Rare Cell Analysis and Sorting by Flow Cytometry

Diether Recktenwald
Independent Professional, dba Desatoya LLC
BD Biosciences, retired
Reno NV 89507
Email: diether.recktenwald@cytometrygroup.org
http://www.desatoya.com

Presentation Outline

- Introduction with list of rare cell applications
- Basic Flow Cytometry
- Points to Consider for Rare Cell Analysis
- Application Examples
- Conclusions
- Key Contributors

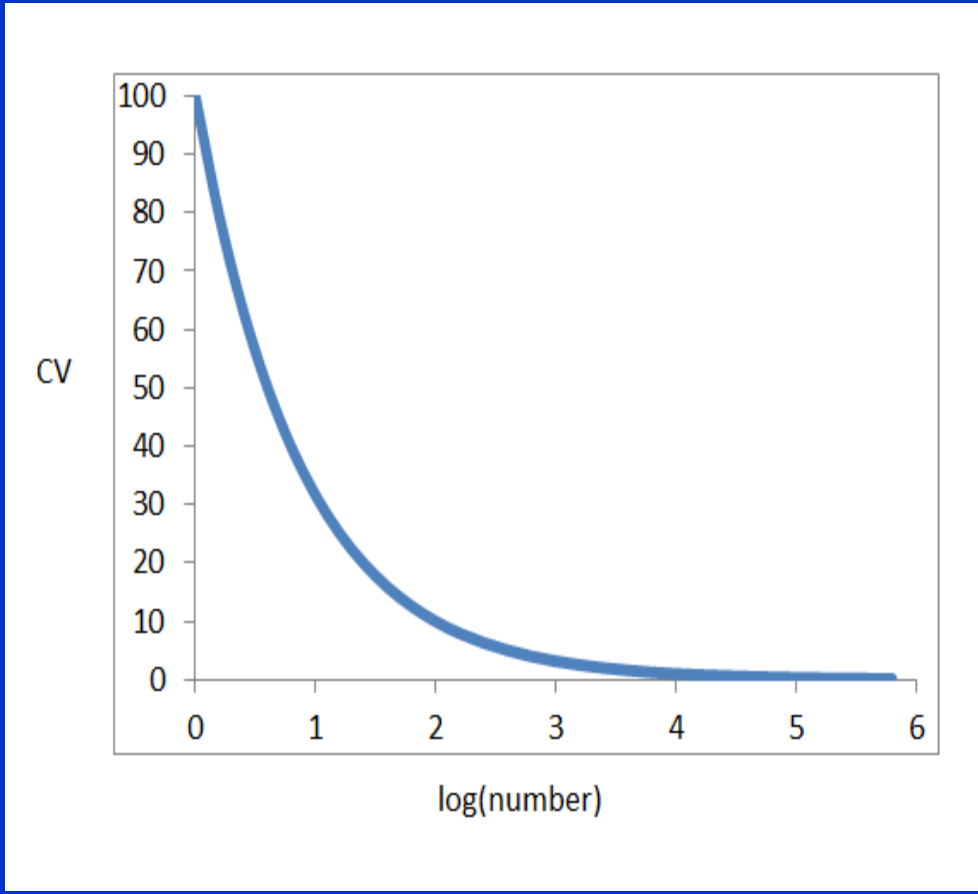
Rare Cell Examples

(<1% of cell sample)

- Epithelial tumor cells, circulating in blood (CTCs)
- Tumor stem cells
- Fetal cells in maternal blood
- Hematopoietic stem cells
- Antigen specific T-cells
- Leukocytes in depleted platelet or erythrocyte preps
- Basophils
- …

Limitations for Accurate and Precise Particle/Cell Counting

- Sampling
- Sample Preparation
- **Counting statistics**



Source : Desatoya LLC

Detection Methods for Rare Cells

- Immunohistochemistry and high speed imaging
- PCR
- **Flow Cytometry**

(rare cell pre-enrichment may be valuable for all approaches, flow cytometry offers an thresholding option, which acts like a pre-enrichment)

Flow Cytometry

Single cell analysis with

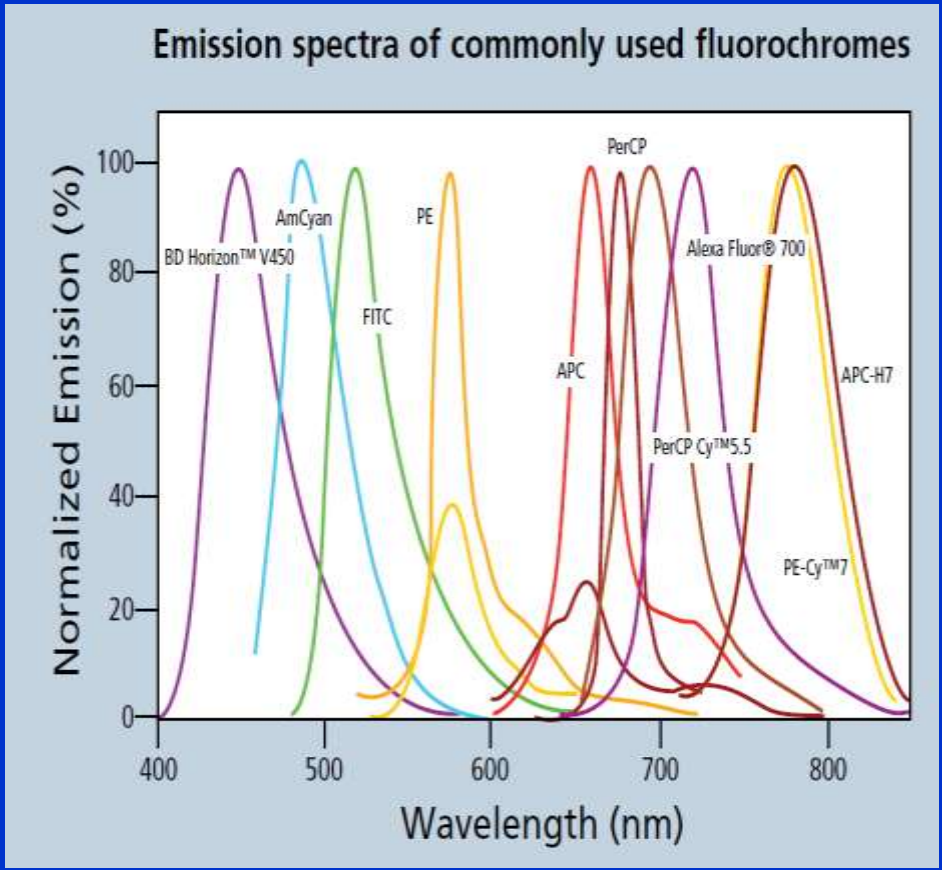
- High sensitivity (single molecule sensitivity by fluorescence)
- Wide dynamic range (100 to 10,000,000 cells mL^{-1})
- High analysis and sorting rates to ~100,000 particles sec^{-1}
- Light scatter for label-free particle analysis and counting
- Multi-color fluorescence, multi-parameter analysis
- Live/dead discrimination
- Recovery of viable cells possible (sorting)
- Ease-of-use for the analysis of cell/particle suspensions

History of 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
- 2003+ many microfluidic flow cytometers and sorters

