

# Evaluation of *Escherichia coli* cell disruption and inclusion body release using nucleic acid binding fluorochromes and flow cytometry

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*BioTechniques* 43:777-782 (December 2007)  
doi 10.2144/000112621

Many types of commercially valuable recombinant proteins produced by fermentation are expressed at high levels in *Escherichia coli*. Often, high-level expression in the host results in the formation of insoluble inclusion bodies. The release of these intracellular inclusion bodies from *E. coli* following cell disruption is a requirement for further downstream recovery. The ability to discern between intact unruptured cells and granules released from broken cells can provide valuable information for improving recovery yields in downstream purification. This paper describes a rapid and sensitive cytometry-based method that allows the simultaneous measurement of intact heat-killed *E. coli* and inclusion bodies using staining with nucleic acid binding fluorochromes.

## INTRODUCTION

The production of therapeutic proteins using *Escherichia coli* as a host organism typically involves growth of the cells in a bioreactor, induction of gene expression and protein production, recovery of cells containing inclusion bodies, cell disruption, inclusion body recovery, and subsequent protein purification via chromatography (1,2). The ability to maximize productivity of the manufacturing process depends upon reliable, sensitive, and rapid methods to assess the efficiency of these unit operations. Although a multitude of methods exist to assess product accumulation during fermentation, relatively few methods measure the disruption of the cells and subsequent recovery of inclusion bodies.

Several biophysical methods including electrical sensing zone (ESZ) methods, photon correlation spectroscopy (PCS), electron microscopy, and centrifugal disc photosedimentation (CDS) have been used by investigators in attempts to characterize and quantify these biological particles (3). These methods, however, lack sensitivity, suffer from lysed cell component interference, require significant sample

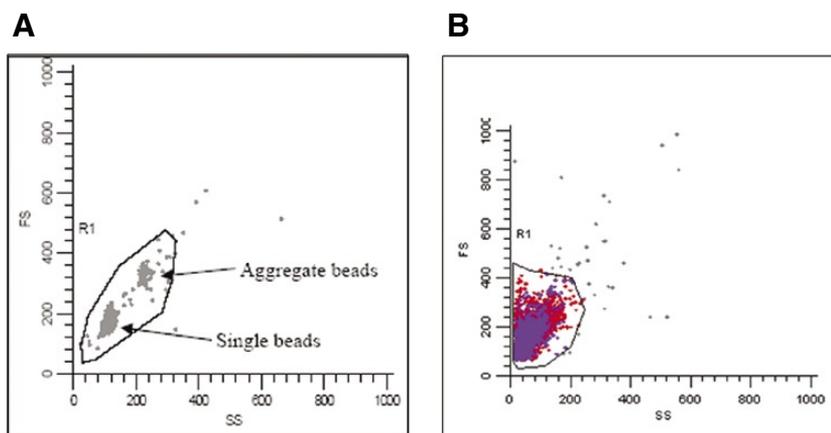
pretreatment, or are not conducive to obtaining statistically significant numbers of events.

Phase contrast microscopy, which enables whole, intact-cell counting relative to free inclusion bodies, serves as the current gold standard for measuring cell disruption efficiency. The small size of both host cells (nominal 3–5  $\mu\text{m} \times 1 \mu\text{m}$ ) and inclusion bodies ( $\sim 1 \mu\text{m}$ ) requires operators to observe several microscope fields containing <100 objects per field at high power (1000 $\times$ ) to produce satisfactory results. Given

these constraints, this microscopic method is time consuming, tedious, and subject to a high degree of error.

Flow cytometric methods designed to assess bacterial cell viability, including *E. coli* (4–9) have also been described. These live/dead cell assays often rely upon fluorochromes with various membrane permeability properties that often target bacterial DNA. The list includes 4',6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), ethidium bromide, BacLight Green and Red, and acridine orange staining (10,11). More recently, cell-impermeant cyanine dyes, such as SYTOX Green, that have a higher affinity for nucleic acids while providing exceptionally bright signals, have been developed (12–14). These dyes easily penetrate cells with compromised plasma membranes, yet will not cross the membranes of live cells. Binding of SYTOX Green stain to nucleic acids results in a 500-fold enhancement in fluorescence emission (absorption and emission maxima at 502 and 523 nm, respectively), rendering bacteria with compromised plasma membranes brightly green fluorescent. SYTOX Green enabled superior detection and discrimination of intact versus permeabilized cells as compared with PI in flow cytometric analysis (14).

