13th Spring School on Immunology

Principles of Flow and Image Cytometry & Emerging Technologies for Single Cell Analysis.

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Biology Research

Key Modern Technologies

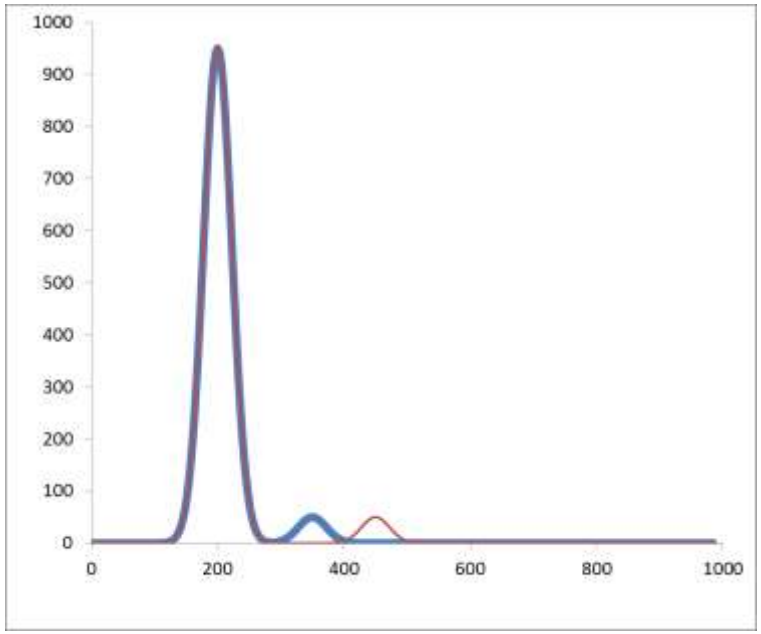
NMR MRI, X-ray imaging, Ultrasound, 2-photon imaging,
In-vivo cytometry, Light microscopy, Electron microscopy,
Flow cytometry, Cell imaging, NA sequencing,
Mass spectrometry, Electrophoresis, ELSISA, …

Information from Cytometric Single Cell Analysis

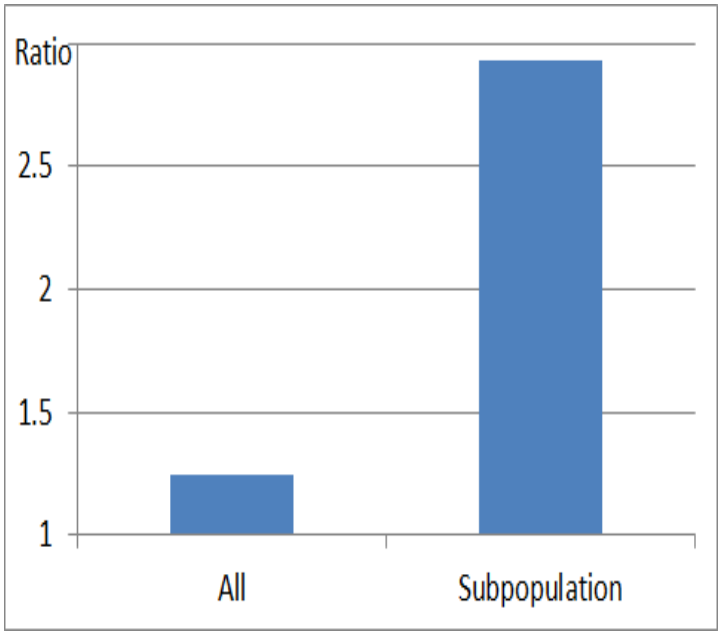
* Cell-concentration
* Subset fractions
* Cell size
* Cell shape
* Cell arrangement in clusters
* Mass of multiple cellular components per cell
* Distribution of component mass in subsets
* Temporal change of the above parameters

Why Subset Specific Analysis

Intensity Histogram



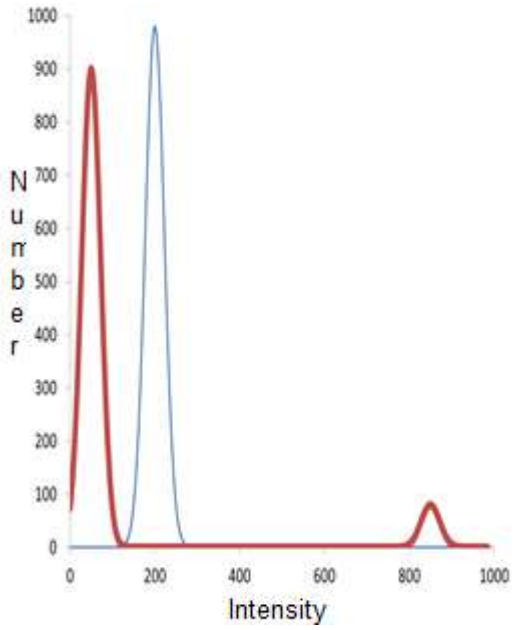
Intensity Ratios



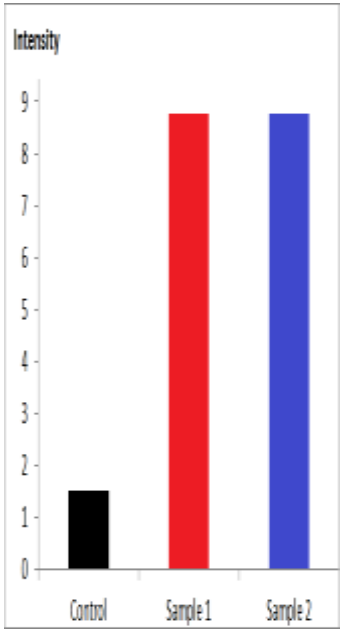
Subpopulation analysis detects changes better, especially for rare subpopulations.

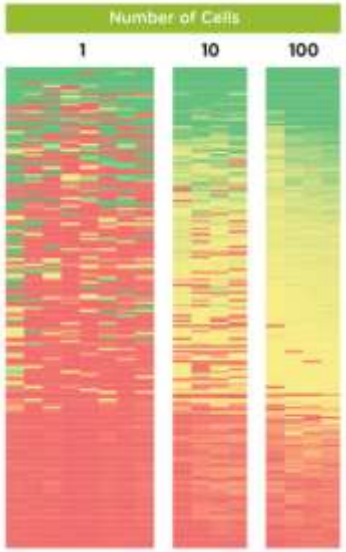
Why Single Cell/Particle Analysis

Intensity Histogram for Single Particles



Intensity per Sample





Source: http://www.nanostring.com

Cell by cell intensity analysis detects population heterogeneity.

