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Confirmatory measurement channels for LIF-based bioaerosol instrumentation

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ABSTRACT

As part of the U.S. Department of Homeland Security Detect-to-Protect (DTP) program, a multilab [Sandia National Laboratories (SNL), Lawrence Livermore National Laboratories (LLNL), Pacific Northwest National Laboratory (PNNL), Oak Ridge National Laboratory (ORNL), and Los Alamos National Laboratory (LANL)] effort is addressing the need for useable detect-to-warn bioaerosol sensors for public facility protection. Towards this end, the SNL team is investigating the use of rapid fluorogenic staining to infer the protein content of bioaerosols. This is being implemented in a flow cytometer wherein each particle detected generates coincident signals of correlated forward scatter, side scatter, and fluorescence. Several thousand such coincident signal sets are typically collected to generate a distribution describing the probability of observing a particle with certain scattering and fluorescence values. These data are collected for sample particles in both a stained and unstained state. A linear unmixing analysis is performed to differentiate components in the mixture. In this paper, we discuss the implementation of the staining process and the cytometric measurement, the results of their application to the analysis of known and blind samples, and a potential instrumental implementations that would use staining.

1. INTRODUCTION

The U.S. Department of Homeland Security (DHS) seeks rapidly acting (1-2 min response time) sensors that indicate the presence of a bioaerosol release in enclosed facilities. Optical measurements are desired for this application because they can rapidly measure intrinsic properties of single particles. Thus, several early warning sensors[1-3] have been developed that measure the laser-induced fluorescence (LIF) and elastic scattering properties of streams of sampled aerosols. Detailed recognition algorithms[4,5] are used to analyze these data to identify significant populations of "threat-like" aerosols (without the requirement to actually identify them) within the facility background aerosol population.

Although their capabilities are continually being improved, current LIF-based sensors are observed to produce false alarms at rates between once per week and once every two months when operated under conditions relevant to facility protection. High-consequence actions (e.g. stopping the flow of traffic in an airport concourse) are likely too costly to incur by facility managers at this rate. Thus, false alarming represents a significant obstacle to the use of the devices.

This paper describes an optical measurement that is intended to provide greater specificity for biological threat agents than those based on intrinsic LIF. It also presents the results of preliminary testing of the method against blind samples provided by the Edgewood Chemical Biological Center (ECBC). The new method employs a fluorogenic stain (i.e., a stain that becomes fluorescent upon reaction) to label proteins in collected aerosols. It is intended to increase the LIF signal of threat particles relative to that of non-biological background interferents, which were found to be prevalent during an earlier phase of this project. The fluorescamine[6] stain that is used reacts quickly ($\leq$ 15 s) with primary amine groups on proteins to form a covalently bound fluorescent product that is $\geq$100 times more fluorescent that the unreacted molecule. It is expected that the methods described here can be extended to the use of other sufficiently rapid stains that

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indicate other particle properties (e.g., the presence of DNA). Moreover, the cytometer provides a platform in which more sophisticated secondary measurements[7,8], such as those based on antibodies, could be immediately performed on a triggering sample.

2. MEASUREMENT DESCRIPTION

The staining measurement has been tested in the laboratory on prepared samples that are measured by a commercial flow cytometer. While consideration was given to modes in which stained particles are measured individually or in aggregate, it was decided that a single-particle measurement would provide the most selective information and that, if desired, an aggregate measurement could be simulated using single-particle data. Thus, a protocol was developed in which particles to be analyzed are stained in bulk and then measured individually in the flow stream of the cytometer.

The cytometric measurement resembles that of the intrinsic LIF sensor (see Fig. 1), except that particles are measured in a hydrodynamically focused liquid stream (i.e., as hydrosols) rather than an air stream (as aerosols). While a cytometer is generally perceived as a laboratory instrument, compact and rugged systems are available that could be adapted to allow the measurement to be made in a fieldable sensor as well. When implemented as such (see Fig. 2), the sample would be collected into a solution using an aerosol-to-hydrosol collector, such as a wetted-wall cyclone. It would then be combined with fluorescamine stain under appropriate conditions for reaction and injected into the cytometer for measurement.

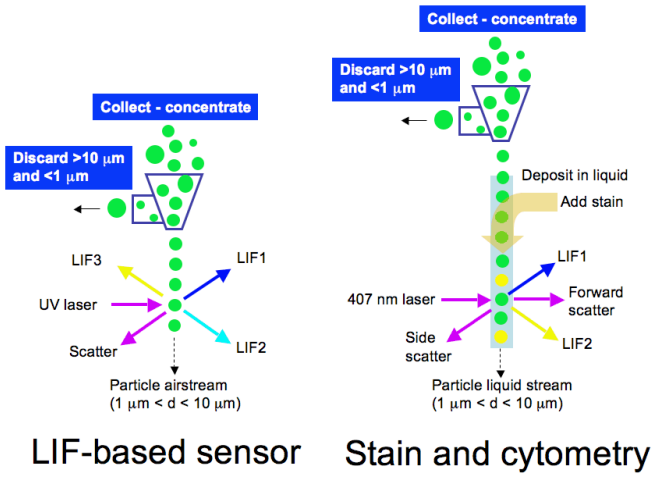

Figure 1 – Diagram of a sensor based on native LIF detection (left) and a sensor based on staining and cytometry (right).

