Flow Cytometry as a Tool for Microbial Analysis

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BD Biosciences
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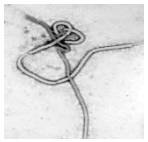
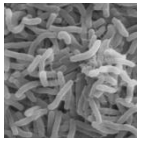
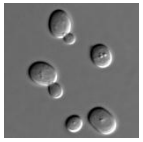
Microbial Analysis: Small Particles Less Than 3 µm



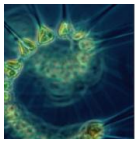
Virus <1 µm



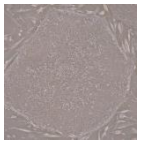
Bacterium ~1 µm



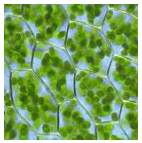
Yeast ~3–4 µm



Algae (highly variable ~0.5–200 µm)



Mammalian Cell Lines ~10 µm



Plant Cell ~100 µm

Uses of Flow Cytometry in Microbial Analysis

Advantages:

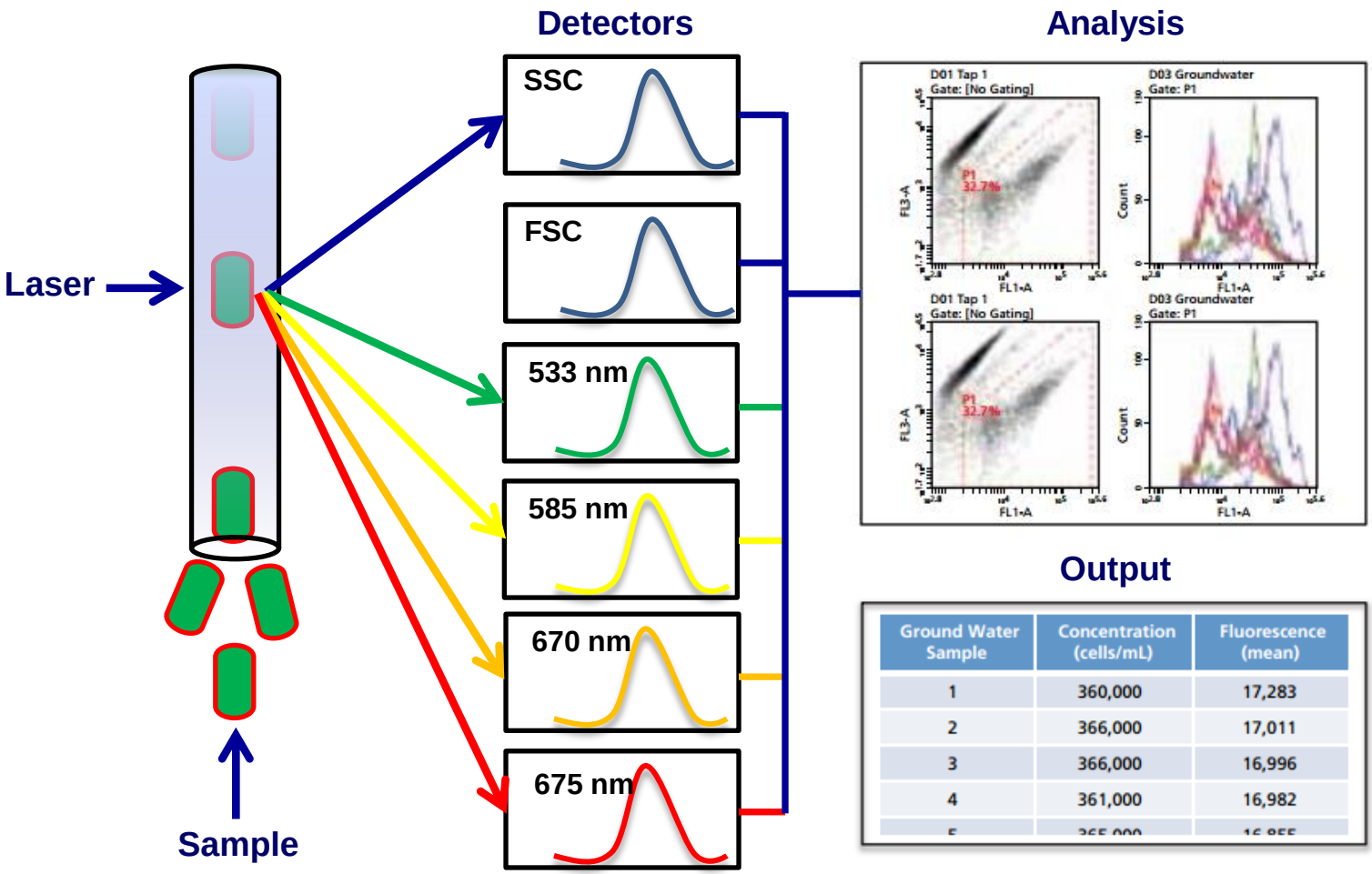
- Rapid real-time analysis of populations
- Multi-parameter single cell analysis
- Analysis irrespective of ability to cultivate
- Cell counting
- Small sampling of large population

BD Accuri C6: Features which Enable Microbial Analysis

- **Small Particle Size:**
  - Ability to threshold based on size and/or fluorescence
- **Wide Size and Fluorescence Range:**
  - Large dynamic range
- **Continuous Sampling:**
  - Open, non-pressurized system
- **Cell Counting:**
  - Direct volume measurement
- **Portability into the Field:**
  - Small, mobile, flow cytometer

Dan Whitely and Maggie Waldron, Antarctica

BD Accuri C6 Flow Cytometer

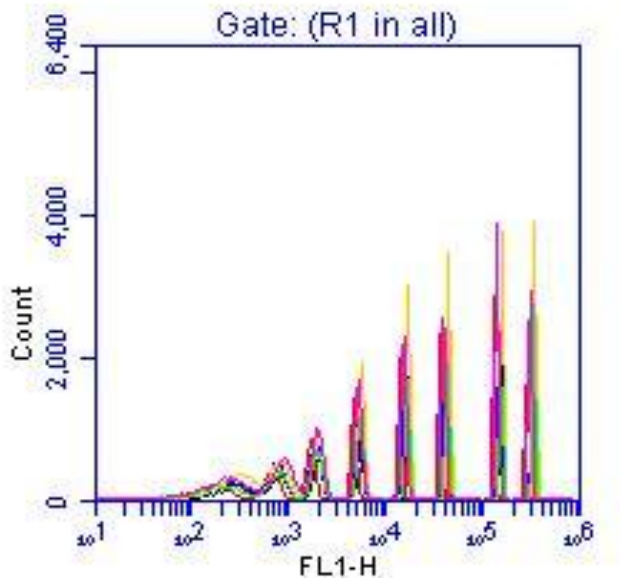


Output

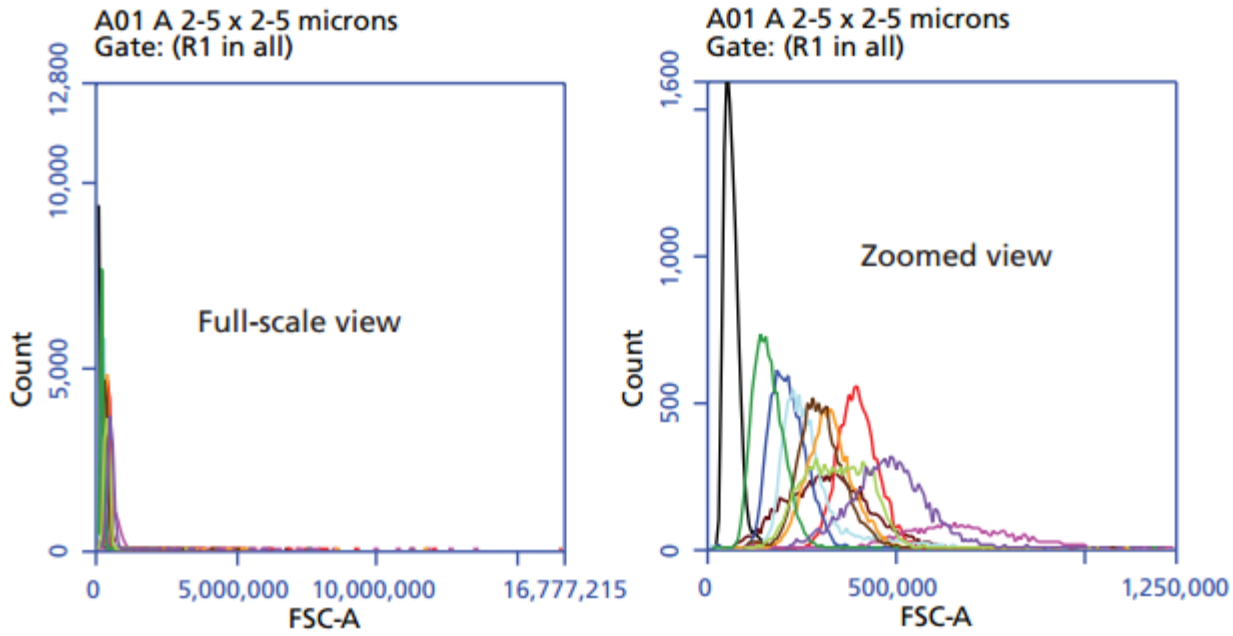
Ground Water Sample	Concentration (cells/mL)	Fluorescence (mean)
1	360,000	17,283
2	366,000	17,011
3	366,000	16,996
4	361,000	16,982
5	365,000	16,855

Advantages of Pre-Optimized Voltage

- **Greatly reduces risk of lost data due to improper setup**
- **Predictable, reproducible analysis relative to sample type and application**
- **Saves time and sample**
- **Allows focus on the *science* of measuring fluorescence, not the *art* of setting voltages**



BD Accuri C6: Aquatic Microorganisms Light-Scatter Profiles



Algae species can be distinguished and characterized by differences in light-scatter properties.

Data courtesy of J. Barker and R.A. Cattolico, PhD, University of Washington

Overview: Flow Cytometry as a Tool for Microbial Analysis

- **Aquatic Microbiome**
  - Environmental science applications
  - Biofuel research applications
- **Bacterial Analysis**
  - Microbial contamination
  - Water quality monitoring
  - Viable but non-culturable cells (VBNCs)

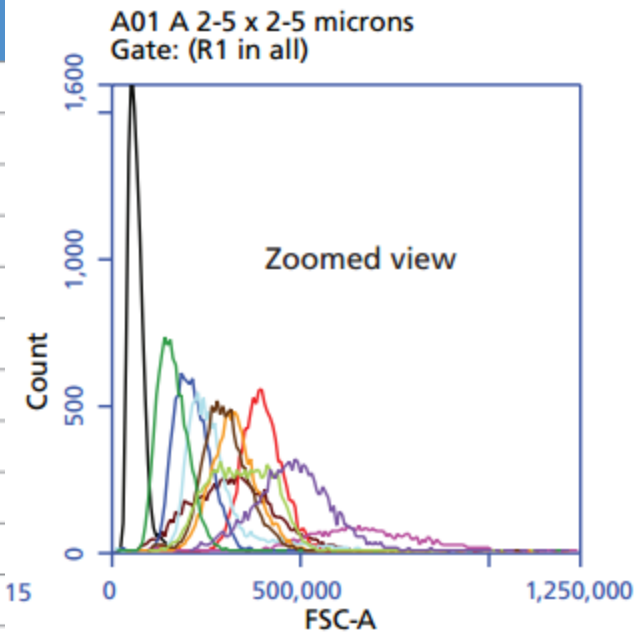
Marine and Freshwater Ecosystems and Biofuel Research

