Extracting an Optical Finger-Print – a New Approach to Single Particle Analysis

Paper # 1187

Eckart Schultz, Ulrich Heimann, Stefan Scharring

German Weather Service, Department of Human-Biometeorology, D-79104 Freiburg; phone: +49 (0) 761 / 2820250; e-mail: eckart.schultz@dwd.de

Albrecht Brandenburg, Markus von Ehr, Gerd Sulz

Fraunhofer Institute of Physical Measurement Techniques, D-79110 Freiburg; phone: +49 (0) 761 / 8857306; albrecht.brandenburg@ipm.fraunhofer.de

Hans Burkhardt, Olaf Ronneberger, Qing Wang

Institute of Computer Sciences, University of Freiburg, D-79110 Freiburg; phone: +49 (0) 761 / 2038260; e-mail: burkhardt@informatik.uni-freiburg.de

Wolfgang Koch, Wilhelm Dunkhorst, Hubert Lödding

Fraunhofer Institute of Toxicology and Experimental Medicine, D-30625 Hannover; phone: +49 (0) 511 / 5350117; e-mail: koch@item.fhg.de

Werner Müller

Helmut Hund GmbH, Wilhelm-Will-Str. 7, D-35580 Wetzlar; phone: +49 (0) 6441 / 200460; e-mail: w.mueller@hund.de

Gernot Breitfuss

Breitfuss Messtechnik GmbH, Danziger Str. 20, D-27243 Harpstedt; +49 (0) 4244 / 967912; e-mail: g.breitfuss@breitfuss.de

ABSTRACT

A fully automated system has been developed for microscope-based single particle analysis by extracting optical finger-prints from individual particles in ambient air samples. For this purpose, light microscopy was developed towards an objective measuring technique by employing a novel pattern recognition technique. Automated particle classification is based on so-called grey scale invariants, extracted from microscopic images of translucent, fluorescent and dark field microscopy. This information was bundled to a feature vector providing a kind of finger print for every particle. In a first step this approach was used for an automated recognition of allergen carriers such as pollen and fungal spores. A leave-one-out test gave a recognition rate of about 95% for 26 for the most frequent pollen species in central Europe. Because no pollen-specific code was used, the recognition software was also employed for an automated recognition of fungal spores without any change. Six of the most frequent airborne fungal spore genera in Central Europe were classified with a mean recognition rate of 93%. These results gave reason to a research project aiming at the development of a fully automated system. The instrument should combine (1) high-volume

sampling of coarse particles >2.5 µm, (2) electrostatic precipitation of this fraction onto a surface suitable for optical analysis, (3) automatic preparation for microscopic single particle analysis, (4) imaging by various microscopic techniques, e.g. transmitted, fluorescence and dark field microscopy, (5) feature extraction by grey scale invariants, (6) classification by self-learning Support Vector Machines and (7) hourly output of number concentration of airborne pollen, fungal spores and other particles of interest. A first demonstrator is presented in early 2005. First field tests are planned for the first half of 2005. A commercialised device should be available as from 2007. The project is funded by the German Ministry of Education and Research.

INTRODUCTION

Health effects of airborne particles, besides concentration, significantly depend on their composition and size distribution.[1] This information is typically not provided by routine particle measurement due to high costs of size fractionated measurement and chemical analysis. Available particle data, therefore, are mostly restricted to the information on mass concentration. Number, size distribution and composition, at least of the coarse fraction are quite easily accessible by light microscopy. Modern techniques of image processing in combination with pattern recognition techniques should make it possible to quantitatively measure these data. Due to optical resolution it has to be considered that microscopic analysis is limited to the super micron particle range. However, a greater number of health effective particles occur in this range, for example allergens like pollen and fungal spores. Even today concentration data of these components world-wide are still derived from tedious microscopic inspection of ambient air samples by eye. These data suffer from considerably varying quality, limited reproducibility and late availability, apart from the high costs arising from the time consuming work. Therefore a project was proposed to develop a microscope-based automatic particle monitor providing reproducible, objective data of known quality. Due to health reasons and public demand in Germany the project firstly was aimed at the monitoring of allergens such as airborne pollen and fungal spores. However, the development from the beginning on was not restricted to these components but open for analysis of any component accessible by light microcopy.

