State of the Art Cytometry & Emerging Technologies for Single Cell Analysis

Diether Recktenwald, BD Biosciences, retired
Desatoya LLC, Reno NV, USA
Email: diether@desatoya.com
http://www.desatoya.com

Biology Research Targets and Tools

Organism

Organ

Tissue

Single Cell

Organelle

Macromolecule

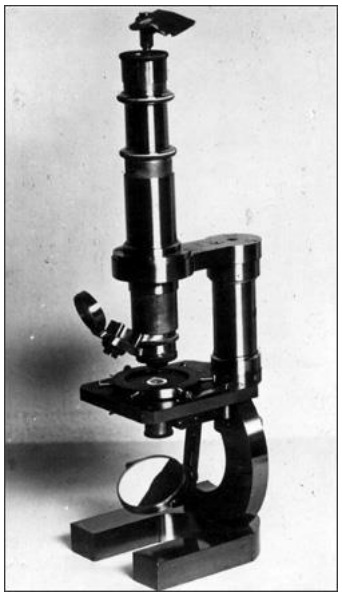
Small molecules

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

Early & Modern Flow Cytometry and Imaging Microscopy



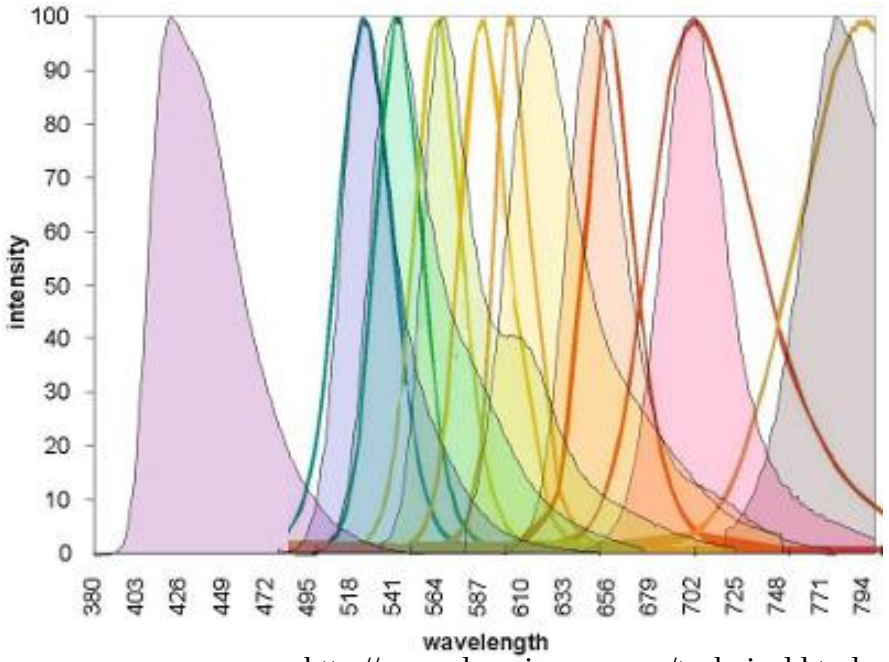
Flow and Imaging Cytometry Features

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

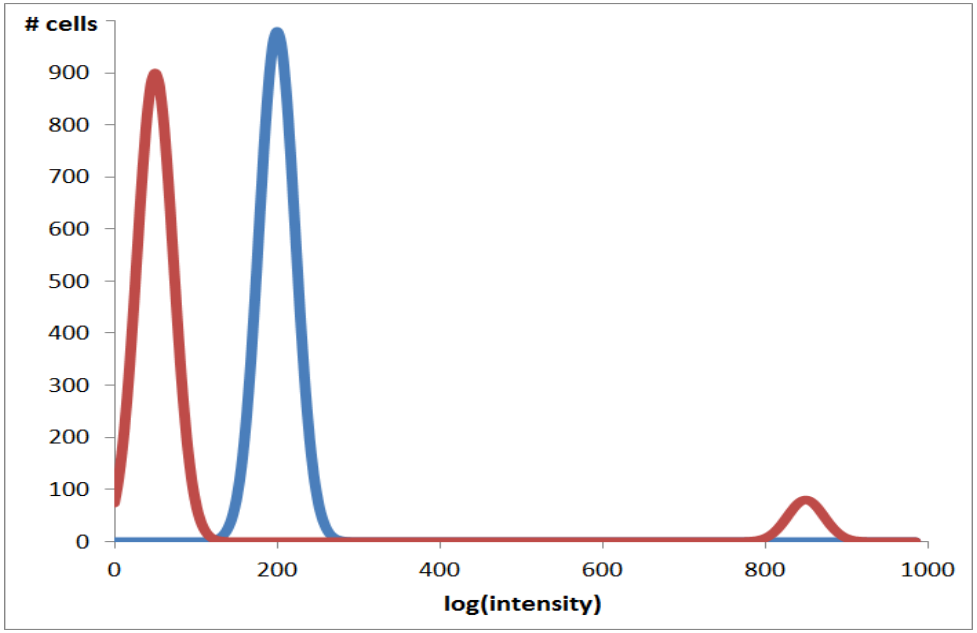
- Light scatter
- Fluorescence
- Phosphorescence
- Raman
- Element mass
- Electrical properties e.g. impedance
- …



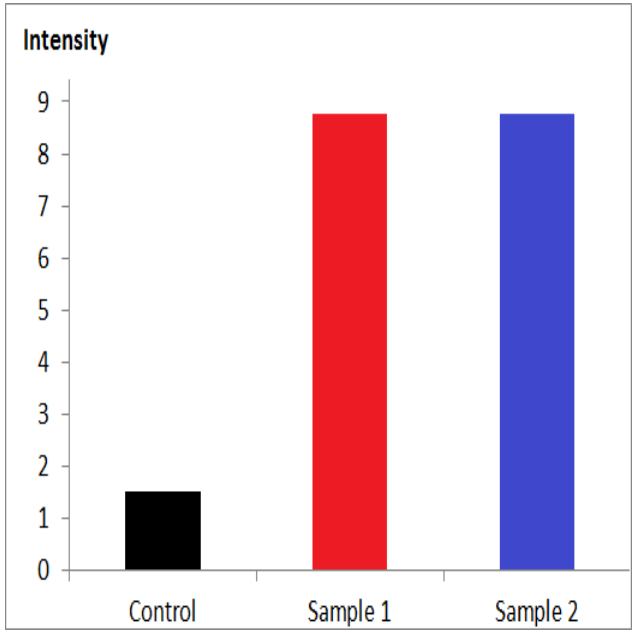
http://www.dvssciences.com/technical.html

Why Single Cell/Particle Analysis

Intensity Histogram for Single Particles



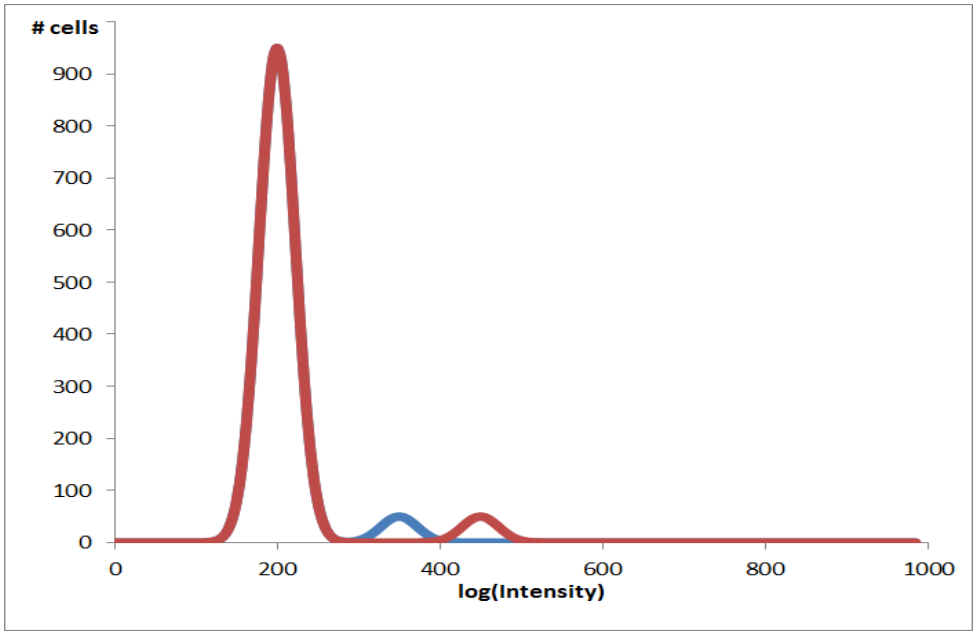
Intensity per Sample



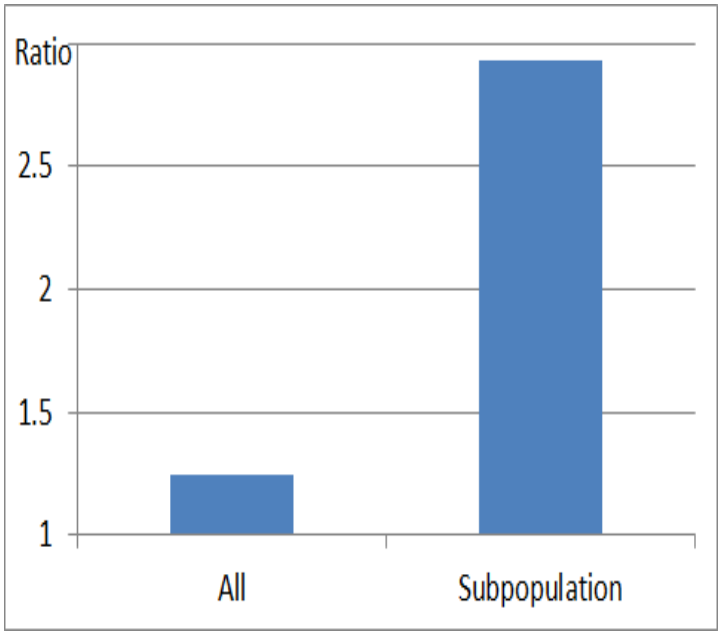
Cell by cell intensity analysis detects population heterogeneity.

