Flow and Image Cytometry

Optimized Single Cell Measurements and Emerging Technologies

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

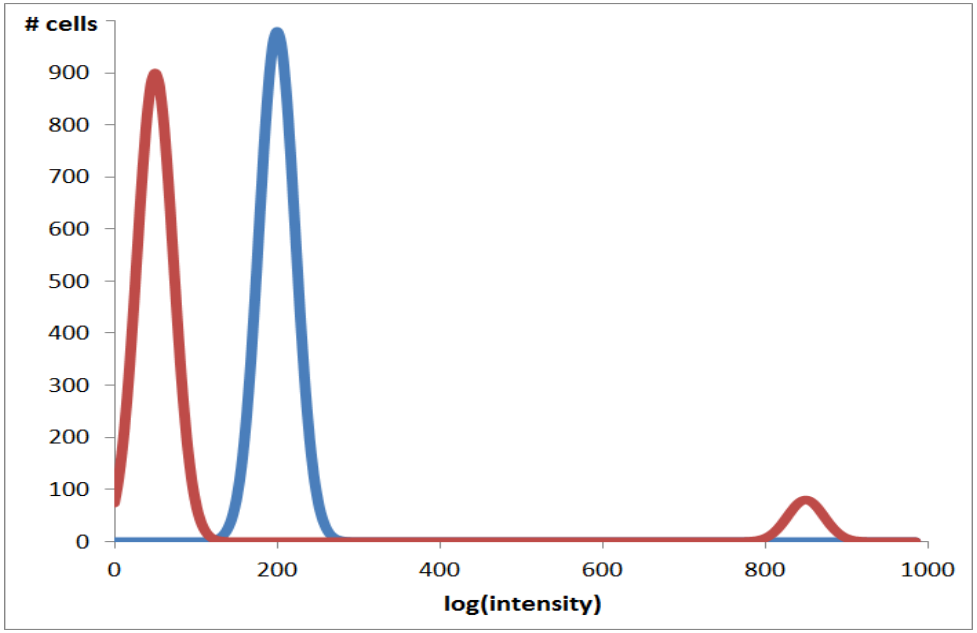
NMR
X-ray imaging
Ultrasound
2-photon imaging
In-vivo cytometry
Light microscopy
Electron microscopy
Flow cytometry
Cell imaging
NA sequencing
Mass spectrometry
TIRF microscopy
Electrophoresis

Contrast agents
Affinity reagents
- antibodies
- probes
Enzyme substrates
Labels
- absorbance
- fluorescence
- element tags

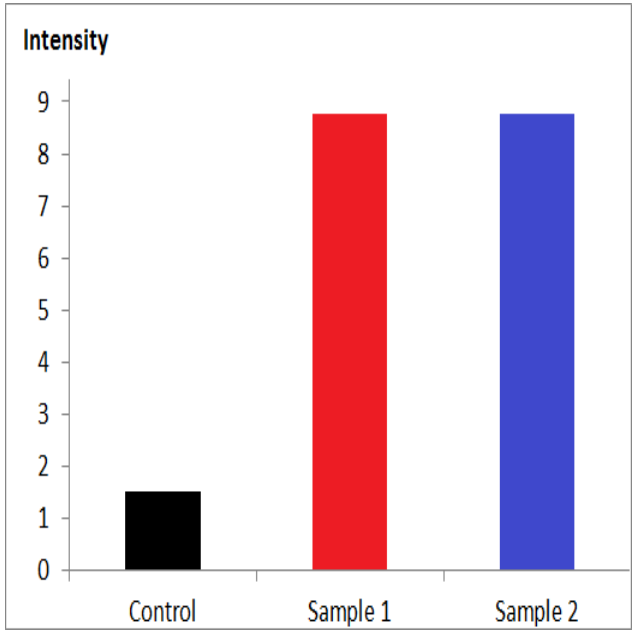
Sample prep

Why Single Cell/Particle Analysis

Intensity Histogram for Single Particles



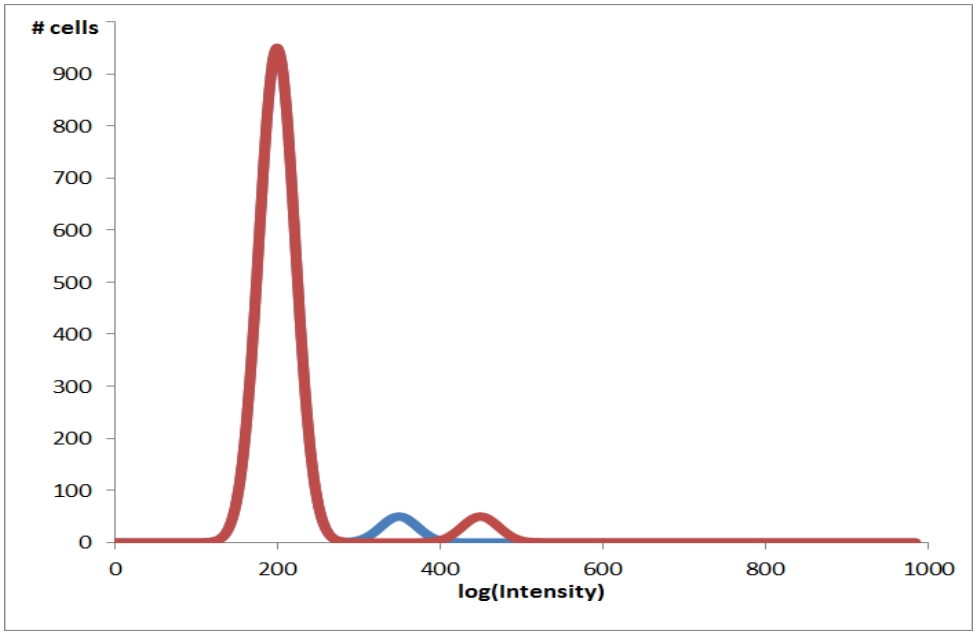
Intensity per Sample



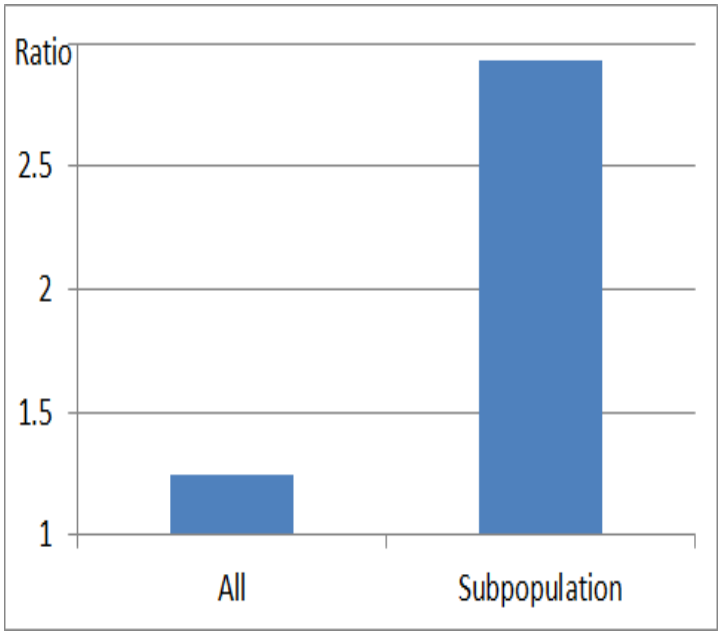
Cell by cell intensity analysis detects population heterogeneity.

Benefits of Subset Specific Analysis

Intensity Histogram



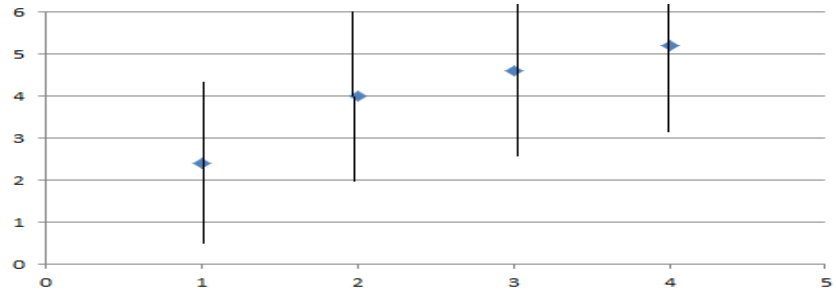
Intensity Ratios



Subpopulation analysis detects changes better, especially for rare subpopulations.

