Exploring Autofluorescent Properties of a Biofilm Extracellular Matrix Using a Spectrofluorometer

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ABSTRACT

Chronic wounds are defined as wounds that fail to progress through the normal stages of wound healing and remain open for six weeks or longer. Chronic wounds are characterized as polymicrobial and biofilm-associated, meaning the bacteria secrete an extracellular matrix that they inhabit. The production of extracellular matrix provides many benefits to bacteria making biofilm-associated infections more difficult to treat and can lead to adverse patient outcomes. A handheld bacterial imaging device called the MolecuLight *i:X* has been developed that can detect autofluorescent properties of bacteria via the exoproduct porphyrin in real-time to aid in diagnosis and treatment of chronic wounds. In addition to detecting the bacteria, there is also interest in developing a mechanism to detect autofluorescent properties of the extracellular matrix associated with biofilm. We used a polymicrobial mixture of *S. aureus, E. coli, and E. cloacae*, which are part of the ESKAPE pathogen group and are associated with chronic wound infections, to evaluate a bacterial suspension versus biofilm to determine shifts or changes in detection of autofluorescence patterns via a spectral emission scan. The goal of this study is to determine if there are any unique autofluorescent signatures of the extracellular matrix that can inform a biofilm mode with the MolecuLight *i:X* device. This would potentially allow clinicians to diagnose not only bacterial burden, but also biofilm-associated infection, which could potentially modify how chronic wounds are treated.

INTRODUCTION

Chronic wounds are typically polymicrobial, or contain many species of bacteria, and tend to be biofilm-associated. Chronic wound biofilms can contain 100 different microbial species [1], which can synergize their activities, leading to more aggressive and virulent infections [2]. Confirmation of bacterial presence is clinically confirmed via microbiological testing. Culture-based diagnostics identify dominant microorganisms as well as their antimicrobial susceptibility, however the lag time to obtain those results (3 days – 4 weeks) significantly impacts wound care and treatment. The MolecuLight *i:X* imaging device visualizes fluorescence from wound **tissues (green)** and **bacteria (red)**, enabling point-of-care localization of regions with moderate-to-heavy bacterial loads. The device shines a safe violet fluorescent light (405nm) on a wound and visualizes resulting endogenous fluorescence from bacterial porphyrins (~630nm) in real-time. This allows physicians to direct specimen sampling to the area with the heaviest bioburden, improving diagnostic capabilities and to direct targeted treatments.

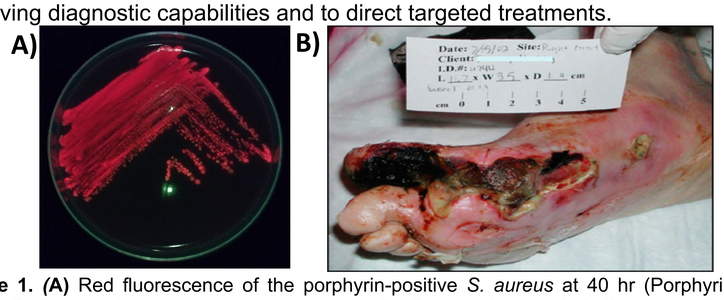


Figure 1. (A) Red fluorescence of the porphyrin-positive *S. aureus* at 40 hr (Porphyrin Test Agar) **(B)** Diabetic Foot Ulcer, photo courtesy of Dr. Randall Wolcott, MD, Southwest Regional Wound Care Center, Lubbock, TX.

BIOFILM-ASSOCIATED INFECTIONS

- Biofilms are polymicrobial communities of microbes that are encased in a self-produced extracellular matrix (EPS)
- Biofilms can be hard to diagnose clinically because biofilms are dynamic and contain many different microorganism. Additionally, the EPS is made up of carbohydrates and sugars similar in chemical structure to host components, making selectively diagnostic or targeted therapies difficult to develop.

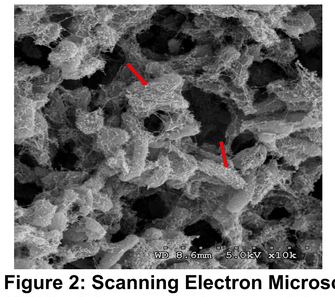


Figure 2: Scanning Electron Microscopy (SEM) of tissue samples containing biofilm. Red arrows note regions of close association between the extracellular matrix and the bacterial cells, indicative of biofilm in tissue.