Benefits of Subset Specific Analysis

Intensity Histogram



Intensity Ratios

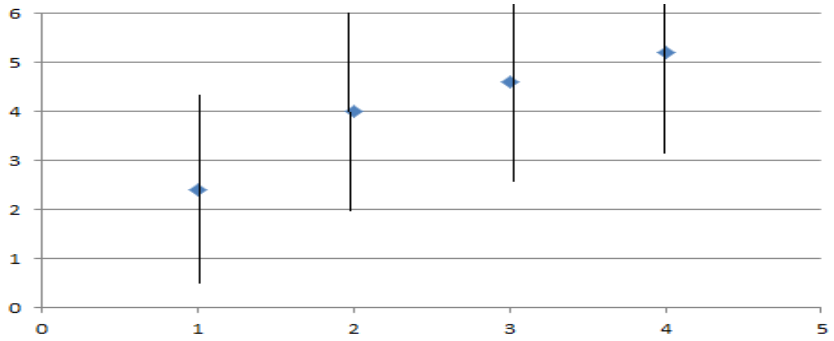


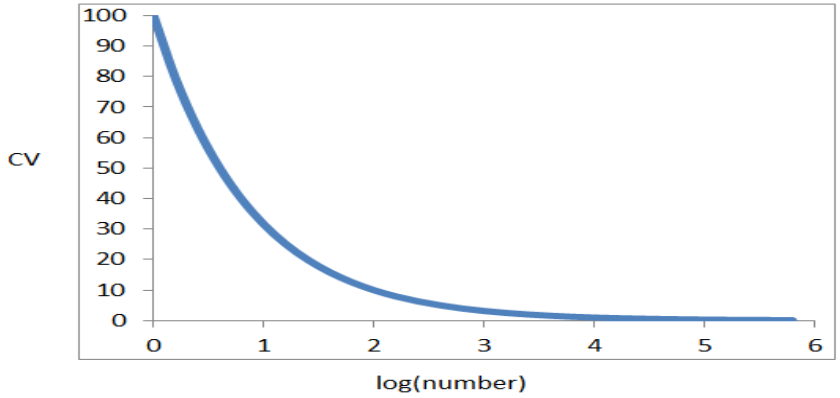
Subpopulation analysis detects changes better, especially for rare subpopulations.

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



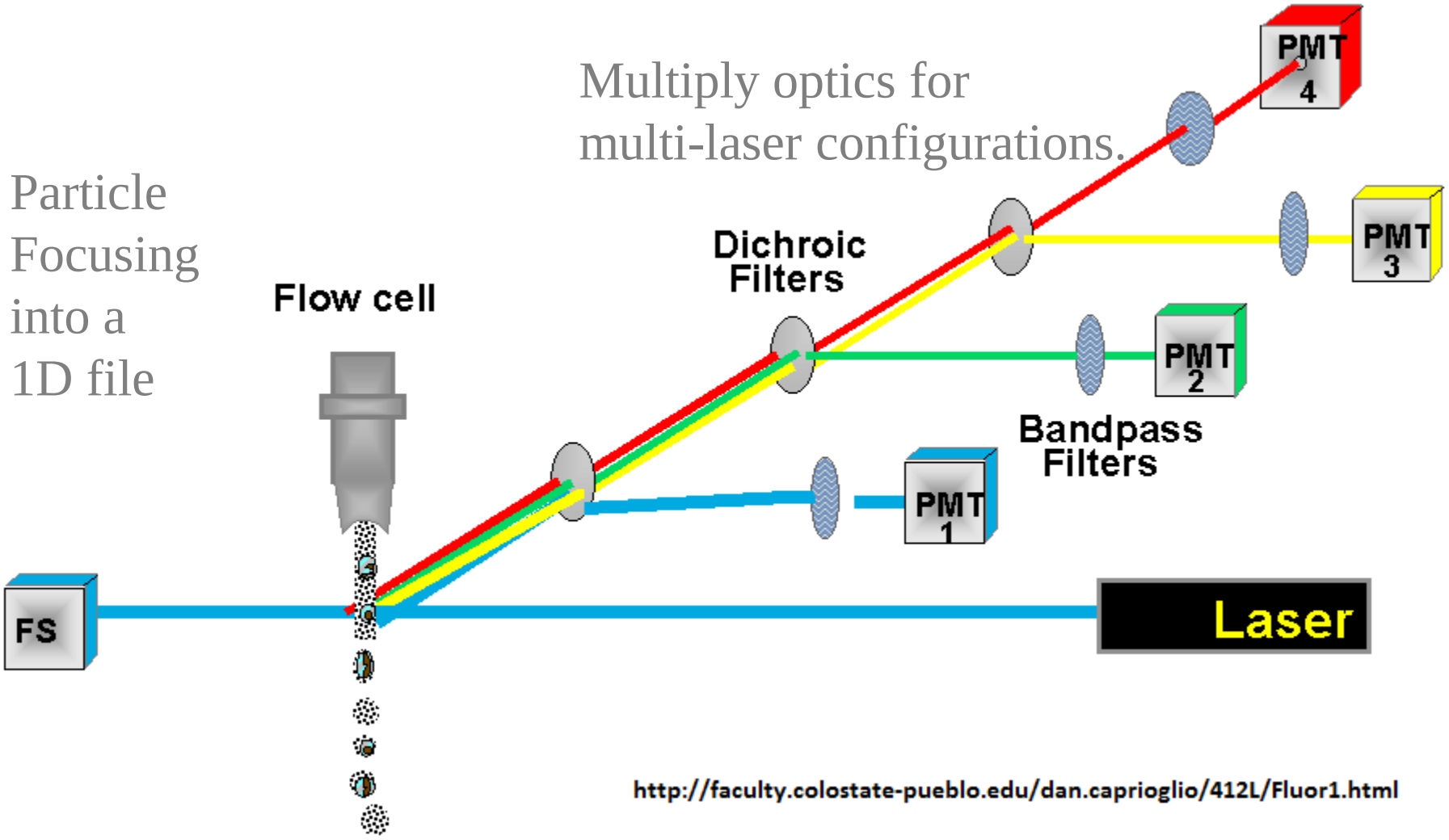


Ignoring Counting Statistics Can Lead to Erroneous Conclusions

Flow Cytometer Components

Particle Focusing into a 1D file

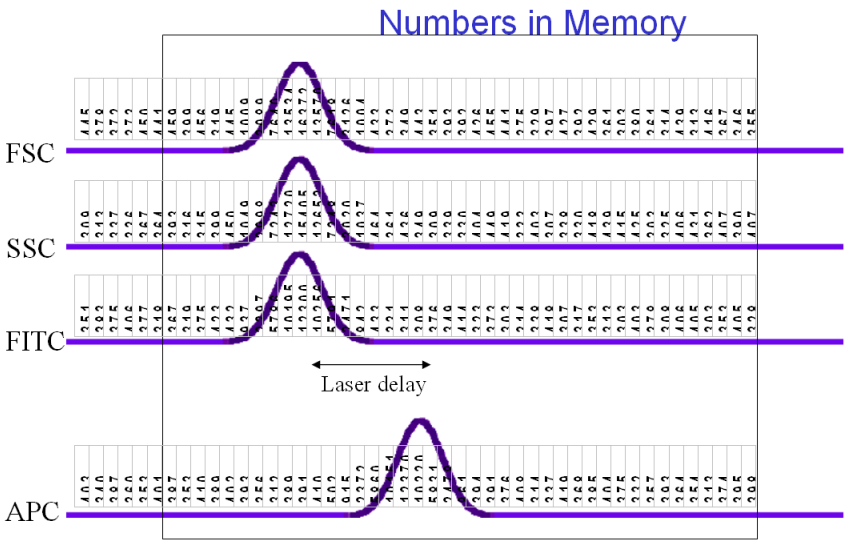
Multiply optics for multi-laser configurations.



