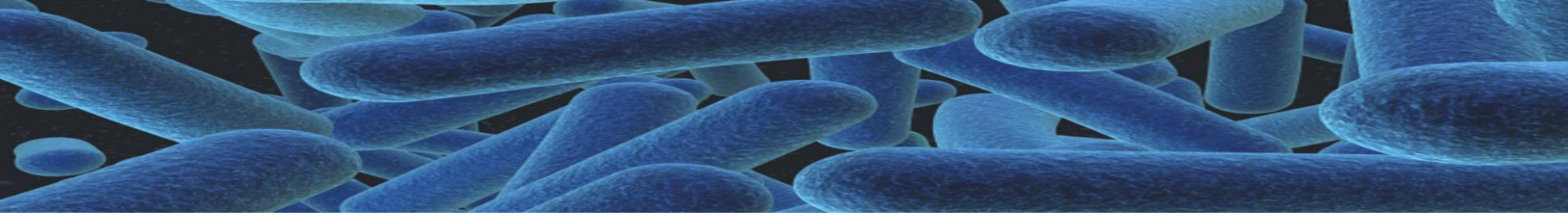

Evaluation, Validation and Implementation of Alternative and Rapid Microbiological Methods

Viability-based Technologies

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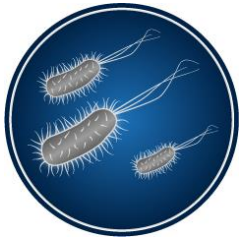
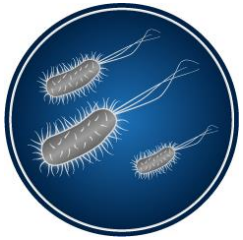


Viability-based Technologies

- Microorganisms are stained with a viability marker
  - Fluorescent labeling that differentiates living from dead cells
  - Direct epifluorescence filter microscopy
- Direct labeling of single cells with *no cell growth required*
- Near real-time results may be realized
- Examples include flow cytometry and solid-phase cytometry
- Usually a destructive assay; some recovery is possible

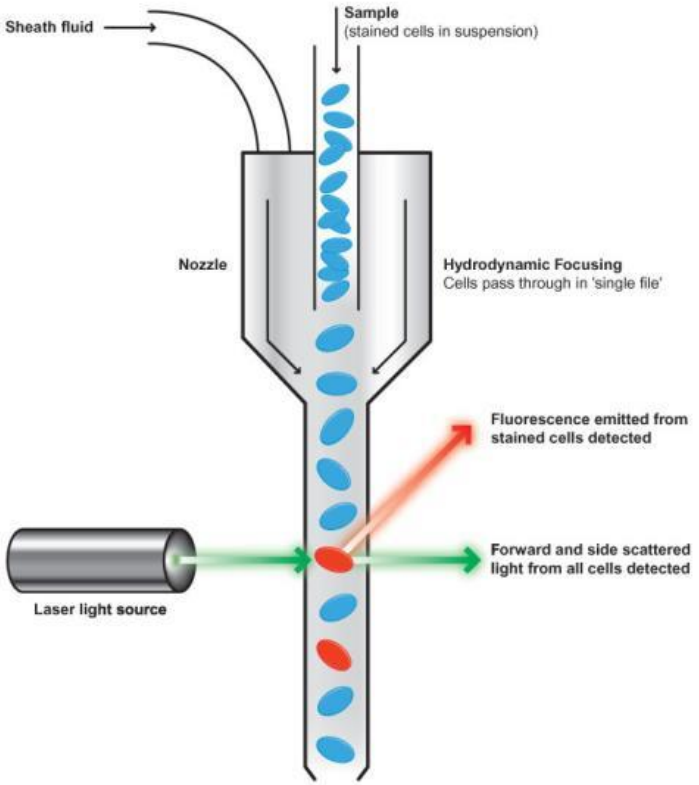


Viability-based Technologies

- Because viability-based RMMs do not rely on microbial growth, microorganisms that are stressed, starved, difficult to culture, or viable but non-culturable (VBNC) may be detected and enumerated
- Could result in a higher count compared with conventional methods
  - In these cases, a correlation between the RMM counts and the conventional counts can be developed
  - The RMM counts can then be used to set new acceptance or specification levels
  - In reality, a significant increase in recovery is normally not observed for samples that are expected to contain low levels of microorganisms

