12th Spring School on Immunology

Practical Flow and Image Cytometry
With
Emerging Technologies for Single Cell Analysis.

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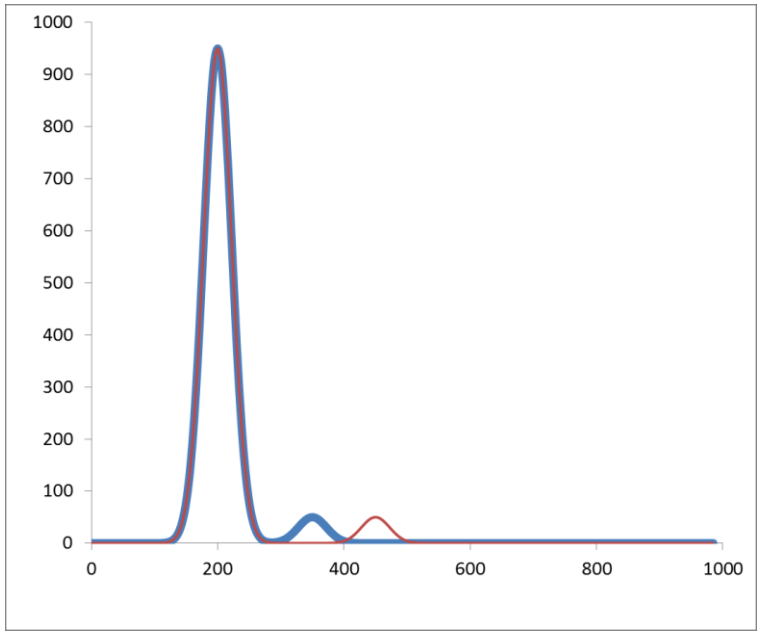
Biology Research

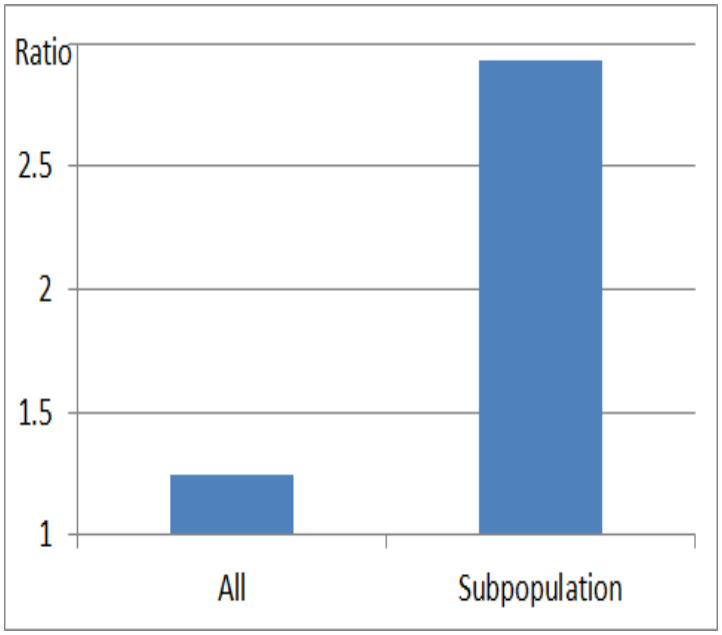
Targets	Tools
Organism	NMR MRI
Organ	X-ray imaging
Tissue	Ultrasound
Single Cell	2-photon imaging
Organelle	In-vivo cytometry
Macromolecule	Light microscopy
Small molecules	Electron microscopy
	Flow cytometry
	Cell imaging
	NA sequencing
	Mass spectrometry
	TIRF microscopy
	Electrophoresis

Why Subset Specific Analysis

Intensity Histogram



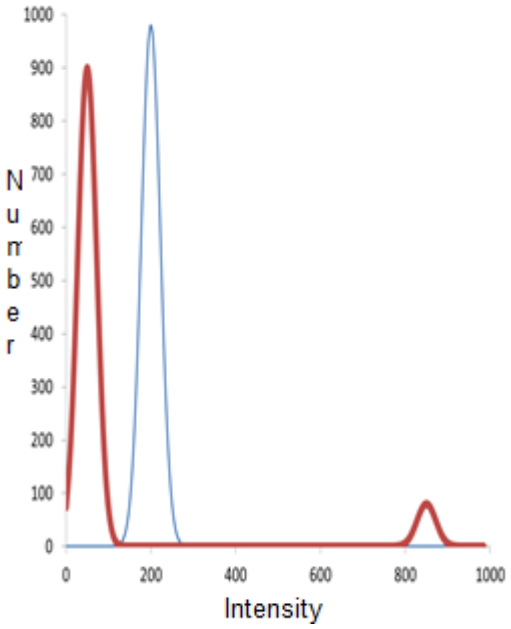
Intensity Ratios



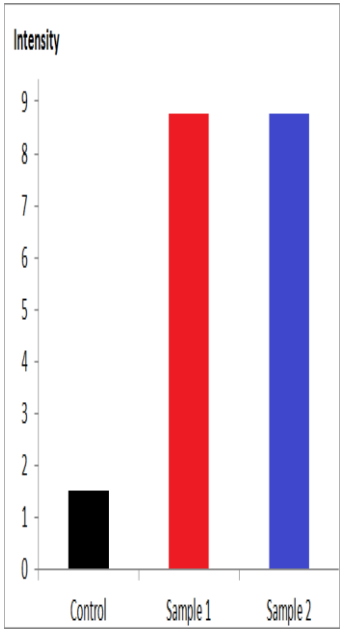
Subpopulation analysis detects changes better, especially for rare subpopulations.

