Bioprocess Monitoring with the BD Accuri™ C6 Flow Cytometer

Erin Gatza,* Pedro V. Peña,† Friedrich Srienc,† Tim Overton,‡
Christian A. Lavarreda,§ and Clare E. Rogers*

*BD Accuri, Ann Arbor, MI, USA
†BioTechnology Institute, University of Minnesota, Minneapolis, MN, USA
‡School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK
§MSP Corporation, Minneapolis, MN, USA

White Paper

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Abstract

Industrial fermentation of bacteria and yeast produces diverse products, but stresses such as aeration, starvation, and changes in oxygen tension, glucose concentration, and pH can induce cellular heterogeneities in bioprocesses that reduce product quantity and quality. Precise control and optimization of microbial fermentation are crucial.

Flow cytometry offers a powerful and effective methodology for monitoring microbial fermentation. Using it, bioprocess engineers can simultaneously analyze a wide range of cellular parameters at the single-cell level, and generate high-throughput, statistically strong data that supports informed decisions for bioprocess control. Offering both performance and simplicity, the BD Accuri™ C6 flow cytometer allows bioprocess engineers to use light scatter signals to discriminate cells and fluorescence signals to measure cell viability, vitality, and other important cell characteristics. The BD Accuri C6 also interfaces smoothly with automated bioreactor sampling systems such as the MSP M5000 FlowCytoPrep™ sample preparation system, giving bioprocess engineers access to real-time, online bioreactor data for pinpoint process control.

Introduction

Overview: Bioprocess monitoring

Industrial microbiology puts microorganisms to work to make a product—often using fermentation, the primary production method in the biotechnology industry. Industrial fermentation of naturally occurring or genetically modified bacteria and yeast produces diverse products, including antibiotics and other pharmaceuticals, foods and additives, alcoholic beverages, vitamins, monoclonal antibodies, and other proteins. Research laboratories often perform fermentations in small volumes of up to five liters. At the production and manufacturing level, large bioreactors are used, each holding up to 200,000 liters or more.

Stresses such as aeration, starvation, and changes in oxygen tension, glucose concentration, and pH that occur with the scale-up of fermentation processes from laboratory to production volumes can induce cell heterogeneities in bioprocesses that reduce product quantity and quality.[1] These stresses can kill the cells or induce a viable but nonculturable (VBNC) state. VBNC cells lose the ability to grow in culture media but maintain at least some metabolic activity and have been shown, in some cases, to actively contribute to fermentation progress.[1] Large numbers of dead or VBNC cells, however, can affect production, impair product quality,[1] and/or raise costs. Precise control and optimization of microbial fermentation are crucial.

Bioprocess monitoring often relies on population-based measures of cell viability or protein production, which fail to reflect the heterogeneity among individual cells within a population[2] and provide results too slowly to allow in-process changes.[3] Multiparametric analysis of single cells performed in real time could provide detailed insight into cell viability, metabolic activity ("vitality"), and productivity in the culture; quantify VBNC, dead, or contaminating cells in the bioreactor; and allow bioprocess engineers to harvest cells or activate inducible systems appropriately to maximize high-value product yield.[1,4,5]

Classical methods for monitoring microbial fermentations

Bioprocess engineers have traditionally used a range of methods to monitor microbial bioprocesses. Each has its advantages and disadvantages.[3] Bulk "enumeration" of bacteria and yeast using dry cell weight or spectrophotometric estimation of optical density (OD) is fast and simple, but requires lengthy drying or is prone to error. Of the true enumerative methods, dilution plating requires culture time and cannot detect VBNC cells, while manual counting of cells stained with methylene blue using a hemocytometer is tedious and prone to variability.

Electrophoresis, fluorescence, and antibody-based methods, such as SDS-PAGE, Western blot (using protein-specific antibodies), and spectrofluorimetry (to detect GFP-tagged proteins) can provide visual evidence of protein production and identify specific proteins, but can be time-consuming and cannot measure culture heterogeneity or cell physiology.

In sum, these classical microbiological and molecular methods typically yield data too slowly to allow in-process changes in microbial fermentations. Those that rely on culturing cells not only take time but also cannot detect VBNC cells. Finally, these methods cannot provide information at the single-cell level or analyze multiple parameters in a single assay to gain a better understanding of cell health. Bioprocessing requires process-independent techniques to study and quantify the heterogeneity intrinsic to cellular fermentation.

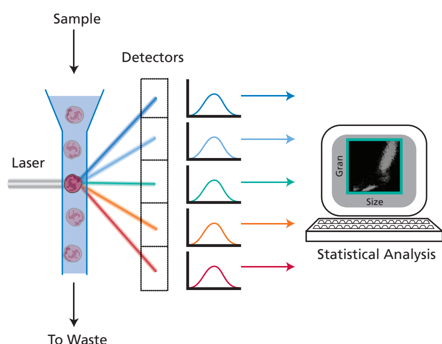

Figure 1. How flow cytometry works.

Impact of flow cytometry

Flow cytometry offers a powerful and effective methodology for monitoring microbial fermentation. In flow cytometry, particles or cells are suspended in a liquid stream that is hydrodynamically focused through beams of laser light. Optical detectors collect scattered laser light and fluorescence, and electronics measure and digitize these light emissions for analysis on a computer. The light scatter data provides basic information about the cells, such as relative size, shape, and surface features. And, when the microbes are labeled with fluorescent dye–conjugated antibodies or proteins, the fluorescence data allows measurement of numerous other cellular properties.

In microbial bioprocessing, microorganisms can be sampled and immediately stained using fluorescent dyes that measure intracellular enzyme activity, membrane integrity, and plasma membrane potential. They can also be genetically modified to express green fluorescent protein (GFP) or other fluorescent proteins, which allows real-time bioprocess monitoring. However the microbes are labeled, bioprocess engineers can then use flow cytometry to analyze multiple cellular parameters simultaneously at the single-cell level. By generating high-throughput, statistically strong data, they can make informed decisions for bioprocess control.

This white paper explores the opportunities and challenges of applying flow cytometry to microbial bioprocessing, and discusses key capabilities and techniques for successful analysis. All research examples in this paper were analyzed on the BD Accuri C6 personal flow cytometer, which is especially well suited for the rapid and efficient assessment of microbial fermentation.