Particle 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



Applications:

- Cell Counting
- Molecule Counting
  - o Digital PCR
  - o Immunoassays

Ignoring Counting Statistics Can Lead to Erroneous Conclusions

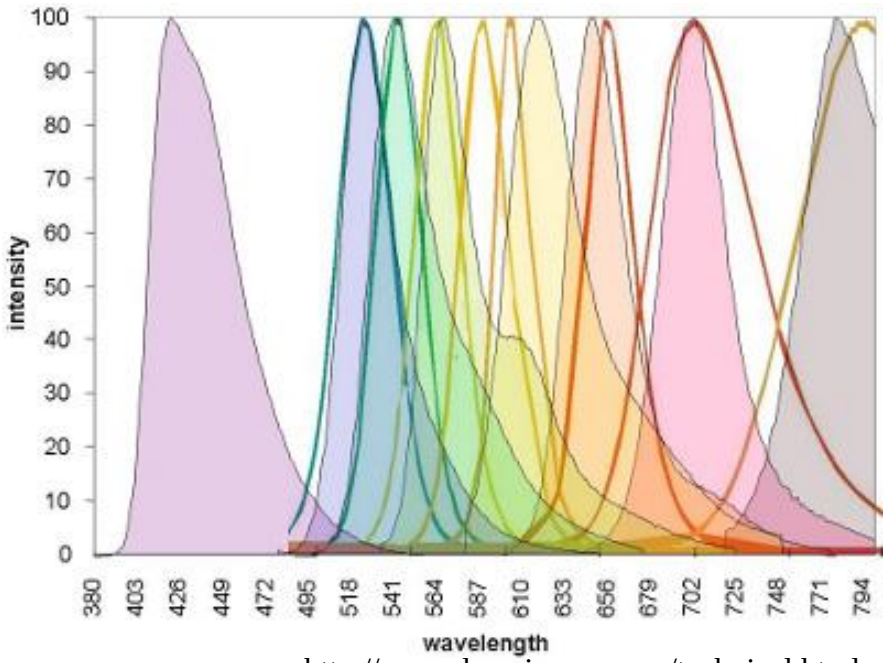
Flow and Imaging Cytometry Features

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 (CyTOF)
- Electrical properties e.g. impedance
- …



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

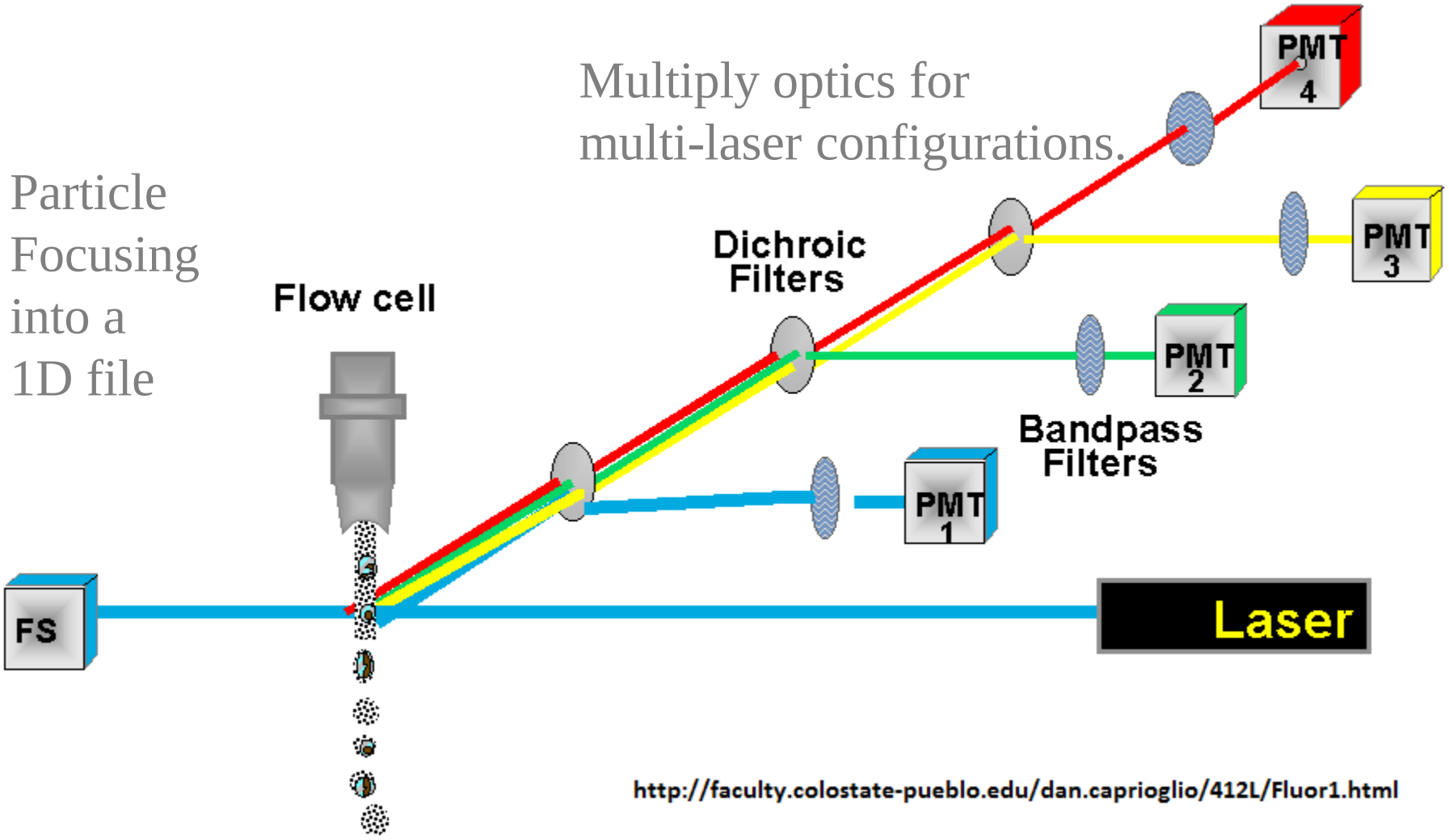
Flow Cytometry Instrument Companies

...

Flow Cytometer Components

Particle Focusing into a 1D file

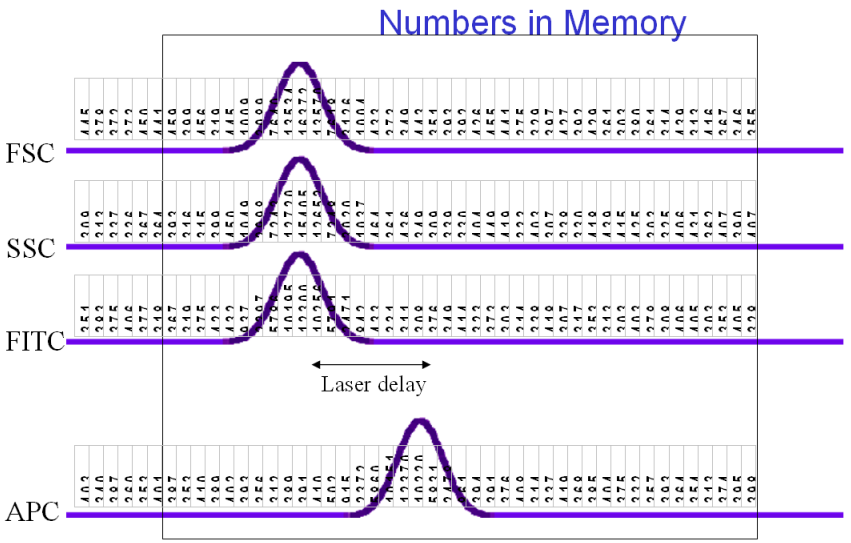
Multiply optics for multi-laser configurations.



