Imaging of the Development and Therapeutic Response of an *In Vivo* Fungal Catheter Biofilm

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Introduction

The majority of human pathogens cause disease in a biofilm lifestyle. Biofilms are communities of cells that remain attached to a foreign surface and to each other. The organisms secrete and become embedded in an extracellular polysaccharide matrix. Biofilm infections occur most commonly on implanted medical devices and are quite recalcitrant to antimicrobial therapy. The most common device involved in these infections is the intravenous catheter. More than 5 million intravenous catheters are inserted into patients in the United States annually of which up to 10% become infected with biofilm producing pathogens.

Prior investigations of this process have been undertaken using *in vitro* models. *In vitro* models are unable to account for a number of factors of suggested importance in the biofilm process, including exposure to host factors such as blood cells and proteins, and flow dynamics of the host vasculature system. We developed an *in vivo* venous catheter biofilm infection model in rats using the fungal pathogen, *Candida albicans*. Our goal was to develop assays to follow the development of the biofilms and to be able to monitor therapeutic response over time. The components of the development process that we wished to capture included the overall architecture of the biofilm, burden of viable fungal cells within the biofilm, the morphology of the fungal cells, and the location and density of the extracellular polysaccharide matrix. The following is a description of the use of confocal and scanning electron microscopy as tools to accomplish these goals.

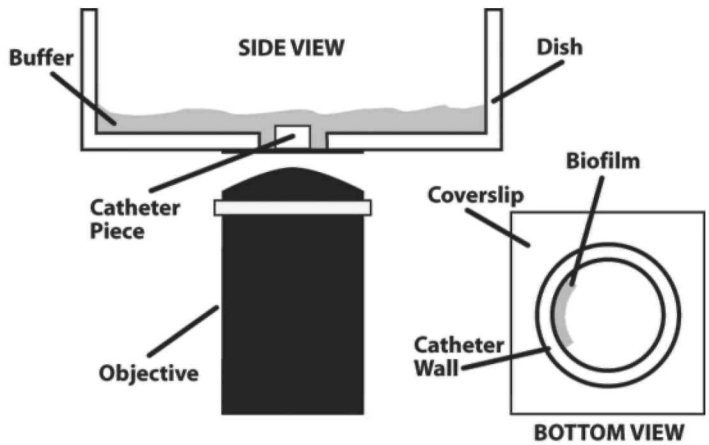
Methods and Results

Gas sterilized, polyethylene, cylindrical tubing (inner diameter 0.76 mm, outer diameter 1.52 mm) was surgically placed 2 cm into the external jugular vein of a rat. The catheter was infected with *C. albicans* via the catheter lumen. The development of the biofilm was monitored at four time points (8, 12, 24, and 48 hr) after infection. Therapy with one of three antifungal drugs was instilled into the catheter lumens after biofilm maturation. The drugs dwelled in the lumen for up to 24 hr. Response to therapy was monitored at three time points (12, 24, and 48 hr). At each monitoring time point in the development and therapeutic studies, rats were euthanized and the catheter removed for analysis.

We identified two fluorescent strains that allow simultaneous imaging of the fungal cell morphology, extracellular matrix, and the viability of the fungal cells within the biofilm. The first is FUN-1, a proprietary stain from Molecular Probes. FUN-1 is a fluorescent two colored probe that allows visualization of the fungal cell wall and provides a measure of fungal viability. The yeast cell wall binds the FUN-1 dye and is visualized with a diffusely distributed yellow-green fluorescence. The stain is also taken up into fungal cell vacuoles and, in metabolically active cells, the strain is altered, resulting in a shift from green to red fluorescence (peak absorbance=508nm, peak emission=590nm). The ability to estimate the number of viable cells per high-power optical field provided a tool to quantify the burden of live and dead cells and determine the effect of various antifungal treatments over time. The cell wall staining properties provided a tool to estimate the density of cells and cell morphology.