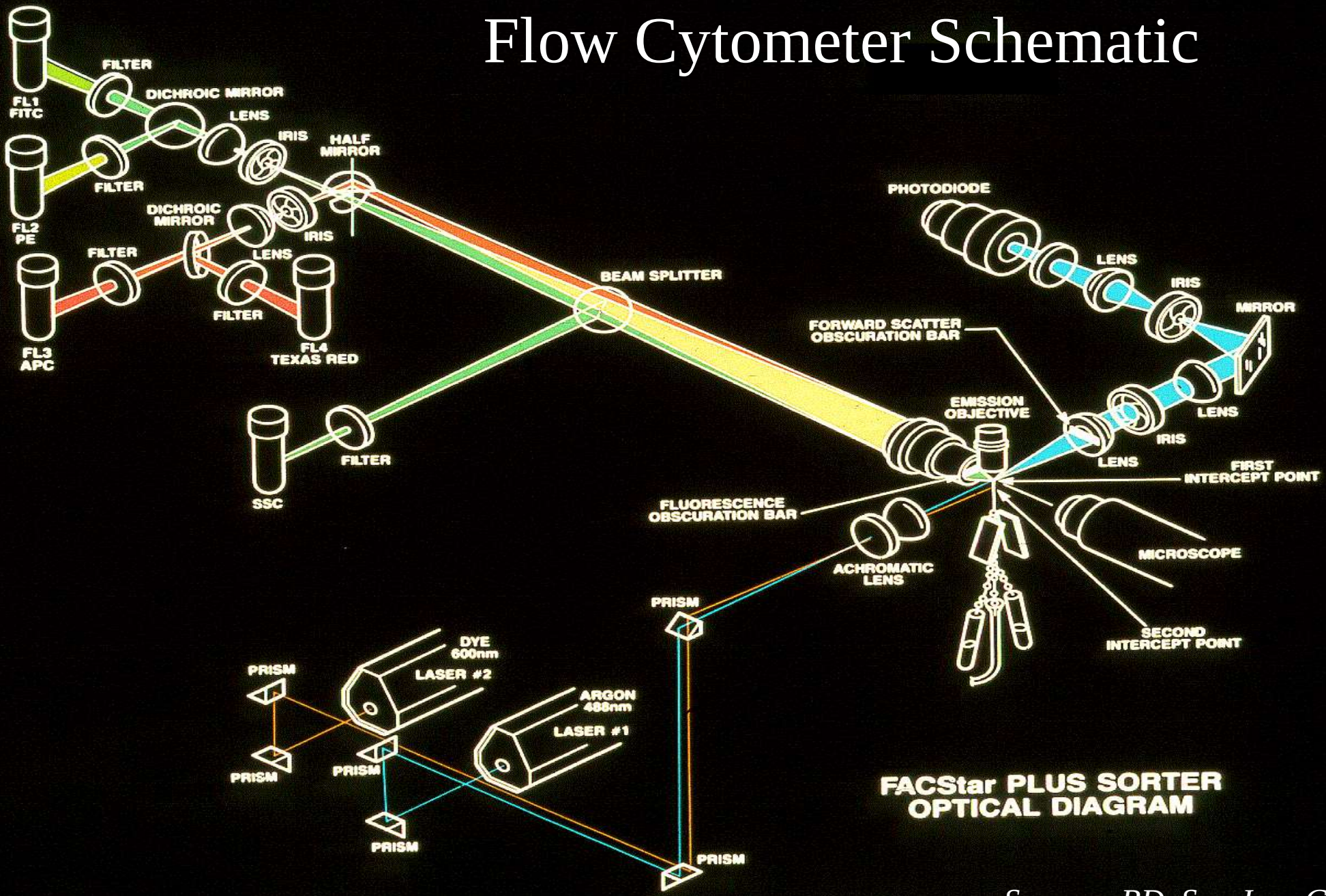
Physical Parameters for Cytometry

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



BD_lsrfortessa_brochure.pdf

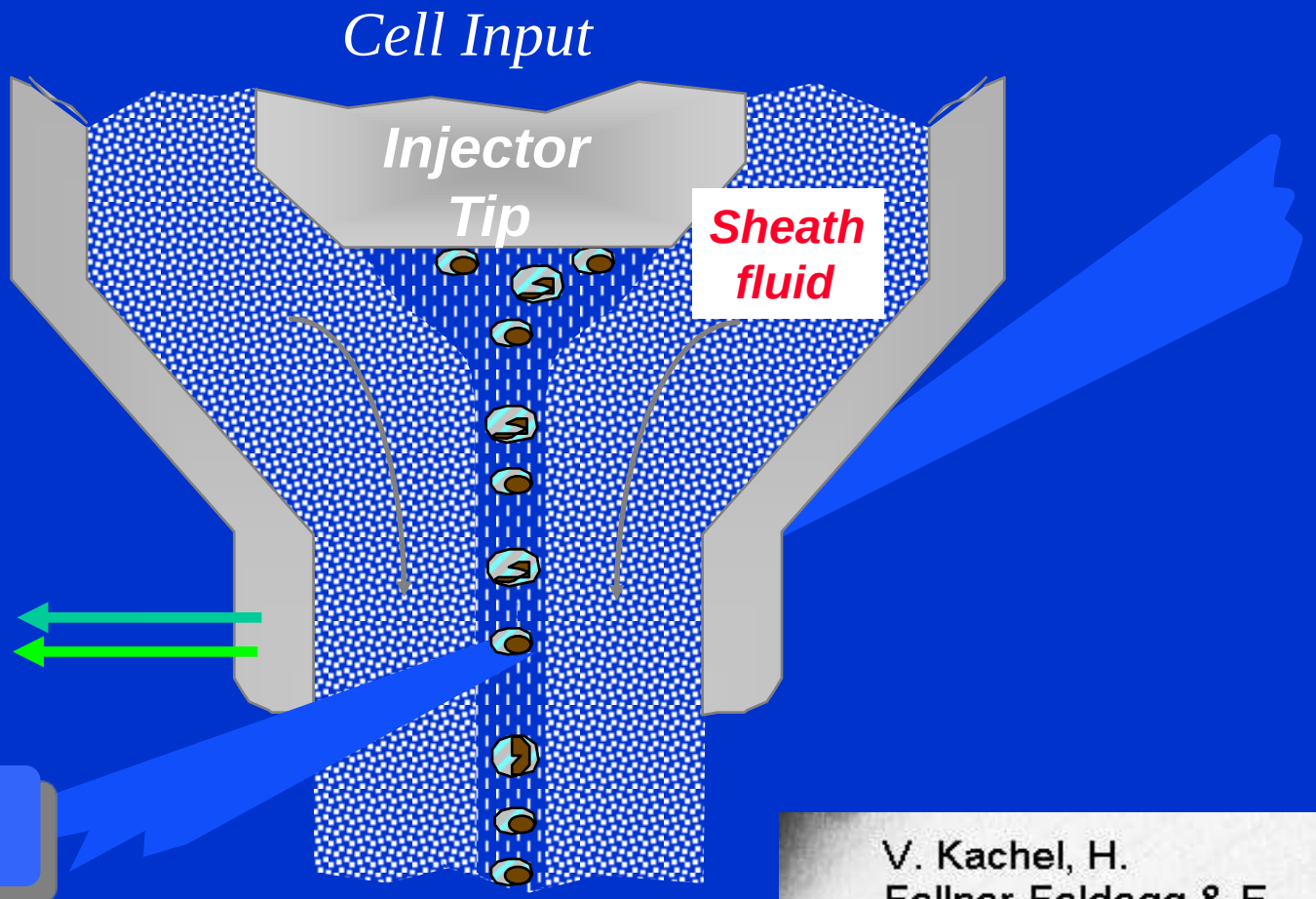
Flow Cytometer Schematic



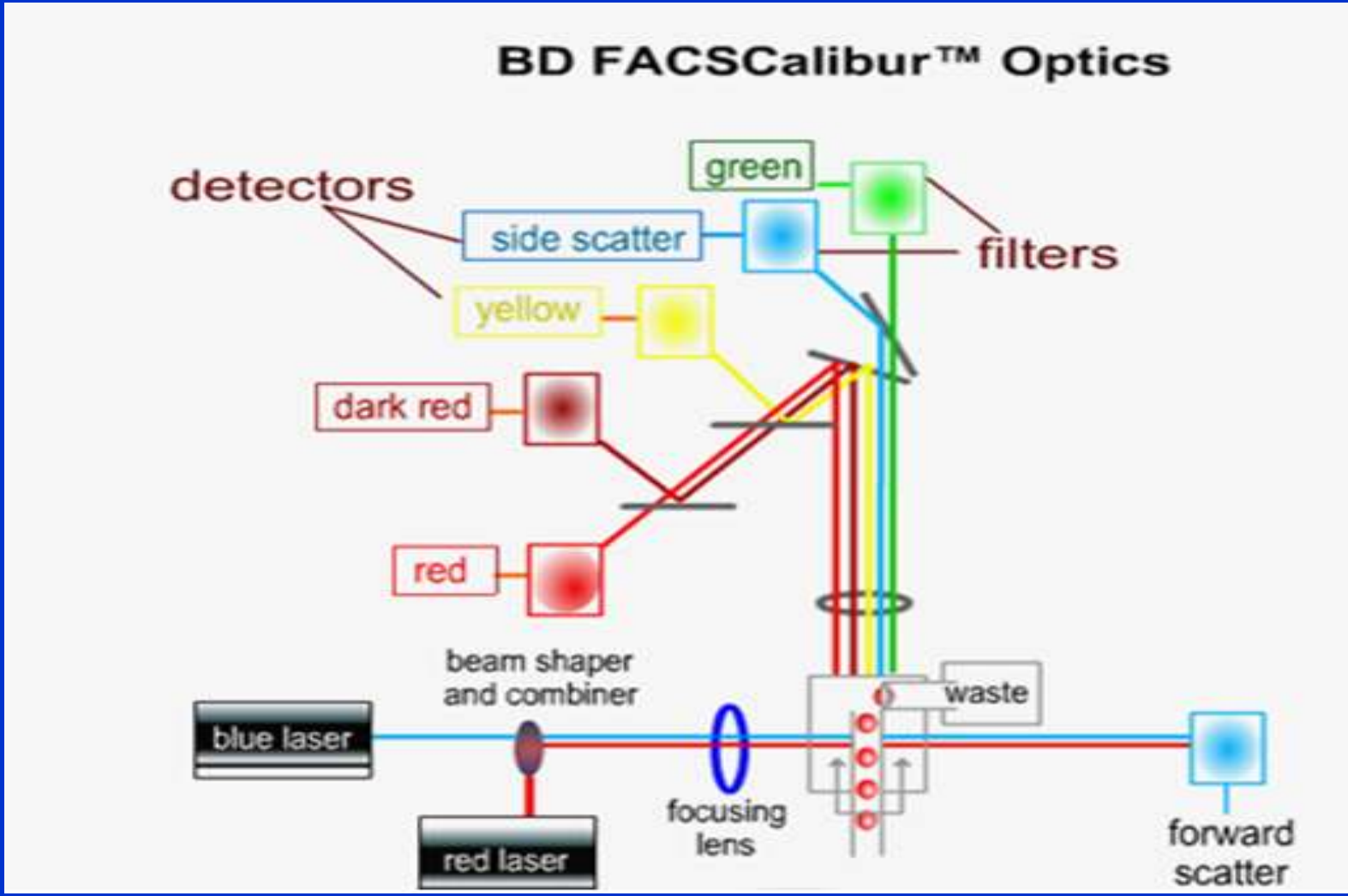
Source : BD, San Jose CA

Flow Cytometer Fluidics

Hydrodynamic or acoustic focusing