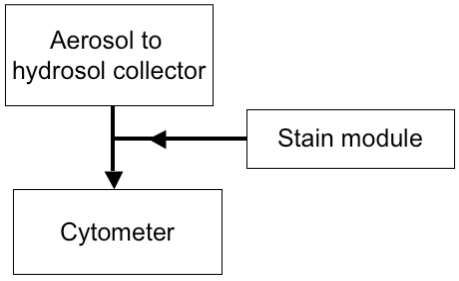

Figure 2 – Elements of a potential fieldable staining sensor.

The four threat types to be measured by this system (cells, spores, viruses, and protein toxins) are expected to arrive at the sensor as respirable-sized (~1-10 μm diameter) clusters of bioagent and binding material

(e.g., clay or salts). Transfer from aerosol to hydrosol state presents the possibility that they would partially or fully fragment prior to measurement. In formulating and testing the staining protocol, it has been assumed that fragmentation would be complete, yielding separated agent species in solution. The cells and spores can be measured directly by the cytometer, while small viruses and protein toxins are too small to trigger the instrument. Their detection is accomplished by measuring the LIF of the stained solution in the absence of particles. As will be described, this measurement of solution LIF was also accomplished using the flow cytometer.

As shown in Fig. 1, the data produced by the cytometer consists of coincident sets of forward- and side-scatter (FSC and SSC, each at 488 nm) and LIF (excited at 407 nm and centered at 545 nm with a 20-nm bandpass) measured for each particle. For example, Fig. 3 contains plots of measurements made on unstained and stained particles of *bacillus subtilis* spores and background particles (Greer house dust). Comparison of the two plots demonstrates that the spores exhibit greater change in the presence of stain than the house dust, thereby separating the two distributions in the plotted space.

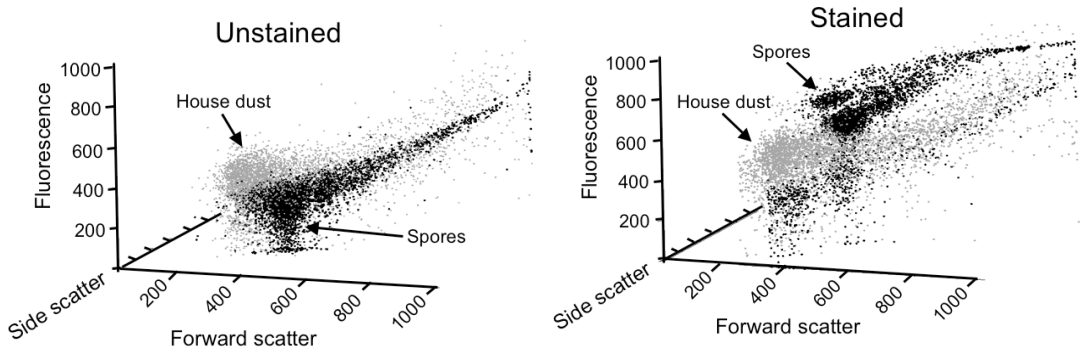

Figure 3 – Cytometric measurements of a mixture of *bacillus subtilis* spores in house dust background. The sample at left is unstained; that at right is stained with fluorescamine. The spores are indicated with dark markers; the house dust with light markers.

Note also that the stained spore data shows two distinct lobes – the main one and then one slightly above and to the left. This is likely caused by either binary spore clusters or by two alignments of the spores in the fluid stream. Because this peak is not correlated with spore concentration it is unlikely to be due to more than one spore in the measurement volume. Evaluation of the hydrosol (spore, vegetative cell) cytometric data is accomplished using a linear unmixing analysis that is described in the following paper of this Proceedings. The procedure is composed of the following steps:

(1) Measurement of a series of stained and unstained n-dimensional (in this case, n=3; FSC, SSC, FL) signature and background data sets
(2) Formation of a signature and background library of these data after binning each to a coarser resolution (26x26x26 elements) than that of the native form from the cytometer (1024x1024x1024) and unfolding to a one-dimensional (17,576 component) vector (to be referred to as the pure vectors **P**).
(3) Collection of similarly unfolded and binned data from unknown samples (**a**)
(3) Analysis of the unknown data by minimizing the residuals (vector **e**) in a linear fit of the pure library components (vectors **P**) to the unknown (**a**) according to $\mathbf{a} = \mathbf{f}\mathbf{P}^T + \mathbf{e}$, where **f** is the fractional profile of the **P** contribution to the unknown.

This method has been successfully applied to the measurement of pure bioagent surrogates (*bacillus subtilis* spores, *bacillus thuringiensis* (BT) spores, *yersinia rohdei* vegetative cells), background materials (Greer house dust), and mixtures of each. It successfully identified the presence of these threat particles in unknown sample mixtures provided by the ECBC and in samples generated by us. The solution LIF method for toxins and viruses was also applied to the ECBC samples using a simple LIF intensity threshold as an indicator of threat material.

