

# Flow Cytometry & Immunofluorescence (Physics and Chemistry)

**Presented by:**  
**Diether Recktenwald, PhD**

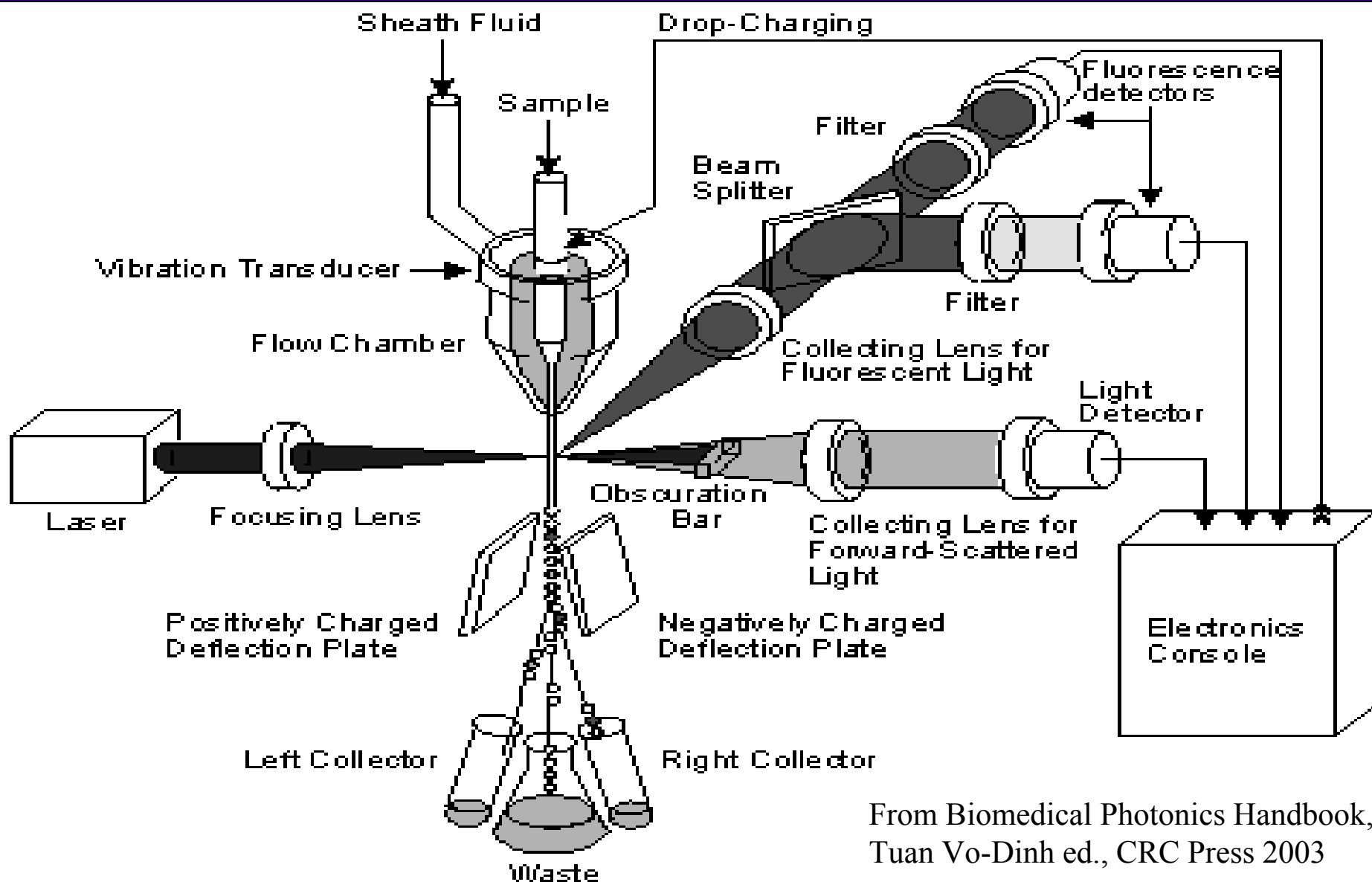
Contact email:  
[diether\\_recktenwald@bd.com](mailto:diether_recktenwald@bd.com) or [diether@att.net](mailto:diether@att.net)

