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Flow cytometric evaluation of the intracellular bacterium, *Wolbachia pipientis*, in mosquito cells

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Abstract

Wolbachia is an obligate intracellular bacterium (*Anaplasmataceae*, *Rickettsiales*) that occurs in arthropods and filarial worms, and spreads by vertical transmission in the oocyte cytoplasm. In insects, reproductive distortions associated with *Wolbachia*, such as cytoplasmic incompatibility in mosquitoes, have potential value for controlling pests, including species that transmit human, animal and plant diseases. *Wolbachia* strains that propagate as a persistent infection in insect cell lines provide an important resource for developing the genetic tools that will facilitate these applications. Here I describe conditions for flow cytometric evaluation of *Wolbachia* growth in persistently infected mosquito cells. Cytometry parameters were established using uninfected mosquito cells and *Escherichia coli* as a surrogate for *Wolbachia*, and quantitation was correlated with cell counts determined with a Coulter electronic cell counter and bacterial counts based on optical density. The protocol was validated by showing depletion of *Wolbachia* in medium containing tetracycline and rifampicin, and sensitivity of *Wolbachia* to treatment of host cells with paraquat, an oxidizing agent, and lumiflavin, an inhibitor of riboflavin uptake. The *Wolbachia* peak on the flow cytometry histogram was shown to contain *Wolbachia* by DNA analysis using the polymerase chain reaction, and by infection of naive recipient cells. This approach will streamline investigation of *Wolbachia* growth in insect cell lines and facilitate identification of culture conditions that select for *Wolbachia*-infected cells.

Keywords

alphaproteobacteria; *Wolbachia*; intracellular microorganism; mosquito cell line; flow cytometry

1. Introduction

Wolbachia is a maternally-inherited, obligate intracellular bacterium (family *Anaplasmataceae*; order, *Rickettsiales*) found in a wide variety of insects and other arthropods, as well as in filarial worms. In arthropods, infection is associated with

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reproductive distortions that facilitate *Wolbachia*'s spread within the host population. *Wolbachia* was first described in mosquitoes, in which it causes cytoplasmic incompatibility (CI), manifested by failure of egg hatch when an uninfected female mates with a *Wolbachia*-infected male. Eggs from *Wolbachia*-infected females, which contain *Wolbachia* in the egg cytoplasm, hatch regardless of the infection status of the male. The reproductive advantage of infected females provides a tool for successful replacement of vector populations (Laven, 1967; Sinkins and Gould, 2006). Recent discovery of the widespread distribution of *Wolbachia* and molecular advances in microbial genetics have stimulated an interest in potential applications of *Wolbachia* for control of pest insects.

Because *Wolbachia* is an obligate intracellular microbe, invertebrate cell lines provide an important tool for investigating *Wolbachia* infection, growth and replication. O'Neill et al. (1997) pioneered in the establishment of a mosquito cell line harboring a natural *Wolbachia* infection, and several strains of *Wolbachia* from insect tissues have been established in heterologous insect cell lines (Noda et al., 2002) and even in insect species that do not harbor *Wolbachia* infections in nature (Hughes and Rasgon, 2014). Although transformation of *Wolbachia* has yet to be reported, limited success with other members of the Rickettsiales (Beare et al., 2011) increases the potential value of in vitro systems to engineer *Wolbachia* for insect control.

Among *Wolbachia* strains that replicate well in insect cell lines, wStr, isolated from the planthopper, *Laodelphax striatellus* (Noda et al., 2002) maintains a particularly robust, persistent infection in a clonal population of *Aedes albopictus* mosquito cells (Fallon et al., 2013a). To facilitate evaluation of conditions that may favor or suppress *Wolbachia* growth in these C/wStr1 cells (Fallon et al 2013b, 2014), we developed a flow cytometric (FC) protocol for simultaneous evaluation of host cell number and *Wolbachia* abundance in persistently-infected host cells.

2. Materials and Methods

2.1. Mosquito cell lines and culture conditions

The C7-10 *Aedes albopictus* mosquito cell line was used as an uninfected control and as a recipient for *Wolbachia* infection; C/wStr1 cells are a population of C7-10 cells persistently infected with *Wolbachia* strain wStr (Fallon et al., 2013a). Cells were maintained at 28 °C in a 5% CO₂ incubator in Eagle's minimal medium containing 5% fetal bovine serum and supplemented as detailed by Shih et al. (1998). To suppress *Wolbachia* growth, tetracycline and rifampicin were added to the culture medium at final concentrations of 5 µg/ml, and 0.4 µg/ml, respectively. Paraquat (Fallon et al., 2013b) and lumiflavin (Fallon et al., 2014) were used as described previously.