Technical considerations for the BD Accuri C6

Operating environment

The BD Accuri C6 flow cytometer offers performance, simplicity, and affordability. Light, rugged, and small enough to fit on a benchtop, it connects to standard electrical circuitry and can be placed in a laminar flow hood. Fixed optics and capillary sheath-flow fluidics make the BD Accuri C6 portable; it can be moved from site to site without realignment.

BD Accuri™ C6 software makes operation intuitive for novice and proficient users alike. Most new users become fluent with the software in less than 30 minutes, assisted only by a 3-page pictorial *Quick Start Guide*. Data files can be analyzed within the program, aided by special tools such as Zoom and VirtualGain™, or exported in FCS 3.0 format into FCS Express™, FlowJo™, and other flow cytometry analysis programs.

Routine instrument cleaning and priming are automated. Maintenance is easy, even for novice users, and requires no tools. The recommended sheath fluid is 0.22-µm filtered, deionized water. These attributes effectively expand flow cytometry beyond the core facility and onto the laboratory bench.

Figure 2. The BD Accuri C6 personal flow cytometer.

The instrument weighs just 13.6 kg (30 lb). Exterior dimensions (H x W x D) are 27.9 x 54.6 x 41.9 cm (11 x 21.5 x 16.5 in.) with fluid tanks in place.

Reliable, high-performance fluidics

Microorganisms can vary greatly in size, which may make them difficult to process through a flow cytometer's fluidics system. The "push-pull" peristaltic pump in the BD Accuri C6 enables independent regulation of both the sheath and sample flow rates. Users can quickly optimize the sample core diameter (adjustable from 5 to 40 µm) based on the anticipated size range of sample microorganisms. The design also allows easy removal of clogs (such as cell clumps) from the flow cell with a brief burst of sheath fluid followed by a high-volume flush, simply by clicking the Unclog and Backflush buttons in the software.

The BD Accuri C6 simplifies bioprocess monitoring by automatically calculating cell concentrations during data collection, eliminating the need to count microorganisms manually. The direct-drive peristaltic pump system meters the sample volume pulled for each run and automatically calculates events per microliter, providing both cell counts and concentration.

The non-pressurized fluidics system of the BD Accuri C6 supports various types of sample tubes, so researchers can use whichever tube is easiest for sample preparation. With open tubes, such as Eppendorf® tubes, researchers can even add reagents or sample during data collection, performing kinetic assays without interruption.[6]

Optical and analytical modalities

The BD Accuri C6 features two lasers, two scatter detectors, and four fluorescence detectors. A state-of-the-art digital signal processing (DSP) system gives the BD Accuri C6 a dynamic range of six full decades. This means that it can finely resolve both faint and bright signals at once and analyze a wide span of variation in fluorescence in a single run. The instrument detects this broad dynamic range using standard factory detector settings, without the need for optimization or tuning.

If additional flexibility is needed, the optical configuration is flexible as shown in Table 1. The Selectable Lasers Module (Cat. No. 653126) allows reassignment of the standard laser/detector associations, and optional filters can modify the effective detector characteristics.

Table 1. Standard and optional optical configuration of the BD Accuri Cytometer system.

Laser/Detector Associations	"3 blue 1 red" (standard)	488 nm (emissions read in: FL1, FL2, FL3) 640 nm (emissions read in: FL4)
	"2 blue, 2 red" (653126 Module)	488 nm (emissions read in: FL1, FL2) 640 nm (emissions read in: FL3, FL4)
	"4 blue" (653126 Module)	488 nm (emissions read in: FL1, FL2, FL3, FL4)
Scatter Detection	Forward (0° ±13°) Side (90° ±13°)	
Emission Detection	Four colors, user-swappable optical filters	
	Standard set: FL1 533 ±15 FL2 585 ±20 FL3 670 LP FL4 675 ±12.5	Optional: 510 ±7.5 (Cat. No. 653184) 540 ±10 (Cat. No. 653528) 565 ±10 (Cat. No. 653185) 610 ±10 (Cat. No. 653186) 780 ±30 (Cat. No. 653187)

Automated data collection

The BD CSampler™ automation accessory (Figure 3) adds simple, unattended, reliable, and easy-to-use automation to the BD Accuri C6 flow cytometer system. Automation can significantly increase sample throughput, allowing bioprocess engineers to optimize fermentations in response to real-time data.

With BD CSampler, samples can be prepared in 96-well plates and analyzed directly on the flow cytometer. This saves time and reduces cost, waste, and potential for operator error. The option is compatible with both 48- and 96-well plates and 24-tube racks. BD CSampler adds minimal footprint to the system—about 3 ft^2 (0.28 m^2) for the pair—keeping the benchtop free for other uses.

Figure 3. The BD Accuri C6 with the BD CSampler option.

The optional BD CSampler provides automated sample processing from a 24-tube rack or from 48- and 96-well plates.

Applications in bioprocess monitoring

Using light scatter signals to discriminate cells

With its broad dynamic range, the BD Accuri C6 system can analyze forward- and side-angle light scatter (FSC and SSC) signals for a broad array of cell types, from bacterial species through eukaryotic cells as large as 30 µm. Because bacteria may be as small as 0.6 µm (compared to 4 µm for a baker's yeast cell and >30 µm for some mammalian cell lines), they may overlap with debris particles in the growth medium. However, an FSC or SSC acquisition threshold can exclude most debris particles from the analysis.[7] Researchers can also use fluorescence as a threshold or gating parameter, or combine the two methods.

Figure 4 shows the detection of *E. coli* BL21 above background debris on the BD Accuri C6 flow cytometer. Figure 4A shows the background debris found in filtered growth medium alone, when the FSC threshold is set at its lowest value of 10. When GFP-expressing *E. coli* are spiked into the sample (Figure 4B), no distinct population is discernible from debris using a light scatter plot (FSC-A vs SSC-A, left plot), but GFP fluorescence, detected in the FL1 channel, confirms that *E. coli* cells are indeed being detected (right plot). Raising the FSC threshold to 11,000 (Figure 4C) excludes most of the debris particles and allows the *E. coli* population to be visualized and gated by scatter as well as GFP fluorescence.

