Bi9393 Analytical cytometry

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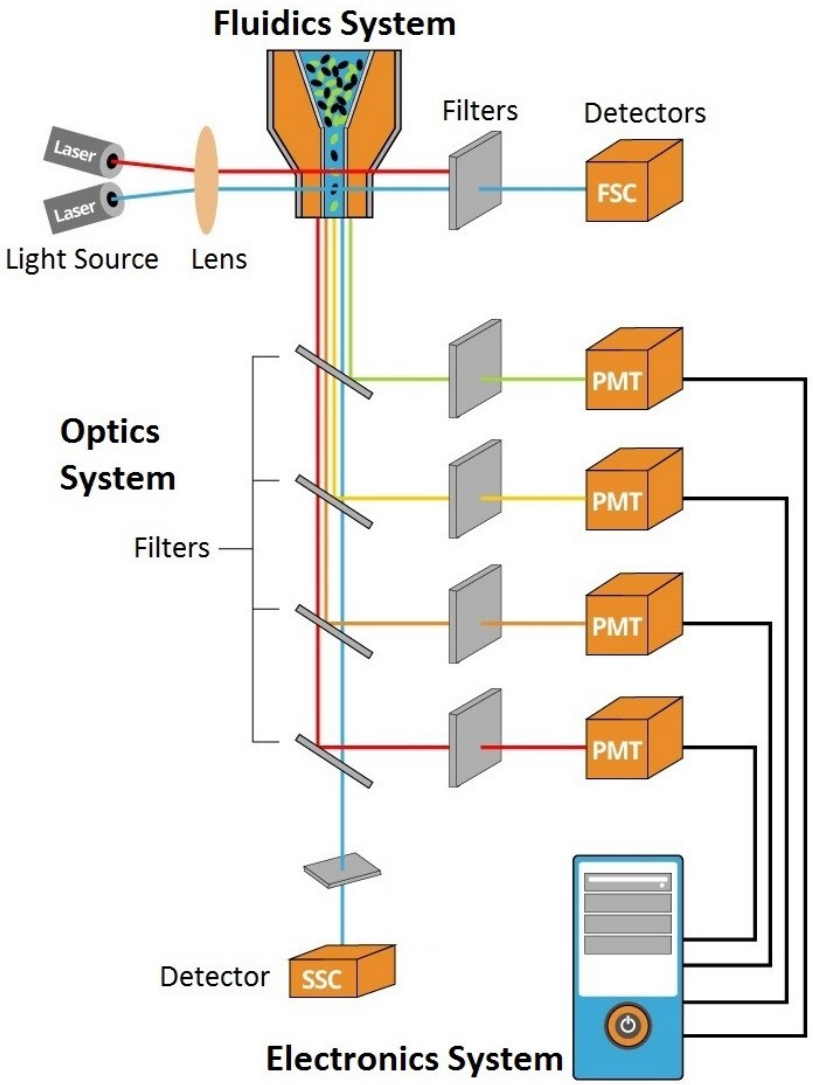
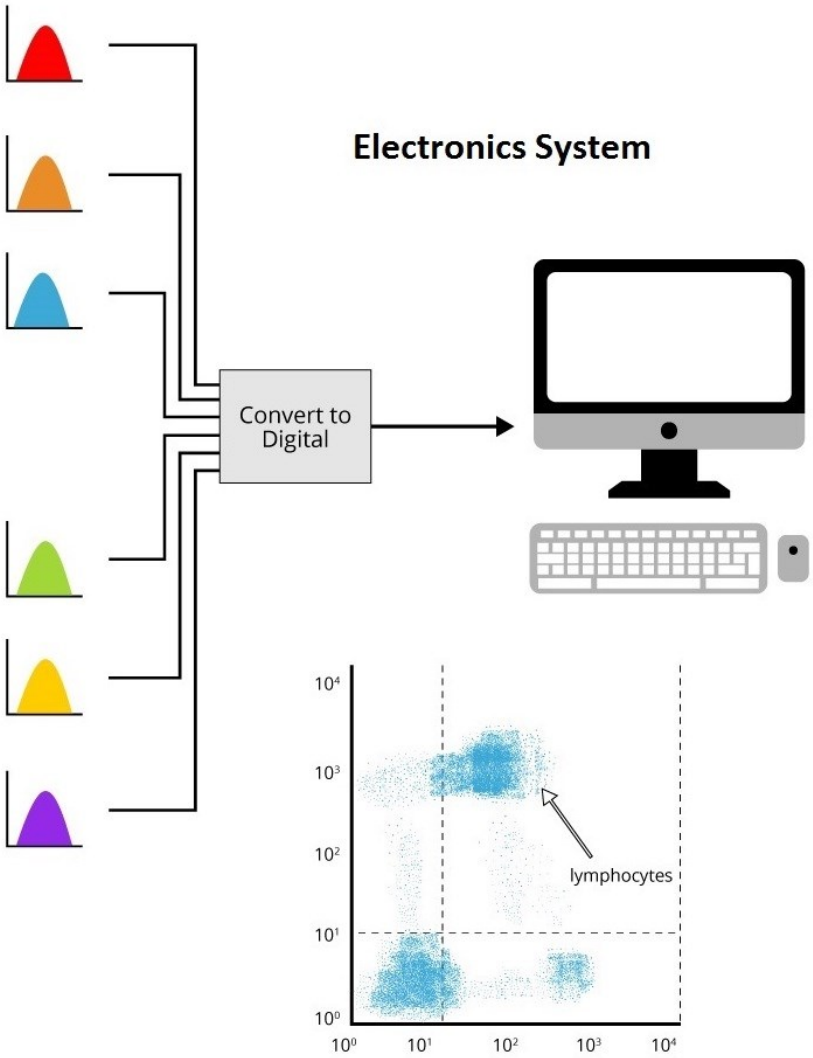
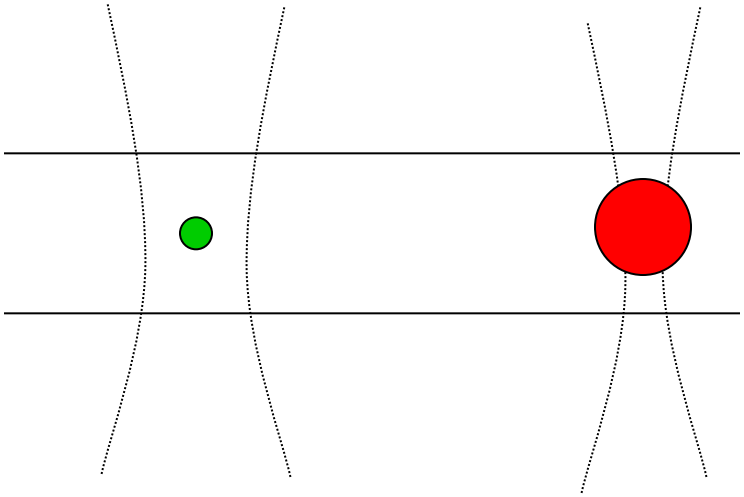
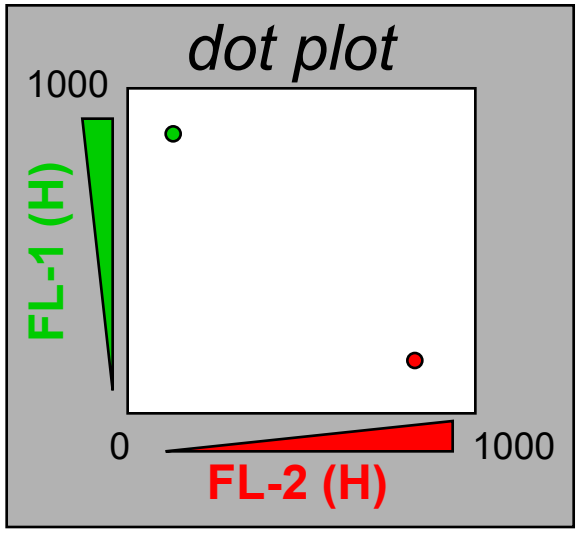




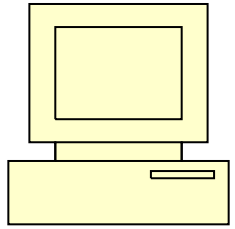
http[illegible]bosterbio.com/protocol-and-troubleshooting/flow-cytometry-principle

Signal processing

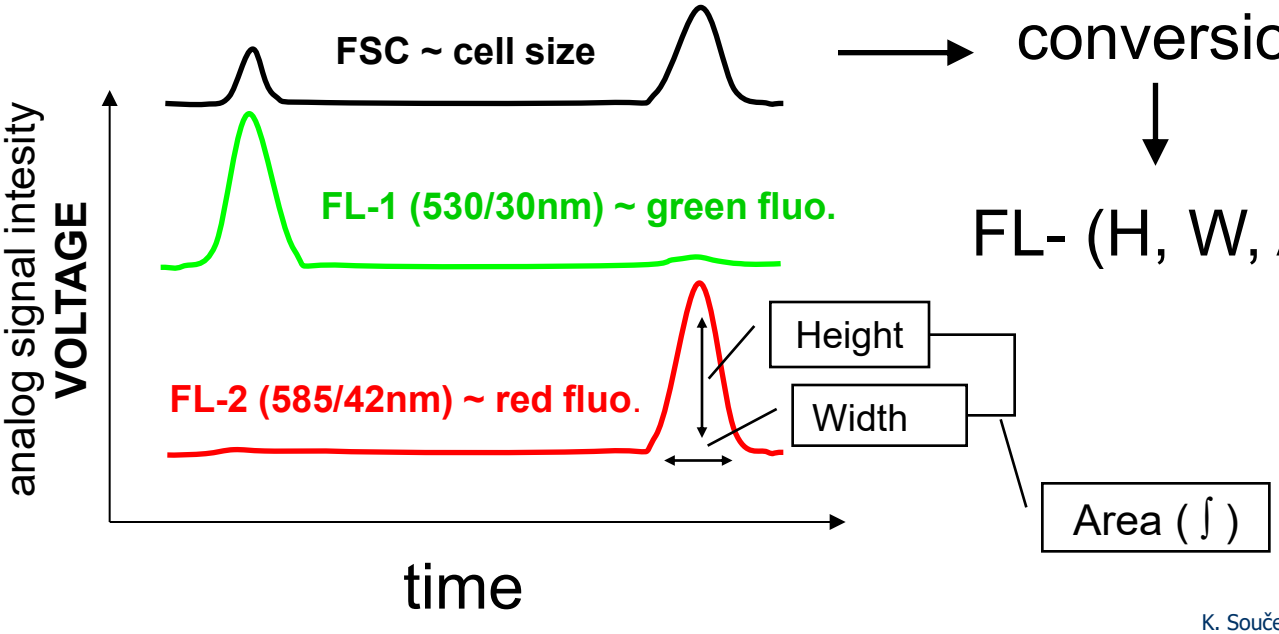




Analog/digital conversion



FL- (H, W, A)



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Help

OK

Date:17-JUL-2015
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Cytometer:FACSAriaII SORP (FACSAriaII)
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$BEGINSTEXT: 0
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AUTOBS: TRUE
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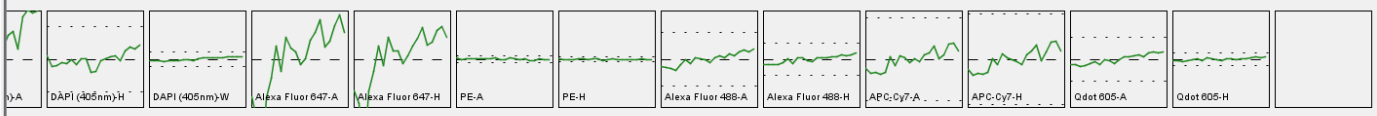
CST BEADS LOT ID: 91725
CST SETUP DATE: 03_25_2015 03:01:55 PM
CST SETUP STATUS: SUCCESS WITH WARNING
CYTNUM: P5Y500001
CYTOMETER CONFIG CREATE DATE: 05_13_2013 01:32:45 PM
CYTOMETER CONFIG NAME: RF_85u 45 psi_SORP Aria_5-laser (2uv-6v-3b-5yg-3r)
EXPERIMENT NAME: DU145_POPRO1_LDYellow_AF488_AF594_PE_APCCy7
EXPORT TIME: 17-JUL-2015-14:30:11
EXPORT USER NAME: fedr
FJ_FCS_VERSION: 3
FSC ASF: 0.57
GUID: dc7612a3-65af-4520-bc0f-51d53273ebea
LASER1ASF: 0.86
LASER1DELAY: 0.00
LASER1NAME: Blue
LASER2ASF: 0.86
LASER2DELAY: -38.47
LASER2NAME: Red
LASER3ASF: 1.02
LASER3DELAY: 77.49
LASER3NAME: UV
LASER4ASF: 0.63
LASER4DELAY: 45.00
LASER4NAME: Violet
LASER5ASF: 0.83
LASER5DELAY: -76.49
LASER5NAME: YG
P10BS: 602
P10DISPLAY: LOG
P10MS: 0
P11BS: 38
P11DISPLAY: LOG
P11MS: 0
P12BS: 5
P12DISPLAY: LOG
P12MS: 0
P13BS: 1118
P13DISPLAY: LOG

Compensation Matrix

	Alexa Fluor 594-A	DAPI (405nm)-A	Alexa Fluor 647-A	PE-A	Alexa Fluor 488-A	APC-Cy7-A	Qdot 605-A
Alexa Fluor 594-A	100	0,42	1,53	1,94	0,02	0,32	9,95
DAPI (405nm)-A	1,1	100	0,27	0,05	0,01	0,08	0,96
Alexa Fluor 647-A	2,45	22,87	100	0,1	0,08	15,14	0,85
PE-A	440,67	0	0,14	100	8,03	0,03	32,23
Alexa Fluor 488-A	-0,01	0,09	0,01	0	100	0	0,05
APC-Cy7-A	0,01	0,04	2,67	0	0,05	100	0,01
Qdot 605-A	0	41,05	0	0	2,34	0	100

Parameters and Stains

Parameter ($PnN)	Stain ($PnS)	Range ($PnR)	Bits ($PnB)	Decades ($PnE)	Gain ($PnG)	Voltage ($PnV)	Derived From
FSC-A		262144	32	0,0	1.0	280	
FSC-H		262144	32	0,0	1.0	280	
SSC-A		262144	32	0,0	1.0	210	
Alexa Fluor 594-A		262144	32	0,0	1.0	460	
Alexa Fluor 594-H		262144	32	0,0	1.0	460	
DAPI (405nm)-A		262144	32	0,0	1.0	650	
DAPI (405nm)-H		262144	32	0,0	1.0	650	
DAPI (405nm)-W		262144	32	0,0	1.0	650	
Alexa Fluor 647-A		262144	32	0,0	1.0	538	
Alexa Fluor 647-H		262144	32	0,0	1.0	538	
PE-A		262144	32	0,0	1.0	330	
PE-H		262144	32	0,0	1.0	330	
Alexa Fluor 488-A		262144	32	0,0	1.0	366	
Alexa Fluor 488-H		262144	32	0,0	1.0	366	
APC-Cy7-A		262144	32	0,0	1.0	700	
APC-Cy7-H		262144	32	0,0	1.0	700	
Qdot 605-A		262144	32	0,0	1.0	410	
Qdot 605-H		262144	32	0,0	1.0	410	
Time		262144	32	0,0	0.01		
Comp-Alexa Fluor 594-A		262144					
Comp-DAPI (405nm)-A		262144					
Comp-Alexa Fluor 647-A		262144					
Comp-PE-A		262144					
Comp-Alexa Fluor 488-A		262144					
Comp-APC-Cy7-A		262144					
Comp-Qdot 605-A		262144					



ORIGINAL ARTICLE

Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

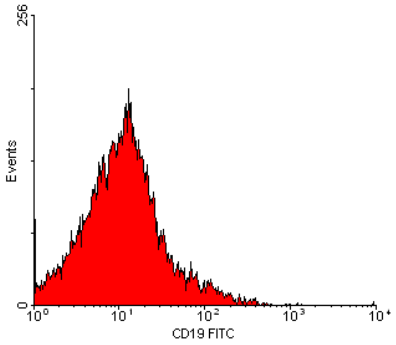
MIFlowCyt: The Minimum Information About a Flow Cytometry Experiment

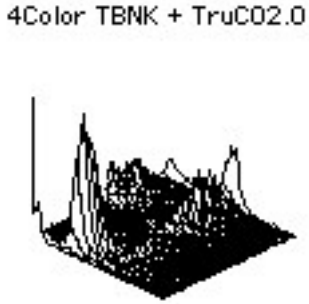
Jamie A. Lee,[1†] Josef Spidlen,[2†] Keith Boyce,[3] Jennifer Cai,[1] Nicholas Crosbie,[4] Mark Dalphin,[5] Jeff Furlong,[6] Maura Gasparetto,[2] Michael Goldberg,[7] Elizabeth M. Goralczyk,[8] Bill Hyun,[9] Kirstin Jansen,[6] Tobias Kollmann,[10] Megan Kong,[1] Robert Leif,[11] Shannon McWeeney,[12,13,14] Thomas D. Moloshok,[8] Wayne Moore,[15] Garry Nolan,[16] John Nolan,[17] Janko Nikolich-Zugich,[18] David Parrish,[3] Barclay Purcell,[19] Yu Qian,[1] Biruntha Selvaraj,[19] Clayton Smith,[2] Olga Tchuvatkina,[7] Anne Wertheimer,[20] Peter Wilkinson,[21] Christopher Wilson,[6] James Wood,[22] Robert Zigon,[23] The International Society for Advancement of Cytometry Data Standards Task Force, Richard H. Scheuermann,[1,24] Ryan R. Brinkman[2*]

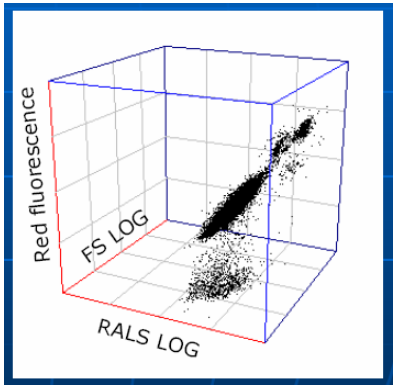
Table 1. Components of a MIFlowCyt-compliant experiment description

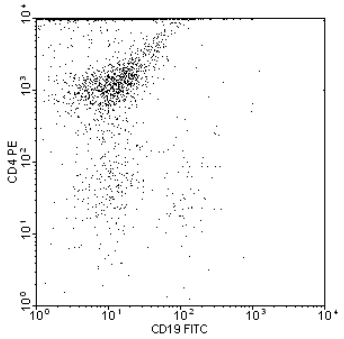
Experiment overview	Purpose/goal/hypothesis
	Experiment variables
	Conclusions
	Quality control
Flow sample (specimen)	Material
	Source/organism/location
	Treatment
	Reagent/analyte/detector/reporter
Data analysis	List-mode data
	Compensation
	Gating
	Descriptive statistics
Instrument details	Instrument identification
	Fluidics configuration
	Optical configuration
	Electronic configuration

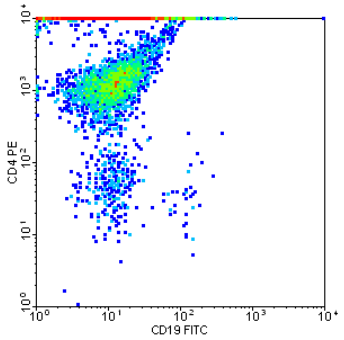
Ways to view data

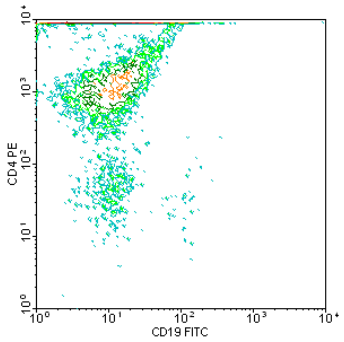


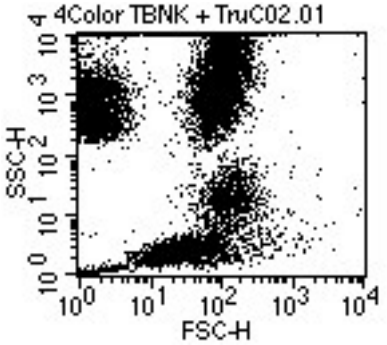


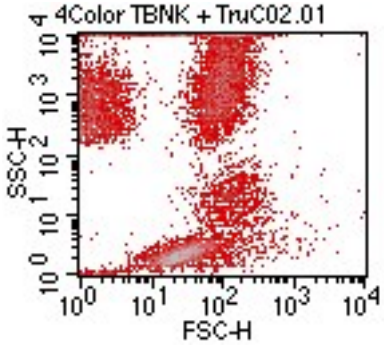


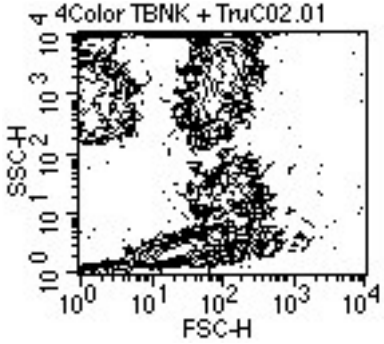






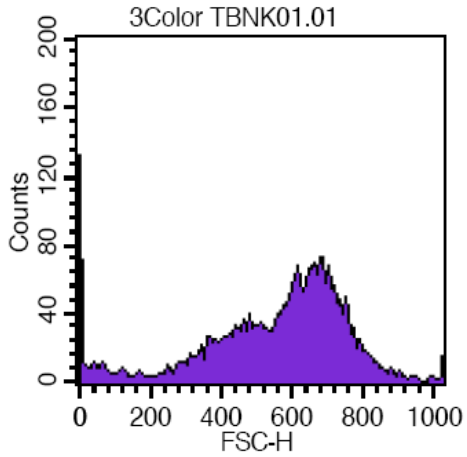


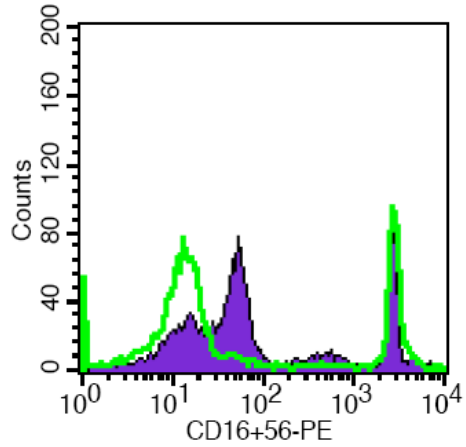




Frequency distribution histogram

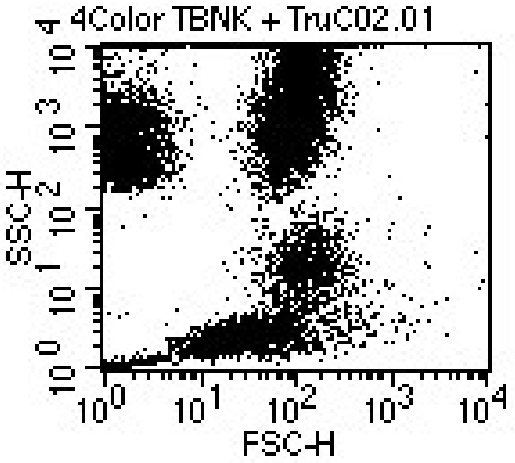
- Histogram shows particle frequency for a single parameter
- Simple Output
- We don't correlate with the next parameter
- The Problem of Identifying Populations





Dot plot

- Displays the correlation of two arbitrary parameters
- The individual dots represent specific measured cells (particles)
- Values for a number of particles can lie in the same location
- We don't have information about the relative density of particles
- Rendering issues with large amounts of data



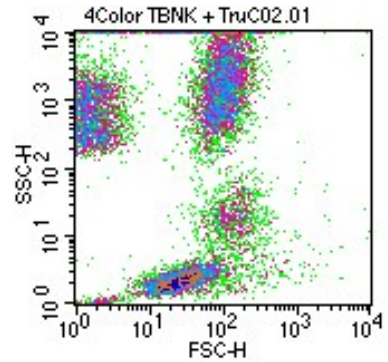
Density & contour plot

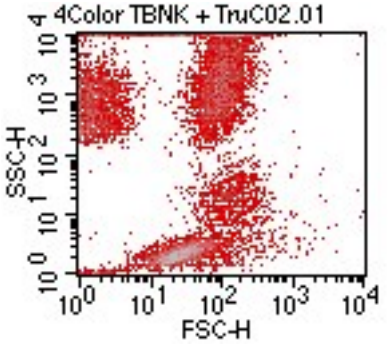
Density plot:

