Intravital Microscopy, Flow Cytometry and Cell Sorting

7 – 13 July 2013 | Berlin, Germany

Cytometry Basics and New Developments

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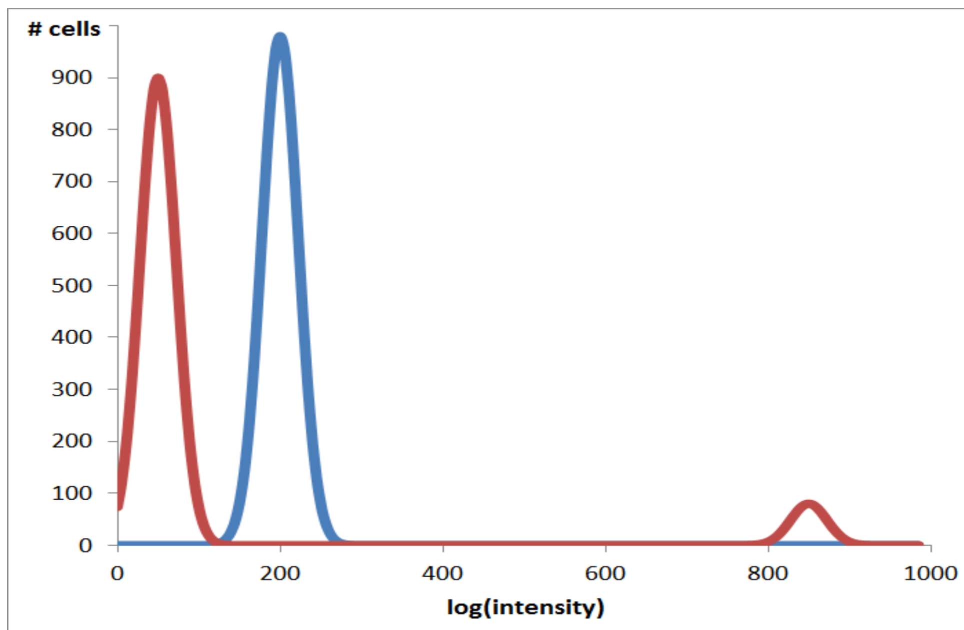
Desatoya LLC, Reno NV, USA

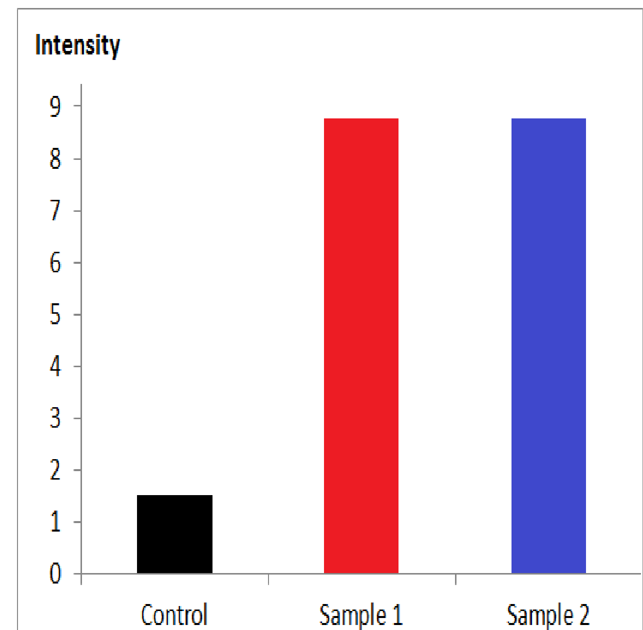
http://www.desatoya.com

Why Single Cell Analysis

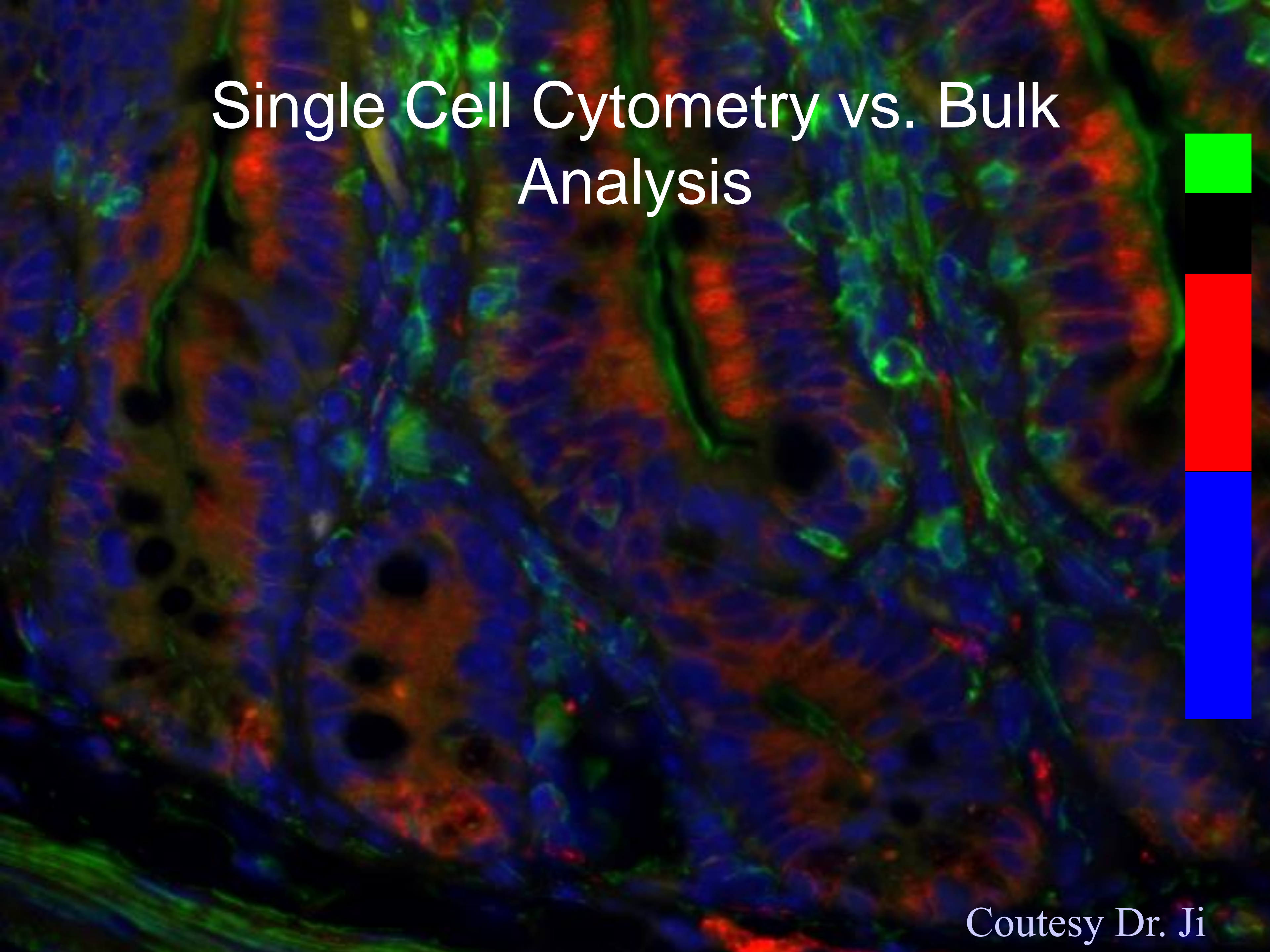
Intensity Histogram for Single Particles



Intensity per Sample

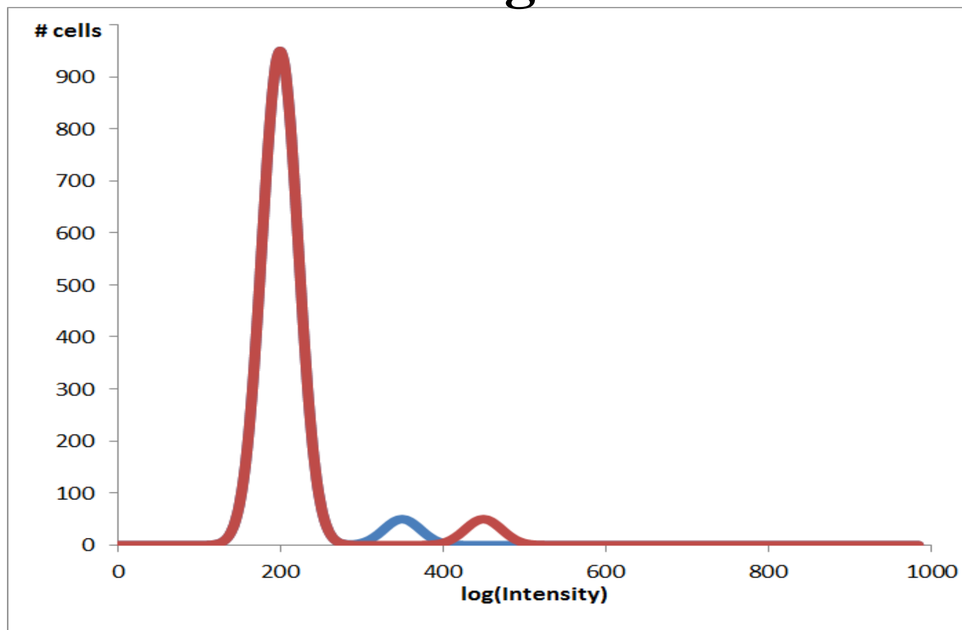


Cell by cell intensity analysis detects population heterogeneity.

