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Quantitative analysis and isolation of *Escherichia coli* O157:H7 in a food matrix using flow cytometry and cell sorting

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Abstract

Flow cytometry is a potentially valuable analytical method in microbiology providing the ability to analyze rapidly large numbers of individual microorganisms by several parameters. With a flow cytometer with enhanced light scatter sensitivity and a conventionally configured sorting cytometer, a series of comparative studies to determine the ability of the two flow systems and the antibody-direct epifluorescent filter technique (Ab-DEFT) to detect and enumerate *Escherichia coli* O157:H7 were made. Initial experiments used culture-derived mixtures of non-pathogenic *E. coli* and serial dilutions of *E. coli* O157:H7. Subsequent studies involved analysis of enrichment cultures from ground beef inoculated with *E. coli* O157:H7. Comparison of flow cytometry with microscopy and plate counts produced similar results at higher concentrations in both culture mixtures and beef enrichments. At the lowest concentrations Ab-DEFT was more sensitive, however, the time required for analysis was much less with flow cytometry. With a cytometer with enhanced light scatter sensitivity designed for bacterial analysis, O157:H7 could be distinguished from *E. coli* strain HB101 on the basis of light scatter. This instrument also provided direct count data for selected populations. In experiments using cell sorting to isolate target organisms, the purity of fluorescent-labeled *E. coli* O157:H7 sorted from beef enrichment cultures and plated was not affected by the level of background organisms, as is often the case in conventional plating procedures. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

Keywords: *Escherichia coli* O157:H7; Flow cytometry; Food

1. Introduction

Contamination of foods including fresh and processed ground beef with enterohemorrhagic *Escherichia coli* (EHEC) of the serogroup and serotype O157:H7 has been responsible for disease outbreaks with major human and economic consequences [1]. The detection of EHEC (and other pathogens) is an important focus in the development of hazard analysis critical control point (HACCP) procedures for meat processing. As a result, many rapid and sensitive detection methods have been developed with DNA and immunologically based approaches in order to reduce the time required for detection of specific pathogens such as *E. coli* O157:H7 by conven-

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tional methods [2]. One antibody-based approach which has been successfully applied to *E. coli* O157:H7 is the antibody-direct epifluorescent filter technique (Ab-DEFT) [3]. This method is sensitive, economical, and takes about 1 h to perform the processing steps prior to microscopic examination of the sample. The drawbacks are that the counting procedure can be laborious and requires well-trained personnel. Because Ab-DEFT is a fluorescence technique and sample processing is liquid-based until the last filtration step, it seemed possible that the sample processing procedures and fluorescence reagents used might be readily adaptable to the use of flow cytometry.

Flow cytometry (FC) offers several potential advantages as a method for detection of bacteria in food and environmental samples. FC is rapid, can be done quantitatively, can be automated and produces real time as well as archival data, all of which are important considerations in rapid detection systems as well as critical control point monitoring. In addition, flow cytometers equipped with cell sorting capability can be used to isolate cells or bacteria of interest for further analysis or confirmatory culture. While FC has been frequently used to analyze bacteria in pure cultures [4,5] it has seen more limited use with heterogeneous natural samples such as those encountered in food microbiology [6]. Food-related applications include detection of *Listeria monocytogenes* in milk [7] and *Salmonella typhimurium* in milk and eggs [8]. The latter investigators also used enrichment cultures and a custom-designed flow cytometer to achieve a higher level of sensitivity [9]. The sensitivity of this approach is a function of the efficacy of the enrichment and the background noise associated with fluorescent staining of non-target particles.

In order to test FC technology in immunofluorescent detection of *E. coli* O157:H7, mixtures of pure cultured *E. coli* O157:H7 and non-EHEC or artificially contaminated non-selective enrichment cultures from ground beef were processed for the Ab-DEFT procedure [3]. Immunofluorescent enumeration of organisms was done in parallel by epifluorescence microscopy and by FC. FC analysis was done with two commercially available flow cytometers with different optical and fluidics configurations. Cell sorting by one instrument was tested as a method for isolation of fluorescent-labeled *E. coli* O157:H7.

2. Materials and methods

2.1. Bacteria

The streptomycin-resistant strain DHS-1 [3] of *E. coli* O157:H7 was used for all experiments. This strain is a variant of ATCC strain 35150. For experiments with bacteria mixtures, the HB101 strain of *E. coli* was used. Bacteria were cultured at 37°C in Luria-Bertani broth. In experiments with sorted O157:H7, bacteria were plated on trypticase soy agar with or without 500 μg ml^{-1} dihydrostreptomycin (Sigma). All media were obtained from Difco.