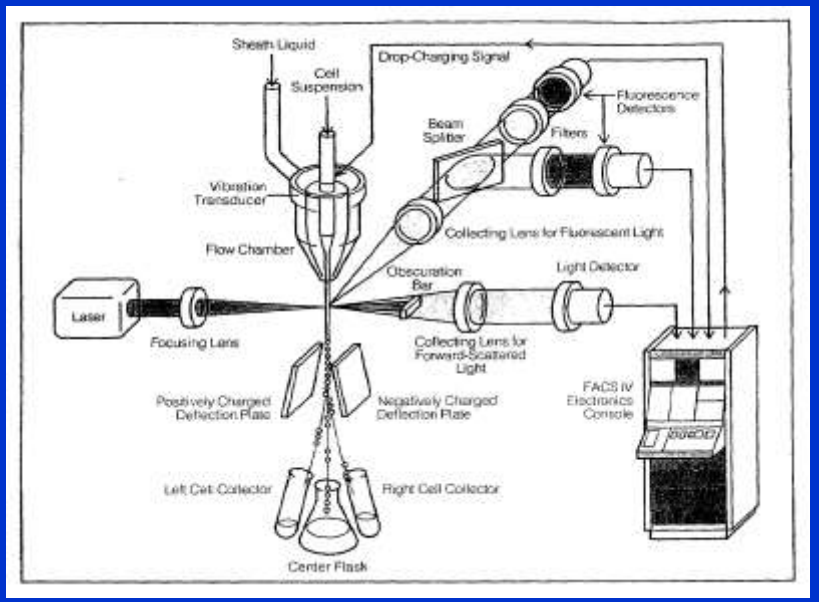


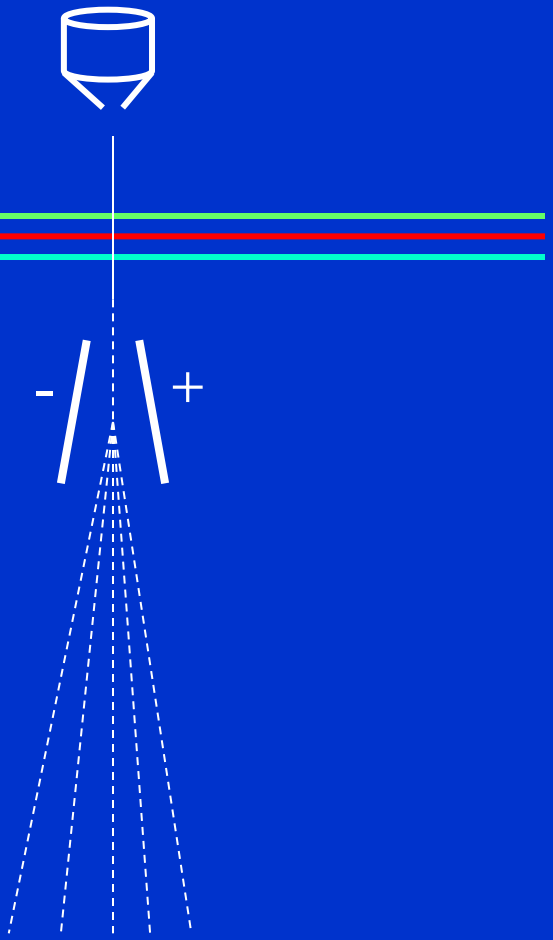
Flow Cytometer Optical Systems



Source : BD, San Jose CA

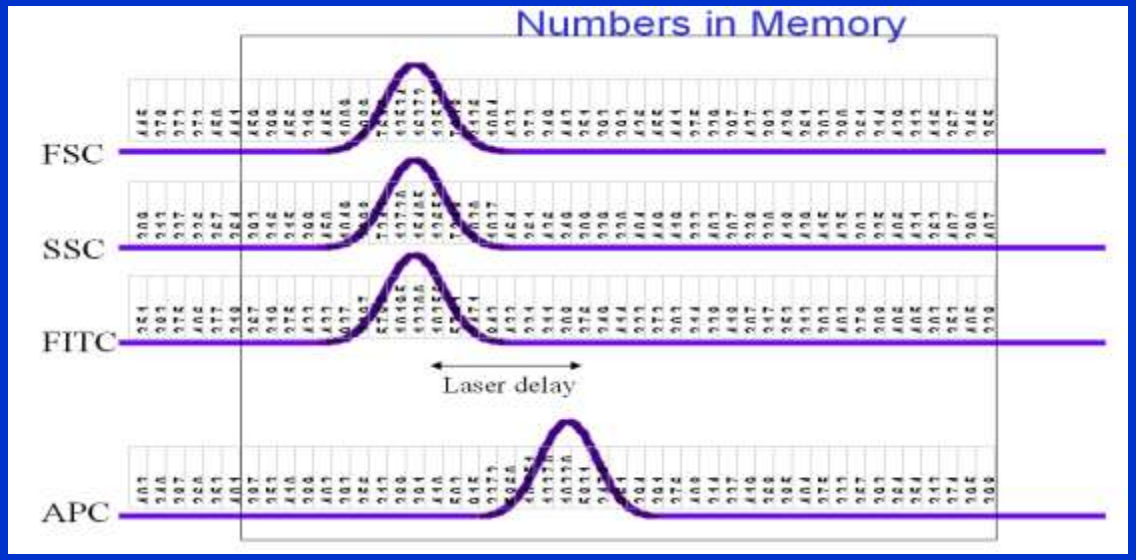
“Droplet-based” Sorting



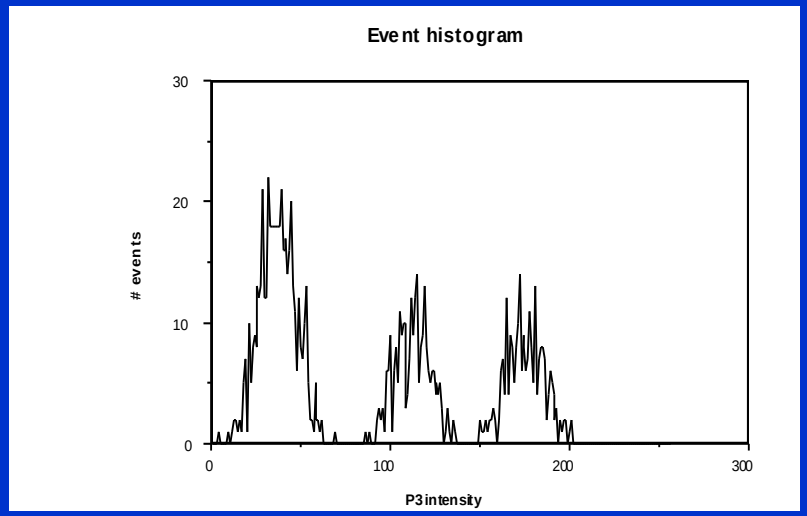


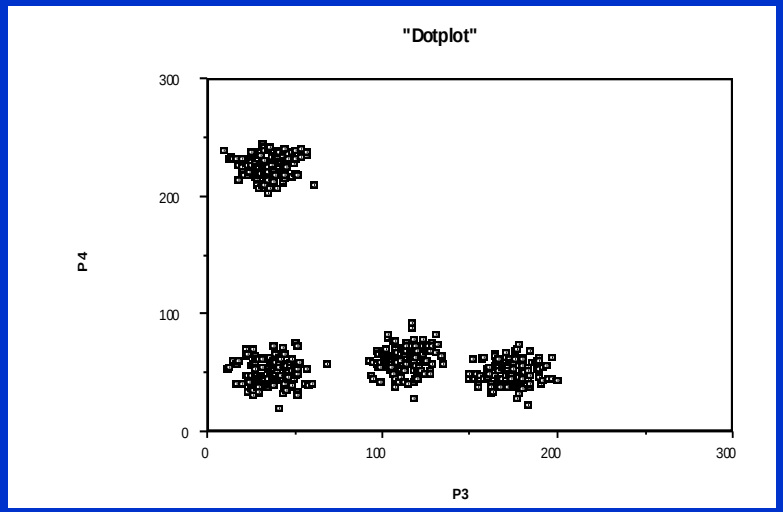
Source : BD, San Jose CA