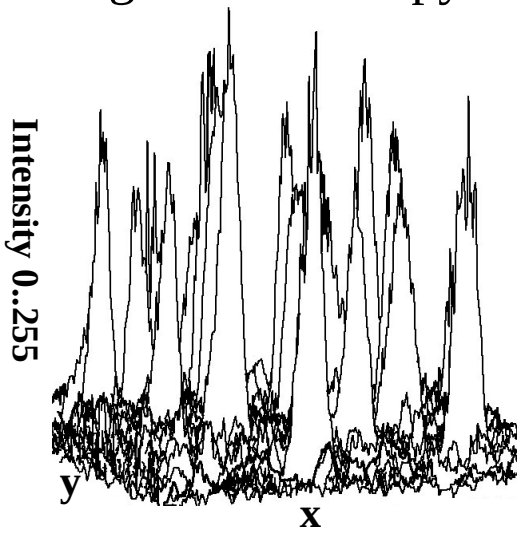
http://faculty.colostate-pueblo.edu/dan.caprioglio/412L/Fluor1.html

Basic Data Processing

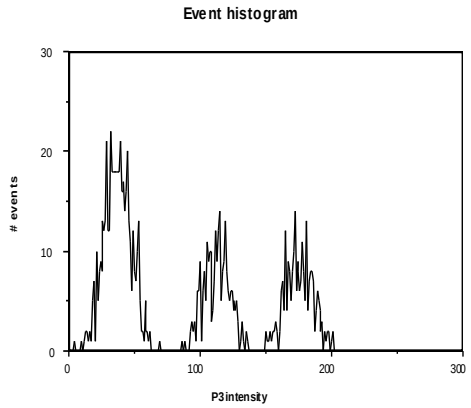
Flow Cytometry

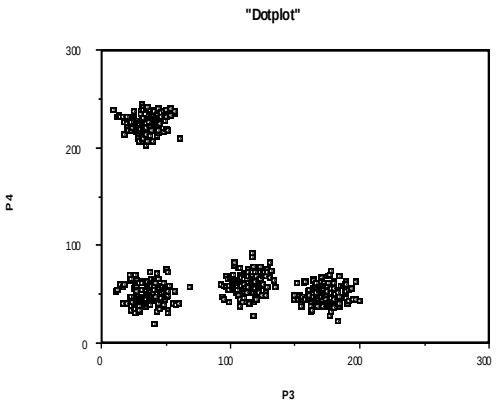


Digital microscopy



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

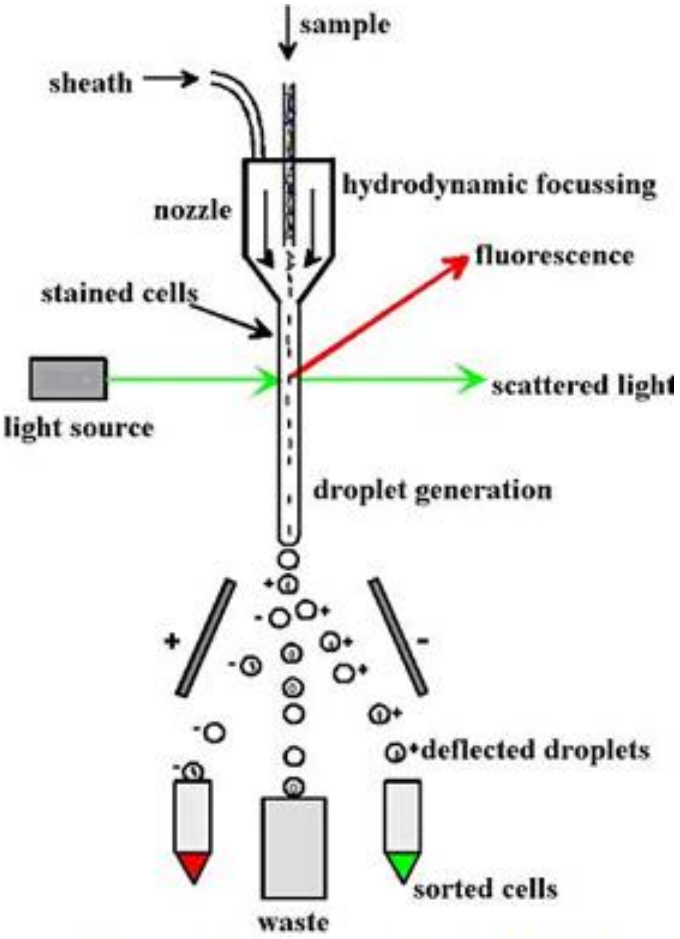




for >2 parameters: gating, cluster analysis, …

For many samples and parameters: bioinformatics

Cell Sorting



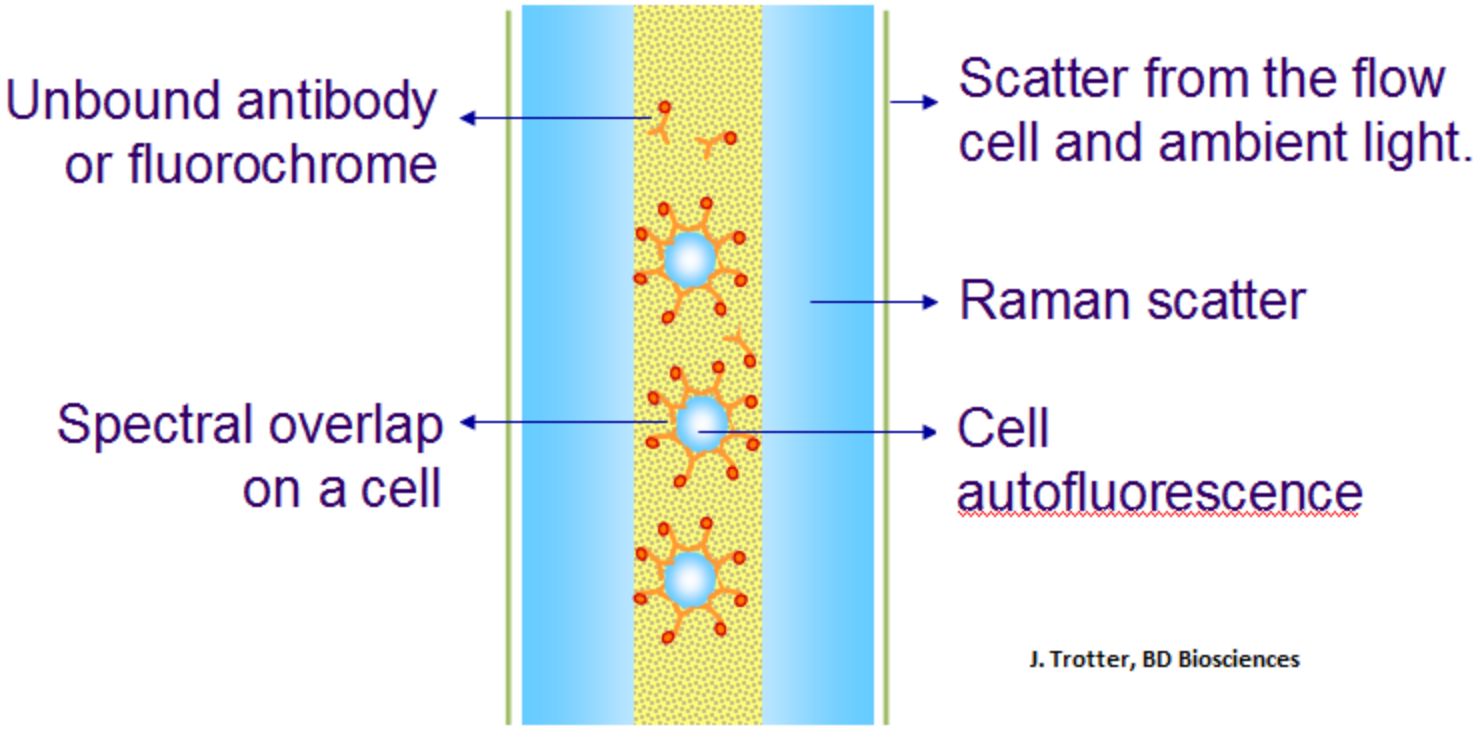
Applications Examples

- Chromosomes
- Strain Improvement
- Genomics (incl. single cells)
- Proteomics (cell subsets)
- …

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

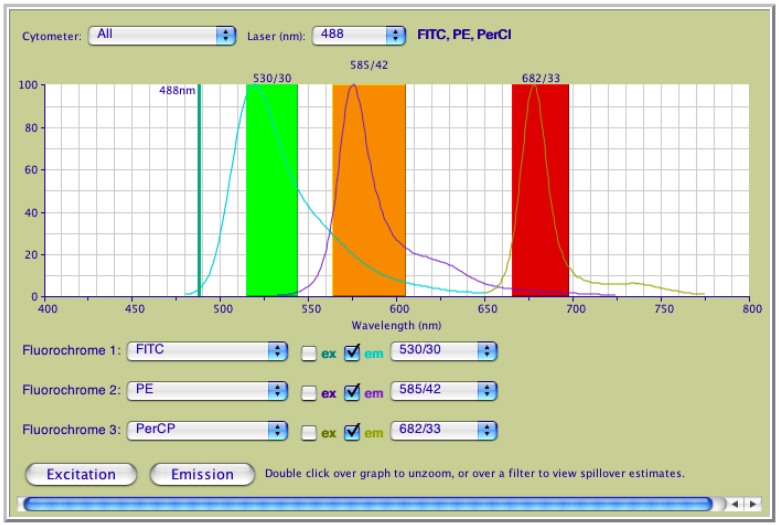
Instrument Evaluation Br

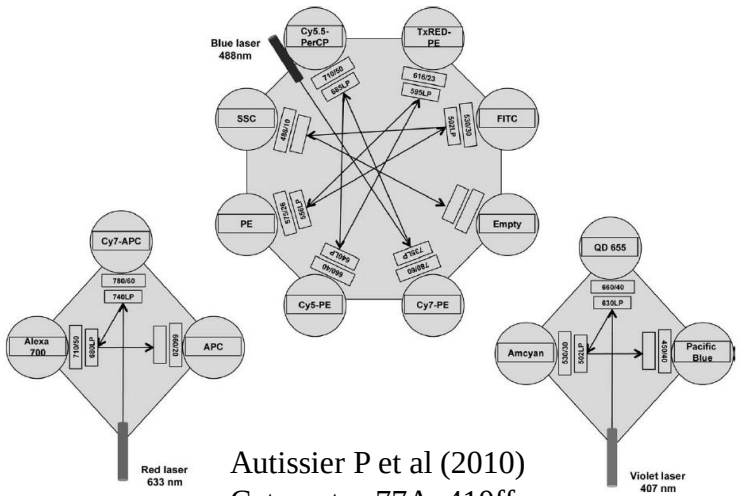
Relative B (Br) is a measure of true optical background in the fluorescence detector.