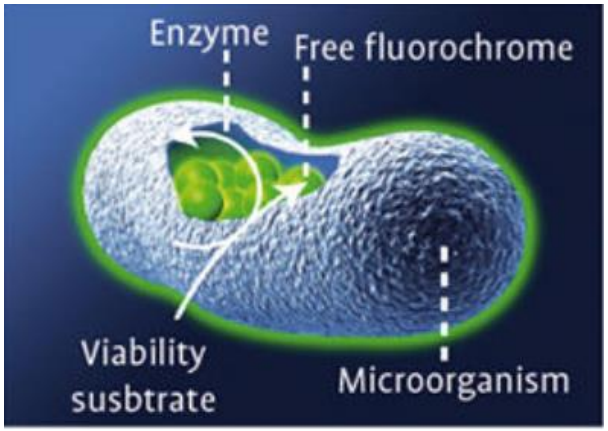
Flow Cytometry

- Counting individual cells as they pass through a laser beam in a very narrow flow cell
- Microorganisms are labeled with a viability stain and then passed through a laser
- Viable cells fluoresce
- Low sample volumes (usually 1 mL or less)
- Sensitivity is 10-50 cells with good accuracy and precision
- Bioburden testing of liquids and non-filterable material



Chemunex / bioMérieux D-Count

- Organisms are stained with a non-fluorescent substrate
- Within the cytoplasm of metabolically active cells, the substrate is enzymatically cleaved (by esterase) to release a fluorochrome
- The fluorochrome will fluoresce when excited by a laser
- Cells with intact membranes will retain the fluorescent label

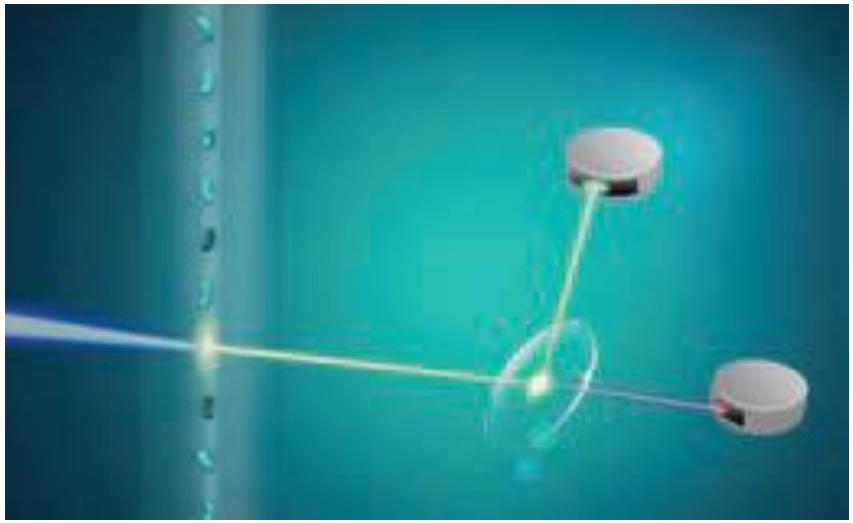


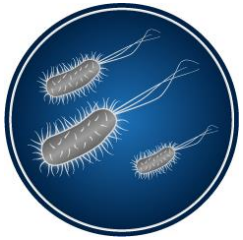
Chemunex / bioMérieux D-Count

- Bacteria, yeast and spores (bacterial and mold) are counted within 20 minutes
- Accurate detection down to 50 organisms per mL
- Up to 64 samples can be analyzed automatically in the D-Count instrument

Chemunex / bioMérieux D-Count

- The labeled organisms pass through the flow cell
  - 488 nm argon ion laser
- Two fluorescence signals (500-530 and >540 nm) are collected by detectors and analyzed
- Results presented as counts and alert/action levels



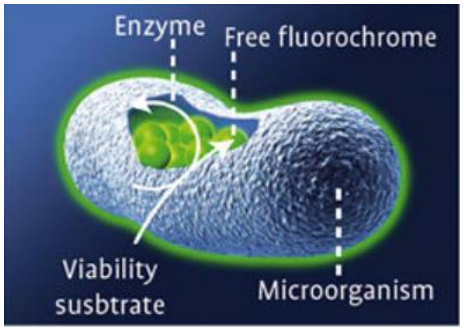


Solid Phase Cytometry

- Counting individual microorganisms that have been captured onto a filter membrane
- Microorganisms are stained and exposed to a laser
- The laser will cause the viability stain to fluoresce
- Sample volumes are higher than those used in flow cytometry (e.g., > 100 mL), but sample must be filterable
- Sensitivity down to a single cell
- Appropriate for bioburden testing, environmental monitoring, water and sterility testing