While several of the staining methods described above have been used to enumerate live/dead *E. coli*, there have been relatively few flow cytometric methods described for the



**Figure 1. Light scatter data from a bead standard and heat-killed *Escherichia coli* preparations.** (A) Forward angle light scatter (FS) versus side angle light scatter (SS) signal data from a 0.99  $\mu\text{m}$  diameter bead standard. (B) Overlay of FS versus SS signal data from heat-killed *E. coli* preparations. Red indicates whole cells; purple indicates isolated protein granules (inclusion bodies).

enumeration of inclusion bodies (15–18). These methods have been used to assess inclusion body formation during fermentation and are largely based upon light scattering (15,16). The lack of methods may be due to the physical size of the inclusion bodies (~1  $\mu\text{m}$  diameter), which is near the threshold for detection based upon light scattering in modern flow cytometers. Light scattering methods are complex, require precise tuning of the optical

systems, and for the reason stated above are prone to error. A flow cytometric method using fluorescently labeled antibodies for detection of inclusion bodies has also been described (18). This method, however, is both time consuming and tedious and requires the generation of highly specific antibodies for the expressed proteins. We recently developed a rapid and sensitive flow cytometric method, based upon nucleic acid binding fluorochromes, with the

capability to resolve intact heat-killed cells from free inclusion bodies in a homogenized preparation.

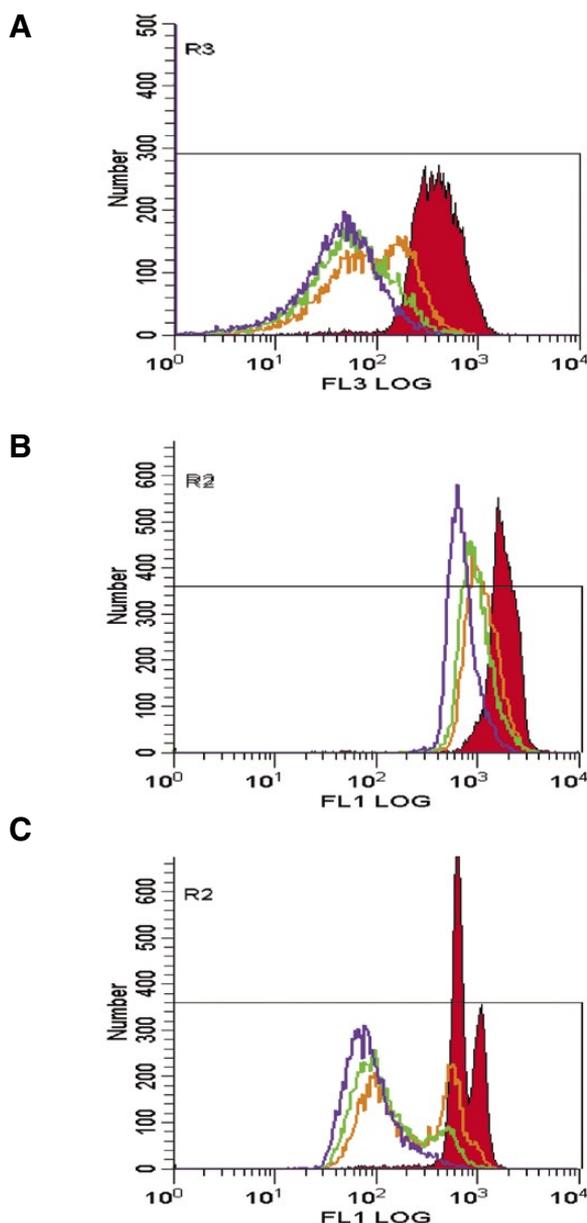
## MATERIALS AND METHODS

### Microorganisms

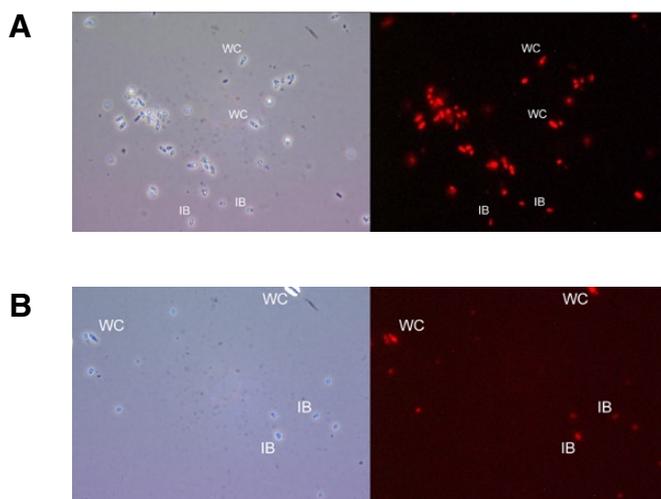
Recombinant *E. coli* produced by fed-batch fermentation were heat-killed at 70°C for 5 min, harvested, and concentrated by continuous disc centrifugation at 8000  $\times g$ . The centrifuged material was then passed through a high-pressure homogenizer at 8000 psig (pound-force per square inch gauge) for the indicated number of passes. Inclusion bodies were harvested from the broken cells and separated from cell debris via continuous disc centrifugation at 8000  $\times g$ . The null strain consisted of the host strain of *E. coli* K12, which does not express the recombinant protein.