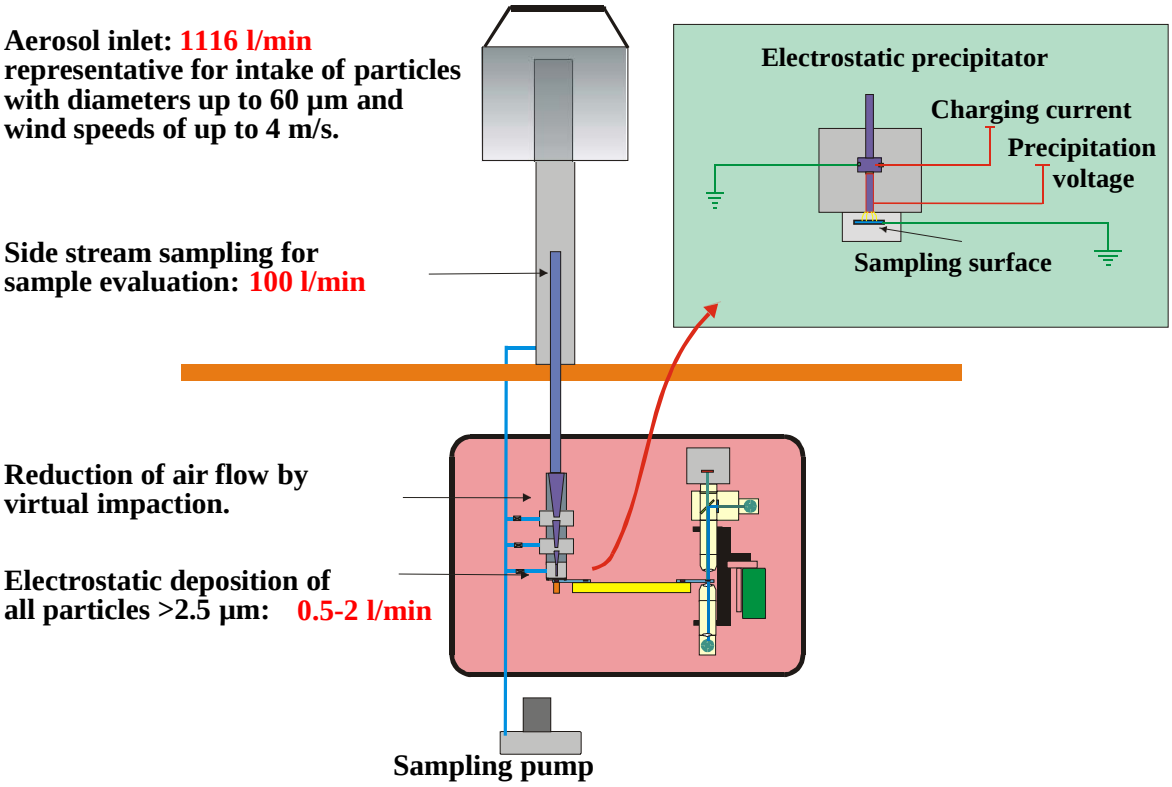
METHODS

Sampling

Outdoor air is collected independent of the wind direction using a suction probe with a radial symmetric inlet. Representative collection of large pollen grains (up to about 60 µm) necessitates fairly large geometric dimensions (probe diameter 4 cm) and a high intake airflow of 70 cubic meters air per hour. By virtual impaction, this large quantity of air is reduced by a factor of about 100 to 1-2 l/min, without reducing the flux of the particles to be investigated, i.e. particles with aerodynamic diameter larger than 2.5 µm.

A two-stage electrostatic precipitator[2] is used to finally deposit the concentrated aerosol onto the sampling surface. In the first step, the particles are charged in an ion cloud created by corona discharge. In the second step, aerosol is deposited by means of an electric field located between the separation nozzle and the conductive glycerine surface. An appropriate nozzle shape and a suitable voltage allow to focus the separation to a defined area of the separation surface underneath the nozzle.

Figure 1: Schematics of the aerosol sampling and deposition unit.