Flow Cytometer Components

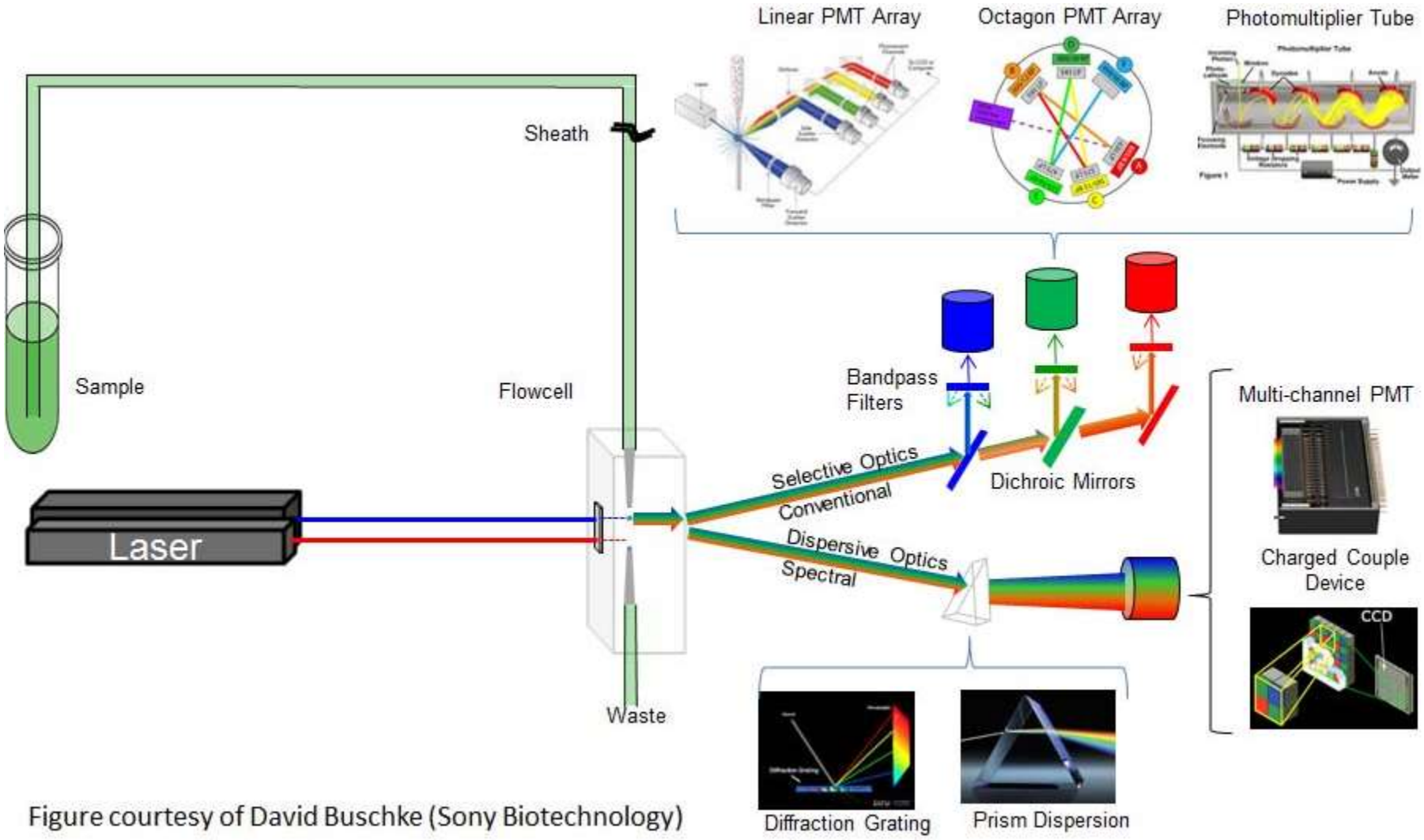
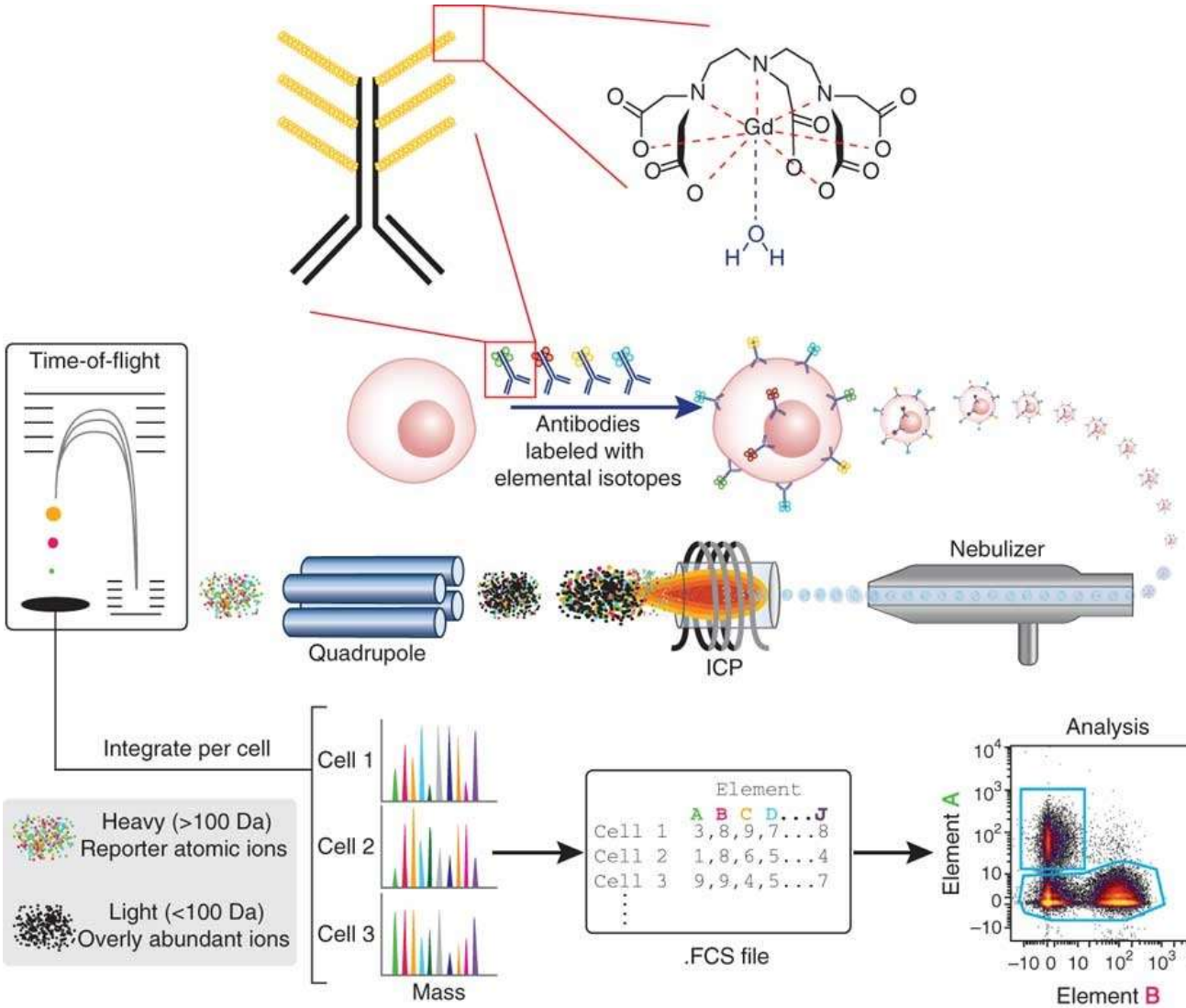


Figure courtesy of David Buschke (Sony Biotechnology)

Dichroic filters vs. Multispectral cytometry: Feher K et al.(2016) Cytometry 89A: 681-9

Mass-Label Cytometer (CyTOF)



Sean C Bendall & Garry P Nolan (2012) Nature Biotechnology 30, 639–647

Imaging Flow Cytometers

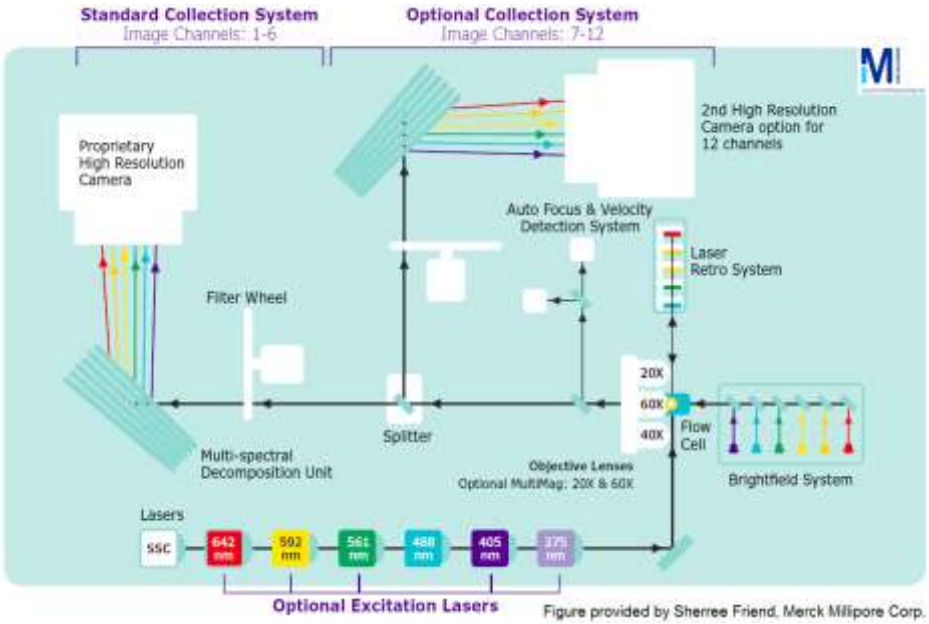
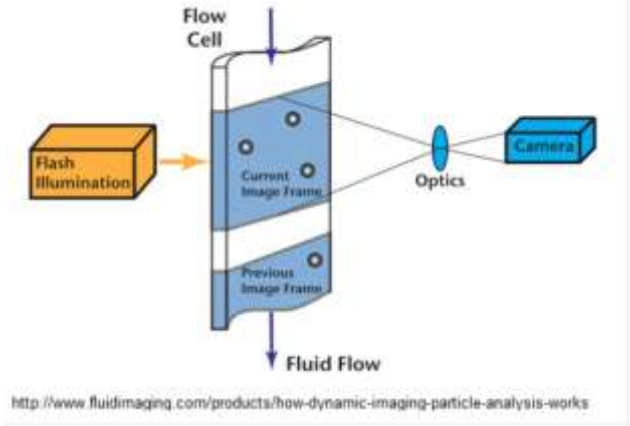
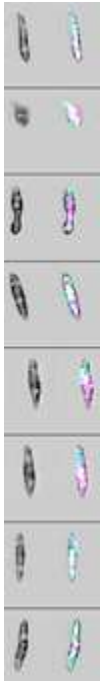
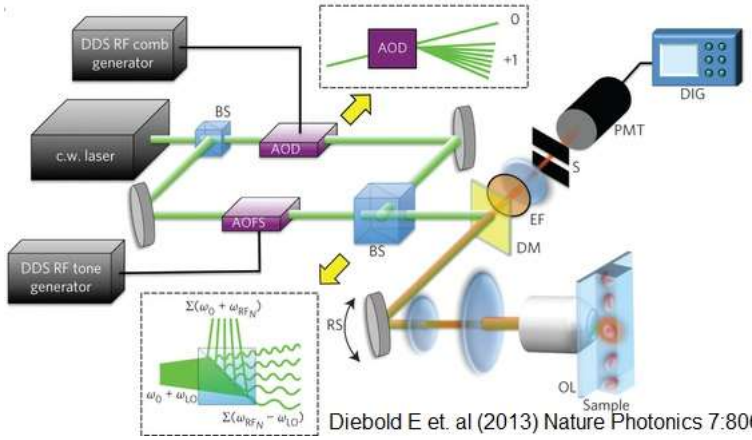


Figure provided by Sherree Friend, Merck Millipore Corp.



