8th ENII Immunology Summer School, May/June 2013

State of the Art in Cytometry

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Presentation Outline

- **Technologies for Biology Research**
- **Flow and Image Cytometry Basics**
- **Examples from Genomics and Proteomics**
- **Optimizing Multi-Parameter Experiments**
- **Additional Considerations for Imaging**
- **Intra-vital Microscopy**
- **In-vivo Flow Cytometry**
- **Evolving Technologies**

Biology Research Targets and Tools

Organism
Organ
Tissue
Single Cell
Organelle
Macromolecule
Small molecules

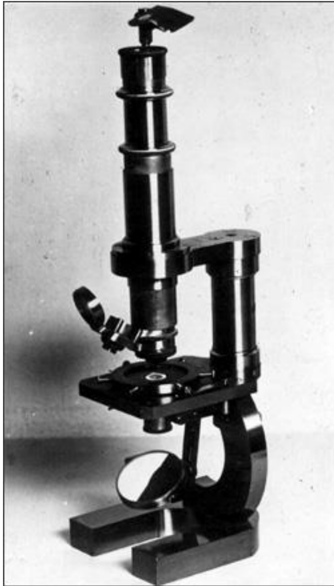
NMR
X-ray imaging
Ultrasound
2-photon imaging
In-vivo cytometry
Light microscopy
Electron microscopy
Flow cytometry
Cell imaging
NA sequencing
Mass spectrometry
TIRF microscopy
Electrophoresis

Contrast agents
Affinity reagents
- antibodies
- probes

Enzyme substrates
Labels
- absorbance
- fluorescence
- element tags

Sample prep

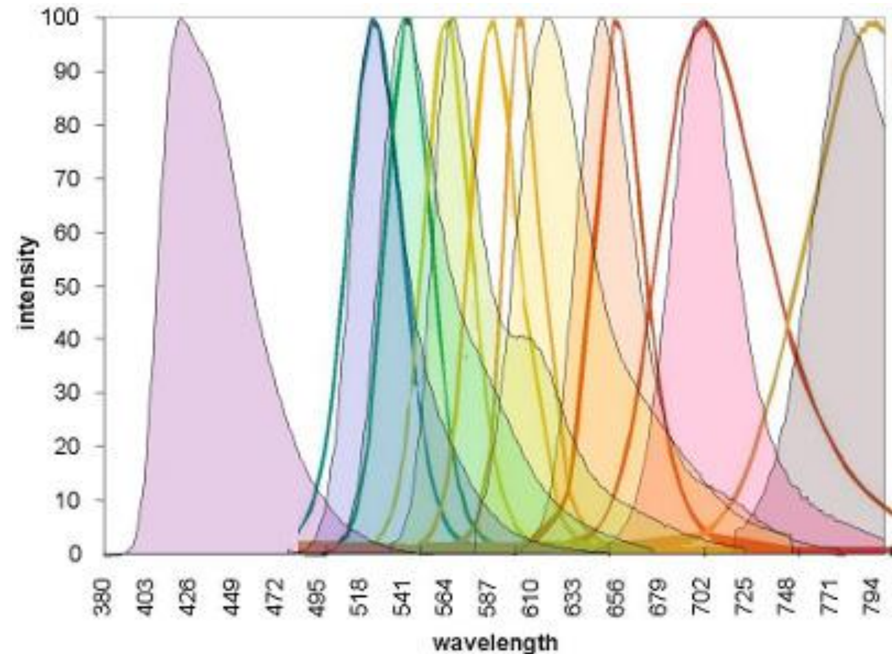
Single Cell Analysis
Microscopy and Flow Cytometry



Cytometry Basics

Physical parameters

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

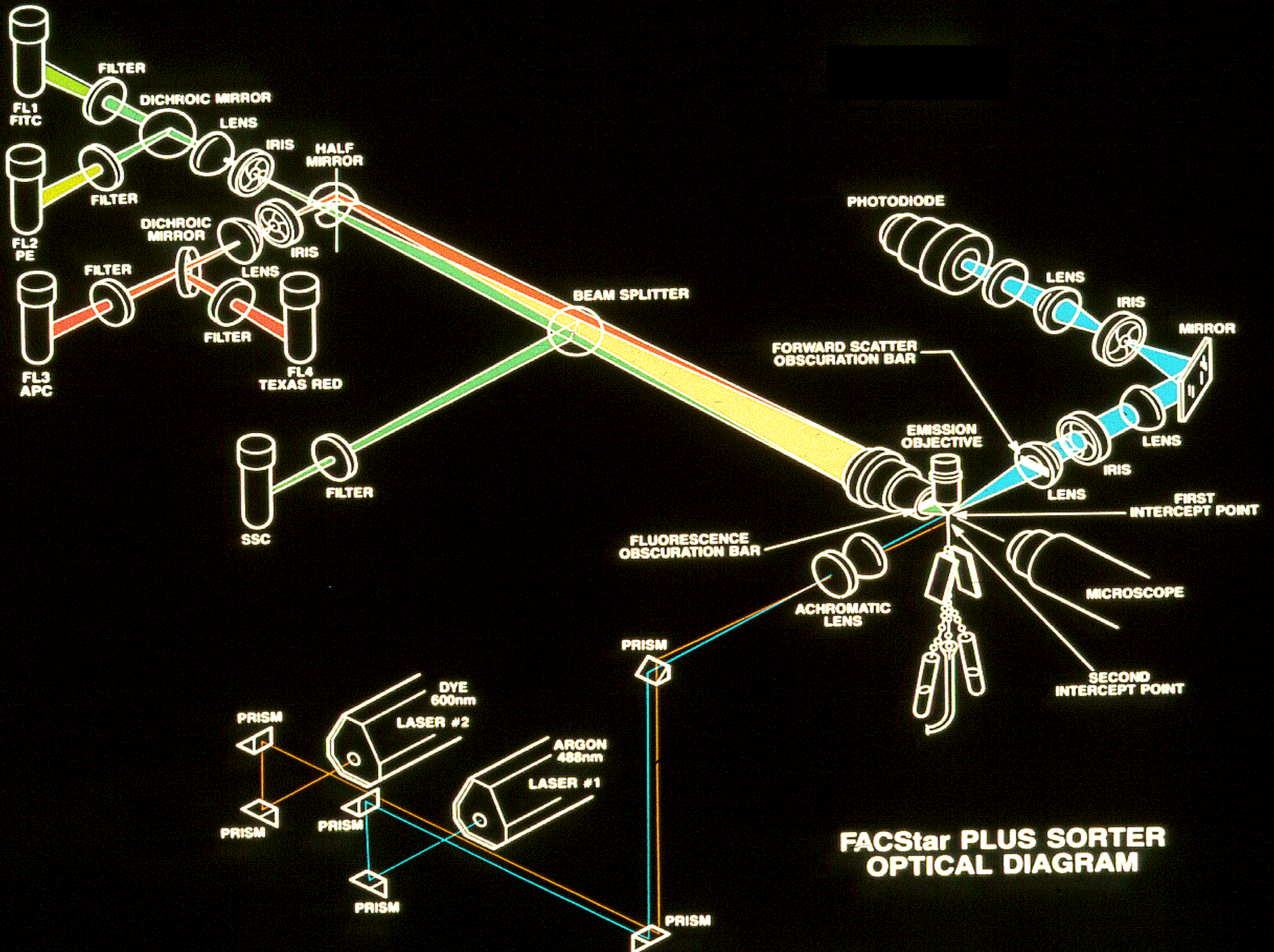


http://www.dvssciences.com/technical.html

Flow and Imaging Cytometry Features

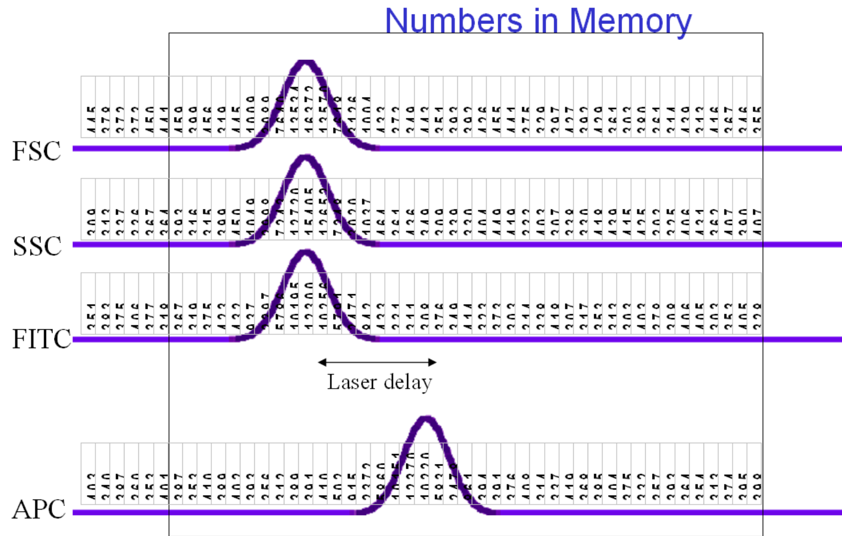
Single particle (cell) analysis with

• 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 microns	I,F
• 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)

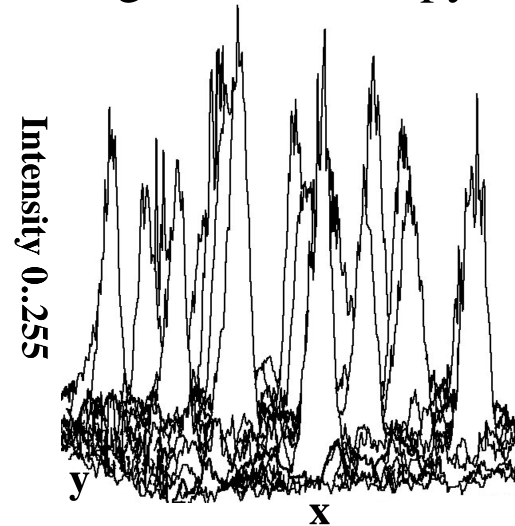

FILTER
FL1
FITC
DICHROIC MIRROR
LENS
IRIS
HALF
MIRROR
FL2
PE
FILTER
DICHROIC
MIRROR
IRIS
FILTER
LENS
FL3
APC
FILTER
FL4
TEXAS RED
BEAM SPLITTER
SSC
FILTER
PHOTODIODE
LENS
IRIS
MIRROR
FORWARD SCATTER
OBSCURATION BAR
EMISSION
OBJECTIVE
LENS
IRIS
LENS
FIRST
INTERCEPT POINT
FLUORESCENCE
OBSCURATION BAR
ACHROMATIC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

