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Technical Report

Flow-through imaging cytometry for characterization of *Salmonella* subpopulations in alfalfa sprouts, a complex food system

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We recently developed an approach combining fluorescence *in situ* hybridization (FISH) and flow cytometry for detecting low levels of *Salmonella* spp. (~10^3 cells/mL sprout wash) against high levels of naturally occurring sprout flora (~10^7–10^8 CFU/g sprouts). Although this "FISH and flow" approach provided rapid presence/absence testing for *Salmonella* in this complex food system, it was not capable of more nuanced tasks, such as probing the phenotypic complexity of the microbes present in sprouts or determining the physical interactions of *Salmonella* with these microbes, or with sprout debris. In the present study, we have combined rapid FISH-based labeling of *Salmonella* spp. in sprout washes with flow-through imaging cytometry (FT-IC), using the ImageStream® 100, a commercial FT-IC instrument. This approach enables image-based characterization of various subpopulations of interest occurring within these samples. Here, we demonstrate the ability of FT-IC to unambiguously identify cells, cell aggregates and other events within these subpopulations based on both cell morphology and hybridization status after reaction with a *Salmonella*-targeted probe cocktail. Our ability to directly explore the nature of these events expands the layers of information possible from cytometric analyses of these complex samples and clearly demonstrates that "a picture is worth a thousand dots".

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1 Introduction

Seed sprouts, including alfalfa, broccoli and radish sprouts are microbiologically and physically complex foods that present real challenges to methods for rapid detection and characterization of pathogens. The same wet, warm, aerobic conditions needed to sprout plant seeds also promote the growth of a robust and varied microflora that grows to levels not found in any other type of non-spoiled produce [1, 2]. Mesophilic plate counts as high as 10^9 CFU/g have been reported for sprouts purchased from retail stores and cells may be present as free-living cells or in biofilms associated with sprout surfaces [1, 3]. Although bacteria are dominant, yeasts and molds can also be found in sprouts, sometimes at concentrations as high as 10^6 CFU/g [4]. Protozoan parasites, including *Cryptosporidium* oocysts and *Giardia* cysts, have also been detected in sprout samples [5]. Despite the high numbers for direct plating, it has been estimated that only about 10% of the microbial flora present on sprouts may be cultivable [3]. Together, these data suggest an almost staggering microbial complexity for this otherwise "fresh" and ready-to-eat food. Culture-independent methods for characterizing bacterial communities occurring on retail alfalfa sprout samples identified 15 families of bacteria and up to 27 dif-

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Abbreviations: FISH, fluorescence *in situ* hybridization; **FT-IC**, flow-through imaging cytometry; **PW**, peptone water; **SEM**, scanning electron microscopy ferent genera, including some potential human pathogens [6]. Gram-negative bacteria, primarily from the families *Enterobacteriaceae* and *Oxalobacteraceae* were most abundant [6]. The prevalence of *Enterobacteriaceae* potentially increases the difficulty for detection of *Salmonella* spp. in sprouts, as background flora in this food mainly consists of non-target cells that are both physiologically and phylogenetically similar to *Salmonella*.