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

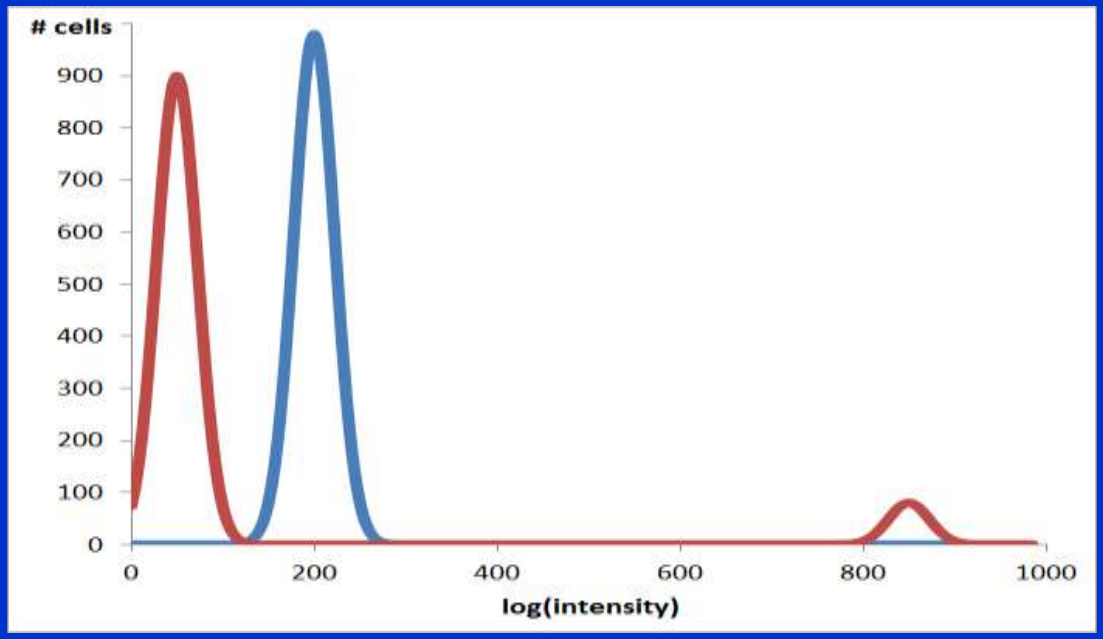




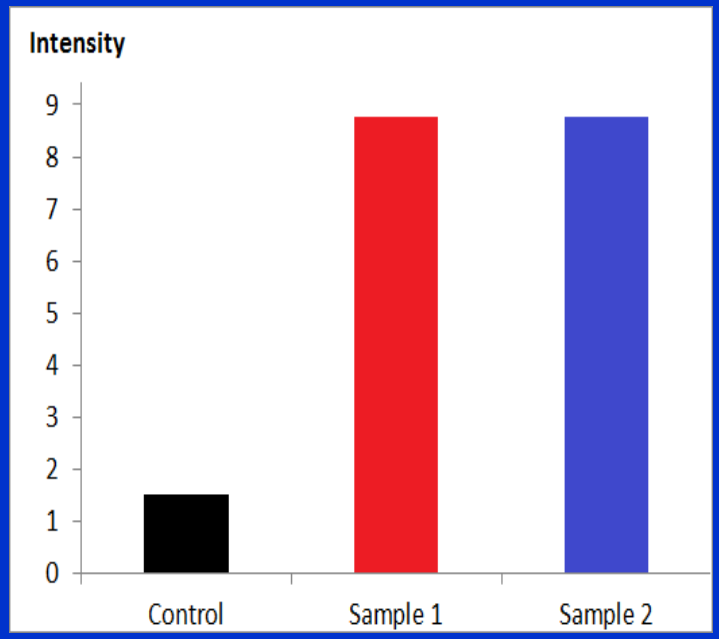
Source : BD, San Jose CA

Why Single Cell Analysis

Intensity Histogram for Single Particles



Intensity per Sample

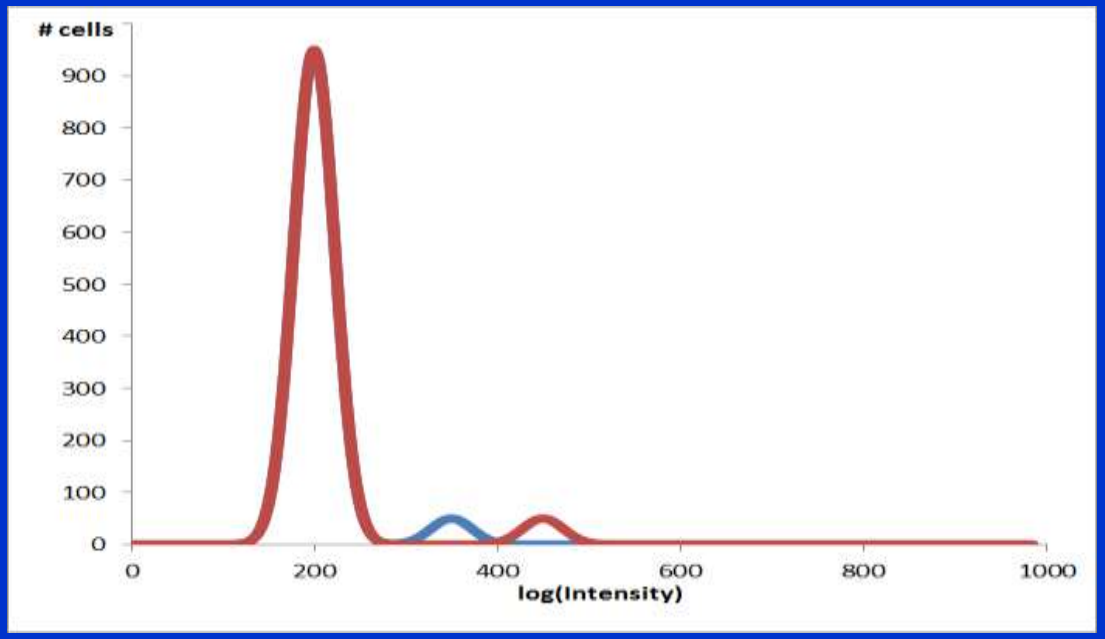


Cell by cell intensity analysis detects population heterogeneity.