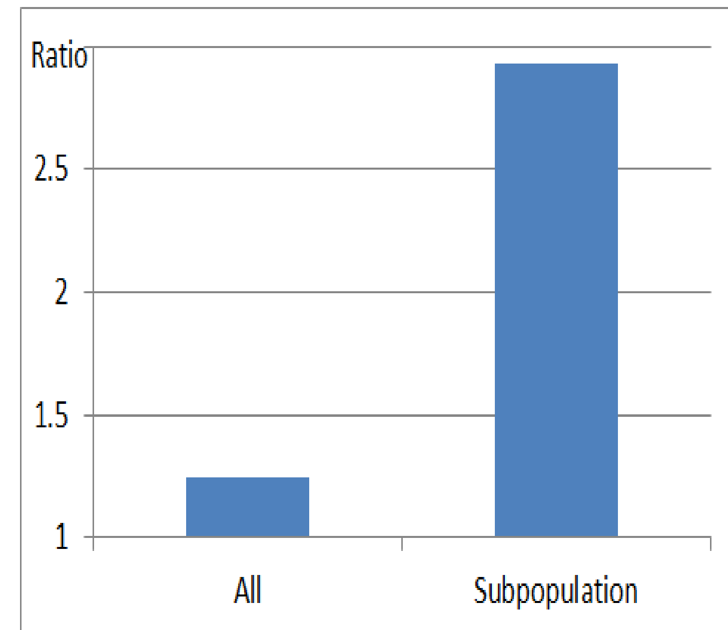

Single Cell Cytometry vs. Bulk Analysis
Coutesy Dr. Ji

Benefits of Subset Analysis

Intensity Histogram

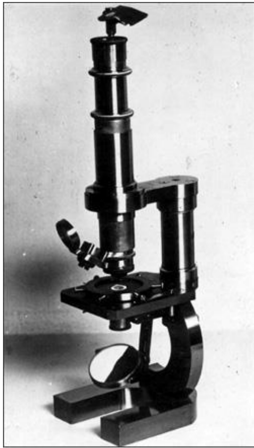


Intensity Ratios



Subpopulation analysis detects changes better, especially for rare subpopulations.

From Early to Present Microscopy and Flow Cytometry

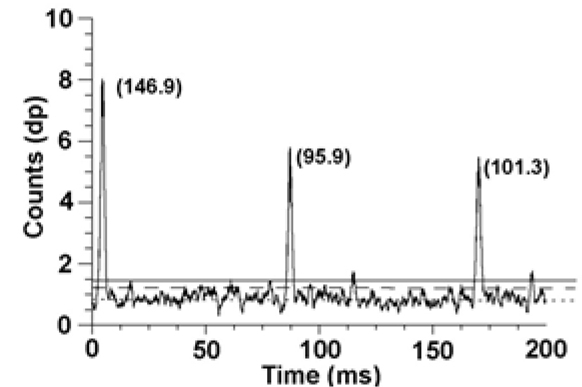


Source: BD Biosciences

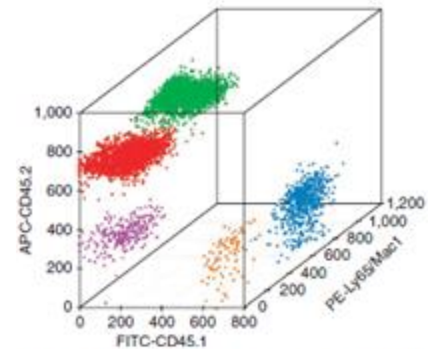
Flow and Imaging Cytometry Features

Single cell analysis with

- High sensitivity (single molecule sensitivity) I,F
- Wide dynamic range ($10^{0 \text{ to } 7}$ mL^{-1}) F
- High analysis rates to ~10^5 sec^{-1} F
- Light scatter F
- Direct size and 3D information I
- Multi-parameter analysis I,F
- Direct kinetic measurements I
- Live/dead discrimination I,F
- Viable cells can be re-covered F,(I)
- Measurement of adherent cells I
- Good ease-of-use F,(I)



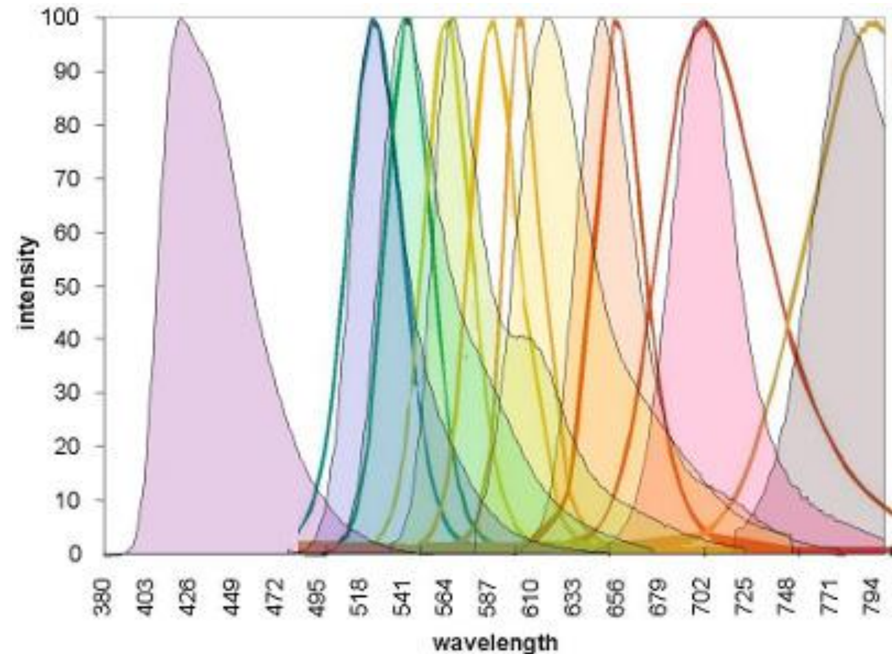
Rob Habbersett & Jim Jett, LANL



FlowCAP Consortium; Nature Methods2013

Physical parameters

- Light scatter
- Fluorescence
- Phosphorescence
- Raman
- Element mass
- Electrical properties
  e.g. impedance
- …