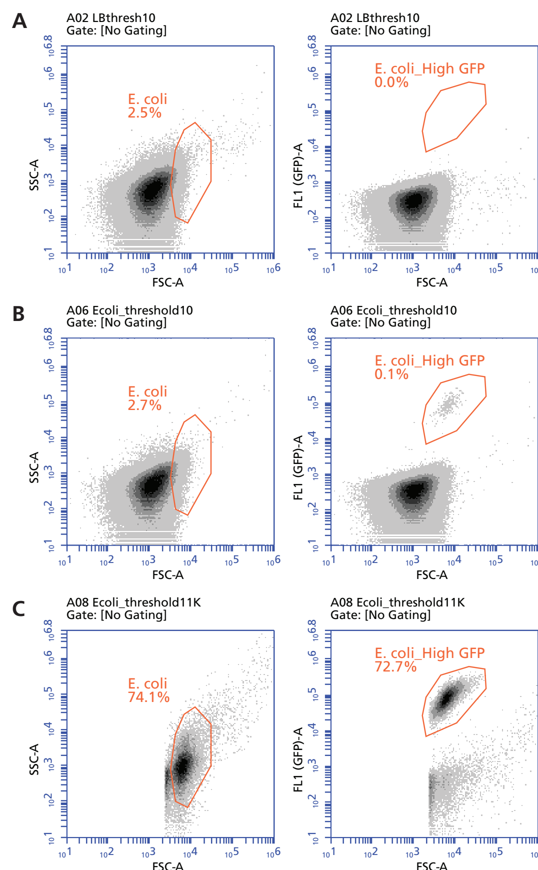

Figure 4. Increasing the FSC threshold enhances *E. coli* detection over background debris.

A 5-mL starter culture of E. coli BL21 cells was grown at 37°C in LB-Amp (1.0% tryptone; 0.5% yeast extract; 1.0% sodium chloride; pH 7; 100 µg/mL ampicillin), and inoculated into 500 mL of LB-Amp at approximately 200 cells/µL in a 2-L bioreactor. The culture was grown at 37°C and stirred at 250 RPM. ***Results*** *show FSC vs SSC (left) and FSC vs FL1 (GFP) density plots of* ***(A)*** *filtered LB medium, FSC-H acquisition threshold = 10;* ***(B)*** *filtered LB medium inoculated with E. coli, FSC-H threshold = 10; and* ***(C)*** *filtered LB medium inoculated with E. coli, FSC-H threshold = 11,000. E. coli regions were drawn based on FSC vs FL1 (GFP) plots (Peña P, Srienc F, unpub).*

In fact, flow cytometry can detect multiple cell populations in the same culture, with important applications in bioprocess control, such as detecting microbial contamination in bioreactors. Figure 5 shows the simultaneous detection and discrimination of both populations in a co-culture of the yeast *S. cerevisiae* S288c and the bacterium *E. coli* BL21, using light scatter signals on the BD Accuri C6. Each population was analyzed individually and then together.

Analysis of the debris in the medium (Figure 5A) shows that, as expected, debris was mostly co-localized in the *E. coli* population gate, with only 16.3% and 0.3% of events recorded within the two population gates. Upon inoculation with *E. coli* alone (Figure 5B), 86.1% of events were recorded in the *E. coli* gate. Similarly, inoculating with *S. cerevisiae* alone (Figure 5C), 68.4% of events were recorded within its gate, and only 5.3% of events in the *E. coli* gate. Finally, when *E. coli* and *S. cerevisiae* were mixed at a 2:1 ratio (Figure 5D), 54.1% and 26.1% of events fell within the *E. coli* and *S. cerevisiae* gates, again as expected.

Simultaneous measurement of viability and vitality

Light scatter signals on the BD Accuri C6 can profile microorganisms based on size and complexity. Adding fluorescent stains can measure cell physiology (including intracellular enzyme activity, membrane integrity, and plasma membrane potential) and detect fluorescent tags such as GFP. Fluorescent stains can add power to bioprocess analysis by providing a more complete picture of the viability, vitality, and productivity of individual cells.[1,3,8]

Viability

Plasma membrane integrity is the most definitive proof of cell viability. Membrane integrity indicates that cells can generate electrochemical gradients and have metabolic activity.[1] Cells lacking an intact membrane cannot generate such gradients, lack metabolic activity, and are considered dead.

Flow cytometry can measure membrane integrity using fluorescent dyes that are impermeable to the cell membrane of healthy cells. Propidium iodide (PI), a fluorescent vital dye often used to stain DNA, is the most common reagent for dye exclusion tests. The BD Accuri C6 detects PI primarily in the FL3 detector (emission filter 670 LP). PI can be used in combination with various other fluorescent markers for multiparametric analysis.

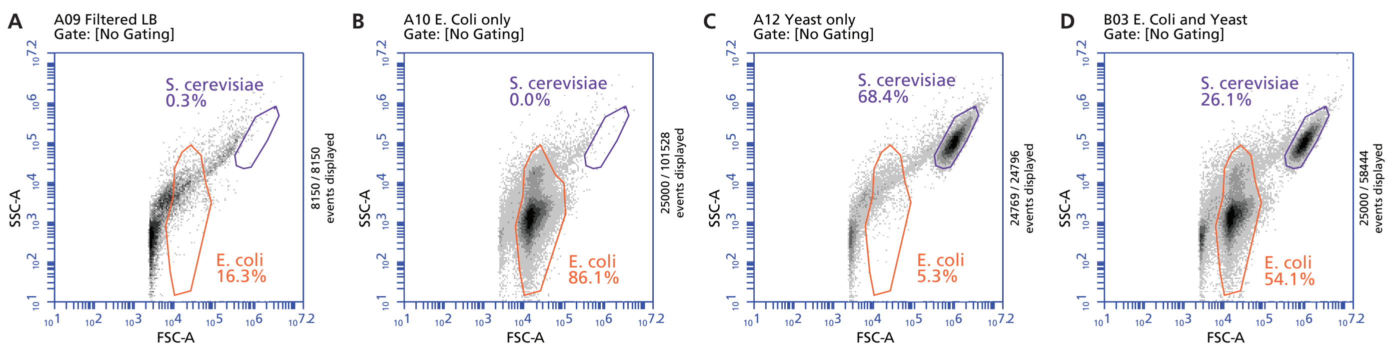

Figure 5. Simultaneous detection of *S. cerevisiae* and *E. coli* in the same culture.

