

Advanced Cytometry Course

A Satellite Symposium of the 4th European Congress of Immunology
ECI, Vienna, Austria

September 6, 2015
9:00 - 16:00 h

Single Cell Analysis Matching Technology with Application Needs

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<http://www.desatoya.com>

Flow Cytometry in the Context of Biological Systems and Other Technologies

Organism

NMR

Contrast agents

X-ray imaging

Affinity reagents

Organ

Ultrasound

- antibodies

2-photon imaging

- probes

Tissue

In-vivo cytometry

Enzyme substrates

Light microscopy

Labels

Single Cell

Electron microscopy

- absorbance

Flow cytometry

- fluorescence

Organelle

Cell imaging

- element tags

Macromolecule

NA sequencing

Mass spectrometry

TIRF microscopy

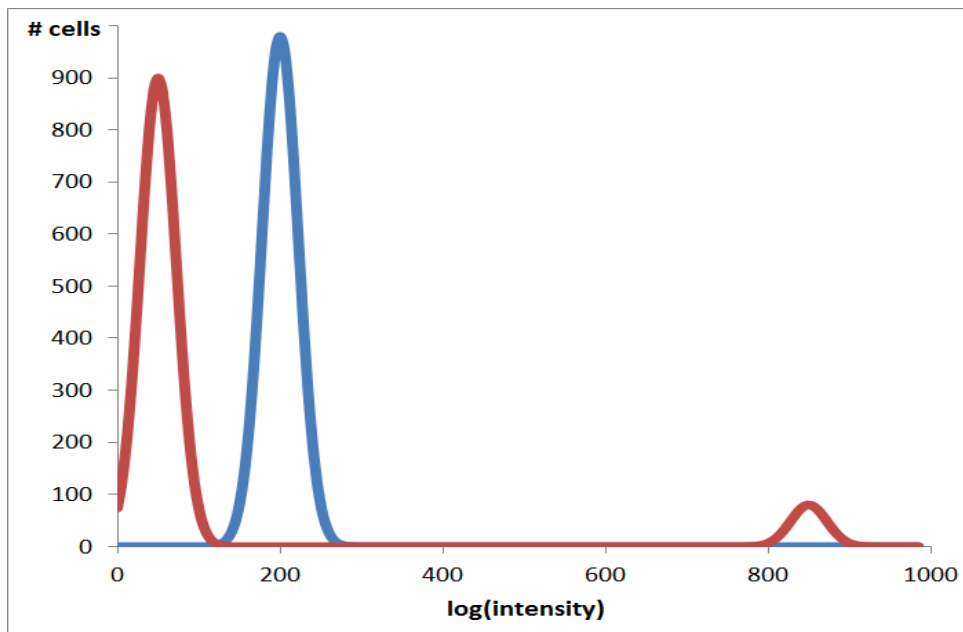
Small molecules

Electrophoresis

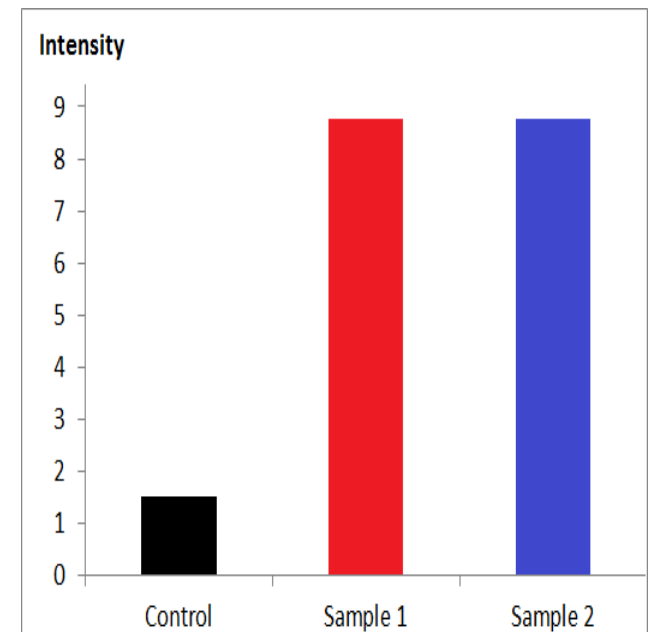
Sample prep

Why Single Cell/Particle Analysis

Intensity Histogram for Single Particles



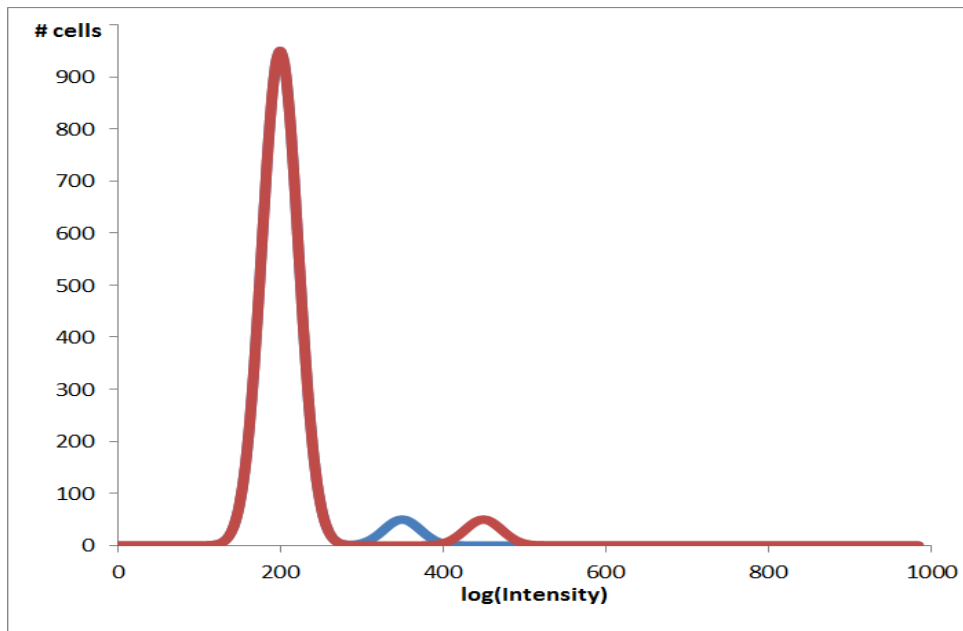
Intensity per Sample



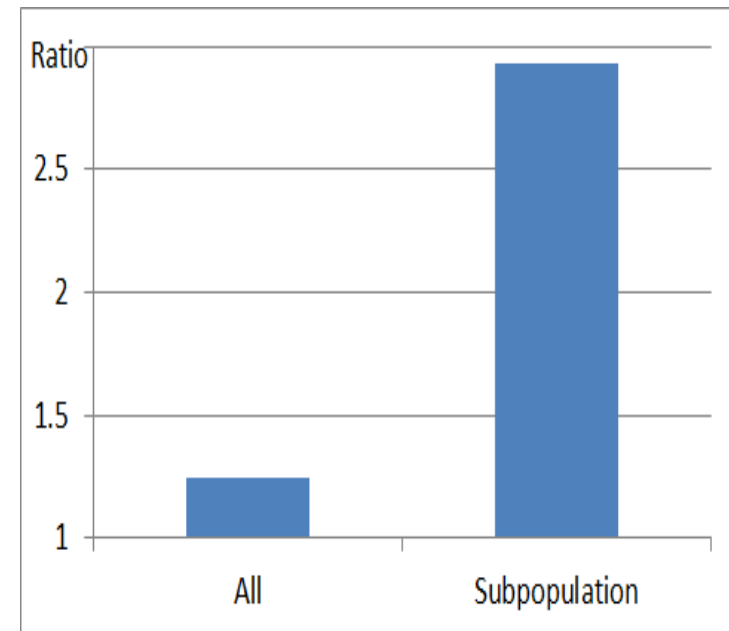
Cell by cell intensity analysis detects population heterogeneity.