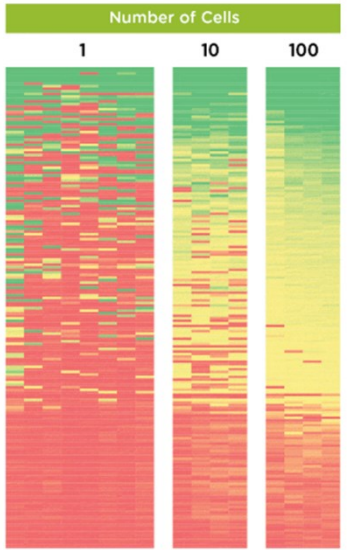
Why Single Cell/Particle Analysis

Intensity Histogram for Single Particles



Intensity per Sample

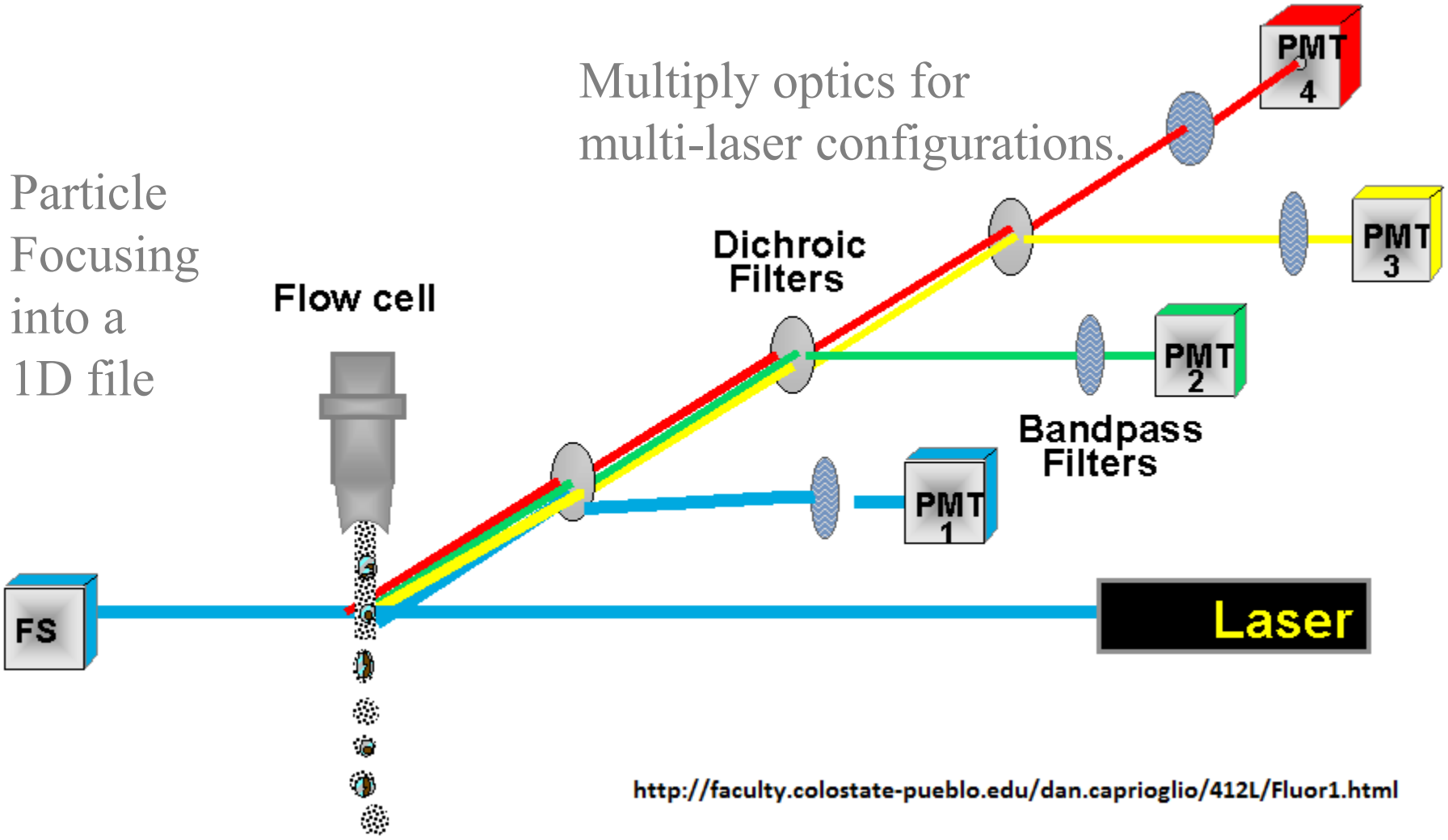




Source: http://www.nanostring.com

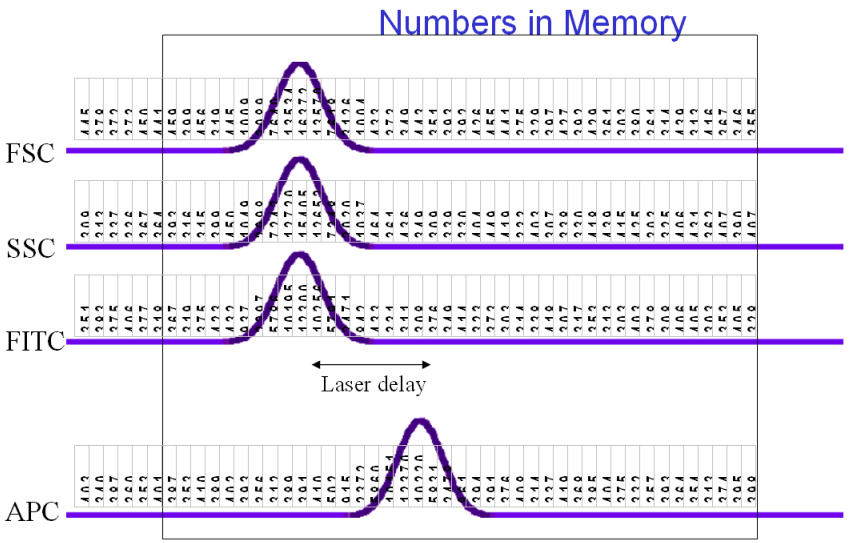
Cell by cell intensity analysis detects population heterogeneity.

Flow Cytometer Components

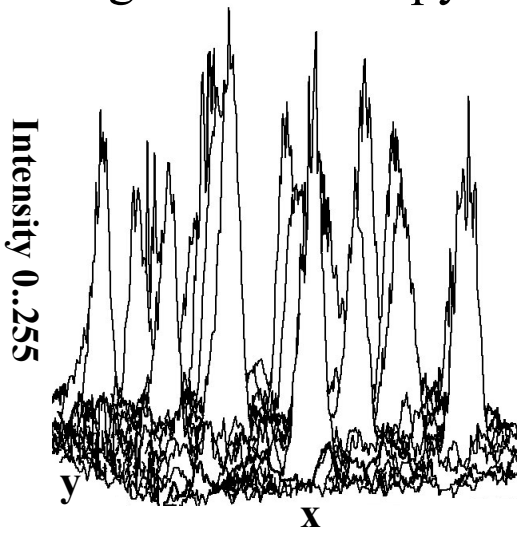


Basic Data Processing

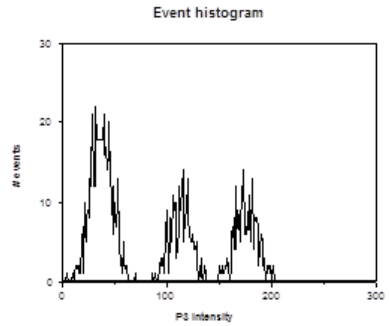
Flow Cytometry

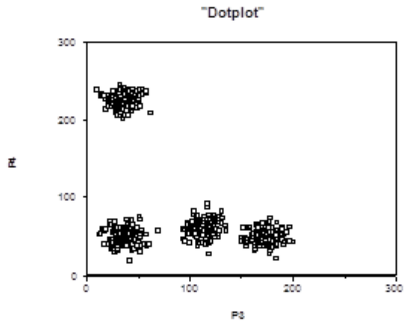


Digital microscopy



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

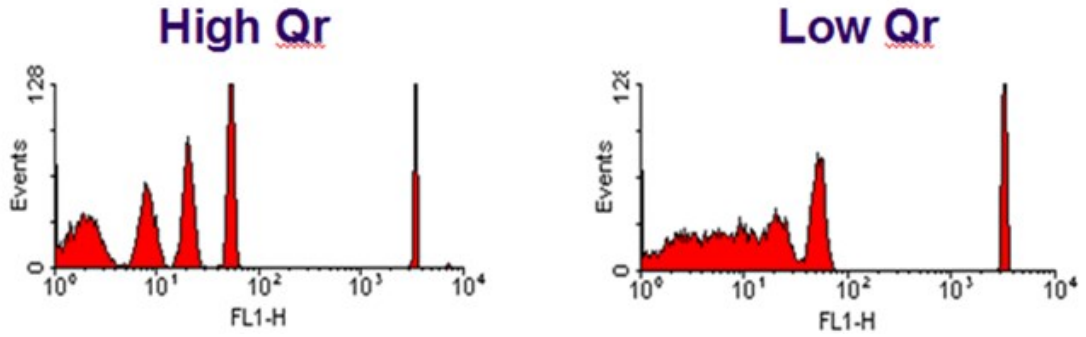
E.Lugli,M.Roederer,A.Cossarizza (2010) Cytometry 77A:705ff

