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Notes & Tips

Dual fluorescent halogen immunoassay for bioaerosols using confocal microscopy

Brett J. Green [a,*], Lyndell L. Millecchia [b], Francoise M. Blachere [a], Euan R. Tovey [c], Donald H. Beezhold [a], Detlef Schmechel [a]

[a] *Allergy and Clinical Immunology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, WV 26505, USA*
[b] *Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, WV 26505, USA*
[c] *Woolcock Institute of Medical Research, Sydney, NSW, Australia*

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The need to measure bioaerosols has increased dramatically in recent years due to the growing incidence of respiratory diseases. Fungi are among the most common bioaerosols that humans inhale and exposure to fungi in domestic and occupational environments has been associated with adverse health effects such as infections, allergies, mycotoxicoses, or irritations [1]. Numerous fungal species are known to produce virulence factors, mycotoxins, and allergens, but many of the fungi that occur naturally in the environment remain uncharacterized. This is partly due to the tremendous diversity of fungal species in the environment, the current lack of analytical methods for the detection and characterization of fungal exposures, and the absence of standardized fungal extracts for the development of immunodiagnostic assays.

Many strategies have evolved to sample, identify, and interpret fungal exposure; however, no strategy serves all purposes, as exposure is a complex and dynamic process confounded by spatial, temporal, and geographic variations in airborne counts. Current techniques for fungal exposure assessment rely on sample cultivation or volumetric air sampling in combination with light microscopy [2]. However, these techniques are often time consuming and subjective and require mycological expertise. More recently, molecular techniques based on PCR and immunoassays have been developed to overcome some of these limitations, although the quantification of viable fungal particles still remains a challenge [2].

In our earlier work [3], we described the development of an enzyme-based halogen immunoassay (HIA).[1] This assay relies on the capture of airborne particles onto protein-binding membranes by volumetric air sampling. Eluted antigens are either detected with monoclonal antibodies (mAbs) to identify specific antigens of interest (environmental monitoring) or immunostained with human serum IgE to identify the patient's sensitization patterns (serological monitoring). Immune complexes are visualized as a halo of immunostaining around the particles using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate staining [4,5]. More recently, this enzyme-based technique has been modified to allow the double immunostaining of monoclonal and human serum antibodies in the same experimental sample [6,7].

The objective of this study was to modify the original enzyme-based HIA to a fluorescent immunoassay format to take advantage of the versatility of confocal laser scanning microscopy. This technology can greatly improve particle resolution and contrast and provide for future quantitative image analysis and automated sample processing capabilities. The improved resolution of confocal microscopy also allows one to demonstrate specific localization and colocalization patterns of the target antigens for diagnostic reagent characterization. Supplementary Fig. 1 shows a schematic illustration of the assay procedure. To demonstrate this approach, we selected experimentally

* Corresponding author. Fax: +1 304 285 6126.
E-mail address: Brett.Green@cdc.hhs.gov (B.J. Green).

[1] *Abbreviations used:* HIA, halogen immunoassay; mAb, monoclonal antibody; MCE, mixed cellulose ester; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DIC, differential interference contrast.

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aerosolized fungal spores of *Stachybotrys chartarum* as a model bioaerosol and two specific mAbs, which recognize molecules produced by *S. chartarum*. The mAb 9B4 (IgG1) is species- and sporulation-stage-specific [8] and the mAb 6D4 (IgM) is directed against Stachylysin, an important hemolysin of *S. chartarum* [9,10].