Benefits of Subset Specific Analysis

Intensity Histogram



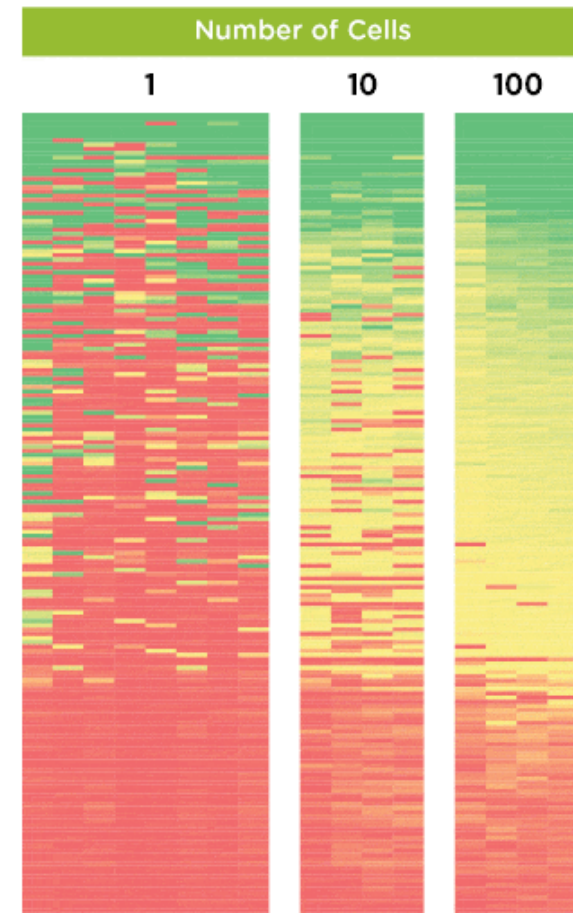
Intensity Ratios



Subpopulation analysis detects changes better, especially for rare subpopulations.

Single Cell Genomics

Single cell analysis reveals heterogeneity, which is masked by averaging, when analyzing groups of cells.



Source: <http://www.nanostring.com>

Single Cell and Subpopulation Cytometry

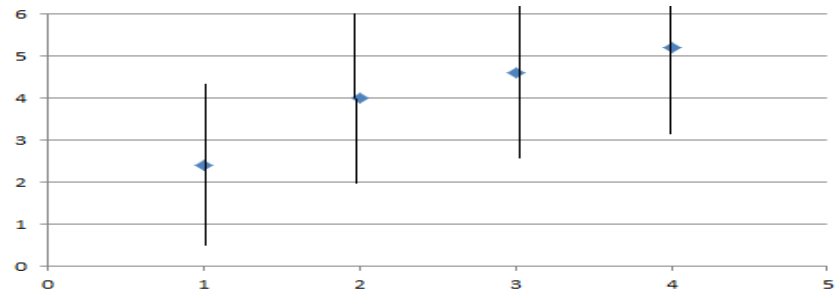
Points To Consider

- Understand the capabilities of your system
- Evaluate effects of specimen collection and sample preparation
- Know the quality of your reagents
- Controls are essential
- Validate data analysis

Particle Counting (abs. counts or percentages)

Counting Statistics

	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



Applications:

- Cell Counting
- Molecule Counting
 - Digital PCR
 - Immunoassays

Ignoring Counting Statistics Can Lead to Erroneous Conclusions

Cytometry Worksteps

- Cell observations (photography including video)
- Cell culture monitoring
- Total Cell Counting
- Cell subset counting
- Multi-parameter Cell Analysis
 - Flow Cytometry
 - Imaging
- In-vivo single cell analysis
- Cell Sorting
- Single Cell Sequencing
- Data Analysis
- Sample Preparation
- Workflow Automation

Low Magnification Video and Photography

- Documentation of changes in cell/tissue culture by digital photography or video recording.
- RGB jpeg data have limited quantitative information
- For subsequent quantitative analysis uncompressed data formats like tiff provide good data
- Intensity depth is generally 8 bits/color, some consumer cameras provide 12 bits/color, scientific cameras 16 bits/color



<http://nanolive.ch/3d-cell-explorer/>

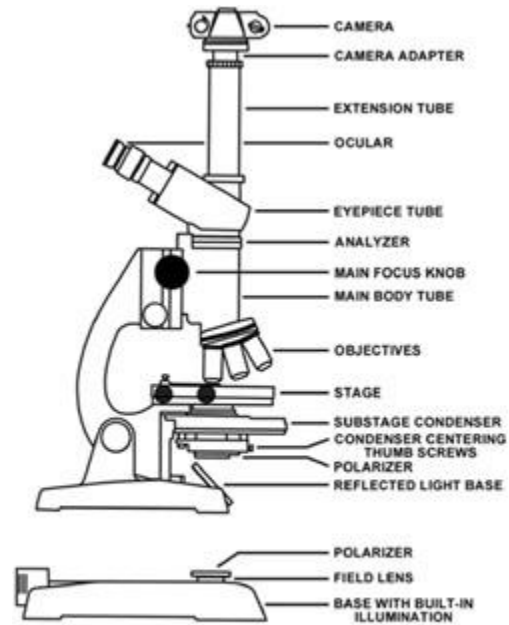
Microscopy

- Brightfield
- Darkfield
- Fluorescence
- Phosphorescence
- Phase contrast
- Interference contrast
- Polarization

Quantitative measurements

see [link](#) ImageJ for microscopy

(select cancel, when prompted for password)



<http://micro.magnet.fsu.edu/publications/pages/introphoto.html>

Cell Growth Monitors/ Tissue Culture

- Low Magnification Microscopy/Imaging
- Impedance

<http://www.aceabio.com/case.aspx>



Low-cost Cell Counters

(many include viability and single color subset analysis)

- Imaging
- Flow
- Impedance



Moxi Flow™
The Worlds First "Smart Flow Cytometer"



& Dual Use
Flow Cell Cassette.

Simple Cell-Subset Counters

- Fluorescence Detection by Imaging (left below)
- Fluorescence Detection by Flow (right on bottom)
- Multi-Frequency Impedance (on right)

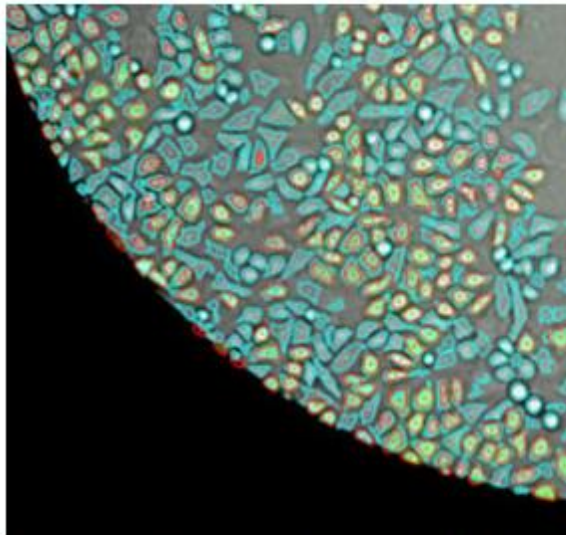
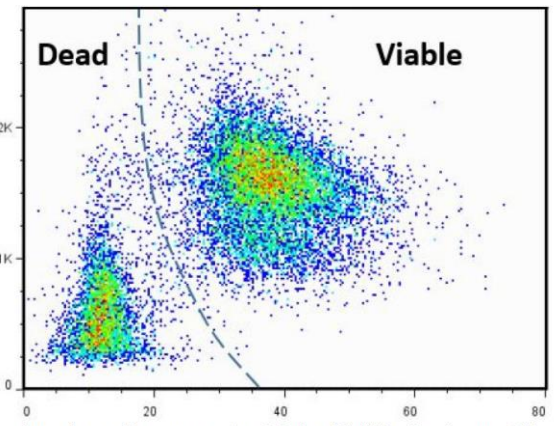


Figure 4: Brightfield image of a well on a 96-well microplate showing counted adherent cells.