Instrument Evaluation Br, Qr

Br, optical background from

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

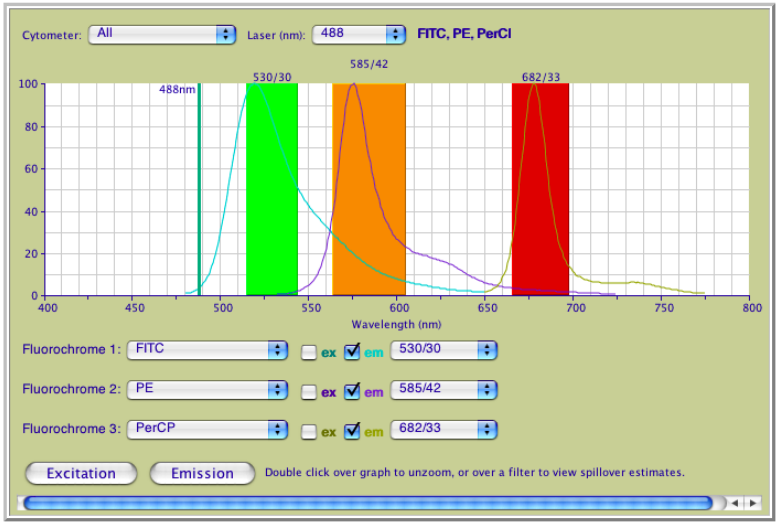
Qr, photon detection efficiency

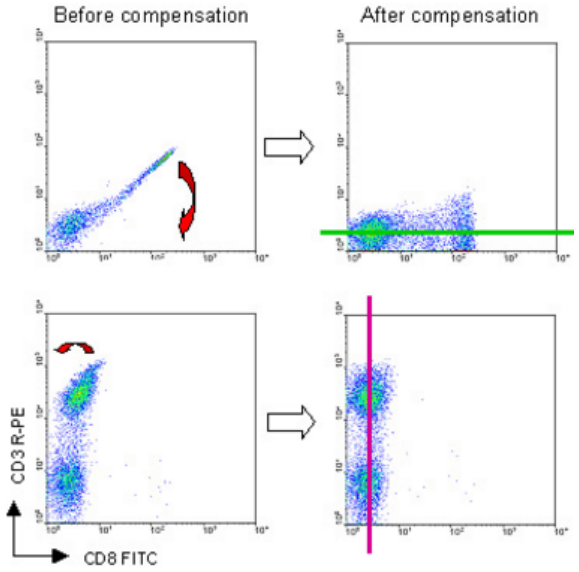


Figures: Joe Trotter, BD Biosciences

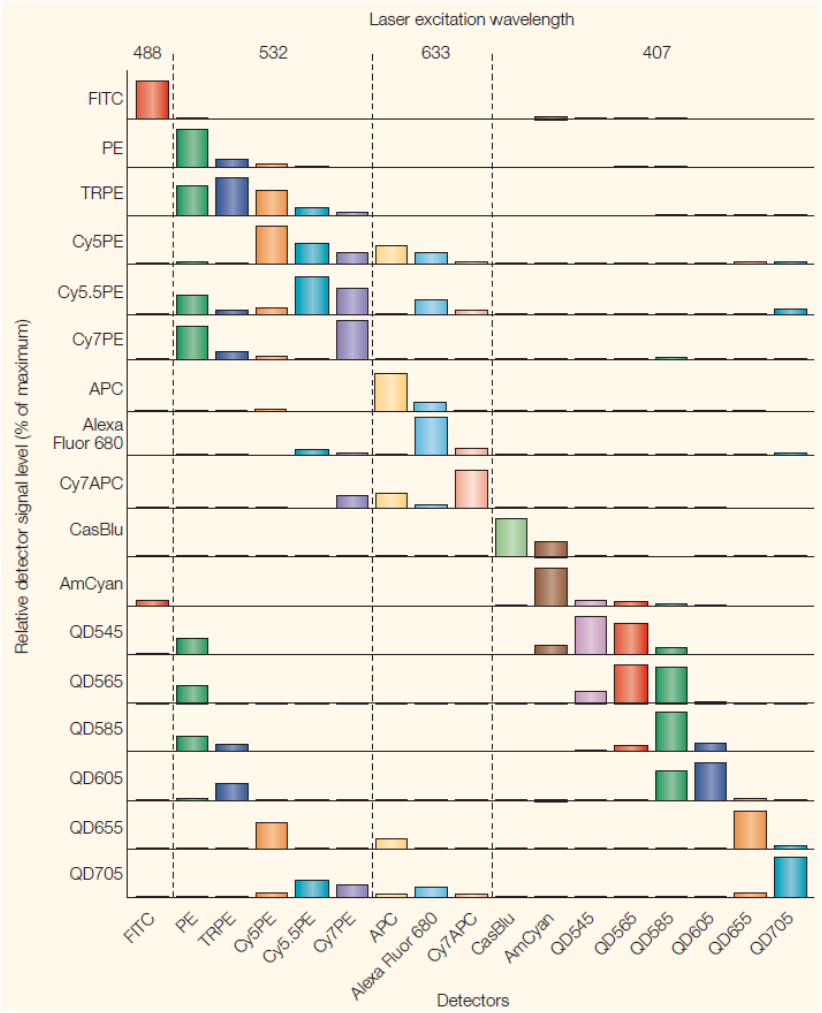
Spectral Overlap and "Compensation"

(not very relevant for element mass cytometry)





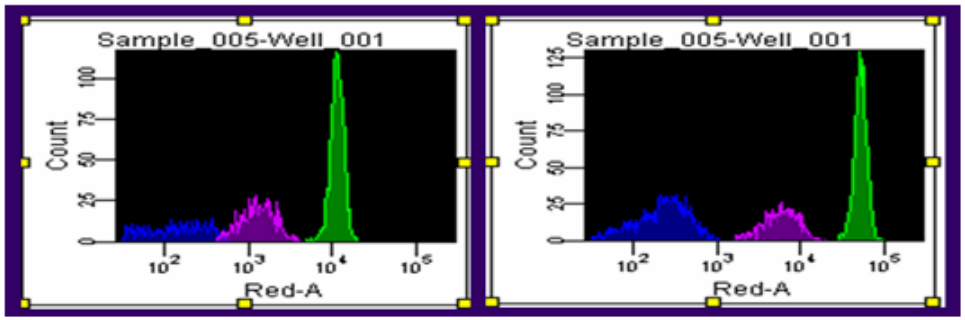
regmed.musc.edu/flowcytometry/Compensation.html



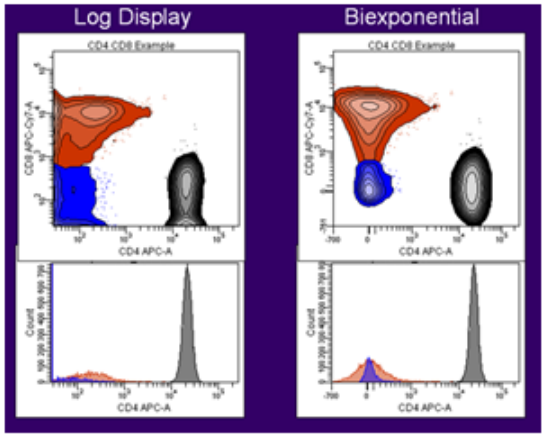
Perfetto SP et al (2004)
Nature Reviews Immunology 4, 648ff

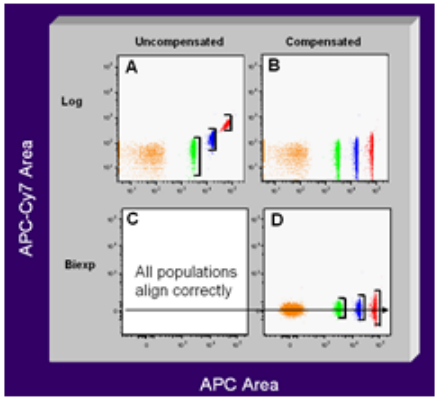
Optimizing cytometry measurements

- Gain (PMT, CMOS, CCD) settings

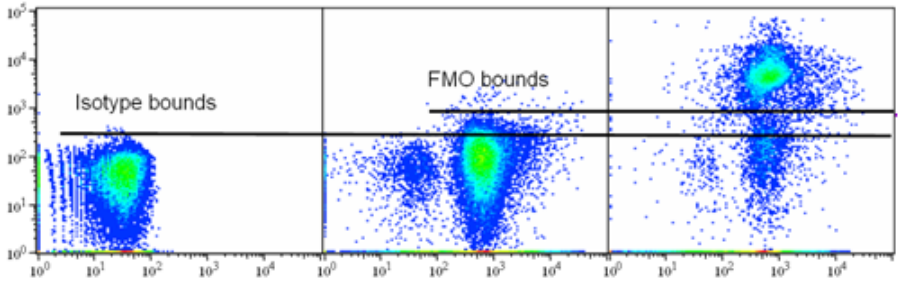


- Data Display





- Controls



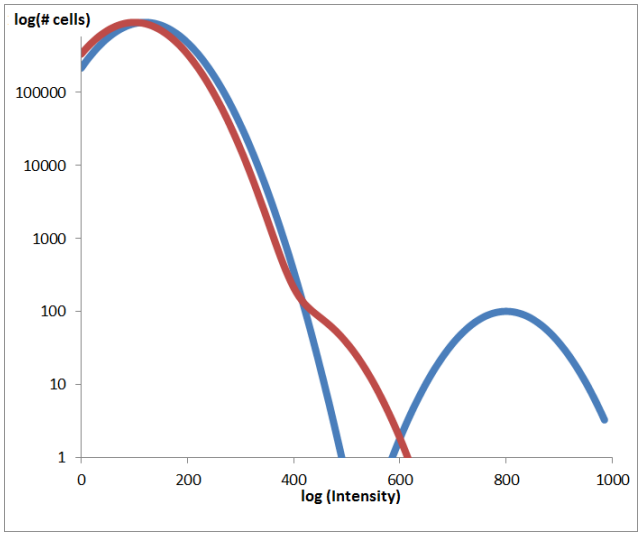
J. Trotter, BD Biosciences

Label Selection

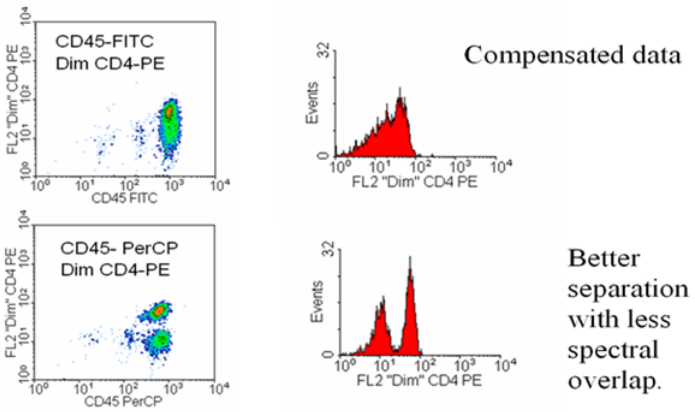
- Detection System
- Brightness
- Spectral Overlap
- Application (surface vs. internal)

