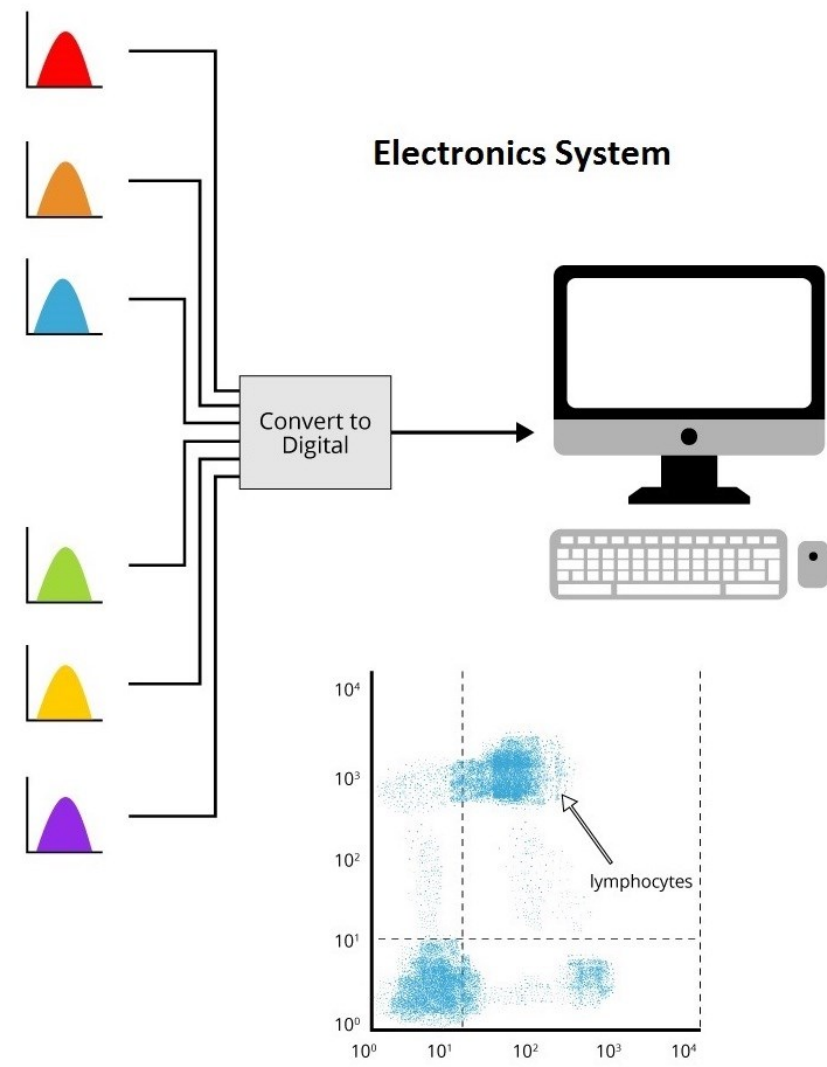
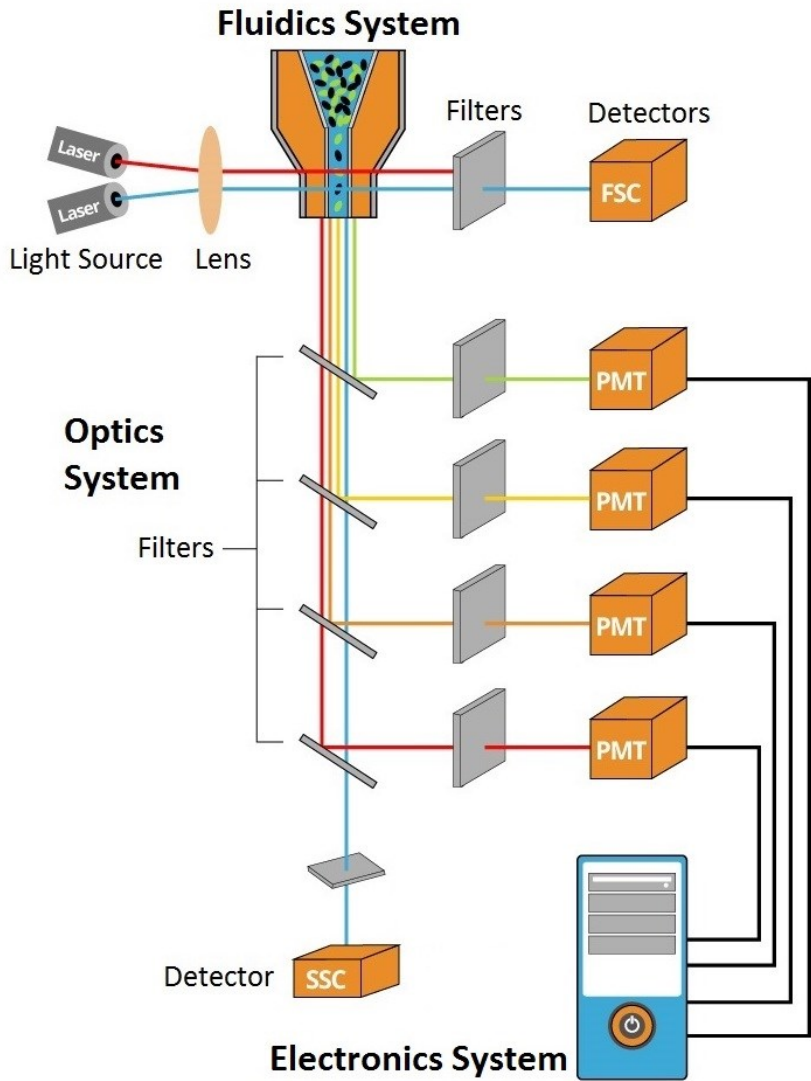


Bi9393 Analytická cytometrie

Karel Souček, Ph.D.

Oddělení cytokinetiky
Biofyzikální ústav AVČR, v.v.i
Královopolská 135
612 65 Brno

e-mail: ksoucek@ibp.cz
tel.: 541 517 166



Date: 17-JUL-2015
 System: Windows XP 5.1
 Cytometer: FACSAriaII SORP (FACSAriaII)
 File: 150717_DU145 Ctrl.fcs
 File URI: file://C:/Users/user/Desktop/install/Infinicyt/150717_DU145%20Ctrl.fcs

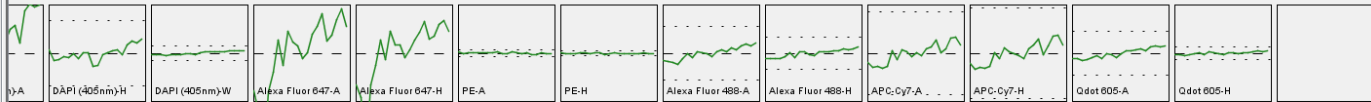
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 \$BEGINDATA: 4148
 \$BEGINTEXT: 0
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 \$BYTEORD: 4,3,2,1
 \$CYT: FACSAriaII SORP (FACSAriaII)
 \$DATATYPE: F
 \$DATE: 17-JUL-2015
 \$ENDANALYSIS: 0
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 \$ENDTEXT: 0
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 \$INST: IBP
 \$MODE: L
 \$NEXTDATA: 0
 \$OP: fedr
 \$PAR: 19
 \$SRC: 150717
 \$SYS: Windows XP 5.1
 \$TIMESTEP: 0.01
 \$TOT: 79620
 APPLY COMPENSATION: TRUE
 AUTOBS: TRUE
 CREATOR: BD FACSDiva Software Version 6.1.3
 CST BASELINE DATE: 03_24_2015 12:52:48 PM
 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 (Zuv-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
 P13MS: 0

Compensation Matrix

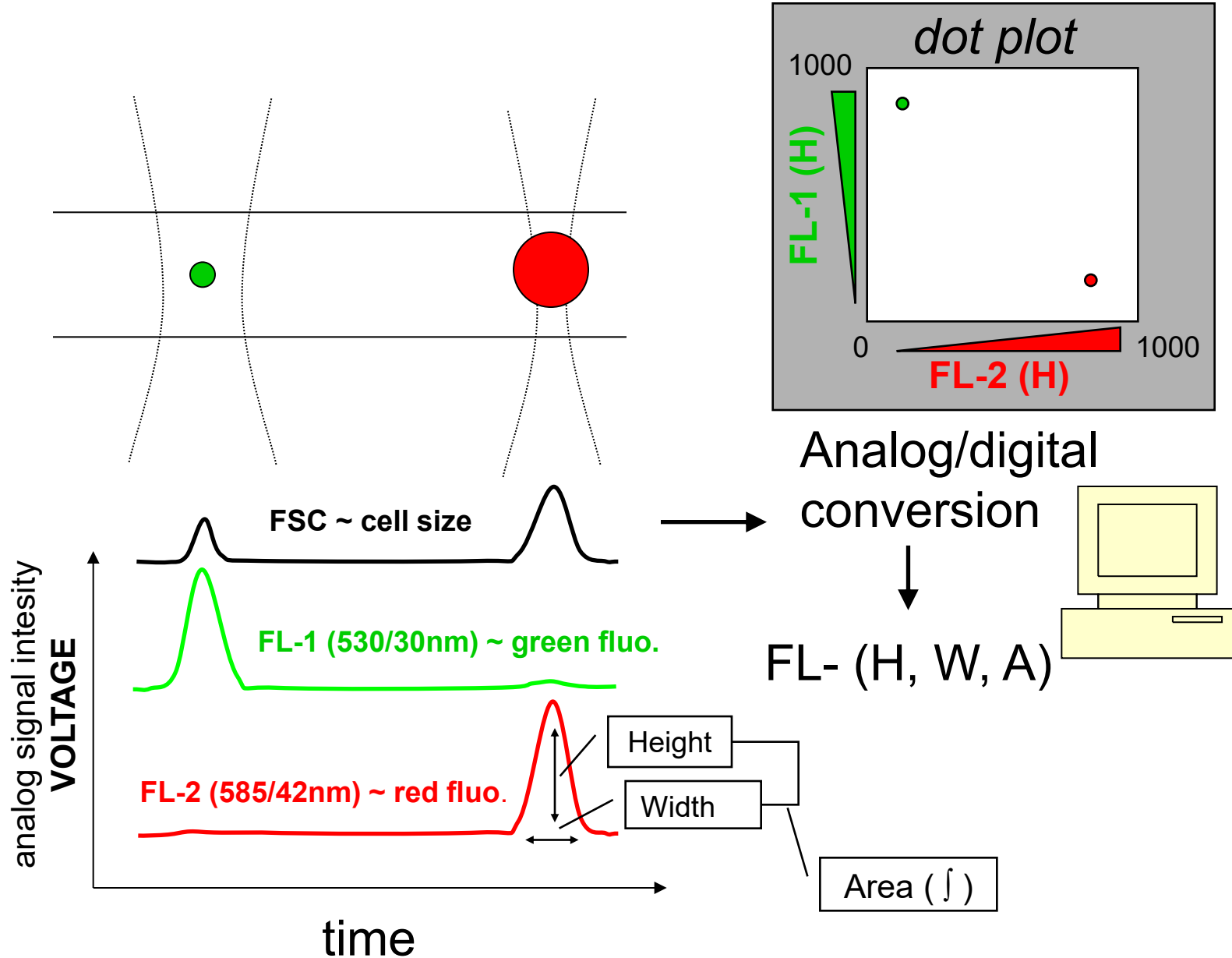
	Alexa Fluor 594-A	DAPI (405nm)-A	Alexa Fluor 647-A	PE-A	Alexa Fluor 488-A	APC-Cy7-A	Qdot 605-A
Alexa Fluor 594-A	100	0.42	1.53	1.94	0.02	0.32	9.95
DAPI (405nm)-A	1.1	100	0.27	0.05	0.01	0.08	0.98
Alexa Fluor 647-A	2.45	22.87	100	0.1	0.08	15.14	0.85
PE-A	440.67	0	0.14	100	8.03	0.03	32.23
Alexa Fluor 488-A	-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 (\$PnI)	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					



Signal processing



Cytometry

MIFlowCyt: The Minimum Information About a Flow Cytometry Experiment

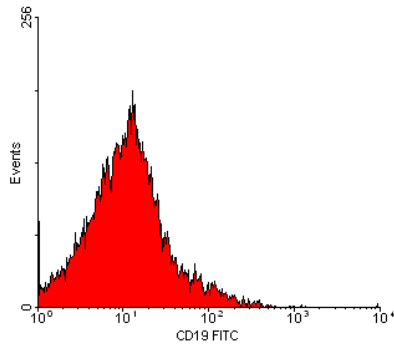
Jamie A. Lee,¹ Josef Spidlen,²¹ Keith Boyce,³ Jennifer Cai,¹ Nicholas Crosbie,⁴ Mark Dalphin,⁵ Jeff Furlong,⁶ Maura Gasparetto,² Michael Goldberg,⁷ Elizabeth M. Goralczyk,⁸ Bill Hyun,⁹ Kirstin Jansen,⁶ Tobias Kollmann,¹⁰ Megan Kong,¹ Robert Leif,¹¹ Shannon McWeeney,^{12,13,14} Thomas D. Moloshok,⁸ Wayne 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. Brinkman²⁴

Table 1. Components of a MIFlowCyt-compliant experiment description

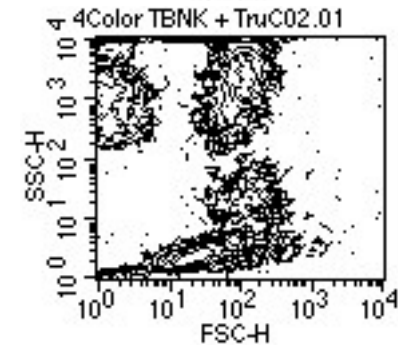
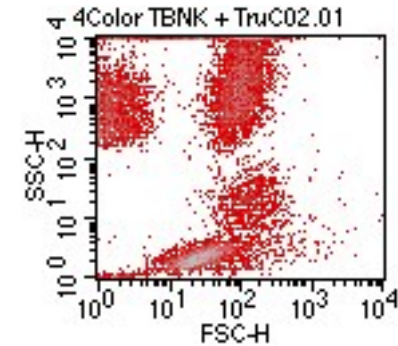
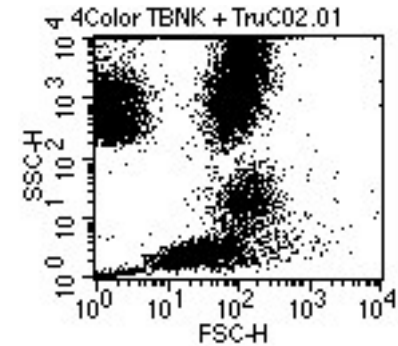
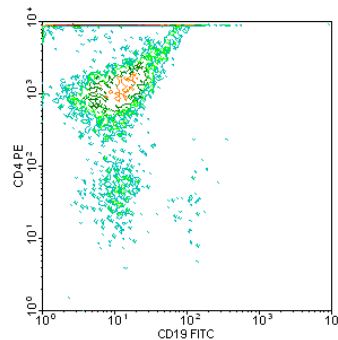
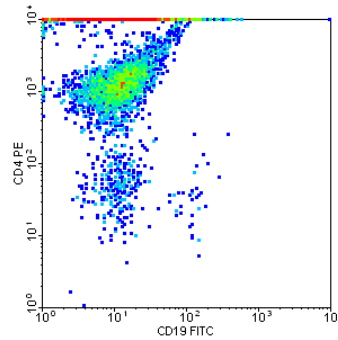
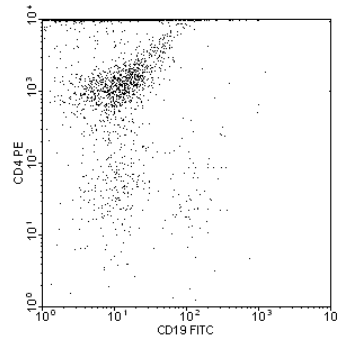
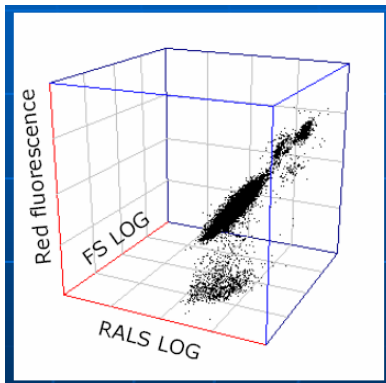
Experiment overview	Purpose/goal/hypothesis Experiment variables Conclusions Quality control
Flow sample (specimen)	Material Source/organism/location Treatment Reagent/analyte/detector/reporter
Data analysis	List-mode data Compensation Gating Descriptive statistics
Instrument details	Instrument identification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



Způsoby pro zobrazení dat

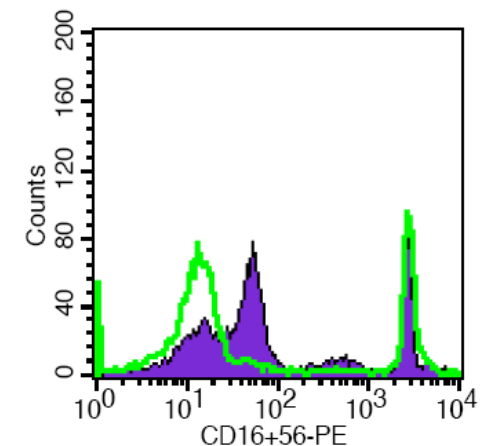
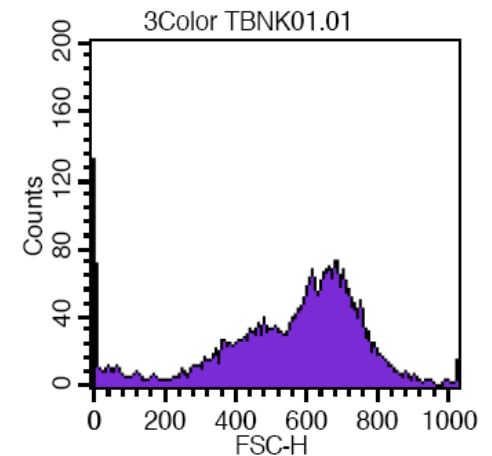


4Color TBNK + TruCO2.01



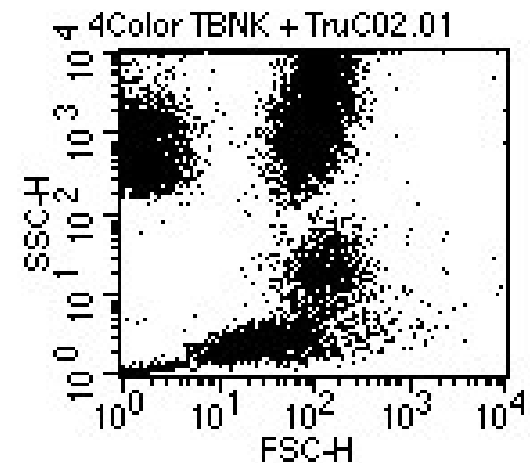
Histogram distribuce četnosti

- Histogram zobrazuje četnost částic pro jeden parametr
- Jednoduchý výstup
- Nekoreluje s dalším parametrem
- Problém s identifikací populací



Dot plot

- Zobrazuje korelaci dvou libovolných parametrů
- Jednotlivé tečky představují konkrétní změřené buňky (částice)
- Hodnoty pro řadu částic mohou ležet ve stejném místě
- Nemáme informaci o relativní denzitě částic
- Problémy s vykreslením v případě velkých objemů dat



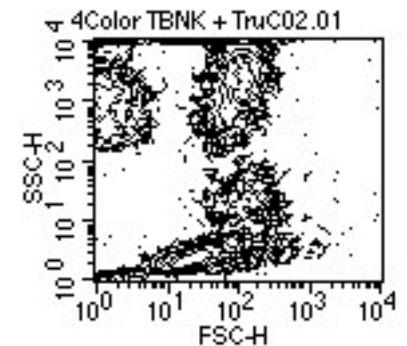
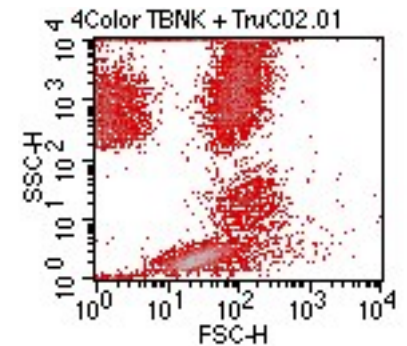
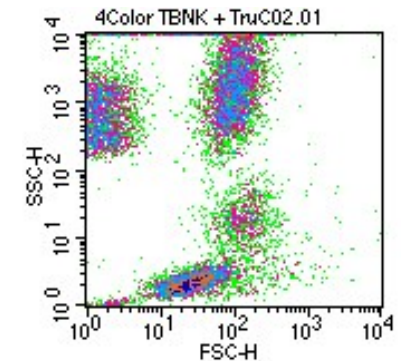
Density & contour plot

Density plot:

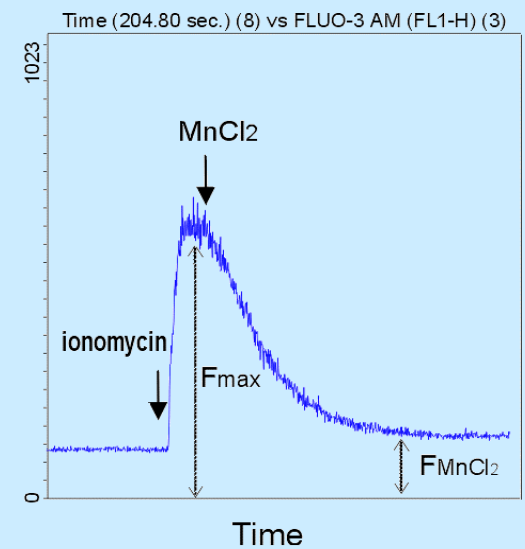
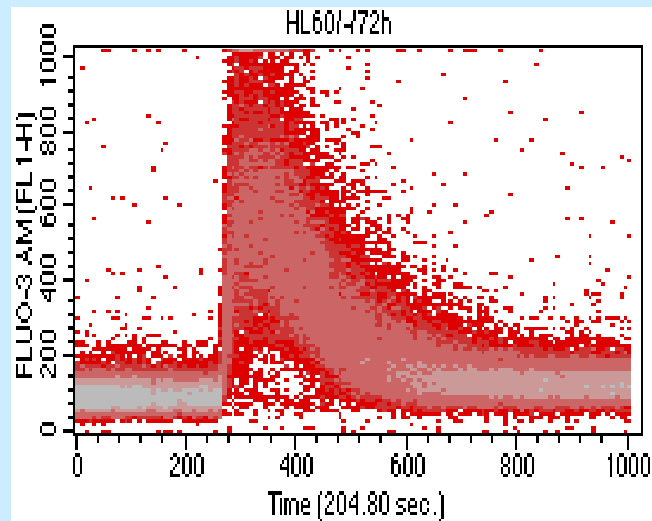
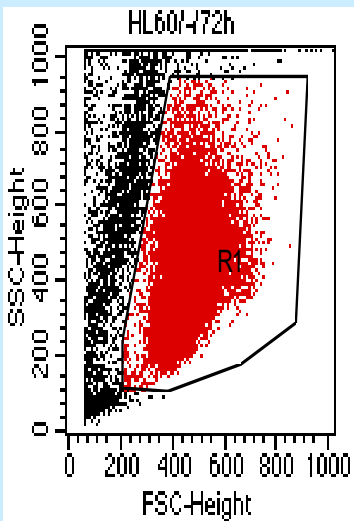
- Zobrazuje dva parametry jako frekvenci četnosti
- barva a nebo její odstín odpovídá četnosti částic

Contour plot:

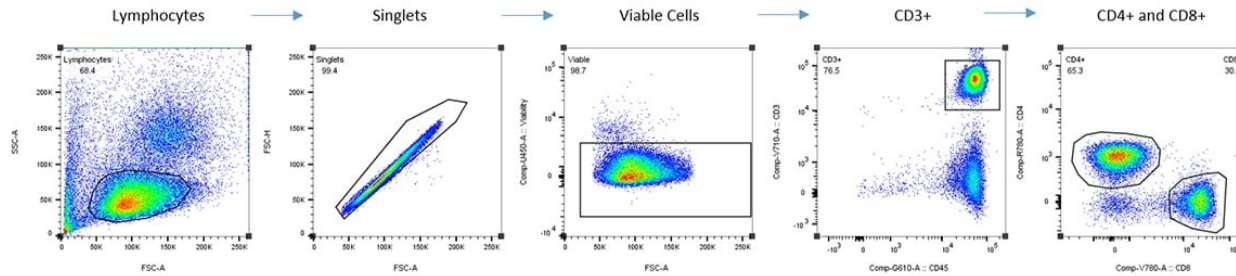
- spojnice spojuje body (částice) se stejnou hodnotou signálu
- V podstatě simulujeme 3D graf – třetí rozměr je frekvence



Čas jako jeden z parametrů



Určení regionů

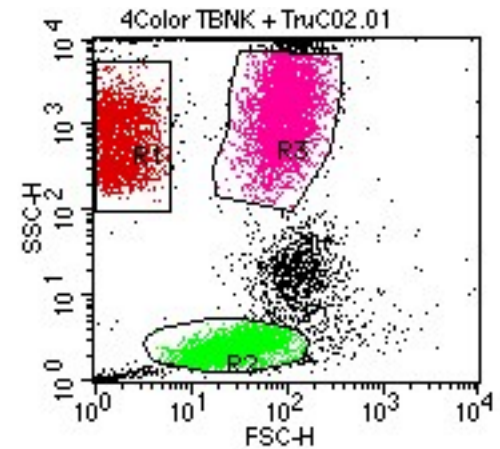


A representative Nested Gating Strategy illustrating lymphocyte population being subgated to the level of CD4+ and CD8+ T Cells.