- **Aquatic Environmental Science Applications:**
  - Productivity of phytoplankton
  - Spatial and temporal distribution of cyanobacteria and other phytoplankton species responsible for algal blooms
- **Biofuel Research Applications:**
  - Real-time monitoring of algal cultures
- **How can we monitor and analyze the aquatic microbiome using flow cytometry?**
  - Visualizing intrinsic size and fluorescence differences
  - Concentration of samples
  - Productivity (for example, lipid levels)

BD Accuri C6: Aquatic Microorganism Size

- **How can we monitor microorganisms which may have a wide range of sizes?**

Strain	Length (µm)	Width (µm)	Mean FSC-A
A	2–5	2–5	61,037
B	6–10	6–8	324,817
C	4–6	4–8	406,640
D	4–6	4–8	213,550
E	3–6	3–6	278,934
F	10–15	10–14	701,813
G	3–6	3–6	343,692
H	5–8	5–8	305,581
J	2–4	2–4	162,400
K	3–10	3–8	345,186
L	6–15	4–10	488,418



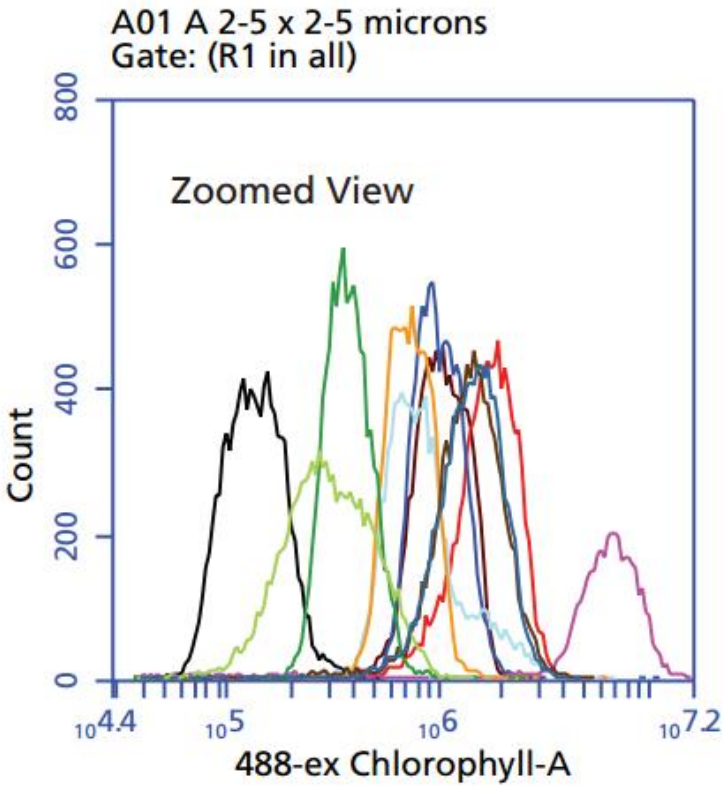
Data courtesy of J. Barker and R.A. Cattolico, PhD, University of Washington

BD Accuri C6: Fluorescence Properties of Phytoplankton Pigments

- Chlorophyll and phycobilins are natural fluorophores with characteristic excitation and emission profiles.

Fluorophore	Exciting Laser	Major Emission Wavelength	BD Accuri C6 Cytometer Detector (filter)
Chlorophyll *a*,*b*	488	>640 nm	FL3 (670 LP)
Phycoerythrin	488	575 nm	FL2 (585 ±20)
C-phycocyanin	640	650 nm	FL4 (675 ±12.5)
R-phycocyanin	640	646 nm	FL4 (675 ±12.5)
Allophycocyanin	640	660 nm	FL4 (675 ±12.5)

BD Accuri C6: Aquatic Microorganism Fluorescence and Dynamic Range



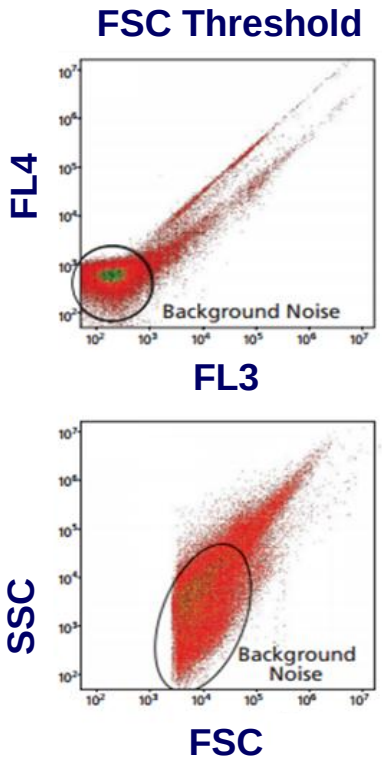
Strain	Mean Chlorophyll-A (FL3-A)
A	139,339
B	1,081,883
C	1,813,107
D	1,044,973
E	974,225
F	7,035,768
G	759,615
H	1,514,209
J	385,538
K	338,308
L	1,523,488

Algae species can be distinguished and characterized by differences in fluorescence properties.

Data courtesy of J. Barker and R.A. Cattolico, PhD, University of Washington

BD Accuri C6: Aquatic Microorganism Light-Scatter Properties

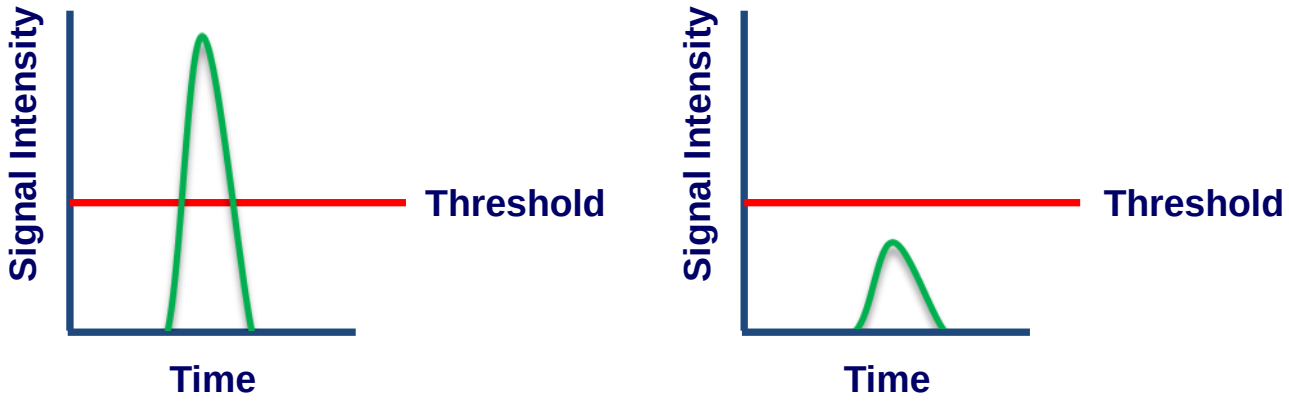
- **How can we delineate the desired cell population from background?**



Surface water samples collected from Lake Erie

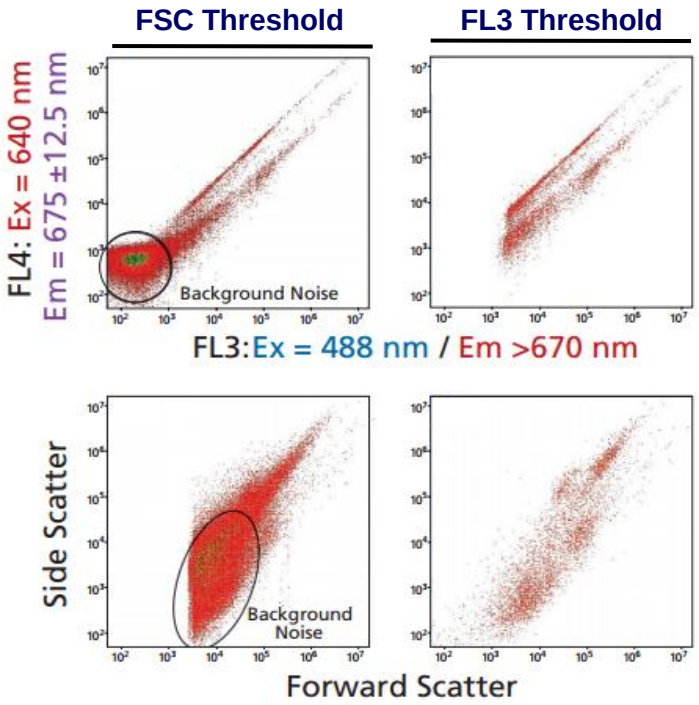
BD Accuri C6: Threshold

- **What is threshold?**
  - Threshold is the lowest signal intensity value an event can have for it to be recorded by the cytometer
- **How can we delineate the desired cell population?**
  - Choose an appropriate threshold to exclude unwanted signals (for example, debris)



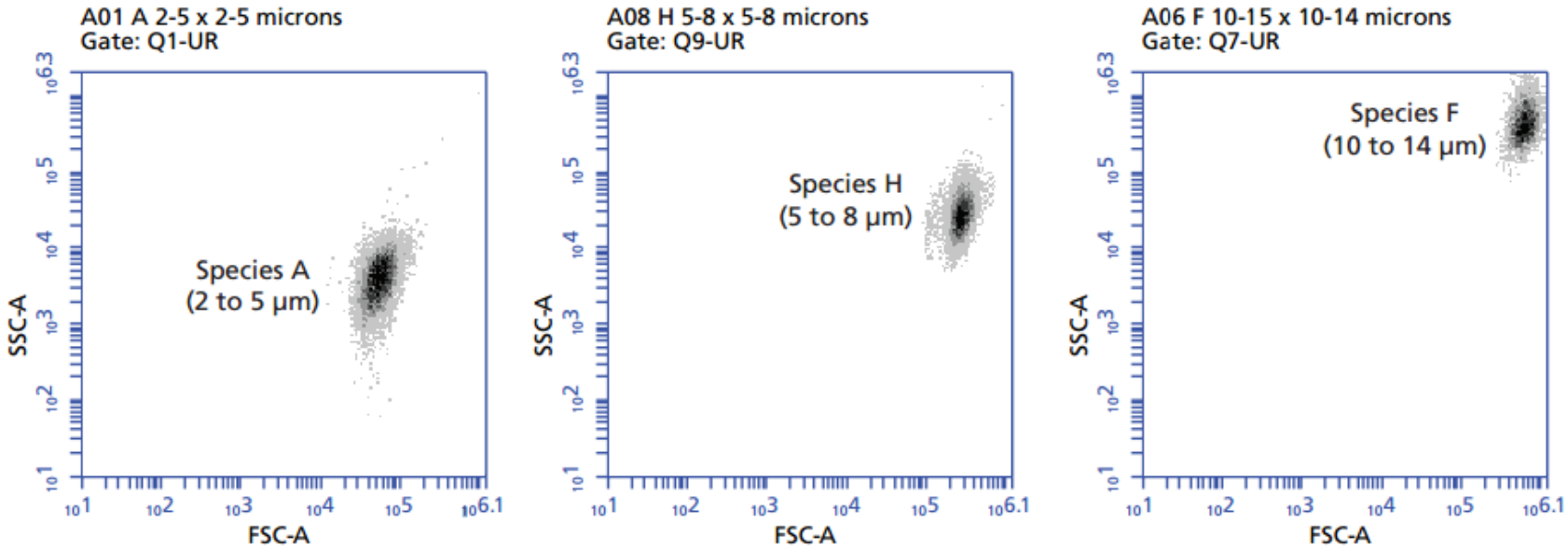
BD Accuri C6: Threshold (cont)

- **How can we delineate the desired cell population from background?**
  - Threshold on fluorescence instead of forward scatter to improve the clear separation of distinct populations.



