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Ettal, Bavaria, March 6 - 11, 2022

Flow and Image Cytometry Essentials with Recent Innovations for Single Cell Analysis

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http://www.desatoya.com

Key Reference Abbreviation

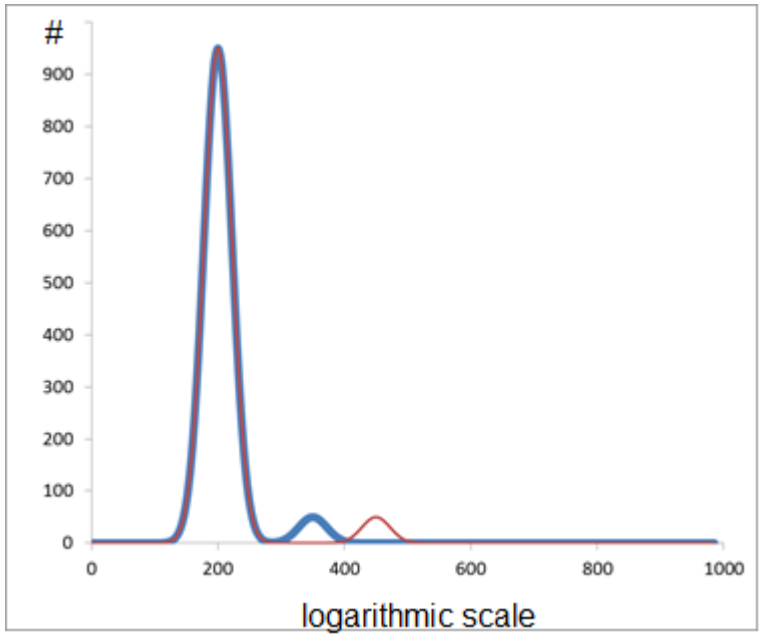
In this document “GUIDELINES” is used for
Cossarizza, Andrea, et al. "Guidelines for the use of flow cytometry and cell sorting in immunological studies." European journal of immunology 49.10 (2019): 1457-1973

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.

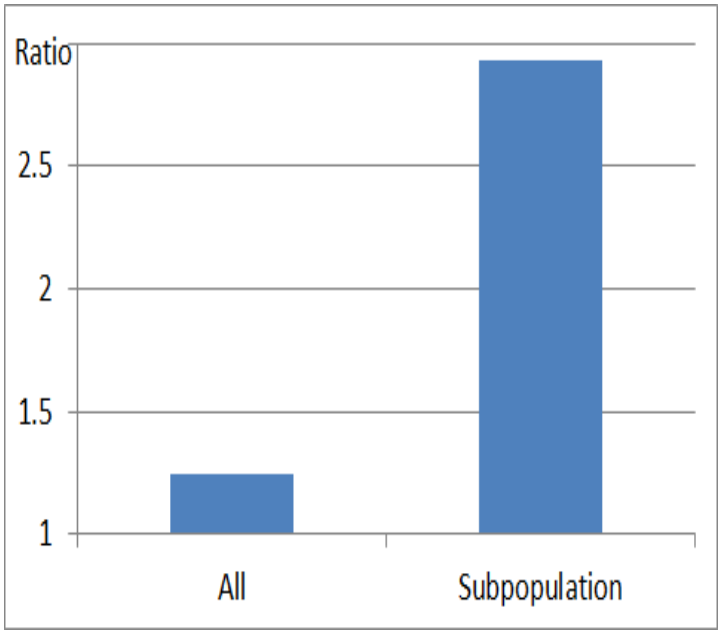
References with a DOI number can be located with an Internet search.

Why Cell Subset Analysis at the Single Cell Level

Intensity Histogram



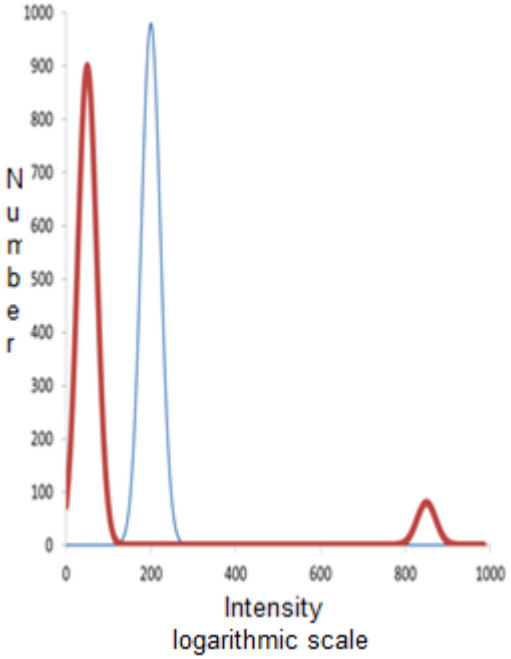
Intensity Ratios



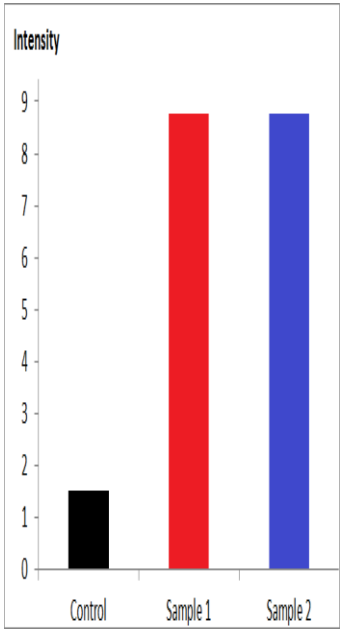
Subpopulation analysis detects changes better, especially for rare subpopulations.