The second strain is a Concanavalin A conjugate, (Alexa Fluor 488 conjugate - 200 mM) also from Molecular Probes. Concanavalin A is a lectin probe used for detection of glyco-conjugates and glycoproteins. The Alexa Fluor 488 conjugate of succinylated Concanavalin A selectively binds to α-mannopyranosyl and α-glucopyranosyl residues of cell wall polysaccharides and is visualized as green fluorescence (peak absorbance=495nm, peak emission=519nm). The stain has been shown to bind to the polysaccharide components secreted in *Candida* biofilm formation.

We anticipated biofilm formation predominantly on the lumen catheter surface. However, we wanted to be able to effectively image both surfaces. The imaging orientation set up provided the first major hurdle. The surface of the tubing is curved, thus making it difficult to acquire an image in the direction of the catheter surface. In addition, the common catheter materials auto-fluoresce and interfere with the capture of a detailed image of the organisms and matrix. To circumvent this, we imaged the catheters with an inverted objective "end on" as shown in Figure 1. We constructed



a cutting device to section the tubing perpendicular to its length. The 2- to 3-mm segments were placed in a glass-bottom Petri dish (coverslip 1.5, 35-mm disk P325G 1.5-14C; MatTek, Ashland, MA). This allowed imaging of both the inner and outer catheter surfaces and minimized the effect of the auto-fluorescent activity of the catheter. The Petri dish also provided a container to bathe the biofilm in a physiologic buffer. The buffer provided a liquid milieu similar to the conditions *in vivo* and prevented the sample from drying during the imaging process.

Prior to imaging, the catheter segments were incubated with each of the dyes (FUN-1 50 μM and Con A 200 mM in phosphate buffered saline pH 7.2 with 2% glucose) for 30 minutes at 30° C protected from ambient light. Biofilms were observed with a Nipkow-disc based confocal microscope (Zeiss Axiovert 200) equipped with a mercury arc lamp. The biofilm cells and matrix can be visualized with the 20X and 40X oil objectives. FUN-1 is excited at a maximum of 508nm. FUN-1 is fluorescent when bound to DNA (Em = 538 nm). Uptake and processing of the dye by live yeast results in red-shifted fluorescence (Em ~590 nm). The Concanavalin A, Alexa Fluor 488 conjugate will have excitation and emission maximums of 496 nm and 518 nm. Confocal images of green (Con A) and red

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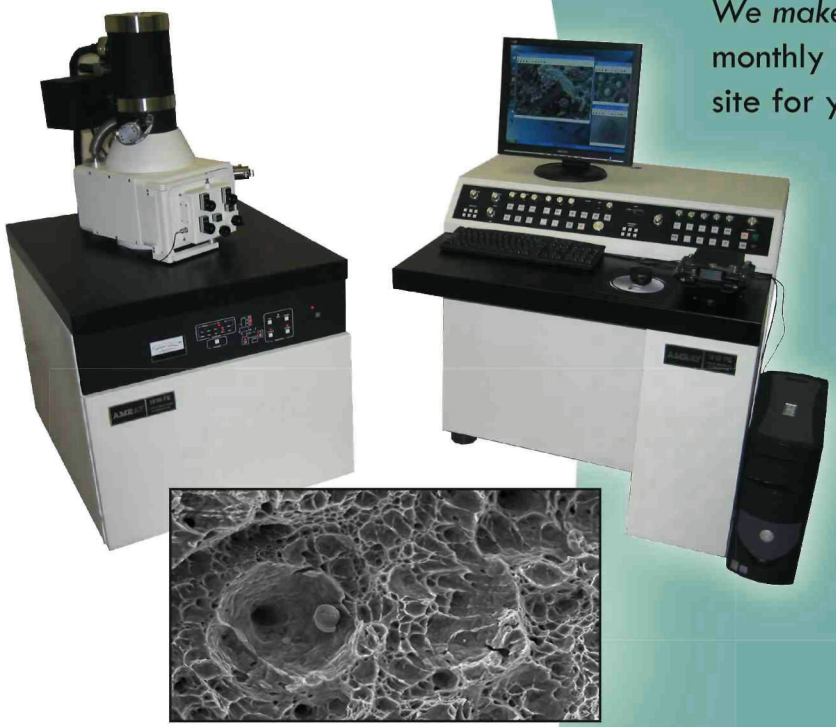