- Objektivní nebo subjektivní?
 - školení/schopnosti/trénink
- Možné tvary:
 - obdelník
 - elipsa
 - “free-hand” (polygon)
 - kvadrant
- Statistika
 - počet
 - podíl (%)
 - průměr, medián, S.D., CV,

Region, gate

- oblast (plocha) v grafu definovaná uživatelem
- mnoho regionů v jedné grafu
- ohraničujeme pomocí nich populace našeho zájmu
- je možné je barevně odlišit
- je definován stejně pro všechny vzorky v analýze
- lze je kombinovat pomocí logických operátorů (AND, OR, NOT; Booleova logika)



Statistika

- Aritmerický průměr
- Geometrický průměr
- Medián
 - odhad střední hodnoty
 - není ovlivněn extrémními hodnotami
- Směrodatná odchylka
- Koeficient variance
- Modus – nejčastější hodnota

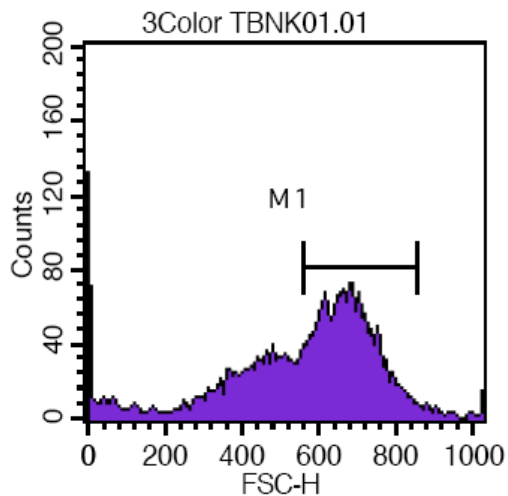
$$\bar{x} = \frac{1}{n} (x_1 + x_2 + \dots + x_n) = \frac{1}{n} \sum_{i=1}^n x_i$$

$$(a_1 \cdot a_2 \cdot \dots \cdot a_n)^{\frac{1}{n}} = \sqrt[n]{a_1 \cdot a_2 \cdot \dots \cdot a_n} = \left(\prod_{i=1}^n a_i \right)^{\frac{1}{n}}$$

$$\int_{-\infty}^m f(x) dx = 0,5$$

$$\bar{x} = \frac{1}{N} \sum_{i=1}^N x_i$$

Statistika pro 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:

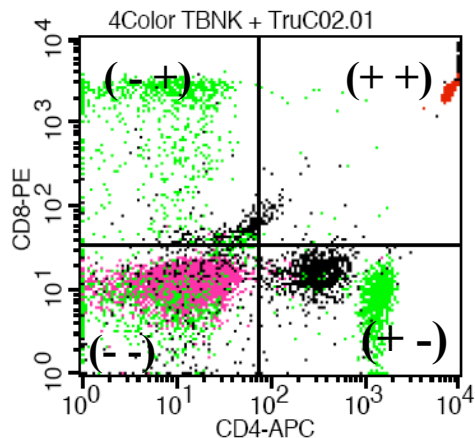
Panel: 3 Color TBNK

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

Analýza kvadrantů



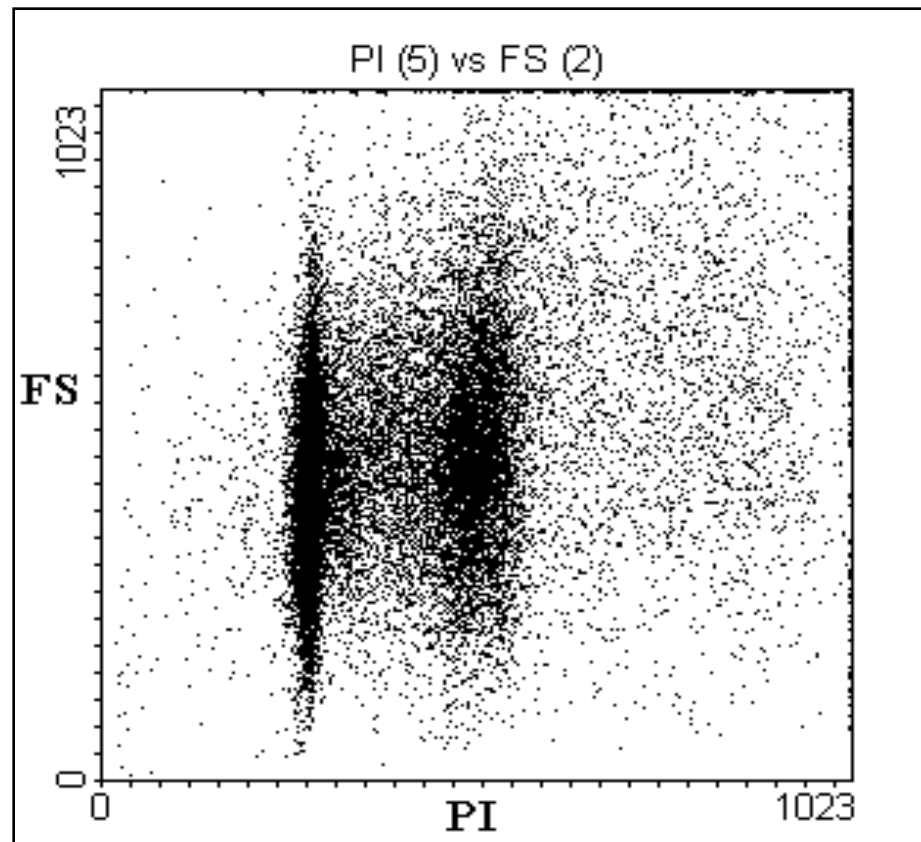
Quadrant Statistics

File: 4Color TBNK + TruC02.01 Log Data Units: Linear Values
 Sample ID: Patient ID:
 Tube: CD8/CD8/CD45/CD4 TruC Panel: 4 Color TBNK + TruC
 Acquisition Date: 08-Oct-98 Gate: No Gate
 Gated Events: 10000 Total Events: 10000
 X Parameter: CD4-APC (Log) Y Parameter: CD8-PE (Log)
 Quad Location: 74, 35

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

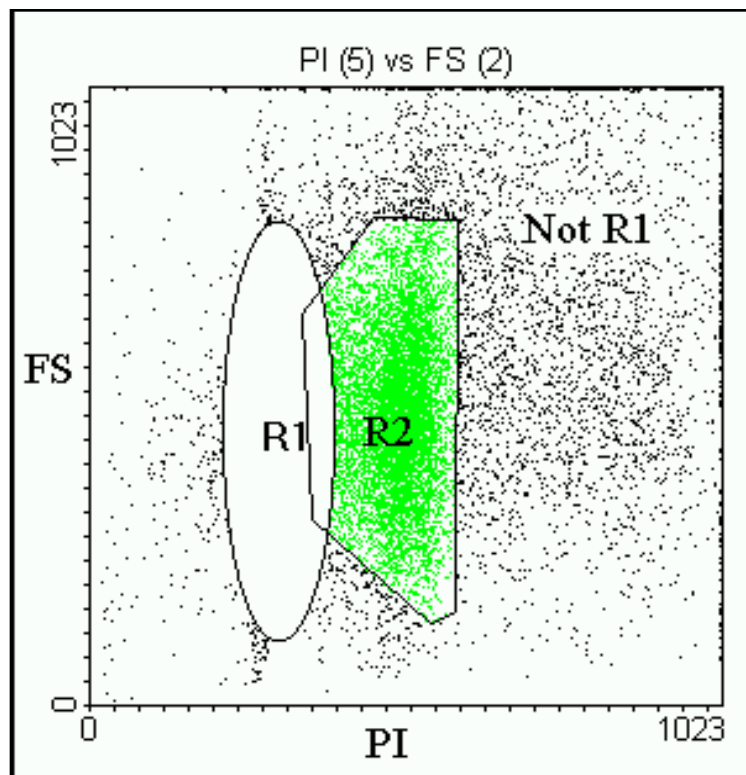
Logický „Gating“ (Booleova logika)

S překrývajícími se oblastmi máme mnoho možností:



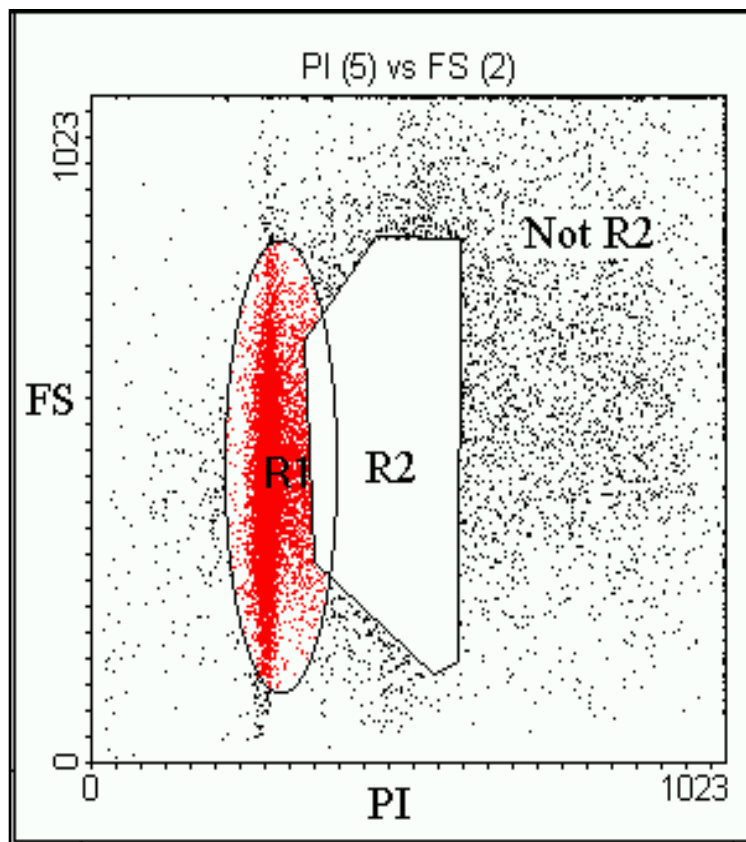
Boolean Gating

Not Region 1:



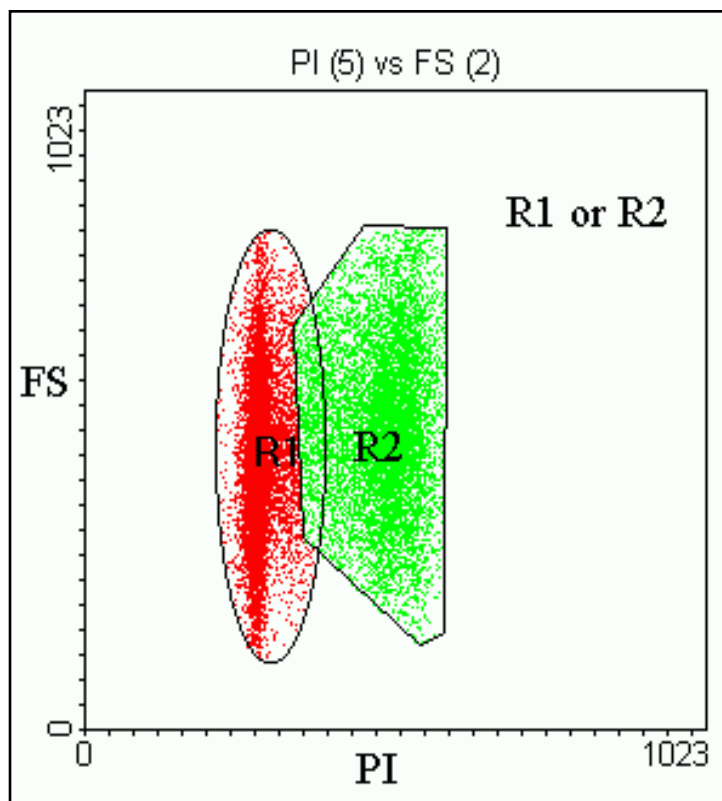
Boolean Gating

Not Region 2:



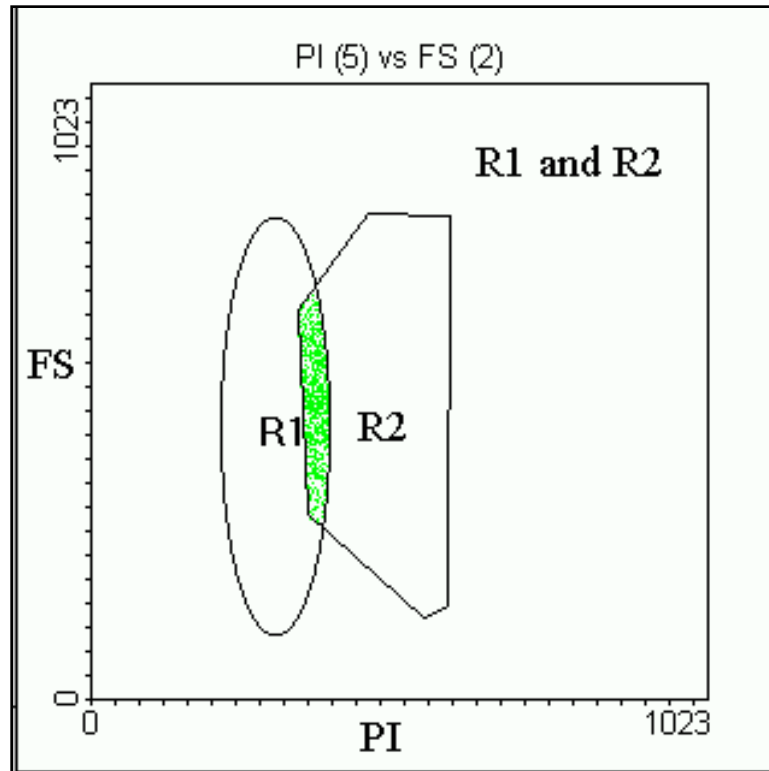
Boolean Gating

Region 1 or Region 2:



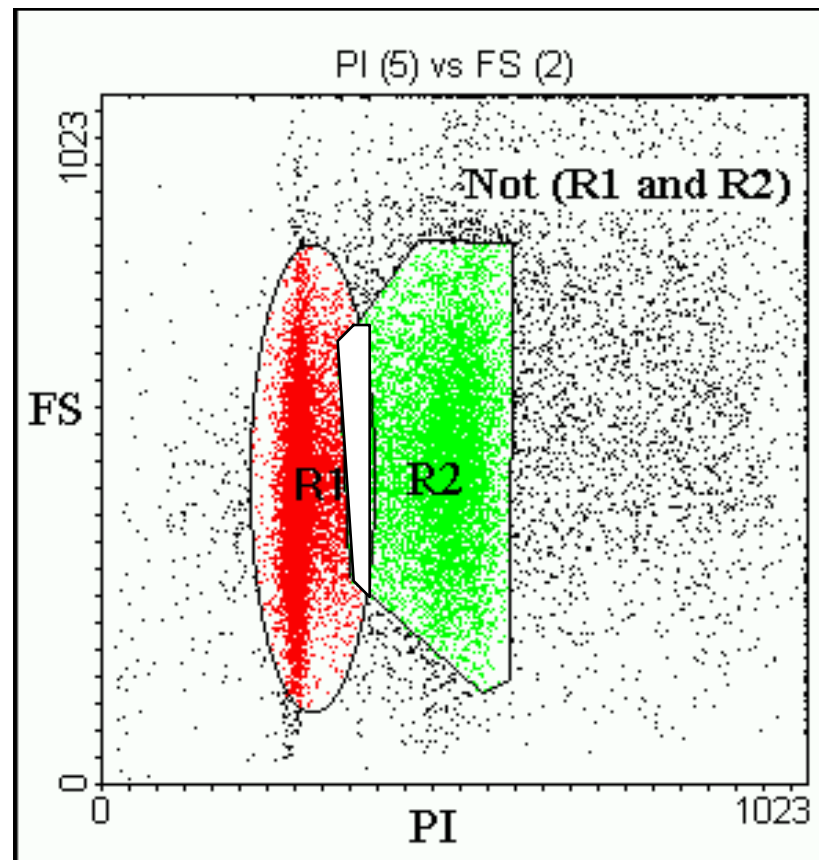
Boolean Gating

Region 1 and Region 2:

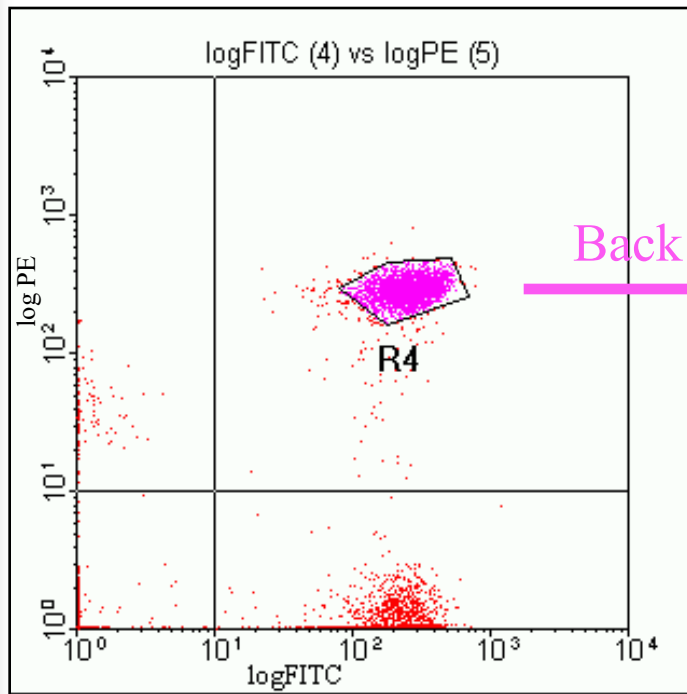


Boolean Gating

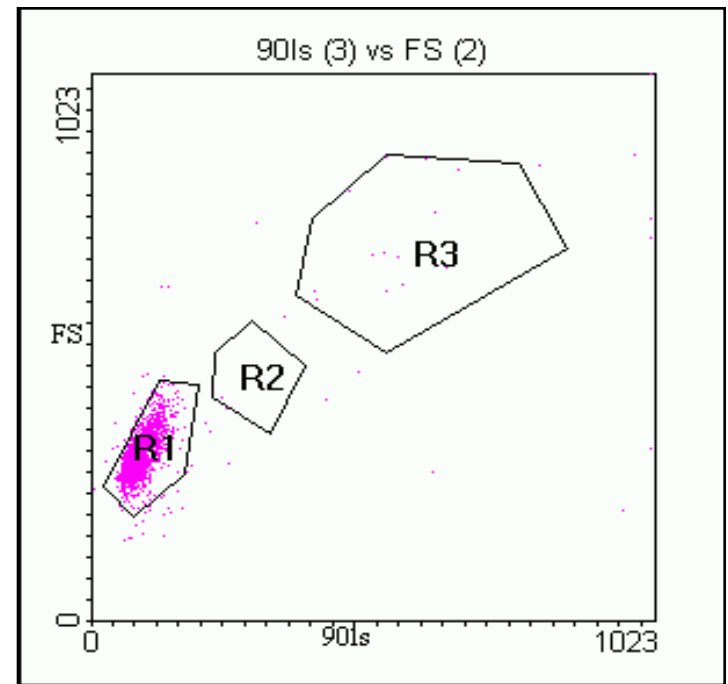
Not (Region1 and Region 2):



Back Gating

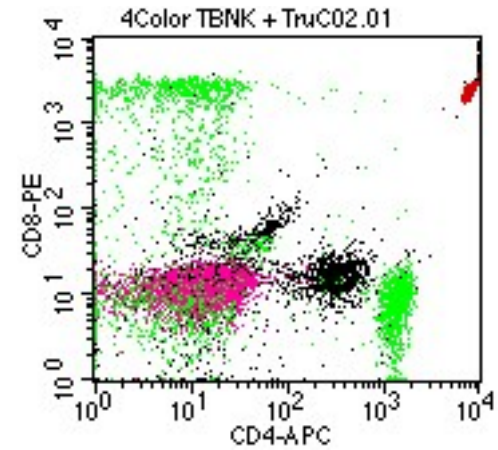
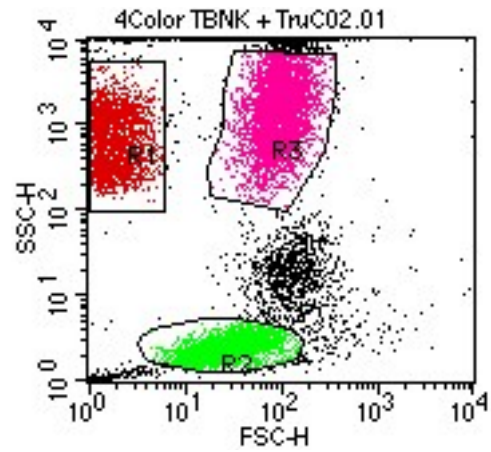


