Micro-fluorimetry for the Biosciences

Diether J. Recktenwald PhD,
Advanced Technology Group
BD Biosciences,
2350 Qume Dr., San Jose CA 95131
Contact email:
diether_recktenwald@bd.com or diether@att.net

BD Biosciences
Clontech
Discovery Labware
Immunocytometry Systems
Pharmingen

BD

Need of Biology Research

BD
BD Biosciences

Biological measurements

Ideally measure all chemicals in an organism with spatial and temporal resolution

- Microscopy

  high spatial resolution to cell substructures, few cells,

  few parallel chemical measurements with subcellular resolution

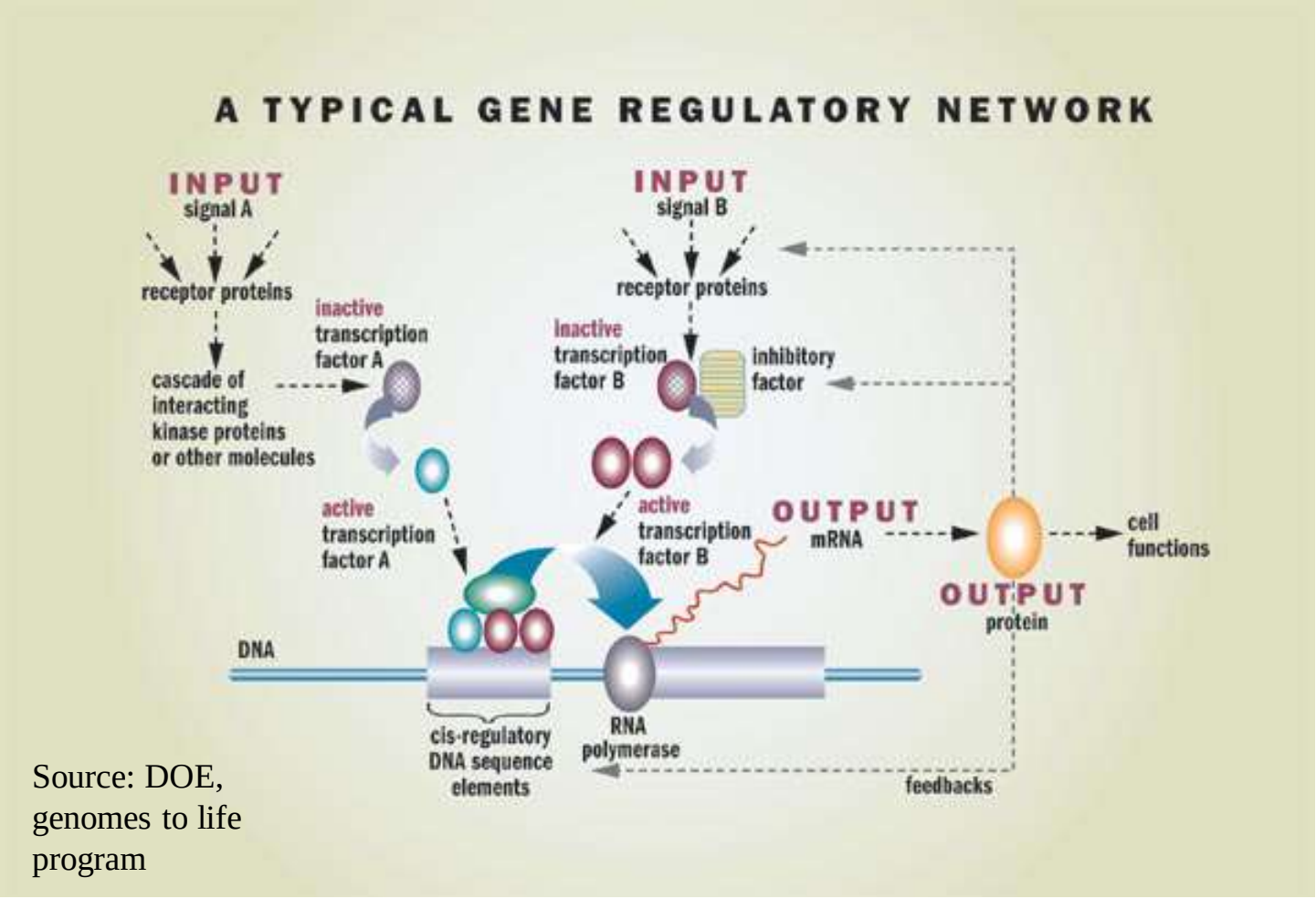
- Flow cytometry

  spatial resolution to one cell, few to many cells, several parallel chemical measurements with one cell resolution

- Arrays

  low spatial resolution to macroscopic cell ensemble level, many cells, many parallel chemical measurements cell sample resolution

Signal Transduction Network



Source: DOE, genomes to life program

BD
BD Biosciences

System Components

BD
BD Biosciences

Detection Instrument Considerations

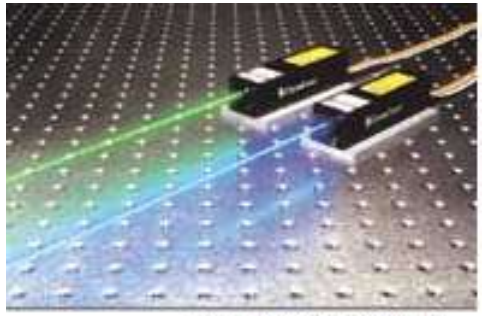
- Light source (wavelength, power, beam-shape)
- Excitation optics (direct beams vs. fiberoptics, multi-laser)
- Emission optics (collection efficiency NA, immersion optics)
- Spectral filtering (dispersive elements, filters, …)
- Detectors (PMT, CCD, …)
- Signal processing (analog vs. digital, data reduction)
- **System background** (electronic noise, photon statistics, …)
- Fluidics (sheath flow vs undiluted sample, edge effects, focal depth)

Excitation light sources

High luminous density emitters

(490nm a desirable wavelength for fluorescein)

- Arc lamps (Hg-arc, …)
- Gas lasers (Ar, Kr, …)
- Solid state lasers



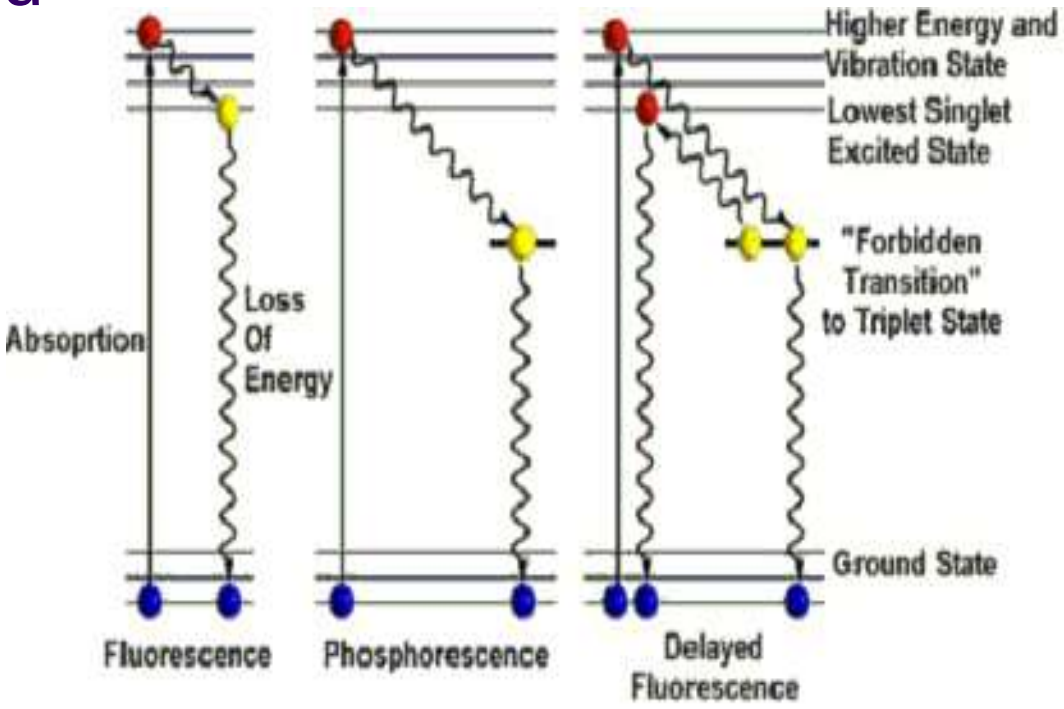
BD

BD Biosciences

Fluorescence

- Absorbance
- Lifetime of excited state
- Stokes shift
- Quantum yield
- Saturation

- Intrinsic
- Extrinsic



Source: www.olympusmicro.com

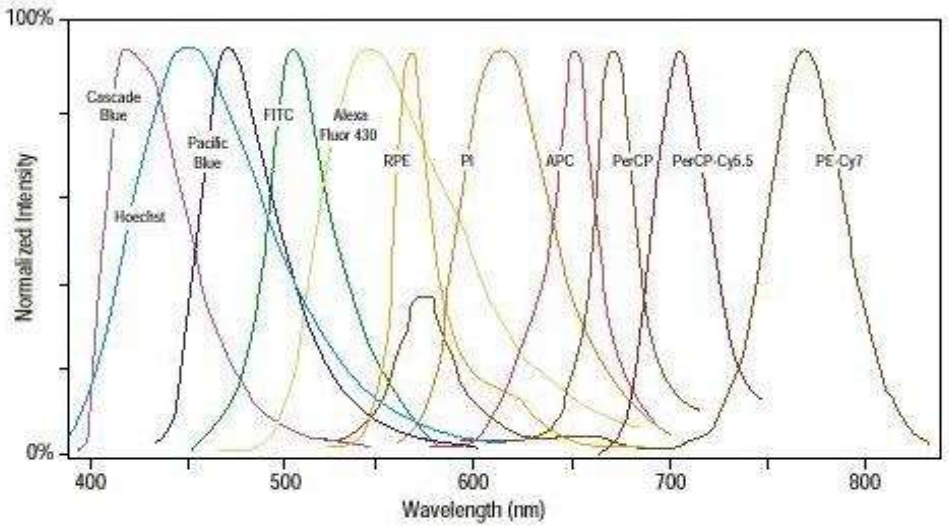
BD
BD Biosciences

Fluorescent Dyes

- Unsaturated organic molecules (photobleaching)
- Energy-transfer dyes
- Lanthanide derived pigments
- Quantum dots