Exponentially growing E. coli BL21 and S. cerevisiae S288c cells were diluted in filtered (0.2 µm) LB medium for individual analysis. Equivalent final dilutions were analyzed in the co-culture. The acquisition threshold was set at FSC-H = 11,000 to exclude debris particles. Samples were analyzed for 2 minutes at the Medium flow rate (35 µL/minute) for a total of 71 µL. ***Results:*** *FSC-A vs SSC-A log-scale density plots show* ***(A)*** *filtered LB medium used as blank sample;* ***(B)*** *inoculation with 460 cells/µL E. coli;* ***(C)*** *inoculation with 240 cells/µL S. cerevisiae;* ***(D)*** *E. coli and S. cerevisiae co-culture (Peña P, Srienc F, unpub).*

In Figure 6, PI was used with thiazole orange (TO) to stain *E. coli* cells that were either untreated (column 1) or treated with isopropanol (column 2) or heat (column 3) to induce cell death. TO is a permeant dye that enters all cells, live and dead, and is detected primarily in the FL1 and FL2 channels of the BD Accuri C6. The fluorescent signal from TO allows clearer distinction between bacteria and debris or noise signals, which improves enumeration of cellular events even when debris contaminates the scatter gate. Figure 6A shows that TO was detected in 71.1% of untreated *E. coli* cells, falling to 53.4% and 3.2%, respectively, in cells treated with isopropanol or heat. Figure 6B shows PI staining of the gated TO^{+} cells. As expected, exposure to isopropanol or heat resulted in increased PI staining, fewer viable cells (Live gate; $TO^{+}PI^{-}$), and more injured ($TO^{+}PI^{int}$) and dead ($TO^{+}PI^{+}$) cells.

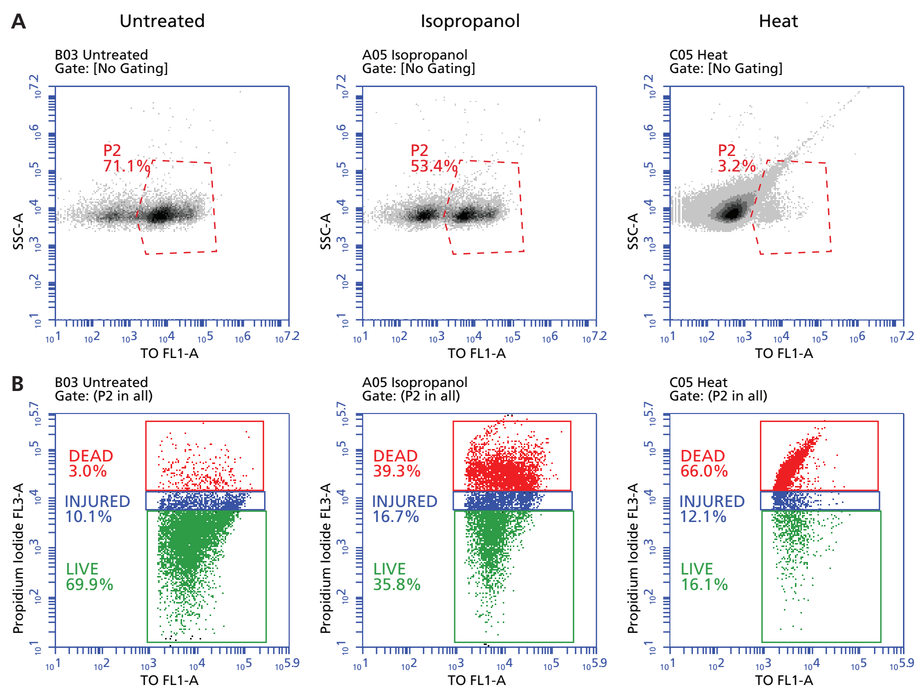

Figure 6. Staining of *E. coli* with TO and PI to assess viability.

Exponentially growing E. coli were harvested and death was induced by treating cells with 17% isopropanol for 30 minutes at room temperature or by exposure to 70–90°C temperatures for 15 minutes. Resulting samples were stained according to the BD™ Cell Viability Kit protocol (Cat. No. 349483). Each sample was acquired on a BD Accuri C6 for 30 seconds on the Medium flow rate (35 µL/min) with SSC-H threshold = 10,000 to exclude debris. ***Results: A.*** *TO (FL1-A) vs SSC-A contour plots show TO staining of (left to right) untreated, isopropanol-treated, and heat-treated E. coli samples. Gate P2 represents* TO^{+} *cells.* ***B.*** *TO (FL1-A) vs PI (FL3-A) log-scale dot plots show gated* TO^{+} *E. coli cells co-stained with PI. Live, Injured, and Dead gates were drawn using single-stained controls (data not shown).*

Tagging yeast and bacteria with fluorescent proteins allows more specific gating and a more focused analysis of the population of interest. GFP and enhanced GFP (eGFP) are commonly used in bacterial and yeast fermentations and can be detected in the FL1 channel of the BD Accuri C6 using the standard emission filter (FL1 533/30).

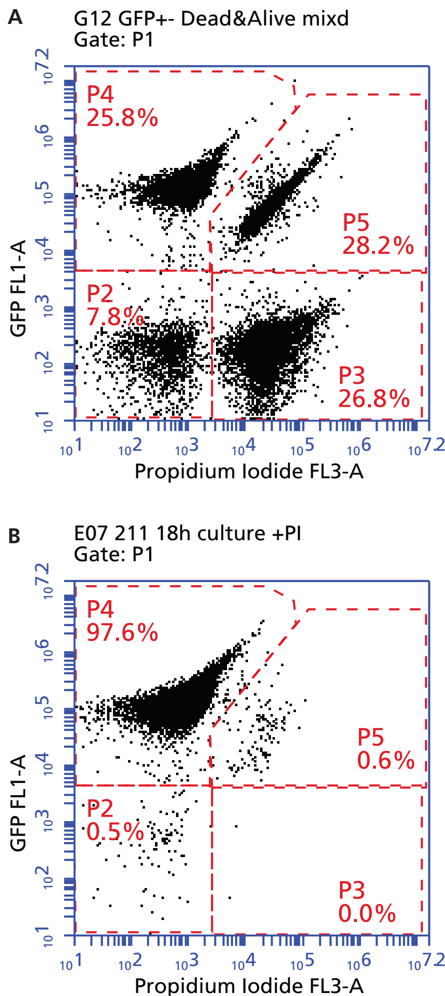

Figure 7. Viability and GFP expression of *E. coli* BL21 following overnight culture.