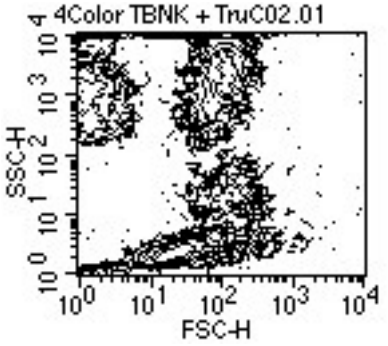
- It displays two parameters as frequency frequency
- the colour or shade corresponds to the particle frequency

Contour plot:

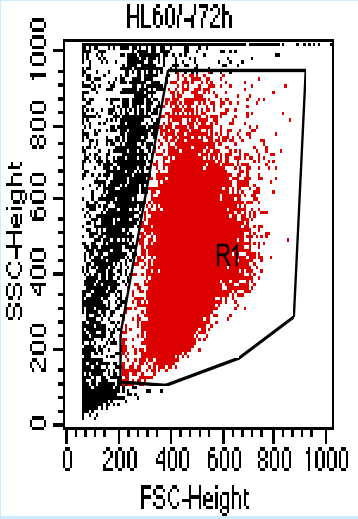
- It connects points (particles) with the same signal value
- Basically, we're simulating a 3D graph – the third dimension is frequency

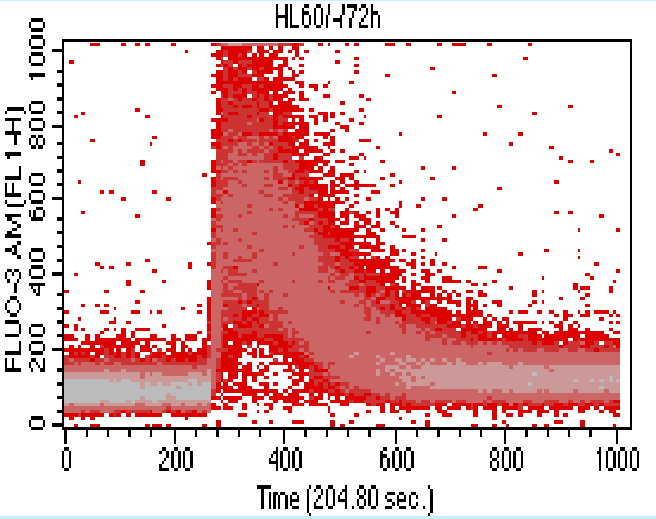


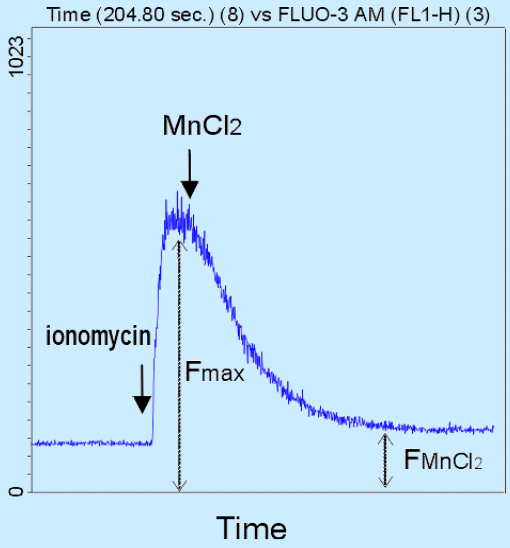




Time as one of the parameters



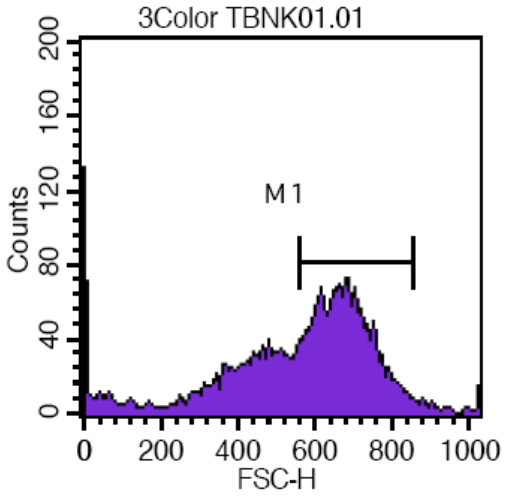




Statistics

- Arithmeric mean $\bar{x} = \frac{1}{n}(x_1 + x_2 + \ldots + x_n) = \frac{1}{n}\sum_{i=1}^{n} x_i$
- Geometric mean $(a_1 \cdot a_2 \cdots a_n)^{\frac{1}{n}} = \sqrt[n]{a_1 \cdot a_2 \cdots a_n} = \left(\prod_{i=1}^{n} a_i\right)^{\frac{1}{n}}$
- Median $\int_{-\infty}^{m} f(x)dx = 0,5$
  - estimation of the mean value
  - is not affected by extreme values
- Standard deviation $\overline{x} = \frac{1}{N}\sum_{i=1}^{N} x_i$
- Coefficient of variance
- Mode – the most common value

Statistics for histogram



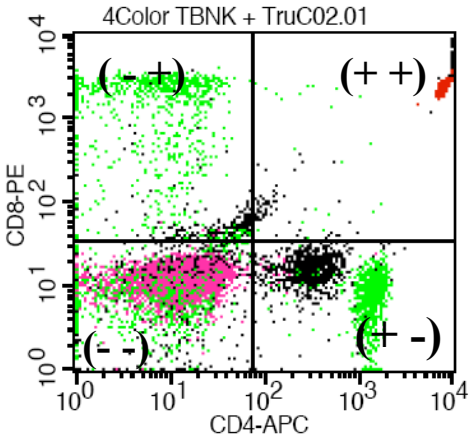
Histogram Statistics

File: 3Color TBNK01.01
Sample ID:
Tube: CD3/CD4/CD45
Acquisition Date: 21-Apr-98
Gated Events: 15000
X Parameter: FSC-H (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: 3 Color TBNK
Gate: No Gate
Total Events: 15000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	15000	100.00	100.00	570.49	500.40	29.98	612.00	0
M1	559, 855	9306	62.04	62.04	670.83	667.81	9.56	667.00	672

Quadrant Analysis



Quadrant Statistics

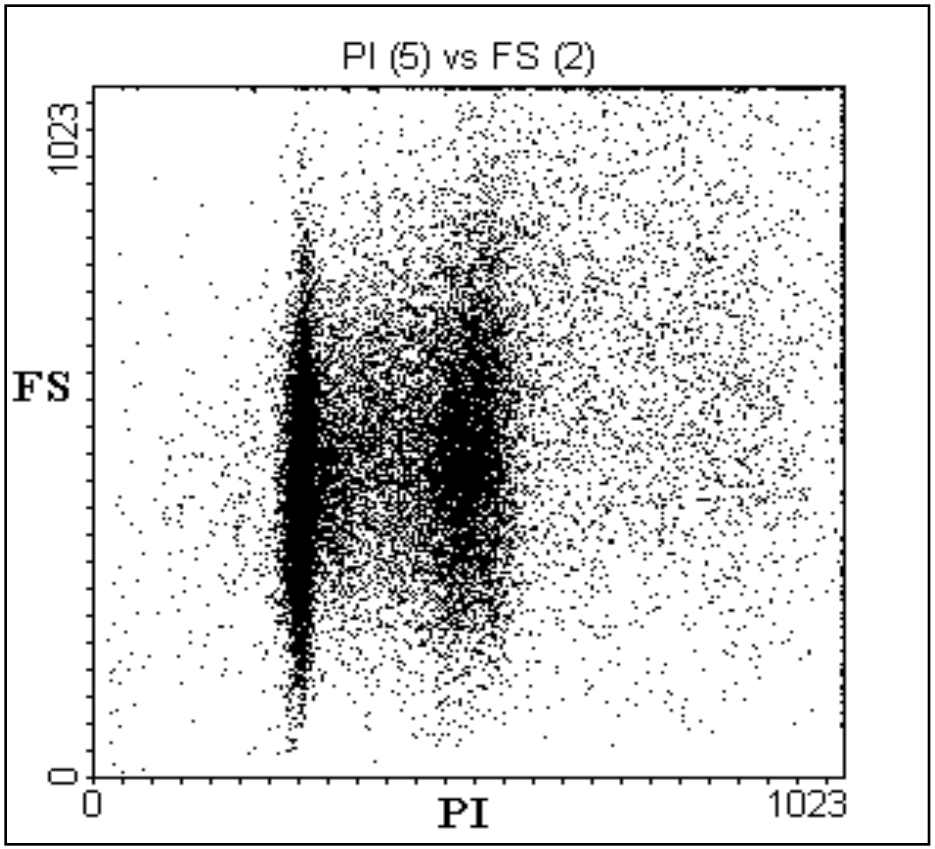
File: 4Color TBNK + TruC02.01
Sample ID:
Tube: CD3/CD8/CD45/CD4 TruC
Acquisition Date: 08-Oct-98
Gated Events: 10000
X Parameter: CD4-APC (Log)
Quad Location: 74, 35

Log Data Units: Linear Values
Patient ID:
Panel: 4 Color TBNK + TruC
Gate: No Gate
Total Events: 10000
Y Parameter: CD8-PE (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	1149	11.49	11.49	16.67	9.14	1474.42	618.99
UR	2222	22.22	22.22	7621.69	6806.34	2386.22	2160.04
LL	4783	47.83	47.83	15.00	10.87	12.01	10.64
LR	1846	18.46	18.46	879.87	646.31	12.24	10.28

Logical "Gating" (Boolean logic)

With overlapping areas, we have many options:



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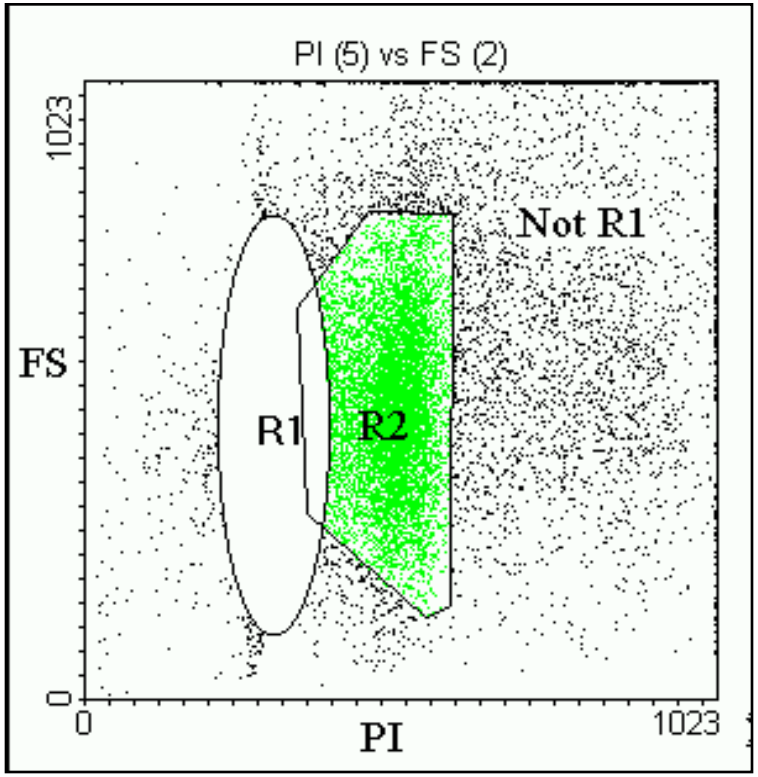
Boolean Gating

Not Region 1:



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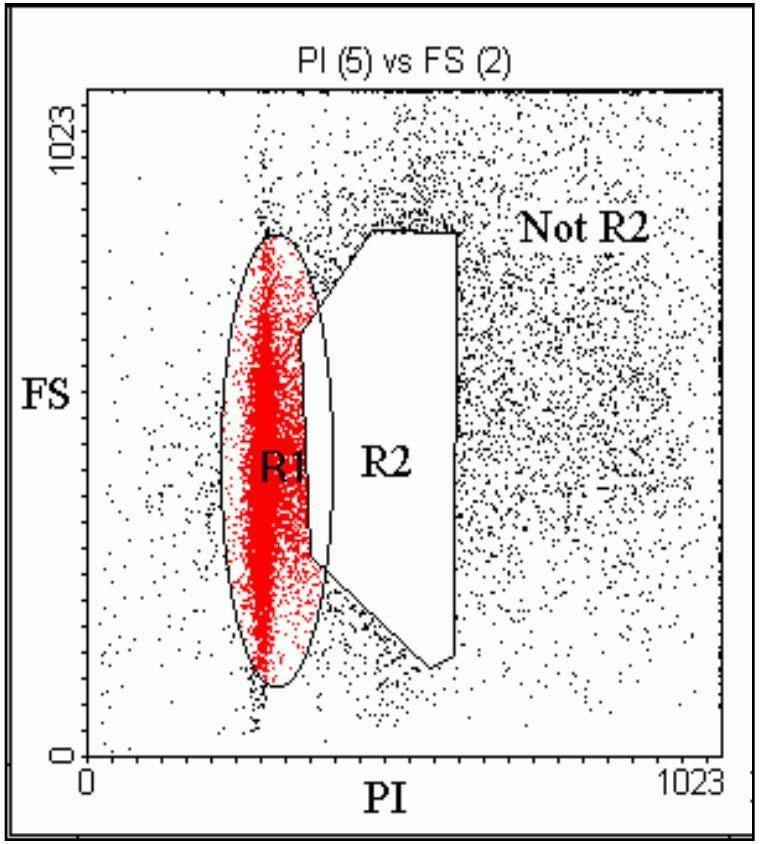
Boolean Gating

Not Region 2:



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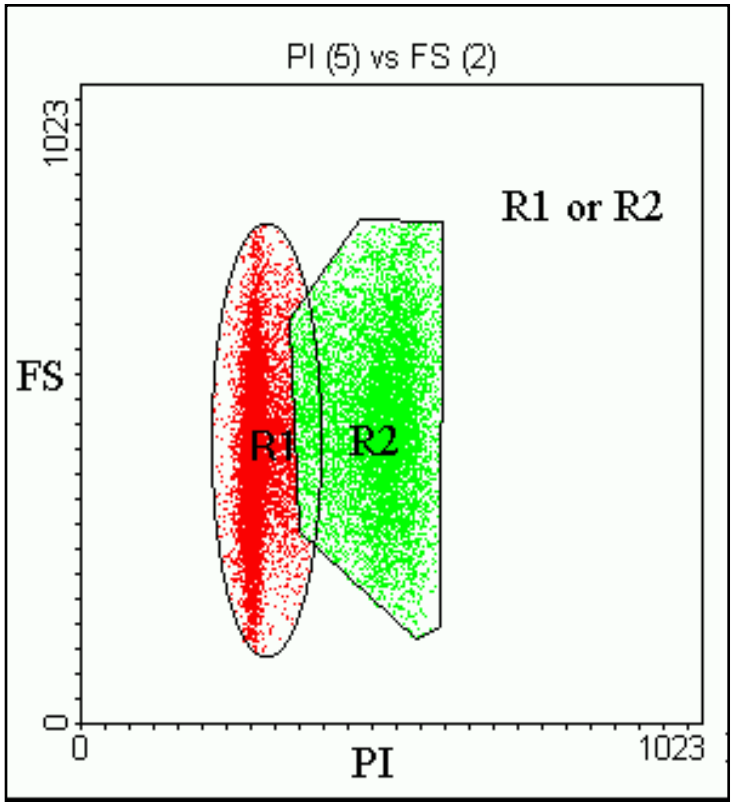
Boolean Gating

Region 1 or Region 2:



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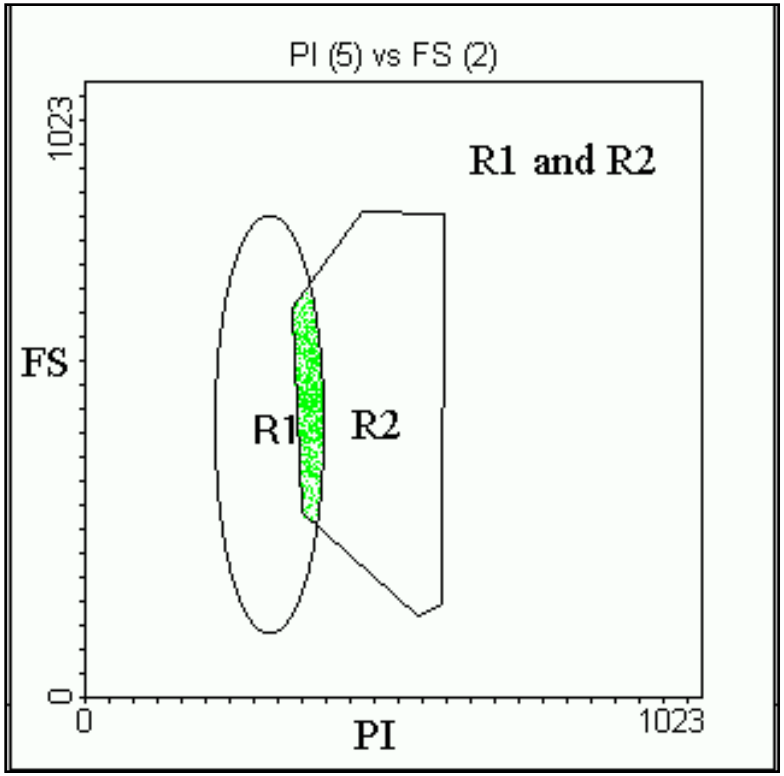
Boolean Gating

Region 1 and Region 2:



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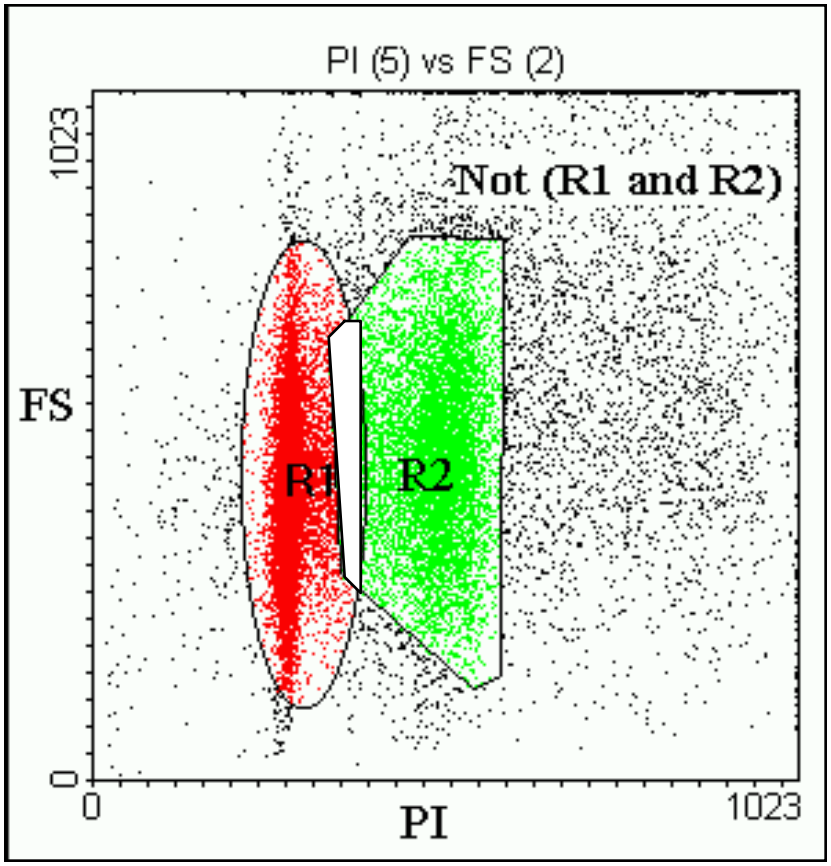
Boolean Gating

Not (Region1 and Region 2):



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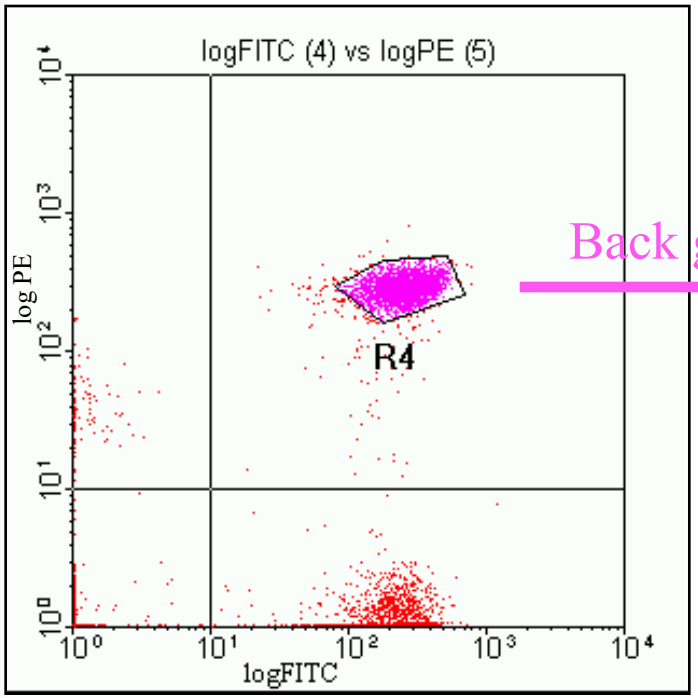
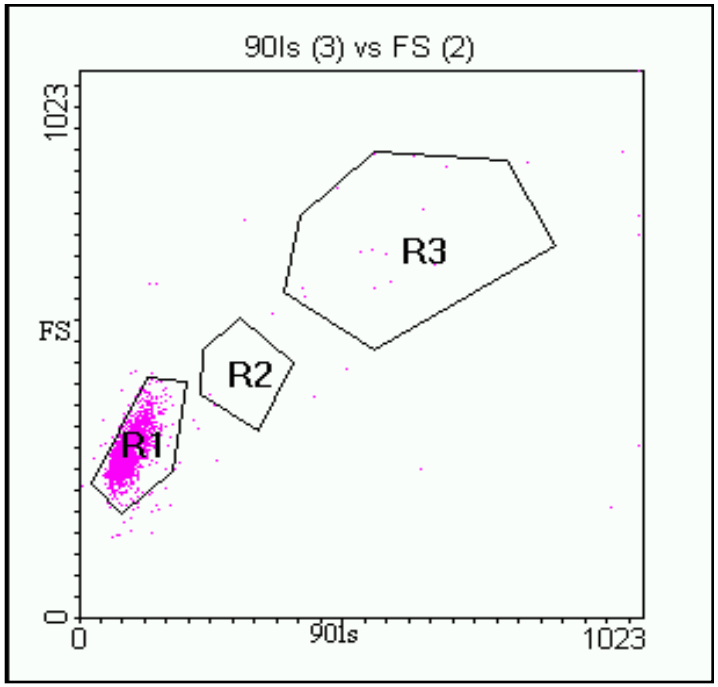
Back Gating



Back gate

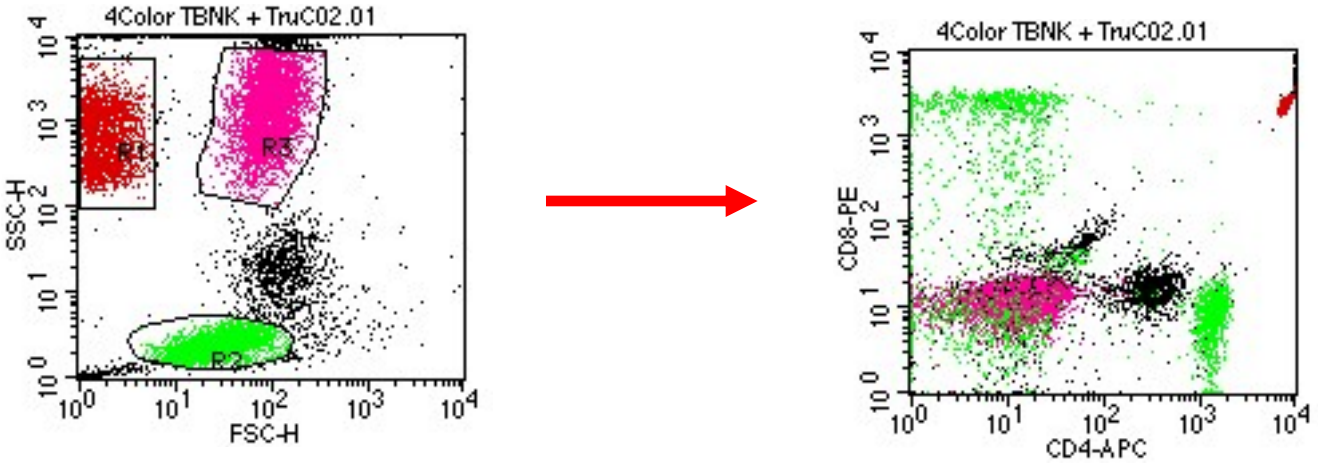


Region 4 established

Back-gating using Region 4

upraveno podle J.P.Robinson

Back Gating


4Color TBNK + TruC02.01
SSC-H
FSC-H
R1
R3
R2
4Color TBNK + TruC02.01
CD8-PE
CD4-APC

Data analysis tools

HW Manufacturers

- Beckman Coulter
    - Kaluza
- Becton Dickinson
    - FACSDiva
    - FACSSuite
    - FlowJo
- BioRad
- Sony
- Milteney
- …

Universal Platforms

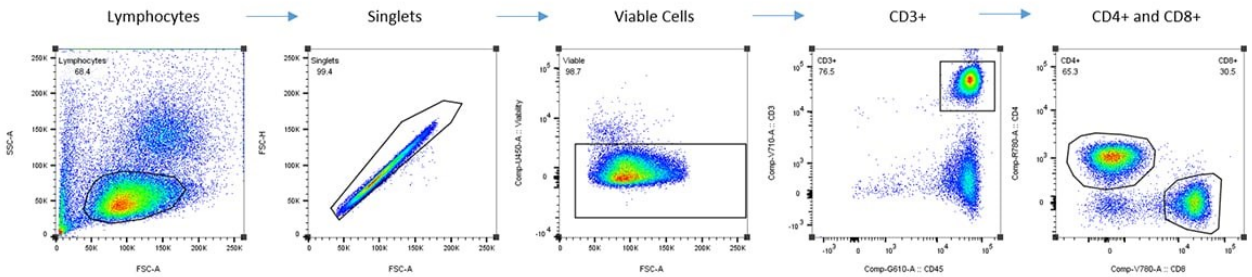
- Commercial
    - FlowJo
    - FCS Express

De Novo Software™ Turning Cytometry Data Into Results

Freeware

- Flowing Software
- Cyflogic
- BioConductor - Flowcore

Specifying Regions/Gates



A representative Nested Gating Strategy illustrating lymphocyte population being subgated to the level of CD4+ and CD8+ T Cells.

