



Principles of Flow and Image Cytometry & Innovations for Single Cell Analysis

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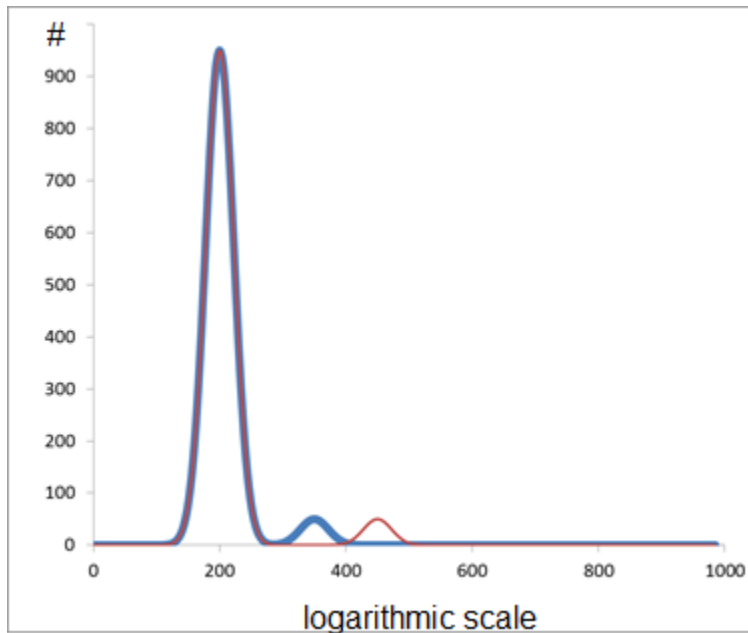
Key Reference Abbreviation

In this document “GUIDELINES” is used for
Cossarizza, Andrea, et al. (2017) "Guidelines for the use of
flow cytometry and cell sorting in immunological studies."
European journal of immunology 47 (10) 1584 - 1797

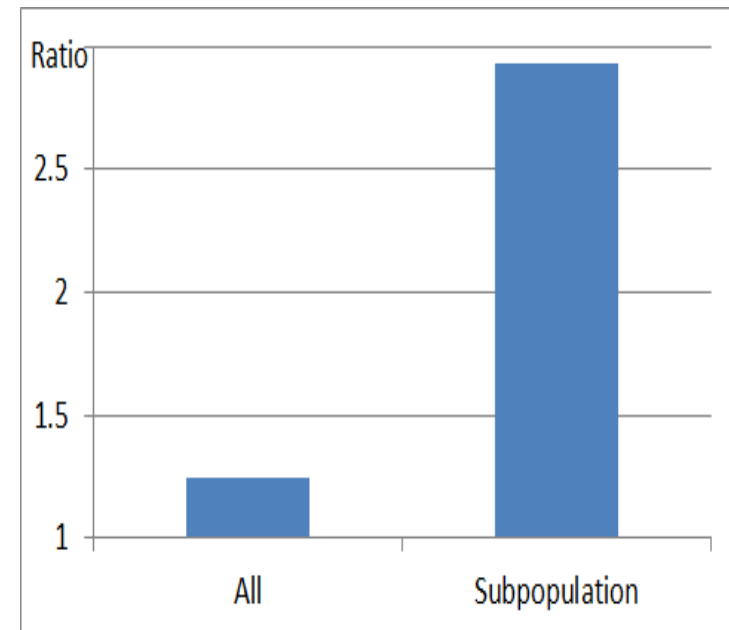
The GUIDELINES contain contributions from 231 experienced
scientists from 192 institutes worldwide, describing their
recommendations for the optimal use of flow cytometry.

Why Cell Subset Analysis at the Single Cell Level

Intensity Histogram



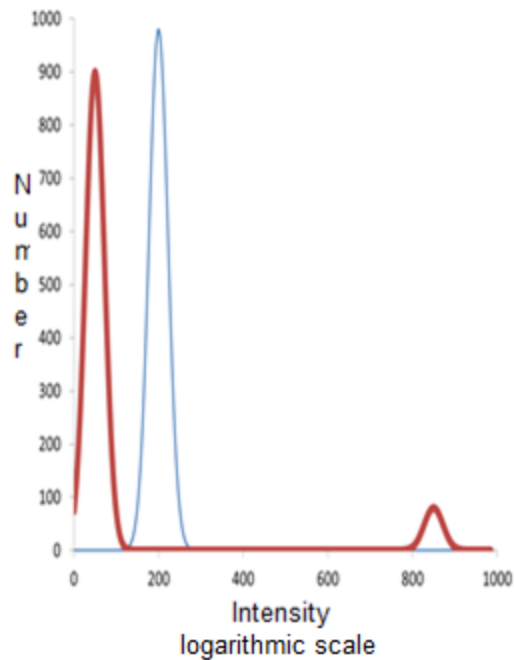
Intensity Ratios



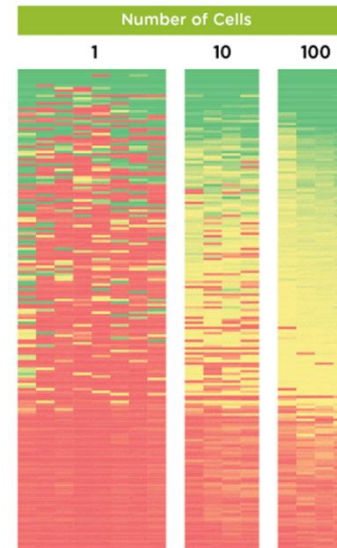
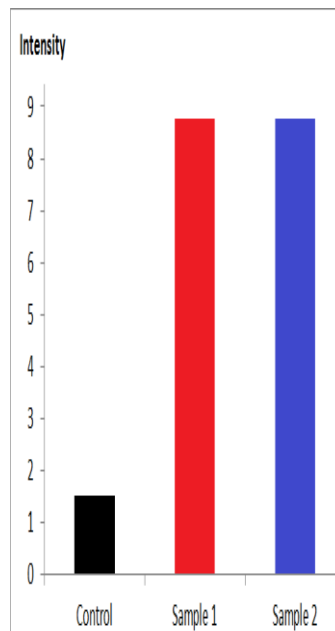
Subpopulation analysis detects changes better, especially for rare subpopulations.