3. EXPERIMENTAL METHOD

3.1 Hydrosol concentrations

The test samples were prepared according to the characteristics of a hypothetical sensor configuration, such as shown in Fig. 3, but based on the specifications of actual devices. The collector assumed is a wetted-wall-cyclone that was developed by Texas A&M University and the University of Texas, is available in configurations that sample air at rates between approximately 100 and 1000 l/min and strip out the entrained aerosols with an efficiency of >90% for particle diameters above a cut point of about 1 μm. The collected aerosols are submerged in a liquid stream that is emitted by the collector at rates of between approximately 100 and 1000 μl/min (depending upon the input flow rate configuration). Thus, the instrument produces an air-to-liquid volumetric concentration of about 10^6.

The cytometer assumed (and used in the lab measurements in this paper) is a Becton Dickson (Franklin Lakes, NJ) FACScan that has been modified by Cytek (Fremont, CA) to extend its operation to include 407 nm excitation. As illustrated in Fig. 4, it contains two continuous wave excitation sources – an argon ion laser emitting at 488 nm and a blue laser diode at 407 nm. Multiple fluorescence channels are associated with each excitation wavelength and scattering data (forward and side) are collected from the 488 nm excitation. While data from the 488 nm and 407 nm sources are correlated on a single particle basis, the beams are vertically offset in the direction of the flow. Further separation between the two excitation wavelengths is provided electronically by timing the data acquisition to coincide with the passage of a particle through each beam.

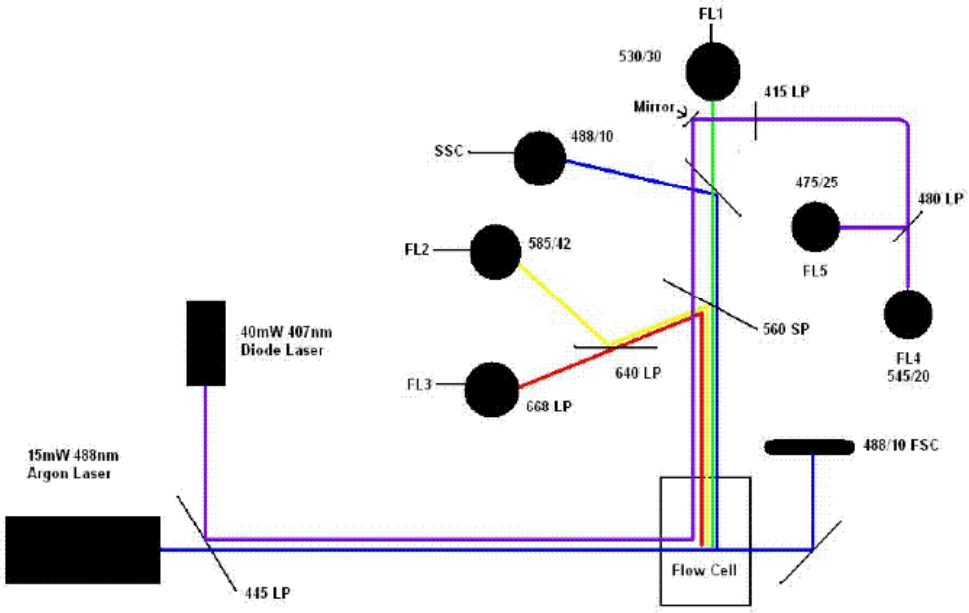

Figure 4 – Optical layout of the BD FACScan cytometer as modified by Cytek. Fluorescence channels FL4 (545nm center +/- 20nm) and FL5 (475nm center +/- 25nm) and are temporally correlated with the 407nm diode laser source. FSC and SSC are correlated with the 488 nm laser. FL4, FSC, and SSC were used to collect the data reported in this study.

The sample is drawn into the cytometer and hydrodynamically focused using sheath fluid to a 20-μm-diameter at the laser probe point. Sample input can occur at two settings (nominally 10 and 60 μl/min).

Optimal data (defined by the narrowest histogram scattering distributions) was observed when operation at the lower flow rate, which was measured to be 23 µl/min.

Expected aerosol conditions can be used in conjunction with the parameters of these instruments (see Table 1) to define useful liquid sample concentrations. We can consider an alarm threshold of 1000 ACPLA. It is assumed that particle breakup may occur, yielding between 1 and 10 hydrosol cells and spores per collected aerosol and between 1 and 30 hydrosol viruses. These values are only estimates and should be characterized more accurately. No breakup of background particles is assumed. The concentration range of protein toxin was generated from DHS test protocols rather than assuming the amount of toxin in an aerosol. The hydrosol concentrations in Table 1 were used as a guide for lab sample preparation and for sample requests made to ECBC during blind sample testing.

3.2 Sample staining protocol

In addition to being fast, the sample staining protocol must be easily applicable to all analyte materials without significantly degrading them or modifying the optical properties of the sample fluid (core fluid) relative to those of the sheath fluid. In the course of developing the staining method several detrimental effects were observed that included cell lysis caused by certain solvents (e.g., acetonitrile), and alterations of the refractive index of the core fluid which caused noisy schlieren scattering at the core-sheath interface. Lysis caused both changes in the cell properties (thus changing its cytometric profile) and the release of interfering stainable and scattering debris.