For infection, inoculum was prepared from three 100 mm plates of confluent C/wStr1 cells. Cells were resuspended in the culture medium and released *Wolbachia* particles were recovered in the cell culture supernatant after centrifugation at 1000 rpm for 10 min in a swinging bucket rotor. The supernatants were pooled and filtered through a 2.7 µm syringe filter into sterile SW41 ultracentrifuge tubes. After centrifugation at 9000 rpm for 30 min, the supernatant was discarded, and pellets were resuspended in E-5 medium, to produce a

10-fold concentration of particles relative to the original volume of supernatant. Samples (0.3 ml) were diluted in 2X PI-MM for FC as detailed below. Exponentially growing C7-10 cells were resuspended in culture medium, counted with a Coulter electronic cell counter, adjusted to 5×10^4 cells/ml in E-5 medium, and the remainder of the resuspended *Wolbachia* was mixed directly with diluted cells. A typical infection involved 60 ml of diluted cells, from which 2 ml samples were added to a series of 35 mm culture dishes. The increase in *Wolbachia* was monitored by fluorescence microscopy and by FC at daily intervals.

2.2 Bacteria

Escherichia coli strain D31 (Monner et al., 1971) was grown in Luria broth, and 1 ml portions of an overnight culture were diluted to 14 ml with distilled water and collected by centrifugation. The pellet was resuspended in 1.0 ml of distilled water and frozen at -20°C . Freezing facilitated propidium iodide (PI) staining, as described below. Turbidity of the bacterial stock was measured with a spectrophotometer, and an OD_{600} of 1.0 was considered equivalent to 8×10^8 bacteria/ml. With *E. coli*, efficiency of PI staining was monitored by noting the position of the WP events with systematic variation of PI concentration. PI concentration was increased until the rightward shift of the WP stabilized. As described below, final PI concentrations were adjusted to accommodate additional uptake of stain by mosquito cell nuclei.

2.3 PI Staining

Because *Wolbachia*-infected mosquito cells become fragile and release bacteria into the culture medium as the infection progresses, we developed a staining protocol that allowed cells, growing as loosely attached monolayers and/or as floating clusters, to be directly resuspended in the culture medium and stained with PI, avoiding centrifugation and fixation procedures commonly employed in cell cycle analyses (see Gerenday and Fallon, 2004). PI concentration and detergents expected to disrupt the host cell plasma membrane and improve staining of intracellular bacteria were systematically varied to arrive at a 2X-Master Mix (20 mM Tris-HCl, pH 7.5, 0.4% Triton X-100, 0.2 mg/ml PI, 0.1 mg/ml boiled RNase A, hereafter abbreviated as 2X PI-MM). Cells in 35 mm plates were resuspended directly in growth medium with a Pasteur pipet, and 0.30 ml was added to 0.30 ml of 2X PI-MM; the final concentration of PI was 0.1 mg/ml (150 μM). Samples were mixed and incubated at room temperature for 0.5 to 2 h. Immediately before analysis, samples were gently vortexed and transferred to a 5 ml polystyrene tube with cell strainer cap (Falcon 3352235, Corning, NY, USA).

Previously-frozen *E. coli* (in 1.0 ml distilled water) were thawed, vortexed, and diluted with 1.0 ml of E-5 cell culture medium and 2.0 ml of 2X PI-MM. The diluted bacteria (0.85 OD_{600} ; 7×10^8 bacteria/ml) were stained at room temperature for 1 h. Bacteria from this stock solution (50 to 400 μl) were added to E-5 medium containing 1X PI-MM to make a final sample volume of 0.6 ml. After an additional 30 min at room temperature, samples were vortexed and analyzed with a Coulter EPICS XL-MCL flow cytometer, which detects propidium iodide (PI) on the FL3 sensor, at a flow rate of 60 $\mu\text{l}/\text{min}$. PI fluorescence of *E. coli*, used to approximate the position of the *Wolbachia* peak (WP), was detected by