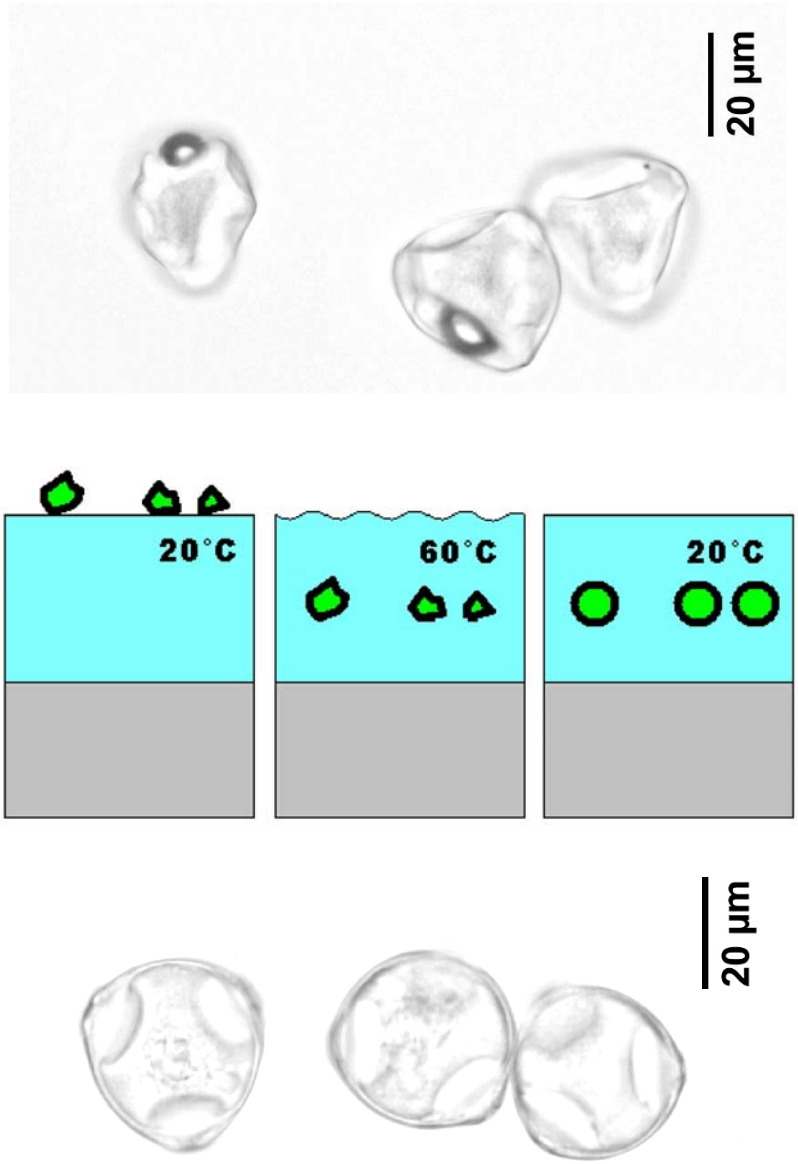


Preparation

Components of the deposition unit and preparation of the particle sample must fulfil certain requirements for microscopic analysis. As a sample carrier, a metal plate with circular punches is used covered on its backside with a highly transparent acrylic glass foil. The resulting shallow cavities are filled to a certain level by an thermoplastic immersion liquid consisting of a mixture of glycerine, gelatine and water. Particles are deposited onto the surface acting as an adhesive. Instead of adding an immersion liquid after sampling the sample is immersed by the same liquid. The immersion is achieved by heating up to about 60°C. At this temperature the liquid is of lower viscosity so that particles get immersed. Additionally, the mixture is adjusted to a refractive index of 1.52 similar to that of most atmospheric particles preventing, by this, light aberration and total reflectance at the particle's boundaries.

When getting airborne pollen dry out and change their shape and size due to the occurring water loss. Pollen recognition requires to re-establish their original shape. This is achieved by the water up-take of pollen grains from the aqueous immersion liquid. This process typically takes only a few seconds.

Figure 2: Micrograph of dried-out hazel pollen (top). Schematic steps of the water up-take when incorporated by the immersion liquid (middle). Hazel pollen re-established after water up-take (bottom).



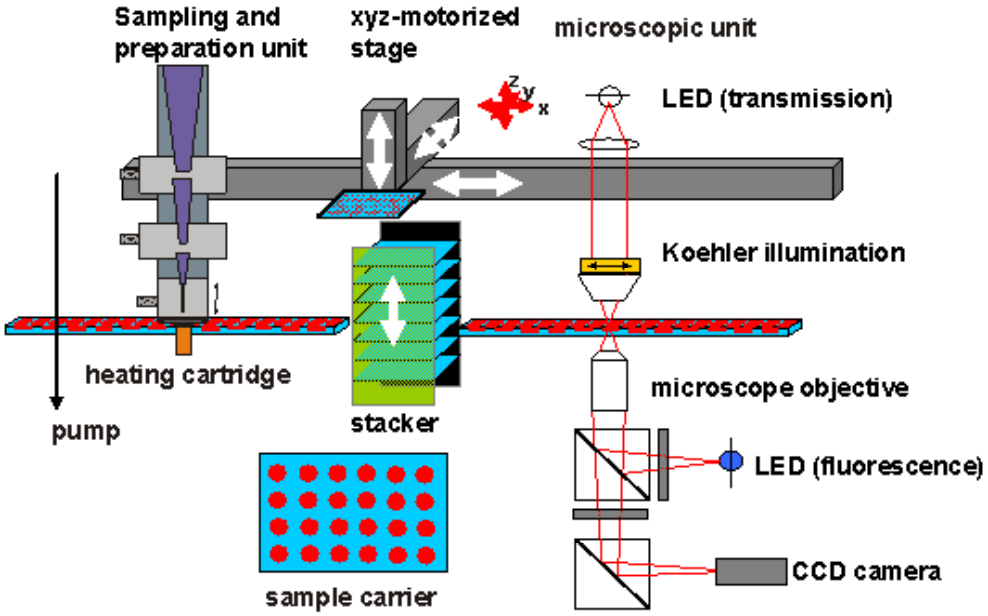
The water content of the embedding medium also provides a conducting surface that is needed for the electrostatic precipitation. Primary fluorescence is a typical feature of bioaerosols. Particularly, pollen grains show a characteristically strong primary fluorescence. At the same time a background fluorescence may arise from the carrier and/or the immersion liquid interfering the recognition of pollen. For this reason acrylic glass was used as carrier and a special gelatine[3] as immersion liquid because of the low fluorescence of both components.