Surface water samples collected from Lake Erie

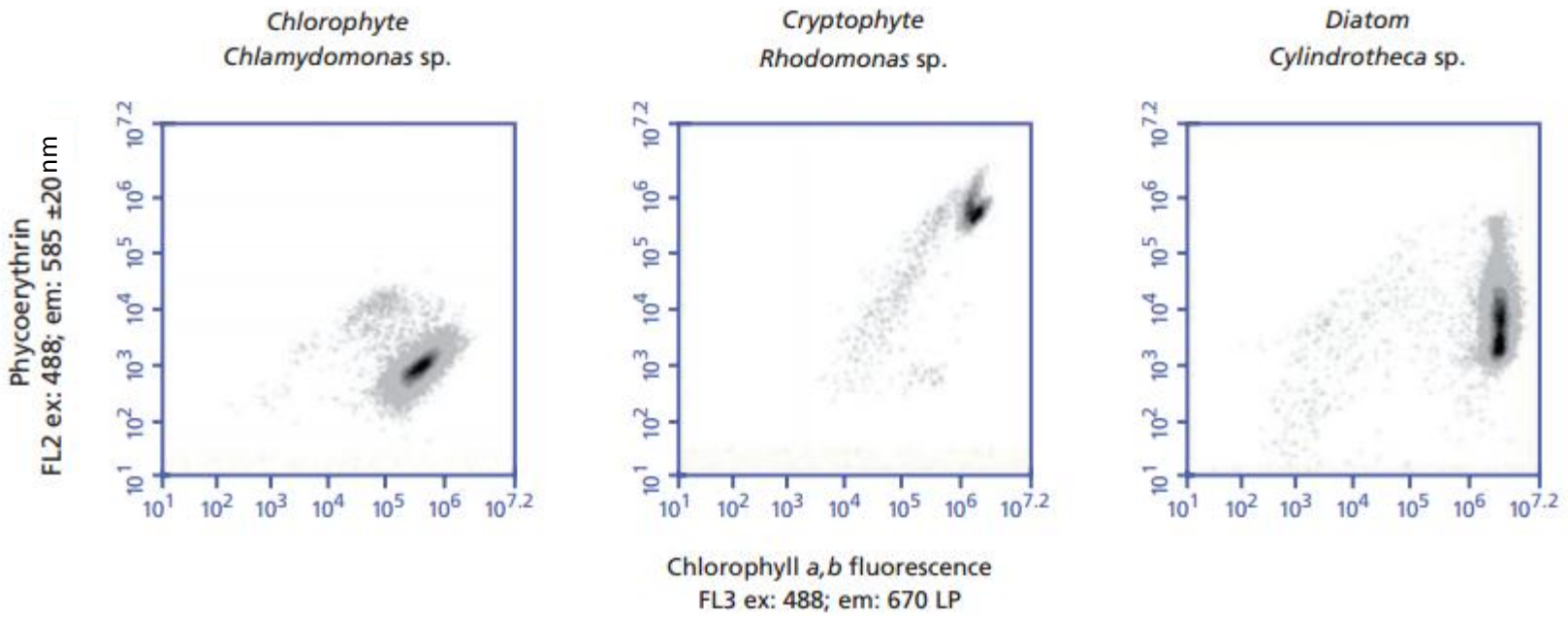
Aquatic Microorganism Light-Scatter Profiles



Algae species can be distinguished and characterized by differences in light-scatter properties.

Data courtesy of J. Barker and R.A. Cattolico, PhD, University of Washington

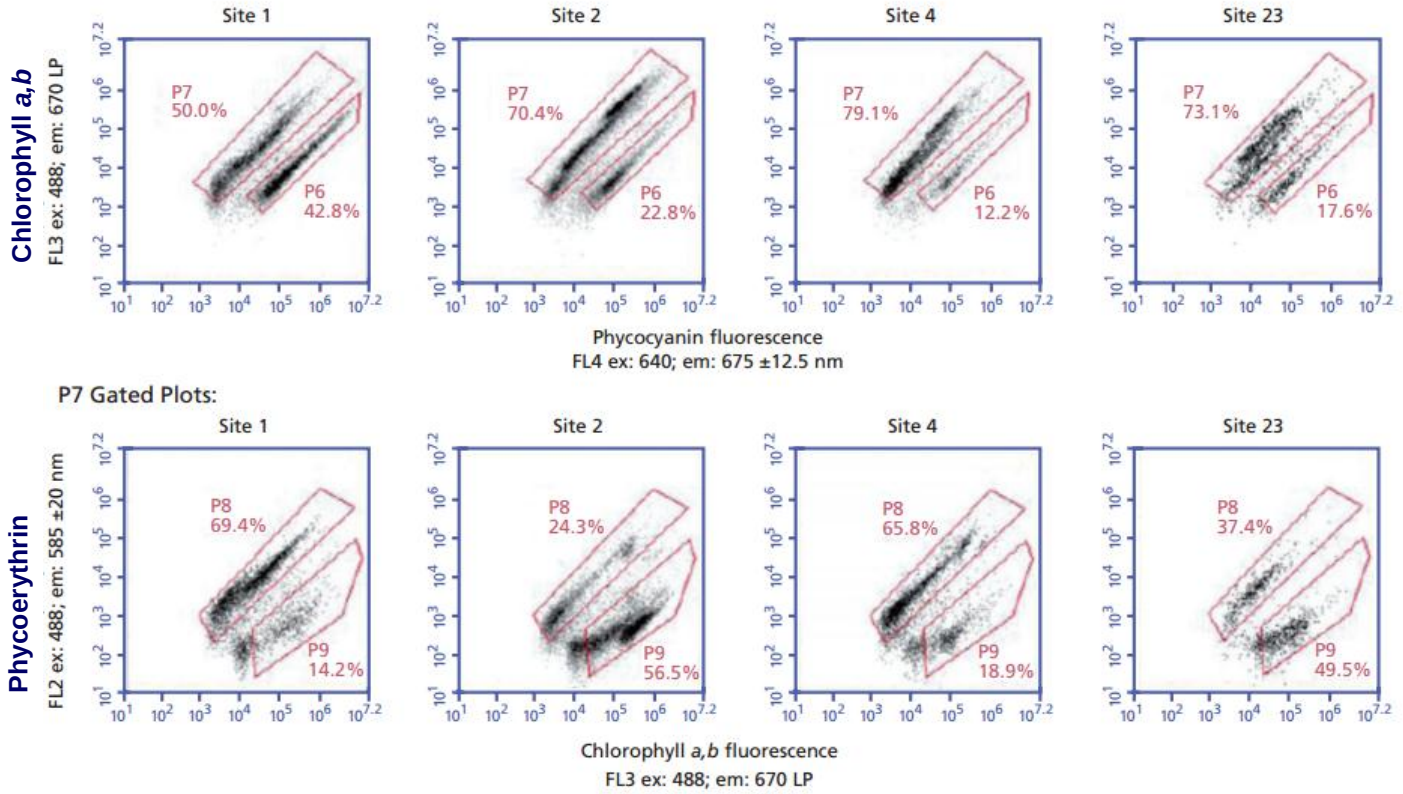
Fluorescence Differences in Phytoplankton



All three species are high in chlorophyll fluorescence but can be distinguished by their phycoerythrin fluorescence.

Data courtesy of J. Adolf, PhD, University of Hawaii, and J.D. Bressie, PhD, NOAA, Seattle, WA

Analysis of Aquatic Samples from Saginaw Bay: Fluorescence



P6: Cyanobacteria
P7: Other phytoplankton

P8:PE fluorescent
P9: Non-PE fluorescent

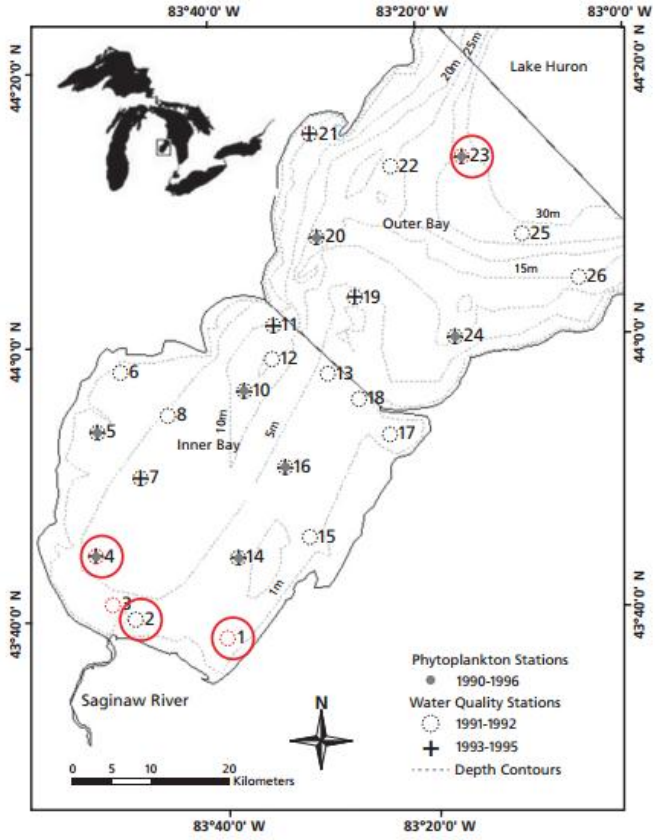
Four types of phytoplankton were identified by fluorescence characteristics.

Data courtesy of J.D. Bressie, PhD, NOAA, Seattle, WA

Analysis of Aquatic Samples from Saginaw Bay: Cell Counting

Site	Population Concentration (per mL)			
Sag. Bay, MI	phycocyanin dominated (P6)	chlorophyll *a,b* dominated (P9)	chlorophyll *a,b* and phycoerythrin (P8)	total fluorescent events/mL sample
1	18,730	3,120	15,200	37,050
2	18,635	32,450	13,955	65,040
4	3,145	3,840	13,390	20,375
23	1,550	3,185	2,405	7,140

Sites 1 and 2, closest to the river, had the highest levels of cyanobacteria.

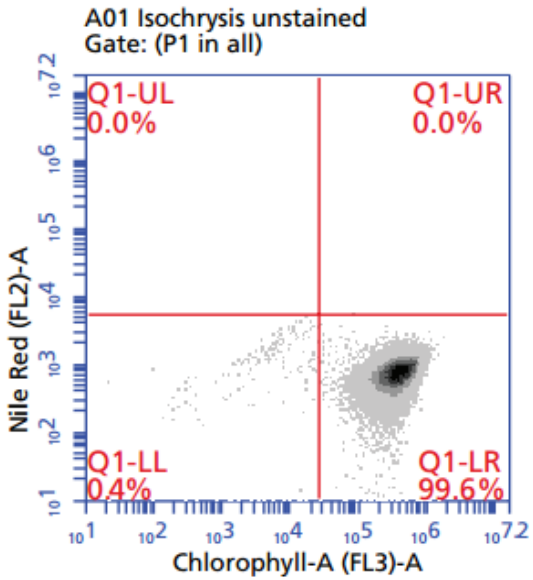


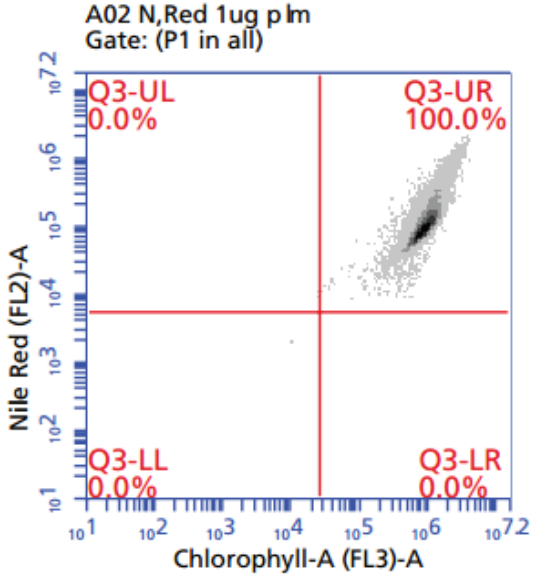
Data courtesy of J.D. Bressie, PhD, NOAA, Seattle, WA