MOLECULIGHT *i:X* IMAGING DEVICE

- The MolecuLight *i:X* fluorescence imaging device detects most medically-relevant species of bacteria via endogenous red fluorescence of porphyrins [3].
- Porphyrin production in bacteria requires δ-aminolaevulinic acid (ALA), an intermediate in heme synthesis.
- In mammalian systems, ALA is endogenous and readily available. In *in vitro* systems, ALA must be exogenously added.
- The device excites a wound with violet light (405nm) and visualizes resultant bacterial fluorescence (~630nm) in real-time, indicated by bright red on images

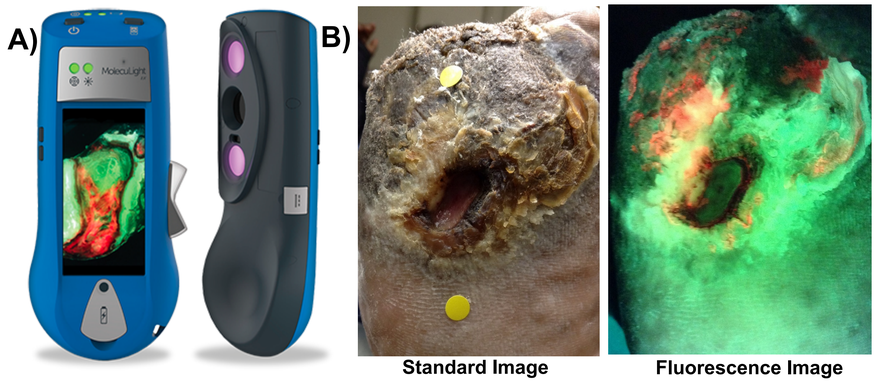
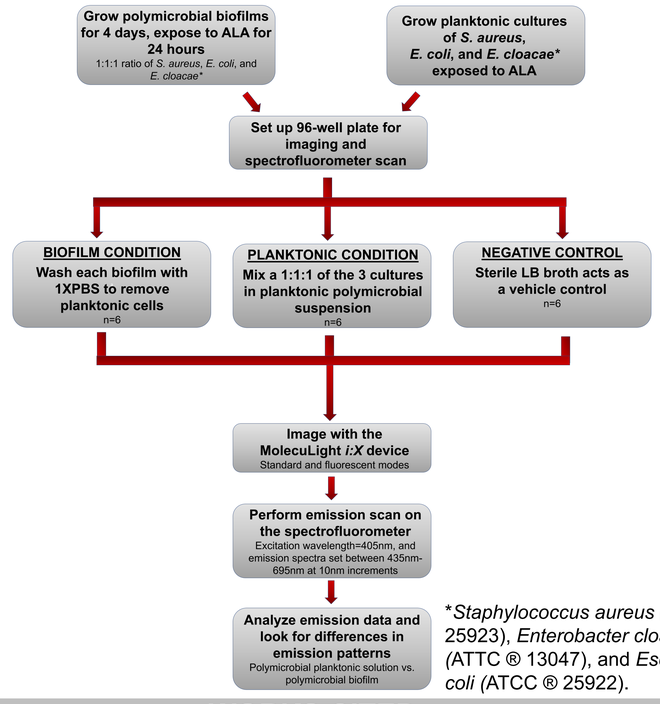


Figure 3: MolecuLight *i:X* Imaging Device (A) Hand-held device and **(B)** images of a chronic foot ulcer wound. Sampled regions of red fluorescence were positive for heavy growth of *Serratia marcescens* [3].

PURPOSE

To determine if the bacterial-derived EPS matrix exhibits any unique fluorescence signature that could be indicative of biofilm, and potentially diagnostic for biofilm with the MolecuLight *i:X* device. This will be determined by conducting an emission spectral scan.

EXPERIMENTAL DESIGN



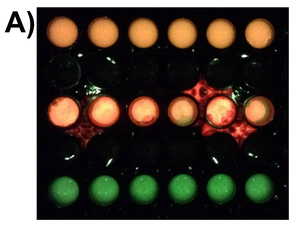
**Staphylococcus aureus* (ATCC ® 25923), *Enterobacter cloacae* (ATTC ® 13047), and *Escherichia coli* (ATCC ® 25922).