E. coli BL21 cells were cultured for 18 hours and analyzed for GFP expression and PI. 20,000 E. coli events/sample (gated on SSC-A vs FSC-A scatter) were acquired on a BD Accuri C6 with FSC-H threshold = 12,000. ***Results*** *show PI (FL3-A) vs GFP (FL1-A) log-scale dot plots for* ***(A)*** *a control sample prepared by mixing live and ethanol-killed plasmid-positive and untransformed cells, and* ***(B)*** *a sample from an 18-hour growth culture. Gates were drawn according to the four populations visible in the control sample: PI^-GFP^- (alive/non-expressing, P2); PI^+GFP^-(dead/non-expressing, P3); PI^-GFP^+ (alive/expressing, P4); and PI^+GFP^+ (dead/expressing, P5) (Wyre C, Anvarian A, Overton T, unpub).*

In Figure 7, *E. coli* BL21 producing a plasmid-encoded recombinant GFP fusion protein were cultured for 18 hours and analyzed for GFP expression and PI staining to determine recombinant protein production and cell viability, respectively. Figure 7A shows a control sample, prepared by mixing live and ethanol-killed populations of plasmid-positive (GFP^+) and untransformed ($GFP^–$) cells, which was used to visualize and gate the four potential cell populations on a PI (FL3-A) vs GFP (FL1-A) plot. Analysis of a sample taken from the overnight culture (Figure 7B) indicated that 97.6% of the bacteria were viable and expressed the GFP-tagged protein ($GFP^+PI^–$, P4). Very small populations of viable/non-expressing (P2) and dead/expressing (P5) cells were also detected.

Vitality

Although PI exclusion indicates an intact cell membrane, implying viability, it does not indicate cellular functionality such as plasma membrane polarity, metabolic activity, or ability to replicate. Understanding the physiological health of living biomass in the culture—the cells' vitality—is crucial in achieving high-efficiency fermentations and recombinant protein production.[2,9] Combining measures of cell membrane integrity (viability) and plasma membrane potential (vitality) in a single sample, for example, allows differentiation between dormant, depolarized cells (such as VBNCs) that retain membrane integrity and may recover if culture conditions are optimized, and dead cells with depolarized membranes and compromised membrane integrity.

Flow cytometry can measure membrane potential using lipophilic dyes that permeate the cell membrane and accumulate according to its charge. Bis-(1,3-dibutylbarbituric acid) trimethine oxonol (also called bis-oxonol, $DiBAC_4(3)$, or BOX) is the preferred membrane potential assay for *E. coli* and Gram-negative bacteria.[8] BOX is lipophilic, anionic, and accumulates within cells with depolarized membranes but is excluded from healthy cells with polarized membranes.[3,8] It is excited at 488 nm and its emission fluorescence can be measured in the FL1 channel of the BD Accuri C6 using the standard 533/30 emission filter.

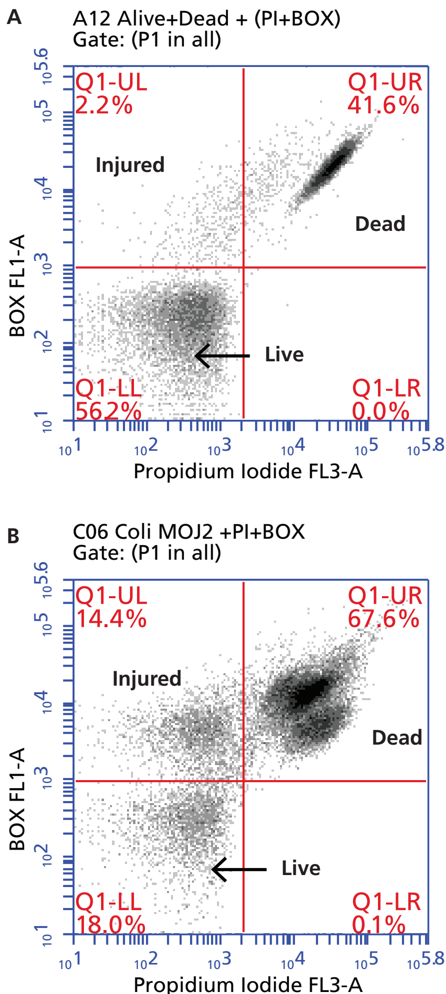

Figure 8. Viability and vitality of *E. coli* MG1655 following chemical stress.

E. coli MG1655 cells were cultured for 18 hours and stained with PI and BOX. 20,000 E. coli events/sample (gated on SSC-A vs FSC-A scatter) were acquired on a BD Accuri C6 with FSC-H threshold = 12,000. ***Results*** *show PI (FL3-A) vs BOX (FL1-A) log-scale dot plots for* ***(A)*** *a control sample prepared by mixing live and ethanol-killed cells and* ***(B)*** *a sample taken following chemical stress to the culture. Live cells are BOX$^-$PI$^-$; injured cells, BOX$^+$PI$^-$; dead cells, BOX$^+$PI$^+$ (Anvarian A, Wyre C, Overton T, unpub).*

In Figure 8, *E. coli* MG1655 cells were co-stained with BOX and PI to simultaneously assess cell viability and vitality. Figure 8A shows a control sample, prepared by mixing live and ethanol-killed *E. coli* cells, on a PI (FL3-A) vs BOX (FL1-A) plot. Populations of live (BOX$^-$PI$^-$) and dead (BOX$^+$PI$^+$) cells are visible. After the culture was subjected to a chemical stress (Figure 8B), a third population of injured cells appeared in the upper-left quadrant. Because their cellular membranes are intact but their plasma membranes are depolarized, these injured cells stain with BOX but not PI (BOX$^+$PI$^-$). Depending on culture conditions, these cells may either remain dormant and injured (BOX$^+$PI$^-$), progress to death (BOX$^+$PI$^+$), or regenerate their membrane potential and move into the live quadrant (BOX$^-$PI$^-$).

