9th Spring School on Immunology

Ettal, Bavaria, March 2013

Flow / Imaging Cytometry and Emerging Technologies for Single Cell Analysis

Diether Recktenwald

Desatoya LLC, Reno NV, USA, (retired from BD)
http://www.desatoya.com

Early Microscopy and Flow Cytometry

Modern Flow Cytometry and Imaging Microscopy

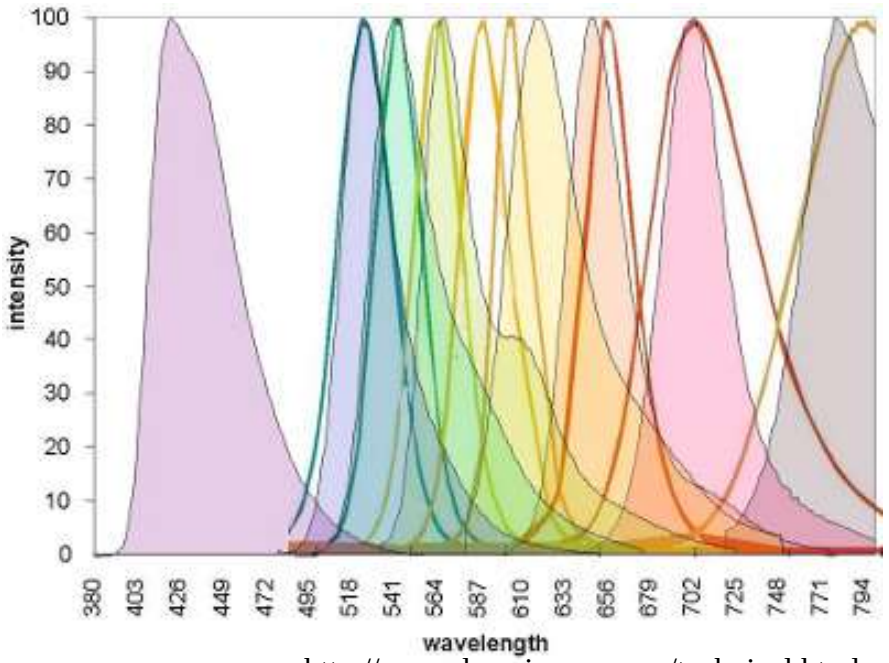
Flow and Imaging Cytometry Features

Single particle (cell) analysis with

• High sensitivity (single molecule sensitivity by fluorescence)	I,F
• Wide dynamic count range (10^3 to 10^7 cells mL^{-1})	F
• Particle sizes from 0.2 to 20 um	F, I
• High analysis rates to ~10^5 particles sec^{-1}	F
• Direct size and 3D spatial information	I
• Multi-color fluorescence, multi-parameter analysis	F,I
• Wide dynamic range for fluorescence (10^5)	F
• Direct kinetic measurements	I
• Viable cells can be re-covered	F,(I)
• Measurement of adherent cells	I
• Good ease-of-use	F,(I)

Physical parameters

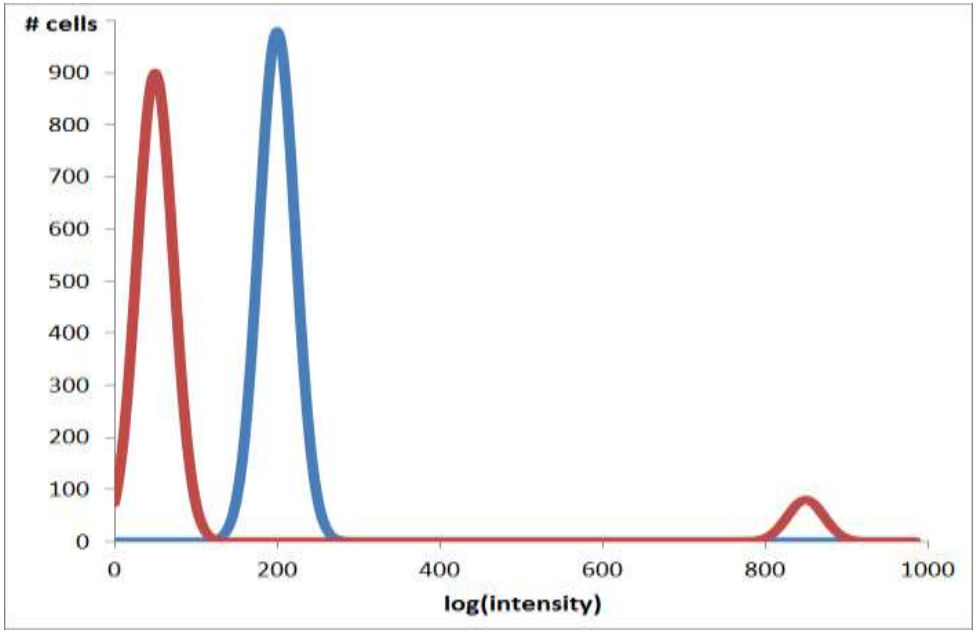
- Light scatter
- Fluorescence
- Phosphorescence
- Raman
- Element mass
- Electrical properties e.g. impedance
- …



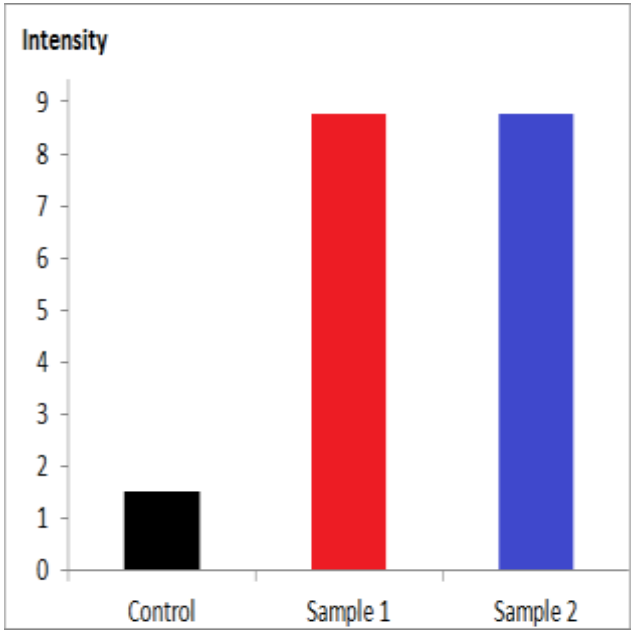
http://www.dvssciences.com/technical.html