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

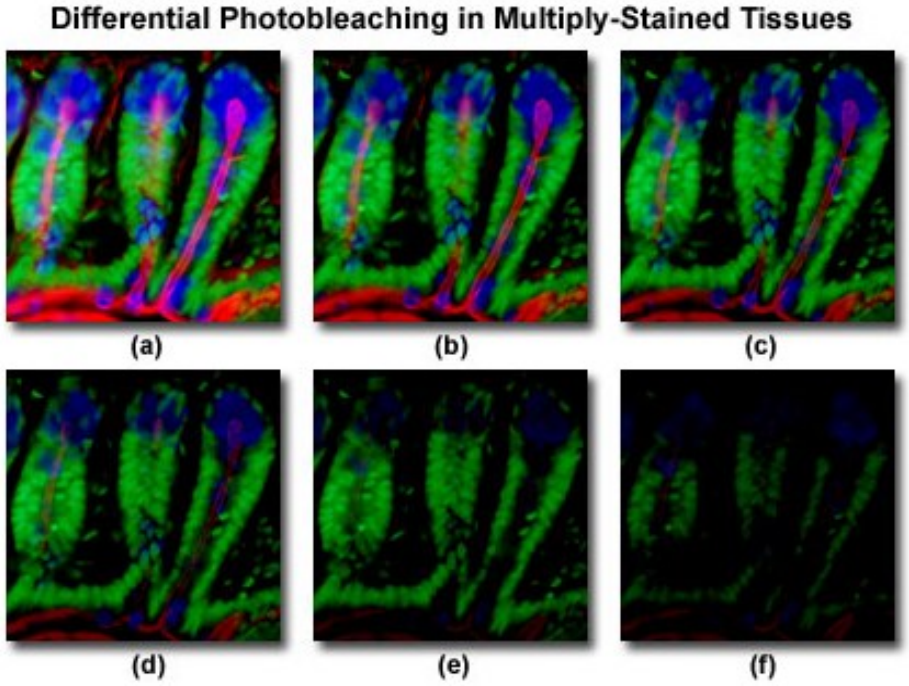
Multi-parameter Fluorescence Cytometry Points To Consider

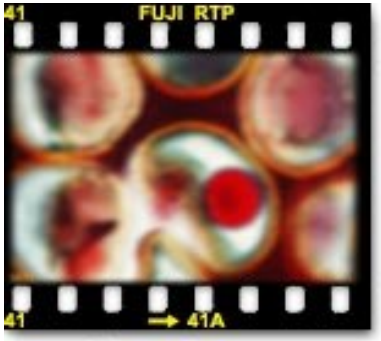
- Know your instrument status e.g. Qr & Br for different channels
- Use high enough gain settings to maximize sensitivity (check to avoid off-scale events)
- An antibody/dye combination with poor separation in a single color assay will not work for a multicolor experiment.
- Avoid spillover from bright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

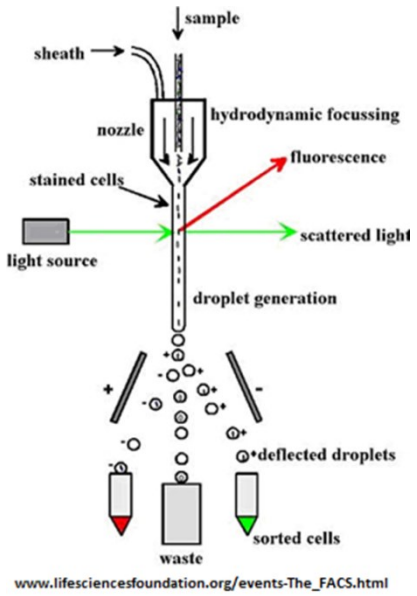
http://micro.magnet.fsu.edu/primer/index.html

Flow and Imaging Cytometry Features

Single particle (cell) analysis with

• High sensitivity (single molecule sensitivity by fluorescence)	I,F
• Wide dynamic count range (10^3 to 10^7 cells mL^{-1})	F
• Particle sizes from 0.2 to 20 um	F, I
• High analysis rates to ~10^5 particles sec^{-1}	F
• Direct size and 3D spatial information	I
• Multi-color fluorescence, multi-parameter analysis	F,I
• Wide dynamic range for fluorescence (10^5)	F
• Direct kinetic measurements	I
• Viable cells can be re-covered	F,(I)
• Measurement of adherent cells	I
• Good ease-of-use	F,(I)

Cell Sorting



Technologies

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

- Bulk Sorting
  - Magnetic
  - Gravity
  - Acoustic
  - …

Application Examples

- Chromosomes
- Cloning
- Strain Improvement
- Genomics
- Proteomics

Cell sorting review: Derek Davies
http://www.facs.ethz.ch/docs/lit

Evaluating Cell Sorting Performance

- Purity, Yield

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

Recktenwald D(1995) unpublished

- Fe Fd

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

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

"Miltenyi S, Schmitz J. High Gradient Magnetic Cell Sorting, pages 218ff 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

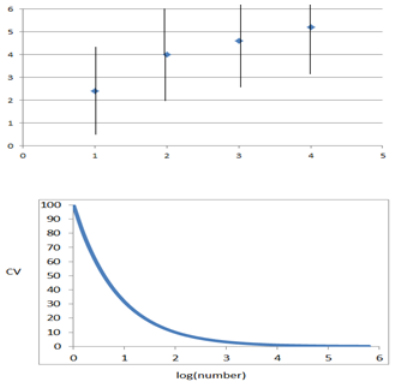
- Multi-parameter immunofluorescence (antibodies)
- Multi-parameter gene expression analysis (NA probes)
- Cell cycle analysis (high resolution FCM, imaging, BrDU)
- Kinetics (population-based flow cytometry, single cell by imaging; Ca++ flux, enzyme activity, cell proliferation)
- Receptor ligand binding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

Rare Cell Analysis and Sorting

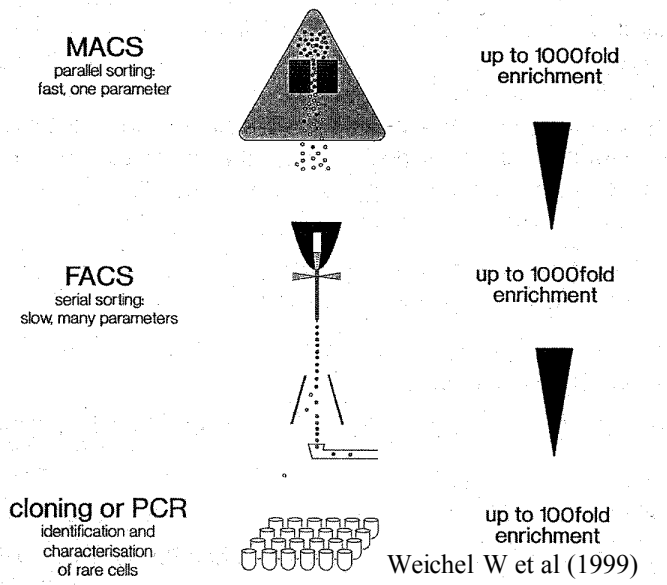
Examples CD34, AC133, antigen specific cells, CTCs

- 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

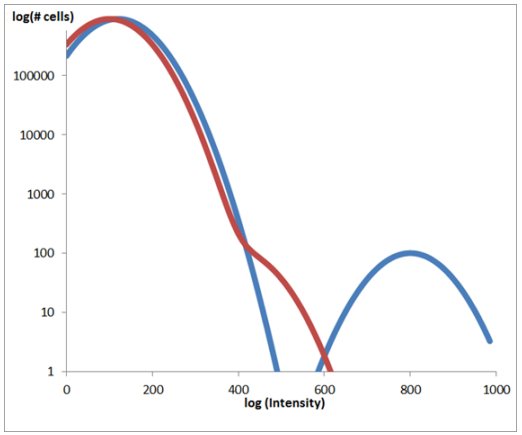


Ignoring Counting Statistics Can Lead to Erroneous Conclusions



Weichel W et al (1999)
Springer Lab Manual

Pre-enrichment



Population Separation

Some Newer Commercially AvailableTechnologies

- Element-Label Flow Cytometry

(CyTOF, addresses fluorescence spectral overlap issue by using elements as labels, Anal. Chem., 2009, 81 (16), pp 6813–6822)

- SERS-Label and Spectral Flow Cytometry

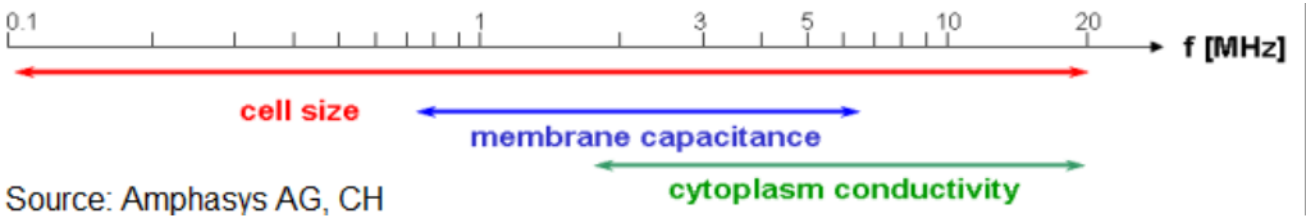
(uses spectral (fine)-structure to distinguish labels, Cytometry, 2008, 73A(2), pp 119-128, SONY cytometer)