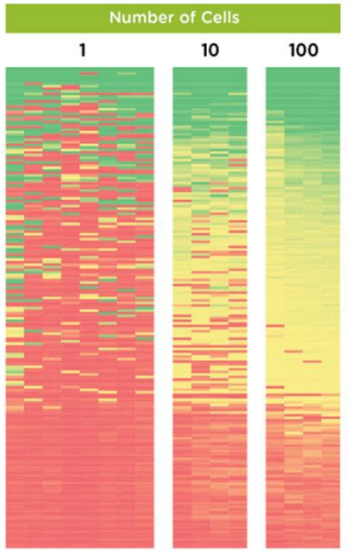
Why Single Cell Analysis

Intensity Histogram for Single Particles



Intensity per Sample





Source: http://www.nanostring.com

Cell by cell intensity analysis detects population heterogeneity.

Technologies for single cell analysis

- Microscopy
  - Super-resolution
  - High parameter cyclical fluorescence
  - In-vivo
- Single cells in separate defined locations
  - Wells of multi-well plates
  - Aqueous droplets in oil
- Flow cytometry
  - Optical property detection incl. in-vivo
  - Mass label detection (CyTOF)

Information from 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
- Gene expression (NGS)

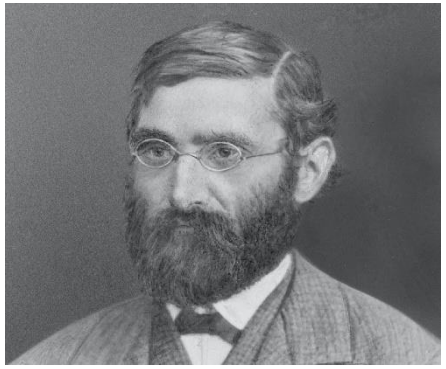
Non direct cell applications

* Highly multiplexed bead-based immunoassays
* Single molecule counting

Microscopy

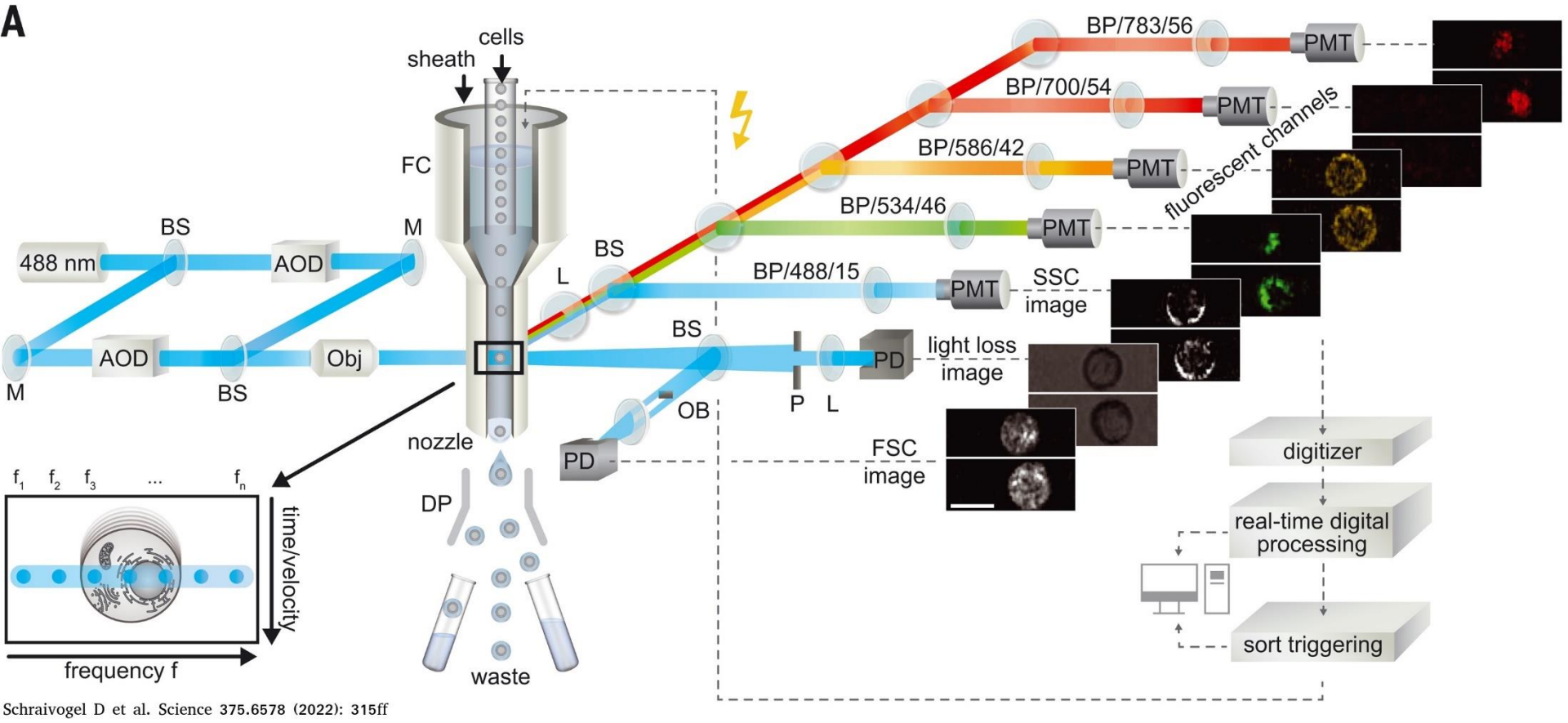
Cell analysis measuring a variety of optical properties of cells and tissue in a fixed location with high spatial resolution with or without labels

e.g. light transmission, light scatter (dark field), polarization, Raman, fluorescence