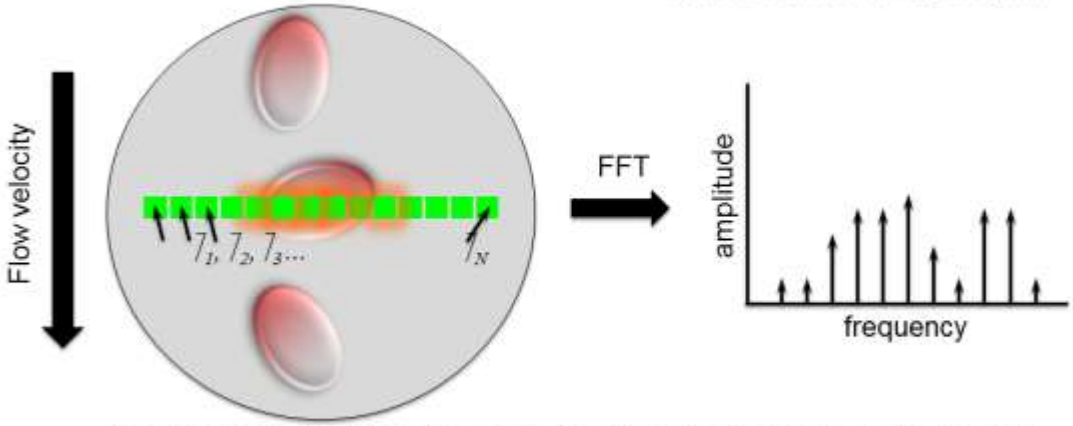




Diebold E et. al (2013) Nature Photonics 7:806ff

Flow cytometer field of view

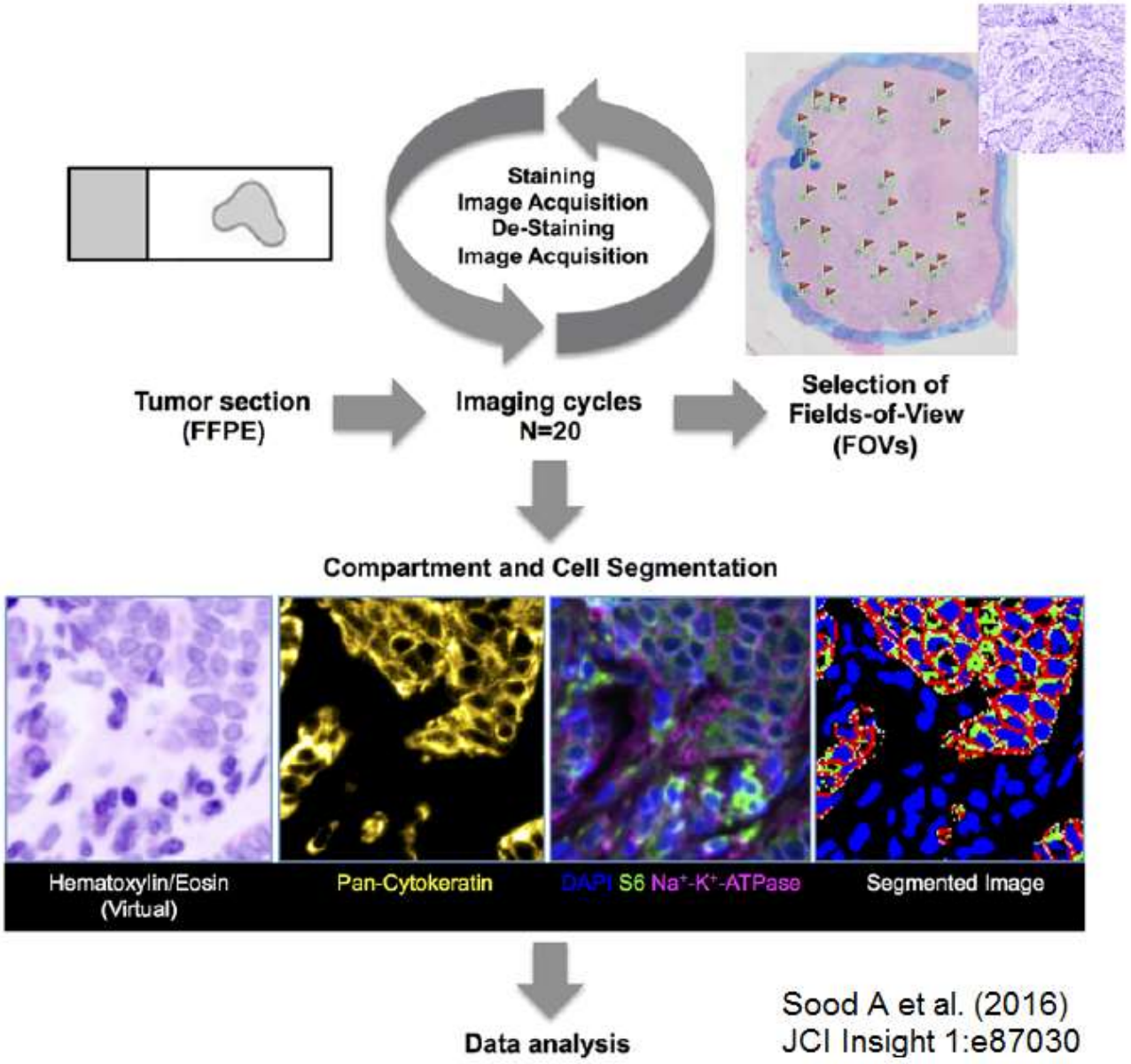
Source: Eric Diebold, Omega Biosystems



Spatial information is contained in frequency domain of fluorescence signal

More info: Barteneva N.S. et al. (2012) Journal of Histochemistry & Cytochemistry 60: 723ff

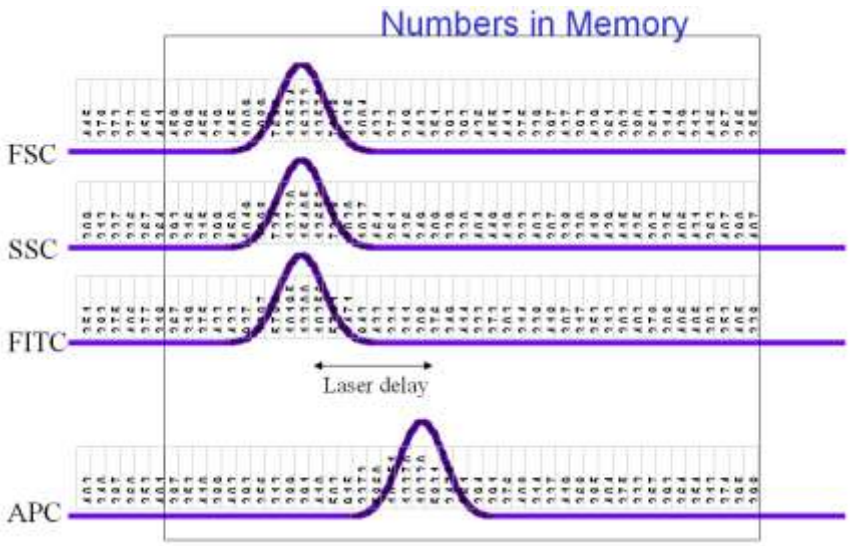
Chip Cytometry



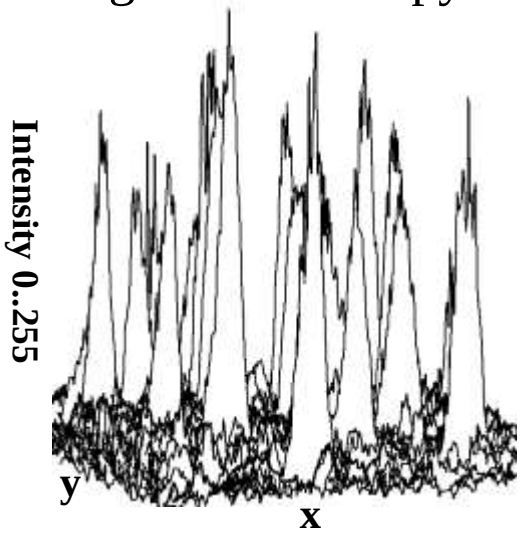
Sood A et al. (2016)
JCI Insight 1:e87030

Basic Data Processing

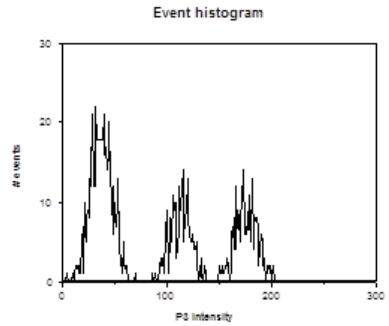
Flow Cytometry

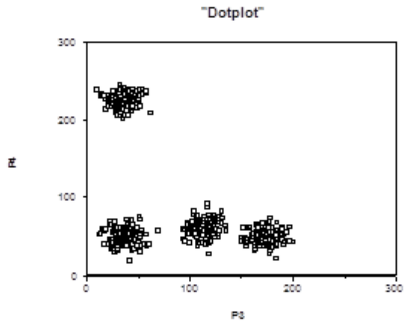


Digital microscopy



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

N. Aghaeepour et al. (2013) Nature Methods 10:228ff

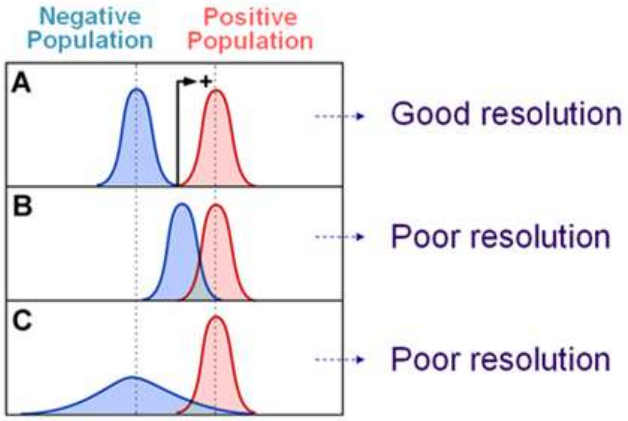
E.Lugli,M.Roederer,A.Cossarizza(2010)Cytometry 77A:705ff

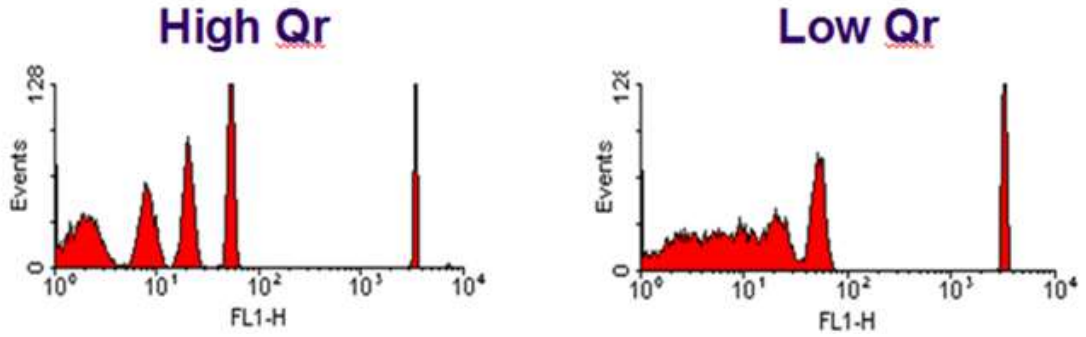
Instrument Evaluation Br, Qr

Br, optical background from

- Free antibody/ fluorochrome
- Flow cell, ambient light
- Raman scatter
- Spectral overlap
- Cell autofluorescence

