Fast detection of air contaminants using immunobiological methods

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ABSTRACT

The fast and direct identification of possibly pathogenic microorganisms in air is gaining increasing interest due to their threat for public health, e.g. in clinical environments or in clean rooms of food or pharmaceutical industries. We present a new detection method allowing the direct recognition of relevant germs or bacteria via fluorescence-labeled antibodies within less than one hour. In detail, an air-sampling unit passes particles in the relevant size range to a substrate which contains antibodies with fluorescence labels for the detection of a specific microorganism. After the removal of the excess antibodies the optical detection unit comprising reflected-light and epifluorescence microscopy can identify the microorganisms by fast image processing on a single-particle level. First measurements with the system to identify various test particles as well as interfering influences have been performed, in particular with respect to autofluorescence of dust particles. Specific antibodies for the detection of *Aspergillus fumigatus* spores have been established. The biological test system consists of protein A-coated polymer particles which are detected by a fluorescence-labeled IgG. Furthermore the influence of interfering particles such as dust or debris is discussed.

Keywords: indoor air quality, microorganism, immunobiological recognition, fluorescence, polymer substrate, electrophoretic separation

1. INTRODUCTION

Methods of fast detection of airborne micro-organisms are of interest for contamination control in various environments such as hospitals, clean rooms in food and pharmaceutical industry, or agriculture. Particularly since the discussion of the risk of bio-threats there has been an increasing activity in the development of suitable detection systems.

There are two fundamentally different approaches to detect micro-organisms: techniques based on the detection of single objects such as microscopic techniques, flow cytometry, and single particle mass spectrometry, and systems based on the detection of chemical agents after lysis of micro-organisms accumulated at a sufficient number in a suitable medium such as PCR, ATP bioluminescence, immune filtration and photometry. Depending on the final read-out method a different degree of specificity is achieved. High specificity is generally attributed to PCR since it detects DNA. Optical and mass spectroscopic methods are less specific; however these are generally techniques with fast response.

In this communication we present a new approach combining the specific coupling of fluorescently labeled antibodies to surface antigens with single particle detection by fluorescence microscopy. The measuring principle integrates sampling,

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sample preparation and detection and involves the following steps: a pre-selected size interval of the airborne particle spectrum is deposited on a gel layer containing the antibodies that are specific to a surface antigen of the micro-organism

or the class of micro-organisms under consideration. After rehydration of the gel, labeling is achieved by diffusion of the mobilized antibodies onto the micro-organism. Free antibodies will subsequently be removed by electrophoresis. The labeled objects are being detected by automatic fluorescence microscopy. The measurement principle is shown schematically in Fig. 1.
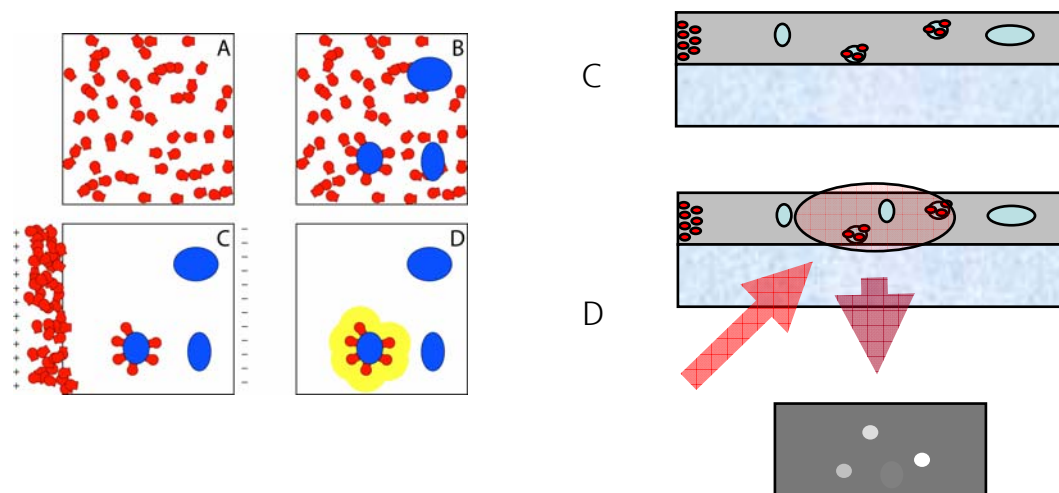

Fig. 1. Measurement principle (A) Gel layer containing fluorescently labeled antibodies (B) Gel after the impaction of micro-organisms were the mobile antibodies specifically bind to their target organisms (C) Electrophoretic removal of the free antibodies (D) Fluorescence detection of the recognized target organisms. The graph on the right illustrates a side view of the substrate with particles in the gel layer.

2. METHODOLOGY

2.1 Air sampling

Sampling, particle size selection and localized particle deposition for microscopy is facilitated by a combination of virtual impaction and direct impaction. The size selection criteria may be an upper boundary, d_u, of 10 µm as these particles are able to penetrate into the lung, and a lower boundary of d_l in the range of 0.8-1 µm as typical minimum diameters of bacteria and spores. Air is sucked in at a flow rate of 50 l/min through an annular slit inlet and is then passed through a first impaction stage removing all particles larger than the upper limit, d_u, of the intended size interval. The volumetric flow rate is split up in a multi nozzle round jet virtual impactor into a major flow of (for example 48 l/min) and a complementary minor flow incorporating all particles larger than d_l. The particles from this small minor flow are deposited on the gel using a conventional slit impactor. The impaction nozzle with dimensions 120 µm x 2 cm is manufactured from a brass plate by an erosion technique. The quasi one-dimensional localized deposition spot allows for very small lateral dimensions of the gel layer i.e. a small amount of antibodies. Furthermore the migration path of free antibodies is short when applying the electric field perpendicular to the narrow deposition strip. In addition the horizontal optical scanning for particle detection can be limited to one dimension.