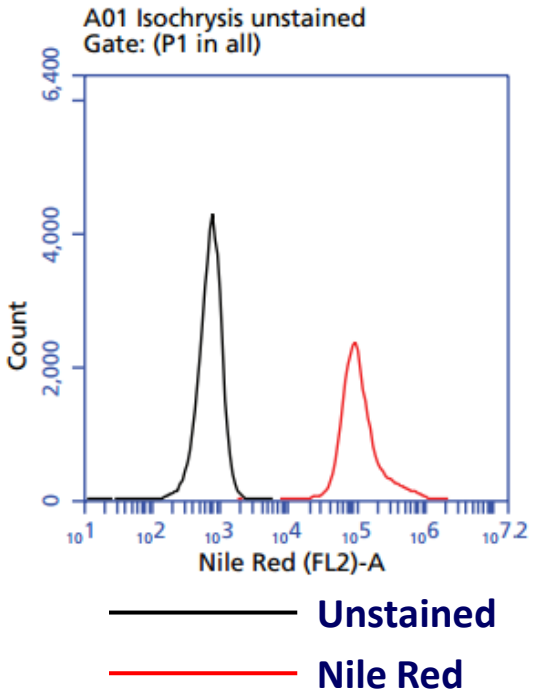
Biofuel: Functional Analysis of Lipid Content

- **Lipid concentration determination in living algal cells**
- **Fatty acid content: extraction, conversion, and measurement**
- **Lipid content using lipophilic dyes**
  - Requires only a small sample (<0.5 mL).
  - Eliminates need for concentration.
  - Cultures can be directly assessed.

Detection of Lipid Content



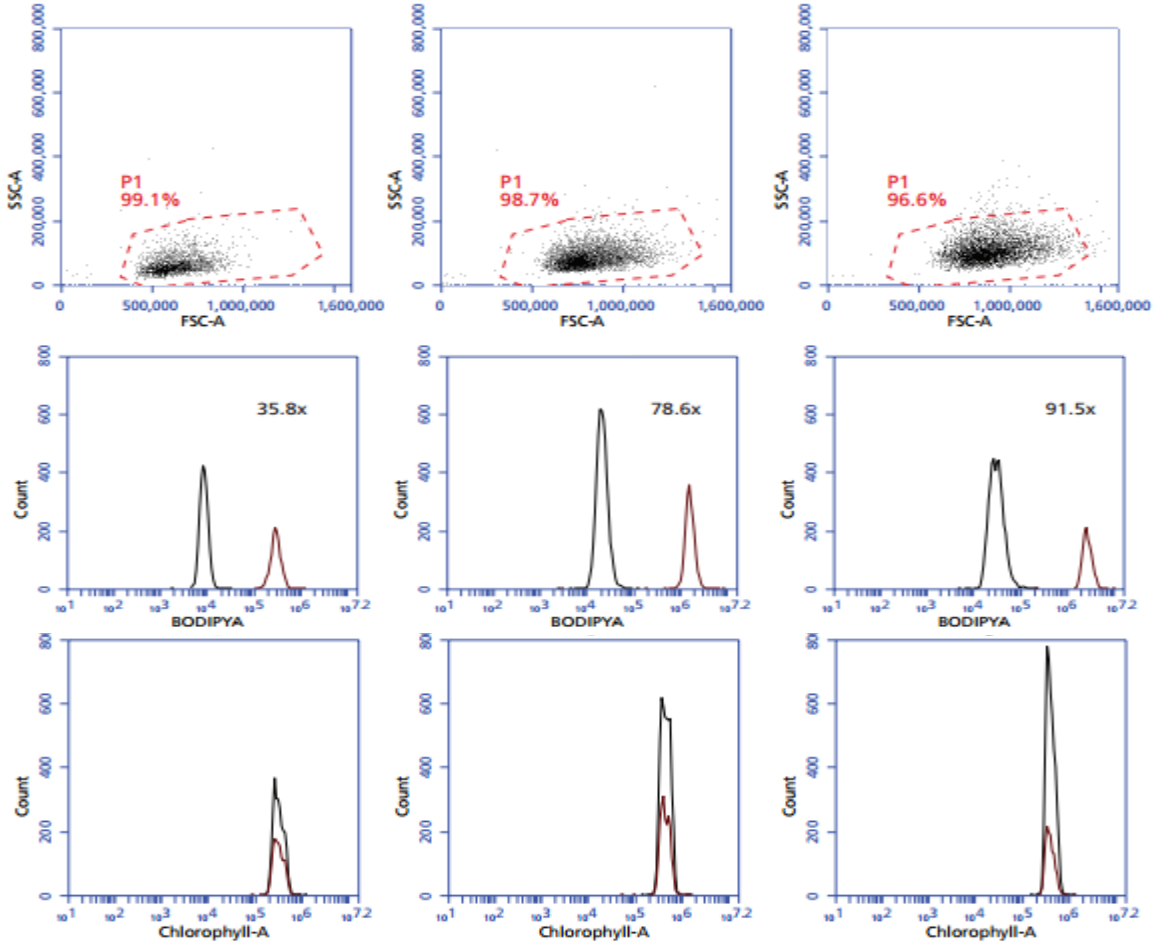




Neutral lipids are labeled in *Isochrysis* algae and can be simultaneously analyzed with chlorophyll fluorescence.

Data courtesy of G. Wolfe, PhD, California State University, Chico

Simultaneous Analysis of Size, Lipid Content, and Chlorophyll Content



Algal size increases over the light cycle.

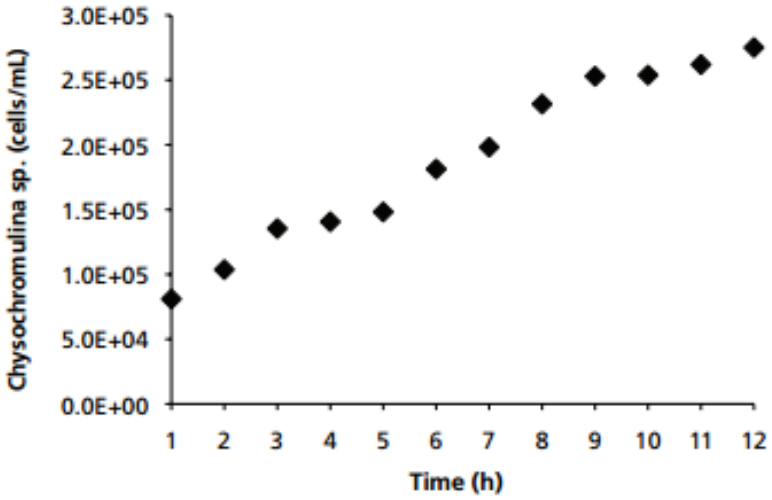
Neutral lipid content increases over the light cycle.

Chlorophyll content remains unchanged over the light cycle.

Data courtesy of J. Barker and R.A. Cattolico, PhD, University of Washington

Biofuel Research and Cell Counting

Multiplexing: light scatter, chlorophyll, lipid content, and cell density



Estimated Time to Count 30 Samples

Method	Time
BD Accuri C6 (with BD CSampler)	22.5–37.5 min
BD Accuri C6 (manual run)	60 min

Data courtesy of J. Barker and R.A. Cattolico, PhD, University of Washington

Marine and Freshwater Ecosystems and Biofuel Research

- **Flow Cytometric Applications in Aquatic Environmental Sciences:**
  - Visualizing intrinsic size differences
  - Visualizing fluorescence differences
  - Determining the concentration of samples
  - Determining the spatial and temporal distribution of the aquatic microbiome
- **Flow Cytometric Applications in Biofuel Research:**
  - Real-time monitoring:
    - Algal size
    - Chlorophyll content
    - Lipid content
    - Cell density

Bacterial Analysis

- **Applications:**
  - Microbial contamination
  - Water quality monitoring
  - Viable but non-culturable cells (VBNCs)

- **How can we monitor and analyze bacteria using flow cytometry?**
  - Visualizing intrinsic size differences as culture contaminant in yeast samples
  - Fluorescent markers
  - The use of fluorescent dyes to measure viability and vitality
  - Concentration of samples
  - Continuous sampling

BD Accuri C6: Bacterial Size

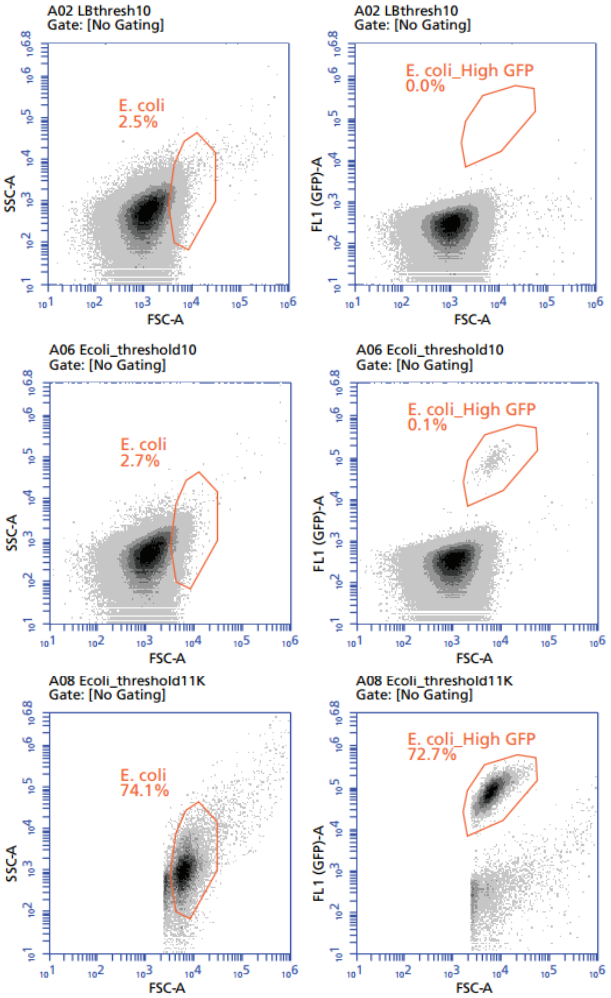
- **How can we monitor microorganisms which may have a small size?**
  - Bacteria can be ~1 µm vs 4 µm for yeast cells.
  - Bacteria can overlap with debris particles.
- **Solution:**
  - Use FSC or SSC acquisition threshold to exclude debris.
  - Use fluorescence as a gating parameter.
  - Use a combination of the two methods above.

