AKADEMIE FÜR IMMUNOLOGIE

SPRING SCHOOL ON IMMUNOLOGY

Ettal, Bavaria, March 29 - April 3, 2020

Date changed to: October 25 - 30

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

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Key Reference Abbreviation

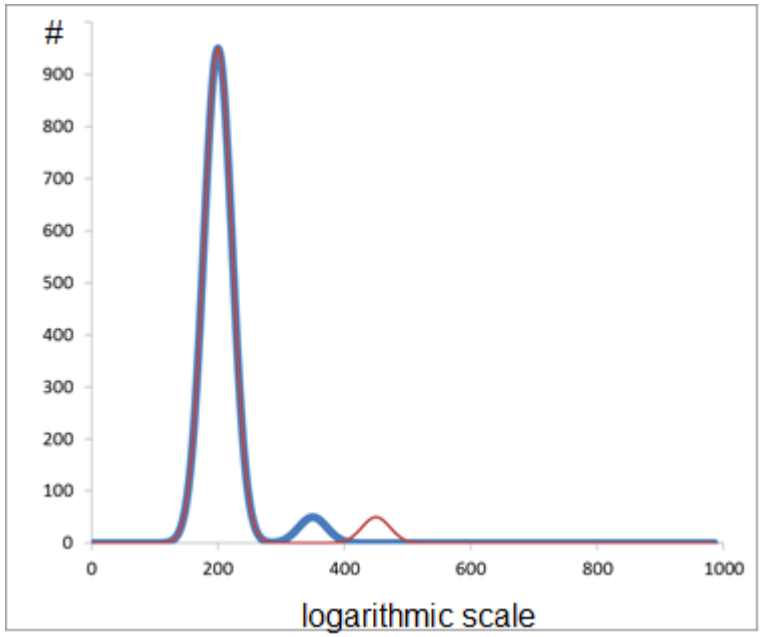
In this document “GUIDELINES” is used for
Cossarizza, Andrea, et al. "Guidelines for the use of flow cytometry and cell sorting in immunological studies." European journal of immunology 49.10 (2019): 1457-1973

The GUIDELINES contain contributions from 337 experienced scientists from more than a hundred institutes worldwide, describing their recommendations for the optimal use of flow cytometry.

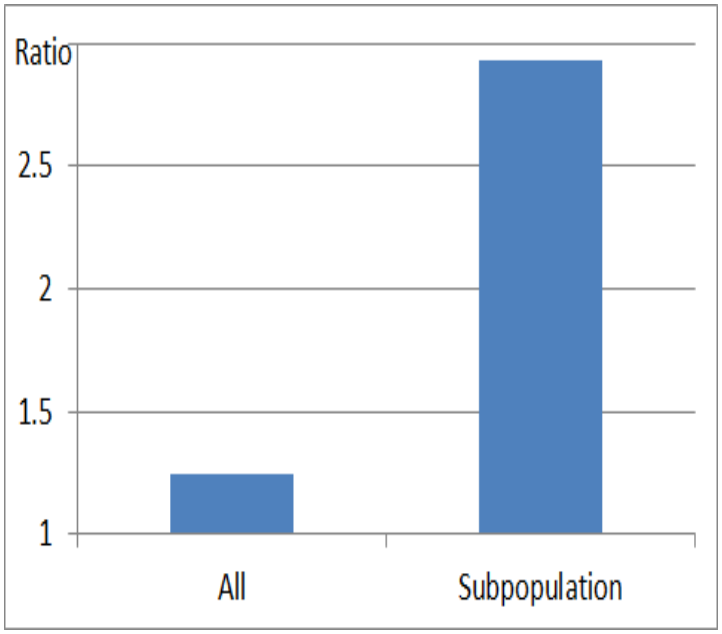
References with a DOI number can be located with an Internet search.

Why Cell Subset Analysis at the Single Cell Level

Intensity Histogram



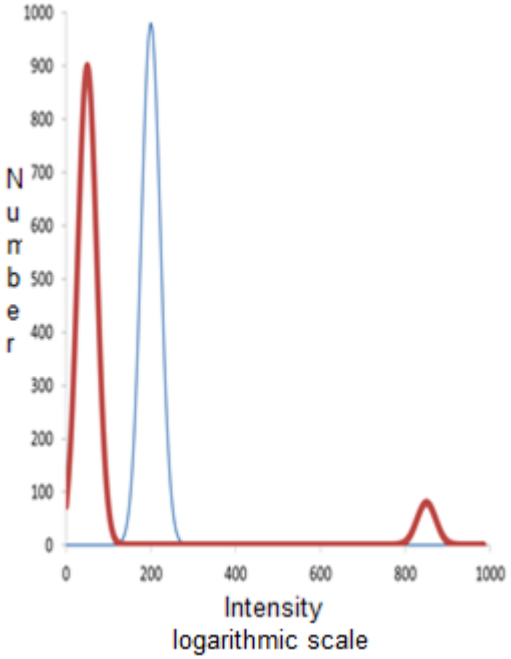
Intensity Ratios



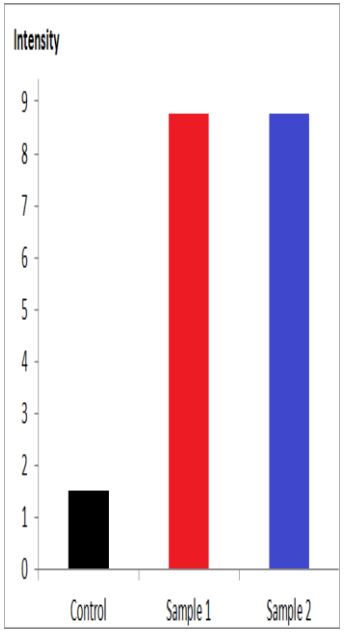
Subpopulation analysis detects changes better, especially for rare subpopulations.

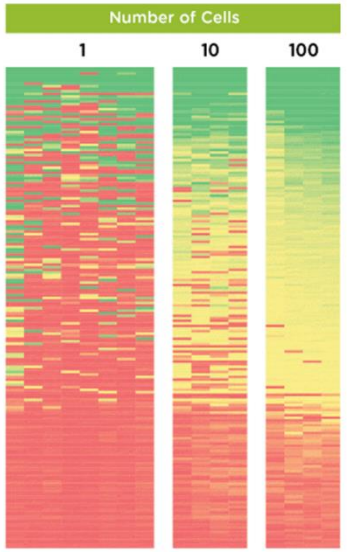
Why Single Cell Analysis

Intensity Histogram for Single Particles



Intensity per Sample





Source: http://www.nanostring.com

Cell by cell intensity analysis detects population heterogeneity.