2.2 Polymer substrate and gel preparation

The gel coatings on the substrate are required to meet a number of demands. It has to hold the incoming micro-organisms. It is the reservoir for all the agents needed for the detection. In particular, it contains the antibodies which are rather sensitive proteins. They tend to denature during storage which results in a loss of their function. The gel must keep at least a part of them functional. For that purpose the gel contains buffer salts, ectoin, glycerine, and another blocking protein. The latter ones are also meant to block surfaces during the analysis.

Besides the function as a carrier for the agents, the gel is required to serve as an environment for the actual analysis, i.e. the recognition of the micro-organisms by the antibodies. After the deposition of the particles from the air on top of the freeze-dried gel coating, the gel is liquefied by a water aerosol. At this time, the blocking proteins and the antibodies are supposed to diffuse to all the particle surfaces. After the binding, the rest of the fluorescently labeled antibodies in the gel and those antibodies which are loosely bound by unspecific interactions will be removed by electrophoresis. For carrying out the electrophoresis, an electrical field is applied to the inter-electrode gap. The field results in an electrical current over the gel which, in turn, heats the gel and produces hydrogen and oxygen at the electrodes. Both these effects stress the substrate. The heating results in an increased evaporation of the water. The increasing viscosity of the gel slows down the migration of the proteins in the electrical field. Since the size of the gel between the electrodes was set by the design of the apparatus to 2 mm, the resistance of the gel must be as high as possible in order to keep the power dissipation low. On the other hand the electrode processes during the electrolysis tend to change the pH value which is prevented by a buffer. The capacity of the buffer must be sufficiently high to keep the pH in a range which does not damage the antibodies for all the time from preparation till the end of the tagging process. A high buffer capacity requires a high salt concentration which, in turn, gives rise to a high conductivity. If we want to keep the conductivity low, the buffer must be prepared from components with small molar conductivities, i.e. with large molecules.

2.3 Antibody fabrication

Monoclonal antibodies were generated according to the hybridoma technique of Milstein & Köhler. As depicted in Fig. 2, mice (BALB/c) were injected with 5×10^5 spores from *Aspergillus fumigatus* i.p. five times with 10 day intervals. Prior to the injection, spores had been inactivated by heat treatment. At day 20, 30, 40, and 50 antibody titers were determined by ELISA or flow cytometry. The spore specific immune response was tested for IgG and IgM class antibodies.

On the day of fusion, the spleen is dissected and spleen cells are fused with cells of the murine myeloma cell line SP2-IL6 (ATCC: CRL-2016) at a ratio of 1:1 in the presence of PEG 1500 (Boehringer, Mannheim, Germany). Fused cells were cultured and selected in RPMI 1640 (Invitrogen, Karlsruhe, Germany) supplemented with 20% bovine calf serum (Biochrom AG, Berlin, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Karlsruhe, Germany), 100 µM hypoxanthine, 16 µM thymidine, and 0,4 µM aminopterin (Sigma-Aldrich, Munich, Germany), 80 U/ml interleukin-6 (FIRMA) and 50 µM ß-mercaptoethanol (FIRMA)on 96-well microtitre plates. Medium was replaced with fresh selection medium after one week. After 12 days culture supernatants were screened for binding antibodies on spores in a direct ELISA. Hybridoma in those wells that resulted in highest ELISA signals were further proceeded to limiting-dilution cloning. Culture medium was exchanged to HT Medium (RPMI 1640, supplemented with 20% bovine calf serum 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µM hypoxanthine, 16 µM thymidine, 80 U/ml interleukin-6 U/ml and 50 µM ß-mercaptoethanol, suppliers as above). Monoclonal hybridoma were cultured in standard growth medium (RPMI 1640 supplemented with 10% bovine calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, suppliers as above). Prior to purification hybridoma were adapted to serum free hybridoma culture medium ISF-1 (Biochrom AG, Berlin, Germany). IgM antibodies were purified from culture supernatants by mannan affinity chromatography kit (Pierce).
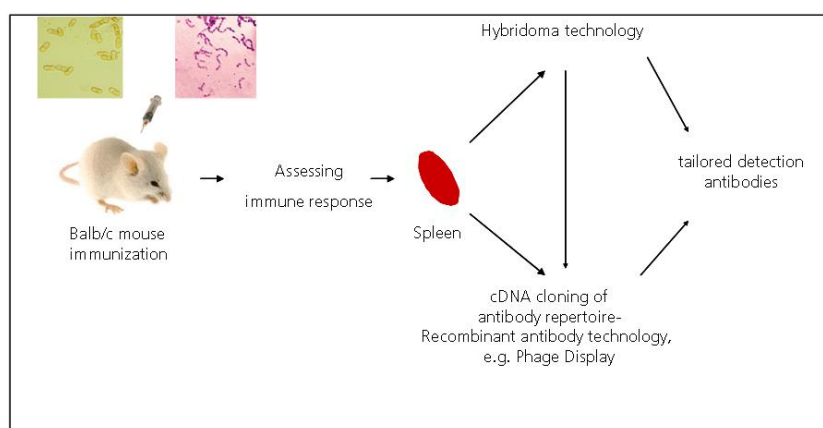

