Cytometry
Past, Present, and Future

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Outline

- History
- Flow Cytometry and Imaging Principles
- Important applications
- New developments
- Opportunities for Acoustic Science
- Outlook
- Summary and Conclusions

History of Cytometry Technologies (Microscopy)

- 1665 – English physicist, Robert Hooke used a microscope lens to observe “pores” in cork
- 1674 – Anton van Leeuwenhoek built a simple microscope with only one lens to examine blood cells
- 1872 – Ernst Abbe calculated the maximum resolution in microscopes
- 1932 – Frits Zernike invented the phase-contrast microscope (label-free observations)
- 1969 – Willard Boyle and George E. Smith at Bell laboratories invented the CCD
- 1971 – Intel launches 4-bit 4004 microprocessor

History of Cytometry Technologies (Flow Cytometry)

1968 1st fluorescence-based flow cytometry device (ICP 11) by Prof. Göhde from the University of Münster, Germany, and first commercialized in 1968/69 by German developer and manufacturer Partec through Phywe AG in Göttingen.

1971 Cytofluorograph, Ortho

1973 PAS 8000, Partec

1974 1st FACS instrument, BD

1977 Epics Instrument,Coulter

2002 Microfluidic Cytometer, Quake, Caltech

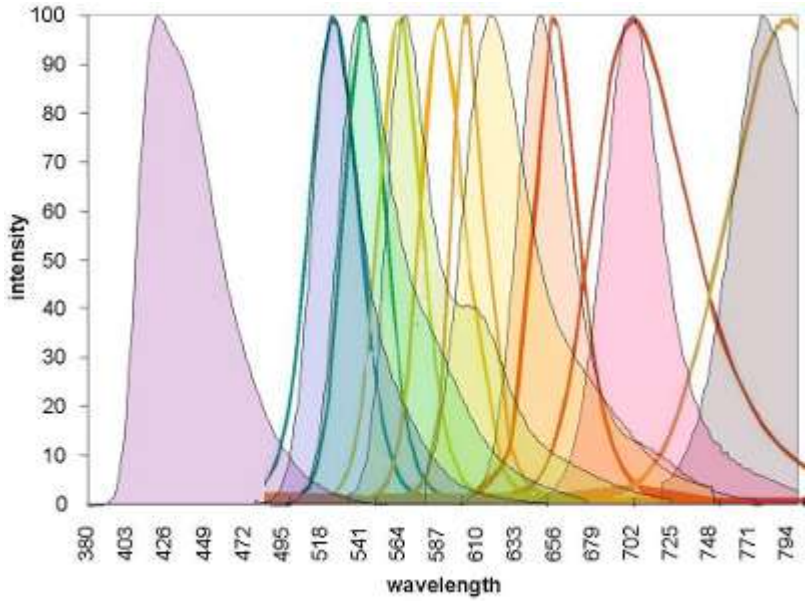
Flow and Imaging Cytometry Features

Single cell analysis with

• High sensitivity (single molecule sensitivity by fluorescence)	I,F
• Wide dynamic range (10^3 to 10^7 cells mL^{-1})	F
• High analysis rates to ~10^5 particles sec^{-1}	F
• Light scatter	F
• Direct size and 3D spatial information	I
• Multi-color fluorescence, 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)

Physical Parameters used for Cytometry

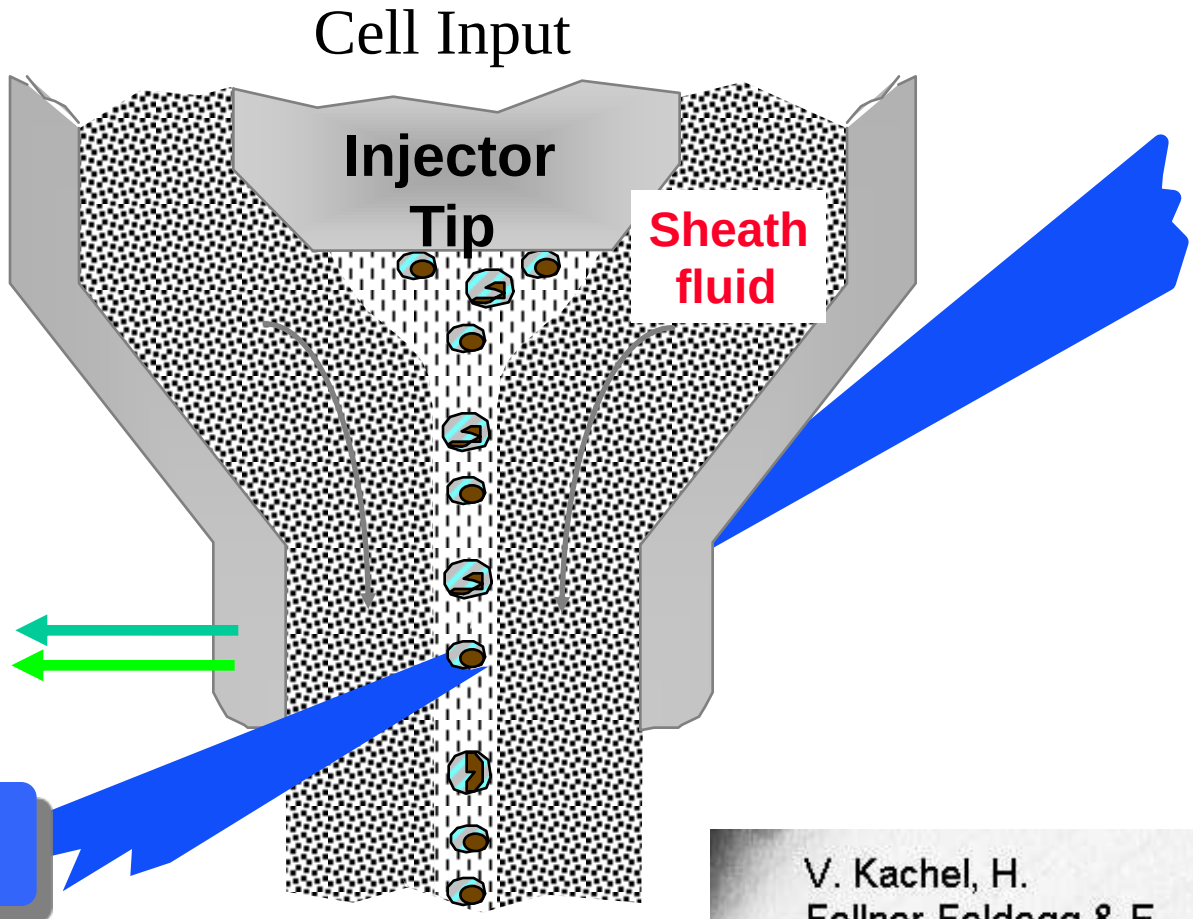
- Light scatter
- Absorbance
- Fluorescence
- Phosphorescence
- Raman
- Electrical properties
- Mechanical properties
- Element mass
- …