Why Single Cell/Particle Analysis

Intensity Histogram for Single Particles



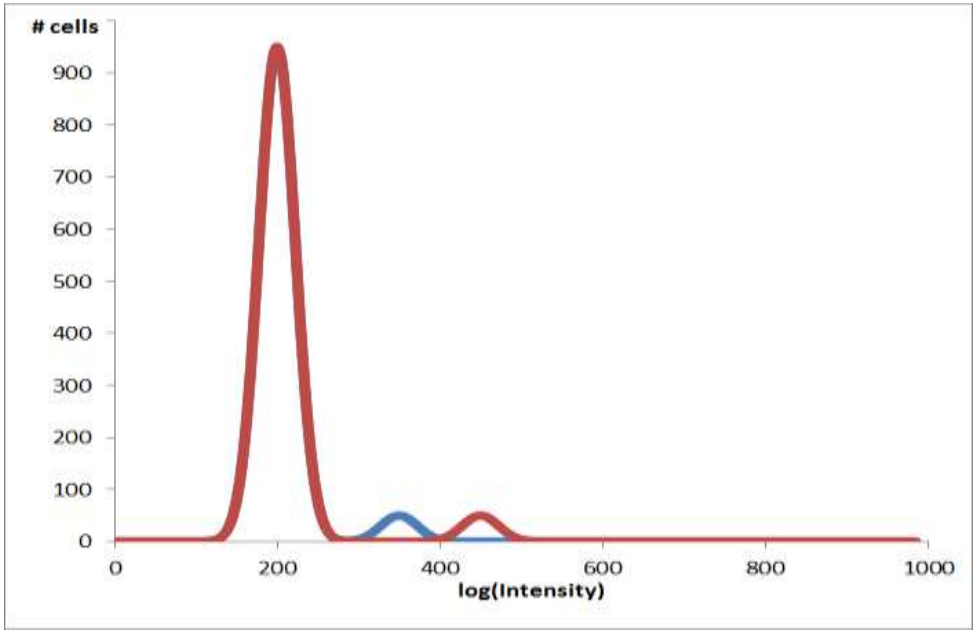
Intensity per Sample



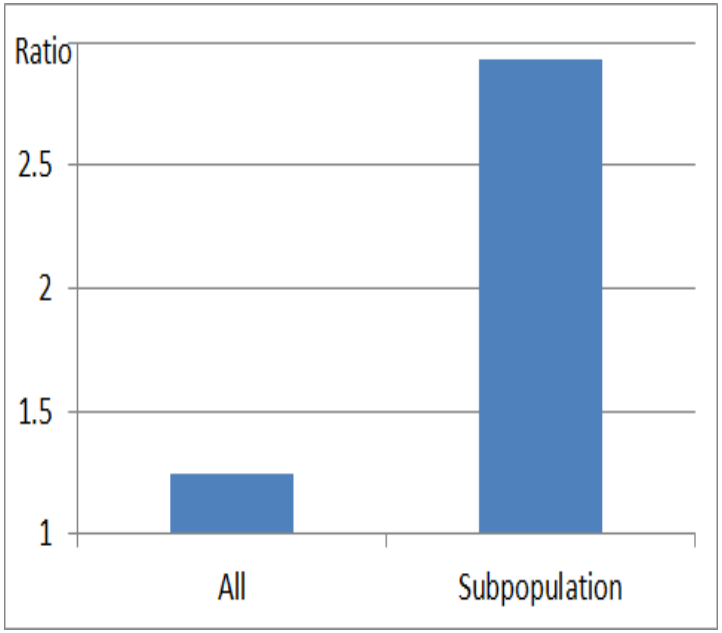
Cell by cell intensity analysis detects population heterogeneity.

Benefits of Subset Specific Analysis

Intensity Histogram



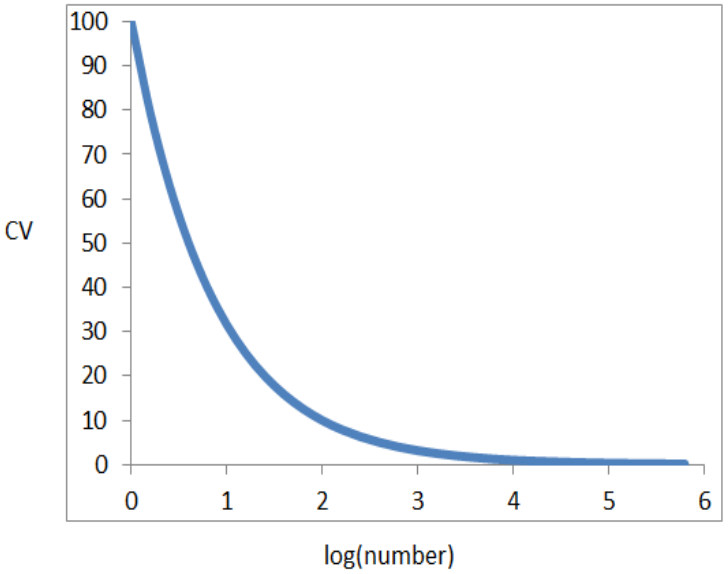
Intensity Ratios



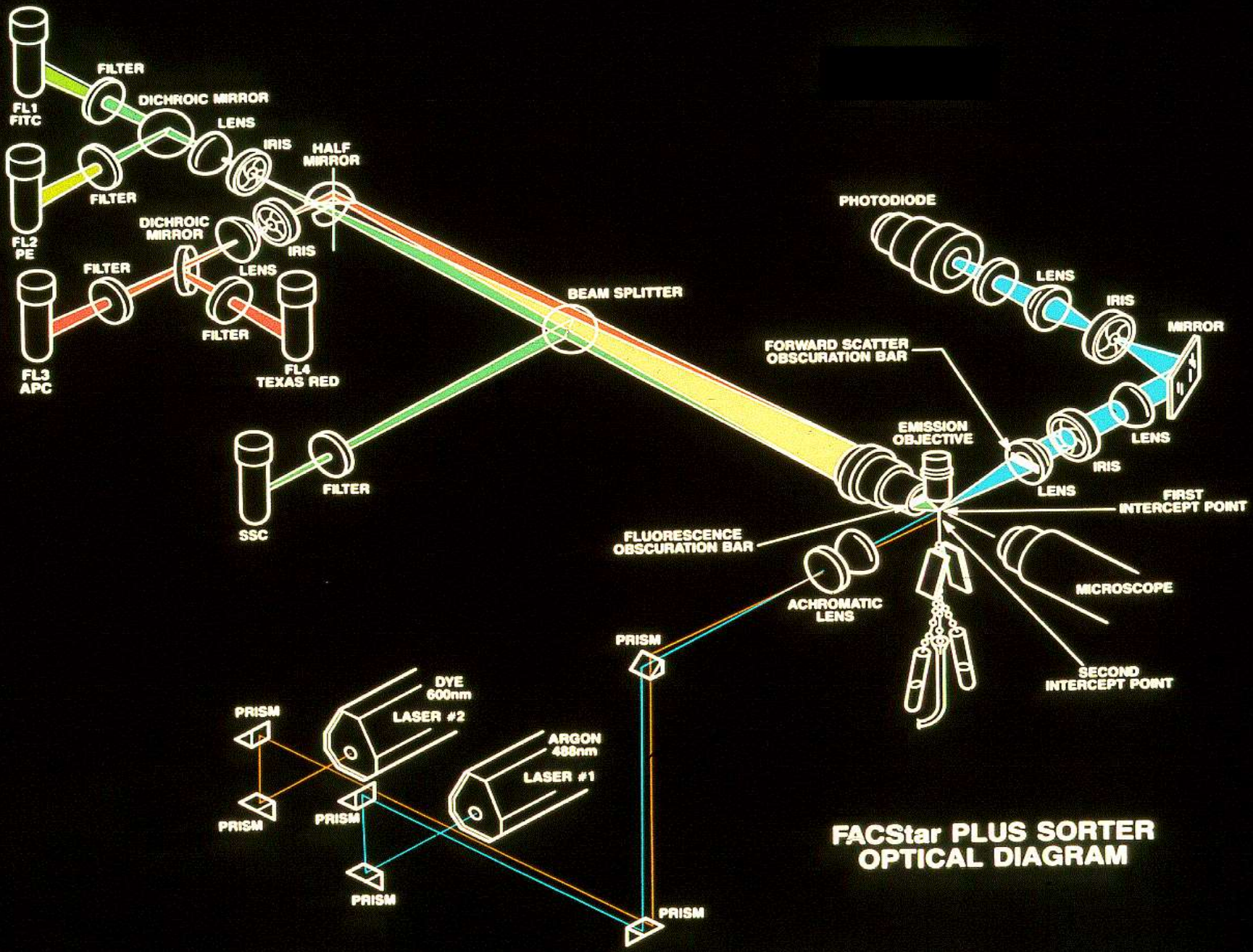
Subpopulation analysis detects changes better, especially for rare subpopulations.

Cell Counting (abs. counts or percentages)
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