Dye combinations for multi-color fluorescence

- Single vs. multi-laser excitation
- Spectral overlap



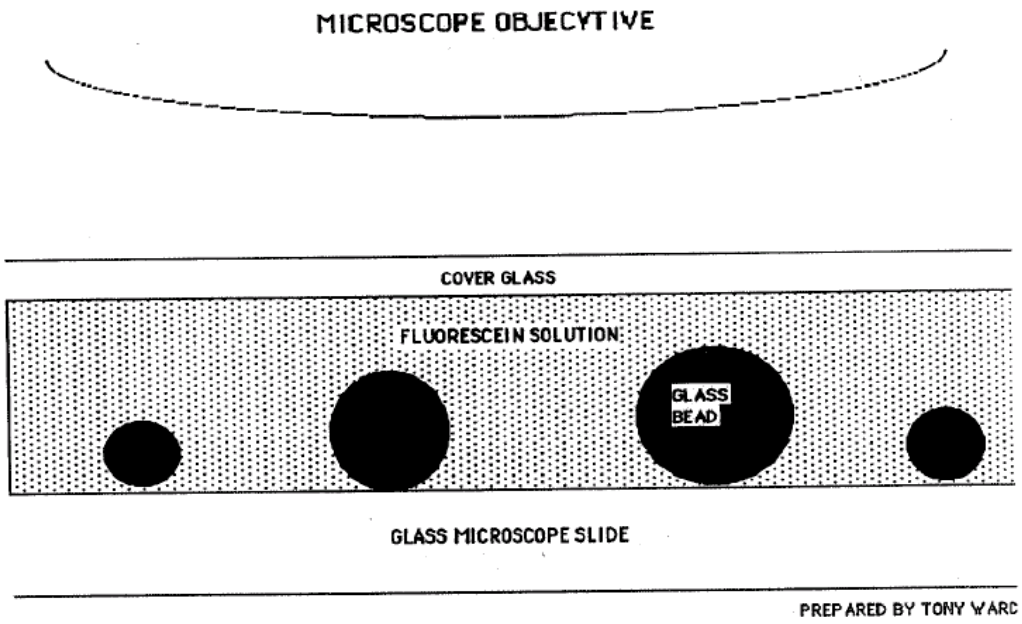
Dye Spectra From the FACSAria User Guide

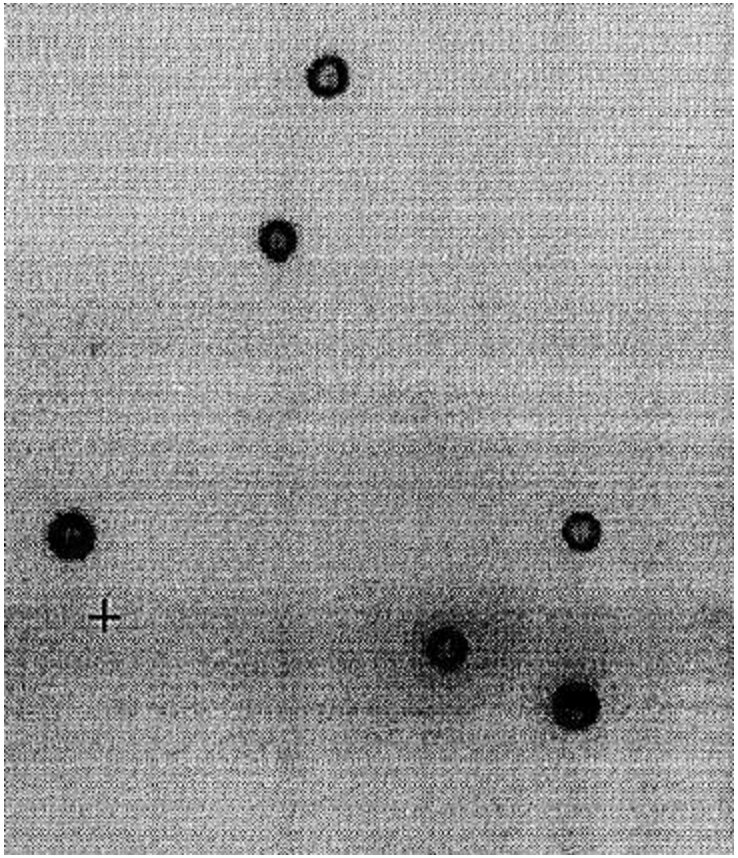
BD Biosciences

Fluorescence Measurements of Chemical Properties

• Intensity	Concentration
• Lifetime	Background reduction
• FRET (energy transfer)	Proximity
• Polarization	Rotational mobility
• Fluorescence correlation	Lateral mobility

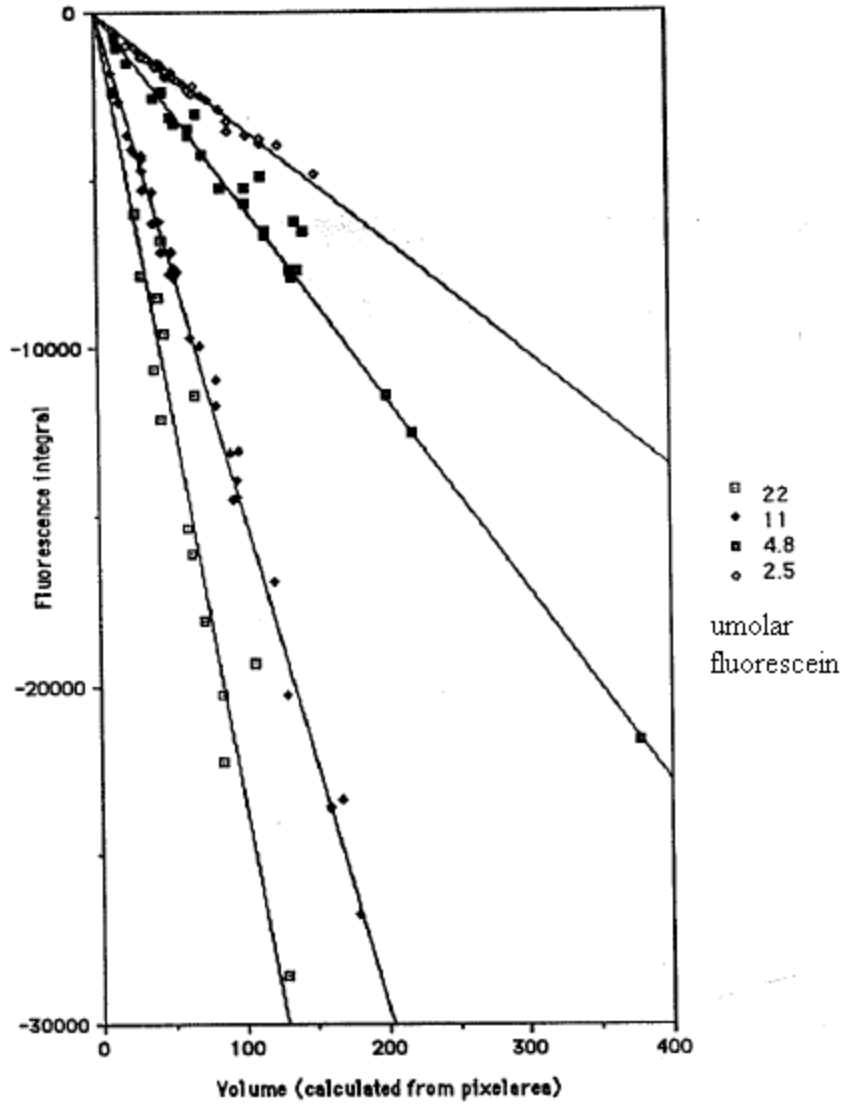
Fluorescence Quantitation by Volume Exclusion (1)

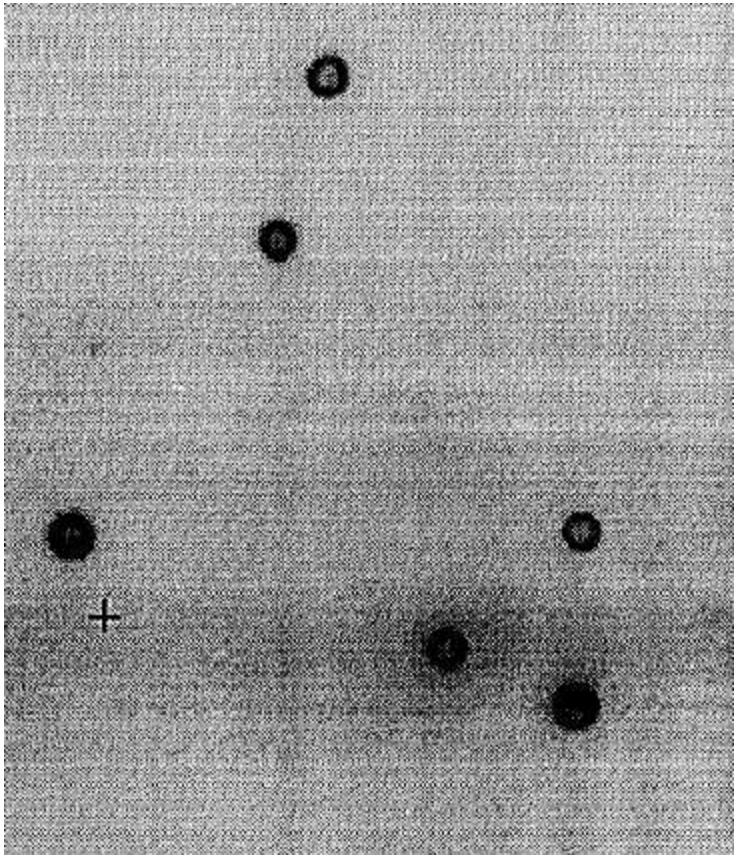




BD
BD Biosciences

Fluorescence Quantitation by Volume Exclusion (2)