Ernst Karl Abbe
Physicist 1840-1905
Zeiss microscopes
(images from zeiss.com)

High speed imaging flow cytometry with droplet sorting capability



Schraivogel D et al. Science 375.6578 (2022): 315ff

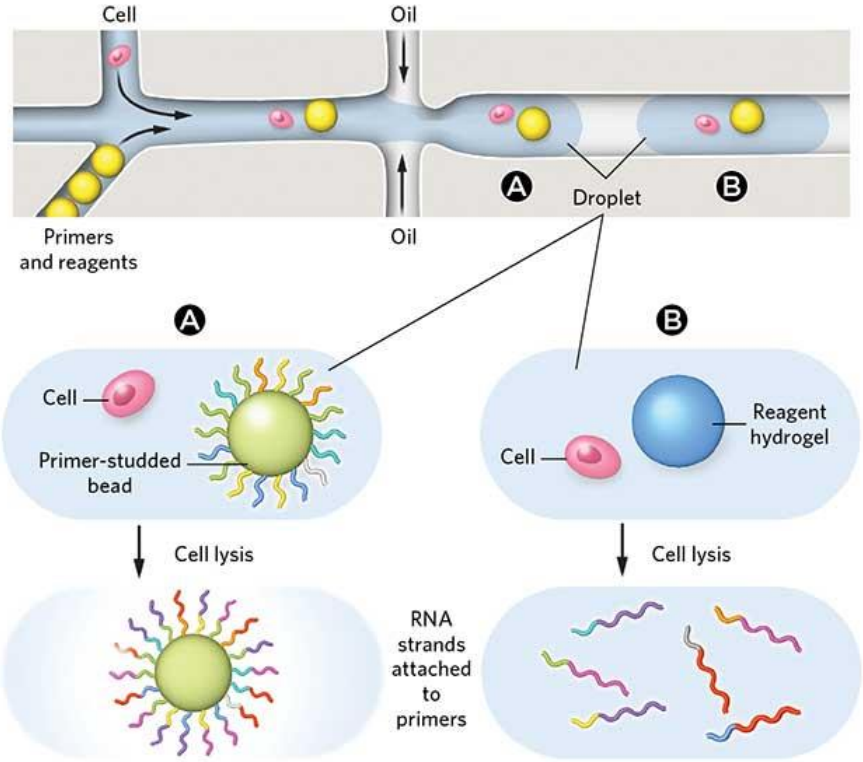
Technology originated at UCLA in Prof. Bahram Jalali's group.
An example of successful physical and life sciences collaboration.

Eric D. Diebold, BrandonW. Buckley, Daniel R. Gossett and Bahram Jalali, Nature Photonics, (2013)

Single cells isolated in aqueous compartments

Dispensing single cells in small microwells or isolating them in aqueous droplets, moving in microfluidic channels micro reaction chambers are generated.

Multi-step chemistry is performed and results are measured by direct microscopic observation or by NA sequencing after barcoding of resulting DNA or RNA to assign sequences to individual cells.