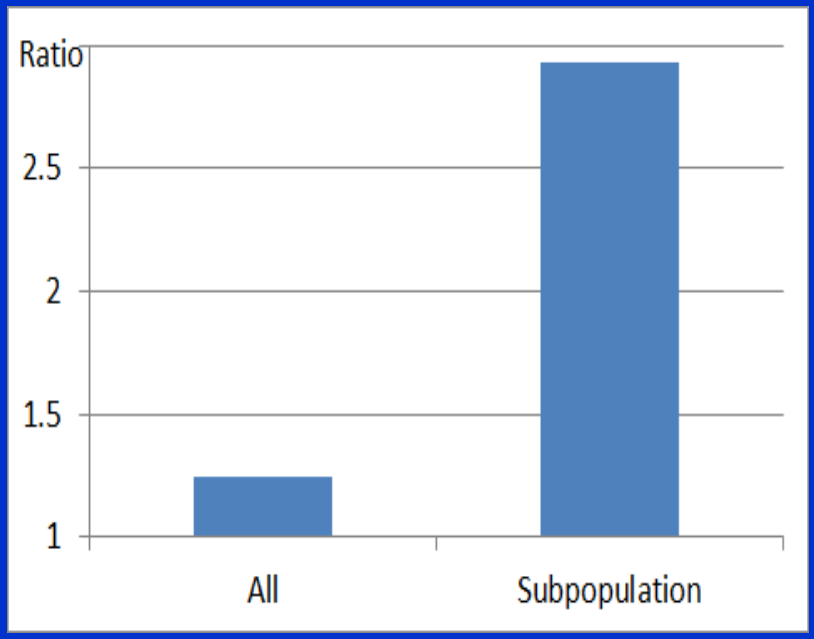
Source : Desatoya LLC

Benefits of Subset Analysis

Intensity Histogram



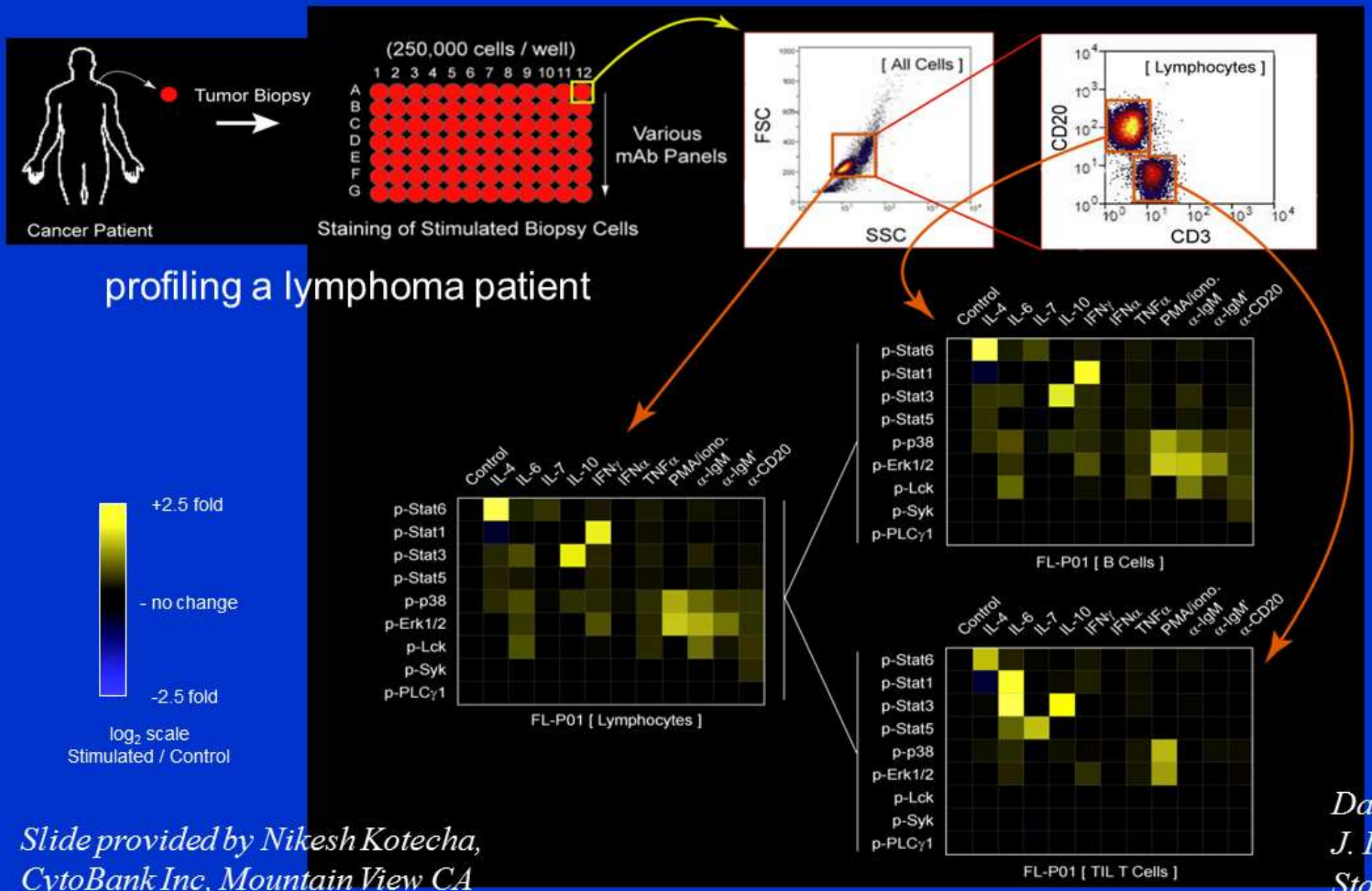
Intensity Ratios



Subpopulation analysis detects changes better, especially for rare subpopulations.

Source : Desatoya LLC

Subset Analysis Example



Slide provided by Nikesh Kotecha, CytoBank Inc, Mountain View CA

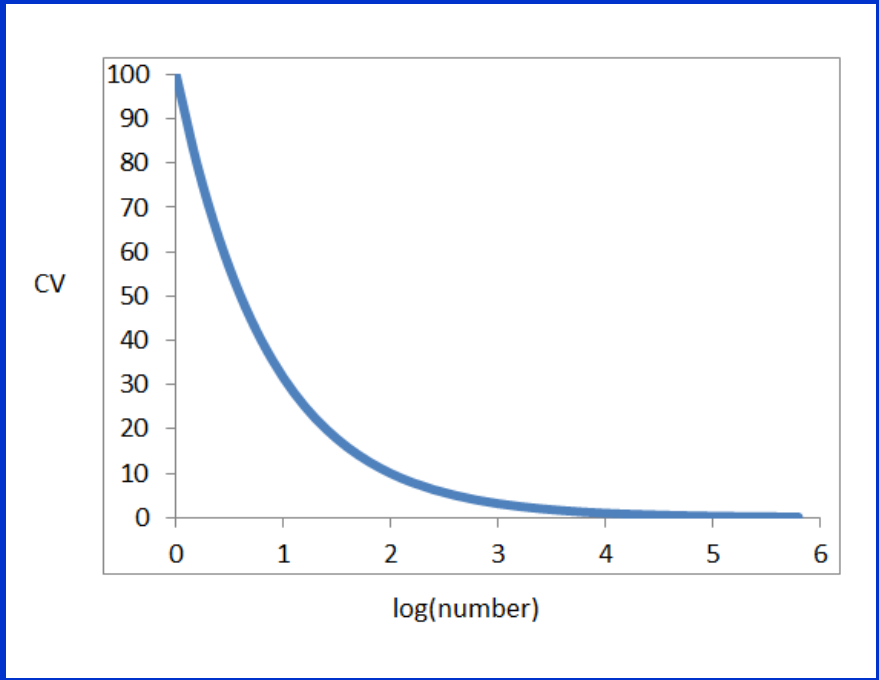
Data: J. Irish, Stanford

Optimizing Flow Cytometry for Rare Cell Analysis and Sorting

- Counting statistics
- Specific Cell Markers
  - Brightness
  - Combinations
- Instrument System Background
  - Carryover
  - System noise
- Raw Data Analysis
- High yield, high purity sorting
  - Pre-enrichment e.g. enrichment sort 1st
- …

