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ORIGINAL ARTICLE

A non-fluidic, fluorometric assay for the detection of fungi on cultural heritage materials

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Abstract Cultural heritage materials are particularly susceptible to biodeterioration by fungi. Improper care and storage of artifacts contaminated with fungal material can promote the growth of these microscopic organisms and the inevitable deterioration that follows. Technology capable of detecting vegetative fungi and their reproductive structures could facilitate the struggle against fungal biodeterioration. Archivists and conservators could be notified of fungal contamination within a collection and apply pre-emptive measures, such as modification of environmental conditions, to prevent biodeterioration. The aim of this study was to improve and simplify a fluorometric assay used for the early detection of minute quantities of fungal biomass on cultural heritage materials. To this end we have successfully developed a non-fluidic assay in which fluid transfers, centrifugation steps, and much of the specialized equipment formerly needed to perform the assay are eliminated. The time required for completion of the assay was reduced to 30 min. Use of the assay was also expanded to include the early detection of viable fungal conidia from several species of fungi. These refinements will expedite implementation of this technology by archivists and conservators as they monitor and combat the fungal deterioration of cultural heritage materials.

Keywords Fungi · Conidia · Non-fluidic · Early detection · Cultural heritage

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Introduction

The contamination and subsequent deterioration of film, paper, and canvas by microscopic fungi is a serious threat to art and archival collections. The removal of established fungal communities from these materials can be costly and risk further damage to artifacts and documents. Storage of these materials in low relative humidity (RH) (<65 %) and low temperature (<20 °C) repositories (Pinzari et al. 2006) can slow fungal growth, but the use of such facilities can be costly, and moisture can infiltrate ill-equipped archival collections via floods, leaky roofs, seeping walls, or condensation (Haines and Kohler 1986). Detection of fungal contamination within repositories of cultural and intellectual artifacts would enable archivists and conservators to identify at risk objects and discern the early stages of fungal growth before visible or lasting damage has occurred.

Fungi and their reproductive structures (conidia) are ubiquitous components of the indoor environment (Burge et al. 1980). A wide variety of the fungi associated with library materials and archives have been identified (Zyska 1997; Rakotonirainy et al. 2007; Zotti et al. 2008). These fungi can introduce blemishes that alter the aesthetic properties of paper (Meynell and Newsam 1978; Florian and Manning 2000; Pinzari et al. 2006), generate destructive acids (Sarantopoulou et al. 2006), and depolymerize the cellulose fibers that give paper its tensile strength (Ponce-Jimenez et al. 2002; Piantanida et al. 2006; Zotti et al. 2008). Fungal growth can induce the decolorization of pigments (Berovic 2003), disintegration of paint layers, and degradation of binding agents in canvas paintings (Ciferri 1999) as they utilize nutrients from the organic material in these substances (Gettens et al. 1941; Strzelczyk 1981). Moreover, cellulolytic and proteolytic enzymes secreted by fungi attack the underlying canvas fibers of the artwork itself. Fungi are also capable of growth on photographic film (Fig. 1), which is composed of polymers and nitrocellulose or cellulose acetate, coated with a gelatin-based emulsion. Numerous fungi secrete

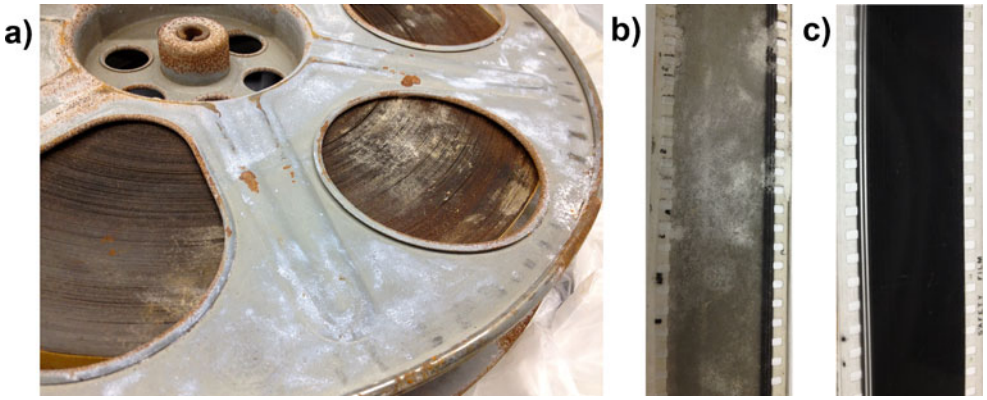

Fig. 1 Biodeterioration of film by fungi. **a** Accumulations of fungi (white deposits) are visible at several locations on the side of the film reel. **b** Biodeterioration of a 20-cm sample of 35 mm film. **c** A relatively pristine sample of 35 mm film included for comparison

enzymes that liquefy this gelatin emulsion as they mine it for organic molecules (Abruscia et al. 2005). This liquefaction deteriorates the film, stimulates further growth of fungi, and obscures any underlying images.