Objective or subjective?

- Training/Skills/Training

Possible shapes:

- rectangle
- ellipse
- "free-hand" (polygon)
- quadrant

Statistics

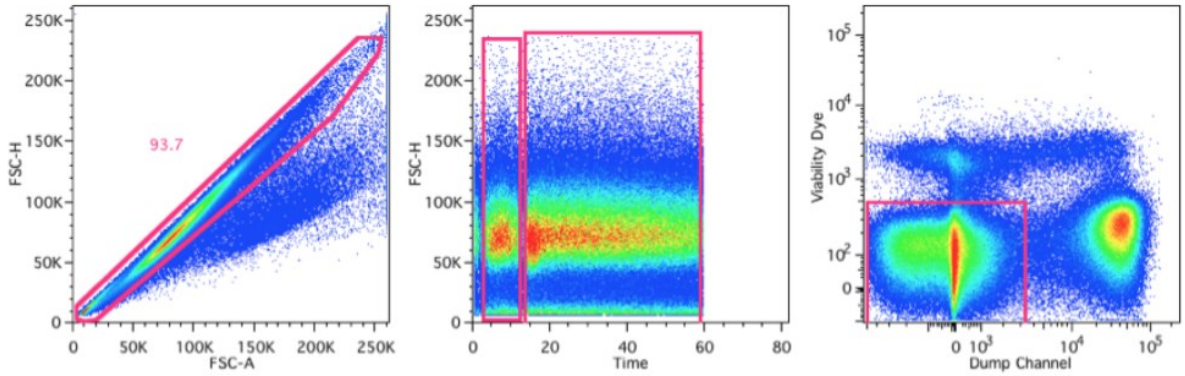
- count
- Share (%)
- mean, median, S.D., CV, ...

Gating – data reduction technique

- Flow stability gating — to capture events once the flow stream has stabilized, eliminating effects of clogging, back-pressure, and other instrument issues.
- Pulse geometry gating — to remove doublets from the dataset.
- Forward and side scatter gating — to remove debris and other events of non-interest while preserving cells based on size and or complexity.
- Subsetting gating — to rely on expression of markers and what they identify. Using viability dyes and dump channels further narrow to the cells of interest. This is where Fluorescence Minus One (FMO) controls become critical in defining the populations of interest.
- Backgating — to provide visualization of cells in final gate at higher level.

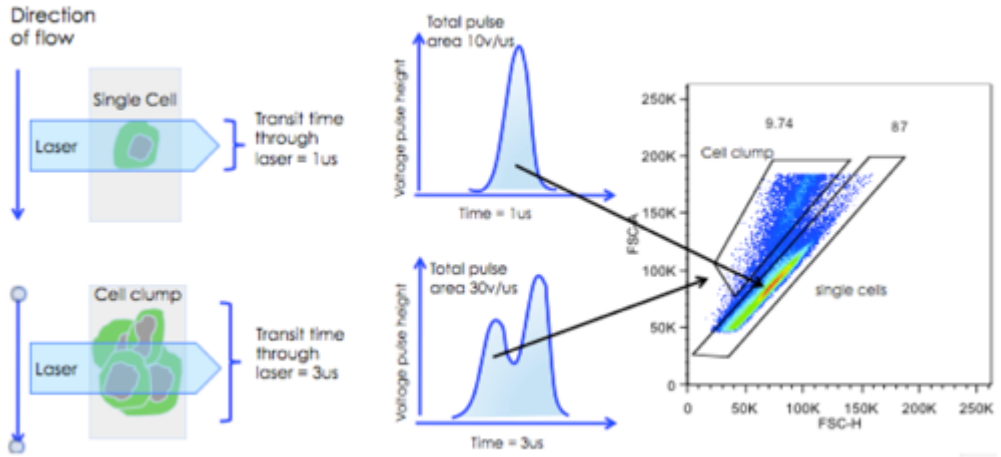
Gating & Quality Control

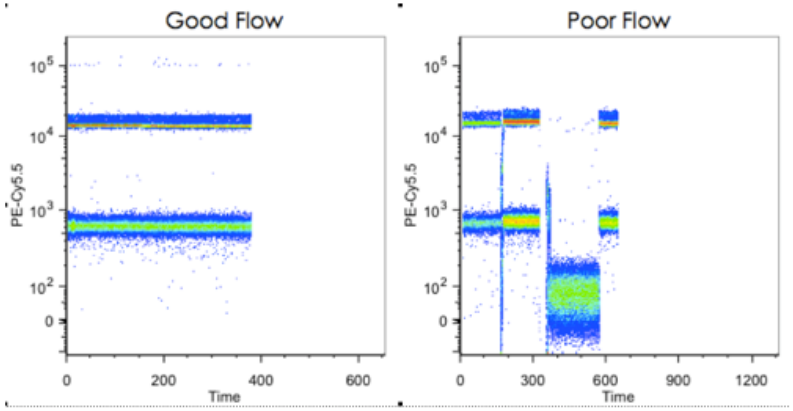
1) Singlets
2) Time
3) viability



http://expertcytometry.com/3-flow-cytometry-gates-that-will-improve-the-accuracy-of-your-facs-data-analysis/

Gating & Quality Control





http://expertcytometry.com/6-flow-cytometry-gating-tips-that-most-scientists-forget/

https://www.flowjo.com/learn/flowjo-university/flowjo/tutorial/33

Back to classes

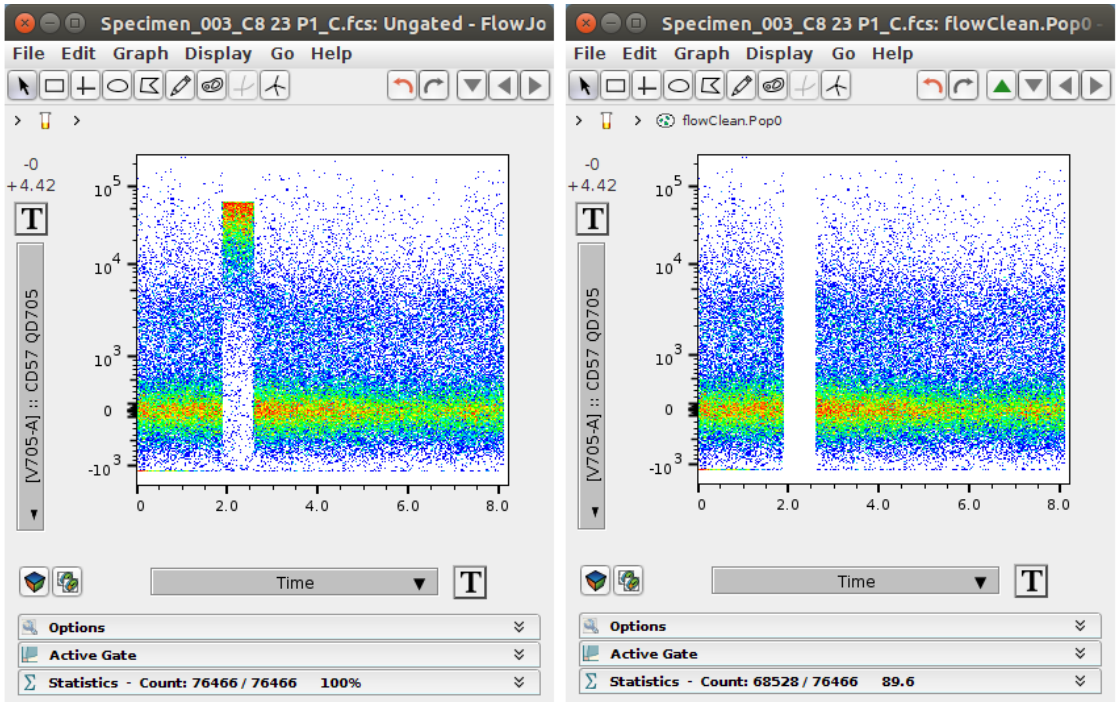
FlowClean Plugin

Josef Spidlen

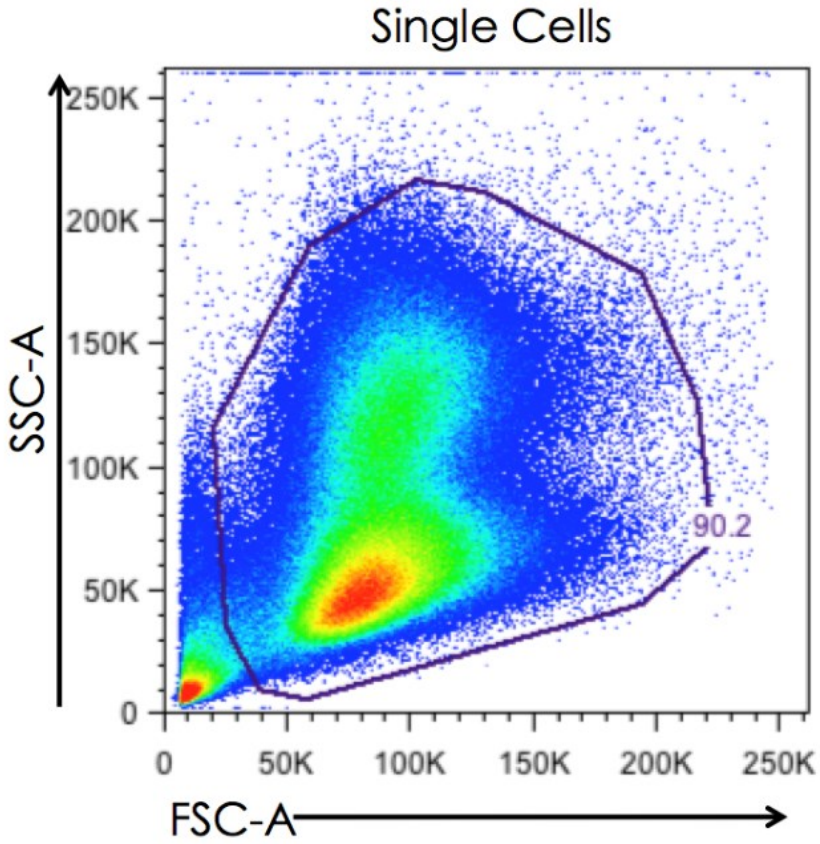
Clean up your data with the FlowClean plugin

Do you analyze a lot of samples? If so, data quality control may be challenging, especially when a large number of parameters is measured. In particular, fluorescence measurements for a sample over the collection time may not remain stable due to fluctuations in fluid dynamics. As many as 13.7% of publicly available FCS files have been shown to have this problem. But don't worry, we are here to help!

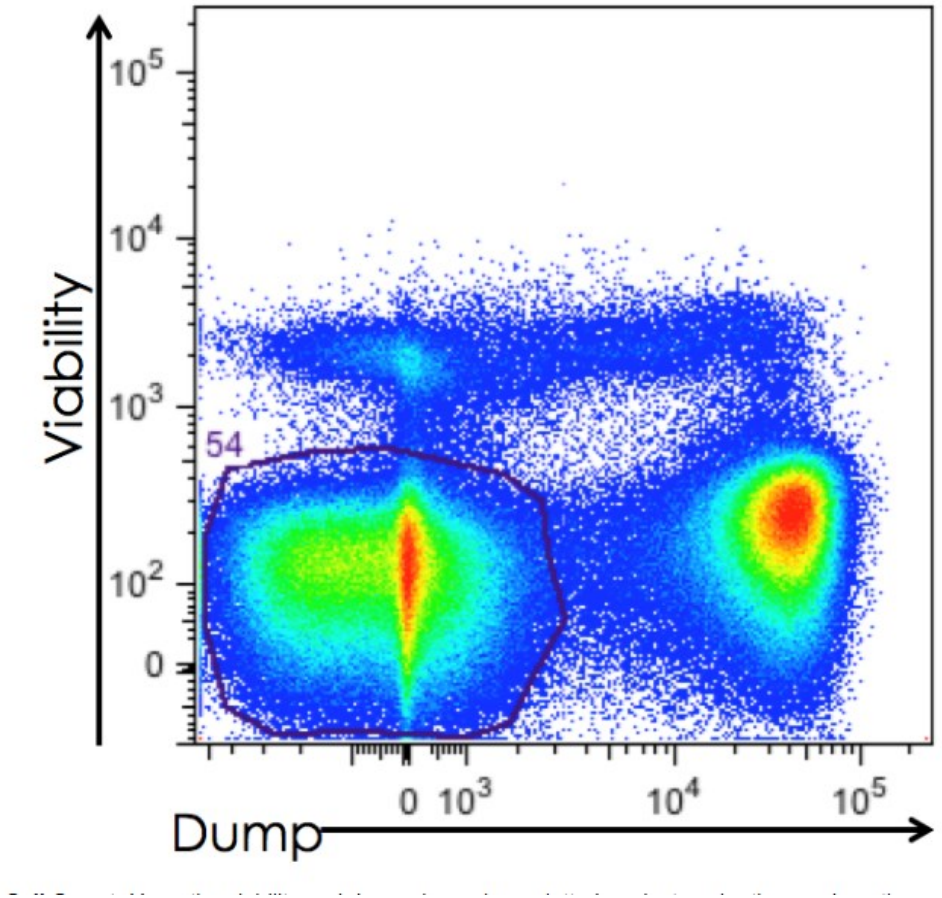
As you may know, our latest release, FlowJo v10.2, contains new and improved architecture for plugins. One of our featured plugins—FlowClean—has been designed to address exactly this issue. It automatically identifies and flags fluorescence anomalies in your FCS files by tracking cell populations in the centered log ratio space. This has been shown to provide a sensitive and consistent method of quality control. Do you want to give it a try?



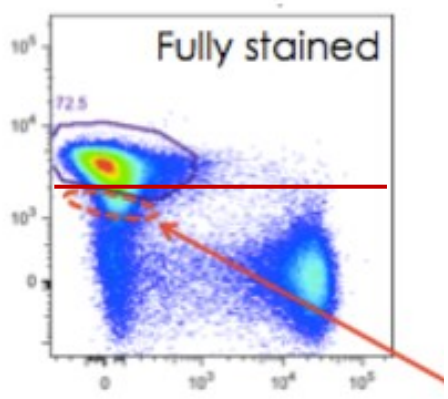
Scatter gating



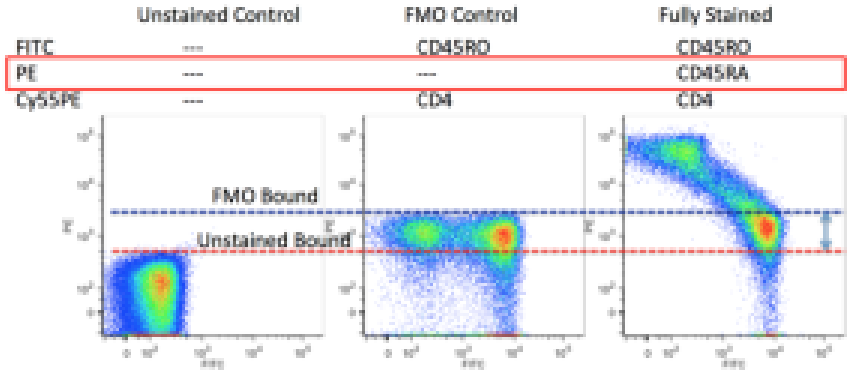
Subset gating



Gating and checking settings



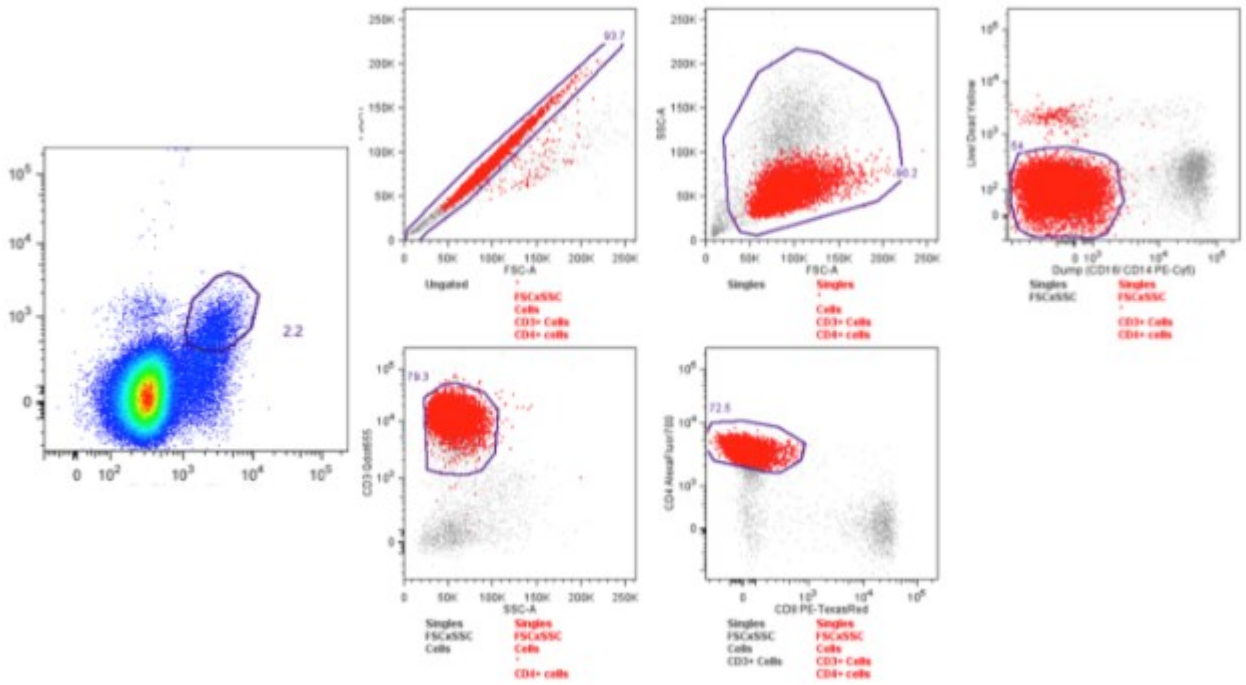
FMO ~ Fluorescence Mines One



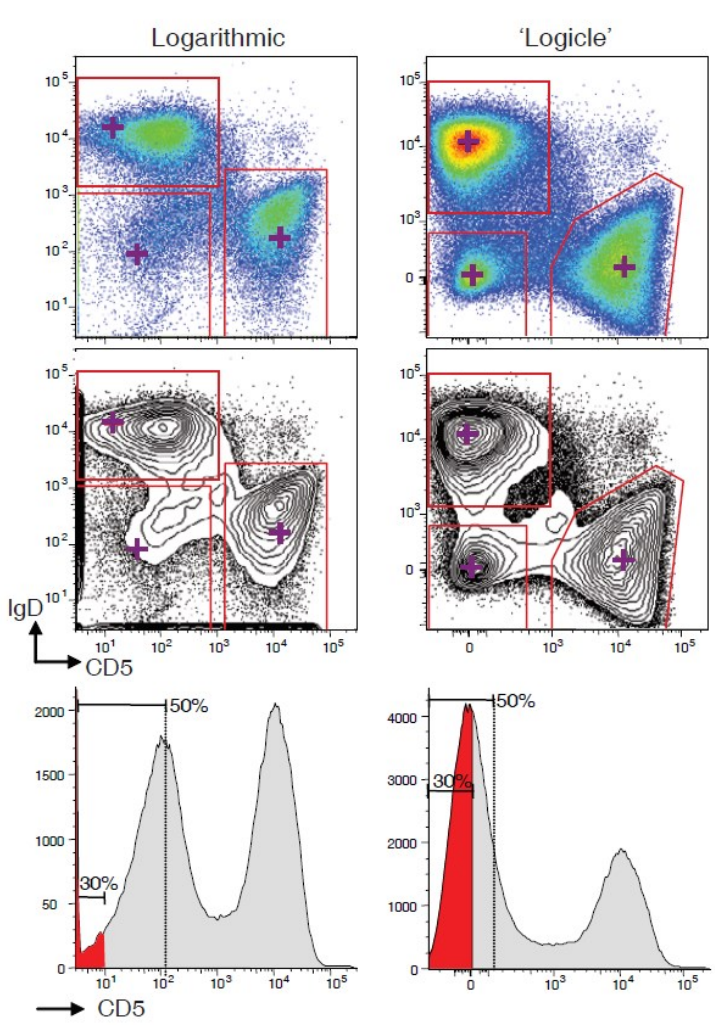
Antigen	FITC	PE	Cy5-PE	APC
CD3 FMO	---	CD4	CD8	CD19
CD4 FMO	CD3	---	CD8	CD19
CD8 FMO	CD3	CD4	---	CD19
CD19 FMO	CD3	CD4	CD8	---

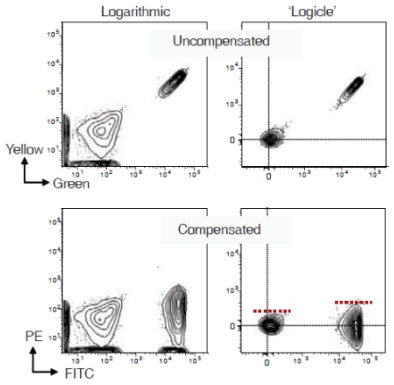
http://expertcytometry.com/6-flow-cytometry-gating-tips-that-most-scientists-forget/

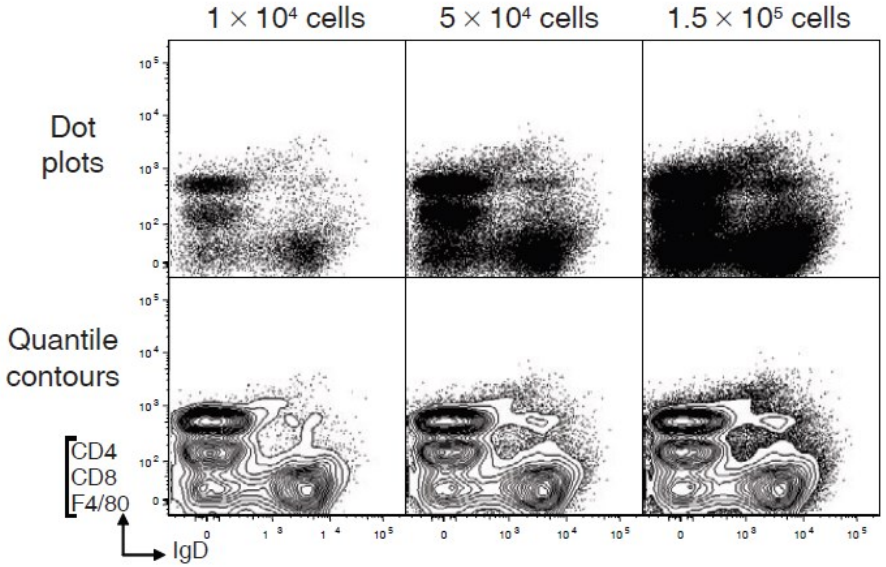
Back gating



Data visualization and data interpretation







Herzenberg LA, Tung J, Moore WA, Herzenberg LA, Parks DR (2006) Interpreting flow cytometry data: a guide for the perplexed. *Nat Immunol* ***7: 681-685***

BOX 1 SUGGESTED GUIDELINES FOR FACS DATA PRESENTATION[4]

Instrument: Identify the FACS instrument and the software used to collect, compensateand analyze the data. Include model and version number where more than one exists.