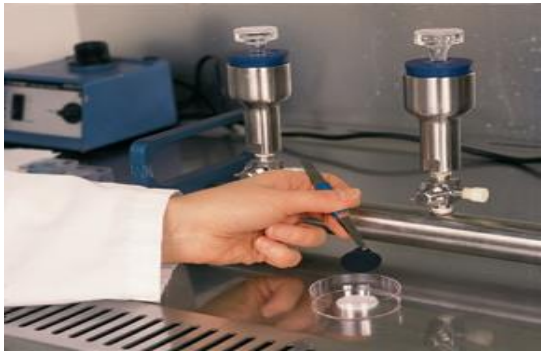
Chemunex / bioMérieux ScanRDI

- Same labeling principle as the Chemunex D-Count
- All viable bacteria, yeast and spores (bacterial and mold) are counted within 2 hours, with single cell sensitivity
- Accurate counting between 1-10^5 bacteria and 1-10^4 for yeast and mold



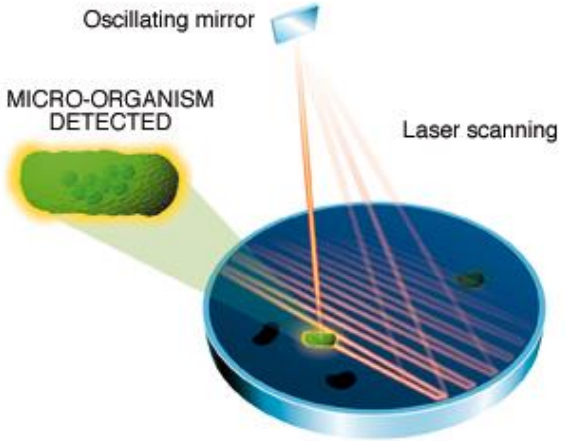
Chemunex / bioMérieux ScanRDI

- Filter the sample through a 0.4 µm polyester membrane
- Activation step (30 min)
- Label with viability substrate, incubate (2+ hours)
- Place membrane into laser scanning chamber



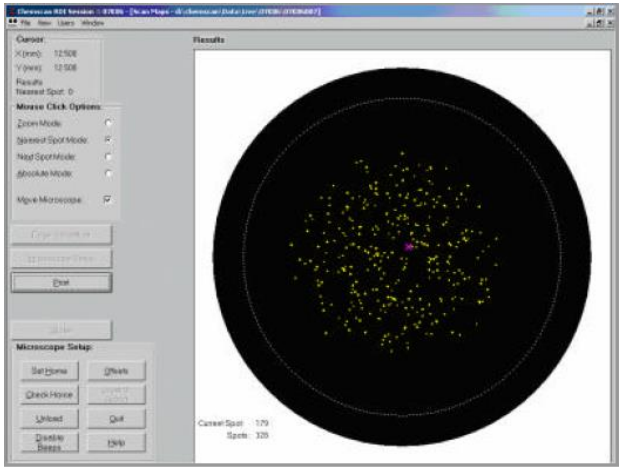
Chemunex / bioMérieux ScanRDI

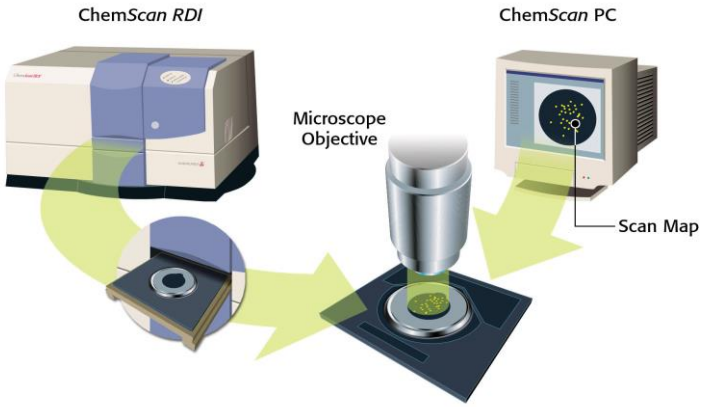
- The membrane is scanned by an argon laser at 488 nm
  - Scan lines are 2.2µm apart to ensure overlap from previous scan
- Photo-multiplier tubes detect emitted fluorescent light within 3 minutes
- Algorithms and discrimination processes determine if the fluorescent signals originate from labeled viable microorganisms or from an auto-fluorescent particle