**BD Biosciences**

Clontech  
Discovery Labware  
Immunocytometry Systems  
Pharmingen

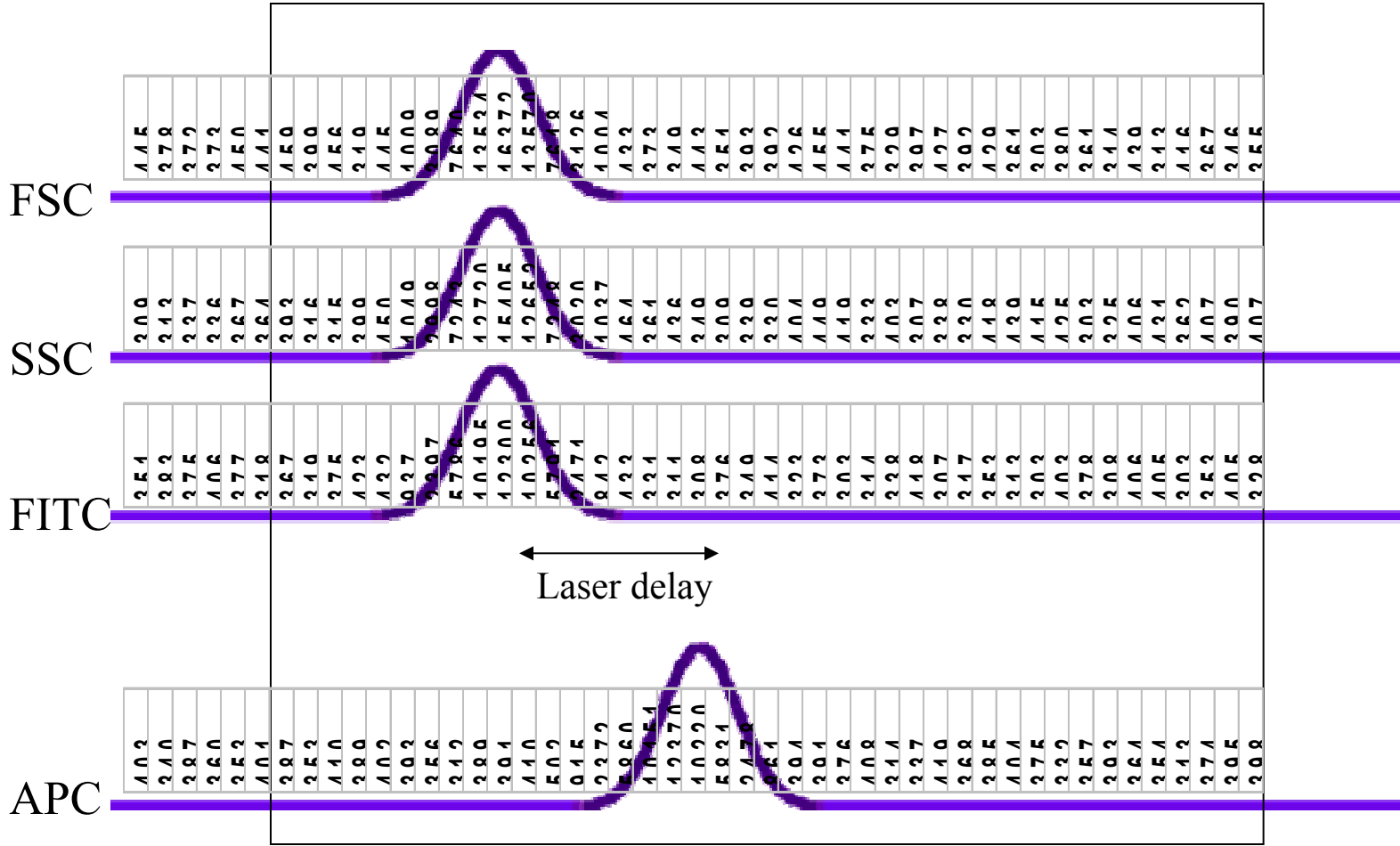


# Flow Cytometer



From Biomedical Photonics Handbook,  
Tuan Vo-Dinh ed., CRC Press 2003

# Numbers in Memory

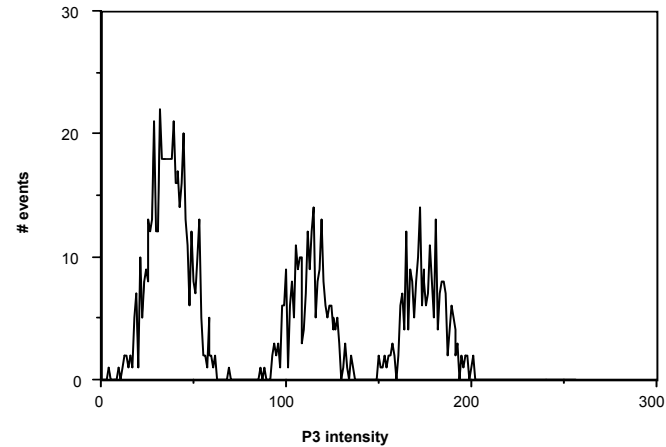


# Flow Cytometry Data Analysis

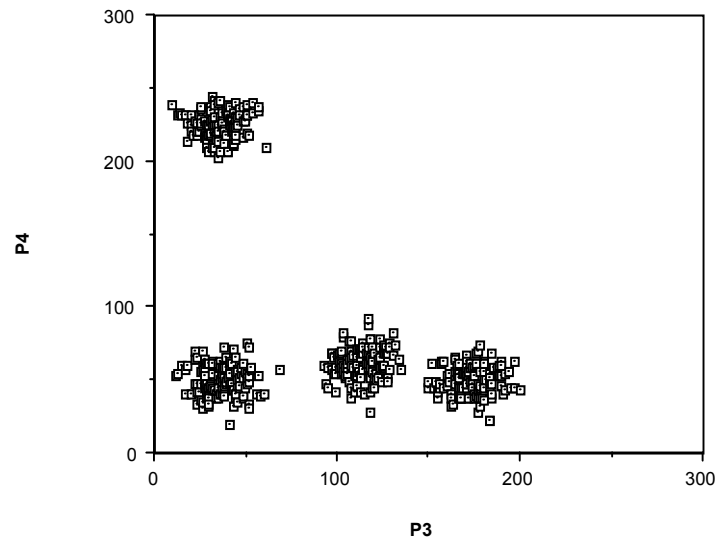
| Cell  | P1  | P2  | P3  | P4   | P5  | Pop# |
|-------|-----|-----|-----|------|-----|------|
| 1     | 242 | 135 | 704 | 175  | 612 | 1    |
| 2     | 146 | 132 | 690 | 178  | 566 | 1    |
| 3     | 269 | 147 | 89  | 206  | 580 | 3    |
| 4     | 442 | 143 | 399 | 250  | 255 | 4    |
| 5     | 212 | 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    |

Note: more than 12 parameters  
in advanced FACS systems

Event histogram

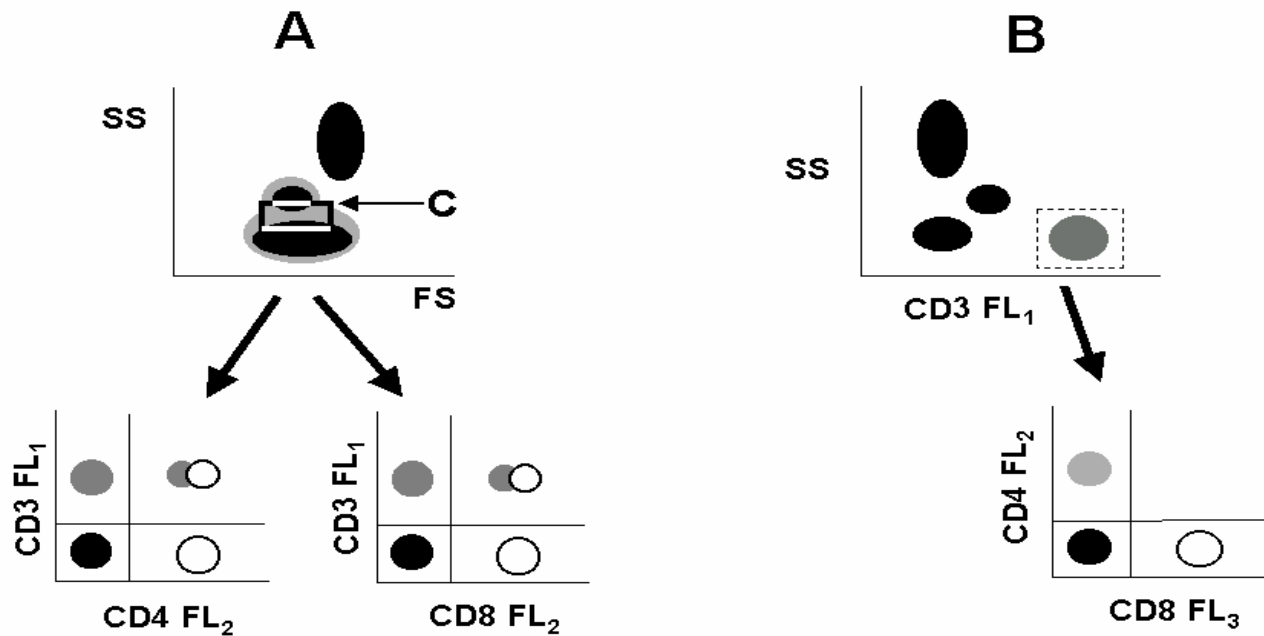


"Dotplot"