Within the past decade, sprouts have been implicated in a number of multistate or international food-borne disease outbreaks caused by pathogens such as *Salmonella*, *Escherichia coli* O157:H7, *Bacillus cereus* and *Yersinia enterocolitica* [7]. We recently developed a FISH-based method for flow cytometric detection of *Salmonella* spp. in seed sprouts (Bisha and Brehm-Stecher, unpublished data). This method combines steps for pre-analytical sample preparation, use of an optimized dual probe cocktail and an abbreviated hybridization step that produces bright staining of *Salmonella* cells within 15 min. Although our "FISH and flow" method proved to be a powerful means for detecting relatively low levels of *Salmonella* against very high levels of natural sprout flora, we could not use this approach for direct observation or characterization of sprout microflora, their phenotypic complexity, or the physical interactions of *Salmonella* cells with these microflora, or with sprout debris. Conventional flow cytometers generate volumes of data and can be used to great advantage in characterizing complex microbial populations. However, these instruments essentially translate cells into a shorthand of pulses and dots that ultimately cannot convey the same level of detail as would a simple microscopic image [8–10]. In response to this limitation, a new generation of "hybrid" cytometers has been developed, capable of collecting both light scatter and fluorescence information as well as image data on the same cells [9, 10]. At least three such hybrid systems are commercially available, including the ImageStream® instrument used here (Amnis Corporation, Seattle, WA, USA). In this study, we combined our FISH-based assay for detection of *Salmonella* spp. in sprouts with flow-through imaging cytometry (FT-IC) using the ImageStream® platform for direct, image-based characterization of six different subpopulations occurring within these samples. As a whole cell method, a key advantage of FISH is its ability to preserve diagnostically important details on cell morphology and on physical or spatial associations between cells and/or non-cellular material. Traditional flow cytometers rely on light scatter and fluorescence to resolve events within a sample. Although light scatter is linked to cell morphology, this relationship is indirect. Non-cellular particulates from the sample matrix, or mineral crystals, dust and bubbles in the buffers used for analysis may give rise to high background levels of scatter-based "noise" that complicate detection of small cells such as bacteria. The inaccessibility of valuable morphological data *via* traditional flow cytometry limits the full diagnostic application of FISH when these two approaches are combined. In contrast, our work highlights the capacity of FT-IC to provide clear visual documentation of discrete events that are not directly observable using conventional flow cytometry. As demonstrated here, FT-IC provides microbiologists with new and exciting opportunities for exploring the complexity of macroscopically mundane, but microscopically fascinating samples such as alfalfa sprouts.

2 Materials and methods

2.1 Bacterial cultures

Salmonella enterica subsp. *enterica* ser. Typhimurium (*S*. Typhimurium) ATCC 14028 was from the American Type Culture Collection (ATCC, Manassas, VA). Working plate cultures stored at 4°C were used to inoculate 10-mL portions of Trypticase Soy Broth and cultures were grown at 30°C for 20–22 h. Cells were harvested *via* centrifugation and washed once in 0.1% peptone water (PW) prior to being used to spike sprout samples (below).

2.2 Preparation of alfalfa sprouts

Sprout samples were spiked with *S*. Typhimurium and processed for cytometry according to a procedure developed recently as part of an assay for *Salmonella* in seed sprouts (Bisha and Brehm-Stecher, unpublished data). Briefly, 25 g of fresh store-bought alfalfa sprouts were aseptically weighed out into a sterile stomacher bag with filter, and inoculated with ~10^7 CFU/g *S*. Typhimurium. Inocula were left in contact with sprouts for ~2 h, then 225 mL 0.1% PW was added and the mixture was homogenized for 1 min at 230 rpm in a Stomacher Circulator® 400 paddle blender (Seward Ltd., Norfolk, UK). The resulting homogenate was vacuum filtered in a single pass through four layers of sterile cheesecloth to remove large particulates (*i.e.*, visible stems, leaves). Small (1.3 mL) portions of the filtered homogenate were centrifuged briefly at low speed (300 × *g*, 30 s) to remove any remaining large particulates. One-milliliter portions of the supernatant were fixed for 30 min in 1 mL 10% buffered formalin, then resuspended in a 50:50 mixture of absolute ethanol and phosphate-buffered saline (PBS) and stored until use at –20°C. Samples without added *Salmonella* were also prepared and analyzed as negative controls.

2.3 Scanning electron microscopy

The physical and microbiological complexity of sprout samples was investigated *via* scanning electron microscopy (SEM) using the following sample preparation procedures. For "uncleaned" samples, 25 g unadulterated sprouts (no *Salmonella* added) were homogenized in 225 mL 0.1% PW, and 1 mL portions of the homogenate were removed and pelleted *via* centrifugation (2000 × *g*, 5 min). The supernatant was discarded and samples were fixed for 15 min in EM-grade glutaraldehyde (2.5% final concentration, Sigma-Aldrich), then resuspended in PBS and shipped to the University of Iowa's Central Microscopy Research Facility (CMRF) for analysis. At CMRF, a drop of the fixed sample was applied to a poly-L-lysine-treated silicon chip, allowed to adhere for 5 min, then samples were fixed further in 1% osmium tetroxide, followed by dehydration in an ethanol series, sputter coating and viewing *via* SEM using an Hitachi S-3400N microscope. For "cleaned" samples, sprout homogenates were filtered through four layers of cheesecloth and subjected to a brief, low speed centrifugation (30 s at 300 × *g*) prior to fixation with glutaraldehyde and subsequent processing at CMRF.