2.2. Preparation of beef enrichment culture mixtures

Enrichment cultures of beef were prepared according to the USDA-recommended method [10], as follows. Ground beef (25 g) was homogenized in 225 ml modified EC broth with novobiocin for 2 min at high speed in a Model 400 Stomacher laboratory blender. The slurry that passed through the mesh lining of the Stomacher bags was harvested and incubated at 35°C for 18–24 h. Bacterial cells from exponential phase cultures were diluted appropriately and added to 5 ml of the beef enrichment culture. The mixtures were subdivided into two aliquots. One aliquot was used for viable plate counts of *E. coli* O157:H7 on MacConkey sorbitol agar (Difco) with dihydrostreptomycin. The other aliquot was further processed for Ab-DEFT and FC analysis.

2.3. Sample processing for Ab-DEFT and FC

The procedure used for sample processing was a modification of a previously reported method [3]. Briefly, 2 ml of enrichment mixture was incubated at 50°C for 10 min with 0.5 ml trypsin and 2 ml 0.5% Triton X-100 (Sigma) and filtered through a 5.0 μm filter. 1 ml of the filtrate or an appropriate dilution prepared in PBS was passed through a 0.4 μm filter, and the filter was stained with a 1 in 2000 dilution of fluorescein-isothiocyanate (FITC)-labeled, affinity-purified rabbit antibody against *E.*

coli O157:H7 (Kirkegard and Perry Laboratories, Gaithersburg, MD, USA) as previously described. Total counts of the beef enrichment microbial population were obtained by staining the filter with acridine orange. A 1 ml aliquot of the 5.0 mM filtrate to be used for FC analysis was twice centrifuged at $5000 \times g$ for 3 min and resuspended in 1 ml Dulbecco's phosphate buffered saline (PBS). This material was diluted between 1 in 5 and 1 in 20 with PBS to achieve a concentration suitable for FC analysis ($1–2 \times 10^6$ bacteria ml^{-1}). 1 ml of the diluted suspension was stained with the labeled antibody at a final concentration of 1 in 2000 for 20 min at 5°C and analyzed by FC. Background controls included samples with no target organisms, and diluent (PBS) samples processed in an identical manner.

2.4. FC analysis

FC was done with a Bryte HS instrument (BioRad Laboratories) and a Epics Elite (Coulter Corporation). The same tube of immunofluorescent stained bacteria suspension was used for both instruments. On the Epics Elite, FITC was excited by an argon