Fig. 2. Diagram of antibody generation: Immunization of Balb/c mice using *A. fumigatus* spores

2.4 Optical detection unit and system integration

Optical microscope-based imaging techniques are the basis for the detection of the specific coupling of fluorescently labeled antibodies to surface antigens. Both reflected light recordings with bright field illumination and reflected fluorescence light images are used for localization and identification purposes. Bright field images provide an overview of the huge variety if deposited particles. Additionally they allow a fast automatic focusing procedure, which is necessary for a fully automatic operation of the optical detection unit. Fluorescence images allow the identification of the fluorescently labelled particles in the gel layer.

The microscopic unit (Fig. 3) consists of a high numerical microscope objective (20x , numerical aperture 0.8, Carl Zeiss AG), an uncooled CCD camera for image acquisition (M300, Jai) and a motorized filter wheel with up to 6 positions for placing different (dichroite) beam splitters in the optical detection path. High power light emitting diodes (LEDs) in the green spectral range (525 nm, 36 mW) for bright field illumination and in the red spectral range (630 nm, 70 mW) for fluorescence image acquisition are used for illumination purposes. As a consequence of the quasi one-dimensional localized deposition of the particles in the gel layer image acquisition is carried out sequentially along this one-dimensional strip. Because one dimension of the image is equal to the maximum deposition width the optical scanning for particle detection can be limited to this dimension.
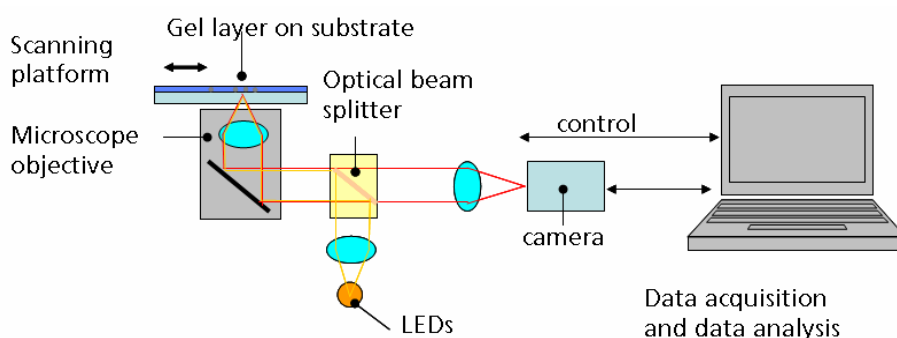

Fig. 3. Principle and structure of optical detection unit

For the automatic and fast detection of contaminants air sampling, preparation of samples (labeling of air contaminants in the gel matrix and electrophoretic separation), sample handling and subsequent microscopic analysis are integrated in a system (Fig. 4). All different parts of the system are controlled by microprocessor based electronics in combination with a personal computer. The control software allows manual, semi-automatic and fully automatic operation of the system.

Fig. 4. Photograph of the integrated detection system with air sampling unit (transparent tower) on top of the chip carrier containing the gel substrate. The LEDs and other optical components are located below the chip carrier.

2.5 Validation

In order to evaluate the new approach under controlled conditions a special "aerosol test environment" (ATE) was developed. The experimental setup allows to control and record important environmental factors, for a sketch of the principle (Fig. 5). The whole set up is realized at laboratory scale (1 m³ and 5 m³) and in real room geometry. Air-temperature, relative humidity and air exchange rate are adjustable within a wide range. As the central prerequisite and as a particular challenge the spore load of the air has to be defined. Therefore a special prepared dry deposit of selected types of spores (one test-germ or a mixture of different species of selected test-germs) is sprayed into the test chamber and ventilated there. In combination with particle measurement of the chamber air at every time the actual spore load within the chamber can be defined. Within the ATE the new approach can be compared with different recent methods for the measurement of biological air contaminants through subsequent measurements. The whole particle load of the test environment is defined by a particle measurement with a portable dust monitor. For aerobiological measurements different devices basing on the impaction method (impinger, short time measurements with a portable impactor and measurements according to the Hirst principle), and the filtration method are used (short time measurement with a portable filtration apparatus and a further device for prolonged measurements) followed partly by a cultivation and analysis of viable germs. All measurements and investigations for the validation are compared with data from current relevant literature, e.g. [9] – [20]. Actually different measurements were done; the detailed results will be presented elsewhere. As a further challenge it is necessary to distinguish between viable resp. colony forming units and nonviable spores/particles in order to compare the different methods. Therefore some experiments with the use of special labeling methods have been undertaken.
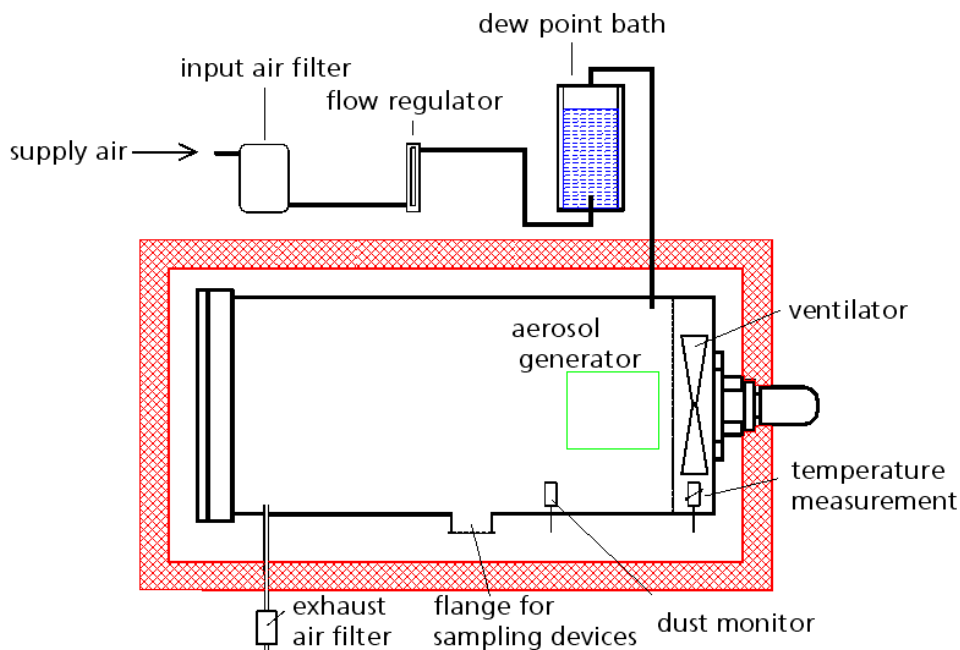

