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Detection and quantification of viable, airborne bacteria and fungi using solid-phase cytometry

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SUMMARY

This protocol describes the use of solid-phase cytometry for the enumeration of airborne bacteria and fungi. In contrast with conventional methods, accurate results can be obtained in real time especially for air samples with low numbers of microorganisms. Air samples are collected by impaction on a water-soluble polymer that is subsequently dissolved. Part of the sample can be filtered over two membrane filters with different pore sizes. One filter is used to obtain a total count of all viable microorganisms, and a second filter is used to determine the number of airborne fungi. Microorganisms present on the filter are labelled with a viability substrate and subsequently detected and quantified using a solid-phase cytometer. The detected spots are microscopically validated using an epifluorescence microscope to discriminate between bacteria, fungi and fluorescent particles. The whole procedure takes 5 hours to complete and results in the accurate quantification of airborne bacteria and fungi for samples with a low or high microbial load.

INTRODUCTION

In solid-phase cytometry (SPC), the principles of epifluorescence microscopy and flow cytometry are combined[1]. Microorganisms are retained on a membrane filter, fluorescently labelled and automatically counted by the Chemscan RDI laser-scanning device. Subsequently, the data for each fluorescent spot are analysed by a computer to differentiate between fluorescent microorganisms and particles. Each retained spot can visually be inspected using an epifluorescence microscope[1,2]. Due to its high dynamic range and speed, SPC seems to solve the shortcomings observed with other methods for quantification of airborne microorganisms. Theoretically, this method would be perfect to enumerate microorganisms in air samples with a very low microbial load.

Overview of SPC

A schematic presentation of the different steps of a SPC protocol is shown in **Figure 1**. First, samples are filtered over a black polyester or polycarbonate membrane filter with an appropriate pore size. These screen filters are used because of their low background fluorescence and the high contrast which facilitates validation using the epifluorescence microscope. Second, the retained cells are fluorescently stained using one or more physiological or taxonomic probes[3]. Cleavage of carboxyfluorescein diacetate (ChemChrome V6) by microbial esterases results in the formation of fluorescent carboxyfluorescein in intact and metabolically active cells only and fluorescently labelled antibodies or oligonucleotide probes target specific microorganisms independent of their physiological state[3].

Next, the fluorescence emitted by the labelled cells is detected using a solid-phase cytometer, which consists of an argon laser, emitting light of 488 nm for

fluorophore excitation and two photomultiplier tubes for signal detection. The produced signals are processed by a computer to differentiate valid signals (labelled microorganisms) from fluorescent particles. To this end, data for several software parameters such as the size of the fluorescent spot, the specific intensity, the color ratio and the signal pattern are used **(Fig. 2)**. Results are displayed as green spots on a membrane filter image in a primary and, after software elimination of background spots, displayed as a secondary scan map[2].

Last, to further analyse the properties of the retained spots (positioned with x and y coordinates), particles on the membrane are visually inspected using an epifluorescence microscope equipped with a computer-driven moving stage. Highlighting of a green spot in the secondary scan map directs the microscope to the respective position on the membrane filter, allowing rapid and accurate validation.

Applications of SPC

SPC has most frequently been used for the detection of highly diluted microorganisms in water:

- Determination of the total viable count (TVC) using the viability stain ChemChrome V6 [1, 4, 5, 6].
- Total viable fungal count by combining viability labelling and lectin labelling [7].
- Specific enumeration of *Escherichia coli* by using a fluorogenic substrate for the target-specific enzyme ß-glucuronidase [8].
- Specific detection of *E. coli* O157:H7 [9], *Cryptosporidium parvum* and *Giardia lamblia* [10, 11], *Legionella pneumophila* [12], *Naegleria fowleri* [13], and the toxic

alga *Prymnesium parvum* [14] by combining SPC with immunofluorescence labelling.

- Specific detection of *Enterobacteriaceae* sp.[15], *E. coli* [16] and *P. parvum* [17] fluorescently labelled by fluorescence *in situ* hybridization.
- Detection of *Campylobacter jejuni* using viability staining [18].