Basic Data Processing

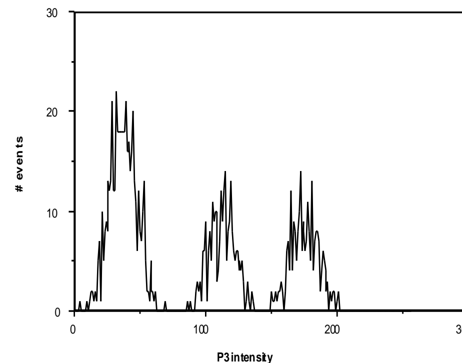
Flow Cytometry

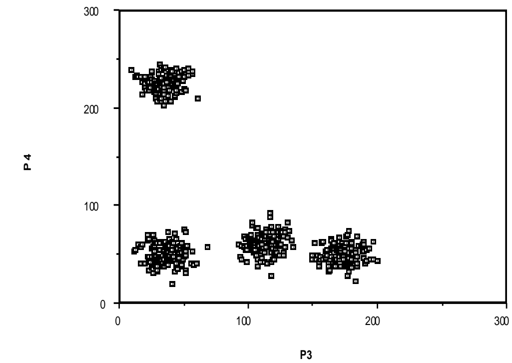


Digital microscopy



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

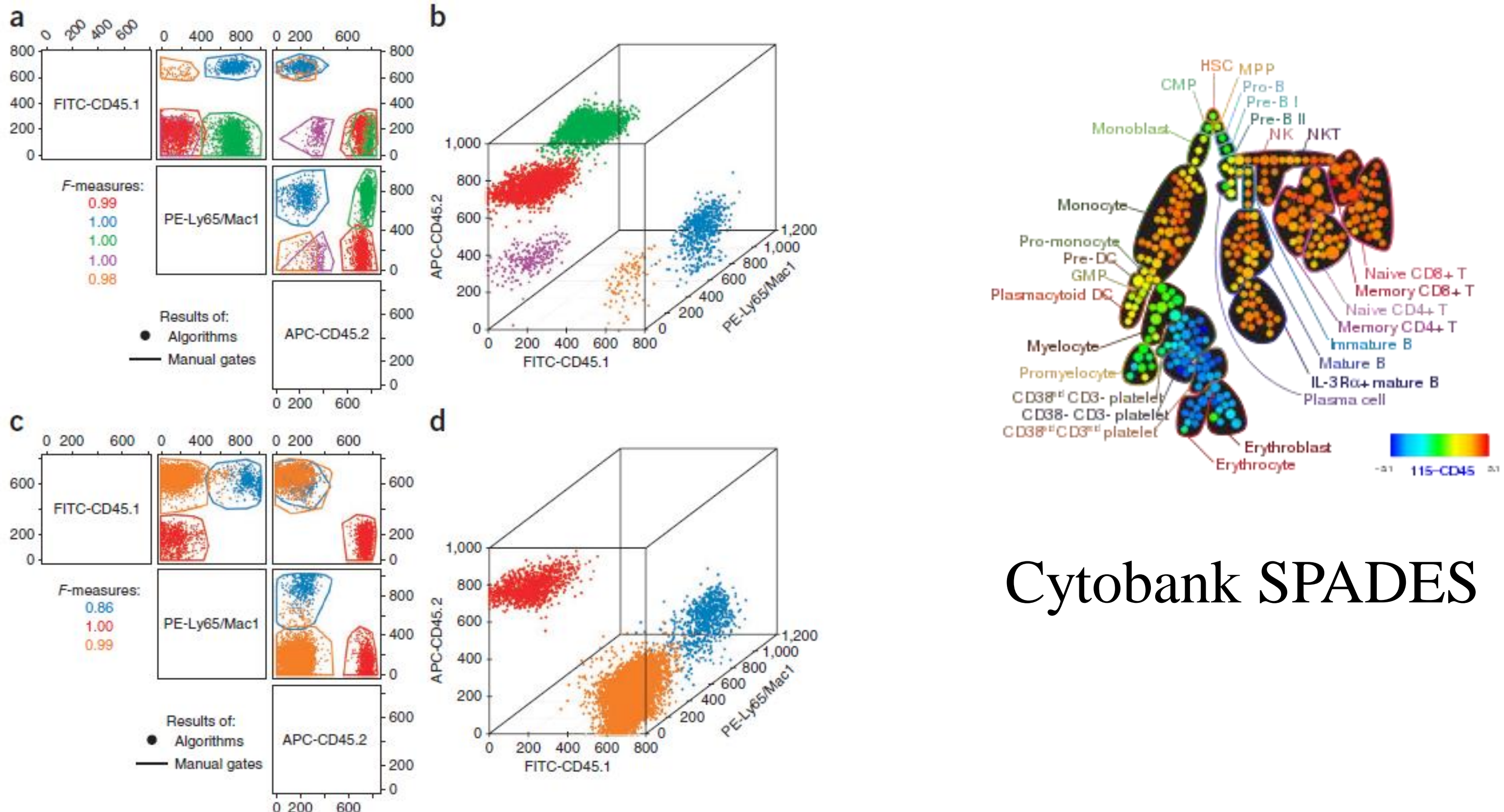




for >2 parameters: gating, cluster analysis, …

For many samples and parameters: bioinformatics

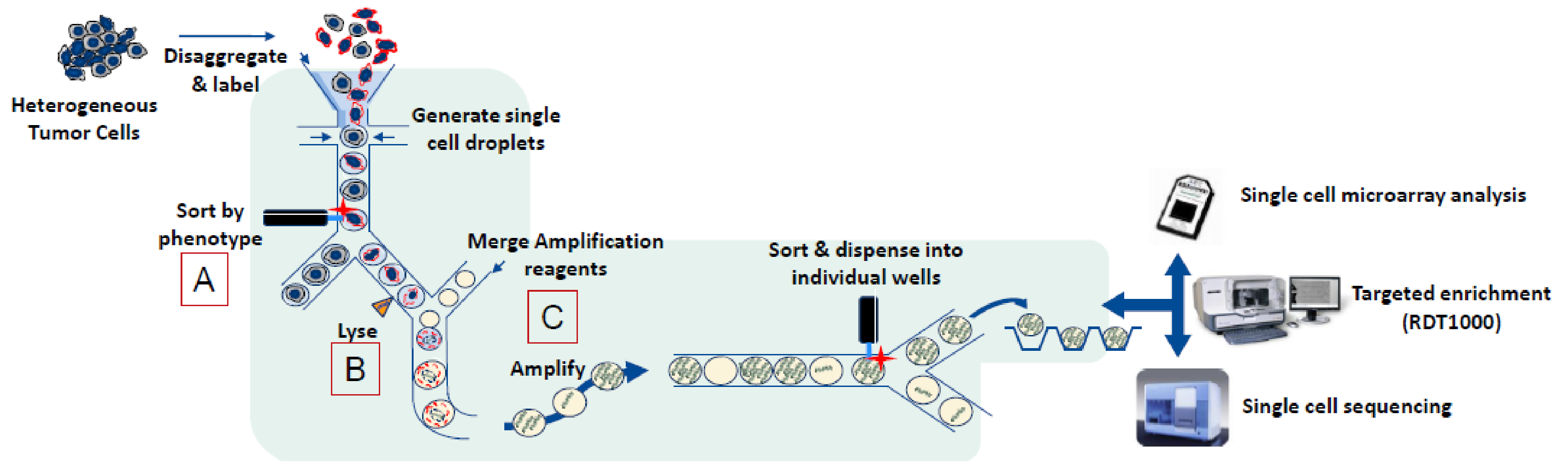
BioInformatics



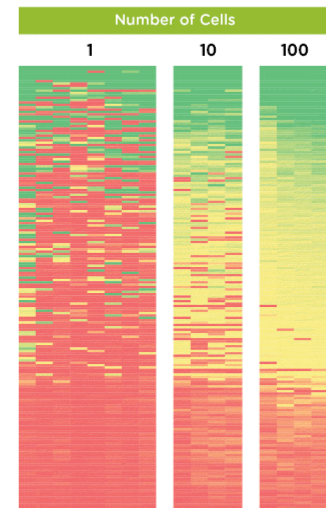
Cytobank SPADES

FlowCAP Consortium;
Nature Methods2013;10, 228ff

Single Cell Genomics



Source: Raindance Technologies



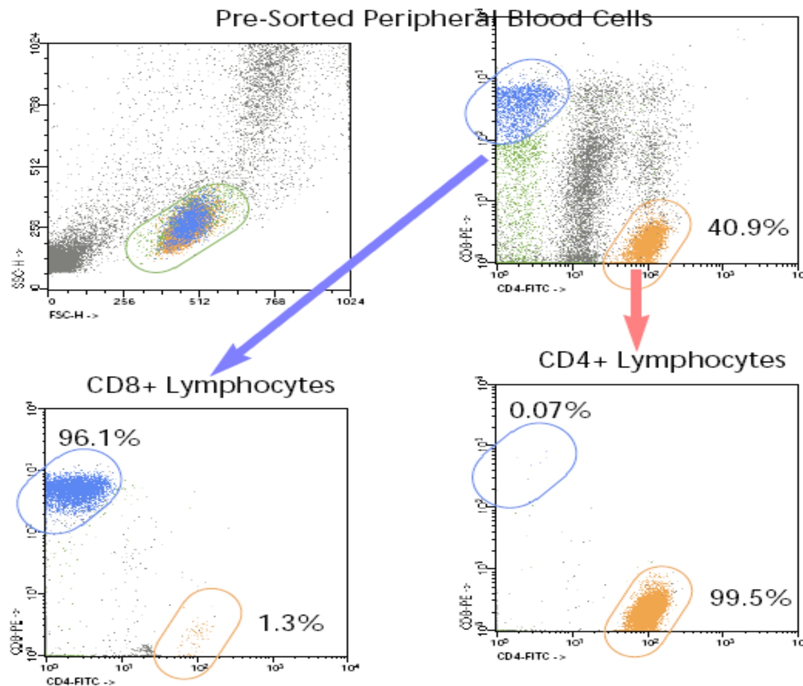
Source: http://www.nanostring.com

Single cell analysis reveals heterogeneity, which is masked by averaging, when analyzing groups of cells.