Fig. 5. Principle of the developed aerosol test environment (ATE) for validation of the new approach at laboratory scale and real room geometry.

3. DATA AND RESULTS

3.1 Validation of air sampling unit

The characteristics of the sampling and deposition device were tested by challenging the instrument with monodisperse abiotic particles of different diameters and measuring the concentration upstream and downstream of the device. The intake air flow rate was 50 l/min. The minor flow rate was adjusted to 3, respectively 6.6 l/min. Fig. 6 shows the collection efficiency i.e. the fraction of the airborne particles sampled on the gel strip underneath the slit impactor. The left branch of the curves is determined by the characteristics of the multi nozzle virtual impactor, the right branch by the inlet impactor. The final slit impactor is not limiting the particle size collected. By changing the design parameters of the inlet impactor and the virtual impactor the collection window can be shifted and/or narrowed. In any case the particles are confined to a very narrow strip on the gel as is shown in Fig. 6.
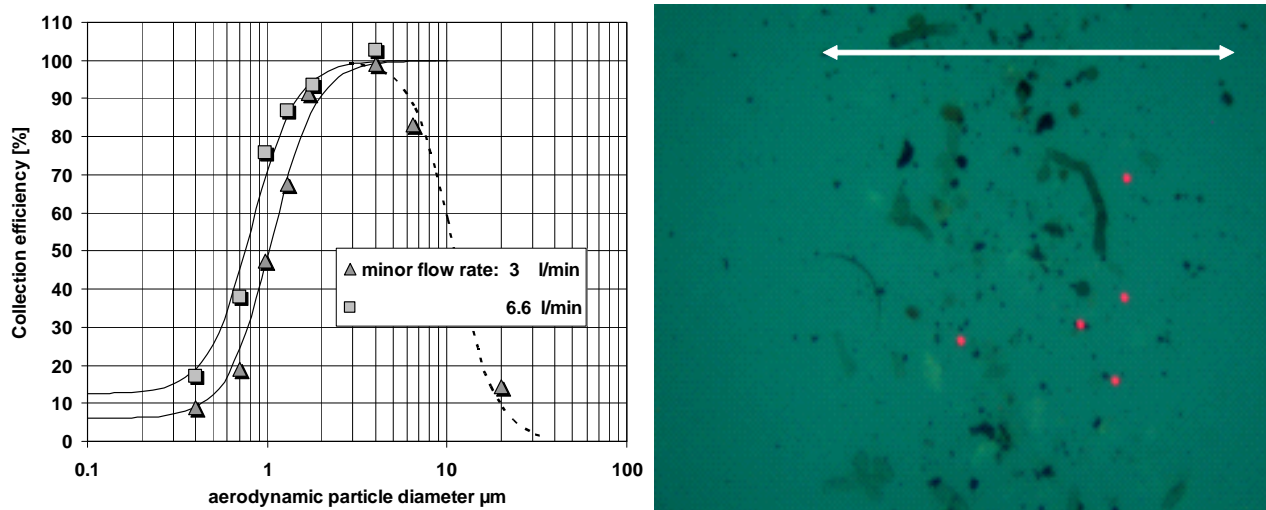

Fig. 6. Particle collection window of the intake and deposition device used in the bio-aerosol monitor (left) and environmental particles and fluorescence beads (6.5 µm) collected on the gel. The width of the arrow is 150 µm (right)

3.2 Characterization of the substrate

Substrates consisting of cyclic olefin copolymer (COC, Aerni-Leuch AG, Switzerland) with a thickness of 200 µm are cleaned in an ultrasonic bath with ethanol. After drying they are covered by an adhesive film at the place of the inter-electrode gap (2 mm width). The masked substrates are treated with a xenon excimer lamp (emission 172 nm, Heraeus Noblelight GmbH, Germany) in an atmosphere of oxygen and nitrogen gas (ratio 1 : 4) at a pressure of 100 mbar for 60 s with an intensity of about 10 mW/cm².