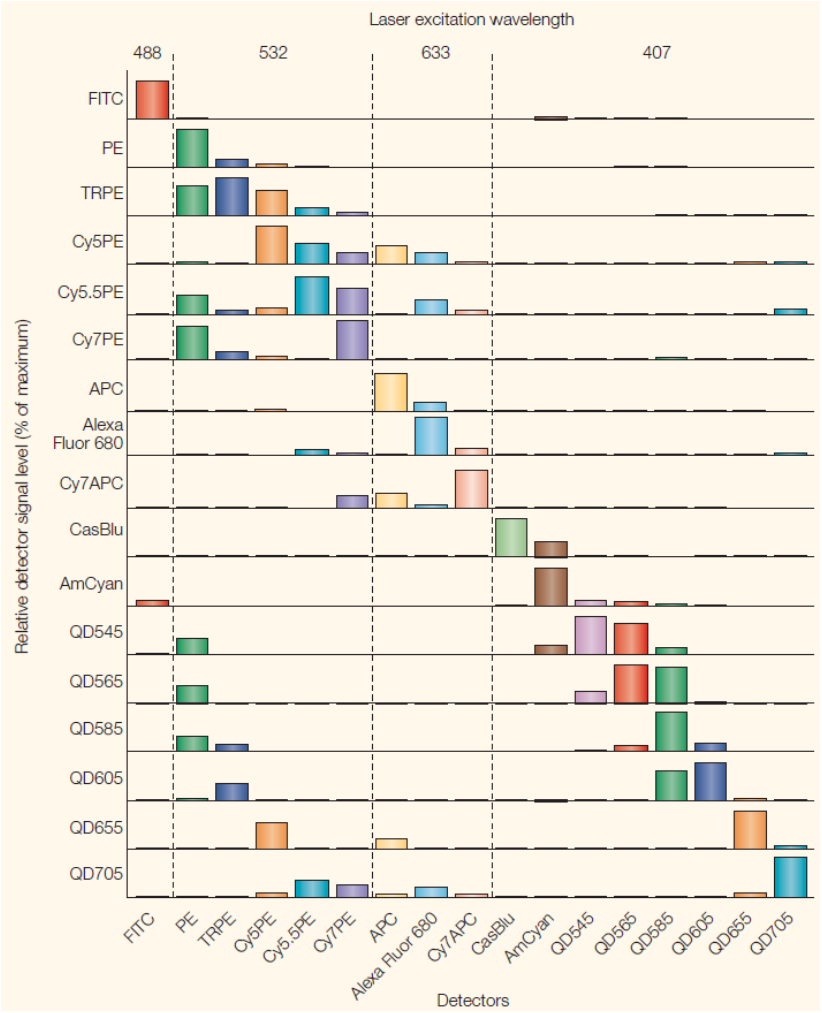
Filter Arrangement and Spectral Overlap

(less relevant for CyTOF element mass cytometry, different for “spectral cytometry”)



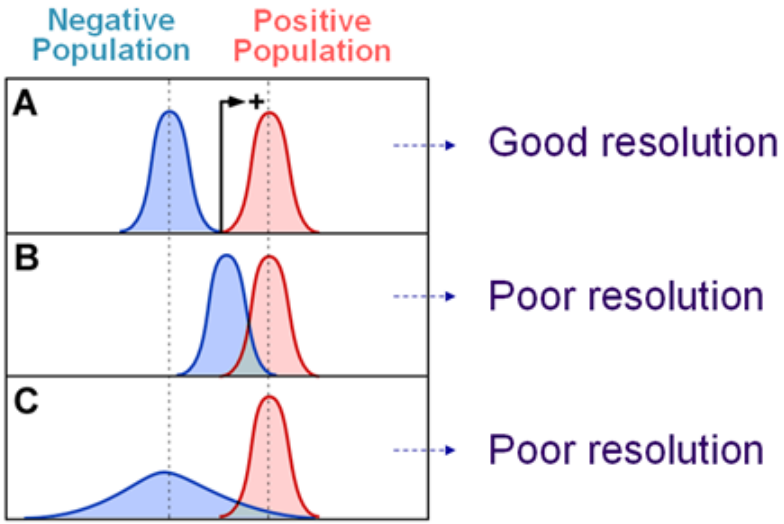


Autissier P et al (2010)
Cytometry 77A, 410ff



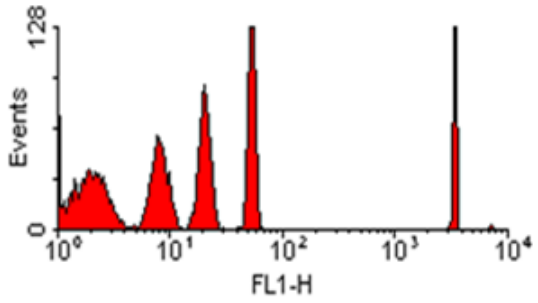
Perfetto SP et al (2004)
Nature Reviews Immunology 4, 648ff

Instrument Evaluation Qr

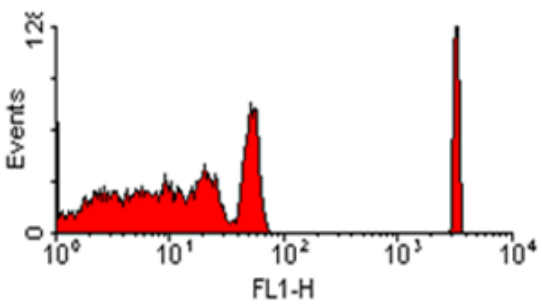


$$Qr = \frac{\#\ \text{photoelectrons}}{\#\ \text{fluorescence molecules}}$$

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:

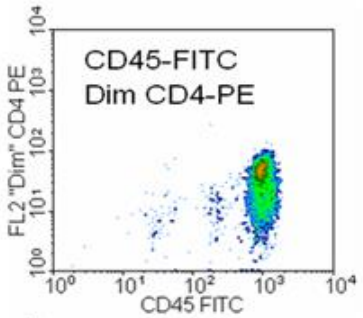
$$SD_{optical} = \sqrt{SD_{PE}^2 + SD_{FITC}^2 + SD_{background}^2}$$

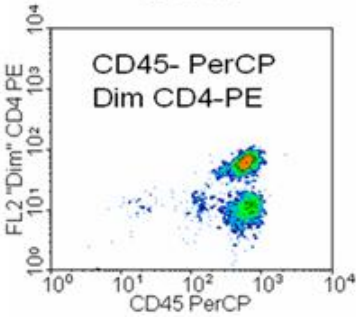
Reagent performance

Stain index

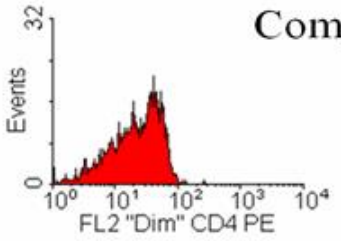
$$\frac{Medium_{pos} - Medium_{neg}}{2 * SD_{neg}}$$

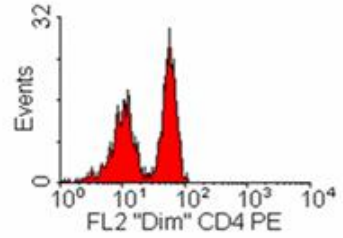
- Dye properties (brightness and spectral overlap)