BD
BD Biosciences

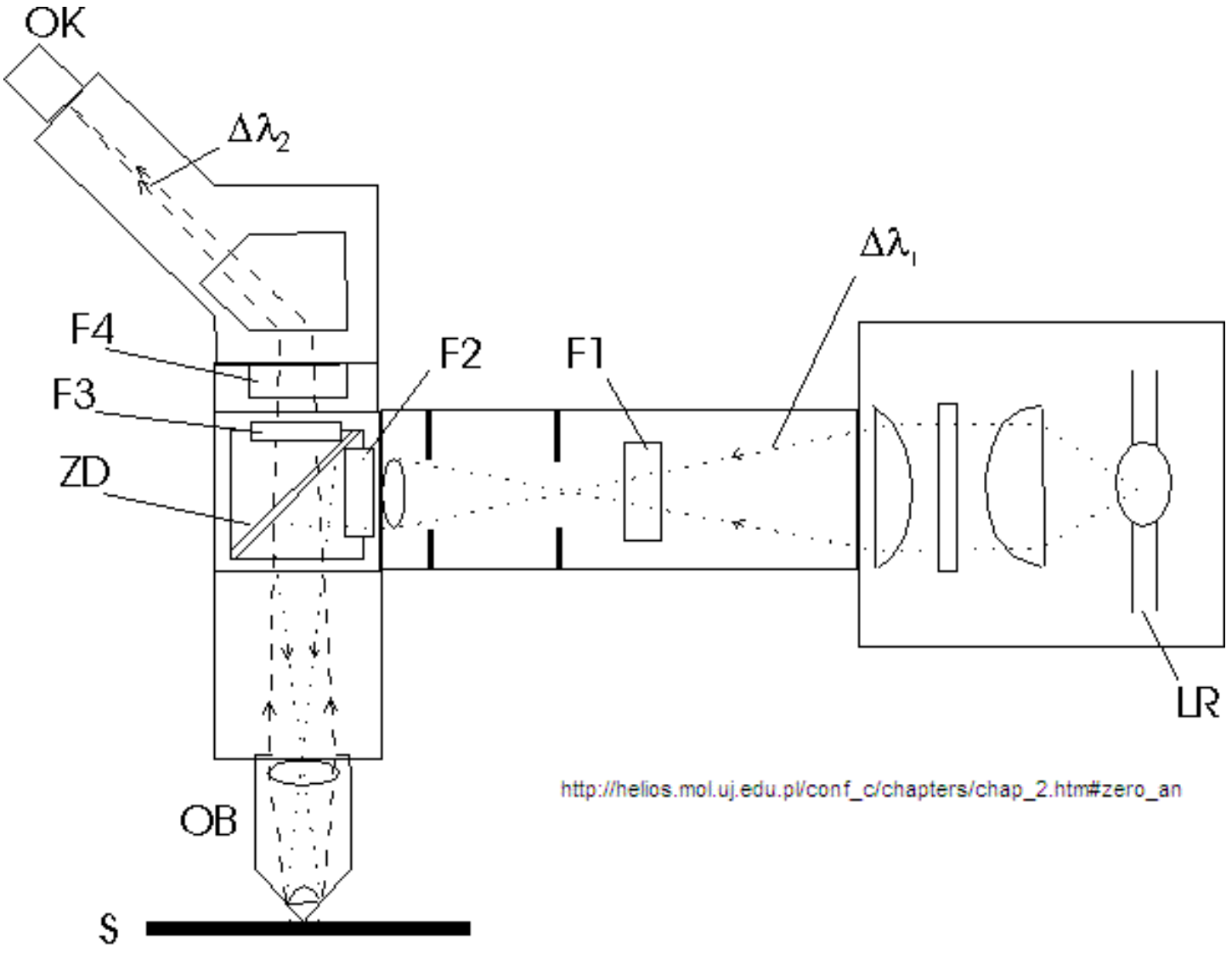
Assay considerations

- Reagent selection
- Selection of labels (spectral properties, overlap)
- Homogeneous vs. non-homogeneous
- Intrinsic fluorescence vs. use of labels
- Specimen and reagent auto-fluorescence
- Reference samples

Systems, which use fluorescence

BD
BD Biosciences

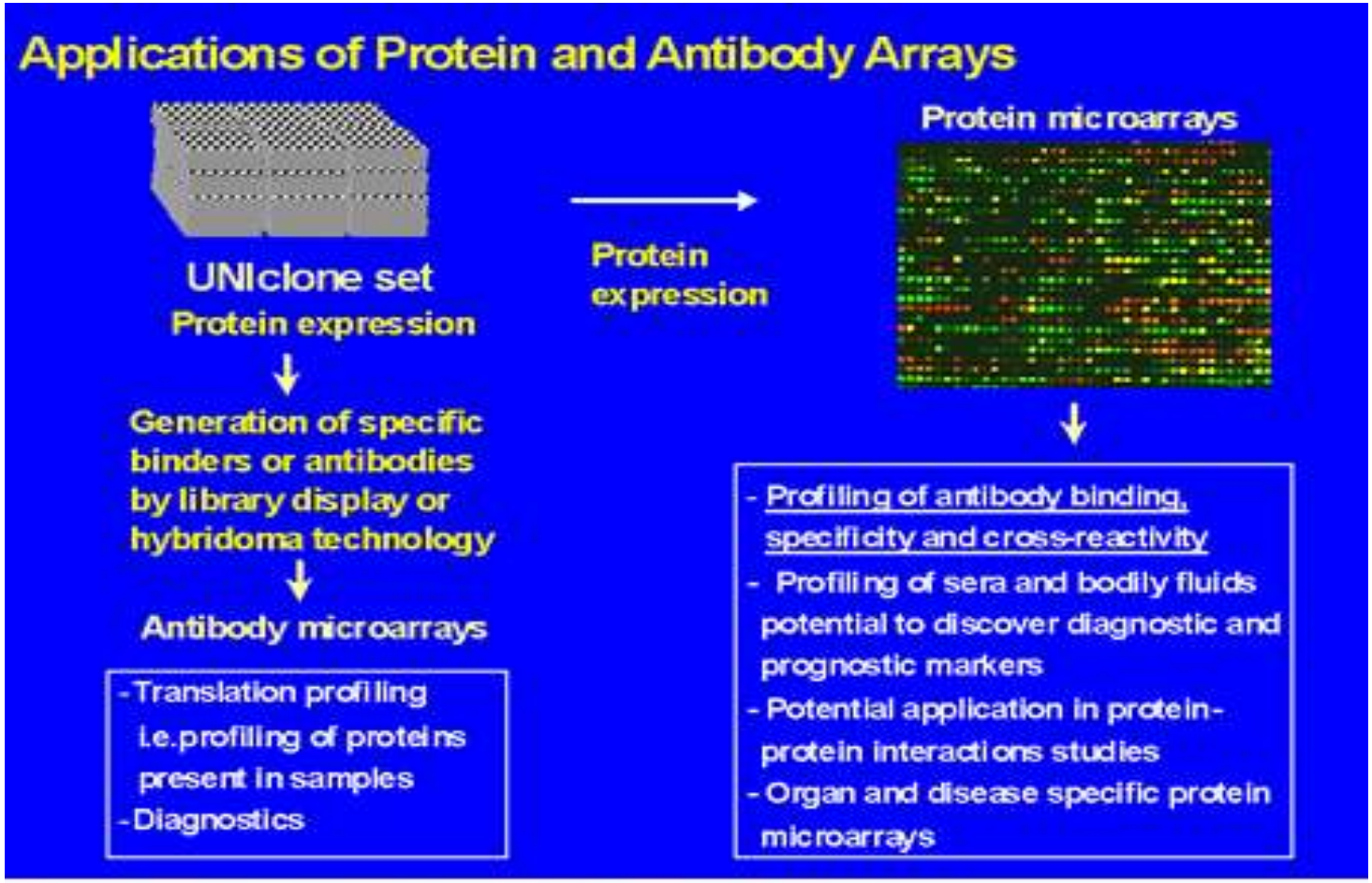
Fluorescence Microscope



http://helios.mol.uj.edu.pl/conf_c/chapters/chap_2.htm#zero_an

BD
BD Biosciences

Micro-arrays

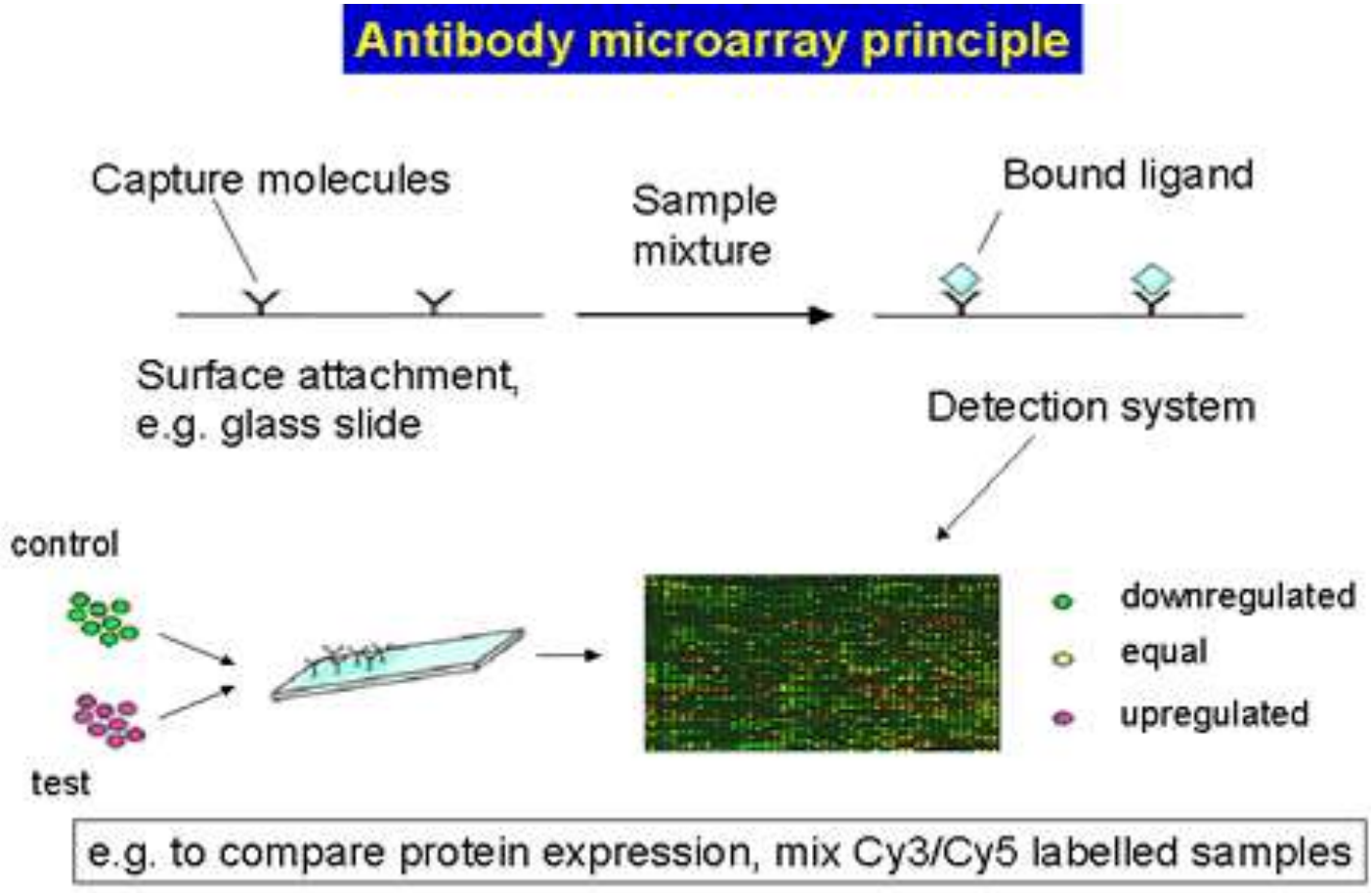


Dolores Cahill, Max-Planck-Institute of Molecular Genetics

BD

BD Biosciences

Micro-arrays



e.g. to compare protein expression, mix Cy3/Cy5 labelled samples

http://www.functionalgenomics.org.uk/sections/resources/protein_arrays.htm#related