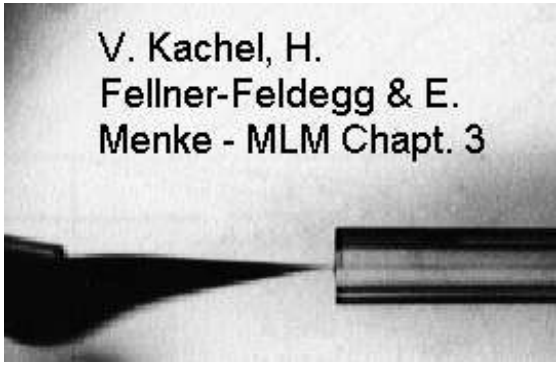


http://www.dvssciences.com/technical.html

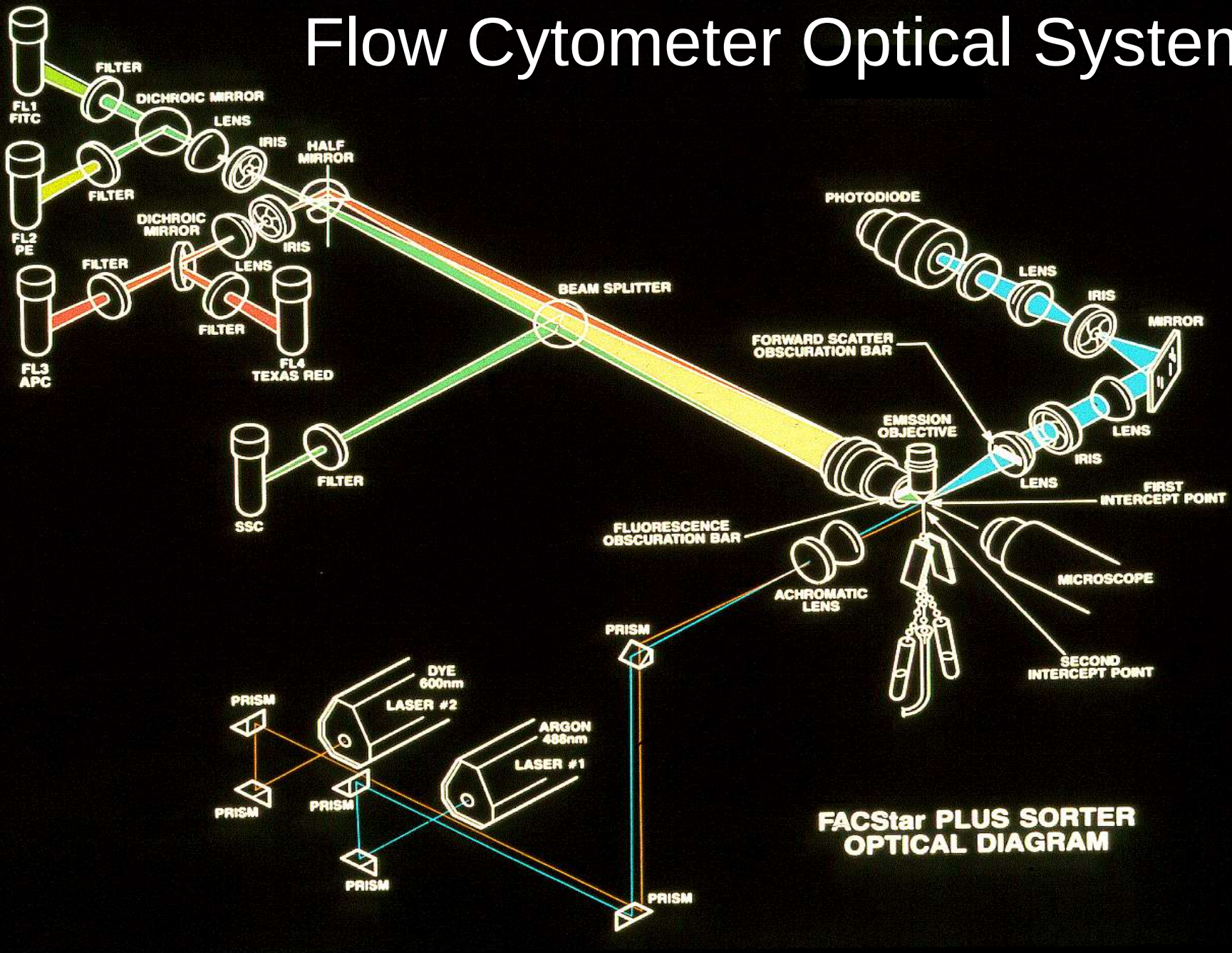
Flow Cytometer Fluidics

- Hydrodynamic focusing
- Acoustic focusing
- …

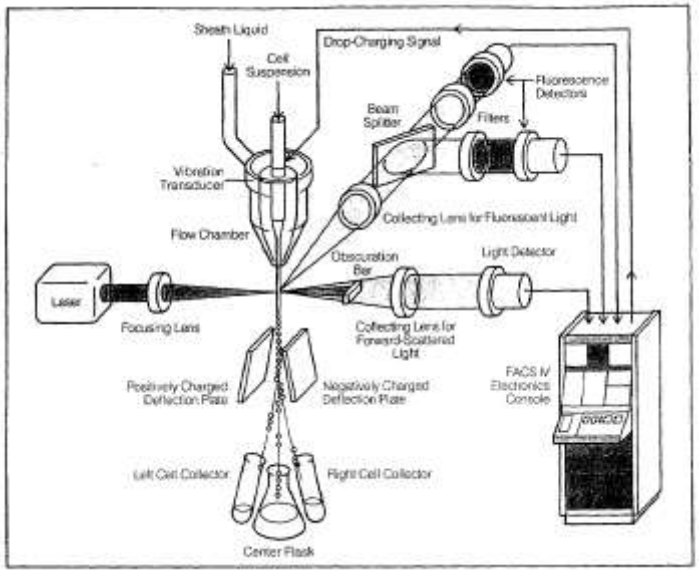


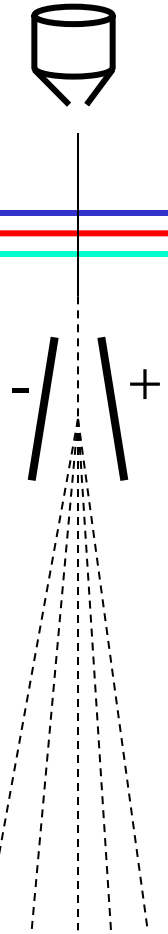


Flow Cytometer Optical System



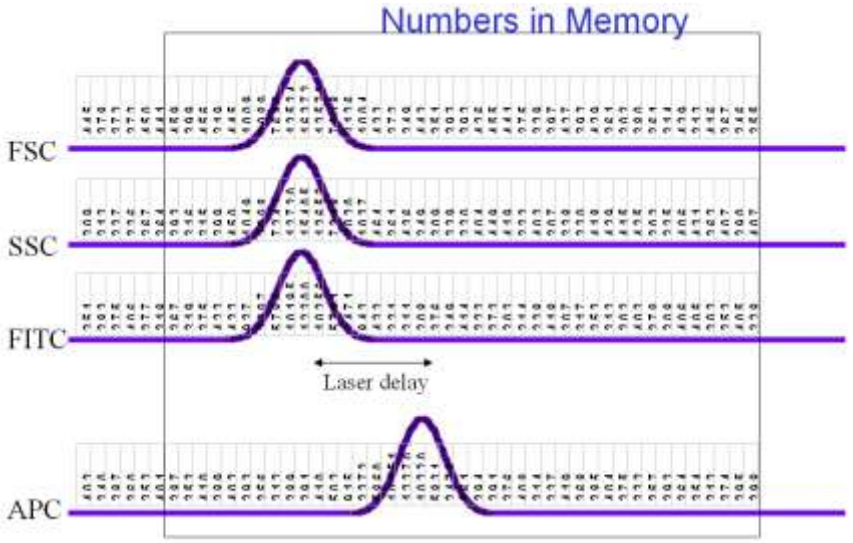
“Droplet-based” Sorting



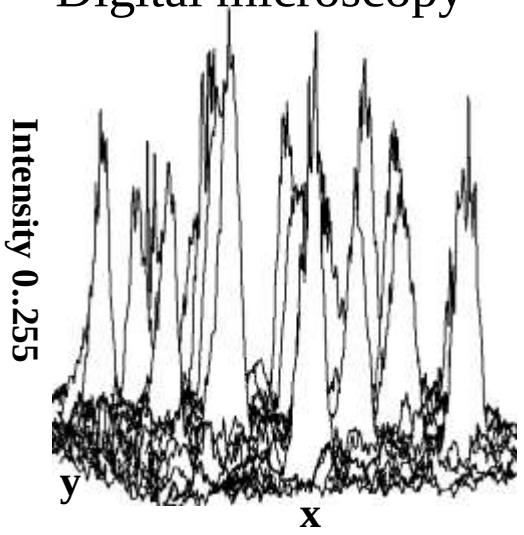


Basic Data Processing

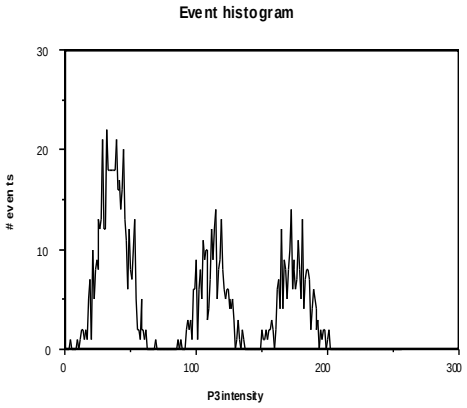
Flow Cytometry

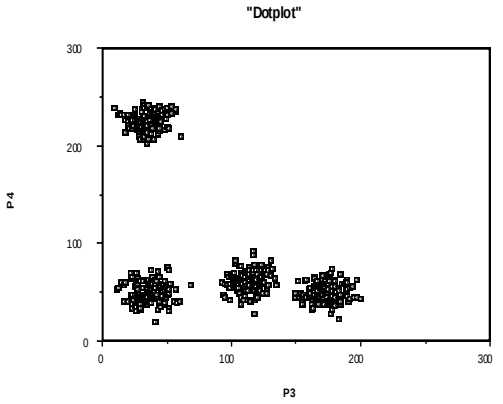


Digital microscopy



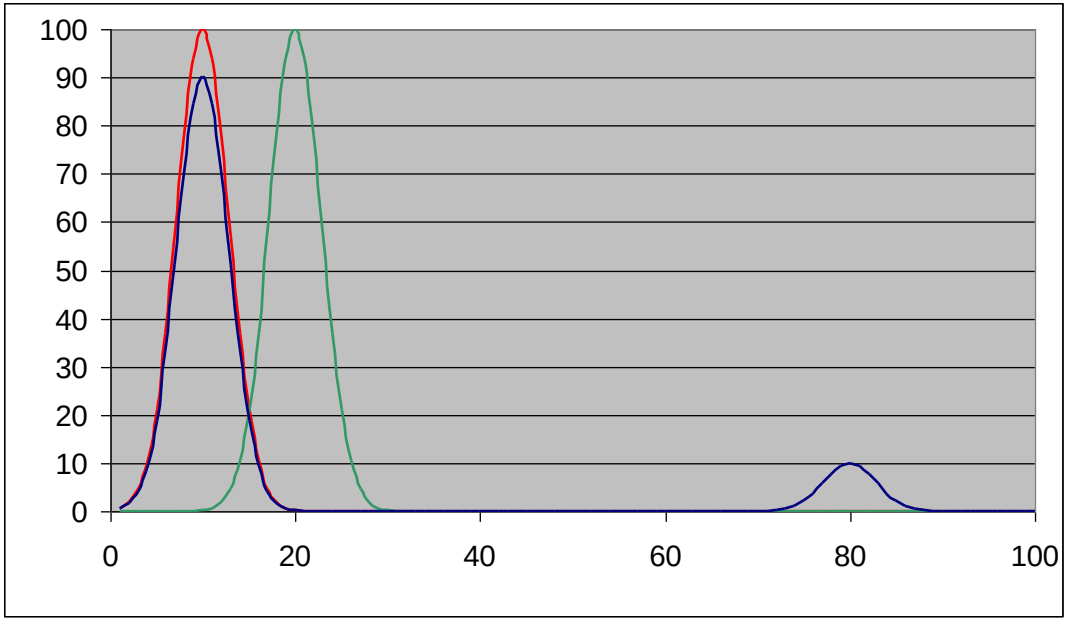
Cell	P1	P2	P3	P4	P5	Pop#
1	242	135	704	175	612	1
2	146	132	690	178	566	1
3	269	147	89	206	580	3
4	442	143	399	250	255	4
5	212	167	155	926	526	2
6	269	2	659	207	575	1
7	204	232	112	171	679	3
8	152	74	160	828	532	2
			…			
9997	215	119	138	936	662	2
9998	244	50	72	261	543	3
9999	214	137	174	1014	597	2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