2.4 Fluorescence *in situ* hybridization

Two 23S rRNA-targeted oligonucleotide probes previously developed for detection of *Salmonella* spp., Sal3 [11], and Salm-63 [12], were combined as described by Lantz *et al*. [13] and used as a dual probe cocktail at a total probe concentration of 5 ng/µL (2.5 ng/µL each probe). Probes were synthesized, end-labeled at the 5′-end with either Cy5 or 6-carboxyfluorescein (FAM) and purified *via* HPLC by Integrated DNA Technologies, Inc. (Coralville, IA). Cy5-labeled probes were used in experiments involving conventional flow cytometry and FAM-labeled probes were used for experiments involving microscopy or imaging cytometry. For each hybridization reaction, 100 µL of fixed sprout samples were pelleted (5 min, 2000 × *g*) and resuspended in 100 µL hybridization buffer (0.7 M NaCl, 0.1 M Tris pH 8.0, 0.1% SDS, 10 mM EDTA) containing the dual probe cocktail. Samples were hybridized for 30 min on a heat block set to 55°C, followed by a 30-min wash step at the same temperature in 500 µL hybridization buffer without probe. Hybridized samples were pelleted and resuspended in a 50:50 mixture of PBS and absolute ethanol, cooled to –20°C and shipped on ice *via* overnight courier to Amnis Corporation. Once received, samples were placed and held at –20°C until used, for up to a week. Samples remained liquid under these storage conditions due to their ethanol content.

2.5 Stability of hybridization (storage study)

As noted above, sprout samples were prepared and hybridized in our lab, then shipped overnight to Amnis for analysis. To determine the lifetime of probe-conferred fluorescence, and thus the acceptable window between hybridization and analysis, we performed an initial storage study comparing two potential storage regimes: frozen and refrigerated. For the frozen treatment, hybridized samples were resuspended in a 50:50 mixture of PBS and absolute ethanol and held at -20°C as described above. Refrigerated samples were stored at 4°C, and received an additional post-hybridization fixation step (30 min in 10% buffered formalin) in an effort to "cement" hybridized probes in place *via* crosslinking, reducing their diffusive loss during storage at this higher temperature. Samples stored using both regimes were held up to a week and assayed periodically for fluorescence intensity *via* fluorescence microscopy and digital microscopy, with a photograph of the "time zero" sample (fresh hybridization) used as a reference for comparison at each sampling interval.

2.6 Flow cytometry

Hybridized sprout samples were examined using either a Becton Dickinson FACSCanto flow cytometer with red (633 nm) excitation or with an ImageStream® 100 FT-IC with blue (488 nm) excitation (Amnis Corporation). Data obtained *via* conventional flow cytometry were analyzed using FlowJo software (v. 8.7.1, Tree Star Inc., Ashland, OR, USA). For FT-IC, data were examined using the Image Data Exploration and Analysis Software package (IDEAS™, v. 3.0, Amnis). As noted above, once samples were received at Amnis, they were placed at –20°C until used. Prior to analysis, tubes were vortexed to resuspend cells and break up loosely associated flocs or aggregates. Samples (100 µL) were pelleted *via* centrifugation (2000 × *g*, 5 min), were washed in 100 µL PBS + 0.5% bovine serum albumin (BSA), centrifuged again, then resuspended in 50 µL PBS + 0.5% BSA prior to running on the ImageStream® system. Prior to collecting data, a compensation matrix was created using single-color controls and was used to correct for spectral crosstalk. Samples were mixed with the SpeedBead™ reagent (an internal control for imaging quality) and files containing 100 000 events were collected. After data collection, the beads were gated out, smaller data files containing ~7000–10 000 events were created and dot plots of side scatter (Y axis) *vs*. fluorescence intensity (X axis) were generated. Six unique subpopulations were chosen from the resulting dot plots for image-based exploration.