- Sequential Stain De-stain Cytometry

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

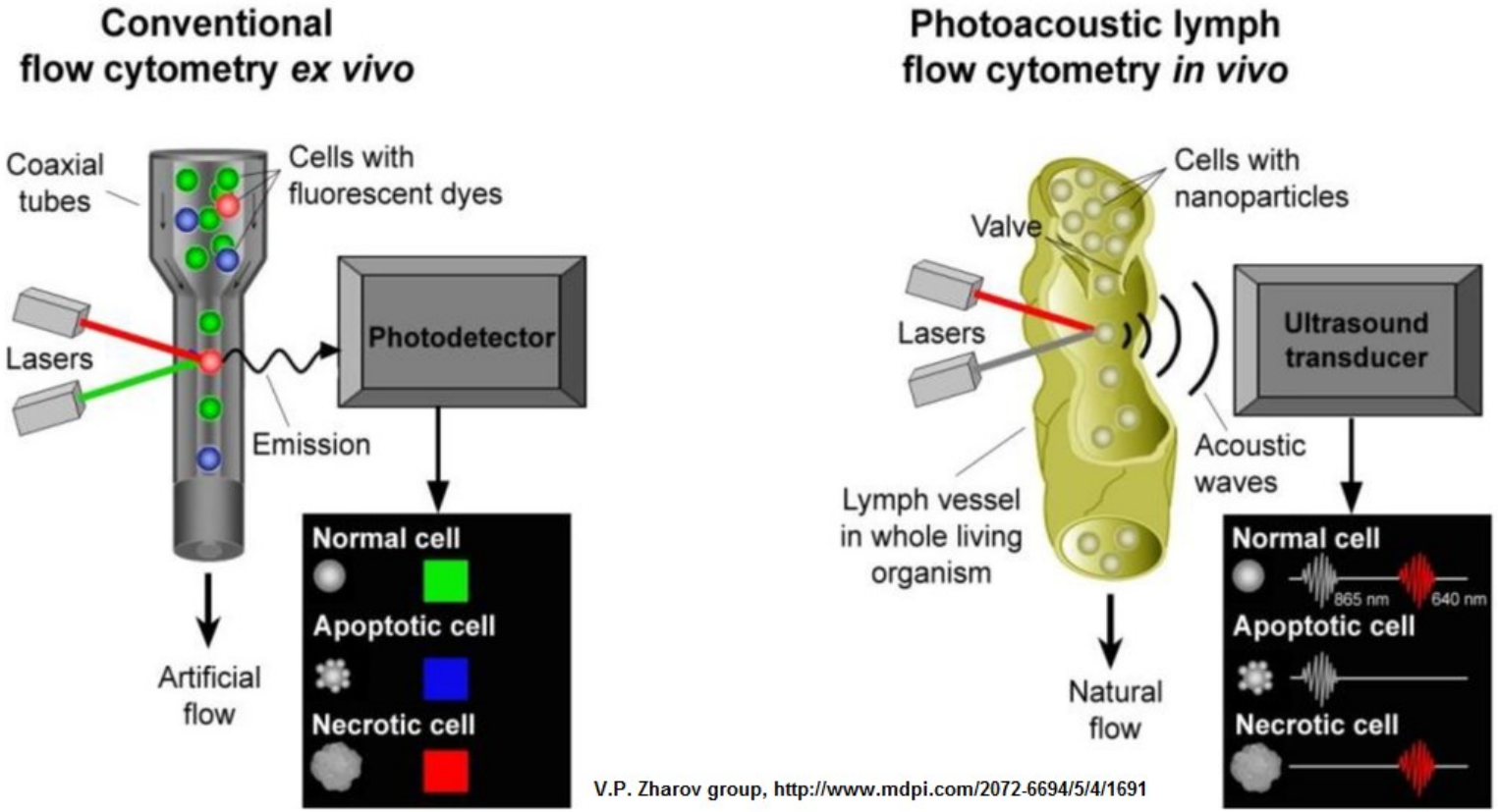
- Impedance Cytometry, electrical cell properties

(Review paper: Chen J et al (2015)Int. J. Mol. Sci. 16, 9804ff)



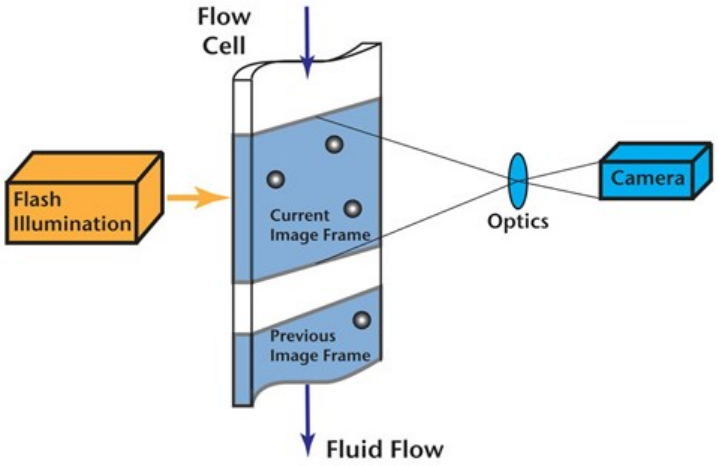
In-vivo Single Cell Analysis

- Intra-vital Imaging
- In-vivo Flow Cytometry

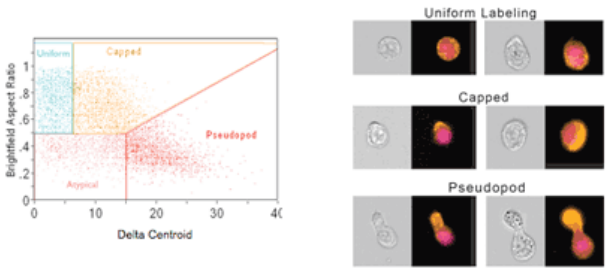


Imaging Flow Cytometry

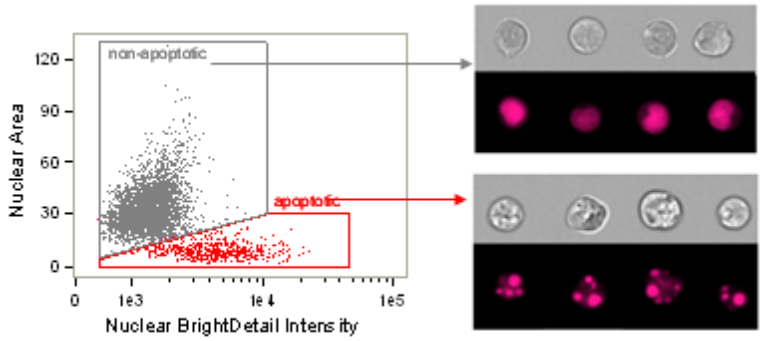
Images of Cells/Particles are captured in a fluid stream and stored individually.