### Flow Cytometry

Samples were analyzed using a Coulter XL MCL flow cytometer (Beckman Coulter, Fullerton, CA, USA) to detect light scattering and fluorescence emissions. An air-cooled argon ion laser (488 nm, 15 mW) illuminated cytometer samples, while fluorescence detection occurred using a standard filter configuration (525-nm band pass filter for FL1, 575-nm band pass filter for FL2, and 620-nm band pass filter for FL3). For each experiment, investigators collected data from ~10,000 cells and processed these data using WinList 5.0 software (Verity Software House, Topsham, ME, USA). Photomultiplier gains were set in the linear mode for light scatter data collection and in the logarithmic mode for fluorescence data collection. Despite the small size of the *E. coli* samples, forward angle light scattering was sufficiently large to trigger the flow cytometer for data collection. Appropriate threshold limits were set to reduce excessive background on stained and unstained whole and homogenized samples.



**Figure 2.** Replicate samples of whole (red), first pass homogenized (orange), second pass homogenized (green), and isolated granules (purple) were stained with the indicated reagents. Reagents are: (A) 20  $\mu\text{L}/\text{mL}$  propidium iodide (PI), (B) 1  $\mu\text{L}/\text{mL}$  BacLight Green, and (C) 4  $\mu\text{L}/\text{mL}$  SYTOX Green. Fluorescence data: FL1, 525 nm emission; FL3, 620 nm emission.



**Figure 3. Fluorescence micrographs of propidium iodide (PI)- and SYTOX Green-stained samples.** (A) Fluorescence microscopy of 10  $\mu\text{L/mL}$  PI-stained sample. Phase contrast (1000 $\times$ ) and fluorescence microscopy photos of *Escherichia coli* after one homogenization pass. These images illustrate that the granules as well as cells take up nuclear stain. (B) Fluorescence microscopy of 4  $\mu\text{L/mL}$  SYTOX Green-stained sample. Phase contrast (1000 $\times$ ) and fluorescence microscopy photos of *E. coli* after one homogenization pass. These images illustrate the brighter signal produced by intact cells versus granules stained by SYTOX Green. WC, whole cells; IB, inclusion bodies.

### Fluorescence Staining

In all cases, samples were diluted with 0.22- $\mu\text{m}$  filtered 0.5 M Tris, pH 8.5, to  $\sim 1 \times 10^6$  cells/mL prior to staining.

A 0.75 M stock solution of PI (Sigma-Aldrich, St. Louis, MO, USA) was prepared in 1 $\times$  phosphate-buffered saline (PBS). This stock was used at 20  $\mu\text{L/mL}$  sample. PI was added directly to dilute culture prior to analysis. Fluorescence data from PI-stained samples were collected in FL3, 620 nm emission.

*BacLight Green Bacterial Stain* (Molecular Probes, Invitrogen, Carlsbad, CA, USA) was prepared as directed by product instructions. The stain was warmed to room temperature. A 1 mM stock solution was made by adding 74  $\mu\text{L}$  dimethyl sulfoxide (DMSO). Finally, a 100  $\mu\text{M}$  working solution was made by adding 2  $\mu\text{L}$  stock to 18  $\mu\text{L}$  DMSO. For cell staining, 1  $\mu\text{L}$  of this 100  $\mu\text{M}$  stock was added directly to 1 mL dilute culture prior to analysis. Fluorescence data from *BacLight Green*-stained samples were collected in FL1, 525 nm emission.

SYTOX Green Nucleic Acid Stain (Molecular Probes, Invitrogen) was

provided and used as a 5 mM solution in DMSO. For cell staining, 4  $\mu\text{L}$  stock solution were added directly to 1 mL dilute culture prior to analysis. Fluorescence data from SYTOX Green-stained samples were collected in FL1, 525 nm emission.