Flow Cytometer Components

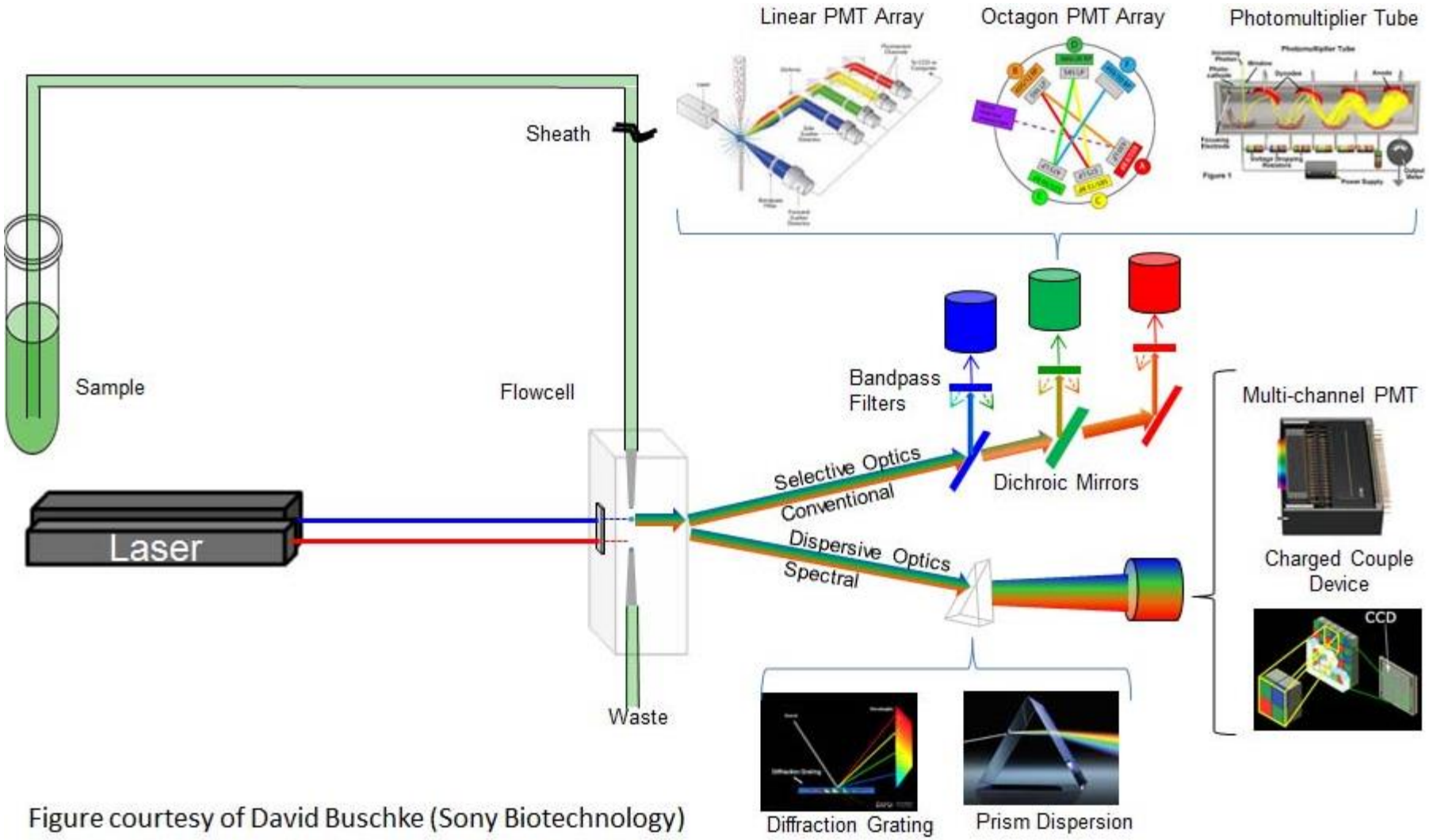


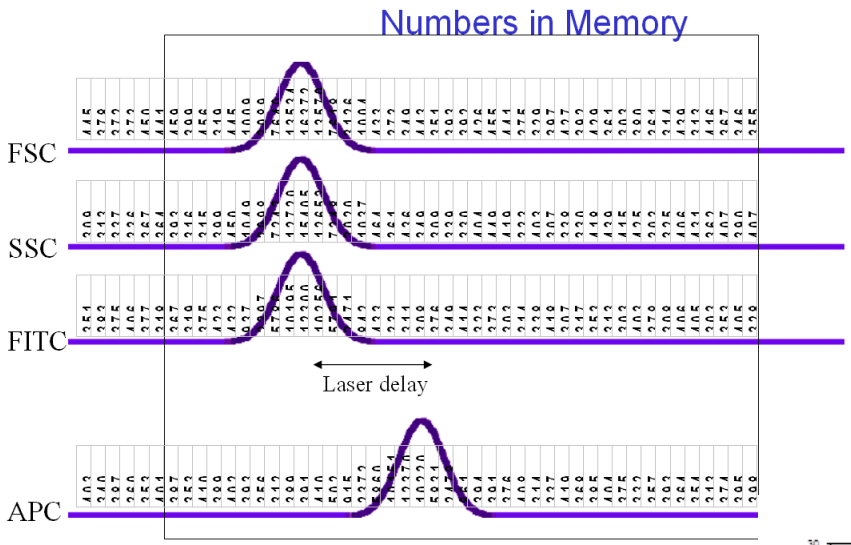
Figure courtesy of David Buschke (Sony Biotechnology)

GUIDELINES Flow cytometers, pages 1478ff

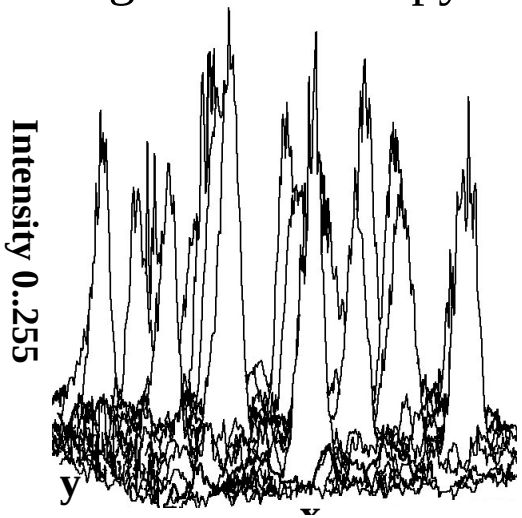
Dichroic filters vs. Multispectral cytometry: Feher K et al.(2016) Cytometry 89A: 681-9

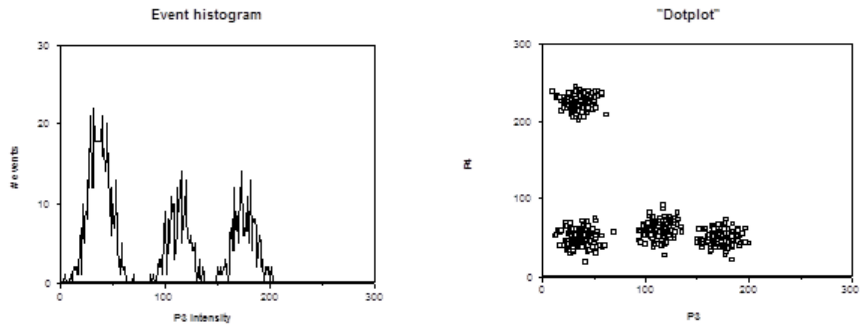
Basic Data Processing

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

GUIDELINES Data Analysis, pages 1829-1854

Hi-Parameter Data Processing

TOOL	PURPOSE
PhenoGraph	Clustering
X-Shift	Clustering
ACCENSE	Clustering
DensVM	Clustering
FlowSOM	Clustering
SPADE	Clustering
Citrus	Clustering, differential abundance analysis
Cydar	Clustering, differential abundance analysis
ACDC	Cell type assignment
SCAFFoLD	Cell type assignment, cellular trajectory mapping
Statistical SCAFFoLD	Cell type assignment, cellular trajectory mapping differential abundance analysis
Wanderlust	Cellular trajectory detection
DREMI/DREVI	Cellular trajectory detection
t-SNE	Dimensionality reduction

Olsen L R et al. (2019) DOI: 10.1002/cyto.a.23621

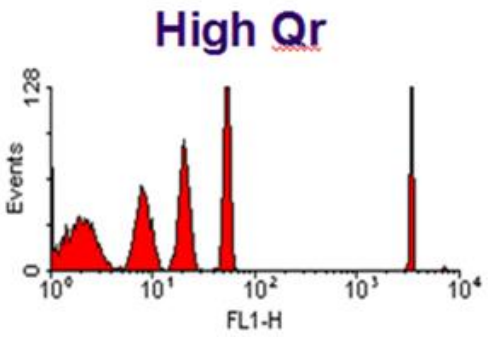
more at: Palit S et al. (2019) doi:http://dx.doi.org/10.1101/473215
Mair F et al. (2016) doi: 10.1002/eji.201545774 (mini-review)

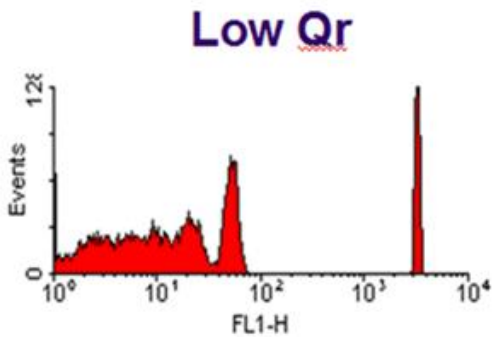
Instrument Evaluation Br, Qr

Qr, photon detection efficiency

Br, optical background from

- Cell autofluorescence
- Flow cell reflections
- Ambient light
- Free fluorochrome
- Raman scatter
- Spectral overlap





Figures: Joe Trotter, BD Biosciences

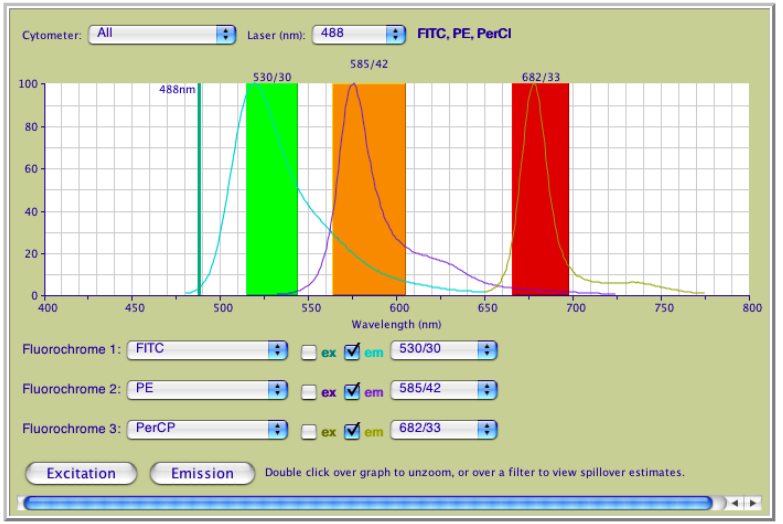
Signal Overlap and "Compensation"