Qr, photon detection efficiency





Figures: Joe Trotter, BD Biosciences

Spectral Overlap and “Compensation”

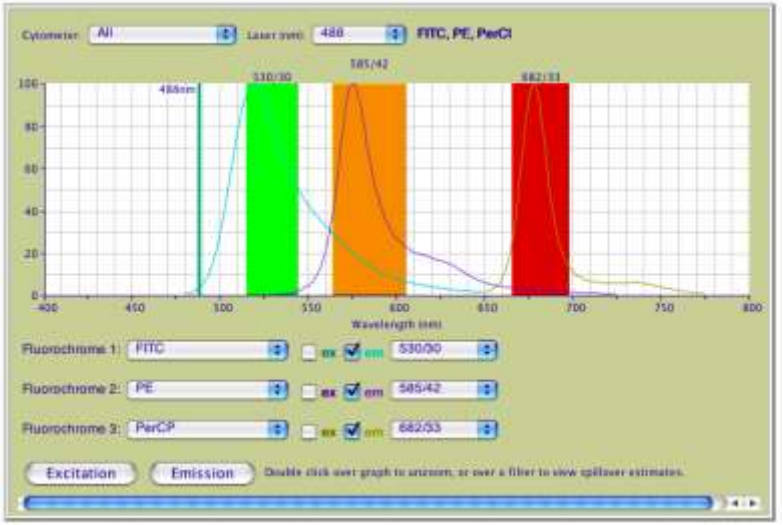
(not very relevant for element mass cytometry)

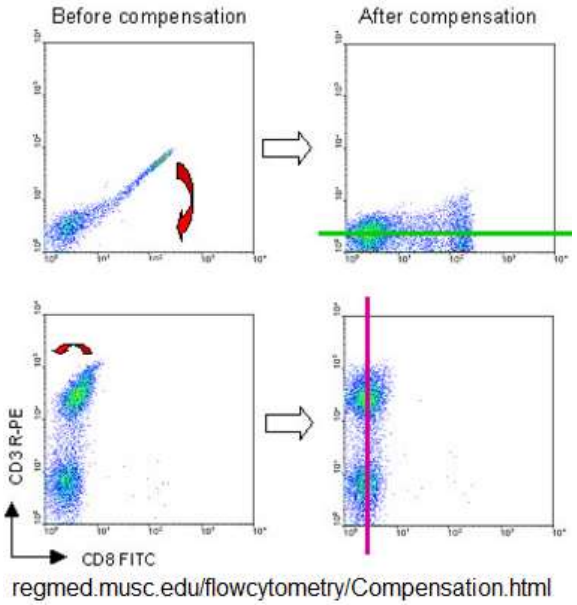
Calculation of concentrations
from optical/mass intensities

$$I_1 = a_{11} * c_1 + a_{12} * c_2 + a_{13} * c_3$$
$$I_2 = a_{21} * c_1 + a_{22} * c_2 + a_{23} * c_3$$
$$I_3 = a_{31} * c_1 + a_{32} * c_2 + a_{33} * c_3$$

a_{ik} : “compensation” matrix numbers
I_i : measured intensities
c_k : label concentrations

Solve n equations with n unknowns
(in spectral cytometry generally
many more equations than unknowns)

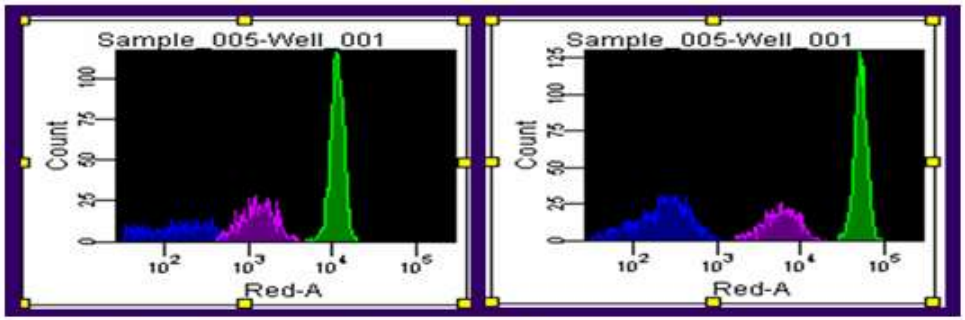




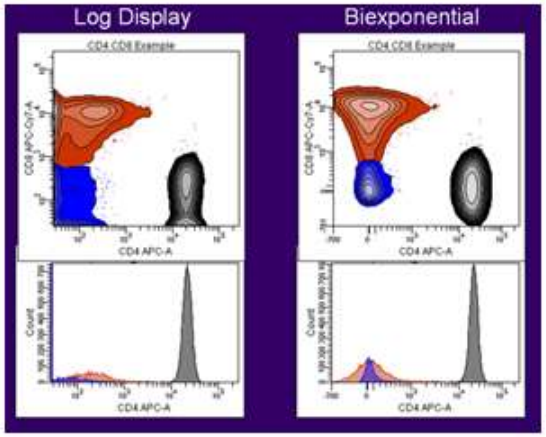
regmed.musc.edu/flowcytometry/Compensation.html

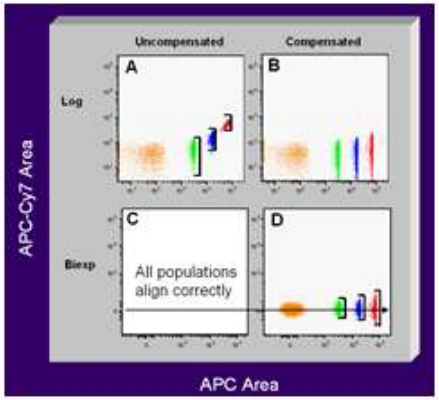
Optimizing cytometry measurements

- Gain (PMT, CMOS, CCD) settings

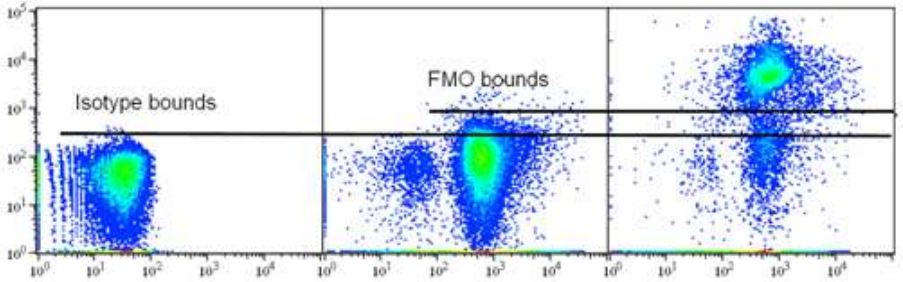


- Data Display





- Controls



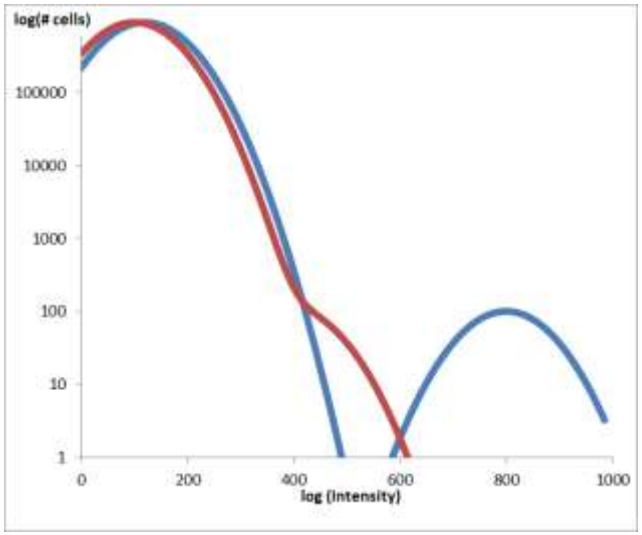
J. Trotter, BD Biosciences

Label Selection

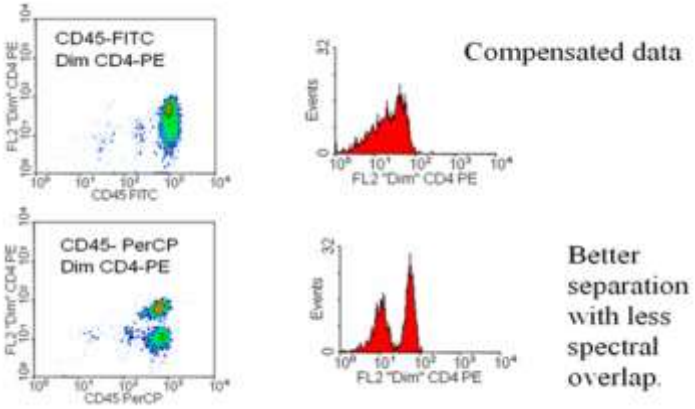
- Detection System
- Brightness
- Spectral Overlap
- Application (surface vs. internal)

