providing the lasers are spatially separated), but as you increase the number of fluorophores this becomes very difficult



Brightness of various fluorochrome conjugates

Relative Brightness	Reagent	Filter
BRIGHTEST	Brilliant Violet™ 421	450/50
	PE	575/26
	Brilliant Violet 605	610/20
	BD Horizon PE-CF594	610/20
	PE-Cy5	670/14
	APC	660/20
BRIGHT	PE-Cy7	780/60
	Alexa Fluor® 647	660/20
	PerCP-Cy5.5	695/40
MODERATE	Alexa Fluor® 488	530/30
	FITC	530/30
	BD Horizon V450	450/50
	Pacific Blue™	450/50
DIM	Alexa Fluor® 700	730/45
	PerCP	695/40
	APC-Cy7	780/60
	AmCyan	525/20
	BD Horizon V500	525/20
	BD APC-H7	780/60

Citometria di Massa: princìpi ed applicazioni

Luca Pasquini

Citometria a flusso

Spettrometria di Massa

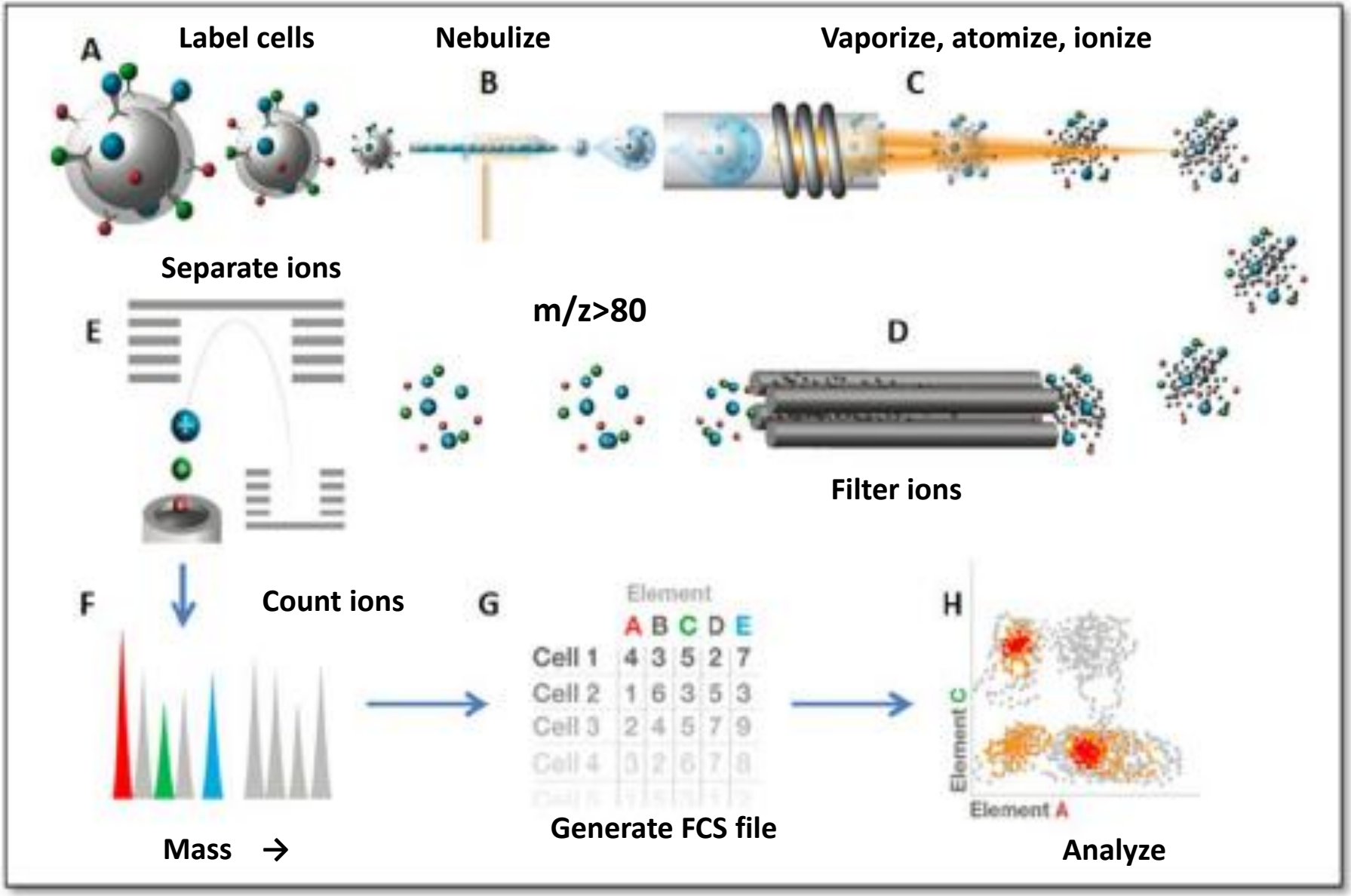
Anticorpi coniugati (con metalli rari)

Risoluzione TOF

Citometria di Massa (CyTOF)

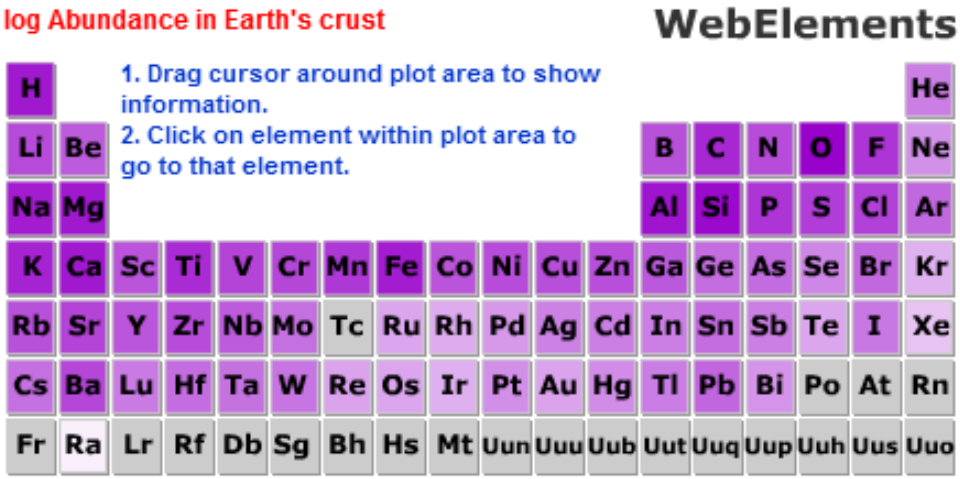
Notevole incremento del numero di parametri valutabili!!!