http://www.dvssciences.com/technical.html

Flow Cytometer Fluidics

Cell Input

Hydrodynamic or

acoustic focusing

Injector Tip

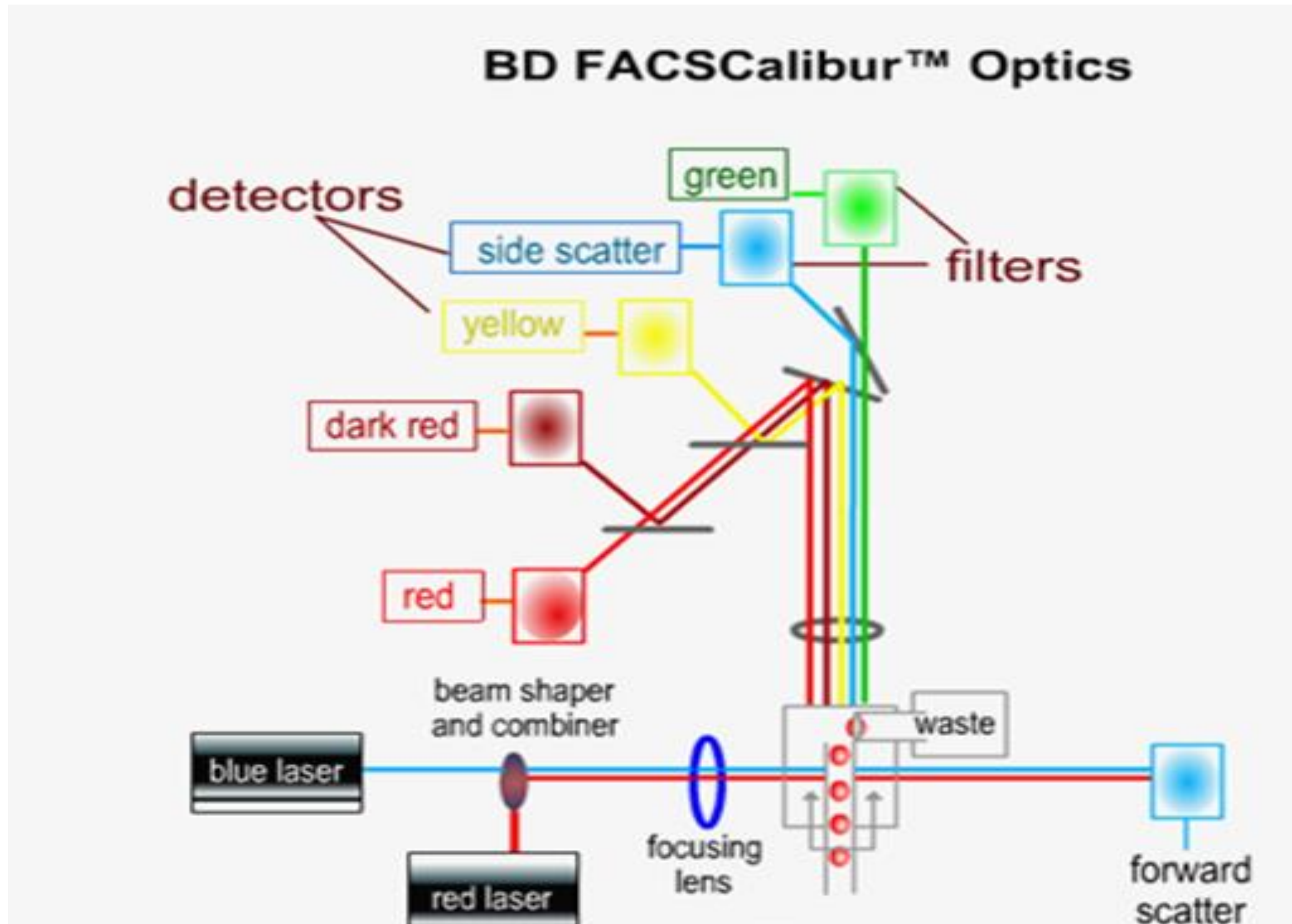
Sheath fluid

Fluorescence signals

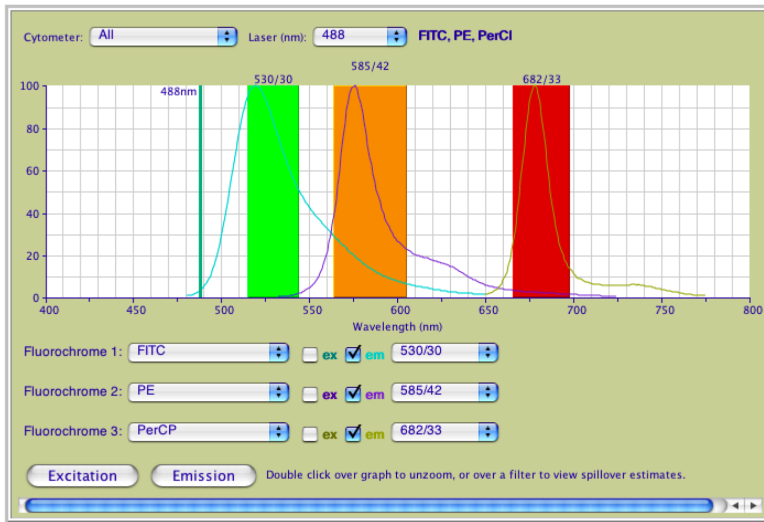
Focused laser beam

Cells after analysis, available for culture

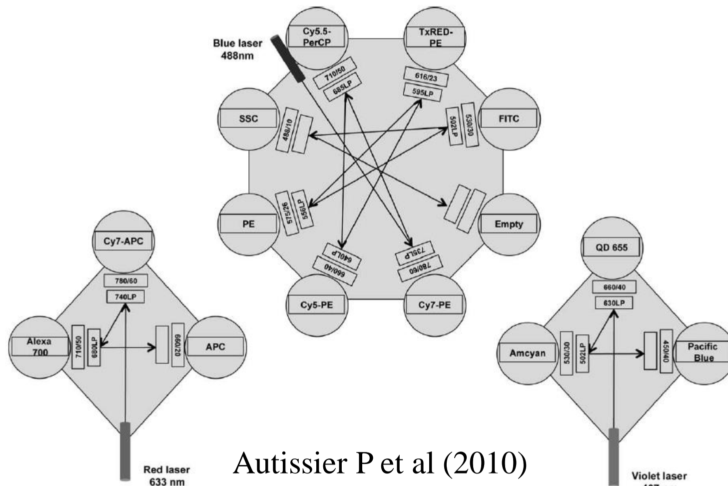
Flow Cytometer Optical Systems



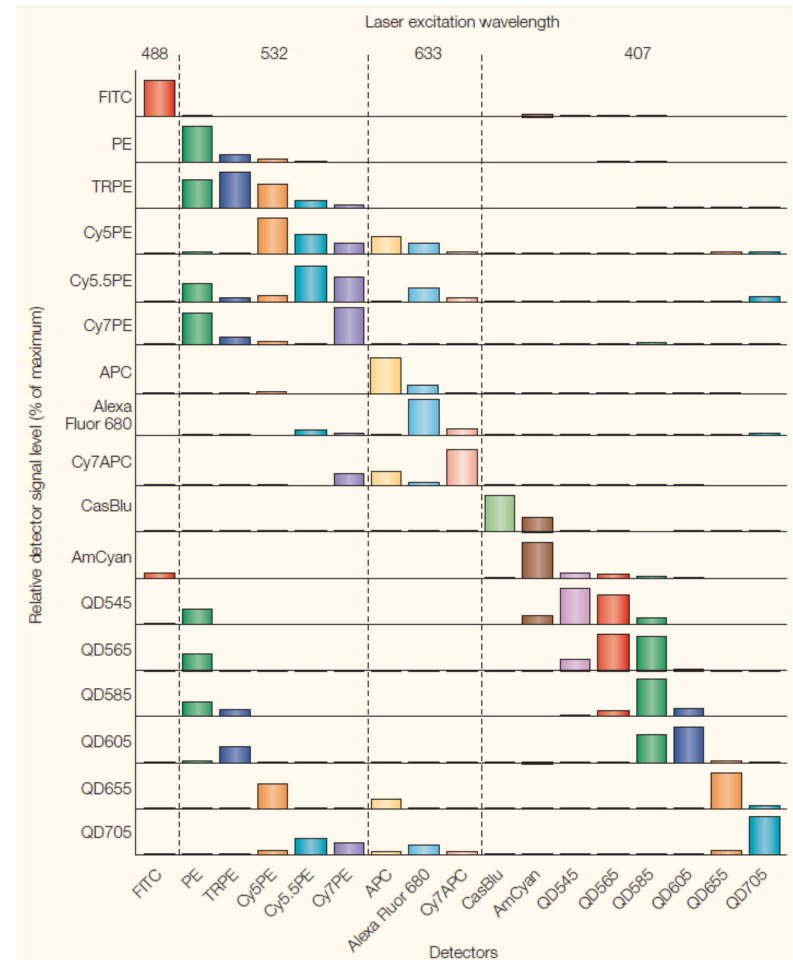
Filter Arrangement and Spectral Overlap



BD Biosciences



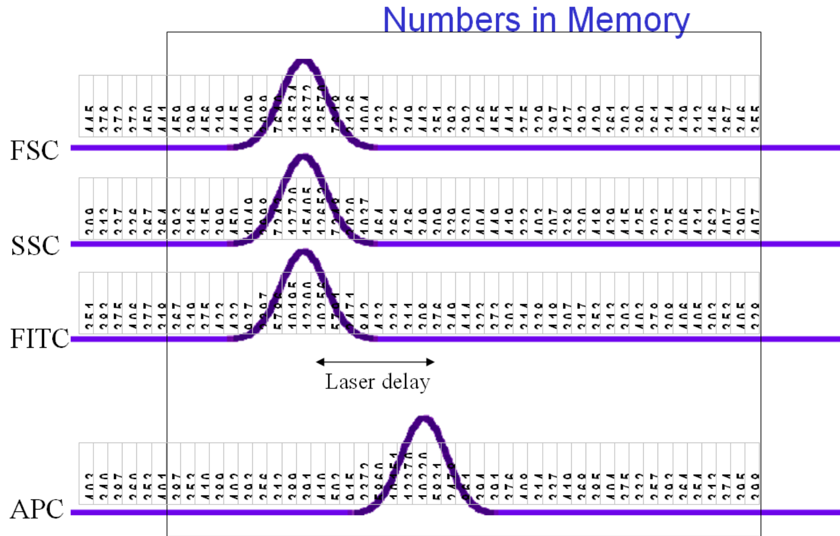
Autissier P et al (2010)
Cytometry 77A, 410ff



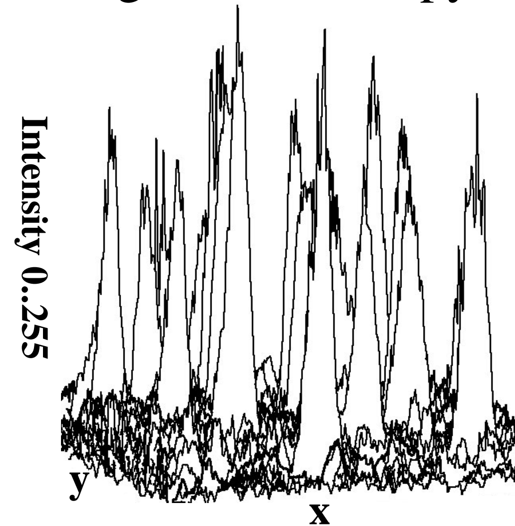
Perfetto SP et al (2004)
Nature Reviews Immunology 4, 648ff

Basic Data Processing

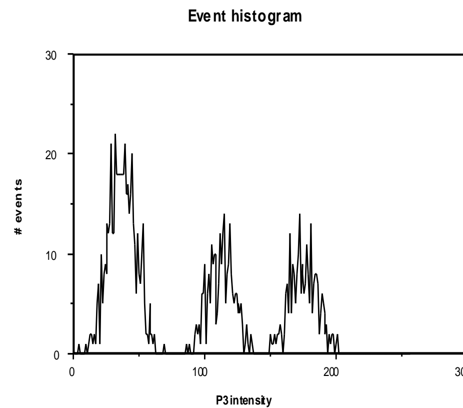
Flow Cytometry

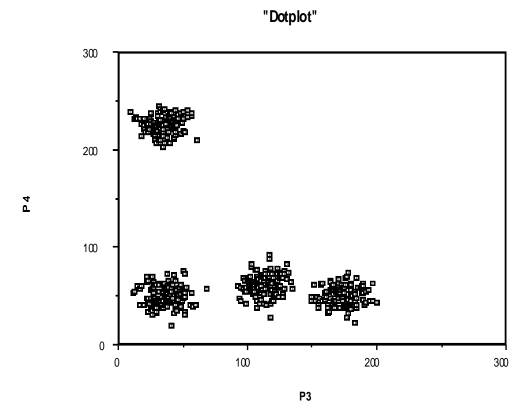


Digital microscopy

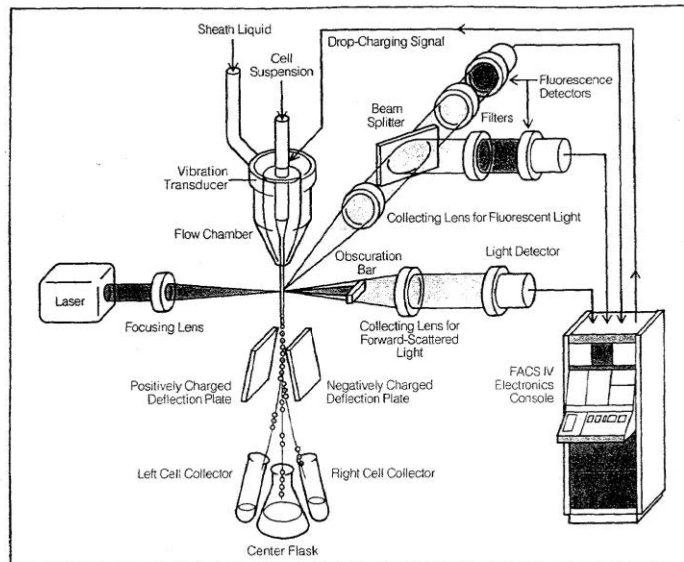


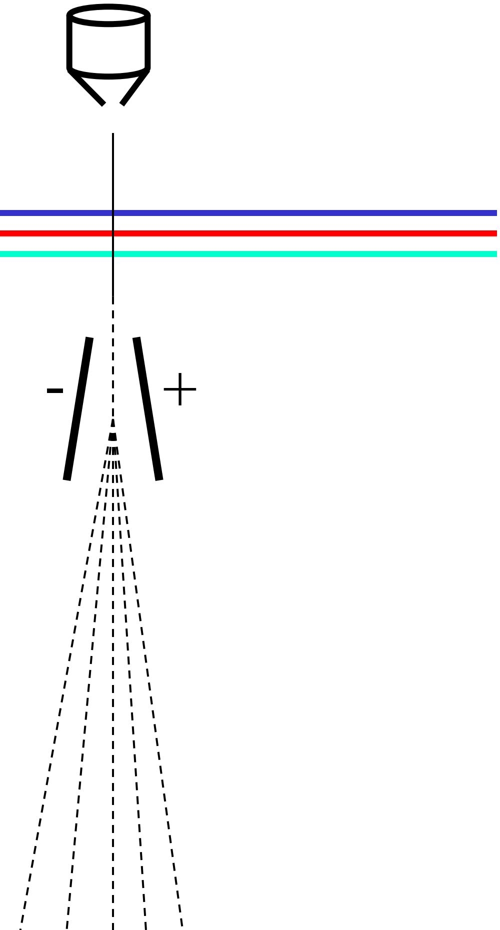
Cell	P1	P2	P3	P4	P5	Pop#
1	242	135	704	175	612	1
2	146	132	690	178	566	1
3	269	147	89	206	580	3
4	442	143	399	250	255	4
5	212	167	155	926	526	2
6	269	2	659	207	575	1
7	204	232	112	171	679	3
8	152	74	160	828	532	2
			...			
9997	215	119	138	936	662	2
9998	244	50	72	261	543	3
9999	214	137	174	1014	597	2
10000	312	87	110	904	560	2