3 Results and discussion

Seed sprouts are surprisingly complex microbial niches, and represent unique challenges to methods for rapid detection and characterization of pathogens such as *Salmonella* spp. Not only are seed sprouts populated by large numbers (up to 10^9 CFU/g) of predominantly Gram-negative bacteria, but they are physically complex, as well, containing a size continuum of particulates. Figure 1 visually depicts these two levels of complexity. Figure 1A (100 μm scale bar) clearly shows the diversity of large sprout particulates present in "uncleaned" sprout samples (see Section 2.3 for details). These plant structures provide a large surface area to which microbial cells can bind. Figure 1B shows a view of "cleaned" sprout samples at higher magnification (10 μm scale bar), highlighting the high load, morphology and adherent nature of the indigenous sprout flora. Although these cells can all be described as "rods", they are varied in size and width, differences that could stem either from cell type or age (or both). Note that even in the "cleaned" sample, there is still ample particulate material to which these cells are bound. As described in previous microscopic studies of sprouts [1, 3] and confirmed in our work, native sprout microflora is present as both loose aggregates of surface-associated cells, and as classically defined biofilms, with several layers of cells embedded in an extracellular matrix (data not shown). No obvious yeast cells were seen using SEM or conventional microscopy, and, although we have previously observed an unidentified, motile, grazing protozoan in fresh sprouts from one manufacturer (Bisha and Brehm-Stecher, unpublished observation), no protozoa were observed in the samples analyzed via SEM or FT-IC. To determine shipping and storage conditions that would maintain the stability of FISH-based staining during transport and pre-analytical storage, we conducted a storage study, as described in Materials and Methods. Results from this study indicated that storage of hybridized cells in a 50:50 mix of ethanol and PBS at –20ºC was superior to post-hybridization fixation with refrigerated storage for maintaining intensity of probe-conferred fluorescence. Cell preparations held at –20ºC remained very bright for up to 1 week after hybridization and these conditions were used throughout the rest of the study.

Next, we sought to compare our results from conventional cytometry with those obtained using FT-IC. The scatter plot in Fig. 2A typifies our hybridization results for alfalfa sprouts contaminated with *S.* Typhimurium when a standard flow cytometer (Becton Dickinson FACSCanto) was used. Several distinct populations can be seen. Population (a), which comprises most of the sample and is thought to consist of both non-target sprout flora and particulate matter, spans a large range of side scatter values. Population (b) increases in fluorescence intensity with increasing scatter values, and may represent large clumps of non-target cells or particulate matter that bind or entrap the *Salmonella* probe cocktail. Despite being markedly more fluorescent than the bulk non-target population, this population was easily differentiated from *S.* Ty-

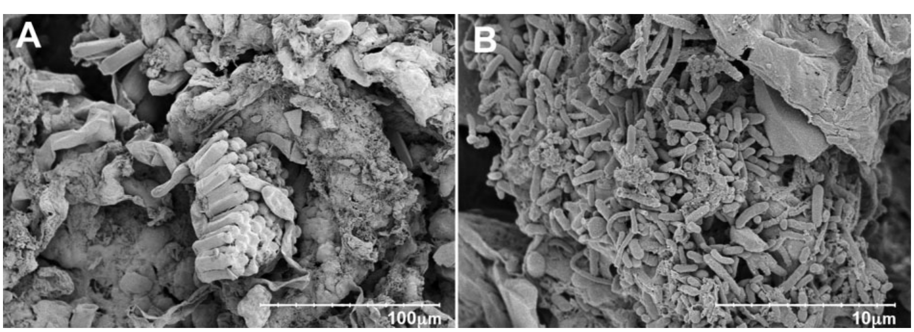

Figure 1. SEM of sprout particulate matter and natural, adherent microflora. (A) The physical complexity of alfalfa sprout samples not treated to remove large particulates. Relatively large alfalfa plant structures can be seen, including columnar palisade parenchymal cells at center and collapsed root or stem structures (scale bar 100 μm). (B) The complex assemblage of rod-shaped bacteria typical of the natural flora of fresh store-bought alfalfa sprouts (scale bar 10 μm).