BD Accuri C6: Changing Threshold to Enhance Bacterial Detection

LB alone
Threshold 10

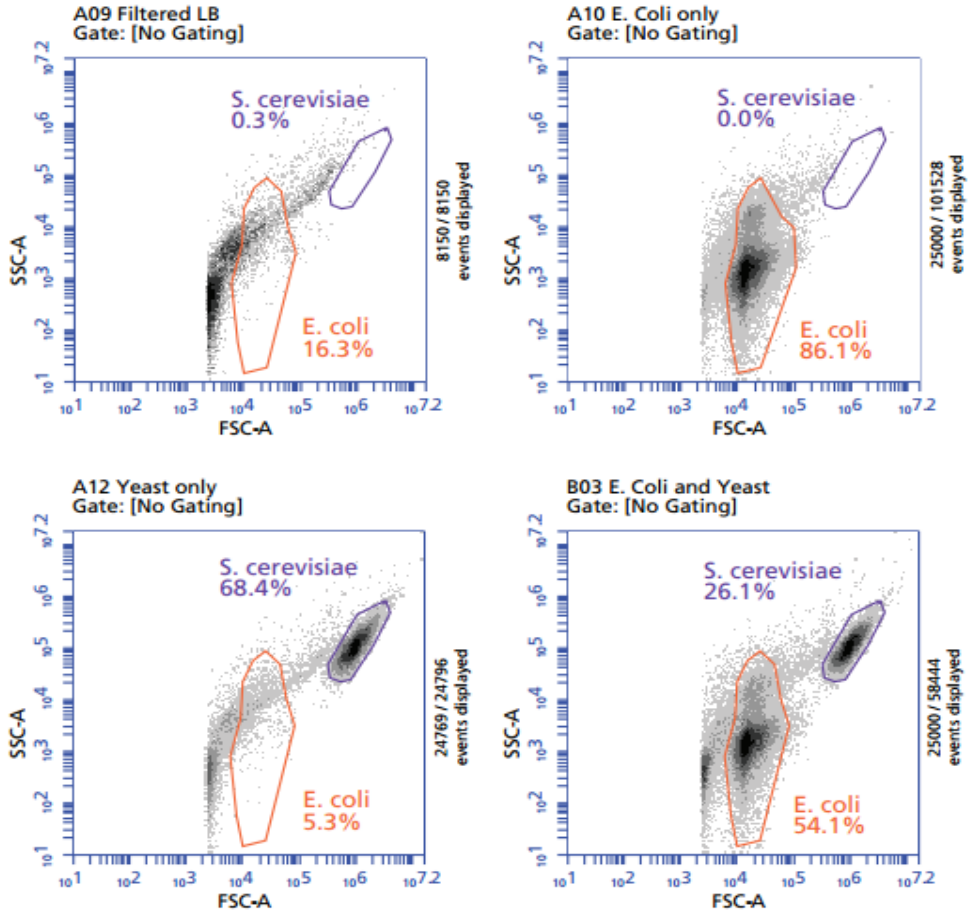
GFP *E.coli* +LB
Threshold 10

GFP *E.coli* +LB
Threshold 11,000



Data courtesy of P. Pena and F. Srienc, University of Minnesota

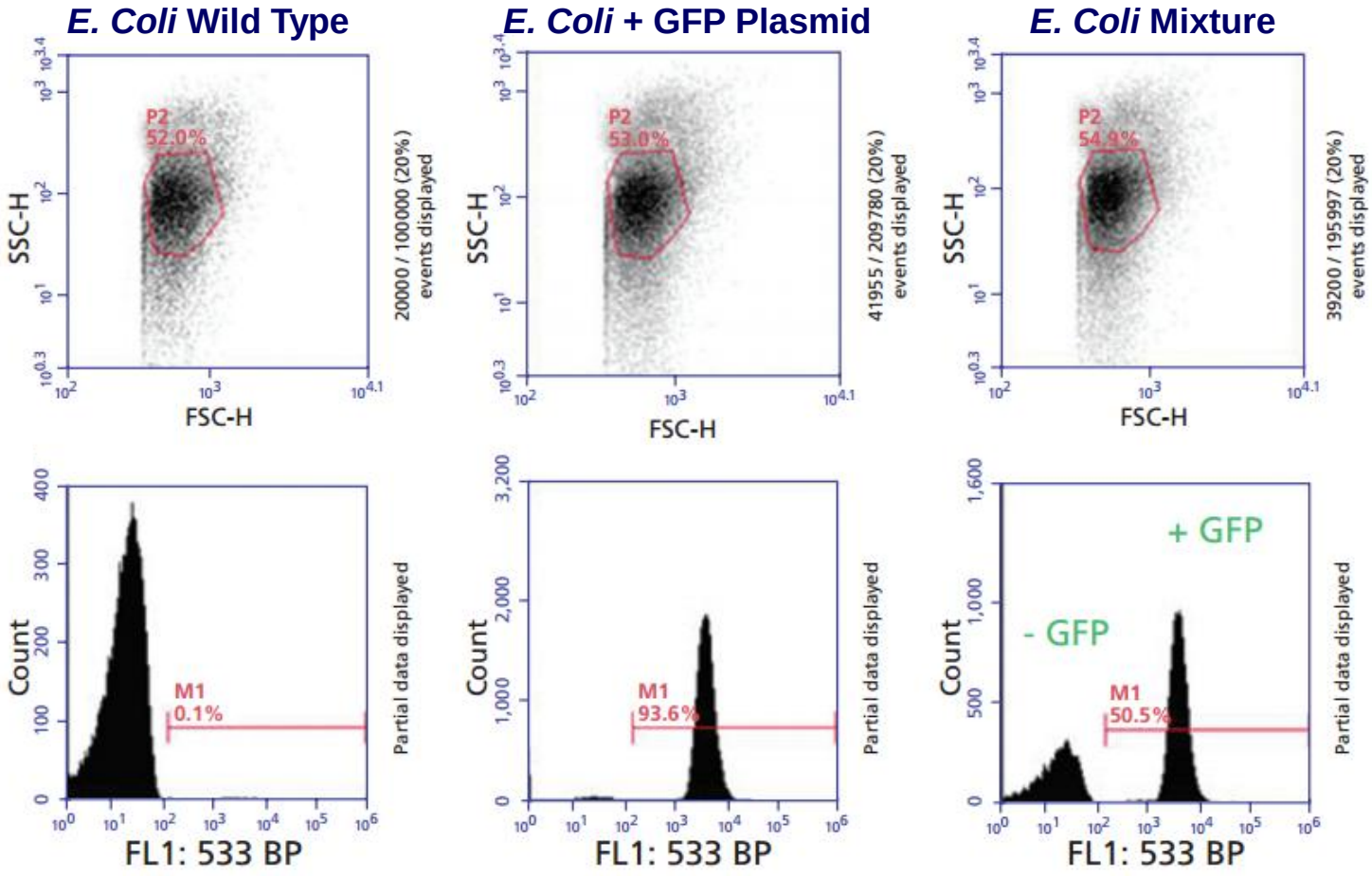
Simultaneous Detection of Yeast and Bacteria in the Same Culture



Multiple populations can be detected in the same culture, enabling the ability to detect microbial contamination.

Data courtesy of P. Pena and F. Srienc, University of Minnesota

Detection of GFP Expression in Bacteria

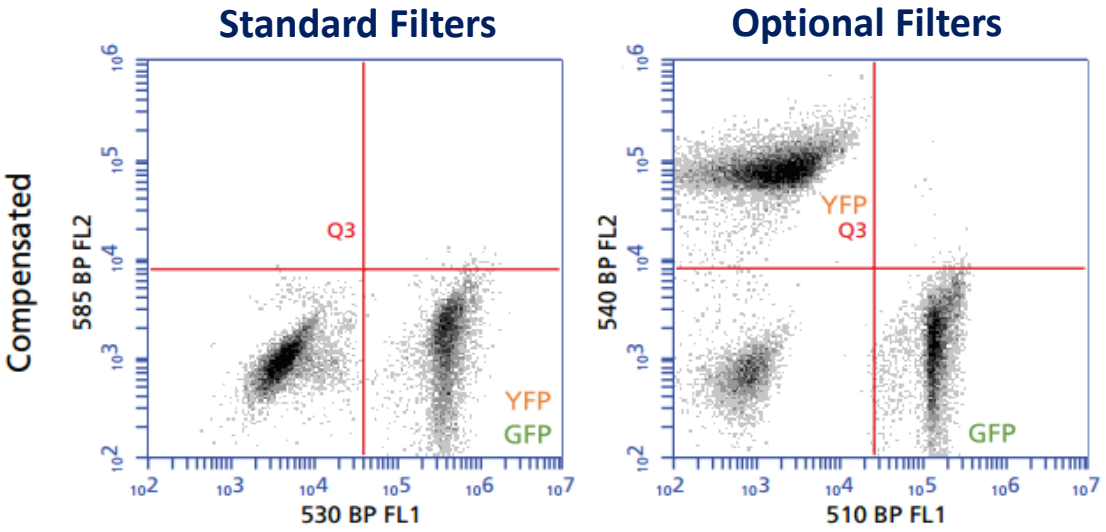


Data courtesy of T.F. Cooper, PhD, University of Houston

Fluorescent Markers

- **Optional filters can increase signal resolution and allow separation of signals that may overlap using the standard configuration.**

Detector	Filter	Fluorescent Proteins
FL1	510/15	GFP
FL2	540/20	YFP, Citrine



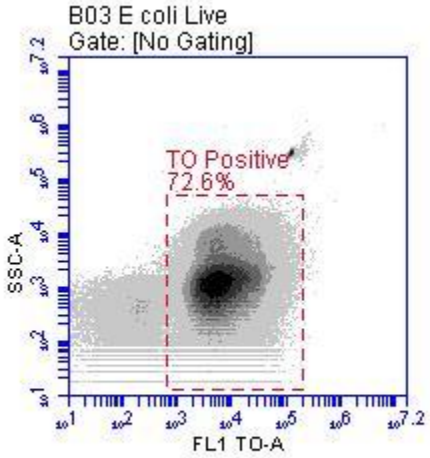
Bacteria: Vitality

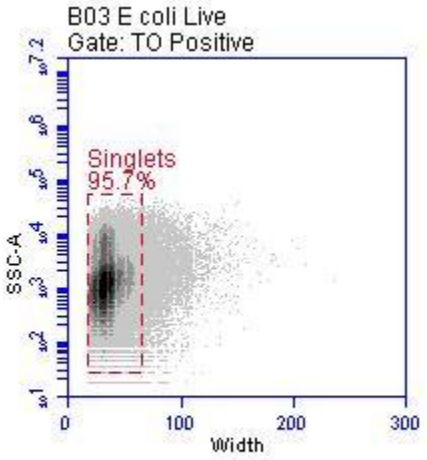
- **Intact membranes imply viability but do not indicate cellular functionality.**
  - Plasma membrane polarity
  - Metabolic activity
  - Replication ability
- **The ability to combine viability (cell membrane permeability) with vitality (for example, plasma membrane polarity) allows the differentiation between VBNCs and dead cells.**
- **Lipophilic dyes can permeate cell membranes and accumulate according to charge.**

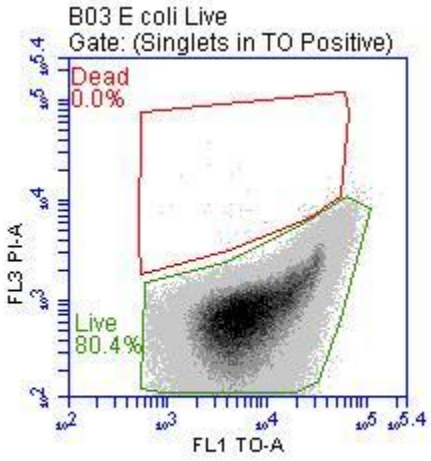
Bacteria: Viability

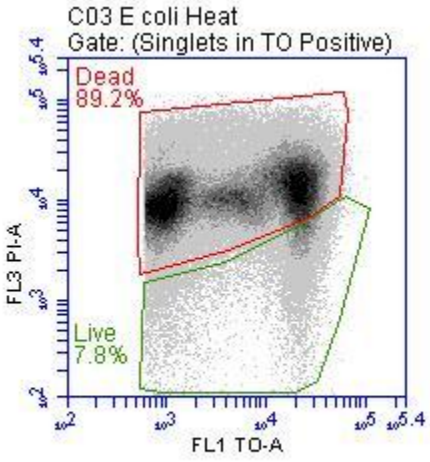
- **Viability can be measured by membrane integrity.**
  - PI (impermeable to cell membrane of living cells)
  - Can be used in combination with dyes that would enter all cells (for example, Thiazole Orange) to help distinguish bacteria from debris