“Droplet-based” Sorting





Source: BD Biosciences

Sorting for gene expression analysis in single cells

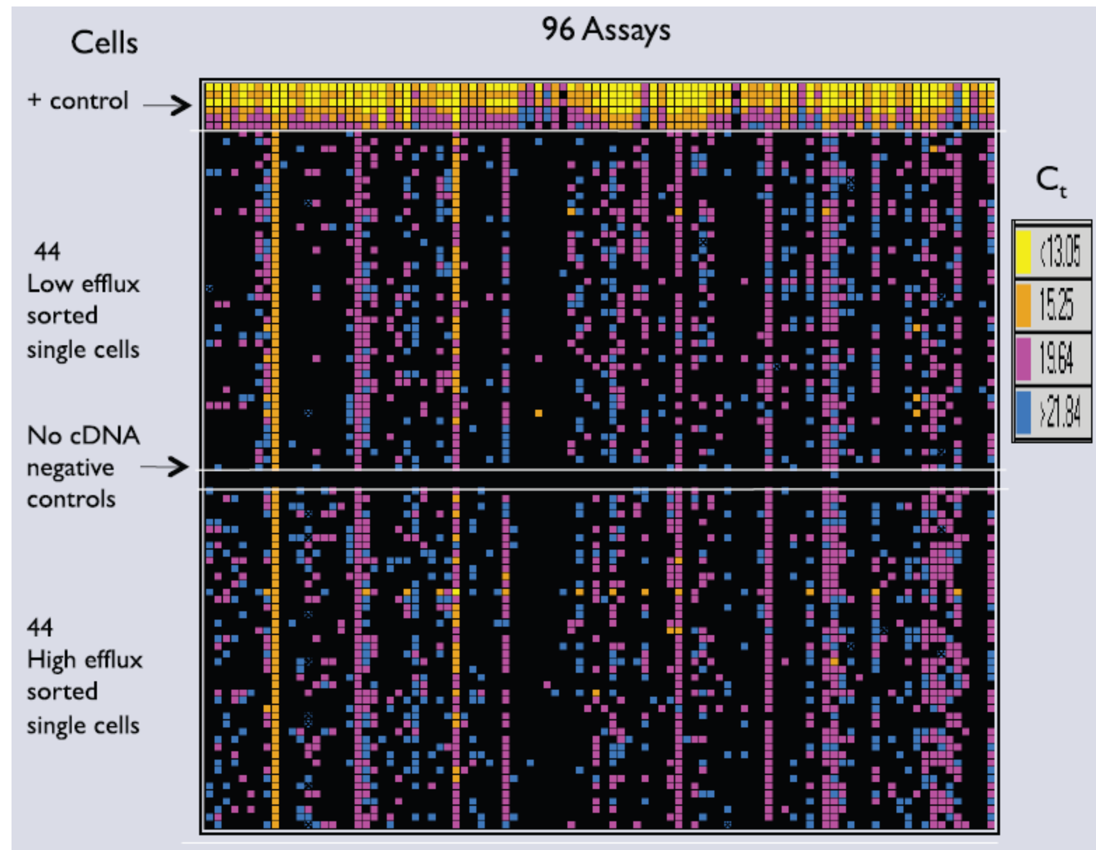


Figure 9: C_t heat map from 96 PCR assays, are shown on a 96.96 chip. Each point represents gene expression within a single cell. Some genes are more highly expressed in SP High than SP Low cell population.

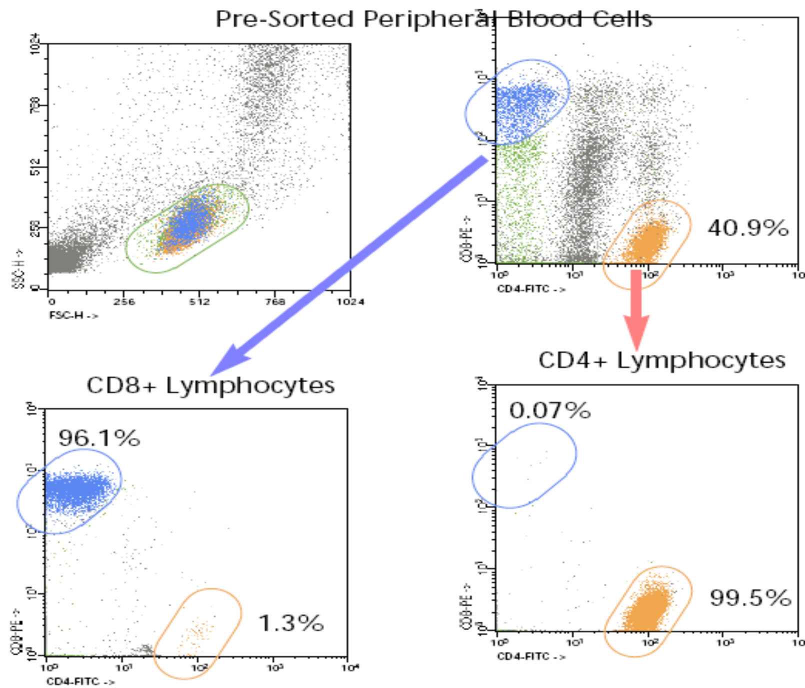
Isolation of Mouse Hematopoietic Stem Cell Side Populations Using SP^{KLS} and Post-Sort Confirmation Using Single-Cell Gene Expression

Susan Reynolds,[1] Gil Reinin,[1] J. Clark Mason,[1] Lisa Isailovic,[2] Krishna Datta,[2] Ken Livak,[2] Chandana Batchu,[2] and Alain Mir[2]
[1]BD Biosciences, 2350 Qume Dr, San Jose, CA, 95131 and [2]Fluidigm Corp, 7000 Shoreline Court, Suite 100, South San Francisco, CA, 94080

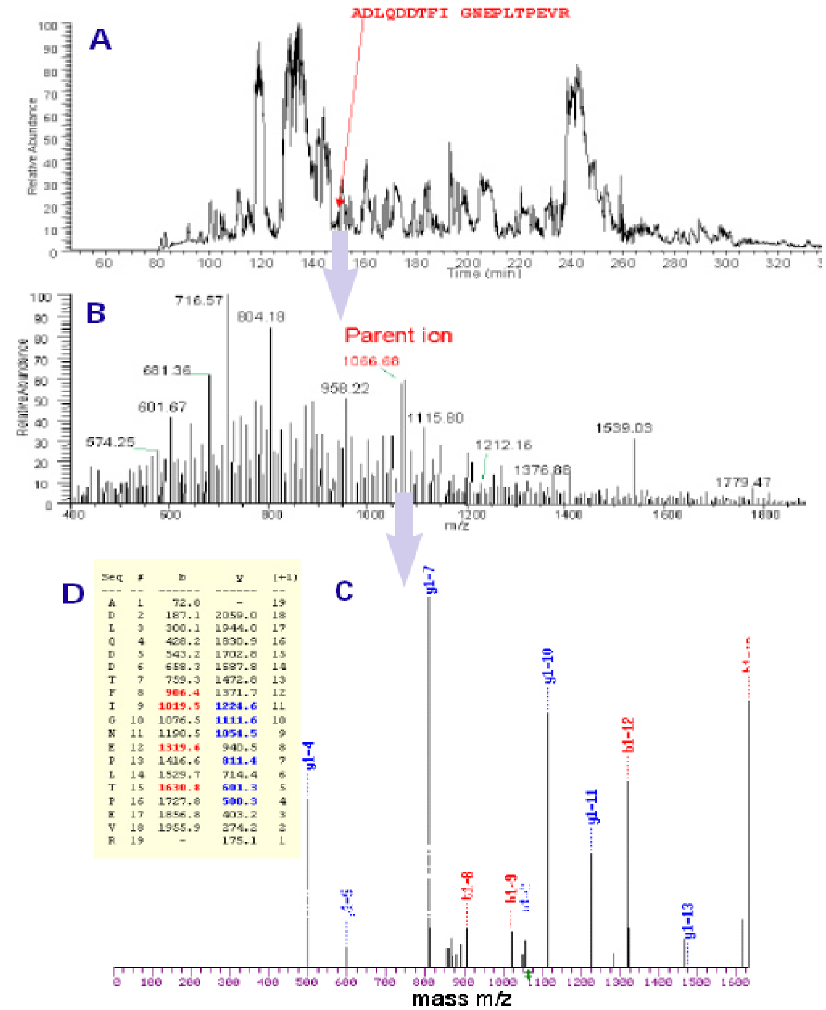
Sorting for Cell Surface Proteomics

Cell surface proteome by FACS sorting, followed by LC MS

(in collaboration with Thermo Finnigan, San Jose, CA)



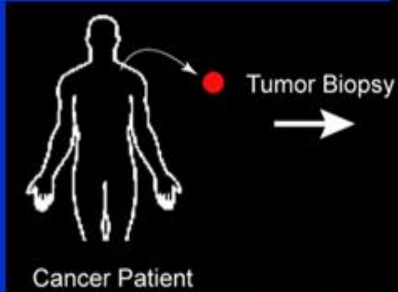
The dot plots show the sorting strategy used for stained peripheral blood cells and population purity after sorting for CD4- and CD8-positive cells. CD4 cells were gated on scatter and FITC fluorescence; CD8 bright cells were gated on scatter and RPE fluorescence. Sorted populations showed >95% purity.