Imaging and System integration

For an automated optical analysis, a system has been developed comprising automated particle sampling, preparation and sample handling for subsequent microscopic analysis and particle recognition. Inside of the system, sample carriers are transferred by means of an x-y-z motorised stage between a stacker, the place of sampling and preparation and the microscopic analysis. The system allows a simultaneous sampling of particles onto a first carrier and a microscopic analysis at a second carrier. By this, a nearly continuous collection of aerosol particles is possible with only short interruptions.

Microscopic analysis is very sensitive to internal vibrations affecting the quality of recorded images. Special care, therefore, was taken to prevent the propagation of mechanical vibrations from the sampling unit containing the pump and the motorised stage.

Figure 3: Schematic of the automatic pollen monitoring system

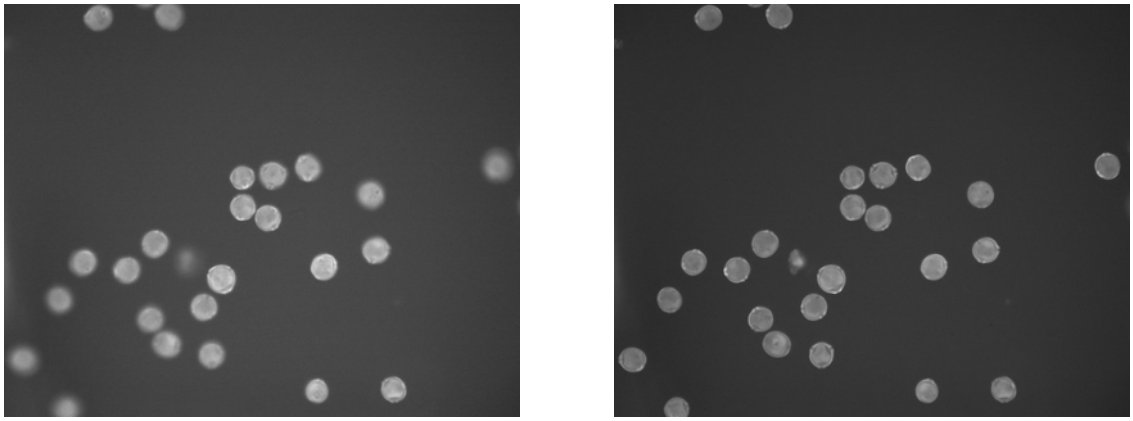


Light microscopy exhibits the great variety of atmospheric particles in size, shape and optical properties.[4] Thus, a fast automatic focusing procedure is necessary for a fully automated image acquisition of a sample area of about 40 mm^2 in a short time (0.5 - 1 h) and of sufficient resolution (< 0.6 µm).
Additionally, it has to be considered that particles are located in quite different levels due to the described automatic preparation. Therefore it was decided to record two image stacks (translucent and fluorescence) at each position with 40 levels (2.5µm distance) each. This results in a full 3D volumetric data set of all particles.
Due to the parallel developing work of the different components in this project, the microscopic unit was not available at the beginning. First results of pollen recognition therefore are basing on images recorded with a conventional microscope (Zeiss Axioplan 2 imaging) using a 20x objective (aperture 0.5). For fluorescence an excitation in the range from 395-440 nm and an emission above 470 nm was used. The sample is moved by a Merzhäuser scanning stage and the images are recorded with a 2/3“ CCD camera with 1392x1040 pixels and a pixel size of 6.45µm x 6.45µm (AVT Dolphin).
Every stack is recorded at the highest possible frame rate of the camera of about 15 images per second in translucent mode and 10 images per second in fluorescence mode due to the required shutter time of 100 ms.