BD Biosciences

Micro-arrays

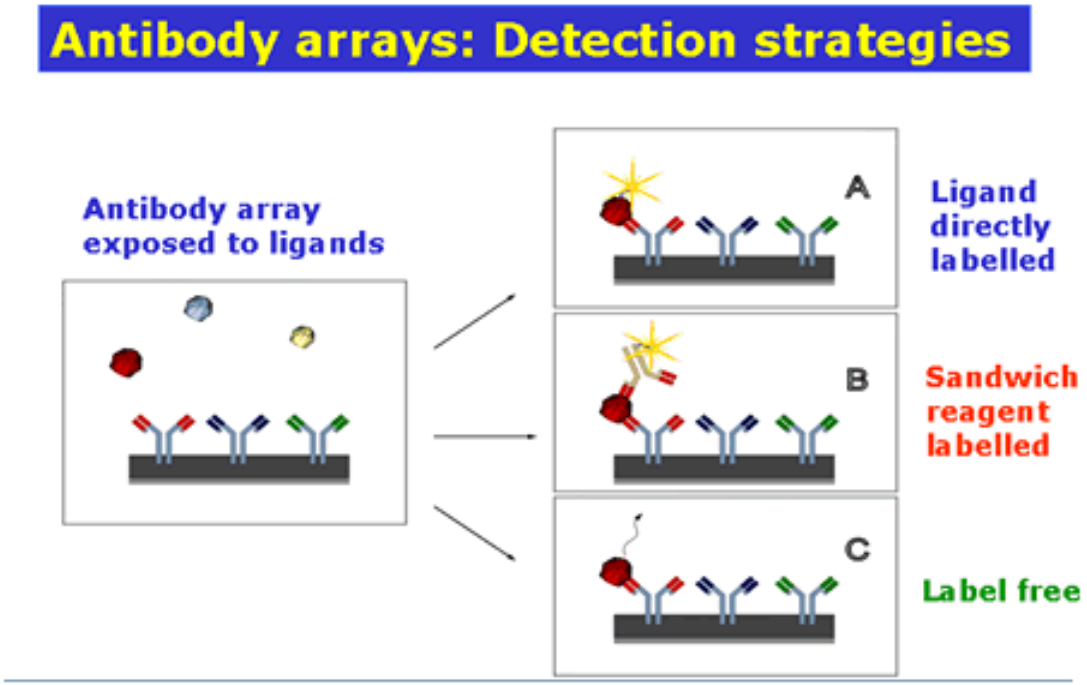
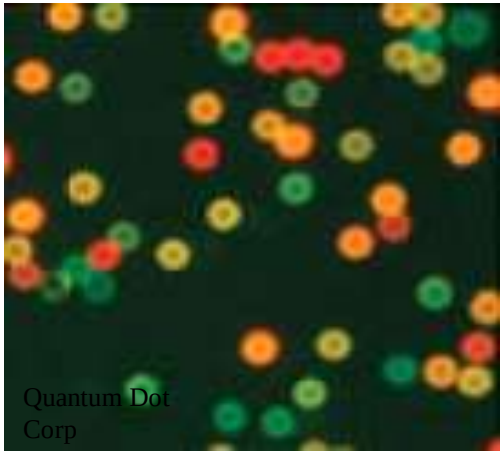
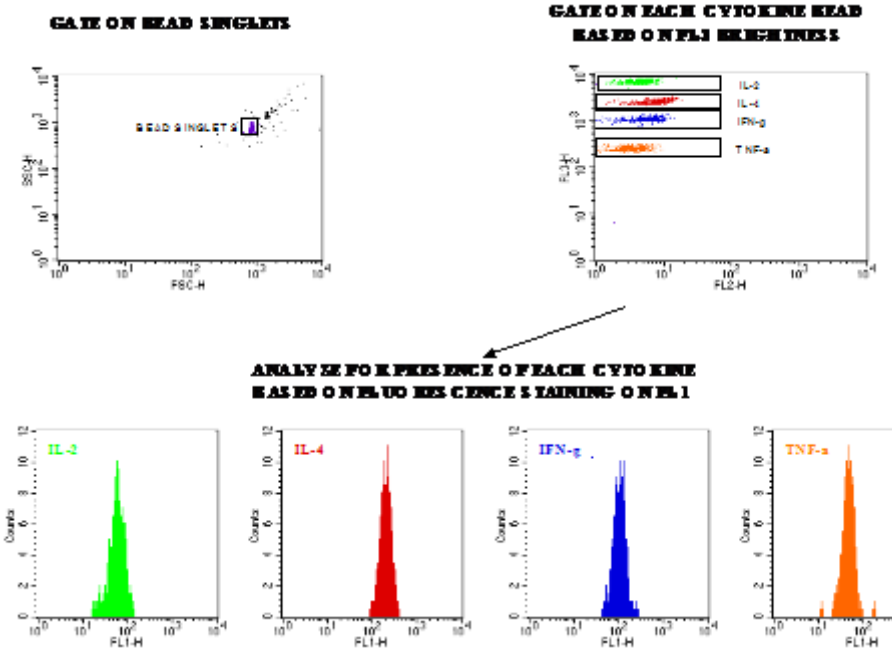


Figure prepared by Jonas Jarvius, Uppsala (from Taussig and Landegren, Targets 2:169-176, 2003)