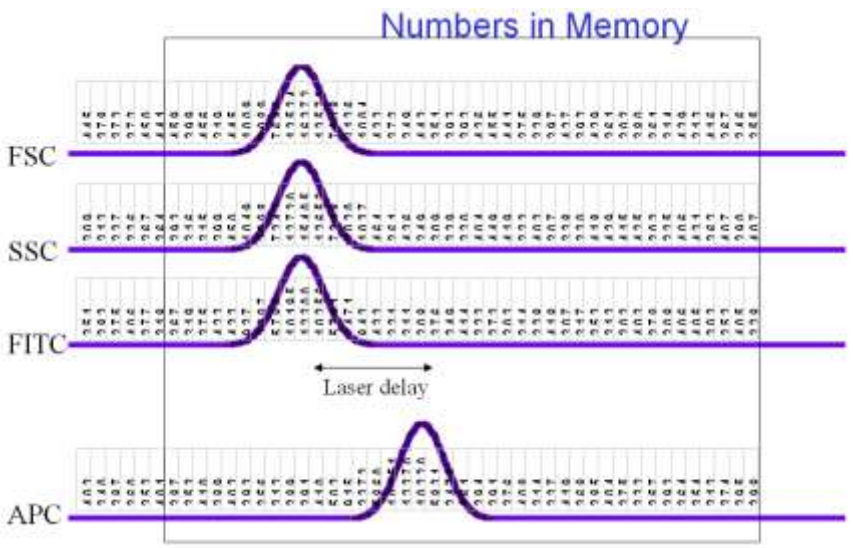


Ignoring Counting Statistics Can Lead to Erroneous Conclusions

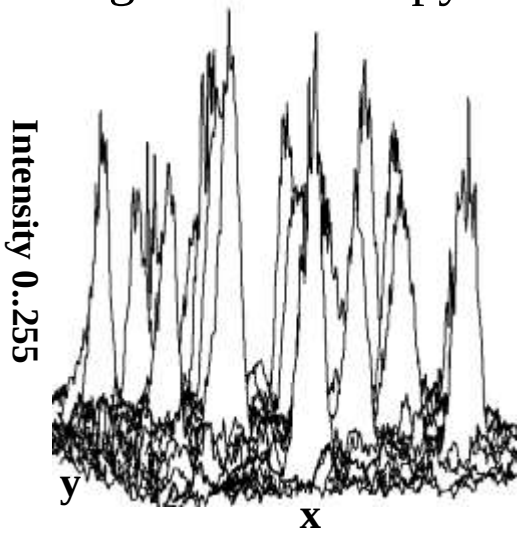

FILTER
FL1
FITC
DICHROIC MIRROR
LENS
IRIS
HALF
MIRROR
FILTER
FL2
PE
DICHROIC
MIRROR
IRIS
FILTER
LENS
FL3
APC
FILTER
FL4
TEXAS RED
BEAM SPLITTER
PHOTODIODE
LENS
IRIS
MIRROR
FORWARD SCATTER
OBSCURATION BAR
EMISSION
OBJECTIVE
LENS
IRIS
LENS
FILTER
SSC
FIRST
INTERCEPT POINT
FLUORESCENCE
OBSCURATION BAR
ACHROMATIC
LENS
MICROSCOPE
PRISM
SECOND
INTERCEPT POINT
DYE
600nm
LASER #2
ARGON
488nm
LASER #1
PRISM
PRISM
PRISM
PRISM
PRISM
FACStar PLUS SORTER
OPTICAL DIAGRAM

Basic Data Processing

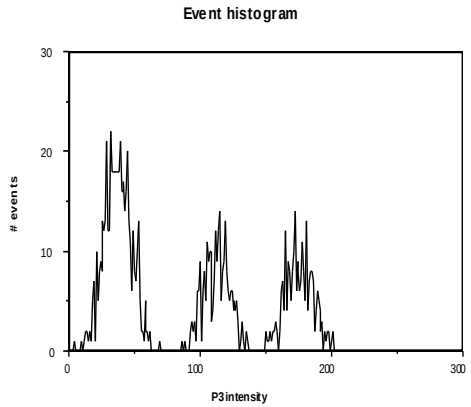
Flow Cytometry

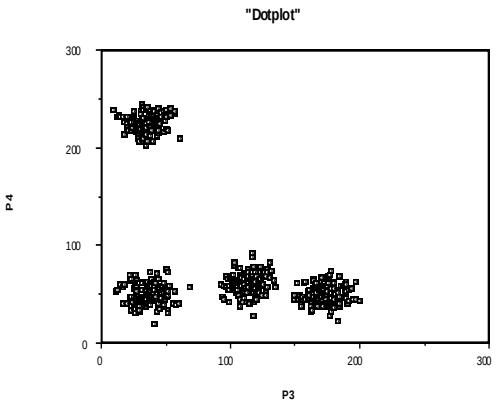


Digital microscopy



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



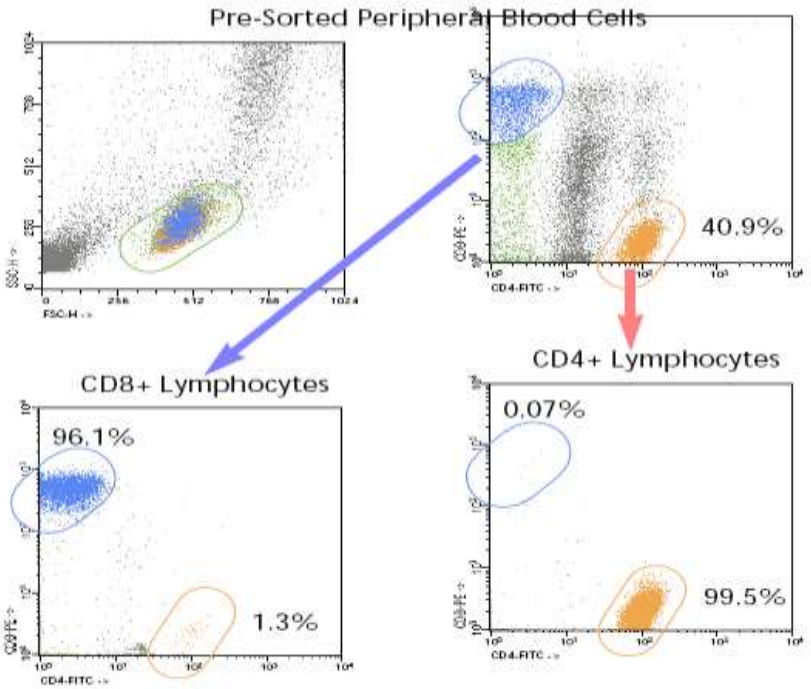


for >2 parameters: gating, cluster analysis, …
For many samples and parameters: bioinformatics