Pattern Recognition

The first step in pattern recognition is to locate and crop the individual particles („segmentation“). Biologic particles such as pollen show a strong primary fluorescence in the UV and blue spectral range allowing a reliable separation from the background and from other simultaneously occurring particles. The segmentation is realised by dividing the shading corrected fluorescence volume into small blocks of 10x10x40 voxels. For each of these blocks the optimal focus plane is found by calculating the energy of Laplacian for each 10x10 pixel image as criterion for sharpness. The layer numbers compose a layer image, which is smoothed and interpolated to get a layer image of original size. A patch image is created from the stack according to the layer image (Fig 4).

Figure 4: Creation of a patch image (right) from an image stack with different focal planes. An example of an image from the middle layer is shown on the left.



This image is used for segmentation. The resulting binary mask for each particle is applied to each plane of the fluorescence and translucent stack to crop each 3D object from the volume. The next step is to calculate a „finger print“ of each recorded object. This finger print is composed of a number of features, extracted from the volumetric data set. The applied feature extraction method uses so-called grey scale invariants[5,6] extracting rotation and translation invariant features of multi-component 2D or 3D data. These data are basing on the Haar integration of free selectable non-linear kernel functions. The main advantages of these features compared to several traditional feature extractors is their direct operation on the grey values of the voxels without any complicated and usually highly application specific pre-processing, like edge detection, searching for pores of pollen grains, etc. The grey scale invariants are therefore applicable to any type of particles not requiring any change of the programs.

The third step in this recognition pipe is the classification of the extracted “finger prints”. As classifier, so-called Support Vector Machines[7] were employed. A support vector machine is able to “learn” how to distinguish between two given classes, e.g. between the finger prints of hazel pollen and the finger prints of miscellaneous particles, just by given examples labelled by experienced personal. The output of this training is a model allowing to classify finger prints of new particles.

Figure 5: Invariants are extracted by linking the grey values of each voxel with a non-linear kernel function (left) and performing a calculation on those results based on the Haar integration (middle and right).

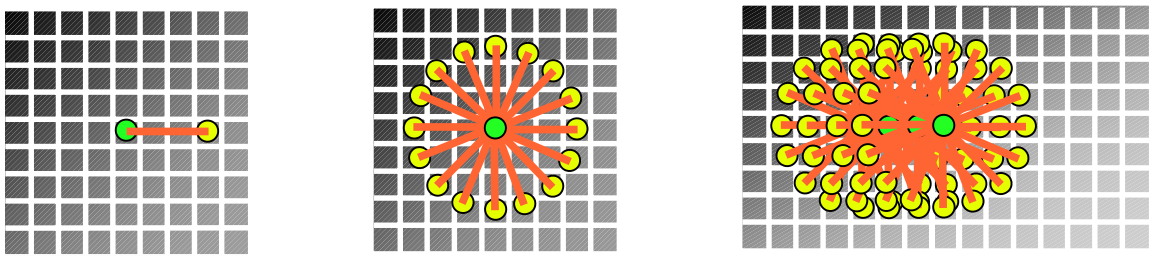
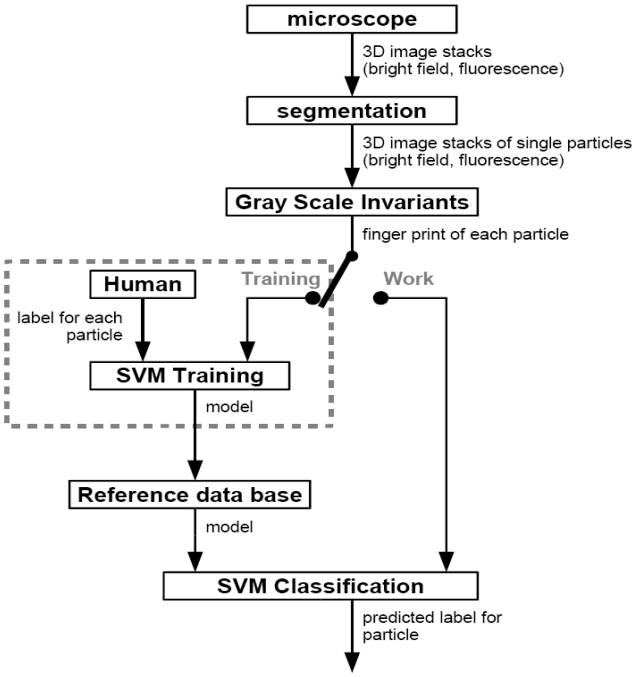