WilliamsR2015 The Scientist

Flow Cytometry

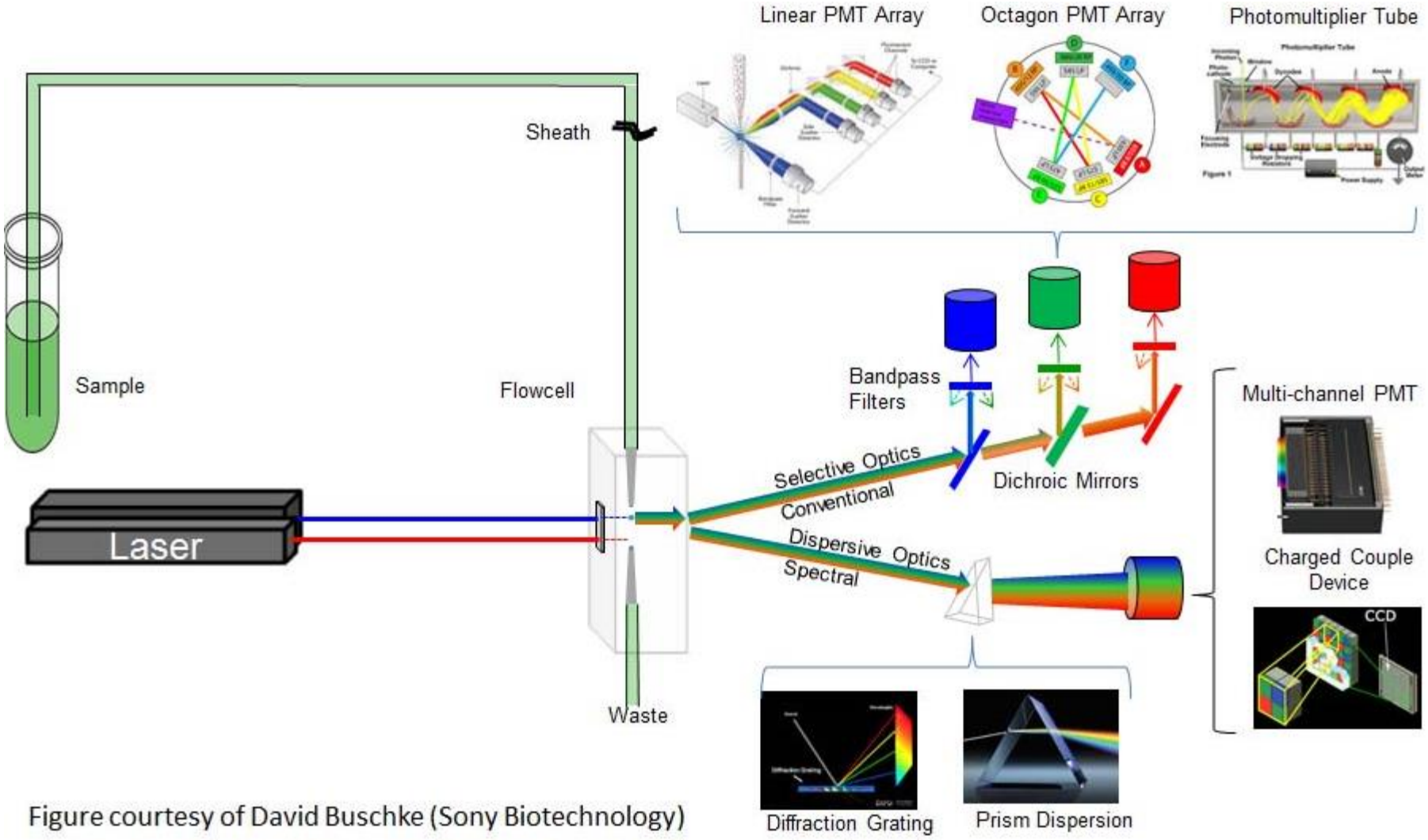


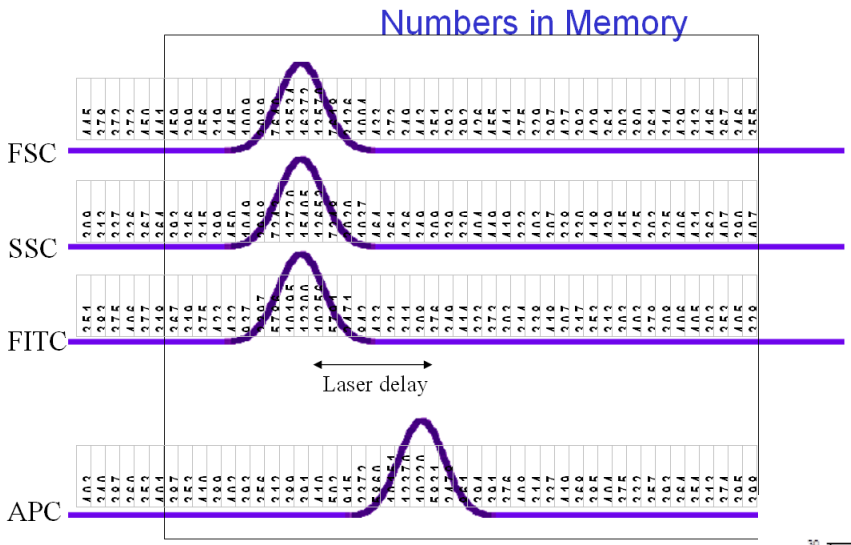
Figure courtesy of David Buschke (Sony Biotechnology)

GUIDELINES Flow cytometers, pages 1478ff

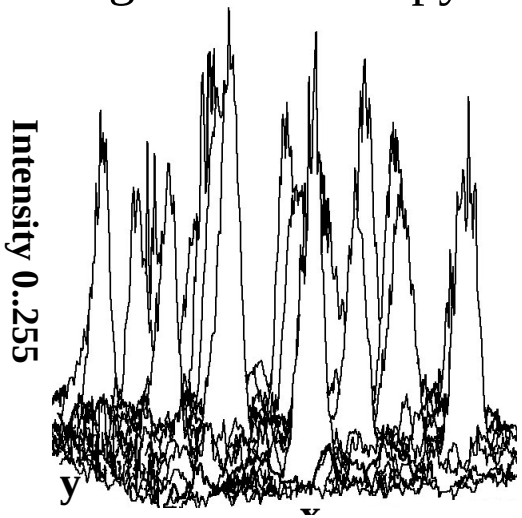
Dichroic filters vs. Multispectral cytometry: Feher K et al.(2016) Cytometry 89A: 681-9

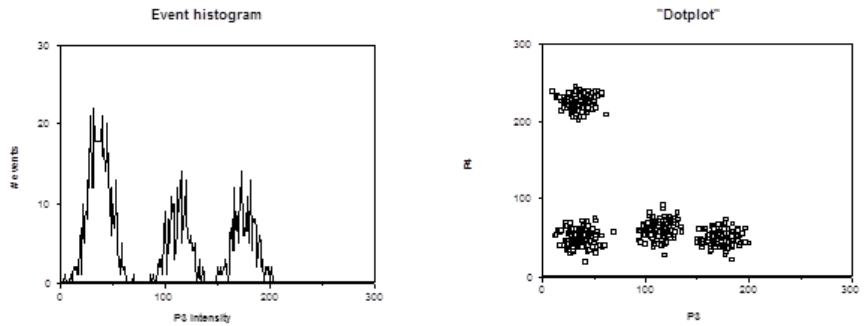
Basic Data Processing

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

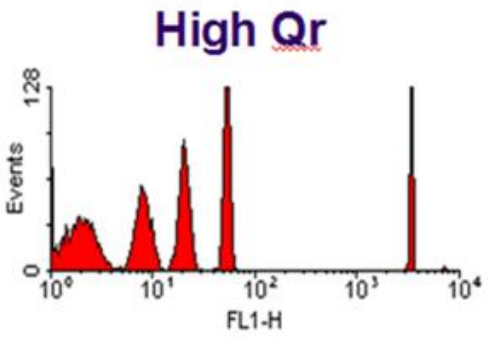
GUIDELINES Data Analysis, pages 1829-1854

