BMS 631 - LECTURE 15
Flow Cytometry: Theory

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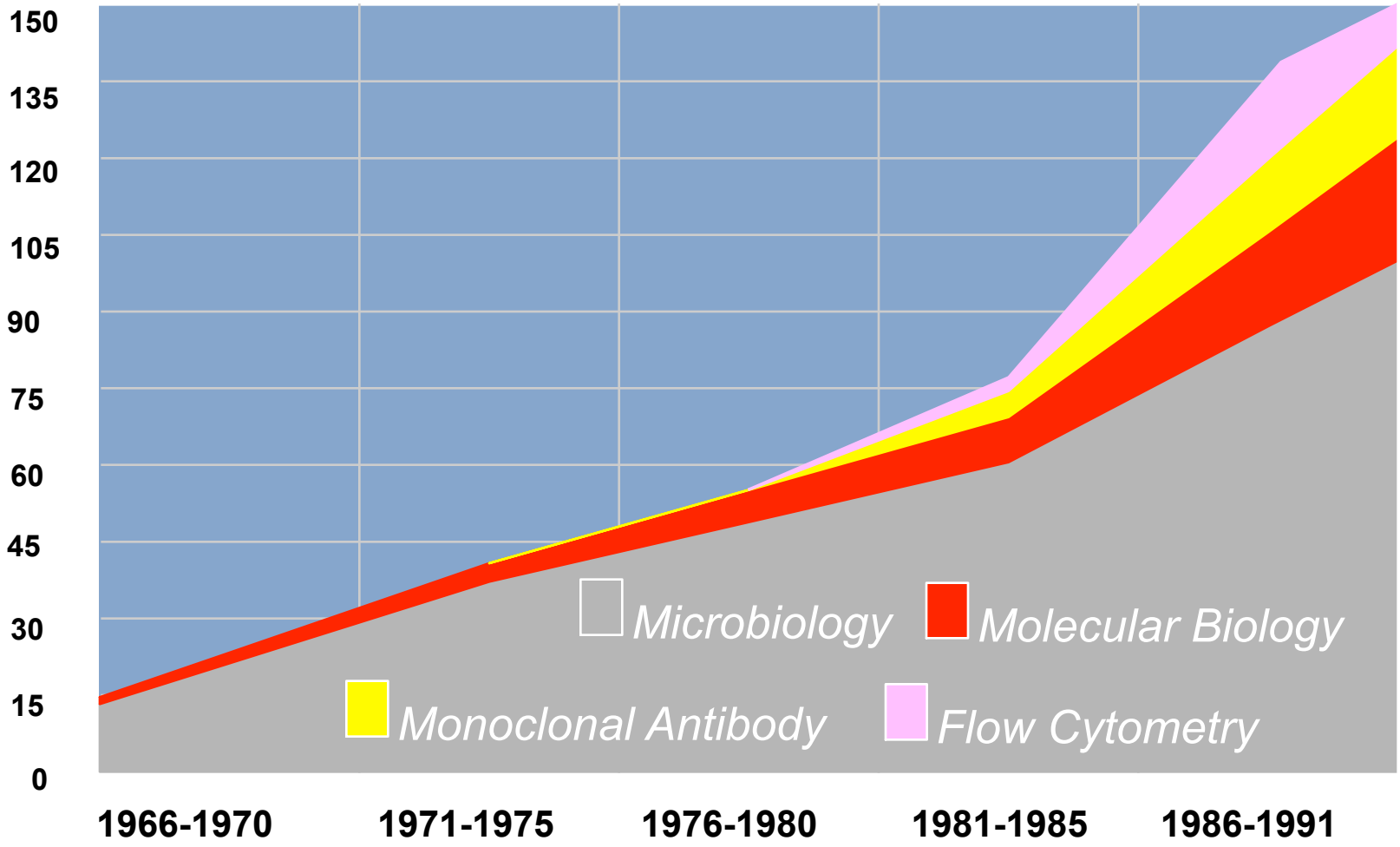
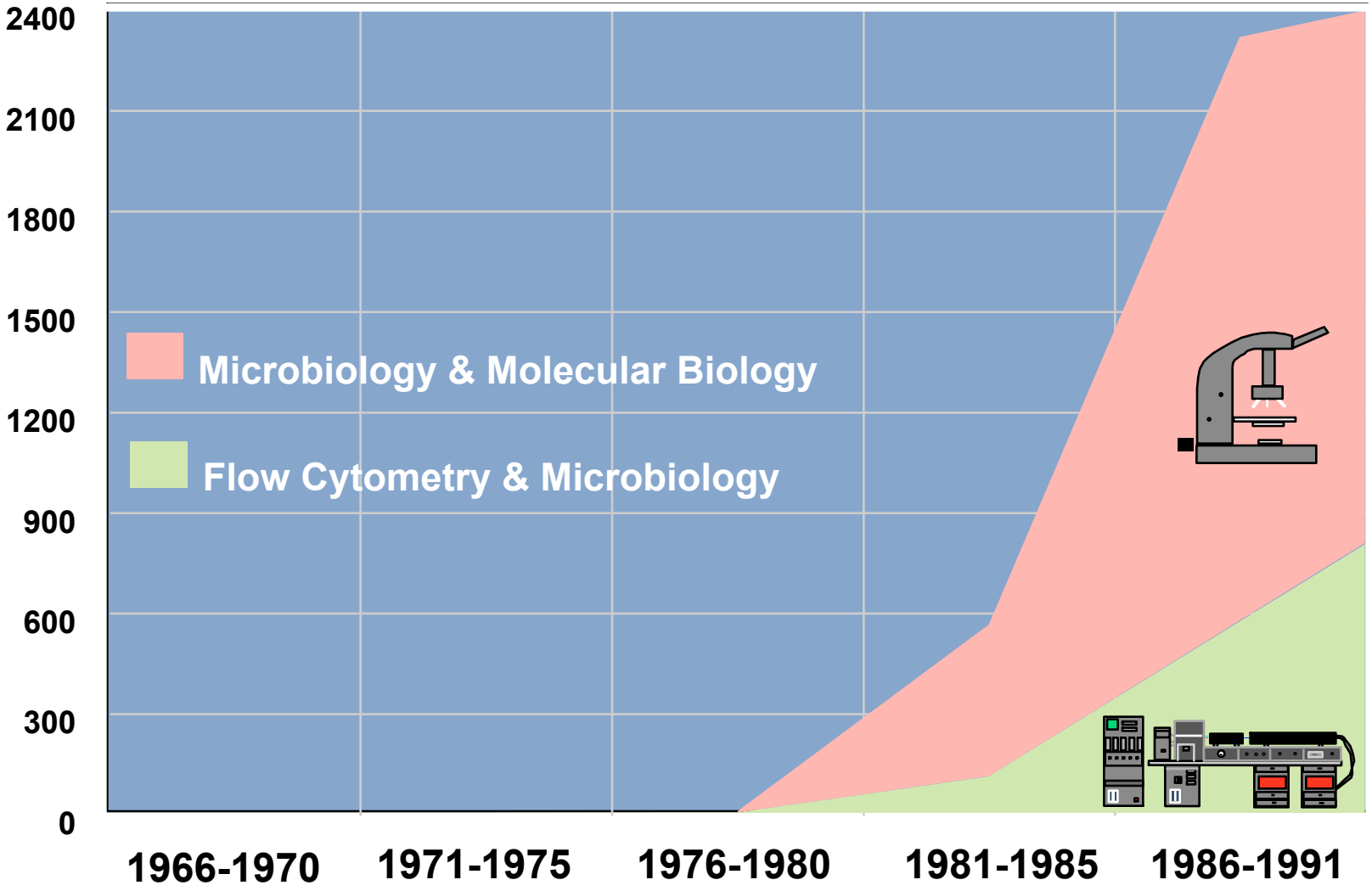
Flow Cytometry & Microbiology

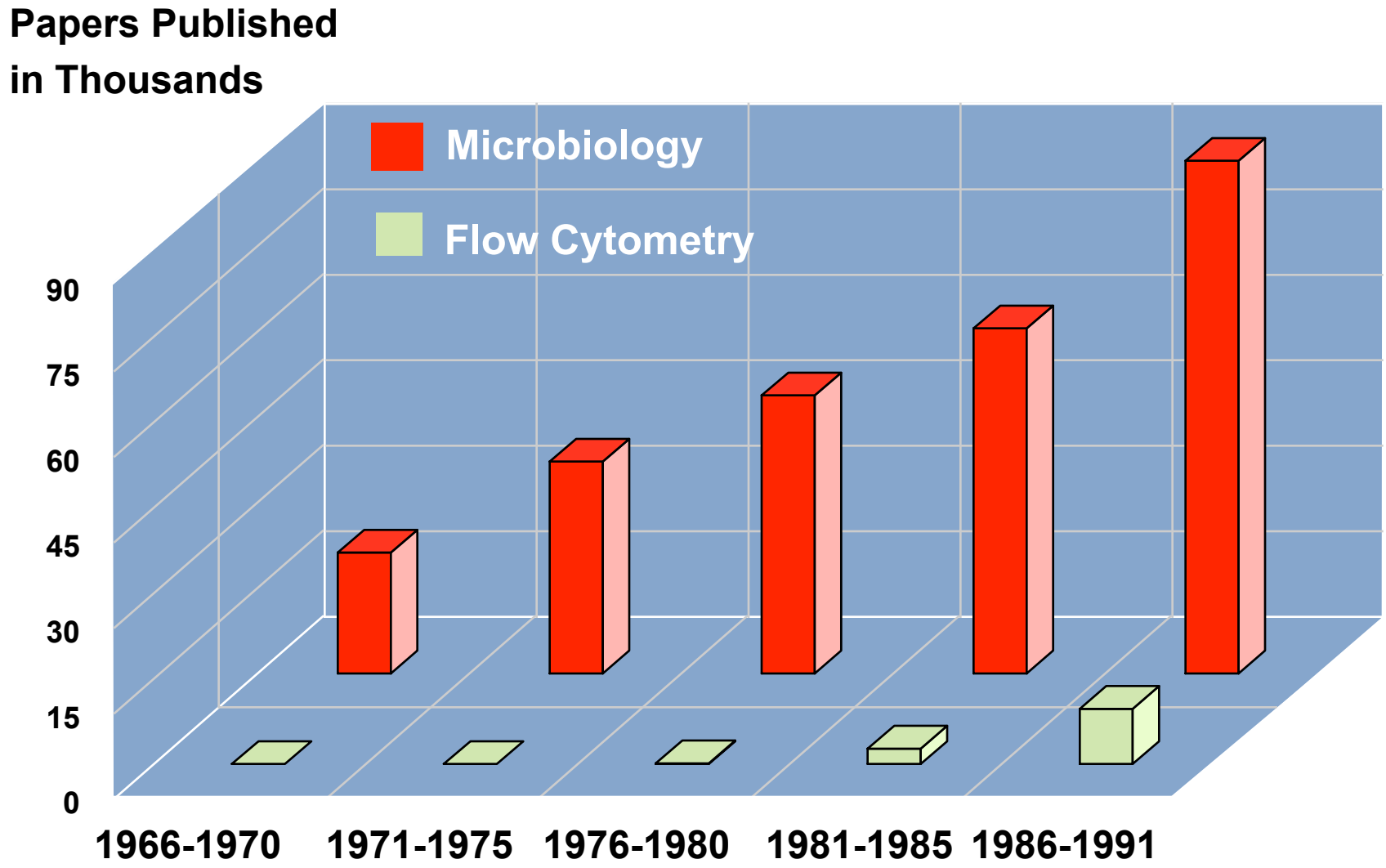
- History
- Major problems
- Potential applications
- Clinical applications
- Future