SPC has also been used for detection of microorganisms in air samples:

- Enumeration of bacteria and fungi [19].
- Specific detection of *Aspergillus fumigatus* in air samples (L.M.E.V., H.J.N. and T.C., submitted).

And SPC has been used for detection of fungi in clinical samples:

- Specific detection of *A. fumigatus* in bronchoalveolar lavage liquid and sputum by combining viability staining and immunofluorescence labelling [20, 21].
- Specific detection of *Cryptococcus neoformans* in serum and cerebrospinal fluid by combining viability staining and immunofluorescence labeling [22].

Advantages and limitations of SPC

One of the important advantages of SPC is its speed and ability to enumerate rare events. As this method does not rely on culturing the microorganisms, quantification results for both culturable and nonculturable, viable microorganisms can be obtained within a few hours. Additionally, the filter membrane is scanned by the laser in only three minutes [1, 2, 3].

SPC has a theoretical detection limit of one cell per filtered volume [1, 2], but SPC can also be used to determine the microbial load of highly contaminated samples

as it has a high dynamic range with an upper limit of approximately 10,000 cells per membrane filter [6].

The applicability of SPC is often restricted by the ability to filter the sample. Previously, intensive procedures had been necessary to obtain a modest improvement in the ability to filter bronchoalveolar lavage and sputum samples [21]. In the present protocol, a filterable air sample is created by impacting a defined volume of air on a water-soluble polymer that is subsequently dissolved [19].

In some samples, the occurrence of fluorescent particles may lead to an aborted scan or to a cumbersome validation when numerous spots are present in the secondary window. By implementing a counterstaining step and/or using a filter with a larger pore size, this problem can sometimes be overcome [23].

Alternatives to SPC for the quantification of airborne microorganisms

Conventional enumeration of airborne microorganisms relies on culture-based or microscopic methods. Culture-based analysis often results in an underestimation of the number of microorganisms owing to the quantification of culturable microorganisms only and differences in growth requirements between microorganisms. Additionally, analysis usually takes at least three days to complete, and fast-growing microorganisms may overgrow slow-growing ones [24].

In contrast, microscopic methods allow the detection of both culturable and nonculturable airborne microorganisms, and results can be obtained within hours of sample collection. However, microscopic enumeration is laborious, requiring a high level of expertise [24] and less sensitive than SPC [1, 25].

Recently flow cytometry (FC) [25, 26], PCR [27] and different biochemical assays targeting, for example, ß,1-3-D-glucan [28], ergosterol [29] and ATP [30], have been

suggested as alternative strategies for the quantification of airborne bacteria and fungi. FC proved to be more precise and reliable than epifluorescence microscopy but it suffered from a relatively high detection limit (10^3 cells/ml). In addition, high background fluorescence was observed for several samples. In contrast, SPC has a theoretical detection limit of one cell per filter. Additionally, the implementation of a counterstaining procedure and visual validation by epifluorescence microscopy allows to easily make the distinction between particles and microorganisms using SPC. Although PCR is a widely used procedure to quantify both viable and non-viable microorganisms, additional reamplification and hybridization steps were necessary to obtain a detection limit of 10 cells when applied to air samples leading to a 9 h procedure. Compared to this method, SPC is much faster and only quantifies the viable cells. Finally, a number of biochemical assays have been developed. However, the applicability of some of these assays is limited (e.g. ß,1-3-D-glucan and ergosterol can be used only for fungi) and it is often difficult or impossible to correlate the results obtained with cell numbers.

EXPERIMENTAL DESIGN

This protocol describes the use of solid-phase cytometry to enumerate viable, airborne microorganisms. Therefore, air is impacted on a water-soluble polymer film present in a standard Petri dish (PVA plate). After dissolution, a measured volume of the obtained suspension can be filtered and viable cells are labelled using a viability stain. Subsequently, the filter is laser scanned and a computer discriminates fluorescent particles from microorganisms using several software parameters. Finally, the retained spots are microscopically validated. A flow diagram of the procedure is presented in **Figure 3**.

Preparation of PVA plates

1 Prepare a 10% (wt/vol) solution of PVA in ultrapure water.

CRITICAL STEP Make sure that the PVA is completely dissolved. Place the solution on a magnetic stirring plate for at least 15 min.