BD
BD Biosciences

Cytometric Bead Array Assays

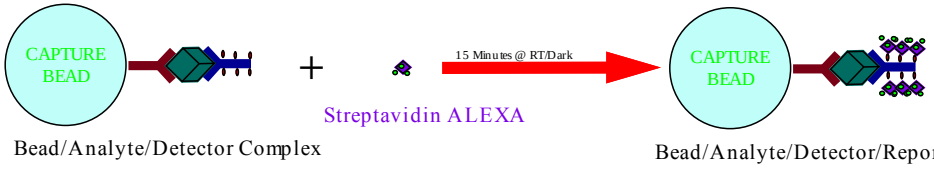




Step 1: Analyte Capture

Step 2: Detector Antibody Binding

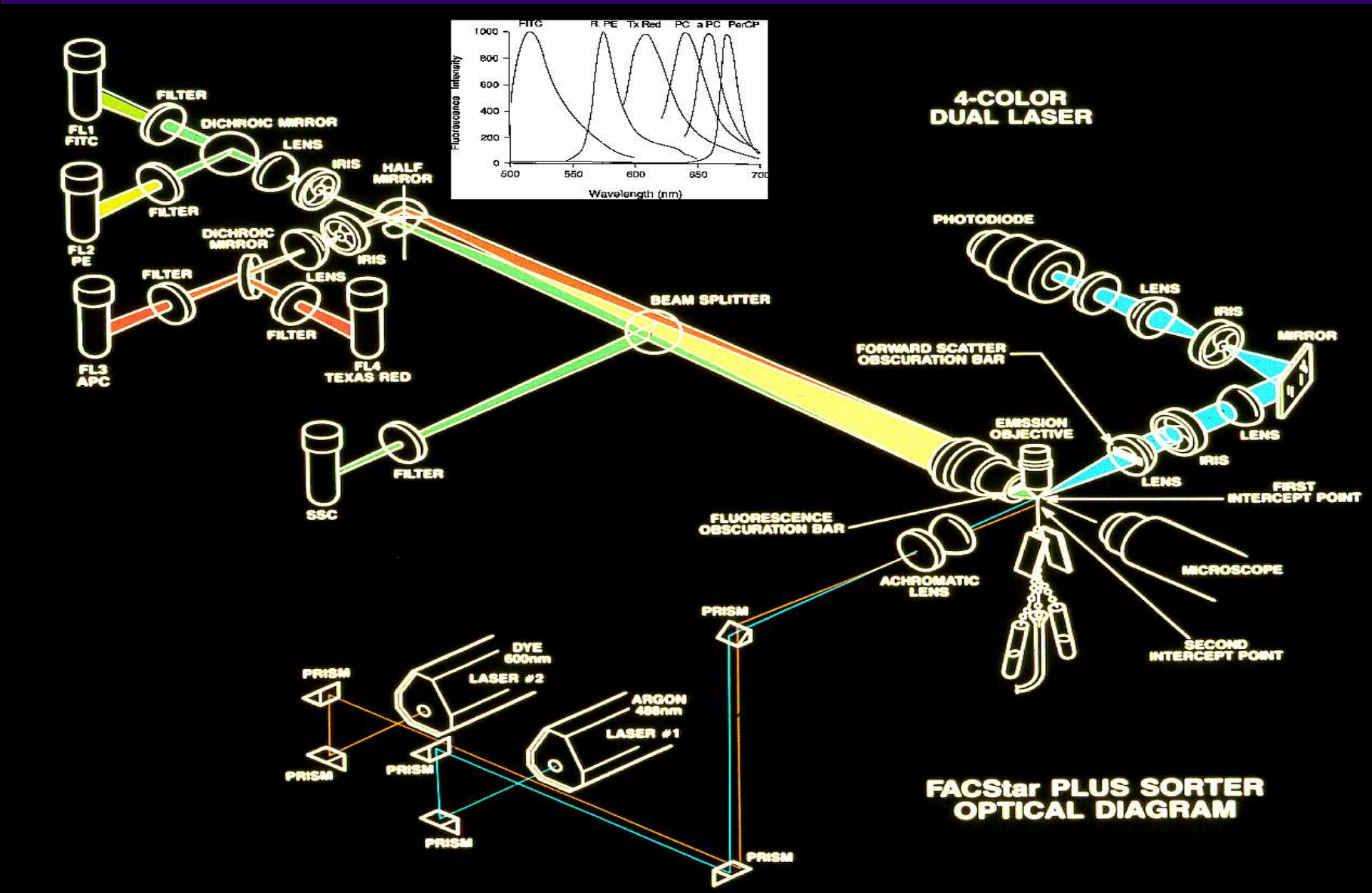
Step 3: Reporter Molecule Binding



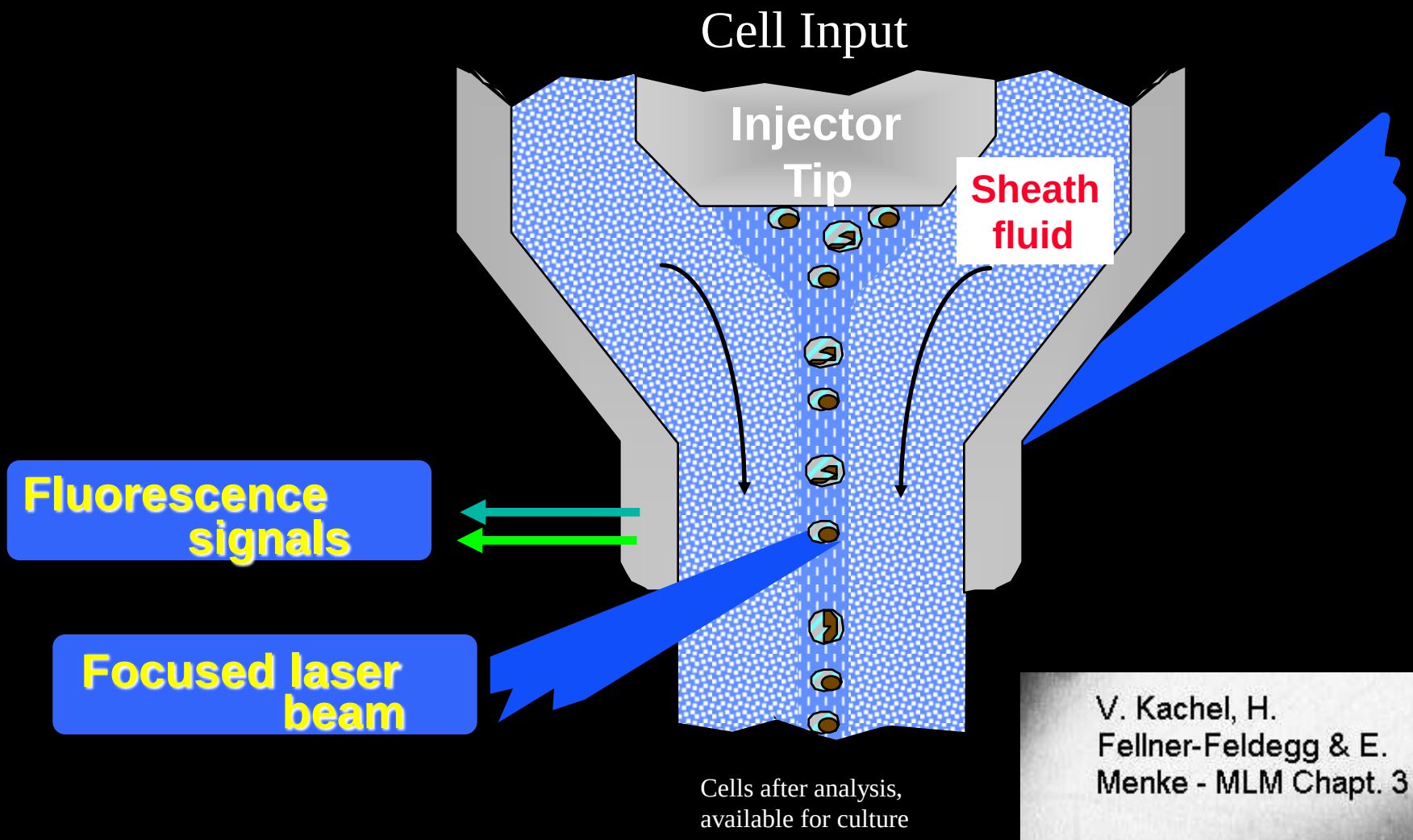
"Bead Array" assays

BD Biosciences

Flow Cytometers



Flow Cytometer Fluidics



CD-ROM Vol 3 Purdue University Cytometry Laboratories

Applications in research

BD
BD Biosciences

Applications of Microfluorimetry

Measurements

- specific structures by immunofluorescence or in-situ hybridization
- protein content
- DNA and RNA with fluorogenic dyes
- gene expression with fluorescent proteins
- auto-fluorescencent components
- enzyme activity with fluorogenic substrates
- pH and other cations with ion-specific probes
- redox potential
- …

Applications of Microfluorimetry

- Cell sorting
- Multi-parameter cell subset analysis
- Organelle visualization
- Protein translocation
- Large scale cell composition changes
- Single fluorescent molecule detection

  (PCR vs. direct)
  - Nucleic acid fragment sizing
  - Protein-ligand interactions
  - Virus counting

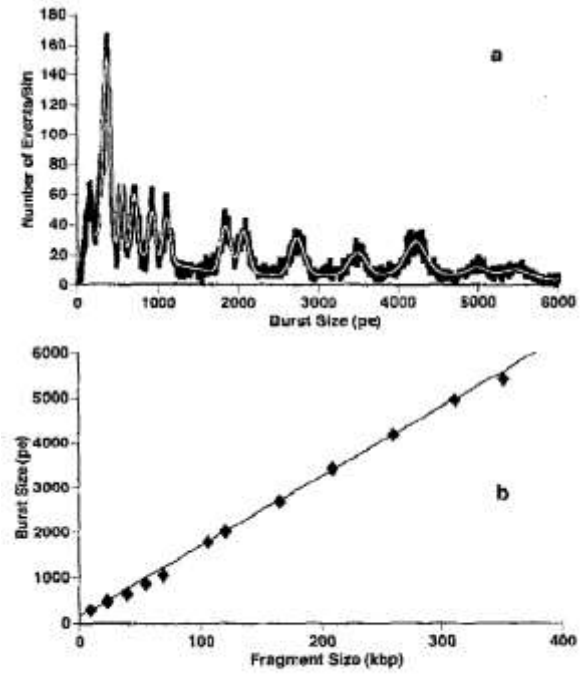
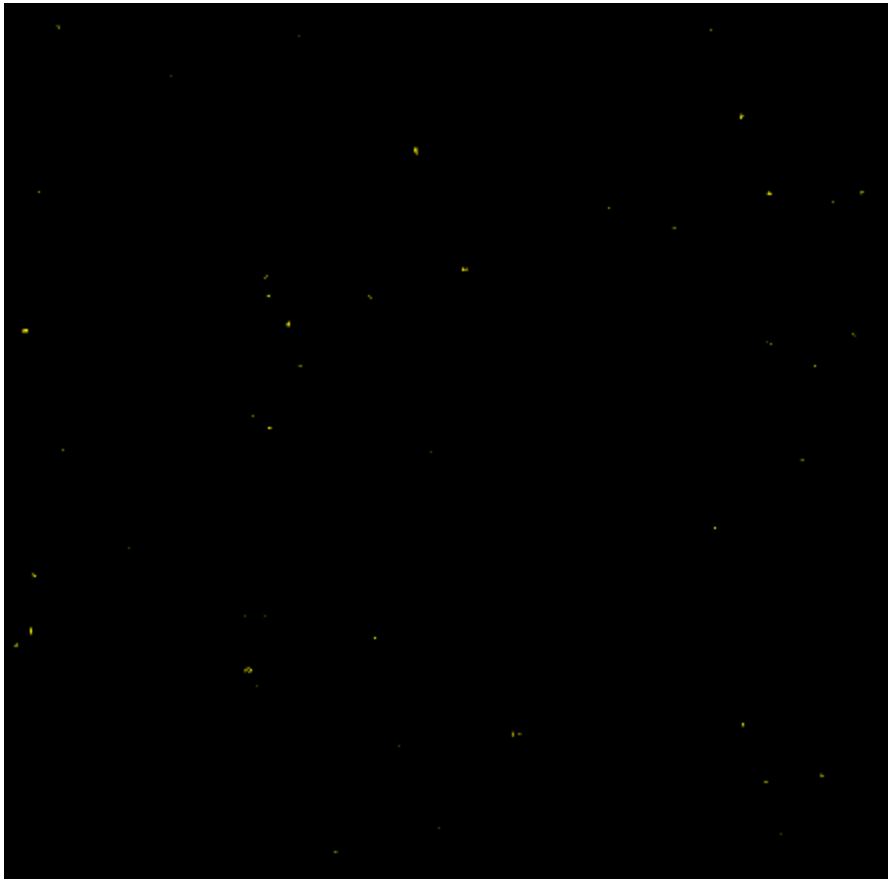


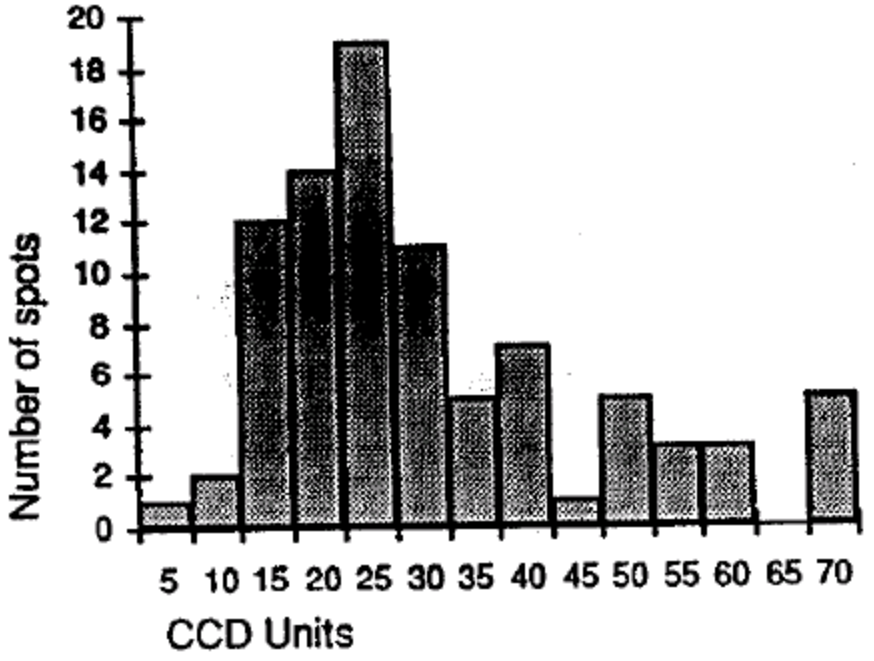
FIG. 2. a: Histogram of the fluorescence burst sizes of *Sma*I digestion of *S. aureus* DNA. Bin width was 10 pe.