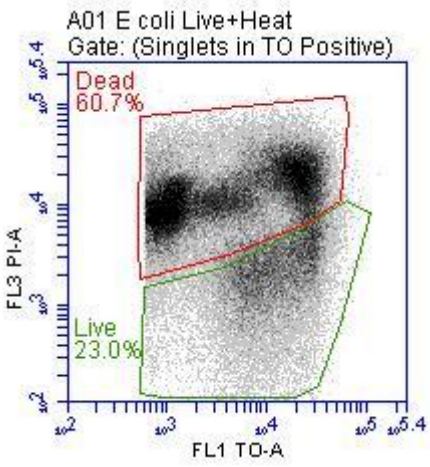
Bacteria: Viability



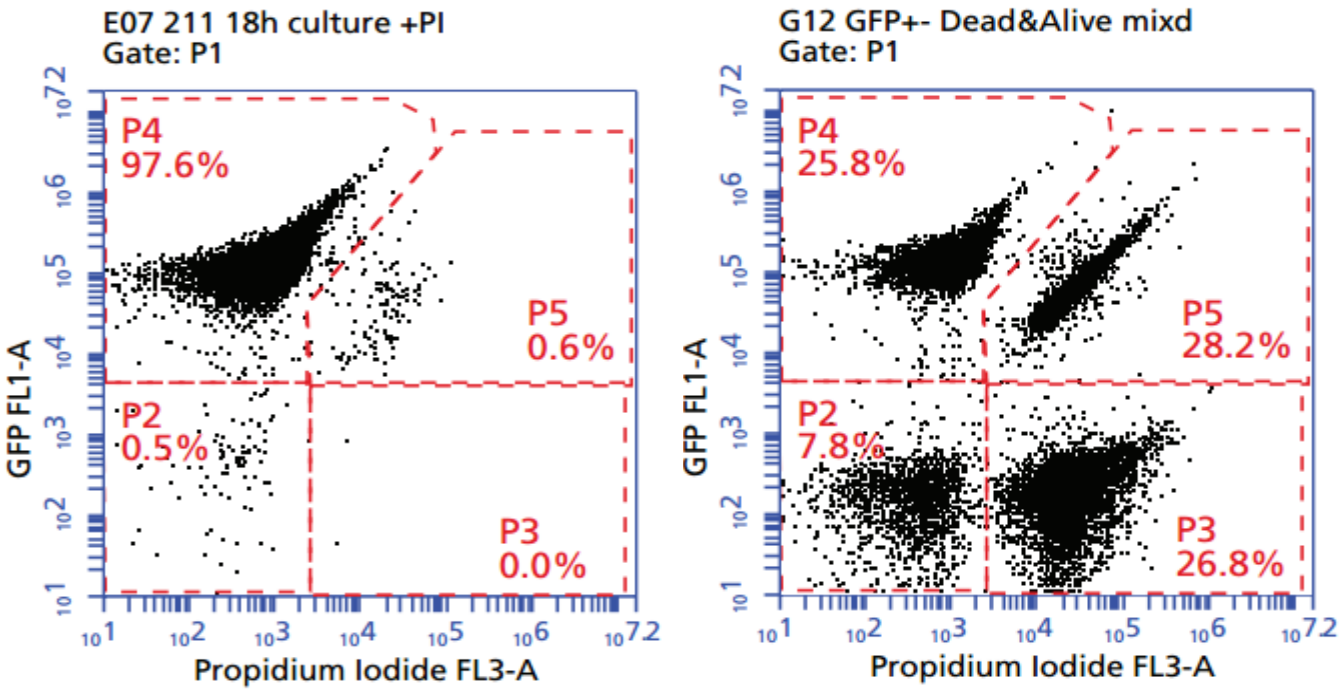








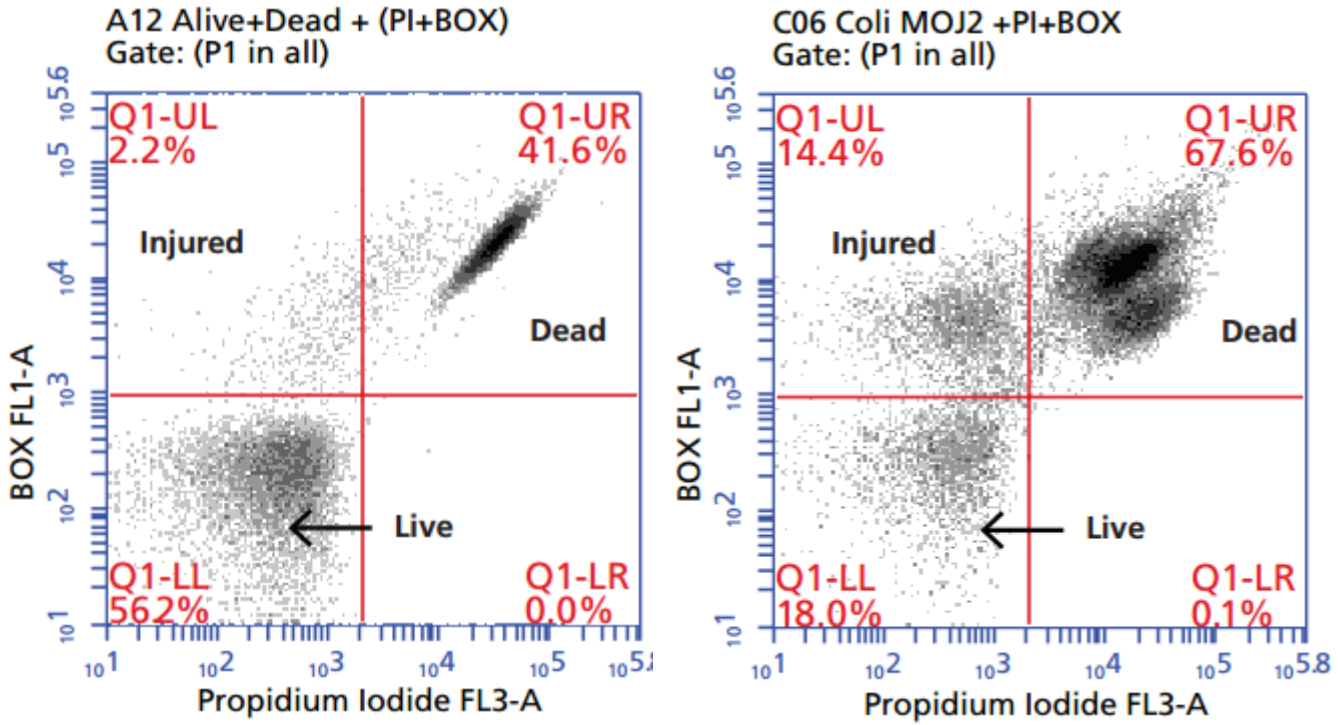
Bacteria: Measuring Viability and GFP



Recombinant protein production (GFP) can be measured simultaneously with cell viability.

Data courtesy of C. Wyre, A. Anvarian, and T. Overton, University of Birmingham, UK

Bacteria: Measuring Viability and Vitality

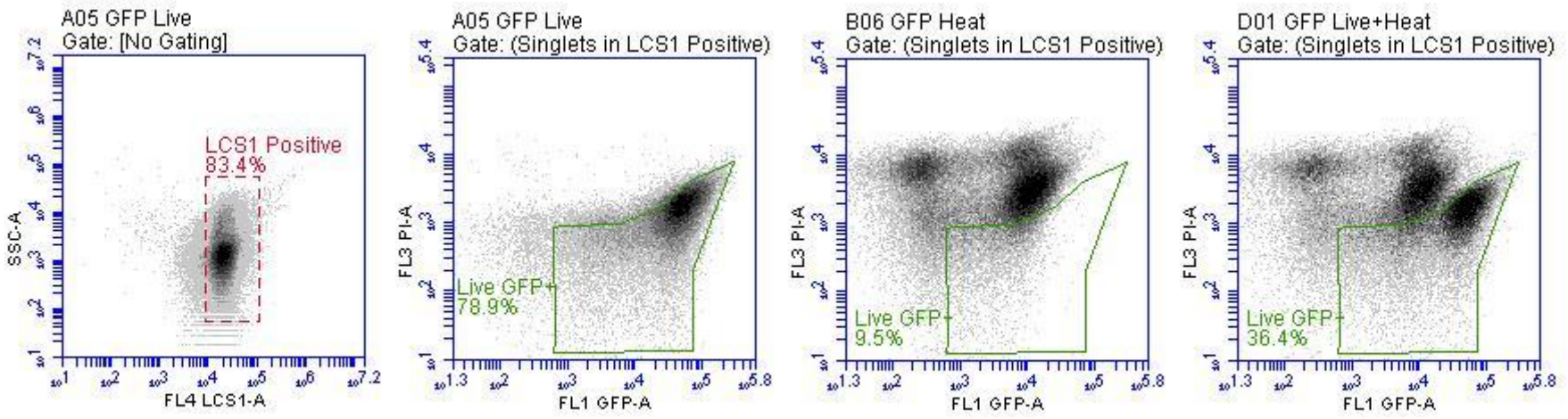


Membrane polarity (vitality) can be measured simultaneously with membrane permeability (viability).

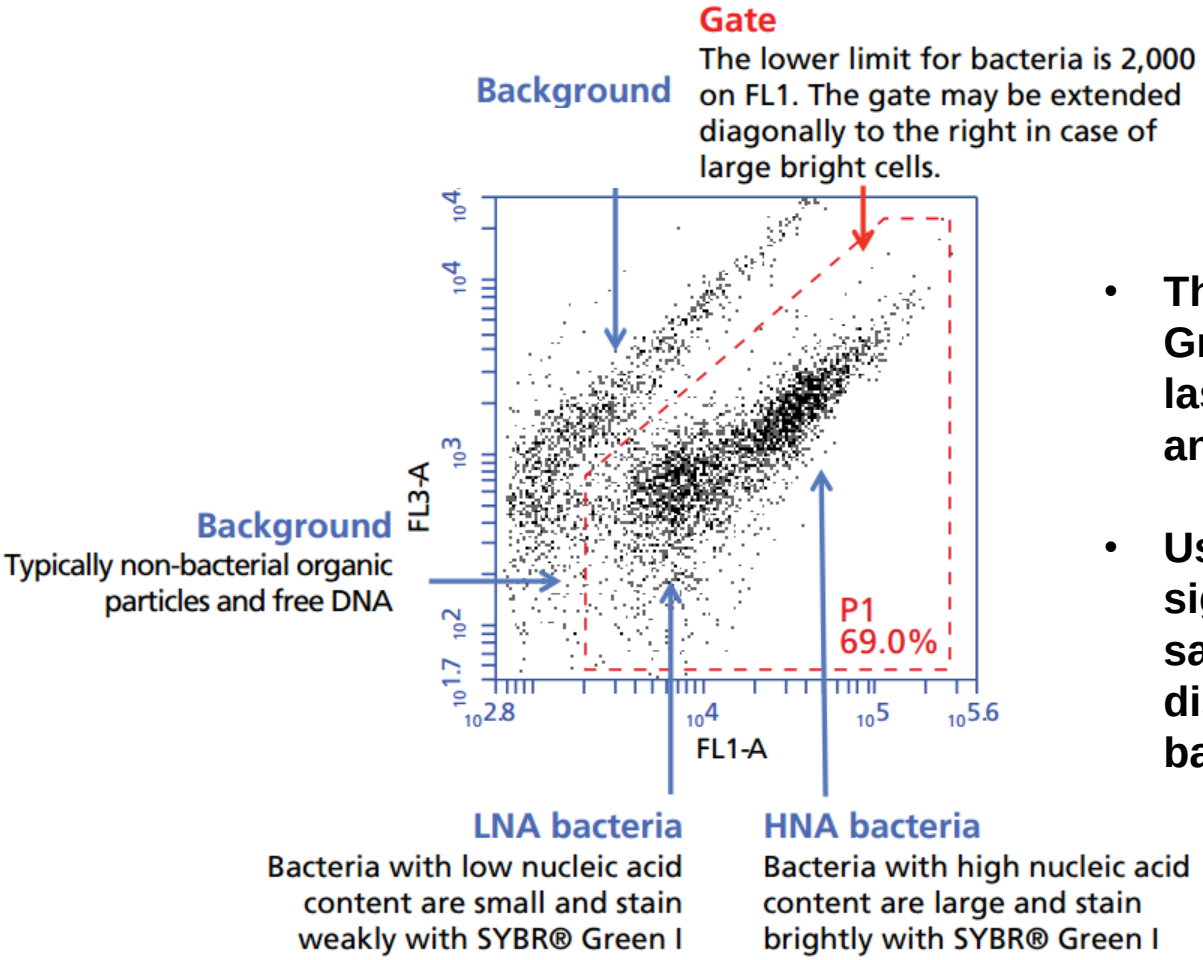
Data courtesy of C. Wyre, A. Anvarian, and T. Overton, University of Birmingham, UK

Bacteria: Expanding the Multiplexing Capabilities

- Conventional green DNA dyes (TO-PRO®, SYBR® Green, SYTO® 9) cannot be used when analyzing GFP cultures or functional dyes detected in FL1.
- Red DNA dyes are detected in FL4 and can be used to discriminate bacteria from noise, while leaving the FL1 channel open.