Region 4 established



Back-gating using Region 4

Back Gating



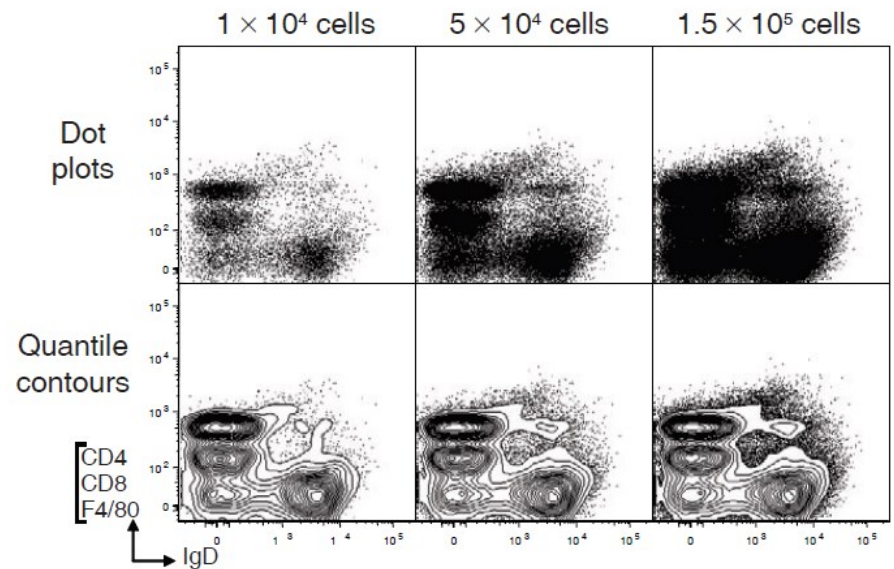
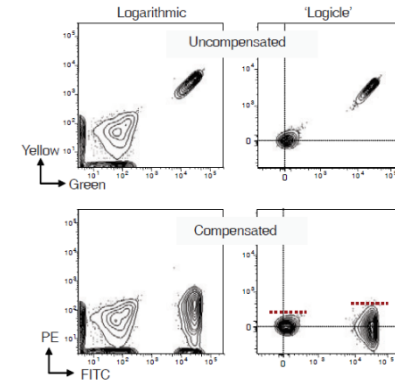
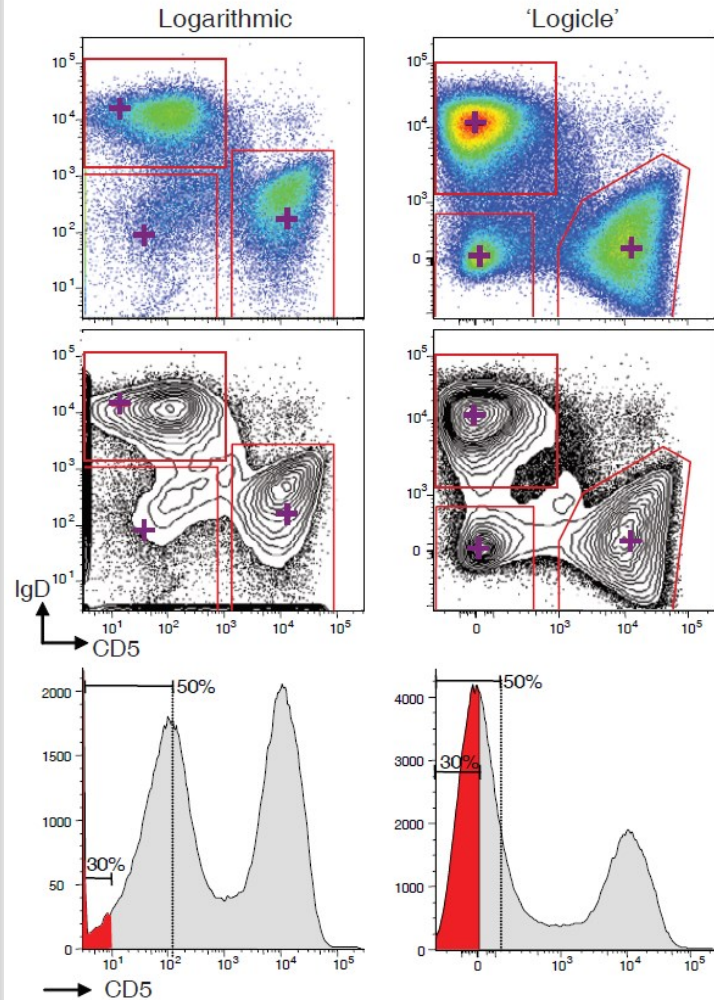
Nástroje pro analýzu dat

- Výrobci HW
 - Beckman Coulter
 - Kaluza
 - Becton Dickinson
 - FACSDiva
 - FACSSuite
 - FlowJo
 - BioRad
 - Sony
 - Milteney
 - ...
- Univerzální platformy
 - Komerční
 - FlowJo
 - FCS Express
 - ...
 - Freeware
 - Flowing Software
 - Cyflogic
 - BioConductor - Flowcore

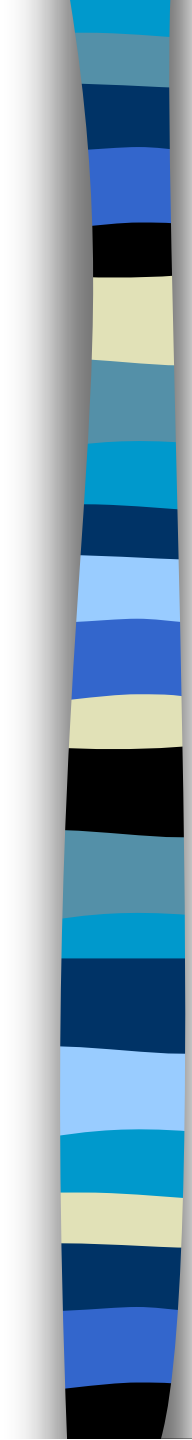


Turning Cytometry Data Into Results

Vizualizace dat a intepretace dat



Herzenberg LA, Tung J, Moore WA, Herzenberg LA, Parks DR (2006) Interpreting flow cytometry data: a guide for the



BOX 1 SUGGESTED GUIDELINES FOR FACS DATA PRESENTATION⁴

Instrument: Identify the FACS instrument and the software used to collect, compensate and 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 (x 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.

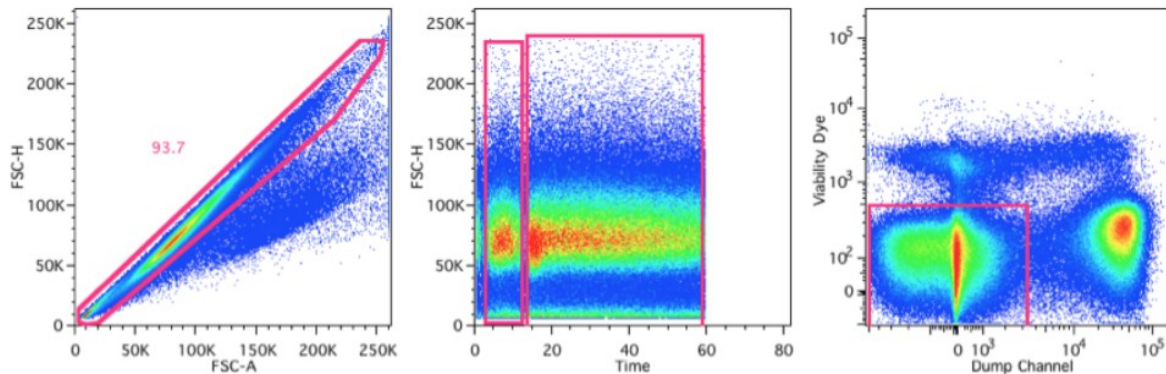


Gating – technika datové redukce

- Flow stability gating — k zachycení událostí, jakmile se průtokový proud stabilizuje, eliminace účinků ucpání, protitlaku a dalších problémů s přístrojem.
- Pulse geometry gating — pro odebrání dubletů z datové sady.
- Forward and side scatter gating — k odstranění „debris“ a dalších nezajímavých událostí při zachování buněk na základě velikosti nebo složitosti.
- Subsetting gating — spoléhat se na vyjádření markerů a na to, co identifikují. Použití viability a dump kanálů se dále zužuje na buňky zájmu. To je místo, kde se kontroly fluorescence minus jedna (FMO) stávají kritickými při definování zájmových populací.
- Backgating — Zajistit vizualizaci buněk v závěrečném gatu na vyšší úrovni.

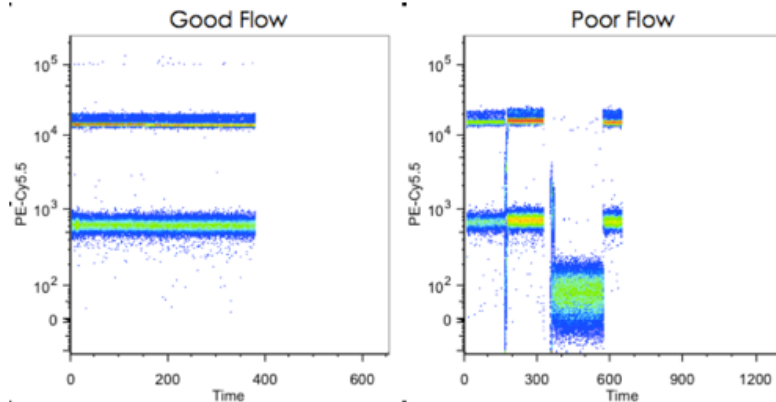
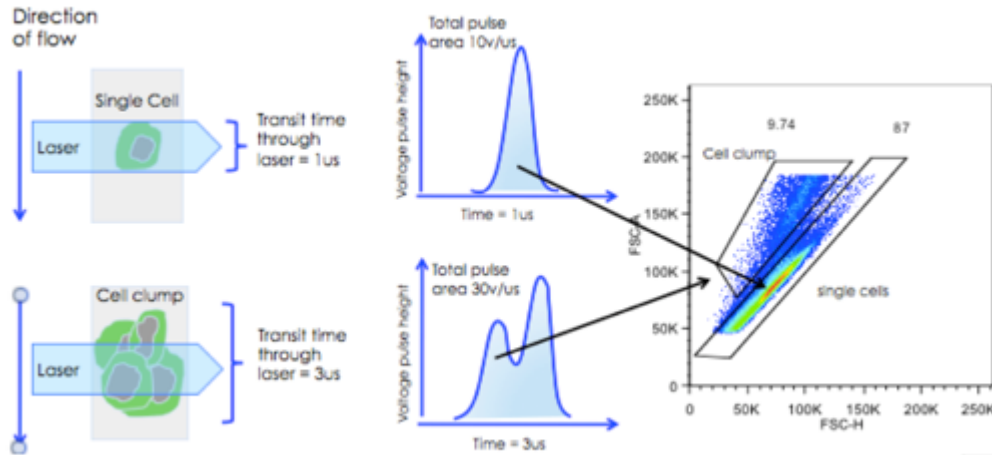
Gating a kontrola kvality

- 1) Singlets
- 2) Time
- 3) viabilita



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

Gating a kontrola kvality



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

FlowClean Plugin

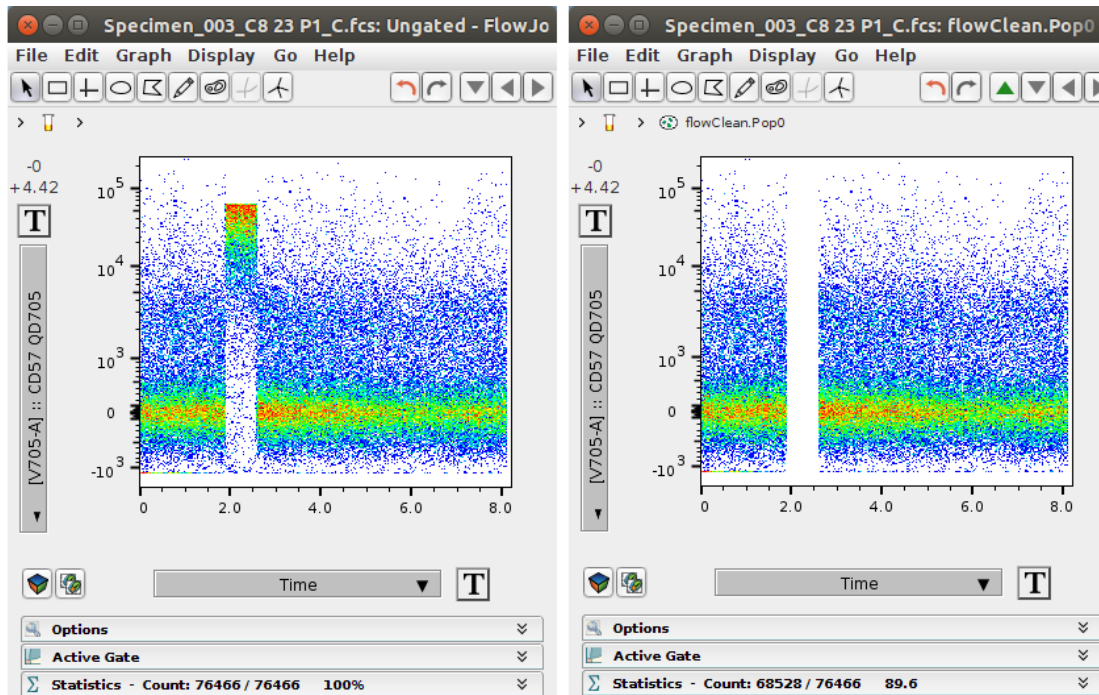


Josef Spidlen

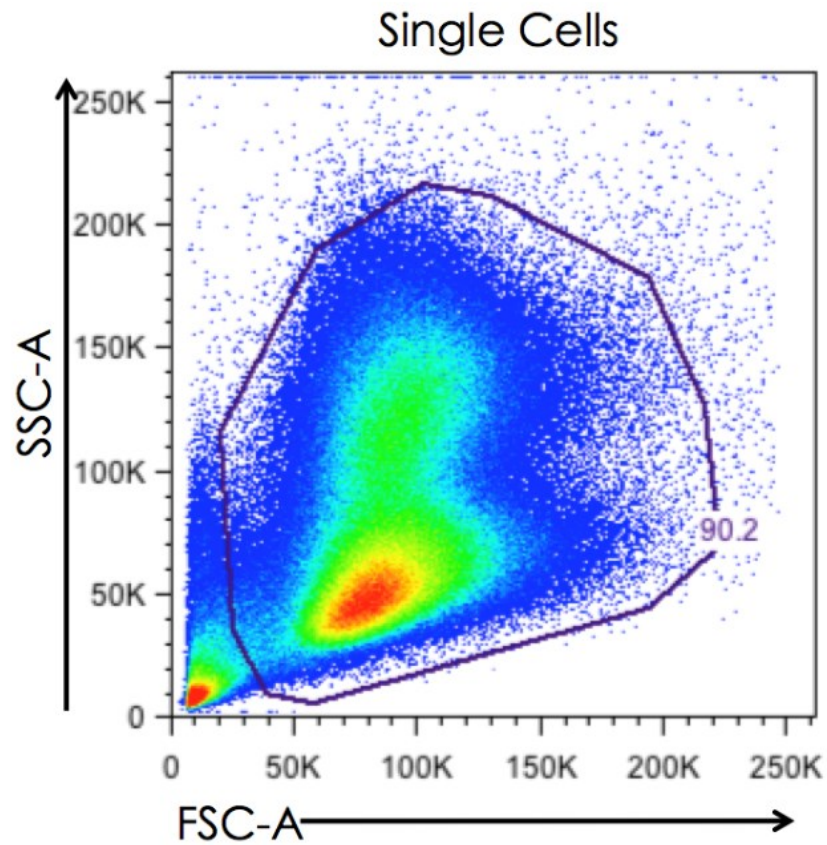
Clean up your data with the FlowClean plugin

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!

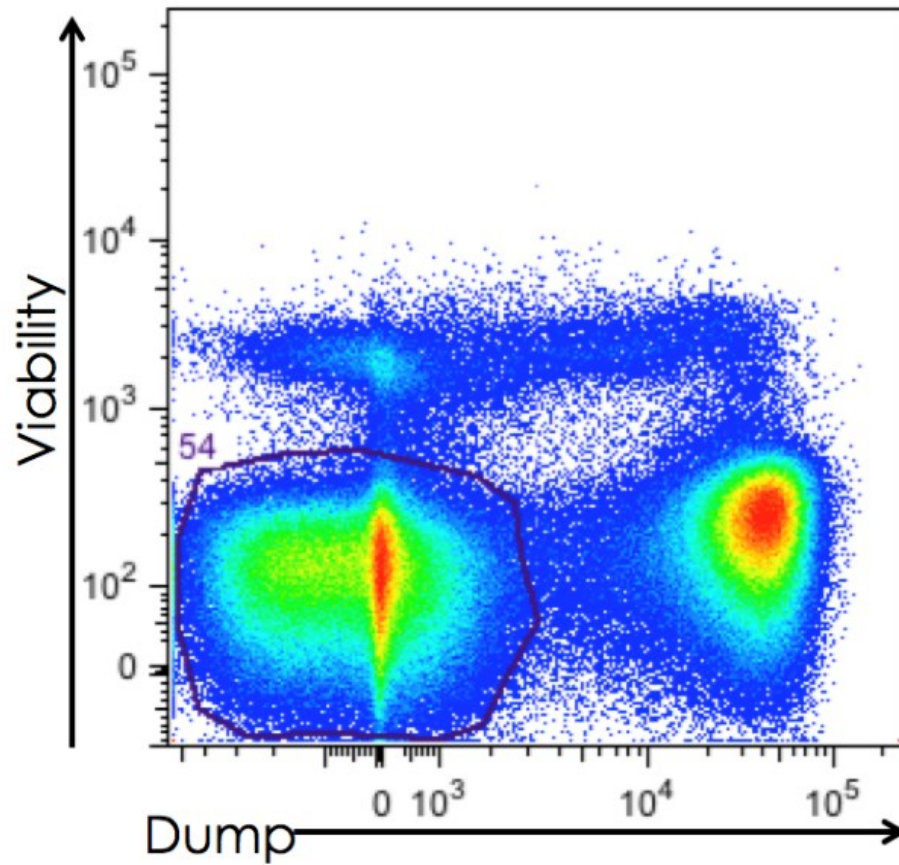
As you may know, our latest release, FlowJo v10.2, contains new and improved architecture for plugins. One of our featured plugins—FlowClean—has been designed to address exactly this issue. It automatically identifies and flags fluorescence anomalies in your FCS files by tracking cell populations in 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?



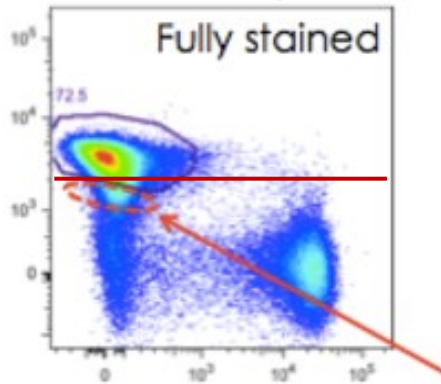
Scatter gating



Subset gating

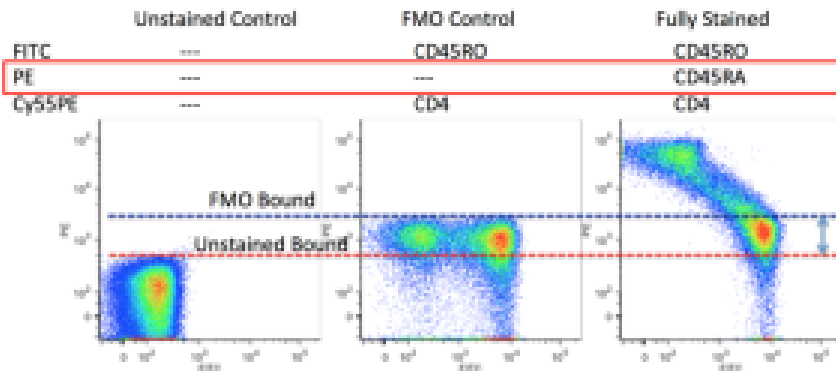


Gating a kontrola nastavení



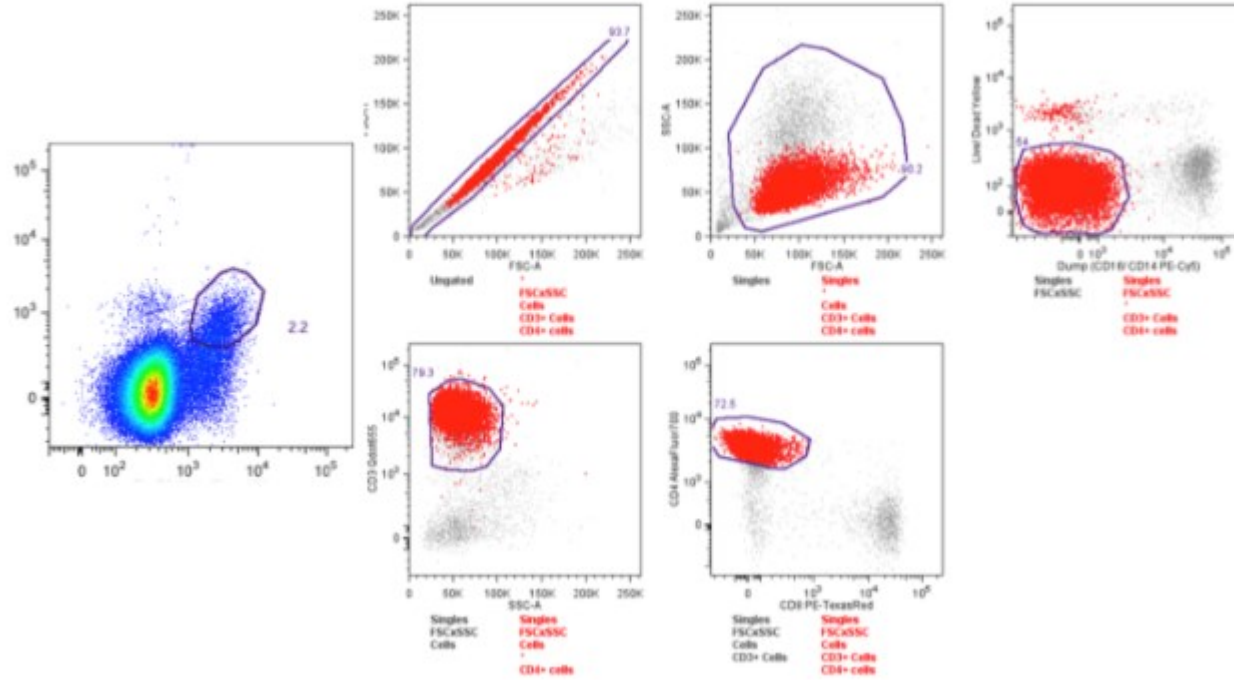
Cells in spread of fluorescence

FMO ~ Fluorescence Mines One



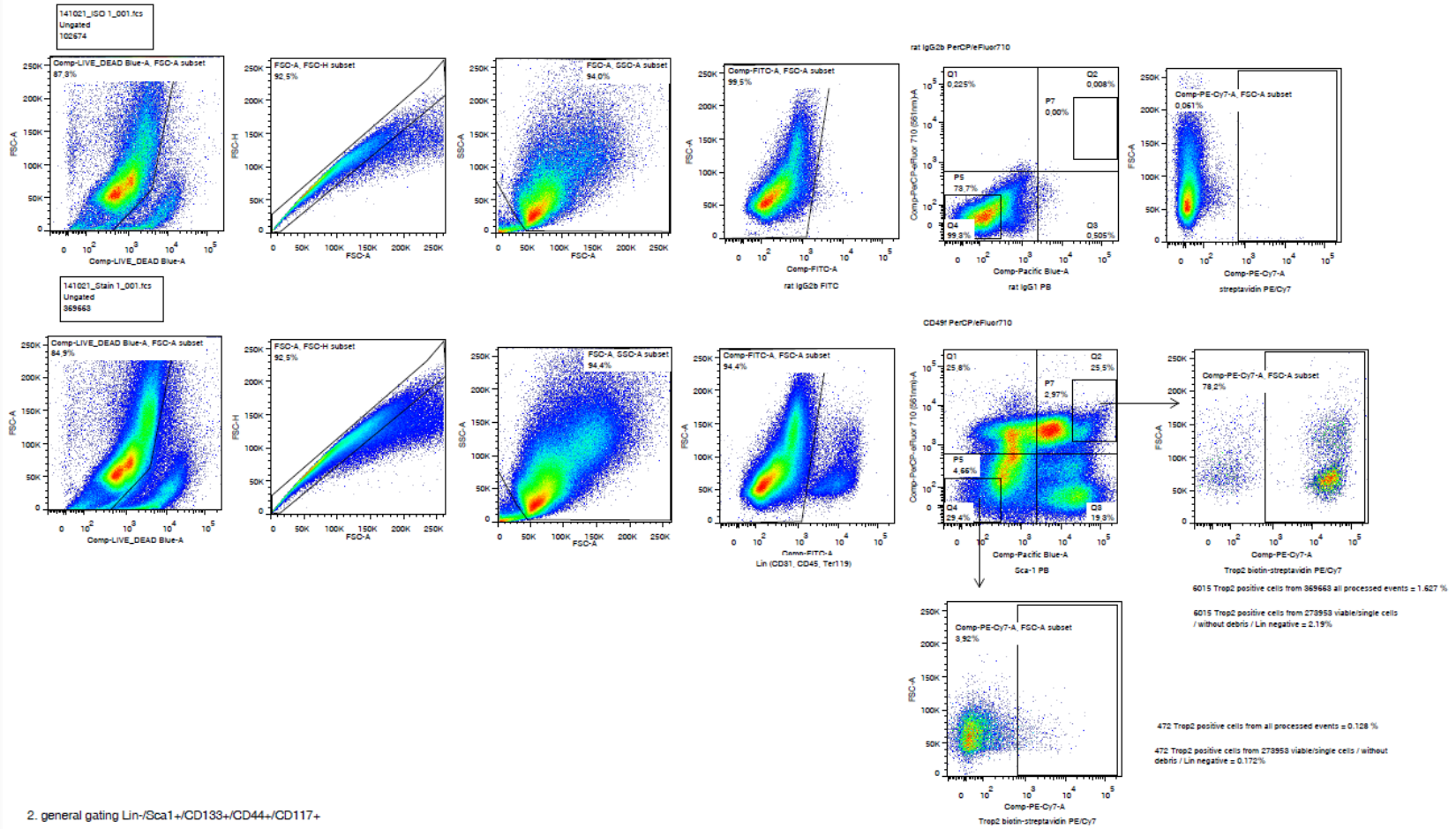
Antigen	FITC	PE	Cy5-PE	APC
CD3 FMO	---	CD4	CD8	CD19
CD4 FMO	CD3	---	CD8	CD19
CD8 FMO	CD3	CD4	---	CD19
CD19 FMO	CD3	CD4	CD8	---

