"Flow Cytometry: Past, Present, and Thoughts about the Future"

36th Annual Research Course in

Flow Cytometry,

Albuquerque, NM, June 13, 2013

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Biology Research Targets and Tools

Organism

Organ

Tissue

Single Cell

Organelle

Macromolecule

Small molecules

NMR
X-ray imaging
Ultrasound
2-photon imaging
In-vivo cytometry
Light microscopy
Electron microscopy
Flow cytometry
Cell imaging
NA sequencing
Mass spectrometry
TIRF microscopy
Electrophoresis

Contrast agents
Affinity reagents
- antibodies
- probes
Enzyme substrates
Labels
- absorbance
- fluorescence
- element tags

Sample prep

Outline

- History
- Flow Cytometry and Imaging Principles
- Important applications
- New developments
- New technologies for single cell analysis and sorting
- Outlook
- Summary and Conclusions

The Past

1966 Kamentsky RCS: Four Sensors, Sorting, Auto Sampling and Computer Data Reduction

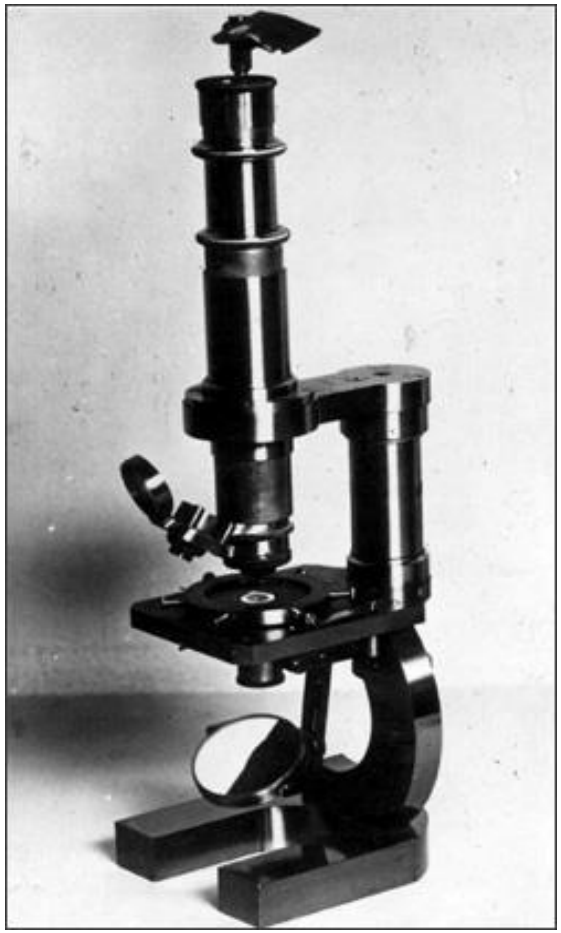
Two analytic instruments were built and one was delivered to LA Herzenberg at Stanford University 1967

ICP 11 (1969) Distributed by Phywe, Göttingen The first commercial flow cytometer PDP 11 computer

Wallace H. Coulter
1913-1998

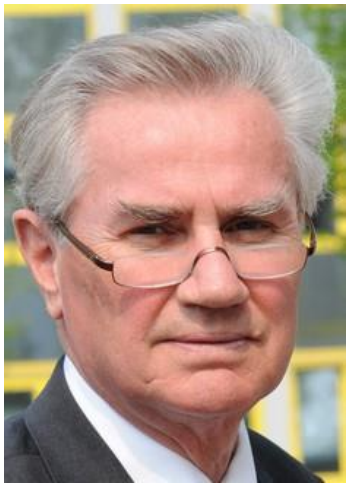
Joseph R. Coulter, Jr.
1924-1995

History of Cytometry Technologies (Microscopy)



- 1665 – English physicist, Robert Hooke used a microscope lens to observe “pores” in cork
- 1674 – Anton van Leeuwenhoek built a simple microscope with only one lens to examine blood cells
- 1872 – Ernst Abbe calculated the maximum resolution in microscopes
- 1932 – Frits Zernike invented the phase-contrast microscope (label-free observations)
- 1969 – Willard Boyle and George E. Smith at Bell laboratories invented the CCD
- 1971 – Intel launches 4-bit 4004 microprocessor

History of Cytometry Technologies (Flow Cytometry)



1968 1st fluorescence-based flow cytometry device (ICP 11) by Prof. Göhde from the University of Münster, Germany, and first commercialized in 1968/69 by German developer and manufacturer Partec through Phywe AG in Göttingen.

1971 Cytofluorograph, Ortho

1973 PAS 8000, Partec

1974 1st FACS instrument, BD

1977 Epics Instrument,Coulter

2002 Microfluidic Cytometer, Quake, Caltech

The Present

MACS Miltenyi Biotec

PARTEC

DVS Sciences

SONY make.believe

Xitogen

BECKMAN COULTER

EMD MILLIPORE

Stratedigm Cytometry Without Compromise

BD

...

handyem™ Democratize cytometry™

BIO-RAD

Bay bioscience Bay bioscience

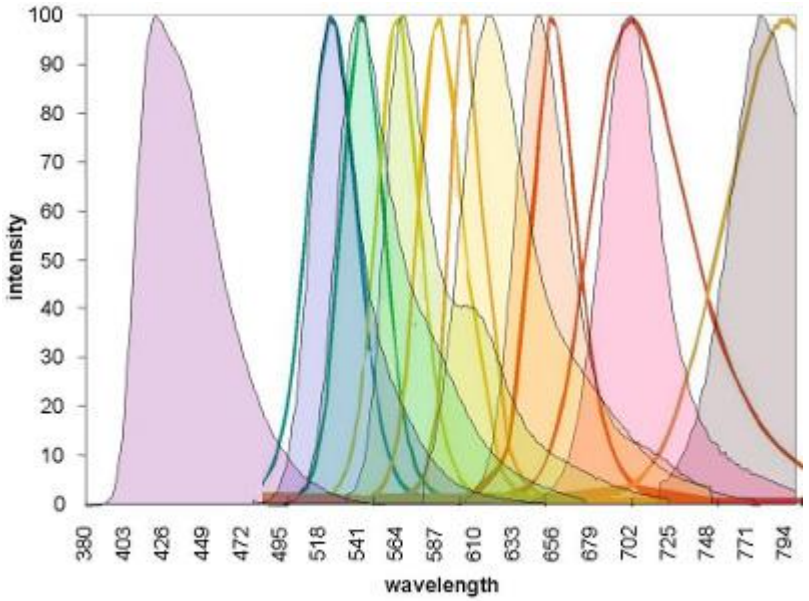
Flow Cytometry Features

Single cell analysis with

- High sensitivity (single molecule sensitivity by fluorescence)
- Wide dynamic range (10^3 to 10^7 cells mL^{-1})
- High analysis rates to ~10^5 particles sec^{-1}
- Light scatter
- Multi-color fluorescence, multi-parameter analysis
- Live/dead discrimination
- Viable cells can be re-covered
- Good ease-of-use

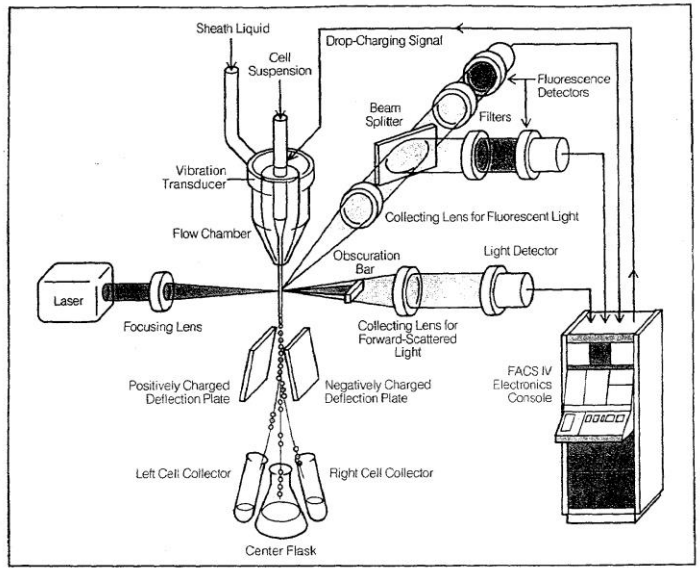
Physical Parameters used for Cytometry

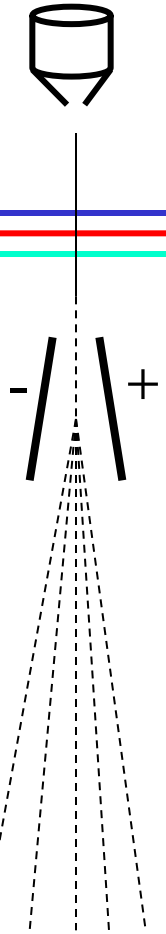
- Light scatter
- Absorbance
- Fluorescence
- Phosphorescence
- Raman
- Electrical properties
- Mechanical properties
- Element mass
- …