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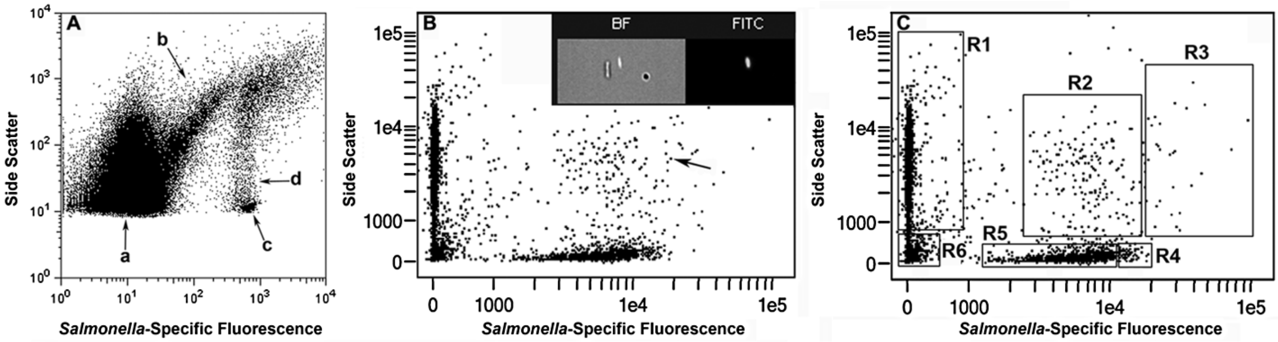

Figure 2. Comparison of sprout samples analyzed *via* both conventional and FT-IC. (A) Typical results for *Salmonella*-spiked alfalfa sprouts hybridized with a Cy5-labeled *Salmonella* probe cocktail and analyzed using a BD FACSCanto cytometer (100 000 events shown). Subpopulations a and b were ascribed to non-target sprout flora and particulate matter, subpopulation c (not present in *Salmonella*-negative control samples) was comprised of hybridized *S.* Typhimurium cells. The identity of subpopulation d, a fluorescent "bridge" connecting the low-scatter *Salmonella* cells to higher scatter events, could not be determined using conventional flow cytometry. (B) The ImageStream® 100 instrument's capacity for direct visual probing of event identity within physically and microbiologically complex samples. The inset shows brightfield and green channel fluorescence images of the multiparticle event highlighted by the arrow. (C) The division of the ImageStream® output into the six distinct regions used for the image-based exploration of this sample shown in Fig. 3.

phimurium (population c) on the combined basis of side scatter and probe-conferred fluorescence.

The exact nature of population (d), which formed a "bridge" between the discrete low-scatter *Salmonella* population and higher-scatter populations, is unknown. Because the fluorescence intensity of this "bridge" coincided with that of the *Salmonella* population, we hypothesized that it could have resulted from *Salmonella* cells bound to a size continuum of sprout particulates, or that it could be an artifact caused by coincidence – one or more non-target cells passing in front of the detector at the same time as a *Salmonella* cell. This latter explanation is more probable – if the "bridge" was formed by binding of *Salmonella* cells to a size-distributed population of sprout particles, larger particles should bind more *Salmonella* cells, which would result in a rightward skew for the bridge at higher scatter values. While this could explain the population at the top of the bridge (upper right hand corner of the plot), the bridge itself is fairly straight. This, along with the high load of non-target flora known to be present in sprouts (~10^8 CFU/g), suggests a coincidence-based explanation for this feature.

Still, for all the power of conventional flow cytometry, its ability to probe deeper and identify the events (cells, particulate matter, or cell-particulate complexes, *etc.*) responsible for these patterns is limited – at some point, a dot is simply a dot. Therefore, we sought to use FT-IC, a "hybrid" approach that combines aspects of conventional flow cytometry with imaging of each event detected, to further explore the nature of these populations [9, 10]. The optical configuration and principles of operation for the ImageStream® 100 are described in detail by Ortyn *et al.* [10]. Briefly, this instrument is capable of imaging cells simultaneously in six different modes: brightfield, darkfield and up to four fluorescence colors. Hydrodynamically focused cells are illuminated with a laser for darkfield and fluorescence imaging, and with a filtered white light for brightfield imaging. An objective lens is used to collect light from the cells, which is then passed through a spectral decomposition unit that separates the composite signal into discrete spectral bands. These are projected onto a charge-coupled detector, with each band trained onto a physically separate vertical "channel" on the chip's surface. Over 35 morphometric and signal intensity features are then calculated for each image using a real time algorithm. Captured images are accessible through an interactive user interface – by highlighting an event (a dot), the operator is able to retrieve stored images of the corresponding cell or object. This approach combines the best aspects of flow cytometry and digital imaging technologies, enabling flow-through multimode imaging of individual cells in liquid suspension [8–10].