reducing the discriminator setting (Aux, FL3 peak) from the setting of 30 used for cell cycle analysis, to a setting of 3. Voltage settings were adjusted to position PI peaks from mosquito nuclei (designated as cell peak, CP) to the rightmost quartile on a histogram showing events vs. FL3 log. Voltage settings were as follows: Forward scatter, 193 volts, gain =1; side scatter, 800 volts, gain =5; FL3 773 volts, gain =1; Aux (FL3 peak height) 155 volts, gain =1. Flow rate was set to the highest setting (60 μ l/min). Validation of conditions with *E. coli*, control mosquito cells, and infected mosquito cells are addressed in the Results section. Like other intracellular bacteria, *Wolbachia* has lost several genes involved in cell wall biosynthesis (Wu et al., 2004), and thus would be expected to stain with PI under conditions suitable for *E. coli*.

3. Results

3.1 Standardization of flow cytometry protocol with mosquito cells and *Escherichia coli*

To facilitate visualization of both bacteria and host cells, we began by placing the nuclear peaks from uninfected control C7-10 mosquito cells in the rightmost quartile of a histogram (Fig. 1A), and established a gate, represented by the horizontal line, that we call the Cell Peak (CP). Within CP, most of the events fall into G1 and G2 peaks of a typical cell cycle, with a small percentage of events at the extreme right representing cells with higher levels of ploidy and/or cell aggregates. Based on a plot of SS log vs. FL3 log, 97.5% of CP events fall in an upper right region (Fig. 1A, Inset). CP events (Fig. 1B, Y axis) showed nearly a 1:1 correlation with cell numbers determined by Coulter counts (Fig. 1B, x axis) up to a density of 1.4×10^6 cells in a 0.6 ml sample volume.

Propidium iodide (PI)-stained *E. coli* was used as a surrogate to establish the position of the bacterial (*Wolbachia*) peak (WP). Because live *E. coli* does not stain readily with PI, we evaluated various methods to enhance staining, including fixation in methanol or formaldehyde, heat treatment, and cold shock (see McClain et al., 2001). Freezing bacteria in distilled water was the most effective way to promote staining while reducing aggregation. The FC histogram (Fig. 2A) showed a single peak, corresponding to events largely in the lower left quartile of the SS log vs FL3 log density plot (Fig. 2A, inset). FC events showed a linear relation to bacterial number from about 4×10^5 events to 2.5×10^6 events per sample. However, events recorded under the FC peak were roughly 150-fold reduced, relative to actual bacterial numbers (compare the axes in Fig. 2B).

In a final comparison, events in the CP and WP regions were evaluated with combinations of cells and bacteria, as would be the case with samples containing *Wolbachia*-infected cells, from which bacteria would be released by exposure to detergent in the 2X PI-MM. Regardless of the presence of *E. coli*, the ratio of cell counts to FC events ranged from 1.0 to 1.3, indicating that FC conditions were sufficiently accurate for evaluation of cell numbers (Table 1). Consistent with Fig. 2B, *E. coli* counts were underestimated by a factor of 140-160 when evaluated alone. In the presence of cells, the underestimation ratio rose to 230-240 fold, and was influenced by flow rate. As detailed in section 2.3, all experiments were conducted using the highest rate available on the EPICS instrument. Because we are primarily interested in comparing changes in *Wolbachia* abundance in response to cellular treatments, rather than absolute numbers of *Wolbachia*, we further tested whether this

systematic error interfered with evaluation of changes in *Wolbachia* levels during growth of infected cells.

3.2 Effect of rifampicin and tetracycline on the *Wolbachia* profile

We proceeded to evaluate FC data on C/wStr1 cells maintained in the presence and absence of tetracycline and rifampicin, which are commonly used to eliminate *Wolbachia* infections from insects and/or cell cultures without damaging host cells or interfering with host cell growth (O'Neill et al., 1997; Fallon et al., 2008; Fallon et al., 2013a). Figure 3 summarizes a representative experiment. Although the Coulter counter gave more accurate data for low cell counts (Fig. 3A), compared to replicate samples evaluated by FC (Fig. 3B) both methods revealed a clear difference between antibiotic-treated (open circles) and untreated (closed circles) cells. As described previously (Fallon, 2008, Fallon et al., 2013a), improved host cell proliferation in the presence of antibiotics suggests that maintenance of the *Wolbachia* infection has a negative effect on overall cell growth, relative to that in "cured" control cells. In infected cells, *Wolbachia* abundance (Fig. 3C, closed squares) increased in proportion to cell counts, reaching a peak of about 10^6 WP events (Panel C) per 3×10^5 cells (Panel B) six days after plating. With a conservative multiplier of 150 for the WP (see Table 1), we estimate about 500 *Wolbachia* per cell at peak density, which is consistent with microscopic observation. Values for the WP in tetracycline-treated cells (Fig. 3C, open squares) ranged from 600 to 4360, or up to 4.6% of total events; note the log scale), and we note that complete elimination of *Wolbachia* requires more than a single treatment (Fallon, 2008).