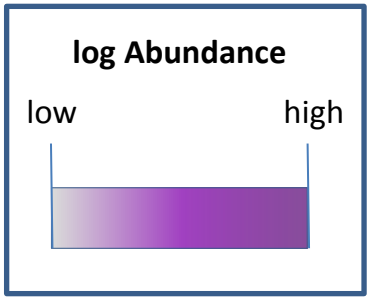
Citometria di Massa (workflow)

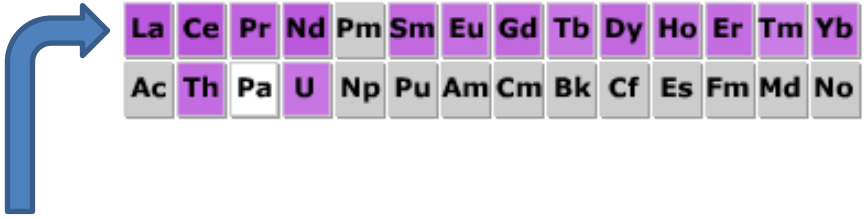


Ref. Fluidigm manuals

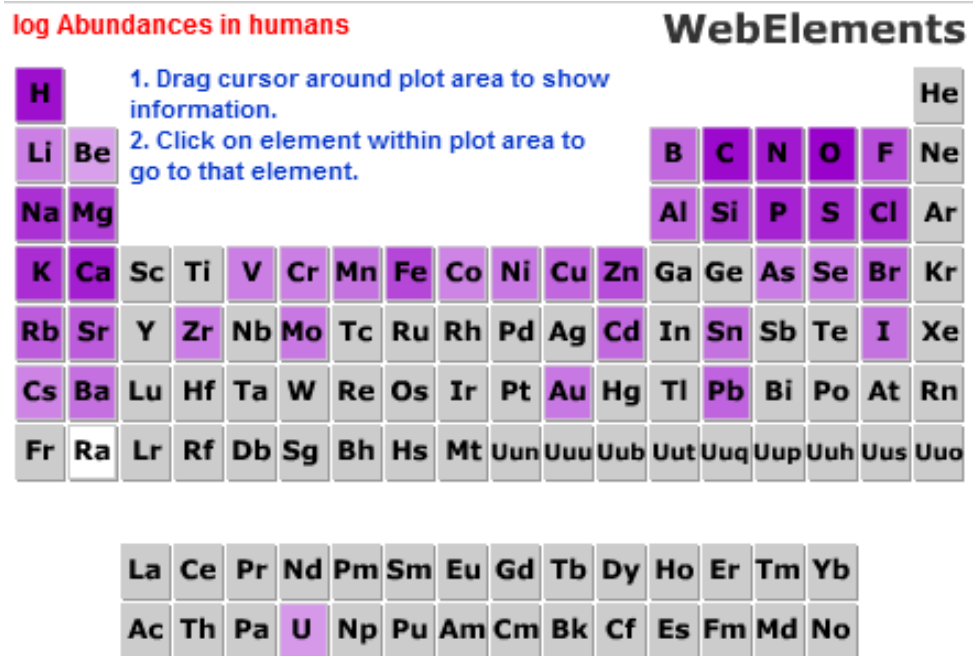
Lantanidi







LANTANIDI



Identificazione degli eventi

Mass detection

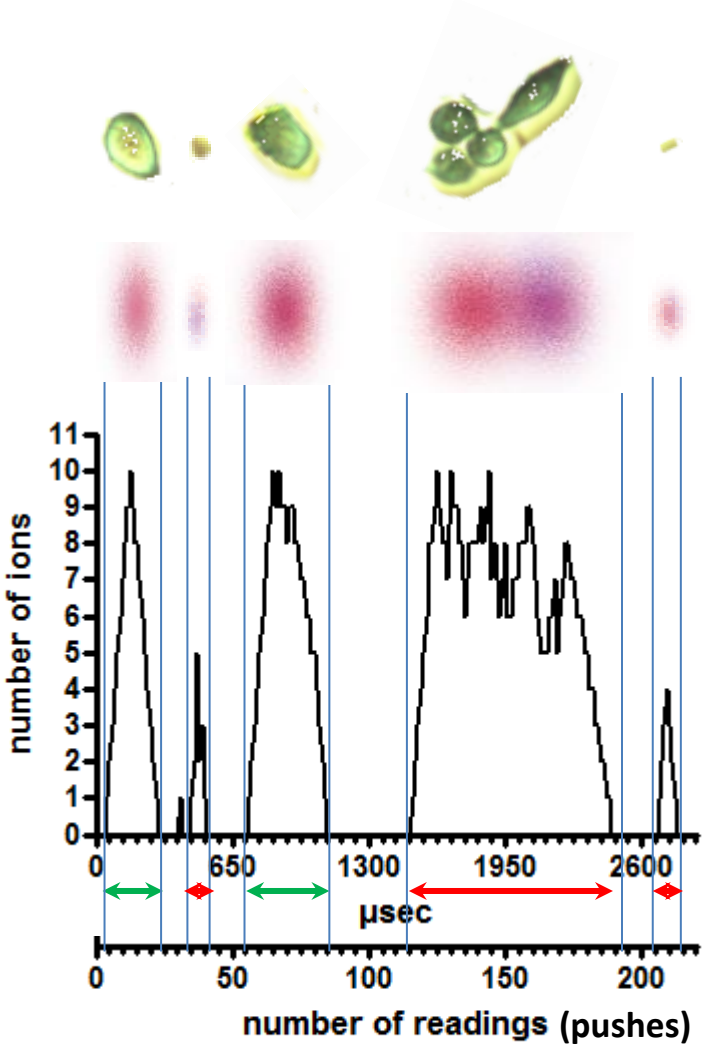
(76800 spectra/s)

↓

1 spectra/13µs = 1 push

Cutoff (cell event):

10-75 pushes



Signal digitization

- 103-193 m/z
- 1ns resolution

Dr. Biffoni M.