Why Single Cell 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

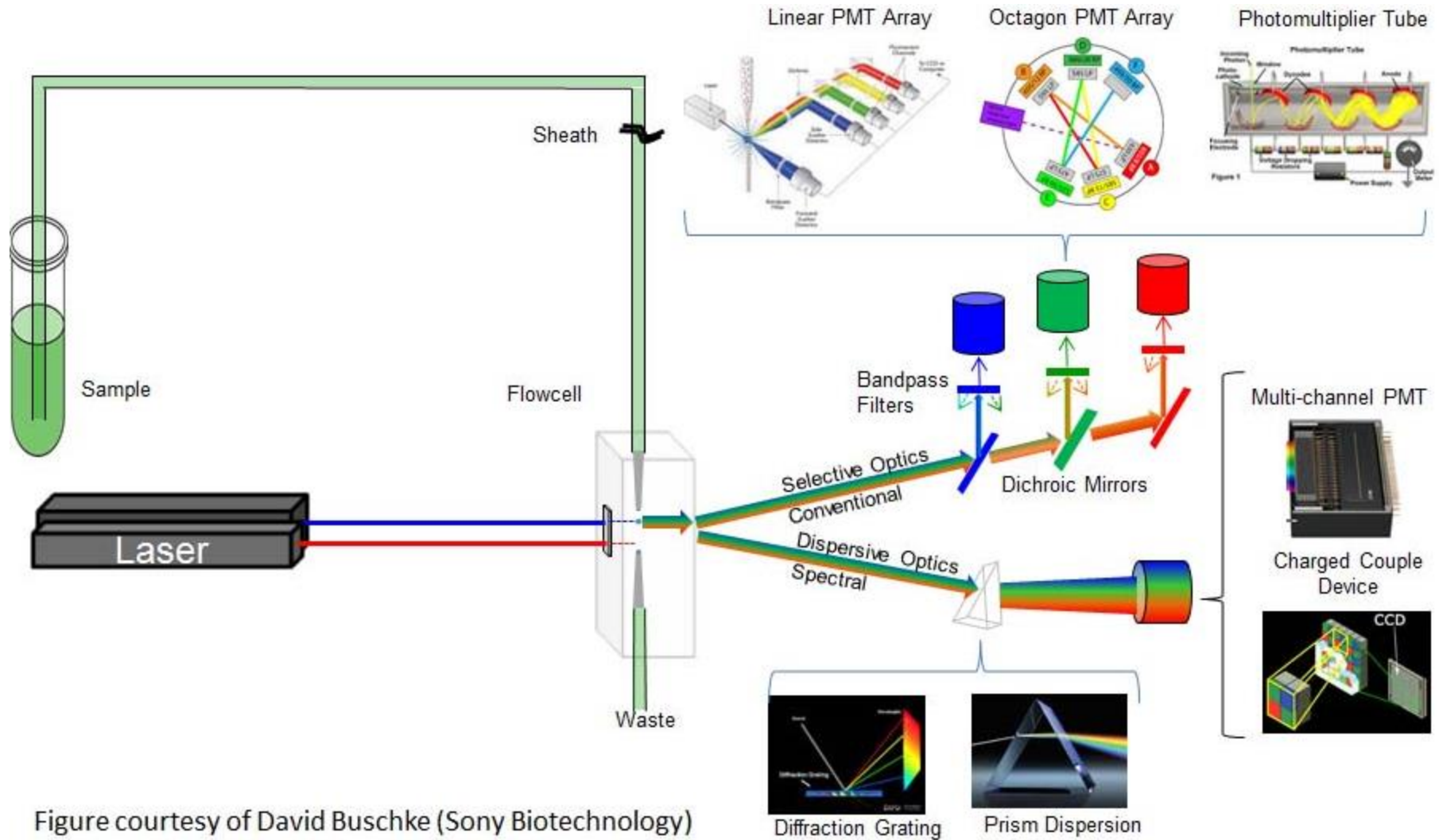


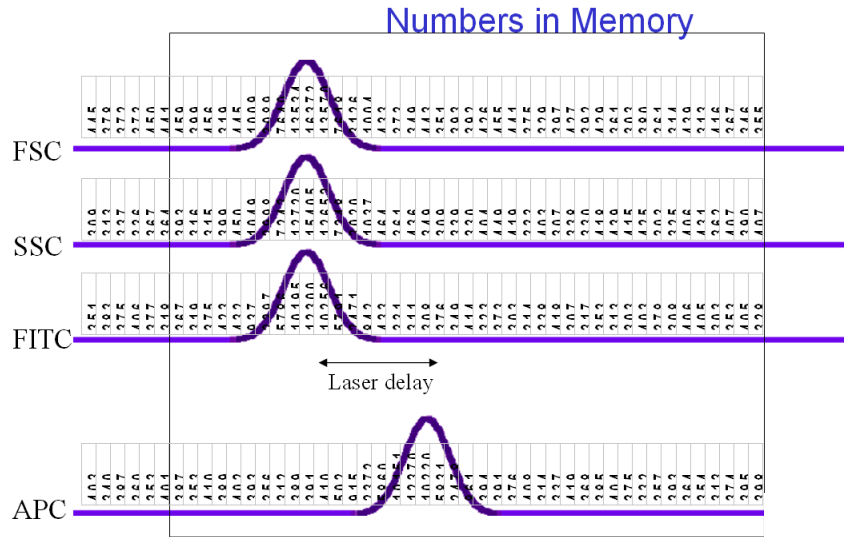
Figure courtesy of David Buschke (Sony Biotechnology)

GUIDELINES Flow cytometers, pages 1596- 1608

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

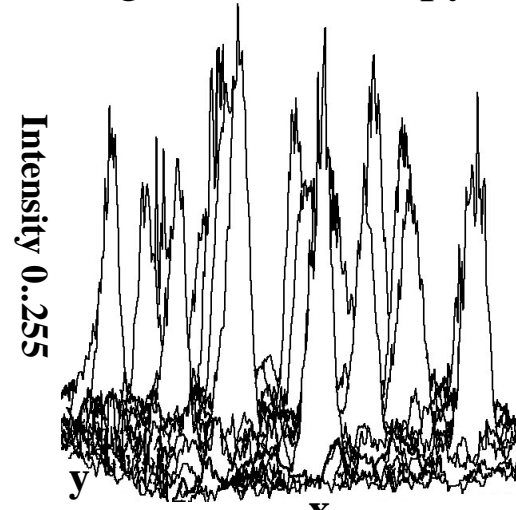
Basic Data Processing

Flow Cytometry

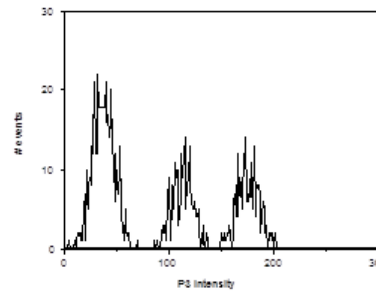


Cell	P1	P2	P3	P4	P5	Pop#
1	242	135	704	175	612	1
2	146	132	690	178	566	1
3	269	147	89	206	580	3
4	442	143	399	250	255	4
5	212	167	155	926	526	2
6	269	2	659	207	575	1
7	204	232	112	171	679	3
8	152	74	160	828	532	2
			...			
9997	215	119	138	936	662	2
9998	244	50	72	261	543	3
9999	214	137	174	1014	597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

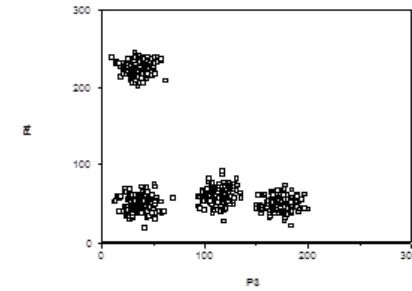
Digital microscopy



Event histogram



"Dotplot"



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

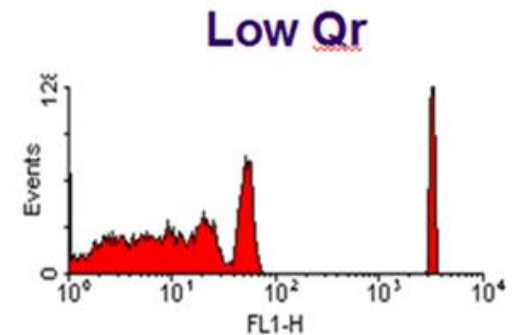
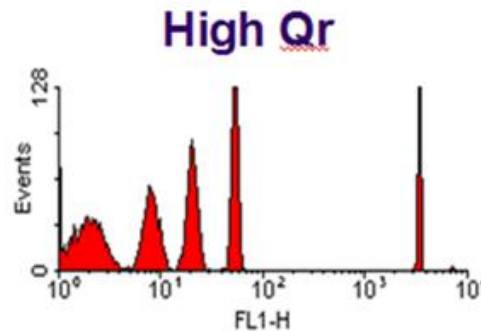
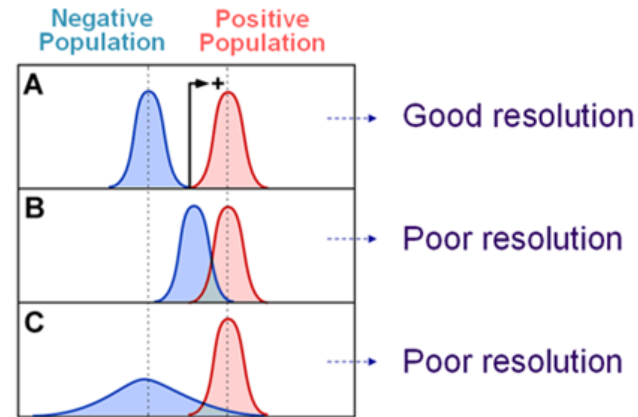
GUIDELINES Data Analysis, pages 1651-62

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



Spectral Overlap and “Compensation”

(not very relevant for element mass cytometry)

Calculation of concentrations
from optical/mass intensities

$$I_1 = a_{11} * c_1 + a_{12} * c_2 + a_{13} * c_3$$

$$I_2 = a_{21} * c_1 + a_{22} * c_2 + a_{23} * c_3$$

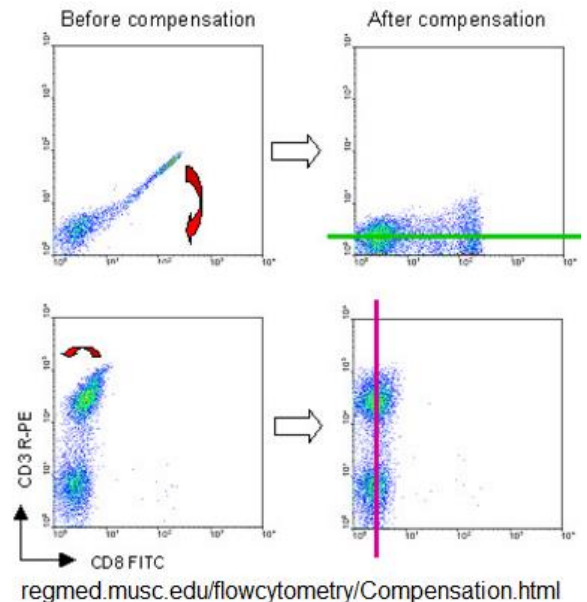
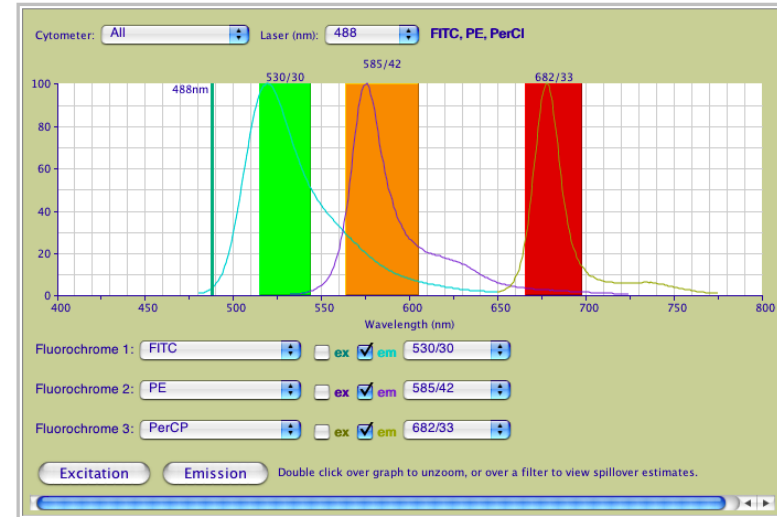
$$I_3 = a_{31} * c_1 + a_{32} * c_2 + a_{33} * c_3$$