Table 1. Instrument parameters and aerosol/hydrosol concentrations

Instrumental parameters	**Values**
Aerosol volumetric concentration factor	10^6
Aerosol collector efficiency (%)	70
Cytometer input flow rate (µl/min)	23
Time allotted for measurement (min)	1
Maximum cytometer particle count rate (cts/s)	5000
Aerosol concentrations	
Assumed alarm level (ACPLA)	1000
Low background (particles/l air)	1000
High background (particles/l air)	30,000
Implied hydrosol parameters and concentrations	
Spore, cell breakup range	1-10:1
Virus breakup range	1-30:1
Spore, cell hydrosol concentration at alarm (particles/µl)	700-7000
Virus hydrosol concentration at alarm (particles/µl)	2100-21,000
Dissolved protein toxin concentration (ng/µl)*	.35-35
Background hydrosol concentration (particles/µl)	700-21,000

*Based on a DHS test range of 0.5-50 ng/l airborne ovalbumin concentration

The sample staining protocol that minimized these interferences was adapted from a method previously used in the SNL µChemLab program. It involves the addition of 50 mM pH 8.5 borate buffer (1:10 buffer-to-sample volume), 1% ethanol, 0.1% (by volume) TWEEN surfactant, and 50 mM fluorescamine in dry acetonitrile (added at a 1:40 dye:sample ratio). The buffer adjusts the solution pH to 8.5, where the dye reaction efficiency is maximum. The ethanol prevents spore activation and was found to have the added benefit of slightly expanding the spores to translate their scattering peak from the cytometer background noise. Although not necessary for the lab tests, the TWEEN was added because it is a necessary reagent in the wetted-wall cyclone collector.

Preservation of the integrity of the blind samples during shipment from ECBC to SNL required that they be frozen on dry ice. In order to prevent ice crystal damage to the vegetative cells, it was requested that all samples contain 10% glycerin. This was found, however, to cause an unacceptable level of schlieren noise in the cytometric measurement. Mitigation of this was achieved by requesting the samples to be provided at concentrations twelve times higher than intended and diluting the samples by a factor of twelve prior to measurement. The resulting ~0.8% glycerin produced negligible scattering.

Other handling precautions were required, particularly for *yersinia rhodei*, which was found to be easily lysed into smaller fragments. For example, it was found that cells stored at 0C still lost approximately half their population to lysis after one day. This was not necessarily a problem for qualitative measurements, but did introduce a new population of stainable, fluorescent fragments that are readily observable at low forward scattering values that are well separated from the main peak.

3.3 Cytometric measurement of cells and spores

Each sample was measured for 1-2 minutes using the BD FACScan cytometer. Fluorescence data were acquired through excitation at 407nm, while correlated FSC and SSC were created at 488nm. The measurement of each particle was triggered when an FSC signal stronger than a certain threshold was detected. The threshold was set to correspond to the scattering level exhibited by the surrogate with weakest FSC. Because there was no discrimination on the basis of LIF strength, signals were acquired for particles with little or weak fluorescence, as well as fluorescent ones. This created large sample sets when samples containing non-fluorescent particles were measured, although these sample sets were useful for developing the analysis methods. Other triggering schemes could be explored in the future. All of the cytometer channel amplification factors were set to span a four decade range which enabled detection of a wide range of threat agent simulants.

Following measurement, the sample data were analyzed using the linear unmixing process described in Section 2 and in the following paper of this Proceedings. In the case of spores and cells, the analysis reported a fraction of the total particles in the sample that is determined to be each of the pure constituents in the library (currently BT spores, *yersinia rohdei* vegetative cells, and Greer house dust). This can be multiplied by the total number of hydrosols measured divided by the sample volume measured to determine the calculated number of a particular hydrosol type per unit volume of processed sample (C_h). This, in turn, can be used to estimate the aerosol concentration (C_a) of that type which would have been measured if a specific instrument configuration were used, according to (assuming that the efficiency of measuring collected hydrosols by the cytometer is 100%):

$$C_a = \frac{C_h}{e_{ht} \cdot c \cdot n}$$

where:

C_a	is the aerosol concentration in air at the inlet (particles/liter)
e_{ht}	is the efficiency of the aerosol-to-hydrosol collection
c	is the volumetric aerosol collector concentration factor
n	is the number of subunits the aerosol breaks into in solution

3.4 Cytometric measurement of viruses and protein toxin simulants

As mentioned previously, the individual viruses (MS2) and proteins that were measured are too small to create a detectable signal in the cytometer. Instead, their presence is inferred by measurement of the change in LIF of the solution (in absence of triggerable particles) that is produced by adding stain. It is desirable that this measurement be made with the cytometer to avoid the need for added hardware. This was accomplished by seeding the fluid with non-fluorescent 6-μm-diameter silica microspheres which create enough scatter to trigger the cytometer. Because they are non-fluorescent, the LIF signal measured

coincidently with that trigger represents the signal from the fluid around the microsphere. The virus and protein toxin measurement was conducted by first measuring the unstained sample solution LIF and then staining the solution and repeating the measurement (each time with the solution containing the microspheres).

4. RESULTS

The method was applied to 20 blind samples formulated by ECBC according to the ranges and protocols described in Section 3 of this paper. The bioagent simulants used were BT spores, *yersinia rohdei* vegetative cells, MS2 virus, and ovalbumin protein (toxin surrogate); the fact that these surrogates was used was known in advance. Each sample contained an unknown amount of simulant (or none) mixed with an unspecified amount and type of background material. Samples of the pure simulants were also provided.