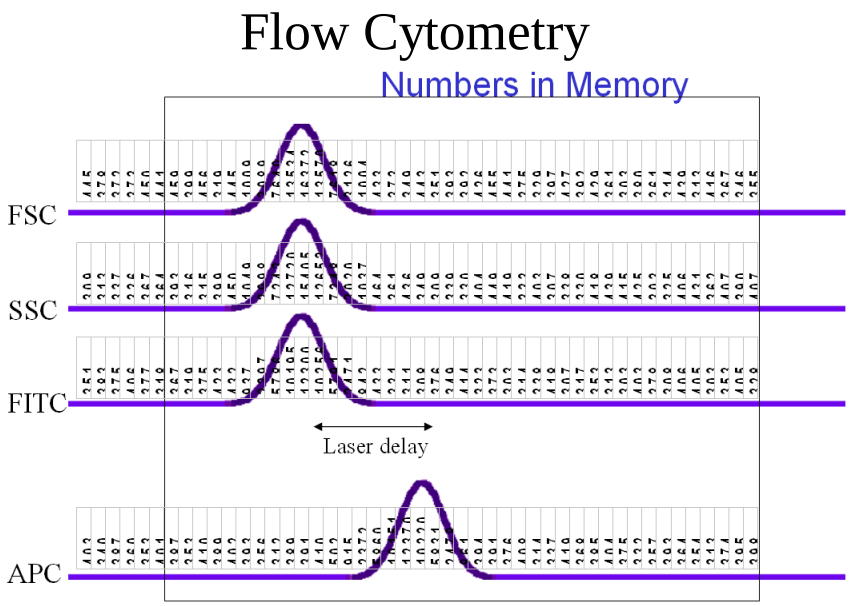
http://www.dvssciences.com/technical.html

“Droplet-based” Sorting

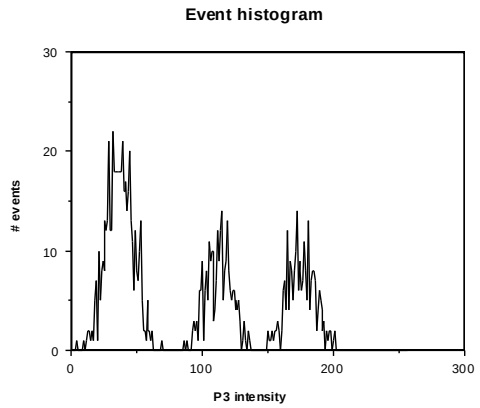


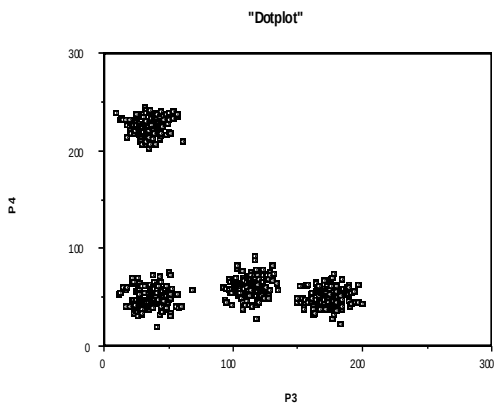


Basic Data Processing



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

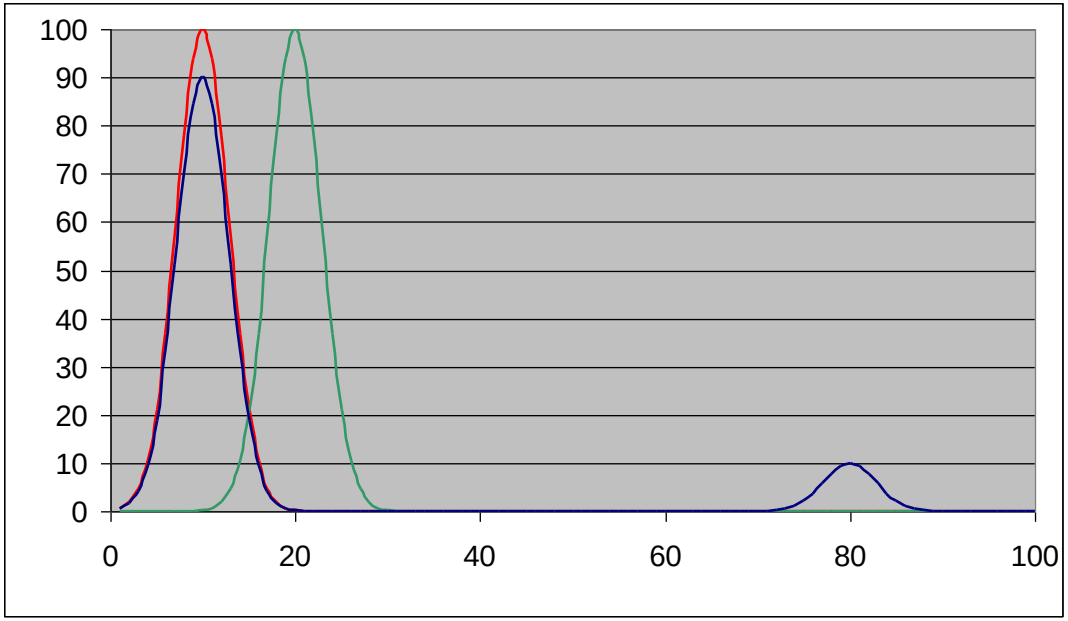




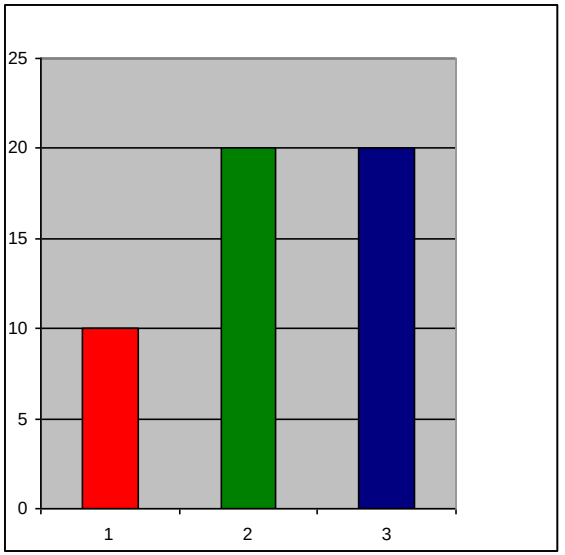
- Gating
- Cluster Analysis
- Other Data Anal.

Single Cell Cytometry vs. Bulk Analysis

Intensity Histogram for Single Particles



Intensity per Sample



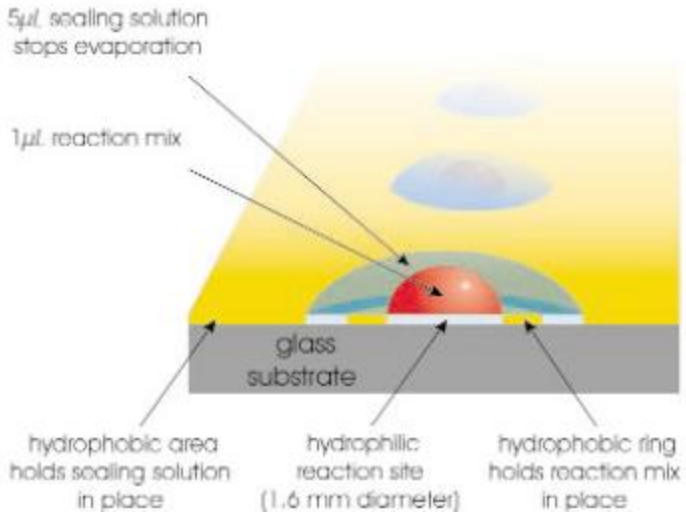
Cell by cell intensity analysis detects population heterogeneity.

Key Applications

- Immunology Research
- Stem Cell Biology
- Clinical Diagnostics
  - Immune status
  - Tumor Cell Cycle
- Cell Sorting
  - Single cell genomics
  - Cell population proteomics
  - Cloning for research and industrial biotechnology
- Marker quantitation
- Molecule counting
- In-vivo molecular analysis

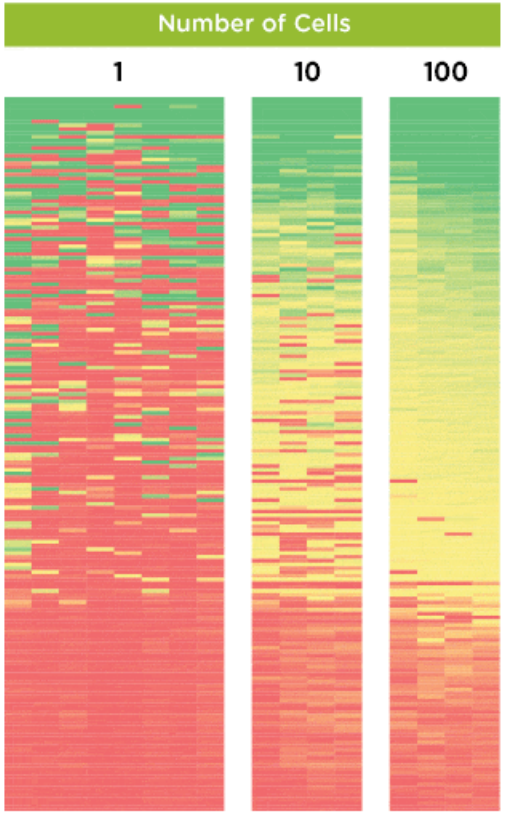
Single Cell Sorting for PCR

FACS sorting of single cells onto a slide followed by automated miniaturized single cell PCR (Advalytix).