### Microscopy

Samples were diluted in 0.5 M Tris buffer, pH 7.5, to obtain  $\sim 10^8$  cells/mL. A 4- $\mu\text{L}$  aliquot of diluted sample was applied to a clean microscope slide, and a coverslip was applied. Samples were then counted using phase contrast at a final magnification of 1000 $\times$  using a Nikon OptiPhot-2 microscope (Nikon, Tokyo, Japan). Three microscopic fields were evaluated for each sample to assess variability.

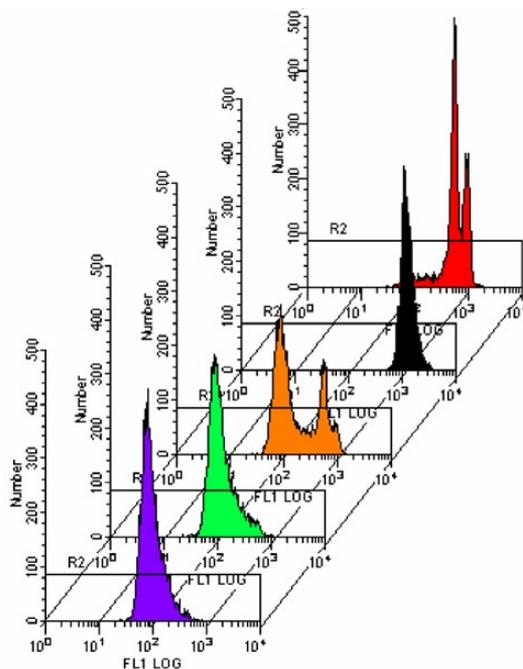
### RESULTS AND DISCUSSION

Although the Coulter XL MCL analyzer had routinely detected intact *E. coli* cells based upon the forward angle light scatter (FS) and side angle light scatter (SS) signals, the samples used in these studies were subjected to high-pressure homogenization, which reduced the particle size from 3–4  $\mu\text{m}$

to 0.8–1.5  $\mu\text{m}$  in diameter as estimated by phase contrast microscopy. Therefore, it was necessary to determine whether light scatter data alone could distinguish an  $\sim 1 \mu\text{m}$  diameter particle signal from noise. If this proved possible, we further wanted to determine whether light scatter could discriminate intact cells from released inclusion bodies. To explore the lower limit of light scatter detection, an analysis of a 0.99  $\mu\text{m}$  diameter Dragon Green-infused bead standard (Bangs Laboratories, Fishers, IN, USA) was performed on the flow cytometer. Successful bead detection (Figure 1A) indicated the analyzer's ability to resolve signal from noise and measure the light scatter signal of particles of comparable size to homogenized *E. coli* samples. We then collected light scatter data from intact heat-killed *E. coli* and compared these data with light scatter data from isolated inclusion bodies (Figure 1B). Although different, the light scatter data from these two samples exhibited extensive overlap.

The results of this experiment showed that, although the analyzer can detect particles of  $\sim 1 \mu\text{m}$  in diameter, the inclusion bodies could not be adequately resolved from unlysed cells using light scatter measurements alone.

In an attempt to visualize the difference between heat-killed intact cells and released inclusion bodies, the following stains were tested: PI, *BacLight Green*, and SYTOX Green. The flow cytometer was set to capture FS, SS, and fluorescence data from a variety of heat-killed *E. coli* samples. Heat-killed intact samples stained with PI exhibited the expected large shift in fluorescence intensity (Figure 2A) relative to unstained control (not shown). Staining of the same heat-killed cells, further subjected to one or two rounds of homogenization, or staining of granules isolated from the heat-killed cells yielded successively lower fluorescence signal (Figure 2A), although all samples yielded a brighter signal than unstained controls. Because intact samples gave the brightest fluorescence signal while isolated granules gave the weakest, it appeared that PI bound both cells and



**Figure 4.** Right to left overlay of fluorescence (FL1, 525 nm emission) histograms comparing whole (red), null (black), first pass homogenized (orange), second pass homogenized (green), and isolated inclusion bodies (purple) stained with SYTOX Green.

