

Alternatives to manual counting for the detection and identification of airborne fungal and actinomycete spores

Janet Macher,^{1*} Sutapa Ghosal,^{1*} Jeff Wagner,^{1*} Kadra Ahmed,² Kaveh Hemati,² Kazukiyo Kumagai,¹ and Stephen Wall¹

¹California Department of Public Health, Environmental Health Laboratory, Richmond, California

²University of California, School of Public Health, Berkeley, California

*Corresponding email: Janet.Macher@cdph.ca.gov, Sutapa.Ghosal@cdph.ca.gov, Jeff.Wagner@cdph.ca.gov

SUMMARY

Raman spectroscopy-based identification of biological materials, automated analysis of spore images, and automated detection of near-monodisperse spore peaks in aerosol size distribution data offer complementary approaches to traditional methods for the identification and counting of fungal and actinomycete spores. An exploratory examination of individual spores for several macro- and microfungi (those with and without large fruiting bodies) by Raman micro-spectroscopy found unique spectral features that can be used to identify fungi to the genus level. Automated analyses of digital spore images accurately recognized and counted single fungal spores and clusters. An automated procedure also was derived to discriminate near-monodisperse bioaerosol peaks from those for polydisperse ambient particulate matter (PM). We anticipate potential application of these approaches in many fields including public health, biological defence, ecology, environmental microbiology, agriculture, and the forensic sciences.

IMPLICATIONS

Easy and accurate identification of fungal and actinomycete spores is relevant to many aspects of human health and comfort as well as plant, animal, and microbial ecology. Light microscopy, a widely used analytical technique, is compatible with a number of air sample formats and requires minimal sample preparation. However, a major drawback is its dependence on a human analyst's ability to distinguish particles and accurately count, size, and identify them. Therefore, automated methods, such as those evaluated in this study, have the potential to provide cost-effective and rapid alternatives if demonstrated to be accurate and reliable.

KEYWORDS

fungal identification, automated analysis, microscopy, Raman micro-spectroscopy, mono-disperse aerosol

INTRODUCTION

Fungi and actinomycetes constitute ubiquitous indoor and outdoor presences and play significant roles in the natural environment and our daily lives. The release of numerous spores into the air is a mechanism by which these organisms disperse over wide regions. For example, Elbert et al. (2007) used air sample data and budget calculations to estimate the global, average, emission rate of basidiospores (~17 Tg/yr) and total fungal spores (~50 Tg/yr). These rates can be compared to estimates of global emissions of ~47 Tg/yr for anthropogenic primary organic aerosols and 12–70 Tg/yr for secondary organic aerosols.

Researchers collect airborne microorganisms using a variety of methods (e.g., impaction, impingement into liquid, and filtration) and analyze air samples by microscopy, culture, biological or chemical assays, and genetic detection. However, these procedures can be time consuming, and analysts may require extensive training and experience. Therefore, alternate methods that combine minimal sample handling with rapid and accurate spore characterization could have broad relevance.

In recent years the application of vibrational, specifically Raman, spectroscopy techniques has attracted considerable interest in the biological sciences, which has led to significant advances in the field of spectroscopy-based biological detection. For example, De Gussem et al. (2007) used this technique to compare spectra for spores from several genera of mushrooms (macrofungi) and for species in the same genus. A vibrational spectroscopy approach to microbial identification offers several advantages in terms of being a relatively rapid and non-destructive technique that requires only small sample volumes and minimal sample preparation while providing access to molecular-level compositional information. One of the goals of this study was to determine the feasibility of a spore identification method based on the Raman spectral signatures of individual spores.

A second focus of this study was to explore the feasibility of substituting automated spore analysis for manual counting to rapidly recognize natural, accidental, or intentional aerosol releases through examination of particle dimensions and identification of nearly monodisperse distributions in the respirable size range. This approach assumed that releases would consist of large numbers of uniformly sized, nearly spherical particles that would be distinguishable from background PM by the atypical narrowness of their size distributions.