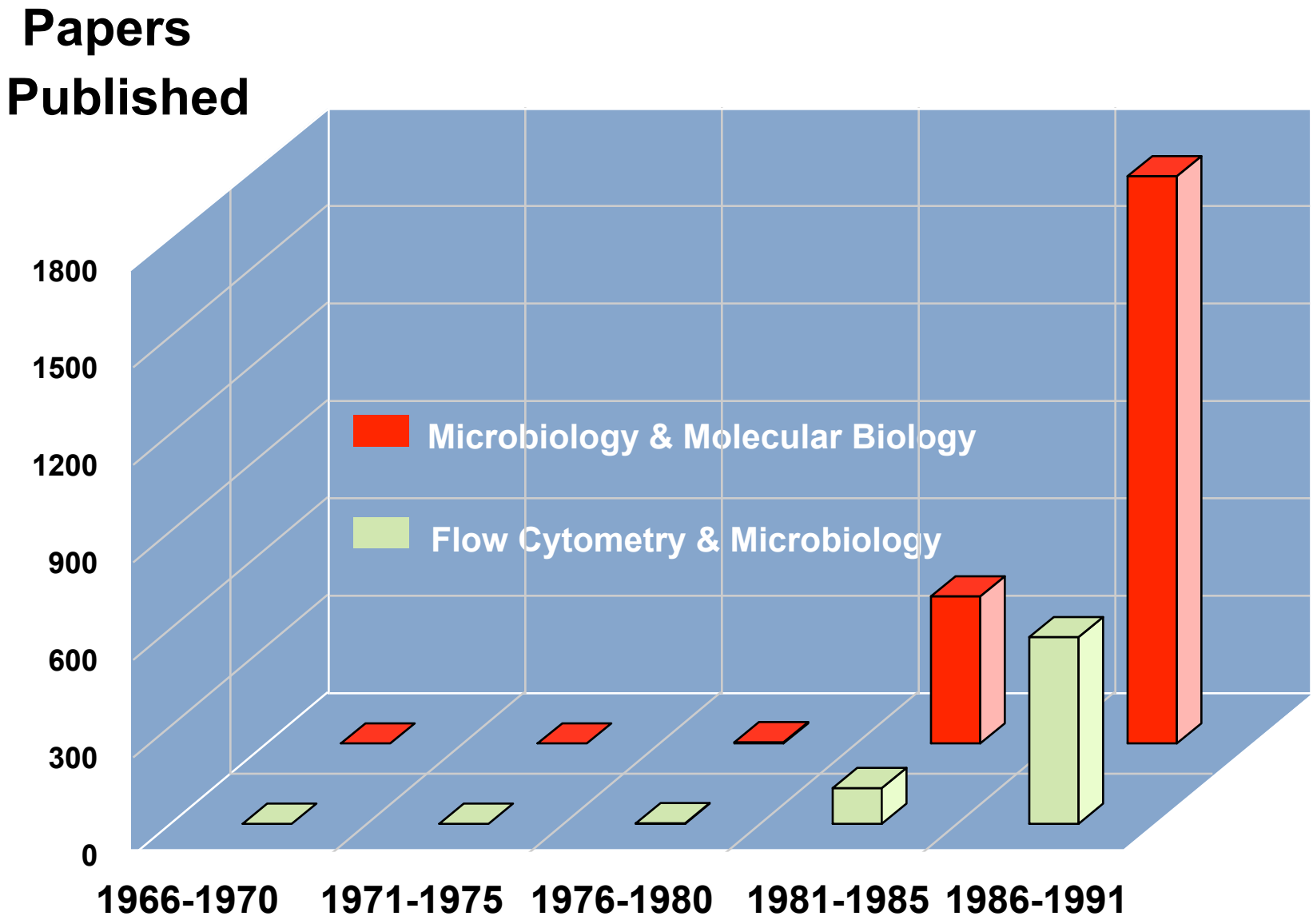
Publications in Thousands



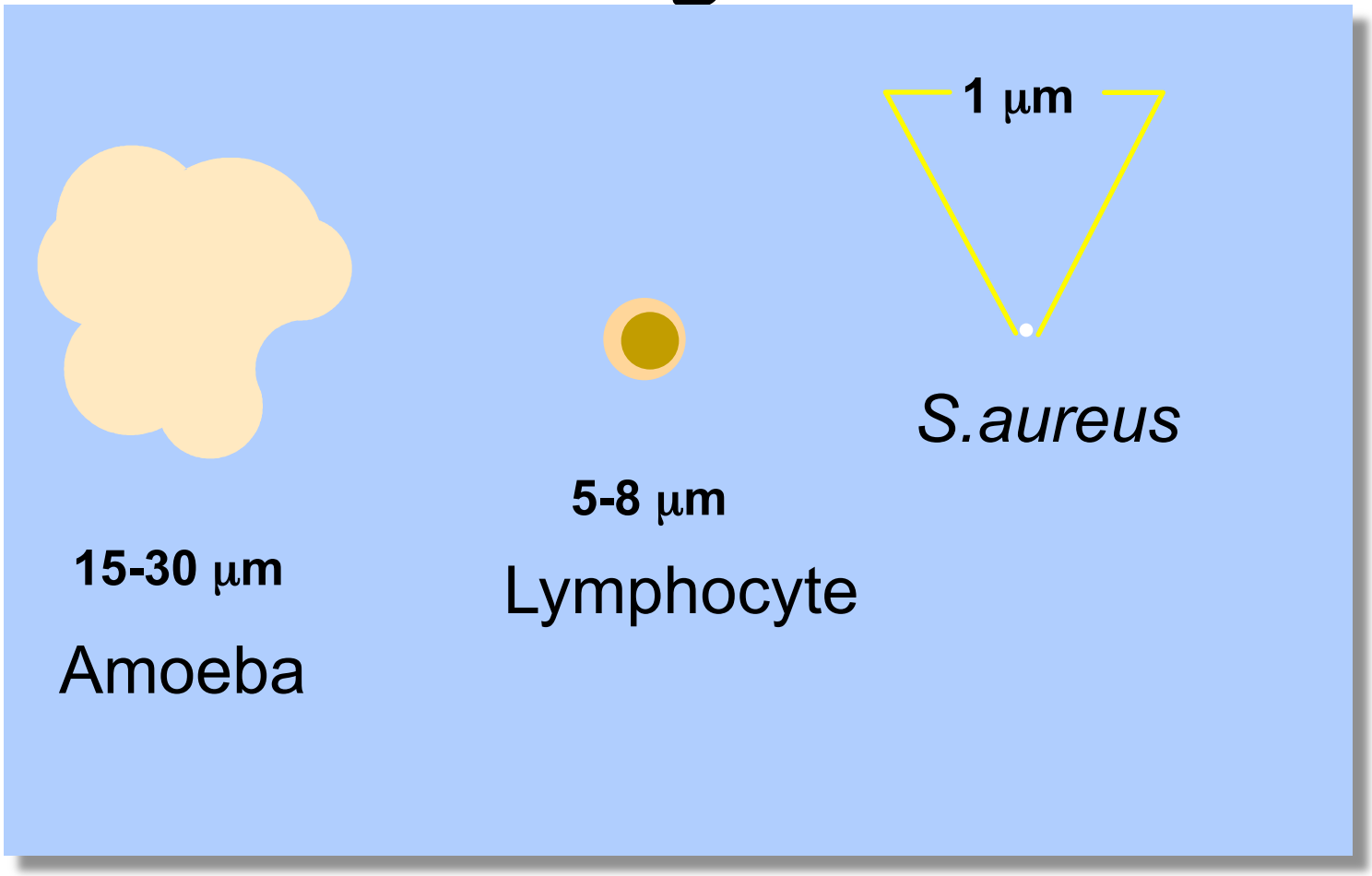
Papers Published







Relative Sizes of Biologicals



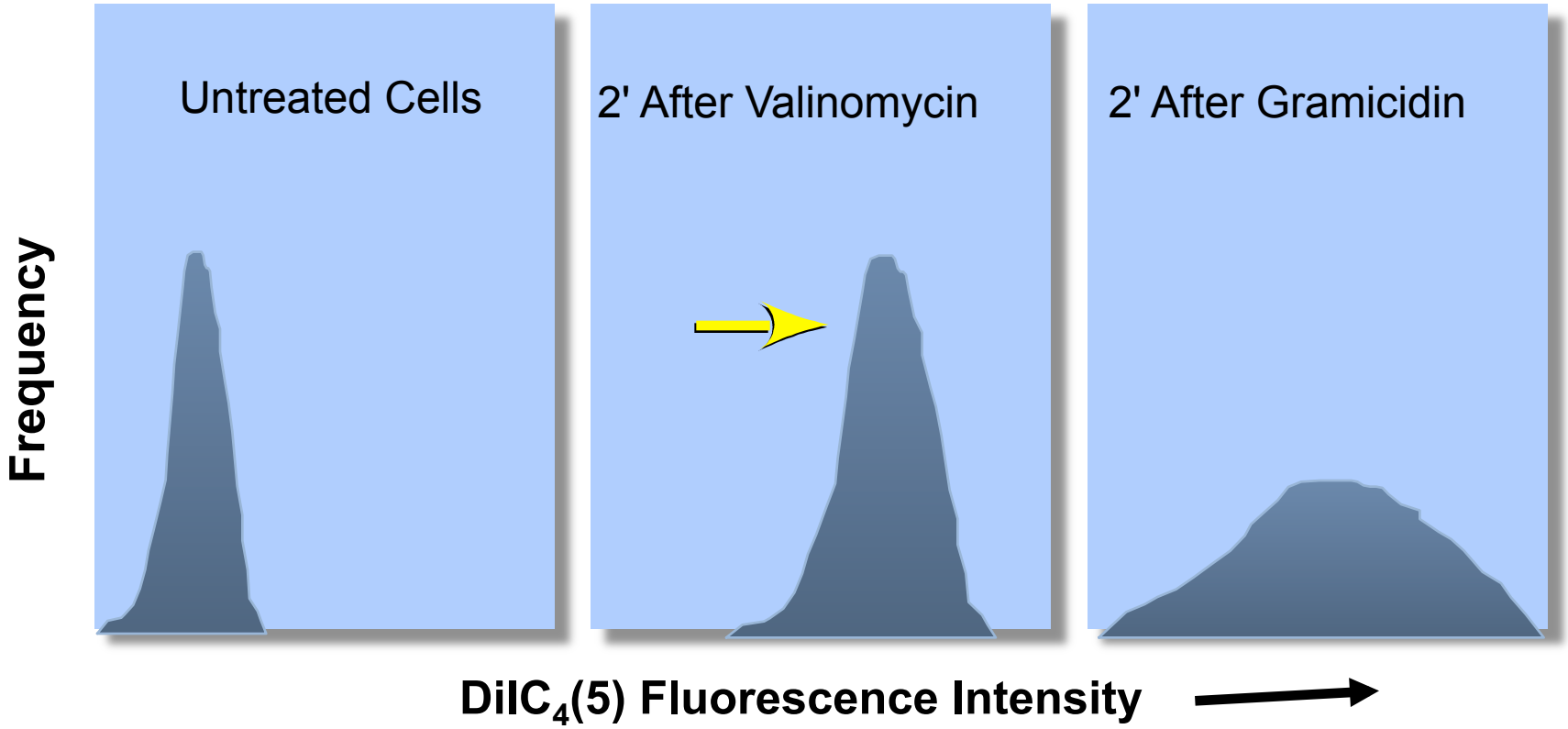
Relative Ratios

Measurement	Bacteria	Yeast	Eukaryotic
Linear	0.5-5	3-5	
Surface $^{10\text{-}30}$	3-12	30-75	300-3000