Compensated data



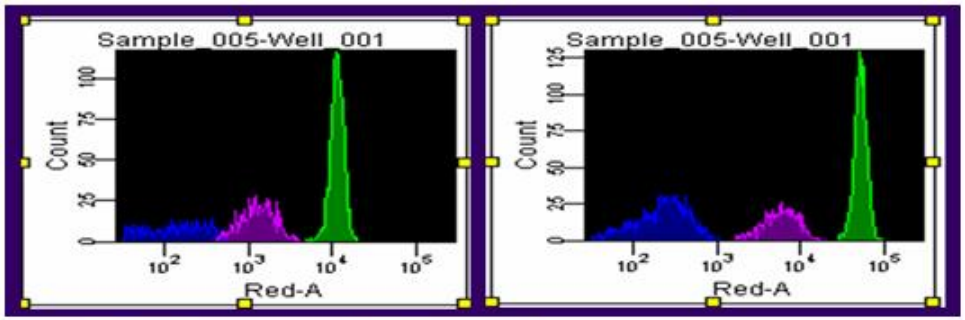


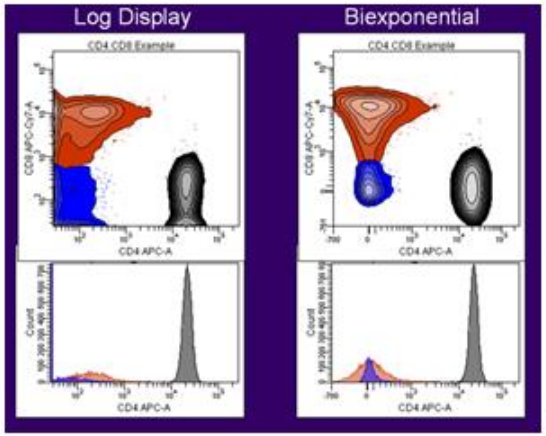
Better separation with less spectral overlap.

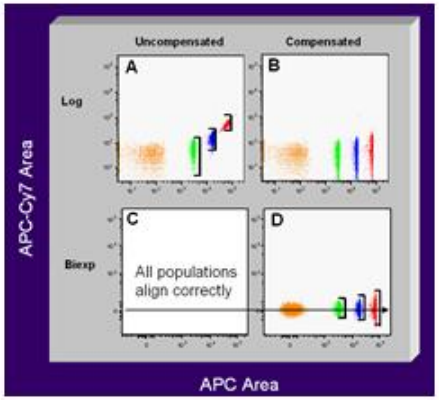
J. Trotter, BD Biosciences

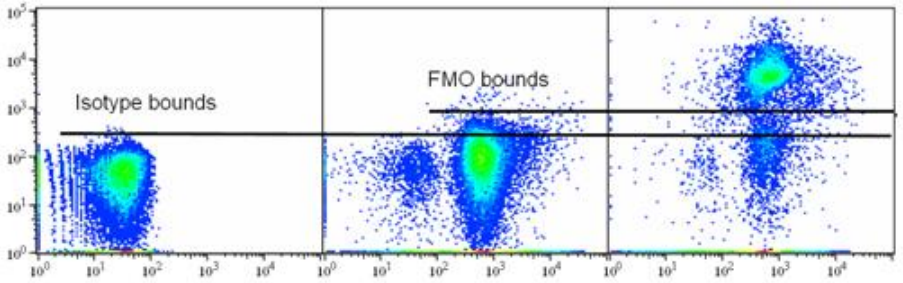
Optimizing cytometry measurements (II)

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









J. Trotter, BD Biosciences

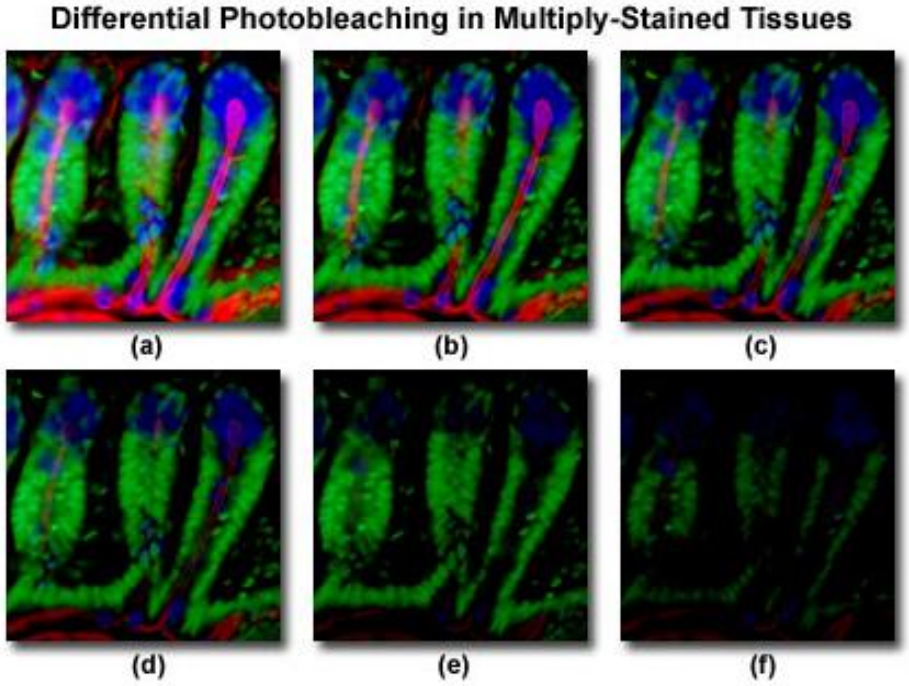
Multi-parameter Fluorescence Cytometry Points To Consider

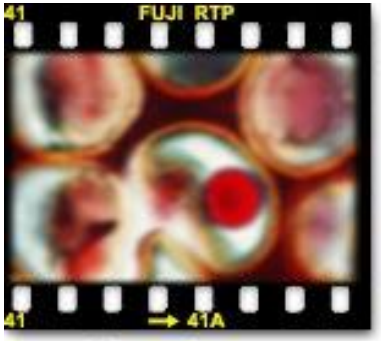
- Know your instrument status e.g. Qr & Br for different channels
- Use high enough gain settings to maximize sensitivity
- An antibody/dye combination that marginally allows discrimination of positives/negatives in a single color assay is unlikely to contribute anything helpful in a multicolor experiment.
- 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

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

- Recent enhancements for more parameters and higher resolution
- Brighter labels
- Novel affinity reagents (antibodies)
- More low complexity cytometers for cell (subset) counting
- Novel cell sorters
- Integrated Cell Analysis System
- Innovative sample preparation
- Intra-vital (in-vivo) microscopy

Recent Systems 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 and Spectral Flow Cytometry

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

- Sequential Stain De-stain Cytometry

(Cytometry, 2009, 75A(4), pp 362-370)

- Highly fluorescent polymer dyes (Brilliant violet, …)

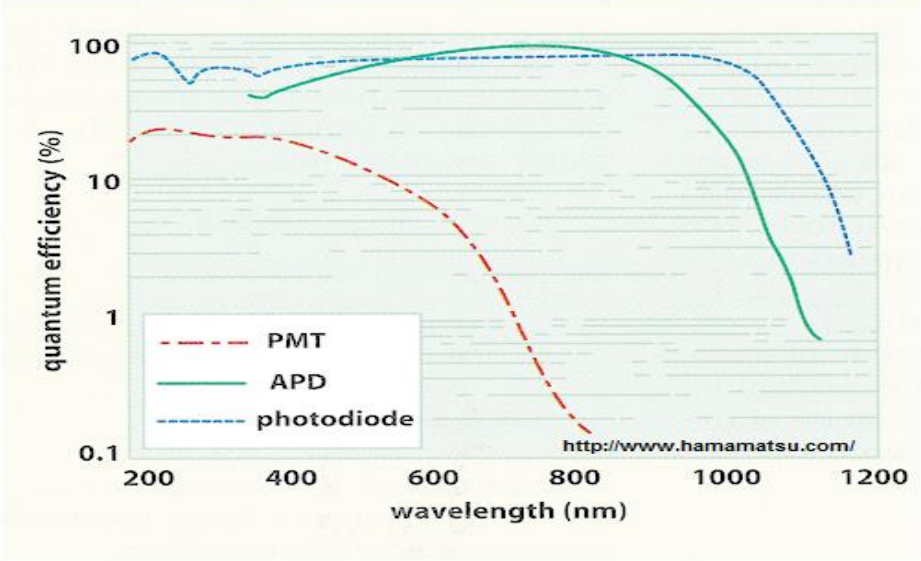
(Cytometry, 2012, 81A(6), pp 456-466, http://www.sirigen.com/sirigen_technology.html

http://www.bdbiosciences.com/documents/multicolor_fluorochrome_laser_chart.pdf)