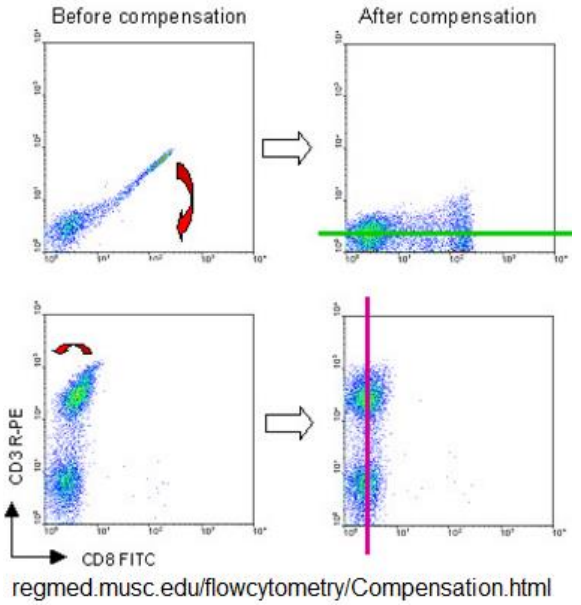
Calculation of concentrations
from optical/mass intensities

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

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)





GUIDELINES Compensation, pages 1484-88

Cytometer Measurements

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

Non-cell applications

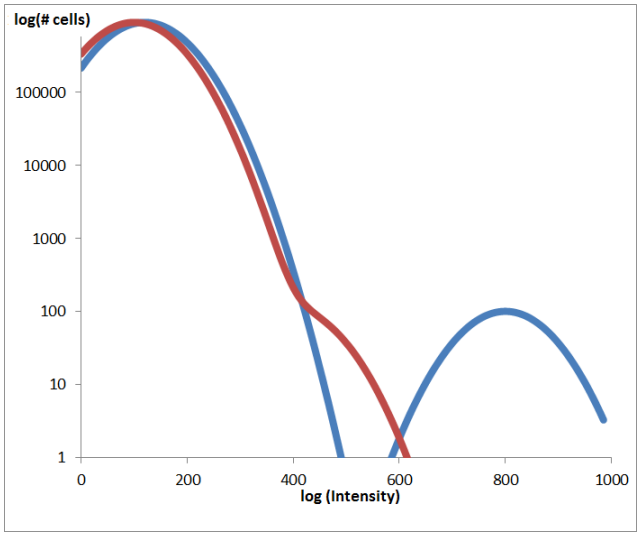
* Highly multiplexed bead-based immunoassays
* Single molecule counting

Label Selection

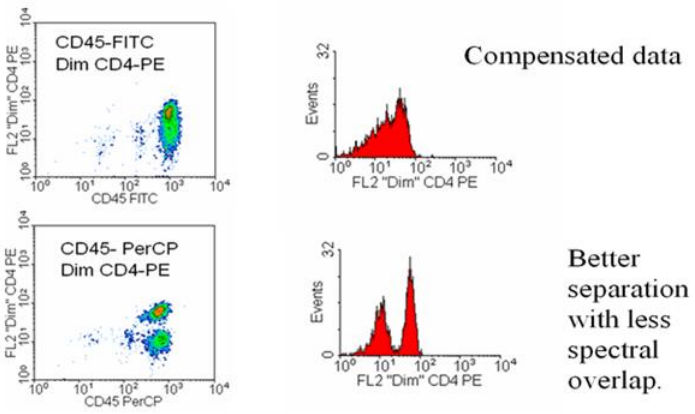
- Detection System
- Brightness
- Spectral Overlap
- Application (surface vs. internal)

Reagent performance

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



Brightness and Separation

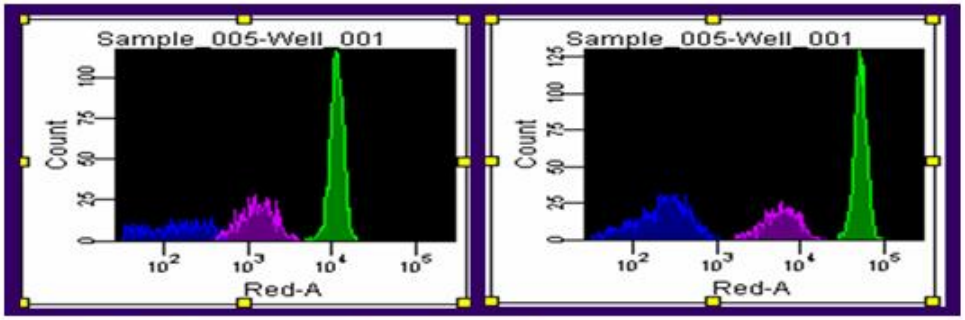


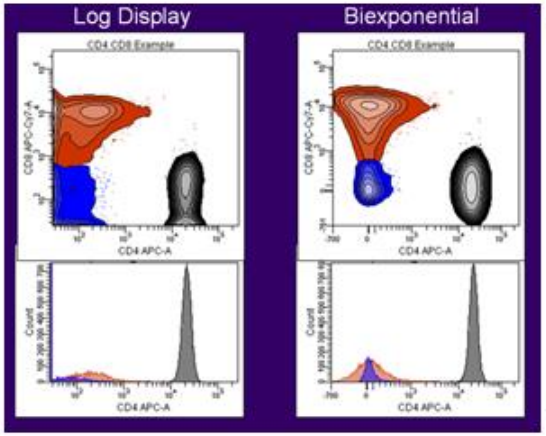
Spectral Overlap and Separation

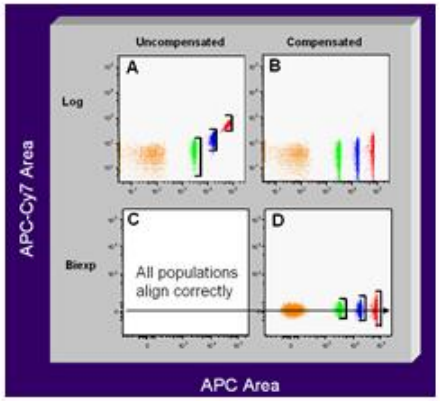
More info: Maecker HT et al. (2004) Cytometry 62A:169-173