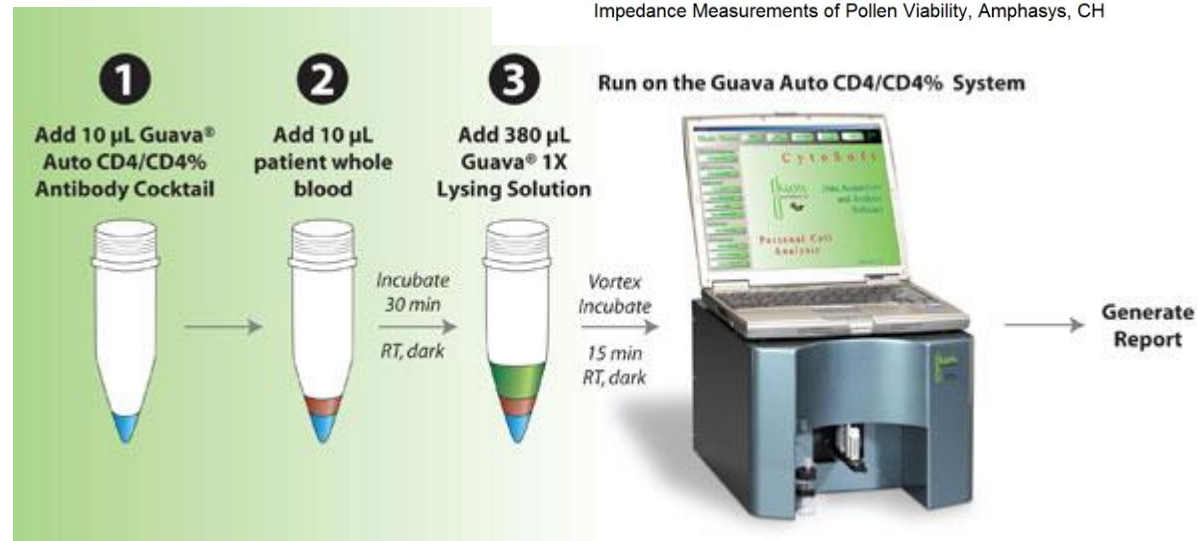
Accurately Measure Adherent Cells without Trypsinization

Analyze your cell sample without trypsinization to avoid losing cells and look at cells right where they grow over multiple scan times.

Nexcelom Celigo
Brightfield and 3-colors of fluorescence

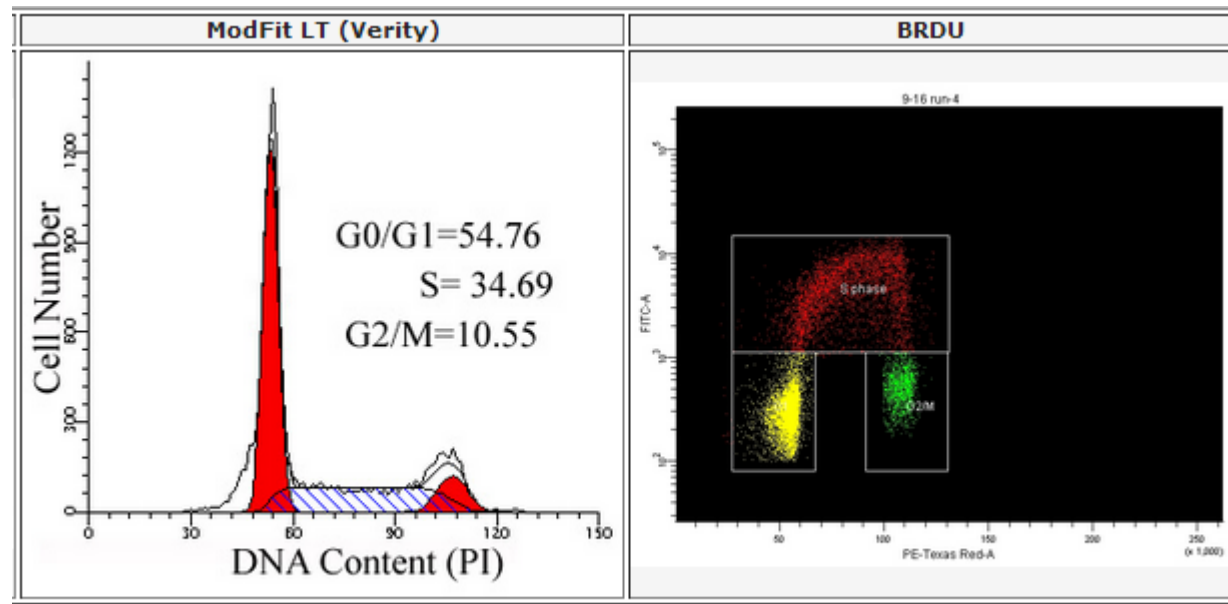


Impedance Measurements of Pollen Viability, Amphasys, CH



Cell Cycle Analysis

- High-resolution Flow Cytometry
- Imaging
- BrDU



From: <http://www.vet.cornell.edu/labs/cytometry/cellcycleprotocols.cfm>

Kinetic Cell Measurements

- Imaging
(single cell kinetics)
- Flow Cytometry
(population kinetics)

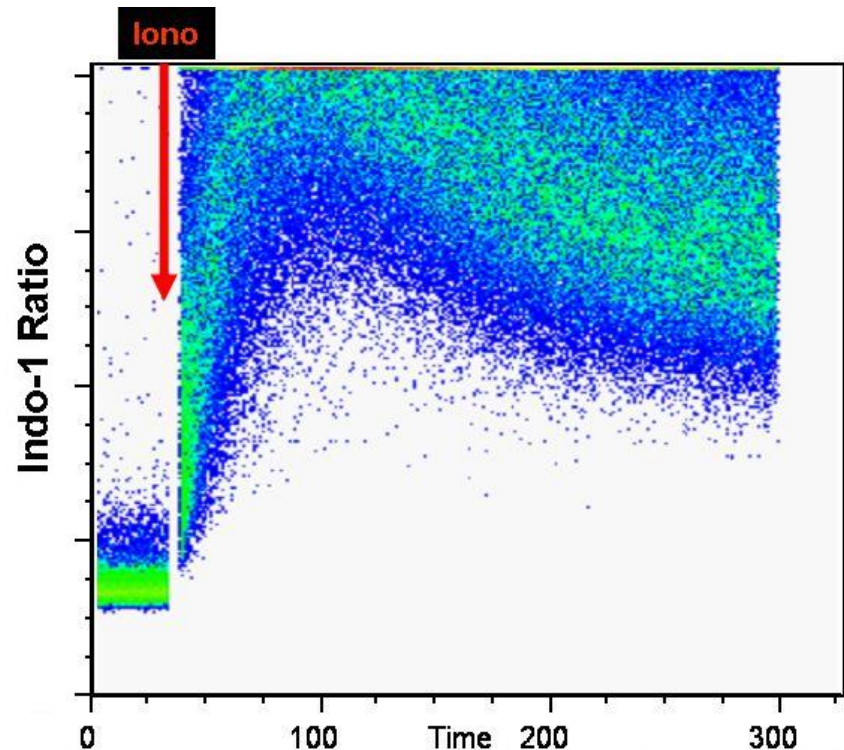
Examples:

Ca⁺⁺ flux

Enzyme kinetics

Cell proliferation

...



Legend. The Indo-1 ratio was calculated from the violet (440 nm) and green (530 nm) emissions and plotted against the Time parameter. Jurkat T-cells were loaded with 1 μ M Indo-1 for 45 min at 37°. Ionomycin (10 μ g/ml) was added after 30 seconds resulting in a rapid rise in Indo-1 Ratio against Time.