Immediately after the VUV activation, the substrates are coated with nickel by electroless plating followed by a galvanic palladium coating. The electroless Ni plating is carried out with chemicals supplied by Atotech Deutschland GmbH according to the protocols of the supplier. The galvanic Pd bath (Anthrapal Palladium bath, Wieland Dental + Technik GmbH & Co KG, Germany) is run at 2.0 V, 10 mA, and 40 °C for 300 s. Finally, the adhesive films are removed and the substrates are cleaned using acetone and oxygen plasma.

Gels are prepared from solutions of the carageenan Satiagel (Cargill Deutschland GmbH), TRIS buffer, ectoin, glycerine, a blocking protein (e.g. casein), and an antibody. All chemicals except the antibodies were obtained from Roth GmbH, Karlsruhe, Germany, unless otherwise noted. A 3 % Satiagel is melted in a buffer solution at 80°C. After cooling to 40°C, the remaining ingredients are added. In the final solution there are typically 0.6 % Satiagel, 10 mmol/l buffer, 100 mmol/l ectoin, 0.2 % glycerine, 1 % blocking protein, and 5 – 50 mg/ l of the antibody.

The substrates are coated with 75 µm of the solution using a wire bar applicator. During the cooling phase the solution solidifies to a gel which can be freeze-dried and stored at room temperature in a dry place.

During the rehydration process and the electrophoresis, all stages of the wet gel can be monitored by the evaluation of the electrical parameters (Fig. 7). For example, the dry gel between the electrodes has a resistance in the order of MΩ. When the gel rehydrates by a flush of a water aerosol, the resistance decreases to a value in the order of 50 kΩ due to the quick water uptake of the porous gel structure, yet the time is depending on the composition.
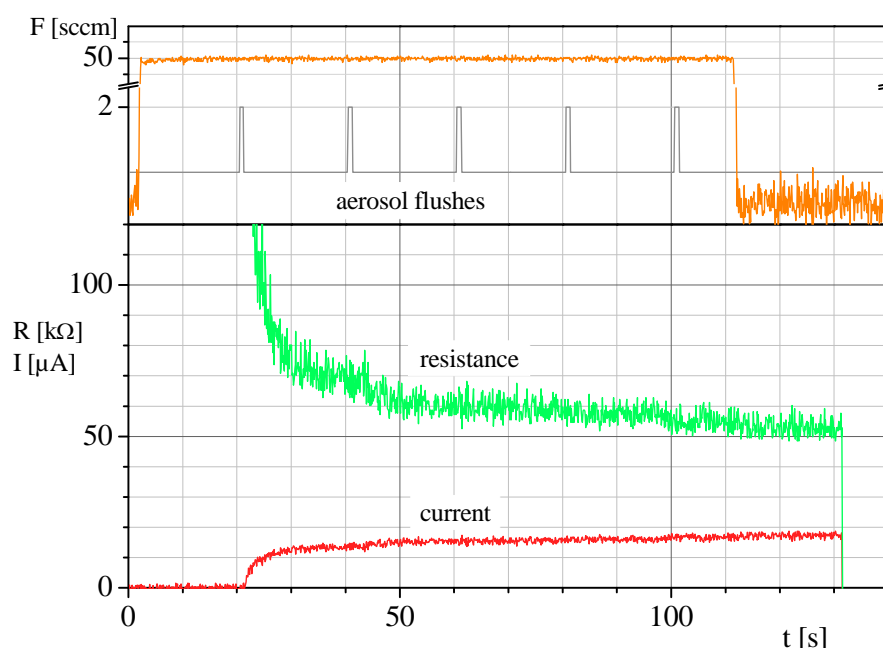

Fig. 7. Wetting and electrical properties of a hydrogel during rehydration.

Applying a small voltage of e.g. 1 V, the electrical properties of the gel and the wetting behaviour can be investigated. Both depend on the composition of the gel. Due to the rather large number of demands, there are many ingredients in the gel. Most of them influence the wetting as well as the electrical properties.

With the small measuring voltage there are no electrochemical reactions going on in a first approximation. This is not true completely. After switching off the voltage a negative voltage and negative current are recorded indicating polarization effects during the measurement. However, for carrying out an electrophoretic separation within a rather short period of time, we need a considerably higher voltage. When 10 V are applied, the gel dissipates about 1.6 mW. A 5 minute electrophoresis would increase the temperature of the gel by 15 K if there would not be any dissipation of the heat. In a real experiment the heating is much smaller since the substrate takes up a lot of the energy.

3.3 Antibody fabrication and testing

Specific antibodies against spores of *A. fumigatus* were generated as depicted in the diagram presented in Fig. 2. Mice were immunized using heat-inactivated spores. Full length monclonal mouse antibodies as well as recombinant V gen single chain format were derived from spleen cell fractions of immunised mice. Both avenues are attractive as each approach has different advantages for the final assay e.g. binding characteristics, modification, labeling strategies, mobility, etc. The immune response during the course of the immunisation was assessed by flow cytometry. This technique allows for the evaluation of the antibody-antigen interaction at conditions that will not affect the conformation of the antibody. In this respect, it reflects in a closer way the conditions of the in gel situation. The results of the flow cytometry analysis clearly show the specific immune response to the spores of *A. fumigatus*. These results are confirmed by immune fluorescence microscopy as shown in Fig. 8. Four monoclonal hybridoma cell lines were established, producing IgM antibodies raised against spores from *A. fumigatus*. The antibodies from clone F38 2MK4 were purified and binding characteristics were analysed by ELISA. The signal to noise ratio could be improved when Casein instead of BSA was used as blocking agent (Fig. 9).