Volume	0.3-3	20-125	500-1500
Dry Cell Mass	1	10	300-3000

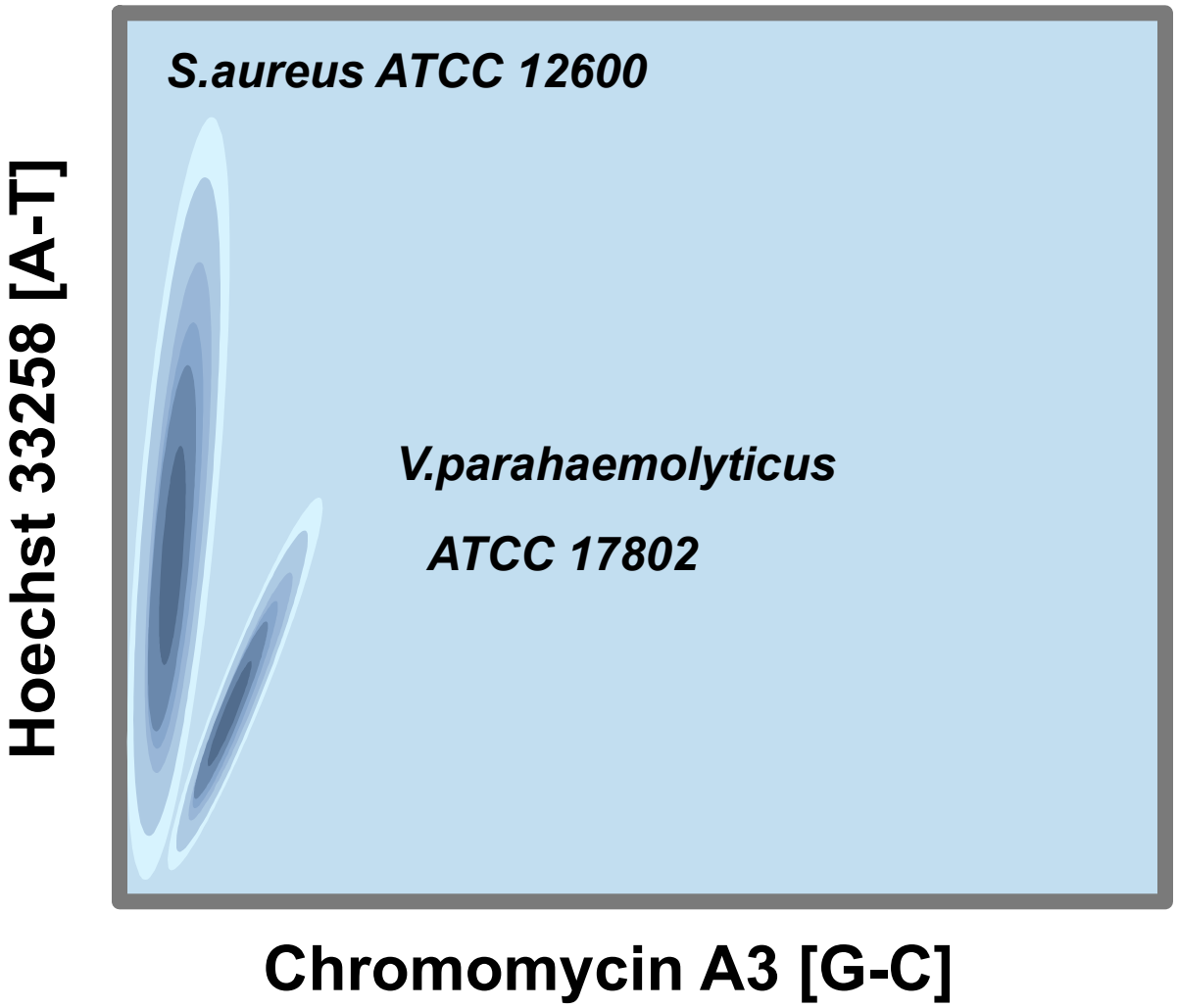
Membrane Potential

1. Presence of live bacteria
2. Partial identification
3. Quantitation
4. Antibiotic sensitivity

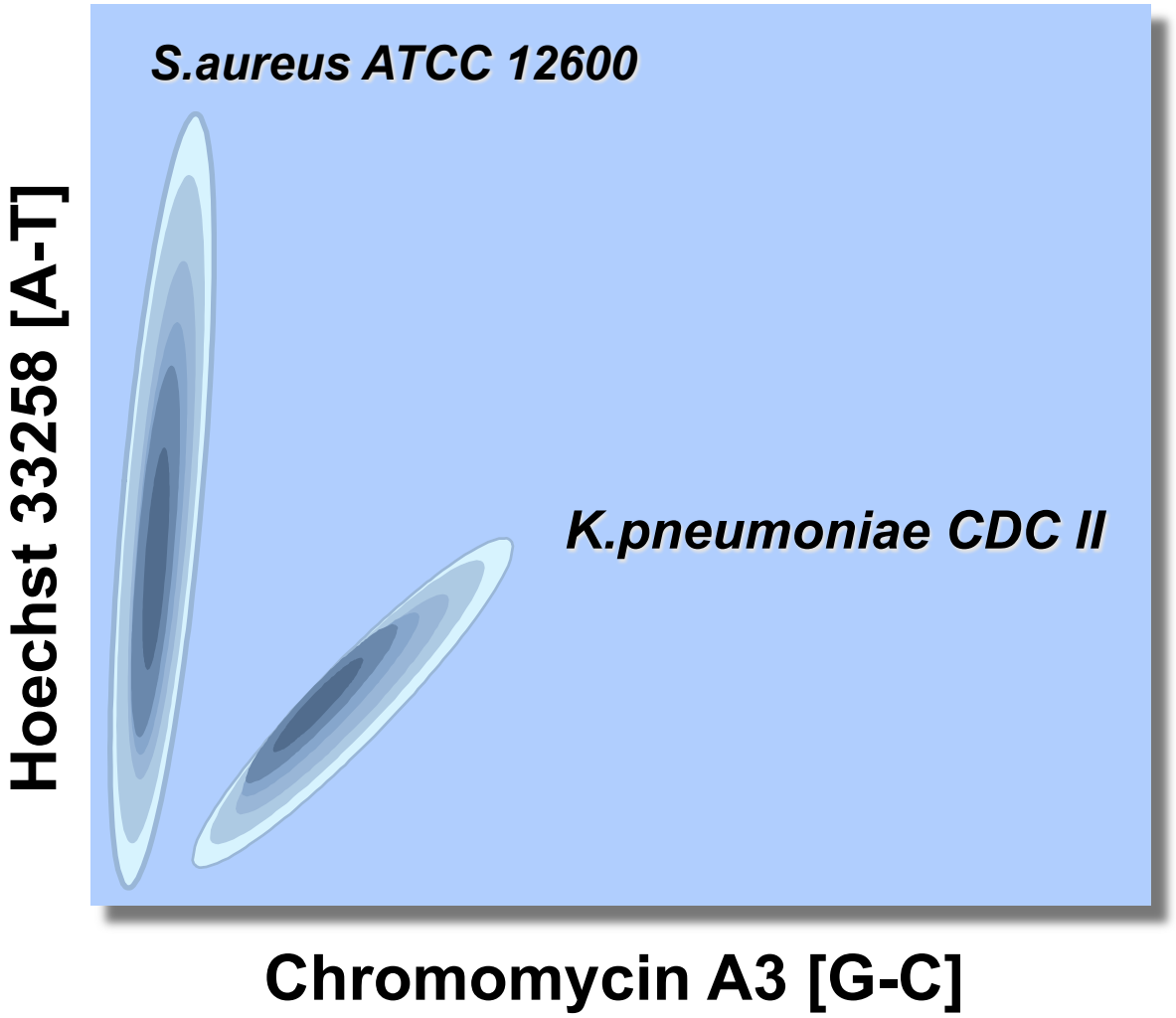
Application of Membrane Potentials in Flow Microbiology