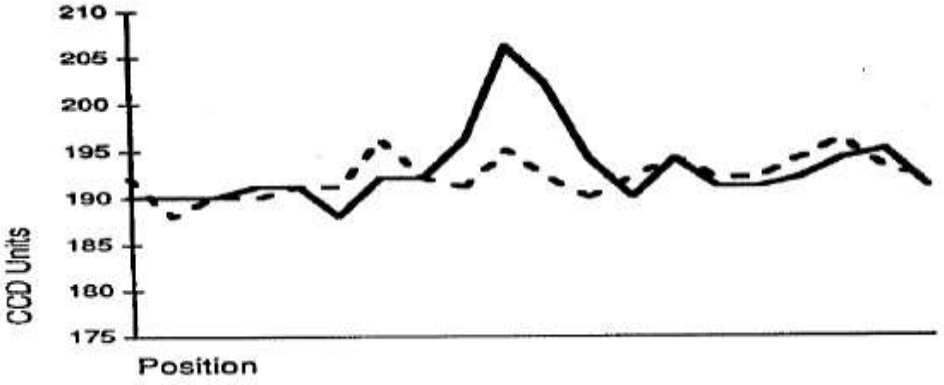
From: Huang Z et al. in Cytometry 35: 169-175 (1999)

BD Biosciences

Single Molecule Detection

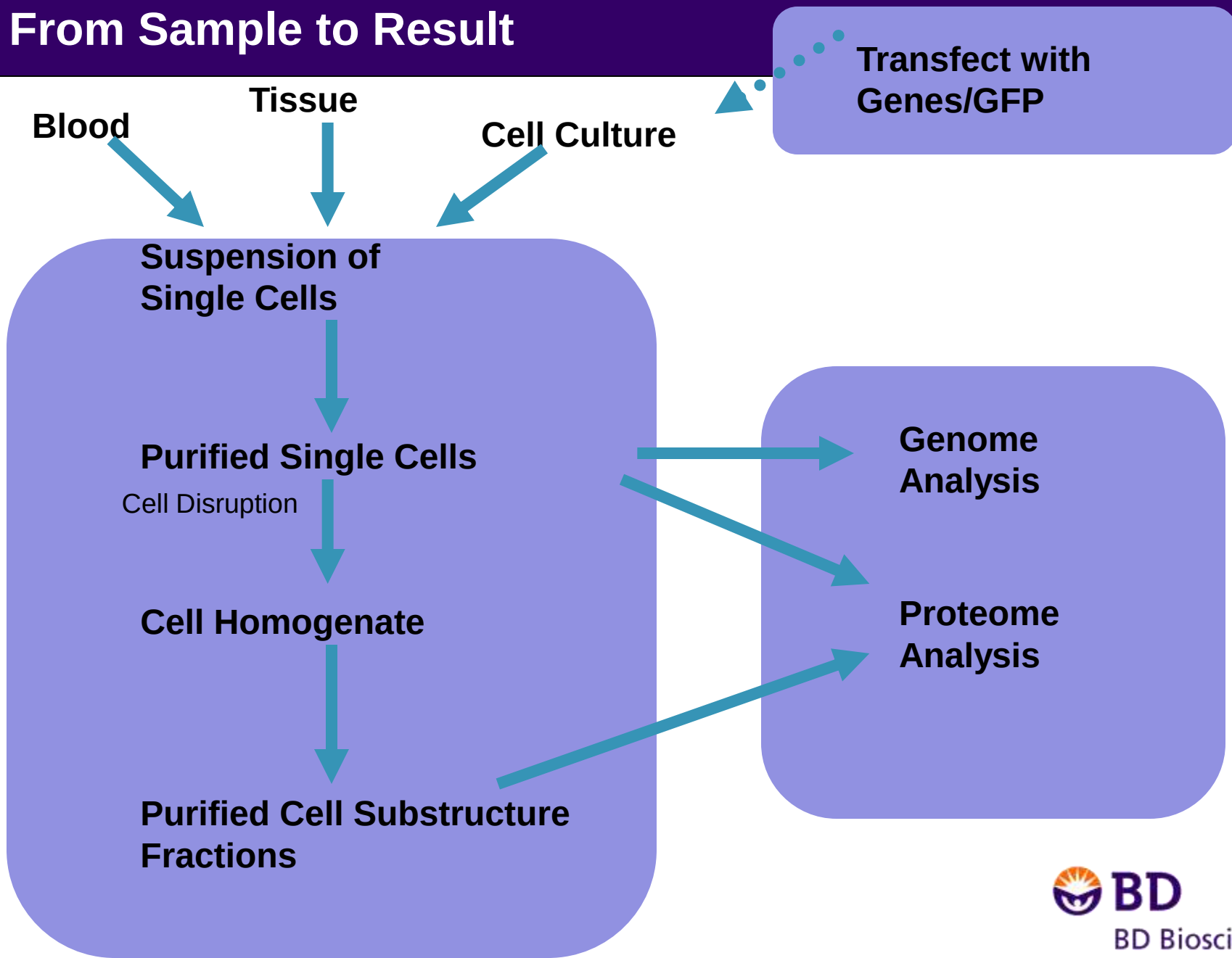






BD
BD Biosciences

Cellular Proteomics: From Sample to Result



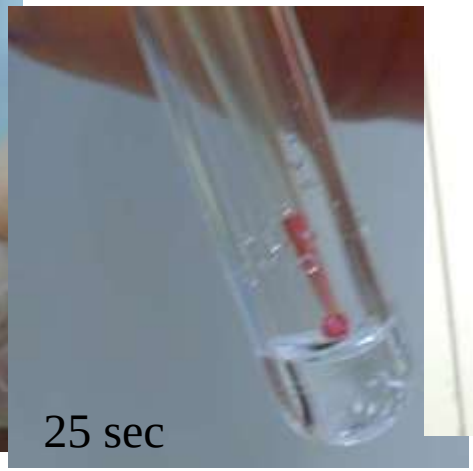
BD Biosciences

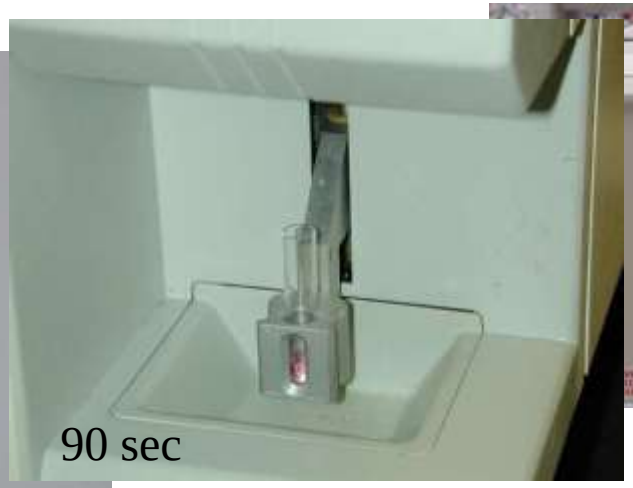
Applications in Bio-defense

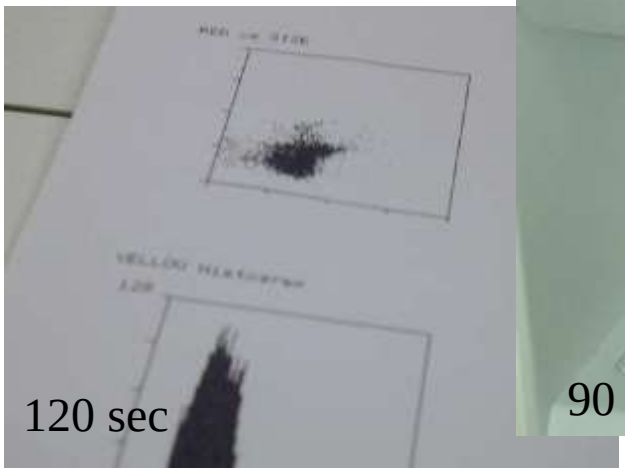
BD
BD Biosciences

Flow Cytometry for Microbiology

Rapid TVC Assay





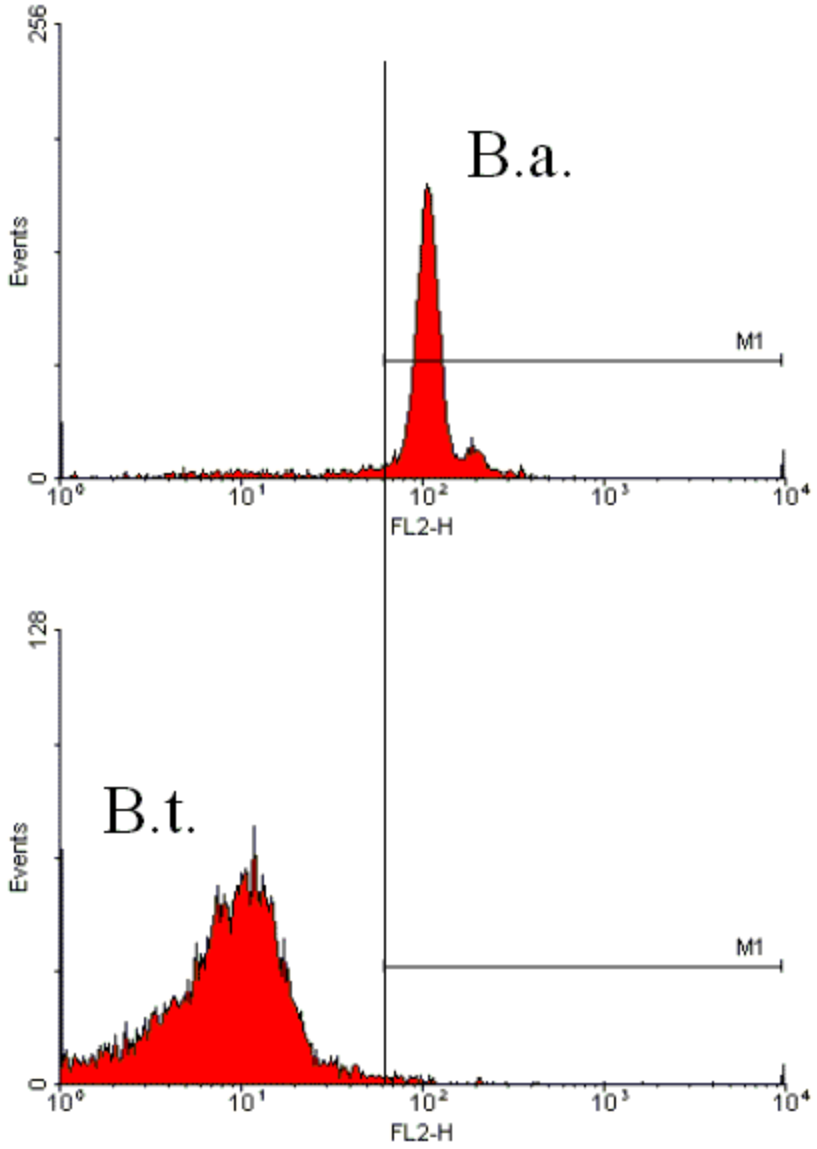


Rana Alsharif, BD Biosciences

BD

BD Biosciences

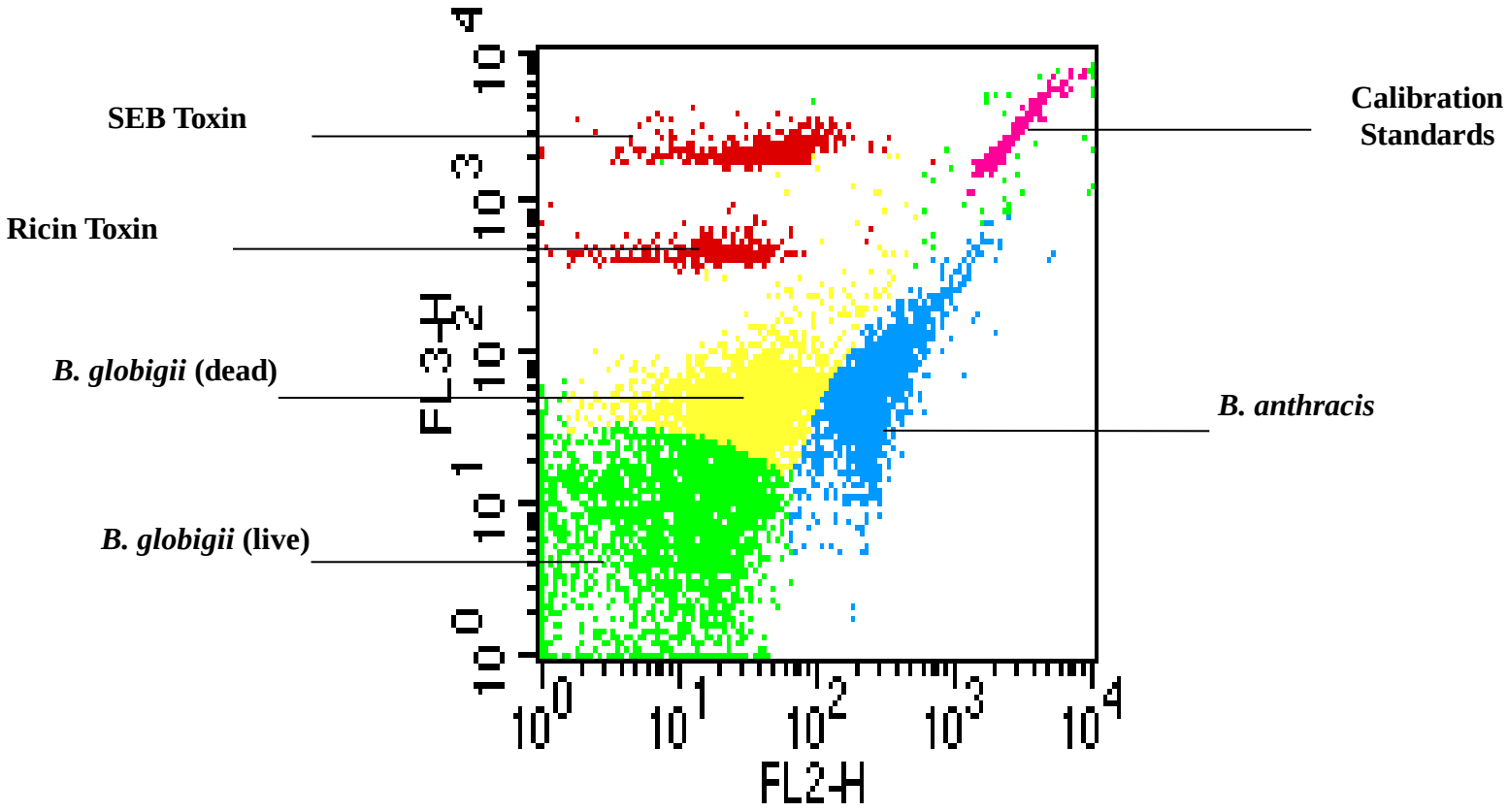
Rapid Bioagent Identification



Fluorescence based detection and identification of Bacillus anthracis within 10 minutes after receiving a sample using widely available instrumentation

BD
BD Biosciences

Simultaneous Measurements of Multiple Agents



BD
BD Biosciences

Applications in Clinical Diagnostics and Monitoring

BD
BD Biosciences

Clinical Tests (CD34 counting)

- Large scale protein analysis in patient serum with arrays

- Cell subset analysis (special hematology)
  - CD4