2 Filter the obtained solution through a 0.22 µm pore size filter/storage bottle system.

3 Pour 15 ml of the sterilized solution into a 90 mm Petri dish.

4 Leave the Petri dish opened in a vertical laminar flow cabinet for 11 hours.

CRITICAL STEP Make sure to respect the timing as plates which are overdried result in the difficult capture of microorganisms during air sampling due to bouncing on the hard surface. Underdried PVA films, on the other hand, are difficult to remove from the Petri dish. It is not necessary to dry for 11 consecutive hours.

PAUSE POINT PVA plates (sealed with parafilm) can be stored for 1 month at 4 °C.

TROUBLESHOOTING

Solid phase cytometer

A solid phase cytometer consists of an argon laser and two photomultiplier tubes. Up till now the only solid phase cytometer commercially available is manufactured by AES-Chemunex and is called the ChemScan *RDI*. After the filter is scanned, the fluorescent spots detected by the photomultiplier tubes are displayed on a primary scan map. Afterwards, the computer analyses the data to discriminate between fluorescent particles and microorganisms and displays the retained spots in a

secondary scan map. The size of the fluorescent spot is reflected in the values for lines (the number of laser lines where the spot is found) and samples (the number of laser spots on the same line where the spot is found). A second important characteristic of a fluorescent spot is the fluorescence intensity which is higher in relation to its size for a microorganisms than for a particle. A third discriminant is the area (color) ratio which refers to the fluorescence intensity found for green and red signals. A microorganism usually has a low red fluorescence intensity. Finally, the pattern of the signal resembles a Gaussian curve for a microorganism while a more irregular pattern is observed for a particle **(Fig. 2)**.

Depending of the application used to perform the computer discrimination, different minimum and maximum values are set for the software discriminants. Two applications, incorporated in the AES-Chemunex software, are used in this protocol. The discriminant settings for both applications are shown in **Table 1**.

Control procedures

From each batch of PVA plates, three unexposed plates were tested for sterility using the total viable count procedure. Only these batches for which sterility was confirmed were used in further experiments.

Before initiation, a control procedure is required to confirm system functionality, laser beam focus, membrane support stage position and detection sensitivity. To this end, filter 100 µl of Standard C3 latex fluorescent beads as five discrete spots through a 0.4 µm Cycloblack-coated polyester membrane filter and initiate a scan using the 'control membrane' application. Check whether the mean peak intensity is within the acceptance range and whether beads are detected in all five

spots. Additionally, set the offsets for the moving stage by scanning the reference membrane and manually positioning the center in the microscope objective.

MATERIALS

REAGENTS

- Poly(vinyl alcohol) (PVA) 80% hydrolyzed (Sigma, cat. no. 360627)
- Ultrapure water (see REAGENT SETUP)
- Ethanol 70% (vol/vol)
- 0.9% (wt/vol) NaCl (see REAGENT SETUP)
- Standard C3 control beads (AES-Chemunex, cat. no. 200-R5070-01)

  CRITICAL Protect this solution from light.
- Counterstaining reagent CSE/2 (AES-Chemunex, cat. no. 200-R4091-01) (see REAGENT SETUP)

  CRITICAL Protect this solution from light as counterstaining properties may be lost upon exposure to light.
- ChemSol A4 (AES-Chemunex, cat. no. 200-R2050-01) (see REAGENT SETUP)
- ChemSol A6 (AES-Chemunex, cat. no. 200-R2053-01) (see REAGENT SETUP)
- ChemSol B2 (AES-Chemunex, cat. no. 200-R2022-02) (see REAGENT SETUP)
- ChemSol B16 (AES-Chemunex, cat. no. 200-R2023-02) (see REAGENT SETUP)
- ChemChrome V6 (AES-Chemunex, cat. no. 200-R1007-03) (see REAGENT SETUP)

  CRITICAL Protect this solution from light.