Graphic displays: Choose smoothing, graph and display options according to the dictates of the study. Be consistent across all displays in an analysis. Indicate the number of cells for which data are displayed and, where applicable, the contour or color density intervals used in the figure.

Scaling: Show all parts of the plot axis necessary to indicate the scaling that was used (such as log, linear or 'logicle'). Numerical values for axis 'ticks' can be eliminated except when necessary to clarify the scaling. For univariate (one-dimensional) histograms, the scale for the abscissa (*y* axis) should be linear and should begin at zero unless otherwise indicated. Numerical axis values should not be included with the zero-based linear axes but should be shown for other axes.

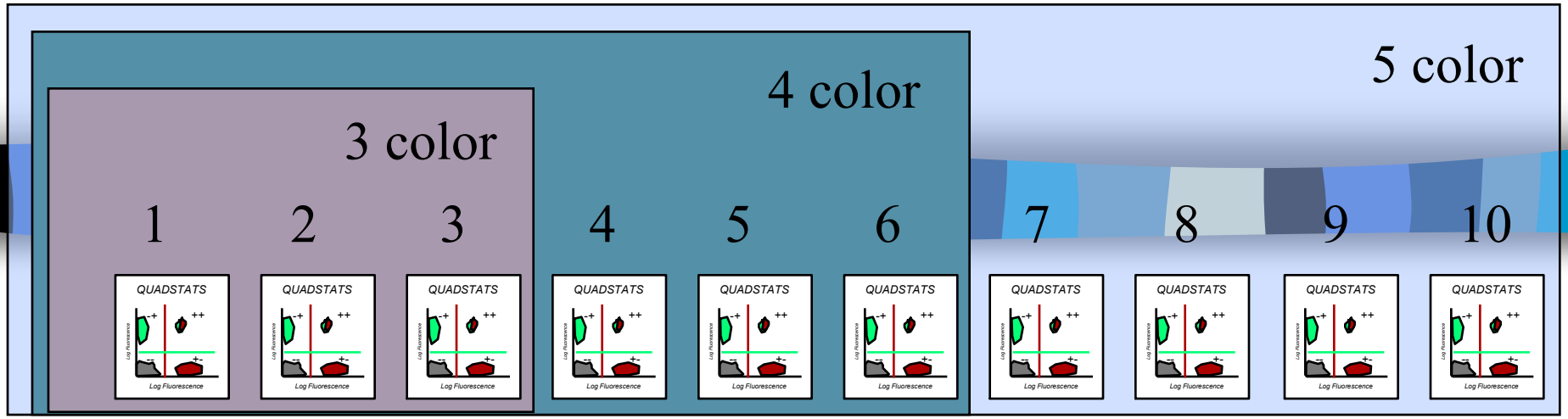
Gating: Display the gates used at each step in the gating sequence when gates are set manually (subjective gating). Show data for control samples when these are used to set gates. If necessary, present this information in supplementary figures. When an algorithm is used to set gates, define it explicitly and state that it has been used. Gating is assumed to be subjective unless otherwise stated.

Frequency measurements: Show the frequencies (or percentages) of cells in gates of importance in the study. Compute these values relative to the total number of cells presented in the display on which the values appear. If a different frequency computation is used, define the method that was used and where it was applied. The graph itself cannot convey this requisite information.

Intensity measurements: Explicitly define the statistic applied (mean, median or a particular percentile). All statistics should be applied to the 'scaled' intensity measurement rather than to 'channel' numbers.

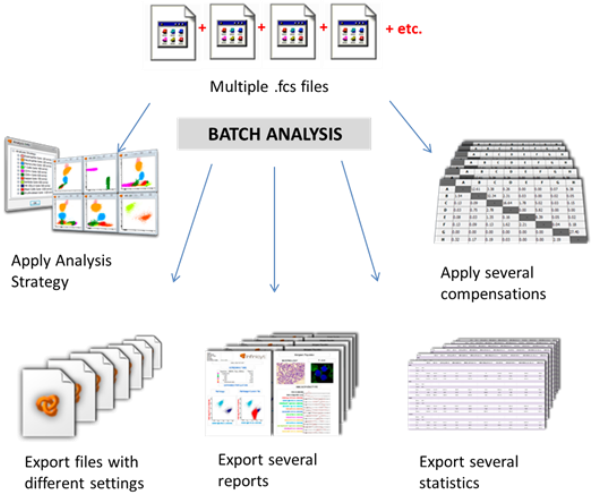
Herzenberg LA, Tung J, Moore WA, Herzenberg LA, Parks DR (2006) Interpreting flow cytometry data: a guide for the perplexed. *Nat Immunol* ***7: 681-685***

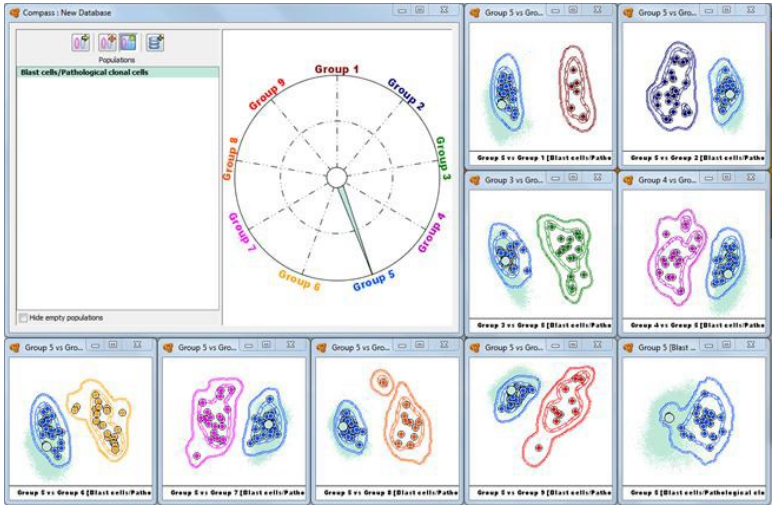
Multi-color analyses generate a lot of data...



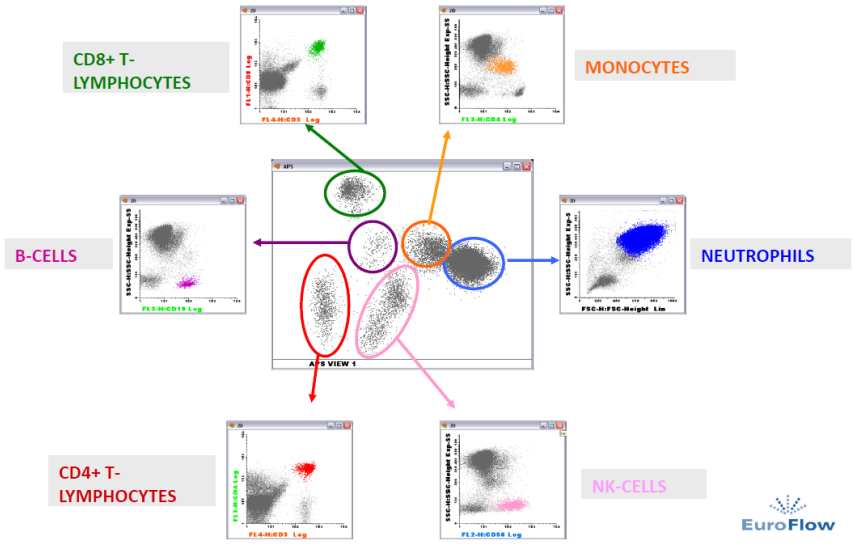
upraveno podle J.P.Robinson

Infinicyt™





Automatic Population Separator



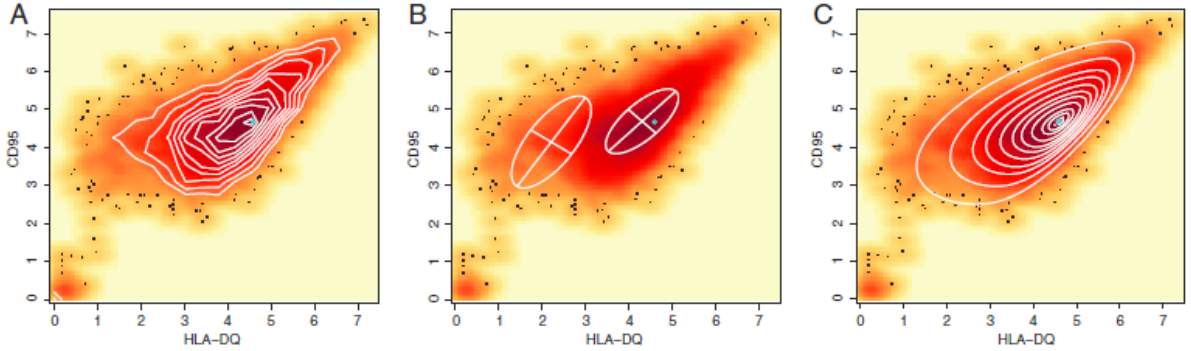
EuroFlow

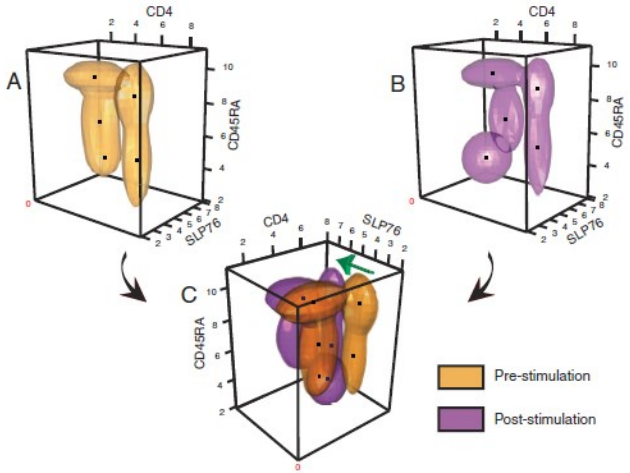
Automated high-dimensional flow cytometric data analysis

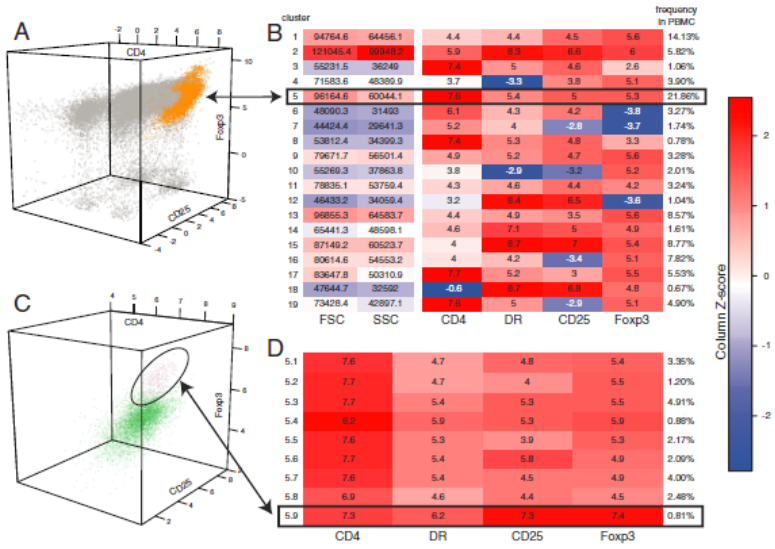
Saumyadipta Pyne[a], Xinli Hu[a,1], Kui Wang[b,1], Elizabeth Rossin[a,1], Tsung-I Lin[c], Lisa M. Maier[a,d], Clare Baecher-Allan[d], Geoffrey J. McLachlan[b,e], Pablo Tamayo[a], David A. Hafler[a,d,2], Philip L. De Jager[a,d,f,3], and Jill P. Mesirov[a,2,3]

[a]Broad Institute of MIT and Harvard, 7 Cambridge Center, Cambridge MA 02142; [b]Department of Mathematics and [e]Institute for Molecular Bioscience, University of Queensland, St. Lucia, Queensland, 4072, Australia; [c]Department of Applied Mathematics, National Chung Hsing University, Taichung 402, Taiwan; [d]Division of Molecular Immunology, Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115; and [f]Partners Center for Personalized Genetic Medicine, Boston, MA 02115

Communicated by Peter J. Bickel, University of California, Berkeley, CA, April 3, 2009 (received for review December 28, 2008)







The Flow Cytometry: Critical Assessment of Population Identification Methods (FlowCAP)

The goal of FlowCAP is to advance the development of computational methods for the identification of cell populations of interest in flow cytometry data. FlowCAP will provide the means to objectively test these methods, first by comparison to manual analysis by experts using common datasets, and second by prediction of a clinical/biological outcome.

Critical assessment of automated flow cytometry data analysis techniques

Nima Aghaeepour[1], Greg Finak[2], The FlowCAP Consortium[3], The DREAM Consortium[3], Holger Hoos[4], Tim R Mosmann[5], Ryan Brinkman[1,7], Raphael Gottardo[2,7] & Richard H Scheuermann[6,7]

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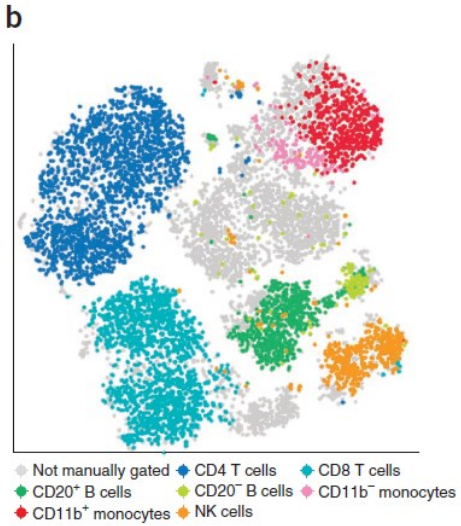
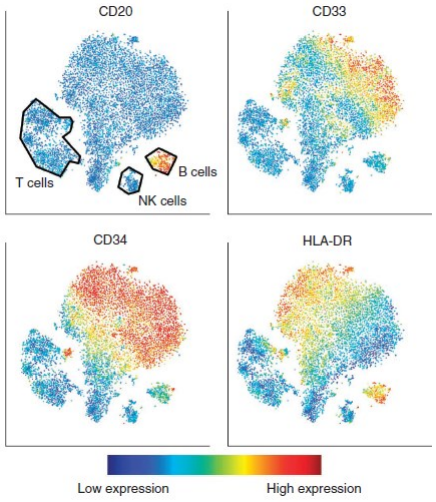
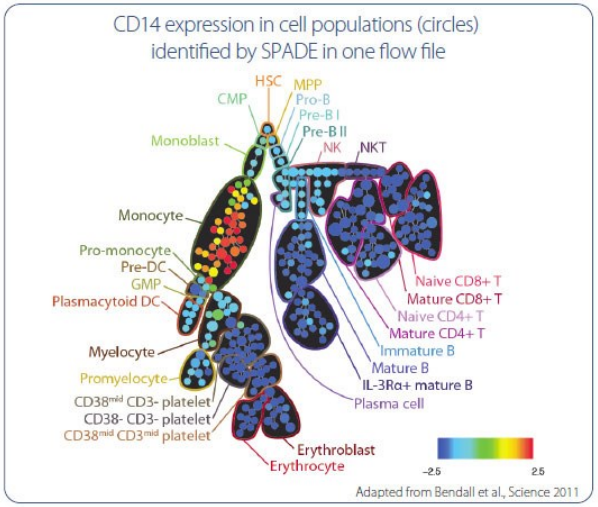
Other ways to visualize multidimensional data

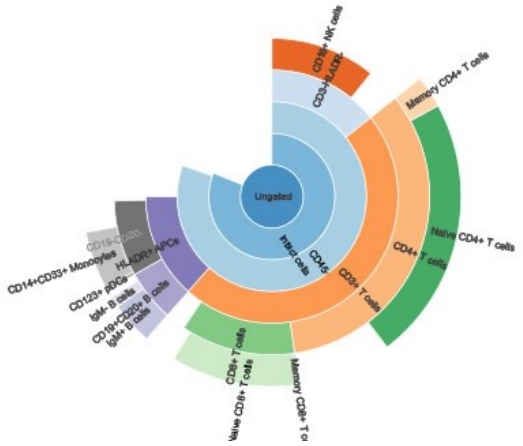
- t-SNE, viSNE
  - t-Distributed Stochastic Neighbor Embedding
  - viSNE is a tool for reducing high-parameter data down to two dimensions
  - visually identify interesting and rare biological subsets
  - allow to gate single cell events across different samples.
- SPADE
  - Spanning-tree Progression Analysis of Density-normalized Events
  - way to automatically identify populations in multidimensional flow cytometry data files
  - clusters cells into populations and then projects them into a tree

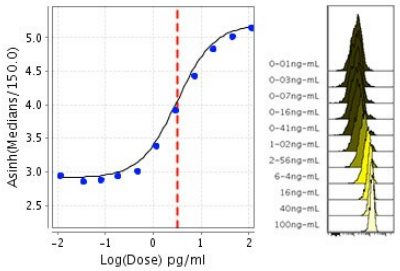


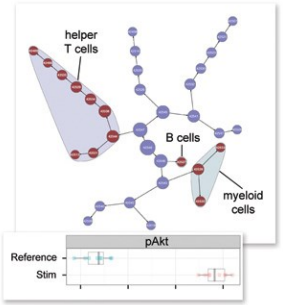


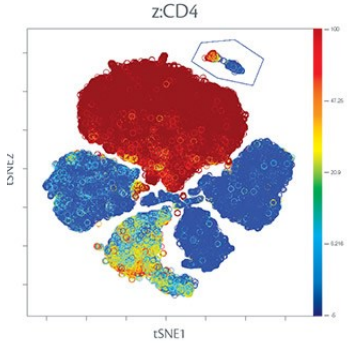


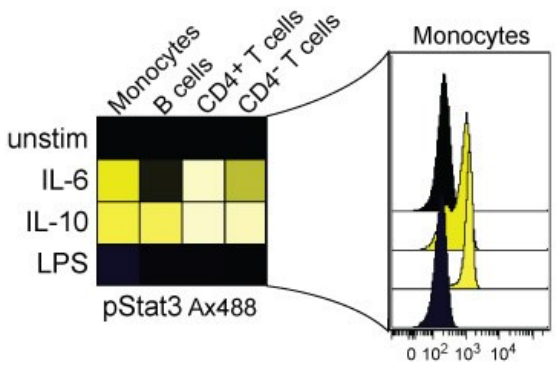
Cytobank

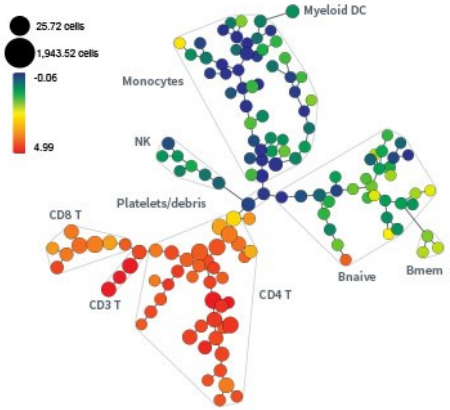

Ungated
Intact cells
CD45+
CD3+ T cells
CD4+ T cells
CD8+ T cells
Naive CD4+ T cells
Memory CD4+ T cells
CD14+CD33+ Monocytes
CD123+ pDCs
IgM- B cells
IgM+ B cells

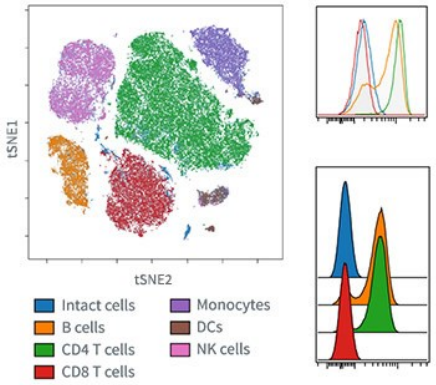

Asinh(Medians/150.0)
Log(Dose) pg/ml
0-01ng-mL
0-03ng-mL
0-07ng-mL
0-16ng-mL
0-41ng-mL
1-02ng-mL
2-56ng-mL
6-4ng-mL
16ng-mL
40ng-mL
100ng-mL


helper T cells
B cells
myeloid cells
pAkt
Reference
Stim


z:CD4
tSNE1
tSNE2


Monocytes
B cells
CD4+ T cells
CD4- T cells
unstim
IL-6
IL-10
LPS
pStat3 Ax488
Monocytes
0 10^2 10^3 10^4


25.72 cells
1,943.52 cells
-0.06
4.99
Myeloid DC
Monocytes
NK
Platelets/debris
CD8 T
CD3 T
CD4 T
Bnaive
Bmem


tSNE1
tSNE2
Intact cells
B cells
CD4 T cells
CD8 T cells
Monocytes
DCs
NK cells

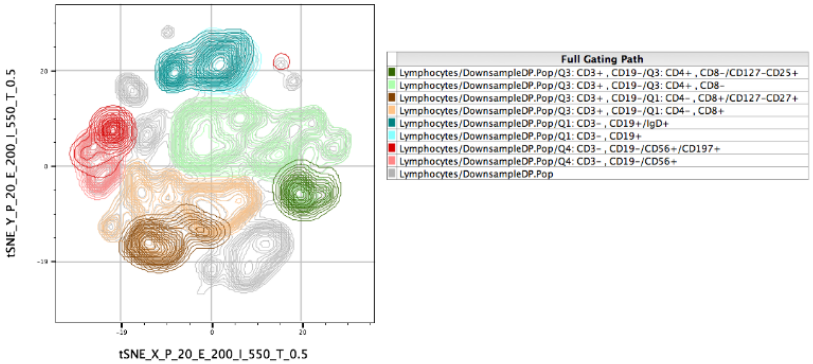
Search the Site...

Search

- Installation
- Getting Acquainted
- Workspaces and Samples
- Graphs and Gating
- Tabular Reports in the Table Editor
- Graphical Reports in the Layout Editor
- Technical FAQ
- Advanced Features
  - Archival Cytometry Standard (ACS) files
  - Templates
  - R-Tools in FlowJo
  - Remote data
  - Dimensionality Reduction
    - tSNE
  - Command Line FlowJo
  - Script Editor
  - Taylor Index
  - Data De-identification Utility
- Platforms
- Plugins
- Setting Your Preferences
- Credits

tSNE

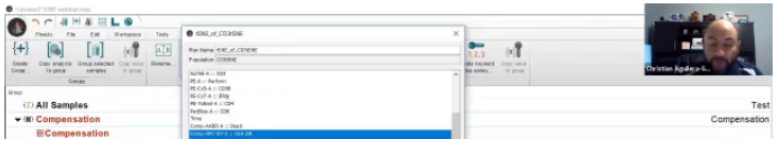
T-Distributed Stochastic Neighbor Embedding (tSNE) is an algorithm for performing dimensionality reduction, allowing visualization of complex multi-dimensional data in fewer dimensions while still maintaining the structure of the data.



tSNE is an unsupervised nonlinear dimensionality reduction algorithm useful for visualizing high dimensional flow or mass cytometry data sets in a dimension-reduced data space. The tSNE platform computes two new derived parameters from a user defined selection of cytometric parameters. The tSNE-generated parameters are optimized in such a way that observations/data points which were close to one another in the raw high dimensional data are close in the reduced data space. Importantly, tSNE can be used as a piece of many different workflows. It can be used independently to visualize an entire data file in an exploratory manner, as a preprocessing step in anticipation of clustering, or in other related workflows. Please see the references section for more details on the tSNE algorithm and its potential applications [1,2].

FlowJo v10 has an extremely powerful native platform for running tSNE. It can be accessed and run through the Populations menu (Workspace tab –> Populations band).

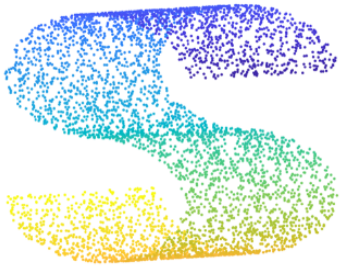
- The native platforms in FlowJo (such as tSNE) *do not* require R.