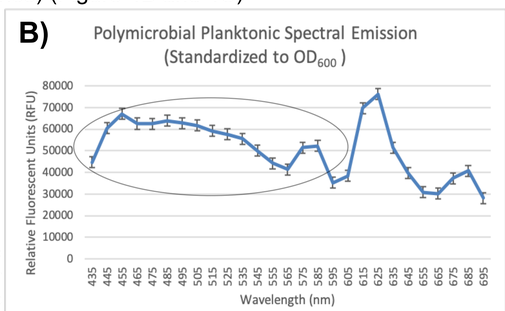
WORKS CITED

[1] Dowd *et al.* 2008. Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. BMC Microbiology. 8: 43
[2] DeLeon *et al.* 2014. Synergistic Interactions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an *In Vitro* Wound Model. Infection and Immunity. 82(11): 4718-4728.
[3] Rennie *et al.* 2019. Understanding Real-Time Fluorescence Signals from Bacteria and Wound Tissues Observed with the MolecuLight *i:X*. Diagnostics. 9(1).

RESULTS

- With the MolecuLight *i:X* device, a fluorescence image was taken to confirm porphyrin producing bacteria were emitting red fluorescence, as expected (Figure 4A)
- A spectrofluorometer (BioTek Synergy H1) was used to scan the full emission spectrum (435-695nm) with an excitation wavelength of 405nm (same as *i:X* device) (Figure 4B and 4C)





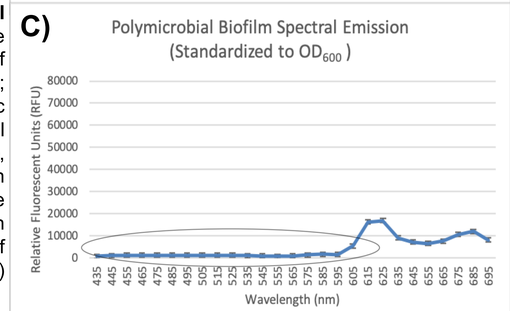


Figure 4: Fluorescence Emission Graphs for Polymicrobial Planktonic Solution vs. Polymicrobial Biofilm. (A) Fluorescence image with *i:X* device of prepared 96-well plate; Polymicrobial planktonic condition on top, polymicrobial biofilm condition in the middle, negative control condition on bottom. Relative fluorescence units (RFUs) over wavelength graphs (corrected for scale) of polymicrobial planktonic (B) versus biofilm (C) conditions.

DISCUSSION

Both the polymicrobial planktonic suspension and the polymicrobial biofilm exhibit fluorescence peaks ~630nm, as expected due to porphyrin production induced by ALA (confirmed by red fluorescence with the *i:X* device (Figure 4). At lower wavelengths, we observe that there is less emission activity in the biofilm condition compared to the planktonic condition (Figure 4B and C). We speculate that the bacterial EPS might be quenching some fluorescence signal, which may be indicative of the presence of EPS, and therefore biofilm, in our samples compared to the planktonic condition. It is also noted that under the same scale, the emissions of the polymicrobial planktonic solution are more robust, consistent with the fluorescence intensity observed with the 96-well plate (Figure 4A), which may also support the EPS signal quenching hypothesis.

FUTURE DIRECTIONS

This work only evaluated the emission spectrum at excitation wavelength 405nm, consistent with current settings with the *i:X* device. Future experimentation will evaluate changes to fluorescence emission patterns across the full spectrum of excitation wavelengths (Figure 5). If there are any differences in the emission patterns comparing polymicrobial planktonic solutions to polymicrobial biofilms, this could inform a "biofilm mode" on the MolecuLight *i:X* device. This would allow physicians to detect not only bacterial burden within a wound but also determine if a wound is biofilm-associated.

Excitation (nM)	Emission range (nM)
400	430-695
405*	435-695
410	440-695
420	450-695
430	460-695
440	470-695
450	480-695
460	490-695
470	500-695
480	510-695
490	520-695
500	530-695
510	540-695
520	550-695
530	560-695
540	570-695
550	580-695
560	590-695
570	600-695
580	610-695
590	620-695
600	630-695
610	640-695
620	650-695
630	660-695
640	670-695
650	680-695
660	690-695

Figure 5: Full Fluorescence Excitation and Emission Spectral Scan

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