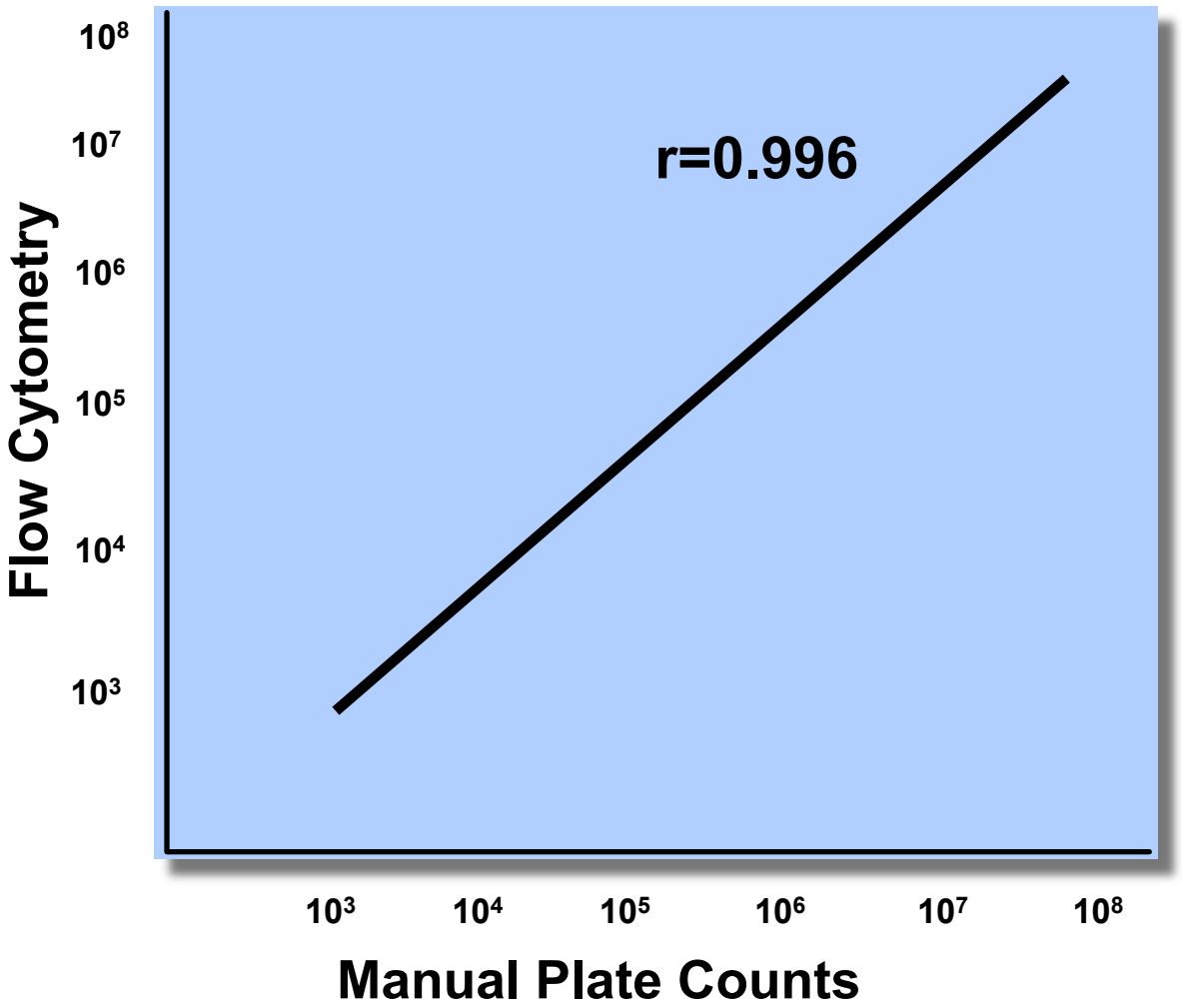
Ratios using DNA Dyes



Ratios using DNA Dyes



Comparison of Flow & Traditional Methods



Clinical Microbiology Applications

Required Information

1. Bacterial presence
2. Concentration/number
3. Identification
4. Antibiotic sensitivity

Blood

- Too many cells
- Too few bacteria

CSF

- Too few organisms
- Blood cells present

Urine

- High organism count
- 50% of specimens

Clinical Microbiology

Infectious Diseases

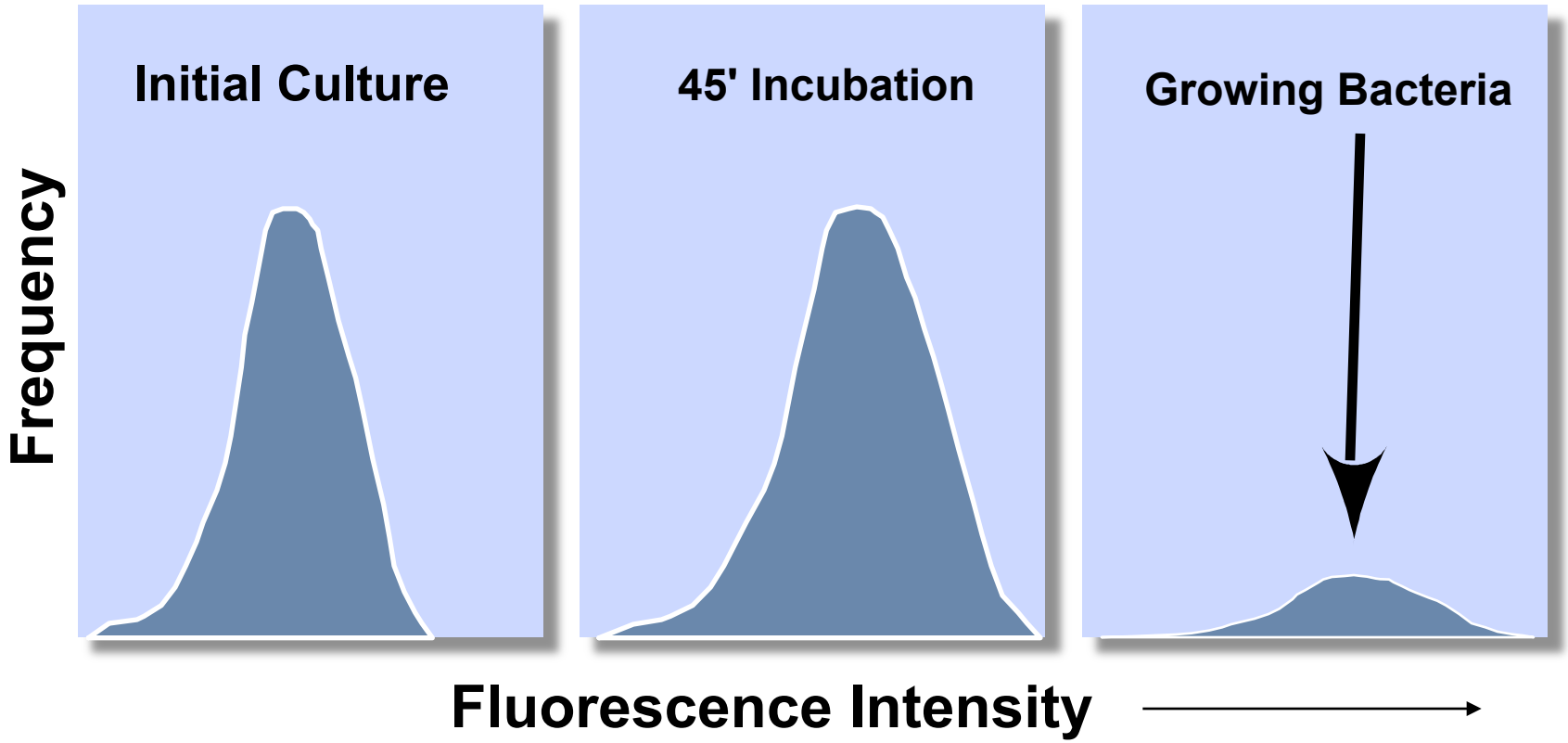
200 x 10^6

Samples/year

Urine Analysis

1. 50% of workload
2. 100 x 10^6
3. ~80% samples negative
4. 5-24 hour detection time

Determination of Growth Rates



Strategies for Detection of Microorganisms by Flow Cytometry

- Detect any microbe present in sample
- Determine if the microbe is viable
- Determine if a particular species or strain of organism is present in sample

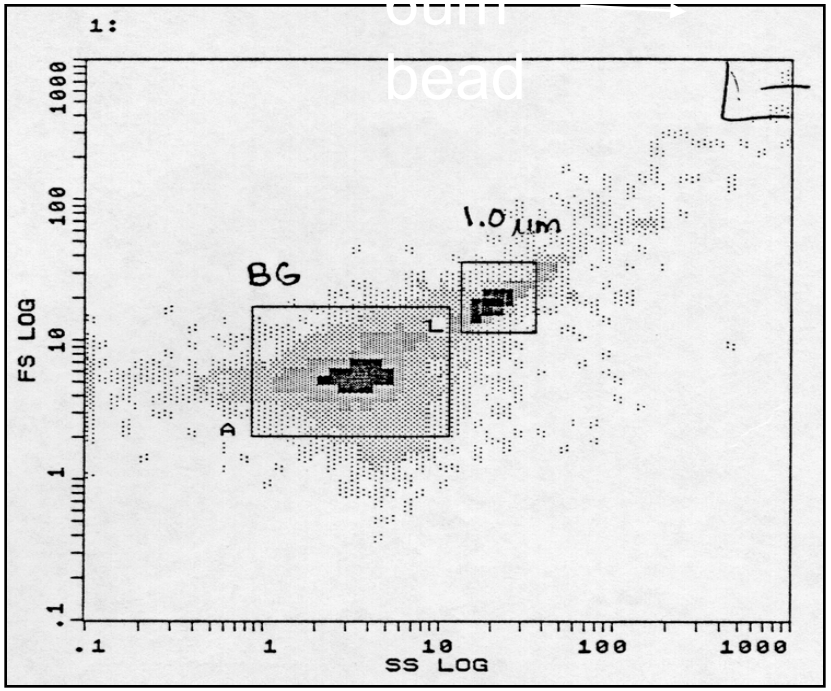
Five Strategic Components

- Quality Control
- Light scatter of bacteria
- Detection of bacteria using fluorescent dyes
- Organism viability
- Specific identification of pathogenic bacteria