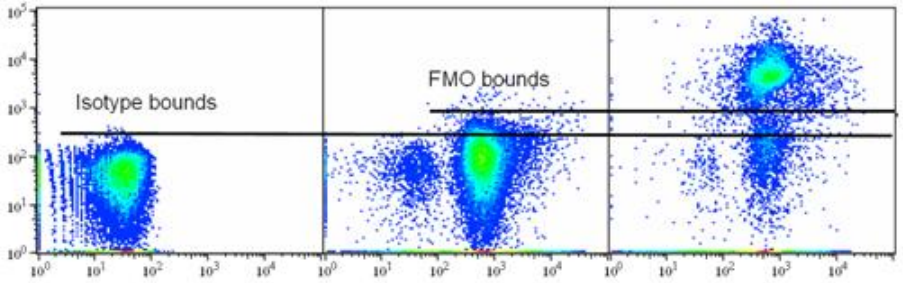
Optimizing cytometry measurements

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







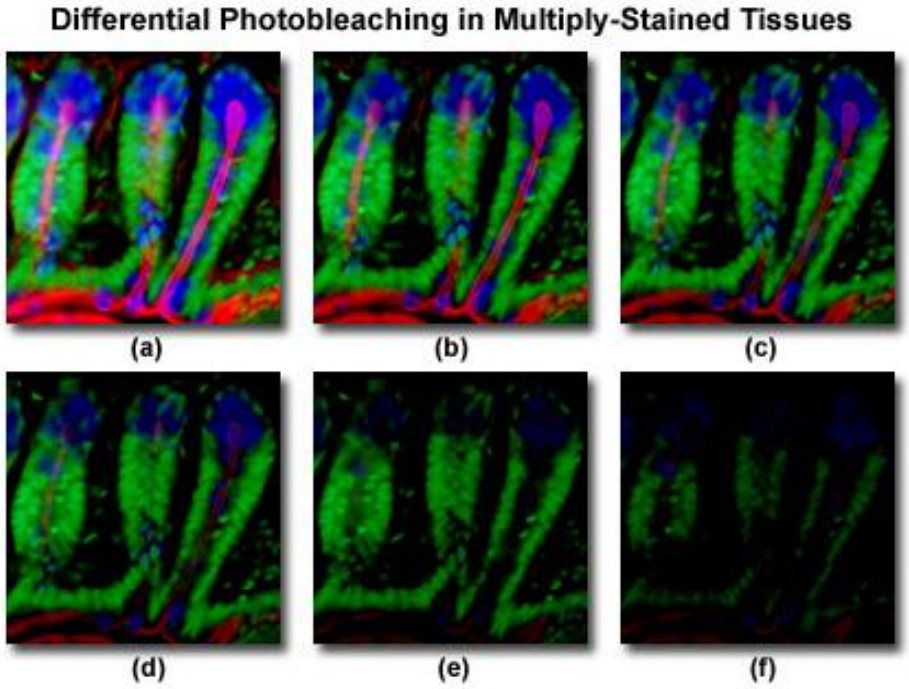


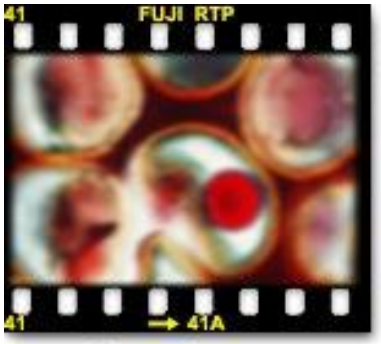
J. Trotter, BD Biosciences

Quantitative Multi-color Microscopy

Additional factors

- Field to field focus
- Photobleaching



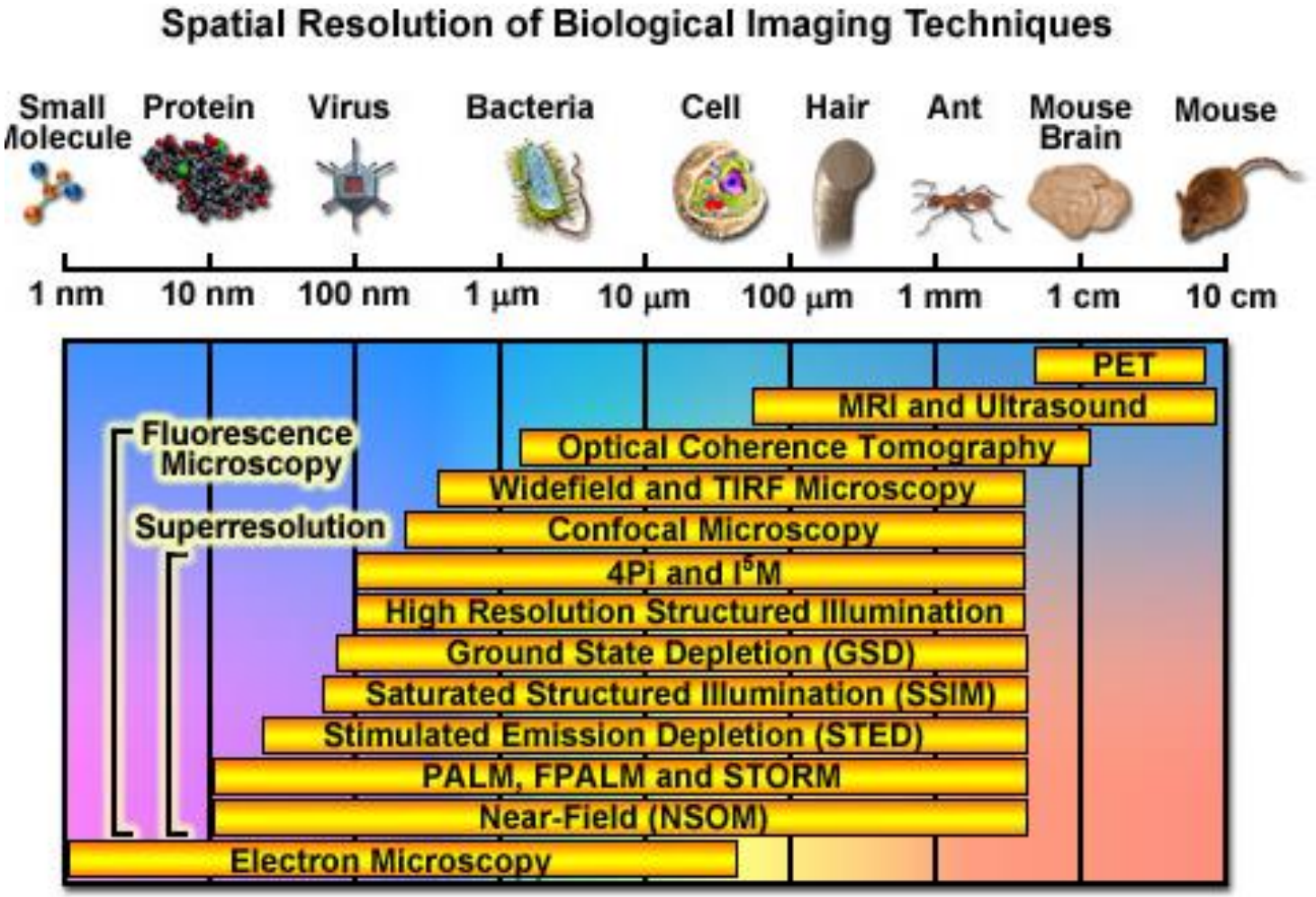


Out of Focus

Images from

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

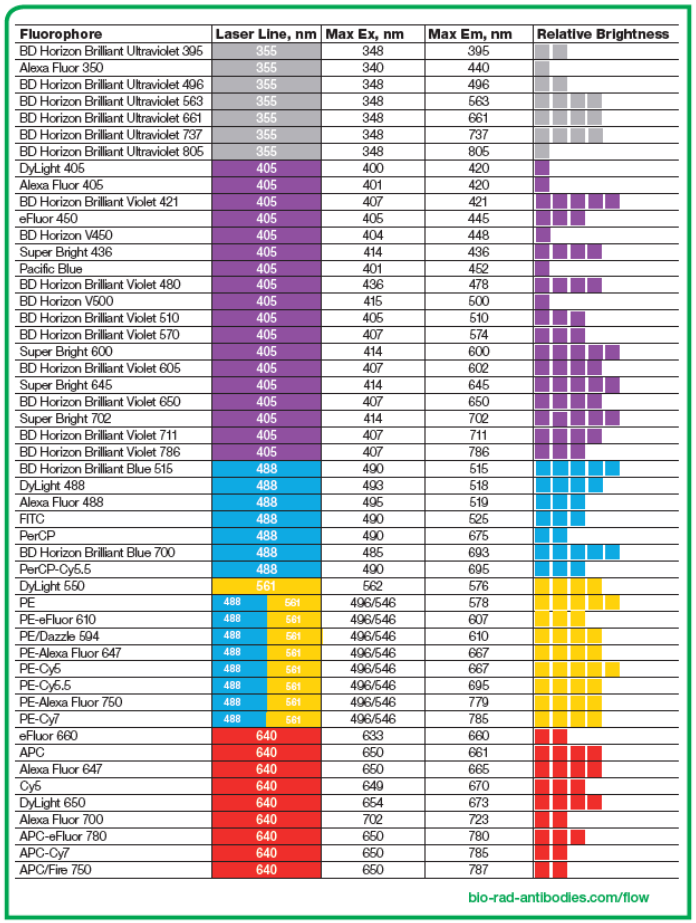
Super-Resolution Microscopy



http://zeiss-campus.magnet.fsu.edu/articles/superresolution/introduction.html

Multiparameter Cell Analysis

- Imaging
  - Immunofluorescence (IF)
  - Sequential Stain-Destain IF
  - …
- Flow Cytometry
  - IF, SERS labels
  - CyTOF
  - Sequence coding
  - Direct Raman
  - Label-free Impedance
  - Imaging e.g. Imagestream
- In-vivo Imaging and Flow Cytometry