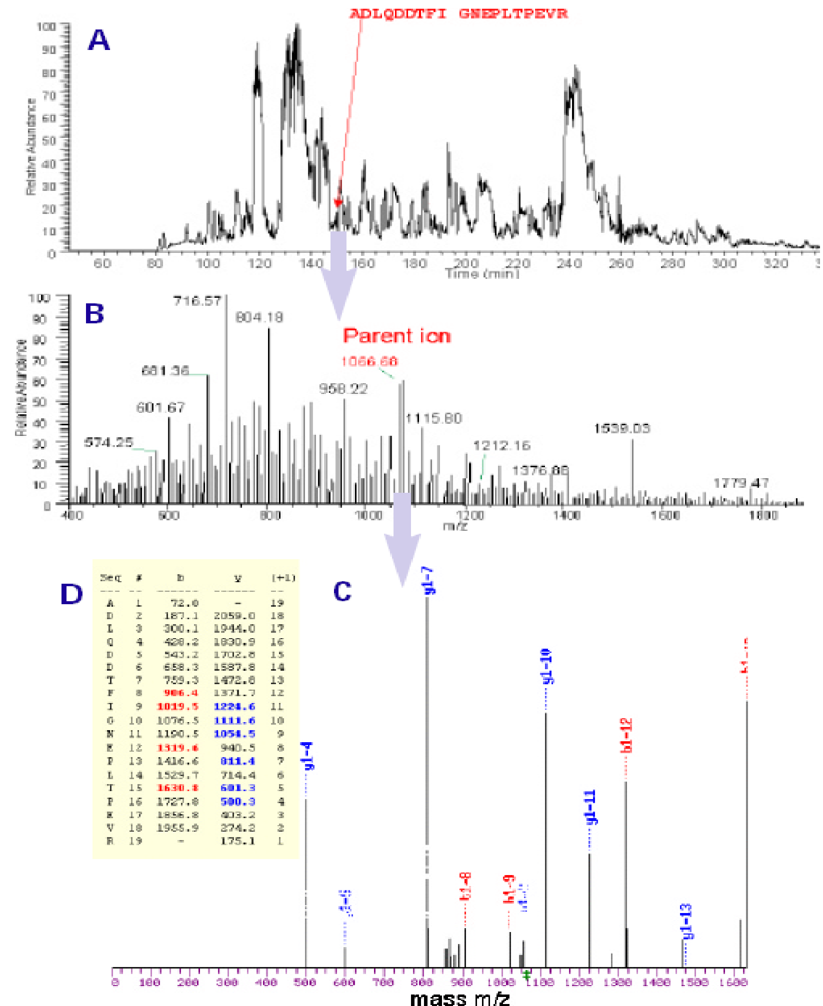
Sorting for Cell Surface Proteomics

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.



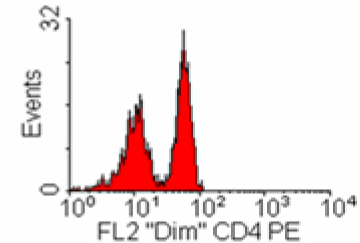
Seq	#	b	y	(+1)
A	1	72.0	-	19
D	2	187.1	2059.0	18
L	3	300.1	1944.0	17
Q	4	428.2	1830.9	16
D	5	543.2	1702.8	15
D	6	658.3	1587.8	14
T	7	759.3	1472.8	13
F	8	906.4	1371.7	12
I	9	1019.5	1224.6	11
G	10	1076.5	1111.6	10
N	11	1190.5	1054.5	9
E	12	1319.6	940.5	8
P	13	1416.6	811.4	7
L	14	1529.7	714.4	6
T	15	1630.8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

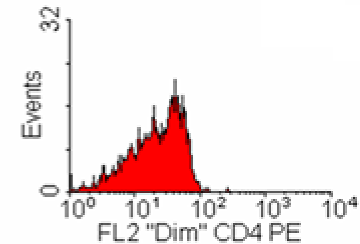
Peptide mixtures were separated by reverse phase HPLC **(A)** as described in Methods. Eluted peptides were subjected to electrospray injection into the mass spectrometer and analyzed for their mass/charge ratio (m/z value) **(B)**. Selected ions were collected in the ion trap. These parent ions were 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)**.

Cell Surface Proteome of Sorted Cells, measured by LC ESI MS

Optimizing Multi-Color Experiments

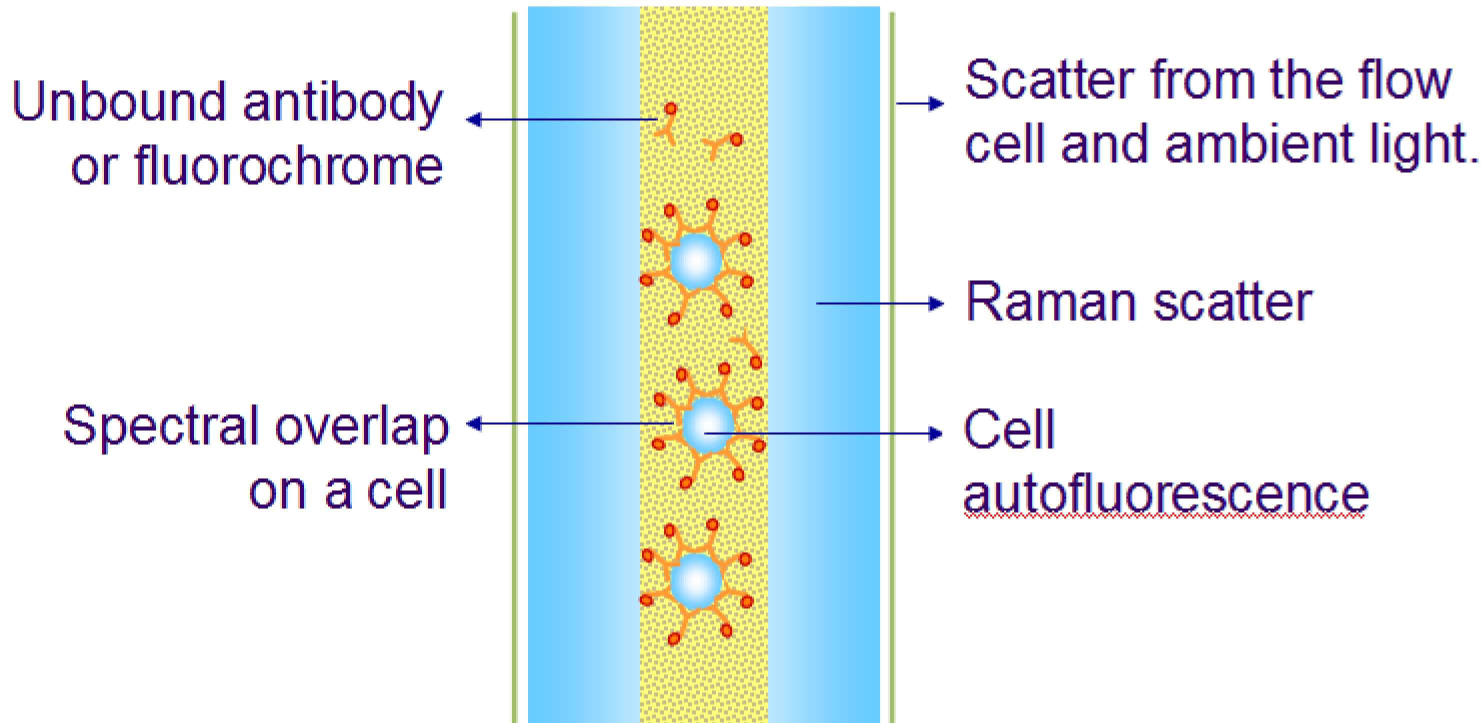
- Population separation
- Br and Qr
- Data Display
- Controls
- Microscopic Imaging



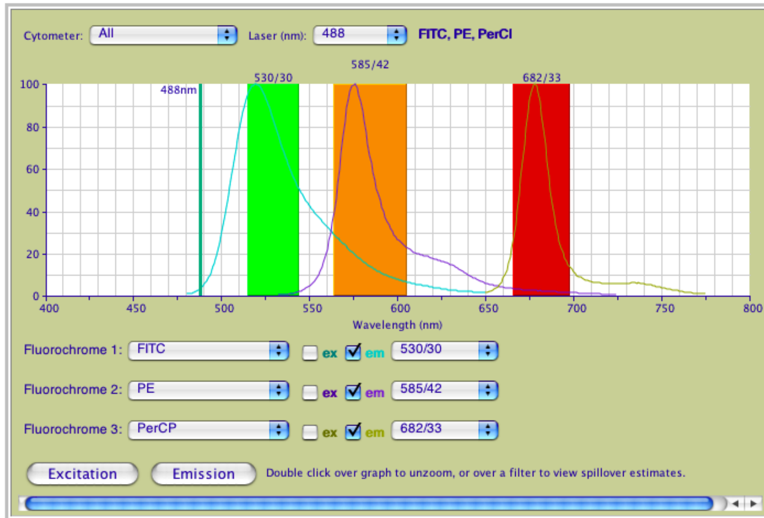


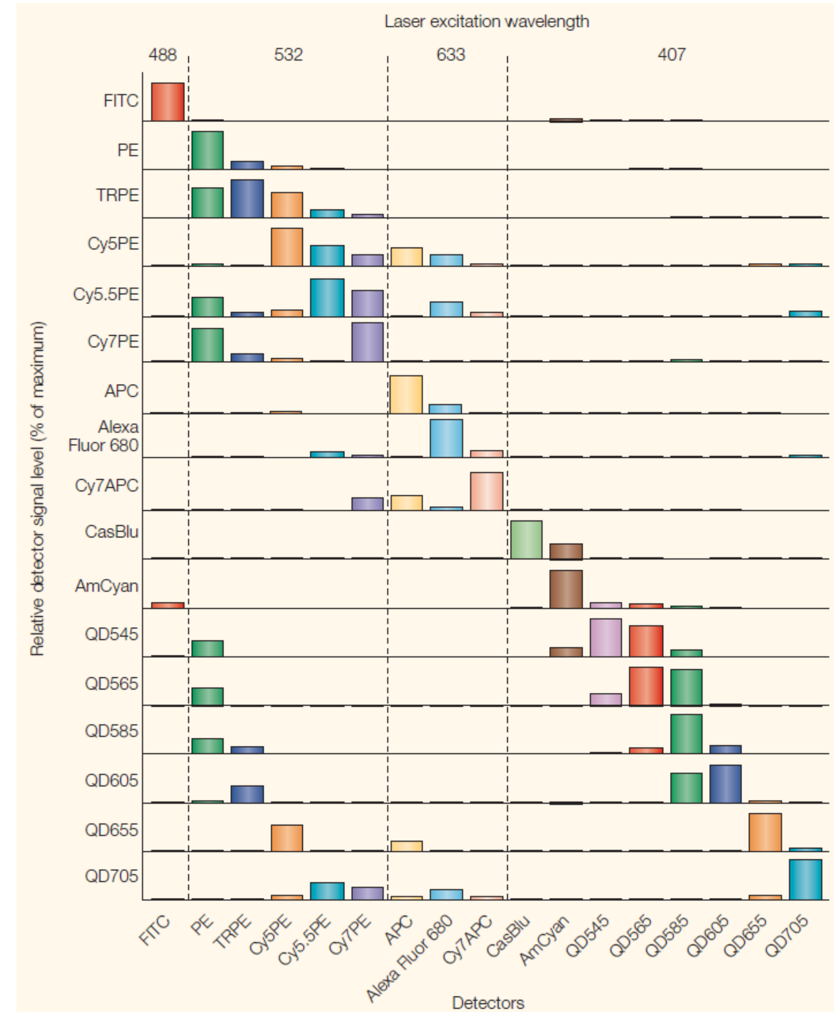
Instrument Evaluation Br

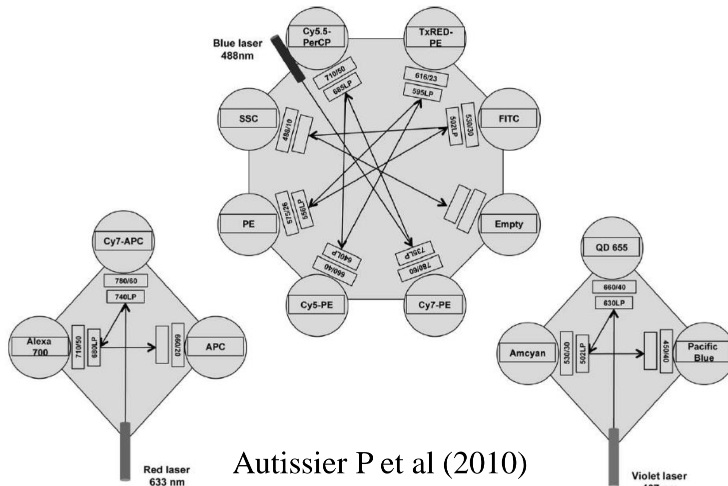
Relative B (Br) is a measure of true optical background in the fluorescence detector.