Recommended Quality Control Procedures for Microbiological Applications of Flow Cytometry

- Standard instrument set-up (alignment beads)
- Filter sheath fluid and buffers with 0.1 um filter
- Spike bacteria samples with latex beads
- Reference standards for bacteria

i.e. Fixed *E.coli* cells, *Bacillus* spores



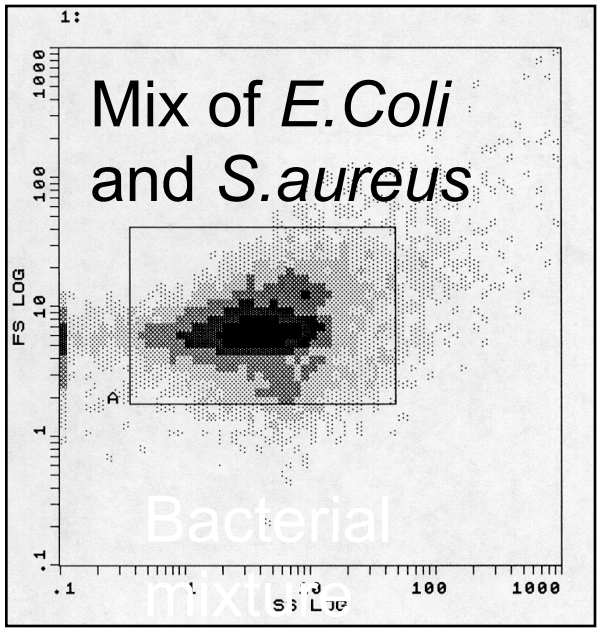
Bacillus subtilis spores spiked with 1.0 um latex beads.

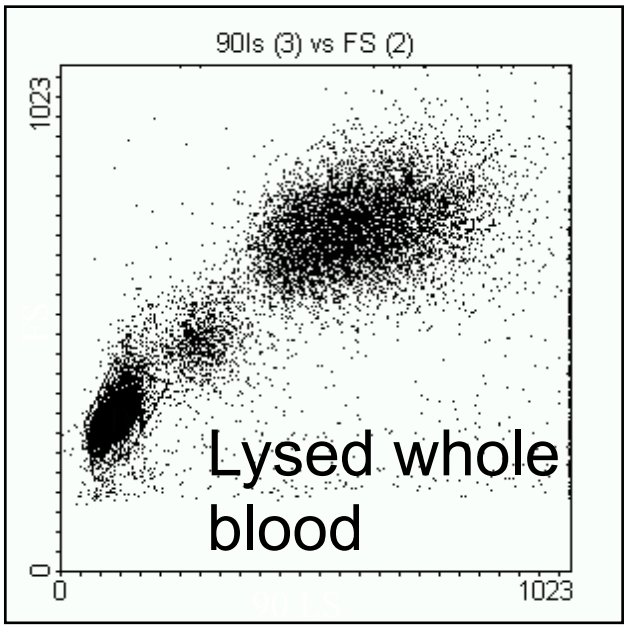
Light scattering profiles for qualitative analysis of pathogenic bacteria

- Set discriminator or threshold to reduce amount of debris
- Establish regions of interest
- Spike bacterial samples with latex beads of known size

Prokaryotes vs. Eukaryotes

Comparison of light scatter profiles of prokaryotes and eukaryotes.





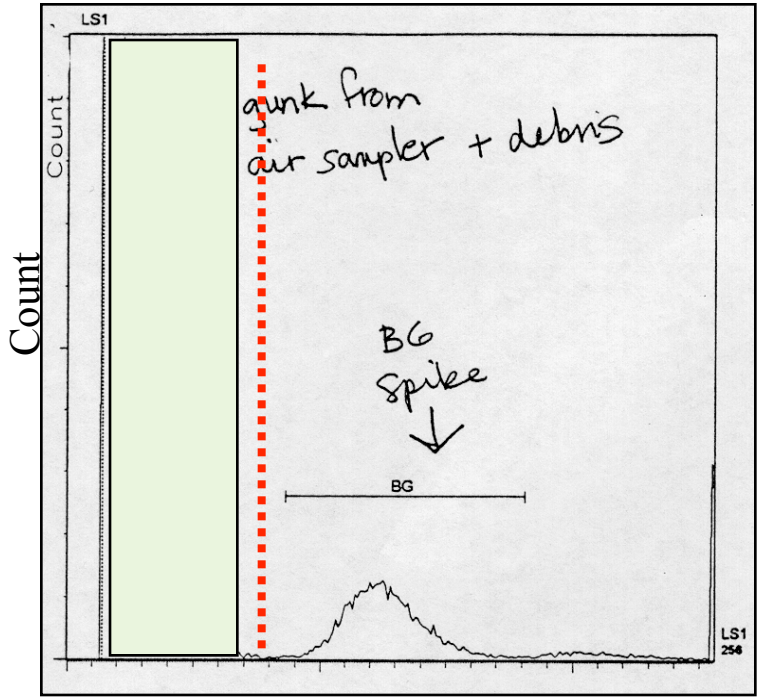
- Size, mass, nucleic acid and protein content of bacteria is 1/1000 of mammalian cells

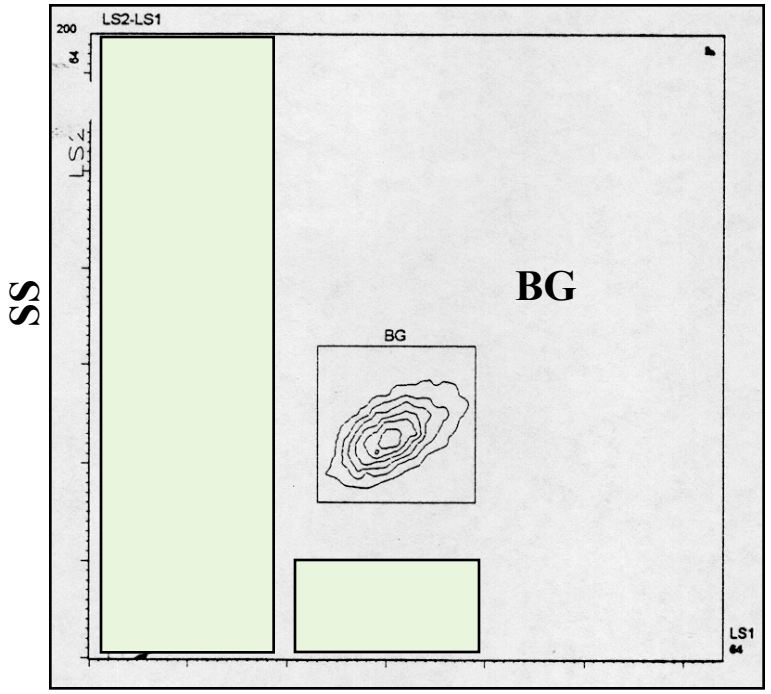
- In bacteria, considerable variation in accessibility of cell interior to dyes
  - -gram-negative vs. gram-positive
  - -vegetative cells vs. spores
  - -capsule formation
  - -efflux pump

Microbial Discrimination and Identification Using Light Scattering

- Debris and nonbiological particulates
- Sample preparation
- Growing bacteria
  - single cells vs. chains/clusters
- Mixed suspensions of bacteria
  - size vs. refractive index
  - vegetative vs. spores