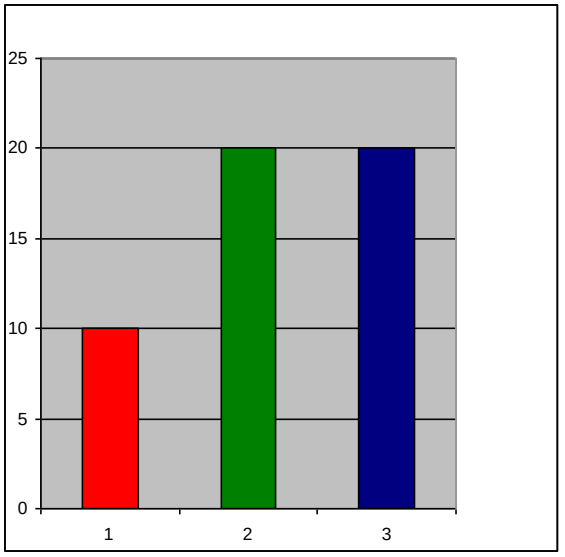


Single Cell Cytometry vs. Bulk Analysis

Intensity Histogram for Single Particles

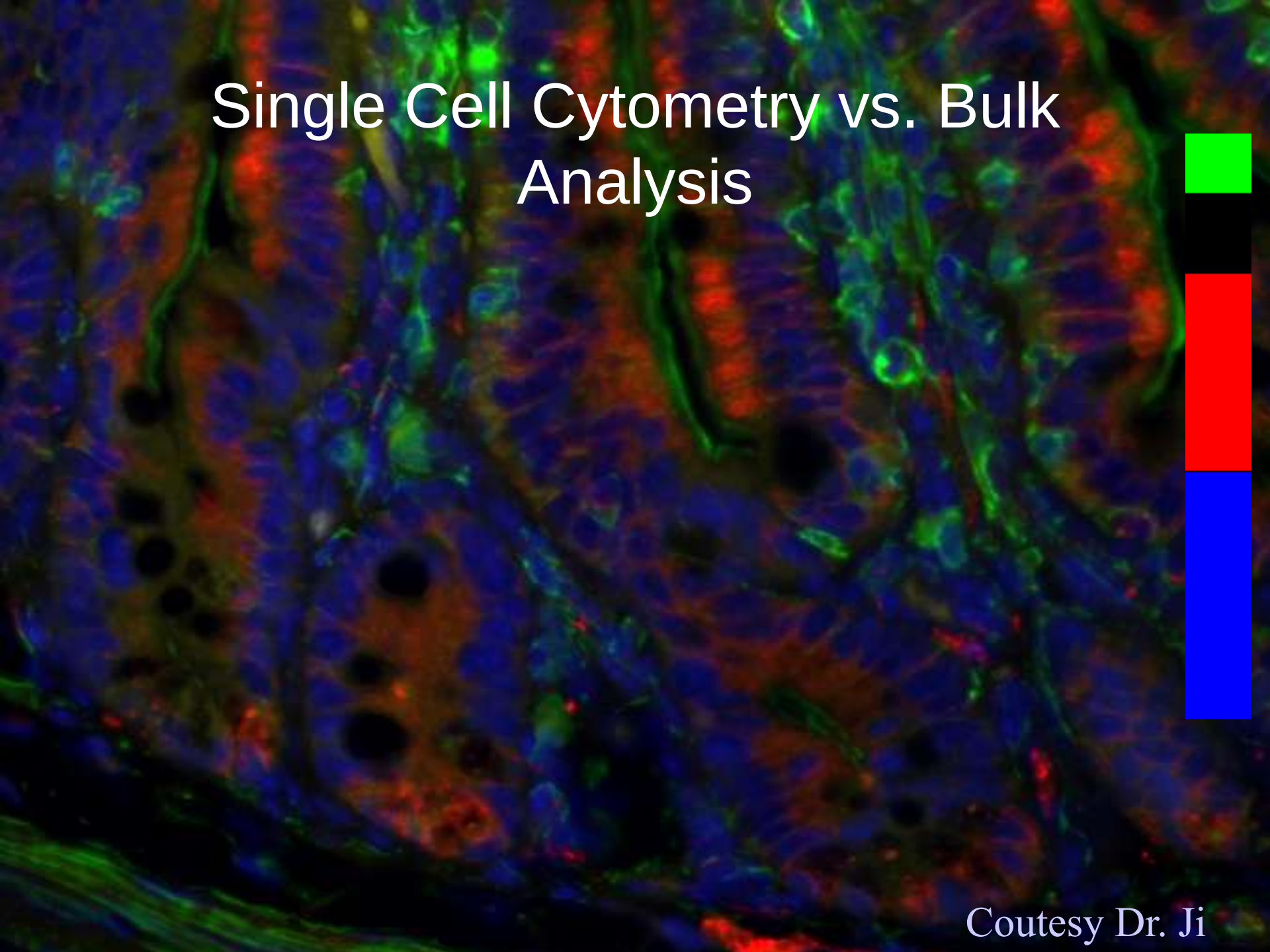


Intensity per Sample



Cell by cell intensity analysis detects population heterogeneity.