Identificazione degli eventi

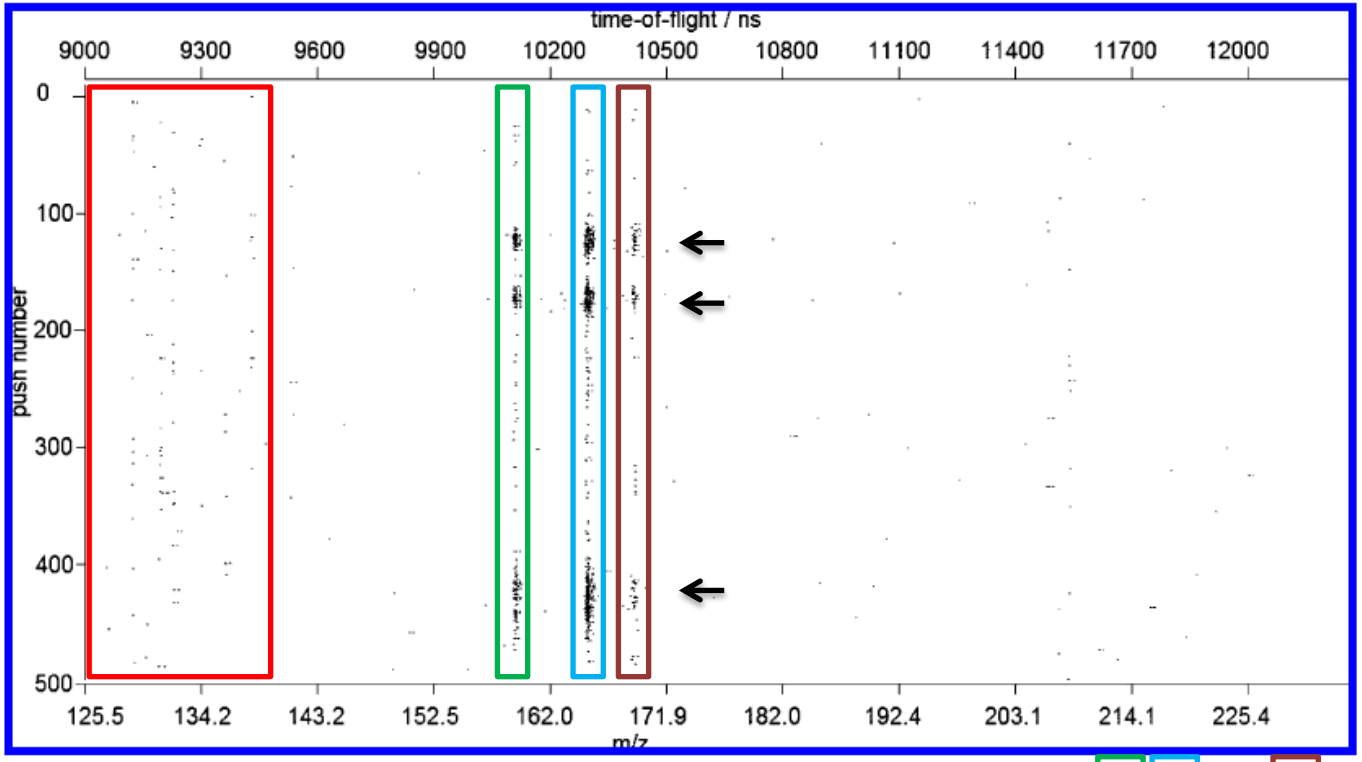
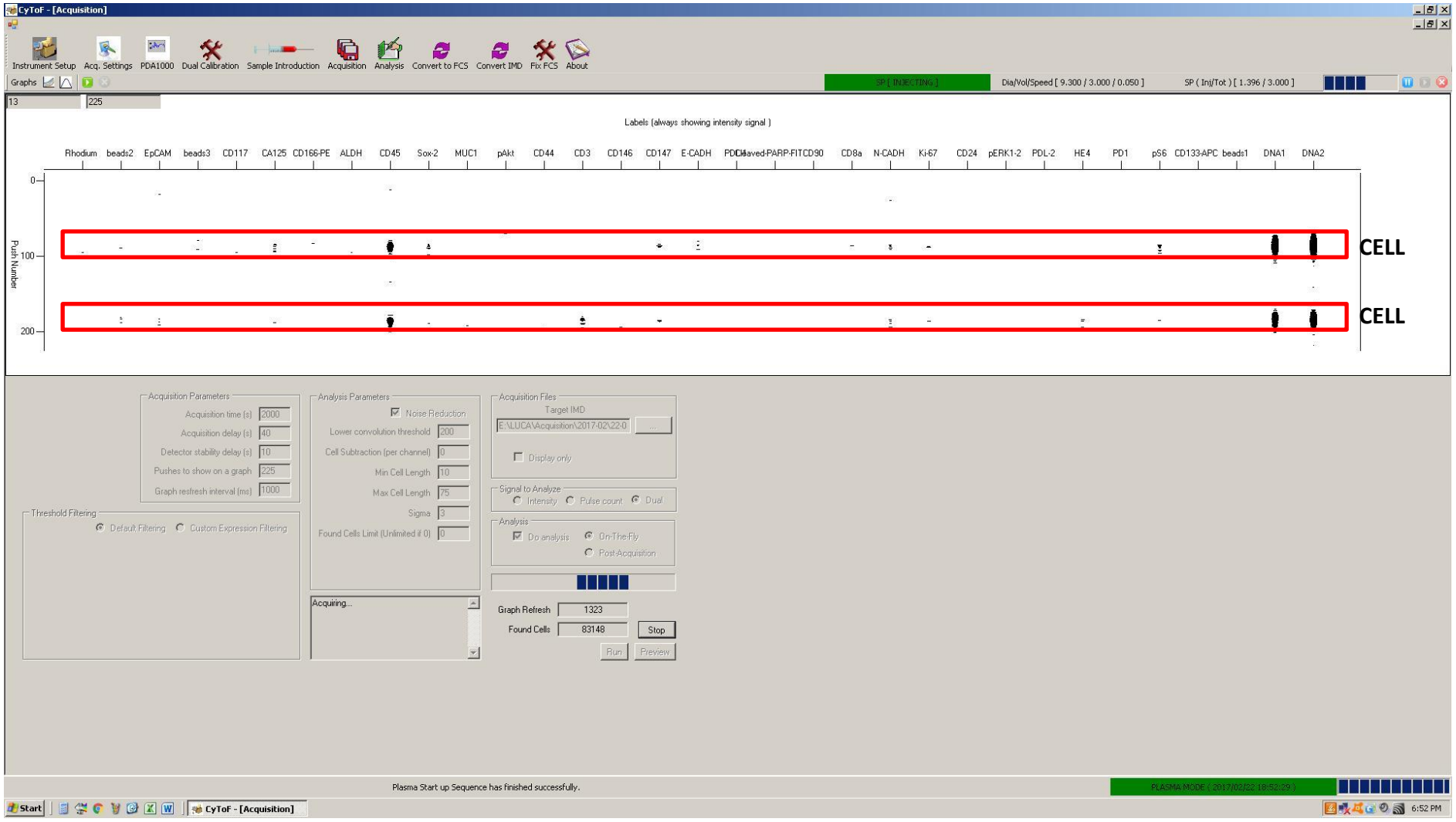