New Detector-Label Combinations

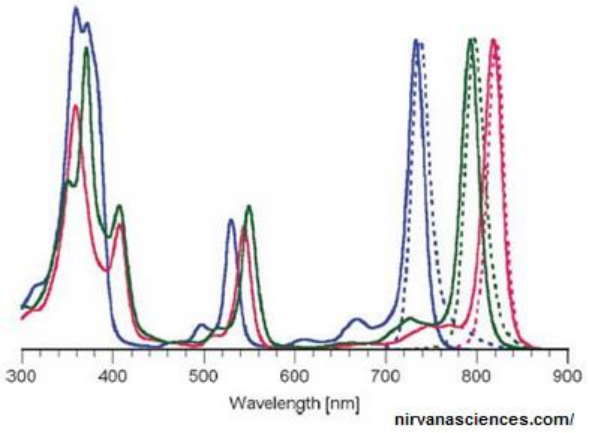
- New photodetectors extend the available spectrum

  (Si avalanche photodiodes extend detection into the far infrared, Xitogen system))



- New dyes add excitation in the UV, some detection in the IR

  (Fluorescent polymers, bacteriochlorins, …)



New Detection Technologies

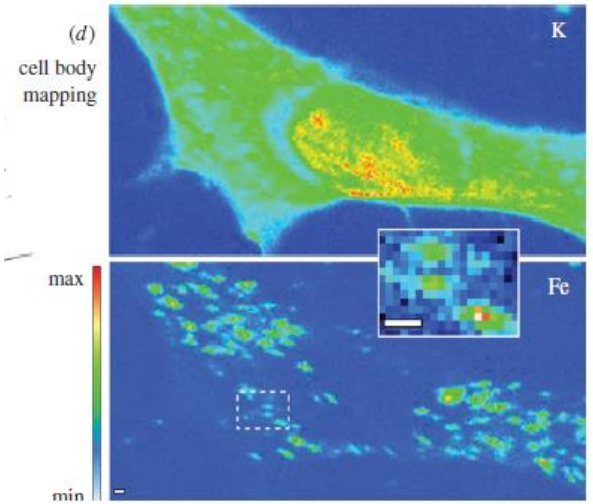
- High spatial resolution and multi-parameter capability with X-ray / synchrotron radiation fluorescence

  (super-high resolution with element labels or direct element imaging)
- Medium resolution, multi-parameter mass spectrometric imaging

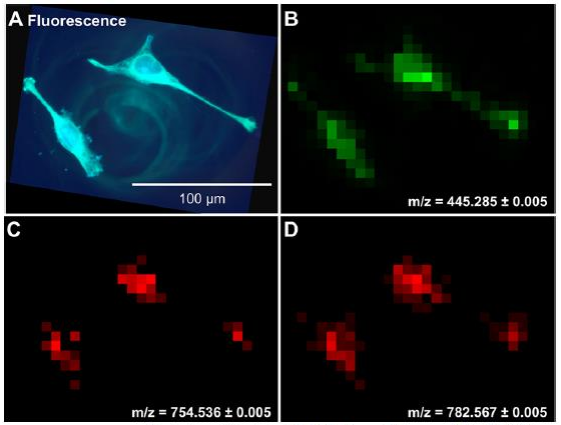
  (CyTOF like element labels, direct metabolite or structural component detection)
- Label-free imaging with Raman

  (measuring cellular components by their Raman spectra)
- Label-free high resolution NMR imaging

  (direct chemical detection)



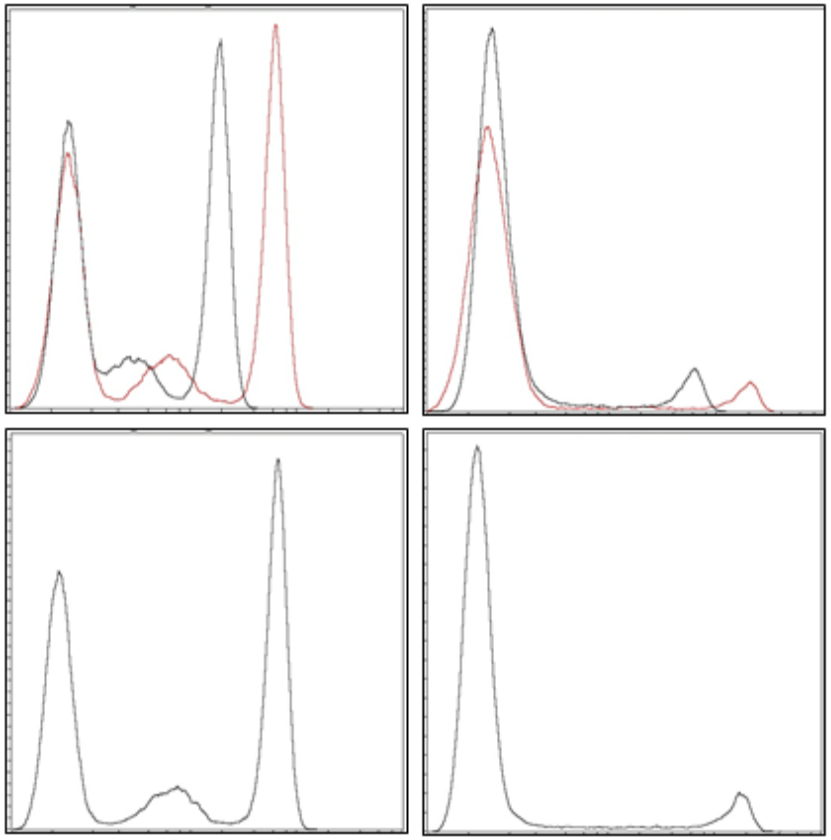
Ortega R et al (2009) J.R.Soc Interface 6: S649-S658



Schober Y et al. (2012) Anal.Chem. 84, 6293ff

More New Dyes

FITC conjugates become as bright as PE-conjugates!



CD4-FITC
CD4-VioBight FITC
(clone Vit 4.3)

CD8-FITC
CD8-VioBright FITC
(clone BW135/80)

CD4-PE
(clone Vit 4.3)

CD8-PE
(clone BW135/80)

Dr. Christian Dose, Miltenyi Biotec

Novel Affinity Reagents

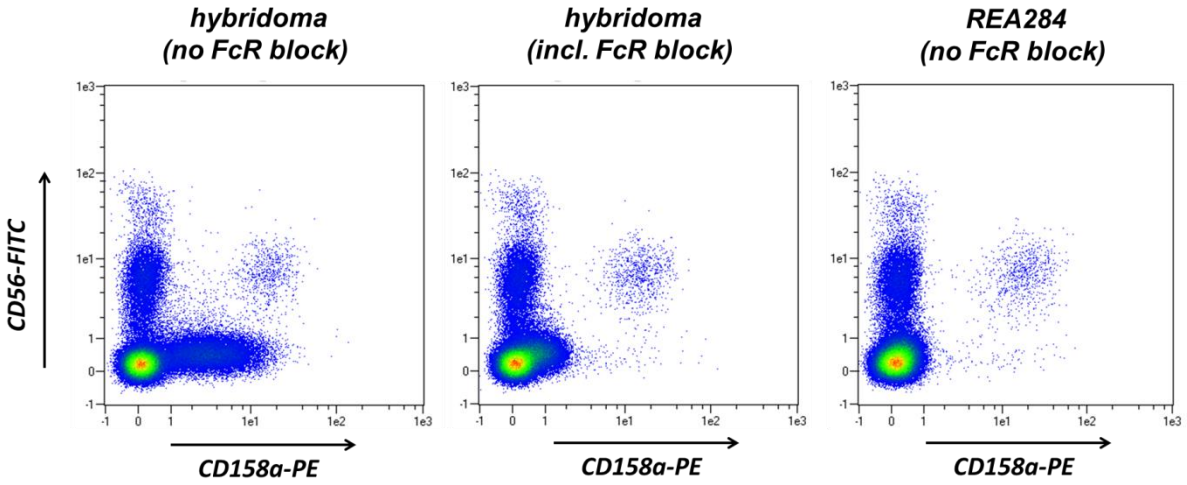
Antibodies

- 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
- …

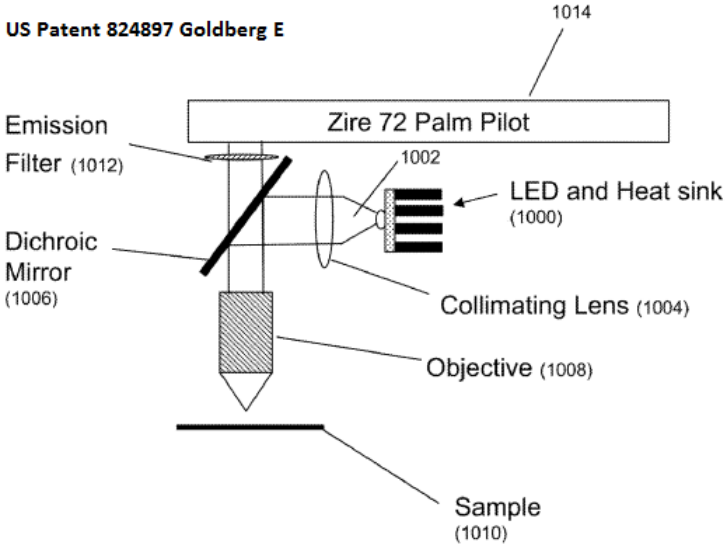
Fodey T et al; Trends in Anal. Chem. 30(2011) 254ff