Reagent performance

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



Brightness and Separation



Spectral Overlap and Separation

More info: Maecker HT et al. (2004) Cytometry 62A:169-173

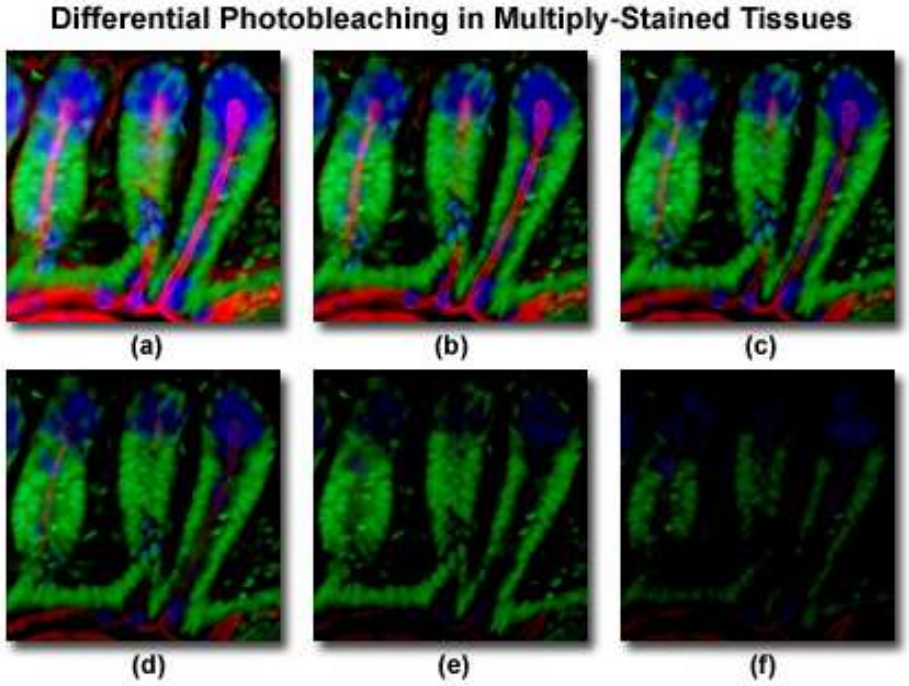
Multi-parameter Fluorescence Cytometry Points To Consider

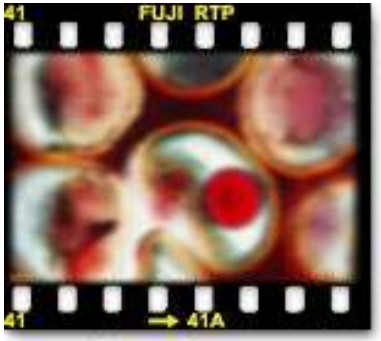
- Know your instrument status e.g. Qr & Br for different channels
- Use high enough gain settings to maximize sensitivity (check to avoid off-scale events)
- An antibody/dye combination with poor separation in a single color assay will not work for a multicolor experiment.
- Avoid spillover from bright 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



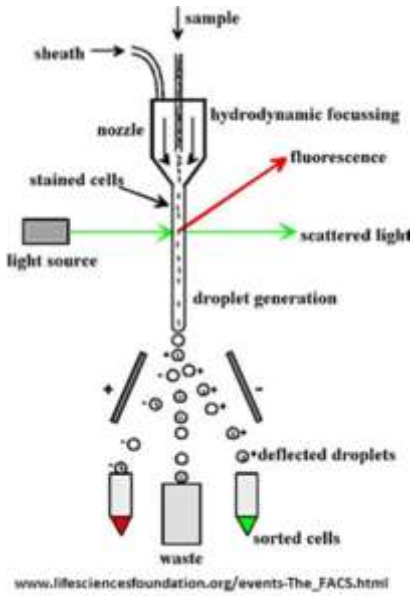


Out of Focus

Images from

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

Cell Sorting



Technologies

- FACS
- Tyto/OWL
- DEP sorter
- Other sorters

- Bulk Sorting
  - Magnetic
  - Gravity
  - Acoustic
  - …

Application Examples

- Chromosomes
- Cloning
- Strain Improvement
- Genomics
- Proteomics

Arnold LW, Lannigan J (2010)... Cell Sorting. Curr Prot Cytom, pp.1-24

Evaluating Cell Sorting Performance

- Purity, Yield

$$Purity = \frac{posFraction * posYield}{posFraction * posYield + negFraction * negYield}$$

http://www.desatoya.com/PostersAndPresentations/SortingPerformanceEvaluation2016Feb.pdf

- Fe Fd

$$\text{Enrichment rate } (f_E) = \frac{\%\text{ neg. cells in orig. sample}}{\%\text{ pos. cells in orig. sample}} \times \frac{\%\text{ pos. cells in pos. fraction}}{\%\text{ neg. cells in pos. fraction}}$$

$$\text{Depletion rate } (f_D) = \frac{\%\text{ pos. cells in orig. sample}}{\%\text{ neg. cells in orig. sample}} \times \frac{\%\text{ neg. cells in neg. fraction}}{\%\text{ pos. cells in neg. fraction}}$$

"Miltenyi S, Schmitz J. High Gradient Magnetic Cell Sorting, pages 216ff in Radbruch A (Ed.) Flow Cytometry and Cell Sorting. 2nd edition. Springer Lab Manual 1999"

Miltenyi S, Schmitz J (1999)

- Rmax

General Eq.

$$Rmax = \frac{\frac{C_{nt}}{C_t} - \frac{O_{nt}}{O_t}}{\frac{C_{nt}}{C_t} - \frac{S_{nt}}{S_t}}$$

Simplified Eq. (Purity≈100%)

$$Rmax = 1 - \frac{O_{nt}}{O_t} \cdot \frac{C_t}{C_{nt}}$$

Riddell A et al. (2015) Methods 82: 64-73

Riddell A et al (2015)

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.

Many systems isolate selected single cells.

New developments in many areas provide more tools for cytometry.

More info: Bendall SC et al. (2012) Nature Biotech. 30:639-47
Maecker H, Trotter J (2011) Multicolor Flow Cytometry Application Note

Applications

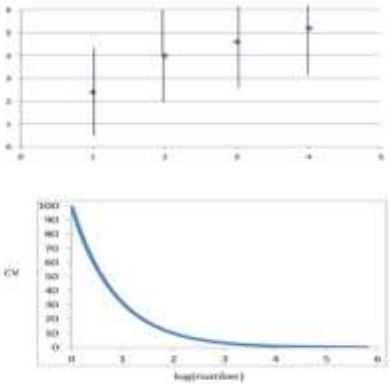
- Multi-parameter immunofluorescence (antibodies)
- Multi-parameter gene expression analysis (NA probes)
- Cell cycle analysis (high resolution FCM, imaging, BrDU)
- Molecular clustering (fluorescence energy transfer FRET)
- Kinetics (population-based flow cytometry, single cell by imaging; Ca++ flux, enzyme activity, cell proliferation)
- Receptor ligand binding (by quantitative fluorescence)
- Single Cell Sequencing (single cell sorting, PCR amplif.)
- Particle-based assays (Luminex-type multiplexed assays)
- Rare Cell Research (*more on next slide*)

Rare Cell Analysis and Sorting

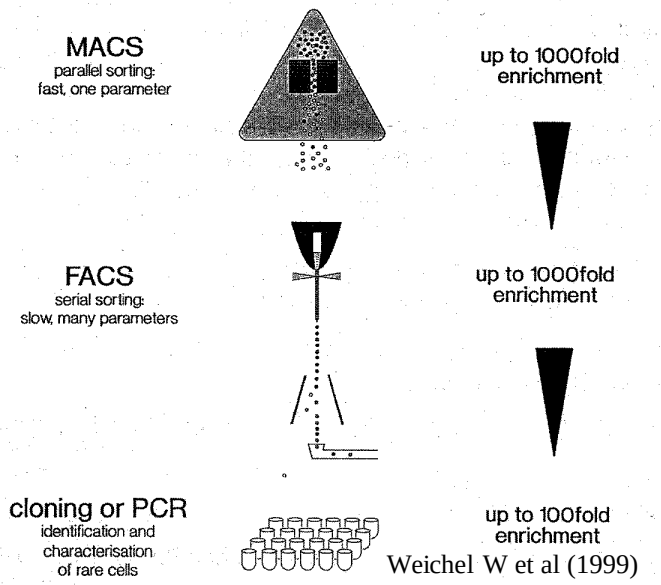
Examples CD34, AC133, antigen specific cells, CTCs

- Poisson count statistics
- Population Separation
- Bulk pre-enrichment or enrichment sorts

	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

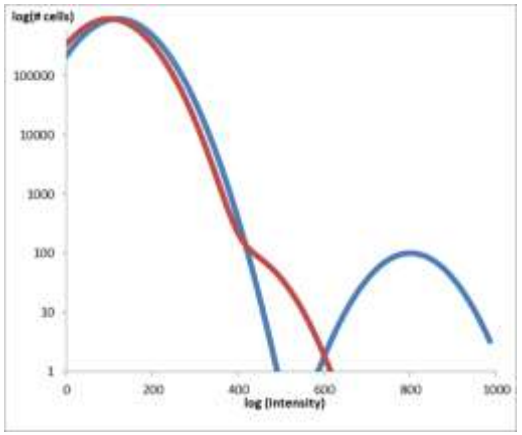


Ignoring Counting Statistics Can Lead to Erroneous Conclusions



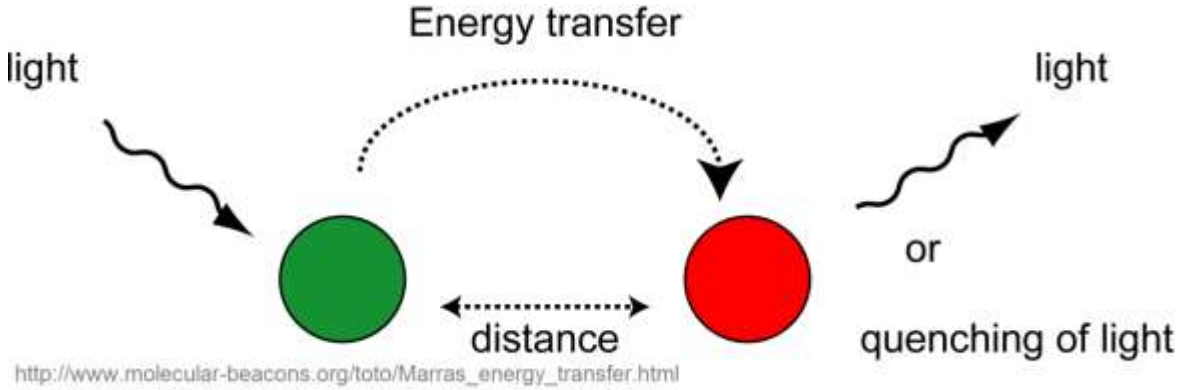
Weichel W et al (1999)
Springer Lab Manual

