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Development and Use of Flow Cytometry for Detection of Airborne Fungi

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Traditional methods for the enumeration of airborne fungi are slow, tedious, and rather imprecise. In this study, the possibility of using flow cytometry (FCM) for the assessment of exposure to the fungus aerosol was evaluated. Epifluorescence microscopy direct counting was adopted as the standard for comparison. Setting up of the method was achieved with pure suspensions of *Aspergillus fumigatus* and *Penicillium brevicompactum* conidia at different concentrations, and then analyses were extended to field samples collected by an impinger device. Detection and quantification of airborne fungi by FCM was obtained combining light scatter and propidium iodide red fluorescence parameters. Since inorganic debris are unstainable with propidium iodide, the biotic component could be recognized, whereas the preanalysis of pure conidia suspensions of some species allowed us to select the area corresponding to the expected fungal population. A close agreement between FCM and epifluorescence microscopy counts was found. Moreover, data processing showed that FCM can be considered more precise and reliable at any of the tested concentrations.

Among the numerous microorganisms present in the air, fungal propagules are the most frequently encountered in both outdoor and indoor environments, where they are sometimes the source of infections, allergic reactions, and toxicoses. Aerobiological surveys of fungi are frequently required for assessment of air quality in such places as hospitals, near sewage works, around or inside industrial plants, in clean room and in areas where people or their livestock and crops are concentrated (10).

The predominant analytical methods for evaluating exposure to mycoaerosol are culture and microscopic techniques. In culture methods, the collected airborne organisms are grown on nutritive media so that visible colonies form, which can be successively enumerated and identified. This method has important limits mainly due to the inability of media to satisfy the specific growth requirements of all species, to the presence of propagule aggregates and to the fungistasis. Moreover, only the viable fungus aerosol can be detected. However, viability is not a necessary requirement for the development of noninfectious diseases, since allergens and toxins are also present in dead cells (13). Microscopy, after the collection of samples with an impinger device, is a currently available method for evaluating the total load of fungus aerosol, including both viable and unculturable or dead propagules. Nevertheless microscopic analytical methods based on the use of hemocytometer chambers are slow, tedious, and time-consuming. When the fungus aerosol is directly examined by microscope on the trapping surface, the assessments are very subjective, and hyaline and/or small propagules such as *Aspergillus* and *Penicillium* conidia are often underestimated or even not recognized. These fungi, however, are the most frequently occurring components of the fungus aerosol (7, 8).

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Flow cytometry (FCM) is a tool with great potential for use in environmental microbiology because of the quantity and quality of the data it provides in a timely fashion, since multiple parameters (e.g., forward light scatter [FSC] and side light scatter [SSC] and fluorescence emission at wavelengths of interest) can be determined individually for a large number of cells in a short time (up to several thousand cells per second). Besides, FCM has the advantage of operating with large samples, enabling collection of statistically reliable results (5).

In recent years, FCM has been widely applied for the enumeration and identification, of both prokaryotic and eukaryotic microorganisms from aquatic environments (see, for example, references 5, 6, and 14).

On the other hand, the use of FCM in aerobiological studies is still unusual, and it has been tested to evaluate the exposure to bacteria only. In this case, comparison with traditional methods highlighted the tremendous potential of this technique, especially when combined with fluorescence in situ hybridization for the detection and quantitative identification of health-threatening organisms (9).

To our knowledge, FCM has never been applied to enumerate naturally occurring airborne fungal spores and conidia. One of the major limitations to the application of this technique to the fungi was the lack of "stainability" of most fungal propagules since they proved to be quite unstainable or fluorescent in a small percentage only (1, 4, 11, 12). Nevertheless, staining with fluorescent dyes is required to distinguish between fungal propagules and abiotic background. Actually, spores and conidia have a very thick and resistant cell wall that protects the protoplast from physical, chemical, and biological injuries in the troposphere, but at the same time, prevents them from staining. To overcome this handicap, we have recently set up a method to avoid the propagule resistance to fluorochromes applying microwave irradiation (V. Prigione and V. Filipello Marchisio, Abstr. 7th Int. Mycol. Cong., abstr. 872, 2002).