Back gating



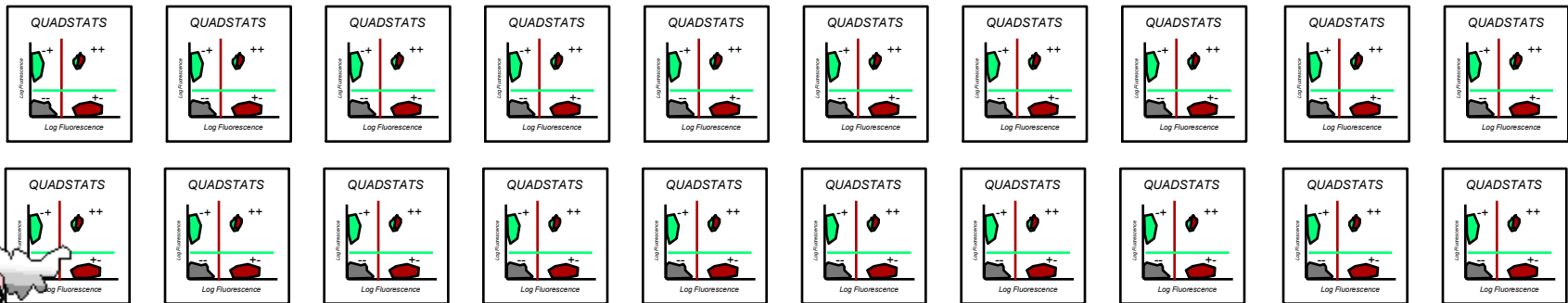
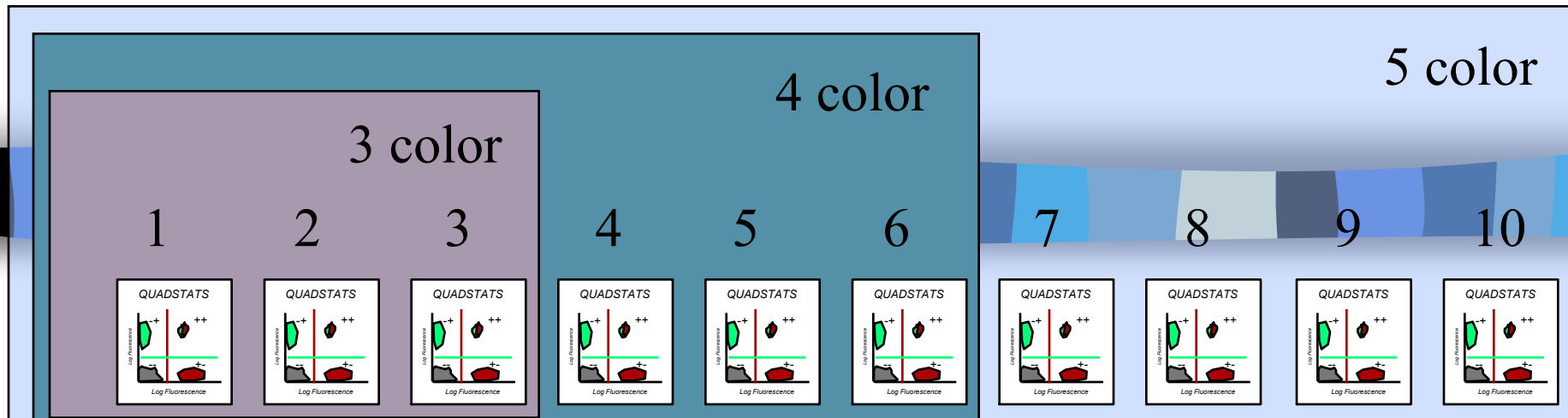
Gating – příklad hodnocení

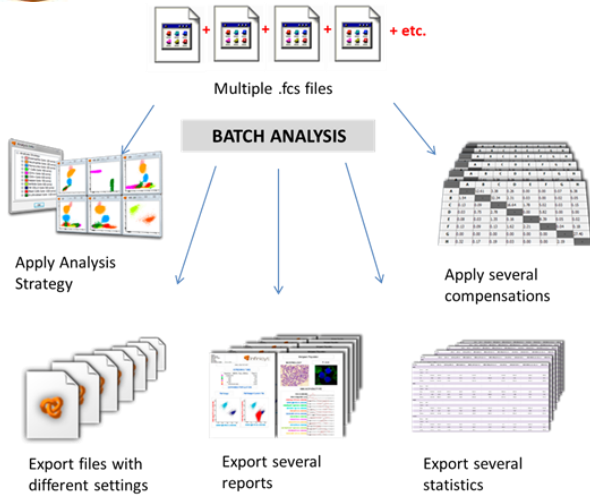
1. general gating Lin-/Sca1+ CD49f+/Trop2+



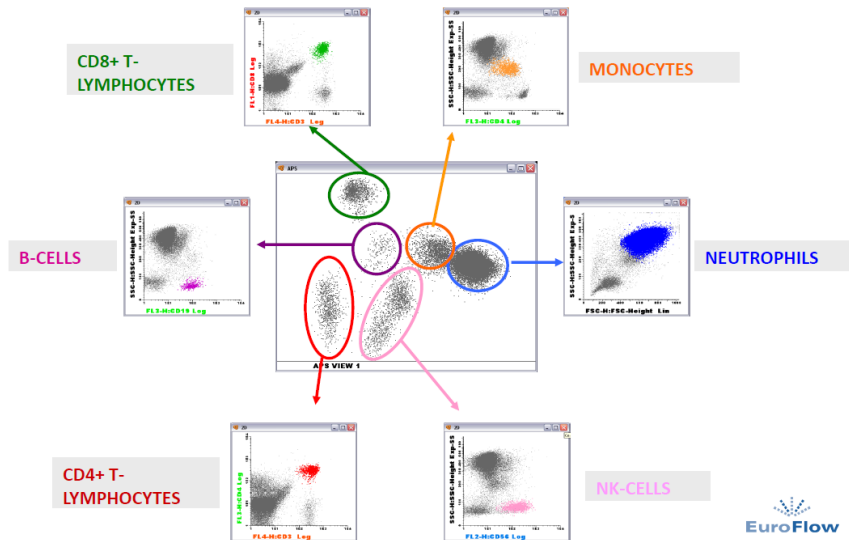
2. general gating Lin-/Sca1+/CD133+/CD44+/CD117+

Vícebarevné analýzy generují mnoho dat...





Automatic Population Separator

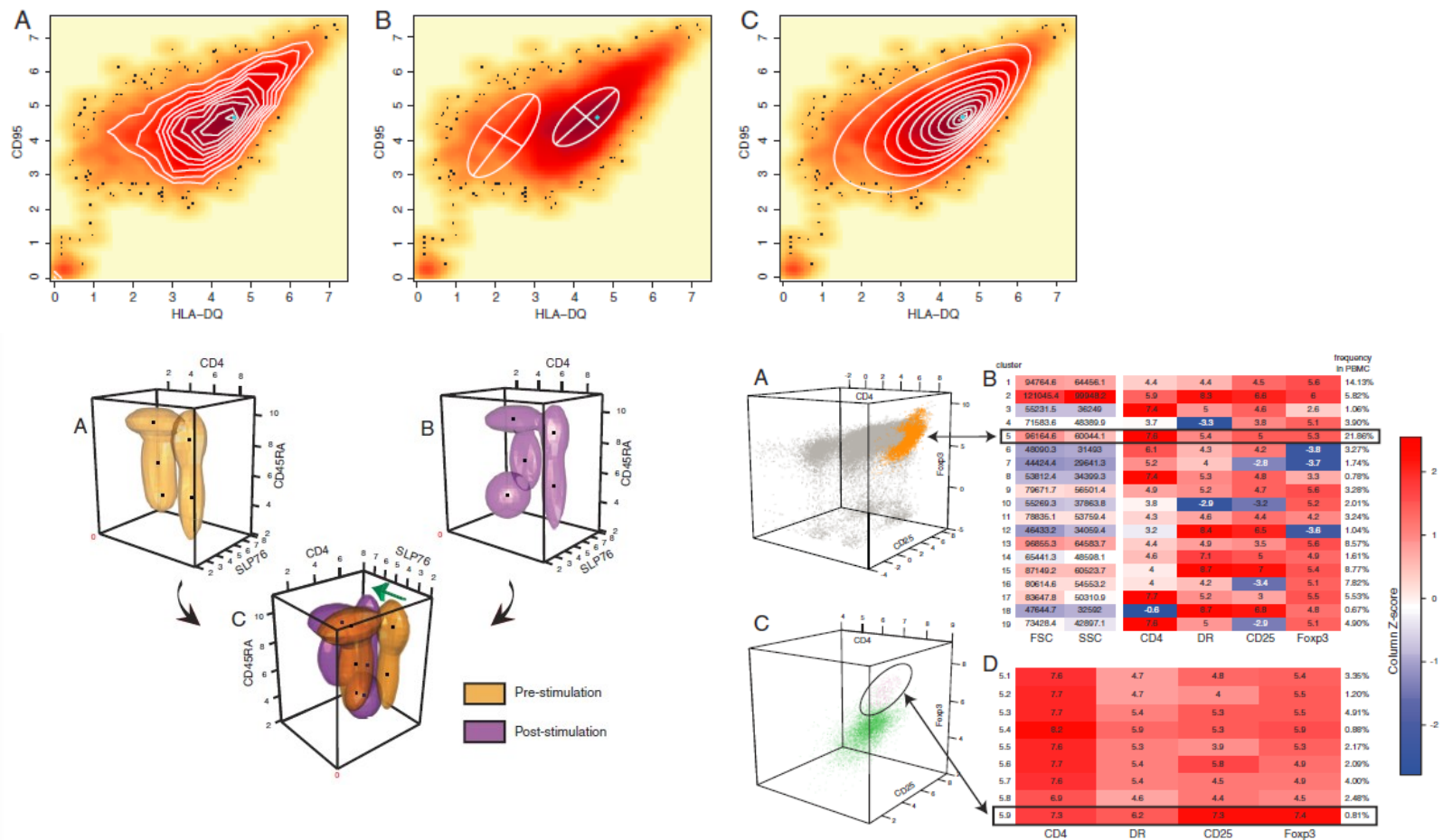


Automated high-dimensional flow cytometric data analysis

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}

^aBroad Institute of MIT and Harvard, 7 Cambridge Center, Cambridge MA 02142; ^bDepartment of Mathematics and ^cInstitute for Molecular Bioscience, University of Queensland, St. Lucia, Queensland, 4072, Australia; ^dDepartment of Applied Mathematics, National Chung Hsing University, Taichung 402, Taiwan; ^eDivision of Molecular Immunology, Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115; and ^fPartners 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.

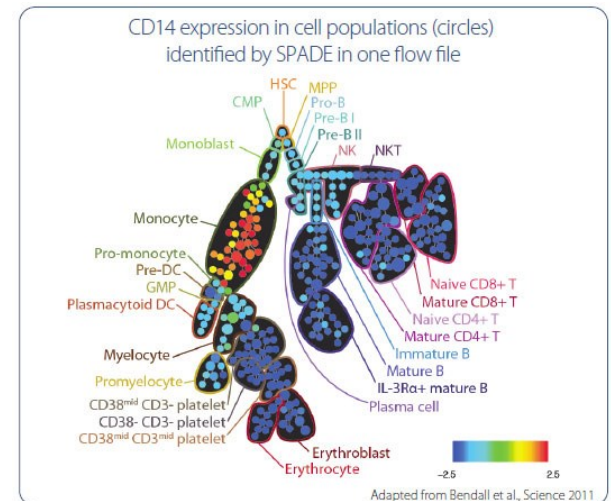
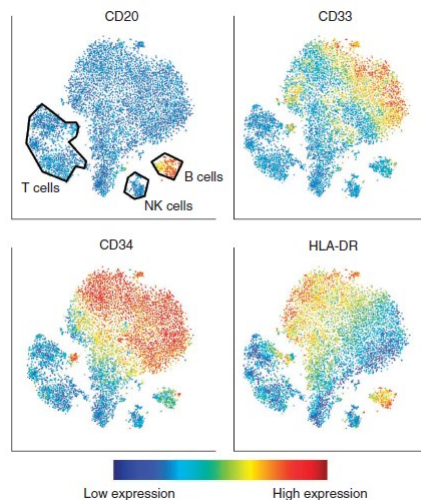
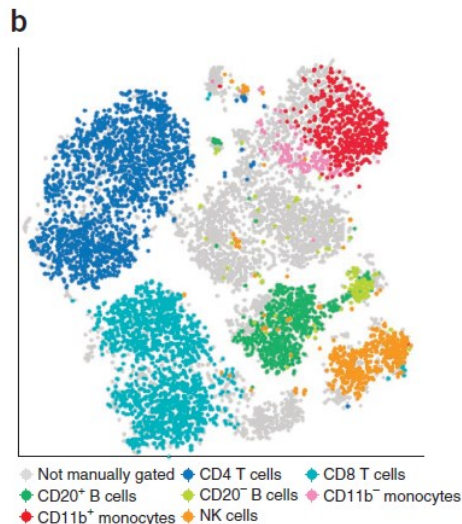
Critical assessment of automated flow cytometry data analysis techniques

Nima Aghaeepour¹, Greg Finak², The FlowCAP Consortium³, The DREAM Consortium³, Holger Hoos⁴, Tim R Mosmann⁵, Ryan Brinkman^{1,7}, Raphael Gottardo^{2,7} & Richard H Scheuermann^{6,7}

Další způsoby vizualizace vícerozměrných dat



- 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



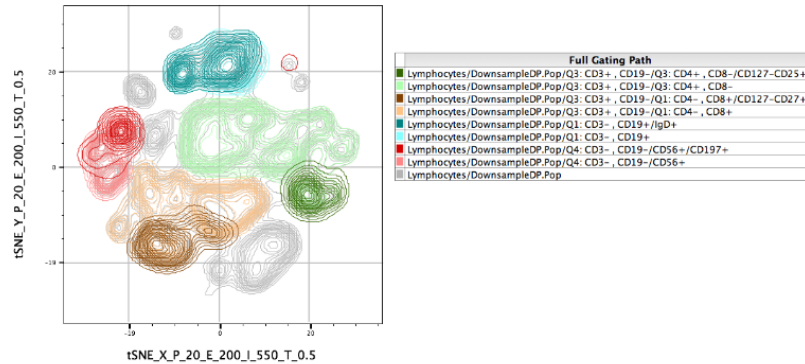
Search the Site...

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

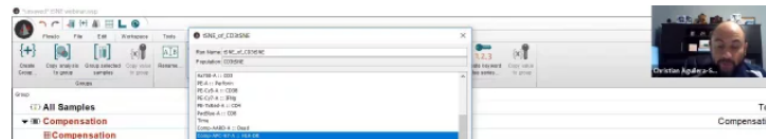
T-Distributed Stochastic Neighbor Embedding (tSNE) is an algorithm for performing dimensionality reduction, allowing visualization of complex multi-dimensional data in fewer dimensions while still maintaining the structure of the data.



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).

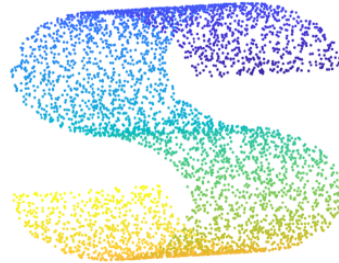
- The native platforms in FlowJo (such as tSNE) do not require R.



- <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**)

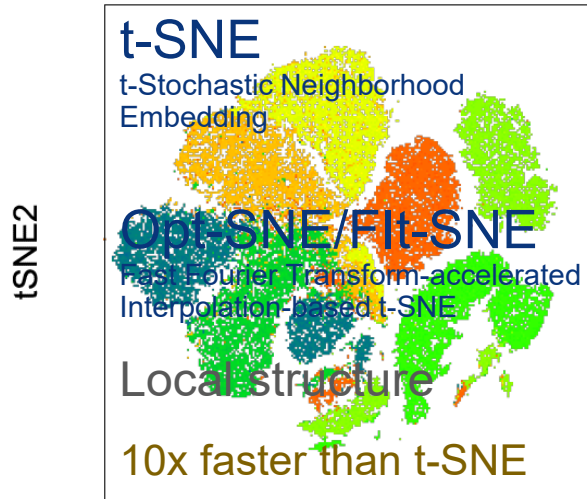


PCA (0.03, 1.00)

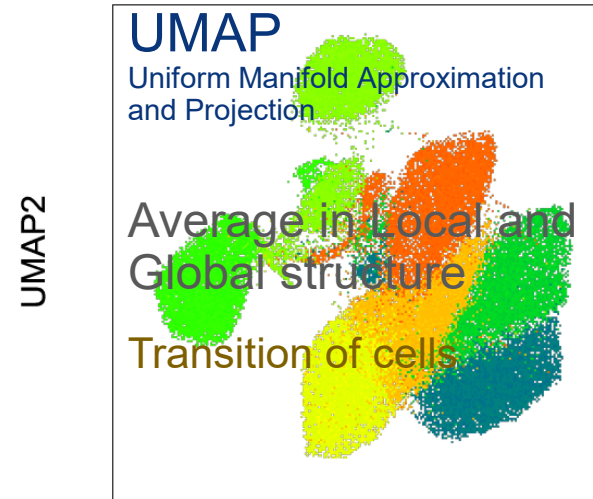


Dimensions reduction

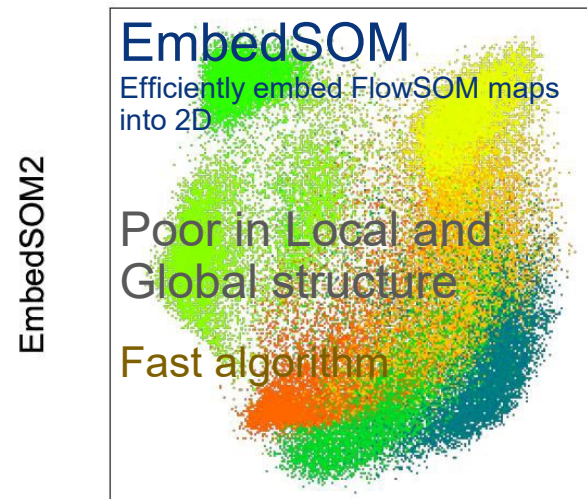
„... to create a map.“



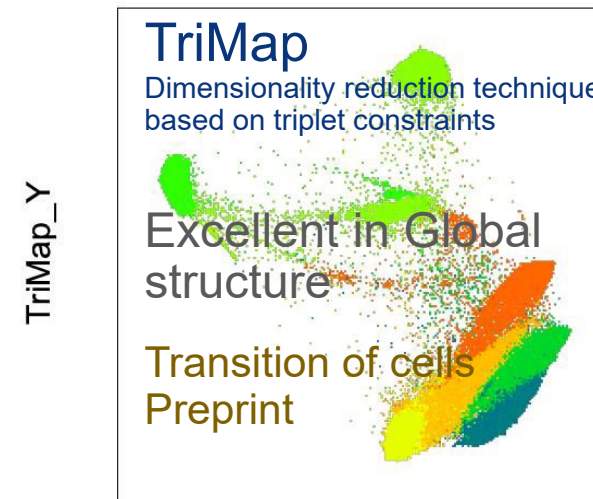
tSNE 1



UMAP1



EmbedSOM1

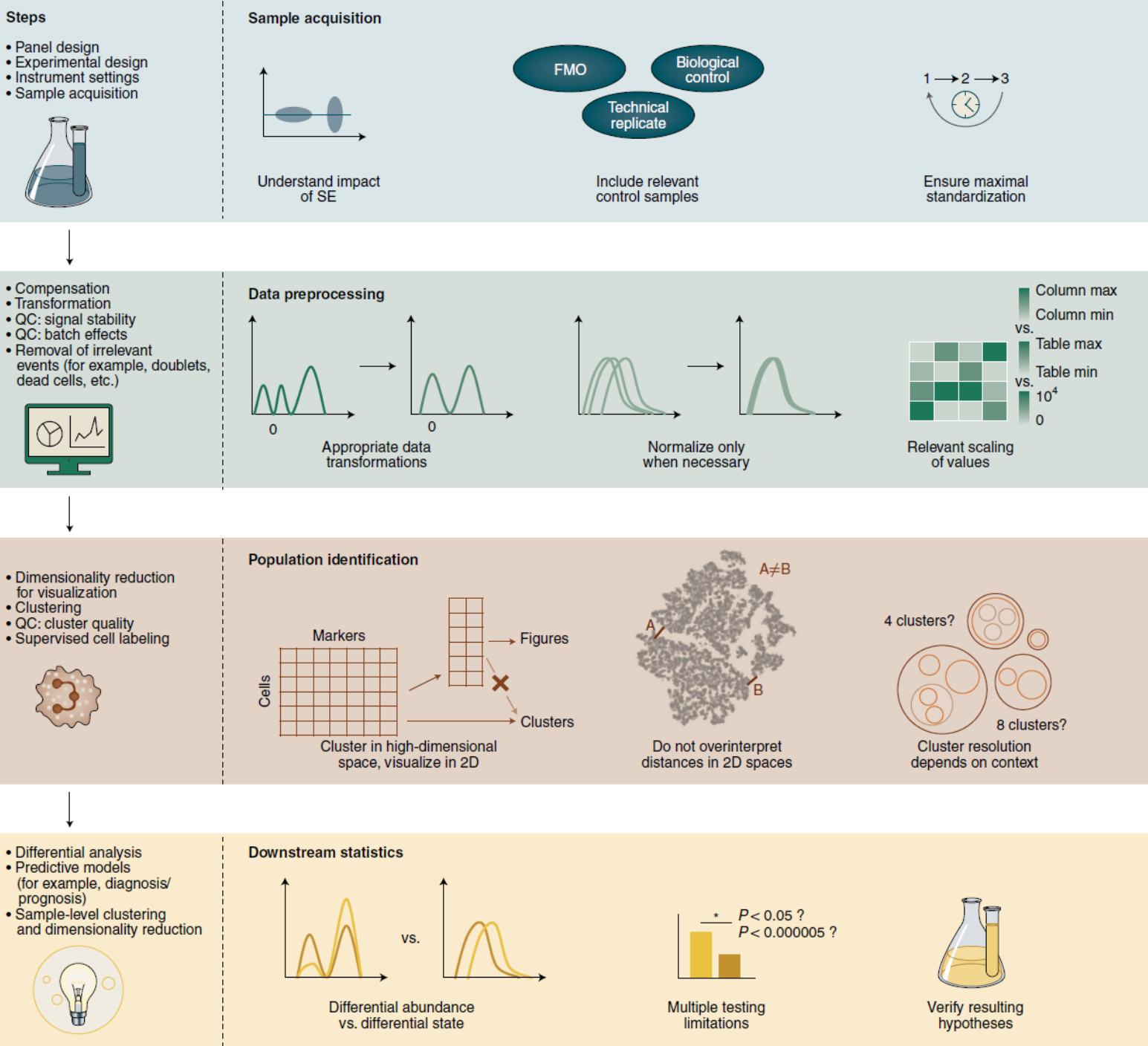


TriMap_X

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 Pric, Sofie Van Gassen and Florian Mair

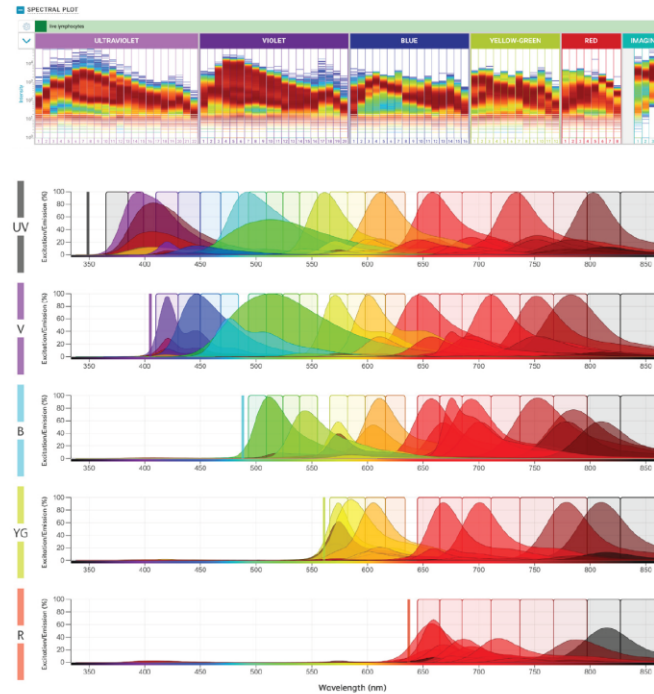


Six-way sorting of deep immunophenotyping panel

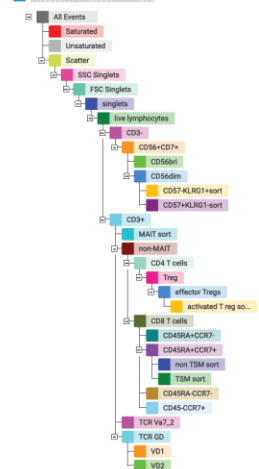
This 38-color spectral panel characterizes and sorts deep lineages of T cell and NK cell subsets.