A nontoxic isolate of *S. chartarum* (JS5105, [8]) was grown on malt extract agar and aerosolized with a jet of air directed across the plate; particles were collected by suction onto mixed cellulose ester (MCE) protein-binding membranes (0.8 μm pore size; Millipore Corp., Bridgewater, MA). Impacted fungal particles were permanently laminated to the MCE by overlaying the sample with an optically clear adhesive/glass coverslip as described previously [4,11]. The laminated samples were immersed in borate buffer (pH 8.2) at room temperature for 3 h to enable antigens and other macromolecules to elute from the fungal particles and bind in close proximity to the membrane. Membranes were blocked in 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 90 min and then incubated overnight at 4 °C with tissue culture supernatant of both the mAbs 9B4 (IgG1) and 6D4 (IgM) diluted 1:50 in 5% BSA/PBS/0.05% Tween 20. Negative control treatments were processed in parallel by substituting the mAbs with (1) hybridoma tissue culture medium [8] diluted 1:50 or (2) control mAbs 16C5 (IgG1) and 1B9 (IgM) diluted 1:50. The control mAbs have been shown to extensively cross-react with the mycelium and spores of a number of fungal species, including several *Aspergillus* and *Penicillium* species but not with *S. chartarum* [12,13].

For double fluorescent detection of mAbs, the membranes were rinsed three times in PBS/0.05% Tween 20 and incubated overnight at 4 °C with both Alexa Fluor 594 goat anti-mouse IgG (red fluorescence) and Alexa Fluor 488 goat anti-mouse IgM (green fluorescence; Molecular Probes, Inc., Eugene, OR) diluted 1:500 in 5% BSA/PBS/0.05% Tween 20. This was followed by rinsing the membranes three times in dH_2O. Each sample was then mounted on a microscope slide in ProLong Gold (Molecular Probes, Inc.) antifade reagent. Confocal laser scanning images were captured using a Zeiss LSM 510 laser scanning confocal system (Carl Zeiss Inc., Thornwood, NY) with an Axioplan 2 microscope, 40X C-Apochromat water immersion objective lens, and argon and HeNe lasers. The images of 9B4-labeled fungal particles were captured using 543-nm excitation and long-pass 560-nm emission, while 6D4-labeled fungal particles were simultaneously visualized using 488-nm excitation and a narrow emission filter bandwidth (505–550 nm). Fluorescent and differential interference contrast images (DIC) were captured using Zeiss software version 3.2 (Carl Zeiss Inc., Thornwood, NY). All settings on the confocal microscope remained constant in the analysis of positive samples and negative controls.

Fig. 1 shows the double immunodetection of *S. chartarum* antigens using confocal laser scanning microscopy. Compared to previous results using the NBT/BCIP precipitating substrate methodology [4,6,7,11], this technique resulted in much higher particle resolution and brighter antibody staining patterns. Antibody staining for the sporulation-stage-specific mAb 9B4 (Figs. 1A–F) resulted in