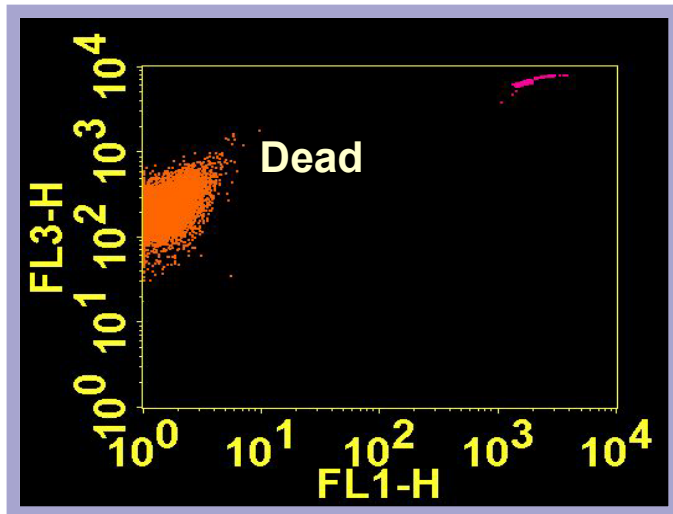


# “Gating”

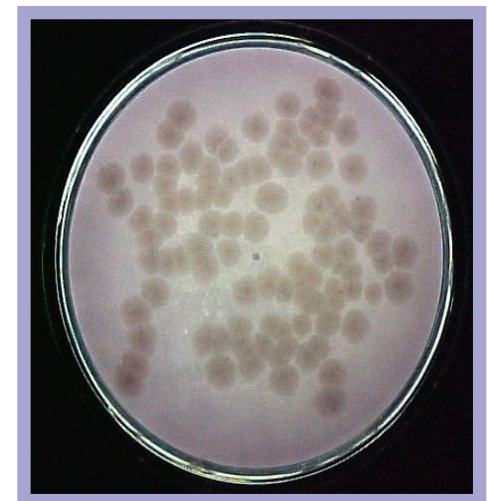
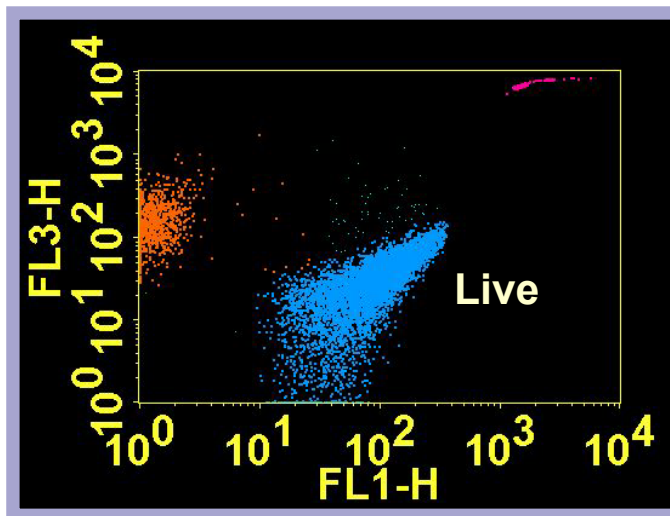
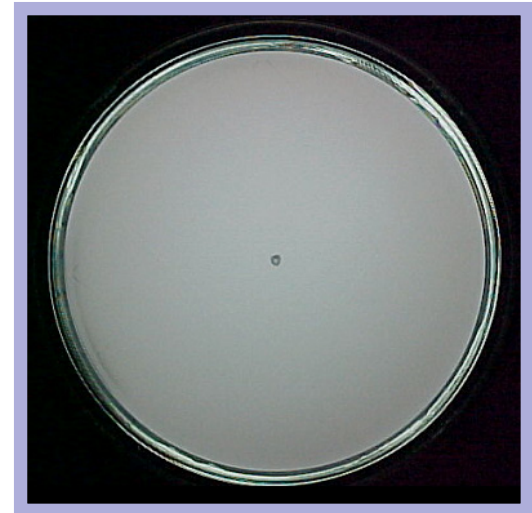
## GATING STRATEGIES BASED ON INTRINSIC AND EXTRINSIC CELL ATTRIBUTES



# Cell Sorting for Functional Studies



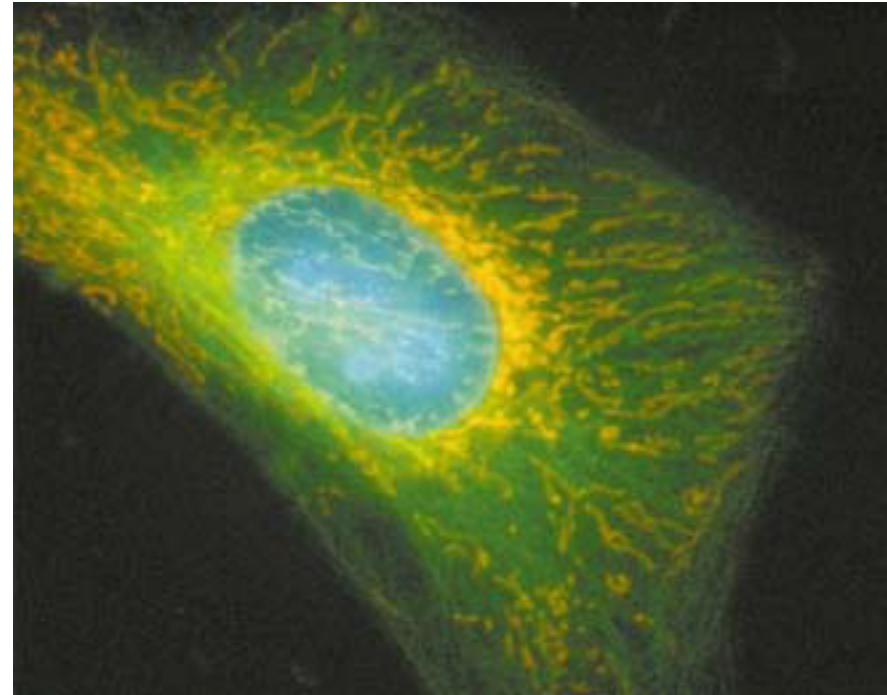
**Flow  
Sorting  
and  
Plate  
Detection**



# Parameters For Cell Analysis by Flow Cytometry

## Analyse and Sort based on:

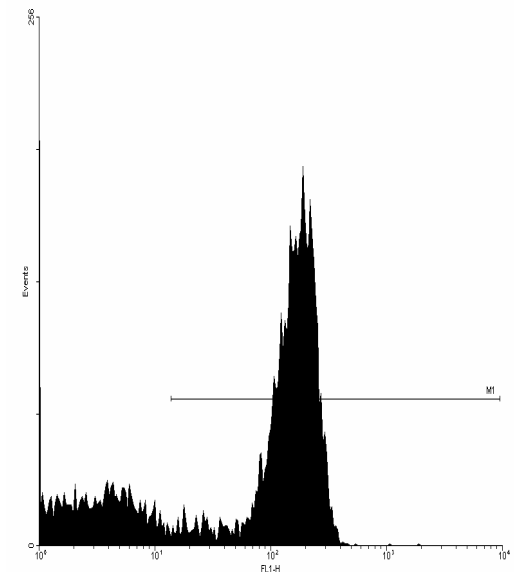
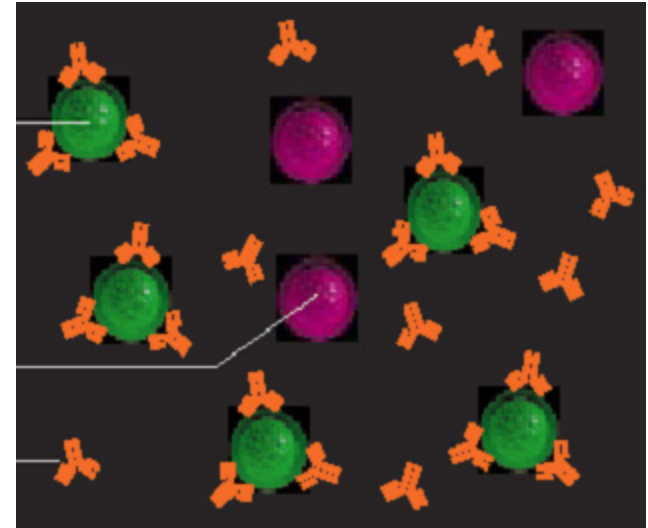
- light scatter
- immunofluorescence
- fluorescent in-situ hybridization
- DNA content
- transfection with fluorescent proteins
- protein content
- auto-fluorescence
- enzyme activity
- pH
- redox potential
- other components detectable by fluorescence



HeLa cells transfected with fluorescent protein vectors for nuclei, mitochondria and tubulin.