The aim of the present study was to develop a reliable method of analysis based on FCM for the rapid enumeration of total airborne fungal load in selected environments that used both light scatter and fluorescence parameters. The sensitivity and reliability of flow cytometric quantitative analyses were demonstrated at first with suspensions of *Aspergillus fumigatus* and *Penicillium brevicompactum* conidia at different concentrations (simulated samples). Then, the analyses were extended to field samples collected by an impinger device. Epifluorescence direct counting was adopted as the standard for comparison.

MATERIALS AND METHODS

Organisms and culture conditions. *A. fumigatus* Fresenius var. *fumigatus* (MUT 1564), *P. brevicompactum* Dierkx (MUT 2430), and *Cladosporium cladosporioides* (Fres.) de Vries (MUT 3155), isolated outdoors and kindly supplied by *Mycotheca Universitatis Taurinensis* (Turin, Italy), were grown on malt extract agar in the darkness at 24°C for 4 weeks in order to obtain completely mature conidia.

Simulated samples. Conidia from fruiting cultures were harvested by gently touching the colony surface by means of a sterile wet Pasteur pipette, and then they were suspended in quarter strength Ringer Solution (Oxoid, Ltd., Basingstoke, England) supplemented with 0.01% Antifoam A and 0.02% Tween 80 (Sigma Aldrich Co., Ltd.) to facilitate the suspension of the hydrophobic conidia. Suspensions were then repeatedly syringed to break conidial chains and adjusted to 10^6 conidia ml^{-1} by using a Bürker chamber. For comparative purpose between flow cytometry and epifluorescence microscopy counting, serial dilutions of suspensions of *A. fumigatus* and *P. brevicompactum* conidia were carried out to work on concentrations of 1×10^6, 5×10^5, 1×10^5, 1×10^4, or 1×10^3 conidia ml^{-1}.

For FCM analyses of field samples, a preanalysis of pure and mixed suspensions of *P. brevicompactum* and *C. cladosporioides* conidia (10^6 conidia ml^{-1}) was performed as a reference to discriminate the relevant fungal population from the background. These two fungal species were chosen as test organisms since they are dominant in the air (7, 8), and the shape and size of their conidia are representative of the morphological and dimensional variability of the most common airborne fungi.

Collection of field samples. Air sampling was performed with an impinger device ("Charlie" model; TCR, Tecora, Italy). The liquid collection medium consisted of 12 ml of sterile quarter-strength Ringer solution (Oxoid) modified as described above. The sampling sites were the composting tunnel of a compost plant (site A, total sampling time 60 min), a woodland in a town area (site B, total sampling time 360 min), and a polluted dwelling house (site C, total sampling time 60 min). At sites A and C one sample was collected; at site B four samples were collected (B1 to B4). Preliminary tests with a surface air sampler (Pool Bioanalyse, Milan, Italy) had shown, at sites A and C, a very high fungal load compared to the outdoor mean values and site B values. Therefore, different volumes of air were sampled to avoid meeting with loads too low to be read by the Bürker chamber. During the sampling, an operator inspected the instrument to avoid the evaporative loss of the collection medium; if this occurred, additional medium was gently added. The sampling flow rate was always 10 liters min^{-1}.

Staining procedures. To optimize propagule staining, samples were microwave treated according to the method proposed by Prigione and Filipello Marchisio (Abstr. 7th Int. Mycol. Cong.). Microwave irradiation was performed by using a domestic Panasonic NN-K257-W microwave oven at the power of 440 W and a frequency of 2,450 MHz, for 30 s. Each 1-ml suspension in Pyrex tube was placed in the center of the oven and irradiated separately to have constant and reproducible conditions. Samples were stained for 2 h at room temperature with propidium iodide (Sigma) at a final concentration of 100 $\mu g\ ml^{-1}$. This fluorochrome is an intercalator that binds with a good specificity to nucleic acids, allowing the user to distinguish between abiotic and biotic particles; it was found very suitable for the staining of fungal propagules after microwave treatment (V. Prigione and V. Filipello Marchisio, Abstr. 7th Int. Mycol. Cong.).

Epifluorescence microscopy. Epifluorescence direct counting was performed by using a Bürker chamber and a Nikon Eclipse E-600 epifluorescence microscope equipped with a 100-W mercury lamp and a 510- to 560-nm filter for the detection of red fluorescence. For each sample, five counts were achieved, each one corresponding to a volume of 0.192 μl, reading three of the nine major squares in the grid. Final data were the mean of the five replicates. Working with simulated samples two sets of experiments were carried out.