Debris vs. Bacteria

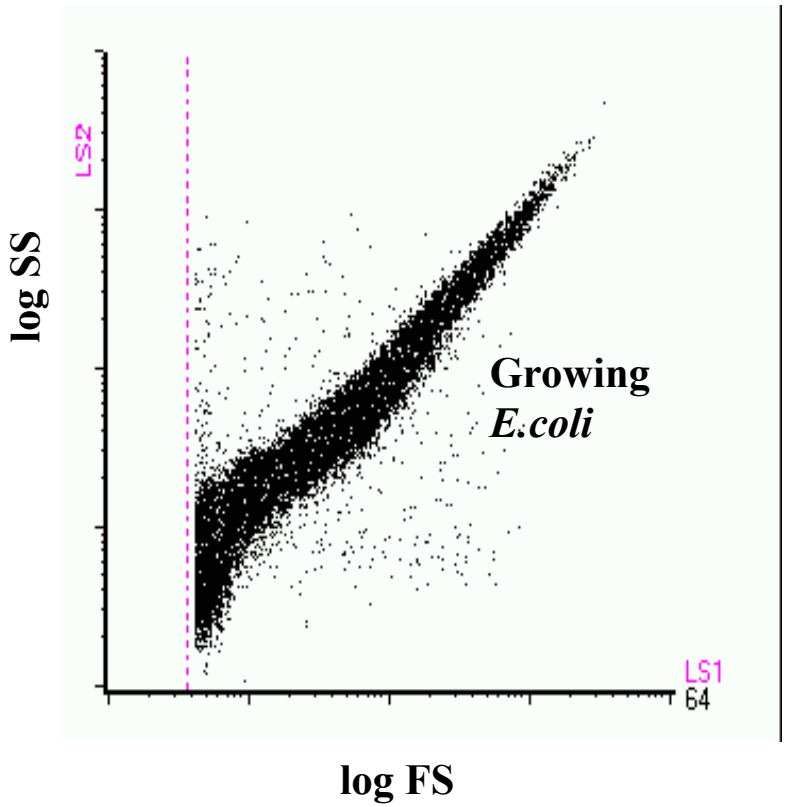




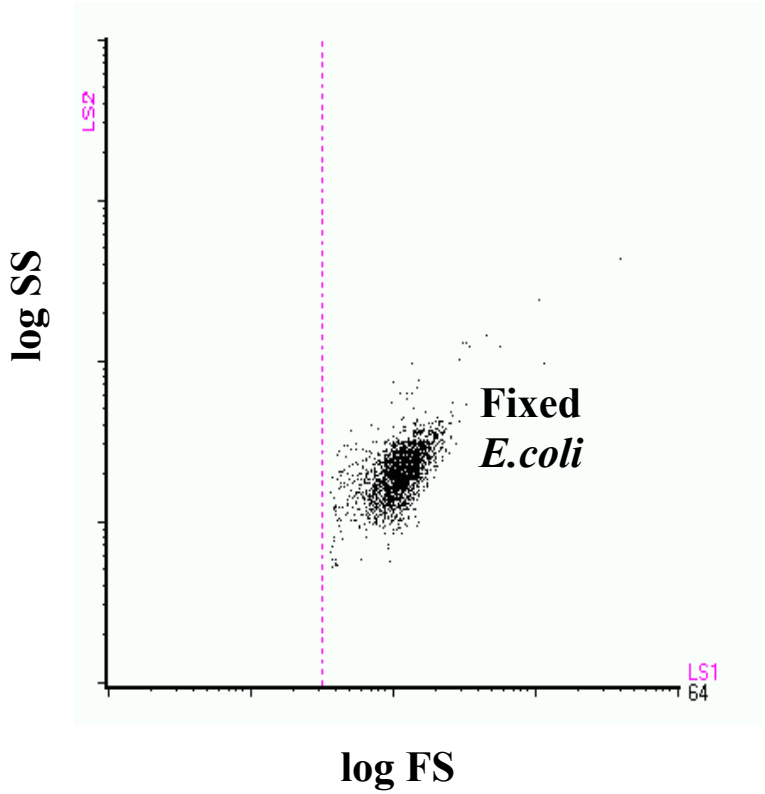
Aerosol sample of *Bacillus subtilis* spores with debris.