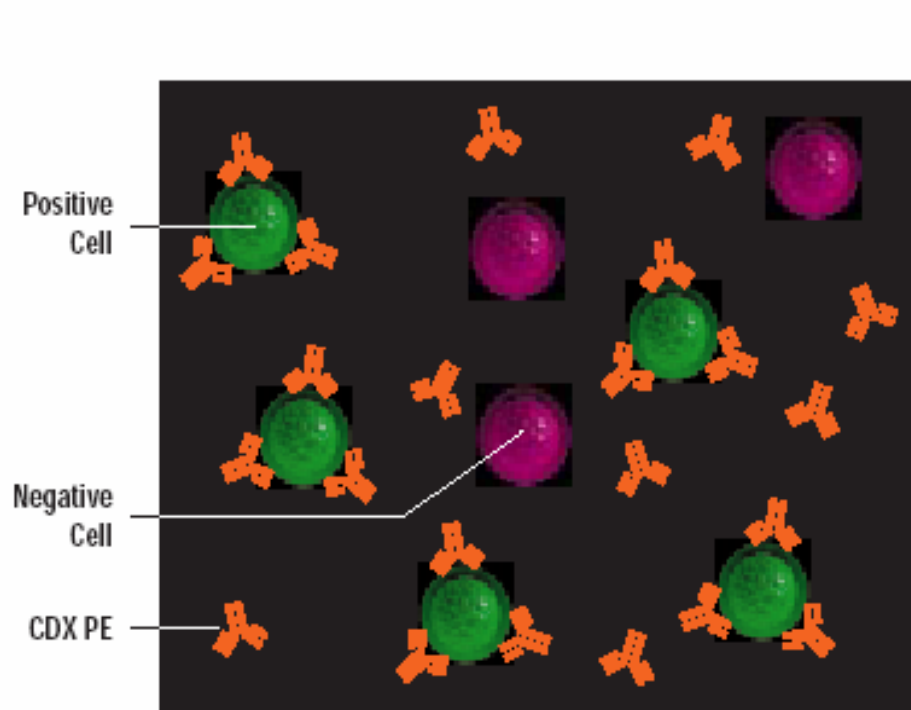
# Immunofluorescence

- Sample conditioning
  - Disaggregation of tissues
  - Pre-enrichment
- Reaction of sample with reagent
  - Direct or indirect immunofluorescence
  - Wash or no-wash
- Multi-color fluorescence measurement
- Data analysis

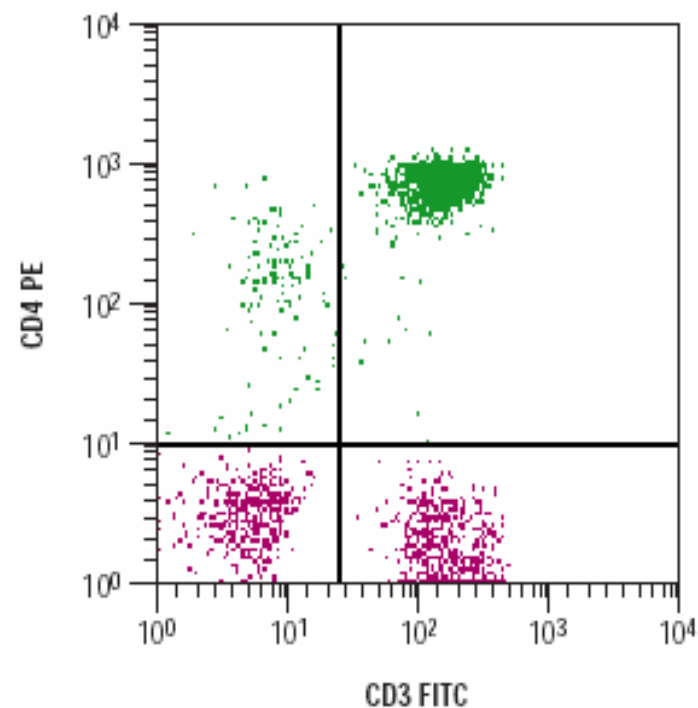




# Immunofluorescence Data (1)

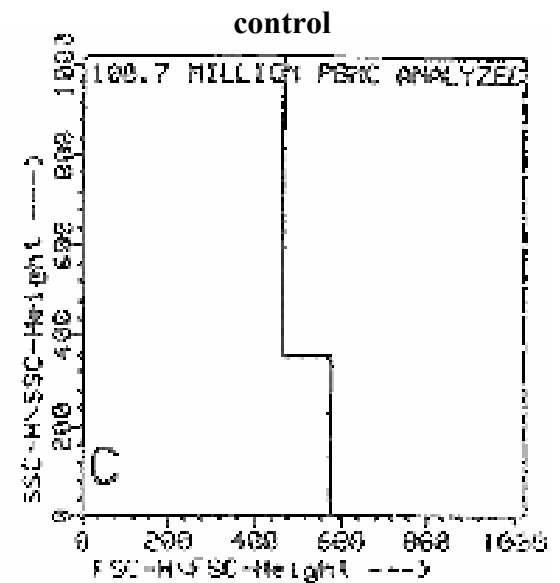
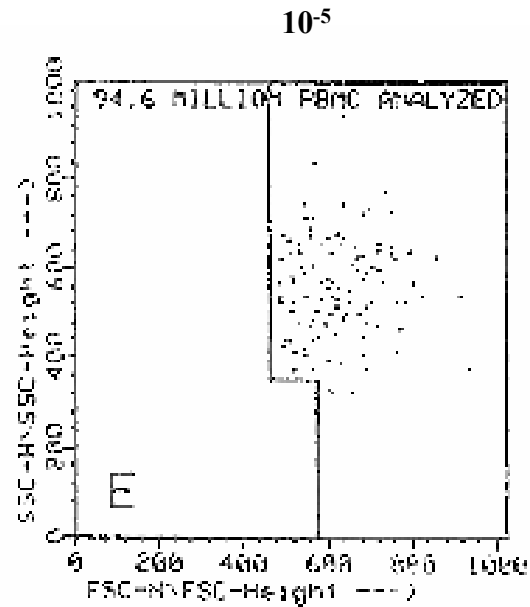
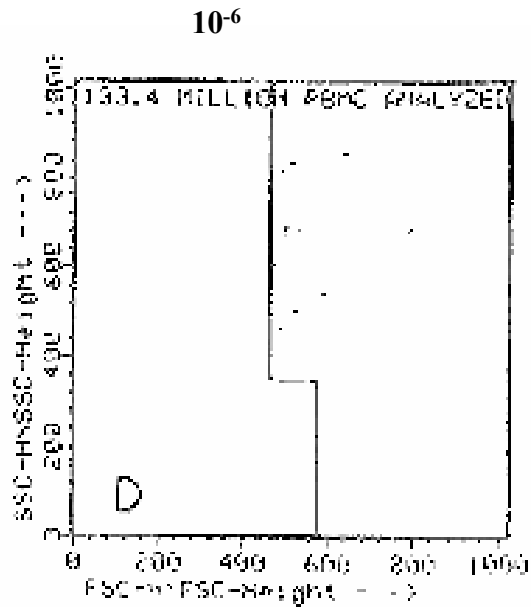