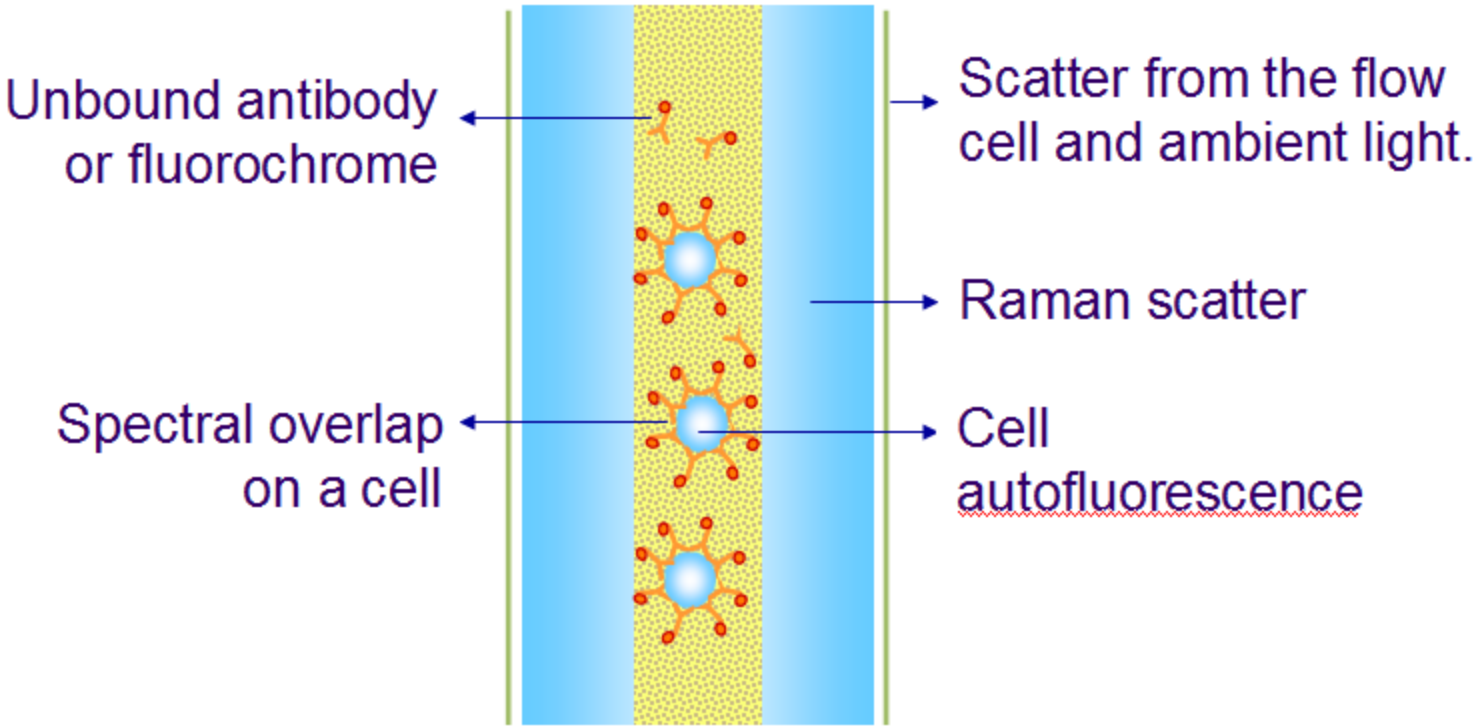
Single Cell Cytometry vs. Bulk Analysis



Coutesy Dr. Ji

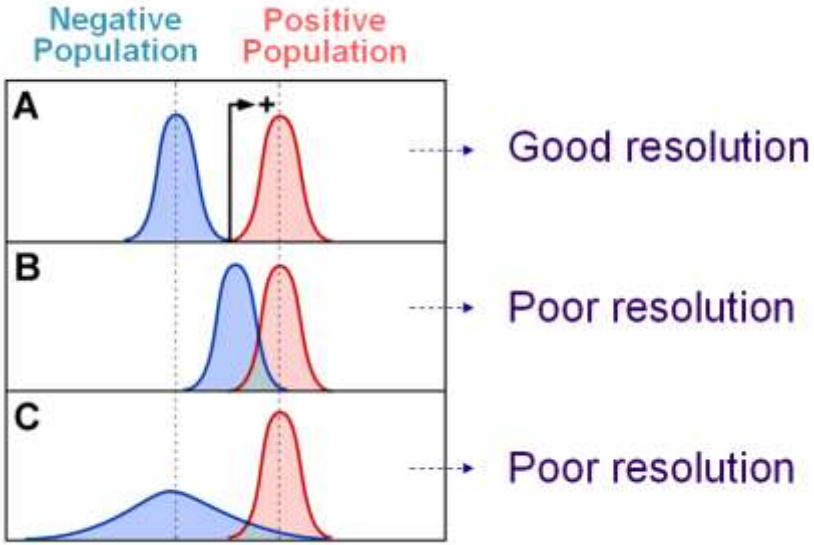
Instrument Evaluation Br

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



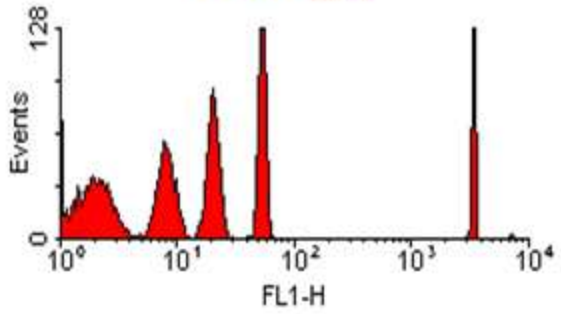
Source: Joseph Trotter

Instrument Evaluation Qr

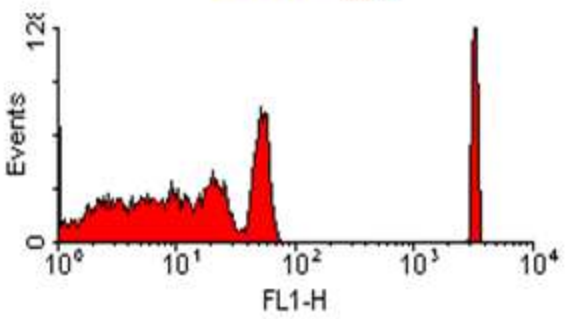


$$Qr = \frac{\#\ \text{photoelectrons}}{\#\ \text{fluorescence molecules}}$$

High Qr



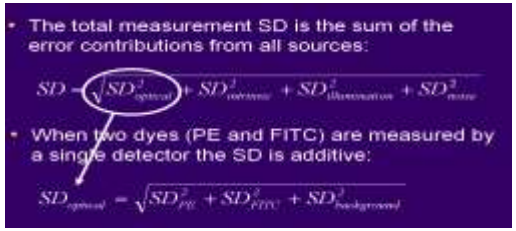
Low Qr



Source: Joseph Trotter

Optimizing cytometry measurements

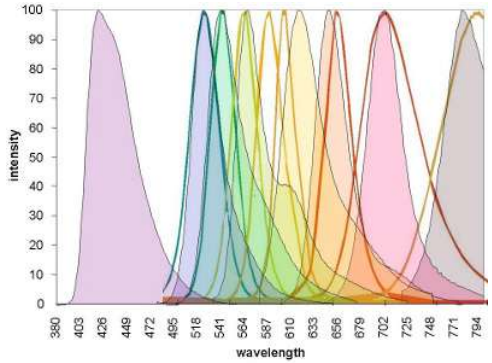
- Background light



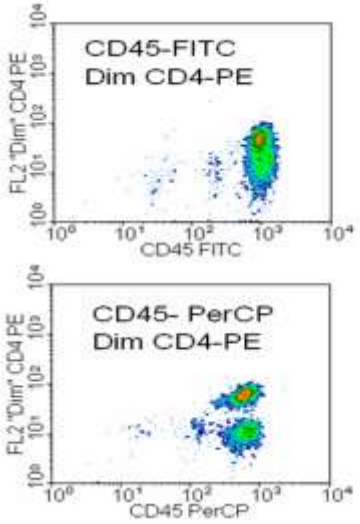
Reagent performance

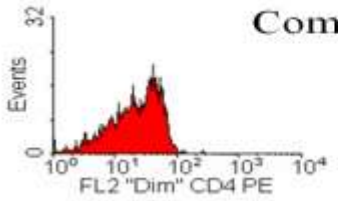
$$\text{Stain index} = \frac{Medium_{pos} - Medium_{neg}}{2 * SD_{neg}}$$

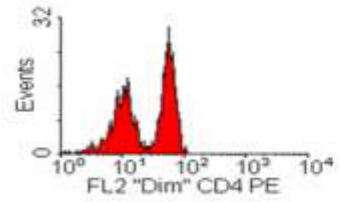
- Dye properties (brightness and spectral overlap)



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







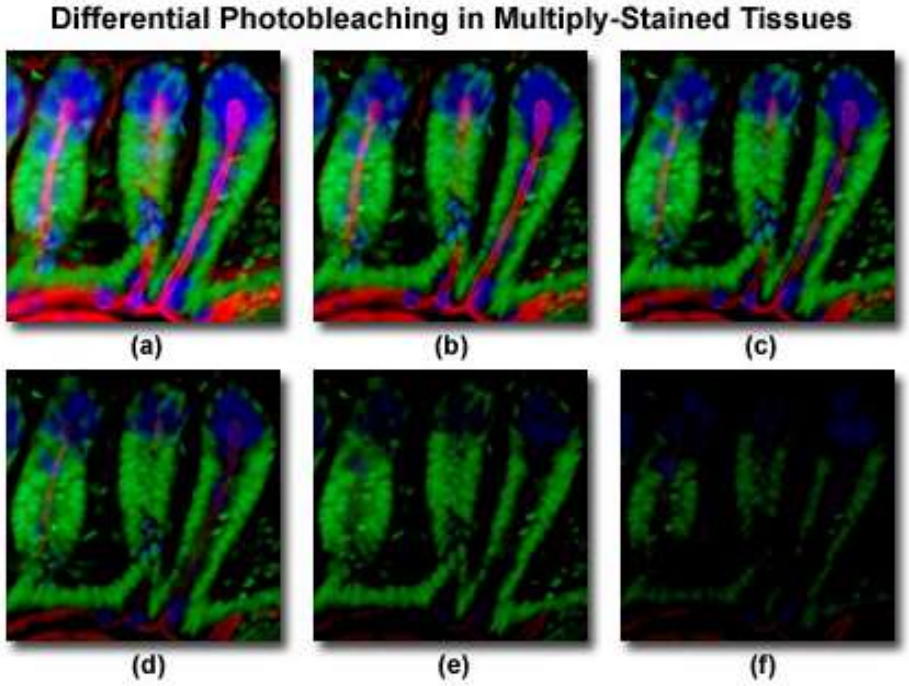
Better separation with less spectral overlap.

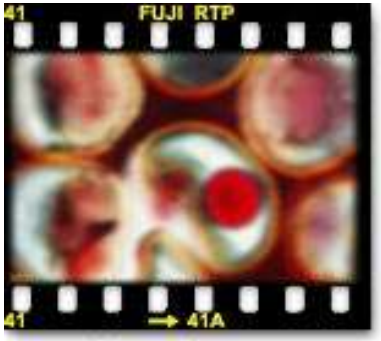
Source: Joseph Trotter

Quantitative Multi-color Microscopy

Additional factors

- Field to field focus
- Photobleaching





Out of Focus

Images from

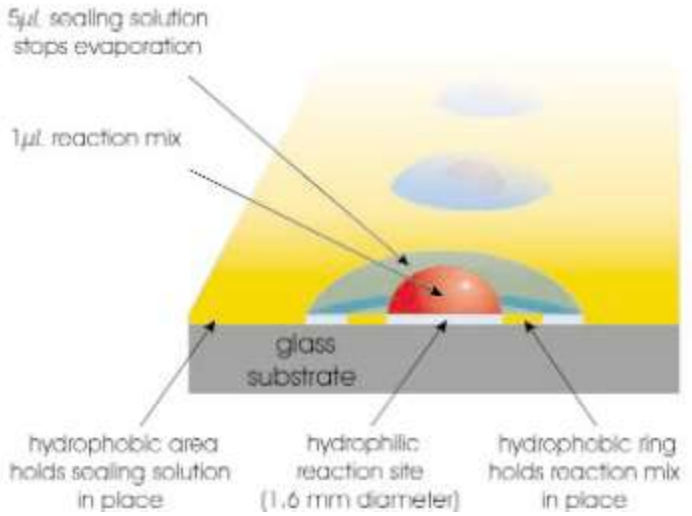
http://micro.magnet.fsu.edu/primer/index.html

Key Applications

- Immunology Research
- Stem Cell Biology
- Clinical Diagnostics
  - Immune status
  - Tumor Cell Cycle
- Cell Sorting
  - Single cell genomics
  - Cell population proteomics
  - Cloning for research and industrial biotechnology
- Marker quantitation
- Molecule counting
- In-vivo molecular analysis

Single Cell Sorting for PCR

FACS sorting of single cells onto a slide followed by automated miniaturized single cell PCR (Advalytix).

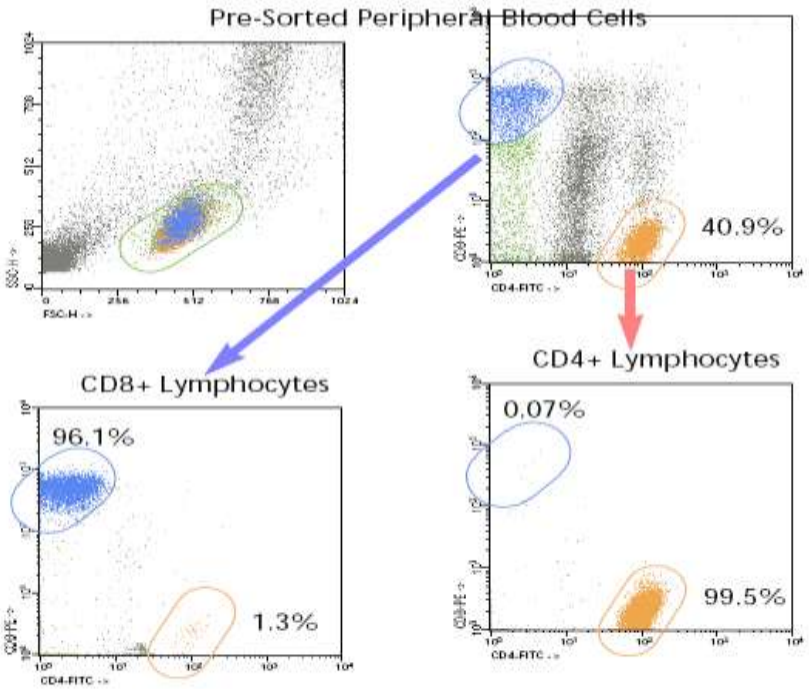


Source: Advalytix

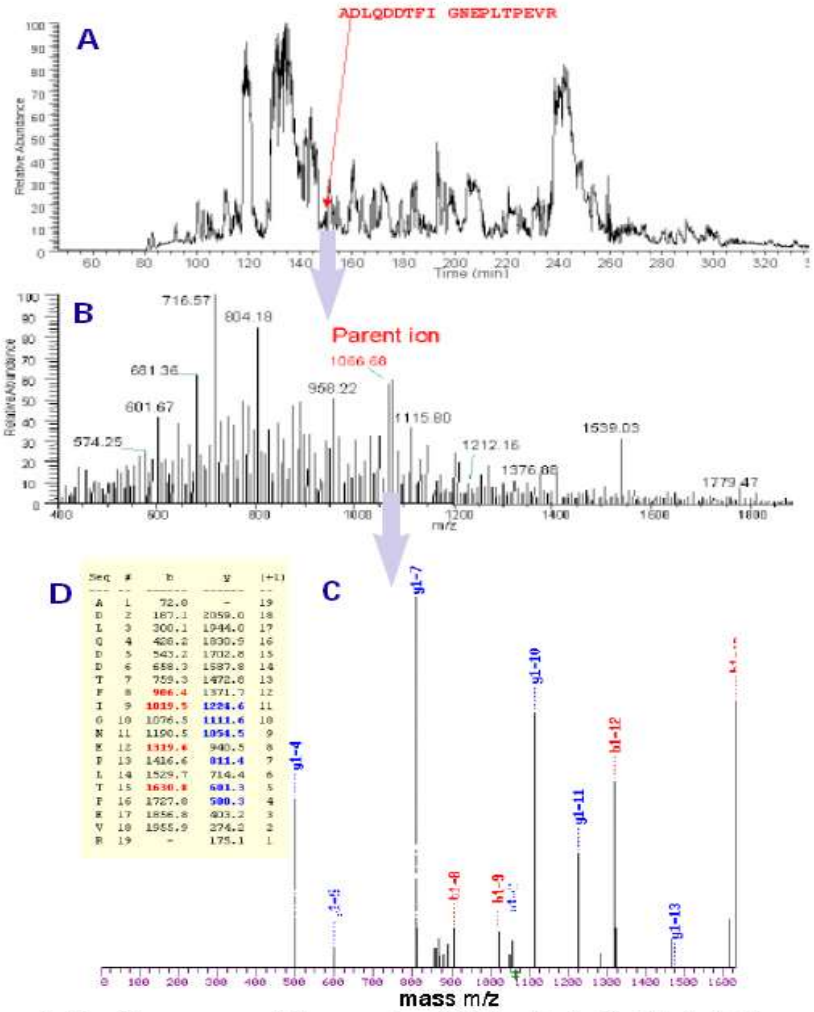
Sorting for Cell Surface Proteomics

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.