FCM. Flow cytometric analyses were performed by using a Partec PAS flow cytometer (Partec GmbH, Münster, Germany) equipped with a 20-mW argon-ion laser and calibrated by using 4.2-μm diameter beads (Polyscience, Warrington, Pa.). The instrument has a built-in volumetric system based on the positioning of two electrodes and is set by the factory to sample a volume of exactly 200 μl. The sensitivity and reliability of the instrument were first tested in simulated samples by collecting data on a logarithmic intensity scale with respect to FSC, the parameter related to the particle size (3), SSC, the parameter related to the cytoplasmic granularity of the particles (3) and red fluorescence (>620 nm) separately and combining the parameters two by two.

The latter method has been used in field samples, where FCM was evaluated for its ability to distinguish relevant fungal population from other biotic and background events of instrument noise and environmental debris; a multiparametric approach is essential in this context (3). The scatter and fluorescence characteristics of pure and mixed laboratory suspensions of *P. brevicompactum* and *C. cladosporioides* were used to set the instrument by selecting the area corresponding to the fungal population. The area where the fungal propagules were localized was noted on a bidimensional scattergram. A polygon drawn to gate the particles falling in this area was used to analyze the field samples.

For each sample, five counts were achieved, each one corresponding to a volume of 200 μl. Final data were the mean of the five replicates. Conidial suspension values were then converted into load values per cubic meter of air. Two sets of experiments were carried out with simulated samples.

Data processing. Acquisition and analysis of FCM data were performed by using the FloMax program, associated with the instrument.

A correlation was calculated between the counting results obtained by FCM and microscopy for each concentration of simulated samples.

The data obtained from microscopy and FCM have been plotted looking for a possible mathematical relation between the percent coefficient of variation (%CV; a value related to the standard deviation and therefore to the variation of the sample) and the log of the concentration of the conidia in suspension. Four kinds of regression (linear, exponential, logistic, and parabolic) were calculated by using the Systat software package.

The nonparametric Mann-Whitney test was run with the Statview II statistical package to assess the significance ($P \leq 0.05$) of differences between results by the two counting methods for field samples.

RESULTS

Simulated samples. During microscopic observation a small portion of coupled conidia was observed even though the conidial suspension had been syringed many times. The FSC frequency histogram allowed us to discriminate between the conidial population going through the flow chamber one by one, represented by the higher peak, and the population of coupled conidia, represented by the lower peak (Fig. 1A and D). Indeed, coupled conidia are able to give a higher FSC signal. The SSC frequency histogram did not allow such a discrimination (Fig. 1C and F). FCM analysis by red fluorescence was able to show single and coupled conidia populations in the case of *A. fumigatus* only (Fig. 1B and E). Therefore, counting of total conidia for each species was possible by calculating the integral of the two FSC histogram peaks of the particles with red fluorescence; that is, the number of conidia was obtained by the addition of the integral of the single conidium peak to twice the integral of the coupled conidium peak.

Tables 1 and 2 show the counting results for each species and set of experiments as determined by both FCM and epifluorescence microscopy. The degree of correlation between the FCM and microscopy counts was quite high: *A. fumigatus* gave R^2 values of 0.97 and 0.99 in the first and second tests, respectively, whereas the *P. brevicompactum* values were 0.99 and 0.95 (one of the plots is shown in Fig. 2).

However, the %CV values of FCM counts were, on average, lower than those of microscopy counts. The best-fitting models