Filter Arrangement and Spectral Overlap
(not relevant for element mass cytometry)



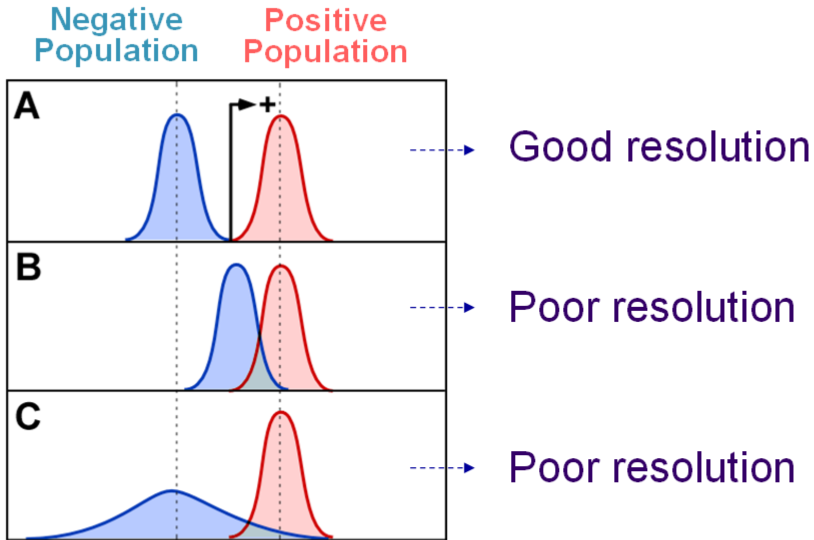




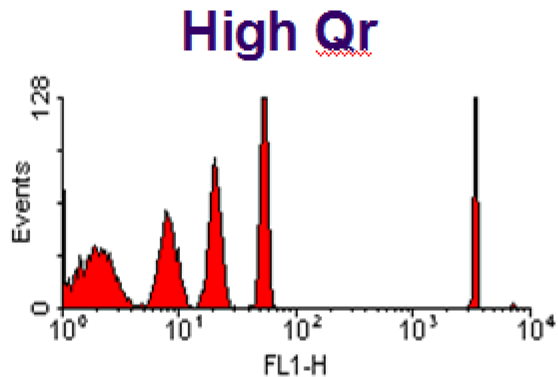
Autissier P et al (2010)
Cytometry 77A, 410ff

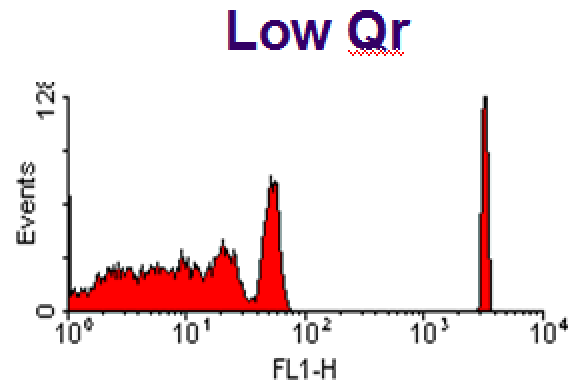
Perfetto SP et al (2004)
Nature Reviews Immunology 4, 648ff

Instrument Evaluation Qr



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





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:

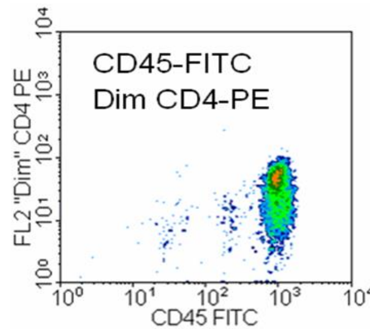
$$SD_{optical} = \sqrt{SD^2_{PE} + SD^2_{FITC} + SD^2_{background}}$$

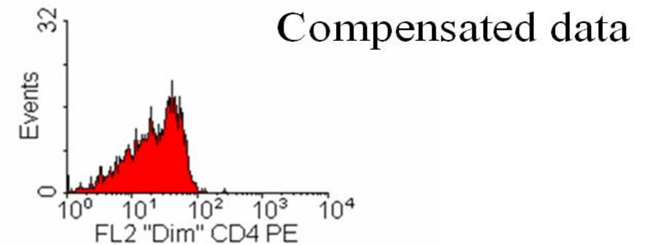
Reagent performance

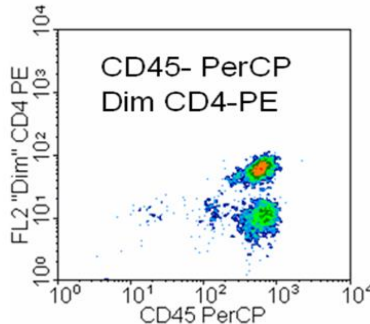
Stain index

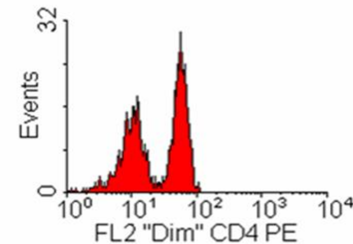
$$\frac{Medium_{pos} - Medium_{neg}}{2 * SD_{neg}}$$

- Dye properties (brightness and spectral overlap)









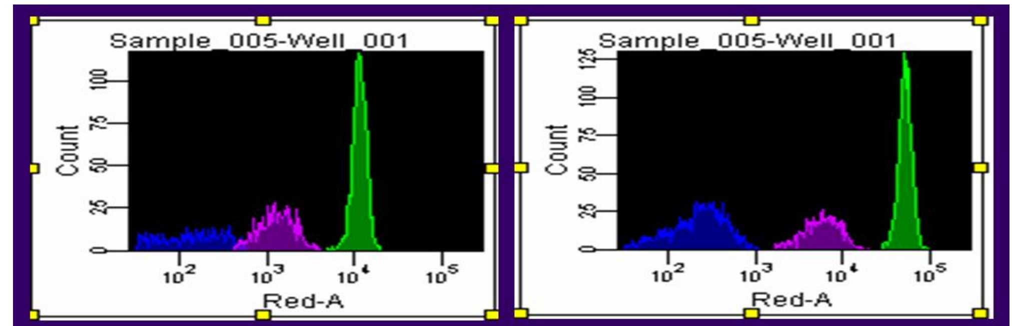
Better separation with less spectral overlap.

next slide:

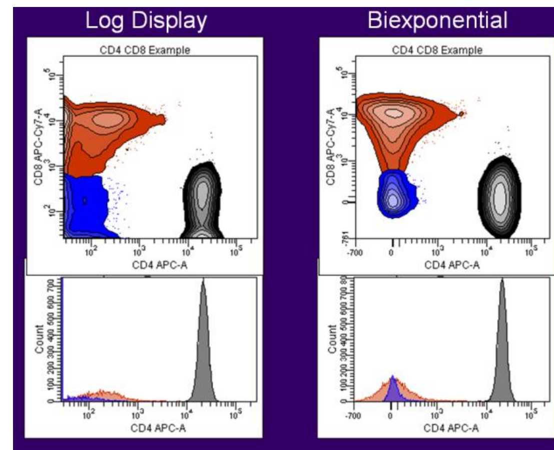
- Gain (PMT, CMOS, CCD) settings
- Data Display
- Controls

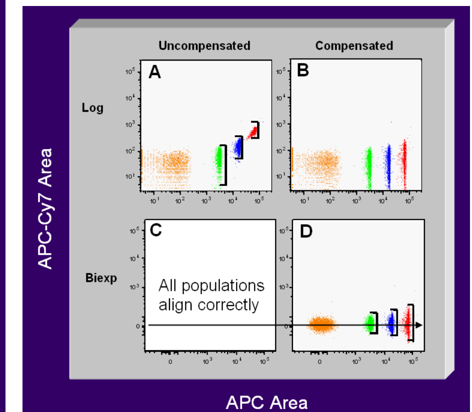
Optimizing cytometry measurements (II)