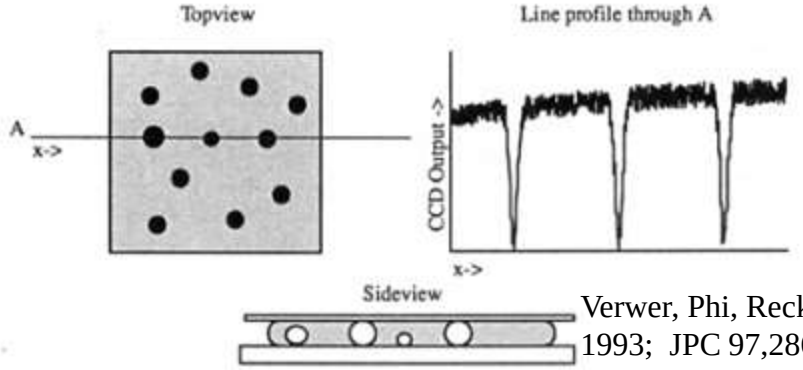
Peptide mixtures were separated by reverse phase HPLC **(A)** as described in Methods. Eluted peptides were subjected to electrospray injection into the mass spectrometer and analyzed for their mass/charge ratio (m/z value) **(B)**. Selected ions were collected in the ion trap. These parent ions were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

Quantitative Multi-Parameter Microscopy

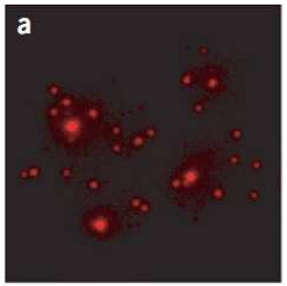
Selected capabilities

- 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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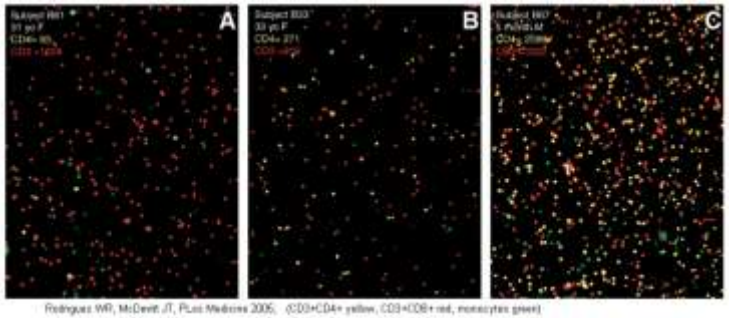
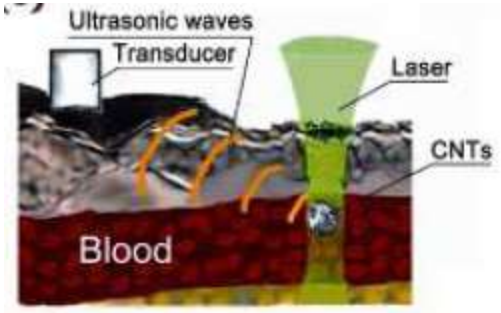


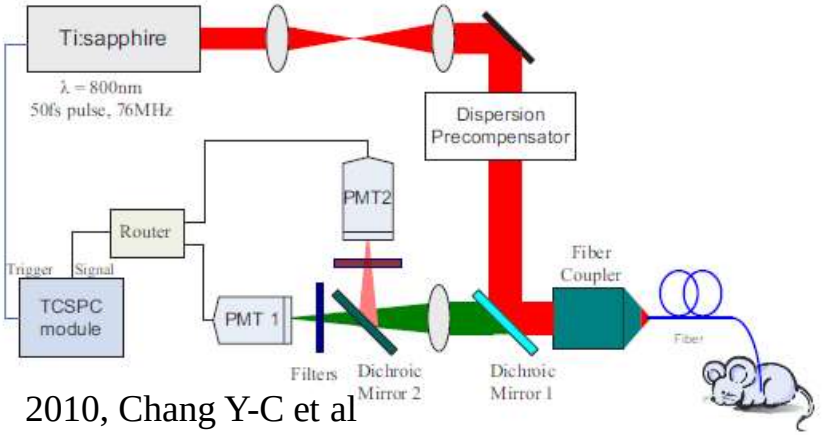
Intra-vital Cytometry

Single cell analysis in living animals

Flow cytometry in blood vessels

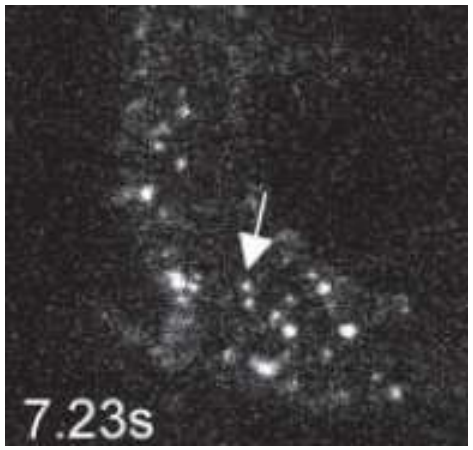


2010, Zharov VP and coworkers



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)

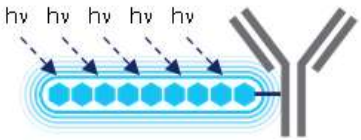
New Developments

- Labels
  - High brightness fluorescent labels
  - Raman labels
- Light sources
  - Solid state lasers
  - LEDs
- Detectors
  - Photomultiplier arrays
  - CMOS
- Fluidics
  - Microfluidic channels for manipulating particles

Bright Fluorescent Polymer Dyes

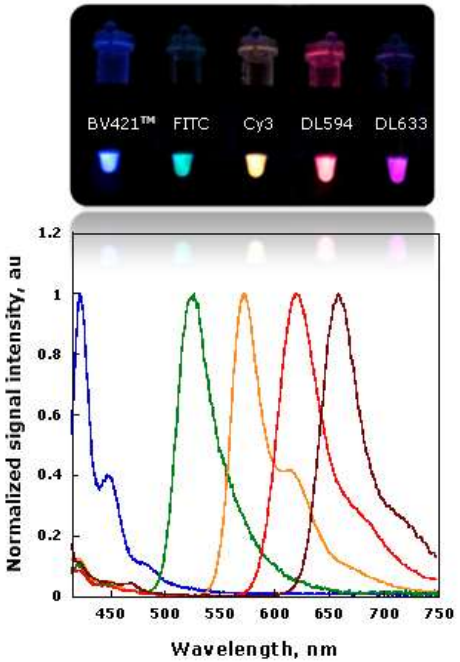
Polymer Based Fluorochromes

- 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



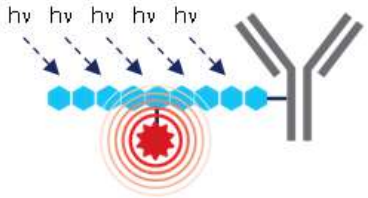
Brilliant Violet 421™

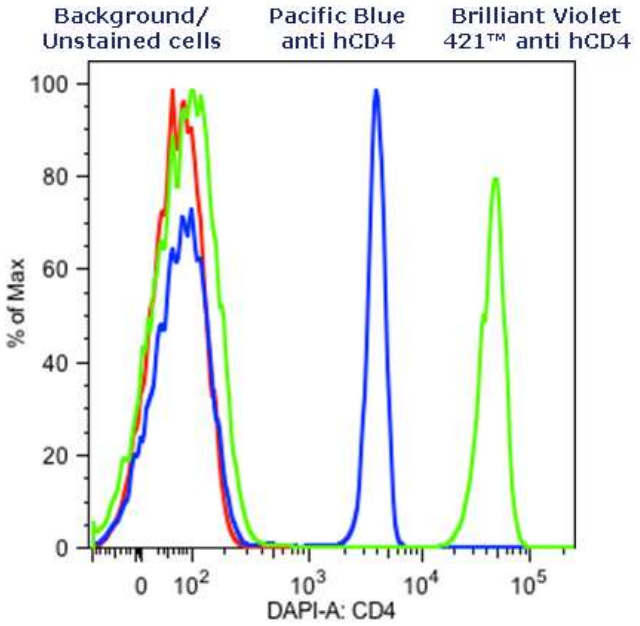
- PE level performance w/ 405nm Laser
- >10x the Stain Index of Pacific Blue
- Enables detection of low abundance targets in multicolor assay panels (e.g. CD56, CD127, etc.)
- Wide range of Ab clones validated



Brilliant Violet Tandems

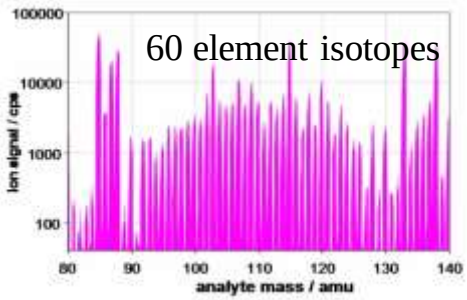
- Provides a wider range of colors spanning the visible spectrum
  - >6 unique colors validated
- Chemically controlled ratio of donor/acceptor provides:
  - Reproducible performance
  - Low (<5%) compensation at 450nm

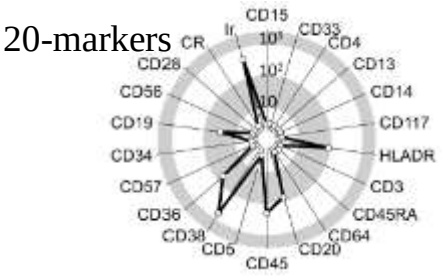




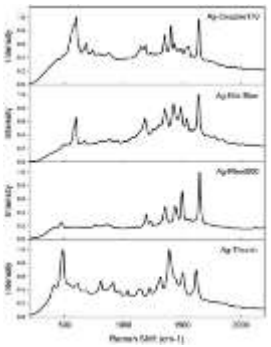
New Developments for Multi-parameter Cytometry