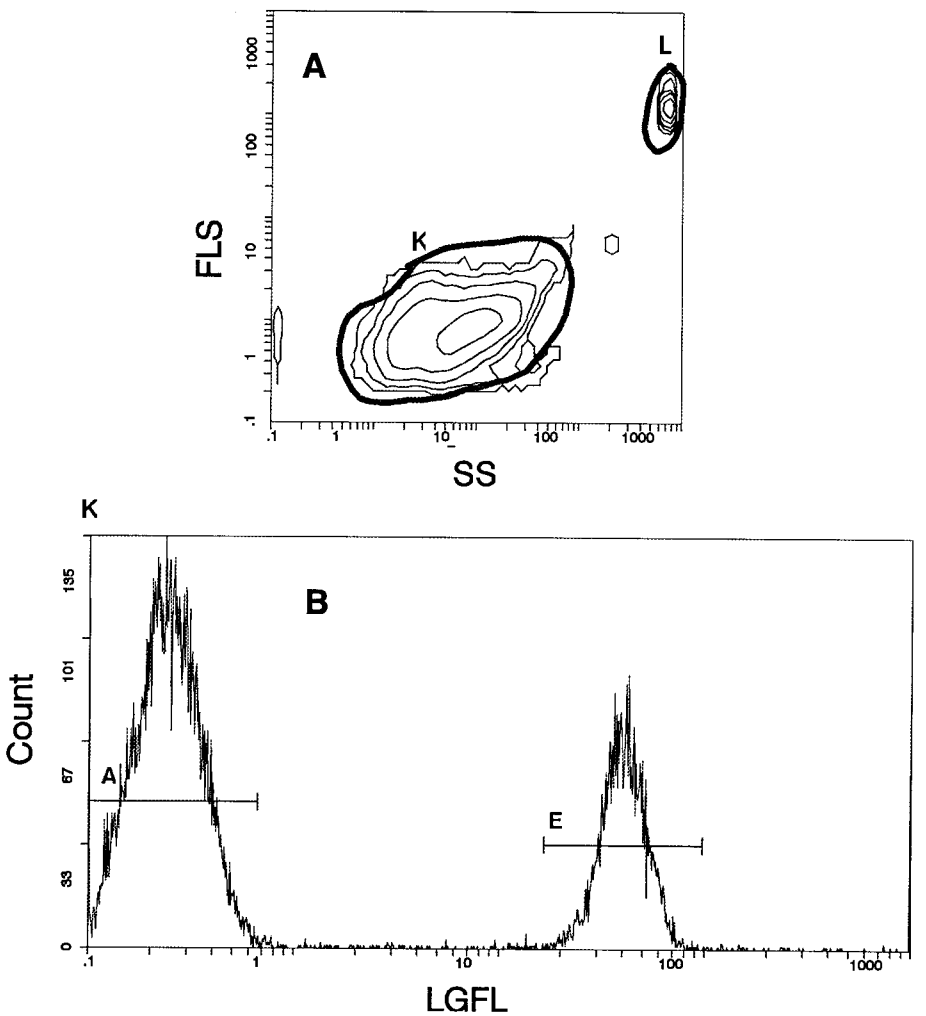

Fig. 1. A: Two parameters (forward scatter vs. side scatter) of amplified light scatter histogram of mixed HB101 and O157:H7 *E. coli* stained with FITC-labeled anti-*E. coli* O157:H7 antibody. Region K is the bacterial cells, region L is 10 μm fluorescent beads added to the mixture. B: Log-amplified green fluorescence histogram derived from gating on region K. Region A is unstained bacteria, region E represents bacteria stained with the FITC-labeled antibody (20 000 events in region K).

laser emitting a 488 nm beam at 15 mW. Beam shaping optics produced an elliptical beam that intersected the sheath/sample stream in a quartz flow cell (stream in quartz). The sheath:sample pressure differential was set to obtain a flow rate of 200–400 particles s^{-1}. For enumeration of bacteria, 10 μm fluorescent polystyrene beads (Coulter Immunocheck) were added to bacteria samples and counted in a hemacytometer using a fluorescence microscope. By FC, the beads were easily distinguished from bacteria on the basis of fluorescence and light scatter. The concentration of fluorescent bacteria was then calculated as a function of the ratio of the number of beads detected by FC vs. the concentration determined microscopically. The complete system architecture of the Bryte HS has been previously described [11]. Briefly, this flow cytometer used epifluorescent dark field optics and stream-on-surface fluidics which results in increased light scatter sensitivity. An excitation beam of 470–490 nm was produced by a xenon lamp with appropriate filters. The sample was delivered by a precision driven microliter syringe at a rate of 2–10 μl min^{-1}. On both instruments, bacteria were detected with log-amplified forward light scatter signals (log FLS) as the trigger. Log FLS histograms or Log FLS vs. side scatter (SS) histograms were used to define the bacterial populations and establish gating regions for fluorescence measurements. Fluorescence emission was measured in log-amplified signals.

2.5. Sorting of labeled bacteria

All sorting was done using the Epics Elite equipped with an Autoclone device for deposition of sorted cells into multi-well plates. Specific numbers of fluorescent labeled bacteria were sorted into sterile 96 well plates containing 100 μl sterile PBS. Four replicate wells were sorted and plated on agar for each sample.

3. Results

3.1. Immunofluorescence detection of E. coli O157:H7 in mixtures of pure cultures

To examine the feasibility and specificity of the immunofluorescent staining procedure and FC analysis and especially the accuracy of the fluorescent bead calibration in the most straightforward manner, mixtures of *E. coli* O157:H7 and HB101 stained with rabbit anti-O157 were analyzed. Bacteria were detectable in log FLS vs. SS plots (Fig. 1A). Gating on this population revealed a distinct fluorescent population that in serial dilutions correlated with the proportion of O157 in the mixture (Fig. 1B). Fluorescent beads added to the mixture for the purpose of extrapolating the number of bacteria per ml are seen as a distinct population. The *E. coli* O157:H7 counts derived in this manner correlated well with those from Ab-DEFT and plate counts (Fig. 2). The background associated with FC, based on analysis of diluent with beads and no added bacteria, was about 3×10^4 ml^{-1}. Quantitation of immunostained bacteria below this level was not feasible.