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)



Cytometer Measurements

Single Cell Analysis

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

Non-cell applications

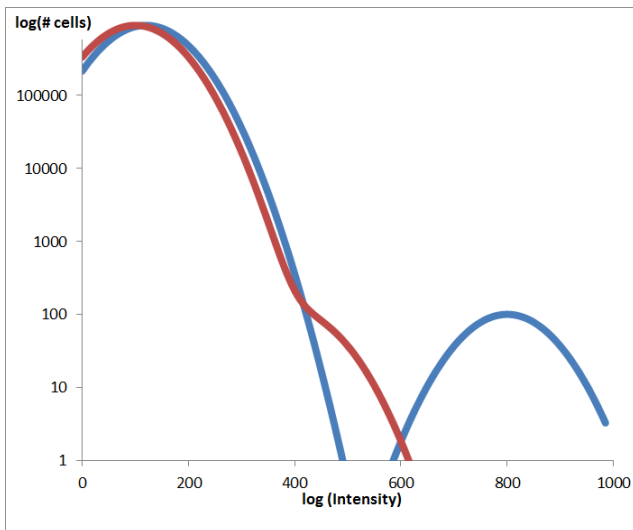
- * Highly multiplexed bead-based immunoassays
- * Single molecule counting

Label Selection

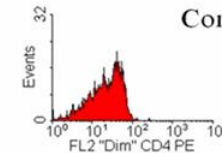
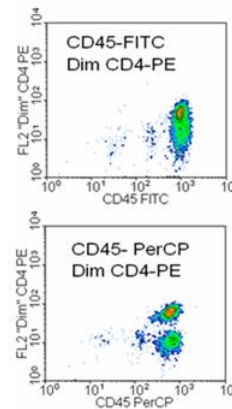
- Detection System
- Brightness
- Spectral Overlap
- Application (surface vs. internal)

Reagent performance $\frac{\text{Stain index}}{2 * SD_{neg}}$

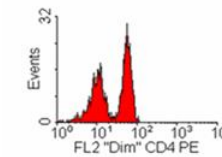
$$\frac{\text{Medium}_{pos} - \text{Medium}_{neg}}{2 * SD_{neg}}$$



Brightness and Separation



Compensated data



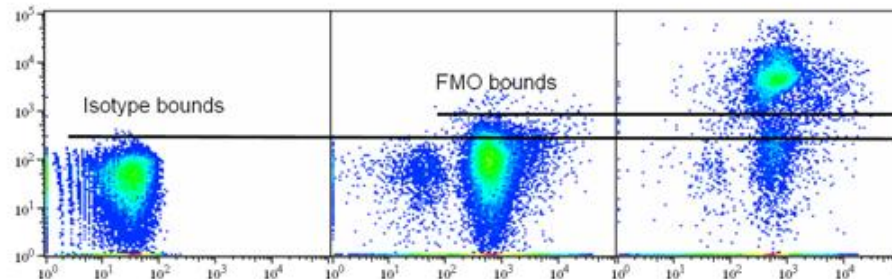
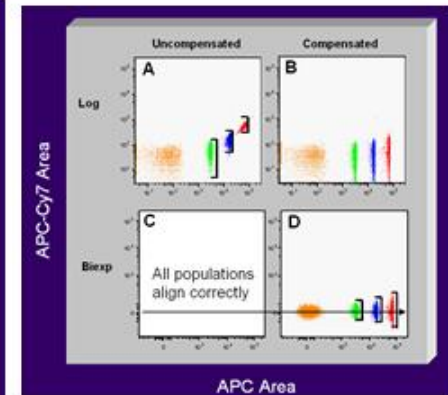
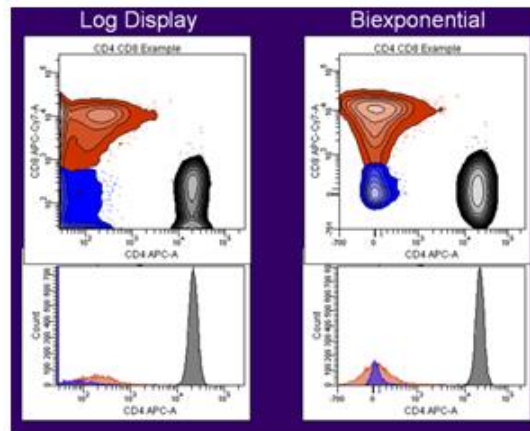
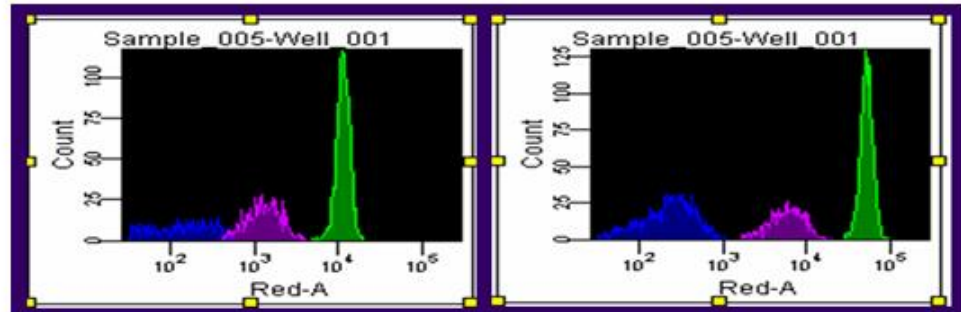
Better separation with less spectral overlap.

Spectral Overlap and Separation

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

Optimizing cytometry measurements

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

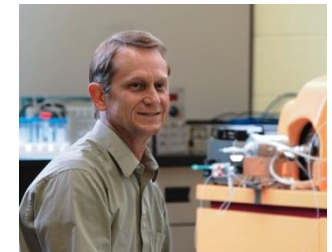
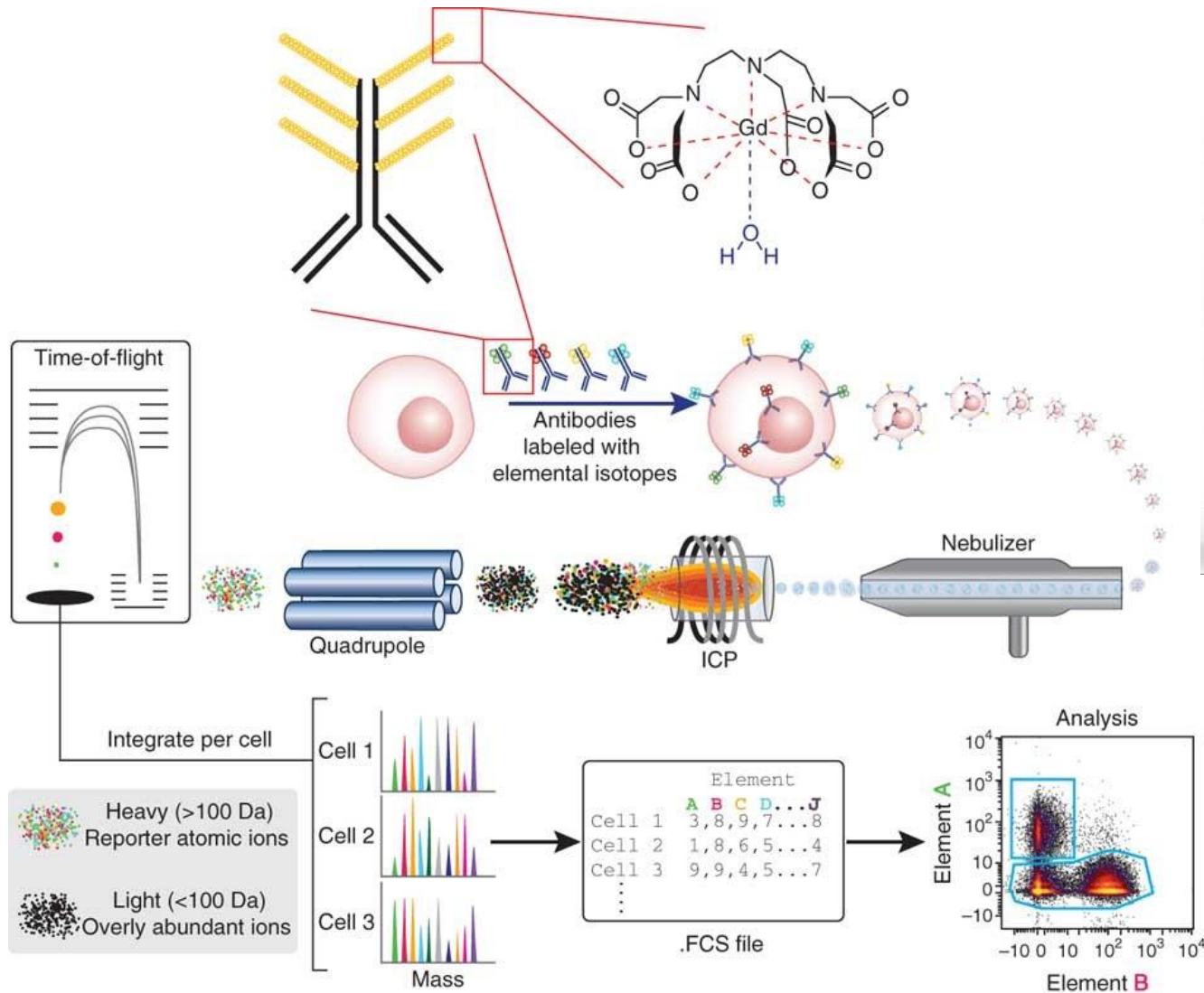