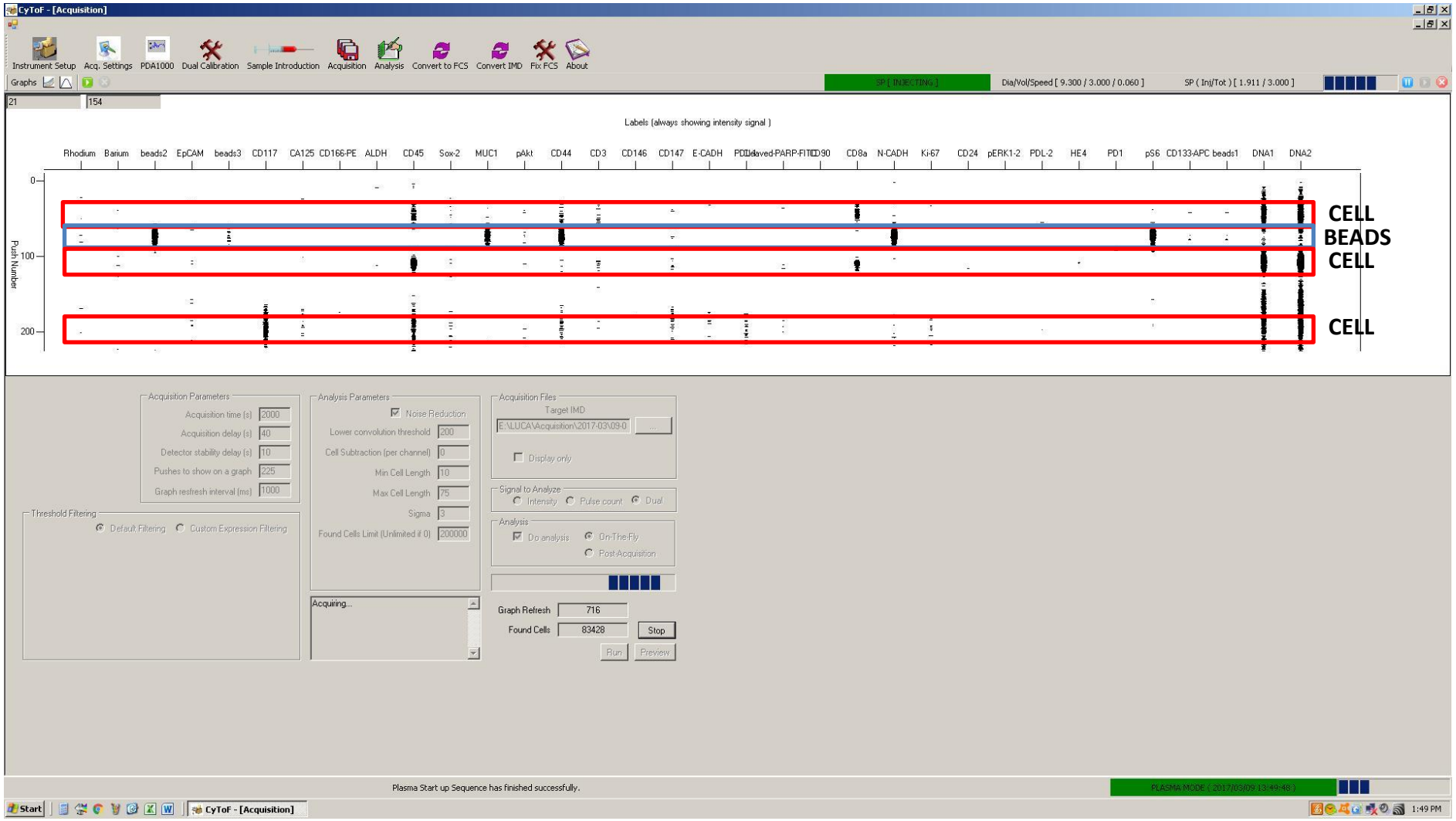
Figure 4. Screenshot of 500 consecutive spectra (total of 6.5 ms) for a sample of beads containing Tb, Ho, and Tm at 1:2:1 concentration.

Modified from: Analytical Chemistry, Vol. 81, No. 16, August 15, 2009

Identificazione degli eventi (densità del campione)



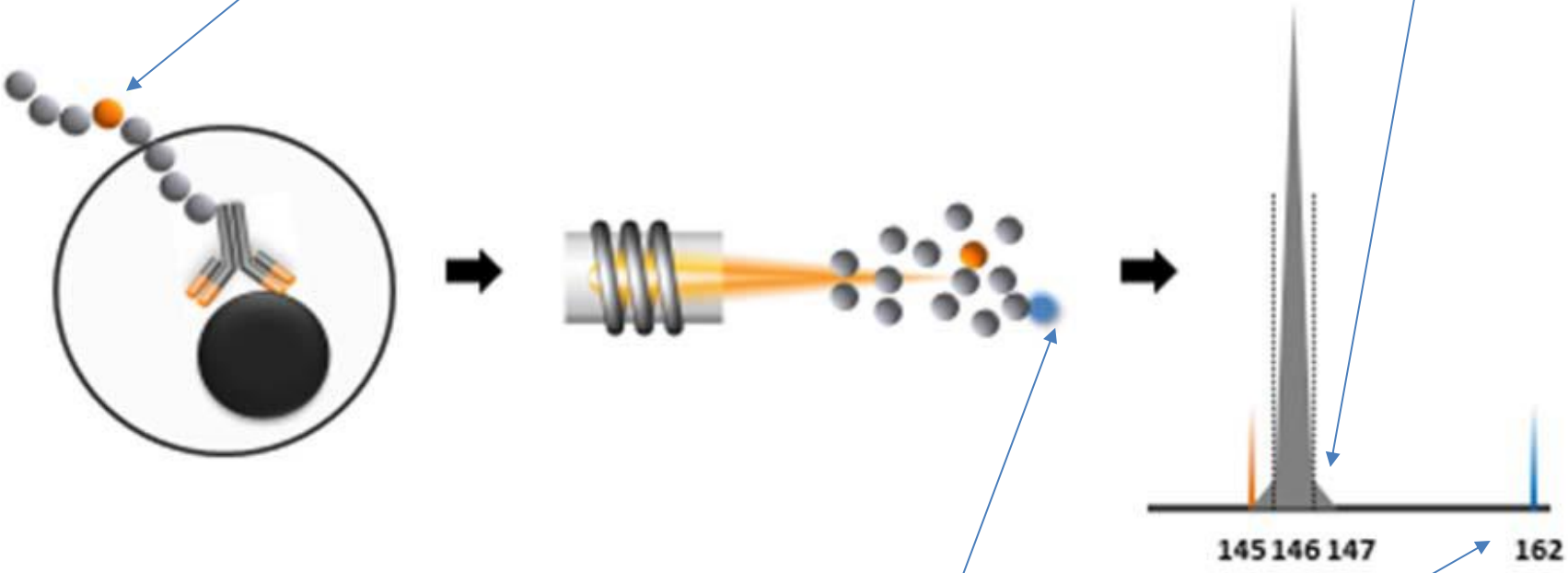
Identificazione degli eventi (densità del campione)



"Signal Overlap"