- Gain (PMT, CMOS, CCD) settings

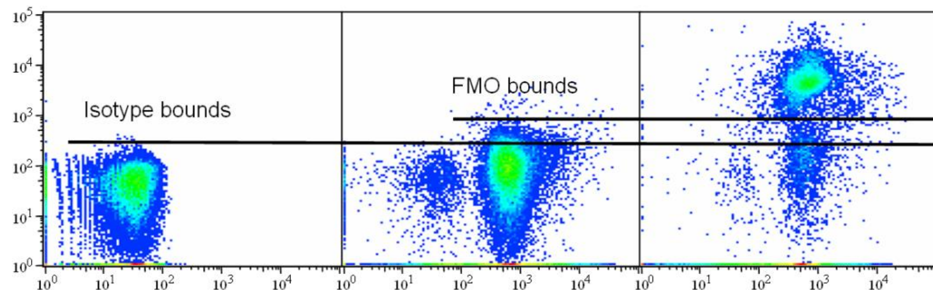


- Data Display

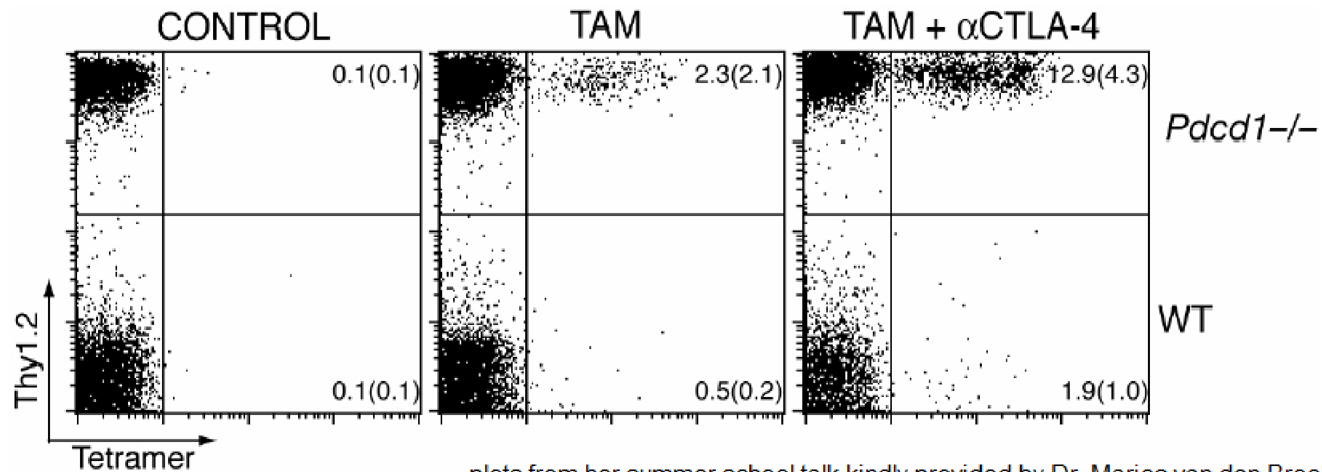




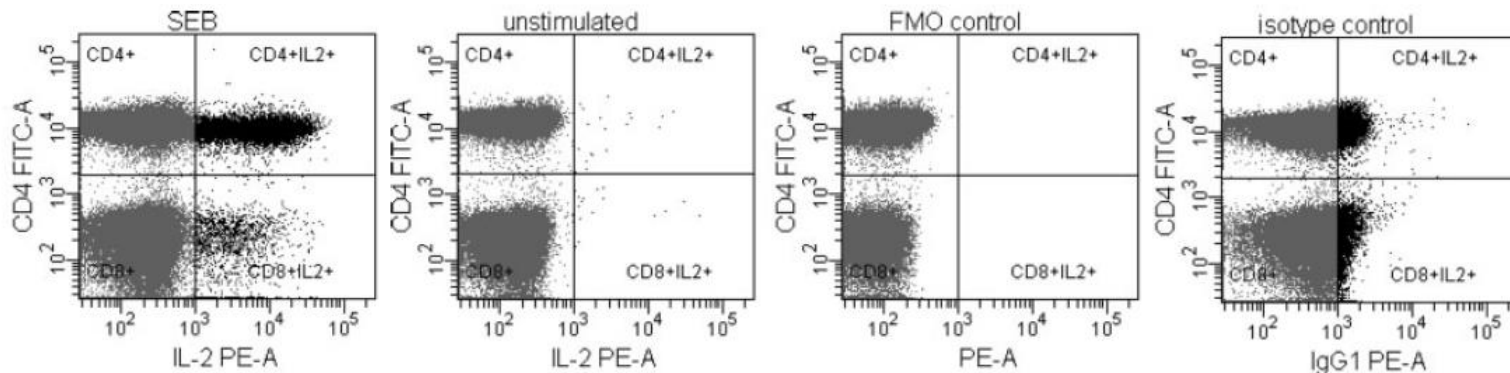
- Controls



Setting Gates and Markers

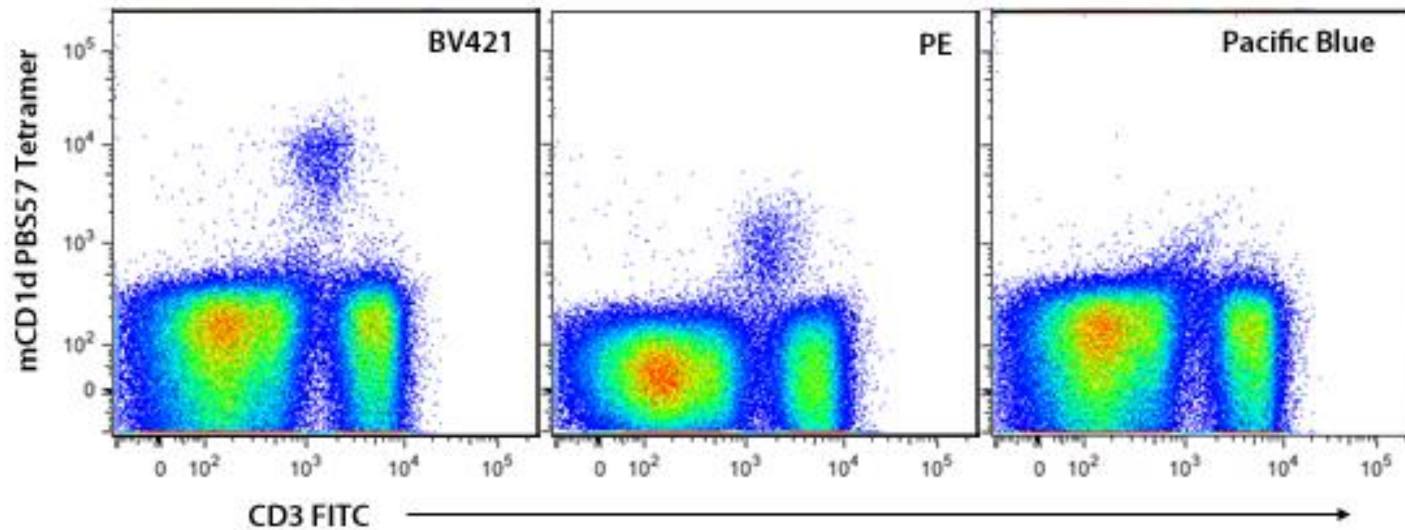


plots from her summer school talk kindly provided by Dr. Maries van den Broek



Adapted from: Maecker HT 2006, Cytometry 69A, 1037ff

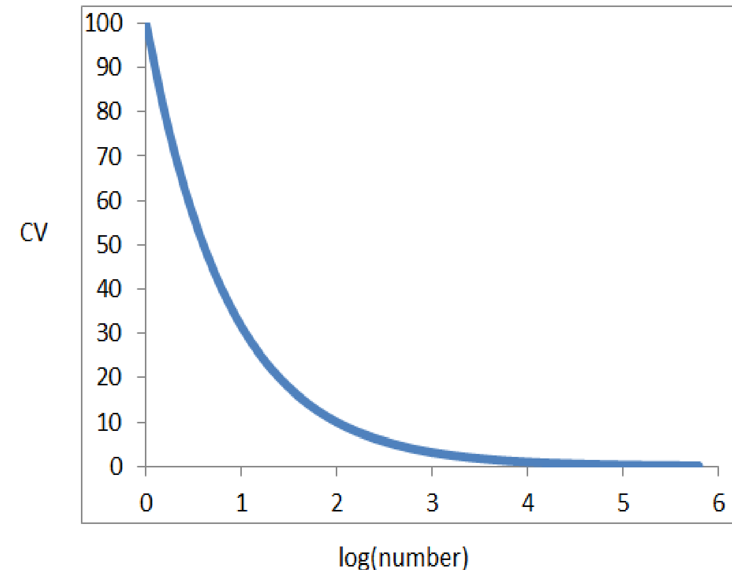
Use of Brighter Labels



http://www.biolegend.com/brilliantviolet

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

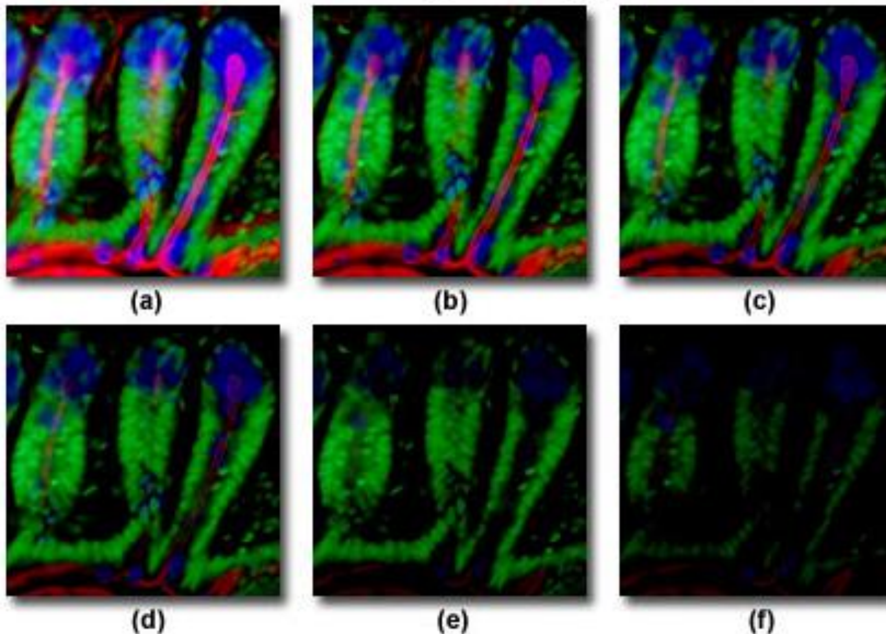
Multi-parameter Fluorescence Cytometry Summary

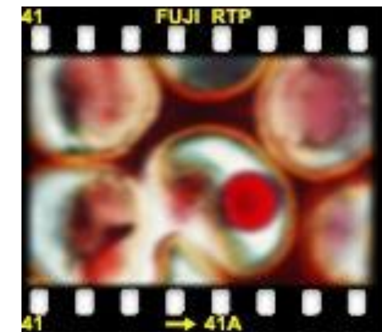
- Know your instrument status e.g. Qr & Br for different channels
- Use high enough gain settings to maximize sensitivity
- An antibody/dye combination that marginally allows discrimination of positives/negatives in a single color assay is unlikely to contribute anything helpful in a multicolor experiment (adequate use of dim and bright labels).
- Avoid spillover from bright cell populations into channels requiring high sensitivity
- Beware of tandem dye degradation
- Internal controls are essential
- Use a gate and marker approach consistent with your experiment objective
- Keep counting statistics limitations in mind

Quantitative Multi-color Microscopy (I)

Additional factors

- Field to field focus
- Photobleaching





Out of Focus

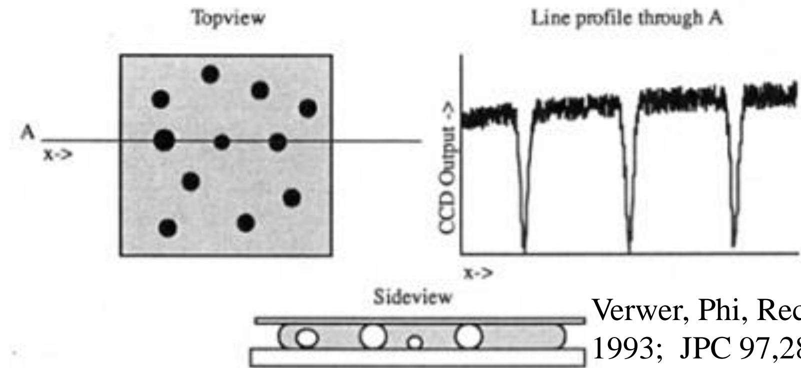
Images from

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

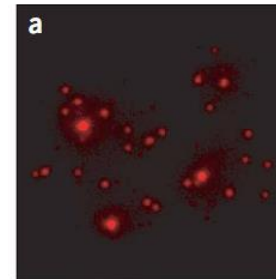
Quantitative Multi-Parameter Microscopy (II)