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

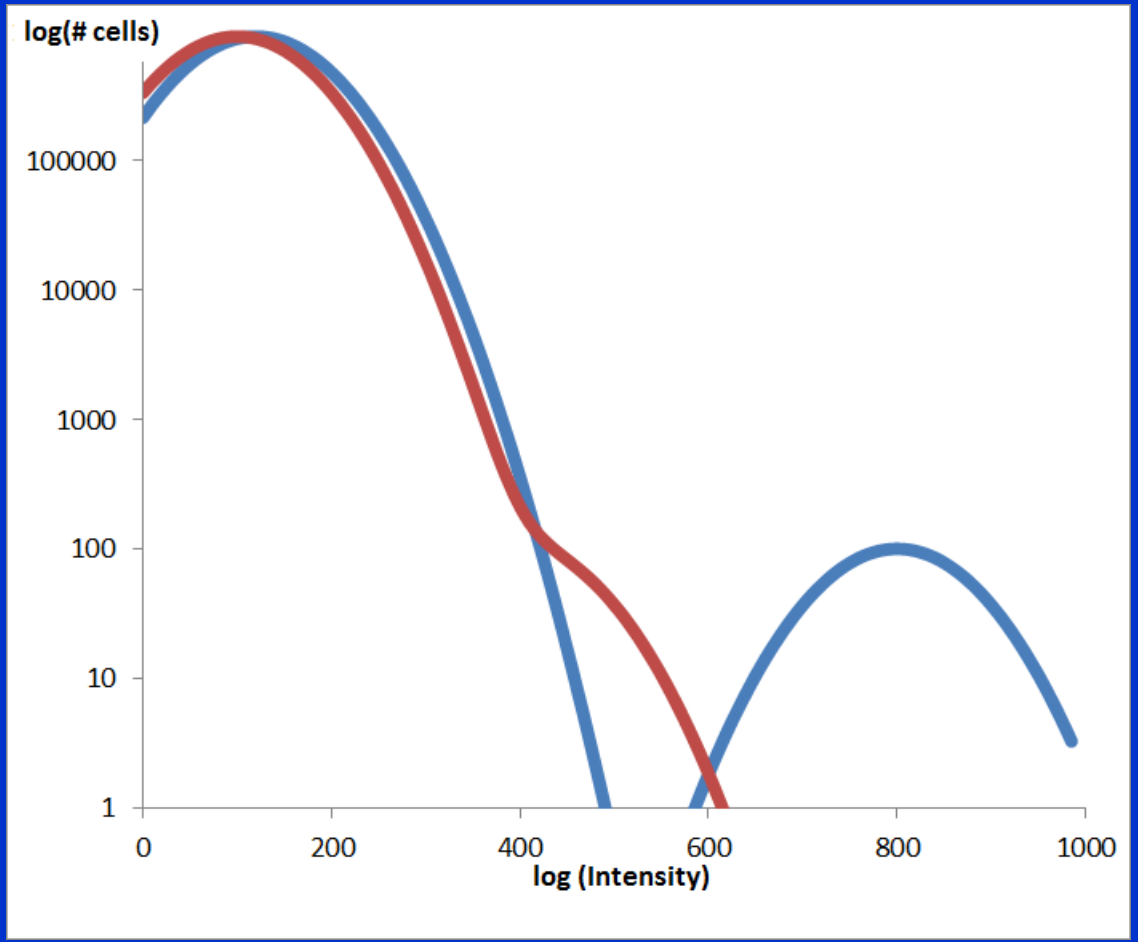


Ignoring Counting Statistics Can Lead to Erroneous Conclusions

Source : Desatoya LLC

Marker Brightness

Brighter markers resolve rare populations better



Source : Desatoya LLC

Marker Combinations

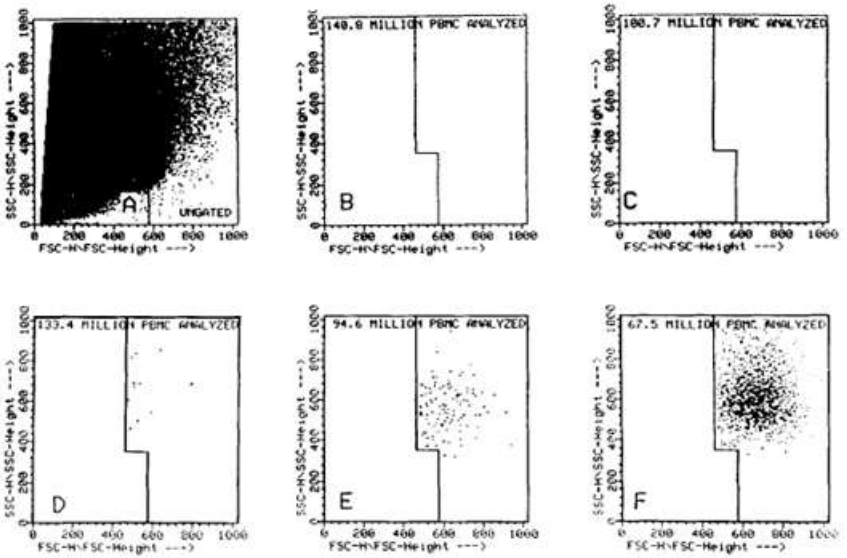


FIG. 3. Results from three-color rare event analysis. **A.** The isotype control (anti-keyhole limpit hemocyanine PE) without REH cells, ungated. **B.** The CD10 PE control, without sorted REH cells. These runs were used to determined the regions R1, R3, and R5 (Fig. 2). **C.** As A, but now gated on regions R1, R3, and R5. **D.** Gated data of PBMCs, frequency REH cells 10^{-6}. **E.** Gated data of PBMCs, frequency REH cells 10^{-5}. **F.** Gated data of PBMCs, frequency REH cells 10^{-4}.

Gross HJ et al, Cytometry 14, 519ff

Background Levels

-	
++ no exc	10^{-4}
+ exc	10^{-6}
++ exc	10^{-8}

CTC Gate

FSC & SSC	cell size
Fl1 & Fl2	CTC specific
Fl3	exclusion markers

Data: Gross HJ et al, Cytometry

An optimized combination of markers, including an exclusion marker decreases background substantially.

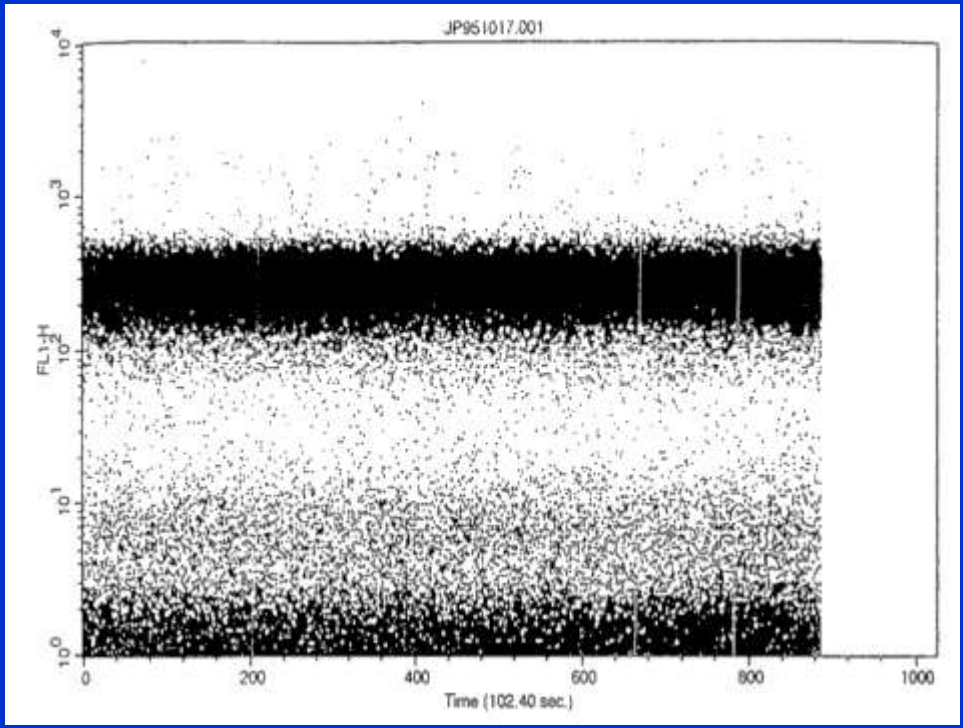
Particle Carry-Over

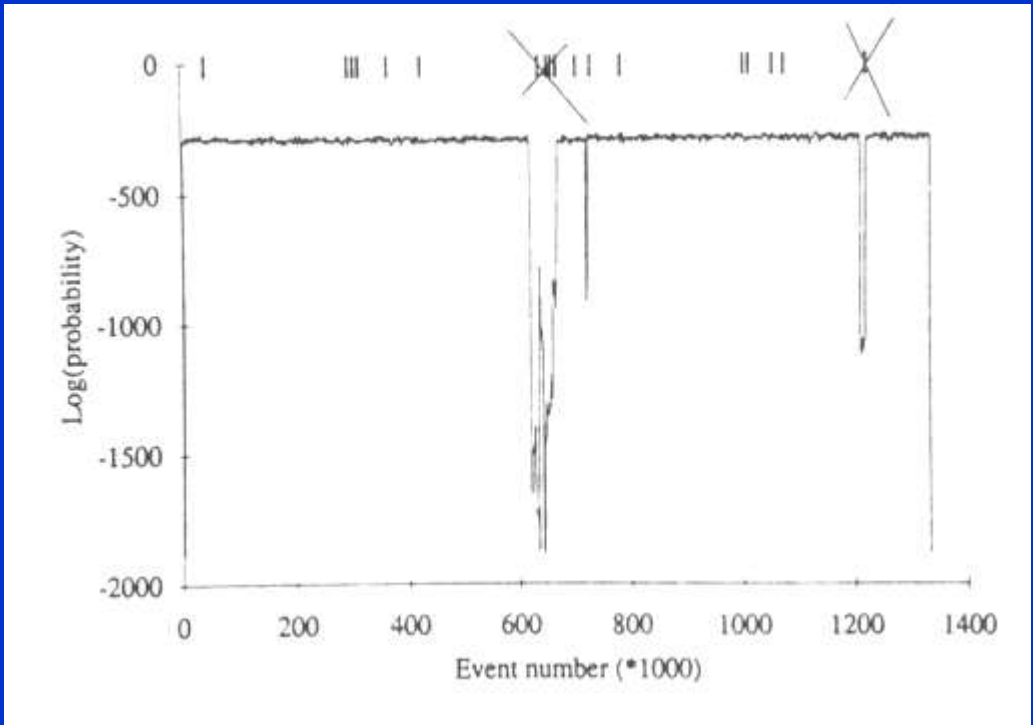
Carry-over specifications		
Beckman Coulter FC500	<1%	
Beckman Coulter	<0.1%	
BD FACSCanto II	<0.1%	loader <1%
BD FACSVerse	<0.5%	
Guava EasyCyte	<0.2%	
Life Technologies Attune		loader <0.5%
Miltenyi MACSQuant	<0.01%	

Sources : Desatoya LLC

Eliminating fluidic system particle carry-over is vital for reliable rare cell analysis.