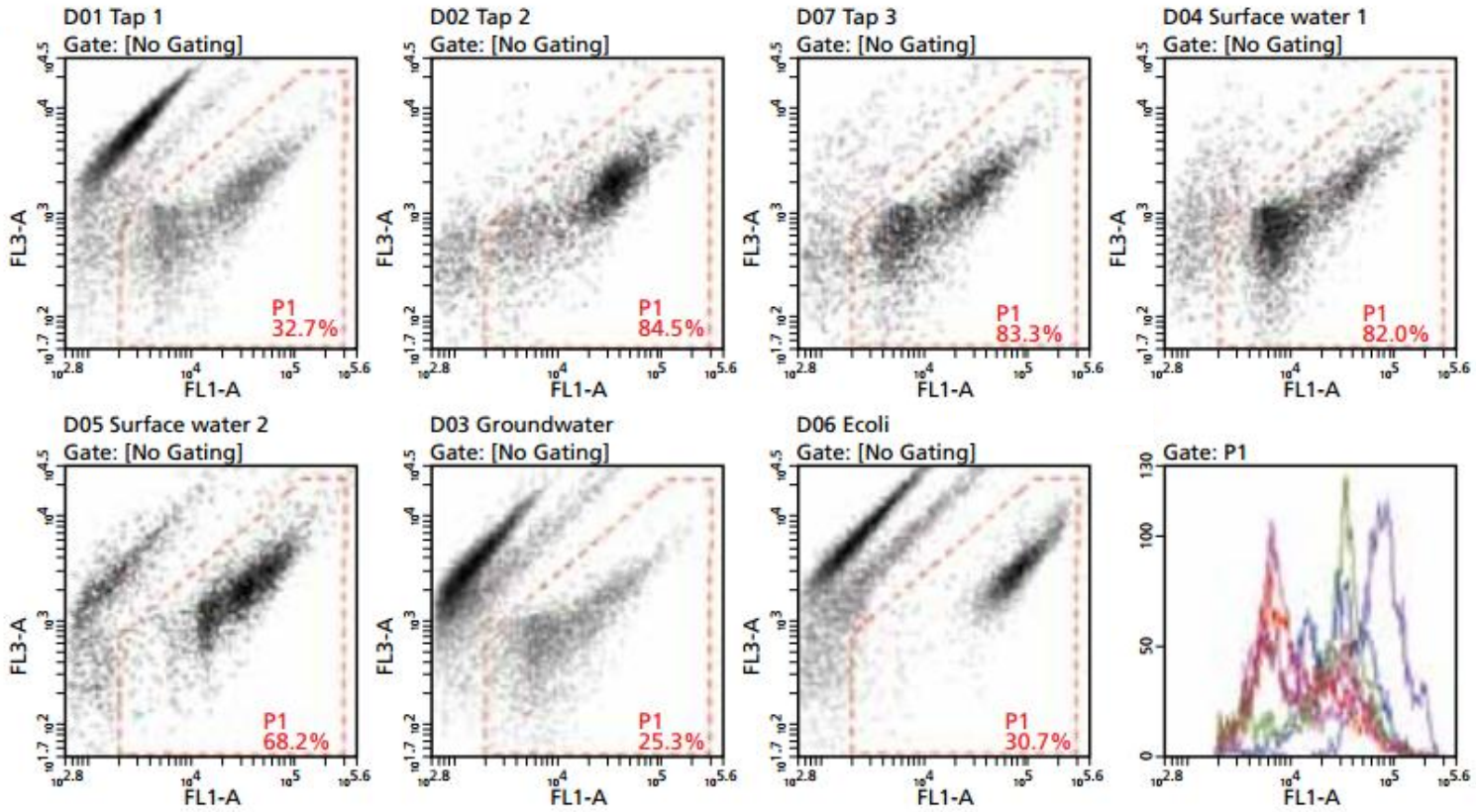


Eawag Method: Using DNA Dye to Detect Bacteria in Water Samples



- **The DNA binding dye, SYBR® Green I is excited by the blue laser and emits in the FL1 and FL3.**
- **Using two fluorescence signals originating from the same dye can help discriminate bacteria from background noise.**

Multiple Water Sample Analysis Using the Eawag Method



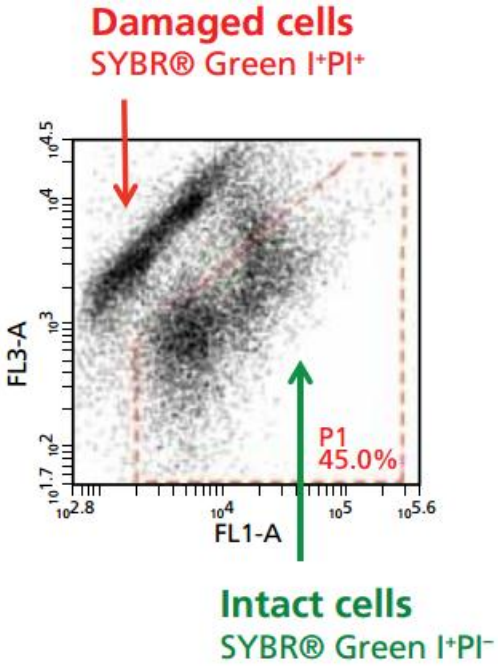
Multiple water samples can be analyzed using the Eawag (Swiss Federal Institute of Aquatic Science and Technology) method, with each water sample displaying a unique footprint.

Multiple Water Sample Analysis Using the Eawag Method: Cell Concentration

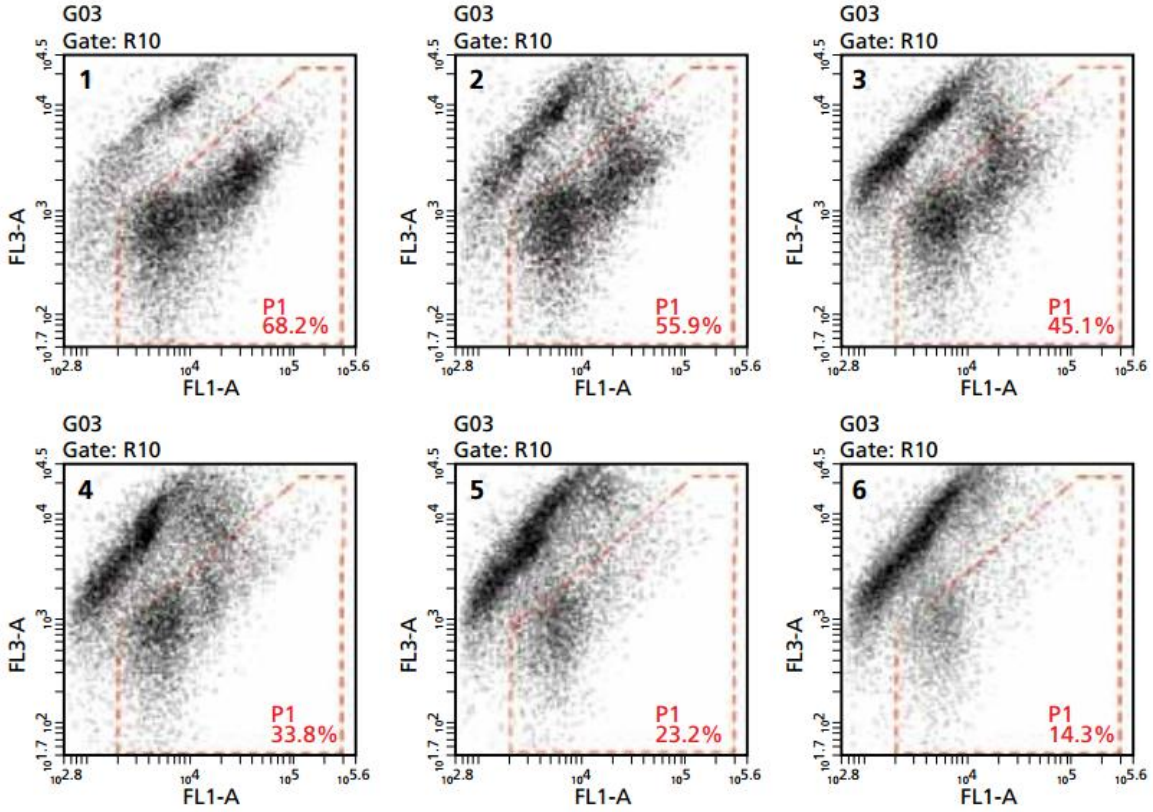
Sample Source	Concentration (cells/mL)	Fluorescence (Mean FL1-A)	Histogram Color
Tap water 1	60,000	23,498	
Tap water 2	217,000	31,326	
Tap water 3	271,000	21,030	
Surface water 1	611,000	19,313	
Surface water 2	309,000	35,687	
Ground water	37,000	17,123	
E. coli	37,000	83,423	

Bacteria in Water Samples: Viability

- **Viability can be measured by membrane integrity.**
  - PI (impermeable to cell membranes of living cells)
  - Can be used in combination with dyes that would enter all cells (for example, SYBR® I Green) to help distinguish bacteria from debris



Bacteria in Multiple Water Samples: Viability and Cell Concentration

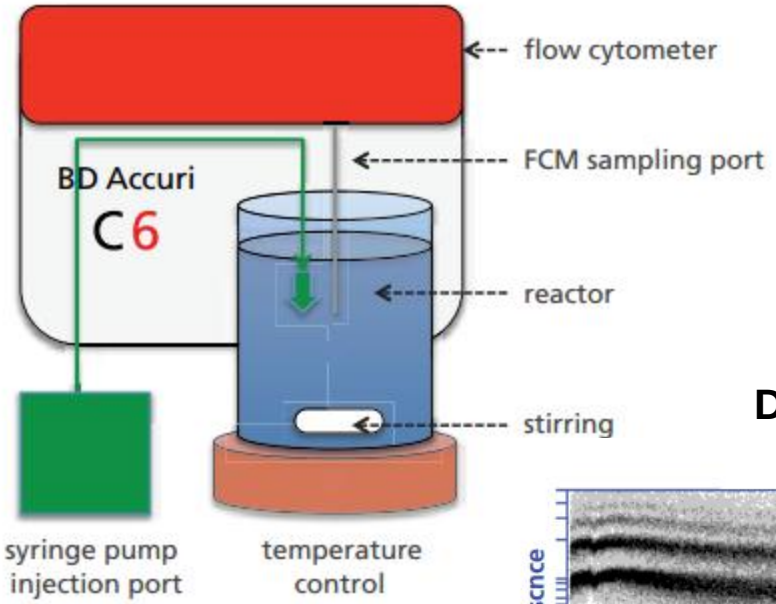


Sample	Concentration (cells/mL)	% cells in P1 (relative to Sample 1)
1 (untreated)	220,000	100%
2	190,000	84%
3	170,000	77%
4	120,000	55%
5	77,000	35%
6	51,000	23%