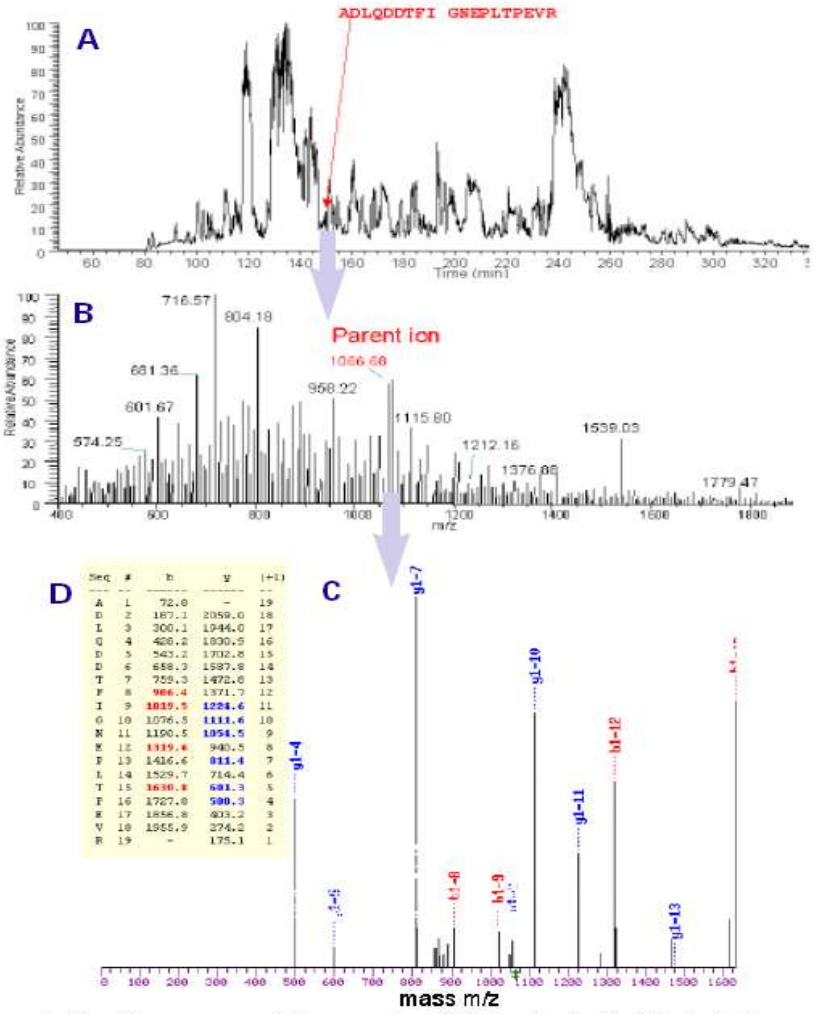
Sorting for Cell Surface Proteomics

Cell surface proteome by FACS sorting, followed by LC MS

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



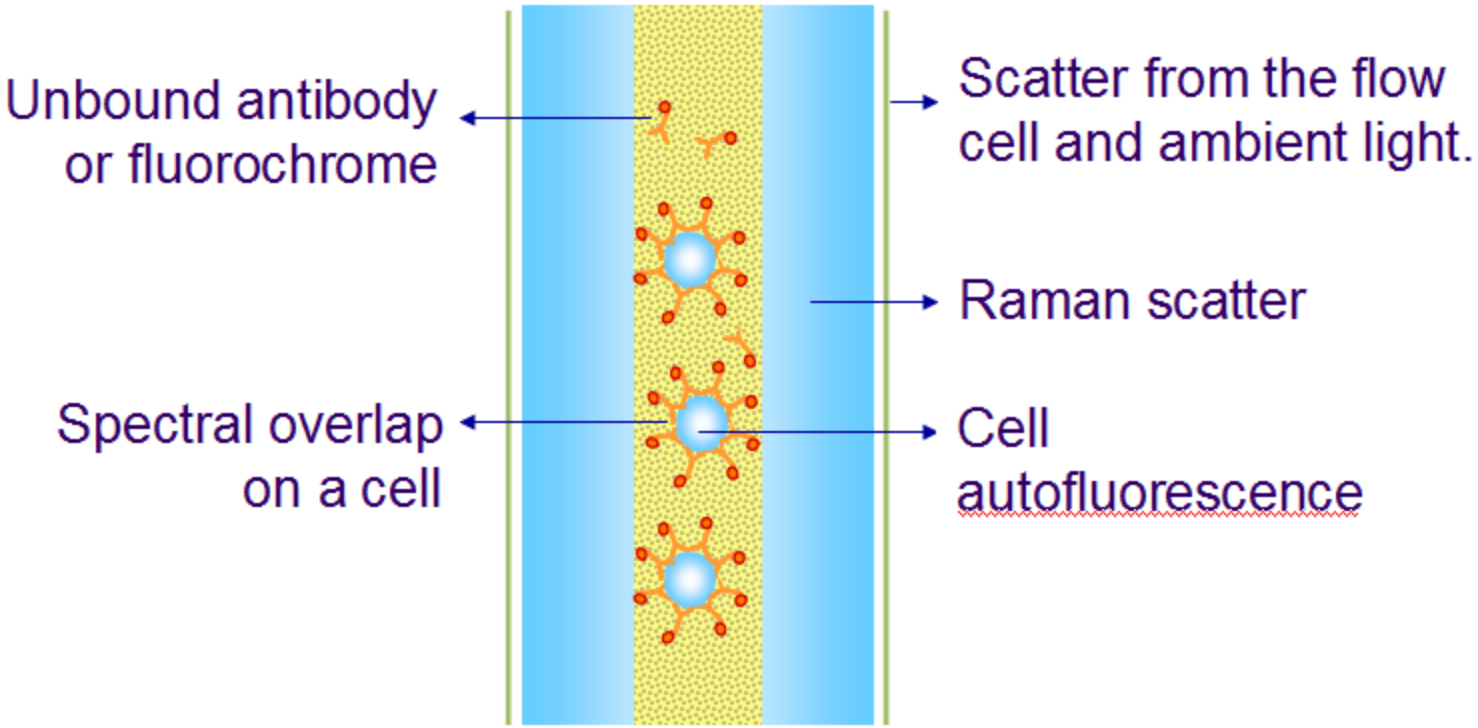
The dot plots show the sorting strategy used for stained peripheral blood cells and population purity after sorting for CD4- and CD8-positive cells. CD4 cells were gated on scatter and FITC fluorescence; CD8 bright cells were gated on scatter and RPE fluorescence. Sorted populations showed >95% purity.



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)**.

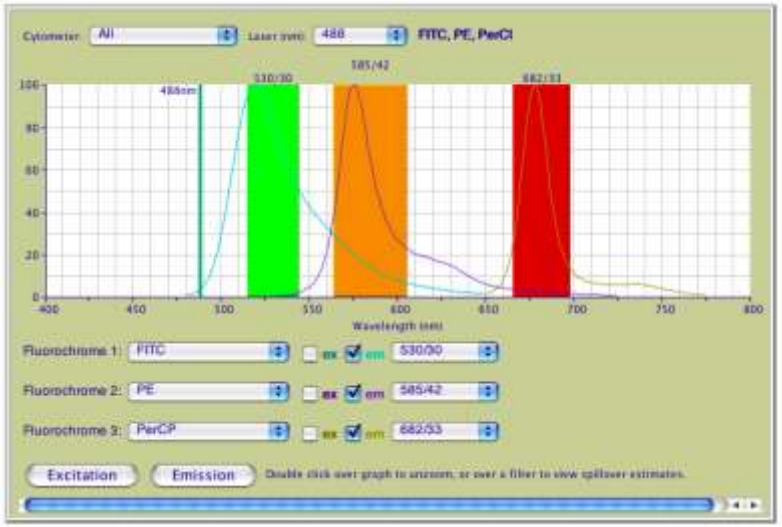
Instrument Evaluation Br

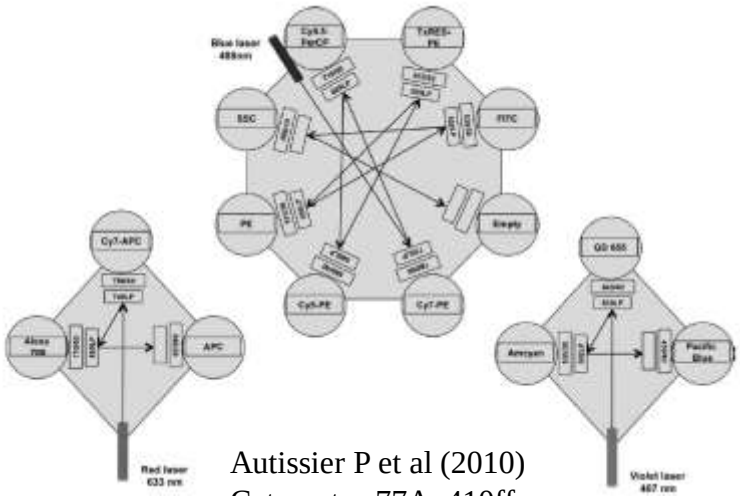
Relative B (Br) is a measure of true optical background in the fluorescence detector.



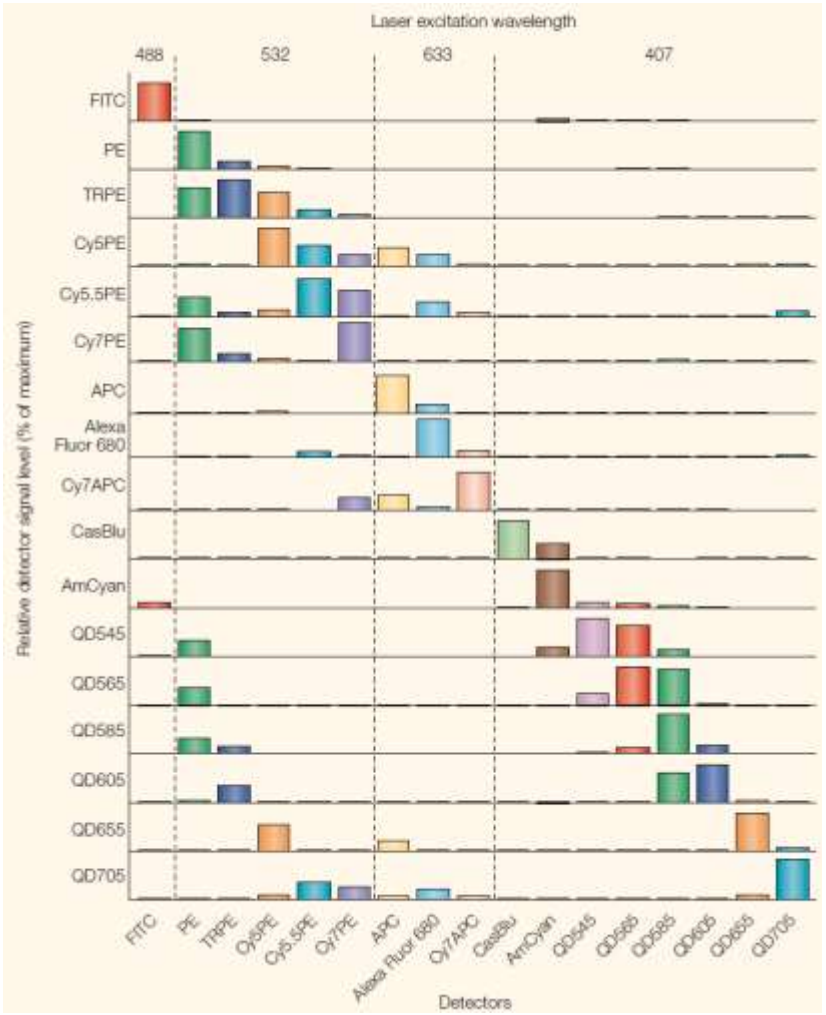
Filter Arrangement and Spectral Overlap

(not relevant for element mass cytometry)