Corresponding FC data for representative infected C/wStr1 samples without and with antibiotic treatment (days 2, 4, and 6 in Fig. 3C) are shown in Fig. 4. *Wolbachia* abundance (WP) rises with cell numbers from days 2 to 6, reaching maximum levels as dividing cells are depleted (Fig. 4, upper panels; note that events corresponding to the S (synthesis) phase of the cell cycle between the G1 and G2 peaks (open arrow, Fig. 4C) drop with increasing time after plating (Fig. 4, compare panels A and C). In the presence of tetracycline and rifampicin, the WP was substantially reduced, although the density plots (insets in Figs. 4D-F) show a low background of events, which increases as cell numbers increase. With antibiotic treatment, the CP numbers reflect growth of the host cells to a higher density, with continued cycling based on S levels on day 6.

3.3. The decline in *Wolbachia* levels is associated with a sub-G1 peak

Towards the end of a growth cycle, WP levels drop (Fig. 3C) and representation of host cells in the S phase of the cell cycle drops to virtually undetectable levels (Fig. 5, compare day 9 with Fig. 4, A - C). Between days 9 and 12, the G1 and G2 peaks broaden, and events begin to accumulate in the area between the WP and CP regions (Fig 5, note the arrow on the day 12 density plot), suggesting onset of apoptotic events associated with degradation of host cell DNA.

3.4. Lumiflavin and paraquat reduce *Wolbachia* levels

In previous studies, we used Coulter cell counts, MTT assays and PCR reactions to demonstrate a reduction in *Wolbachia* levels in host cells exposed to the oxidizing agent,

paraquat (Fallon et al., 2013b), and the riboflavin transport inhibitor, lumiflavin (Fallon et al., 2014). As a final check on the reliability of FC data, these experiments were repeated by quantifying WP and CP events. FC data replicated previous results (Fig. 6), showing an improvement in cell numbers as *Wolbachia* levels drop, followed by a decline in cell numbers as drug concentrations become toxic to host cells. *Wolbachia* levels were sharply decreased at paraquat concentrations as low as 0.015 μ M (Fig. 6), with a corresponding increase in CP events, which reached a maximum at approximately 0.1 μ M paraquat, consistent with data based on cell counts and MTT assays described previously. Likewise, treatment of host cells with lumiflavin at concentrations greater than 2.5 μ M reduced *Wolbachia* levels, with a concomitant increase in CP events. Based on molar concentrations, note that *Wolbachia* infection is substantially more sensitive to paraquat than to lumiflavin.

Validation of the presence of *Wolbachia* in the WP—In insect tissues, *Wolbachia* are pleomorphic in size and shape (Hertig, 1936) and cannot be physically separated from mitochondria (Sun et al., 2001), which have a DNA genome ~75-fold smaller than that of *Wolbachia*. Because *Wolbachia* are no longer recoverable by centrifugation after treatment with 2X PI-MM, presumably due to detergent-associated damage to the bacterial cell wall, we validated the presence of *Wolbachia* in the WP by showing that material from filtered cell culture supernatant recovered by centrifugation is associated with a cell-free WP with 99% of events in the lower left quartile of the SSlog/FL3 histogram (Fig. 7A), contains *Wolbachia* DNA as assayed by the polymerase chain reaction (see Fallon, 2008) and is infectious, reestablishing a WP that increased from 2200 to 104000 events between days two and eight (Fig. 7B, C). In the CP, note that the proportion of cells in the "S" phase of the cell cycle drops with increasing cell number as *Wolbachia* levels approach a maximum.