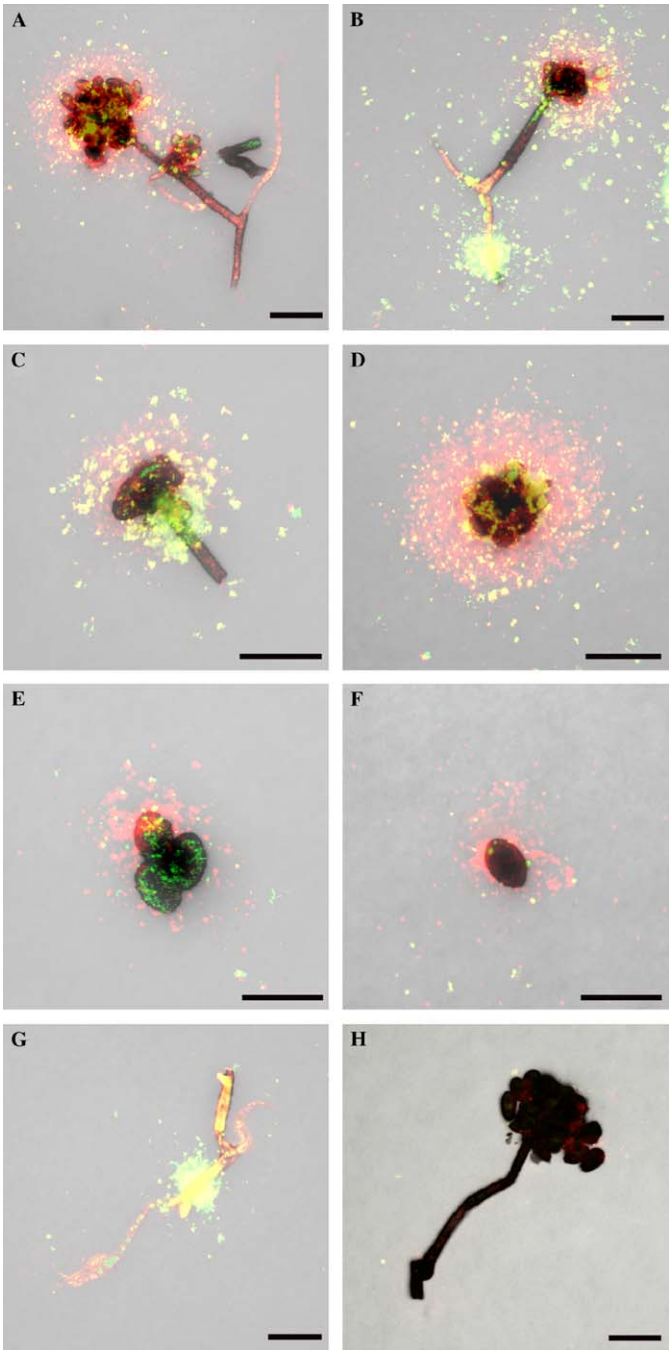

Fig. 1. High-resolution images of dual HIA colocalized immunostaining (yellow fluorescence) of culture-derived *S. chartarum* using mAbs 9B4 (red fluorescence) and 6D4 (green fluorescence). Each image is a composite of two focal image planes, membrane level and fungus level (8-μm interval) superimposed on the DIC image of the fungal particulates. Conidiophores (A and B); detached groups of phialides (C and D); phialoconidia (E and F); and a hyphal fragment (G) labeled with 9B4 (red), 6D4 (green) or expressing their colocalized immunostaining (yellow). Negative control (H) using mAbs 16C5 (IgG1) and 1B9 (IgM) diluted 1:50, showed no specific localized immunostaining, however, faint nonspecific staining was observed on the surface of the particles. No immunostaining was observed in negative controls using hybridoma tissue culture medium (data not shown). Scale bar, 20 μm.

distinct homogenous haloes around conidiophores with phialides, detached phialides, and phialoconidia but not melanized hyphae, whereas for mAb 6D4 (Stachylysin) immunostaining was heterogeneous with numerous intense spots, which were primarily localized around phialides (Figs. 1A–D) and septal wall junctions in hyphae (Figs. 1B and G). Fig. 1 also demonstrates that the extension of the HIA to confocal laser scanning microscopy with double immunofluorescence allows the colocalization of two distinct fungal antigens using mAbs, which can be visualized as bright yellow fluorescence by digitally merging the two separate antibody images and the DIC image. Given the availability of additional species-specific mAbs to other fungi in the future, multiple species can potentially be labeled in the same sample using this technique.

In summary, the dual fluorescent HIA has several distinct advantages over conventional immunoassays. It enables the colocalization of different mAb pairs (Supplementary Figs. 1A–D) or mAbs in combination with human serum antibodies (Supplementary Figs. 1E–H). The fluorescent HIA also provides novel insight into the localization of antigen expression patterns of fungi, which in turn will aid in the characterization and selection of stage- and species-specific mAbs for the production of much needed immunodiagnostic reagents and the development of automated immunoassays for airborne fungal pathogens in the future. Our approach avoids many of the potential problems associated with traditional airborne fungal measurement techniques and future work will investigate computer-based quantification of fluorescent HIA images. The combination of environmental and serological monitoring will provide patient-specific exposure and sensitization profiles that will help to elucidate adverse health effects due to fungal aerosols and ultimately contribute to better patient management. Future applications of this approach may also be relevant for the detection of other bioaerosols in plant pathology, human health, and biodefence.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2006.03.035.

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