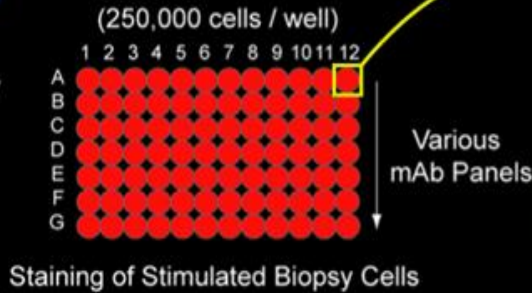


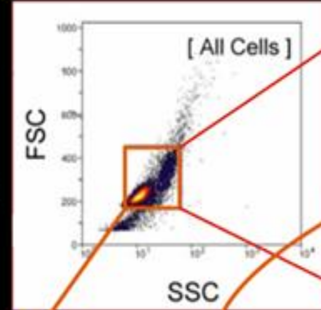
Seq	#	b	y	(+1)
A	1	72.0	-	19
D	2	187.1	2059.0	18
L	3	300.1	1944.0	17
Q	4	428.2	1830.9	16
D	5	543.2	1702.8	15
D	6	658.3	1587.8	14
T	7	759.3	1472.8	13
F	8	906.4	1371.7	12
I	9	1019.5	1224.6	11
G	10	1076.5	1111.6	10
N	11	1190.5	1054.5	9
E	12	1319.6	940.5	8
P	13	1416.6	811.4	7
L	14	1529.7	714.4	6
T	15	1630.8	601.3	5
P	16	1727.8	500.3	4
E	17	1856.8	403.2	3
V	18	1955.9	274.2	2
R	19	-	175.1	1

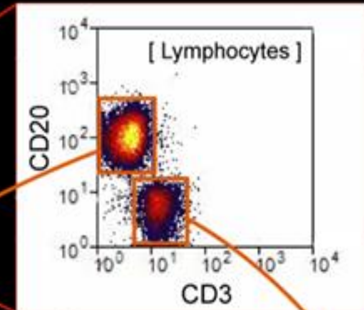
Peptide mixtures were separated by reverse phase HPLC **(A)** as described in Methods. Eluted peptides were subjected to electrospray injection into the mass spectrometer and analyzed for their mass/charge ratio (m/z value) **(B)**. Selected ions were collected in the ion trap. These parent ions were cracked by collision ion dissociation to produce a range of fragment sizes **(C)** that were compared to predicted peptide sequences in the human database using TurboSequest **(D)**.