The cytometric staining protocol was found to correctly recognize the presence of a threat in all cases, as evident in Table 2, which lists the cytometrically determined analyte identity (Column 2), its inferred aerosol concentration (Column 3), the cytometrically determined presence of soluble protein (Yes or --) (Column 4), the actual analyte that was present in the sample as reported by ECBC (Column 1), and the relative amount (high/low) of background material reported to be in the sample by ECBC (Column 5). Note that a fast response sensor is not required to identify the sample, but only to report that a threat is present.

The data that indicated cell and spore population in the samples are shown in Figs. 5 and 6. Note that each column of graphs corresponds to a particular analyte, with the first column representing the pure samples and subsequent columns corresponding to blind samples labeled with the same numbers as in Table 2. While the determinations were ultimately made by the linear analysis method, it is clear that visual inspection allows direct identification of those materials because the concentrations of background appear to be relatively low.

Table 2 – ECBC blind sample test results

ECBC Antigen	SNL Cell/spore assignment	Inferred particle per liter air	SNL soluble protein	ECBC Background H=High L=Low
Yersinia	Yersinia	6600	Y	H
Yersinia	Yersinia	5700	Y	L
Yersinia	Yersinia	1300	Y	H
Yersinia	Yersinia	6600	Y	L
Bt	Bt	37,000	--	L
Bt	Bt	≤45,000	N	L
Bt	Bt	39,000	--	H
Bt	Bt	14,000	--	H
MS2	--	≤0.9	Y	L
MS2	--	≤12	Y	L
MS2	--	≤0.7	Y	H
MS2	--	≤4	Y	H
OVA	--	≤0.1	Y	H
OVA	--	≤0.08	Y	H
OVA	--	≤0.1	Y	L
OVA	--	≤3	Y	L
None	--	≤0.02	--	L
None	--	≤4	--	H
None	--	≤4	--	H
None	--	≤12	--	L

The aerosol counts inferred from Column 3 in Table 2 indicate that the apparent spore aerosol concentrations ranged from 14,000 to 45,000 per liter of air and that the cell concentrations ranged from 1300 to 6600 per liter of air. These values were calculated using Eq. 1 with the parameters of the University of Texas cyclone collector (e_{ht} = 0.7, c = 10^6) and assuming no aerosol breakup (n = 1). If cluster breakup were to occur, the tabulated numbers would be lower. Note also that these numbers refer to actual particle counts without consideration of viability.

Figure 7 contains cytometric data that were collected on samples created in the laboratory in which BT spores were diluted in various amounts of house dust. These were created to test the ability of the linear analysis to retrieve simulant signals in the presence of higher concentrations of house dust. Figure 8 shows the correlation of the amount of BT indicated by the analysis, plotted against the actual concentration of the samples. The good correlation supports the ability of the cytometric analysis to identify BT at as low as a 1% dilution in house dust.

Figure 8 shows the stained liquid LIF intensity as measured (using the bead method described in Section 3.4) for each of the ECBC samples. The dashed line indicates the agent/no-agent threshold used in evaluating the data. The data show that all ovalbumin and MS2 samples correctly alarmed. They also show that the *yersinia rohdei* samples alarmed, which is consistent with the presence of some lysed cells and the presence of their inner proteins in solution.

5. CONCLUSIONS

The results of this study demonstrate that fluorogenic stains can be used to alter the LIF properties of surrogate biological agents in controlled manner associated with some characteristic of those particles, such as the presence of proteins. Moreover, this response was shown to be different for certain surrogates than for background and serves, therefore, as a basis for improving the discrimination of biological agents from backgrounds. The method was demonstrated to be effectively implemented in a cytometric format in which the LIF and scattering properties of individual hydrosols are measured and can be assembled to produce a multidimensional (three in the demonstrated case) function that describes the sample. As demonstrated in the following paper, linear analysis can be applied to this function to separate out contributions for individual components. In the case of soluble analytes (small viruses and protein toxins), a separate staining method was devised that used the cytometer to measure the staining of portions of the fluid that were free of large particles. It should be investigated whether some viruses may be large enough (>100 nm diameter) to be detected by a cytometer.

In order to meet the needs of a fieldable, rapid-response sensor, certain constraints must be adhered to. One is that the stain must be capable of rapid response. It is desirable that it be fluorogenic (i.e. having negligible LIF in the unreacted state) so that it is not necessary to rinse unreacted starting material. Provided that they meet these requirements, other characteristics that could be probed for include presence of other molecules (e.g., DNA, RNA, carbohydrates), enzymatic activity, and affinity to antibodies.

Additional constraints are associated with the use of a cytometer and a hydrosol collector. While the laboratory system used for this analysis is not fieldworth, low-cost and compact cytometers that could be configured for a deployable format are becoming available. The use of a fluid-based system is clearly less desirable than one addressing only aerosols; however, with this cost comes the benefit of being able to perform aqueous reactions. In using a cytometer it is necessary to manage the fluid streams associated with the core and, most significantly, the sheath fluid. Rather than consume these fluids in a single pass, as is done in most laboratories, it would be desirable to filter and recycle them. This is currently done on deployable cytometer-based sensors, such as are used in oceanography. It is also possible to mitigate fluid consumption by using a very low-cost trigger mechanism to activate the cytometer at only selected instances.