Figure 6: Schematic of the recognition process including human-aided training of particle recognition for the set-up of a reference database



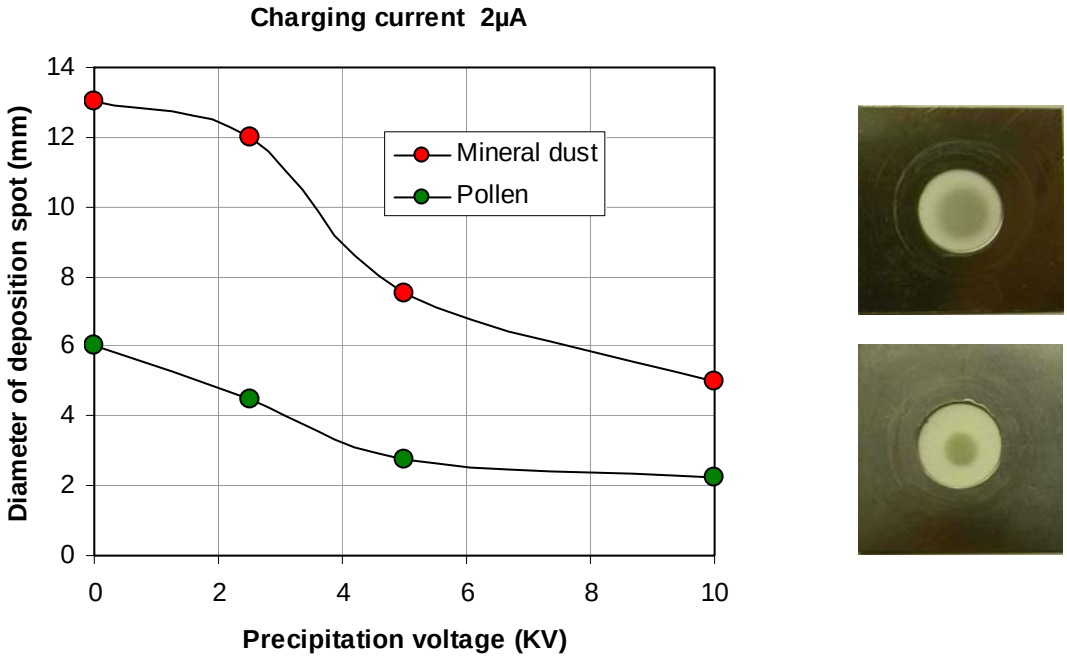
RESULTS AND DISCUSSION

Sampling

The measurement of the aspiration and the deposition characteristics of the sampling unit was carried out by exposing the instrument to different test aerosols (including grass pollen (30 µm), mineral dust (2-10 µm) and glass beads (10-100 µm)) in an environmental test chamber as well as in a small wind tunnel. The aspiration efficiency proved to be constant and nearly 100 % for particles up to 70 µm and dropped to zero for particles as large as 200 µm aerodynamic diameter. The aspiration curves were shown to only weakly depend on the wind speed in the range tested covering 0-4 m/s.
The combination of virtual impaction and electrostatic precipitation showed optimum results for an intake flow rate of 100 l/m through the virtual impactor and a flow rate of 1.5 l/min through the electrostatic precipitator. The optimum value of the charging current was between

1 and 2 µA. The size of the deposition spot on the gelatine surface can be adjusted by changing the precipitation voltage applied between the nozzle and the surface.

Fig. 7: Size of the deposition spot as function of precipitation voltage. Photographs on the right demonstrating this effect for mineral dust at low voltage (up) and high voltage (below).