Percent Positives



# Immunofluorescence (1)

## Limit of Detection for Rare Cells



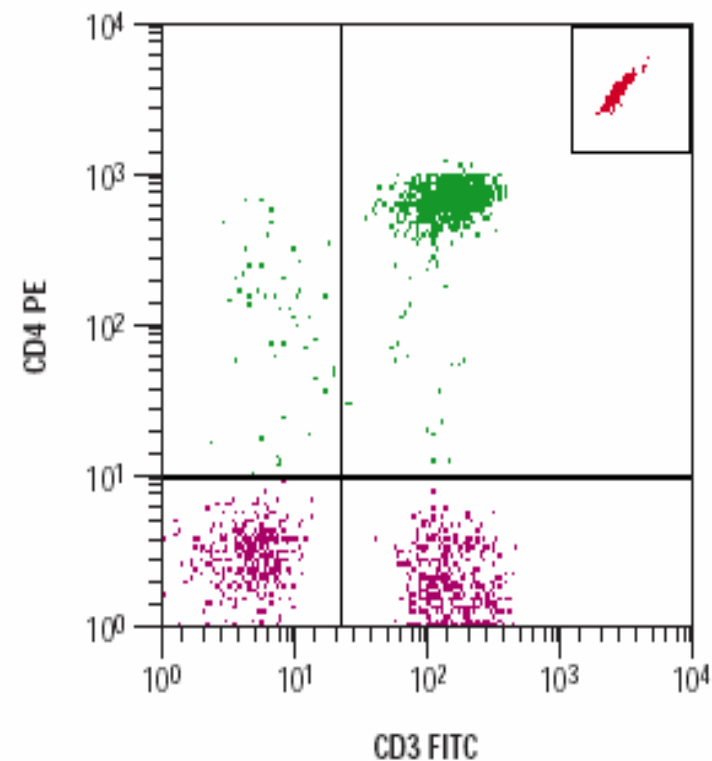
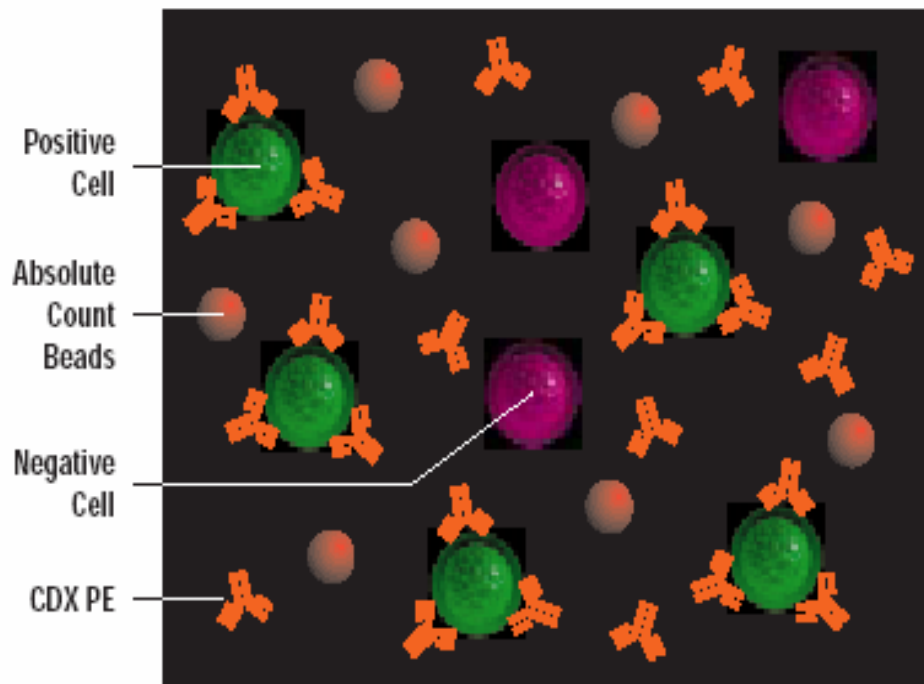
Routine >0.2%

Optimized instrument >0.01%

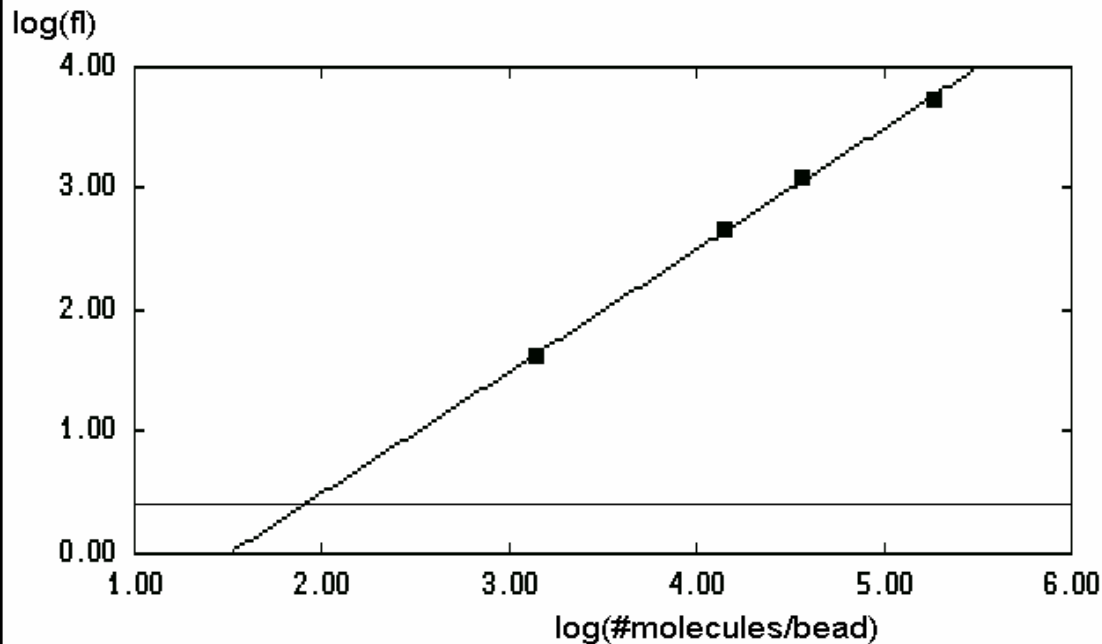
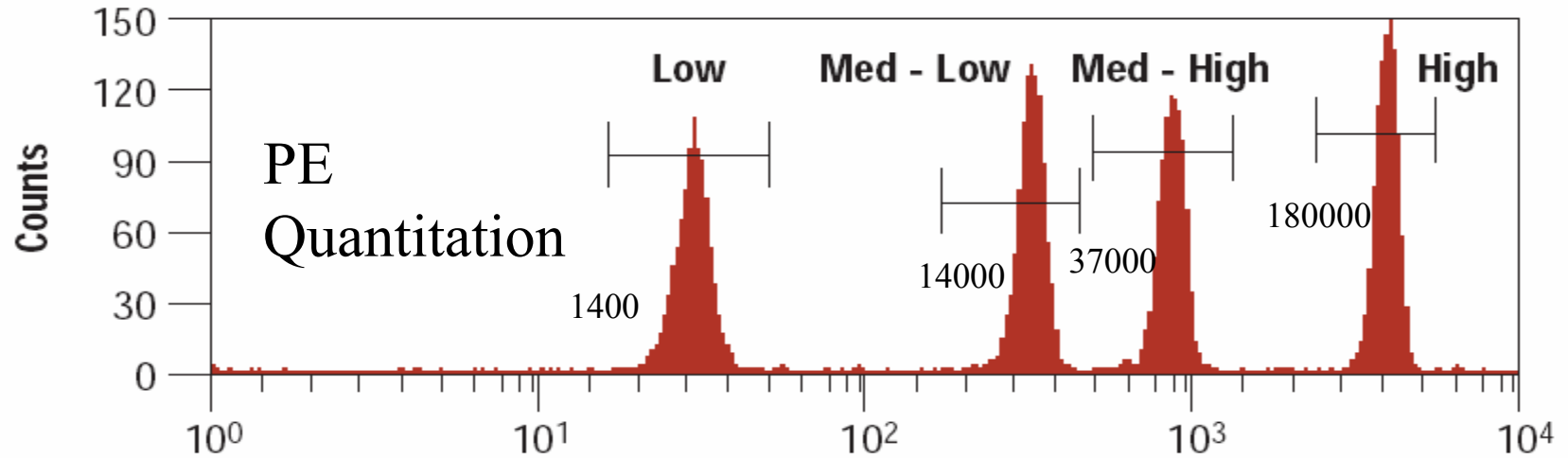
Optimized system >10<sup>-7</sup>