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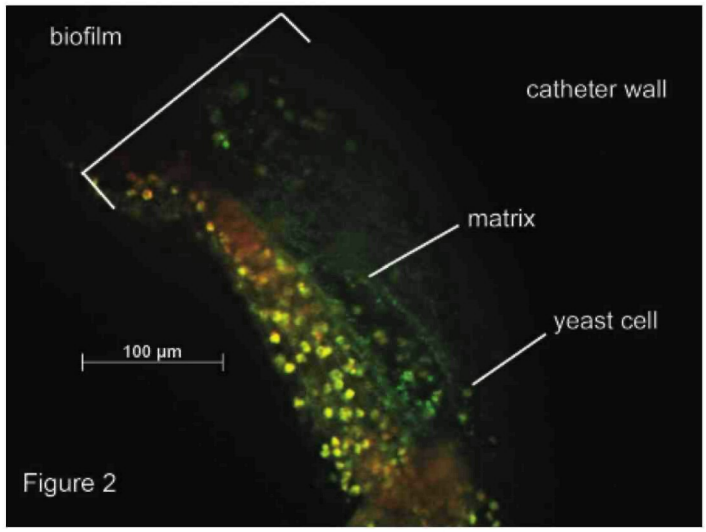

Figure 2

(FUN-1) fluorescence were captured simultaneously using Z-stack mode with fluorescent filters 490/528 nm and 555/617 nm, respectively. Imagining was performed at slice intervals appropriate for Nyquist sampling. The intensity of the dyes is great enough so that the mercury arc lamp provides more than adequate excitation. The exposure times for each of the channels ranged from as short as 50 to 2,000 ms for the 490/528 nm filters. The exposure times required for adequate visualization with the 555/617 nm were much longer (range 3,000 to 12,000 ms).

Figure 2 is a representative 40X image of a *C. albicans in vivo* biofilm on the luminal surface of a polyethylene catheter after 24 hr of growth in rats. Yeast cells range in size from 4 to 10 µm and are visualized throughout the biofilm thickness. Due to co-localization of fluorescent dyes (FUN-1 and Con A), the metabolically active cells appear yellow to orange during multichannel image capture. The matrix appears strand-like and is most dense adjacent to the catheter wall. The density of the matrix makes it more difficult to visualize individual yeast cells. The thickness of the process in most areas is nearly 200 µm. At the 8 and 12 hr time points, the biofilm is only 3 to 4 cell layers thick and the matrix is not as apparent. The differences in the biofilm between 24 and 48 h are minimal.

Scanning electron microscopy

Scanning electron microscopy was used to confirm and expand the architectural investigation of the biofilm process. The predominant hurdle in electron microscopy imaging of the biofilm is the risk of alteration of the architecture during sample processing. However, the images obtained using the following preparative process identified a complex architecture that included maintenance of channels between cells and the fibrous nature of portions of the matrix.

After removal from the animals, the catheter was similarly transected. Catheter segments were washed with PBS pH 7.2 and placed in fixative (1% glutaraldehyde and 4% formaldehyde) overnight. The samples were then washed with PBS for 5 minutes and placed in 1% osmium tetroxide for 30 minutes. Next, the samples were dehydrated in a series of ethanol washes (30%, 50%, 70%, 85%, 95%, 100%, and molecular sieve dried 100%x2), each for 10 minutes. Final desiccation was accomplished by critical point drying. Specimens were mounted on aluminum stubs and sputter-coated with gold for 3 minutes. Samples were then observed in a scanning electron microscope (Hitachi S-5700) at 10 kV.