Automated online sampling for real-time process control

Automated monitoring of a cell culture using flow cytometry can provide high-resolution analysis of the growth and phenotype of a population of cells. BD Biosciences has partnered with MSP Corporation to interconnect the BD Accuri C6 with the MSP M5000 FlowCytoPrep sample preparation system. The FlowCytoPrep is a new microreactor technology platform that enables user-initiated or fully automated sterile bioreactor sampling, sample preparation including cellular staining with fluorescent dyes, and sample injection into the BD Accuri C6 flow cytometer. Together, the two instruments can perform rapid online measurement of single-cell properties, which is not feasible by classical methods.[10,11] The interface between the two instruments is seamless, resulting in a turnkey system for real-time, online monitoring of bioreactors.

Figure 9. The MSP M5000 FlowCytoPrep connected to the BD Accuri C6.

To test its performance, the BD Accuri C6/MSP FlowCytoPrep system was used to measure the kinetics of batch growth and GFP expression of *E. coli* BL21 cells cultured in either LB or M9 minimal media. GFP was expressed from the pRSET-GFP expression plasmid, which was propagated in the cells using the ampicillin selectable marker.

E. coli cultured in LB-Amp medium

For LB cultures, a 5-mL starter culture of *E. coli* BL21 cells was grown at 37°C in LB-Amp (1.0% tryptone; 0.5% yeast extract; 1.0% sodium chloride; pH 7; 100 µg/mL ampicillin), and inoculated into 500 mL of LB-Amp at approximately 200 cells/µL in a 2-L bioreactor. The culture was grown at 37°C and stirred at 250 RPM. The MSP FlowCytoPrep acquired and delivered the samples, automatically executing acquisition and cleaning commands on the BD Accuri C6.

Samples were analyzed for exponential growth and GFP expression every 15 minutes following inoculation. Monitoring GFP expression over time allows discrimination between producing and non-producing cells in a fermentation. One can tell, for example, whether reduced overall production is due to reduced production in every cell of a homogeneous population, or to a subset of low- or non-producers.

As shown in Figure 10, the concentration of cells in the bioreactor (▲) expanded from 192 cells/µL at inoculation to 18,557 cells/µL over 8 hours of monitoring. (Beyond this point, cell concentrations in undiluted samples fall outside the linear range of the BD Accuri C6.) Concurrently, GFP expression (■) decreased over time.

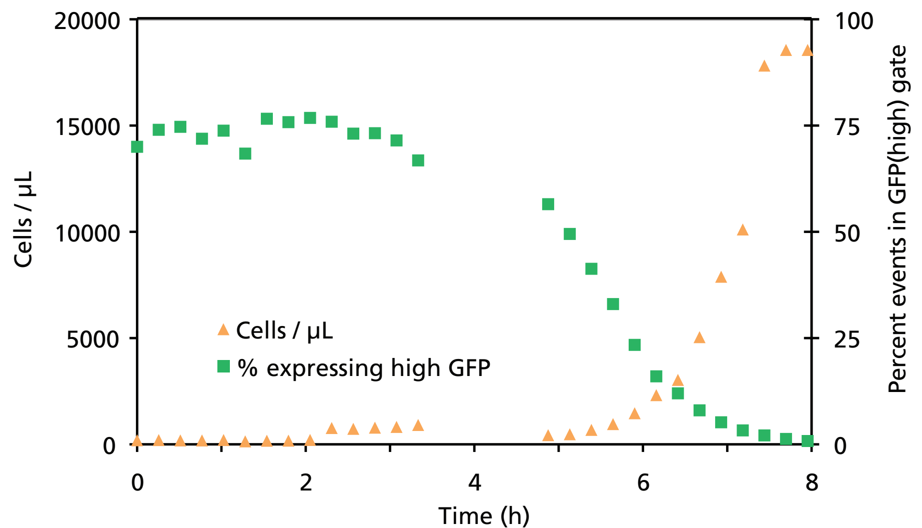

Figure 10. Batch growth and GFP fluorescence of *E. coli* BL21 pRSET-GFP in LB medium.

E. coli BL21 cells were cultured in LB-Amp, acquired, and analyzed on the MSP FlowCytoPrep and BD Accuri C6 as described in the text. Samples were analyzed for 1.5 minutes at the Medium flow rate (35 µL/min), with an acquisition threshold of FSC-H = 11,000 to exclude debris. GFP fluorescence was detected in FL1 (533/30) using the standard emission filter. ***Results:*** *Cell concentrations (in cells/µL, ▲) increased rapidly toward the end of culturing, while the percentage of GFP+ events (■) decreased (Lavarreda C, Peña P, Srienc F, unpub).*

Further analysis revealed a relationship between GFP expression and growth phase, as shown in Figure 11. Upon inoculation from a late-exponential/stationary overnight culture (column 1), GFP expression was high in 83.6% of cells, and the cells were relatively small (median FSC channel value = 7,635). As the cells approached their exponential growth phase (column 2), however, the GFP^+ population waned and a GFP^- population appeared with higher FSC (median FSC signal = 18,776 GFP^- vs 9,781 GFP^+). This trend continued throughout the exponential growth phase, resulting in a progressive drop in GFP^+ events to 1.4% of gated *E. coli* cells over the course of the experiment (columns 3 and 4). Thus, two populations were observed during the exponential phase, smaller cells with high GFP fluorescence and larger cells with low GFP fluorescence. This suggests that GFP expression is induced during the late-exponential phase, and that as the cells resume normal growth, the induction decreases until GFP expression is nearly absent.