- https://docs.flowjo.com/flowjo/advanced-features/dimensionality-reduction/tsne/

Dimensions reduction

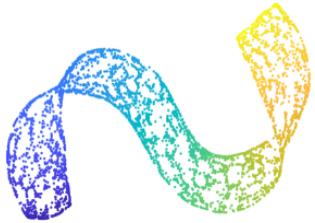
Original (AUC, GS)



t-SNE (**0.18**, 0.18)

UMAP (0.16, 0.13)

TriMap (0.15, **0.80**)

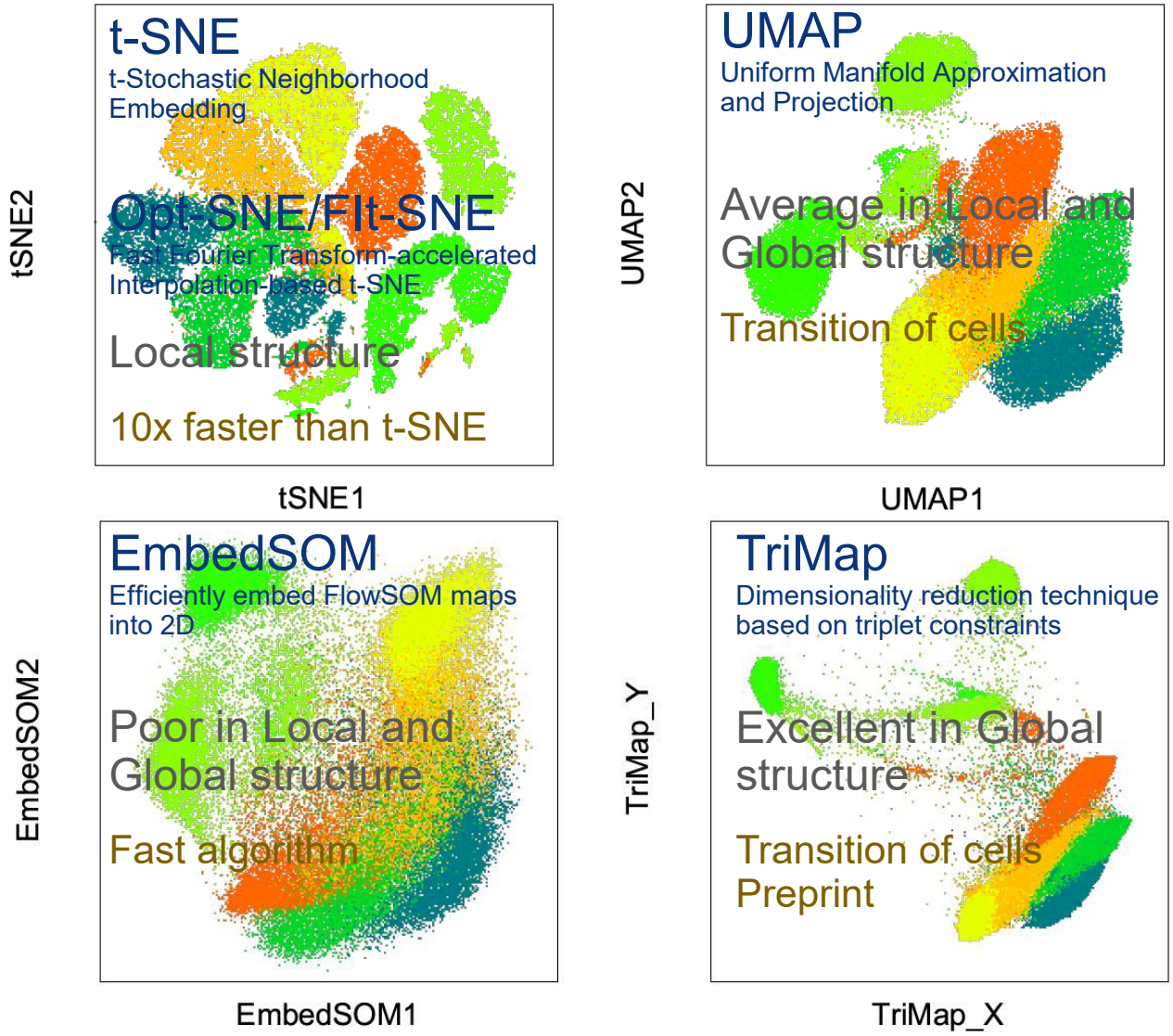


PCA (0.03, 1.00)

AMID, Ehsan; WARMUTH, Manfred K. TriMap: Large-scale dimensionality reduction using triplets. arXiv preprint arXiv:1910.00204, 2019.

Dimensions reduction

„… to create a map.“



Courtesy of B. Kvokačková, R. Fedr

comment

An updated guide for the perplexed: cytometry in the high-dimensional era

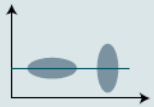
High-dimensional cytometry experiments measuring 20–50 cellular markers have become routine in many laboratories. The increased complexity of these datasets requires added rigor during the experimental planning and the subsequent manual and computational data analysis to avoid artefacts and misinterpretation of results. Here we discuss pitfalls frequently encountered during high-dimensional cytometry data analysis and aim to provide a basic framework and recommendations for reporting and analyzing these datasets.

Thomas Liechti, Lukas M. Weber, Thomas M. Ashhurst, Natalie Stanley, Martin Prlic, Sofie Van Gassen and Florian Mair

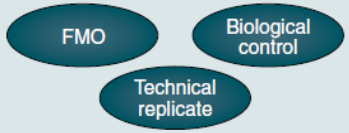
Steps

- Panel design
- Experimental design
- Instrument settings
- Sample acquisition

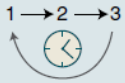
Sample acquisition



Understand impact of SE



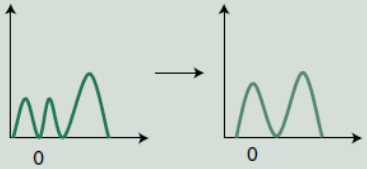
Include relevant control samples



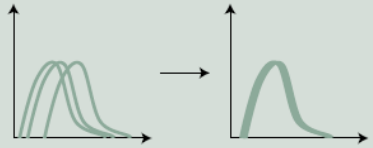
Ensure maximal standardization

- Compensation
- Transformation
- QC: signal stability
- QC: batch effects
- Removal of irrelevant events (for example, doublets, dead cells, etc.)

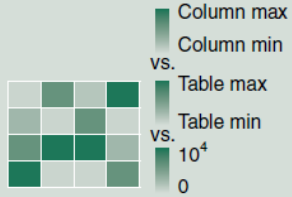
Data preprocessing



Appropriate data transformations



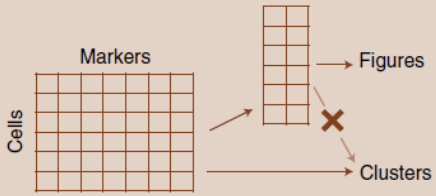
Normalize only when necessary



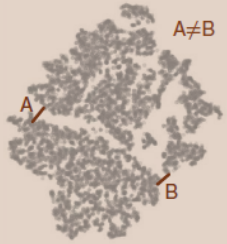
Relevant scaling of values

- Dimensionality reduction for visualization
- Clustering
- QC: cluster quality
- Supervised cell labeling

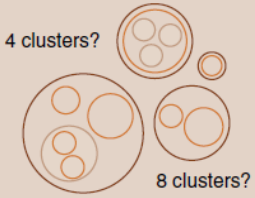
Population identification



Cluster in high-dimensional space, visualize in 2D



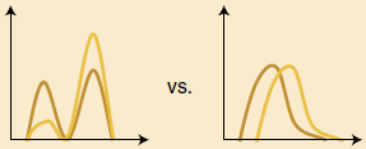
Do not overinterpret distances in 2D spaces



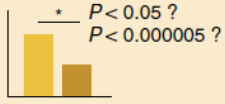
Cluster resolution depends on context

- Differential analysis
- Predictive models (for example, diagnosis/ prognosis)
- Sample-level clustering and dimensionality reduction

Downstream statistics



Differential abundance vs. differential state



Multiple testing limitations

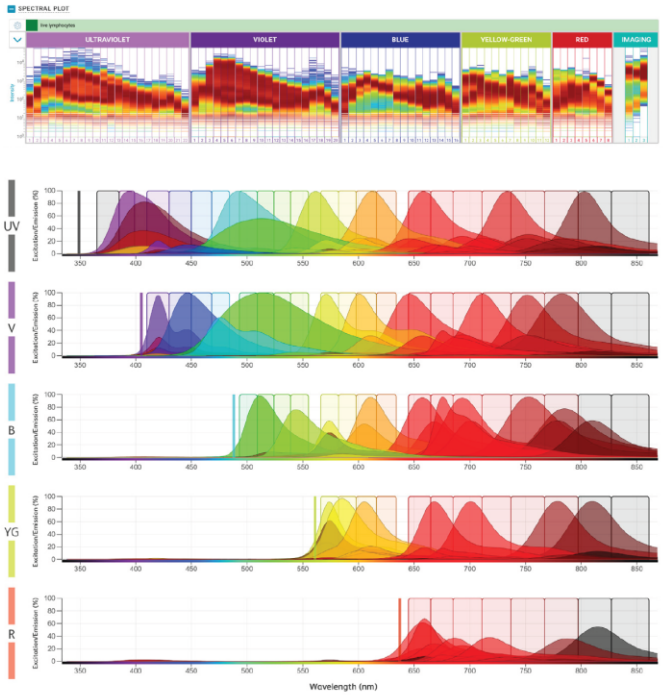
Verify resulting hypotheses

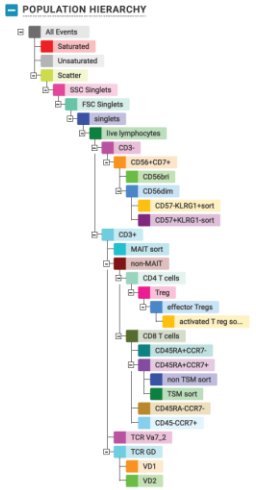
Six-way sorting of deep immunophenotyping panel

This 38-color spectral panel characterizes and sorts deep lineages of T cell and NK cell subsets.

The panel includes BD Horizon RealYellow™ and BD Horizon RealBlue™ Dye technology, engineered to work in tandem with the BD FACSDiscover™ S8 Cell Sorter for high-parameter spectral analysis to reveal biological information.

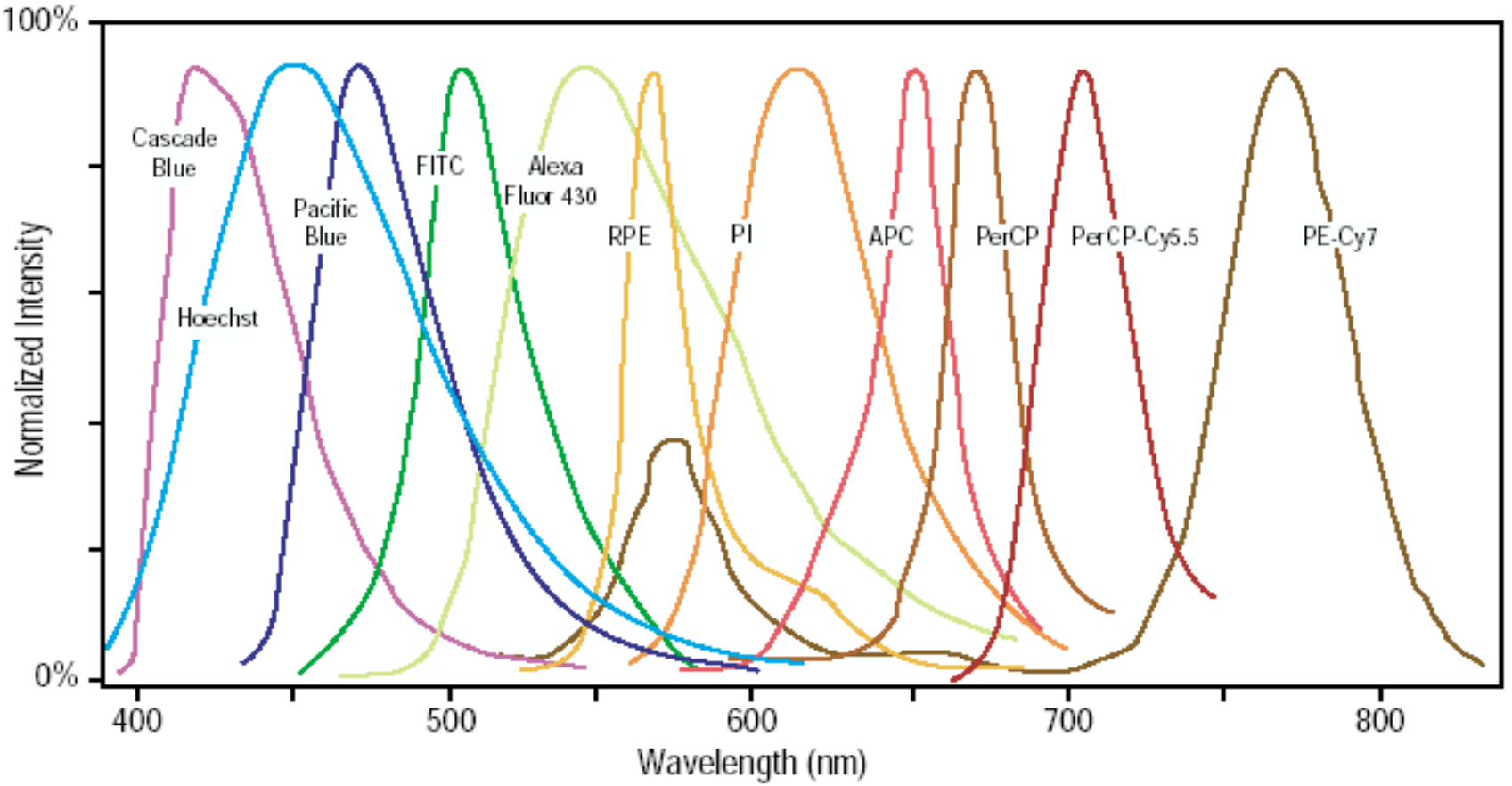
Laser	#	Fluorochrome	Marker
UV	1	BUV395	CD27
	2	FVS440UV	FVS440UV
	3	BUV496	CD8
	4	BUV563	CD16
	5	BUV615	CCR7 (CD197)
	6	BUV663	NKG2C
	7	BUV737	CCR5
	8	BUV805	CD161
Violet	9	BV421	PD1
	10	V450	CD7
	11	BV480	CD45RA
	12	BV510	CD15s
	13	BV570	CD57
	14	BV605	TCRgd
	15	BV650	TCR V-α24
	16	BV711	NKG2A
	17	BV750	NKG2D
	18	BV786	CD28
Blue	19	BB515	HLA-DR
	20	BB630	CD94
	21	BB660	CD194
	22	PerCP-Cy5.5	TCR Vg9
	23	BB700	TCR Vα7.2
	24	BB755	CD196
	25	RB780	CD95
	26	RB545	CD3
Yellow/Green	27	PE	CD25
	28	PE-Cy5	CD185
	29	PE-Cy7	CD38
	30	RY586	KLRG1
	31	PE-Fire 810	CD39
	32	PE-eFluor 610	TCR VD1
	33	PE-Fire 700	CD127
Red	34	APC	TCR VD2
	35	R718	CD183
	36	APC-H7	CD4
	37	SNIR-685	CD56
	38	APC-Fire 810	CD14_CD19



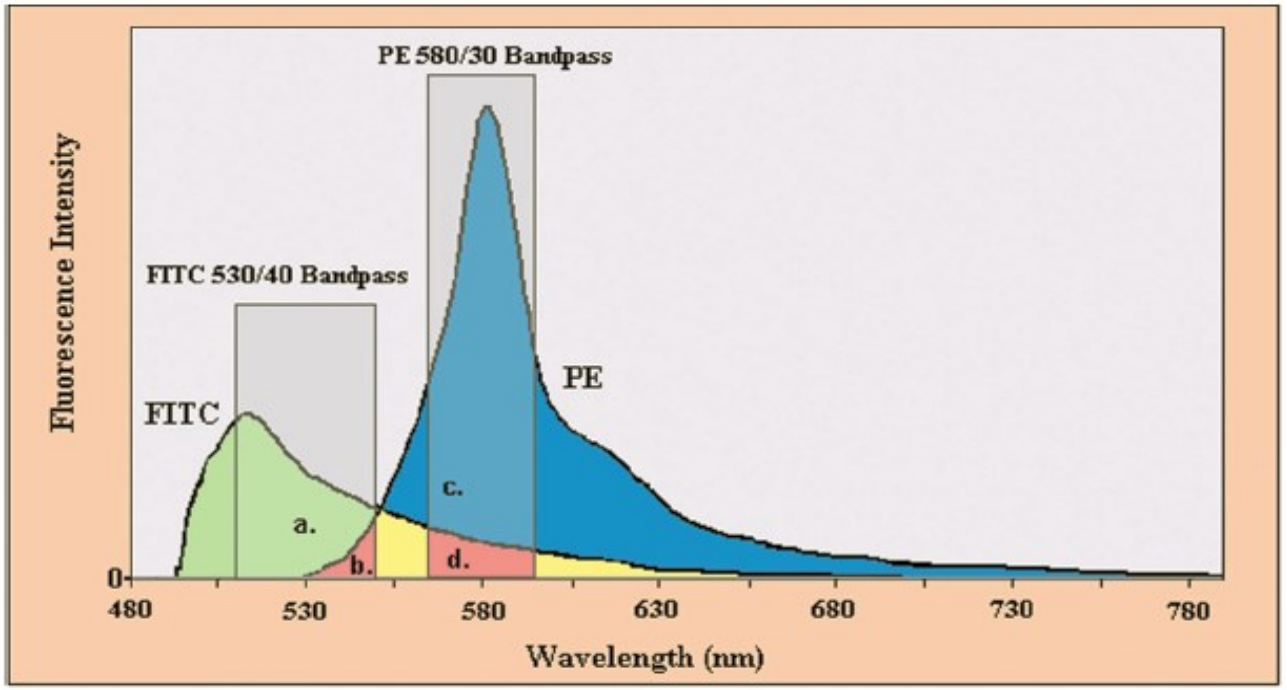


APC
APC-H7
APC-Fire™ 810
BB515
BB630-P2
BB660-P2
BB700
BB755-P
BUV395
BUV496
BUV563
BUV615
BUV661
BUV737
BUV805
BV421
BV480
BV510
BV570
BV605
BV650
BV711
BV750
BV786
PE
PE-Cy5
PE-Cy7
PE-eFluor 610
PE/Fire™ 700
PE/Fire™ 810
PerCP-Cy5.5
R718
RB545
RB780
RY586
Spark NIR™ 685
V450

Emission Spectra–Spectral Overlap



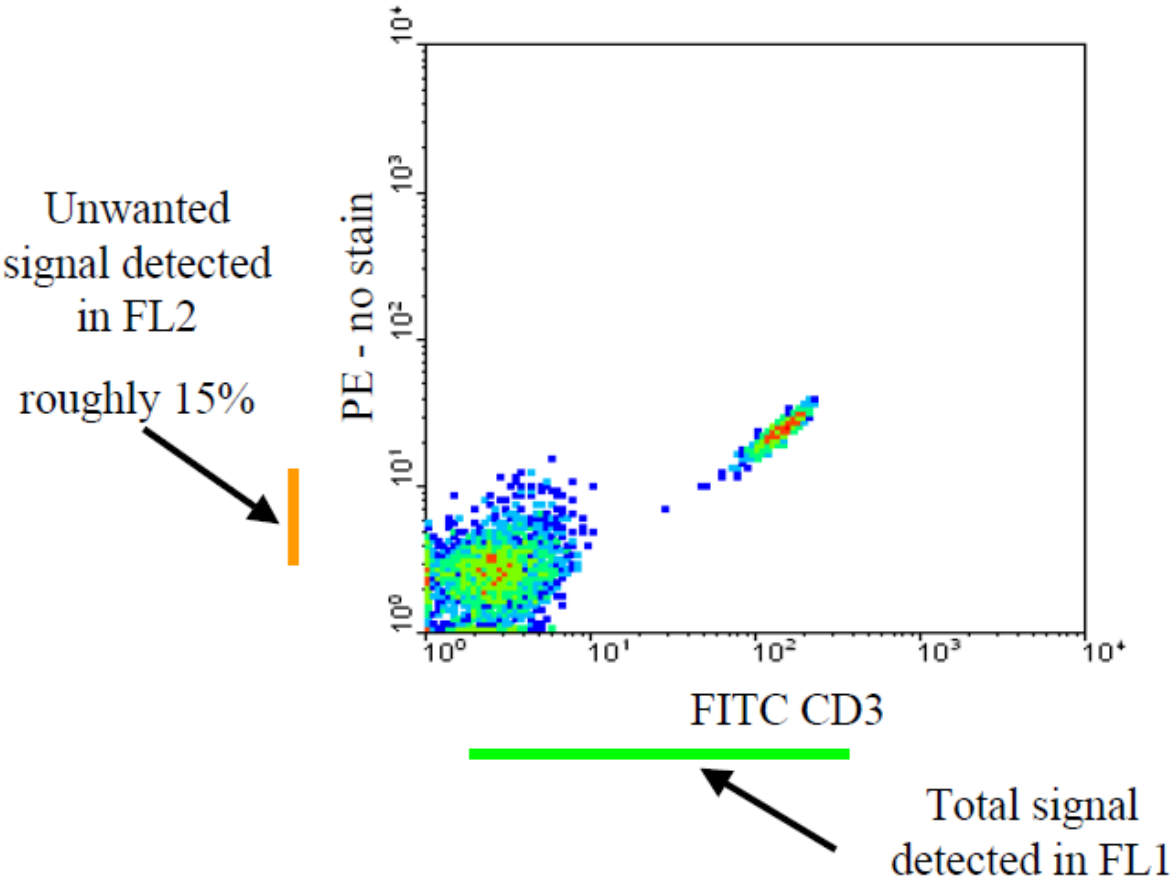
What is the problem with multi-color detection?



Fluorescence signal compensation in multicolor detection

- A process in which all fluorescence signals are eliminated except for the fluorochrome signal to be detected on the detector
- Adjustment using a mix of microparticles or cells labeled/unlabeled with the appropriate fluorochromes.