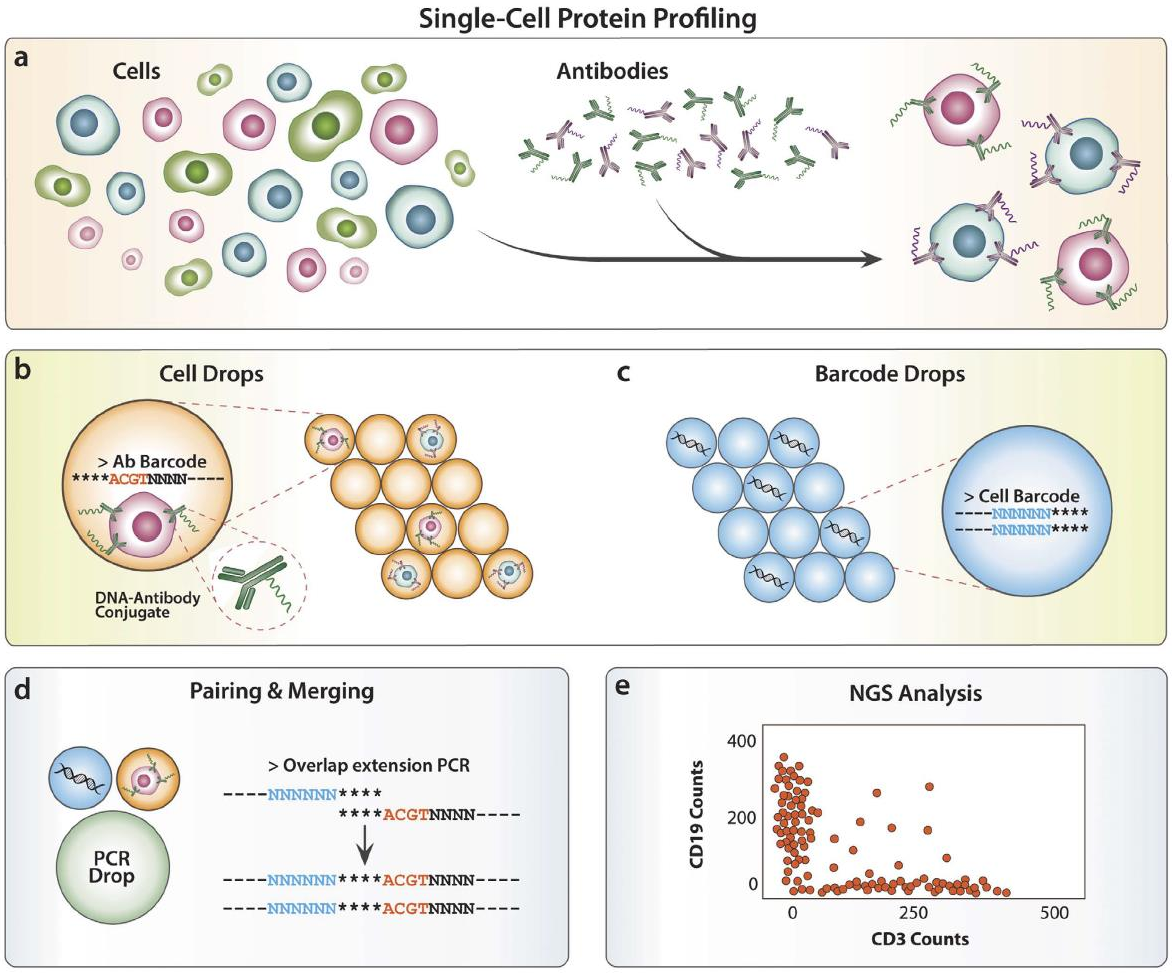


Fluorophore	Laser Line, nm	Max Ex, nm	Max Em, nm	Relative Brightness
BD Horizon Brilliant Ultraviolet 395	355	348	395	
Alexa Fluor 350	355	340	440	
BD Horizon Brilliant Ultraviolet 496	355	348	496	
BD Horizon Brilliant Ultraviolet 563	355	348	563	
BD Horizon Brilliant Ultraviolet 661	355	348	661	
BD Horizon Brilliant Ultraviolet 737	355	348	737	
BD Horizon Brilliant Ultraviolet 805	355	348	805	
DyLight 405	405	400	420	
Alexa Fluor 405	405	401	420	
BD Horizon Brilliant Violet 421	405	407	421	
eFluor 450	405	405	445	
BD Horizon V450	405	404	448	
Super Bright 436	405	414	436	
Pacific Blue	405	401	452	
BD Horizon Brilliant Violet 480	405	436	478	
BD Horizon V500	405	415	500	
BD Horizon Brilliant Violet 510	405	405	510	
BD Horizon Brilliant Violet 570	405	407	574	
Super Bright 600	405	414	600	
BD Horizon Brilliant Violet 605	405	407	602	
Super Bright 645	405	414	645	
BD Horizon Brilliant Violet 650	405	407	650	
Super Bright 702	405	414	702	
BD Horizon Brilliant Violet 711	405	407	711	
BD Horizon Brilliant Violet 786	405	407	786	
BD Horizon Brilliant Blue 515	488	490	515	
DyLight 488	488	493	518	
Alexa Fluor 488	488	495	519	
FITC	488	490	525	
PerCP	488	490	675	
BD Horizon Brilliant Blue 700	488	485	693	
PerCP-Cy5.5	488	490	695	
DyLight 550	561	562	576	
PE	488 561	496/546	578	
PE-eFluor 610	488 561	496/546	607	
PE/Dazzle 594	488 561	496/546	610	
PE-Alexa Fluor 647	488 561	496/546	667	
PE-Cy5	488 561	496/546	667	
PE-Cy5.5	488 561	496/546	695	
PE-Alexa Fluor 750	488 561	496/546	779	
PE-Cy7	488 561	496/546	785	
eFluor 660	640	633	660	
APC	640	650	661	
Alexa Fluor 647	640	650	665	
Cy5	640	649	670	
DyLight 650	640	654	673	
Alexa Fluor 700	640	702	723	
APC-eFluor 780	640	650	780	
APC-Cy7	640	650	785	
APC/Fire 750	640	650	787	

bio-rad-antibodies.com/flow

More info: Bendall SC et al. (2012) Nature Biotech. 30:639-47
Maecker H, Trotter J (2011) Multicolor Flow Cytometry Application Note
http://www.desatoya.com/ScienceTechnology/CytometryWithSorting.htm

Multiparameter Single Cell Analysis using Sequence Barcodes

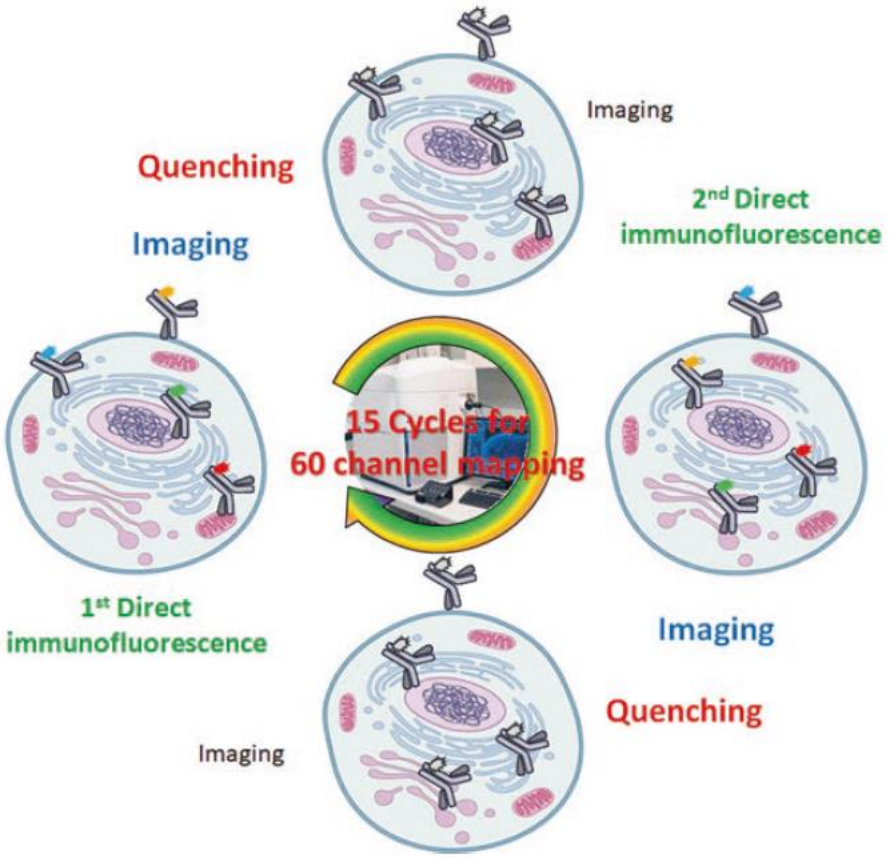


Shahi P et al. (2017) Abseq; DOI: 10.1038/srep44447

Conceived to measure unlimited number of markers with single molecule sensitivity (PCR).

Analysis of the details reveals important limitations e.g. limited dynamic range, issues combining low and high expression markers in addition to common staining artefacts.