<http://www.icms.qmul.ac.uk/flowcytometry/uses/functionalanalysis/calciumflux/ratiometric/index.html>

Multiparameter Cell Analysis

- Imaging
 - Immunocytochemistry
 - Multi-color Immunofluorescence
 - Sequential Stain-Destain Immunofluorescence
 - Raman including SERS
- Flow Cytometry
 - Multi-color Immunofluorescence
 - Filter-based
 - Spectral Analysis
 - Raman including SERS
 - CyTOF
- Imaging Flow Cytometry (ImageStream)

More info: Bendall SC et al. (2012) Nature Biotech. 30:639-47

Maecker H, Trotter J (2011) Multicolor Flow Cytometry Application Note

Recent Systems for Multi-parameter Cytometry

- 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)

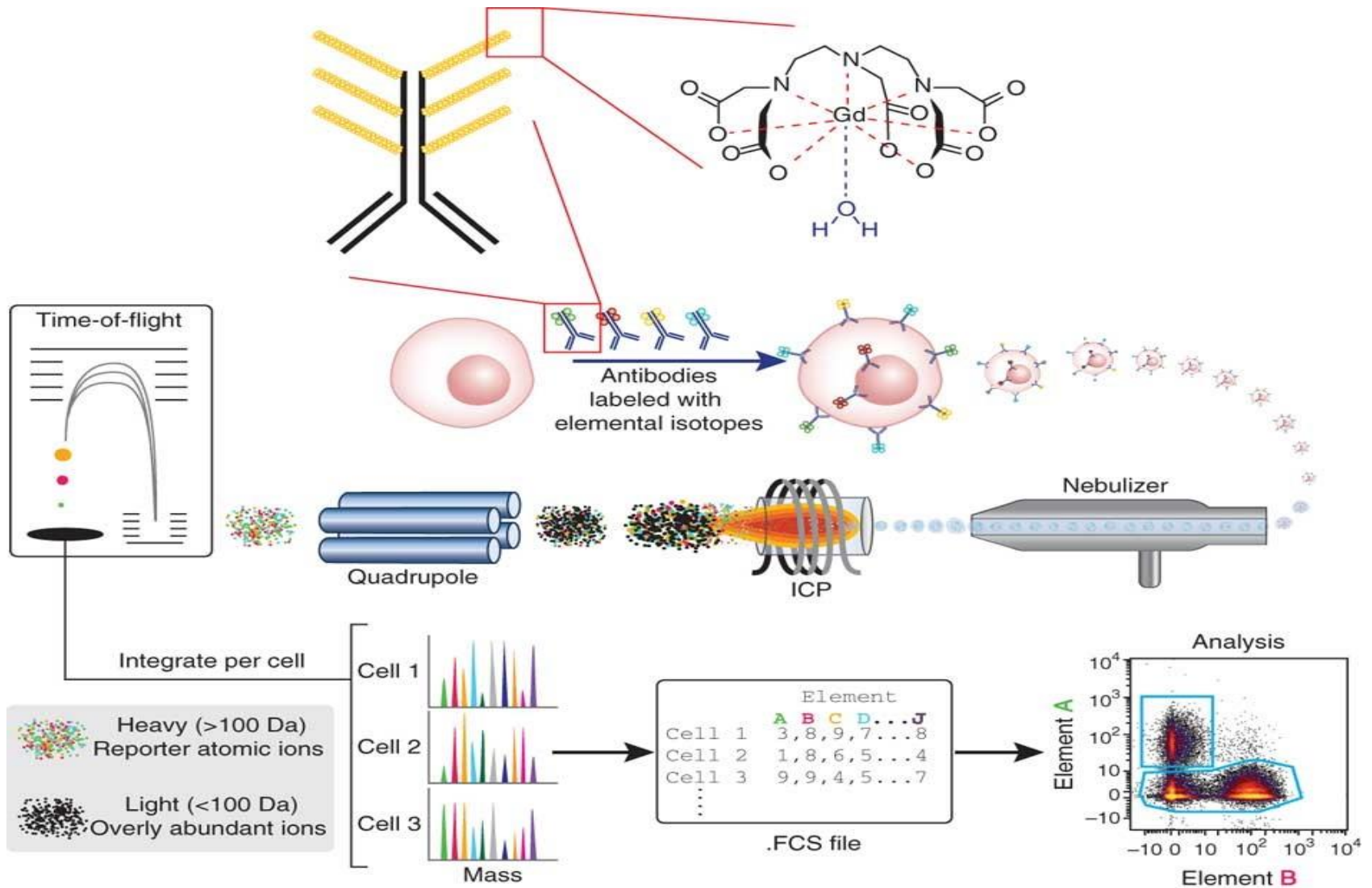
- Highly fluorescent polymer dyes (Brilliant violet, ...)

(Cytometry, 2012, 81A(6), pp 456-466,

http://www.sirigen.com/sirigen_technology.html

http://www.bdbiosciences.com/documents/multicolor_fluorochrome_laser_chart.pdf)

CyTOF Cytometer



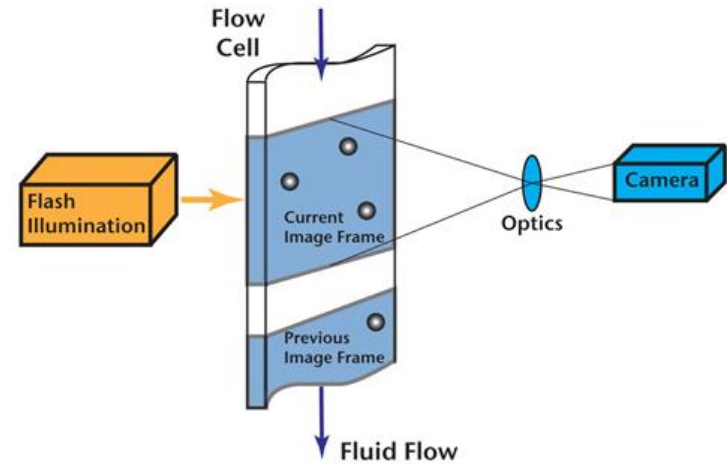
From single cells to deep phenotypes in cancer

Sean C Bendall & Garry P Nolan

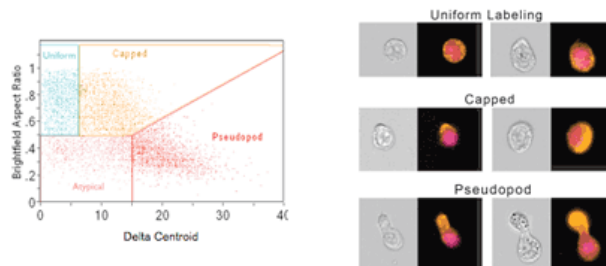
Nature Biotechnology 30, 639–647 (2012) | doi:10.1038/nbt.2283

Imaging Flow Cytometer

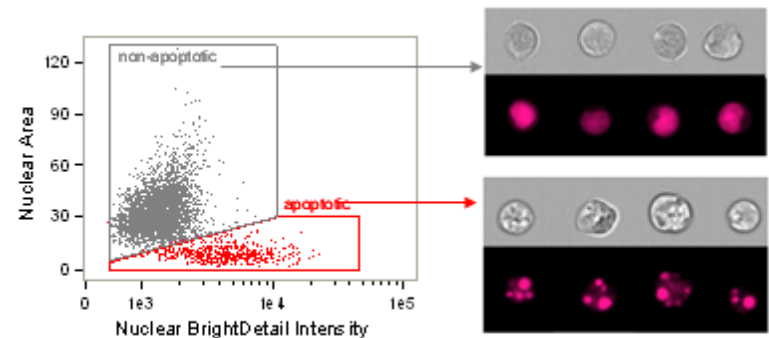
Images of Cells/Particles are captured in a fluid stream and stored individually.