# Immunofluorescence Data (2)

Absolute Counts — Cells per  $\mu\text{L}$



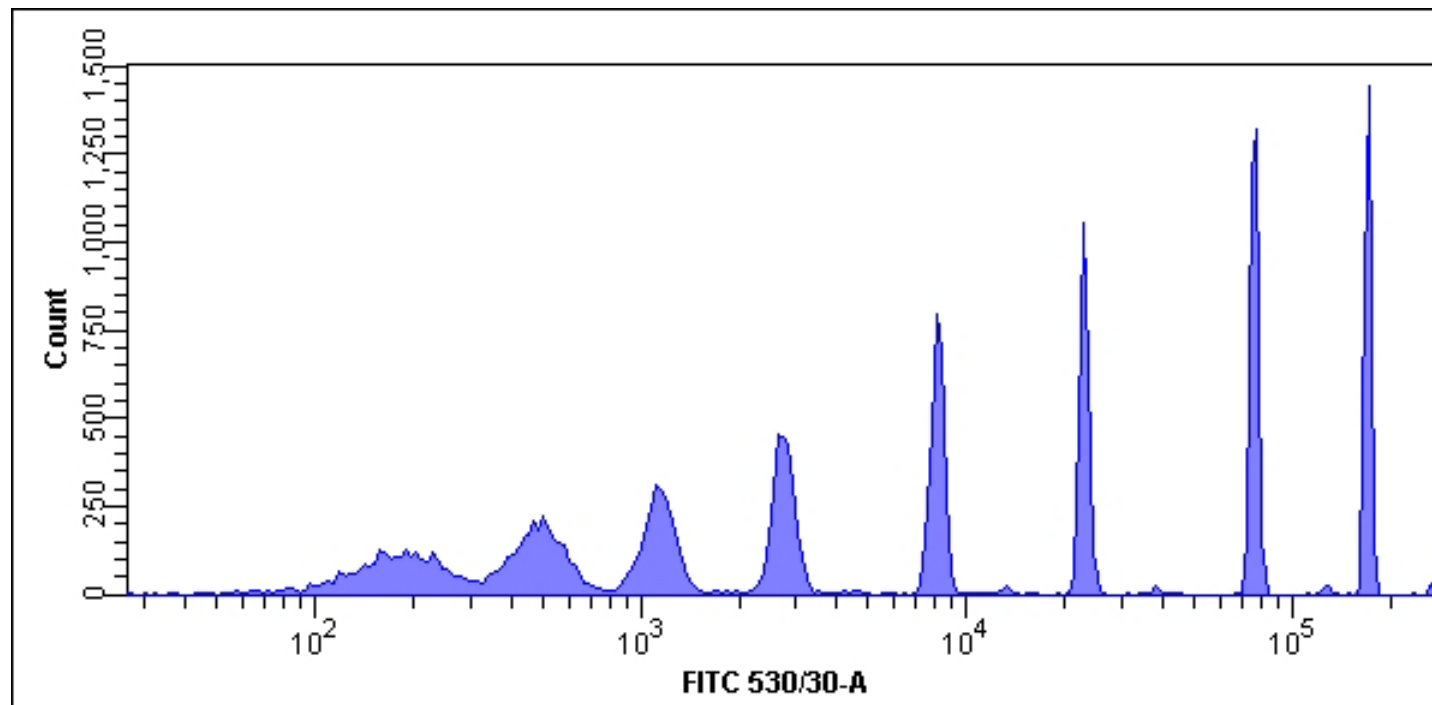
# Immunofluorescence Data (3)



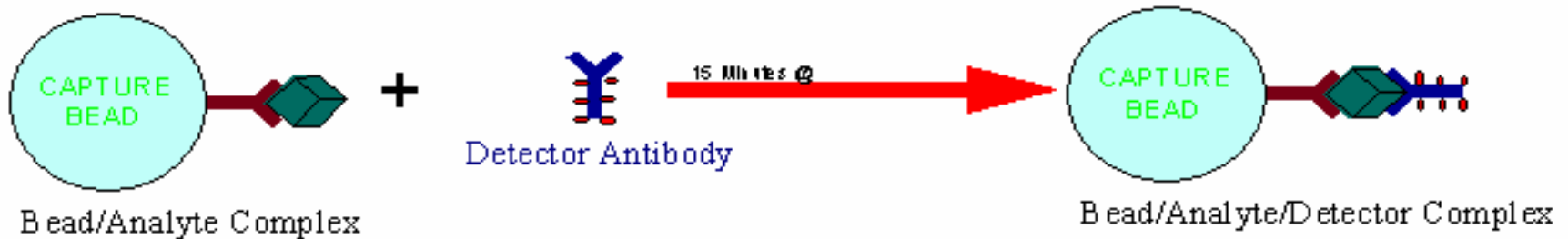
| Molecule | #/cell            |
|----------|-------------------|
| CD3      | $8.1 \times 10^4$ |
| CD4      | $5.9 \times 10^4$ |
| CD8      | $1.4 \times 10^5$ |
| CD11a    | $2.7 \times 10^4$ |
| CD16     | $7.9 \times 10^4$ |
| CD18     | $3.1 \times 10^4$ |
| CD45     | $1.9 \times 10^5$ |

From:  
 Appendix A, Cell Separation Methods  
 and Applications. Marcel Dekker 1998.  
 Reckenwald D and Radbruch A, eds.

# Sensitivity

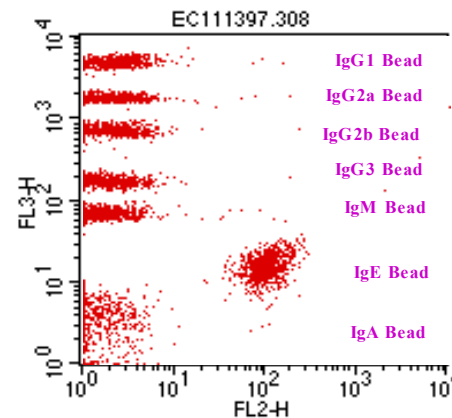
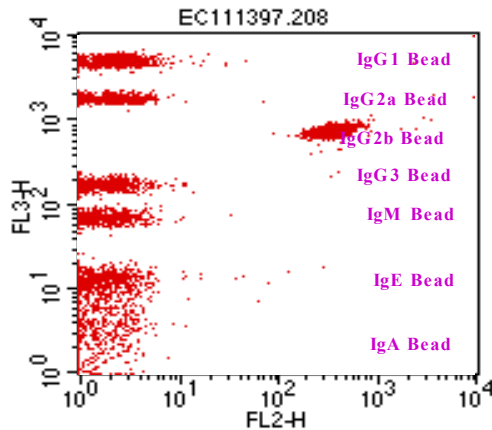