Previous studies have described the use of a fluorometric method based on the activity of beta-*N*-acetylhexosaminidase (3.2.1.52) (NAGase), an enzyme constitutively expressed by a plethora of filamentous fungi, to detect and estimate vegetative fungal biomass on various cultural heritage materials (Konkol et al. 2010, 2012). This method offers several advantages over detection methods that involve microscopic estimation of hyphal biovolume, determination of ergosterol content, ATP activity, and quantitative immunological techniques (Gessner and Newell 2002). However, early versions of the fluorometric method, which detected the NAGase-catalyzed release of flourogenic 4-methylumbelliferyl (MUF) from the compound *N*-acetyl-beta-*D*-glucosamine (NAG) found in fungal cell walls, required the preparation and transfer of several chemical solutions, multiple centrifugation steps, and specialized laboratory equipment, all of which made its use impractical for conservation scientists.

The non-fluidic fluorometric assay detailed below utilizes self-contained assay strips infused with the fluorogenic MUF-labeled NAG (MUF-NAG) substrate. Fluorescence of any liberated MUF molecules in the presence of NAGase is stimulated with UV light and recorded with a digital camera. Extended exposure times (1.6 s) and subsequent analysis of the digital images revealed a linear relationship between the number of *Aspergillus niger* conidia and MUG fluorescence and established a lower detection limit of a few hundred viable *A. niger* conidia. The subsequent growth of vegetative fungal biomass from these conidia was also monitored on film, paper, and canvas coupons. Use of the assay was expanded to several previously untested fungal species known to cause biodeterioration of cultural heritage materials. The NAGase activity of viable and non-viable *A. niger* conidia was also assayed and confirmed NAGase activity was absent in non-viable conidia.

Materials and methods

Filter paper assay strips

Squares (1 cm^2) of Whatman #5 filter paper (Whatman, Maidstone, Kent, UK) were sterilized at 121 °C for 15 min. A 30-μl aliquot of 200 μM MUF-NAG (Sigma-Aldrich, St. Louis, MO) was added to each square, and the filter paper strips were allowed to air dry for at least 12 h before use. The prepared assay strips were wrapped in aluminum foil and stored at room temperature for up to 8 weeks prior to use.

Construction of the standard curve

Conidia were harvested from 7- to 10-day-old cultures of *A. niger* grown on Difco™ Tryptic Soy Agar (Becton-Dickinson and Co., Sparks, MD) by pipetting sterile H_2O onto the surface of the mycelium. The conidial suspension was removed from the surface of the TSA plate and transferred to a sterile 1.5-ml microfuge tube and briefly centrifuged. The supernatant was removed, and the pellet was resuspended in sterile H_2O. Conidia were suspended in sterile H_2O at the following concentrations: 350, 560, 933, 1,400, and 1,750 conidia/μl. A direct count of conidia was conducted in a hemocytometer. A 1-μl aliquot of each suspension was dropped in the center of the 1-cm^2 filter paper assay strips containing 30 μl of 200 μM MUF-NAG, and the assay strips were allowed to air dry for 5 min. Six replicates of each dilution and a negative control assay strip were tested. The assay strips were placed on a sheet of wet filter paper, and the wicking action of the assay strips resulted in a uniform moistening of each test strip. The moist assay strips were incubated at 30 °C for 30 min.

Images were acquired under UV light (1.6 s exposure). The intensity of fluorescence was measured in an area of 19.104 mm^2 using a Molecular Imager® Gel Doc™ XR System with Quantity One software (Bio-Rad Laboratories, Hercules, CA). The average background fluorescence was subtracted from all subsequent measurements, and the

resulting numbers were plotted against the direct count of conidia measured using a hemocytometer.