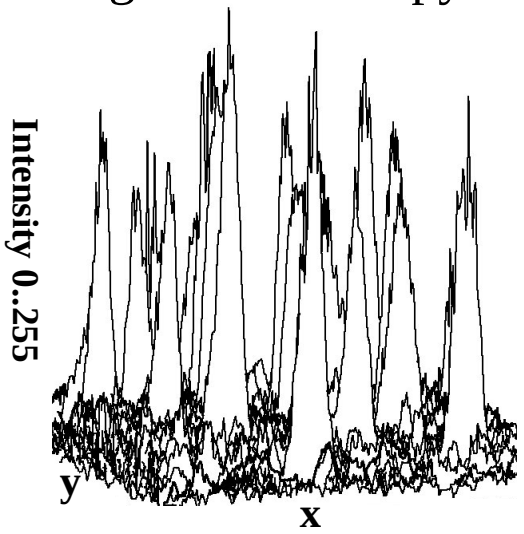
http://faculty.colostate-pueblo.edu/dan.caprioglio/412L/Fluor1.html

Basic Data Processing

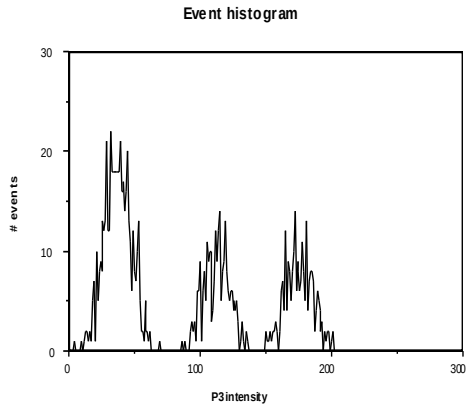
Flow Cytometry

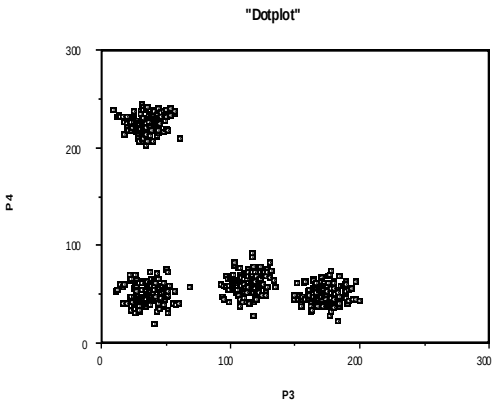


Digital microscopy



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

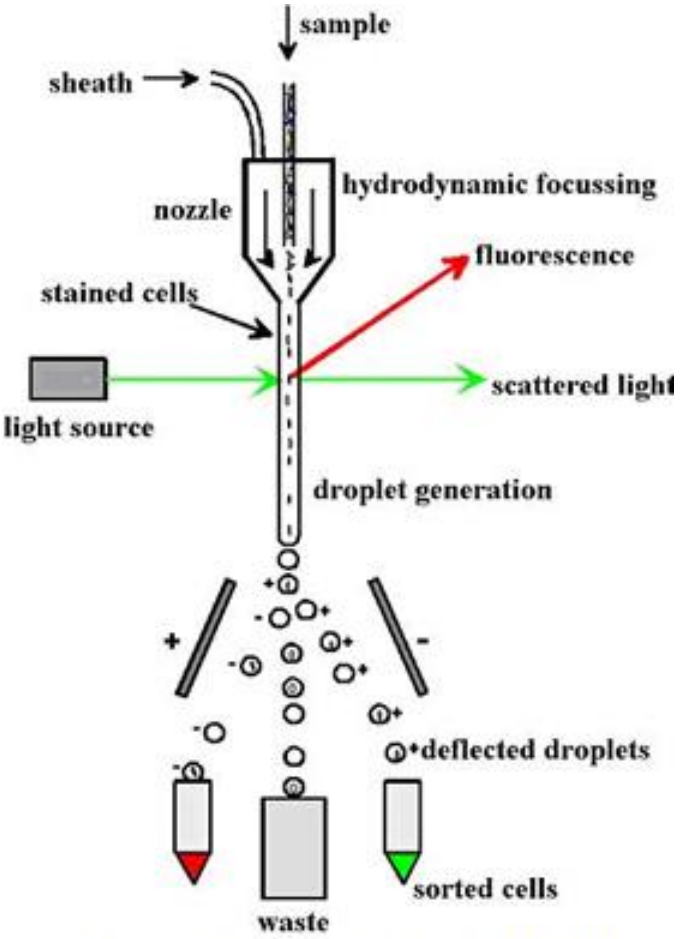




for >2 parameters: gating, cluster analysis, …

For many samples and parameters: bioinformatics

Cell Sorting



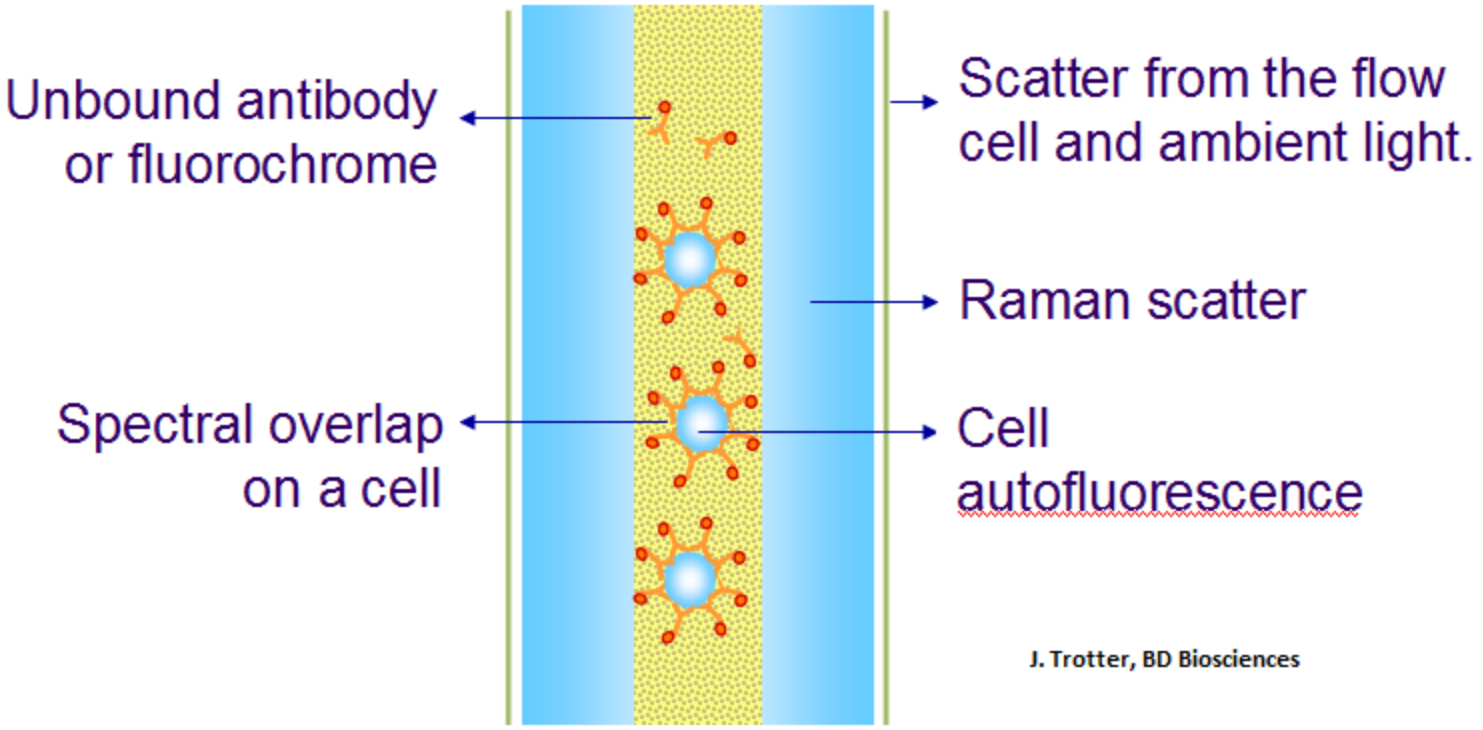
Applications Examples

- Chromosomes
- Strain Improvement
- Genomics
- Proteomics
- …

Cell sorting review: Derek Davies
http://www.facs.ethz.ch/docs/lit
see also:
http://www.desatoya.com/ScienceTechnology/CytometryWithSorting.htm

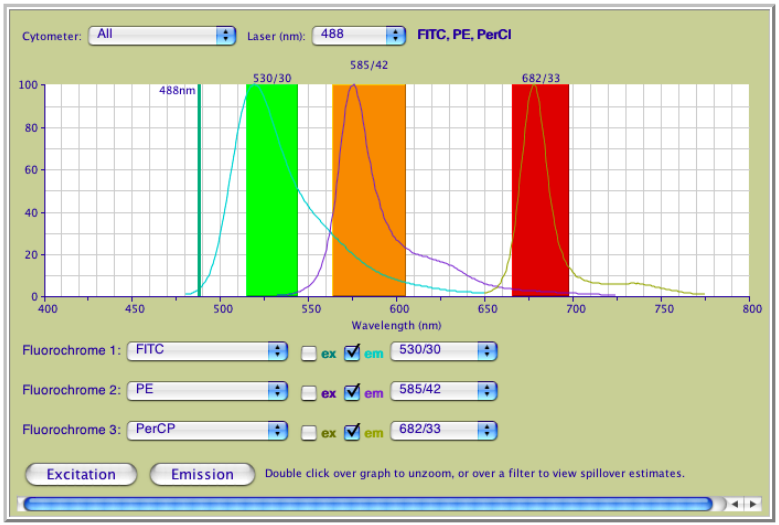
Instrument Evaluation Br

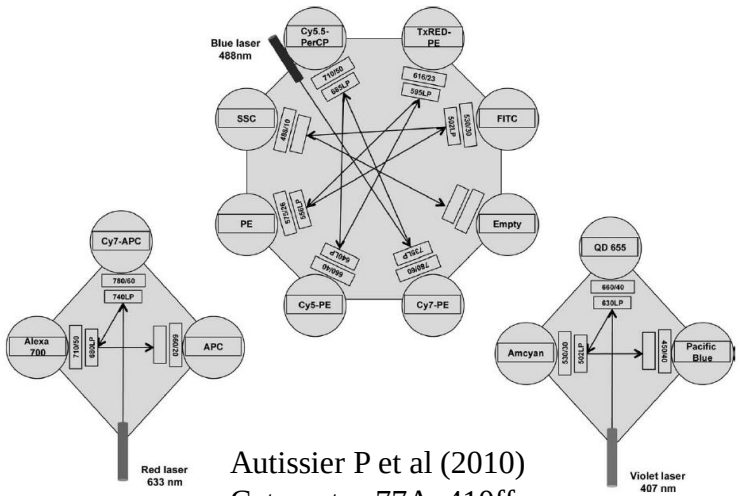
Relative B (Br) is a measure of true optical background in the fluorescence detector.