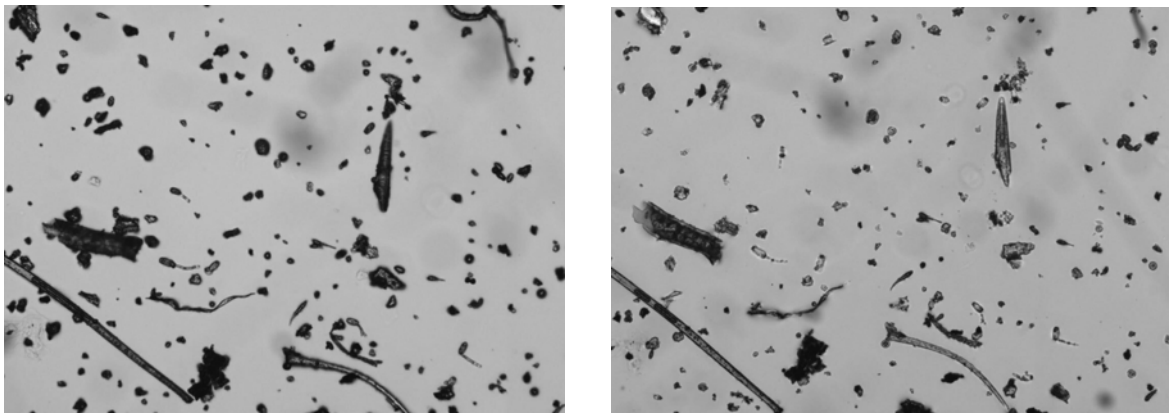


Preparation

The sampling plate was prepared by filling up the circular wholes with the glycerine gelatine solution. For microscopic analysis a layer of about 100 µm turned out as the appropriate fill level. A thicker layer was found to affect image quality, a thinner layer tended to dry-out and to limit particle incorporation.
Samples loaded with grass pollen and mineral dust by the above described sampling technique showed a circular, widely homogeneous coverage of particles sticking to the surface layer.
After heating up, particles are immersed in the solution due to the considerably lowered viscosity and are embedded, by this, for microscopic analysis. Microscopic inspection by confocal laser scanning microscopy showed that particles did not enter the solution deeper than 100 µm. Simultaneously, the pollen swelled up to their original shape and size due to a fast water up-take from the aqueous solution.
For quality assurance of microscopic analysis the optical behaviour of samples was studied as a function of time. The experiments indicated that even a long-term storage of several months did not lead to changes in pollen morphology due to water losses if environmental conditions were specifically controlled. On the other hand, there is evidence that optical properties may change. Fluorescence can weaken and/or change in activity within the pollen individual. Such effects have to be considered when analysing aged samples.
Microscopic inspection proved that samples collected without electrostatic precipitation contain only particles of an aerodynamic diameter > 10 µm while a mixed sampling by impaction and electrostatic precipitation leads to a particle deposition down to 2.5 µm. This

demonstrates the capability of electrostatic precipitation for a sampling of smaller particles, i.e. fungal spores.

Fig. 8: Appearance (bright field, transmitted light) of an aerosol sample before (left) and after (right) preparation indicating that the immersion of particles minimises light aberration and supports reliable particle analysis.



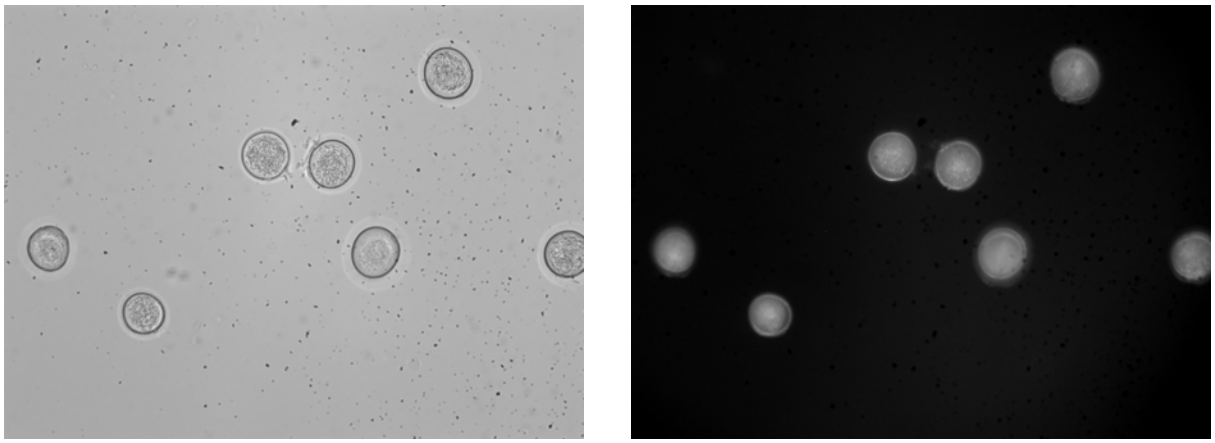
Imaging and system integration

The fully automated monitoring system is shown in Fig 9. Sample carriers can be transferred within 15 sec between the different units (stacker, sampling and preparation unit, microscopic unit). Therefore a nearly continuous sampling and optical analysis is possible with a clock pulse of about one hour.