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.

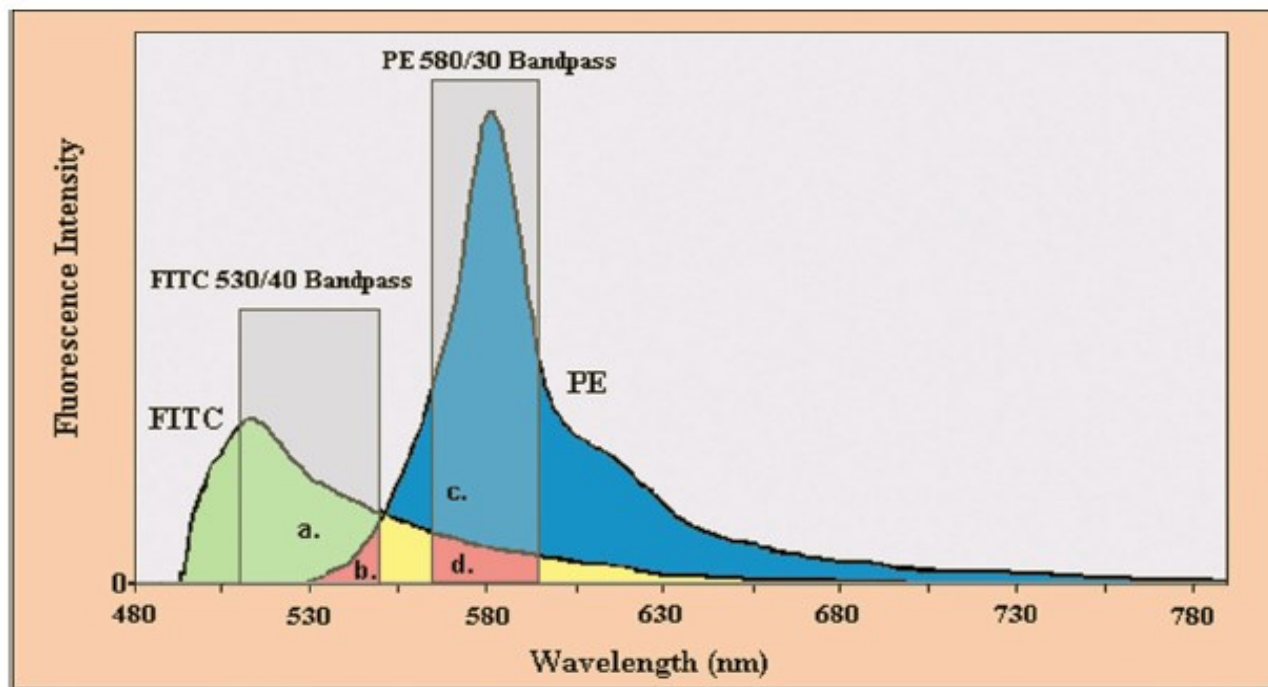
Laser	#	Fluorochrome	Marker
UV	1	BLV395	CD27
	2	FV5440UV	FV5440UV
	3	BLV496	CD8
	4	BLV563	CD16
	5	BLV615	CCR7 (CD197)
	6	BLV663	NG2C
	7	BLV737	CCR5
	8	BLV805	CD161
Violet	9	BV421	PD1
	10	V450	CD7
	11	BV480	CD45RA
	12	BV510	CD15c
	13	BV570	CD57
	14	BV605	TCRgd
	15	BV650	TCR V-α24
	16	BV711	NG2A
	17	BV750	NG2D
	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	BB780	CD95
	26	BB545	CD3
Yellow/Green	27	PE	CD25
	28	PE-Cy5	CD185
	29	PE-Cy7	CD38
	30	RYS86	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



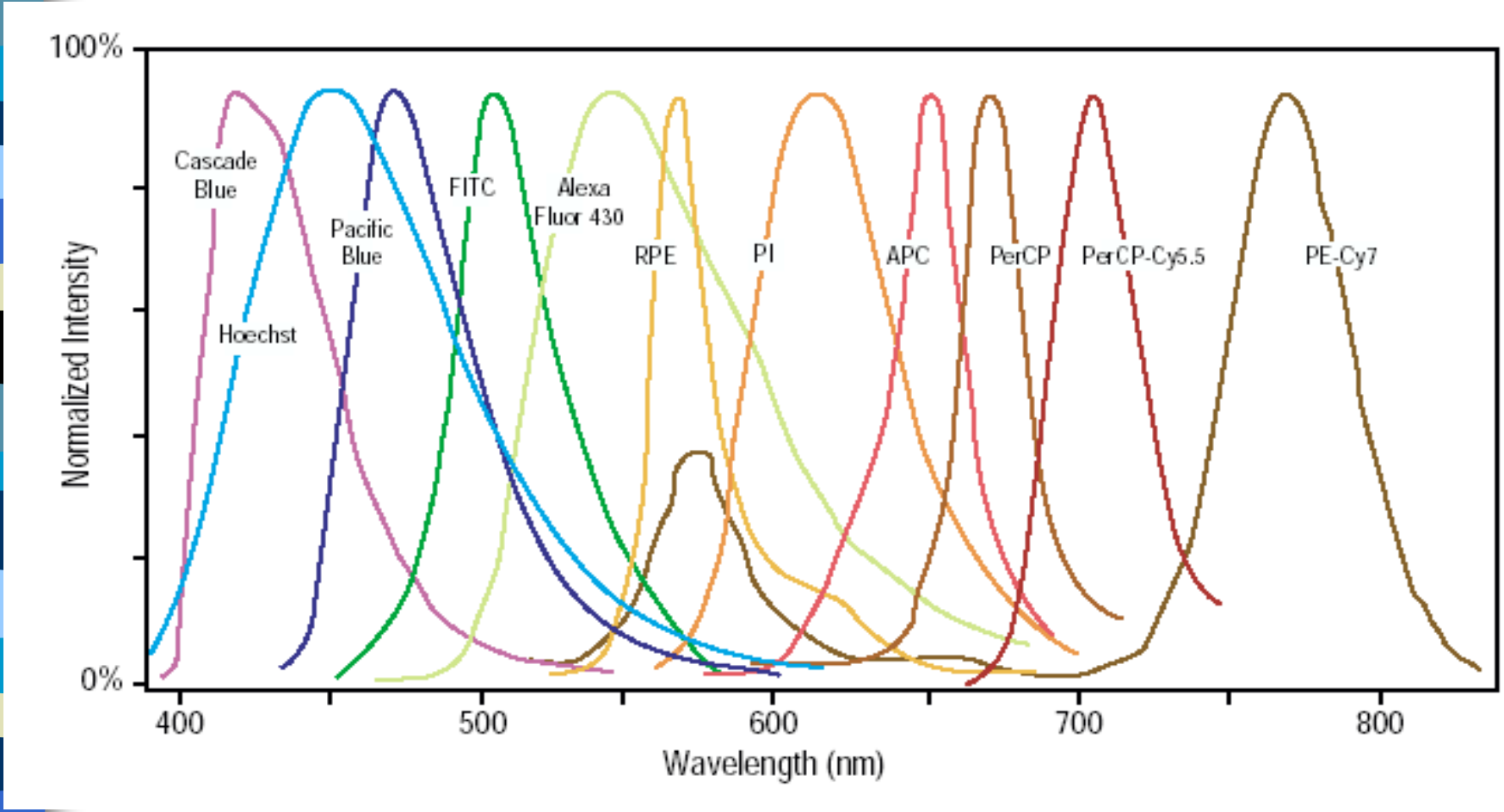
POPULATION HIERARCHY



Co je problém při vícebarevné detekci?



Emission Spectra–Spectral Overlap

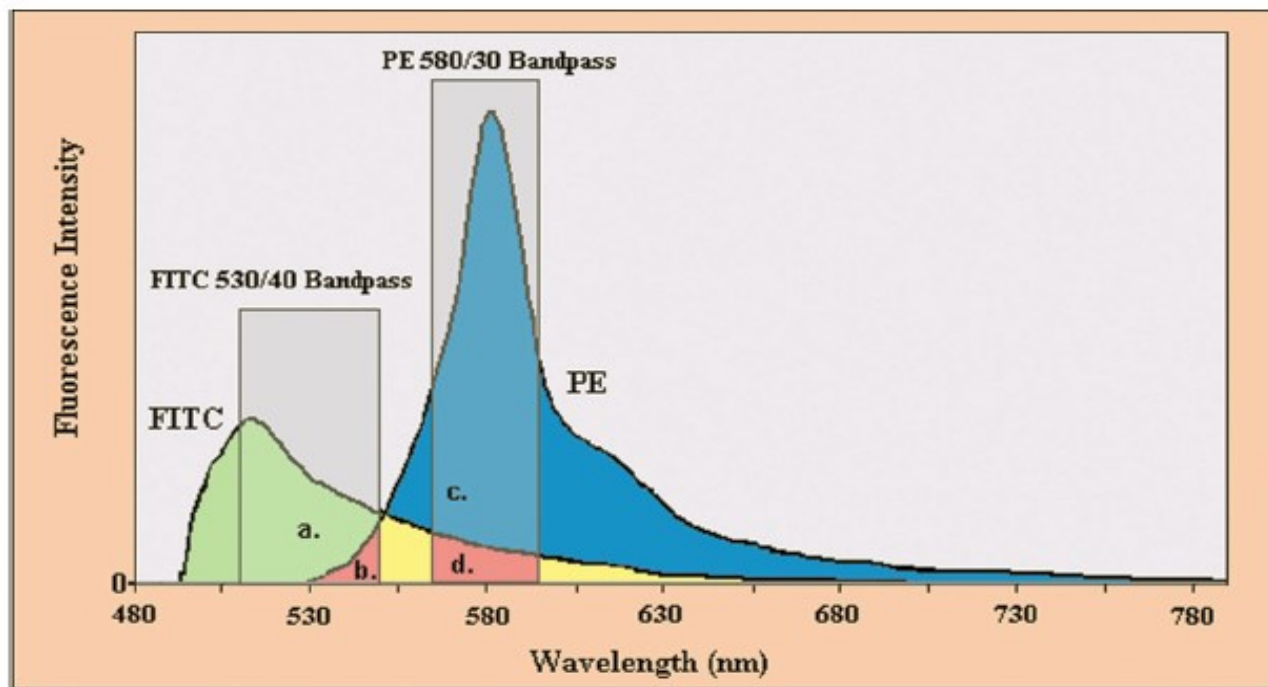




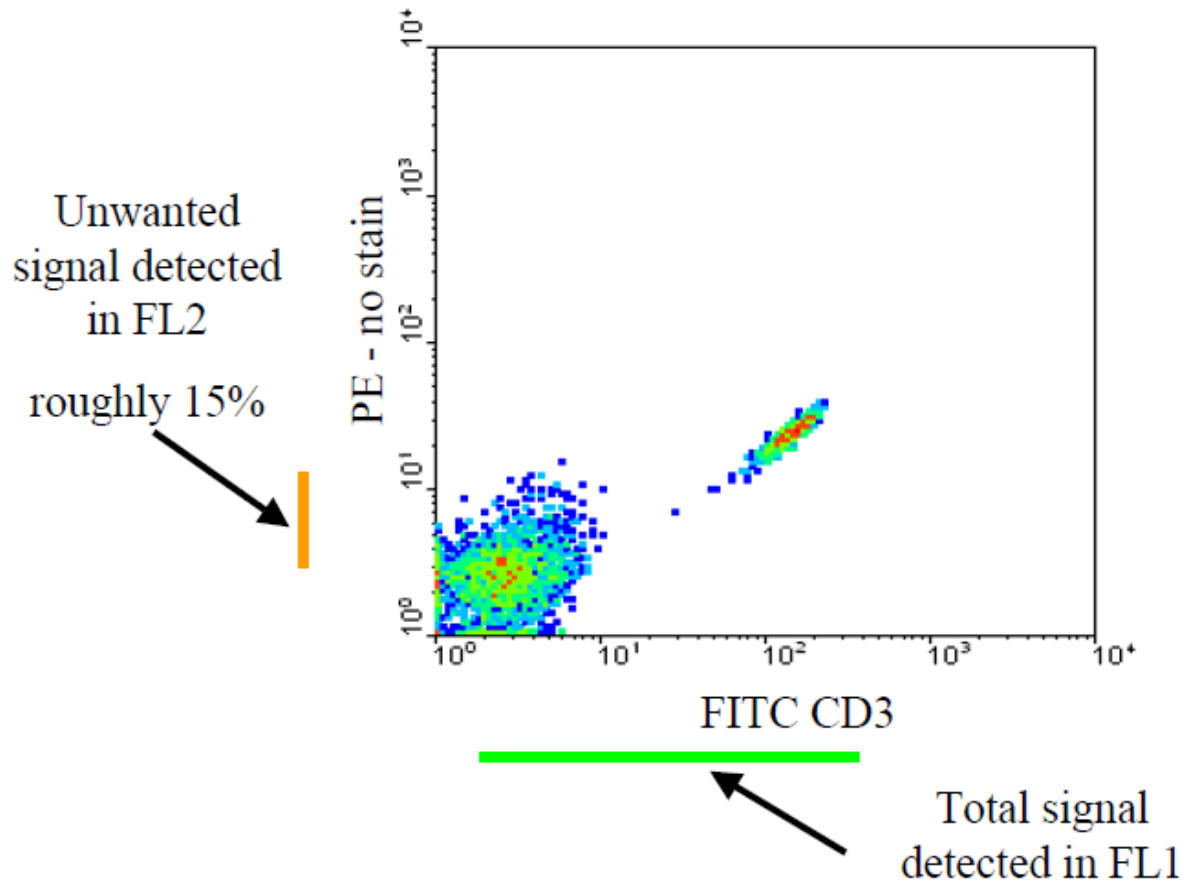
Kompenzace fluorescenčního signálu při vícebarevné detekci

- Proces při kterém dochází k eliminaci všech fluorescenčních signálů kromě signálu z fluorochromu který má být na příslušném detektoru detekován
- Nastavení pomocí mixu mikročástic či buněk označených/neoznačených příslušnými fluorochromy.

Co je problém při vícebarevné detekci?

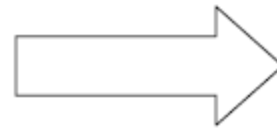
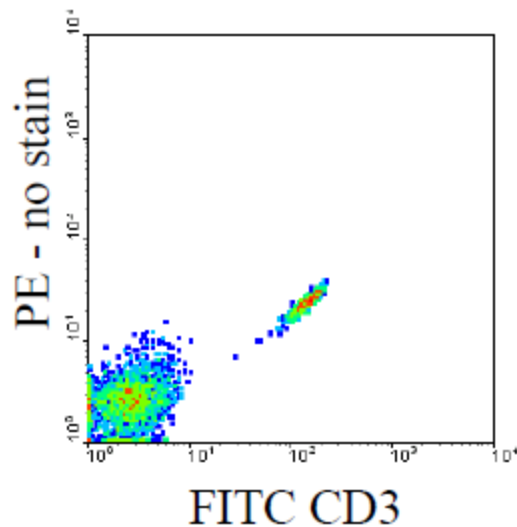


Uncompensated FITC Single stain Control



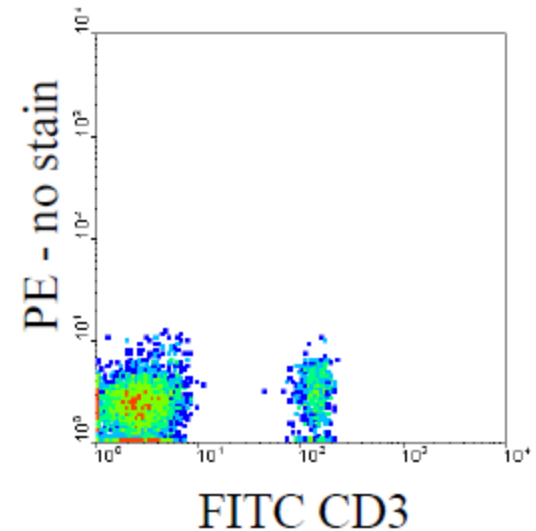
FITC Single Stain Control

Uncompensated

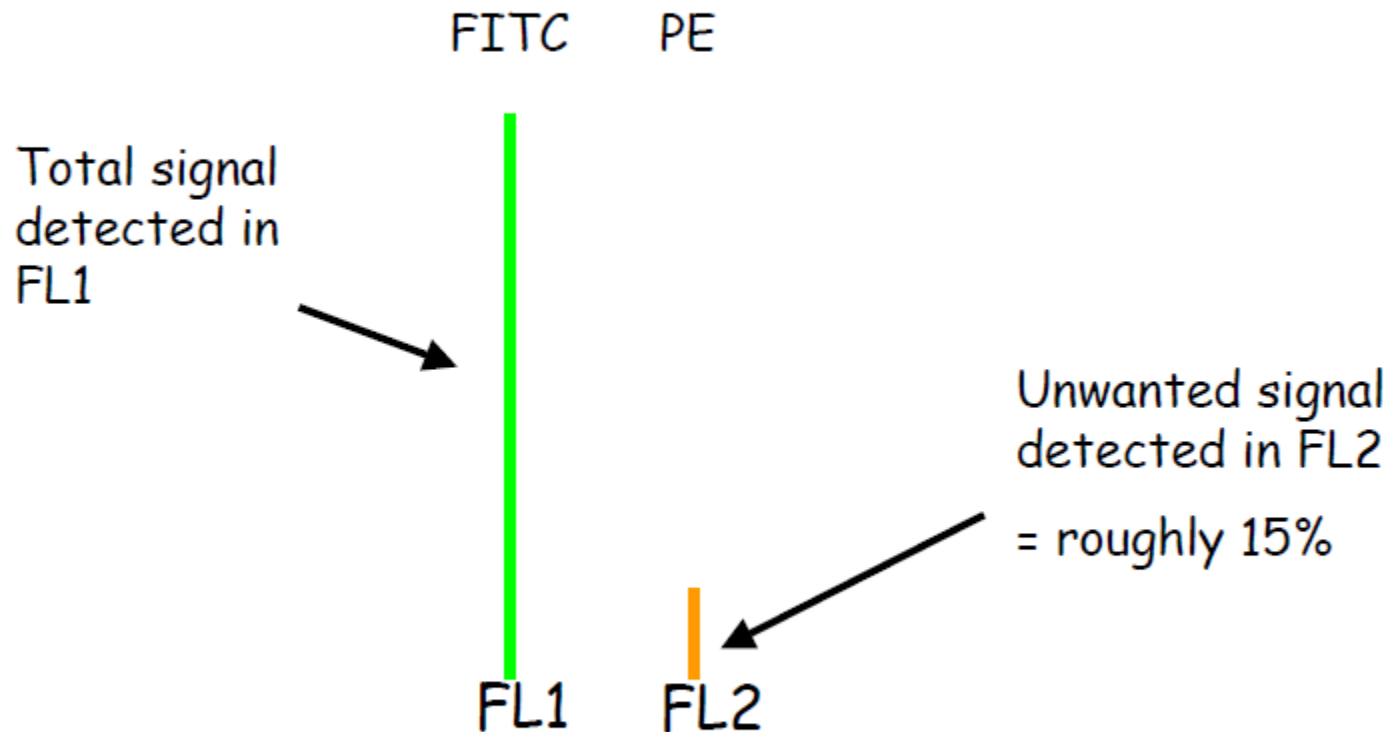


FL2-15%FL1

Compensated



FITC Single Stain Control



$$\text{True PE} = \text{Total FL2} - 15\% \text{ FL1}$$

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

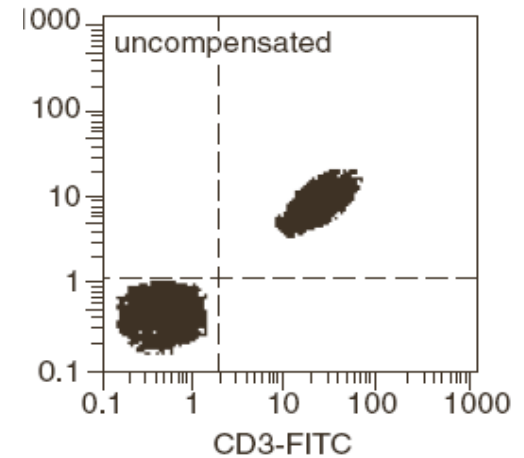
Reagent	Clone	Filter	Stain Index
PE	RPA-T4	585/40	356.3
Alexa 647	RPA-T4	660/20	313.1
APC	RPA-T4	660/20	279.2
PE-Cy7	RPA-T4	780/60	278.5
PE-Cy5	RPA-T4	695/40	222.1
PerCP-Cy5.5	Leu-3a	695/40	92.7
PE-Alexa 610	RPA-T4	610/20	80.4
Alexa 488	RPA-T4	530/30	75.4
FITC	RPA-T4	530/30	68.9
PerCP	Leu-3a	695/40	64.4
APC-Cy7	RPA-T4	780/60	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



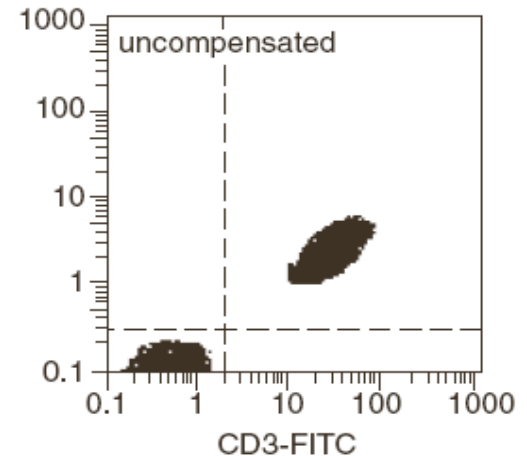
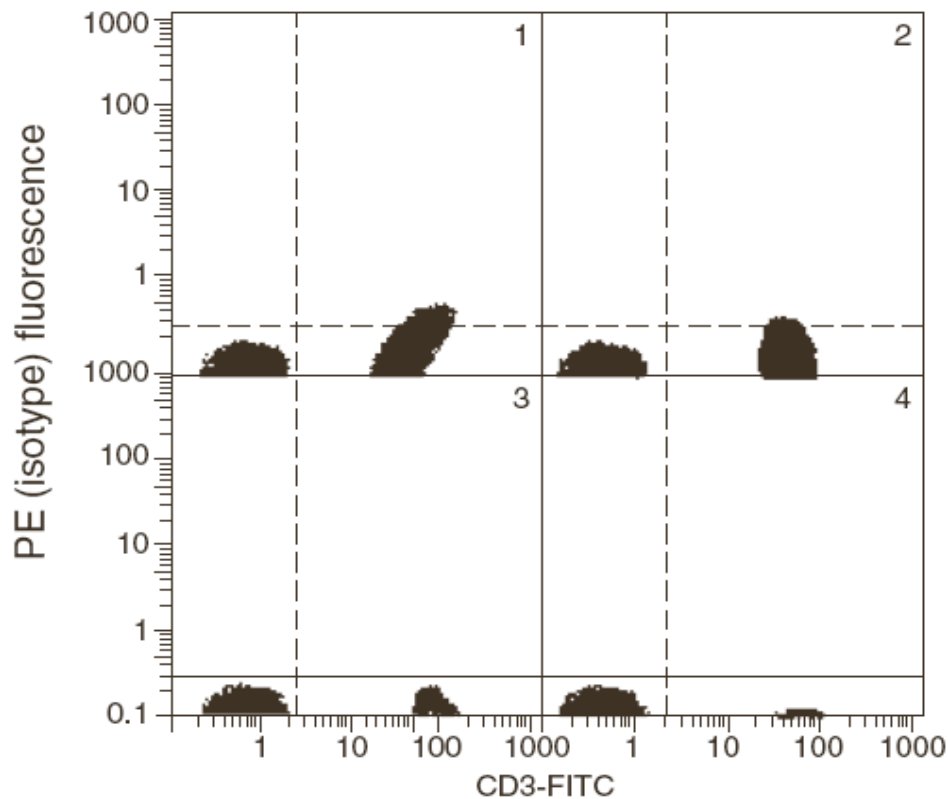
Current Protocols in Cytometry