Single Cell Genomics

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

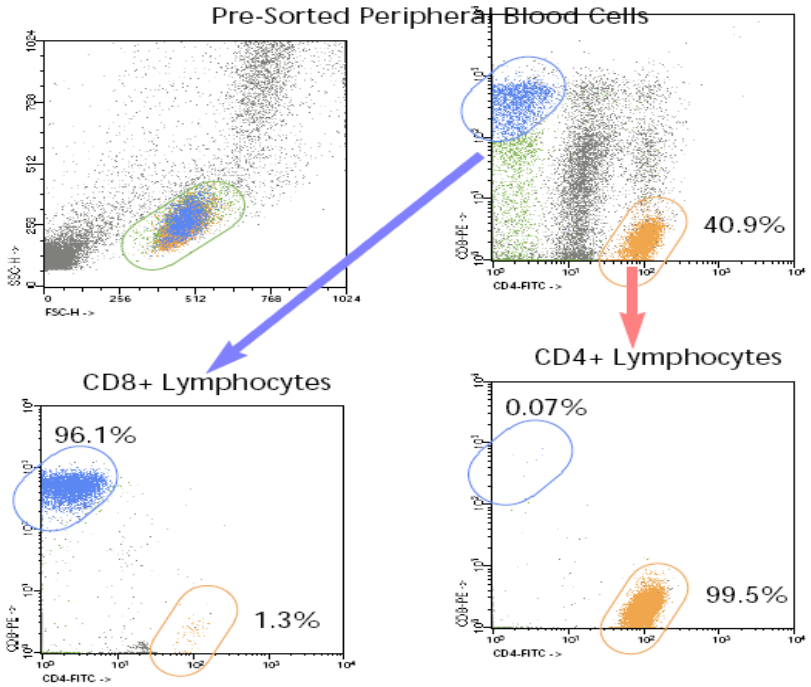


Source:
http://www.nanostring.com

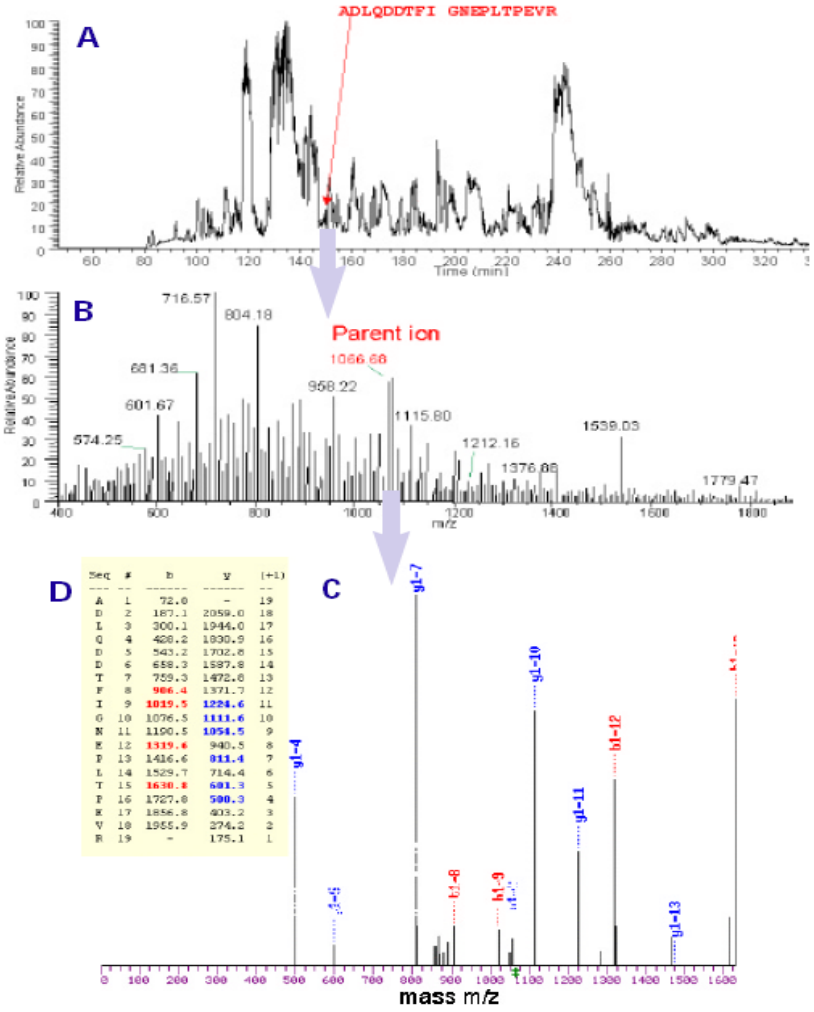
Sorting for Cell Surface Proteomics

Cell surface proteome by FACS sorting, followed by LC MS

(in collaboration with Thermo Finnigan, San Jose, CA)



The dot plots show the sorting strategy used for stained peripheral blood cells and population purity after sorting for CD4- and CD8-positive cells. CD4 cells were gated on scatter and FITC fluorescence; CD8 bright cells were gated on scatter and RPE fluorescence. Sorted populations showed >95% purity.

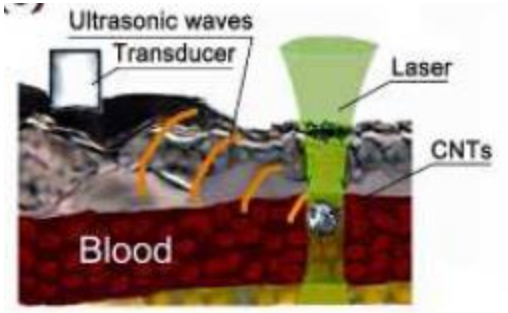


Peptide mixtures were separated by reverse phase HPLC **(A)** as described in Methods. Eluted peptides were subjected to electrospray injection into the mass spectrometer and analyzed for their mass/charge ratio (m/z value) **(B)**. Selected ions were collected in the ion trap. These parent ions were cracked by collision ion dissociation to produce a range of fragment sizes **(C)** that were compared to predicted peptide sequences in the human database using TurboSequest **(D)**.

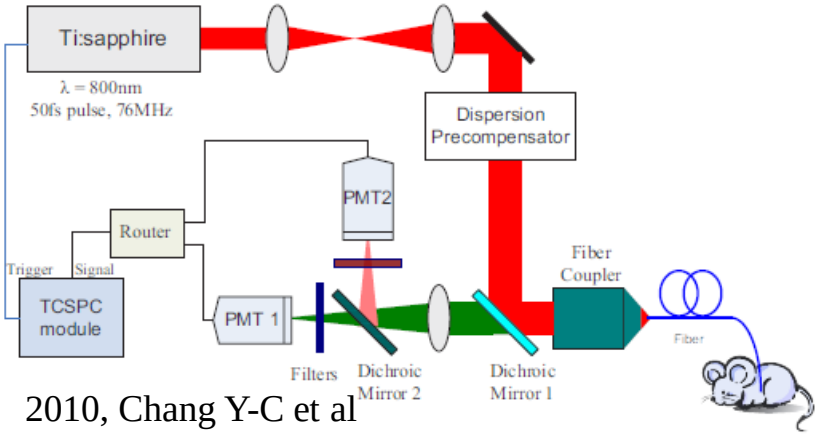
Intra-vital Cytometry

Single cell analysis in living animals

Flow cytometry in blood vessels

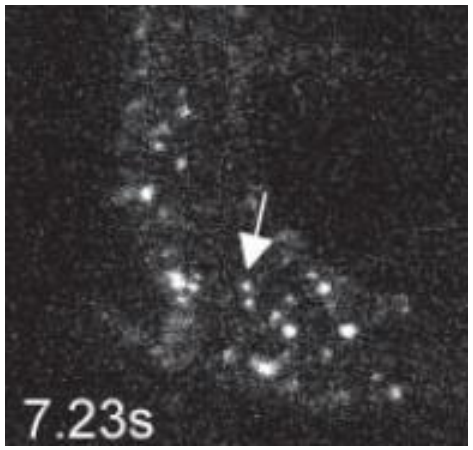


2010, Zharov VP and coworkers



2010, Chang Y-C et al

Microscopy



2011, Runnels JM et al; homing of multiple myeloma cells in bone marrow

Signals from

- 2-photon fluorescence
- bioluminescence
- photo-acoustic effect
- …

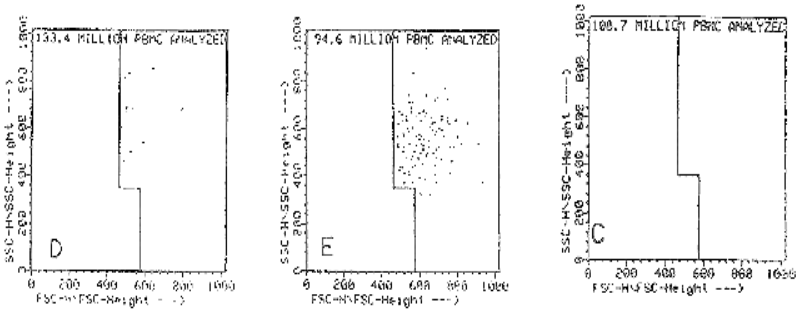
Review paper:
Niesner RA, Cytometry 79A (2011)

Limit of Detection for Rare Cells

Routine >0.2%

Optimized instrument >0.01%

Optimized system >10^{-7}



10^{-6} **10^{-5}** **control**

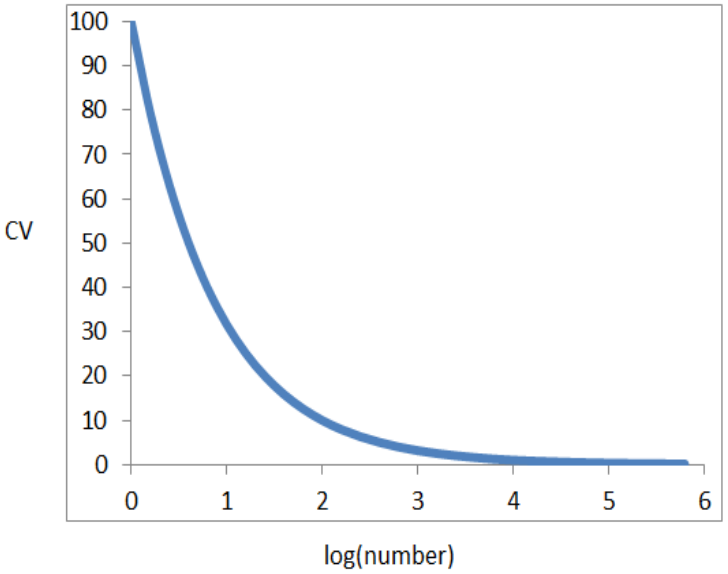
Gross HJ et al, Cytometry 14 (1993) 519-526