EQUIPMENT

- MAS-100 Eco impaction air sampler (Merck) (see EQUIPMENT SETUP)
- Petri dish, 90 mm diameter
- Petri dish, 55 mm diameter
- Magnetic stirring plate
- Laminar flow cabinet, biohazard type II (= downward laminar flow with exhaust air passing through a HEPA filter. A class II safety cabinet therefore provides protection for the technician, environment and experiment.)
- 0.22 µm pore size filter/storage bottle system (Corning Inc., cat. no. 430767)
- Vortex mixer
- Tweezers
- 0.4 µm Cycloblack-coated polyester membrane filter, 25 mm diameter (AES-Chemunex, cat. no. 200-C2010-01)
- 2.0 µm Cycloblack-coated polyester membrane filter, 25 mm diameter (AES-Chemunex, cat. no. 200-C2011-01)
- Filtration unit with 3 ports with sintered glass filter supports and vacuum release valves
- Vacuum pump capable of sustaining 400 millibars
- Sterile syringes with needles
- 0.2 µm pore size cellulose acetate syringe filter (Whatman Schleicher and Schuell, cat. no. 10 462 200)
- Labelling pad (AES-Chemunex, cat. no. 200-C3012-02)
- Incubator at 30 °C
- Incubator at 37 °C
- Support pad (AES-Chemunex, cat. no. 200-C2107-01)
- Refrigerator 4 °C

- Filter holder (AES-Chemunex, supplied with the ChemScan RDI)
- ChemScan RDI (AES-Chemunex) (see EQUIPMENT SETUP)
- PC equipped with the ChemScan user interface (AES-Chemunex) (see EQUIPMENT SETUP)
- Epifluorescence microscope (e.g. Olympus BX40) equipped with a compatible computer-driven moving stage (e.g. Olympus) (see EQUIPMENT SETUP)

REAGENT SETUP

0.9% NaCl Sterilize by filtration through a 0.22 µm filter/storage bottle system. This solution can be stored for one month at 4 °C.

Labeling reagents Filter the counterstaining reagent, ChemSol A4, A6, B2 and B16 and ultrapure water through a 0.22 µm pore size cellulose acetate syringe filter. These solutions should be prepared fresh each day and stored at 4 °C between experiments.

EQUIPMENT SETUP

MAS-100 Eco This impaction air sampler has a constant airflow of 100 l min^{-1}. Samples of 10–1,000 l of air can be collected (collection times ranging between 6 s and 10 min). In the MAS-100 *Eco*, air is drawn through a perforated lid and particles present in the air are impacted onto a solid material.

ChemScan RDI This solid phase cytometer is equipped with a laser for excitation at 488 nm and two photomultiplier tubes with wavelength windows set for the green (500–530 nm) and amber (540–585 nm) regions of the fluorescein emission spectrum.

Fluorescence microscope The Olympus BX40 is equipped with a moving stage directed by a computer via the Chemscan user interface. Components of the microscope include a ×40/0.75 and ×10/0.25 objective, a 10/22 eyepiece, a 100 W mercury lamp and a filter block containing a filter cube consisting of a 500 nm dichroic mirror, a 450–490 nm bandpass excitation and a 515 nm cut-off emission filter (Olympus, type UMWD).

PROCEDURE

Air sampling

TIMING 1 - 10 min

1 Wipe the perforated lid and dust cover of the air sampler with 70% ethanol (vol/vol) before and between sampling cycles.

CRITICAL STEP Make sure that the holes in the perforated lid are not clogged by controlling if all ethanol is evaporated before continuing to the next step.

2 Collect air samples (10–1,000 l) in triplicate on PVA plates using the MAS-100 Eco.

CRITICAL STEP In order to minimize the effect of desiccation of the microorganisms during air sampling, the sampling volume should be kept to a minimum after initial determination of the bioaerosol concentration.

TROUBLESHOOTING

PAUSE POINT The PVA plates are sealed with parafilm and transported to the lab. A period of 4 hours between sample collection and sample preparation does not lead to a reduction in the number of microorganisms. The effect of a longer period between sampling and dissolution of the PVA plate has not been investigated.

Preparation of the samples

TIMING 16 min

(all steps need to be performed under sterile conditions in the laminar flow cabinet)

3 Remove the polymer from the Petri dish using sterile tweezers.

CRITICAL STEP Do not flame the tweezers but use ethanol (70% vol/vol) to sterilize them.