Purezza degli isotopi

Sensibilità dello strumento

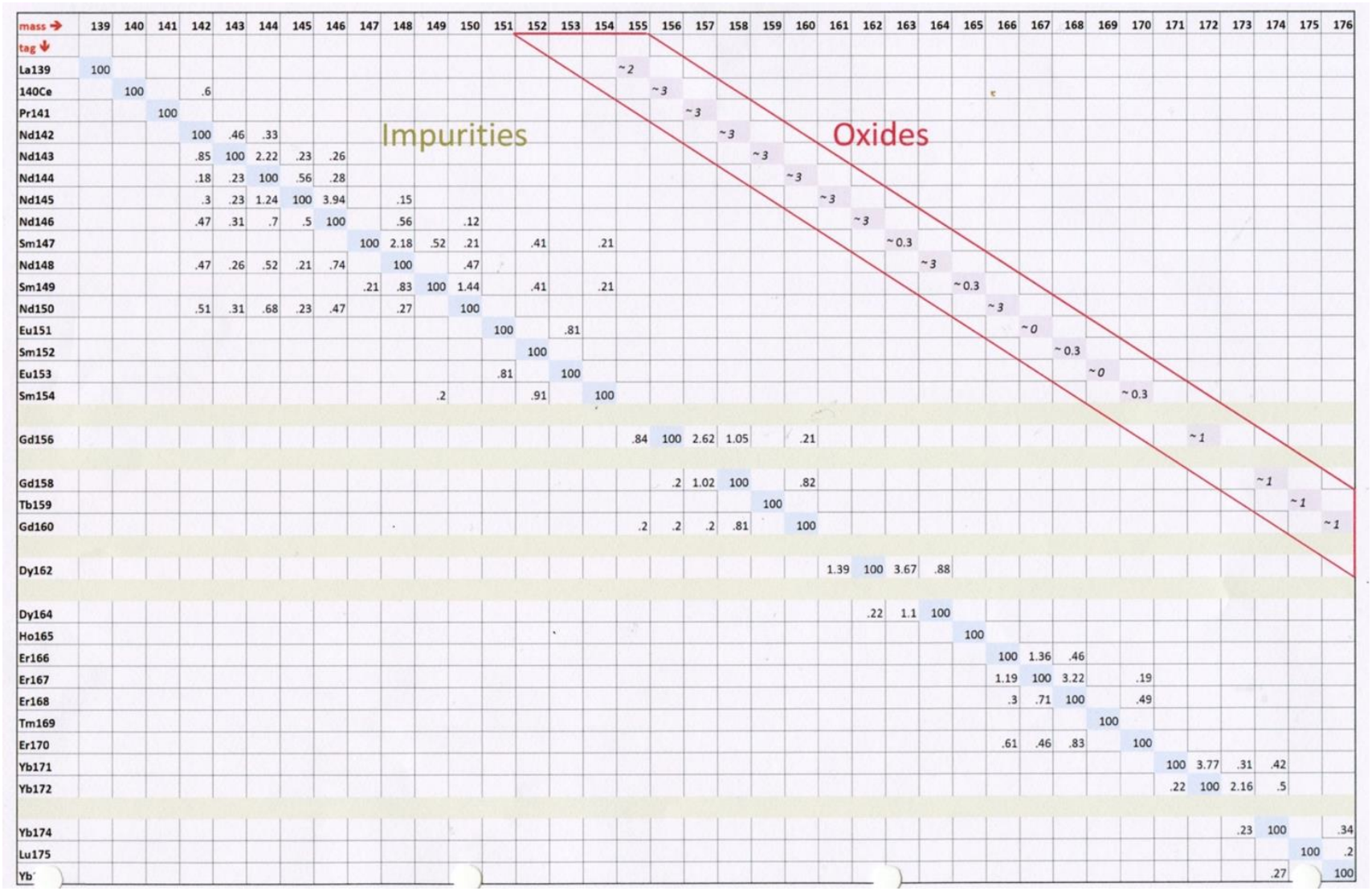


Formazione degli ossidi (M+16)

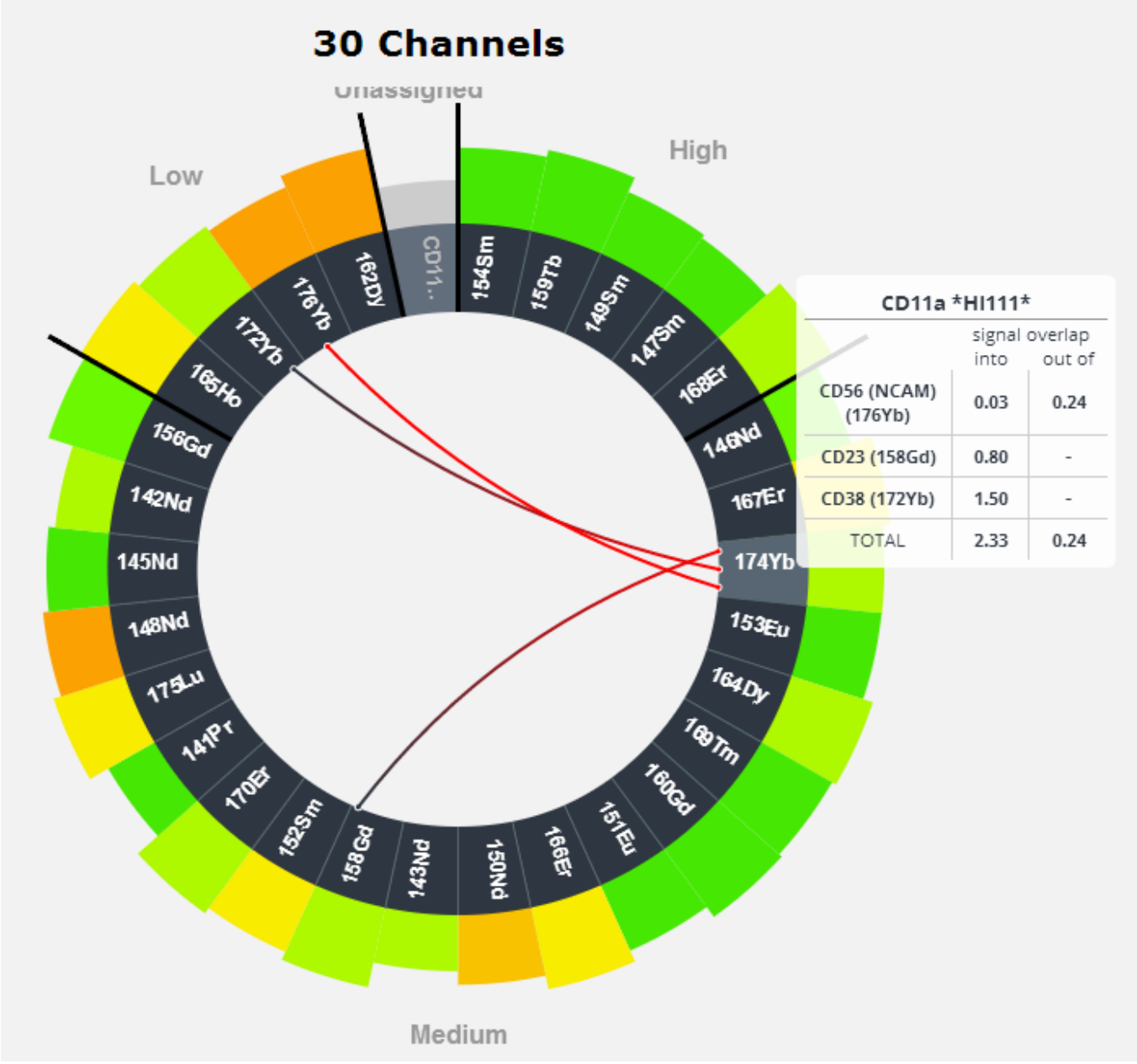
"Signal Overlap"