Selected capabilities

- Intensity calibration by volume exclusion
- Single molecule observation
- Low complexity, low resolution cytometry
  (Shapiro H, “Cellular Astronomy”)



Verwer, Phi, Recktenwald 1993; JPC 97,2868-70



Single m-RNA molecule analysis. Robert H Singer’s group, Nature S&MB 2008

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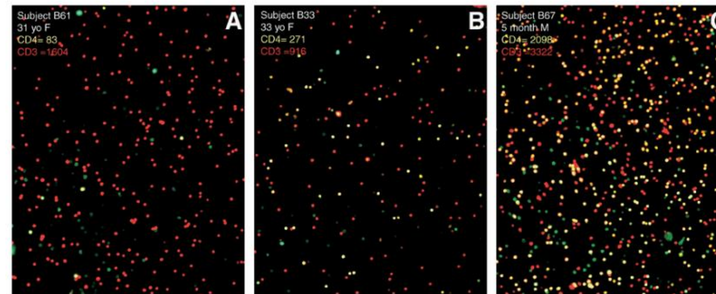
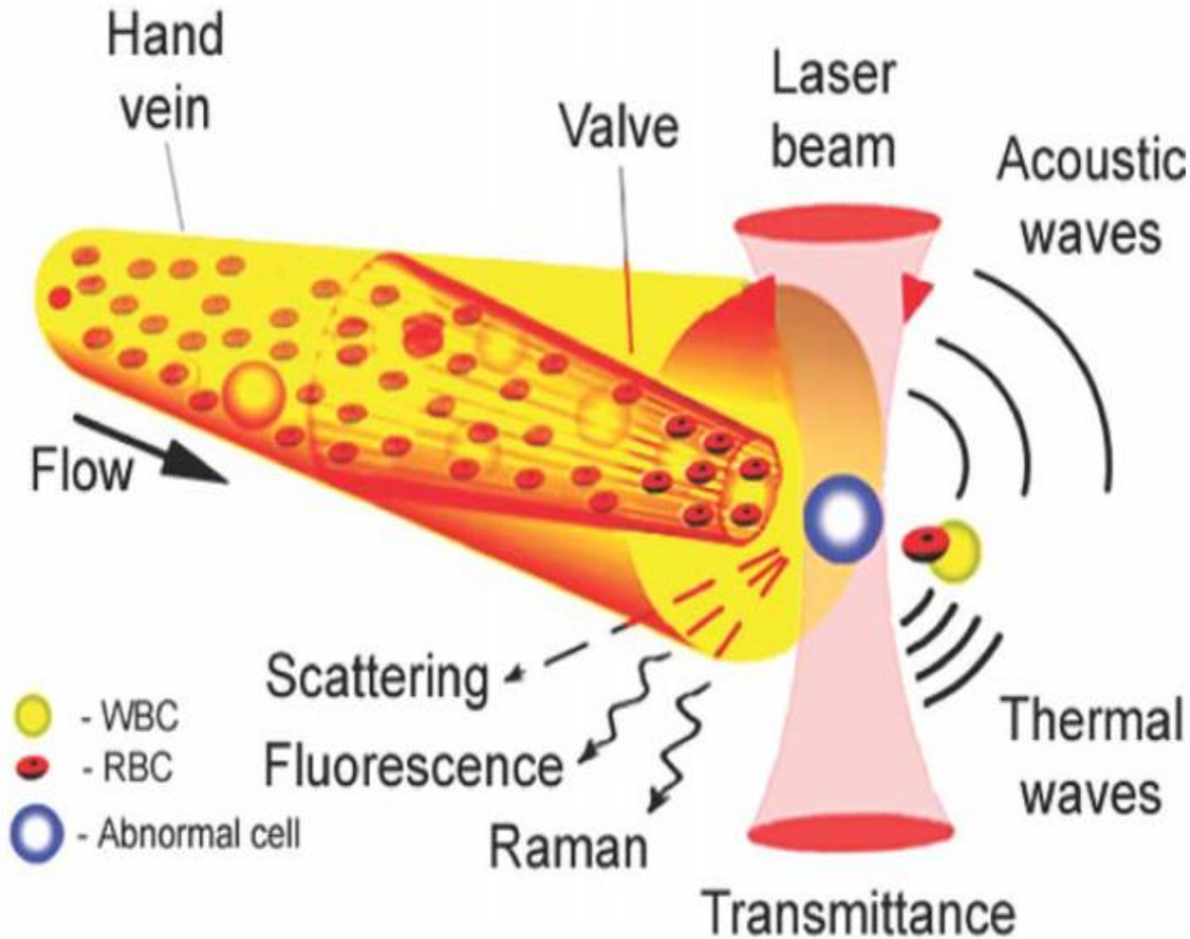


Rodriguez WR, McDevitt JT, PLos Medicine 2005; (CD3+CD4+ yellow, CD3+CD8+ red, monocytes green)

In-vivo Multi-parameter Cytometry

Single cell analysis in living animals



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

Issues:

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

Intravital Microscopy

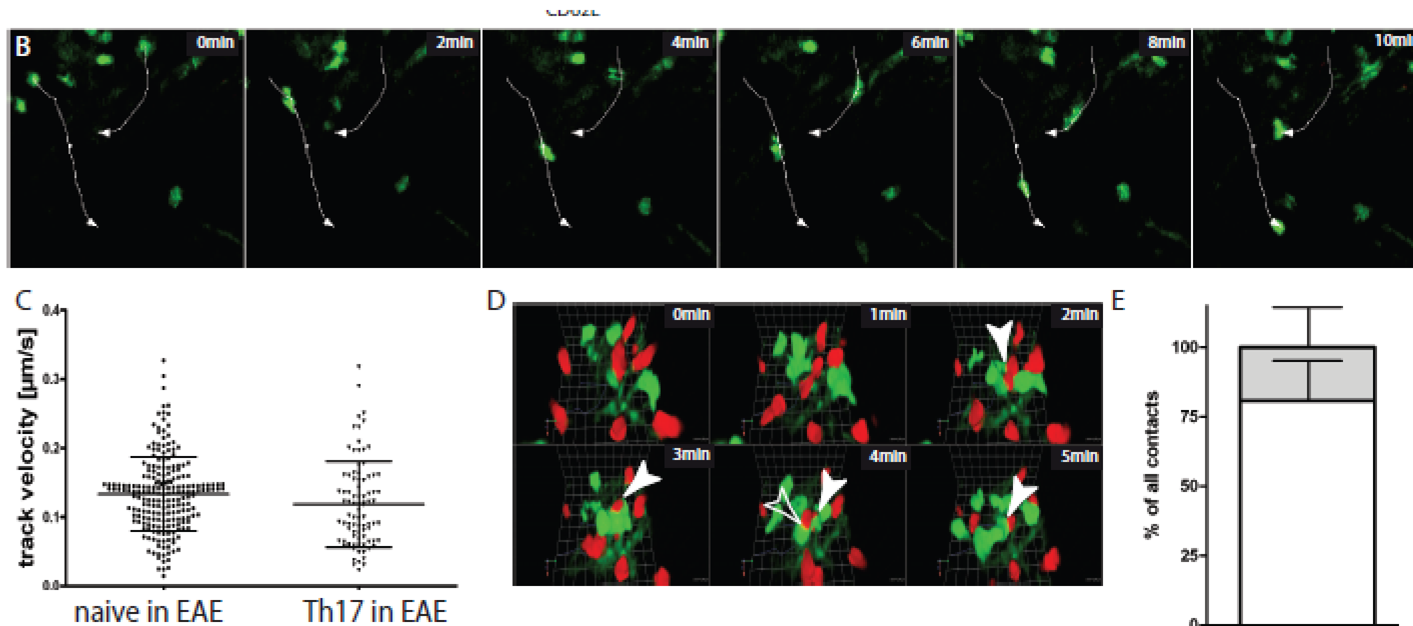


Figure 1 Intravital imaging reveals high motility of naïve CD4+ T cells in the inflamed CNS. Naïve OT2 EGFP T cells were intravenously injected into EAE affected mice at the peak of disease (clinical score 2.5) or locally applied onto the imaging field. Intravital TPLSM on the brain stem of these mice was performed 12 - 24 hours after naïve T cell injection or 30 minutes after local application. Adoptive EAE was induced by transfer of *in vitro* generated encephalitogenic 2d2 Th17 T cells into *C57BL/6 RAG1-/-* mice. **(A)** T cell phenotype of MACS isolated naïve T cells was confirmed by FACS analysis prior to experiments. Surface antigen expression of CD62L, CD25, CD69 and CD44 was determined on CD4+ lymphocytes. **(B)** A representative time lapse series derived from intravital TPLSM demonstrates rapid movement of naïve T cells deep in CNS tissue (100-150 µm). Two cell tracks are shown exemplarily by white arrows. For further details see also Additional File 1 (Scale bar: 10 µm.). **(C)** Cell track velocities of naïve OT2 (N = 212) and effector 2d2 Th17 (N = 87) cells at the peak of disease in the inflamed CNS were quantified. The mean track velocities from 4 independent experiments are shown (± SD). **(D)** Contacts (arrowheads) between encephalitogenic 2d2 Th17 effector T cells (EGFP,green) and naïve OT2 (tdRFP red) could be observed during intravital TPLSM, as revealed by the reconstructed 3D time lapse series (80-110 µm). These interactions were mainly short and random like (open arrowhead) although some static long-lasting contacts (filled arrowhead) could be also detected. **(E)** To quantify effector-naïve T cell interactions we analyse the co-localisation area of EGFP and tdRFP as previously described [19]. We discriminated short (random) contacts (< 5 min) from long-lasting (most probably non-random) interactions (≥ 5 min) and observed that 81% ± 14 formed short interactions with effector T cells (white bar) and 19% ± 14 (gray bar) formed long-lasting interactions. Data are shwon as percentage of all contacts from two independent experiments (± SD).

Herz *et al. Journal of Neuroinflammation* 2011, **8**:131
http://www.jneuroinflammation.com/content/8/1/131

Conclusions

Multi-parameter cytometry

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

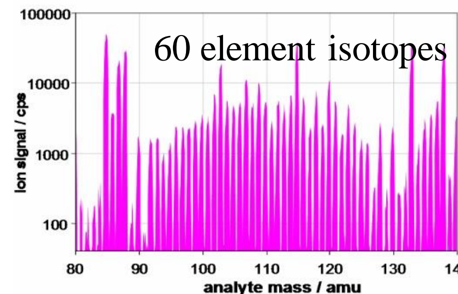
New developments in many areas have simplified the tools for the biologist.