Multi-parameter Fluorescence Cytometry

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

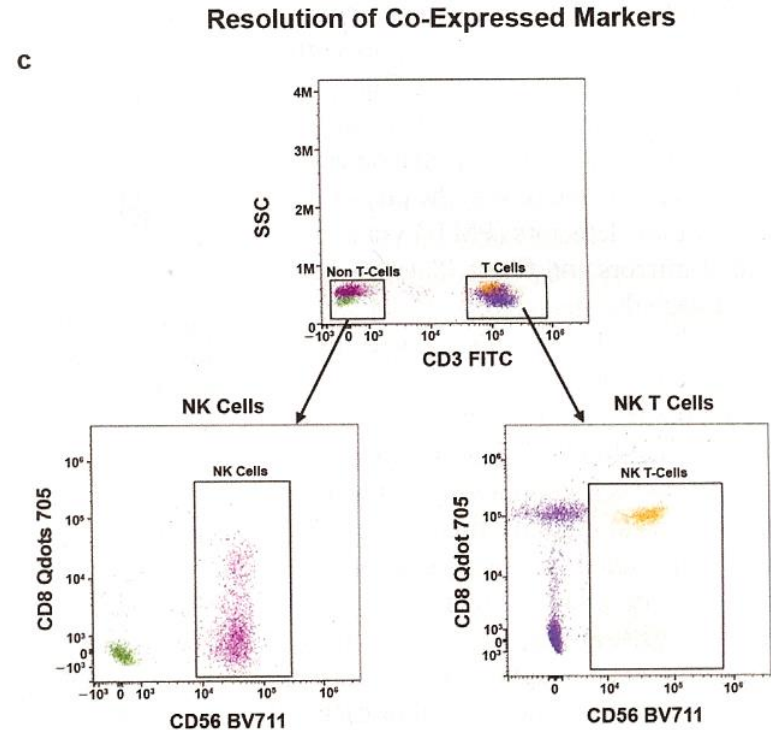
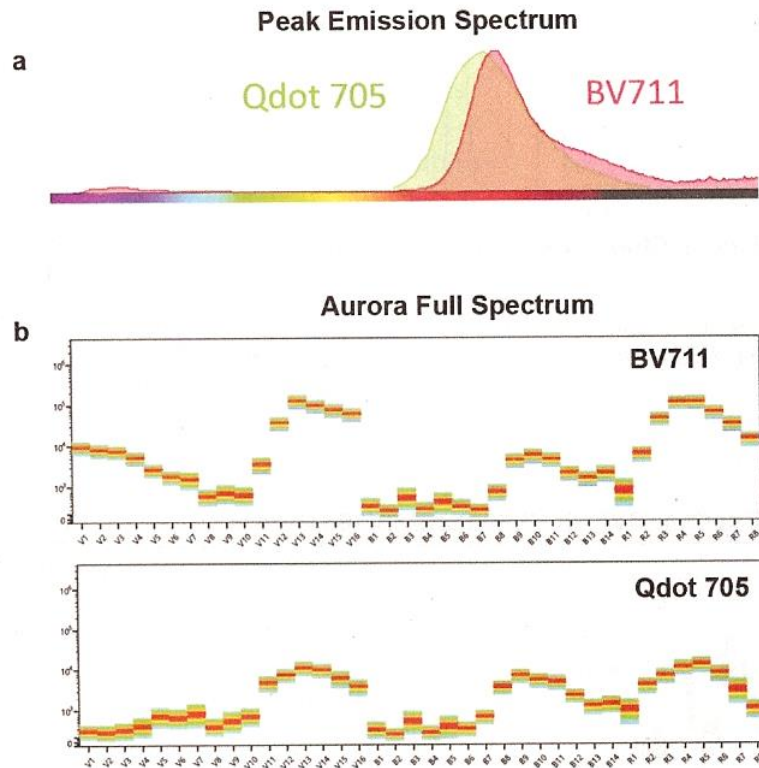
Mass-Label Cytometer (CyTOF)



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

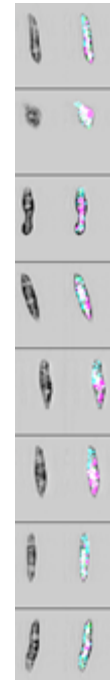
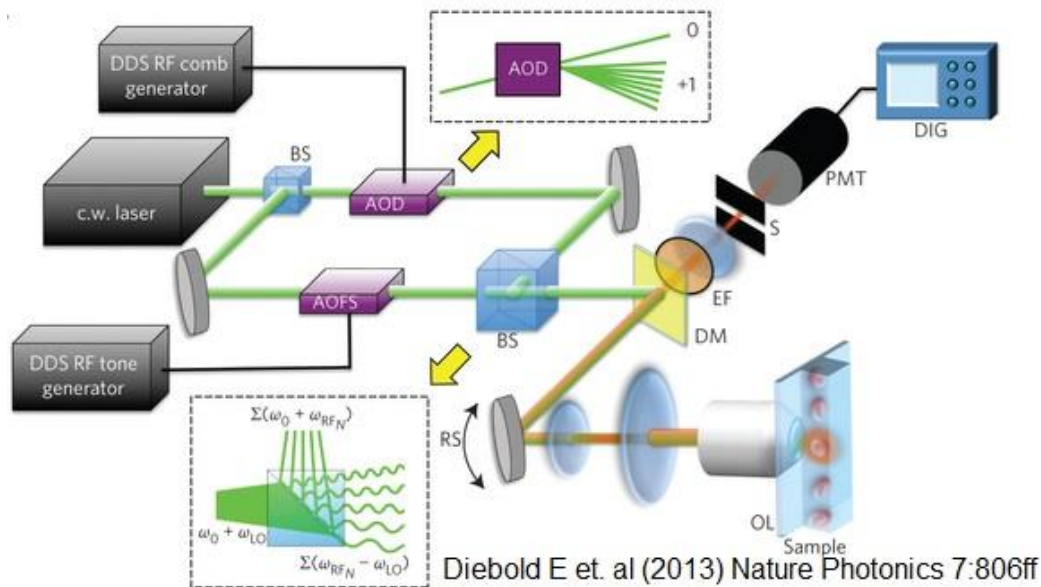
GUIDELINES Mass Cytometry,
pages 1604- 08

Full Fluorescence Emission Spectra by Flow Cytometry



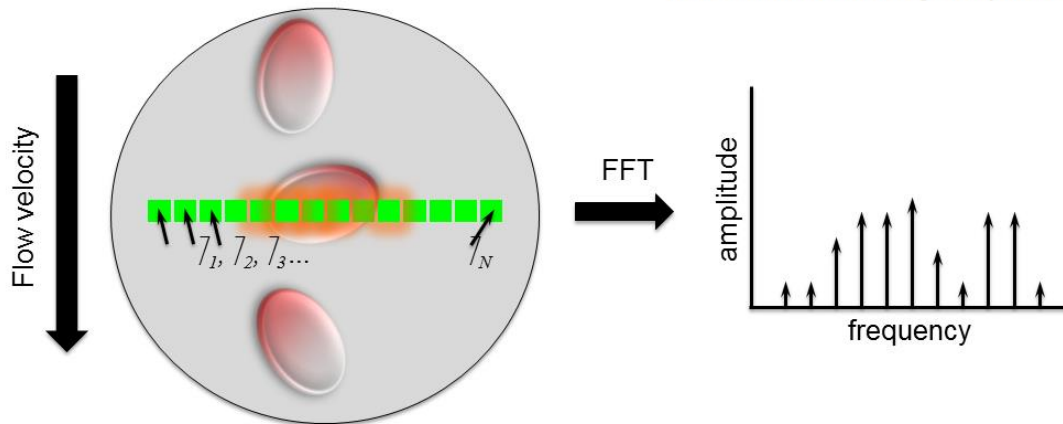
Ming Y et al. ; Biophotonics, Nov/Dec 2017, p 28-31