Mass channel

Mass tag



Signal Overlap (Panel Designer)



CD11a *HI111*

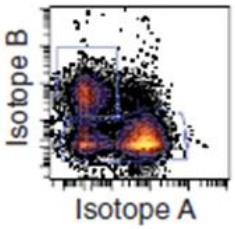
	signal overlap into	signal overlap out of
CD56 (NCAM) (176Yb)	0.03	0.24
CD23 (158Gd)	0.80	-
CD38 (172Yb)	1.50	-
TOTAL	2.33	0.24

Analisi dei dati

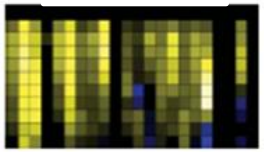
Analysis Software
or
Services (Cytobank)

.FCS files
(Mass Cytometry data)

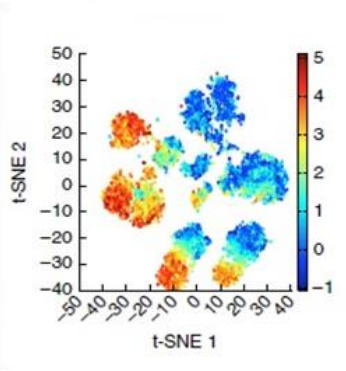
2D Plots (FlowJo)



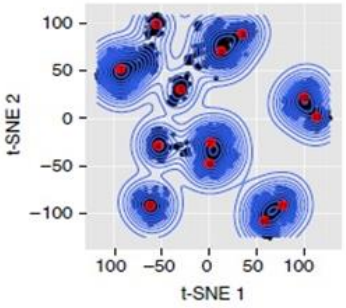
Expression &
Fold-Changes



SPADE



viSNE



ACCENSE

Citometria di massa – CyTOF

Principali Vantaggi:

- Analisi multiparametrica >35-40 parametri;
  [attualmente in ISS compresa tra i 25 e 30 parametri, Progetto HERCULES (H2020)]
- Analisi contemporanea di antigeni di superfice, intracellulari e fosfoproteine a livello di singola cellula;
- Notevole riduzione del fenomeno di sovrapposizione tra i segnali (non è richiesta compensazione);
- Il costo degli anticorpi (già coniugati con il metallo) è paragonabile a quelli per citofluorimetria;
- Mediante l'utilizzo di kit specifici di coniugazione è possibile produrre Ab-Met "*in-house*";

Limiti:

- Velocità di acquisizione molto inferiore alla citofluorimetria (~500 cell/s);
- Non è possibile effettuare "cell sorting";
- Solo il 30-40% del campione iniettato viene analizzato;

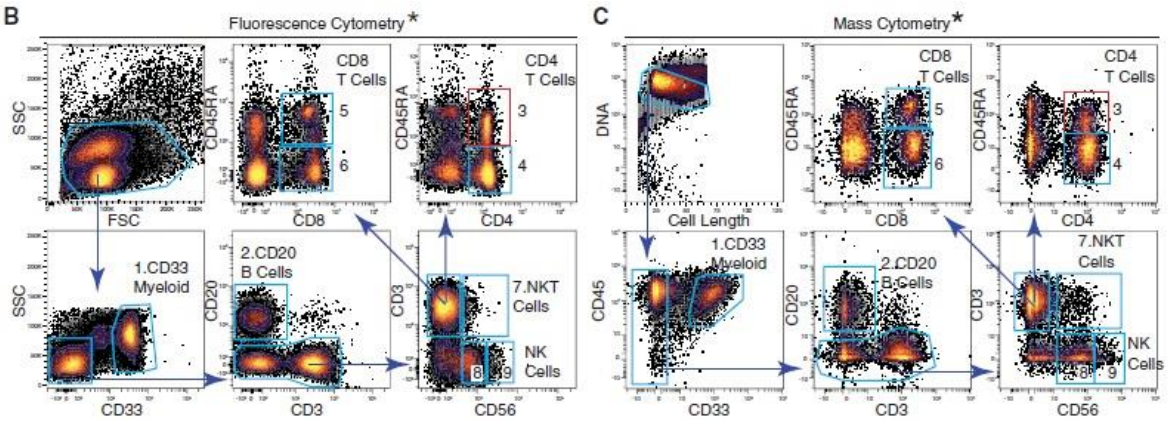
Principali Applicazioni:

- Immunofenotipo (Caratterizzazione di popolazioni cellulari e definizione del signaling a livello delle singole sottopopolazioni cellulari)
- Biomarcatori (Identificazione di biomarcatori predittivi della risposta alle terapie anche in specifiche sottopopolazioni)
- Famacodinamica (Screening farmaci ed identificazione del meccanismo di azione)

Esperimenti ben costruiti forniscono un numero enorme di informazioni!

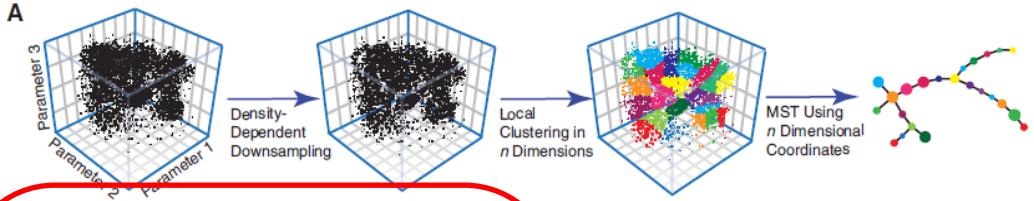
"Single-Cell Mass Cytometry of Differential Immune and Drug Responses Across a Human Hematopoietic Continuum - *Bendall SC et al. Science. 2011*"

