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Aspergillus fumigatus Biofilms: a Comparison of Processing Techniques for Scanning Electron Microscopy of Fungal Mycelium and Extracellular Matrix.

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Introduction: Biofilms are matrix-enclosed microbial populations adherent to each other and/or to surfaces or interfaces [1,2]. It has recently been shown that *Aspergillus* produces *in vitro* an extracellular matrix with typical biofilm characteristics under static and shaken, submerged conditions [3]. *Aspergillus fumigatus* is frequently isolated from cystic fibrosis (CF) patients, and *Aspergillus* biofilms may be one of the most important virulence factors in CF and invasive pulmonary aspergillosis [4, 5]. In-depth analysis of *Aspergillus* biofilms is therefore necessary to improve antifungal targets for treating complex *A. fumigatus* biofilm-associated diseases [6]. SEM analysis of the 3D architecture of hydrated biofilms is commonly affected by standard fixation and drying techniques [7], and stabilization of proteins through aldehyde cross-linking, with post-fixation of lipids with osmium-tetroxide (OsO_4), help maintain overall biofilm structure. Retention of fine features is generally accomplished through critical point drying (CPD) or hexamethyldisilazane (HMDS) [8]. Environmental SEM, using Ruthenium Red as contrasting agent, or Variable Pressure (VP)-SEM using ionic liquids, have been reported to improve imaging of hydrated biofilms and their natural *in situ* 3D architecture [9, 10, 11].

In this study we investigated the effect of processing techniques and reagents on SEM analysis of the cellular mycelium and extracellular matrix (ECM) of two modes of biofilm growth of *A. fumigatus.* Processing parameters evaluated were (1) time in primary aldehyde fixatives, (2) including OsO_4 as secondary fixative, (3) final drying through CPD or HMDS and (4) hydrated structure with VP-SEM.

Methods: *A. fumigatus* biofilms were grown in RPMI 1640 culture medium on 12mm circular plastic rotating bioreactor disks, or as a floating biofilm mat close to the water-air interface. After 2 days of growth, disks and biofilm mats were removed from culture medium, rinsed in phosphate-buffered saline and fixed in 4% paraformaldehyde (PFA) with 2% glutaraldehyde (GA) in 0.1M sodium cacodylate buffer. Table 1 summarizes the processing parameters evaluated. Hydrated samples were observed with a Hitachi 3400-N SEM operated at 15kV, 60Pa, using Backscattered Electron (BSE) detection. Dried samples were sputter-coated (50Å, Au/Pd) before imaging with a Hitachi 3400N SEM operated at 10kV under high vacuum, and a Zeiss Sigma FESEM using InLens Secondary Electron (SE) detection at 2kV.

Results: Post-fixation with OsO_4 generally improved ultrastructure, while also enhancing SE and BSE detection for SEM analysis. Also, shorter periods (less than 1hr) in both aldehyde and OsO_4 fixatives resulted in improved separation of fine structural features (Figure 1), while longer fixation times caused a collapse of fungal mycelium and fibers in the extracellular matrix. CPD resulted in improved preservation of especially ECM, whereas biofilms dried with HMDS showed more collapsed hyphae connected by sheets of ECM lacking fibrous ultrastructure. Inherent to VP-SEM is the poor signal to noise ratio due to gas and moisture in the specimen chamber, as well as the occurrence of water coating hyphae as an electron dense sheet, which obscures fine cellular features while retaining 3D structure.

Conclusions: Revisiting standard processing protocols for EM analysis of microbial biofilms emphasizes the complexities involved in visualizing the attached lifestyle of microbial communities. Fixation and dehydration-induced artifacts should be thoroughly analyzed to determine the appropriate combination of techniques that will best reveal specific structural aspects of biofilms. Using VP-SEM instead of high-vacuum SEM may better reveal hydrated 3D architecture, but limit ultrastructural analysis of individual cells and extracellular matrix. Our results suggest that shortened times of aldehyde fixation and OsO_4 post-fixation, followed by CPD, is optimal for high-resolution ultrastructural SEM analysis of cellular features and extracellular matrix of *Aspergillus* biofilms.

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BIOFILM CULTURE (x2): FLASK versus DISK							
2%GA + 4%PFA + 1%OsO_4				2%GA + 4% PFA (no OsO_4)			
24hrs fix		45min fix		24hrs fix		45min fix	
CPD	HMDS	CPD	HMDS	CPD	HMDS	CPD	HMDS
Hydrated: VP-SEM (60Pa)				Hydrated: VP-SEM (60Pa)			

Table 1: Summary of fixation and drying parameters used to process *A. fumigatus* biofilms for SEM.

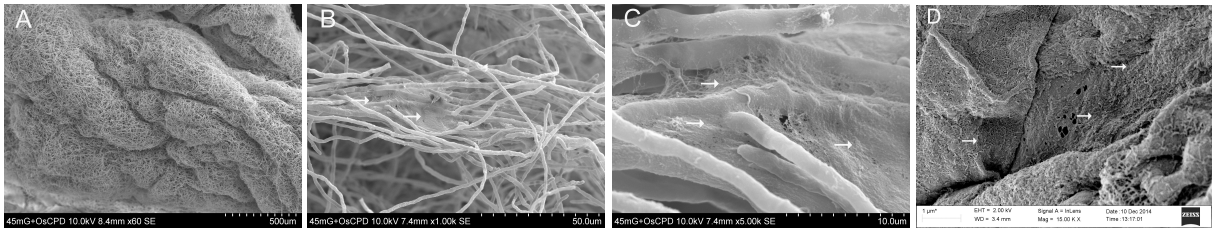

Figure 1: SEM images illustrating typical biofilm characteristics after optimal fixation periods in PFA, GA and OsO_4, followed by CPD. In the dense mycelium (A) hyphae are connected by fibrous ECM (arrows) (B, C), which can be seen closely adherent to the cell surface at high magnification (D).