Figure 9: Automatic monitoring system with sampling, preparation and microscopic detection unit

A high power LED was used as light source both for transmitted light and fluorescence microscopy. There are several advantages by using LED's:
1) a lifetime of about 50,000 -100,000 hours, which is especially important for an automatically operating, unsupervised system,
2) very low power consumption in comparison to conventionally used bulbs – therefore the heat generation is negligible,
3) high stability of emitted light spectra.
For transmitted light microscopy a green LED (~ 533 nm; 35 mW) was used to achieve sufficient optical resolution. The uniformity of the bright field illumination was better than 90%.
In the fluorescence mode, good results were obtained with a LED having an excitation wavelength of about 380 nm, the emission was recorded above 455 nm.
Examples for transmitted light and fluorescence images are shown in figure 10. These images built up the basis for pattern recognition. The images were taken by a 2/3" CCD camera (1392 x 1040 pixels, pixel size 6.45 µm), and a magnification of 20 x (NA 0.5).
An important aspect is the time needed for recording the image stacks with different focus depths within the sample area. The time needed is primarily ascertained by the integration of the fluorescence images. A further reduction of this time is expected by increasing the power of the fluorescence illumination.

Figure 10: Transmitted light (left) and fluorescent (right) microscopic images of grass pollen grains



Pattern recognition

1751 pollen from manual prepared samples were recorded for a test of the pattern recognition and a prediction its performance. A so-called leave-one-out test was carried out by taking one particle out of the data set, train the classifier with the remaining N-1 particles and classify the left out particle with the resulting model. This procedure is repreated for each particle.

Table 1. Recognition rates for pollen in a leave-one-out test

Taxa	N samples	N correct	N wrong	% correct
Alnus	120	110	10	*91.7*
Betula	335	330	5	*98.5*
Carpinus	156	156	0	*100*
Corylus	209	205	4	*98.1*
Juglans	134	128	6	*95.5*
Fraxinus	122	117	5	*95.9*
Plantago	53	37	16	*69.8*
Platanus	151	147	4	*97.4*
Gramineae/Poaceae	83	81	2	*97.6*
Secale	68	67	1	*98.5*
Rumex	165	161	4	*97.6*
Taxus	155	155	0	*100*
Total	**1751**	**1694**	**57**	***96.7***

CONCLUSIONS

Sampling

The sampling unit fulfils the specifications with respect to the desired size range of scavenging from about 2.5 µm (fungal spores) to even large airborne pollen up to about 60 µm (maize). A uniform deposition of particles is provided onto the preparation surface suitable for automated microscopic analysis.

Preparation

The used impaction surface simultaneously acts both as an adhesive and an embedding liquid due to its thermoplastic properties. Subsequent microscopic analysis is possible without any additional preparative efforts. The water content of the adhesive affords the restoring of the collected pollen in size and shape and provides a conducting surface that is needed for the electrostatic precipitation.

Imaging and system integration

The combination of transmitted light microscopy (bright field and dark field) and fluorescence microscopy is an effective approach to automated particle recognition, e.g. pollen and fungal spores. High power LED’s are well suited as light source for the employed microscopic techniques. Additionally LED’s fulfil the requirements for outdoor operation, i.e. long term stability (power, wavelength, and lifetime), low power consumption and low-maintenance.

Pattern recognition

The recognition rates for manually prepared samples are very promising and are noticably higher than 90%. The low recognition rate for Plantago of only 69% supposably results from the low number of only 53 samples, which may not yet represent all possible variations of objects in size, shape and morphology.

ACKNOWLEDGEMENTS

The project is funded by the German Ministry of Education and Research (BMBF). The financial support by the BMBF in the Biophotonik framework under grant No. 13N8367, 13N8368, 13N8372, 13N8437 and 13N8487 is gratefully acknowledged.

REFERENCES

1. Lin, M.; Chen, Y.; Burnett, R.T.; Villeneuve, P.J.; Krewski, D. *Env. Health Perspectives* **2002**, 110, 575-581.

2. Mainelis, G.; Adhikari, A.; Willeke, K.; Lee, S.; Reponen, T.; Grinshpun, S. *J. Aerosol Sci.* **2002**, 33, 1417-1432.

3. Cole, C.G.B; Roberts J.J. *S.Afr. J. Food Sci. Nutr.* **1996**, 8, 139-143.

4. McCrone, W.C.; Delly, J.G. *The Particle Atlas*; Ann Arbor Science Publishers, Inc.: Ann Arbor, Michigan, 1973.

5. Burkhardt, H.; Siggelkow, S. In *Nonlinear Model-Based Image/Video Processing and Analysis*; Kotropoulos, C.; Pitas, I., Ed.; John Wiley & Sons, 2001; pp 269-307.

6. Ronneberger, O.; Schultz, E.; Burkhardt, H. *Aerobiologia* **2002**, 18, 107-115.

7. Vapnik, V. N.; *The Nature of Statistical Learning Theory*; Springer, Germany, 1995

KEY WORDS

Pollen, fungal spores, online monitoring, light microscopy, pattern recognition, grey scale invariants, particle classification