Flow Cytometry

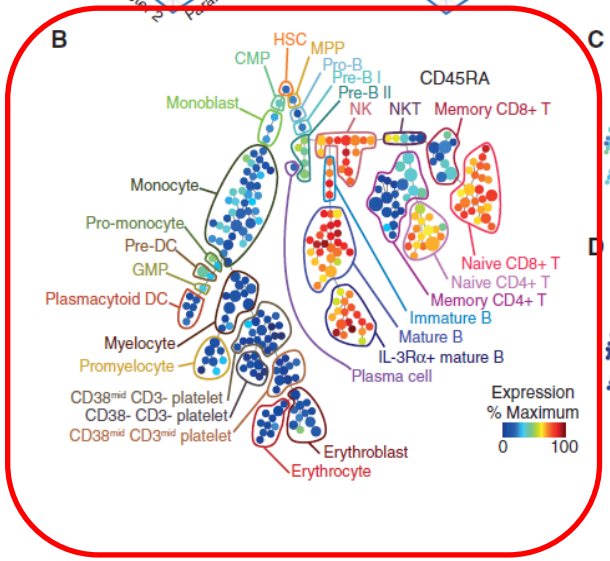


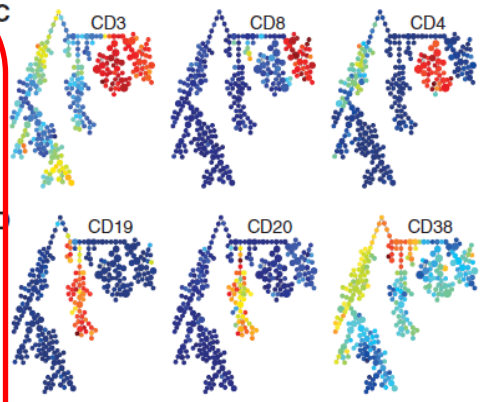
Mass Cytometry

SPADE analysis



13 marcatori
di superficie
+ 18 intracellulari
(comprese
fosfoproteine)



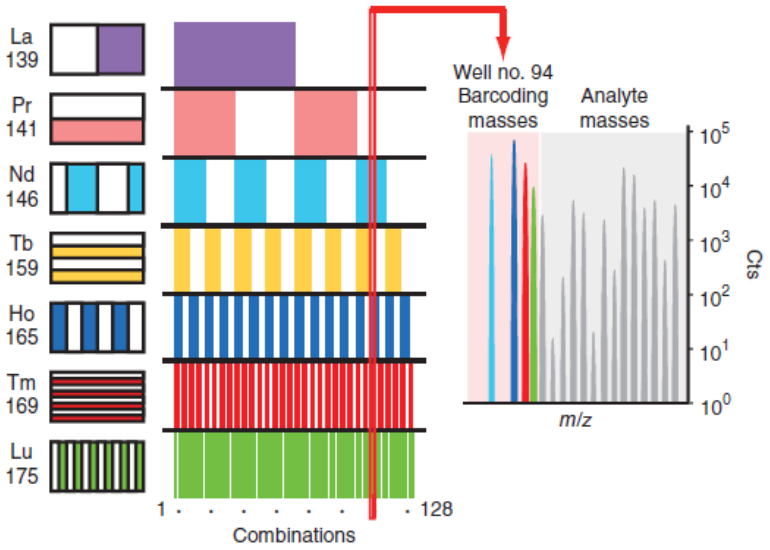


T cell
lineage

B cell
lineage

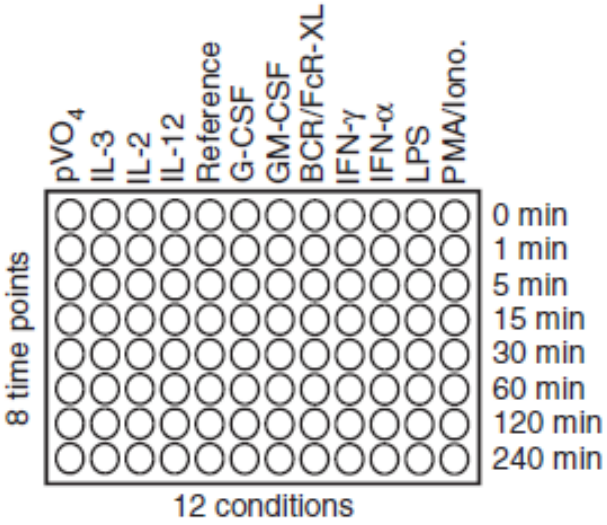
Multiplexed mass cytometry profiling of cellular states perturbed by small-molecule regulators - *Bodenmiller et al. Nature Biotechnology 30 (9) 2012*

"CELL BARCODING"



Le cellule presenti in ogni pozzetto sono etichettate con una combinazione unica di 7 Lantanidi (2^n possibili combinazioni generate)

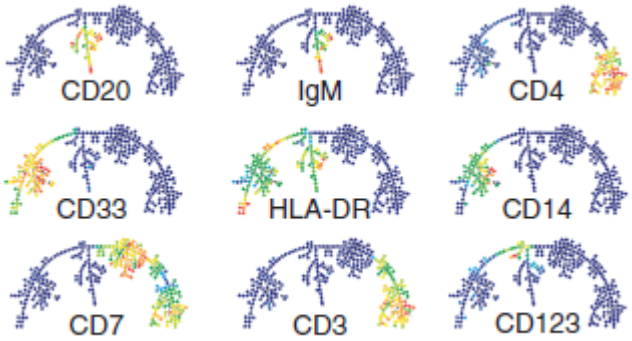
Vantaggi del Barcoding:
- Riduzione dell'errore di meccanico (pipetta)
- Variazioni nello staining
- Consumo di anticorpi
- Riduzione tempo di acquisizione



12 differenti stimoli - 8 differenti incubazioni

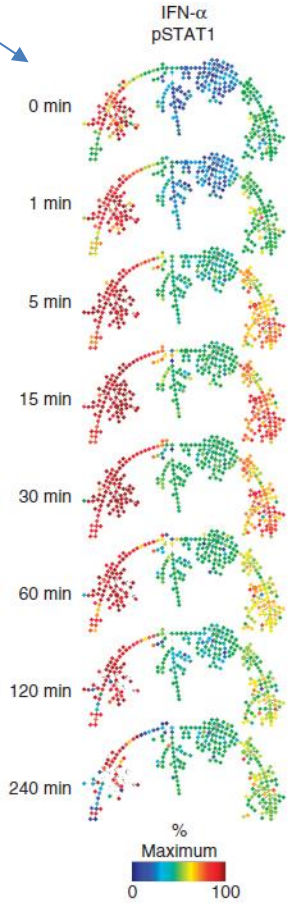
Cellule raccolte e colorate con 9 marcatori di superficie e 14 marcatori del signaling

Multiplexed mass cytometry profiling of cellular states perturbed by small-molecule regulators - *Bodenmiller et al. Nature Biotechnology 30 (9) 2012*