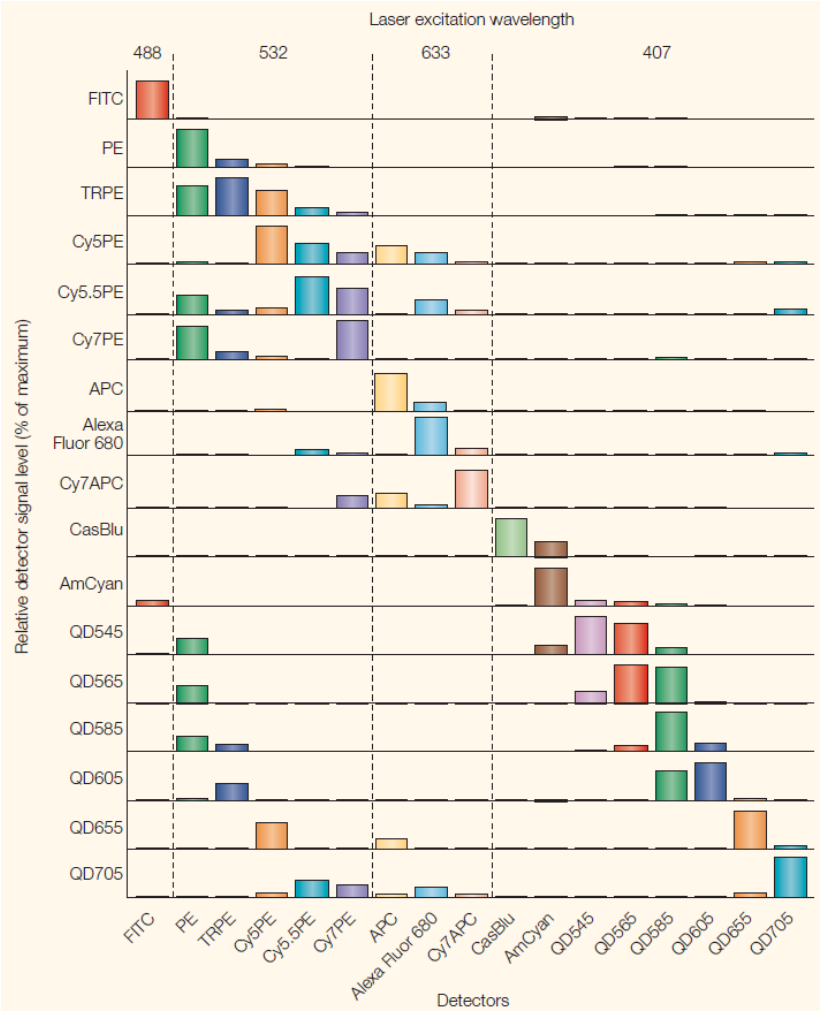
Filter Arrangement and Spectral Overlap

(not relevant for element mass cytometry)



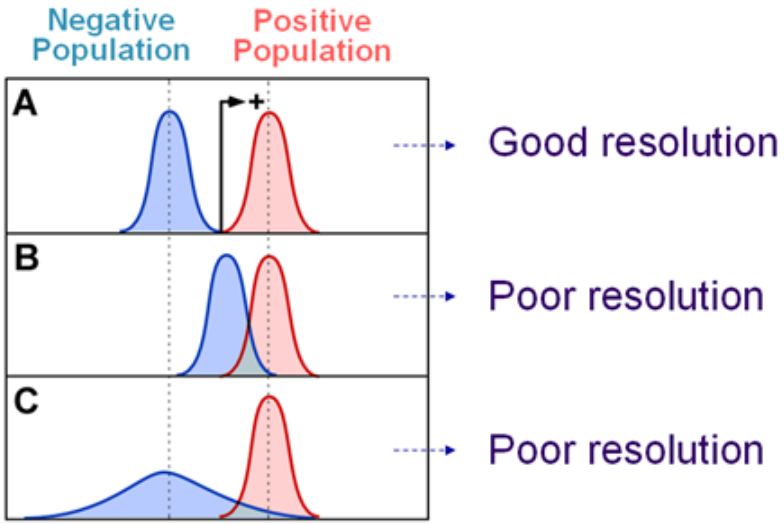


Autissier P et al (2010)
Cytometry 77A, 410ff



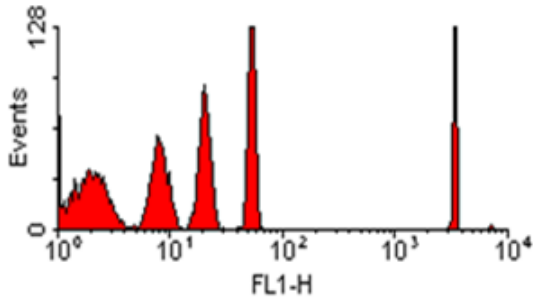
Perfetto SP et al (2004)
Nature Reviews Immunology 4, 648ff

Instrument Evaluation Qr

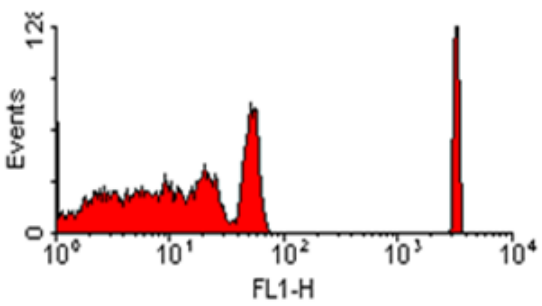


$$Qr = \frac{\#\ \text{photoelectrons}}{\#\ \text{fluorescence molecules}}$$

High Qr



Low Qr



J. Trotter, BD Biosciences

Optimizing cytometry measurements (I)

- Background light

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

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

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

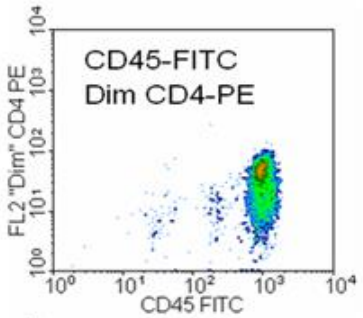
$$SD_{optical} = \sqrt{SD_{PE}^2 + SD_{FITC}^2 + SD_{background}^2}$$

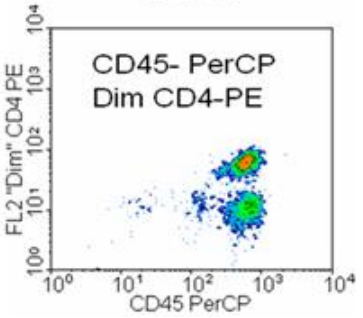
Reagent performance

Stain index

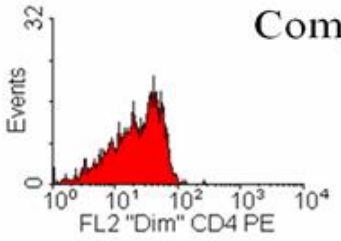
$$\frac{Medium_{pos} - Medium_{neg}}{2 * SD_{neg}}$$

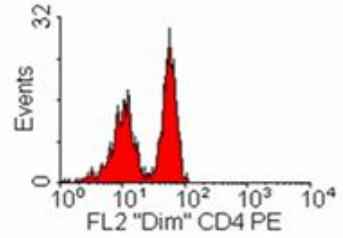
- Dye properties (brightness and spectral overlap)





Compensated data



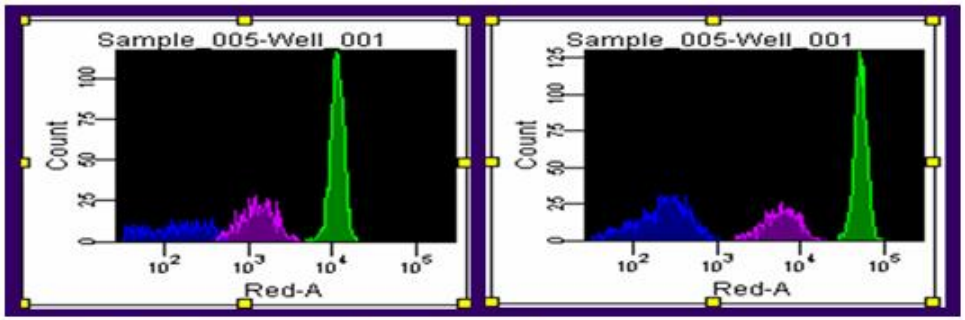


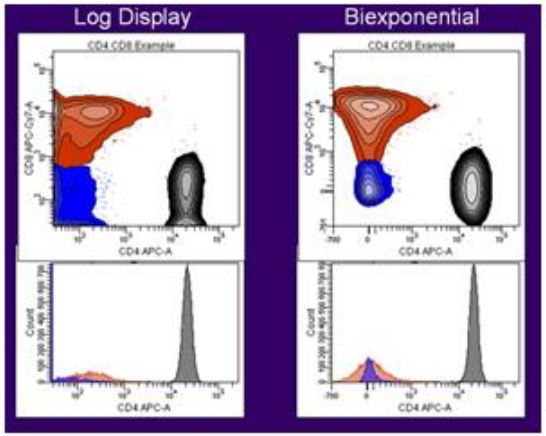
Better separation with less spectral overlap.