Figure 2B highlights the power of the FT-IC approach for image-based confirmation of "dot" identity. With the FT-IC system we used (the ImageStream® 100 instrument from Amnis), highlighting of an event using a crosshair-shaped cursor calls up the images collected of this event and displays them according to operator-defined specifications. Images possible include bright field, dark field, up to four fluorescence colors and automatically generated overlays of individual image channels. The inset in Fig. 2B shows bright field (BF) and green channel fluorescence (FITC, or fluorescein) images collected of the multi-particle event high-

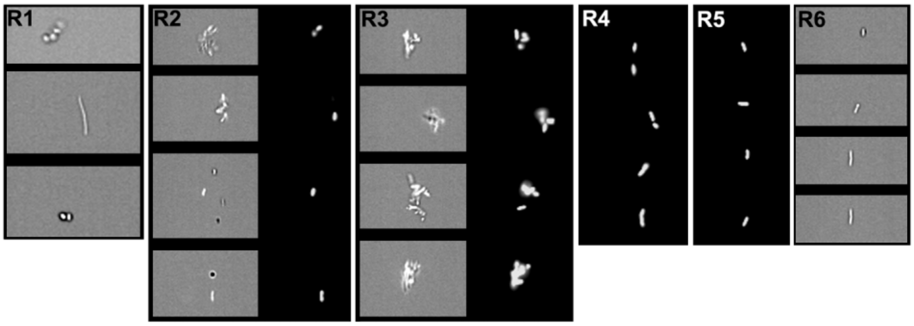

Figure 3. Representative images of events occurring in regions R1–6. Image-based exploration of events occurring within each of the six regions shown in Fig. 2 provided a unique window into cell or particle morphology and interactions of non-target events with FISH-labeled *Salmonella* cells.

lighted with the arrow on the scatter plot. Although this event appeared as a single dot on the side scatter *vs*. fluorescence plot, the corresponding images provide further layers of informational content. Specifically, these images establish that this "single" event arose from the simultaneous detection of three particles: one non-target rod-shaped bacterium (left hand particle), one *S.* Typhimurium cell (center particle, stained green with the *Salmonella*-targeted FISH probe cocktail), and one small high-contrast sphere (right hand particle), possibly either a 1-μm calibration bead (SpeedBead™ reagent) or an end-on rod having a diameter of ~1 μm. Although the exact identity of this particle is not known, this example clearly shows that this image-based cytometry approach has substantial advantages over conventional cytometry for further exploring subpopulations of interest within physically and microbiologically complex samples such as alfalfa sprouts.

When we examined the side scatter *vs*. fluorescence plot of a *Salmonella*-contaminated alfalfa sprout sample, we identified at least six unique regions that we chose to explore further *via* FT-IC-based imagery (Fig. 2C). Clockwise from top, these regions are: R1 (high scatter, no fluorescence), R2 (high scatter, medium fluorescence), R3 (high scatter, intense fluorescence), R4 (low scatter, high fluorescence), R5 (low scatter, medium fluorescence) and R6 (low scatter, no fluorescence). Using the IDEAS™ software, we explored images of events occurring within these regions, looking for images that typified each region.

Figure 3 is a montage of images of events occurring in R1–6. Probe-positive cells are shown here in these black and 6 white images as bright cells against a black background. Brightfield images from R1 show that this region was typified by large (high-scatter) non-target bacteria, unidentified microbes or sprout debris. Because these cells or particles did not react with the *Salmonella* probe cocktail, these events remained dark in the green (fluorescein) channel. R2 was populated by large aggregates of non-target flora containing one or two *S.* Typhimurium cells or physically separate, but coincident events comprised of one *S.* Typhimurium cell and one or more non-target particles. The lack of green channel fluorescence for non-target cells in this panel highlights the specificity of the *Salmonella* probe cocktail. Although FT-IC provides additional data on particle morphology, the identities of some particles remain unclear. For example, the dark sphere immediately above the *S.* Typhimurium cell in the bottom frame of R2 may either be an end-on rod or a SpeedBead™ tracking particle, two objects having an expected diameter of ~1 μm. Additional labeling, such as nucleic acid staining, could help resolve the identity of this particle. R3 contained large (high-scatter) aggregates of non-target bacteria or sprout debris and four or more *S.* Typhimurium cells. The large number of *Salmonella* cells contained within these aggregates led to their intense fluorescence signatures. It is not clear if this type of event is an artifact stemming from how we spiked *Salmonella* into the sprouts, or if natural biofilm-based growth of *Salmonella* spp. would result in similar subpopulations.