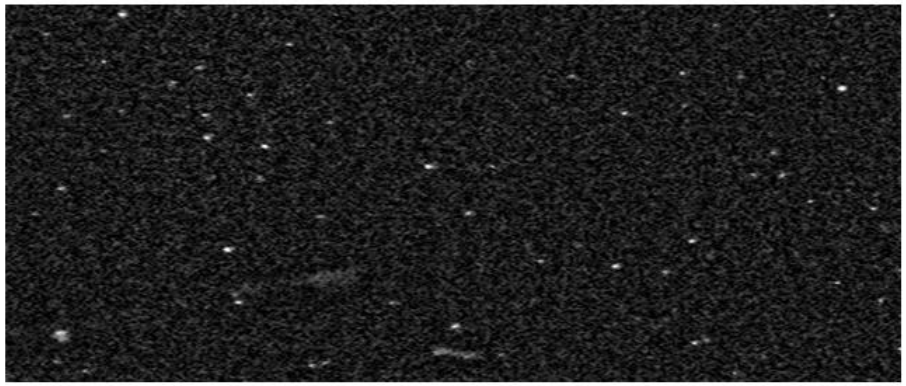
Fc-receptor binding:
CD158a-PE on PBMC

Dr. Christian Dose, Miltenyi Biotec

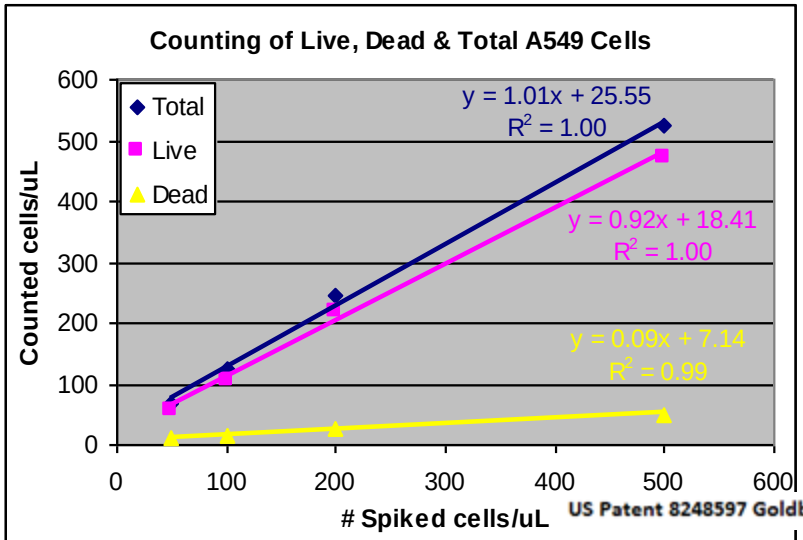
CCD Technology for Low-complexity Cytometers for Cell Counting