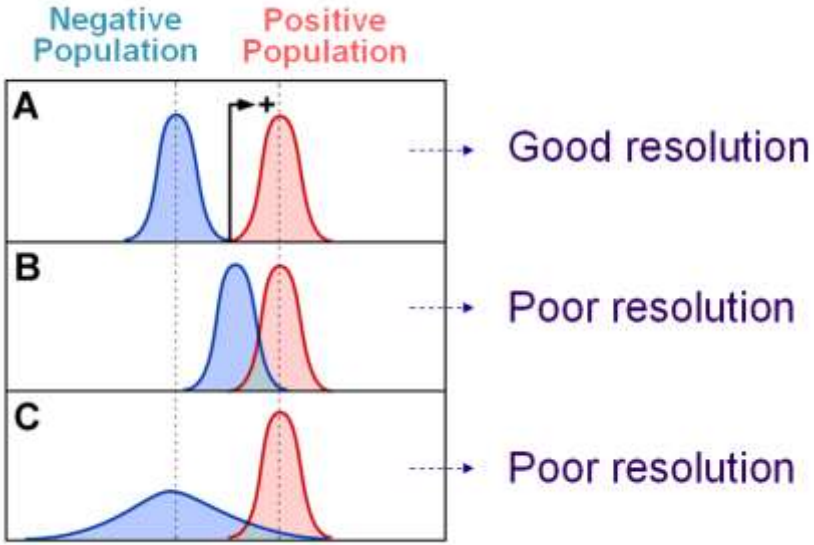


Autissier P et al (2010)
Cytometry 77A, 410ff



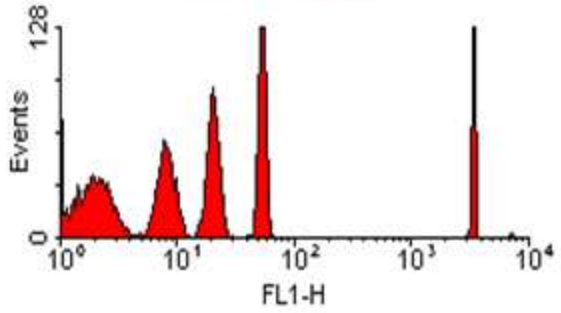
Perfetto SP et al (2004)
Nature Reviews Immunology 4, 648ff

Instrument Evaluation Qr

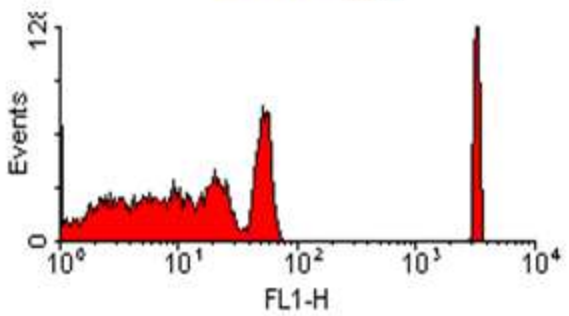


$$Qr = \frac{\#\ \text{photoelectrons}}{\#\ \text{fluorescence molecules}}$$

High Qr



Low Qr



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:

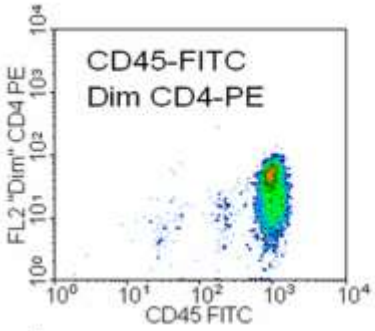
$$SD_{optical} = \sqrt{SD^2_{PE} + SD^2_{FITC} + SD^2_{background}}$$

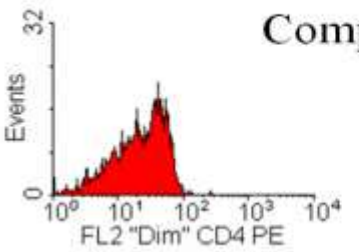
Reagent performance

Stain index

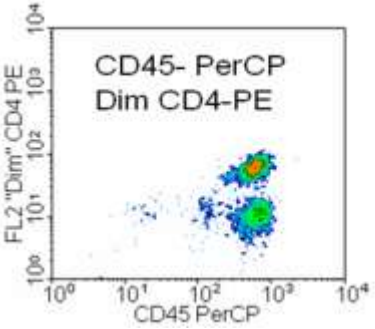
$$\frac{Medium_{pos} - Medium_{neg}}{2 * SD_{neg}}$$

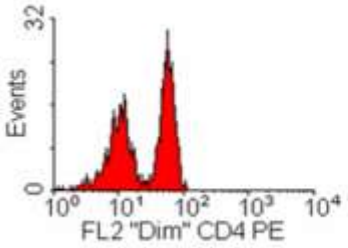
- Dye properties (brightness and spectral overlap)





Compensated data

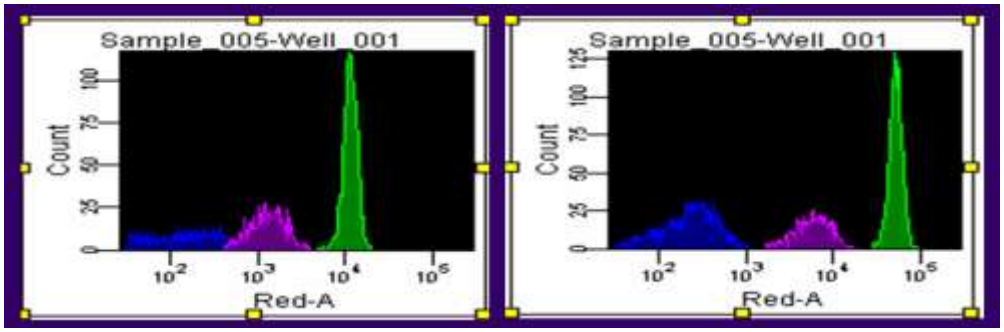




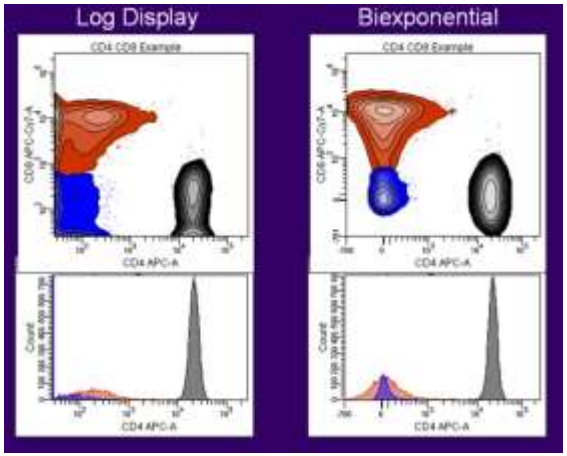
Better separation with less spectral overlap.

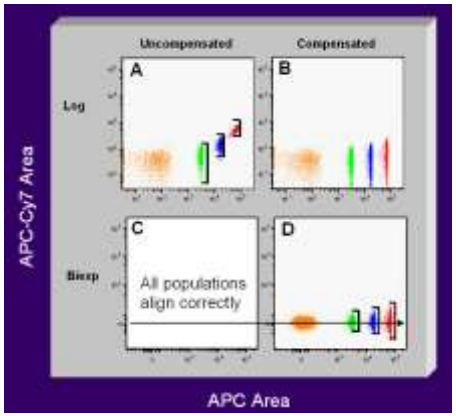
Optimizing cytometry measurements (II)