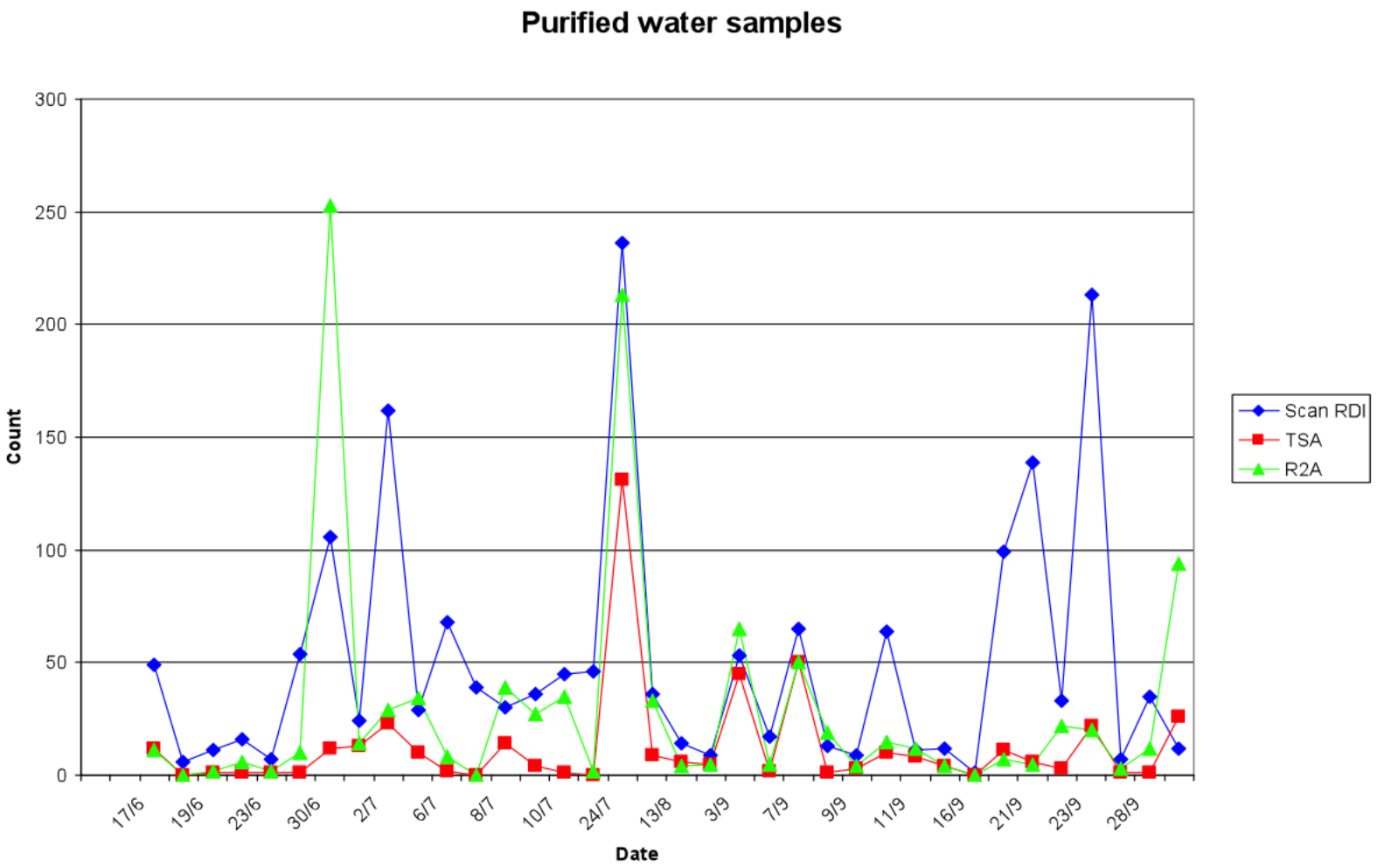
Chemunex / bioMérieux ScanRDI

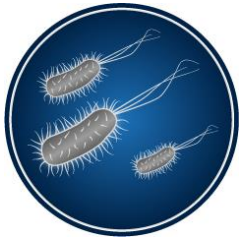
- Auto-fluorescent particles, membrane fluorescence and background noise are rejected and a total viable count is displayed
- Actual cells may be visually observed using a phase-contrast microscope and automated stage