Gross HJ et al, PNAS 92 (1995) 537-541

Cell 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



Ignoring Counting Statistics Can Lead to Erroneous Conclusions

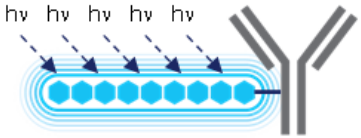
Recent Novel Products

- Labels
  - High brightness fluorescent labels
- Fluidics
  - New particle focusing technologies
- Sorting
  - New single cell sorter
- Systems
  - More parameters

Bright Fluorescent Polymer Dyes

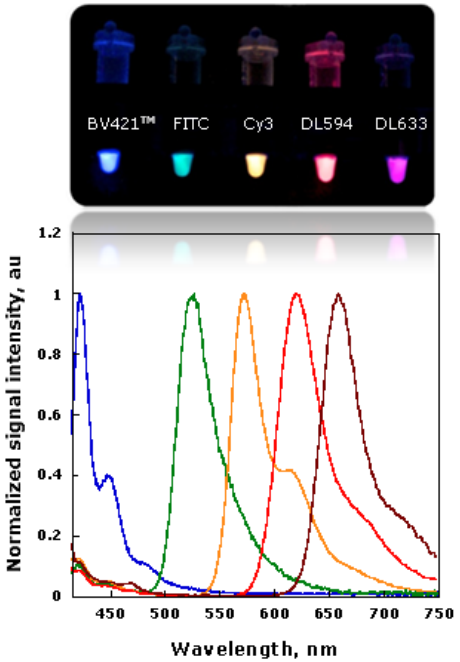
Polymer Based Fluorochromes

- Well defined synthetic organic polymer structures
  - Single conjugation site, defined size, etc.
- Backbone comprised of π-conjugated repeat units
  - Affords massive light harvesting ($\varepsilon > 10^6$) materials with high quantum yields
- Tunable architecture adapted for low NSB, high aqueous solubility and spectral performance



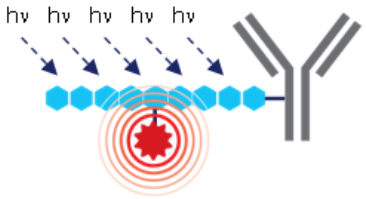
Brilliant Violet 421™

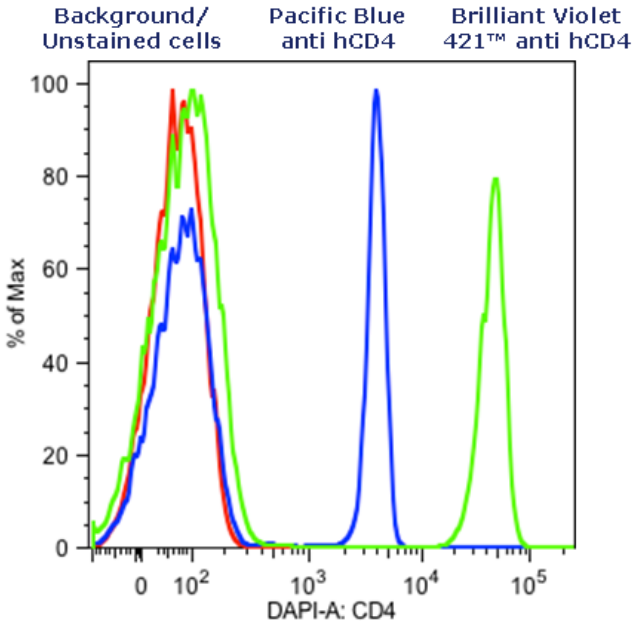
- PE level performance w/ 405nm Laser
- >10x the Stain Index of Pacific Blue
- Enables detection of low abundance targets in multicolor assay panels (e.g. CD56, CD127, etc.)
- Wide range of Ab clones validated



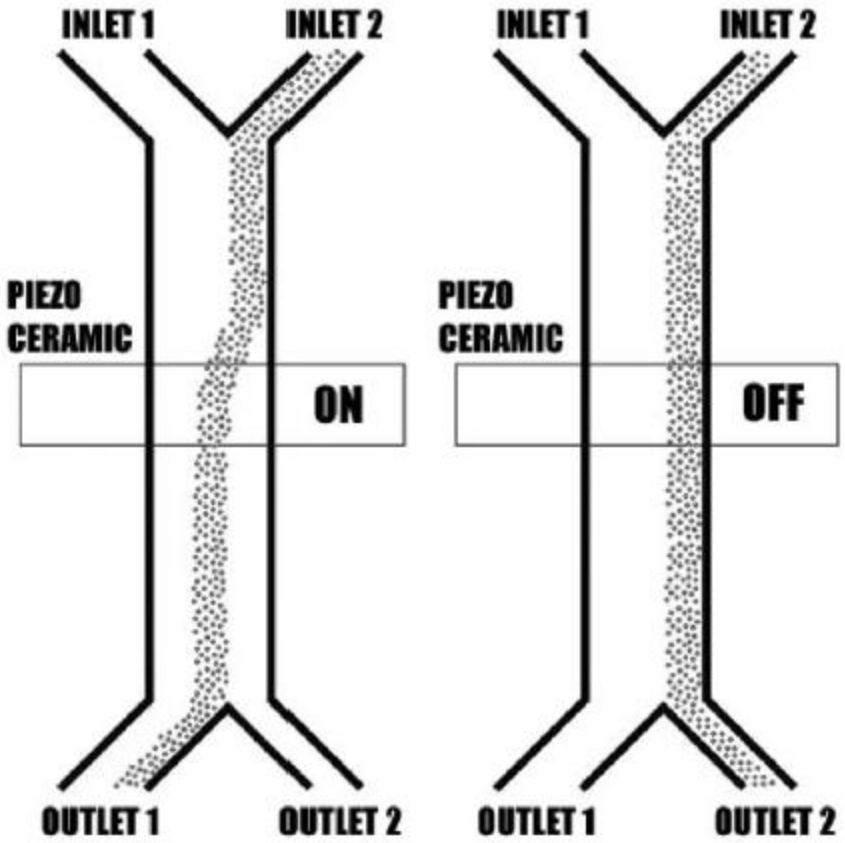
Brilliant Violet Tandems

- Provides a wider range of colors spanning the visible spectrum
  - >6 unique colors validated
- Chemically controlled ratio of donor/acceptor provides:
  - Reproducible performance
  - Low (<5%) compensation at 450nm

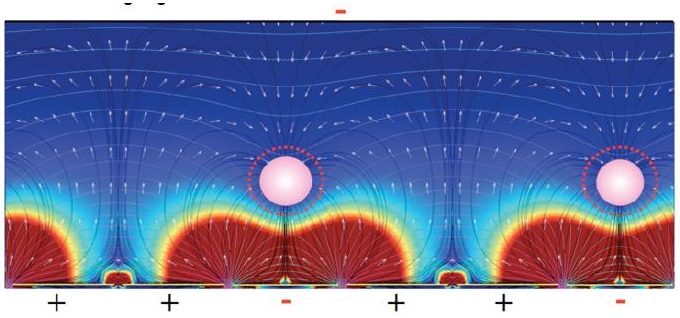




Acoustic Particle Focusing



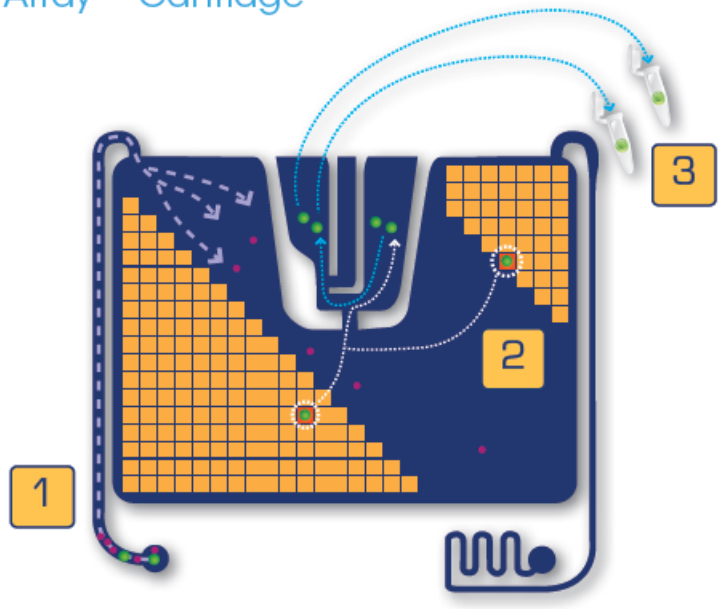
Laurell T et al 2006, Chem. Soc. Reviews



Single Cell Sorter with Microscopic Detection

Cell movement with dielectric forces.
DEPArray
Silicon Biosystems, Bologna, IT

The DEPArray™ Cartridge



1
Inject, trap and image cells

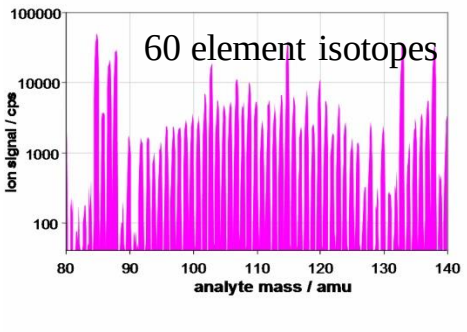
2
Move cells of interest into parking chamber

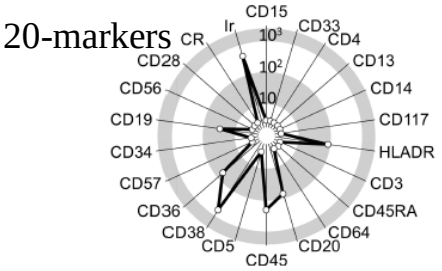
3
Move individual or multiple cells into recovery chamber and flush

Cells are transferred to a special slide with 40,000 “cages”. Cells of interest are identified by fluorescence microscopy and sorted by the instrument.