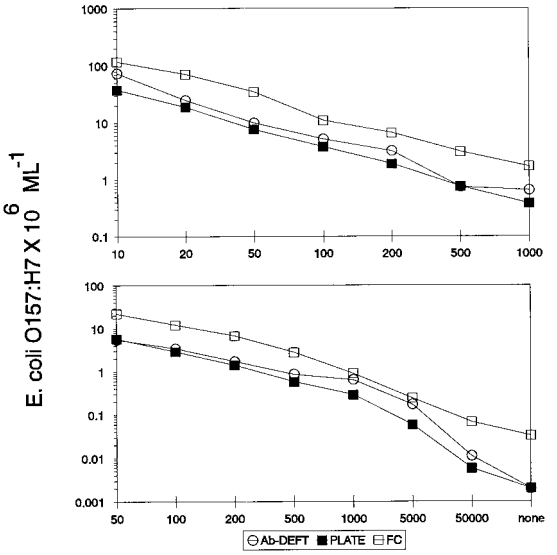

Fig. 2. Comparison of the number of *E. coli* O157:H7 detected by FC and Ab-DEFT in a series of mixtures with a constant number of HB101 and a decreasing proportion of O157:H7. Reciprocal dilution of *E.coli* O157:H7 in the mixture is shown on *x* axis; none means no target organisms added. Plate represents the calculated viable plate count of the added *E. coli* O157:H7 for each mixture. Results of two experiments.

The increased FLS resolution achieved with dark field, stream-on-surface architecture revealed two distinct populations. The higher light scattering population correlated with the population stained by the

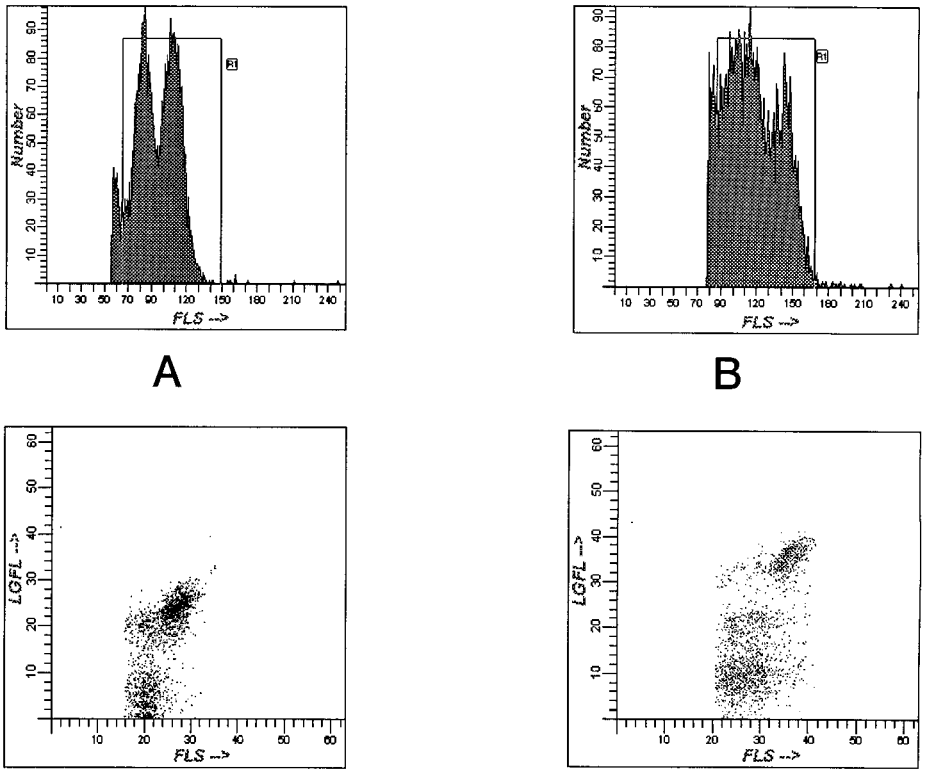

Fig. 3. Analysis of mixed HB101 and O157:H7 *E. coli* (A) or beef enrichment and O157:H7 (B) stained with FITC-labeled anti-O157:H7 antibody using a flow cytometer with enhanced light scatter resolution. Upper panels are log forward light scatter (*x* axis) vs. cell number (*y* axis), lower panels are log green fluorescence (*y* axis) vs. log forward light scatter (*x* axis).

anti-*E. coli* O157 fluorescent antibody (Fig. 3A). In control experiments, however, binding of the antibody itself had no effect on light scatter. Because this instrument uses a precision motor driven micro syringe to deliver the sample, direct counts of particles per ml are obtained. For this reason fluorescent bead calibration of this instrument was not required.