- Gain (PMT, CMOS, CCD) settings

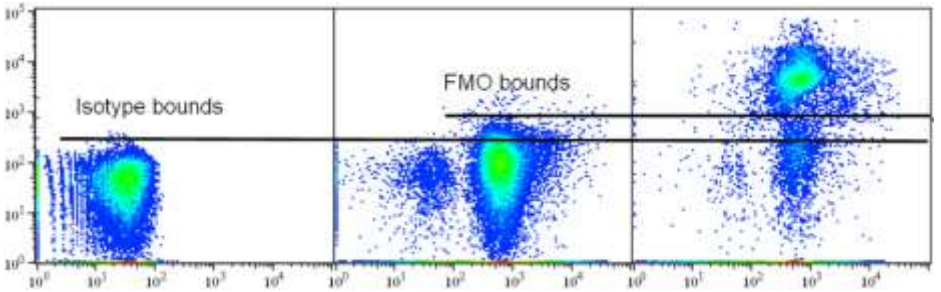


- Data Display





- Controls



Multi-parameter Fluorescence Cytometry Points To Consider

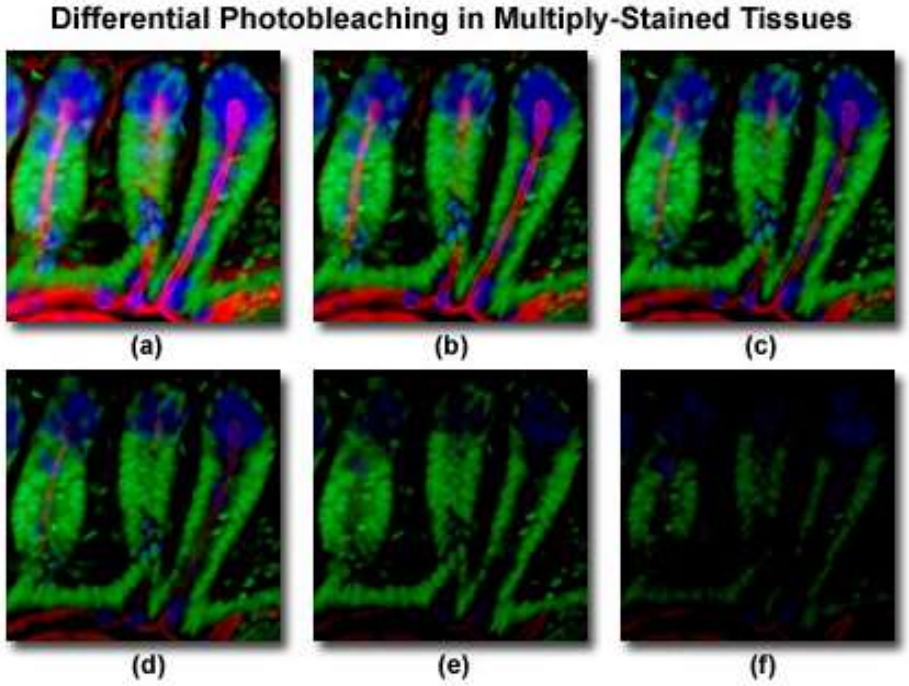
Summary

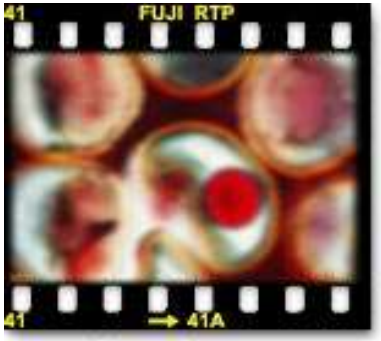
- Know your instrument status e.g. Qr & Br for different channels
- Use high enough gain settings to maximize sensitivity
- An antibody/dye combination that marginally allows discrimination of positives/negatives in a single color assay is unlikely to contribute anything helpful in a multicolor experiment.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

Quantitative Multi-color Microscopy (I)

Additional factors

- Field to field focus
- Photobleaching





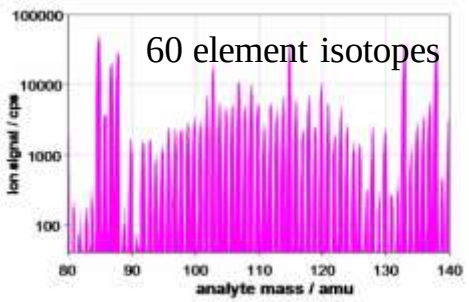
Out of Focus

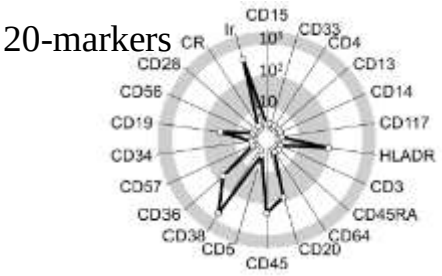
Images from

http://micro.magnet.fsu.edu/primer/index.html

New Developments for in-vitro Multi-parameter Cytometry

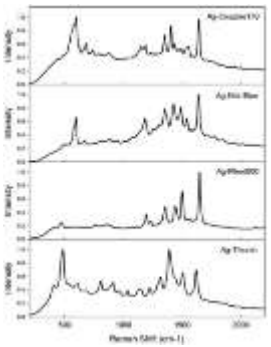
- Element-Label Flow Cytometry (CyTOF, addresses fluorescence spectral overlap issue by using elements as labels, Anal. Chem., 2009, 81 (16), pp 6813–6822)

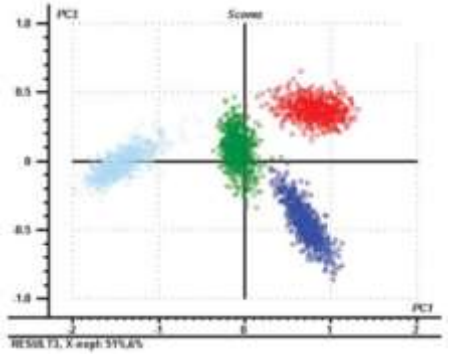




Scott Tanner, DVS Sciences Inc

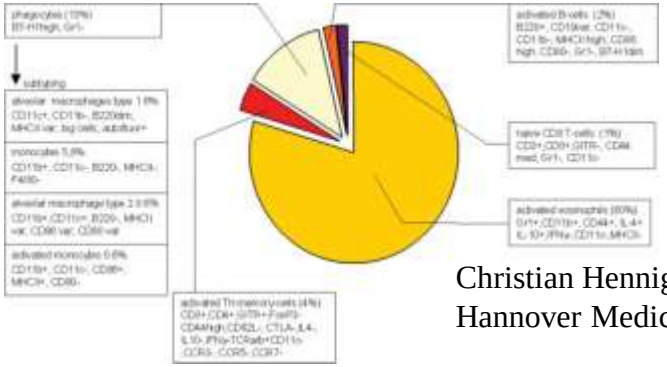
- SERS-Label Flow Cytometry (uses spectral fine-structure to distinguish labels, Cytometry, 2008, 73A(2), pp 119-128)