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



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



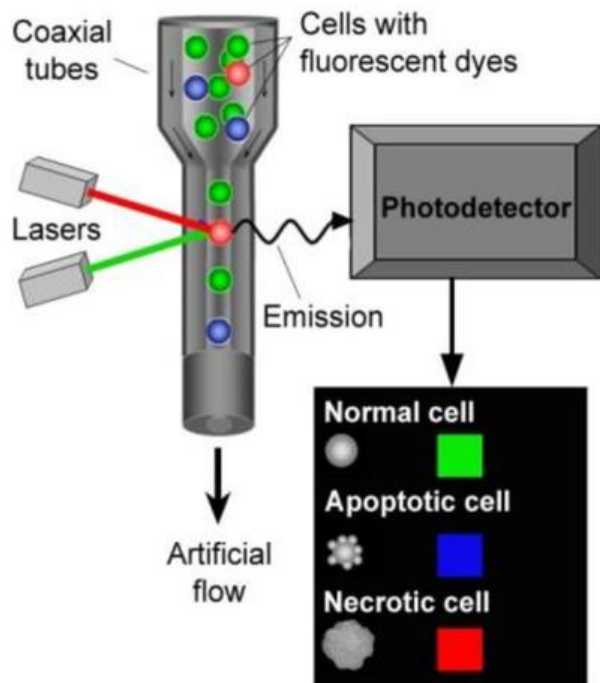
<https://www.amnis.com/images/ApoptoticIndex.png>

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

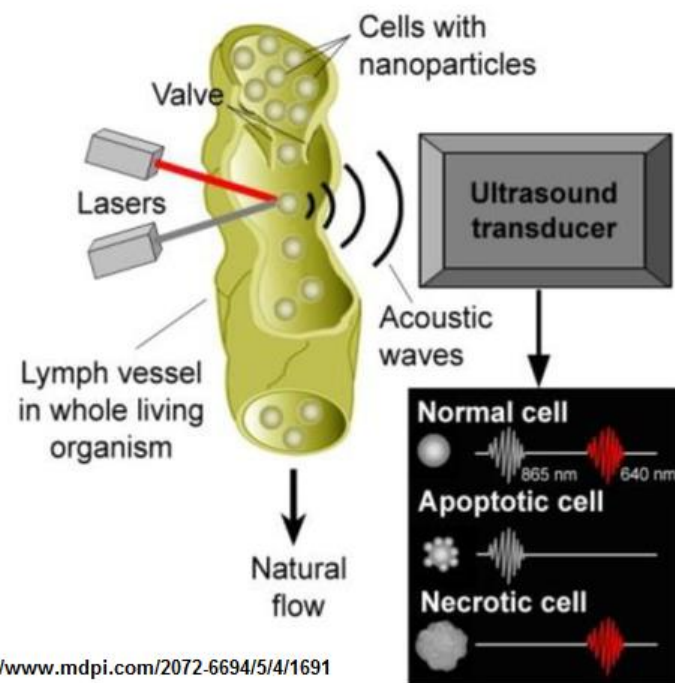
In-vivo Single Cell Analysis

- Intra-vital Imaging
- In-vivo Flow Cytometry

**Conventional
flow cytometry *ex vivo***



**Photoacoustic lymph
flow cytometry *in vivo***



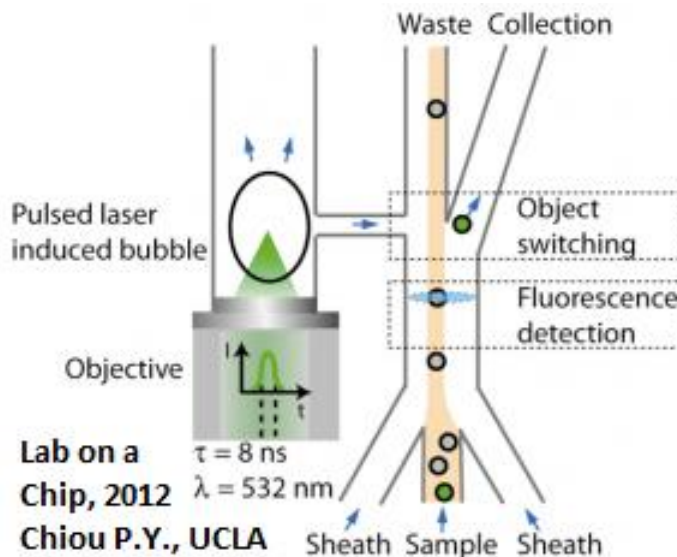
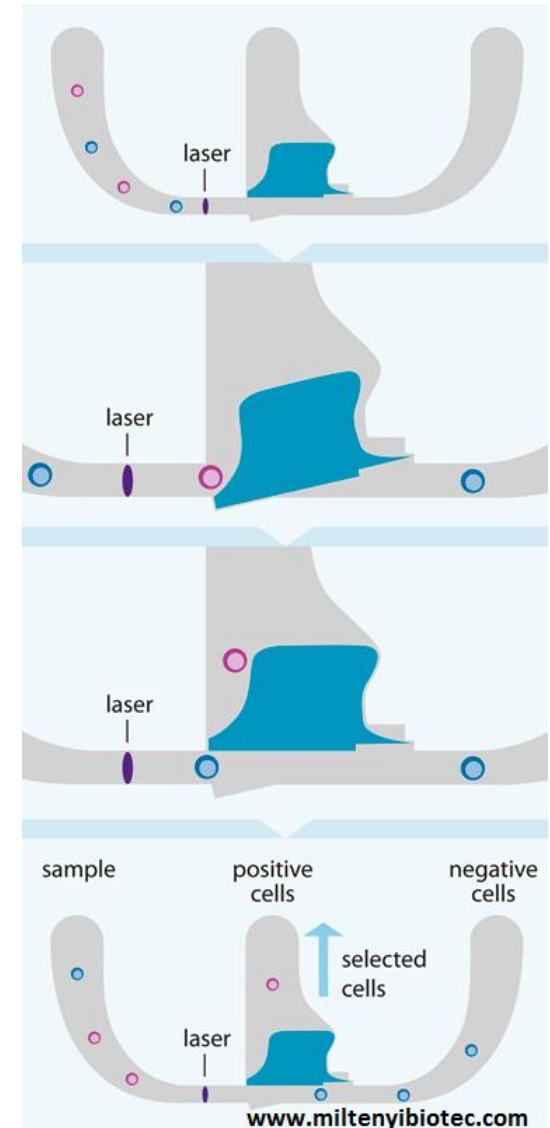
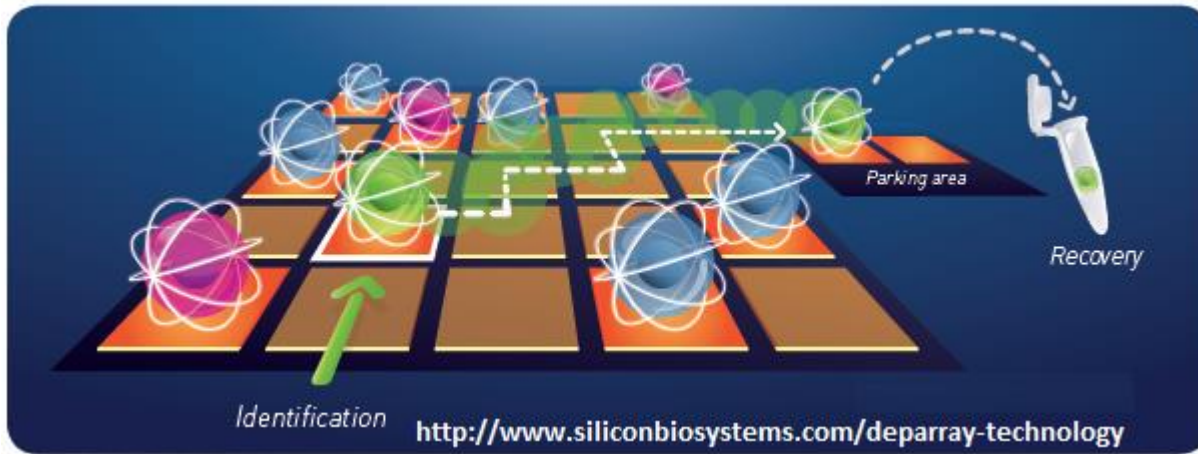
V.P. Zharov group, <http://www.mdpi.com/2072-6694/5/4/1691>

Cell Sorting

- Bulk Sorting
 - Magnetic
 - Gravity
 - Acoustic
 - ...
- FACS
- Tyto/OWL
- DEP sorter
- Other sorters