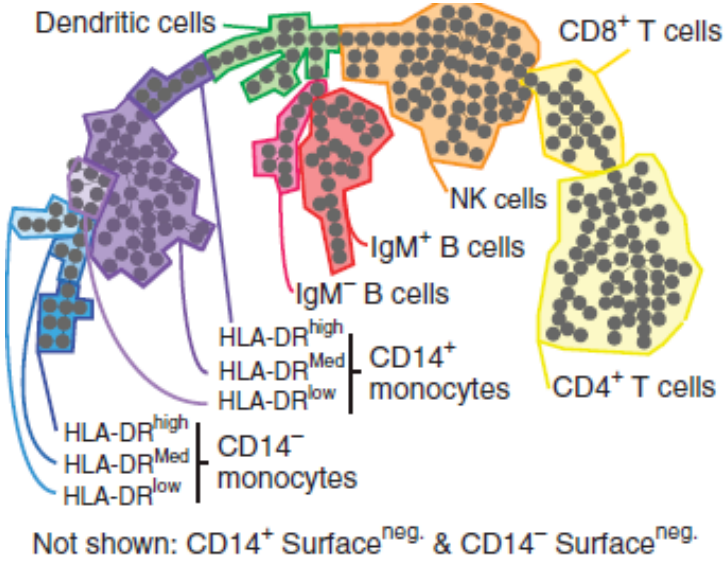


Espressione dei 9 marcatori di superficie

Valutazione dell'attivazione del signaling a livello di ogni sottopopolazione ed in base al tempo di incubazione per ogni stimolo

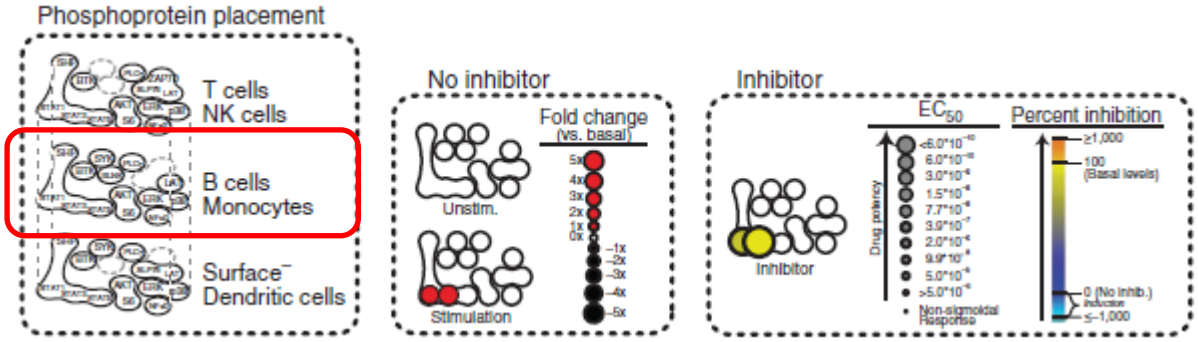


Identificazione delle sottopopolazioni

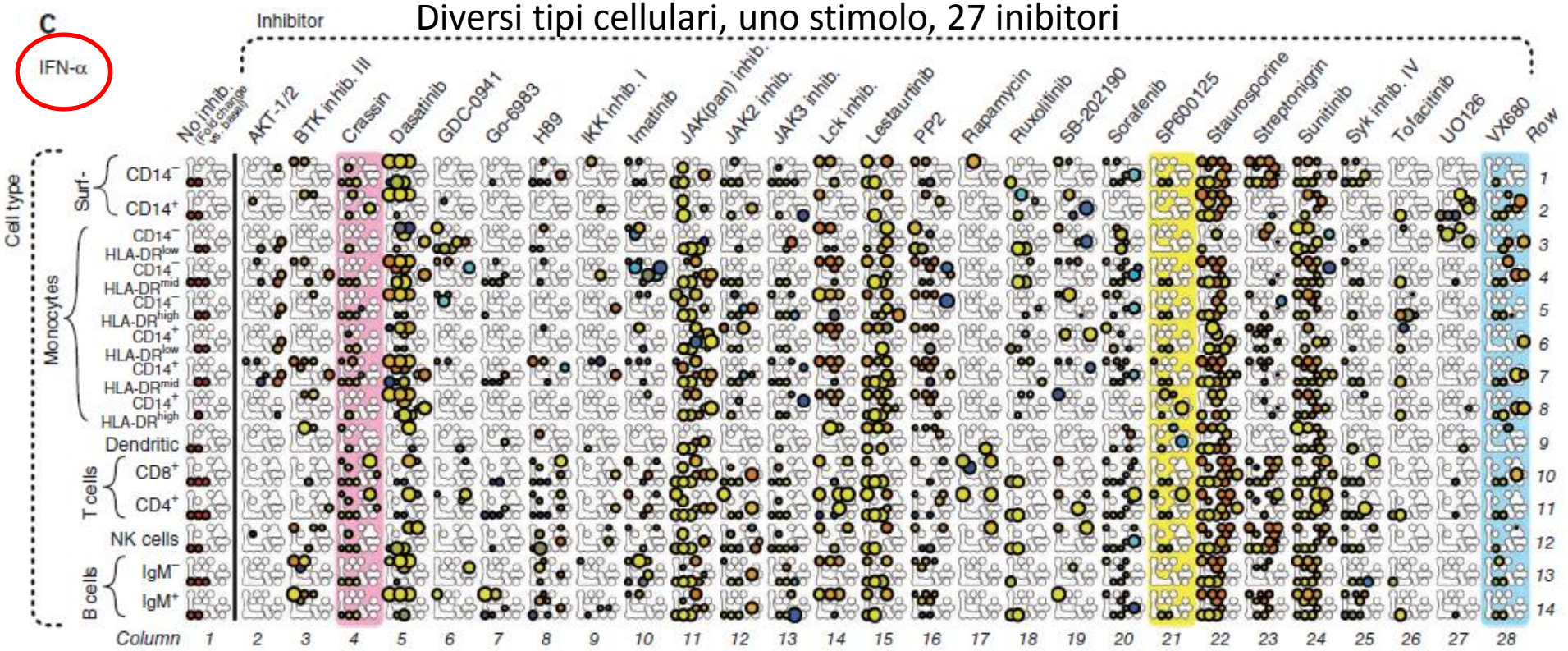


Multiplexed mass cytometry profiling of cellular states perturbed by small-molecule regulators - *Bodenmiller et al. Nature Biotechnology 30 (9) 2012*

Signaling network



Diversi tipi cellulari, uno stimolo, 27 inibitori



Thank you!

Valentina Tirelli - Ed. 15, piano A, st. 12 - t. 2576

Luca Pasquini - Ed.1, piano G, st.16-19 - t. 2422-2632

Prenotazioni Analisi Citometria:
http://gscf.iss.it/dw/doku.php?id=aree:citometria:prenotazioni