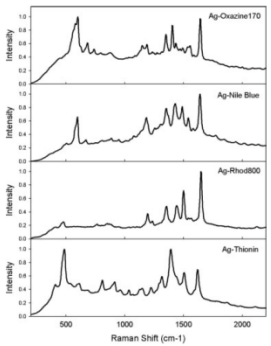
New Developments for Multi-parameter Cytometry

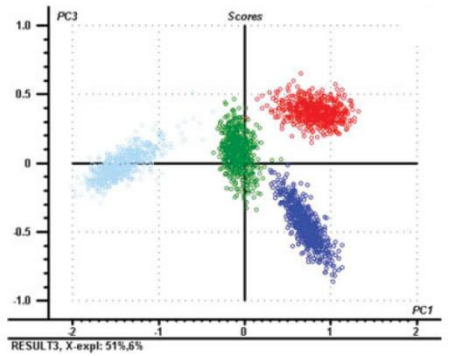
- Element-Label Flow Cytometry (CyTOF, addresses fluorescence spectral overlap issue by using elements as labels, Anal. Chem., 2009, 81 (16), pp 6813–6822)
- SERS-Label Flow Cytometry (uses spectral fine-structure to distinguish labels, Cytometry, 2008, 73A(2), pp 119-128)
- Sequential Stain De-stain Cytometry (Cytometry, 2009, 75A(4), pp 362-370)
- Spectral analysis, SONY



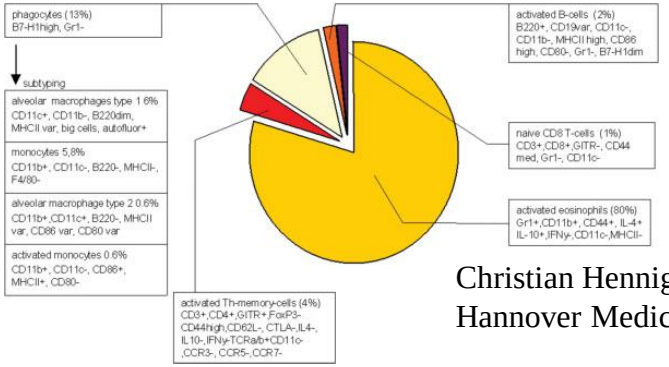


Scott Tanner, DVS Sciences Inc





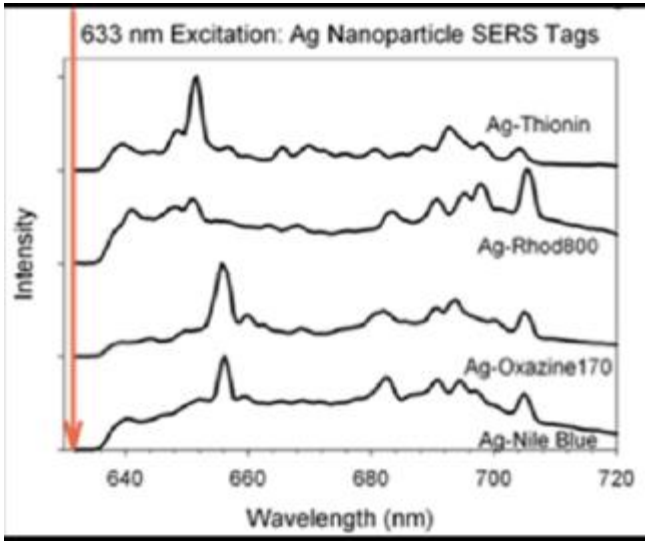
John Nolan, La Jolla Bioengineering Institute

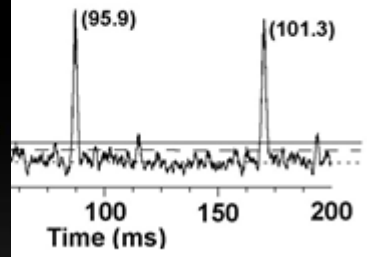


31-marker analysis

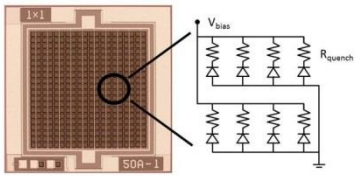
Christian Hennig, ChipCytometry
Hannover Medical School

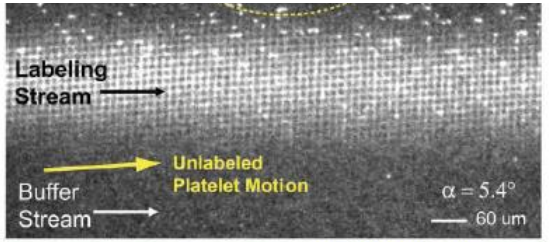
Thoughts About the Future

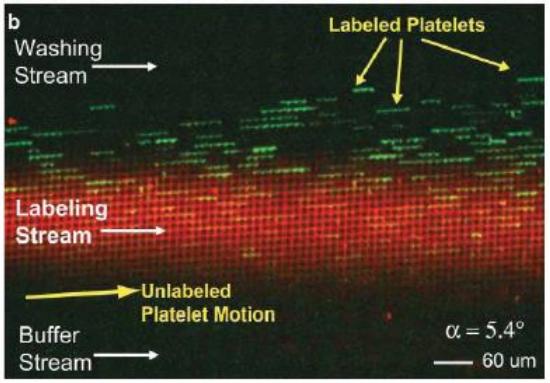


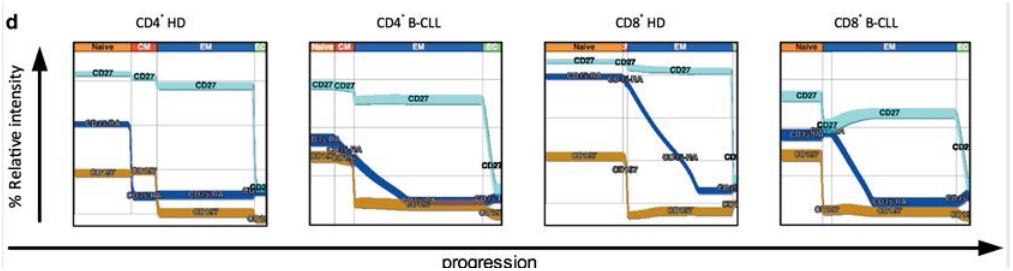


Rob Habbersett & Jim Jett, LANL









Technology Developments For Changes in Cytometry

- Labels
  - High brightness fluorescent labels ,e.g. polymers, nanoparticles
  - Raman labels
- Light sources
  - Solid state lasers
  - LEDs
- Detectors
  - Photomultiplier arrays
  - CMOS detectors
- Fluidics
  - Microfluidic channels for manipulating particles
- Computing
  - Fast multi-parallel processing

The Future Of:

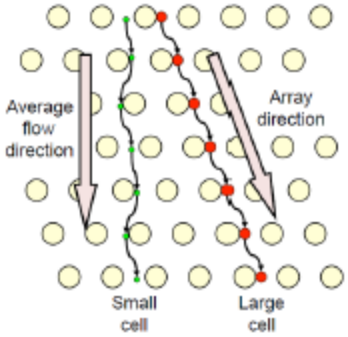
- Sample Handling and Preparation
- Instrumentation including Calibration
- Cell Sorting
- Reagents
- Software and Algorithms
- Systems

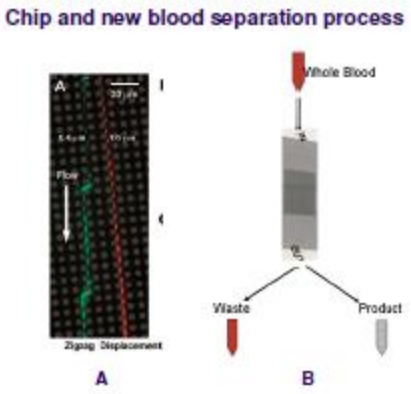
Particle Control for Sample Handling

- Acoustic Forces e.g. UNM, Lund U, …
- Mechanical Forces e.g. Aviva filters
- Photon Pressure
- Dielectric Forces
- Hydrodynamic forces e.g. Princeton, UCLA
- …