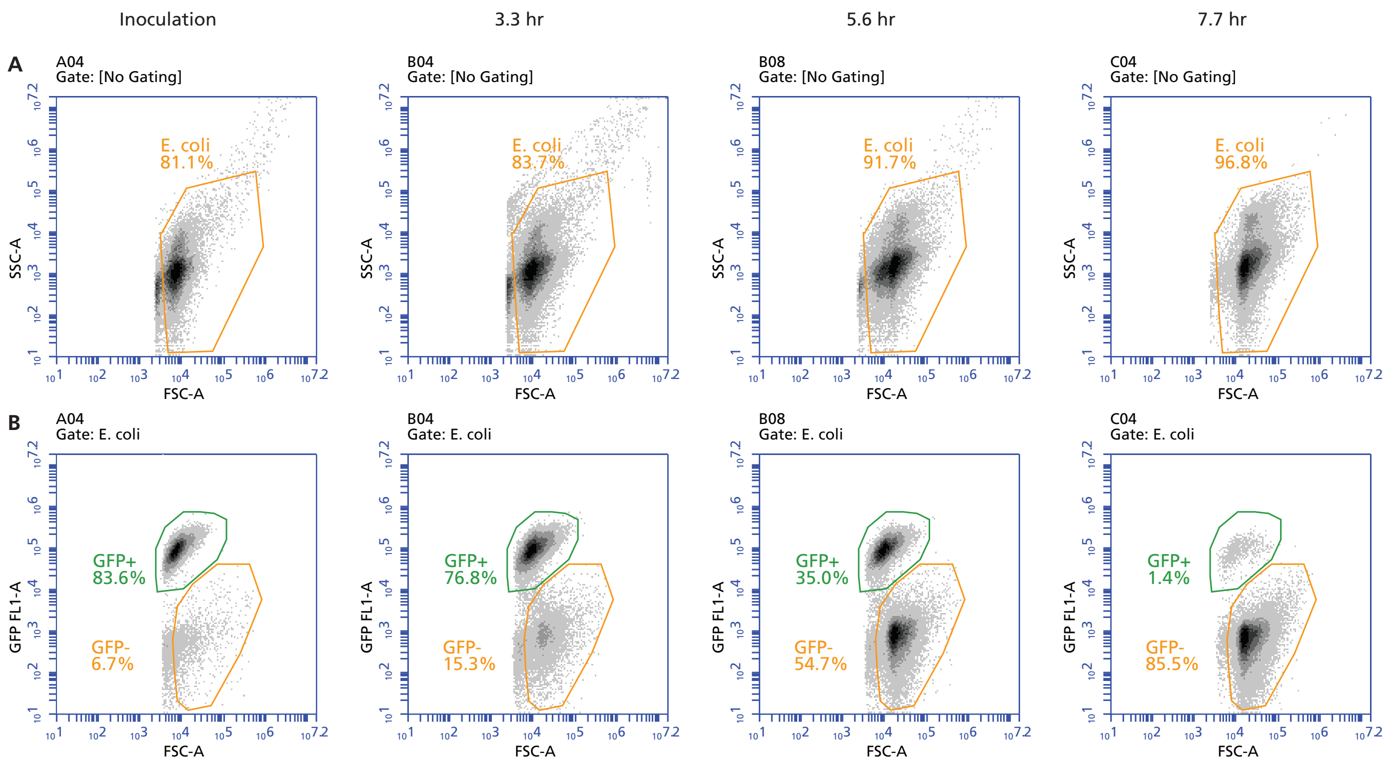

Figure 11. *E. coli* BL21 GFP^+ and GFP^- populations in LB medium over time.

E. coli BL21 cells were cultured in LB-Amp, acquired, and analyzed on the MSP FlowCytoPrep and BD Accuri C6 as described in the text and Figure 10. ***Results*** *show* ***(A)*** *FSC-A vs SSC-A and* ***(B)*** *FSC-A vs GFP (FL1-A) density plots of samples acquired during batch growth at (left to right) inoculation, 3.3 h, 5.6 h, and 7.7 h. FSC vs GFP plots display 50,000 events/sample and are gated on E. coli (Lavarreda C, Peña P, Srienc F, unpub).*

E. coli cultured in M9 minimal medium

To assess growth in M9 minimal medium (5 g/L glucose), the *E. coli* BL21 cells were transferred from LB to M9 minimal medium and grown overnight before similarly inoculating the bioreactor and analyzing samples over time. In M9 minimal medium, growth was much slower than in LB (▲, Figure 12), taking nearly 30 hours to expand from approximately 150 cells/µL to 18,000 cells/µL.

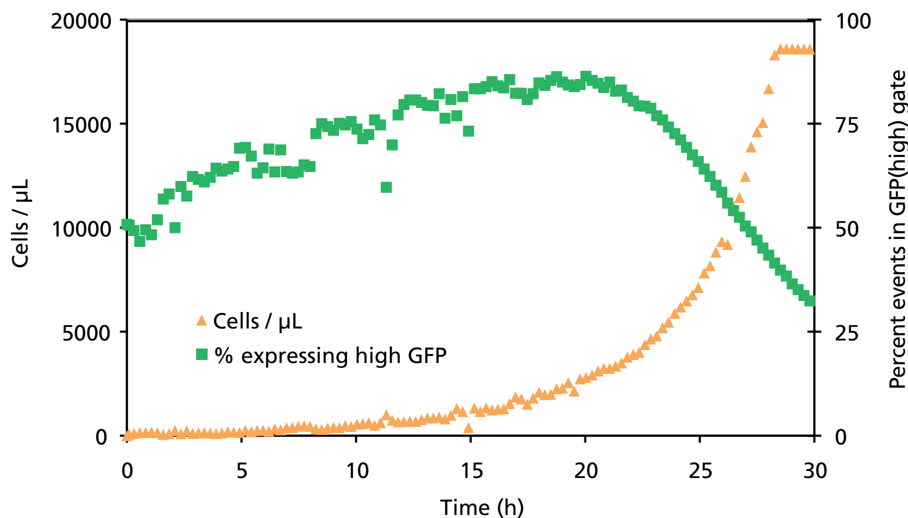

Figure 12. Batch growth and GFP fluorescence of *E. coli* BL21 pRSET-GFP in M9 minimal medium.

E. coli BL21 cells were cultured in M9 minimal medium, acquired, and analyzed on the MSP FlowCytoPrep and BD Accuri C6 as described in the text. Samples were analyzed for 1.5 minutes at the Medium flow rate (35 µL/min), with an acquisition threshold of FSC-H = 11,000 to exclude debris. GFP fluorescence was detected in FL1 (533/30) using the standard emission filter. ***Results:*** *Cell concentrations (in cells/µL, ▲) increased over time, while the percentage of GFP+ events (■) increased, reached a plateau, and then decreased (Lavarreda C, Peña P, Srienc F, unpub).*

Figure 13 shows that cells grown in M9 were smaller than those grown in LB, and the percentage expressing GFP at inoculation was lower, with only ~65% of cells falling into the GFP+ gate (column 1). However, the GFP+ expression phenotype was retained during early exponential phase (10–20 hours post-inoculation); the percentage of GFP+ cells increased transiently, only to decrease rapidly later on (columns 2–4 and Figure 12, ■). Compared to LB, where the GFP– cells were bigger than the GFP+ cells, the two populations did not exhibit size differences when grown in M9 minimal medium.