Case Study – Purified Water



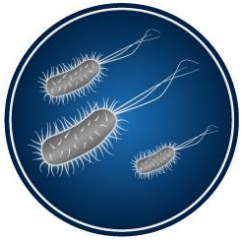


Case Study – Wyeth Batch Bioreactor

- Detection of contamination in mammalian (CHO) cell culture
- Batch 1
  - Scan RDI: 2 fe/mL at 3 hours (fe=fluorescent events)
  - Plate count: 1 CFU at 7 days
- Batch 2
  - Scan RDI: 298 fe/mL at 3 hours
  - Plate count: 126 CFU at 3 days
- Batch 3
  - Scan RDI: 15,000 fe/mL at 3 hours
  - Plate count: Not reported
- Batch 4
  - Scan RDI: TNTC at 3 hours
  - Plate count: TNTC at 1 day

Case Study

- Alcon Laboratories received FDA approval to use the Scan RDI as an alternative to the USP Sterility Test
- Testing is performed inside an isolator environment

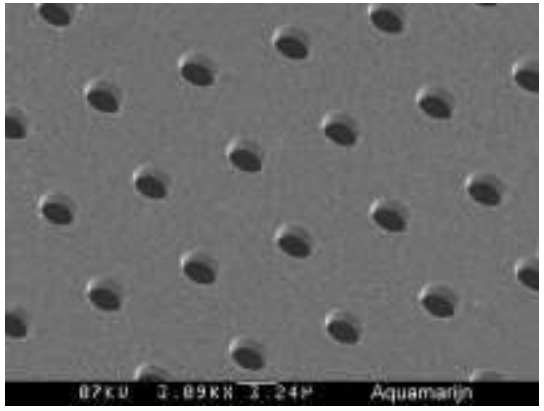


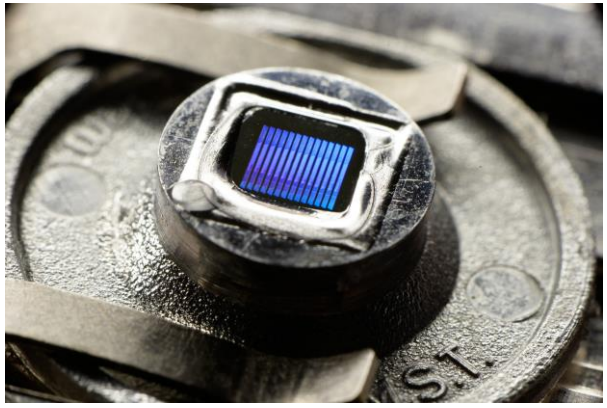
Case Study

- Eight microorganisms were evaluated
  - *Clostridium sporogenes, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Aspergillus niger, Candida albicans) and the Gram-positive anaerobe Propionibacterium acnes*
- The number of viable organisms was estimated using the Scan RDI method and the conventional sterility test method using most probable number technique
- 95% confidence intervals around the mean difference were estimated
- The Scan RDI was found to be numerically superior and statistically non-inferior to the compendial sterility test with respect to the limits of detection for all organisms tested

Innosieve Diagnostics muScan

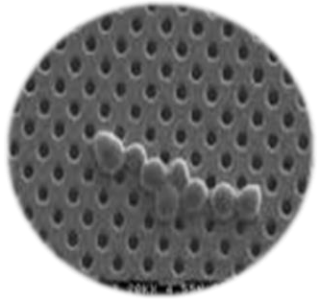
- Organisms are filtered through a novel, microfiltration membrane
- 0.45 um microsieve; precision etched in an ultra thin silicon nitride membrane

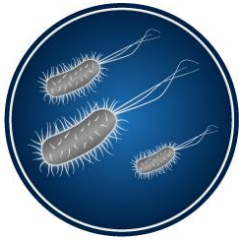




Innosieve Diagnostics muScan

- Filter the sample
- Add staining reagent (esterase-based); 30 minute incubation
- Insert microsieve tube into muScan instrument
- Fluorescently-labeled cells are enumerated by an LED-based detector
- 260 connecting scans
- Image analysis based on size, shape, fluorescent intensity





Innosieve Diagnostics muScan

- Staining or fluorescent tagging of specific microorganisms
  - DNA probes (FISH), PNA-probes, antibodies, target specific dyes
  - Legionella pneumophila, Cronobacter spp., Salmonella spp., E. coli, Enterococci, Borrelia spp.
- Sensitivity: 1 cell
- Time-to result: ~ 60 minutes