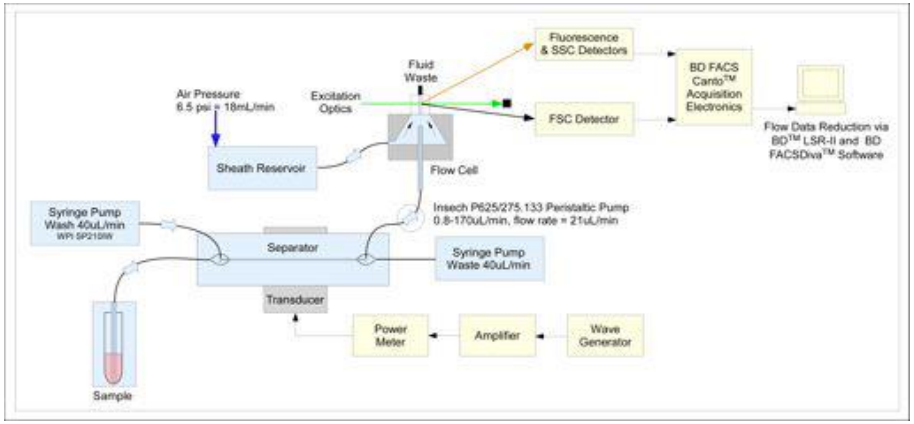
Innovative Sample Preparation

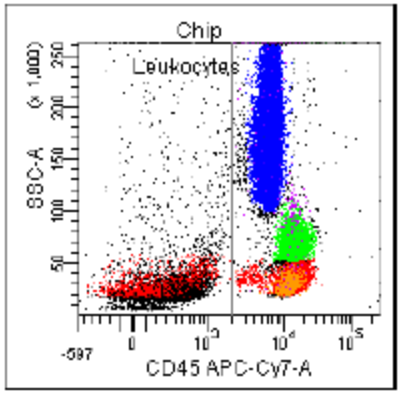
Microfluidic system for leukocyte isolation (deterministic lateral displacement)

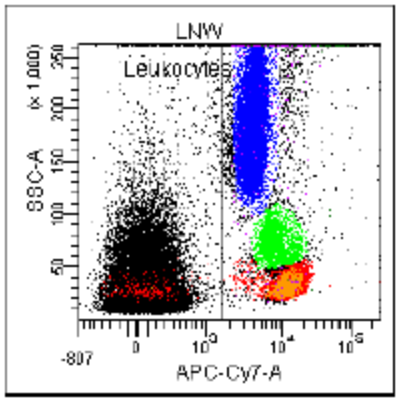




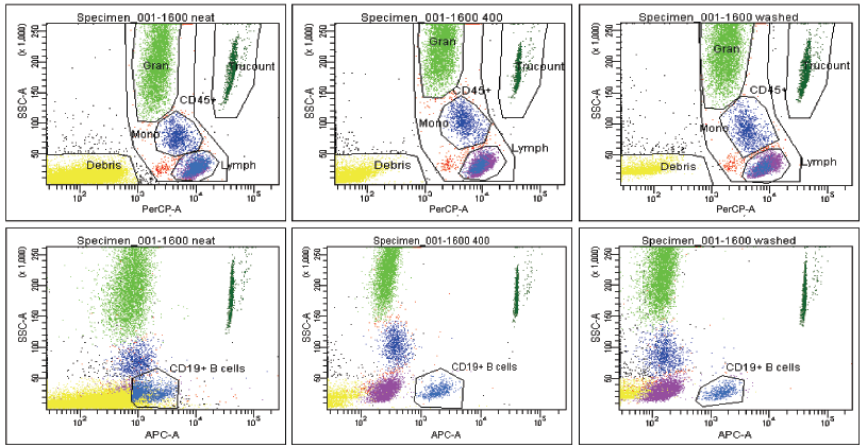
Acoustic particle focusing for cell washing







Lyse no wash | Chip wash | Centr. wash



Cyto 2012 poster, Liping Yu et al, GPB and BD Biosciences

2010, Laurell group, Lund University & Brian Warner, BD Biosciences

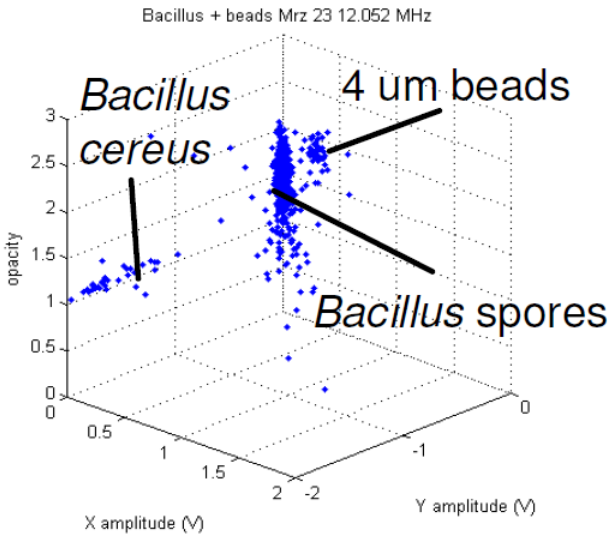
Instrumentation including Calibration

- Spectral Analysis e.g. SONY
- Raman Labels
- Label-free Analysis
- High speed imaging in flow
- Single molecule sensitivity
- Universal Setup e.g. BD FACSVerse™

- …

Label-free Cell Analysis

LEISTER : Axetries Impedance flow cytometry



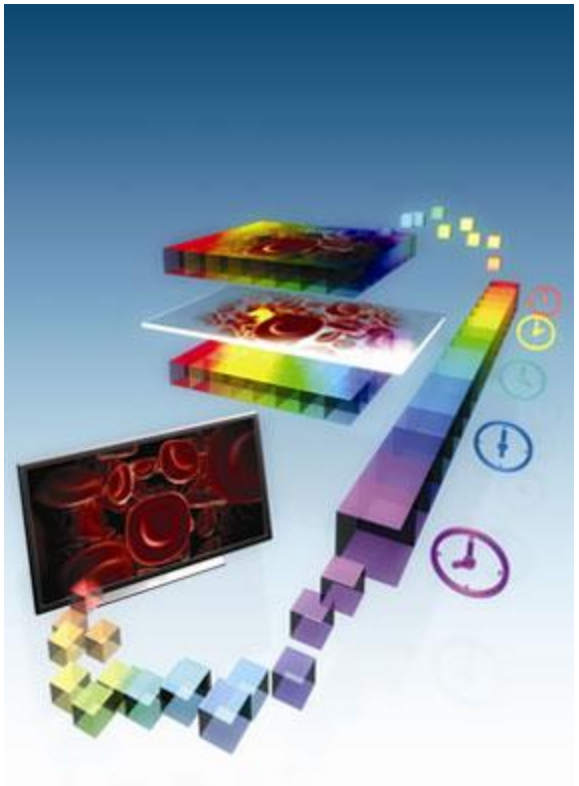
Marco DiBeradino, Leister Axetris

Electrical parameters of living cells (no label required).

Other parameters: fluorescence polarization, fluorescence lifetime, compressibility, …

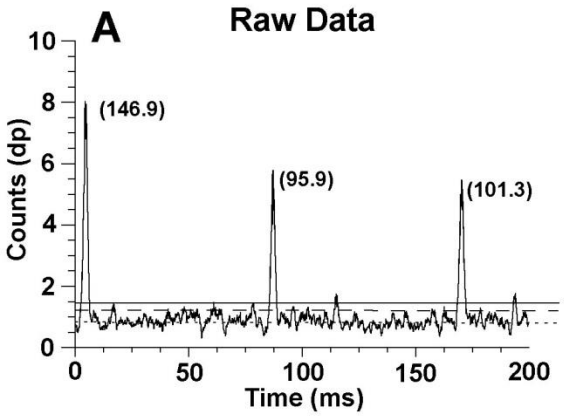
High speed imaging in flow

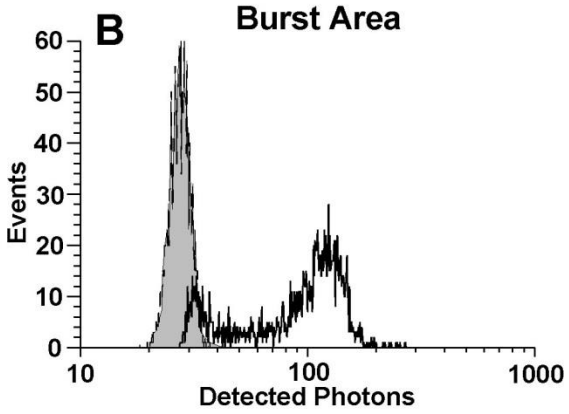
- ImageStream (EM Merck)
- Bahram Jalali group, UCLA
- …

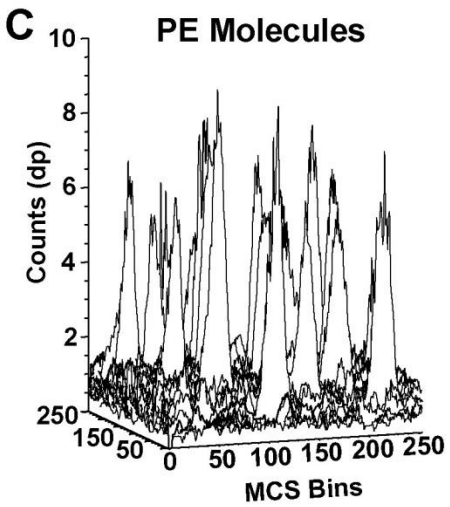


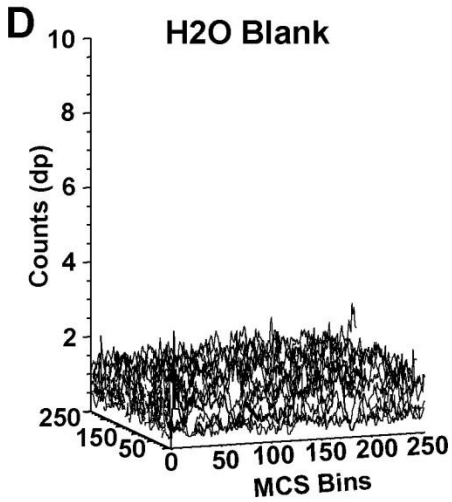
http://www1.ee.ucla.edu/Research-highlights-jalali-4.htm