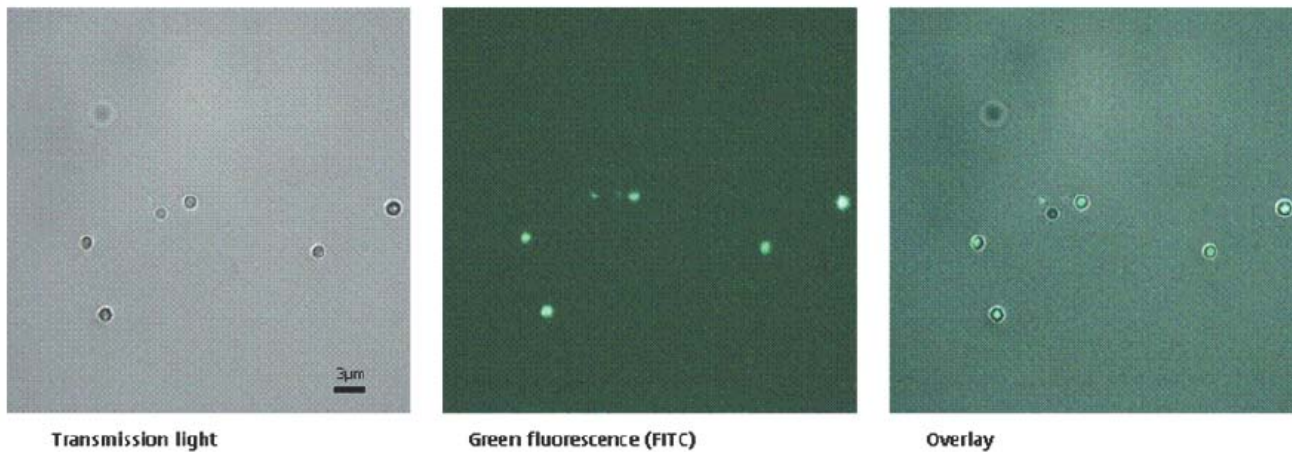| Transmission light | Green fluorescence (FITC) | Overlay |

Fig. 8: Fluorescence microscopy of *A. fumigatus* detected with sera from i.p. immunized mouse.
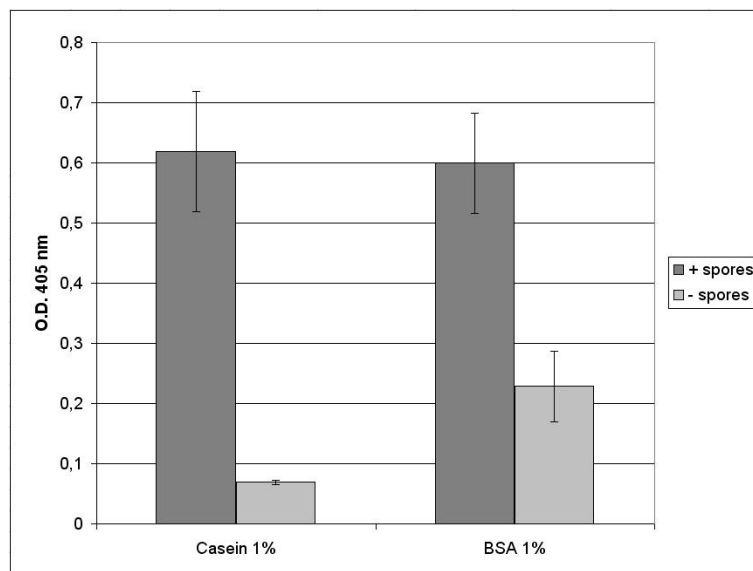

Fig. 9. Binding of purified monoclonal IgM antibodies to spores of *A. fumigatus* as demonstrated by ELISA. Unspecific background signals were reduced by replacing BSA with Casein

3.4 System validation and testing

In a first step of an evaluation of the system focusing on the electrophoretic separation of bound and free antibodies, we used protein A-coated polystyrene beads with a mean diameter of 6.78µm (Spherotech Inc., Lake Forest, USA) in combination with an IgG (Sigma-Aldrich, Taufkirchen, Germany) labeled with AlexaFluor647 (Invitrogen, Karlsruhe, Germany). The COC substrate was pretreated as described in section 3.2, yet Cu electrodes were used instead of Ni/Pd. The gel was prepared from 0.5% Satiagel with an addition of 1:100 IgG. The dry gel was then inserted into the detection system, and 5µl buffer solution containing 0.05‰ polystyrene beads impacted onto the gel at a flow rate of 3.3 l/min. After the rehydration of the gel, a voltage of 5V was applied and the electrophoresis phase monitored in the fluorescence readout mode of the system. The figures in Video 1 give exemplarily four stages during the electrophoretic removal of free antibodies: in the rehydrated gel, the protein A-coated particles are recognized by the fluorescently labeled IgG (a), when a voltage is applied, the electrophoresis starts (at the top of the image, b and c), clearly noticeable by a darkening