Detection of *A. niger* on cultural heritage materials

Samples of film, paper, and canvas were cut into 3×6-cm coupons. The front and back sides of the film were each sterilized under UV light for 15 min. Paper was sterilized at 121 °C for 15 min. Each material was assayed in triplicate. Individual coupons of each material were placed in sterile petri dishes, and each coupon was inoculated with 2.7×10^5 *A. niger* conidia suspended in 20 μl malt extract broth. The negative control coupons were inoculated with 20 μl each of sterile de-ionized water. All dishes were placed inside a humidity chamber (70 %) and incubated at 30 °C. The materials were assayed for conidia at 0 h and for vegetative fungal biomass after 16, 24, and 42 h by rubbing the 1-cm^2 assay strips infused with 30 μl of a 200 μM MUF-NAG solution across the surface of each coupon with sterile forceps in a circular motion for 5 s. Images were acquired under UV illumination and analyzed as described above.

Detection of various fungal species

Fungal material, including spores and hyphae, were removed from plates using a toothpick and transferred to the 1-cm^2 assay strips previously inoculated with 30 μl of a 200 μM MUF-NAG solution. The assay strips were moistened with sterile H_2O as detailed above and incubated for 15 min at 30 °C. Fluorescence was visualized with a benchtop UV transilluminator.

Ability to differentiate between viable and non-viable conidia

Conidia were harvested from 7- to 10-day-old cultures of *A. niger* grown on TSA by pipetting sterile H_2O onto the surface of the mycelium. The conidial suspension was removed from the surface of the TSA plate, transferred to a sterile 1.5-ml microfuge tube, and briefly mixed using a vortex mixer. The suspension was divided into three equal 0.5-ml aliquots. The conidia in each suspension were pelleted by centrifugation (30 s) and the supernatant removed. The pellet that served as the untreated control was resuspended in sterile H_2O and held at room temperature. One pellet was resuspended in H_2O and heated at 65 °C for 2 h. The third pellet was resuspended in 70 % EtOH and held at room temperature for 2 h. Each aliquot was centrifuged for 30 s, the supernatant was removed, and all pellets were resuspended in sterile H_2O. A 1-μl aliquot of each suspension was applied to the center of the 1-cm^2 assay strip and analyzed as detailed above. Each condition was tested in duplicate.

Results and discussion

Construction of the standard curve

The number of *A. niger* conidia in each sample was related to fluorescence intensity through the construction of a calibration curve (Fig. 2). A consistent linear relationship (R^2=0.9931) between NAGase activity and fluorescence was maintained as

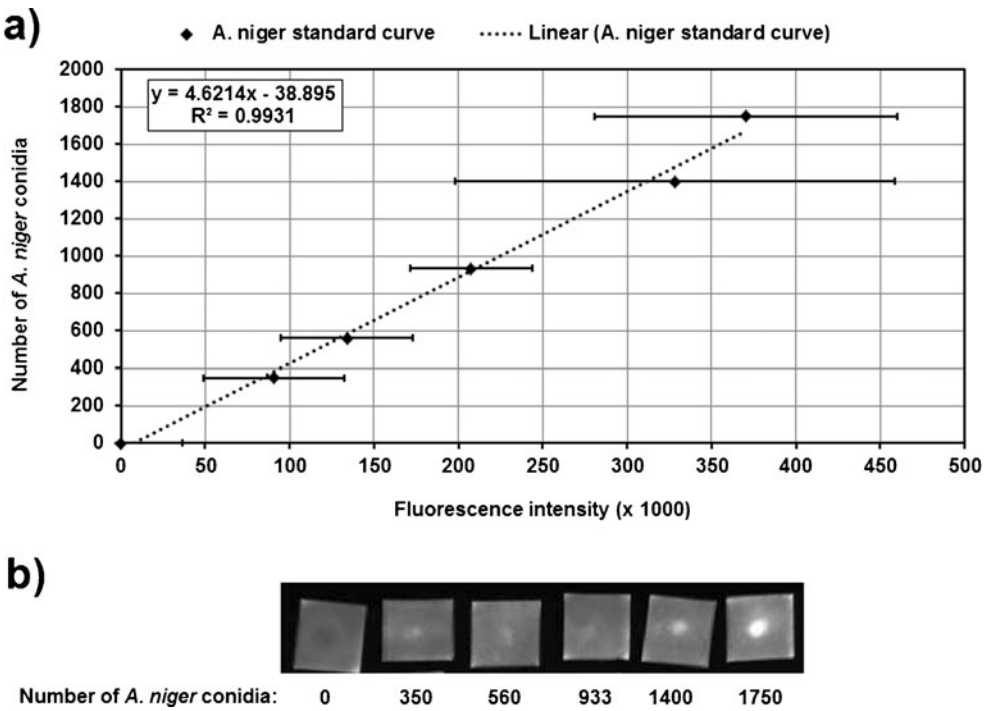

Fig. 2 **a** Graphic representation of the relationship between the number of *Aspergillus niger* conidia and fluorescence intensity. From *left to right* each data point represents the fluorescence generated by 0, 350, 560, 933, 1,400, and 1,750 *A. niger* conidia, respectively (R^2=0.9931, p<0.001). **b** Fluorescence of 0, 350, 560, 933, 1,400 and 1,750 *A. niger* conidia, respectively