Single molecule sensitivity with a special flow cytometer









A: 200 ms corrected data showing 3 molecules of B-PE

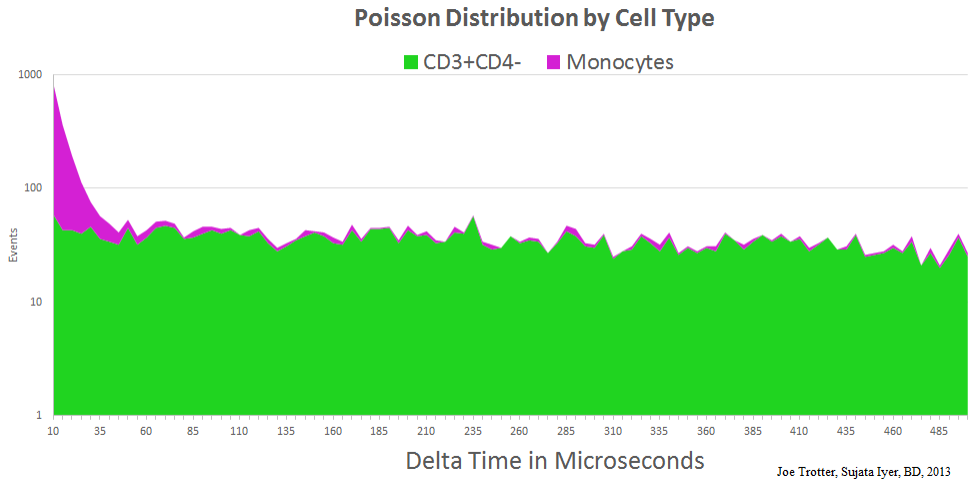
B: 2645 photon burst areas (background-grey)

C,D: each 256 bin (row) = 25.6 ms data. **C** is B-PE showing single molecules. **D** is H_20 control

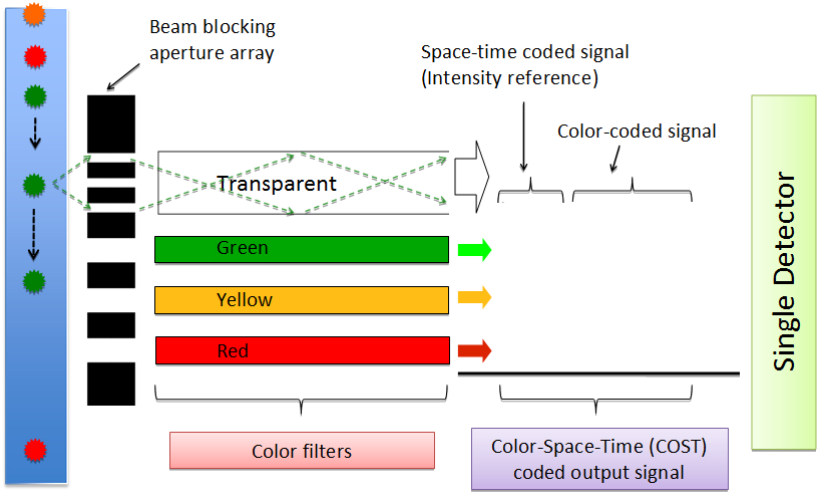
(Rob Habbersett & Jim Jett, LANL)

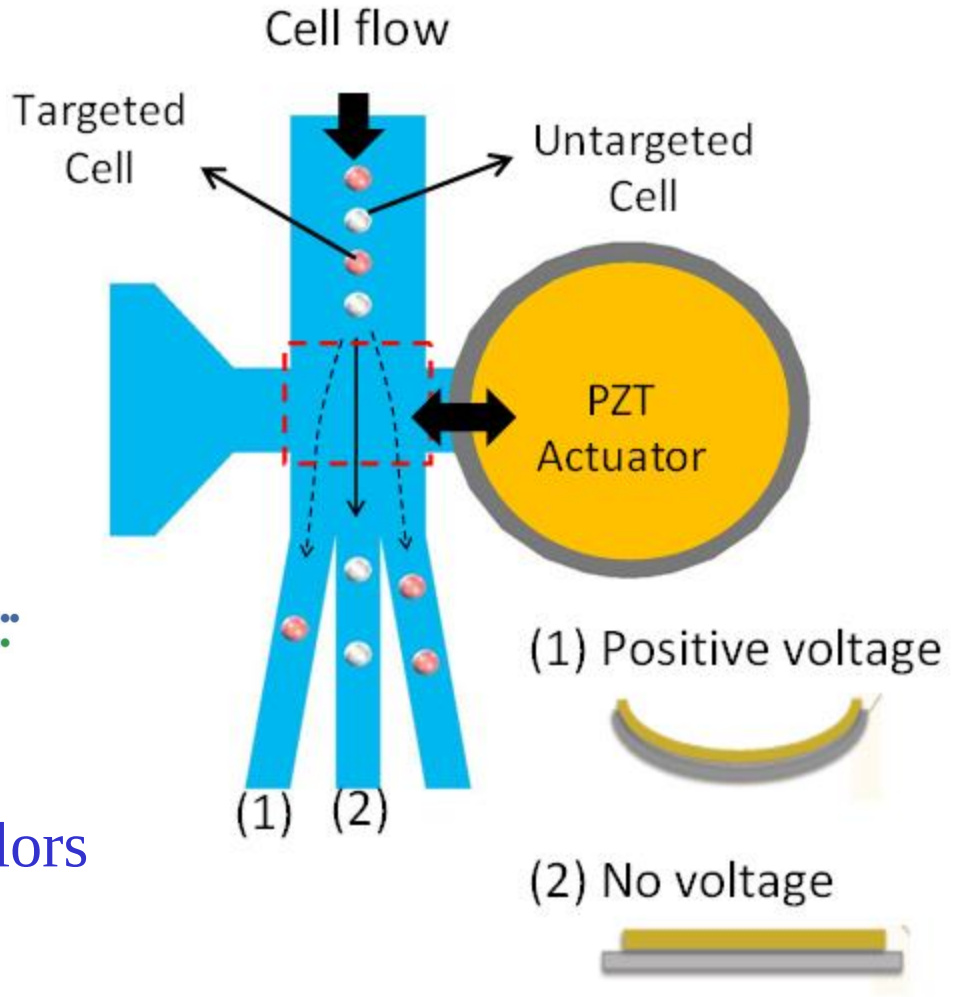
Cell Sorting

- Optimized position control in droplets
- Specialized microfluidics sorters
- New sorting technologies e.g. OWL
- . . .



Microfluidic Analyzer/Sorter





nanocellect
Biomedical, Inc.

- microfluidics fabrication
- single detector for multiple colors
- in-channel cell deflection

Reagents

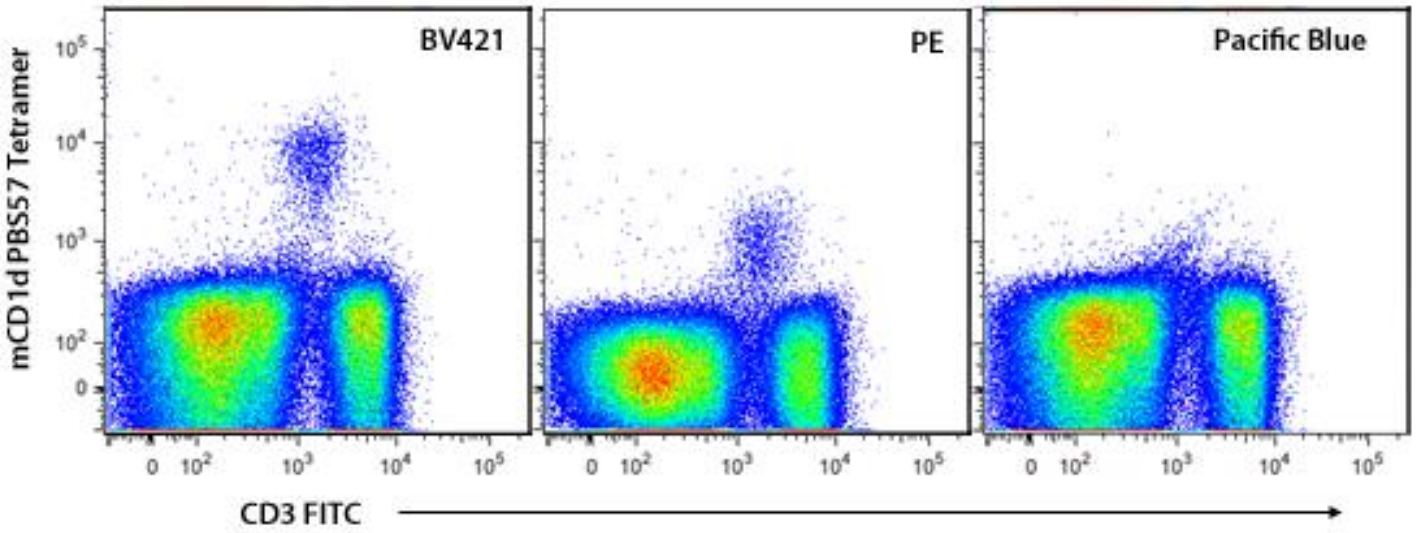
- Advances in affinity reagents
- New amplification methods for single molecule sensitivity
- More and brighter polymer and nano-particle dyes
- Concentration measurments by molecule counting

Novel Affinity Reagents

- Antibodies
  - Antibodies from different species (e.g. Llama 15 kDalton fragments with 10^{-9}M Kd and high stability, potential for intracllular use)
  - Recombinant antibody fragments
  - …
- Synthetic affinity reagents
  - Aptamers
  - Protein scaffolds
  - Molecular Imprinted Polymers