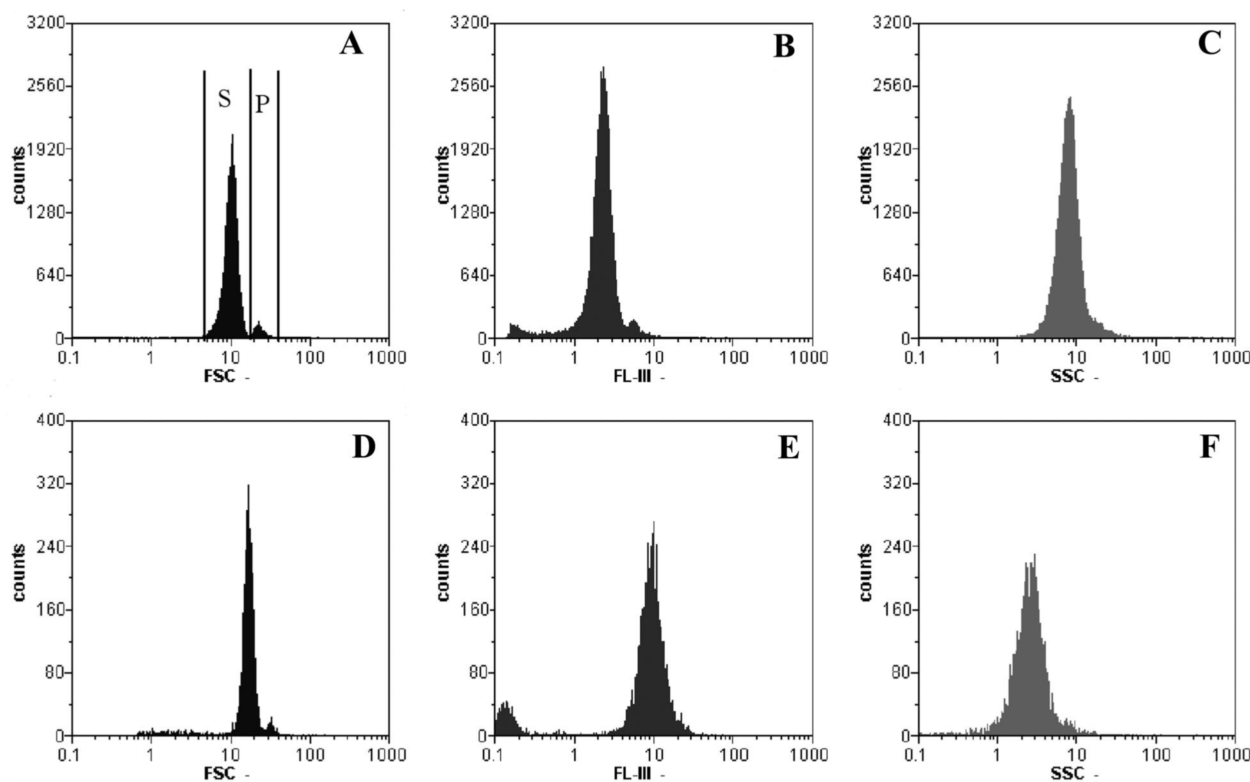

FIG. 1. Histograms obtained by flow cytometric analysis of simulated samples after microwave irradiation and propidium iodide staining. (A to C) *A. fumigatus*; (D to F) *P. brevicompactum*. Counts, number of conidia; FL-III, red fluorescence; S, conidia passing singly through the flow cell; P, conidia passing in pairs through the flow cell.

for microscopy data were represented by the linear regression (one of the plots is shown in Fig. 3). Consistently high and repeatable R^2 values ($0.85 < R^2 < 0.95$) were obtained in all of the tests. The curves showed a negative angular coefficient, indicating a negative correlation between the concentration of the conidia in the suspension and the error in the measure. None of the tested regressions adequately described the distribution of the dot plot for the FCM data (R^2 was always lower than 0.36), suggesting that the distribution of the datum points is random and not dependent on the concentration of the conidia.

Field samples. Histograms and dot plots obtained by the combination of SSC and FSC displayed an abundant background that did not allow us to discriminate the fungal population. The staining with propidium iodide could gate the abiotic background, leaving it out from further data acquisition.

TABLE 1. Quantitative analysis of simulated samples of *A. fumigatus* conidia by FCM and epifluorescence microscopy[a]

Dilution	Expt set	FCM		Microscopy	
		Mean no. of conidia ml^{-1}	%CV	Mean no. of conidia ml^{-1}	%CV
1×10^3	I	5.04×10^3	60.91	1.04×10^3	224.04
	II	3.75×10^3	44.27	1.04×10^3	224.04
1×10^4	I	1.17×10^4	20.51	1.14×10^4	74.56
	II	1.52×10^4	9.87	0.93×10^4	91.40
1×10^5	I	1.00×10^5	9.00	1.39×10^5	20.14
	II	1.20×10^5	20.00	1.72×10^5	20.35
5×10^5	I	3.24×10^5	34.26	5.79×10^5	5.70
	II	5.32×10^5	24.44	8.35×10^5	10.30
1×10^6	I	0.82×10^6	20.99	1.13×10^6	8.67
	II	1.18×10^6	25.42	1.55×10^6	18.71

[a] Mean values are the means of five replicates.