5. ACKNOWLEDGMENTS

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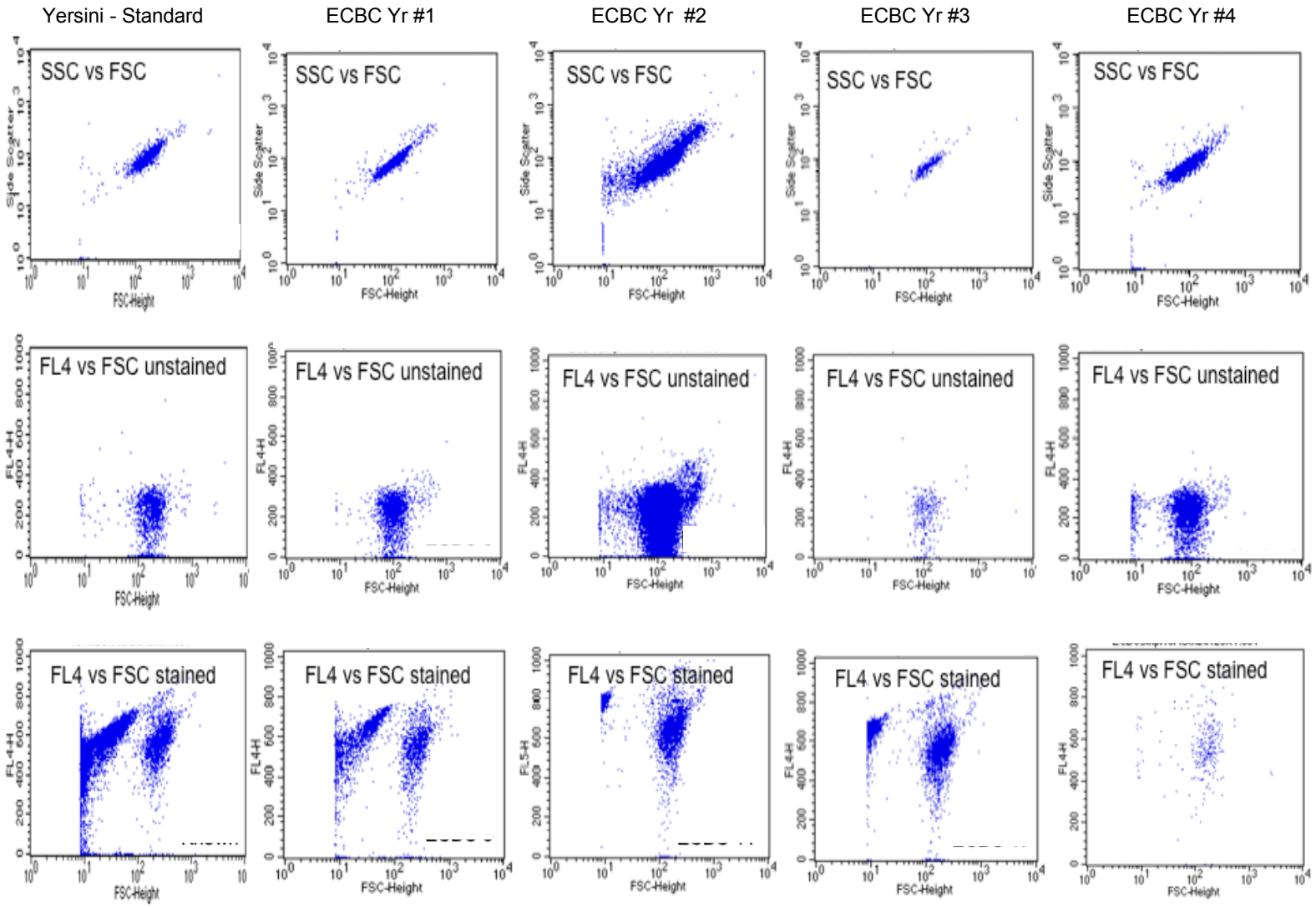

Figure 5 – Plots of the cytometric data of pure *Yersinia rohdei* vegetative cells (leftmost column) and the four blind samples that indicated the presence of that simulant (right four columns). The top row shows forward scatter (FSC) plotted against side scatter (SSC). The second row shows the cytometer LIF channel used (FL4) plotted against FSC for an unstained sample; the bottom row shows the same for a stained sample.