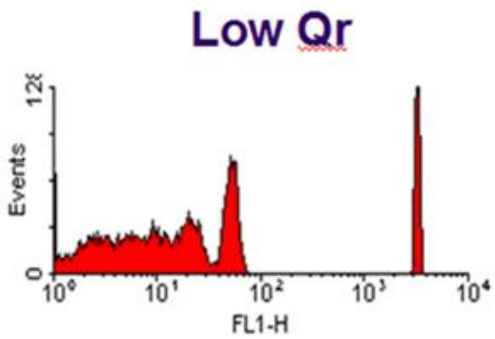
Instrument Evaluation Br, Qr

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

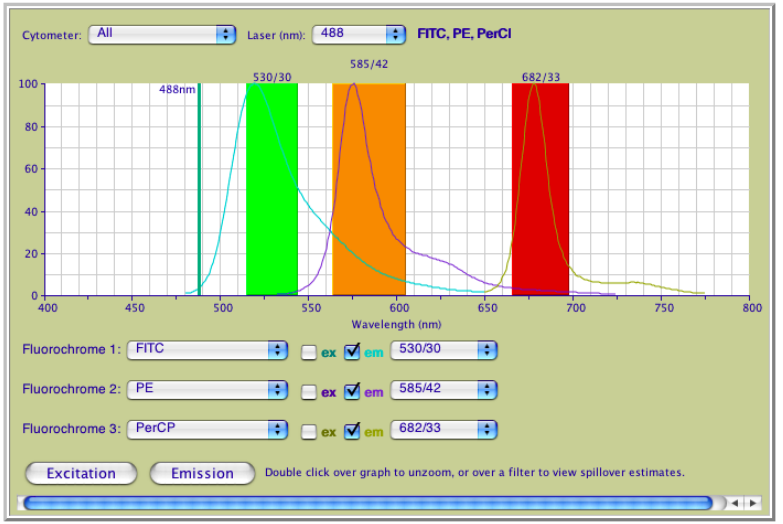
Signal Overlap and "Compensation"

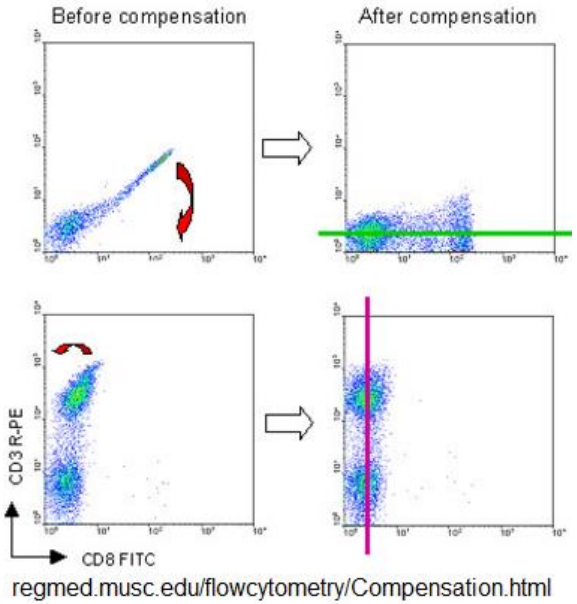
Calculation of concentrations from optical/mass intensities

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

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

Solve n equations with n unknowns (in spectral cytometry more complex calculations are performed.)

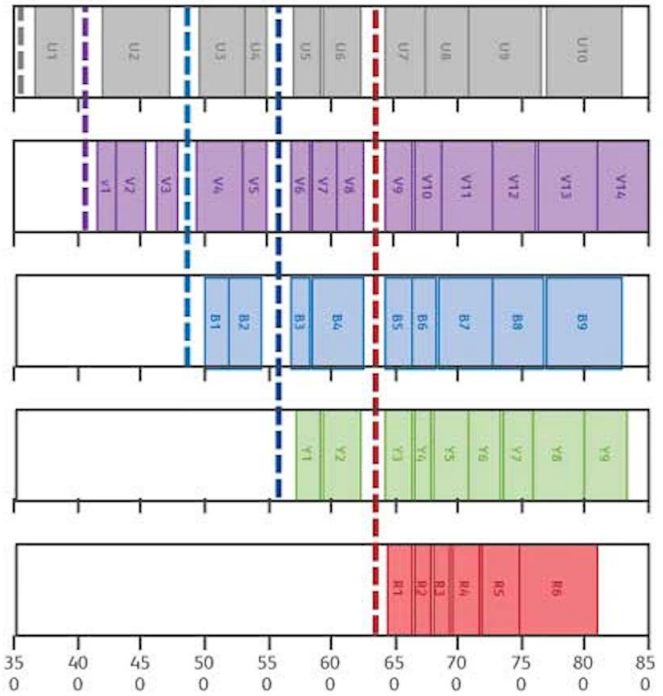




GUIDELINES Compensation, pages 1484-88

“Spectral” Flow Cytometry

BD FACSymphony! A5 SE Analyzer



BD Biosciences website

Table 3. Comparison of background (B), Q value, and detection limit (DL) of standard filter setting and multispectral filter setting for QSC microspheres stained with CD4 FITC or CD4 PE