4 Transfer the film to a sterile sample pot and add 20 ml of physiological saline to ensure maximum recovery of the microorganisms.

CRITICAL STEP Close but do not shake the recipient and allow the polymer film to dissolve for 15 min.

PAUSE POINT Polymer suspensions (sealed with parafilm) can be stored for 5 h at 4 °C.

Filtration and labelling

(all steps need to be performed under sterile conditions in the laminar flow cabinet)

5 Determine the TVC (A) or the fungal count (B) as follows:

(A) Total viable count

TIMING 3 h 30 min

(i) Successively wash the filter support 3 times with 70% ethanol and 3 times with filtered (0.22 µm) ultrapure water.

(ii) Place a 0.4 µm Cycloblack-coated polyester membrane filter in the center of the filter support using sterile tweezers.

(iii) Switch on the vacuum pump and filter a measured volume of the suspension over the membrane.

CRITICAL STEP Homogenize the suspension by inverting it. Do not use the vortex mixer as the solution will foam excessively.

CRITICAL STEP Depending on the expected bioaerosol concentration, different volumes of the PVA suspension need to be filtered. Preliminary tests may indicate whether filtration of larger volumes is necessary, or in contrast, dilution of the suspension before filtration is necessary.

CRITICAL STEP Distribute the sample over the entire surface of the filter in order to obtain a maximal spread of the microorganisms and hence make validation easier.

CRITICAL STEP As soon as the sample has been filtered, switch the vacuum pump off.

(iv) Counterstain interfering particles by filtering 1 ml of CSE/2.

CRITICAL STEP Protect this solution from light.

(v) Place a labelling pad with 600 µl of ChemSol A4 pipetted onto it in a 55 mm Petri dish. Transfer the filter to the labelling pad and incubate at 37 °C for 3 h.

CRITICAL STEP Before incubation, put a labelling pad soaked with 600 µl physiological saline in the lid of the Petri dish to create a humid atmosphere.

(vi) After vortexing, dilute ChemChrome V6 1:100 in ChemSol B16.

CRITICAL STEP This solution can be stored for 5 h at 4 °C, but needs to be protected from light.

(vii) Incubate the filter for 30 min at 30 °C on a labelling pad with 600 µl ChemChrome V6 solution pipetted onto it.

PAUSE POINT After incubation, filters can be stored at 4 °C on the ChemChrome V6 solution for 12 h.

(B) Fungal count

TIMING 4 h

(i) Successively wash the filter support 3 times with 70% ethanol and 3 times with filtered ultrapure water.

(ii) Place a 2.0 µm Cycloblack-coated polyester membrane filter in the center of the filter support using sterile tweezers.

(iii) Switch on the vacuum pump and filter a measured volume of the suspension over the membrane.

CRITICAL STEP Homogenize the suspension by inverting it. Do not use the vortex mixer as the solution will foam excessively.

CRITICAL STEP Depending on the expected bioaerosol concentration, different volumes of the PVA suspension need to be filtered. Preliminary tests may indicate whether filtration of larger volumes is necessary, or in contrast, dilution of the suspension before filtration is necessary.

CRITICAL STEP Distribute the sample over the entire surface of the filter in order to obtain a maximal spread of the microorganisms and hence make validation easier.

CRITICAL STEP As soon as the sample has been filtered, switch the vacuum pump off.

(iv) Place a labelling pad with 600 µl of ChemSol A6 pipetted onto it in a 55 mm Petri dish. Transfer the filter to the labelling pad and incubate at 37 °C for 3 h.

CRITICAL STEP Before incubation, put a labelling pad soaked with 600 µl physiological saline in the lid of the Petri dish in order to create a humid atmosphere.

(v) After vortexing, dilute ChemChrome V6 1:100 in ChemSol B2.

CRITICAL STEP This solution can be stored for 5 h at 4 °C but needs to be protected from light.

(vi) Incubate the filter for 1 h at 37 °C on a labelling pad with 600 µl ChemChrome V6 solution pipetted onto it.

PAUSE POINT After incubation, filters can be stored at 4 °C on the ChemChrome V6 solution for 12 h.