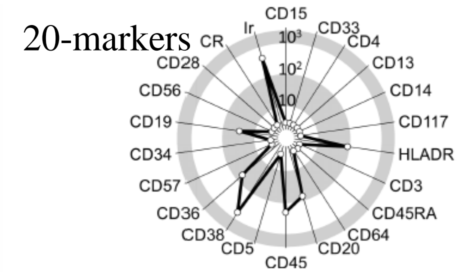
Evolving Technologies for Cytometry

- Multi-parameter cytometry approaches
- High speed imaging in flow
- Single cell sorting
- Low complexity cytometers for cell (subset) counting
- “Label-free” cell analysis
- Sample preparation micro-fluidics
- Fluorescent polymers as labels for high sensitivity
- Affinity reagents (antibodies)

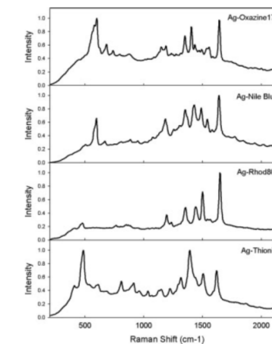
New Developments for in-vitro Multi-parameter Cytometry

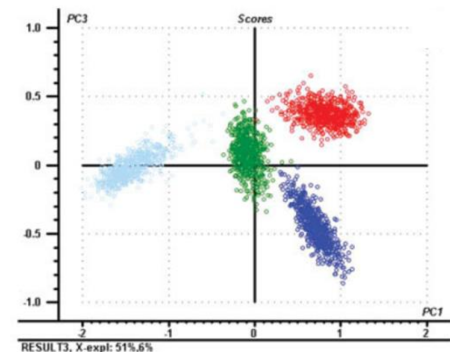
- Element-Label Flow Cytometry (CyTOF, addresses fluorescence spectral overlap issue by using elements as labels, Anal. Chem., 2009, 81 (16), pp 6813–6822)
- Sequential Stain De-stain Cytometry (Cytometry, 2009, 75A(4), pp 362-370)
- SERS-Label Flow Cytometry (uses spectral fine-structure to distinguish labels, Cytometry, 2008, 73A(2), pp 119-128)
- SONY spectral analysis



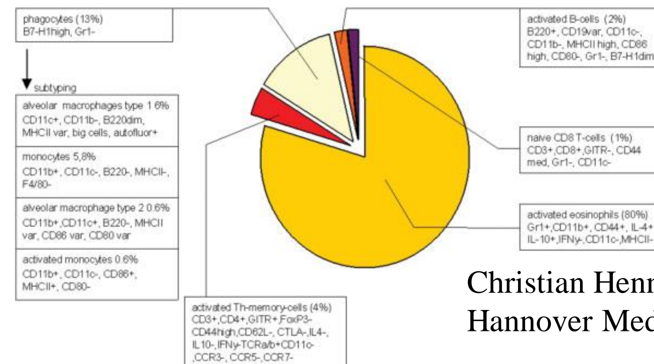


Scott Tanner, DVS Sciences Inc





John Nolan, La Jolla Bioengineering Institute

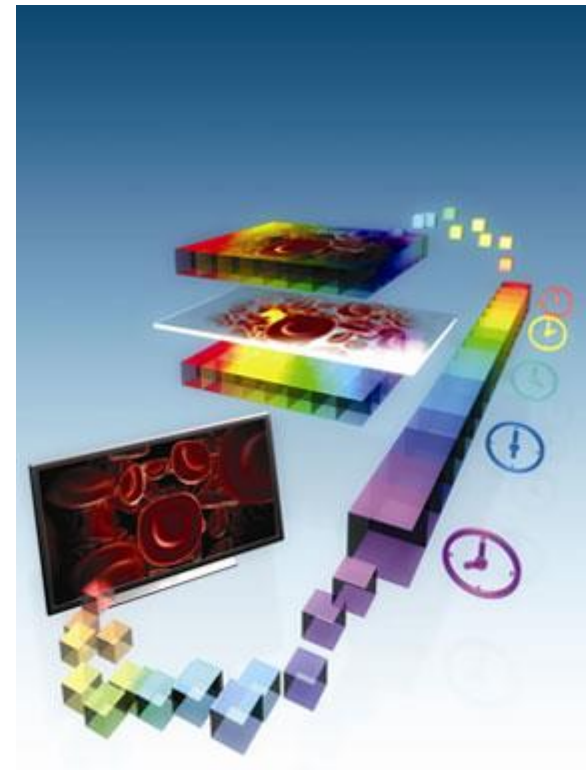


31-marker analysis

Christian Hennig, ChipCytometry
Hannover Medical School

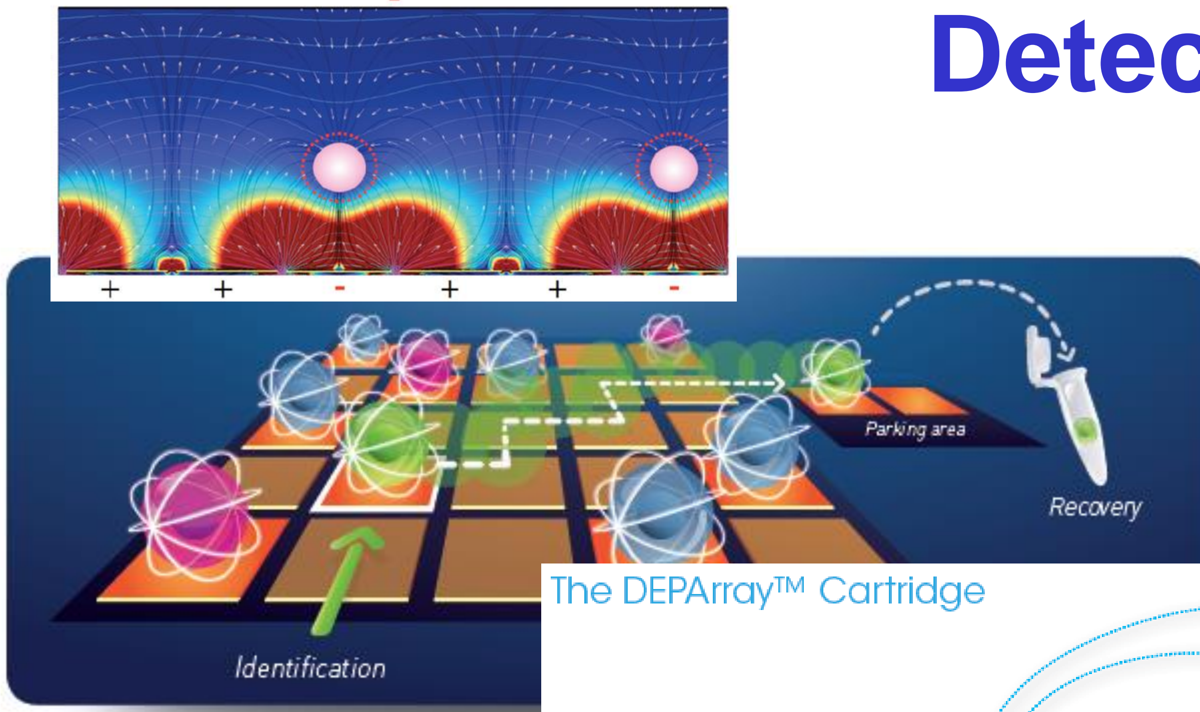
High speed imaging in flow

- ImageStream (EM Merck)
- Bahram Jalali group, UCLA
- …

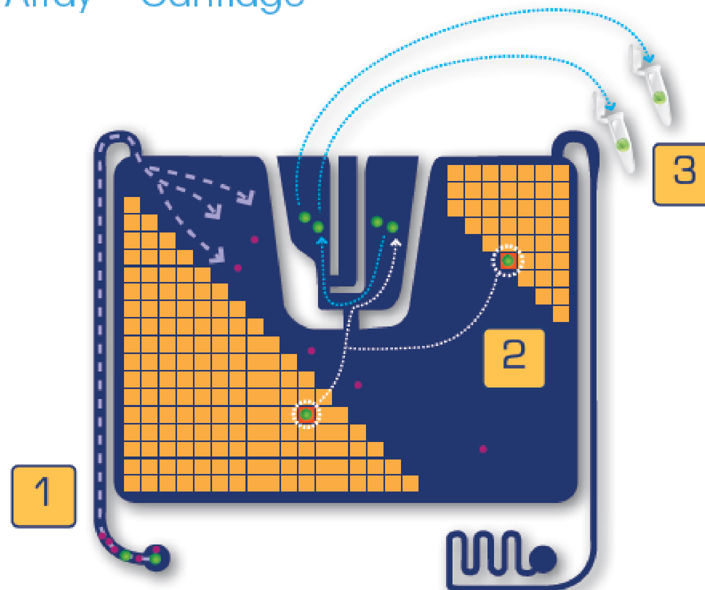


http://www1.ee.ucla.edu/Research-highlights-jalali-4.htm

Single Cell Sorter with Microscopic Detection



Cell movement with dielectric forces.
DEPArray
Silicon Biosystems, Bologna, IT



1 Inject, trap and image cells

2 Move cells of interest into parking chamber

3 Move individual or multiple cells into recovery chamber and flush

Cells are transferred to a special slide with 40,000 “cages”. Cells of interest are identified by fluorescence microscopy and sorted by the instrument.

Advanced Single Cell Analysis in Droplets

Generate | Co-Flow | Merge | Split

Mix | Timing | Thermal Zones

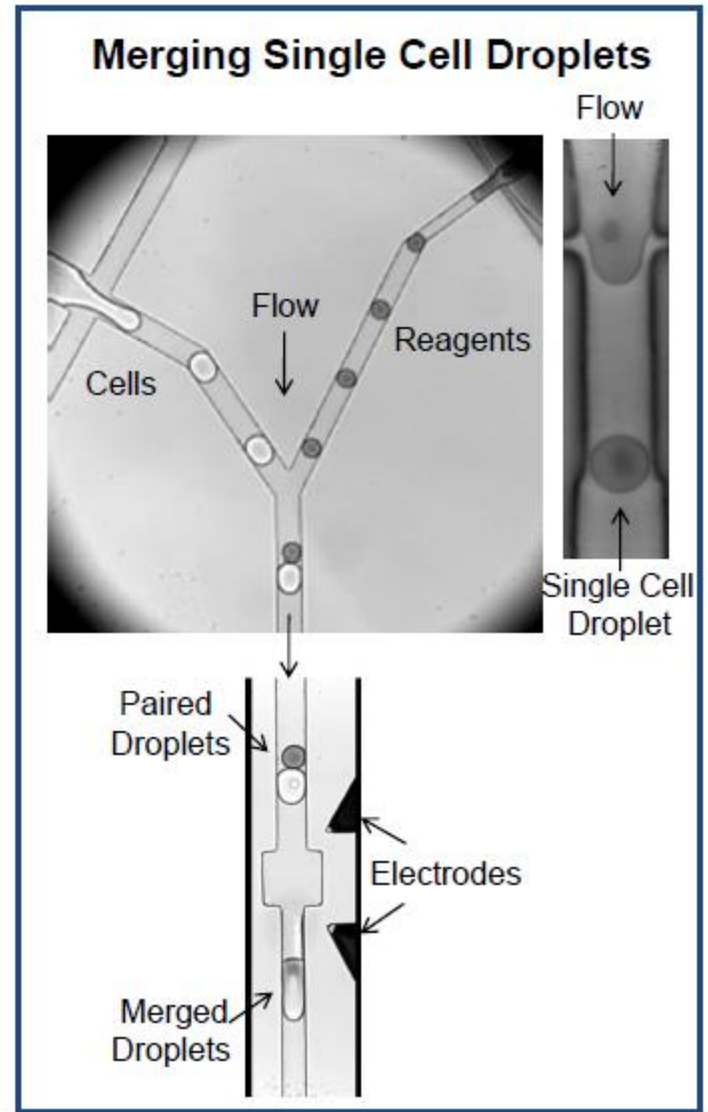
Detect | Sort | Collect | Library Introduction