Cyclic Immunofluorescence Microscopy



Eng J (2020) DOI: 10.1007/978-1-4939-9773-2_24

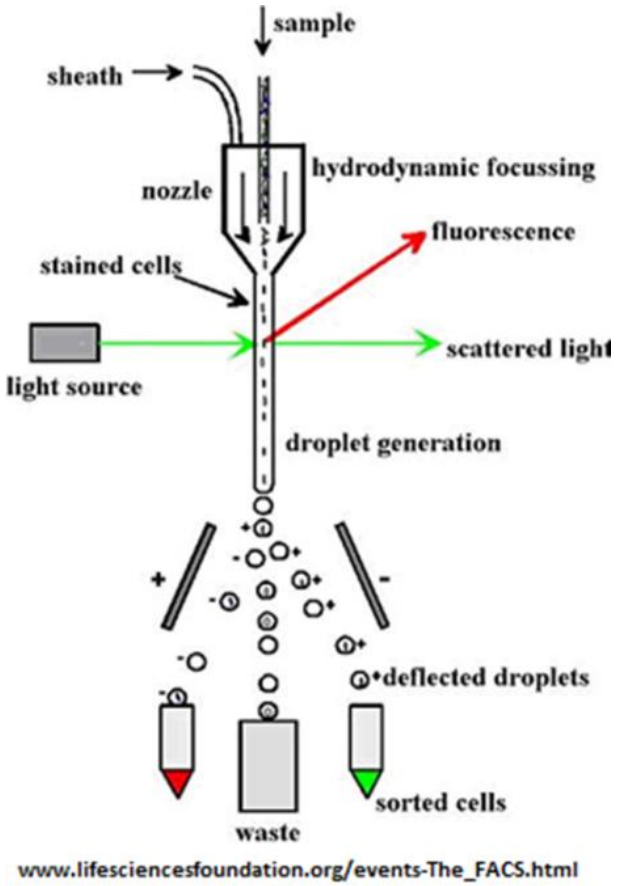
Capable of measuring a very large number of markers on a stationary cell/tissue sample by sequentially staining, observing, quenching.

Very high sensitivity with fluorescent labels

Multi-marker Cytometry Measurements Points To Consider

- Know your instrument status e.g. Qr & Br for different channels
- Use optimal detector settings e.g. high gain to maximize sensitivity (check to avoid off-scale events)
- An antibody/dye combination with poor separation for a single marker assay will not work for a multi-marker measurement
- Use high sensitivity labels for low expression markers and vice versa
- For energy transfer fluorophors beware of spectral drifts by photo-degradation
- Internal controls are essential

Cell Sorting
(FACS droplet sorter)



Application Examples

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

GUIDELINES Cell sorting, pages 1513 - 1523

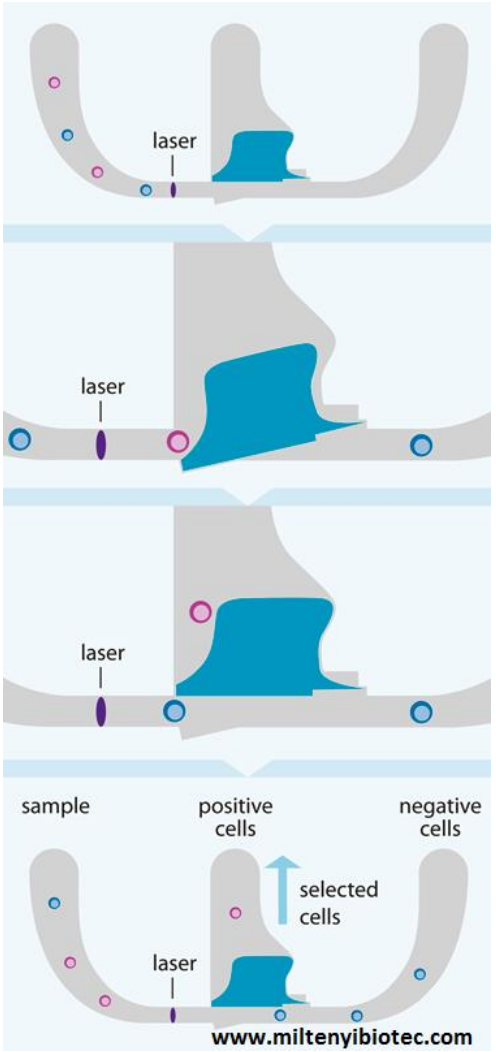
Other Cell Sorting Technologies

- Single Cell dispensers
- Tyto/OWL
- DEP sorter

- BulkSorting

(Magnetic, Gravity, Acoustic, …)

DEPArray™ System

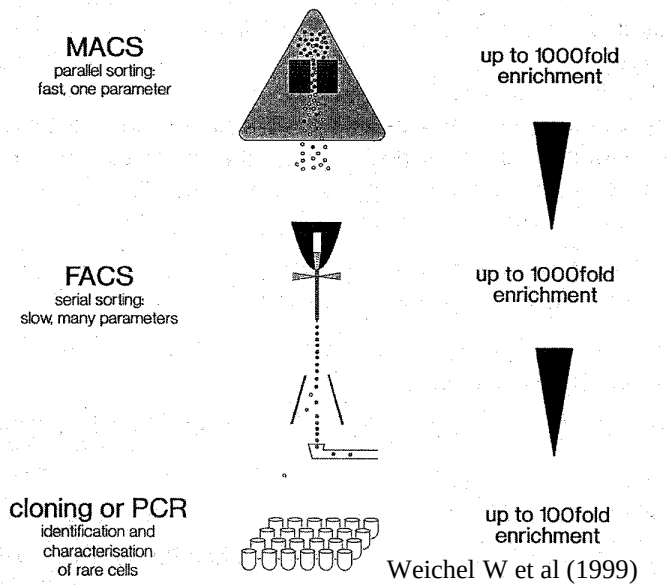


MACSQuant®Tyto™

Rare Cell Analysis and Sorting

Examples CD34, AC133, antigen specific cells, CTCs

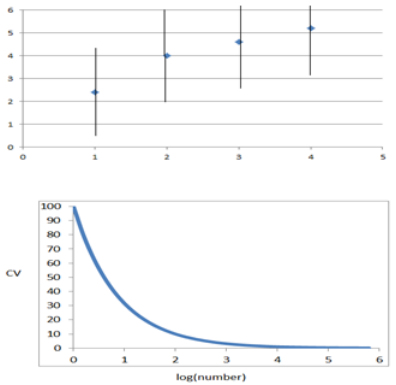
- Poisson count statistics
- Population Separation
- Bulk pre-enrichment or enrichment sorts



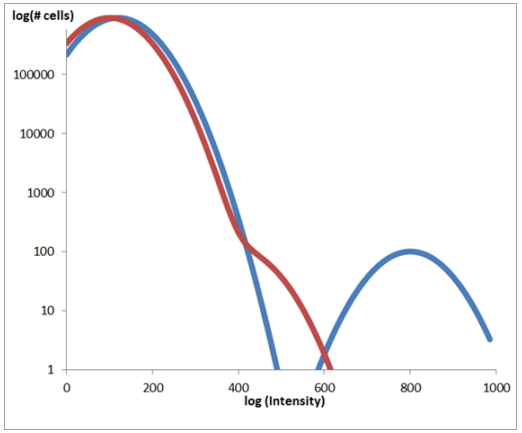
Weichel W et al (1999)
Springer Lab Manual

Pre-enrichment

	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

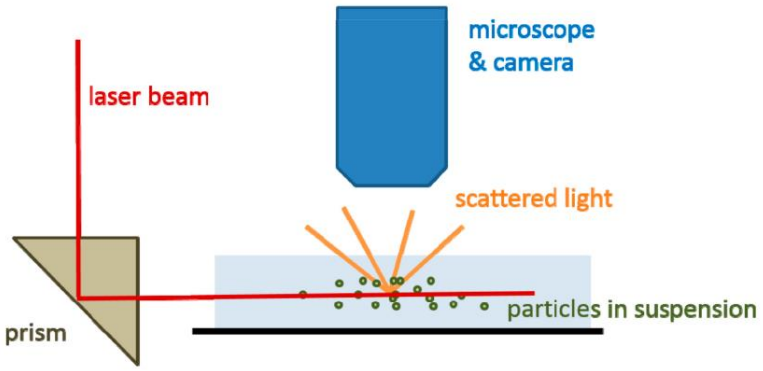
GUIDELINES Rare Cells: General Rules, pages 1523 – 26, 1846-7

Small Particle Analysis

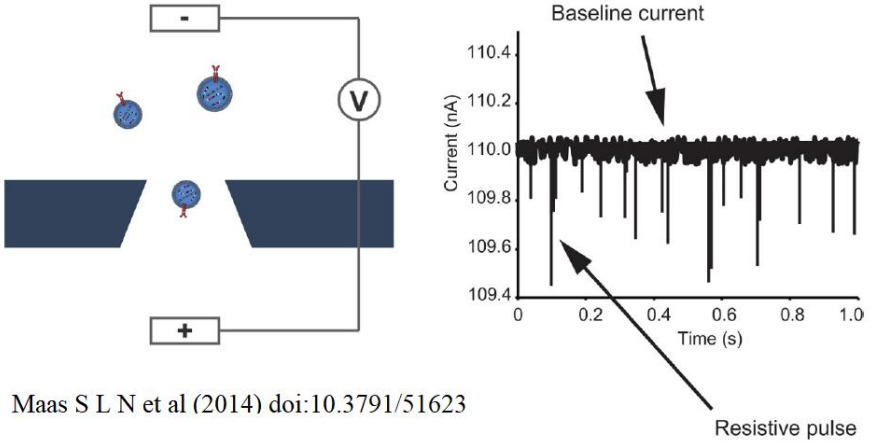
Microorganisms, Organelles, Exosomes

Flow Cytometry
Light microscopy
Electron microscopy
Atomic force microscopy

Nanoparticle tracking analysis
Tunable resistive pulse sensing
Dynamic light scattering