http://www.fluidimaging.com/products/how-dynamic-imaging-particle-analysis-works



http://www.sharpedgelabs.com/sharp-edge-radar/imaging-cytometry/

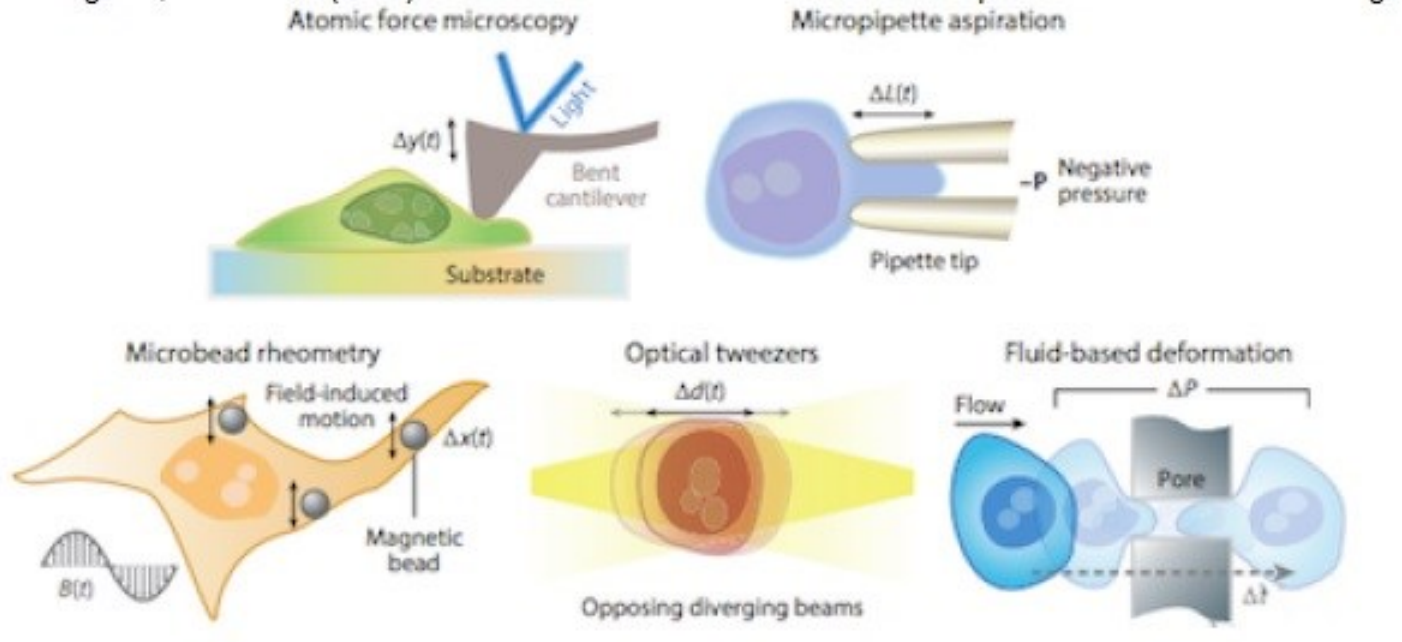


https://www.amnis.com/images/ApoptoticIndex.png

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

Single Cell Analysis
Mechanical Properties

Darling EM, Di Carlo D (2015) Measurement of CellularMechanical Properties. Ann.Rev.Biomed.Eng.

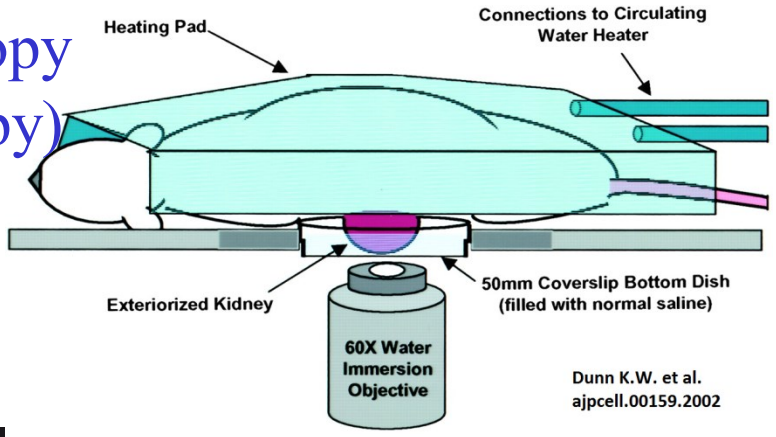


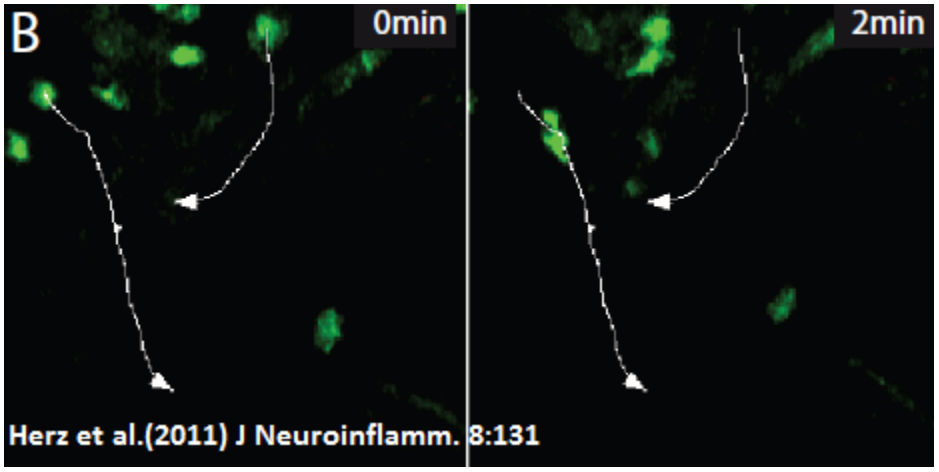
http://biomicrofluidics.com/

See also: Jochen Guck, TU Dresden(2015) Nat Methods, 12:199ff
http://www.biotec.tu-dresden.de/research/guck.html

Intra-vital Imaging

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



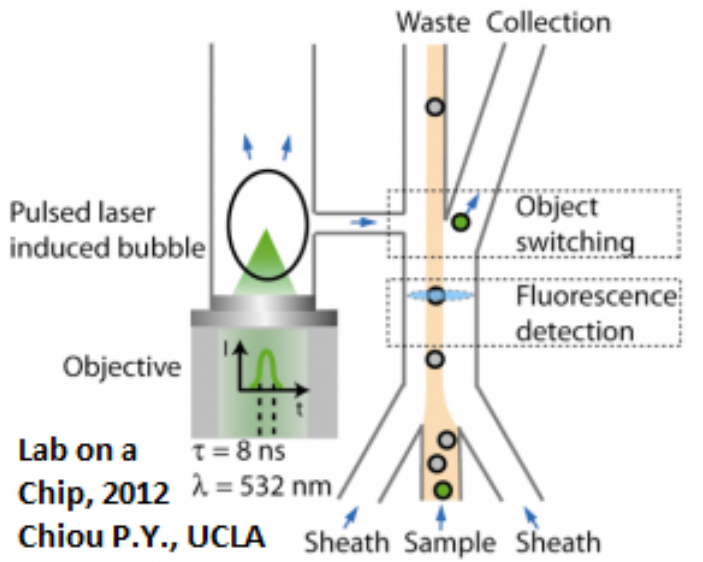


Issues:

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

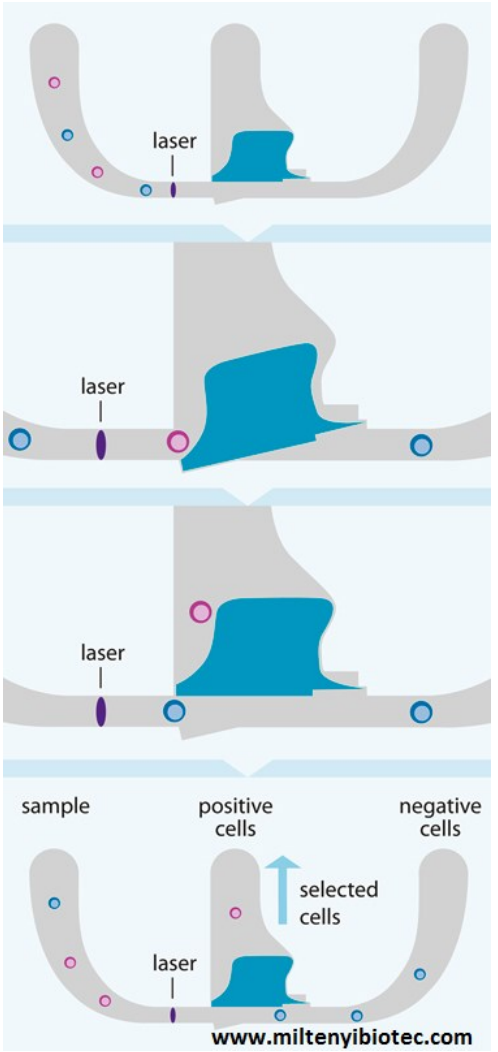
Recent review of in-vivo microscopy: Andresen V, et al. (2012) High-Resolution Intravital Microscopy. PLoS ONE 7(12): e50915