CryoSEM methods commonly used for biofilm studies were not used in this instance because of size constraints. The tubing plus biofilm samples were too big to fit into the available Balzers HPF-010 high-pressure freezer, and could not be cut down to size without severely altering their morphology and losing their orientation. Similarly, the samples were necessarily too large for freeze-fixing by other methods, without creating serious freezing artifacts.

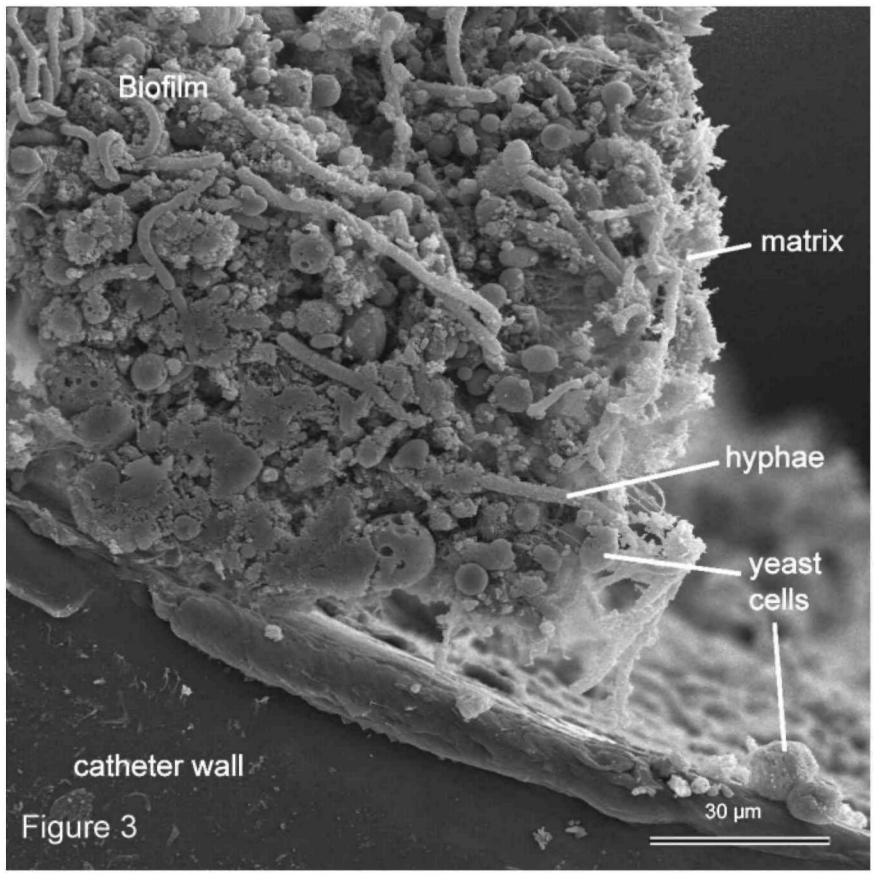

Figure 3

Figure 3 shows a similar end-on orientation of the biofilm at 1000X magnification. Similar to the confocal images, the biofilm process adjacent to the catheter was dense with matrix concretions. Host blood cells (more than 4-fold larger than the yeast cells) were also identified and appeared to be embedded in the matrix.

Conclusions

The confocal and scanning electron microscopy methods provided the tools to characterize *in vivo C. albicans* catheter biofilm development and treatment response. In addition to the use of the combination of fluorescent dyes to visualize the fungal cells and polysaccharide matrix, the unique imaging orientation provided a means to capture this process on a concave medical device surface. ■

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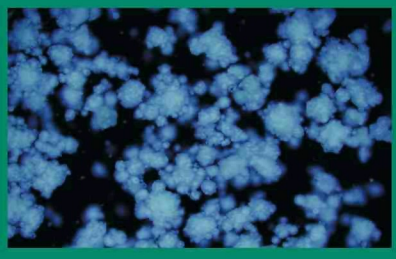

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