Kompenzace fluorescenčního signálu

FITC positive & negative

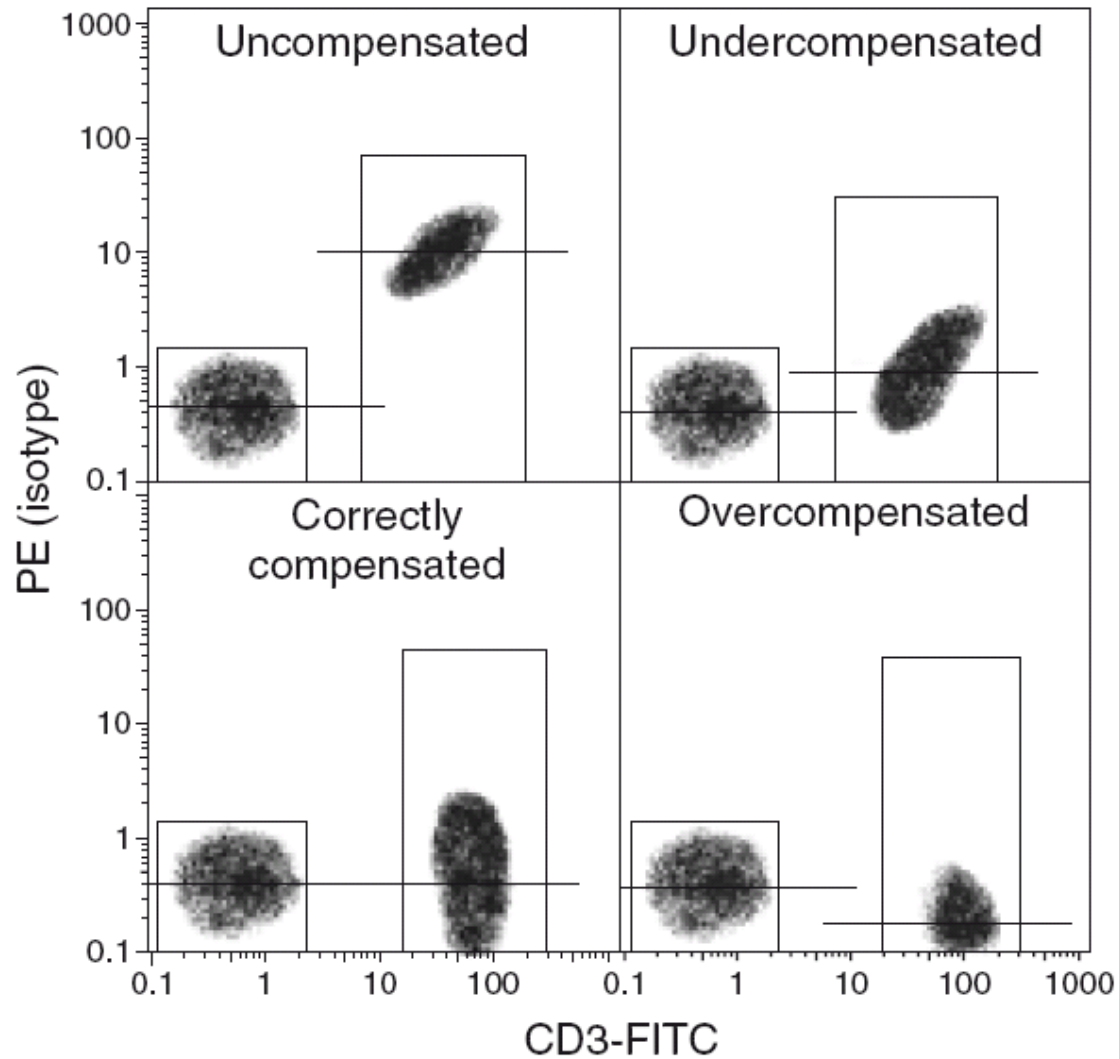
PE negative beads

NONE!



Current Protocols in Cytometry

Kompenzace fluorescenčního signálu



Nastavení kompenzací

- značené mikročástice – pro běžně konjugované fluorochromy



CaliBRITE Beads

CaliBRITE 3 three-color kit–Catalog No. 340486

CaliBRITE two-color kit–Catalog No. 349502

CaliBRITE PerCP Beads–Catalog No. 340497

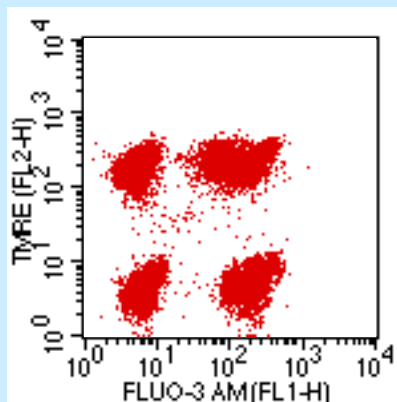
CaliBRITE APC Beads–Catalog No. 340487

CaliBRITE PerCP-Cy5.5 Beads with Bead Dilution Buffer–Catalog No. 345036

For In Vitro Diagnostic Use with FACS brand flow cytometers

Setup	Tube ^a	Unlabeled	FITC	PE	PerCP or PerCP-Cy5.5 ^b	APC
two-color	A	1 drop				
	B	1 drop	1 drop	1 drop		
three-color	A	1 drop				
	B	1 drop	1 drop	1 drop	1 drop	
four-color	A	1 drop				1 drop
	B	1 drop	1 drop	1 drop	1 drop	1 drop

- značené buňky – pro vitální značení



parametr - detektor amp.

FL1 - 544

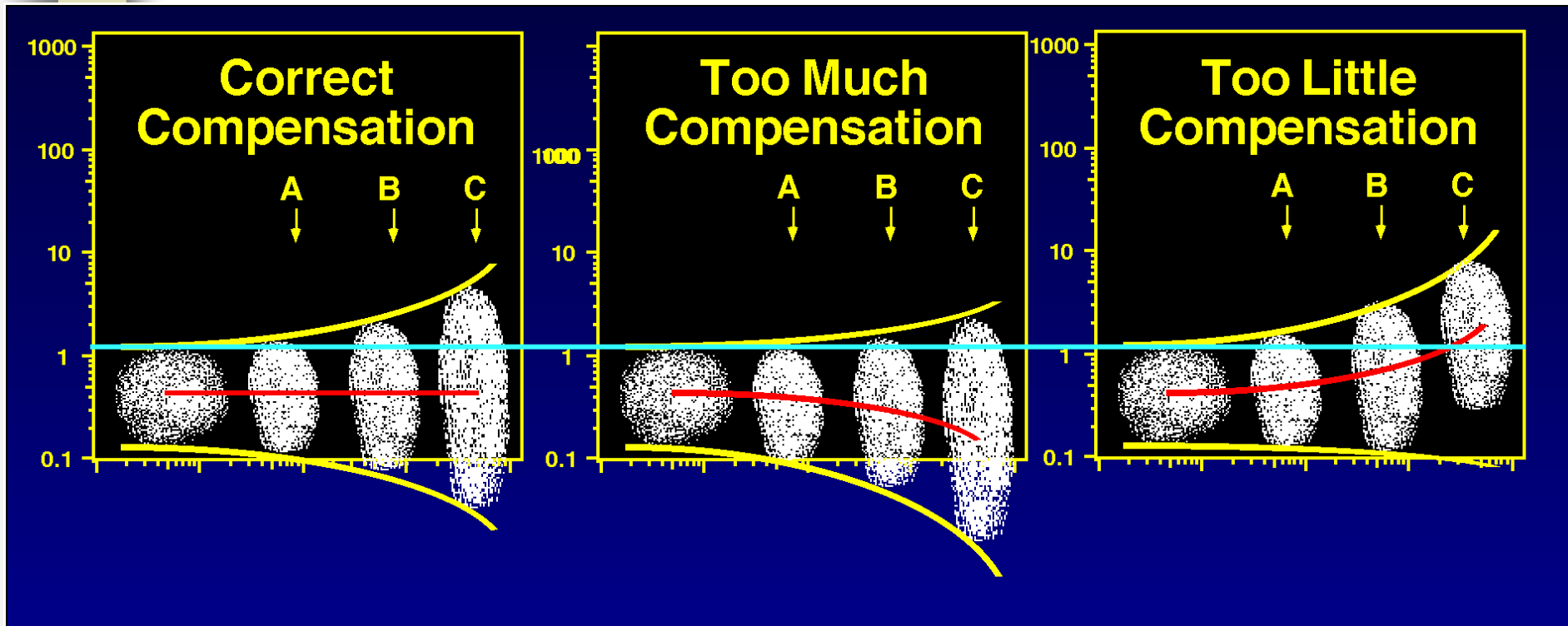
FL2 - 434

kompenzace

FL1 - 1.1%FL2

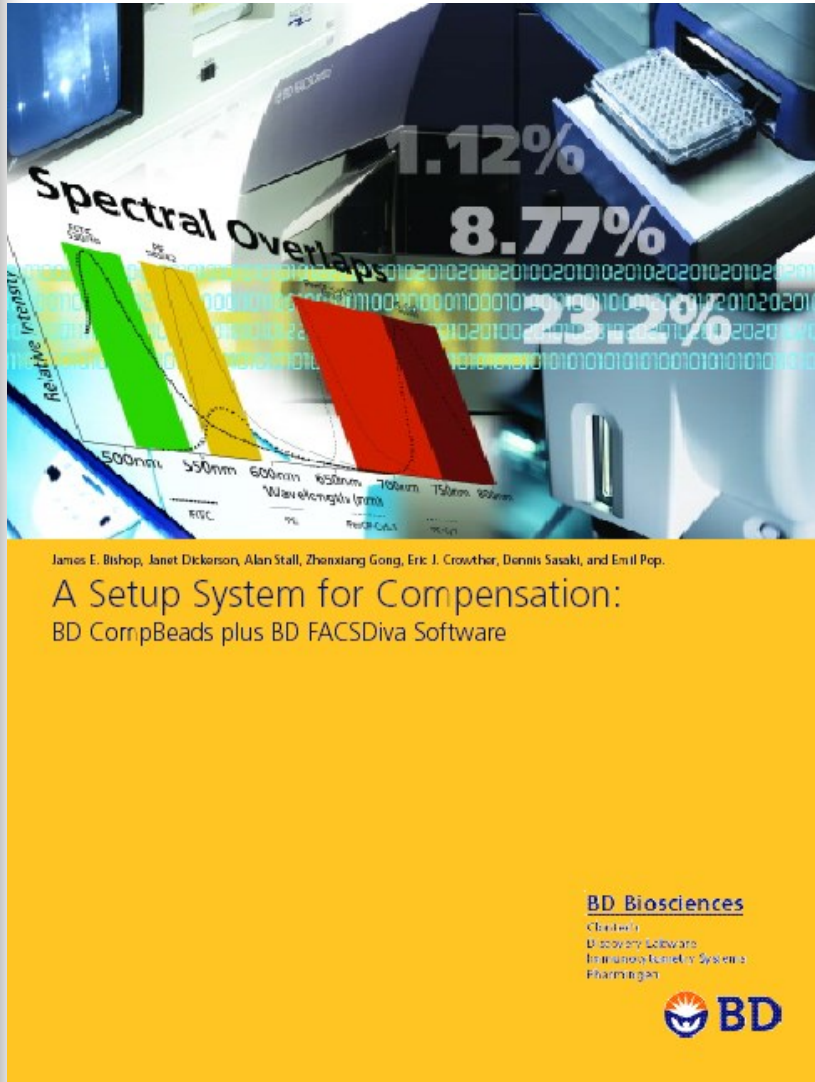
FL2 - 17.5%FL1

Which marker for compensation?



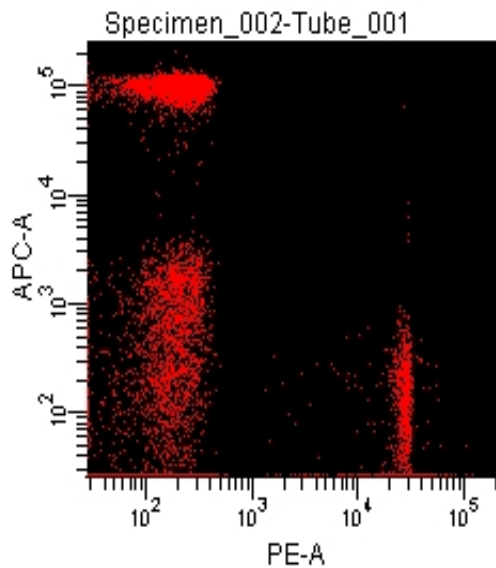
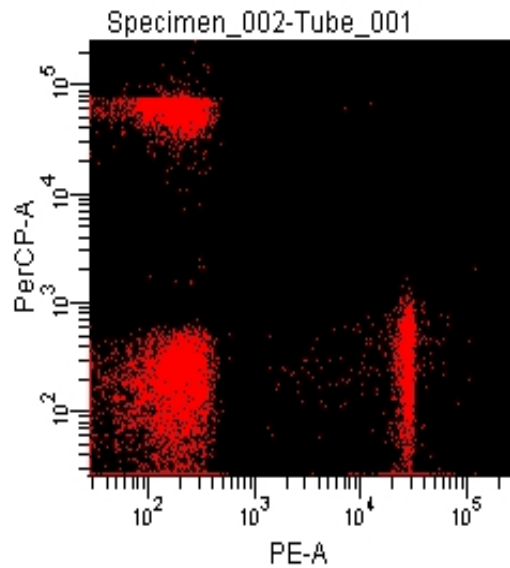
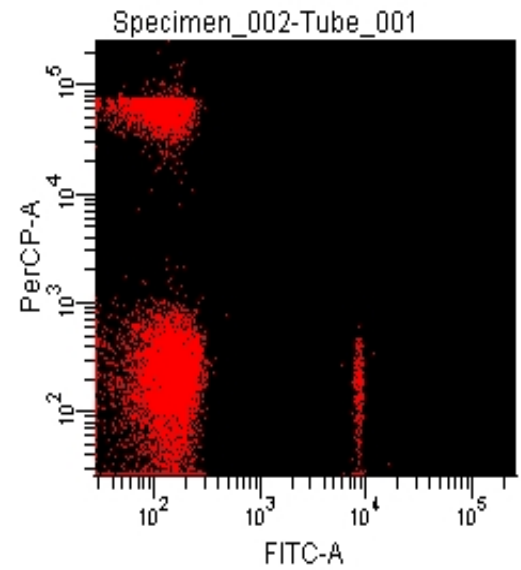
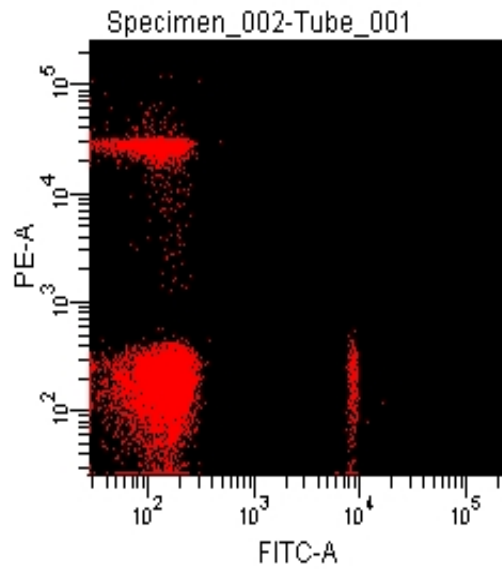
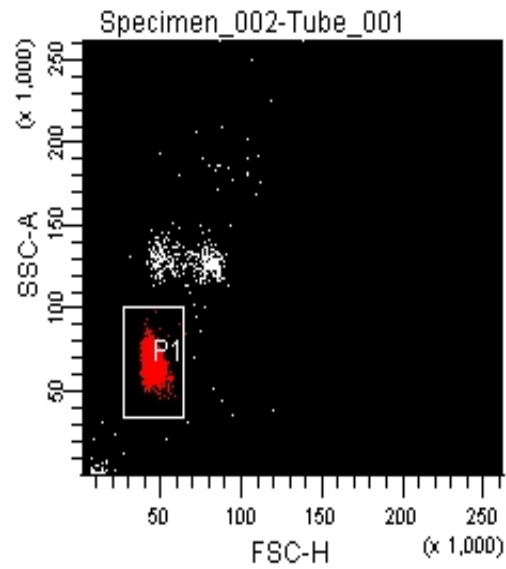
Small errors in compensation of a dim control (A) can result in large compensation errors with bright reagents (B & C).
Use bright markers to setup proper compensation.

BD Comp Beads

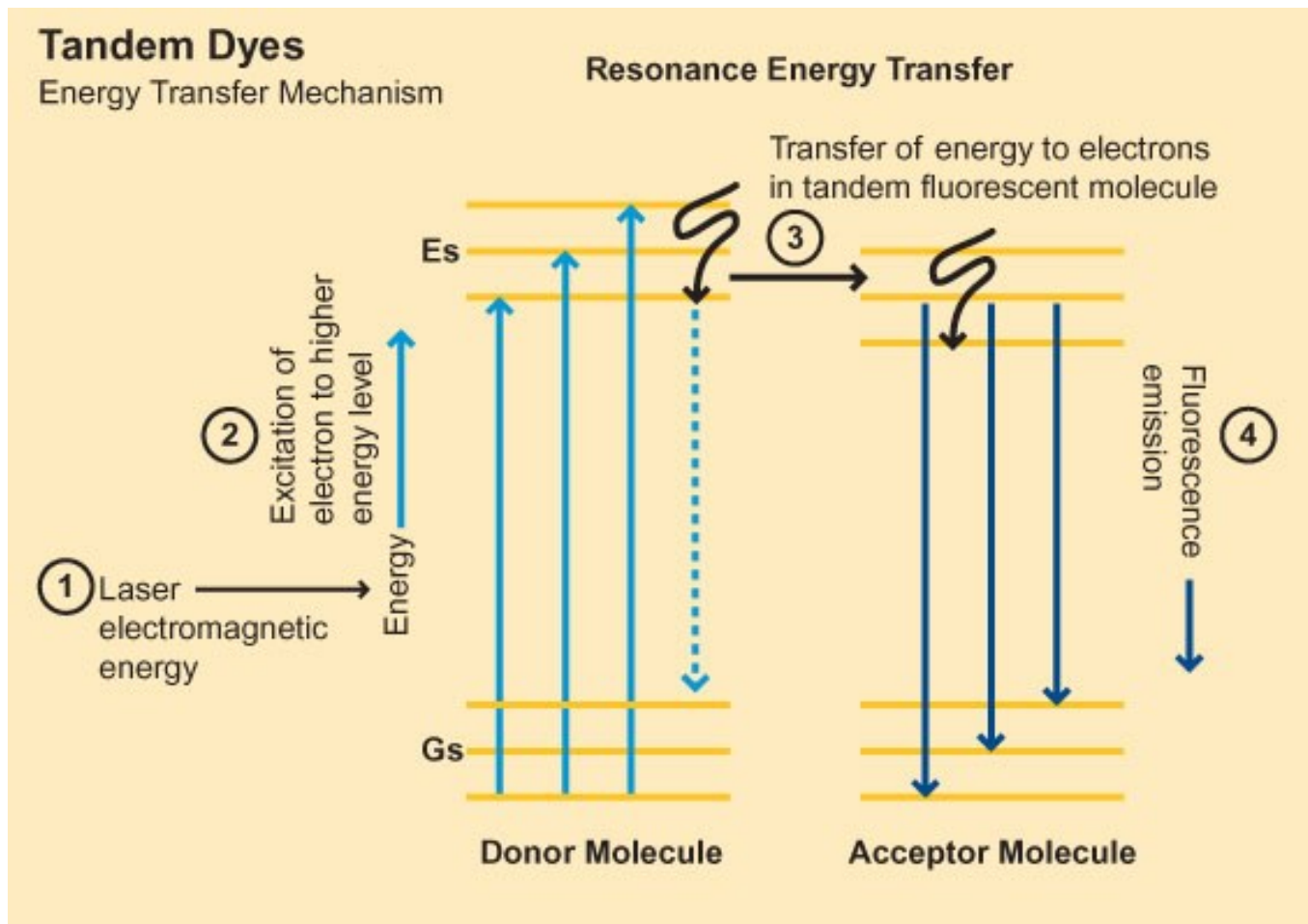


- Always positive
- Bright staining
- Save sample (HIV patients)
- Use the same antibody for compensation and the real experiment

BD Comp Beads

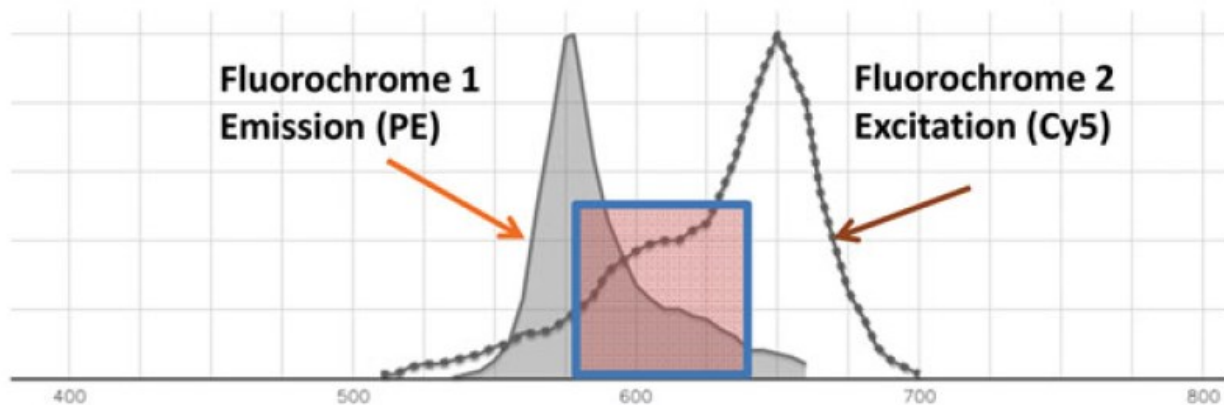
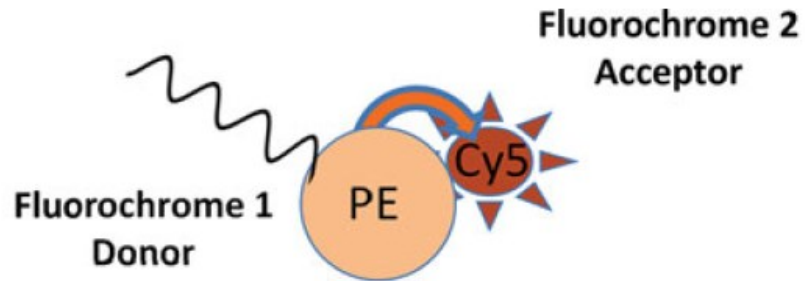