Subset Analysis Example

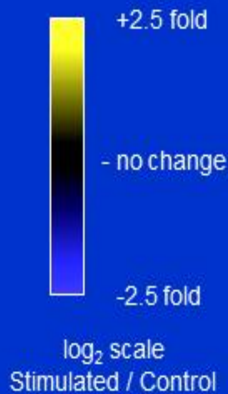


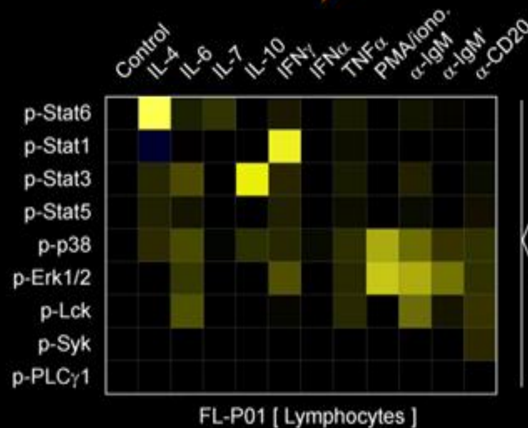


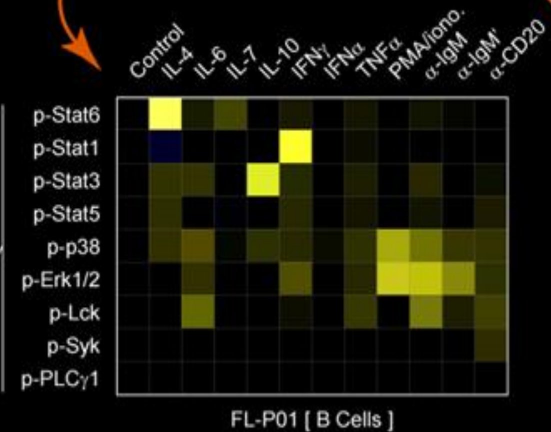


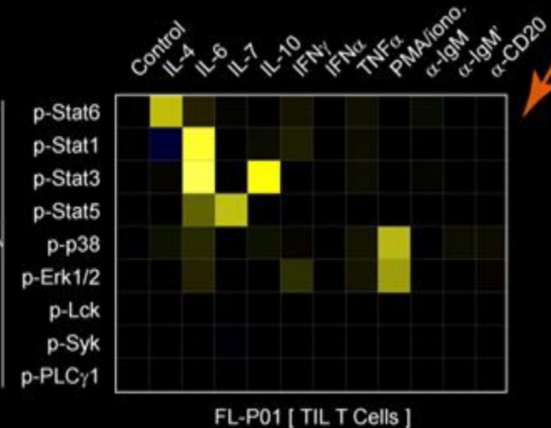


profiling a lymphoma patient







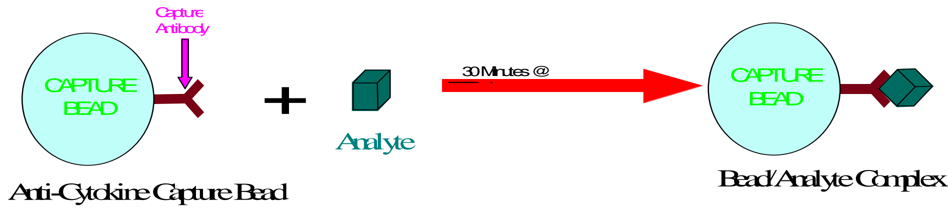


Slide provided by Nikesh Kotecha, CytoBank Inc, Mountain View CA

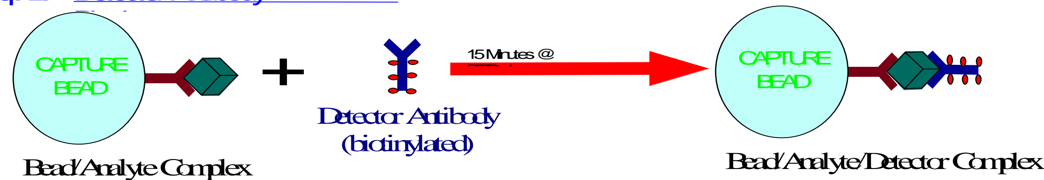
Data: J. Irish, Stanford

BeadArray Assays

Step 1: Analyte

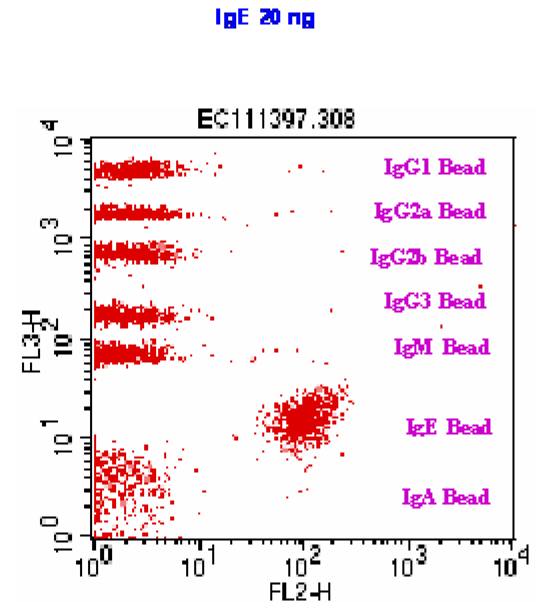


Step 2: Detector Antibody



Step 3: Reporter Molecule

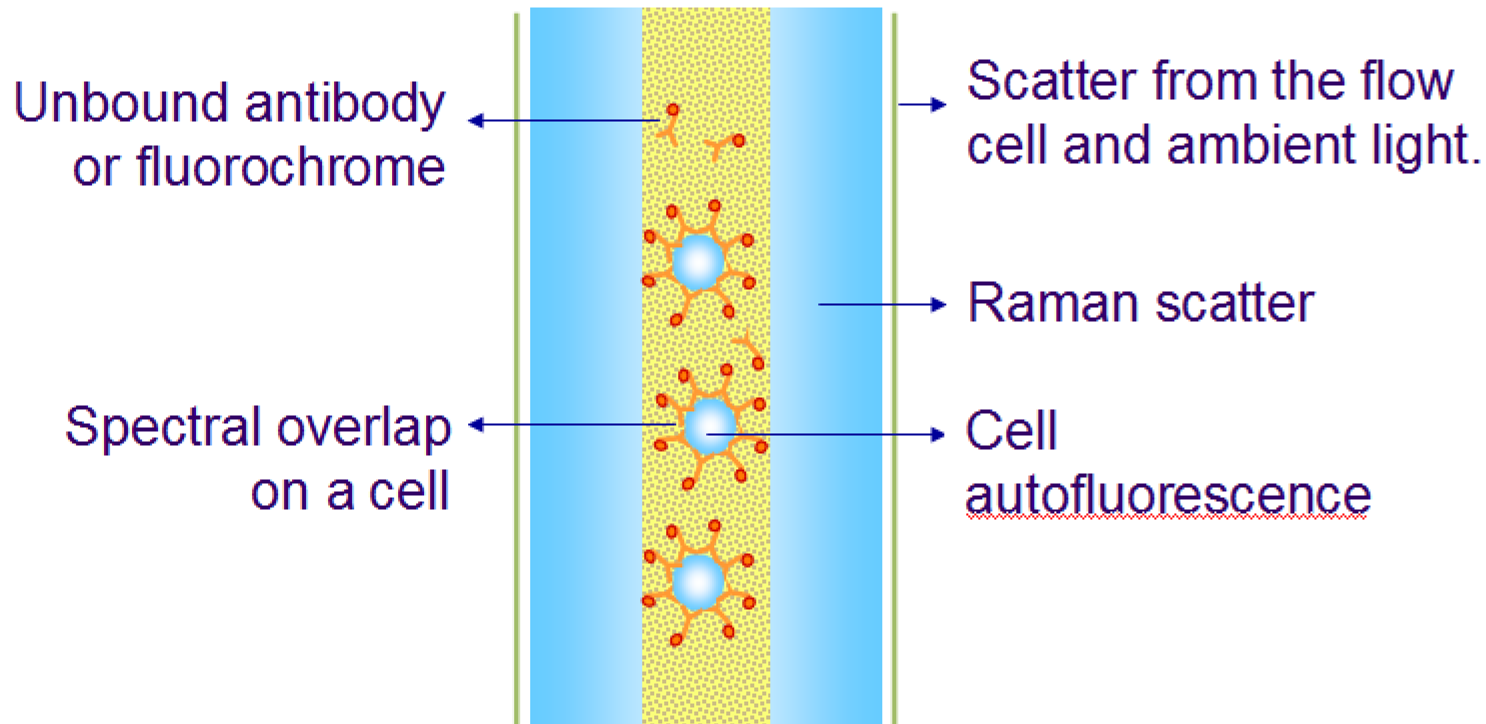
IgE 20 ng



Dr. Rudi Varro, BD Biosciences

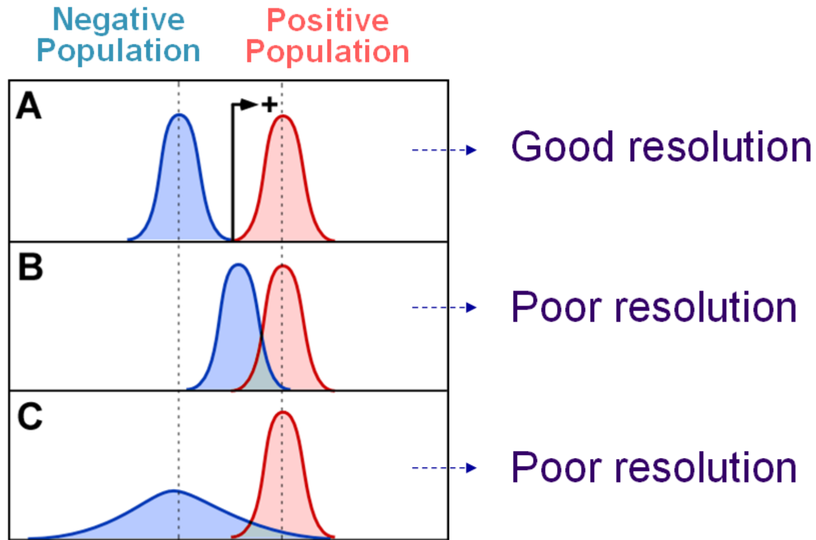
Instrument Evaluation Br

Relative B (Br) is a measure of true optical background in the fluorescence detector.



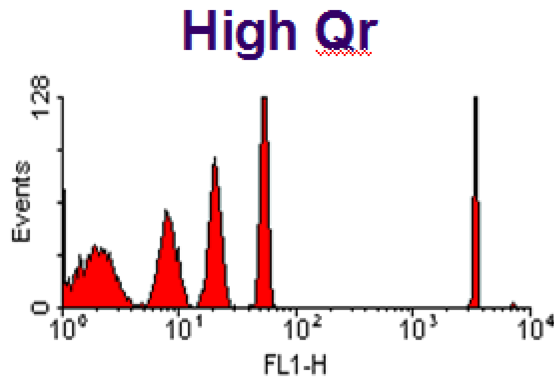
Source: Joe Trotter, BD Biosciences

Instrument Evaluation Qr

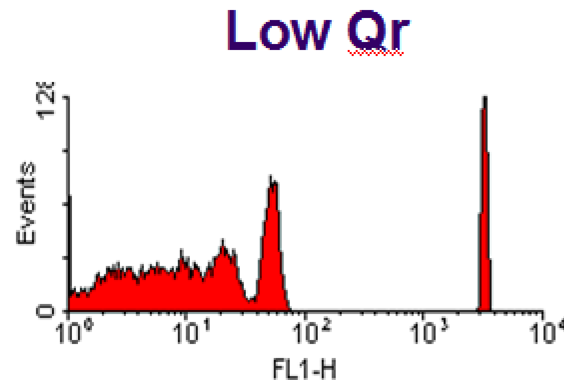


$$Qr = \frac{\#\ \text{photoelectrons}}{\#\ \text{fluorescence molecules}}$$

High Qr



Low Qr



Source: Joe Trotter, BD Biosciences

Optimizing cytometry measurements (I)

- Background light

- The total measurement SD is the sum of the error contributions from all sources:

$$SD = \sqrt{SD^2_{optical} + SD^2_{intrinsic} + SD^2_{illumination} + SD^2_{noise}}$$

- When two dyes (PE and FITC) are measured by a single detector the SD is additive:

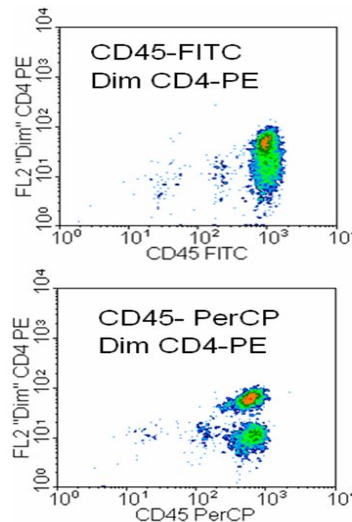
$$SD_{optical} = \sqrt{SD^2_{PE} + SD^2_{FITC} + SD^2_{background}}$$

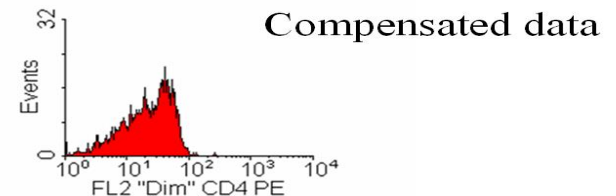
Reagent performance

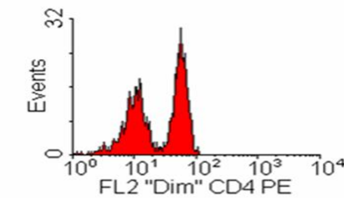
Stain index

$$\frac{Medium_{pos} - Medium_{neg}}{2 * SD_{neg}}$$

- Dye properties (brightness and spectral overlap)







Better separation with less spectral overlap.

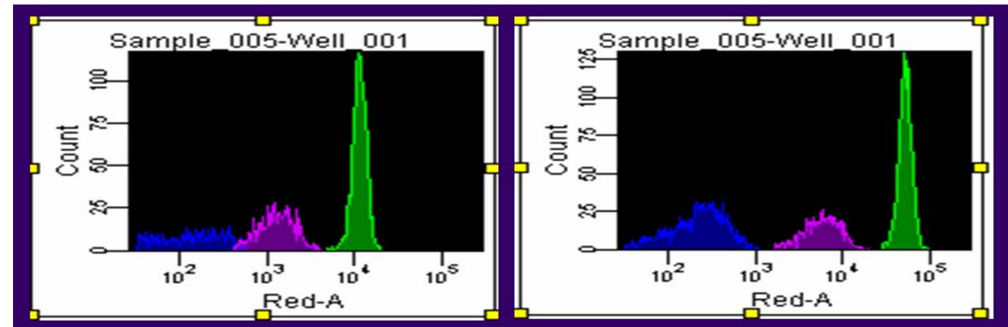
next slides:

- Multi-color dye overlap
- Gain (PMT, CMOS, CCD) settings
- Data Display
- Controls

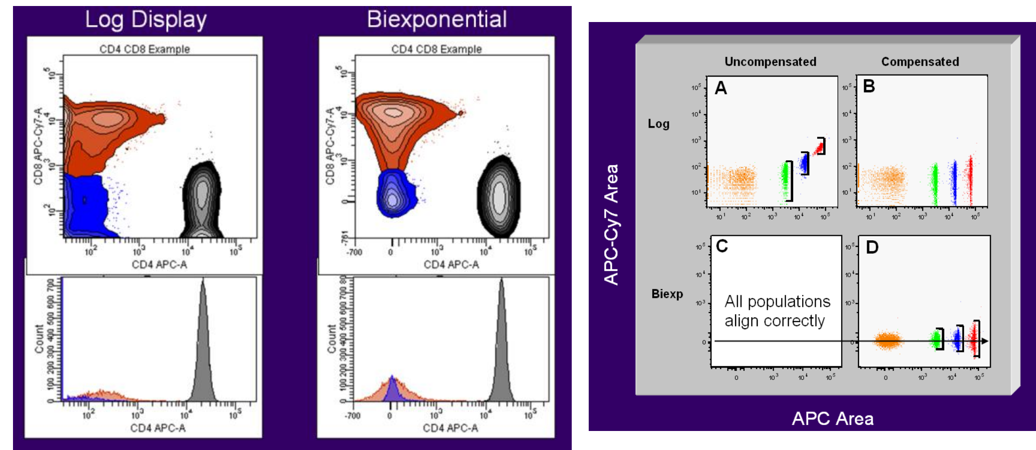
Source: Joe Trotter, BD Biosciences

Optimizing cytometry measurements (II)