Light Scatter Changes
Growing Culture vs. Fixed cells

Growing culture of *E.coli*

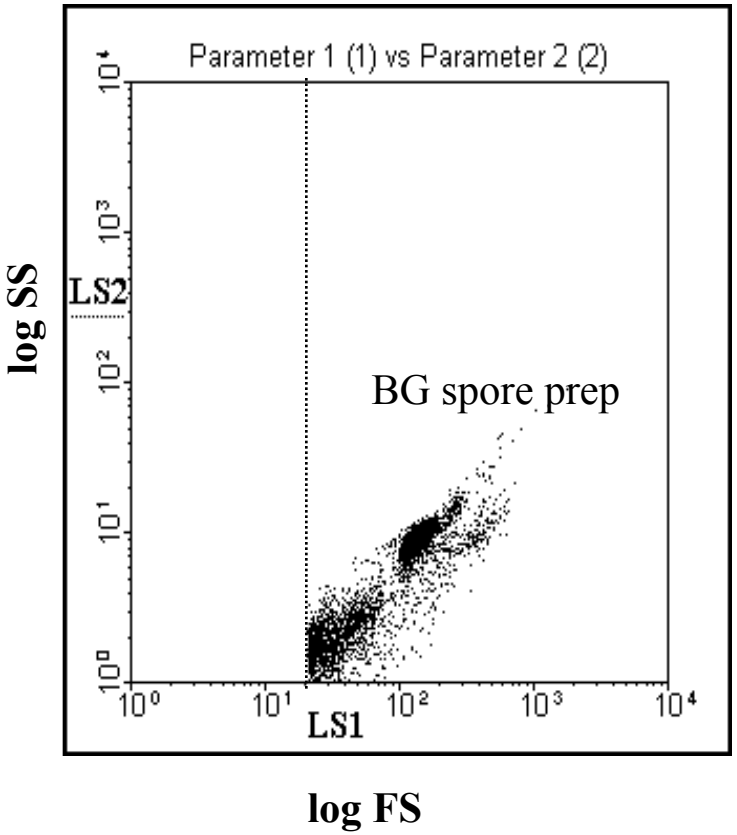


Fixed *E.coli* cells

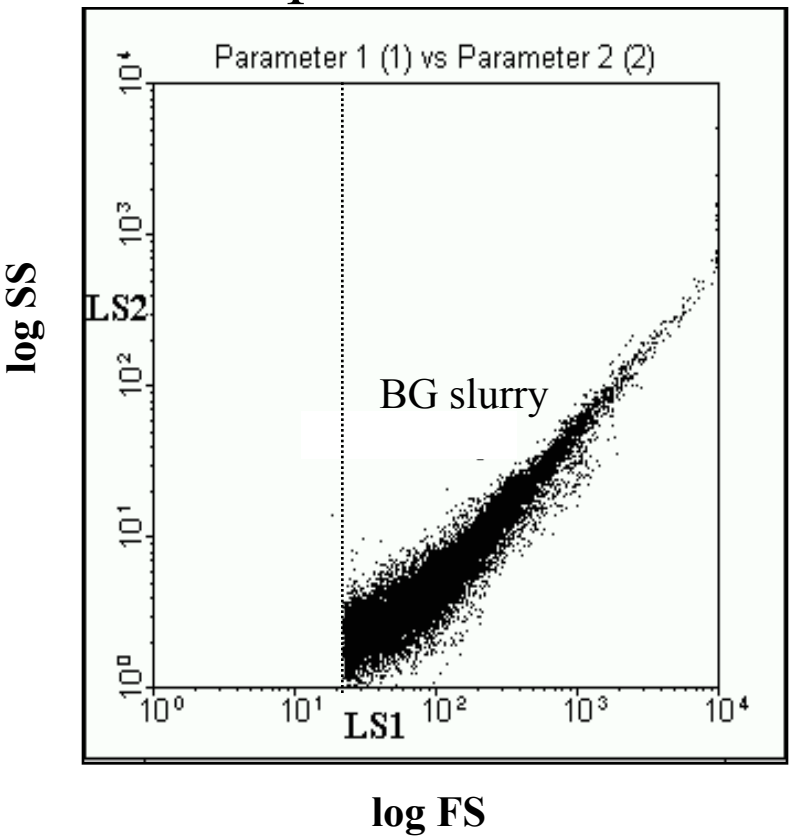


Light scatter changes due to Sample Preparation

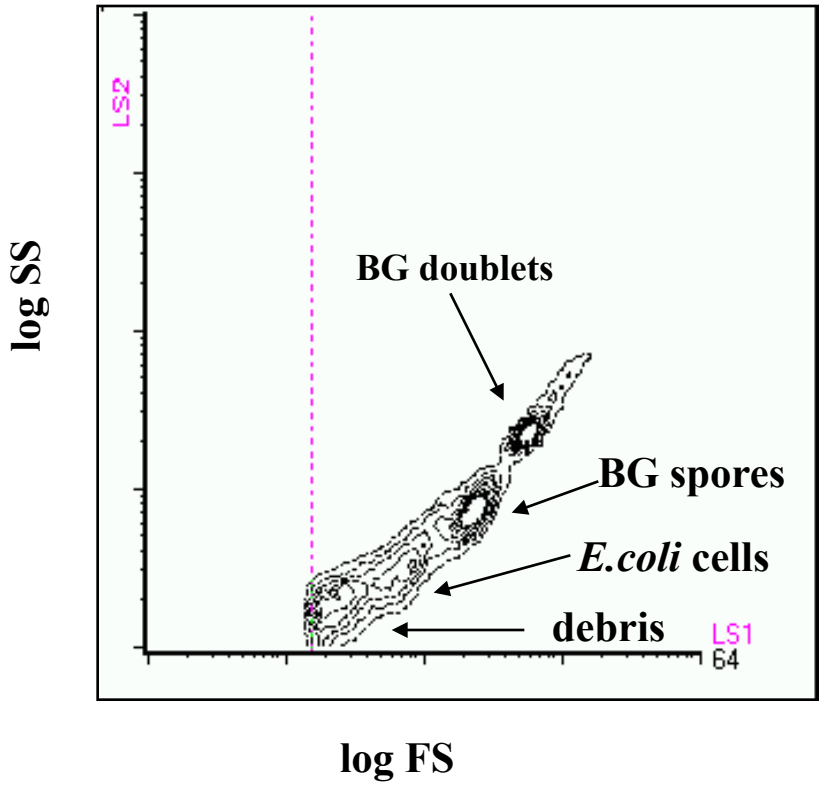
B.subtilis (BG) spores washed

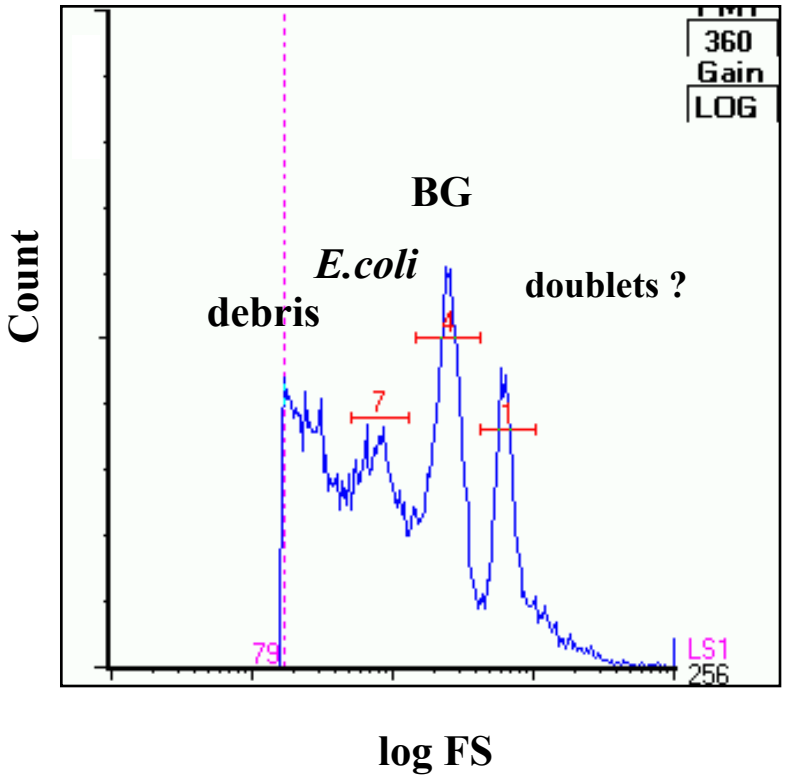


BG spore slurry air sampler



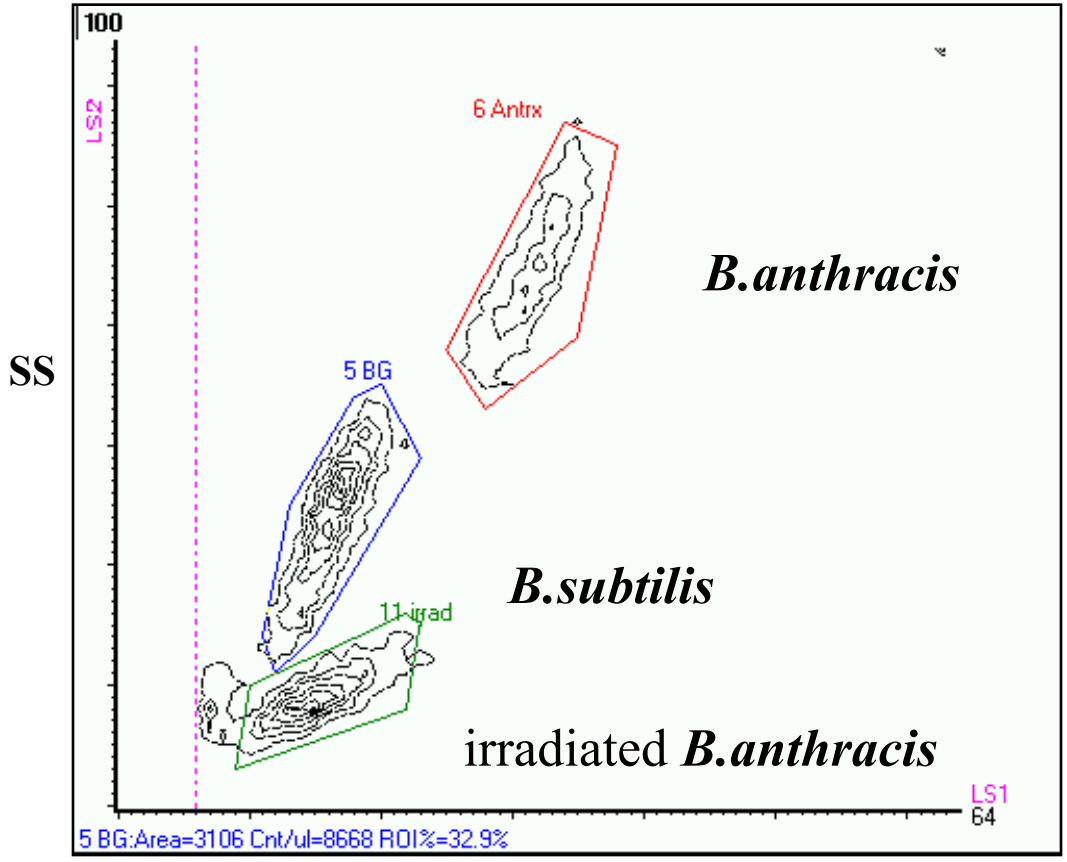
Mixed suspensions of bacteria Identification on scatter alone?





Light scatter signature of a mixture of *B.subtilis* spores (BG) and *E.coli* cells.

Light Scatter of Bacterial Spores



SS

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Light scatter signals from a mixture of live *B.anthracis* spores, live *B. subtilis* spores and gamma irradiated *B. anthracis* spores.

Rapid Detection of Pathogenic Bacteria Using Fluorescent Dyes

Purpose:

To determine if bacteria are present or not in unknown sample

Method:

To fix or not to fix??

-Maintain morphological integrity

-Fluorescent probe must enter the cell

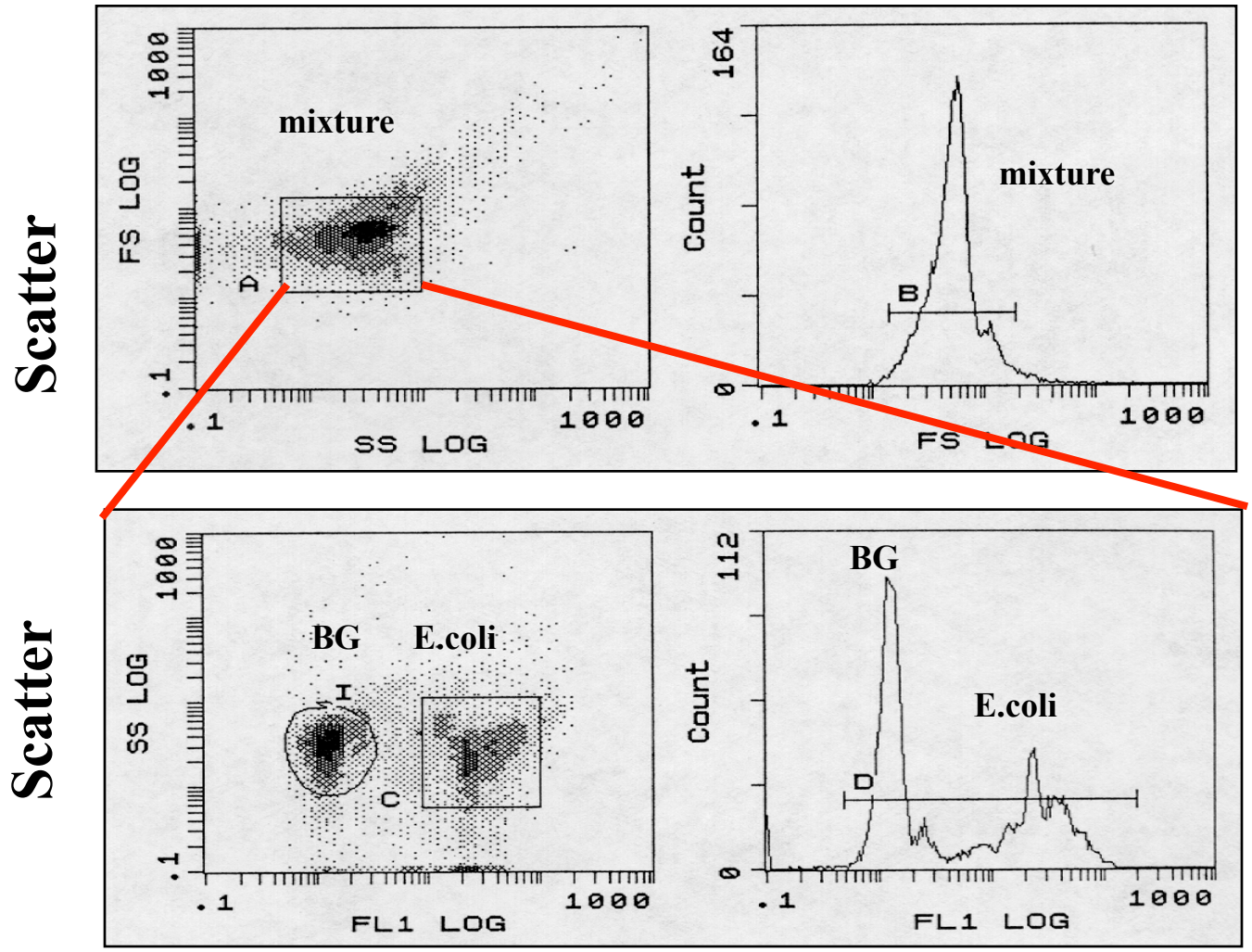
Nucleic Acid Content

- Distinguish bacteria from particles of similar size by their nucleic acid content

- Fluorescent dyes
  - -must be relatively specific for nucleic acids
  - -must be fluorescent only when bound to nucleic acids

Examples

- -DAPI
- -Hoechst 33342
- -cyanine dyes YoYo-1, YoPro-1, ToTo-1



Run on Coulter XL cytometer

Fluorescence

YoYo-1 stained mixture of 70% ethanol fixed *E.coli* cells and *B.subtilis* (BG) spores.

Specific Identification of Pathogenic Bacteria

- Flow Cytometric Immunoassays
  - Polyclonal vs. Monoclonal Antibodies
  - Enrichment Cultures
  - Microsphere beads assays for toxins

- Nucleic Acid Sequences

Microbial Identification Using Antibodies

Enumeration & identification of target organisms in mixed populations

Examples include:

- *Legionella* spp. in water cooling towers
- *Cryptosporidium* & *Giardia* in water reservoirs
- *Listeria monocytogenes* in milk
- *E.coli* O157:H7 in contaminated meat
- *Bacillus anthracis* & *Yersinia pestis* biowarfare agents