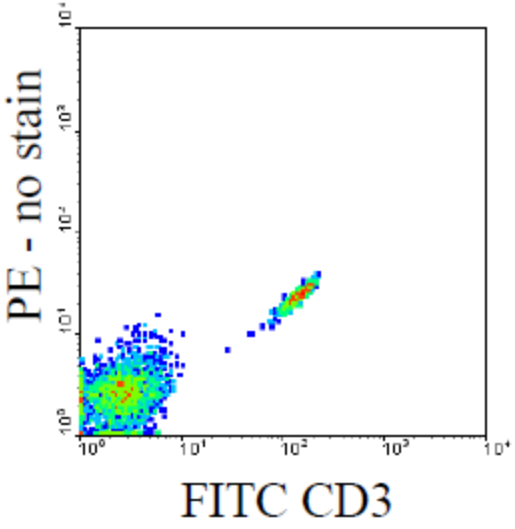
Uncompensated FITC Single stain Control



Jennifer Wilshire, PhD
jennifer@flowjo.com

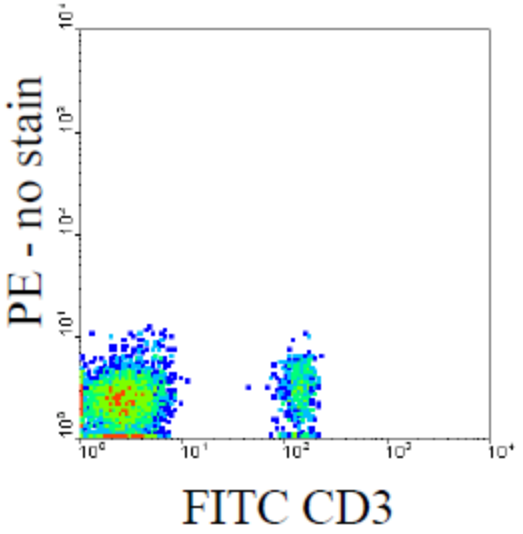
FITC Single Stain Control

Uncompensated



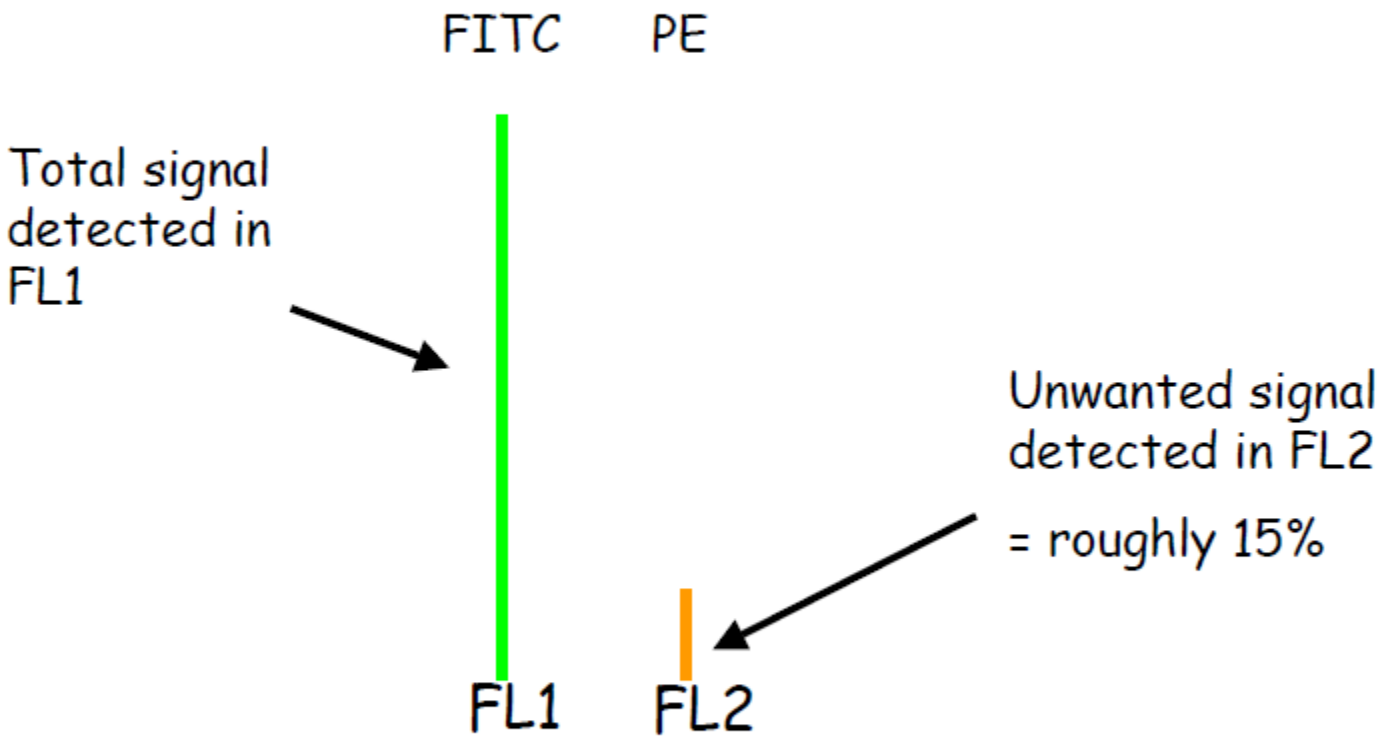
FL2-15%FL1

Compensated



Jennifer Wilshire, PhD
jennifer@flowjo.com

FITC Single Stain Control



True PE = Total FL2 – 15% FL1

Jennifer Wilshire, PhD
jennifer@flowjo.com

Choices for 6,- 8,- 10,- and more colors

6-color	8-color	10-color	Additional
FITC or Alexa 488	FITC or Alexa 488	FITC or Alexa 488	FITC or Alexa 488
PE	PE	PE	PE
		PE-Texas Red or PE-Alexa 610	PE-Texas Red or PE-Alexa 610
PerCP-Cy5.5	PerCP-Cy5.5	PerCP-Cy5.5	PerCP-Cy5.5
PE-Cy7	PE-Cy7	PE-Cy7	PE-Cy7
APC or Alexa 647	APC or Alexa 647	APC or Alexa 647	APC or Alexa 647
		Alexa 680 or 700	Alexa 680 or 700
APC-Cy7	APC-Cy7	APC-Cy7	APC-Cy7
	AmCyan	AmCyan	AmCyan
	Pacific Blue	Pacific Blue	Pacific Blue
			Q-dot 655, 705…

Fluorochrome selection considerations

"Bright" antibodies go on "dim" fluorochromes

Avoid spillover from bright cell populations into channels requiring high sensitivity

Beware of tandem dye degradation

Various fluorochromes-stain index

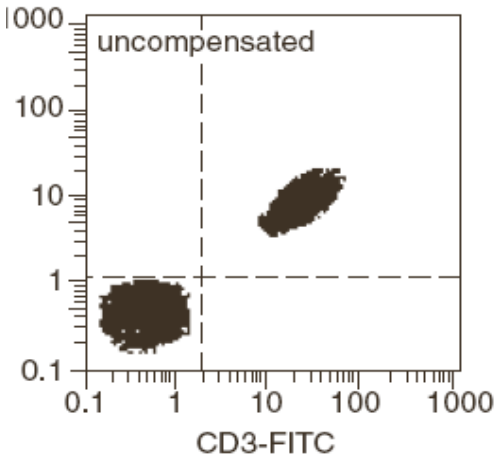
Reagent	Clone	Filter	Stain Index
PE	RPA-T4	585/40	356.3
Alexa 647	RPA-T4	660/20	313.1
APC	RPA-T4	660/20	279.2
PE-Cy7	RPA-T4	780/60	278.5
PE-Cy5	RPA-T4	695/40	222.1
PerCP-Cy5.5	Leu-3a	695/40	92.7
PE-Alexa 610	RPA-T4	610/20	80.4
Alexa 488	RPA-T4	530/30	75.4
FITC	RPA-T4	530/30	68.9
PerCP	Leu-3a	695/40	64.4
APC-Cy7	RPA-T4	7801/60	42.2
Alexa 700	RPA-T4	720/45	39.9
Pacific Blue	RPA-T4	440/40	22.5
AmCyan	RPA-T4	525/50	20.2

Kompenzace fluorescenčního signálu

#2

FITC positive & negative

PE negative beads



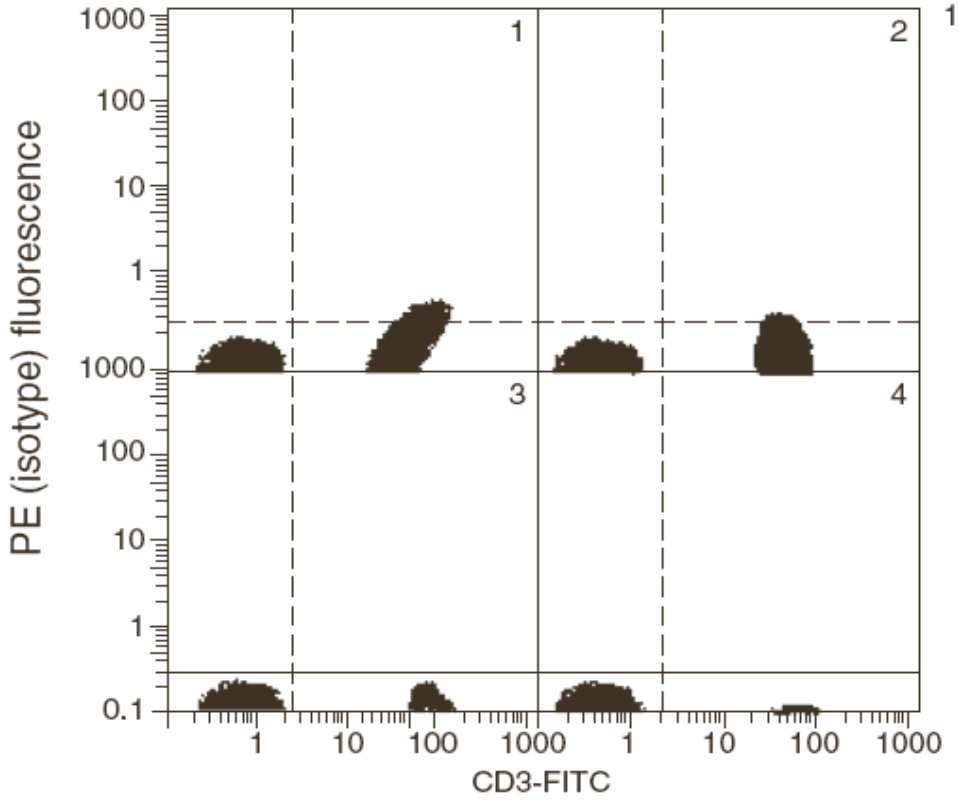
Current Protocols in Cytometry

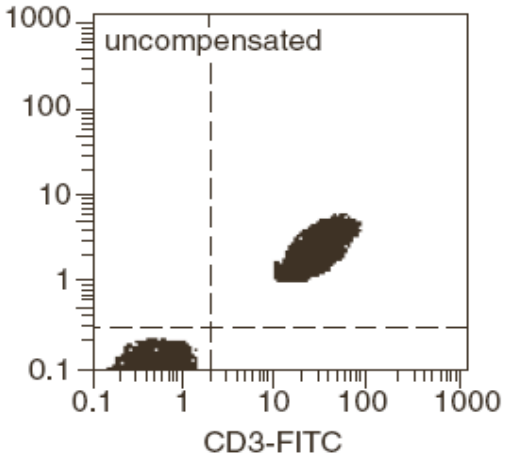
Kompenzace fluorescenčního signálu

FITC positive & negative

PE negative beads

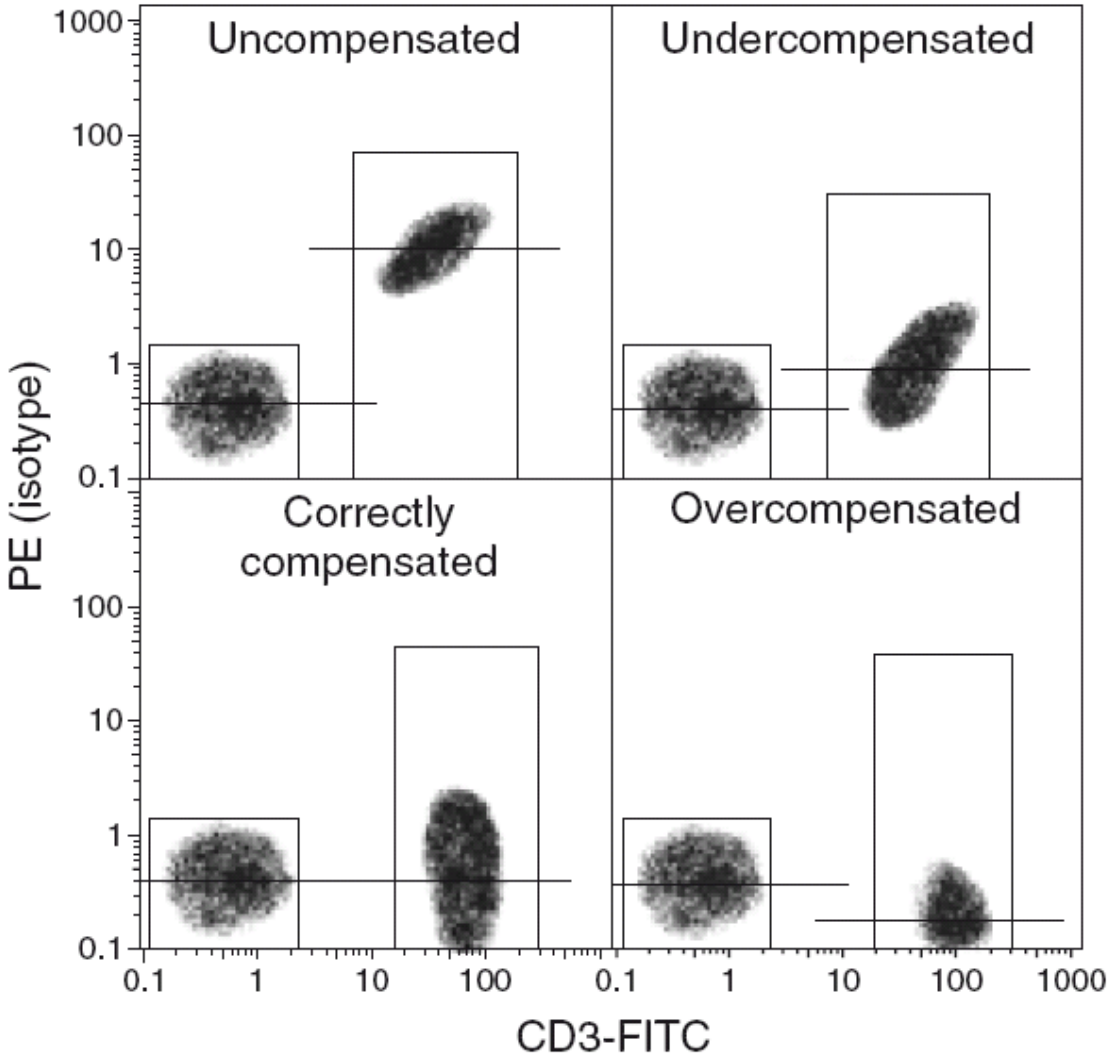
NONE!



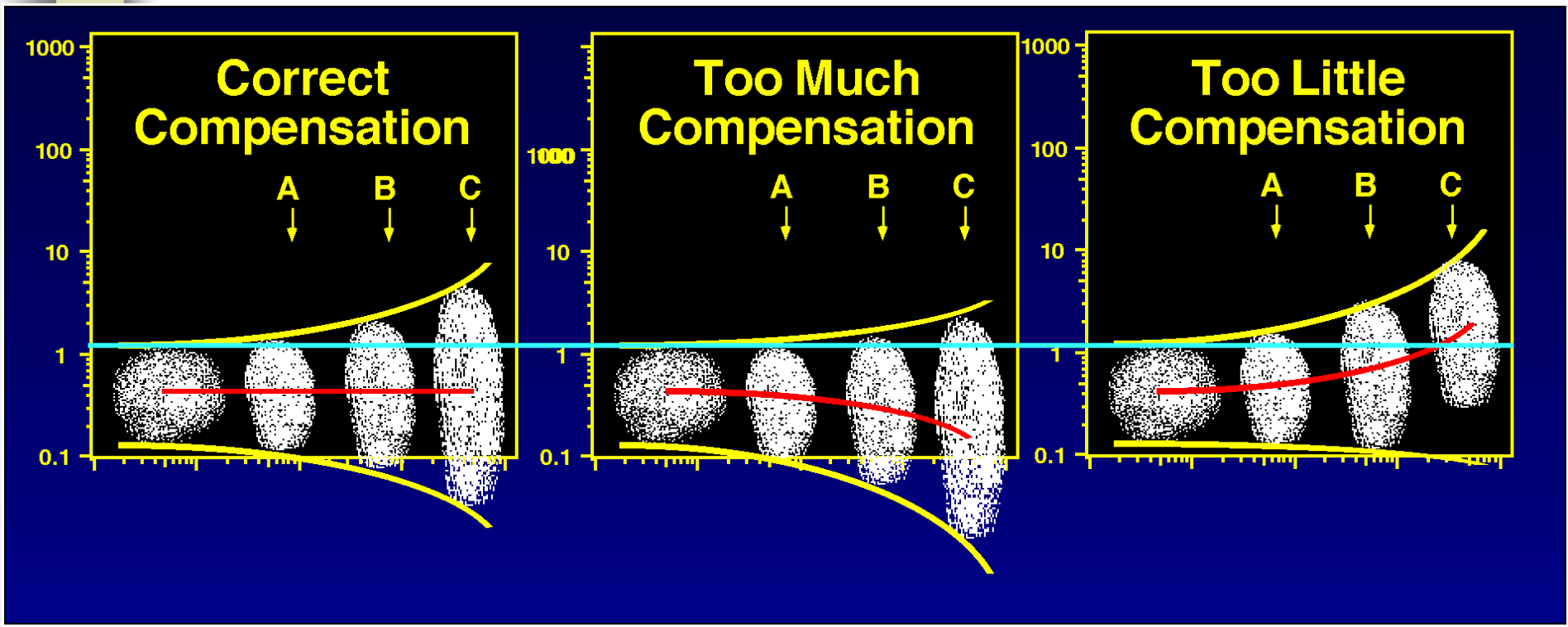


Current Protocols in Cytometry

Kompenzace fluorescenčního signálu



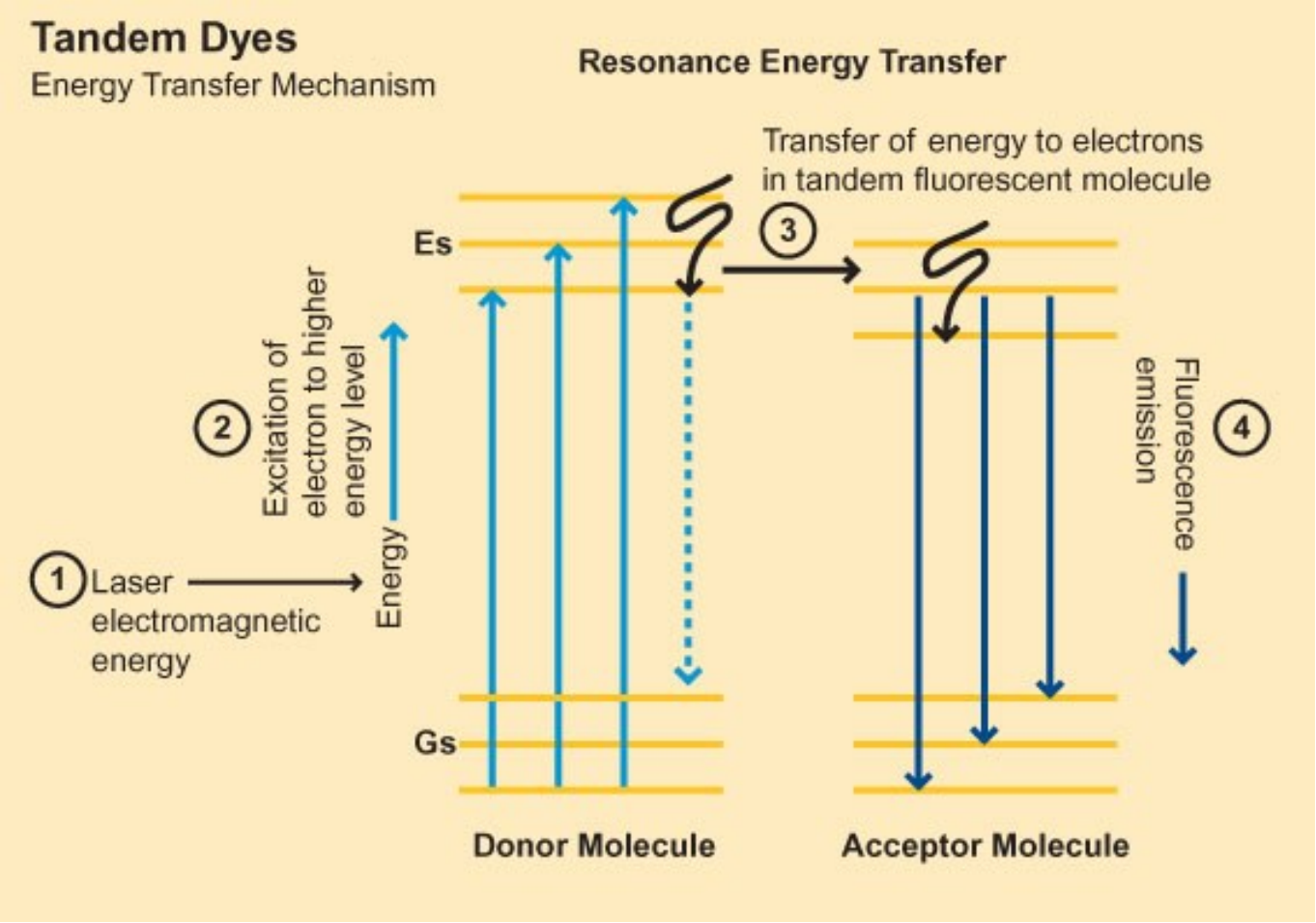
Which marker for compensation?



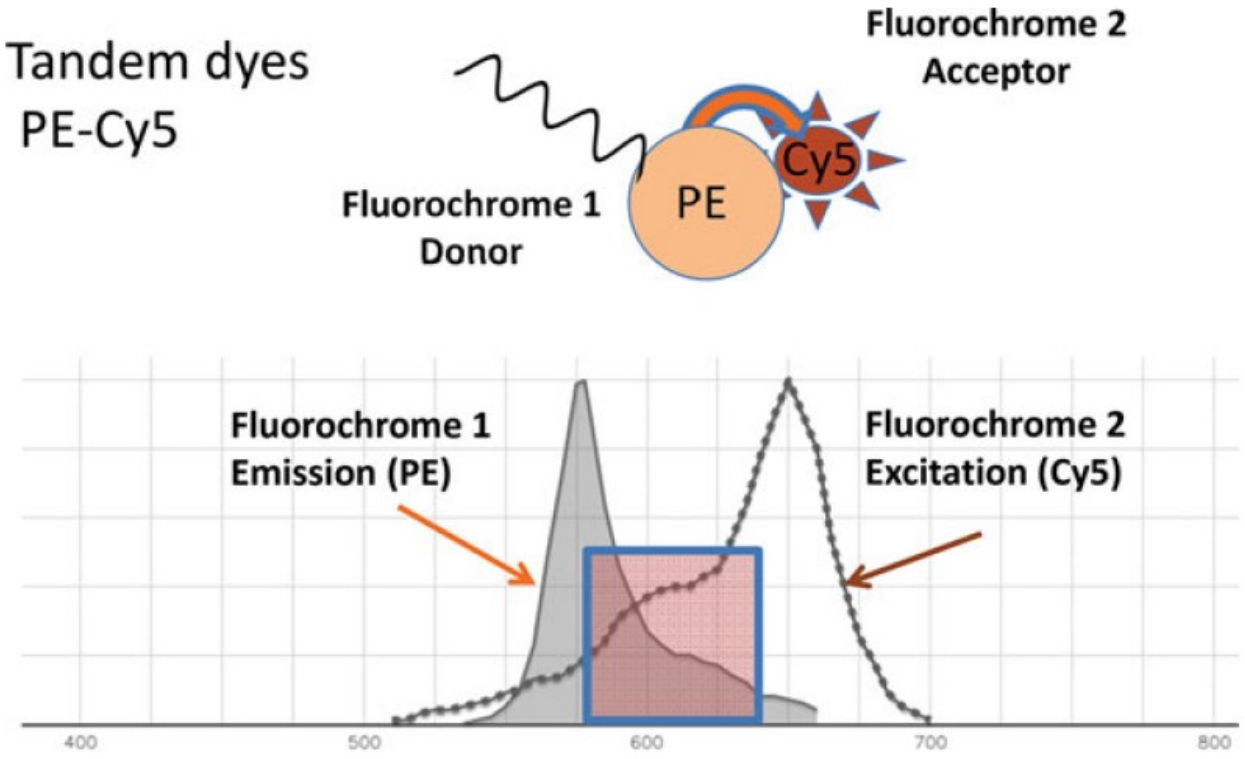
Small errors in compensation of a dim control (A) can result in large compensation errors with bright reagents (B & C).

Use bright markers to set up proper compensation .