Imaging Flow Cytometers



Flow cytometer field of view

Source: Eric Diebold, Omega Biosystems



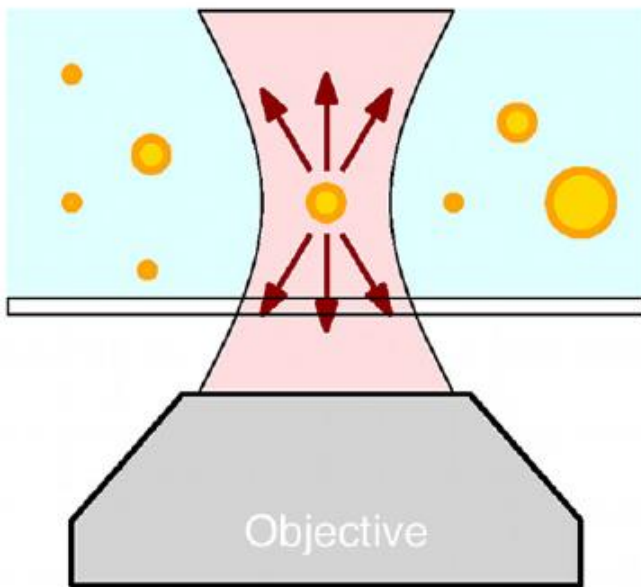
Spatial information is contained in frequency domain of fluorescence signal

GUIDELINES

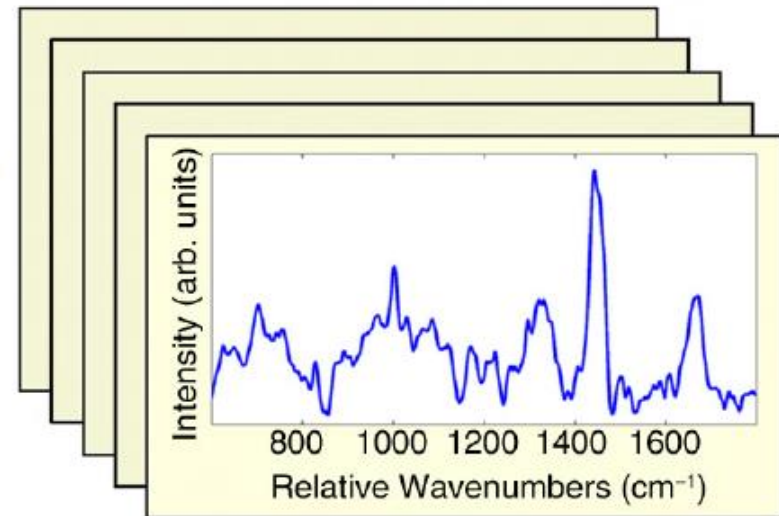
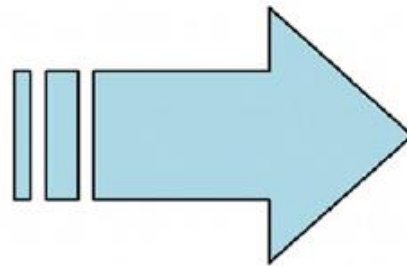
Imaging Flow Cytometry,
pages 1602- 04
(technology above not covered)

Label-Free Cytometry

- Autofluorescence
- Light Scatter
- Optical trap RAMAN
- Impedance
- Optical trap RAMAN
- RAMAN imaging
- ...



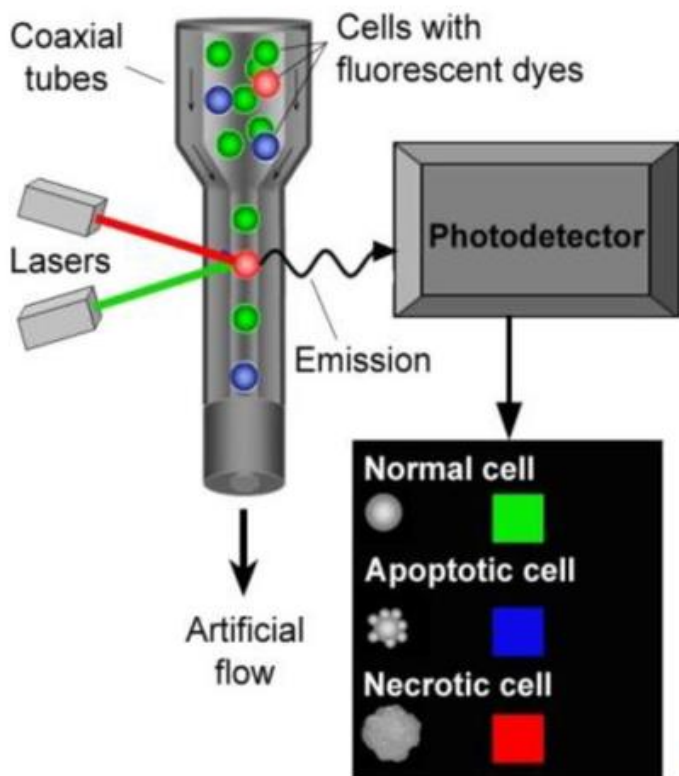
Laser Trapping of
Single Exosomes



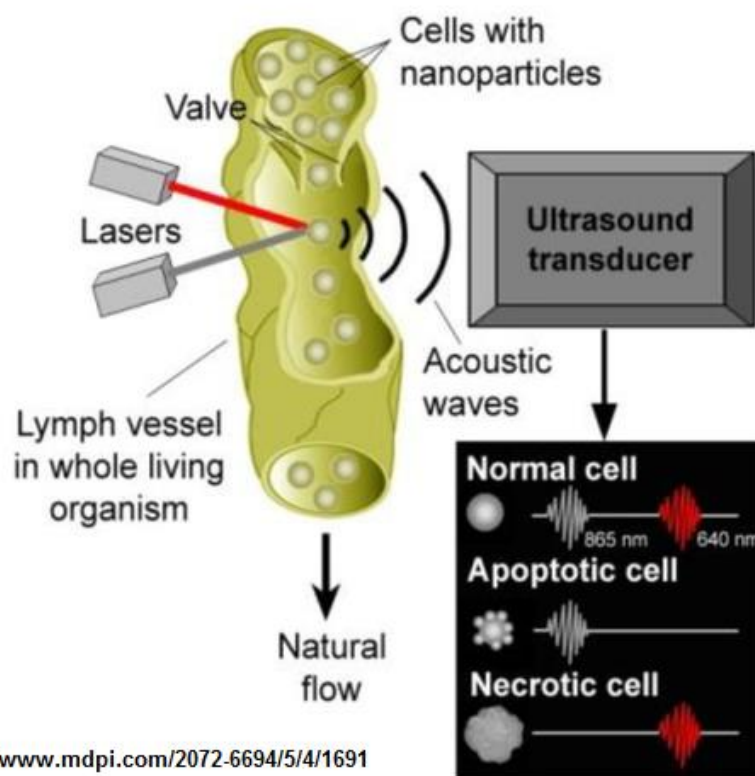
Raman spectra of
Single Exosomes

In-vivo Single Cell Analysis by Flow Cytometry

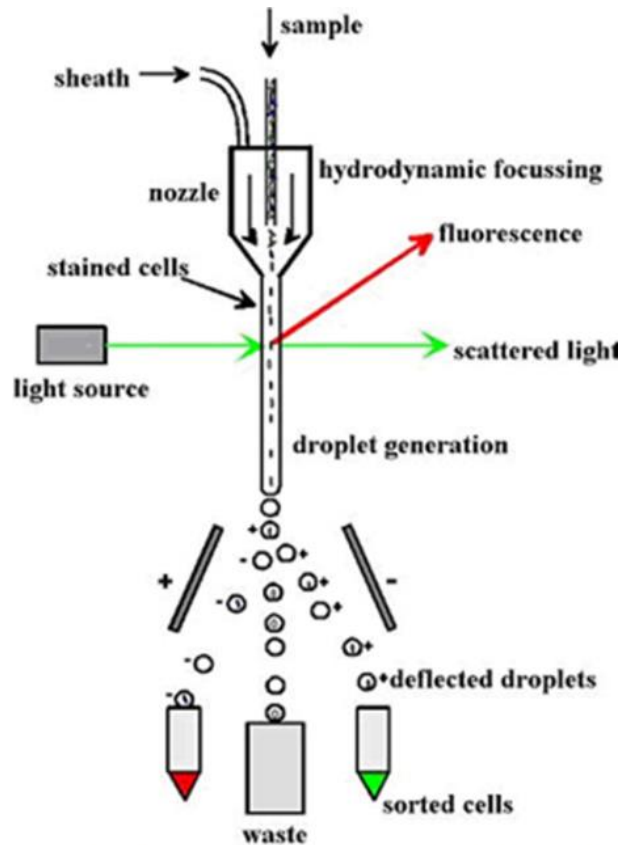
Conventional flow cytometry *ex vivo*



Photoacoustic lymph flow cytometry *in vivo*



Cell Sorting (FACS droplet sorter)