Laser scanning

TIMING 6 min

6 Place 100 µl of ChemSol B16 or ChemSol B2 onto the membrane holder to determine the TVC or the fungal count, respectively.

7 Place a support pad on the membrane holder and wait until the pad has absorbed the ChemSol B16 or ChemSol B2 completely.

8 Transfer the labelled membrane from the labelling pad to the support pad.

CRITICAL STEP Make sure that no bubbles are trapped under the membrane.

9 Initiate a scan using the 'tvc' or 'fungi' application, respectively (see **Table 1**).

10 Depending of the number of fluorescent spots, next proceed to microscopic validation (A) or reanalysis (B). If the scan is aborted because too many fluorescent spots were detected by the laser or if the validation would be too cumbersome because of the high number of fluorescent spots in the secondary window, continue with a reanalysis of the samples. Otherwise complete the analysis with the microscopic validation of each fluorescent spot in the secondary window. This window displays the fluorescent spots which are retained after software discrimination by the computer (see Experimental design).

TROUBLESHOOTING

(A) Microscopic validation

TIMING 6 min

(i) Remove the filter holder from the ChemScan RDI and place it on the moving stage of the epifluorescence microscope in exactly the same orientation.

(ii) Validate all spots in the secondary window based on fluorescence intensity, characteristic shape and line amplitudes. Discriminate between bacteria, fungi and fluorescent particles (see **Fig. 4**).

TROUBLESHOOTING

(B) Reanalysis

TIMING 4 h 12 min

(i) Repeat the protocol starting from step 5 and filter the volume necessary to obtain a lower number of fluorescent spots in the secondary window.

CRITICAL STEP As polymer solutions can only be stored for 5 h, reanalysis is only possible if no delays were encountered during the protocol.

Data analysis

TIMING 1 min

11 Calculate the number of microorganisms per square meter based on the results from the TVC count using the following formula:

$$\text{Number of microorganisms/m}^3 = \frac{\text{(number of validated microorganisms} \times 20{,}000)}{\text{(filtered volume in ml)} \times \text{(sampled air volume in l)}}$$

CRITICAL STEP Do not use the positive hole conversion table supplied with the air sampler as this is only valid for impaction on culture media.

12 Calculate the number of fungi per square meter based on the results from the fungal count using the following formula:

$$\text{Number of fungi per m}^3 = \frac{\text{(number of validated fungi} \times 20{,}000)}{\text{(filtered volume in ml)} \times \text{(sampled air volume in l)}}$$

13 Calculate the number of bacteria per square meter based on the previous results using the following formula:

$$\text{Number of bacteria per m}^3 = \text{number of microorganisms per m}^3 - \text{number of fungi per m}^3$$

TIMING

A summary of the approximate time necessary to complete the various stages of the SPC procedure is presented in **Figure 3**.

TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

ANTICIPATED RESULTS

After labelling with ChemChrome V6, intensely labelled, green fluorescent microorganisms can be observed using the epifluorescence microscope. Typical microscopy images seen during validation are shown in **Figure 4**. Validation is easy to perform as bacteria and fungi can clearly be discriminated in fluorescence images based on their characteristic shape. Additionally, typically less than ten particles are retained in the secondary window after counterstaining with CSE/2 or filtering through a 2.0 µm membrane filter. Fungal counts obtained with the TVC protocol and the fungi protocol are comparable. Consequently, the use of the fungi protocol can be avoided and counts for both bacteria and fungi can be obtained using one protocol, making analysis even less complicated and expensive.

In **Figure 5** the average log number of bacterial and fungal cells is shown for air samples collected in triplicate at ten various locations. Initially, we collected 100-l air samples at these locations and filtered 9 ml of the polymer solution to obtain a TVC count and fungal count. Depending on the number of airborne bacteria and fungi, we reanalyzed (different filtered volume) and/or resampled (different sampled air volume), leading to an accurate, quantitative result for all samples (**Fig. 5**). Bacterial counts ranged from 185 to 930,000 cells/m^3 whereas fungal counts were usually lower, ranging from 30 to 12,000 for the locations shown in **Figure 5**. The low detection limit of one cell per filter and hence per filtered volume makes the SPC method particularly suited for accurate analysis of air samples containing low numbers of microorganisms. Additionally, the possibility to dilute the polymer suspension also enables the analysis of high microbial load samples. Comparison of the standard errors of the mean obtained for triplicate analysis of samples obtained at 50 locations with the SPC method and a traditional culture-based method revealed

485 that although the SPC method requires more manipulation the variation of the results
486 is similar (Kolmogorrov-Smirnov test).