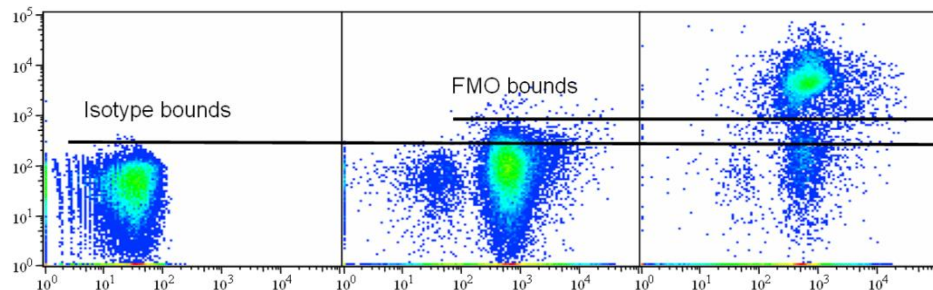
- Gain (PMT, CMOS, CCD) settings



- Data Display



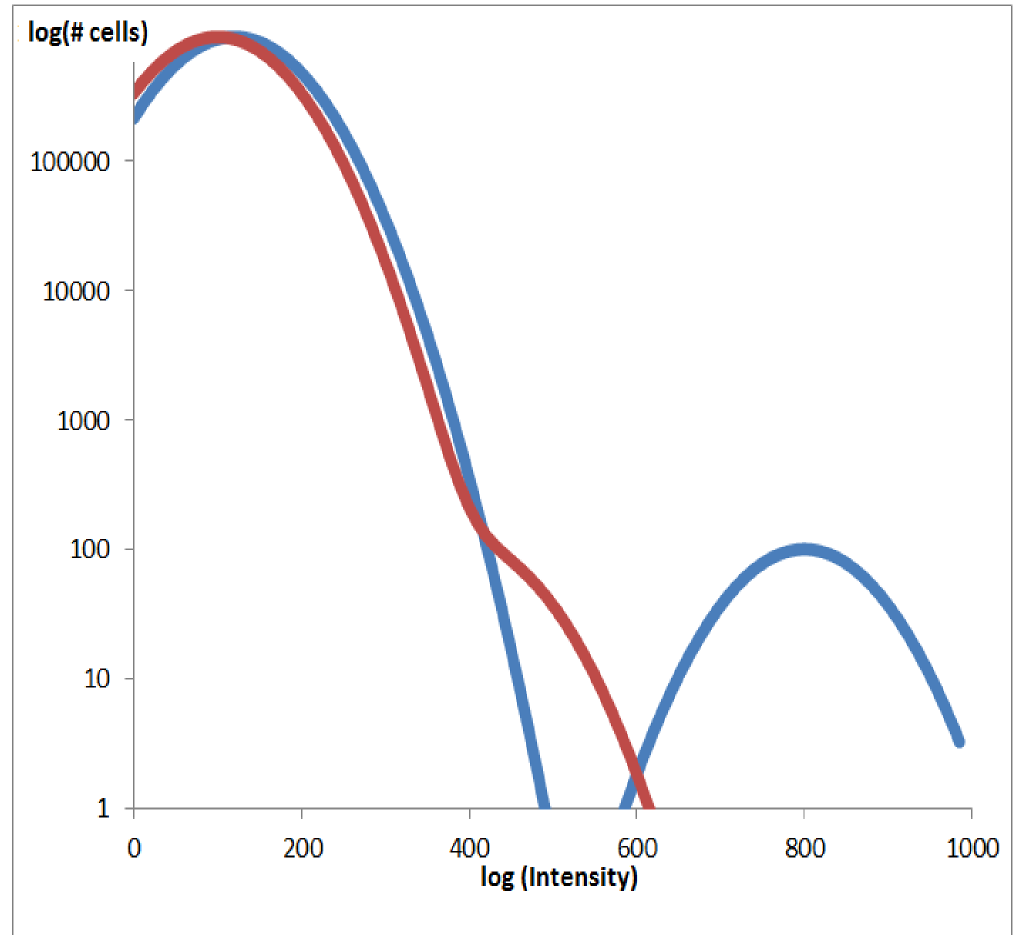
- Controls



Source: Joe Trotter, BD Biosciences and others

Marker Brightness

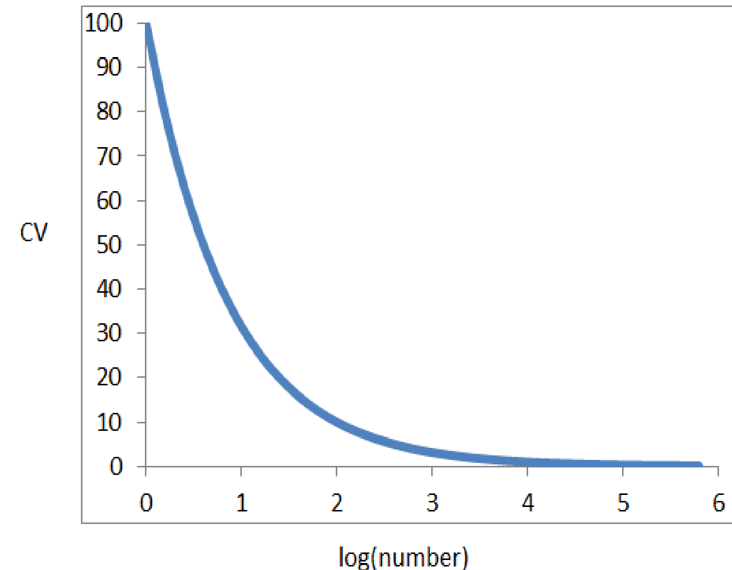
Brighter markers resolve rare populations better



Rare Subpopulation Analysis

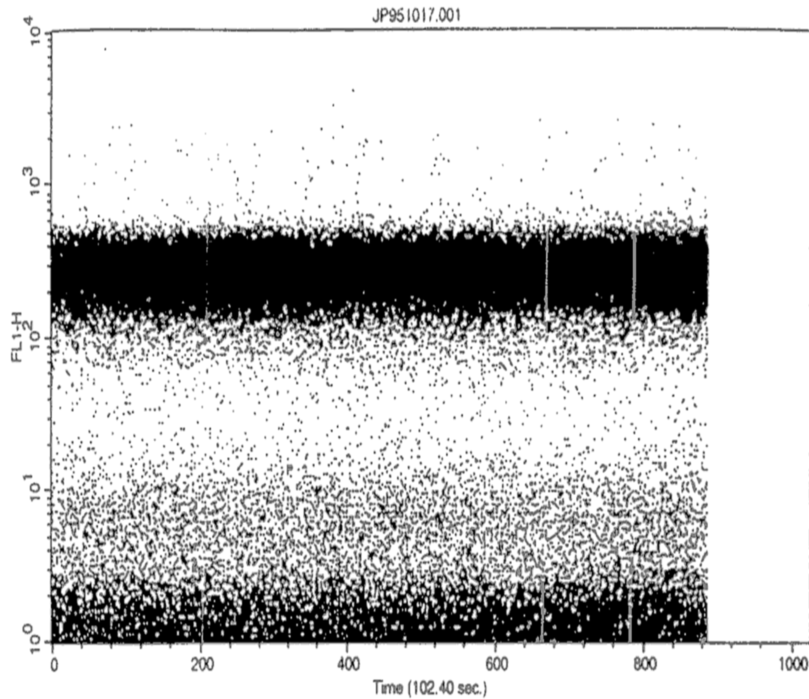
Counting Statistics

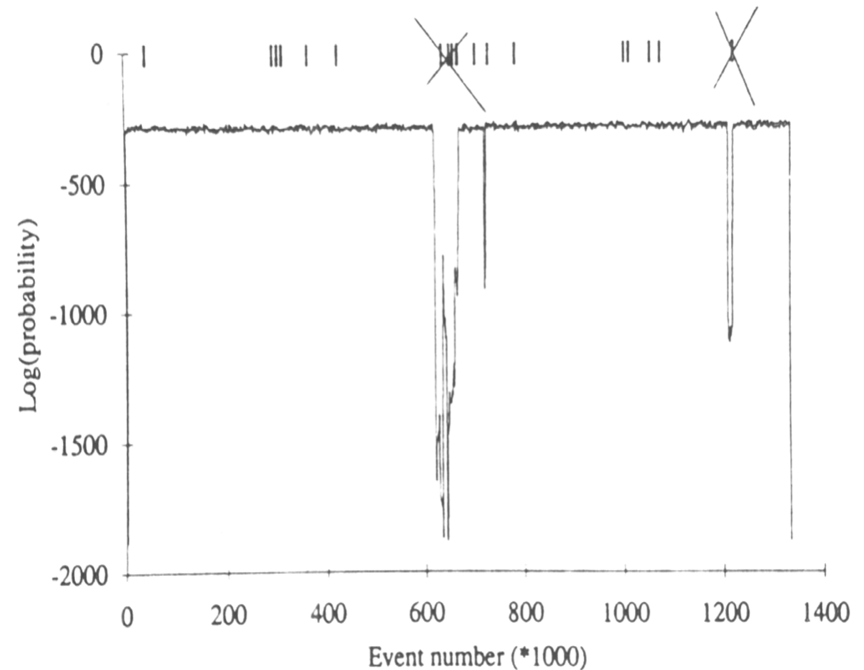
	Sample 1	Sample 2	Sample 3	Sample 4
	6	2	6	8
	3	7	1	6
	1	3	5	3
	1	4	5	6
	1	4	6	3
Mean	2.4	4	4.6	5.2
St.Dev	2.2	1.9	2.1	2.2
		Overall	Mean	4.1
			St.Dev	2.2



Ignoring Counting Statistics Can Lead to Erroneous Conclusions

System Noise





Gross HJ et al, Cytometry 1993

Eliminating system noise from fluidics perturbations and other sources improves the limit of detection for rare cell analysis.

Particle Carry-Over

Carry-over specification examples
(from manufacturer's info)

Beckman Coulter FC500	<1%	
Beckman Coulter	<0.1%	
BD FACSCanto II	<0.1%	loader <1%
BD FACSVerse	<0.5%	
Guava EasyCyte	<0.2%	
Life Technologies Attune		loader <0.5%
Miltenyi MACSQuant	<0.01%	

Eliminating fluidic system particle carry-over is vital for reliable rare cell analysis.