TABLE 2. Quantitative analysis of simulated samples of *P. brevicompactum* conidia by FCM and epifluorescence microscopy[a]

Dilution	Expt set	FCM		Microscopy	
		Mean no. of conidia ml^{-1}	%CV	Mean no. of conidia ml^{-1}	%CV
1×10^3	I	1.33×10^3	15.67	3.13×10^3	91.26
	II	0.55×10^3	12.68	3.12×10^3	149.05
1×10^4	I	1.08×10^4	9.54	1.56×10^4	33.33
	II	0.75×10^4	28.33	0.62×10^4	108.66
1×10^5	I	0.86×10^5	10.26	1.04×10^5	18.02
	II	0.66×10^5	14.47	0.73×10^5	28.09
5×10^5	I	3.78×10^5	25.02	4.68×10^5	9.94
	II	3.61×10^5	30.70	2.98×10^5	13.73
1×10^6	I	0.63×10^6	10.88	1.00×10^6	6.33
	II	0.49×10^6	13.53	0.66×10^6	9.64

[a] See Table 1, footnote *a*.

Preanalysis of pure and mixed laboratory suspensions of *P. brevicompactum* and *C. cladosporioides* enabled us to gate the area corresponding to the expected fungal population. Figure 4 shows the area gated for counting in the B1 field sample.

Table 3 shows the counting results by FCM and epifluorescence microscopy for each field sample. It was not possible to distinguish between single and coupled conidia owing to the greater heterogeneity of airborne particles in comparison with simulated samples; on the other hand, a negligible share of coupled conidia had been observed by microscopy. The Mann-Whitney test showed that the results by FCM were always not significantly different from those by epifluorescence microscopy. As for simulated samples, the %CV values of FCM counts were, on average, lower than those of microscopy counts.

DISCUSSION

Detection and quantification by FCM of the dominant and stable component of the airborne fungi, as described by Filipello Marchisio and Airaudi (7), are feasible, by combining light scatter and propidium iodide red fluorescence and pretreating the suspensions by microwave irradiation to achieve the cell wall permeabilization of fungal propagules (Prigione and Filipello Marchisio, Abstr. 7th Int. Mycol. Cong.).

Working with simulated samples at different concentrations of conidia, a close agreement between the FCM and epifluorescence microscopy results was always found. Moreover, data processing shows that FCM can be considered more precise and reliable at any of the tested concentrations. In addition to

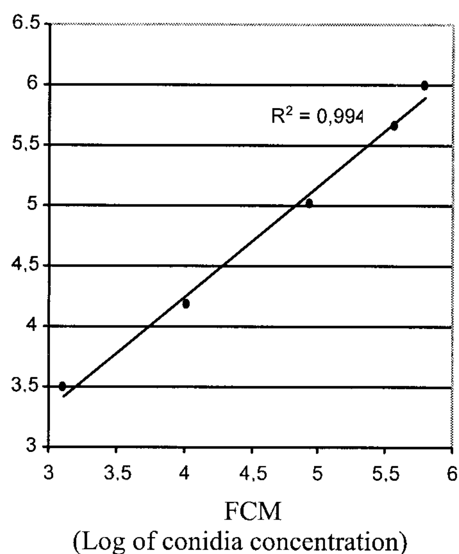

FIG. 2. Correlation between FCM and microscopy counting results (log of the conidia concentration) for one of the experiments with *P. brevicompactum*. A linear least-squares best-fit line was calculated and is shown with correlation coefficient R^2.

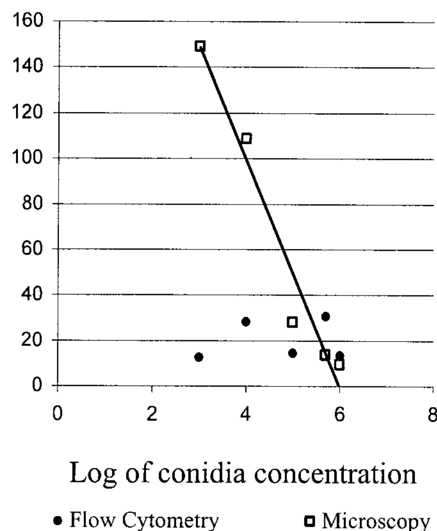

FIG. 3. Dot plot of the log of the conidium concentration in the analyzed suspension versus the %CV for one of the experiments with *P. brevicompactum*. A line with equation $y = -49.931x + 298.49$ and $R^2 = 0.9597$ fitted the microscopy data, whereas no curve fitted the dot distribution of flow cytometry data. Plots and lines with similar results were obtained for all of the other experiments as well (not shown).