PARAMETER	530/30	585/40	MULTISPECTRAL
Detection wavelength (nm)	515–545	565–605	505–810
Q_{FITC} (phe$^-$/ABC)	0.004	–	0.04
Q_{PE} (phe$^-$/ABC)	–	0.02	0.14
B (phe$^-$)	9	32	63
DL_{FITC} (ABC)	320	–	59
DL_{PE} (ABC)	–	875	231

FeherK2016 DOI: 10.1002/cyto.a.22888

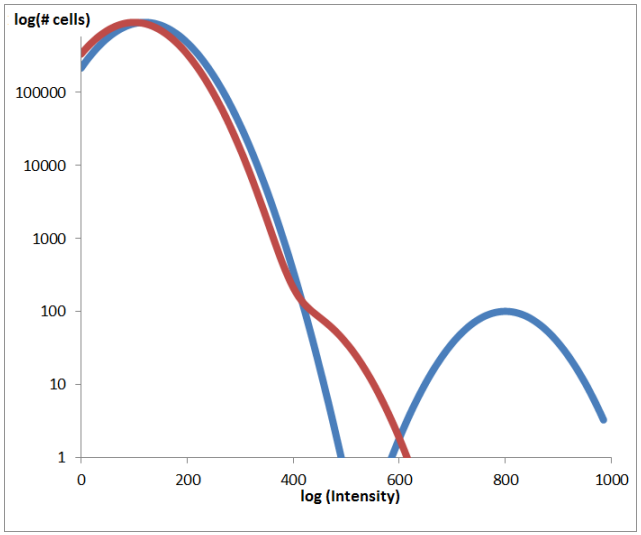
Full spectrum analysis generally collects more photons and as a result a lower limit of detection for fluorescence is achieved.

Label Selection

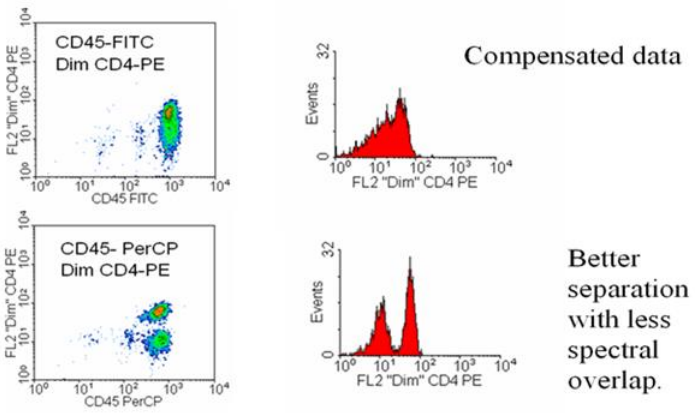
- Detection System
- Brightness
- Spectral Overlap
- Application (surface vs. internal)

Reagent performance

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



Brightness and Separation

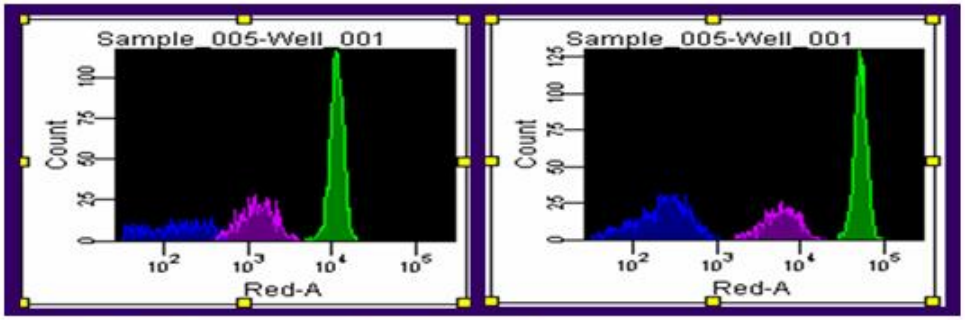


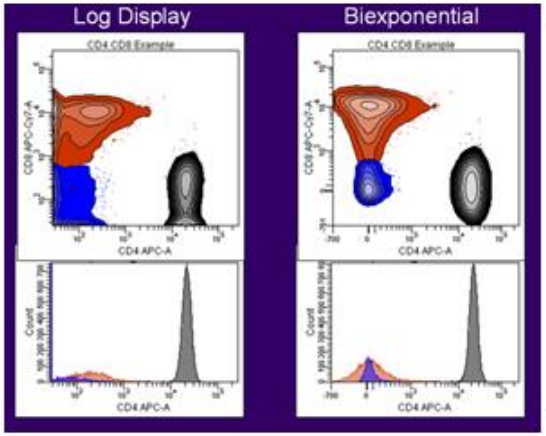
Spectral Overlap and Separation

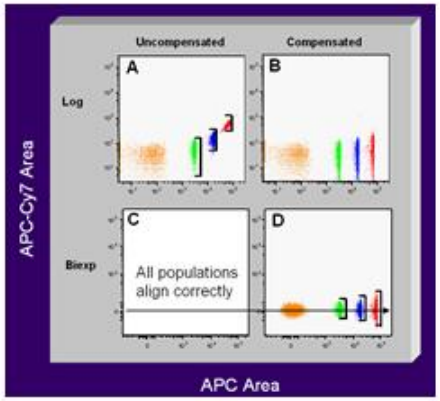
More info: Maecker HT et al. (2004) Cytometry 62A:169-173