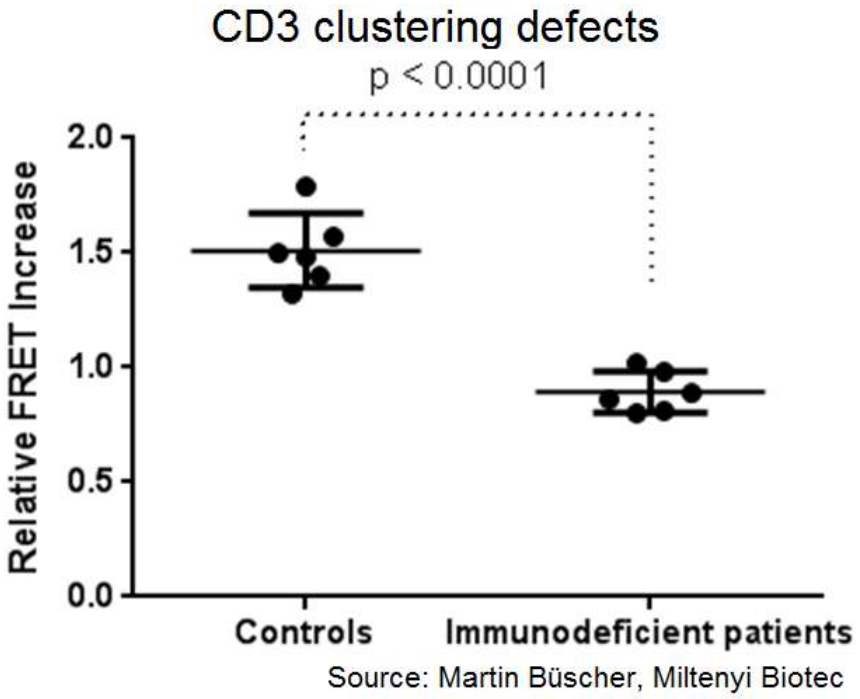
Pre-enrichment



Population Separation

Fluorescence Resonant Energy Transfer (FRET)



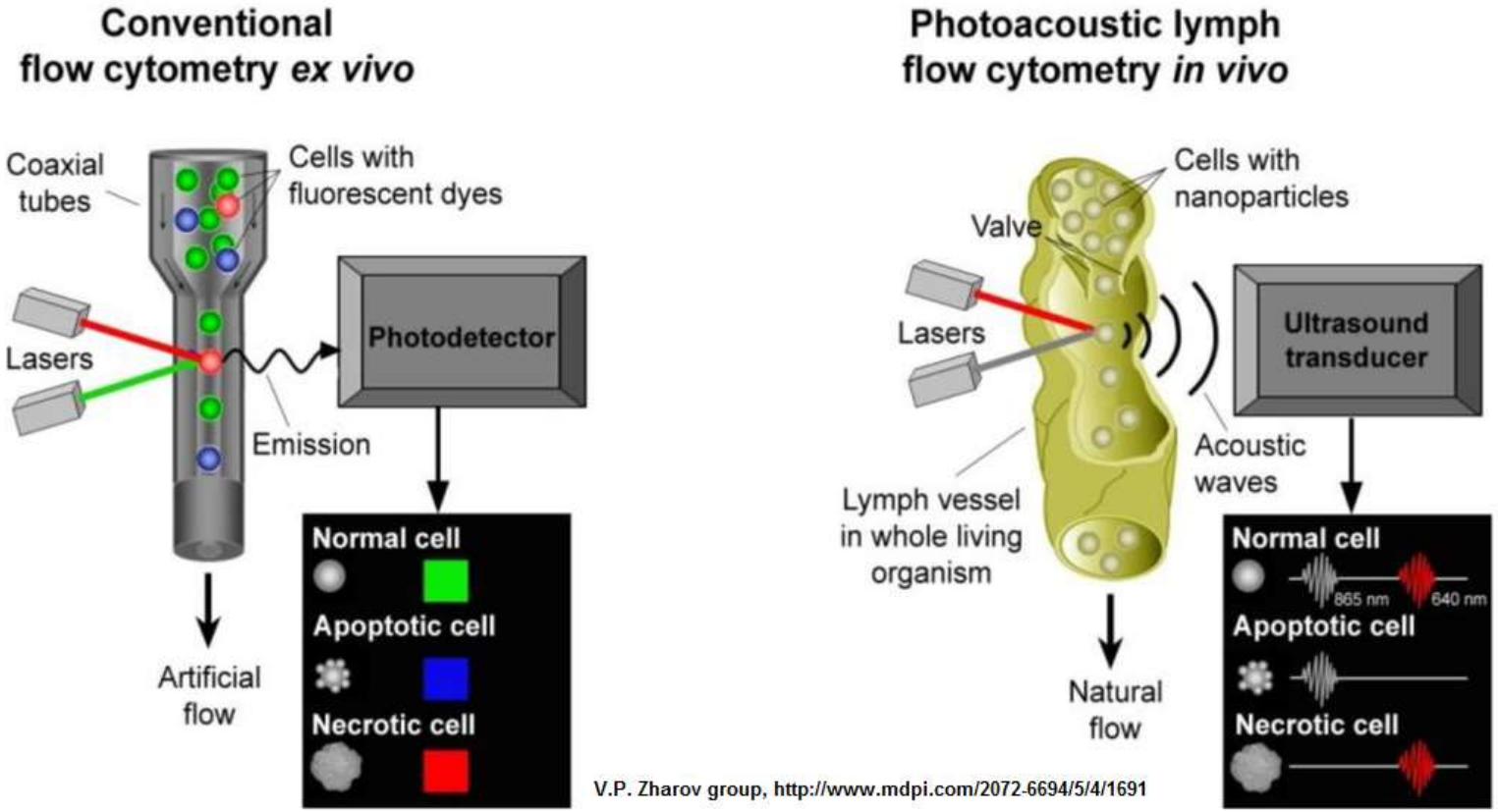


FRET measures the distance between molecules e.g. to detect clustering.

von Kolontaj, K. et al. (2016) *Automated measurement of protein–protein interactions*. Cytometry 89A:835ff

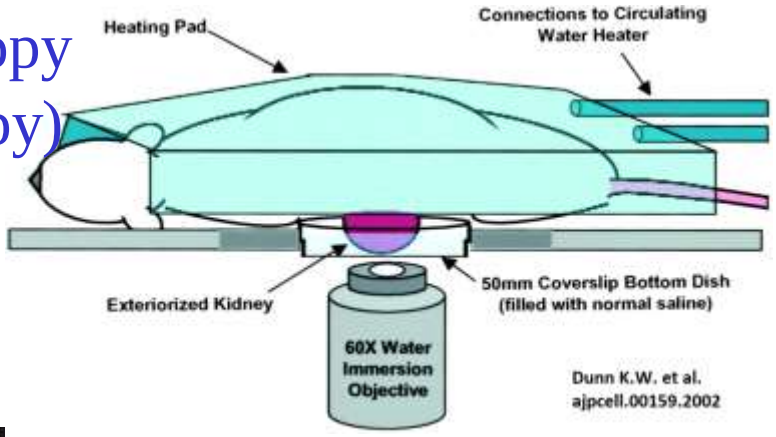
In-vivo Single Cell Analysis

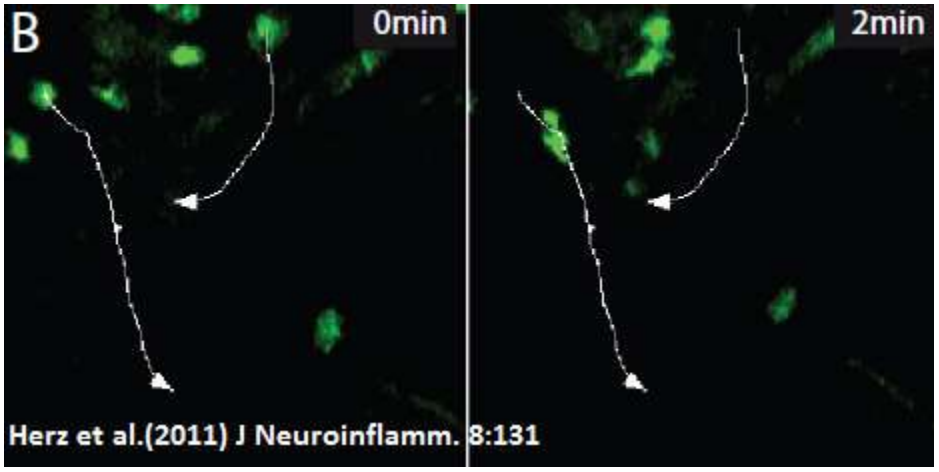
- Intra-vital Imaging
- In-vivo Flow Cytometry



Intra-vital Imaging

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





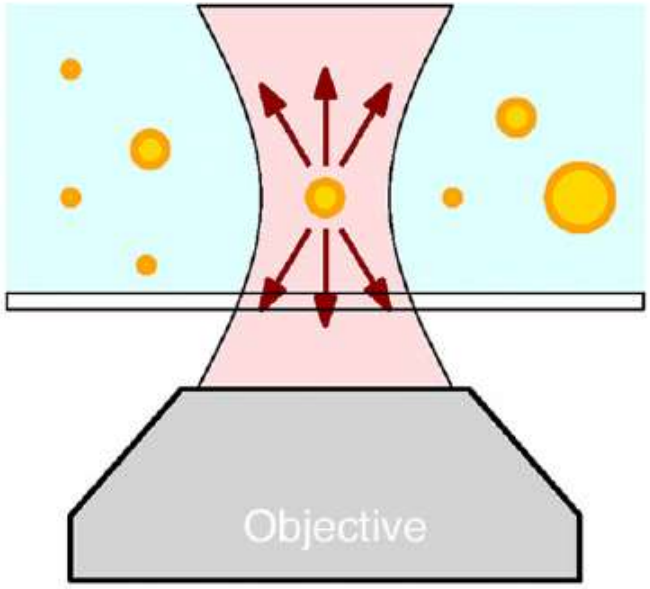
Issues:

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

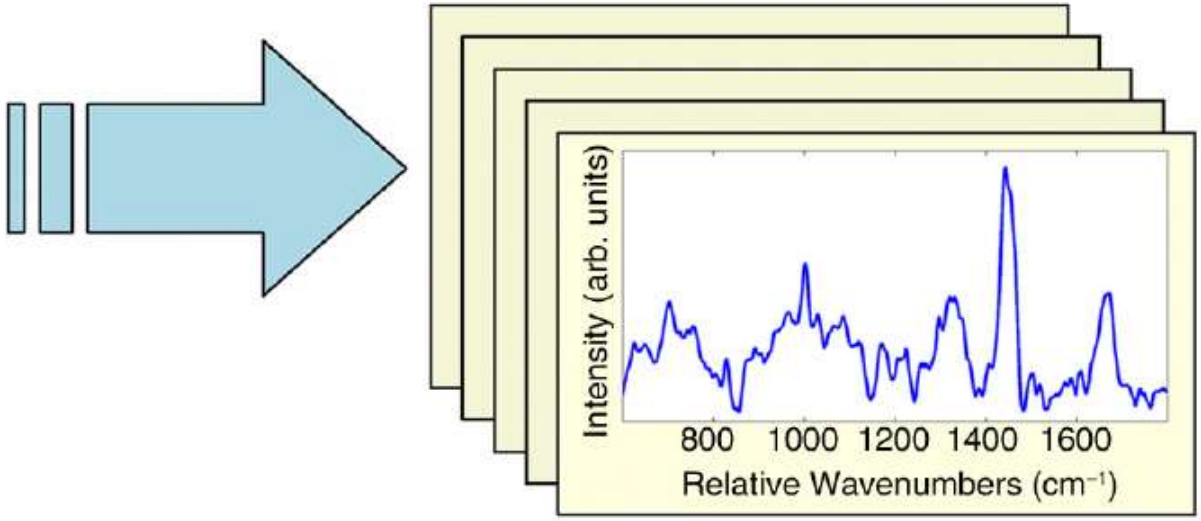
Recent review of in-vivo microscopy: Andresen V, et al. (2012) High-Resolution Intravital Microscopy. PLoS ONE 7(12): e50915

Label-Free Cytometry

- Autofluorescense
- Light Scatter
- Optical trap RAMAN
- Impedance
- Optical trap RAMAN
- RAMAN imaging
- …



Laser Trapping of
Single Exosomes

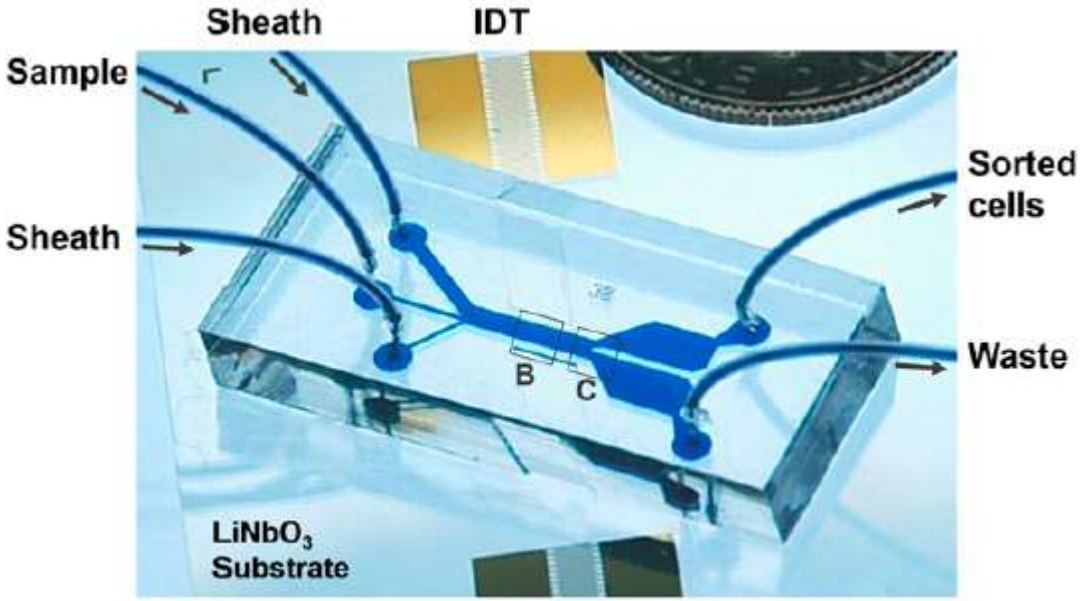


Raman spectra of
Single Exosomes

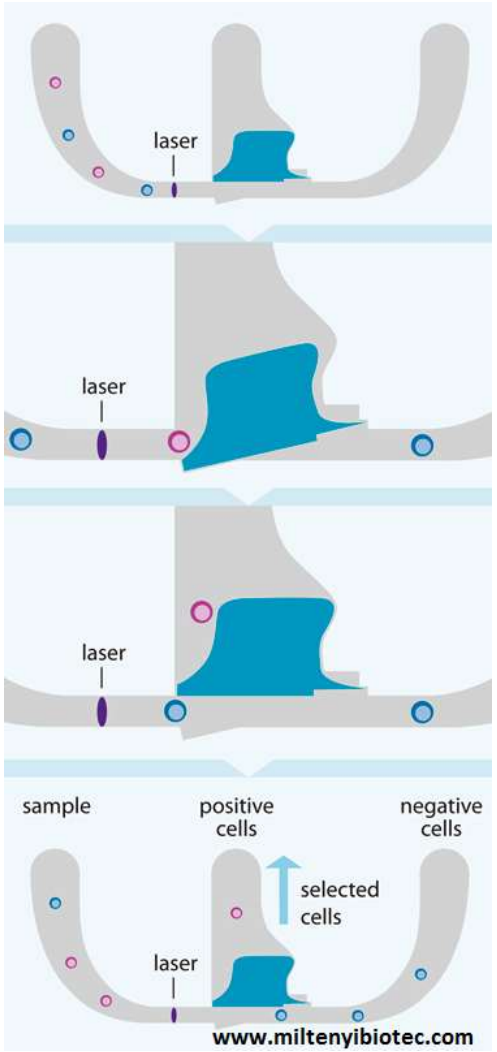
Smith, Zachary J., et al. (2014) J. extracellular vesicles 4: 28533-28533.

More Cell Sorting Technologies

DEPArray™ System

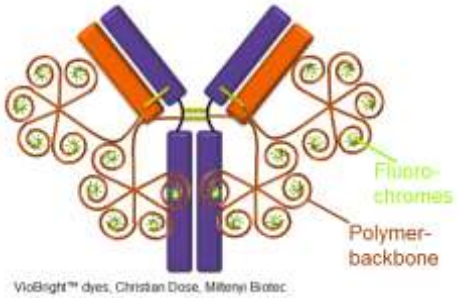


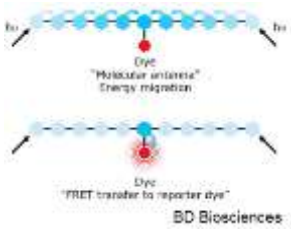
Ding X et al (2014) PNAS 111:12993ff; SAW cell sorting