METHODS

We describe three methods to detect and identify fungal spores in environmental samples. All procedures offer automated sample analysis, while providing information comparable to traditional methods, and represent significant reductions in the time and labor required for sample analysis.

Raman micro-spectroscopy This vibrational spectroscopy technique is based on the inelastic (Raman) scattering of incident, monochromatic light that results in a characteristic energy shift through interaction with molecules in a sample. Individual spores from several micro- and macrofungi were analyzed by Raman micro-spectroscopy with the goal of developing a robust, automated method for the identification of fungal spores based on their distinct Raman spectral signatures. Method development steps included 1) selection of an optimal sampling substrate, 2) acquisition of Raman spectral signatures for spores from known micro- and macrofungi, and 3) automation of searches to compare the spectra from unknown specimens with those in a library of known fungi.

Spores samples were placed on disks of commercially available, aluminum foil mounted on glass slides with double-sided tape. Samples were dispensed either as water suspensions and air dried in a biological safety cabinet or transferred dry with wooden sticks from spore prints. This sampling format was used to enable micro-analysis of individual spores.

Isolates of microfungi were obtained from the American Type Culture Collection (Manassas, VA; www.atcc.org) and the U.S. Department of Agriculture (Agricultural Research Service, Washington, DC; www.ars.usda.gov) (*Alternaria alternata*, *Aspergillus versicolor*, *Cladosporium herbarum*, *Epicoccum nigrum*, *Eurotium amstelodami*, and *Scopulariopsis brevi-*

caulis). Microfungi were grown on water, cornmeal, oatmeal, and malt extract agar (ASM, 2007). Macrofungi were outdoor and commercial mushrooms (an unidentified Basidiomycete, *Agricus bisporus*, *Hebeloma crustuliniforme*, and an *Inocybe* species).

Spectroscopic measurements were made with a Senterra Dispersive Raman Microscope (Bruker Optics Inc., Billerica MA) equipped with 785- and 532-nm excitation wavelengths. Samples were excited using 1–100 mW of 785-nm laser light focused through a 100× objective (MPlan 100×/0.90; Olympus, Center Valley, PA) with signal acquisition times of 60–90 s per measurement. All spectra were recorded and analyzed using OPUS 6.0.72 dll 3.0.1.1 software (Opus Software Inc., San Rafael, CA).

Automated spore counting and sizing Two image-analysis-based methods were evaluated by comparing automated spore counts with those made by a human counter. High-contrast, near-binary images of monodisperse *S. brevicaulis* spores (count median diameter, CMD: 6.9 μm ; geometric standard deviation, GSD: 1.2 μm) were acquired at 200× magnification using plane-polarized transmitted light (Figure 1a). One trained counter (K. Hemati) examined 35 images for each of five impactor air sample slides and recorded the number of single spores and clusters.

An image processing and analysis software in Java format was used on the digital images (Version 1.37, ImageJ, National Institutes of Health, Bethesda, MD; <http://rsb.info.nih.gov/ij/>). The program scanned an image until it found an object's edge then outlined and numbered the object (Figure 1b). Feret's diameter (d_F), area (A), perimeter (P), and circularity (C , $4\pi \cdot A \cdot P^{-2}$) were measured for each particle. Values of C range from 0–1, where 1.0 indicates a perfect circle and values approaching 0.0 are increasingly elongated polygons.