# Microsphere-based assays for soluble analytes



IgG2b 20 ng

IgE 20 ng



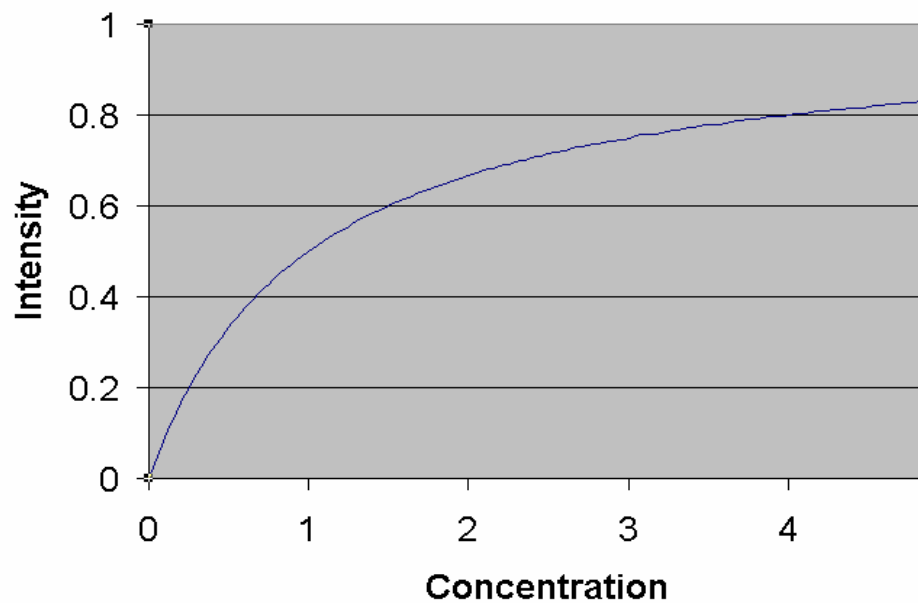
Dr. Rudi Varro, BD Biosciences

# Immunofluorescence Issues

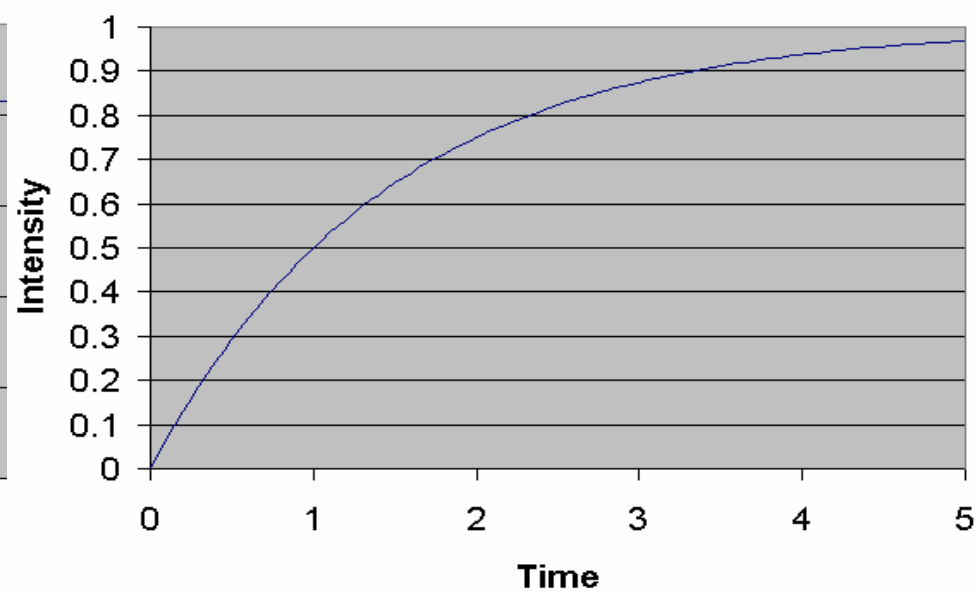
- **Label selection** (sensitivity and compensation)
- **Photobleaching** (especially energy transfer conjugates)
- **Environment sensitive fluorescence** (i.e. FITC)
- **Fixation**
- **Dead cells** (PI, EMA)
- **Reagent equilibrium binding**
- **Binding kinetics**
- **“Non-specific” reactions**