More Cell Sorting Technologies



UCLA Sorter, Chiou lab

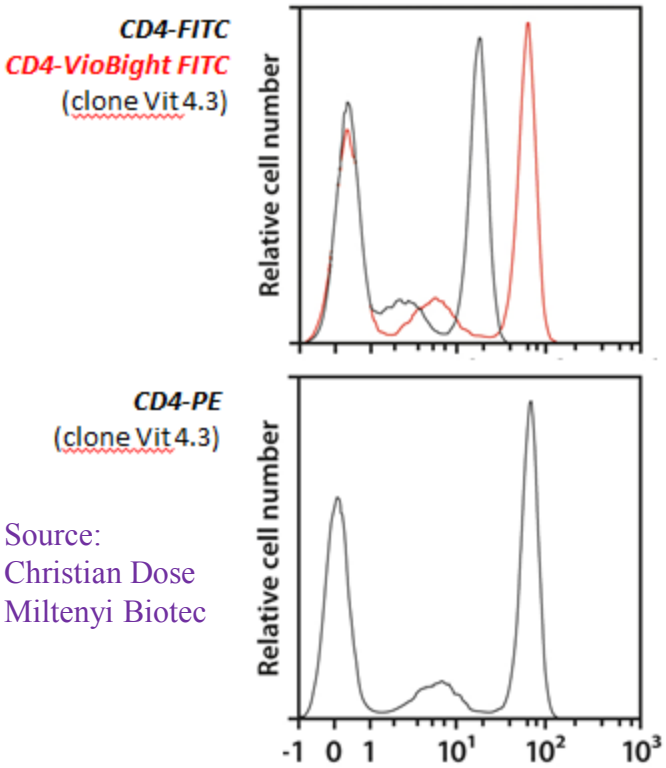
DEPArray™ System



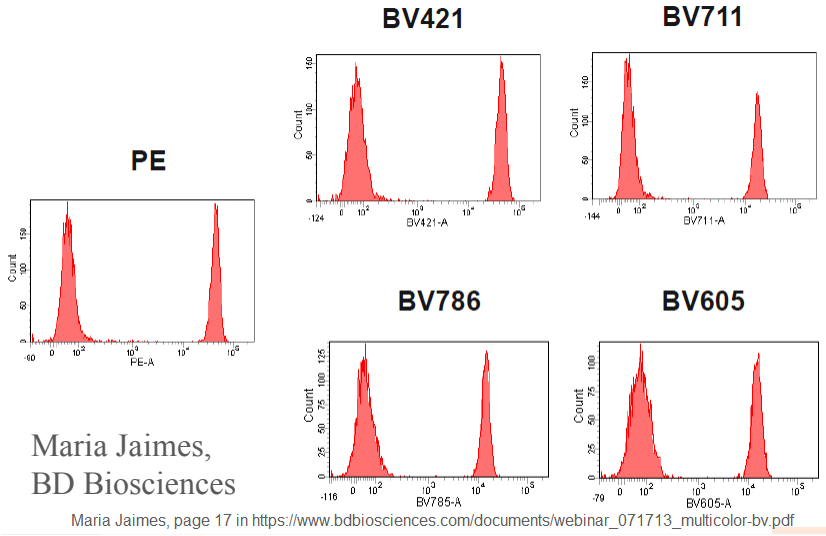
MACSQuant®Tyto™

New Bright Dyes

More bright label systems are available in addition to phycobiliproteins.