SzatanekR2017 doi:10.3390/ijms18061153



Maas S L N et al (2014) doi:10.3791/51623

GUIDELINES Mitochondria, Exosomes 1527-35; microbes 1883-7

Organelles

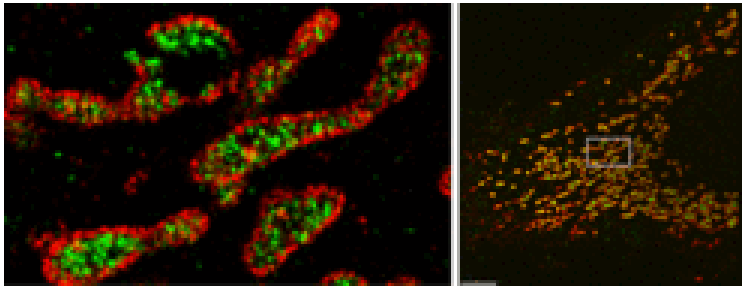
Observation

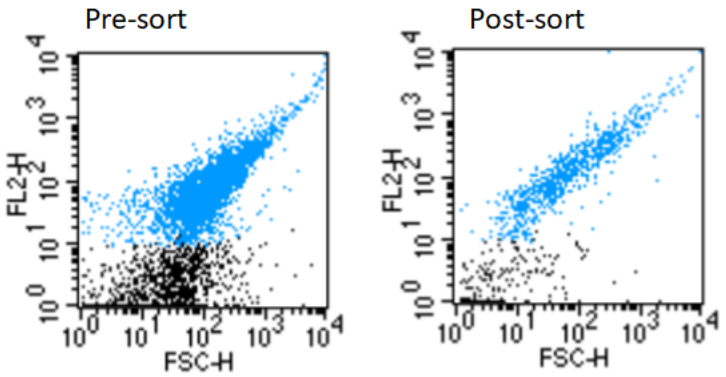
Fluorescence microscopy
Superresolution microscopy
Electron microscopy
Atomic force microscopy
Flow Cytometry

Purification

Centrifugation
Optical trapping
Field flow fractionation
Inertial flow microfluidics
Affinity binding to beads
FAOS (fluorescence assisted organelle sorting)

www.biochem.mpg.de/en/20171219-schueder-jungmann





Sorting of metaxin-stained liver mitochondria

Data from BD Biosciences

Conclusions / Caveats

- For optimal results use an adequate technology

  (flow cytometry has enormous capabilities, but is not always the adequate technology to use e.g. single cell kinetics)

- Understand the limitations of the system

  (complexity, limits of detection, non-specific binding of reagents, …)

- Use appropriate statistical methods

  (understand variance of very low counts during rare cell analysis)

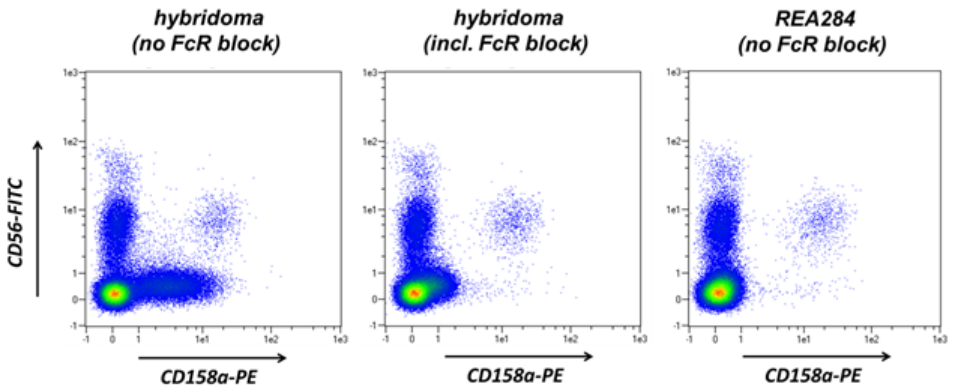
- Validate results with appropriate controls

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, Raman, mass, sequence “barcodes”, microlasers)

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

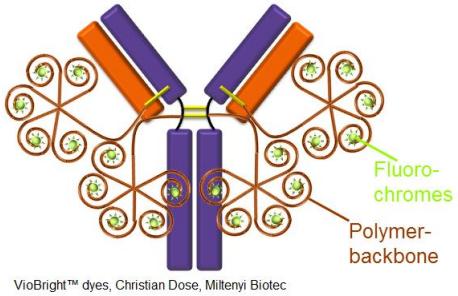
Fodey T et al; Trends in Anal.Chem. 30(2011) 254ff



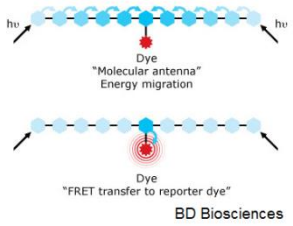
Fc-receptor binding:
CD158a-PE on PBMC

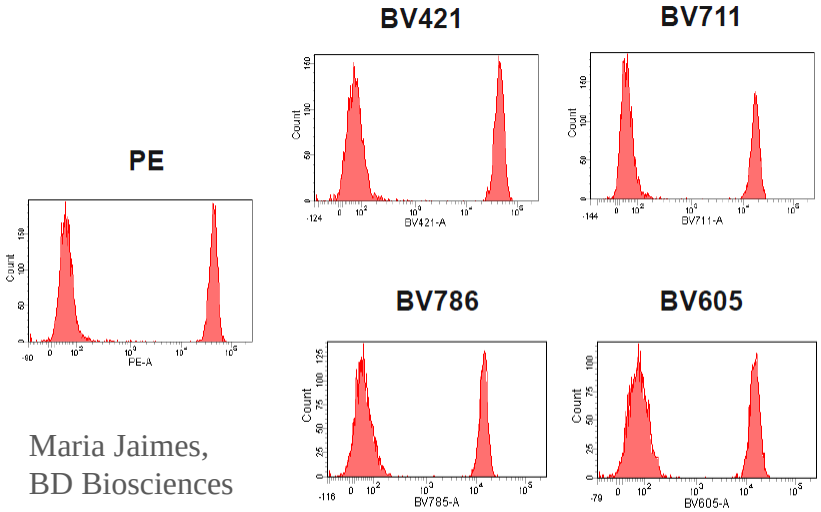
Source: Dr. Christian Dose,
Miltenyi Biotec

New Bright Dyes



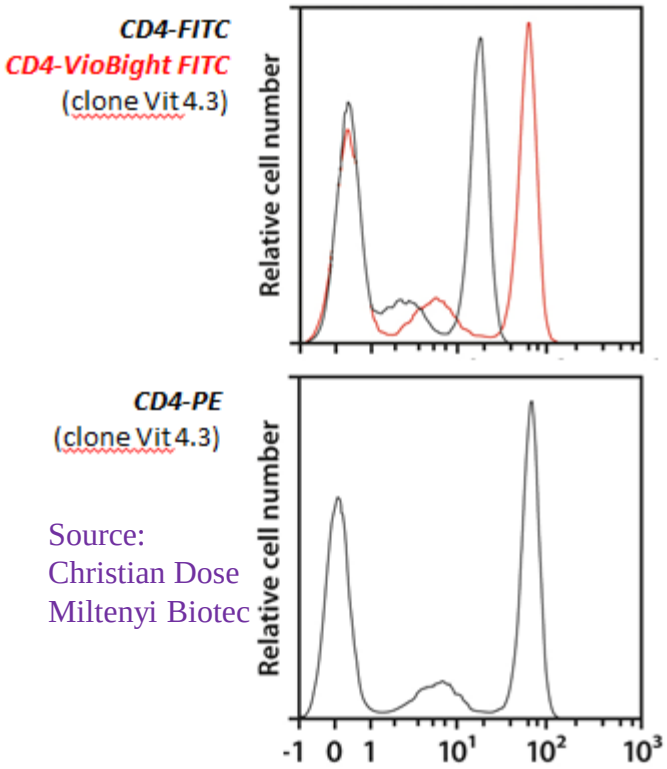
VioBright™ dyes, Christian Dose, Miltenyi Biotec