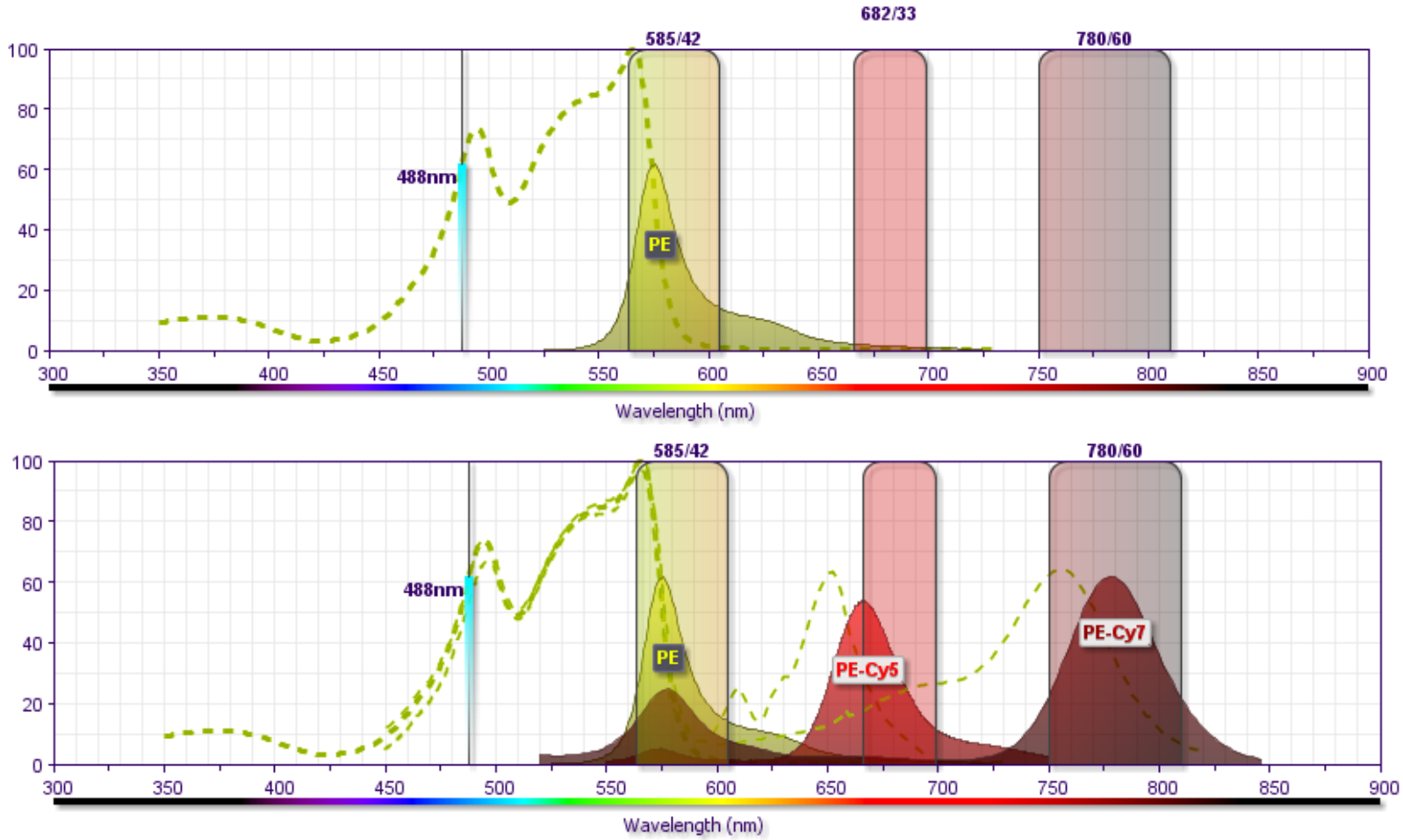
Tandem Dyes


Tandem Dyes
Energy Transfer Mechanism
Resonance Energy Transfer
Transfer of energy to electrons in tandem fluorescent molecule
3
Es
2
Excitation of electron to higher energy level
Energy
1
Laser electromagnetic energy
Fluorescence emission
4
Gs
Donor Molecule
Acceptor Molecule

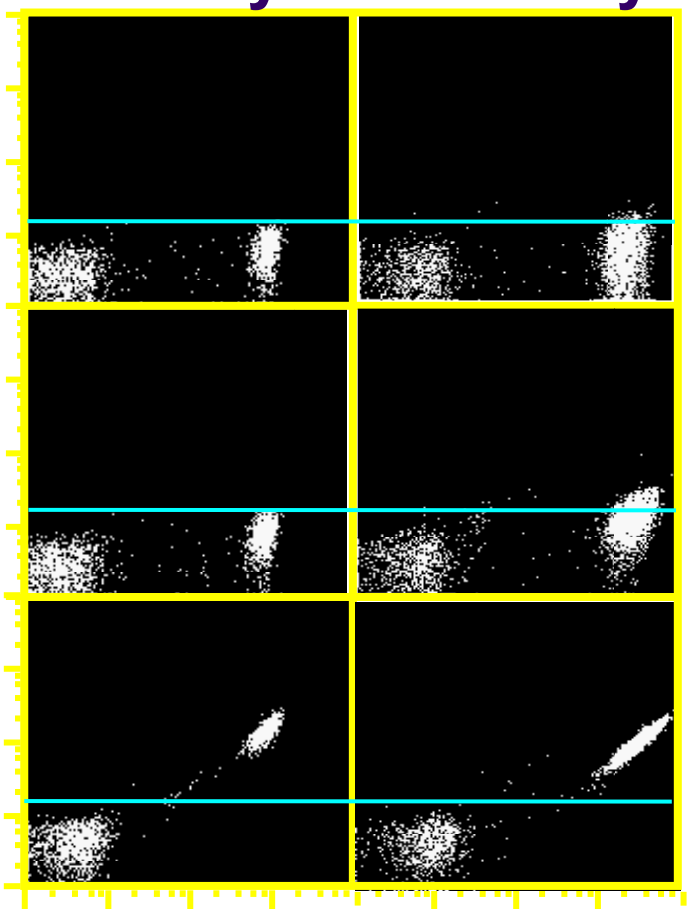
Tandem Dyes

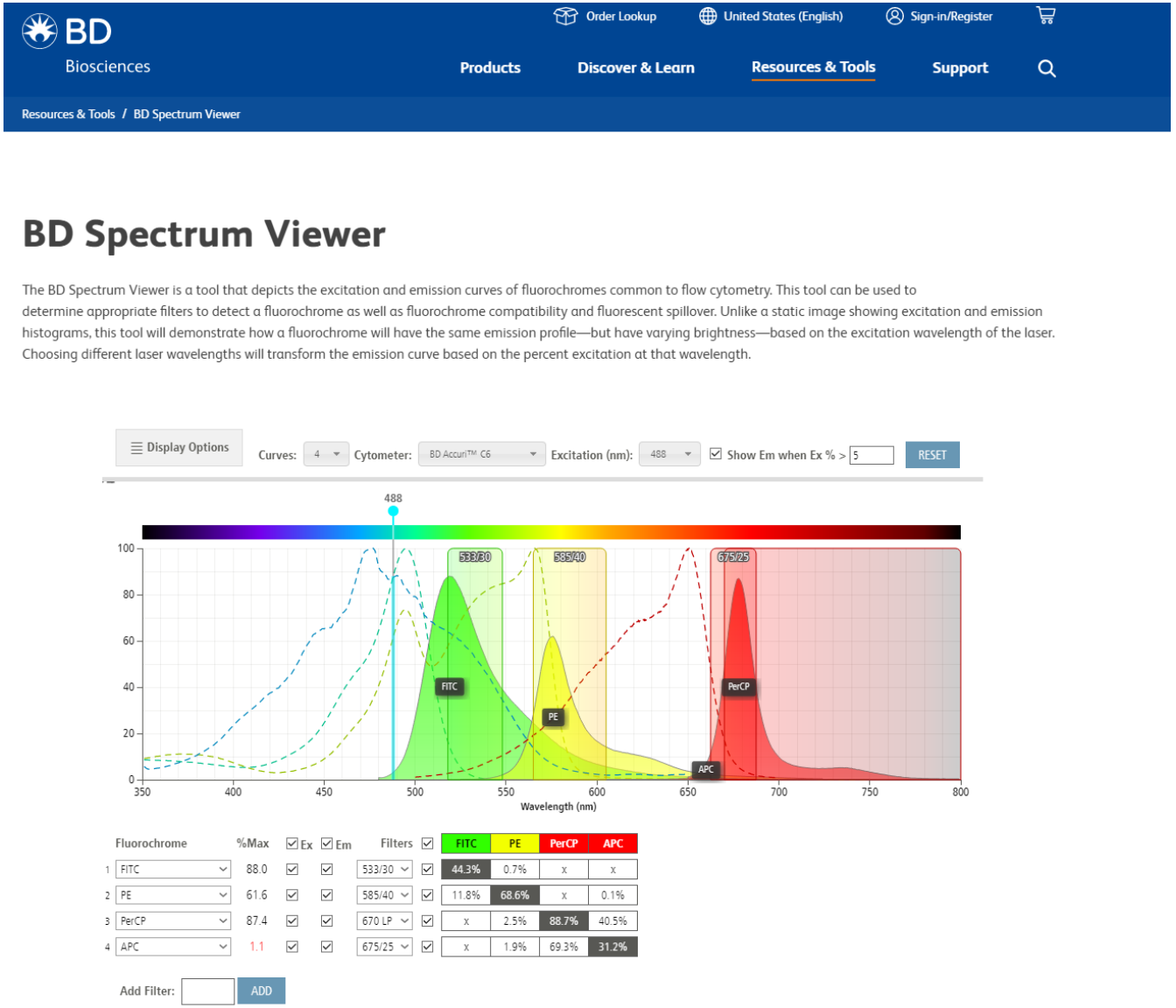

Tandem dyes
PE-Cy5
Fluorochrome 2
Acceptor
Cy5
PE
Fluorochrome 1
Donor
Fluorochrome 1
Emission (PE)
Fluorochrome 2
Excitation (Cy5)
400
500
600
700
800

Tandem Dyes - examples



Tandems are light sensitive





https://www.bdbiosciences.com/en-us/resources/bd-spectrum-viewer

Factors that Effect Compensation

- Reagent Lot-to-Lot Variation
- Fluorochrome Stability
- Sample-to-Sample Variation
- Assay Staining Conditions

- Another solution ?

  #1

The Idea (1931)

" Simplicissimus Karl Arnold Mobile Telephony" by Source (WP:NFCC#4). Licensed under Fair use via Wikipedia

Invention (1973)

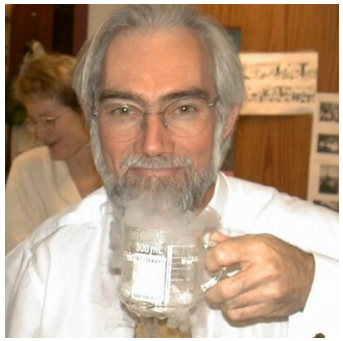
Martin Cooper , Motorola

Innovation (2007)

Steve Jobs , Apple

Spectral flow cytometry

JP Robinson, Purdue University



Cytometry Part A • 81A: 35–44, 2012

ORIGINAL ARTICLE

Cytometry PART A Journal of the International Society for Advancement of Cytometry

MIFlowCyt

Hyperspectral Cytometry at the Single-Cell Level Using a 32-Channel Photodetector

Gérald Grégori,[1,2] Valery Patsekin,[1,3] Bartek Rajwa,[1,3] James Jones,[4] Kathy Ragheb,[1,3] Cheryl Holdman,[1,3] J. Paul Robinson[1,3,4]*

2
DOI: 10.1017/S1431927605510328

Microsc Microanal 11(Suppl 2), 2005

Multispectral Flow Cytometry: Next Generation Tools For Automated Classification

J. Paul Robinson[a,b], Valery Patsekin[a] , Gerald Gregori[a], Bartek Rajwa[a,b], and James Jones[a,b]

[a]Department of Basic Medical Science, School of Veterinary Medicine, and [b]Weldon Department of Biomedical Engineering, Purdue University, West Lafayette, IN, 47907, USA

US007280204B2

(12) **United States Patent**
Robinson et al.

(10) **Patent No.: US 7,280,204 B2**
(45) **Date of Patent: Oct. 9, 2007**

(54) **MULTI-SPECTRAL DETECTOR AND ANALYSIS SYSTEM**

(75) Inventors: **Joseph Paul Robinson**, West Lafayette, IN (US); **Bartlomiej Rajwa**, West Lafayette, IN (US); **Gérald Grégori**, Marseille (FR); **Valery Patsekin**, West Lafayette, IN (US)

(73) Assignee: **Purdue Research Foundation**, West Lafayette, IN (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 139 days.

5,394,237 A	2/1995	Chang et al.	188/79.51
5,422,712 A	6/1995	Ogino	356/73
5,675,517 A	10/1997	Stokdijk	702/85
5,719,667 A *	2/1998	Miers	356/73
6,249,341 B1 *	6/2001	Basiji et al.	356/73
6,630,307 B2 *	10/2003	Bruchez et al.	435/6
6,885,440 B2 *	4/2005	Silcott et al.	356/73
6,947,134 B2 *	9/2005	Chang et al.	356/318
7,057,712 B2 *	6/2006	Beck et al.	356/72

(Continued)

FOREIGN PATENT DOCUMENTS

EP	0 315 939	5/1989

(Continued)

Spectral flow cytometry

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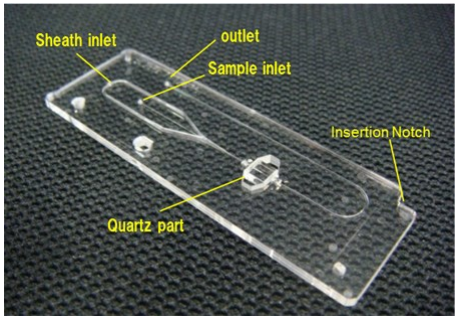
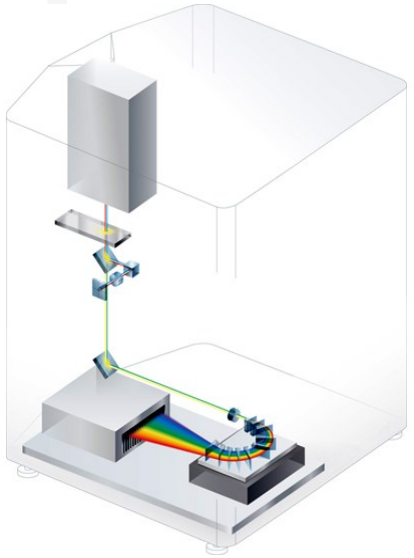
Overview Features Applications Specifications Literature

See Everything

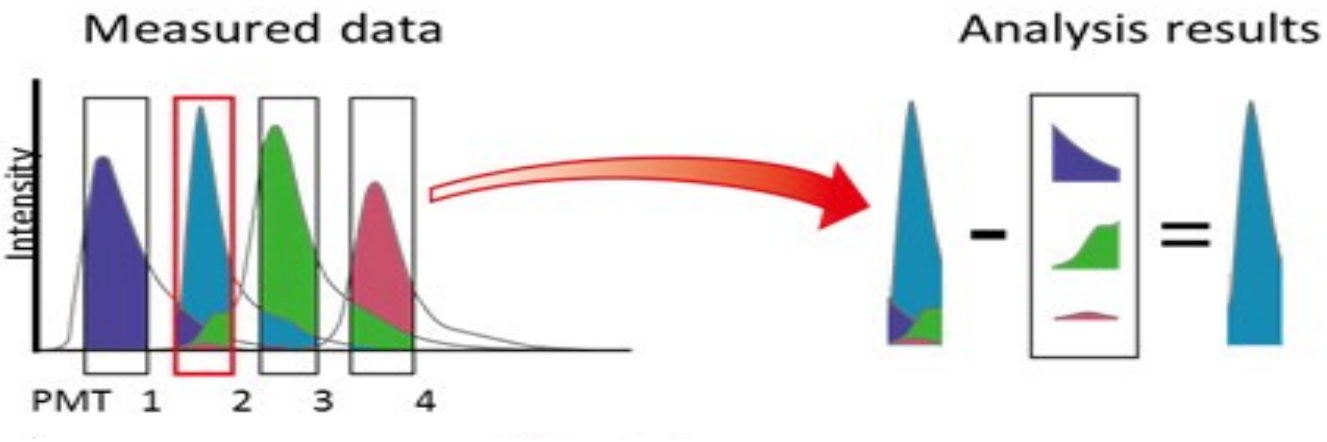
The SP6800 Spectral Analyzer is Sony Biotechnology Inc.'s newest innovative life science system fundamentally expanding the way cell and biomarker analysis can be performed. This system incorporates a unique optical bench, Blu-ray™ disc technology, and advanced algorithms to deliver some of the most accurate and precise data available.

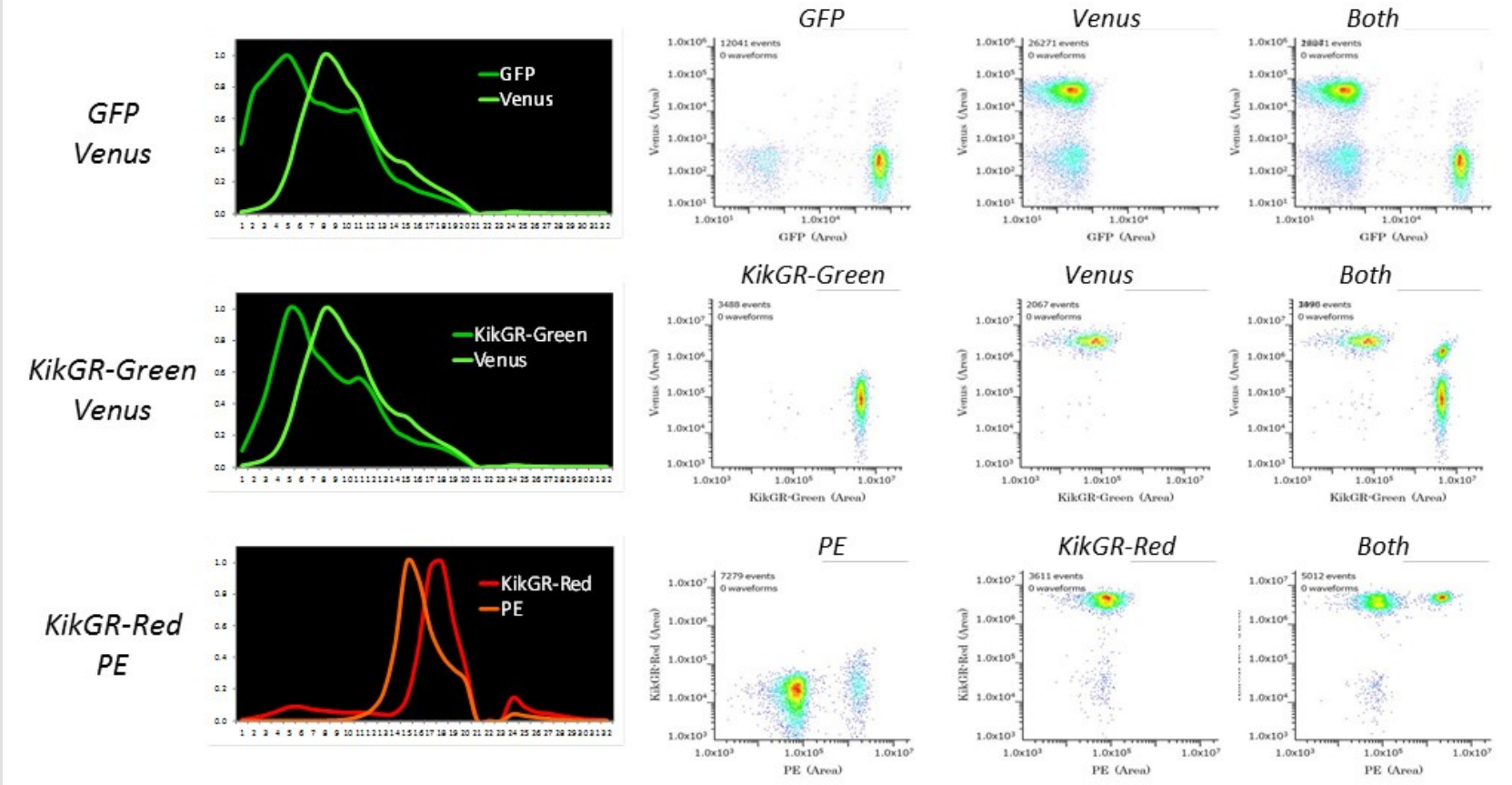
The SP6800 Spectral Analyzer also introduces new Flow Point technology to analyze core stream and sample event location within the flow cell. To improve accuracy of data, this system also provides unique functions to display and analyze cellular autofluorescence and allows the user to easily automatically remove.





Conventional vs. spectral analysis





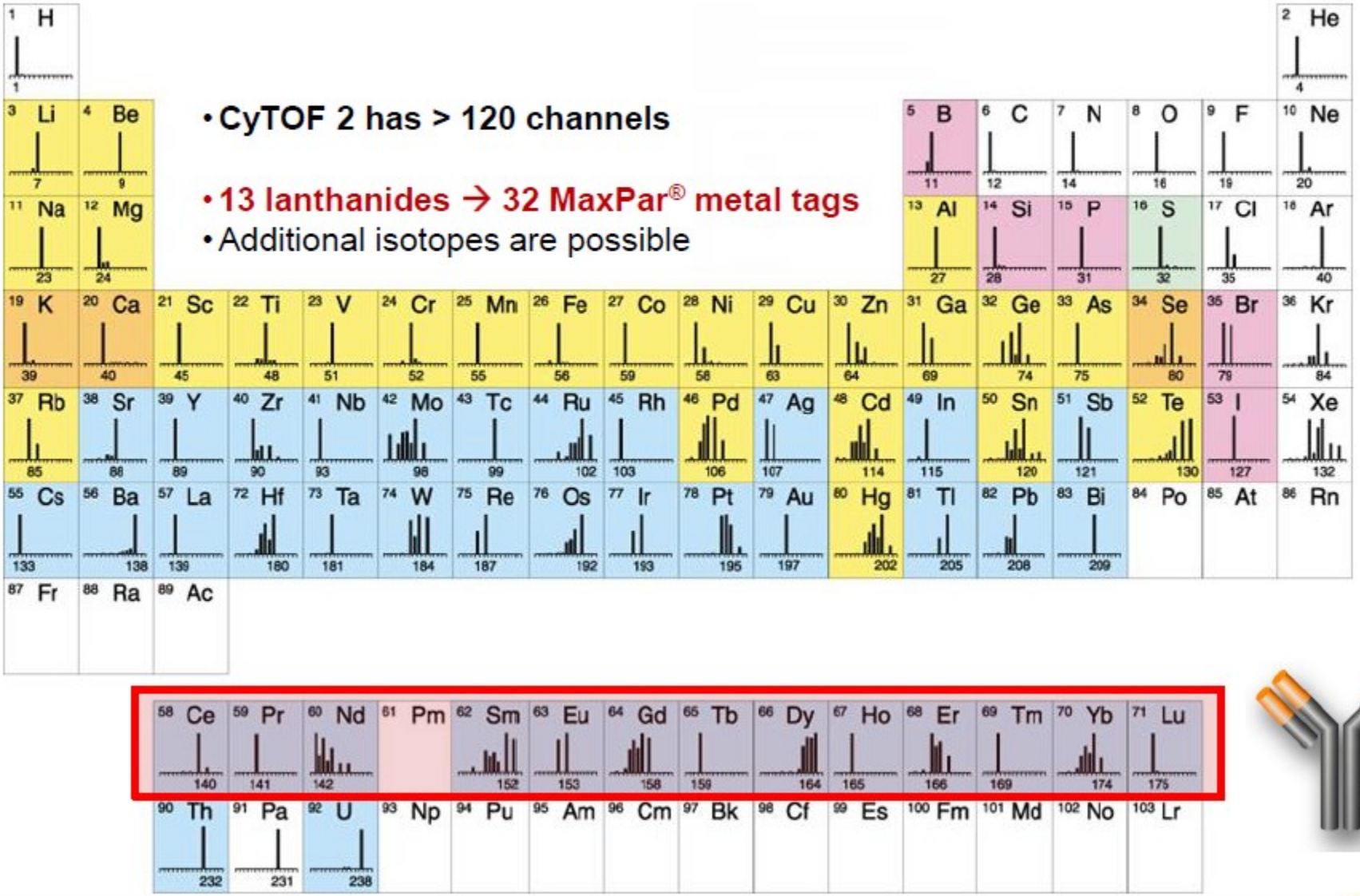
For revealing spatiotemporal regulation of immune cells, fluorescent proteins are very useful, which can be difficult to analyze with traditional flow cytometry technologies. These figures show how easily the SP6800 Spectral Analyzer can separate overlapping spectra of fluorescent proteins and fluorochromes. Data courtesy of M. Tomura of Kyoto University.

http://www.sonybiotechnology.com/images/sp6800/Fluorescent_Proteins_and_Fluorochromes.jpg

- Another solution ?

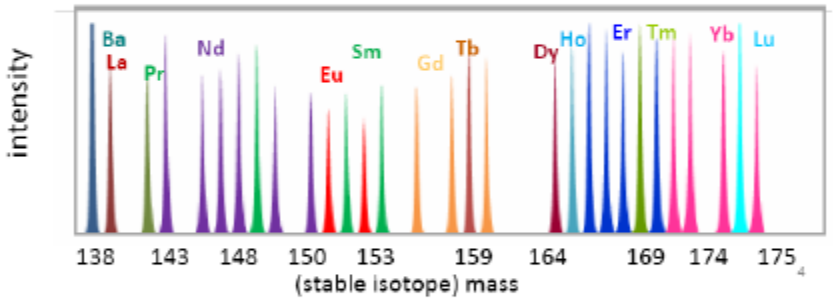
  #2

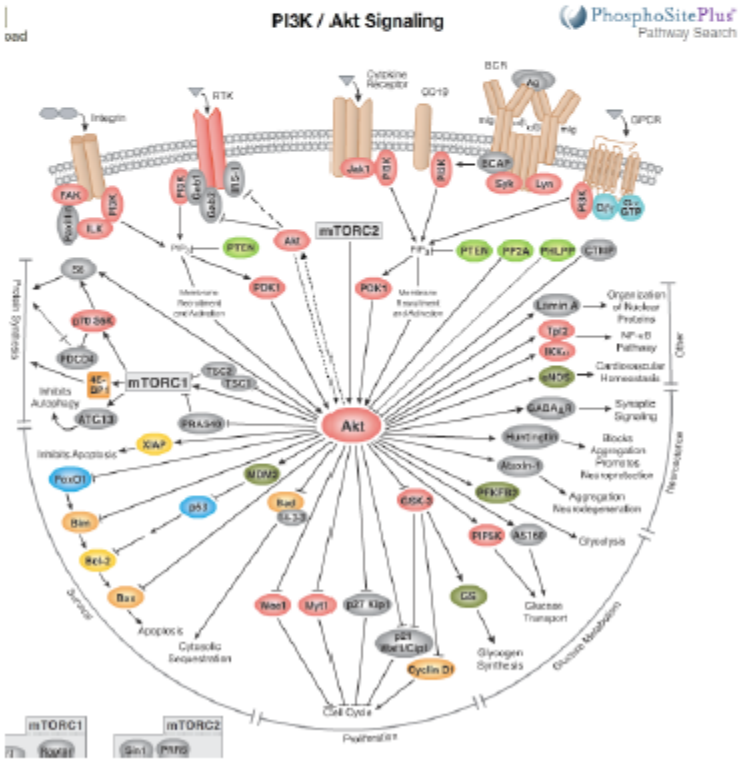
Probing with Isotopes



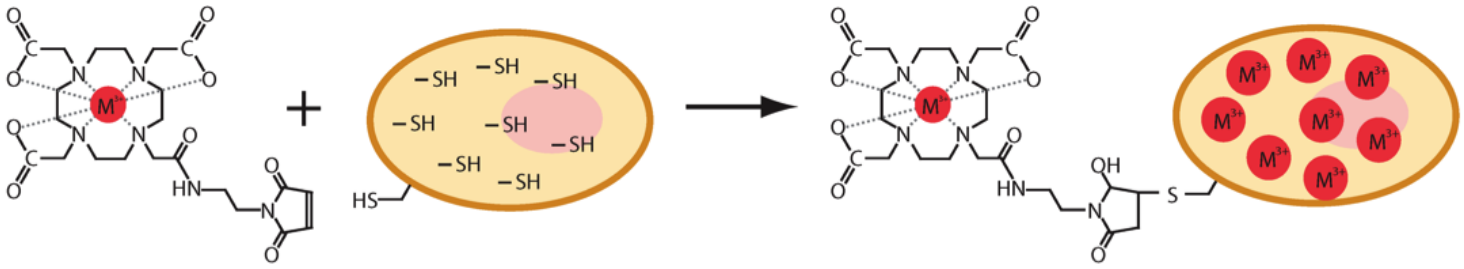
Why Mass Cytometry?