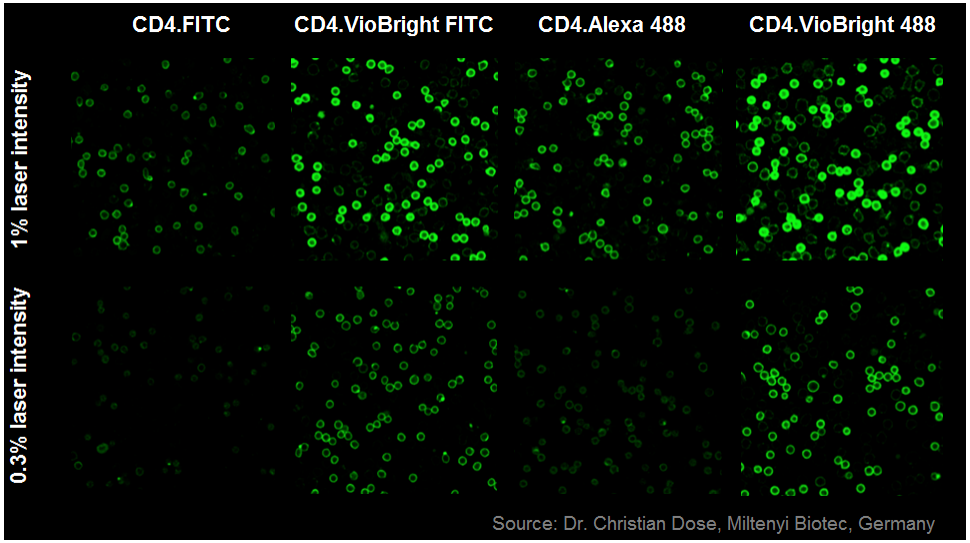


Source:
Christian Dose
Miltenyi Biotec



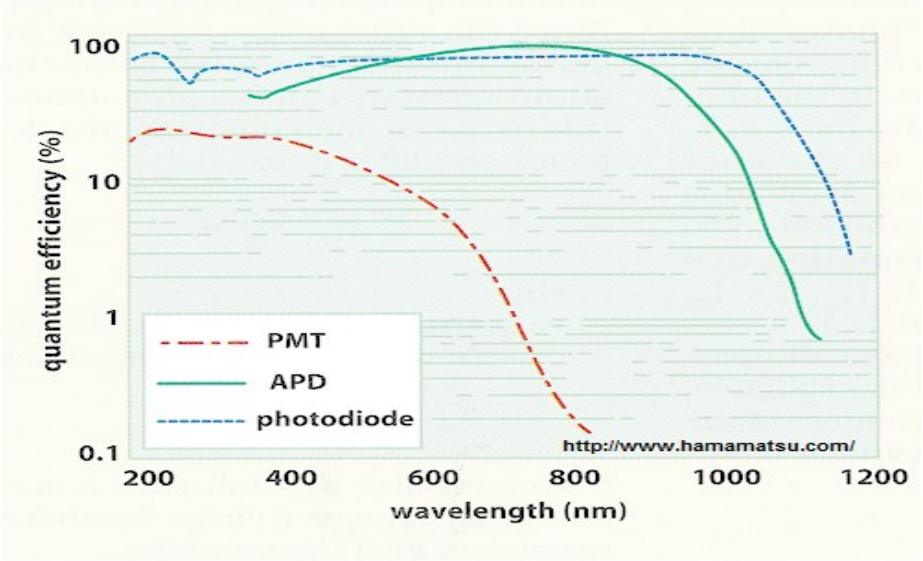
Maria Jaimes,
BD Biosciences

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

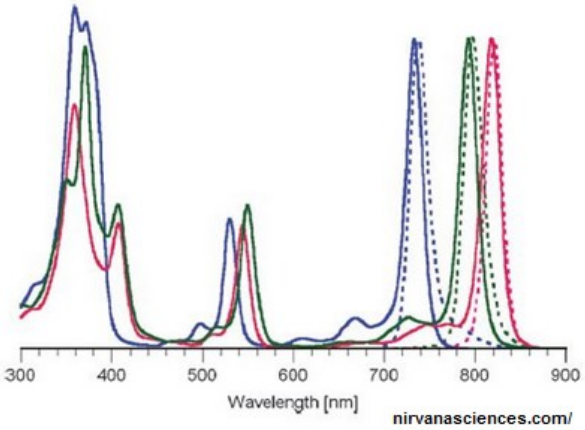


New Detector-Label Combinations

- 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

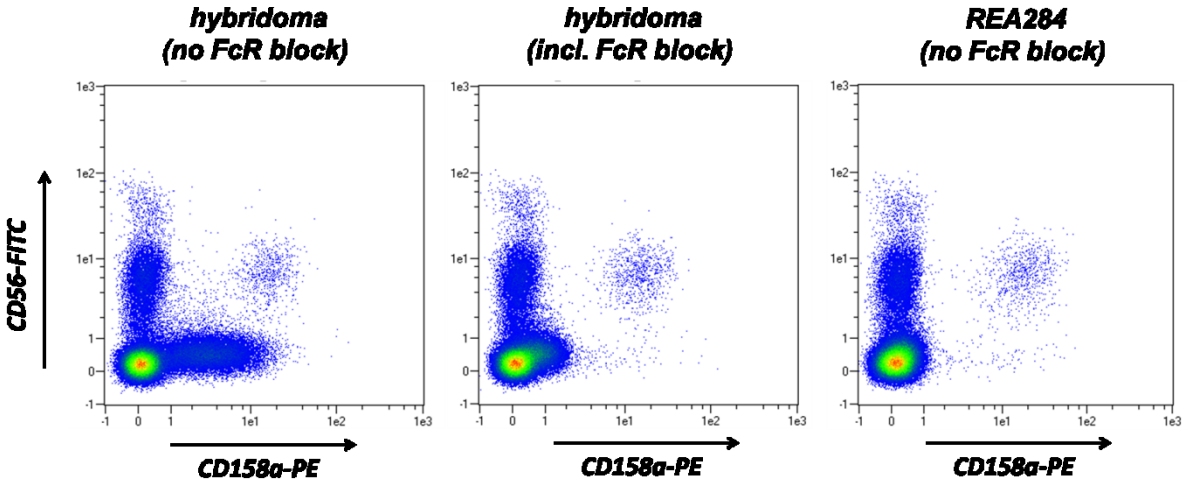
Antibodies

- 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

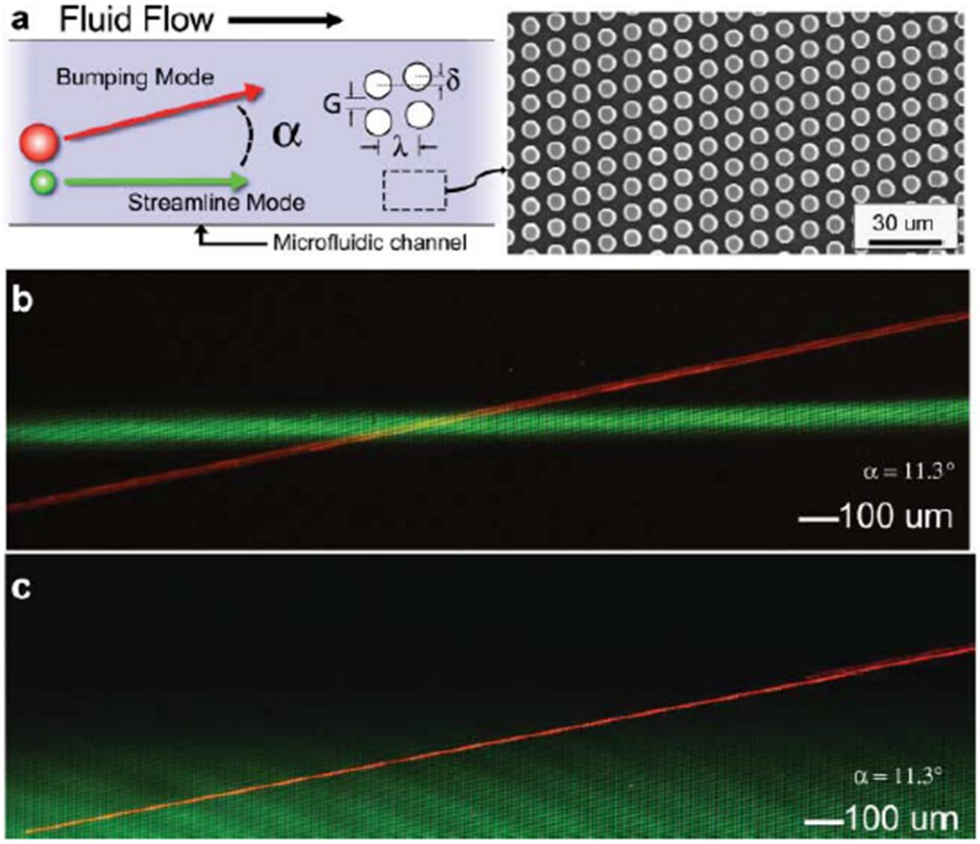


Fc-receptor binding:
CD158a-PE on PBMC

Source: Dr. Christian Dose, Miltenyi Biotec

Innovative Sample Preparation

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

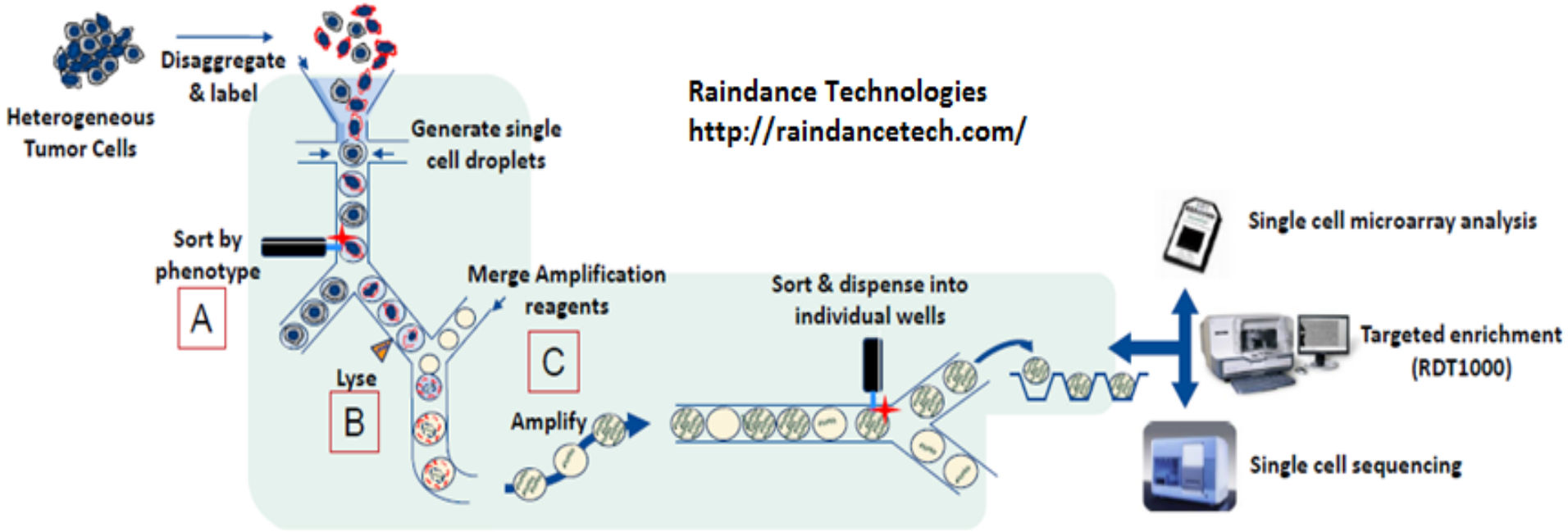


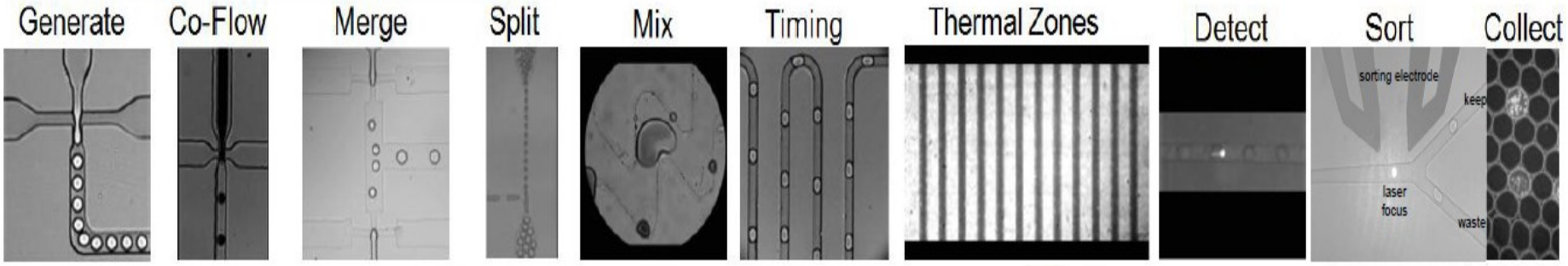
also:

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

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

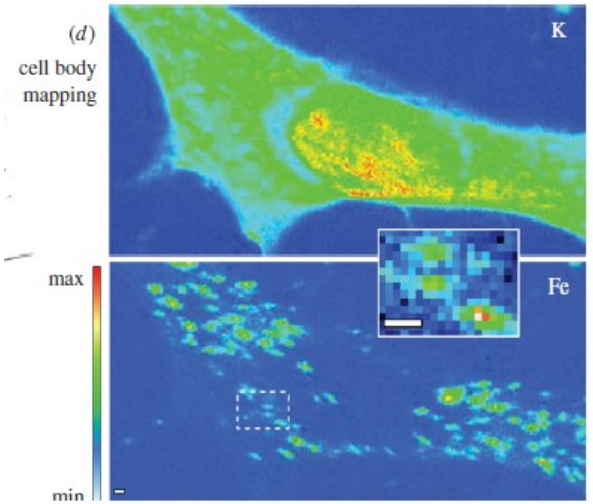
Droplet-based Integrated Bio-Assay System Technology



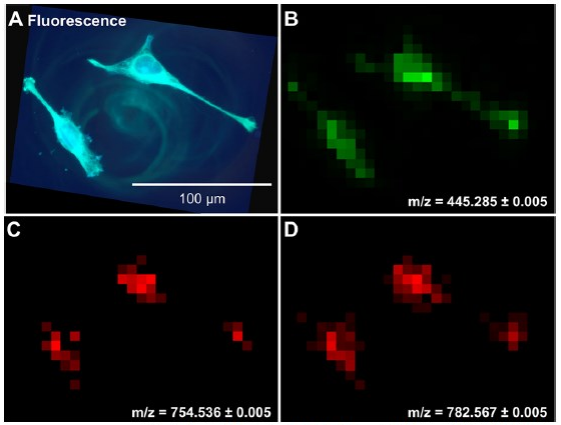


New Detection Technologies

- High spatial resolution and multi-parameter capability with X-ray / synchrotron radiation fluorescence
  (super-high resolution with element labels or direct element imaging)
- Medium resolution, multi-parameter mass spectrometric imaging
  (CyTOF like element labels, direct metabolite or structural component detection)
- Label-free imaging with Raman
  (measuring cellular components by their Raman spectra)
- Label-free high resolution NMR imaging
  (direct chemical detection)



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



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

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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