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

Use of Brighter Labels



http://www.biolegend.com/brilliantviolet

Software and Algorithms/ BioInformatics

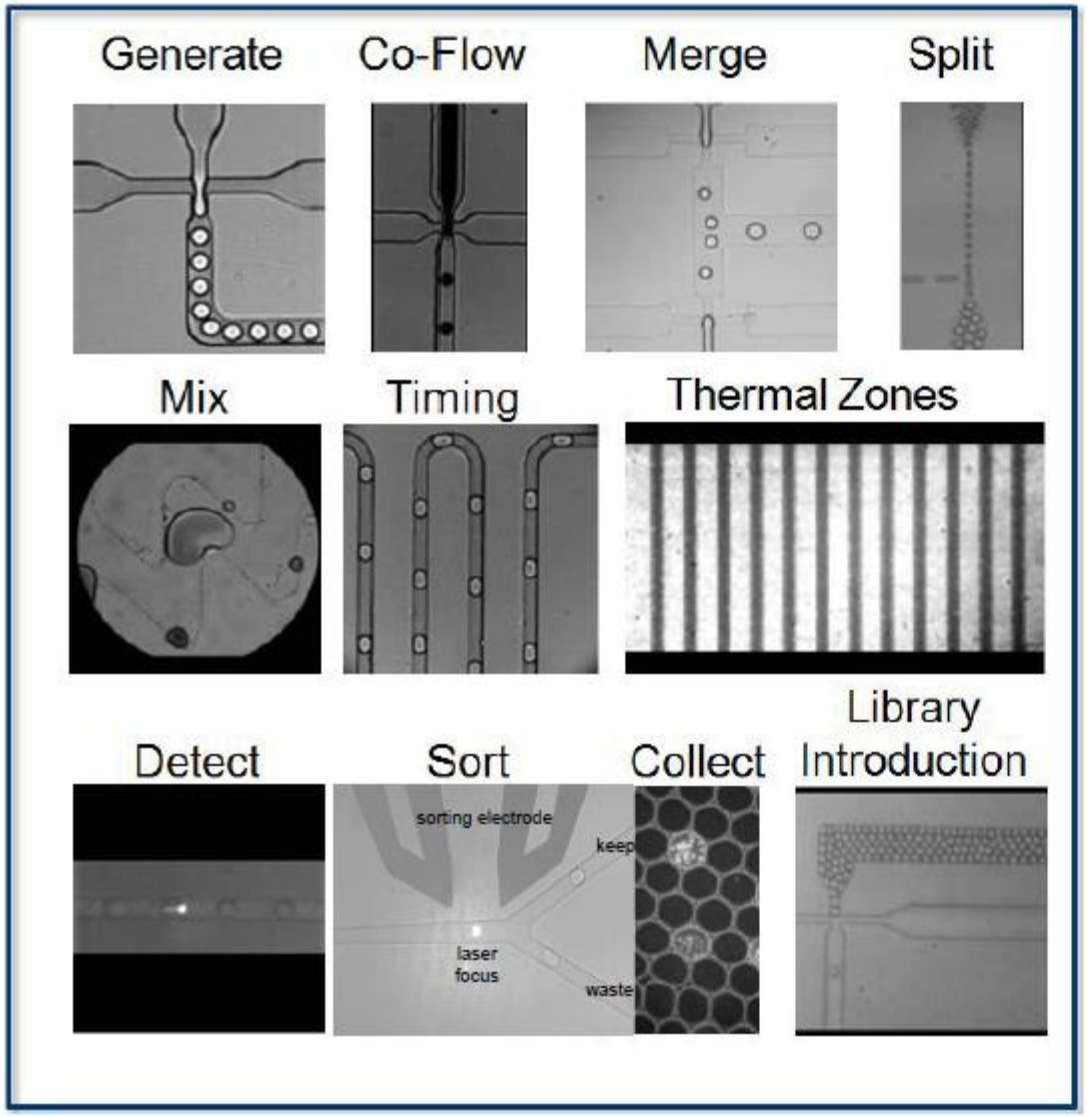
Integration, enhancements, and additions to:

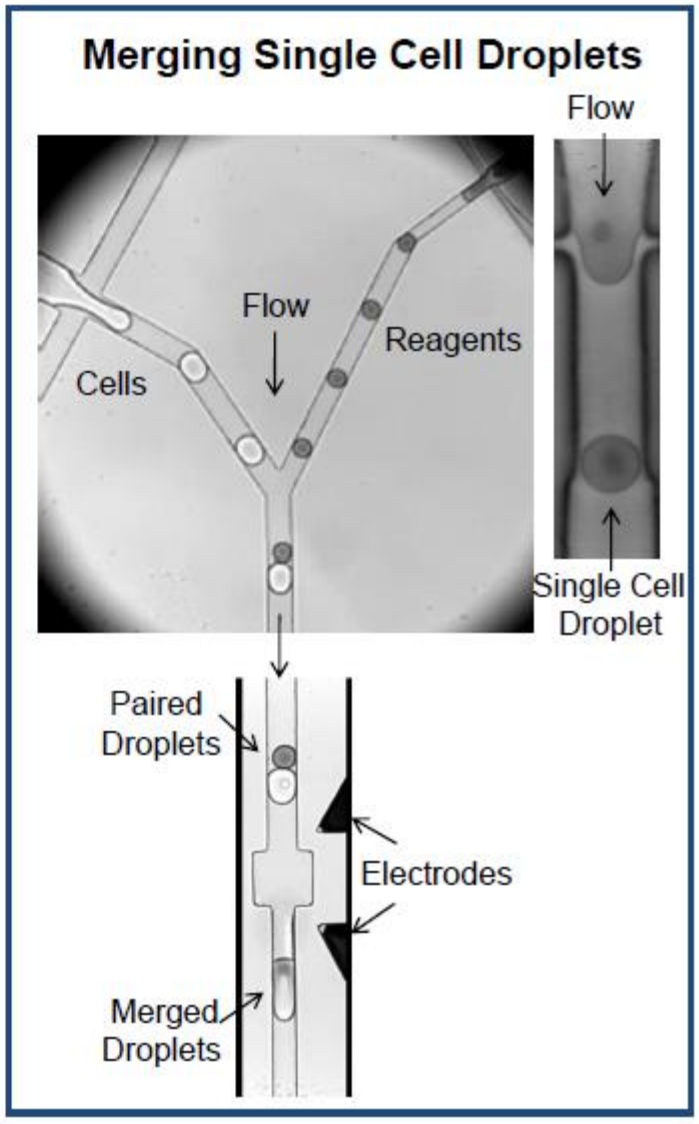
- FLOCK
- Gemstone
- Spades
- Cytobank
- …

Systems

- Fully integrated user-programmable research systems
- Fully automated, pre-programmed, validated clinical systems
- …

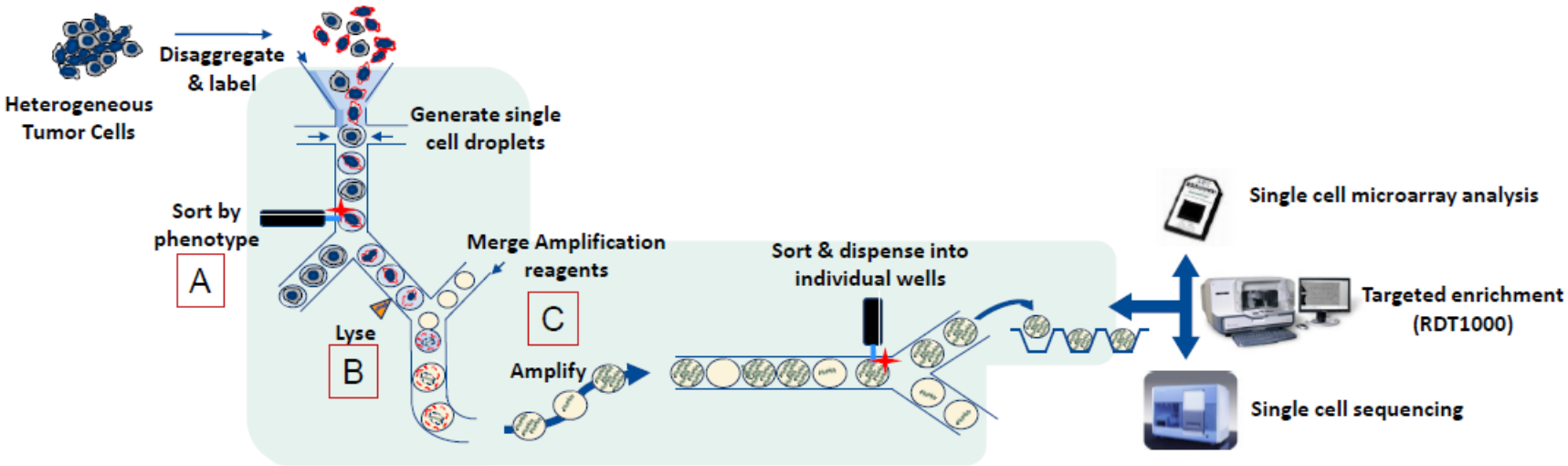
Advanced Single Cell Analysis in Droplets





Source: RainDance Technologies

Fully Integrated Single Cell Analysis



Source: Raindance Technologies

Conclusion

After more than 30 years, cytometry is at the beginning of new era to enable revolutionary discoveries in biology, higher quality in monitoring of biotechnological processes, and better patient care through clinical diagnostics and cellular therapy.

Acknowledgements

- Joe Trotter
- Brian Warner
- Liping Yu
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- Brent Gaylord

all BD

- Bob Hoffman (retired)
- Thomas Laurell (Lund University)
- Janette Phi (now IntelliCyt)
- Ken Davis (retired)
- Bill Godfrey (now Beckmann Coulter)
- Holden Maecker (now Stanford U)
- Collette Rudd (Thermo)
- ...
- BD

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END