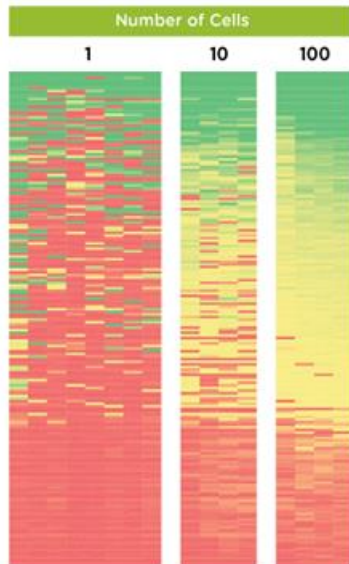
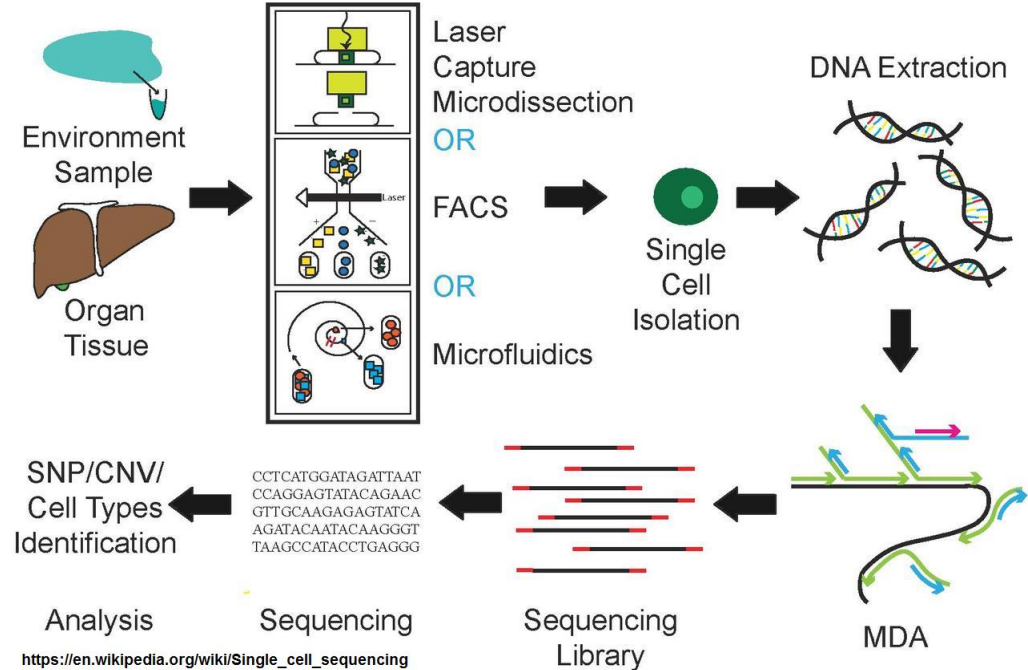
New Cell Sorting Technologies



Single Cell Sequencing

Gene expression analysis of single cells from blood or tissues

Single Cell Genome Sequencing Workflow



Novel Affinity Reagents

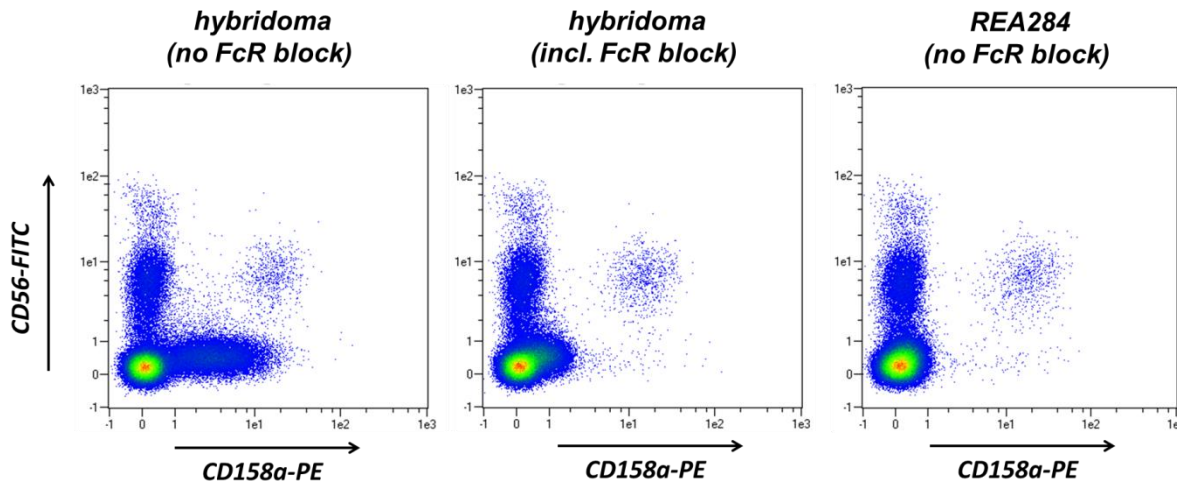
Antibodies

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

Gene Expression Analysis RNA Flow Cytometry

RNA Probes:

- Detection of specific RNA sequences after hybridization and amplification
- Single copy limit of detection

More info: Hanley MB et al. (2013)
PLoS ONE 8:e57002

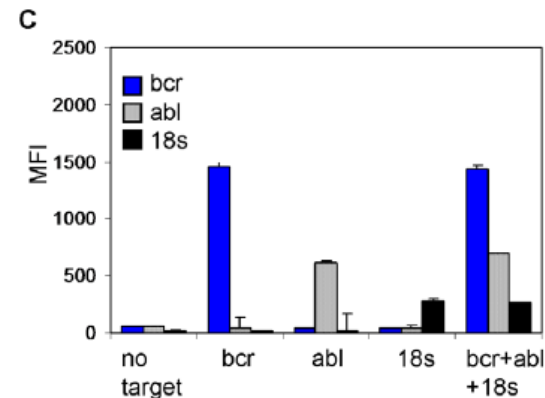
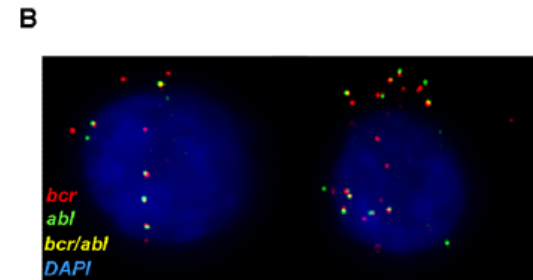
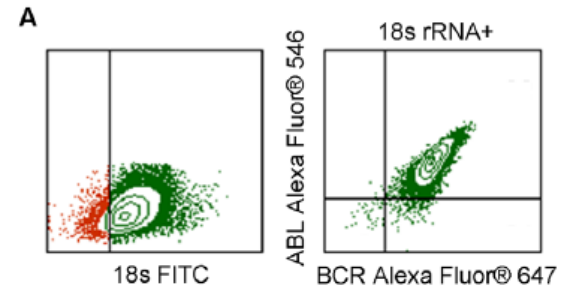
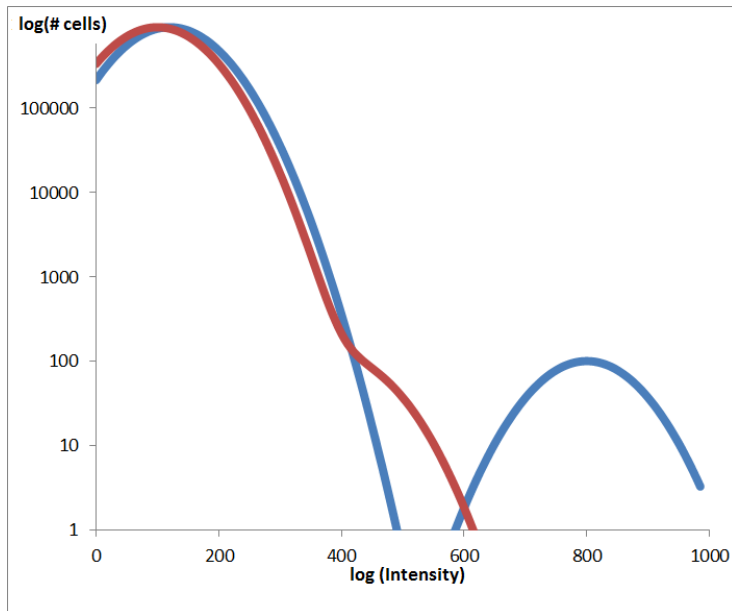


Figure 4. Assessment of RNA flow cytometry probe multiplexing.