- Highly multi-parametric, on a single cell basis
- Facilitates exploration of complex pathways
- Enables discovery of cellular relationships, responses, and developmental pathways
- Allows deep-profiling of your cell system of interest





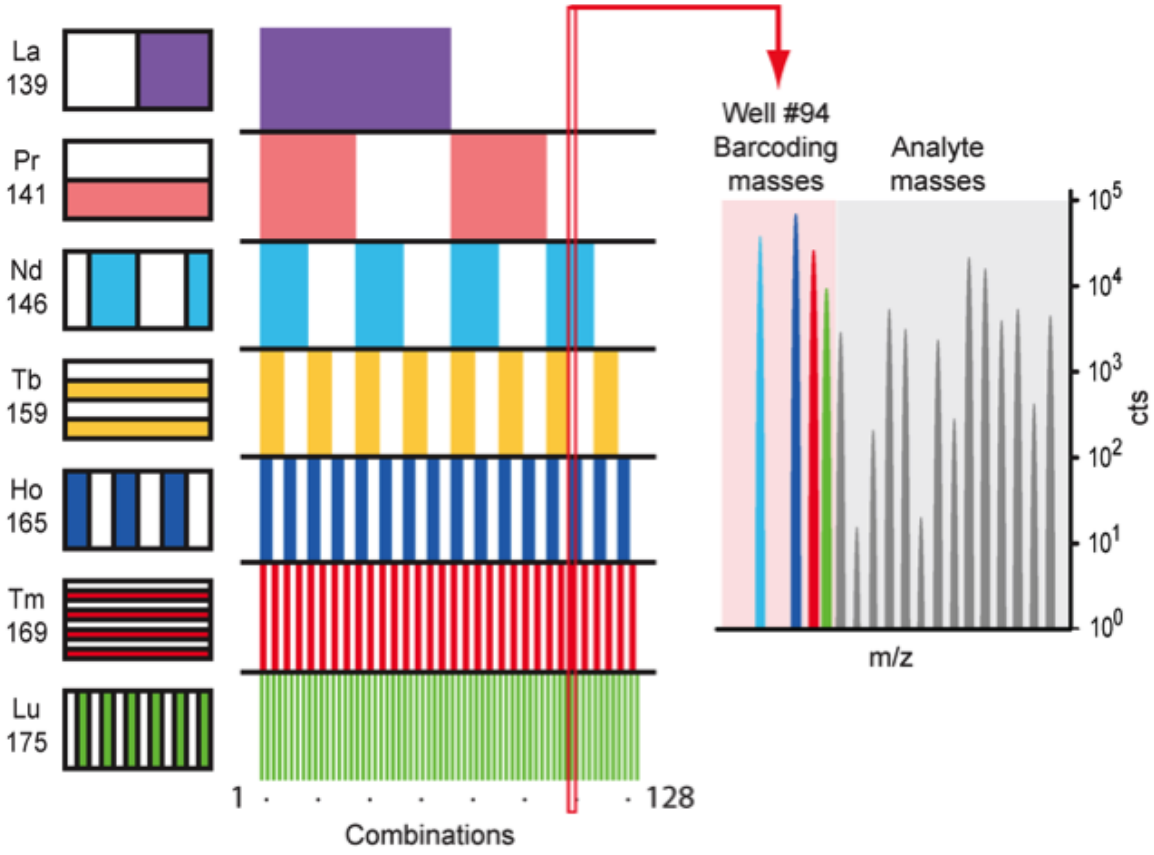
Single Cell Mass Cytometry



Cells were covalently labeled with a bifunctional compound, maleimido-mono-amide-DOTA (mDOTA). This compound can be loaded with a lanthanide(III) isotope ion, and reacts covalently with cellular thiol groups through the maleimide moiety.

Single-Cell Mass Cytometry of Differential Immune and Drug Responses Across a Human Hematopoietic Continuum
Sean C. Bendall, *et al.*
Science **332**, 687 (2011);

Single Cell Mass Cytometry



Seven unique lanthanide isotopes were used to generate 128 combinations, enough to barcode each sample in a 96-well plate. The seven lanthanide isotopes, their masses and their locations on the 96-well plate are shown.

Single-Cell Mass Cytometry of Differential Immune and Drug Responses Across a Human Hematopoietic Continuum
Sean C. Bendall, *et al.*
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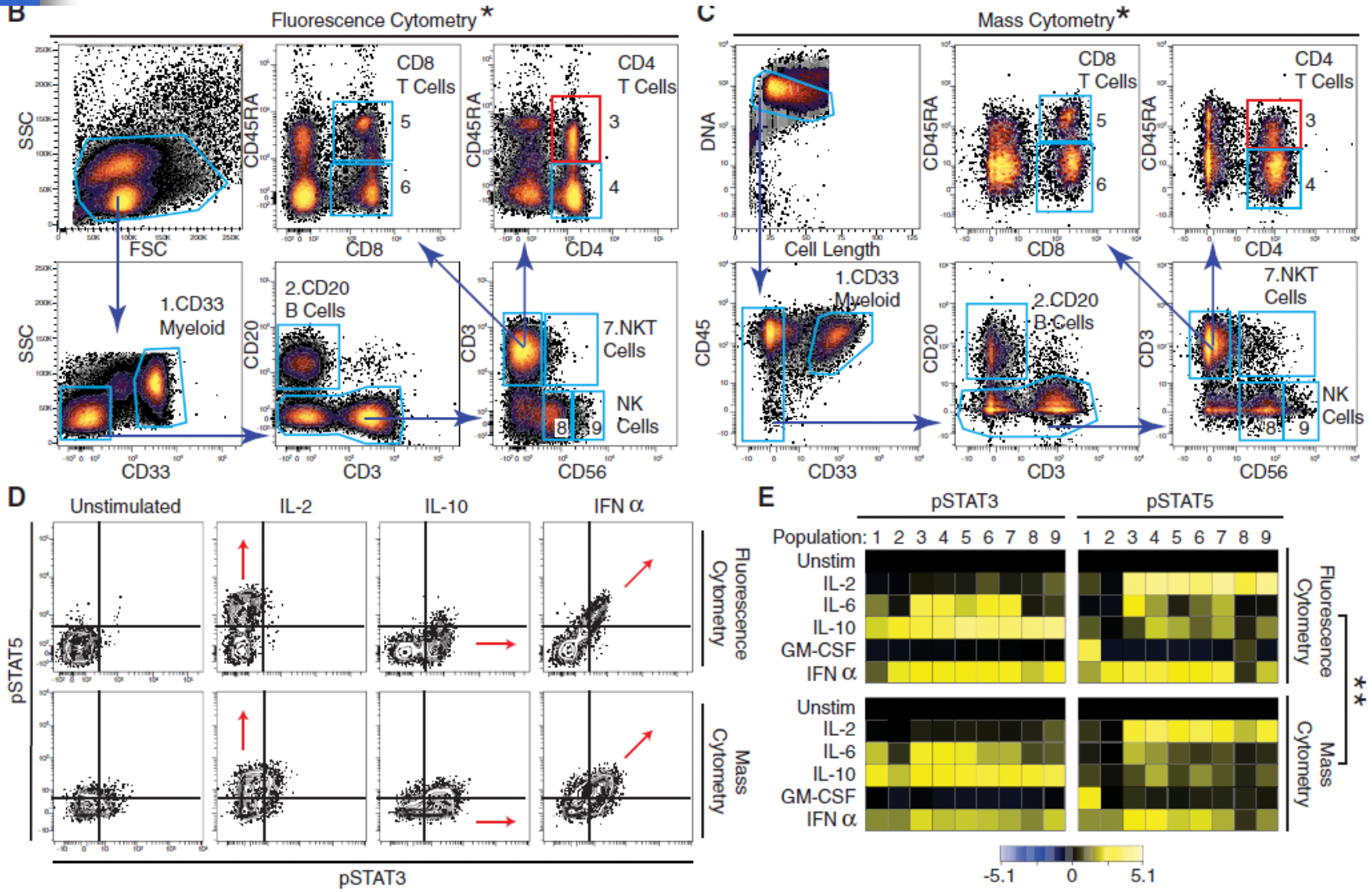
Single Cell Mass Cytometry



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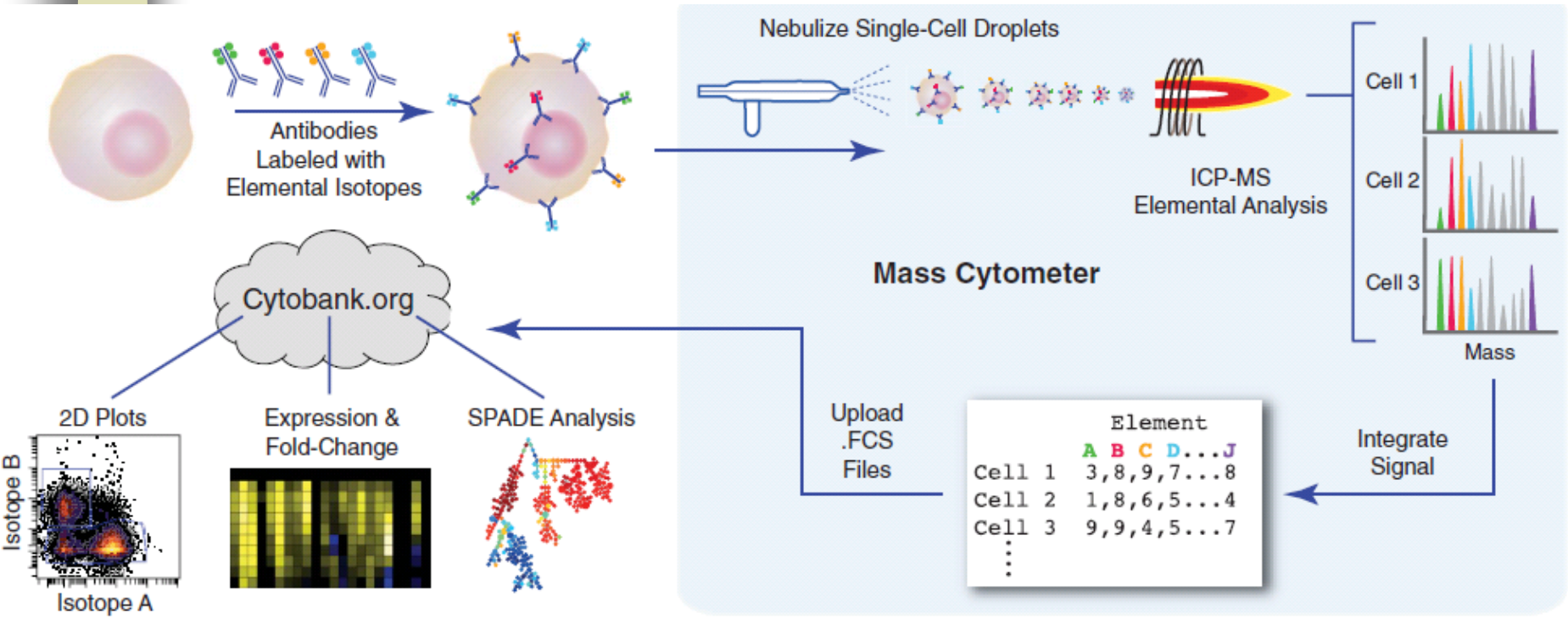
Single Cell Mass Cytometry



Single-Cell Mass Cytometry of Differential Immune and Drug Responses Across a Human Hematopoietic Continuum
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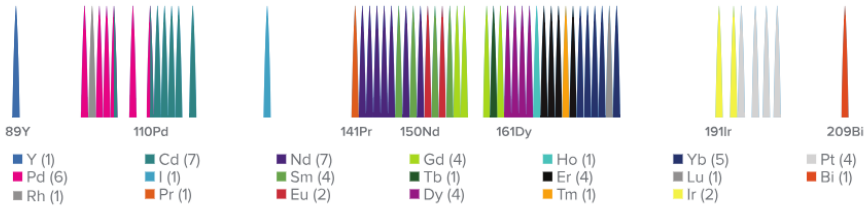


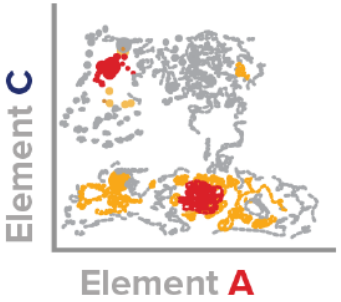
Mass Cytometry: 50+ Parameters on Millions of Cells

Discovery of new biology
Comprehensive functional profiling

Basic research
Drug discovery
Clinical research

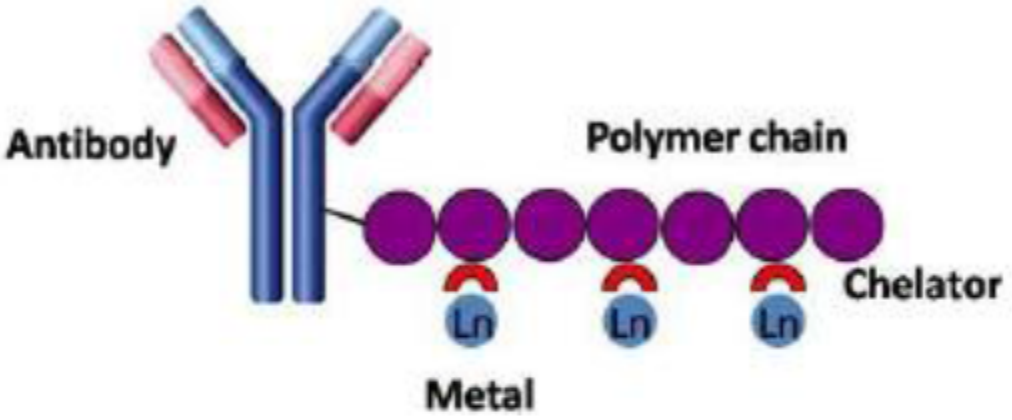
63 Eu

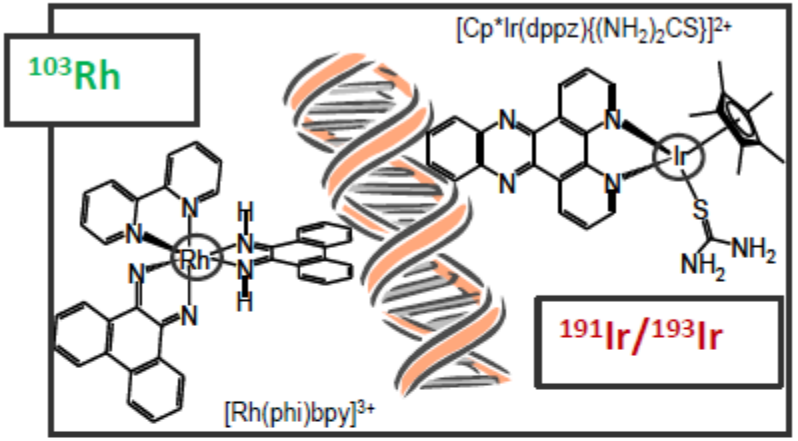
STANDARD BIOTOOLS



FLUIDIGM

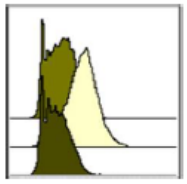
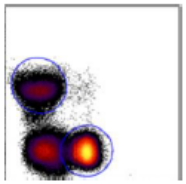
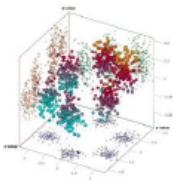
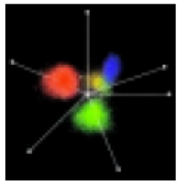
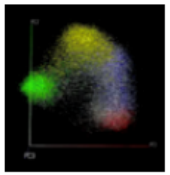
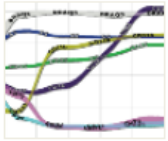
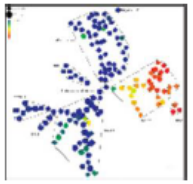
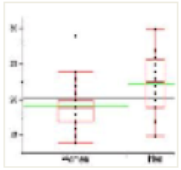
MaxPar® metal-tagged probes

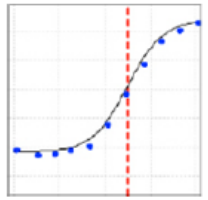




- Lanthanide tags: 32 isotopes from 13 elements
- IgG antibody probes:
  - Pre-conjugated antibodies (220 currently available and growing)
  - MAXPAR® labeling kits (for 32 stable isotopes)
- Nucleic acid-binding metallo-intercalators
  - Identifies single cell events
  - Live/dead indicator

Analyze: Cytobank

Plot raw data	 Histogram	 Biaxial plot	 3D plot	 Radar
Reduce dimensionality	 PCA	 Gemstone	 SPADE	 viSNE
Summarize statistics	 Box plot	 Heat map	 Network	 Sunburst

 Dose curve

Cytobank

BECKMAN COULTER Life Sciences

Analyze: Cytobank

Analysis toolkit designed for mass cytometry

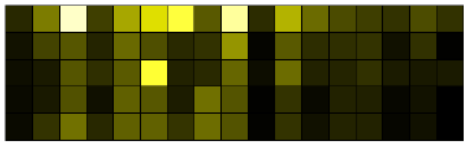
Cloud-based—accessible from anywhere

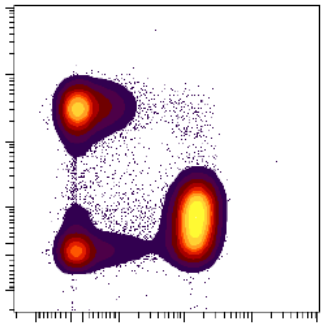
Data storage and backup included

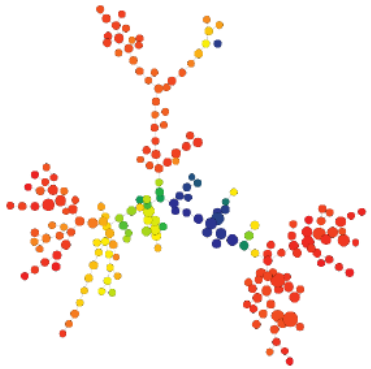
Demo datasets and tutorials

Strong scientific support

fluidigm.cytobank.org







Cytobank

STANDARD BIOTOOLS

Hyperion XTi
IMAGING SYSTEM

Imaging Mass Cytometry

Automated slide loader
Image 40 slides per day

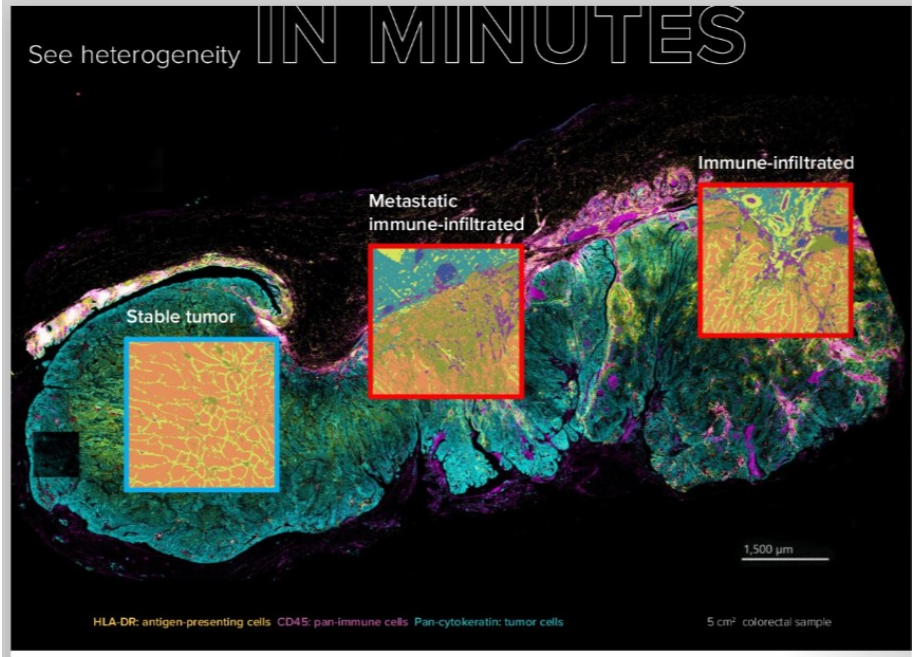
1 **Modularized panels**
Swap markers without panel revalidation.

2 **Batch staining**
Stable antibodies enable stained slides to be acquired at any time.

3 **Bulk slide loading**
Load up to 40 slides for automated imaging.

4 **Walk-away acquisition**
Automatically process slides, imaging 40-plus markers per sample simultaneously.

5 **Real-time data**
Visualize 40-plus markers in minutes.



WALK-AWAY AUTOMATION

Incorporating a new level of throughput and efficiency, an integrated slide loader enables researchers to **load up to 40 slides and walk away**.

High capacity to load up to 40 slides at once

Unattended acquisition increases sample throughput 2–3x.

Reliable sample identification with integrated barcode reader

Visualize 40-plus markers **in real time.**

Understanding the complex tissue microenvironment is essential to the timely evaluation of disease progression and the response to therapeutics. Imaging Mass Cytometry (IMC™) uniquely enables 40-plus protein and RNA markers to be **simultaneously acquired and visualized**, without time-consuming acquisition cycles.

A ONE-STEP MULTIPLEXED IMAGING APPROACH

Simultaneous detection
n=40-plus markers

Real-time data

Compensation - literature

Mario Roederer - Compensation in Flow Cytometry
Current Protocols in Cytometry (2002) 1.14.1-1.14.20 John Wiley & Sons, Inc.

M. Loken, D. R. Parks, & L. A. Herzenberg (1977). Two-color immunofluorescence using a fluorescence-activated cell sorter. *J. Histochem. Cytochem.* **25**:899-907.

M. Roederer & R. F. Murphy (1986). Cell-by-cell autofluorescence correction for low signal-to-noise systems: application to EGF endocytosis by 3T3 fibroblasts. *Cytometry* **7**:558-565.

S. Alberti, D. R. Parks, & L. A. Herzenberg (1987). A single laser method for subtraction of cell autofluorescence in flow cytometry. *Cytometry* **8**:114-119.

C. B. Bagwell & E. G. Adams (1993). Fluorescence spectral overlap compensation for any number of flow cytometry parameters. *in:* **Annals of the New York Academy of Sciences**, **677**:167-184.

Maciorowski, Z., Chattopadhyay, P.K., & Jain, P. (2017). Basic multicolor flow cytometry. **Current Protocols in Immunology**, 117, 5.4.1–5.4.38. doi: 10.1002/cpim.26

No Data Analysis Technique Can Make Good Data Out of Bad Data!

Shapiro's 7th Law of Flow Cytometry

Summary of the lecture

- Data analysis
- Compensation
- Quality control, principles

At the end of today's lecture, you should know:

1. Pronciples of gating and data analysis
2. Principles of compensations
3. What are the basic principles of multispectral and mass cytometry