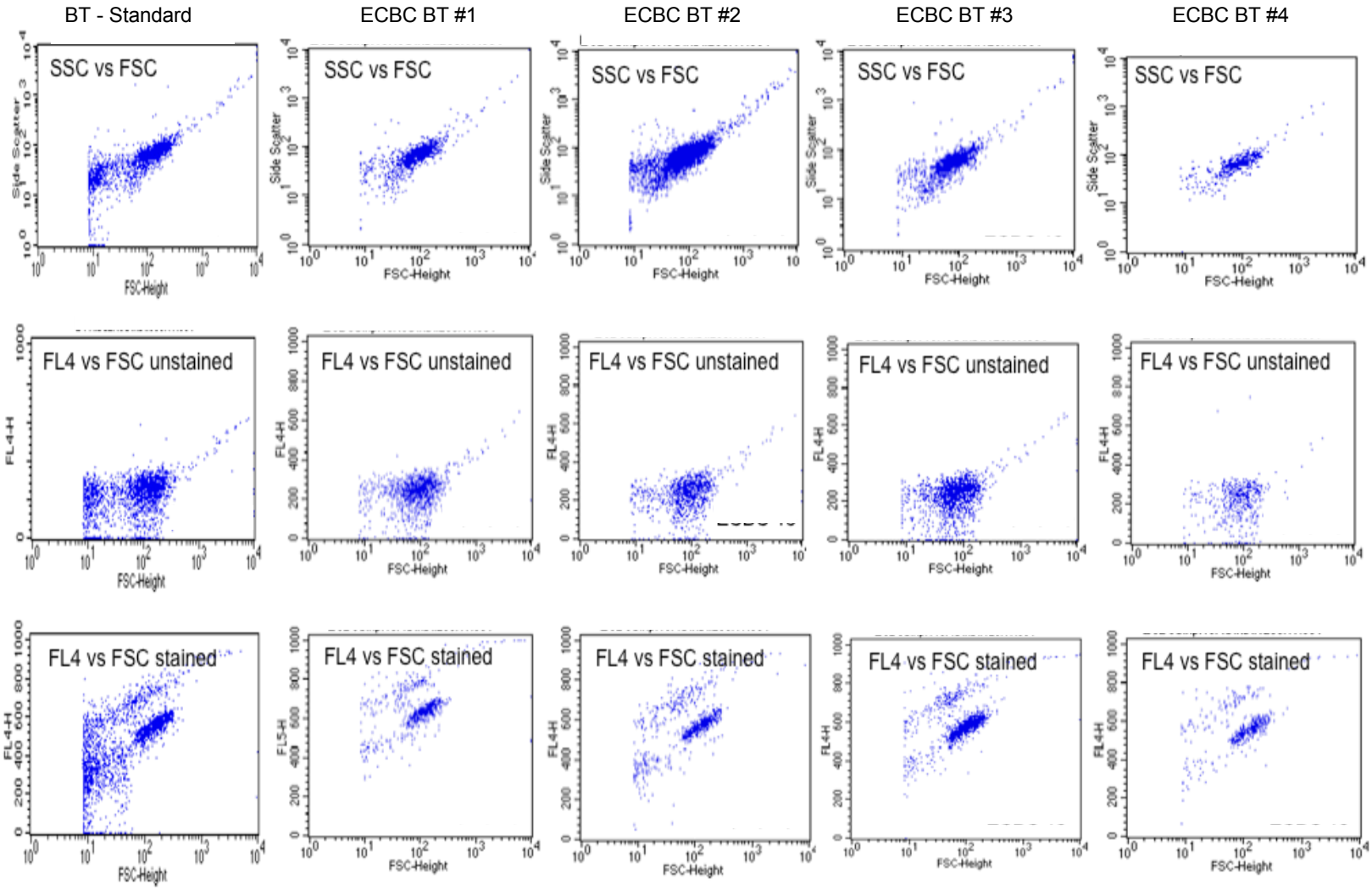

Figure 6 – Plots of the cytometric data of pure *bacillus thurigiensis* spores (leftmost column) and the four blind samples that indicated the presence of that simulant (right four columns). The top row shows forward scatter (FSC) plotted against side scatter (SSC). The second row shows the cytometer LIF channel used (FL4) plotted against FSC for an unstained sample; the bottom row shows the same for a stained sample.