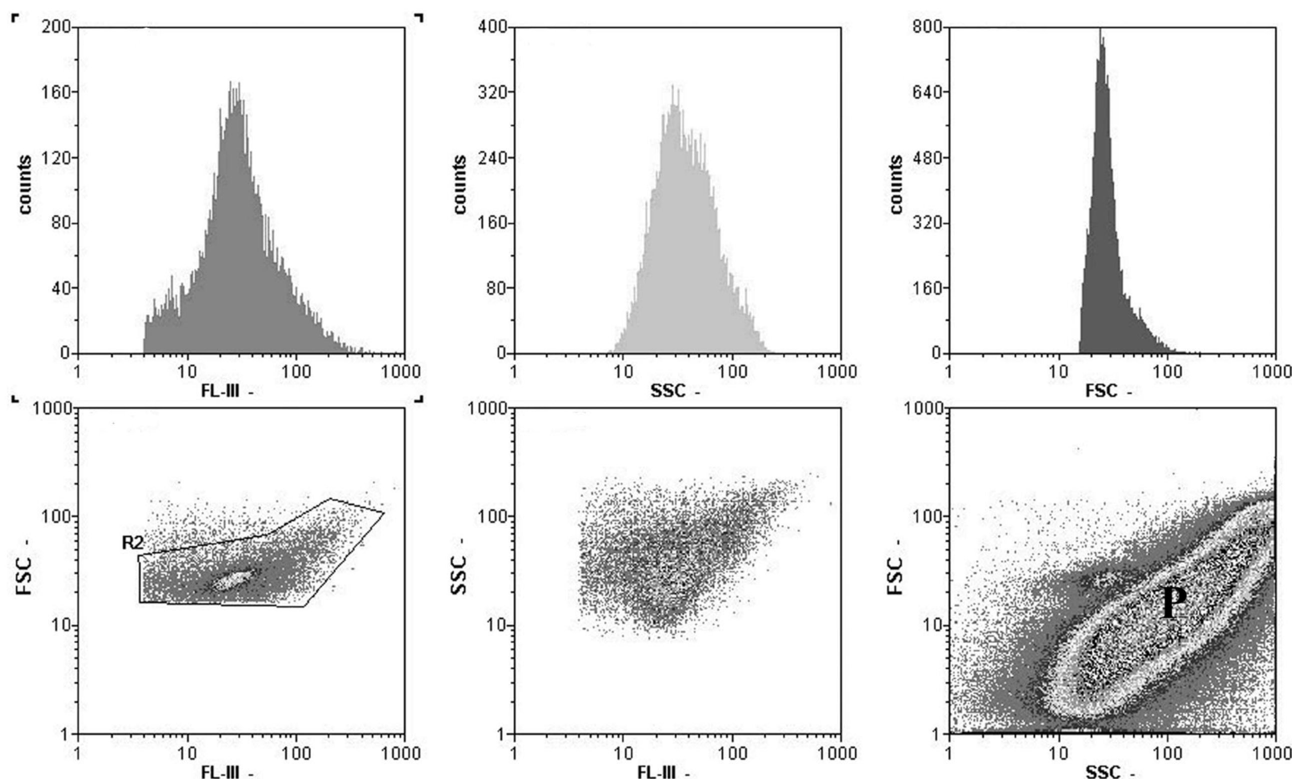

FIG. 4. Histograms and dot plots obtained by flow cytometric analysis of B1 field sample after microwave irradiation and propidium iodide staining. Counts, number of particles; FL-III, red fluorescence; R2, area gated for counting; P, background.