System Noise

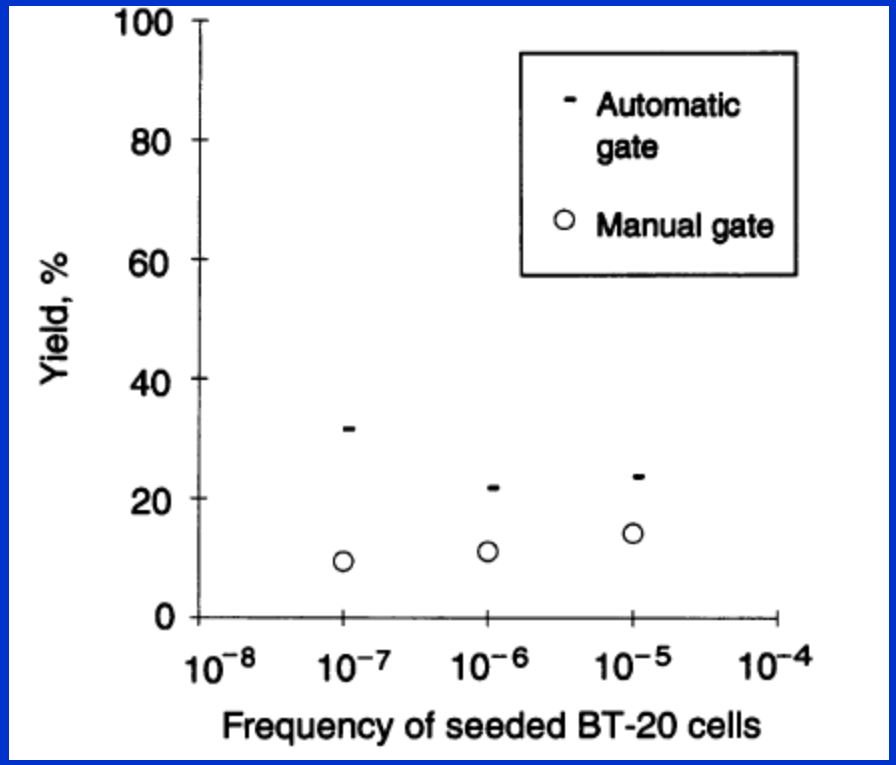




Gross HJ et al, Cytometry

Eliminating system noise from fluidics perturbations and other sources improves the limit of detection for rare cell analysis.

Raw Data Analysis



A "genetic algorithm" finds more rare cells than manual analysis by an expert (30% vs. 10% yield at low frequencies).

Data: Gross HJ et al, PNAS

Computer algorithms allow better raw data extraction for multi-parameter rare cell analysis.

High yield, high purity sorting

Rare CTC sorting from peripheral blood leukocytes

	Target fraction	Yield	Time [min]
Original sample	1.00E-06		
Enrichment	0.20	0.90	30
Purification	0.98	0.70	10

Source : Desatoya LLC calculation

Useful sorting Guidelines:

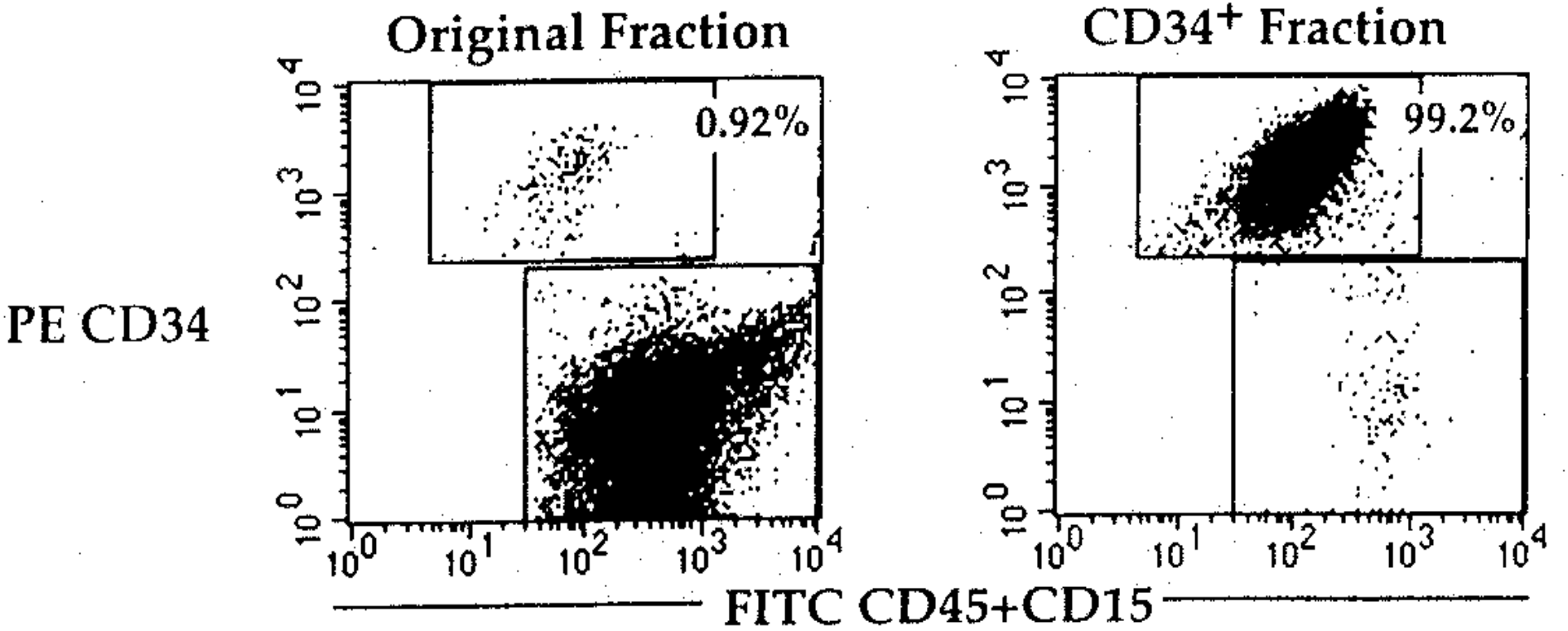
http://hcc.musc.edu/research/resources/flowcytometry/FACSAria%20Guidelines%20070208.pdf

Pre-enrichment helps sample throughput, for sorting.

Example Data for Rare Cell Applications

- Leukocyte-subset analysis without erythrocyte lysis
- Counting of hematopoietic progenitor cells
- Measuring Basophil Activation
- CTC isolation for gene expression analysis

Counting of hematopoietic progenitor cells



Aaron B. Kantor, Ian Gibbons, Stefan Miltenyi, and Jürgen Schmitz Magnetic Cell Sorting with Colloidal Superparamagnetic Particles. in: Diether Recktenwald, and Andreas Radbruch edts.;Cell Separation Methods and Applications; Marcel Dekker Inc., New York, 1997