Advantages

<10 min. direct assay

<40 min. with enrichment broth

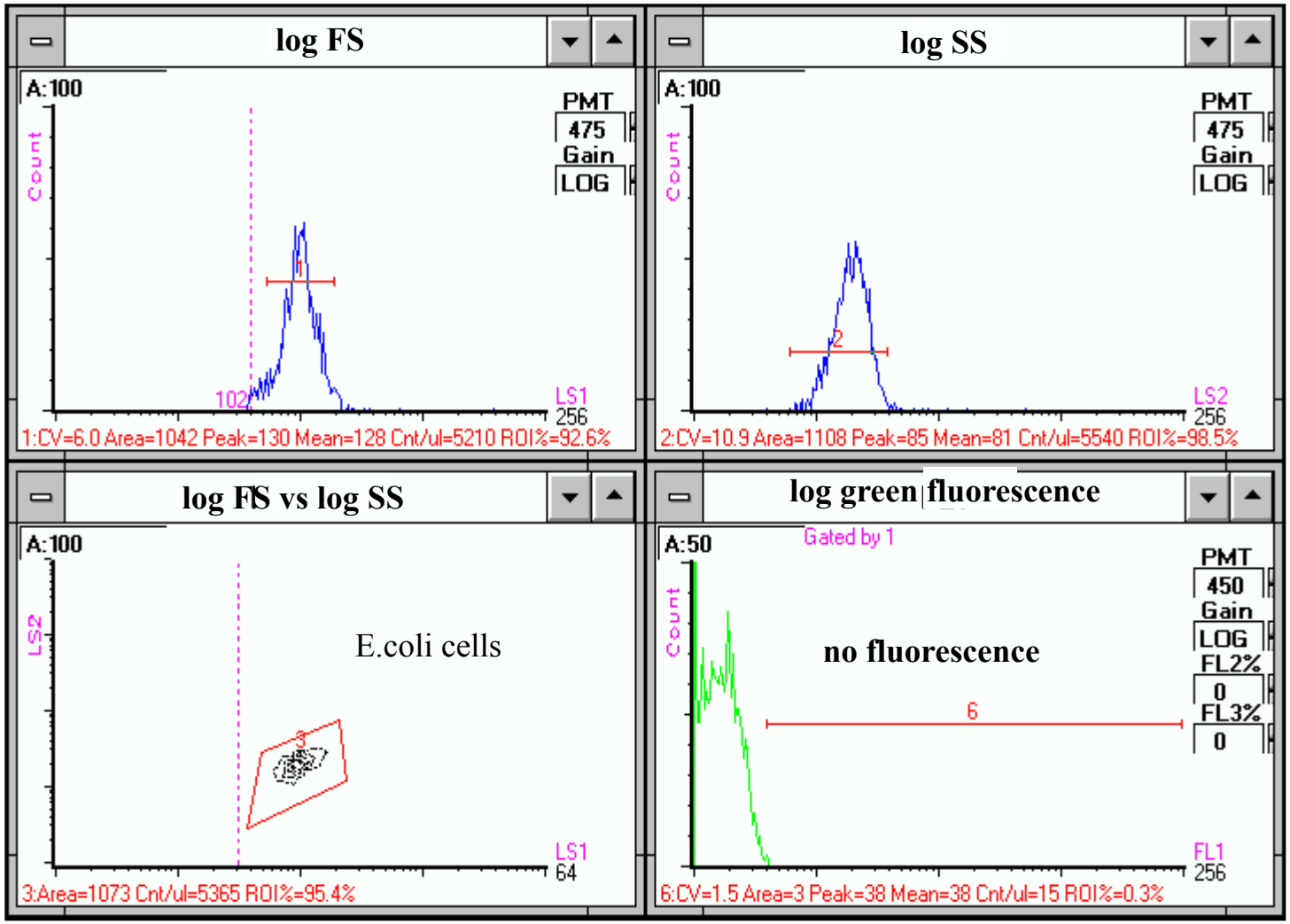
E.coli 10^4 cells/ml

B.anthracis 10^5 cells/ml

- Can be combined with viability probes
- Fixation is not always necessary
- Applications include clinical, water, food, etc.

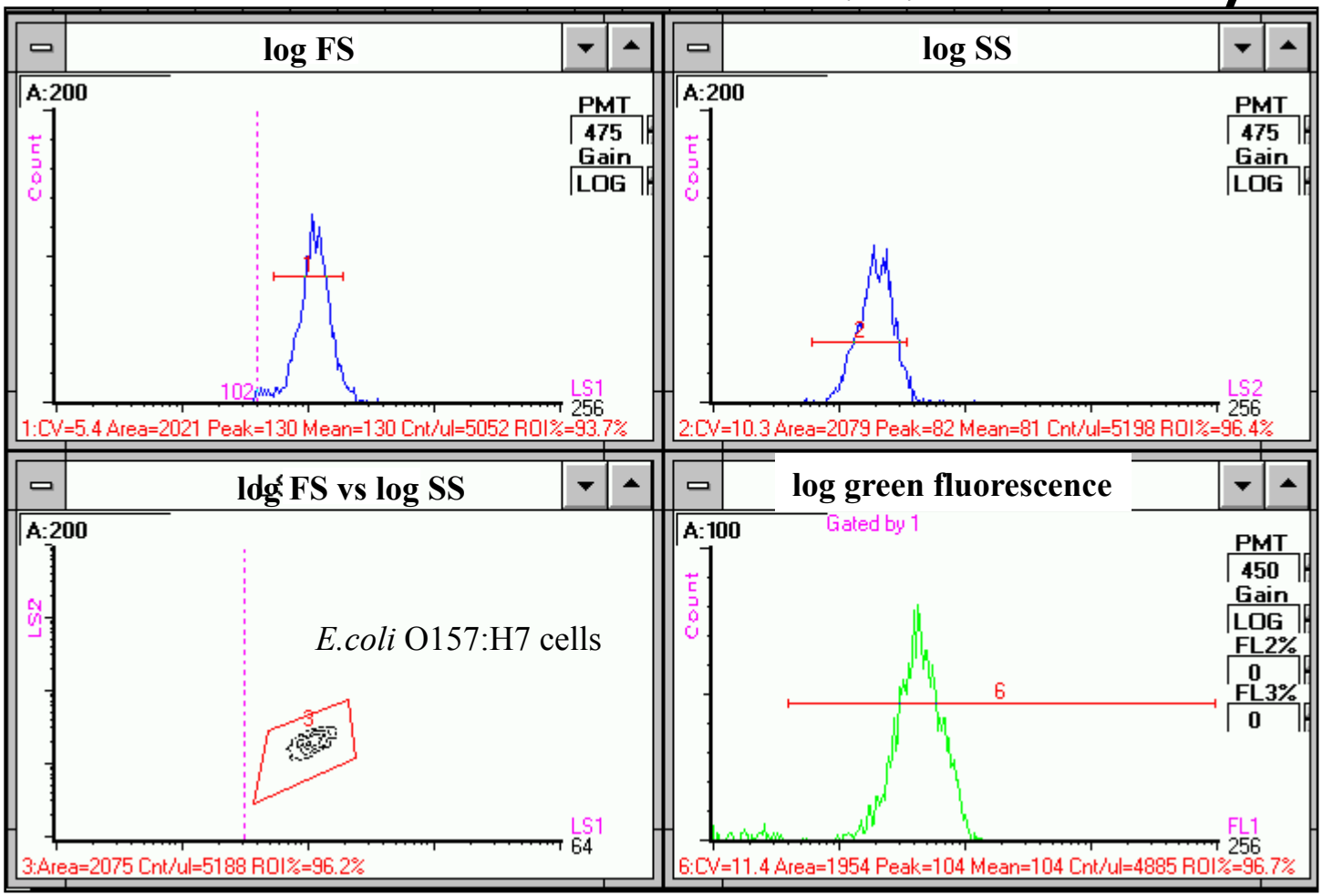
Disadvantages

- Sensitivity, specificity and reliability of assay depends on antibody quality
- Very few commercially available antibodies for bacteria
- MAb preferred but expensive to prepare
- PCAb easy/cheap to prepare but not specific
- Genetic variability of bacteria



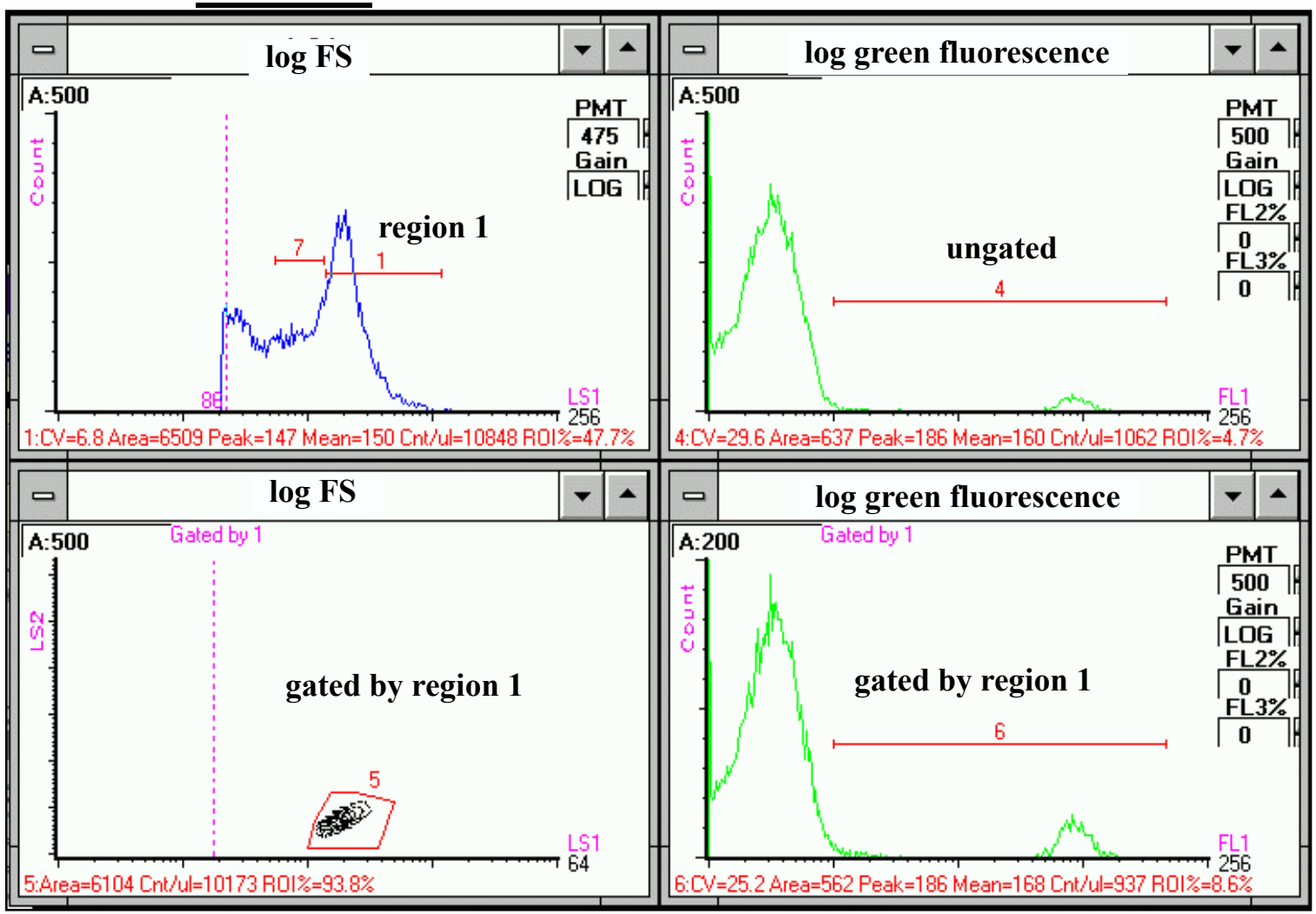
Unstained *E.coli* O157:H7.

E.coli O157:H7 Flow Immunoassay



Flow cytometric identification of *E.coli* O157:H7 stained with FITC-labeled anti-*E.coli* O157:H7 polyclonal antibody.

E.coli O157:H7 in Ground Beef



Flow cytometric identification of *E.coli* O157:H7 stained with FITC-labeled anti-*E.coli* O157:H7 polyclonal antibody in beef.