4. Discussion

Drawing from recent applications of flow cytometry to free-living bacteria (Vivas-Rego et al., 2000; Button et al., 2001; Tracy et al., 2010), we investigated use of FC to study an obligate intracellular bacterium, *Wolbachia pipientis*, in insect host cells. Although we are unaware of a systematic study such as ours with other intracellular bacteria, Tombolini et al. (1997) successfully used FC to examine expression of green fluorescent protein in *Pseudomonas*, and Ryan et al. (2002) determined *E. coli* genome equivalents using FC, albeit with ethanol-fixed samples. Pils et al. (2006) recently demonstrated that flow cytometry was comparable to antibiotic protection assays for evaluating invasion of bacterial pathogens into eukaryotic cells, and Messick and Rikihisa (1993) described a qualitative evaluation of a related intracellular alphaproteobacterium, *Ehrlichia risticii*, using flow cytometry.

Because *Wolbachia*-infected mosquito cells become fragile as bacteria proliferate, we used fluorescence microscopy together with FC to develop a PI-staining solution that did not require prior manipulation of infected cells by centrifugation or fixation. With host cells, it was important that lysis be sufficiently gentle enough to avoid widespread dispersion of cytoplasmic contents, which can interfere with quantitation of the WP, and it is recommended that the FC profiles be verified for individual host lines, which may require modification of Triton-X-100 levels in the staining solution. Although we have shown that

FC reliably tracks establishment of a *Wolbachia* infection in naive cells, we note that further efforts to identify fluors and/or antibodies that allow FC-based discrimination between live and dead *Wolbachia* will enhance efforts to transform *Wolbachia* and evaluate subsequent recovery of genetically manipulated strains in host cells (see Beare et al., 2011).

The ease and rapidity of FC has advantages over methods based on cell numbers, biochemical assessment of metabolic activity, fluorescence microscopy and polymerase chain reactions (PCR), used previously to evaluate effects of potential selective agents on growth of *Wolbachia* in mosquito cells. Although fluorescence microscopy is equally rapid, day-to-day comparisons based on photographic records are limited by the relatively small proportion of a population that can be photographed, by loss of fluorescence intensity during examination, and by changes in properties of the host cell, such as adherence to substrate, cell-cell aggregation, and membrane fragility, which can vary over the course of an experiment. The time required for extraction and amplification of *Wolbachia* DNA make the PCR reaction less than ideal for timely quantification of inocula immediately before application to host cells, and does not provide information on apoptotic events in host cells, which may affect the quality of an inoculum. We anticipate that further refinement of FC approaches using commercially available fluorescent probes will facilitate exploration *Wolbachia*'s interactions with its host cells, and that the approach described here may be extended to other intracellular microorganisms.

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Highlights

Flow cytometry can be used to track Wolbachia infections in mosquito cells.

Flow cytometry can be used to evaluate Wolbachia preparations used for infection.

Wolbachia is sensitive to the antibiotics rifampicin and tetracycline.

Wolbachia is sensitive to paraquat and lumiflavin.

Flow cytometry provides a tool to facilitate Wolbachia transformation.

Fig. 1.

FC analysis of uninfected C7-10 cells. Panel A. Histogram showing profile from a sample containing 2.4×10^5 cells in an 0.6 ml sample. CP, Cell peak; G1, G2: cells in the G1 and G2 phases of the cell cycle, respectively. The inset shows a corresponding density plot of side scatter (SS log) vs. propidium iodide (FL3 log). Values indicate percentage of events in each region. Panel B. Relation between FC and Coulter counts. Cells were counted with a coulter electronic cell counter, diluted in E-5 medium, and duplicate samples containing 6.0×10^4 to 7.4×10^5 cells in 0.3 ml were added to 0.3 ml of 2x PI-MM. For the final two points, cells were concentrated by centrifugation and resuspended in 1X PI-MM diluted with E-5 medium. After 30 min at room temperature, samples were strained through a 40 μ m mesh and analyzed by FC for 60 sec, at a flow rate of 1 μ l/sec. Events under the CP-region (See Fig. 1A) were averaged and multiplied by 10 to obtain events per 0.6 ml sample (Y axis). Values represent averages of duplicate samples; error bars showing ranges between values are obscured by the data points.

Fig. 2. FC analysis of *E. coli* as a surrogate for *Wolbachia*. A. Density plot from the data point indicated by the arrow in Panel B. WP, *Wolbachia* Peak (180,000 events), CP, Cell Peak. Values in the histogram designate percentage of events in each region. B. FC events in the

WP peak, relative to input bacteria. Bacteria were stained as described in the Materials and Methods. Numbers of bacteria in an 0.6 ml sample (X-axis) were calculated according to absorbance at 600 nm. The Y-axis shows events per 0.6 ml sample, averaged from duplicate readings; where visible, bars show the range of variation between duplicates. Background in the absence of bacteria ranged from 463-504 events. Comparison of the axis scales indicates that number of events detected in the WP is about 150-fold lower than the actual bacterial count.