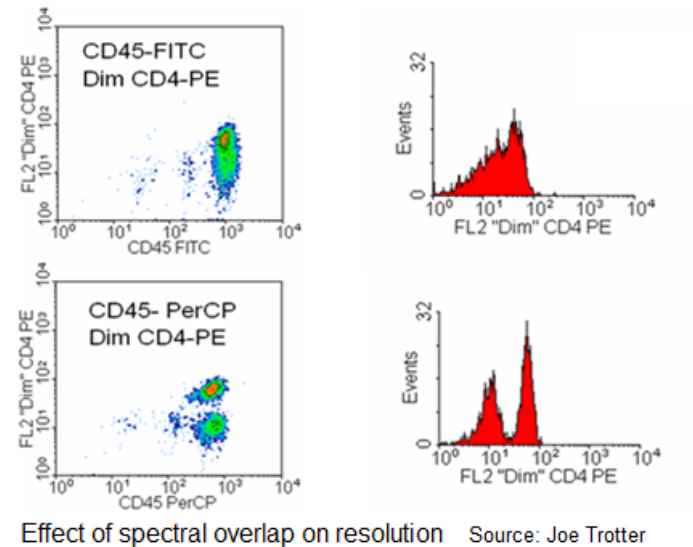
Hanley MB et al. (2013) PLoS ONE 8:e57002

Label Selection

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



Brightness and Separation

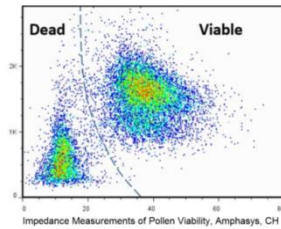


Spectral Overlap and Separation

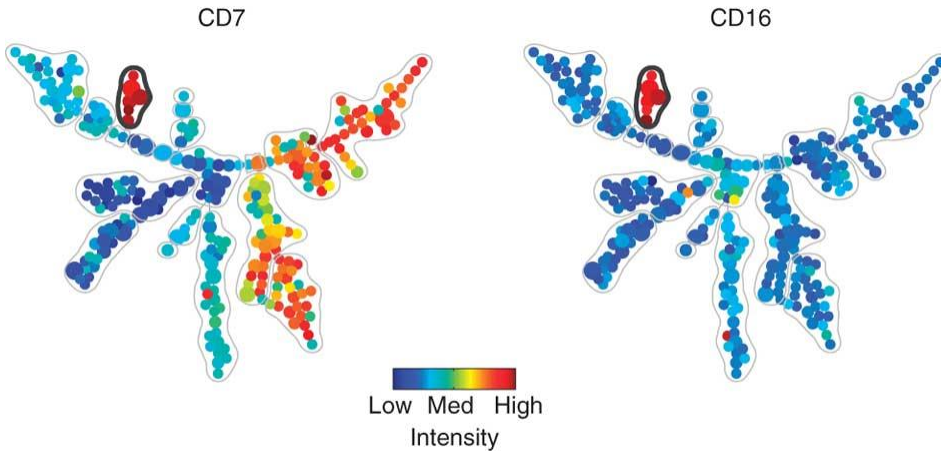
More info: Maecker HT et al. (2004) Cytometry 62A:169-173