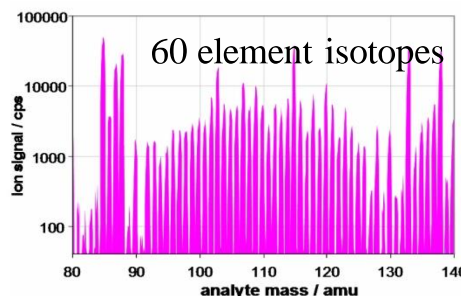
Multi-parameter Fluorescence Cytometry Points To Consider

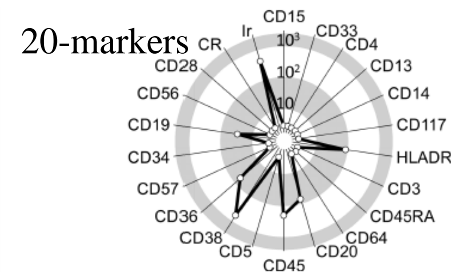
Summary

- Know your instrument e.g. Qr & Br for different channels, carrover, …
- Use high enough gain settings to maximize sensitivity
- An antibody/dye combination that marginally allows discrimination of positives/negatives in a single color assay is unlikely to contribute anything helpful in a multicolor experiment.
- Avoid spillover from bright cell populations into channels requiring high sensitivity
- Beware of tandem dye degradation
- Internal controls are essential
- Take advantage of bright markers for “dim markers”

New Developments for Multi-parameter Cytometry

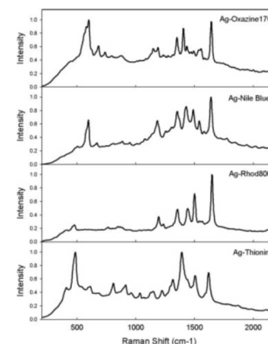
- Element-Label Flow Cytometry (CyTOF, addresses fluorescence spectral overlap issue by using elements as labels, Anal. Chem., 2009, 81 (16), pp 6813–6822)

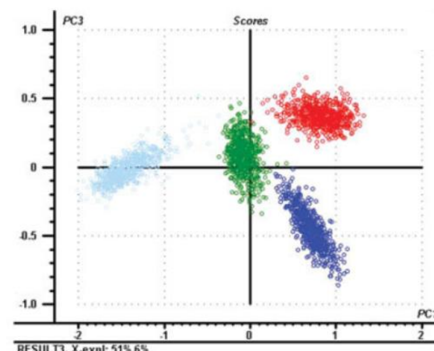




Scott Tanner, DVS Sciences Inc

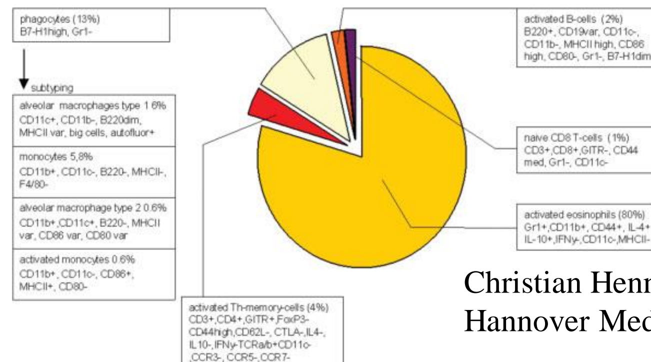
- SERS-Label Flow Cytometry (uses spectral fine-structure to distinguish labels, Cytometry, 2008, 73A(2), pp 119-128)





John Nolan, La Jolla Bioengineering Institute

- Sequential Stain De-stain Cytometry (Cytometry, 2009, 75A(4), pp 362-370)
- SONY spectral analysis



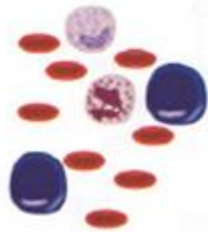
31-marker analysis

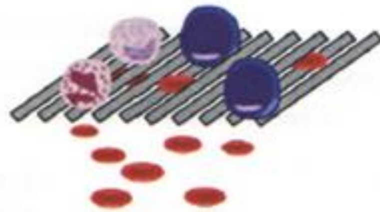
Christian Hennig, ChipCytometry
Hannover Medical School

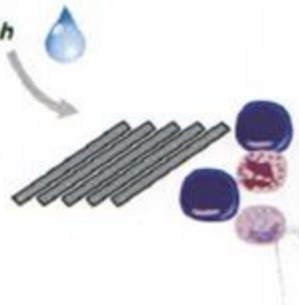
New Developments for Sample Preparation

Removing erythrocytes and/or free reagent without centrifugation

Silicon filter for leukocyte/ erythrocyte separation





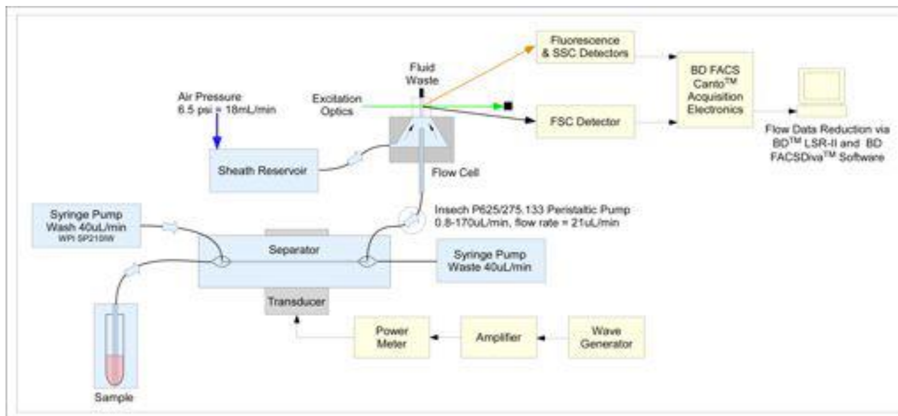


Leukocyte recovery

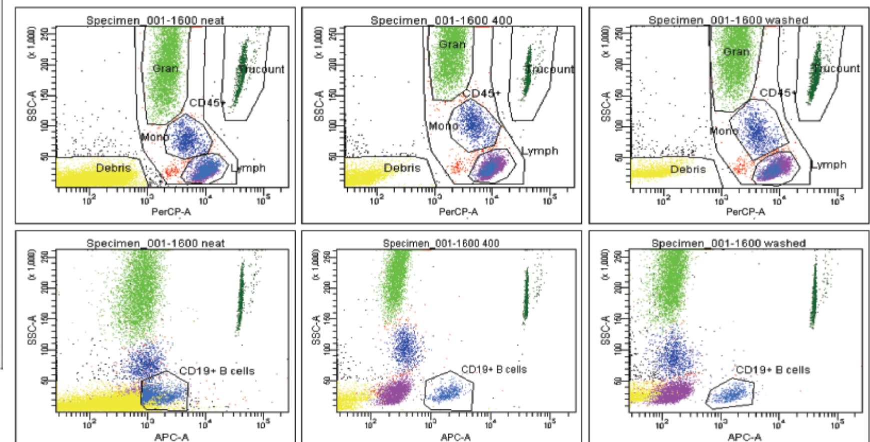
Cell load \ Flow rate	0.18ml/min	0.33ml/min
10ul (51.1±7.5)×1000 cells	0.98±0.04	0.92±0.07
50ul (350±14.1)×1000 cells	0.75±0.18	0.35±0.15

2011 Liping Yu et al, BD Biosciences and Aviva Inc.

Acoustic particle focusing



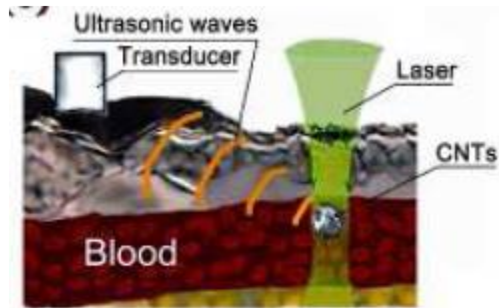
2010, Laurell group, Lund University
& Brian Warner, BD Biosciences



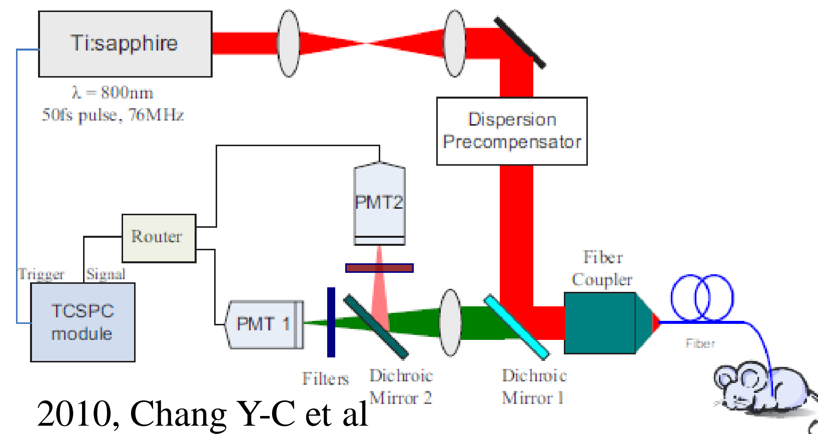
Intra-vital Cytometry

Single cell analysis in living animals

Flow cytometry in blood vessels

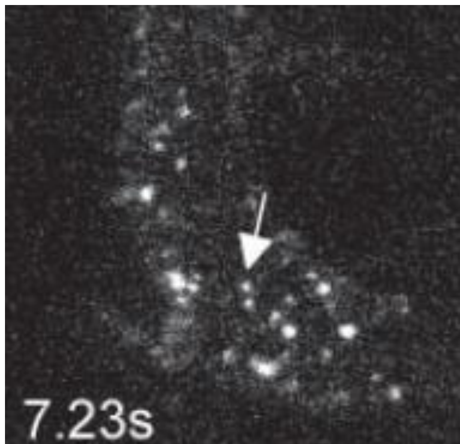


2010, Zharov VP and coworkers



2010, Chang Y-C et al

Microscopy



2011, Runnels JM et al; homing of multiple myeloma cells in bone marrow

Signals from
- 2-photon fluorescence
- bioluminescence
- photo-acoustic effect
- …

Review paper:
Niesner RA, Cytometry 79A (2011)

Conclusions

New technologies from many fields are
rapidly generating
new opportunities for cytometry.

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- BD

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