Fig. 3.

Changes in cell numbers and *Wolbachia* abundance during a 12-day growth cycle. *C/wStr1* cells at passage 77 were plated at 1×10^5 cells/ml, 2 ml per 35 mm plate. Tetracycline and rifampicin were added to one set of plates, at final concentrations of 5 $\mu\text{g/ml}$, and 0.4 $\mu\text{g/ml}$, respectively. On the indicated days after plating, duplicate plates were resuspended and analyzed with a Coulter electronic cell counter (Panel A), or by FC (Panel B, CP; Panel C, WP). Circles, cell counts (CP); squares, *Wolbachia* counts (WP). Open symbols with dashed lines, antibiotic treated plates; solid symbols, *C/wStr1* cells without antibiotics.

Fig. 4.

FC histograms from the exponential phase of *Wolbachia* growth. Representative plots from days 2, 4, and 6 of Fig. 3 are shown. WP and CP values are indicated below the panels. Panels A-C, cells without antibiotics, supporting normal *Wolbachia* growth; Panels D-F, duplicate plates treated with tetracycline and rifampicin. In panel C, the open arrow indicates the position of cells in the S phase of the cell cycle. Insets show density plots.

Fig. 5.

Decline in *Wolbachia* abundance correlates with accumulation of subcellular events. Representative panels for days 9 and 12 from Fig. 3 are shown. The open arrow in the lower right panel designates events associated with deterioration of host cells. Day 9: CP = 36,000; WP = 121,000; day 12: CP = 17,000; WP = 29,000.

Fig. 6.

Wolbachia is more sensitive to paraquat and lumiflavin than its host cells. *C/wStr1* cells (passage 76) were plated at 1×10^5 cells/ml, 2 ml/35 mm plate, in the presence of the indicated concentrations of paraquat or lumiflavin. After 5 days, cells from duplicate plates were resuspended in the culture medium, and 0.3 ml was added to 0.3 ml of 2X PI-MM. After 30-60 min, cells were gently vortexed, strained and assayed by FC. Values on the Y-axis represent raw sample counts $\times 10^{-3}$. Where visible, bars indicate the range from duplicate samples.

Fig. 7.

The WP contains infectious *Wolbachia*. Inoculum containing *Wolbachia* released by natural lysis of host cells (or breakage during manipulation) was recovered from cell culture supernatant after passage through a 2.7 μ m filter to remove cell fragments. *Wolbachia* was recovered by centrifugation at 9000 rpm for 30 min in an SW41 rotor at ambient temperature. A. FC analysis of the inoculum; WP = 62,000; inset: histogram showing 99% of events in the lower left quartile. B. Infected cells at day 2; WP = 2200 ; CP = 7700 . D. Infected cells at day 10 (WP = 104,000; CP = 59,000).

Table 1

Cells Coulter	CP events FACS	Ratio Cells/Events	Bacterial numbers	WP events FACS	Ratio Bacteria/WP
1.3 e4	1.3 e4	1.0	0	0.07 e4	na
2.5 e4	2.2 e4	1.1	0	0.06 e4	na
3.8 e4	3.2 e4	1.2	0	0.05 e4	na
7.5 e4	6.0 e4	1.2	0	0.05 e4	na
1.3 e4	1.1 e4	1.2	4.5 e7	1.9 e5	240
2.5 e4	2.0 e4	1.2	3.6 e7	1.6 e5	230
3.8 e4	2.9 e4	1.3	2.7 e7	1.1 e5	240
7.5 e4	5.9 e4	1.3	0	0.009 e5	na
0	0.0001 e5	na	0	0.004 e5	na
0	0.0002 e5	na	1.8 e7	1.1 e5	160
0	0.0002 e5	na	3.6 e7	2.5 e5	140
0	0.0002 e5	na	5.3 e7	3.1 e5	170

Columns labeled Cells and Bacteria indicate calculated events per 60 μ l, based on Coulter counts for cells, and OD₆₀₀ for bacteria. FC events in CP and WP columns represent raw data from FACS output under CP and WP peaks, respectively. Ratio, number of calculated cells or bacteria, divided by number of FC events.