Data Analysis

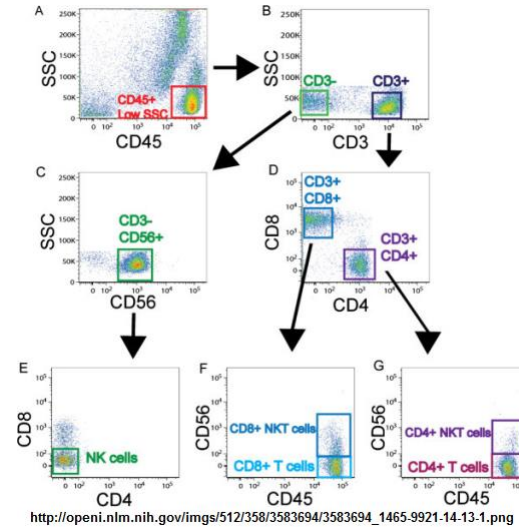
- Manual Gating



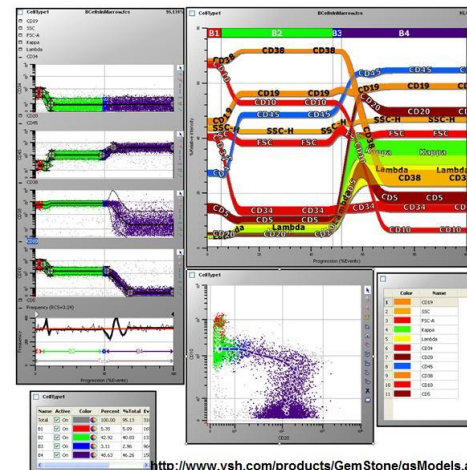
- Automated Analysis



<http://www.nature.com/nbt/journal/v29/n10/pdf/nbt.1991.pdf>



http://openi.nlm.nih.gov/imgs/512/358/3583694/3583694_1465-9921-14-13-1.png



<http://www.vsh.com/products/GemStone/gemModels.asp>

More info: Nima Aghaeepour et al. (2013) Nature Methods 10:228ff

27 Enrico Lugli, Mario Roederer, Andrea Cossarizza (2010) Cytometry 77A:705ff

Automated Sample preparation

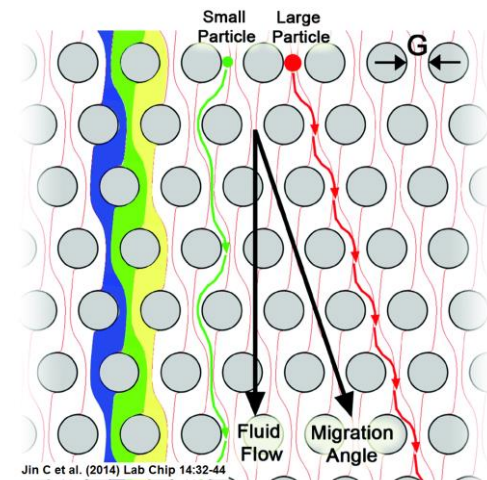


Presently available

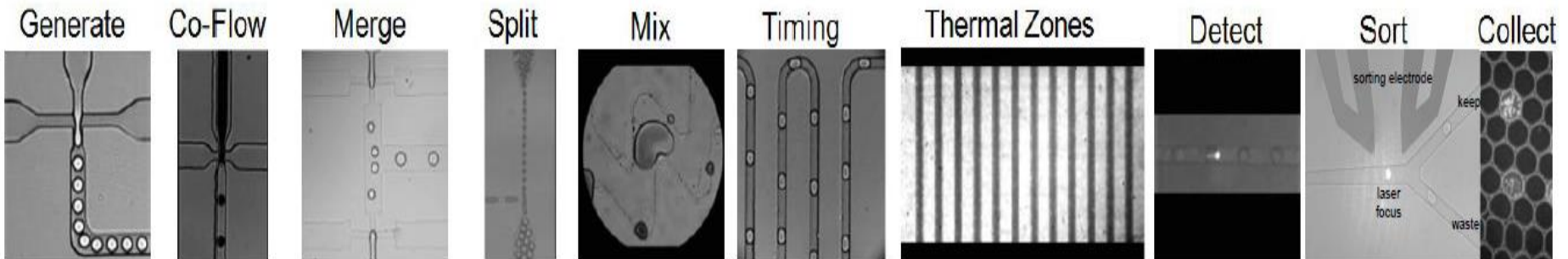
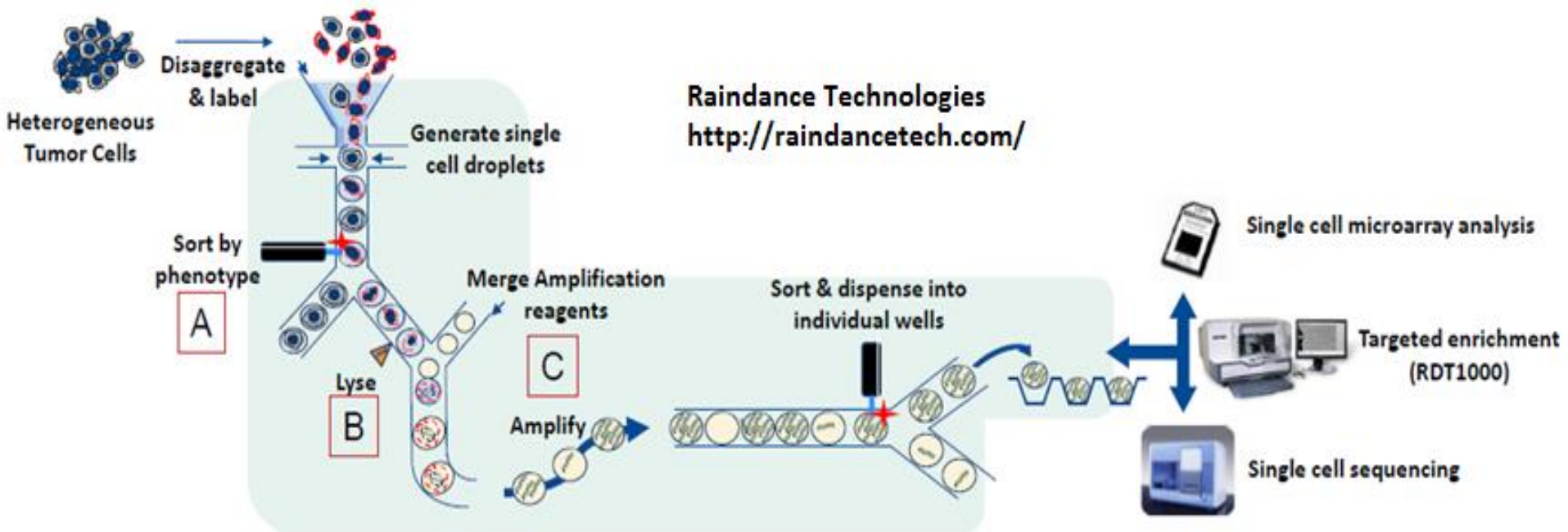
- Fully automated sample to result e.g. BC Acquios CL, MACSQuant
- xyz-fluid handling robots e.g. BD SPA or BC Biomek
- Washing by special centrifugation e.g. BD LWA
- Membrane Filtration e.g. MSP FlowCytoPrep

Promising future technologies

- Deterministic lateral displacement
- Acoustic focusing
- Inertial flow

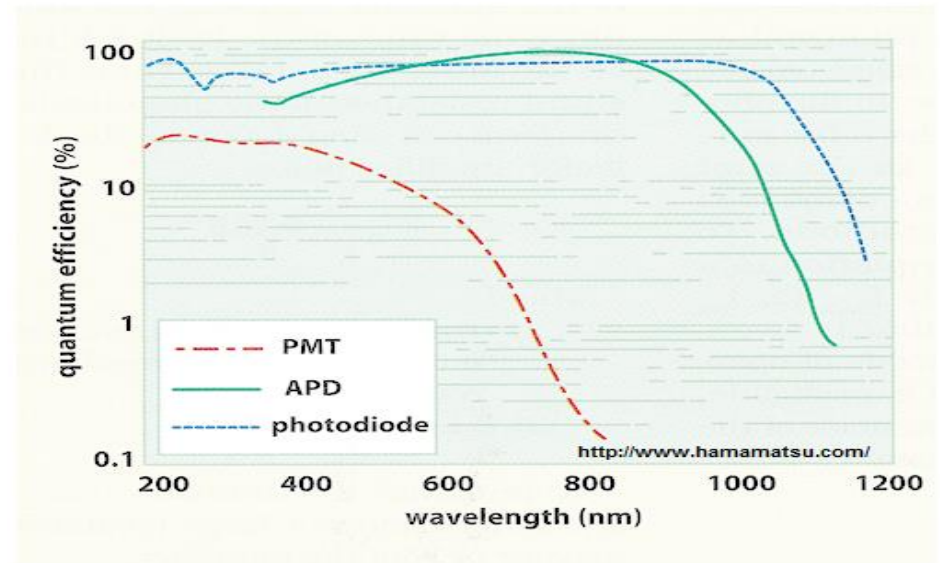


Droplet-based Integrated Bio-Assay System Technology

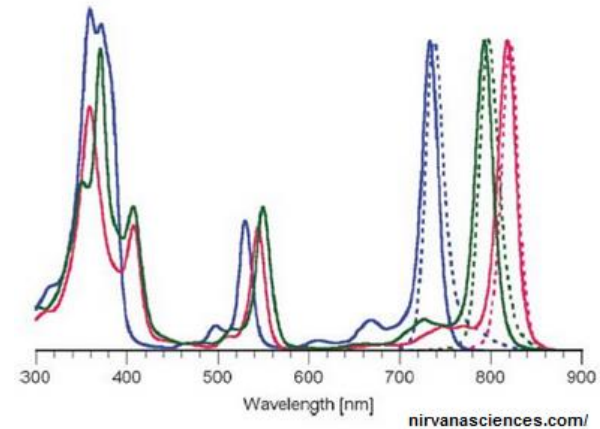


New Detector-Label Combinations

- New photodetectors extend the available spectrum
(Si avalanche photodiodes extend detection into the far infrared, Xitogen system))

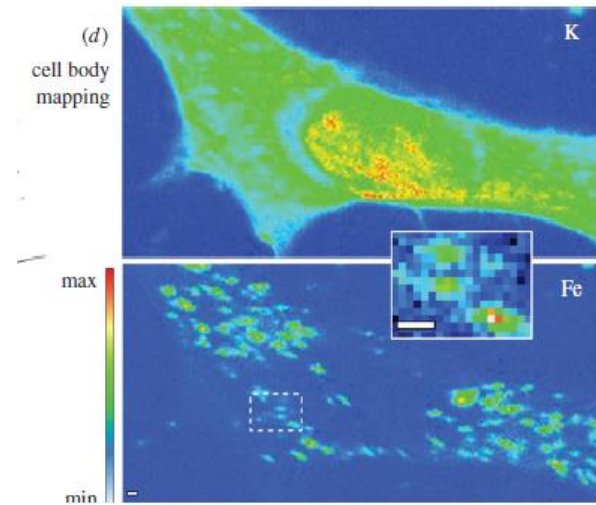


- New dyes add excitation in the UV, some detection in the IR
(Fluorescent polymers, bacteriochlorins, ...)

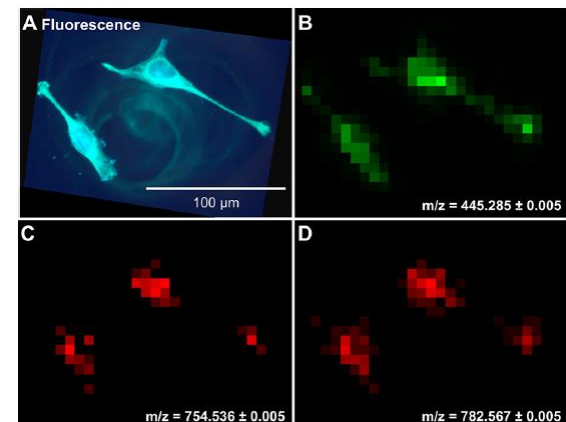


New Detection Technologies

- 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

- **For optimal results use an adequate technology**
(flow cytometry has enormous capabilities, but is not always the technology to use e.g. single cell kinetics)
- **Understand the limitations of the system**
(limits of detection, non-specific binding of reagents, ...)
- **Validate results with appropriate controls**

Acknowledgements

- Joe Trotter
 - Ed Goldberg
 - Liping Yu
 - Brent Gaylord
- above all BD Biosciences

Input from companies:

- BD Biosciences
- Miltenyi Biotec
- Beckman Coulter
- Thermo Fisher
- ...

- Martin Buescher, Miltenyi
- Christian Dose, Miltenyi
- Holden Maecker, Stanford
- Bob Hoffman
- Ming Yan
- Maria Jaimes
- Hrair Kirakossian
- ...

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URL (for presentation and links):
<http://www.desatoya.com>