granules and that lower fluorescence intensity indicated increased homogenization and granule release. To test this hypothesis, PI-stained homogenized material was examined by epifluorescence microscopy (Figure 3A). These images verify that granules as well as cells take up nuclear stain. It is possible that the observed fluorescence of the inclusion bodies could, in theory, be caused by contamination with nucleic acids either as a result of entrapment during expression or as a result of sticking to the inclusion bodies following cell disruption. To test this, we have treated the granules with a DNase preparation and did not observe a significant decrease in the fluorescence (data not shown). Therefore, it is more likely that the dye molecules diffuse into the submicron pores in the inclusion bodies and become trapped without binding to nucleic acid. As per the fluorescence histograms (Figures 2C and 4) and as illustrated in Figure 3A, the relative amount of fluorescence is substantially lower than that caused by the binding of the dye to nucleic acids within intact cells. As noted above,

the relative fluorescence of SYTOX is ~500-fold greater when bound to nucleic acids. As per the histograms, this is approximately the difference observed between the fluorescence of the intact cells versus inclusion bodies, suggesting that dye entrapment is the mechanism.

Based upon these experiments, it appeared that flow cytometric analysis in conjunction with nucleic acid staining could distinguish isolated granules from intact heat-killed *E. coli*. Additionally, based on histogram results, this staining seemed sensitive enough to detect sample differences in the intervening homogenization rounds leading to granule isolation.

While PI staining proved promising, there was a need to further resolve the signal overlap seen among intact *E. coli*, homogenized *E. coli*, and isolated granule samples. Therefore, several other stains were tested (*BacLight* Green and SYTOX Green) to determine whether greater resolution could be achieved. In comparison with PI, sample resolution did not improve when stained with *BacLight* Green (Figure 2B). However, sample resolution did improve dramati-

cally when stained with SYTOX Green (Figures 2C and 3B). Of the several concentrations of the stain tested (2, 4, 20, and 30  $\mu\text{L}/\text{mL}$ ), 4  $\mu\text{L}/\text{mL}$  yielded the best results.

Additional experiments (data not shown) demonstrated that the flow cytometric method using SYTOX Green is capable of distinguishing free inclusion bodies from unruptured (intact) cells and cell debris. Purified inclusion bodies recovered from the manufacturing process stream following centrifugation and washing were used as a standard in the experiments that were performed. We have verified that these inclusion bodies are aligned with the histogram peaks for fluorescence and also populate the identified region in a dot density plot of FSS versus fluorescence. The inclusion body region is clearly distinguishable from both intact cells and debris allowing the events to be enumerated. Furthermore, we have demonstrated the identity of the inclusion body peak in the fluorescence histogram by spiking the samples with the purified inclusion bodies and observing the number of events associated with this peak increase accordingly.

During the course of these studies, it was observed that the histogram associated with intact heat-killed *E. coli* exhibited bimodality upon SYTOX Green staining. During fermentation, the gene expression induction process does not cause all cells to produce inclusion bodies. To explore whether SYTOX Green could enable resolution of the producing from nonproducing populations, the *E. coli* host strain (ungranulated) was stained with SYTOX Green and analyzed along with samples from a standard production run, containing intact as well as homogenized and isolated granule samples. The results are summarized in Figure 4.

The results show the whole null strain fluorescent emission as distinct from that of the induced (granulated) strain, as well as distinct from all other homogenized or isolated granule samples. Since the alignment of the null strain histogram peak consistently overlays well with the brighter of the two doublet peaks, the data support that this method can resolve productive (i.e., granulated) versus nonproductive

cells. The relative correlation of the number of cells in the dimmer and brighter peaks with manual count ratios of producing and nonproducing *E. coli* further supports this hypothesis (data not shown).

Several methods have been described in the past to assess cell breakage, including release of biochemical markers (DNA, protein), direct microscopy, and photosedimentation (3). All of these methods, however, include significant drawbacks. We describe here a flow cytometric method that accurately and rapidly evaluates *E. coli* breakage during purification processes.

The method described can also distinguish productive from nonproductive cells within a fermentation culture. The ability to distinguish the two populations of cells was only observed with SYTOX Green and not with the other nucleic acid stains tested. The SYTOX dye reportedly has a higher affinity for nucleic acids than other stains and also is considered as a brighter stain (15). These properties, combined with its ~500-fold fluorescent enhancement upon nucleic acid binding and high quantum yield, apparently allow the separation of the nongranulated versus granulated cells based on fluorescence intensity.

## ACKNOWLEDGMENTS

*The authors would like to thank Graham Tulloch, Donald Herbert, and Christopher C. Frye for their input in the review of this manuscript and Carroll Teater for assistance in preparation of the manuscript.*

## COMPETING INTERESTS STATEMENTS

*The authors declare no competing interests.*

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Received 13 June 2007; accepted 17 September 2007.

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