John Nolan, La Jolla Bioengineering Institute

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

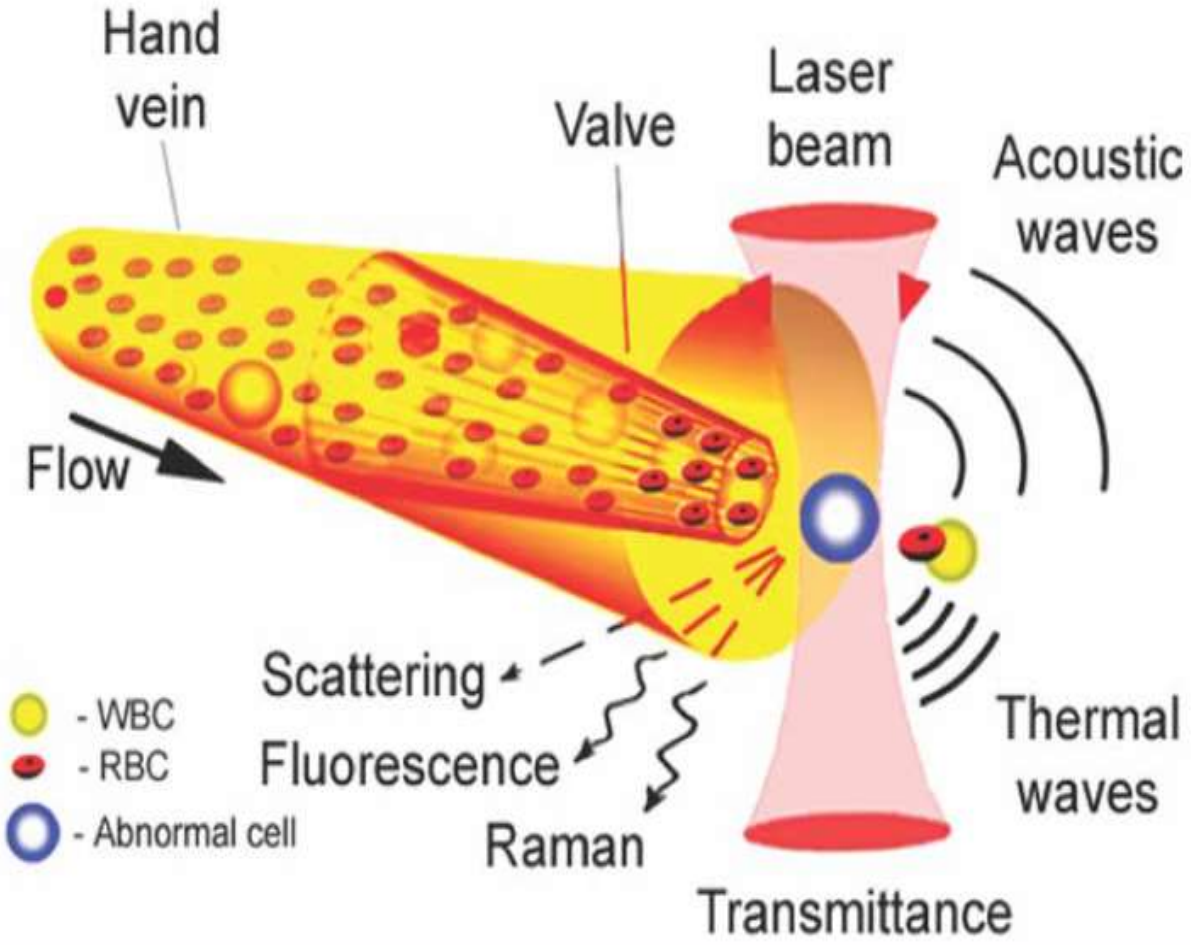


31-marker analysis

Christian Hennig, ChipCytometry
Hannover Medical School

In-vivo Multi-parameter Cytometry

Single cell analysis in living animals



Issues:

- tissue optics
- object motion
- flow rate
- labelling
- …

Tuchin VV et al: Cytometry 79A (2011) 737ff

Conclusions

Multi-parameter cytometry

Optimized flow and imaging single cell cytometry with adequate bio-informatics tools provide quantitative molecular measurements into biological processes at organism, cellular and sub-cellular levels.

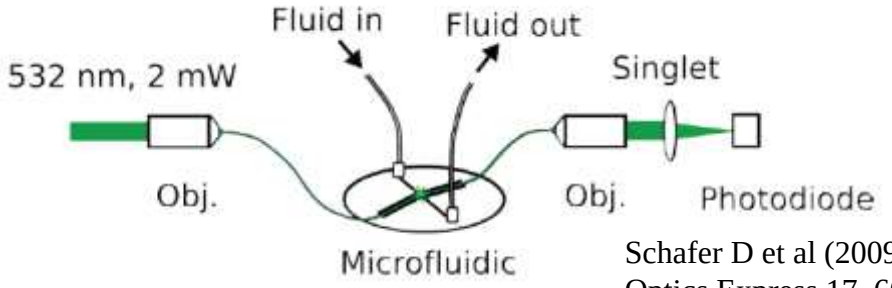
New developments in many areas have simplified the tools for the biologist.

Evolving Technologies for Cytometry

- More low complexity cytometers for cell (subset) counting
- Impedance as a cell analysis parameter
- Innovative automated sample preparation
- Fluorescent polymers for high sensitivity
- Novel affinity reagents (antibodies)

Low-complexity Cytometers for Cell Counting

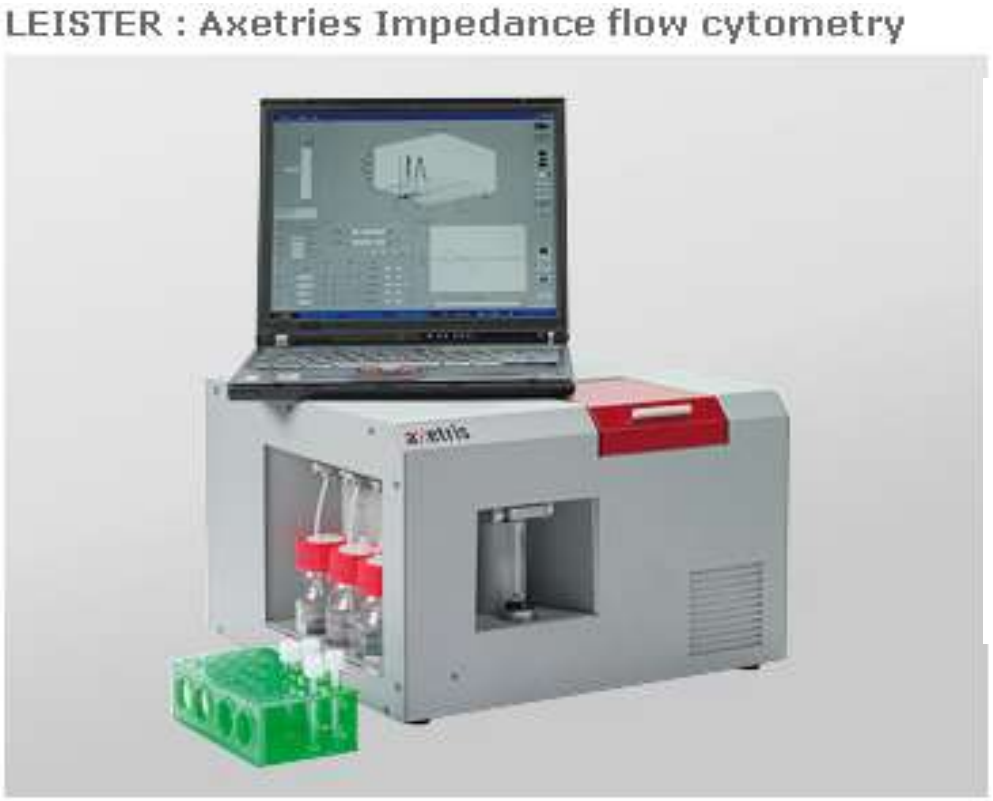
Low-end cell counters

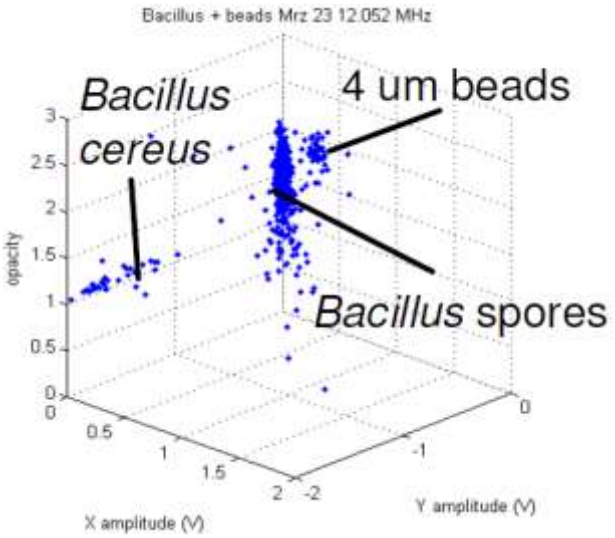


Schafer D et al (2009)
Optics Express 17, 6068ff

Merck Millipore MUSE™

Impedance Flow Cytometry





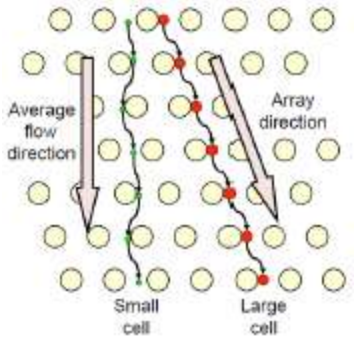
Marco DiBeradino, Leister Axetris, Amphasys AG

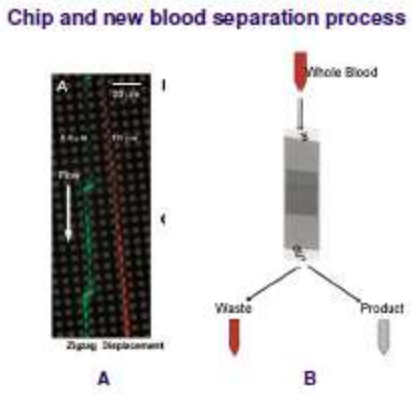
Electrical parameters of living cells (no label required).