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COMPETING INTERESTS STATEMENTS

The authors declare no competing financial interests.

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615 **Table 1** Summary of the software discriminants and their values used in the two scan

616 applications of the ChemScan RDI

Software discriminants	**Scan application**			
	TVC		**Fungi**	
	Minimum	**Maximum**	**Minimum**	**Maximum**
Lines	1	50	1	60
Samples	1	250	1	250
Area (color) ratio	0	1.1	0	1.2
Peak intensity value	250	NA	250	NA
Half width	NA	15	NA	15
Specific intensity (AS)	10	NA	3	N/A
Specific intensity (HW)	25	NA	20	NA
2D gaussian	NA	850	NA	1,800
Multi peaks	NA	1	NA	2
Multi wiggles	NA	3	NA	5

617 **Table 2** Troubleshooting table.

Step	Problem	Possible cause	Solution
EXP. DESIGN	The polymer film does not solidify	Incomplete dissolution of PVA before filtration	Slowly add the ultrapure water to the PVA powder. Make sure no gelatinous masses are formed. Make sure to leave the PVA solution on the magnetic stirring plate for 15 min.
2	'AIRBLOCK' appears on the display of the MAS-100 Eco	Impeded airflow because the perforated lid is blocked	Make sure no ethanol is left in the holes of the perforated lid. If a lot of particles are suspected in the sampled air, clean the perforated lid with compressed air between sampling cycles.
10	Scan aborted because too many fluorescent spots are detected by the laser	Too many microorganisms present in the sample	Reanalyze the sample by diluting it or filtering a smaller volume. Collect a smaller volume of air onto the PVA plates.
		Too many particles present in the sample	Make sure all reagents used in the protocol are filtered through a 0.22 µm

		filter.
		Make sure the tweezers used to manipulate the membrane filter are not flamed.
		Make sure a counterstaining step is included in the TVC labelling protocol.
		Check if the right application was used and if the discriminant settings were not altered.
Very low number of fluorescent spots in the secondary window	Air sample with low bioaerosol concentration	Sample a larger volume of air at this location.
	Vacuum pump switched on too long	Switch off the vacuum pump immediately after filtering.
No microorganims visible during validation	Wrong offsets	Check with the reference membrane if the offsets are set correctly.
	Bleaching	Make sure the microorganisms are not extensively illuminated.

Figure 1 Schematic overview of solid phase cytometry. Steps include: membrane filtration, fluorescent labelling, scanning, data analysis by a computer and microscopic validation. After scanning of the filter all fluorescent spots are displayed in a primary scan map image. Data obtained during a scan include size, fluorescence intensity, color ratio and signal pattern for each fluorescent spot. Based on these values, the computer differentiates between fluorescent particles and microorganisms and displays the latter in a secondary scan map. Finally, the filter is placed under an epifluorescence microscope in exactly the same orientation and each spot in the secondary window can be inspected.

Figure 2 Overview of the software parameters used by the computer to discriminate among microorganisms. The four main discriminants are size, specific intensity, color ratio and signal pattern of the detected fluorescent spot.

Figure 3 Flow diagram of the procedure. Time required for each step is indicated on the right side. Pause points are indicated by red arrows and specific information about length and conditions of storage is shown.

Figure 4 Anticipated results. (**a**–**d**) Microscope images of *Pseudomonas aeruginosa* (**a**), *Candida albicans* (**b**), *Aspergillus fumigatus* (**c**) and an autofluorescent particle (**d**) viewed during validation. Scale bars represent 7 µm.

Figure 5 Anticipated results. The average log number of bacterial and fungal cells per m^3 (n = 3) for air samples collected at ten various locations. For each location, the sampled air volume and filtered volume used to obtain the quantitative results are indicated in the table below the figure. Error bars represent standard error of the mean.