  - CD34
  - L&L

- Histopathology

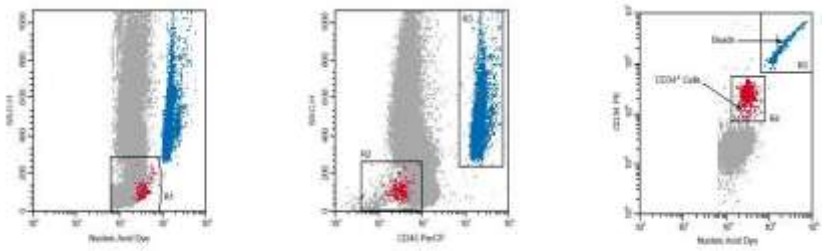


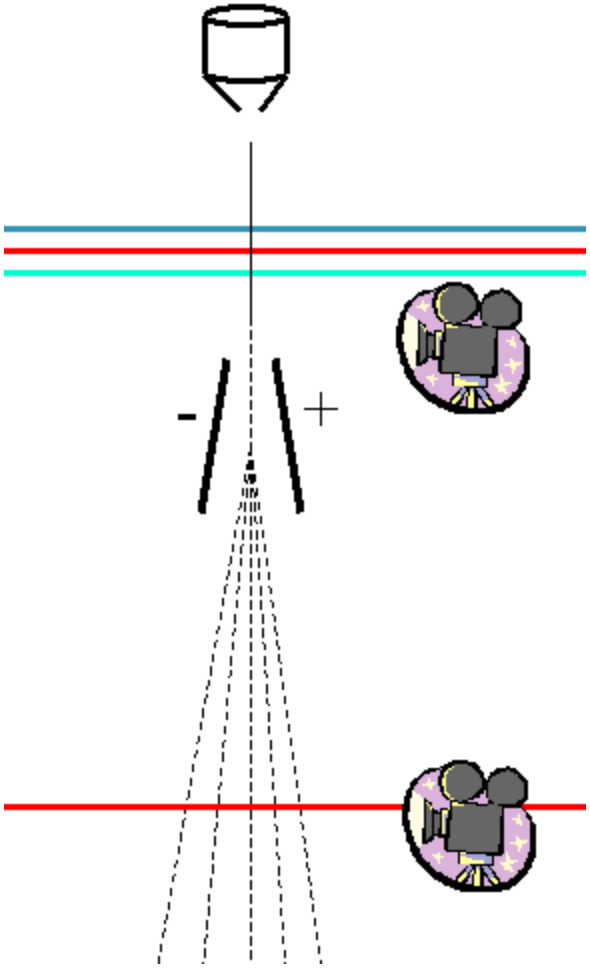
Figure 3. ProCOUNT Multiparameter Data Analysis Gating Strategy

BD
BD Biosciences

Therapy

BD
BD Biosciences

Cell Sorting



BD
BD Biosciences

CD34 progenitor cell sorting

Isolate human blood progenitor cells for cell transplantation

- CD34 at about 1% in mobilized peripheral blood
- for high purity several parameters are used
- 2-5*10^6 CD34 cells needed for treatment
- analysis rate maximally at 10^5 cells sec^{-1}
- sort rate at 2*10^4 cells sec^{-1}
- several hours of sorting required

BD
BD Biosciences

Outlook

BD
BD Biosciences

Challenges

- Better use of single molecule sensitivity
- Absolute quantitation
- Better multiplexing
- Efficient use of intrinsic fluorescence

END

BD
BD Biosciences

BACKUP SLIDES

BD
BD Biosciences

Limits of Detection by CCD Microscopy

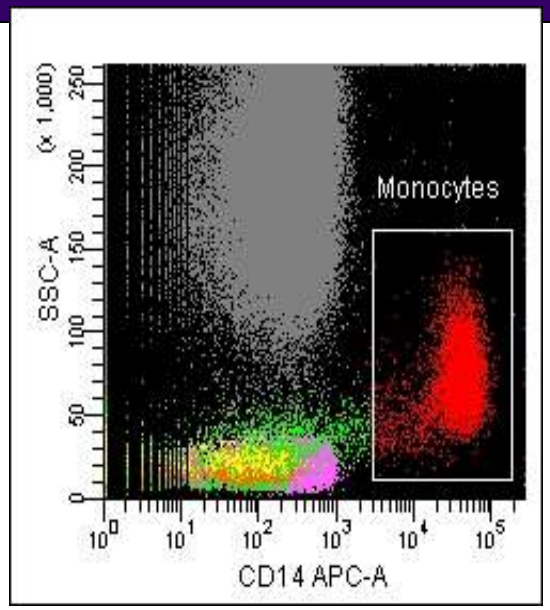
Diether Recktenwald, Janette Phi-Wilson, Ben Verwer 1993

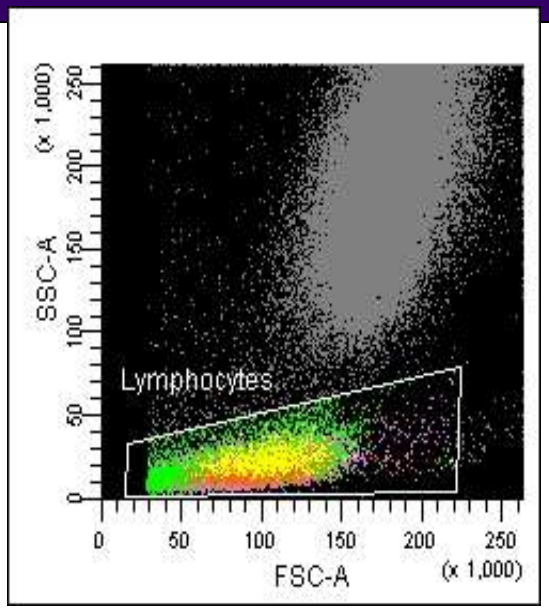
Fluorescein	1000 Molecules/Pixel
R.-Phycoerythrin	15 Molecules/Pixel