Tandemové značky

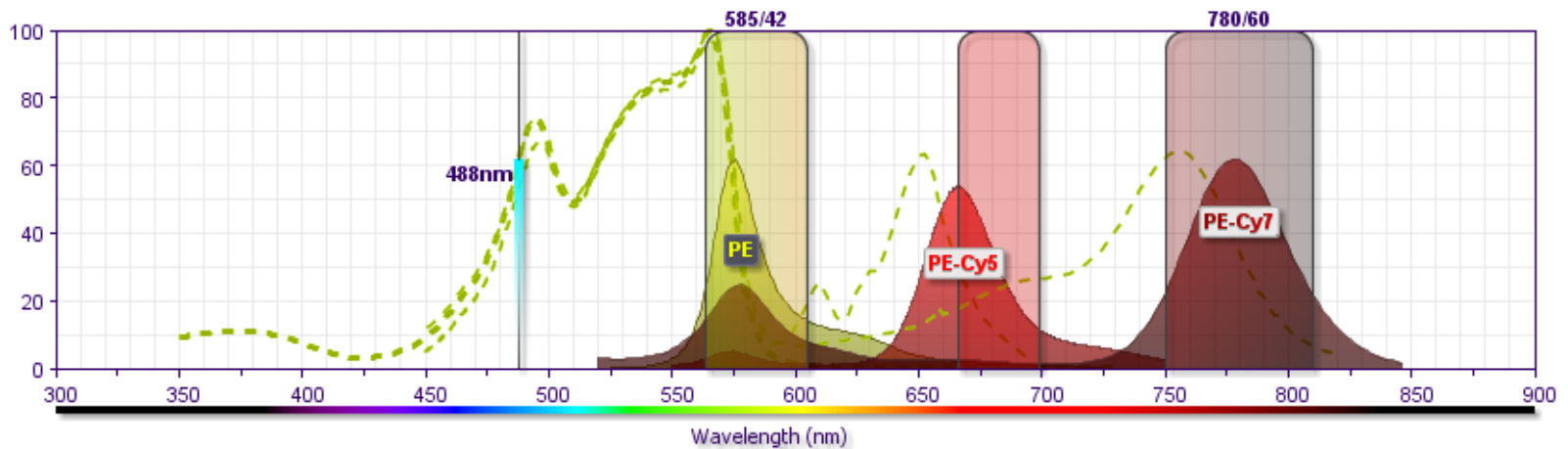
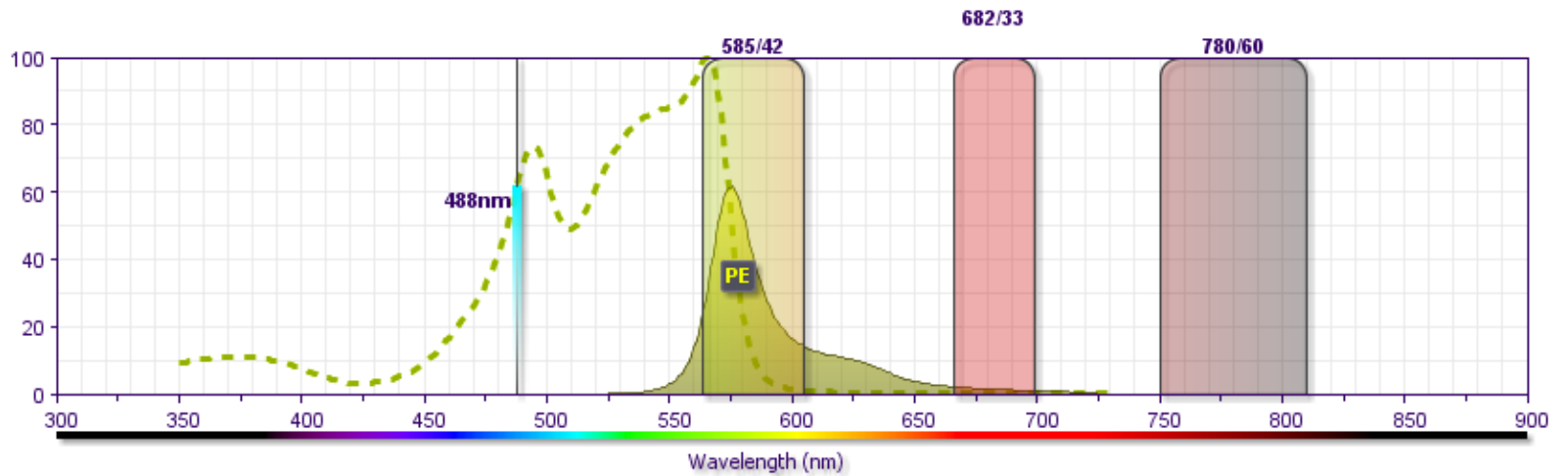


Tandemové značky

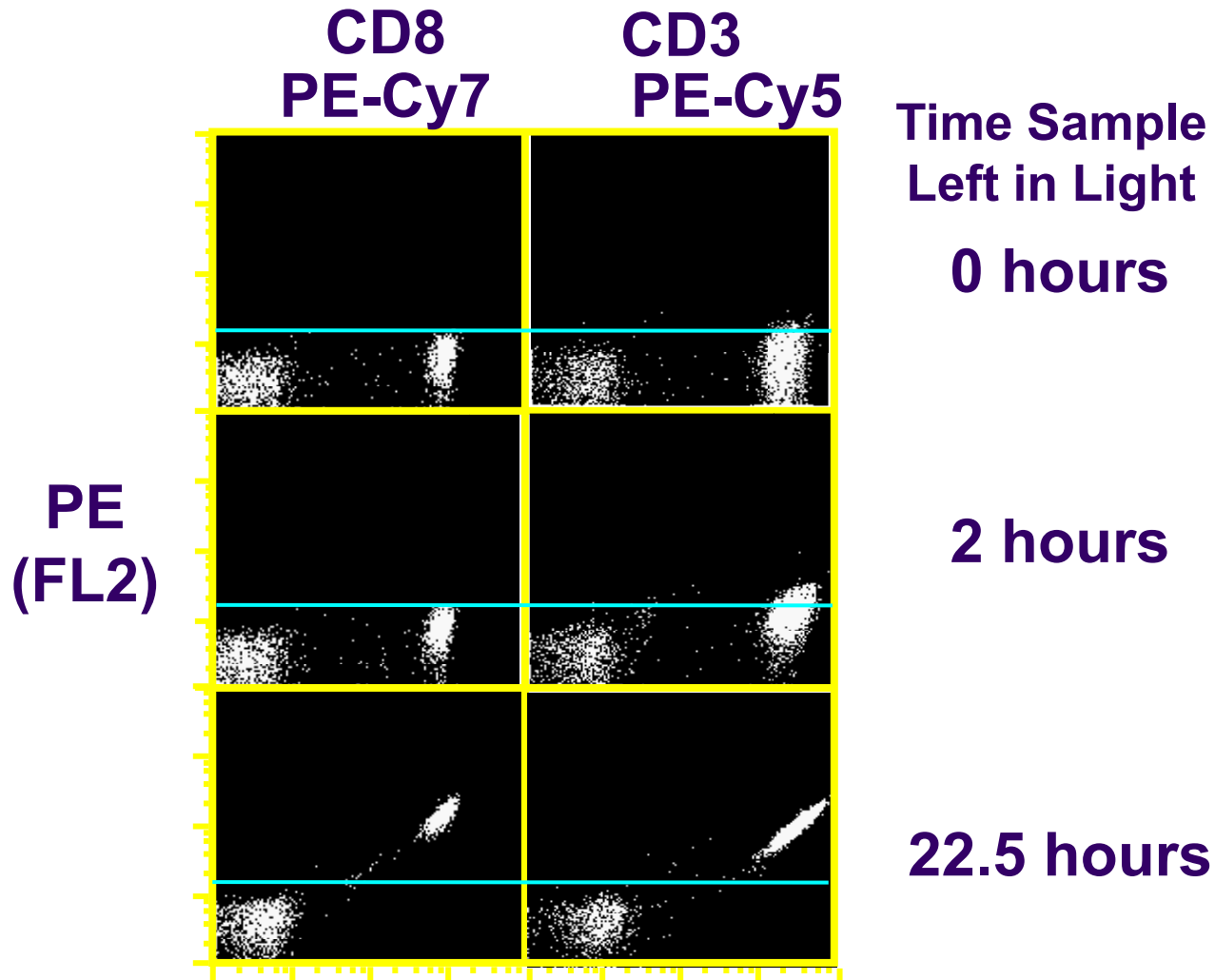
Tandem dyes
PE-Cy5



Tandemové značky - příklad

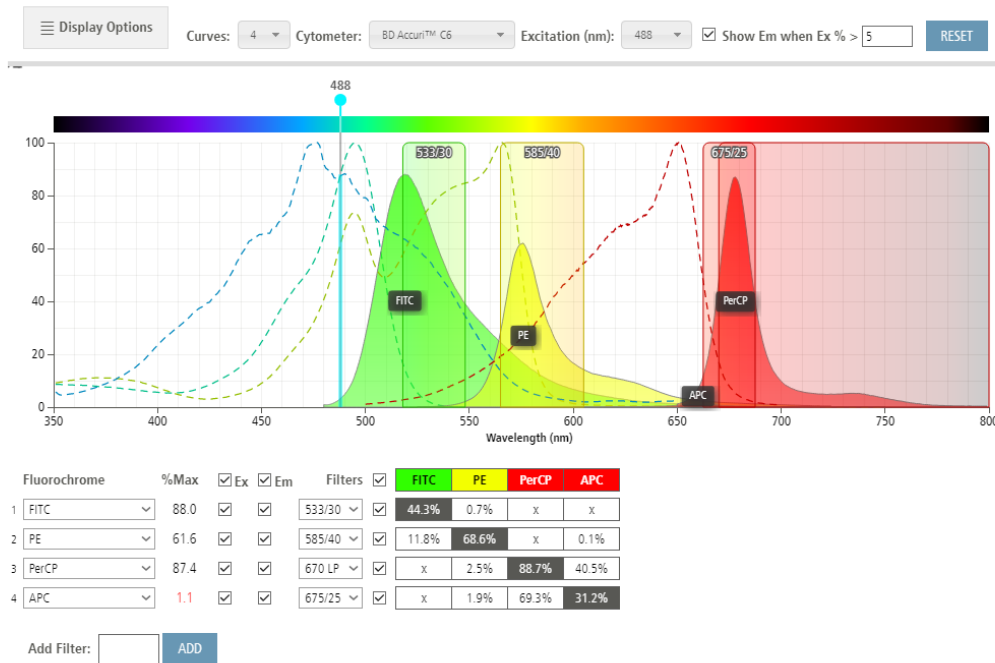


Tandems are light sensitive



BD Spectrum Viewer

The BD Spectrum Viewer is a tool that depicts the excitation and emission curves of fluorochromes common to flow cytometry. This tool can be used to determine appropriate filters to detect a fluorochrome as well as fluorochrome compatibility and fluorescent spillover. Unlike a static image showing excitation and emission histograms, this tool will demonstrate how a fluorochrome will have the same emission profile—but have varying brightness—based on the excitation wavelength of the laser. Choosing different laser wavelengths will transform the emission curve based on the percent excitation at that wavelength.



<https://wwwbdbiosciences.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



- Jiné řešení?
#1

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)



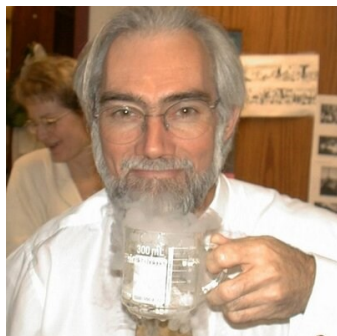
Steve Jobs, Apple

Spectral flow cytometry

J.P. Robinson, Purdue University

Cytometry Part A • 81A: 35–44, 2012

ORIGINAL ARTICLE



Cytometry

PART A
Journal of the
International Society for
Advancement of Cytometry



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

Gérald Grégori,^{1,2} Valery Patsekina,^{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
Copyright 2005 Microscopy Society of America

Multispectral Flow Cytometry: Next Generation Tools For Automated Classification

J. Paul Robinson^{a,b}, Valery Patsekina^a, Gerald Grégori^a, Bartek Rajwa^{a,b}, and James Jones^{a,b}

^aDepartment of Basic Medical Science, School of Veterinary Medicine, and ^bWeldon Department of Biomedical Engineering, Purdue University, West Lafayette, IN, 47907, USA



(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	5,394,237 A	2/1995	Chang et al.	188/79.51
	5,422,712 A	6/1995	Ogano	356/73
	5,675,517 A	10/1997	Stokdjik	702/85
(75) Inventors: Joseph Paul Robinson , West Lafayette, IN (US); Bartłomiej Rajwa , West Lafayette, IN (US); Gérald Grégori , Marseille (FR); Valery Patsekina , West Lafayette, IN (US)	5,719,667 A *	2/1998	Miers	356/73
	6,240,541 B1 *	6/2001	Baraji et al.	356/73
	6,630,307 B2 *	10/2003	Broschek 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

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

(Continued)



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

FOREIGN PATENT DOCUMENTS

EP 0 315 939 5/1989

(Continued)

Spectral flow cytometry

SONY Sony Biotechnology Inc.   US

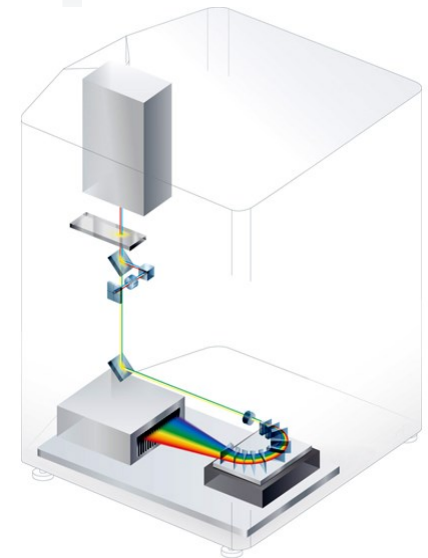
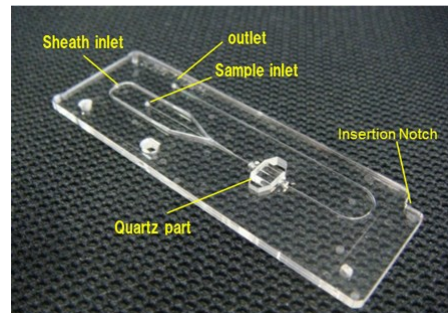
Products ▸ Resources ▸ Support ▸ About Us ▸ Contact Us ▸ Order Info ▸ Site Q

Overview Features Applications Specifications Literature

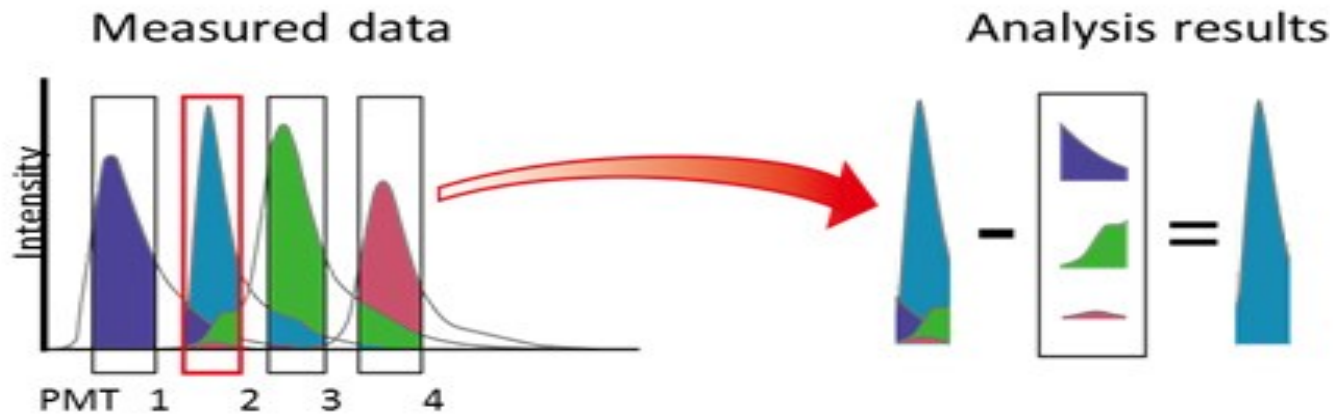
See Everything

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.

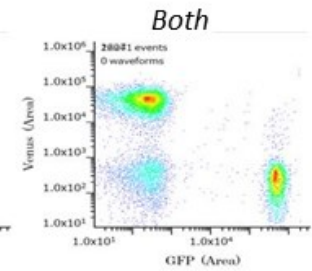
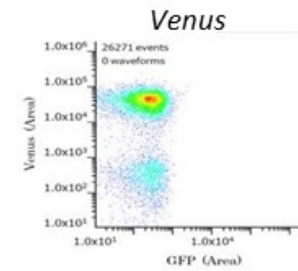
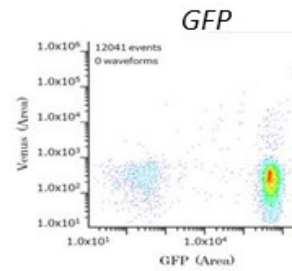
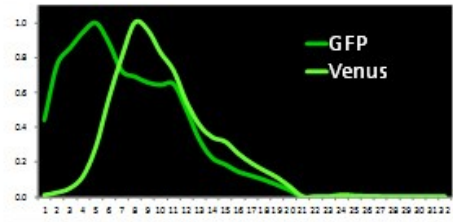
The SP6800 Spectral Analyzer also introduces new Flow Point technology to analyze core stream and sample event location within the flow cell. To improve accuracy of data, this system also provides unique functions to display and analyze cellular autofluorescence and allows the user to easily automatically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



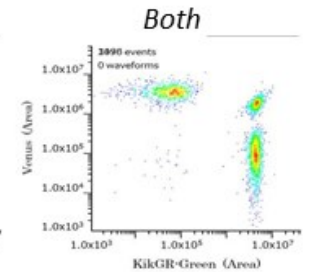
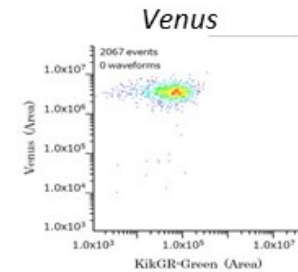
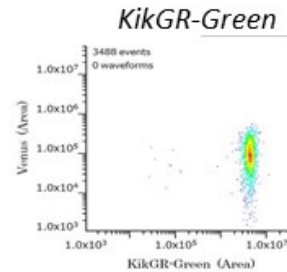
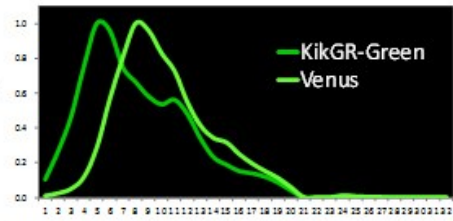
Conventional vs. spectral analysis



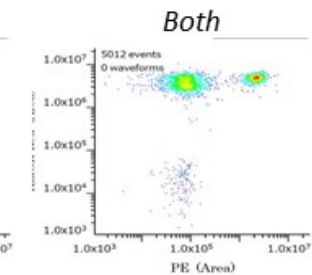
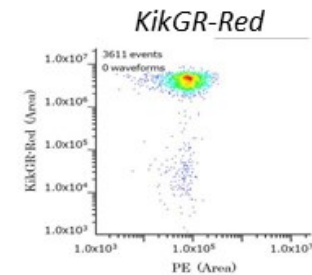
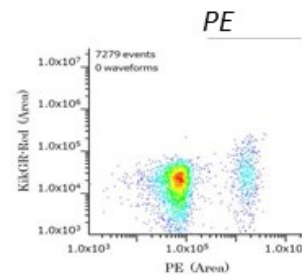
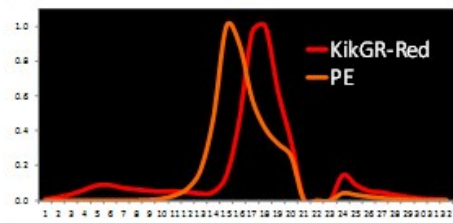
GFP
Venus



KikGR-Green
Venus



KikGR-Red
PE



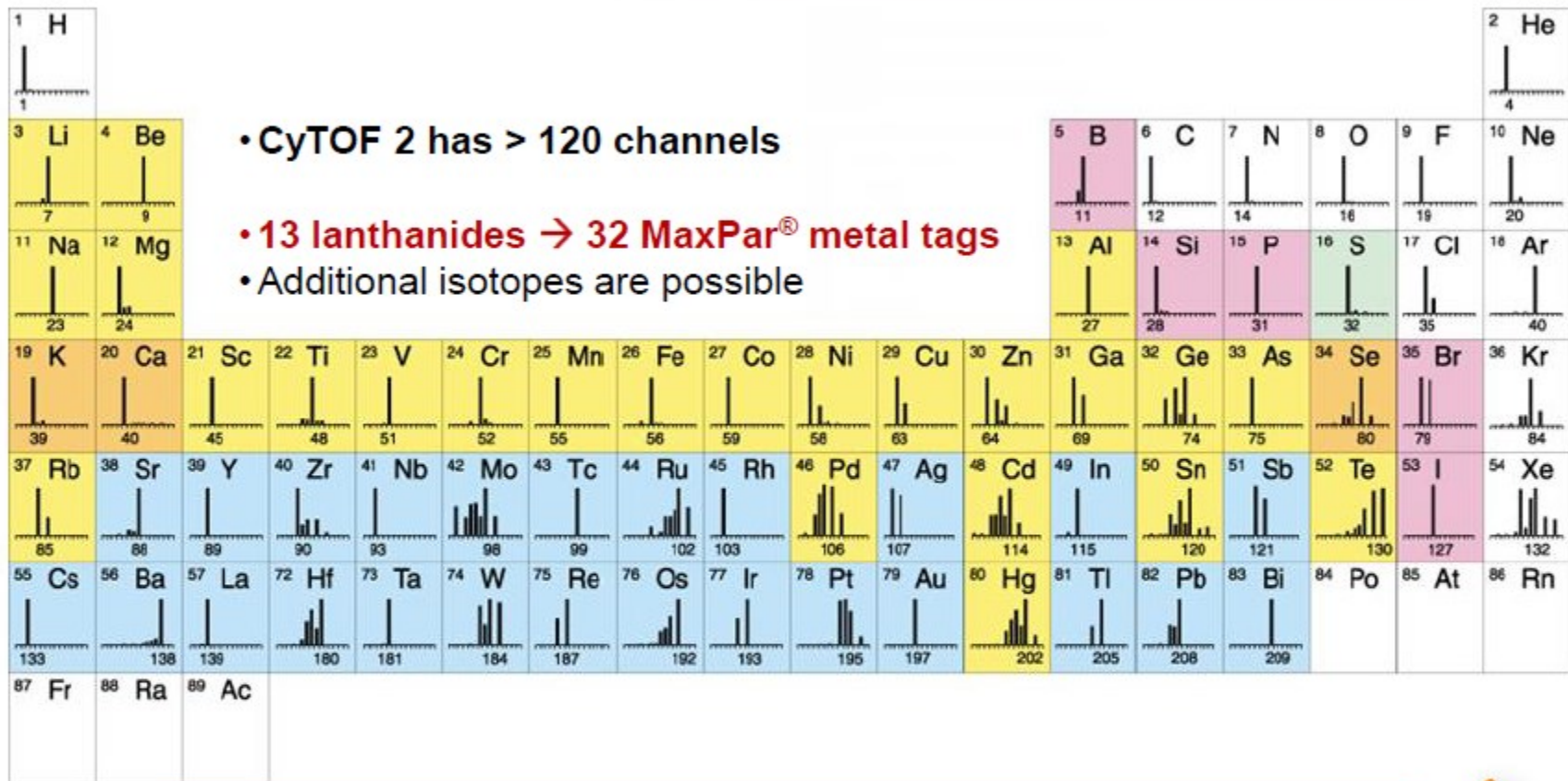
For revealing spatiotemporal regulation of immune cells, fluorescent proteins are very useful, which can be difficult to analyze with traditional flow cytometry technologies. These figures show how easily the SP6800 Spectral Analyzer can separate overlapping spectra of fluorescent proteins and fluorochromes.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

