REAL-TIME LABEL-FREE MONITORING *STAPHYLOCOCCUS AUREUS* ANTIBIOTIC SUSCEPTIBILITY USING SURFACE PLASMON RESONANCE IMAGING

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ABSTRACT
In this study, antibiotic susceptibility of *Staphylococcus aureus* was determined by combining Surface Plasmon Resonance imaging (SPRi) with conventional microfluidics. SPRi is an outstanding technique for studying antibiotic resistance and bacterial behavior due to its large field of view and real-time, label-free monitoring capabilities. Initially, varying concentrations of *S. aureus* were measured to ascertain the sensitivity of SPRi. Then, the minimum inhibitory concentration (MIC) of antibiotics for *S. aureus* was determined using this system. Finally, different antibiotic concentrations were allowed to diffuse into a microchamber filled with bacterial culture to monitor the effects of the antibiotic on bacterial behavior.

KEYWORDS: Surface Plasmon Resonance imaging, *S. aureu*s, Antibiotic susceptibility, Microfluidics

INTRODUCTION
Rapid identification of bacterial susceptibility to antibiotics is critical for improving patient outcomes, as treatment with ineffective or broad-spectrum antibiotics slows recovery and leads to the emergence of multidrug resistant species, such as Methicillin-resistant *Staphylococcus aureus* (MRSA) [1]. We present here a unique, high-resolution approach that combines microfluidics with Surface Plasmon Resonance imaging (SPRi) to monitor *S. aureus* growth and chemotaxis in real-time when exposed to varying concentrations of penicillin/streptomycin. Since SPRi rapidly determines changes in both the amount and location of biomass on a surface without labels, our approach can be extended to any bacterial species. By employing custom microfluidics in this type of large-area imaging system (1 cm^2), bacterial samples can be exposed to multiple concentrations and types of antibiotics in a single run. The individual 10 μm^2 pixels with biomass accumulation appear bright in the difference images, which are obtained every 15 seconds. As we have demonstrated recently, the brightness is directly proportional to the bacterial surface coverage [2]. Low-cost PDMS replica molding, employing a glass master patterned with Kapton® tape and Wite-Out® correctional fluid, was used to demonstrate the proof-of-concept.

EXPERIMENTAL
SPRi experiments were carried out with microfluidic channels placed on a gold-coated prism. Microchannels were prepared using 3 layers of tape, resulting in a thickness of 240 μm, while constrictions were created with correctional fluid, 17 μm dry thickness, on a glass master and molded with polydimethylsiloxane (PDMS). Bacterial samples were loaded into the channels and inserted into the SPRi system to monitor the changes.

Prior to each experiment, *S. aureus* was cultured overnight at 37 °C in 6 mL of Lysogeny Broth (LB) and cell counts were obtained with a hemocytometer. Antibiotic mixtures were prepared with 10,000 units/mL penicillin and 10,000 μg/mL of streptomycin, and necessary dilutions performed for each experiment.

RESULTS AND DISCUSSION
Initially, different concentrations of *S. aureus* were loaded into microchannels to quantify SPRi signal and to evaluate its sensitivity to detect *S. aureus* growth at room temperature. Three parallel microchannels were placed on the sensor surface. Channels initially were filled with Lysogeny Broth (LB) as control, 5.4 $x10^6$, and 5.4 $x10^8$ cells/mL of *S. aureus* (*Figure 1-A*). Changes in the brightness of the three channels were continuously monitored for 10 hours and quantified using image analysis options

in Adobe Photoshop CS5. A statistically significant change in the brightness, due to bacterial growth at room temperature, was recorded within 2 hours of incubation (*Figure 1-B*).

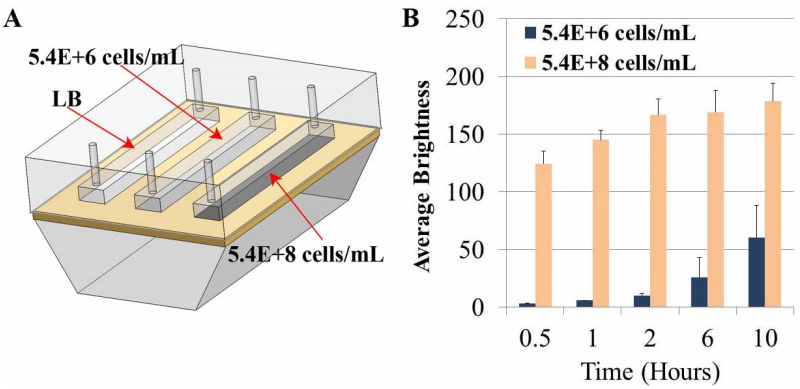

Figure 1: (A) Schematic of the SPRi setup. (B) The average brightness in the channels initially filled with 5.4 x10^6 and 5.4x10^8 cells/mL of S. aureus vs. time. The average brightness of the negative control channel filled with LB has been subtracted from the average brightness of bacteria filled channels for each time point to adjust for random signal drift in the SPRi. The results show that bacterial growth can be detected within 2 hours by comparing the brightness against the control channel. The error bars show the standard deviation of 3 runs.