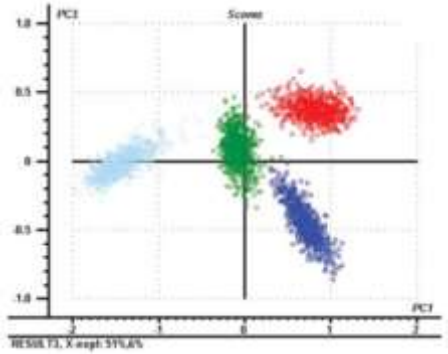
- Element-Label Flow Cytometry (CyTOF, addresses fluorescence spectral overlap issue by using elements as labels, Anal. Chem., 2009, 81 (16), pp 6813–6822)
- SERS-Label Flow Cytometry (uses spectral fine-structure to distinguish labels, Cytometry, 2008, 73A(2), pp 119-128)
- Sequential Stain De-stain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



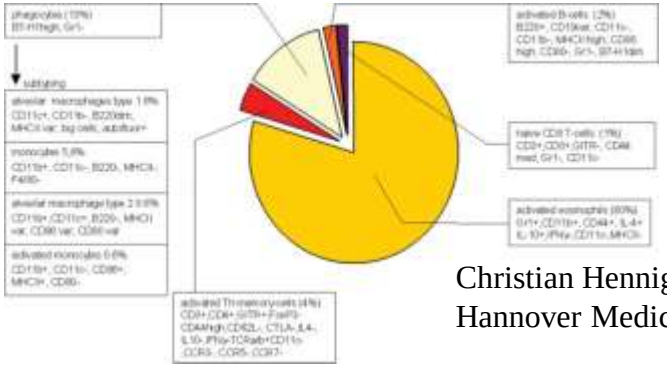


Scott Tanner, DVS Sciences Inc





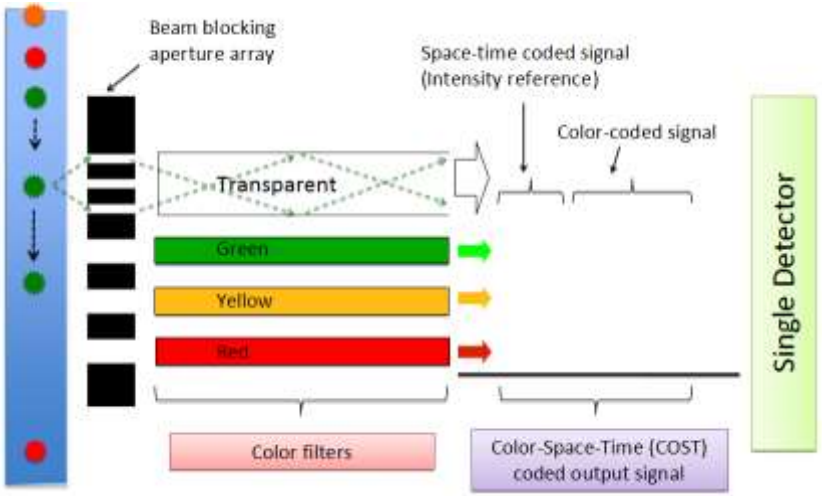
John Nolan, La Jolla Bioengineering Institute

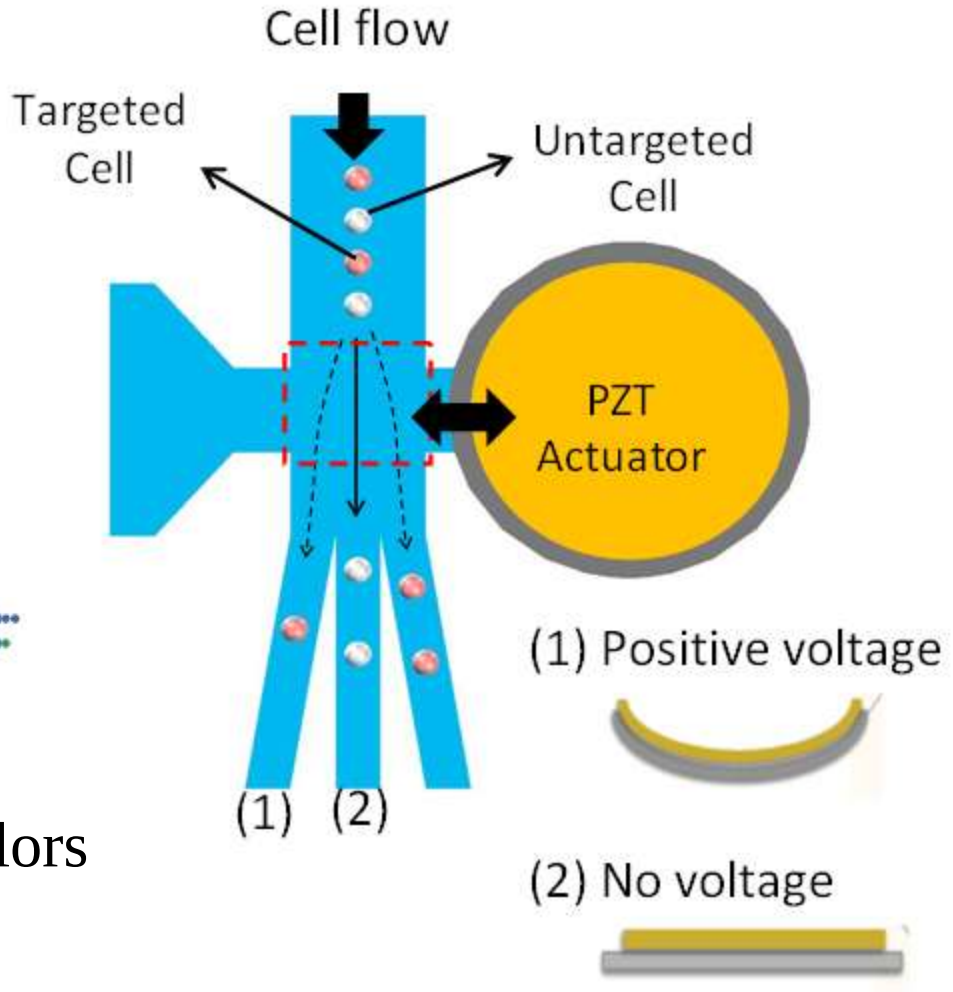


31-marker analysis

Christian Hennig, ChipCytometry
Hannover Medical School

Microfluidic Analyzer/Sorter Example





nanocellect Biomedical, Inc.

- microfluidics fabrication
- single detector for multiple colors
- in-channel cell deflection

nanocellect Biomedical, Inc.