of the area where the antibody is already removed, until upon complete removal of the free antibody the remaining particles can be detected by their fluorescence (d).
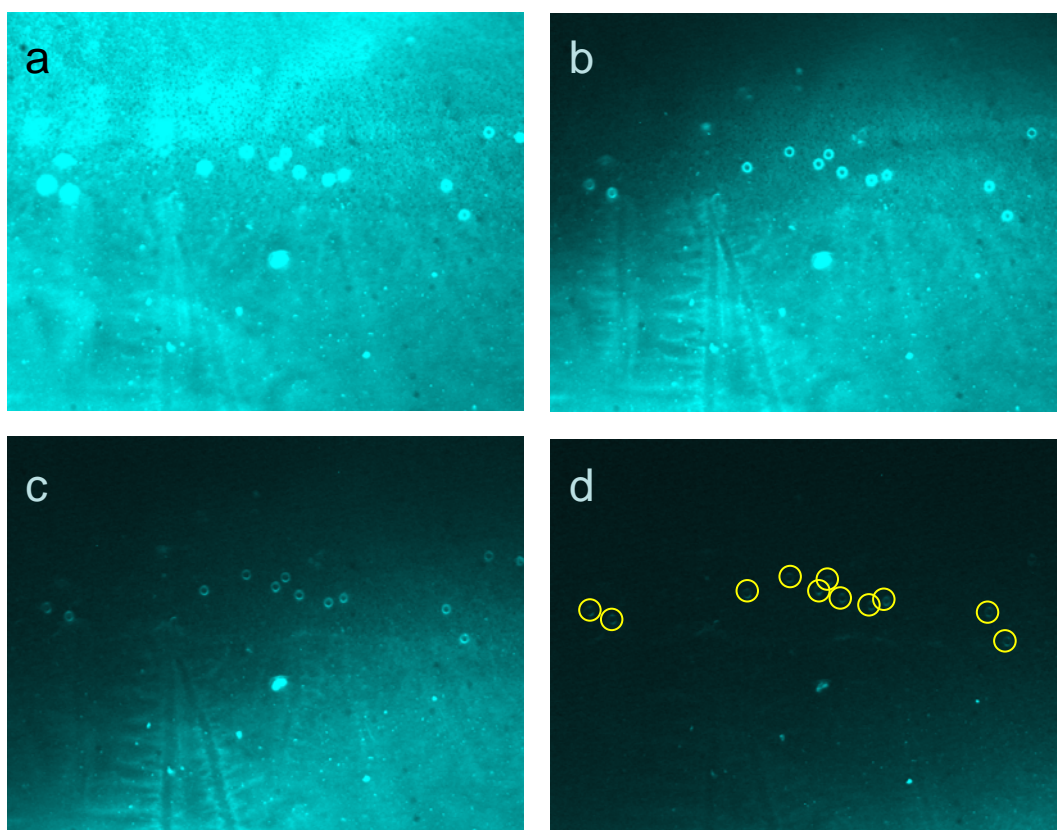
Video 1: Experimental run with protein A-coated test particles, recognized by an AlexaFluor647-labeled IgG, recorded in the fluorescence readout mode of the system (800ms integration time/image, retroactively colored for better visualization). a) The particles are recognized by the antibodies in the rehydrated gel b+c) During the electrophoresis the free antibodies are removed from the field of view, starting at the top of the image d) after the complete removal of the antibodies, the remaining fluorescently labeled particles are detected by the software. The complete video can be seen under http://dx.doi.org/doi.number.goes.here

The decreasing fluorescence intensity of the recognized particles can be attributed to a large extend to a washing of loosely bound antibodies on the particle surface. A control experiment, where the particle solution was directly mixed with the labeled antibodies, and after incubation washed twice with buffer, supports this hypothesis. In this case the fluorescence intensity was decreased to roughly the same extend when compared to the particles that were exposed to the electrophoretic washing.

Another issue is the discrimination of detected particles from interfering particles such as dust or mineral particles. Both biotic and abiotic particles show autofluorescence to a certain degree in the visible spectrum, mainly when excited by an ultraviolet or blue light source. To assess the influence of dust particles on the specific detection of micro-organisms by fluorescently labeled antibodies, indoor air was sampled for 2h and the fluorescence intensity compared at blue excitation (λ=365nm) with excitation in the red spectral range (λ=640nm). Fig. 10 shows the results for an integration time of 2s. With blue excitation, a total number of 102 particles were counted compared to only 17 at red excitation for the same threshold value. This implies a significantly higher level of autofluorescence from interfering particles in the blue spectral region. Additionally, the autofluorescence in the red spectral region should be sufficiently low not to interfere with the detection of the fluorescently labeled particles, as can be seen from Video 1 compared to Fig. 10, as in Fig. 10 the integration time was set to 2s instead of 800ms.
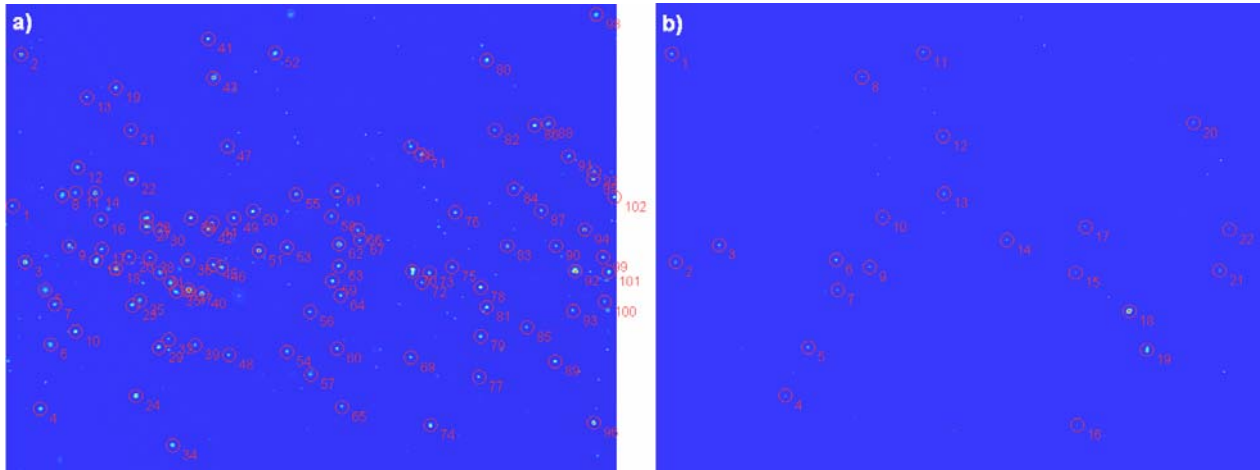
Fig. 10: Determination of autofluorescence from indoor dust (int. time 2s) (a) excitation: λ=365nm, detection at λ>405nm longpass, (b) excitation: λ=640nm, detection at λ>660nm. The threshold for particle recognition was set to the same value indicating a significantly higher autofluorescence in the blue spectral region compared to red.