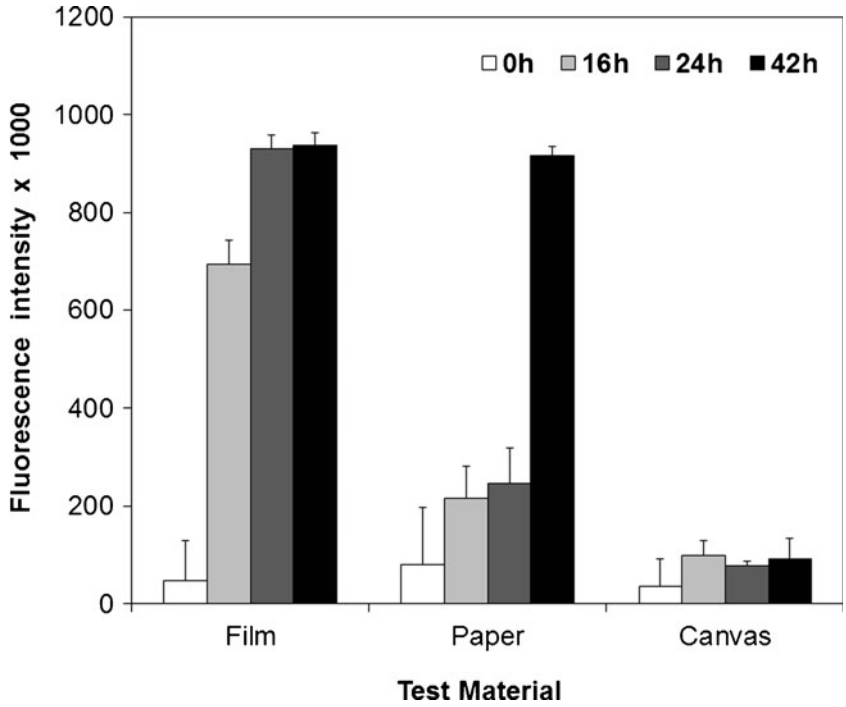

Fig. 3 Fluorescence generated by *A. niger* conidia (0 h) and vegetative biomass (16, 24, 42 h) on film, paper, and canvas coupons incubated at 30 °C. Data points represent the means of six replicates. *Bars* Standard deviation (SD)

the total number of conidia assayed increased from 350 to 1,750.

The construction of a standard curve permits the quantification of unknown numbers of conidia on cultural heritage materials. The fluorescence generated by an unknown quantity of conidia on an artifact may be plotted against the fluorescence generated by known quantities of conida on the standard curve. An estimate of the number of conidia may then be obtained at the point where the recorded fluorescence and the standard curve intersect.

Lower detection limit and resolution

The fluorescence generated from the NAGase cleavage of the MUF-NAG substrate by progressively smaller numbers of *A. niger* conidia in a defined area (19.104 mm^2) was used to resolve the lower detection limit of the assay (Fig. 2a). The mean fluorescence generated by 350 conidia/19.104 mm^2 was significantly higher ($p<0.05$) than the mean background fluorescence according to an analysis of variance (ANOVA) and established this number as the lower detection limit. Statistical analysis using ANOVA demonstrated that the mean fluorescence produced by the samples of 350 conidia and 560 conidia was not statistically different ($p=0.09$) nor was the mean fluorescence produced by any adjacent pair of data points in the series. Therefore, the estimated resolution of this method was 583 conidia/19.104 mm^2; the difference between 933 and 350 conidia/19.104 mm^2. However, visual analysis of fluorescence intensity displayed in the individual digital images used to generate the standard curve was distinguishable between different quantities of spores (Fig 2b), particularly when the concentration of conidia exceeded 933/19.104 mm^2.

The assay strips were capable of distinguishing between populations of *A. niger* conidia that vary by more than 583 conidia/19.104 mm^2. Quantitative analysis of the assay strips was unable to resolve some differences in the number of conidia tested (Fig. 2a), but digital images of the assay strips captured with a 1.6-s exposure clearly permitted these differences to be distinguished (Fig. 2b). Therefore, the difficulties experienced with the quantitative analysis should not preclude the use of the assay strips as a rapid, semi-quantitative means of detecting the fungal contamination of cultural heritage materials.