sorting electrode

keep

laser focus

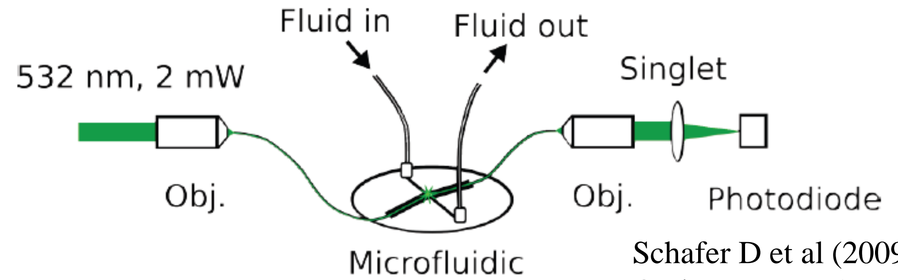
waste



Source: RainDance Technologies

Low-complexity Cytometers for Cell Counting

Low-end cell counters

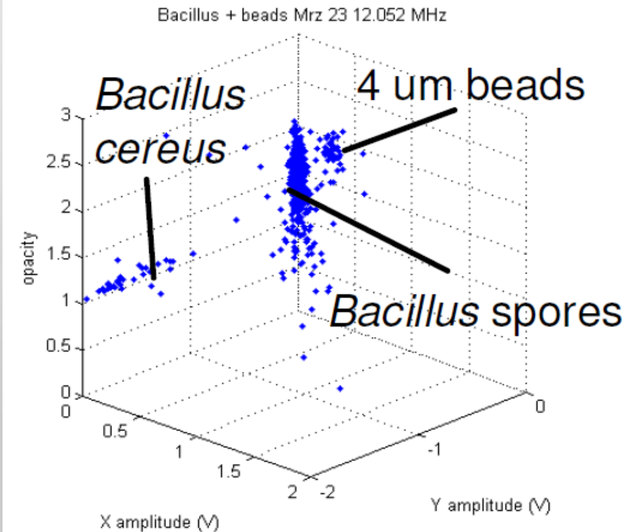


Schafer D et al (2009)
Optics Express 17, 6068ff

Merck Millipore MUSE™

Label-free Cell Analysis

LEISTER : Axetries Impedance flow cytometry



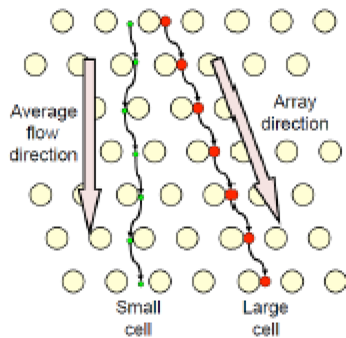
Marco DiBeradino, Leister Axetris

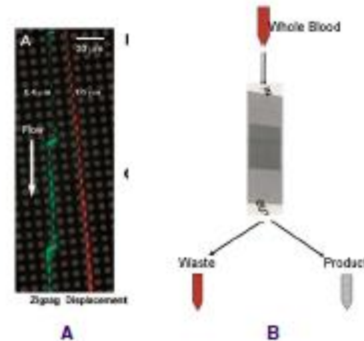
Electrical parameters of living cells (no label required).

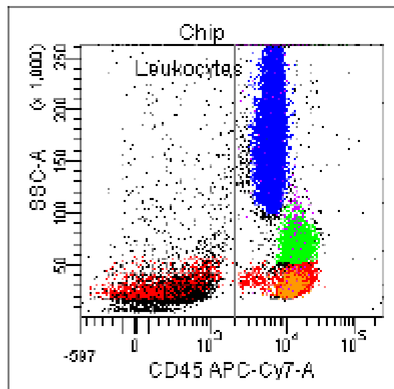
Other parameters: fluorescence polarization, fluorescence lifetime, compressibility, …

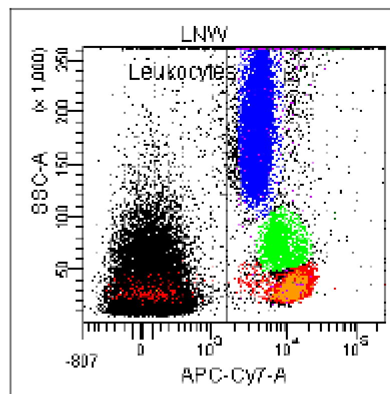
Innovative Sample Preparation

Microfluidic system for leukocyte isolation (deterministic lateral displacement)



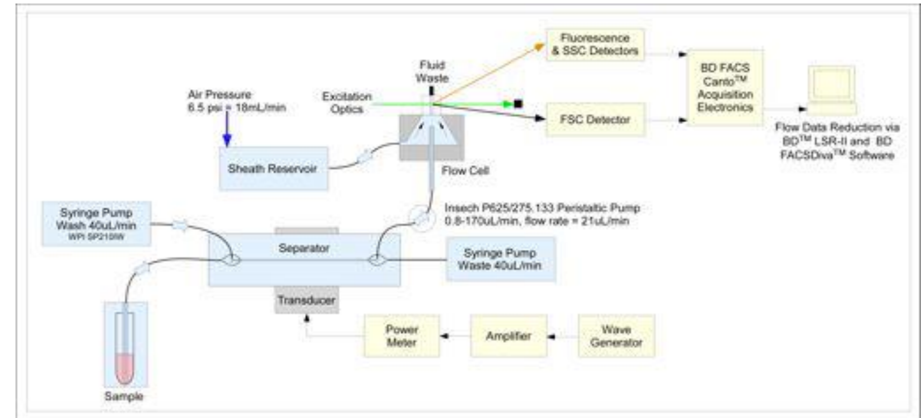




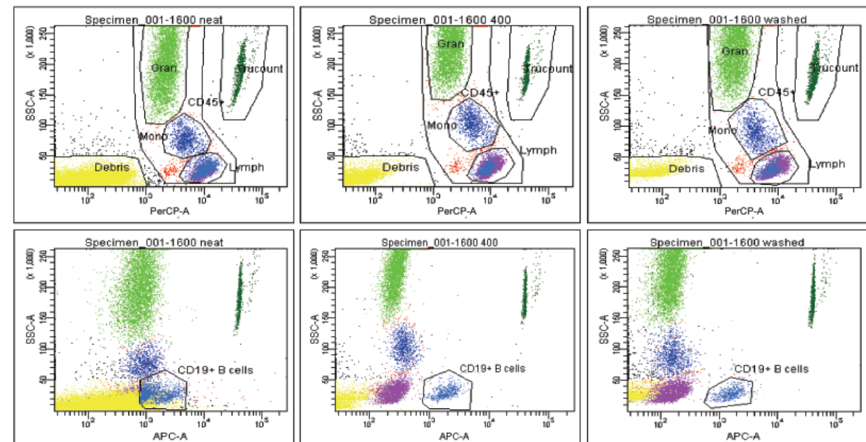


Cyto 2012 poster, Liping Yu et al, GPB and BD Biosciences

Acoustic particle focusing for cell washing



Lyse no wash Chip wash Centr. wash

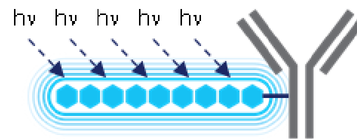


2010, Laurell group, Lund University & Brian Warner, BD Biosciences

Bright Fluorescent Polymer Dyes

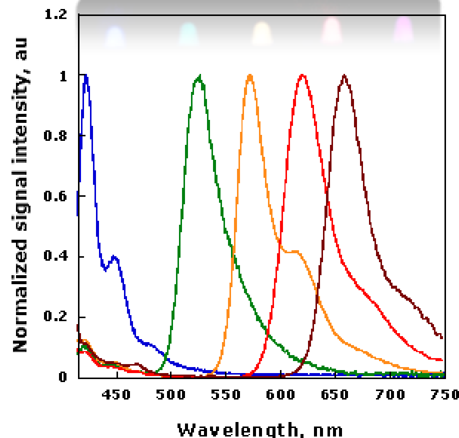
Polymer Based Fluorochromes

- 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



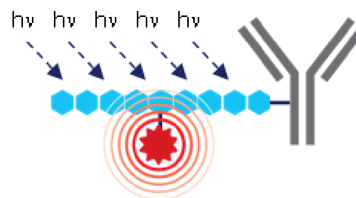
Brilliant Violet 421™

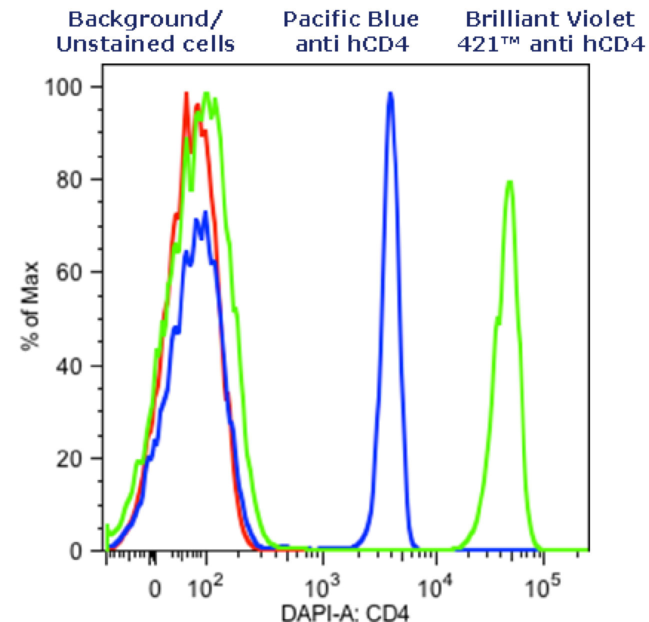
- PE level performance w/ 405nm Laser
- >10x the Stain Index of Pacific Blue
- Enables detection of low abundance targets in multicolor assay panels (e.g. CD56, CD127, etc.)
- Wide range of Ab clones validated



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





http://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 the physical sciences provide tools for deeper molecular understanding of living systems.

Acknowledgements

- Joe Trotter
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- Liping Yu
- Mike Brasch
- Ben Verwer

  above all BD

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

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