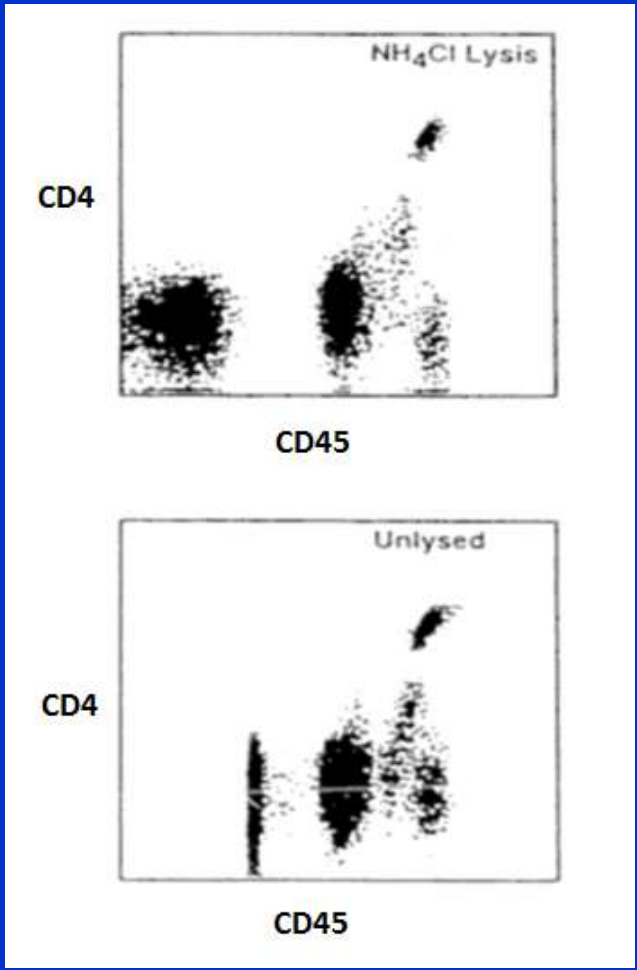
CTC isolation for genomic analysis

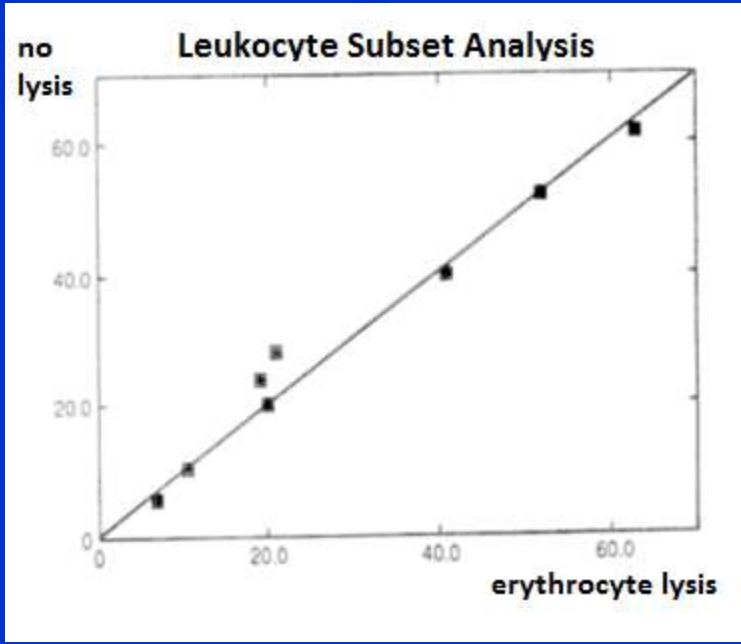
1. Cancer patient blood 5 – 20 mL
2. Immunomagnetic enrichment of epithelial cells
3. Flow cytometric analysis and sorting, based on nuclear stain, epithelial marker, and leukocyte exclusion marker
4. Genomic analysis on gene arrays after whole genome amplification

Result: Genomic alterations in CTCs from prostate cancer were detected. Removal of non-CTC cells is essential for the analysis.

Source : Magbanua M G M et al (2012), BMC Cancer 12: 78ff

Leukocyte-subset analysis without erythrocyte lysis





- increase of analysis rate with a fluorescence threshold
- good correlation between lysis and no-lyse method

Source : BD, San Jose CA

Measuring Basophil Activation

Basophils are activated by stimulating with Anti-IgE or fMLP which cross-link the IgE receptors on the basophil cell membranes

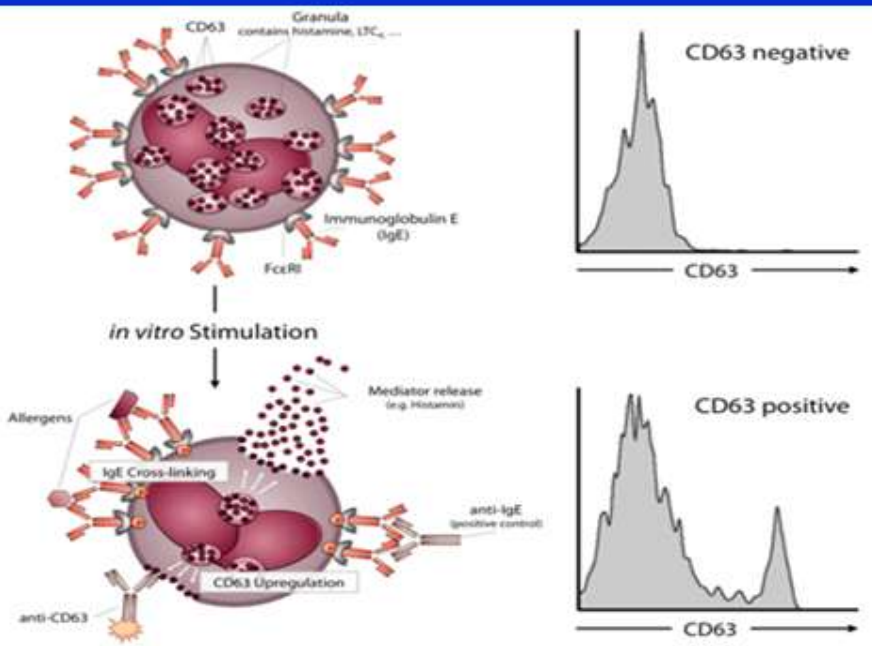
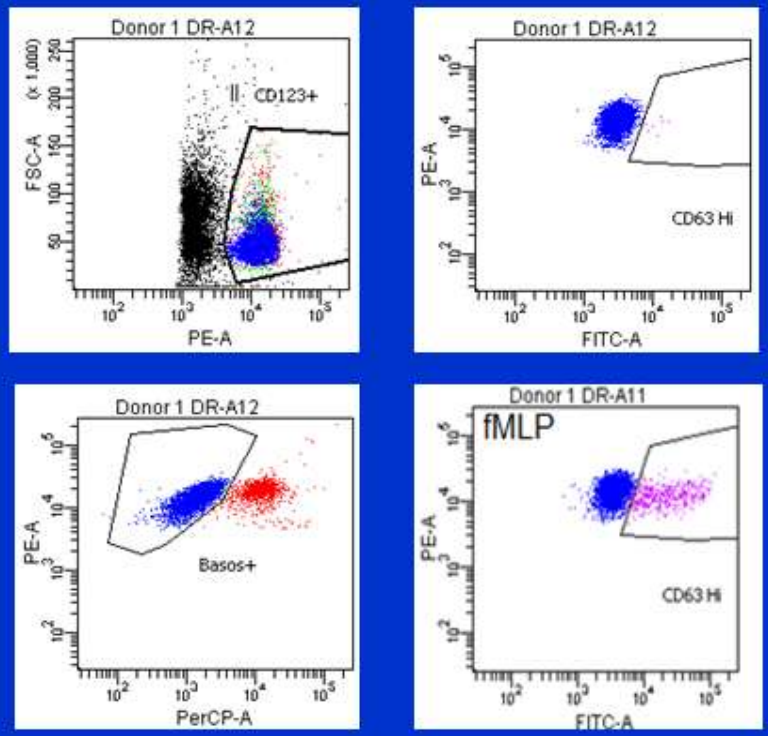


Figure 1. Principle of the basophil activation test.
Upon cross-linking of membrane-bound IgE, basophils upregulate the expression of specific activation markers such as CD63. These phenotypic alterations can be acquired by flow cytometry using monoclonal staining antibodies.



CD63 is an activation marker in basophils which is upregulated upon stimulation by IgE . This causes the granules within the basophil to move to the cell membrane and degranulate, releasing several inflammatory chemical mediators including Histamine & IL-4

Source: IntelliCyt Corp.

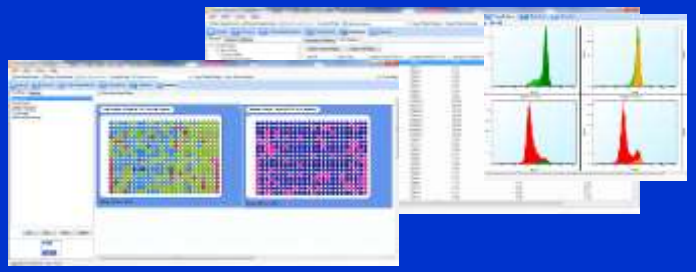
An Integrated Solution

iQue™ Screening System

- **High Throughput**
- **High Content**
- **Multiplexed**
- **Cells and Beads**

Automated high sample through-put flow cytometry system capable of rare cell analysis.

Integrated Application Solution



ForeCyt® *and* *i*DM® Software

Rapidly transforms massive data sets into actionable results

Assay Reagent Kits

Robust, plug & play application specific kits

Source : IntelliCyt Corp

Conclusions

- With a high analysis rate of up to 100,000 cells/sec, flow cytometry performs robust rare cell analysis and isolation.
- Thresholding allows focusing at a cell subset for higher analysis rates.
- Optimized systems are commercially available.
- New technology developments will further help to enhance the use of automated single cell analysis.

Key contributors

- Hans-Joachim Gross, David Houck (BD)
  rare cell analysis by flow concept and data
- Ben Verwer (BD) algorithms
- Chia-Huei Chen (BD) un-lysed blood analysis
- Janette Phi (AmCell) CD34 counting
- Hrair Kirakossian, Liping Yu (BD) CTC analysis and sorting
- Nikesh Kotecha (CytoBank) data analysis
- …

For more details email: diether.recktenwald@cytometrygroup.org or contact me through http://www.desatoya.com