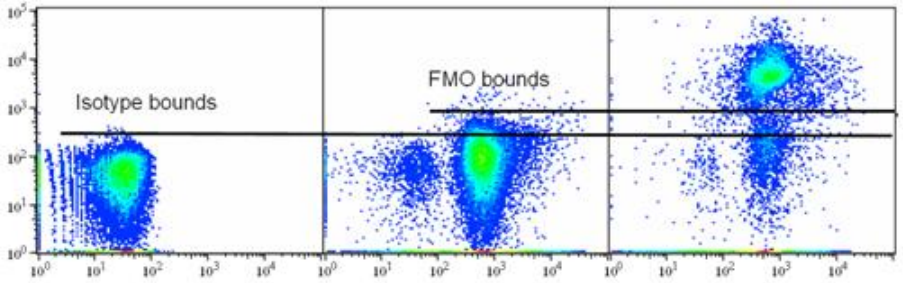
Optimizing cytometry measurements

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









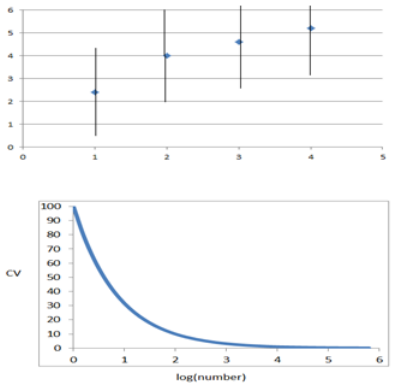
J. Trotter, BD Biosciences

Rare Cell Analysis

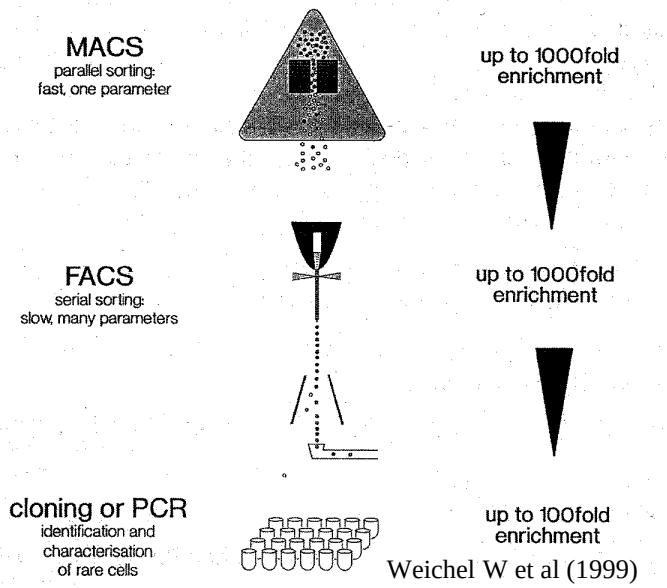
Examples CD34, AC133, antigen specific cells, CTCs

- Poisson count statistics
- Population Separation
- Subset 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

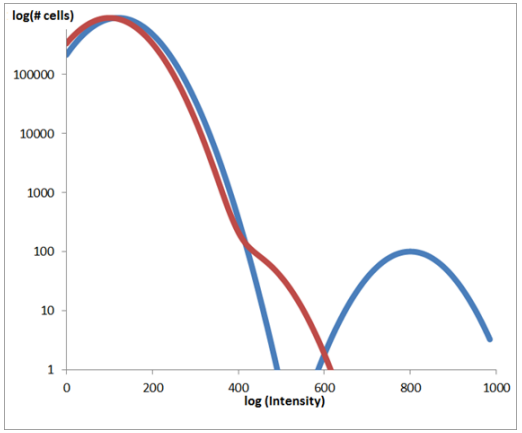


Ignoring Counting Statistics Can Lead to Erroneous Conclusions



Weichel W et al (1999)
Springer Lab Manual

Pre-enrichment



Population Separation

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

Multi-marker Cell Analysis
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 poor separation conditions for a single marker analysis will be even worse for a multi-marker measurement
- Use high sensitivity labels for low expression markers
- High sensitivity does not help against non-specific binding
- For energy transfer fluorophors beware of spectral drifts by photo-degradation
- Internal controls are essential
- Be aware of counting statistics limitations for low count populations

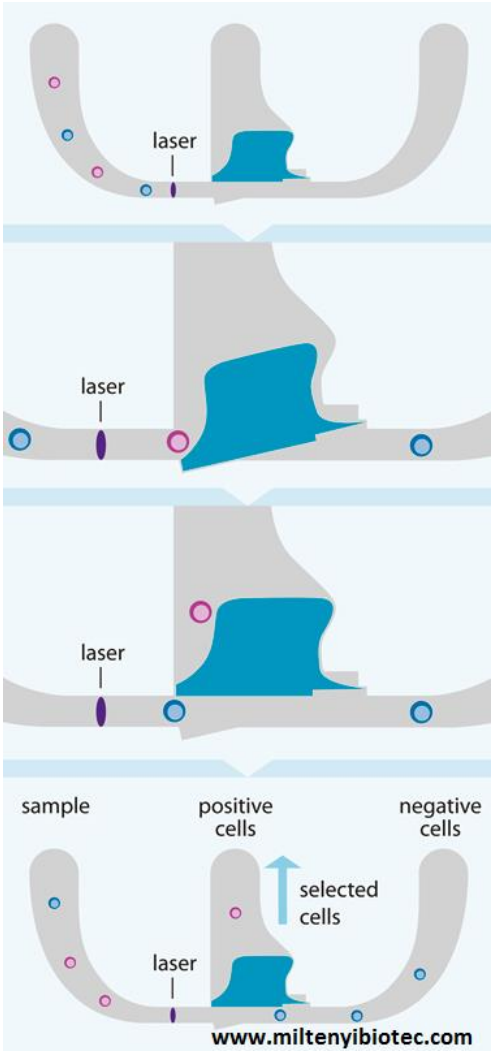
Cell Sorting Technologies

- Classical droplet sorters (FACS™)
- Single Cell dispensers
- Tyto/OWL
- DEP sorter
- …