www.lifesciencesfoundation.org/events-The_FACS.html

Application Examples

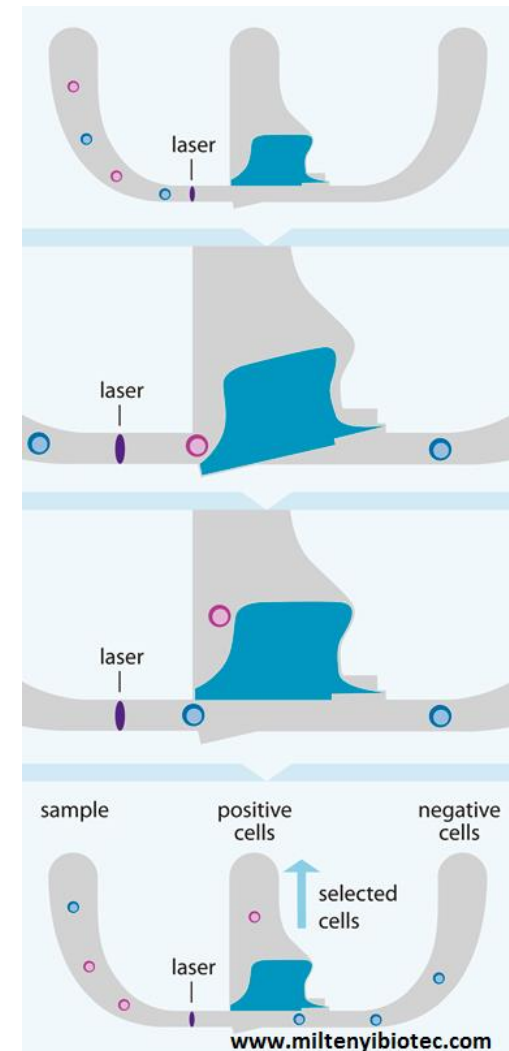
- Chromosomes
- Cloning, Strain Improvement
- Genomics, Proteomics
- Comprehensive single cell analyses (heterogeneity)

Other Cell Sorting Technologies

- Single Cell dispensers
- Tyto/OWL
- DEP sorter
- NanoCollect
- BulkSorting
(Magnetic, Gravity, Acoustic, ...)



DEPArray™ System



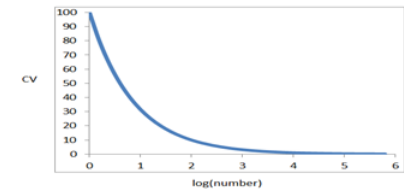
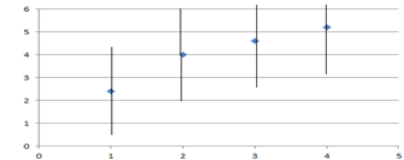
MACSQuant®Tyto™

Rare Cell Analysis and Sorting

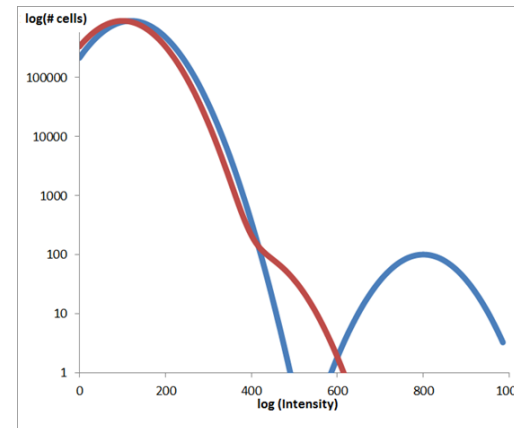
Examples CD34, AC133, antigen specific cells, CTCs

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

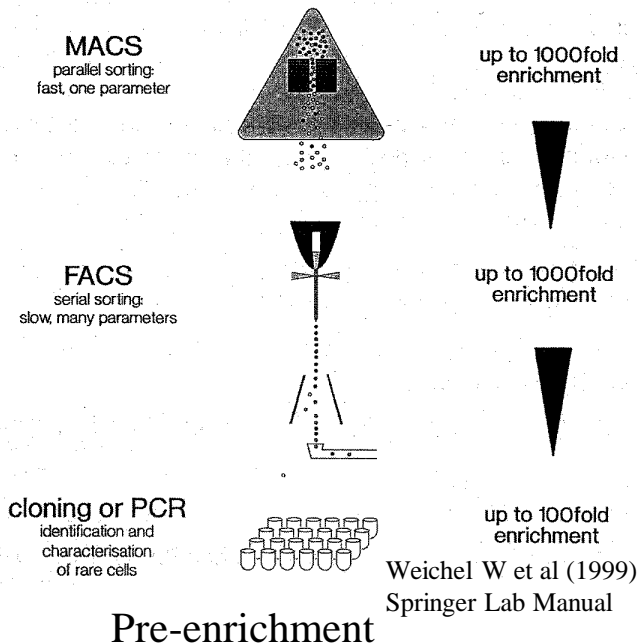
	Sample 1	Sample 2	Sample 3	Sample 4
	6	2	6	8
	3	7	1	6
	1	3	5	3
	1	4	5	6
	1	4	6	3
Mean	2.4	4	4.6	5.2
St.Dev	2.2	1.9	2.1	2.2
		Overall	Mean	4.1
			St.Dev	2.2



Ignoring Counting Statistics Can Lead to Erroneous Conclusions



Population Separation



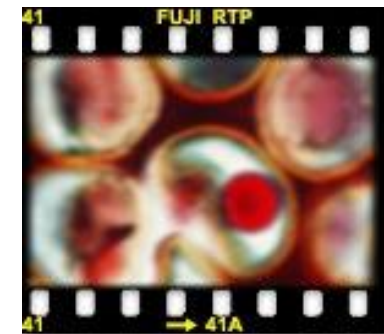
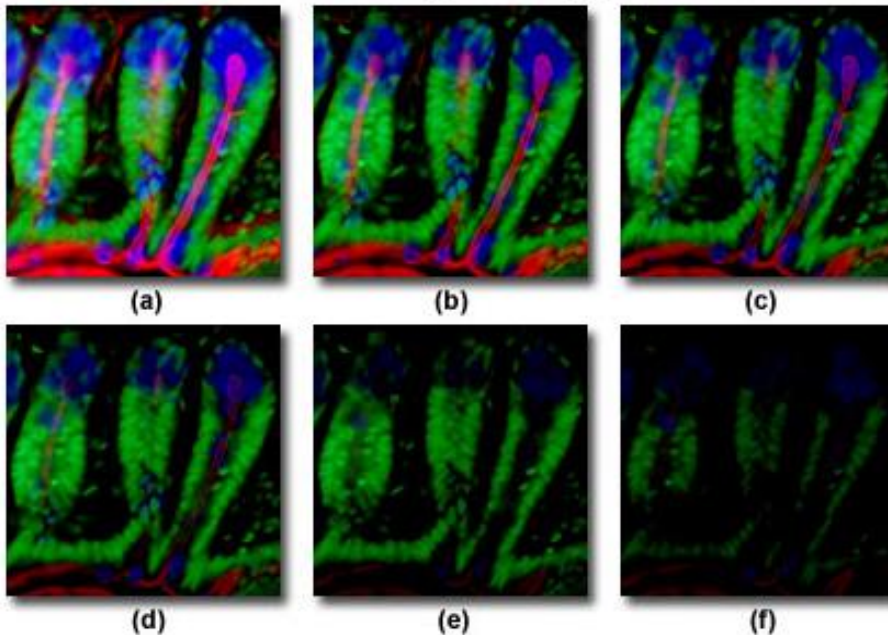
Pre-enrichment