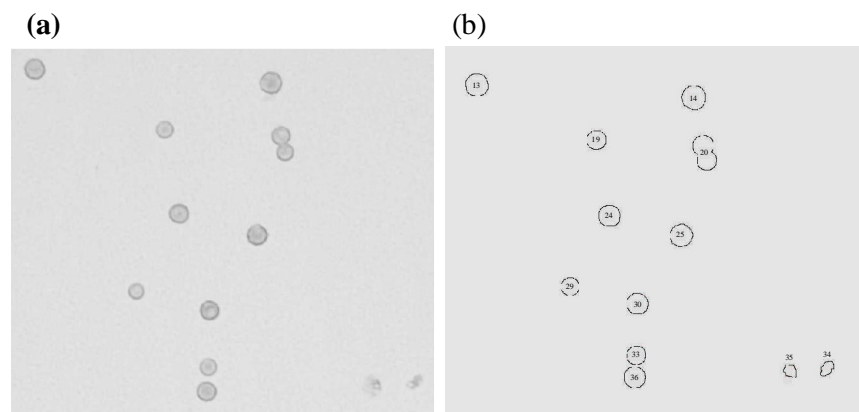


Figure 1. Examples of (a) photographic and (b) ImageJ pictures of larger, circular, *S. brevicaulis* spores and smaller, irregular, debris particles.

For the first automated analysis, spores could be distinguished from debris and recognized as singlets or doublets if d_F and A were between the 1st and 99th percentiles and C exceeded the 1st percentile of the respective distributions for known spores. An upper/lower d_F cutpoint of 9.5 μm was used to avoid counting a particle as both a singlet and doublet because the respective 99th and 1st percentiles were similar (9.6 and 9.5 μm). Inclusion of a criterion for P did not improve distinction between singlets and doublets, therefore, was needed only for clusters of three or more spores. In larger clusters, spores could be in straight chains or more compact configurations. Therefore, circularity was not informative for identification of clusters larger than two spores.

For the second automated analysis, the number of spores of known diameter in a particle (N) was estimated from Equation 1. This equation has been used to recover three-dimensional particle properties from two-dimensional projected images (Fang et al., 1998; Hu et al., 2003; Hu et al., 2004). The constant 1.15 and exponent 1.09 approximately account for particle overlap.

$$N = 1.15 \left[\frac{A}{\pi \bar{d}_F^2 / 4} \right]^{1.09} \quad (1)$$

Automated detection of near-monodisperse peaks A computer program to detect near-monodisperse “spore peaks” within ambient particle size distribution data was written in Visual Basic for Applications (VBA, Microsoft, Redmond, WA) using Excel spread-sheet inputs and outputs. The feasibility of using this program to identify bacterial and fungal spore aerosols was evaluated with real and simulated data. For the two sets of ImageJ data, the program automatically extracted particle measurements, compiled them into size bins, converted the counts into mass size distributions, and calculated spore distributions. For real *S. brevicaulis* data, the program detected distinct peaks for single and double spores and a less well-defined peak for triple spores. For simulated ambient PM and a bacterial spore release, the program detected the three largest peaks, fit a lognormal distribution to each peak, determined whether it met the criterion for a near-monodisperse aerosol, and output its estimated mass concentration. Near-monodisperse spore peaks were discriminated from polydisperse ambient PM using the criterion $GSD \leq 1.7$ developed from typical values in the literature. The spore detection algorithms were designed to work with mass rather than count distributions, which avoided problems with fine count modes dominating other peaks.

RESULTS

Raman micro-spectroscopy As a part of method development, several substrates (aluminum foil, Tedlar[®] film, and KBr crystal, mica, and quartz slides) were surveyed to identify the optimal substrate for spectral analysis. Commercially available aluminum foil was selected for spectrum acquisition due to the absence of interfering peaks in the spectral region of interest, ease of preparation, low cost, and ready availability.

Figure 2 shows the Raman spectra of fungal spores from a) an unidentified mushroom and b) *Aspergillus versicolor* as well as c) a blank aluminum substrate. The fungal spore spectra correspond to measurements at the single spore level. Blank aluminum foil had no background peaks in contrast to distinct peaks for the fungal spores. Each examined spore type displayed a distinct spectral signature that was readily distinguishable from others, demonstrating our ability to differentiate among spore types based on Raman spectra of single spores. Furthermore, the unidentified mushroom’s spectrum was consistent with spectra in the literature for *Laccaria* species (De Gussem et al., 2007). Typically, we could readily distinguish fungal genera, although differentiation at the species level has proven to be challenging thus far. These findings confirmed our ability to identify single fungal spores to the genus level using Raman micro-spectroscopy.