# Ligand binding (1)

## Equilibrium Binding



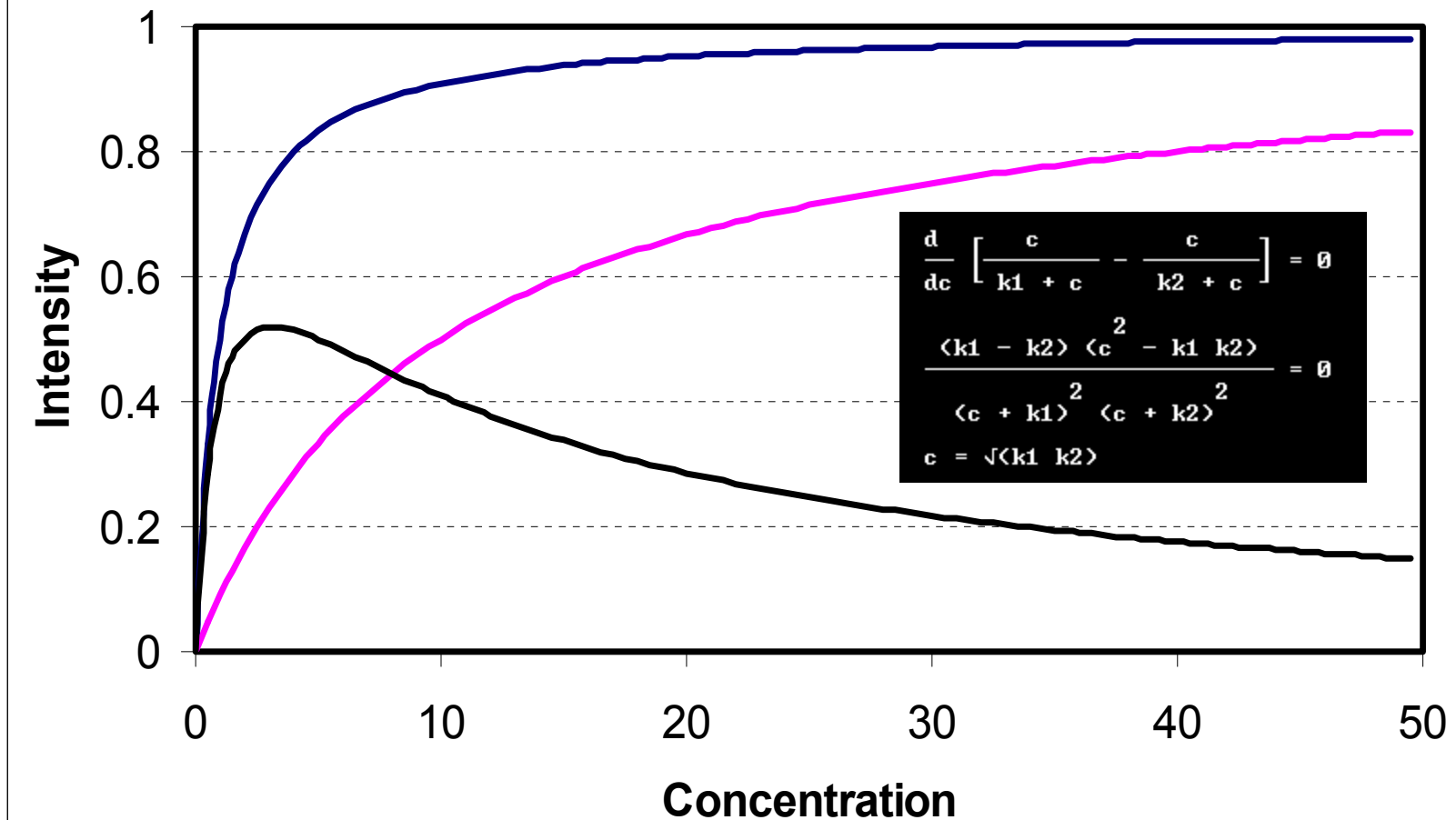
## Kinetics





# Ligand binding (2)

## Effect of "non-specific" binding

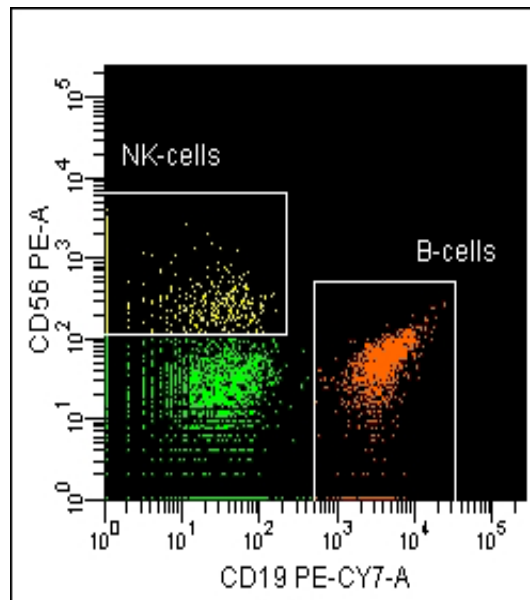
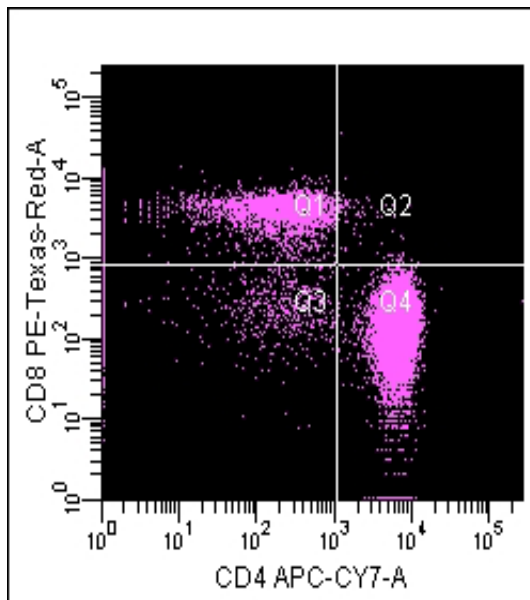
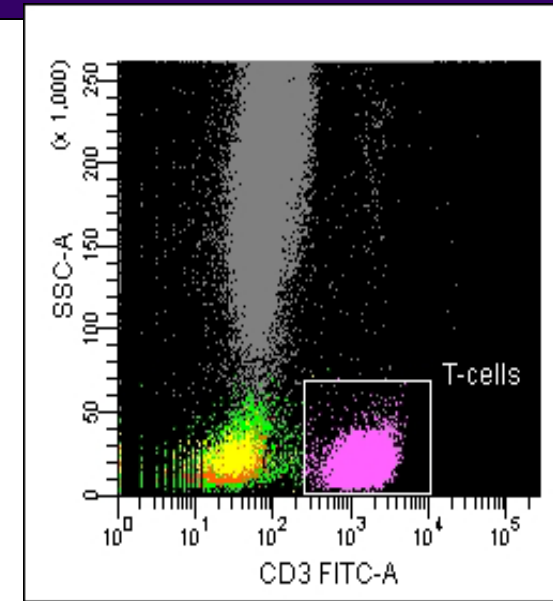
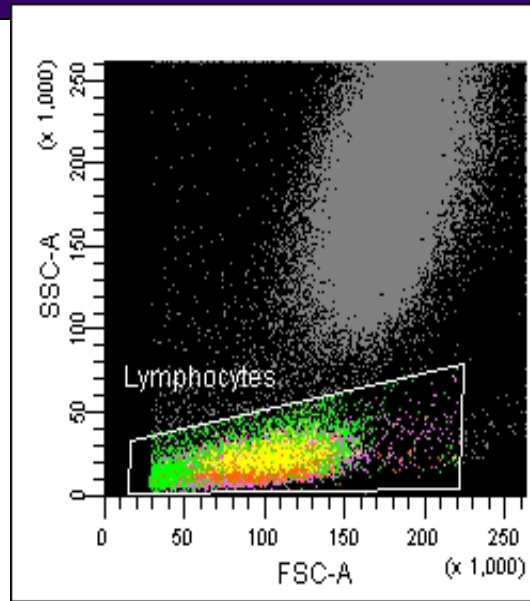
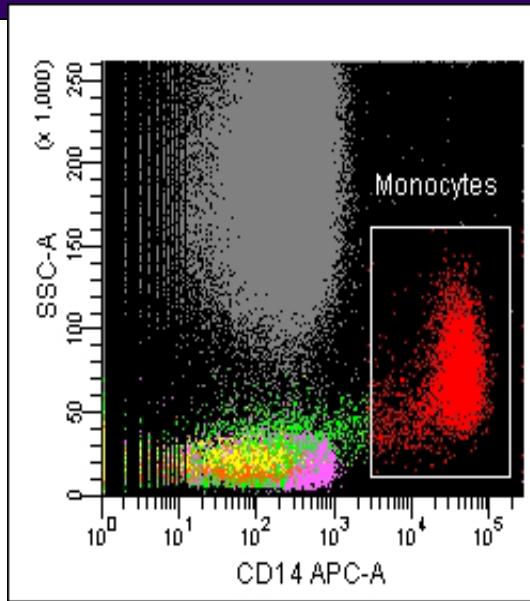


# Immunofluorescence Multi-color Analysis

| DYE     | Excitation with       |                       | Emission<br>(nm) | Extinction<br>Coefficient<br>( $\text{cm}^{-1} \text{M}^{-1}$ ) | Quantum<br>yield | MW<br>(Da) |
|---------|-----------------------|-----------------------|------------------|---|------------------|------------|
|         | 488                   | 635                   |                  |   |                  |            |
|         | 1 <sup>st</sup> laser | 2 <sup>nd</sup> laser |                  |   |                  |            |
| FTTC    | x                     |                       | 519              | 67,000  | 0.71             | 389        |
| PE      | x                     |                       | 578              | 1,960,000   | 0.68             | 240,000    |
| PerCP   | x                     |                       | 675              | na  | 1                | 35,000     |
| ECD     | x                     |                       | 613              | na  | na               | 250,000    |
| PE-Cy5  | x                     |                       | 675              | 1,960,000   | na               | 241,000    |
| APC     |                       | x                     | 660              | 700,000   | 0.68             | 104,000    |
| Cy5     |                       | x                     | 670              | 250,000   | 0.28             | 792        |
| APC-Cy7 |                       | x                     | 767              | 700,000   | na               | 105,000    |

From Biomedical Photonics Handbook, Tuan Vo-Dinh ed., CRC Press 2003

# Six color example



- CD3 FITC
- CD56 PE
- CD8 PE-Texas Red
- CD19 PE-Cy7
- CD14 APC
- CD4 APC-Cy7

# End

address questions to:

Diether Recktenwald PhD

BD Biosciences Immunocytometry Systems

2350 Qume Dr.

San Jose CA 95131-1807, USA

Phone: 408-954-2191(o)

FAX: 928-441-2245(efax)

Email: [Diether\\_Recktenwald@bdis.com](mailto:Diether_Recktenwald@bdis.com) or

[diether@att.net](mailto:diether@att.net)

**BD Biosciences**

Clontech  
Discovery Labware  
Immunocytometry Systems  
Pharmingen