The events in R4 formed a discrete, low scatter/high fluorescence subpopulation, distinct from R5. Images revealed that R4 was comprised of large *S.* Typhimurium cells or *S.* Typhimurium cells in various stages of division. Large or dividing cells would be expected to contain higher levels of rRNA, in agreement with our observation of correspondingly brighter probe-conferred intensities for these cells. The bulk of the *Salmonella* from these samples was contained in R5. These were present as single, non-dividing *S.* Typhimurium cells. Lastly, the non-fluorescent, low scatter subpopulation in R6 was comprised of non-target cells of various shapes and sizes.

As shown in Figs. 2A and B, the two side scatter *vs*. fluorescence outputs from the ImageStream® 100 and the BD FACSCanto are not superimposable, but contain similar elements, including a large non-target population, at least one clearly separat-

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ed subpopulation of FISH-stained *Salmonella*, and a density of high-scatter/high fluorescence events immediately above the main *Salmonella* population. The FACSCanto output shown in Fig. 2A is comprised of ~100 000 events, the plot density level at which the "bridge" immediately above the *Salmonella* subpopulation becomes visually apparent. It was the inaccessibility of this bridge feature to direct characterization *via* conventional flow cytometry that led us to seek FT-IC as an alternate means of exploring the subpopulations present in *Salmonella*-spiked sprouts. Although Image Stream® files plotted at a density of 100 000 events more closely resembled the output from the FACSCanto, the type of manual image screening we performed in our lab necessitated the use of smaller "bite-sized" files of ~7000 events (Fig. 2B). The ability to directly explore the identities of individual events using FT-IC provided us with a unique window on the interactions of *Salmonella* with non-target cells occurring within this complex, heterogeneous food sample. Our image-based analysis of events in R2 of the ImageStream® output supports the theory that the bridge feature from the FACSCanto output can be explained by both clumping of target and non-target cells and by coincidence – passage of one or more non-target cells in front of the detector at the same time as a *Salmonella* cell.

4 Concluding remarks

A time-honored saying among flow cytometrists is that "dots don't lie". Although this may be true, our results suggest that they still can withhold information. We have shown here that FT-IC, combined with "phylogenetic staining" using *Salmonella*-targeted DNA-FISH probes, enables a more complete and direct visual exploration of this physiologically and phylogenetically complex food system than is possible using either traditional imaging approaches, such as microscopy, or conventional flow cytometry. The ImageStream® instrument used here was originally developed to bridge the gap between the relatively slow, but detailed imaging capabilities of confocal microscopy and the faster, but less information-rich analyses provided by traditional flow cytometry [10]. Because they enable high-throughput imaging of liquid sample suspensions, the use of such "hybrid" cytometry systems can provide unique insights into discrete phenomena occurring in complex samples such as alfalfa sprout washes, including information on cell-cell and cell-particle interactions and coincidence. As we have shown here, the use of *Salmonella*-specific FISH probes provides an additional layer of information, allowing differentiation of this organism within mixed populations. This can be especially useful in samples such as alfalfa sprouts, where physiological differences between target and non-target cells are either limited or nonexistent.

Recent studies on the prevalence of protozoa in foods and food processing environments and the potential protective effects of these protozoa on internalized pathogens suggest other areas in food microbiology where use of a combined FISH and FT-IC approach could provide valuable information. Protozoa such as *Tetrahymena pyriformis*, *Glaucoma* spp. and others have been isolated from spinach, lettuce and food contact surfaces within meat-cutting plants [14, 15]. These protozoa can engulf food-borne pathogens such as *Salmonella*, and serve as hosts, enabling the bacteria to multiply while simultaneously protecting them against inactivation by antimicrobials or physical treatments such as heat [14, 15]. The role that such protozoan hosts may play in the ecology of food-borne pathogens, or in protection of these pathogens against traditional methods of inactivation could be examined in further detail through the combination of FISH, additional physiological stains and analysis via FT-IC. The ability of instruments such as the ImageStream® to provide high-throughput, visually rich information on complex samples is expected to be of great basic value for observing the activities of native, inoculated or contaminant microbial populations in foods, fermentations or other materials of interest to food biotechnologists.

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