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

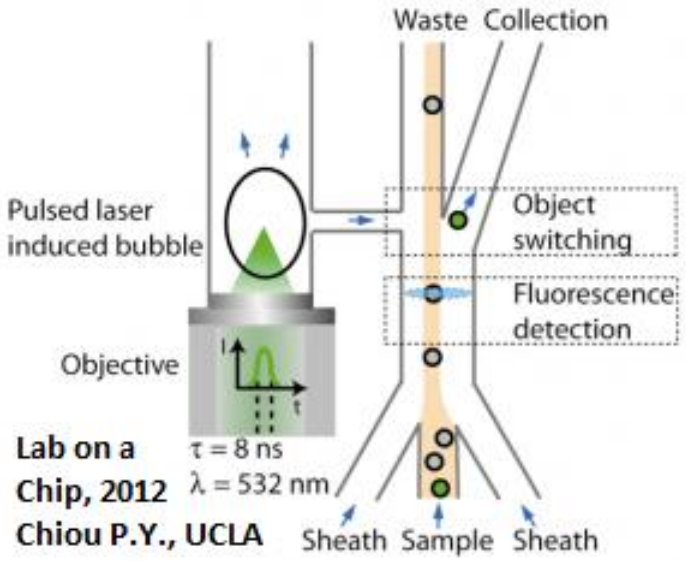


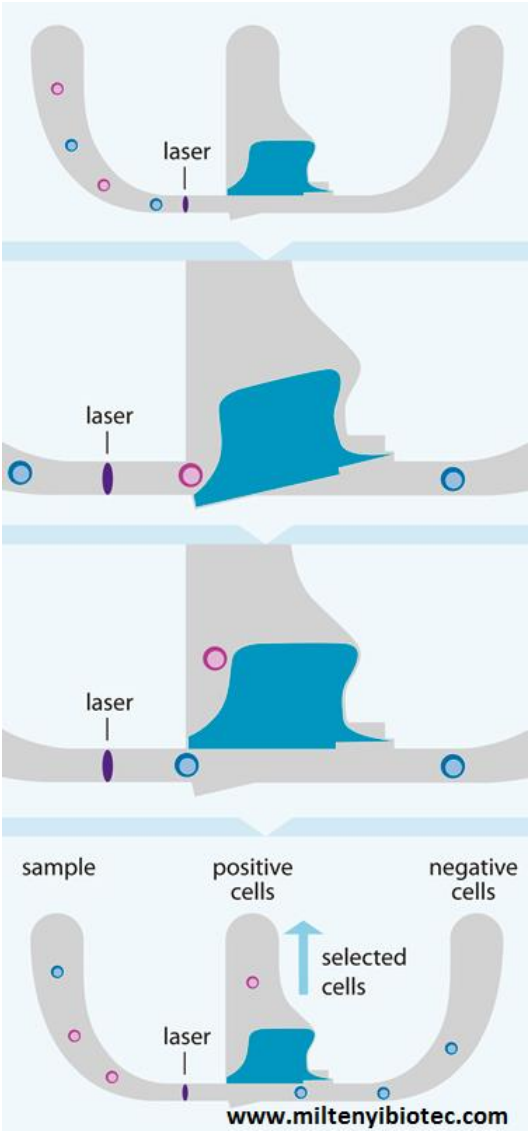
US Patent 8248597 Goldberg E



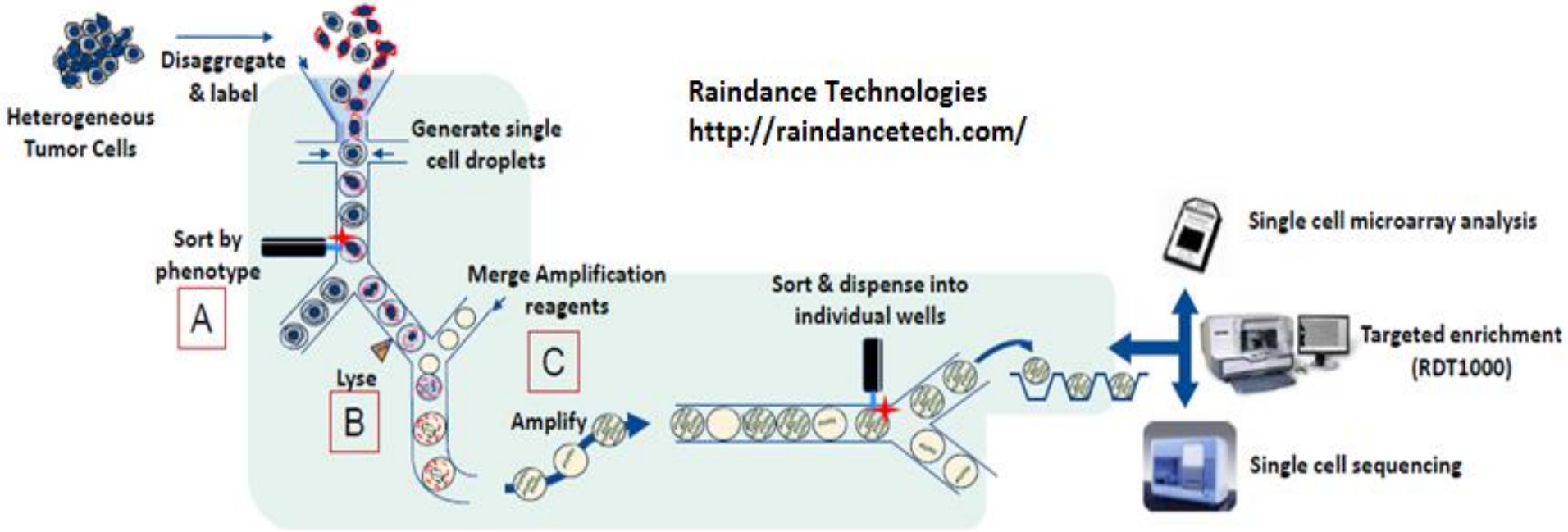
US Patent 8248597 Goldberg E

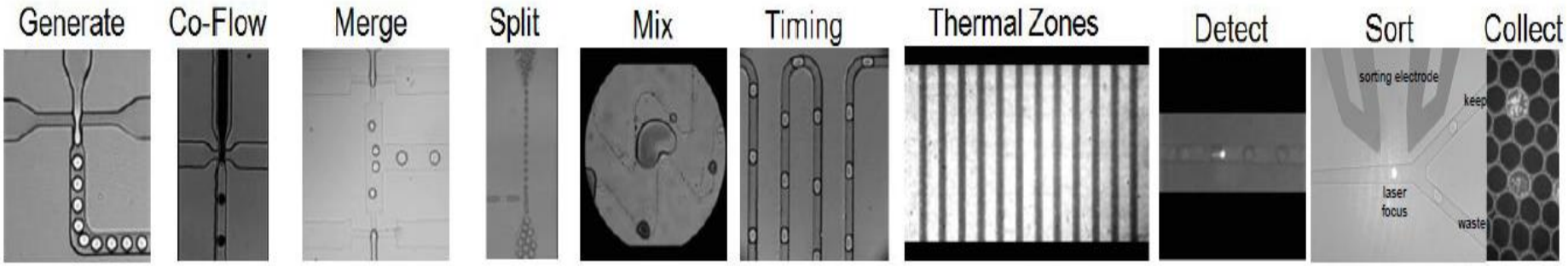
More Cell Sorting Technologies





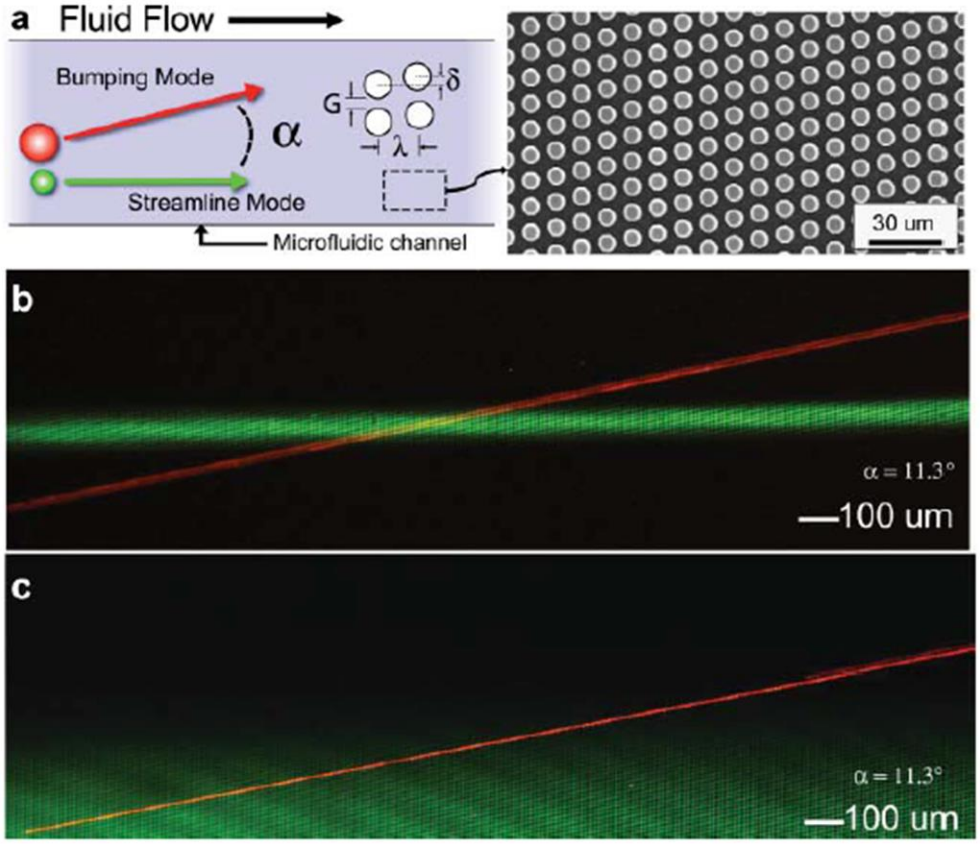
Droplet-based Integrated Bio-Assay System Technology





Innovative Sample Preparation

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



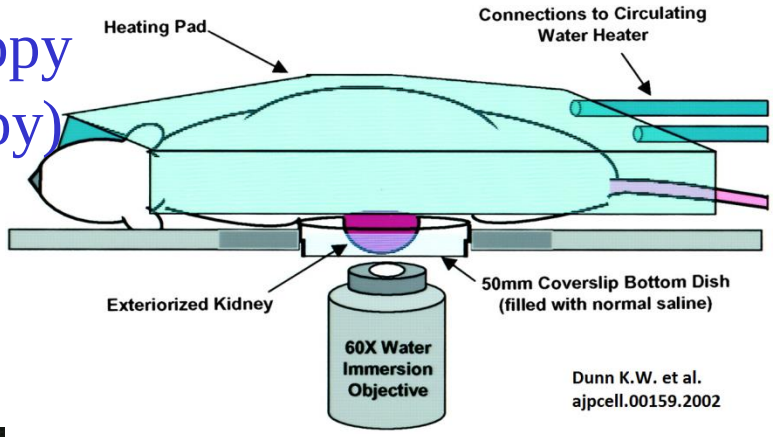
also:

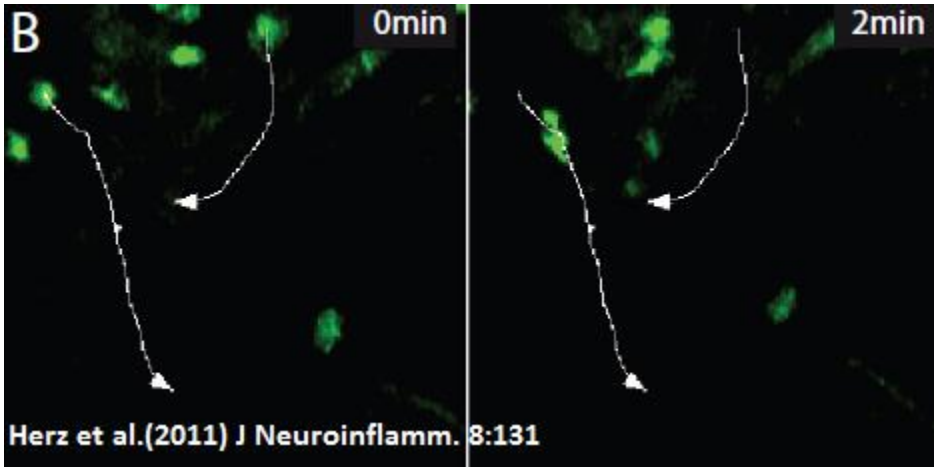
- acoustic focusing
- microfluidic filters
- inertial flow
- magnetic nanoparticles
- high density particles
- dielectropheresis
- optical traps
- …

1. Davis JA et al (2006) PNAS 103: 14779ff
2. Morton KJ et al (2008) Lab on a Chip 8: 1448ff
3. Cyto 2012 poster, Liping Yu et al,
4. Sturm JC et al. (2014) Interface Focus 4: 1-9

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

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
- Maria Jaimes
- Ed Goldberg
- Liping Yu
- Brent Gaylord

above all BD Biosciences

Input from companies:

- **BD Biosciences**
- Miltenyi Biotec
- Beckman Coulter
- Thermo Fisher
- …

- Martin Buescher, Miltenyi
- Christian Dose, Miltenyi
- Holden Maecker, Stanford
- Bob Hoffman
- Ming Yan
- Hrair Kirakossian
- **…**

Contact

Email: diether@desatoya.com

Phone: USA-408-658-6074

URL: http://www.desatoya.com