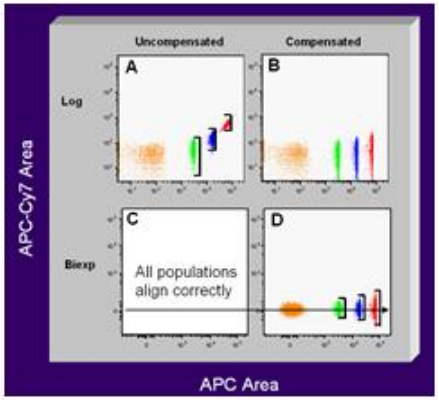
J. Trotter, BD Biosciences

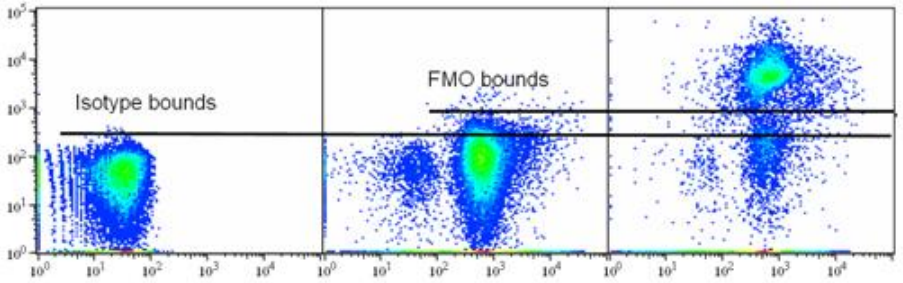
Optimizing cytometry measurements (II)

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









J. Trotter, BD Biosciences

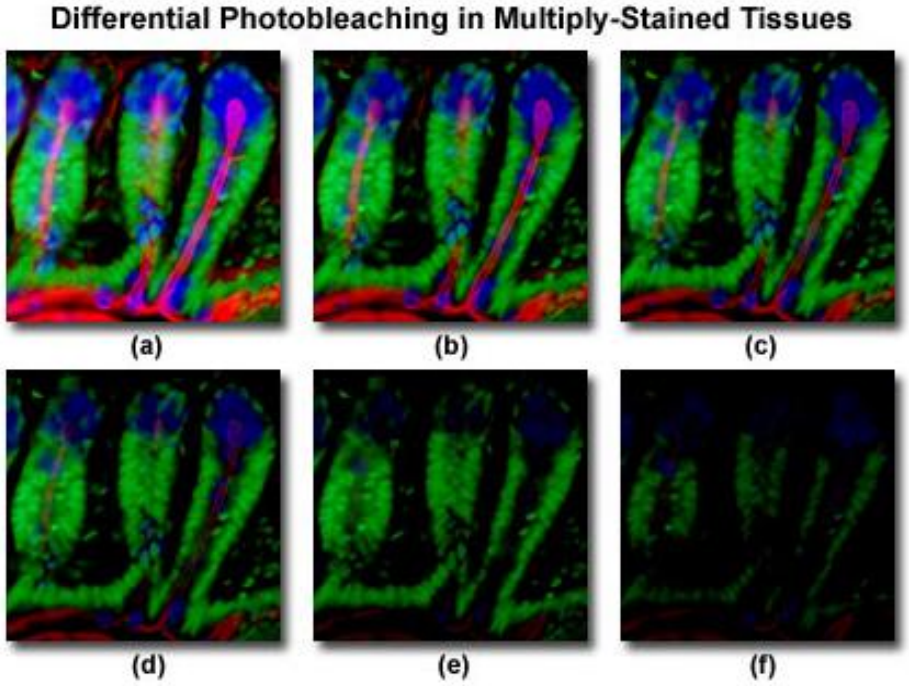
Multi-parameter Fluorescence Cytometry Points To Consider

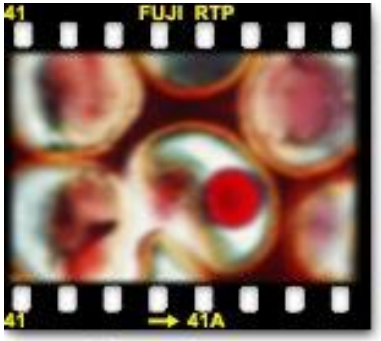
- Know your instrument status e.g. Qr & Br for different channels
- Use high enough gain settings to maximize sensitivity
- An antibody/dye combination that marginally allows discrimination of positives/negatives in a single color assay is unlikely to contribute anything helpful in a multicolor experiment.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

Quantitative Multi-color Microscopy

Additional factors

- Field to field focus
- Photobleaching





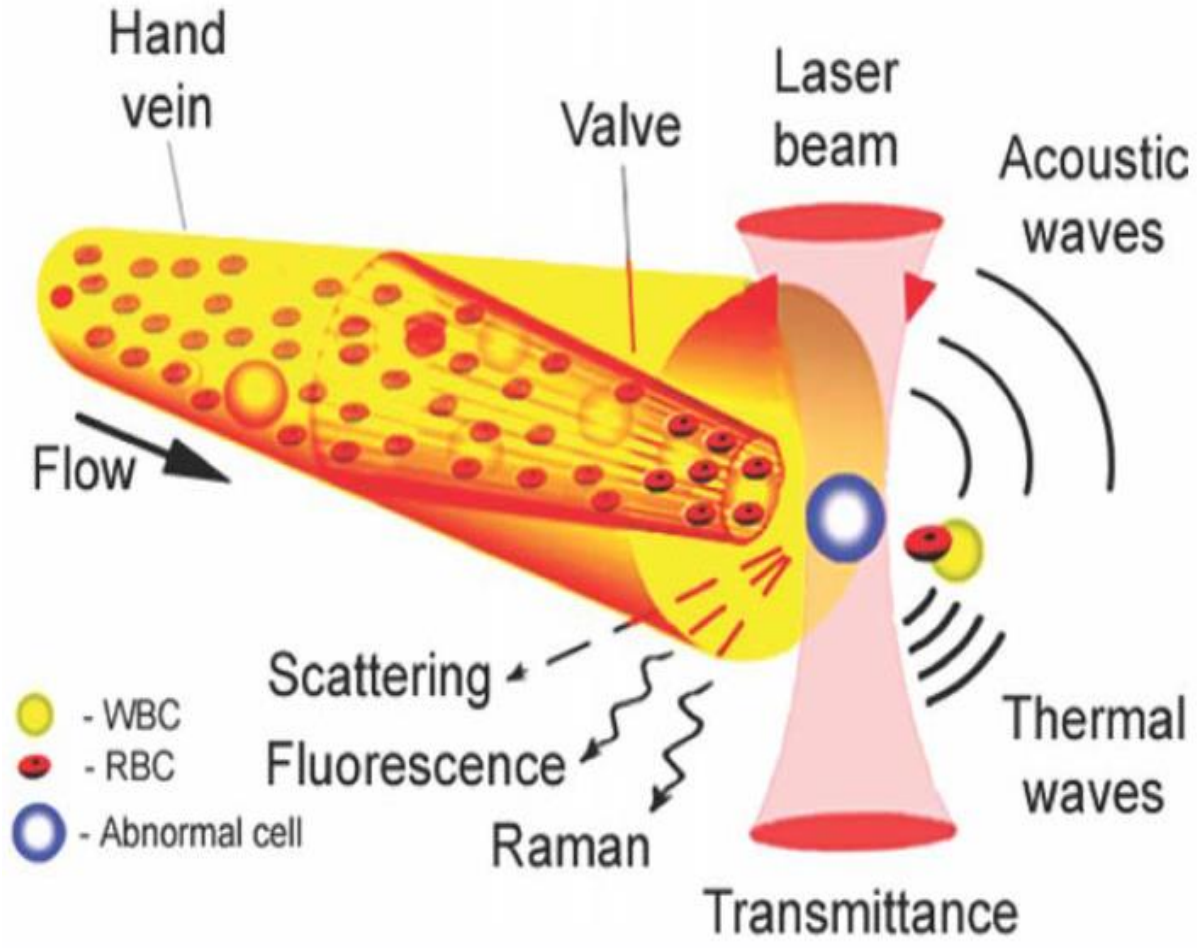
Out of Focus

Images from

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

In-vivo Multi-parameter Cytometry

Single cell analysis in living animals



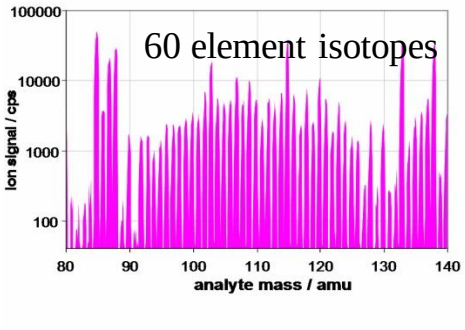
Issues:

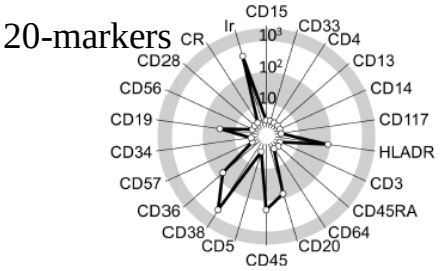
- tissue optics
- object motion
- flow rate
- labelling
- …