Automated spore counting and sizing Correlations among manual and automated counts of *S. brevicaulis* were 0.99–1.00, 0.86–0.93, and 0.84–0.90 for singles, doubles, and ≥ 3 spores, respectively, and total counts did not differ significantly for the three methods. Therefore, we concluded that automated particle counting and sizing reasonably could be substituted for manual examination of particles for which size distribution data is available.

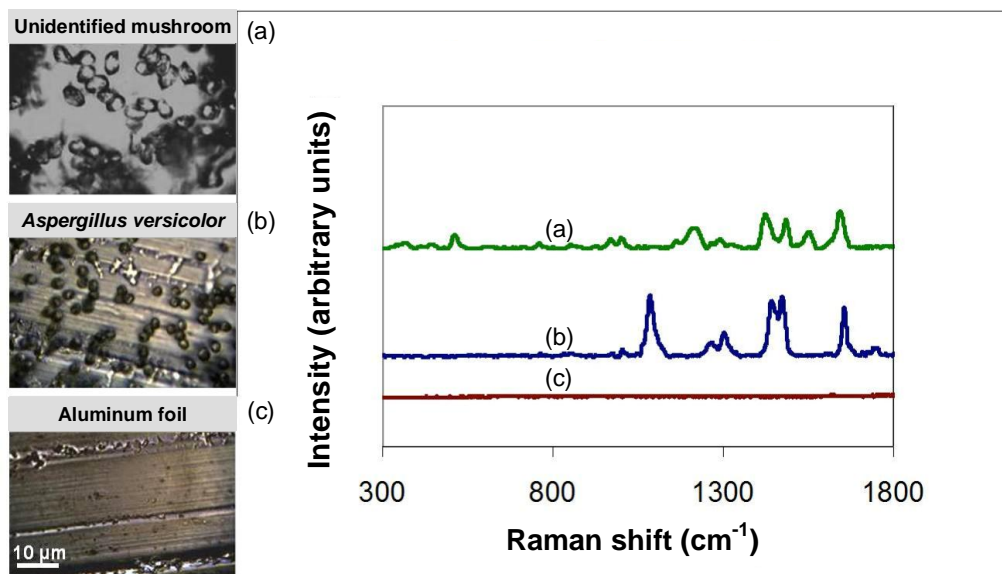


Figure 2. Optical images and corresponding Raman spectra for macro- and microfungi and the aluminum foil substrate.

Automated detection of near-monodisperse peaks Figure 3 shows simulated particle size distribution data for a typical bacterial spore (*Streptomyces albus*) and bimodal, ambient PM. The spore mass median diameter (MMD) lies distinctly between the upper and lower tails for fine and coarse PM, respectively, facilitating successful peak detection. Much smaller or larger spores could overlap problematically with ambient PM peaks. However, many fungal and actinomycete spores are in the 1–4- μm size range and have narrow size distributions (Reponen *et al.*, 2001), thus would be distinguishable from ambient particles from anthropogenic and natural sources.