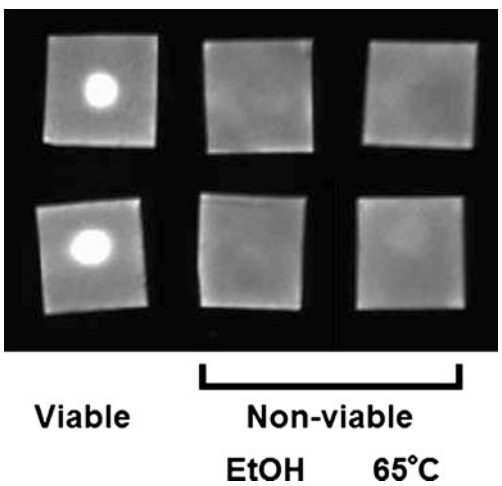

Fig. 4 Fluorescence of viable and non-viable *A. niger* conidia in duplicate. Non-viable conidia were either submerged in EtOH or heated to 65 °C

Detection of *A. niger* on cultural heritage materials

The utility of our non-fluidic assay and its application to cultural heritage materials was tested on film, paper, and canvas coupons inoculated with equal numbers of *A. niger* conidia. Immediately after inoculation of the test coupons at 0 h, the assay strips were able to discern the fluorescence generated by 2.7×10^5 *A. niger* conidia from background fluorescence (Fig. 3). Vegetative fungi could also be detected at all subsequent time points on all three materials. Over the length of the incubation period, as the conidia germinated and vegetative fungal biomass increased, there was a corresponding increase in fluorescence. The first time point used to assay vegetative fungal biomass was at 16 h; 2 h earlier than previous studies that utilized less refined iterations of the assay (Konkol et al. 2012). These data demonstrate the ability of the assay strip to detect both conidia, used by the fungi for reproduction and survival during harsh conditions, and active growth of vegetative fungi that predominate under more optimal growth conditions, such as high RH. The detection of both forms is important, as the presence of conidia demonstrates the potential for biodeterioration, while the detection of vegetative fungal biomass signifies that conditions favoring fungal growth do exist, that fungal-induced biodeterioration is likely underway, and that urgent action is required.

This series of experiments demonstrated the utility of the non-fluidic assay strips. The detection of both reproductive and vegetative fungal structures on remote or immobile artifacts should be possible. Such artifacts could be sampled with the assay strips on-site, and the strips stowed for transport. Reactions could then be initiated by exposing the strips to moisture and viewed under a UV light source at a suitable location. The use of digital imaging equipment and image analysis software in our study were for documentary purposes, and this step could be eliminated when such documentation is not required. In many cultural heritage settings a simple UV transilluminator will be sufficient for the generation of fluorescence from MUF.

Range of fungal species

A diverse assortment of filamentous fungi that belong to the divisions Ascomycota (*Aspergillus niger*, *Hormoconis resinae*, *Penicillium chrysogenum*, *Penicillium pinophilum*), and Deuteromycotina (*Epicoccum niger*) were detected with the assay strips. Conidia from all of the fungi listed above generated high fluorescence intensity when exposed to the assay strips. Members of *Aspergillus*, *Hormoconis*, and *Penicillium* have been implicated in the degradation of canvas and paper artifacts (Zyska 1997).

It is important to state that the assay strips have the capability to detect disparate genera of fungi. Previous studies demonstrated the capacity of the MUF-NAG substrate to detect a wide assortment of fungi (Miller et al. 1998). The inclusion of the fungus *Epicoccum niger* in this study provides additional evidence of the ability of the assay strip to detect a diverse assemblage of fungi with the potential for deterioration of cultural heritage materials.

Viable versus non-viable conidia

NAGase activity appears only in viable fungal conidia. Non-viable conida, inactivated with EtOH or heat, do not generate NAGase activity and are not detectable with the assay strips (Fig. 4). This eliminates the possibility of false positive reactions that detect dead fungal material incapable of further growth even under optimal growing conditions.

Conclusion

Fungal growth on a cultural artifact can lead to irreparable damage if not detected and treated. Detection is often delayed until altered aesthetics or odors, the first outward signs of fungal contamination, are noticed (Garg et al. 1995; Ciferri 1999; Rakotonirainy et al. 2003). The capability of our non-fluidic assay to detect both conidia and vegetative fungal growth at an early stage provides an important new tool for the protection of cultural heritage materials. Additional research is required to determine the shelf-life of the assay strips and to develop a more flexible and inexpensive system for semi-quantitative detection and analysis.

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