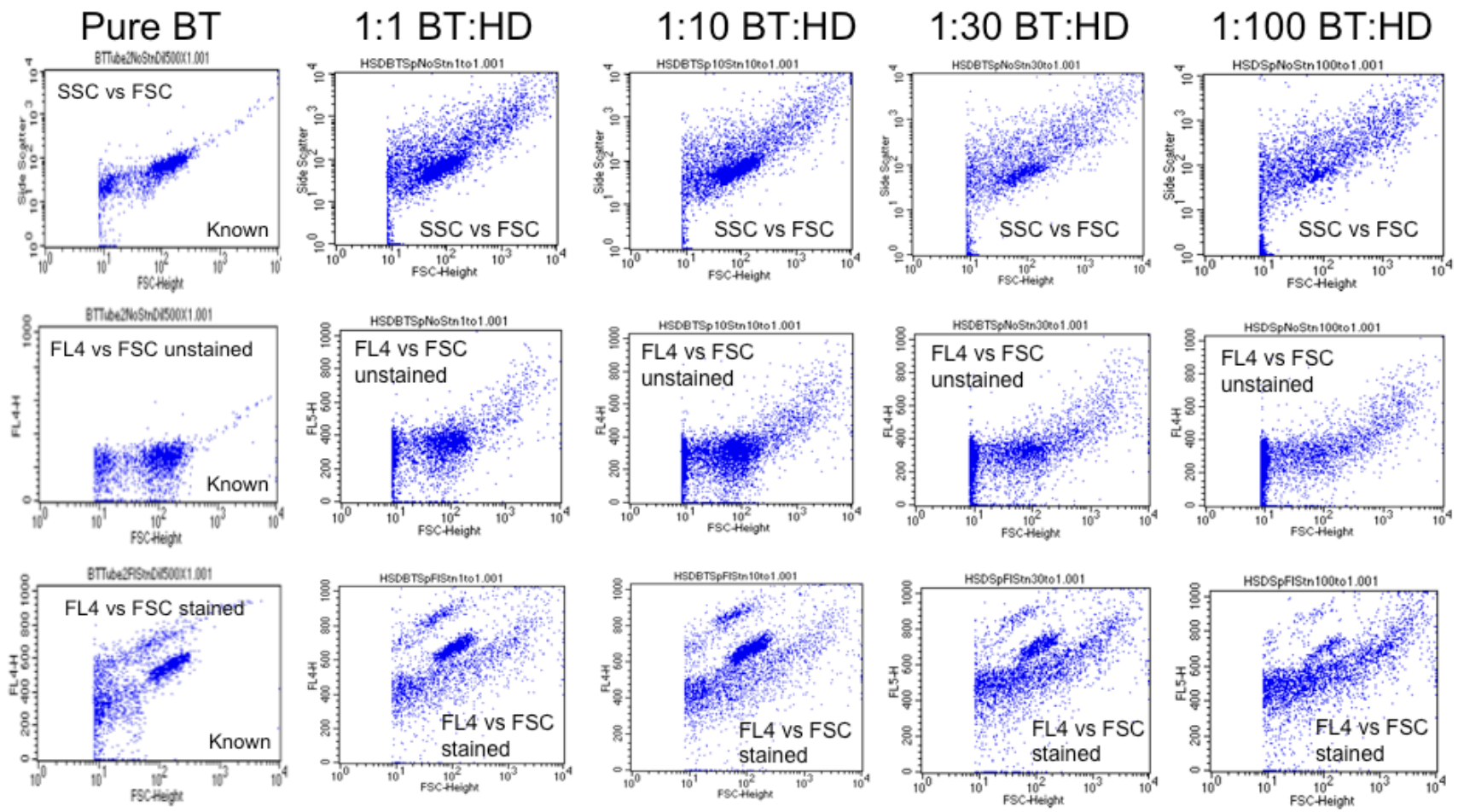

Figure 7 – Plots of the cytometric data obtained when analyzing a series of dilutions of BT spores in house dust. The leftmost column is pure BT. The other columns correspond to the dilutions of BT in house dust indicated at top. The data types plotted in each row are the same as for the previous two papers.

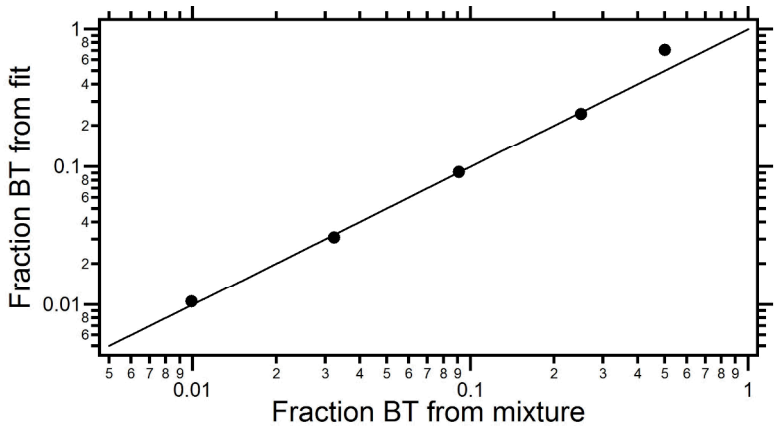

Figure 8 – Plot of the BT fraction in the samples of Fig. 7, plotted against the actual fraction determined by the dilution process.

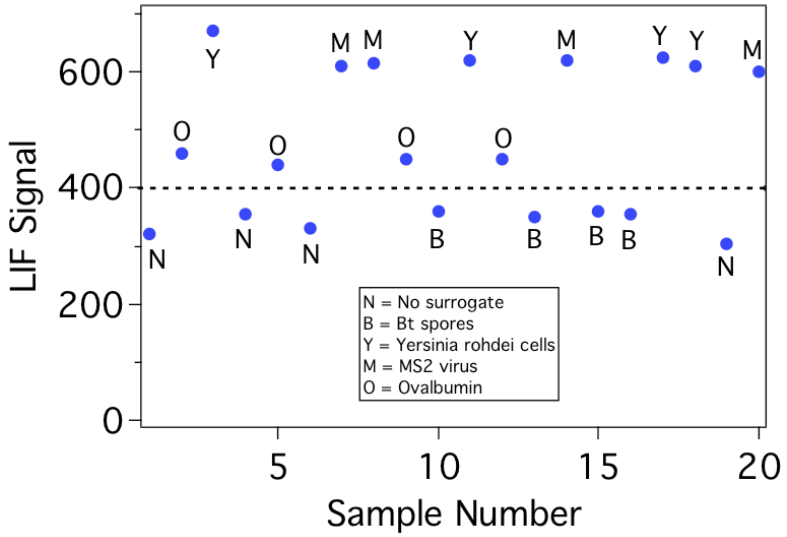

Figure 9 – Plot of the solution LIF signal generated for each ECBC sample using the bead method described in Section 3.4. The dashed line indicates the threshold used in analyzing the data.