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

New Developments for in-vitro Multi-parameter Cytometry

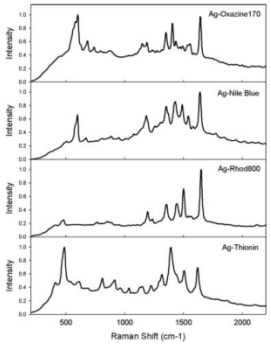
- Element-Label Flow Cytometry (CyTOF, addresses fluorescence spectral overlap issue by using elements as labels, Anal. Chem., 2009, 81 (16), pp 6813–6822)

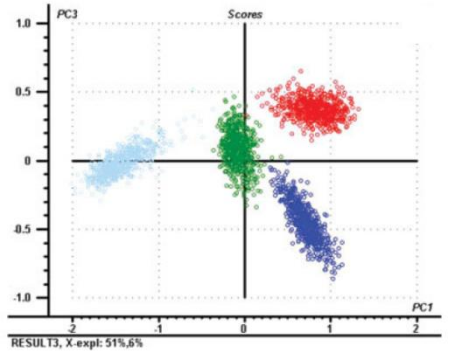




Scott Tanner, DVS Sciences Inc

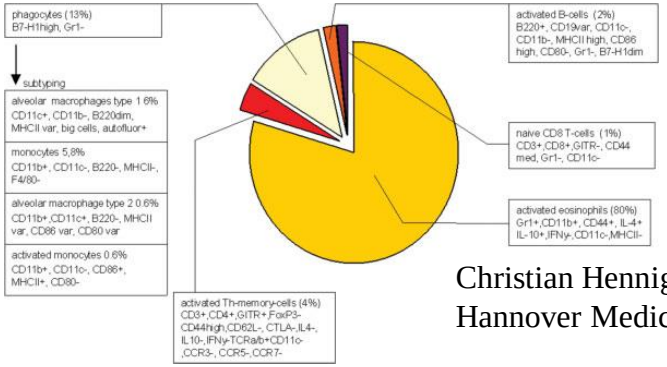
- SERS-Label and Spectral Flow Cytometry (uses spectral (fine)-structure to distinguish labels, Cytometry, 2008, 73A(2), pp 119-128, SONY cytometer)





John Nolan, La Jolla Bioengineering Institute

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



31-marker analysis

Christian Hennig, ChipCytometry
Hannover Medical School

Conclusions

Multi-parameter cytometry

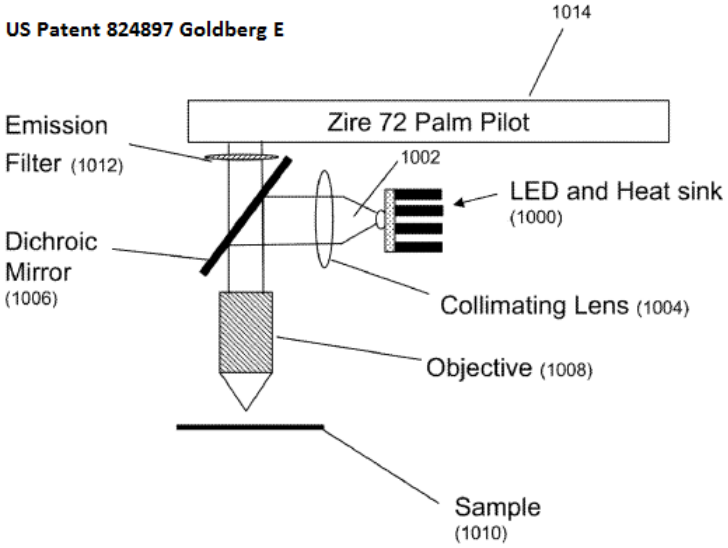
Optimized flow and imaging single cell cytometry with adequate bio-informatics tools provide quantitative molecular measurements into biological processes at organism, cellular and sub-cellular levels.

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

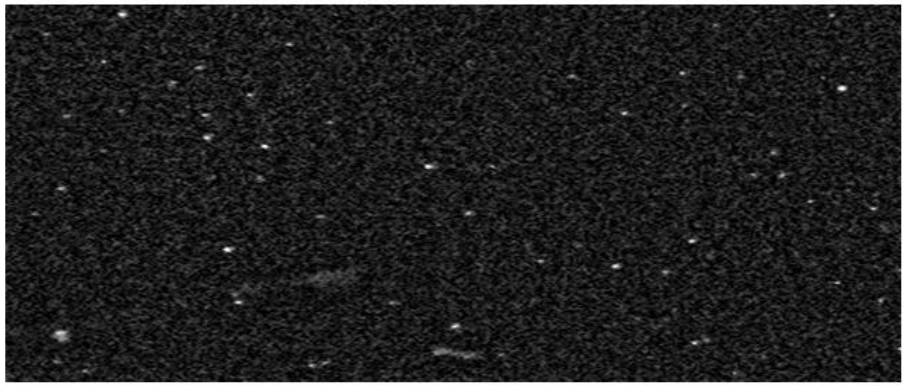
Evolving Technologies for Cytometry

- More low complexity cytometers for cell (subset) counting
- Novel cell sorters
- Integrated Cell Analysis System
- Intra-vital imaging
- Innovative sample preparation
- Fluorescent polymers for high sensitivity
- Novel affinity reagents (antibodies)

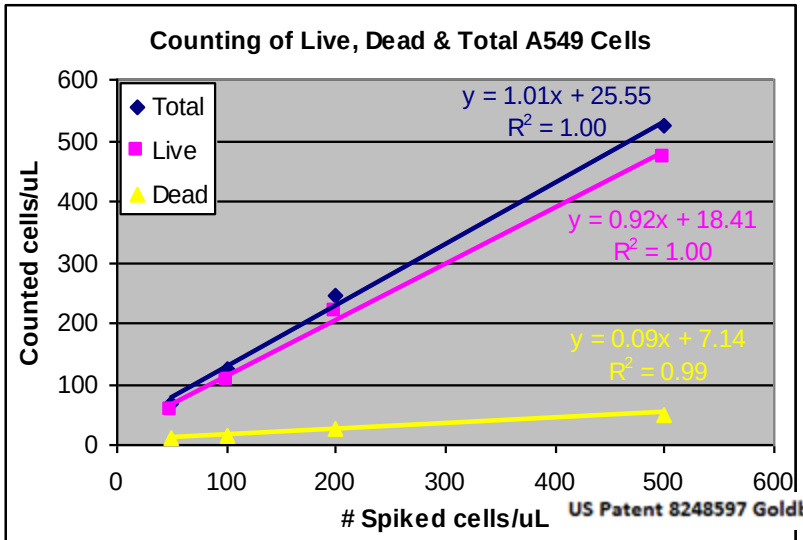
Low-complexity Cytometers for Cell Counting



Counting Nucleated Cells
Propidium Iodide (impermeant) + SYTO-17 (permeant)