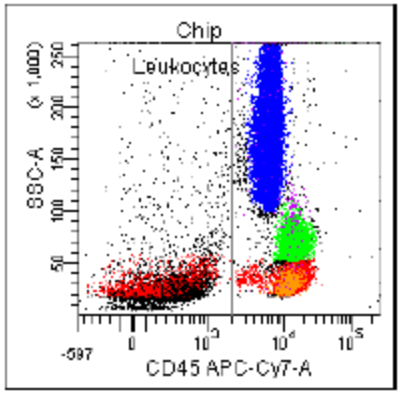
Other parameters: fluorescence polarization, fluorescence lifetime, compressibility, …

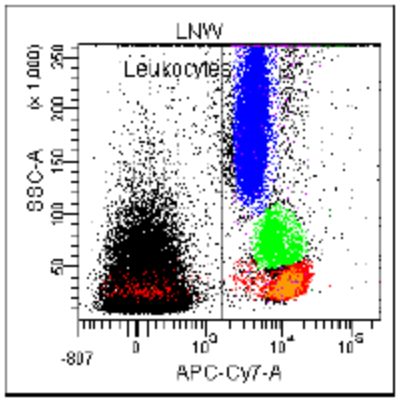
Innovative Sample Preparation

Microfluidic system for leukocyte isolation (deterministic lateral displacement)



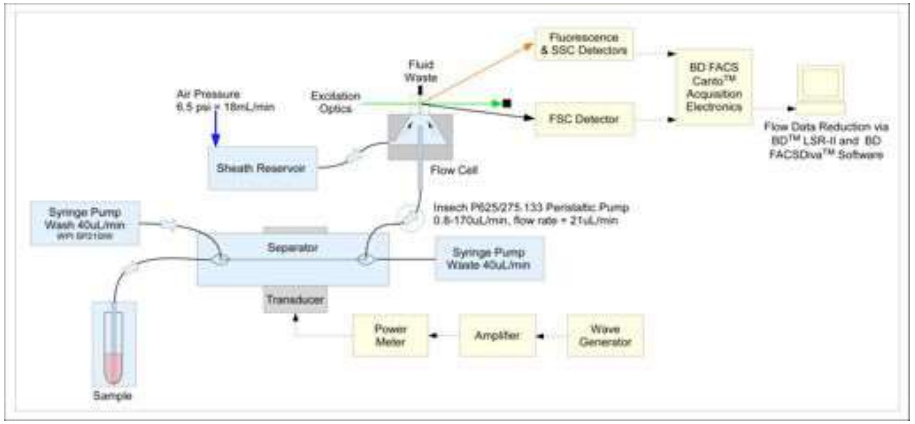


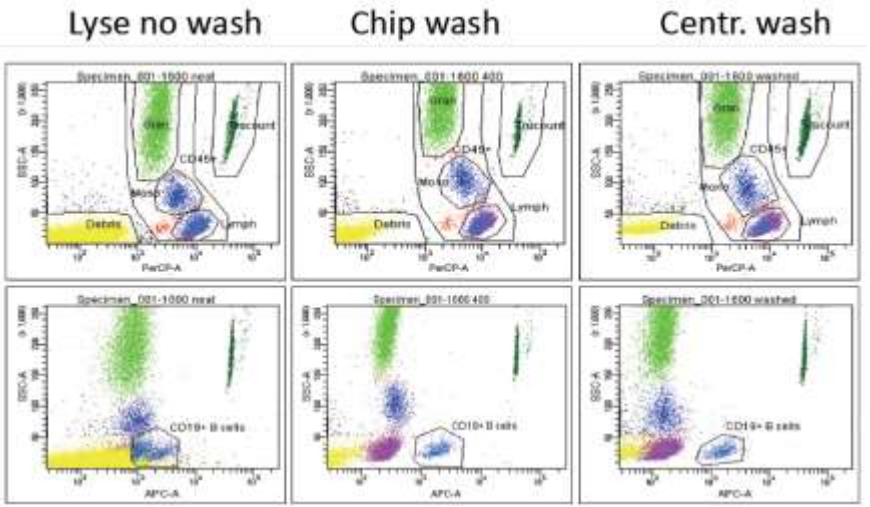




Cyto 2012 poster, Liping Yu et al, GPB and BD Biosciences

Acoustic particle focusing for cell washing



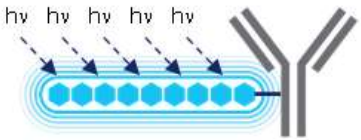


2010, Laurell group, Lund University & Brian Warner, BD Biosciences

Bright Fluorescent Polymer Dyes (Sirigen)

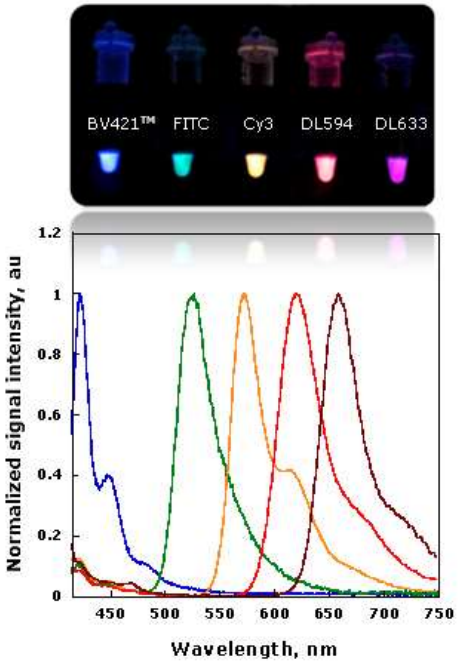
Polymer Based Fluorochromes

- Well defined synthetic organic polymer structures
  - Single conjugation site, defined size, etc.
- Backbone comprised of π-conjugated repeat units
  - Affords massive light harvesting ($\varepsilon > 10^6$) materials with high quantum yields
- Tunable architecture adapted for low NSB, high aqueous solubility and spectral performance



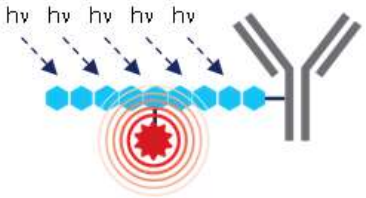
Brilliant Violet 421™

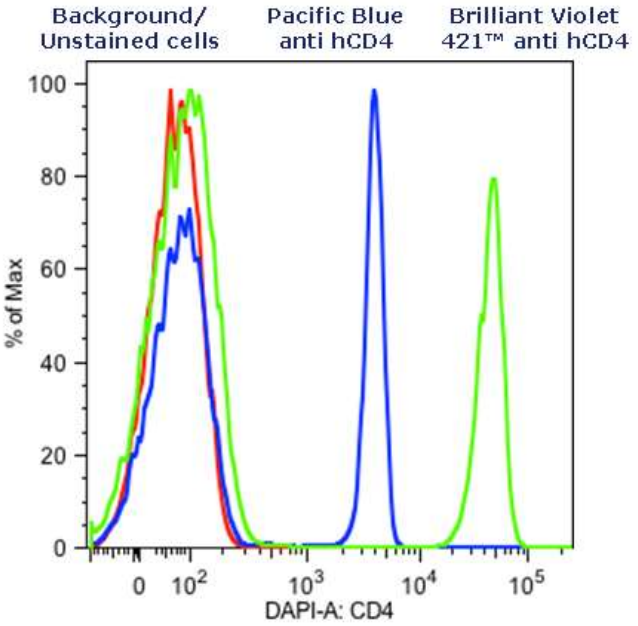
- PE level performance w/ 405nm Laser
- >10x the Stain Index of Pacific Blue
- Enables detection of low abundance targets in multicolor assay panels (e.g. CD56, CD127, etc.)
- Wide range of Ab clones validated



Brilliant Violet Tandems

- Provides a wider range of colors spanning the visible spectrum
  - >6 unique colors validated
- Chemically controlled ratio of donor/acceptor provides:
  - Reproducible performance
  - Low (<5%) compensation at 450nm





www.sirigen.com

Novel Affinity Reagents

- Antibodies
  - Antibodies from different species (e.g. Llama 15 kDalton fragments with 10^{-9}M Kd and high stability, potential for intracllular use)
  - Recombinant antibody fragments
  - …
- Synthetic affinity reagents
  - Aptamers
  - Protein scaffolds
  - Molecular Imprinted Polymers

Recent review: Fodey T et al; Trends in Anal. Chem. 30(2011) 254ff

Conclusions

Evolving Technologies

Technology developments in algorithms, computing, detectors, electronics, nanotechnology, microfluidics, organic chemistry, and recombinant protein technology create the basis for new reliable analytical approaches for a deeper molecular understanding of living systems.

There is value in working with other scientific disciplines.

Acknowledgements

- Joe Trotter
- Ming Yan
- Maria Jaimes
- Brian Warner
- Ed Goldberg
- Hrair Kirakossian
- Liping Yu
- Mike Brasch
- Ben Verwer

  above all BD

- Holden Maecker, Stanford
- Bob Hoffman, consultant
- Ken Davis, retired
- Bill Godfrey, Beckman Coulter
- Brent Gaylord, Sirigen > BD
- Collette Rudd, Thermo

Contact

Diether@Desatoya.com **Phone**: USA-408-658-6074

http://www.desatoya.com