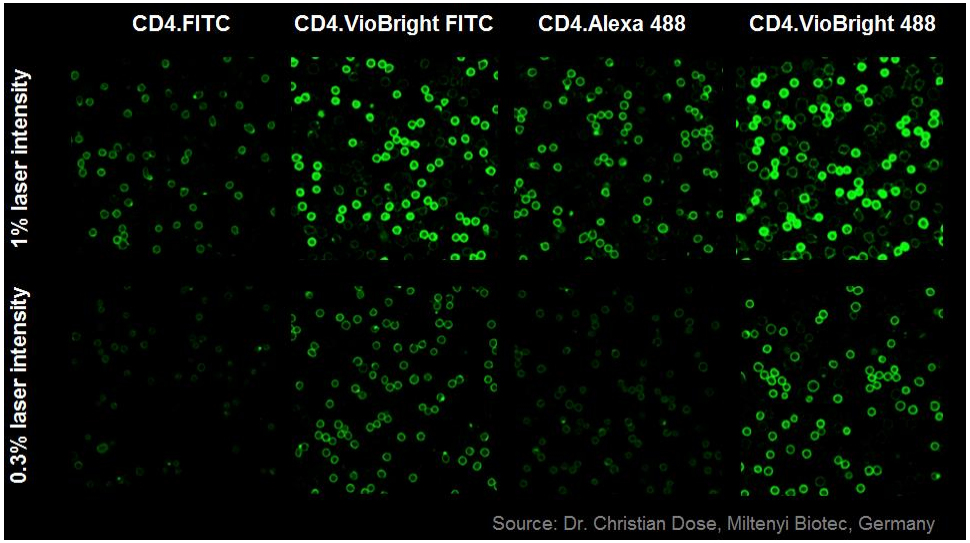


Maria Jaimes,
BD Biosciences

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



Source:
Christian Dose
Miltenyi Biotec



Conclusions

Multi-parameter cytometry

- Optimized flow and imaging single cell cytometry with well controlled sample collection and preparation, well characterized reagents and appropriate bio-informatics tools provide quantitative molecular insights into biological processes at 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

Key Applications

- Multi-parameter immunofluorescence (antibodies)
- Multi-parameter gene expression analysis (NA probes)
- Exosome analysis and sorting
- Single cell sorting for analysis with other technologies
- 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)

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

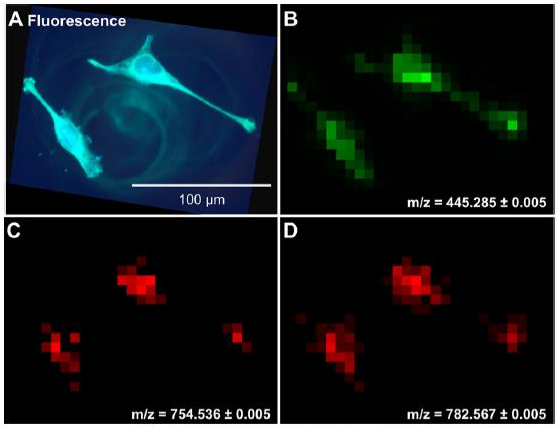
Microfluidics for WBC Isolation From Blood

Method		Dilution factor	WBC Separation efficiency	RBC depletion	Purity		WBC Enrichment	Sample throughput**
					WBC	MNC		
Cross-Flow filter	8	Undiluted	~98%	~99.975%	~70.5%	~28%*	~2000	0.06 µl/min
	12	Undiluted	~97.2%	NA	~96.9%	~39%*	NA	0.33 µl/min
Hydrodynamic filtration	13	10x	NA	NA	~3.6%	~1.4%*	~29	2 µl/min
Hydrophoretic filtration	15	20x (rat blood)	NA	NA	~58%	~23.2%*	~210	0.05 µl/min
Deterministic lateral displacement	9	Undiluted	~96% (WBC) ~95% (MNC)	~99.1%	~9%	~5.5%	~110	0.018 µl/min
Microfiltration using rarchets	26	Undiluted	~98% (WBC)	~100%	~100%	~40%*	NA	0.083 µl/min
Inertial focusing	16	500x	~95%	~94%	NA	NA	NA	3.6 µl/min
	18	400x	~89.7%	~99.8%	~91%	~36.4%*	NA	0.375 µl/min
	19	20x	NA	NA	~48%	~19.2%*	NA	240 µl/min (30 µl/min per channel)
Dielectrophoresis	21	5x	~92.1%	~87%	NA	NA	NA	0.16 µl/min
Leukocyte margination	22	Undiluted	NA	NA	NA	NA	~34	NA
Continuous erythrocyte lysis	23	Undiluted	~100%	>99.5%	NA	NA	NA	0.5 µl/min
	24	10x	~99%	NA	NA	NA	NA	100 µl/min
Slanted hydrodynamic filtration	27	20x	~85%	NA	~80%	~32%*	NA	2 µl/min
Acoustophoresis	This work	20x	>43% (WBC) >87% (MNC)	>99.95%	~54%	~53%	~1000 (WBC) ~2800 (MNC)	5 µl/min

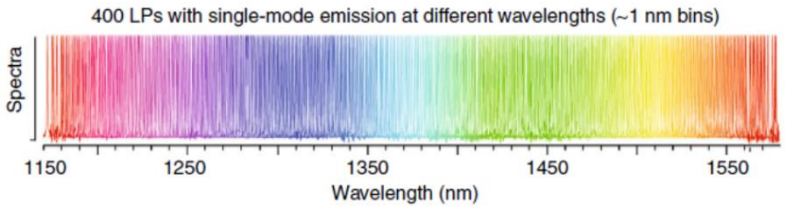
Label-free, continuous separation of WBC from blood using microfluidics Urbansky A et al (2017) DOI:10.1038/s41598-017-17200-9

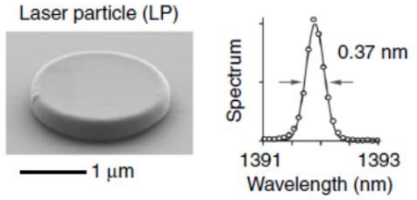
Examples of New Detection Technologies

- MALDI imaging
  (high parameter in-vitro imaging using mass spectrometry)
- Label-free imaging with Raman
  (measuring cellular components by their Raman spectra)
- Microlasers for high parameter cytometry
  (ultra-narrow bands of light emission)
- Label-free medium resolution NMR imaging
  (chemical environment sensing)



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





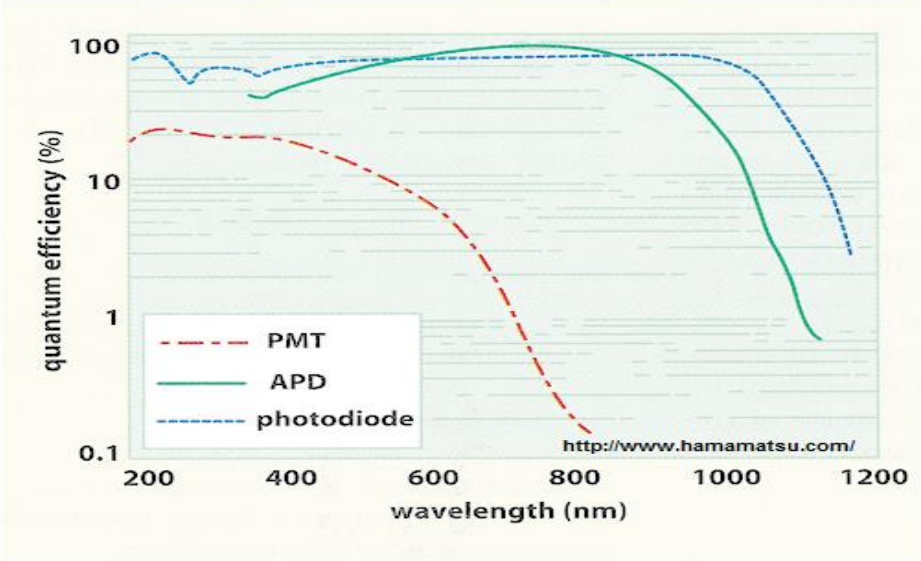
Laser Particles as Labels for Cell Analysis
Kwok S.J.J. et al (2019)

https://doi.org/10.1038/s41377-019-0183-5

New Detector-Label Combinations

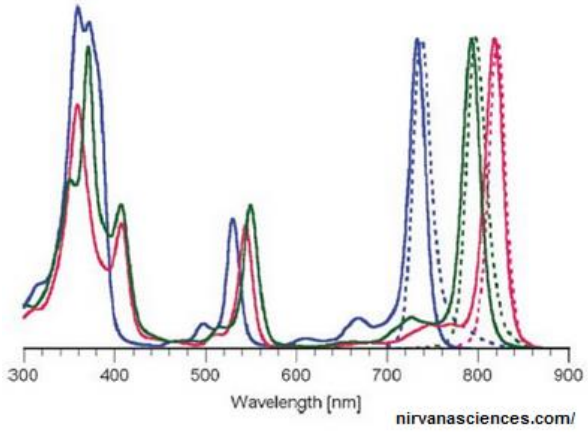
- New photodetectors extend the available spectrum

  (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 substsantial value in working with other scientific disciplines.

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  above all BD

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- Brian Warner
- David Basiji, BioCare Medical
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More science detail and references: http://www.desatoya.com