the fact that the range of the %CV was always lower in the FCM counts than in the microscopy counts, the search for the best-fitting curves showed that the two methods provide results with completely different reliabilities. A systematic error was introduced when microscopy was used for the count of conidia: the lower their concentration in the suspension, the higher the %CV (i.e., the error in the count). On the other hand, no curve could be consistently and repeatedly fitted to the dot distribution of the FCM data, showing that results were not dependent on the concentration of the conidia nor subject to a systematic instrumental error. This fact is important for the development of reliable routine methods for counting total airborne fungal load, but of special significance in cases in which rare particles, such as the propagules of a potentially pathogenic species, must be detected in a sample. Since FCM is performed on a sample size ca. 1,000 times greater (200 μl versus 0.192 μl) and its results are not as much dependent on particle concentration as those of microscopy, this technique could greatly increase the chance of detecting rare conidia. Highly species-specific detection of rare conidia will require further development and improvement of the method through the combination of FCM with immunochemical and/or molecular methods. This approach, besides increasing the analysis specificity, could also reduce the instrumental limit of quantification that, in the present study, was not investigated below a concentration of 10^3 propagules ml^{-1}. Actually, the aim of the present study was to verify the capability of surveying the total load of airborne fungi in selected environments which, in the specific instance, had loads of about 10^4 to 10^5 propagules m^{-3} of air. According to some researchers, the limit of quantification in aquatic environments is 10^2 cells ml^{-1} (9, 14).

The application of FCM to samples collected in some nat-

TABLE 3. Quantitative analysis of fungal propagules in field samples by FCM and microscopy[a]

Sample	FCM			Microscopy		
	Mean no. of fungal propagules ml^{-1}	Mean no. of fungal propagules m^{-3}	%CV	Mean no. of fungal propagules ml^{-1}	Mean no. of fungal propagules m^{-3}	%CV
A	2.65×10^4	5.30×10^5	30.57	3.85×10^4	7.70×10^5	18.96
B1	4.49×10^4	1.50×10^5	30.08	5.60×10^4	1.87×10^5	30.71
B2	1.14×10^4	3.80×10^4	39.47	1.04×10^4	3.46×10^4	61.53
B3	1.56×10^4	5.20×10^4	24.36	1.35×10^4	4.50×10^4	43.70
B4	1.41×10^4	4.70×10^4	9.93	1.67×10^4	5.57×10^4	71.26
C	0.98×10^4	1.96×10^5	7.29	1.04×10^4	2.08×10^5	93.54

[a] See Table 1, footnote *a*.

ural environments by using an impinger device, the most suitable instrument for this kind of analysis, gave good results as well. FCM analysis of field samples involves two main difficulties: the possibility that the background events hide the fungal propagules in the sample and the need for selecting the coordinates of the region in which fungal population is included. Since propidium iodide is a nucleic acid-specific dye, the biotic component could be recognized, whereas the preanalysis (concerning propidium fluorescence, FSC, and SSC) of *P. brevicompactum* and *C. cladosporioides* suspended conidia allowed us to select the area corresponding to the expected fungal population. Propidium iodide is not specific for fungi; therefore, labeled particles might not be only fungal propagules. However, strong support of the correct identification of the particles came, in the first place, from the close agreement between microscopy and FCM counting results. Moreover, the particles considered for the count were selected by relying on three parameters (propidium iodide staining, FSC, and SSC) and not only on the basis of red fluorescence. The use of multiple parameters to detect and select the particles of interest represents one of the technical key features of FCM, allowing researchers to differentiate fungal propagules from different cells and biotic debris in field samples. In environments different from the ones we have considered, and especially in agricultural environments, pollens could pose a problem due to their abundance in some periods of the year. The size range of some of the smallest pollens, in fact, overlaps that of some fungal propagules. Nevertheless, most are much larger than the most common airborne fungal propagules (2). In the present study pollens did not cause problems since they were never observed during microscopic counts and analyses of our field samples. Day et al. (4), working with simulated samples and combining light scatter and intrinsic fluorescence parameters, could differentiate sporangia of the late-blight pathogen *Phytophthora infestans* from conidia of *Alternaria* and *Botrytis* spp., rust urediniospores, and the pollen of grasses and plantain. Differentiation between *P. infestans* sporangia and powdery mildew conidia could also be achieved in the same study after the samples were stained with Calcofluor White M2R and analytical rules evolved by genetic programming methods were applied. These data suggest that FCM could also be used to detect and quantify airborne fungi in environments different from the ones we chose to examine, including agricultural environments.

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