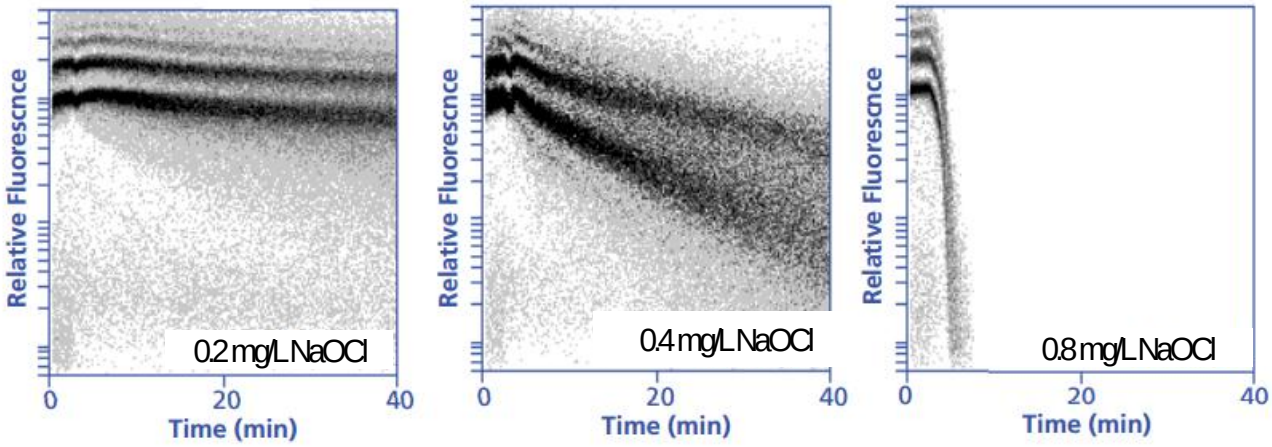
Software templates available at bdbiosciences.com/go/templates

Description
BD Accuri™ C6 Eawag Water Quality Template (zip file) containing:
Eawag Water Quality Template for BD Accuri C6.c6t file
Eawag Water Quality Template_ReadMe.txt file

Real-time Effects of Chlorination Concentrations on Bacteria



Data can be monitored in real time.



Bacterial Analysis

- **Flow Cytometric Applications in Bacterial Analysis:**
  - Visualizing intrinsic size differences as culture contaminant in yeast samples
  - Fluorescent markers
  - The use of fluorescent dyes to measure viability and vitality
  - Concentration of samples
  - Continuous sampling
  - Water sample analysis

BD Accuri C6: Features which Enable Microbial Analysis

- **Wide Fluorescence Range:**
  - Expanded dynamic range
- **Wide Size Range:**
  - Ability to adjust core diameter through flow rate adjustment
- **Small Size Detection:**
  - Ability to threshold based on size and/or fluorescence
- **Real-Time Analysis:**
  - Open, non-pressurized system
- **Cell Counting:**
  - Direct volume measurement
- **Small, Transportable Flow Cytometer**

BD Accuri C6 Software: Ease of Use

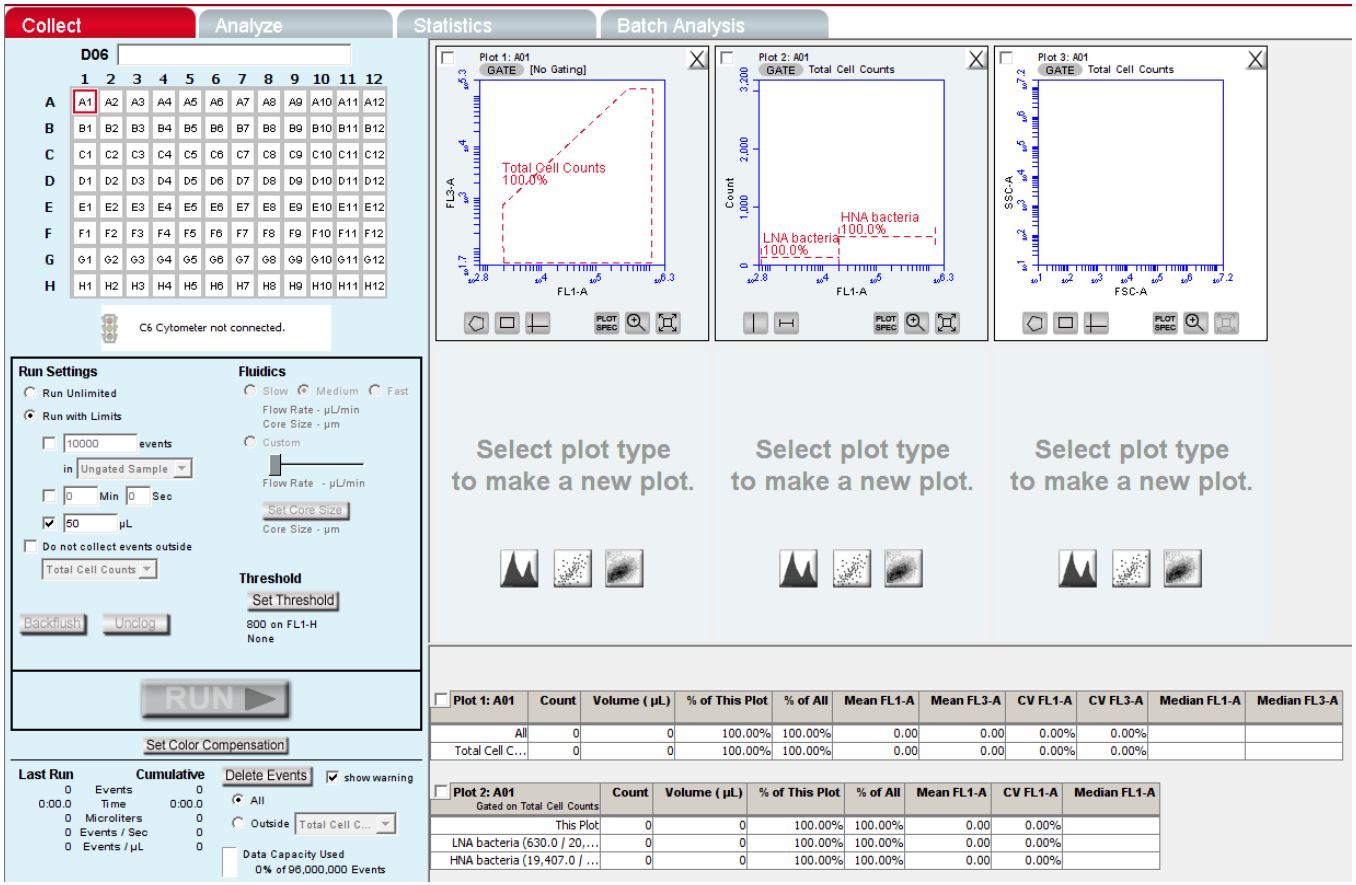
Sample Grid

Cytometer Status

Fluidics Controls

Run Criteria

Real-Time Updates

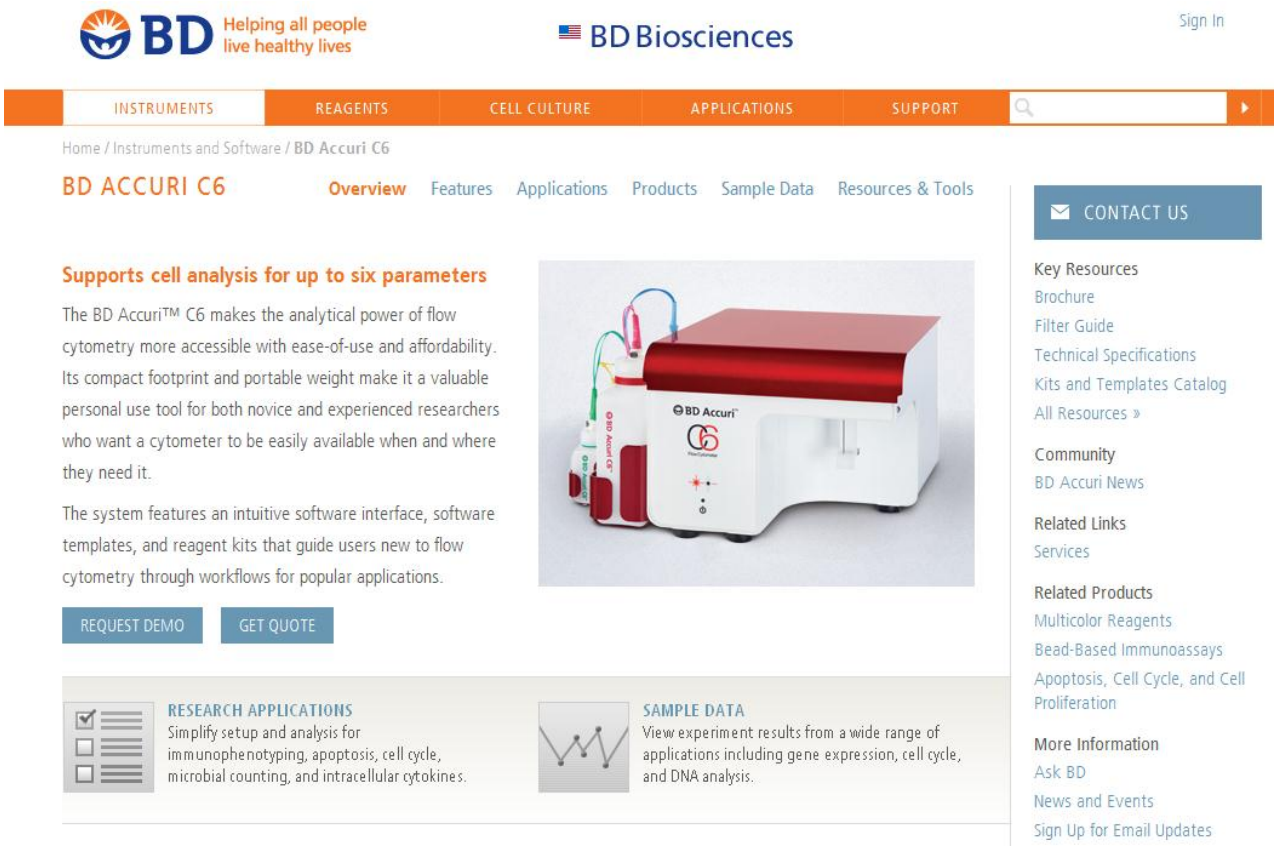


Histogram, Dot Plot, and Density Plot Display Area

Analysis and Gating Tools

Plot Statistics

Additional Resources



www.bdbiosciences.com/resources/accuri

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- California State University, Chico
- University of Houston
- Eawag, Swiss Federal Institute of Aquatic Science and Technology
- University of Birmingham
- University of Minnesota

Questions?

Flow Cytometry within Reach™

The BD Accuri™ C6 Personal Flow Cytometry Tour

BD