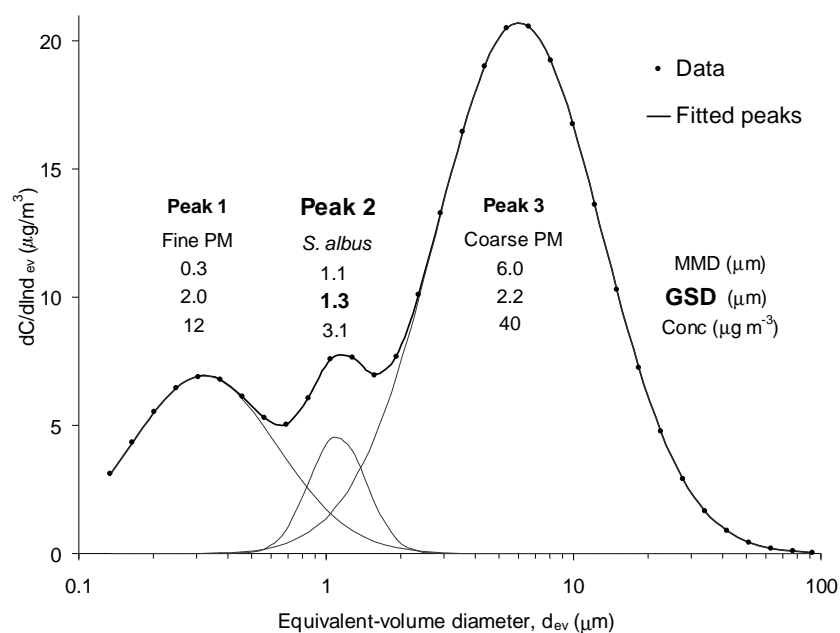


Figure 3. Simulated bimodal ambient PM mass distribution plus a peak representing an actinomycete spore release (discrete data points). Our program automatically fitted three log-

normal peaks as well as their sum (solid distribution curves), then flagged Peak 2 as a potential “spore peak” because its GSD was <1.7.

DISCUSSION AND CONCLUSIONS

The rapid detection of hazardous aerosols would enable public officials to respond quickly and appropriately to releases of infectious agents; biological toxins; or non-volatile chemical, mineral, or radioactive particles. Particle recognition based on Raman spectra and automated image analysis and peak detection show promise for rapid and accurate bioaerosol detection if spectra and images can be acquired for sequential samples and the information fed to a data analysis device.

We have described the application of Raman micro-spectroscopy for identification of fungal spores in environmental samples based on their distinct spectral signatures. The next tasks are to compile a library of spectra for fungal spores commonly found in indoor and outdoor environments (in particular those present as a result of indoor, moisture-related, microbial growth) and to examine additional bioaerosol particles (e.g., actinomycete spores).

Two automated methods to count single spores and clusters were shown to produce results comparable to manual sample evaluation. A program was developed to detect and measure the concentration of near-monodisperse spore peaks from within polydisperse ambient PM size distributions, and was shown to perform successfully using simulated size distribution data. Hypothetically, this algorithm can be used for data obtained with any size-resolved method, e.g., optical particle counters, scanning mobility particle sizers, gravimetrically analyzed impactor samples, and microscope examination of filters and slides. However, the potential success of this approach depends significantly on the separation of bioaerosol peaks from ambient PM peaks and the size resolution of a given technique.

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REFERENCES

- American Society for Microbiology. 2007. Media, Chapter 130. In: *Manual of Clinical Microbiology*, 9th ed., Murray, P.R., Baron, E.J., Jorgensen, J.H., Landry, M.L., and M.A. Tenover, Eds., ASM Press, Washington, DC.
- De Gussem K., Vandenabeele, P., Verbeken, A., and Moens, L. 2007. Chemotaxonomical identification of spores of macrofungi: possibilities of Raman spectroscopy. *Anal. Bioanal. Chem.*, 387, 2823–2832.
- Elbert W., Taylor P.E., Andreae M.O., et al. 2007. Contribution of fungi to primary biogenic aerosols in the atmosphere: wet and dry discharged spores, carbohydrates, and inorganic ions. *Atmos. Chem. Phys.*, 7, 4569–4588.
- Fang T.C., Megaridis C.M., Sowa W.A., and Samuelsen G.S. 1998. Soot morphology in a liquid-fueled, swirl-stabilized combustor. *Combust. Flame*, 112, 312–328.
- Hu B., Yang B., and Koylu O.U. 2003. Soot measurements at the axis of an ethylene/air non-premixed turbulent jet flame. *Combust. Flame*, 134, 93–106.
- Hu B. and Koylu U.O. 2004. Size and morphology of soot particulates sampled from a turbulent nonpremixed acetylene flame. *Aerosol Sci. Technol.*, 38, 1009–1018.
- Reponen T., Grinshpun S., Conwell K., et al. 2001. Aerodynamic versus physical size of spores: Measurement and implication for respiratory deposition. *Grana*, 40, 119–125.