Next, the MIC of an antibiotic cocktail, known to kill *S. aureus*, was determined by using SPRi. A known effective kill dose is 10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin. Four separate PDMS microchambers on the gold sensing surface were filled with sterile LB, 5.4 x10^6 cells/mL of *S. aureus* with no antibiotics, 5.4 x10^6 cells/mL of *S. aureus* with a 1000X diluted dose, and 5.4 x10^6 cells/mL of *S. aureus* with a 200X diluted dose (*Figure 2-A*). Initial experiments with a 1X dose exhibited no bacterial growth (data not shown). Bacteria grew rapidly in the chamber without antibiotics, while growth was suppressed for at least 6 hours in chambers containing 200X and 1000X dilutions of the antibiotic cocktail (*Figure 2-B*). After 12 hours of incubation, growth was observed in the chamber containing the 1000X antibiotic dilution. The drift in the signal of the system is shown in the raw LB values.

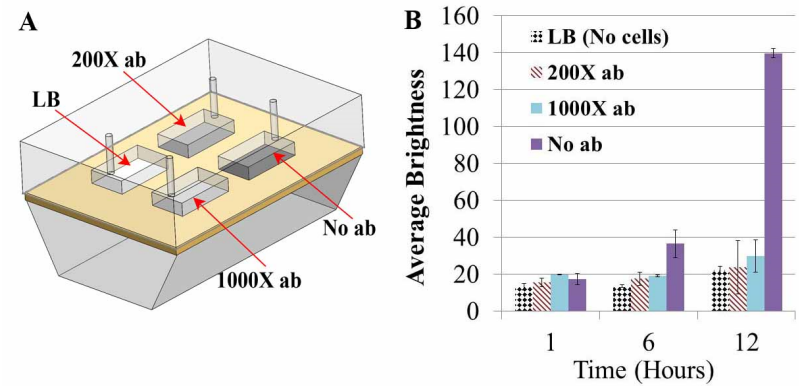

Figure 2: (A) Schematic of the SPRi setup for antibiotic resistance experiments. (B) The average brightness change in the channels filled with LB without cells (diamonds), or 5.4 x10^6 cells/mL S. aureus with no antibiotic (purple), with 1000X diluted antibiotic (blue), with 200X diluted antibiotic (sloping lines). The experiment was repeated four times. The error bars show the standard deviation of the data.

Lastly, the effect of antibiotic concentrations on bacterial behavior was tested by designing a microsystem consisting of a main chamber connected to two microchambers via constrictions made with correctional fluid. Bacterial growth was monitored continuously as antibiotics diluted 1000X and 200X in LB diffused from small microchambers to the main channel via the constrictions. SPRi difference images after 21 hours of bacterial growth reveal that the area surrounding the microchannel with 200X diluted antibiotic is darker compared to the remainder of the chamber. In contrast, the region near the 1000X diluted antibiotic chamber is brighter than average (*Figure 3-A*). Based on a mass balance for the

system, after 21 hours, the nutrients in the main chamber were consumed. *Figure 4* confirms that fewer cells were around the 200X constriction and suggests that bacteria are willing to tolerate low levels of antibiotics (1000X) to be closer to a source of fresh food.

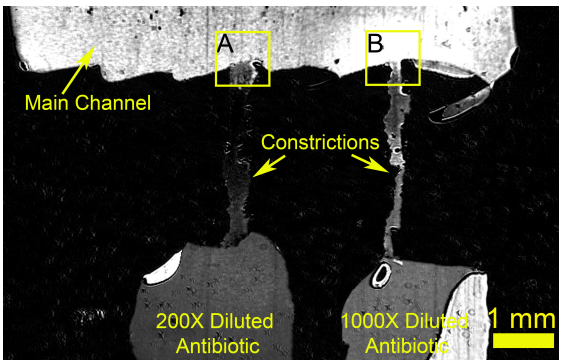

Figure 3: SPRi difference image 21 hours after the start of the experiment. The main channel on top was filled initially with 5.4 x10^6 cells/mL of S. aureus. The microchamber on the bottom left was filled with 200X diluted penicillin/streptomycin in LB and the microchamber on the bottom right was filled with 1000X diluted penicillin/streptomycin in LB. The lower intensity (A) at the entrance of 200X diluted antibiotic chamber indicates less biomass accumulation in that region than at (B) where the cells can survive and are trying to reach the unconsumed LB despite the presence of antibiotic.

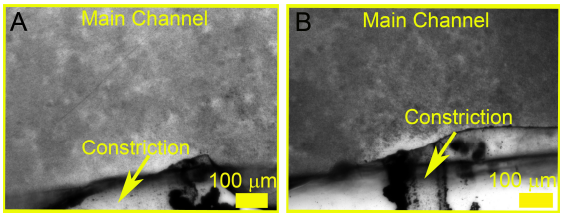

Figure 4: Brightfield optical micrographs of the regions shown in Figure 3 (A) and (B) after the fluidic setup was removed from the SPRi instrument. In these images, a darker color represents a greater biomass density, since the incident light is scattered by the biomass and not reflected off the gold SPRi sensor substrate. The images confirm the SPRi results in Figure 3.

CONCLUSION

In this study, antibiotic susceptibility of *S. aureus* is investigated using SPRi. SPRi can detect different concentrations of *S. aureus* from variations in brightness of the difference images. Initial findings regarding the MIC of penicillin/streptomycin elucidate that 1000X diluted antibiotic concentration could not prevent the *S. aureus* growth after 12 hours of incubation whereas a 200X dilution of the dose suppressed the growth. Finally, SPRi and optical microscopy images illustrate that as nutrients become scarce in the surroundings, bacteria will tolerate a low antibiotic dose to have access to fresh nutrients. This novel approach can potentially be optimized to rapidly screen antibiotic resistance of unidentified pathogens.

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