Opportunities for Acoustic Science

Sample Preparation

- particle trapping
- particle concentration
- particle washing

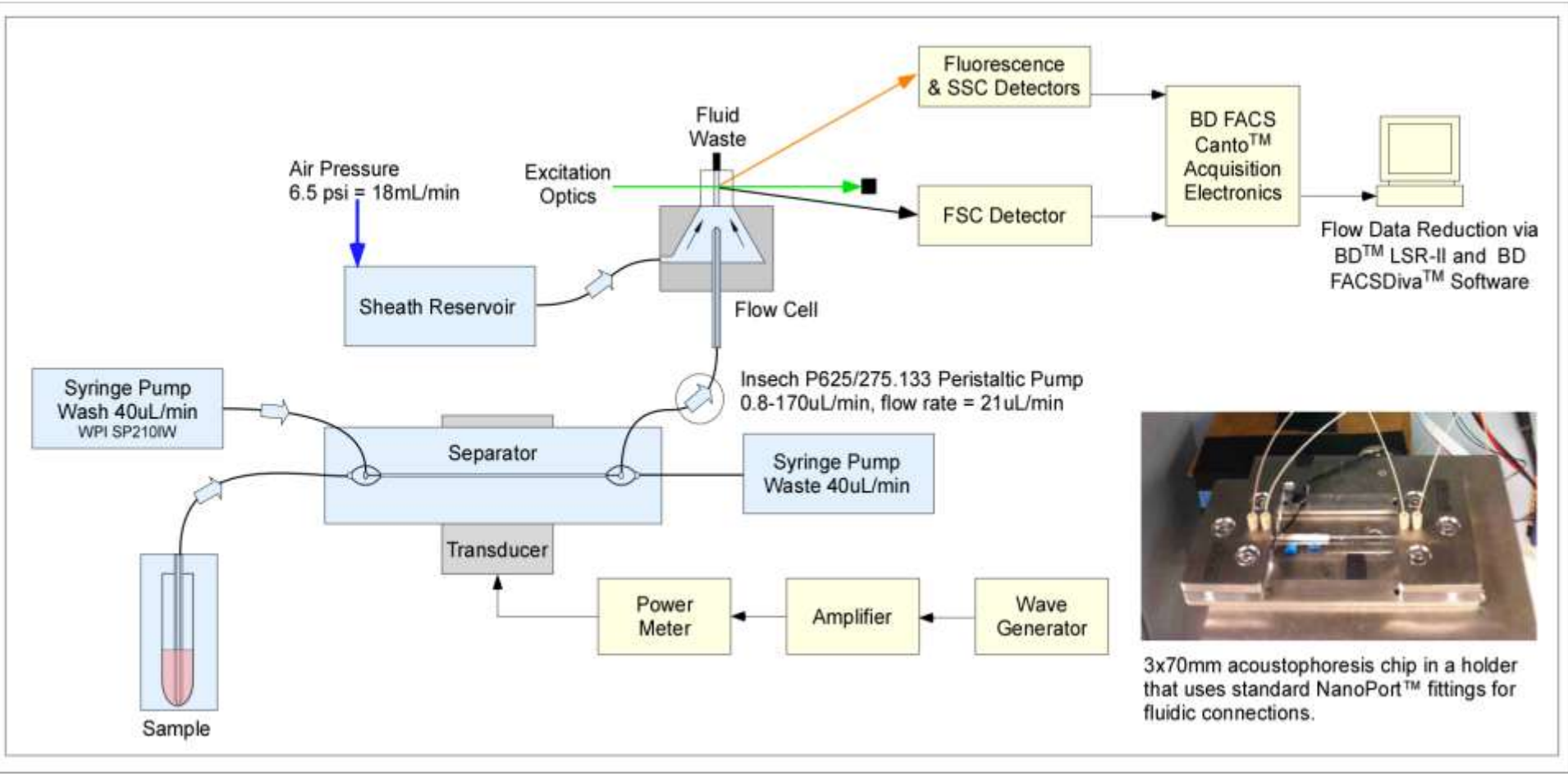
Particle/cell sorting

- label free
- special labels

Cell Analysis

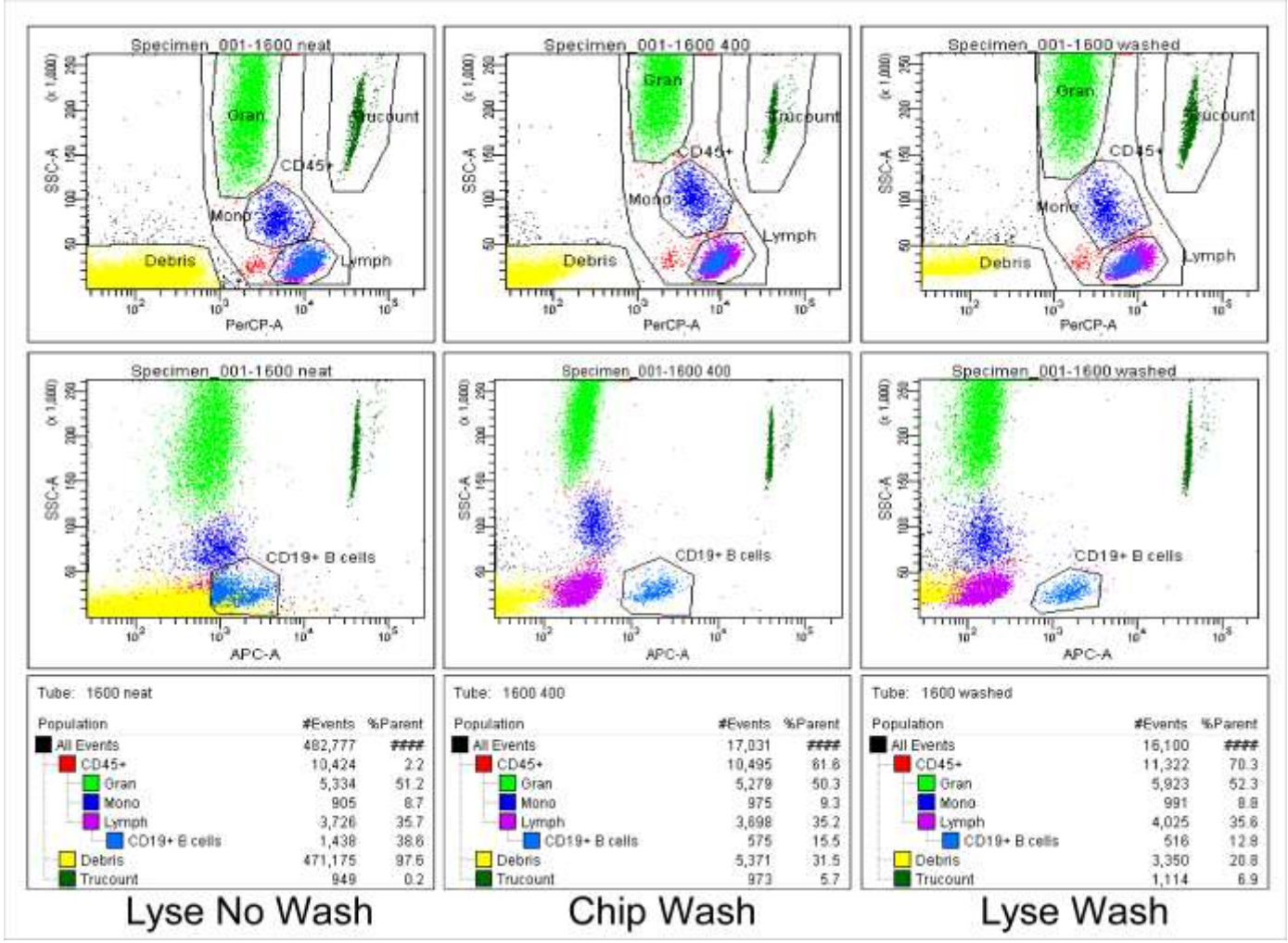
- acoustic particle focusing
- acoustic parameters

Cell Washing System



From: Warner, Yu, Blom, Buesink, Lenshof, and Laurell; Improving Flow Cytometric Performance Using Modular In-Line Acoustophoretic Washing; CYTO 2012; Leipzig, Germany; Poster 184

Cell Washing Results



From: Warner, Yu, Blom, Buesink, Lenshof, and Laurell; Improving Flow Cytometric Performance Using Modular In-Line Acoustophoretic Washing; CYTO 2012; Leipzig, Germany; Poster 184

Conclusions
Evolving Technologies

Technology developments in fields outside of biology e.g. acoustics, create the basis for new reliable analytical approaches for cell biology / diagnostic medicine.

Automation of critical steps (sample preparation, instrument setup, data analysis) makes cytometry more widely useable.

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- Holden Maecker (now Stanford U)
- Collette Rudd (Thermo)
- ...

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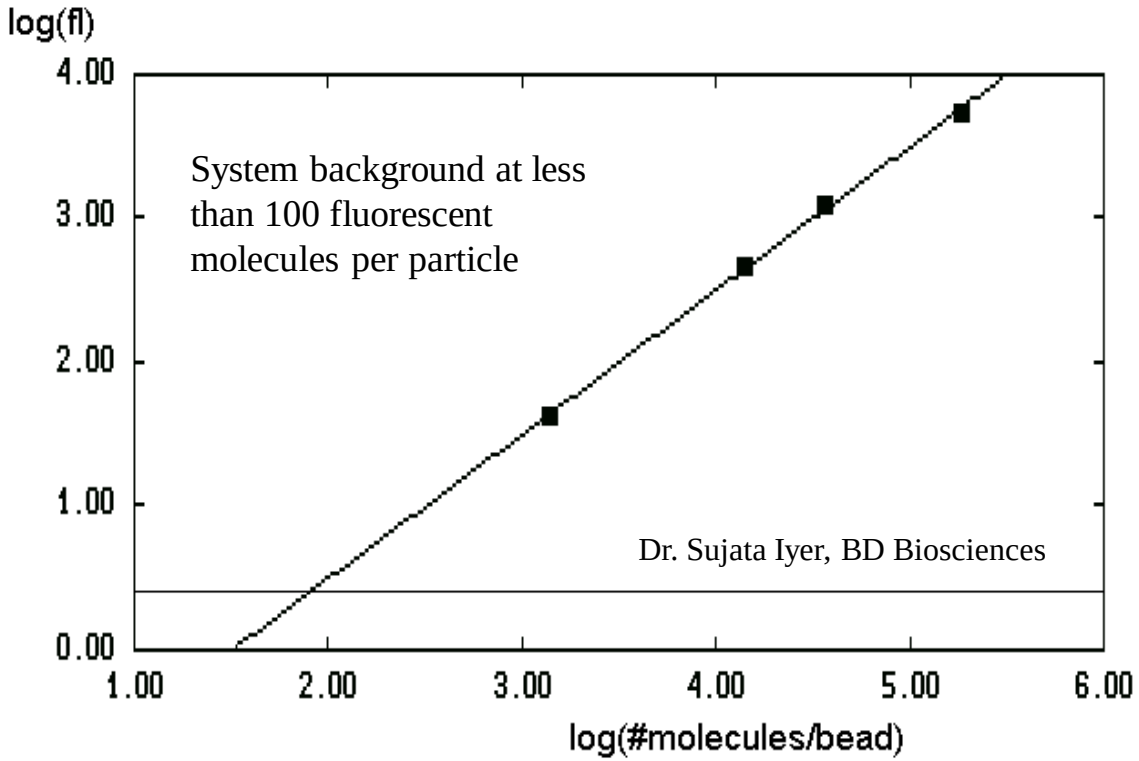
END

Backup Slides

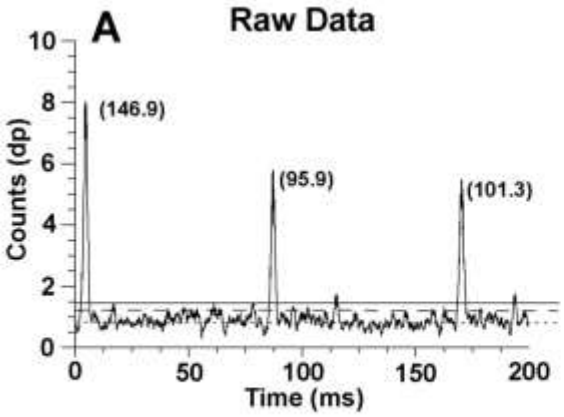
Quantitation and Limit of Detection for Particle Fluorescence (routine flow cytometer)

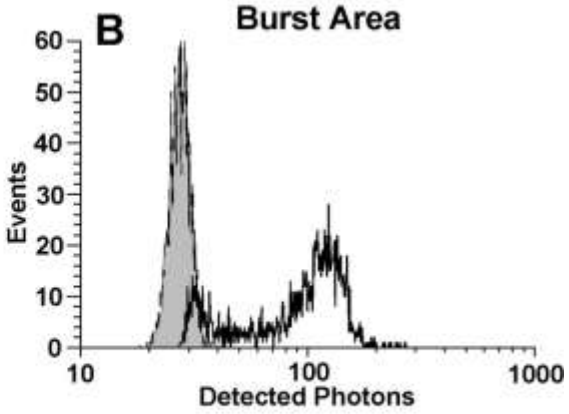
Molecule	#/cell
CD3	8.1×10^4
CD4	5.0×10^4
CD8	1.4×10^5
CD11a	2.7×10^4
CD16	7.9×10^4
CD18	3.1×10^4
CD45	1.9×10^5

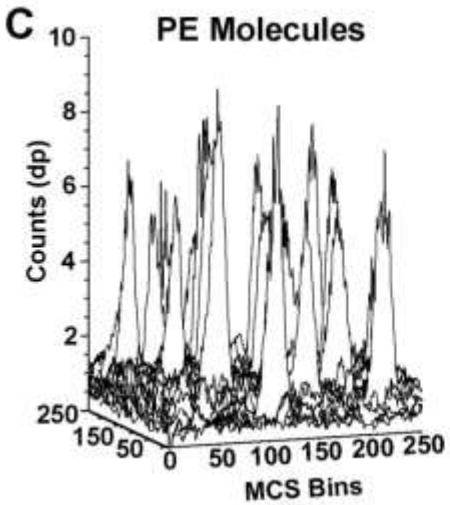
Appendix A, Cell Separation Methods and Applications. Marcel Dekker 1998. Recktenwald D and Radbruch A, eds.

