BMS 631 - LECTURE 15
Flow Cytometry: Theory

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Flow Cytometry & Microbiology

- History
- Major problems
- Potential applications
- Clinical applications
- Future
Relative Sizes of Biologicals

Amoeba: 15-30 µm
Lymphocyte: 5-8 µm
S. aureus: 1 µm
### Relative Ratios

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Bacteria</th>
<th>Yeast</th>
<th>Eukaryotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td>0.5-5</td>
<td>3-5</td>
<td></td>
</tr>
<tr>
<td>Surface</td>
<td>10-30</td>
<td>3-12</td>
<td>300-3000</td>
</tr>
<tr>
<td>Volume</td>
<td>0.3-3</td>
<td>20-125</td>
<td>500-1500</td>
</tr>
<tr>
<td>Dry Cell Mass</td>
<td>1</td>
<td>10</td>
<td>300-3000</td>
</tr>
</tbody>
</table>
Membrane Potential

1. Presence of live bacteria
2. Partial identification
3. Quantitation
4. Antibiotic sensitivity
Application of Membrane Potentials in Flow Microbiology

- Untreated Cells
- 2' After Valinomycin
- 2' After Gramicidin

Frequency

DilC₄(5) Fluorescence Intensity
Ratios using DNA Dyes

S.aureus ATCC 12600

V.parahaemolyticus
ATCC 17802

Chromomycin A3 [G-C]

Hoechst 33258 [A-T]
Ratios using DNA Dyes

S. aureus ATCC 12600

K. pneumoniae CDC II

Hoechst 33258 [A-T]

Chromomycin A3 [G-C]
Comparison of Flow & Traditional Methods

Flow Cytometry vs. Manual Plate Counts

$r=0.996$
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

Initial Culture  
45' Incubation  
Growing Bacteria

Frequency  
Fluorescence Intensity
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
• 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.

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