3.2. Detection and enumeration of *E. coli* O157:H7 in beef enrichment cultures

Because the lower limit of quantitation by FC in pure culture mixtures is far in excess of the required sensitivity for assurance of food safety, enrichment cultures would be an essential step in detection of low numbers of *E. coli* O157:H7 in ground beef, as

Table 1
Growth of presumptive streptomycin-resistant *E. coli* O157:H7 sorted from beef enrichment cultures on streptomycin containing and antibiotic-free agar (CFU mean of three replicate plates)

O157:H7 frequency	100 sorted	Strep[a]	500 sorted	Strep
44.4%	48	54	243	292
13.6%	55	58	277	311
2.6%	30	43	ND	ND

[a]Growth on streptomycin (500 μg ml^{-1}) agar.

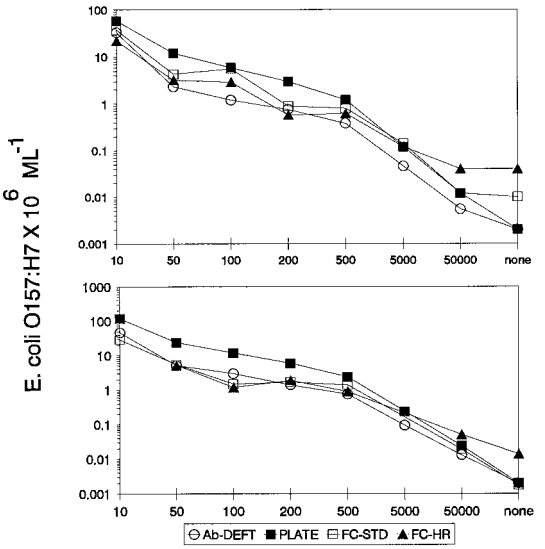

Fig. 4. Comparison of the number of *E. coli* O157:H7 detected by Ab-DEFT and by FC using a flow cytometer with conventional light scatter detection (FC-STD) or a flow cytometer with enhanced light scatter resolution and metered sample volume (FC-HR) in a series of beef enrichment mixtures with a decreasing proportion of O157:H7. Reciprocal dilution of *E. coli* O157:H7 in the mixture is shown on *x* axis; none means no target organisms added. Plate represents the viable plate count of the added O157:H7 for each mixture. Results of two experiments.

they are in other rapid methods [12]. The background bacterial population associated with this procedure is heterogeneous compared to pure culture. Acridine orange staining of control (uninoculated) beef enrichment cultures showed the indigenous microbial population of the beef at an enrichment level of approx. $1–4\times10^8$ organisms ml^{-1}. To test the FC procedure in this milieu, beef enrichment cultures produced using the USDA protocol [10] and seeded with serial dilutions of *E. coli* O157:H7 strain DHS 1 were processed for FC and Ab-DEFT analysis. The major difference between fluorescence histograms from beef enrichment cultures and mixtures of pure cultures was the presence of a small population of intermediate fluorescence between the negative bacteria and the *E. coli* O157:H7 that was seen on both flow cytometers (Fig. 3B). This population appeared to be associated with the background bacterial flora as it did not correlate with the addition of *E. coli* O157:H7. Neither the immunofluorescent staining nor the increased FLS of the *E. coli* O157:H7 seen in pure cultures was affected by the enzyme and detergent used in processing. There was excellent correlation between the FC procedures, Ab-DEFT and viable plate counts; however, the lower limit for the FC procedures was again limited by background fluorescence noise in baseline controls containing no *E. coli* O157:H7 (Fig. 4).

3.3. Sorting *E. coli* O157:H7 from beef enrichment cultures

A sorting region was established centered on the brightest immunostained population of a series of enrichment cultures that contained decreasing numbers of streptomycin-resistant *E. coli* O157:H7. From this population, 100 or 500 presumptive *E. coli* O157:H7 were sorted into wells of a sterile 96 well plate and cultured on agar with or without streptomycin. The number of colonies on streptomycin agar was equivalent to those on non-antibiotic agar (Table 1). The number of colonies reflected the five-fold difference in the number of sorted cells. These observations were the same at all frequencies of *E. coli* O157:H7 tested indicating that the purity of the sorting was not effected by decreasing frequency of target organisms in the population.