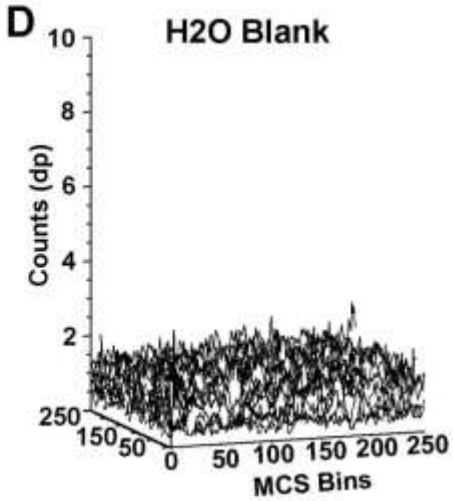


Single molecule sensitivity with a special flow cytometer









A: 200 ms corrected data showing 3 molecules of B-PE

B: 2645 photon burst areas (background-grey)

C,D: each 256 bin (row) = 25.6 ms data. **C** is B-PE showing single molecules. **D** is H_20 control

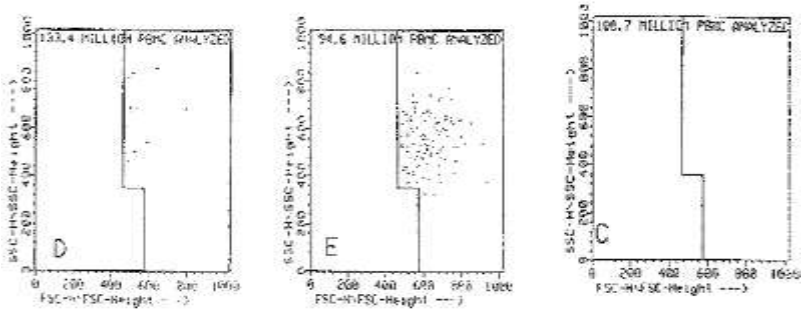
(Rob Habbersett & Jim Jett, LANL)

Limit of Detection for Rare Cells

Routine >0.2%

Optimized instrument >0.01%

Optimized system $>10^{-7}$

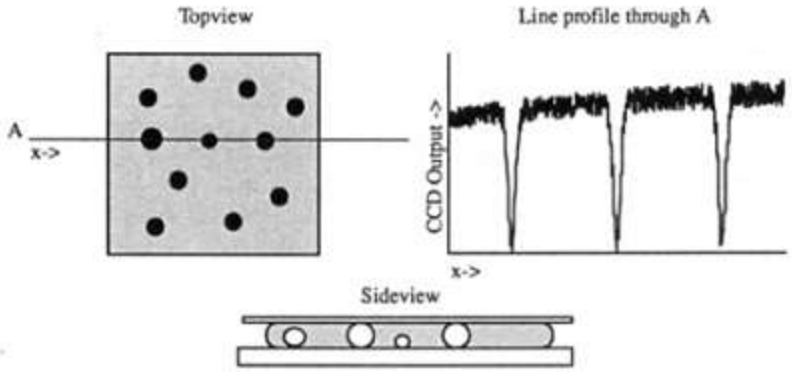


10^{-6} **10^{-5}** **control**

Gross HJ et al, Cytometry 14 (1993) 519-526

Gross HJ et al, PNAS 92 (1995) 537-541

Fluorescence Microscopy
Quantitation



System calibration in number of molecules by volume exclusion fluorescence microscopy

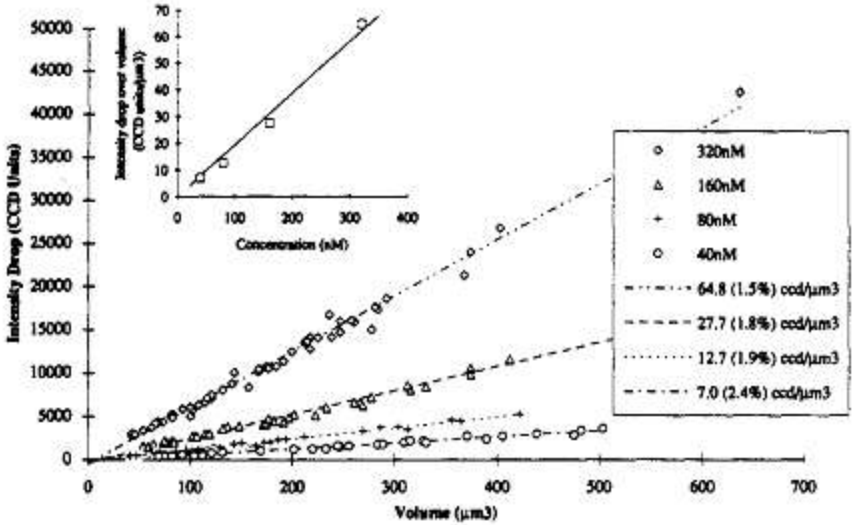
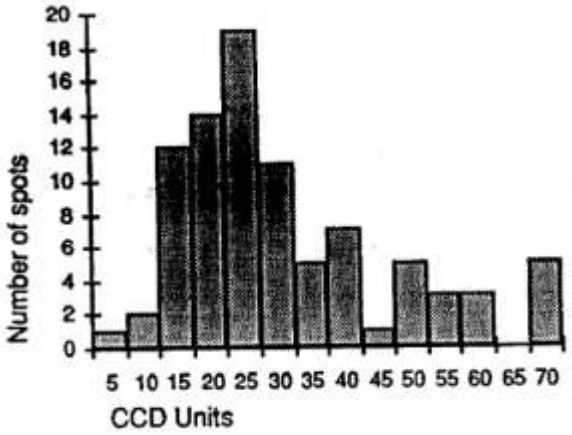
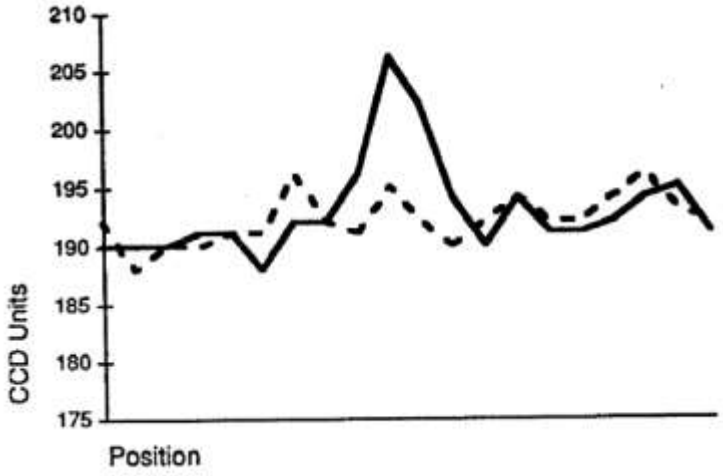


Figure 4. Intensity drop versus volume for four solutions of (*R*)-phycoerythrin (numbers in parentheses) after slope estimates are coefficients of variation. Inset shows intensity drop over volume versus concentration. Relation is 0.30 CCD units/molecule.

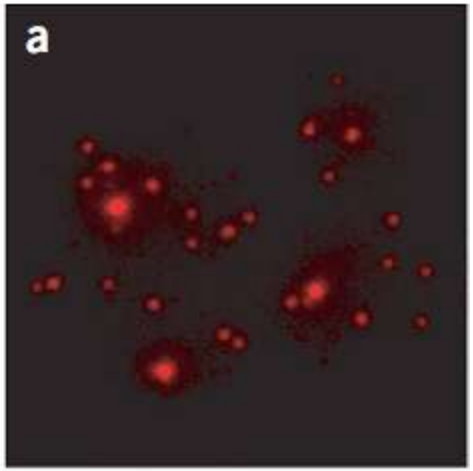
Verwer, Phi, Recktenwald 1993; JPC 97,2868-70

Fluorescence Microscopy
Single Molecule Analysis





Single PE molecule microscopy, Verwer, Phi, Recktenwald 1994



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

Fluorescence Microscopy
for Low Cost Cytometry

+

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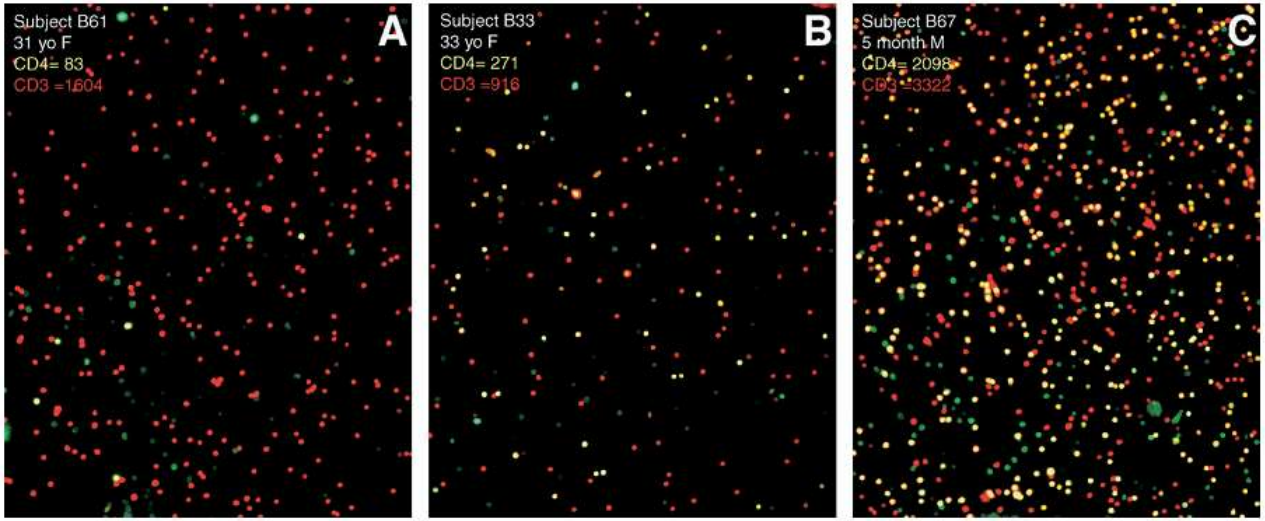

Rodriguez WR, McDevitt JT, PLos Medicine 2005; (CD3+CD4+ yellow, CD3+CD8+ red, monocytes green)