- BulkSorting

(Magnetic, Gravity, Acoustic, …)

DEPArray™ System



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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

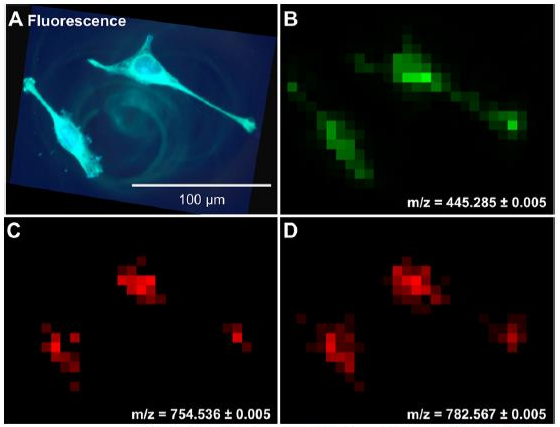
  (understand variance of very low counts during rare cell analysis)

- Validate results with appropriate controls

Examples of New Detection Technologies

MALDI imaging

(high parameter in-vitro imaging using mass spectrometry)



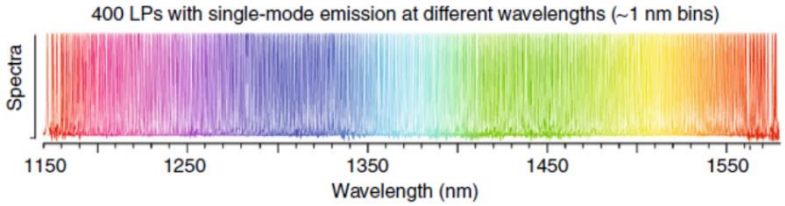
Schober Y et al. (2012) Anal.Chem. 84, 6293ff

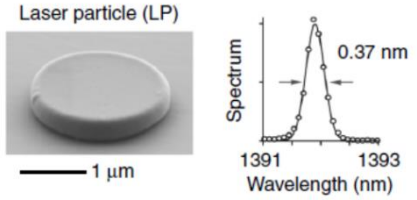
Label-free imaging with Raman

(measuring cellular components by their Raman spectra)

Microlasers for high parameter cytometry

(ultra-narrow bands of light emission)





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

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

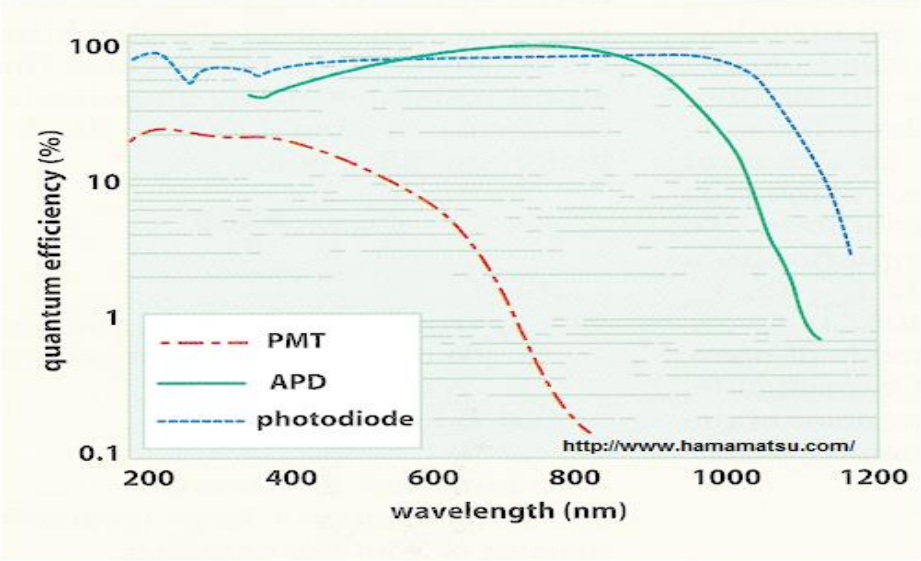
Label-free medium resolution NMR imaging

(chemical environment sensing)

New Detector-Label Combinations

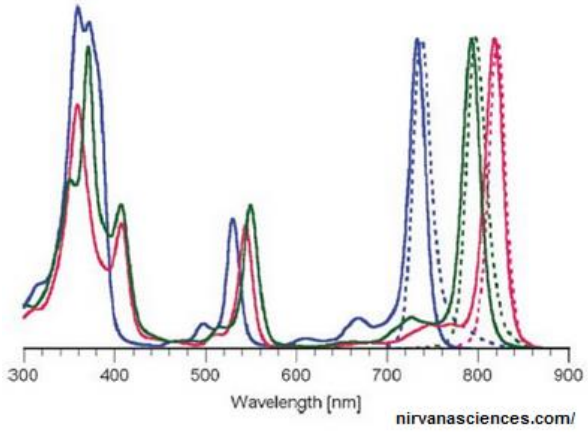
- New photodetectors extend the available spectrum

  (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 substantial value in working with other scientific disciplines.

Acknowledgements

- Joe Trotter
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- Brian Warner
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(all while at BD)

- Holden Maecker, Stanford
- Martin Büscher, Miltenyi
- Christian Dose, Miltenyi
- Ming Yan, CYTEK
- Eric Chase, CYTEK
- Maria Jaimes, CYTEK
- Janette Phi, Applied Cells
- **…**

- BD Biosciences
- Miltenyi Biotec
- CYTEK Biosciences
- …

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