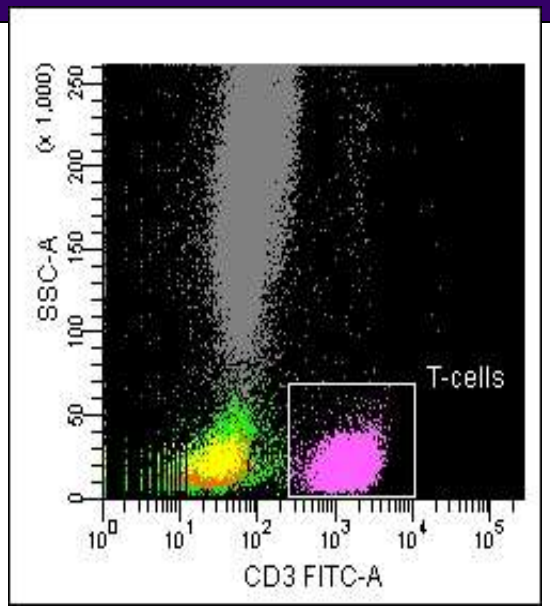
Factors limiting practical use:

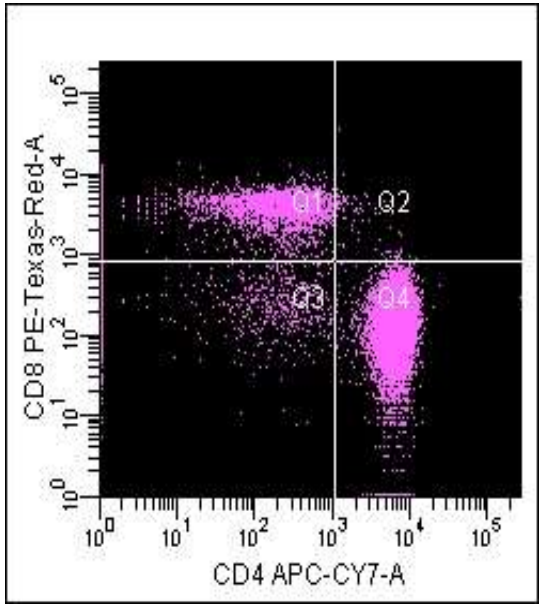
- biological autofluorescence
- photo-bleaching
- diffusion

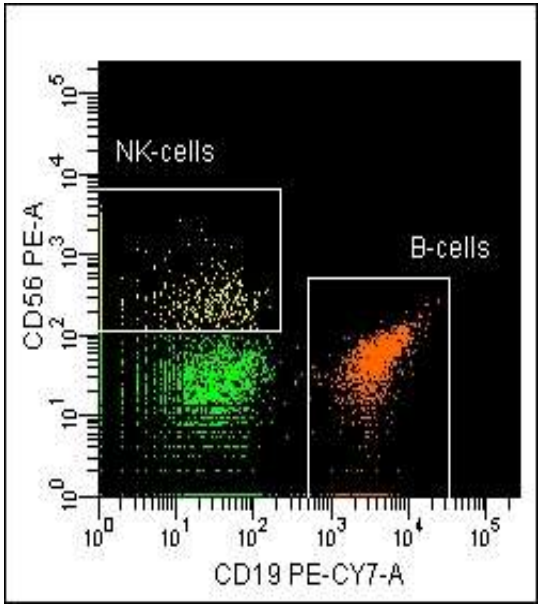
Multi-color analysis (6-color example)





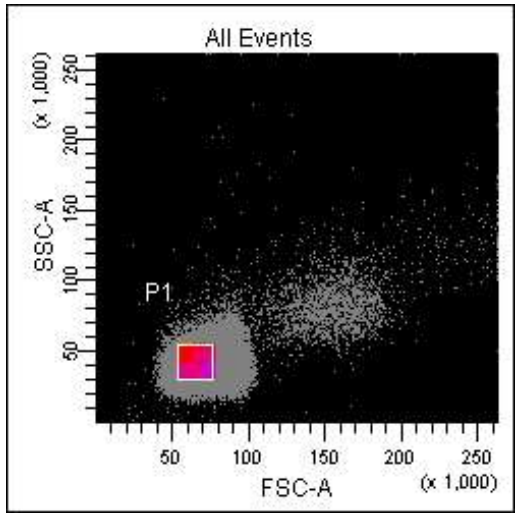


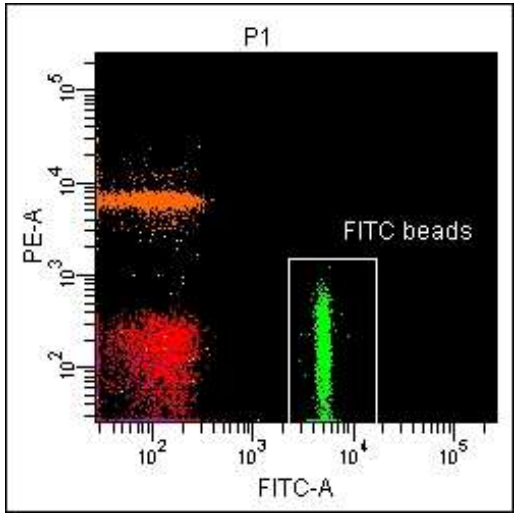


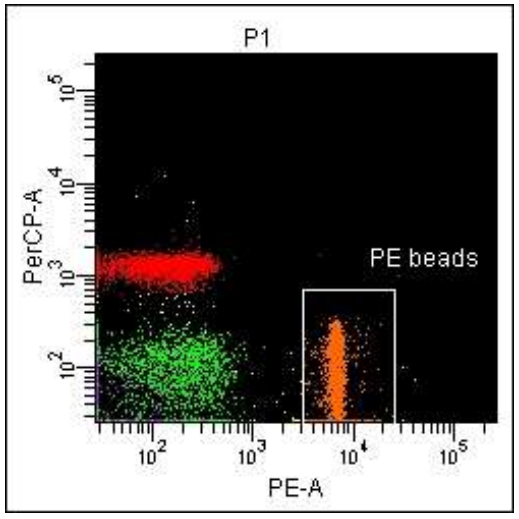


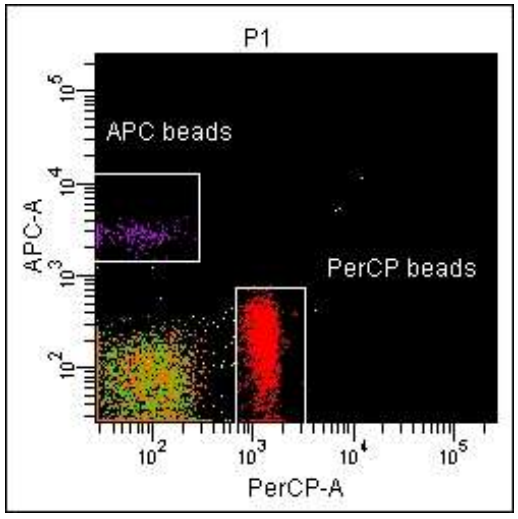
BD
BD Biosciences

Digital Cytometry FACS (Pre- Sort)





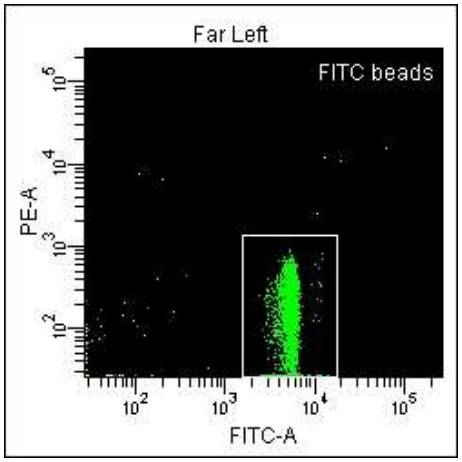


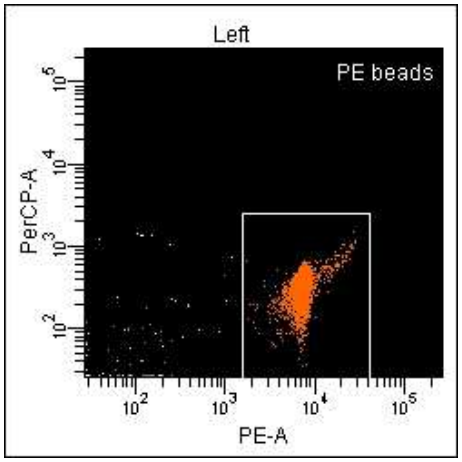


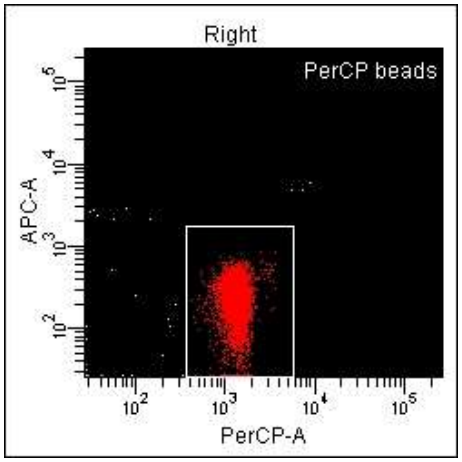
45 psi
64 kHz
20,000 events/s

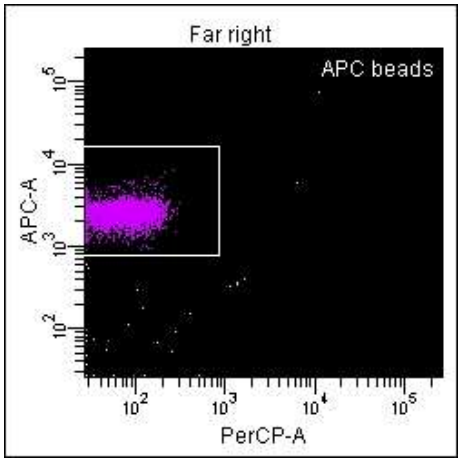
BD
BD Biosciences

Digital Cytometry FACS (Purity)









FITC	PE	PerCP	APC
8.2%	9.5%	8.0%	0.6%
99.1%	99.2%	99.5%	99.4%

BD
BD Biosciences

Abstract

- The understanding of complex biological systems requires the quantitative measurement of DNA, RNA, proteins, and metabolites in-vivo with high sensitivity. Micro-fluorimetry with modern light sources and detectors has demonstrated single molecule sensitivity. Instrumentation and assays with these capabilities for the measurement of cellular properties and secreted proteins will be discussed, including diagnostic and therapeutic uses of the technology.

- Suggested reading:
- 1.Mandy F, Varro R, Recktenwald D (2003) Flow Cytometry Principles in Biomedical Photonics Handbook, Tuan Vo-Dinh ed, CRC Press 2003
- 2.Enderlein J., Ambrose WP, Goodwin PM, Keller R (1999) Fluorescence Detection of Single Molecules, http://www.joerg-enderlein.de/pubs/book99.pdf
- 3.Baumgarth M, Roederer M (2000), JIM 243, 77. A practical approach to multicolor flow cytometry for immunophenotyping (http://facs.scripps.edu/BRJIM.pdf)

BD

BD Biosciences