Quantitative Multi-color Microscopy

Additional factors

- Field to field focus
- Photobleaching

Differential Photobleaching in Multiply-Stained Tissues

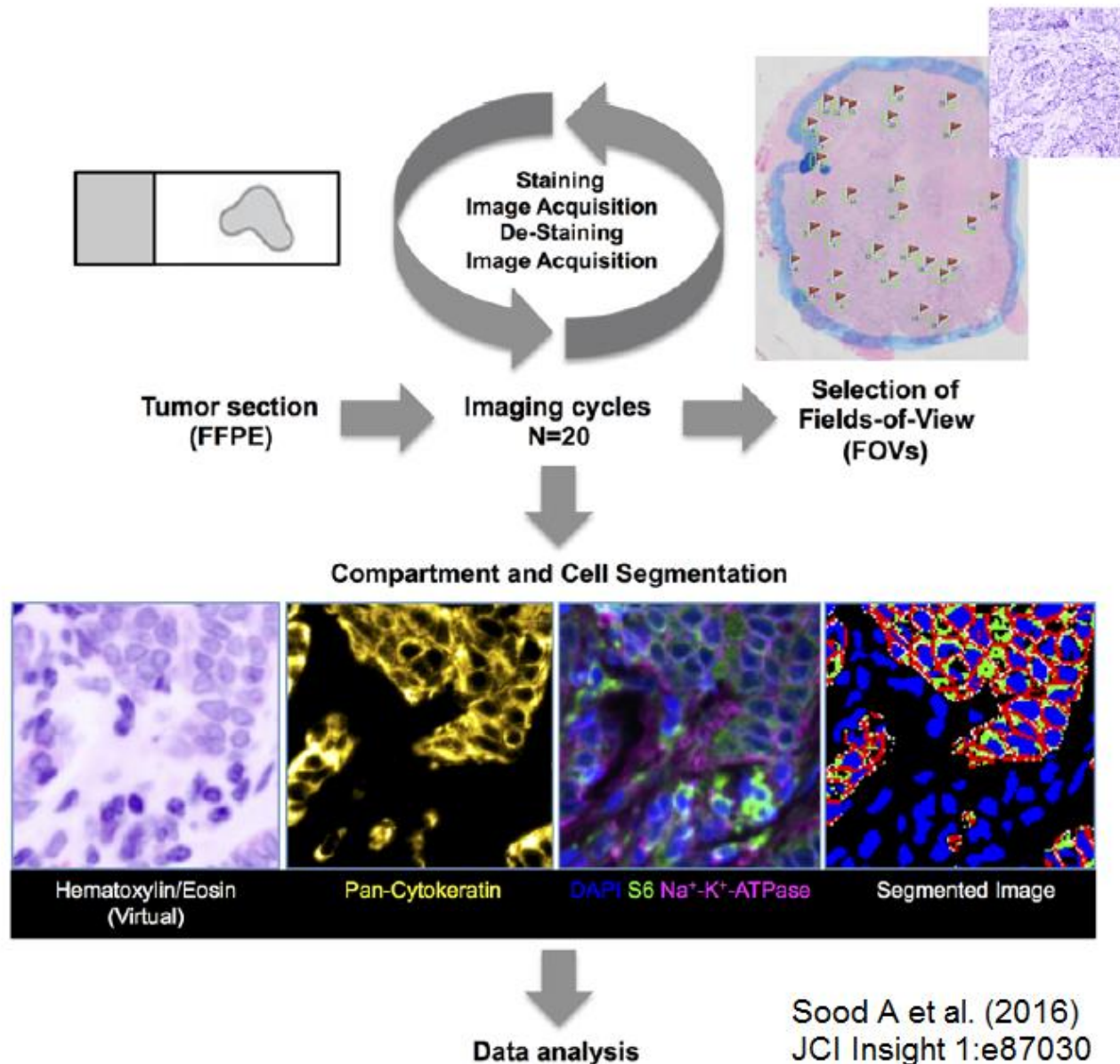


Out of Focus

Images from

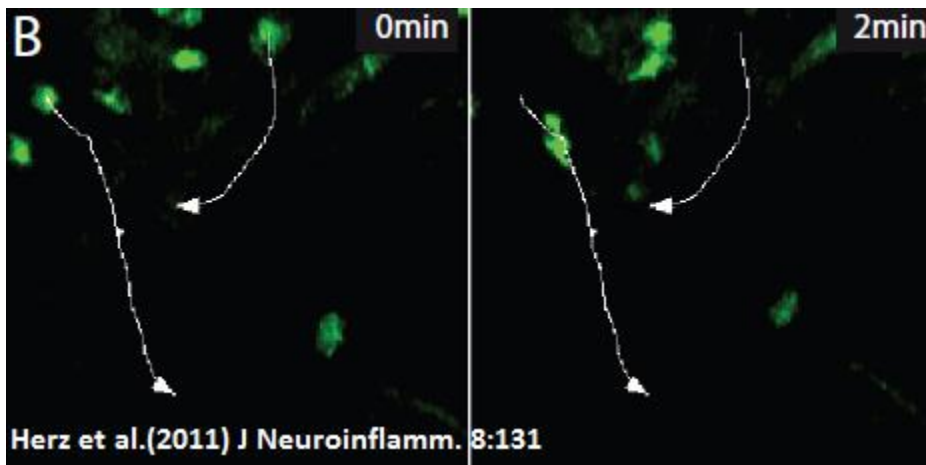
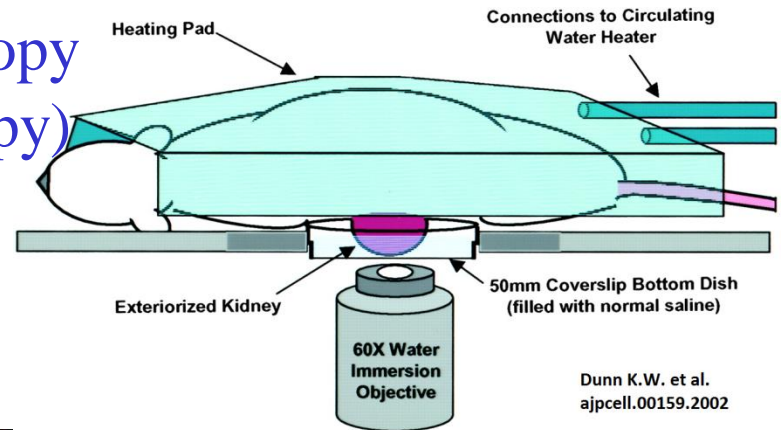
<http://micro.magnet.fsu.edu/primer/index.html>

Cyclic Multicolor Immunofluorescence



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

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

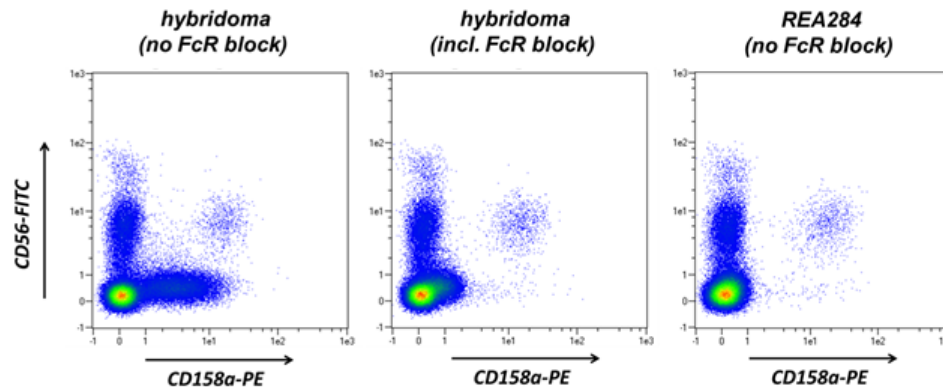
- Multi-parameter immunofluorescence (antibodies)
- Multi-parameter gene expression analysis (NA probes)
- Cell cycle analysis (high resolution FCM, imaging, BrDU)
- Molecular clustering (fluorescence energy transfer FRET)
- Kinetics (population-based flow cytometry, single cell by imaging; Ca⁺⁺ flux, 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)

Reagents

- Nucleic acid Probes
- Antibodies (Human, Mouse, Rabbit; Camel, Llama, Shark)
- Recombinant Antibodies
- Aptamers
- Molecular Imprinted Polymers
- Environment sensitive dyes (DNA dyes, pH probes)
- Enzyme reaction probes (fluorogenic substrates)
- Labels (fluorescent dyes, Raman labels, mass labels)

Llama: 15 kDalton antibodies
 10^{-9} M Kd, high stability

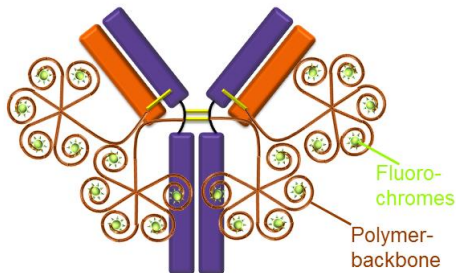
Fodey T et al; Trends in Anal.Chem. 30(2011) 254ff



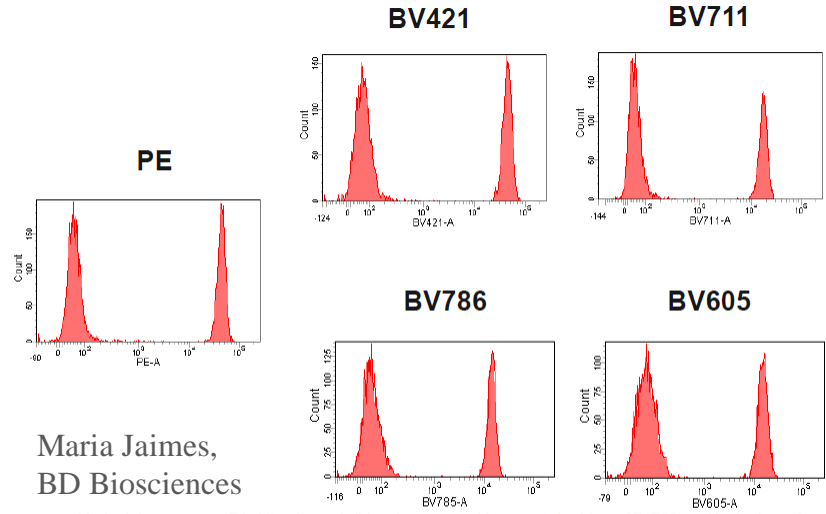
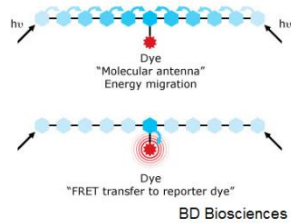
Fc-receptor binding:
CD158a-PE on PBMC

Source: Dr. Christian Dose,
Miltenyi Biotec

New Bright Dyes



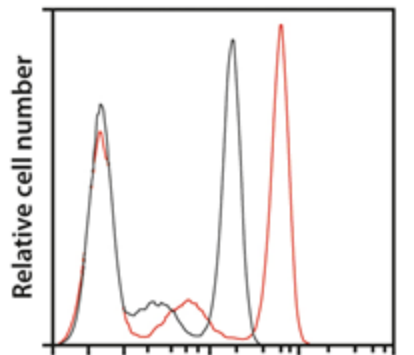
VioBright™ dyes, Christian Dose, Miltenyi Biotec