3.5 Characterization of the validation chamber

Within the ATE environmental conditions are controlled accurate and recorded automatically. Several tests showed that it is possible to generate a very stable spore aerosol that is in the range of typical indoor air values. After spraying of, e.g., 0,1 g dry spores of the *Penicillium*/*Aspergillus*-type there is an exponential decrease in spore concentration in the ATE with 1 m³ total volume for about 60 – 90 min after spraying (depending on the type of spores) until an almost constant level of indoor-typical spore concentration is reached and hold for several hours. The time behavior of measurement with sterilized spores of *A. fumigatus* is shown in Figure 11. When stable conditions are reached different methods for documentation of indoor air spore load can be used and compared. In the example (Fig. 11) there are shown the times when measurements according to the Hirst principle versus the filtration method where applied. The ATE setup can not only be used to validate the new approach but it is also applicable in further scientific questions connected to assessment of biological air contaminants.
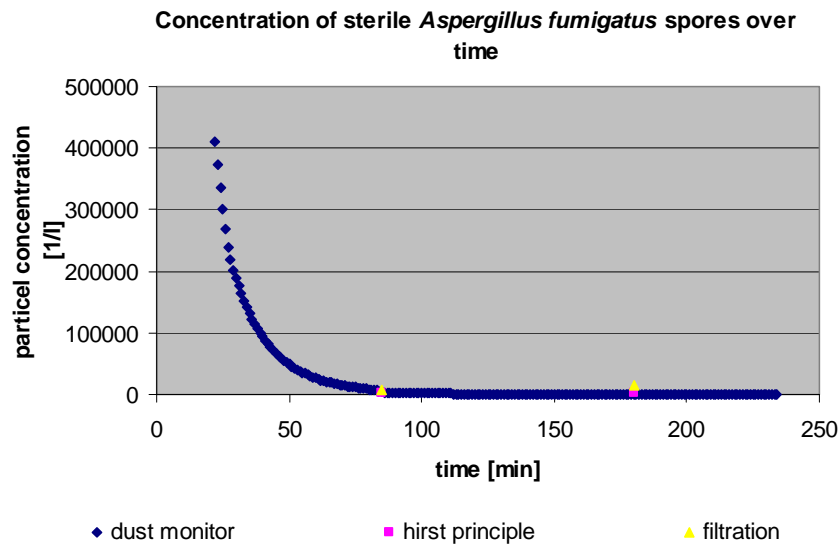

Fig. 11: Documentation of spore load within the ATE. Measurements with different methods for investigation of air contaminants are made, when almost a constant particle load is reached.

4. CONCLUSIONS

In this paper a new approach for the detection and classification of airborne micro-organisms is presented. The principle is based on immunobiological detection, i.e. specific antibodies bind to the surface of the micro-organisms. The antibodies carry a fluorescent label emitting in the far-red end of the visible spectrum, thereby minimizing interfering autofluorescence effects, e.g. from dust particles. A first integrated system has been set up, which samples particles in the relevant size from 1µm to 10µm and allows the identification by fluorescence detection within less than an hour. In detail, an air sampling unit has been characterized and optimized to sample the relevant particle size and to deposit the particles locally on the gel substrate. A suitable gel composition has been found with respect to antibody viability and parameters for the electrophoretic separation of unbound antibodies. First measurements on a test system comprising protein A-coated polystyrene beads to be recognized by fluorescently labeled IgG were performed. The measurements showed the proof-of-principle of the proposed detection method with a complete experimental run involving the deposition of test particles with subsequent removal of free antibodies. The immunization of Balb/c mice yielded specific antibodies suited for the detection of *A. fumigatus*. In a next step the detection system will be tested involving the antibodies produced against *A. fumigatus* and further relevant germs or bacteria. With the setup of an aerosol test environment (ATE), the detection system can be further tested providing real environmental conditions.

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