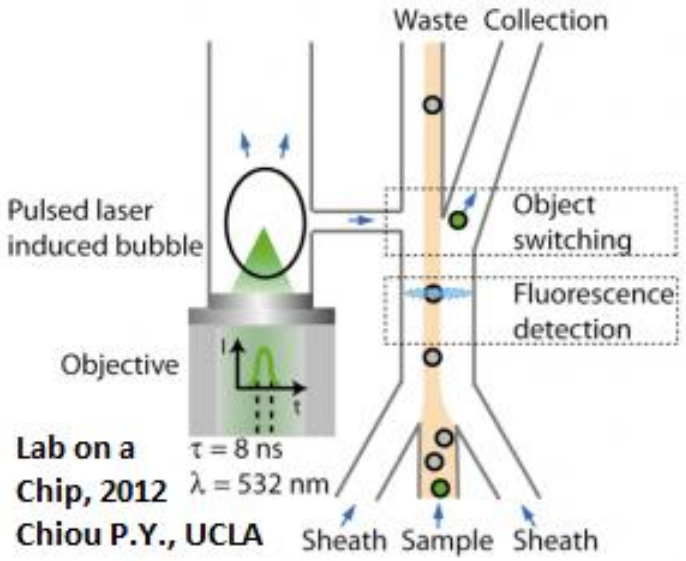


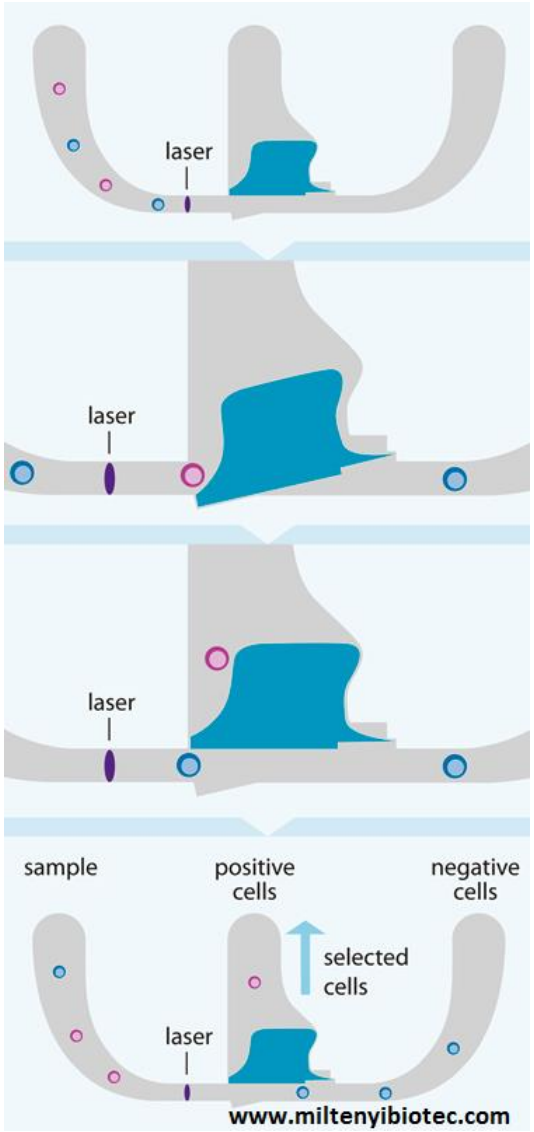
US Patent 8248597 Goldberg E



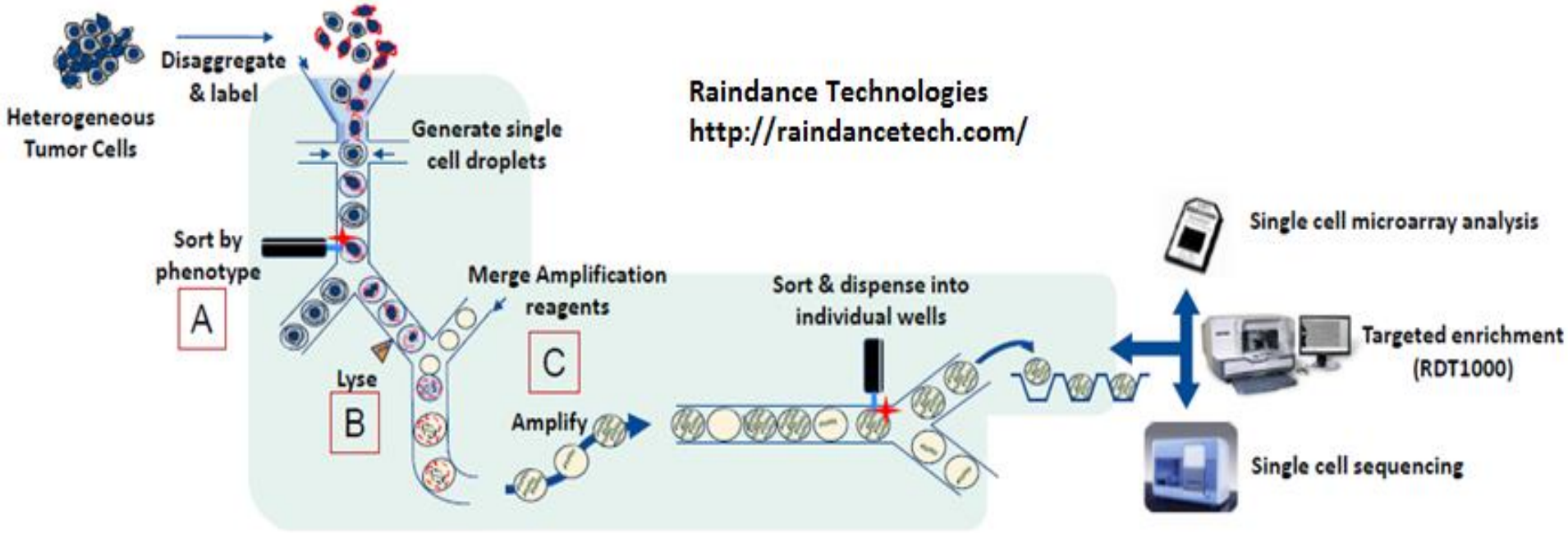
US Patent 8248597 Goldberg E

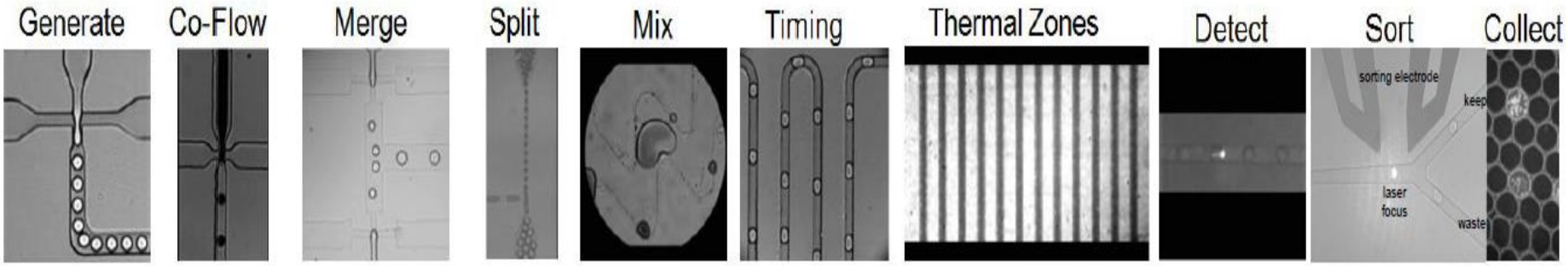
Novel Cell Sorters





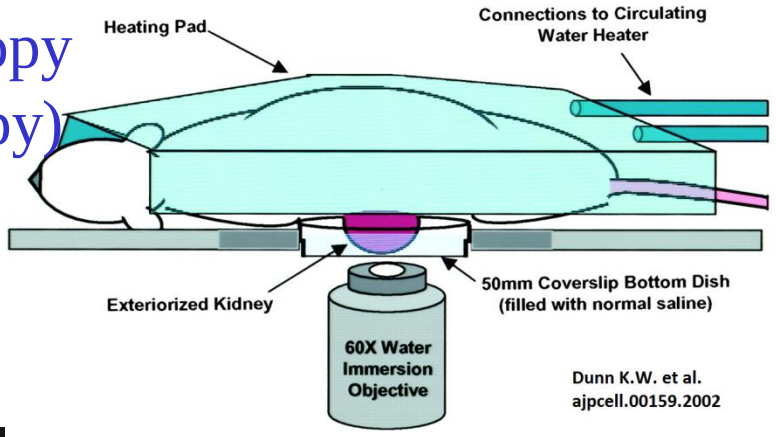
Integrated Bio-Assay System

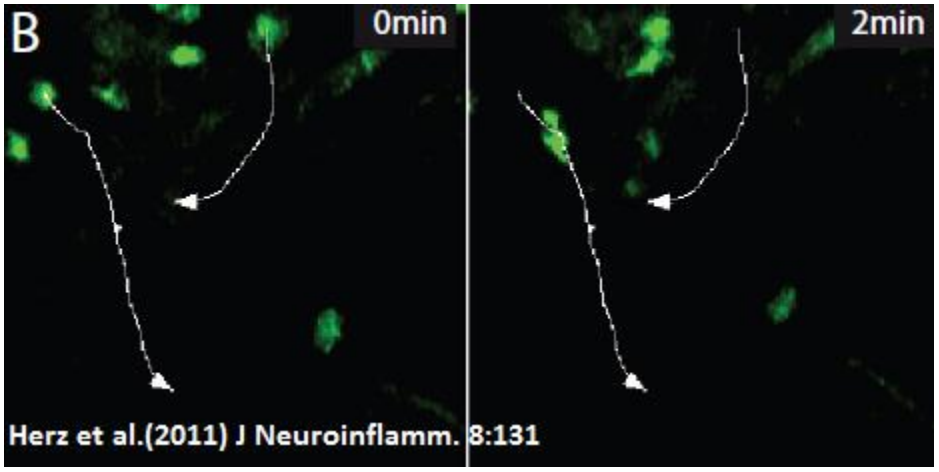




Intra-vital Imaging

- Two-photon laser scanning microscopy
- Raman (SERS and CARS microscopy)
- Positron emission tomography
- Ultrasound, x-Rays
- …





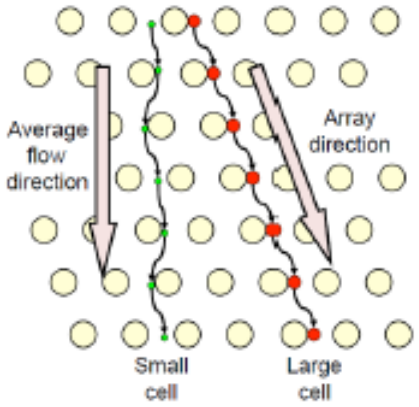
Issues:

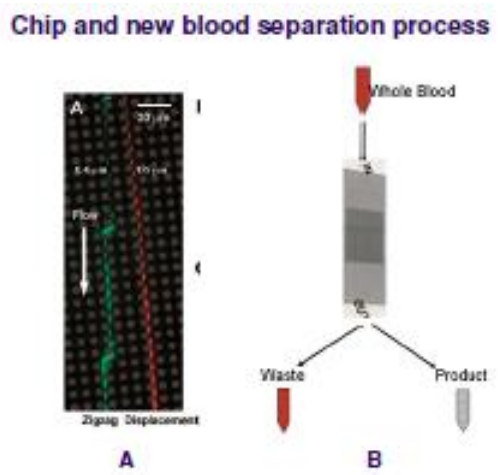
- tissue optics
- object motion
- flow rate
- labeling
- …