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



- Jiné řešení?
#2

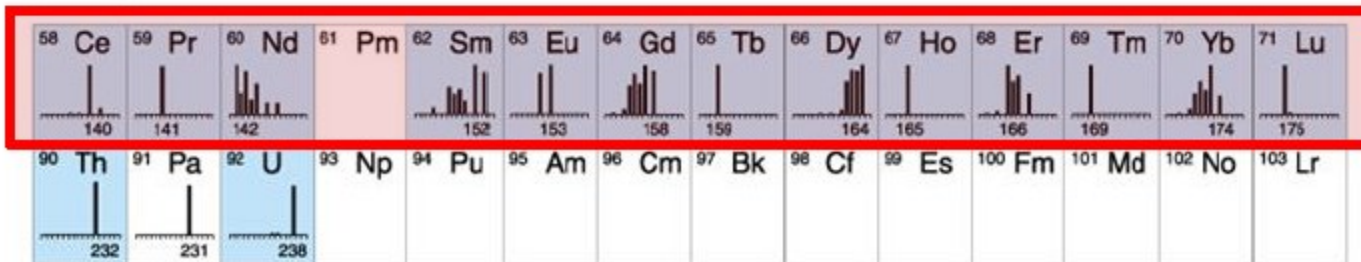
Probing with Isotopes



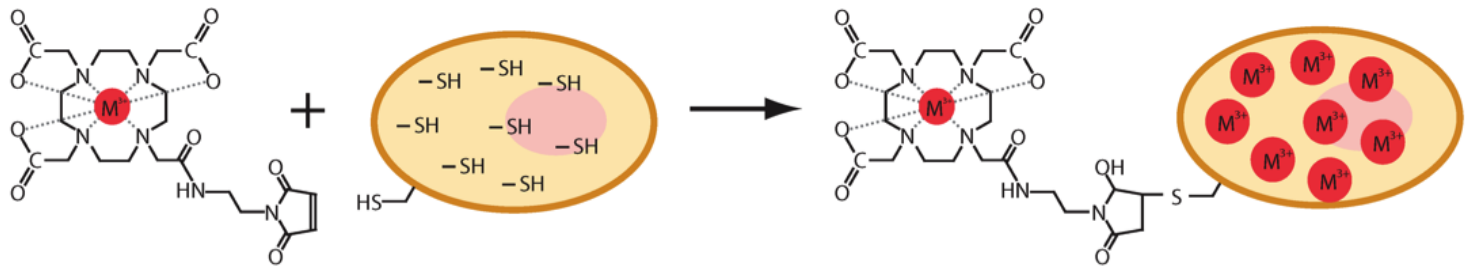
• CyTOF 2 has > 120 channels

• 13 lanthanides → 32 MaxPar[®] metal tags

• Additional isotopes are possible



Single Cell Mass Cytometry



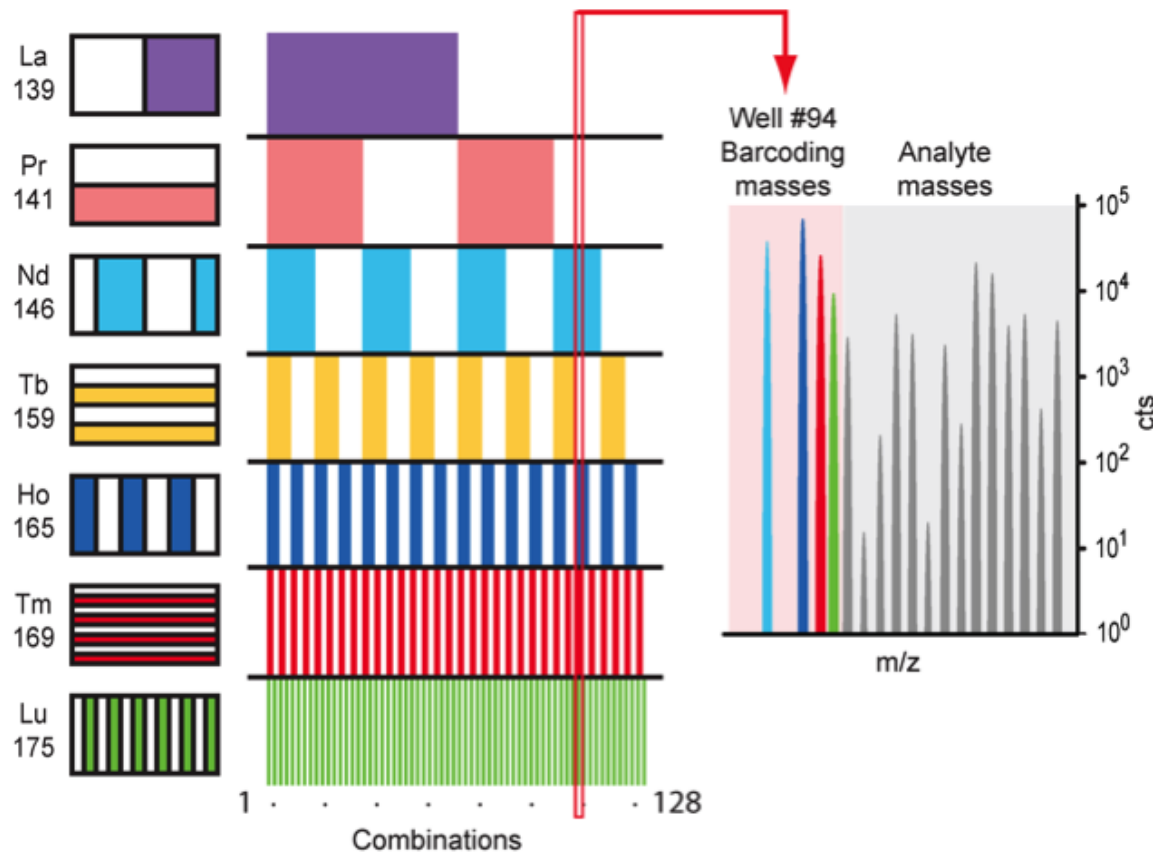
Cells were covalently labeled with a bifunctional compound, maleimido-mono-amide-DOTA (mDOTA). This compound can be loaded with a lanthanide(III) isotope ion, and reacts covalently with cellular thiol groups through the maleimide moiety.

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

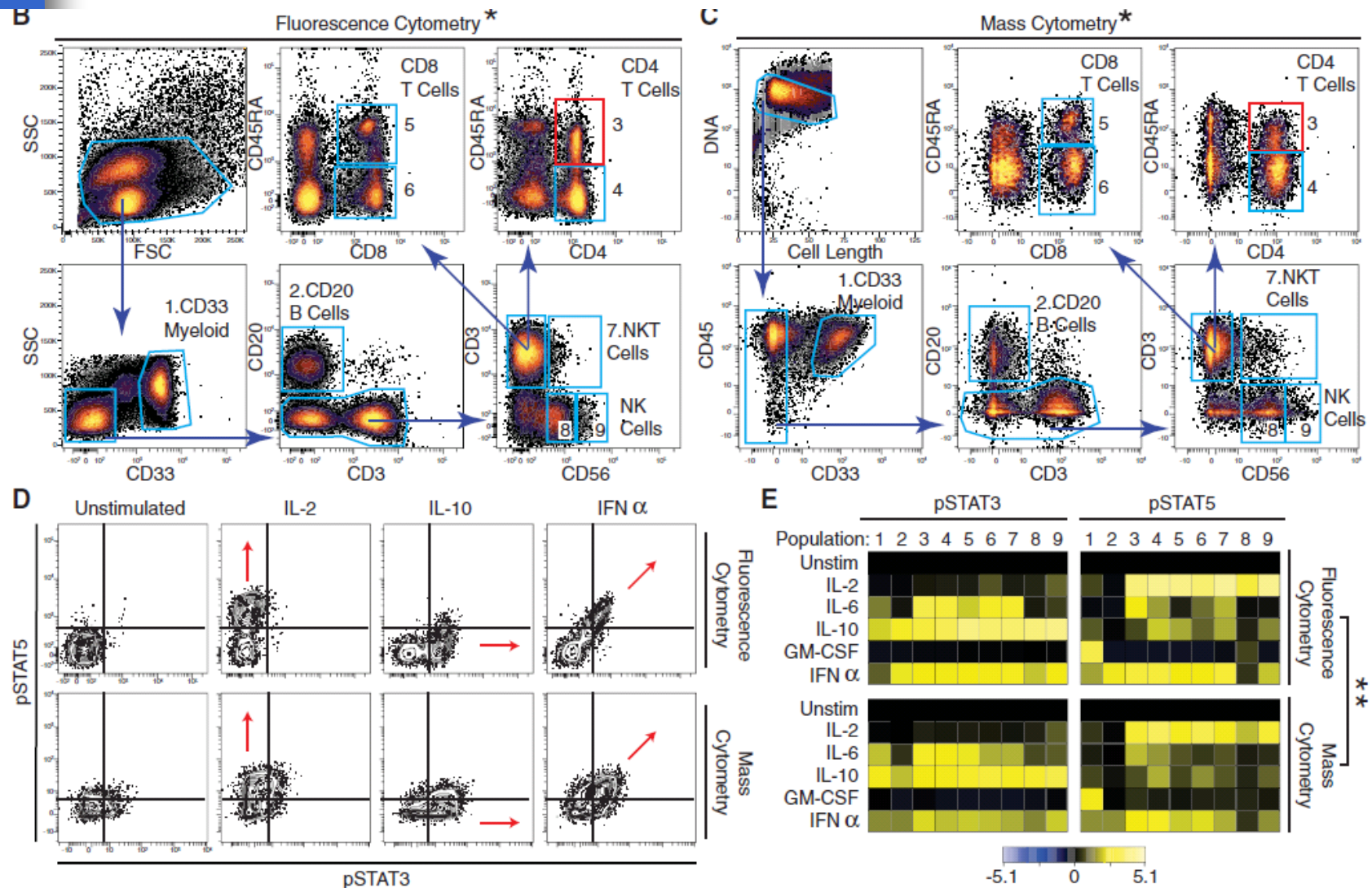


Seven unique lanthanide isotopes were used to generate 128 combinations, enough to barcode each sample in a 96-well plate. The seven lanthanide isotopes, their masses and their locations on the 96-well plate are shown.

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

Sean C. Bendall, *et al.*
Science **332**, 687 (2011);

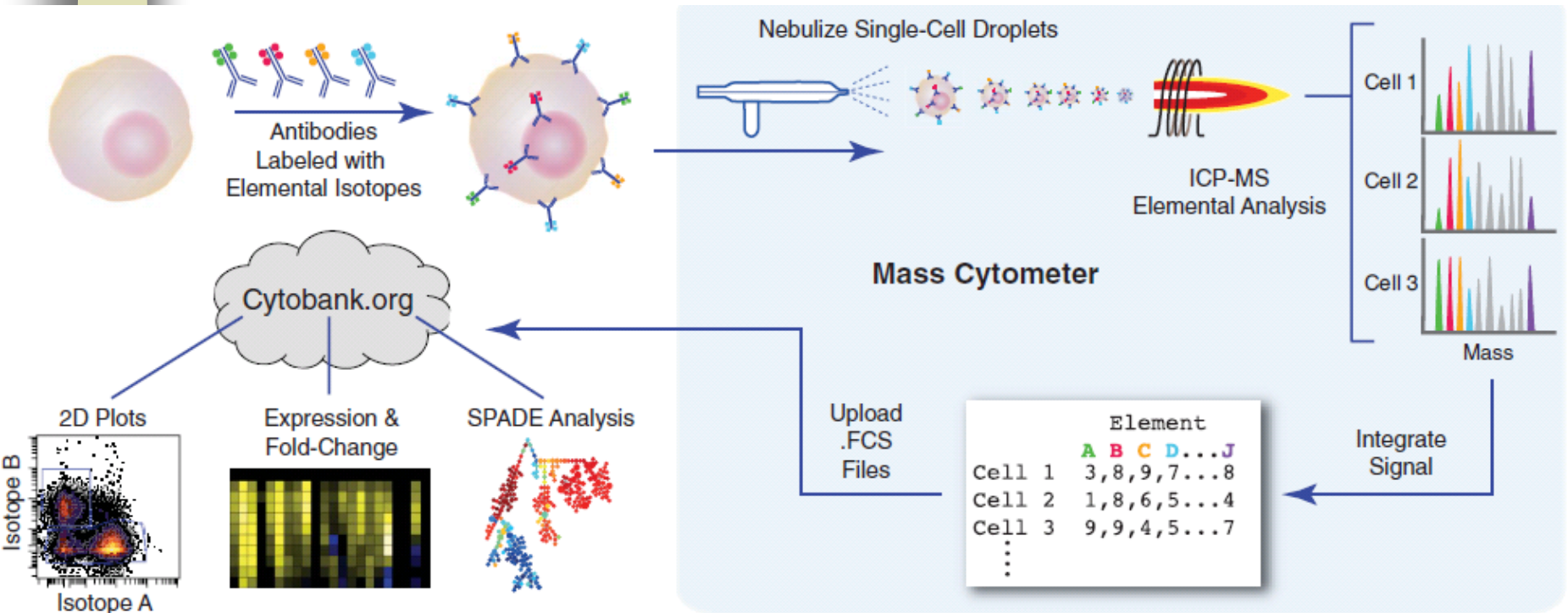
Single Cell Mass Cytometry



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

Sean C. Bendall, *et al.*
Science **332**, 687 (2011);

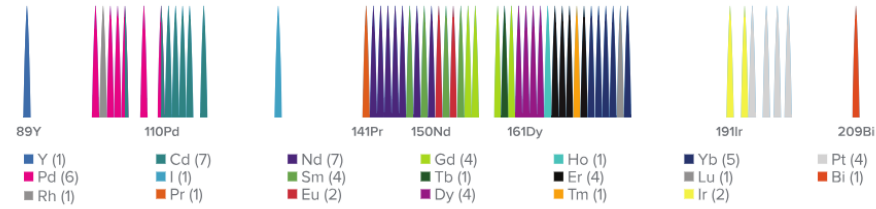
Single Cell Mass Cytometry



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

Sean C. Bendall, *et al.*

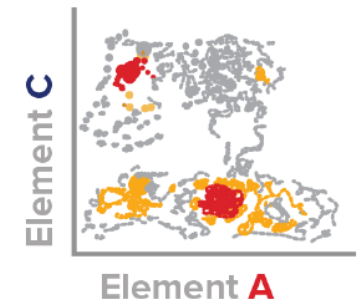
Science **332**, 687 (2011);



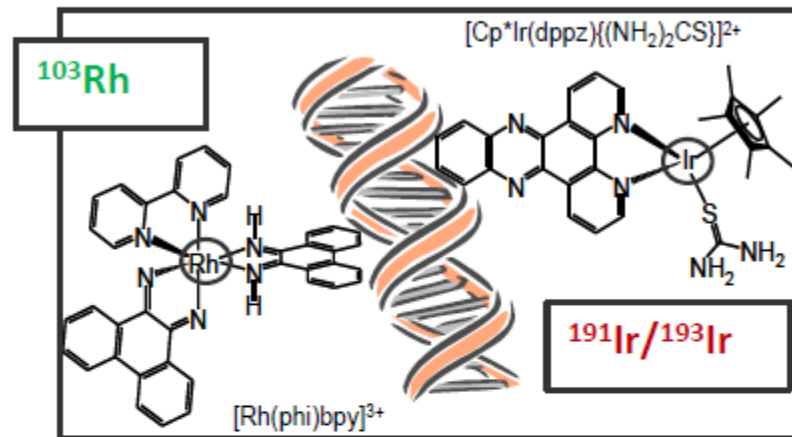
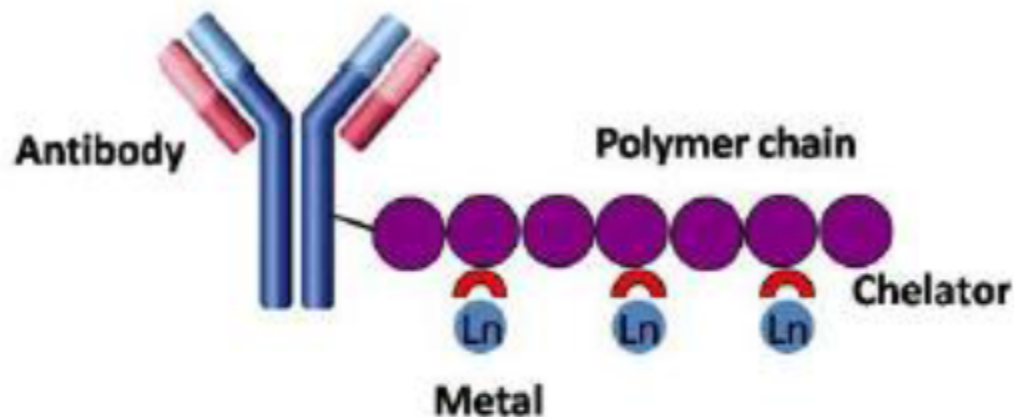
Mass Cytometry: 50+ Parameters on Millions of Cells

Discovery of new biology
Comprehensive functional profiling

Basic research
Drug discovery
Clinical research



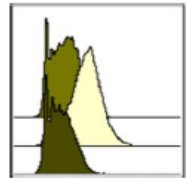
MaxPar[®] metal-tagged probes



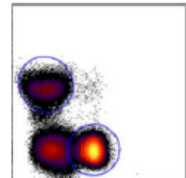
- Lanthanide tags: 32 isotopes from 13 elements
- IgG antibody probes:
 - Pre-conjugated antibodies (220 currently available and growing)
 - MAXPAR[®] labeling kits (for 32 stable isotopes)
- Nucleic acid-binding metallo-intercalators
 - Identifies single cell events
 - Live/dead indicator

Analyze: Cytobank

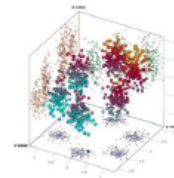
Plot raw data



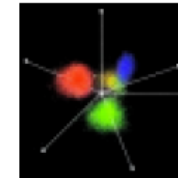
Histogram



Biaxial plot

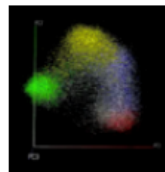


3D plot

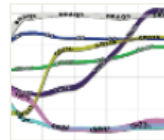


Radar

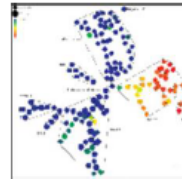
Reduce dimensionality



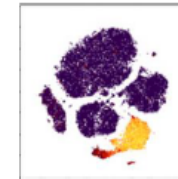
PCA



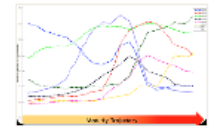
Gemstone



SPADE

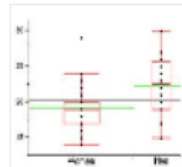


viSNE

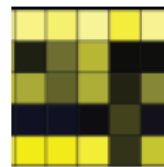


Wanderlust

Summarize statistics



Box plot



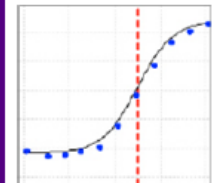
Heat map



Network



Sunburst



Dose curve



Analyze: Cytobank

Analysis toolkit designed for mass cytometry

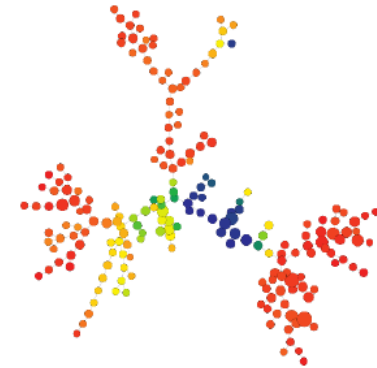
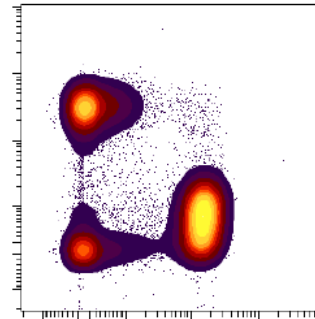
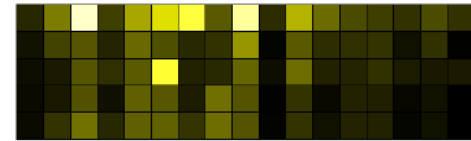
Cloud-based—accessible from anywhere

Data storage and backup included

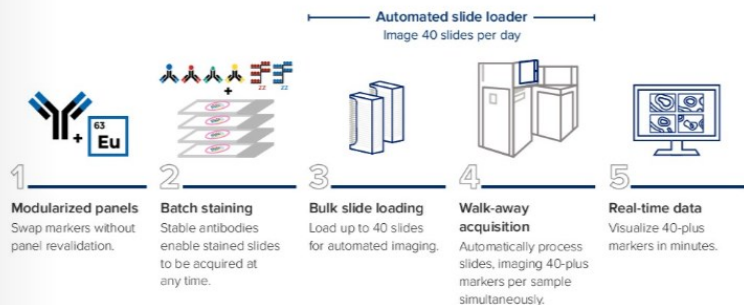
Demo datasets and tutorials

Strong scientific support

fluidigm.cytobank.org

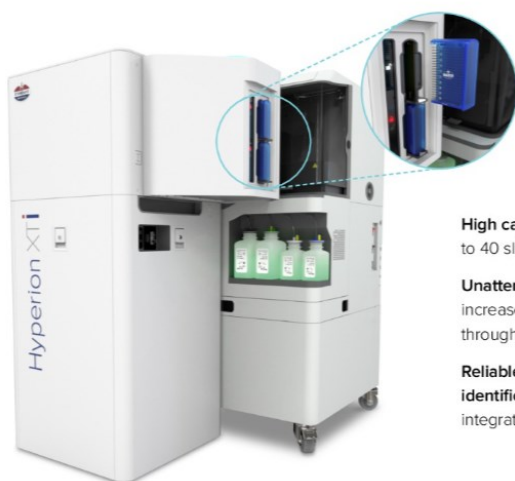


Imaging Mass Cytometry



WALK-AWAY AUTOMATION

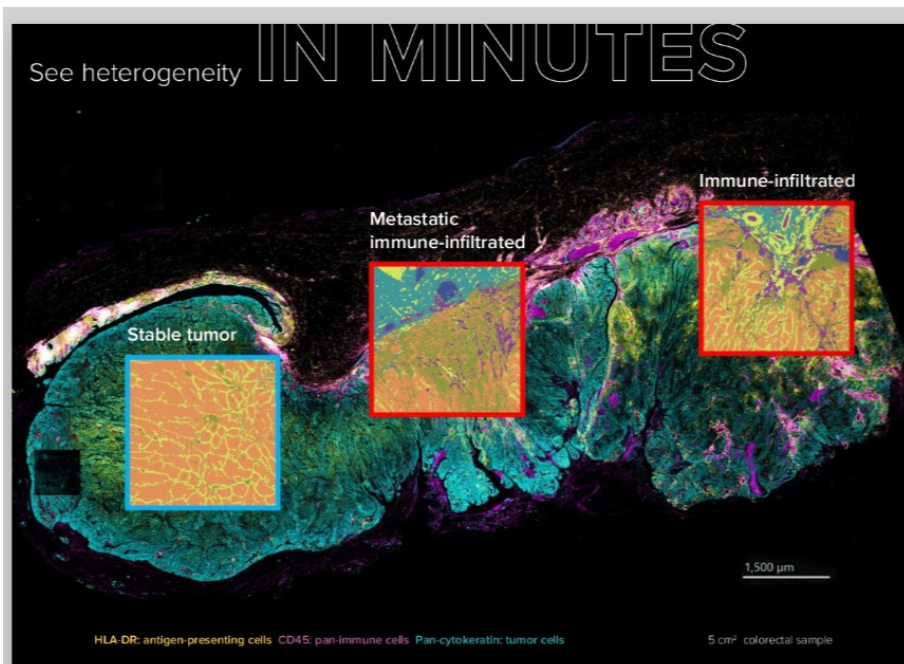
Incorporating a new level of throughput and efficiency, an integrated slide loader enables researchers to **load up to 40 slides and walk away**.



High capacity to load up to 40 slides at once

Unattended acquisition increases sample throughput 2–3x.

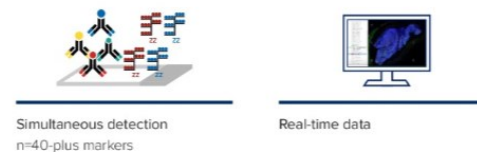
Reliable sample identification with integrated barcode reader



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





Kompenzace - literatura

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



Shrnutí

- zpracování signálu
- vizualizace dat a „gating“
- Kompenzace
- Spektrální a hmotnostní cytometrie

Na konci dnešní přednášky byste měli:

1. rozeznat jednotlivé způsoby vizualizace dat,
2. chápat základní principy „gatingu“,
3. znát princip kompenzace signálu při vícebarevné detekci,
4. Rozumět principům spektrální analýzy a hmotnostní cytometrie