4. Discussion

We have applied processing techniques used for Ab-DEFT detection of *E. coli* O157:H7 in ground beef in order to test their applicability to FC detection using two commercially available FC systems. Cell sorting of immunofluorescent stained *E. coli* O157:H7 from ground beef preparations was also accomplished. In addition to establishing the lower limits of quantitation with this system, we also compared FC and Ab-DEFT for the ability to determine counts of *E. coli* O157:H7 across a broad range of concentration in the presence of high levels of background organisms. Based on comparison to viable plate counts of streptomycin resistant *E. coli* O157:H7, FC was generally equivalent in accuracy to Ab-DEFT, until counts of the target organism reached a low enough level that signal:noise ratio problems were encountered. This threshold, which occurred in the range of 10^4 organisms ml^{-1}, was

similar to that reported in studies using FC to detect *Salmonella* spp. in eggs and milk [8]. Interestingly, the problem appeared to be unrelated to the fact that the samples were derived from a food matrix with a heterogeneous background flora, since studies with mixtures of pure cultures in PBS had a similar lower limit of *E. coli* O157:H7 measurement. Based on FC analysis of diluent (PBS), a more likely cause appeared to be the presence of fluorescent (possibly autofluorescent) particles present in instrument fluidics or associated with diluents and reagents used in the staining process and which fall within the light scatter region used for gating. Similar phenomena have been described in other related studies [9] and represent an obstacle to increased sensitivity with this technology. In contrast, the Ab-DEFT relies on operator recognition of bacterial morphology to distinguish such debris from the target organism, and although recognition problems occur to some extent, a lower limit of detection by two orders of magnitude is achieved [3]. This level of sensitivity is of vital importance in the realm of direct detection, however, acceptable enrichment procedures generally increase the concentration to well above the threshold for FC detection at which point issues of analysis time and sample through-put come into play. Our emphasis was not to determine the efficacy of the enrichment procedure in terms of the minimum starting number of target organisms that could be detected, but to determine the accuracy and sensitivity of the FC procedure in the enrichment milieu.

Sorting of stained *E. coli* O157:H7 from a diverse background such as beef enrichment cultures demonstrates that this is a practical approach to isolation of the organisms for further study or culture confirmation. Using the streptomycin-resistant strain made it unnecessary to do confirmation and demonstrated that the sorted organisms were essentially pure *E. coli* O157:H7, since the number of colonies with and without streptomycin selection were equivalent. Based on colony numbers with and without streptomycin the purity of the sorting was not affected by decreasing frequency of the target cells down to levels usually achievable with growth enrichment. In terms of food microbiology, this ability to have the organism in hand has important implications with regard to regulatory or quality control issues.

Forward light scatter is the most common parameter for detection of particles by FC. Bacteria are at the limits of the light scatter sensitivity of most FC instruments designed for analysis of larger cells. One approach to this problem has been the use of multiparameter analysis in which a strong fluorescence signal for all bacteria has been provided by nucleic acid binding dyes or by labeled antibody. A second fluorescence signal such as FITC was then used to identify the organism of interest [8]. Through the use of log-amplified FLS signals together with careful alignment and cleaning of optical and fluidic systems of the stream in quartz FC system we were able to achieve acceptable light scatter sensitivity without using nucleic acid binding dyes which would been incompatible with viable cell sorting. With the stream on surface, dark field FC system, FLS sensitivity was greater and the increased FLS resolution of the system indicated a distinctive light scatter profile for *E. coli* O157:H7. This suggests that enhanced light scatter resolution associated with this optical configuration may render light scatter analysis itself useful as an additional approach to multiparameter analysis of food samples. The increased light scatter associated with *E. coli* O157:H7 was an intrinsic feature of the strain, probably related to surface properties such as LPS side chains, but not associated with antibody binding. Distinct light scatter signatures have been shown in other examples of microbial analysis where multiple species of Gram-negative and Gram-positive bacteria were found to be distinguishable on the basis of light scatter [13]. Use of light scatter as an additional component of multiparameter analysis, in combination with nucleic acid binding dyes and specific immunofluorescence has potential to further refine FC methodology.

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