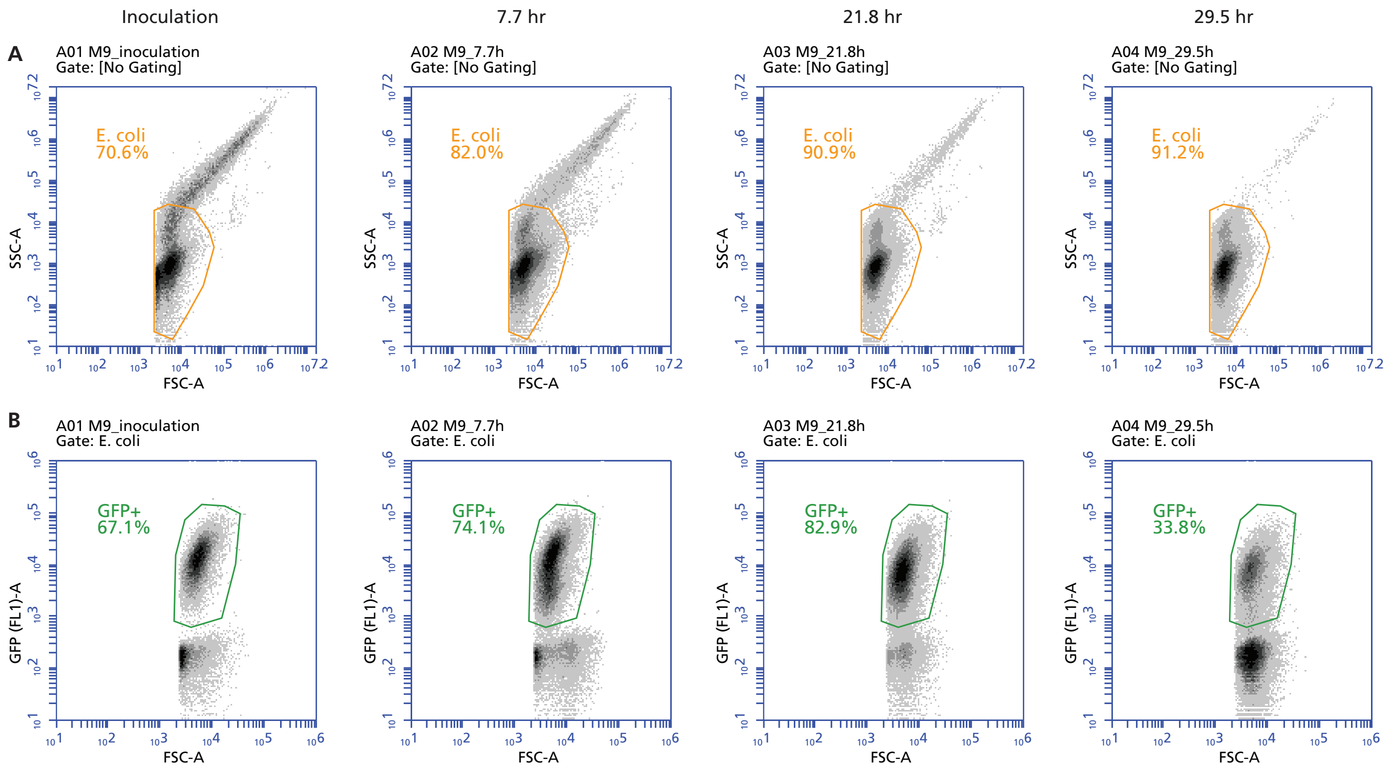

Figure 13. *E. coli* BL21 GFP+ and GFP– populations in M9 minimal medium over time.

E. coli BL21 cells were cultured in M9 minimal medium, acquired, and analyzed on the MSP FlowCytoPrep and BD Accuri C6 as described in the text and Figure 12. ***Results*** *show* ***(A)*** *FSC-A vs SSC-A and* ***(B)*** *FSC-A vs GFP (FL1-A) density plots of samples acquired during batch growth at (left to right) inoculation, 7.7 h, 21.8 h, and 29.5 h. FSC vs GFP plots display 50,000 events/sample and are gated on E. coli (Lavarreda C, Peña P, Srienc F, unpub).*

In this data, automated flow cytometric analysis of the *E. coli* BL21 cell culture not only measured growth kinetics, but also identified subtle differences in cell size and GFP expression. In both LB and M9 minimal media, GFP expression decreased as the cultures entered exponential growth, suggesting that the stationary cells used for inoculation had auto-induced expression from the T7 promoter of the pRSET-GFP expression vector. This induction mechanism seemed to be deactivated under growth conditions. Interestingly, cells growing in M9 minimal medium retained the induction longer than those grown in nutrient-rich LB medium, indicating that the deactivation may be driven by nutrient availability or growth rate. Alternatively, the decrease in fluorescence may be a dilution effect as the components involved in activation, and the GFP protein itself, are diluted with each cell division.

Automated flow cytometric analysis of growth and other phenotypic parameters throughout the life of a cell culture could unveil novel characteristics of cellular function. The pairing of the MSP FlowCytoPrep with the BD Accuri C6 offers high value for bioprocess scientists and engineers, increasing process knowledge, enabling better yields, and facilitating more efficient and profitable operations. This combination also promises to aid regulatory compliance, such as the US Food and Drug Administration's new Process Analytical Technology (PAT) initiative for quality by design.[12]

Summary

With flow cytometry, bioprocess engineers can simultaneously analyze a wide range of cellular parameters at the single-cell level, and generate high-throughput, statistically strong data that supports informed decisions for bioprocess control. Offering both performance and simplicity, the BD Accuri C6 flow cytometer allows bioprocess engineers to use light scatter signals to discriminate cells and fluorescence signals to measure cell viability, vitality, and other important cell characteristics. The BD Accuri C6 also interfaces smoothly with automated bioreactor sampling systems such as the MSP M5000 FlowCytoPrep, giving bioprocess engineers access to real-time bioreactor data for pinpoint process control.

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BD Biosciences
2350 Qume Drive
San Jose, CA 95131
US Orders: 855.236.2772
BD Accuri Technical Support: 734.994.8000
TechSupport@AccuriCytometers.com
bdbiosciences.com

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