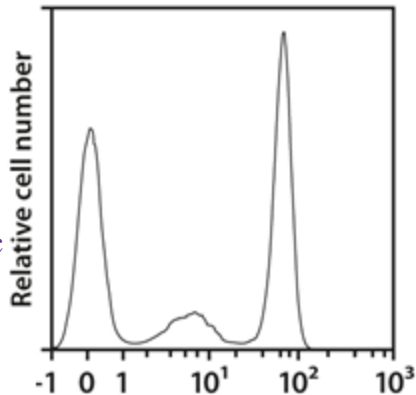
Maria Jaimes,
BD Biosciences

Maria Jaimes, page 17 in https://www.bdbiosciences.com/documents/webinar_071713_multicolor-bv.pdf

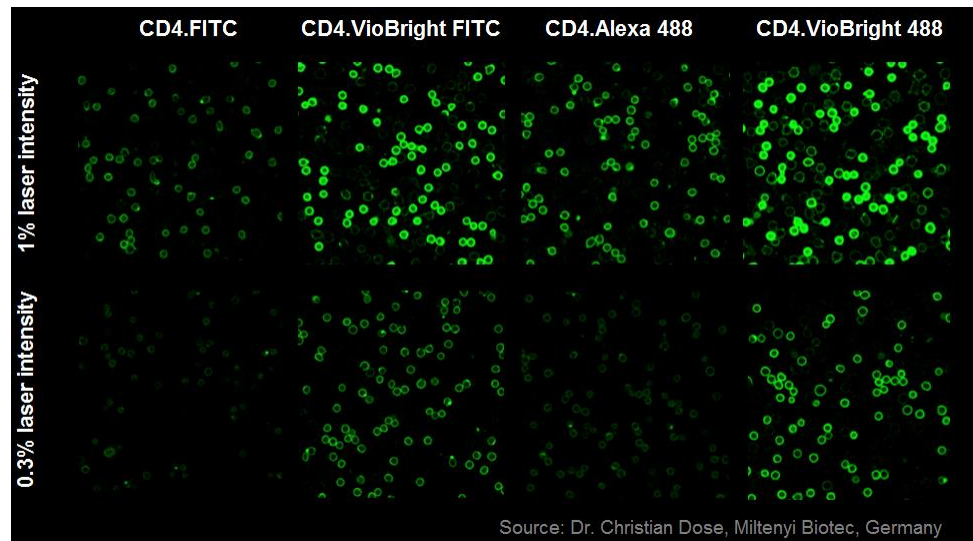
CD4-FITC
CD4-VioBright FITC
(clone Vit 4.3)



CD4-PE
(clone Vit 4.3)



Source:
Christian Dose
Miltenyi Biotec



Source: Dr. Christian Dose, Miltenyi Biotec, Germany

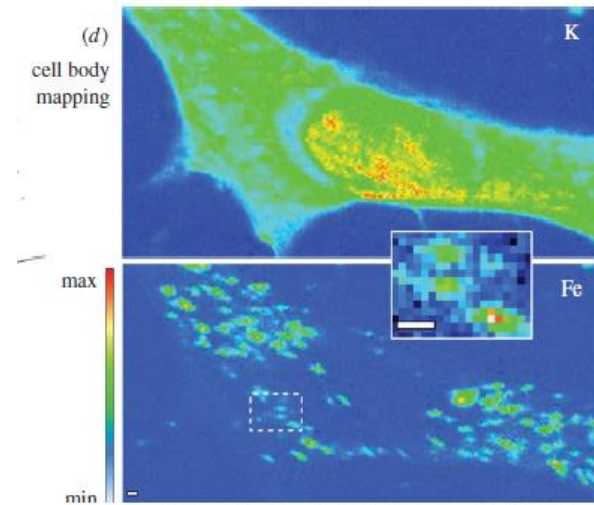
Technologies for Cell Analysis

	LOD	Mult	Envi	Id	Morph	Res	vivo	lbl-free
Flow cytometry	+++	+	+	+	-	-	+	+
Digital microscopy	+++	++	++	+	+++	++	-	++
2-photon imaging	+++	+	+	+	++	++	+	-
Electron microscopy	+	-	-	+	+++	+++	---	-
NGS	++	+++	---	+++	---	---	---	+++
ELISA	++	+	--	+++	---	---	---	---
Electrophoresis	++	++	+	+	---	---	---	+++
Mass spectrometry	+	+++	---	+++	+	-	---	+++
NMR MRI MRM	--	++	++++	++	++	---	+++	+++
Acoustic imaging	--	--	--	--	++	-	+++	+++
X-ray imaging	--	-	-	-	+++	++	+++	+++
...	na	na	na	na	na	na	na	na

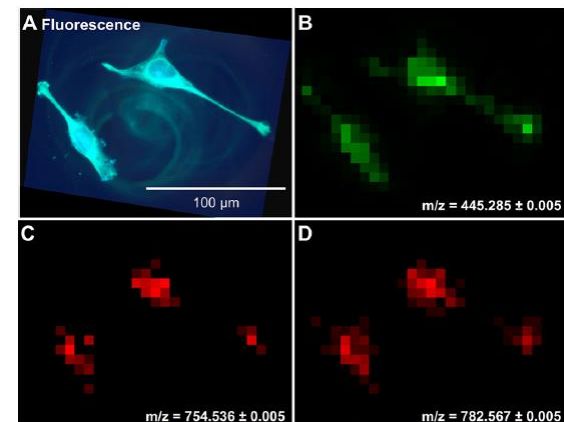
Details at: <http://www.desatoya.com/ScienceAndTechnology.htm>

Examples of 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
(chemical environment sensing)



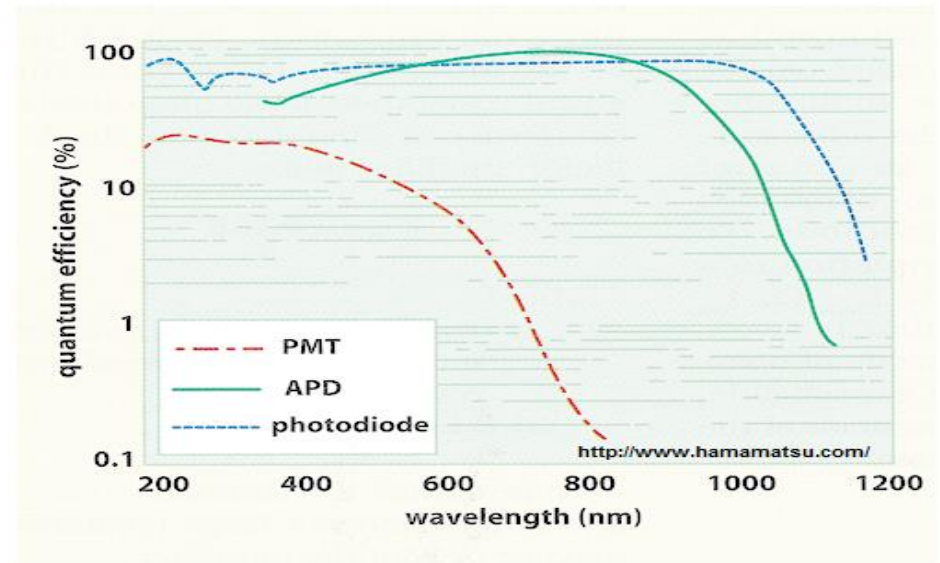
Ortega R et al (2009) J.R.Soc Interface 6: S649-S658



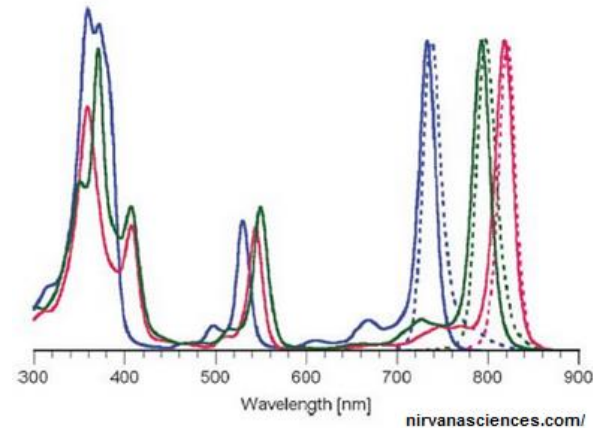
Schober Y et al. (2012) Anal.Chem. 84, 6293ff

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, ...)



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
- Ed Goldberg
- Liping Yu
- Brent Gaylord
- Mike Brasch
- Ben Verwer
- Eric Diebold
- ...
 above all BD
- BD Biosciences
- Miltenyi Biotec
- CYTEK Biosciences
- ...
- Holden Maecker, Stanford
- Bob Hoffman, consultant
- Martin Büscher, Miltenyi
- Christian Dose, Miltenyi
- Ming Yan, CYTEK
- Eric Chase, CYTEK
- Hrair Kirakossian, consultant
- Maria Jaimes, CYTEK
- Brian Warner
- David Basiji, BioCare Medical
- ...

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