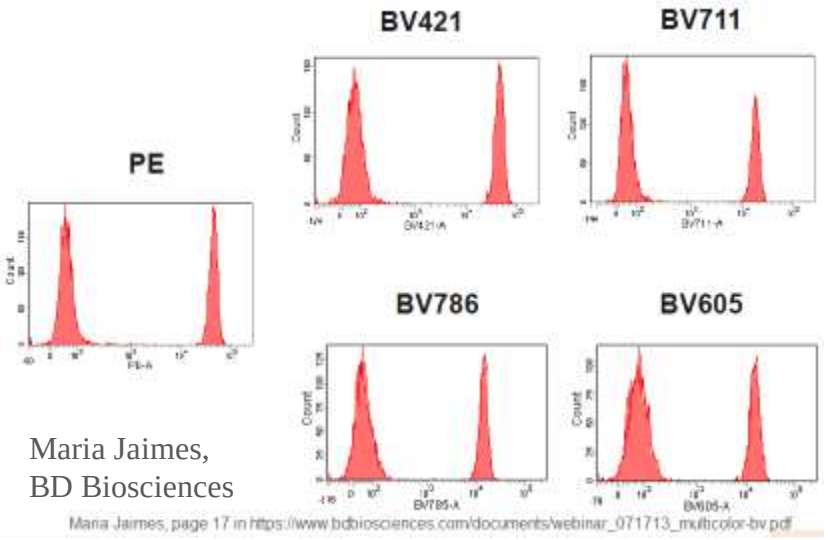


MACSQuant®Tyto™

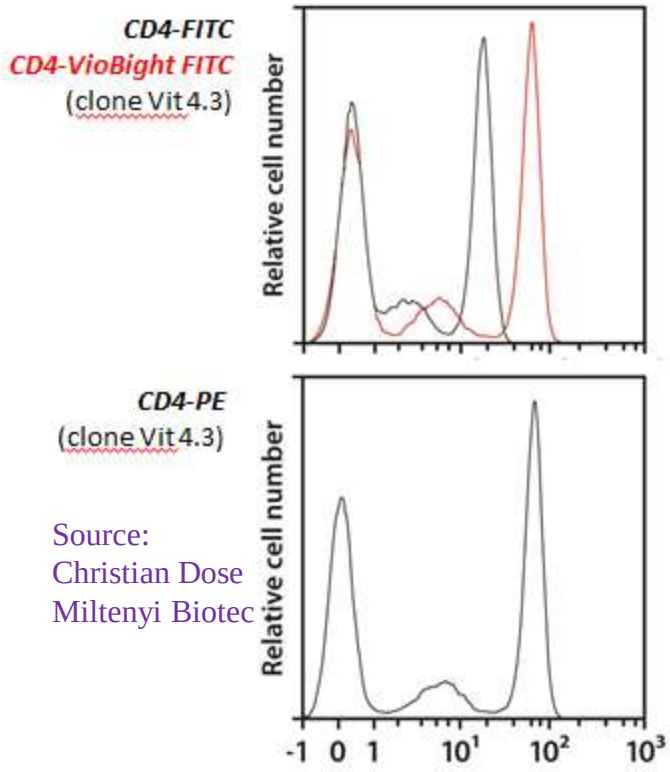
New Bright Dyes



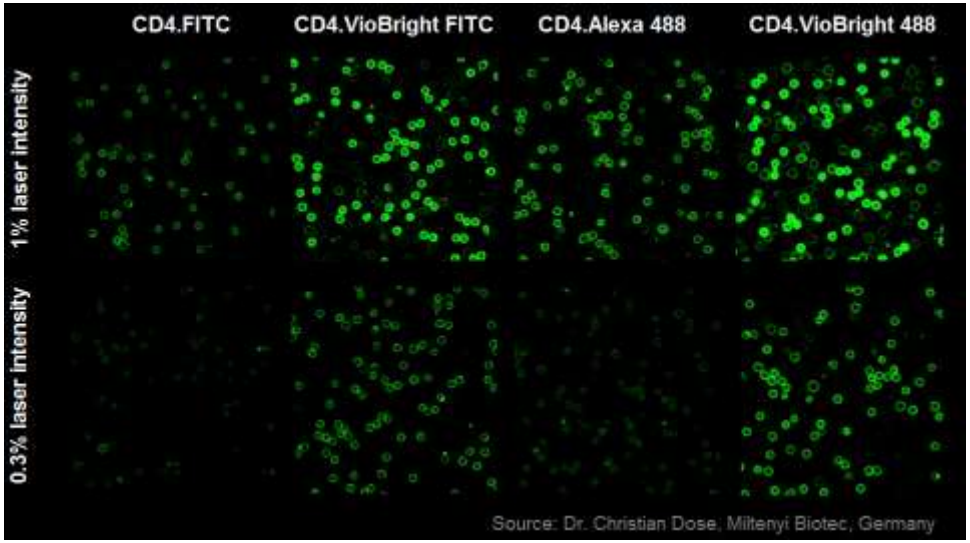




Maria Jaimes,
BD Biosciences



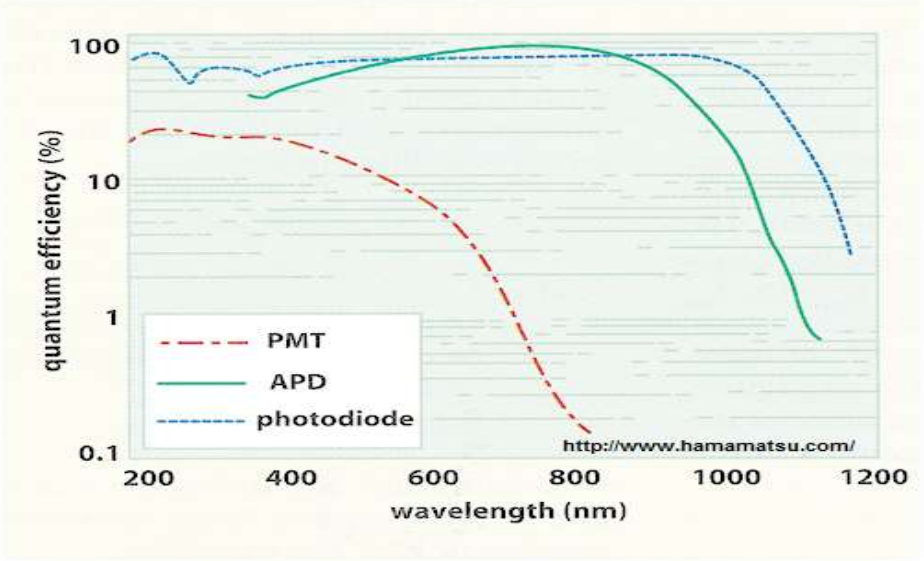
Source:
Christian Dose
Miltenyi Biotec



New Detector-Label Combinations

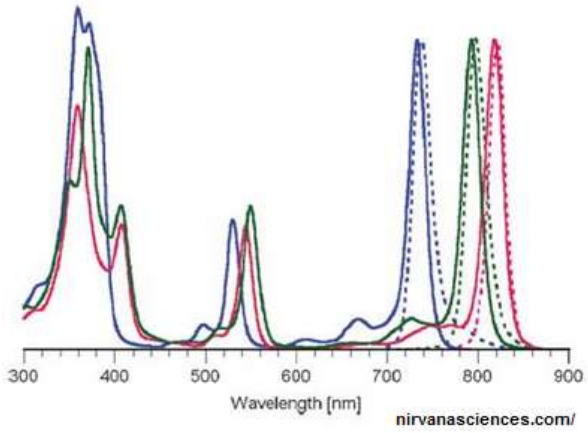
- New photodetectors extend the available spectrum

  (Si avalanche photodiodes extend detection into the far infrared)



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

  (Fluorescent polymers, bacteriochlorins, …)



Novel Affinity Reagents

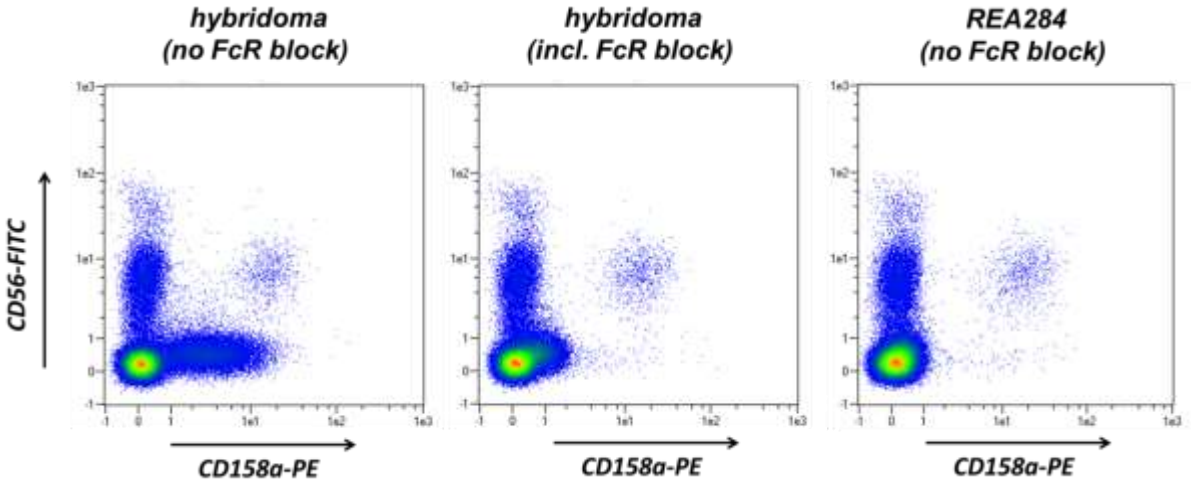
Antibodies

- Antibodies from different species
  (e.g. Llama 15 kDalton fragments
  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

Source: Dr. Christian Dose, Miltenyi Biotec

New Detection Technologies

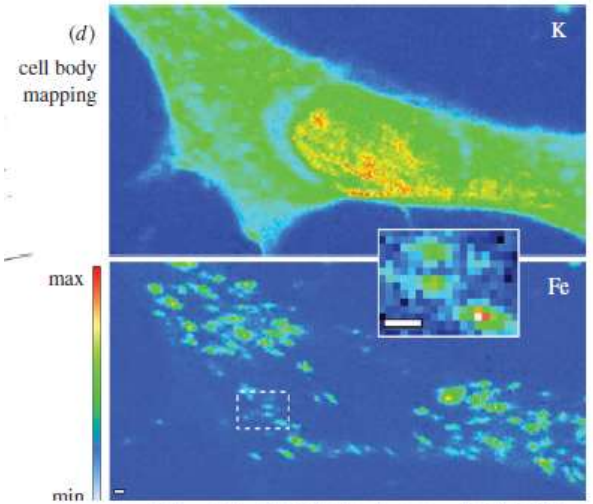
- High spatial resolution and multi-parameter capability with X-ray / synchrotron radiation fluorescence

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

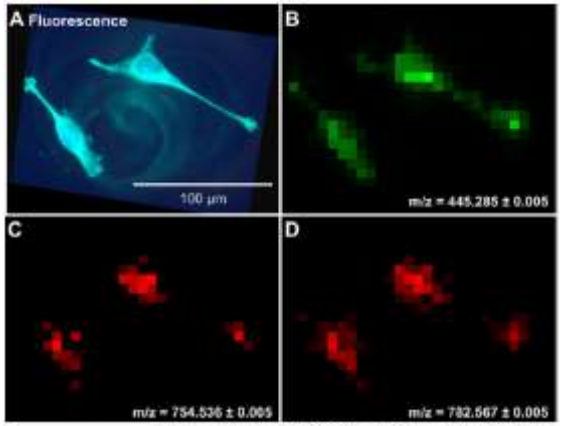
  (CyTOF like element labels, direct metabolite or structural component detection)
- Label-free imaging with Raman

  (measuring cellular components by their Raman spectra)
- Label-free medium 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

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.

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- David Basiji
- **…**

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More science detail and references: http://www.desatoya.com