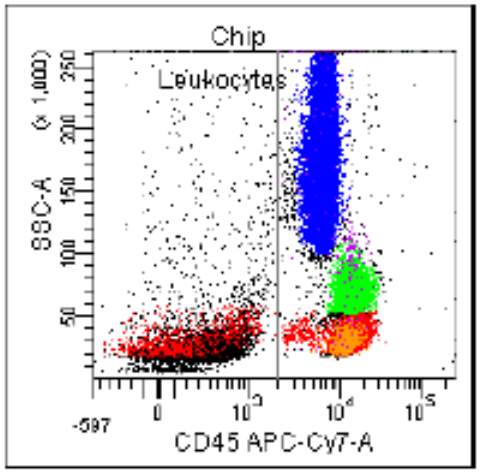
Recent review of in-vivo microscopy: Andresen V, et al. (2012) High-Resolution Intravital Microscopy. PLoS ONE 7(12): e50915

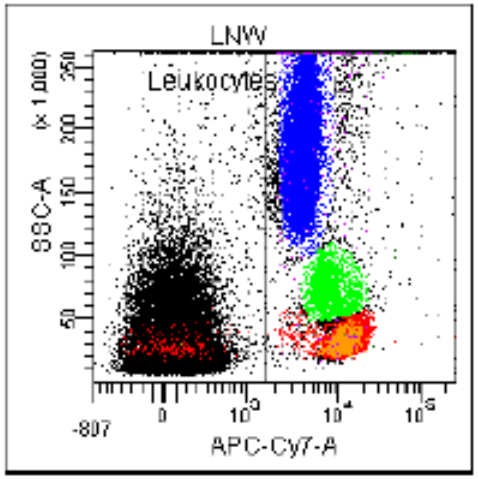
Innovative Sample Preparation

Microfluidic system for leukocyte isolation and cell washing (deterministic lateral displacement)







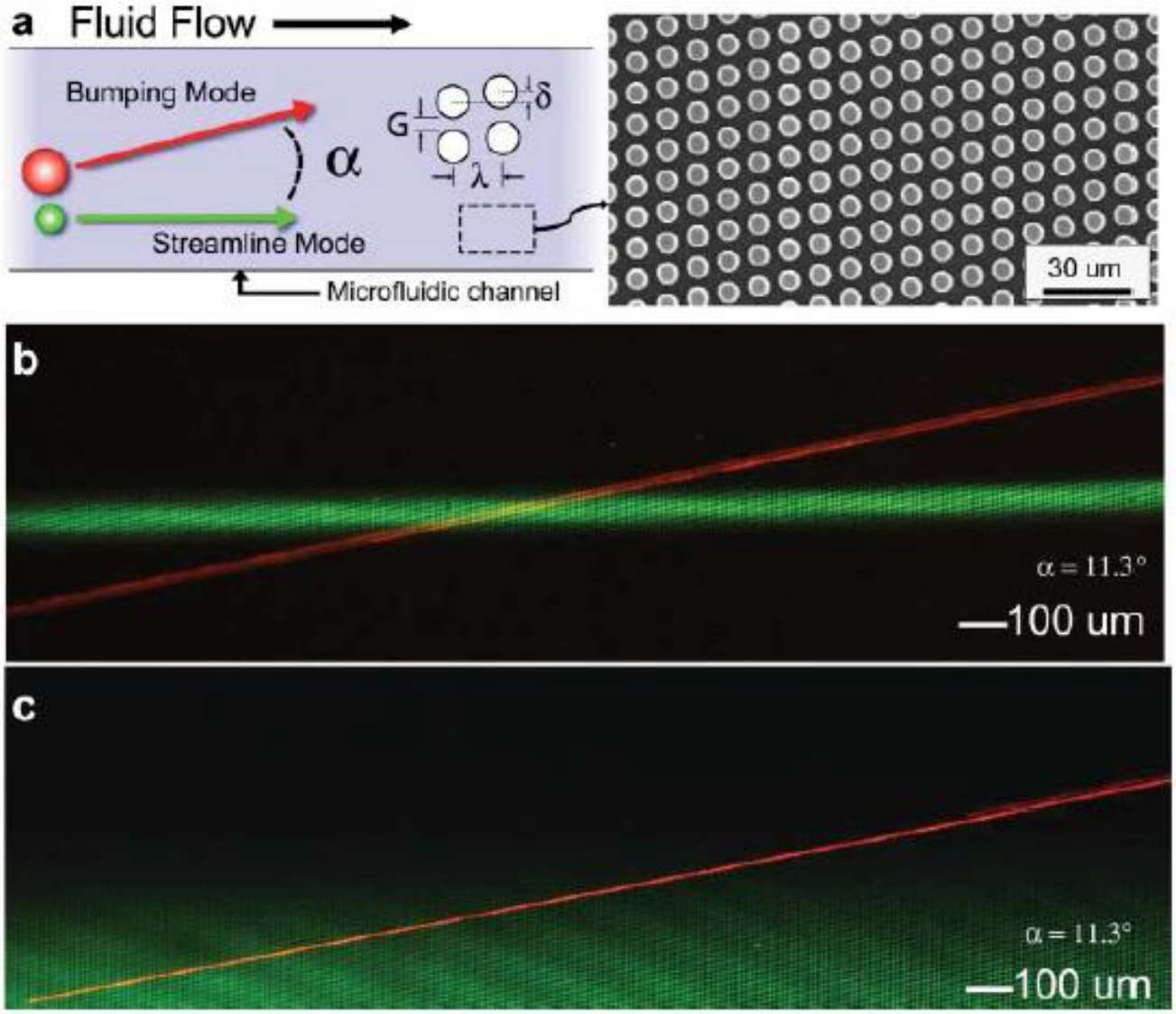


also:

- acoustic focusing
- microfluidic filters
- inertial flow
- magnetic nanoparticles
- high density particles
- dielectropheresis
- optical traps
- …

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

Deterministic Lateral Displacement



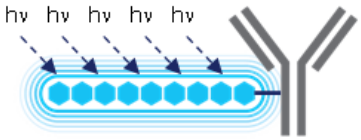
Separating particles by size, adding reagent, mixing, and washing

From: Morton K J et al. Lab on a Chip 2008

Bright Fluorescent Polymer Dyes (BD Sirigen)

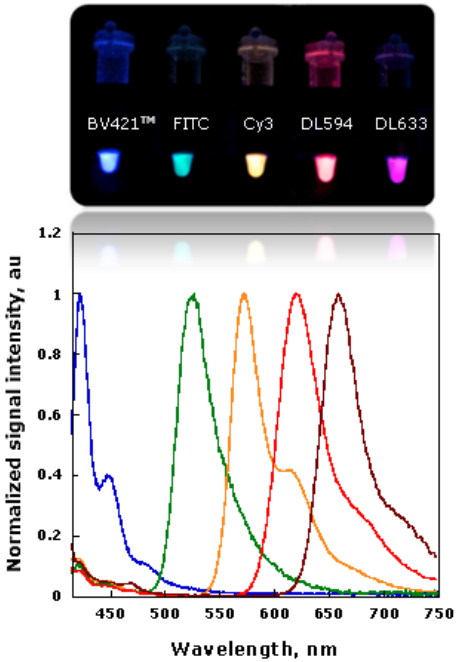
Polymer Based Fluorochromes

- 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



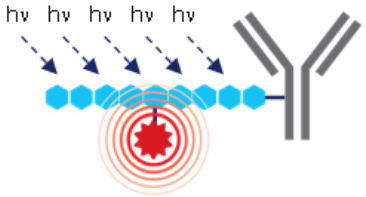
Brilliant Violet 421™

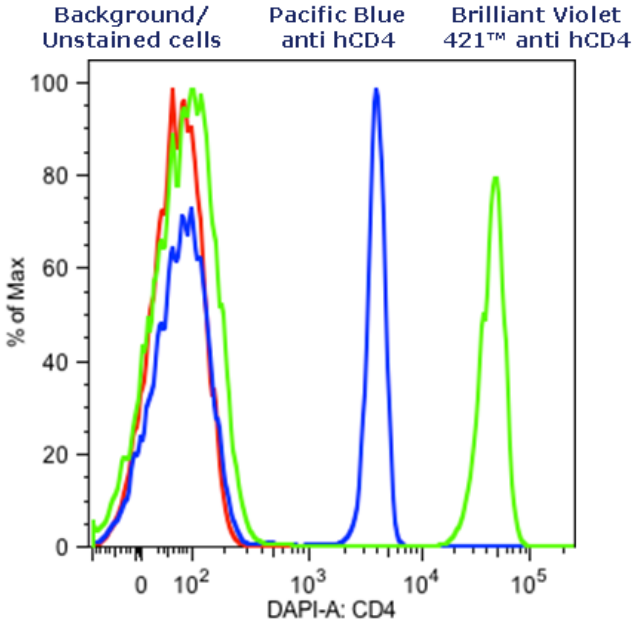
- PE level performance w/ 405nm Laser
- >10x the Stain Index of Pacific Blue
- Enables detection of low abundance targets in multicolor assay panels (e.g. CD56, CD127, etc.)
- Wide range of Ab clones validated



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





see also: Cytometry A. **81**: 45ff

http://sirigen.com/flow_cytometry.html

Novel Affinity Reagents

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

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
- Brent Gaylord
- Mike Brasch
- Ben Verwer
- ...

  above all BD

- Holden Maecker, Stanford
- Bob Hoffman, consultant
- Martin Buescher, Miltenyi
- ...

- BD Biosciences
- AmCell Corp/ Miltenyi Biotec
- …

Contact

Email: diether@desatoya.com

Phone: USA-408-658-6074

URL: http://www.desatoya.com